Structure-Based Design of New Constrained Cyclic Agonists of the Cholecystokinin CCK-B Receptor

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New constrained cyclic pseudopeptide cholecystokinin-B (CCK-B) agonists have been designed on the basis of conformational characteristics of the potent and selective CCK-B agonist Boc-Trp-(NMe)Nle-Asp-Phe-NH_2 (K_i = 0.8 nM, selectivity ratio CCK-A/CCK-B > 6000) (Goudreau *et al. Biopolymers,* **1994**, *34,* 155–169). These compounds are among the first successful examples of macrocyclic constrained CCK_4 analogs endowed with agonist properties and as such may be of value for the development of nonpeptide CCK-B agonists. The affinities and selectivities of these compounds for CCK-B and CCK-A receptors have been determined *in vitro* by measuring the displacement of [^{3}H]pCCK_8 binding to guinea pig cortex and pancreas membranes, respectively. The most potent compound, **8b**, *N*-(cycloamido)-α-Me(R)Trp-[(2*S*)-2-amino-9-((cycloamido)carbonyl)nonanoyl]-Asp-Phe-NH_2, has a K_i value of 15 ± 1 nM for guinea pig cortex membranes with a good CCK-B selectivity ratio (CCK-A/CCK-B = 147). Furthermore, **8b** behaved as a potent and full agonist in a functional assay which measures the stimulation of inositol phosphate accumulation in CHO cells transfected with the rat CCK-B receptor (EC_{50} = 7 nM). The *in vivo* affinity of **8b** for mouse brain CCK-B receptors was determined following intracerebroventricular injection (ID_{50} ∼ 29 nmol/kg). **8b** was also shown to cross the blood–brain barrier (0.16%), after intravenous administration in mice. **8b** also increased gastric acid secretion measured in anesthetized rats after intravenous injection. Therefore, **8b** appears to be an interesting pharmacological tool and is currently under investigation as a lead for further development of nonpeptide CCK-B agonists.

Introduction

The C-terminal octapeptide of cholecystokinin (CCK_8 or CCK_{26-33}, H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) has well-established biological activities in the periphery and the central nervous system which are mediated through CCK-A[1,2] and CCK-B/gastrin receptors.[3,4] In the central nervous system CCK_8 is reported to be involved in motivation and anxiety,[5,6] analgesia,[7,8] memory processes,[9] and neuropsychiatric disorders,[10,11] most of these responses being related to CCK-B receptor or receptor subsite activation.[12,13] Accordingly, the CCK-B receptor constitutes an interesting therapeutic target. Thus in humans, CCK-B antagonists have been shown to block panic attacks triggered by CCK_4 (Trp-Met-Asp-Phe-NH_2),[14] to induce anxiolytic effects,[15,16] and to potentiate antinociceptive responses induced by exogenous opiates such as morphine[17] or by endogenous enkephalins[18] in various animal models. Moreover, CCK-B antagonists exhibit antidepressant-like effects in rodents.[19] The latter results show the existence of a physiological antagonism between the opioid and CCK systems modulated by δ opioid and CCK-B receptors.[8] In addition, systemic administration of microgram doses of the potent and selective CCK-B agonist BC 264,[20] led to hypervigilance effects in rodents and monkeys, suggesting that CCK-B agonists could have interesting clinical applications.[21] Although, several potent and selective CCK-B antagonists (peptide, pseudopeptide, and nonpeptide) have been designed, no potent nonpeptide agonists are yet available for the CCK-B receptor, although benzodiazepine derivatives have recently been reported to behave as CCK-A agonists with affinities in the micromolar range.[22] However, the selectivity of these compounds versus the CCK-B receptor is poor, and their agonist or antagonist profile for this receptor has not been analyzed. It remains therefore an intriguing and challenging problem to design nonpeptide compounds able to satisfy the structural requirements for activation of the CCK-B receptor. An accurate model of a CCK-B agonist receptor-bound conformation would be interesting for the rational development of such compounds. However, the exact three-dimensional structure of the G-protein-coupled CCK-B receptor is unknown, and structural information is limited to the recently determined amino acid sequence[23,24] and the results of site-directed mutagenesis experiments.[25–31]

In the absence of this structural information, CCK_4 which constitutes the smallest CCK peptide fragment endowed with a good selectivity for brain over peripheral receptors, was used, following a deletion approach, to design the CCK-B peptoid antagonist PD-134,308.[32]

Several modifications were made to CCK_4 which increased its CCK-B selectivity, such as the N-terminal protection of the tetrapeptide in Boc-CCK_4[33] or suc-CCK_4 and modifications of the different amino acids such as the replacement of Met by Nle and NMeNle.[34]

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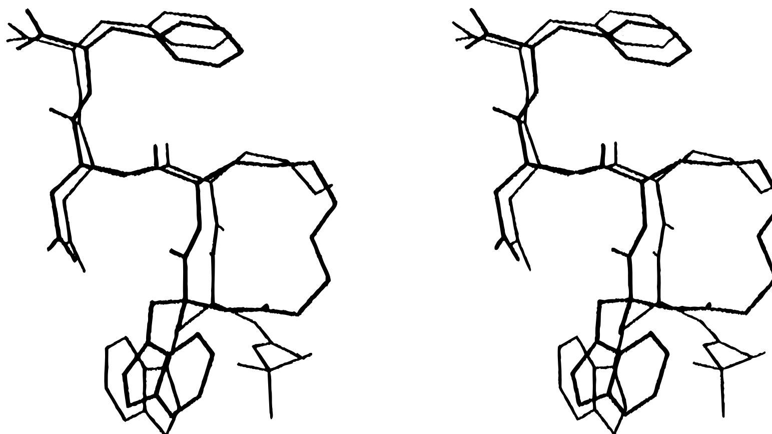


Figure 1. Stereoview of the superposition (heavy atoms only) of the preferential conformation of Boc-Trp-(NMe)Nle-Asp-PheNH$_2$ (normal) and its derived macrocyclic analog **8b** (bold).

Finally, restrained analogs of CCK$_4$ containing a diketopiperazine nucleus were prepared to provide rigid templates for the eventual design of peptidomimetics.[35,36] Recent ^{1}H-NMR and molecular dynamics studies performed in our laboratory indicated that the potent and CCK-B selective CCK$_4$ agonist, Boc-Trp-(NMe)Nle-Asp-Phe-NH$_2$ (K_i = 0.8 nM, selectivity ratio CCK-A/CCK-B > 6000) adopts an S-shaped conformation (Figure 1) with a relatively well defined orientation of the side chains.[37] On the basis of these results, we have already reported the synthesis of novel cyclic CCK-B agonists involving a diketopiperazine ring in place of the two N-terminal residues.[38]

As shown in Figure 1, the side chain of Nle, together with the N-terminus of tryptophan appeared as good candidates for another possible cyclization. Thus, cyclic compounds were designed by molecular modeling in order to mimic the proposed biologically active conformation of Boc-Trp-(NMe)Nle-Asp-Phe-NH$_2$. These compounds behave as potent and selective CCK-B agonists able to cross the blood−brain barrier and may be used for the further design of nonpeptide CCK-B agonists. Their synthesis and binding properties are reported in this study with some pharmacological responses observed with the most potent compounds.

Results

Molecular Design. The aromatic and acidic side chains of CCK$_4$ are known to play a key role in the interaction with the CCK-B receptor.[32,39,40] In the proposed biologically "S-shaped" active conformation of Boc-Trp-(NMe)Nle-Asp-Phe-NH$_2$ (Figure 1), the aliphatic side chain of Nle is oriented such that it might be extended and anchored to the N-terminus of tryptophan by omitting the bulky Boc substituent.

This proposed macrocyclic peptide was built by molecular modeling, initially preserving *S* stereochemistry for the four amino acid residues. The modeling suggested that inversion of the Trp stereochemistry from *S* to *R* was required to preserve the preferred orientation for the indole residue. This conformation appeared further stabilized by incorporation of the αMeTrp residue. The same cyclic peptide with a αMe(L)Trp, in place of the D isomer, was shown by molecular modeling to give a much less satisfactory orientation of the Trp side chain. This is not totally surprising, since changing LTrp for αMe(L)Trp and αMe(D)Trp, in CCK$_4$-derived compounds, bearing bulky N-protected groups, resulted in affinity increases (by a factor 5 and 35) for the CCK-B receptor.[41]

A 13-membered heterocyclic ring system appeared to be suitable for mimicking the conformational and structural features of the tetrapeptide Boc-Trp-(NMe)-Nle-Asp-Phe-NH$_2$[37] (Figure 1). The initial cyclic analog **8** (RB 360) was synthesized by replacing the (NMe)Nle residue with the non-natural and racemic amino acid (2*R*,*S*)-2-aminononane-1,9-dicarboxylate, H$_2$NCH[(CH$_2$)$_7$-COOH]COOH, which allowed ring closure by formation of an amide bond between the carboxylate of its side chain and the amino group of αMeD-Trp. A further reduction of the flexibility of the macrocyclic ring system, in order to probe the structural tolerance of the receptor binding site, was achieved by introducing an additional amide bond (compound **16** or RB 370) or a disulfide bridge (**24** or RB 380) into the 13-membered ring (Schemes 2 and 3), and by changing the size of the ring (Table 1, compounds **43** and **45**).

Synthesis. The cyclic tetrapeptides, listed in Table 1, were synthesized by standard liquid phase peptide synthesis, following the synthetic pathways summarized in Schemes 1−4 and Table 2.

Physical and chemical data for all the final compounds and intermediates are also given in Table 2. The non-natural amino acid **2**, methyl[(2*R*,*S*)-2-amino-9-(*tert*-butyloxycarbonyl)nonanoate, Xaa(OtBu)OMe, was obtained as a racemate in high yield by simple alkylation of the benzylidene derivative of glycine methyl ester with *tert*-butyl 8-bromooctanoic acid ester as the alkylating agent and subsequent acidic hydrolysis of the imine, according to a procedure described by Stork *et al.*[42]

Condensation of **2** (Scheme 1) with Boc-αMe(R)Trp, which was prepared as described by Horwell *et al.*,[43] led to intermediate **3** which was subsequently deprotected by treatment with TFA to give intermediate **4**. Intramolecular cyclization of **4** was then achieved by means of amidification with BOP, NaHCO$_3$ using a dilute (<10^{-2} M) solution[44] of the linear precursor in DMF. The 13-membered cyclic dipeptide **5** which structure was confirmed by ^{1}H-NMR spectroscopy, and mass spectrometry was hence prepared in a 29% yield. The expected acid derivative **6** was obtained by hydrolysis of **5** with an aqueous solution of NaOH, followed by coupling to the C-terminal dipeptide Asp(OBzl)PheNH$_2$

Table 1. Affinities and Potencies of CCK Ligands in Inhibiting [^{3}H]pCCK$_8$ Specific Binding to Guinea Pig Cortex Membranes, Pancreatic Membranes, or CHO Cells Transfected with the Rat CCK-B Receptor and in the Stimulation of Inositol Phosphate Accumulation in CHO Cells

	Compound	*	Name	K_I,[a] nM Brain (CCK-B)	K_I,[a] nM Pancreas (CCK-A)	Ratio A/B	K_I,[b] nM CHO Cells	EC_{50},[c] nM CHO Cells
	Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2		CCK$_8$	0.64 ± 0.04	0.28 ± 0.01	0.44	0.60 ± 0.12	0.63 ± 0.12
	Trp-Met-Asp-Phe-NH_2		CCK$_4$	19.5 ± 1.9	36133 ± 660	186	93 ± 8	-
	Boc-Trp-NMeNle-Asp-Phe-NH_2			0.8 ± 0.01	> 5.10^{-6} M	-	8.7 ± 2	1.5 ± 0.3
8	α-Me (R)Trp-NH-C*H-CO-Asp-Phe-NH_2 (cyclic: CO-(CH_2)$_7$)	R	8a RB 360-I	4372 ± 255	> 10^{-5} M	-	-	-
		S	8b RB 360-II	15 ± 1	2210 ± 432	147	18 ± 3	7 ± 1
16	α-Me (R)Trp-NH-C*H-CO-Asp-Phe-NH_2 (cyclic: CO-CH_2-CONH-(CH_2)$_4$)	S	RB 370	40 ± 4	2293 ± 163	57	-	-
24	α-Me (R)Trp-NH-C*H-CO-Asp-Phe-NH_2 (cyclic: CO-(CH_2)$_4$-S-S-(CH_2)$_2$)	S	RB 380	51 ± 7	271 ± 71	5	80 ± 10	7 ± 2
44	α-Me (R)Trp-NH-C*H-CO-Asp-Phe-NH_2 (cyclic: CO-(CH_2)$_6$)	S		62 ± 6	2880 ± 800	46	142 ± 15	-
45	α-Me (R)Trp-NH-C*H-CO-Asp-Phe-NH_2 (cyclic: CO-(CH_2)$_8$)	S		15 ± 1	2472 ± 300	165	23 ± 4	4 ± 1

[a] The K_I values represent the arithmetic means ± SEM of three separate experiments, each in triplicate performed on guinea pig cortex membranes (CCK-B), pancreatic membranes (CCK-A) and [b], on the rat brain CCK-B receptor expressed in CHO cells. [c] Results are the mean ± SEM of three separate measurements of inositol phosphate production, each in triplicate. The asterisk indicates the carbon whose configuration is indicated.

to give **7**. Finally, after deprotection of the Asp residue by catalytic hydrogenolysis, the expected compound **8** was obtained as a mixture of two diastereoisomers which were separated by semipreparative HPLC to give the optically pure isomers **8a** and **8b** (Scheme 1).

The assignment of their configuration has been proposed for both isomers by using a previously reported method[45] based on the differences in chemical shifts of side chain protons for dipeptides containing one aromatic moiety adjacent to an aliphatic one. For conformational reasons, a closer proximity between the side chains exists for *R,S* and *S,R* isomers in these dipeptides, leading the β-CH_2 protons of the aliphatic amino acid with these configurations to be more shielded by the aromatic moiety than the corresponding protons of *S,S* or *R,R* isomers, in which the side chains are further apart.[45] In the case of **8a** and **8b**, a simple molecular model shows that, due to cyclization-induced distorsion, a closer proximity occurs between the side chains for *R,R* isomers of αMeTrp and Xaa, than for *R,S* isomers. As the β-CH_2 protons of the non-natural amino acid (Xaa) are more deshielded in **8b** than in **8a** (respectively 1.50 and 1.35 ppm), we assumed that **8b** corresponds to the *R,S,S,S* isomer whereas **8a** corresponds with the *R,R,S,S* isomer.

The synthesis of the cyclic tetrapeptide **16**, outlined in Scheme 2, also consisted of the condensation of two dipeptide fragments using the optically pure *S* isomer of Lys(Z)OMe instead of the non-natural amino acid **2** used in the synthesis of **8**. Condensation with Boc-αMe-(R)Trp gave **9**, which was deprotected by treatment with TFA to give the dipeptide **10**. A monobenzylmalonic residue was introduced on the N-terminal free amino group of this dipeptide to give the intermediate **11**, from which protecting groups were simultaneously removed by catalytic hydrogenolysis. The intramolecular cyclization was then performed by using BOP in highly diluted conditions ($<10^{-2}$ M) to yield 38% of cyclic dipeptide **13**. Saponification of **13** gave the corresponding acid **14** which was coupled to the C-terminal dipeptide Asp(OBzl)PheNH$_2$ to give **15**. An unusually high rate of epimerization (~20%) was observed at the chiral center of the Lys residue following the coupling step. This can only be explained by the formation of an oxazolone intermediate,[46] presumably favored by steric and/or electronic effects of the methyl group on the α-carbon atom of Trp. Chromatographic purification to homogeneity, however, permitted the pure *R,S,S,S* diastereoisomer of **15** to be obtained, which was subsequently converted into the final product **16** by catalytic hydrogenolysis (Scheme 2).

The cyclic peptide **24** was obtained by a stepwise synthesis as outlined in Scheme 3 using the protecting groups 4-methylbenzyl (4-MeBzl) and cyclohexyl (cHex) for the side chains of residues Cys and Asp, respectively. Standard coupling and deprotection procedures (see the Experimental Section) permitted the linear tetrapeptide **22** to be obtained, which was further condensed with *S*-(carbomethoxysulfenyl)-5-thiopentanoic acid on its N-terminal free amino group using *N,N'*-dicyclohexylcarbodiimide (DCC), providing **23** with a yield of 69%.

S-(carbomethoxysulfenyl)-5-thiopentanoic acid had been obtained by the action of carbomethoxysulfenyl chloride (Scm-Cl)[47] on *tert*-butyl 5-thiopentanoic acid ester and subsequent removal of the *tert*-butyl ester by

Table 2. Physical and Chemical Data for All Compounds[a]

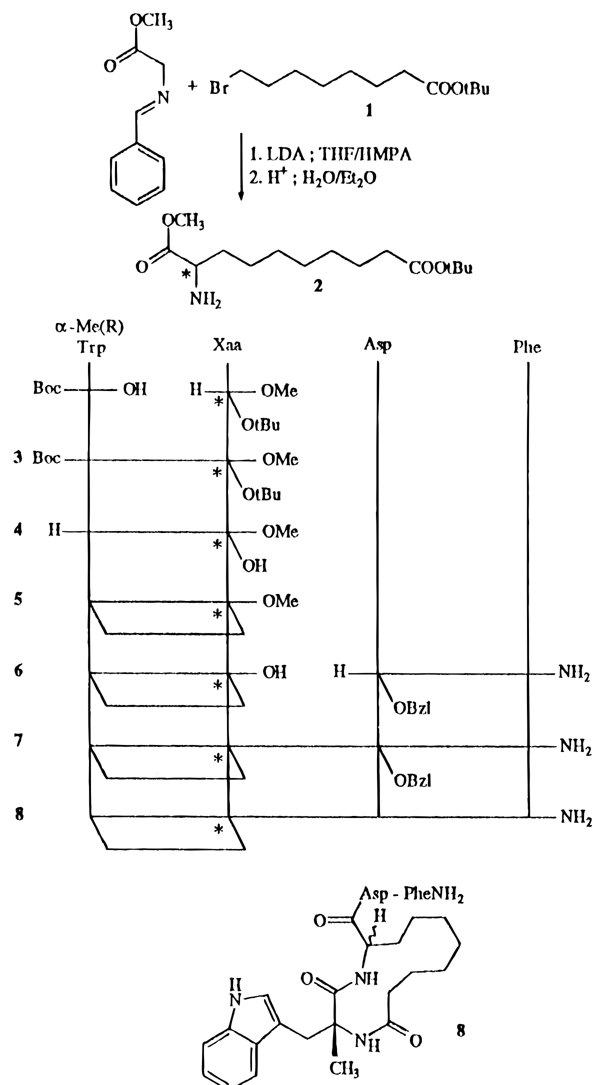
compd	scheme; procedure[b]	yield (%)	TLC[c] R_f	mp (°C)	MS (M^+)
1	I; A	95	0.90(B)	oil	
2	I; B	60	0.58(D)	oil	
3	I; C	64	0.69(C)	175–179	
4	I; D	100	0.21(C)	>250	
5	I; E	29	0.53(C)	177–187	414
6	I; F	100	0.38(E)	196–198	
7	I; C	62	0.52(C)	177–189	
8	I; G	77			
8a			0.68(E)	194–201	660
8b			0.71(E)	202–211	660
9	II; C	45	0.71(C)	169–174	
10	II; D	100	0.38(C)	145–153	
11	II; H	93	0.67(C)	177–181	
12	II; G	100	0.33(E)	193–199	
13	II; E	38	0.50(C)	167–173	
14	II; F	100	0.60(D)	201–209	
15	II; C	36	0.81(C)		
16	II; G	96	0.48(E)	192–207	675
17	III; H	95	0.49(B)	195–204	
18	III; D	94	0.46(C)	177–178	
19	III; H	62	0.38(B)	174–183	
20	III; D	94	0.39(C)	182–187	
21	III; C	59	0.59(C)	167–179	
22	III; D	85	0.54(C)	164–170	
23	III; I	69	0.66(C)	175–193	
24	III; J	27	0.62(E)	202–213	696
25[d]	IV; K	55	0.67(D)		
26	IV; K	50	0.25(B)		
27[e]	IV; K	31	0.30(B)		
28	IV; C	57	0.60(C)	165	
29	IV; C	66	0.60(C)	165	
30	IV; C	68	0.60(C)	159	
31	IV; G	100	0.41(C)	167–175	
32	IV; G	100	0.50(C)		
33	IV; G	100	0.55(C)		
34	IV; C	95	0.59(C)	170–179	
35	IV; C	98	0.55(C)	198	
36	IV; C	46	0.50(C)	192	
37	IV; D	100	0.10(C)	187–196	
38	IV; D	98	0.15(C)	64	
39	IV; D	92	0.30(C)	138	
40	IV; C	29	0.56(C)	173–186	742
41	IV; C	30		152	728
42	IV; C	37			756
43	IV; J	81	0.71(E)	202–211	660
44	IV; J	40	0.71(E)	204–211	646
45	IV; J	30	0.76(E)	203–215	674

[a] ^{1}H NMR data and elemental analysis for the final compounds are included in the Experimental Section. [b] Refers to the scheme in which the synthesis of the compound is outlined; procedures are detailed in the Experimental Section. [c] Solvent systems are indicated in parentheses and explained in the Experimental Section. [d] $[\alpha]^{20}_D = -10.0°$ (c 1.0, MeOH). [e] $[\alpha]^{20}_D = -7.2°$ (c 1.0, MeOH).

the action of TFA (see the Experimental Section). The Scm group, as described by Hiskey *et al.*,[47] offers the advantage of acting both as a protecting and as an activating group, yielding a disulfide when treated with thiols, with release of the volatile carbonyl sulfide and methanol. Final deprotection on the cysteine side chain of **23** using hydrogen fluoride (HF)[48] allowed the formation of a disulfide bridge and consequent intramolecular cyclization, with simultaneous cleavage of the cyclohexyl ester[49] leading to the final optically pure compound **24** in a 27% yield (Scheme 3). The formation of a significant amount (25%) of dimerized product was observed, as shown by HPLC and mass spectrometry, which is probably due to the concentrated conditions ($\pm 10^{-1}$ M) required for the handling of HF.

In order to confirm the exact stereochemistry of the isomers **8a** and **8b**, and to optimize the size of the ring

Scheme 1. Preparation of Compound **8**[a]

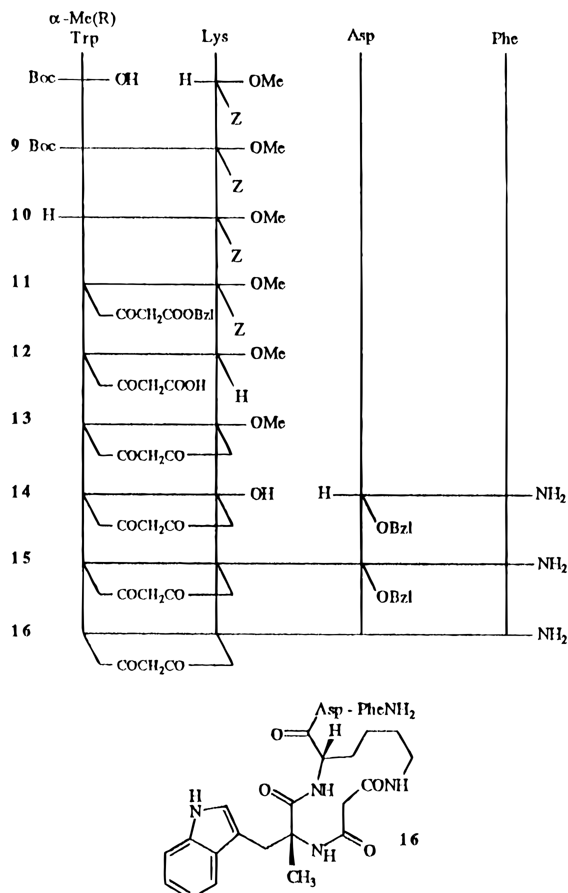


[a] All amino acids have an (S)-configuration unless indicated otherwise. An asterisk (*) means (R,S)-mixture.

system, a stereoselective stepwise synthesis (Scheme 4) was finally performed using the S isomer of the protected non-natural amino acids **25**–**27** (ZNH-CH[$(CH_2)_n$-COOtBu]COOH) with n = 6–8, which were obtained by an asymmetric alkylation of methyl N-(diphenylmethylene)glycinate using the chiral inductor of Oppolzer.[50] This chiral glycine equivalent was prepared as previously described[51] and easily alkylated with *tert*-butyl bromoalkanoic acid esters followed by acidic hydrolysis of the imine with overall yields of around 50%. Subsequent amino protection with a benzyloxycarbonyl group and cleavage of the sultam auxiliary gave the non-natural amino acids **25**–**27.** Stepwise elongation of the peptide chain by incorporation of compounds **25**–**27** and α-Me(R)Trp using classical coupling and deprotection procedures, followed by cyclization and a final deprotection, gave the optical pure final compounds **43**–**45** (Scheme 4). Compound **43** was characterized by ^{1}H-NMR spectroscopy and mass spectrometry and proved to be identical to **8b**, supporting our previously discussed assignment of the two separated isomers.

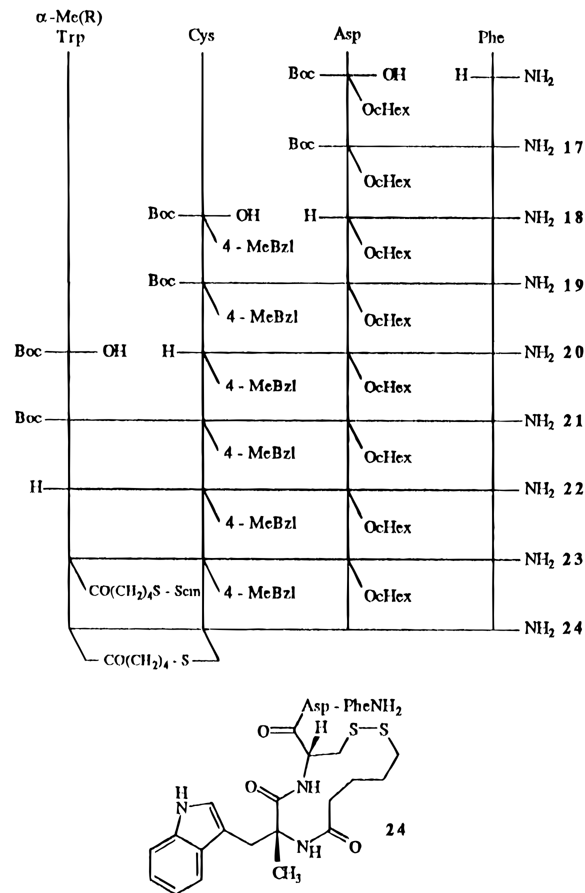
Biological Evaluation. The apparent affinities of the synthesized compounds for CCK-A and CCK-B

Scheme 2. Preparation of Compound **16**[a]



[a] All amino acids have an (*S*)-configuration unless indicated otherwise.

Scheme 3. Preparation of Compound **24**[a]



[a] All amino acids have an (*S*)-configuration unless indicated otherwise. Scm means carbomethoxysulfenyl (SCOOMe).

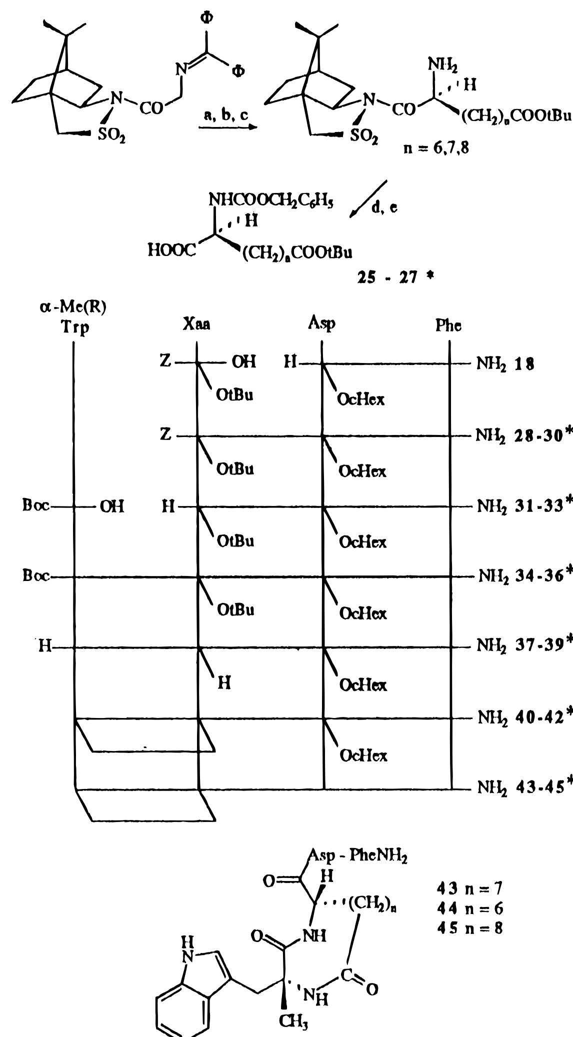
binding sites were determined by measuring the displacement of [^{3}H]pCCK$_8$ from guinea pig pancreatic and brain cortex membranes, respectively, as described previously.[20] These data are summarized in Table 1 and show that most of the compounds designed have a good CCK-B affinity and selectivity. A comparison of CCK-B receptor affinities between **8b** (K_i = 15 ± 1 nM), **16** (K_i = 40 ± 4 nM), and **24** (K_i = 51 ± 7 nM), which contain a similar 13-membered cycle, shows that the best affinity is obtained with the alkyl chain. Thus, incorporation of an amide (**16**) or disulfide bond (**24**) in the cycle does not improve the CCK-B affinity, and in the case of **24**, the selectivity has been even decreased (ratio CCK-A/CCK-B = 5). This could be due to steric constraints imposed on the macrocycle. A comparison of **8b** (K_i = 15 ± 1 nM) and compounds **44** (K_i = 62 ± 6 nM) and **45** (K_i = 15 ± 1 nM) suggests that a 13- or 14-membered ring size is optimal for binding to the CCK-B receptor. In agreement with the molecular modeling studies, **8b** (Figure 1) with a *R,S,S,S,* configuration has a better affinity for the CCK-B receptor than the *R,R,S,S,* isomer (**8a**).

The apparent *in vivo* affinity of **8b** in mouse brain was determined by measuring the displacement of [^{3}H]-pBC 264 as previously described.[52] Inhibition of the specific binding of 10 pmol of [^{3}H]pBC 264 by increasing concentrations of **8b** was studied 15 min after icv coinjection of both compounds. The apparent ID$_{50}$ value of **8b**, calculated from the competition curve (not shown), was 19 μg/kg (29 ± 6.5 nmol/kg). Moreover a significant inhibition (32 ± 5% and 87 ± 8%) of *in vivo* [^{3}H]pBC 264 specific binding was observed after iv injection of 5 and 20 mg/kg of **8b**, respectively, showing that this compound is able to enter the mouse brain and to displace the selective CCK-B agonist [^{3}H]pBC 264 from its binding sites. The ability of **8b** to cross the blood−brain barrier was estimated to be around 0.16%. This was calculated, as previously described in detail,[52] by establishing the ratio of the doses, administered either centrally (7.8 μg/kg) or peripherally (5 mg/kg), which produced the same percentage of inhibition (32%).

The pharmacological profile of the most potent compounds was investigated by measuring the formation of inositol phosphates (IP) in CHO cells, stably transfected with the rat brain CCK-B receptor (Table 1, Figure 2). All the compounds tested behaved as agonists and were able to induce the IP accumulation at doses inferior to those expected when their CCK-B affinities are taken into account. Moreover, they were devoid of antagonist properties at concentrations up to 10^{-5} M (not shown).

In order to confirm their pharmacological agonist profile on the CCK-B receptor *in vivo*, **8b** and **24** were tested for their ability to stimulate the CCK-B/gastrin receptor by measuring gastric acid output in the anesthetized perfused rat stomach. As shown in Figure 3, iv injected **8b** and **24** stimulated gastric acid output in a dose-dependent manner. On the basis of the ED$_{50}$ values estimated from the dose−response curves, **8b** (ED$_{50}$ = 7.6 ± 1.6 nmol/kg·h) was almost as potent as CCK$_8$ (ED$_{50}$ = 4 ± 2 nmol/kg·h), whereas **24** (ED$_{50}$ = 52 ± 2.3 nmol/kg·h) was 10-fold less potent than CCK$_8$.

Scheme 4. Diastereoselective Synthesis of **8b** and Analogs[a]



[a] (a) nBuLi, THF, −78 °C; (b) $Br(CH_2)_nCOOtBu$, THF/HMPA, −55 °C to rt; (c) citric acid, H_2O/THF; (d) $C_6H_5CH_2O_2CCl$, Et_3N, THF; (e) LiOH, H_2O/THF. All amino acids have an (*S*)-configuration unless indicated otherwise. An asterisk (*) shows *n* being 7, 6, and 8, respectively.

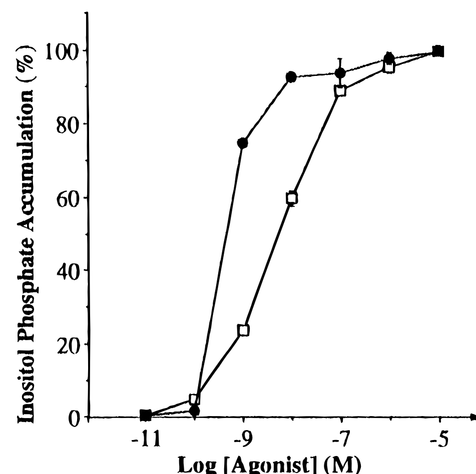


Figure 2. Stimulation of inositol phosphate accumulation in CHO cells transfected with the rat CCK-B receptor. Cells were treated with either CCK_8 (●) or compound **8b** (□), as described in the Experimental Section. Results are the mean ± SEM of three separate measurements of inositol phosphate production, each in triplicate. EC_{50} values are given in Table 1.

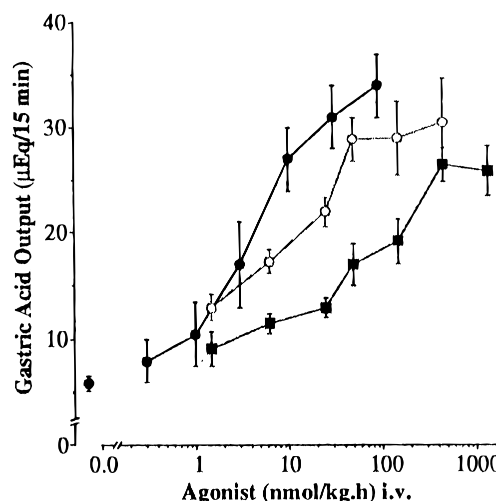


Figure 3. Ability of CCK_8 (●), **8b** (○) and **24** (■) to increase the gastric acid output. ED_{50} values are respectively 4 ± 2, 7.6 ± 1.6, and 52 ± 2.3 nmol/kg·h for CCK_8, **8b**, and **24**.

In this pharmacological model, the observed effects of **8b** and **24** were in agreement with their affinities measured on CHO cells transfected with the CCK-B receptor (see Table 1). The less than expected ED_{50} of CCK_8 might reflect its enzymatic degradation or its capacity to activate CCK-A receptor, thus inducing the release of somatostatin, which in turn could inhibit gastric acid secretion.[53]

Discussion

The goal of this study was to stabilize the bioactive conformation of CCK-B agonists in order to aid the design of non peptide ligands. As shown in Table 1 and Figures 1−3, cyclic CCK-B agonists have been obtained using a molecular modeling approach based on the proposed biologically active conformation of Boc-Trp-(NMe)Nle-Asp-Phe-NH_2[37] (Figure 1). In agreement with our results, the same type of folded structures have been reported for several potent agonists derived from CCK_4 and containing a [*trans*-3-propyl-L-proline],[35] a diketopiperazine skeleton,[36] or a [(alkylthio)proline[31]] residue.[54] It is noteworthy that all these CCK-B agonists have a common C-terminal dipeptide: Asp-Phe-NH_2.

Compounds **8b**, **16**, and **24**, which have good affinities for central CCK receptors, are an interesting series of cyclic peptidomimetic agonists. To date only a few CCK_4-derived cyclic compounds have been reported,[36,38,55] most of them behaving as antagonists. This is the case for peptoid analogs, such as PD-134,308,[32] whose cyclization resulted in derivatives with reduced affinities.[56] In our compounds, the spatial constraints imposed by the formation of a 13-membered ring also reduced the CCK-B receptor affinity as compared to Boc-Trp-(NMe)Nle-Asp-Phe-NH_2 and even to the nonmethylated Boc-Trp-Nle-Asp-Phe-NH_2.[37] Nevertheless, the agonist profile of these molecules is maintained, and **8b** was found to have a similar affinity *in vitro* as CCK_4 (K_i = 20 nM).[57]

A slight reduction in affinity was observed for **16** and **24**, when compared with **8b**, suggesting that only a certain degree of constraint is tolerated by the CCK-B receptor agonist site. Interestingly, binding to the

CCK-B versus CCK-A receptors was influenced by changes in the nature of the ring system. A cyclic moiety with a disulfide bridge in **24** increases CCK-A receptor recognition (Table 1). This is not surprising since the nature of amino acid 31 was previously shown to be important for recognition of central versus peripheral receptors. Thus, replacement of the Met^{31} residue in Boc-CCK_4 with a lysine bearing a phenylurea at the amino group place resulted in a series of potent and selective CCK-A agonists exemplified by A-71623.[58] Potent CCK-B receptor affinity was restored, but a loss in selectivity was obtained upon simple substitution of the lysine amino group.[59] On the other hand, introduction of a Phe^{31} residue in CCK_8 analogs was found to favor recognition of the CCK-B receptor site.[60]

As listed in Table 1, some of the compounds show slight differences in binding affinities using either the CCK-B receptor from guinea pig brain or CHO cells stably transfected with the rat CCK-B receptor, in accordance with variations observed in various species.[61]

In vivo binding experiments on mice, performed using [^{3}H]pBC 264, gave an ID_{50} value of (29 ± 6.5 nmol/kg) for **8b**, while in the same conditions an ID_{50} value of 8.5 ± 0.4 nmol was obtained for CCK_8.[52] Due to its low solubility, CCK_4 could only be used at a maximal dose of 10 nmol where it inhibited 40% of the *in vivo* [^{3}H]-pBC264 binding. Since CCK_8 has an *in vitro* affinity higher than that of **8b** and CCK_4 for the CCK-B receptor (Table 1), these results suggest that *in vivo* **8b** is enzymatically much more stable than CCK_8 and even CCK_4.

The capacity of **8b** to cross the blood−brain barrier, after iv injection, was evaluated in mice and found to be 0.16%. This is better than the percentage (0.02%) found for pBC 264, a highly potent CCK-B agonist (K_i = 0.17 nM in mouse brain).[52] The higher capacity of **8b** to cross the blood−brain barrier is illustrated by the similar dose (20 mg/kg, iv) of **8b** and pBC 264[52] able to inhibit 87% of the *in vivo* binding of [^{3}H]pBC 264. Interestingly, under the same conditions, 20 mg/kg of CCK_4 was unable to modify the binding of [^{3}H]pBC 264.[52] Since following iv, ip, and sc administration CCK_4 provokes anxiogenic responses[62,63] and is even able to induce panic attacks when iv administered to human subjects at doses as low as 10−50 μg,[64] these results suggest that CCK_4 might interact with regions which are not fully shielded by the blood−brain barrier,[65] such as the brainstem.

Finally, in two functional assays, our macrocyclic compounds behaved as full agonists. Their *in vivo* potencies for gastric acid induced secretion by stimulation of the rat CCK-B/gastrin receptor correlate well with their *in vitro* binding affinities on CHO cells expressing the rat CCK-B receptor. In the case of the stimulation of IP accumulation in CHO cells, these compounds have better efficiencies than would be expected from their binding affinities to the same cells.

In conclusion, we have shown that, in the case of CCK-B receptor, it is possible, by rational design of relatively rigid molecules, to stabilize the bioactive conformation of CCK_4 while conserving agonist properties. In addition, the new bioactive macrocyclic analogs of CCK_4 exemplified by **8b** proved to be relatively easy to synthesize. Further studies of **8b** are being carried out in the laboratory, aimed at achieving a better definition of the structural and conformational requirements for CCK-B receptor recognition and signal transduction.

Experimental Section

Computational Procedures. All calculations were performed on a Silicon Graphics Workstation using the Insight/Discover Software package (Biosym Technologies Inc.). The calculations on Boc-Trp-(NMe)Nle-Asp-$PheNH_2$ were performed as previously described.[37] Conformational analyses were performed through high-temperature molecular dynamics. The initial structure of **8** (*R,S,S,S* isomer) was minimized using steepest descents and conjugate gradients algorithms to be subjected afterward to 100 ps of dynamics at 600 K. An integration time step of 1 fs was used. Molecular geometries were stored at 1 ps intervals, rendering a set of 100 structures. All stored structures were then retrieved and cooled down to 300 K by contact with a heat bath using a temperature coupling constant of 1 ps. The dynamic stimulation continued for an additional 20 ps under these conditions, and the instantaneous structures at the end of the annealing phase were energy minimized using a combination of steepest descents/conjugate gradients algorithms. The 100 resulting energy-minimized structures were then clustered according to conformational similarities (heavy atom superposition) using the insight/analysis module. Finally the representative conformer of each family was then compared with the preferential conformation of Boc-Trp-(NMe)Nle-Asp-$PheNH_2$[37] by using a template-forcing procedure (Insight II User Guide, Version 2.1.0. Copyright 1992, Biosym Technologies Inc., San Diego, CA). The atoms taken into account for the template-forcing procedure were matched pairs of atoms which were identical for both molecules.

Synthesis. Commercial chemicals were used without further purification. When appropriate, solvents were purified and dried by standard methods before use. All compounds in Table 2 were dried *in vacuo* over KOH and silica gel. Flash column chromatography was performed using Merck silica gel (230−400 mesh). Merck plates precoated with F254 silica gel were used for thin layer chromatography with the following solvent systems (by volume): A, CH_2Cl_2/MeOH (50/1); B, CH_2Cl_2/MeOH (19/1); C, CH_2Cl_2/MeOH (9/1); D, AcOEt/CH_2Cl_2/MeOH/H_2O/AcOH (110/70/30/6/3); E, CH_2Cl_2/MeOH/H_2O/AcOH (70/30/6/3). Plates were developed with UV, iodine vapor, ninhydrin, or Ehrlich's reagent. Melting points were performed on an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer polarimeter (model 241). Mass spectra were recorded on a quadrupole NERMAG R10-10C apparatus. ^{1}H-NMR spectra were recorded on a Bruker AC 270-MHz or AM 400-MHz instrument in DMSO-d_6. Chemical shifts were measured in ppm with HMDS as internal standard. The purity (>98%) of all final compounds was checked by HPLC (Shimadzu apparatus) on a 250 × 4.6-mm Kromasil C_8-5 μm column with a mixture of CH_3CN and $H_2O/TFA_{0.05\%}$ as eluent (flow rate 1.2 mL/min, UV detection 214 nm). Elemental analyses, performed by "Service Régional de Microanalyses" (Paris, France), were within ±0.4% of the theoretical values unless noted otherwise.

Procedure A: *tert*-Butyl 8-Bromooctanoate (1). A solution of 8-bromooctanoic acid (112 mol) was treated with 2-methylpropene (784 mmol) in 20 mL of Et_2O and 1.12 mL of sulfuric acid using the method of McCloskey *et al.*[66] to give 29.8 g of *tert*-butyl 8-bromooctanoate with a 95% yield: R_f = 0.90 (B); ^{1}H-NMR (DMSO) ∂ 1.15−1.48 (17H, m, $(CH_2)_4$ + tBu), 1.70 (2H, m, CH_2), 2.10 (2H, t, CH_2), 3.45 (2H, t, CH_2).

Procedure B: 1-Methyl-(2*R,S*)-2-amino-9-(*tert*)-butyloxycarbonyl)nonanoate (2). *tert*-Butyl 8-bromooctanoate (16.0 g, 57.3 mmol) was used to alkylate 10.15 g of the benzylidene derivative of glycine ethyl ester (57.3 mmol) using lithium diisopropylamine (LDA) and hexamethylphosphoramide (HMPA) at −78 °C in dry tetrahydrofuran (THF) as described by Stork *et al.*[42] After the mixture was stirred overnight at room temperature, the pH was adjusted to 7 with 1 N HCl, the solvent was removed *in vacuo*, and 200 mL of

Et_2O was added. The organic layer was washed with H_2O and brine, dried with Na_2SO_4, filtered, and evaporated to yield 21 g of orange oil, which was redissolved in 30 mL of Et_2O, and 100 mL of an aqueous 10% citric acid solution was added. This mixture was stirred for 48 h at room temperature (rt). The pH of the aqueous layer was adjusted to 12 with $NaHCO_3$, and it was extracted with EtOAc (3 × 30 mL). The combined organic layers were washed with H_2O and brine, dried with Na_2SO_4, filtered, and evaporated to yield **2** (9.9 g, 60%) as a pale yellow oil: ^{1}H-NMR (DMSO+TFA) ∂ 1.15−1.50 (19H, m, 5 × CH_2 + tBu), 1.75 (2H, m, CH_2), 2.14 (2H, t, CH_2), 3.75 (3H, s, CH_3), 4.00 (1H, m, α-CH), 8.30 (3H, s, NH_3^+).

Procedure C: Boc-αMe(R)Trp-NHCH[$(CH_2)_7$COOtBu]-$COOCH_3$ (3). Boc-αMe(R)Trp (1.66 g, 5.2 mmol), which was prepared as previously described,[43] was dissolved in 3 mL of dimethylformamide (DMF), and at 0 °C were subsequently added diisopropylethylamine (DIEA, 5.2 mmol) and (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 5.7 mmol). After 30 min of stirring at 0 °C, the amine **2** was added (1.50 g, 5.2 mmol), plus one additional equivalent (5.2 mmol) of DIEA, if the amine was used as a salt. Stirring was continued overnight at rt, the solvent removed *in vacuo*, and 50 mL of EtOAc added to the residue. The organic phase was washed with an aqueous 10% citric acid solution, H_2O, $NaHCO_3$ solution (10%), H_2O, and brine, dried with Na_2SO_4, filtered, and evaporated. The residual solid was then further purified by flash column chromatography (CH_2Cl_2/EtOAc elution (9/1)) to give **3** (1.97 g, 64%): ^{1}H-NMR (DMSO) ∂ 1.10−1.65 (33H, m, $(CH_2)_6$ + tBu + Boc + α-CH_3), 2.10 (2H, t, CH_2CO), 3.02−3.40 (2H, m, β-CH_2 (Trp)), 3.54 (3H, s, CH_3), 4.08−4.25 (1H, m, α-CH), 6.40 (1H, s, BocNH), 6.85 (1H, t, indole H_5), 6.96 (2H, m, indole H_2 + H_6), 7.25 (1H, d, indole H_7), 7.44 (1H, d, indole H_4), 8.30 (1H, m, NH), 10.84 (1H, s, indole NH).

Procedure D: TFA·Me(R)Trp-NHCH[$(CH_2)_7$COOH]-$COOCH_3$ (4). The carbamate **3** (1.9g, 3.2 mmol), dissolved in a mixture of CH_2Cl_2 (10 mL), trifluoroacetic acid (TFA, 10 mL), and anisole (1 mL), was treated for 1 h at 0 °C and an additional 2 h at rt. After evaporation and trituration with dry Et_2O, the residual solid was dried to provide quantitatively **4** (1.76 g): ^{1}H-NMR (DMSO) ∂ 1.10−1.80 (15H, m, $(CH_2)_6$ + α-CH_3), 2.10 (2H, m, CH_2CO), 3.15−3.30 (2H, m, β-CH_2 (Trp)), 3.55 and 3.60 (3H, s, CH_3), 4.14 and 4.40 (1H, m, α-CH), 6.95 (1H, t, indole H_5), 7.04 (1H, t, indole H_6), 7.12 and 7.20 (1H, d, indole H_2), 7.30 (1H, d, indole H_7), 7.58 and 7.65 (1H, d, indole H_4), 7.94 and 8.00 (3H, s, NH_3^+), 8.58 and 8.68 (1H, d, NH), 11.02 and 11.08 (1H, s, indole NH).

Procedure E: *N*-(Cycloamino)-αMe(R)Trp-[1-methyl-(*2R,S*)-2-amino-9-((cycloamido)carbonyl)nonanoate] (5). To a solution at 0 °C of the linear precursor **4** (1.5 g, 2.75 mmol) in 550 mL of DMF were subsequently added $NaHCO_3$ (8.25 mmol) and BOP (3.03 mmol). The mixture was stirred for 30 min at 0 °C and for 5 h at rt. After evaporation, 50 mL of EtOAc was added to the residue. The organic phase was washed with an aqueous 10% citric acid solution, H_2O, $NaHCO_3$ solution (10%), H_2O, and brine, dried with Na_2SO_4, filtered, and evaporated. The residual solid was then further purified by flash column chromatography (CH_2Cl_2/MeOH elution (50/1)) and provided **5** (325 mg, 29%): ^{1}H-NMR (DMSO) ∂ 1.10−1.70 (15H, m, $(CH_2)_6$ + α-CH_3), 1.88−2.10 (2H, m, CH_2CO), 2.90, 3.18, 3.24, and 3.68 (2H, d, β-CH_2 (Trp)), 3.54 and 3.60 (3H, s, CH_3), 4.14 and 4.22 (1H, t, α-CH), 6.82−7.00 (2H, m, indole H_5 + H_6), 6.90 and 7.10 (1H, s, indole H_2), 7.24 (1H, d, indole H_7), 7.26 and 7.98 (1H, d, NH), 7.32 and 7.42 (1H, d, indole H_4), 7.64 and 7.80 (1H, s, NH (Trp)), 10.78 and 10.82 (1H, s, indole NH).

Procedure F: N-(Cycloamido)-αMe(R)Trp-[(*2R,S*)-2-amino-9-((cycloamido)carbonyl)nonanoic acid] (6). To a cold (0 °C) solution of the ester **5** (107 mg, 0.26 mmol) in 5 mL of MeOH was added 1 N NaOH (0.77 mL, 3 equiv). The reaction mixture was stirred overnight at rt, the solvents were evaporated, and H_2O was added. The pH was adjusted to 3 with an aqueous 10% citric acid solution and the product extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with an aqueous 10% citric acid solution, H_2O, and brine, dried with Na_2SO_4, filtered, and evaporated to provide quantitatively **6** (103 mg): ^{1}H-NMR (DMSO) ∂ 1.10−1.75 (15H, m, $(CH_2)_6$ + α-CH_3), 1.90−2.15 (2H, m, CH_2CO), 2.92, 3.72, 3.20, and 3.40 (2H, d, β-CH_2 (Trp)), 4.14 and 4.20 (1H, t, α-CH), 6.85−7.04 (2H, m, indole H_5 + H_6), 6.95 and 7.74 (1H, s, indole H_2), 7.10 and 7.95 (1H, d, NH), 7.28 (1H, d, indole H_7), 7.38 and 7.50 (1H, d, indole H_4), 7.68 and 7.82 (1H, s, NH (Trp)), 10.80 (1H, s, indole NH).

Procedure G: [N-(Cycloamido)-αMe(R)Trp-[(*2R,S*)-2-amino-9-((cycloamido)carbonyl)nonanoyl]]-Asp-Phe-NH_2 (8, RB 360). A solution of the benzyl ester (or carbamate) **7** (100 mg, 0.13 mmol) in 7 mL of MeOH was treated overnight with 10% palladium on carbon (60 mg/mmol) at room temperature under hydrogen atmosphere. After filtration through Celite, the filtrate was concentrated to give **8** (68 mg, 77%) as mixture of two diastereomeric isomers after purification by flash column chromatography (EtOAc/CH_2Cl_2/MeOH/H_2O/AcOH elution (220/70/30/6/3)). Anal. ($C_{35}H_{44}N_6O_7$·0.3H_2O) C, H, N.

Separation of the Diastereoisomers. The diastereoisomers of **8** were separated by semipreparative HPLC (column 330 × 7-mm Kromasil C_8; $CH_3CN/H_2O-TFA_{0.05\%}$ = 72:28 (isocratic); flow 2.5 mL/min) to give the optically pure **8a** and **8b**, the latter corresponding to the higher retention time, in TLC.

8a: HPLC ($CH_3CN/H_2O_{TFA-0.05\%}$ = 35/65) t_R = 11.2 min; ^{1}H-NMR (DMSO) ∂ 1.06−1.70 (15H, m, $(CH_2)_6$ + α-CH_3), 1.90−2.05 (2H, m, CH_2CO), 2.18−2.50 (2H, m, β-CH_2 (Asp)), 2.74−3.08 (2H, m, β-CH_2 (Phe)); 2.88 and 3.67 (2H, d, β-CH_2 (Trp)), 3.94 (1H, m, α-CH (Xaa)), 4.22 (1H, m, α-CH (Phe)), 4.36 (1H, m, α-CH (Asp)), 6.87 (1H, t, indole H_5), 6.91 (1H, s, indole H_2), 6.96 (1H, t, indole H_6), 7.04−7.22 (8H, m, ArH (Phe) + NH_2 + NH (Xaa)), 7.25 (1H, d, indole H_7), 7.32 (1H, d, indole H_4), 7.78 (1H, s, NH (Trp)), 8.05 (2H, d, NH (Asp + Phe)), 10.82 (1H, s, indole NH). Anal. ($C_{35}H_{44}N_6O_7$·0.3H_2O) C, H, N.

8b: HPLC ($CH_3CN/H_2O_{TFA-0.05\%}$ = 35/65) t_R = 12.0 min; ^{1}H-NMR (DMSO) ∂ 1.06−1.70 (15H, m, $(CH_2)_6$ + α-CH_3), 1.90−2.15 (2H, m, CH_2CO), 2.33−2.54 (2H, m, β-CH_2 (Asp)), 2.72−3.08 (2H, m, β-CH_2 (Phe)), 3.14 and 3.30 (2H, d, β-CH_2 (Trp)), 4.15 (1H, m, α-CH (Xaa)), 4.28 (1H, m, α-CH (Phe)), 4.59 (1H, m, α-CH (Asp)), 6.87 (1H, t, indole H_5), 6.96 (1H, t, indole H_6), 7.02 (1H, s, indole H_2), 7.04−7.22 (7H, m, ArH (Phe) + NH_2), 7.25 (1H, d, indole H_7), 7.42 (1H, d, indole H_4), 7.60 (1H, d, NH (Xaa)), 7.99 (1H, s, NH (Trp)), 8.18 (2H, d, NH (Asp + Phe)), 11.02 (1H, s, indole NH). Anal. ($C_{35}H_{44}N_6O_7$·0.5H_2O) C, H, N.

Procedure H: (Benzyl)malonyl-αMe(R)Trp-Lys(Z)-OMe (11). To a cold solution (0 °C) of the carboxylic acid, monobenzylmalonate (352 mg, 1.81 mmol), which was prepared as described by Strube[67] in 25 mL of CH_2Cl_2, were subsequently added DCC (2.0 mmol), Et_3N (2.0 mmol), and the amine **10** (1 g, 1.81 mmol), plus one additional equivalent (1.81 mmol) of Et_3N if the amine was used as a salt. The mixture was stirred at 0 °C for 1 h and at rt overnight. After filtration of the *N,N'*-dicyclohexylurea (DCU), the solvent was removed *in vacuo* and 30 mL of EtOAc was added. The organic layer was washed with an aqueous 10% citric acid solution, H_2O, saturated $NaHCO_3$ solution, H_2O, and brine, dried with Na_2SO_4, filtered, and evaporated to yield **11** (1.12 g, 93%): ^{1}H-NMR (DMSO) ∂ 1.10−1.70 (9H, m, $(CH_2)_3$ + α-CH_3), 2.88 (2H, m, β-CH_2 (Lys)), 3.18−3.30 (4H, m, β-CH_2 (Trp) + $COCH_2CO$), 3.54 (3H, s, OCH_3), 4.08 (1H, m, α-CH), 4.92 (2H, s, CH_2 (Bzl)), 5.08 (2H, s, CH_2 (Bzl)), 6.86 (1H, t, indole H_5), 6.96 (1H, t, indole H_6), 7.05 (1H, d, indole H_2), 7.15 (1H, t, NH (Lys)), 7.20−7.38 (11H, m, ArH (Bzl) + indole H_7), 7.42 (1H, d, indole H_4), 7.82 (1H, d, NH (Lys), 7.94 (1H, s, NH (Trp), 10.84 (1H, s, indole NH).

[Cycloamido[malonyl-αMe(R)Trp-Lys]]-Asp-Phe-NH_2 (16, RB 370). **16** (42 mg) was obtained in 96% yield according to procedure G from 50 mg of **15** (0.065 mmol) after purification by flash column chromatography (EtOAc/CH_2Cl_2/MeOH/H_2O/AcOH elution (220/70/30/6/3)): ^{1}H-NMR (DMSO) ∂ 1.10−1.65 (9H, m, $(CH_2)_3$ + α-CH_3), 2.30−2.64 (2H, m, β-CH_2 (Asp)), 2.75−3.56 (8H, m, β-CH_2 (Trp + Phe) + $COCH_2CO$ + CH_2 (Lys)), 4.00 (1H, t, α-CH (Lys)), 4.28 (1H, m, α-CH (Phe)), 4.54 (1H, m, α-CH (Asp)), 6.88 (1H, t, indole H_5), 6.96 (1H, m, indole H_6), 7.04−7.28 (9H, m, ArH (Phe) + indole H_2, H_7 + amide

NH + NH (Lys)), 7.40 (1H, d, indole H_4), 7.48 (1H, s, amide NH), 8.04 (1H, d, NH (Lys)), 8.15 (2H, m, NH (Phe + Asp)), 8.36 (1H, s, NH (Trp)), 11.00 (1H, s, indole NH). Anal. ($C_{34}H_{41}N_7O_8$) C, H, N.

Procedure I: [*S*-(Carbomethoxysulfenyl)-5-thiopentanoyl]-αMe(R)Trp-Cys(4-MeBzl)-Asp(OcHex)-Phe-NH_2 (23). Synthesis of $HS(CH_2)_4COOtBu$. A solution of 5.0 g of $Br(CH_2)_4COOtBu$ (21 mmol) in 10 mL of EtOH was treated with 1.6 g of thiourea (21 mmol) following a procedure described by Urquhart *et al.*[68] The reaction mixture was refluxed for 3 h, a solution of 1.25 g of NaOH in 5 mL of H_2O was then added, and stirring was continued for another 2 h at reflux temperature. After evaporation of the EtOH, ether (30 mL) was added. The organic phase was separated, washed with brine, dried on Na_2SO_4, filtered, and evaporated to dryness to give 2.91 g of pale yellow oil (73%): $HS(CH_2)_4$-COOtBu; R_f = 0.90 (C); ^{1}H-NMR (DMSO) ∂ 1.30 (9H, s, tBu), 1.50 (4H, m, CH_2), 2.10−2.45 (5H, m, CH_2 + SH).

Synthesis of $CH_3OOC-S-S-(CH_2)_4COOtBu$. To a cold (0 °C) solution of $HS(CH_2)_4COOtBu$ (1.5 g, 7.9 mmol) in 50 mL of MeOH was added at once 1.0 g of carbomethoxysulfenyl chloride (Scm-Cl, 7.9 mmol).[47] The reaction mixture was stirred for 3 h at rt, the solvent was then removed *in vacuo*, and the residue was purified by flash column chromatography (100% CH_2Cl_2 elution) to give 2.36 g of a colorless oil (95%): $CH_3OOC-S-S-(CH_2)_4COOtBu$; R_f = 0.75 (A); ^{1}H-NMR (DMSO) ∂ 1.35 (9H, s, tBu), 1.52 (4H, m, CH_2), 2.25 (2H, t, CH_2), 2.75 (2H, t, CH_2), 3.80 (3H, s, CH_3).

Synthesis of $CH_3OOC-S-S-(CH_2)_4COOH$. A 600 mg sample of $CH_3OOC-S-S-(CH_2)_4COOtBu$ (1.9 mmol) was stirred at 0 °C with a mixture of TFA/CH_2Cl_2 (1 mL/2 mL) for 2 h and at rt, and then solvents were evaporated to give in quantative yield a colorless oil: $CH_3OOC-S-S-(CH_2)_4COOH$; R_f = 0.38 (C); ^{1}H-NMR (DMSO) ∂ 1.50 (4H, m, CH_2), 2.15 (2H, t, CH_2), 2.75 (2H, t, CH_2), 3.78 (3H, s, CH_3).

Synthesis of 23. To a cold solution (0 °C) of **22** (1.23 g, 1.39 mmol) in 5 mL of DMF were subsequently added DIEA (0.27 mL, 1.53 mmol), DCC (316 mg, 1.53 mmol), and CH_3-$OOC-S-S-(CH_2)_4COOH$ (344 mg, 1.53 mmol). The mixture was stirred at 0 °C for 1 h, and after the mixture was warmed to rt, stirring was continued overnight. After filtration of the DCU, the solvent was removed *in vacuo* and 50 mL of EtOAc were added. The organic layer was washed with an aqueous 10% citric acid solution, H_2O, saturated $NaHCO_3$ solution, H_2O, and brine, dried with Na_2SO_4, filtered, and evaporated. The residual solid was further purified by flash column chromatography (CH_2Cl_2/MeOH elution (100/3)) to yield 936 mg of **23** (69%) as a white solid: ^{1}H-NMR (DMSO) ∂ 1.10−1.75 (21H, m, cHex + $(CH_2)_4$ + α-CH_3), 2.20 (3H, s, CH_3), 2.55−3.55 (8H, m, β-CH_2), 3.60 (2H, s, CH_2(Bzl)), 3.80 (3H, s, OCH_3), 4.20−4.65 (5H, m, α-CH + OCH), 6.85−7.55 (17H, m, ArH + NH_2 + NH), 8.08 (1H, d, NH), 8.20 (2H, m, NH), 10.90 (1H, s, indole NH).

Procedure J: [Cyclo-*S,S*-[(5-thiopentanoyl)-αMe(R)-Trp-Cys]]-Asp-Phe-NH_2 (24, RB 380). **23** (800 mg, 0.82 mmol) was treated at 0 °C with 15 mL of anhydrous hydrogen fluoride (HF) in the presence of 0.8 mL of anisole during 75 min following the procedure of Sakakibara *et al.*[48] After removal of the HF by vacuum distillation, the residue was precipitated and washed with Et_2O. Purification by flash chromatography (CH_2Cl_2/MeOH/AcOH elution (18/2/1)) gave **24** (1.55 mg, 27%) as a white solid: ^{1}H-NMR (DMSO) ∂ 1.25 (3H, s, α-CH_3), 1.54−1.75 (4H, m, CH_2), 2.10−2.24 (2H, m, CH_2), 2.35−3.45 (10H, m, β-CH_2 + CH_2), 4.30 (2H, m, α-CH), 4.48 (1H, m, α-CH), 6.90 (1H, t, indole H_5), 6.98 (1H, m, indole H_6), 7.03 (1H, s, indole H_2), 7.05−7.23 (7H, m, ArH (Phe) + NH_2), 7.28 (1H, d, indole H_7), 7.40 (1H, d, indole H_4), 7.84 (1H, s, NH), 7.92 (1H, d, NH), 8.05 (1H, s, NH (Trp)), 8.25 (1H, d, NH), 10.88 (1H, s, indole NH). Anal. ($C_{33}H_{40}N_6O_7S_2$) C, H; N: calcd, 12.06; found, 12.67.

Procedure K: *N*-(Benzyloxycarbonyl)-(2*S*)-2-amino-9-(*tert*-butyloxycarbonyl)nonanoic Acid (25). Synthesis of the Sultam Intermediate. According to Josien *et al.*,[51] ((−)-10,2-bornane)sultam *N*-(diphenylmethylene)glycinate (the chiral inductor of Oppolzer[50]) (5.51 g, 12.6 mmol) was solubilized in 50 mL of THF, and 9.47 mL of nBuLi (1.6 M/hexane, 15.2 mmol) was added at −78 °C. The mixture was stirred for 1 h while being warmed to −55 °C. Afterward *tert*-butyl 8-bromooctanoate (6.7 g, 25.3 mmol) and HMPA (12.0 mL, 68 mmol) in 40 mL of THF were added, and stirring was continued at rt for 16 h. The pH was adjusted to 6 with a solution of acetic acid in THF (1 mL/4 mL). Et_2O (200 mL) was added, and the organic phase was washed successively with saturated solutions of NH_4Cl, $NaHCO_3$, water, and brine, dried over Na_2SO_4, and evaporated to dryness. The residue was redissolved in a solution of aqueous 10% citric acid and THF (70 mL/70 mL). After the mixture was stirred for 48 h at rt, the solvents were evaporated, and a mixture of H_2O and Et_2O was added. The aqueous layer was basified with $NaHCO_3$ (pH = 8) and extracted with EtOAc. The organic phase was then washed with H_2O and brine, dried over Na_2-SO_4, and evaporated to dryness. The residue was further purified by chromatography using CH_2Cl_2/MeOH (100/2) elution to yield 2.88 g of a yellow oil (50%): ((−)-10,2-bornane)-sultam (2*S*)-2-amino-9-(*tert*-butyloxycarbonyl)nonanoate: R_f = 0.50 (D); ^{1}H-NMR (DMSO) ∂ 0.88 (3H, s, CH_3), 0.98 (3H, s, CH_3), 1.10−1.90 (31H, m, CH_2 + CH + tBu + NH_2), 2.08 (2H, t, CH_2), 3.58−3.80 (3H, m, α-CH + CH_2).

N-Protection of the Sultam Intermediate. A solution of 5.6 g of ((−)-10,2-bornane)sultam (2*S*)-2-amino-9-(*tert*-butyloxycarbonyl)nonanoate (12.3 mmol) in 115 mL of THF was treated at 0 °C with 1.62 g of benzyl chloroformate (13.5 mmol) and 2.36 g of Et_3N (13.5 mmol). The mixture is stirred at rt for 2 h. After filtration and evaporation to dryness, the residue was redissolved in EtOAc and the organic phase washed with an aqueous 10% citric acid solution, H_2O, a saturated solution of $NaHCO_3$, H_2O, and brine, dried over Na_2-SO_4, and evaporated to dryness to give 8.21 g of a colorless oil (99%): ((−)-10,2-bornane)sultam *N*-(benzyloxycarbonyl)-(2*S*)-2-amino-9-(*tert*-butyloxycarbonyl)nonanoate: R_f = 0.28 (hexane/acetone = 8/2); ^{1}H-NMR (DMSO) ∂ 0.88 (3H, s, CH_3), 0.98 (3H, s, CH_3), 1.10−1.90 (29H, m, CH_2 + CH + tBu), 2.08 (2H, t, CH_2), 3.54−3.80 (2H, m, CH_2), 4.50 (1H, m, α-CH), 4.98 (2H, s, CH_2), 7.30 (5H, m, ArH), 7.54 (1H, d, NH).

Synthesis of 25. A solution of 8.2 g of ((−)-10,2-bornane)-sultam *N*-(benzyloxycarbonyl)-(2*S*)-2-amino-9-(*tert*-butyloxycarbonyl)nonanoate (13.9 mmol) in 40 mL of THF was treated with 36 mL of 0.5 N LiOH (18.1 mmol) at rt for 4 h. After evaporation to dryness, EtOAc was added and the organic phase was washed with an aqueous 10% solution of citric acid, H_2O, and brine. Then the organic phase was dried over sodium sulfate and evaporated to dryness. The resulting residue was purified by chromatography using CH_2Cl_2/MeOH (100/2) elution to give **25** (2.66 g, 55%) as a colorless oil: *N*-(benzyloxycarbonyl)-(2*S*)-2-amino-9-(*tert*-butyloxycarbonyl)-nonanoic acid: ^{1}H-NMR (DMSO) ∂ 1.10−1.60 (21H, m, CH_2 + tBu), 2.08 (2H, t, CH_2), 3.85 (1H, m, α-CH), 4.98 (2H, s, CH_2), 7.28 (5H, m, ArH), 7.48 (1H, d, NH).

[N-(Cycloamido)-αMe(R)Trp-[(2S)-2-amino-9-((cycloamido)carbonyl)nonanoyl]]-Asp-Phe-NH_2 (43). **40** (35 mg, 0.047 mmol) was treated at 0 °C with 15 mL of anhydrous HF and 0.4 mL of anisole during 75 min following procedure J. After removal of the HF by vacuum distillation, the residue was precipitated and washed with Et_2O. Purification by semipreparative HPLC (column 330 × 7-mm Kromasil C_8; CH_3-$CN/H_2O-TFA_{0.05\%}$ = 72:28 (isocratic); flow 2.5 mL/min) gave 25 mg of the final product **43** (81%) showing the same physical characteristics as does **8b**.

[N-(Cycloamido)-αMe(R)Trp-[(2S)-2-amino-8-((cycloamido)carbonyl)octanoyl]]-Asp-Phe-NH_2 (44). Compound **44** was obtained in the same way as **43**: ^{1}H NMR (DMSO) ∂ 1.06−1.65 (13H, m, $(CH_2)_5$ + α-CH_3), 1.95−2.10 (2H, m, CH_2-CO), 2.60−2.80 (2H, m, β-CH_2 (Asp)), 2.75−3.00 (2H, m, β-CH_2 (Phe)), 3.20 and 3.41 (2H, d, β-CH_2 (Trp)), 4.10 (1H, m, α-CH (Xaa)), 4.35 (1H, m, α-CH (Phe)), 4.50 (1H, m, α-CH (Asp)), 6.87 (1H, t, indole H_5), 6.96 (1H, t, indole H_6), 7.02 (1H, s, indole H_2), 7.04−7.22 (7H, m, ArH (Phe) + NH_2), 7.25 (1H, d, indole H_7), 7.42 (1H, d, indole H_4), 7.55 (1H, d, NH (Phe)), 7.80 (1H, d, NH (Xaa)), 7.95 (1H, s, NH (Trp)), 8.25 (1H, d, NH (Asp)), 10.80 (1H, s, indole NH). Anal. ($C_{34}H_{42}N_6O_7$) C, H, N.

[*N*-(Cycloamido)-αMe(R)Trp-[(2*S*)-2-amino-10-((cycloamido)carbonyl)-decanoyl]]-Asp-Phe-NH_2 (45). Compound

45 was obtained in the same way as **43**: ^{1}H NMR (DMSO) ∂ 1.10−1.65 (17H, m, $(CH_2)_7$ + α-CH_3), 2.10 (2H, m, CH_2CO), 2.40−3.40 (6H, m, β-CH_2 (Asp + Phe + Trp)), 4.10−4.60 (3H, m, α-CH (Asp + Phe + Xaa)), 6.90 (1H, t, indole H_5), 6.95 (1H, t, indole H_6), 7.10−7.20 (8H, m, indole H_2 + ArH (Phe) + NH_2), 7.25 (1H, d, indole H_7), 7.40 (1H, d, NH (Phe)), 7.60 (1H, d, NH (Xaa)), 7.90 (1H, s, NH (Trp)), 8.20 (1H, d, NH), 10.85 (1H, s, indole NH). Anal. ($C_{36}H_{46}N_6O_7$·$0.3H_2O$) C, H, N.

***In Vitro* Binding Assays.** [^{3}H]pCCK_8 (specific activity 60 Ci/mmol) was purchased from Amersham. Brain cortex and pancreas homogenates were prepared from guinea pig as previously described.[20] Protein concentration was estimated using the Pierce bicinchoninic acid protein assay reagent with bovin serum albumin as a standard. Incubations (final volume 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). [^{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. Nonspecific 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.

Cell Growth, Transfection, and Transformant Cell Selection. Chinese hamster ovary (CHO) cells were grown in HAM-F12 medium containing 10% fetal calf serum, 50 μg/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 μg of the pcDNA3/RKB vector, using the calcium phosphate method.[70] 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 by the limit dilution method.

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 100000*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 $MgCl_2$ containing 0.02% of bacitracin. The membranes were aliquoted and frozen at −80 °C. The binding assays were performed as with brain membranes with 40 μg of protein/tube and 0.4 nM of [^{3}H]pCCK_8.

Inositol Phosphate Assays. CHO cells stably expressing wild-type CCK-B rat receptor were assayed for agonists stimulated membrane phosphoinositides hydrolysis essentially as previously described.[70] Cells were plated in 24-well microtiter plates. Before confluency cells were grown in the presence of 1 μCi/mL myo-[γ-^{3}H]inositol for 16 h at 37 °C. Cells were treated with 10 mM LiCl for 30 min at 37 °C, and various concentrations of agonists were then added to the cells. After 45 min at 37 °C, the incubation medium was removed and the cells were washed twice with 1 mL of PBS. The reaction was stopped by adding 200 μL of ice-cold 67% methanol and 300 μL of 0.125% Triton. The cells were scraped, the wells were then rinsed with 200 μL of ice-cold 67% methanol, and the suspension was subjected to chloroform extraction. Then 0.5 mL of the aqueous phase was added to 4.5 mL of water. The column of 0.5 mL anion exchange resin (Dowex AG1 × 8 200−400 mesh from BioRad) 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 the compounds.

***In Vivo* Binding Assays.** The experiments with icv and iv injections were performed as previously described.[52] Briefly, mice were killed by cervical dislocation 15 min after icv injection of [^{3}H]pBC 264 (10 pmol, 1 μCi) alone or with **8b**, and their brains were quickly removed. A delay of 15 min was chosen, as previous studies showed that specific binding of [^{3}H]-pBC 264 is maximum at this time.[52] The brain (minus cerebellum) was homogenized in 10 mL of cold 50 mM Tris-HCl buffer containing 0.02% bacitracin. Aliquots (0.15 mL) of the homogenate were immediately filtered through Whatman GF/B glass filters and rinsed twice with 5 mL of cold buffer. Free radioactivity was calculated as the difference between total radioactivity and radioactivity retained on the filters, which was considered as bound radioactivity. **8b** was prepared as a stock solution in ethanol/Chremophor EL/H_2O (1/1/8) and diluted in saline before icv coinjection with [^{3}H]-pBC 264 or iv administration at different concentrations 5 min prior to [^{3}H]pBC 264.

Gastric Acid Secretion. Experiments were performed using male Sprague−Dawley rats weighting 300 ± 25 g, fasted for 18 h with free access to water.[71] The technical aspects of the operation have been described by Ghosh and Schild[72] and modified by Lai.[73] Briefly, the rats were anesthetized by intramuscular injection of urethane (0.6−0.7 mL of 25% solution per 100 g of body weight). A polyethylene catheter introduced in the oesophagus and passed to the cardia 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.

The tests started after stabilization of the gastric perfusion, which was achieved usually between 30 and 60 min after completion of the surgical preparation. During this period, an intravenous infusion of physiological saline at 2.4 mL/min (perfusor Braun, Roucaire) was made into the dorsal vein of the penis. Stimulation by the tested compounds was obtained by continuous infusion through the same route diluted in saline as described.[37] Gastric secretion was collected every 15 min, and the H^+ levels were evaluated by titrating the entire sample with 0.01 N NaOH to pH 7. The results are expressed as a percentage (mean of the two experimental points preceding administration of the antagonist) of gastric acid output induced by each compound. Statistical evaluations were performed using Student's *t*-test.

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