DOI: 10.1002/cmdc.200600054

Conformationally Constrained CCK8 Analogues Obtained from a Rationally Designed Peptide Library as Ligands for Cholecystokinin Type B Receptor

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*A library of 14 cyclic peptide analogues derived from the octapeptide C-terminal sequence of the human cholecystokinin hormone (CCK(26–33), or CCK8) was designed, synthesized, and characterized. The 14 peptide analogues were rationally designed to specifically interact with the CCK type B receptor (CCK_B-R) on the basis of the structure of the bimolecular complex between CCK8 and the third extracellular loop of CCK_B-R, namely CCK_B-R-(352–379). The rational design of new ligands for CCK_B-R has relied on stabilization by cyclic constraints of the structural motifs that bring the key residues of the ligand (especially Trp 30, Met 31, and Phe 33) in the proper spatial orientation for optimal interaction with the receptor. The binding affinity of the new ligands for CCK_B-R was assessed by displacement experiments of ^{111}In-radiolabeled CCK8 in cells that overexpress the CCK_B receptor. The new ligands generally showed binding affinities lower than that of parent CCK8, with the best compounds having IC_{50} values around 10 μM. Structure–activity relationship data show that preservation of the Trp 30–Met 31 motif is essential and that the Phe 33 side chain must be present. NMR conformational studies of the compound with maximal binding affinity (*cyclo-*B11*, $IC_{50} = 11$ μM) in DPC micelles shows that this compound presents a turn-like conformation centered at the Trp 30–Met 31 segment, as planned by rational design. Such a conformation is stabilized by its interaction with the micelle rather than by the cyclic constraint.*

Introduction

There is considerable interest in the pharmacology of the two cholecystokinin (CCK) receptors CCK_A-R (or CCK-1) and CCK_B-R (or CCK-2) that mediate the biological action of the CCK hormone.[1] CCK_A-R and CCK_B-R are membrane bound receptors belonging to the superfamily of G-protein-coupled receptors (GPCRs) and are predominantly located in the gastrointestinal tract and the central nervous system, respectively. Many efforts have been put into developing selective CCK analogues with either agonist or antagonist activity.[2–6] Most of them are based on the fact that the CCK C-terminal octapeptide (CCK-(26–33), or CCK8) is the main determinant for high affinity receptor binding, and that modifications of the octapeptide can alter its binding properties, thus introducing a selectivity for binding to either the type A or the type B receptor subtype.[7–11] Only in recent years has high-resolution structural information on the bimolecular complexes between the CCK8 ligand and receptor fragments appeared in literature: the NMR structure of the bimolecular complex between CCK8 and the N-terminal extracellular loop of the CCK_A receptor (fragment CCK_A-R(1–47)) has been solved by Pellegrini and Mierke.[12] Later, the same research group published the NMR structure of the complex formed by CCK8 with the third extracellular loop (EL3) of the CCK_B receptor.[13] These studies indicate a slightly different binding mode of CCK8 with the two receptor subtypes. CCK8 binds to the CCK_A receptor such that the C terminus of the ligand is accommodated within the seven-helix bundle and the N terminus projects out between the transmembrane α helices TM1 and TM7, thereby forming specific interactions with the N terminus of the CCK_A receptor. In contrast, CCK8 binds to the CCK_B receptor by interacting specifically with residues belonging to the third extracellular loop, CCK_B-R(352–379).[14] Recently published fluorescence spectroscopy

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studies have analyzed the interaction of CCK4 (a tetrapeptide corresponding to fragment 30–33 of native CCK) and CCK8 with both type A and type B CCK receptors.[15] These studies provided direct evidence that CCK8 binds to the highly homologous type A and type B CCK receptors in distinct manners, even though binding affinities and potencies are identical. Namely, the amino terminus of CCK8 is more exposed to the aqueous milieu when bound to the type B CCK receptor than when bound to the type A CCK receptor. An analogous binding topology in the bimolecular complex between CCK4 and the CCK_B receptor has been hypothesized. This could explain why modifications at the CCK4 N terminus with a fluorescent substituent is tolerated in the case of binding to the type B CCK receptor, whereas the same structural modification negatively affects CCK4 binding to the type A CCK receptor (in this case, steric hindrance is thought to be detrimental to binding).[15] The structural details of the CCK8–CCK_A-R complex allowed us to design a new CCK8 analogue[16] characterized by a cyclic skeleton introduced to stabilize the bioactive conformation of the peptide. Such cyclic analogues show selective binding properties toward CCK_A-R. We have now used a similar rational approach, based on the structural details of the complex between CCK8 and the CCK_B receptor fragment, to design a library of peptide ligands with binding properties toward CCK_B-R. Herein, we describe the design and synthesis of a peptide library that has been rationally designed to allow us to identify the structural requirements needed for preferential binding to the CCK_B-R receptor. Conformational NMR studies on the most active peptide within this library are also reported.

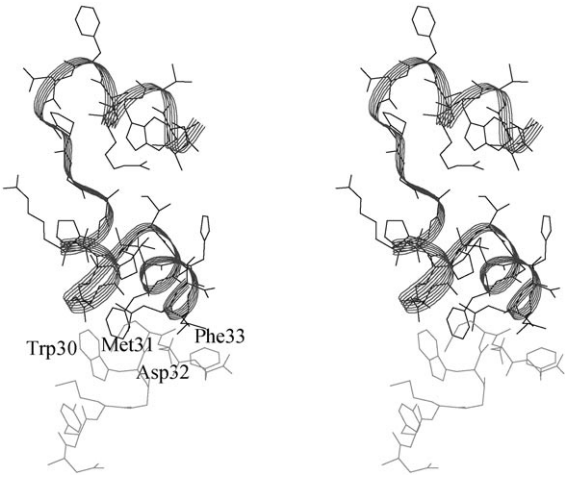

Figure 1. Stereographic view of the molecular model of the CCK8–CCK_B-R-(352–379) complex from reference [5]. CCK_B-R(352–379) is shown in ribbon representation. Some CCK8 residues relevant for the interaction are labeled.

Results and Discussion

Peptide design

The starting point for the rational design of CCK8 peptidomimetic analogues was the NMR structure of the complex between CCK8 and the third extracellular loop (EL3) of the cholecystokinin type B receptor, CCK_B-R(352–379) (Figure 1), resolved by Giragossian and Mierke.[14] The coordinates of the complex were provided by Professor D. F. Mierke. The residue numbering scheme of CCK8, and of its new analogues, is based on the numbering of the 33-mer natural cholecystokinin, with CCK8 as its C-terminal end (encompassing residues 26–33). As the first step of the modeling procedure, we identified those residues of CCK8 which make contacts with the CCK_B-R(352–379) receptor fragment using structural analysis. The structure of the complex placed the pseudohelix of CCK8 nearly perpendicular to the C-terminal helix (TM7) of the receptor fragment, with the C-terminal Phe 33 residue directed toward the N-terminal helix (TM6). The side chains of the Trp 30, Met 31, and Phe 33 residues of the ligand were observed to intercalate in a hydrophobic pocket formed by the side chains of the P371, S373, F374, L377, and L378 residues in the C-terminal helix (TM7) of the receptor fragment. The experimentally based model of the CCK8–CCK_B-R(352–379) complex, proposed by Mierke, underscores that the complex is further stabilized by other hydrophobic and coulombic interactions. In particular, the hydrophobic interactions involve the side chains of Met 31 and Phe 33, while the coulombic interactions involve the Asp 32 residue. The complex is stabilized by H-bond interactions involving Tyr 27 OH, Asp 32 $C^{\gamma}O$, Asp 32 CO, and Phe 33 NH_2 of CCK8. The CCK8 conformation is also partially stabilized by a weak intramolecular 4→1 H-bond, with the formation of a β-turn structure between Gly 29 CO and Asp 32 NH. In conclusion, this analysis shows that the essential contact region between the CCK8 and the CCK_B-R(352–379) involves the Trp 30, Met 31, and Phe 33 residues of the ligand. This analysis is supported by fluorescence spectroscopy studies of the interaction between CCK4 and CCK8 with both type A and type B CCK receptors.[15] As the second step, rational design of peptidomimetic analogues was carried out according to the following criteria: 1) to preserve the major interactions between the Trp 30–Phe 33 tract of the ligand and the CCK_B-R(352–379) receptor fragment, 2) to stabilize the bioactive turn-like conformation in the Trp 30–Phe 33 region, and 3) to preserve as far as possible any further interactions between the ligand and the whole receptor. As a result, we have designed the four classes of cyclic peptidomimetic molecules that are sketched in Figure 2. In the first class of compounds (*cyclo*-B1 and *cyclo*-B2), we substituted Gly 29 of the CCK8 peptide with a diaminopropionic acid (Dap) residue, allowing introduction of a cyclic constraint between the $C^{\gamma}O$ of the Asp 32 side chain and the NH of the Dap side chain. This cyclic constraint is expected to preserve the spatial orientation of the side chains belonging to the CCK8 C terminus (Phe 33), yet not modify the bioactive turn conformation of the Trp 30–Met 31 residues. Furthermore, the residues preceding the Dap residue, that, in principle, are not involved in binding with CCK_B-R(352–379), were deleted. Two variants have been synthesized, one having a free N terminus (*cyclo*-B1), and the other acetylated on the N terminus (*cyclo*-B2). In the second class of compounds (*cyclo*-B7–*cyclo*-B10), we substituted Gly 29 in the CCK8 peptide with either an L-Cys (*cyclo*-B7 and *cyclo*-B9) or a D-Cys residue (*cyclo*-B8 and *cyclo*-B10), and Asp 32 with a L-cysteine residue to allow the

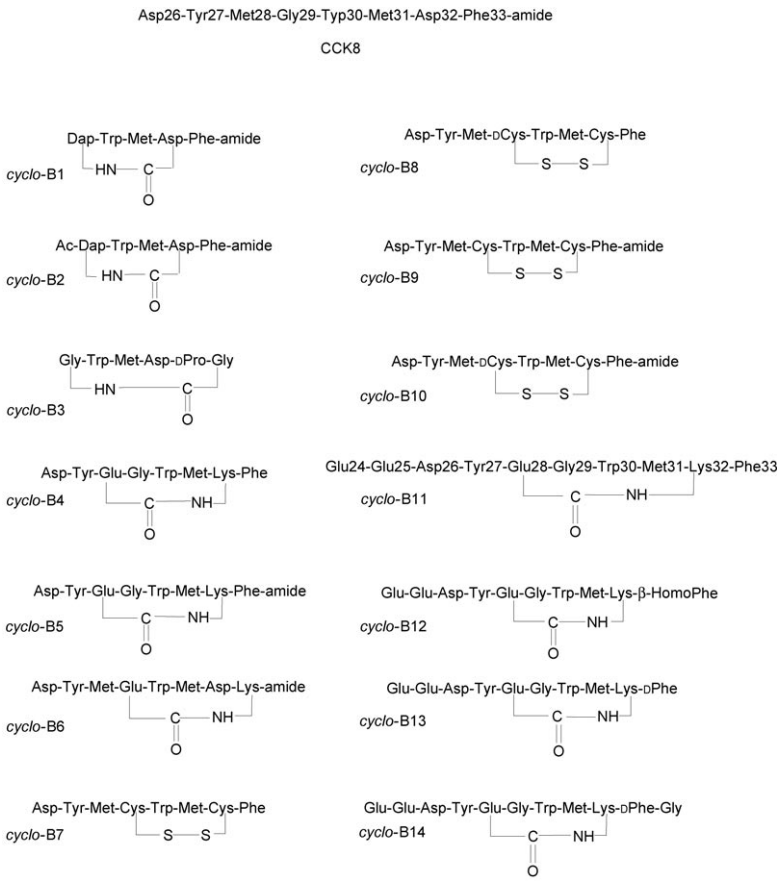

Figure 2. Amino acid sequence of the CCK8 peptide and schematic representation of the 14 CCK8 cyclic peptide analogues. The residue-numbering scheme for CCK8 and *cyclo*-B11, based on the numbering of the 33 residues of natural cholecystokinin, is also shown.

formation of a disulphide bridge. Two sets of compounds were synthesized having the C terminus in the free carboxylic form (*cyclo*-B7 and *cyclo*-B8) or in the amide form (*cyclo*-B9 and *cyclo*-B10). In the third class (*cyclo*-B4, *cyclo*-B5, and *cyclo*-B11–*cyclo*-B14), we have substituted Met 28 in the CCK8 peptide with a Glu residue, and Asp 32 with a Lys residue. This permits formation of an amide bond between the side chains of the Lys and Glu residues. Within this class, compound *cyclo*-B4 has a free carboxyl C terminus, whereas compound *cyclo*-B5 has the C terminus in the amide form. Compounds *cyclo*-B11–*cyclo*-B14 were designed to assess: 1) the effect of the positioning of the Phe 33 side chain and 2) the capability of the C terminus to restore the coulombic interactions that are lost because of the replacement of Asp 32 with a Lys residue. Regarding the role of Phe 33, the residue has been replaced by β-HomoPhe (*cyclo*-B12), by D-Phe (*cyclo*-B13), or by a D-Phe-Gly dipeptide (*cyclo*-B14). Moreover, two Glu residues in the N-terminal part of the ligand have been introduced to improve the solubility of the *cyclo*-B11–*cyclo*-B14 compounds. The fourth class of molecules (*cyclo*-B3 and *cyclo*-B6) was designed to conserve the Trp 30–Asp 32 tract using two different strategies to form a cyclic structure. In the *cyclo*-B3 molecule, the CCK8 sequence was truncated at the Gly 29 residue, the Phe 33 was replaced with a D-Pro, and a Gly residue was added after the D-Pro. Then, the ring structure was closed by formation of a peptide bond between the N- and C-terminal amino acid residues. In the *cyclo*-B6 molecule, the Gly 29 residue in the CCK8 peptide was substituted with a Glu residue and the Phe 33 with a Lys residue, with the aim of performing a N↔C cyclization between the side chains of the Lys and Glu residues. In both cases, the constraint introduced by cyclization is expected to stabilize the bioactive conformation of the Trp 30–Asp 32 tract. The models of the complexes between all new molecules and the CCK_B-R(352–379) receptor were energy minimized to refine the structures with a two-step procedure. First, all compounds were energy minimized to eliminate "hot spots" introduced in the design phase, keeping the residues of the receptor at a fixed position. Then, the restraints were removed and further energy minimizations were performed. The structures obtained were then superimposed onto the NMR structure of CCK8 to verify whether they could maintain the network of interactions with the receptor as predicted by the design procedure. The minimized models kept the desired interactions despite some distortions due, partially, to the cyclic constraints introduced in the molecules.

Peptide synthesis

A 14-membered library consisting of different cyclic peptides was synthesized on solid phase by using Fmoc chemistry standard protocols. Different solid supports were employed to construct the peptidic moieties and different types of reaction were performed to obtain the cyclic peptides. Rink Amide resin was employed to synthesize *cyclo*-B1, *cyclo*-B2, *cyclo*-B5, and *cyclo*-B6 bearing an amido group at the C terminus. The N↔C cyclization reaction was performed on resin after removal of the Mtt and 2-Ph*i*Pr protecting groups from Dap and Asp, respectively, for *cyclo*-B1 and *cyclo*-B2, and from Lys and Glu, respectively, for *cyclo*-B5 and *cyclo*-B6. *Cyclo*-B3 was synthesized by using the superacid-labile 2-chlorotrityl resin, to obtain the whole peptide completely protected upon cleavage from the resin. The cyclization reactions were performed in solution between the N- and C-terminal amino acid residues of the fully protected peptides. Wang resin was used to prepare *cyclo*-B4, *cyclo*-B11, *cyclo*-B12, *cyclo*-B13, and *cyclo*-B14, as this solid support releases peptide acids upon treatment with a

high percentage of trifluoroacetic acid. The N↔C cyclization was performed on resin between the side chains of Lys and Glu residues, as previously described for *cyclo*-B5 and *cyclo*-B6.

Cyclo-B7 and *cyclo*-B8 were obtained using Wang resin as solid support, while *cyclo*-B9 and *cyclo*-B10 were synthesized on Rink Amide resin. All the cyclic peptides were obtained by disulphide bridge formation between two Cys residues. The oxidation reaction was performed under basic conditions. The final yields for all compounds were in the range of 30–40%.

Biological assays

A preliminary biological assay was performed to verify the binding ability of the cyclic CCK8 analogues *cyclo*-B1–*cyclo*-B14 towards the entire CCK_B receptor expressed in cultured cells. The binding measurements were obtained by competition with the labeled linear peptide ^{111}In]DTPAGlu-Gly-CCK8 (DTPA-Glu = *N*,*N*-bis[2-[bis(carboxyethyl)amino]ethyl]-L-glutamic acid), which displays a high affinity for the CCK_B receptor (K_d = 10^{-8} M).[17] The data obtained (Figure 3) show a typical pattern

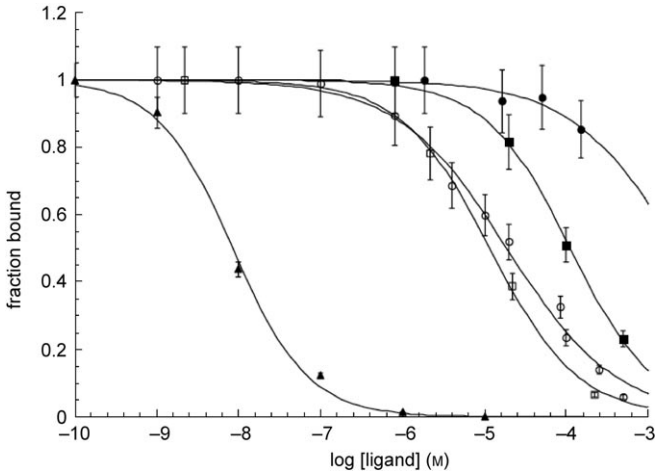

Figure 3. Competitive binding of *cyclo*-B-selected compounds and the CCK8 agonist ^{111}[In]DTPAGlu-CCK8: *cyclo*-B1, ●; *cyclo*-B4, ■; *cyclo*-B8, ○; *cyclo*-B11, □; CCK8, ▲.

of competitive interaction with a decrease in binding of the radiolabeled tracer at relatively high concentrations of unlabeled CCK8, used as control. Quantitative analysis of the data yielded the 50% inhibitory concentration (IC_{50}). The IC_{50} values for each compound are reported in Table 1. These values indicate that all compounds are much less active than the endogenous CCK8 (IC_{50} of CCK8 is in the low nanomolar range). However, use of the IC_{50} values of the whole series of 14 peptides allows us to identify the factors underlying binding affinity to CCK_B-R. The first observation concerns peptides of the first class of compounds (*cyclo*-B1 and *cyclo*-B2). These peptides are substantially inactive in CCK_B-R binding even though the cyclic constraint does not affect the bioactive turn-like conformation of the Trp 30–Met 31 residues. If it is considered that the C-terminal part of these peptides has not been changed with respect to the parent CCK8, it can be inferred that the binding

Table 1. IC_{50} values for the *cyclo*-B compounds.

Compd	IC_{50} [μM]	SE [μM]
CCK8	0.019	±0.002
cyclo-B1	7351	±7
cyclo-B2	>10000	–
cyclo-B3	>10000	–
cyclo-B4	112	±1
cyclo-B5	41	±1
cyclo-B6	278	±1
cyclo-B7	17	±3
cyclo-B8	38	±1
cyclo-B9	271	±12
cyclo-B10	185	±3
cyclo-B11	11	±2
cyclo-B12	39	±1
cyclo-B13	9	±1
cyclo-B14	9	±2

affinity is very dependent on the presence of the segment preceding Dap 29. As a matter of fact, compound *cyclo*-B3 (fourth class) which also has a large truncation of the N terminus, shows virtually no binding.

The lower values for the free carboxyl C terminus peptides (*cyclo*-B7 and *cyclo*-B8) with respect to the amidated analogues (*cyclo*-B9 and *cyclo*-B10) suggest the importance of a negatively charged group close to the C-terminal portion of the molecule in this class of compounds. In fact, the negative charge due to the Asp 32 side chain of these analogues is lost with respect to CCK8 because of its substitution with the noncharged Cys residue, moreover the partially polar amide bond obtained by N–C side-chain cyclization for all the other compounds is absent in this class. The four peptides *cyclo*-B11–*cyclo*-B14 belonging to the third class are the most active analogues of the entire library, as IC_{50} values are in the lower μM range. All of them contain a free carboxyl at the C terminus and are characterized by an elongation at the N terminus with charged Glu residues. The different orientation and backbone distance of the Phe aromatic ring and of a free carboxylic function at the C terminus (see *cyclo*-B11–*cyclo*-B14) only affect their binding properties slightly. Compounds *cyclo*-B4 and *cyclo*-B5, lacking the hydrophilic N-terminus elongation, present a slightly lower affinity. Finally, IC_{50} values for compounds belonging to the fourth class indicate that the absence of the aromatic Phe 33 residue substantially decreases the binding affinity. On the basis of the results reported herein, the structure of *cyclo*-28,32[Glu 24, Glu 25, Glu 28, Lys 32]-CCK8 (*cyclo*-B11) was resolved using NMR, as this peptide is representative of the class of peptides showing maximal binding affinity. The resulting experimental model has then been compared with that obtained by theoretical methods according to the rational design procedure.

^{1}H NMR studies

The NMR spectra of *cyclo*-B11 in aqueous solution (peptide 3.0 mM, H_2O/D_2O 95%, pH 6.4, Figure 4 A) are generally characterized by sharp resonances within the temperature range

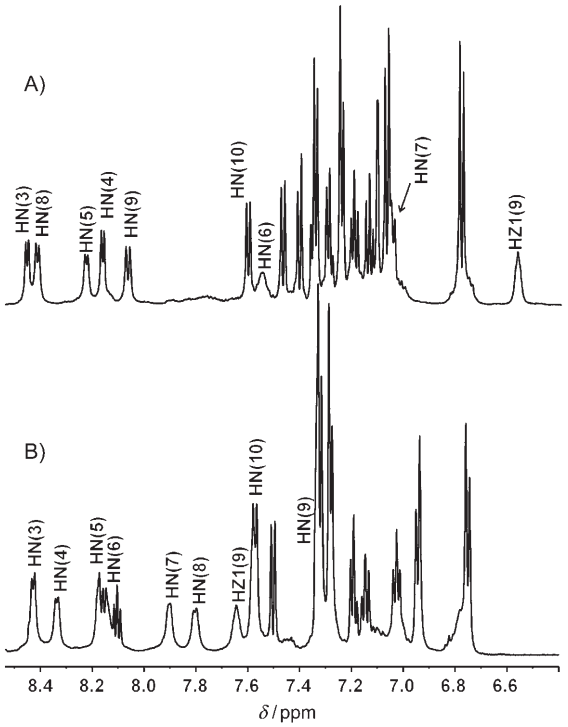

Figure 4. Expansion of the aromatic/amide regions of the ^{1}H NMR spectra of *cyclo*-B11 (3.0 mM, H_2O/D_2O 94%, pH 6.4, $T = 298$ K) in A) aqueous solution and B) in the presence of 170 mM [D_{38}]DPC. The assignment of HN amide protons is shown.

285–305 K. In particular the resonances of the HN protons within segment 25–32 (except that of Gly 29) are sharp enough to clearly show the multiplet structure, from which $^3J_{HN-H^\alpha}$ coupling constants in the range 6.1–9.2 Hz can be directly read. A small number of NOE interactions (63) can be found in the NOESY spectrum (mixing time 400 ms), most of them being intra-residue (37) or sequential (24). This indicates that the structure is flexible in these conditions. ROESY experiments (with mixing times up 400 ms) provide no correlations other than those found in NOESY spectra. The spectral features of the peptide change remarkably when [D_{38}]DPC (fully deuterated dodecylphosphocholine) at a concentration higher than that of the critical micelle concentration (CMC) is added. The ^{1}H NMR spectrum of *cyclo*-B11 in the presence of 170 mM [D_{38}]DPC (peptide 3.0 mM, H_2O/D_2O 95%, pH 6.4) is shown in Figure 4B. On going from aqueous solution to micelles, the line-width of the proton resonances (especially those of backbone HN protons) increases and the signals of the amide protons belonging to segment 29–32 (including the side chain amide proton of Lys 32) are remarkably shifted. These features strongly indicate that the peptide interacts with the micelle. The NOESY spectra of *cyclo*-B11 give more intense and more abundant cross-peaks than found in neat water, consistent with the lengthening of τC because of the peptide–micelle interaction and the stabilization of the peptide in a preferred conformation. A single set of resonances for each proton is detected within the temperature range 285–305 K, indicating

Table 2. ^{1}H NMR chemical shifts of *cyclo*-B11 in aqueous solution and DPC/water.[a]

Residue	Proton	Water[b]	DPC/Water[c]
Glu 24	H^α	4.16	4.05
	others	1.97/1.82(H^β), 2.26(H^γ)	2.10/2.07(H^β), 2.40/2.37(H^γ)
Glu 25	HN	8.78(br)	8.85(br)
	H^α	4.32	4.30
	others	1.86(H^β), 2.23(H^γ)	1.90/1.81(H^β), 2.21/2.19(H^γ)
Asp 26	HN	8.45	8.42
	H^α	4.54	4.55
	others	2.61/2.56(H^β)	2.62/2.60(H^β)
Tyr 27	HN	8.16	8.33
	H^α	4.49	4.34
	others	2.98/2.90(H^β), 7.06(H^δ), 6.78(H^ε)	3.00/2.92(H^β), 6.94(H^δ), 6.74(H^ε)
Glu 28	NH	8.23	8.17
	H^α	4.00	3.94
	others	1.67/1.38(H^β,H^γ)	2.11/1.93(H^β), 2.28/2.25(H^γ)
Gly 29	NH	7.56	8.14
	H^α	3.96/3.74	3.80/3.72
Trp 30	NH	7.04	7.90
	H^α	4.85	4.47
	others	3.36/3.32(H^β), 7.10($H^\delta1$), 7.47($H^\varepsilon3$), 10.18($H^\varepsilon1$), 7.14($H^\zeta3$), 7.41($H^\zeta2$), 7.19($H^\eta2$)	3.39/3.37(H^β), 7.33($H^\delta1$), 7.56($H^\varepsilon3$), 10.56($H^\varepsilon1$), 7.02($H^\zeta3$), 7.49($H^\zeta2$), 7.14($H^\eta2$)
Met 31	NH	8.42	7.80
	H^α	4.33	4.45
	others	1.98/1.96(H^β), 2.50/2.42(H^γ), 2.06(H^ε)	2.10/2.08(H^β), 2.54/2.42(H^γ), 2.10(H^ε)
Lys 32	NH	8.07	7.32
	H^α	4.33	4.32
	others	1.78/1.56(H^β), 1.27/1.34(H^γ,H^δ), 3.10/2.86(H^ε), 6.56(H^ζ)	1.78/1.61(H^β), 1.26/1.42(H^γ, H^δ), 3.33/2.87(H^ε), 7.64(H^ζ)
Phe 33	NH	7.60	7.57
	H^α	4.44	4.36
	others	3.20/2.96(H^β), 7.25(H^δ), 7.34(H^ε), 7.29(H^ζ)	3.16/2.96(H^β), 7.28(H^δ), 7.32(H^ε), 7.18(H^ζ)

[a] The assignment of diastereotopic atom pairs is not stereospecific. [b] *Cyclo*-B11 3.0 mM, H_2O/D_2O 94%, pH 6.4, $T = 298$ K. [c] *Cyclo*-B11 3.0 mM, [D_{38}]DPC 170 mM, H_2O/D_2O 94%, pH 6.4, $T = 298$ K.

that there is no exchange between different peptide conformers during a slow NMR timescale. The iterative comparison of NOESY, COSY, and TOCSY spectra according to the sequence-specific method[18] allowed us to unambiguously assign almost all ^{1}H NMR signals (but no stereospecific assignments have been attempted, see Table 2). The analysis of a NOESY spectrum acquired at 298 K, with a mixing time of 150 ms, allowed us to obtain a total of 193 geometric constraints, amongst which 72 were inter-residue nonsequential (however, 18 of these 72 constraints involve residues Glu 28/Lys 32, whose side chains are covalently linked to close the cyclic moiety). After structure minimization (see experimental section for details), a bundle of 30 structures was selected as representative of the peptide structure. This bundle of conformers was characterized by an average residual target function of 0.048 Å^2, and by no violations larger than 0.2 Å of the geometric or van der Waals upper/lower limit bounds. The calculation statistics indicate that the optimized structures are both sterically and geometrically consistent with the NMR derived constraints. The 30 optimized structures are characterized by a RMSD of 0.94 Å calculated over the superposition of the backbone atoms of segment 25–32 (the RMSD is 1.76 Å when calculated over all the heavy atoms of segment 25–32, see Table 3). These values are quite large, indicating that the NMR constraints cannot define a very well-resolved global structure. However, the analysis of local RMSD values, calculated over three-residue-long segments, indicates that residues 28–32 (that is, those residues which are part of the cyclic portion of the molecule) define an ordered peptide segment (Figure 5). The conformation of the N-terminal segment can be defined to a lesser extent because of the lack of structurally relevant NOE constraints, and is probably locally more flexible/disordered than the remaining part of the molecule. A subgroup of 15 of the total 30 structures is shown in Figure 6, superposed so as to minimize the RMSD over the heavy atoms of the cyclic backbone (backbone atoms of residues 28–32, and side chain heavy atoms of residues Glu 28 and Lys 32). Another view of the structure of the peptide is shown in Figure 7, where 15 optimized NMR structures have been superposed by minimizing the RMSD between the backbone atoms of the residues forming the cyclic moiety (residues 28–32). The conformation of the peptide backbone in the DPC–water solution defines a turn-like motif encompassing residues Trp 30 and Met 31, with the cyclic moiety adopting a boat-shaped conformation (Figure 6) similar to that found for

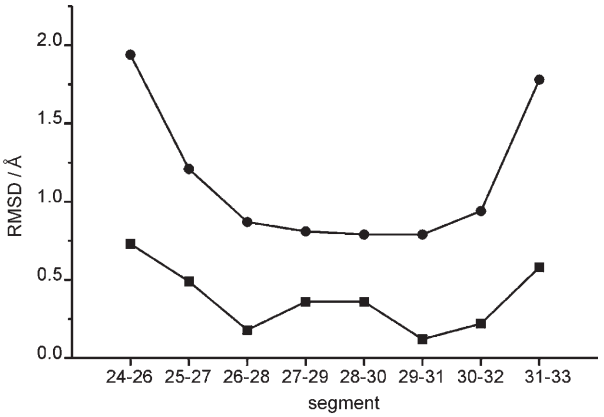

Figure 5. Plot of the atomic backbone RMSD (■: backbone heavy atoms; ●: all heavy atoms) calculated over the superposition of consecutive three-residue segments versus the position of the segment in the amino acid sequence. The RMSDs were calculated over superposition of 30 structures of *cyclo*-B11.

Table 3. Summary of the NMR-derived constraints used for torsion angle dynamics (TAD) with simulated annealing calculations and results from structure optimization of *cyclo*-B11 (DPC micelles).[a]

Inter-proton Upper Distance Bounds from NOE Interactions	
Total	193
Intra-residue	48
I, *i*+1	73
I, *i*+2	30
I, *i*+3	22
I, *i*+4	20[d]
Structure Calculation[b]	
Residual target function (±SD)	0.05±0.02 [Å^2]
Violations of upper-distance bounds[c]	
Violations > *0.2 Å*	0
Violations > *0.1 Å*	0
Violations of van der Waals lower bounds > 0.1 Å[c]	0
Global RMSD ±SD [Å]	
Segment 25–33 (backbone)	0.94±0.26
Segment 25–33 (heavy atoms)	1.76±0.33
Segment 25–32 (backbone)	0.74±0.24
Segment 25–32 (heavy atoms)	1.38±0.27
Segment 28–32 (backbone)	0.45±0.21
Segment 28–32 (heavy atoms)	1.05±0.26

[a] *Cyclo*-B11 3.0 mM, [D_{38}]DPC 170 mM, H_2O/D_2O 94%, pH 6.4, NOESY mixing time 150 ms. [b] Statistics calculated from over 30 structures out of 500 minimized conformers endowed with minimal residual target functions. [c] Violations consistently found in at least one-third of the analyzed structures. [d] Amongst these NOE-derived distances, 18 are between Glu 28 and Lys 32.

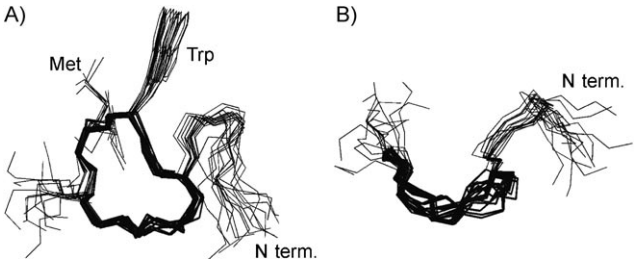

Figure 6. NMR structures of *cyclo*-B11 (DPC micelle) obtained from NOE-derived constraints by means of TAD with simulated annealing, restrained molecular dynamics, and energy minimization. View of 15 structures superposed to minimize the RMSD over the heavy atoms of the cyclic structure (the bonds involving backbone heavy atoms of segment 5–9 and those involving the side chain heavy atoms of residues Glu 28 and Lys 32 are shown as thicker lines). A) Top view showing the side chains of Trp 30 and Met 31 and the side chains of Glu 28 and Lys 32 closing the ring. B) Side view showing the backbone and the side chains of Glu 28 and Lys 32 closing the ring.

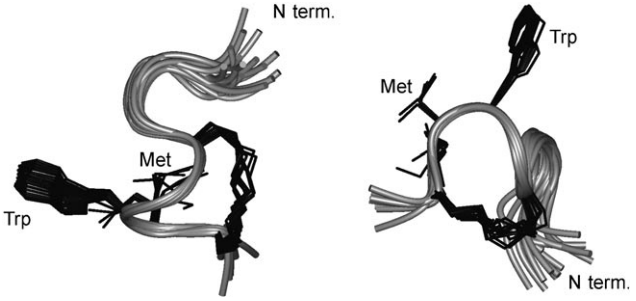

Figure 7. Superposition of 15 NMR structures of *cyclo*-B11 for which the RMSD between the backbone heavy atom of residues 28–32 has been minimized (two views). The peptide backbone is represented as a grey ribbon; the side chains of Glu 28 and Lys 32 as well as the side chains of Trp 30 and Met 31 are shown as black lines.

the *cyclo*-29,34[Dap 29,Lys 34]-CCK8 analogue.[16] The comparison of the NMR structure of segment 28–32 of *cyclo*-B11 with the corresponding segment of the linear parent CCK8 molecule[12] shows that the turn conformation involving the Trp 30–Met 31 region is essentially preserved (Figure 8). As expected

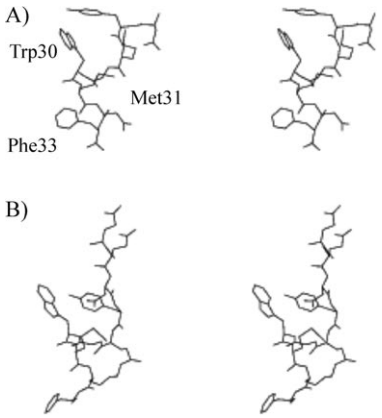

Figure 8. Stereographic view of A) the NMR structure of CCK8[5] and of B) the NMR structure of *cyclo*-B11.

from rational design, the side chains of Trp 30 and Met 31 extend outside the ring defined by the cyclic backbone, and their spatial orientation is compatible with that described for an optimal interaction with the receptor.[14,16] The finding that *cyclo*-B11 is essentially unstructured in water, whereas it becomes properly structured in the presence of DPC micelles, suggests that the restriction of conformational freedom due to the cyclic structure cannot provide by itself a very strong stabilization of the bioactive conformation. Besides the cyclic constraint, the interaction with the micelle still provides the critical structure-promoting driving force. Thus, the key residues are the hydrophobic aromatic residues belonging to the C-terminal segment of *cyclo*-B11, of which Phe 33 and Trp 30 play a major role (in particular, the chemical shifts of the Trp 30 side chain aromatic protons are greatly influenced by the micelles). The key role of these aromatic residues is supported from published data, as the molecular model of an analogue of CCK8 (namely, DM-[Thr 28, Nle 31]-CCK9) placed at the interface of a water–lipid system showed that the tryptophan and phenylalanine residues are buried within the hydrophobic phase.[19]

Conclusions

The NMR structure of the complex between CCK8 and the third loop fragment of CCK type B receptor, CCK_B-R(352–379),[14] has been used as the starting point to design a library of peptidomimetic, conformationally constrained analogues of CCK8 by using a rational approach. The binding assays performed on the whole series of 14 peptides indicate that the Trp 30–Phe 33 residues are important for the interaction. In fact, the most active molecules maintain the turn region centered around Trp 30–Met 31. Our findings also support that the Phe 33 side chain must be conserved. The NMR conformational studies on *cyclo*-B11 show that the turn conformation in the Trp 30–Met 31 region is preserved, giving rise to the correct side chain orientation of Trp 30 and Met 31 residues for interaction with the CCK_B receptor as planned by rational design. The comparison of the conformational features of *cyclo*-B11 with those published for the parent CCK8 molecule[12] and other analogues[16,19] retaining significant levels of receptor binding affinity, allows discussion of the factors underlying the stabilization of the bioactive conformation. Several studies indicate that the most important contribution to the stabilization of the bioactive conformation of linear analogues of CCK8 arises from the molecular interactions between the peptide hydrophobic residues and the cell membrane bilayer (or its model systems).[12,19] Moreover, in the case of the [Thr 28, Nle 31]-CCK9 analogue, lateral penetration of receptor structures by the membrane bound form of the ligand has been suggested as a possible mechanism for receptor activation.[19] The introduction of cyclic constraints to decrease the conformational flexibility of the ligand is expected to further stabilize the bioactive conformation. However, the *cyclo*-B11 derivative gives no evidence of a well-defined structure in water whereas it becomes properly structured (at least in the C-terminal segment) in the presence of DPC micelles. In this regard, the *cyclo*-29,34[Dap 29, Lys 34]-CCK8 has been shown to behave in a very similar way.[16] Thus, the restriction of conformational freedom due to the cyclic structure cannot provide by itself a very strong stabilization of the bioactive conformation. The evaluation of the contribution of conformational restraining of the cyclic peptide bioactive fold is difficult because of the greater contribution to structural stability afforded by the micelle interaction. The structure-promoting role of aromatic residues is supported by the finding that both Trp 30 and a phenyl ring at the C-terminal end are required for high affinity binding to the receptor.

Experimental Section

Design: Energy minimizations were carried out with the program DISCOVER, version 2.98, implemented in the BIOSYM software package. Calculations were performed using the Consistent Valence Force Field (CVFF)[20–22] without cross- and Morse terms on a Silicon Graphics Octene workstation. The package INSIGHT/DIS-

COVER (Biosym Technologies, San Diego, CA, USA), version 2000, was employed for model-building procedures, as a graphic interface, and for energy minimization procedures. The minimization was carried out using the conjugate gradient method until the energy difference between two subsequent steps was lower then 0.01 kJ mol^{-1}.

Peptide synthesis: Solid-phase peptide synthesis were performed on a fully automated Shimadzu (Kyoto, Japan) SPPS-8 synthesizer. PyBOP, HOBt, HATU, Fmoc-amino acid derivatives, Wang resin, 2-chlorotrityl chloride resin, Rink Amide MBHA resin were purchased from Calbiochem-Novabiochem. All other chemicals were obtained from Aldrich, Fluka, or LabScan and were used without further purification, unless otherwise stated. Analytical RP HPLC runs were carried out on a HP Agilent Series 1100 apparatus using a Phenomenex, C_{18} column, 4.6×250 mm with a flow rate of 1.0 mL min^{-1}. Preparative RP HPLC was carried out on a Shimadzu 8 A apparatus equipped with a UV Shimadzu detector using a Phenomenex, C_{18} column, 22×250 mm with a flow rate of 20 mL min^{-1}. For all the RP HPLC procedures the system solvent used was: H_2O 0.1% TFA (eluent A) and CH_3CN 0.1% TFA (eluent B), with a linear gradient from 5 to 70% B in 30 min (gradient 1) or from 20 to 80% B in 20 min (gradient 2). Mass spectral analysis was carried out on MALDI-TOF Voyager-DE and on MALDI-TOF Voyager-DE PRO mass spectrometers (PerSeptive Biosystems). The cyclic peptide analogues were synthesized by a solid-phase method using the standard Fmoc procedure. All couplings were performed twice for 1 h by using an excess (4 equiv) of each amino acid derivative. The amino acids were activated in situ by the standard HOBt/PyBOP/DIPEA protocol. Fmoc deprotection was performed with 20% piperidine in DMF for 5+10 min. The Kaiser test[23] was employed to monitor the N↔C cyclization reaction performed on resin. Fully protected linear peptides were cleaved from the solid support by suspending the resin in 1% CF_3COOH in CH_2Cl_2 with stirring for 1 min. The resin was then filtered and the filtrate was poured into 10% pyridine in CH_3OH. This procedure was repeated 10 times. After being filtered, the mixture was concentrated under reduced pressure and the crude product was isolated by precipitation into cold water. The precipitate was collected by centrifugation and dried in vacuo (over P_2O_5 pellets). The peptide cleavage from the solid support and the simultaneous removal of all protecting groups from the amino acid residues was carried out by suspending the fully protected compound-resins in TFA/H_2O/EDT (94:4:2) for 3 h followed by filtration. The solution was then concentrated and the crude product isolated by precipitation into cold diethyl ether. The precipitate was collected by centrifugation and dried in vacuo (over KOH pellets). The cyclization reaction, by disulphide bond formation between the two Cys residues, was performed by dissolving the crude peptide (final concentration 10^{-4} M) in 0.1 M solution of NH_4HCO_3 in water to promote the oxidation reaction. After 4 h the reaction mixture was concentrated and the desired compound isolated by chromatographic purification. All peptides were fully characterized for their identity by mass spectrometry, and for their purity by analytical HPLC. The compounds used in biological assays were all at >98% HPLC purity.

Cyclo-**B1**, *cyclo*-**B2**, *cyclo*-**B5**, *cyclo*-**B6**: For each compound 0.150 g of Rink Amide MBHA resin (0.28 mmol g^{-1} substitution; 42 μmol scale) were used. The synthesis of the peptide chains was performed by sequential coupling and Fmoc deprotection of the appropriate amino acids (Fmoc-Phe-OH, Fmoc-Asp(O-2-Ph*i*Pr)-OH, Fmoc-Met-OH, Fmoc-Trp(Boc)-OH, and Fmoc-Dap(Mtt)-OH were used for *cyclo*-B1 and *cyclo*-B2; Fmoc-Phe-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Met-OH, Fmoc-Trp(Boc)-OH, Fmoc-Gly-OH, Fmoc-Glu(O-2-Ph*i*Pr)-OH, Fmoc-Tyr(*t*Bu)-OH, and Fmoc-Asp(O*t*Bu)-OH were used for *cyclo*-B5; Fmoc-Lys(Mtt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Met-OH, Fmoc-Trp(Boc)-OH, Fmoc-Glu(O-2-Ph*i*Pr)-OH, Fmoc-Met-OH, Fmoc-Tyr(*t*Bu)-OH, and Fmoc-Asp(O*t*Bu)-OH were used for *cyclo*-B6), the synthesis was ended with the acetylation of the N-terminal amino acid residue for *cyclo*-B2. After completion of the synthesis, the resins were treated ten times for 1 min with 1% CF_3COOH in CH_2Cl_2. This procedure allowed the removal of the Mtt and 2-Ph*i*Pr protecting groups from Dap and Asp for *cyclo*-B1 and *cyclo*-B2, respectively, and from Lys and Glu for *cyclo*-B5 and *cyclo*-B6, respectively. The successive N↔C cyclization reaction on resin was performed as a typical coupling reaction (HOBt/PyBOP/DIPEA in DMF). Following the protocol described in the general procedures paragraph, the peptides were cleaved from the solid support and freed from all the protecting groups at the same time. RP HPLC purifications and the MALDI-TOF mass spectrometry analysis confirmed the presence of the desired compounds. *Cyclo*-B1: t_R = 24.48 min (gradient 1); $[M+H]^+$ = 666.10 (calcd = 666.68); *cyclo*-B2: t_R = 25.20 min (gradient 1); $[M+H]^+$ = 707.96 (calcd = 708.68); *cyclo*-B5: t_R = 17.12 min (gradient 2); $[M+H]^+$ = 1057.95 (calcd = 1057.18); *cyclo*-B6: t_R = 16.23 min (gradient 2); $[M+H]^+$ = 1099.87 (calcd = 1099.24).

Cyclo-**B3**: The first amino acid derivative Fmoc-Gly-OH was coupled to the 2-Chlorotrityl chloride resin (1.04 mmol g^{-1} substitution; 0.100 g resin) under basic conditions (4 equiv DIPEA) in CH_2Cl_2. The loading was evaluated by Fmoc test (0.70 mmol g^{-1} calcd substitution; 0.07 mmol scale). The peptide chain was than elongated by sequential coupling and Fmoc deprotection of Fmoc-Dap-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Met-OH, Fmoc-Trp(Boc)-OH and Fmoc-Gly-OH. The fully protected peptide was cleaved from the solid support by following the protocol described in the general procedures paragraph. The backbone N↔C cyclization reaction was performed by dissolving the peptide in CH_2Cl_2 (final concentration 10^{-4} M) and using PyBOP/DIPEA to promote the cyclization reaction. The cyclized peptide was than freed from the protecting groups by treatment with high percentage of trifluoroacetic acid. The final steps of purification and characterization afforded the desired compound. t_R = 21.75 min (gradient 1); $[M+H]^+$ = 644.79 (calcd = 644.72).

Cyclo-**B4**, *cyclo*-**B11**, *cyclo*-**B12**, *cyclo*-**B13**, *cyclo*-**B14**: The synthesis was performed for each compound on 0.100 g of Wang resin. The first amino acid derivative (Fmoc-Phe-OH for *cyclo*-B4 and *cyclo*-B11; Fmoc-β-HomoPhe-OH for *cyclo*-B12; Fmoc-D-Phe-OH for *cyclo*-B13; Fmoc-Gly-OH for *cyclo*-B14) was loaded on the resin according to the published protocol,[24] and the loading was evaluated by the Fmoc test (0.70 mmol g^{-1} calculated substitution; 0.07 mmol scale). The peptide chains were synthesized by sequential coupling and Fmoc deprotection of the following Fmoc-amino acid derivatives: Fmoc-Lys(Mtt)-OH; Fmoc-Met-OH; Fmoc-Trp(Boc)-OH; Fmoc-Gly-OH; Fmoc-Glu(O-2-Ph*i*Pr)-OH; Fmoc-Tyr(*t*Bu)-OH; Fmoc-Asp(O*t*Bu)-OH; Fmoc-Glu(O*t*Bu)-OH; Fmoc-Glu(O*t*Bu)-OH. Concerning *cyclo*-B4, the peptide chain elongation was stopped at the Asp residue. After completion of the synthesis, the same procedure described above was used to remove the Mtt and 2-Ph*i*Pr protecting groups from Lys and Glu respectively. For the N↔C cyclization, the peptide cleavage from the solid support and the deprotection of all amino acid residues were carried out as described in the previous paragraph. Full analysis, purification, and characterization allowed us to identify the compounds synthesized. *cyclo*-B4: t_R = 17.21 min (gradient 2); $[M+H]^+$ = 1057.85 (calcd = 1057.18); *cyclo*-B11: t_R = 23.95 min (gradient 1); $[M+H]^+$ = 1316.60 (calcd = 1316.40); *cyclo*-B12: t_R = 24.81 min (gradient 1); $[M+H]^+$ = 1330.24 (calcd =

1330.43); *cyclo*-B13: $t_R = 24.17$ min (gradient 1); $[M+H]^+ = 1316.20$ (calcd = 1316.40); *cyclo*-B14: $t_R = 23.87$ min (gradient 1); $[M+H]^+ = 1372.91$ (calcd = 1373.45).

Cyclo-**B7**, *cyclo*-**B8**, *cyclo*-**B9**, *cyclo*-**B10**: The same procedure followed to functionalize the Wang resin for *cyclo*-B4 and *cyclo*-B11–*cyclo*-B14 was used to prepare *cyclo*-B7 and *cyclo*-B8 (0.100 g resin; 0.60 mmol g^{-1} calcd substitution; 0.06 mmol scale). Instead, the synthesis of *cyclo*-B9 and *cyclo*-B10 was performed by using the Rink Amide MBHA resin (0.150 g; 0.28 mmol g^{-1} substitution; 42 μmol scale). All peptides were synthesized using the same Fmoc-amino acid derivatives (Fmoc-Phe-OH; Fmoc-Cys(Trt)-OH; Fmoc-Met-OH; Fmoc-Trp(Boc)-OH; Fmoc-D/L-Cys(Trt)-OH; Fmoc-Met-OH; Fmoc-Tyr(*t*Bu)-OH; Fmoc-Asp(O*t*Bu)-OH) except the second Cys residue which was a Fmoc-Cys(Trt)-OH for *cyclo*-B7 and *cyclo*-B9, and a Fmoc-D-Cys(Trt)-OH for *cyclo*-B8 and *cyclo*-B10. Following the protocols described in the general procedures paragraph, the peptides were cleaved from the resin and fully deprotected at the same time. The final oxidation reaction led to cyclized peptides by disulphide bond formation. The compounds were purified and characterized as for the other molecules previously described. *cyclo*-B7: $t_R = 18.22$ min (gradient 2); $[M+H]^+ = 1096.27$ (calcd = 1096.35); *cyclo*-B8: $t_R = 18.90$ min (gradient 2); $[M+H]^+ = 1096.19$ (calcd = 1096.35); *cyclo*-B9: $t_R = 18.21$ min (gradient 2); $[M+H]^+ = 1095.32$ (calcd = 1095.35); *cyclo*-B10: $t_R = 18.24$ min (gradient 2); $[M+H]^+ = 1095.27$ (calcd = 1095.35).

In vitro cellular assays: Competition binding experiments were performed on A431 cells that had been stably transfected with a plasmid containing the full coding sequence for the human CCK_B receptor.[17] Assays were performed on cells that had been grown to 80–90% confluency, trypsinized, resuspended in tissue culture medium and subsequently incubated for at least 1 h at 37 °C to allow recovery of receptor expression on the cell surface. The cell suspension was cooled to 4 °C and equal aliquots were distributed into 1.5 mL tubes. Tracer amounts of ^{111}In-labeled DTPAGlu-CCK8[17] were incubated with cells in the presence of each *cyclo*-B compound at concentrations ranging from 10^{-10} to 10^{-3} M for 1 h while rotating at 4 °C. Bound radioactivity was then separated by centrifuging the cells through dibutylphthalate (Sigma), freezing the tubes on dry ice, and excising the cell pellet containing tips of the tubes. Bound and free radioactivity were determined with a Wallac Wizard gamma counter. Nonlinear regression analysis using a model for homologous competition binding was performed using GraphPad Prism (version 3.0a for Macintosh, GraphPad Software, http://www.graphpad.com) to derive the 50% inhibitory concentrations (IC_{50}) for each *cyclo*-B compound.

^{1}H NMR spectroscopy: [D_{38}]DPC (98.96% isotopic enrichment) was purchased from CDN Isotopes Inc. and was used without further purification. NMR spectra of *cyclo*-B11 in aqueous solution, 2.3 mg of the peptide were dissolved in 560 μL of deionized water and 40 μL of D_2O (or dissolved into 600 μL of D_2O) was added to obtain a final peptide concentration of 3.0 mM. The pH was adjusted to 6.4 ± 0.1 by adding small amounts of NaOH. For experiments with DPC micelles, the aqueous solution (or D_2O solution) of 3.0 mM *cyclo*-B11 was added with a suitable amount of solid [D_{38}]DPC to give final concentration of 170 mM [D_{38}]DPC, and the pH was adjusted to 6.4 ± 0.1. Samples were transferred into 5 mm tubes for NMR experiments that were carried out on a Bruker Avance 600 spectrometer operating at 14 T (corresponding to a proton Larmor frequency of 600.13 MHz) equipped with a triple axis-PFG (Pulse Field Gradient) probe optimized for ^{1}H detection. Water suppression was achieved by means of the WATERGATE 3-9-19 pulse train[25,26] in the case of H_2O/D_2O 95% mixtures or by presaturation of the solvent line during the recycle delay for samples dissolved into D_2O. Chemical shifts were referenced to external TMS (0.03% in deuterated chloroform, coaxial insert). 2D-TOCSY (total correlation spectroscopy) experiments[27] were carried out by means of a MLEV17 spin-lock pulse sequence[28] flanked by two 2.5 ms trim-pulses with a spin locking field strength of 10 KHz and a duration of 75–100 ms. The STATES-TPPI (time-proportional phase increment) phase cycling was used to obtain complex data points in the t1 dimension. 2D-NOESY (nuclear Overhauser effect spectroscopy) experiments[29] were carried out by the standard pulse sequence with the STATES-TPPI phase cycling scheme with mixing times ranging from 100 to 450 ms. 2D-DQF-COSY (double quantum-filtered correlation spectroscopy) experiments[30] were obtained in the phase sensitive mode by means of the TPPI method with the standard double quantum filtered pulse sequence coupled with a combination of PFG at the magic angle, and selective water excitation to achieve optimal water suppression. Typically, the following instrumental settings were used for 2D experiments: spectral width 8400 Hz; 512 and 2048 complex data points in the t1 and t2 dimensions, respectively; 16–32 scans per t1 increment; relaxation delay 2.5 s. The data were multiplied by a square cosine window function and zero filled to a matrix sized 1024 × 1024 prior to FT and baseline correction.

Molecular dynamics: All calculations were carried out on a Silicon Graphics Octane workstation. The assignment of NMR signals and integration of NOE peaks were done by means of the XEASY[31] software package. The assignment of ^{1}H NMR resonances was carried out by the sequence-specific method,[18] that is, by iterative comparison of TOCSY, NOESY, and DQF-COSY spectra. A number of ambiguities in the assignment because of severe signal overlap could be resolved by comparing experiments carried out at different temperatures (between 285 and 305 K). The structure optimization based on NMR constraints was carried out by the program DYANA[32] (energy minimization by torsion angle dynamics and simulated annealing) and structure analysis by means of MOLMOL[33] (molecular graphics). Peak volumes were obtained from NOESY spectra acquired with mixing times of 150 ms. The derivation of the geometric constraints and the calculation of optimized structures was performed according to the protocol described in reference [16], in which another cyclic analogue of CCK8 was studied. Briefly, NOE signal intensities have been converted into upper limit distance constraints between a given pair of atoms, whereas the sum of the atomic van der Waals radii has been imposed as the lower limit distance constraint. The geometry around the amide bond formed by the side chains of Glu 28 and Lys 32 was fixed to trans by setting the distance between Glu 28 C^{δ} and Lys 32 N^{ζ} to the fixed value of 1.35 ± 0.1 Å, and by setting the distance between Glu 28 $O^{\varepsilon}1$ and Lys 32 $H^{\zeta}1$ to the fixed value of 3.17 ± 0.1 Å. Structure optimization has been carried out on an initial set of 500 random conformations (bond length and bond angles were fixed at their optimal values according to the ECEPP/2[34] standard geometry) that were minimized by the DYANA standard protocol. A bundle of 30 conformers having the lowest residual target function values after structure optimization and further energy minimization were selected as representative of the peptide conformation and forwarded to the MACROMODEL 6.5 software package[35] for further molecular dynamics minimizations with the AMBER* force field[36,37] and final energy minimization by means of the Polak–Ribiere conjugate gradient minimization mode.

Abbreviations: Boc, *tert*-butoxycarbonyl; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; EDT, 1,2-ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-

1,1,3,3-tetramethyluronium; HOBt, 1-hydroxybenzotriazole; Mtt, 4-methyltrityl; 2-Ph*i*Pr, 2-phenylisopropyl; PyBOP, benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; Trt, trityl.

Acknowledgements

This work was supported by grants from Italian MIUR (Ministry of Education, University and Research) (Progetto Oncologia), and from Italian CNR. We are grateful to Professor Dale F. Mierke for providing the coordinates of the NMR structure of the complex between CCK8 and CCKB-R(352-379).

Keywords: cholecystokinin · cyclic constraints · NMR structures · peptides · receptors

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Received: March 3, 2006
Revised: May 29, 2006
Published online on August 17, 2006

