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Synthesis and biological evaluation of cyclic and branched peptide analogues as ligands for cholecystokinin type 1 receptor

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Abstract—A library of cyclic CCK8 analogues, containing unnatural amino acids in the peptide sequence, is prepared using solid-phase synthesis. The structure of these cyclic peptides is based on a previously synthesised compound, *cyclo*-CCK8, selective for CCK_1 receptor. Structure–activity investigations are performed by evaluating the binding properties of the new analogues. In particular, the binding ability of the cyclic CCK8 analogues is tested by nuclear medicine studies on cell line transfected with CCK_1 receptor. Compounds named *cyclo*-A4–*cyclo*-A7 show binding constant in the range 6.0–8.0 μM, with an improved affinity over the previous described *cyclo*-CCK8, but almost comparable IC_{50} values among new analogues towards CCK_1 were obtained.

1. Introduction

Cholecystokinin (CCK), a peptide hormone found in the central nervous system and gastrointestinal tract, occurs in multiple biologically active forms (CCK58, CCK39, CCK33, CCK8, CCK4) with CCK8 predominating.[1,2] CCK mediates many diverse hormonal and neuromodulatory functions through the action of two specific G protein coupled receptor subtypes, termed CCK_1 and CCK_2.[3,4]

The CCK_1 receptor subtype binds sulfated CCK8 with significantly higher affinity than either the desulfated form of CCK8 or CCK4, whereas the CCK_2 receptor binds all three of these CCK peptides with comparable affinities.[5]

The variety of physiological effects of CCK and its possible role in some pathological disorders have stimulated research in this area.[6,7] Over the past decade, a number of potent and selective nonpeptide or peptidomimetic CCK_1 and CCK_2 receptor agonists and antagonists have been developed.[8,9] Some of these ligands have been useful tools for gaining insight into the functional roles of both CCK receptor subtypes. However, beside the interest for shedding further light on CCK receptors' functionality, the research activity in this area is equally devoted to search for selective ligands of CCK receptors to be used as therapeutic agents. For example, there is an increasing interest for oncological applications of drugs able to target GPCRs, like CCK receptors, due to their overexpression in cancer cells.[10]

As with other GPCRs, a detailed knowledge of the structure of the complex between the peptide ligand and its CCK receptor is crucial in the development of peptide derivatives for pharmaceutical applications. In particular, adequate structural information could help in the design of new peptides or peptide analogues endowed with higher in vivo stability and improved selectivity for particular receptor subtypes.

Abbreviations: Boc, *tert*-butoxycarbonyl; DBU, (1,8-diazabicyclo[5.4.0]-undec-7-ene); DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DTPA-Glu, *N*,*N*-bis[2-[bis(carboxyethyl)amino]-ethyl]-L-glutamic acid; EDT, 1,2-ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; Mtt, 4-methyltrityl; *o*-NBS, 2-nitrobenzenesulfonamide; PyBOP, benzotriazol-1-yloxy-tris-pyrrolidino-phosphonium; ^{t}Bu, *tert*-butyl; TFA, trifluoroaceticacid.

Keywords: Cholecystokinin; Cyclic constraints; Cyclic peptides; Receptor binding affinity.

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Unfortunately, structural characterization of the ligand/receptor complex utilising the entire receptor molecule is a presently elusive task by high resolution NMR or X-ray crystallography, so that other strategies have been developed to clarify the peptide-receptor binding modalities. In fact, NMR spectroscopy has been recently employed to study the molecular basis of the interaction between the peptide ligand and its CCK receptor, using the cholecystokinin endogenous octapeptide CCK8 and a series of synthetic protein fragments derived from the extracellular portions of the CCK receptors. This approach was adopted by Mierke and coworkers who published the NMR structure of the bimolecular complex between CCK8 and the N-terminal domain of the cholecystokinin type 1 receptor.[11] Later on, the same authors published the NMR structure of the complex formed by CCK8 with the third extracellular loop of the cholecystokinin type B receptor.[12] These structural information together with other studies, such as mutagenesis, photoaffinity labelling and fluorescence spectroscopy, suggest differences in the binding mode of the same ligand with the two receptor subtypes.[11–14]

Our work has been focused on the development of peptide analogues selective for CCK_1 receptor on the basis of the NMR structure of the complex between CCK8 and the N-terminal extracellular arm of CCK_1-R. We have already published[15] the studies performed on a newly developed CCK8 analogue, Cyclo^{29,34}[Dap^{29}, Lys^{34}]-CCK8 (*cyclo*-CCK8), selective for CCK_1 receptor. In particular, we tested the binding affinity of *cyclo*-CCK8 towards the N-terminal receptor fragment CCK_1-R(1-47) by fluorescence spectroscopy. The dissociation constant found for the complex *cyclo*-CCK8/CCK_1-R(1-47) was in the same nanomolar range of that found for the complex CCK8/CCK_1-R(1-47). This result indicated that the rationally designed *cyclo*-CCK8 fully recovered the binding affinity of the template (CCK8) towards the receptor system model (CCK_1-R(1-47)). However, the biological evaluations performed in vitro, on cells expressing the entire CCK_1 receptor, revealed that *cyclo*-CCK8 had binding affinity (IC_{50} = 15 μM) much lower than that of the endogenous ligand conjugated to a chelating agent DTPAGlu-Gly-CCK8 (IC_{50} = 0.032 μM). It is likely that the cyclic constraint, in spite of having stabilised a selective conformation of the cyclic analogue towards CCK_1 receptor,[15] induces a lower binding affinity as a consequence of the reduced flexibility of the peptide backbone.

Our efforts have been focused on the design and synthesis of a library of cyclic CCK8 analogues containing unnatural amino acids in the peptide sequence, in order to improve the knowledge of the structure–activity relationships within this class of compounds. The chemical formula for the new CCK8 analogues is reported in Figure 1 together with the chemical sequence of the already studied *cyclo*-CCK8. Successively, the binding properties of the new CCK8 analogues were tested by nuclear medicine studies on cell line transfected with CCK_1 receptor.

2. Results and discussion

2.1. Peptide library design

In a previous paper, we have described the rational design of a CCK8 peptidometic cyclic analogue, *cyclo*-CCK8, using the NMR structure of the complex between CCK8 and the N-terminal fragment CCK_1-R(1-47) as starting point.[15]

After superimposition of the energy-minimised *cyclo*-CCK8 model onto the CCK8 NMR structure, the analysis of the spatial three-dimensional arrangement of each residue involved in the receptor binding process showed that a significant distortion is only present in Phe^{33} residue.[15] This distortion seems to be due to the cyclic constraint introduced into the molecule and was confirmed by the NMR studies performed on *cyclo*-CCK8 in water containing DPC micelles.

In *cyclo*-CCK8 peptide,[15] the cyclic constraint was introduced between the Dap side-chain group and the CCK8 carboxyl terminus (Lys^{34}) in order to decrease the backbone flexibility of the molecule and to stabilise the bioactive conformation selective for CCK_1 receptor. The choice of a lysine residue was dictated by the possibility of using its side-chain amino group to bind a chelating agent. However, in order to evaluate potential effects of this side chain on binding affinity, a new peptide with Lys^{34} replaced by Ala residue was synthesised (compound *cyclo*-A1). The substantial conservation of affinity of this peptide implied a limited role of lisine side chain and allowed further replacements of this residue, aimed at inducing different cyclic structural constraints.

In fact, the distortion observed in phenylalanine side-chain orientation, after peptide cyclisation, could be responsible for the in vitro low binding affinity (IC_{50} = 15 μM) of *cyclo*-CCK8 with respect to the endogenous ligand conjugated to a chelating agent DTPAGlu-Gly-CCK8 (IC_{50} = 0.032 μM).

Therefore, two new cyclic peptide libraries have then been designed, in order to evaluate the effects of the cyclic constraint size/flexibility (compounds *cyclo*-A2–*cyclo*-A3) and of the side-chain length (compounds *cyclo*-A4–*cyclo*-A7) onto Phe^{33} orientation and, consequently, onto their receptor binding affinity.

In particular, the flexibility and the size of the cycle were increased by replacing Lys^{34} with a Gly (compound *cyclo*-A2), or with a β-Ala residue (compound *cyclo*-A3), respectively. The comparable affinity observed for compounds *cyclo*-A2–*cyclo*-A3, also similar to those measured for *cyclo*-CCK8, evidenced a limited effect of the cyclic constraint size/flexibility onto the binding affinity for this class of CCK8 analogues.

Concerning the second class of compounds (*cyclo*-A4–*cyclo*-A7), we decided to maintain the Gly as C-terminal peptide residue. Then, we designed other peptide analogues bearing an unnatural amino acid in place of the Phe^{33} residue. The replacement of the Phe residue with

Figure 1. Amino acid sequence of the CCK8 peptide, *cyclo*-CCK8, and schematic representation of the eight CCK8 cyclic peptide analogues. The residue-numbering scheme for all peptides is based on the numbering of the 33 residues of natural cholecystokinin.

amino benzyl derivatives of several amino acid residues, such as lysine (compound *cyclo*-A4), ornithine (compound *cyclo*-A5), di-aminobutyric acid (compound *cyclo*-A6) or di-aminopropionic acid (compound *cyclo*-A7), enables location of the benzyl moiety at different distances from the backbone, so that the aromatic ring could better dock in the binding site of the receptor.

The moderately increased IC_{50} found for these peptides could derive either from an only marginal improvement of interactions involving the aromatic residue within this compound series, or from a possible replacement of the aromatic ring by other groups of *cyclo*-CCK8 in the interaction with receptor.

In order to discern between these two possible sceneries, compound *cyclo*-A8 was synthesised by replacing Phe^{33} with an Ala residue. The dramatic fall in affinity associated to this replacement strongly supports the former interpretation of the observed results for compounds *cyclo*-A4 through *cyclo*-A7.

In Figure 2 are reported the NMR structure of CCK8[11] and of *cyclo*-CCK8,[15] and the hypothetical conformation of cyclo-A4 in which the Phe residue is replaced by the amino benzyl derivative of lysine. The hypothetical model of the interaction between this class of amino benzyl derivative analogues and CCK_1-R(1-47) is reported in Figure 3.

2.2. Peptide synthesis and characterization

The peptide library, consisting of two different classes of cyclic peptides, was synthesised on solid phase using Fmoc chemistry standard protocols. The full synthetic strategy is reported in detail in Scheme 1.

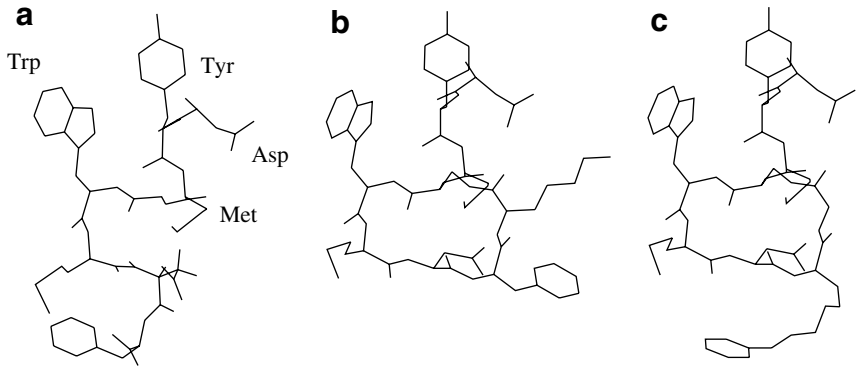


Figure 2. Molecular models of NMR structure of CCK8 (a) and of *cyclo*-CCK8 (b). (c) Picture of the hypothetical conformation of *cyclo*-A4. The sulfate group was omitted for clarity.

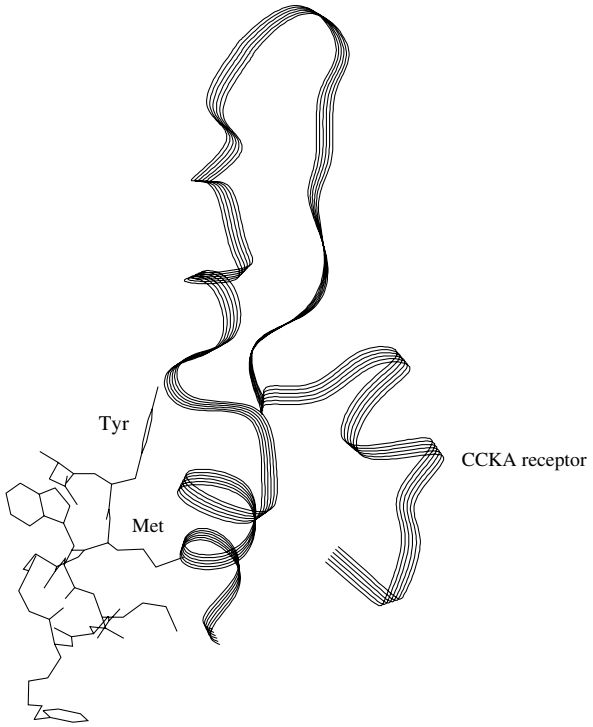


Figure 3. View of the hypothetical model of interaction between *cyclo*-A4 and CCK_1-R(1-47). The CCK_1-R(1-47) fragment is represented with a ribbon.

The procedure employed to obtain the four different *o*-NBS protected Fmoc-amino acid derivatives has been already described.[16]

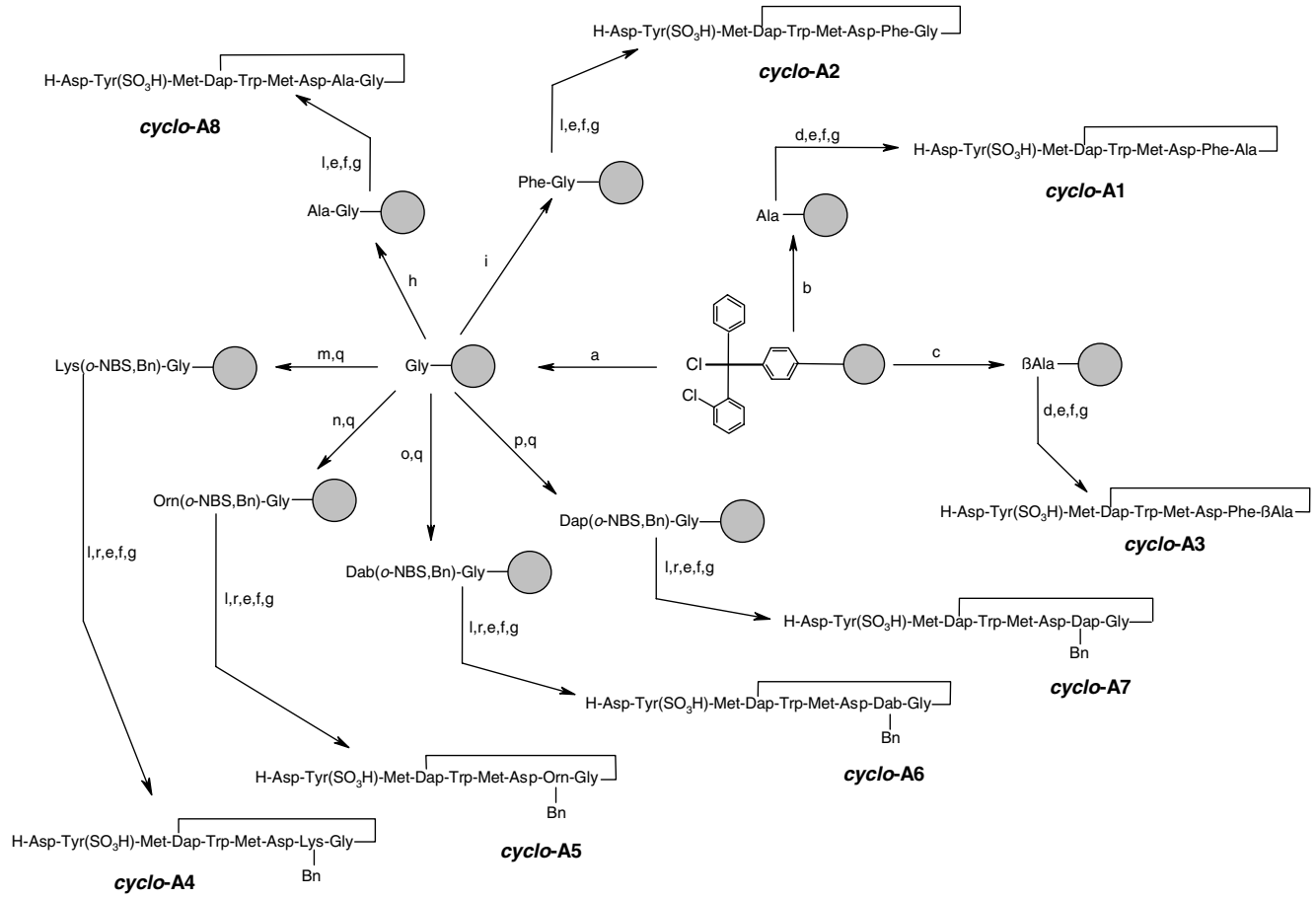
The 2-chlorotrityl chloride resin was employed as solid support. After the complete assembly of the peptide chain, the linear peptides were cleaved from the resin and the Dap residue was simultaneously freed from its protecting group. The $N \leftrightarrow C$ cyclisation reaction, between the carboxylic peptide terminal function and the Dap β-NH_2 function, was performed in solution on the protected molecules. Concerning the mono-benzylated peptides, they were prepared utilising the following very efficient strategy: after binding the four *o*-NBS protected Fmoc amino acids to the growing peptide chain, the benzylation reaction was performed with benzyl bromide in presence of potassium carbonate as the next step. After the complete assembly of the peptide onto the solid support, the *o*-NBS protecting group was easily removed by using 2-mercapto-ethanol in combination with DBU.

All compounds were obtained in good yield and with high purity grade (>95%) after RP-HPLC purification. They were fully characterized for their identity by MALDI-TOF and ^{1}H NMR.

Concerning the NMR analysis, by a careful inspection of TOCSY, NOESY and DQF-COSY spectra, the peptides' proton resonances were partly assigned, following standard procedures.[17] Proton chemical shifts for all the resonances in water, at 285 K, are listed in Section 4. The NOESY spectra of *cyclo*-A8, *cyclo*-A1, *cyclo*-A2 and *cyclo*-A3 do not indicate the presence of a clear preferred conformation for any of them in the explored experimental conditions. In the case of *cyclo*-A4, *cyclo*-A5, *cyclo*-A6 and *cyclo*-A7, TOCSY and NOESY spectra indicate the presence of multiple preferred conformations. Moreover, the $^3J_{HN\alpha}$ spin–spin coupling constant values ($6.0\ Hz < {}^3J_{HN\alpha} < 8.0\ Hz$), extracted from the 1D and DQF-COSY spectra, support the already evident flexible secondary structure of these peptides (data not shown).[17]

2.3. Biological assays

Biological assays were carried out by competition binding experiments, in order to estimate the binding ability of the cyclic CCK8 analogues towards the entire CCK_1 receptor expressed in cultured cells. Fixed tracer amounts of the labelled linear peptide [^{111}In]DTPAGlu-Gly-[$Tyr^{27}(SO_3H)$]-CCK8 were incubated with the receptor-expressing cells in the presence of the synthetic analogues at different concentrations. The data obtained (Fig. 4) show a typical pattern of competitive interaction with reduction in binding of the radiolabelled tracer, that is, of the radiation emitted, at relatively high concentration of the unlabelled cyclic CCK8 analogues. Nonlinear regression analysis



Scheme 1. Peptide synthesis according to the Fmoc solid phase protocol. Reagents and conditions: 2-chlorotritylchloride resin, loading 0.5 mmol g^{-1}; (a, b, c) protected amino acids, PyBop/DIPEA in CH_2Cl_2, 1 h; (d, h, i, l, m, n, o, p) protected amino acids, PyBop/HOBt/DIPEA in DMF, 1 h; (e) CH_2Cl_2/TFA/TIS (tris-iso-propylsilane) (98:1:1), 1.5 h; (f) PyBop/DIPEA in CH_2Cl_2, 2 h; (g) TFA/H_2O/TIS (97:2:1), 1.5 h; (q) K_2CO_3 (2 equiv), benzyl bromide (2 equiv) in DMF, 1 h; (r) DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) (10 equiv), 2-mercapto-ethanol (5 equiv), in DMF, 30 min.

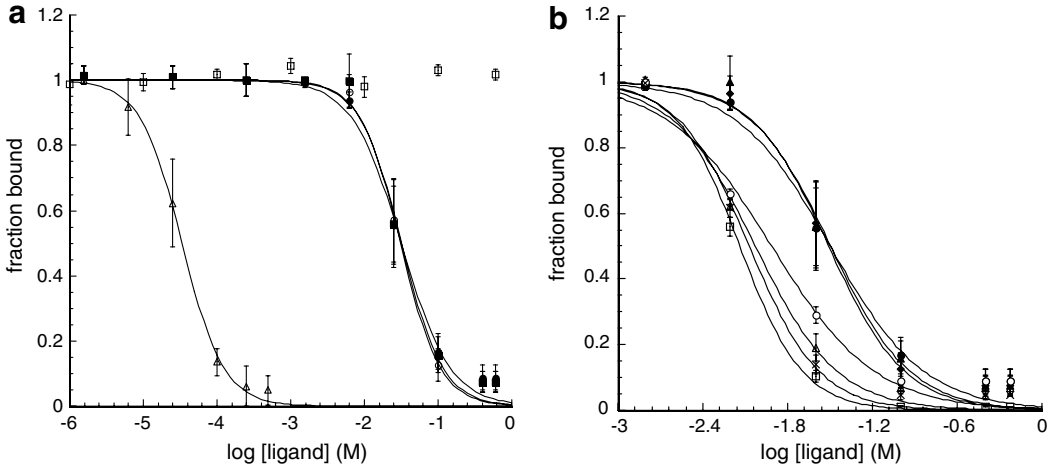


Figure 4. Competitive binding of all compounds and the conjugate DTPAGlu-Gly-CCK8: (a) *cyclo*-A2 (●), *cyclo*-A1 (■); *cyclo*-A3 (○),*cyclo*-A8 (□), DTPAGlu-Gly-CCK8 (◇); (b) *cyclo*-A2 (●),*cyclo*-A1 (▲); *cyclo*-A3 (♦), *cyclo*-A4 (□), *cyclo*-A6 (◊), *cyclo*-A7 (○), *cyclo*-A5 (×).

allowed us to calculate the 50% inhibitory concentration (IC_{50}) for all synthesised compounds. The IC_{50} values are reported in Table 1 and revealed that all compounds are less active than the parent endogenous CCK8.

Compound *cyclo*-A1 showed an IC_{50} value comparable to that of *cyclo*-CCK8, it allowed to replace the C-terminal residue Lys^{34} in the peptide sequence, since the lysine side chain was proved to have any effect onto binding affinity.

Table 1. IC_{50} values, with their standard errors, for DTPAGlu-G-CCK8 (*), *cyclo*-CCK8 and the eight new compounds

Compound	IC_{50} (μM)	SE (μM)
DTPAGlu-G-CCK8 (*)	0.032	$\pm 1.153.10^{-3}$
cyclo-CCK8	15	
cyclo-A1	>1000	
cyclo-A2	26.34	±1.114
cyclo-A3	26.66	±1.105
cyclo-A4	24.75	±1.087
cyclo-A5	6.45	±1.008
cyclo-A6	6.94	±1.049
cyclo-A7	7.46	±1.159
cyclo-A8	7.2	±1.007

(*) DTPAGlu-G-CCK8 refers to the derivative DTPAGlu-Gly-[$Tyr^{27}(SO_3H)$]-CCK8 in which the DTPAGlu chelating agent is bound on the N-terminal end of Gly-CCK8.

Concerning compounds *cyclo*-A2 and *cyclo*-A3, IC_{50} with no substantial differences among them were found, their affinities all spanning the same micromolar range observed in the original *cyclo*-CCK8. So, the new cyclic constraints apparently failed to induce a better orientation of the aromatic ring of Phe^{33} and, as a consequence, to improve the binding affinity towards the CCK_1 receptor.

The second class of analogues (compounds *cyclo*-A4–*cyclo*-A7) showed an improved affinity towards CCK_1 receptor over *cyclo*-CCK8 (see Table 1), but almost comparable IC_{50} values among each other. These data can be interpreted by assuming that the gradual elongation of the chain bearing the benzyl moiety has allowed a better accommodation of the aromatic group into the receptor site, consequently increasing the favourable enthalpic contribution to binding across the analogue series. On the other side, the immobilization of an increasingly longer and intrinsically more flexible side chain upon binding is associated to a growing unfavourable entropic contribution. In this view, the observed binding affinities could result from a perfect balance between enthalpy and entropy, so that no macroscopic difference is observed among the binding ability of the new analogues towards CCK_1 receptor.

A complete loss of affinity has been observed for compound *cyclo*-A8. This result definitely clarified that, in the binding process, the side-chain aromatic ring in position 33 of all new CCK8 analogues prepared plays the same role of Phe^{33} in CCK8.[18,19]

3. Conclusions

In conclusion, two classes of CCK8 peptide analogues as ligands for CCK_1 receptor have been designed and synthesised, using the structure of the previously synthesised ligand *cyclo*-CCK8 as starting point. The comparative analysis of the binding data concerning the compounds reported in the present work and that of the previous paper[15] allowed us to gain further insights into the structural requirements for preparing new ligands for CCK_1 receptor.

The cyclic constraint, introduced into the peptide backbone of each analogue, induces a distortion of the Phe^{33} side chain, as confirmed by the already reported NMR structure of *cyclo*-CCK8[15] and as suggested by the low binding affinity found for the new classes of compounds. In fact, any success in recovering a better orientation of this aromatic amino acid residue was obtained by modifying the cyclic constraint size. The location of the benzyl moiety at different distances from the peptide backbone was enabled by the replacement of the Phe residue with amino benzyl derivatives of several amino acid residues. This structural modification has improved the affinity of the second class of CCK8 analogues (compounds *cyclo*-A4–*cyclo*-A7) towards CCK_1. Unfortunately, no macroscopic difference is observed among the binding abilities of the new analogues towards CCK_1, but it is likely that the Phe^{33} aromatic ring of this class of compounds can be better docked in the binding site of the receptor target with respect to *cyclo*-CCK8. The evidence that all compounds present a binding affinity lower than CCK8 indicates that these compounds do not keep the three-dimensional requirements to interact with the entire CCK_1 receptor. Nevertheless, these observations can represent an important starting point to design new peptidomimetic analogues with improved affinity towards CCK_1 receptor.

4. Experimental

4.1. Peptide synthesis and characterization

Solid-phase peptide synthesis was performed on a fully automated Shimadzu (Kyoto, Japan) SPPS-8 synthesizer. Analytical RP-HPLC runs were carried out on a HP Agilent Series 1100 apparatus using a Phenomenex (Torrance, CA) C18 column, 4.6×250 mm with a flow rate of 1.0 mL min^{-1}. Preparative RP-HPLC was carried out on a Shimadzu 8A apparatus equipped with an UV Shimadzu detector using a Phenomenex (Torrance, CA) C18 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 (A) and CH_3CN 0.1% TFA (B), with a linear gradient from 20% to 80% B in 20 min. Mass spectral analyses were carried out on MALDI-TOF Voyager-DE mass spectrometer (Perseptive Biosystems).

The peptide synthesis was performed by solid phase method using the standard Fmoc procedures. PyBOP, HOBt, all Fmoc-amino acid derivatives and the 2-chlorotrityl chloride resin were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). All other chemicals were obtained from Aldrich (St. Louis, MI), Fluka (Milwaukee, WI) or LabScan (Stillorgan, Dublin, Ireland) and were used without further purification, unless otherwise stated.

For all compounds the first amino acid derivatives were loaded on a low concentration acid-labile solid support, the 2-chlorotrityl chloride resin (1.04 mmol/g substitution), according to a standard protocol (4 equiv of DIPEA in CH_2Cl_2). The loading was evaluated by Fmoc test. All couplings were performed twice for 30 min in DMF, by using an excess of 4 equiv for each amino acid

derivative activated in situ by the standard HOBt/PyBOP/DIPEA procedure. Amino acid couplings were monitored by Kaiser test.

All peptides were dissolved in an aqueous solution mixture (H_2O/D_2O 90%, pH 6.4) at concentration of approximately 1.5 mM. NMR experiments were acquired at 285 K by using a 600 MHz Varian Inova spectrometer (Istituto di Biostrutture e Bioimmagini, CNR, Napoli, Italy). One-dimensional (1D) NMR spectra were acquired using typically 64 scans with 32 K data size. Pulse programs of the standard Varian software library were used for the two-dimensional (2D) experiments. All 2D experiments were performed by the States–Haberkorn method and water suppression for spectra in H_2O was obtained by the selective echo refocusing by means of excitation sculpting.[20] Spin-system identification and assignment of individual peptide resonances were carried out by using a combination of 2D-TOCSY (total correlation spectroscopy)[21] and DQF-COSY (double quantum-filtered correlation spectroscopy).[22] Mixing times for 2D-NOESY (nuclear Overhauser effect spectroscopy) experiments were set from 100 to 450 ms.[23] 2D-TOCSY experiments were recorded with mixing times of 30 and 70 ms. The data were apodized with a square sine or Gaussian multiplication window functions and zero filled to a 1024 in f1 prior to Fourier transformation. Chemical shifts were referenced to TSP (3-(trimethylsilyl)-propionic acid-D4, sodium salt) and measurements of coupling constants were obtained from 1D and DQF-COSY spectra. Data were transformed with the standard Varian software and processed with the CARA program.[24]

4.2. Compounds *cyclo*-A1–*cyclo*-A3 and *cyclo*-A8

After the first amino acid derivative, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-βAla-OH, Fmoc-Gly-OH for compounds *cyclo*-A1, *cyclo*-A2, *cyclo*-A3 and *cyclo*-A8, respectively, was attached to the resin (0.50 mmol/g calcd substitution; 0.150 g resin; 0.075 mmol scale), the peptide chains were elongated by sequential coupling and Fmoc deprotection of Fmoc-Phe-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Met-OH, Fmoc-Trp(Boc)-OH, Fmoc-Dap(Mtt)-OH, Fmoc-Met-OH, Fmoc-Tyr(SO_3H)-OH, Boc-Asp(O^{t}Bu)-OH. Concerning compound *cyclo*-A8, the amino acid derivative for the second coupling was Fmoc-Ala-OH.

The linear peptides were cleaved from the solid support and the Dap residue was simultaneously freed from its protecting group (Mtt) by suspending the resin in a mixture of TFA/DCM/TIS (1:98:1, 1 mL total volume) for 1 min. The resin was then filtered and the filtrate poured into a 10% solution of pyridine in CH_3OH. This procedure was repeated 10 times. After the combined filtrate was concentrated under reduced pressure, the crude product was isolated by precipitation into cold water, collected by centrifugation and dried in vacuo (P_2O_5 pellets). The N ↔ C cyclisation, between the C-terminal carboxylic group and the Dap β-amine function, was performed by dissolving the protected peptides in CH_2Cl_2 (final concentration 10^{-4}M) and using PyBOP/DIPEA C-activation with stirring for 4 h. The cyclised peptides were then freed from their protecting group by treatment with a mixture of TFA/EDT/H_2O (94:4:2) for 3 h. The crude products were analysed and purified by using RP-HPLC, the main peaks of the analytical chromatogram were confirmed by MALDI-Tof mass spectral and by ^{1}H NMR analysis.

^{1}H NMR spectral data were indicated in the form: name of aminoacid, δ (ppm).

cyclo-A8: Asp26: 4.10 (Hα), 2.70/2.67 (Hβ); Tyr(SO_3H) 27: 8.26 (HN), 4.50 (Hα), 3.2 (Hβ), 7.11(Hδ), 6.6 (Hε); Met28: 8.20 (HN), 4.25 (Hα), 1.75 (Hβ), 2.22 (Hγ), 1.89 (Hε); Dap29: 8.07 (HN), 4.33 (Hα), 3.63/3.50 (Hβ), 7.87 (NHβ); Trp30: 8.16 (HN), 4.56 (Hα), 3.2 (Hβ), 7.23 (Hδ1), 7.47 (Hε3), 10.5 (Hε1), 7.17 (Hζ3), 7.57 (Hζ2), 7.23 (Hη2); Met31: 8.0 (HN), 4.18 (Hα), 2.0 (Hβ), 1.71 (Hγ), 1.58 (Hε); Asp32: 8.14 HN, 4.20 (Hα), 2.73 (Hβ); Ala33: 8.09 (HN), 4.20 (Hα), 1.09 (Hβ), Gly34: 8.10 (HN), 3.89/3.78 (Hα).

t_R = 17.213; $[M-H]^-$ = 1225.20 (calcd = 1225.46); final HPLC purity ≈95%.

cyclo-A1: Asp26: 4.11 (Hα), 2.72/2.60 (Hβ); Tyr(SO_3H) 27: 8.28 (HN), 4.50 (Hα), 3.1 (Hβ), 7.11 (Hδ), 6.6 (Hε); Met28: 8.18 (HN), 4.25 (Hα), 1.74 (Hβ), 2.23 (Hγ), 1.95 (Hε); Dap29: 8.0 (HN), 4.32 (Hα), 3.62/3.52 (Hβ), 7.82 (NHβ); Trp30: 8.07 (HN), 4.40 (Hα), 3.3 (Hβ), 7.21 (Hδ1), 7.54 (Hε3), 10.2 (Hε1), 7.11 (Hζ3), 7.53 (Hζ2), 7.22 (Hη2); Met31: 8.00 (HN), 4.21 (Hα), 2.10 (Hβ), 1.70 (Hγ), 1.66 (Hε); Asp32: 8.16 HN, 4.33 (Hα), 2.60 (Hβ); Phe33: 8.19 (HN), 4.25 (Hα), 3.20/3.10 (Hβ), 7.20 (Hδ1), 7.28 (Hε1), 7.30 (Hζ); Ala34: 8.10 (HN), 4.4 Hα, 1.2 (Hβ).

t_R = 16.430; $[M-H]^-$ = 1211.78 (calcd = 1211.43); final HPLC purity ≈95%.

cyclo-A2: Asp26: 4.10 (Hα), 2.70/2.60 (Hβ); Tyr(SO_3H) 27: 8.24 (HN), 4.55 (Hα), 3.0 (Hβ), 7.10 (Hδ), 6.8 (Hε); Met28: 8.18 (HN), 4.27 (Hα), 1.79 (Hβ), 2.28(Hγ), 1.90 (Hε); Dap29: 8.0 (HN), 4.35 (Hα), 3.62/3.52 (Hβ), 7.82 (NHβ); Trp30: 8.07 (HN), 4.48 (Hα), 3.62/3.52 (Hβ), 7.20 (Hδ1), 7.50 (Hε3), 10.3 (Hε1), 7.10 (Hζ3), 7.50 (Hζ2), 7.20 (Hη2); Met31: 8.08 (HN), 4.20 (Hα), 2.07 (Hβ), 1.65 (Hγ), 1.79 (Hε); Asp32: 8.16 HN, 4.30 (Hα), 2.60 (Hβ); Phe33: 8.23 (HN), 4.30 (Hα), 3.22/3.18 (Hβ), 7.20 (Hδ1), 7.31 (Hε1), 7.29 (Hζ); Gly34: 7.95 (HN), 3.97/3.62 (Hα).

t_R = 17.123; $[M-H]^-$ = 1225.31 (calcd = 1225.46); final HPLC purity ≈95%.

cyclo-A3: Asp26: 4.18 (Hα), 2.67/2.61 (Hβ); Tyr(SO_3H) 27: 8.23 (HN), 4.53 (Hα), 3.3 (Hβ), 7.12 (Hδ), 6.4 (Hε); Met28: 8.23 (HN), 4.27 (Hα), 1.71 (Hβ), 2.20 (Hγ), 1.90 (Hε); Dap29: 8.1 (HN), 4.30 (Hα), 3.60/3.50 (Hβ), 7.81 (NHβ); Trp30: 8.10 (HN), 4.46 (Hα), 3.1 (Hβ), 7.21 (Hδ1), 7.52 (Hε3), 10.2 (Hε1), 7.12 (Hζ3), 7.55 (Hζ2), 7.25 (Hη2); Met31: 8.12 (HN), 4.10 (Hα), 2.11 (Hβ), 1.77 (Hγ), 1.68 (Hε); Asp32: 8.20 HN, 4.35 (Hα), 2.70 (Hβ); Phe33: 8.12 (HN), 4.15 (Hα), 3.22/3.12 (Hβ),

7.21 (Hδ1), 7.29 (Hε1), 7.32 (Hζ); βAla; 8.10 (HN), 3.99/3.97 (Hα), 3.78 (Hβ).

t_R = 12.892; $[M-H]^-$ = 1135.93 (calcd = 1135.33); final HPLC purity ≈95%.

4.3. Compounds *cyclo*-A4–*cyclo*-A7

Fmoc-Gly-OH was the first amino acid derivative attached to the resin (0.50 mmol/g calcd substitution; 0.150 g resin; 0.075 mmol scale) for each compound, then Fmoc-Lys(*o*-NBS)-OH, Fmoc-Orn(*o*-NBS)-OH and Fmoc-Dab(*o*-NBS)-OH, Fmoc-Dap(*o*-NBS)-OH, previously synthesised by using the already published protocol,[15] were coupled in order to prepare compounds *cyclo*-A4, *cyclo*-A5, *cyclo*-A6 and *cyclo*-A7, respectively. The next step consisted in the benzylation reaction performed in DMF containing 2 equiv of benzyl bromide and K_2CO_3. The peptide chains were then elongated by sequential coupling and Fmoc deprotection of Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Met-OH, Fmoc-Trp(Boc)-OH, Fmoc-Dap(Mtt)-OH, Fmoc-Met-OH, Fmoc-Tyr(SO_3H)-OH and Boc-Asp(O^{t}Bu)-OH. After the complete assembly of the peptide chain, the *o*-NBS protecting group was removed using DBU (10 equiv) and 2-mercapto-ethanol (5 equiv) in DMF with stirring for 30 min.

Following the procedures described in the previous paragraph, the peptides were cleaved from the solid support, cyclised and freed from all protecting. After RP-HPLC purification, mass spectrometry and ^{1}H NMR analysis confirmed the obtainment of the desired compounds.

^{1}H NMR spectral data were indicated in the form: name of aminoacid, δ (ppm).

cyclo-A4: Asp26: 4.14 (Hα), 2.77/2.66 (Hβ); Tyr(SO_3H) 27: 8.60 (HN), 4.53 (Hα), 2.98 (Hβ), 7.11 (Hδ), 6.81 (Hε); Met28: 8.28 (HN), 4.37 (Hα), 1.82 (Hβ), 2.35/2.30 (Hγ), 1.94 (Hε); Dap29: 8.11 (HN), 4.38 (Hα), 3.56 (Hβ), 7.89 (Hβ); Trp30: 8.02 (HN), 4.60 (Hα), 3.28 (Hβ), 7.20 (Hδ1), 7.56 (Hε3), 10.5 (Hε1), 7.13 (Hζ3), 7.46 (Hζ2), 7.21 (Hη2); Met31: 8.11 (HN), 4.17 (Hα), 1.82/1.68 (Hβ), 1.99/1.91(Hγ), 2.0 (Hε); Asp32: 8.16 HN, 4.30 (Hα), 2.62/2.65(Hβ); Lys33(Bn): 8.27 (HN), 4.0 (Hα), 1.87/1.76 (Hβ), 1.46 (Hγ), 1.70 (Hδ), 3.02 (Hε), 7.98 (NHε), 7.30–7.15 (H-Bz); Gly34: 8.0 (HN), 3.94/3.80. (Hα).

t_R = 17.92; $[M-H]^-$ = 1283.95 (calcd = 1282.49); final HPLC purity ≈95%.

cyclo-A5: Asp26: 4.16 (Hα), 2.76/2.56 (Hβ); Tyr(SO_3H) 27: 8.58 (HN), 4.51 (Hα), 2.98 (Hβ), 7.12 (Hδ), 6.84 (Hε); Met28: 8.27 (HN), 4.31 (Hα), 1.80 (Hβ), 2.35/2.30 (Hγ), 1.95 (Hε); Dap29: 8.12 (HN), 4.33 (Hα), 3.58 (Hβ), 7.90 (NHβ); Trp30: 8.17 (HN), 4.57 (Hα), 3.28 (Hβ), 7.24 (Hδ1), 7.56 (Hε3), 10.2 (Hε1), 7.14 (Hζ3), 7.44 (Hζ2), 7.23 (Hη2); Met31: 8.12 (HN), 4.14 (Hα), 1.83/1.67 (Hβ), 1.99/1.92 (Hγ), 2.1 (Hε); Asp32: 8.15 HN, 4.33 (Hα), 2.70/2.68 (Hβ); Orn33(Bn): 8.19 (HN), 3.98 (Hα), 1.90/1.85 (Hβ), 1.70 (Hγ), 3.5 (Hδ), 7.80 (NHδ), 7.31–7.10 (H-Bz); Gly34: 8.2 (HN), 4.07/3.99. (Hα).

t_R = 17.32; $[M-H]^-$ = 1268.21 (calcd = 1268.47); final HPLC purity ≈95%.

cyclo-A6: Asp26: 4.13 (Hα), 2.89 (Hβ); Tyr(SO_3H)27: 8.50 (HN), 4.61 (Hα), 3.01 (Hβ), 7.10 (Hδ), 6.67 (Hε); Met28: 8.22 (HN), 4.23 (Hα), 1.80 (Hβ), 2.33/2.30 (Hγ), 1.93 (Hε); Dap29: 8.00 (HN), 4.23 (Hα), 3.59 (Hβ), 7.90 (NHβ); Trp30: 8.17 (HN), 4.54 (Hα), 3.22 (Hβ), 7.21 (Hδ1), 7.56 (Hε3), 10.2 (Hε1), 7.12 (Hζ3), 7.45 (Hζ2), 7.21(Hη2); Met31: 8.11 (HN), 4.11 (Hα), 1.83/1.66 (Hβ), 1.99/1.90 (Hγ), 2.2 (Hε); Asp32: 8.15 HN, 4.31 (Hα), 2.70/2.68 (Hβ); Dab33(Bn): 8.20 (HN), 4.05 (Hα), 3.67/3.59 (Hβ) 3.7 (Hγ).

7.85 (NHγ) 7.31–7.10 (H-Bz); Gly34: 8.18 (HN), 4.0/3.89. (Hα).

t_R = 17.51; $[M-H]^-$ = 1254.65 (calcd = 1254.44); final HPLC purity ≈95%.

cyclo-A7: Asp26: 4.15 (Hα), 2.87 (Hβ); Tyr(SO_3H)27: 8.53 (HN), 4.63 (Hα), 2.98 (Hβ), 7.11 (Hδ), 6.68 (Hε); Met28: 8.27 (HN), 4.22 (Hα), 1.70 (Hβ), 2.05/2.30 (Hγ), 1.90 (Hε); Dap29: 8.00 (HN), 4.20 (Hα), 3.55 (Hβ), 7.90 (NHβ); Trp30: 8.15 (HN), 4.51 (Hα), 3.28 (Hβ), 7.24 (Hδ1), 7.51 (Hε3), 10.2 (Hε1), 7.11 (Hζ3), 7.40 (Hζ2), 7.20 (Hη2); Met31: 8.07 (HN), 4.16 (Hα), 1.85/1.63 (Hβ), 1.99 (Hγ), 2.3 (Hε); Asp32: 8.17 HN, 4.36 (Hα), 2.77/2.67 (Hβ); Dap33(Bn): 8.25 (HN), 3.98 (Hα), 3.62/3.55 (Hβ) 7.90 (NHβ); 7.33–7.12 (H-Bz); Gly34: 8.28 (HN), 3.95/3.88 (Hα).

t_R = 16.85; $[M-H]^-$ = 1239.14 (calcd = 1240.42); final HPLC purity ≈95%.

4.4. 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_1 receptor. 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]DTPAGlu-Gly-[Tyr^{27}(SO_3H)]-CCK8 were incubated with cells in the presence of each compound at concentrations ranging from 10^{-8} to 10^{-1} 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 compound.

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