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Pro-Pro Amide Bond Configuration and the Immunosuppressive Activity of Cyclolinopeptide A

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The comparison of conformational preferences of cyclolinopeptide A (CLA) analogues containing the twisted *cis*-amide bond mimetics, based on CD, molecular modeling, and their immunosuppressive activity was performed. The results suggest that there exists a twisted *cis*-amide bond between two proline residues in the biologically active conformation of CLA.

Key words: *cis*-amide bond mimetics, *cis*/*trans* isomerization, cyclophilin A, cyclolinopeptide A analogues

Recently we introduced 3-hydroxy- and 3-oxo- derivatives of 4-amino-1-cyclohexanecarboxylic acid as mimetics of the dipeptide moiety with a twisted *cis*-amide bond. Previously we presented syntheses of all stereoisomers of c-4-amino-t-3--hydroxy-r-1-cyclohexanecarboxylic acid and *cis*-4-amino-3-oxo-1-cyclohexanecarboxylic acid [1], as well as t-4-amino-c-3-hydroxy-r-1-cyclohexanecarboxylic acid and *trans*-4-amino-3-oxo-1-cyclohexanecarboxylic acid [2]. Our aim was to obtain the structural analogues of cyclolinopeptide A (CLA) – a nonapeptide, isolated from linseed, with a sequence c-(Leu-Ile-Ile-Leu-Val-**Pro-Pro**-Phe-Phe), which exhibits a distinct immunosuppressive activity [3]. The Pro-Pro fragment of CLA in the analogues was exchanged for these mimetics. The analysis of the X-ray structure of CLA [4] as well as of its tyrosine analogue [5] showed that the peptide crystallizes in the form possessing the *cis*-amide bond between two proline residues. However, in $CDCl_3$ solution of CLA there exist both *cis*- and *trans*- isomers of the Pro-Pro moiety of the peptide, and in the NMR spectra of CLA-Ba^{+2} complex only the *trans*-isomer is observed [6].

The molecular mechanism of immunosuppressive action of CLA consists – like the mechanism of action of known immunosuppressor, cyclosporin A (CsA) – in the loss of enzymatic activity of calcineurin via the formation of cyclophilin A (CyPA) – CLA – calcineurin complex [7]. The CLA target protein – CyPA is one of peptidyl-prolyl *cis*/*trans* isomerases (PPI-ases), the enzymes that catalyze *cis*/*trans* isomerization of the Xaa-Pro amide bond. The peptide substrates accept the nonplanar

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cis-amide bond conformation, on forming complexes with PPI-ases, with ω angle up to 45° in their Xaa-Pro fragment [8]. On purpose to see if such a conformation within the Pro-Pro fragment of CLA is of importance for the biological effect of CLA, we synthesized series of linear CLA analogues in which the Pro-Pro fragment was exchanged for (1R, 3S, 4S)-, (1S, 3R, 4R)-, (1R, 3R, 4R)-, and (1S, 3S, 4S)- stereoisomers of 4-amino-3-hydroxy-1-cyclohexanecarboxylic acid (we denote them as ct(R)AHC, ct(S)AHC, tc(R)AHC, and tc(S)AHC, respectively). In this way the following linear octapeptides were prepared:

Val-ct(R)AHC-Phe-Phe-Leu-Ile-Ile-Leu-OH Ia
Val-ct(S)AHC-Phe-Phe-Leu-Ile-Ile-Leu-OH IIa
Val-tc(R)AHC-Phe-Phe-Leu-Ile-Ile-Leu-OH IIIa
Val-tc(S)AHC-Phe-Phe-Leu-Ile-Ile-Leu-OH IVa

The peptides were subjected to cyclization. This operation being successful in the case of peptides containing ct(R)AHC and ct(S)AHC, but failing in the case of peptides with tc(R)AHC and tc(S)AHC (tcAHC residues induce the extended conformations of the peptide chain, what disfavors the cyclization).

The obtained cyclic peptides were oxidized at the 3-hydroxyl group of AHC residues with pyridinium dichromate (PDC), giving the peptides with (1R, 4S)- and (1S, 4R)- enantiomers of the 4-amino-3-oxo-1-cyclohexanecarboxylic acid residue (c(R)AOC and c(S)AOC, respectively).

Thus, the following cyclic peptides were obtained:

c-(-Val-ct(R)AHC-Phe-Phe-Leu-Ile-Ile-Leu-) Ib
c-(-Val-ct(S)AHC-Phe-Phe-Leu-Ile-Ile-Leu-) IIb
c-(-Val-c(R)AOC-Phe-Phe-Leu-Ile-Ile-Leu-) Ic
c-(-Val-c(S)AOC-Phe-Phe-Leu-Ile-Ile-Leu-) IIc

The syntheses of all these peptides are described in [2].

In this paper the biological tests, CD measurements, and molecular modeling studies, performed on the peptides shown above, are presented. They lead to the conclusion that the nonplanar *cis*-amide bond conformation within the -Pro-Pro- fragment of CLA can be really needed for biological activity of these compounds.

EXPERIMENTAL

The syntheses of investigated CLA analogues were described in our previous publication [2]. CD spectra were recorded on a Jasco J-600 spectropolarimeter. All spectra were recorded at room temperature in methanol. Path lengths of 1 mm and 10 mm were used for the peptide and aromatic region, respectively. Concentrations of the solutions were 0.06–0.09 mg/ml for the peptide region and 0.2–0.5 mg/ml for the aromatic region. Data are presented as molar ellipticity [Θ]. The immunosuppressive activity of all analogues was tested using "plaque forming cells" (PFC) *in vitro* test; this test was performed according to Mishell and Dutton modification of Jerne's procedure [9]. Details of this test are described in ref. [3]. Molecular modeling simulations of the cyclic CLA analogues were performed using an AMBER force field (program Hyper Chem 4.5). As a starting structure we chose the X-ray structure of CLA from Cambridge Structural Database (code: GIPCAR10 [4]). This structure, after insertion of hydrogen atoms (not present in the X-ray structure) in geometrically predicted positions, was optimized using AM1 semiempirical method and then using AMBER force field, with charges on atoms from AM1 method and dielectric constant 78.4. The obtained conformation was very similar to the starting one. The models of analogues Ic

and IIc were obtained, from the optimized conformation of CLA, by replacement of the -Pro-Pro- fragment by the c(R)AOC and c(S)AOC residues, respectively. For the c(R)AOC and c(S)AOC residues there are two possible conformations as a result of the ring inversion, so we took both of them into consideration, and we created two models of Ic and two models of IIc analogue. After replacement of the -Pro-Pro- fragment by the mimetic the structures were optimized again using AMBER force field.

The model of CLA with the nonplanar ($\omega = 90°$) Pro-Pro amide bond was also generated by optimization with a fixed ω torsion angle and used in the analysis.

RESULTS AND DISCUSSION

All the peptides indicated were tested for their immunosuppressive activity in the humoral "plaque forming cell" (PFC) *in vitro* test. The results of these experiments are shown in Fig. 1. In the experiments cyclosporin A (CsA) was used as the reference. All the peptides are distinctly less active than CsA in this test. Because the activity of CLA in this test at low doses is comparable to that of CsA (see ref. [3]), the results show that the exchange of the Pro-Pro moiety in CLA for AHC or AOC in the analogues reduces the immunosuppressive activity of the peptide. However, a total lack of the activity was not observed for any analogue. It is also of note that the activity of linear tcAHC analogues exceeds that of ctAHC analogues. The cyclization of peptides increases the immunosuppressive activity (see Ib and IIb). However, oxidized cyclic peptide IIc is the least active in the whole series, whereas its counterpart Ic is the most active. The activity depends distinctly on a configuration of the mimetic residue, all the analogues with the (R) configuration on the C1 atom of AHC or AOC being much more potent than the analogues with enantiomeric residues. The results show that the mimetics which mimic CLA with the twisted *cis*-amide bond between two proline residues can, like CLA, produce immunosuppressive effects. It suggests that CLA in complexes with PPI-ases may posses the twisted Pro-Pro *cis*-amide bond.

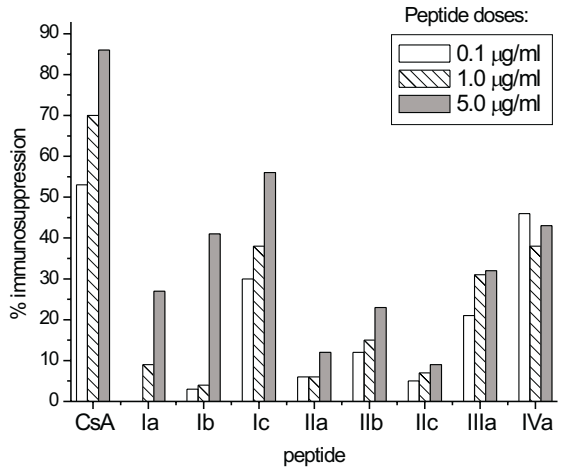

Figure 1. The results of PFC *in vitro* test of the linear and cyclic CLA analogues (% immunosuppression = 100*(1-exp*control^{-1}); exp – number of PFC for analogue; control – number of PFC for control).

Circular dichroism (CD) measurements: The CD spectra of cyclic peptides Ib, IIb, Ic, IIc, and CLA in methanol are shown in Figs. 2 and 3. It can be seen from Fig. 2 that the overall conformation of IIc (the analogue containing the c(S)AOC residue) is the most similar to the CLA conformation from the whole investigated series. However, this compound was of the lowest immunosuppressive activity. In the spectrum of Ic the shoulders on the CD curve corresponding with the maxima on CLA CD curve are visible. However, they are superimposed on the strong negative effect below 200 nm, what can indicate a dominance of unordered conformations in the conformational equilibrium [10]. Unordered conformations also seem to dominate in the case of peptides Ib and IIb.

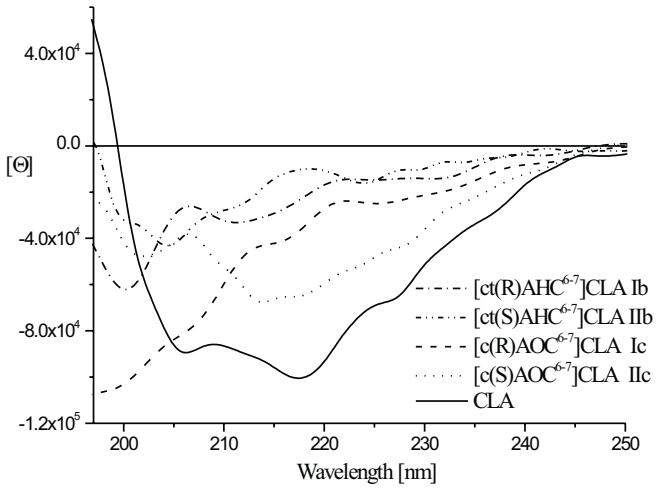

Figure 2. The CD spectra of CLA and its cyclic analogues Ib, IIb, Ic, and IIc in the amide region.

The aromatic region of the CD spectra (Fig. 3) in the case of Ic and IIc is dominated by the effects connected to the $n \rightarrow \pi^*$ transition of the carbonyl group of AOC residues. The signals connected to the $1L_b$ absorption band of Phe residues are superimposed on the negative (in the case of IIc) or positive (in the case of Ic) background, resulted from the $n \rightarrow \pi^*$ carbonyl transition. It is, however, of interest that in the case of [ct(S)AHC^{6,7}]CLA (IIb) and [ct(R)AHC^{6,7}]CLA (Ib) the aromatic CD bands are – in comparison with CLA – strongly reduced. It supports the conclusion expressed above that these peptides are characterized by strong conformational flexibility.

It was found by us that the protected ctAHC residue exists in the chair conformation in the solid state, with the equatorial amino- and hydroxy- groups and the axial carboxyl group [1,2]. NMR experiments suggest that the same conformation predominates in solution. In the case of the protected cAOC residue the NMR experiments suggest similar conformational preferences, *i.e.* the chair conformation with the equatorial amino- and axial carboxyl group [1].

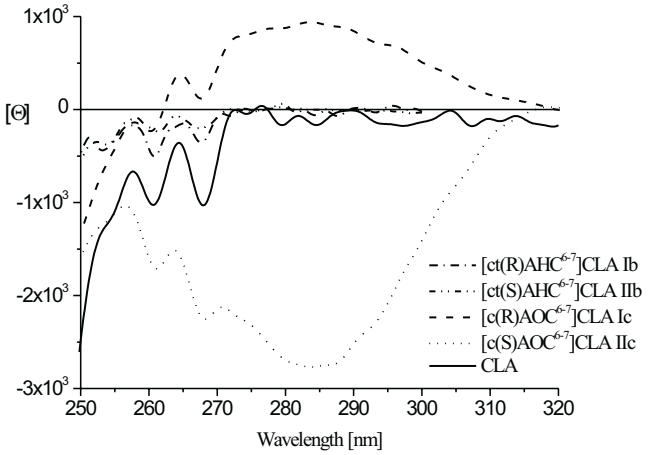

Figure 3. The CD spectra of CLA and its cyclic analogues Ib, IIb, Ic, and IIc in the aromatic region.

The application of the octant rule for the respective fragments of cAOC-CLA analogues suggests, however, that the conformations of cAOC residues after incorporation into the cyclic peptides must be different: equatorial for the carboxyl group and axial for the amino- group. Only in such a case the signs of Cotton effects, predicted for the $n \rightarrow \pi^*$ carbonyl transition of cAOC residues, agree with the observed ones for Ic and IIc (see Figs. 4 and 5).

Molecular modeling studies: The comparison of the spatial structure of investigated analogues with that of CLA was performed by using the X-ray data (from CLA structure collected in Cambridge Structural Database) as a base. A geometry of the starting structure of CLA was optimized after introduction of H atoms. After the ex-

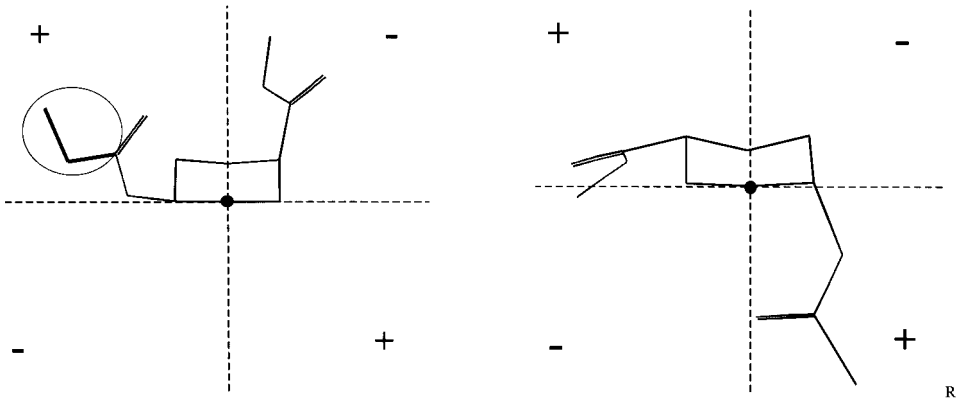

Figure 4. The prediction of the Cotton effect sign for the cyclic peptide with the c(R)AOC residue (Ic). Projections of the c(R)AOC residue along C=O bond with axial (left) and equatorial (right) carboxamide group are shown. The rear octant signs are shown, fragment in the circle is located in the front octant.

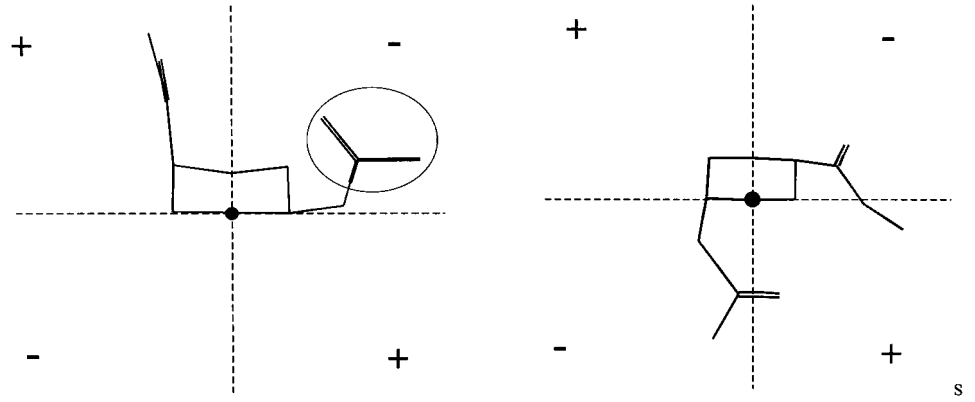

Figure 5. The prediction of the Cotton effect sign for the cyclic peptide with the c(S)AOC residue (IIc). Projections of the c(S)AOC residue along C=O bond with axial (left) and equatorial (right) carboxamide group are shown. The rear octant signs are shown, fragment in the circle is located in the front octant.

change of the Pro-Pro fragment by c(R)AOC or c(S)AOC residues (in the optimized structure) the geometry optimization was performed again. The results of comparison of the optimized structures of CLA and cyclic analogues are shown in Figs. 6–8. It can be seen from Figs. 6 and 7 that in the case when the carboxamide substituent of c(S)AOC and c(R)AOC occupies an equatorial position, what was predicted from the CD spectra, the geometry of the CLA -Pro-Pro- fragment with a *cis*-amide bond (especially C=O bonds positions) is better reflected by c(S)AOC. This is in a good agreement with the CD spectra of the amide bond region, because the CD spectrum of the analogue with c(S)AOC is most similar to the spectrum of CLA. On the other hand, this analogue has the lowest biological activity from the whole series. However, when we compared the same models of analogues with the CLA structure with a nonplanar Pro-Pro amide bond (Figs. 8 and 9) the results were completely different – in the case of the analogue with the c(R)AOC residue all C=O bonds appear now in the proximity and are oriented in the same direction as in CLA, whereas for the analogue with the c(S)AOC residue the ketone group of this residue and the C=O group of the Pro-Pro amide bond are oriented in two different directions and are distant from each other.

Thus, the most biologically active analogue of CLA demonstrates the best fitting to the spatial model of CLA molecule with a twisted Pro-Pro amide bond. It suggests that the biologically active conformation of CLA may be really of such a nature. Our experiments also suggest that the spatial structure of the -Pro-Pro- fragment of CLA can be of greater importance for its biological activity than the conformation of remaining parts of the molecule.

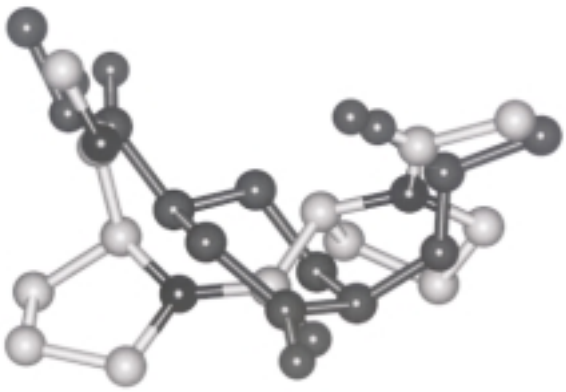

Figure 6. The superposition of optimized structures of CLA and its analogue with the c(S)AOC residue (equatorial carboxamide group); the -Pro-Pro- fragment and the c(S)AOC residue (black) are shown.

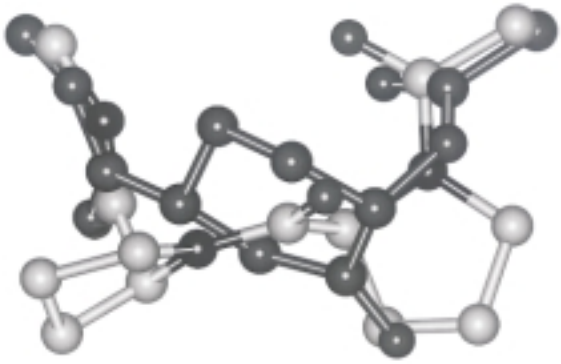

Figure 7. The superposition of optimized structures of CLA and its analogue with the c(R)AOC residue (equatorial carboxamide group); the -Pro-Pro- fragment and the c(R)AOC residue (black) are shown.

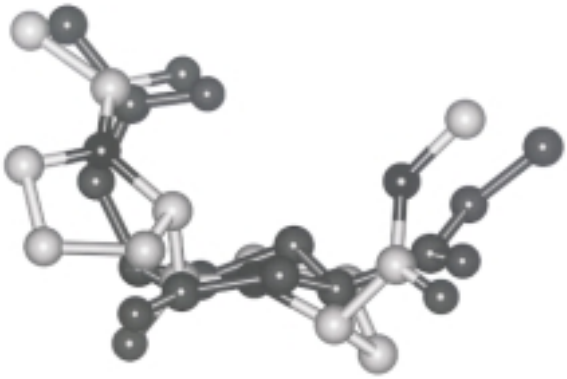

Figure 8. The superposition of optimized structures of CLA (with the twisted Pro-Pro amide bond, $\omega = 90°$) and its analogue with the c(R)AOC residue (equatorial carboxamide group); the -Pro-Pro- fragment and the c(R)AOC residue (black) are shown.

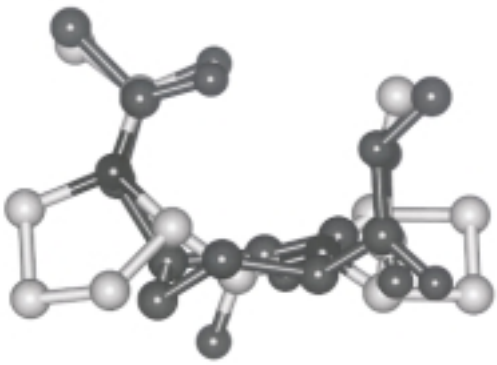

Figure 9. The superposition of optimized structures of CLA (with the twisted Pro-Pro amide bond, ω = 90°) and its analogue with the c(S)AOC residue (equatorial carboxamide group); the -Pro-Pro- fragment and the c(S)AOC residue (black) are shown.

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