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Review

Cyclopeptides of *Linum usitatissimum*

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Abstract: Cyclolinopeptide A (CLA), a cyclic nonapeptide from linseed, possesses strong immunosuppressive and antimalarial activity along with the ability to inhibit cholate uptake into hepatocytes. The structure of the peptide was studied extensively in solution as well as in the solid state. It is postulated that both the Pro–Pro *cis*-amide bond and an 'edge-to-face' interaction between the aromatic rings of two adjacent Phe residues are important for biological activity. Structure–activity relationship studies of many linear and cyclic analogues of CLA suggest that the Pro-Xxx-Phe sequence and the flexibility of the peptide are important for the immunosuppressive activity. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: edge-to-face; cyclolinopeptide; cyclosporin; immunomodulation; immunosuppression

DISCOVERY

In 1959, cyclolinopeptide A (CLA) was isolated by Kaufmann and Tobschirbel from the sediments deposited from crude linseed oil [1]. Ten years later, Prox and Weygand determined the primary structure of CLA as a cyclic hydrophobic nonapeptide with the following sequence [2]:

cyclo(Pro^1-Pro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)

Another similar cyclic nonapeptide named CLB was isolated in Weygand's laboratory. It differs from CLA by the presence of a Met residue and has the following sequence: cyclo (-Ile-Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-) [3]. The syntheses of both of these peptides were performed by Obermeier. The linear precursors of the peptides with a *C*-terminal Leu^6 residue were cyclized by the azide activation method [4]. CLB was rediscovered by Morita *et al.* at a later time [5]. The same research group described other cyclic peptides from Linum [6,7]:

CLC cyclo (-Pro-Pro-Phe-Phe-Val-Ile-Mso-Leu-Ile-)
CLD cyclo (-Pro-Phe-Phe-Trp-Ile-Mso-Leu-Leu-)
CLE cyclo (-Pro-Leu-Phe-Ile-Mso-Leu-Val-Phe-)
CLF cyclo (-Pro-Phe-Phe-Trp-Val-Mso-Leu-Mso-)
CLG cyclo (-Pro-Phe-Phe-Trp-Ile-Mso-Leu-Mso-)
CLH cyclo (-Pro-Phe-Phe-Trp-Ile-Mso-Leu-Met-)
CLI cyclo (-Pro-Phe-Phe-Trp-Val-Met-Leu-Mso-)

Mso – methionine sulfoxide

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At the same time Picur *et al.* isolated from *Linum album* a cyclic peptide CLX, containing non-proteinaceous amino acid *N*-methyl-4-aminoproline [8]. To evaluate the configuration of the residue X, the circular dichroism (CD) spectra of natural CLX was compared with the spectra of the cyclic peptides containing all four stereoisomers of the amino acid. It followed from the investigations that the X residue in the natural peptide corresponds to (2S, 4R) *N*-methyl-4-aminoproline (see Figure 1) [9].

STRUCTURE

The conformation of CLA in solution was studied by CD and NMR spectroscopy, and conformational energy calculations by Naider *et al.*, Brewster *et al.*, and Tonelli, respectively [10–12]. The conformational preferences predicted in this early work were not confirmed by later investigations. Naider *et al.* pointed at the structural similarity of CLA and antamanide, a cyclic decapeptide present in *Amanita phalloides* tissue but concluded that CLA is much more flexible in solution than antamanide. Later Siemion *et al.* found evidence for the existence of a *cis*-amide bond between the two Pro residues in the CLA molecule [13]. This proposition was supported by X-ray studies

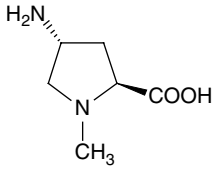

Figure 1 The structure of the 'X' residue of cyclolinopeptide X (CLX) [9].

by Di Blasio *et al.* The authors concluded that 'the solid state and solution conformations of CLA are essentially identical, even if this cyclic system tends to give rise to a complex mixture of quasi-isoenergetic conformations, favoured by the flexibility of the ring enhanced by the isomerism of the Pro–Pro bond and by the polar solvents [14]. The details of the crystal structure of CLA are summarized in the recent article of Benedetti and Pedone [15]. The structure of CLA in several solvents was studied by NMR methods by Tancredi *et al.* [16]. At room temperature 1H-NMR spectra show very broad lines, indicating the presence of chemical exchange among several conformers. However, studies in $CDCl_3$ solution at low temperatures allowed freezing a single conformational state consistent with the main features of the solid-state structure. The X-ray study of CLA was also performed by Neela *et al.* [17] and its conformation in d_6-DMSO solution via NMR by Raghothama *et al.* [18]. A structure very similar to the crystal-state structures of CLA was also obtained in a case of its [Aib^{5,6}, D-Ala^{8}]CLA analogue. However, the presence of two α-amino-isobutyric acid (Aib) residues results in a very significant enhancement of molecular rigidity of the compound, even in solution [19]. The X-ray structure of [Tyr^{4}]CLA could also be superimposed upon that of CLA [20]. A different structure was found for CLX, where (2S, 4R) *N*-methyl-4-aminoproline residue closes a ring formed by the – Pro-Pro-Phe-Phe-Ile-Leu-Leu- sequence. The X residue plays the role of a dipeptide moiety with a non-planar *cis*-peptidomimetic bond. All other amide bonds are in the trans configuration [9]. Distance-geometry calculations and molecular dynamics simulations were also applied in the investigation of the conformation of CLA and the results for the most stable conformations resembled those in the crystal state [21]. Molecular dynamics simulations *in vacuo* and in solution performed by Saviano *et al.* showed that CLA exists in water in several conformations favoured by the intrinsic flexibility of the molecule caused by the *cis*-trans isomerism of the two Xxx-Pro amide bonds. The results of the simulations for a periodic crystal were in agreement with the X-ray data and NMR data in $CDCl_3$ solution at low temperature [22].

The introduction of the $C^{\alpha\alpha}$- dialkylated glycine residue into the peptide sequence (achieved by the synthesis of cyclo (-Pro-Pro-Phe-Phe-Ac_6c-Ile-D-Ala-Val-), where Ac_6c is 1-aminocyclohexane-1-carboxylic acid) resulted in decreased flexibility of the peptide in solution [23]. In the crystal state, the peptide possesses a 'banana-twisted conformation with a *cis*-amide bond between the two Pro residues' [24].

An interesting feature of the CLA conformation is the reciprocal orientation of the aromatic rings of the two Phe residues. The rings are perpendicular to each other, forming an edge-to-face interaction [25]. The experiments with tyrosine analogues of CLA unequivocally showed that the residue in the position 4 plays a role of the 'edge' and the one in position 3 a role of the 'face' in this interaction. It is also of interest that in the CD spectra the residue 3 is optically active and that in position 4 inactive, probably due to the different side chain conformation of both aromatic residues. This effect was not observed in the case of linear precursors of CLA [26].

As we have already noted, CLA can be considered as an analogue of a cyclic decapeptide, antamanide, which is known for its ability to form complexes with metal ions of IA and IIA groups [27]. In the case of CLA, this tendency for metal ions complexation is strongly reduced [28]. However, the conductivity measurements showed that CLA forms a 1 : 1 complex with potassium cation in methanol solution [29]. NMR and CD conformational studies performed by Tancredi *et al.* show that complexes of CLA with Ba^{+2} ions are stronger than those with K^{+}, Na^{+}, Mg^{+2}, and Ca^{+2}. CD data indicate that two types of such complexes are present in acetonitrile solution depending on the concentration: 1 : 2 sandwiches and 1 : 1 equimolar complexes. NMR data were consistent with an equimolar form [30]. Addition of bivalent metal ions to the CLA solution induces drastic structural changes. In the equimolar Ba^{+2}/CLA complex the peptide backbone contains all-trans peptide bonds and the global shape of the complexed peptide can be described as a bowl with a concave (polar) side hosting Ba^{+2} and the opposite convex side predominantly apolar. Zanotti *et al.* studied a structural analogue of CLA called CYS7 and found that the peptide preferentially binds calcium ions forming an equimolar complex similar in its overall shape to that of Ba^{+2}/CLA complex with a clear separation of two hydrophobic/hydrophilic surfaces [31]. The conformational features of both free and Ca^{+2}-complexed CLA analogues: cyclo-[Pro-Phe-Phe-Ala-Xaa]$_2$ have been recently determined by NMR spectroscopy and extensive distance-geometry calculations. The Ca^{+2}-complexed peptides presented two *cis*-peptide bonds and were generally similar to those observed for the metal-complexed forms of antamanide and related analogues [32].

BIOLOGICAL ACTIVITY

The original role of CLA in linseed remains unknown. The first biological activity found for this peptide was its ability to inhibit cholate uptake into hepatocytes. In this regard, CLA resembles antamanide and somatostatin. The -Pro-Phe-Phe- tripeptide block was identified as a sequence responsible for this activity [33]. This effect was also studied by Rossi *et al.* [34]. In 1991 Siemion *et al.* reported that CLA possesses a strong immunosuppressant activity [35]. Details of this finding were presented in a paper by Wieczorek *et al.* [36]. The influence

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of CLA on the humoral response was determined by the plaque forming cell (PFC) test and the influence on the cellular immune response by the delayed-type hypersensitivity (DTH) test. It was also found that CLA influences human lymphocyte proliferation *in vitro* and tempers post adjuvant polyarthritis in rats and haemolytic anaemia of New Zealand Black mice. The effects of CLA in these tests were comparable with those exerted by a known immunosuppressant – cyclosporin A (CsA).

In 1997 Gaymes *et al.* found that CLA, along with CsA, inhibits calcium dependent activation of T lymphocytes. However, the CLA concentration required for complete inhibition was ten times as high as that of CsA. It was also demonstrated that calcineurin, a phosphatase involved in T-lymphocyte signalling, is inhibited by CLA by a mechanism dependent on cyclophilin A – one of the peptidyl-prolyl *cis-trans* isomerases. The direct binding of CLA to cyclophilin A was confirmed by the studies of tryptophane fluorescence and PPIase assays. This suggests that the molecular mechanism of CLA action is the same as that of CsA [37,38]. Gallo *et al.* who studied the binding of CLA to cyclophilin A, speculated that the CLA sequence – Val-Pro-Pro-Phe-Phe- was responsible for this interaction [39]. Using a hydrophilic CLA analogue: cyclo (-Ala-Lys-Pro-Phe-Phe-Ala-Lys-Pro-Phe-Phe-) Kemmer *et al.* isolated three hepatocellular peptide-binding proteins from the integral part of plasma membranes and the cytosol. They were identified as cytochromes P450IIC13 and P450IIC22, and 3-hydroxy-androgen-UDP-glucuronosyltransferase, proteins known for their ability to bind bile acids [40]. Some years ago, Bell *et al.* discovered the antimalarial activity of CLA and its analogues. It was found that the substitution of hydrophobic residues by less hydrophobic ones results in a decrease of the antimalarial activity although such peptides retained the immunosuppressive properties. This suggests that the antimalarial activity of CLA does not depend on its binding with cyclophilin-like receptors. The introduction of D-aromatic residues into the CLA molecule leads to a decrease in the immunosuppressive activity but has little effect on antimalarial activity [41].

Analogues

A great number of structural analogues of CLA were synthesized and examined for their immunosuppressant activity. On the basis of the investigation of rigid analogues of CLA, Benedetti and Pedone concluded that the flexibility of the peptide structure plays an important role in the biological function of this class of peptides [15]. The results of the Polish group working on this subject were revised by Siemion *et al.* The results obtained for other natural cyclic peptides displaying structural similarity with CLA, such as antamanide, cycloamanides, hymenistatin, and hymenamides are summarized in that work [42]. It was found that many of the linear analogues of CLA show immunosuppressive activity and the preservation of the intact Pro–Phe amide bond is of importance for this biological effect. The substitution of successive amino acid residues in the linear peptide Leu-Ile-Ile-Leu-Val-Pro-Phe-Phe by Gly, with the exception of Gly^1-sequence, leads to inactive compounds [43]. Shortening of the linear sequence from the *N*-terminus results in the decrease of immunomodulatory effects, but the tetrapeptide Pro-Pro-Phe-Phe was found to be quite potent [44]. Closing of the sequence by the disulphide bridge between Mpa (Mpa – mercaptopropionic acid) and Cys residues situated on the *N*- and *C*-terminus of the peptide, respectively, resulted in an active analogue. The all-D isomer was also active. The acetylation of the Gly-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe peptide and its *N*-terminal elongation with additional Gly residues evoked a distinct increase in the immunosuppressive potency [45]. The substitution of the successive residues by Ala does not abolish the biological activity of CLA [46]. In addition, the linear and cyclic analogues of CLA in which one or both Phe residues were exchanged by Tyr, were active immunosuppressants, though their activity was lower than that of the native peptide [47]. The aromatic region of CLA was also modified by the substitution of Phe residues by D-Phe, D-Tyr, Trp, and D-Trp residues. It was found that the change of configuration leads to a distinct decrease in the immunosuppressive potency [48]. The CD spectra of these analogues suggest that this decrease may result from the change of the conformational preferences of these peptides [49,50].

To increase the solubility of CLA, the linear and cyclic analogues with Thr residue occupying the successive positions in the -Leu-Ile-Ile- Leu-Val- fragment of the peptide were synthesized. It was found, however, that the presence of a single Thr residue does not sufficiently change the solubility of the resulting peptides. This substitution does not influence the conformation of the peptide and preserves its biological activity [51]. Better water-soluble peptides were obtained when one or two of the Phe residues of CLA were replaced by their sulphonated derivatives. All linear and cyclic analogues of this kind were active as immunosuppressors in PFC and DTH tests [52]. Another approach to increase the bioavailability of CLA, CLX, and their analogues, was to link them covalently to the cell-permeable peptides based on HIV-1 Tat protein sequence [53].

Zabrocki *et al.* enhanced solubility of linear and cyclic CLA analogues in water by the substitution of Leu^1, Leu^5 or Val^9 residues in the molecule by their α-hydroxymethyl derivatives. The peptides were four times soluble in water as the native peptide. However, in the lymphocyte proliferation assays only peptides containing α-hydroxymethylleucine (HmL) showed the inhibitory activity. The most promising, in this respect, was HmL^8-peptide. It was found that the conformation

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of this peptide was closest to the conformation of CLA in the solid state [54]. The same group synthesized a cyclolinopeptide B analogue with Met residue substituted by α-hydroxymethylmethionine. The peptide was non-toxic and inhibited humoral and cellular immune response (PFC and DTH tests, respectively) at a degree comparable to cyclosporine A [55].

To examine if the *cis*-amide bond in the Pro–Pro moiety of CLA is important for its inhibitory activity, the analogues in which two dipeptide segments, Val–Pro and Pro–Pro, respectively, were replaced by their tetrazole derivatives, were synthesized by Zabrocki *et al* [56]. The tetrazole moiety functions as a *cis*-amide bond mimetic in solution, as shown in Figure 2. In the humoral response test, the cyclic peptides of this type showed activity that was equal, at the low doses, to those exerted by CLA and CsA. It was also shown that the conformation of cyclo-(Leu-Ile-Ile-Leu-Val-Pro-$\Psi(CN)_4$-Ala-Phe-Phe) analogue of CLA resembles that of CLA in the solid state. The same methodology was applied to antamanide and hymenistatin I analogues [57,58].

The (2S, 4R)-4-aminopyroglutaminic acid was also used as a surrogate for a dipeptide moiety with a fixed *cis*-amide bond. The CLA analogue resulting from the cyclization of a structure presented in Figure 3 inhibited the lymphocyte proliferation much less efficiently than CsA [59].

To elucidate if the edge-to-face orientation of the two Phe residues of CLA influences its biological activity, analogues with one or both Phe residues substituted by *N*-benzylglycine were synthesized [60]. It was found that the edge-to-face orientation as well as the distance between Phe aromatic rings is important for the biological activity [61].

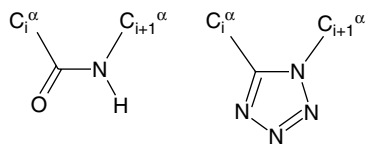

Figure 2 Tetrazole moiety as a *cis*-amide bond mimetic.

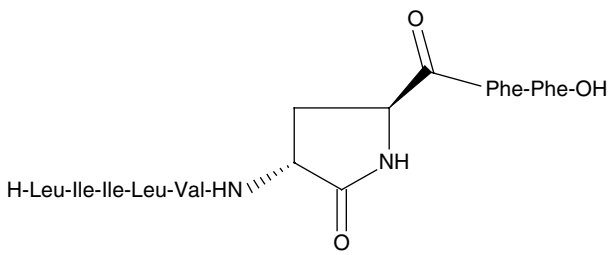

Figure 3 CLA analogue containing (2S, 4R)-4-aminopyroglutaminic acid.

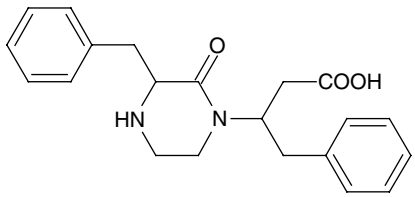

Figure 4 The structure of the dipeptide unit containing an ethylene link bridging two phenylalanine nitrogens [62].

A CLA analogue with the Phe–Phe fragment conformationally constrained by an ethylene linker between the two nitrogens within the moiety was also synthesized (see Figure 4) [62].

In summary, studies have shown that many modifications of the initial structure of CLA do not abolish its immunosuppressive potency. However, no analogue possessing activity higher than CLA was found during these investigations. The results also suggest that the – Pro-Xxx-Phe- sequence of CLA, where Xxx is a hydrophobic aliphatic (e.g. Leu, Val), or aromatic residue, is of importance for the immunosuppressive activity of CLA analogues.

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