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Review

Cyclolinopeptides and Their Analogs – a New Family of Peptide Immunosuppressants Affecting the Calcineurin System

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Abstract. The results of the investigation of immunosuppressive activity of cyclolinopeptide A (CLA – cyclic hydrophobic nonapeptide present in the linseeds) and its analogs are discussed. The results obtained for other natural cyclic peptides showing structural similarities with CLA (antamanide, cycloamanides, hymenistatin, hymenamides) are also reviewed. It results from these investigations that the molecular mechanism of the CLA action is the same as that of cyclosporin A and FK-506 compound, i.e. it consists in formation of the complex with cyclophilin and inhibition – in this form – of the phosphatase activity of calcineurin. The results also suggest that the immunosuppressive activity of these compounds resides in their –Pro-Xxx-Phe- fragment, where Xxx is a hydrophobic (e.g. Leu, Val) or aromatic amino acid residue.

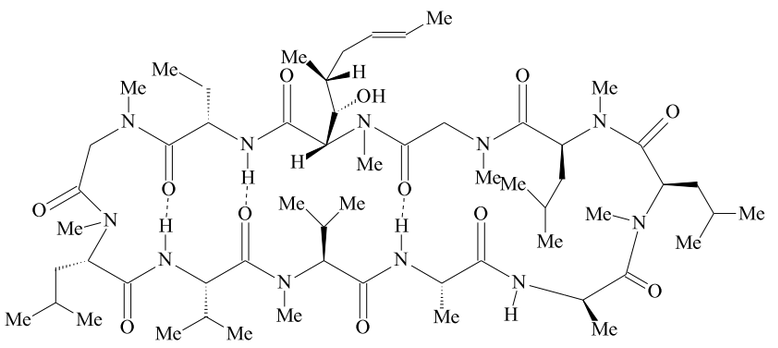
Key words: antamanide; cycloamanides; cyclolinopeptide A; hymenamides, hymenistatin; peptide immunosuppressants.

Introduction

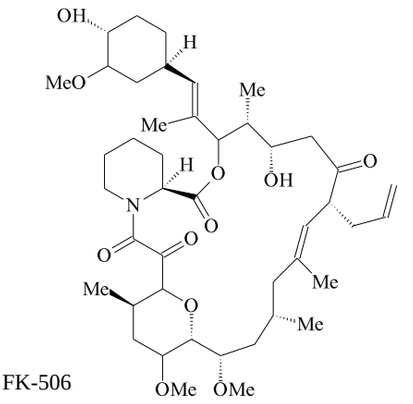
The problem of transplanted organ survival is one of the very serious issues of the contemporary medicine. Two effective immunosuppressants, cyclosporin A (CsA, Sandimmun®)[6] and FK-506[29], both producing a selective inhibition of T lymphocyte activation, are used in the medical praxis as the potent drugs for the prevention of graft rejection after organ transplantation and in the therapy of some autoimmune diseases. CsA is produced by fungi *Cylindrocarpon lucidum* and *Tolypocladium inflatum* whereas FK-506 is of the bacterial origin (it is produced by the soil bacteria *Streptomyces tsukubaensis*). The chemical structures of CsA and FK-506 are very different; the first being a cyclic undecapeptide bearing a non-proteinaceous amino acid (2S, 3R, 4R, 6E) -2-amino-3-hydroxy-4-methyl-6-octenoic acid), the second one is a macrolide compound with just one non-proteinaceous amino acid (pipecolic acid) residue.

Despite the dissimilarity of the chemical structures, the mechanism of biological action of these two compounds is similar and consists in formation of the complex with cytoplasmatic target proteins: cyclophilin and

Abbreviations used: ANT – antamanide, ARFC – autologous rosette forming cell, CLA – cyclolinopeptide A, CLB – cyclolinopeptide B, CsA – cyclosporin A, DTH – delayed type hypersensitivity, GvH – graft versus host, HIR – humoral immune response, IL – inteleukin, Mpa – mercaptopropionic acid, NS – not significant statistical data, PFC – plaque forming cells, p – Student's *t*-test statistical coefficient, s-ANT – symmetrical antamanide, % sup. – percent of suppresion (% sup. = [1–(result for peptide/result for control)] × 100).



CsA



FK-506

FK-506 binding protein (FKBP), respectively. These complexes bind to calcineurin inhibiting its phosphatase activity[14, 15, 21]. Calcineurin plays a crucial role in T lymphocyte activation by dephosphorylating the nuclear factor of activated T cells (NF-AT) which in turn translocates from the cytoplasm to the nucleus where it is required for the induction of transcription of mRNA for interleukin 2 and other cytokines[16].

Because of the side effects of CsA and FK-506 therapy, a wider use of these drugs meets a great limitations[29]. Thus, the search for new immunosuppressants with the similar mechanism of action but with lower toxicity is an important task for the medicinal chemistry.

The Discovery of Cyclolinopeptide A Immunosuppressive Activity

In the 80's we worked on the peptide immunomodulator from ovine colostrum, nonapeptide Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro[13]. It was found that the C-terminal hexapeptide of this immunomodulator (Tyr--Val-Pro-Leu-Phe-Pro) is as potent as the parent nonapeptide in biological tests[9]. The sequence, as well as the retrosequence of this hydrophobic peptide shows some similarity to cyclolinopeptide A (CLA), a cyclic nonapeptide present in linseeds:

Tyr-Val-Pro-Leu-Phe-----Pro	sequence of hexapeptide
c-(-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val-Pro-)	CLA
Pro-----Phe-Leu-Pro-Val-Tyr	retrosequence of hexapeptide

CLA was isolated from linseed by KAUFMANN and TOBSCHIRBEL[10]. Its chemical constitution was solved by PROX and WEYGAND[20].

We participated in the early stages of CLA investigation by our conformational studies[26] and the work on the ion complexing ability of CLA[12]. One of our findings was that the Pro-Pro peptide bond in the peptide is of the *cis*-configuration. This finding was confirmed by the X-ray analysis of CLA performed by DI BLASIO et al[5].

The first biological activity reported for CLA was the ability to inhibit the hepatocyte cell transport system, which may produce the cytoprotective effects against the cell poisoning[11]. An effect of phalloidin transport inhibition by CLA into the rat hepatocytes was investigated by MUNTER et al[18].

The sequential similarity between CLA and immunoactive peptide from ovine colostrum prompted us to examine CLA for a possible immunomodulatory activity. The results of these investigations were published in 1991[23]. It was found that CLA possesses an immunosuppressive activity which, at the low doses, is equal to that exerted by CsA. CLA affects both the humoral and the cellular immune response: its activity in the humoral immune response (HIR) was tested by Jerne's PFC test (plaque forming cell number determination) and in the cellular immune response by delayed type hypersensitivity (DTH) reaction (inductive phase). It was found that CLA significantly prolongs the skin allograft rejection time and diminishes the graft versus host (GvH) index in mice. The peptide inhibits the proliferation of human lymphocytes caused *in vitro* by phytohemagglutinin. The effects of CLA in two immune diseases: the post-adjuvant polyarthritis induced in rats and spontaneous hemolytic anemia in NZB mice were also investigated. CLA shows a pronounced protective effects in both these abnormalities[32]. In the experiments, different procedures of CLA administration to animals were examined (i. p., i. v., and p. o.). For the p. o. administration, the solution of CLA in olive

oil, 2% gelatin solution, and intralipid were used. It was found that CLA administered p. o. to mice and rats at the doses of 4 g/kg (mice) and 3 g/kg (rats) of CLA in 2% gelatin solution was non-toxic. The same result was obtained after the i. v. injection to mice of 230 mg/kg of CLA. In the test for oral toxicity in rats treated through 13 weeks with 15, 45, and 135 mg/kg/day of CLA all animals survived the experiment without harm[32].

CLA, like CsA, inhibits the action of both interleukin 1α (IL-1α) and interleukin 2 (IL-2). The effects were observed not only in the HIR test (PFC), but also in autologous rosette forming cell (ARFC) number determination. As it is known, IL-1α decreases the ARFC number[37]. CLA applied together with IL-1α to the cell culture completely inhibits this IL-1α action.

The similarity of the activities presented by CLA and CsA prompted us to formulate a hypothesis that the molecular mechanism of action of both these substances should be similar. Such a possibility was supported by the results of GALLO et al[7]. They found that CLA binds specifically to cyclophilin A, a cellular target of cyclosporin A. The direct evidence concerning the mechanism of action of CLA on the molecular level was recently presented by the group of Prof. J. E. Kay, in collaboration with our research team[8]. It has been demonstrated that the complex of CLA with cyclophilin really interacts with the calcineurin system, inhibiting its phosphatase activity. However, CsA is 10 times as potent calcineurin phosphatase inhibitor as CLA. It was also found that CLA does not form a complex with the FKBP protein.

Thus, CLA, a peptide produced by a plant, seems to be the third natural product, besides CsA produced by fungi and FK-506 of bacterial origin, which targets calcineurin via a specific protein receptor.

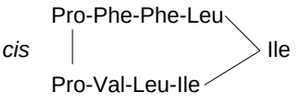
Besides CLA, another cyclic nonapeptide, a cyclolinopeptide B (CLB) was found in linseeds[31]. It differs from CLA by the small changes in the amino acid composition and sequence:

c-(-Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-Ile-) CLB

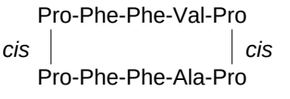
As it has been found recently (unpublished results), CLB suppresses the humoral immune response (PFC test) and the inductive phase of DTH reaction to a lower degree than CLA. However, it is a stronger suppressor of the effector phase of DTH reaction than CLA. These results are in accordance with those reported recently by MORITA et al[17] who found that CLB shows the inhibiting effect on concanavalin induced response of human peripheral-blood lymphocytes.

The Immunosuppressive Activity of Some Other CLA-Like Natural Peptides

The structural similarity to CLA is shown by a cyclic decapeptide, antamanide (ANT), isolated by WIELAND et al[36] from the mushroom *Amanita phalloides*. The similarity consists not only in the presence of fragment -Pro-Pro-Phe-Phe- in both peptides, but also in similar stereochemical characteristics – both peptides possess the *cis*-amide bonds between two Pro residues:

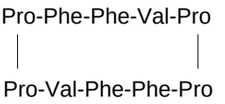


CLA



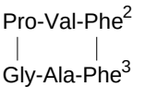
ANT

The immunosuppressive properties of antamanide and many of its analogs, including symmetrical antamanide and linear antamanide partial sequences, were investigated.

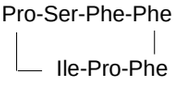


symmetrical antamanide (s-ANT)

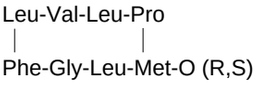
Antamanide and symmetrical antamanide exhibits lower immunosuppressive activity than CLA. Linear antamanide fragments are also active, however, at higher concentrations of these peptides some toxic effects occur [28]. Antamanide is accompanied in the mushroom tissue by several cyclic peptides of smaller ring size – cycloamanides A, B, C, and D:



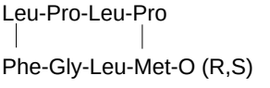
cycloamanide A



cycloamanide B



cycloamanide C



cycloamanide D

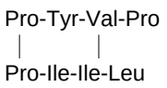
It was shown that cycloamanides possess immunosuppressive activity in humoral and cell mediated immune responses (PFC and foot pad tests) comparable to that of CLA and ANT. Cycloamanide A and its D-Phe[3] analog are the most active compounds in the whole series of cycloamanides[34]. It is of interest that

the retrosequence of cycloamanide A is similar to the partial sequence of antamanide and to the sequence of the immunoactive hexapeptide of ovine colostrum, mentioned above:

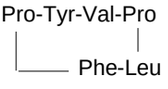
-Phe-Phe-Val-Pro-Pro-Ala-	the partial sequence of antamanide
-Phe-Phe-Val-Pro-Gly-Ala-	the linear retrosequence of cycloamanide A
Tyr-Val-Pro-Leu-Phe-Pro	the hexapeptide of ovine colostrum

Taking this into account we compared the immunomodulatory effects of a series of linear and cyclic hexapeptides, related to the hexapeptide of ovine colostrum and to the retrosequence of cycloamanide A. It was found that substitution of one or both Phe residues of the linear retrosequence of cycloamanide A by Tyr enhances the immunosuppressive activity of the resulting peptide and that a change of the configuration of the N-terminal aromatic residues does not affect the immunosuppressive activity. The highest immunosuppressive potency was found for the c-(-Phe-Tyr-Val-Pro-Gly-Ala-) hexapeptide[1].

In 1990 a new cyclic octapeptide, hymenistatin 1, was isolated from Pacific Ocean *Hymeniacidon* sponge[19]. It possesses a structure very similar to that of the synthetic cyclic hexapeptide obtained from the hexapeptide of ovine colostrum.



hymenistatin I



cyclic hexapeptide derived from peptide of ovine colostrum

It was found that this natural peptide also suppresses the immune response at the range almost reaching that of CsA. However, both these substances have a very different influence on the production of cytokines (IL-1, lL-2, lL-6, and TNF-α)[4].

In collaboration with a group of Prof. J. Zabrocki we investigated the hymenamides E, G, and H, cyclic peptides isolated from the sponge *Hymeniacidon.* The sequences of these peptides are as follows:

c-(-Phe-Phe-Tyr-Pro-Thr-Thr-Pro-)	hymenamide E
c-(-Leu-Pro-Val-Tyr-Pro-Pro-Leu-Ile-)	hymenamide G
c-(-Leu-Pro-Thr-Leu-Pro-Val-Trp-Pro-)	hymenamide H

Although this group of peptides was sequentially similar to the peptides mentioned above, it was practically devoid of the immunosuppressive activity in the PFC test [38].

The Structure-Activity Relationship in CLA Series

On purpose to determine the structural features, which are of importance for the development of CLA immunosuppressive effects, a vast number of linear and cyclic CLA analogs was investigated. The results of these investigations are shortly summarized in Tables 1–7. At first, it was examined whether the activity is preserved when the cyclic structure of the peptide is destroyed. In this connection the following linear peptides were synthesized:

1. Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe
2. Phe-Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe
3. Phe-Phe-Leu-Ile-Ile-Leu-Val-Pro-Pro
4. Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val-Pro
5. Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val
6. Val-Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu
7. Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile-Ile
8. Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile
9. Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu

It was found that the linear CLA analogs preserve the immunosuppressive activity. However, whereas in the GvH test peptides **2** and **4** were active, peptide **3** was inactive. This suggests that the intact Pro-Phe bond is important for the activity preservation and indicates the importance of the -Pro-Phe-Phe- moiety for the evolving of immunosuppressive effects.

Substitution of the successive residues in the linear sequence of **1** by Gly leads, except for the substitution of N-terminal Leu, to inactive compounds[22].

To limit the conformational flexibility of the linear analogs, some of them were installed into the heterodetic ring closed by the disulfide bridge formed between mercaptopropionic acid (Mpa) and cysteine amide, situated at the ends of respective sequences. As a result the following CLA analogs were obtained:

10. Mpa-Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-Cys-NH_2
11. Mpa-Val-Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Cys-NH_2
12. Mpa-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile-Ile-Cys-NH_2
13. Mpa-Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile-Cys-NH_2
14. Mpa-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Cys-NH_2

In this series peptide **10** was the most active suppressor of the humoral as well as the cellular immune response. The heterodetic cyclic analog **15** of all-D type:

15. Mpa-D-Leu-D-Ile-D-Ile-D-Leu-D-Val-D-Pro-D-Pro-D-Phe-D-Phe-Cys-NH_2

Table 1. Immunosuppressive activity of linear analogues of CLA with a shortened peptide chain

Peptide	HIR [a] *in vitro*				DTH			
	% sup.[b] 10 μg	p[c]	% sup. 100 μg	p	% sup. 10 μg	p	% sup. 100 μg	p
CsA	34.69	<0.05	68.37	<0.001	32.52	<0.02	53.66	<0.001
16	49.48	<0.01	70.99	<0.001	51.22	<0.001	52.85	<0.001
17	71.07	<0.001	27.23	<0.05	39.02	<0.01	53.66	<0.001
18	64.40	<0.001	65.62	<0.001	33.33	<0.02	44.72	<0.01
19	56.85	<0.01	55.28	<0.01	40.65	<0.01	50.41	<0.001
20	81.11	<0.001	53.10	<0.01	60.16	<0.001	60.16	<0.001
21	51.31	<0.01	20.81	NS[d]	36.59	<0.02	23.58	NS

[a] Humoral immune response (PFC test).
[b] Percentage of the immune response suppression: % sup. = [1–(result for peptide/result for control)] × 100.
c Student's *t*-test p statistical coefficient.
[d] Result statistically insignificant.
The *in vivo* experiments were performed for groups of 5–7 mice and those *in vitro* for 4–6 wells.

was also active as an immunosuppressor in HIR and DTH tests.

The shortening of the sequence of **10** (being still a part of a heterodetic cyclic system) resulted in a decrease of the immunomodulatory effects [22]. The following linear CLA sequences with a shortened peptide chain were also tested[27]:

16. Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe
17. Ile-Leu-Val-Pro-Pro-Phe-Phe
18. Leu-Val-Pro-Pro-Phe-Phe
19. Val-Pro-Pro-Phe-Phe
20. Pro-Pro-Phe-Phe
21. Pro-Phe-Phe

The results of HIR and DTH experiments for this series of peptides are summarized in Table 1. Octapeptide **16** was found to be the most active in the whole series. The immunosuppressive potency of these peptides diminishes with shortening the peptide chain and increases again for a tetrapeptide **20** (Pro-Pro-Phe-Phe). It was also found that peptide **16** distinctly suppresses the production of IL-1, IL-6, and TNF-α. These results seem to confirm the conclusion on the importance of the -Pro-Phe-Phe- moiety for immunosuppression.

Taking into account the finding that the linear sequence Gly-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe (peptide **22**) showed quite a strong immunosuppressive activity during the preliminary investigation, a series of its derivatives, protected at the N- and/or C-terminus by acetyl (Ac), succinyl (Suc), and amide functions, and elongated at the N- and/or C-terminus by additional glycine residues was synthesized[25].

22. **Gly**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe
23. **Gly**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-**NH_2**
24. **Ac-Gly**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe
25. **Ac-Gly**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-**NH_2**
26. **Suc-Gly**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe
27. **Ac-Gly**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-**Gly**
28. **Ac-Gly-Gly**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-**Gly**
29. **Ac-Gly-Gly-Gly**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-**Gly**
30. **Ac-Gly**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-**Gly-Gly-Gly**

The selected results of the investigation of these peptides are shown in Table 2. It is evident from this Table that the protection of the terminal and/or carboxyl groups of a peptide slightly increases its immunosuppressive potency. At the same time the elongation of the peptide chain at both termini by additional Gly residues evokes a distinct increase of the activity (see Table 2).

The role of a particular side chain of amino acids which form CLA was studied by the classical approach of substitution of successive residues of the linear nonapeptide Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe by Ala. In the course of this investigation the following peptides were obtained:

31. **Ala**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe
32. Leu-**Ala**-Ile-Leu-Val-Pro-Pro-Phe-Phe
33. Leu-Ile-**Ala**-Leu-Val-Pro-Pro-Phe-Phe

Table 2. Immunosuppressive activity of glycine containing linear analogs of CLA in the DTH test (dose 100 μg)

Peptide	CsA	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
% sup.[a]	47.3	16.7	25.3	17.3	36.7	22.7	35.0	60.0	58.4	36.6
p[b]	<0.001	NS[c]	<0.05	NS	<0.01	<0.05	<0.01	<0.001	<0.001	<0.01

[a] Percentage of the immune response suppression: % sup. = [1–(result for peptide/result for control)] × 100.
[b] Student's t-test p statistical coefficient.
[c] Result statistically insignificant.

34. Leu-Ile-Ile-**Ala**-Val-Pro-Pro-Phe-Phe
35. Leu-Ile-Ile-Leu-**Ala**-Pro-Pro-Phe-Phe
36. Leu-Ile-Ile-Leu-Val-**Ala**-Pro-Phe-Phe
37. Leu-Ile-Ile-Leu-Val-Pro-**Ala**-Phe-Phe
38. Leu-Ile-Ile-Leu-Val-Pro-Phe-**Ala**-Phe
39. Leu-Ile-Ile-Leu-Val-Pro-Phe-Phe-**Ala**

All these analogs of CLA showed the immunosuppressive potencies with respect to the HIR tested *in vitro* (see Table 3). Their activities were diminishing in the following order: **38**, **39**>**31**, **36**>**35**, **37**, **33**>**32**, **34**, i.e. the activity was lower when the inner residues of the parent compound were substituted by Ala; substitution of the residues occupying the N- and C- terminus produced more active compounds. In the HIR *ex vivo* PFC test as well as in the DTH – foot pad test (where the activities were strongly reduced) the activities were far less differentiated, which made the speculations on the side chain structure-activity relationship practically impossible. The data show that the aromatic Phe side chains, as well as Pro residues, are not absolutely necessary for evoking the immunosuppressive effects (see Table 3). However, according to the results of the *in vivo* experiments, the changes within the -Phe-Phe- fragment produce less active compounds than the corresponding changes within the N-terminal -Leu-Ile- fragment[35].

As we noted above, the -Pro-Phe-Phe- moiety of CLA seems to be especially important for immunosuppressive activity of the peptides of the CLA group. However, the experiments performed with Ala analogs of CLA showed that even in this part of the molecule some modifications of the structure are possible.

The same conclusion results from the experiments with Tyr analogs of CLA. The following peptides containing such a modification were examined for immunosuppressive potency:

40. Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-**Tyr**
41. Leu-Ile-Ile-Leu-Val-Pro-Pro-**Tyr**-Phe
42. Leu-Ile-Ile-Leu-Val-Pro-Pro-**Tyr**-**Tyr**
43. c-(-Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-**Tyr**-)
44. c-(-Leu-Ile-Ile-Leu-Val-Pro-Pro-**Tyr**-Phe-)
45. c-(-Leu-Ile-Ile-Leu-Val-Pro-Pro-**Tyr**-**Tyr**-)

The results of PFC number and DTH reaction determinations are shown in Table 4. All the investigated peptides are active immunosuppressants in the HIR test, although none of them reaches the level of activity demonstrated by CLA and CsA. In the DTH test, all

Table 3. Immunosuppressive activity of alanine containing linear analog

Peptide	HIR[a] *in vitro*				HIR *in vivo*				DTH	
	% sup.[b] 1 µg	p[c]	% sup. 5 µg	p	% sup. 10 µg	p	% sup. 100 µg	p	% sup. 100 µg	p
31	61.2	<0.001	88.6	<0.001	53.9	<0.001	54.0	<0.001	35.6	<0.01
32	30.4	<0.05	75.8	<0.001	56.6	<0.001	63.7	<0.001	30.1	<0.01
33	29.0	<0.02	82.8	<0.001	67.9	<0.001	79.0	<0.001	28.1	<0.01
34	33.0	<0.02	75.3	<0.001	60.9	<0.01	64.1	<0.001	37.0	<0.01
35	48.1	<0.01	81.8	<0.001	70.6	<0.001	75.6	<0.001	39.7	<0.001
36	60.0	<0.001	87.4	<0.001	53.7	<0.01	68.0	<0.001	41.1	<0.001
37	32.8	<0.02	81.9	<0.001	66.0	<0.001	75.6	<0.001	43.8	<0.001
38	62.7	<0.001	91.8	<0.001	52.2	<0.01	64.4	<0.001	41.1	<0.001
39	62.9	<0.001	90.2	<0.001	57.7	<0.01	41.5	<0.02	37.0	<0.001

Explanations see under Table 1.

Table 4. Immunosuppressive activity of tyrosine analogues of CLA

Peptide	HIR[a] *in vitro*				DTH	
	% sup.[b] 1 µg	p[c]	% sup. 5 µg	p	% sup. 50 µg	p
CsA	90.1	<0.001	91.6	<0.001	42.6	<0.01
CLA	88.0	<0.001	91.1	<0.001	67.0	<0.001
40	75.6	<0.001	89.8	<0.001	73.4	<0.001
41	45.9	<0.02	63.8	<0.01	45.7	<0.01
42	83.1	<0.001	84.3	<0.001	55.3	<0.001
43	69.5	<0.001	81.3	<0.001	56.4	<0.001
44	74.1	<0.001	70.2	<0.001	56.4	<0.001
45	83.8	<0.001	87.9	<0.001	70.2	<0.001

Explanations see under Table 1.

the peptides studied exceeded the activity of CsA. The most active compound was linear peptide **40**. It must be pointed out, however, that peptides **42**, **43**, and **45** showed highly toxic effects during the experiments [33].

The series of CLA analogs modified within the aromatic segment of the peptide chain was recently enlarged by the following 18 compounds:

46. Ile-Ile-Leu-Val-Pro-Pro-**D-Phe**-Phe-Leu
47. Ile-Ile-Leu-Val-Pro-Pro-Phe-**D-Phe**-Leu
48. c-(Ile-Ile-Leu-Val-Pro-Pro-**D-Phe**-Phe-Leu)
49. c-(Ile-Ile-Leu-Val-Pro-Pro-Phe-**D-Phe**-Leu)
50. Ile-Ile-Leu-Val-Pro-Pro-**D-Tyr**-Phe-Leu
51. Ile-Ile-Leu-Val-Pro-Pro-Phe-**D-Tyr**-Leu
52. c-(Ile-Ile-Leu-Val-Pro-Pro-**D-Tyr**-Phe-Leu)
53. c-(Ile-Ile-Leu-Val-Pro-Pro-Phe-**D-Tyr**-Leu)
54. Ile-Ile-Leu-Val-Pro-Pro-**Trp**-Phe-Leu
55. Ile-Ile-Leu-Val-Pro-Pro-Phe-**Trp**-Leu
56. Ile-Ile-Leu-Val-Pro-Pro-Trp-**Trp**-Leu
57. c-(Ile-Ile-Leu-Val-Pro-Pro-**Trp**-Phe-Leu)
58. c-(Ile-Ile-Leu-Val-Pro-Pro-Phe-**Trp**-Leu)
59. c-(Ile-Ile-Leu-Val-Pro-Pro-**Trp**-**Trp**-Leu)
60. Ile-Ile-Leu-Val-Pro-Pro-**D-Trp**-Phe-Leu
61. Ile-Ile-Leu-Val-Pro-Pro-Phe-**D-Trp**-Leu
62. c-(Ile-Ile-Leu-Val-Pro-Pro-**D-Trp**-Phe-Leu)
63. c-(Ile-Ile-Leu-Val-Pro-Pro-Phe-**D-Trp**-Leu)

The aims of this work were: 1) to see whether the configurational change within the aromatic sector of CLA would preserve the immunomodulatory activity of the peptides; 2) to elucidate what kind of changes of the peptide backbone conformation will be induced by configurational changes. We found that the exchange of Phe residues by D-Phe, D-Tyr, Trp, and D-Trp residues leads to a distinct decrease in the immunosuppressive activity of resulting CLA analogs (see Table 5). The Trp analogs, however, are more potent than their D-Trp counterparts. The CD data show that the decrease in biological activity of these peptides may result from the change of their conformational preferences, as compared with CLA. It follows from the data collected in Table 5 that the analogs modified in position 9 are usually more active than those modified in position 8. Peptide **49** ([D-Phe^9] CLA) was the most active in the HIR tests. It corresponds very well with the preservation of the CLA peptide backbone conformation indicated by CD spectra of this compound. In general the results show that a change of the aromatic residue configuration in position 8 destabilizes CLA conformation much more than the respective change in position 9 [2].

In this connection the question of importance of the *cis*-amide bond present between both Pro residues of CLA for the immunosuppression exerted by this compound was examined. For this purpose the group of Prof. J. Zabrocki have synthesized the CLA analogs with the tetrazole moiety introduced into the respective positions of a peptide chain.

The tetrazole moiety mimics well the rigid *cis*-amide bond:

The tetrazole group was introduced in the place of Val-Ala and Pro-Ala amide bonds of cyclic peptides **64** and **65**:

64. c-(-Leu-Ile-Ile-Leu-**Val-Ala**-Pro-Phe-Phe-)
65. c-(-Leu-Ile-Ile-Leu-Val-**Pro-Ala**-Phe-Phe-)

Both resulting peptides were active in the HIR test with peptide **64** being more effective than **65**. This observation suggests that the *cis*-geometry of Pro-Pro amide bond of CLA may be really of importance for its biological effects. However, it seems that the spatial requirements for the fitting of CLA into its protein receptor are not precisely defined. The receptor can probably interact with the analog possessing a *cis*-amide bond situated not only between Pro residues, but also between Val and Pro residues of CLA (unpublished results).

The Search for CLA Analogs Soluble in Water

The limitation of the possible use of CLA in the medical practice consists in its very poor solubility in the water media. To enhance the hydrophilicity of the peptides of the CLA group Thr residues in different positions of the -Leu-Ile-Ile-Leu-Val- fragment of the CLA peptide chain were introduced. The series of cyclic Thr-analogs and their linear precursors consisted of the following compounds:

66. **Thr**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe
67. Leu-**Thr**-Ile-Leu-Val-Pro-Pro-Phe-Phe
68. Leu-Ile-**Thr**-Leu-Val-Pro-Pro-Phe-Phe
69. Leu-Ile-Ile-**Thr**-Val-Pro-Pro-Phe-Phe
70. Leu-Ile-Ile-Leu-**Thr**-Pro-Pro-Phe-Phe
71. c-(-**Thr**-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-)
72. c-(-Leu-**Thr**-Ile-Leu-Val-Pro-Pro-Phe-Phe-)
73. c-(-Leu-Ile-**Thr**-Leu-Val-Pro-Pro-Phe-Phe-)
74. c-(-Leu-Ile-Ile-**Thr**-Val-Pro-Pro-Phe-Phe-)
75. c-(-Leu-Ile-Ile-Leu-**Thr**-Pro-Pro-Phe-Phe-)

It was found, however, that the presence of a single Thr residue in the CLA sequence does not sufficiently change the solubility of the resulting peptides. The CD spectra measured for the cyclic CLA analogs of this series show that the analogs preserve the characteristic conformation of CLA. The CD spectra of the linear

Table 6. Immunosuppressive activity of threonine containing analogs of CLA

Peptide	HIR[a] *in vitro*				DTH	
	% sup.[b] 1 μg	p[c]	% sup. 5 μg	p	% sup.[b] 10 μg	p
CsA	87.2	<0.001	89.7	<0.001	27.1	<0.02
66	64.2	<0.02	79.1	<0.01	49.2	<0.01
67	59.3	<0.02	88.2	<0.001	30.5	<0.02
68	66.4	<0.02	70.5	<0.01	34.7	<0.02
69	73.9	<0.01	82.6	<0.001	34.7	<0.02
70	49.5	<0.05	81.5	<0.001	24.6	<0.05
71	58.2	<0.01	77.0	<0.001	15.1	NS[d]
72	53.1	<0.02	38.5	<0.05	27.1	<0.02
73	41.3	<0.02	74.8	<0.001	1.9	NS
74	60.7	<0.02	76.6	<0.01	28.0	<0.02
75	66.8	<0.02	75.5	<0.01	48.3	<0.01

Explanations see under Table 1.

Table 7. Immunosuppressive activity of sulfonated analogs of CLA

Peptide	HIR[a] *in vitro*		HIR *in vivo*				DTH			
	% sup.[b] 1 μg	p[c]	% sup. 10 μg	p	% sup. 100 μg	p	% sup. 10 μg	p	% sup. 100 μg	p
CsA	64.5	<0.001	22.9	<0.05	60.5	<0.001	28.0	<0.05	39.0	<0.01
76	–10.4	NS[d]	23.2	<0.05	32.4	<0.01	67.2	<0.05	64.3	<0.01
77	27.2	<0.05	18.2	NS	53.1	<0.01	69.8	<0.001	69.0	<0.001
78	31.1	<0.05	33.1	<0.001	51.7	<0.001	43.3	<0.02	48.9	<0.01
79	16.3	NS	22.7	NS	42.2	<0.001	41.4	<0.05	86.4	<0.001
80	55.8	<0.001	26.6	<0.05	61.8	<0.001	31.0	<0.05	51.0	<0.01
81	44.6	<0.01	17.3	NS	57.5	<0.01	29.3	<0.05	56.0	<0.01

Explanations see under Table 1.

precursors (**66–70**) of the cyclic compounds also demonstrate the conformational similarity to CLA. The linear, as well as cyclic analogs preserve also the immunosuppressive activity both in humoral and cellular immune response (Table 6) [24].

The substitution of Phe residues of CLA by their sulfonated derivatives gave far better results with respect to the water solubility of peptides. A series of 6 such peptides was synthesized:

76. Ile-Ile-Leu-Val-Pro-Pro-**Phe(SO_3Na)**-Phe-Leu
77. Ile-Ile-Leu-Val-Pro-Pro-Phe-**Phe(SO_3Na)**-Leu
78. Ile-Ile-Leu-Val-Pro-Pro-**Phe(SO_3Na)-Phe(SO_3Na)**-Leu
79. c-(-Ile-Ile-Leu-Val-Pro-Pro-**Phe(SO_3Na)**-Phe-Leu-)
80. c-(-Ile-Ile-Leu-Val-Pro-Pro-Phe-**Phe(SO_3Na)**-Leu-)
81. c-(-Ile-Ile-Leu-Val-Pro-Pro-**Phe(SO_3Na)-Phe(SO_3Na)**-Leu-)

All these analogs show some immunosuppressive activity in the HIR test. The highest activity in both *in vitro* and *ex vivo* test exhibits the compound **80** (see Table 7). It produces effects similar to those of CsA. As a rule, the presence of sulfonated residue in position 9 of the peptide chain favors the immunosuppressive activity in HIR test, while the presence of such a residue in position 8 removes it. However, in the DTH test analog **79** is the most potent. The linear compounds **76–78** are also active in this test, but their activities show a very limited dose dependence.

Thus, the introduction of the -SO_3Na group into the Phe residue does not abolish the immunosuppressive activity of the peptide, at the same time greatly enhancing its solubility in water[3].

In conclusions, the studies performed with different series of cyclolinopeptide A analogs showed that a big number of different modifications of the initial structure of the peptide does not abolish its immunosuppressive potency. However, during this investigation no peptide possessing distinctly better immunomodulatory properties than CLA itself was found. The observations suggest that the -Pro-Phe-Phe- segment of CLA may be more important for its biological activity than other parts of the molecule. It is especially noticeable from the experiments performed with the shortened CLA analogs and from the data on the activity of a series of CLA nonapeptide analogs formed by the splitting of the CLA peptide ring in nine successive amide bond positions (see above).

A similar triad of successive amino acids, composed of proline, a hydrophobic residue, and aromatic

or hydrophobic one, can be noticed in the sequences (or retrosequences) of all the natural peptide immunosuppressors examined by us:

nonapeptide of ovine colostrum	-Pro-Leu-Phe-
CLA	-Pro-Phe-Phe-
CLB	-Pro-Phe-Phe-
antamanide	-Pro-Phe-Phe-
antamanide	-Pro-Ala-Phe-
symmetrical antamanide	-Pro-Phe-Phe-
cycloamanide A	-Pro-Val-Phe-
cycloamanide B	-Pro-Ser-Phe-
the retrosequence of cycloamanide B	-Pro-Phe-Phe-
the retrosequence of cycloamanide C	-Pro-Leu-Val-
the retrosequence of cycloamanide D	-Pro-Leu-Phe-
hymenistatin I	-Pro-Tyr-Val-

This suggests that the immunosuppressive activity may reside in such triads of amino acids. Although the sequence -Pro-Ser-Phe- appearing in cycloamanide B does not fit this regularity, in this case the retrosequence -Pro-Phe-Phe- could play a role of the active site of the peptide. In the case of cycloamanides C and D the same role could be ascribed to -Pro-Leu-Val (cycloamanide C) and -Pro-Leu-Phe- (cycloamanide D) partial retrosequences. However, the segments of this type can also be found within the structures of hymenamides E, G, and H, and these natural compounds are devoid of any activity in the PFC test:

hymenamide E	-Pro-Phe-Phe
hymenamide G	-Pro-Val-Tyr
hymenamide H	-Pro-Val-Tyr

This suggests that the presence of the above mentioned peptide triad does not automatically evoke the activity; it also depends probably on the conformational possibilities of fitting of a given peptide to the receptor site of the target protein, and those could be different in e. g. CLA and hymenamides.

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