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Synthesis, conformational analysis and immunological activity of β^3Phe-substituted Cyclolinopeptide A analogues[‡]

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CLA, a natural, highly hydrophobic cyclic nonapeptide with sequence *c*(Pro[1]-Pro[2]-Phe[3]-Phe[4]-Leu[5]-Ile[6]-Ile[7]-Leu[8]-Val[9]-), isolated from linseed oil, was found to possess a strong immunosuppressive activity comparable, in low doses, with that of CsA, with a mechanism that depends on the inhibition of the interleukin-1 and interleukin-2 action. Structural analysis of CLA and its related compounds has underlined that the presence of the tetrapeptide Pro-Pro-Phe-Phe sequence, the Pro-Pro *cis* amide bond, and the 'edge-to-face' interaction are possible important features for the immunosuppressive activity of CLA. To evaluate the role and significance of 'edge-to-face' interaction in the process of molecular recognition by receptors, we have synthesised three linear precursors and three cyclic analogues of CLA, in which one or both Phe residues have been replaced by β^3Phe residues. A conformational analysis by NMR in CD_3CN/H_2O mixture has been carried out on the CLA analogue, in which Phe[3] has been replaced by a βPhe, to study the influence of the mutation on the three-dimensional structure. All linear and cyclic CLA analogues containing βPhe have been tested in the humoral and cellular immune response *in vivo* assays in mice. The peptide activities have been compared with CsA, as a reference drug. **Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: CLA; β^3Phe; NMR spectroscopy; immunosuppressive activity

Introduction

Inhibitors of PPI-ases such as CsA [1], tacrolimus (FK-506) [2] and rapamycin [1] are effective immunosuppressants that are able to evoke a selective inhibition of lymphocyte T activation during immune response. All three of them are used in medicine as potent drugs, preventing graft rejection after organ transplantation and in the therapy of some autoimmune diseases.

Because of the significant side effects of CsA and tacrolimus therapy, a wider use of these drugs meets great limitation [2]. Thus, the search for new immunosuppressants with a similar mechanism of action but lower toxicity, especially in the group of naturally existing immunomodulatory peptides and their analogues, is an important task for today's medicinal chemistry.

CLA, a natural, highly hydrophobic cyclic nonapeptide with sequence *c*(Pro[1]-Pro[2]-Phe[3]-Phe[4]-Leu[5]-Ile[6]-Ile[7]-Leu[8]-Val[9]-) [3], isolated from linseed oil [4], was found to possess a strong immunosuppressive activity comparable, in low doses, with that of CsA [5,6], with a mechanism that depends on the inhibition of the interleukin-1 and interleukin-2 action. The determination of the x-ray structure and the NMR structural analysis of CLA [7–9] gave the basis to study the biological, structural, and conformational properties of CLA and related compounds [10–20]. In solution, at room temperature, CLA exhibits marked conformational mobility leading to the presence of a mixture of several conformers. A suitable NMR analysis of CLA has been carried out in $CDCl_3$ at low temperature (214 K). In these conditions CLA exists as a single

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Abbreviations used: *acid; AFC, antibody-forming-cells; CsA, cyclosporin A; CLA, cyclolinopeptide A; ConA, concanavalin A; DQF, double quantum filtered; EDC, N-(3-dimethylaminopropyl)N′-ethylcarbodiimide; EtOAc, ethyl acetate; TPPI, time-proportional phase incrementation; HF, hydrogen fluoride; PFG, pulsed field gradient; DPFGSE, double pulsed field gradient spin echo; SRBC, sheep red blood cell; OVA, ovalbumin; DTH, delayed type hypersensitivity.*

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'frozen' conformer [8], whose structure is consistent with that found in the solid state. It has been postulated that the following structural features are important for the immunosuppressive activity of CLA: presence of the tetrapeptide Pro-Pro-Phe-Phe sequence, the Pro-Pro *cis* amide bond [21] and the 'edge-to-face' interaction between the aromatic rings [22].

In order to evaluate the role and significance of 'edge-to-face' interaction in the process of molecular recognition by receptors, we have synthesised three linear precursors and three cyclic analogues of CLA, in which one or both Phe residues have been replaced by β^3Phe residues (Figure 1).

β^3Phe belongs to the class of β-amino acids. Recent studies by Seebach [23] and researchers from Japan [24] demonstrated that some of these amino acids, found in nature, possess a biological activity and constitute an integral part of many naturally occurring peptides bearing important pharmacological properties. Moreover, replacement of α-amino acids by β-amino acids in peptides may lead to (i) restriction of the conformational freedom and (ii) stabilisation of respective secondary structures. Peptide bonds, modified with the inclusion of a β-amino acid in the sequence, are resistant to enzymatic degradation, so that they may exhibit a prolonged biological activity. In addition, the definition of factors which determine conformational preference of β-amino acids could have a great importance in designing peptide sequences of strictly defined structure.

An NMR conformational analysis in CD_3CN/H_2O mixture has been performed on one CLA analogue, in which Phe^3 has been replaced by a βPhe; the predominant conformation was determined and compared with the known x-ray and solution structure of the parent peptide.

All linear and cyclic CLA analogues, containing β^3Phe, have been tested in the humoral and cellular immune response *in vivo* assays in mice. The activities of the peptides have been compared with that of CsA, as reference drug.

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

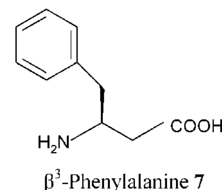
2. Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-Phe^3-**β^3Phe^4**-Leu^5

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

4. *c*(Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-**β^3Phe^3**-Phe^4-Leu^5-)

5. *c*(Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-Phe^3-**β^3Phe^4**-Leu^5-)

6. *c*(Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-**β^3Phe^3**-**β^3Phe^4**-Leu^5-)



β^3-Phenylalanine **7**

Figure 1. Sequences of newly synthesised linear precursor **1–3** and CLA analogues **4–6** containing (*S*)-β^3-Phe-OH **7**.

Materials and Methods

General Remarks

All solvents were purified by conventional methods. Evaporations were carried out under reduced pressure. Melting points were determined on a capillary melting point apparatus and are uncorrected. HPLCs were performed on an LDC/Milton-Roy and Thermo Separation HPLC analytical instruments using a Vydac C18 column (0.46 × 25 cm): flow 1.0 ml/min, detection at 220 nm and eluants (A) 0.05% TFA in water and (B) 0.038% TFA in acetonitrile/water 90 : 10 with a gradient application. Purification of peptides was performed by preparative reversed-phase HPLC on a Vydac C18 column (2.2 × 25 cm): flow rate 16 ml/min, UV detection at 220 nm. The structures of the pure peptides were confirmed by Esquire 3000 mass spectrometer (Bruker Saxonia, Leipzig, Germany) equipped with an ion-trap analyser. Samples were mixed with 30% methanol in water, supplemented with 0.1% formic acid and introduced into the instrument using a syringe pump. Scan range was set between 50 and 1200 *m/z*. Spectra were taken in the positive-ion mode.

All amino acid derivatives and peptide bond-forming reagents were purchased from IRIS Biotech (Germany).

Peptide Synthesis and Purification

The linear peptides **1–3** (Figure 1) were synthesised by the manual solid-phase method using chloromethylated Merrifield resin as a solid support. Attachment of the Boc-Leu-OH to the resin was performed according to cesium salt procedure [25] and the substitution level was determined by weight gain measurements (Boc-Leu-Ⓟ, 0.372 mM/g). Synthesis of desired peptides was achieved by stepwise coupling of Boc-amino acids to the growing peptide chain on the resin. Starting with 0.4 mM of the substituted resin, standard single TBTU/HOBt-coupling protocol (but with each deprotonation step omitted) was used for all amino acids and was repeated if Kaiser test [26] or Chloranil test (proline residue) [27] was found positive. In all cases where, after second coupling, the test was slightly positive, all remaining free amino groups were acetylated with the aid of acetic anhydride in 4-dicyanomethylene-6-(p-dimethylaminostyryl)-2-methyl-4H-pyran (DCM). After the synthesis had been completed, the Boc protecting group was removed with 50% TFA in DCM. The peptide resin was treated with liquid HF (10 ml) containing anisole (0.5 ml) at −70 °C and stirred for 60 min at 0 °C [28]. After the removal of HF and anisole *in vacuo*, the mixture was washed with diethyl ether, then the crude peptide was extracted from the resin with 10% AcOH and lyophilised. The analytical samples were purified by preparative HPLC (gradient 50–100% B).

The crude linear precursors **1–3** (1 eq.) were cyclised (progress of the cyclisation reaction was monitored by HPLC) by means of EDC (3 eq.) in the presence of HOAt (3 eq.) and DIPEA in DCM at much lower concentration (40 mg of linear peptide in 800 ml of DCM) than usually described for 'head to tail' peptide cyclisation reactions [29,30]. Crude cyclic peptides **4–6** were purified in the same way as their linear precursors using preparative HPLC (gradient 50–100% B). The physicochemical properties of all synthesised analogues are summarised in Table 1.

NMR Spectroscopy

The NMR samples of CLA analogue **4** were prepared by dissolving the peptide in $CDCl_3$ (500 µl) at a concentration of $1.0–6.0 \times 10^{-3}$ M and in CD_3CN/H_2O mixture (500 µl, 90 : 10 v/v) at a concentration of 1.5×10^{-3} M.

NMR experiments were carried out on a Varian Inova 600 MHz spectrometer, equipped with a triple axis PFG or a cryogenic

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Table 1. Physicochemical properties of synthesised peptides **1–6**

Compound	Yield[a] (%)	HPLC[b,c] t_R (min)	Molecular formula	Molecular weight	ESI-MS
[β^3Phe^3]CLA **1**	92.3	14.11	$C_{58}H_{89}N_9O_{10}$	1071.3	1072.6 [M + H$^+$]
[β^3Phe^4]CLA **2**	96.5	15.18	$C_{58}H_{89}N_9O_{10}$	1071.3	1072.6 [M + H$^+$]
[β^3Phe^{3,4}]CLA **3**	86.7	14.67	$C_{59}H_{91}N_9O_{10}$	1085.4	1086.6 [M + H$^+$]
[β^3Phe^3]CLA **4**	17.5	20.52	$C_{58}H_{87}N_9O_9$	1053.3	1054.4 [M + H$^+$]
[β^3Phe^4]CLA **5**	28.5	20.26	$C_{58}H_{87}N_9O_9$	1053.3	1054.4 [M + H$^+$]
[β^3Phe^{3,4}]CLA **6**	43.7	18.97	$C_{59}H_{89}N_9O_9$	1067.4	1068.2 [M + H$^+$]

[a] Yields of crude peptides were calculated on the basis of amino acid content of the resin.
[b] All peptides were at least 99% pure.
[c] Eluation system: (50–90% B).

probe optimised for ^{1}H detection. For the one-dimensional (1D) ^{1}H spectra, 64 scans were acquired with a spectral width of 6714.8 Hz, relaxation delay 1.0 s, 16 384 data points for acquisition and 32 768 for transformation. The 1D spectra in $CDCl_3$ were recorded in the temperature range of 277–298 K, the spectra in CD_3CN/H_2O at 298 K. The two-dimensional (2D) [^{1}H,^{1}H] spectra DQF-COSY [31], TOCSY [32], NOESY [33] and ROESY [34] were acquired using the TPPI method to obtain complex data points in the t_1 dimension. Typically, 32 or 64 scans per t_1 increment were collected with a spectral width of 6714.8 Hz along both f_1 and f_2, 2048 × 256 data points in t_2 and t_1, respectively, and recycle delay 1.5 s. Water suppression in CD_3CN/H_2O mixture was achieved by means of DPFGSE sequence [35,36]. The TOCSY experiment was recorded using a DIPSI-2 mixing scheme of 70 ms with 7.7 KHz spin-lock field strength. The NOESY spectra were carried out at 298 K with a mixing time in the range of 250–500 ms. The mixing time of the ROESY experiment was set to 200 ms. The data were typically apodised with a square cosine window function and zero filled to a matrix of size 4096 × 1024 prior to Fourier transformation and baseline correction. Chemical shifts were referenced to internal tetramethylsilane. All NMR data were processed with Varian VNMRJ 1.1.D software and analysed using XEASY [37], a tool of CARA (http://www.nmr.ch) [38]. Measurements of vicinal coupling constants were obtained from 1D and DQF-COSY spectra. The temperature coefficients were measured from TOCSY spectra recorded in the temperature range of 298–318 K.

Experimental distance restraints for structure calculation were derived from the cross-peak volumes in the NOESY spectrum recorded in CD_3CN/H_2O with a mixing time of 400 ms. NOESY cross-peaks were manually integrated using the XEASY software and converted to upper distance constraints according to an inverse sixth power peak volume-to-distance relationship for the backbone and an inverse forth power function for side chains by using the CALIBA module of the CYANA v.2.1 program [39].

Distance constraints together with $^3J_{HNCH}$ coupling constant were then used by the GRIDSEARCH module, implemented in CYANA, to generate a set of allowable dihedral angles; the structure calculations, using the torsion angle dynamics protocol of CYANA, were then started from 100 randomized conformers.

The molecular graphics program MOLMOL [40] was used to analyse and represent the NMR structure ensembles of analogue **4**.

Biological Assays

Mice. Ten to twelve week old CBA mice of both sexes were used for the studies. The animals were fed a commercial pelleted food and filtered tap water *ad libitum*. The local ethics committee approved the studies.

Reagents. SRBCs were delivered by Wroclaw University of Environmental and Life Sciences. SRBCs were stored in Alsever's solution until use. CsA was from Sandoz (Switzerland) and OVA from Sigma (Germany).

The humoral immune response in vivo. The peptides (10 and 100 µg/mouse in 0.2 ml of saline) were administered to mice intraperitoneally (i.p.) 2 h before and 24 h after immunisation of mice with 0.2 ml of 10% SRBC suspension. After 4 days the number of PFC in the spleens was determined according to Mishell and Dutton [41]. The results are presented as the mean ± SE from five mice determinations.

The DTH. The peptides (10 and 100 µg/mouse) were administered to mice i.p., 2 h before and 24 h after a subcutaneous sensitisation of mice with 5 µg of OVA in Freund's complete adjuvant into tail base (influence on the inductive phase of DTH) or 2 h before elicitation of the response (influence on the effector phase of DTH). After 4 days the eliciting dose of antigen (50 µg of OVA in Freund's incomplete adjuvant into hind foot pads) was given. Following next 24 h the magnitude of DTH reaction was measured with a caliper as a foot pad oedema [42] and presented in DTH units (1 unit = 10^{-2} cm). The results are expressed as the mean ± SE from eight mice.

Statistics. The results were presented as the mean values ± SE using Student's *t*-test. The effects were regarded as significant when $p \leq 0.05$.

Results and Discussion

Peptide Synthesis

The three new, linear analogues of CLA (**1–3**), modified with β^3Phe residue in positions 3 or 4 and both 3 and 4, were

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synthesised by stepwise coupling of Boc-amino acids to the growing peptide chain on Merrifield resin. The couplings were mediated by the TBTU/HOBt method. On completion of the synthesis, the unprotected resins were cleaved with the HF in the presence of anisole at 0 °C and the crude linear precursors were cyclised by means of EDC in the presence of HOAt and DIPEA. The cyclisation reactions were carried out in very diluted solutions to avoid dimer formation. The homogeneity of the purified cyclic peptides was checked by analytical HPLC and their structures were confirmed by ESI-MS and NMR spectroscopy.

NMR Conformational Studies

Previous NMR conformational studies showed that, in chloroform solution, it is possible to freeze a unique conformation of CLA at 214 K [9], while several conformers in intermediate chemical exchange are present in water and in other polar solvents. In particular, the room-temperature ^{1}H spectrum of CLA in polar solvents is characterised by very broad resonances and only at high temperature it is possible to observe sharp resonances, resulting from a fast-exchange between two or more conformer. To compare the solution conformational behaviour of βPhe analogues with CLA, we began an ^{1}H NMR study of the analogue **4** in chloroform. The proton spectrum of peptide **4** in $CDCl_3$ at 298 K, in the range of 1–6 mM concentration, is characterised by very broad resonances, which are not sharpened after the lowering of the temperature to 277 K (Figure 2(a) and (b)). These results suggest the existence of several conformers in intermediate chemical exchange in $CDCl_3$ solvent, as observed for the parent peptide. Interestingly, analogue **4**, when dissolved in a CD_3CN/H_2O mixture, showed much sharper resonances just at 298 K (Figure 2(c)) with the HN resonances well separated and spread within a range of 1.1 ppm. This may suggest that the incorporation of a β-aminoacid in CLA can stabilise a single conformation, as observed in other peptides modified with β-aminoacids [23,24], or can induce, at room temperature, two or more conformations in fast exchange. To evaluate whether a single conformation predominates, we have performed a more detailed NMR conformational analysis. This analysis has indeed shown (see below) that the number of collected NMR experimental constraints of CLA analogue in CD_3CN/H_2O is sufficient to derive meaningful structural features, which are well comparable with previous CLA structural data, obtained either in solution and in the solid state [8]. The choice to perform the NMR solution analysis of the CLA analogue in polar environment at room temperature allows to evaluate its conformational preferences in more biologically relevant conditions and nonetheless to compare them with the previous structural results.

Resonance assignment, collection of conformational constraints and structure calculations

Proton spin system identification and assignment of individual resonances of analogue **4** in CD_3CN/H_2O solution were carried out by using a combination of TOCSY [32] and DQF-COSY [31] spectra. Sequence-specific assignment was obtained by NOESY [33] and ROESY [34] experiments, according to standard procedures [43]. Proton chemical shifts for all resonances are listed in Table 2. Under the experimental conditions employed, the 600-MHz NOESY spectra do show positive NOEs (extreme narrowing limit). Totally, 163 NOE cross-peaks were assigned and integrated; stereospecific assignments for the βCH_2s of Phe^4 and Leu^5 were obtained using the CYANA v2.1 software. Moreover, $^3J_{HNCH}$ coupling constants of the amide protons of peptide **4** were measured from ^{1}H and DQF-COSY spectra (Table 3). Temperature dependence of NH chemical shift was determined in order to probe the solvent accessibility and the possible existence of hydrogen bonds (Table 3). The lower $-\Delta\delta/\Delta T$ values were observed for Val^9 and Ile^7 indicating their possible involvement in hydrogen bonds. On the contrary, Phe^4, Leu^5 and Leu^8 show coefficients higher than 4.5 ppb/K that are typical of amide proton exposed to the solvent [44].

The configuration of the two Xxx-Pro peptide bonds was determined from NOEs. The strong NOE between the two α protons of Pro^1 and Pro^2 and those between Pro^2 αH and β and β' protons of Pro^1 are characteristic of a *cis* Pro^1-Pro^2 peptide bond, feature common to CLA and other cyclopeptides containing the sequence Pro-Pro-Phe-Phe (CLA analogues [22], antamanide [45,46]). On the other hand, the strong cross-peaks connecting Val^9 αH to the δ, δ' protons of Pro^1 indicate a *trans* arrangement of

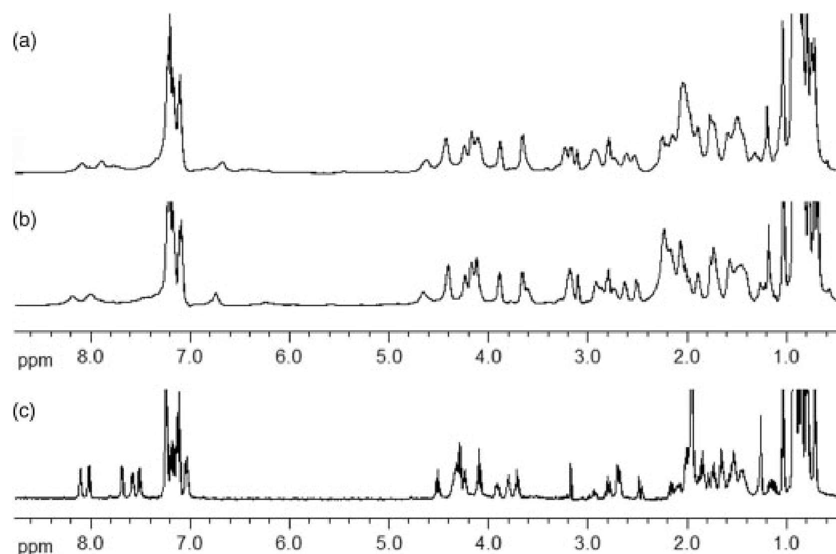

Figure 2. The 600-MHz ^{1}H NMR spectra of *c*(Pro-Pro-β^3Phe-Phe-Leu-Ile-Ile-Leu-Val-) in chloroform at 298 K (a) and 277 K (b) as compared with the spectrum in a 90/10 (v/v) CD_3CN/H_2O mixture at 298 K (c).

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Table 2. ^{1}H Chemical shifts of CLA analogue **4** in CD_3CN/H_2O mixture

Residue	HN	αH	βH	Others
Pro¹		4.20	2.12, 1.69	γCH_2 1.98, 1.81
				δCH_2 3.76, 3.67
Pro²		4.25	1.81, 1.53	γCH_2 1.53, 0.85
				δCH_2 3.22, 2.95
∗∗∗**βPhe³**	7.99	2.65, 2.43	4.26	2, 6 H 7.07
				3, 5 H 7.21
				4 H 7.15
Phe⁴	8.08	4.32	3.01, 2.89	2, 6 H 7.09
				3, 5 H 7.20
				4 H 7.12
Leu⁵	7.66	3.87	1.62, 1.49	γH 1.49
				δCH_3 0.87, 0.81
Ile⁶	7.01	4.06	1.92	γCH_2 1.42, 1.10
				γCH_3 0.89
				δCH_3 0.82
Ile⁷	7.48	4.29	2.06	γCH_2 1.39, 1.12
				γCH_3 0.90
				δCH_3 0.76
Leu⁸	7.55	4.06	1.71, 1.61	γH 1.42
				δCH_3 0.77, 0.68
Val⁹	7.00	4.47	1.98	γCH_3 1.00, 0.89

Table 3. Temperature dependence of the HN chemical shift and vicinal coupling constants of the analogue **4** of CLA in CD_3CN/H_2O mixture

Residue	$-\Delta\delta/\Delta T$ (ppb/K)	$^3J_{HNCH}$ (Hz)
βPhe³	4.0	9.0
Phe⁴	6.8	6.1
Leu⁵	7.9	6.1
Ile⁶	3.6	8[a]
Ile⁷	3.3	9.6
Leu⁸	4.9	7.8
Val⁹	3.3	9[a]

[a] Obtained from DQF-COSY.

the Val^9-Pro^1 bond. The existence of a single set of NOE signals for Xxx-Pro allows to exclude slow *cis-trans* equilibria around Xxx-Pro bonds.

The final input file for the CYANA structure calculation software contained 99 meaningful distance constraints (45 intraresidue, 34 short-, 15 medium- and 5 long-range) and 42 angle constraints, which were derived from intraresidue and sequential NOEs and the $^3J_{HNCH}$ vicinal coupling constants. Moreover, two upper and lower distance constraints between the Val^9 carbonyl carbon and the Pro^1 amide nitrogen and the Val^9 carbonyl oxygen and the Pro^1 δ carbon, were imposed to allow peptide cyclisation. These constraints were, then, used to generate a total of 100 structures and among them the 20 structures with the lowest target function values were selected. The backbone superposition of 20 conformers is reported in Figure 3. Structures with higher target function were also analysed, in order to probe the existence of other conformations, but they were not considered because of the violation of the distance constraints imposed by cyclisation.

The presence of only one residual violation indicates that the input data set is self-consistent and the constraints are well satisfied by the 20 best CYANA structures (Table 4). Moreover, most of the distances shorter than 3.5 Å, occuring in more than half of the best CYANA structures, give rise to cross-peaks in the NOESY spectrum. This observation is particularly indicative of the presence of a predominant conformer for analogue 4 in the acetonitrile/water mixture [47].

The ensemble of the 20 structures of analogue **4** (Figure 3) is well defined since the rms deviation values of the backbone and of the heavy atoms are 0.37 and 1.07 Å, respectively. Backbone dihedral

Table 4. Statistics for the 20 model NMR solution structures of CLA analogue **4**

Number of residues	9
NOE upper distance limits	99
Dihedral angle constraints	42
Average CYANA target function (Å^2)	0.41 ± 0.15
Distance restraints	
All	
Short-range, $\|i-j\| \leq 1$	81
Medium-range, $1 < \|i-j\| < 5$	15
Long-range, $\|i-j\| > 5$	5
Distance bounds (# restraints)	
−2.99	16
3.00–3.99	35
4.00–4.99	5
5.00–5.99	9
6.00–	36
RMSD	
All residues, backbone (Å)	0.37 ± 0.13
All residues, heavy atoms (Å)	1.07 ± 0.20
Ramachandran plot statistics	
Most favoured regions	45.8%
Additional allowed regions	53.3%
Generously allowed regions	0.8%
Disallowed regions	0.0%

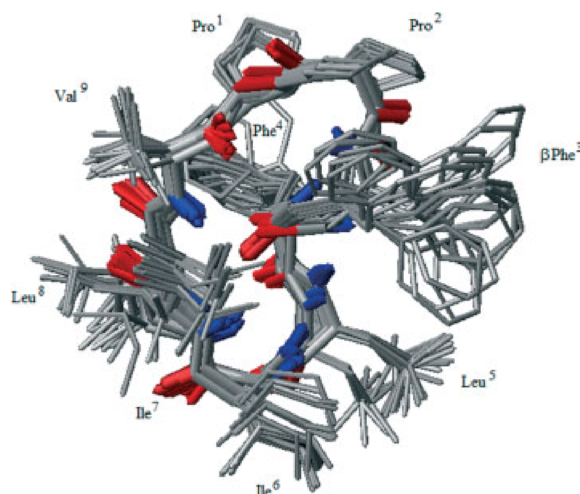

Figure 3. Superimposition on the backbone atoms of the best 20 CYANA structures of CLA analogue **4**. The CO bonds are shown in black.

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Table 5. Dihedral angle statistics for the 20 best CYANA structures of CLA analogue **4**

Residue	ϕ	ψ	μ
Pro^1	−69.8 (1.00)	168.8 (1.00)	
Pro^2	−72.7 (1.00)	−20.0 (1.00)	
βPhe^3	−128.7 (0.96)	−14.5 (0.84)	90.6 (1.00)
Phe^4	−78.0 (0.98)	−2.8 (1.00)	
Leu^5	−84.1 (0.97)	−59.7 (1.00)	
Ile^6	−95.8 (0.95)	−45.5 (0.96)	
Ile^7	−85.6 (1.00)	−0.9 (1.00)	
Leu^8	49.7 (0.99)	29.3 (0.99)	
Val^9	−145.9 (0.98)	112.0 (0.99)	

Average values for each angle are in degrees. In parentheses the angular order parameter values are reported. μ angle in βPhe^3 is defined as *N*-C$^\beta$-C$^\alpha$-C′.

angles of the 20 structures are reported in Table 5. All backbone ϕ/ψ pairs lie mainly within the most favoured and additional allowed regions of the Ramachandran plot [48] (Table 4). The calculated ϕ values are in good agreement with that obtained by the measured vicinal coupling constants. All ϕ/ψ pair values fall within the right-handed helical regions except for Leu^8 whose ϕ and ψ values lie within the left-handed helical region. The dihedral angles of Pro^1 and Pro^2 residues in the Val^{9-}-Pro^1-Pro^2-βPhe^3 segment are close to those proper of a type VIa β-turn, with one *cis* peptide bond between the two proline residues, but the intramolecular 4 → 1 H-bond, involving the HN of βPhe^3 and the CO of Val^9 does not form. The inspection of hydrogen bonds present in more than eight structures shows the occurrence of two H-bonds:

1. An intramolecular 7 → 1 H-bond, involving Val^9 HN and βPhe^3 carbonyl oxygen, supported by the Val^9 HN temperature coefficient value, which stabilises a C_{19} ring structure.
2. An intramolecular 3 → 1 H-bond between Phe^4 HN and Pro^2 CO (not supported by the temperature coefficient, probably because of an exchange of the Phe^4 HN with water).

Side chains of the analogue **4** residues are generally well defined with exception of βPhe^3 (Figure 3), which tends to form a hydrophobic interaction with Pro^2 ring as confirmed by the upfielded chemical shift of one Pro^2 Hγ. Also Phe^4 and Leu^8 side chains fold close, giving rise to a hydrophobic interaction also supported by the upfielded chemical shift of the two Leu^8 Hδ protons.

Comparison with CLA structure

To compare the conformation of analogue **4** with that found for CLA [9], the two structures were superimposed (Figure 4). The structural comparison clearly indicates that the main structural differences reside, as expected from the presence of the additional CH_2 in the polypeptide backbone, around the βPhe^3 mutation. Indeed, the Ile^6-Pro^2 segments give a quite good superimposition (backbone rmsd = 0.77 Å), whereas the βPhe^3-Phe^4-Leu^5 structure largely differs from the corresponding Phe^3-Phe^4-Leu^5 in CLA. In particular, in compound **4**, Phe^4 side chain points outside the peptide ring, forming a hydrophobic interaction with Leu^8, whereas in CLA it points inside. Moreover, the two cyclopeptides present a different intramolecular hydrogen-bond network. Indeed, the different conformation of the βPhe^3-Phe^4-Leu^5 region is responsible for the loss of a β-turn between Leu^8 NH and Leu^5 CO and of the intramolecular Phe^3 HN → Val^9 CO H-bond present in CLA structure. In addition, the intramolecular Val^9 HN → Phe^4 CO H-bond, stabilising a C_{16} ring structure in CLA, is not present in the peptide **4** and is substituted, as described above, by the Val^9 HN → βPhe^3 CO H-bond that give rise to the formation of a C_{19} ring structure (Figure 5). It is worth noting that, even thought the Phe^3 has been replaced by the corresponding β^3-aminoacid, the Val^9-Pro^1-Pro^2-Phe^3-Phe^4 segment preserves important structural features of CLA, such as the *cis* geometry of Pro^1-Pro^2 amide bond and the stacking of the rings Pro^2-Phe^3, but contains a different structure of Phe^4 side chain that is located on the opposite side of the peptide plane. Therefore, analogue **4** does not manifest the face to edge interaction of phenyl rings, as in CLA. The different relative orientation of the two rings is also evident from the analysis of βPhe^3 HN chemical shift. The close proximity of the aromatic rings in CLA causes a strong signal shift of the Phe^3 HN to 5.96 ppm [9]. Such upfield is not observed for βPhe^3 HN in peptide **4**, indeed the HN of the two Phe are placed in the same region of the ^{1}H NMR spectrum (Table 2).

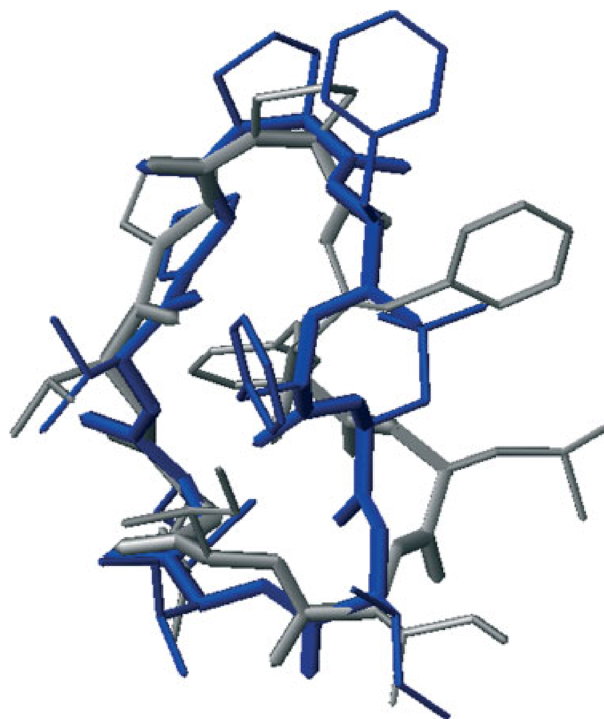

Figure 4. Superimposition of Ile^6-Pro^2 segments between a single most representative NMR structure of *c*(Pro-Pro-β^3Phe-Phe-Leu-Ile-Ile-Leu-Val-) (grey) and the molecular model of CLA (black) obtained by x-ray diffraction analysis [8].

Biological Assays

In the inductive phase of the DTH response to OVA in mice (Figure 6) the studied linear and cyclic CLA analogues, modified with the β^3Phe residue, exhibited strong suppressive effects, comparable with that of CsA. The strongest inhibitory actions were found in the cases of analogues **4**, **5** and **6**. Interestingly, the suppressive actions of the peptides were, generally, not distinctly stronger at the higher (100 µg) dose except of compound **6**. Moreover, peptide **3** was more active at 10 µg/mouse dose.

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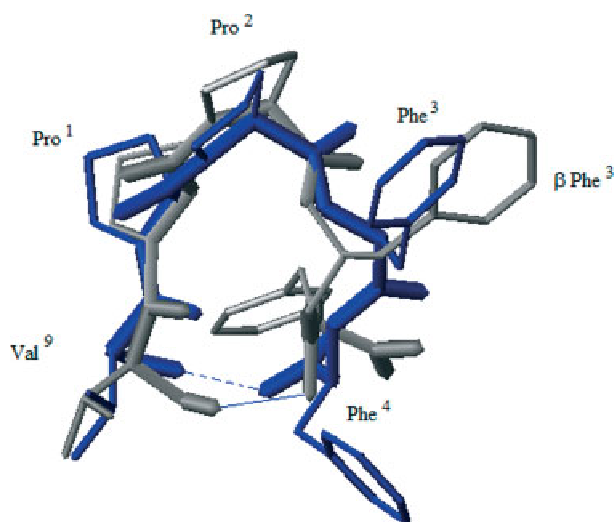

Figure 5. Superimposition between the Val-Pro-Pro-Phe-Phe moiety of a single most representative NMR structure of CLA analogue **4** (grey) and of CLA x-ray structure (black). The hydrogen bonds involving HN of Val^9 and CO of β^3Phe^3 and Phe^4 in peptide **4** and in CLA, respectively, are reported as dotted and thin black lines.

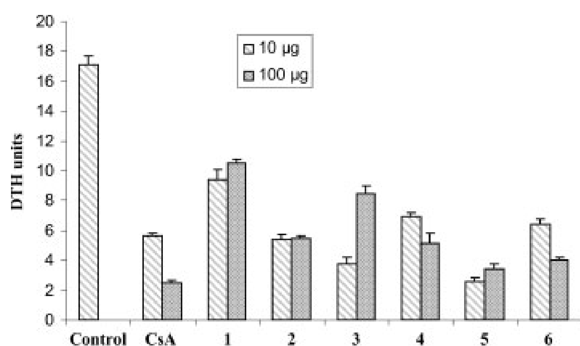

Figure 6. Effects of CLA analogues on the inductive phase of the DTH to OVA. Mice were given the compounds at 10 or 100 µg doses, 2 h before and 24 h after immunisation of mice with OVA. Four days after immunisation the mice were given the eliciting dose of antigen (OVA) and 24 h later the magnitude of the DTH reaction was measured as the footpad swelling. The results are presented as mean values from ten determinations (foot pads) ± SE and expressed as DTH units.

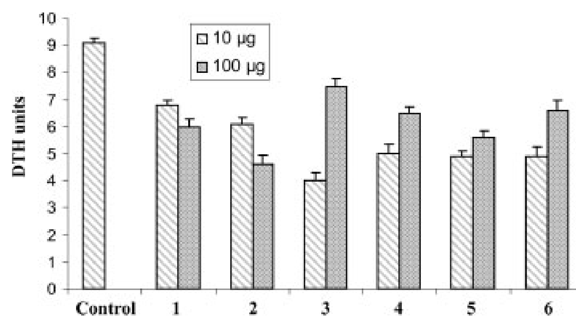

Figure 7. Effects of CLA analogues on the effector phase of the DTH to OVA. Mice were immunised with OVA and after 4 days, 2 h before administration of the eliciting dose of antigen, the mice were given the compounds (10 or 100 µg doses). The measurement of the DTH reaction and presentation of the results are as described in Figure 6.

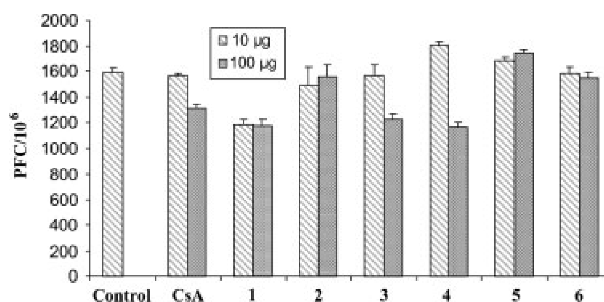

Figure 8. Effects of CLA analogues on the humoral immune response *in vivo* to sheep erythrocytes mice were treated with 10 or 100 µg of the compounds, 2 h before and 24 h after immunisation of animals with SRBC. Four days later the number of cells producing anti-SRBC antibodies (PFC) in the spleens was determined. The results are presented as mean PFC values from 5 mice ± SE.

In the effector phase of the DTH (Figure 7) all peptides demonstrated moderate, but statistically significant suppression of the cellular response. Generally, the same compounds display similar effects in both DTH experimental protocols. In this protocol, CsA was not used since, according to our hitherto experience, CsA does not affect the effector phase of DTH. The ability of the analogues to suppress the manifestation of the cellular immune response is a valuable feature, potentially allowing to apply them to control ongoing inflammatory reactions mediated by Th1 cells.

The peptides, administered before immunisation, had differential effect on the humoral immune response to SRBC (Figure 8). Compound **1** showed a moderate inhibitory activity at both doses and compounds **3** and **4** were active only at 100 µg/mouse. Other peptides did not alter the immune response. It is worth to note that CsA, the reference compound, was only slightly inhibitory at the dose of 100 µg. It has to be, however, mentioned that the humoral immune response to SRBC is subject to a regular biorhythm [49] and the action of CsA and other immunosuppressive agents may vary depending on a magnitude of the immune response. In the case of CsA the suppressive effect on the inductive phase of the humoral immune response to SRBC is strong when the control responses are high, not low-to-moderate [50]. Nevertheless, compounds **1**, **3** and **4** at 100 µg dose were equally effective as CsA in suppressing the humoral response.

Conclusions

In the present work, we have studied three linear and three cyclic analogues of CLA with very interesting immunosuppressive properties. The strongest inhibitory actions, comparable with that of CsA, were found in the cases of analogues **4**, **5** and **6** and were best pronounced in the inductive phase of the cellular response. The effector phase of DTH was inhibited to a lesser degree. On the other hand, the humoral immune response was slightly inhibited, suggesting that the compounds expressed directional suppressive action towards the cellular immune response. The conformational analysis carried out on peptide **4**, in which with respect to the natural peptide molecule, CLA, we have replaced the Phe residue in position 3 by β^3Phe, shows conformational changes of the nonapeptide around the βPhe^3 mutation, while the Ile^6-Pro^2 segment gives a quite good superimposition with CLA. Peptide **4** shows also a similar stacking of the rings Pro^2-Phe^3 with respect to the homologue sequence of CLA, but the observation that the Phe^4 side chain is located on the opposite side of the peptide plane

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seems to indicate that the orientation of this side chains does not influence the biological response for this class of compounds.

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