European Journal of Medicinal Chemistry 44 (2009) 3731–3738

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and immunosuppressive activity of cyclolinopeptide A analogues containing homophenylalanine

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ARTICLE INFO

Article history:
Received 25 April 2008
Received in revised form
17 March 2009
Accepted 26 March 2009
Available online 5 April 2009

Keywords:
Cyclolinopeptide A
Immune suppression
Homophenylalanine
Cytotoxity

ABSTRACT

Immune response suppressors are used in the medical praxis to prevent graft rejection after organ transplantation and in the therapy of some autoimmune diseases. Cyclolinopeptide A, naturally existing immunomodulatory peptide, was modified with homophenylalanine in positions 3 (**4**), 4 (**5**) or both 3 and 4 (**6**). The conformational influence of the replacement of Phe by Hphe was analyzed by NMR spectroscopy. Peptides **4**–**6** exist as single isomers with all *trans* peptide bonds except *cis* Pro–Pro peptide bond. The peptides were tested for their ability to suppress the proliferative response of mouse splenocytes to T- and B-cell mitogens and the secondary humoral immune response to sheep erythrocytes in vitro in parallel with a reference drug – cyclosporine A. The substitution of Phe with Hphe in positions 3 and 4 of CLA led to three different activities in the studied immunological assays. Very potent inhibition of AFC number of peptide **4** was not associated with cell toxicity. This compound caused a complete block of T- and B-cell proliferation. Peptides **5** and **6**, containing Hphe in position 3 or 3 and 4, respectively, gave similar effects on the proliferative response of splenocytes to mitogens. Peptide **6** was a moderate suppressor of the humoral immune response, peptide **5** was exceptionally inhibitory. The presence of Hphe in position 4 of CLA backbone markedly reduced the viability of the tested cell line, however addition of the second Hphe in position 3 improved cell survival in comparison with the solvent.

1. Introduction

The problem of transplantology, a dynamically expanding branch of contemporary medicine, is not merely the problem of exquisite surgical technique, but also, and even more importantly, the problem of transplanted organ survival. The inhibitors of PPIases, such as cyclosporine A (CsA) [1], Tacrolimus (FK-506) [2] and rapamycin [1] are effective immune suppressors able to cause a selective inhibition of lymphocyte T activation during immune response. All three compounds are 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 therapies, a wider use of these drugs meets great limitation [2]. Thus, the search for new immune suppressors with a comparable efficacy but with lower toxicity, especially in the group of naturally existing immunomodulatory peptides and its analogues, is an important task for medicinal chemistry.

Cyclolinopeptide A (CLA), a natural highly hydrophobic cyclic nonapeptide: 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 at 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].

As a part of structure–activity relationships studies a great number of CLA analogues were synthesized and their biological activity was evaluated, but none of these analogues showed the immunosuppressive potency higher than the native peptide [21,22]. The conformational flexibility of CLA molecule is believed to be one of the most important factors responsible for its biological activity. In addition, it was suggested, that the presence of the Pro-Pro-Phe-Phe tetrapeptide unit sequence, containing the Pro–Pro peptide bond in *cis* geometry and the "edge to face" interaction

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doi:10.1016/j.ejmech.2009.03.037

between the two phenylalanine's aromatic rings, was important for the immunosuppressive activity of CLA.

In order to evaluate the role and significance of "edge to face" interaction between the two phenylalanine's aromatic rings for immunosuppressive activity of the native peptide, we synthesized three linear precursors and three cyclic analogues of CLA, in which one or both phenylalanine residues were replaced by homophenylalanine residues (Fig. 1).

This particular, unnatural amino acid, a homologue of phenylalanine with elongated phenylalkyl side chain, is an agent of interest to pharmacologists since a long time. It was used as a precursor for the synthesis of many antihypertensive drugs acting as angiotensin-converting enzyme inhibitors [23,24] and many other biologically active compounds including opioid peptides and proteinase inhibitors [25–29]. Hphe residue, as a non-proteinogenic amino acid, should enhance the stability of the peptide toward the enzymatic degradation with preservation of the aromatic character of Phe. The elongation of the side chain may change the mutual orientation of aromatic rings and change the lipophilicity of modified peptide molecule. The effects exerted by new CLA analogues on the humoral immune response in mice and the proliferative response of splenocytes to T-cell and B-cell mitogens will be compared with those produced by CsA.

2. Materials and methods

2.1. 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. The optical rotation was measured in a 1 dm cell (1 ml) on a Horiba high speed automatic polarimeter at 589 nm. For thin layer chromatography 250 nm silica gel GF precoated uniplates (Merck) were used with following solvent systems: (C) acetonitrile:water (5:1), (D) butan-1-ol:acetic acid:water (4:1:1), (E) chloroform:methanol:acetic acid (16:4:1), (F) ethyl acetate, (G) chloroform:methanol (9:1), (H) chloroform:methanol (19:1). The chromatograms were visualized with chlorine followed by starch/KI and/or ninhydrin. HPLC was performed on an LDC/Milton-Roy analytical instrument using a Vydac C18 column (0.46 × 25 cm): flow 1.0 ml/min., detection at 220 nm and eluants (A) 0.05% trifluoroacetic acid in water and (B) 0.038% trifluoroacetic acid in acetonitrile/water 90:10 with a gradient application. Purification of peptides was performed by the preparative reversed-phase HPLC on a Vydac C18 column (2.2 × 25 cm): flow rate 16 ml/min, UV detection at 220 nm. The identities of the pure peptides were confirmed by Maldi-TOF mass spectrometry on Voyager Elite, Perseptive Biosystems using α-cyano-4-hydroxycinnamic acid as a matrix. Cyclic peptides were additionally analyzed by LSI MS on Finnigan MAT 95 mass spectrometer using glycerol matrix.

The one- and two-dimensional ^{1}H NMR spectra were recorded on a Bruker Avance II Plus spectrometer at 700.4 MHz in DMSO-d_6 or $CDCl_3$, using as an internal standard DMSO signal 2.50 ppm or TMS (0 ppm), respectively. Spin-lock time in TOCSY experiments was 80 ms and NOESY experiments were recorded with 300 ms mixing time. 2D NMR spectra were processed with NMRPipe [30] and analyzed by CARA [31] software.

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

2.2. Peptide synthesis and purification

The linear peptides **1**–**3** were synthesized by the manual solid-phase method using chloromethylated Merrifield resin as a solid support. Attachment of the first Boc-AA-OH (Boc-Phe-OH, Boc-Leu-OH and Boc-Hphe-OH) to the resin was performed according to cesium salt procedure [32] and the substitution level was determined by weight gain measurements (Boc-Phe-Ⓟ, 0.65 mmol/g; Boc-Leu-Ⓟ, 0.59 mmol/g; Boc-Hphe-Ⓟ, 0.66 mmol/g). Synthesis of desired peptides was achieved by stepwise coupling of Boc-amino acids to the growing peptide chain on the resin. Starting with 0.2 mmol of Boc protected amino acid attached to the resin, standard single TBTU/HOBt/DIPEA coupling protocol (but with each deprotonation step omitted) was used for all amino acids and was repeated if Kaiser test [33] or Chloranil test (proline residue) [34] was found positive. In all cases where after second coupling test was slightly positive, remaining free amino groups were acetylated with the aid of acetic anhydride (100 μl) in the presence of diisopropylethylamine (100 μl). After coupling step the Boc protecting group was removed with 50% TFA in methylene chloride. The peptide resin was treated with TFMSA (1 ml), TFA (10 ml) and anisole (0.5 ml) at 0 °C and stirred for 60 min at room temperature. After the resin had been filtered off and washed with TFA the crude peptide was precipitated upon concentration of solvents and addition of diethyl ether. The analytical samples were purified by the preparative HPLC. The physicochemical properties of the synthesized linear peptide are summarized in Table 1.

The crude linear precursors **1**–**3** (1 eq.) were cyclized (progress of the cyclization reaction was monitored by HPLC) by means of EDC (3 eq.) in the presence of HOAt (3 eq.) and diisopropylethylamine (1 eq. to deprotonate the amino group of linear precursor) in methylene chloride at much lower concentration (40 mg of the linear peptide in 800 ml of methylene chloride) than usually described for "head to tail" peptide cyclization reactions, to avoid the dimer formation [35,36]. Crude cyclic peptides **4**–**6** were purified in the same way as their linear precursors. The physicochemical properties of the synthesized cyclic peptide are summarized in Table 1.

2.3. ^{1}H NMR analysis of cyclopeptides *4*–*6*

c**(Pro^1-Pro^2-Hphe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)* (4***). (DMSO-d_6, 25 °C) *δ*: 0.69 (3H, H$^\delta$, Ile^7), 0.71 (3H, H$^\delta$, Leu^8), 0.75 (3H, H$^\gamma$, Val^9), 0.76 (3H, H$^\delta$, Ile^6), 0.78 (3H, H$^\gamma$, Ile^7), 0.78 (3H, H$^\delta$, Leu^5), 0.80

1 H-Leu^5-Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-**Hphe^3**-Phe^4-OH

2 H-**Hphe^4**-Leu^5-Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-Phe^3-OH

3 H-Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-**Hphe^3-Hphe^4**-Leu^5-OH

4 **c**(Pro^1-Pro^2-**Hphe^3**-Phe^4 -Leu^5-Ile^6-Ile^7-Leu^8-Val^9) [Hphe^3]CLA

5 **c**(Pro^1-Pro^2-Phe^3-**Hphe^4**-Leu^5-Ile^6-Ile^7-Leu^8-Val^9) [Hphe^4]CLA

6 **c**(Pro^1-Pro^2-**Hphe^3-Hphe^4**-Leu^5-Ile^6-Ile^7-Leu^8-Val^9) [Hphe^{3,4}]CLA

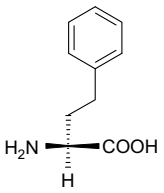

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Fig. 1. The sequences of newly synthesized CLA analogues **1**–**6** containing (*S*)-Hphe-OH **7**.

Table 1
Physicochemical properties of CLA analogues **4**–**6** used in the present study and their linear precursors **1**–**3**.

Peptide	Yield[a] (%)	HPLC		TLC, R_f[b]			Mol. form.	Formula mass	MALDI MS, *m/z*	LSI MS, *m/z*
		Purity (%)	t_R (min)							
1	78.9	95.6	10.33 (40–80% B)	0.45 (C)	0.34 (D)	0.47 (E)	$C_{58}H_{89}N_9O_{10}$	1071.67	$[M+H]^+$ 1073.3 $[M+Na]^+$ 1095.1	
2	100	100	8.43 (45–65% B)	0.46 (C)	0.37 (D)	0.42 (E)	$C_{58}H_{89}N_9O_{10}$	1071.67	$[M+K]^+$ 1125.9	
3	100	97.3	5.75 (50–90% B)	0.54 (C)	0.50 (D)	0.56 (E)	$C_{59}H_{91}N_9O_{10}$	1085.69	$[M+K]^+$ 1125.9	
4	33.2	99	16.53 (50–90% B)	0.41 (F)	0.59 (G)	0.33 (H)	$C_{58}H_{87}N_9O_9$	1053.66	$[M+Na]^+$ 1076.1 $[M+K]^+$ 1092.1	$[M+H]^+$ 1054.7 $[M-H]^-$ 1052.6
5	33.2	99	16.37 (50–90% B)	0.56 (F)	0.52 (G)	0.30 (H)	$C_{58}H_{87}N_9O_9$	1053.66	$[M+Na]^+$ 1076.1 $[M+K]^+$ 1092.1	$[M+H]^+$ 1054.7 $[M-H]^-$ 1052.6
6	42.1	99	16.73 (50–90% B)	0.39 (F)	0.56 (G)	0.32 (H)	$C_{59}H_{89}N_9O_9$	1067.68	$[M+Na]^+$ 1090.6	$[M+H]^+$ 1068.6 $[M-H]^-$ 1066.6

[a] Yields of crude peptides were calculated on the basis of the loading of the first amino acid.
[b] Solvent systems and conditions are given in the Materials and methods section.

(3H, H^{δ}, Leu^5), 0.80 (3H, H^{δ}, Leu^8), 0.83 (3H, H^{γ}, Ile^6), 0.90 (3H, H^{γ}, Val^9), 1.00 (1H, H^{γ}, Ile^7), 1.07 (1H, H^{γ}, Ile^6), 1.35 (1H, H^{γ}, Ile^7), 1.38 (1H, H^{γ}, Leu^8), 1.39 (1H, H^{γ}, Ile^6), 1.39 (1H, H^{β}, Leu^5), 1.47 (1H, H^{β}, Leu^5), 1.58 (1H, H^{γ}, Leu^5), 1.61 (1H, H^{β}, Leu^8), 1.72 (1H, H^{γ}, Pro^2), 1.75 (1H, H^{β}, Pro^2), 1.75 (1H, H^{β}, Pro^1), 1.75 (1H, H^{β}, Leu^8), 1.82 (1H, H^{γ}, Pro^1), 1.85 (1H, H^{β}, Ile^7), 1.90 (1H, H^{β}, Val^9), 1.91 (1H, H^{γ}, Pro^2), 1.93 (1H, H^{γ}, Pro^1), 1.95 (1H, H^{γ}, Hphe^3), 2.03 (1H, H^{β}, Ile^6), 2.04 (1H, H^{γ}, Hphe^3); 2.20 (1H, H^{β}, Pro^2), 2.20 (1H, H^{β}, Pro^1), 2.46 (1H, H^{β}, Hphe^3), 2.54 (1H, H^{β}, Hphe^3), 2.88 (1H, H^{β}, Phe^4), 3.05 (1H, H^{β}, Phe^4), 3.39 (1H, H^{δ}, Pro^2), 3.55 (1H, H^{δ}, Pro^1), 3.56 (1H, H^{δ}, Pro^2), 3.59 (1H, H^{δ}, Pro^1), 3.62 (1H, H^{α}, Ile^6), 3.81 (1H, H^{α}, Leu^8), 4.04 (1H, H^{α}, Leu^5), 4.08 (1H, H^{α}, Pro^1), 4.11 (1H, H^{α}, Phe^4), 4.33 (1H, H^{α}, Ile^7), 4.43 (1H, H^{α}, Pro^2), 4.46 (1H, H^{α}, Val^9), 4.63 (1H, H^{α}, Hphe^3), 6.84 (2H, $H^{2,6}$, Phe^4), 7.14 (1H, H^4, Phe^4), 7.14 (2H, $H^{2,6}$, Hphe^3), 7.14 (2H, $H^{3,5}$, Phe^4), 7.18 (2H, $H^{3,5}$, Hphe^3), 7.24 (1H, H^N, Val^9), 7.27 (1H, H^4, Hphe^3), 7.35 (1H, H^N, Leu^5), 7.75 (1H, H^N, Ile^7), 7.82 (1H, H^N, Leu^8), 8.03 (1H, H^N, Ile^6), 8.06 (1H, H^N, Phe^4), 8.41 (1H, H^N, Hphe^3).

($CDCl_3$, −60 °C, TMS) δ: 0.68 (3H, H^{γ}, Val^9), 0.74 (3H, H^{δ}, Ile^7), 0.74 (3H, H^{δ}, Leu^5), 0.78 (3H, H^{γ}, Ile^7), 0.78 (3H, H^{γ}, Val^9), 0.84 (3H, H^{δ}, Leu^8), 0.84 (3H, H^{δ}, Leu^8), 0.90 (3H, H^{δ}, Leu^5), 0.94 (3H, H^{δ}, Ile^6), 0.96 (3H, H^{γ}, Ile^6), 0.99 (1H, H^{γ}, Ile^7), 1.10 (1H, H^{γ}, Ile^7), 1.15 (1H, H^{γ}, Ile^6), 1.46 (1H, H^{γ}, Ile^6), 1.51 (2H, H^{β}, Leu^5), 1.54 (1H, H^{γ}, Leu^5), 1.65 (1H, H^{β}, Hphe^3), 1.66 (1H, H^{γ}, Leu^8), 1.66 (1H, H^{γ}, Pro^2), 1.69 (1H, H^{β}, Hphe^3), 1.73 (1H, H^{γ}, Hphe^3), 1.79 (1H, H^{β}, Pro^2), 1.84 (1H, H^{γ}, Hphe^3), 1.94 (1H, H^{β}, Ile^7), 1.96 (1H, H^{γ}, Pro^1), 2.01 (1H, H^{γ}, Pro^1), 2.02 (1H, H^{β}, Ile^6), 2.05 (1H, H^{γ}, Pro^2), 2.15 (1H, H^{β}, Pro^2), 2.16 (1H, H^{β}, Leu^8), 2.16 (1H, H^{β}, Pro^1), 2.26 (1H, H^{β}, Pro^1), 2.34 (1H, H^{β}, Leu^8), 2.38 (1H, H^{β}, Val^9), 2.92 (1H, H^{β}, Phe^4), 3.53 (1H, H^{β}, Phe^4), 3.55 (1H, H^{α}, Leu^8), 3.58 (1H, H^{δ}, Pro^2), 3.66 (1H, H^{δ}, Pro^2), 3.69 (1H, H^{α}, Leu^5), 3.88 (2H, H^{δ}, Pro^1), 4.16 (1H, H^{α}, Ile^6), 4.30 (1H, H^{α}, Pro^2), 4.37 (1H, H^{α}, Pro^1), 4.73 (1H, H^{α}, Val^9), 4.73 (1H, H^{α}, Ile^7), 4.78 (1H, H^{α}, Hphe^3), 4.94 (1H, H^{α}, Phe^4), 6.27 (1H, H^N, Ile^6), 6.46 (1H, H^N, Hphe^3), 6.86 (1H, H^N, Val^9), 6.90 (2H, $H^{2,6}$, Hphe^3), 7.10 (1H, H^4, Hphe^3), 7.10 (2H, $H^{2,6}$, Phe^4), 7.19 (3H, $H^{3,4,5}$, Phe^4), 7.25 (2H, $H^{3,5}$, Hphe^3), 7.27 (1H, H^N, Ile^7), 7.33 (1H, H^N, Leu^5), 7.56 (1H, H^N, Leu^8), 8.10 (1H, H^N, Phe^4).

***c*(*Pro*^{1}-*Pro*^{2}-*Phe*^{3}-*Hphe*^{4}-*Leu*^{5}-*Ile*^{6}-*Ile*^{7}-*Leu*^{8}-*Val*^{9}) (*5*).** (DMSO-d_6, 25 °C) δ: 0.73 (3H, H^{δ}, Ile^7); 0.77 (1H, H^{γ}, Pro^2), 0.81 (3H, H^{δ}, Leu^8), 0.82 (3H, H^{δ}, Leu^5), 0.82 (3H, H^{γ}, Ile^7), 0.83 (3H, H^{δ}, Leu^8), 0.83 (3H, H^{δ}, Ile^6), 0.84 (3H, H^{γ}, Val^9), 0.87 (3H, H^{γ}, Ile^6), 0.90 (3H, H^{δ}, Leu^5), 0.94 (3H, H^{γ}, Val^9), 1.04 (1H, H^{γ}, Ile^7), 1.15 (1H, H^{γ}, Ile^6), 1.38 (1H, H^{γ}, Ile^7), 1.43 (1H, H^{γ}, Leu^8), 1.48 (1H, H^{γ}, Pro^2), 1.49 (1H, H^{γ}, Ile^6), 1.56 (1H, H^{β}, Leu^5), 1.60 (1H, H^{γ}, Pro^1), 1.62 (1H, H^{γ}, Leu^5), 1.64 (1H, H^{β}, Pro^1), 1.65 (1H, H^{β}, Leu^5), 1.69 (1H, H^{β}, Leu^8), 1.78 (1H, H^{β}, Pro^2), 1.87 (1H, H^{β}, Ile^6), 1.88 (1H, H^{γ}, Hphe^4), 1.88 (1H, H^{γ}, Pro^1), 1.89 (1H, H^{β}, Leu^8), 1.90 (1H, H^{β}, Val^9), 1.95 (1H, H^{γ}, Hphe^4), 2.04 (1H, H^{β}, Ile^7), 2.05 (1H, H^{β}, Pro^1), 2.40 (1H, H^{β}, Hphe^4); 2.55 (1H, H^{β}, Hphe^4), 2.94 (1H, H^{β}, Phe^3), 3.14 (1H, H^{δ}, Pro^2), 3.24 (1H, H^{δ}, Pro^2), 3.24 (1H, H^{β}, Phe^3), 3.51 (1H, H^{δ}, Pro^1), 3.58 (1H, H^{δ}, Pro^1), 3.95 (1H, H^{α}, Ile^6), 4.05 (1H, H^{α}, Leu^8), 4.06 (1H, H^{α}, Leu^5), 4.13 (1H, H^{α}, Hphe^4), 4.30 (1H, H^{α}, Pro^2), 4.30 (1H, H^{α}, Pro^1), 4.39 (1H, H^{α}, Ile^7), 4.49 (1H, H^{α}, Val^9), 4.97 (1H, H^{α}, Phe^3), 7.08 (1H, H^N, Val^9), 7.12 (2H, $H^{2,6}$, Hphe^4), 7.16 (2H, $H^{2,6}$, Phe^3), 7.17 (1H, H^4, Phe^3), 7.17 (1H, H^4, Hphe^4), 7.26 (2H, $H^{3,5}$, Phe^3), 7.27 (2H, $H^{3,5}$, Hphe^4), 7.52 (1H, H^N, Ile^7), 7.68 (1H, H^N, Leu^8), 7.75 (1H, H^N, Leu^5), 7.78 (1H, H^N, Ile^6), 8.28 (1H, H^N, Hphe^4), 8.43 (1H, H^N, Phe^3).

($CDCl_3$, −60 °C, TMS) δ: 0.47 (3H, H^{δ}, Ile^7), 0.49 (1H, H^{γ}, Pro^2), 0.56 (1H, H^{γ}, Ile^7), 0.61 (3H, H^{δ}, Leu^5), 0.74 (3H, H^{γ}, Ile^7), 0.77 (3H, H^{γ}, Ile^6), 0.81 (6H, H^{δ}, Leu^8), 0.88 (3H, H^{γ}, Val^9), 0.89 (3H, H^{δ}, Leu^5), 0.90 (3H, H^{δ}, Ile^6), 0.94 (3H, H^{γ}, Val^9), 0.95 (1H, H^{β}, Pro^2), 1.07 (1H, H^{γ}, Ile^6), 1.28 (1H, H^{γ}, Ile^7), 1.35 (1H, H^{γ}, Pro^2), 1.40 (1H, H^{γ}, Leu^8), 1.41 (1H, H^{γ}, Ile^6), 1.49 (1H, H^{γ}, Leu^5), 1.53 (2H, H^{β}, Leu^5), 1.56 (1H, H^{β}, Leu^8), 1.64 (1H, H^{β}, Pro^1), 1.86 (1H, H^{β}, Val^9), 1.90 (1H, H^{β}, Ile^6), 1.93 (2H, H^{γ}, Pro^1), 1.97 (1H, H^{β}, Pro^2), 1.98 (1H, H^{β}, Pro^1), 2.02 (1H, H^{β}, Hphe^4), 2.28 (1H, H^{β}, Hphe^4), 2.28 (1H, H^{β}, Ile^7), 2.30 (1H, H^{β}, Leu^8), 2.47 (1H, H^{γ}, Hphe^4), 2.68 (1H, H^{γ}, Hphe^4), 2.69 (1H, H^{β}, Phe^3), 3.14 (1H, H^{δ}, Pro^2), 3.51 (1H, H^{α}, Leu^5), 3.54 (1H, H^{α}, Leu^8), 3.55 (1H, H^{δ}, Pro^2), 3.62 (1H, H^{β}, Phe^3), 3.83 (2H, H^{δ}, Pro^1), 4.14 (1H, H^{α}, Ile^6), 4.15 (1H, H^{α}, Pro^2), 4.26 (1H, H^{α}, Pro^1), 4.66 (1H, H^{α}, Val^9), 4.67 (1H, H^{α}, Ile^7), 4.75 (1H, H^{α}, Hphe^4), 5.07 (1H, H^{α}, Phe^3), 6.30 (1H, H^N, Ile^6), 6.98 (1H, H^N, Ile^7), 7.11 (2H, $H^{2,6}$, Hphe^4), 7.18 (2H, $H^{2,6}$, Phe^3), 7.18 (2H, $H^{3,5}$, Hphe^4), 7.21 (1H, H^N, Val^9), 7.26 (2H, $H^{3,5}$, Phe^3), 7.26 (1H, H^4, Hphe^4), 7.34 (1H, H^4, Phe^3), 7.51 (1H, H^N, Leu^8), 8.03 (1H, H^N, Hphe^4).

***c*(*Pro*^{1}-*Pro*^{2}-*Hphe*^{3}-*Hphe*^{4}-*Leu*^{5}-*Ile*^{6}-*Ile*^{7}-*Leu*^{8}-*Val*^{9}) (*6*).** (DMSO-d_6, 25 °C) δ: 0.61 (3H, H^{δ}, Ile^7), 0.72 (3H, H^{δ}, Ile^6), 0.75 (3H, H^{γ}, Ile^7), 0.78 (3H, H^{δ}, Leu^5), 0.78 (3H, H^{γ}, Ile^6), 0.79 (3H, H^{γ}, Val^9), 0.79 (3H, H^{δ}, Leu^8), 0.81 (3H, H^{δ}, Leu^8), 0.88 (3H, H^{δ}, Leu^5), 0.92 (3H, H^{γ}, Val^9), 0.98 (1H, H^{γ}, Ile^7), 1.09 (1H, H^{γ}, Ile^6), 1.30 (1H, H^{γ}, Ile^7), 1.35 (1H, H^{γ}, Ile^6), 1.39 (1H, H^{γ}, Leu^8), 1.52 (1H, H^{β}, Leu^5), 1.58 (1H, H^{γ}, Pro^1), 1.60 (1H, H^{β}, Leu^5), 1.61 (1H, H^{γ}, Leu^5), 1.66 (1H, H^{γ}, Pro^2), 1.68 (1H, H^{β}, Leu^8), 1.73 (1H, H^{β}, Ile^6), 1.74 (1H, H^{β}, Pro^1), 1.79 (1H, H^{γ}, Hphe^4), 1.87 (1H, H^{β}, Val^9), 1.90 (1H, H^{γ}, Hphe^4), 1.91 (1H, H^{γ}, Pro^2); 1.91 (1H, H^{γ}, Pro^1); 1.92 (1H, H^{γ}, Hphe^3), 2.01 (1H, H^{β}, Leu^8); 2.05 (1H, H^{β}, Ile^7), 2.10 (1H, H^{β}, Pro^2), 2.14 (1H, H^{γ}, Hphe^3), 2.16 (1H, H^{β}, Pro^1), 2.34 (1H, H^{β}, Hphe^4), 2.39 (1H, H^{β}, Pro^2), 2.52 (2H, H^{β}, Hphe^3), 2.52 (1H, H^{β}, Hphe^4), 3.43 (2H, H^{δ}, Pro^2), 3.49 (1H, H^{δ}, Pro^1), 3.58 (1H, H^{δ}, Pro^1), 3.91 (1H, H^{α}, Hphe^4), 3.95 (1H, H^{α}, Ile^6), 4.00 (1H, H^{α}, Leu^5), 4.05 (1H, H^{α}, Leu^8), 4.40 (1H, H^{α}, Ile^7), 4.47 (1H, H^{α}, Pro^1), 4.51 (1H, H^{α}, Val^9), 4.52 (1H, H^{α}, Pro^2), 4.80 (1H, H^{α}, Hphe^3), 6.95 (1H, H^N, Val^9), 7.10 (2H, $H^{2,6}$, Hphe^4), 7.14 (2H, $H^{2,6}$, Hphe^3), 7.18 (1H, H^4, Hphe^4), 7.18 (1H, H^4, Hphe^3), 7.27 (2H, $H^{3,5}$, Hphe^3), 7.27 (2H, $H^{3,5}$, Hphe^4), 7.38 (1H, H^N, Leu^8), 7.49 (1H, H^N, Ile^7), 7.55 (1H, H^N, Ile^6), 7.81 (1H, H^N, Leu^5), 8.47 (1H, H^N, Hphe^4), 8.50 (d, $J = 8.5$ Hz, 1H, H^N, Hphe^3).

($CDCl_3$, −60 °C, TMS) δ: 0.61 (3H, H^{δ}, Ile^7), 0.66 (1H, H^{γ}, Ile^7), 0.78 (3H, H^{δ}, Leu^5), 0.85 (3H, H^{γ}, Ile^7), 0.90 (3H, H^{γ}, Ile^6), 0.93 (6H, H^{δ}, Leu^8), 0.99 (3H, H^{γ}, Val^9), 0.99 (3H, H^{δ}, Ile^6), 1.01 (3H, H^{δ}, Leu^5), 1.06 (3H, H^{γ}, Val^9), 1.15 (1H, H^{γ}, Ile^6), 1.38 (1H, H^{γ}, Ile^7), 1.50 (1H, H^{γ}, Ile^6),

1.51 (1H, H^{γ}, Leu^{8}), 1.60 (1H, H^{γ}, Leu^{5}), 1.62 (1H, H^{β}, Leu^{5}), 1.67 (1H, H^{β}, Leu^{8}), 1.88 (1H, H^{β}, Pro^{2}), 1.96 (1H, H^{β}, Val^{9}), 1.98 (1H, H^{β}, $Hphe^{3}$), 1.98 (1H, H^{β}, Ile^{6}), 2.00 (1H, H^{γ}, Pro^{2}), 2.07 (1H, H^{γ}, Pro^{1}), 2.11 (1H, H^{β}, $Hphe^{4}$), 2.12 (1H, H^{γ}, Pro^{1}), 2.19 (1H, H^{γ}, Pro^{2}), 2.21 (1H, H^{β}, Pro^{2}), 2.36 (1H, H^{β}, Pro^{1}), 2.41 (1H, H^{β}, Ile^{7}), 2.42 (1H, H^{β}, $Hphe^{4}$), 2.42 (1H, H^{β}, Leu^{8}), 2.42 (1H, H^{β}, $Hphe^{3}$), 2.47 (1H, H^{β}, Pro^{1}), 2.52 (1H, H^{δ}, $Hphe^{4}$), 2.68 (1H, H^{δ}, $Hphe^{3}$), 2.70 (1H, H^{δ}, $Hphe^{3}$), 2.75 (1H, H^{δ}, $Hphe^{4}$), 3.64 (1H, H^{α}, Leu^{5}), 3.65 (1H, H^{α}, Leu^{8}), 3.74 (1H, H^{δ}, Pro^{2}), 3.82 (1H, H^{δ}, Pro^{2}), 3.94 (1H, H^{δ}, Pro^{1}), 3.98 (1H, H^{δ}, Pro^{1}), 4.23 (1H, H^{α}, Ile^{6}), 4.42 (1H, H^{α}, Pro^{2}), 4.54 (1H, H^{α}, Pro^{1}), 4.77 (1H, H^{α}, Ile^{7}), 4.79 (1H, H^{α}, $Hphe^{4}$), 4.79 (1H, H^{α}, Val^{9}), 4.91 (1H, H^{α}, $Hphe^{3}$), 6.32 (1H, H^{N}, Ile^{6}), 6.96 (1H, H^{N}, Ile^{7}), 7.13 (1H, H^{N}, Leu^{5}), 7.17 (2H, $H^{2,6}$, $Hphe^{4}$), 7.24 (2H, $H^{2,6}$, $Hphe^{3}$), 7.27 (1H, H^{4}, $Hphe^{3}$), 7.34 (3H, $H^{3,4,5}$, $Hphe^{4}$), 7.34 (1H, H^{N}, Val^{9}), 7.41 (2H, $H^{3,5}$, $Hphe^{3}$), 7.61 (1H, H^{N}, Leu^{8}), 8.05 (1H, H^{N}, $Hphe^{4}$).

2.4. Biological methods

Mice. 12-week-old CBA mice of both sexes were used for the study. Mice were fed with a commercial, pelleted food and filtered tap water *ad libitum*.

Reagents and media. Concanavalin A (ConA), pokeweed mitogen (PWM), 93-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and DMSO were from Sigma, RPMI-1640 and Hanks' medium from Cibi/Life Technologies, UK, and fetal calf serum from Gibco. Sheep red blood cells (SRBC) were delivered by Wroclaw University of Environmental and Life Sciences.

Preparations of the peptides for the tests. The compounds were initially dissolved in DMSO and subsequently in the culture medium. Then, the solutions (1 mg/ml) were sterilized by filtration, aliquoted and stored at −20 °C until use.

Proliferative response of mouse splenocytes to mitogens. Spleens were homogenized by pressing the organs through a plastic screen into Hanks' medium. After centrifugation the cells were treated for 5 min with 0.83% ammonium chloride to lyze erythrocytes. Then, the cells were washed twice by centrifugation in Hanks' medium and filtered through a sterile cotton wool to remove dead cells and debris. Eventually, the cells were re-suspended in a medium consisting of RPMI 1640, supplemented with FCS, L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics (referred later to as the culture medium). The cells were distributed to flat-bottom 96-well culture plates at density of 10^5 cells/100 μl/well. ConA or PWM were used at a dose of 2.5 μg/ml and the studied peptides at 1–100 μg/ml concentrations. The cells were incubated for 3 days in a cell culture incubator and the rate of cell proliferation was determined using colorimetric MTT method [37]. The results were presented as mean optical density (OD) values at 550/630 nm wavelength from quadruplicate wells (determinations) ± standard error (SE). The studied effects of the compounds were compared with appropriate DMSO controls.

Determination of the humoral immune response in vitro. Mice were sensitized with 0.2 ml of 1% SRBC suspension. Four days later the animals were sacrificed and the splenocyte suspension was prepared as described above. The cells were distributed to flat-bottom, 24-well culture plates (5×10^6/ml/well), followed by addition of SRBC (0.1 ml of 0.005% suspension). After four-day incubation the number of antibody-forming cells (AFC) was determined [38]. The results were presented as mean AFC numbers from quadruplicate wells ± SE (standard error).

Determination of toxicity of the compounds for WEHI 164.13 cells. The toxicity of the compounds was determined with respect to WEHI 164.13 cell line [39]. The compounds were dissolved in DMSO, then in the culture medium at the concentration range of 0.1–100 μg/ml. Appropriate concentrations of DMSO served as respective controls. The monolayers of WEHI 164.13 cells (2×10^5/well) in flat-bottom, 96-well plates were incubated with DMSO control solutions or the compounds for 24 h in a cell culture incubator. After the incubation the cell viability was determined by MTT colorimetric method [36]. The results are presented as the percentage of OD values in relation to appropriate solvent dilutions, where the cell viability was regarded as 100%.

Statistics. The data were statistically evaluated using Student's *t*-test. The results were presented as mean values ± SE and regarded significant when $p < 0.05$.

3. Results and discussion

3.1. Chemistry

The three new analogues of CLA (**4**–**6**), modified with homophenyalanine residue in positions 3, 4 and both 3 and 4, were synthesized by stepwise coupling of Boc-amino acids to the growing peptide chain on Merrifield resin. The couplings were mediated by the TBTU/HOBt in the presence of diisopropylethylamine. On completion of the synthesis, the unprotected peptides were cleaved with the TFMA/TFA reagent and the crude linear precursors (**1**–**3**) were cyclized by means of EDC in the presence of HOAt and DIPEA. The cyclization reactions were carried out in very diluted solutions to avoid the dimer formation. The homogeneity of the purified cyclic peptides was checked by analytical HPLC and their structures were confirmed by Maldi-TOF MS, LSI MS and NMR spectroscopy. ^{1}H NMR measurements indicated absence of epimerization during the synthesis and confirmed that pure peptides **4**–**6** contained only one isomer as in the native peptide.

3.2. NMR measurements

The structure of CLA molecule was studied in the solid state and in solution [8]. The geometry of peptide bonds is *trans* except the *cis* Pro^1–Pro^2 bond ($\omega = 10°$). The backbone conformation of CLA is stabilized in the solid state by five hydrogen bonds NH···O═C: $HN^5 \rightarrow CO^3$, $HN^7 \rightarrow CO^4$, $HN^8 \rightarrow CO^5$, $HN^4 \rightarrow CO^9$ and $NH^9 \rightarrow CO^4$. The conformational flexibility of CLA molecule in solution is comparable to the flexibility of linear peptides.

CLA in chloroform solution at 214 K exists as a single conformer with well-defined structure. In ^{1}H NMR spectrum NH resonances were recorded within 2 ppm region and the NH–CHα coupling constants were determined for all residues except Leu^5. The temperature dependence of NH chemical shifts indicated that NHs of Phe^4, Ile^7, Leu^8 and Val^9 are hydrogen bonded and Ile^6 is exposed to the solvent. The close proximity of Pro^2 side chain and aromatic Phe^3 ring in the CLA molecule causes a strong signal shift of one of Pro^2 γ proton to 0.33 ppm [8].

Peptides **4**–**6** were examined by ^{1}H NMR in DMSO or $CDCl_3$ solutions. In both solvents peptides **4**–**6** exist as one isomer with all *trans* peptide bonds except *cis* Pro–Pro peptide bond. The diagnostic NOE correlations between CαH atoms of Pro^1 and Pro^2 were found for each peptide.

The replacement of one or both Phe residues by Hphe in peptides **4**–**6** changed their ^{1}H NMR spectra recorded in $CDCl_3$ at 213 K in comparison to that recorded for the unmodified CLA [8]. The most noticeable changes in chemical shifts are observed for residues **1**–**4**. The presence of Hphe in position 3 as in **4** and **6** causes the increasing of Pro^1 CαH chemical shift of 0.15 and 0.21 ppm, respectively. The difference in chemical shifts between the β protons of Pro^1 is about 0.1 ppm for **4** and **6**, smaller than observed for these protons in **5** and in CLA (0.34 and 0.37 ppm, respectively). The aliphatic protons of Pro^2 in peptides **4** and **6** are strongly shifted down-field in comparison to CLA. Particularly, the γ′ protons of Pro^2 in **4** and **6** are observed at 1.71 and 1.94 ppm,

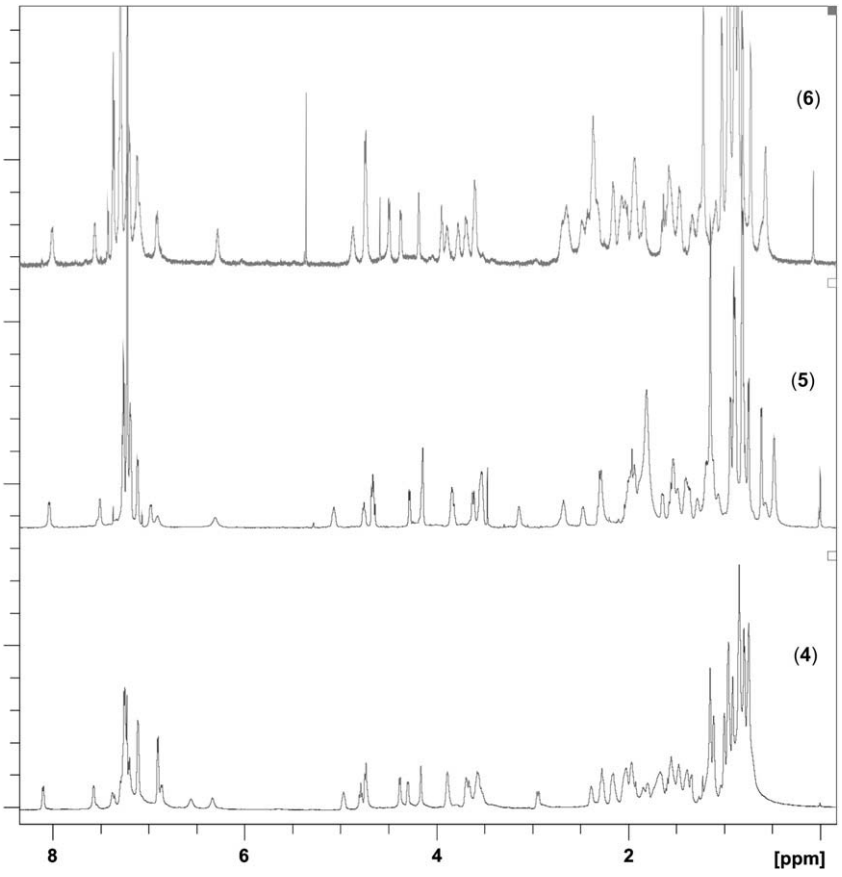

Fig. 2. The 700 MHz ^{1}H NMR spectra of peptides **4**–**6** in $CDCl_3$ recorded at 213 K.

respectively. It means much smaller shielding effect of Hphe phenyl rings in comparison to Phe. The mutual orientation of Pro^2 and Phe^3 in **5** is similar to that in CLA. All resonances of Pro^2 are placed in similar positions, however γ′ proton (0.53 ppm) do not experience unusually large high-field shift observed for CLA (0.33 ppm). The majority of Leu^5, Ile^6, Ile^7, Leu^8 and Val^9 protons in **4**–**6** have resonances placed very close or slightly down-field shifted (<0.25 ppm) relatively to chemical shifts observed for these residues in CLA. Only few exceptions are noticeable. The presence of Hphe in position 4 as in 5 and 6 causes the down-field shift of Ile^7 NH signal of 0.32 and 0.44 ppm, respectively (Fig. 2).

The replacement of $CDCl_3$ by DMSO and increase of temperature to 333 K simplified proton spectra in a way characteristic for the chemical exchange among conformers of similar energy (Fig. 4). However, the rate of exchange for peptide **4** at elevated temperature remained slow and only Leu^5 NH signal became sharp. For peptide **5** and **6** the exchange was faster and more NH signals became well resolved, except for NH signals of Hphe^4, Leu^5 and Ile^6 for peptide **5** and Hphe^4 for peptide **6**.

The NH–CαH coupling constants determined at 213 K in chloroform and at 333 K in DMSO are summarized in Table 2. Most of $^3J_{NHC\alpha H}$ values of about 7 Hz were measured. Low number of vicinal couplings determined at low temperature in $CDCl_3$ indicate lack of preferred conformation in the solution, contrary to that observed for CLA. At elevated temperature in DMSO the fast exchange between quasi-energetic conformers led to the coalesced peaks (Fig. 3).

The solvent exposure of NH protons was probed by determining the temperature coefficients ($\Delta\delta/\Delta T$) in DMSO from ^{1}H NMR spectra or in the case of the overlapping signals, COSY DQF spectra recorded in the range of 298–333 K (Table 3). All NH resonances, except of NH of Leu^5 (**1**), Leu^8 and Val^9 (**5**), which moved linearly upfield with temperatures. The low temperature coefficients (<3 ppb/K) are characteristic for the presence of intramolecular hydrogen bonds [40]. Peptides **5** and **6** are stabilized by six intramolecular hydrogen bonds involving all NH atoms except of NH Hphe^4 atom. For peptide **4** only three NH (Phe^4, Leu^5 and Val^9) atoms formed hydrogen bonds with carbonyl oxygen atoms.

Signals of Phe and HPhe aromatic rings in $CDCl_3$ and DMSO are placed in similar range of about 0.2 ppm. Only *ortho* atoms of Hphe^3 in peptide **4** in are shifted up-field to 6.84 ppm (DMSO, 298 K) and 6.95 ppm ($CDCl_3$, 213 K). This may suggest, for this CLA analogue, the "face to edge" interaction of phenyl rings, similarly as in the native CLA [8,41]. The close proximity of Pro^2 side chain and aromatic Phe^3 ring in peptide **5** is consistent with observed strong up-field shifts of Pro^2 γ′ signal to 0.77 ppm (DMSO, 298 K) and 0.53 ppm ($CDCl_3$, 213 K).

Table 2
Vicinal coupling constants $^3J_{NHC\alpha H}$ [Hz] of peptides **4**–**6** in DMSO at 333 K and in chloroform at 213 K.

	4		**5**		**6**		CLA[a]
	DMSO	$CDCl_3$	DMSO	$CDCl_3$	DMSO	$CDCl_3$	$CDCl_3$
Phe^3/Hphe^3	8.31	nd	8.57	nd	8.62	nd	8.82
Phe^4/Hphe^4	nd	7.86	9.10	7.84	nd	6.63	10.01
Leu^5	4.96	nd	8.40	nd	5.62	nd	<2
Ile^6	nd	nd	9.10	nd	6.61	nd	7.96
Ile^7	nd	nd	8.40	9.09	10.08	8.49	11.02
Leu^8	nd	nd	6.93	3.73	6.68	nd	7.81
Val^9	nd	9.31	9.12	nd	8.96	nd	11.23

[a] Data from Ref. [10].

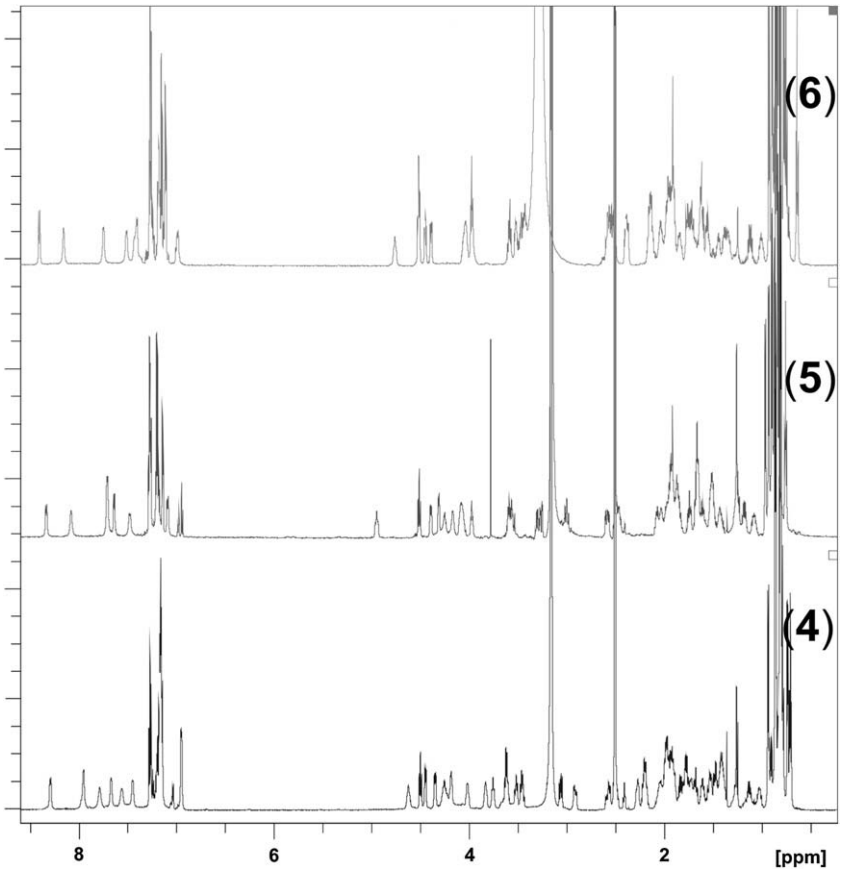

Fig. 3. The 700 MHz ^{1}H NMR spectra of peptides **4**–**6** in DMSO recorded at 333 K.

From parameters obtained from NMR it may be concluded that peptides **4**–**6** exist in solutions as mixtures of conformers. Peptide **4** was the most flexible CLA analogue in this study.

3.3. *Biology*

The immunomodulatory activities of three cyclic nanopeptides modified with homophenylalanine residue (**4**–**6**) were examined and compared to that of cyclosporine A. The peptides exhibited interesting, differential properties in the response of splenocytes to T- and B-cell mitogens and in the model of secondary humoral immune response to sheep erythrocytes in vitro. The results showed that the peptides **5** and **6**, containing Hphe in position 3 or 3 and 4, respectively, exhibited similar effects on the proliferative response of splenocytes to mitogens (Figs. 4 and 5). Both peptides stimulated T-cell proliferation, the B-cell proliferation was, however, inhibited. Whereas **6** was a moderate suppressor of the humoral immune response (Fig. 6), peptide **5** was exceptionally inhibitory (92% inhibition at 100 μg/ml). That effect was correlated with relatively high cell toxicity (47.19% at 100 μg/ml concentration) – Fig. 7. Interestingly, very potent inhibition of AFC number (94.5% at 100 μg/ml) of peptide **4** was not associated with cell toxicity (6.03%). This compound caused a complete block of T- and B-cell proliferation at 100 μg/ml (Figs. 4 and 5). The presented data clearly showed that modification of the tetrapeptide sequence (Pro^1-Pro^2-Phe^3-Phe^4) in CLA, by substitution of Phe with Hphe in positions 3 and 4, led to appearance of three different activities in the studied immunological assays. Moreover, Hphe included in position 4 markedly reduced the viability of the tested cell line, however

Table 3
Temperature dependence of the NH chemical shits ($-\Delta\delta/\Delta T$, ppb/K) of major isomers of peptides **4**–**6** in DMSO, in the range 298–338 K.

Peptide	Phe^3 or Hphe^3	Phe^4 or Hphe^4	Leu^5	Ile^6	Ile^7	Leu^8	Val^9
4	3.6	2.7	−2.7	7.3	5.4	4.4	2.5
5	2.9	6.2	1.4	2.5	1.4	1.7	0.05
6	2.7	6.4	2.0	1.2	2.0	−0.06	−0.9

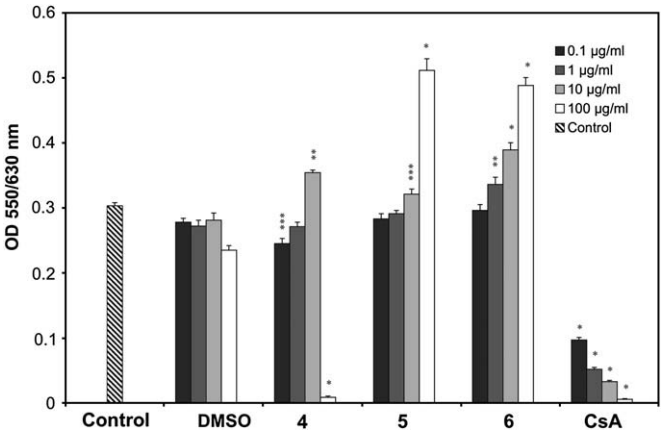

Fig. 4. Effects of peptides **4**–**6** on concanavalin A-induced splenocyte proliferation. Mouse splenocytes were induced by addition of 2.5 μg/ml of ConA and peptides **4**–**6** were applied at concentrations of 0.1–100 μg/ml. * Indicates $p < 0.05$, ** indicates $p < 0.02$ and *** indicates $p < 0.001$.

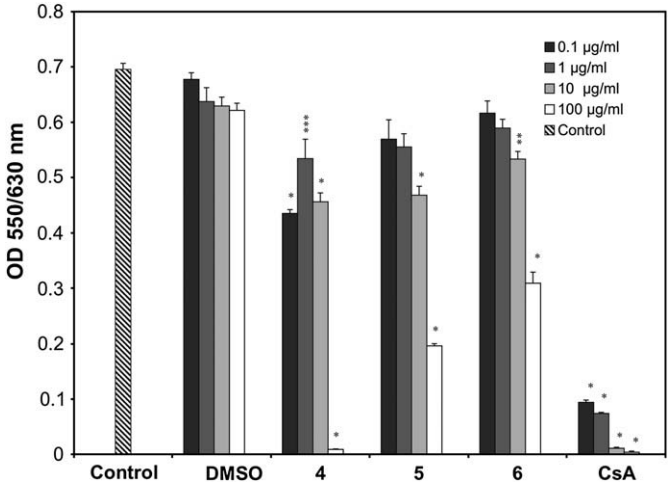

Fig. 5. Effects of peptides **4**–**6** on pokeweed mitogen-induced splenocyte proliferation. Mouse splenocytes were induced by addition of 2.5 µg/ml of PWM and peptides **4**–**6** were applied at concentrations of 0.1–100 µg/ml. * Indicates $p < 0.05$, ** indicates $p < 0.02$ and *** indicates $p < 0.001$.

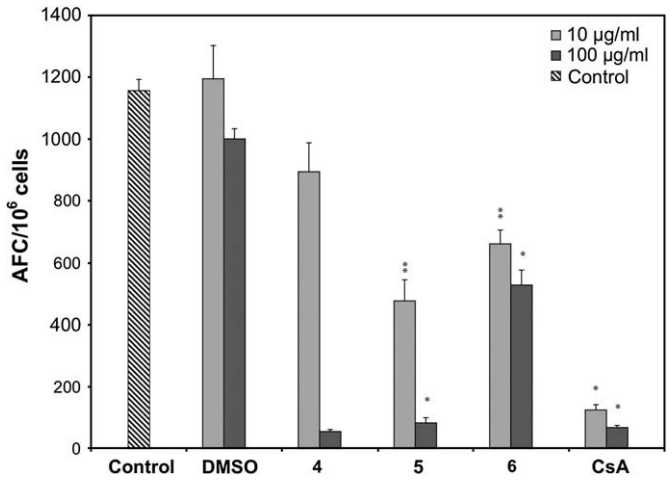

Fig. 6. Effects of peptides **4**–**6** on the secondary humoral immune response to SRBC *in vitro*. Mouse splenocytes were culturated with antigen (0.1 ml of 0.005% SRBC suspension) and peptides **4**–**6** (10 and 100 µg/ml concentrations) for four days. * Indicates $p < 0.05$ and ** indicates $p < 0.02$. The number of antibody-forming cells are presented as mean values from four determinations ± SE.

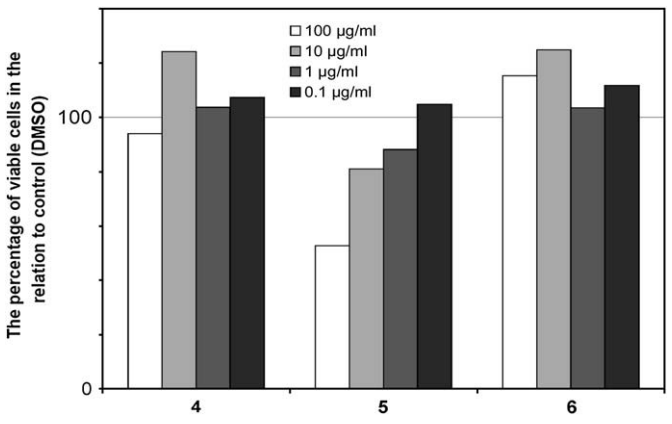

Fig. 7. The toxicity of peptides **4**–**6** with regard to WEHI 164.13 cells. The peptides were used at concentrations 0.1–100 µg/ml. The cell toxicity of the peptides was compared with toxicity of the respective dilutions of the solvent (DMSO), which was regarded as having 100% viability (0% toxicity).

addition of the second Hphe in position 3 improved, in fact, cell survival in comparison with the solvent.

4. Conclusions

The replacement of one or both Phe residues by Hphe led to three different biological activities. This observation supports the view that the balance of the phenyl rings and their relative angle may control the biological activity of CLA molecule, not only the immunosuppressive activity, but also its toxicity. Elongation of the side chain in position 3, as in peptide **4**, resulted in a very potent inhibition of AFC number, not associated with cell toxicity, as well as a complete block of T- and B-cell proliferation. Peptides **5** and **6** with Hphe in position 3 or 3 and 4, respectively, gave similar effects on the proliferative response of splenocytes to mitogens. The viability of cell lines is lower when Hphe is present in both positions 3 and 4 and the lowest when Hphe is present in position 4. Only peptide **6** was a moderate suppressor of the humoral immune response, peptide **5**, was, in turn, exceptionally inhibitory. All peptides (**4**–**6**) are attractive for more detailed investigations in other immunological models; peptide **4** deserves more attention because it appears to be a universal suppressor of the immune response and is devoid of toxicity.

Acknowledgements

This work was supported by the Ministry of Science and Higher Education, Grant No. 2 505F 035 28.

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