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Original article

Synthesis and biological activity of cyclolinopeptide A analogues modified with γ^3-bis(homophenylalanine)

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ABSTRACT

Cyclolinopeptide A, naturally occurring immunomodulatory nonapeptide, was modified with *S* or *R*-γ^3-bis(homophenylalanine) in positions 3 or 4, or both 3 and 4. The replacement of one or both Phe residues by γ^3-hhPhe led to decrease of their conformational flexibility in the analogues in comparison to CLA. All cyclic peptides, except **11**, exist as isomers with the *cis* Pro–Pro peptide bond. Cyclic peptide **11** with single modification *S*-γ^3-hhPhe^4 exists as a mixture of two isomers and the major isomer (89%) contains all peptide bonds of the *trans* geometry.

The peptides were subjected to several immunological tests *in vitro* and *in vivo*. Linear peptides **1**–**8**, precursors of CLA analogues **9**–**16**, were not toxic against human peripheral blood mononuclear cells (PBMC) but cyclic analogues showed dose-dependent toxicity with exception of peptide **11**. Linear peptides did not inhibit mitogen-induced PBMC proliferation whereas cyclic ones inhibited the proliferation in a dose-dependent manner. The actions of linear and cyclic peptides with regard to lipopolysaccharide (LPS) -induced tumour necrosis factor alpha (TNF α) production in whole human blood cultures were differential but particularly suppressive in the case of linear compound **6**. Therefore, for *in vivo* tests compounds **6** and **11** were selected. The compounds showed comparable, suppressive actions in induction and effector phases of delayed type hypersensitivity as well as in the carrageenan-induced foot pad edema in mouse models. In summary, linear peptide **6** and cyclic peptide **11** are attractive as potential immune suppressor drugs.

1. Introduction

Naturally occurring and synthetic cyclopeptides exhibit different activities: anti-cancer [1,2], anti-HIV [3], antinoceptive [4,5], cytotoxic [6,7], anti-bacterial [8], antifungal [9] or insecticidal [10]. Cyclic immunosuppressive peptides derived from natural sources are regarded as a new class of pharmaceuticals [11].

Cyclolinopeptide A (cyclo-(Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val), CLA), discovered in linseed oil in 1959 [12], possesses a range of biological activities including the ability to inhibit hepatocyte cell transport [13] and their immunosuppressive activity at low doses is equal to that of cyclosporine A, that has been confirmed in a number of assays [14,15]. All aspects of the biological activity of CLA and its analogues based on the structure–activity relationship have been discussed in detail in reviewed papers [16,17]. According to the well-known hypothesis the Pro-Pro-Phe-Phe sequence and conformational flexibility are the most important factors responsible for its biological activity. We applied several modifications of CLA using (S)-β^2-isoproline or (S)-β^3-homo-proline instead of proline [18], or replacement of phenylalanine by β^3-phenylalanine [19], or homophenylalanine [20]. The introduction of ethylene bridge between phenylalanine nitrogens resulted in

Abbreviations: ANOVA, analysis of variance; Boc, *tert*-Butoxycarbonyl; C8, octyl stationary phase; C18, octadecyl stationary phase; CLA, cyclolinopeptide A; COSY-DQF, double quantum filtered proton spin correlation; DEX, dexamethasone; DIPEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; hhPhe, bis(homophenylalanine); HOBt, 1-hydroxybezotriazole hydrate; Ile, isoleucine; Leu, leucine; LPS, lipopolysaccharide; MTT, 93-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBMC, peripheral blood mononucleated cell; PHA, Phytohemagglutinin A; Phe, phenylalanie; Pro, proline; TBTU, *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; TNF-α, tumour necrosis factor alpha; Val, valine.

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conformational flexibility of CLA molecule being probably a cause of its antagonistic action towards methotrexate [21].

γ-Amino acids are homologues of α-amino acids with two additional carbon atoms separating amino and carboxyl groups. Such a modification leads to the resistance against enzymatic degradation [22]. α,γ-Peptide hybrids display antibiotic activity [23] as well as antiviral [24] and anticancer properties [25,26]. γ-Amino acids are also important elements of foldamers [27] and self-assembling nanotubes [28]. Despite their significant flexibility, γ-amino acids form well-defined secondary structures in solutions. In particular, homogenous oligomers containing monosubstituted γ-amino acids can form stable helical conformations in solution [29].

In order to evaluate the role and significance of the presence of γ-amino acid residue on the biological activity we have synthetized 8 linear and 8 cyclic analogues of CLA, in which one or both phenylalanines were replaced by one or two S or R γ^3-bis(homophenylalanine) residues (Fig. 1).

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 in a capillary melting point apparatus Büchi SMP-20. The optical rotation was measured in a 1 dm cell (1 mL) on a PolAAr 3001 automatic polarimeter at 589 nm. For thin layer chromatography 254 nm silica gel on TLC plates (Fluka) was used with ethyl acetate: hexane (4:1) solvent system. The chromatograms were visualized by $KMnO_4$ or ninhydrin. HPLC was performed on a Thermo Spectra System, a Vydac C8 column (0.46 × 25 cm): flow 1.5 mL/min or 1 mL/min, detection at 220 nm and eluents (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 Gilson 322 pump and UV/Vis-152 detector on a Vydac C8 column (2.21 × 25 cm): flow rate 20 mL/min, UV detection at 220 nm. The identities of the pure peptides were confirmed by Maldi-TOF mass spectrometry on Voyager Elite, Perceptive Biosystems using α-cyano-4-hydroxy-cinnamic acid as a 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, $CDCl_3$, MeOD or CD_3CN using as an internal shift standard solvent signals of 2.50 ppm, 7.26 ppm, 4.87 ppm, CD_3CN 1.94 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 TopSpin 2.1 (Bruker) and analyzed by Sparky software [30]. All amino acid derivatives and peptide bond forming reagents were purchased from IRIS Biotech (Germany). All reagents were purchased from **S**igma–**A**ldrich and solvent were purchased from POCh.

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

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

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

4 H-Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-***R*-γ^3-hhPhe^3-*S*-γ^3-hhPhe^4**-Leu^5-OH

5 H-Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-***S*-γ^3-hhPhe^3-*R*-γ^3-hhPhe^4**-Leu^5-OH

6 H-Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-***R*-γ^{3}hhPhe^3-*R*-γ^{3}hhPhe4**-Leu^5-OH

7 H-Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-***R*-γ^{3}hhPhe^3**-Phe^4-Leu^5-OH

8 H-Ile^6-Ile^7-Leu^8-Val^9-Pro^1-Pro^2-Phe^3-***R*-γ^{3}hhPhe^4**-Leu^5-OH

9 c(Pro^1-Pro^2-***S*-γ^3-hhPhe^3-*S*-γ^3-hhPhe^4**-Leu^5- Ile^6-Ile^7-Leu^8-Val^9)

10 c(Pro^1-Pro^2-***S*-γ^3-hhPhe^3**-Phe^4-Leu^5- Ile^6-Ile^7-Leu^8-Val^9)

11 c(Pro^1-Pro^2-Phe^3-***S*-γ^3-hhPhe^4**-Leu^5- Ile^6-Ile^7-Leu^8-Val^9)

12 c(Pro^1-Pro^2-***R*-γ^3-hhPhe^3-*S*-γ^3-hhPhe^4**-Leu^5- Ile^6-Ile^7-Leu^8-Val^9)

13 c(Pro^1-Pro^2-***S*-γ^3-hhPhe^3-*R*-γ^3-hhPhe^4**-Leu^5- Ile^6-Ile^7-Leu^8-Val^9)

14 c(Pro^1-Pro^2-*R*-γ^{3}hhPhe^3-*R*-γ^{3}hhPhe^4-Leu^5- Ile^6-Ile^7-Leu^8-Val^9)

15 c(Pro^1-Pro^2-***R*-γ^{3}hhPhe^3-Phe^4-Leu^5- Ile^6-Ile^7-Leu^8-Val^9)

16 c(Pro^1-Pro^2-Phe^3-***R*-γ^{3}hhPhe^4-Leu^5- Ile^6-Ile^7-Leu^8-Val^9)

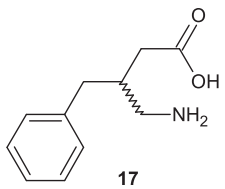

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Fig. 1. The sequences of linear (**1**–**8**) and cyclic (**9**–**16**) analogues of CLA containing γ^3-hhPhe **17**.

2.2. Peptide synthesis and purification

The linear peptides **1**–**8** were synthesized by the manual solid-phase method using chloromethylated Merrifield resin as a solid support. Attachment of the first Boc-AA-OH (Boc-Leu) to the resin was performed according to cesium salt procedure [31] and the substitution level was determined by weight gain measurements (Boc-Leu-Ⓟ, 0.62 mmol for peptides **1**–**3** or 0.72 mmol for peptides **4**–**8**). Synthesis of 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 (with deprotonation steps omitted) was used for all amino acids and was repeated if Kaiser test [32] or Chloranil test (proline residue) [33] was found positive. In all cases, when second coupling test was slightly positive, remaining free amino groups were acetylated with the aid of acetic anhydride (190 μl) in the presence of diisopropylethylamine (350 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. The resin had been filtered off and washed with TFA, and 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.

2.3. ^{1}H NMR analysis of cyclolinopeptides **9**–**16**

2.3.1. *c(Pro^1-Pro^2-**S**-γ^3-**hhPhe^3**-**S**-γ^3-**hhPhe^4**-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)* (**9**)

(DMSO-d_6, 50 °C) δ: 0.75 (3H, H$^\gamma$, Ile^7), 0.77 (3H, H$^\delta$, Leu^8), 0.80 (3H, H$^\delta$, Ile^7), 0.81 (3H, H$^\delta$, Ile^6), 0.82 (3H, H$^\gamma$, Ile^6), 0.85 (3H, H$^\gamma$, Val^9), 0.86 (3H, H$^\delta$, Leu^8), 0.89 (6H, H$^\delta$, Leu^5), 0.94 (3H, H$^\gamma$, Val^9), 1.00 (1H, H$^\gamma$, Ile^7), 1.06 (1H, H$^\gamma$, Ile^6), 1.37 (1H, H$^\gamma$, Ile^7), 1.40 (1H, H$^\gamma$, Ile^6), 1.47 (1H, H$^\beta$, Leu^5), 1.49 (2H, H$^\beta$, Leu^8), 1.53 (1H, H$^\beta$, Leu^5), 1.55 (1H, H$^\gamma$, Leu^8), 1.62 (1H, H$^\gamma$, Leu^5), 1.66 (1H, H$^\gamma$, Pro^2), 1.70 (1H, H$^\beta$, Pro^1), 1.71 (1H, H$^\beta$, Ile^7), 1.80 (1H, H$^\beta$, Ile^6), 1.80 (1H, H$^\gamma$, Pro^2), 1.80 (1H, H$^\gamma$, Pro^1), 1.97 (1H, H$^\gamma$, Pro^1), 1.99 (1H, H$^\beta$, Val^9), 2.03 (1H, H$^\alpha$, hhPhe^4), 2.03 (1H, H$^\beta$, Pro^2), 2.06 (2H, H$^\alpha$, hhPhe^3), 2.07 (1H, H$^\beta$, hhPhe^4), 2.21 (1H, H$^\beta$,Pro^2), 2.24 (1H, H$^\beta$, Pro^1), 2.34 (1H, H$^\alpha$, hhPhe^4), 2.45 (1H, H$^{\gamma''}$, hhPhe^3), 2.53 (1H, H$^\beta$, hhPhe^3), 2.57 (1H, H$^{\gamma''}$, hhPhe^4),

Table 1
Physicochemical properties of peptides **1**–**16**.

Peptide	Yield [%]	HPLC			Molecular formula	Formula mass	MALDI MS, *m/z*	
		t_R (min)	Purity [%]				$[M+H]^+$	$[M+Na]^+$
			Crude	Purified				
1	74[a]	13.233	86.57	98.43	$C_{61}H_{95}N_9O_{10}$	1113.72	1114.35	1136.29
2	96[a]	12.690	90.58	99.08	$C_{59}H_{91}N_9O_{10}$	1085.69	1086.51	1108.41
3	88[a]	13.177	85.26	95.97	$C_{59}H_{91}N_9O_{10}$	1085.69	1086.50	1108.48
4	91[a]	13.085	91.24	97.23	$C_{61}H_{95}N_9O_{10}$	1113.72	1114.64	1136.61
5	90[a]	13.938	92.25	95.74	$C_{61}H_{95}N_9O_{10}$	1113.72	1114.66	1136.61
6	90[a]	14.073	94.16	96.52	$C_{61}H_{95}N_9O_{10}$	1113.72	1114.61	1136.54
7	90[a]	13.383	90.20	97.98	$C_{59}H_{91}N_9O_{10}$	1085.69	1086.48	1108.47
8	89[a]	14.168	89.73	100.00	$C_{59}H_{91}N_9O_{10}$	1085.69	1086.55	1108.51
9	12[b]	10.110		98.39	$C_{61}H_{93}N_9O_9$	1095.71	1096.38	1118.34
10	12[b]	9.890		100.00	$C_{59}H_{89}N_9O_9$	1067.68	1068.50	1090.47
11	18[b]	11.630		95.32	$C_{59}H_{89}N_9O_9$	1067.68	1068.53	1090.50
12	25[b]	9.605		98.09	$C_{61}H_{93}N_9O_9$	1095.71	1096.60	1118.57
13	40[b]	10.543		100.00	$C_{61}H_{93}N_9O_9$	1095.71	1096.62	1118.56
14	25[b]	9.242		96.82	$C_{61}H_{93}N_9O_9$	1095.71	1096.67	1118.62
15	36[b]	10.033		98.43	$C_{59}H_{89}N_9O_9$	1067.68	1068.69	1090.63
16	46[b]	11.870		97.07	$C_{59}H_{89}N_9O_9$	1067.68	1068.52	1090.49

[a] Before HPLC purification.
[b] Yield of cyclization.

2.62 (1H, $H^{\gamma''}$, hhPhe^3), 2.65 (1H, $H^{\gamma''}$, hhPhe^3), 3.04 (1H, H^{γ}, hhPhe^3), 3.15 (1H, H^{γ}, hhPhe^4), 3.21 (1H, H^{γ}, hhPhe^3), 3.31 (1H, H^{γ}, hhPhe^4), 3.39 (1H, H^{δ}, Pro^2), 3.41 (1H, H^{δ}, Pro^2), 3.58 (1H, H^{δ}, Pro^1), 3.67 (1H, H^{δ}, Pro^1), 4.13 (1H, H^{α}, Ile^6), 4.19 (1H, H^{α}, Leu^5), 4.20 (1H, H^{α}, Ile^7), 4.23 (1H, H^{α}, Pro^1), 4.34 (1H, H^{α}, leu^8), 4.42 (1H, H^{α}, Val^9), 4.44 (1H H^{α}, Pro^2), 7.11 (2H, $H^{2,6}$, hhPhe^4), 7.18 (1H, H^4, hhPhe^3), 7.20 (2H, $H^{2,6}$, hhPhe^3), 7.22 (2H, $H^{3,5}$, hhPhe^4),7.24 (1H, H^4, hhPhe^4), 7.22 (2H, $H^{3,5}$, hhPhe^4), 7.27 (2H, $H^{3,5}$, hhPhe^3), 7.46 (1H, H^N, Ile^7), 7.53 (1H, H^N, Val^9), 7.64 (1H, H^N, Ile^7), 7.78 (1H, H^N, Leu^5), 7.81 (1H, H^N, hhPhe^4), 8.05 (1H, H^N, Leu^8), 8.07 (1H, H^N, hhPhe^3).

*2.3.2. c(Pro^1-Pro^2-**S-γ^3-hhPhe^3**-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9) (**10**)*

(DMSO-d_6, 50 °C) δ: 0.70 (6H, H^{δ}, Leu^5), 0.73 (3H, H^{δ}, Ile^6), 0.80 (3H, H^{δ}, Ile^7), 0.81 (3H, H^{γ}, Ile^6),0.81 (6H, H^{δ}, Leu^5), 0.86 (3H, H^{γ}, Ile^7), 0.86 (3H, H^{γ}, Val^9), 0.96 (3H, H^{γ}, Val^9), 0.99 (1H, H^{γ}, Ile^7), 1.04 (1H, H^{γ}, Ile^6), 1.34 (1H, H^{γ}, Ile^6), 1.37 (1H, H^{γ}, Leu^5), 1.40 (1H, H^{β}, Leu^8), 1.40 (1H, H^{γ}, Ile^7), 1.47 (1H, H^{γ}, Leu^5), 1.56 (2H, H^{β}, Leu^5), 1.58 (1H, H^{β}, Leu^5), 1.58 (1H, H^{γ}, Pro^2), 1.69 (1H, H^{β}, Pro^1), 1.81 (1H, H^{γ}, Pro^1), 1.82 (1H, H^{γ}, Pro^2), 1.88 (1H, H^{β}, Ile^7), 1.88 (1H, H^{β}, Ile^6), 2.01 (1H, H^{β}, Pro^2), 2.02 (1H, H^{γ}, Pro^1), 2.06 (2H, H^{α}, hhPhe^3), 2.07 (1H, H^{β}, Val^9), 2.29 (1H, H^{β}, Pro^2), 2.29 (1H, $H^{\gamma''}$, hhPhe^3), 2.33 (1H, H^{β}, Pro^1), 2.39 (1H, H^{β}, hhPhe^3), 2.57 (1H, $H^{\gamma''}$, hhPhe^3), 2.76 (1H, H^{γ}, hhPhe^3), 2.81 (1H, H^{β}, Phe^4), 3.04 (1H, H^{γ}, hhPhe^3), 3.05 (1H, H^{β}, Phe^4), 3.31 (1H, H^{δ}, Pro^2), 3.42 (1H, H^{δ}, Pro^2), 3.58 (1H, H^{δ}, Pro^1), 3.75 (1H, H^{δ}, Pro^1), 3.75 (1H, H^{α}, Ile^6), 4.15 (1H, H^{α}, Leu^5), 4.20 (1H, H^{α}, Pro^1), 4.26 (1H, H^{α}, Leu^8), 4.35 (1H, H^{α}, Ile^7), 4.48 (1H, H^{α}, Pro^2), 4.48 (1H,H^{α}, Val^9), 4.68 (1H, H^{α}, Phe^4), 7.11 (1H, $H^{2,6}$, hhPhe^3), 7.12 (1H, H^4, Phe^4), 7.14 (2H, $H^{2,6}$, Phe^4), 7.16 (2H, $H^{3,5}$, Phe^4), 7.18 (1H, H^4, hhPhe^3), 7.25 (2H, $H^{3,5}$,hhPhe^3), 7.54 (1H, H^N, Ile^7), 7.55 (1H, H^N, Val^9), 7.78 (1H, H^N, Ile^6), 7.82 (1H, H^N, Leu^5), 7.95 (1H, H^N, Leu^8), 8.06 (1H, H^N, hhPhe^3), 8.34 (1H, H^N, Phe^4).

*2.3.3. c(Pro^1-Pro^2-Phe^3**-S-γ^3-hhPhe^4**-Leu^5-Ile^6-Ile^7-Leu^8-Val^9) (**11a**) **(89.3%)***

(DMSO-d_6, 50 °C) δ:0.75 (3H, H^{δ}, Leu^8), 0.80 (3H, H^{δ}, Ile^7), 0.82 (3H, H^{γ}, Ile^6), 0.84 (3H, H^{γ}, Ile^7), 0.85 (3H, H^{δ}, Leu^8), 0.86 (3H, H^{γ}, Val^9), 0.86 (3H, H^{δ}, Leu^5), 0.90 (3H, H^{δ}, Leu^5), 0.96 (3H, H^{γ}, Val^9), 1.00 (1H, H^{γ}, Ile^6), 1.02 (1H, H^{γ}, Pro^2), 1.09 (1H, H^{γ}, Ile^6), 1.11 (1H, H^{γ}, Ile^7), 1.44 (3H, H^{δ}, Ile^6), 1.47 (1H, H^{γ}, Ile^7), 1.52 (1H, H^{β}, Leu^8), 1.55 (1H, H^{β}, Leu^8), 1.56 (1H, H^{γ}, Leu^8), 1.57 (1H, H^{β}, Leu^5), 1.59 (1H, H^{γ}, Leu^5), 1.61 (1H, H^{γ}, Pro^2), 1.62 (1H, H^{β}, Pro^1), 1.64 (1H, H^{β}, Leu^5), 1.78 (1H, H^{γ}, Pro^1), 1.81 (1H, H^{β}, Ile^6), 1.85 (1H, H^{β}, Pro^2), 1.92 (1H, H^{β}, Ile^7), 1.95 (1H, H^{γ}, Pro^1), 2.02 (1H, H^{β}, Val^9), 2.10 (1H, H^{α}, hhPhe^4), 2.13 (1H, H^{β}, Pro^1), 2.16 (1H, H^{β}, Pro^2), 2.23 (1H, H^{α}, hhPhe^4), 2.26 (1H, H^{β}, hhPhe^4), 2.56 (1H, $H^{\gamma''}$, hhPhe^4), 2.61 (1H, $H^{\gamma''}$, hhPhe^4), 2.97 (1H, H^{δ}, Pro^2), 3.08 (1H, H^{γ}, hhPhe^4), 3.23 (1H, H^{γ}, hhPhe^4), 3.22 (1H, H^{β}, Phe^3), 3.26 (1H, H^{δ}, Pro^2), 3.28 (1H, H^{β}, Phe^3), 3.35 (1H, H^{δ}, Pro^1), 3.61 (1H, H^{δ}, Pro^1), 4.02 (1H, H^{α}, Ile^7), 4.14 (1H, H^{α}, Leu^5), 4.19 (1H, H^{α}, Ile^6), 4.20 (1H, H^{α}, Leu^8), 4.36 (1H, H^{α}, Pro^1), 4.36 (1H, H^{α}, Pro^2), 4.41 (1H, H^{α}, Phe^3), 4.44 (1H, H^{α}, Val^9), 7.18 (1H, H^N, Val^9), 7.18 (1H, H^4, Phe^3), 7.19 (2H, $H^{2,6}$, hhPhe^4), 7.23 (2H, $H^{3,5}$, Phe^3), 7.26 (2H, $H^{2,6}$, Phe^3), 7.26 (1H, H^4, hhPhe^4), 7.26 (2H, $H^{3,5}$, hhPhe^4), 7.67 (1H, H^N, Ile^6), 7.79 (1H, H^N, Ile^7), 7.81 (1H, H^N, Leu^5), 7.81 (1H, H^N, hhPhe^4), 8.07 (1H, H^N, Leu^8), 8.47 (1H, H^N, Phe^3)

*2.3.4. c(Pro^1-Pro^2-Phe^3**-S-γ^3-hhPhe^4**-Leu^5-Ile^6-Ile^7-Leu^8-Val^9) (**11b**) (**10.7%**)*

(DMSO-d_6, 50 °C) δ: 0.71 (3H, H^{δ}, Ile^6), 0.76 (3H, H^{δ}, Ile^7), 0.77 (3H, H^{δ}, Leu^8), 0.85 (3H, H^{γ}, Ile^6), 0.85 (3H, H^{γ}, Ile^7), 0.86 (3H, H^{δ}, Leu^8), 0.87 (3H, H^{γ}, Val^9), 0.87 (6H, H^{δ}, Leu^5), 0.93 (3H, H^{γ}, Val^9), 1.02 (1H, H^{γ}, Ile^6), 1.06 (1H, H^{γ}, Ile^7), 1.16 (1H, H^{γ}, Pro^2), 1.54 (2H, H^{β}, Leu^5), 1.55 (1H, H^{γ}, Ile^7), 1.56 (1H, H^{γ}, Leu^8), 1.61 (1H, H^{γ}, Ile^6), 1.65 (2H, H^{β}, Leu^8), 1.65 (1H, H^{γ}, Pro^1), 1.66 (1H, H^{γ}, Pro^2), 1.67 (1H, H^{β}, Pro^1), 1.68 (1H, H^{γ}, Leu^5), 1.69 (1H, H^{γ}, Pro^1), 1.87 (1H, H^{β}, Ile^6), 1.90 (1H, H^{β}, Pro^2), 1.94 (1H, H^{β}, Val^9), 1.96 (1H, H^{α}, hhPhe^4),1.96 (1H, H^{β}, Ile^7), 2.11 (1H, H^{α}, hhPhe^4), 2.17 (1H, H^{β}, Pro^2), 2.17 (1H, H^{β}, hhPhe^4), 2.23 (1H, H^{β}, Pro^1), 2.53 (2H, $H^{\gamma''}$, hhPhe^4), 2.71 (1H, H^{γ}, hhPhe^4), 3.06 (1H, H^{δ}, Pro^2), 3.19 (1H, H^{β}, Phe^3), 3.25 (1H, H^{γ}, hhPhe^4), 3.27 (1H, H^{δ},Pro^2), 3.29 (1H, H^{β}, Phe^3), 3.33 (1H, H^{δ}, Pro^1), 4.40 (1H, H^{δ}, Pro^1), 3.96 (1H, H^{α}, Leu^8), 3.97 (1H, H^{α}, Ile^7), 4.13 (1H, H^{α}, Ile^6) 4.15 (1H, H^{α}, Val^9), 4.30 (1H, H^{α}, Pro^2), 4.41 (1H, H^{α}, Phe^3), 4.43 (1H, H^{α}, Leu^5), 4.77 (1H, H^{α}, Pro^1), 7.04–7.35 (10H, $H^{2,6}$, Phe^3, $H^{3,5}$, Phe^3, H^4, Phe^3, $H^{2,6}$, hhPhe^4, $H^{3,5}$, hhPhe^4, H^4, hhPhe^4), 7.55 (1H, H^N, Val^9), 7.73 (1H, H^N, hhPhe^4), 7.73 (1H, H^N, Leu^8), 8.06 (1H, H^N, Ile^7), 8.14 (1H, H^N, Leu^5), 8.17 (1H, H^N, Ile^6), 8.43 (1H, H^N, Phe^3).

*2.3.5. c(Pro^1-Pro^2-**R-γ^3-hhPhe^3-S-γ^3-hhPhe^4**-Leu^5-Ile^6-Ile^7-Leu^8-Val^9) (**12**)*

(DMSO-d_6, 50 °C) δ: 0.72 (3H, H^{δ}, Leu^8), 0.79 (3H, H^{γ}, Ile^7), 0.80 (3H, H^{δ}, Ile^7), 0.81 (3H, H^{γ}, Val^9), 0.81 (3H, H^{δ}, Leu^8), 0.82 (3H, H^{δ}, Ile^6), 0.83 (3H, H^{γ}, Ile^6), 0.88 (3H, H^{δ}, Leu^5), 0.91 (3H, H^{δ}, Leu^5), 0.92 (3H, H^{γ}, Val^9), 1.01 (1H, H^{γ}, Ile^7), 1.06 (1H, H^{γ}, Ile^6), 1.36 (1H, H^{β}, Leu^8), 1.40 (1H, H^{γ}, Ile^7), 1.41 (1H, H^{γ}, Ile^6), 1.47 (1H, H^{β}, Leu^5), 1.50 (1H, H^{β}, Leu^8), 1.54 (1H, H^{β}, Leu^5), 1.55 (1H, H^{γ}, Leu^8), 1.62 (1H, H^{γ},

Leu^5), 1.64 (1H, H$^\gamma$, Pro^2), 1.68 (1H, H$^\beta$, Pro^1), 1.72 (1H, H$^\beta$, Ile^7), 1.80 (1H, H$^\gamma$, Pro^2), 1.81 (1H, H$^\gamma$, Pro^1), 1.82 (1H, H$^\beta$, Ile^6), 1.90 (1H, H$^\beta$, Val^9), 1.98 (1H, H$^\gamma$, Pro^1), 1.99 (1H, H$^\beta$, Pro^2), 2.03 (1H, H$^\alpha$, hhPhe^3), 2.06 (1H, H$^\beta$, hhPhe^4), 2.17 (1H, H$^\alpha$, hhPhe^4), 2.21 (1H,H$^\alpha$, hhPhe^3), 2.23 (1H,H$^\beta$, Pro^2), 2.24 (1H,H$^\beta$, Pro^1), 2.30 (1H,H$^\alpha$, hhPhe^4), 2.36 (1H, H$^\beta$, hhPhe^3), 2.50 (1H, H$^{\gamma''}$, hhPhe^3), 2.58 (1H, H$^{\gamma''}$, hhPhe^3), 2.66 (1H, H$^{\gamma''}$, hhPhe^4), 2.95 (1H, H$^{\gamma''}$, hhPhe^4), 2.98 (1H, H$^\gamma$, hhPhe^3), 3.17 (1H, H$^\gamma$, hhPhe^3), 3.17 (1H, H$^\gamma$, hhPhe^4), 3.30 (1H, H$^\delta$, Pro^2), 3.34 (1H, H$^\gamma$, hhPhe^4), 3.40 (1H, H$^\delta$, Pro^2), 3.59 (1H, H$^\delta$, Pro^1), 3.67 (1H, H$^\delta$, Pro^1), 4.10 (1H, H$^\alpha$, Ile^6), 4.22 (1H, H$^\alpha$, Ile^7), 4.23 (1H, H$^\alpha$, Leu^5), 4.39 (1H, H$^\alpha$, Leu^8), 4.43 (1H, H$^\alpha$, Val^9), 4.44 (1H, H$^\alpha$, Pro^2), 4.29 (1H, H$^\alpha$, Pro^1), 7.14 (2H, H^{3,5}, hhPhe^3), 7.16 (2H, H^{3,5}, hhPhe^4), 7.17 (1H, H^4, hhPhe^4), 7.18 (1H, H^{2,6}, hhPhe^3), 7.19 (2H, H^{2,6}, hhPhe^4), 7.19 (1H, H^4, hhPhe^3), 7.51 (1H, H^N, Ile^7), 7.58 (1H, H^N, Ile^6), 7.66 (1H, H^N, Val^9), 7.77 (1H, H^N, Leu^5), 7.88 (1H, H^N, hhPhe^4), 8.10 (1H, H^N, Leu^8), 8.23 (1H, H^N, hhPhe^3).

2.3.6. *c(Pro^1-Pro^2-**S-γ^3-hhPhe^3-R-γ^3-hhPhe^4**-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)* (**13**)

(DMSO-d$_6$, 50 °C) δ: 0.67 (3H, H$^\gamma$, Ile^6), 0.78 (3H, H$^\gamma$, Ile^7), 0.79 (3H, H$^\delta$, Ile^6), 0.80 (3H, H$^\delta$, Leu^5), 0.81 (3H, H$^\delta$, Ile^7), 0.84 (3H, H$^\delta$, Leu^8), 0.84 (3H, H$^\delta$, Leu^8), 0.86 (3H, H$^\delta$, Leu^5), 0.88 (3H, H$^\gamma$, Val^9), 0.95 (1H, H$^\gamma$, Ile^6), 0.96 (3H, H$^\gamma$, Val^9), 0.98 (1H, H$^\gamma$, Ile^7), 1.36 (1H, H$^\gamma$, Ile^7), 1.39 (1H, H$^\gamma$, Ile^6), 1.44 (2H, H$^\beta$, Leu^5), 1.44 (1H, H$^\beta$, Leu^8), 1.56 (1H, H$^\gamma$, Leu^5), 1.53 (1H, H$^\beta$, Leu^8), 1.59 (1H, H$^\gamma$, Leu^8), 1.64 (1H, H$^\beta$, Ile^6), 1.68 (1H, H$^\gamma$, Pro^2), 1.70 (1H, H$^\beta$, Ile^7), 1.73 (1H, H$^\beta$, Pro^1), 1.81 (1H, H$^\gamma$, Pro^1), 1.81 (1H, H$^\gamma$, Pro^2), 1.91 (1H, H$^\gamma$, Pro^1), 1.97 (1H, H$^\alpha$, hhPhe^3), 2.00 (1H, H$^\beta$, Val^9), 2.01 (1H, H$^\alpha$, hhPhe^4), 2.06 (1H, H$^\beta$, Pro^2), 2.07 (1H, H$^\alpha$, hhPhe^4), 2.10 (1H, H$^\alpha$, hhPhe^3), 2.20 (1H, H$^\beta$, Pro^2), 2.22 (1H, H$^\beta$, Pro^1), 2.26 (1H, H$^\beta$, hhPhe^4), 2.26 (1H, H$^{\gamma''}$, hhPhe^4), 2.28 (1H, H$^{\gamma''}$, hhPhe^3), 2.56 (1H, H$^\beta$, hhPhe^3), 2.64 (1H, H$^{\gamma''}$, hhPhe^4), 2.69 (1H, H$^{\gamma''}$, hhPhe^3), 2.86 (1H, H$^\gamma$, hhPhe^4), 3.08 (1H, H$^\gamma$, hhPhe^3), 3.19 (1H, H$^\gamma$, hhPhe^4), 3.22 (1H, H$^\gamma$, hhPhe^3), 3.40 (1H, H$^\delta$, Pro^2), 3.44 (1H, H$^\delta$, Pro^2), 3.60 (1H, H$^\delta$, Pro^1), 3.63 (1H, H$^\delta$, Pro^1), 4.01 (1H, H$^\alpha$, Ile^6), 4.09 (1H, H$^\alpha$, Leu^5), 4.15 (1H, H$^\alpha$, Ile^7), 4.20 (1H, H$^\alpha$, Pro^1), 4.33 (1H, H$^\alpha$, Leu^8), 4.43 (1H, H$^\alpha$, Val^9), 4.43 (1H, H$^\alpha$, Pro^2), 7.14 (2H, H$^\alpha$, hhPhe^4), 7.17 (2H, H^{2,6}, hhPhe^3),7.19 (1H, H^4, hhPhe^4), 7.21 (1H, H$^\alpha$, hhPhe^3), 7.24 (2H, H^{3,5}, hhPhe^4),7.25 (2H, H$^\alpha$, hhPhe^3), 7.25 (1H, H^N, Ile^7),7.53 (1H, H^N, Val^9),7.85 (1H, H^N, hhPhe^4), 7.90 (1H, H^N, Ile^6), 7.96 (1H, H^N, Leu^5), 8.06 (1H, H^N, hhPhe^3), 8.15 (1H, H^N, Leu^8).

2.3.7. *c(Pro^1-Pro^2-**R-γ^{3}hhPhe^3-R-γ^{3}hhPhe^4**-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)* (**14**)

(DMSO-d$_6$, 50 °C) δ: 0. 75 (3H, H$^\delta$, Leu^5), 0.76 (3H, H$^\gamma$, Ile^7), 0.80 (3H, H$^\delta$, Ile^6), 0.81 (3H, H$^\delta$, Ile^7), 0.81 (3H, H$^\gamma$, Ile^6), 0.82 (3H, H$^\delta$, Leu^8), 0.83 (3H, H$^\delta$, Leu^5), 0.86 (3H, H$^\gamma$, Val^9), 0.89 (3H, H$^\delta$, Leu^5), 0.93 (3H, H$^\gamma$, Val^9), 1.02 (1H, H$^\gamma$, Ile^7), 1.04 (1H, H$^\gamma$, Ile^6), 1.37 (1H, H$^\beta$, Leu^8), 1.40 (1H, H$^\gamma$, Ile^7), 1.42 (1H, H$^\gamma$, Ile^6), 1.46 (2H, H$^\beta$, Leu^5) 1.50 (1H, H$^\beta$, Leu^8), 1.57 (1H, H$^\gamma$, Leu^8), 1.59 (1H, H$^\gamma$, Leu^5), 1.65 (1H, H$^\alpha$ Pro^2), 1.68 (1H, H$^\beta$, Pro^1), 1.69 (1H, H$^\beta$, Ile^7), 1.79 (1H, H$^\gamma$, Pro^1), 1.80 (1H, H$^\beta$, Ile^6), 1.81 (1H, H$^\gamma$, Pro^1), 1.85 (1H, H$^\gamma$, Pro^2), 1.94 (1H, H$^\beta$, Val^9), 1.98 (1H, H$^\beta$, Pro^2), 2.01 (1H, H$^\alpha$, hhPhe^3), 2.01 (1H, H$^\alpha$, hhPhe^4), 2.22 (1H, H$^\alpha$, hhPhe^3), 2.22 (1H, H$^\beta$, Pro^2), 2.23 (1H, H$^\beta$, Pro^1), 2.24 (1H, H$^\alpha$, hhPhe^4), 2.28 (1H, H$^\beta$, hhPhe^3), 2.33 (1H, H$^\beta$, hhPhe^4), 2.42 (1H, H$^{\gamma''}$, hhPhe^4), 2.59 (2H, H$^\gamma$, hhPhe^3), 2.67 (1H, H$^{\gamma''}$, hhPhe^4), 2.98 (1H, H$^\gamma$, hhPhe^4), 3.02 (1H, H$^\gamma$, hhPhe^3), 3.16 (1H, H$^\gamma$, hhPhe^4), 3.20 (1H, H$^\gamma$, hhPhe^3), 3.31 (1H, H$^\delta$, Pro^2), 3.41 (1H,H$^\alpha$ Pro^1), 3.49 (1H, H$^\delta$, Pro^2), 3.61 (1H, H$^\delta$, Pro^1), 4.13 (1H, H$^\alpha$, Ile^6), 4.17 (1H, H$^\alpha$, Ile^7), 4.20 (1H, H$^\alpha$,Leu^5), 4.27 (1H,H$^\alpha$, Pro^1), 4.42 (1H, H$^\alpha$, Pro^2), 4.44 (1H, H$^\alpha$, Val^9), 4.44 (1H, H$^\alpha$, Leu^8), 7.14 (2H, H^{2,6}, hhPhe^3), 7.17 (1H, H^4, hhPhe^3), 7.17 (1H, H^4, hhPhe^4), 7.18 (2H, H^{2,6}, hhPhe^4), 7.24 (2H, H^{3,5}, hhPhe^3), 7.26 (2H, H^{3,5}, hhPhe^4), 7.40 (1H, H^N, Ile^6), 7.54 (1H, H^N, Ile^7), 7.71 (1H, H^N, Val^9), 7.76 (1H, H^N, Leu^5), 7.82 (1H, H^N, hhPhe^4), 8.08 (1H, H^N, Leu^8), 8.13 (1H, H^N, hhPhe^3).

2.3.8. *c(Pro^1-Pro^2-**R-γ^{3}hhPhe^3**-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)* (**15**)

(DMSO-d$_6$, 50 °C) δ: 0.76 (3H, H$^\delta$, Leu^5), 0.78 (3H, H$^\delta$, Ile^7), 0.82 (3H, H$^\delta$, Ile^6), 0.83 (3H, H$^\gamma$, Ile^6), 0.83 (3H, H$^\delta$, Leu^5),0.84 (3H, H$^\delta$, Leu^8), 0.86 (3H, H$^\gamma$, Ile^7), 0.87 (3H, H$^\gamma$, Val^9), 0.88 (3H, H$^\delta$, Leu^8), 0.97 (3H, H$^\gamma$, Val^9), 1.04 (1H, H$^\gamma$, Ile^7), 1.10 (1H, H$^\gamma$,Ile^6), 1.41 (1H, H$^\gamma$, Ile^7), 1.43 (1H, H$^\beta$, Leu^5), 1.45 (1H, H$^\gamma$, Ile^6), 1.52 (1H, H$^\beta$, Leu^5), 1.53 (1H, H$^\gamma$, Leu^8), 1.54 (1H, H$^\beta$, Leu^8), 1.55 (1H, H$^\gamma$, Leu^5), 1.63 (1H, H$^\beta$, Leu^8), 1.66 (1H, H$^\gamma$, Pro^2), 1.70 (1H, H$^\beta$, Pro^1), 1.81 (1H, H$^\beta$, Ile^7), 1.84 (1H, H$^\gamma$, Pro^2), 1.86 (1H, H$^\gamma$, Pro^1), 1.97 (1H, H$^\beta$, Ile^6), 1.97 (1H, H$^\beta$, Val^9), 1.99 (1H, H$^\gamma$, Pro^1), 2.06 (1H, H$^\alpha$, hhPhe^3), 2.06 (1H, H$^\beta$, Pro^2), 2.16 (1H, H$^\alpha$, hhPhe^3), 2.22 (1H, H$^\beta$, hhPhe^3), 2.24 (1H, H$^\beta$, Pro^2), 2.28 (1H, H$^\beta$, Pro^1), 2.37 (1H, H$^{\gamma''}$, hhPhe^3), 2.45 (1H, H$^{\gamma''}$, hhPhe^3), 2.85 (1H, H$^\beta$, Phe^4), 2.93 (2H, H$^\gamma$, hhPhe^3), 3.05 (1H, H$^\beta$, Phe^4), 3.37 (1H, H$^\delta$, Pro^2), 3.44 (1H, H$^\delta$, Pro^2), 3.59 (1H, H$^\delta$, Pro^1), 3.72 (1H, H$^\delta$, Pro^1), 3.39 (1H, H$^\alpha$, Ile^6), 4.18 (1H, H$^\alpha$, Leu^8), 4.25 (1H, H$^\alpha$, Ile^7), 4.25 (1H, H$^\alpha$, Pro^1), 4.26 (1H, H$^\alpha$, Leu^5), 4.40 (1H, H$^\alpha$, Pro^2), 4.48 (1H, H$^\alpha$, Val^9), 4.61 (1H, H$^\alpha$, Phe^4), 7.09 (1H, H^4, Phe^4), 7.10 (2H, H^{2,6}, hhPhe^3), 7.16 (1H, H^4, hhPhe^3), 7.16 (2H, H^{3,5}, Phe^4), 7.23 (2H, H^{2,6}, Phe^4), 7.23 (2H, H^{3,5}, hhPhe^3), 7.38 (1H, H^N, Val^9), 7.64 (1H, H^N, Ile^7), 7.85 (1H,H^N, Ile^6), 7.86 (1H, H^N, Leu^8), 7.87 (1H, H^N, Leu^5), 7.96 (1H, H^N, Phe^3), 8.03 (1H, H^N, hhPhe^3).

2.3.9. *c(Pro^1-Pro^2-Phe^3-**R-γ^{3}hhPhe^4**-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)* (**16**)

(DMSO-d$_6$, 50 °C) δ: 0.76 (3H, H$^\gamma$, Ile^7), 0.79 (3H, H$^\delta$, Leu^8), 0.80 (3H, H$^\delta$, Ile^7), 0.82 (3H, H$^\delta$, Ile^6), 0.84 (3H, H$^\gamma$, Ile^6), 0.84 (3H, H$^\delta$, Leu^8), 0.84 (3H, H$^\delta$, Leu^5), 0.86 (1H, H$^\gamma$, Pro^2), 0.89 (3H, H$^\delta$, Leu^5), 0.92 (3H, H$^\gamma$, Val^9), 0.99 (3H, H$^\gamma$, Val^9), 1.05 (1H, H$^\gamma$, Ile^7), 1.08 (1H, H$^\gamma$, Ile^6), 1.41 (1H, H$^\gamma$, Ile^7), 1.42 (1H, H$^\gamma$, Ile^6), 1.50 (2H, H$^\beta$, Leu^5), 1.52 (2H, H$^\beta$, Leu^8), 1.55 (1H, H$^\gamma$, Pro^2), 1.60 (1H, H$^\gamma$, Leu^5), 1.62 (1H, H$^\gamma$, Leu^5), 1.67 (1H, H$^\beta$, Pro^1), 1.79 (1H, H$^\beta$, Pro^2), 1.80 (1H, H$^\beta$, Ile^7), 1.82 (1H, H$^\gamma$, Pro^1), 1.91 (1H, H$^\beta$, Ile^6), 1.99 (1H, H$^\gamma$, Pro^1), 2.05 (1H, H$^\beta$, Val^9), 2.10 (1H, H$^\alpha$, hhPhe^4), 2.13 (1H, H$^\beta$, Pro^2), 2.18 (1H, H$^\alpha$, hhPhe^4), 2.19 (1H, H$^\beta$, Pro^1), 2.30 (1H, H$^\beta$, hhPhe^4), 2.39 (1H, H$^{\gamma''}$, hhPhe^4), 2.69 (1H, H$^{\gamma''}$, hhPhe^4), 2.85 (1H, H$^\delta$, Pro^2), 2.99 (1H, H$^\gamma$, hhPhe^4), 3.09 (1H, H$^\beta$, Phe^3), 3.18 (1H, H$^\beta$, Phe^3), 3.24 (1H, H$^\delta$, Pro^2), 3.30 (1H, H$^\gamma$, hhPhe^4), 3.58 (1H, H$^\delta$, Pro^1), 3.69 (1H, H$^\delta$, Pro^1), 3.99 (1H, H$^\alpha$, Ile^7), 4.00 (1H, H$^\alpha$, Ile^6), 4.19 (1H, H$^\alpha$, Leu^5), 4.28 (1H, H$^\alpha$, Leu^8), 4.3 (1H, H$^\alpha$, Pro^1), 4.40 (1H, H$^\alpha$, Pro^2), 4.40 (1H, H$^\alpha$, Val^9), 4.49 (1H, H$^\alpha$, Phe^3), 7.16 (1H, H^4, Phe^3), 7.19 (2H, H^{2,6}, hhPhe^4), 7.21 (2H, H^{3,5}, Phe^3), 7.25 (2H, H$^\alpha$, Phe^3), 7.27 (2H, H^{3,5}, hhPhe^4), 7.28 (1H, H^4, hhPhe^4), 7.33 (1H, H^N, Val^9), 7.63 (1H, H^N, Ile^6), 7.65 (1H, H^N, Ile^7), 7.75 (1H, H^N, hhPhe^4), 7.92 (1H, H^N, Leu^5), 7.93 (1H, H^N, Leu^8), 8.52 (1H, H^N, Phe^3).

2.4. Synthetic procedures

(*3S*)- and (*3R*)-4-((*tert*-Butoxycarbonyl)amino-)-3-benzyl-butanoic acid **17** was obtained from racemic (±)-3-aminomethyl-4-phenylbutanoic acid hydrochloride, which was synthesized according to the earlier published procedure [34] with some modifications. Ethyl (±)-3-nitromethyl-4-phenylbutanoate was hydrolysed and then hydrogenated using 10% Pd/C to get acid, which was transformed into Boc-derivative. The products were purified by crystallization from ethyl acetate/hexane to yield crystalline solids. The enantiomeric purity was determined according to the known procedure using N_α-(2,4-dinitro-5-fluorophenyl)-L-valinamide as a derivatizing reagent [35]. Diastereomeric derivatives were detected at different retention times (min): 12.67 and 13.62 for **17-*3R*** and **17-*3S***, respectively.

2.4.1. Synthesis and enantiomeric resolution of **17**

2.4.1.1. (3RS)-4-((tert-Butoxycarbonyl)amino-)-3-benzyl-butanoic acid, ***17***. (±)-3-Aminomethyl-4-phenylbutanoic acid hydrochloride (6.66 g, 29 mmol) and Boc$_2$O (7 g, 32.1 mmol) in 1,4-dioxane (30 mL) and 2 N NaOH (30 mL) were stirred at RT for 24 h. After

the solvents removal the residue was dissolved in water (40 mL), pH was adjusted to 9–10 with 1 N $NaHSO_4$ and solution was washed with EtOAc (3 × 50 mL). The solution was then washed with dichloromethane (3 × 50 mL) at pH 3–4 and (2 × 50 mL) at pH 1. The organic phases were combined, washed with brine (2 × 50 mL) and dried over $MgSO_4$. After evaporation of the solvent the racemic acid **17** was obtained in 63.6% yield (5.41 g, 95.3% purity).

Enantiomeric resolution of **17** was achieved by crystallization in the presence of (*R*)-(+)-methylbenzylamine or (*S*)-(−)-methylbenzylamine starting from 5409 g of 4-((*tert*-butoxycarbonyl) amino-)-3-phenyl-pentanoic acid **17-*3R,S***. For crystallization of 1 g of racemic acid **17** ethyl acetate (110 mL) was applied. (*S*)-(−)-Methylbenzylamine was used to obtain (*3S*)-(+)-4-((*tert*-butoxycarbonyl)amino-)-3-phenyl-pentanoic acid **17-*3R***, then the solid was recrystallized three times to obtain final product with ee = 97.4%. (*R*)-(+)-Methylbenzylamine was applied to the mother liquor after the first crystallization of **17-*3S*** and (*3R*)-(−)-4-((*tert*-butoxycarbonyl)amino-)-3-phenyl-pentanoic acid **17-*3R*** and ee = 98.1% was obtained. Each time the solutions were left for 24 h at +5 °C for crystallization. After each crystallization, 3–5 mg were used for enantiomeric excess examination using Marfey's reagent.

Acids **17-*3R*** and **17-*3S*** were generated from the ethyl acetate solution by treatment with 1 M $NaHSO_4$.

2.4.1.2. (3RS)-4-((tert-Butoxycarbonyl)amino-)-3-benzyl-butanoic acid, ***17***. Mp 64–66 °C; HPLC (50–90% B, 15 min), t_R = 7.65 min (95.3%); IR ν 3332, 2977, 2930, 1701, 1656, 1514, 1453, 1406, 1366, 1249, 1161, 909, 731, 699, 504, 461 cm^{-1}; ^1H NMR (700 MHz, CD_3Cl) (78%) δ 1.44 (s, 9H, $(CH_3)_3C$), 2.29–2.39 (m, 3H, *CH,* HOOC−CH_2), 2.65 (d, 2H, *J* = 6.3 Hz, Ph-CH_2), 3.06–3.14 (m, 1H, NH−CH*H*), 3.20–3.27 (m, 1H, NH−*CH*H), 4.71 (bs, 1H, N*H*), 7.17 (d, 2H, *J* = 7.5 Hz, Ph), 7.21 (t, 1H, *J* = 7.5 Hz, Ph), 7.29 (t, 2H, *J* = 7.5 Hz, Ph); ^1H NMR (700 MHz, CD_3Cl) minor rotamer (22%) δ 1.44 (s, 9H, $(CH_3)_3C$), 2.21 (bs, 1H, CH, HOOC−*CH*H), 2.30–2.40 (m, 1H, CH, HOOC−*CH*H), 2.45 (bs, 1H, C*H*), 2.57 (bs, 1H, Ph-CH*H*), 2.59–2.68 (m, 1H, Ph-*CH*H), 2.95 (bs, 1H, NH−CH*H*), 3.21–3.27 (m, 1H, NH−*CH*H), 5.89 (bs, 1H, N*H*), 7.17 (d, 2H, *J* = 7.5 Hz, Ph), 7.21 (t, 1H, *J* = 7.5 Hz, Ph), 7.29 (t, 2H, *J* = 7.5 Hz, Ph); ^{13}C NMR (175 MHz, CD_3Cl) 28.5, 36.4, 38.2, 38.5, 43.6, 79.9, 126.6, 128.7, 129.3, 139.4, 156.7, 177.6.

2.4.1.3. (3R)-(−)-4-((tert-Butoxycarbonyl)amino-)-3-benzyl-butanoic acid, ***17-*3R***. Mp 84–86 °C; $[\alpha]_D^{25} = -14.3°$; HPLC (40–70% B, 15 min), t_R = 10.67 min (100%), ee = 98.10%; IR ν 3372, 2977, 2966, 2931, 1705, 1656, 1455, 1441, 1398, 1365, 1200, 1169, 1138, 1106, 1083.47, 991, 863, 774, 738, 698, 658, 596, 496, 437 cm^{-1}; ^1H NMR (700 MHz, $CDCl_3$ 300 K) major rotamer (76%) δ 1.43 (s, 9H, $(CH_3)_3C$), 2.29–2.39 (m, 3H, *CH,* HOOC−CH_2), 2.65 (d, 2H, *J* = 4.2 Hz, Ph-CH_2), 3.06–3.14 (m, 1H, NH−CH*H*), 3.20–3.27 (m, 1H, NH−*CH*H), 4.71 (bs, 1H, N*H*), 7.17 (d, 2H, *J* = 7.5 Hz, Ph), 7.21 (t, 1H, *J* = 7.5 Hz, Ph), 7.29 (t, 2H, *J* = 7.5 Hz, Ph), 10.81 (bs, 1H, OH); ^1H NMR (700 MHz, $CDCl_3$ 300 K) minor rotamer (24%) δ 1.44 (s, 9H, $(CH_3)_3C$), 2.20 (bs, 1H, CH, HOOC−*CH*H), 2.30–2.40 (m, 1H, CH, HOOC−*CH*H), 2.48 (bs, 1H, C*H*), 2.57 (bs, 1H, Ph-CH*H*), 2.59–2.68 (m, 1H, Ph-*CH*H), 2.91 (bs, 1H, NH−CH*H*), 3.21–3.27 (m, 1H, NH−*CH*H), 6.07 (bs, 1H, N*H*), 7.17 (d, 2H, *J* = 7.5 Hz, Ph), 7.21 (t, 1H, *J* = 7.5 Hz, Ph), 7.29 (t, 2H, *J* = 7.5 Hz, Ph) 10.81 (bs, 1H, OH); ^{13}C NMR (175 MHz, $CDCl_3$) major rotamer δ 28.5, 36.3, 38.1, 38.5, 43.7, 79.8, 126.5, 128.7, 129.3, 139.3, 156.6, 178.0; ^{13}C NMR (175 MHz, $CDCl_3$) minor rotamer δ 28.4, 37.0, 37.7, 38.7, 45.3, 81.0, 126.5, 128.7, 129.3, 139.3, 157.9, 178.0; ^1H NMR (700 MHz, CD_3CN)) major/minor rotamer (88/(12) δ 1.40 (s, 9H, $C(CH_3)_3$), 2.16 (dd, 1H, *J* = 5.00, 14.4 Hz, HOOC−CH*H*), 2.19–2.26 (m, 2H, HOOC−*CHH*, C*H*), 2.56 (dd, 1H, *J* = 6.9, 13.7 Hz, Phe-CH*H*), 2.61 (dd, 1H, *J* = 6.6, 13.7 Hz, Phe-CH*H*), 2.94–3.00 (m, 1H, NH−CH*H*), 3.04–3.09 (m, 1H, NH−CH*H*), (minor 5.00 (bs, 1H, NH), major 5.36 (bs, 1H, NH)) 7.19–7.22 (m, 3H, Ar), 7.28–7.31 (m, 2H, Ar) 9.52 (s, 1H, O*H*); ^{13}C NMR (175 MHz, CD_3CN) δ 29.0, 36.9, 39.1, 39.3, 44.5, 79.8, 127.5, 129.7, 130.6, 141.5, 157.8, 174.9. ^1H NMR (700 MHz, DMSO) major rotamer (90%) δ 1.38 (s, 9H, $(CH_3)_3C$), 1.99–2.04 (m, 1H, HOOC−CH_2), 2.11–2.20 (m, 2H, HOOC−CH_2, C*H*), 2.47–2.51 (m, 1H, Ph-CH*H*), 2.59 (dd, *J* = 6.7, 14.0 Hz, 1H, Ph-C*H*H), 2.82–2.87 (m, 1H, NH−CH*H*), 2.91–2.97 (m, 1H, NH−C*H*H), 6.87 (t, *J* = 5.4 Hz, 1H, N*H*), 7.16–7.20 (m, 3H, $Ph_{2,6;4}$), 7.26–7.29 (m, 2H, $Ph_{3,5}$), 12.06 (bs, 1H, O*H*); ^1H NMR (700 MHz, DMSO-d_6) minor rotamer (10%) δ 1.33 (s, 9H, $(CH_3)_3C$), 1.99–2.04 (m, 1H,HOOC−CH_2), 2.11–2.20 (m, 2H, HOOC−CH_2, C*H*), 2.47–2.51 (m, 1H, Ph-CH*H*), 2.53–2.58 (m, 1H, Ph-C*H*H), 2.75–2.83 (m, 1H, NH−CH*H*), 2.91–2.97 (m, 1H, NH−C*H*H), 6.55 (t, *J* = 5.4 Hz, 1H, N*H*), 7.16–7.20 (m, 3H, $Ph_{2,6;4}$), 7.26–7.29 (m, 2H, $Ph_{3,5}$), 12.06 (bs, 1H, O*H*); ^{13}C NMR (175 MHz, DMSO) δ 28.2, 35.7, 37.0, 37.2, 42.8, 77.5, 125.9, 128.2, 129.1, 139.8, 155.8, 173.7; CI MS *m/z* (%) 294.3 $[M+1]^+$, 294.2 (100), 238.1 (46), 194.1 (26), 176.1 (3). For $C_{16}H_{23}NO_4$ calculated 293.162.

2.4.1.4. (3S)-(+)-4-((tert-Butoxycarbonyl)amino-)-3-benzyl-butanoic acid, ***17-*3S***. Mp 84–86 °C; $[\alpha]_D^{25} = 14.0°$; HPLC (40–80% B, 15 min), t_R = 10.64 min (97.4%), ee = 97.36%. All spectroscopic data (NMR, IR, MS) were identical as recorded for **17-*3R***.

2.5. Biological methods

2.5.1. Reagents

DMSO, RPMI-1640, PHA, MTT, LPS, OVA, Freund's complete adjuvant, dexamethasone, carrageenan and all other reagents were supplied from Sigma–Aldrich (USA). LSM 1077 was obtained from CytoGen, Germany. Human TNF-α ELISA Ready-SET-Go was provided by eBioscience, USA.

2.5.2. Preparation of the compounds for biological assays

The compounds were dissolved in DMSO (5 mg/200 μl) and subsequently diluted to 5 mL of RPMI-1640 medium for *in vitro* studies or in 0.9% NaCl for injections. As a control appropriate dilutions of DMSO in RMPI-1640 medium or in 0.9% NaCl were used.

2.5.3. Mice

CBA female and male mice, 8–12 weeks old were supplied by the Animal Breeding Center in Ilkowice, Poland. The mice were kept in 12 h light and dark cycles with free access to water and granulated food. The local ethics committee approved the study.

2.5.4. Isolation of the peripheral blood mononuclear cells (PBMC)

Venous blood from a single donor was withdrawn into heparinized syringes and diluted twice with PBS. PBMC were isolated by centrifugation on ficoll-uropoline gradient (density 1.077 g/mL) and centrifuged at 800 × *g* for 20 min at 4 °C. The interphase cells, consisting of lymphocytes (20%) and monocytes (80%), were then washed three times with Hanks' medium and re-suspended in a culture medium, referred to below as the culture medium, consisting of RPMI-1640, supplemented with 10% foetal calf serum, L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics, at density of 2 × 10^6 cells/mL.

2.5.5. Phytohemagglutinin A (PHA)-induced proliferation of human blood mononuclear cells

PBMC were distributed into 96-well flat-bottom plates in 100 μl aliquots (2 × 10^5 cells/well). PHA was added at a concentration of 5 μg/mL. The compounds were tested at doses of 1, 10 and 100 μg/mL. DMSO at appropriate dilutions served as control. After a four-day incubation in a cell culture incubator, the proliferative response of the cells was determined by the colorimetric MTT

method [36]. The data are presented as a mean OD value from quadruplicate wells ± standard error (SE).

2.5.6. Cytotoxicity of the compounds against human blood mononuclear cells

PBMC at density of 3×10^5/100 μL/well, re-suspended in the culture medium, were cultured for 24 h in a cell culture incubator with the preparations at 1, 10 and 100 μg/mL concentrations. Cell survival was determined by MTT colorimetric method. The results are given in percentage of viable cells as compared with appropriate DMSO controls.

2.5.7. Lipopolysaccharide (LPS)-induced tumour necrosis factor alpha (TNF-α) production in whole blood cell culture

The test was performed as described elsewhere [37]. In brief, venous blood from a single donor was diluted 10 × with RPMI-1640 medium and distributed in 1 mL aliquots in 24-well culture plates (Nunc). The cultures were stimulated by addition of 1 μg/mL of LPS from *Escherichia coli*, serotype O:111:B4. The compounds were added to the cultures at concentrations of 5 and 25 μg/mL. Higher concentrations of the compounds could not be used because of inhibitory effects on TNF-α production by corresponding DMSO (the solvent) dilutions. Appropriate dilutions of DMSO served as controls. After overnight incubation in a cell culture incubator, the supernatants were harvested and frozen at −20 °C until cytokine determination by immunoassay.

2.5.8. Delayed type hypersensitivity to ovalbumin (OVA)

The test was performed as described previously [38]. Mice were sensitized subcutaneously into tail base with 5 μg OVA in complete Freund's adjuvant. After 4 days the mice received 50 μg OVA in incomplete Freund's adjuvant in a volume of 50 μl in the hind foot pads. After 24 h the foot pad edema was measured using a spring caliper. Control (background group) mice were not sensitized but received the eliciting dose of antigen. The compounds were given to mice intraperitoneally, in 100 μg doses, 2 h before sensitization or 2 h before elicitation of the DTH reaction with the second dose of antigen. Control mice received appropriate doses of the solvent (DMSO). The results were presented as mean values of antigen-specific increase of foot pad swelling measured in 5 mice per group (10 determinations from 10 feet) and expressed in DTH units ±standard error (SE). One DTH unit $= 10^{-2}$ cm.

2.5.9. Carrageenan-induced paw inflammation

We followed the procedure as recently published [39]. Mice were administered 0.1 mL of 2% carrageenan in 0.9% NaCl in hind foot pads. The studied compounds (100 μg doses) were injected intraperitoneally at 24 h or 24 h and 30 min before administration of carrageenan. The foot pad edema was measured using a spring caliper after: 2, 3 and 4 h after carrageenan administration. Dexamethasone (50 μg/ml), administered 30 min before carrageenan, was served as a reference drug. The results were presented as a mean increase in foot pad swelling from 5 mice per group (10 determinations from 10 feet) ± standard error.

2.5.10. Statistics

The results are presented as mean values ± standard error (SE). Brown-Forsyth's test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (One-way ANOVA) was applied, followed by *post hoc* comparisons with the Tukey's test to estimate the significance of the difference between groups. Nonparametric data were evaluated with the Kruskal–Wallis' analysis of variance, as indicated in the text. Significance was determined at $p < 0.05$. Statistical analysis was performed using STATISTICA 6.1 for Windows.

Table 2
Vicinal coupling constants $^3J_{NHC\alpha H}$ [Hz] of peptides **9**–**16** in DMSO-d_6 at 323 K.

Peptide	Phe^3 or γ^3-hhPhe^3	Phe^4 or γ^3-hhPhe^4	Leu^5	Ile^6	Ile^7	Leu^8	Val^9
9	5.8	5.8	8.1	8.9	8.4	8.6	9.2
10	5.9	6.7	8.0	7.3	8.5	6.2	8.8
11a	7.4	8.5[a]	8.1[a]	10.9[a]	9.9[a]	8.8[a]	9.3[a]
11b	7.5[a]	10.2[a]	8.0[a]	8.7[a]	7.5[a]	7.0[a]	8.7[a]
12	5.4	6.0	8.3	8.9	8.6	8.1	9.2
13	7.4	7.8	8.1	8.1	–	8.8	9.2
14	5.4	5.5	8.3	9.1	8.5	8.8	9.2
15	7.4	5.6	8.2	8.2	8.2	8.2	8.9
16	7.6	–	8.0	8.5	7.8	7.8	8.3

[a] Vicinal coupling constants ^3JNHCαH [Hz] of peptides determined from COSY-DQF spectra.

3. Results and discussion

3.1. Chemistry

All analogues were modified with γ^3-hhPhe residue in position 3 or 4 and both 3 and 4. All analogues were synthetized according to manual SPPS procedure based on Boc procedure. The scale of synthesis was 0.2 mmol for each analogue. The deprotection took place with 50% solution TFA in DCM through 10 min. TBTU was used as a coupling reagent, HOBt as an antiracemic additive and DIPEA as an amin. Resin was washed with DCM, methanol and DCM, then the synthetic procedure (coupling, deprotection, acylation) was continued. Standard Boc-protected amino acids were used in 3 eq and Boc-γ^3-hhPhe-OH was used in 1.5 eq in coupling to the resin. The cleavage took place in 30 min using TFMSA, TFA and anisol. The solution was evaporated under vacuum and the resulting peptide was achieved by precipitation with diethyl ether. The cyclisation was achieved with syringe pump and commonly occurred through 24 h with 12–46% yield. For biological assays all peptides were purified by HPLC.

3.2. NMR measurements

^{1}H NMR spectra of cyclic peptides **9**–**16** in DMSO-d_6 at 300 K are well resolved, much better than those recorded for the unmodified CLA [40]. The NH resonances are spread within the narrow range of 0.5–0.8 ppm, except range of 1.2 ppm for peptides **11a** and **16**. Most of the NH signals are well resolved, enabling determination of NH-CHα coupling constants.

For CLA analogues **9**–**16** the geometries of peptide bonds were established from ROESY spectra. The ROE between C^α–H and C^δ-H proline atoms is present for *trans* Pro–Pro peptide bond and between C^α–H and C^α-H for *cis* Pro–Pro bonds. In addition the difference in chemical shifts between C^β and C^γ in prolines was examined. The differences of about 9 ppm and about 5 ppm are

Table 3
Temperature dependence of the NH chemical shifts ($-\Delta\delta/\Delta T$, ppb/K) of peptides **9**–**16** in DMSO-d_6, in the range of 300–340 K.

Peptide	Phe^3 or γ^3-hhPhe^3	Phe^4 or γ^3-hhPhe^4	Leu^5	Ile^6	Ile^7	Leu^8	Val^9
9	0.1	5.1	3.5	0.1	5.6	5.7	4.9
10	1.3	0.7	2.6	4.2	2.1	2.9	3.3
11a[a]	3.8	3.5	3.5	4.2	3.5	3.9	0.1
12	3.7	5.5	2.5	1.8	3.6	3.6	5.3
13	0.8	5.0	5.2	8.9	?	5.6	3.6
14	2.6	5.1	3.8	1.2	5.3	4.3	4.3
15	3.4	5.4	3.8	3.8	4.9	3.8	1.8
16	5.5	5.3	2.1	2.5	3.4	4.4	4.3

[a] The temperature dependence of the NH chemical shifts for cyclopeptide **11b** was unable to determine and is omitted.

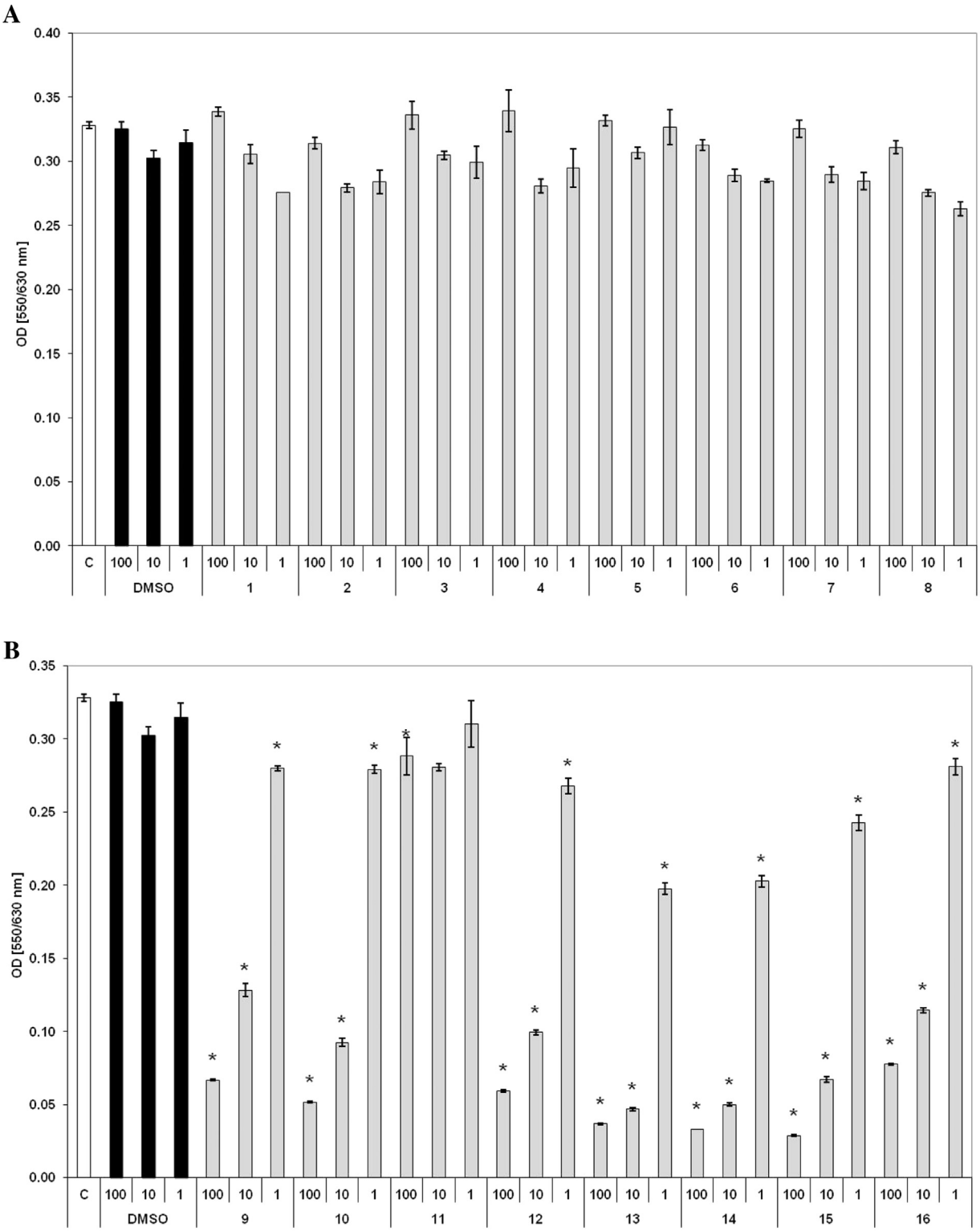

Fig. 2. The toxicity of linear peptides **1**–**8** (**A**) and cyclic peptides **9**–**16** (**B**) with regard to human peripheral blood mononuclear cells. The peptides were used at concentrations of 1–100 μg/mL. The cell toxicity was compared with toxicity of DMSO used as solvent.

characteristic for *trans* and *cis* Xxx-Pro geometries, respectively [41]. All cyclic peptides, except major isomer of peptide **11**, exist as isomers with the *cis* Pro–Pro peptide bond, similarly as in CLA [40]. The CLA analogue **11** with single modification *S*-γ^3-hhPhe^4 is a mixture of two isomers in the ratio 89:11. The major isomer contains all peptide bonds of the *trans* geometry.

Chemical shifts of the γ protons of Pro^2 are very similar for all cyclopeptides except **11** and **16** containing fragment of Phe^3-*R*-γ^3-hhPhe^4. For these peptides one of γ protons experiences a high-

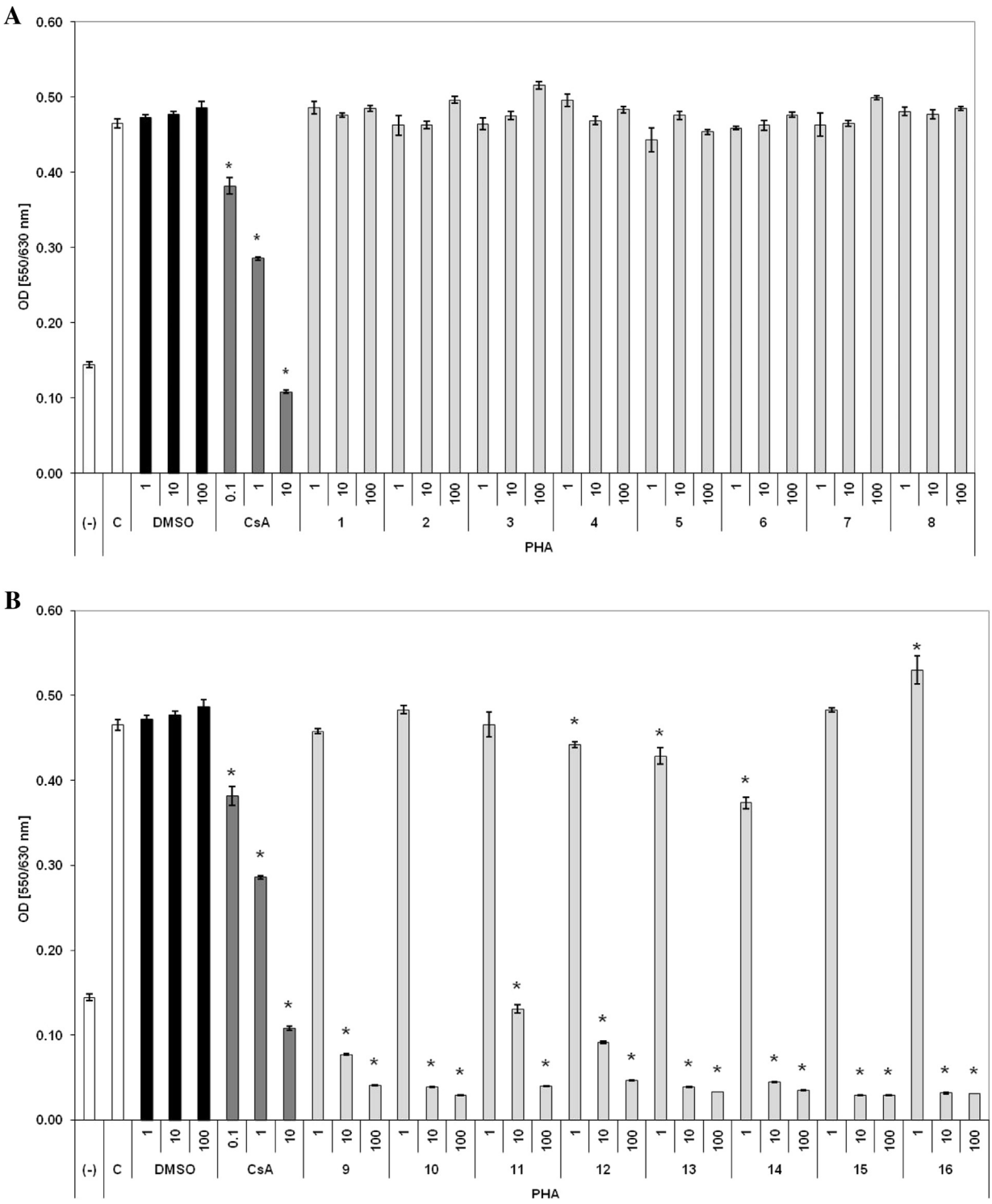

Fig. 3. Effects of linear peptides **1**–**8** (**A**) and cyclic peptides **9**–**16** (**B**) on PHA-induced PBMC proliferation. The peptides were used at concentrations of 1–100 μg/mL and cyclosporine A was applied as reference drug.

field shift up to 1.02 ppm (**11a**), 1.16 (**11b**) and 0.86 ppm (**16**). The observed up-field shift is not as large as observed for CLA (Pro^2 γ proton observed at 0.33 ppm), but evidences much closer proximity of the Phe^3 aromatic rings and Pro^2 side chains in peptides **11** and **16**, than in other cyclopeptides.

For all studied cyclopeptides' signals of both Phe aromatic rings are placed in narrow and similar range. This suggests that peptides modified with γ-hhPhe do not manifest the face-to-edge interaction of phenyl rings as in native CLA [40,42,43] or its analogues modified with tyrosine [44].

Vicinal coupling constants $^3J_{NHC\alpha H}$ were measured directly from ^{1}H NMR spectra or from COSY-DQF spectrum (peptide **11**) and are summarized in Table 2. Most of $^3J_{NHC\alpha H}$ vicinal couplings constants are about 8 Hz. For Phe or hhPhe residues $^3J_{NHC\alpha H}$ are about 6 Hz,

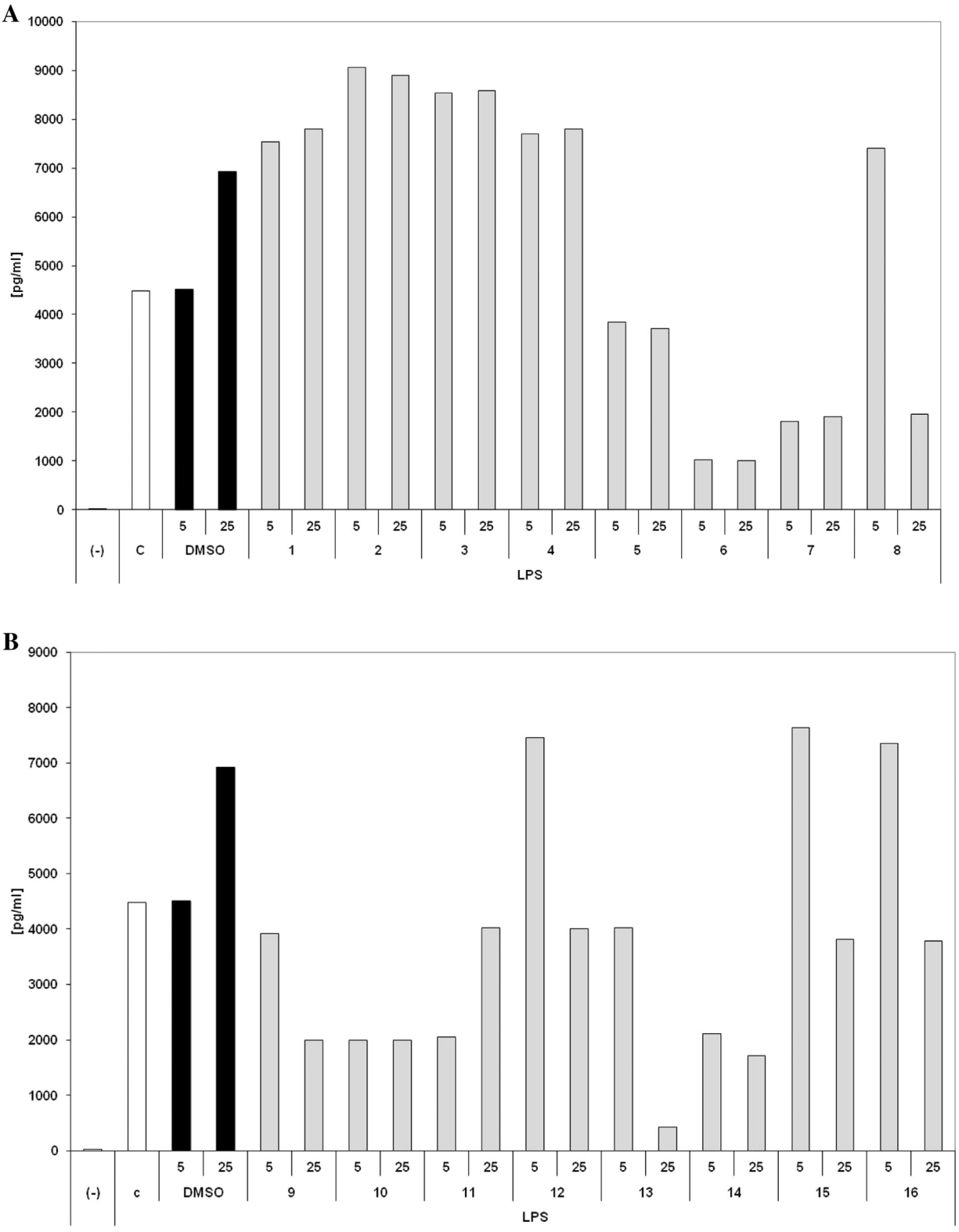

Fig. 4. Effects of linear peptides **1**–**8** (A) and cyclic peptides **9**–**16** (**B**) on LPS-induced TNF-α production in human whole blood cultures. The peptides were used at concentrations 5 and 25 μg/mL.

except peptides **11**, **13** and **16**. Vicinal couplings indicates the similarity of the NH-CαH moieties with φ angles in the range −80°/−160° [45].

The solvent exposure of NH protons was probed by measuring the temperature effect coefficients ($\Delta\delta/\Delta T$) (Table 3). The low temperature coefficients ($\Delta\delta/\Delta T < 3$ ppb/K) indicate the presence of intramolecular hydrogen bonds [30]. For CLA the NH's of Phe^4, Ile^7,

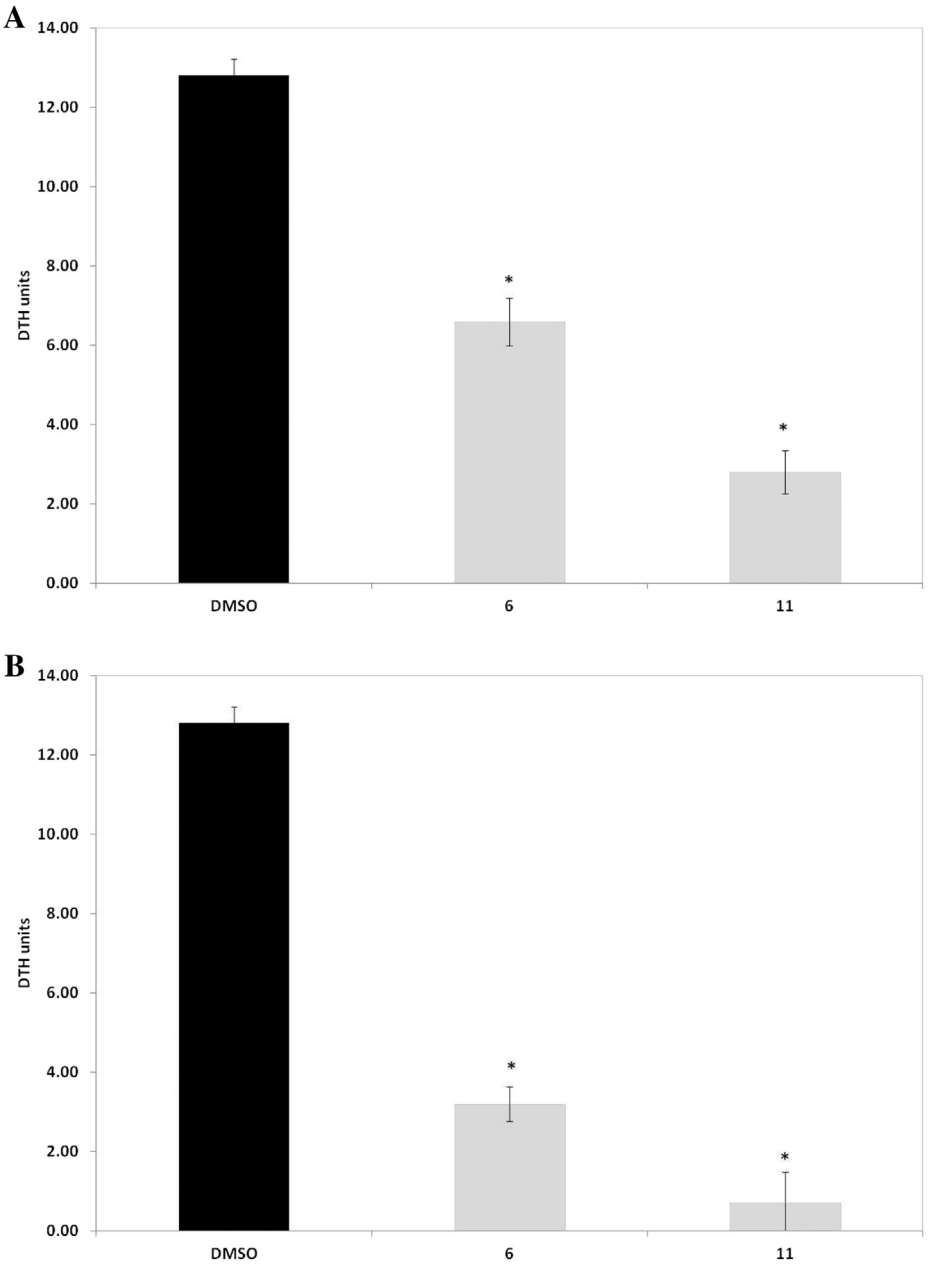

Fig. 5. Inhibitory effects of peptides **6** and **11** on delayed type hypersensitivity to ovalbumin. **A**: the compound administered before sensitization, **B**: the compound administered before elicitation of DTH.

Leu^8, and Val^9 were found engaged in intramolecular hydrogen bonds in chloroform in the range of 204–264 K. The analogues of CLA modified with γ^3-hhPhe are characterized in DMSO by different numbers of hydrogen bonds: five hydrogen bonds (**10** – *S*-γ^3-hhPhe^3, Phe^4, Leu^5, Ile^7, Leu^8), two hydrogen bonds (**9** – *S*-γ^3-hhPhe^3, Ile^6, **12** – Leu^5, Ile^6, **14** – *R*-γ^3-hhPhe^3, Ile^6, **16** – Leu^5, Ile^6) or one hydrogen bond (**11a** – Val^9, **13** – *S*-γ^3-hhPhe^3, **15** – Val^9).

In comparison to native CLA the replacement of one or both Phe residues by γ^3-hhPhe increased the number of stabilizing hydrogen bonds for all analyzed analogues, except peptide **10**. ^{1}H NMR spectra are better resolved, probably due to lower exchange between conformers in the NMR scale. All cyclic peptides, except **11**, exist as isomers with the *cis* Pro–Pro peptide bond. The CLA analogue **11** with single modification *S*-γ^3-hhPhe^3 is a mixture of two isomers and the major isomer (89%) contains all peptide bonds of the *trans* geometry.

3.3. Biology

3.3.1. Effects of the compounds on viability of PBMC

The cytotoxic effects of the compounds, at the concentration range of 1–100 μg/mL, were evaluated in the cultures of PBMC. For linear peptides **1**–**8** we have not found any effects on the viability of cells in the studied concentration range (Fig. 2A). All cyclic analogues **9**–**16,** except for **11**, appeared to be toxic in a dose-dependent manner. Typically, a significant toxicity occurred at 10 μg/mL (Fig. 2B. Interestingly, peptide **11** was virtually devoid of toxicity.

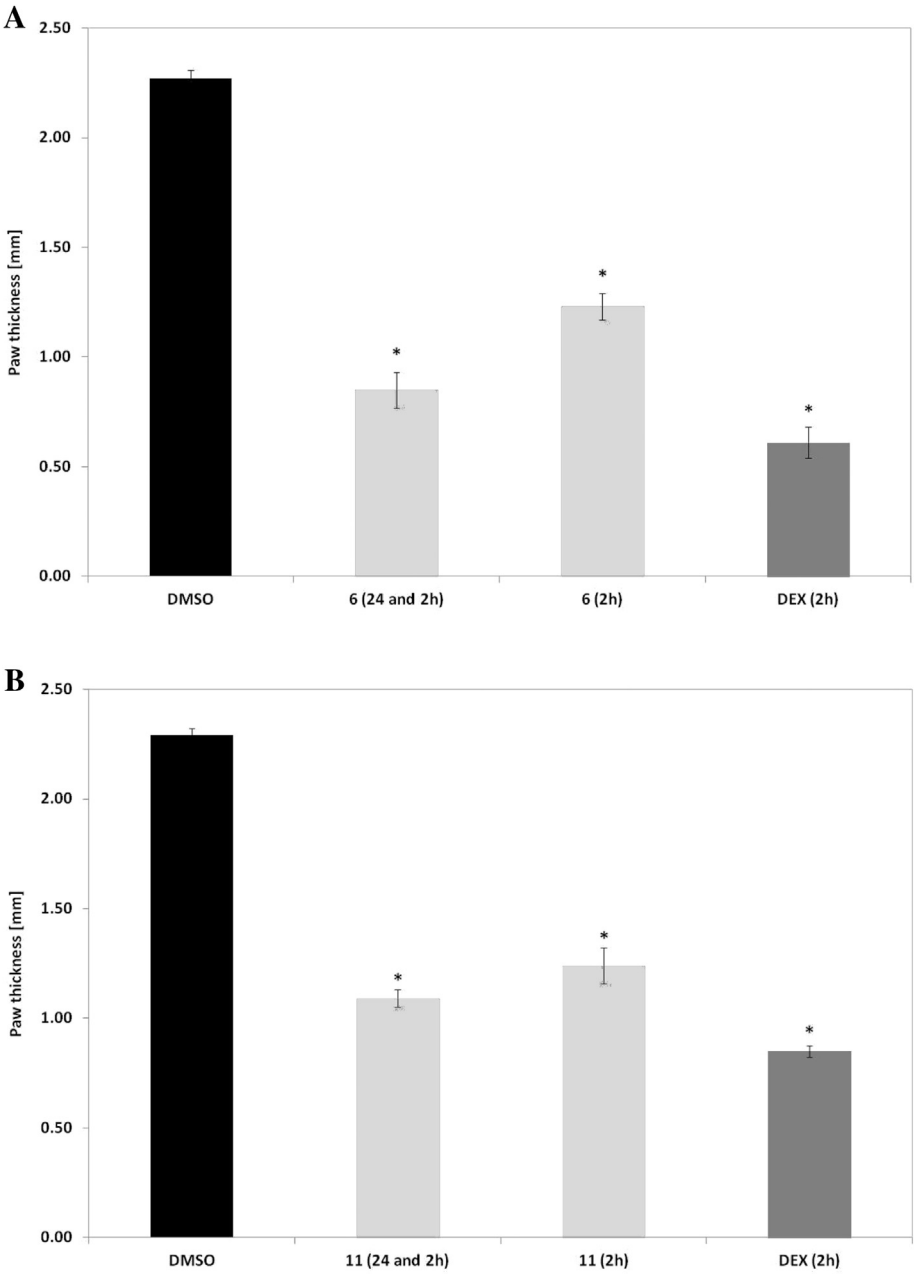

Fig. 6. The anti-inflammatory actions of peptide 6 (**A**) and 11 (**B**) on carrageenan-induced foot pad inflammation in mice. Dexamethasone (DEX) was applied as reference drug.

Peptides **1**–**8** at the concentration range of 1–100 μg/mL did not affect proliferation of PBMC to PHA (Fig. 3A). Cyclosporine A, used as a reference drug, showed a typical, strong anti-proliferative activity already at 1 μg/mL. On the other hand, the cyclic peptides exhibited strong anti-proliferative activities in the model of PHA-induced PMBC proliferation (Fig. 3B). Although that strong inhibition of cell proliferation could be attributed to high cell toxicity of majority of the peptides, the anti-proliferative action of peptide **11** (the non-toxic peptide) was probably associated with a different mechanism.

The effects of the linear peptides on LPS-induced TNF-α production are shown in Fig. 4A. The peptides were used at 5 and 25 μg/mL. Among them the peptides **1**–**4** showed some stimulatory activities and peptide **8** was strongly inhibitory at 25 μg/mL. The two remaining linear compounds – **6** and **7** were strongly inhibitory at both concentrations.

The actions of the cyclic CLA analogues on LPS-induced TNF-α production (Fig. 4B) were differentiated but predominantly inhibitory, depending on a dose. Providing that at 25 μg/mL concentration and in part at 5 μg/mL, the compounds, except for peptide **11**, were cytotoxic, in these cases the suppressive effects on TNF-α production could be due to toxicity. Interestingly, the non-toxic **11** displayed about 55% inhibition of TNF-α production at 5 μg/mL and 42% inhibition at 25 μg/mL.

For the *in vivo* experiments peptides **6** and **11** were chosen considering their strong antiproliferative and anti-inflammatory actions *in vitro*. Peptide **6** and **11** were administered at doses of 100 μg to mice 2 h before sensitization of animals with OVA (Fig. 5A) or 2 h before elicitation of the antigen-specific cellular

response with a second dose of antigen (Fig. 5B). The results revealed a substantial (about 50%) inhibition of the DTH reaction upon administration of compound **6** before sensitization and even deeper inhibition of the immune response when **6** was given before elicitation of the reaction. CLA analogue **11** exerted even stronger suppressive actions on DTH reaction to OVA, both on the sensitization (Fig. 5A) and on elicitation phase of the response (Fig. 5B). In the latter case it virtually blocked the response.

Next, peptides **6** and **11** were tested in the model of carrageenan-induced foot pad inflammation in mice. The compounds were administered intraperitoneally at 100 μg dose, 24 h and 2 h before carrageenan or only 2 h before carrageenan injection. Dexamethasone, a control drug (50 μg), was given i.p. 2 h before application of carrageenan. Fig. 6 shows the results of measurements of foot pad edema after 3 h following carrageenan administration. It appeared that both compounds were strongly inhibitory in this model, particularly when given at two doses.

In summary, among the studied compounds two of them (**6** and **11**) showed distinct anti-inflammatory actions. The ability of **6** to strongly inhibit LPS-induced TNF-α production *in vitro* may, in part, explain its anti-inflammatory properties *in vivo*. These properties seem to be universal regarding the nature and mechanism of action of pro-inflammatory stimuli (Freund's complete adjuvant or carrageenan) leading to inhibition of respective immune responses of involving different cell types and mediators. Such non-specific anti-inflammatory actions may be useful both in prevention or treatment of inflammatory processes.

In contrast to completely non-toxic linear peptides, the cyclic peptides synthesized and investigated in this work, bear differential characteristics, i.e. part of them are highly cytotoxic, thus inhibition of PHA-induced proliferation in these cases is probably solely due to toxicity. Some of them have also ability to inhibit TNF-α production. Importantly, we found that peptide **11**, the cyclic counterpart of compound **6**, appears to be attractive enough for further research since it is non-toxic at the studied concentration range and its immunosuppressive activities *in vivo* are very strong. The compound exhibited stronger suppressive effects on the DTH reaction and somewhat weaker ones in the carrageenan test as compared to compound **6**. However, hydrophobicity of compound **11** and resistance to proteolysis predisposes this peptide as a better candidate for a potential therapeutic drug than its linear counterpart.

4. Conclusions

The replacement of one or two Phe residues in the CLA molecule by γ^3-hhPhe led to the expansion of backbone of peptides **11**, **15** and **16** by two carbon atoms, and peptides **10**, **12**–**14** by four carbon atoms. Consequently the modified CLA molecules are less conformationally flexible or the exchange between conformers is much slower in the NMR time scale. Peptides **9**, **10** and **12**–**16** are single isomers with the *cis* Pro–Pro bond and other peptide bonds of the *trans* geometry. Peptide **11** is a mixture of two isomers and the major isomer (89%) contains all *trans* peptide bonds.

Linear peptides **1**–**8**, precursors of CLA analogues **9**–**16**, were not toxic against PBMC but the cyclic analogues showed dose-dependent toxicity with the exception of peptide **11**. Linear peptides did not inhibit mitogen-induced PBMC proliferation, whereas cyclic ones inhibited the proliferation in a dose-dependent manner. The actions of linear and cyclic peptides with regard to LPS –induced TNF α production in whole human blood cultures were differential but particularly suppressive in the case of linear compound **6**. Therefore, for the *in vivo* investigations compounds **6** and **11** were selected. The compounds showed comparable, suppressive actions in the inductive and effector phases of the delayed type hypersensitivity as well as in the carrageenan-induced foot pad edema. In conclusion, linear peptide **6** and cyclic peptide **11** are attractive as potential drugs, although the cyclic structure of compound **11** predisposes the peptide as a better candidate as an anti-inflammatory and immunosuppressive therapeutic.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.09.014.

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