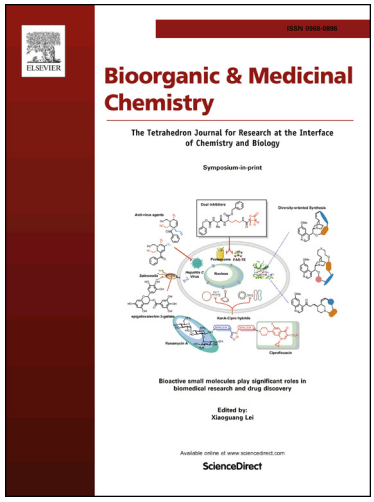

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Synthesis and biological activity of cyclolinopeptide A analogues modified with γ^4-bis(homo-phenylalanine)

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Abstract

Cyclolinopeptide A (CLA), an immunosuppressive nonapeptide derived from linen seeds, was modified with *S* or *R*-γ^4-bis(homo-phenylalanine) in positions 3 or 4, or both 3 and 4. These modifications changed the flexibility of new analogues and distribution of intramolecular hydrogen bonds. Analogues **11** c(Pro[1]-Pro[2]-Phe[3]-***S*-γ[4]-hhPhe[4]**-Leu[5]-Ile[6]-Ile[7]-Leu[8]-Val[9])**, 13** c(Pro[1]-Pro[2]-***S*-γ[4]-hhPhe[3]-*R*-γ[4]-hhPhe[4]**-Leu[5]-Ile[6]-Ile[7]-Leu[8]-Val[9]) and **15** c(Pro[1]-Pro[2]-***R*-γ[4]-hhPhe[3]**-Phe[4]-Leu[5]-Ile[6]-Ile[7]-Leu[8]-Val[9]) existed as a mixture of stable *cis*/*trans* isomers of Pro-Pro peptide bond. The comparison of the relative spatial orientations in crystal state of the two carbonyl groups, neighboring γ-amino acids, revealed conformational similarities to α-peptides. The addition of two -CH_2- groups in γ-amino acids led to a more rigid conformation, although a more flexible one was expected. A significant difference in the relative orientation of the carbonyl groups was found for cyclic γ-peptides with a dominance of an antiparallel arrangement. As carbonyl groups may be engaged in the interactions with plausible receptors through hydrogen bonds, a similar biological activity of the modified peptides was expected. Our biological studies showed that certain cyclic, but not the corresponding linear peptides, lowered the viability of peripheral blood mononuclear cells (PBMC) at 100 μg/mL concentration. The proliferation of PBMC induced by phytohemagglutinin A (PHA) was strongly inhibited by cyclic peptides only, in a dose-dependant manner. On the other hand, lipopolysaccharide (LPS)-induced tumor necrosis factor alpha (TNF-α) production in whole blood cell cultures was inhibited by both linear and cyclic peptides. Peptide **15** c(Pro[1]-Pro[2]-*R*-γ[4]-hhPhe[3]-Phe[4]-Leu[5]-Ile[6]-Ile[7]-Leu[8]-Val[9]) blocked the expression of caspase-3, inhibited the expression of caspases-8 and -9 in 24h culture of Jurkat cells, and caused DNA fragmentation

in these cells, as an indicator of apoptosis. Thus, we revealed a new mechanism of immunosuppressive action of a nonapeptide.

Keywords: Cyclolinopeptide A, γ-amino acids, cyclic peptides, immunosuppression, apoptosis, PBMC, caspase.

Abbreviations:

Boc, *tert*-Butoxycarbonyl; C8, octyl stationary phase; C18, octadecyl stationary phase; CLA, cyclolinopeptide A; COSY-DQF, double quantum filtered proton spin correlation; CsA, cyclosporine A; DIPEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; hhPhe, γ-bis(homo-phenylalanine); HOBt, 1-hydroxybenzotriazole 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, phenylalanine; Pro, proline; TBTU, *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; TNF-α, tumor necrosis factor alpha; Val, valine.

1. Introduction

Cyclolinopeptide A (CLA), bearing the sequence c(Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val), a naturally occurring peptide from linseed oil [1] was shown to display an immunosuppressive activity comparable to that of cyclosporine A [2, 3]. As proposed, its immunosuppressive activity was related to inhibition of cyclophilin A (peptidyl-prolyl *cis* - *trans* isomerase). [4]. Nevertheless, the affinity of CLA to cyclophilin was lower in comparison to CsA [5].

Studies on structure-activity relationship revealed that -Pro-Pro-Phe-Phe- fragment was responsible for the immunosuppressive activity of CLA [6]. The modifications of this peptide fragment had an impact on immunosuppressive activity, suggesting an association with conformational flexibility [7]. Preliminary studies with non-physiological amino acids showed that the modifications with β or homo -amino acids caused increases in the immunosuppressive and anti-inflammatory activities [8, 9, 10]. Of interest, the analogues of CLA exhibited also synergic immunosuppressive action with methotrexate [11].

Our recent report, describing CLA analogues modified with γ^3-bis(homo-phenylalanine) residues, indicated that the peptide backbone's elongation by an ethylene fragment may

increase the immunosuppressive activity. However, such a modification was also associated with a decreased viability of peripheral blood mononuclear cells through the new analogues. Only one analogue c(Pro[1]-Pro[2]-Phe[3]-*S*-γ[3]-hhPhe[4]-Leu[5]-Ile[6]-Ile[7]-Leu[8]-Val[9]) did not impact on cell viability. It appeared that the lack of cell death was correlated with its capability to form *cis*, *trans* isomers within the -Pro-Pro- fragment [12].

A ^{1}H NMR examination of Boc-γ[4]-bis(homo-phenylalanine) showed that it was possible to stop the rotation in the NH-CO region of the amino acid, which caused two possible syn/anti rotamers of the amide bond, visible in the NMR spectrum [13]. ^{1}H NMR analysis of that molecule shed light on the conformation in solution. However, to fully examine three-dimensional features we performed an X-ray analysis and compared it with all available X-ray data of peptides containing γ-amino acids to find some common conformational patterns.

In order to confirm the influence of ethylene bridges incorporated into the peptide backbone by γ-amino acid and to evaluate the biological activity, 8 new linear and 8 new cyclic analogues of CLA have been synthesized. In these new peptides one or both phenylalanines were replaced by one or two *S* or *R*-γ[4]-bis(homo-phenylalanine) residues (Fig. 1). In addition, we intended to determine the crystal structures of N-protected γ-amino acids, applied in the study, for evaluation of conformational preferences of γ-amino acids in comparison to respective α-amino acids, based on crystallographic data.

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

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

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

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

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

7 H-Ile[6]-Ile[7]-Leu[8]-Val[9]-Pro[1]-Pro[2]-***R*-γ[4]-**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[4]-**hhPhe**[4]-Leu[5]-OH

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

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

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

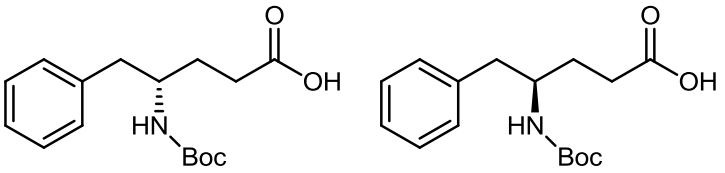
12 c(Pro[1]-Pro[2]-***R*-γ[4]-**hhPhe**[3]-***S*-γ[4]-**hhPhe**[4]-Leu[5]- Ile[6]-Ile[7]-Leu[8]-Val[9])

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

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

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

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



(4*S*)-17 **(4*R*)-17**

Fig. 1. The sequences of linear (**1**-**8**) and cyclic (**9**–**16**) analogues of CLA modified with the use of **(4*S*)** and/or **(4*R*)**-Boc-γ[4]-hhPhe **17**.

2. Materials and methods

2.1. *General remarks*

2.1.1. Chemistry

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 a following solvent system: ethyl acetate:hexane (4:1). 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 1mL/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. The 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. In the peptides cyclisation process Ascor AP22 pump was used. The identities of the pure peptides were confirmed by Maldi-TOF mass spectrometry on Voyager Elite, Perseptive Biosystems using α-cyano-4-hydroxy-cinnamic acid as a matrix. The one- and two-dimensional ^1H 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 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 ROESY experiments were recorded with 300 ms mixing time. 2D NMR spectra (COSY, ROESY, TOCSY, HSQC) were processed with TopSpin 2.1 (Bruker)

and analyzed by Sparky software [14]. All amino acid derivatives and peptide bond forming reagents were purchased from IRIS Biotech (Germany). All reagents were purchased from Sigma-Aldrich and solvents were purchased from POCh.

2.1.2. Biology

RPMI 1640 medium, Hanks' medium and Lymphocyte Separation Medium (LSM) were from Cytogen Sinn, Germany. MTT (93-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide), phytohemagglutinin A (PHA) and other reagents were from Sigma, USA. Fetal calf serum was from Gibco, USA. TNF-α was measured using ELISA kits manufactured by RD System, Minneapolis, USA & Canada. In separations of PBMC cells Heraeus Sepatech Varifuge 3.0R centrifuge was used. The MTT assays were performed with the use of Elisa reader, Opsys MR Dynex Technologies. Cells were incubated in New Brunswick™ Galaxy® 48 R incubator. Density of cells was determined with the use of Bürker hemocytometer cell counter.

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 the cesium salt procedure [15] and the substitution level was determined by weight gain measurements (Boc-Leu-Ⓟ, 0.62 mmol for peptides **1**-**8**). Synthesis of the 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 [16] or Chloranil test (proline residue) [17] was found positive. In all cases, where the 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 the concentration of solvents and addition of diethyl ether as a TFA*peptide salt.

Cyclisations of the peptides were achieved with the use of medical syringe pump equipped with two syringes A and B according to the idea presented in the reference [18] with some modifications. Syringe A contains solution of 60 mg of the linear peptide dissolved in 10ml of DMF, and syringe B contains solution with an equivalent amount of the HATU/HOAT in 10ml of DMF. Solutions from syringe A and B were injected with a speed 0.42 ml/h into a flask containing approximately 15 ml of DMF and 3 eq of DIPEA. Solution in the flask was stirred with a magnetic stirrer. After cyclisation the mixtures were analyzed by HPLC. DMF was evaporated under reduced pressure and crude peptides were purified by preparative HPLC.

All the peptides sent to biological trials were purified by the preparative HPLC and once again were examined by HPLC and additionally by MS techniques. The physicochemical properties of the synthesized linear and cyclic peptide are summarized in Table 1.

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	96	12.98[a]	97.20	99.64	$C_{61}H_{95}N_9O_{10}$	1113.72	1114.18	1136.14
2	88	12.76[a]	87.47	99.79	$C_{59}H_{91}N_9O_{10}$	1085.69	1086.41	1108.33
3	100	13.38[a]	95.78	97.70	$C_{59}H_{91}N_9O_{10}$	1085.69	1086.49	1108.42
4	94	13.54[a]	99.79	96.26	$C_{61}H_{95}N_9O_{10}$	1113.72	1114.54	1136.53
5	81	12.76[a]	94.56	99.11	$C_{61}H_{95}N_9O_{10}$	1113.72	1114.50	1136.54
6	70	12.96[a]	82.89	99.00	$C_{61}H_{95}N_9O_{10}$	1113.72	1114.76	1136.76
7	84	13.55[a]	88.37	100.00	$C_{59}H_{91}N_9O_{10}$	1085.69	-	1109.03
8	82[b]	12.94[a]	94.59	97.35	$C_{59}H_{91}N_9O_{10}$	1085.69	1086.88	1108.81
9	45[b]	8.23		100.00	$C_{61}H_{93}N_9O_9$	1095.71	1096.44	1118.40
10	40[b]	9.90		99.20	$C_{59}H_{89}N_9O_9$	1067.68	1068.58	1090.54
11	22[b]	10.38		100.00	$C_{59}H_{89}N_9O_9$	1067.68	1068.52	1090.51
12	19[b]	10.46		100.00	$C_{61}H_{93}N_9O_9$	1095.71	1096.64	1118.58

13	61[b]	9.12	96.20	$C_{61}H_{93}N_9O_9$	1095.71	1096.68	1118.59
14	35[b]	9.61	95.20	$C_{61}H_{93}N_9O_9$	1095.71	1097.02[*]	1118.98
15	24[b]	10.71	96.47	$C_{59}H_{89}N_9O_9$	1067.68	1068.61	1090.60
16	23[b]	10.61	100.00	$C_{59}H_{89}N_9O_9$	1067.68	1068.98	-

a) before HPLC purification; b) yield of cyclization

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

All NMR spectra (COSY, TOCSY, HSQC and ROESY) of synthesized peptides can be found in supplementary material file as well as ^{1}H NMR description assigned based on these spectra.

2.4. *Synthesis procedures*

(*4S*)- and (*4R*)-4-((*tert*-Butoxycarbonyl)amino-)-4-benzyl-butanoic acid **17** was obtained from L or D-Boc-phenylalanine in parallel synthesis according to known procedure [13] with some modifications. Boc-phenylalanine was transformed into a mixed anhydride by using ClCOOEt/TEA and reduced to Boc-phenylalaninol using $NaBH_4$ in THF. Boc-phenylalaninol was oxidized to Boc-phenylalaninal by using PCC in DCM in a presence of Å4 molecular sieves. Side products of oxidation were removed by crystallization with EtOAc/hexane mixture. The solution containing Boc-phenylalaninal after evaporation of the solvents was used in the next step reaction without additional purification. The product of oxidation was used in Horner-Wadsworth-Emmons reaction with triethyl phosphonoacetate and KOH as a base in a mixture of H_2O/1,4-dioxane (10/90%). Product (E)-4-[(tert-butoxycarbonyl)amine]-5-phenyl-2-pentenoic ethyl ester was then hydrogenated by using 10% Pd/C and hydrolyzed with 2N KOH in methanol giving derivative **17** with S or R configuration. The products were purified by crystallization from ethyl acetate/hexane to yield crystalline solids. The enantiomeric purity was determined according to the procedure using N_α-(2,4-dinitro-5-fluorophenyl)-L-valinamide as a derivatizing reagent [19]. Diastereomeric derivatives were detected at different retention times (min): 8.84 and 7.45 for **(*4R*)-17** and **(*4S*)-17**, respectively. Samples intended for crystallographic studies were recrystallized from pure acetonitrile. All spectra (^{1}H, ^{13}C, IR), as well as specific rotation and melting points of synthetized intermidiates and **(*4R*)-17** and **(*4S*)-17,** can be found in supplementary material file.

2.5. *Crystallographic studies*

Single crystals of the two enantiomers **(4*S*)-17** and **(4*R*)-17** were measured with Bruker SMART APEX II CCD diffractometer [20] at 100 K with Cu Kα radiation. Other details concerning the crystal data and refinement are available in supplementary material section (Table 14s,15s). The applied crystallographic programs were as follows: SAINT-Plus for cell refinement and data reduction [21], SADABS for multi-scan absorption correction [22], SHELXS97 for structure solution (direct methods) and refinement (full-matrix least-squares on F2) [23]. The absolute structures were verified with Flack parameter [24] and the material for publication was made with PLATON [25]. The statistical X-ray analysis was performed with the use of CCDS conquest software, version 1.18 using Cambridge Structural Database [39].

In order to confirm representativeness of the studied single crystals, the powder X-ray diffraction of **(4*S*)-17** and **(4*R*)-17** was performed using a PANalytical X'Pert Pro MPD diffractometer in Bragg-Brentano reflecting geometry and CuK_{α} radiation at room temperature (Fig. 115s). Due to the very small amount of enantiomeric samples available, the examination was done with a Silicon single crystal substrate. The data were collected in the range 2-50° 2Θ with step of 0.0167° and exposition per one step of 50 s.

2.6. *Biological assays*

2.6.1. *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. As a control, appropriate dilutions of DMSO in RPMI-1640 medium were used.

2.6.2. *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 LSM and centrifuged at 800 × g for 20 min at 4 °C. The interphase cells were then washed three times with Hanks' medium and re-suspended in a culture medium, consisting of RPMI-1640, supplemented with 10%

fetal calf serum, L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics (referred to below as the culture medium), at density of 2×10^6 cells/mL.

2.6.3. *Phytohemagglutinin A (PHA)-induced proliferation of human peripheral 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 concentrations of 1, 10 and 100 µg/mL. DMSO at appropriate dilutions served as control. After fourth-day incubation in a cell culture incubator, the proliferative response of the cells was determined by the colorimetric MTT method [26]. The data are presented as a mean optical density (OD) value from quadruplicate wells ± standard error (SE).

2.6.4. *Effects of the compounds on viability of human blood mononuclear cells*

PBMC at density of 3×10^5/100 µL/well, re-suspended in the culture medium, were cultured for 24h 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.

2.6.5. *Lipopolysaccharide (LPS)-induced tumor necrosis factor alpha (TNF-α) production in whole blood cell culture*

The test was performed as described elsewhere [27]. 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. The cultures were stimulated by addition of 1 µg/mL of LPS from *E. coli*, serotype O:111:B4. The compounds were added to the cultures at concentrations of 1 and 10 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 (representing 100% cytokine production). After overnight incubation in a cell culture incubator, the supernatants were harvested and frozen at -20 °C until cytokine determination by immunoassay.

2.6.6. Cultures of Jurkat cells and total RNA isolation

Jurkat cells (10^6/mL) in the culture medium were cultured overnight with the compound 15 (10 µg/mL). Total RNA isolation was carried with TRIzol Reagent (Ambion) accordingly to manufacturer's recommendations. The cell pellet (2×10^6 cells) was suspended in 1 mL of

TRIzol Reagent, shaken, incubated for 10 min at room temperature (RT), supplemented with 0.2 mL of chloroform, shaken vigorously for 15 sec, incubated for 3 min at RT and centrifuged at 12000 × g for 15 min at 4 ^{o}C. The water phase was collected, transferred to a new tube, supplemented with 0.5 mL of isopropanol, incubated at RT for 10 min and centrifuged at 12000 × g for 10 min at 4 ^{o}C. The RNA pellet was washed with 1 mL of 75% ethanol, dried in air and dissolved in 20-30 µL of sterile diethyl pyrocarbonate-treated Mili-Q water. RNA samples were stored at -20 ^{o}C.

2.6.7. Reverse transcription

Single stranded complementary DNA (cDNA) was synthesized with oligo (dT)12-18 primers from 5 µg of total RNA using Novazym VerteKit, accordingly to the manufacturer's instruction.

2.6.8. Quantitation of gene expression by Real Time PCR

Expression of the genes, i.e. β-actin, caspase-3, caspase-8 and caspase-9, was measured using APA SYBR FAST qPCR Kit. The sequences of primers are enclosed in the supplementary data. The reaction was performed in Applied Biosystems ViiA7 thermocycler starting with 5 min of preincubation at 95 ^{o}C followed by 35 amplification cycles as follows: 95 ^{o}C for 30 sec and simultaneous annealing-extension-data acquisition for 45 sec and 60 ^{o}C. Beta-actin was used as a housekeeping gene for arbitrary unit calculation for every tested gen.

2.6.9. Analysis of DNA fragmentation

Approximately 2×10^{6} cells were lysed overnight in 37 ^{o}C, in 500 µL 50 mM Tris-HCl buffer pH 8.5 containing 10 mM EDTA, 5% SDS and 500 µg/mL proteinase K with occasional shaking. Proteinase K was deactivated by 30 min heating in 80 ^{o}C, than samples were cooled on ice, shortly centrifuged, supplemented with RNase (50 µg/mL) and incubated for 1h at 37 ^{o}C. DNA was precipitated with 750 µL of isopropanol, and after centrifugation at 12 000×g at 4 ^{o}C for 15 min, washed with 75% ethanol, and dissolved in water. The cleavage of DNA fragmentation was analyzed by electrophoresis fractionation in 2% agarose gel.

3. Results and discussion

3.1. *Chemistry*

All analogues were synthesized using Boc-γ^4-hhPhe-OH. The resin was exactly the same as in the reference [12] to give the readers possibility to compare the yield of the synthesis. This amino acid was incorporated into 3 or 4 and both 3 and 4 position of CLA. Linear precursors of CLA **1-8** were synthesized according to SPPS procedure based on Boc amine protecting group. Each analogue was synthesized at scale 0.2 mmol. The removal of the Boc amino protecting group was achieved using 50% TFA in DCM through 15 min. In the coupling reactions TBTU was used as a coupling reagent, DIPEA as an amine and HOBt as an antiracemic additive. After each step (coupling reaction, acylation of unreacted amine groups, deprotection of amine) a resin was washed three times with DCM, MeOH and DCM each. In the coupling reactions standard Boc-protected amino acids were used in 3 eq**,** and Boc-γ^4-hhPhe-OH was used in 1.5 eq to the resin. 1 mL of TFMSA, 10 mL of TFA and 0.5 mL of anisol were used for 30 min to cleavage the peptide from the resin. After filtration, the obtained solution was concentrated under vacuum and peptide was precipitated by diethyl ether addition. In the cyclization reaction, to promote high dilution of reacting peptides, a syringe pump was used. The reaction of cyclization took place through 24h with yield from 19% for analogue **12** to 61% for analogue **13**. Prior to biological assays all peptides were purified by preparative HPLC.

3.2. *NMR measurements*

^{1}H NMR spectra of new analogues **9-16** recorded in DMSO-d_6 at 300 K had better resolution in comparison with CLA, which probably correlated with its lower exchange rate between conformers in the NMR scale [28]. However, by the subtraction of COSY spectra from ROESY spectra we observed large number of long-range correlations in comparison to CLA. That fact confirmed us that the structures of new analogues are more rigid in comparison to CLA molecule (please see supporting material file).

The NH resonances were located within the narrow range of 0.5-0.8 ppm, as compared to the range of 0.9, 1.0 and 1.4 ppm for peptides **10**, **11a** and **16**. The resolution of most of the NH signals enabled to determine the NH-CHα coupling constants.

In order to determine the geometries of peptide bonds for CLA analogues **9-16** the ROESY spectra were recorded. In the fragment Pro-Pro, the geometry was also confirmed by ^{13}C chemical shifts between C^β and C^γ in prolines. According to the literature the differences of about 9 ppm are characteristic for *trans* Xxx-Pro, whereas about 5 ppm are characteristic for *cis* Xxx-Pro geometries [29]. In DMSO solution**,** with the use of NMR spectroscopy, it was found that all cyclic peptides, except peptides **11**, **13** and **15**, existed as single isomers

with one *cis* peptide bond in the -Pro-Pro- fragment. In case of peptides **11**, **13** and **15** the content of *cis*/*trans* isomers was 50/50, 70/30 and 70/30, respectively.

In almost all cyclic analogues the chemical shifts of the γ protons of Pro^2 were very similar. The differences were found in analogues **14**, **15a** and **16** modified with ***R*-γ^4-hhPhe** or **12** containing fragment of ***R*-γ^4-hhPhe^3-*S*-γ^4-hhPhe^4**. In case of these peptides one of γ protons exhibited a high-field shift up to 0.86 ppm (**12**), 0.87 (**11a**), 0.95 (**14**), 1.02 ppm (**15a**) and 0.69 ppm (**16**). Notably, up-field shift was not as large as in native CLA (Pro^2 γ proton observed at 0.33 ppm), but still indicated a proximity of the Phe^3 aromatic rings and Pro^2 side chains in these peptides [30].

The signals of aromatic rings of both phenylalanine residues were located in a narrow range for cyclopeptides **11a**, **12**, **14**, **15a**, **16,** but for analogues **9**, **10**, **11b**, **13**, **15b** a wide range was observed, allowing a possibility for arrangement of the phenyl ring to the edge-to-face geometry. The observed arrangement of phenyl rings for these analogues corresponded to the geometry of native CLA [28, 31, 32] or analogues modified with tyrosine [33] with characteristic pattern of the aromatic range (Fig. 52s, 53s) containing one signal shift to high-field (lower ppm unit).

Vicinal coupling constants $^3J_{NHC\alpha H}$ were determined directly from ^{1}H NMR or from COSY-DQF spectra (peptide **11** and **15**) and are summarized in Table 2. Almost all $^3J_{NHC\alpha H}$ vicinal couplings constants were about 8 Hz. For two analogues **11** and **15** vicinal coupling constants $^3J_{NHC\alpha H}$ were about 10~12 Hz suggesting an almost perpendicular arrangement [34].

Table 2. Vicinal coupling constants $^3J_{NHC\alpha H}$ [Hz] of peptides **9-16** in DMSO-d_6 at 300 K and 348 K.

Peptide	Phe^3 or γ^4-hhPhe^3	Phe^4 or γ^4-hhPhe^4	Leu^5	Ile^6	Ile^7	Leu^8	Val^9
9	8.4	8.8	8.4	9.3	8.8	8.4	8.4
10	9.3	7.9	7.9	7.9	7.7	6.6	9.3
11a	8.9	8.4	8.3	9.2	14.2	10.7	12.2
11b	11.6*	8.8*	10.6*	9.6*	8.2*	7.7*	13.7*
12	8.2	8.8	8.7	9.0	8.2	8.7	8.4

13a	8.8	8.1	7.8	8.0	8.1	7.1	8.7
14	8.6	8.2	8.2	8.4	7.8	8.2	8.0
15a	8.3*	10.0*	10.0*	9.7*	10.3*	9.1*	10.8*
15b	7.3	8.6	-	8.8	7.7	6.5	8.0
16	8.4	8.0	7.8	8.4	8.6	7.6	-

* vicinal coupling constants 3*J*NHCαH [Hz] of peptides determined from COSY-DQF spectra.

The presence of intramolecular hydrogen bonds was determined by measuring the NH's temperature coefficients (Δδ/ΔT) (Table 3).

Table 3. Temperature dependence of the NH chemical shifts (-Δδ/ΔT, ppb/K) of peptides **9-16** in DMSO-d_6, in the range of 300-340 K.

Peptide	Phe[3] or γ[4]-hhPhe[3]	Phe[4] or γ[4]-hhPhe[4]	Leu[5]	Ile[6]	Ile[7]	Leu[8]	Val[9]	Solvent	Ref.
CLA	-2.8	-6.2	0.9	-4.1	-4.7	-4.7	-2.8	DMSO	[37]
CLA	-6.6	-5.4	1.2	-6.8	-2.5	-4.2	-1.8	DMSO/$CDCl_3$	[35]
CLA	a	0.8	b	2.8	1.6	2.0	0.9	$CDCl_3$	[28]
9	1.6	6.0	5.4	1.8	4.9	3.8	3.9	DMSO	
10	2.1	6.7	0.1	5.3	0.4	5.0	3.0	DMSO	
12	3.4	5.5	3.0	3.5	1.9	2.3	4.4	DMSO	
13a	5.1	2.4	4.3	2.8	3.7	3.7	4.0	DMSO	
14	3.1	4.9	5.7	2.7	2.0	2.5	4.7	DMSO	
15a	4.8	5.7	0.3	3.1	5.0	3.1	0.2	DMSO	
16	4.8	4.8	4.7	3.1	3.9	3.2	-	DMSO	

The temperature dependence of the NH chemical shifts for cyclopeptides **11, 13b** and **15b** were unable to determine and is omitted. a - non-linear, b - absent

According to literature, the coefficient ($\Delta\delta/\Delta T$ < 3 ppb/K) in DMSO is characteristic for the presence of intramolecular hydrogen bonds [36]. For the natural CLA molecule, the intramolecular hydrogen bonds involve NH's of Phe^4, Ile^7, Leu^8, and Val^9 [28], whereas when dissolved in DMSO**,** the NH's of Phe^3, Leu^5 and Val^9 were engaged in intramolecular hydrogen bonds [37]. In the case of the CLA analogues modified with γ^4-hhPhe (analyses in DMSO) different types of controllers determining their stability were found. Analogue **10** contained four hydrogen bonds covering *S*-γ^4-hhPhe^3, Leu^5, Ile^7, Val^9 residues, for peptide **14** three hydrogen bonds were observed (Ile^6, Ile^7, Leu^8), for peptides **9**, **12**, **13**, **15** in stabilization two hydrogen bonds were involved, including NH protons from (*S*-γ^4-hhPhe^3, Ile^6), (Ile^7, Leu^8), (Phe^4, Ile^6) and (Leu^5, Val^9) respectively. For peptide **16**, it was not possible to determine temperature dependence of the NH of Val^9 residue, what suggests that it is the least stabilized analogue.

The replacement of one or both Phe residues by incorporation of γ^4-hhPhe residue(s) changed the network of stabilizing hydrogen bonds relative to native CLA. This relation was observed for all analyzed analogues. Only peptide **14** contained the same number of stabilizing hydrogen bonds as native CLA in DMSO, but they interacted with different NH's residues, i.e. Ile^6, Ile^7, Leu^8. Peptide **10** had the same pattern of hydrogen bond as native CLA, but additional, Ile7 residue was involved in the structure's stabilization.

All cyclic peptides, except **11**, **13** and **15**, existed as isomers with the *cis* configuration peptide bond between the Pro-Pro motif. Peptide **11,** containing only one residue *S*-γ^4-hhPhe^3**,** existed as a mixture of equal amount of *cis* and *trans* geometry isomers. In case of two peptides, **13** modified with *S*-γ^4-hhPhe^3-*R*-γ^4-hhPhe^4 and **15** contained single *R*-γ^4-hhPhe^3 residue, two conformers with major *cis* isomer in 70% of population were observed.

3.3. *Crystallography*

Resolution of the crystal structures of the Boc-γ^4-hhPhe-OH derivatives **(4*R*)-17** and **(4*S*)-17** allowed to determine their absolute configurations and their conformations (Fig. 2). Torsion angles listed in Table 4 showed the presence of *trans* geometry of the amide bond for both enantiomers and very similar conformations of their main chains. The orientation of the carboxyl group was defined by respective torsion angle t1. The differences were related to different hydrogen bond networks having an impact on different packing of **(4*R*)-17** and **(4*S*)-17** in crystal state (Fig. 3). In Table 4 we compared torsion angles of the known γ-amino acids obtained from X-ray analysis. The restricted number of observed values of torsion angles, also

in other structures than **(4*R*)-17** and **(4*S*)-17**, suggested a rather rigid conformation of the fragment, despite the cis/trans isomerism. In the known crystals of γ-amino acids presented in Table 4 only intermolecular hydrogen bonds were observed as well as in **(4*R*)-17** and **(4*S*)-17**.

The enantiomeric Boc-γ^4-hhPhe-OH derivatives **17** were characterized by inversed polymorphs. This observation was confirmed by different melting points of derivatives, 108-110 °C for **(4*S*)-17** enantiomer and 122-124 °C for the **(4*R*)-17** enantiomer. Literature values were reported for **(4*R*)-17** crystals, 132-133 °C by Loukas *et al.* [13] and 114 °C by Hanessian & Yang [38]. They all might be still other polymorphs of **17**, because the crystallization conditions in these three cases were different. However, the differences in crystal state, to some degree, could result in the presence of other factors, such as small amounts of impurities indicated by powder diffraction profile of **(4*R*)-17** (Fig. 115s).

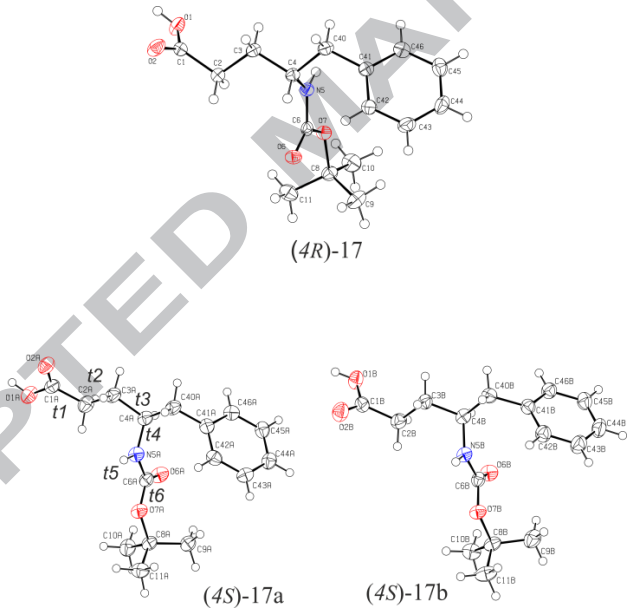


Fig. 2. The molecular structures of Boc-γ^4-hhPhe-OH **17** in their enantiomeric crystals.

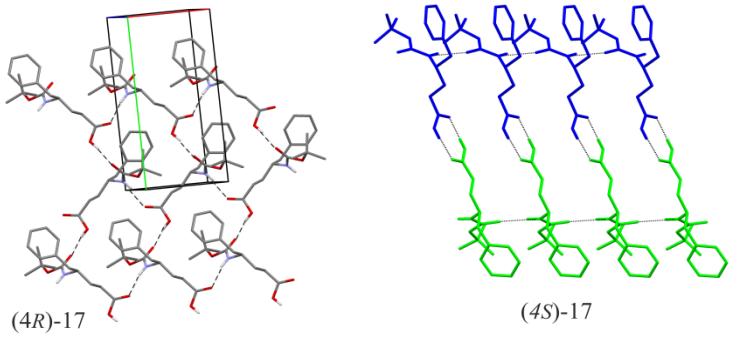


Fig. 3. Packing of the molecules in the enantiomeric crystals of the **17** amino acid.

Table 4. Conformation of the Boc-N-protected γ-amino acids in the crystal state (this study and CSD [39]). The definitions of the torsion angles are as follows: HO-C1-C2-C3 (t1), C1-C2-C3-C4 (t2), C2-C3-C4-N5 (t3), C3-C4-N5-C6 (t4), C4-N5-C6-O7 (t5) and N5-C6-O7-C8 (t6). The values of the torsion angles are in degrees and rounded. The compounds comprising substituents participating in strong hydrogen bonds or rigid systems incorporated into their main chain were omitted in the analysis. All structures were enantiomeric.

Structure/ REFCODE	Substituents	*t1*	*t2*	*t3*	*t4* *(a)*	*t5* *cis/ trans*	*t6*	HB type *(b)*	Literature
(4*S*)-17, Z'=2	4-Bz	94	-176	-59	127	176	-179	aa(A)+aa(B)	
		-179	173	-60	131	170	-177	cc(AB)	
(4*R*)-17	4-Bz	-86	-176	-62	111	177	177	ac(en)	
18 HOMYOZ	3-Bz	-174	-168	71	90	-14	177	ac(en)	[40]
19 NIFPOH	2-Me; 4-Me	119	-71	-69	122	3	-169	ac(en)	[41]

Notations: (a) The torsion angles were unified by making t4 positive; (b) Hydrogen bond network notation (aa, cc and ac) refers respectively to amide-amide (-N-H···O=C-NH···), carboxyl-carboxyl (-C-OH···O=C-OH···), amide-carboxyl (-N-H···O=C-OH···) hydrogen bonds. In the case of enantiomeric structure 1 (Z'=2), A and B are used to distinguish the symmetrically independent molecules.

The observed torsion angles (Table 4) indicated that the conformation of N-protected γ-amino acids is rather conservative, despite the presence of a very flexible fragment comprising three single bonds which separate amine and carboxylic groups. Thus, it seemed interesting to compare the conformational predisposition of γ-amino acids with α-amino acids in the peptides according to available X-ray structures. Some light on the subject gives a comparison of preferred orientations of the carbonyl groups participating in the hydrogen bonds in the α-peptides with peptides containing γ-amino acids [39] (Fig. 4).

The distribution of θ angles (Fig. 116s) between C-O vectors representing two neighbouring carbonyl groups (being H acceptors) in α-peptides was significantly different from peptides comprising γ-amino acids (Fig. 4a,b). The range of preferable values for peptides derivatives of γ-amino acids was extremely narrow and the average value is much higher (Fig. 4b). For 2230 appropriate fragments found in Crystallographic Structural Data [39] for peptides built from α-amino acids (excluding glycine), the 56% of the θ angles were

in the range of 20-90° (Fig. 4a). For this range the average angle was equal to 39°. In the case of peptides built from γ-residue (Fig. 4b) the 88% (136 examples) of the θ angles were in the narrow range of 40-60°, and the average value was equal to 49° [39]. Additionally, much smaller maximum of θ values of γ-peptides was observed for antiparallel orientation of carbonyl groups (Fig. 4b).

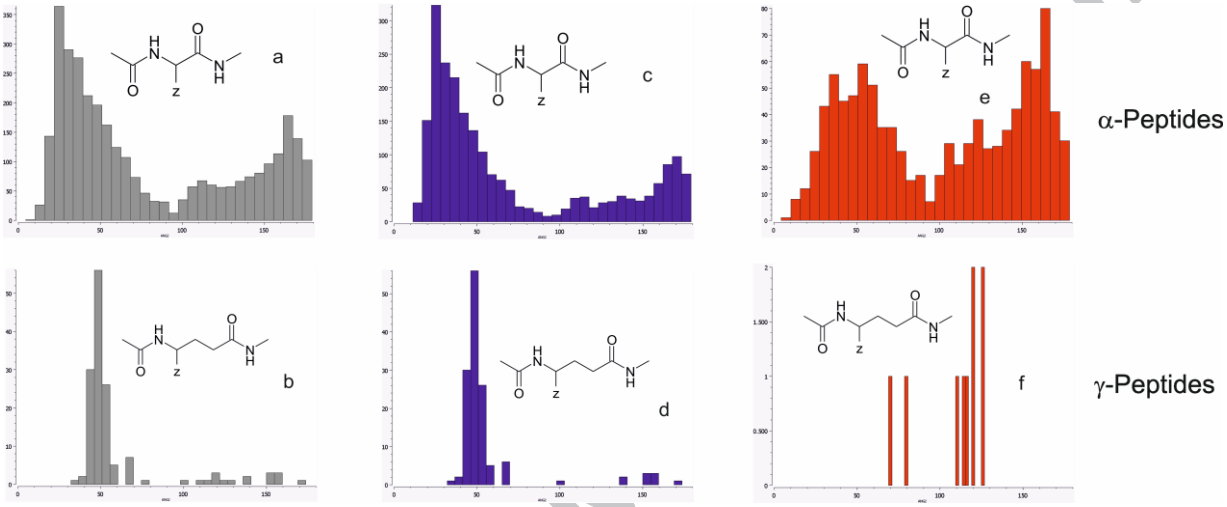


Fig. 4. Distribution of θ angles for structures deposited in the CSD [α-peptides – a (linear and cyclic peptides), c (linear peptides), e (cyclic peptides); γ-peptides – b (linear and cyclic peptides), d (linear peptides), f (cyclic peptides)]. In each graph X axis represents θ angle (range 0^o – left, 180^o –right) and Y axis represents a number of the analysed X-ray structures.

However, comparison of linear and cyclic peptides showed more clearly the differences in distribution of θ angles (Fig. 4c-f). In the case of α-amino acids, creating linear peptides, the θ angles were predominantly in the range of 20-80° (Fig. 4c), whereas in cyclic α-peptides the orientation was more balanced between antiparallel and parallel orientation (Fig. 4e). Even more dramatic change in distribution of θ angles was visible for the γ-peptides (Fig. 4d,f). In rare cyclic peptide examples all the θ angles were over 70°, most of them over 110° (Fig. 4f). The difference in θ values between peptides containing γ- and α-amino acids could be one of the factors causing different PHA-induced PBMC proliferation between new linear and cyclic analogues of CLA reported in this work.

3.4. *Biology*

3.4.1. *Effects of the compounds on viability of PBMC.*

The effects of the linear and cyclic compounds on the cell viability are presented in (Fig. 5a and 5b). The compounds were tested at concentration of 1, 10 and 100 μg/mL in a 24h culture of PBMC and the cell viability was determined by the MTT colorimetric method.

Fig. 5A.

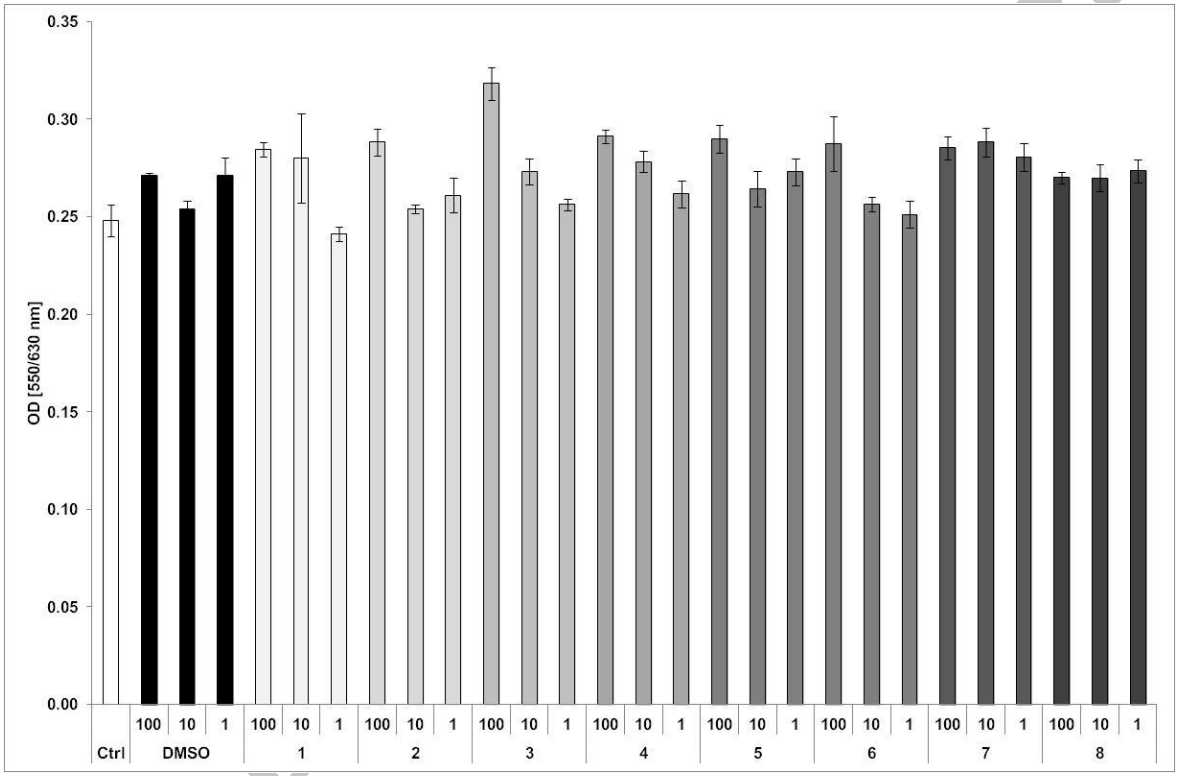


Fig. 5B.

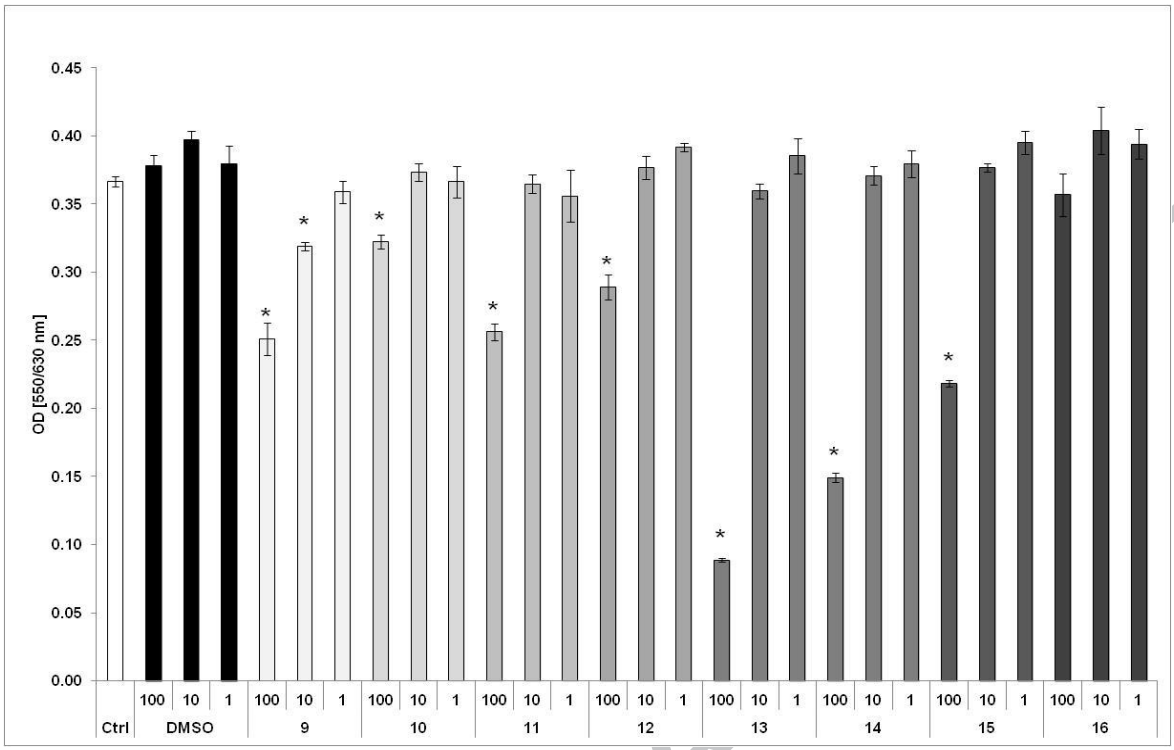


Fig. 5. The effects of linear peptides **1** – **8** (A) and cyclic peptides **9** – **16** (B) on the viability of human peripheral blood mononuclear cells. The peptides were used at concentrations of 1, 10 and 100 μg/mL. The viability of cells upon culture with the compounds was compared to the effects of DMSO used as solvent (filled bars). The open bar represents PHA-stimulated cultures without DMSO addition.

The linear peptides (Fig. 5a) did not lower cell viability at the studied concentrations but the cyclic ones showed some inhibitory effects on the cell survival at 100 μg/mL, in particular compounds **9, 10** and **11** (Fig. 5b).

3.4.2. Effects of the compounds on PHA-induced PBMC proliferation

Fig. 6A.

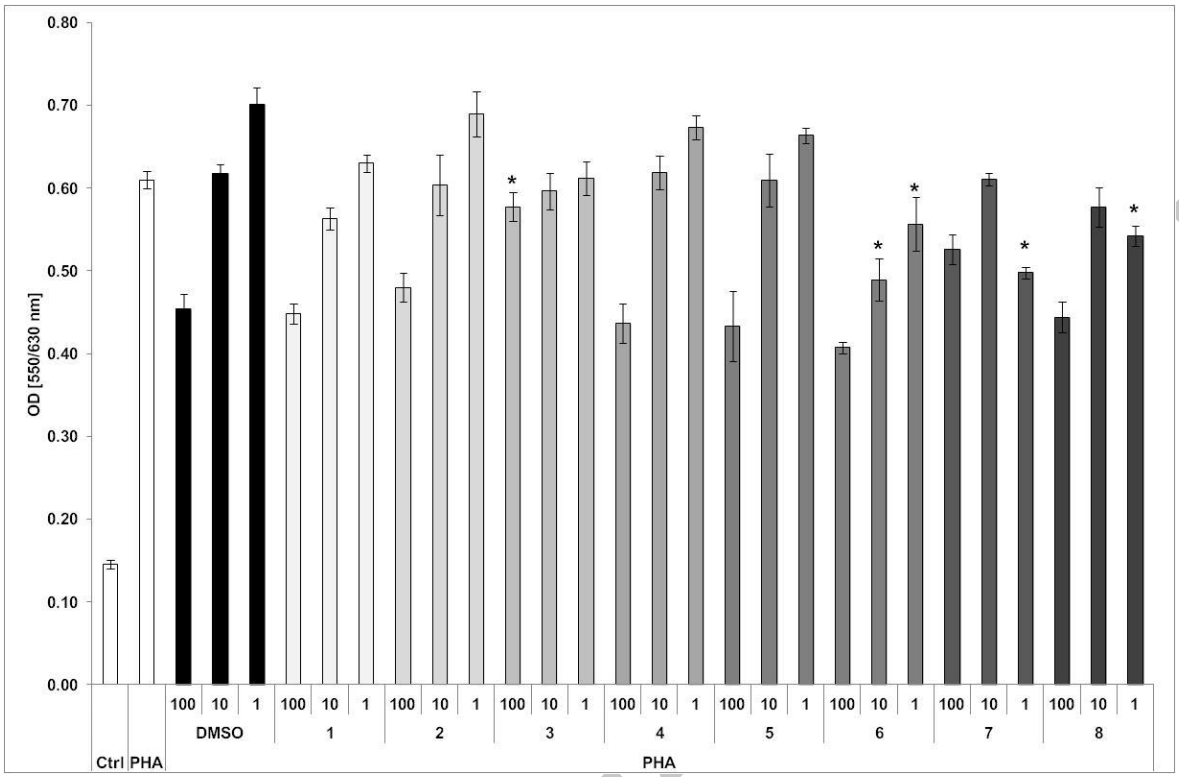


Fig. 6B.

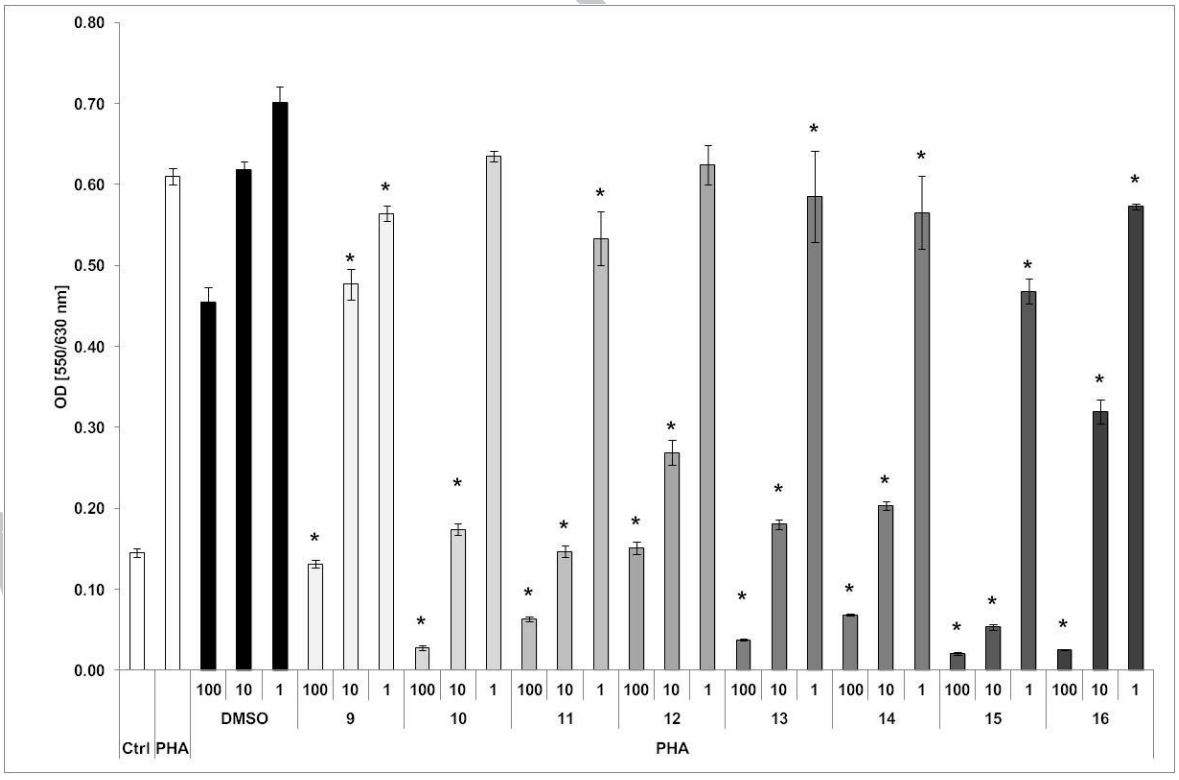


Fig. 6. Effects of linear peptides **1** – **8** (A) and cyclic peptides **9** – **16** (B) on PHA-induced PBMC proliferation. The peptides were used at concentrations of 1, 10 and 100 μg/mL. The viability of cells upon culture with the compounds was compared to the effects of DMSO

used as solvent (filled bars). The open bar represents PHA-stimulated cultures without DMSO addition. "Ctrl" – no PHA added.

The effects of the compounds on PHA-induced proliferation of human PBMC in the concentration range 1-100 μg/mL are presented in Fig 6ab. The linear peptides (Fig. 6a) were not suppressive, whereas the cyclic peptides (Fig. 6b) showed differential, dose–dependent ability to inhibit the mitogen-induced cell proliferation. Considerable inhibitory effects were already observed at concentration of 10 μg/mL for almost all cyclic peptides. Compound **15** was the most suppressive taking into account its best effects at all tested concentrations in comparison to other compounds.

3.4.3. Effects of the compounds on LPS-induced TNF-α production in whole blood cultures

The effects of the peptides on LPS-induced TNF-α production in whole blood cell cultures are shown in (Fig. 7a and 7b**)**. The linear peptides exhibited about 45% inhibitory effects on the cytokine production at 10 μg/mL concentration with exception of peptide **2**. The cyclic compounds also inhibited TNF-α production at 10 μg/mL. In addition, peptide **14** was strongly inhibitory at both concentrations and peptide **13** also at 10 μg/mL. The inhibitory effects of the peptides on TNF-α production correlate well with their antiproliferative actions since TNF-α augments mitogen-induced PBMC proliferation [42].

Fig. 7A.

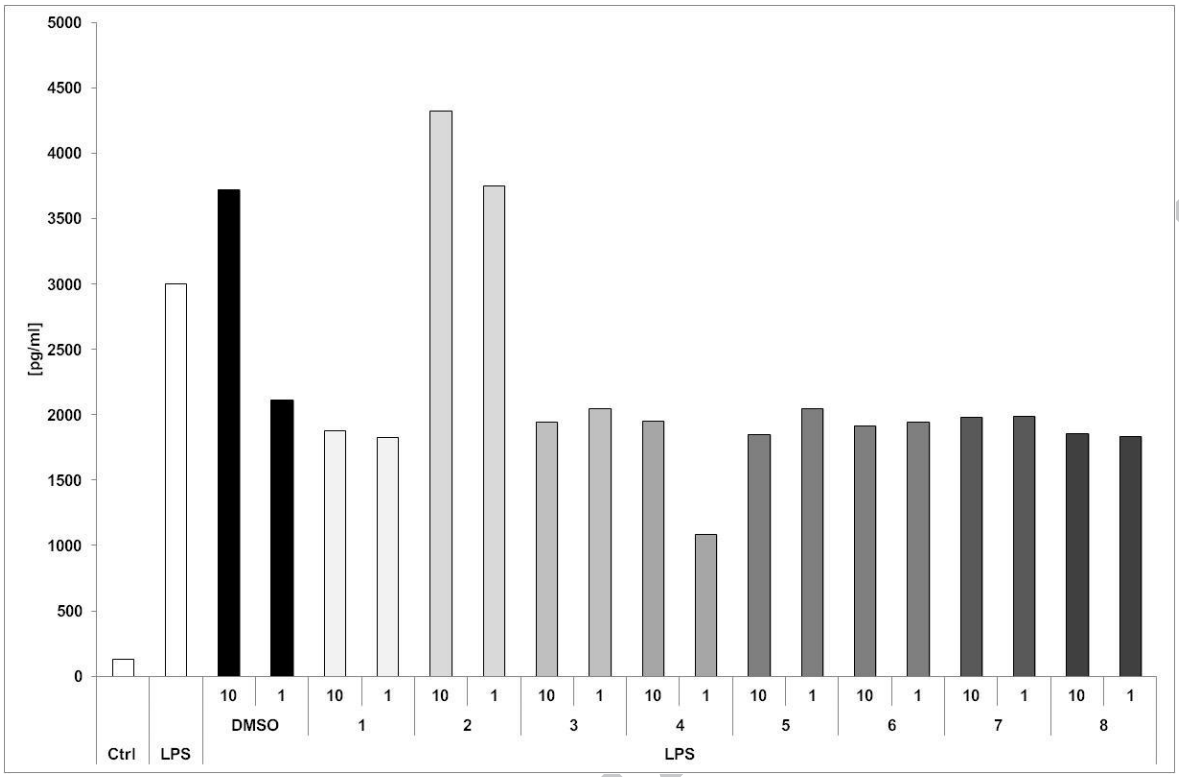


Fig. 7B.

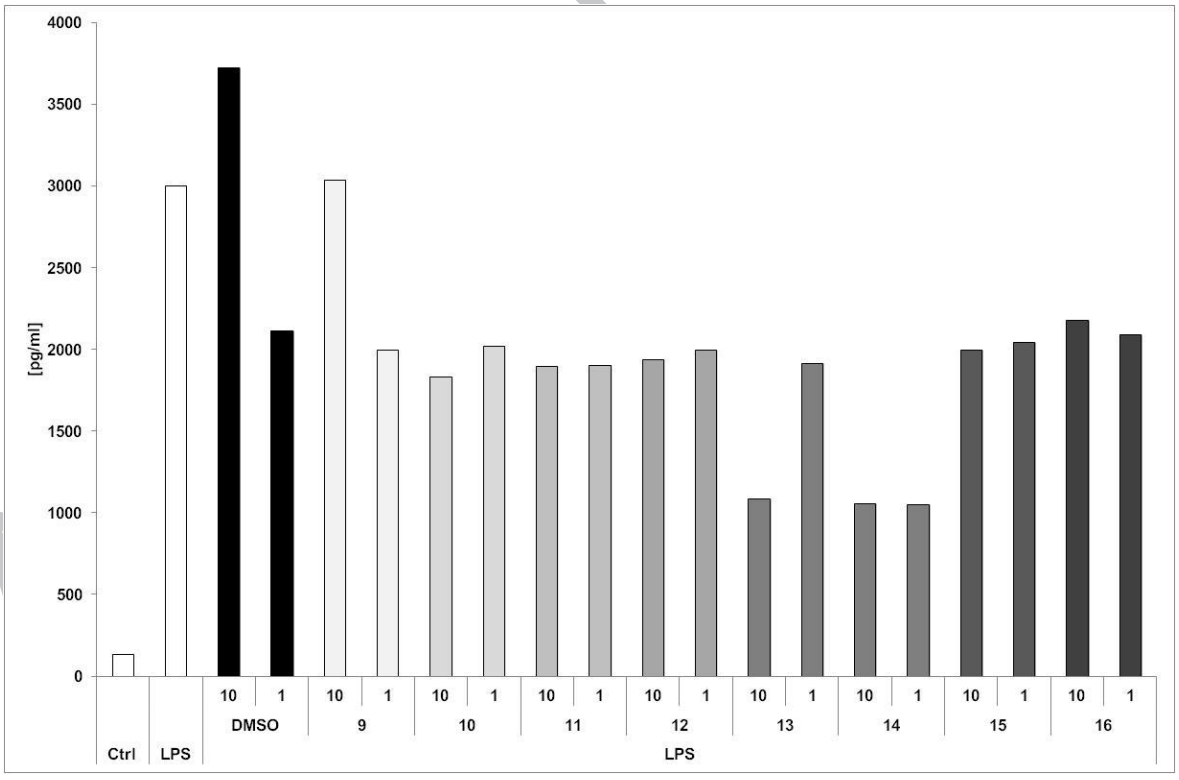


Fig. 7. Effects of linear peptides **1** – **8** (A) and cyclic peptides **9** – 1**6** (B) on LPS-induced TNF-α production in human whole blood cultures. The peptides were used at concentrations

of 1 and 10 μg/mL. The filled bars represent solvent (DMSO) controls. The oper bar ("LPS") – the cultures with addition of LPS only. "Ctrl" – no LPS added.

3.4.4. Description of structure-activity relationships in the previously investigated CLA analogues

In an effort to increase the suppressive potency of CLA, natural, modified or not physiologic amino acids were substituted outside or within the "active" Pro-Pro-Phe-Phe sequence of the cyclolinopeptide. The actions of such peptides were compared with native CLA and/or CsA. Three tyrosine analogues were synthesized by replacement of phenylalanine in the -Pro-Pro-Phe-Phe- fragment of the CLA: **IV** -Pro-Pro-Phe-Tyr-, **V** -Pro-Pro-Tyr-Phe- and **VI** -Pro-Pro-Tyr-Tyr- [43]. The analogues were tested for their effects on the inductive phase of the delayed type hypersensitivity (DTH) in mice. It appeared that compounds **IV** and **V** were weaker but peptide **VI** was a stronger inhibitor than the parent CLA peptide. It seems, therefore, that the replacement of both phenylalanines with tyrosines may convert the parent cyclopeptide into a more potent inhibitor. In this model native CLA displayed a stronger immunosuppressive action than CsA. In the same experimental model [44] several CLA analogues were tested with substitution of threonine in the -Leu-Ile-Ile-Leu-Val- fragment. The analogues with the following sequences: c(Thr-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe), c(Leu-Ile-Thr-Leu-Val-Pro-Pro-Phe-Phe) and c(Leu-Ile-Ile-Leu-Thr-Pro-Pro-Phe-Phe) displayed stronger immunosuppressive activities than CsA in the DTH model. In another attempt CLA was modified with (S)-β^2-iso-proline or with (S)-β^3-homoproline [10]. In the mouse models of the proliferative response of lymphocytes to mitogens and in the humoral, secondary immune response *in vitro*, a peptide bearing the Pro^1-β^3hPro^2 fragment exhibited a strongest immunosuppressive action. In another approach, [8] the authors found that a CLA analogue containg Phe^3-β^3Phe^4 fragment appeared to be the most effective (better than CsA) in inhibition of the inductive phase of DTH to ovalbumin. A comparison of three CLA, analogues bearing: $Hphe^3$-Phe^4, Phe^3-$Hphe^4$ and $Hphe^3$-$Hphe^4$ substitutions, showed that the first analogue exhibited the strongest inhibitory activities in the models of mitogen-induced splenocyte proliferation and secondary humoral immune response *in vitro* [9]. Lastly, two cyclic analogues of CLA were synthesized, containing conformationally restricted dipeptide fragment Phe-Phe or d-Phe-d-Phe with ethylene brigde -CH_2-CH_2- between phenylalanine nitrogens [11] of the following sequences: -Pro^3-Pro^4-Phe-Phe- and -Pro^3-Pro^4-D-Phe-D-Phe-. The peptides were tested in the model of the secondary humoral immune response suppressed

by methotrexate (MTX). It was found that although native CLA augmented the suppressive action of MTX, the modified peptides counteracted the action of MTX, in particular the second one. Taken into consideration the structure-activity relationships and suppressive potencies of the hitherto described CLA analogues, out of the herein presented peptides, **15** appears to be most promising, even we had examined different biological assays. Compound **15**, c(Pro^{1}-Pro^{2}-***R***-**γ^{4}-hhPhe^{3}**-Phe^{4}-Leu^{5}-Ile^{6}-Ile^{7}-Leu^{8}-Val^{9}), showed the strongest effect in the model of the PHA-induced PMBC proliferation, where a complete inhibition of proliferation was achieved at 10 μg/mL and some, statistically significant inhibition at 1μg/mL concentration. Such a conclusion may be drawn based on our thorough analysis on the results cited above and generated exclusively in the Institute of Immunology, Wrocław, in collaboration with biochemists from other laboratories.

All new analogues had less impact on viability of PBMC than analogues modified with γ^{3}-bis(homo-phenylalanine) [12] which is associated with the relocation of the benzyl group. In the case of γ^{4}-bis(homo-phenylalanine) analogues, the fragment -Pro^{2}-γ^{4}-hhPhe^{3}- or -Pro-Phe-γ^{4}-hhPhe^{3}- is very similar to the native CLA. That similarity gives a possibility to the edge-to-face arrangement. In the case of the most active peptide **15** we reported *cis*/*trans* isomerism together with the edge-to-face arrangement of phenyl rings and additional more rigid structure in comparison to CLA. The most important feature of the new incorporated modifications and in the previous one [12] is that the modification is incorporated into the peptide backbone. That induces large conformational changes in comparison to the side chain modifications and gives new conformations by relocation of stabilizing hydrogen bonds.
In summary, the above described modifications of the native CLA sequence indicate that some substitutions may lead to synthesis of new analogues of a better immunosuppressive potency, and that the naturally occurring -Pro-Pro-Phe-Phe- sequence, regarded as essential for the inhibitory property of native CLA, may be modified to obtain stronger immunosuppressive effects. In addition, CLA analogues of unexpected immunorestoring activities may be also obtained.

3.4.5. Molecular studies

The hitherto conducted studies on a plausible mechanism of action of cyclic peptides have not provided satisfactory explanation on this matter. As induction of apoptosis in activated T cells seemed to be very likely, we exposed Jurkat cells for 24h to **15** peptide at concentration of 10

μg/mL (at this concentration the viability of PBMC was not affected). DMSO (the solvent) was used in control culture. RNA and DNA were isolated from the cell pellets to determine expression of relevant caspases by RT-PCR and DNA fragmentation in gel electrophoresis. The results shown in Table 5 revealed a block of caspase-3 expression and diminution of caspase-8 and -9 expressions. The demonstration of inhibition of caspase expression in Jurkat cells by **15** allows to explain its suppressive effect in PHA-induced proliferation of PBMC, since caspases are essential for IL-2 release upon T cell activation [45]. In addition, T-cell receptor-induced NF-κB activation and subsequent IL-2 production are also dependent on caspase cascade [46]. Inhibition of caspases may also result in an alternative cell death pathway termed necroptosis [47]. Such a process is not excluded in the case of compound **15** action since DNA fragmentation was observed in Jurkat cells (Fig. 114s). The fragmentation of DNA in Jurkat cells was described also for another bacteria-derived peptide – nicin [48]. Several compounds are known to interfere with the TNF-α production and several mechanisms of action was have been considered [47, 49]. Thus, the inhibition of caspase expression, possibly associated with an inhibition of NF-kappaB activity, could also explain the inhibition of the TNF-α production by peptide **15**.

Table 5. Effects of peptide **15** on expression of caspases in 24h culture of Jurkat cells.

Cell line	Protein mRNA expression	DMSO	15
Jurkat	β-actin	1	1
	caspase-3	0.686	0
	caspase-8	0.895	0.591
	caspase-9	0.829	0.538

3.4.6. Additional biological studies

For additional studies we chose the most potent cyclic peptide **15** and the native CLA molecule. We revealed that the peptide did not inhibit the activity of cyclooxygenases (supplementary data, Table 12s.). On the other hand (supplementary data Fig. 112s. and 113s) compound **15** did not inhibit growth of HT-29 colon cell line and A-431 epidermal tumor cell line at a concentration of 25 μg/mL. CLA, the parent nonapeptide, was still significantly suppressive in the models at the concentrations of 12 μg/mL and 6 μg/mL, respectively. We

hope that these additional biological studies would be helpful information for future applied studies with CLA or CLA analogues.

4. Conclusions

Incorporation of one or two γ^4-hhPhe residues into the peptide chains of CLA changes the length of their backbone by two or four methylene group. This modification dramatically changes the distribution of intramolecular hydrogen bonds stabilizing conformation of the obtained derivatives. For three peptides, **11, 13** and **15**, the inappropriate resolution in ^{1}H NMR spectra was correlated with *cis*/*trans* isomerism between the two prolines in positions 3 and 4. Peptide **11** exists as an equal mixture of *cis* and *trans* isomers, whereas peptides **13** and **15** exist as a mixture containing 70% of *cis* isomer. The increase in the resolution of ^{1}H NMR spectra is associated with more rigid structure of new analogues. This finding is also consistent with the statistic examination of θ angles in γ-peptides. The result obtained in X-ray studies shows also that in γ-peptides modified with the use of Boc-γ^4-hhPhe-OH residue the θ angles should be in the narrow ranges. Thus incorporation of γ-bis(homo-aminoacids) into the peptide sequence in our opinion leads to a more rigid structure of the modified peptides.

Evaluation of the biological activities revealed that the cyclic peptides, but not linear ones, were potent inhibitors of mitogen-induced proliferation of human peripheral blood mononuclear cells. However, both types of peptides demonstrated the ability to suppress TNF-α production in blood cell cultures. Compound **15**, a representative of cyclic peptides, was shown to inhibit caspase expression and induce DNA fragmentation in Jurkat cells, thus revealing the alternative mechanism of action of this group of compounds. The CLA analogues may therefore represent model compounds with a potential therapeutic value in control of autoimmune diseases or prevention of allogeneic graft rejection and other clinical states associated with abnormal recruitment and proliferation of T cells.

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Supplementary data

Supplementary data associated with this article can be found, in the online version at ;

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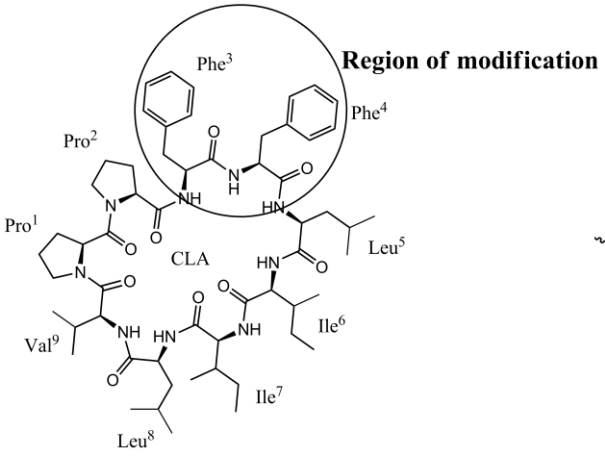
Highlights

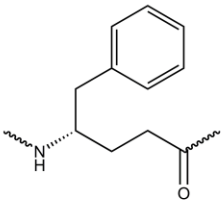
CLA was modified using γ^4-bis(homophenylalanine)

All analogues were examined for their *in vitro* immunosuppressive activities

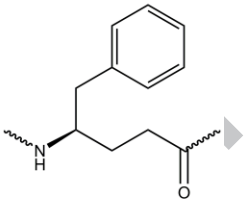
A selected peptide strongly inhibited caspase expression in Jurkat cells

DNA fragmentation indicates apoptosis as the mechanism of action





(*S*)-γ^4–HHPhe



(*R*)-γ^4–HHPhe