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Synthesis and immunosuppressive activity of new cyclolinopeptide A analogs modified with β-prolines

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Abstract: Immune response suppressors are used in the medical praxis to prevent graft rejection after organ transplantation and in the therapy of some autoimmune diseases. As a continuation of our previous work searching for new, effective suppressors devoid of toxicity, we present the synthesis, conformational analysis, and biological activity of nonapeptides 1–6, analogs of naturally existing immunomodulatory peptide CLA. New CLA analogs were modified with (*S*)-β^2-iso-proline 7 or (*S*)-β^3-homo-proline 8, respectively. The conformational influence of the β-iso-proline and β-homo-proline building blocks was analyzed by NMR spectroscopy. Peptides 1–6 exist as a mixture of four isomers due to *cis*/*trans* isomerization of the Xxx–Pro peptide bond. The major isomers of peptides 1, 3, and 4 contain all peptide bonds of the *trans* geometry. The geometry of the proline–proline bond of the second populated isomer of peptides 3 and 4 is *cis*. The proline–proline peptide bond is *cis* for the major isomers of peptides 2, 5, and 6. The peptides were tested for their ability to suppress the proliferative response of mouse splenocytes to T- and B-cell mitogens and the secondary humoral immune response to sheep erythrocytes *in vitro* in parallel with a reference drug – cyclosporine A. The immunoregulatory actions of the peptides depended on the position and content of proline isomers and were, with some exceptions, strongly inhibitory at the highest dose tested (100 μg/ml). In addition, the peptides were practically devoid of toxicity at that dose. In conclusion, the replacement of Pro by β-Pro may be useful for fine-tuning CLA immunosuppressive potency and undesirable toxicity. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: cyclolinopeptide A; immune response suppresors; β-prolines; NMR

INTRODUCTION

Transplanted organ survival is a very serious problem for transplantology, a dynamically expanded branch of contemporary medicine. Two effective immunosuppressants, CsA [1] and Tacrolimus (FK-506) [2], both producing a selective inhibition of T-lymphocyte activation are used in the medical praxis as potent drugs for the prevention of graft rejection after organ transplantation and in the therapy of some autoimmune diseases.

Despite the differences in the chemical structures (CsA being a cyclic undecapeptide and Tacrolimus a macrolide), the mechanism of a biological action of these two compounds is similar and consists of the formation of the complex with the cytoplasmatic target protein (cyclophilin and FK-506 binding protein). These complexes bind to calcineurin to inhibit its phosphatase activity [3–5]. Calcineurin plays a crucial role in T-lymphocyte activation by dephosphorylating the nuclear factor of activated T cells (NF-AT), which in turn translocates from the cytoplasm to the nucleus, where it is required for the induction of mRNA transcription for interleukin 2 (IL-2) and other cytokines [6].

A wider use of these therapeutics is, however, limited by the significant side effects of CsA and Tacrolimus [2]. Thus, the search for new immunosuppressants with a similar mechanism of action but with lower toxicity, especially in the group of naturally existing immunomodulatory peptides and their analogs, is an important challenge for medicinal chemistry.

CLA, a natural highly hydrophobic cyclic nonapeptide: cyclo(–Pro^1-Pro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9-) [7], isolated from linseed oil [8] was found to possess a strong immunosuppressive activity comparable in low doses with that of CsA [9,10], with a mechanism that depends on the inhibition of the IL-1 and IL-2 action. CLA inhibits calcium-dependent, but not calcium-independent, activation of T lymphocytes comparably to the activity of CsA and FK506. However, the concentration required for the complete inhibition is 10 times higher than that of CsA. Direct binding of CLA to cyclophilin A was confirmed in tryptophan fluorescence

Abbreviations: AFC, antibody-forming-cells; CsA, cyclosporin A; CLA, cyclolinopeptide A; ConA, concanavalin A; EDC, N-(3-dimethylaminopropyl)N′-ethylcarbodiimide; PWM, pokeweed mitogen.

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studies and PPIase assays [11]. The biology and structure–activity relationships have been reviewed in detail recently [12,13]. A large number of CLA analogs were synthesized and their biological activity was evaluated, but none of these analogs showed immunosuppressive potency higher than native CLA. The conformational flexibility of the CLA molecule is believed to be one of the most important factors responsible for CLA biological activity. It has been postulated that the following structural features are important for the immunosuppressive activity of CLA: presence of the tetrapeptide Pro-Pro-Phe-Phe sequence containing the Pro–Pro *cis* amide bond [14], the 'edge-to-face' interaction, and distance between the aromatic rings [15]. The question whether the complex CLA–cyclophilin A is able to bind calcineurin is still not answered.

In order to evaluate the role and significance of this tetrapeptide unit for biological activity of the native peptide, we have synthesized six cyclic analogs of CLA (1–6), in which one or both proline residues have been replaced by (*S*)-β^2-iso-proline **7** or (*S*)-β^3-homo-proline **8** residues (Figure 1).

Peptides consisting solely of β-amino acids form helical structures [16]. The conformational behavior of mixed peptides containing both α- and β-amino acids depends on the nature of the modified amino acids used. In short linear peptides, the incorporation of β-homo amino acids does not alter the overall conformation of the peptide backbone [17]. Incorporation of β-homo-Leu or β-homo-D-Phe into tetra-, penta-, and hexapeptides resulted in stabilization of the overall secondary structure [18]. The replacement of proline by β-homo-Pro increased the flexibility of the peptide backbone [19]. The β-amino acids incorporated in cyclic peptides preferably occupy the position $(i + 1)$ in β- and γ-turns, which are called *pseudoturns*, because they are expanded by one C-atom [18,19]. The effects exerted by new CLA analogs on the humoral immune response in mice and the proliferative response of splenocytes to T-cell and B-cell mitogens are compared with those produced by CsA.

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

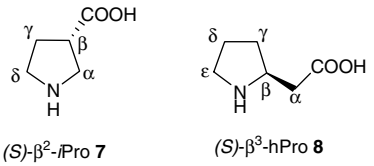
cyclo(Pro^1-β^3hPro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9) **2**

cyclo(β^3hPro^1-β^3hPro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9) **3**

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

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

cyclo(β^2iPro^1-β^2iPro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9) **6**



(*S*)-β^2-*i*Pro **7** (*S*)-β^3-hPro **8**

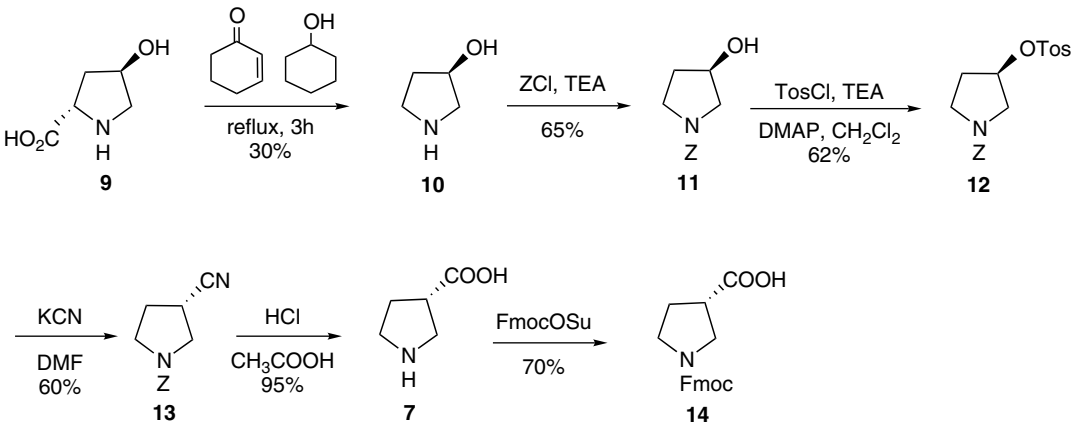
Figure 1 Sequences of newly synthesized CLA analogs **1**–**6** containing (*S*)-β^2-iso-proline **7** or (*S*)-β^3-homo-proline **8**.

RESULTS AND DISCUSSION

Synthesis

The synthesis of (*S*)-β^2-iso-proline **7** was performed according to known procedures [20–22]. Displacement of the tosylate by cyanide resulted in the formation of the nitrile **13** with inversion of configuration at the C-4 carbon atom. Subsequent acidic hydrolysis of **13** and substitution of Fmoc for Z, using 9-fluorenylmethoxy succinimide (Fmoc-OSu), as the nitrogen atom protection, gave the desired (*S*)-β^2-iso-proline derivative **14** (Scheme 1).

The synthesis of (*S*)-β^3-homo-proline was achieved in a similar way (Scheme 2) based on a slightly modified procedure described by Cardillo *et al.* [23]. The starting material Boc-prolinol **16** was obtained by the reduction of Boc-(*S*)-Pro **15** using mixed anhydride (isobutylchloroformate) with sodium borohydride [24]. Following deprotection of the Boc group, the resulting prolinol hydrochloride **17** was converted to its Z



Scheme 1 Synthesis of (*S*)-β^2-iso-proline **7**.

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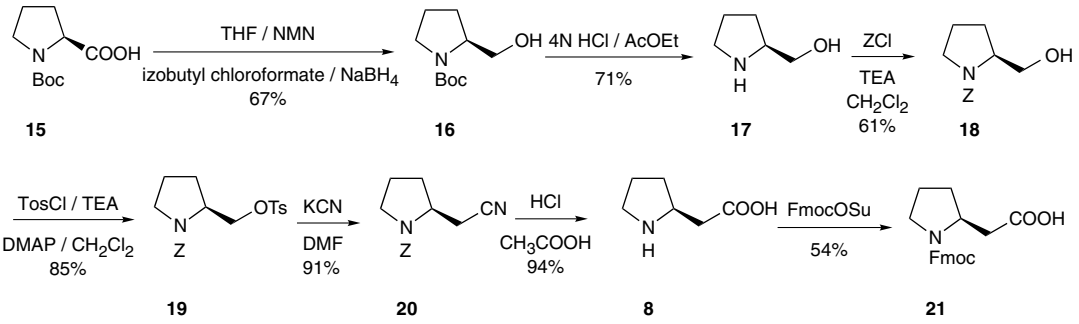

Scheme 2 Synthesis of (*S*)-β^3-homo-proline **8**.

derivative **18**. The hydroxyl group was then converted to its tosylate **19** and displaced by cyanide to give *N*-Z-pyrolidylacetonitrile **20** in an excellent yield (91%). The same reaction carried out using *N*-Boc tosylate produced the desired nitrile only in 30% (we noticed that the prolongation of the reaction time caused both the total displacement by cyanide and the deprotection of the Boc group in a great part). In the next step, the acid hydrolysis simultaneously gave Z-deprotection and cyano group hydrolysis to afford (*S*)-homoproline **8**. The final treatment with Fmoc-OSu gave *N*-Fmoc-(*S*)-β^3-homo-proline **21**.

The enantiomeric purity of the resulting β-amino acids was determined by HPLC, using Marfey's method [25,26]. (*S*)-β^2-Iso-proline was proved to have 94% ee, while (*S*)-β^3-homo-proline had 98% ee.

The linear peptides were prepared manually using a standard solid-phase procedure on the *p*-alkoxybenzyl-alcohol (Wang) resin, using the Fmoc group for *N*-amino protection. The crude peptides were purified by reverse-phase HPLC using an acetonitrile/water gradient buffered with TFA. Structures of all compounds were characterized by MS and NMR spectroscopy.

Conformational Studies in Solution

An X-ray analysis revealed that all CLA crystals are of similar conformation independently on polymorphic forms [27]. The geometry of peptide bonds is *trans* except the *cis* Pro^1–Pro^2 bond ($\omega = 10^\circ$). The backbone conformation of CLA in the solid state is stabilized by five hydrogen bonds NH···O=C. The following turns were found: γ-turn stabilized by $3 \rightarrow 1$ hydrogen bond $HN^5 \rightarrow CO^3$, two β-turns, one of type I and one of type III, with $4 \rightarrow 1$ hydrogen bonds $HN^7 \rightarrow CO^4$ and $HN^8 \rightarrow CO^5$, respectively, one $5 \rightarrow 1$ H-bond (α-turn) involving the $HN^4 \rightarrow CO^9$ and C_{17} ring structure stabilized by intramolecular hydrogen bond between the NH of Val^9 and C=O of Phe^4. The conformational flexibility of CLA molecule in solution is comparable to the flexibility of linear peptides. At 214 K in the $CDCl_3$ solution, the conformation is similar to that of the solid state. At higher temperature or in polar solvents, the fast exchange between conformers makes it difficult or even impossible for conformational analysis using NMR.

^{1}H NMR spectra in $CDCl_3$ at 298 K are poorly resolved with very broad resonances, which do not become sharper even at 217 K, as observed in the CLA spectra. However, the proton spectra of peptides **1**–**6** in the DMSO solution are much better resolved than those recorded for the unmodified CLA [27]. This may suggest a lower rate of exchange in the NMR time scale in DMSO than in $CDCl_3$, and conformational analysis was based on proton spectra in a more polar solvent. The NH resonances of peptides **1**–**6** in DMSO are spread within the range of 0.8–1.1 ppm and most of them are well separated, enabling the determination of NH-C^α-H coupling constants. However, the NH signals for peptide **1** were broad, except the Phe^3 and Ile^7 signals, and determination of NH–C^α-H coupling constants was possible at a temperature elevated to 318 K. The inspection of NH–C^β-H correlations for Phe^3 and Phe^4 residues in TOCSY spectra recorded at 298 K, except the spectra recorded for **1** and **3** at 318 K, revealed the presence of four isomers in the ratio: 74 : 16 : 7 : 3 (**1**), 39 : 36 : 20 : 4 (**3**), 46 : 28 : 14 : 12 (**4**), 69 : 16 : 8: 7 (**5**) and 67 : 17 : 10 : 6 (**6**) for each of the peptides **1**–**6**. Peptide **2** also exists as a mixture of four isomers with very high content of a major isomer (95%) but the ratio of other isomers cannot be precisely established.

The four isomers result from the *cis*/*trans* isomerization of the Pro–Pro peptide bonds. The geometries of peptide bonds were easily established by means of NOESY/ROESY spectra for isomers with content above 30%. For minor isomers, unambiguous assignment was not possible due to overlapping of signals. The NOE between C^α-H and C^δ-H proline atoms is present for *trans* Pro–Xxx bond and the correlation between C^α-H and C^α-H for *cis* Pro–Xxx bonds, where Xxx represents βhPro^1 or βiPro^2, as in **1** and **5**. Diagnostics of βhPro^2 are NOE interactions of its C^ε-H atoms (isomer *trans*) or C^β-H (isomer *cis*) with C^α-H atoms of Pro^1 or β-proline in position 1, as in analogs **2** and **3**. In the case of βiPro^1, the diagnostics are the NOE correlations of CβH atoms with C^α-H (isomer *cis*) or C^δ-H (isomer *trans*) atoms, as in **4** and **6**.

The major isomers of peptide **1**, **3,** and **4** contain all peptide bonds of the *trans* geometry and the proline–proline peptide bond is *cis* for major isomers

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of peptides **2**, **5,** and **6.** The geometry of the proline–proline bond of the second-largest quantity of isomers of peptides **3** and **4** is *cis*.

The solvent exposure of NH protons was probed by determining the temperature coefficients ($\Delta\delta/\Delta T$) from ^{1}H NMR spectra or in the case of the overlapping signals, from COSY DQF spectra. All spectra were recorded for eight different temperatures in the range of 298–338 K (Table 1) with 5 K increment. All NH resonances, except NH of Leu^5 (**1**), Phe^4 (**2**), and Leu^8 (**6**), moved linearly upfield with temperatures. The low temperature coefficients (<3 ppb/K) are characteristic of strong solvent shielding and may indicate the presence of intramolecular hydrogen bonds. The high $\Delta\delta/\Delta T$ values (>4.5 ppb/K) indicate the exposure of NH atoms to the solvent [28]. Thus, peptides modified with β-hPro are characterized by at least two hydrogen bonds including NH protons (**1** – Phe^3, Leu^5; **2** – Phe^4, Ile^6; **3** – Phe^3, Leu^5, Ile^7, Val^9). Each of βiPro analogs of CLA contain only one NH proton engaged in intramolecular hydrogen bond: **4** – Val^9; **5** – Leu^5; **6** – Leu^8.

Signals of both Phe aromatic rings are placed in a narrow and similar range. This suggests that peptides modified by β^3-homo or β^2-iso-prolines do not manifest the face-to-edge interaction of phenyl rings, as in native CLA [15,27]. The different orientation of the Phe^3 ring is also evident from the analysis of the Pro^2 chemical shifts. The close proximity of the Pro^2 side chain and the aromatic Phe^3 ring in the CLA molecule causes a strong signal shift of one of the Pro^2 γ proton to 0.33 ppm. Such upfield shift is not observed for any of peptides **1**–**6**. To examine the mutual orientation of phenyl rings, the MD calculations were carried out for the major isomer (96%) of peptide **2** with the proline–proline *cis* peptide bond. The calculated structures were verified by means of interproton distances derived from the NOESY spectrum. The violations of NOE bounds were observed for Phe^3 C^{2,6}H-Leu^5 CδH (0.5 Å), Val^9 C$^\gamma$-H- Phe^4 HN (0.2 Å), and Ile^7 HN- Leu^8 C$^\beta$-H (0.4 Å) only. The average values of the backbone φ/ψ pairs lie within the favored regions of the Ramachandran plot. The analysis of hydrogen bonds reveals the presence of two intramolecular $3 \rightarrow 1$ hydrogen bonds involving HNVal^9–Ile^7 CO and HNPhe^4-β-hPro^2 CO (supported by the temperature coefficient).

Table 1 Temperature dependence of the NH chemical shifts ($-\Delta\delta/\Delta T$, ppb/K) of major isomers of peptides **1–6** in DMSO, in the range 298–338 K

Peptide	Phe^3	Phe^4	Leu^5	Ile^6	Ile^7	Leu^8	Val^9
1	1.9	5.2	−2.1	7.5	11.9	3.8	4.0
2	4.2	−1.3	3.9	0.1	7.8	4.2	3.9
3	2.6	3.8	2.6	4.5	2.7	3.3	0.8
4	4.3	3.7	4.3	7.8	4.3	3.6	2.2
5	4.7	3.1	1.5	5.4	3.5	3.2	4.1
6	4.7	4.0	4.5	3.8	4.5	−0.4	4.5

The comparison of the NMR structure of peptide **2** and the X-ray structure of CLA [27] shows different spatial orientation of phenyl rings. The side chains of Phe^3 and Phe^4 in **2** are located at opposite sides of the backbone with a 7.0 Å distance between C-1 atoms (Figure 2). The structural comparison of **2** and CLA backbones indicates C$^\alpha$ RMSD of 2 Å.

The MD calculations for peptides **1** and **3–6** are in progress and results will be published elsewhere.

Evaluation of the Biological Activity

CLA has been assayed in a variety of *in vitro* and *in vivo* experimental models, and its activity as well as the mechanism of action was similar to that of CsA. In particular, CLA was shown to exhibit the immunosuppressive actions in the following: delayed-type hypersensitivity reaction, graft-versus-host reaction and skin allograft rejection in mice, adjuvant-induced arthritis in rats, and hemolytic anemia in New Zealand Black Mice [10]. In this study, we applied sensitive immunological assays *in vitro*, detecting the activities of CsA and CLA. In the model of the *in vitro* humoral immune response, the suppressive effects of CsA and CLA are probably associated with inhibition of antigen-specific expansion of T-helper cells as well as precursors of antibody-forming cells.

Figure 3 presents the effects of the peptides on ConA-induced proliferation of mouse splenocytes. The compounds were tested over a wide (0.1–100 μg/ml) concentration range with CsA serving as a reference compound. All immunomodulatory effects of the compounds were related to the appropriate dilution of DMSO (the solvent). The actions of the peptides were differential and dose dependent, and, in general, were strongly inhibitory at the highest (100 μg/ml) concentration. There were however some exceptions, i.e. peptide **1** was, at that dose, stimulatory and the inhibitory action of peptide **6** was rather weak. Also, quite unexpectedly, compound **2** in 10 μg/ml concentration showed some immunostimulatory activity. The compounds, as well as CsA, also inhibited at higher concentrations the background, spontaneous proliferation of splenocytes, in this case being the result of syngeneic mixed lymphocyte reaction also mediated by Il-2 [29].

The strongly inhibitory actions of compounds **3** and **4** at the 100 μg/ml concentration can, by no means, be explained by cell toxicity since, at that dose, the compounds were devoid of toxicity (as shown in 4). This finding is also in agreement with our previous data with native CLA, which showed no significant toxic effects even at relatively large doses [9]. On the other hand, we have found that a dose of 400 μg/mouse

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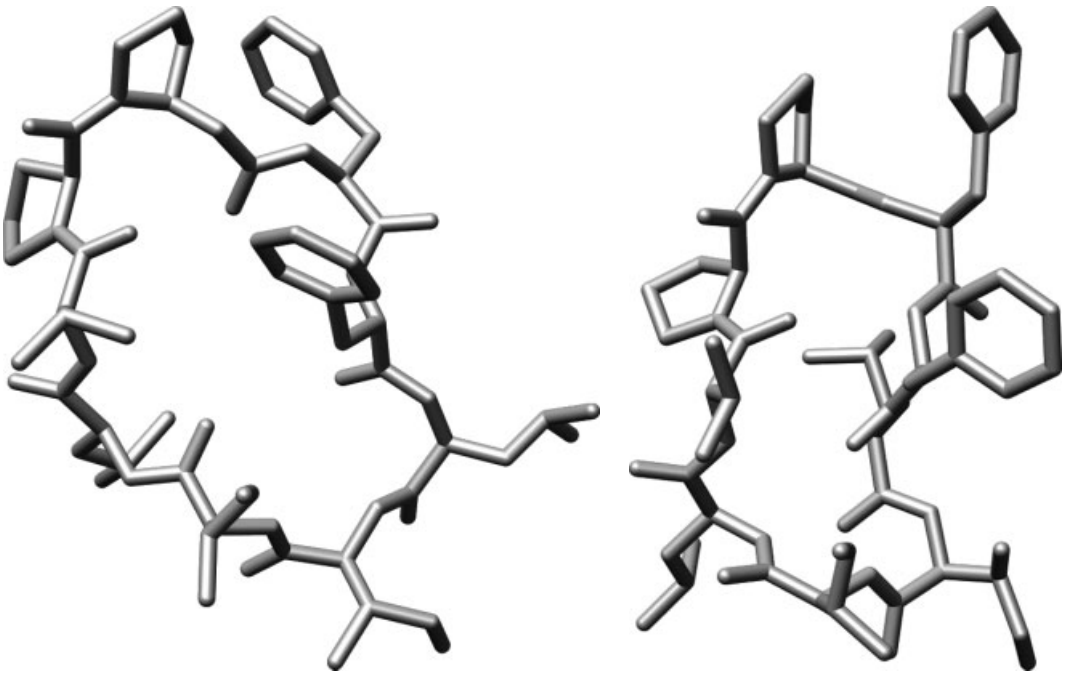

Figure 2 NMR structure of peptide **2** in comparison with the X-ray structure of CLA.

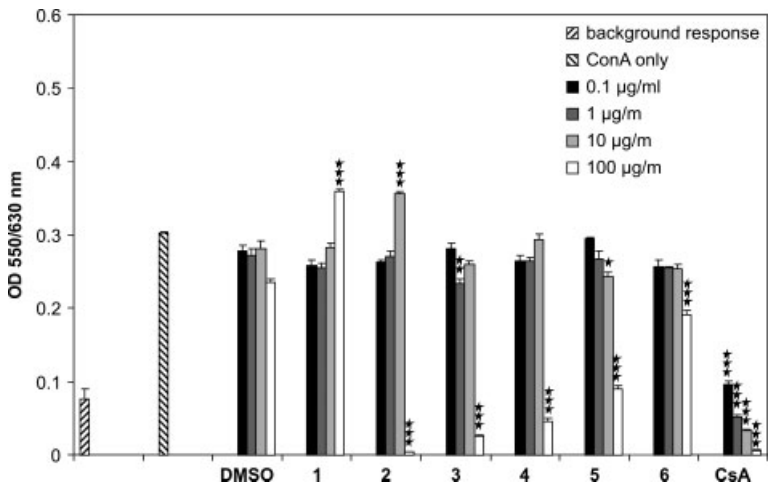

Figure 3 Effects of peptides **1–6** on the proliferative response of mouse splenocytes induced by ConA. Mouse splenocytes were induced to proliferate by addition of 2.5 µg/ml of ConA over a 3-day culture period. The compounds were applied at concentrations of 0.1–100 µg/ml. * indicates $p < 0.05$, ** indicates $p < 0.02$ and *** indicates $p < 0.001$.

of CsA, given intraperitoneally, was lethal (unpublished observations). In the model of B-cell proliferation, induced by PWM (Figure 5), the compounds exhibited somewhat different properties, and no cases of stimulation were observed. Most notably, compound **2** exhibited distinct, dose-dependent inhibitory activity suppressing the proliferative response of splenocytes already at 10 µg/ml (60% inhibition) and completely blocking the response at 100 µg/ml. Compounds **3** and **4** were also strongly inhibitory at 100 µg/ml, similar to that in the ConA-induced proliferation.

Although very strong inhibition of both T- and B-cell proliferation at 100 µg/ml by compounds **2** and **4** is correlated with the profound inhibition of the AFC number (Figure 6), it was not the case with compound **3** (stimulatory at 10 µg/ml). This suggests a different mechanism of action of this peptide which, in addition, was completely devoid of cell toxicity (Figure 4). Nevertheless, the suppressive action of compounds **2** and **4** is much weaker compared to that of CsA, the reference drug. The same relates to the suppressive actions of the peptides in the proliferation assays. Such results may well be explained by the

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fact that the calcium-dependent complete inhibition of T-lymphocyte activation by CLA requires 10 times higher concentration than in the case of CsA [11]. The stimulatory activity (2.2-fold) of compound **6** at 100 μg/ml concentration in the humoral immune response is also intriguing because the peptide shows some cell toxicity at that concentration and a moderate inhibition of both T- and B-cell proliferation. Most likely, in the model of secondary immune response *in vitro*, the expansion of antigen-specific T memory cells is not affected by compounds **3** and **6**.

Such compounds like **3** and **6** may be of potential therapeutic value since inhibition of T- and B-cell proliferation relevant to suppressing some autoimmune disorders or preventing allograft rejection should not cause inhibition of the immune response to pathogens or vaccines.

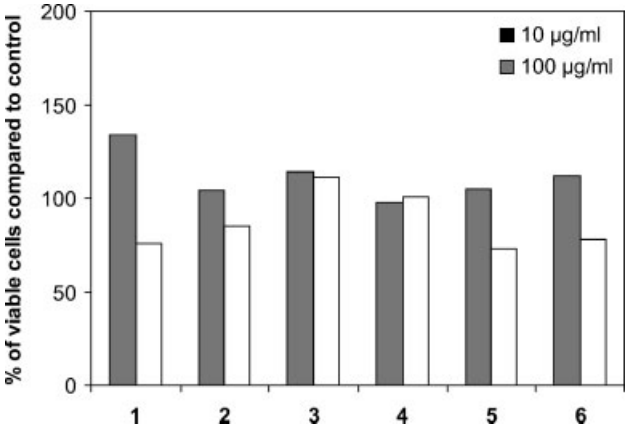

Figure 4 The toxicity of peptides **1–6** with regard to WEHI 164.13 cells. The peptides were used at concentrations of 10 and 100 μg/ml. The cell toxicity of the peptides was compared with toxicity of the respective dilutions of the solvent (DMSO), which were regarded as having 100% viability (0% toxicity).

CONCLUSIONS

The replacement of one or both proline residues in the CLA molecule by βiPro or βhPro caused changes in the conformational behavior of the nonapeptide. The backbones of peptides **1**, **2**, **4,** and **5** are expanded by one carbon atom, and backbones of peptides **3** and **6** by two carbon atoms. The modified CLA molecules are less conformationally flexible or the exchange between conformers is much slower in the NMR time scale than observed for CLA molecule and, moreover, all of them exist as mixtures of four isomers due to *cis*/*trans* isomerization of the Xxx–Pro bonds. The lowest immunosuppressive activity of peptide **1** is not surprising because of its highest content of all *trans* isomers. Also, the biological activity of peptide **2** containing 95% of the isomer with *cis* Pro–βhPro bond is consistent with geometric requirements for immunosuppressive activity of CLA. Peptides **3** and **4** contain much lower amounts of both isomers with *trans* and *cis* peptide bonds between prolines. These peptides are slightly less potent than peptide **2**, but almost completely devoid of toxicity. The expansion of the CLA backbone by one or two carbon atoms has an effect on the mutual orientation of Phe aromatic rings, and their edge-to-face orientation is not present. The lack of spatial proximity of benzene rings is not transmitted into significant decrease of immunosuppressive activity of CLA analogs, as would be expected on the basis of previous findings. In our opinion, the replacement of Pro by β-Pro is a useful tool for fine-tuning of CLA immunosuppressive potency and undesirable toxicity.

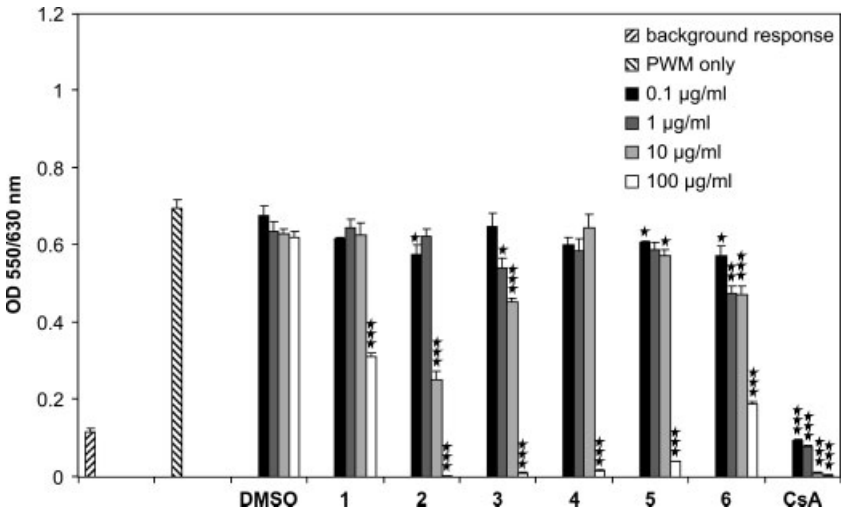

Figure 5 Effects of peptides **1–6** on the proliferative response of mouse splenocytes induced by PWM. Mouse splenocytes were induced to proliferate by addition of 2.5 ug/ml of PWM over a 3-day culture period. The results are presented as the mean OD values from four determinations (culture wells) ± SE. The compounds were applied at concentrations of 0.1–100 μg/ml. * indicates $p < 0.05$, ** indicates $p < 0.02$, and *** indicates $p < 0.001$.

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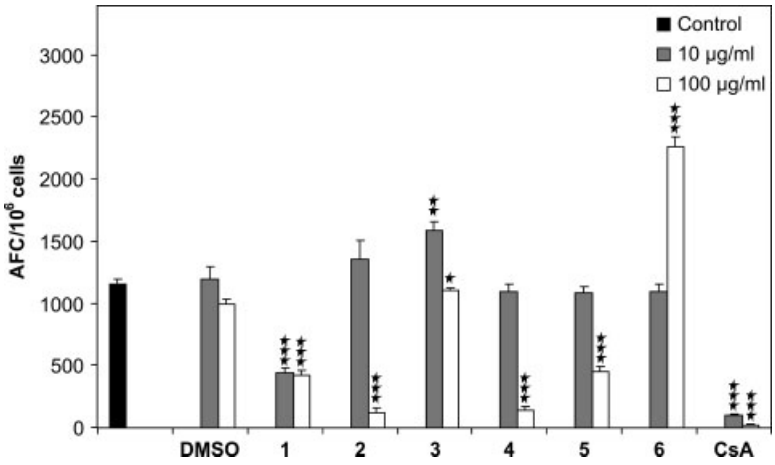

Figure 6 Effects of peptides **1–6** on the secondary humoral immune response to SRBC *in vitro*. Mouse splenocytes from SRBC-primed mice were cultured with antigen (0.1 ml of 0.005% SRBC suspension) and the compounds (10 and 100 µg/ml concentrations) for 4 days. * indicates $p < 0.05$, ** indicates $p < 0.02$, and *** indicates $p < 0.001$. The number of antibody-forming cells was determined by the method of local hemolysis in agar gel and presented as mean values from four determinations (wells) ± SE.

Peptide **3** would be attractive to explore in other experimental models such as mixed lymphocyte reaction, graft rejection, and collagen-induced arthritis because of lack of toxicity, strong inhibition of T- and B-cell proliferation, and no suppressive effect in the humoral immune response.

MATERIALS AND METHODS

General

All solvents were purified by conventional methods. Evaporations were carried out under reduced pressure. Melting points were determined on a capillary melting point apparatus and were uncorrected. The optical rotation was measured in a 1-dm cell (1 ml) on a Horiba high speed automatic polarimeter at 589 nm. For thin-layer chromatography, 250-nm silica gel GF precoated uniplates (Merck) were used with the following solvent systems: (A) $CHCl_3$/MeOH/AcOH (90 : 10 : 2), (B) EtOAc/hexane/AcOH (90 : 10 : 1), (C) $CHCl_3$/MeOH (95 : 5), (D) EtOAc/hexane (1 : 1), (E) EtOAc/hexane (7 : 3), (F) BuOH/AcOH/H_2O (4 : 1 : 1), (G) BuOH/AcOH/AcOEt/H_2O (1 : 1 : 1 : 1). The chromatograms were visualized with chlorine followed by starch/KI and/or ninhydrin. Flash chromatography was performed with Merck silica gel 60 (230–400 mesh). HPLC was performed on an LDC/Milton–Roy analytical instrument using a Vydac C18 column (0.46 × 25 cm): flow 1.0 ml/min, detection at 220 nm and eluants (A) 0.05% TFA in water and (B) 0.038% TFA in acetonitrile/water 90 : 10 with a gradient application. Purification of peptides was performed by preparative reversed-phase HPLC on a Vydac C18 column (2.2 × 25 cm): flow rate 16 ml/min, UV detection at 220 nm. The structures of the pure peptides were confirmed by FAB mass spectroscopy on APO Electron, MI 1201E and Finningan MAT 95 mass spectrometer using thioglycerol as the matrix.

NMR Spectroscopy

NMR measurements were carried out on Bruker spectrometers. ^{1}H NMR spectra (250.13 MHz) were recorded at 25 °C in D_2O or $CDCl_3$, with DSS or tetramethylsilane as the internal standards respectively. The samples of peptides **1**–**6** for the NMR measurements were prepared by dissolving 6 mg of the peptide in 0.6 ml of DMSO-d_6 and 1D/2D NMR experiments were recorded at 700.08 MHz. Spin-lock time was 80 ms and 300 ms in TOCSY and ROESY experiments respectively, and NOESY experiments were recorded with 300 ms mixing time. 2D NMR spectra were processed with NMRPipe [30] and analyzed by CARA software [31].

Molecular Dynamics

The starting coordinates of a cyclic peptide **2** were constructed using default simulated annealing protocol implemented within XPLOR-NIH program [32,33]. The solvent was treated explicitly as a truncated octahedron box of 832 DMSO molecules. The box vector length was set at 55, which is large enough to prevent cyclic peptide interactions with its periodic image. The system was equilibrated by using 5000 steps with the conjugate gradient algorithm and subsequent NPT molecular dynamics (200 ps) with restrained cartesian coordinates of peptide atoms. MD simulation was performed using the GROMACS 3.3.2 package [34,35] and GROMOS 53A6 force field [36]. The total simulation time was 500 ns. In order to maintain a temperature of 300 K and pressure of 1 bar, Berendsen weak coupling [37] algorithm was used, with coupling constants of 0.1 ps for the temperature and 1 ps for the pressure. Coulomb interactions were evaluated using Particle–Mesh Ewald algorithm [38] with switching distance of 1.0 Å. A twin-range cutoff of 1.0/1.4 Å for the van der Waals interactions was applied. Neighbor lists were utilized and updated every tenth integration step. Integration time step was 2 fs and all bonds were constrained by means of SHAKE algorithm [39]. A relative dielectric constant of 1.0 was used.

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In the NOESY spectrum recorded with mixing time 138, cross peaks were assigned (94 intraresidual, 33 sequential and 11 inter-residual), integrated, and then applied for verification of the calculated structures. Cross-peak volumes were classified on the basis of their volumes into three categories: strong (1.8–2.5 Å), medium (1.8–3.5 Å), and weak (1.8–5.0 Å). Pseudo-atom corrections (0.9 Å for CH_2, 1.0 Å for CH_3, and 2.2 Å for $(CH_3)_2$ were applied for experimental upper bounds [40]. NOE distance bounds were checked for the last 300 ns of trajectory using $< r^{-6} >$ distance averaging. NOE violations higher than 0.5 Å were not observed. Clustering based on the root mean square difference (RMSD) of backbone atoms positions performed with cutoff of 1.0 Å resulted in one main cluster with a population of 69%.

Synthetic Procedures

(R)-3-Hydroxypyrrolidine (10). The mixture of L-hydroxy proline **9** (30 g, 228 mM) and 2-cyclohexen-1-one (3 ml, 31 mmol) in cyclohexanol (150 ml) was refluxed for 3 h. Cyclohexanol was removed by distillation and the residue was purified by distillation under reduced pressure to give the product as a colorless oil (6 g, 69 mmol, 30%); bp 108 ÷ 110 °C/8 mmHg (lit. [41]: bp 100 °C/1.5 mmHg); $[\alpha]_D$ = +3.79° (c = 1.5; MeOH) {lit. [42]: $[\alpha]_D$ = +5.7° (c = 0.7; MeOH)}, ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.68–2.05 (m, 2H, C^{γ}-H), 2.78–3.18 (m, 4H, C^{α}-H, C^{δ}-H), 4.37–4.42 (m, 1H, C^{β}-H). The oily product was treated by the solution of 4N HCl in ethyl acetate and (*R*)-3-hydroxypyrrolidine hydrochloride was obtained (4.6 g, 52.8 mmol, 23%) as a solid crystallized from acetone; mp 106–109 °C (lit. [22]: 109 °C); $[\alpha]_D$ = −8.29° (c = 1; MeOH) {lit. [22]: $[\alpha]_D$ = −7.60° (c = 3.45; MeOH)}; $R_f(F)$ = 0.29, $R_f(G)$ = 0.34; ^{1}H-NMR (250 MHz, D_2O) δ_H 1.96–2.12 (m, 2H, C^{γ}-H), 3.19–3.33 (m, 4H, C^{α}-H, C^{δ}-H), 4.51 (s, 1H, C^{β}-H).

(R)-1-Z-3-hydroxypyrrolidine (11). To the vigorously stirred suspension of (*R*)-3-hydroxypyrrolidine hydrochloride (5.4 g, 44 mmol) and TEA (12.4 ml, 88 mmol) in DCM (350 ml), benzyl chloroformate (9.4 ml, 66 mmol) was added dropwise at 0 °C; then the reaction mixture was stirred for 3 h at the temperature 5–10 °C. The DCM was evaporated; the residue was diluted with EtOAc (100 ml) and washed with 0.5 M HCl (3 × 30 ml), with saturated $NaHCO_3$ (3 × 30 ml), and water (2 × 30 ml) and dried over $MgSO_4$. The solution was concentrated and the crude product was purified by flash chromatography (eluant: EtOAc-hexane 7/3, $R_f(E)$ = 0.34) affording **11** as an oil (6.3 g, 28.47 mM, 65%). $[\alpha]_D$ = −19.69° (c = 1, $CHCl_3$) {lit. [43]: $[\alpha]_D$ = −21.2° (c = 1, $CHCl_3$)}; $R_f(D)$ = 0.19, $R_f(C)$ = 0.7, HPLC purity = 100% (t_R = 6.39 min, 30–70%B, 25 min); ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.93–2.17 (m, 3H, C^{γ}-H, O-H), 3.39–3.62 (m, 4H, C^{α}-H, C^{δ}-H), 4.43–4.45 (m, 1H, C^{β}-H), 5.13 (s, 2H, Ph-CH_2-O), 7.26–7.37 (m, 5H, C-H arom).

(R)-1-Z-3-tosyloxypyrrolidine (12). To the stirred solution of **11** (7.08 g, 32 mmol), TEA (4.5 ml, 32 mmol), and DMAP (782 mg, 6.4 mmol) in DCM (250 ml), tosyl chloride (7.87 g, 41.3 mM) was added in portions at 0 °C. After being stirred at this temperature for 3 h, the reaction mixture was washed with saturated K_2CO_3 (1 × 80 ml) and then the water layer was extracted twice with DCM (2 × 30 ml). Organic layers were collected and dried over Na_2SO_4 and $MgSO_4$. Solvents were evaporated at reduced pressure and the resulting crude residue was purified by flash chromatography (eluant: EtOAc-hexane 1/1). The pure compound **12** was isolated as an oil (7.4 g, 19.7 mM, 62%). $[\alpha]_D$ = −14.44° (c = 1, $CHCl_3$) {lit. [44]: $[\alpha]_D$ = −11.80° (c = 1, $CHCl_3$)}; $R_f(E)$ = 0.66, $R_f(C)$ = 0.75, $R_f(D)$ = 0.46, HPLC purity = 100% (t_R = 9.55 min, 50–90%B, 25 min); ^{1}H-NMR (250 MHz, $CDCl_3$) δ 2.01–2.17 (m, 2H, C^{γ}-H), 2.45 (s, 3H, CH_3 Ts), 3.43–3.59 (m, 4H, C^{α}-H, C^{δ}-H), 5.06–5.11 (m, 3H, Ph-CH_2-O, C^{β}-H), 7.26–7.34 (m, 7H, C-H arom Cbz and Ts), 7.78 (d, 2H, *J* = 10 Hz, C-H arom Ts).

(S)-1-Z-3-cyanopyrrolidine (13). To the solution of **12** (3.5 g, 9.3 mM) in DMF (50 ml), KCN (1.2 g, 18.6 mM) was added and the mixture was stirred at 90 °C for 30 h. The progress of the reaction was monitored by HPLC (40–80%B, 25 min). After the reaction was completed, EtOAc (150 ml) was added and the organic layer was washed with water (3 × 50 ml). The combined water layers were treated with $KMnO_4$ before disposal. The organic layer was dried over $MgSO_4$ and the solvent was evaporated at reduced pressure. The crude residue was purified by flash chromatography over silica gel (eluant: EtOAc-hexane 1/1, $R_f(D)$ = 0.40) to give pure compound **12** as an oil (1.45 g, 6.3 mM, 60%, $[\alpha]_D$ = +29.60° (c = 2, $CHCl_3$) {lit. [44]: $[\alpha]_D$ = +25.40° (c = 1.3, $CHCl_3$)}, HPLC purity = 97% (t_R = 10.61 min, 30–70%B, 25 min,), $R_f(E)$ = 0.58, $R_f(C)$ = 0.67, ^{1}H-NMR (250 MHz, $CDCl_3$) δ_H 2.22–2.30 (m, 2H, C^{γ}-H), 3.06–3.16 (m, 1H, C^{β}-H), 3.47–3.79 (m, 4H, C^{α}-H, C^{δ}-H), 5.14 (s, 2H, Ph-CH_2-O), 7.34–7.38 (m, 5H, C-H arom), ^{13}C-NMR (62.5 MHz, $CDCl_3$) δ_C 23.61 (C^{β}-H), 30.27 (C^{γ}-H), 46.86 (C^{δ}-H),54.12 (C^{α}-H), 67.22 (Ph-CH_2-O), 117.72 (CN), 127.75 (C-H arom), 127.98 (C-H arom),128.43 (C-H arom),136.39 (O-CH_2-C arom), 154.80 (C=O).

(S)-1-Pyrrolidine-3-carboxylic acid ((S)-β^2-iso-proline) (7). The mixture of **13** (2.64 g, 11.5 mmol), concentrated HCl (100 ml), and concentrated CH_3COOH (23 ml) was refluxed with stirring for 7 h. After being cooled to room temperature, the solution was washed with Et_2O (3 × 30 ml), evaporated, and dried under reduced pressure over KOH pellets and P_2O_5. The crude (*S*)-β^2-iso-proline hydrochloride without any further purification was used in the next reaction (1.6 g, 10.6 mM, 92%); $R_f(F)$ = 0.26, $R_f(G)$ = 0.29; ^{1}H-NMR (250 MHz, D_2O) δ 2.06–2.33 (m, 2H, C^{γ}-H), 3.19–3.52 (m, 4H, C^{α}-H, C^{δ}-H), 4.56–4.73 (m, 1H, C^{β}-H). A small amount of (*S*)-β^2-iso-proline hydrochloride (30 mg, 0.2 mM) was purified using ion exchange chromatography (Amberlite IR 120, Fluka, 1.91 meq/ml). Pure (*S*)-β^2-isoproline was isolated (15.22 mg, 133 mM, 67%) and its optical purity was checked using Marfey's reagent [25], HPLC = 94% ee (t_R = 30.97, 10–50%B, 60 min, λ = 340 nm), $[\alpha]_D$ = +17.53° (c = 2, H_2O) {lit. [44]: $[\alpha]_D$ = +18.20° (c = 2.5, H_2O)}.

(S)-1-(Fmoc)pyrrolidine-3-carboxylic acid ((S)-Fmoc-β^2-iso-proline) (14). To the suspension of (*S*)-β^2-iso-proline hydrochloride (1.65 g 11 mmol) and K_2CO_3 (3.35 g 24.2 mM) in water (44 ml) and acetone (55 ml), Fmoc-OSu (4.1 g 12 mM) was added in several portions under vigorous stirring while pH was maintained in the range 8–9. After the reaction mixture had been stirred overnight at room temperature, the acetone was evaporated, and the reaction solution was diluted with water, extracted with Et_2O (3 × 30 ml), and the ether extracts were discarded. The water layer was then acidified at 0 °C with 6N HCl to reach the pH range 2–3 and the white suspension was extracted with EtOAc (3 × 40 ml). The

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combined organic extracts were washed with water (3 × 30 ml) and dried over $MgSO_4$. After solvent evaporation, the crude product was treated with hexane to give the desired product as a white powder (2.60 g, 7.75 mM, 70%); $[\alpha]_D = +10.94°$ (c = 1, MeOH), $R_f(A) = 0.68$, $R_f(B) = 0.55$; HPLC purity = 100% (t_R = 10.68 min, 40–80%B, 25 min); ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.17–2.22 (m, 2H, C^{γ}-H), 3.08–3.21 (m, 1H, C^{β}-H), 3.44–3.74 (m, 4H, C^{α}-H, C^{δ}-H), 4.21–4.43 (m, 3H, CH-CH_2 Fmoc), 7.25–7.44 (m, 4H, C-H arom), 7.60 (d, J = 7,5 Hz, 2H, C-H arom), 7.76 (d, J = 7,5 Hz, 2H, C-H arom); ^{1}H-NMR (250 MHz, DMSO) δ 1.96–2.13 (m, 2H, C^{γ}-H), 2.97–3.13 (m, 1H, C^{β}-H), 3.32–3.48 (m, 4H, C^{α}-H, C^{δ}-H), 4.19–4.35 (m, 3H, CH-CH_2 Fmoc), 7.41 (dd, J = 1.25, 8.5 Hz, 2H, CH arom), 7.33 (dd, J = 1.25, 8.5 Hz, 2H, CH arom); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 42.90 (C^{β}-H), 44.12 (C^{γ}-H), 47.30 (CH-Fmoc), 48.04 (C^{δ}-H), 49.89 (C^{α}-H), 67.33 (CH_2 Fmoc), 119.98 (CH arom), 125.06 (CH arom), 127.04 (CH arom), 127.69 (CH arom), FAB-MS $[M + H]^+ = 338.1$, calcd. for $C_{20}H_{20}O_4N$ 338.34.

(S)-1-tert-Butoxycarbonyl-2-hydroxymethylpyrrolidine (16). To the stirred solution of Boc-L-proline **15** (21.5 g, 100 mM) and NMM (11.1 ml, 100 mM) in THF (100 ml), isobutylchloroformate (13.6 ml, 100 mM) was added dropwise at −5 °C. After 10 min, the mixture was filtered over Celite and the precipitate was washed with THF (3 × 20 ml). The collected organic filters were cooled to 0 °C and the solution of $NaBH_4$ (5.7 g 150 mM) in water (50 ml) was added carefully (a gas was released rapidly). After dilution with water (1 l), the solution was washed with EtOAc (3×). The organic layers were collected and dried over $MgSO_4$. The solvent was evaporated at reduced pressure. The residue was separated by flash chromatography over silica gel (eluant: hexane/EtOAc 7 : 3, R_f = 0.32), giving pure (*S*)-Boc-prolinol **16** as a white solid; (13.5 g, 67 mM, 67%), $[\alpha]_D = -50.69°$ (c = 1; $CHCl_3$), {lit. [45] (*R*)-*N*-Boc-prolinol: $[\alpha]_D = +47.50°$ (c = 1; $CHCl_3$)}, $R_f(C)$ = 0,56; $R_f(D)$ = 0,41}; HPLC purity = 99% (t_R = 6.83 min 30–70%B, 25 min), ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.39–1.55 (s, 9H, $3CH_3$ ^{t}Bu), 1.77–1.84 (m, 4H, C^{γ}-H_2, C^{δ}-H_2), 3.28–3.48 (m, 5H, C^{β}-H, C^{α}-H_2, C^{ε}-H_2).

(S)-2-Hydroxymethylpyrrolidine hydrocloride (17). Boc–prolinol **16** (3.2 g 16 mM) was treated at room temperature with a solution of 4N HCl in AcOEt (48 ml) for 1 h. The solvent was evaporated and the residue was washed with Et_2O (2 × 15 ml), giving the crude (*S*)-prolinol hydrochloride **15** (1.57 g, 15.5 mM, 71%) which was used for the next reaction without any further purification. $[\alpha]_D = +11.69°$ (c = 1,3, $CHCl_3$), $R_f(F) = 0.26$, $R_f(G) = 0.33$, ^{1}H NMR (250 MHz, D_2O) δ_H 1.66–1.84 (m, 2H, C^{ε}-H_2), 2.04–2.21 (m, 4H, C^{γ}-H_2, C^{δ}-H_2), 3.29–3.32 (m, 3H, C^{α}-H_2, C^{β}-H).

(S)-1-Z-2-hydroxymethylpyrrolidine (18). The same procedure as for synthesis of **11** was applied to obtain **18**. Starting from (*S*)-2-hydroxymethylpyrrolidine hydrochloride **17** (1.57 g, 15.5 mM), the desired product was isolated as an oil (2.24 g, 9.5 mM, 61%) after being purified by flash chromatography over silica gel (eluant: hexane/EtOAc 7 : 3, R_f = 0.31). $[\alpha]_D = -40.6°$ (c = 1.5, $CHCl_3$); $R_f(D) = 0.45$. $R_f(C) = 0.58$; HPLC purity = 98% (t_R = 8.45 min 30–70%B, 25 min), ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.61–1.91 (m, 4H, C^{δ}-H_2, C^{γ}-H_2), 1.99–2.04 (m, 2H, C^{ε}-H_2), 3.37–3.44 (m, 1H, C^{β}-$^-$H), 3.50–3.68 (m, 2H, CH_2-OH), 4.00–4.03 (m, 1H, OH), 5.15 (s, 2H, CH_2 Cbz), 7.26–7.28 (m, 5H, C-H arom.).

(S)-1-Z-2-tosyloxymethylpyrrolidine (19). Starting from **18** (1.8 g, 7.7 mM) and following the same procedure as for compound **11**, derivative **19** was isolated as an oil (2.5 g, 6.4 mM, 85%) after being purified by flash chromatography over silica gel (eluant: EtOAc/hexane 1 : 1, $R_f(D)$ = 0.57). $[\alpha]_D = -42.77°$ (c = 2, $CHCl_3$); $R_f(C) = 0.68$, $R_f(E) = 0.76$; HPLC purity = 97% (t_R = 10.97 min 50–90% B, 25 min); ^{1}H NMR ($CDCl_3$) δ_H 1.78–1.95 (m, 4H, C^{γ}-H_2, C^{δ}-H_2), 2.42 (s, 3H, CH_3-OTs), 3.34–3.39 (m, 2H, C^{ε}-H_2), 4.01–4.16 (m, 3H, C^{β}-H, CH_2-OTs), 5.00–5.06 (m, 2H, CH_2 Cbz), 7.26–7.33 (m, 7H, C-H arom, Cbz, Ts), 7.68–7.78 (m, 2H, C-H arom, Ts).

(S)-1-Z-2-cyanomethylpyrrolidine (20). Starting from **19** (2.5 g, 6.4 mM) and following the same procedure as for **12,** derivative **20** was isolated as an oil (1.54 g, 5.9 mM, 93%), after being purified by flash chromatography over silica gel (eluant: EtOAc/hexane 7 : 3). $[\alpha]_D = -86.72°$ (c = 2, $CHCl_3$); $R_f(C) = 0.58$, $R_f(E) = 0.73$; HPLC purity = 100% (t_R = 13.81 min 30–70%B, 25 min); ^{1}H NMR ($CDCl_3$) δ_H 1.83–2.04 (m, 4H, C^{γ}-H_2, C^{δ}-H_2), 2.78–2.85 (m, 2H, CH_2-CN), 3.48–3.53 (m, 2H, C^{β}-H_2), 4.05–4.11 (m, 1H, C^{γ}-H), 5.08–5.19 (m, 2H, CH_2 Cbz), 7.25–7.44 (m, 5H, CH Cbz), ^{13}C NMR ($CDCl_3$) δ_C 28.22 (CH_2-CN), 30.01 (C^{δ}-H_2), 44.78 (C^{γ}-H_2), 49.03 (C^{ε}-H_2), 67.06 (C^{β}-H), 77.49 (CH_2 Cbz), 119.58 (CN), 127.87 (C-H arom), 128.09 (C-H arom), 128.40 (C-H arom), 136.26 (O-CH_2-C arom), 154.20 (C=O).

(S)-1-Pyrrolidine-2-methylenecarboxylic acid ((S)-β^3-homo-proline) (8). Starting from **20** (2.5 g, 6.4 mM) and following the same procedure as for the synthesis of **7**, compound **8** was isolated (1.6 g, 10.6 mM, 92%) and used for the next synthesis without any further purification. $R_f(F) = 0.29$, $\mathbf{R}_f(G) = 0.27$; ^{1}H NMR (250 MHz, D_2O) δ 1.53–2.15 (m, 4H, C^{δ}-H_2, C^{γ}-H_2), 2.58–2.79 (m, 2H, CH_2-COOH), 3.19 (t, J = 7.5 Hz, C^{ε}-H), 3.68–3.80 (m, 1H, C^{β}-H), 4.32–4.85 (m, 2H, NH, OH).

A small amount of (*S*)- β^3-homo-proline hydrochloride (33 mg, 0.2 mM) was purified using ion exchange chromatography (Amberlite IR 120, Fluka, 1.91 meq/ml, mesh). Pure (*S*)-β^3-homoproline was isolated (14 mg, 108 mM, 65%) and its optical purity was checked using Marfey's method [25], HPLC = 98% ee (t_R = 36.32, 10–50%B, 60 min, λ = 340 nm), $[\alpha]_D = +34.24°$ (c = 1, 2N HCl) {lit. [46]: $[\alpha]_D = +35.00°$ (c = 1; 2N HCl)}.

(S)-1-(Fmoc)pyrrolidine-2-methylenecarboxylic acid (Fmoc-(S)-β^3-homoproline) (21). Starting from **8** (0.93 g, 5.6 mM) and following the same procedure as for **14**, compound **21** was isolated as a white powder (1.1 g, 3.1 mM, 54%). $[\alpha] = -28.99°$ (c = 1, MeOH); $R_f(A) = 0.68$, $R_f(B) = 0.7$; HPLC purity = 100% (t_R = 11.25 min, 40–80% B, 25 min); ^{1}H NMR (250 MHz, DMSO) δ 1.16–2.26 (m, 6H, C^{δ}-H_2, C^{γ}-H_2, CH_2-COOH), 3.24–3.40 (m, 2H, C^{ε}-H), 3.92–3.95 (m, 1H, C^{β}-H), 4.29–4.32 (m, 3H, CH-CH_2 Fmoc), 7.32–7.42 (m, 4H, C-H arom. Fmoc), 7.63 (d, 2H, J = 7.5 Hz, C-H arom. Fmoc), 7.87 (d, 2H, J = 7.5 Hz, C-H arom. Fmoc), ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.80–1.90 (m, 4H, C^{δ}-H_2, C^{γ}-H_2), 2.04–2.17 (m, 2H, CH_2-COOH), 2.40–2.45 (m,1H, C^{β}-H), 3.39–3.41 (m, 2H, C^{ε}-H), 4.11–4.25 (m, 1H, CH-Fmoc), 4.41–4.54 (m, 2H, CH_2 Fmoc), 7.26–7.42 (m, 4H, C-H arom Fmoc), 7.59 (d, 2H, J = 7.5 Hz, C-H arom Fmoc), 7.74 (d, 2H, J = 7.5 Hz, C-H arom Fmoc), ^{13}C NMR ($CDCl_3$) δ_C 23.62 (C^{β}-H_2), 31.16 (C^{γ}-H_2), 38.65 (C^{ε}-H_2), 46.57 (CH_2-COOH), 47.36 (CH-Fmoc), 67.18

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(C^{β}-H), 77.51 (CH_2 Fmoc), 119.99 (C-H arom Fmoc), 127.05 (C-H arom Fmoc), 127.69 (C-H arom Fmoc), 127.99 (C-H arom Fmoc), 141.39 (C arom. Fmoc), 143.99 (C arom. Fmoc), 155.06 (C=O Fmoc), 175.43 (C=O), FAB-MS $[M+H]^+ = 352.1$, calcd. for $C_{21}H_{22}O_4N$ 352.34.

Solid-Phase Peptide Synthesis

The linear peptides were prepared manually using standard solid-phase procedure on the *p*-alkoxybenzylalcohol (Wang) resin. Fmoc-Ile attached to Wang resin was prepared through esterification reaction using Mitsunobu methods [47,48]. Unreacted hydroxyl groups were acetylated with the aid of acetic anhydride, pyridine, and DCM (1 : 2 : 3). A standard single TBTU protocol (3 eq) was used for all single amino acid derivatives (3 eq) and was repeated if Kaiser [49] or chloranil [50] (for proline residue) tests were slightly positive. Deprotection of Fmoc groups was accomplished by treating the resin with 20% piperidine solution in DMF (twice: 20 and 10 min). Standard washing of resin was accomplished using DMF (3 × 10 ml), DCM (3 × 10 ml), MeOH (3 × 10 ml), and DCM (3 × 10 ml). The peptide resin, following drying under reduced pressure over KOH and P_2O_5, was cleaved with a mixture of TFA/H_2O/anisol (95 : 5 : 3) for 3 h at room temperature. Crude peptides were obtained after TFA removal by evaporation and washing several times with diethyl ether. Purification of linear precursors was achieved by preparative HPLC with a linear gradient.

Synthesis of Cyclic Peptides

Pure linear peptides were lyophilized from *tert*-butanol/water and were cyclized by means of EDC (3 eq) in the presence of HOBt (3 eq) and DIEA (3 eq) in DCM at much lower concentrations (40 mg of peptide in 800 ml of DCM) than usually described for 'head-to-tail' peptide cyclization reactions. The solution was stirred for 10 days at ambient temperature and was then washed with water (3 × 200 ml) and evaporated. The crude residue was dried under reduced pressure over KOH and P_2O_5 and then purified using preparative HPLC. Crude cyclic peptides were purified by preparative HPLC with linear gradient 50–95% B in A in 40 min, $\lambda = 220$ nm (B: 0.038 TFA in acetonitrile/water 90 : 10, A: 0.05% TFA in water). The homogeneity of all peptides was checked by analytical HPLC and structures were confirmed by MALDI and NMR spectra. ^{1}H NMR chemical shifts for peptides 1–6 are provided as Supporting Information.

Cyclo(βhPro^1-Pro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)

(1). The crude linear peptide: 305 mg (HPLC purity = 75%, $t_R = 12.26$ min, 40–80%B in 25 min). Purification using preparative HPLC ($t_R = 22.09$ min, 40–80%B in 40 min) gave nonapeptide (13.3 mg, 44%, HPLC purity = 100%, t_R = 10.99 min, 40–80% B in 25 min); MALDI MS: 1094.3 $[M+Na]^+$, 1110.3 $[M+K]^+$, calcd. for $C_{58}H_{89}O_{10}N_9$ 1071.67.

The crude cyclic peptide: 42 mg (HPLC purity = 49%, t_R = 19.02 min, 50–90%B in 25 min). Purification by preparative HPLC ($t_R = 28.72$ min, 50–95% B in 40 min), yielded **3** (10 mg, 25%, HPLC purity = 100%, $t_R = 17.60$ min, 50–90% B in 25 min). MALDI MS: 1078.9 $[M+Na]^+$, 1094.8 $[M+K]^+$, calcd. for $C_{58}H_{87}O_9N_9$ 1053.66.

Cyclo(Pro^1-βhPro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)

(2). The crude linear peptide: 320 mg (HPLC purity = 77%, $t_R = 11.56$ min, 40–80% B in 25 min). Purification using preparative HPLC ($t_R = 23.42$ min, 40–80% B in 40 min), gave nonapeptide (10.4 mg, 35%, HPLC purity = 100%, t_R = 9.42 min, 40–80% B in 25 min); MALDI: 1094.4 $[M+Na]^+$, 1110.4 $[M+K]^+$ calcd. for $C_{58}H_{89}O_{10}N_9$ 1071.67.

The crude cyclic peptide: 40 mg (HPLC purity = 65%, t_R = 14.22 min, 50–90%B in 25 min). Purification by preparative HPLC ($t_R = 20.96$ min, 50–95% B in 40 min), yielded **4** (11.9 mg, 30%, HPLC purity = 100%, $t_R = 12.53$ min, 50–90% B in 25 min). MALDI MS: $[M+Na]^+$, 1094.9 $[M+K]^+$ calcd. for $C_{58}H_{87}O_9N_9$ 1053.66.

Cyclo(βhPro^1-βhPro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)

(3). The crude linear peptide: 331 mg (HPLC purity = 79%, $t_R = 15.06$ min, 35–75% B in 25 min). Purification using preparative HPLC ($t_R = 23.56$ min, 35–75%B in 40 min) gave nonapeptide (13.7 mg, 46%, HPLC purity = 100%, t_R = 10.79 min, 40–80% B in 25 min); MALDI MS: 1108.4 $[M+Na]^+$, 1124.4 $[M+K]^+$ calcd. for $C_{59}H_{91}O_{10}N_9$ 1086.40.

The crude cyclic peptide: 38 mg (HPLC purity = 56%, t_R = 16.92 min, 50–90%B in 25 min). Purification by preparative HPLC yielded **5** (11 mg, 28%, HPLC purity = 100%, t_R = 16.67 min, 50–90% B in 25 min). MALDI MS: 1090.4 $[M+Na]^+$, 1106.4 $[M+K]^+$ calcd. for $C_{59}H_{89}O_9N_9$ 1067.68.

Cyclo(βiPro^1-Pro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)

(4). The crude linear peptide: 258 mg (HPLC purity = 79%, $t_R = 21.34$ min, 35–75% B in 25 min). Purification using preparative HPLC ($t_R = 31.56$ min, 35–75%B in 40 min) gave nonapeptide (7.7 mg, 39%, HPLC purity = 100%, t_R = 9.23 min, 40–80% B in 25 min); MALDI MS: 1080.4 $[M+Na]^+$, 1096.3 $[M+K]^+$ calcd. for $C_{57}H_{87}O_{10}N_9$ 1057.66.

The crude cyclic peptide: 60 mg (HPLC purity = 38%, $t_R = 16.02$ min, 50–90% B in 25 min). Purification using preparative HPLC ($t_R = 28.12$ min, 50–95%B in 40 min), yielded **6** (10 mg, 25%, HPLC purity = 100%, $t_R = 13.54$ min, 50–90% B in 25 min). MALDI MS: 1062.2 $[M+Na]^+$, 1078.4 $[M+K]^+$ calcd. for $C_{57}H_{85}O_9N_9$ 1039.65.

Cyclo(Pro^1-βiPro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)

(5). The crude linear peptide: 278 mg (HPLC purity = 76%, $t_R = 12.49$ min, 35–75% B in 25 min). Purification using preparative HPLC ($t_R = 27.08$ min, 35–55% B in 40 min), yielded nonapeptide (13 mg, 43%, HPLC purity = 100%, t_R = 8.34 min, 40–80%B in 25 min); MALDI MS: 1080.4 $[M+Na]^+$, 1096.4 $[M+K]^+$ calcd. for $C_{57}H_{87}O_{10}N_9$ 1057.66.

The crude cyclic peptide: 50 mg (HPLC purity = 49%, t_R = 13.09 min, 50–90% B in 25 min). Purification using preparative HPLC ($t_R = 19.89$ min, 50–95% B in 40 min), yielded **7** (16 mg, 41%, HPLC purity = 100%, $t_R = 12.05$ min, 50–90% B in 25 min). MALDI MS: 1062.2 $[M+Na]^+$, 1078.4 $[M+K]^+$ calcd. for $C_{57}H_{85}O_9N_9$ 1039.65.

Cyclo(βiPro^1-βiPro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9)

(6). The crude linear peptide: 267 mg (HPLC purity = 73%, $t_R = 13.27$ min, 35–75%B in 25 min). Purification using preparative HPLC ($t_R = 27.19$ min, 35–55% B in 40 min) gave nonapeptide (13 mg, 43%, HPLC purity = 100%, t_R = 8.44 min, 40–80% B in 25 min); MALDI MS: 1080.4 $[M+Na]^+$, 1096.3 $[M+K]^+$ calcd. for $C_{57}H_{87}O_{10}N_9$ 1057.66.

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The crude cyclic peptide: 64 mg (HPLC purity = 43%, t_R = 12.02 min, 50–90%B in 25 min). Purification using preparative HPLC (t_R = 17.92 min, 50–95% B in 40 min), yielded **8** (5 mg, 13%, HPLC purity = 100%, t_R = 9.66 min, 50–90% B in 25 min); MALDI MS: 1062.2 $[M + Na]^+$, 1078.4 $[M + K]^+$ calcd. for $C_{57}H_{85}O_9N_9$ 1039.65.

Biological Tests

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

Reagents and media. ConA, PWM, 93-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), and DMSO were from Sigma, RPMI-1640 and Hanks' medium from Cibi/Life Technologies, UK, and fetal calf serum (FCS) from Gibco. SRBC were delivered by Wroclaw Agriculture University (Wroclaw, Poland).

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

The proliferative response of mouse splenocytes to mitogens. Spleens were homogenized by pressing the organs through a plastic screen into Hanks' medium. After centrifugation, the cells were treated for 5 min with 0.83% ammonium chloride to lyse the erythrocytes. Then, the cells were washed twice by centrifugation in Hanks' medium and filtered through sterile cotton wool to remove dead cells and debris. Eventually, the cells were resuspended in a medium consisting of RPMI-1640, supplemented with FCS, L-glutamine, sodium pyruvate, 2-mercaptoethanol, and antibiotics (referred later as the culture medium). The cells were distributed to flat-bottom 96-well culture plates at density of 10^5 cells/100 μl/well. ConA or PWM was used at a dose of 2.5 μg/ml and the studied peptides at 1–100 μg/ml concentrations. The cells were incubated for 3 days in a cell culture incubator and the rate of cell proliferation was determined using colorimetric MTT method [51]. The results were presented as mean optical density (OD) values at 550-/630-nm wavelength from quadruplicate wells (determinations) ± standard error (SE). The studied effects of the compounds were compared with appropriate DMSO controls (dilutions).

Determination of the humoral immune response in vitro. Mice were sensitized with 0.2 ml of 1% SRBC suspension. Four days later, the animals were sacrificed and the splenocyte suspension was prepared as described above. The cells were distributed to flat-bottom, 24-well culture plates (5×10^6/ml/well), followed by addition of SRBC (0.1 ml of 0.005% suspension). The peptides were used at concentrations of 10 and 100 μg/ml. After a 4-day incubation, the number of AFC was determined [52]. The results were presented as mean AFC numbers from quadruplicate wells ± SE.

Determination of toxicity of the compounds for WEHI 164.13 cells. Toxicity of the compounds was determined with respect to WEHI 164.13 cells [53]. The compounds were dissolved in DMSO, then in the culture medium at the concentration range 0.1–100 μg/ml. Appropriate concentrations of DMSO served as respective controls. The monolayers of WEHI 164.13 cells (2×10^5/well) were incubated with DMSO control solutions or the compounds for 24 h in a cell culture incubator. After incubation the cell viability was determined by MTT colorimetric method [52]. The results are presented as percentage of the OD values in relation to appropriate solvent (DMSO) concentrations, where the cell viability was regarded as 100%.

Statistics. The data were statistically evaluated using the Student's *t*-test. The results were presented as mean values from four determinations (culture wells) ± SE and regarded significant when $p < 0.05$. In the figures the significance was presented as follows: $^*p < 0.05$, $^{**}p < 0.02$, $^{***}p < 0.001$. Other differences were not significant.

Supporting Information

Supporting information may be found in the online version of this article.

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