JOURNAL OF PHYSICAL ORGANIC CHEMISTRY
J. Phys. Org. Chem. 2004; **17:** 625–630
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/poc.782

Correlating biological activity with calculated geometric motifs in cyclolinopeptide A analogs†

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Received 23 May 2003; revised 22 September 2003; accepted 25 September 2003

ABSTRACT: Three linear and three cyclic analogs of cyclolinopeptide A, with phenylalanine residues in position 8 and/or 9 replaced by *N*-benzylglycine, were synthesized using the SPPS method and cyclization with TBTU reagent. The peptides were examined for their immunosuppressive activity in a lymphocyte proliferation test. In order to test the importance of the *edge-to-face* interactions between Phe[8]–Phe[9] aromatic rings, molecular modeling studies were carried out. The results support its importance of the *edge-to-face* interactions for the biological activity of these compounds and indicate that the distance between the two rings also plays an essential role. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: cyclolinopeptide A; geometric motifs; biological activity; molecular modeling

INTRODUCTION

A serious problem in transplantology, a dynamically expanding brunch of contemporary medicine, is the survival of the transplanted organ. A wider use of two existing and effective immunosuppressants, cyclosporin A (CsA, Sandimmun)[1] and FK-506,[2] as a potent drugs for the prevention of graft rejection is limited by their side-effects. The search for new immunosuppressants, exhibiting the similar mechanism of action but devoid of toxicity, especially in the group of naturally existing immunomodulatory peptides and their analogs, is therefore an important challenge for medicinal chemists.

Cyclolinopeptide A (CLA) is a highly hydrophobic, cyclic nonapeptide of the sequence cyclo(Leu[1]–Ile[2]–Ile[3]–Leu[4]–Val[5]–Pro[6]–Pro[7]–Phe[8]–Phe[9]) isolated from linseed oil by Kaufmann and Tobschirbel.[3] A peculiarity of CLA structure is the presence of *cis*-configurated peptide bond between Pro[6]–Pro[7] residues and the existence of an *edge-to-face* interaction between Phe[8]–Phe[9] aromatic residues.[4–7] Recently, a cyclosporin-like immunosuppressive activity has been attributed to CLA and antamanide (ANT).[8–11] In particular, CLA was found to possess an immunosuppressive activity range comparable to that of CsA, with the mechanism that depends on the inhibition of the interleukin-1 and interleukin-2 action. This biological activity was assessed by the *in vitro* plaque-forming cell test (PFC) and the *in vivo* delayed-type hypersensitivity test (DTH).

The results of our recent work[12] on CLA analogs modified in positions 6–7 by a 1,5-disubstituted tetrazole ring, a good *cis*-petide bond mimetic, and comparison with data reported in literature makes it possible to conclude that the Pro[6]–Pro[7]–Phe[8]–Phe[9] segment and the preservation of the CLA backbone conformation seem to be important for immunosuppressive activity. For further evaluation of the significance of this particular unit for the CLA biological activity, we have synthesized three linear and three cyclic analogs in which phenylalanine residue in position 8 and/or 9 has been replaced by *N*-benzylglycine (N-BzlGly):

Leu–Val–Pro–Pro–**N–BzlGly**–Phe–Leu–Ile–Ile **1**

Leu–Val–Pro–Pro–Phe–**N–BzlGly**–Leu–Ile–Ile **2**

Leu–Val–Pro–Pro–**N–BzlGly–N–BzlGly**–Leu–Ile–Ile **3**

cyclo(–Leu–Val–Pro–Pro–**N–BzlGly**–Phe–Leu–Ile–Ile–) **4**

cyclo(–Leu–Val–Pro–Pro–Phe–**N–BzlGly**–Leu–Ile–Ile–) **5**

cyclo(–Leu–Val–Pro–Pro–**N–BzlGly–N–BzlGly**–Leu–Ile–Ile–) **6**

The effects exerted by cyclic peptides, examined for their immunosuppressive activity in a lymphocyte proliferation test (LPT), were compared with those produced by their linear precursors and by CsA. In order to test the importance of the *edge-to-face* interaction between Phe[8]–Phe[9] aromatics molecular modeling studies

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Contract/grant sponsor: Polish State Committee for Scientific Research; *Contract/grant number:* 4 PO5F 004 13.
†Selected paper part of a special issue entitled 'Biological Applications of Physical Organic Chemistry dedicated to Prof. William P. Jencks'.

were carried out aimed at correlating the relative positions of these two rings and the biological activity of the corresponding cyclopeptides.

EXPERIMENTAL

All solvents were purified by conventional methods. Evaporations were carried out under reduced pressure. HPLC for all compounds was performed on an LDC/Milton-Roy Analytical instrument using a Vydac C_{18} column (25×0.46 cm i.d.), flow-rate 1.0 ml min^{-1}, detection at 220 nm and gradient elution with solvents (A) 0.05% trifluoroacetic acid in water and (B) 0.038% trifluoroacetic acid in acetonitrile–H_2O (90:10) N^{α}-*tert*-Butoxycarbonyl (Boc)-protected amino acids were obtained from ChemImpex International. Coupling reagents (TBTU and HOBt) were obtained from Richelieu Biotechnologies and Fluka, respectively. CsA was purchased in Sigma-Aldrich and used as a dimethyl sulfoxide (DMSO) solution. The preparation of Boc-N-BzlGly-OH (**9**), N-BzlGly-OBzl·Tos-OH (**10**) and the dipeptide Boc-N-BzlGly-N-BzlGly-OH (**12**) has been described elsewhere.[13]

Solid-phase peptide synthesis

Boc-Ile attached to chloromethylated Merrifield resin was prepared through esterification reaction performed in DMF in the presence of dry KF (6 equiv.) at 50 °C for 72 h. The Boc-Ile-polymer after washing (5 × DMF, 5 × water, 5 × MeOH, 5 × DCM, 5 × MeOH) was dried under reduced pressure over KOH and P_2O_5 and a substitution level of 0.432 mmol g^{-1} was determined by weight gain measurements. The linear peptides were synthesized by the standard SPPS methodology starting from 0.834 g (0.4 mmol) of Boc-Ile-resin. The standard single TBTU/HOBt protocol was used for all single amino acid derivatives and was repeated whenever the Kaiser test was found positive. In all cases, where the Kaiser test was slightly positive after second coupling, remaining free amino groups were acetylated with the aid of acetic anhydride in DCM. The peptide resin was cleaved with anhydrous HF in the presence of anisole (~10%) at 0 °C for 60 min. After HF removal under reduced pressure, the resin was washed several times with diethyl ether and then extracted with aqueous acetonitrile. Lyophilization of the extracts yielded crude linear peptides: (N-BzlGly8)LA, 381 mg (0.376 mmol, 94% yield, 85.7% purity by HPLC); (N-BzlGly9)LA, 386 mg (0.385 mM, 96.2% yield, 89.9% purity by HPLC); and (N-BzlGly8,9)LA, 390 mg (0.368 mmol, 92% yield, 84.97% purity by HPLC).

Purification of the crude linear precursors was achieved by preparative HPLC (LCD Analytical) on a reversed-phase column [Vydac C18, 250 × 25 mm i.d., 10 µm column, $\lambda = 214$ nm, flow-rate 16 ml min^{-1} with a linear gradient from 45 to 80% B in A (B, 0.038% TFA in 82% acetonitryle–water; A, 0.05% TFA in water) in 40 min]. ESI-mass spectra confirmed the expected structure of main products in crude (N-BzlGly8)LA, (N-BzlGly9)LA and (N-BzlGly8,9)LA. Linear precursors were cyclized by means of TBTU in the presence of HOBt and DIPEA in DCM. Crude cyclic peptides were purified in the same way as their linear precursors.

The homogeneity of the purified peptides were checked by analytical HPLC [Vydac C_{18}, 250 × 4.6 mm i.d., 5 µm column, $\lambda = 214$ nm, flow-rate 1 ml min^{-1} with a linear gradient from 50 to 95% B in A (B, 0.038% TFA in 82% acetonitrile–water; A, 0.05% TFA in water) in 25 min] for cyclic and linear peptides on an LDC/Milton-Roy Analytical instrument. The structures of the pure peptides were confirmed by ESI-msss spectra, which were recorded with an Esquire 3000 apparatus (Bruker, Karlsruhe, Germany), equipped with an ESI ionization source with an ion-trap detector.

Biological tests

The immunosuppressive activity of the linear and cyclic analogs of CLA was investigated by the lymphocyte proliferation test (LPT) and compared with the immunosuppressive effect of CsA as a reference immunosuppressant.

Assessment of cell viability. Peripheral blood mononuclear cells (PBMC) were cultured in presence of serial dilutions of drugs for 24, 48 and 72 h. After incubation, the cells were mixed with 0.2% trypan blue and examined on microscopic slides. Blue cells were considered to be dead and percentage viability was referred to 200 examined cells.

Assessment of immunosuppressive activity. LPT was performed according to the following methodology.[14] Lymphocytes (PBMC) were isolated from heparinized blood of healthy volunteers by gradient centrifugation on Ficoll–Hypaque (Pharmacia).[15] After three washes in culture medium (RPMI-1640), PBMC were resuspended to a final concentration 2×10^6 cells ml^{-1} in RPMI-1640 supplement with 10% fetal calf serum, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (all reagents from GIBCO, Germany). PBMC were cultured in four replicates at 200 µl per well in 96-well microtiter plates using different concentrations of the examined analogs of CsA and CLA. Cells were stimulated with phytohemagglutinine (PHA) at a final concentration of 10 µg ml^{-1}. Plates were incubated at 37 °C in a 5% CO_2 humidified atmosphere. After 48 h of incubation, 1 µCi per well of radiolabeled [methyl-^{3}H]thymidine (Lacomed, Czech Republic) was added; 24 h later, cultures were harvested on to glass filters and thymidine incorporation into DNA was measured using liquid scintillation counter (LKB) and expressed as counts per minute

(CPM). The suppressive effect of the studied compounds were expressed as percentage of PHA-stimulated CPM values (without drugs).

Theoretical calculations

The structure of the reference compound, CLA, was optimized at the molecular mechanics levels Amber,[16] OPLS[17] and CHARMM22[18] as implemented in Hyperchem 7.0,[19] and MMFF94[20] and SYBYL[21] as implemented in Titan.[22] Full conformational searches were performed using the Monte Carlo approach and OPLS and MMFF94 force fields. The conformational space of 14 400 structures was studied using the MMFF94 force field. The eight most stable conformations within a 10 kcal mol^{-1} range (1 kcal = 4.184 kJ) were identified. A conformational search using OPLS was performed over all torsion angles of the macrocycle using a 30° increment. The ten most stable conformations were identified within a 10 kcal mol^{-1} range.

For comparison, the structure of CLA was optimized using PM3,[23] PM5[24] and PM6[25] semi-empirical parameterizationsas implemented in MOPAC2002.[26] Additionally, for the optimization using the PM5 method, the continuum solvent model COSMO[27] was used. The geometries of **4**–**6** were optimized using the OPLS force field. In all cases the convergence criterion was set at 0.01 kcal mol^{-1} Å^{-1}.

RESULTS AND DISCUSSION

Syntheses

The preparation of *N*-benzylglycine was based on the previously reported strategy of reductive alkylation.[28,29] First, glycine was converted into its *N*-benzyl analog **8** using benzaldehyde and sodium borohydride as a reducing reagent (Fig. 1). The *N*-benzylglycine **8** was obtained in moderate yield (37%) and was accompanied by a significant amount of *N*,*N*-dibenzylglycine as a byproduct. To protect the amino function of **8**, the Boc group was introduced using di-*tert*-butyl pyrocarbonate as a reagent under the standard reaction conditions.[30] The synthesis of the *N*-benzylglycine benzyl ester *p*-toluenosulfonates **10** was achieved using an esterification procedure catalyzed by *p*-toluenesulfonic acid.[31]

Compound **9** was coupled to **10** giving the fully protected dipeptide Boc-N-BzlGly-N-BzlGly-OBzl (**11**) using TBTU [*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate] in the presence of HOAt (1-hydroxy-7-azabenzotriazole) as a condensing reagent.[32] The observed low yield of the desired dipeptide **11** was probably due to the high steric hindrance of both amino and carboxyl components. Removal of the benzyl ester group from **11** by catalytic hydrogenolysis in methanol over 10% Pd on charcoal yielded the dipeptide **12** with an unprotected carboxylic group.

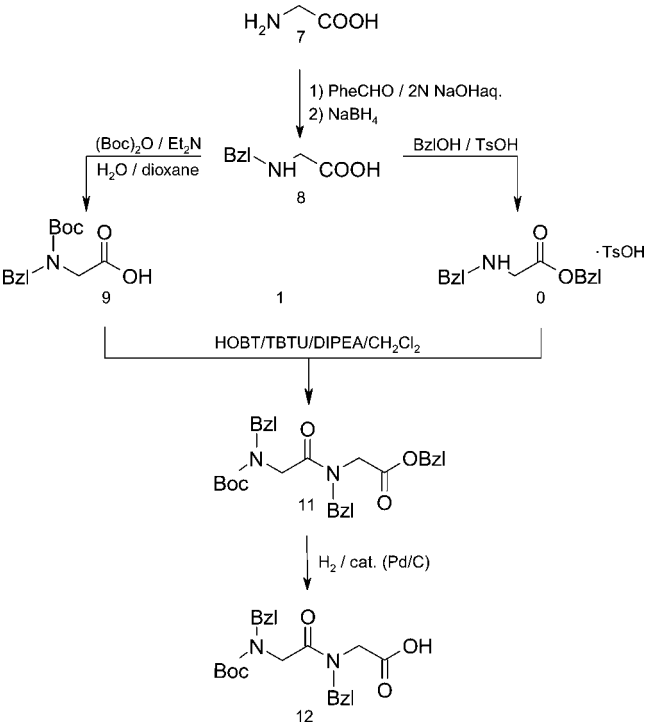

Figure 1. Synthesis of *N*-benzylglycine and derivatives

The synthesis of the desired peptides **4**, **5** and **6** was achieved according to the synthetic scheme shown in Fig. 2 on a polymeric support (Merrifield type resin) using TBTU as the coupling reagent. In the case of the peptide **6**, the dipeptide Boc-N-BzlGly-N-BzlGly-OH was used to avoid possible difficulties in this particular coupling step. After being cleaved from the resin, the crude linear precursors **1**, **2** and **3** were cyclized by means of TBTU in the presence of HOAt and DIPEA (*N*,*N*-diispropylethylamine) in dichloromethane (DCM) at a much lower concentration than described for peptide cyclization reactions. Crude cyclic peptides **4**, **5** and **6** and their linear precursors **1**, **2** and **3** were purified by HPLC and characterized by MS and chromatographic techniques (Table 1).

Biological studies

To evaluate the immunosuppressive activity of the synthesized compounds, the lymphocyte proliferation test (LPT) was employed, which is a standard method of assessment of the effect of drugs on immunological processes.[33] In contrast to earlier studies using animal models, human cell system (lymphocytes from healthy human volunteers) was used for the assessment of the immunosuppressive activity. To exclude the toxic effects of the synthesized compounds, as a prerequisite for LPT, the effect of the synthesized compounds on the viability of lymphocytes was assessed. Only the concentrations of compounds that secured at least 90% cell viability, found by the dye exclusion method, were considered as potentially useful for the immunosuppressive activity testing (Table 2).

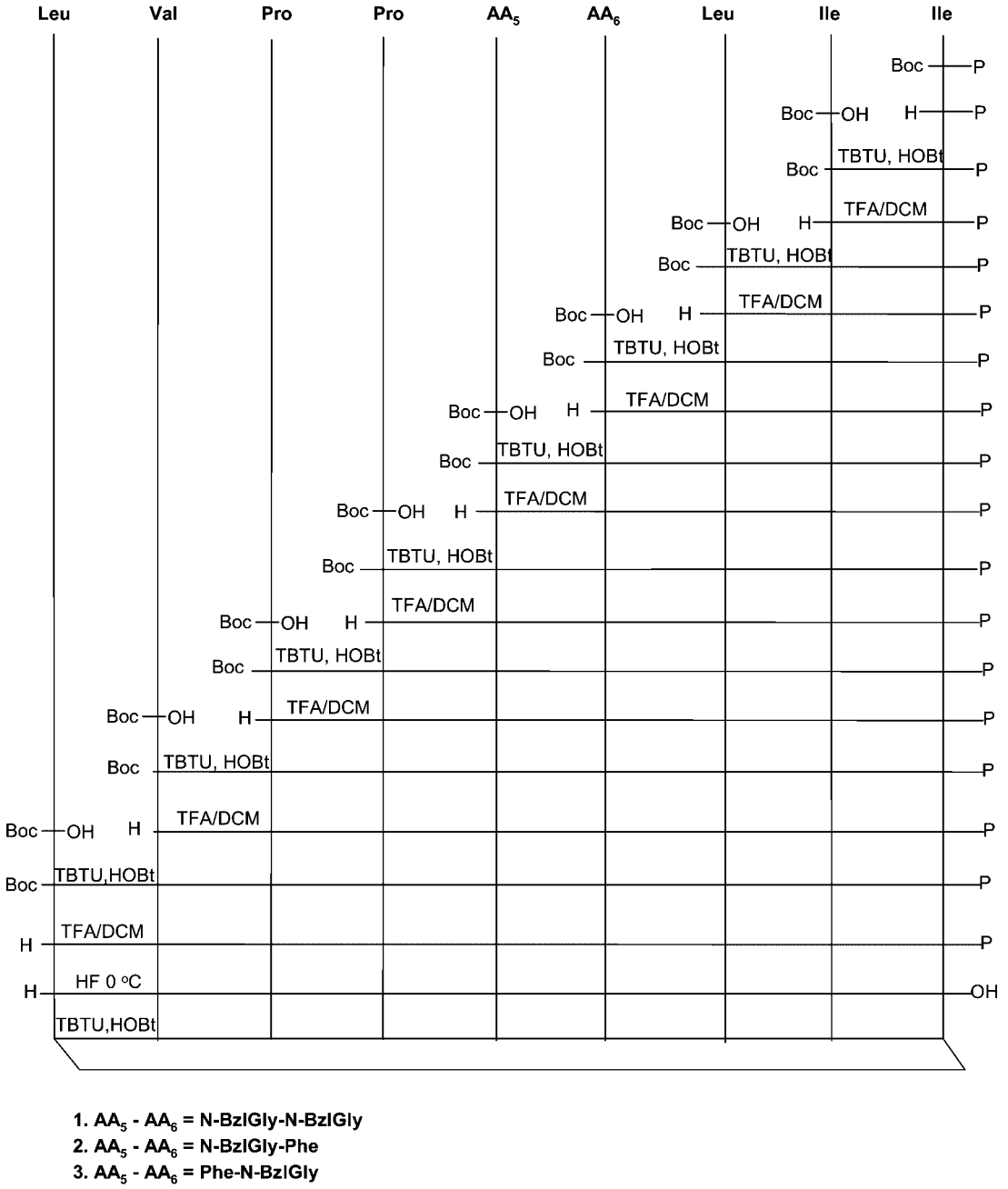

Figure 2. Synthetic scheme for CLA analogs containing N-BzlGly residues

Table 1. Analytical data for peptoid CLA analogs

No.	Peptide	Yield[a] (%)	t_R^b(min)	MW[c]
1	(N-BzlGly8)LA	85.7	11.15	1058.3/1058.6
2	(N-BzlGly9)LA	89.9	11.38	1058.3/1058.9
3	(N-BzlGly8,9)LA	84.97	11.87	1058.3/1058.7
4	(N-BzlGly8)CLA	22.97	23.33	1040.3/1040.6
5	(N-BzlGly9)CLA	28.48	21.56	1040.37/1040.5
6	(N-BzlGly8,9)CLA	33.61	18.81	1040.37/1040.6

[a] Peptide content in crude product as shown by analytical HPLC with linear gradient from 40 to 90% B in 25 min. (A) 0.05% trifluoroacetic acid in water and (B) 0.038% trifluoroacetic acid in acetonitrile–H2O (90:10).
[b] Gradient as above.
[c] Molecular weight calculated/found by ESI-MS measurements.

The linear compounds (**1**, **2** and **3**) did not affect cell viability over the range of concentrations from 0.2 to 25 $\mu g\,ml^{-1}$. Cyclic compounds (**4**, **5** and **6**), on the other hand, demonstrated toxic activity at concentrations above 2 $\mu g\,ml^{-1}$.

The reference drug CsA and the native CLA demonstrated a dose-dependent inhibition of PHA-stimulated proliferation of lymphocytes: 42, 50, 87 and 98% for CsA and 34, 41, 93 and 98% for CLA when tested in concentrations 1, 2, 10 and 20 $\mu g\,ml^{-1}$, respectively (Table 3). In contrast, none of the synthesized analogs demonstrated any significant immunosuppressive activity over the range of non-toxic concentrations.

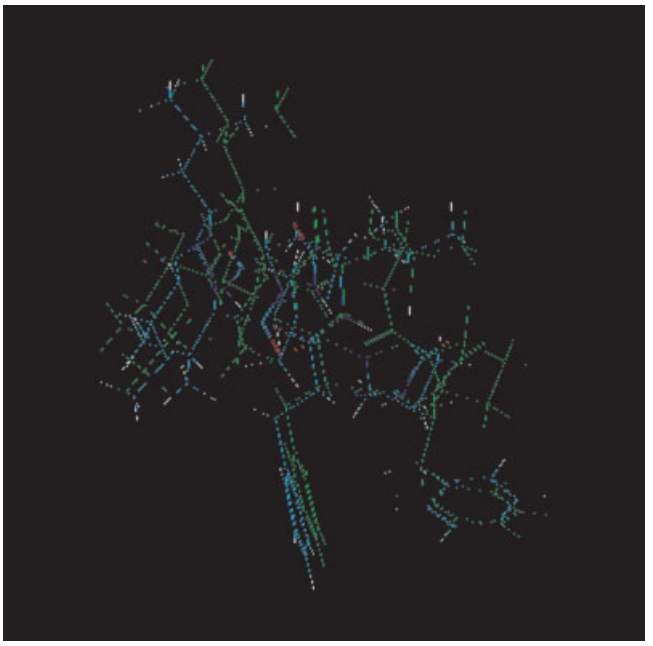

Plate 1. Superposition of the OPLS-optimized and NMR-based structures of CLA (shown in green)

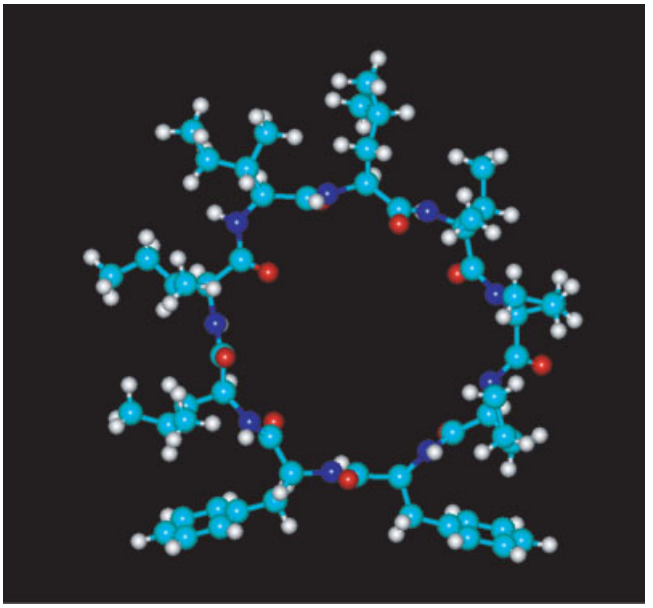

Plate 2. The best conformation of CLA obtained using the MMFF94 force field

Table 2. Viability of human peripheral blood lymphocytes after incubation (% of lymphocyte viability)

	(N-BzlGly^x)LA									(N-BzlGly^x)CLA								
	$x=8$			$x=8, 9$			$x=9$			$x=8$			$x=8, 9$			$x=9$		
c ($\mu g\,ml^{-1}$)	*24*[a]	*48*	*72*	*24*	*48*	*72*	*24*	*48*	*72*	*24*	*48*	*72*	*24*	*48*	*72*	*24*	*48*	*72*
0.2	100	99	98	100	97	99	99	97	98									
1.0	100	98	96	100	97	96	99	96	95	100	96	89	100	98	63	99	97	90
2.0										99	90	82	98	96	56	96	94	84
2.5	100	98	95	99	97	94	99	96	94									
5.0	100	96	88	99	95	89	99	96	89									
10	99	95	87	99	96	88	99	96	88	78	68	51	87	85	45	75	68	46
20										56	46	35	76	71	39	55	45	32
25	96	92	80	96	94	86	99	94	87									

[a] Values in italics: duration in hours.

Assuming that all new compounds with potential application in medicine should demonstrate immunosuppressive activity at least comparable to that of CsA, we conclude that the synthesized CLA analogs are not likely to be suitable as immunosuppressants. Our study does not exclude, that the examined compounds could have an immunomodulatory effect on other immunological functions.

Computer modeling

For the model compound, CLA, the quality of several force fields (and semi-empirical Hamiltonians for comparison) was tested by comparing the structure elucidated from NMR measurements with structures optimized using the corresponding methods. Calculations using the SYBYL force field failed in reaching convergence. Among other methods used, the best results were obtained for the OPLS and MMFF94 force fields. The results are given in Table 4.

Plate 1 shows the structure obtained using OPLS superimposed on the reference structure of CLA generated based on the NMR results. Semi-empirical calculations using PM3 and PM5 parameterizations yielded geometries of quality comparable to those with the best molecular mechanics methods whereas the PM6 parameterization performed slightly worse. It should be noted, however, that the PM6 method applied here is not finalized. Our experience indicates that it yields high-quality geometries for the corrinoid systems (to be published elsewhere). There was no change in the quality of the geometry when the COSMO continuum solvent model was included with the PM5 method.

These results suggest that OPLS and MMFF94 are the best methods (of those studied) for describing this class of system. For both of them full conformation analysis using the Monte Carlo method was performed. This analysis using the MMFF94 force field yielded two conformers, which differ in energy by $0.3\,kcal\,mol^{-1}$. Both have circular nine-peptide ring as illustrated in Plate 2. As can be seen, the phenyl rings are placed nearly in the same plane rotated outwards from each other. Also, all hydrogen bonds across the macrocyclic ring are neglected. On the basis of these results we decided to use the OPLS force field for the comparison of geometries of **4–6**. From the optimized structures we calculated the angle between the planes of two phenyl rings that is equal to about 90° in the reference NMR-based structure. The results are listed in Table 5.

All three cyclic compound **4–6** show negligible bioactivity within the range of non-toxic concentrations. The angle between the phenyl rings, although much smaller than 90° for all three compounds, shows large variations that do not parallel the biological activity, although it should be noted that this angle in **6** is very close to one found for CLA. The importance of the through-space interactions between π-systems in

Table 3. Effect of studied compounds on lymphocyte proliferation (% inhibition of lymphocyte proliferation)

		Concentration ($\mu g\,ml^{-1}$)			
No.	Compound	1	2	10[a]	20[a]
	CsA	42	50	87	98
	CLA	34	41	93	98
1	(N-BzlGly8)LA	0	0	0	0
2	(N-BzlGly8,9)LA	0	0	0	0
3	(N-BzlGly9)LA	0	0	0	0
4	(N-BzlGly8)CLA	4	11	25	47
5	(N-BzlGly8,9)CLA	7	12	31	57
6	(N-BzlGly9)CLA	5	10	27	50

[a] Toxic concentrations.

Table 4. R.m.s. difference between CLA geometry optimized using different force fields and semi-empirical parametrizations

Method	R.m.s. distance (Å)
Amber	1.004
OPLS	0.781
CHARMM22	1.64
MMFF94	0.769
PM3	0.793
PM5	0.795
PM6	0.961

Table 5. Angles between the phenyl rings calculated using the OPLS force field

Compound	Angle (°)	C—C (Å)
CLA (from NMR)	89.6	6.6
CLA	62.8	6.5
(N-BzlGly8)CLA (**4**)	33.7	8.3
(N-BzlGly8,9)CLA (**5**)	49.4	7.3
(N-BzlGly9)CLA (**6**)	59.1	10.1

molecular recognition, and also other chemical phenomena, has been recognized earlier.[34] Our results, however, clearly indicate that the *edge-to-face* position is not the sole factor influencing the bioactivity of the compounds studied. Inspection of the optimized structures of all the compounds hinted at the distance between the two rings being an additional factor. In Table 5, the distances between the *para*-carbons of the rings are listed, illustrating that these rings are much further apart in **6** than in the other three compounds, and that **5** has this distance closest to that found in CLA. Hence the balance of the phenyl rings distance and their relative angle seems to control the biological activity of the compound. This is not surprising as both phenyl rings are probably involved in interactions with the receptor. Based on the findings described above, we surveyed a number of cyclic CLA analogs, identifying those that are characterized by the optimal angle and distance between the two phenyl rings. The most promising analogs were synthesized. The preliminary results for their biological activity are very encouraging,[35] showing a fruitful interplay between theoretical calculations and the practice of rational synthesis of bioactive compounds.

CONCLUSIONS

Finding new compounds of desired biological activity is a tedious and expensive task. Computational tools, on the other hand, are becoming widely available owing to advances in computer technology and the software development. It is therefore desirable to explore *a priori* structures of the compounds to be synthesized and to compare their geometric motifs with those of compounds of known bioactivity. In this work we have tested the importance of the *edge-to-face* interactions between Phe8–Phe9 of CLAs. All synthesized analogs are devoided of significant biological activity; however, our computational results strongly support the importance for biological activity of both the *edge-to-face* arrangement and the distance between the two rings.

Acknowledgments

We thank Professor Hiroshi Morita (Faculty of Pharmaceutical Sciences, Hokkaido University, Japan) for providing us with the NMR structure of CLA. This work was partially supported by the Polish State Committee for Scientific Research, grant No. 4 PO5F 004 13, and through university funds.

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