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New analogue of cyclolinopeptide B modified by amphiphilic residue of α-hydroxymethylmethionine★✪

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In order to evaluate the role and influence of the methionine residue on the biological activity of cyclolinopeptide B, an analogue with methionine residue in position 7 replaced by the amphiphilic (*S*)-α-hydroxymethylmethionine residue was synthesized. This peptide exhibits high immunosuppressive activity in the cellular, and to a lesser degree in the humoral immune response, comparable to that of CsA. In addition, the peptide was devoid of toxicity, even at high doses.

The use of cyclosporine A (CsA) and FK-506 in transplantology is limited by their side-effects (Soy *et al.*, 1995; Hojo *et al.*, 1999). The search for new immunosuppressants, exhibiting a similar mechanism of action but devoid of toxicity, especially in the group of natural immunomodulatory peptides and their analogues, is an important challenge for medicinal chemistry.

Cyclolinopeptide B (CLB), a natural cyclic nonapeptide *cyclo*(-Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-Ile-), was isolated together with the very similar CLA *cyclo*(Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val) from seeds of *Linum*

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Abbreviations: Boc, *tert*-butyloxycarbonyl; Boc_2O, *tert*-butyloxycarbonyl anhydride; CLB, cyclolinopeptide B; CsA, cyclosporine A; DIEA, diisopropylethylamine; α-HmM, α-hydroxymethylmethionine; HF, hydrogen fluoride; HPLC, high performance liquid chromatography; MALDI, Matrix-assisted laser desorption ionisation; OVA, ovalbumin, PFC, plaque forming cells; SRBC, sheep red blood cells; DTH, delayed type hypersensitivity, TBTU, *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TLC, thin layer chromatography; $TMAH{\times}5H_2O$, tetramethylammonium hydroxide pentahydrate.

usitatissimum (*Linaceae*) in 1959 (Kaufman & Tobschirbel, 1959). Sequence analysis of both peptides was performed by Prox and Weygand (Prox & Weygand, 1967; Weygand, 1968) and the CLB sequence was additionally supported by Morita *et al.* in 1997 (Morita *et al.*, 1997). This peptide reveals an inhibitory effect on a mitogen (concanavalin A), comparable to that of cyclosporin A (Morita *et al.*, 1999). The three-dimensional structure of CLB (Fig. 1) was established by Takeya *et al.*

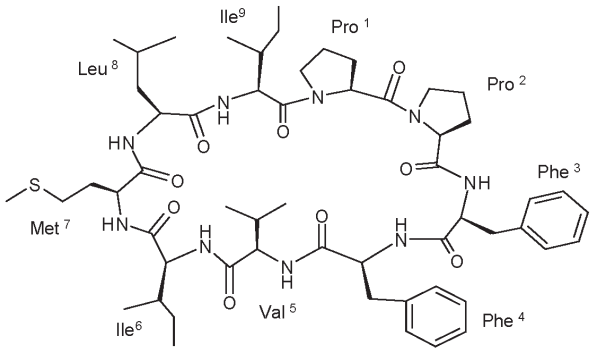

Figure 1. Structure of cyclolinopeptide B.

(Morita *et al.*, 1999; Matsumoto *et al.*, 2002) showing similarity to the known CLA structure with a *cis* amide bond between two proline residues. A recent report by Picur *et al.* (2002) has shown that natural CLB can adopt two different structural forms with opposite thermal effects concluded, from their CD and NMR spectra.

In this paper we describe the synthesis of an analogue of CLB with the methionine residue in position 7 replaced by the amphiphilic (*S*)-α-hydroxymethylmethionine in order to evaluate the role of methionine residue and the influence of the hydrophilic hydroxymethyl group on the peptide biological activity. It is worth to note that (*S*)-α-hydroxymethylmethionine might be considered as a hybrid of two amino acids, serine and methionine.

MATERIALS AND METHODS

Chemistry

Racemic α-hydroxymethylmethionine was synthesized by selective α-hydroxymetylation, and resolved into enantiomers by fractional crystallization of diastereomeric salts of their N-benzoyl derivatives with (–)-quinine, using the method previously described (Witkowska *et al.*, 2001). (*S*)-Boc-HmM was prepared according to published procedure (Khalil *et al.*, 1996) in acetonitrile using TMAH $\times$ $5H_2O$ and Boc_2O.

The peptide was synthesized by the manual solid-phase method using Boc-Val-Merrifield resin (200–400 mesh, 0.59 mmole/g, Novabiochem). N^{α}-Boc-protected amino acids were obtained from commercial sources. Starting with 0.68 g (0.4 mmol) of resin the following protected amino acids were added in a stepwise fashion to the growing peptide chain: N^{α}-Boc-Phe, N^{α}-Boc-Phe, N^{α}-Boc-Pro, N^{α}-Boc-Pro, N^{α}-Boc-Ile, N^{α}-Boc-Leu, N^{α}-Boc-(*S*)-HmM, N^{α}-Boc-Ile. The amino acids were coupled in 3-fold excess using TBTU (3 equiv.) in the presence of DIEA (6 equiv.). When α-hydroxymethylamino acid was acylated or used as an acylating component, prolonged reaction times (24 h for repeated coupling) was necessary. Removal of the Boc protecting group was performed with 50% (v/v) TFA in CH_2Cl_2 for 5 and 20 min, followed by CH_2Cl_2 washes (3 $\times$ 1 min), a neutralization with 10% DIEA in CH_2Cl_2 for 5 and 10 min and CH_2Cl_2 washes (3 $\times$ 1 min). Completion of coupling reactions was monitored by the ninhydrin test (Kaiser *et al.*, 1970). The linear peptide was cleaved from the resin using anhydrous HF (5 ml/g resin) procedure with anisole added as a scavenger (1 ml/g resin) for 1 h at 0°C. After evaporation of HF, the resin was extracted three times with anhydrous ethyl ether and, subsequently with 50% aqueous solution of acetic acid. The crude linear peptide was obtained in solid form by lyophilization of the acetic acid extracts and was purified by

preparative reversed-phase HPLC on a Vydac C_{18} column (25 × 2.2 cm) with a linear gradient 40–80% B at a flow rate 12 ml/min. The linear peptide was cyclized by means of TBTU reagent. The cyclic peptide was >99% pure as determined by analytical reversed-phase HPLC on a Vydac C_{18} column (25 × 0.46 cm) using a linear gradient of 40–80% B in 25 min at a flow rate of 1 ml/min, with UV detection at 220 nm. The relative molecular mass (M_r) was confirmed by MALDI-TOF MS. Analytical data of the linear and cyclic analogues are presented in Table 1.

Table 1. Analytical data of linear and cyclic [(*S*)-HmM^7]CLB

Compound	Total formula	M_r	MALDI	HPLC t_R[a] (min)	TLC R_f[b]
linear [(*S*)-HmM^7]CLB	$C_{57}H_{87}O_{11}N_9S$	1106.4	1107	13.20	0.50
cyclic [(*S*)-HmM^7]CLB	$C_{57}H_{85}O_{10}N_9S$	1088.4	1089	19.71	0.91

[a]Linear gradient of 20–50% B over 25 min at a flow rate of 1 ml/min; (A) 0.05% trifluoroacetic acid in water and (B) 0.038% trifluoroacetic acid in acetonitrile/H_2O (90:10, v/v); [b]n-BuOH/AcOH/AcOEt/H_2O (1:1:1:1, by vol.)

Biology

Effects of the peptide on the induction phase and the effector phase of the delayed type hypersensitivity (DTH) in CBA mice as well as the effects of the peptide on the induction phase of the humoral immune response to sheep red blood cells (SRBC) were examined.

Animals. CBA mice (males and females, 10–12 weeks old) were delivered by the Animal Facility of the Institute of Immunology and Experimental Therapy, Wrocław, Poland. Mice were fed commercial, pelleted food and water *ad libitum*.

Generation of the cellular immune response to ovalbumin (OVA). Mice were immunized subcutaneously (s.c.) into tail base with 5 μg OVA in complete Freund's adjuvant (cFa). After 4 days the DTH reaction was elicited by s.c. injection of 50 μg OVA in incomplete Freund's adjuvant (iFa) into hind feet. Specific DTH reaction was calculated by subtracting the foot pad thickness of naïve mice given the eliciting dose of the antigen from the DTH reaction of sensitised mice.

Determination of the effect of the p*eptides on the inductive and effector phase of DTHG response*. *To* establish the effect of the peptides on the inductive phase of the DTH response (events leading to generation of antigen-specific T cells, such as antigen processing and presentation) the compounds were given 2 h before and 24 h after immunization. In order to evaluate the influence of the peptides on the effector phase of the DTH reaction (administration of an eliciting dose of antigen leading to attraction of already existing antigen-specific T cells and accompanying cells (neutrophils and macrophages to the site of antigen depot), the compounds were given i.p. 2 h before the eliciting dose of the antigen.

Reagents. SRBC were supplied by the Laboratory of General Chemistry, Institute of Immunology. SRBC were stored in Alsever's solution for at least 3–4 days before use. Cyclosporine A (CsA) was purchased from Sandoz (Switzerland) and ovalbumin from Sigma. For application in mice CsA, dissolved in a commercial solvent (Cremophor), was further diluted in saline.

Immunization of mice for studies of humoral immune response and determination of plaque forming cells. CBA mice were immunized intraperitoneally (i.p.) with a single 0.2 ml dose of 5% SRBC suspension

Table 2. Effects of [(*S*)-HmM^7]CLB on the induction phase of the delayed type hypersensitivity (DTH) in CBA mice

Compound	Dose (μg/mouse)	DTH Units	Iinhibition (%)	±S.E.	*P* Student's test
Control		17.1			
[(*S*)-HmM^7]CLB	10	5.6	67	0.37	<0.001
	100	4.9	71	0.27	<0.001
CsA	10	5.6	67	0.25	<0.001
	100	2.5	85	0.18	<0.001

The preparations were given intraperitoneally 2 h before and 24 h after the sensitizing dose of antigen (OVA).

Table 3. Effects of [(*S*)-HmM^7]CLB on the effector phase of the delayed type hypersensitivity (DTH) in CBA mice

Compound	Dose (μg/mouse)	DTH Units	Inhibition (%)	±S.E.	*P* Student's test
Control		9.1			
[(*S*)-HmM^7]CLB	20	6.3	30	0.32	<0.001
	200	5.1	44	0.32	<0.001

The preparation was given intraperitoneally 2 h before the eliciting dose of antigen.

Table 4. Effects of [(*S*)-HmM^7]CLB on the induction phase of the humoral immune response to sheep red blood cells (SRBC).

Compound	Dose (μg/mouse)	PFC/10^6	Percent of control	±S.E.	*P* Student's test
Control		1589			
[(*S*)-HmM^7]CLB	10	1411	89	30.69	<0.02
	100	1476	93	59.17	NS
CsA	10	1564	98	16.39	NS
	100	1310	82	35.59	<0.01

The preparations were given intraperitoneally 2 h before and 24 h after immunization of mice with SRBC.

in 0.9% saline. After 4 days the spleens were isolated, single cell suspension was prepared and the number of plaque forming cells (PFC) was determined according to Mishell and Dutton (1967) and expressed as the PFC number per 10^6 viable spleen cells.

Statistics

For evaluation of data the Student's test was applied. The studied groups of mice consisted of 5 mice. The results are presented as mean values from 5 mice (humoral immune response) and 10 determinations (two hind foot pads — from 5 mice): DTH ± standard error (S.E.). The results were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

[(*S*)-HmM^7]CLB strongly inhibited the magnitude of the delayed type hypersensitivity response (Table 2). CsA, a reference drug, was

Table 5. Mice mortality after immunization

Compound	Dose (μg/mouse)	Mice mortality (%)
Control		0
[(*S*)-HmM^7]CLB	50	0
	500	0
CsA	50	0
	500	12.5

The compounds were given i.p. into mice at the indicated doses. The mice were monitored for signs of toxicity and for mortality for 10 days. Eight mice per group were used.

also a strong inhibitor showing a dose dependent effect. As far as the effector phase of DTH was concerned, the peptide exhibited statistically significant inhibition of the DTH reaction ($P < 0.001$), although to a lesser degree as compared to the effects exerted on the inductive phase of DTH (Table 3). CsA was not used in this case because previous studies showed no effect of CsA on the effector phase of DTH. The effect of the peptide on the inductive phase of the humoral immune response was comparable to that of CsA (Table 4), but was minor and significant only at the dose of 10 μg/mouse. Interestingly the synthesized analogue of CLB modified by (*S*)-HmM demonstrated no toxicity (Table 5) even in high doses. These results encourage us to continue the project on construction and synthesis of a new analogues of CLB in the hope of obtaining potent immunosuppressants showing no side-effects. The latter is particularly relevant in the light of the recently reported carcinogenic properties of CsA *via* the cell-autonomous mechanism (Hojo *et al.*, 1999).

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