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A NEW IMMUNOSUPPRESSIVE CYCLIC NONAPEPTIDE, CYCLOLINOPEPTIDE B FROM *LINUM USITATISSIMUM* [1)]

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Abstract: A new cyclic nonapeptide, cyclolinopeptide B: *cyclo* (-Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-Ile-), has been isolated from the seeds of *Linum usitatissimum* and its structure was elucidated by extensive 2D NMR methods and chemical degradations. Cyclolinopeptide B showed potent immunosuppressive activity comparable with that of cyclosporin A.

In 1959, cyclolinopeptide A (CLA), a cyclic nonapeptide, has been isolated from linseed oil as one of the first isolated natural cyclic peptides.[2)] So far, the presence of the other peptides in the linseed oil has not been known. Recently, CLA was found to exhibit a distinct immunosuppressive activity[3)] and is currently intensively investigated chemically, biologically, and pharmacologically.[4)]

As part of our continuing investigation of new biologically active cyclic peptides from higher plants,[5)] we have focused our attention to the isolation of new cyclic peptides from the seeds of *Linum usitatissimum* (Linaceae). Chromatographic purification of the extract prepared from the seeds of *L. usitatissimum* resulted in the isolation of a new cyclic peptide, named cyclolinopeptide B (CLB). CLB showed the inhibitory effect on mitogen (concanavalin A) - induced response of human peripheral-blood lymphocytes. In this communication, we report the structure elucidation of CLB by extensive 2D NMR methods and chemical degradations, and its immunosuppressive activity against human peripheral-blood lymphocytes.

The methanolic extract, prepared from the seeds of *L. usitatissimum* was subjected to Diaion HP-20 column (H_2O-MeOH), and MeOH eluted fraction was chromatographed on a silica gel column ($CHCl_3$-MeOH), followed by HPLC on ODS (80% MeOH) to yield a peptidic compound, cyclolinopeptide B (CLB: 0.0002%), together with CLA.

Cyclolinopeptide B,[6)] white powder, $[\alpha]_D$ -104.1° (c 0.20, MeOH), showed a high-resolution FAB-MS spectral quasimolecular ion peak at *m/z* 1058.6031 $(M+H)^+$, corresponding to the molecular formula, $C_{56}H_{82}N_9O_9S$. The IR absorptions at 3436 and 1659 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. The nonapeptide nature of CLB was evident from its ^{13}C NMR spectrum, showing nine

amide carbonyl groups (δ 173.17, 172.57×2, 171.65, 171.16, 170.72×2, 169.99, and 169.89), as shown in Table 1. In ^{1}H NMR spectrum, however, the signals due to seven amide protons (δ 7.89, 7.82, 7.73, 7.71, 7.55, 7.45, and 7.27) were only observed. In order to elucidate the amino acid composition, CLB was subjected to complete hydrolysis with 6N HCl by heating at 110 °C for 24 h in a sealed tube. The hydrolysate was then analyzed by HPLC and the amino acid composition was shown to be one leucine (Leu), one valine (Val), one methionine (Met), two isoleucines (Ile), two phenylalanines (Phe) and two prolines (Pro) per molecule. These nine amino acid units accounted for the above observed amide protons and amide carbonyl groups. Further, the lack of terminal amino group protons in the ^{1}H NMR and the observed mass molecular weight suggested that CLB might be a cyclic nonapeptide.

The presence of Pro residues in the primary sequence, in general, is promising to produce *cis/trans* conformers which interconvert at a rate slow enough to give the separate signals. However, the NMR spectra in $CDCl_3$-DMSO-d_6 (1:1) at 330K gave well-resolved sharp signals and the presence of minor conformers was not observed. The ^{1}H-^{1}H COSY spectrum allowed the coupling sequence of each amino acid resonance and the corresponding carbon resonances were elucidated on the basis of HMQC spectra as shown in Table 1. The gross structure including the sequence of the amino acids was assembled by connecting the individual amino acids on the basis of connectivities observed in a phase sensitive ROESY and HMBC experiments. The HMBC correlations between each amide carbonyl carbon and neighboring amide NH and Hα protons, and NOEs between neighboring amino acid protons as shown in Fig. 1 indicated the structure of CLB to be *cyclo* (-Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-Ile-). The absolute stereochemistry of the component amino acids was determined to be L-configuration by derivatization of the acid hydrolysate with Marfey's reagent,[7] followed by HPLC analysis.

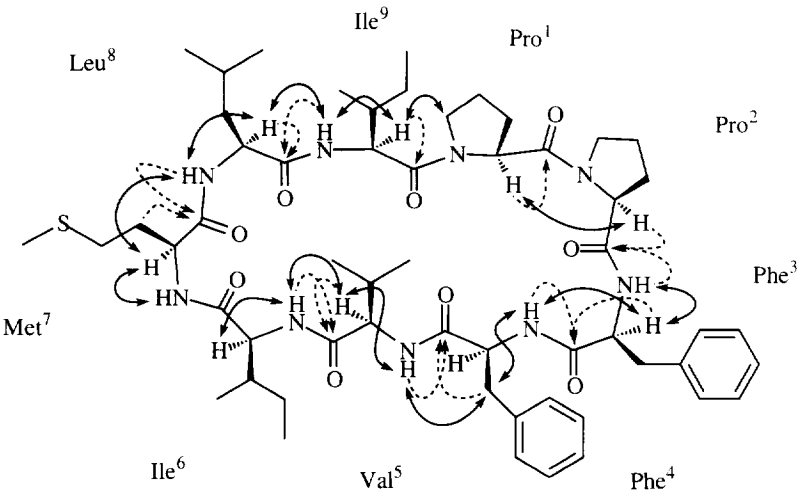

Fig. 1. The structure of cyclolinopeptide B, The arrows show selected NOE correlations, and dashed arrows selected HMBC ones which connect the individual amino acids.

One of the proline amide bonds (the geometry between Pro[1] and Pro[2]) was shown to be *cis* by the strong NOE correlation between Hα in Pro[1] and Hα in Pro[2], the ^{13}C chemical shifts (δ 31.13 and 21.41) of β and γ positions in Pro[2] residue,[8] and the occurrence of a doublet signal of Hα in Pro[2].[9]

CLB showed the inhibitory effect on mitogen (concanavalin A) - induced response of human peripheral-blood lymphocytes (IC_{50}: 44 ng/ml), which is comparable to that of cyclosporin A.[10] It is known that the biological activity of CLA is critically dependent on the sequence and conformation.[11] Studies on the conformational analyses and further biological evaluations of CLA and CLB, possessing different sequencing each other, are currently being done in our laboratories.

Table 1. ^{1}H and ^{13}C NMR Signal Assignments of CLB in $CDCl_3$-DMSO-d_6 (1:1) at 330K.

assignment		^{1}H NMR δ_H [int. mult, J(Hz)]	^{13}C NMR δ_C	assignment		δ_H	δ_C
Pro[1]	α	3.97(1H, dd, 6.0, 9.5)	58.28	Ile[6]	α	3.93(1H, t, 8.8)	60.31
	β	2.10(1H, m)	28.34		β	2.00(1H, m)	35.25
		1.71(1H, m)			γ	1.51(1H, m)	25.29
	γ	2.01(1H, m)	24.21			1.25(1H, m)	
		1.86(1H, m)			γ_{Me}	0.91(3H, d, 6.9)	15.63
	δ	3.67(2H, m)	47.63		δ_{Me}	0.85(3H, d, 6.8)	10.92
	C=O		171.16		NH	7.71(1H, d, 8.8)	
Pro[2]	α	4.14(1H, d, 6.9)	60.54		C=O		169.99
	β	1.93(1H, d, 8.0)	31.13				
		1.65(1H, m)		Met[7]	α	4.46(1H, dt, 4.0, 8.3)	51.82
	γ	1.49(1H, m)	21.41		β	2.14(1H, m)	32.02
		1.00(1H, m)				1.80(1H, m)	
	δ	3.30(2H, m)	46.67		γ	2.44(2H, dt, 4.2, 9.4)	30.34
	C=O		171.65		ε_{Me}	1.98(3H, s)	14.80
Phe[3]	α	4.84(1H, ddd, 3.9, 8.8, 12.3)	54.54		NH	7.55(1H, d, 8.3)	
	β	3.16(1H, m)	36.73		C=O		170.72
		2.96(1H, t, 12.3)					
	γ		137.78	Leu[8]	α	3.84(1H, m)	53.00
	δ	7.18(2H, m)	128.89		β	1.89(2H, m)	37.29
	ε	7.22(2H, m)	128.13		γ	1.54(1H, m)	24.64
	ζ	7.17(1H, m)	126.37		δ_{Me}	0.89(3H, d, 7.6)	23.16
	NH	7.89(1H, d, 8.8)				0.83(3H, d, 7.5)	21.41
	C=O		172.57		NH	7.73(1H, d, 7.3)	
Phe[4]	α	4.57(1H, m)	55.13		C=O		170.72
	β	3.12(2H, m)	35.80				
	γ		137.71	Ile[9]	α	4.56(1H, t, 9.0)	54.54
	δ	7.10(2H, d, 7.3)	128.89		β	1.76(1H, m)	37.29
	ε	7.22(2H, m)	128.13		γ	1.62(1H, m)	23.86
	ζ	7.17(1H, m)	126.15			0.98(2H, m)	
	NH	7.82(1H, d, 7.0)			γ_{Me}	0.96(3H, d, 6.5)	15.37
	C=O		173.17		δ_{Me}	0.88(3H, d, 6.6)	10.74
Val[5]	α	3.72(1H, dd, 6.0, 12.4)	61.61		NH	7.27(1H, d, 9.0)	
	β	2.08(1H, m)	29.41		C=O		169.89
	γ_{Me}	0.97(3H, d, 6.7)	19.25				
		0.97(3H, d, 6.7)	18.89				
	NH	7.45(1H, 6.0)					
	C=O		172.57				

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6. Cyclolinopeptide B: Colorless powder, $[\alpha]_D$ -104.1° (c 0.20, MeOH); *m/z* 1058 [Found: $(M+H)^+$, 1058.6031. $C_{56}H_{83}N_9O_9S$ requires, 1058.6113]; ν_{max} (KBr)/cm^{-1} 3436 (NH) and 1659 (amide C=O); λ_{max} (MeOH) / nm 267 (ε 250) and 218 (ε 10050). ^{1}H- and ^{13}C-NMR ($CDCl_3$:DMSO-d_6 1:1): see Table 1.
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10. Venous blood (10 ml) was taken from a healthy volunteer, heparinized, and mononuclear lymphocytes were separated by a Ficoll-Hypaque density gradient. The cells were suspended in RPMI 1640 medium containing 10% of fetal calf-serum to a cell density of 1×10^6 cells/ml. Two hundred microliters of this suspension were placed into each well of a microtiter plate with 96 flatbottom wells. Concanavalin A was added to each well to a final concentration of 5.0 μg/ml. Subsequently, 4 μl of an ethanol solution containing the test compounds were added to a final concentration of 1-10000 ng/ml. The plate was incubated for 4 days in 5 % CO_2/95% air at 37°C. After termination of cell culture, 10 μl of 5 mg/ml MTT (Sigma, USA) in phosphate buffered saline was added to every well and the plate reincubated at 37°C in 5% CO_2/air for a further 4h. The plate was then centrifuged at 800 × g for 5 min to precipitate cells and formazan. 150 ml of the supernatant was removed from every well, and 175 μl of DMSO was added to dissolve the formazan crystals. The plate was mixed on a microshaker for 10 min, and then read on a microplate reader (Corona MT P-32, Corona Co., Japan) at 550 nm. A dose response curve was plotted for each drug, and a concentration which gave 50% inhibition of cell growth (IC_{50}) was calculated. The viability of lymphocytes was assessed by a dye exclusion test using trypane blue as a dye as described elsewhere. In this assay, cyclosporin A showed the inhibitory effect with IC_{50} of 3 ng/ml.
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