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Cyclolinopeptides B - E, New Cyclic Peptides from *Linum usitatissimum*[1]

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Abstract: New cyclic nona and octapeptides, cyclolinopeptides B - E were isolated from the seeds of *Linum usitatissimum* and their structures were elucidated as *cyclo* (-Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-Ile-), *cyclo* (-Pro-Pro-Phe-Phe-Val-Ile-Mso-Leu-Ile-), *cyclo* (-Pro-Phe-Phe-Trp-Ile-Mso-Leu-Leu-), and *cyclo* (-Pro-Leu-Phe-Ile-Mso-Leu-Val-Phe-), respectively, by extensive 2D NMR methods and chemical degradations. Cyclolinopeptides B and E showed immunosuppressive activity.

Cyclolinopeptide A (CLA), a cyclic nonapeptide, which exhibits a potent immunosuppressive activity,[2] has been isolated from linseed oil in 1959 as one of the first naturally occurring cyclic peptides isolated.[3]

we have isolated and studied on various cyclic peptides from higher plants having various biological activities.[4] As part of our continuing investigation of new biologically active cyclic peptides from higher plants, in the present study, we isolated new cyclic peptides from the seeds of *Linum usitatissimum* (Linaceae). Chromatographic purification of a MeOH extract from the seeds gave four new cyclic peptides, cyclolinopeptides B - E (CLB - CLE) in the yields of 0.0002%, 0.0037%, 0.0015%, and 0.0058%, respectively. CLB and CLE showed an inhibitory effect on the mitogen (concanavalin A) - induced response of peripheral-blood lymphocytes. This paper reports the structure elucidation of CLB - CLE by the extensive 2D NMR methods and chemical degradations, and their immunosuppressive activities on human and mouse peripheral-blood lymphocytes.

The methanolic extract of the seeds of *L. usitatissimum* was subjected to Diaion HP-20 column (H_2O-MeOH), and the MeOH eluate was chromatographed on a silica gel column, followed by HPLC on ODS to yield peptidic compounds, CLA - CLE.

Cyclolinopeptide B (CLB), white powder, $[\alpha]_D$ -104.1° (*c* 0.20, MeOH), showed a HR-FABMS quasimolecular ion peak at *m/z* 1058.6031 (MH^+), corresponding to the molecular formula, $C_{56}H_{83}N_9O_9S$. 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) (Table 1). In ^1H NMR spectrum, however, only seven amide proton signals (δ 7.89, 7.82, 7.73, 7.71, 7.55, 7.45, and 7.27) were observed. The amino acid analysis of the acid hydrolysate of CLB by HPLC showed the presence of 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 NMR signals due to amide protons and amide carbonyl carbons. The lack of terminal amino group protons in the ^1H NMR and the observed mass molecular weight suggested that CLB was a cyclic nonapeptide.

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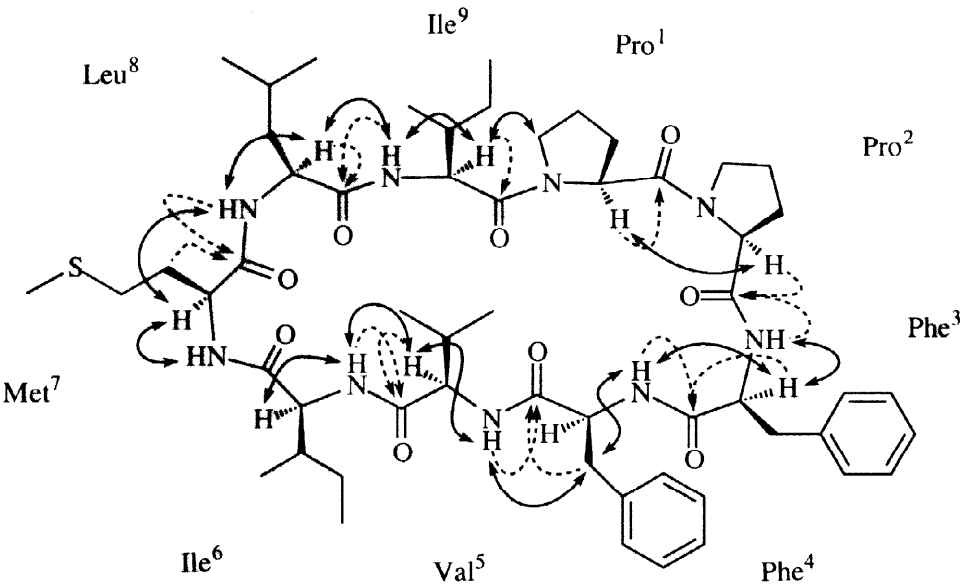

Fig. 1. Structure of cyclolinopeptide B. Arrows show selected NOE correlations, and dashed arrows selected HMBC correlations.

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

assignment		δ_H [int. mult, J(Hz)]	δ_C			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, t, 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, t, 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				

The presence of a Pro residue in the primary sequence, generally, produces *cis/trans* conformers which convert into each other at a rate slow enough to give separate signals. However, its NMR spectra in $CDCl_3$-

DMSO-d_6 (1:1) at 330K gave well-resolved sharp signals without implying the presence of minor conformers. The proton signals and the corresponding carbon signals were assigned by the ^{1}H-^{1}H COSY and HMQC spectra as shown in Table 1. The gross structure including the amino acid sequence was determined by linking the individual amino acids according to the phase sensitive ROESY and HMBC experiments. The HMBC correlations between each amide carbonyl carbon and neighboring amide NH and Hα protons, and NOEs between the neighboring amino acid protons finally determined the structure as *cyclo* (-Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-Ile-) (Fig. 1). All of the component amino acids was determined to be of L-configuration by the derivatization of the acid hydrolysate with Marfey's reagent,[5] followed by HPLC analysis.

The amide bond geometry between Pro^1 and Pro^2 was determined to be *cis* on the basis of the strong NOE correlation between Hα in Pro^1 and Hα in Pro^2, and the chemical shifts (δ 31.13 and 21.41) of β and γ carbons of Pro^2 residue,[6] and the doublet signal of Hα of Pro^2.[7]

Cyclolinopeptide C (CLC), white powder, $[\alpha]_D$ -109.7° (c 0.21, MeOH), showed a HR-FABMS quasimolecular ion peak at *m/z* 1074.6039 (MH^+, Δ -2.3 mmu), corresponding to molecular formula, $C_{56}H_{84}N_9O_{10}S$. The IR absorptions at 3329 and 1663 cm^{-1} were attributed to amino and amide carbonyl groups, respectively.

The ^{1}H and ^{13}C NMR signal assignments were made on the basis of the COSY, HMQC and HMBC spectra (Table 2), which indicated that CLC was similar to CLB in the structure. However, the singlet methyl signal of Met in CLC was at δ 2.45, whereas the methyl signal of Met in CLB was at δ 1.98. In addition, the signals ascribable to the γ and methyl carbons of Met in CLC were at δ 49.31 (γ) and 37.60 (Mε), whereas the corresponding signals of CLB were at δ 30.34 (γ) and 14.80 (Mε). Therefore, CLC was considered to contain a methionine sulfoxide in the place of methionine in CLB. Reductive transformation of CLC by thioglycolic acid gave CLB to show the presence of methionine sulfoxide in CLC.[8] The absolute stereochemistry of the amino acids in CLC was determined by Marfey's method[6] to be L except for the configuration of sulfoxide. The amino acid sequence of CLC was shown to be the same as that of CLB by the HMBC and NOE studies (Fig. 2).

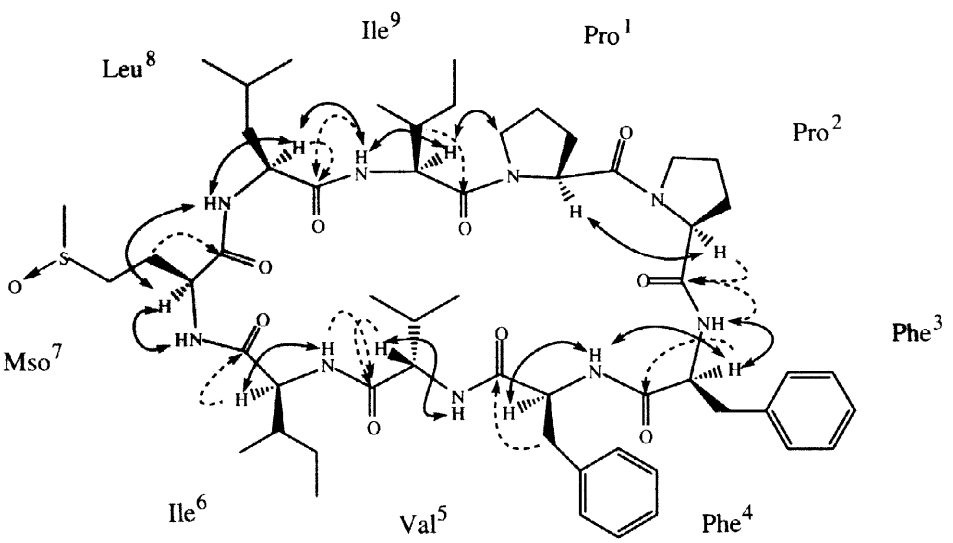

Fig. 2. Structure of cyclolinopeptide C. Arrows show selected NOE correlations, and dashed arrows selected HMBC correlations.

Table 2. ^{1}H and ^{13}C NMR Signal Assignments of CLC in DMSO-d_6 at 320K.

assignment		δ_H [int. mult, J(Hz)]	δ_C		δ_H	δ_C
Pro[1]				Ile[6]		
	α	3.77(1H, br.s)	58.03	α	4.47(1H, m)	54.04
	β	2.06(1H, m)	27.73	β	1.78(1H, m)	36.70
		1.62(1H, m)		γ	1.46(2H, m)	23.19
	γ	1.91(1H, m)	23.98	γ_{Me}	0.86(3H, m)	15.08
		1.81(1H, m)		δ_{Me}	0.79(3H, m)	10.26
	δ	3.55(2H, m)	47.12	NH	7.26(1H, br.s)	
	C=O		171.73	C=O		168.78
Pro[2]				Mso[7]		
	α	4.14(1H, d, 7.7)	60.00	α	4.40(1H, m)	51.20
	β	1.86(1H, m)	30.59	β	2.10(1H, m)	25.36
		1.58(1H, m)			1.88(1H, m)	
	γ	1.45(1H, m)	21.09	γ	2.77(1H, m)	49.31
		0.93(1H, m)			2.62(1H, m)	
	δ	3.25(2H, m)	46.25	ε_{Me}	2.45(3H, s)	37.60
	C=O		171.13	NH	7.89(1H, br.s)	
Phe[3]				C=O		169.52
	α	4.51(1H, m)	55.01	Leu[8]		
	β	3.06(2H, m)	35.26	α	4.02(1H, m)	51.90
	γ		137.54	β	1.75(2H, m)	37.57
	δ	7.02(2H, m)	127.69	γ	1.49(1H, m)	24.12
	ε	7.21(2H, m)	128.61	δ_{Me}	0.85(3H, m)	22.62
	ζ	7.18(1H, m)	125.75		0.83(3H, m)	21.28
	NH	7.94(1H, d, 6.8)		NH	8.15(1H, br.s)	
	C=O		170.40	C=O		170.17
Phe[4]				Ile[9]		
	α	4.75(1H, br.s)	54.19	α	3.77(1H, br.s)	60.16
	β	3.08(2H, m)	36.30	β	2.07(1H, m)	34.51
	γ		137.79	γ	1.47(1H, m)	24.82
	δ	7.16(2H, m)	127.79		1.17(1H, dt, 7.9, 13.7)	
	ε	7.21(2H, m)	128.76	γ_{Me}	0.86(3H, m)	15.45
	ζ	7.18(1H, m)	126.00	δ_{Me}	0.79(3H, m)	10.69
	NH	8.12(1H, br.s)		NH	7.97(1H, d, 7.2)	
	C=O		171.45	C=O		171.07
Val[5]						
	α	3.88(1H, t, 7.0)	60.16			
	β	2.04(1H, m)	29.49			
	γ_{Me}	0.90(3H, m)	18.97			
		0.88(3H, m)	18.47			
	NH	7.39(1H, d, 7.0)				
	C=O		171.79			

Cyclolinopeptide D (CLD) was isolated as white powder, $[\alpha]_D$ -75.0° (*c* 0.20, MeOH) which showed a quasimolecular ion peak at *m/z* 1064.5682 (MH^+, Δ +3.9 mmu) in HR-FABMS, corresponding to $C_{57}H_{78}N_9O_9S$. Since the presence of UV absorption max. at 280 nm suggested the presence of tryptophan residue, it was hydrolyzed with 6N HCl containing 4% thioglycolic acid. HPLC analysis of the hydrolysate showed that CLD was a cyclic octapeptide consisting of two phenylalanines (Phe), two leucines (Leu), one proline (Pro), one isoleucine (Ile), one tryptophan (Trp), and one methionine (Met) per molecule. The IR absorption at 1029 cm^{-1} implied the presence of sulfoxide. The molecular formula corresponded to a cyclic peptide containing the above amino acids and one oxygen which was due to methionine sulfoxide.

In the ^{1}H and ^{13}C NMR spectra, seven amide NH and eight amide carbonyl carbons of the cyclic octapeptide were observed (Table 3). Since information about the NOE data and the long range $^{2}J_{H\text{-}C}$ and $^{3}J_{H\text{-}C}$ correlations by HMBC was not sufficient for the structure elucidation, the compound was transformed chemically to an acyclic derivative. Reductive transformation of methionine sulfoxide by thioglycolic acid, followed by cyanogen bromide decomposition, gave the corresponding acyclic peptide, which showed an absorption IR band at 1774 cm^{-1} of homoserine lactone.[8] Sequential analysis by HMBC and NOE correlations

indicated by arrows in Fig. 4 determined the structure of the acyclic peptide as Leu-Leu-Pro-Phe-Phe-Trp-Ile-HomoSer lactone and thus the structure of CLD as *cyclo* (-Leu-Leu-Pro-Phe-Phe-Trp-Ile-Mso-).

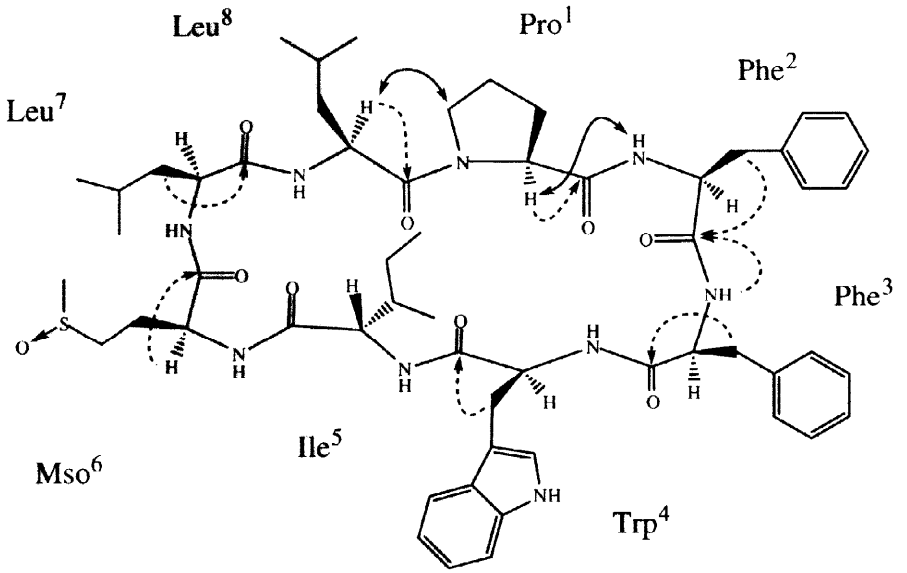

Fig. 3. Structure of cyclolinopeptide D. Arrows show selected NOE correlations, and dashed arrows selected HMBC correlations.

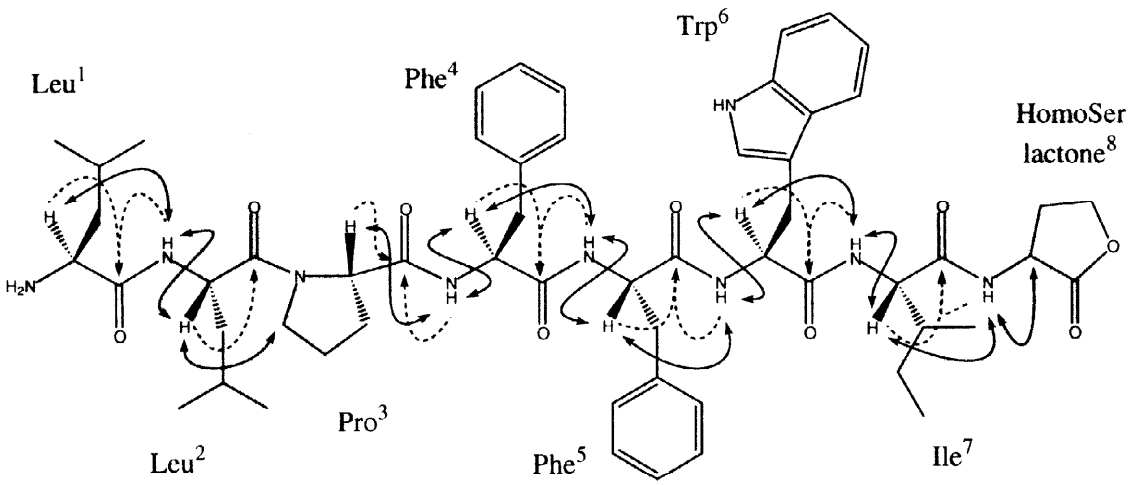

Fig. 4. Structure of acyclic CLD. Arrows show selected NOE correlations, and dashed arrows selected HMBC correlations.

Cyclolinopeptide E (CLE) was isolated as white powder, $[\alpha]_D$ -75.5° (*c* 0.20, MeOH). Molecular formula, $C_{57}H_{78}N_9O_9S$ was determined by HR-FABMS [*m/z* 977.5491 (MH^+, Δ +5.7 mmu)]. HPLC of the acid hydrolysate showed that CLE was an octapeptide consisting of two Phe, two Leu, one Pro, one Ile, one Val, and one Met per molecule. Each of these amino acid was shown to be of L-configuration by derivatization

Table 3. ^{1}H and ^{13}C NMR Signal Assignments of CLD in DMSO-d_6 at 320K.

assignment	δ_H [int. mult, J(Hz)]	δ_C	assignment	δ_H	δ_C
Pro[1]			Ile[5]		
α	4.16(1H, d, 7.9)	61.90	α	4.27(1H, m)	57.61
β	2.07(1H, m)	28.63	β	1.83(1H, m)	36.60
	1.68(1H, m)		γ	1.42(1H, m)	24.31
γ	1.96(2H, m)	24.42		1.14(1H, m)	
δ	3.59(2H, m)	46.86	γ_{Me}	0.87(3H, d, 5.9)	15.28
C=O		173.02*	δ_{Me}	0.84(3H, t, 5.9)	11.13
Phe[2]			NH	7.59(1H, br.s)	
α	4.09(1H, m)	53.45	C=O		174.14*
β	3.01(1H, t, 13.7)	35.10	Mso[6]		
	2.91(1H, m)		α	4.10(1H, m)	56.03
γ		137.92	β	2.18(1H, m)	23.68
δ	7.23(2H, m)	128.49		1.91(1H, m)	
ε	7.01(2H, m)	127.87	γ	2.67(2H, m)	49.95
ζ	7.24(1H, m)	126.01	ε_{Me}	2.46(3H, s)	37.94
NH	8.14(1H, d, 5.6)		NH	8.04(1H, d, 6.1)	
C=O		170.47	C=O		170.07*
Phe[3]			Leu[7]		
α	4.60(1H, m)	53.45	α	3.76(1H, m)	52.90
β	3.30(1H, m)	36.27	β	1.86(1H, m)	37.59
	2.80(1H, m)			1.57(1H, m)	
γ		138.22	γ	1.63(1H, m)	23.49
δ	7.23(2H, m)	128.56	δ_{Me}	0.86(3H, d, 6.2)	22.78
ε	7.25(2H, m)	127.87		0.82(3H, d, 6.0)	20.94
ζ	7.24(1H, m)	126.01	NH	8.03(1H, d, 6.2)	
NH	7.87(1H, d, 8.5)		C=O		171.60*
C=O		171.29*	Leu[8]		
Trp[4]			α	4.76(1H, dt, 6.3, 8.0)	48.37
α	4.18(1H, m)	55.75	β	1.55(2H, m)	40.65
β	3.31(2H, m)	24.51	γ	1.26(1H, m)	28.31
1NH	10.56(1H, s)		δ_{Me}	0.86(3H, d, 6.2)	23.29
2	7.04(1H, d, 7.1)	120.63		0.94(3H, d, 6.1)	21.57
3		110.20	NH	7.27(1H, d, 8.0)	
4	7.46(1H, d, 8.0)	117.68	C=O		171.50*
5	7.21(1H, t, 8.0)	122.89			
6	6.95(1H, t, 8.0)	118.00			
7	7.33(1H, d, 8.0)	111.09			
8		135.85			
9		127.21			
NH	8.29(1H, br.s)				
C=O		172.03*			

*Assignments may be interchanged.

of the acid hydrolysate with Marfey's reagent and subsequent HPLC analysis. As the presence of Mso was indicated by the IR absorption band at 1028 cm^{-1}, and the definite amino acid sequence was not obtained by the NMR methods, CLE was transformed into an acyclic derivative following the procedure in CLD.[8] The NMR spectra were taken in DMSO-d_6 given the best dispersion of signals for analysis. The sequence of the amino acids was determined by the NMR methods as shown in Fig. 6. Accordingly, the structure of CLE was determined to be *cyclo*(-Pro-Leu-Phe-Ile-Mso-Leu-Val-Phe-) (Fig. 5).

CLB showed an 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.[9] Furthermore, CLB and CLE possessing different amino acid sequence showed a moderate inhibitory effect of about the same potency on the mouse lymphocyte proliferation induced by Con. A (IC_{50}: CLB 39 μg/mL; CLE 43 μg/mL). It is known that the biological activity of CLA is critically dependent on the sequence and conformation.[10] The conformational analyses and further conformation-biological activity relationship of CLB and CLE, are currently conducted in our laboratories.

Table 4. ^{1}H and ^{13}C NMR Signal Assignments of CLE in DMSO-d_6 at 320K.

assignment		δ_H [int. mult, J(Hz)]	δ_C		δ_H	δ_C
Pro[1]				Mso[5]		
	α	4.35(1H, t, 7.7)	62.14	α	4.22(1H, m)	57.12
	β	2.18(1H, m)	28.89	β	2.19(1H, m)	24.15
		1.83(1H, m)			2.01(1H, m)	
	γ	1.90(2H, m)	24.15	γ	2.96(1H, m)	49.73
	δ	3.55(1H, m)	47.16		2.67(1H, m)	
		3.50(1H, m)		ε_{Me}	2.56(3H, s)	38.04
	C=O		170.11	NH	8.04(1H, br.s)	
Leu[2]				C=O		169.98*
	α	3.87(1H, m)	54.66	Leu[6]		
	β	1.76(1H, m)	38.77	α	4.51(1H, m)	51.37
		1.54(1H, m)		β	2.51(1H, m)	24.68
	γ	1.66(1H, m)	24.09		1.95(1H, m)	
	δ_{Me}	0.89(3H, m)	22.39	γ	1.96(1H, m)	28.79
		0.79(3H, m)	21.12	δ_{Me}	0.89(3H, m)	22.47
	NH	8.16(1H, br.s)			0.79(3H, m)	21.39
	C=O		171.94*	NH	7.75(1H, br.s)	
Phe[3]				C=O		171.75*
	α	4.40(1H, m)	52.72	Val[7]		
	β	3.35(1H, dd, 4.9, 14.3)	35.38	α	3.79(1H, m)	59.36
		2.81(1H, m)		β	1.98(1H, m)	29.95
	γ		137.07	γ_{Me}	0.63(3H, d, 6.6)	19.16
	δ	7.20(2H, m)	127.75		0.70(3H, d, 6.6)	18.35
	ε	7.11(2H, m)	128.47	NH	7.75(1H, br.s)	
	ζ	7.15(1H, m)	125.86	C=O		171.28*
	NH	7.81(1H, d, 7.7)		Phe[8]		
	C=O		170.56*	α	4.90(1H, m)	51.37
Ile[4]				β	3.12(1H, dd, 5.2, 14.4)	36.42
	α	3.87(1H, m)	53.37		2.92(1H, m)	
	β	1.39(1H, m)	38.43	γ		138.12
	γ	1.47(1H, m)	24.46	δ	7.20(2H, m)	127.89
		1.02(1H, m)		ε	7.24(2H, m)	128.96
	γ_{Me}	0.96(3H, d, 6.6)	16.73	ζ	7.15(1H, m)	126.01
	δ_{Me}	0.86(3H, m)	10.12	NH	8.51(1H, br.s)	
	NH	7.90(1H, d, 7.7)		C=O		170.86*
	C=O		172.38*			

*Assignments may be interchanged.

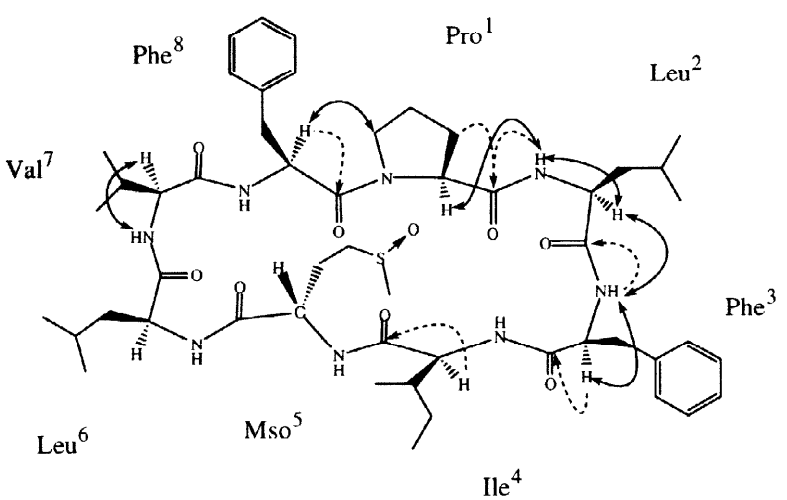

Fig. 5. Structure of cyclolinopeptide E. Arrows show selected NOE correlations, and dashed arrows selected HMBC correlations.

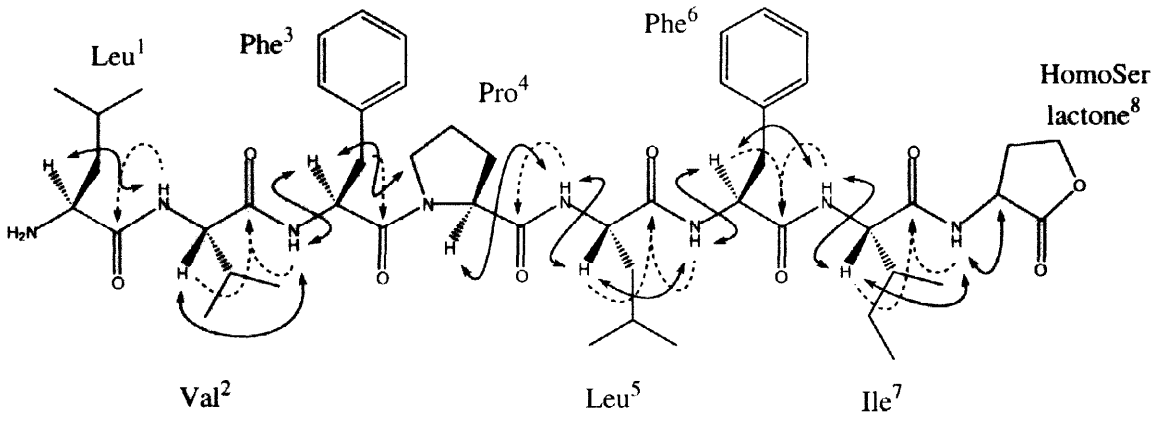

Fig. 6. Structure of acyclic CLE Arrows show selected NOE correlations, and dashed arrows selected HMBC correlations.

EXPERIMENTAL

M.p.s were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 spectrometer and the $[\alpha]_D$ values are given in 10^{-1}deg cm^2 g^{-1}. FAB mass spectra were taken with a VG Autospec spectrometer, IR spectrum with a JASCO A-302 spectrophotometer and high-pressure liquid chromatography (HPLC) with an Inertsil PREP-ODS column (20 mm i.d. × 250 mm and 30 mm i.d. × 250 mm, GL Science Inc.) packed with 10 μm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck) and the spots were detected by spraying Dragendorff reagent. Proton and carbon NMR spectra were recorded on Bruker spectrometers (AM400 and AM500) and Varian Unity 400 spectrometer. 10 mg each sample of cyclolinopeptides B - E in a 5 mm tube [0.5 mL DMSO-d_6 or DMSO-d_6-$CDCl_3$ (1:1) degassed] was used for the homonuclear and heteronuclear measurements. The spectra were recorded at 303K. Phase sensitive NOESY experiments were acquired with mixing times of 0.6 sec and ROESY experiments 90 msec. The value of the delay to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 3.2 Hz and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 msec.

Extraction and Isolation

Dry roots of *Linum usitatissimum* (30 kg) was extracted with hot MeOH three times to give a MeOH extract (4 kg), which was subjected to Diaion HP-20 column chromatography using a water - MeOH gradient system (1:0 - 0:1). The fractions eluted by 100% MeOH was further subjected to silica gel column chromatography using a $CHCl_3$ - MeOH gradient system (1:0 - 0:1). The fraction eluted by 10% MeOH was subjected to ODS HPLC with 70 - 80 % MeOH, and 40 - 60 % CH_3CN solvent systems to give CLA (0.007%), CLB (0.0004%), CLC (0.003%), CLD (0.0012%) and CLE (0.0002%) as colorless needles or colorless powder.

Cyclolinopeptide A (CLA). - Colorless needles, m.p. 243°C, $[\alpha]_D$ -111.1° (*c* 0.23, MeOH); *m/z* 1040 (Found: MH$^+$, 1040.6562. $C_{57}H_{86}N_9O_9$ requires, 1040.6549); ν_{max} (KBr)/cm^{-1} 3332 (NH) and 1668 (amide C=O); λ_{max} (MeOH) / nm 275 (ε 209) and 218 (ε 10050).

Cyclolinopeptide B (CLB).- Colorless powder, $[\alpha]_D$ -104.1° (*c* 0.20, MeOH); *m/z* 1058 (Found: MH$^+$, 1058.6031. $C_{56}H_{84}N_9O_9S$ requires, 1058.6113); ν_{max} (KBr)/cm^{-1} 3436 (NH) and 1659 (amide C=O); λ_{max} (MeOH) / nm 267 (ε 243) and 218 (ε 10600).

Cyclolinopeptide C (CLC). - Colorless powder, $[\alpha]_D$ -109.7° (*c* 0.21, MeOH); *m/z* 1074 (Found: MH$^+$, 1074.6039. $C_{56}H_{84}N_9O_{10}S$ requires, 1074.6062); ν_{max} (KBr)/cm^{-1} 3329 (NH) and 1663 (amide C=O); λ_{max} (MeOH) / nm 268 (ε 245) and 218 (ε 11000).

Cyclolinopeptide D (CLD).- Colorless powder, $[\alpha]_D$ -75.0° (*c* 0.20, MeOH); *m/z* 1064 (Found: MH$^+$, 1064.5682. $C_{57}H_{78}N_9O_9S$ requires, 1064.5643); ν_{max} (KBr)/cm^{-1} 3332 (NH), 1657 (amide C=O) and 1029 (sulfoxide); λ_{max} (MeOH) / nm 280 (ε 4631) and 229 (ε 10070).

Cyclolinopeptide E (CLE). - Colorless powder, $[\alpha]_D$ -75.5° (*c* 0.20, MeOH); *m/z* 977 (Found: MH$^+$, 977.5491. $C_{51}H_{77}N_8O_9S$ requires, 977.5534); ν_{max} (KBr)/cm^{-1} 3314 (NH), 1657 (amide C=O) and 1028 (sulfoxide); λ_{max} (MeOH) / nm 267 (ε 303) and 218 (ε 8690).

Acid Hydrolysis of CLA - CLE

A solution of peptide (1 mg) in 6N HCl was heated at 110 °C for 24 h in a sealed tube. In the case of CLD, 4% thioglycolic acid was also added. After cooling, each solution was concentrated to dryness. The hydrolysate was dissolved in 0.02N HCl and applied to the amino acid analyzer (Hitachi L-8500 Amino acid Analyzer).

Absolute Configuration of Amino Acids

A solutions of peptide (1 mg) in 6N HCl was heated at 110 °C for 12 h in a sealed tube. After being cooled, each solution was concentrated to dryness. The residue was dissolved in water and treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) and 1M $NaHCO_3$ at 35 °C for 1 h. After being cooled, it was treated with 2M HCl and then concentrated to dryness. This residue was subjected to HPLC [Lichrospher 100, RP-18 (10 μm, Merck), flow rate 1 mL/min, detection 340 nm, solvent : 10 - 80% CH_3OH (80 min, gradient) / 50 mM triethylamine phosphate (TEAP) buffer (pH 3.2), t_R (min): L-Pro (42.5), D-Pro (47.7), L-Met (51.4), D-Met (63.5), L-Val (52.9), D-Val (65.6), L-Trp (56.3), D-Trp (64.4), L-Phe (58.8), D-Phe (69.7), L-Ile (60.3), D-Ile (72.6), L-Leu (61.7), D-Leu (73.3)].

Reaction of CLD and CLE with Thioglycolic Acid and Cyanogen Bromide

A solution of CLD (21.2 mg) and a solution of CLE (10.6 mg) in 40% thioglycolic acid (1.0 ml) were heated at 50 °C for 24 h. The reaction mixture was subjected to HP-20 column and purified by HPLC to give the reduction product of methionine sulfoxide. The product was treated with 100M cyanogen bromide and 70% formic acid (1.0 mL) for 24 h at room temperature. The reaction mixture was lyophilized and purified by HPLC to give acyclic homoserine lactonic product (6.8 mg and 4.7 mg), respectively.

Compound **6** - Colorless powder; *m/z* 1018 (MH^+); ν_{max} (KBr)/cm^{-1} 3401 (NH), 1774 (γ-lactone) and 1655 (amide C=O). ^{1}H-NMR (DMSO-d_6, 300K): Leu^1 4.02(1H, m, α), 1.61(2H, m, β), 1.50(1H, m, γ), 0.90(3H, m, δ), 0.88(3H, m, δ), 8.08(1H, br.s, NH); Leu^2 4.55(1H, m, α), 1.65(2H, m, β), 1.39(1H, m, γ), 0.90(3H, m, δ), 0.88(3H, m, δ), 8.61(1H, d, 7.9, NH); Pro^3 4.29(1H, dd, 2.7, 7.9, α), 3.00(1H, m, β), 2.78(1H, m, β), 1.74(2H, m, γ), 3.57(1H, dd, 7.3, 13.8, δ), 3.41(1H, dd, 7.1, 13.8, δ); Phe^4 4.39(1H, m, α), 2.91(1H, m, β), 2.76(1H, m, β), 7.20(2H, m, δ), 7.18(2H, m, ε), 7.15(1H, m, ζ), 7.78(1H, d, 7.7, NH); Phe^5 4.52(1H, m, α), 2.98(1H, m, β), 2.79(1H, m, β), 7.20(2H, m, δ), 7.18(2H, m, ε), 7.15(1H, m, ζ), 7.99(1H, d, 7.7, NH); Trp^6 4.66(1H, dt, 6.0, 7.2, α), 3.15(1H, dd, 6.0, 14.4, β), 3.01(1H, m, β), 10.79(1H, s, NH), 7.12(1H, d, 7.1), 7.58(1H, d, 8.0), 7.05(1H, t, 8.0), 6.96(1H, t, 8.0), 7.31(1H, d, 8.0), 8.16(1H, d, 7.2, NH); Ile^7 4.20(1H, t, 7.8, α), 1.68(1H, m, β), 1.45(1H, m, γ), 1.08(1H, m, γ), 0.86(3H, d, 6.2, γ), 0.81(3H, t, 7.2, δ), 7.97(1H, d, 7.8); HomoSer lactone^8 4.60(1H, m, α), 2.38(1H, m, β), 2.14(1H, m, β), 4.36(1H, m, γ), 4.22(1H, m, γ), 8.38(1H, d, 8.0, NH). ^{13}C-NMR (DMSO-d_6, 300K): Leu^1 50.57 (α), 39.92 (β), 23.36 (γ), 22.68 (δ), 21.28 (δ), 168.74 (C=O); Leu^2 48.81 (α), 39.71 (β), 23.82 (γ), 23.17 (δ), 21.75 (δ), 169.88 (C=O); Pro^3 59.11 (α), 28.65 (β), 24.20 (γ), 46.59 (δ), 170.94 (C=O); Phe^4 53.63 (α), 37.22 (β), 137.52 (γ), 127.84 (δ), 129.13 (ε), 126.04 (ζ), 170.52 (C=O); Phe^5 53.73 (α), 37.54 (β), 137.52 (γ), 127.88 (δ), 129.20 (ε), 126.10 (ζ), 170.62 (C=O); Trp^6 53.18 (α), 27.52 (β), 123.53, 118.43, 120.72, 118.14, 111.15, 136.00, 127.40, 170.77 (C=O); Ile^7 56.69 (α), 36.87 (β), 24.28 (γ), 15.13 (γ), 11.02 (δ), 170.87 (C=O); HomoSer lactone^8 47.72 (α), 27.89 (β), 65.22 (γ), 174.92 (C=O).

Compound **7** - Colorless powder; *m/z* 932 (MH^+); ν_{max} (KBr)/cm^{-1} 3425 (NH), 1777 (γ-lactone) and 1649 (amide C=O). ^{1}H-NMR (DMSO-d_6, 300K): Leu^1 3.85(1H, m, α), 1.44(2H, m, β), 1.55(1H, m, γ), 0.87(3H, m, δ), 0.83(3H, m, δ), 8.06(1H, br.s, NH); Val^2 4.25(1H, t, 9.0, α), 1.92(1H, m, β), 0.85(3H, m, γ), 0.82(3H, m, γ), 8.34(1H, d, 9.0, NH); Phe^3 4.68(1H, dt, 4.6, 8.1, α), 2.99(1H, m, β), 2.78(1H, m, β), 7.20(2H, m, δ), 7.18(2H, m, ε), 7.15(1H, m, ζ), 8.30(1H, d, 8.1, NH); Pro^4 4.34(1H, m, α), 1.85(2H, m, β), 1.95(1H, m, γ), 1.78(1H, m, γ), 3.62(1H, m, δ), 3.48(1H, m, δ); Leu^5 4.20(1H, m, α), 1.60(2H, m, β), 1.40(1H, m, γ), 0.87(3H, m, δ), 0.83(3H, m, δ), 7.93(1H, d, 7.9, NH); Phe^6 4.60(1H, m, α), 3.04(1H, m, β), 2.83(1H, m, β), 7.20(2H, m, δ), 7.18(2H, m, ε), 7.15(1H, m, ζ), 7.81(1H, d, 8.0, NH); Ile^7 4.16(1H, t, 8.9, α), 1.73(1H, m, β), 1.43(1H, m, γ), 1.09(1H, m, γ), 0.86(3H, d, 6.6, γ), 0.80(3H, t, 7.3, δ), 7.89(1H, d, 8.9, NH); HomoSer lactone^8 4.59(1H, m, α), 2.40(1H, m, β), 4.38(1H, m, γ), 4.22(1H, m, γ), 8.43(1H, d, 8.1, NH). ^{13}C-NMR (DMSO-d_6, 300K): Leu^1 50.64 (α), 40.13 (β), 23.40 (γ), 22.71 (δ), 21.70 (δ), 168.74 (C=O); Val^2 57.43 (α), 30.88 (β), 19.12 (γ), 18.18 (γ), 169.88 (C=O); Phe^3 51.77 (α), 36.48 (β), 137.46 (γ), 127.88 (δ), 129.07 (ε), 126.08 (ζ), 169.36 (C=O); Pro^4 59.11 (α), 28.87 (β), 24.39 (γ), 46.74 (δ), 171.04 (C=O); Leu^5 51.21 (α), 40.77 (β), 24.01 (γ), 22.93 (δ), 21.79 (δ), 171.65 (C=O); Phe^6 53.12 (α), 37.05 (β), 137.53 (γ), 127.98 (δ), 129.18 (ε), 126.08 (ζ), 170.37 (C=O); Ile^7 56.53 (α), 36.87 (β), 24.18 (γ), 15.13 (γ), 10.90 (δ), 170.70 (C=O); HomoSer lactone^8 47.72 (α), 27.88 (β), 65.22 (γ), 174.89 (C=O).

Biological Assay

Heparinized human blood (10 mL) collected from a healthy volunteer, was centrifuged to remove the red cells at 1300 × g for 15 min at room temperature. Mononuclear lymphocytes were separated by using a Ficoll-Hypaque density gradient and suspended at a cell density of 1×10^6 cells/ml in RPMI 1640 medium containing 100,000 IU/L of penicillin, 100 μg/L of streptomycin, and 10% of fetal calf-serum. 200 μL of this suspension was placed in 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 and subsequently, 4 μL of serially diluted EtOH of the test compounds was added to a final concentration of 1 - 10000 ng/mL. 4 μL of EtOH was added to the control well. The plate was incubated for 4 days in 5 % CO_2 / 95% air at 37 °C. The cells were pulsed with 0.5 μCi/well of [^3H]-thymidine for the last 16 h of incubation and then collected on a glass fiber filter paper by using a multiharvester device and dried. The radioactivity retained on the filter was counted by the liquid scintillation counter. The mean of the counts of triplicate measurements for each sample was recorded. The concentration of the test compounds giving 50% inhibition of lymphocyte mitosis (IC_{50}) was obtained from the dose response curves. The viability of lymphocytes was assessed by the dye exclusion test using trypane blue as a dye as described elsewhere.

The method using mouse lymphocyte was as follows. ICR mice aged 5 to 8 weeks were dislocated and the spleen was removed, mashed and the cells were suspended in RPMI 1640 medium and passed through a stainless steel mesh. The single cell suspension was washed twice with the medium and finally suspended in RPMI 1640 medium containing 10% fetal calf serum, and 10 mg/mL Kanamycin to give 3.5×10^6 cells/mL. 200 μL of this suspension was placed in each well of a microtiter plate with 96 flatbottom wells. Concanavalin A was added to each well to a final concentration of 2.5 μg/mL. Subsequently, 4 μL of serially diluted EtOH solution of test compound was added to a final concentration of 10 - 100000 ng/mL. The plate was incubated for 3 days in 5% CO_2 / 95% air at 37 °C. After termination of cell culture, 20 μL of 5 mM 1-methoxy PMS and 0.2 mM WST-1 (DOJINDO Laboratories) in phosphate buffered saline was added to every well and the plate was incubated again at 37 °C in 5% CO_2 / air for a 4 h. The plate was read on a microplate reader (Corona MT P-32, Corona Co., Japan) at 415 nm. A dose response curve was plotted for each drug, and the concentration which gave 50% inhibition of cell growth (IC_{50}) was recorded.

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