Curcacycline A – a novel cyclic octapeptide isolated from the latex of *Jatropha curcas* L.

A.J.J. van den Berg[a,*], S.F.A.J. Horsten[a], J.J. Kettenes-van den Bosch[b], B.H. Kroes[a], C.J. Beukelman[a], B.R. Leeflang[c], R.P. Labadie[a]

[a]*Department of Pharmacognosy, Faculty of Pharmacy, Universiteit Utrecht, P.O. Box 80082, 3508 TB Utrecht, The Netherlands*
[b]*Department of Pharmaceutical Analysis, Faculty of Pharmacy, Universiteit Utrecht, The Netherlands*
[c]*Department of NMR Spectroscopy, Bijvoet Center, Faculty of Chemistry, Universiteit Utrecht, The Netherlands*

Received 24 October 1994; revised version received 30 November 1994

Abstract From the latex of *Jatropha curcas* L. (Euphorbiaceae) a novel cyclic octapeptide was isolated, which we named curcacycline A. The compound was found to contain one threonine, one valine, two glycine, and four leucine residues. By two-dimensional ^{1}H-NMR spectroscopy (HOHAHA and ROESY), its sequence was determined to be Gly^1-Leu^2-Leu^3-Gly^4-Thr^5-Val^6-Leu^7-Leu^8-Gly^1. Curcacycline A displays a moderate inhibition of (i) classical pathway activity of human complement and (ii) proliferation of human T-cells.

Key words: Jatropha curcas; Euphorbiaceae; Curcacycline A; Cyclic peptide

1. Introduction

Application of *Jatropha* species (Euphorbiaceae) in traditional medicine has been frequently reported, e.g. *Jatropha curcas* is used in Nepal to treat malarial fever [1], and in Egypt to treat arthritis, gout and jaundice [2]. In Indonesia, the latex of *J. multifida* is externally applied to (infected) wounds and ulcers [3]. Although seed oils of *J. multifida*, *J. podagrica*, *J. curcas*, and *J. gossypifolia* are known to possess toxic, skin irritant and tumour-promoting properties due to the presence of phorbol esters [4,5], several compounds with anti-tumour activities have also been found in *Jatropha* species. For instance, from the roots of *J. gossypifolia*, jatrophone (a macrocyclic diterpenoid) and 2α- and 2β-hydroxyjatrophone were isolated [6,7], and from *J. macrorrhiza* acetylaleuritolic acid (a triterpenoid) and jatropham (a lactam), which all displayed antileukemic activity [8,9].

In our laboratory two immunomodulatory cyclic peptides were isolated from the latex of *J. multifida* which both selectively inhibit classical pathway activity of human complement [3]. This led us to investigate other Euphorbiaceous plants for the presence of (biologically active) peptides. As a result, a novel cyclic peptide was isolated from the latex of *Jatropha curcas*, which we named curcacycline A. Curcacycline A also inhibits classical pathway complement activity, although in a lesser degree than the *J. multifida* cyclic peptides mentioned above. In addition, curcacycline A shows a moderate dose-dependent inhibition of human T-cell proliferation; direct cytotoxic effects could not be observed. This paper describes the determination of the primary structure of curcacycline A by a combination of amino acid analysis, FAB mass spectrometry, and two-dimensional ^{1}H-NMR spectroscopy (HOHAHA and ROESY).

*Corresponding author.

2. Experimental

2.1. Plant material

Jatropha curcas L. plants grown as hedges in Jepara (near Kudus, Java, Indonesia) were identified. Crude latex was obtained by cutting off leaf stalks, adding a few drops of EtOH to prevent the latex from excessive foaming. The latex was stored at −20°C until use.

2.2. Isolation procedure

To 50 ml of crude latex 200 ml of demineralized H_2O and 2 ml of EtOH were added, after which the mixture was extracted with 3 × 500 ml of EtOAc. EtOAc was removed from the extract under reduced pressure. The residue was dissolved in 1 ml of MeOH and fractionated on Sephadex LH-20 (Pharmacia; column dimensions: 1.4 cm i.d. × 40 cm) with MeOH as eluting solvent at a flow rate of 0.35 ml/min; fractions of 8 ml were collected. Peptide-containing fractions were traced by TLC on precoated TLC plates 60 F254 (Merck) using a solvent system consisting of $CHCl_3$/MeOH/H_2O = 5:4:1 in a saturated chamber. Peptides were detected with Cl_2/*o*-tolidine reagent [10]. The peptide-containing fractions were combined, and further fractionated by preparative column chromatography on silica gel H (Merck, No. 7736) using a Miniprep LC apparatus (Jobin Yvon, France; column dimensions: 1.4 cm i.d. × 40 cm; column pressure 8 bar) with $CHCl_3$/MeOH = 7:2 as eluting solvent (solvent pressure 2 bar); fractions of 16 ml were collected. Fractions containing single peptides were combined; the solvent was evaporated under N_2. The residue was taken up in a few drops of MeOH and dissolved in H_2O. Subsequently, the aqueous solution was lyophilized yielding 26 mg of curcacycline A.

2.3. Amino acid analysis

Curcacycline A (0.5 mg, accurately weighed) was hydrolysed in 1 ml of 6 N HCl in a sealed vial at 110°C for 24 h. HCl was removed under reduced pressure, and the residue dissolved in 0.2 M Li-citrate buffer (pH 2.2). Amino acids were determined by cation-exchange chromatography on an LKB 4151 Alpha plus Amino Acid Analyser (Li-system) with ninhydrin detection.

2.4. Mass spectrometry

A positive ion spectrum was obtained by fast atom bombardment (FAB) using a JEOL SX102 mass spectrometer. Glycerol was used as the matrix. The FAB mass spectrum of curcacycline A showed an $[M+H]^+$ at *m/z* 767, and immonium ions at *m/z* 30, 72, 74, and 86.

2.5. ^{1}H-NMR spectroscopy

Prior to ^{1}H-NMR spectroscopy, curcacycline A was dissolved in DMSO-d_6 (2 mg/ml). All experiments were performed at 300K.

Two-dimensional ^{1}H-NMR experiments were carried out at 500 MHz with a Bruker AMX-500 (Dept. of NMR spectroscopy, Bijvoet Center, Universiteit Utrecht). The processing of the spectra was performed on a Silicon Graphics workstation using the 'TRITON' software package (R. Boelens and R. Kaptein, Dept. of NMR Spectroscopy, Bijvoet Center, Universiteit Utrecht).

A 2D HOHAHA spectrum was recorded using a 53 ms MLEV-17 pulse sequence [11] with a field strength of 9,600 Hz. The time propor-

SSDI 0014-5793(94)01405-1

tional phase increment (TPPI) method [12,13] was used for t_1 amplitude modulation, and 512 free induction decays (FIDs) of 1,024 complex data points each were collected. The spectral width was 5,000 Hz in both dimensions. The t_1 period was incremented from 3 μs to 54.2 ms. The time domain data in both dimensions were weighted with a sine-bell function shifted by $\pi/3$ radians. The data were processed to give a phase sensitive spectrum of 1,024 × 1,024 real data points.

A 2D ROESY spectrum was recorded using a simple 150 ms CW spin-lock pulse [14,15] with a field strength of 2,500 Hz. The carrier frequency was positioned at 4.64 ppm. Amplitude modulation in t_1 was accomplished with TPPI, and 640 FIDs of 1,024 complex data points each were recorded. The spectral width in both dimensions was 5,434 Hz. The t_1 period was incremented from 3 μs to 61.9 ms. The time domain data in both dimensions were weighted with a sine-bell function shifted by $\pi/3$ radians. The data were processed to give a phase sensitive spectrum of 1,024 × 2,048 real data points.

In addition to the 2D experiments described above, ^{1}H-NMR spectra were recorded at 400 MHz on a Bruker MSL-400 (NMR Department, Gorlaeus Laboratories, State University Leiden); for the exchange of amide and OH (threonine) protons D_2O was added.

^{1}H-Chemical shifts of amino acid residues expressed in ppm are listed in Table 1.

3. Results and discussion

3.1. Amino acid analysis and mass spectrometry

A compound was isolated from the latex of *Jatropha curcas*, which we named curcacycline A. The compound showed a Cl_2/*o*-tolidine positive reaction which is indicative of (an) amide group(s) [10]. Amino acid analysis showed curcacycline A to contain (molar ratio in parenthesis) Gly (glycine; 39.2), Val (valine; 22.5), Thr (threonine; 22.1), and Leu (leucine; 77.9). Immonium ions (R-CH = NH_2^+) in the FAB mass spectrum indicated the presence of Gly (*m/z* 30), Val (*m/z* 72), Thr (*m/z* 74) and Leu/Ile(isoleucine) (*m/z* 86). These results indicate curcacycline A to be an octapeptide consisting of 2 Gly, 1 Val, 1 Thr and 4 Leu for which in case of a linear structure a molecular mass of 784 can be calculated. FAB mass spectrometry, however, revealed a molecular mass of 766 ($[M+H]^+$ at *m/z* 767), indicating an extra loss (18) of one H_2O. Together with the observation that sequence fragments were absent in the FAB spectrum, this indicates a cyclic structure. It was concluded that curcacycline A is a cyclic octapeptide containing 2 Gly, 1 Val, 1 Thr and 4 Leu residues.

3.2. NMR spectroscopy

The ^{1}H-NMR spectrum of curcacycline A showed three clusters of resonances characteristic of peptides (i.e. downfield amide, αH, and upfield side-chain protons). In agreement with the results from amino acid analysis, nine resonances were observed which disappeared upon addition of D_2O, i.e. eight amide protons (8.69–7.42 ppm), and one proton of Thr-OH (5.36 ppm).

Table 1
^{1}H-Chemical shifts expressed in ppm for curcacycline A in DMSO-d_6 (500 MHz)

Residue	NH	αH	βH	others
Gly[1]	8.59	3.92[a]		
		3.44[b]		
Leu[2]	7.54	4.62	1.42[c]	δCH$_3$ 0.83[d]
			1.34[c]	
Leu[3]	8.52	3.92[a]	1.55[c]	δCH3 0.89[d], 0.85[d]
			1.38[c]	
Gly[4]	8.69	3.92[a]		
		3.44[b]		
Thr[5]	7.42	4.77	4.32[e]	γCH$_3$ 0.91
				OH 5.36
Val[6]	7.75	4.10	2.34	γCH$_3$ 0.89[d], 0.85[d]
Leu[7]	8.19	4.32[e]	1.63[c]	γCH 1.55[c]
			1.26[c]	δCH3 0.86[d], 0.78[d]
Leu[8]	8.04	3.92[a]	1.43[c]	δCH3 0.87[d], 0.80[d]

[a–e]Indicate overlapping chemical shifts.

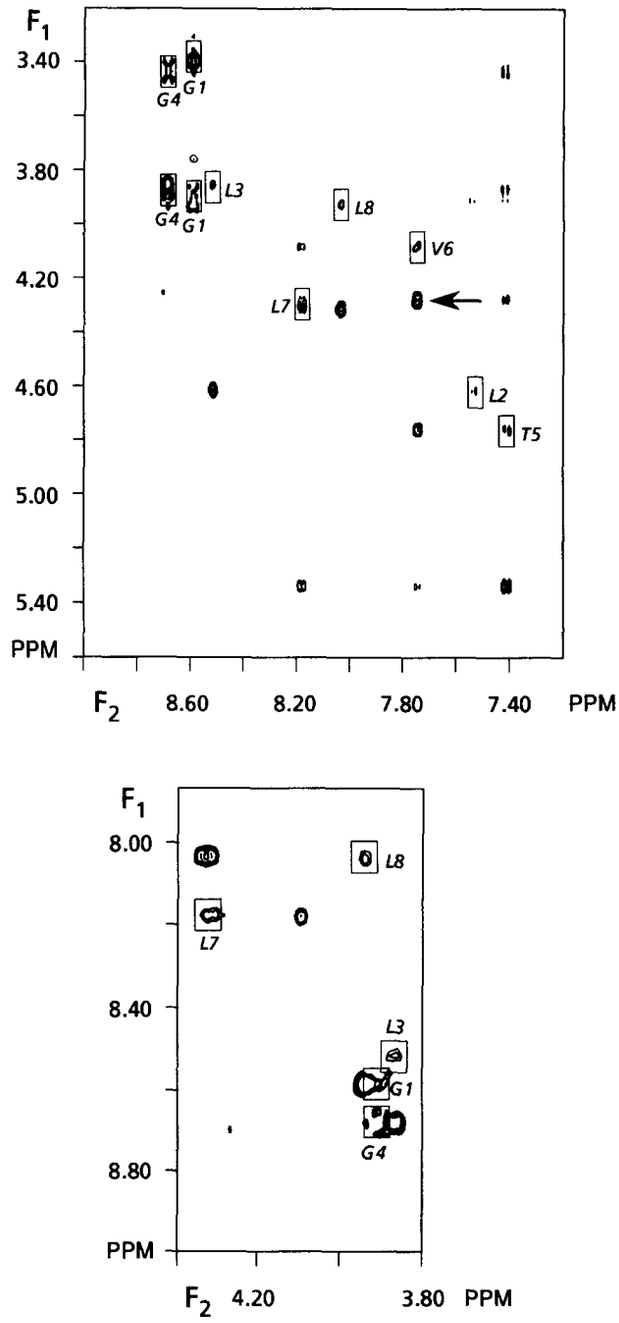

Fig. 1. Regions of the ROESY spectrum of curcacycline A (in DMSO-d_6) showing inter-residue connectivities between α-protons (F_1: 4.8–3.4 ppm) and amide protons (F_2: 8.7–7.4 ppm). Intra- residue cross peaks, which also appeared in the HOHAHA spectrum, are boxed; unboxed signals represent sequential $d_{\alpha N}(i,i+1)$ connectivities with the exception of cross peaks between Thr^5-NH (7.42 ppm) and Thr^5-βH (4.32 ppm), Thr^5-OH (5.36 ppm) and Thr^5-NH, Thr^5-OH and Val^6-NH (7.75 ppm), and Thr^5-OH and Leu^7-NH (8.19 ppm). The arrow indicates the $d_{\beta N}(i,i+1)$ coupling between Thr^5-βH and Val^6-NH.

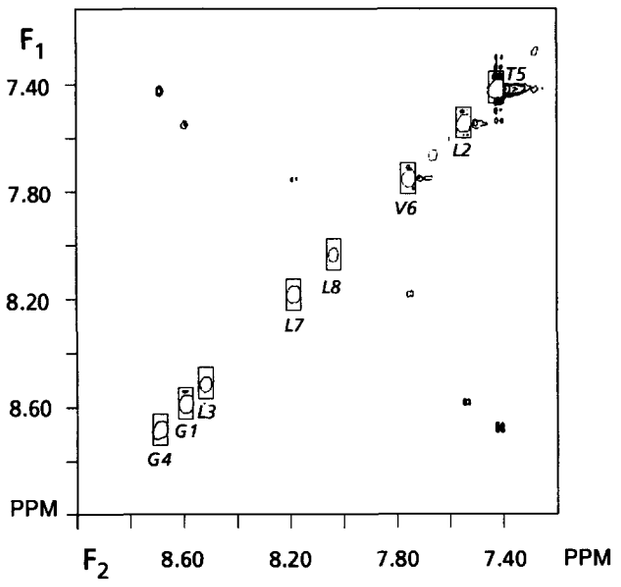

Fig. 2. Region of the ROESY spectrum of curcacycline A (in DMSO-d_6) showing inter-residue $d_{NN}(i,i+1)$ connectivities between amide protons (F_1, F_2: 8.7–7.4 ppm); the diagonal peaks are boxed.

The amino acid sequence of curcacycline A was determined by two-dimensional ^{1}H-NMR experiments [16,17]. Intra-residue J-couplings displayed in the spectrum obtained by Homonuclear Hartmann-Hahn spectroscopy (HOHAHA) [11] were used to assign ^{1}H-chemical shifts to the specific protons of individual amino acid residues; in particular the assignment of amide and Cα protons is a prerequisite for sequence determination. The HOHAHA spectrum showed complete spin-spin coupling patterns for two Gly, one Thr, and one Val; for the four Leu residues most of the J-coupling connectivities could be

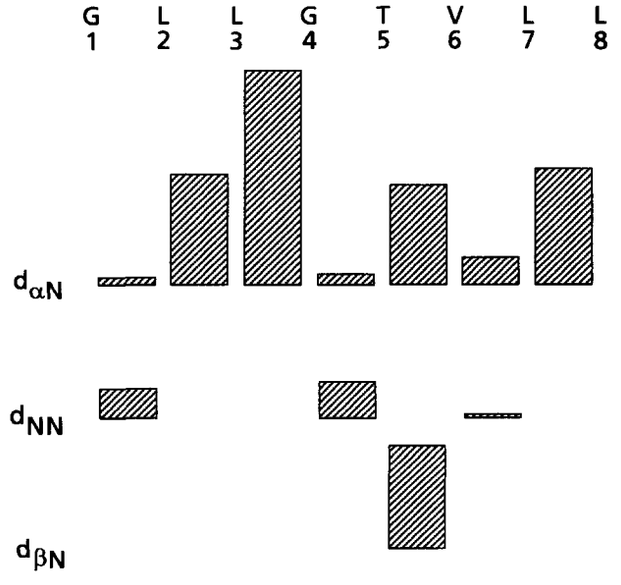

Fig. 3. Intensities of cross peaks due to sequential $d_{\alpha N}$, d_{NN} and $d_{\beta N}$ connectivities in the ROESY spectrum of curcacycline A corrected for the offset dependency according to [19].

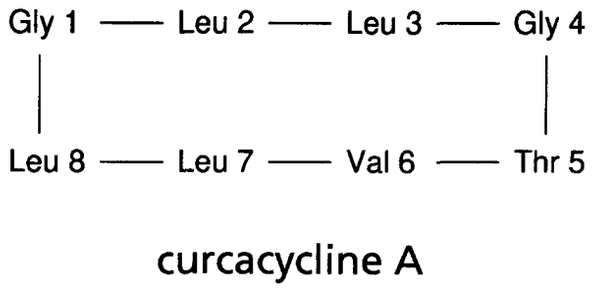

Fig. 4. Structure of curcacycline A.

distinguised. ^{1}H-Chemical shift assignments for the amino acid residues of curcacycline A in DMSO-d_6 are listed in Table 1.

The assignment of cross peaks in the ROESY (two-dimensional rotating frame Overhauser effect spectroscopy) spectrum of curcacycline A in DMSO-d_6 allowed the sequential assignment of amino acid residues and was based on the fact that irrespective of the secondary structure of proteins (or peptides), at least one distance between amide-NH, αH and βH protons on adjacent residues is less than 3 Å, thus giving rise to connectivities through space (NOEs) [18]. For curcacycline A, $d_{\alpha N}(i,i+1)$ in particular, and less frequently $d_{NN}(i,i+1)$ and $d_{\beta N}(i,i+1)$ connectivities were observed between adjacent residues. Inter-residue $d_{\alpha N}(i,i+1)$ connectivities were found between one Gly^1-α proton and Leu^2-NH, Leu^2-αH and Leu^3-NH, Leu^3-αH and Gly^4-NH, two Gly^4-α protons and Thr^5-NH, Thr^5-αH and Val^6-NH, Val^6-αH and Leu^7-NH, Leu^7-αH and Leu^8-NH, and Leu^8-αH and Gly^1-NH (Fig. 1). In addition, $d_{NN}(i,i+1)$ connectivities were found between Gly^1-NH and Leu^2-NH, Gly^4-NH and Thr^5-NH, and Val^6-NH and Leu^7-NH (Fig. 2), and a cross peak as a result of $d_{\beta N}(i,i+1)$ coupling between Thr^5-βH (4.32 ppm) and Val^6-NH (Fig. 1). The sequential $d_{\alpha N}$, d_{NN} and $d_{\beta N}$ connectivities for curcacycline A are summarized in Fig. 3, and presented as intensities of the corresponding cross peaks (in the ROESY spectrum) corrected according to [19]. The proposed structure for curcacycline A, consistent with these results, is shown in Fig. 4.

In addition to the sequential connectivities mentioned above, cross peaks in the ROESY spectrum were also found between Thr^5-NH (7.42 ppm) and Thr^5-βH (4.32 ppm), Thr^5-OH (5.36 ppm) and Thr^5-NH, Thr^5-OH and Val^6-NH (7.75 ppm), and Thr^5-OH and Leu^7-NH (8.19 ppm) (Fig. 1).

The coupling constants $J_{NH\alpha H}$ of Leu^3 and Leu^8 (J = 4.4 and 4.3 Hz, respectively) were found to be rather small in comparison with those of Leu^7 and Leu^2 (J = 8.5 and 8.8 Hz, respectively) or J = 8.0 Hz reported for protected linear tetrapeptides in DMSO-d_6 [20]. This indicates the *cis* rather than the *trans* configuration between Leu^3-αH and Leu^3-NH, and Leu^8-αH and Leu^8-NH.

Since many plants are known to synthesize both L- and D-amino acids, our current research is focused on the determination of the absolute configurations of constituting amino acid residues. Subsequently, computerized modelling studies will be performed to establish the tertiary structure of curcacycline A. Finally, together with other structurally elucidated cyclic peptides from the genus *Jatropha* (e.g. labaditin [3]), curcacycline A will be subjected to structure-activity relationship (SAR) studies to find possible leads for the development of (immunomodulatory) drugs.

Acknowledgements: The authors gratefully acknowledge Mr. C. Versluis (Analytical Molecular Spectrometry, Universiteit Utrecht) for recording the mass spectrum, Drs. C. Erkelens (NMR Dept., Gorlaeus Laboratories, Leiden) for recording ^{1}H-NMR spectra at 400 MHz, Mr. H.J.L. Ravestein (Zoology, Universiteit Utrecht) for the amino acid analysis, and Mr. P.W. van Dorp van Vliet for preparing the drawings. The study was financially supported by a grant from the Van Leersum Fonds (KNAW).

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