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PODACYCLINE A AND B, TWO CYCLIC PEPTIDES IN THE LATEX OF *JATROPHA PODAGRICA*

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Key Word Index—*Jatropha podagrica*; Euphorbiaceae; cyclic peptides; podacycline A; podacycline B.

Abstract—Two novel cyclic peptides were isolated from the latex of *Jatropha podagrica*, which we named podacycline A and B. Podacycline A is a cyclic nonapeptide with the sequence Gly1-Leu2-Leu3-Gly4-Ala5-Val6-Trp7-Ala8-Gly9-Gly1. The sequence of podacycline B, a cyclic heptapeptide, was determined to be Phe1-Ala2-Gly-3-Thr4-Ile5-Phe6-Gly7-Phe1. The amino acid residues of both compounds were found to have the L-configuration.

INTRODUCTION

In our search for immunomodulating plant constituents, a cyclic decapeptide and a cyclic nonapeptide were isolated from the latex of *Jatropha multifida* by an activity-guided fractionation procedure; both compounds inhibit classical pathway activity of human complement [1]. This led us to investigate also other *Jatropha* species for the presence of such peptides. As a result, several cyclic peptides were obtained, e.g. curcacycline A, a cyclic octapeptide, and cyclogossine A, a cyclic heptapeptide, isolated from the latices of *J. curcas* and *J. gossypifolia*, respectively [2, 3]. This paper deals with the isolation of two novel cyclic peptides from the latex of *J. podagrica*, which we named podacycline A and B, and the determination of their primary structures by a combination of amino acid analysis, FAB-mass spectrometry, and two-dimensional ^{1}H NMR spectroscopy (HOHAHA and ROESY). The absolute configurations of the amino acid residues were also determined.

RESULTS AND DISCUSSION

Podacycline A and B were isolated from the latex of *J. podagrica* Hook. Both compounds showed a positive reaction with chlorine/*o*-tolidine reagent, which indicates the presence of amide groups [4]. By amino acid analysis, the acid hydrolysate of podacycline A was found to contain (molar ratio in parenthesis): Gly (glycine; 33.9), Ala (alanine; 27.8), Val (valine; 16.1), Leu (leucine; 32.2) and Trp (tryptophan; 11.9); immonium ions ($R{=}CH{=}NH_2^+$) in the FAB-mass spectrum indicated the presence of Gly (*m/z* 30), Ala (*m/z* 44), Val (*m/z* 72), Leu/Ile(isoleucine) (*m/z* 86), and Trp (*m/z* 130 and 159). The results of the amino acid analysis suggested that podacycline A contained two Gly, two Ala, one Val, two Leu, and one Trp, corresponding with a M_r of 785. The FAB-mass spectrum, however, showed a $[M+H]^+$ at *m/z* 825, whereas in the HOHAHA spectrum [5] of podacycline A three spin systems characteristic of glycine could be assigned. Although (repeated) amino acid analysis resulted in rather low molar ratio values for glycine, it was concluded that podacycline A is a nonapeptide consisting of three (and not two) Gly, two Ala, one Val, two Leu, and one Trp, for which linear structure a M_r of 842 is calculated. The actual M_r of 824, however, indicates a loss of one H_2O; in addition, no sequence ions were observed in the FAB-mass spectrum. Therefore, it was concluded that podacycline A is a cyclic nonapeptide, containing three Gly, two Ala, one Val, two Leu, and one Trp.

In the ^{1}H NMR spectrum of podacycline A (and also B) three clusters of resonances could be observed characteristic of peptides, i.e. downfield amide and aromatic protons (δ 9–7), αH protons (δ 5–3), and upfield side-chain protons (δ 2–0). In agreement with

exchanged for deuterium, i.e. nine amide protons (δ 8.33–7.48) and one indole-NH (Trp) (δ 10.85). The sequence of amino acid residues was determined by two-dimensional ^{1}H NMR experiments [6, 7]. The HOHAHA spectrum [5] was used to assign ^{1}H-chemical shifts to the specific protons of individual amino acid residues; the assignment of amide and Cα protons in particular is a prerequisite for sequence determination. The spectrum showed spin systems corresponding to three Gly, two Ala, one Val, two Leu, and the aliphatic part of one Trp. In addition, the aromatic spin system of one 3-indolyl moiety (Trp) was identified, whereas in the ROESY spectrum ROEs were observed between the aliphatic Cα and Cβ protons of Trp 7 (δ 4.48, and 3.14 and 3.04, respectively) and the aromatic Trp7-C4H proton (δ 7.52), thus providing evidence for the presence of the Trp residue. ^{1}H chemical-shift assignments for the amino acid residues of podacycline A in DMSO-d_6 are listed in Table 1.

The sequence of amino acid residues was determined by specific ROE assignments in the ROESY spectrum of podacycline A in DMSO-d_6, and was based on the observation that, irrespective of the tertiary structure of a peptide, at least one distance between NH, αH and βH protons on adjacent residues is less than 3 Å [8], which results in cross-peaks in the ROESY spectrum. In particular, $d_{\alpha N}(i, i+1)$ but also $d_{NN}(i, i+1)$ and less frequently $d_{\beta N}(i, i+1)$ connectivities were found between adjacent residues. Thus, sequence-specific $d_{\alpha N}(i, i+1)$ connectivities were observed between two Gly1-αH protons and Leu2-NH, Leu2-αH and Leu3-NH, Leu3-αH and Gly4-NH, two Gly4-αH protons and Ala5-NH, Ala5-αH and Val6-NH, Val6-αH and Trp7 (amide)-NH, Trp7-αH and Ala8-NH, and Ala8-αH and Gly9-NH (Fig. 1A). In addition, $d_{NN}(i, i+1)$ connectivities between Gly1-NH and Leu2-NH, Leu2-NH and Leu3-NH, Gly4-NH and Ala5-NH, Val6-NH and Trp7-NH, Ala8-NH and Gly9-NH, and Gly9-NH and Gly1-NH were found (Fig. 1B), as well as cross-peaks due to $d_{\beta N}(i, i+1)$ couplings between Leu 3-βH and Gly4-NH, Ala5-βH and Val6-NH, Val6-βH and Trp7-NH, and Ala8-βH and Gly9-NH. Figure 2 shows the sequential $d_{\alpha N}$, d_{NN}, and $d_{\beta N}$ connectivities for podacycline A, presented as intensities of the corresponding cross-peaks corrected for the offset dependency [9]. The proposed structure of podacycline A, consistent with these connectivities, is shown in Fig. 3. Nonsequential cross-peaks observed in the ROESY spectrum of podacycline A were found between Val6-NH (δ 7.94) and Val6-γCH$_3$ (δ 0.75 and 0.73), Val6-NH and Val6-βH (δ 2.00), Leu2-NH (δ 7.48) and Leu2-γCH (δ 1.63).

In addition to the L-enantiomers, D-amino acids are frequently found in higher plants. Therefore, the abso-

Table 1. ^{1}H Chemical shifts (δ) for podacycline A in DMSO-d_6 (500 MHz)

Residue	NH	αH	βH	Others	
Gly1	7.90	3.75[a]			
		3.57[b]			
Leu2	7.48	4.19[c]	1.48[d]	γCH	1.63
				δ_1CH$_3$	0.89
				δ_2CH$_3$	0.84
Leu3	8.33	3.96	1.48[d]	γCH	1.48[d]
				δ_1CH$_3$	0.82
				δ_2CH$_3$	0.78
Gly4	8.14[e]	3.85			
		3.46			
Ala5	7.87	4.27	1.19		
Val6	7.94	3.88	2.00	γ_1CH$_3$	0.75
				γ_2CH$_3$	0.73
Trp7	7.72	4.48	3.14	N1H	10.85
			3.04	C2H	7.15
				C4H	7.52
				C5H	6.98
				C6H	7.06
				C7H	7.32
Ala8	7.74	4.19[c]	1.13		

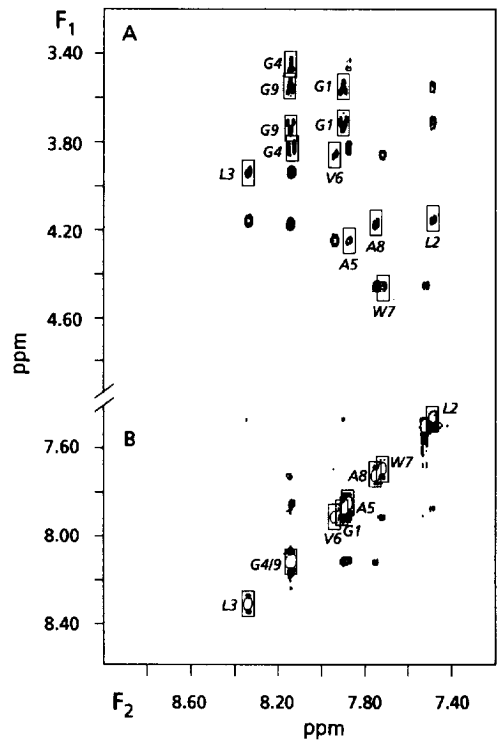


Fig. 1. Sequential $d_{\alpha N}(i, i+1)$ and $d_{NN}(i, i+1)$ connectivities observed as cross-peaks in the ROESY spectrum of podacycline A (in DMSO-d_6). (A) Region of the ROESY spectrum showing inter-residue connectivities between α-protons (F_1: δ 4.5–3.4) and amide protons (F_2: δ 8.3–7.4). Intra-residue 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 the cross-peak between Trp7-αH (δ 4.48) and Trp7-C4H (δ 7.52)

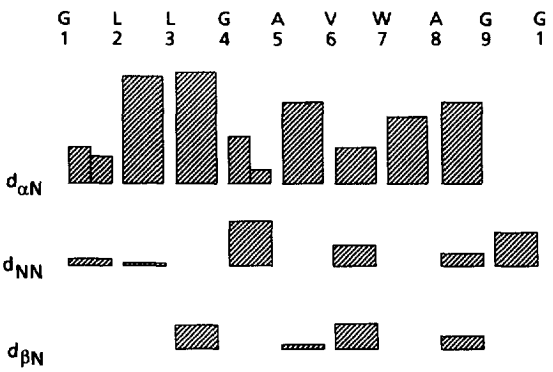
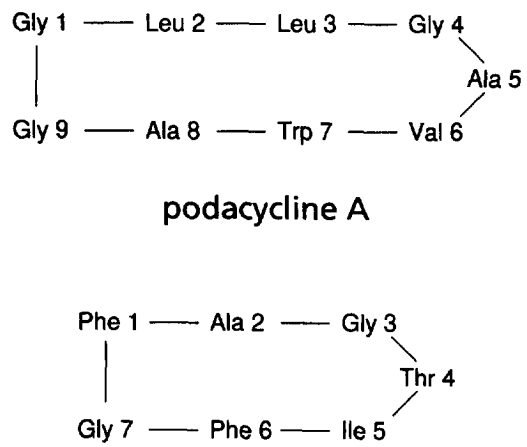


Fig. 2. Intensities of cross-peaks due to sequential $d_{\alpha N}$, d_{NN} and $d_{\beta N}$ connectivities in the ROESY spectrum of podacycline A corrected for the offset dependency [9]. The connectivities between the two α protons of each glycine residue (Gly1-$\alpha H_{1,2}$ and Gly4-$\alpha H_{1,2}$) and the adjacent amide protons (Leu2-NH and Ala5-NH, respectively) are presented by double bars.

lute configurations of the Trp, Leu, Val, and Ala residues were determined. It was found, that podacycline A only contained L-enantiomers.

Amino acid analysis showed podacycline B to contain (molar ratio in parentheses): Gly (39.6), Ala (28.4), Thr (threonine; 27.3), Ile (29.8), and Phe (phenylalanine; 49.9); the FAB-mass spectrum showed immonium ions at m/z 30 (Gly), 44 (Ala), 74 (Thr), 86 (Ile), and 120 (Phe). These results suggest podacycline B to be a heptapeptide consisting of two Gly, one Ala, one Thr, one Ile, and two Phe residues corresponding with a M_r of 711. FAB-mass spectrometry, however, revealed a M_r of 693 ($[M + H]^+$ at m/z 694), the difference (18) being one H_2O. Together with the fact that no fragments other than immonium ions were observed in the FAB-mass spectrum, this indicates a cyclic structure. It was concluded that podacycline B is a cyclic heptapeptide, containing two Gly, one Ala, one Thr, one Ile, and two Phe residues.



The ^1H NMR spectrum of podacycline B showed eight resonances which disappeared upon addition of D_2O, i.e. seven amide protons (δ 8.92–7.06) and one Thr-OH proton (δ 4.86). Spin systems corresponding to two Gly, one Ala, one Thr, one Ile, and the aliphatic parts of two Phe residues could be identified in the HOHAHA spectrum. ^1H chemical shifts of 10 aromatic protons for two Phe residues were found at δ 7.29–7.12. Cross-peaks (ROEs) in the ROESY spectrum of podacycline B indicated spatial proximities between aliphatic Cα and Cβ protons of Phe6 and Phe1 (Phe6-αH: δ 4.32, Phe6-βH: δ 3.20 and 2.69; Phe1-αH: δ 4.68, Phe1-βH: δ 2.92 and δ 2.81), and the aromatic protons of these residues (δ 7.29–7.12). In this way, the presence of two Phe residues indicated by amino acid analysis and FAB-mass spectrometry was confirmed. ^1H chemical-shift assignments for the amino acid residues of podacycline B in DMSO-d_6 are listed in Table 2.

In the ROESY spectrum of podacyline B in DMSO-d_6, inter-residue $d_{\alpha N}(i, i + 1)$ connectivities were observed between Phe1-αH and Ala2-NH, Ala2-αH and Gly3-NH, one Gly3-α proton and Thr4-NH, Thr4-αH and Ile5-NH, Ile5-αH and Phe6-NH, and Phe6-αH and Gly7-NH (Fig. 4A; the sequential connectivity between Ile5-αH and Phe6-NH did not appear in the region of the ROESY spectrum shown, but was clearly visible at the other side of the diagonal). In addition, $d_{NN}(i, i + 1)$ connectivities were found between Gly3-NH and Thr4NH, and Thr4-NH and Ile5-NH (Fig. 4B), and a cross-peak due to a $d_{\beta N}(i, i + 1)$ connectivity between Thr4-βH and Ile5-NH (Fig. 4A). The sequential $d_{\alpha N}$, d_{NN} and $d_{\beta N}$ connectivities for podacycline B are summarized in Fig. 5. The structure proposal for podacycline B is shown in Fig. 3. In addition to the sequential connectivities mentioned above, cross-peaks in the ROESY spectrum of podacycline B were also found between Thr4-OH (δ 4.86) and Thr4-NH (δ 7.56), Thr4-OH and Ile5-NH (δ 8.09), Ile5-NH and Ile5-βH (δ 1.82), Ile5-NH and Ile5-γCH_2 (δ 0.97), and Ile5-NH and Ile5-γCH_3 and/or Ile5-δCH_3 (δ 0.70

Table 2. ^1H Chemical shifts (δ) for podacycline B in DMSO-d_6 (500 MHz)

Residue	NH	αH	βH	Others	
Phe1	7.06	4.68	2.92		
			2.81		
Ala2	8.92	3.98[a]	1.23		
Gly3	8.62	3.90			
		3.45			
Thr4	7.56[b]	4.56	4.32[c]	OH	4.86
				γCH_3	1.09
Ile5	8.09	3.98[a]	1.82	γCH_2	0.97
				γCH_3	0.70
				δCH_3	0.67
Phe6	7.56[b]	4.32[c]	3.20		
			2.69		

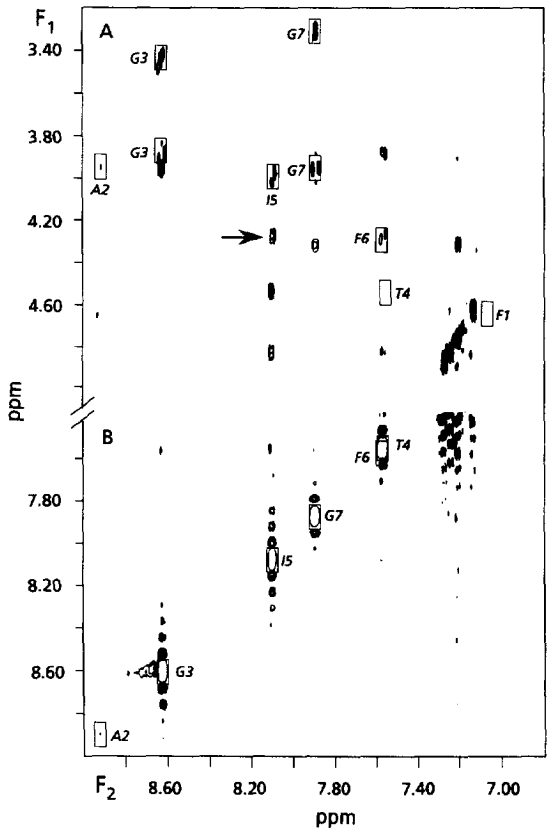


Fig. 4. Sequential $d_{\alpha N}(i, i+1)$, $d_{NN}(i, i+1)$ and $d_{\beta N}(i, i+1)$ connectivities observed as cross-peaks in the ROESY spectrum of podacycline B (in DMSO-d_6). (A) Region of the ROESY spectrum showing inter-residue connectivities between α-protons (F_1: δ 4.7–3.3) and amide protons (F_2: δ 9.0–7.0). 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 the $d_{\beta N}(i, i+1)$ coupling between Thr4-βH and Ile5-NH (arrow) and cross-peaks between Thr4-OH (δ 4.86) and Thr4-NH (δ 7.56), Thr 4-OH and Ile 5-NH (δ 8.09), and Cα protons of Phe6 and Phe1 (δ 4.32 and 4.68, respectively) and aromatic protons (δ 7.29–7.12) of the Phe residues. (B) Region showing inter-residue $d_{NN}(i, i+1)$ connectivities between amide protons (F_1, F_2: δ 9.0–7.4); the diagonal peaks are boxed.

and δ 0.67, respectively). The amino acid residues Phe, Thr, Ala, and Ile in podacycline B were found to have the L-configuration.

Podacycline A and B are two novel cyclic peptides isolated from the latex of *J. podagrica*. Until now, only a limited number of cyclic peptides have been found in higher plants. In addition to *Jatropha* species [1–3], the presence of cyclic peptides has been reported in *Rubia cordifolia*, *R. akane* (Rubiaceae) [10], and in *Laportea moroides* (Urticaceae) [11].

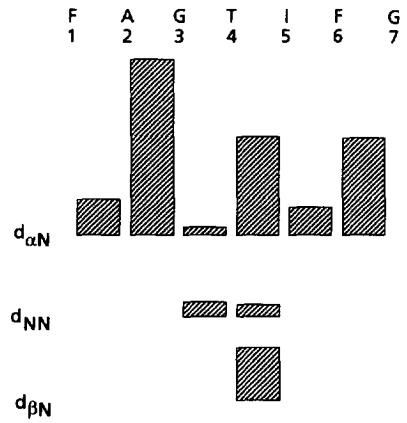


Fig. 5. Intensities of cross-peaks due to sequential $d_{\alpha N}$, d_{NN} and $d_{\beta N}$ connectivities in the ROESY spectrum of podacycline B corrected for the offset dependency [9].

imported from Kenya and commercially grown in a greenhouse (Jorre, Aalsmeer, The Netherlands) for ornamental purposes. The latex was stored at −20° until use.

Isolation procedure. From 50 ml of crude latex, 24 mg of podacycline A and 37 mg of podacycline B were obtained following the isolation procedure described in ref. [2].

Amino acid analysis. Amino acids were determined by cation-exchange chromatography using an automatic amino acid analyser (LKB 4151 Alpha plus, Li-system), after hydrolysis of the peptide (0.5–1 mg, accurately weighed) in 1 ml of 6 M HCl in a sealed vial at 110° for 24 hr. For the determination of tryptophan, hydrolysis was performed in 1 ml of 6 M HCl containing 4% thioglycolic acid. HCl and thioglycolic acid were removed under red. pres., and the residues dissolved in 0.2 M Li-citrate buffer (pH 2.2). Amino acids were detected with ninhydrin.

Mass spectrometry. Positive-ion spectra were obtained by fast atom bombardment (FAB), using a JEOL SX102 mass spectrometer. Glycerol was used as the matrix. The FAB mass spectra of podacycline A and B showed $[M+H]^+$ ions at m/z 825, and m/z 694, respectively.

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

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).

2D-HOHAHA spectra of both compounds were

amplitude modulation, and 512 free induction decays (FIDs) of 1024 complex data points each were collected. The spectral width was 4504 Hz in both dimensions. The t_1 period was incremented from 3 μsec to 56.8 msec. 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 phase-sensitive spectra of 1024×1024 real data points.

2D ROESY spectra were recorded using simple 150 ms CW spin-lock pulses [15, 16] with field strengths of 2500 Hz. The carrier frequency was positioned at δ 4.64. Amplitude modulation in t_1 was accomplished with TPPI; for podacycline A, 640, and for podacycline B, 256 FIDs of 1024 complex data points each were recorded. The spectral width in both dimensions was 5000 Hz. The t_1 was incremented from 3 μsec to 64.0 msec for podacycline A, and from 3 μsec to 25.6 msec for podacycline B. 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 phase-sensitive spectra of 1024×2048 real data points.

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

Determination of absolute configurations of amino acids. Podacycline A or B (1 mg) were hydrolysed in a sealed vial in 1 ml 6M HCl with 4% thioglycolic acid (determination of D- or L-Trp) or in 1 ml of 6M HCl (determination of other D- or L-amino acids) at 110° for 24 hr. Thioglycolic acid and HCl were removed under red. pres. and the residue was dissolved in 1 ml of H_2O; the aq. soln was lyophilized. Subsequently, the hydrolysate and amino acid standards (D- and L-enantiomers) were converted into their *N*-trifluoroacetyl/iso-PrOH derivatives by a procedure adapted from [17]. Iso-PrOH (75 μl) and trifluoroacetic anhydride (75 μl) were added to the dry samples. The mixts were heated in sealed vials at 85° for 15 min. Excess reagent was removed under N_2 and residues were dissolved in 0.5 ml of EtOAc. Aliquots of 2 μl were subjected to GC.

For GC, a Carlo Erba HRGC 5300 gas chromatograph was used equipped with a WCOT fused silica capillary column (25 m × 0.25 mm) coated with Chirasil-L-Val (film thickness 0.12 μm; Chrompack Cat. no. 7496), and a NPD detector. Injector and detector temps were set at 250°; the splitting ratio was 3:50. H_2 was used as carrier gas at an inlet pres. of 70 kPa. Upon injection of a sample, the column temp. of 75° was kept isothermal for 5 min. Subsequently, temp. was increased at 5°/min to 200°; the latter temp. was maintained for 25 min. Peak areas were computed with an electronic integrator (CE Instruments DP 700).

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