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Labaditin, a novel cyclic decapeptide from the latex of *Jatropha multifida* L. (Euphorbiaceae)

Isolation and sequence determination by means of two-dimensional NMR

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An immunologically active novel cyclic decapeptide, consisting of 1 Ala, 2 Gly, 1 Ile, 2 Thr, 2 Trp and 2 Val, has been isolated from the latex of *Jatropha multifida* L. (Euphorbiaceae). The structure was elucidated by means of amino acid analysis and FAB-mass spectroscopy. The amino acid sequence was obtained by two-dimensional ^{1}H-NMR spectroscopy (COSY and NOESY).

Latex; Decapeptide, cyclic; Labaditin; NMR, ^{1}H-; Peptide isolation; (*Jatropha multifida*, Euphorbiaceae)

1. INTRODUCTION

Jatropha multifida L. (Euphorbiaceae), a shrub of 2–3 m in height is endemic in South America but is widely grown for its ornamental foliage and flowers in Southeast Asia [1]. Its latex is used in folkloric medicine for the treatment of infected wounds, skin infections, scabies and is commonly known as 'Jodium' in East Java (Surabaya). Its reputation as a medicine is reflected by many names, some of which are: 'jarak gurita' or 'jarak cina' (Indonesia), 'mana' (Philippines), 'manaku' (Malaysia), 'kuthinervala' (Tamil), and 'bhadradanti' (Sanskrit) [2–5].

Although the curative properties of the latex have been known for generations, very little scientific data are available on the chemistry of the latex [6]. As far as the biological activity of the latex is concerned, no scientific studies have been carried out. To verify the empirical data for its possible mode of action, an immunomodulatory-activity guided isolation [7] of the active latex constituents was conducted.

The presence of a proanthrocyanidine with selective calcium-binding capacity mediated the inhibition of human complement activation through the classical pathway [8]. Two acylphloroglucinol derivatives, named multifidol and multifidol glucoside [9], have been found to inhibit the luminol-dependent chemiluminescence mediated by reactive oxygen species (ROS) produced by activated polymorphonuclear leukocytes (PMNs). In the same series of activity-guided fractionation studies we isolated two cyclic peptides, a decapeptide and a nonapeptide, which show selective inhibition of the classical pathway of human complement activation in vitro. The activity of both cyclic peptides was found to be based on an interaction with C1 of the human complement system. The results of the study on these immunomodulatory effects will be published

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Abbreviations: NMR, nuclear magnetic resonance; 2D, two-dimensional; COSY, 2D shift correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; FAB-ms, fast atom bombardment-mass spectroscopy; TLC, thin-layer chromatography

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elsewhere. In the present paper we report on the isolation and the primary structure of the cyclic decapeptide using amino acid analysis. FAB-mass spectroscopy, two-dimensional ^{1}H-NMR (COSY and NOESY). We named this novel cyclic decapeptide labaditin.

2. MATERIALS AND METHODS

2.1. *Plant material*

Latex was collected from the leaf stalks of two-week-old plants of *Jatropha multifida*, cultivated in the garden of the first author in Surabaya (Indonesia). To that end young stalks were cut off at two weeks intervals at 5 a.m. from June to September 1986. The harvested crude latex was kept at −20°C.

Plants were also cultivated in the green house of Utrecht University, The Netherlands and in the botanical garden of Santo Tomas University, The Philippines, from seeds of the Surabaya population and identified.

2.2. *Peptide isolation*

Crude latex was mixed with an equal amount of water, centrifuged and the supernatant was freeze-dried yielding a solid which is about 10% of the latex. 500 mg dried water-soluble material was chromatographed on a polyamide column (4.5 × 25 cm, MN-Polyamide-CC 6, grain size 0.16 μm) successively with 500 ml of the following mixtures of MeOH/H_2O (0:100, 20:80, 40:60, 60:40 and 100:0) as eluting solvents, yielding 161, 30, 93, 42 and 16 mg lyophilized material, respectively.

The concentrated MeOH/H_2O (60:40) fraction (42 mg) was dissolved in 1% $NaHCO_3$ and extracted repeatedly with ethyl acetate.

The combined ethyl acetate fractions were evaporated under reduced pressure to dryness, dissolved in 2 ml MeOH and chromatographed over Sephadex LH-20 with MeOH as eluting solvent and collecting 300 drops per fraction. Fractions 4, 5 and 6, showing a single spot on TLC, were evaporated under reduced pressure and freeze-dried yielding 14 mg of a white solid.

2.3. *Amino acid analysis*

The amino acid composition was determined with an automatic amino acid analyser (LKB 4151 Alpha plus, Na-system 20 cm column) after hydrolysis in 6 N HCl at 110°C for 48 h and, for tryptophan determination, in 6 N HCl with 4% thioglycolic acid at 110°C for 24 h.

2.4. *Thin-layer chromatography (TLC)*

Silica gel 60 F-254 TLC plates were used with $CHCl_3$/MeOH/H_2O (65:50:10) as solvent. Spots were visualized under UV at 254 nm and with vanillin/sulphuric acid reagent.

2.5. *NMR spectroscopy*

For the NMR experiments the purified peptide was dissolved in DMSO-d6 (conc. 30 mg/ml). For some experiments 5% D_2O was added to exchange the amide protons. ^{1}H-NMR spectra were recorded on a Bruker WM-300 spectrometer at 303 and 338 K. Two-dimensional ^{1}H-NMR spectra were obtained at 303 K. For the COSY spectrum 257 records of 2K data were recorded at 400 MHz on a Bruker MSL-400. The phase-sensitive NOESY experiment, containing 350 records of 2K data, was obtained at 600 MHz on a Bruker AM-600.

The NOESY data were multiplied with sinebell windows and Fourier transformed in both domains. The COSY spectrum was displayed in the absolute value mode.

2.6. *FAB-MS*

For the FAB experiments a ZAB-2F VG instrument was used.

3. RESULTS AND DISCUSSION

3.1. *Characterization and amino acid analysis*

The immunomodulatory activity-guided isolation of the 60% MeOH fraction resulted in one compound. In TLC analysis of labaditin the substance exhibits a single spot (R_f value, 0.69) and detected as a purple spot after sprayed with vanillin reagent. The presence of tryptophan in labaditin is responsible for the colour reaction.

Amino acid analysis and the molecular mass, found by FAB ms, proved unambiguously that labaditin is a cyclic decapeptide consisting of one Ala, two Gly, one Ile, two Thr, two Trp and two Val (table 1). In agreement with this the chemical shifts due to 10 amide protons were found in the NMR spectra.

3.2. *NMR spectra*

Assignments of the ^{1}H-chemical shifts of the individual amino acid residue were achieved by means of a COSY spectrum. In such a spectrum correlations can be seen between the *J*-coupled

Table 1

Amino acid composition of labaditin[a]

	Value of HPLC measurements			Molecular mass (FAB)
	[b]	[c]	Mass	
Thr	1.9 (2)	– (2)	238	1070
Gly	2.0 (2)	2.0 (2)	150	
Ala	1.1 (1)	1.0 (1)	89	
Val	2.0 (2)	2.0 (2)	234	
Ile	1.0 (1)	1.0 (1)	131	
Trp		1.5 (2)	408	
				1250 − (10 × 18) = 1070

[a] The numbers of parentheses are deduced assuming labaditin is a single peptide

[b] Hydrolysis with 6 N HCl at 110°C for 48 h

[c] Hydrolysis with 6 N HCl + 4% thioglycolic acid at 110°C for 24 h

protons of each amino acid residue [10,11]. Thus one spin system corresponding to a valine was identified by cross-peaks between the NH and the Cα proton, between Cα and Cβ protons, between the Cβ and the Cγ and the Cγ1 protons (data not shown). In this way the spin systems corresponding to one Ala, two Gly, one Ile, two Thr, two Val and the aliphatic part of two Trp systems were obtained. Also in the aromatic region of the ^{1}H-NMR spectrum two aromatic spin systems corresponding to Trp could be identified. On the basis of NOEs observed in the NOESY spectrum between the aromatic ring resonances and the Cβ protons the complete Trp spin systems were identified. Thus a complete correspondence between the amino acid composition and the spin system analysis was obtained.

The individual amino acid numbering is according to their amide proton chemical shift; the amide proton chemical shift at lowest field being

Table 2

Chemical shifts of the individual amino acid moieties of labaditin in DMSO-d6 at different temperatures

Proton	δ 303 K (ppm)	δ 338 K (high) (ppm)	Δδ 303 K/l [12] (ppm)	Δδ 303/338 K (ppm)
Ala 01				
Hα	3.78	3.92	−0.6	−0.1
Hβ	1.27	1.29	0.0	0.0
NH	8.97	8.59	0.1	0.4
Gly 07				
Hα1	3.29	3.44	−0.5	−0.2
Hα2	3.85	3.90	0.1	0.0
NH	7.78	7.67	−0.4	0.1
Gly 09				
Hα1	3.44	3.48	−0.3	0.0
Hα2	4.44		0.7	
NH	7.52	7.60	−0.6	−0.1
Ile 04				
Hα	4.22	0.00	0.0	0.0
Hβ	1.64	1.73	−0.3	−0.1
Hγ1a	0.90	1.05	−0.3	−0.1
Hγ1b	1.49	1.50	0.0	0.0
Hγ2	0.80		−0.1	
Hδ	0.79		0.0	
NH	8.43	8.09	0.2	0.4
Thr 03				
Hα	4.66	4.58	0.4	0.1
Hβ	3.83	3.94	−0.1	−0.1
Hγ	0.91	0.95	−0.2	0.0
NH	8.70	8.24	0.9	0.5
Thr 10				
Hα	4.71	4.64	0.6	0.1
Hβ	4.35	0.00	0.4	0.0
Hγ	1.16	1.13	0.1	0.0
NH	7.30	7.34	−0.5	0.0

Table 2 (continued)

Chemical shifts of the individual amino acid moieties of labaditin in DMSO-d6 at different temperatures

Proton	δ 303 K (ppm)	δ 338 K (high) (ppm)	Δδ 303 K/l [12] (ppm)	Δδ 303/338 K (ppm)
Trp 02				
CH2	7.15	7.14	0.0	0.0
CH4	7.44	7.46	−0.2	0.0
CH5	6.94	6.94	−0.1	0.0
CH6	7.02	7.03	−0.1	0.0
CH7	7.27	7.28	−0.1	0.0
CNH	10.77		0.0	
Hα	4.54	0.00	0.0	0.0
Hβ1	2.96	3.00	0.0	0.0
Hβ2	3.07		−0.1	
NH	8.78	8.47	0.7	0.3
Trp 06				
CH2	7.02	7.03	−0.2	0.0
CH4	7.50	7.51	−0.2	0.0
CH5	6.97	6.96	0.0	0.0
CH6	7.04	7.04	−0.1	0.0
CH7	7.28	7.30	−0.1	0.0
CNH	10.77		0.0	
Hα	4.51		−0.1	
Hβ1	2.69	2.79	−0.2	−0.1
Hβ2	3.32	3.32	0.3	0.0
NH	7.83	7.76	−0.3	0.1
Val 05				
Hα	3.67	3.81	−0.6	−0.1
Hβ	1.82	1.86	−0.2	0.0
Hγ1	0.32	0.51	−0.6	−0.2
Hγ2	0.63	0.67	−0.3	−0.1
NH	8.20	7.98	0.3	0.2
Val 08				
Hα	4.22		0.0	
Hβ	2.06	2.07	0.1	0.0
Hγ1	0.82	0.83	−0.1	0.0
Hγ2	0.83	0.83	−0.2	0.0
NH	7.68	7.63	−0.2	0.0

number 1. The chemical shifts at 303 K and 338 K, their delta values, and the difference in chemical shifts, compared with those given in the literature for the same amino acid moiety in protected small linear peptides [10] are given in table 2. The chemical shifts of many resonances deviate significantly from those in the linear peptides, indicating that the molecule has a well defined three-dimensional structure. Concerning the 10 amide NH-chemical shifts, the five between 8.2 and 9.0 are at a normal field and are temperature-dependent (>0.2 ppm difference with the 338 K spectrum). The other five shifts appear at a relative high field and are not or almost not temperature dependent, probably indicating hydrogen bonds [10] (especially from 7 Gly, 9 Gly and 10 Thr).

The sequence of the individual amino acid in the cyclic decapeptide was determined by means of sequence-specific NOE assignments in the NOESY spectrum [11]. In the NOESY spectrum cross peaks can be observed between protons which are closer to each other (<4 Å). Thus apart from

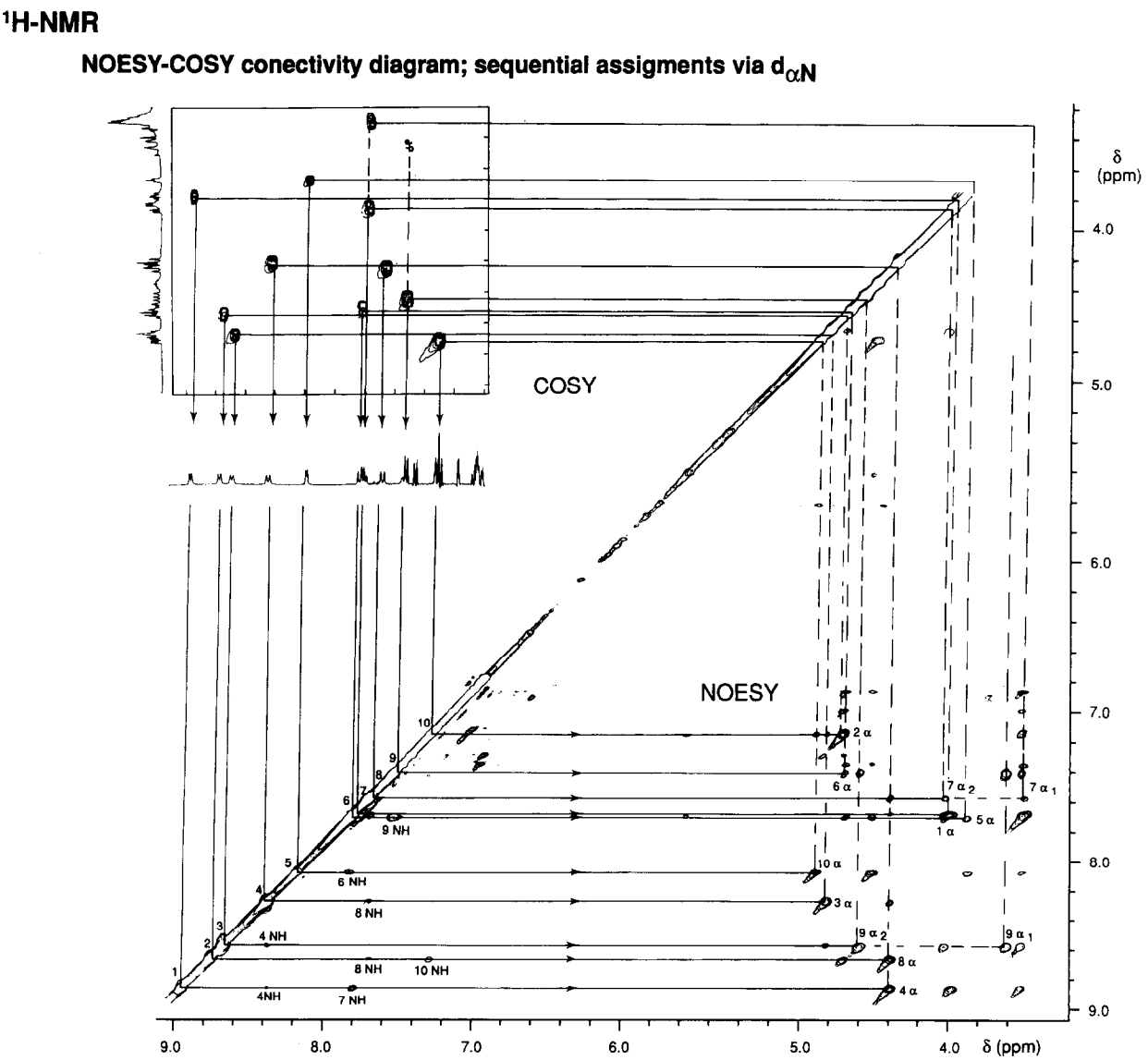

Fig.1. COSY-NOESY connectivity diagram. Indicated are the sequential assignments of labaditin via $d_{\alpha N}$ (cf. fig.3). An NOE indicated as 4α on the line of 1 identifies the contact of the amide proton of 1 with the α proton of 4.

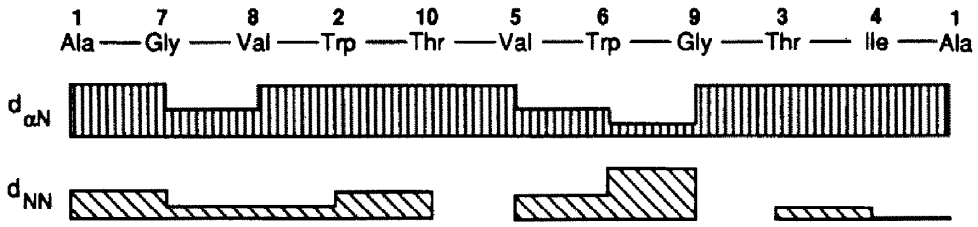

Fig.2. Summary of the sequential $d_{\alpha N}$ and d_{NN} connectivities in the NOESY. The width of the bars indicate the infinity of the NOE cross peaks.

NOEs between protons belonging to the same amino acid, also NOEs between protons belonging to adjacent amino acids can be observed.

Depending on the exact structure strong $d_{\alpha N}(i,i+1)$ connectivities (for extended structures) or relatively strong $d_{NN}(i,i+1)$ connectivities are almost always found between neighbouring amino acids [11]. These connectivities form the basis for the sequential assignment methods of protein ^{1}H-NMR spectra [11]. In this paper, however, we use these sequential connectivities in a reversed way: the $d_{\alpha N}$ and d_{NN} connectivities are used to establish the amino acid sequence of the cyclic decapeptide [12].

All amide protons and most α protons showed separate chemical shifts in the NOESY spectrum. Only the α protons of 4 Ile and 8 Val have the same chemical shifts in this spectrum. Assignments of the connectivities with these protons, however, could be made through the co-occurrence of NOEs with other protons of these particular amino acids. In this way connectivities were assigned between 1 Ala-NH and 4 Ile-αH and between 2 NH and 8 Val-αH.

A combined COSY and NOESY spectrum (high field region only), plotted at the same scale, showing the sequential $d_{\alpha N}$ connectivities of the ten amide NH protons, is given in fig.1. Thus starting at the NH frequency of the unique 1 Ala residue, we observe a $d_{\alpha N}$ connectivity to the unique 4 Ile spin system in addition to the internal αN cross peak of Ala. Thus the 4 Ile is likely to be adjacent in the sequence to the 1 Ala residue. Now we continue on the NH frequency of 4 Ile and identify a $d_{\alpha N}$ connectivity to 3 Thr. On the 3 Thr-NH frequency we observe two connectivities to the next 9 Gly. On the 9 NH frequency we observe a weak $d_{\alpha N}$ connectivity to 6 Trp.

In the same way we found $d_{\alpha N}$ connectivities from 6 Trp-NH to 5 Val-αH, from 5 Val-NH to 10 Thr-α H, from 10 Thr-NH to 2 Trp-α H, from 2 NH to 8 Val-αH, from 8 Val-NH to two 7 Gly-α protons and from 7 NH to 1 Ala-αH.

A sequence consistent with these observations is given in fig.2 (with observed d_{NN} and $d_{\alpha N}$ values) and in fig.3. The $J_{NH\alpha H}$ of 5 Val (J = 4.1 Hz) is rather unusual, indicating a *cis* rather than a *trans*-configuration between the 5 NH and 5 αH.

Whereas both the α protons of 7 Gly have almost the same $J_{NH\alpha H}$ (~6 Hz) those of 9 Gly are quite different (J = 2.7 and 9.1 Hz) indicating a *cis*- and a *trans*-configuration, respectively, and thus a more rigid conformation.

Thus the molecule probably contains two turns containing 5 Val and 7 Gly, respectively. This is in agreement with the two observed d_{NN} connectivities at these residues. The extended structure between the two turns form probably an antiparallel β-structure stabilized by hydrogen bonds. The presence of these H-bonds explains the low dependency of the chemical shift on temperature for a number of the NH protons. However a more complete description of the structure of the cyclic decapeptide has to await a more quantitative analysis of all NOEs from the NOESY spectrum. FAB-MS $[M+H]^+$ = 1071.

Finally it is stated that labaditin is the first cyclic decapeptide found in higher plants. Its most in-

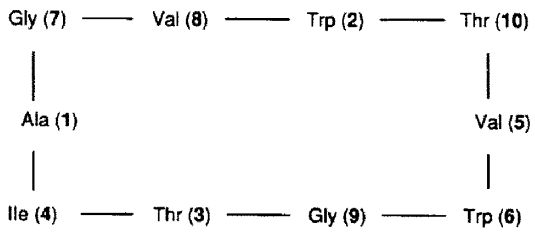

Fig.3. Structural formula for labaditin.

teresting occurrence prompted us to check whether freshly collected latex also contained this compound. This could be confirmed by direct TLC analysis of the freshly collected latex from plants grown in the green house of Utrecht University.

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