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Structure of syringotoxin, a bioactive metabolite of *Pseudomonas syringae* pv. *syringae*

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The covalent structure of syringotoxin, a bioactive metabolite of *Pseudomonas syringae* pv. *syringae* isolates, pathogenic on various species of citrus trees, has been deduced from 1D and 2D ^{1}H- and ^{13}C-NMR spectra combined with extensive FAB-MS data and results of some chemical reactions. Similarly to syringomicins and syringostatins, produced by other plant pathogenic strains of *P. syringae* pv. *syringae*, syringotoxin is a lipodepsinonapeptide. Its peptide moiety corresponds to Ser-Dab-Gly-Hse-Orn-aThr-Dhb-(3-OH)Asp-(4-Cl)Thr with the terminal carboxy group closing a macrocyclic ring on the OH group of the N-terminal Ser, which in turn is N-acetylated by 3-hydroxytetradecanoic acid.

Phytotoxin; Lipodepsipeptide; Syringotoxin; *Pseudomonas syringae* pv. *syringae*

1. INTRODUCTION

Among plant pathogenic strains of *Pseudomonas syringae* pv. *syringae*, some isolated from species of citrus trees produce syringotoxin (ST), a bioactive compound with an undefined peptide structure [1-3]. In previous papers dealing with the chemistry of syringomycins (SRs), we have shown that several isolates of the above bacterium pathogenic on stone fruits, pear and grass hosts synthesize a group of structurally related peptides [4], and we have proposed a structure for the major component of the mixture (SR-E), and for a lower and a higher homologue of it (SR-A_1 and SR-G, respectively) [5]. Recently, a japanese group has reported on syringostatins, a group of antifungal metabolites of a *P. syringae* pv. *syringae* strain isolated from lilac [6], and has described the structures of the two major components, syringostatins A and B [7], as well as that of a syringomycin closely related to our SR-E, produced by a sugar cane isolate of the same bacterium [8].

All above mentioned bacterial metabolites are lipodepsinonapeptides sharing a number of structural features. Particularly characteristic is the C-terminal part of the nonapeptide moiety, which contains a cluster of three uncommon amino acids (2,3-dehydro-2-aminobutyric acid, 4-chlorothreonine and 3-hydroxyaspartic acid), with the terminal carboxyl closing a large lactone ring on the N-terminal Ser, which is in turn N-acylated by a long chain 3-hydroxy fatty acid.

Recently the amino acid sequence of the C- terminal peptide moiety proposed for SRs [4] has been questioned: it was found to be Dhb-(3-OH)Asp-(4-Cl)Thr (with the α-carboxyl of (3-OH)Asp implied in the peptide linkage) in syringostatins A and B [6], as well as in SR isolated from the sugar cane pathogenic bacterium [7]. The present paper demonstrates that ST differs from syringostatin A for a single amino acid substitution; furthermore it provides conclusive evidence that the sequence of the two C-terminal amino acids in SRs is identical to that of all the so far identified lipodepsipeptides of plant pathogenic *P. syringae* pv. *syringae*.

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Abbreviations: DMSO, dimethyl sulfoxide; FAB-MS, fast atom bombardment mass spectrometry; TBDMS, *t*-butyldimethylsilyl; (3-OH)Asp, 3-hydroxyaspartic acid; Dab, 2,4-diaminobutyric acid; Dhb, 2,3-dehydro-2-aminobutyric acid; (4-Cl)Thr, 4-chlorothreonine; Hse, homoserine; Hse>, homoserine lactone; (4-OH)Thr, 4-hydroxythreonine; aThr, allo-threonine; DQF-COSY, double quantum filtered correlated spectroscopy; TOCSY, total correlated spectroscopy; NOESY, nuclear Overhauser effect correlated spectroscopy; SR, syringomycin; ST, syringotoxin

2. MATERIALS AND METHODS

2.1. *Preparation of syringotoxins*

P. syringae pv. *syringae* strain B427 (from lemon) was grown in still culture for 6 days in medium IMM [9]. Two ml of a 24 h shake culture were used to inoculate 150 ml of the medium in 1 liter Roux bottles. After incubation at 25°C, cultures were acidified at pH 2, and ST was extracted and purified according to Surico and DeVay [10]. Further

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fractionation was carried out by HPLC as reported previously for SRs [4].

2.2. *Analytical methods*

The antibiotic activity was assayed on *Rodotorula pilimanae* [11]. Amino acid analyses were carried out with an LKB Alpha plus 4151 analyzer after hydrolysis with 6 N HCl at 110°C for 24 h in vacuo, or by GC-MS after transformation into TBDMS derivatives [12]. N-terminal analyses were performed by a dansyl chloride method [13]. The chirality of amino acid residues was determined by a modified (unpublished) Marfey's method [14]. FAB-MS spectra were mostly obtained as reported in [4]. 1D and 2D NMR (^{1}H-^{1}H DQF-COSY, TOCSY-MLEV17, ROESY with two different mixing times 100 and 140 ms) experiments were performed on 200 and 400 MHz Bruker spectrometers. The spectra were run at 25°C in D_2O, DMSO and CD_3CN/H_2O [7] solutions containing 1 mg·ml^{-1} without any pH correction. NMR data were processed with a program kindly provided by Prof. R. Kaptein, Dept. of Organic Chemistry, Afd. NMR, University of Utrecht, The Netherlands.

2.3. *Chemical methods*

Partial acid hydrolyses were performed in 60 mM HCl for 90 min at 110°C; after lyophilization the hydrolysate was fractionated by HPLC. The opening of the lactone, accompanied by substitution of Cl with OH, was achieved by incubation with 0.1 M ammonium bicarbonate at 37°C for 5 h.

Table I

^{1}H Chemical shifts and relative assignments for syringotoxin in three different solvents

Residue	Position	D_2O	DMSO	CD_3CN/H_2O
Fatty acid	C14	0.83	0.83	0.83
	C5-C13	≃ 1.2	1.21	1.23
	C4	1.47	≃ 1.3	1.41
	C3	3.94	3.89	3.71
	C2	2.43-2.38	2.22	2.01
Ser	NH	-	8.2	8.38
	Cα	4.85	4.65	4.76
	Cβ	4.65	4.15	4.60-4.26
Dab	NH	-	8.66	8.43
	Cα	4.21	4.3	4.15
	Cβ	≃ 2.2	1.97	2.05
	Cγ	3.09	2.80	2.99
Gly	NH	-	8.47	7.95
	Cα	4.07-3.86	3.83-3.63	≃ 4.05
Hse	NH	-	8.20	7.50
	Cα	4.37	4.13	4.28
	Cβ	≃ 2.1	1.83	2.05-1.90
	Cγ	3.37-3.48	3.51	3.28
Orn	NH	-	8.0	8.83
	Cα	4.45	4.30	4.61
	Cβ	≃ 2.0	1.74	≃ 1.85
	Cγ	1.68	1.53	1.51
	Cδ	3.00	2.80	2.94
Thr	NH	-	8.12	8.18
	Cα	4.15	4.10	4.08
	Cβ	4.10	3.74	4.02
	Cγ	1.36	1.16	1.27
Dhb	NH	-	-	9.31
	Cβ	6.93	6.60	6.82
	Cγ	1.75	1.62	1.63
(3-OH)Asp	NH	-	7.6	7.65
	Cα	5.03	4.72	4.92
	Cβ	4.70	4.06	4.58
(4-Cl)Thr	NH	-	8.13	8.38
	Cα	5.01	4.68	4.86
	Cβ	4.42	≃ 4.14	4.34
	Cγ	3.57-3.52	3.56-3.49	≃ 3.44

3. RESULTS AND DISCUSSION

At variance with partially purified samples of SR, ST yielded in HPLC an elution profile with only one main peak (a more hydrophobic peptide with a different amino acid composition was evidenced on washing the column); a number of small peaks represented less than 10 per cent of the total eluate. The main peak gave after freeze-drying a white amorphous residue, soluble in water, acetic acid, DMSO, and very sparingly soluble in alcohols. Its activity against *Rhodotorula pilimanae* was 38.4 U·μg^{-1}. The UV spectrum showed only end absorption. The IR spectrum in KBr contained a strong amide CO band at 1672 cm^{-1} and a weaker band at 1745 cm^{-1}. FAB-MS showed a doublet MH^+ ion at 1136–1138 with an intensity suggestive of the presence of one chlorine atom. 1D and 2D COSY ^{1}H-NMR spectra in D_2O, and in particular a TOCSY-MLEV-17 spectrum in the same solvent [15,16], made possible the assignment of resonances to all spin systems of amino acids using a well established procedure [17] (Table I). The only two degenerate systems are the two AX and the two AM_2X_2, which should be due to Gly and (3-OH)Asp and to Dab and Hse respectively; these can be identified on the basis of coupling constants as well as chemical shift considerations. Sequential assignment, see below, confirm the above considerations. This agreed with those found by conventional amino acid determinations and/or by GC-MS analysis of their TBDMS derivatives [11]. In particular, five corresponded to those constantly present in the previously

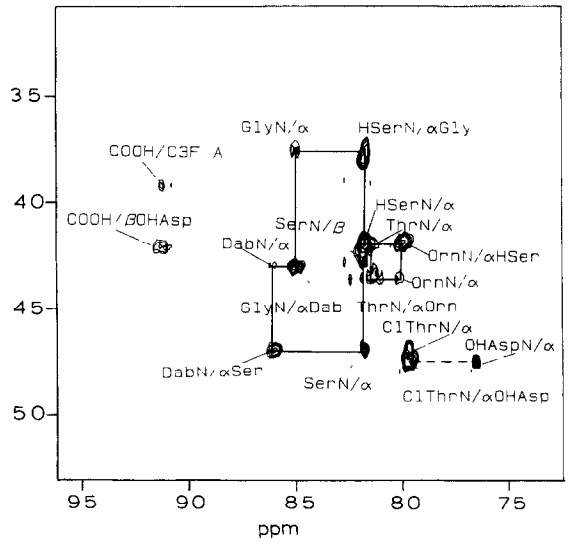

Fig. 1. Rotating frame NOE connectivities between resonances of protons in the amide range (9.7–7.3 ppm) and CHα range (5.5–3.0 ppm) obtained by ROESY experiment with a mixing time of 100 ms in DMSO solution. ^{1}H-NMR assignments obtained by DQF-COSY and MLEV17-TOCSY used to identify the spin systems (not shown) are indicated. The solid line shows the sequential assignment pathway for the esapeptide segment. The dashed line indicates dipeptide segment.

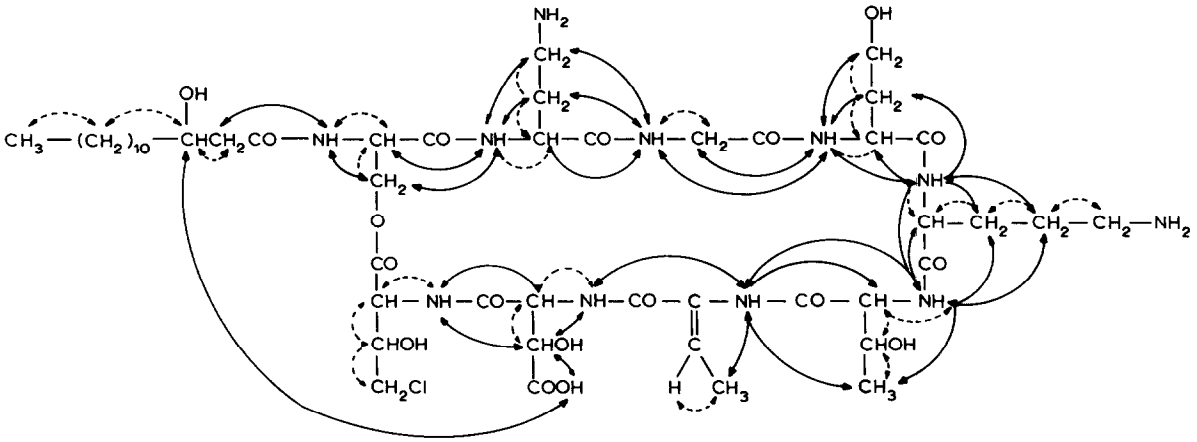

Fig. 2. Structure of syringotoxin. Arrows represent the proton-proton NOE couplings in the ROESY spectra in two different solvents: DMSO and CD_3CN/H_2O. TOCSY-MLEV17 (or COSY) cross peaks are indicated by dotted lines in two different solvents: D_2O and CD_3CN/H_2O.

investigated *P. syringae* pv. *syringae* metabolites [5–8], namely (4-Cl)Thr, Dab, Dhb, (3-OH)Asp and Ser; the remaining four amino acids were Gly, Hse, Orn and Thr, and corresponded, with the exception of Gly, to those found in syringostatins A and B [6,7]. The ^{13}C-NMR spectrum in D_2O demonstrated furthermore the presence in ST of a 3-hydroxytetradecanoyl group, as in SR-G [5] and in syringostatin A [6,7]. The molecular mass determined by FAB-MS is accounted for by the condensation of the above components to give a lactone structure, which is supported by the IR absorption at 1745 cm^{-1}.

The ^1H-NMR analysis in DMSO yielded information on the protons of some NH peptide groups and on the free carboxy group of (3-OH)Asp (Table I). The ROESY spectrum in the same solvent showed NOE peaks between the protons of one amino acid (identified by DQF-COSY and TOCSY-MLEV-17) and the peptide NH of the adjacent amino acid and thus provided the proof of the sequences Dab-Gly-Hse-Orn-Thr and (3-OH)Asp-(4-Cl)Thr (Fig. 1). These results are in agreement with those of automated Edman degradation carried out on partial acid hydrolysis fragments. The first sequence was further extended after having assigned the NH group to each amino acid on the basis of ^1H-NMR spectra run in CD_3CN-H_2 O (1D, DQF-COSY, TOCSY-MLEV17 and ROESY at two mixing times; see Table I) and is reported in Fig. 2 together with all NMR contacts. The sequence is in full agreement with the fragmentation observed in the FAB-MS spectrum of ST hydrolysed with ammonium bicarbonate, as well as with the nature of the products formed by mild acid hydrolysis of ST (Table II). Derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide [14] has allowed to establish the L-configuration for Ser, Orn, aThr, (3-OH)Asp and the D-configuration for Dab and Hse. The presence of the alloisomer of threonine has been confirmed by NMR data. In fact the value of $^3J\alpha\beta = 7.74$ Hz indicates a predominant *trans* conformation of the α-β protons in this residue. With this major conformer, a strong NOE contact, found between the NH group and the methyl, is compatible only with the alloisomer. Chirality has not yet been assigned to (4-Cl)Thr and to the 3-hydroxy fatty acid moiety. Some medium range NOE cross peaks were also observed, they will be discussed in a forthcoming paper with the discussion of the conformation of side chains.

Inspection of the structure so far proposed for the *P. syringae* pv. *syringae* lipodepsipeptides discloses in SRs a reversed order of the terminal and subterminal amino acid residues as compared to the other analogous metabolites. This has prompted a critical re-examina-

Table II

Some partial acid hydrolysis products of syringotoxin

Peptide	MH^+
CH_3-$(CH_2)_{10}$-CHOH-CH_2-CO-Ser-Dab-Gly-Hse-Orn-Thr-Dhb-(3-OH)Asp-(4-OH)Thr-OH	1136
CH_3-$(CH_2)_{10}$-CHOH-CH_2-CO-Ser-Dab-Gly-Hse-Orn-Thr-Dhb-(3-OH)Asp-OH	1019
CH_3-$(CH_2)_{10}$-CHOH-CH_2-CO-Ser-Dab-Gly-Hse-Orn-Thr-Dhb-OH	888
CH_3-$(CH_2)_{10}$-CHOH-CH_2-CO-Ser-Dab-Gly-Hse-Orn-Thr-OH	805
CH_3-$(CH_2)_{10}$-CHOH-CH_2-CO-Ser-Dab-Gly-Hse-Orn-OH	704
CH_3-$(CH_2)_{10}$-CHOH-CH_2-CO-Ser-Dab-Gly-Hse-OH	590
CH_3-$(CH_2)_{10}$-CHOH-CH_2-CO-Ser-Dab-Gly-Hse>	572
CH_3-$(CH_2)_{10}$-CHOH-CH_2-CO-Ser-Dab-Gly-OH	489
CH_3-$(CH_2)_{10}$-CHOH-CH_2-CO-Ser-Dab-OH	432
CH_3-$(CH_2)_{10}$-CHOH-CH_2-CO-Ser-OH	332

tion of SR-E NMR data which has led to conclude that the position of (4-Cl)Thr and (3-OH)Asp in SRs must indeed be reversed. We believe that the error in the interpretation reported [5] derived from the marked chemical instability of SRs in the solvent used (not observed with ST) with consequent progressive broadening of their CH-α signals which furthermore displayed very close chemical shift values. The final demonstration that (4-Cl)Thr occupies the C-terminal position also in SRs was obtained by FAB-MS of SR-E hydrolysed with ammonium bicarbonate (a sample was kindly supplied by Dr. I. Grgurina); the fragmentation spectrum showed losses from the MH^+ -ion identical to those reported by Fukuchi *et al.* for sugar cane SR [8].

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