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The structure of syringomycins A_1, E and G

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By a combination of 1D and 2D ^{1}H- and ^{13}C-NMR, FAB-MS, and chemical and enzymatic reactions carried out at the milligram level, it has been demonstrated that syringomycin E, the major phytotoxic antibiotic produced by *Pseudomonas syringae* pv. *syringae*, is a new lipodepsipeptide. Its amino acid sequence is Ser-Ser-Dab-Dab-Arg-Phe-Dhb-4(Cl)Thr-3(OH)Asp with the β-carboxy group of the C-terminal residue closing a macrocyclic ring on the OH group of the N-terminal Ser, which in turn is *N*-acylated by 3-hydroxydodecanoic acid. Syringomycins A_1 and G, two other metabolites of the same bacterium, differ from syringomycin E only in their fatty acid moieties corresponding, respectively, to 3-hydroxydecanoic and 3-hydroxytetradecanoic acid.

Phytotoxin; Lipodepsipeptide; Syringomycin A_1; Syringomycin E; Syringomycin G; *Pseudomonas syringae* pv. *syringae*

1. INTRODUCTION

Pseudomonas syringae pv. *syringae* is a bacterial pathogen of numerous monocot and dicot plants [1]. Most strains of this organism produce a non-host-specific toxin called syringomycin (SR) which significantly contributes to virulence [2–4]. The first paper on SR production by pathogenic isolates of *P. syringae* [2] was followed by a wide interest in the role of the toxin in plant pathogenesis, but surprisingly this has not been paralleled by studies on its structure. In fact, until recently, the only available information was derived from preliminary analyses carried out on preparations which appeared homogeneous by disc electrophoresis [5,6]; they showed that SR was a low molecular mass, peptide-containing substance which on acid hydrolysis yielded Arg, Phe, Ser and an unidentified basic amino acid in a 1:1:2:2 molar ratio [6]. More recently, it has been reported that the above preparations are mixtures which can be resolved by reverse-phase HPLC into a number of components, all containing Arg, Phe, Ser and Dab in the molar ratio 1:1:2:2, as well as some other unidentified amino acids and a long chain 3-hydroxy fatty acid [7]. Here, we demonstrate that SR-E and SR-G, the two most active and abundant components of the mixtures, and SR-A_1, a minor component, are new cyclic lipodepsinonapeptide phytotoxins.

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Abbreviations: DMSO, dimethyl sulfoxide; FAB-MS, fast atom bombardment mass spectrometry; Abu, 2-aminobutyric acid; 3(OH)Asp, 3-hydroxyaspartic acid; Dab, 2,4-diaminobutyric acid; Dhb, 2,3-dehydro-2-aminobutyric acid; 4(Cl)Thr, 4-chlorothreonine; 4(OH)Thr, 4-hydroxythreonine; SR, syringomycin

2. MATERIALS AND METHODS

2.1. *Syringomycins*

SR-A_1, SR-E and SR-G were obtained by HPLC fractiona-

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tion of SR mixtures [7]. Each batch was quantitated by amino acid analysis after HCl hydrolysis [7].

2.2. *Analytical methods*

Amino acid analyses were carried out with an LKB Alpha plus 2231 analyser after hydrolysis with 6 N HCl at 110°C for 24 h in vacuo. *N,O*-Trifluoroacetyl isopropyl esters of amino acids were prepared according to Bayer et al. [8] and analysed by GC-MS on an LKB 2091 instrument equipped with an OV-101 capillary column operated either isothermally at 115°C or programmed from 100 to 280°C at 10°C/min.

N-terminal analyses were performed by the dansyl chloride method of Gray [9].

3-Hydroxydodecanoic acid methyl ester was analysed by an HP-5058 gas chromatograph connected to an HP-5970 mass spectrometer; a silica capillary column crosslinked with methylsilicone and programmed from 100 to 250°C at 3°C/min was used. FAB-MS spectra were mostly run as reported in [7]. The set of intensity measurements of the MH^+ and $MH^+ + 2$ signals for SR-E were obtained by using 3-nitrobenzyl alcohol as a matrix [10]. High-resolution and negative ion FAB-MS of TNP were recorded on an MAT 731 spectrometer equipped with an Ion Tech BF-11 gun with ionization by 6 keV Xe.

1D and 2D NMR (^1H-^1H COSY, COSYLR, NOESY and ^1H-^{13}C heterocorrelation XHCORRDC, and WALTZ decoupled [11] 1D-^{13}C) experiments were performed on 200, 300, 400 and 500 MHz spectrometers. Unless otherwise stated all spectra were run at 55°C and on D_2O solutions containing 1 or 8 mg in 0.5 ml for ^1H or ^{13}C, respectively.

2.3. *Chemical methods*

Catalytic hydrogenations were performed at room temperature and 1 atm pressure on 10% Pd on C in 50% aqueous ethanol. SR-E was oxidised with DMSO in acetic anhydride [12]. SR-E methyl ester was prepared by 15 h treatment with 14% BF_3 in methanol at room temperature.

Methyl 3-hydroxydodecanoate was prepared by adding ethereal diazomethane to an ethyl acetate extract of hydrolysed SR-E. Partial acid hydrolysis of SR-E was performed in 120 mM HCl for 14 h at 110°C; after lyophilization the sample was dissolved in 0.2% trifluoroacetic acid and fractionated by reverse-phase HPLC [7].

2.4. *Enzymatic hydrolyses*

Digestion with trypsin (TPCK-treated, Worthington) was carried out in 0.1 M ammonium bicarbonate at 37°C for 5 h at an enzyme/substrate ratio of 1:30 (w/w). Digestion with thermolysin (Serva, Heidelberg) was carried out in 0.2 M *N*-ethylmorpholine buffer (pH 7.6) at either 37 or 60°C for 12 h at an enzyme/substrate ratio of 1:2 (w/w). Digestion with carboxypeptidase B or Y (Boehringer, Mannheim) was performed in 0.1 M ammonium bicarbonate or *N*-ethylmorpholine buffer (pH 8.0) at 37°C for 1–4 h at an enzyme/substrate ratio of 1:30 (w/w).

3. RESULTS AND DISCUSSION

Pure SR-E was obtained by HPLC [7] as a white amorphous solid, soluble in water, acetic acid, pyridine or DMSO, and very sparingly soluble in methanol and ethanol. Its UV spectrum showed only end absorption. The IR spectrum in KBr contained a strong amide CO band at 1670 cm^{-1} and a weaker band at 1737 cm^{-1}. FAB-MS showed a doublet MH^+ ion at 1225–1227; when the matrix was 3-nitrobenzyl alcohol the intensity of the isotopic quasimolecular ion peaks suggested the presence of one chlorine atom, confirmed by qualitative elemental microanalysis. By employing homo- and heteronuclear 1D and 2D NMR techniques, all proton and carbon resonances were assigned (table 1). In particular, nine peptide carbonyls, out of a total of 11 CO groups were detected, suggesting a peptide moiety composed of nine amino acid residues, namely three more than those identified in previous investigations [6,7].

A careful inspection of NMR spectra revealed the nature of the missing residues. Resonances ascribed to a $CH_3CH=C$ group indicated a Dhb residue, an assignment confirmed by the formation of 2-oxobutyric acid on HCl hydrolysis of SR-E, and of Abu on acid hydrolysis of dihydro-SR-E. The strongly upfield-shifted signals with respect to literature data [13] indicated moreover the proximity of this residue to Phe. The partial structure $C_\alpha H$-CH(OH)-CH_2X was derived for the second unknown residue, with X remaining at the end of the structural analysis the only possible assignment for the chlorine atom in SR-E. The ^1H-NMR spectrum of a sample of *N*-Cbz-4-chlorothreonine methyl ester, prepared according to Shaw et al. [14], was also in keeping with this assignment. Finally, the third unknown residue was identified as 3(OH)Asp by comparing its shifts and coupling constants with those of a commercial sample (Sigma) of the amino acid. The occurrence of 4(Cl)Thr and 3(OH)Asp residues in SR-E was confirmed by GC-MS analysis of the *N,O*-trifluoroacetyl isopropyl ester derivatives of the amino acids present in acid hydrolysates of SR-E.

^1H-NMR shows that each amino acid, as well as the C_{12}-hydroxy-fatty acid (see below), is represented once in SR-E, except for Ser and Dab which contribute two residues [7]. Thus, on the assumption that the components of SR-E have a cyclic arrangement, the composition $C_{53}H_{85}N_{14}O_{18}Cl$, which matches the molecular mass determined by FAB-MS, can be calculated for SR-E. Support for this assumption required at first the assessment of the amino acid sequence in

Table 1

^{13}C- and ^{1}H-NMR signals of SR-E in D_2O and relative assignments[a]

Residue[b]	^{13}C	δ	^{1}H J
1α	56.64	4.76	$J_{\alpha\beta} = J_{\alpha\beta'} = 3.5$
β	65.69	4.60; 4.85	$J_{\beta\beta'} = -11.0$
2α	55.61	4.20	$J_{\alpha\beta} = 5.5$; $J_{\alpha\beta'} = 6.5$
β	61.27	3.95; 3.82	$J_{\beta\beta'} = -11.5$
3α	52.83	4.31 d,d	(10.0, 5.0)
β	28.80	2.00; 2.30 m	
γ	37.46	2.89; 2.98 m	
4α	52.54	4.29 d,d	(10.0, 5.0)
β	29.18	2.00; 2.30 m	
γ	37.46	2.89; 2.98 m	
5α	53.14	4.53 d,d	(9.5, 5.5)
β	28.52	1.75; 1.88 m	
γ	26.00	1.54 m	
δ	41.38	3.18 t	$J_{\gamma\delta} = 7.0$
guanidino-C	157.53		
6α	53.87	4.66	$J_{\alpha\beta} = 7.0$; $J_{\alpha\beta} = 9.5$
β	37.46	3.24; 3.06	$J_{\beta\beta'} = -12.0$
ring-1	136.44	7.2–7.4 m	
ring-2,6	130.19		
ring-3,5	129.72		
ring-4	128.27		
7α	128.10		
β	135.40	6.60 q	$J_{\beta\gamma} = 7.2$
γ	13.46	1.47 d	
8α	57.84	5.05 d	$J_{\alpha\beta} = 2.5$
β	72.57	4.45 d,t	$J_{\beta\gamma} = J_{\beta\gamma'} = 7.0$
γ	46.00	3.58; 3.54 d,d	$J_{gem} = -11.5$
9α	57.27	5.02	$J_{\alpha\beta} = 2.5$
α-COOH	176.23		
β	72.20	4.72	
β-CO	167.51		
Fatty acid C-2	43.78	2.48; 2.42 d,d	$J_{gem} = -14.5$
C-3	69.68	3.97 m	$J_{vic} = 4.5$; $J_{vic} = 8.5$
C-4	37.46	1.47 m	
C-5	25.87	1.2	
C-6,7,8	29.90	1.2	
C-9	29.64	1.2	
C-10	32.27	1.2	
C-11	23.08	1.2	
C-12	14.43	0.83 t	
Amide CO[c]	171.14		
	171.97		
	172.21		
	172.39		
	172.84		
	173.06		
	173.53		
	174.36		
	175.13		

[a] Chemical shifts (δ) are expressed in ppm from TMS, but actually are measured from 10^{-6} M acetone assumed at 2.225 ppm

[b] Arabic numerals refer to residue position in the peptide moiety (see formula in text); Greek letters to side chain positions [15]

[c] Nine unassigned signals

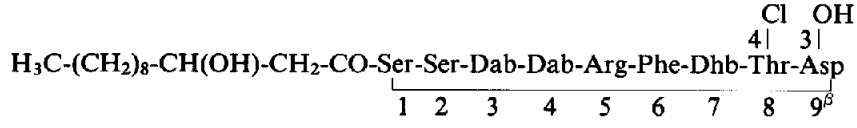
the peptide moiety of SR-E. Edman degradation and carboxypeptidase treatment indicated that both N- and C-terminals were non-reactive. Mild acid hydrolysis, followed by FAB-MS of the products partially fractionated by reverse-phase HPLC, provided evidence for the linear arrangement of seven out of nine amino acid residues and for the *N*-acylation of the N-terminal Ser by 3-hydroxydodecanoic acid (table 2). Trypsin or thermolysin cleaved SR-E into two fragments called TPP and TNP. The structure of TPP, deduced by positive ion FAB-MS, corresponded to 3-hydroxydodecanoyl-Ser-Ser-Dab-Dab-Arg-OH, with Arg removable by carboxypeptidase B. The structure of TNP, obtained by negative ion FAB-MS and supported by high-resolution FAB-MS (MH^+ calc. 496.1805, found 496.1800), was Phe-Dhb-4(OH)Thr-3(OH)Asp-OH with Phe removable by Edman degradation. The sequences of TPP and TNP are in complete agreement with the results of mild acid hydrolysis reported in table 2. The replacement of Cl by OH in TNP is ascribed to the lability of the C-Cl bond at the pH of the buffer. In fact, SR-E incubated in the absence of enzyme and monitored by FAB-MS shows an immediate uptake of one mole of water (MH^+ at 1243–1245), which suggests the presence of a lactone, followed by the replacement of Cl with OH (MH^+ at 1225, singlet ion peak). The formation of a lactone closing a macrocyclic ring between the β-carboxy group of the C-terminal 3(OH)Asp residue and the OH group of the N-terminal Ser is supported by the following facts: (i) the absorbance at 1737 cm^{-1} disappears when SR-E is treated with base, (ii) one Ser is protected from oxidation in SR-E, (iii) only one carboxy group is available for methylation in SR-E, (iv) the ^1H- and ^{13}C-NMR signals of the N-terminal Ser residue are strongly perturbed in SR-E as compared to those of the same residue in SR-E cleaved by base. The involvement of the β-carboxy group of 3(OH)Asp in the lactone function is proved by the pK_a value of the free carboxy group in SR-E (2.8 ± 0.1), as well as by the simultaneous broadening of the ^1H-NMR signals for the -CH_2O- group of the N-terminal Ser residue (δ 4.60 and 4.85 ppm) and the -CHOH group of the 3(OH)Asp residue (δ 4.72 ppm,d), when the temperature is lowered from 55 to 25°C.

Table 2

Some partial acid hydrolysis products of SR-E

Peptide	MH^+
3(OH)C_{11}CO-Ser-Ser-Dab-Dab-Arg-Phe-Dhb-OH	977
3(OH)C_{11}CO-Ser-Ser-Dab-Dab-Arg-Phe-OH	894
H-Ser-Ser-Dab-Dab-Arg-Phe-Dhb-OH	779
H-Ser-Ser-Dab-Dab-Arg-Phe-OH	696
H-Ser-Dab-Dab-Arg-Phe-Dhb-OH	692
H-Ser-Dab-Dab-Arg-Phe-OH	609
H-Dab-Dab-Arg-Phe-Dhb-OH	605
H-Dab-Arg-Phe-OH	422
H-Arg-Phe-Dhb-OH	405

Thus, the structure of SR-E is established as in the following abbreviated formula



which, according to IUPAC-IUB recommendations [15], has the peptide residues numbered 1–9 from the N-terminus; the upper numbers in Thr and Asp designate the carbon atom bearing the indicated substituent.

Work is in progress to assign the correct stereochemistry to all chiral carbons; NMR spectra demonstrate that both 3(OH)Asp and 4(Cl)Thr have the *threo* configuration and that Dhb is the *Z* isomer. The suggestion that SR-A_1 and SR-G are, respectively, a lower and a higher homologue of SR-E [7] has been confirmed by their ^1H-NMR spectra which coincide with those of SR-E, except for their fatty acid moieties which are 3-hydroxydecanoic and 3-hydroxytetradecanoic acids, respectively.

In conclusion, the three SRs described in the present paper are new members of the lipodepsinonapeptide family of bacterial metabolites which comprehends polypeptins A and B [16], permetins A and B [17] and herbicolins A and B [18].

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