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STRUCTURE OF PHYTOTOXIN SYRINGOMYCIN PRODUCED BY A SUGAR CANE ISOLATE OF PSEUDOMONAS SYRINGAE PV. SYRINGAE

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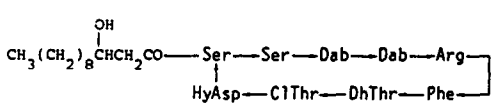
Abstract. The structure of the phytotoxin syringomycin produced by a sugar cane isolate of Pseudomonas syringae pv. syringae was determined as 1 with NMR and mass spectrometry.

Syringomycins are phytotoxins produced by the phytopathogenic bacterium, Pseudomonas syringae pv. syringae and show wide antimicrobial activity. The toxins are thought to be virulence determinants, but their structures have been uncertain for about two decades.[1,2] Recently, Segre et al. proposed the structure of syringomycin E, the major component of a mixture of these toxins, as 2.[3,4] In our research for the phytotoxins produced by P. syringae pv. syringae, we found that one strain of this bacterium produced new toxins named syringostatins,[5] and the structure of syringostatins were established as 3.[6] The structures 2 for syringomycin E[3,4] and the structures of syringostatins (3)[6] are closely related. Besides the replacement of several amino acids, 2 differs from 3 in two other major ways: the arrangement of the C-terminal two amino acids and the linkage of β-hydroxyaspartic acid residue. In recent investigation, we found that P. syringae pv. syringae SC1, isolated from sugar cane in Japan, produced syringomycins. In this communication, we report the isolation and the structural determination of the major syringomycin of this isolate. The structure differs slightly from that of syringomycin E reported by Segre et al.[3,4]

Pseudomonas syringae pv. syringae SC1. cultured to stationary phase in the potato medium reported by Zhang and Takemoto[7] for 9 days, indicated antimicrobial activity to Saccharomyces cerevisiae. The culture supernatant fluid, acidified with acetic acid, was passed through an XAD-2 column. And the effluent was adsorbed on an XAD-7 column, washed



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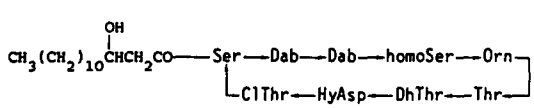

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Table 1 ^{13}C and ^{1}H spectrum of syringomycin (1) (600MHz).

Unit	C No.	δ_C	δ_H	Unit	C No.	δ_C	δ_H
A	1	174.8		F	1	174.0	
	2	43.9	2.36 (dd, 9,15), 2.44 (dd, 4,15)		2	53.9	4.37 (m)
	3	69.7	3.93 (m)		3	29.2	1.70 (m), 1.81 (m)
	4	37.8	1.47 (m)		4	25.4	1.52 (m)
	5	26.2	1.33 (m)		5	41.7	3.14 (m)
	6-8	30.2	1.30 (m)		αNH		8.05 (d, 7)
	9	29.9			δNH		7.24 (br.t, 6)
	10	32.5			$C(NH_2)_2$	158.0	6.63 (br.)
	11	23.2		G	1	172.6	
	12	14.3	0.90 (t, 7)		2	56.6	4.64 (m)
B	1	172.1			3	37.9	3.11 (m)
	2	53.3	4.67 (ddd, 5,5,8)		φ1	137.1	
	3	65.5	4.52 (dd, 5,12), 4.56 (dd, 5,12)		φ2,6	130.3	7.31 (d, 7)
	αNH		8.22 (d, 8)		φ3,5	129.6	7.35 (t, 7)
C	1	173.3			φ4	127.9	7.28 (t, 7)
	2	58.1	4.21 (ddd, 6,6,6)		αNH		7.99 (d, 6)
	3	61.8	3.81 (dd, 6,12), 3.90 (dd, 6,12)	H	1	166.7	
	αNH		8.23 (d, 6)		2	129.2	
D	1	173.0			3	134.0	6.52 (q, 7)
	2	52.7	4.34 (m)		4	13.3	1.36 (d, 7)
	3	29.2	2.11 (m), 2.27 (m)		αNH		9.18 (s)
	4	37.9	2.99 (m), 3.06 (m)	I	1	171.9	
	αNH		8.26 (d, 7)		2	57.0	5.01 (dd, 2,9)
	γNH_2		7.58 (br.)		3	72.3	4.77 (d, 2)
E	1	172.4			4	175.2	
	2	52.7	4.31 (m)		αNH		7.70 (d, 9)
	3	29.1	2.08 (m), 2.25 (m)	J	1	170.9	
	4	37.9	2.99 (m), 3.06 (m)		2	55.8	4.93 (dd, 2,10)
	αNH		8.14 (d, 7)		3	72.3	4.39 (m)
	γNH_2		7.58 (br.)		4	45.8	3.53 (dd,7,12), 3.58 (dd, 6,12)
					αNH		8.16 (d,10)

with 10% ethanol in 0.2% acetic acid and eluted with 80% ethanol in 0.2% acetic acid. Most of the antimicrobial activity was recovered in the 80% ethanolic fraction. The toxins were further purified by Sephadex G-25 column chromatography, and many components of the toxins were finally isolated by preparative HPLC on an ODS column. The most abundant component (17.2mg), obtained from 4 liters broth, was similar to syringomycin E when comparing the mass and ^{1}H-NMR spectra and the HPLC retention times with the authentic specimen. The FABMS spectrum of the syringomycin gave an $(M+H)^+$ ion at m/z 1225, whose isotopic ion pattern indicated the presence of one chlorine atom. Amino acid analysis of the hydrolysate and NMR spectrometry revealed all the constituents to be each two residues of serine and 2,4-diaminobutanoic acid (Dab), and each one residue of arginine, phenylalanine, dehydrothreonine (DhThr), β-hydroxyaspartic acid (HyAsp) and γ-chlorothreonine (ClThr) as amino acids, and 3-hydroxydodecanoic acid as an acid residue. A cyclic structure composed of all these constituents led to the molecular formula, $C_{53}H_{85}ClN_{14}O_{17}$, which matched the observed molecular weight.

The sequence of the residues was examined by the HMBC spectrum[8] in CD_3CN-H_2O (7:2 v/v) as in the case of syringostatins (3)(Fig.1).[6] The observed long-range couplings between carbonyl carbons, and neighboring α-protons and amide protons clarified the peptide bonds, establishing the sequence from the acid moiety (unit A) to the γ-chlorothreonyl residue (unit J). Furthermore, the long-range couplings between carbonyl carbon of unit J and the hydroxymethylene protons of the O-acylated seryl residue (unit B) indicated that the ester-bond existed between these two groups. The remaining ambiguous point was whether the peptide-linkage of the β-hydroxyaspartic acid residue (unit I) was α or β.

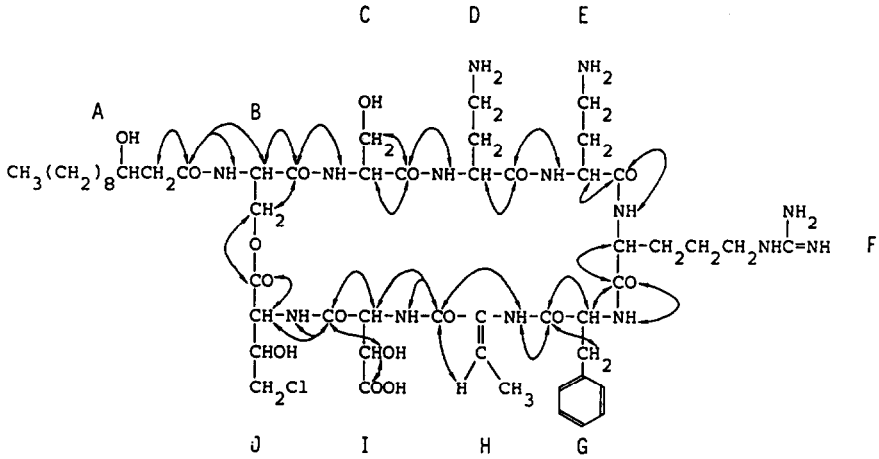

Fig.1 The structure of syringomycin (1). Arrows represent the carbon-proton long-range couplings between carbonyl and the protons in the HMBC spectrum of 1. The gothic letters A-J indicated the names of the units.

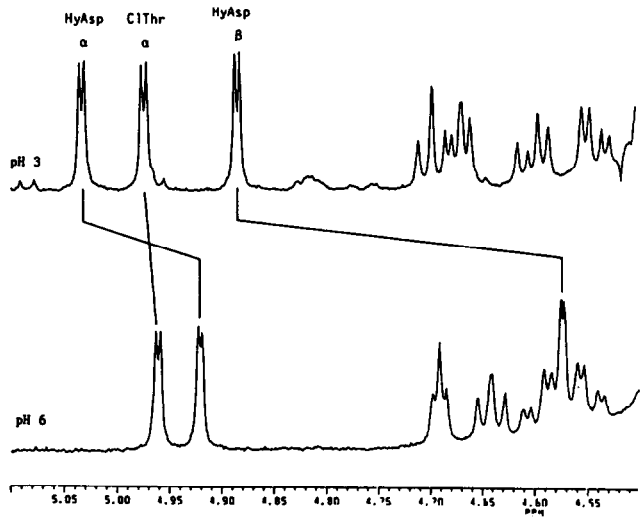

Fig. 2 The ^{1}H-NMR spectrum of syringomycin (1) in different pH values.

This was resolved by observing the varying chemical shifts of α- and β-protons of the residue in different pH. In the 0.025N DCl solution of syringomycin, α-proton experienced a sensitive down field shift (Δδ +0.31) whereas the shift of β-proton was small (Δδ +0.11), in comparison of the chemical shifts in the neutral solution (pH 6)(Fig.2). The other proton signals were hardly affected. These data proved the linkage of the β-hydroxyaspartic acid residue to be α. As a result, the structure of the major syringomycin was decided as 1. The NOESY spectrum was also in consistent with this sequence.

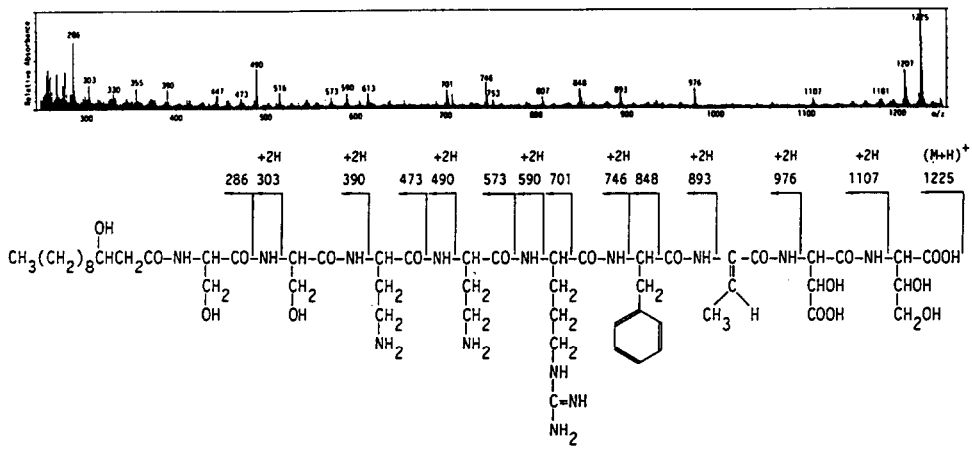

Fig. 3 Fragment peaks in the FAB mass spectrum of 4. The mass numbers represent the fragment peaks observed.

In the FAB mass spectrum of the linear peptide (4), obtained by alkaline hydrolysis of syringomycin in 0.1M Tris-HCl buffer (pH 8.5) for 20 hours,[6] $(M+H)^+$ ion were observed at m/z 1225 and also many N-terminal fragments appeared as shown in Fig. 3. The fragmentation indicated by arrows confirmed the sequence for syringomycin as 1.

The structure (1) proposed here differs from that of syringomycin E (2) in the sequence of units J and I and the α-peptide linkage of unit I. Syringomycin E was obtained from a stone fruit isolate of P. syringae pv. syringae. Only further analysis of syringomycin E will determine if these two syringomycins are analogs or if the proposed structure for syringomycin E[4] needs modification. The syringomycin reported here resembles the syringostatins (3) except for replacement of four of the nine amino acids.

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