Isolation and Structural Elucidation of Syringostatins, Phytotoxins Produced by *Pseudomonas syringae pv. syringae* Lilac Isolate

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A bacterial strain of *Pseudomonas syringae pv. syringae* isolated from lilac was found to produce a homologous mixture of phytotoxins different from syringomycin and syringotoxin. The toxins were termed syringostatins and the structures of the main components, syringostatins A and B, were determined by 2D-NMR spectroscopy and mass spectrometry. Minor component structures were elucidated from mass/mass spectra.

Pseudomonas syringae pv. syringae is a phytopathogenic bacterium which damages crops, fruit trees and various plants world wide. This bacterium is known to produce at least two different phytotoxins as virulence determinants.[1,2] Syringomycin is produced by the strains from various diseased plants, and syringotoxin is produced by strains from fruit trees. Although long recognized as peptides[3] and known to be toxic to plant cells,[4] little structural information was known until Segre *et al.*[5] reported the partial structure of syringomycin E.

In the 1980s, lilac blight spread through the central parts of Japan, and *Pseudomonas syringae pv. syringae* was isolated as the causative bacterium of the disease.[6] We made initial attempts to isolate phytotoxins produced by the bacterium (designated strain SY12) by monitoring the inhibitory activity to lettuce seedlings. These efforts yielded indole-3-acetic acid as the active substance. Then, we screened further for toxic metabolites in the cultured medium of the bacterium by detecting antimicrobial activity to *Penicillium chrysogenum*.

As a result, we succeeded in isolating peptidal toxins which differed from the known two toxins, syringomycin and syringotoxin. The toxin preparations were composed of several components and termed syringostatins.[7] The structures of the main components have been reported in preliminary form.[8] In this paper, we describe the production, isolation and structural elucidation of the syringostatins in detail.

The production level of syringostatins by strain SY12 was sensitive to culture conditions. We established the optimal conditions to be as follows: A nutrient broth culture (2 cm^3) was seeded into modified potato–dextrose–broth (200 cm^3) in 500 cm^3 capacity Erlenmeyer flasks. The flasks were incubated without shaking at 26.5 °C for 7 days. The quantity of the toxin produced was estimated by antifungal activity to *Penicillium chrysogenum*.

The toxins were labile under basic condition and lost antifungal activity completely by alkaline treatment. Therefore, the entire isolation procedure was performed under neutral or acidic conditions. The toxins were isolated by successive XAD-7. Sephadex G-25 and Sephadex LH-20 column chromatography. The final purification was performed by HPLC using a reverse-phase ODS column. The major components, syringostatin A **1** and a mixture of syringostatins B **2** and C **3**, and minor components syringostatins D **4**, E **5**, F **6**, G **7** and H **8** were isolated as shown in Fig. 1. Compound **1** and **2** were further separated by HPLC with another reverse-phase column. The yields were 7.4, 5.5 and 4.5 mg from 9 dm^3 of the broth culture for **1**, **2** and **3**, respectively, and less than 1 mg for the other minor components.

The FAB-MS spectra of all the syringostatins revealed $(M + H)^+$ ions around *m/z* 1200. Their isotopic patterns could be divided into two types; one suggested the presence of a chlorine atom and the other was normal. The mass spectral features are summarized in Table 1 and Fig. 2. The high resolution FAB-MS spectra of **1**, **2** and **4** confirmed the above estimation and suggested their molecular formulae as $C_{50}H_{87}ClN_{12}O_{18}$, $C_{50}H_{87}ClN_{12}O_{19}$ and $C_{50}H_{88}N_{12}O_{20}$, respectively.

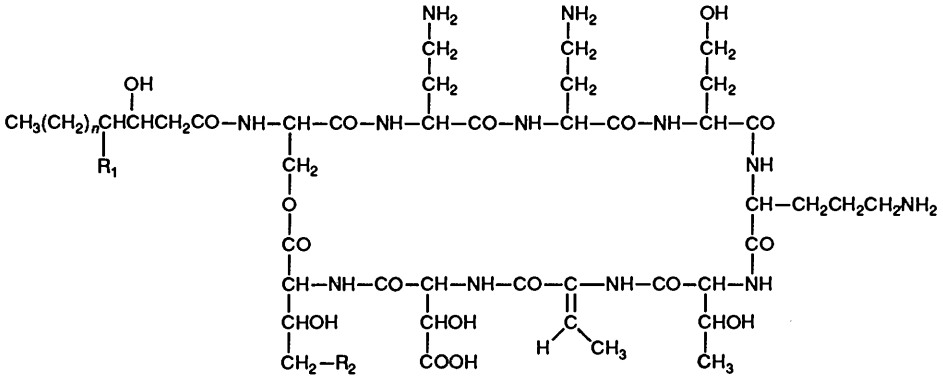
Amino acid analyses of **1** and **2** showed that each had one mole of serine (Ser),* allothreonine (alloThr), ornithine (Orn), two moles of 2,4-diaminobutanoic acid (Dab) and some unknown amino acids.

The ^1H NMR spectra of **1** and **2** showed only broad signals in D_2O and in $[^2H_6]$-DMSO. The proton signals containing amide NH signals resonated sharply in a mixture of CD_3CN and H_2O (7:2 v/v). The COSY spectra and HOHAHA spectra indicated the presence of homoserine (homoSer), β-hydroxyaspartic acid (HyAsp) and 2-aminobut-2-enoic acid (dehydrothreonine; DhThr) residues and an NH-CHOH-CH_2X unit besides the above amino acid residues determined by the amino acid analyses. Further analyses of COSY spectra established the presence of long chain aliphatic residues in **1** and **2**, and showed that they were 3-hydroxyfatty acid and 3,4-dihydroxyfatty acid residues, respectively. Except for the signals due to the fatty acid residues, the ^1H NMR spectra of **1** and **2** were identical.

Fatty acid analyses of the acid hydrolysates of **1** and **2** by GC-MS indicated the presence of 3-hydroxytetradecanoic acid and 3,4-dihydroxytetradecanoic acid residues in **1** and **2**, respectively.

^{13}C NMR and HMQC spectra of **2** confirmed all of the non-carbonyl carbon assignments, and further indicated that the remaining unit was 2-amino-4-chloro-3-hydroxybutanoic acid (4-chlorothreonine; ClThr).[9] Because of the higher chemical shift (δ_C 46.5) for the C-4 carbon (for which the protons resonated at δ_H 3.51 and 3.56) the chlorine atom should be attached to the C-4 carbon. Similarly the presence of this unique amino acid in **1** was established. The above units could explain all of the signals in ^1H and ^{13}C NMR spectra of **1** and **2**, and satisfied the molecular formula suggested by the high resolution FAB-MS spectra when considering one dehydration in the molecule. Thus, the molecular formulae of **1** and **2** indicated cyclic peptides composed of one hydroxyfatty acid and nine amino acid residues.

* *Abbreviations:* serine (Ser), allothreonine (alloThr), ornithine (Orn), 2,4-diaminobutanoic acid (Dab), homoserine (homoSer), β-hydroxyaspartic acid (HyAsp), dehydrothreonine (DhThr) and 4-chlorothreonine (ClThr).



		n	R_1	R_2
Syringostatin	A	9	H	Cl
	B	9	OH	Cl
	D	9	OH	OH
	E	7	H	Cl
	F	9	H	OH
	H	11	OH	Cl

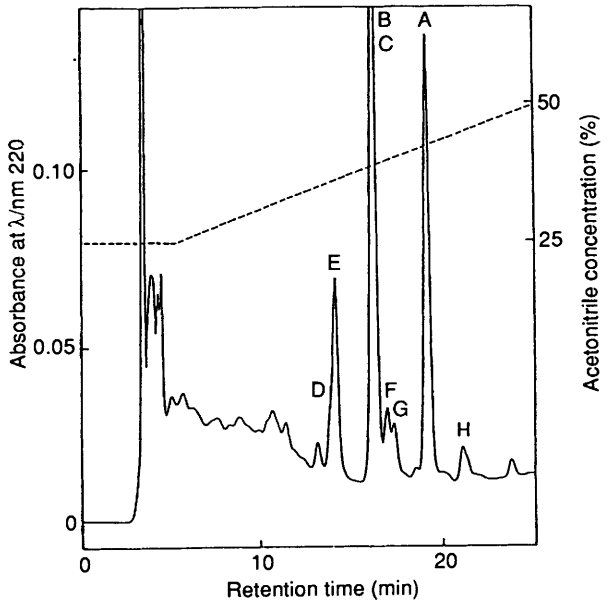


Fig. 1 Chromatogram of syringostatins from reversed phase HPLC. Column: SSC-ODS-H-3201 (250 × 8 mm); eluting solvent: 25–50% acetonitrile in 0.1% TFA (linear gradient)

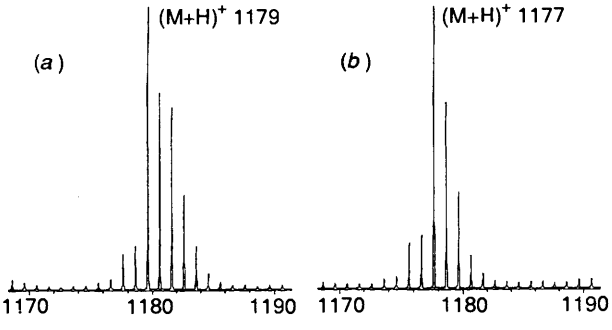


Fig. 2 Isotopic ion patterns of $(M + H)^+$ ions in the FAB mass spectra of syringostatins A **1** (*a*) and D **4** (*b*)

The sequence of these units in **2** was determined by NOESY and HMBC spectra. NOE correlations between α-protons and amide NH protons beyond carbonyl carbons, and two amide NH protons in the neighbouring two residues suggested the sequence from the 3-hydroxy acid residue to ClThr residue as shown in Fig. 3. NOE correlation between the methyl proton and the β-proton in DhThr residue suggested that this residue was (*Z*)-form. The carbon–proton long-range couplings observed in the HMBC spectrum enabled the assignment of all the carbonyl carbons as shown in Table 2 and confirmed the above peptide sequence. Furthermore, the long-range couplings between the β-protons of Ser and carbonyl carbon of ClThr in the HMBC spectrum indicated that the hydroxy group of Ser and the carboxy group of ClThr should form an ester bond. The involvement of the hydroxymethyl group of Ser was confirmed by the lower field shift of the corresponding protons (δ_H: 4.41, 4.52). Similar data were obtained in the spectra of **1**.

The remaining problem in establishing the covalent structures of **1** and **2** was whether the α- or β-carbonyl in the HyAsp residue should be involved in the amide bond with the ClThr residue. This was solved by measuring the chemical shift change of α- and β-protons of HyAsp residue at different pHs. The ^1H NMR spectrum of **2** was measured first in D_2O (pH *ca.* 6.0), then the spectrum was run again after addition of DCl (0.025 mol dm^{-3} DCl), which changed the pH of the solution to *ca.* 3.0. Both the α- and β-protons had similar sharp doublets and similar chemical shifts in this solvent. In 90% H_2O–D_2O (pH *ca.* 6.0), the α-proton gave a double-doublet signal due to the coupling with the amide NH proton. The downfield shift of the β-proton (0.28 ppm) was more sensitive to the pH shift than that of the α-proton (0.14 ppm), showing that the β-carbonyl was the free carboxylic acid and the α-carbonyl was involved in the amide bond. The same result was obtained for **1**. From the above data, the covalent structures of **1** and **2** were determined.

The syringostatins were labile under alkaline condition. Incubation of **1** in 0.1 mol dm^{-3} Tris-HCl buffer (pH 8.5) at 37 °C for half an hour yielded a derivative named SYL-A **9**; further treatment for several hours yielded nearly quantitatively another derivative named SYH-A **10**. In the same way, **2** gave derivatives SYL-B **11** and SYH-B **12**. From the FAB mass spectra, **9** and **11** gave $(M + H)^+$ ions at 1197 and 1213 with ion patterns showing the preservation of the chlorine atom. ^1H NMR spectra of **9** showed hydroxymethyl protons of Ser residue shifted upfield (δ_H 3.78, 3.83). These data indicated that **9** and **11** were linear derivatives formed by hydrolysis of the ester bonds. On the other hand, the derivatives **10** and **12** gave $(M + H)^+$ ions at 1179 and 1195 in FAB mass spectra, respectively, and these ions lost the typical isotopic ion patterns due to a chlorine atom. Therefore, these compounds were generated by replacement of chlorine atoms by hydroxy groups probably through epoxide formation. Fragment ions commonly observed in FAB mass spectra of both **9** and **10** showed many *N*-terminal ions as shown in Fig. 4 and those ions confirmed the proposed

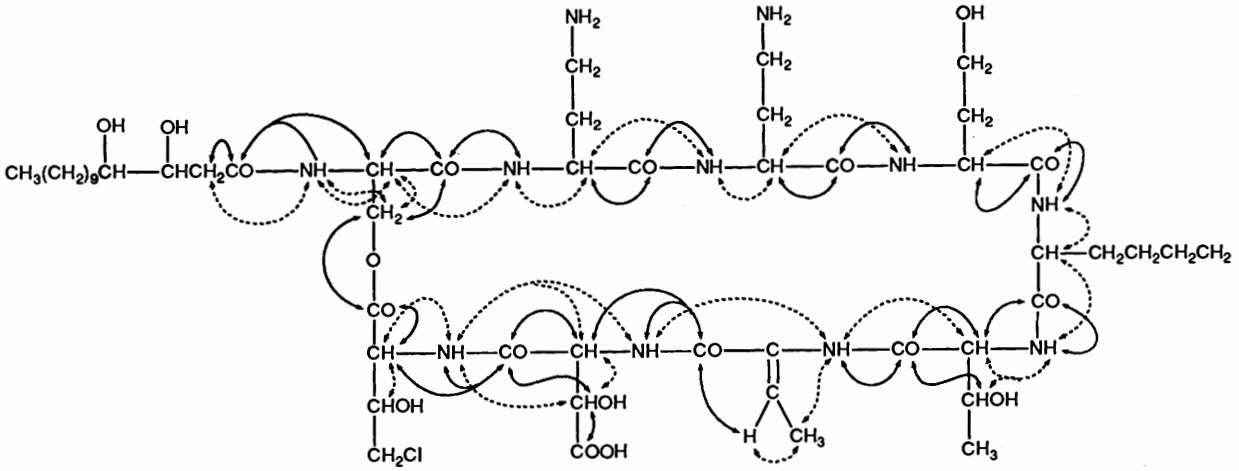


Fig. 3 Structure of syringostatin B **2**. The heavy arrows represent the carbon–proton long-range couplings observed in the HMBC spectrum, and the broken arrows represent the NOE correlations observed in the NOSEY spectrum.

Table 1 FAB mass spectra of syringostatins

Component names	$(M + H)^+$	Ion patterns	Proposed formula	Mass error (mmu)
Syringostatin A	1179, 5970	chlorine	$C_{50}H_{88}ClN_{12}O_{18}$	−5.7
Syringostatin B	1195, 5890	chlorine	$C_{50}H_{88}ClN_{12}O_{19}$	−8.7
Syringostatin C	1211	chlorine		
Syringostatin D	1177, 6230	normal	$C_{50}H_{89}N_{12}O_{20}$	−8.5
Syringostatin E	1151	chlorine		
Syringostatin F	1161	normal		
Syringostatin G	1211	chlorine		
Syringostatin H	1223	chlorine		

Table 2 ^{13}C and ^{1}H NMR spectroscopic data for syringostatin B **2**[a]

Unit	Carbon	δ_C	δ_H	Unit	Carbon	δ_C	δ_H
A	1	175.1		E	1	174.4	
	2	39.2	2.36 (dd, *J* 10, 15)		2	53.1	4.35 (m)
			2.53 (dd, *J* 3, 15)		3	34.1	2.01 (m)
	3	72.7	3.85 (m)		4	58.8	3.51 m)
	4	75.1	3.48 (m)				3.58 (m)
	5	33.3	1.30 (m)		NH		7.99 (d, *J* 7)
			1.52 (m)	F	1	174.2	
	6	26.4	1.30 (m)		2	53.2	4.47 (ddd, *J* 5, 9, 11)
			1.48 (m)		3	28.9	1.76 (m)
	7–10	30.2					1.99 (m)
	11	29.9	1.30 (m)		4	24.2	1.66 (m)
	12	32.4			5	39.9	2.97 (m)
	13	23.2			NH_2		7.49 (br)[b]
	14	14.3	0.90 (t, *J* 6)		NH		8.25 (d, *J* 9)
B	1	171.8		G	1	172.5	
	2	53.6	4.67 (ddd, *J* 6, 6, 6)		2	61.5	4.18 (dd, *J* 6, 7)
	3	65.4	4.41 (dd, *J* 6, 11)		3	67.6	4.09 (dq, *J* 7, 6)
			4.52 (dd, *J* 6, 11)		4	20.7	1.31 (d, *J* 6)
	NH		8.31 (d, *J* 6)		NH		8.07 (d, *J* 6)
C	1	173.4		H	1	166.2	
	2	52.9	4.29 (ddd, *J* 7, 7, 9)		2	128.8	
	3	29.2	2.08 (m)		3	136.5	6.81 (q, *J* 7)
			2.17 (m)		4	13.8	1.75 (d, *J* 7)
	4	37.6	3.03 (m)		NH		9.31 (s)
	NH_2		7.58 (br)[b]	I	1	171.8	
	NH		8.46 (d, *J* 7)			56.9	5.06 (dd, *J* 2, 9)
D	1	172.9			3	72.4	4.76 (d, *J* 2)
	2	52.2	4.37 (m)		4	175.3	
	3	29.5	2.10 (m)		NH		7.75 (d, *J* 9)
			2.24 (m)	J	1	170.8	
	4	37.6	3.01 (m)		2	55.8	4.87 (dd, *J* 3, 9)
	NH_2		—		3	72.1	4.36 (m)
	NH		8.17 (d, *J* 7)		4	45.8	3.54 (dd, *J* 7, 11)
							3.59 (dd, *J* 6, 11)
					NH		8.12 (d, *J* 9)

[a] Solvent: CD_3CN–H_2O (7:2 v/v). *J* values are given in Hz. [b] Broadened signal.

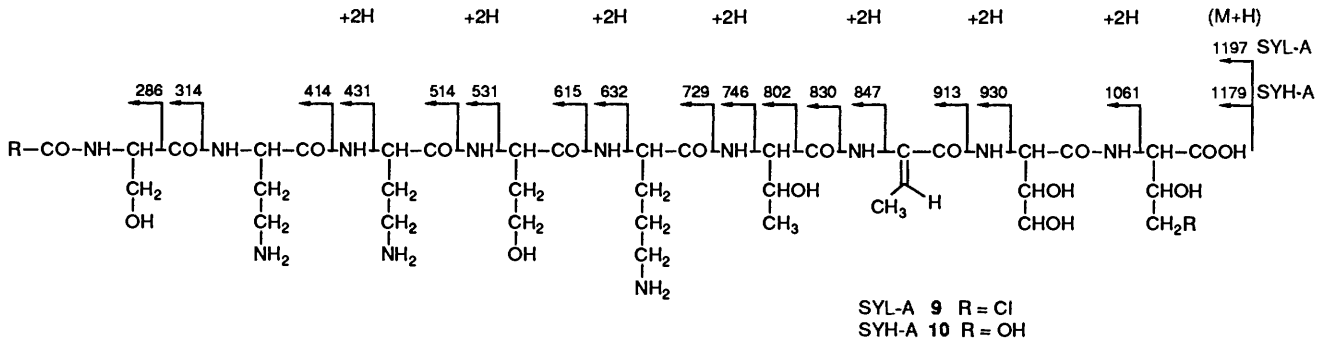


Fig. 4 Fragment ions observed in the FAB mass spectra of SYL-A **9** and SYH-A **10**

sequences of syringostatin A **1**. Similar *N*-terminal fragment ions in the FAB mass spectra of **11** and **12** (all 16 mass units less than those of **9** and **10** respectively) were also consistent with the proposed structure for **2**.

In the FAB mass spectra of **1** and **2**, several fragment ions were observed, but they were not so useful for sequence analyses of these cyclic depsipeptides. Also in the mass/mass spectra, the sequences of **1** and **2** could not be deduced, although more fragment ions were detected than in the FAB mass spectra. On the other hand, the linear derivative such as **9**, **10**, **11** and **12** gave many fragments in the FAB mass spectra, useful for sequence analysis as shown above. But, these were restricted to *N*-terminal fragments, and *C*-terminal fragments were not observed. For **9** and **10**, fragments not observed in the FAB mass spectra were observed in the mass/mass spectra and these could be assigned to *C*-terminal fragments; the data of **10** is shown in Fig. 5 as an example. The *N*- and *C*-terminal fragments were identified according to fragmentation patterns known for linear peptides.[10] The structures of the minor components of the syringostatins were then deduced by FAB mass and mass/mass spectra of the linear derivatives of the minor syringostatins. Fig. 6 shows the fragment ions and the fragmentation patterns of these linear derivatives. The FAB mass and mass/mass spectra of the linear peptides **13** and **14** obtained from **4** and **6**, respectively, were the same as those of **12** and **10**. Therefore, **4** and **6** should have HyThr instead of ClThr in **2** and **1**. The retention times of **13** and **14** in the HPLC were also the same as those of **12** and **10**, respectively. The linear derivative **15** obtained from **5** gave the *N*-terminal fragments which were 28 mass units less than those of **9**, indicating that **5** bears a 3-hydroxydodecanoyl residue instead of 3-hydroxytetradecanoyl in **1**. The derivative **16** obtained by hydrolysis of **8** showed $(M + H)^+$ ion at *m/z* 1241, which was 28 mass units more than that of **11**, and the C-terminal fragments of **16** were all identical to those of **11**. Therefore the difference between **8** and **2** was in the acid residue, with **8** having the 3,4-dihydroxyhexadecanoyl residue. Thus, the structures of most of the minor syringostatins were determined. The minor components, **3** and **7**, gave unassignable signals in mass spectra, and therefore their structures could not be determined.

Recently, Segre *et al.*[9] proposed structures for the syringomycins, which are cyclic lipodepsipeptides. The structures of these latter toxins closely resemble those of the syringostatins reported here. However, significant differences in the amino acid content occur. A discrepancy in the *C*-terminal sequence is also apparent between that reported for syringomycin E and for the syringostatin. In the subsequent paper, a comparative structural study is reported for these toxins including syringotoxin. Additionally, an evaluation of the *C*-terminal sequence of syringomycin is detailed as also discussed recently by Ballio *et al.*[11]

Experimental

FAB Mass Spectrum.—The FAB mass spectra were measured on a JEOL JMS-DX303 mass spectrometer, using glycerol containing hydrogen chloride as matrix and ionized by the impact of the accelerated Xe atom. The high resolution FAB

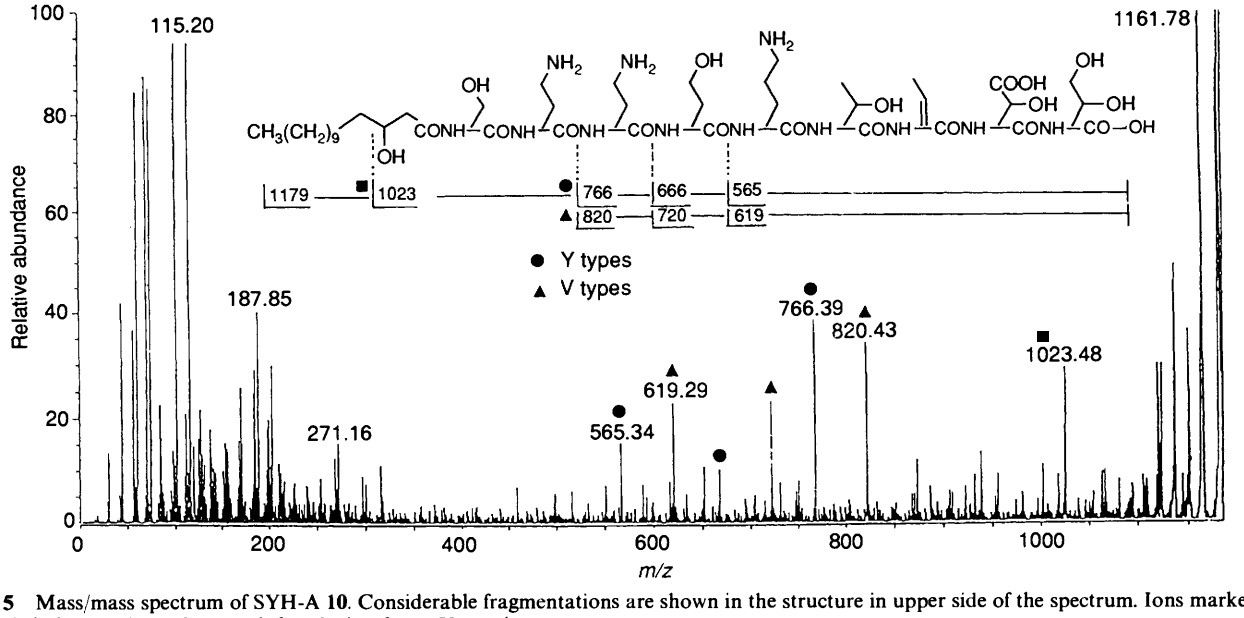


Fig. 5 Mass/mass spectrum of SYH-A **10**. Considerable fragmentations are shown in the structure in upper side of the spectrum. Ions marked by closed circles are Y type ions, and closed triangle are V type ions.

mass spectra were measured on a JEOL JMS-SX102 mass spectrometer, using a mixture of glycerol and thioglycerol as matrix and ionized by the impact of the accelerated Xe atom. The exact mass was calculated by comparison with polyethyleneglycol #1000 as mass marker. The mass/mass spectra were also measured on the JEOL JMS-SX102/SX102 mass spectrometer.

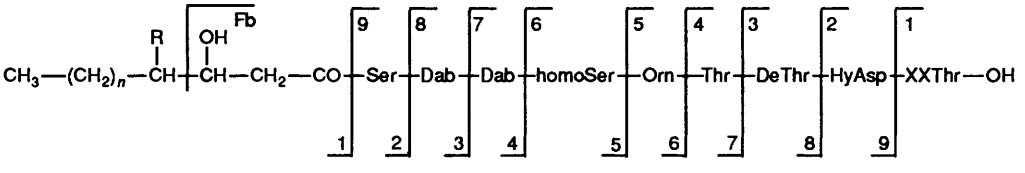
NMR Spectra.—The NMR spectra were measured with Bruker AM-600 and Bruker ACP-300 spectrometers. ^1H NMR spectra were recorded with 32K (AM-600) or 16K (ACP-300) data points, and normalized with residual CHD_2CN (δ_H 2.00) in CD_3CN–H_2O (7:2 v/v), HDO (δ_H 4.80) in D_2O, $CHCl_3$ (δ_H 7.24) in $CDCl_3$ and CHD_2OD (δ_H 3.55) in CD_3OD as internal standards. ^{13}C NMR spectra were recorded with 64K (AM-600) or 32K (ACP-300) data points, and normalized with the signals of CD_3CN (δ_C 1.2), $CDCl_3$ (δ_C 77.0) and CD_3OD (δ_C 49.0) as the internal standards in each solvent and the external standard of dioxane (δ_C 67.8) in D_2O. *J* values are given in Hz throughout.

COSY and DEPT spectra were performed by conventional methods. HOHAHA spectra were measured by the method of Davis and Bax[12] using spin lock pulses (2.5 ms) before and after the 40 times of MLEV17 cycles (mixing time, 80 ms). HMQC spectra and HMBC spectra were measured by the method of Bax and Summer,[13] adopting 3.6 ms as $1/2^1J_{CH}$ and 60 ms as $1/2^nJ_{CH}$ as the developing times. In the HMQC spectra, carbon signals were decoupled by 14 cycles of GARP1 pulses. Phase-sensitive NOESY spectra were obtained by the method of Bodenhauser *et al.*[14] using 800 ms as the mixing time.

Isolation of Indole Acetic Acid.—The activity was assayed by the inhibitory activity on the growth of lettuce seedlings.

P. syringae pv. syringae SY12 was cultured in 5 dm^3 volume Erlenmeyer flasks (2 dm^3 medium in each flask) with Bennett medium (1% glucose, 0.2% polypeptone, 0.1% meat extract and 0.1% yeast extract) at 26.5 °C for 4 d with shaking. The centrifuged supernatant (7000 rpm, 10 °C, 20 min) was adsorbed on a charcoal column (19.7 × 3.6 cm), washed with 20% ethanol (400 cm^3), and the activity was eluted with 20% pyridine (1 dm^3). This eluate was diluted to twice its volume and applied to a DEAE-Sephadex A-25 column (buffered with 10% pyridine), washed with 10% pyridine (200 cm^3), and eluted with a linear gradient of acetic acid (0.02–1.0 mol dm^{-3}, 200 cm^3) and further 1 mol dm^{-3} acetic acid (200 cm^3). The activity was recovered around 1 mol dm^{-3} acetic acid eluate, and the active fractions were concentrated and lyophilysed. The residue was dissolved in small volumes of water and further purified by HPLC with an ODS column (SSC-ODS-H-3201, 200 × 8 mm, Senshu Kagaku Co.), eluted with an acetonitrile gradient (0–20% in 2 min, 20% isocratic for 20 min) in 0.1% TFA (trifluoroacetic acid). The active substance, indole-3-acetic acid was obtained (31.8 mg) from 13 dm^3 of broth; FD-MS; *m/z* 176 $[(M + H)^+]$, $\delta_H(D_2O)$ 3.72 (s, 2 H), 7.00 (ddd, *J* 1, 7, 8), 7.09 (ddd, *J* 1, 7, 8), 7.16 (s), 7.33 (ddd, *J* 1, 1, 8) and 7.53 (ddd, *J* 1, 1, 8).

Production and Isolation of Syringostatins.—*P. syringae pv. syringae* SY12 was cultured in a medium containing 1% potato extract (Difco), 1.5% glucose and 0.4% casamino acid (Difco), adjusted to pH 7.0. The cultivation was performed without



	R	XXTHr	*n*
9	H	ClThr	9
11	OH	ClThr	9
10 = **15**	H	HyThr	9
12 = **13**	OH	HyThr	9
14	H	ClThr	7
16	OH	ClThr	11

N-terminal fragments in FAB mass spectrum

Compounds	M + H	A2	B2	C2	B3	C3	B4	C4	B5	C5	C6	A7	C7	C8	C9
9 (SYL-A)	1197	286	314	331	414	431	514	531	615	632	746	802	847	930	1061
11 (SYL-B)	1213		330			447	530	547	631	648	762		863	946	1077
10 (SYH-A)	1179		314		414	431	514	531	615	729	746	802	847	930	1061
12 (SYH-B)	1195		330			447	530	547	631	648	762	818	863	946	1077
14	1169		286		386	403	486	503	587	604	718		819	902	1034
16	1241														

15 = **10** (SYH-A), **13** = **12** (SYH-B)

C-terminal fragments in mass/mass spectrum

Compounds	M + H	Y5	Y6	V6	Y7	V7	V8	Fb
15	1179	565	666	619	766	720	820	1023
13	1195	565	666	619	766	720	820	1023
14	1169	583	684	637	784	737	838	1041
16	1241				784	738	838	1041

Fig. 6 Structures and the fragment ions observed in the FAB mass and mass/mass spectrum of syringostatins. Derivatives **13**, **14**, **15** and **16** were obtained from syringostatins D **4**, E **5**, F **6** and H **8**, respectively.

stirring in 500 cm^3 volume Erlenmeyer flasks containing medium (200 cm^3) at 26.5 °C for 7 d. During the process of the isolation, syringostatins were assayed by their antibiotic activity to fungi, *Penicillium chrysogenum*, on potato–dextrose–agar plates. The centrifuged supernatant (8000 rpm, 4 °C, 20 min) of the cultured broth (9 dm^3) was adsorbed on an XAD-7 column (40 × 3.6 cm), which was rinsed preliminarily with 0.2% TFA. After washing with 20% ethanol (1.5 dm^3), the activity was eluted with 80% ethanol (900 cm^3). The concentrated active fractions were dissolved with 0.2% TFA (30 cm^3), stirred for 30 min and centrifuged (16 000 rpm, 4 °C, 15 min), then the supernatant was applied to a Sephadex G-25 column (53 × 2.3 cm) with 0.2% TFA. The column was eluted with 0.2% TFA. The active material was recovered in the fractions of elution volumes 180–230 cm^3. After lyophilization, the active material was chromatographed on a Sephadex LH-20 column (55 × 2.3 cm) with 45% ethanol and 0.5% acetic acid, and fractions (5 cm^3) collected. Fractions 29–36 were concentrated under reduced pressure and lyophiliysed. The residue, dissolved in water, was applied to an HPLC ODS column (SSC–H–3210, 200 × 8 mm, Senshu Kagaku Co.) and eluted with an acetonitrile gradient (25–50% in 20 min) in 0.1% TFA. The main component, syringostatin A (7.4 mg), the mixture of syringostatins B and C (10.0 mg), and the minor syringostatins from D to H (each less than 1 mg) were isolated. The mixture of syringostatins B and C was further resolved by HPLC using another reverse-phase column (SSC–CN–4251–N, 250 × 10 mm, Senshu Kagaku Co.). The yields of syringostatins B and C were 5.5 and 4.5 mg, respectively.

Amino Acid Analysis of Syringostatins.—Syringostatins (*ca.* 120 μg) were hydrolysed in sealed glass tybes with hydrochloric acid (6 mol dm^{-3}; 200 mm^3) at 110 °C for 18 h. The hydrolysate was concentrated under reduced pressure and dissolved in hydrochloric acid (0.02 mol dm^{-3}; 500 mm^3). The sample was analysed for amino acids (Hitachi model-835). The detected known amino acids were allothreonine (1.1 mole, an unknown peak overlapped), serine (0.5 mole), 2,4-diaminobutanoic acid (1.8 mole) and ornithine (0.7 mole) from one mole of syringostatin B **2**.

Analysis of Fatty Acid in Syringostatins.—Hydrolysed syringostatins (200 μg) were dried, dissolved in hydrochloric acid (1 mol dm^{-3}; 200 mm^3), and extracted with ethyl acetate (100 mm^3). After removal of the solvent, the residue was methylated with CH_2N_2 in ether, and the reactant was applied to a GC-MS (JEOL JMS-DX303) with a packed column of OV-17. The column temperature was increased from 80 to 250 °C in 21 min, and the residue ionized by the electron-impact (EI) method. The methylated acid residue of the hydrolysate of syringostatin A **1** was eluted at 13.8 min, and gave fragment ions at *m/z* 241, 209, 208, 183, 166, 103, 71 and 55. Methylated derivative from syringostatin B **2** was eluted at 14.2 min, and gave ions at *m/z* 225, 169, 143, 130, 115, 111, 98 and 55. The value of *m/z* 241 for syringostatin A was explained by the deletion of an hydroxy group from the methyl ester form, and *m/z* 225 by deletion of an hydroxy group from the γ-lactone form.

Synthesis of 3-Hydroxytetradecanoic Acid.—A solution of anhydrous THF (50 cm^3) was refluxed and vigorously stirred with powdered zinc (3.2 g). Dodecanal (2.0 g, 10.9 mmol) and bromoethylacetate (4.1 g, 24.6 mmol) in anhydrous THF was added dropwise. After refluxing for 1 h, the supernatant was decanted off, and the solvent removed under reduced pressure; further traces of solvent were removed from the reaction mixture at 120–130 °C. Benzene (50 cm^3) was added to the reaction mixture, which was then refluxed for 2 h. After removal of the solvent, 2 mol dm^{-3} HCl was added to the residue and it was extracted with ethyl acetate (3 × 300 cm^3). The residual solid remaining after removal of the solvent was then hydrolysed with 90% ethanol (100 cm^3) and KOH (4.0 g) at room temperature for 40 h. Extraction with ethyl acetate and evaporation of solvent yielded 3-hydroxytetradecanoic acid as a yellow amorphous solid (*ca.* 350 mg), $\delta_C(CDCl_3)$ 14.1, 22.7, 25.4, 29.3, 29.5–29.6 (some signals were overlapped), 31.9, 36.5, 41.1, 66.1 and 177.7, $\delta_H(CDCl_3)$ 0.82 (t, *J* 7), 1.1–1.5 (m), 2.40 (dd, *J* 8, 17), 2.51 (dd, *J* 3, 17). The methylated derivative of this compound showed the same GC-MS peaks and ions as that from the hydrolysate of syringostatin A as shown above.

Base-hydrolysis of Syringostatin A and B.—A solution of syringostatin A (500 μg in 500 mm^3) was combined with Tris-HCl buffer (0.2 mol dm^{-3}; 500 mm^3; pH 8.5), and incubated at 37 °C for 1 h. The reaction was stopped by adding hydrochloric acid (1 mol dm^{-3}; 100 mm^3). The solvent was removed under reduced pressure, and the sample was applied to an ODS column (SSC-ODS-H-3201, 20 × 8 mm, Senshu Kagaku Co.), and the hydrolysed derivative SYL-A was recovered. Similarly the derivative SYH-A was obtained by incubation for 18 h. Syringostatin B and minor compounds of syringostatins were treated in the same way.

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