Structure and Stereochemistry of Three Phytotoxins, Syringomycin, Syringotoxin and Syringostatin, Produced by *Pseudomonas syringae* pv. *syringae*

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The structures of two phytotoxins, syringomycin and syringotoxin, produced by *Pseudomonas syringae* pv. *syringae*, were determined. Several amino acid residues of syringomycin were different from those in the syringostatins. Syringotoxin B proved to be [Gly^3]syringostatin A. The three kinds of phytotoxins showed close structural similarity, and the stereochemistry of their components was deduced and compared.

The phytopathogenic bacterium, *Pseudomonas syringae* pv. *syringae*, is known to produce the phytotoxins syringomycin and syringotoxin.[1,2] In addition to these we recently found another set of toxins produced by this organism isolated from lilacs suffering from blight.[3] The new toxins were termed syringostatins and their structures have been reported in previous papers.[4,5] Segre *et al.* first[6] reported the structure of syringomycin, which is a cyclic lipodepsipeptide. We independently isolated syringomycin from the cultured filtrate of a sugar cane isolate of *P. syringae* pv. *syringae*.[7] Structural analysis indicated a discrepancy in the sequence of the *C*-terminal pair of amino acids from that reported by Segre *et al.*[6] We further studied the structure of syringotoxin B from a toxin preparation provided by J. E. DeVay (Davis, CA, USA). Syringotoxin B was shown to be a glycine analogue of syringostatin A.[8] These observations of the structures including their stereochemistry have been reported in a preliminary communication.[9] In this paper we describe the details of the structural elucidation of these two toxins, and the stereochemistry of all three members of this phytotoxin family.

We isolated the syringostatins from culture filtrates of *P. syringae* pv. *syringae* as reported in our previous paper.[5] When extracellular metabolites of another strain of the bacterium isolated from Japanese sugar cane were analysed, antifungal substances different from the syringostatins were found. This strain was cultured on the stationary phase in the potato–dextrose medium described by Zhang and Takemoto[10] at 26.5 °C for 9 days, when the production of antibiotic activity against *Saccharomyces cerevisiae* was maximal. The substances were tentatively named SCs. The culture supernatant (4 dm^3), acidified by acetic acid (finally 0.2% acetic acid), was passed through an XAD-2 column, and purified by chromatography on XAD-7 and Sephadex G-25 columns, successively. Finally, the active substances SC-1 to -10 were obtained by HPLC with an ODS column as shown in Fig. 1. Major compounds were SC-6 (17.2 mg) and SC-10 (5.0 mg); the others were minor compounds (each less than 1 mg).

The most abundant compound, SC-6, gave an $(M + H)^+$ ion at 1225 in its FAB mass spectra. The spectral isotopic patterns

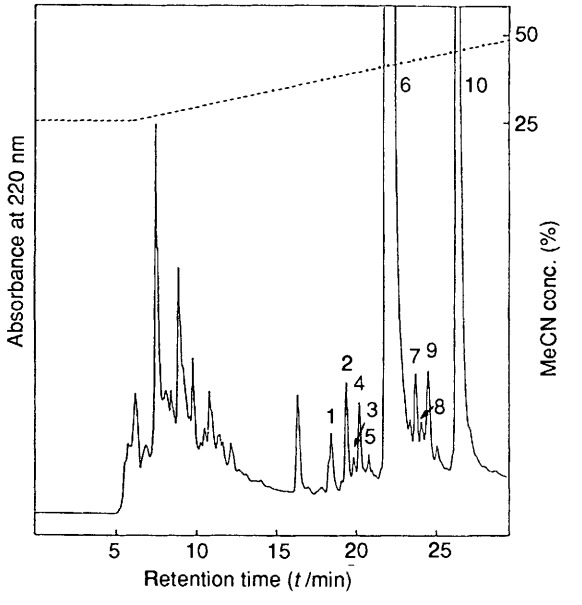
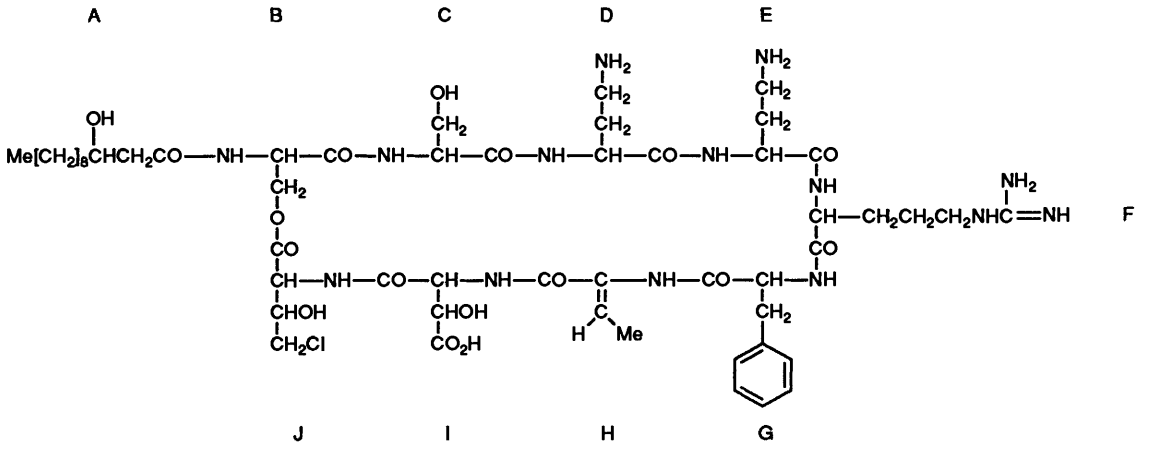


Fig. 1 Chromatogram of SCs (SC-1 to -10) from reversed-phase HPLC. Column: SSC-ODS-H-5251 (250 × 20 mm); eluting solvent: 25–50% acetonitrile in 0.1% trifluoroacetic acid (TFA) (linear gradient). Flow rate: 8.0 dm^3 min^{-1}.

were characteristic of the presence of one chlorine atom. The mass and ^{1}H NMR spectra of SC-6 and the retention time of SC-6 on HPLC with a reversed-phase column were the same as those of syringomycin E[6] purified independently from *P. syringae* pv. *syringae* strain B3010.[10] The second major component, SC-10, which showed an $(M + H)^+$ ion at *m/z* 1253 with a chlorine isotopic pattern, was found to correspond to syringomycin G.[6] SC-6 was referred to as syringomycin **1** to distinguish it from syringomycin E **1′** whose structure had been reported by Segre *et al.*[6] The structure **1′** was homologous to those of the syringostatins.[7] However, the *C*-terminal sequence of compound **1′** differed fundamentally from those of the syringostatins in the sequence of the two *C*-terminal amino acids, ClThr and HyAsp, and the β linkage of the latter. Because of these differences, we decided to re-evaluate the structure of compound **1** and have determined its structure.

From amino acid analyses of syringomycin **1**, the presence of Arg, Phe, two moles of Ser and two moles of Dab was

Abbreviations: Amino acids are abbreviated as follows: arginine (Arg), phenylalanine (Phe), β-hydroxyaspartic acid (HyAsp), serine (Ser), homoserine (homoSer), allothreonine (alloThr), 2,4-diaminobutanoic acid (Dab), glycine (Gly), dehydrothreonine (DhThr), ornithine (Orn), 4-chlorothreonine (ClThr) and 4-hydroxythreonine (HyThr).



Structure of syringomycin **1**

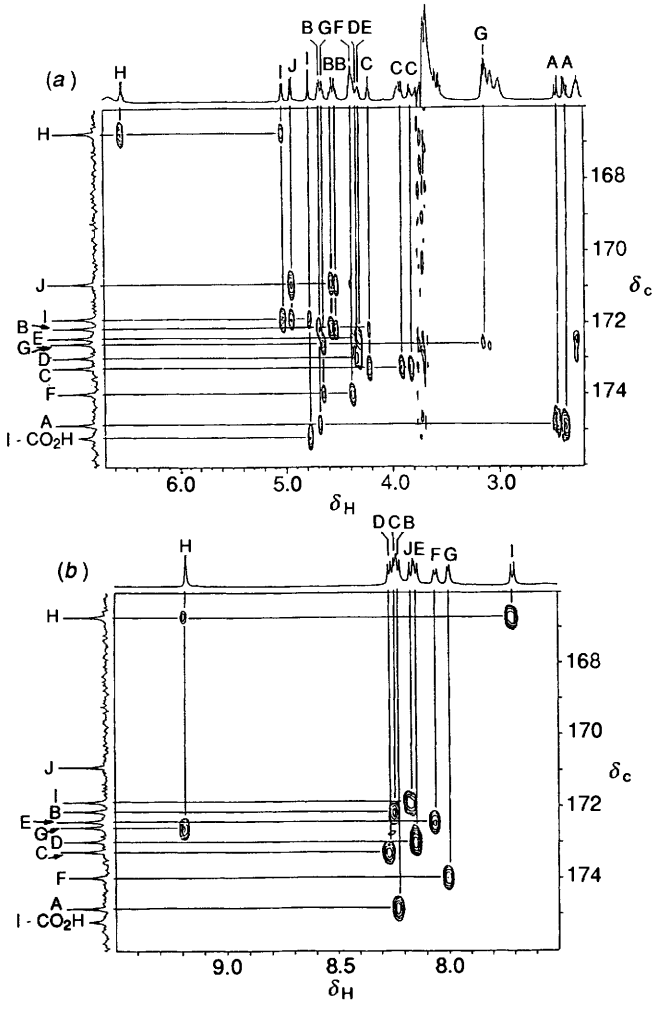


Fig. 2 HMBC (heteronuclear multiple bond connectivity) spectrum of syringomycin **1** (600 MHz). (a) Carbonyl carbon and amide proton; (b) carbonyl carbon and aliphatic proton. The letters A–J indicate that the signals are assigned to the corresponding residues shown in the structure of syringomycin **1**. A: 3-Hydroxydodecanoic acid; B: Ser1; C: Ser2; D: Dab1; E: Dab2; F: Arg; G: Phe; H: DhThr; I: ClThr; J: HyAsp.

ascertained. Fatty acid analysis indicated the presence of 3-hydroxydodecanoic acid. As for the syringostatins, ^1H and ^{13}C NMR spectra of syringomycin **1** in CD_3CN–water (7:2 v/v), along with COSY, HOHAHA (homonuclear Hartmann–Hahn) and HMQC (heteronuclear multiple quantum coherence) spectra, suggested the presence of HyAsp, DhThr and ClThr moieties, which are commonly components of the syringostatins. Summation of the masses of these components satisfied the molecular weight of syringomycin **1**, provided that they constituted a cyclic structure. Assignment of the carbonyl carbons and the sequence of the ten units was determined from the HMBC spectrum, which also indicated the presence of an ester linkage between the ClThr and *N*-terminal Ser residues (Fig. 2). The linkage of the HyAsp residue was determined by the pH-sensitive chemical shift, as observed for the syringostatins.[5] As with the syringostatins, the β-proton (+0.31 ppm) of the HyAsp residue was much more sensitive than that of the α-proton (+0.11 ppm), showing the presence of an α-linkage. The proton and carbon assignments are summarized in Table 1.

Alkaline treatment of syringomycin **1** in Tris-HCl buffer (pH 8.5) yielded two derivatives, SRL **2** and SRH **3**, which gave $(M + H)^+$ ions at *m/z* 1243 and 1225, respectively. Derivative **2** was a linear peptide formed by hydrolysis of the ester bond, and **3** occurred by the replacement of a chlorine atom in compound **2** by an hydroxy group. Fragment ions observed in the FAB mass spectra of these two derivatives confirmed the structures as shown in Fig. 3.

A preparation of syringotoxin (120 mg) was generously provided by J. E. DeVay, University of California, Davis. Two active components, designated syringotoxin A (3 mg) and syringotoxin B (10 mg), were isolated by HPLC with an ODS column. The activity of the purified toxin to *Geotrichum candidum* was 2048 units/μg, which was almost equivalent to that of syringomycin (J. E. DeVay, personal communication). Syringotoxin A **4** and B **5** gave $(M + H)^+$ ions at *m/z* 1118 and 1136, respectively. The latter showed the presence of one chlorine atom in FAB mass spectroscopy, but the former did not; because of this we chose to determine the structure of syringotoxin B **5** by methods similar to those used for syringostatins[5] and syringomycin. Alkaline hydrolysis of syringotoxin B **5** gave a linear peptide, STL-B **6**, and a non-chlorinated molecule, STH-B **7**. The fragmentation patterns in the FAB mass spectrum of compound **7** suggested that syringotoxin B **5** was [Gly^3]syringostatin A (Fig. 4). Amino acid analysis and ^1H and ^{13}C NMR spectra confirmed the presence of one residue each of Gly and Dab. The assignments from ^1H and ^{13}C NMR spectra are summarized in Table 2, and the HMBC spectrum of syringotoxin B established the sequence of the units (Fig. 5). The mass difference between structures **4** and **5** (18 mass units) and the isotopic ion patterns of their $(M + H)^+$ ions suggested that syringotoxin A **4** might be [HyThr^9]syringotoxin B. Alkaline hydrolysis of compound **4** gave a linear peptide which was the same as that obtained by prolonged alkaline hydrolysis of syringotoxin B **5**, confirming the covalent structure of syringotoxin A **4**. The structures of all

Table 1 ^{13}C and ^{1}H NMR spectral data for syringomycin **1**

Unit	Carbon	δ_C	δ_H (multiplicity, *J*/Hz)	Unit	Carbon	δ_C	δ_H(multiplicity, *J*/Hz)
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)

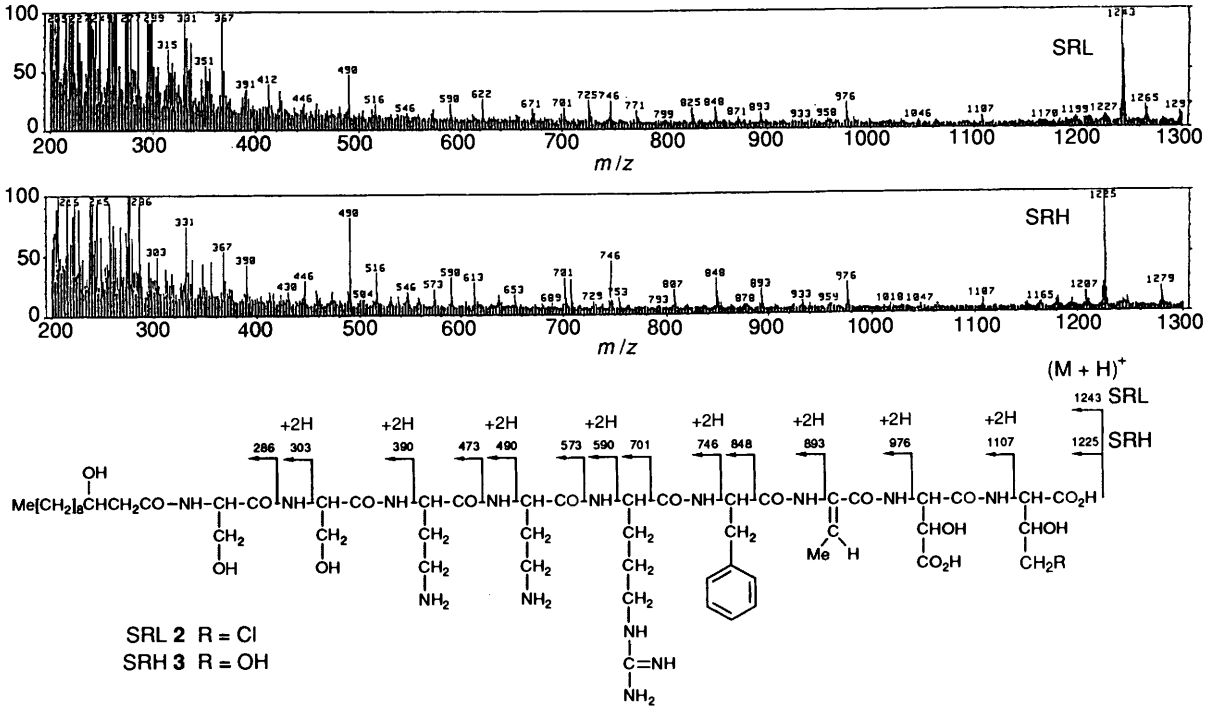


Fig. 3 FAB mass spectra of SRL **2** and SRH **3**, and their *N*-terminal fragment ions observed. The upper spectrum shows that of SRL **2**, and the lower is that of SRH **3**. The arrows in the structure represent the *N*-terminal fragment ions observed.

three kinds of phytotoxins, **1**, **5**, and the syringostatin A, are summarized in Fig. 6. They are structural homologues and identical in the three C-terminal residues, with the ClThr ester-linked to Ser.

The configuration of DhThr residue commonly present in the three toxins was shown to be (*Z*) from NOESY experiments.[5] The stereochemistry of the other amino acids was investigated by the method of Marfey.[11] The method involves derivatization of amino acids with an optically active fluorobenzene derivative, *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA) into diastereoisomeric compounds and separation by HPLC with a reversed-phase column. L- and DL-forms of the common amino acids, and DL-*threo*, L-*threo*- and DL-*erythro*-β-hydroxyaspartic acid were used as standards. The diastereoisomeric

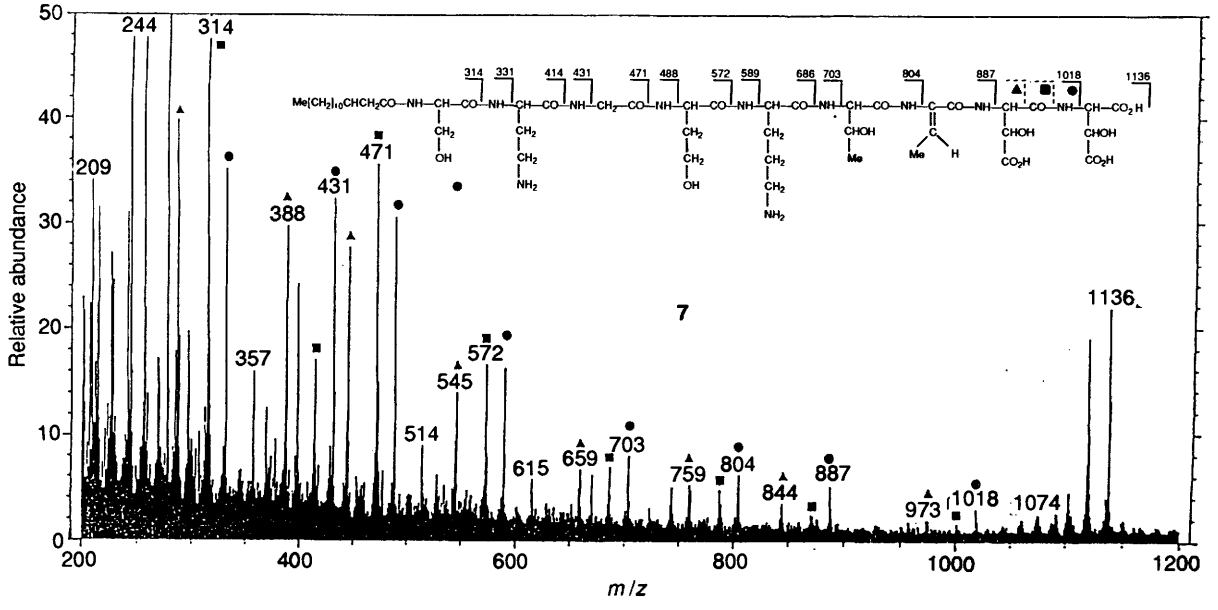


Fig. 4 FAB mass spectrum and fragmentation pattern of STH-B 7. Fragment ions marked ●, ▲ and ■ indicate *N*-terminal fragment ions produced by cleavage at the NH–CH bond, CO–NH bond and CH–CO bond, respectively.

Table 2 ^{13}C and ^{1}H NMR spectral data for syringotoxin B **5** (125 MHz for carbons and 600 MHz for protons)

Unit	Carbon	δ_C	δ_H(multiplicity, *J*/Hz)	Unit	Carbon	δ_C	δ_H(multiplicity, *J*/Hz)
A	1	174.7		F	1	174.2	
	2	43.9	2.34 (dd, 9, 15),		2	53.5	4.43 (ddd, 6, 8, 12)
			2.44 (dd, 3, 15)		3	28.9	1.78 (m)
	3	69.7	3.93 (m)				1.95 (m)
	4	37.9	1.47 (m)		4	24.4	1.70 (m)
	5	26.2	1.40 (m)		5	40.1	2.98 (m)
			1.30 (m)		NH		8.14 (d, 8)
	6–10	30.2					
	11	29.9		G	1	172.2	
	12	32.5	1.30 (m)		2	61.3	4.23 (dd, 6, 6)
	13	23.2			3	68.1	4.10 (dq, 6, 6)
	14	14.3	0.90 (t, 7)		4	20.6	1.31 (d, 6)
					NH		7.91 (d, 6)
B	1	171.6					
	2	53.7	4.71 (ddd, 4, 5, 7)	H	1	166.1	
	3	65.6	4.47 (dd, 4, 11)		2	129.1	
			4.53 (dd, 5, 11)		3	136.4	6.81 (q, 7)
	NH		8.09 (d, 7)		4	13.8	1.75 (d, 7)
					NH		9.13 (s)
C	1	173.5					
	2	52.9	4.32 (ddd, 6, 7, 10)	I	1	171.8	
	3	29.4	2.06 (m)		2	57.0	5.07 (dd, 2, 9)
			2.20 (m)		3	72.4	4.75 (d, 2)
	4	37.7	3.04 (m)		4	175.1	
	NH		8.29 (d, 6)		NH		7.73 (d, 9)
D	1	172.2		J	1	170.7	
	2	43.8	3.88 (dd, 6, 17),		2	55.9	4.87 (dd, 3, 9)
			3.95 (dd, 6, 17)		3	72.3	4.36 (m)
	NH		8.03 (t, 6)		4	45.9	3.55 (m)*
					NH		7.97 (d, 9)
E	1	174.6					
	2	53.0	4.37 (m)				
	3	34.2	1.97 (m)				
	4	59.0	3.55 (m)*				
	NH		7.78 (d, 6)				

The data were obtained in CD_3CN–water (7:2 v/v) at 313 K. * Data at 303 K, these being hidden in the HO signal at 313 K.

derivatives were separated by using three solvent systems. For ClThr, four diastereoisomers were prepared from L- and DL-methionines by the methods of Ohfune and Kurokawa,[12] Afzari-Aldakani and Rapoport,[13] and Shaw *et al.*[14] Upon epoxidation of benzyloxycarbonylvinylglycine methyl ester, the *syn*- and *anti*-forms were produced as a 3:1 mixture. Purified L-*syn*-derivative (*syn*-**11**) was converted into L-*threo*-4-chlorothreonine by hydrolysis in 6 mol dm^{-3} HCl at 110 °C. Also, the L-*erythro* and DL-*threo*/*erythro* forms were prepared. When these isomers of ClThr were treated with FDAA and the

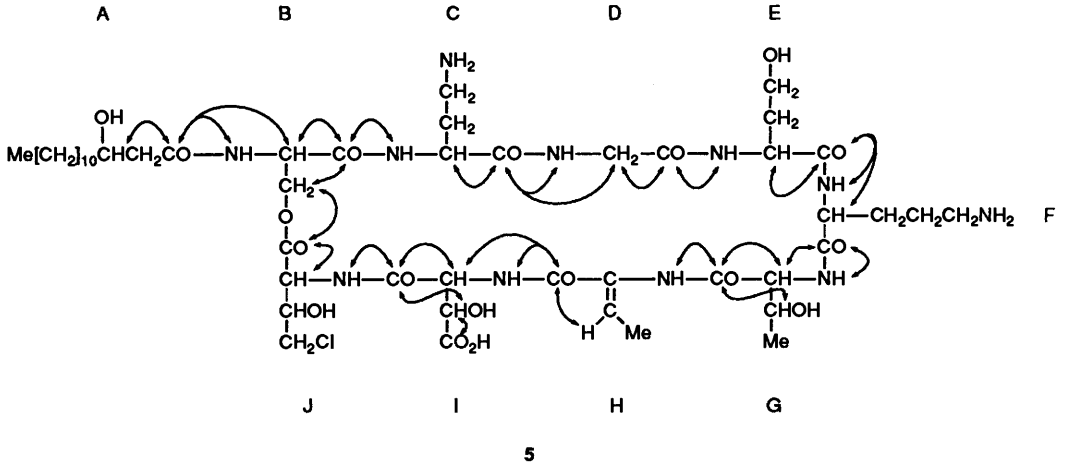
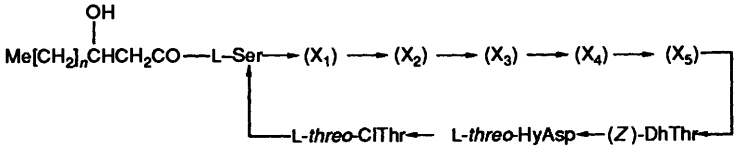
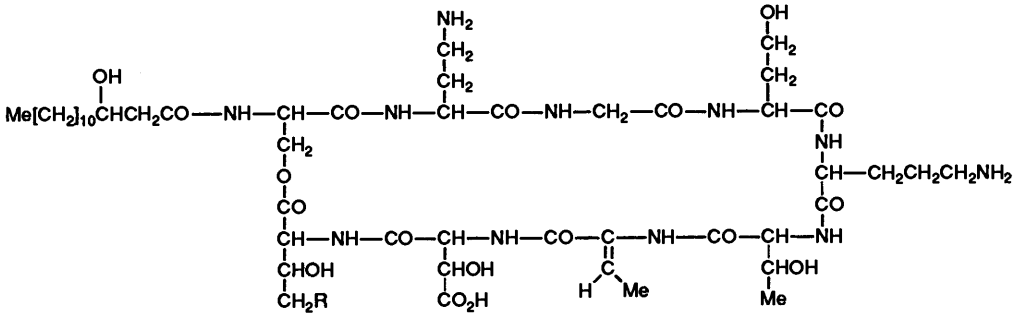


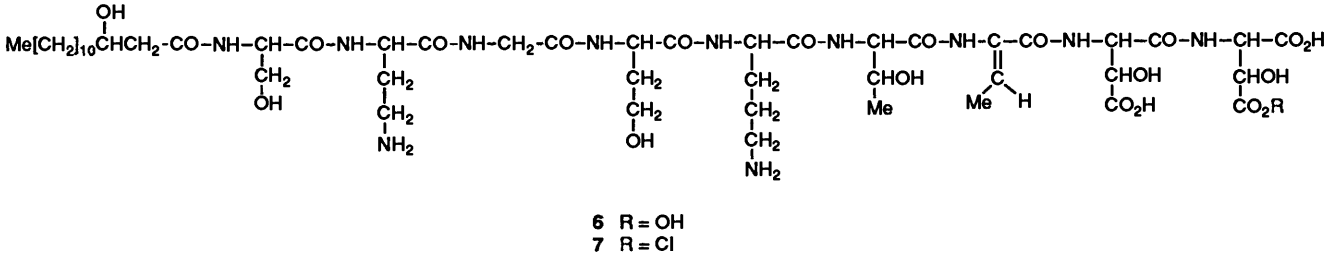
Fig. 5 Structure of syringotoxin B 5. Arrows represent the carbon–proton long-range couplings observed in the HMBC spectrum.



	n	X_1	X_2	X_3	X_4	X_5
Syringostatin A	10	D-Dab	L-Dab	D-homoSer	L-Orn	L-alloThr
Syringomycin **1**	8	D-Ser	L-Dab	D-Dab	L-Arg	L-Phe
Syringotoxin B **5**	10	D-Dab	Gly	D-homoSer	L-Orn	L-alloThr

Fig. 6 Structure of syringostatin A, syringomycin **1** and syringotoxin B **5**.



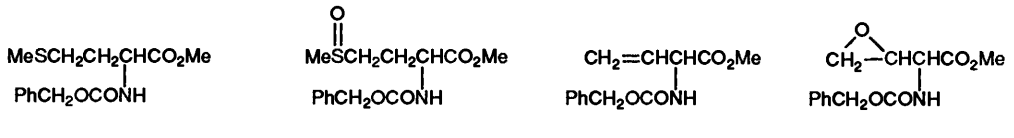


products analysed by reversed-phase chromatography, L(D)-*threo* and L(D)-*erythro* isomers were found to have the same retention times. Therefore, it was only possible to differentiate the D- and L-form of ClThr. Four isomers of HyThr were then produced by alkaline treatment of the corresponding ClThr that preserved the original stereochemistry. The derivatives of these four diastereoisomers of HyThr were clearly separated by HPLC as shown in Fig. 7.

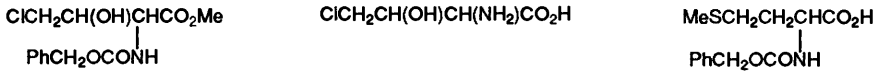
Marfey's method was applied to the acid hydrolysates of syringostatin A, syringomycin **1**, and syringotoxin B **5** and the results are shown in Table 3. The two unusual amino acids, HyAsp and ClThr, were both L-*threo* forms. The configurations of the other amino acids in syringotoxin B **5** were established as D for Dab and homoSer and L for Orn and alloThr. Similarly, Arg and Phe in syringomycin **1**, and Orn, alloThr and Ser in syringostatin A, were L, and homoSer in the latter was D. The remaining Dabs in syringostatin A, and Dabs and Sers in syringomycin **1**, were each a 1:1 mixture, and therefore their configurations could not be directly determined. However, the configurations of these amino acids may be deduced from comparison with the amino acids in the analogous positions in syringotoxin B **5** and the syringostatins. Thus, the *N*-terminal seryl residue of syringomycin **1** was deduced to be the L-form, and the configurations of the second, third and

Table 3 Stereochemistry of amino acids in the hydrolysates of syringostatin A, syringomycin **1** and syringotoxin B **5**

	Ser	Dab	homoSer	Orn	Arg	Thr	Phe	HyAsp	ClThr
Syringostatin A	L	D,L	D	L		L-*allo*		L-*threo*	L-*threo*
Syringomycin **1**	D,L	D,L			L		L	L-*threo*	L-*threo*
Syringotoxin B **5**	L	D	D	L		L-*allo*		L-*threo*	L-*threo*



8 **9** **10** **11**



12a, b **13** **14**

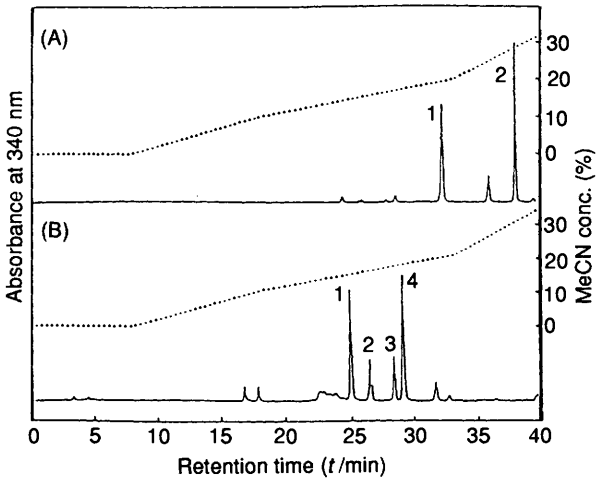


Fig. 7 Chromatograms of FDAA derivatives of the diastereoisomers of 4-chlorothreonine and 4-hydroxythreonine. (A) 4-Chlorothreonine; 1: L-(*threo*/*erythro*)-; 2: D-(*threo*/*erythro*)-. (B) 4-Hydroxythreonine; 1: L-*threo*-; 2: L-*erythro*-; 3: D-*erythro*-; 4: D-*threo*-. Column: SSC-ODS-1151-N (150 × 4.6 mm); eluting solvent: acetonitrile gradient with acetic acid–sodium acetate buffer (pH 6.5).

fourth residues were presumed to be the D-, L- and D-form, respectively. Similarly, the first and second Dab residues in syringostatin A were thought to be D and L forms, respectively. These stereochemical considerations are summarized in Fig. 6.

The stereochemistry of 3-hydroxydodecanoic acid in syringomycin **1** was determined by analysis of its α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) ester[15] by HPLC on a silica gel column. In this HPLC system, (*R*)-MTPA esters of authentic D- and L-3-hydroxydecanoic acids were eluted at 10.8 and 11.5 min, respectively. The MTPA derivative of the isolated acid eluted at the same retention time as that of the authentic D-form. With mass spectrometry, this material yielded the expected $(M + H)^+$ ion at *m*/*z* 447. A small amount of material was observed having the same retention time as the MTPA derivative of the L-form acid, which must be due to partial racemization through dehydration and hydration during hydrolysis. Considering the homology of the acid components and the total structure of these phytotoxins, 3-hydroxytetradecanoic acid in syringostatin A and syringotoxin B **5** were deduced to be D-forms as well. The stereochemistry of the 3-position of 3,4-dihydroxytetradecanoic acid in syringostatin B **5**[5] was probably the same, but that of the 4-position was uncertain.

The structures of the phytotoxins produced by *P. syringae* pv. *syringae* have been uncertain until recently. In this report, and in our previous report,[5] we established the covalent structures of syringostatins, syringomycin, and syringotoxin B by using the techniques of 2D-NMR spectroscopy and mass spectrometry fragmentation of linear derivatives. We further propose the stereochemistry of their components.

Only one strain, isolated in Japan, is known to produce the syringostatins; several other strains of *P. syringae* pv. *syringae* from around the world are known to produce syringomycins or syringotoxins. This raises the possibility that other isolates of *P. syringae* pv. *syringae* may produce other cyclic lipodepsipeptidal toxins with homologous structures. The methods and approaches described here involving mass spectra of the linearized peptides by alkaline hydrolysis and amino acid analysis may be generally applied to these toxins.

Bachmann and Takemoto[16] deduced the stereochemistry of Arg, Phe and Dab in syringomycin from the results of enzymic digestion. Recently, Ballio *et al.*[17] reported independently the structure of syringotoxin and amino acid stereochemistries except that for ClThr, their results agreeing with our present results. Also, a re-evaluation of the structure of syringomycin E by Ballio *et al.* agrees with the present findings.

Experimental

Isolation of Syringomycins.—*Pseudomonas syringae* pv. *syringae* SC1, isolated from Japanese sugar cane, was cultured in the medium reported by Zhang and Takemoto[10] (200 cm^3 of medium in 500 cm^3 capacity Erlenmeyer flasks) at 26.5 °C for nine days. For the detection of the toxins in the isolation procedure, the antifungal activity against *Saccharomyces cerevisiae* 8A-1B, provided by Prof. T. Miyakawa, Hiroshima University, Japan, was assayed. The cultured broth was centrifuged, and to the supernatant (4 dm^3) acetic acid was added to a final concentration of 0.2%. The acidified supernatant was passed through an XAD-2 column (28 × 3.9 cm) and the column was washed with 0.2% acetic acid (200 cm^3). The effluent was adsorbed on an XAD-7 column (39 × 1.8 cm). The column was washed with 0.2% acetic acid and was then eluted with 80% ethanol. Most of the ethanol of the eluent was evaporated off and the residue was applied to a Sephadex G-25 column (53 × 2.3 cm) and chromatographed using 0.1% TFA as eluent. Fractions (10 cm^3) were collected. The antifungal activity was recovered in fractions 24–28 (50 cm^3), and these active fractions were lyophilysed. The toxins were finally purified by using a preparative reversed-phase ODS

column (SSC-ODS-H-5251, 250 × 20 mm, Senshu Kagaku Co.) and a linear gradient of 25–50% of acetonitrile–0.1% TFA in 20 min. The eluates were monitored at 220 nm (Fig. 1). SC-6 and authentic syringomycin E were eluted at the same retention time with an analytical ODS column (SSC-ODS-H-3201, 200 × 8 mm, Senshu Kagaku Co.) using a system 25–50% acetonitrile–0.1% TFA linear gradient in 20 min. Yields were SC-6 (syringomycin) (17.2 mg), SC-10 (5.0 mg), SC-1 to -5 and SC-7 to -9 (less than 1 mg).

FAB Mass Spectrum and NMR Spectrum.—FAB mass spectra and NMR spectra of isolated SCs were obtained as described in the previous paper. $(M + H)^+$ Ions and isotopic ion patterns in the FAB mass spectra of SCs were as follows: SC-1 (*m/z* 1225, Cl), SC-2 (1207), SC-3 (1241, Cl), SC-4 (1189), SC-5 (1191), SC-6 (1225, Cl), SC-7 (1235), SC-8 (1239, Cl), SC-9 (1251, Cl) and SC-10 (1253, Cl) (Cl in parentheses indicates the presence of a chlorine atom).

Isolation of Syringotoxins A and B.—A preparation of syringotoxin (120 mg) was generously provided by Prof. J. E. DeVay, University of California, Davis, and was purified by the same HPLC method used for syringomycin. Yields of syringotoxins A and B were ~3.0 and 10 mg, respectively. FAB mass spectra of syringotoxins A and B gave $(M + H)^+$ ions at *m/z* 1118 and 1136, respectively, the latter indicating the presence of a chlorine atom.

Alkaline Hydrolysis of Syringomycin and Syringotoxin B.—The alkaline hydrolysis procedures previously described for the syringostatins[5] were used.

Amino Acid and Fatty Acid Analyses.—Amino acid and fatty acid analyses were performed as described previously.[5] The detected known amino acids were Ser (1.2 mol), Phe (0.6 mol), Dab (1.8 mol) and Arg (0.7 mol) from one mol of syringomycin. For syringotoxin B, PTC amino acid analysis was applied on the acid hydrolysate of syringotoxin B as described by Antherton.[18] The proportions of the detected amino acids were 1:1:1:1 for Gly, Ser, Orn and Dab.

For the fatty acid standard, 3-hydroxydodecanoic acid was synthesized from decanal (2.0 g, 12.9 mmol), ethyl bromoacetate (4.8 g, 28.8 mmol) and powdered zinc (3.8 g) by methods used previously for the synthesis of 3-hydroxytetradecanoic acid.[5] The yield was ~300 mg of a DL-mixture; $\delta_C(CDCl_3)$ 14.1, 22.7, 25.4, 29.3, 29.5, 29.6, 29.6, 31.9, 36.5, 41.1, 66.1 and 177.7. Both the methylated acid residue from the acid hydrolysate of syringomycin and the synthesized methyl 3-hydroxydodecanoate were eluted at 11.2 min in GC-MS as described in the previous paper.[5] These gave fragment ions at *m/z* 213 $(M - OH)^+$, 181, 180, 155, 138, 103, 71 and 55.

Determination of Amino Acid Configurations.—Determination of amino acid configurations was performed by Marfey's method.[11] A 50 mmol dm^{-3} solution of amino acid mixture (10 mm^3), 6% triethylamine (4 mm^3) and 1% *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA) in acetone (20 mm^3) were combined and incubated at 40 °C for 1 h in a polypropylene microtube. The reactant was diluted into water (1 cm^3), and an aliquot of the solution (200 pmol for each amino acid) was applied to an ODS column (SSC-ODS-1151-N, 150 × 4.6 mm, Senshu Kagaku Co.). For resolution of the standard amino acid derivatives, three solvent systems were used at a flow rate of 1.0 cm^3 min^{-1}. For basic amino acids in syringostatin A and syringotoxin B (Dab and Orn), 10 mmol dm^{-3} triethylamine–phosphate buffer (pH 3.0) with an acetonitrile gradient (16–30% in 18 min and 30–50% in 12 min) was used. For separation of Dab and Phe in syringomycin, 0.1% TFA with an acetonitrile gradient (15–40% in 25 min and 40–50% in 7 min) was used. For other amino acids (Ser, homoSer, alloThr, HyAsp, ClThr, Arg and Gly), 10 mmol dm^{-3} acetic acid–sodium acetate buffer (pH 6.5) with an acetonitrile gradient (0–10%, 10 min; 10–20%, 15 min; 20–31.5%, 7 min; and 31.5–50%, 2 min) was used. Derivatized amino acids were detected by their absorbance at 340 nm.

N-[(*Benzyloxy*)*carbonyl*]-L-*methionine Methyl Ether* **8**.—*N*-[(Benzyloxy)carbonyl]-L-methionine (Peptide Industry Inc.) (1.00 g, 3.53 mmol) was dissolved in diethyl ether (10 cm^3). To this vigorously stirred solution was added a solution of CH_2N_2 in diethyl ether (~20 cm^3). After the reaction was complete, the solvent was removed under reduced pressure to yield compound **8** quantitatively as a light-yellow solid; δ_H(300 MHz; $CDCl_3$) 1.97 (1 H, m), 2.07 (3 H, s), 2.12 (1 H, m), 2.53 (2 H, t, *J* 7), 3.74 (3 H, s), 4.50 (1 H, m), 5.11 (2 H, s), 5.40 (1 H, br d, *J* 7) and 7.34 (5 H, m).

Methyl L-2-[(*Benzyloxy*)*carbonylamino*]-4-(*methylsulfinyl*)*butanoate* **9**.—To a stirred solution of the sulfide **8** in methanol at 0 °C was slowly added $NaIO_4$ (0.726 g, 3.39 mmol). Further quantities of methanol (6 cm^3) and water (3 cm^3) were added. After the mixture had been stirred at 0 °C for another 2 h the mixture was filtered to remove insoluble material. The filtrate was concentrated to 5 cm^3 under reduced pressure, and extracted with $CHCl_3$ (10 cm^3 × 3), and the extract was washed with water (10 cm^3 × 3) and then dried, to yield sulfoxide **9** quantitatively as an oil; δ_H(300 MHz; $CDCl_3$) 2.12 (1 H, m), 2.36 (1 H, m), 2.52 (3 H, s), 2.62–2.80 (2 H, m), 3.73 (3 H, s), 4.47 (1 H, m), 5.08 (2 H, s), 5.79 (1 H, m) and 7.33 (5 H, m).

Methyl L-2-[(*Benzyloxy*)*carbonylamino*]*but*-3-*enoate* **10**.—The sulfoxide **9** (0.726 g, 2.32 mmol) was dissolved in xylene (10 cm^3) and the solution was refluxed in an oil-bath (156 °C) for 36 h, to afford a brown oil after removal of the solvent. This residual oil was dissolved in a small volume of hexane–ethyl acetate (9:1 v/v) and applied to a Wacogel C-100 silica gel column (100 cm^3). The column was eluted stepwise with hexane–ethyl acetate (9:1 v/v; 200 cm^3), (8:2 v/v; 200 cm^3) and (7:3 v/v; 200 cm^3), to yield crude ester **10** in the latter two fractions. The crude product was further purified by silica gel (Wacogel C-100) column chromatography, developed isochratically with hexane–ethyl acetate (9:1 v/v). Pure ester **10** was obtained as a light yellow oil (414 mg, 72%); δ_H(300 MHz; $CDCl_3$) 3.75 (3 H, s), 4.93 (1 H, m), 5.12 (2 H, s), 5.27 (1 H, dd, *J* 2, 10), 5.35 (1 H, dd, *J* 2, 20), 5.51 (1 H, br d, *J* 7), 5.89 (1 H, ddd, *J* 5, 10, 20) and 7.34 (5 H, m).

Methyl (2S,3S)-2-[(*Benzyloxy*)*carbonylamino*]-3,4-*epoxybutanoate* syn-**11** *and* (2S,3R)-*form* anti-**11**.—The mixed solution of ester **10** (377 mg, 1.50 mmol) and *m*-chloroperbenzoic acid (MCPBA) (1.00 g) in methylene dichloride (15 cm^3) were allowed to react in a vigorously stirred mixture at room temperature. Insoluble material was removed with a glass filter, and the solution was washed successively with 10% aq. sodium hydrogen sulfite (10 cm^3 × 2) and 10% aq. sodium hydrogen carbonate (10 cm^3 × 3). The organic layer was dried and evaporated to yield a yellow oil. The oil was dissolved in hexane–ethyl acetate (7:3 v/v), and then applied to a silica gel column (Wacogel C-100, 100 cm^3). The column was eluted with the same solvent and fractionated into 100 cm^3 portions. The mixture of *syn*-**11** and *anti*-**11** (4:1) (233 mg, 59%) was recovered in fractions 3 and 4 (200 cm^3). The resulting residue was further purified by repetition of the chromatography and collection of 50 cm^3 fractions. The *syn* and *anti* mixture of esters **11** was recovered in fractions 5–9. The ratio of *syn*-**11** and *anti*-**11** in

fraction 5 (50 cm^3) was 10:1 (crude *syn*-**11**, 82.9 mg) and that of combined fractions 8 and 9 was 3:1 (mixed **11**, 30.4 mg). For *syn*-**11**: δ_H(300 MHz; $CDCl_3$) 2.64 (1 H, dd, *J* 2, 5), 2.76 (1 H, dd, *J* 5, 5), 3.43 (1 H, ddd, *J* 1, 2, 5), 3.79 (3 H, s), 4.69 (1 H, dd, *J* 1, 9), 5.10 (2 H, s), 5.29 (1 H, br d, *J* 9) and 7.34 (5 H, m); δ_C(75 MHz; $CDCl_3$) 43.8, 51.1, 52.9, 53.0, 67.2, 128.0 (× 2), 128.2 (× 2), 128.5, 135.9, 156.1 and 170.1. For *anti*-**11**: δ_H(300 MHz; $CDCl_3$) 2.76 (2 H, m), 3.20 (1 H, ddd, *J* 4, 4, 5), 3.77 (3 H, s), 4.48 (1 H, dd, *J* 5, 7), 5.10 (2 H, s), 5.52 (1 H, br d, *J* 7) and 7.34 (5 H, m).

These data of *syn*-**11** and *anti*-**11** were identical with those reported by Shaw *et al.*[14]

Methyl (2S,3S)-2-[(*Benzyloxy*)*carbonylamino*]-4-*chloro*-3-*hydroxybutanoate* (Z-4-*chloro*-L-threo-*threonine*-*Me*) **12a**.—Crude ester *syn*-**11** (82.9 mg) was dissolved in methanol (1 cm^3) and 5% hydrogen chloride–methanol (2 cm^3) was added dropwise at room temperature. The mixture was kept stationary for 1.5 h before being poured into ice-cold chloroform (20 cm^3), washed with water (10 cm^3 × 3), and dried. The resulting residue was purified on a silica gel column (Wacogel C-100; 100 cm^3) with hexane–ethyl acetate (1:1 v/v) as solvent and collection of 50 cm^3 fractions. Compound **12a** was recovered in fractions 6–10 (250 cm^3). Recrystallization from ethyl acetate–hexane–light petroleum (30–70 °C) yielded needles of title compound **12a** (39.0 mg); δ_H(300 MHz; $CDCl_3$) 2.64 (1 H, br d, *J* 5), 3.52 (1 H, dd, *J* 8, 12), 3.64 (1 H, dd, *J* 5, 12), 3.78 (3 H, s), 4.35 (1 H, m), 4.54 (1 H, dd, *J* 2, 9), 5.12 (2 H, s), 5.52 (1 H, br d, *J* 9) and 7.34 (5 H, m); δ_C(75 MHz; $CDCl_3$) 36.4 (t), 52.9 (q), 56.0 (d), 67.4 (t), 71.8 (d), 128.1 (× 2) (d), 128.3 (× 2) (d), 135.8 (s), 156.7 (s) and 170.5 (s).

Mixture of Methyl (2S,3S)/(2S,3R)-2-[(*Benzyloxy*)*carbonylamino*]-4-*chloro*-3-*hydroxybutanoate* (*Mixture of* Z-4-*Chloro*-L-threo- *and* -erythro-*threonine*) **12a** *and* **12b**.—Mixed ester **11** (30.4 mg) yielded a mixture of esters **12a** and **12b** as needles (29.0 mg) by using the same purification procedure used for ester **12a**; δ_H for **12b** (300 MHz; $CDCl_3$) 2.82 (1 H, br d, *J* 5), 3.6–3.7 (2 H, m), 3.78 (3 H, s), 4.18 (1 H, m), 4.42 (1 H, m), 5.12 (2 H, s), 5.58 (1 H, br d, *J* 9) and 7.34 (5 H, m).

(2S,3S)-2-*Amino*-4-*chloro*-3-*hydroxybutanoic Acid* (4-*Chloro*-L-threo-*threonine*; L-threo-*ClThr*) **13a**.—Hydrolysis of ester **12a** (5.0 mg) with 6 mol dm^{-3} hydrochloric acid in a sealed tube at 110 °C for 13 h gave a quantitative yield of acid **13a**; δ_H(300 MHz; D_2O) 3.77 (2 H, d, *J* 6), 4.29 (1 H, d, *J* 5) and 4.45 (1 H, dt, *J* 5, 6); δ_C(75 MHz; D_2O) 46.3 (t), 56.5 (d), 69.9 (d) and 171.5 (s).

(2S,3S)/(2S,3R)-2-*Amino*-4-*chloro*-3-*hydroxybutanoic Acid* (*Mixture of* 4-*Chloro*-L-threo- *and* -erythro-*threonine*; L-*mixed*-*ClThr*) **13a** *and* **13b**.—A mixture of acids **13a** and **13b** (3:1) was obtained from a mixture of esters **12a** and **12b** (3:1) by the method described above.

Synthesis of DL-threo/erythro-2-*Amino*-4-*chloro*-3-*hydroxybutanoic Acid* (DL-4-*Chlorothreonine*; DL-*ClThr*).—The DL-form of acid **13** was synthesized, in the same way that the L-form of acid **13** (**13a** and **13b**) was obtained, from DL-[(benzyloxy)carbonyl]methionine (DL-**14**). Compound DL-**14** was prepared in the following way: DL-methionine (1.50 g, 10.1 mmol) was suspended in aq. sodium hydrogen carbonate (2.10 g, 25 mmol in 20 cm^3) and benzyloxycarbonyl chloride (ZCl) (Nakarai Tesk Co.) (1 cm^3, 6.25 mmol) was added dropwise to the vigorously stirred suspension in an ice-cold bath. Diethyl ether (10 cm^3) was added, and after the mixture had been stirred for 2 h ZCl (1 cm^3), diethyl ether (10 cm^3) and water (10 cm^3) were added to the reaction solution. The solution was then stirred for 3 h. After being washed with diethyl ether (15 cm^3), the resulting water phase was acidified with 6 mol dm^{-3} hydrochloric acid and extracted with ethyl acetate (15 cm^3 × 3). The solvent was removed and the residue was recrystallized with ethyl acetate–diethyl ether–light petroleum to give an amorphous solid in quantitative yield.

Conversion of 4-*Chlorothreonine into* 4-*Hydroxythreonine*.—L-*threo*-ClThr, L-Mixed-ClThr and DL-ClThr (each 2.5 μmol) were dissolved in water (13 mm^3) in microtubes. 1 mol dm^{-3} aq. sodium hydroxide (10 cm^3) was added and the solution was incubated at 40 °C for 18 h. The reactant solution was neutralized with dil. hydrochloric acid and yielded the corresponding 4-hydroxythreonine quantitatively. The 4-hydroxythreonine was detected by HPLC analysis after derivatization by Marfey's method.[11]

Analysis of Stereochemistry of 3-*Hydroxydodecanoic Acid*.—Syringomycin **1** (6.0 mg) was hydrolysed in a sealed tube with 6 mol dm^{-3} HCl (1.2 cm^3) for 18 h at 110 °C. The reaction mixture was diluted with water (2 cm^3) and ethyl acetate (3 cm^3), and extracted with 10% aq. Na_2CO_3. The aqueous solution was adjusted to pH 3 with HCl and extracted with ethyl acetate. After evaporation of the ethyl acetate extracts, an ethereal solution of diazomethane (1 cm^3) was added to esterify the carboxylic group of the fatty acid. The sample was dried at room temperature. Dry pyridine (100 mm^3) and MTPA-Cl (30 cm^3) [from (*R*)-MTPA acid] were added to the dry sample to prepare the (*R*)-MTPA ester. The reaction mixture was vortexed and kept at room temperature overnight until the reaction was complete. The mixture was diluted with water (3 cm^3) and extracted with ethyl acetate. This extract was washed with dil. HCl and dried with Na_2SO_4. The filtered solution was concentrated, and diluted with hexane (1 cm^3). It was then adsorbed on a Sep-Pac silica column, washed with hexane, and eluted with 5% ethyl acetate in hexane. The evaporated eluate was diluted with hexane and analysed by HPLC on a silica-1251-N column. Hexane–tetrahydrofuran–methanol (6000:100:1) was used as the solvent with a flow rate of 1.5 cm^3 min^{-1}, and detection was carried out at 210 nm. (*R*)-MTPA esters of L-3-hydroxydodecanoic acid and synthetic DL-3-hydroxydodecanoic acid were used as standards for determination of relative retention times between D- and L-3-hydroxydodecanoic acid.

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