Articles

The Structures of Syringolides 1 and 2, Novel C-Glycosidic Elicitors from *Pseudomonas syringae* pv. *tomato*†

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The isolation and structure determination of two bacterial signal molecules which elicit active plant defense responses are reported. The production of these molecules by Gram-negative bacteria requires the action of avirulence gene D (*avrD*), cloned from *Pseudomonas syringae* pv. *tomato*. The structures of syringolide 1 (**1a**) and syringolide 2 (**1b**) are determined by a combination of NMR experiments, biosynthetic arguments, molecular modeling, and X-ray crystallography. A proposed biosynthetic scheme based on the condensation of D-xylulose with a β-ketoalkanoic acid is presented. Further cyclization of the biosynthetic intermediates leads to C-glycosides with a novel tricyclic ring system. These are the first nonproteinaceous specific elicitors of a plant hypersensitive response.

In the plant kingdom one widespread type of resistance to microbial pathogens is the hypersensitive response (HR). This active defense reaction involves a rapid, localized cell death followed by accumulation around the infection site of antimicrobial compounds called phytoalexins.[1] Occurrence of the HR often requires the presence of genetic elements in the plant called resistance genes and matching or complementary avirulence genes in the pathogen, i.e., "gene-for-gene" complementarity.[1,2] Resistance to the pathogen occurs only when the plant expresses a dominant resistance gene and the pathogen expresses a complementary avirulence gene.

Indirect evidence suggests that plant disease resistance gene products may function as receptors for signal molecules produced by the pathogen, called elicitors. The production of specific elicitors has been firmly associated with avirulence gene function in only three cases.[3,4] One of these cases, that of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* and various soybean cultivars, has been the object of intensive scrutiny in our laboratories.[1,4-6] In the two other known examples,[3] the protein produced by the avirulence gene is itself the elicitor. However, the elicitors produced by *P. syringae* pv. *tomato* are not proteins; the direct protein product of the avirulence gene in *P. syringae* pv. *tomato* is devoid of elicitor activity.

Avirulence gene D, *avrD*, cloned from *P. syringae* pv. *tomato*,[5] causes Gram-negative bacteria expressing the gene to elicit an HR on soybean plants carrying the resistance gene, *Rpg4*.[4] Bacteria expressing *avrD* have furthermore been shown to produce, extracellularly, two nonproteinaceous low molecular weight metabolites each of which functions as a specific signal molecule that causes HR specifically in soybean plants harboring *Rpg4*.[6] These *avrD* elicitors have considerable biological interest since, in constituting the molecular signal recognized by plants expressing a resistance gene, they have features in common with antigens that are recognized by the immune systems of vertebrates.

We report here unique chemical structures for the *avrD* elicitor-active molecules, the first of their class to be elucidated. They are C-glycosides possessing a new ring

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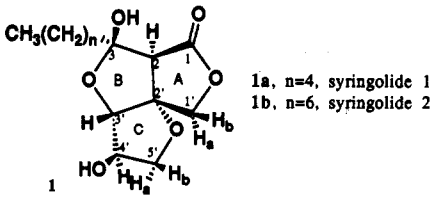

1a, n=4, syringolide 1
1b, n=6, syringolide 2

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Figure 1. Elicitors from *P. syringae* pv. *tomato*.

system.[7] Their structures, deduced from a combination of spectroscopic and biosynthetic arguments, were finally confirmed by X-ray crystallography.

Results and Discussion

Ethyl acetate extracts of culture fluids of *P. syringae* pv. *tomato* or *P. syringae* pv. *glycinea* carrying plasmid pAVRD12/519 or *Escherichia coli* carrying plasmid pAVRD12 were purified by HPLC with guidance from the soybean HR assay[4] to afford the two elicitors, syringolide 1 (**1a**) and syringolide 2 (**1b**), whose structures are shown in Figure 1. The same two compounds could be obtained from either *E. coli* or *P. syringae* preparations in respective yields of about 2 or 0.5 mg of each per liter of culture. Because of the apparent sensitivity of the syringolides to both acid and base, poor recoveries were obtained from many chromatographic systems. Best separations and recoveries of up to 80% were achieved on normal-phase silica, but silica columns varied, and some irreversibly adsorbed only the two active elicitors from the preparations. Isolated **1a** and **1b** were air-stable waxy solids.

The purified elicitors had nearly identical IR and UV spectra, but the mass spectrum of **1a** showed a molecular ion of 28 mass units less than that of **1b**. The difference was found to be that **1a** contains a hydrocarbon chain with two less methylene units than that of **1b**. Precise mass measurement of **1b** indicated a molecular formula of $C_{15}H_{24}O_6$ (HREIMS *m/z* 300.15700 obsd, 300.15729 calcd; $\Delta = 0.97$ ppm). ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, and PSCSCM spectra (Table I) suggested a C_7H_{15} chain[8] and a $C_8H_9O_6$ tricyclic ring system. The IR band at 1773 cm^{-1} and the ^{13}C peak at 172 ppm defined the carbonyl as a γ-lactone. The ring system included two hydroxyl protons, identified as D_2O-exchangeable peaks at δ 5.31 and 4.27 in acetone-d_6. The location of one of the hydroxyls was determined by a secondary deuterium isotope shift experiment. Upfield deuterium isotope shifts[9,10] were observed by ^{13}C NMR at δ 108.8 (77 ppb), 59.7 (40 ppb), and 39.4 (60 ppb); this indicates that the quaternary δ 108.8 carbon bears a hydroxyl substituent and is flanked by the methine at δ 59.7 and the methylene at δ 39.4. Since this three-carbon unit and the hydrocarbon chain both contain the δ 39.4 methylene, they are linked (unit **a**, Figure 2). The carbon at δ 108.8 is doubly oxygenated, as shown by formation of a methyl ketal when **1b** was stirred at room temperature with anhydrous methanol and trace HCl. The presence of a bond between δ 108.8 and 59.7 was established by INAPT[11] irradiations at δ 1.87 and 3.08 (Table I). Since its proton portion shows no vicinal coupling, the δ 59.7 methine must also be attached to the remaining quaternary carbons at δ 99.0 and 172.7, as suggested by INAPT at δ 3.08 and chemical shift values.

The location of the second hydroxyl group could not be verified by deuterium isotope shift of its ^{13}C NMR signal in acetone-d_6, perhaps due to fast exchange of the OH proton. We were unsuccessful in our attempts to slow the exchange. Substitution of dimethyl sulfoxide-d_6 for acetone-d_6 caused decomposition, and the use of drying agents[9] such as $CaSO_4$ or 3-Å molecular sieves was ineffective. Elicitor **1b** was unstable to acylation conditions, but an acetate was produced from its methyl ketal using acetyl chloride and a trace of pyridine. Observed acetylation shifts confirmed the presence of hydroxyl at the δ 75.4/δ 4.13 methine of **1b**. ^{1}H–^{1}H COSY shows a weakly coupled spin system consisting of methines at δ 4.48 and 4.13 and a methylene at δ 3.94 and 3.81. This system is further defined by ^{13}C NMR acetylation shifts; the alcohol carbon must be central (unit **b**, Figure 2).

Units **a**, **b** and the isolated δ 75.6 methylene (unit **c**, Figure 2) with ^{1}H NMR peaks at δ 4.65 and 4.30 complete the structure. The methine at δ 92.2, the methylenes at δ 75.6 and 74.9, and the quaternary carbon at δ 99.0 must all be oxygenated to justify their chemical shift values, but none of these can have more than one oxygen based on the molecular formula. Experimental $^1J_{C-H}$ values (Table I) preclude the existence of 3-membered rings,[12] but there are still several plausible structures. The computer program, SESAMI,[13] which generates structures based on input NMR data, initially excluded structures 1 until the ^{13}C NMR chemical shift limit for an sp^3 carbon with one oxygen was increased to 100 ppm. These structures do contain unusually large downfield chemical shift values of δ 92.2 and 99.0 which must be attributed to singly oxygenated carbons; the large number of deshielding β-substituents could explain the apparent anomaly. We have found only a few examples.[14] Among the chemically reasonable possibilities generated, the attractiveness of 1 and 2 (Figure 5) is greatly enhanced by the availability of a simple and intriguing biosynthetic pathway.

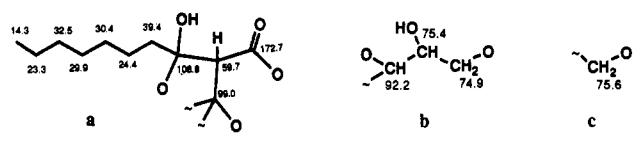

Figure 2. Structural units of syringolide 2.

Spectroscopically it is difficult to distinguish between 1 and 2; the biggest differences should be found in long-range correlations between the C3 hemiketal or the C1 carbonyl and the H3′ methine or the H1′ methylene. INAPT irradiations of H1′a and H1′b each gave transfer

(7) Verification of the novelty of this ring system was obtained from R. W. White of Chemical Abstracts Service.

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Table I. NMR Data for Syringolide 2 (1b)[a]

position	δ^1H(multiplicity, Hz)	δ^{13}C(multiplicity)	INAPT correlations	^{1}H–^{1}H COSY
1		172.7 (s)		
2	3.08 (bs)	59.7 (d, J = 141.5)	1,3,4,1′,2′	5.3
3		108.8 (s)		
4	1.87 (dd, J = 8.4)	39.4 (t, J = 125.1)	2,3,6[c]	1.5, 1.6
5a	1.48 (m)	24.4 (t, J = 122.3)		1.3, 1.6, 1.9
5b	1.60 (m)			1.3, 1.5, 1.9
6	1.29 (m)	30.4 (t, J = 124.8)		
7	1.29 (m)	29.9 (t, J = 124.5)		
8	1.29 (m)	32.5 (t, J = 121.0)		
9	1.29 (m)	23.3 (t, J = 123.8)		
10	0.88 (t, J = 7)	14.3 (q, J = 123.8)		1.3
1′a	4.30 (d, J = 10.3)	75.6 (t, J = 152.9)	1,2′,3′[b],4′[b,c]	4.7
1′b	4.65 (d, J = 10.3)		1,2[b,c],3[b,c],2′[b,c],3′,4′[b,c]	4.30
2′		99.0 (s)		
3′	4.48 (bs)	92.2 (d, J = 159.2)	1′[b],2′,4′[b],5′	3.9
4′	4.13 (dd, J = 4.1, 2.9)	75.4 (d, J = 153.2)	1′[b,c],2′,3′[c],5′[b]	3.8, 3.9, 4.27
5′a	3.81 (dd, J = 10, 2.9)	74.9 (t, J = 147.0)	4′	3.9, 4.1
5′b	3.94 (dd, J = 10, 1.1)		2′,3′,4′	3.8, 4.1, 4.5
3-OH	5.31 (d, J = 1.8)		2,3,4[b,c]	3.1
4′-OH	4.27 (d, J = 4.1)			4.1

[a] Spectra were taken in acetone-d_6. Carbon–proton correlations were made by PSCSCM. [b] These correlations were not observed by HMBC. [c] These correlations were not observed by CSCMLR.

to C1 in support of structure 1, although in one case a weak hemiketal transfer was also observed, supporting structure 2. INAPT irradiation of H3′ did not produce polarization transfer to C3 *or* to C1. Proton chemical shift differences were induced at H1′a, H1′b, and H3′ by methylation of the hemiketal at C3. Methylation effects at H3′ were slightly stronger, also weak evidence for 1. Unfortunately, 2 still could not be rigorously excluded.

Each of the possible stereochemical isomers of 1 and 2, with n = 1, was examined with the conformational search program BAKMDL for all stable conformations within 3 kcal of the global minimum.[15] For each stereochemical isomer, the computed lowest energy conformer and the Boltzmann-weighted aggregate conformer were then used to calculate dihedral angles, interproton distances, and vicinal ^{1}H–^{1}H coupling constants. There was only one isomer of 1 (and none of 2) whose Boltzmann-weighted aggregate had all vicinal coupling constants less than 4 Hz. This isomer also had the lowest calculated energy of the four possible epimers at C4′ and C3, and it predicted our observed J's within 1 Hz.[16] The higher energy conformations, comprising about 15% of the weighted average of 1 (and 37% of 2), contain large J's due to an alternate pucker at C5′. The relative stereochemistry is as shown for 1 (Figure 1). The C3 epimer was ruled out based on the calculated coupling constants for the protons of the C-ring and the observed W-coupling between H2 and OH3.

The identity of the elicitors as structure 1 was confirmed by X-ray crystallography[17] of **1b**. The structure, solved by direct methods, is shown in Figure 3. The relative stereochemistry of crystalline syringolide 2 was precisely as predicted by modeling calculations. There were no intramolecular hydrogen bonds in the crystal structure. The X-ray conformation differed from the lowest energy model only in a twist of the side chain. The predicted conformation of OH3 allowing W-coupling to H2 was maintained in the crystalline form. The relative stereochemistry observed indicates that the syringolides could, in fact, be derived from xylulose, the natural *threo*-2-ketopentose which we propose as a biosynthetic precursor.

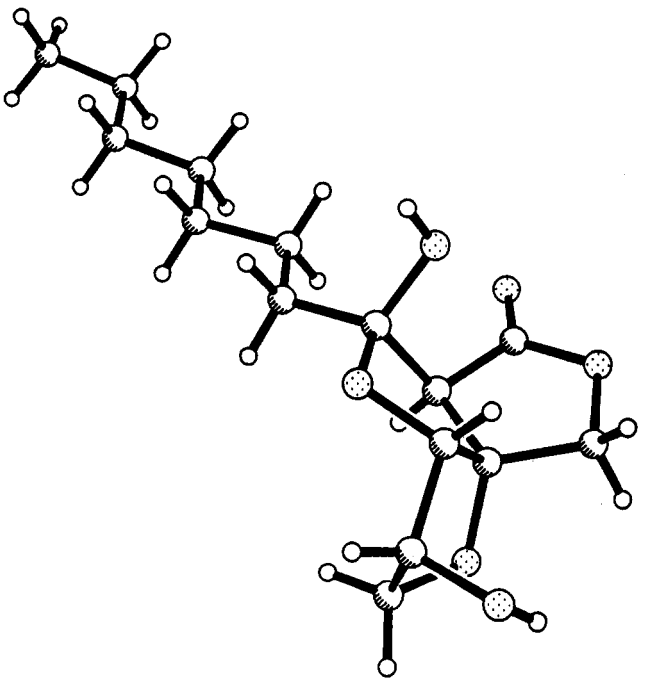

Figure 3. Computer-generated perspective drawing of the final X-ray model of syringolide 2. The absolute configuration was not determined in the X-ray experiment.

Proposed Biosynthesis

The structures of the syringolides can be visualized as combinations of a β-keto acid unit and a pentose unit. While it is common to find acylated sugars in nature, the combination of molecules from these two major metabolic pathways via a carbon–carbon bond to make C-glycosidic lipids, as in the syringolides, does not seem to have been observed before. Examples for biologic condensation of 3-carbon ketones with either β-keto acids or 2-ketopentoses

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(16) Dihedral angles from H4′ to H3′, H5′a, and H5′b were 88, 42, and 80°, respectively, corresponding to calculated J values of 0.8, 2.8, and 1.4 Hz.

(17) Siemens Analytical X-ray Instruments, Inc. SHELXTL-PLUS Version 4.0, 1990.

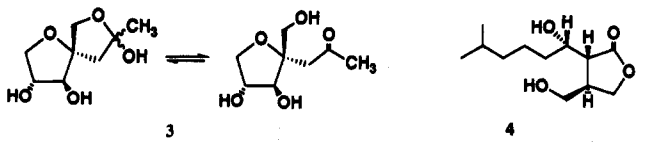

Figure 4. Molecules related to the syringolides by biosynthesis.

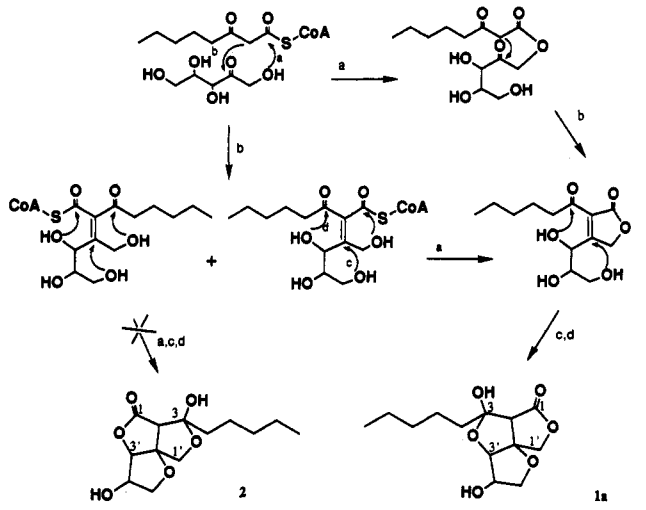

Figure 5. Possible reaction scheme for biosynthesis of syringolide 1: a, acylation; b, aldol (Knoevenagel) condensation; c, Michael addition; d, hemiketalization.

have been found, however, in secondary metabolites isolated from *Streptomyces*. Sphydrofuran (**3**, Figure 4)[18] can be considered to be a condensation product of acetone and D-xylulose. **3** has no known biological activity. Virginiae butanolide A (**4**, Figure 4) has been biosynthetically shown to result from a condensation between a β-keto acid derivative and a unit such as dihydroxyacetone phosphate.[19] The virginiae butanolides are signal molecules which regulate both secondary metabolite production and cytodifferentiation.

Possible reaction pathways for the biosynthesis of the syringolides, using **1a** as an example, are proposed in Figure 5. Two alternative first steps are illustrated: first, acylation followed by Knoevenagel condensation or, alternatively, Knoevenagel condensation followed by lactone formation. Initial Knoevenagel condensation could produce two stereoisomers which, theoretically, could subsequently cyclize to produce structure **1a** or **2**. (Structure **2** has not been found experimentally.) Initial acylation of xylulose as shown allows only one condensation product leading directly to structure **1a**. After condensation and acylation, Michael addition and hemiketal formation are thermodynamically favored. O-acylation of sugars usually by β-hydroxy acids, is known in *Pseudomonas* species.[20]

The relative stereochemistry of the syringolides has been determined spectroscopically and by X-ray crystallography. Assignment of the absolute configuration of any portion of the molecule therefore defines the whole structure. Models that incorporate the naturally occurring D-xylulose as the precursor of the C-ring of the tricyclic system must adopt the absolute configuration depicted in Figure 1. Thus syringolides 1 and 2 are predicted to both possess the following absolute configuration: 2*S*,3*R*,2′*R*,3′*S*,4′*R*.

Concluding Remarks

A single protein is encoded by *avrD*.[4] If this protein is catalytic in the biosynthesis of elicitors, one might expect that it would control a single reaction, i.e., either the acylation of xylulose or the condensation of xylulose with the β-keto acid. Common aldolases[21] have an apparent three-carbon limitation on the anionic component in the aldol condensations they catalyze. Further work will be necessary to determine the role of the protein.

Syringolides 1 and 2 are extraordinary molecules both chemically and biologically, as demonstrated by their structures and their role as nonproteinaceous elicitors of plant hypersensitivity. One interesting observation is the presence of a 2,3-disubstituted γ-lactone moiety in signal molecules of both *Pseudomonas* and *Streptomyces*. Ongoing investigations address the relationship between plant resistance and pathogenic avirulence, the chemistry and mode of action of syringolides 1 and 2, and their interaction with the products of plant disease resistance genes.

Experimental Section

General Procedures. *E. coli* (strain DH5α) cells carrying pAVRD12 were obtained by procedures which have been previously described.[4] *P. syringae* pv. *tomato* (isolate PT23) and *P. syringae* pv. *glycinea* (isolate R4) were transformed with the broad-host-range plasmid pAVRD12/519. This latter plasmid was constructed by cloning a ca. 3.3-kb *Pst*I/*Sal*I DNA fragment from pAVRD12 (containing the *avrD* gene, triple *lac* UV5 promoters and transcriptional terminators of pAVRD12) into the same sites of pDSK519.[4] Mass spectra were determined using a Fissons VG autospec Q or a VG ZAB IFHF (high resolution) or a VG 7070 EHF instrument. UV spectra were recorded on a Perkin-Elmer 202. Infrared spectra were taken on a Nicolet FTIR or on a Perkin-Elmer Model 137. Optical rotations were obtained with a Perkin-Elmer Model 241 polarimeter with a 10-cm cell. NMR experiments were conducted using a GE-NMR QE300 or a Bruker 500-MHz instrument (ASU); chemical shifts are reported relative to Me_4Si. *J* values are reported in Hz. Carbon multiplicities were determined using APT or DEPT. One-bond C–H correlations were obtained using PSCSCM or HMQC optimized for 120 Hz and $^1J_{C-H}$ were measured by gated decoupling of ^{13}C spectra. Long-range C–H correlations were obtained by selective INEPT (INAPT) experiments and by HMBC and by CSCMLR, normally optimized at 10, 7, and 6.25 Hz, respectively.

Soybean Hypersensitive Response Determination. The elicitor activity of culture fluids, column fractions, etc. was determined by the soybean leaf bioassay.[4] Generally, column fractions were dried, weighed, and redissolved at known concentrations in 95% ethanol. The resultant solutions were bioassayed by infiltrating into leaves of several soybean cultivars. These were scored after 24 h for the appearance of necrosis only on leaves of cultivars carrying the *Rpg4* disease resistance gene. Reproducible elicitor activity was obtained from 10 μL of a 6.67×10^{-5} M solution of **1a** or **1b**.

Purification of 1a and 1b. The *P. syringae* strains carrying pAVRD12/519 were maintained on KMB agar plates containing kanamycin sulfate at 50 μg/mL for ca. 36 h. *E. coli* DH5α cells carrying pAVRD12 were grown on L ampicillin plates for ca. 19

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h. Synthetic liquid M9 medium[4] was used for elicitor production and contained, in addition to inorganic components, glucose at 0.4% and thiamine at 5 μg/mL. For *E. coli* cultures, 5 mL/L of 0.1 M isopropyl β-D-thiogalactopyranoside (IPTG) and 1.5 mL/L of 5% ampicillin was added to the M9 medium. For *P. syringae* isolates, kanamycin sulfate was added to the liquid medium to 50 μg/mL. Erlenmeyer culture flasks (2 L) with 1 L of medium were seeded relatively heavily with bacteria taken from fresh agar plates, and the flasks were shaken for ca. 21 h at 28 °C. The contents were then centrifuged for 5 min at 7500*g* to remove bacterial cells. The supernatant fluids were decanted, and the pH was adjusted from the ambient value of 6.6–7.1 to 5.0 with concentrated HCl. Each liter of fluid was extracted three times with 500-mL portions of freshly distilled EtOAc; the extracts were then taken to dryness in vacuo at 40 °C. The crude yield from *E. coli* preparations averaged 55 mg/L of fluid and from *P. syringae* about 25 mg/L.

The crude extract was further purified by vacuum liquid chromatography.[22] A 30-mL sintered glass filter funnel tightly packed with 4 cm of Kieselgel 60H (EM no. 7736) was used. Combined extracts (1 g, from about 18 L of fluids) dissolved in 5 mL of $CHCl_3$ were loaded on the column after equilibration with $CHCl_3$ and successively eluted with 25 mL of $CHCl_3$, 50 mL of 60% EtOAc/$CHCl_3$, 50 mL 100% EtOAc, and 50 mL of 50% EtOAc/MeOH. Fraction 2 (60% EtOAc/$CHCl_3$) contained most of the crude elicitor activity (300 mg). This fraction was further purified by HPLC using 2-propanol/EtOAc/hexanes (1.5:43.5:55) on a Dynamax silica gel column (1 in. × 25 cm). At 8 mL/min, **1a** and **1b** eluted with retention times of 51 and 44 min, respectively, and 6 and 5.5 mg of each was obtained per 50-mg injection. They were stored as waxy solids. **1b** was more readily crystallized than **1a**, and eventually crystals suitable for X-ray analysis were produced over several weeks by vapor diffusion of hexane into a concentrated butyl acetate solution of **1b**.

Syringolide 1 (1a) was obtained in purified yield of about 1.8 mg per liter of *E. coli* or 0.5 mg per liter of *P. syringae* culture fluids: mp 112.5–114.5 °C; $[\alpha]^{24}_D$ = −83.66° (*c* = 0.15, $CHCl_3$); UV (MeOH) 223 nm (ε = 680), 245 (544), 315 (227); IR ($CHCl_3$) 3580, 2950, 2930, 2860, 1775, 1470, 1360, 1240, 1190, 1150, 1075, 1050 980, 910 cm^{-1}; ^1H NMR (acetone-d_6, 300 MHz), δ 0.88 (3 H, t, *J* = 8, H8), 1.3 (4 H, bm, H6,7), 1.46 (1 H, dtt, *J* = 10.7, 6.7, 6.7, H5a), 1.6 (1 H, dtt, *J* = 10.7, 6.7, 6.7, H5b), 1.87 (2 H, m, H4), 3.08 (1 H, s, H2), 3.81 (1 H, dd, *J* = 10, 2.9, H5′a), 3.93 (1 H, dd, *J* = 10, 1.1, H5′b), 4.12 (1 H, bt, *J* = 4, H4′), 4.30 (1 H, d, *J* = 10.3, H1′a), 4.48 (1 H, s, H3′), 4.65 (1 H, d, *J* = 10.3, H1′b), 4.30 (1 H, bs, 4′OH), 5.34 (1 H, d, *J* = 1.8, 3-OH); ^{13}C NMR δ 14.3 (C8), 23.2 (C7), 24.0 (C5), 32.6 (C6), 39.4 (C4), 59.7 (C2), 74.9 (C5′), 75.4 (C4′), 75.6 (C1′), 92.2 (C3′), 99.0 (C2′), 108.8 (C3), 172.7 (C1); ^1H–^1H COSY correlations δ 0.88–1.3; 1.5–1.3, 1.6, 1.9; 1.6–1.3, 1.5, 1.9; 1.9–1.5, 1.6; 3.1–5.3; 3.8–3.9, 4.1; 3.9–3.8, 4.5; 4.1–3.8, 3.9, 4.3; 4.3–4.1, 4.5, 4.7; 4.7–4.3; INAPT correlations δ 3.1–39.4, 75.6, 99.0, 108.8, 172.7; 3.8–75.4; 3.9–75.4, 92.2, 99.0; 4.1–74.9, 75.6, 92.2, 99.0; 4.3–75.4, 92.2, 99.0, 172.7; 4.5–74.9, 75.4, 75.6, 99.0; 4.7–92.2, 172.7; 5.3–59.7, 108.8; MS (CI^+, NH_3) *m/z* 290 (12), 272 (51), 264 (10), 256 (19), 255 (100), 254 (10), 239 (15), 236 (16), 230 (23), 229 (12), 228 (10), 212 (12), 211 (47), 210 (10), 200 (18), 197 (10), 195 (13), 186 (20), 183 (12), 181 (10), 169 (16), 116 (13), 99 (13), 72 (11), 58 (19), 45 (14), 44 (25); HRMS (CI^+, NH_3), 290.1614 ($C_{13}H_{24}NO_6$ = 290.160 36, Δ = 3.6 ppm).

Syringolide 2 (1b) was obtained in purified yield of 2 mg per L of *E. coli* or 0.5 mg per L of *P. syringae* culture fluids: mp 123–124 °C; $[\alpha]^{24}_D$ = −75.91° (*c* = 0.22, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz), δ 0.9 (3 H, t, *J* = 8, H10), 1.3 (8 H, bm, H6,7,8,9), 1.5 (2 H, m, H5), 1.92 (2 H, m, H4), 3.09 (1 H, s, H2), 3.85 (1 H, dd, *J* = 10.4, 2.9, H5′a), 4.05 (1 H, dd, *J* = 10.4, 1.1, H5′b), 4.31 (1 H, d, *J* = 2.9, H4′), 4.45 (1 H, bs, H3′), 4.48 (1 H, d, *J* = 10.5, H1′a), 4.72 (1 H, d, *J* = 10.5, H1′b); ^{13}C NMR δ 14.08 (C10), 22.61 (C9), 23.48 (C5), 29.09 (C7), 29.39 (C6), 31.72 (C8), 38.86 (C4), 59.08 (C2), 74.26 (C5′), 74.74 (C1′), 74.66 (C4′), 91.42 (C3′), 97.63 (C2′), 108.20 (C3), 172.22 (C1); UV (MeOH) 220 nm (ε = 355), 240 (255), 317 (248); IR ($CDCl_3$) 3583, 2956, 2930, 2873, 2857, 1773, 1466, 1377, 1244, 1186, 1152, 1074, 1047, 1026 cm^{-1}; MS (CI^+, CH_4) *m/z* 301 (9), 283 (16), 241 (30), 239 (20), 223 (9), 211 (17), 197 (14), 145 (9), 127 (72), 85 (13), 69 (12), 61 (100); HRMS (CI^+, CH_4) 301.1638 ($C_{15}H_{25}O_6$ = 301.1651, Δ = 4.7 ppm); NMR data for **1b** in acetone-d_6 are given in Table I.

Methylation of 1b. Dowex 50W-X8 (5 g) was converted to its acid form by treatment with HCl. The excess acid and water were removed from the resin as completely as possible by washing with anhydrous methanol three times and then finally drying by vacuum filtration. **1b** (5 mg) and anhydrous methanol (5 mL) were added to the dry Dowex 50, and the mixture was stoppered and stirred at room temperature for 16 h (or alternatively refluxed for 2 h). The solution was filtered, and solvent was removed, affording 5.1 mg of relatively clean methyl ketal. The product was further purified by HPLC using a Maxsil 5 silica column (250 × 4.6 mm) with 40% EtOAc in hexanes, eluting with t_R of 10.3 min at 1.5 mL/min. 3-Methoxysyringolide 2 has the following characteristics: ^1H NMR ($CDCl_3$, 300 MHz), δ 0.9 (3 H, t, *J* = 8, H10), 1.3 (8 H, bm, H6,7,8,9), 1.6 (2 H, m, H5), 1.78 (1 H, m, H4a), 1.92 (1 H, m, H4b), 3.10 (1 H, s, H2), 3.25 (3 H, s, OMe3), 3.85 (1 H, dd, *J* = 10.8, 3.2, H5′a), 4.04 (1 H, dd, *J* = 10.8, 1, H5′b), 4.30 (2 H, s, H3′ and H4′), 4.43 (1 H, d, *J* = 10, H1′a), 4.65 (1 H, d, *J* = 10, H1′b); ^{13}C NMR δ 14.08 (C10), 22.63 (C9), 23.59 (C5), 29.08 (C7), 29.62 (C6), 31.78 (C8), 32.43 (C4), 48.47 (3-OMe), 59.50 (C2), 74.18 (C5′), 74.71 (C1′), 74.75 (C4′), 91.01 (C3′), 98.13 (C2′), 111.29 (C3), 171.80 (C1); INAPT correlations δ 3.1–171.8, 111.3, 98.1; 3.3–111.3, 59.5; MS (DCI^+, CH_4) *m/z* 315 (12), 285 (7), 284 (16), 283 (100), 282 (6), 281 (11), 239 (12), 215 (61), 211 (9), 183 (8), 179 (16), 127 (6), 41 (20); HRMS (CI^+, CH_4), 315.1819 ($C_{16}H_{27}O_6$ = 315.1808, Δ = 3.5 ppm).

Acetylation of 3-Methoxysyringolide 2. To 4.5 mg of **1b** in 50 μL of $CDCl_3$ was added 7.5 μL of dry methanol and 5 μL of acetyl chloride. After 5 min the solution was diluted by addition of 600 μL of $CDCl_3$ for NMR analysis. Formation of 3-methoxysyringolide 2 was complete within 30 min. The crude mixture was evaporated to dryness and then redissolved in 600 μL of $CDCl_3$ and treated with 25 μL of acetyl chloride and 10 μL of pyridine. Followed by NMR, the acetylation reaction required about 18 h at room temperature. The product was isolated from reagents by eluting with a 5-mL EtOAc rinse through a silica SEP-PAK. Evaporation of solvent left 5.5 mg of crude material. HPLC on Maxsil 5 with 15% EtOAc in hexanes (t_R = 12 min, μ = 1.5) afforded 2.5 mg of 4′-acetyl-3-methoxysyringolide 2: ^1H NMR ($CDCl_3$, 300 MHz), δ 0.9 (3, H, t, *J* = 8, H10), 1.3 (8 H, bm, H6,7,8,9), 1.5 (2 H, m, H5), 1.9 (2 H, m, H4), 2.10 (3 H, s, 4′-OAc), 3.12 (1 H, s, H2), 3.24 (3 H, s, OMe3), 3.90 (1 H, dd, *J* = 11, 2.9, H5′a), 4.11 (1 H, dd, *J* = 11, 1.4, H5′b), 4.37 (1 H, bd, H3′), 4.42 (1 H, d, *J* = 10.3, H1′a), 4.61 (1 H, d, *J* = 10.3, H1′b), 5.15 (1 H, d, *J* = 2.9, H4′); ^{13}C NMR δ 14.09 (C10), 20.93 (4′-OAc), 22.63 (C9), 23.60 (C5), 29.09 (C7), 29.61 (C6), 31.78 (C8), 32.34 (C4), 48.62 (3-OMe), 59.35 (C2), 71.87 (C5′), 74.58 (C1′), 76.71 (C4′), 88.86 (C3′), 98.50 (C2′), 111.52 (C3), 169.99 (4′-OAc), 171.61 (C1); INAPT correlation δ 5.15–170.0, 98.5, 88.9, 71.9; MS (CI^+, NH_3) *m/z* 374 (7), 343 (7), 342 (34), 326 (19), 325 (100), 257 (13), 77 (10), 72 (10), 60 (25), 58 (22), 45 (26), 44 (41); HRMS (CI^+, NH_3), 374.2195 ($C_{18}H_{32}NO_7$ = 374.217 88, Δ = 4.5 ppm).

Single-Crystal X-ray Structure Determination of 1b. Syringolide 2 (**1b**) crystallized as colorless plates from *n*-butyl acetate/hexane, and a single crystal with dimensions 0.05 × 0.2 × 0.55 mm was selected for analysis. All measurements were done on a Siemens R3m diffractometer using graphite-monochromated Cu Kα radiation (λ = 1.541 80 Å). Preliminary diffraction photographs displayed monoclinic symmetry, and accurate lattice constants of *a* = 8.0720(10) Å, *b* = 5.7850(10) Å, *c* = 16.773(4) Å, β = 90.39(2)° were determined by a least-squares fit of 25 diffractometer-measured 2θ values in the range of 35–45°. Systematic extinctions, crystal density, and optical activity were uniquely consistent with space group $P2_1$ with one molecule of composition $C_{15}H_{24}O_6$ forming the asymmetric unit. A total of 1247 reflections with 2θ ≤ 116° were collected using a variable speed θ:2θ scan. No absorption or decomposition corrections were made. After correction for Lorentz, polarization and background effects, 1173 of the 1247 unique reflections (94%) were judged observed ($|F_o| \geq 4\sigma|F_o|$). The structure was solved by direct methods and refined by full-matrix least-squares methods using the SHELXTL program set. Anisotropic thermal parameters were employed for the non-hydrogen atoms, and the

(22) Coll, J. C.; Mitchell, S. J.; Stokie, G. J. *Aust. J. Chem.* **1977**, *30*, 1859. (b) Pelletier, S. W.; Chokshi, H. P.; Desai, H. K. *J. Nat. Proc.* **1986**, *49*, 892.

hydrogen atoms rode on the heavy atoms with fixed geometry. The final agreement was $R = 0.051$. Additional crystallographic details are available. The authors have deposited atomic coordinates for syringolide 1 with the Cambridge Crystallographic Centre. The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK.

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Supplementary Material Available: ^{1}H, ^{13}C NMR, and APT spectra and COSY and PSCSCM 2D-NMR maps for syringolide 2 and ^{1}H and ^{13}C NMR for syringolide 1, 3-methoxysyringolide 2, and 4-acetyl-3-methoxysyringolide 2 (15 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.