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Note

Structural characterization of an O-linked tetrasaccharide from *Pseudomonas syringae* pv. tabaci flagellin

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ABSTRACT

The flagellin of *Pseudomonas syringae* pv. tabaci is a glycoprotein that contains O-linked oligosaccharides composed of rhamnosyl and 4,6-dideoxy-4-(3-hydroxybutanamido)-2-*O*-methylglucosyl residues. These O-linked glycans are released by hydrazinolysis and then labeled at their reducing ends with 2-aminopyridine (PA). A PA-labeled trisaccharide and a PA-labeled tetrasaccharide are isolated by normal-phase high-performance liquid chromatography. These oligosaccharides are structurally characterized using mass spectrometry and NMR spectroscopy. Our data show that *P. syringae* pv. tabaci flagellin is glycosylated with a tetrasaccharide, 4,6-dideoxy-4-(3-hydroxybutanamido)-2-*O*-methyl-Glc*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha-(1→, as well a trisaccharide, 4,6-dideoxy-4-(3-hydroxybutanamido)-2-*O*-methyl-Glc*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha-(1→, which was identified in a previous study.

The phytopathogenic bacterium *Pseudomonas syringae* is classified into at least 50 pathovars on the basis of its virulence toward different host plant species.[1,2] Flagellin is a protein that is an essential component of the flagellum filament of many bacteria including *P. syringae*. Flagellins of numerous plant pathogens including *P. syringae* pv. tabaci 6605, *P. syringae* pv. glycinea race 4, *P. syringae* pv. tomato DC 3000, and *Acidovorax avenae* are glycosylated.[3,4] Recently we demonstrated that the flagellin of *P. syringae* pv. glycinea, but not *P. syringae* pv. tabaci, causes hypersensitive cell death in tobacco cells,[3] even though the flagellins from both pathovars have identical amino acid sequences. Such results when taken together with the results of biological and mutational studies have led to the suggestion that flagellin glycosylation has a role in *P. syringae* virulence and host specificity.[5–8] In previous studies we showed that six serine residues (S143, S164, S176, S183, S193, and S201) are glycosylated in the flagellins from *P. syringae* pv. tabaci and *P. syringae* pv. glycinea.[7,9] We reported that S201 of the flagellin from *P. syringae* pv. tabaci and *P. syringae* pv. glycinea is O-glycosylated with a trisaccharide.[9] To determine if structurally different oligosaccharides are attached to the other serine residues, we subjected *P. syringae* pv. tabaci flagellin to hydrazinolysis and labeled the released oligosaccharides with 2-aminopyridine (PA). A PA-labeled trisaccharide and a PA-labeled tetrasaccharide were isolated and structurally characterized by mass spectrometry (MS) and NMR spectroscopy.

In a previous study we showed that *P. syringae* pv. tabaci flagellin contained L-Rha and a modified 4-amino-4,6-dideoxyglucose.[9] We confirmed that the Rha was L using GC and GC–MS analysis of the trimethylsilylated (TMS) (*S*)-(+)-2-butyl glycosides and by reversed-phase HPLC analysis of the *S*-(+)-2-*tert*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid glycose derivatives[10] (data not shown). We also confirmed the presence of the acid-labile dideoxy sugar[11,12] by treating the flagellin with anhydrous hydrogen fluoride (HF). This procedure cleaves the glycosidic bond of dideoxyglycoses with limited degradation of the released glycose.[12] The products generated by HF treatment were reduced with $NaBD_4$, O-peracetylated, and then analyzed by GC–MS. The electron- impact mass spectrum (EIMS) of the acetylated alditol derivative of the 4,6-dideoxyglycose contained fragment ions at *m/z* 118 and 347, indicating that an *O*-methyl and a 3-hydroxybutanamido group are linked to C-2 and C-4, respectively (Fig. 1). These results, together with chemical ionization MS (CIMS) analysis, confirmed that the glycose was 4,6-dideoxy-4-(3-hydroxybutanamido)-2-*O*-

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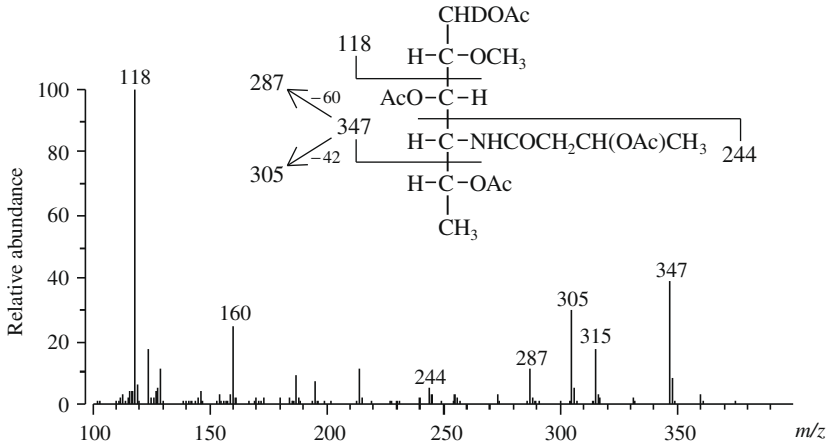

Figure 1. EIMS spectrum of the alditol acetate derivative of 4,6-dideoxy 4-(3-hydroxybutanamido)-2-*O*-methylglucopyranose (mVio). The origins of the predominant primary and secondary fragment ions are shown.

methylglycopyranose. This dideoxyglycose will be referred to as modified viosamine (mVio) as it is structurally related to 4,6-dideoxy-4-(3-hydroxy-3-methylbutanamido)-2-*O*-methylglucopyranose (viosamine) that has been identified in other bacteria.[13] Rhamnose and mVio were also detected when the *P. syringae* pv. tabaci flagellin was treated with methanolic HCl, and the resulting methyl glycosides were then O-peracetylated and analyzed by GC and GC–MS (data not shown). Thus, we conclude that *P. syringae* pv. tabaci flagellin glycans are composed of only L-Rha and mVio.

The O-linked oligosaccharides of *P. syringae* pv. tabaci flagellin were released by hydrazinolysis and labeled at their reducing ends with PA. Two quantitatively major and one minor peaks were detected when the labeled products were analyzed by normal-phase high-performance liquid chromatography (HPLC) with fluores-

Table 1
NMR signal assignment of compound **1** and **2**

Residue	Position	Compound **1**				Compound **2**			
		^{1}H NMR			^{13}C NMR	^{1}H NMR			^{13}C NMR
		δ (ppm)	Multiplicity	J (Hz)	δ (ppm)	δ (ppm)	Multiplicity	J (Hz)	δ (ppm)*
L-Rhamnitol **R**	1	3.635	dd	14.5, 3.1	43.87	3.641	nd	nd	43.8
	1	3.588	dd	14.5, 6.0		3.553	nd	nd	
	2	3.949	ddd	6.6. 6.0, 3.1	78.86	3.951	nd	nd	79.2
	3	3.967	dd	6.6, 2.2	70.28	3.986	dd	6.0, 2.3	70.2
	4	3.478	dd	7.4, 2.2	75.59	3.489	dd	7.4, 2.3	75.7
	5	3.883	dq	7.4, 6.3	68.76	3.882	dq	7.4, 6.3	68.8
	6	1.243	d	6.3	20.25	1.256	d	6.3	20.2
α-L-Rha*p* **A**	1	4.911	d	1.6	101.32	4.996	br s		100.2
	2	4.129	dd	3.1, 1.6	72.00	3.950	d	3.3	80.5
	3	3.786	dd	9.7, 3.1	81.30	3.834	dd	9.8, 3.3	71.8
	4	3.543	dd	9.7, 9.6	72.72	3.412	dd	9.7, 9.7	73.7
	5	3.733	dq	9.6, 6.3	71.11	3.686	dq	9.7, 6.4	71.2
	6	1.063	d	6.3	18.20	1.042	d	6.4	18.3
α-L-Rha*p* **E**	1	—				4.966	d	1.1	103.9
	2	—				4.255	dd	3.1, 1.1	71.3
	3	—				3.878	dd	9.7, 3.1	81.2
	4	—				3.587	nd	nd	72.8
	5	—				3.715	nd	nd	71.2
	6	—				1.256	d	6.3	18.4
4,6-Dideoxy-2-*O*-Me-glucopyranosyl **T**	1	4.655	d	8.0	105.43	4.710	d	8.1	105.2
	2	3.118	dd	9.5, 8.0	84.87	3.120	dd	9.1, 8.1	85.0
	3	3.523	dd	9.7, 9.5	74.44	3.508	nd	nd	74.3
	4	3.609	dd	10.0, 9.7	58.27	3.611	nd	nd	58.4
	5	3.529	dd	10.0, 6.2	72.54	3.528	nd	nd	72.7
	6	1.204	d	6.2	18.66	1.189	d	6.3	18.6
	2-*O*-Me	3.605	s		61.68	3.613	s		61.8
3-Hydroxy-butanamido **HBA**	1′	—			176.19	—			nd
	2′	2.432	dd	14.1, 5.7	46.85	2.399	dd	12.8, 5.6	46.9
	2′	2.409	dd	14.1, 7.7		2.423	dd	12.8, 7.4	
	3′	4.185	ddq	7.7, 5.7, 6.3	66.71	4.174	ddq	7.4, 5.6, 6.3	66.7
	4′	1.224	d	6.3	23.75	1.217	d	6.3	23.8

The signals of PA were assigned as follows: H-3 (δ 6.678 ppm, d, 8.2 Hz), H-4 (δ 7.562 ppm, ddd, 8.2, 7.0, 1.7 Hz), H-5 (δ 6.697 ppm, dd, 7.0, 5.2 Hz) and H-6 (δ 7.968 ppm, dd, 5.2, 1.7 Hz), C-2 (δ 160.36 ppm), C-3 (δ 114.94 ppm), C-4 (δ 140.45 ppm), C-5 (δ 110.72 ppm), and C-6 (δ 148.36 ppm).
* ^{13}C data were obtained from HSQC analysis. nd, not determined; br s, broad singlet; s, singlet.

cence detection. These peaks were collected and then analyzed by positive-ion mode electrospray ionization mass spectrometry (ESIMS) and by NMR spectroscopy. The first peak co-eluted with PA and was not further analyzed. The ESIMS spectrum of the second peak (compound **1**), together with ^{1}H and ^{13}C NMR spectroscopic data (see Table 1 and Figs. 2A and 3A), is consistent with the sequence *N*-[4,6-dideoxy-4-(3-hydroxybutanamido)-2-*O*-methyl-Glc*p*-(1→3)-α-L-Rha*p*-(1→2)-L-rhamnitol]-2-aminopyridine (Fig. 2A). A trisaccharide with this sequence has previously shown to be attached to serine 201 of the flagellins from *P. syringae* pv. tabaci 6605 and *P. syringae* pv. glycinea race 4.[9]

The ESIMS spectrum of the third peak (compound **2**) contained an ion at *m/z* 780 that corresponds to the $[M+H]^{+}$ ion of an oligosaccharide that is composed of one mVio, two rhamnosyl, one rhamnitol, and one PA unit. The product ion spectrum of the protonated ion at *m/z* 780 contained Y_n type ions[14] at *m/z* 535, 389, and 243. Such results are consistent with the sequence mVio-Rha-Rha-rhamnitol-PA. The primary structure of compound **2** was determined by ^{1}H NMR spectroscopy. The complete assignments of chemical shifts are given in Table 1. Three signals were detected in anomeric region (Fig. 2B). The broad signals at δ 4.996 and 4.966 were assigned to H-1 of the internal Rha*p* residues **A** and **E**, respectively. These chemical shifts and their small coupling constants were consistent with α-linkages.[15] The H-1 resonance of the non-reducing terminal mVio, **T** was at δ 4.710, and its large coupling (*J* 8.1 Hz) is consistent with β-linkage[13,15] (Fig. 2B, Table 1). Other signals of these residues were assigned on the basis of the correlations in the DQF-COSY and TOCSY spectra (Table 1). Some of the ^{1}H signals of L-rhamnitol residue **R** could not be unambiguously assigned because of contaminating material. Due to the small amount of compound **2** available, a ^{13}C NMR spectrum was not obtained; rather the ^{13}C chemical shifts were deduced from an HSQC experiment (Fig. 3B, Table 1). The two H-1 protons of L-rhamnitol residue derived from the former reducing end of the oligosaccharide released by hydrazinolysis were assigned in the HSQC spectrum (Fig. 3B, Table 1). The second L-Rha*p* residue **A** was deduced to be linked to O-2 of the PA-labeled L-rhamnitol, **R** by a HMBC correlation of H-1–C-2 and the low-field shift of C-2 (δ 79.2) (Fig. 3B, Table 1). The third L-Rha*p* residue **E** was shown to be linked to O-2 of the second L-Rha*p* residue **A** by HMBC correlations of H-1–C-2 and the low-field shift of C-2 (δ 80.5) (Fig. 3B, Table 1). The structure of the non-reducing terminal glycosyl residue **T** was identified as 4-amino-4,6-dideoxyglucose by comparison of the ^{1}H and ^{13}C NMR data (Table 1) with those of 4,6-dideoxy-4-(3-hydroxy-3-methylbutanamido)-2-*O*-methylglucopyranose.[13] The mVio was shown to be linked to O-3 of Rha*p* residue, **E** by HMBC correlations of mVio H-1 to L-rhamnosyl residue **E** C-3 and the low-field shift of C-3 (δ 81.2, Fig. 3B, Table 1). Modification of the viosamine residue by O-methylation on C-2 was confirmed by a HMBC experiment. Attachment of the 3-hydroxybutyl group to viosamine through amide linkage was deduced from the high similarity of ^{1}H and ^{13}C NMR chemical shifts to those of compound **1** although HMBC correlation of the carbonyl to H-4 of mVio was not observed for compound **2**. We conclude that compound **2** is *N*-[4,6-dideoxy-4-(3-hydroxybutanamido)-2-*O*-methyl-Glc*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-L-rhamnitol]-2-aminopyridine (Fig. 2B).

Our present results demonstrate that *P. syringae* pv. tabaci flagellin is O-glycosylated with a trisaccharide (compound **1**) and a tetrasaccharide (compound **2**). Six serine residues (S143, S164, S176, S183, S193, and S201) are known to be glycosylated in the flagellin protein from *P. syringae* pv. tabaci and *P. syringae* pv. glycinea.[7,9] At least one of these residues (S201) is glycosylated with the trisaccharide (compound **1**).[9] The ratios of a trisaccharide (compound **1**) and a tetrasaccharide (compound **2**) on LC by fluorescence detection were about 5:1, indicating that about five serine residues were glycosylated with a trisaccharide (compound **1**).

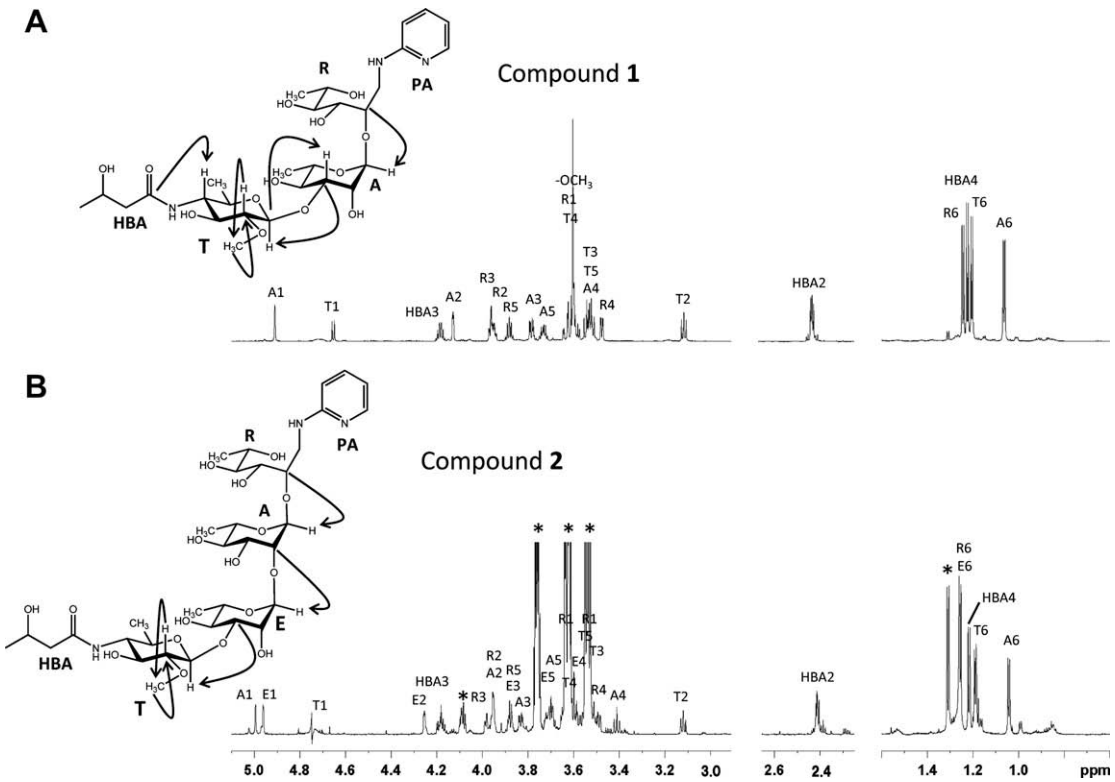

Figure 2. ^{1}H NMR and chemical structure of compounds **1** and **2**. (A) compound **1**; (B) compound **2**. Arrows show HMBC correlations indicating the linkage pattern between sugars. Letters **A**, **E**, **R**, **T,** and **HBA** on the spectra show sugars and a modification group on the oligosaccharides as indicated in Table 1. Numbers after the letters show positions on each sugar. Asterisks show peaks due to contaminating material.

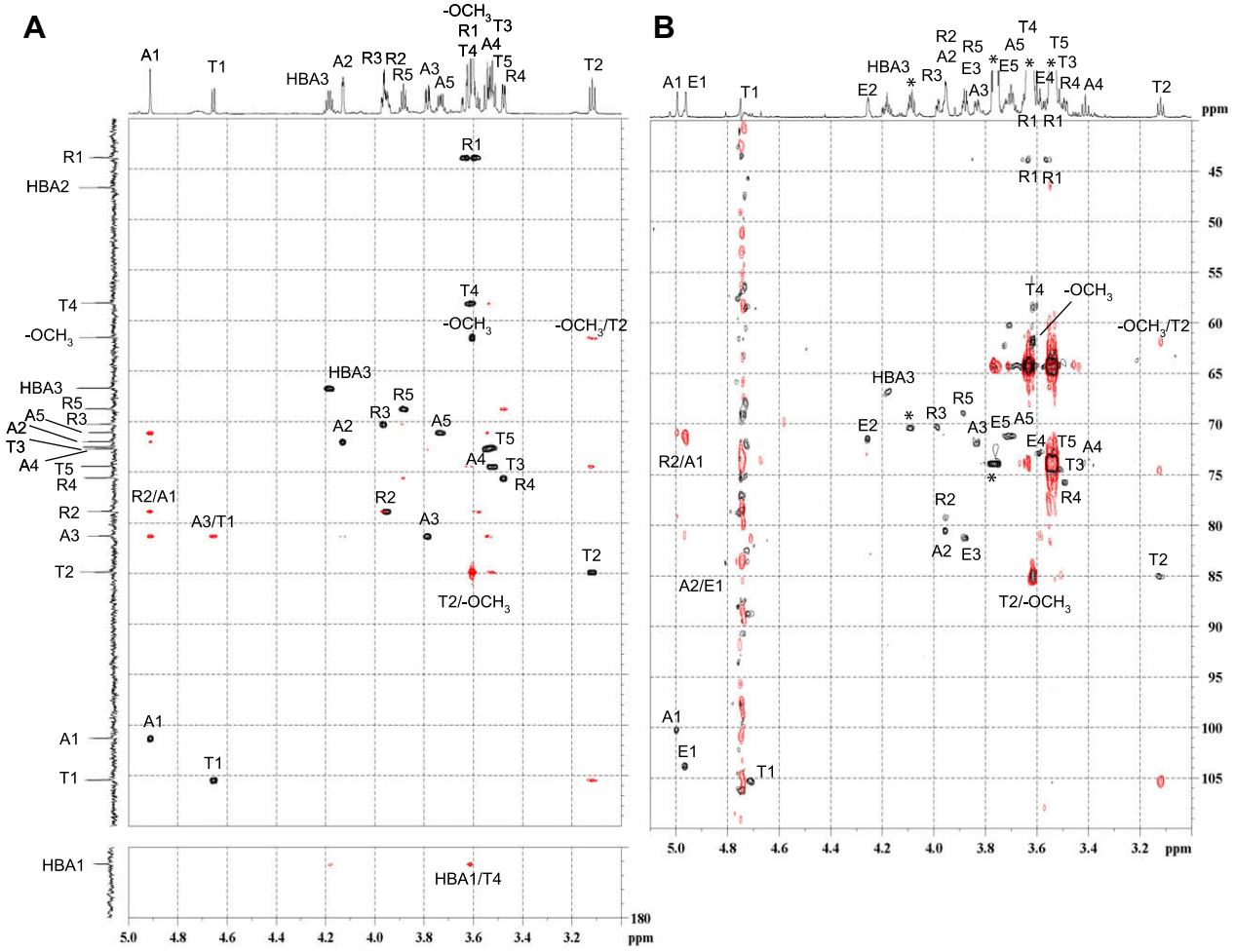

Figure 3. Partial overlap of HSQC (black) and HMBC (red) spectra of compounds **1** and **2**. (A) Compound **1**; (B) compound **2**. Letters **A**, **E**, **R**, **T,** and **HBA** on the spectra show sugars and a modification group on the oligosaccharides as indicated in Table 1. Numbers after the letters show positions on each sugar. Asterisks show peaks due to contaminating material.

Elucidating the glycosylation patterns of these pathovars will require the complete structural characterization of the glycopeptides generated from the flagellin proteins. The glycans of the flagellin of *P. syringae* pv. tabaci contain L-Rha, whereas the *P. syringae* pv. glycinea flagellin contains D and L Rha.[9] Thus, the possibility cannot be discounted that the chirality of the Rha residues together with the glycosylation pattern and the degree of polymerization of the flagellin oligosaccharide side chains are factors that determine host specificity and virulence.

1. Experimental

(*S*)-(+)-2-butanol was purchased from Sigma–Aldrich Japan (Tokyo, Japan). TMSI-H (trimethylsilyling reagent) was obtained from GL Science (Tokyo, Japan). D_2O was obtained from Kanto Kagaku (Tokyo, Japan). (*R*)-(−)-2-butanol, 5% methanolic hydrogen chloride solution, and other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

Pseudomonas syringae pv. tabaci 6605 was maintained in King's B medium at 27 °C.[3] It was grown in LB medium containing 10 mM $MgCl_2$ for 48 h at 25 °C.[3] The cells were harvested by centrifugation, resuspended in one-third volume of minimal medium [50 mM potassium phosphate buffer, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, and 1.7 mM NaCl (pH 5.7)] supplemented with mannitol and fructose (10 mM each), and then kept for 24 h at 23 °C. Flagellin was purified by the method of Taguchi et al.[3]

GC–MS was performed with a JEOL JMS-DX303HF mass spectrometer interfaced with an Agilent Technologies 6890N gas chromatograph. GC-chemical ionization MS (GC–CIMS) was performed with ammonia as the reagent gas and a source temperature at 180 °C. GC–electron impact ionization MS (GC–EIMS) was performed with an ionization current of 70 eV and an ion source temperature of 180 °C. ESIMS spectra were recorded with a LCQ DUO mass spectrometer (Thermo Quest, Tokyo, Japan) operated in the positive-ion mode with a spray voltage of 4.55 kV, a capillary voltage of 3.1 V, and a capillary temperature of 180 °C. Solutions of PA oligosaccharides in 50% aq (v/v) MeOH containing 0.5% (v/v) HCOOH were infused into the electrospray source at 5 μL min^{-1} with a syringe pump. Spectra were obtained between *m/z* 150 and 2000 with a step size of 0.1 amu and a dwell time of 25 ms.

HF treatment was performed at the Peptide Institute (Osaka, Japan). Isolated flagellin (10 mg) was dried in vacuo over P_2O_5 for 16 h. The dry residue was dissolved in anhyd HF (1 mL) in a HF apparatus and kept for 3 h at room temperature.[12] The HF was removed under diminished pressure, and the last traces of HF were removed by co-distillation with Et_2O.[12] The resulting monosaccharides were reduced with $NaBD_4$ and converted into their alditol acetate derivatives.[16]

The monosaccharide compositions of flagellin glycans were determined by GC analysis of the acetate derivatives of the methyl glycosides. Samples were dried by stream of dry air, suspended in 250 μL of 5% methanolic HCl, and heated at 80 °C overnight.

tert-Butanol (200 μL) was added to the tube, and the methanolic HCl was removed by stream of dry air. The methyl glycosides were acetylated with Ac_2O and pyridine (1:1 v/v) for 20 min at 121 °C. The acetylated glycosides were separated by GC–MS on a DB-1 column (15 m × 0.25 mm) as described.[16] The absolute configuration of rhamnose was determined by GC and GC–MS analyses of the trimethylsilylated (*S*)-(+)-2-butyl glycosides of L-rhamnose as described.[17] The retention time of D-rhamnose, for which a standard was not available, was determined by chromatography of the trimethylsilylated (*R*)-(−)-2-butyl glycosides of L-rhamnose. Absolute configuration of rhamnose was also determined by fluorescent labeling with (*S*)-(+)-2-*tert*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid (TBMB), followed by separation of the fluorescent-labeled rhamnose by reversed-phase HPLC.[10]

Selective hydrazinolysis was used to release O-linked oligosaccharides from flagellin of *P. syringae* pv. tabaci 6605. Hydrazinolysis was performed at Masuda Chemicals Inc. Ltd (Kagawa, Japan). Briefly, solutions of flagellin (10–15 mg) were concentrated to dryness using a vacuum centrifuge and then stored for 16 h under vacuum over P_2O_5. The material was then subjected to hydrazinolysis for 5 h at 60 °C according to the method of Patel et al.[18] The released oligosaccharides were labeled with PA according to the method of Hase et al.[19]

PA-labeled oligosaccharides from flagellin were analyzed by normal-phase HPLC using fluorescence (λ_{ex} = 320 nm, λ_{em} = 400 nm) detection. Normal-phase HPLC was performed using a TOSOH Amide-80 column (4.6 × 250 mm, TOSOH, Tokyo, Japan) eluted at 0.5 mL min^{-1} at 40 °C. The column was eluted as follows: eluent A, 90% (v/v) of 50 mM ammonium formate (pH 4.5) and 10% CH_3CN; eluent B, 10% of 50 mM ammonium formate (pH 4.5) and 90% CH_3CN, and a linear gradient of eluent B from 80% (v/v) to 70% (v/v) in 20 min. The fluorescent-positive fractions (retention time 12.5 min and 15.0 min for compounds **1** and **2,** respectively) were collected manually, concentrated by rotary evaporation, and then freeze-dried. The purified oligosaccharides were analyzed by ESIMS, ^1H and ^{13}C NMR spectroscopies, and their glycosyl residue compositions were determined by GC analysis.

The purified PA-labeled oligosaccharides were dissolved in 99.96% isotopically enriched D_2O and then freeze-dried. The samples were dissolved in 99.96% enriched D_2O. 1D ^1H NMR and 2D-DQFCOSY, 2D-TOCSY, HSQC, and HMBC spectra were recorded at 800.13 MHz with a Bruker Avance 800 spectrometer with a three-channel inverse ($^1H/^{13}C$ [^{15}N]) CryoProbe (Bruker Biospin, Karlsruhe, Germany) at a temperature of 303 K. 1D ^{13}C NMR spectra were recorded at 125.77 MHz on a Bruker Avance 500 spectrometer with a dual ^{13}C [^1H] CryoProbe (Bruker Biospin) at 303 K. The methyl signals of 2-methyl-2-propanol, H δ 1.230 and C δ 31.30 were used as references for ^1H and ^{13}C chemical shifts.

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