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Novel acetylated α-cyclosophorotridecaose produced by *Ralstonia solanacearum*

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Abstract—α-Cyclosophorotridecaose (α-C13) produced by *Ralstonia solanacearum* is isolated by trichloroacetic acid treatment and subjected to various chromatographic techniques. Here, we report for the first time that *R. solanacearum* produces acetylated α-C13. Structural analyses of the acetylated α-C13 were performed with 1D or 2D NMR spectroscopy, MALDI-TOF MS and HPLC. The results show that the α-C13 is substituted by mainly one acetyl residue at the C-6 position of the glucose unit.

Keywords: Acetylated α-cyclosophorotridecaose; Periplasmic cyclic glucan; *Ralstonia solanacearum*; Structural analysis

1. Introduction

Ralstonia solanacearum is the causative pathogen of plant wilt. It generally enters a plant through the roots, penetrates the xylem, systemically colonizes the stem, and causes wilt symptoms.[1] It is considered to be a serious disease because the damaged hosts[2] are crop plants such as tomato, potato, tobacco, peanuts, and bananas, as well as many native plant species in the warm temperate and tropical regions of the world, causing great economic losses worldwide.

Generally, the cell-surface carbohydrates of this microorganism are known to be involved in bacterium–plant interactions[3] in pathogenesis, and the carbohydrates produced by *R. solanacearum* are extracellular polysaccharides (EPSs), lipopolysaccharides (LPSs), osmoregulated periplasmic glucans (OPGs), etc. EPSs are acidic heteropolymers involving *N*-acetylgalactosamine, *N*-acetylgalactosaminuronic acid, and rhamnose, and they play an important role in the pathogenicity of *R. solanacearum*.[4,5] LPSs are the major components of the outer membrane of Gram-negative bacteria and have long been demonstrated to be biologically active in the interaction with the host plants.[6,7] α-Cyclosophorotridecaose (α-C13), general constituent of the periplasmic space of Gram-negative bacteria, is a cellular cyclic glucan consisting of 13 glucose residues with one α-(1→6) linkage and 12 β-(1→2) linkages.[8–10]

In some bacterial species, OPGs are modified by non-glucose residues that originate from the membrane phospholipids (phosphoglycerol, phosphoethanolamine, and phosphocholine)[11–13] or from intermediate metabolism (acetyl, succinyl, and methylmalonyl).[14–16] Recently, studies on the presence of the novel glycerophosphorylated α-cyclosophorohexadecaose (α-C16) from *Xanthomonas campestris*[17] and the succinylated cyclic β-(1→2) glucan from *Brucella abortus*[15] have been also reported. Even though the presence of acetylated glucans in OPGs has been known in *Erwinia chrisanthemi*[18] and *Rhodobacter sphaeroides*,[16] no study is reported in *Ralstonia* species as yet.

In this study, we first report that acetylated α-cyclosophorotridecaose (α-C13) is synthesized by the plant pathogenic bacterium, *R. solanacearum*. The exact structure of the novel acetylated α-C13 was elucidated using 1D or 2D NMR spectroscopy, MALDI-TOF MS and HPLC analyses.

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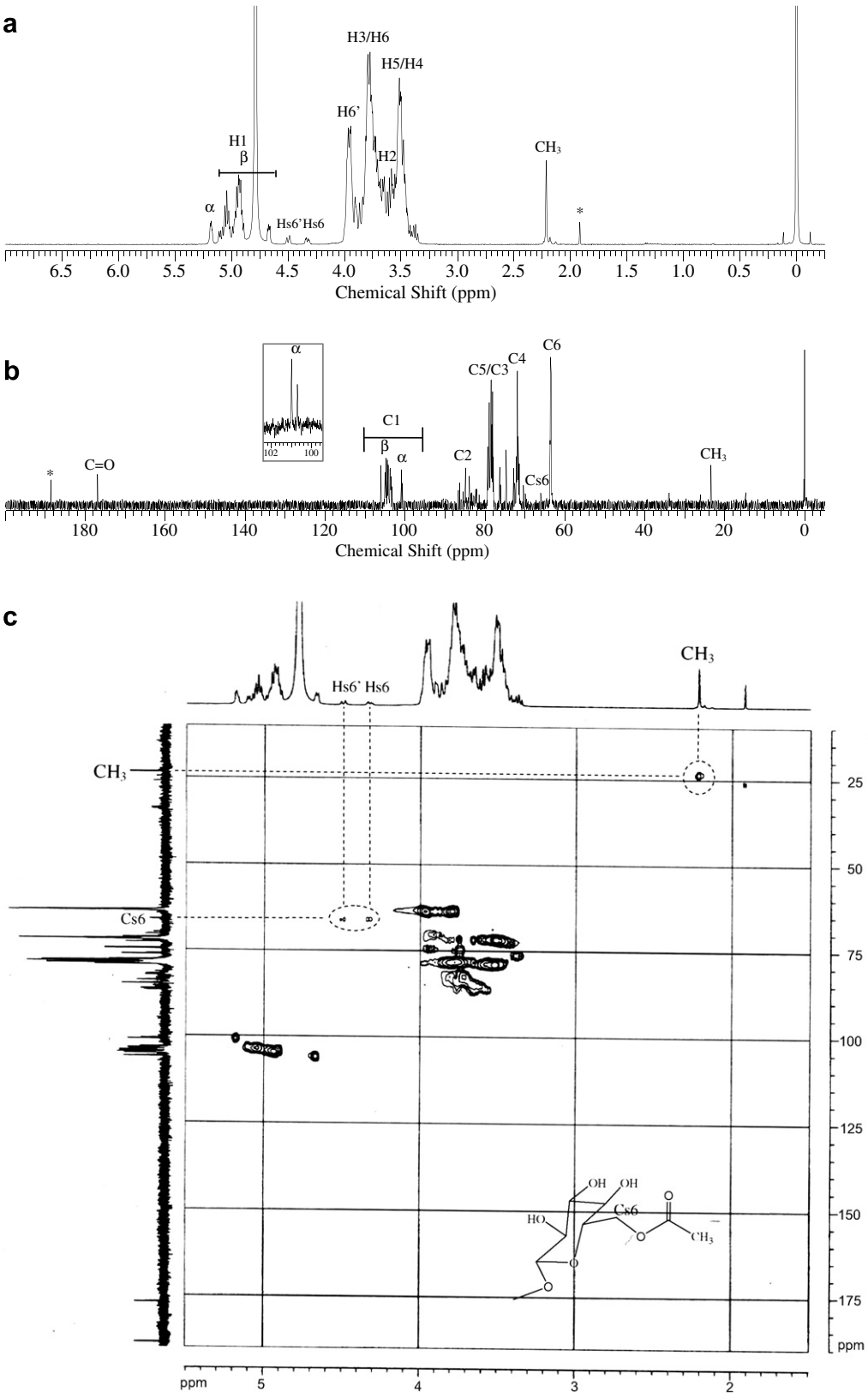

Figure 1. (a) ^{1}H NMR spectrum of the purified α-C13 of *R. solanacearum*. The asterisk (∗) peak at 1.92 ppm is an impurity in the sample. (b) ^{13}C NMR spectrum of the purified α-C13 of *R. solanacearum*. The inset spectrum from 100.00 to 102.00 ppm is enlarged to examine the resonances of C-1 carbons of α-glucose residue. The asterisk (∗) peak at 188.61 ppm is an impurity in the sample. (c) ^{1}H–^{13}C HSQC spectrum of the purified α-C13 from *R. solanacearum*. The H_s-6′, H_s-6 and C_s-6 indicate that the protons and the carbons of the glucose residues with acetyl ester are linked at position 6, respectively. The inset indicates the glucose acetylated at C_s-6.

2. Results and discussion

We analyzed the structure of acetylated α-cyclosophorotridecaose (α-C13) from *R. solanacearum* with various spectroscopic or spectrometric methods such as Fourier-transform infrared (FT-IR), ^{1}H NMR, ^{13}C NMR, ^{1}H–^{13}C-heteronuclear single quantum correlation (HSQC), ^{1}H–^{13}C-heteronuclear multiple bond correlation (HMBC) spectroscopy, MALDI-TOF mass spectrometry and HPLC.

In the ^{1}H NMR spectrum (Fig. 1a), the characteristic peaks between 4.30 and 4.60 ppm are assigned to the two protons of the glucose residues substituted with an acetyl ester at position 6. A singlet at 2.22 ppm is determined to be the protons of the acetyl group. The peak at 5.19 ppm is indicative of the anomeric proton (H-1) of the α-glucose residue, and the peaks from 4.66 to 5.11 ppm are indicative of the H-1 protons of the glucose residues engaged in β-(1→2) linkages. Other proton peaks of the glucose units are also designated as shown in Figure 1a. In the ^{13}C NMR spectrum (Fig. 1b), a characteristic peak at 176.93 ppm is assigned to the carbon resonance of the C═O group from the acetyl substituent, and a peak at 23.51 ppm is indicative of carbon of CH_3 group from acetyl substituents. We also assigned the C-1 carbons of the α-glucose residue as 100.98 ppm and 100.70 ppm, and the two peaks are due to the different C-1 carbons of the α-glucose residue of acetylated and unsubstituted cyclic OPGs. Furthermore, a peak at 66.07 ppm is indicative of the carbons of the glucose residues substituted with an acetyl ester at position 6. The resonances in the vicinity of 103.24–106.00 ppm are indicative of anomeric carbons, and no peak in the range of 92.00–96.00 ppm indicates the presence of nonreducing terminal glycosyl groups of the cyclic backbone structure in this molecule. The C-2 resonances were assigned in the ranges from 81.48 to 86.71 ppm and the peaks in the range of 76.10–76.35 ppm were also assigned as the upfield-shifted C-2 carbons not involved in the glycosidic linkages. The C-4 carbons of the β-glucose residues were assigned to the peaks ranging from 71.45 to 72.87 ppm, the C-3 and C-5 carbons of the β-glucose residues to the peaks ranging from 77.95 to 79.34 ppm, and the C-3 and C-5 carbons of the α-glucose residues to the peaks at 74.70 and 74.73 ppm, respectively. All the C-6 resonances are assigned to the range of 63.47–63.71 ppm except for downfield-shifted C-6 carbons involving

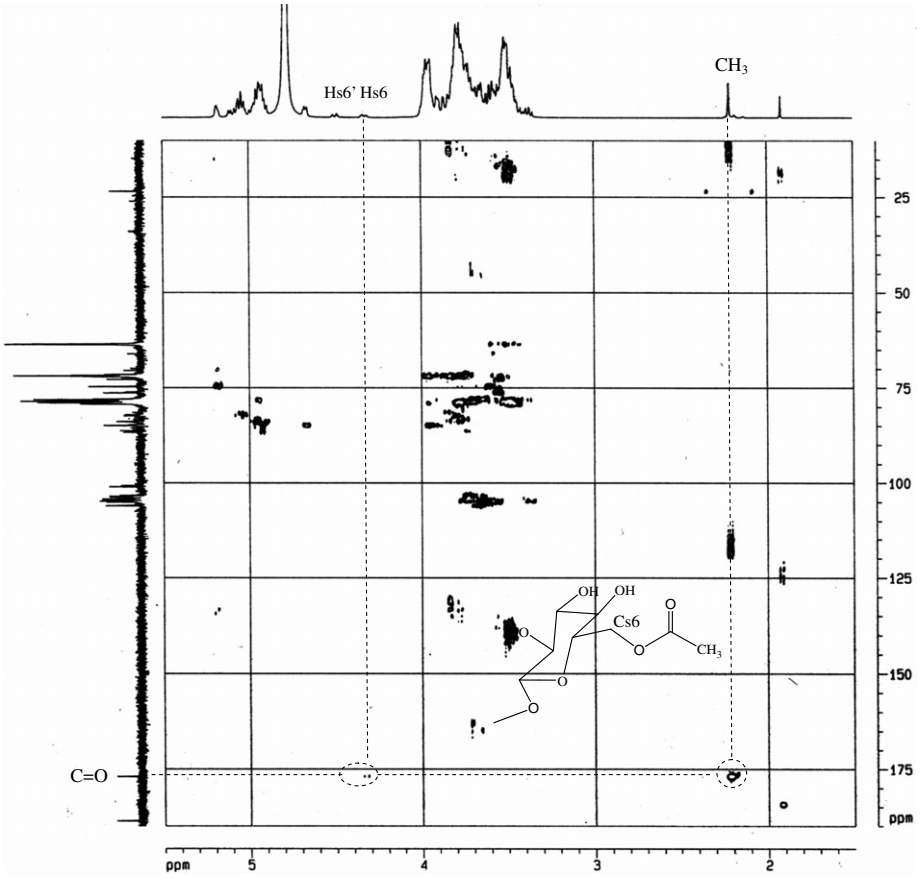

Figure 2. ^{1}H–^{13}C HMBC spectrum of the purified α-C13 from *R. solanacearum*. The H_s-6′ and H_s-6 indicate the protons of the glucose residues with acetyl ester linked at position 6. The inset indicates the glucose acetylated at C_s6.

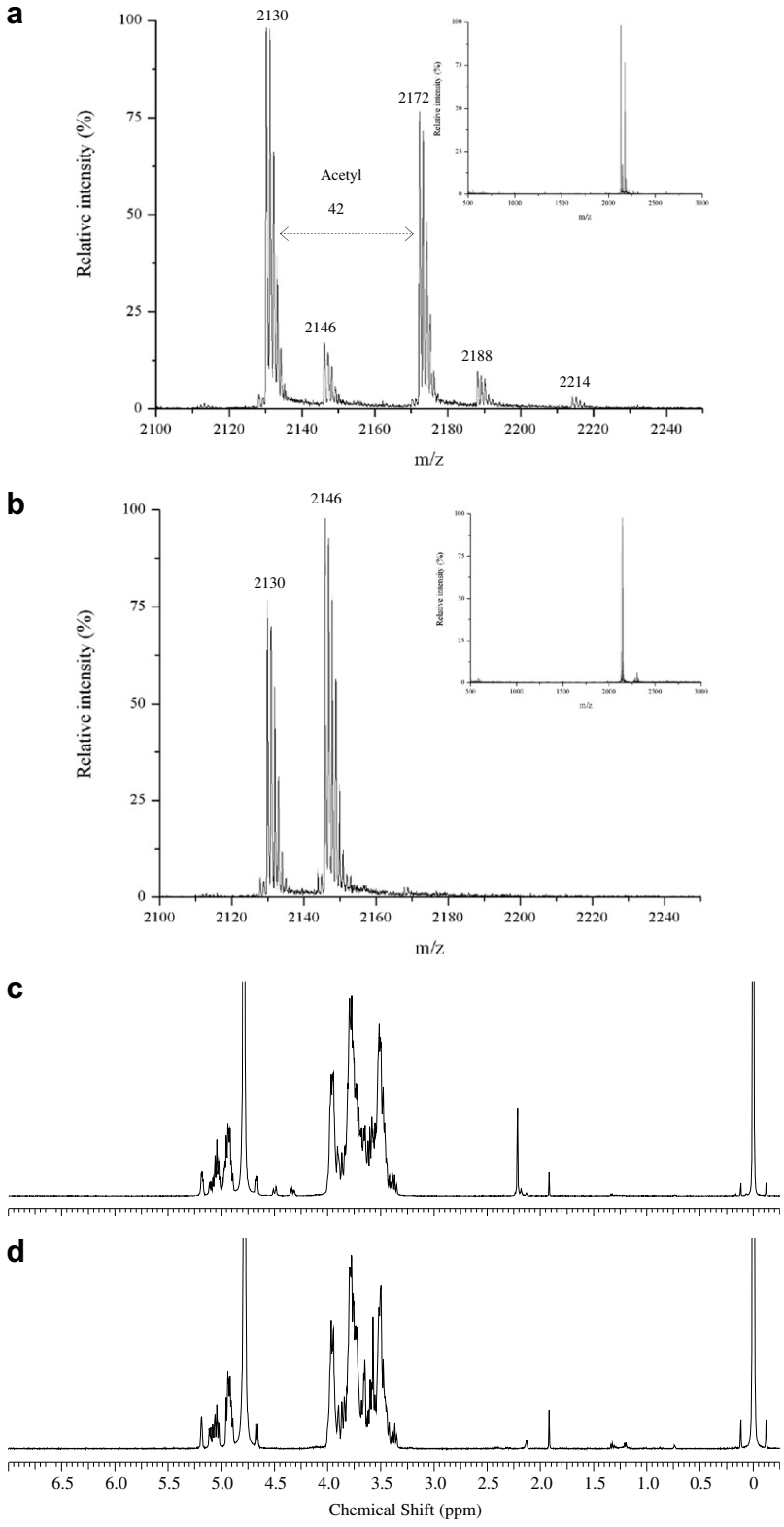

Figure 3. MALDI-TOF spectra and ^{1}H NMR spectra before and after mild alkaline treatment of α-C13 from *R. solanacearum*. (a) MALDI-TOF spectrum of native α-C13 from *R. solanacearum*. Each mass unit at *m*/*z* 2130, 2146, 2172, 2188, and 2214 corresponds to [unsubstituted α-C13 + Na^+ + H^+], [unsubstituted α-C13 + K^+ + H^+], [one acetylated α-C13 + Na^+ + H^+], [one acetylated α-C13 + K^+ + H^+], and [two acetylated α-C13 + Na^+ + H^+], respectively. The inset is the spectrum with full range from *m*/*z* 500 to 3000. (b) MALDI-TOF spectrum of 0.1 M KOH-treated α-C13 from *R. solanacearum*. The inset is the spectrum with full range from *m*/*z* 500 to 3000. (c) ^{1}H NMR spectrum of native α-C13 from *R. solanacearum*. (d) ^{1}H NMR spectrum of 0.1 M KOH-treated α-C13 from *R. solanacearum*. In (c) and (d), the peaks at 1.92 and 2.13 ppm are due to impurities in the sample.

α-(1→6) linkages at 69.92 and 70.43 ppm. The two peaks at 69.92 and 70.43 ppm are also due to the different C-6 carbons of the α-glucose residues of acetylated and unsubstituted α-C13. In the HSQC spectrum (Fig. 1c), the C_s-6 carbons shifted downfield to 66.07 ppm compared with C-6 carbons (63.47–63.71 ppm) with the free OH group, at which point the H_s-6′ and H_s-6 resonances attached to the C_s-6 carbons were correlated. Also, the correlations at 2.22 ppm for ^1H and at 23.51 ppm for ^{13}C were assigned.

HMBC analysis was performed to confirm the substituted position of the acetyl group on the glucose residue of α-C13. As shown in Figure 2, a cross-peak (2.22/176.93) by the two-bond correlation between protons of the CH_3 group and carbons of the C=O group was detected, and also the three-bond correlations between the H_s-6′ or H_s-6 resonances and the same carbons were weakly observed. As a result, the 2D NMR data clearly show that the acetyl residue is attached to the unsubstituted α-C13 of *R. solanacearum*. The ^1H or ^{13}C NMR peak assignments of unsubstituted α-C13 of *R. solanacearum* are based on the previous report.[8]

The presence of the acetyl group attached to the α-C13 was also confirmed by the FT-IR spectroscopy, where the characteristic C=O stretching group corresponding to the acetyl group within the acetylated α-C13 was detected around 1716 cm^{-1} in the FT-IR spectrum (data not shown).

Figure 3a shows the MALDI-TOF spectrum of the acetylated α-C13. Molecular ions at *m*/*z* 2130 and 2172 were detected, which indicate the [unsubstituted α-C13 + $Na^+ + H^+$] and [one acetylated α-C13 + $Na^+ + H^+$], respectively. The mass increments are 42, which corresponds to the *O*-ester-linked acetyl residue. Also, the other sodium-cationized molecular ion at *m*/*z* 2214 corresponding to [two acetylated α-C13 + $Na^+ + H^+$] was weakly detected. The presence of *O*-ester-linked[16a] acetyl residues within α-C13 was confirmed after mild alkaline treatment to that of a substituted α-C13 fraction where only the unsubstituted α-C13 was observed as shown in Figure 3b and d. Figure 3b shows that the molecular ions of the acetylated α-C13 at *m*/*z* 2172 and 2214 disappears. In the ^1H NMR spectrum (Fig. 3d), the characteristic peaks (4.30–4.60 ppm) of the H_s-6′ and H_s-6 protons in acetylated α-C13 and the single peak (2.22 ppm) of the acetyl protons disappeared after the alkaline treatment.

As a quantitative analytical tool, we used high-performance liquid chromatography (HPLC) with RI detector. Figure 4 shows the HPLC elution profile of α-C13 in which the chromatogram shows the presence of two peaks with retention times of 13.0 min (A) and 16.1 min (B). After the mild alkaline treatment, we confirmed that the peak A with retention time at 13.0 min disappeared, whereas the one at 16.1 min remained (data not shown). Therefore, the HPLC profile suggests

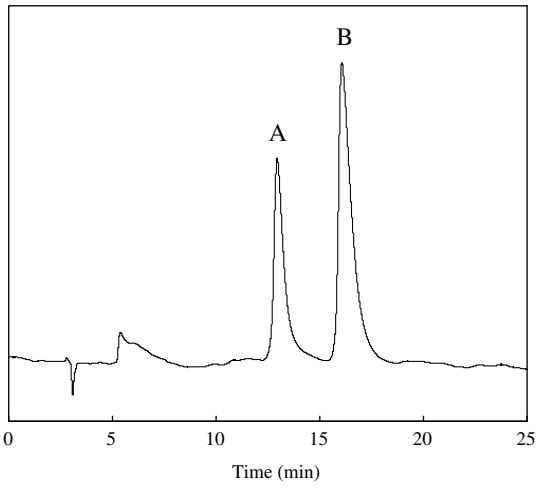

Figure 4. HPLC elution profile of native α-C13 from *R. solanacearum*. A and B correspond to the peak of acetylated α-C13 and the peak of unsubstituted α-C13, respectively.

that the peak A indicates α-C13 with one acetyl group (37%), and peak B is indicative of unsubstituted α-C13 (63%), and there is no peak corresponding to diacetylated α-C13 due to very low abundance in sample or low detection limits. This might be the reason why there are only two structures visible in the NMR spectra.

In conclusion, we show here that *R. solanacearum* produces the acetylated α-C13 as well as the unsubstituted one, where α-C13 having an acetyl group seems to be the major one based on NMR spectroscopic, MALDI-TOF MS and HPLC analysis. A proposed structure of the acetylated α-C13 is shown in Figure 5. According to the previous studies, the acetylated glucan was found with succinyl ester as the substituents within their structures in *R. sphaeroides*[16] and *E. chrisanthemi*.[18] In *R. sphaeroides*,[16] a competition between acetyl and succinyl substitution could be suggested, and in *E. chrisanthemi*,[18]

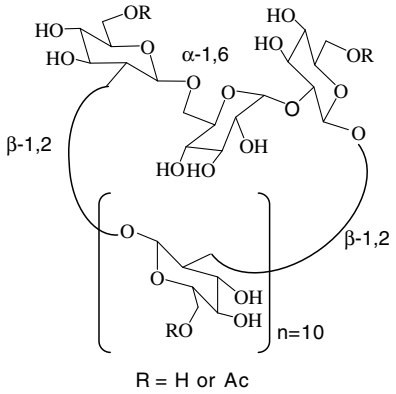

Figure 5. Proposed structure of acetylated α-cyclosophorotridecaose (α-C13) from *R. solanacearum*. α-C13 contains 13 glucose units, all β-(1→2) linked except for one α-(1→6) linkage (R = H or Ac). Based on the MALDI-TOF MS, one acetyl residue is mainly substituted at the C-6 position of the glucose in one molecule of unsubstituted α-C13.

the degree of substitution by acetyl ester was constant regardless of the growth conditions. However, in this study, we found out that the α-C13 substituted with an acetyl group rather than other substituents could be produced by *R. solanacearum*.

It is generally known that the physiological functions of the periplasmic cyclic glucan of Gram-negative bacteria are related with the osmotic pressure between the cell and external media. In *E. chrisanthemi*,[18] it is suggested that the OPG substitutions have played a role for the bacterial virulence or osmoregulation. The acetylated α-cyclosophorotridecaose (α-C13) produced from *R. solanacearum*, a plant pathogen, may biologically function similar to the OPGs of *E. chrisanthemi*. Structural information on the acetylated α-cyclosophorotridecaose performed here will be very useful for further study on the exact physiological role of α-C13 in Nature.

3. Experimental

3.1. Bacterial cultures and conditions

R. solanacearum KACC 10698 were from the Korean Agricultural Culture Collection (KACC), and they were grown in a TGY medium [5 g of tryptone, 3 g of yeast extract, 5 g of glucose, 700 mg of K_2HPO_4, and 250 mg of $MgSO_4{\cdot}7H_2O$ (pH 7.2)] at 24 °C with agitation.

3.2. Isolation of α-cyclosophorotridecaose (α-C13) from *R. solanacearum*

The microorganisms were collected during the early exponential growth phase by centrifugation at 4 °C for 10 min at 8000 rpm. The cell pellets were extracted with 1% trichloroacetic acid,[10] and after centrifugation, the supernatant was neutralized with NH_4OH[17] and desalted on a Sephadex G-25 column. The fractions containing the putative cyclic glucan were pooled, concentrated, and then applied to a column (2 × 35 cm) of DEAE-Sephadex to separate the anionic contaminants. The neutral one is collected and desalted on a Bio-Gel P-4 column (Bio-Rad). The column (2.4 × 54 cm) was eluted at room temperature with distilled water at a flow rate of 25 mL/h and then the desalted material was finally lyophilized.

3.3. NMR spectroscopy

For NMR spectroscopic analysis, a Bruker Avance 500 spectrometer was used to record the ^1H NMR, ^{13}C NMR, and HSQC, and HMBC spectra. All NMR spectroscopic analyses were done in D_2O at room temperature. The HSQC and HMBC spectra were recorded using 256/2048 complex data points and 28298/3255 Hz spectral widths in t_1 and t_2, respectively.

3.4. MALDI-TOF MS

The mass spectra of the oligosaccharides were obtained with a MALDI-TOF mass spectrometer (Voyager-DE™ STR BioSpectrometry, PerSeptive Biosystems, Framingham, MA, USA) in the positive-ion mode using 2,5-dihydroxybenzoic acid (DHB) as the matrix.

3.5. Deacetylation of α-cyclosophorotridecaose (α-C13)

The acetylated and unsubstituted α-C13 were treated with 0.1 M KOH at 37 °C for 1 h as previously described[16a] for removing nonsugar substituents of OPGs. The mixture was neutralized, and finally was desalted on a Bio-Gel P4 column.

3.6. HPLC

Heterogeneity of the purified α-C13 was determined by HPLC. The native α-C13 of *R. solanacearum* was subjected to HPLC on an NH_2 column (5 μm, 250 × 4.6 mm; Agilent ZORBAX) at 65 °C and detected with an RI detector ERC-7520 (Erma Optical Works, Tokyo, Japan) using 55:45 (v/v) acetonitrile–water as the solvent system at a flow rate of 1 mL/min.

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