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ISOLATION AND CHARACTERIZATION OF 3-*C*-CARBOXY-5-DEOXY-L-XYLOSE, A NATURALLY OCCURRING, BRANCHED-CHAIN, ACIDIC MONOSACCHARIDE[†,*]

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ABSTRACT

A branched-chain acidic monosaccharide, 3-*C*-carboxy-5-deoxy-L-xylose, has been identified for the first time as a component of plant cell-walls. This sugar was first observed as a constituent of rhamnogalacturonan II, a primary cell-wall, pectic polysaccharide. The sugar was shown to be a 3-*C*-carboxy-5-deoxypentose by nuclear magnetic resonance spectroscopy and mass spectrometry of the underivatized sugar and of several derivatives. X-Ray crystallography of one of the derivatives confirmed these structural features, and established that the sugar has the *xylo* configuration. The absolute configuration of the sugar was elucidated by the identification of L-lactic acid as the major product of periodate oxidation of the methyl glycoside. "Aceric acid" (AceA) has been proposed as a trivial name for this, the first branched-chain, acidic sugar to be found as a natural product.

INTRODUCTION

Rhamnogalacturonan II (RG-II) is a pectic polysaccharide released from primary cell-walls of suspension-cultured, sycamore (*Acer pseudoplatanus*) cells by incubation with an endopolygalacturonase purified from the culture fluid of the fungus *Colletotrichum lindemuthianum*. RG-II is an extremely complex polysaccharide that constitutes ~3% of the plant cell-wall and contains at least 10 different monosaccharides, including apiose [3-*C*-(hydroxymethyl)-D-*glycero*-tetrose], 2-*O*-

[†]Structure of Plant Cell Walls, Part XIII. For Part XII, see ref. 1.
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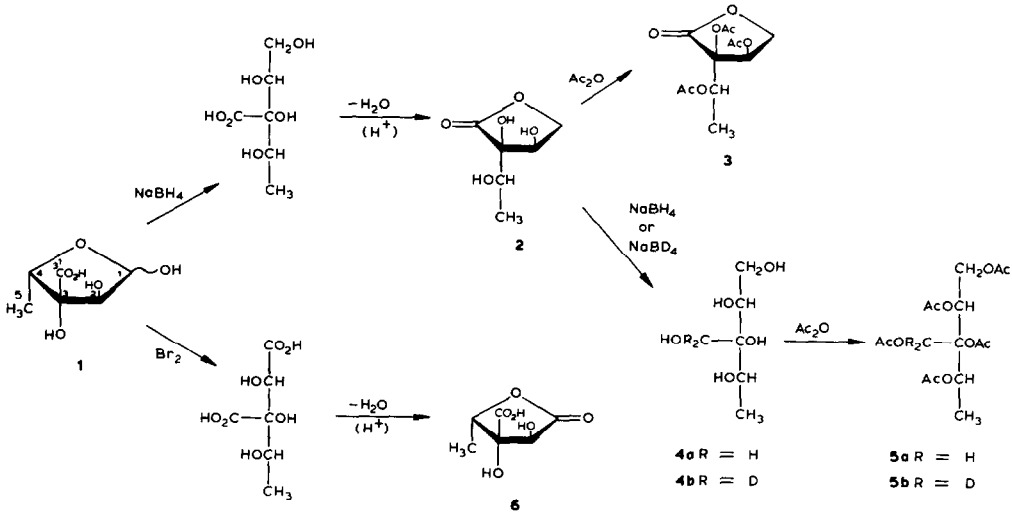
methylfucose, and 2-*O*-methylxylose. The glycosyl residues of RG-II are interconnected by a great variety of glycosyl linkages[2].

The presence in RG-II of two unidentified glycosyl residues has been reported[2]. These residues were detected by gas–liquid chromatography (g.l.c.) analysis after hydrolysis of RG-II, reduction of the resulting aldoses to alditols, and acetylation. One of the unidentified components was subsequently found[2a] to be an underacetylated derivative of apiose.

The electron impact-mass spectrometry (e.i.-m.s.) fragmentation-patterns of the second unidentified component did not allow its identification as an alditol acetate. However, the gross features of the mass-spectral data were consistent with those of an acetylated carbohydrate derivative. The e.i.-mass spectrum was characterized by losses of ketene, acetic acid, and acetic anhydride[3,4]. The component could only be reduced with borohydride after acid hydrolysis; therefore, the unknown compound appeared to be an internal glycosidic residue of RG-II. We now describe the isolation and characterization of this sugar.

EXPERIMENTAL

^{1}H-N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra were recorded with a Bruker WM-250, Fourier-transform, n.m.r. spectrometer operated at 250 MHz. Water-soluble samples were lyophilized twice from deuterium oxide (99.7 atom% D), and then dissolved in deuterium oxide (99.997 atom% D). Chemical shifts were assessed relative to an external standard (capillary) of hexamethyldisilazane (δ 0.6995). The spectrum of the acetylated sample (compound **3**) was recorded for a solution in deuteriochloroform (99.997 atom% D), and chemical shifts were assigned relative to internal chloroform (δ 7.26).



^{13}C-*N.m.r. spectroscopy.* — ^{13}C-N.m.r. spectra were recorded with a Bruker WM-250, Fourier-transform, n.m.r. spectrometer operated at 62.9 MHz, for solutions of samples in deuterium oxide (99.7 atom% D). Chemical shifts were assessed relative to internal 1,4-dioxane (δ 67.4). The ^{13}C-n.m.r. spectra of compounds **1** and **6** were recorded at pH 1.5. At this pH, the carboxyl resonances were sharper and more intense than those observed when the spectra were recorded at neutral pH.

Fast-atom bombardment–mass spectrometry (f.a.b.–m.s.). — F.a.b.–m.s.[5] spectra were recorded with a VG Analytical HF-ZAB 1F mass spectrometer fitted with a f.a.b. source. Samples were dissolved in 1:19 (v/v) acetic acid– or methanol–water (native and derivatized samples, respectively) (5 to 10 μg/μL), and a 1-μL aliquot was added to a drop of glycerol on the stainless-steel target. Xenon was used as the bombarding gas. Spectra were recorded on u.v.-sensitive chart-paper, and *m/z* ratios were determined by manual counting.

G.l.c. and g.l.c.–m.s. — All g.l.c. and g.l.c.–m.s. conditions were as described[6,7].

Isolation of RG-II. — Cell walls were prepared from suspension-cultured, sycamore (*Acer pseudoplatanus*) cells[8]. Isolated cell-walls were incubated with *C. lindemuthianum* endopolygalacturonase[9], as described. The material solubilized from the cell walls by the enzyme was dialyzed against H_2O, lyophilized, and de-esterified with NaOH (pH 12) for 2 h at 2°. The pH was adjusted to 5.2 with acetic acid, and 100 units of *C. lindemuthianum* endopolygalacturonase were added to the de-esterified wall-polymer. Thimerosal (0.1% w/v) was added as an antimicrobial agent. The solution was incubated overnight at 30°, dialyzed against H_2O, and then lyophilized. The lyophilized material was dissolved in 50mM sodium acetate buffer, pH 5.2 (5 mL), and chromatographed on a column of Bio-Gel P-10, as described[2].

Isolation of 3-C-carboxy-5-deoxy-L-xylose (**1**) *from RG-II.* — Isolated RG-II (4 mg) was hydrolyzed in 2M trifluoroacetic acid (TFA) for 1 h at 120°, and the TFA was removed by evaporation. The hydrolyzate was applied to a column (1 × 70 cm) of Bio-Rad AG1-X8 (acetate form) anion-exchange resin (200–400 mesh) that had been pre-equilibrated with 0.2M acetic acid. Galacturonic acid and glucuronic acid were eluted from the column by a gradient consisting of 0.5M acetic acid (200 mL) and 2M acetic acid[10] (200 mL). The column was then eluted with 4M acetic acid (200 mL). Compound **1**, which was detected by g.l.c. and e.i.-m.s. after reduction and acetylation, was eluted from the column in the last eluate.

Preparation of a polysaccharide-enriched fraction from Pectinol AC. — Pectinol AC (600 g) was suspended in H_2O (2.5 L), insoluble material was removed by filtration through a GF/C glass-fiber filter (Whatman), and the filtrate was concentrated to 800 mL by rotary evaporation. Solid trichloroacetic acid (TCA; 80 g) was added, the solution was kept for 2 h at 4°, and precipitated protein was removed by centrifugation.

Absolute ethanol was added to the supernatant liquor to a final concentration

of 63% (v/v). After 12 h at 4°, the suspension was centrifuged, and the precipitate discarded. The ethanol concentration of the supernatant liquor was brought to 86% (v/v), the mixture was kept for 12 h at 4°, and the suspension centrifuged. The material that was soluble in 63% ethanol but insoluble in 86% ethanol constituted >60% of the orcinol-positive[11] carbohydrate initially present in the Pectinol AC.

*Preparation of 3-C-carboxy-5-deoxy-*L*-xylitol-3*^{1}*,1-lactone* (**2**). — Sodium borohydride (1 mg) in M NH_4OH (0.1 mL) was added to **1** (1 mg). The solution was kept for 1 h at room temperature, the excess of borohydride was decomposed by adding glacial acetic acid, and the solution was evaporated to dryness with a stream of filtered air. Boric acid was removed by successive addition and evaporation of 4 portions (0.5 mL each) of 10% (v/v) acetic acid in methanol and 4 0.5-mL portions of methanol[12]. The residue was dissolved in H_2O (0.5 mL), the solution was passed through a column (2 mL) of Dowex-50 (H^+) cation-exchange resin, and the eluate was lyophilized. Compound **2** was isolated as a syrup.

*Preparation of 2,3,4-tri-*O*-acetyl-3-*C*-carboxy-5-deoxy-*L*-xylitol-3*^{1}*,1-lactone* (**3**). — Compound **2** (0.25 mg) was treated for 3 h at 120° with acetic anhydride (0.2 mL) containing sodium acetate (2 mg), and the excess of acetic anhydride was decomposed by adding $NaHCO_3$. The sample was partitioned between CH_2Cl_2 (0.5 mL) and H_2O (0.5 mL); the CH_2Cl_2 layer was washed with 2 portions (0.5 mL) of H_2O, and then evaporated to dryness with a stream of filtered air, yielding compound **3**.

Preparation of 1,2,3,3^1*,4-penta-*O*-acetyl-5-deoxy-3-*C*-(hydroxymethyl)-*L*-xylitol* (**5a**). — A carboxyl-reduced alditol (compound **4a**) was prepared by reduction of compound **2**, using the procedure of Jones and Albersheim[13]. To a solution of compound **2** (0.2 mg) in a few drops of 10mM sodium borate buffer (pH 7.5) was added a solution of sodium borohydride (2.5 mg) in 0.25 mL of the same buffer. After 1 h at room temperature, the reaction was quenched with acetic acid, and the boric acid was removed as already described. The sample was per-*O*-acetylated by treatment with 1:1 pyridine–acetic anhydride (0.2 mL) for 30 min at 120°, to yield compound **5a**.

Compound **4b** was prepared by an analogous procedure, using sodium borodeuteride and 10mM sodium borate buffer made with D_2O, and then per-*O*-acetylated to yield compound **5b**.

*Preparation of 3-*C*-carboxy-5-deoxy-*L*-xylono-1,4-lactone* (**6**). — To a solution of compound **1** (20 mg) in H_2O (3 mL) was added bromine (0.05 mL), with vigorous stirring, and the solution was kept for 72 h in the dark. The excess of bromine was removed by rotary evaporation, the acid was neutralized with solid Ag_2CO_3, the AgBr was removed by filtration through a GF/C glass-fiber filter, and the filtrate was lyophilized, yielding a syrup. The syrup was dissolved in 2-pentanone, and then crystallized from a mixture[14] of 2-pentanone and $CHCl_3$, using vapor diffusion[15]. The sample was recrystallized twice from 2-pentanone–$CHCl_3$, yieding 15 mg (75%) of **6**, m.p. 120–122° (uncorr.).

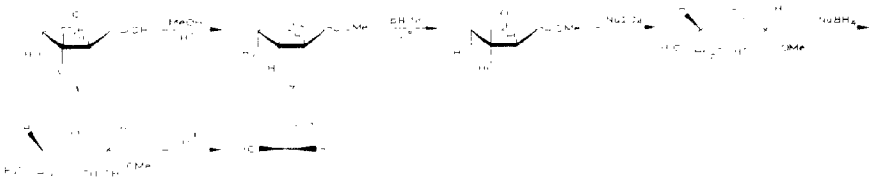
X-Ray crystallography of compound* **6**. — $C_6H_8O_6 \cdot H_2O$, M_r = 194.14, monoclinic, space group $P2_1$, a = 7.697(2), b = 7.414(2), c = 7.336(2) Å, β = 101.88(2)°, Z = 2, $F(000)$ = 204, D_c = 1.567 g.cm^{-3}, 787 reflections, [524; $I > 3\sigma(I)$], in the range $3 < \theta < 25°$. The conformation is ^3E(L).

A crystal with dimensions 0.14 × 0.12 × 0.12 mm was selected. The crystal data showed the systematic absence $0k0$ ($k = 2n + 1$) that established the space group as $P2_1$ (for an optically active compound). Intensity data were collected on a Philips PW1100 diffractometer with MoKα radiation (λ = 0.7106 Å) and a $\theta - 2\theta$ scan-mode. Three standard reflections were measured every 2 h during the course of data collection to monitor crystal quality and alignment; these were constant within ±5%. Absorption corrections were not applied. Equivalents were averaged to give 787 unique reflections.

The structure was solved by standard, multisolution, tangent refinement with SHELX76[16]. Using E > 1.2, the second E map from the sign expansion (R_a = 0.073) revealed the positions of all 13 nonhydrogen atoms. The H atoms were found in a subsequent, difference-Fourier synthesis. The nonhydrogen atoms were refined assuming anisotropic motion, and the H atoms on isotropic motion, using full-matrix, least-squares methods. Final refinement of the model in the L configuration gave a conventional R value of 0.0421, and a weighted R value of 0.0401 using a weighting factor, $w = 1/^2(F_0)$. The final Hamilton R_g factor[17] was 0.0425. Neutral atom scattering-factors were used[18]. A final difference-map showed no significant features, with a maximum residual density of 0.22 e Å^{-3}. Final refinement of the model in the D configuration from the same starting parameters gave, of course, identical values of R, R_w, and R_g, making therefrom an assignment of the optical isomer impossible.

Determination of the absolute configuration of **1**. — Compound **1** (2 μmol) was dried under diminished pressure over P_2O_5. The sample was treated with anhydrous M methanolic hydrogen chloride (1 mL) for 16 h at 85°, the acid neutralized with solid Ag_2CO_3, and the suspension centrifuged. The insoluble material was washed with three 1-mL portions of methanol, and the supernatant liquors were combined and evaporated[19]. The product was de-esterified with NaOH (pH 12) during 2 h at 2°, the base neutralized with acetic acid, the solution passed through a column (2 mL) of Dowex 50 (H^+) resin, and the eluate lyophilized, yielding a mixture of anomeric methyl glycosides (**7**; see Scheme 1). To a solution of the methyl glycosides in 0.05M sodium acetate buffer, pH 5.2 (2.5 mL) was added 0.1M aqueous $NaIO_4$ (0.5 mL), and the mixture was kept in the dark for 68 h at 22°. The excess of periodate was decomposed by adding M glycerol (40 μL). The sample was reduced with sodium borohydride as already described, the prod-

*Supplementary data: Fractional atomic coordinates and anisotropic or isotropic thermal parameters for nonhydrogen atoms and hydrogen atoms, bond lengths, bond angles, and observed and calculated structure factors for **6** can be obtained from Elsevier Scientific Publishing Company, BBA Deposition, P.O. Box 1527, Amsterdam, The Netherlands. Reference should be made to BBA/DD/261/*Carbohydr. Res.*, 122 (1983) 115–129.



Scheme 1. Reaction sequence used to determine the absolute configuration of compound **1**.

uct hydrolyzed in M HCl (0.5 mL) for 12 h at 22°, and the acids neutralized with NaOH.

The content of L-lactic acid (**8**) was determined enzymically in 0.2M glycine buffer (pH 9.2) containing 0.16M hydrazine, nicotinamide adenine dinucleotide (0.8 mg/mL), and beef-heart L-(+)-lactic acid dehydrogenase (Sigma, 15 units/mL). Total assay volume was 1.5 mL. Substrate-dependent formation of NaDH was determined spectrophotometrically (A_{340}). The content of D-lactic acid was determined in the same way, except that *Lactobacillus leichmannii* D-(−)-lactic acid dehydrogenase (Sigma; 20 units) was used instead of the L-(+)-lactic acid dehydrogenase. Readings of A_{340} were corrected by subtracting the A_{340} value of a solution containing all of the assay components except the enzyme.

RESULTS AND DISCUSSION

Isolation of compound **1**. — Compound **1** was bound by anion-exchange resins, and it could be eluted only under conditions stronger than those under which uronic acids are eluted. These results demonstrated that **1** was an acidic compound, and suggested that it could be purified by anion-exchange chromatography of the mixture of monosaccharides produced by acid hydrolysis of RG-II. Compound **1** (25–50 μg) was isolated from acid-hydrolyzed, sycamore cell-wall RG-II (3 mg) by chromatography in acetic acid on a column of Bio-Rad AG1-X8 (acetate) anion-exchange resin.

Compound **1** constitutes ~7% of purified RG-II, and RG-II, ~3% of isolated, sycamore cell-walls[2]. Therefore, it was impractical to use sycamore cell-wall RG-II as the starting material for the isolation of sufficient **1** for structural characterization. Fortunately, a more abundant and readily accessible source of **1** was found in our laboratory. Pectinol AC (Corning, Inc.), a commercial preparation of the enzymes secreted by the fungus *Aspergillus niger* when the fungus is grown by using plant cell-walls as the carbon source, was found to contain **1**. Experiments (J. Thomas, W. S. York, A. G. Darvill, and P. Albersheim, unpublished results) have shown that Pectinol AC contains ~3% of carbohydrate by weight, ~50% of which is a polysaccharide having glycosyl-residue composition, glycosyl-linkage composition, and a size very similar to those of sycamore cell-wall RG-II. The Pectinol AC RG-II contains residues of apiose, 2-*O*-methylfucose, 2-*O*-methylxylose, and com-

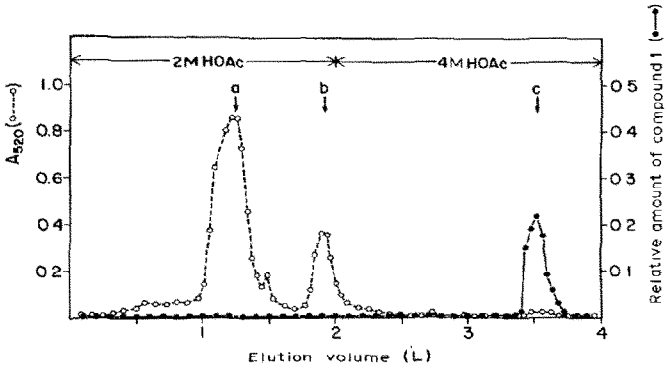


Fig. 1. Chromatography of the acid hydrolyzate of the Pectinol AC polysaccharide on Bio-Rad AG1-X8 (acetate) anion-exchange resin. [The column (2 × 70 cm) was equilibrated with 0.2M acetic acid. The acid-hydrolyzed Pectinol AC polysaccharide (~5 g) was dissolved in H_2O (1.2 L), and the pH of the solution was adjusted to 9.0 with 0.1M NaOH. After application of the sample, the column was washed with 0.2M acetic acid (500 mL). The column was eluted with 2M acetic acid (2 L), followed by 4M acetic acid (2 L). The 2M and 4M acetic acid effluents were collected in 10-mL fractions, and aliquots of the fractions were assayed[19] for uronic acid content (A_{520}). The relative content of compound **1** was assayed as follows. Aliquots (20 μL) of the fractions were reduced with $NaBD_4$, and the products acetylated; this treatment converted compound **1** into compound **3**, which was quantitated by g.l.c. The relative content of **1** was calculated by dividing the g.l.c. peak-area of compound **3** by the peak area of an internal standard of *myo*-inositol hexaacetate.]

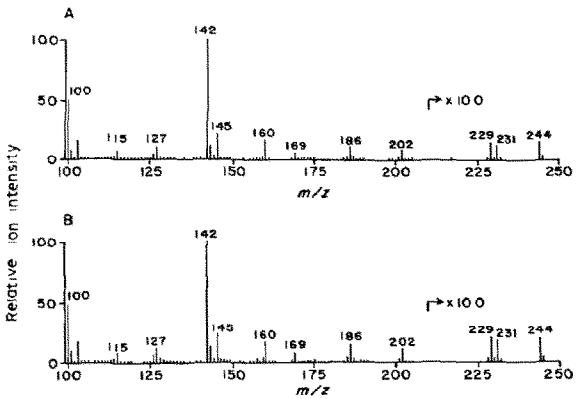


Fig. 2. E.i.-mass spectra of compound **3**, formed by reduction and per-*O*-acetylation of: (A) compound **1** isolated from sycamore cell-wall RG-II, and (B) compound **1** isolated from the Pectinol AC polysaccharide.

pound **1**, the rare glycosyl residues that characterize RG-II. Therefore, Pectinol AC was selected as an inexpensive, plentiful starting-material from which sufficient compound **1** could be isolated to permit its structural characterization.

A polysaccharide-enriched fraction was prepared from Pectinol AC (600 g) as described in the Experimental section. A solution of this material in 2M TFA (500 mL) was heated under reflux for 18 h at 94°, the TFA removed by rotary evaporation, and the hydrolyzate applied to a column (2 × 70 cm) of AG1-X8 (acetate form) anion-exchange resin (see Fig. 1). Material that was adsorbed to the column was eluted with 2M acetic acid, followed by 4M acetic acid. Galacturonic acid (peak a) and glucuronic acid (peak b, Fig. 1) were eluted by the 2M acetic acid. Compound **1** was detected by g.l.c. after aliquots from fractions had been reduced with borohydride, and the products acetylated. Compound **1** was eluted from the column by the 4M acetic acid (peak c, Fig. 1) and was isolated as a syrup (60 mg).

Comparison of **1** *isolated from sycamore cell-wall RG-II to* **1** *isolated from Pectinol AC polysaccharide.* — Samples of **1** isolated from sycamore cell-wall RG-II and from Pectinol AC polysaccharide were reduced and the products acetylated[8]. The acetates were indistinguishable by g.l.c. on two different capillary columns (SP 2330, Supelco, and DB 1, J & W Scientific). The e.i.-mass spectra of the alditol acetates were identical (see Fig. 2).

The procedure of Gerwig *et al.*[20] was used to demonstrate that only one optical isomer of **1** was present in the Pectinol AC polysaccharide, and that the same optical isomer of **1** was present in sycamore-cell RG-II. Aliquots of **1** isolated from Pectinol AC were treated with (+)-2-butanol containing HCl, and with (−)-2-butanol containing HCl, and the per-*O*-(trimethylsilyl) derivatives of the products were analyzed by g.l.c. The patterns of peaks obtained with (−)-2-butanol was different from that obtained with (+)-2-butanol. In both cases, peaks that would have corresponded to the other optical isomer of **1** were not present. Therefore, it was concluded that a single optical isomer of **1** was present in Pectinol AC.

A heptasaccharide isolated from sycamore cell-wall RG-II and containing **1** as an internal glycosyl residue[21] was treated with (+)-2-butanol–HCl, the product per-*O*-(trimethylsilyl)ated and the material analyzed by g.l.c., giving a profile that contained, *inter alia*, the same pattern of peaks as observed with compound **1** isolated from the Pectinol AC polysaccharide. The combined results demonstrated that compound **1** isolated from the Pectinol AC polysaccharide was identical to compound **1** from sycamore cell-wall RG-II.

Characterization of underivatized **1**

Colorimetric assays. — Compound **1** gave a positive response in the Nelson–Somogyi[22] and the *p*-hydroxybenzoic hydrazide[23] assays for reducing sugars. It did not give a positive response in the anthrone[11], orcinol[11], or *m*-hydroxybiphenyl[24] assays. These results indicated that **1** was a reducing sugar that could not be transformed into a 2-furaldehyde derivative[11].

Fast-atom bombardment–mass spectrometry. — The molecular weight of **1** was found by f.a.b.–m.s. to be 178. The f.a.b.–mass spectrum of compound **1** did

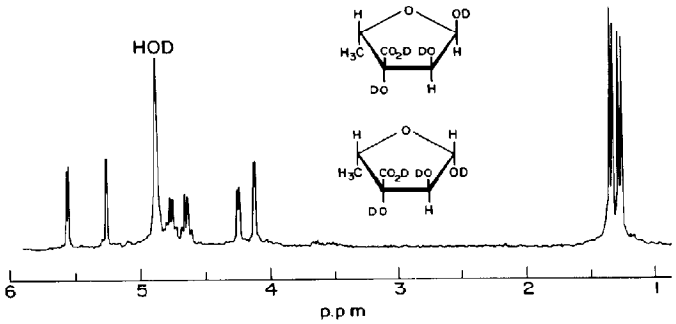


Fig. 3. ^{1}H-N.m.r spectrum of a solution in D_2O of the mixture of anomers of compound 1. Chemical shifts were assessed relative to an external standard (capillary) of hexamethyldisilazane (δ 0 6995).

not contain the characteristic, isotope ratios that would indicate the presence of silicon or sulfur in the molecule[25]. Compound **1** did not contain phosphate, as assayed by a modification of the Fisk-SubbaRow procedure[26]. Therefore, it was concluded that the acidic moiety in **1** is a carboxyl group.

^{1}H-N.m.r. spectroscopy. — The ^{1}H-n.m.r. spectrum of **1** is shown in Fig. 3. This spectrum provided evidence that **1** was a 3-C-carboxy-5-deoxypentose. In solution, 1 existed as a mixture of its anomers, present in the ratio of ~1.2:1. This difference in concentration was large enough to allow each resonance to be assigned to one of the anomers on the basis of its area in the n.m.r. spectrum. Each anomer gave rise to an upfield, 3-proton doublet (δ 1.27, and δ 1.34); this indicated that the molecule contained a methyl group (C-5). In each anomer, H-4 was split by the methyl protons, and gave rise to a quartet (δ 4.76 and 4.64, respectively). The resonances at δ 5.56 and 5.27 arose from the hemiacetal protons (H-1). The hemiacetal proton of each anomer was coupled to one other proton (δ 4.25 and 4.13, respectively), which was not further split. The magnitude of the coupling between H-1 and H-2 of a furanoid sugar can be used to distinguish the anomer in which H-1 and H-2 are *cis* from that in which they are *trans*[27,28]. Therefore, the resonance at δ 5.56 ($J_{1,2}$ 4.3 Hz) arises from H-1 of the anomer containing a *cis*-1,2-diol grouping, and the resonance at δ 5.27 ($J_{1,2}$ 2.6) arises from H-1 of the anomer containing a *trans*-1,2-diol grouping.

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectrum of the mixture of anomers of **1** was consistent with the proposed structure (see Table I). The chemical shifts of the C-1 atoms of the anomers differed by 5 p.p.m. This difference is characteristic of a furanose; the hemiacetal carbon atom of the anomer containing a *trans*-1,2-diol grouping resonates 5–6 p.p.m. downfield of the corresponding signal of the anomer containing a *cis*-1,2-diol grouping[29]. The resonances that have been assigned as those of the C-3^1 atom of the anomers had chemical shifts and in-

TABLE I

^{13}C-N.M.R. CHEMICAL-SHIFTS OF THE MIXTURE OF ANOMERS OF COMPOUND **1**

	Chemical shift[a] *(p.p.m.)*	
	cis[b] *Anomer*	*trans*[b] *Anomer*
C-1	97.3	102.2
C-2	79.8[c]	83.5
C-3	83.6	81.0
C-3[1]	172.2	174.2
C-4	79.2[c]	79.7
C-5	11.4	14.6

[a]Chemical shifts were assessed relative to internal 1,4-dioxane at δ 67.4. [b]The designation "*cis*" refers to the anomer containing a *cis*-1,2-diol grouping, and "*trans*" refers to the anomer having a *trans*-1,2-diol grouping. [c]These assignments may have to be reversed for the *cis* anomer.

tensities characteristic of carbonyl carbon atoms (in this case, carboxyl). These signals had ~30% of the intensity of those arising from the methyl carbon atoms; low signal-intensity is usually observed with carbon atoms that have no hydrogen atoms bonded to them. The resonances assigned as those of C-3 had chemical shifts typical of carbon atoms of alcohols, and had intensities comparable to those of the carboxyl carbon atoms. These results indicated that C-3 of each anomer was that of a tertiary-alcohol carbon atom, and provided additional evidence for the branched-chain structure of compound **1**.

Preparation and characterization of reduction products of **1**

Compound **2**. — Reduction of **1** with borohydride yielded a 3-C-carboxy-5-deoxypentitol, which readily formed a γ-lactone (**2**) on acidification. Evidence for the formation of **2** was obtained by f.a.b.–m.s. The molecular weight of borohydride-reduced **1** was found to be 162, which is the molecular weight calculated for **2**. No ion with *m/z* 181 was observed that would correspond to the $(M + H^+)$ ion of the free-acid form of the hydride-reduced derivative. The ^1H-n.m.r. spectrum of **2** in D_2O contained a 3-proton doublet (J 6.5 Hz) at δ 1.36, and a 1-proton quartet (J 6.5 Hz) at δ 4.11. These signals arose from the methyl protons and H-4, respectively. The other three nonexchangeable protons (2 protons on C-1, and 1 proton on C-2) gave rise to a complex, 3-proton multiplet between δ 4.33 and 4.70. The ^{13}C-n.m.r. spectrum of **2** in D_2O contained, as expected, resonances at δ 16.5 (methyl); 68.5, 68.6, 74.3 (alcohol); 79.2 (tertiary alcohol); and 180.1 (carbonyl).

Compound **3**. — Acetylation of compound **2** yielded a volatile, tri-*O*-acetylated lactone (**3**). The molecular weight of **3** was 288, as determined by c.i.–m.s. (isobutane) and f.a.b.–m.s. The molecular weight of the corresponding derivative that was per-*O*-(deuterioacetyl)ated by using hexadeuterioacetic anhydride was 297. The difference of nine mass units indicated that three *O*-acetyl groups had been incorporated.

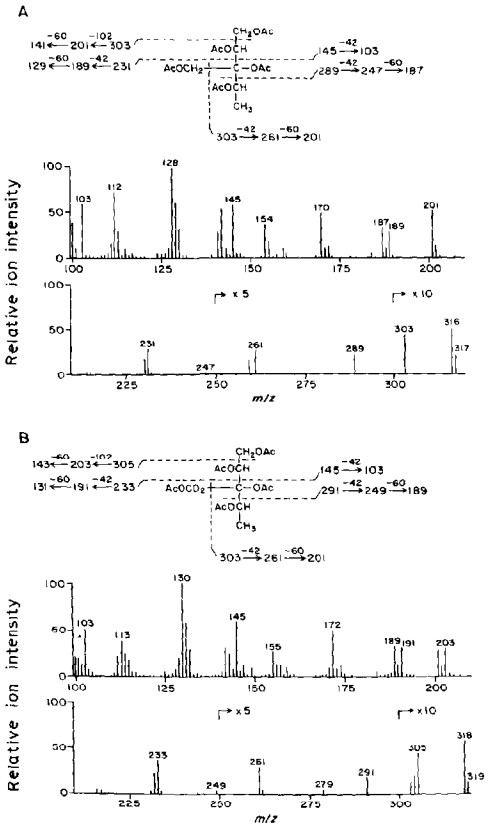


Fig. 4. E.i.-mass spectra of (A) compound **5a**, and (B) compound **5b**. Secondary fragment-ions, formed by eliminations of ketene (42 mass units), acetic acid (60 mass units), and acetic anhydride (102 mass units) have been indicated.

The e.i.-mass spectrum of **3** is shown in Fig. 2. The ion with *m/z* 244 (M − 44) corresponded to loss of carbon dioxide from the molecular ion; this is characteristic of a lactone[30]. The M − CO_2 ion sequentially eliminated two molecules of ketene, to yield the ions with *m/z* 202 and *m/z* 160. The base peak, with *m/z* 142, arose from the elimination of acetic anhydride from the M − CO_2 ion. The ion with *m/z* 231 arose from the sequential losses of a methyl radical and ketene from

the molecular ion. The other features of the mass spectrum were typical of those of alditol acetates[3,4]. The ion with *m/z* 229 resulted from the loss of an acetoxyl radical from the molecular ion. This ion lost acetic acid to generate the ion with *m/z* 169, which, in turn, eliminated ketene to yield the ion with *m/z* 127. Loss of acetic acid from the molecular ion yielded the ion with *m/z* 228, which, in turn, lost ketene to yield the ion with *m/z* 186.

The ^{1}H-n.m.r. spectrum of **3** in $CDCl_3$ contained a three-proton doublet (J 6.3 Hz) at δ 1.38 and a one-proton quartet (J 6.3 Hz) at δ 5.24, arising from the methyl group and H-4, respectively. The methyl protons of the *O*-acetyl groups gave rise to three-proton singlets at δ 2.04, 2.11, and 2.18. The other three, nonexchangeable protons comprised an AMX spin-system, and gave rise to one-proton, doublets of doublets at δ 4.27 (J 9.7 and 5.5 Hz), 4.74 (J 9.7 and 8.2 Hz), and 5.54 (J 5.5 and 8.2 Hz).

Carboxyl-reduced alditols. — Borohydride reduction of lactone **2** yielded compound **4a**, a branched-chain deoxyalditol. Reduction of **2** with borodeuteride in borate buffer yielded compound **4b**, the dideuterio-labeled derivative of **4a**. Compound **4a** and **4b** were per-*O*-acetylated, yielding compounds **5a** and **5b**, respectively, which were analyzed by g.l.c.–m.s. The e.i.-mass spectrum of compound **5a** is shown in Fig. 4A. The highest ions in the spectrum (*m/z* 317 and *m/z* 316) were formed by eliminating from the molecular ion an acetoxyl radical and acetic acid, respectively. The other primary fragment-ions arose from fission of the alditol chain, as indicated in Fig. 4A. Secondary fragment-ions were formed by eliminations of ketene, acetic acid, and acetic anhydride from the primary fragment-ions. The base peak at *m/z* 128 and the ion at *m/z* 170 are present in the e.i.-mass spectra of all hexa-*O*-acetylhexitols and penta-*O*-acetyl-6-deoxyhexitols[31], and are, therefore, of no diagnostic value.

Comparison of the e.i.-mass spectrum of compound **5a** to that of compound **5b** (Fig. 4B) confirmed the location and nature of the branch in compound **1**. The primary ions with *m/z* 317, 316, 289, and 231 in the mass spectrum of **5a** were shifted to *m/z* 319, 318, 291, and 233 in the mass spectrum of **5b**. The secondary ions derived from these primary fragments were also shifted by two mass units. The primary ion at *m/z* 145 and the secondary ion derived from it were not changed by carboxyl reduction with borodeuteride. Compound **5a** gave rise to two different, primary fragment-ions with *m/z* 303. In the mass spectrum of compound **5b**, one of these ions had *m/z* 303, whereas the other had shifted to *m/z* 305. These results confirmed that compound **1** had a carboxyl branch at C-3.

Determination of the relative configurations of the three asymmetric carbon atoms of compound **1**. — The results just presented clearly established that compound **1** was a 3-*C*-carboxy-5-deoxypentose. The acyclic form of such a sugar has three asymmetric carbon atoms; therefore, compound **1** could exist as one of eight stereoisomers (four L isomers and four D isomers). The relative configurations of the three asymmetric carbon atoms were established by X-ray crystallography of compound **6**, an oxidation product of compound **1**.

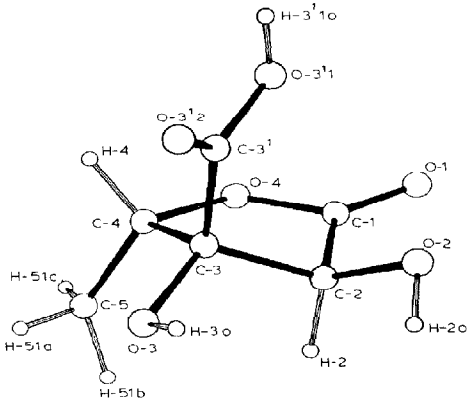


Fig. 5. Structure of compound **6**, as determined by X-ray crystallography.

Compound **6** was prepared by bromine oxidation of compound **1**. The general structure of compound **6** was confirmed by ^{1}H-n.m.r. and ^{13}C-n.m.r. spectroscopy. The ^{1}H-n.m.r. spectrum of **6** in D_2O contained a three-proton doublet (J 6.6 Hz) at δ 1.40 and a 1-proton quartet at δ 4.70, arising from the methyl group and H-4, respectively; H-2 gave rise to a one-proton singlet at δ 4.62. The ^{13}C-n.m.r. spectrum of **6** contained, as expected, the following signals: δ 15.7 (methyl); 73.4 and 80.5 (alcohol); 81.0 (tertiary alcohol); and 173.9 and 175.9 (carbonyl).

Compound **6** crystallized from a mixture of 2-pentanone and chloroform. X-Ray crystallography of compound **6** confirmed all of the previously determined structural features, and revealed that the sugar has the *xylo* configuration (Fig. 5). It was not, of course, possible to determine the absolute configuration of **6** from the crystallographic data.

Determination of the absolute configuration of **1**. — The absolute configuration of **1** was determined by periodate oxidation of the anomeric mixture of its methyl glycosides, prepared by methanolysis of **1**, followed by de-esterification with base. The mixed methyl glycosides were subjected to Smith degradation (periodate oxidation, followed by borohydride reduction and mild hydrolysis with acid)[32]. The product expected of this series of reactions would be D-lactic acid if **1** has the D configuration, or L-lactic acid if **1** has the L configuration (see Scheme 1).

Enzymic analysis was used in order to determine the concentrations of D- and L-lactic acid obtained by Smith degradation of the methyl glycosides of **1**. L-Lactic acid (0.6 μmol of L-lactic acid per μmol of starting material) was the major product recovered. Therefore, it was concluded hat **1** is an L sugar. A small proportion of D-lactic acid (0.1 μmol/μmol of starting material) was also detected. The origin of

the D-lactic acid is not understood, because compound **1** was shown to be optically pure by the method of Gerwig *et al.*[30] (see earlier). A possible explanation for the formation of D-lactic acid is that some racemization occurred during the series of reactions used to form the lactic acid.

GENERAL DISCUSSION

Compound **1** is 3-*C*-carboxy-5-deoxy-L-xylose. We propose the trivial name "aceric acid" for this sugar, because it was first observed[7] as a constituent of cell walls isolated from suspension-cultured cells of *Acer pseudoplatanus* (sycamore). More than a dozen branched-chain sugars have now been found in Nature, frequently as components of antibiotics[33,34]. Of these, streptose (3-*C*-formyl-5-deoxy-L-lyxose)[11], a neutral-sugar component of the antibiotic streptomycin, is the most similar to aceric acid, but it has a formyl, not a carboxyl, group on C-3, and the *lyxo*, not the *xylo*, group configuration. Only two other branched-chain sugars have previously been found in plant tissue[35]; these are apiose [3-*C*-(hydroxymethyl)-D-*glycero*-tetrose] and hamamelose [2-*C*-(hydroxymethyl)-D-ribose]. Aceric acid is the first branched-chain, acidic sugar to be found as a natural product.

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REFERENCES

1 M. McNEIL, A. G. DARVILL, AND P. ALBERSHEIM *Plant Physiol.*, 70 (1982) 1586–1591
2 A. G. DARVILL, M. McNEIL AND P. ALBERSHEIM, *Plant Physiol.*, 62 (1978) 418–422
2a W. S. YORK, M. McNEIL, A. G. DARVILL, AND P. ALBERSHEIM, unpublished results.
3 I. S. GOLOVKINA, O. S. CHIZHOV, AND N. S. WULFSON, *Izv. Akad. Nauk SSSR, Ser. Khim.*, (1966) 1915–1926.
4 J. LONNGREN AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.
5 M. BARBER, R. S. BORDOLI, R. D. SEDGWICK, AND A. N. TYLER, *Nature (London)*, 293 (1981) 270–275.
6 B. S. VALENT, A. G. DARVILL, M. McNEIL, B. K. ROBERTSON, AND P. ALBERSHEIM, *Carbohydr. Res.*, 79 (1980) 165–192
7 M. McNEIL AND P. ALBERSHEIM, *Carbohydr. Res.*, 56 (1977) 239–248.
8 K. W. TALMADGE, K. KEEGSTRA, W. D. BAUER, AND P. ALBERSHEIM, *Plant Physiol.*, 51 (1973) 158–173
9 P. D. ENGLISH, A. MAGLOTHLIN, K. KEEGSTRA, AND P. ALBERSHEIM, *Plant Physiol.*, 49 (1972) 293–297
10 B. LARSEN AND A. HAUG *Acta Chem. Scand.*, 15 (1961) 1397–1398
11 Z. DISCHE *Methods Carbohydr. Chem.*, 1 (1962) 477–512.
12 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohydr. Res.*, 5 (1967) 340–345
13 T. M. JONES AND P. ALBERSHEIM, *Plant Physiol.*, 49 (1972) 926–936.
14 F. J. KUEHL JR., *Methods Carbohydr. Chem.*, 1 (1962) 268–272

15 G. H. STOUT AND L. H JENSEN, *X-ray Structure Determination*, Macmillan, New York, 1968, p. 65.
16 G. M. SHELDRICK, *SHELX76, Program for Crystal Structure Determination*, University of Cambridge, England, 1976.
17 W C. HAMILTON, *Acta Crystallogr.*, 18 (1965) 502–510.
18 D. T. CROMER AND J B. MANN, *Acta Crystallogr., Ser. A*, 24 (1968) 321–324.
19 R. E. CHAMBERS AND J. R. CLAMP, *Biochem J*, 125 (1971) 1009–1018.
20 G. J GERWIG, J. P. KAMERLING, AND J. F. G. VLIEGENTHART, *Carbohydr Res.*, 77 (1979) 1–7.
21 M. W. SPELLMAN, M. MCNEIL, A. G DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 122 (1983) 131–153.
22 N. J. NELSON, *J. Biol. Chem.*, 153 (1944) 375–380.
23 M. LEVER, *Anal Biochem.*, 47 (1972) 273–279.
24 N. BLUMENKRANTZ AND G. ASBOE-HANSEN, *Anal Biochem.*, 54 (1973) 484–489.
25 R. M. SILVERSTEIN, G. C. BASSLER, AND T C. MORRILL, *Spectrometric Identification of Organic Compounds*, Wiley, New York, 1974, p. 13.
26 B. N AMES, *Methods Enzymol*, 8 (1966) 115–118.
27 J. D. STEVENS AND H. G. FLETCHER, JR, *J Org. Chem*, 33 (1968) 1799.
28 D. R. BUNDLE AND R. U. LEMIEUX, *Methods Carbohydr. Chem.*, 7 (1976) 79–86.
29 A. S PERLIN, N. CYR, H. J. KOCH, AND B. KORSCH, *Ann. N. Y. Acad. Sci.*, 222 (1973) 935–942.
30 F. W. MCLAFFERTY, *Interpretation of Mass Spectra*, University Science Books, Mill Valley, California, 1980, p. 283.
31 P.-E. JANSSON AND B. LINDBERG, *Carbohydr. Res.*, 86 (1980) 287–292.
32 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
33 H. GRISEBACH AND R. SCHMID, *Angew Chem., Int Ed. Engl*, 11 (1972) 159–173
34 N. R. WILLIAMS AND J D. WANDER, in W PIGMAN AND D. HORTON (Eds.), *The Carbohydrates*, Vol. 1B, 2nd edn., Academic Press, New York, 1980, pp. 761–798.
35 H. GRISEBACH, in J. PREISS (Ed.), *The Biohemistry of Plants*, Vol. 3, Academic Press, New York, 1980, pp. 171–197.