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Synthesis and characterization of authentic standards for the analysis of ribofuranose-containing carbohydrates by the reductive-cleavage method

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Abstract

Described herein is the synthesis of all positional isomers of partially methylated and acetylated or benzoylated 1,4-anhydro-D-ribitol. The benzoates are generated simultaneously from 1,4-anhydro-D-ribitol by sequential partial methylation and benzoylation or sequential partial benzoylation and methylation. The individual isomers are obtained in pure form by high-performance liquid chromatography. Debenzoylation and acetylation provided the corresponding acetates. Reported herein are the ^{1}H NMR spectra of the benzoates and the electron-ionization mass spectra of the acetates and the tri-*O*-methyl derivative and also for the acetates and the tri-*O*-methyl derivative, their linear temperature programmed gas–liquid chromatography retention indices on three different capillary columns.

Keywords: Reductive-cleavage; D-Ribitol, 1,4-anhydro, partially methylated and acylated

1. Introduction

D-Ribose is found as a component of many bacterial polysaccharides, including lipopolysaccharides, capsular polysaccharides, and teichoic acids, where it generally occurs in the β-furanosyl form [1]. In order to better understand the biological roles of these carbohydrates, their primary structures must be fully determined. The reductive-cleavage method is a very useful technique in this regard since it is capable of

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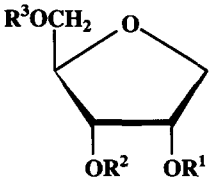
simultaneously establishing the identity, ratio, ring form, and linkage position(s) of each monomeric residue [2–4]. However, in order to fully implement the reductive-cleavage method, a database of authentic standards is needed. Since the independent synthesis of each authentic standard is very time consuming and laborious [5–12], we have recently developed two efficient procedures for their synthesis [13,14]. Described herein is the application of these two procedures to the synthesis of all possible standards for D-ribofuranosyl residues, namely the positional isomers of partially methylated and acetylated or benzoylated 1,4-anhydro-D-ribitol. As an aid to those who wish to use the reductive-cleavage method, ^{1}H NMR spectra of the seven methylated and benzoylated positional isomers (**2b–8b**) are reported, as are the electron-ionization mass spectra (EIMS) and GLC retention indices of the corresponding acetates (**2a–8a**) and the tri-*O*-methyl derivative (**1**).



	R^1	R^2	R^3
1	Me	Me	Me
2a	Ac	Me	Me
2b	Bz	Me	Me
3a	Me	Ac	Me
3b	Me	Bz	Me
4a	Me	Me	Ac
4b	Me	Me	Bz
5a	Ac	Ac	Me
5b	Bz	Bz	Me
6a	Ac	Me	Ac
6b	Bz	Me	Bz
7a	Me	Ac	Ac
7b	Me	Bz	Bz
8a	Ac	Ac	Ac
8b	Bz	Bz	Bz

2. Results and discussion

Synthesis.—The tri-*O*-methyl (**1**), tri-*O*-acetyl (**8a**), and tri-*O*-benzoyl (**8b**) derivatives of 1,4-anhydro-D-ribitol were prepared from the latter by total methylation [15], acetylation, and benzoylation, respectively.

The partially methylated and benzoylated positional isomers (**2b–7b**) were prepared from 1,4-anhydro-D-ribitol by sequential partial methylation [16] and benzoylation in situ [13], or by sequential partial benzoylation and methylation [14]. A small portion of the reaction mixture generated by the former method was subjected to acetylation prior to benzoylation, and a small portion of the benzoate mixture generated by the latter method was subjected to debenzoylation and acetylation. The resulting *O*-acetyl derivatives were analyzed by GLC and GLC combined with chemical-ionization mass spectrometry (CIMS) (see Figs. 1a and 1b, respectively). It was evident from these results that both methods were able to produce the three possible diacetates (**5a–7a**), but the three possible monoacetates (**2a–4a**) were formed in significant proportions only in the former method (Fig. 1a).

The mixtures of partially methylated 1,4-anhydro-D-ribitol benzoates derived by the

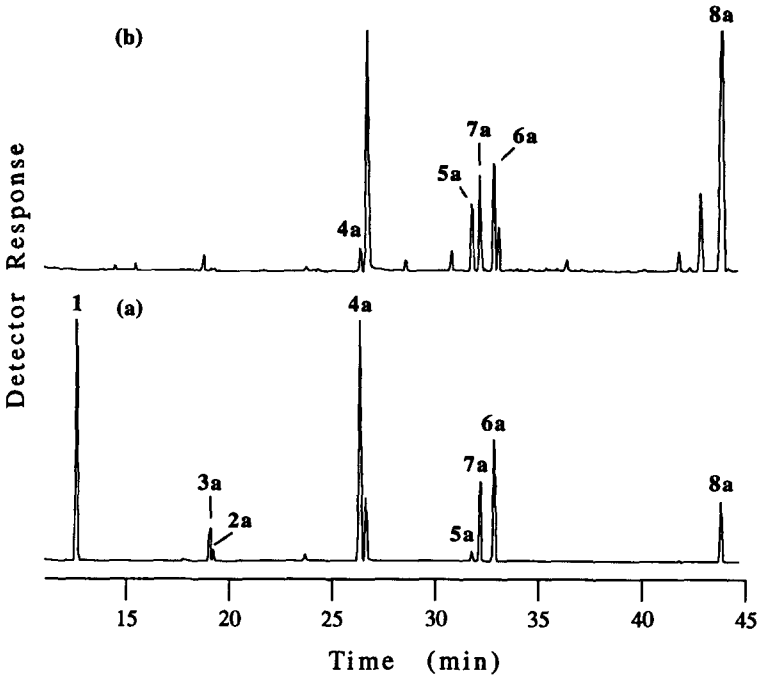


Fig. 1. Gas–liquid chromatogram of the partially methylated 1,4-anhydro-D-ribitol acetates derived from 1,4-anhydro-D-ribitol by sequential partial methylation and acetylation in situ (*a*), or by sequential partial benzoylation and methylation, then debenzoylation and acetylation (*b*). The peaks are numbered with the compound numbers. A Restek RTx-200 fused silica capillary column (0.25 mm×30 m, 0.25-μm film thickness) and a guard column (0.25 mm×1 m) were used. The temperature of the column was programmed from 80 to 210°C at 2°C/min, with no initial hold.

two methods were fractionated by semipreparative reversed-phase HPLC using a Rainin C_{18} column. The individual components (see Table 1 for capacity factors) were collected and analyzed by ^{1}H NMR spectroscopy. Where needed, compounds were further purified by normal-phase HPLC using a Regis Spherisorb silica gel column (Table 1). A portion of each benzoate was then debenzoylated, and the product was acetylated to afford the partially methylated 1,4-anhydro-D-ribitol acetate in chromatographically pure form.

^{1}H NMR spectra of partially methylated 1,4-anhydro-D-ribitol benzoates (**2b–8b**).— Given in Table 2 are ^{1}H NMR spectral data which provide unique and unambiguous assignments for compounds **2b–8b**. The individual components of the mixture (see Table 1) were identified based upon analysis of the chemical shifts, multiplicities, and coupling constants of the ring hydrogen resonances. The positions of the benzoyl groups were readily discerned by the large downfield chemical shift of the corresponding ring hydrogen resonances. Overlapping signals were assigned by selective decoupling experiments, and the H-1 protons were assigned using 1D NOE experiments by saturating at each H-1 proton and viewing the effects on H-2 and the other H-1 proton. The NOE experiments were successful for the tribenzoate (**8b**) and two of the dibenzoates (**5b,**

6b), and in each case the further downfield H-1 proton was shown to have a stronger effect on H-2 and was thus assigned as H-1β. For the monobenzoyl derivatives, the individual H-1 resonances were not assigned due to lack of sufficient amounts of material.

The ^{1}H NMR spectra of the 3,5-di-*O*-benzoyl (**7b**) and 5-*O*-benzoyl (**4b**) derivatives were very complex when obtained in deuteriochloroform. In acetonitrile, however, all proton resonances were well resolved and were easily assigned by COSY and selective decoupling experiments.

Mass spectra of the methylated 1,4-anhydro-D-*ribitol acetates* (**1**, **2a–8a**).—Compounds **1** and **2a–8a** were analyzed by GLC–CIMS and –EIMS. The CI (ammonia) mass spectra of all compounds displayed the expected $(M + H)^+$ and $(M + NH_4)^+$ ions, which identifies them as anhydropentitol derivatives due to their unique molecular weights. Their EI mass spectra (Fig. 2) are characteristic of 1,4-anhydropentitol derivatives, because all spectra display either an (M − 45) peak, or an (M − 73) peak, arising by loss [6,17] of the exocyclic CH_2OCH_3 or CH_2OAc group, respectively. The EI mass spectra of the positional isomers are also diagnostically different and are very similar to those of the respective positional isomers of 1,4-anhydro-D-xylitol [14].

One of the major fragmentation pathways (see Scheme 1) for derivatives of this type [14] begins with loss of the exocyclic methoxymethyl group (M − 45) or acetoxymethyl group (M − 73), to give fragment ions **9a** and **9b**, respectively. The further elimination of methanol or acetic acid from the (M − 45) ion (**9a**) gives rise to fragment ions **10a** or **11a** (M − 77) and **10b** or **11b** (M − 105), respectively, whereas the elimination of methanol or acetic acid from the (M − 73) ion (**9b**) gives rise to fragment ions **10b** or **11b** (M − 105) and **10c** or **11c** (M − 133), respectively. Some other key ions are those

Table 1
Reversed-phase and normal phase HPLC capacity factors for partially methylated 1,4-anhydro-D-ribitol benzoates **2b–8b**

Compound	Position of benzoate	Capacity factor (k') [a]	
		Reversed-phase [b]	Normal-phase [c]
2b	2-	1.37	
3b	3-	1.44	
4b	5-	0.83	
5b	2,3-	3.81	6.85
6b	2,5-	4.04	7.44
7b	3,5-	3.91	8.88
8b	2,3,5-	5.07	

[a] Capacity factors (k') were calculated from the equation $k' = (t_r - t_0)/t_0$ where t_r is the absolute retention time of the compound of interest and t_0 is the dead time (5.0 min for reversed-phase and 0.88 min for normal-phase) of the column. The dead time was calculated from the equation $t_0 = (0.5Ld_c^2)/F$ where 0.5 is a unitless constant, L is the length of the column in cm, d_c is the column diameter in cm, and F is the column flow rate in mL/min.

[b] Reversed-phase HPLC was conducted on a Rainin Dynamax semipreparative C_{18} column as described in the text.

[c] Normal phase HPLC was conducted on a Regis Spherisorb silica gel column as described in the text. The column was eluted with 90:10 hexane–EtOAc.

Table 2
^{1}H NMR data (δ in ppm, J in Hz in brackets) for partially methylated 1,4-anhydro-D-ribitol benzoates **2b–8b** [a,b]

Compound	H-1β [c]	H-1α [c]	H-2 [d]	H-3 [d]	H-4 [d]	H-5	H-5′	*O*-Me
2b	4.28 dd	4.04 dd	5.62 dt	3.90 dd	4.07 m	3.68 dd	3.54 dd	3.44, 3.42
	(4.7, 10.6)	(2.4, 10.6)	(2.5, 4.7)	(5.0, 7.9)		(2.4, 10.7)	(4.6, 10.7)	
3b	4.20 dd	3.88 dd	4.13 q	5.34 t	4.25 m	3.63 dd	3.57 dd	3.42, 3.38
	(5.9, 9.0)	(6.1, 9.0)	(5.6)	(5.1)		(2.9, 10.6)	(4.3, 10.6)	
4b	3.90 dd	3.83 dd [e]	3.96 br q	3.81 dd [e]	4.04 ddd	4.44 dd	4.27 dd	3.38, 3.37
(CD_3CN)	(4.1, 9.8)	(3.0, 9.8)	(4.0)	(4.6, 7.1)	(3.4, 5.2, 7.1)	(3.4, 11.8)	(5.2, 11.8)	
5b	4.47 dd	4.07 dd	5.69 q	5.57 t	4.34 dt	3.71 dd	3.63 dd	3.45
	(5.5, 10.1)	(4.7, 10.1)	(5.2)	(5.7)	(2.9, 5.2)	(2.8, 10.6)	(4.7, 10.6)	
6b	4.31 dd [e]	4.11 dd	5.68, dt	3.93 dd	4.29 ddd [e]	4.65 dd	4.44 dd	3.43
	(4.3, 10.7)	(2.3, 10.7)	(2.1, 4.6)	(4.8, 8.0)	(3.0, 5.2, 8.0)	(3.0, 11.9)	(5.2, 11.9)	
7b	4.13 dd	3.86 dd	4.19 q	5.40 t	4.39 dt [e]	4.48 dd	4.42 dd [e]	3.32
(CD_3CN)	(5.2, 9.4)	(4.9, 9.4)	(5.1)	(5.2)	(4.1, 4.9)	(4.0, 11.5)	(4.7, 11.5)	
8b	4.50 dd	4.15 dd	5.79 q	5.64 t	4.55–4.59	4.71 dd	4.54 dd	
	(5.1, 10.3)	(4.0, 10.3)	(4.8)	(5.9)	m	(2.9, 11.4)	(4.7, 11.4)	

[a] Additional resonances were observed for benzoyl hydrogens at δ 6.9–8.2; spectra were acquired in $CDCl_3$ unless otherwise indicated.
[b] Multiplicities include br, broad; dd, doublet of doublets; ddd, doublet of doublets of doublets; q, quartet; t, triplet; dt, doublet of triplets; m, multiplet.
[c] Assignments may be reversed for compounds **2b–4b**.
[d] The resonances assigned as a doublet of triplets (dt) and triplet (t), were actually a doublet of doublets of doublets (ddd) and a doublet of doublets (dd) respectively, with two coupling constants having nearly equal magnitude and the resonances assigned as a quartet (q) were actually doublet of doublets of doublets (ddd) with three coupling constants having nearly equal magnitude.
[e] Resonance partially obscured.

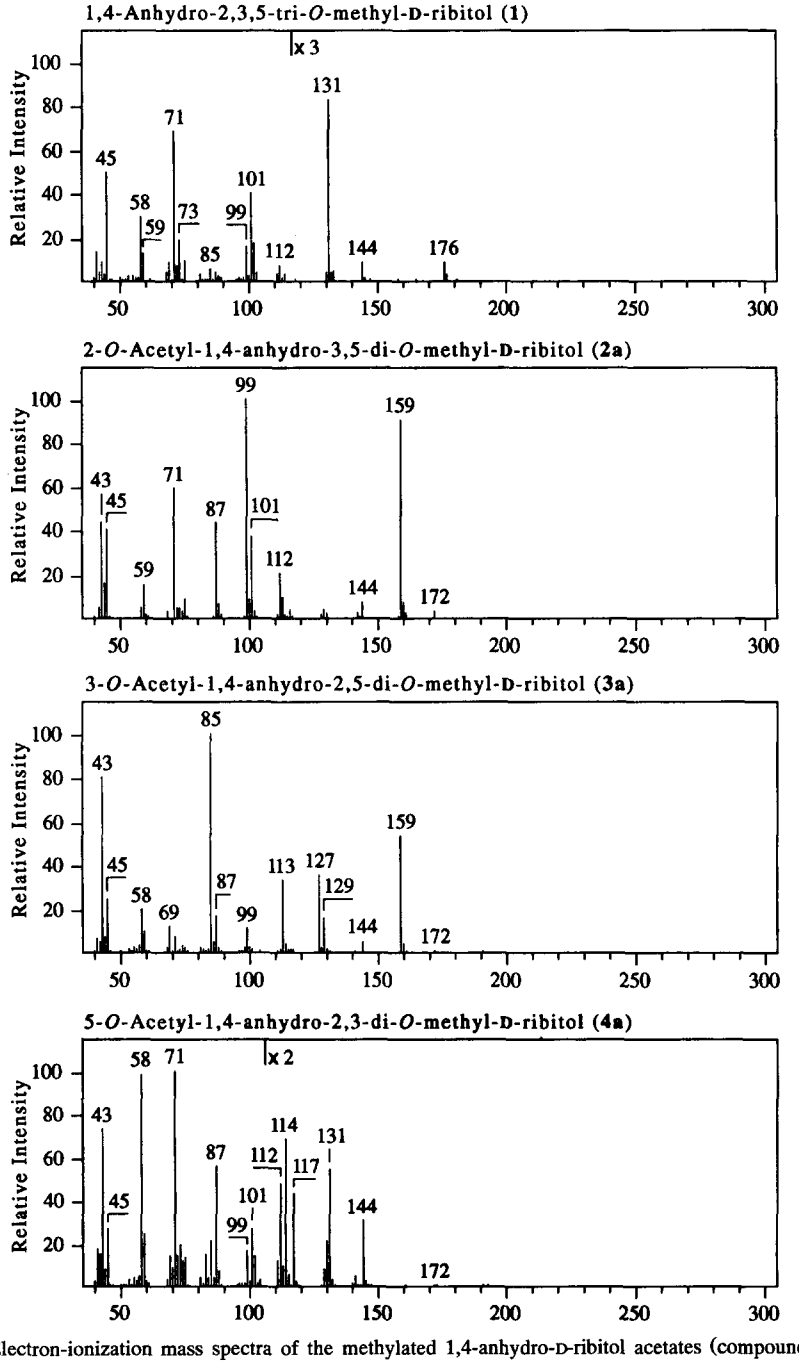


Fig. 2. Electron-ionization mass spectra of the methylated 1,4-anhydro-D-ribitol acetates (compounds **1** and **2a–8a**).

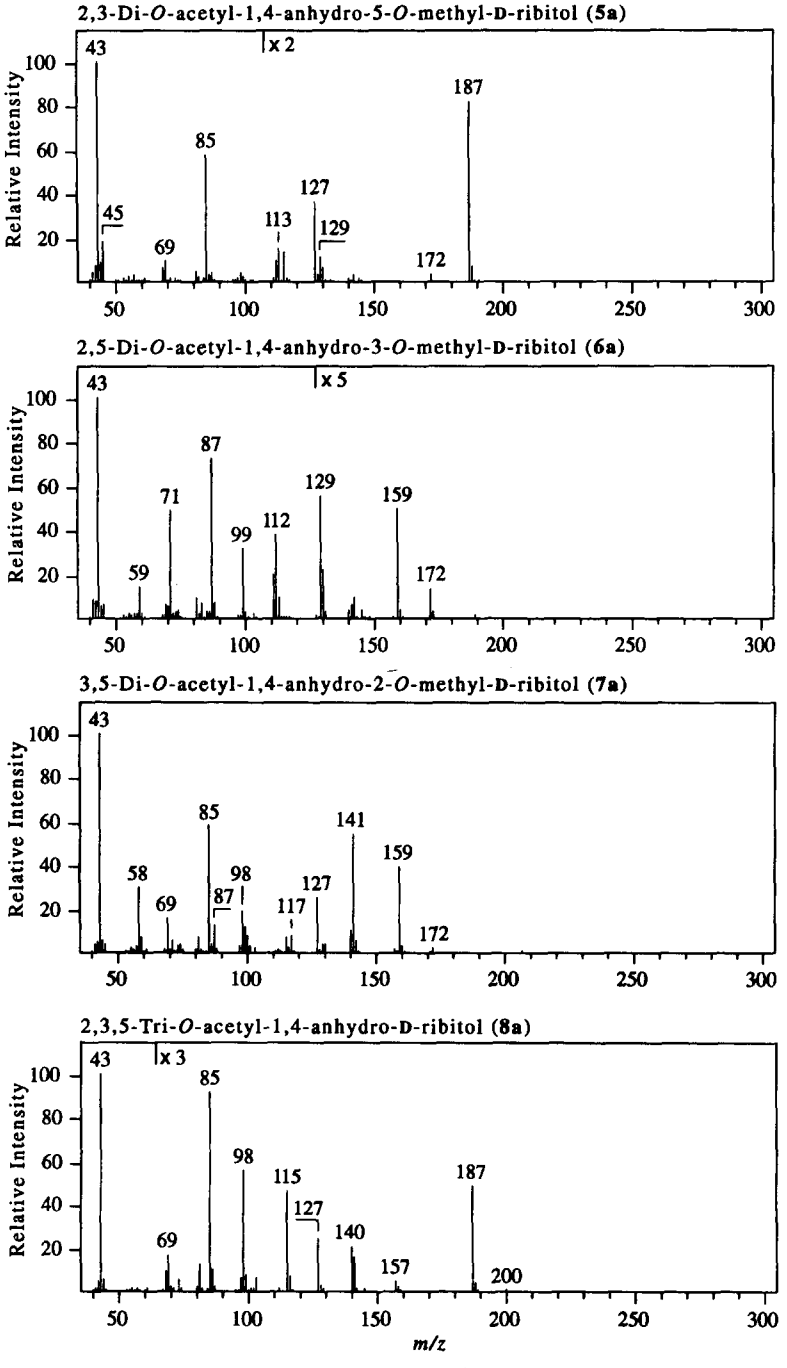
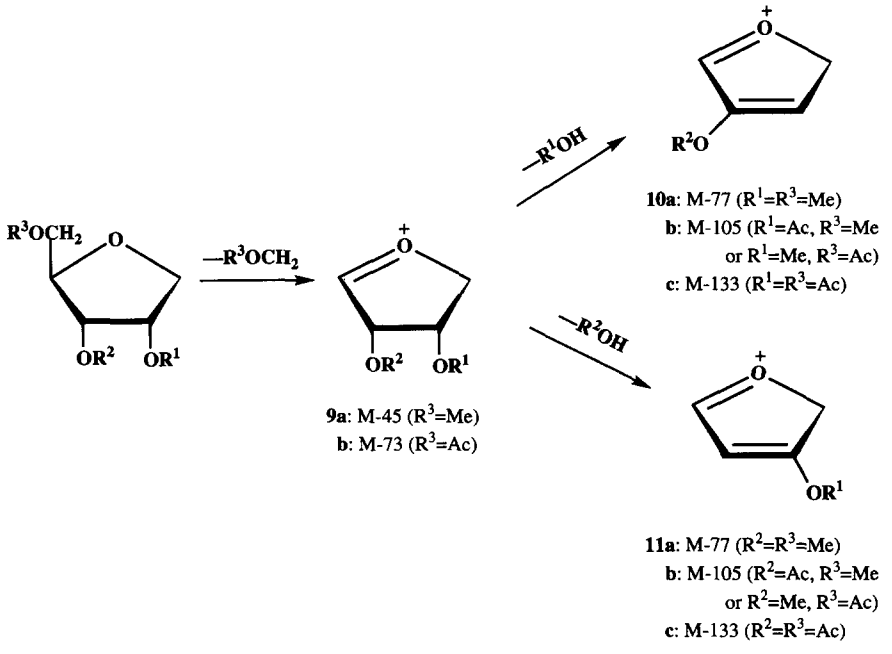


Fig. 2 (continued).



Scheme 1.

that arise from elimination of methanol (M − 32) or acetic acid (M − 60) from the molecular ion. It should be noted that there are other ions in the spectra of as yet unknown origin that are also diagnostic for identification of a particular positional isomer. For example, the *m/z* 71 ion is always prominent in the spectra of 3-*O*-methyl derivatives, whereas the *m/z* 85 ion is always prominent in the spectra of 3-*O*-acetyl derivatives. The same correlations were made for the partially methylated and acetylated derivatives of 1,4-anhydro-D-xylitol [14] and 1,4-anhydro-L-fucitol [18].

Table 3
Selected fragments observed in the electron-ionization mass spectra of compounds **1** and **2a–8a**

Compound [a]	Mol wt	(M−32)	(M−45)	(M−60)	(M−73)	(M−77)	(M−105)
1 (None)	176	+	+	— [b]	+ [c]	+	+ [d]
2a (2)	204	+	+	+	—	—	+
3a (3)	204	+	+	+	—	+	+
4a (5)	204	+	—	+	+	—	+
5a (2,3)	232	—	+	+	—	—	+
6a (2,5)	232	—	—	+	+	—	—
7a (3,5)	232	—	—	+	+	—	+
8a (2,3,5)	260	—	—	+	+	—	—

[a] Position of *O*-acetyl group is in parenthesis.
[b] Not observed.
[c] Isotope peak of *m/z* 102.
[d] Derived from a fragmentation pathway other than that depicted in Scheme 1.

Table 4
Linear temperature programmed gas–liquid chromatography retention indices (LTPGLCRI) of compounds **1** and **2a–8a** [a]

Compound [b]	Stationary phase		
	DB-5	DB-17	RTx-200
1 (None)	1215.73 [c]	1441.09	1425.94
3a (3)	1320.42	1565.30	1572.15
2a (2)	1320.42	1569.13	1575.89
4a (5)	1394.75	1663.78	1725.60
5a (2,3)	1460.43	1731.62	1837.81
7a (3,5)	1480.59	1767.38	1846.13
6a (2,5)	1492.57	1781.03	1860.55
8a (2,3,5)	1617.28	1923.81	2100.00

[a] Indices were determined using a mixture of all compounds co-injected with the homologous series of *n*-alkanes from $C_{11}H_{24}$ to $C_{26}H_{54}$. Values were calculated from the equation $LTPGLCRI_{(x)} = 100n + [100\Delta n (t_{R(x)} - t_{R(n)})/(t_{R(n+\Delta n)} - t_{R(n)})]$ where $LTPGLCRI_{(x)}$ is the linear temperature programmed gas–liquid chromatography retention index of the compound of interest (x), n is the carbon number of the *n*-alkane eluting just before the compound of interest (x), Δn is the difference in carbon number between the *n*-alkane standard eluting just before and just after the compound of interest (x), and $t_{R(n)}$ and $t_{R(n+\Delta n)}$ are the absolute retention times of the *n*-alkanes eluting just before and just after the compound of interest (x).
[b] Position of *O*-acetyl group is in parenthesis.
[c] Values are listed according to increasing retention index on the DB-5 column.

As summarized in Table 3, there was excellent correlation between the presence or absence of fragment ions at (M − 32), (M − 45), (M − 60), (M − 73), (M − 77) and (M − 105) and the positions of substitution of *O*-methyl and *O*-acetyl groups. The (M − 45) ion (**9a**) is present only in the mass spectra of 5-*O*-methyl derivatives and is always much greater in intensity than the (M − 73) ion, if indeed the latter is even present. In contrast, the (M − 73) ion (**9b**) is prominent only in the spectra of 5-*O*-acetyl derivatives, and the (M − 45) ion is absent. Mono-*O*-acetyl derivatives (**2a** and **3a**) that give an (M − 45) ion (**9a**) are readily distinguished by the relative intensities of the ions at m/z 85 and m/z 127 (**10a** or **11a**), which are prominent only in the 3-*O*-acetyl derivative (**3a**). The two di-*O*-acetyl regioisomers (**6a** and **7a**) that give an (M − 73) ion (**9b**) are also easily distinguished by the intensities of the ions at m/z 85 and m/z 127 (**10b** or **11b**) which, again, are prominent only in the 3,5-di-*O*-acetyl derivative (**7a**).

GLC retention indices of methylated 1,4-anhydro-D-*ribitol acetates* (**1, 2a–8a**).—GLC analysis based upon linear temperature programmed gas–liquid chromatography retention indices [19,20] (LTPGLCRI) values provides the most convenient way in which to routinely analyze carbohydrates when the reductive-cleavage method is used. Research in our laboratory has indicated that the use of three different types of capillary columns is necessary in order to generate a unique set of LTPGLCRI values for each authentic standard [21] and that LTPGLCRI values are a much more accurate and reliable way to report retention data than relative retention time values. Therefore, the retention data for compounds **1** and **2a–8a** were obtained on DB-5, DB-17, and RTx-200 capillary columns under the conditions reported previously [13], and their LTPGLCRI values were then calculated (Table 4). In all cases, the standard deviations were less than 0.1 units, which corresponds to an absolute retention time difference of less than 0.3 s.

3. Experimental

General.—Reagents and HPLC solvents were purified and stored as previously described [14].

Instrumentation.—HPLC was performed using a Beckman model 338 System Gold chromatograph. Reversed-phase HPLC was conducted on a 5-μm particle-size Rainin Dynamax Microsorb Semipreparative C_{18} column (1 × 25 cm) connected to a guard column (1 × 5 cm) having the same packing. Normal-phase HPLC was performed on a 5-μm particle-size Regis Spherisorb silica gel column (4.6 mm × 25 cm). Both systems were fitted with a 2.0-μm pore-size stainless steel in-line filter frit installed between the solvent mixing chamber and the injector, and a 0.50-μm pore-size stainless steel filter frit installed between the injector and the guard column or the silica column. Chromatography was conducted at a flow rate of 3 mL/min.

Gas–liquid chromatograms were obtained using a Hewlett–Packard 5890 gas–liquid chromatograph using the same columns and conditions as previously described [13]. GLC–MS analyses were performed using a Finnegan-MAT 95 high-resolution, double-focusing, reverse-geometry mass spectrometer equipped with a Hewlett–Packard 5890A Series II gas–liquid chromatograph and a Digital Equipment Corporation model 2100 workstation. EI mass spectra and CI mass spectra with ammonia as the reagent gas were acquired under the same conditions used previously [13].

^{1}H NMR spectra were recorded on a Varian VXR-500S NMR spectrometer at room temperature in $CDCl_3$ or CD_3CN and were referenced to internal tetramethylsilane or acetonitrile (δ 1.93), respectively.

*Partially methylated 1,4-anhydro-*D*-ribitol benzoates* (**2b–8b**).—1,4-Anhydro-D-ribitol, prepared by the method of Heard et al. [22] was a generous gift from Dr Robert Barker. Its purity was confirmed by analysis of its acetate by ^{1}H NMR spectroscopy and GLC. The sample was dried under high vacuum before use.

I. Partial methylation followed by benzoylation in situ [13].—1,4-Anhydro-D-ribitol (20 mg) was placed into each of three flame-dried round bottom flasks. Dry Me_2SO (1 mL) was added to each, and the flasks were sealed and vented with nitrogen. To each reaction was added 0.75, 1.5, and 2.5 equiv, respectively, of lithium methylsulfinylmethanide, then, after stirring for 60 min, iodomethane (0.2 mL) was added to each reaction. After stirring for an additional 30 min, excess CH_3I was removed by blowing dry nitrogen gas above the reaction solution for 30 min. A portion (ca. 10%) of each reaction mixture was saved, and the remainder was benzoylated as previously described [13].

II. Partial benzoylation followed by methylation [14].—After drying under high vacuum in a flame-dried 50-mL conical flask, 59.5 mg of 1,4-anhydro-D-ribitol was dissolved in 6 mL of dry pyridine (stored over CaH_2) and then 103.1 μL (2.0 equiv) of benzoyl chloride and 50 μL of *N*-methylimidazole were added. After stirring for 3 h, cold satd aq $NaHCO_3$ (6 mL) was added, and the reaction mixture was stirred vigorously for 3–4 h. The reaction solution was evaporated to dryness under vacuum to yield a white solid that was partitioned between dichloromethane (20 mL) and water (15 mL). The organic layer was extracted three times with deionized water, dried (anhyd Na_2SO_4), and concentrated to a clear syrup. After drying under high vacuum overnight,

the syrup was dissolved in 3 mL of freshly distilled dichloromethane and 131.4 mg of $BF_4 \cdot OMe_3$ (2 equiv) was then added. After stirring vigorously overnight, 6 mL of MeOH was added, the reaction solution was stirred for 1 h, and was then evaporated under vacuum to give a mixture of partially methylated 1,4-anhydro-D-ribitol benzoates as a clear syrup.

Before HPLC separation, the mixtures were dissolved in acetonitrile, passed through a Waters Sep-Pak® Vac RC (500 mg) C_{18} cartridge, and then filtered through a 0.2-μm pore-size Acrodisc into a 4-mL screwcap vial fitted with a Teflon liner. Separation of the benzoates (**2b–8b**) was accomplished by reversed-phase HPLC (see Table 1) using a semipreparative C_{18} column. Aliquots (20 μL) of the mixtures were applied to the column, which was equilibrated in 50:50 MeCN–H_2O. After injection, the column was eluted for 10 min with 50:50 MeCN–H_2O, followed by a linear gradient to 95:5 MeCN–H_2O over 20 min. The individual components from eight or more applications were collected, combined and, after removal of solvent by evaporation under vacuum, were dissolved in $CDCl_3$ and identified by ^1H NMR spectroscopy.

*Methylated 1,4-anhydro-*D*-ribitol acetates* (**1, 2a–8a**).—About one-third of each pure benzoate, obtained as described above, was subjected to debenzoylation (NaOMe, MeOH) then acetylation as previously described [13]. The pure standards so obtained, and the tri-*O*-methyl derivative (**1**), which was synthesized independently, were then chromatographed individually on the three aforementioned GLC columns under the conditions already described [13]. In this way, the relative order of elution of the standards on each column was determined.

*Determination of LTPGLCRI values of methylated 1,4-anhydro-*D*-ribitol acetates* (**1, 2a–8a**).—In order to expedite acquisition of their mass spectra and retention time data and to be sure that the mixture of standards contained only the title compounds, further studies used a mixture prepared by combining aliquots of the individual pure standards [13]. The methylated anhydroalditol acetate standard solution was co-injected with the stock solution of *n*-alkanes from $C_{11}H_{24}$ to $C_{26}H_{54}$ in such a way that their area responses were comparable. The LTPGLCRI values were determined in triplicate on each of the columns using the equation depicted in Table 4.

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