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Authentic standards for the reductive-cleavage method. The positional isomers of partially methylated and acetylated or benzoylated 1,5-anhydro-D-fucitol

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ABSTRACT

Described herein is an efficient method for the synthesis of the eight positional isomers of methylated and acetylated or benzoylated 1,5-anhydro-D-fucitol. The compounds are generated simultaneously by partial methylation of 1,5-anhydro-D-fucitol and subsequent benzoylation, and the individual isomers are obtained in pure form by high-performance liquid chromatography. Debenzoylation of the latter and acetylation yielded the desired 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. Also reported for the acetates and the tri-*O*-methyl derivative are their linear temperature programmed gas–liquid chromatography retention indices on three different capillary columns.

INTRODUCTION

The reductive-cleavage method[1] for glycosyl-linkage analysis is based upon methylation analysis but departs from it significantly in that reductive cleavage, rather than hydrolysis, of glycosidic linkages in the fully methylated glycan is performed. The partially methylated *anhydroalditols* so obtained are either converted to their benzoates, which are separated by high-performance liquid chromatography (HPLC) and characterized by ^{1}H NMR spectroscopy[2–5], or to their acetates, which are separated by gas–liquid chromatography (GLC) and analyzed by mass spectrometry (MS)[6–12]. Although the former method is laborious and requires greater amounts of sample, it does not depend upon the availability of authentic standards. The latter method, which is much less laborious and much more sensitive, depends upon the availability of authentic standards. In past work, authentic standards required for the analysis were synthesized independently[7–10,13–16], but such syntheses were themselves very laborious and, in spite of

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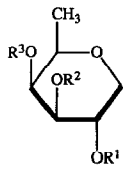
these efforts, standards representing all possible combinations of ring form and position(s) of linkage for even one sugar have yet to be derived.

Clearly, a new approach to the synthesis of such standards is needed. Described herein is such an approach, as illustrated for the synthesis of standards (**1–8**) representing all possible positions of linkage in D-fucopyranosyl residues. As an aid to those who wish to use the reductive-cleavage method in either of its forms, ^{1}H NMR spectra of the seven methylated and benzoylated positional isomers of 1,5-anhydro-D-fucitol (**2b–8b**) are reported, as are the electron-ionization (EI) mass spectra of the corresponding acetates (**2a–8a**) and the tri-*O*-methyl derivative (**1**). In addition, the retention indices of the acetates (**2a–8a**) and the tri-*O*-methyl derivative (**1**) on three different GLC columns are reported, using a much more accurate method but one not heretofore used for carbohydrate derivatives useful in glycosyl-linkage analysis.



	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

RESULTS

Synthesis.—The tri-*O*-methyl (**1**), tri-*O*-acetyl (**8a**), and tri-O-benzoyl (**8b**) derivatives of 1,5-anhydro-D-fucitol were prepared from the latter by total methylation[17], acetylation, and benzoylation, respectively. The remaining six partially methylated and benzoylated positional isomers (**2b–7b**) were prepared by partial methylation[18] of 1,5-anhydro-D-fucitol, followed by benzoylation in situ. The resultant mixture of partially methylated 1,5-anhydro-D-fucitol benzoates was then separated by semipreparative reversed-phase HPLC using a Rainin C_{18} column (Fig. 1). The individual components were isolated in pure form and, after removal of solvent, were identified by ^{1}H NMR spectroscopy. A portion of each benzoate was then debenzoylated (NaOMe in MeOH), and the product was acetylated, affording the partially methylated 1,5-anhydro-D-fucitol acetate in chromatographically pure form.

^{1}H NMR spectra of partially methylated 1,5-anhydro-D-fucitol benzoates (**2b–8b**). —Given in Table I are ^{1}H NMR spectral data for compounds **2b–8b**. The individual components of the mixture (see Fig. 1) were easily identified based upon a straightforward analysis of the chemical shifts and coupling constants of the ring hydrogen resonances. All resonances displayed the multiplicities and coupling constants expected for a tetrahydropyran derivative of the *galacto* configuration in

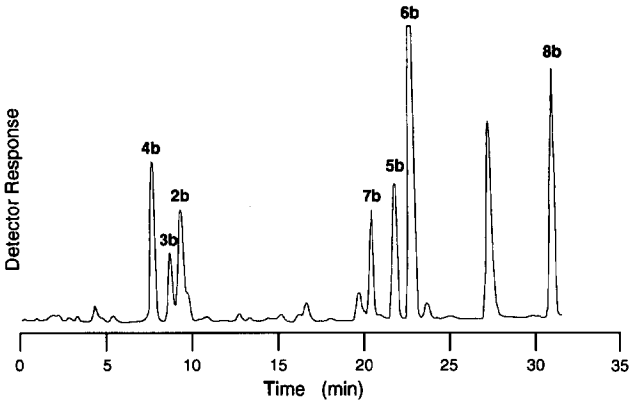


Fig. 1. High-performance liquid chromatogram of the partially methylated 1,5-anhydro-D-fucitol benzoates derived from 1,5-anhydro-D-fucitol by sequential partial methylation and benzoylation in situ. The peaks are numbered with the compound numbers.

the $^4C_1$ conformation, and, in addition, the positions of substitution of benzoyl groups were readily discerned based upon the large downfield shift of the respective ring hydrogen resonances.

Mass spectra of the methylated 1,5-anhydro-D-fucitol acetates (**1**, **2a–8a**).—Compounds **1** and **2a–8a** were analyzed by chemical ionization (CI) mass spectrometry with ammonia as the reagent gas and by EI mass spectrometry. The CI (NH_3) mass spectra of all compounds displayed the expected $(M + H)^+$ and $(M + NH_4)^+$ ions,

TABLE I

^{1}H NMR data (δ in ppm, J in Hz in brackets) for partially methylated 1,5-anhydro-D-fucitol benzoates **2b–8b** [a,b]

Compound	H-1e	H-1a [c]	H-2 [c]	H-3	H-4	H-5	H-6	*O*-Me
2b	4.21 dd (5.5, 11.0)	3.26 t (10.5)	5.45 dt (5.5, 9.8)	3.49 dd (2.9, 9.6)	3.54–3.56 complex	3.54–3.56 complex	1.33 d (6.3)	3.52, 3.64
3b	4.16 d (5.4, 11.4)	3.23 t (10.9)	3.86 dt (5.4, 10.0)	5.08 dd (3.2, 9.7)	3.59 br d (3.2)	3.61 br q (6.4)	1.29 d (6.4)	3.45, 3.51
4b	4.19 dd (5.3, 11.3)	3.21 t (10.9)	3.63 dt (5.3, 9.9)	3.35 dd (3.4, 9.3)	5.58 br d (3.4)	3.68 br q (6.4)	1.2 d (6.4)	3.46, 3.51
5b	4.31 dd (5.6, 11.1)	3.44 t (10.7)	5.72 dt (5.6, 10.3)	5.41 dd (3.0, 10.1)	3.69 br d (3.2)	3.72 br q (6.4)	1.35 d (6.4)	3.56
6b	4.32 dd (5.5, 11.1)	3.40 t (10.8)	5.46 dt (5.5, 10.1)	3.66 dd (3.4, 9.8)	5.69 br d (3.4)	3.79 br q (6.4)	1.26 d (6.4)	3.45
7b	4.28 dd (5.3, 11.5)	3.38 t (10.9)	3.85–3.91 complex	5.30 dd (3.5, 9.8)	5.64 br d (3.5)	3.85–3.91 complex	1.24 d (6.4)	3.44
8b	4.43 dd (5.4, 11.2)	3.56 t (10.7)	5.72 dt (5.4, 10.2)	5.65 dd (3.4, 10.1)	5.75 br d (3.4)	3.98 br q (6.4)	1.30 d (6.4)	

[a] Additional resonances were observed for benzoyl hydrogens at δ 7.25–8.20. [b] Multiplicities include br, broad; d, doublet; dd, doublet of doublets; q, quartet; t, triplet; dt, doublet of triplets. [c] The resonances assigned as a triplet (t) and a doublet of triplets (dt) were actually a doublet of doublets (dd) and doublet of doublets of doublets (ddd), respectively, with a pair of coupling constants having nearly equal magnitude.

which, because of their unique molecular weights, readily identifies them as deoxyanhydrohexitol derivatives. The EI mass spectra (Fig. 2) of the compounds (**1, 2a–8a**) readily identified them as 6-deoxyanhydrohexitol derivatives since none displayed characteristic fragment ions for loss[19] of exocyclic methoxymethyl ($MeOCH_2$, M − 45) or acetoxymethyl ($AcOCH_2$, M − 73) groups. Although fragmentation pathways for derivatives of this type have not been worked out, it is clear from inspection of their mass spectra (Fig. 2) that they are diagnostically different.

*GLC retention indices of methylated 1,5-anhydro-*D*-fucitol acetates* (**1, 2a–8a**).—In order to develop a database of GLC retention indices that would truly be useful, it was necessary at the outset to develop a set of separation parameters for potential reductive-cleavage products of widely differing structure. Therefore, a series of studies were performed using a mixture containing the title compounds as well as those derivable from various pentose, hexose, and aminosugar residues. These

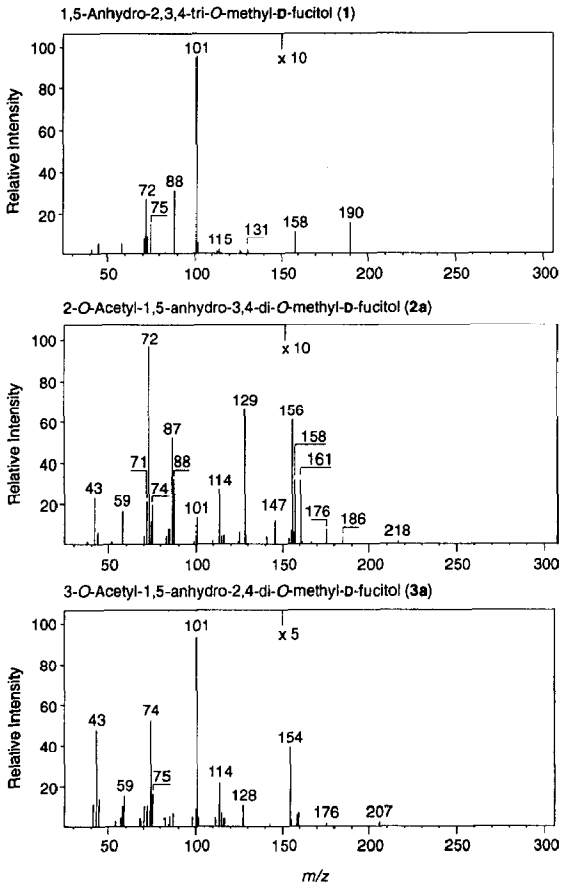


Fig. 2. Electron-ionization mass spectra of the methylated 1,5-anhydro-D-fucitol acetates (compounds **1** and **2a–8a**).

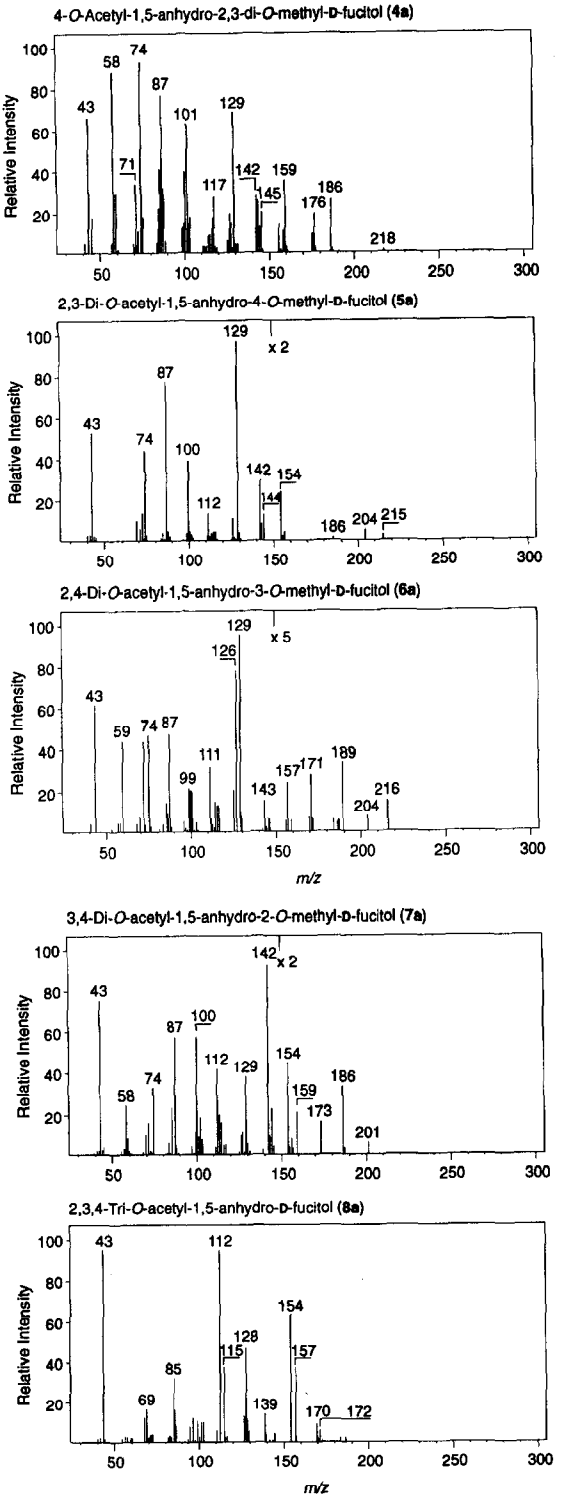


Fig. 2 (continued).

compounds were chromatographed on several different GLC columns using a variety of temperature programs, and from these studies three different capillary GLC columns giving substantially different separation parameters were selected. Of the three columns, one (DB-5) had a relatively nonpolar stationary phase (5% phenyl–95% methyl polysiloxane), one (DB-17) a more polarizable stationary phase (50% phenyl–50% methyl polysiloxane), and one (RT_X-200) a relatively polar stationary phase (50% trifluoropropyl–50% methyl polysiloxane). A single linear temperature program suitable for all three columns was selected so that chromatography could be conducted on any two of the columns at the same time by using a two-way splitter.

A series of studies were also performed (data not reported) in order to identify the most accurate and reproducible way to report retention time data. The relative retention times[20] for the compounds were measured using *myo*-inositol hexaacetate as an internal standard[21] and for comparison, their linear temperature programmed gas–liquid chromatography retention indices (LTPGLCRI) were measured using a mixture of the homologous series of *n*-alkanes from $C_{11}H_{24}$ to $C_{26}H_{54}$ as internal standards[22]. From these studies, it was clear that LTPGLCRI values were more precise (standard deviation) by a factor of ten, much less dependent upon carrier gas linear velocity, and more reliably reproduced on a different instrument equipped with a different column of the same type.

Given in Table II are the LTPGLCRI values for compounds **1** and **2a–8a** as determined under these conditions on DB-5, DB-17, and RT_X-200 capillary columns. Analyses were performed in triplicate on each column using a mixture of

TABLE II

Linear temperature programmed gas–liquid chromatography retention indices (LTPGLCRI, ±standard deviation) of compounds **1** and **2a–8a** [a]

Compound	Stationary Phase		
	DB-5	DB-17	RT_X-200
1	1226.52 (±0.02) [b]	1439.33 (±0.03)	1387.49 (±0.05)
4a	1327.30 (±0.02)	1580.01 (±0.03)	1536.59 (±0.03)
3a	1381.16 (±0.03)	1639.09 (±0.03)	1662.98 (±0.03)
2a	1419.35 (±0.03)	1665.56 (±0.06)	1753.81 (±0.01)
7a	1476.51 (±0.04)	1763.47 (±0.03)	1831.70 (±0.02)
6a	1503.28 (±0.05)	1796.46 (±0.03)	1856.63 (±0.01)
5a	1515.80 (±0.05)	1790.20 (±0.00)	1935.33 (±0.03)
8a	1588.91 (±0.06)	1895.83 (±0.05)	2038.11 (±0.02)

[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 $LTPGLCRI_{(x)} = 100n + 100 \cdot \Delta n \cdot (t_{R(x)} - t_{R(n)} / t_{R(n+\Delta n)} - t_{R(n)})$ 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] Values are listed according to increasing retention index on the DB-5 column.

all eight compounds. As is evident, the standard derivations were less than 0.1 units in all cases, which corresponds to an absolute retention time difference of less than 0.3 s.

DISCUSSION

This is the first of a series of papers describing the synthesis and spectral characterization of authentic standards for the reductive-cleavage method. The goal of these studies is to provide such data for standards representing all possible combinations of position(s) of linkage and ring form for the most frequently encountered sugars. For such an effort to be successful, a simple, rapid method for the synthesis of these compounds was needed, and, moreover, a method of synthesis was needed wherein the ring form (furan, pyran) of the anhydroalditol product was not in question. As illustrated herein for the synthesis of all the methylated and acetylated positional isomers derivable from D-fucopyranosyl residues, these requirements are easily met by a strategy involving partial methylation of the corresponding 1,5-anhydroalditol. This strategy also has the advantage of providing both the benzoyl and acetyl derivatives of the standards, as both types of derivatives are useful for structural analysis.

In choosing a method for reporting GLC retention data for the methylated/acetylated compounds, we rejected the use of relative retention times in favor of retention indices. Retention indices, as developed for isothermal GLC by Kováts[23], are considered to be the most accurate, reliable, and most easily standardized means of reporting retention data[24]. A modification of Kováts' retention index formula by van den Dool and Kratz[22] readily allows retention indices to be determined using a linear temperature program. It has been proven[24,25] for many classes of compounds that retention indices obtained in this way are more accurate over the entire range of the temperature program and less dependent on carrier gas flow rate and other instrument parameters than relative retention times. These observations were confirmed (data not reported) using partially methylated/acetylated anhydroalditols; therefore, we chose to report all retention data as linear temperature programmed gas–liquid chromatography retention indices[22]. It cannot yet be determined what the reproducibility of these values will be when determined on different lots of the same column. However, values for the title compounds have been determined on a different lot of the DB-5 column, and the retention index values were found to agree with those reported herein within a range of 0.8 units for compound **1** to 1.5 units for compound **8a**. Only experience can tell whether the values reported herein will be reproducible in different laboratories or whether some calibration of columns will be required. However, as an aid to those who wish to use the method, it is our intent to determine retention index data for all compounds using columns derived from the same manufacturer's lot, so that their relative order of elution from the columns will be known.

EXPERIMENTAL

General.—Dimethyl sulfoxide (Me_2SO) was refluxed over $LiAlH_4$ then distilled and stored over 4A molecular sieves. Lithium methylsulfinyl carbanion, prepared as previously described[11], was standardized[26] and stored at 0°C in 2-mL aliquots in 4-mL screwcap vials fitted with Teflon septa. Iodomethane was passed over a small column of silica gel, then distilled and stored over copper at 3°C under N_2. Acetic anhydride and 1-methylimidazole were separately distilled and stored under N_2 at room temperature. Benzoic anhydride was dissolved in $CHCl_3$, the solution was extracted with aq $NaHCO_3$, the $CHCl_3$ layer was evaporated to dryness under vacuum, and the product was recrystallized from petroleum ether. Reagent grade MeOH was refluxed over magnesium methoxide, distilled, and stored at room temperature over 3A molecular sieves under N_2. Chloroform was obtained from EM Science and used without further purification. Dowex-50 (H^+) cation-exchange resin was washed with MeOH, charged by elution with M HCl, then thoroughly rinsed with distilled water.

All alkane standards were obtained from Aldrich Chemical Company. A stock solution of the homologous series of alkanes from $C_{11}H_{24}$ to $C_{26}H_{54}$ was prepared by combining 20–30 mg of each alkane and diluting to 10 mL with hexane.

Instrumentation.—HPLC was performed using a Beckman model 338 System Gold chromatograph. Chromatography was performed on a 5-μm particle-size Rainin Dynamax Microsorb semi-preparative C_{18} reversed phase column (1 × 25 cm) equipped with a guard column (1 × 5 cm) having the same packing. The system was fitted with a 2.0-mm stainless steel in-line filter frit installed between the solvent mixing chamber and the injector and a 0.50-μm stainless steel filter frit installed between the injector and the guard column. Chromatography was conducted at a flow rate of 3.0 mL/min.

Analytical GLC was performed on a Hewlett–Packard 5890 gas–liquid chromatograph equipped with split/splitless injection ports, two flame ionization detectors, and Hewlett Packard 3392A or 3390A integrators. The columns used were a J&W Scientific DB-5 fused silica capillary column (0.25 mm × 30 m, 0.25-μm film thickness), a J&W Scientific DB-17 fused silica capillary column (0.25 mm × 30 m, 0.25-μm film thickness), and a Restek RT_X-200 fused silica capillary column (0.25 mm × 30 m, 0.25-μm film thickness). Each column was fitted with a J&W fused silica guard column (0.25 mm × 1 m) via a press-tight connector (J&W or Restek). Chromatography on the DB-5 and RT_X-200 columns was performed simultaneously by inserting a two-way (Y) press-tight capillary column splitter (Restek) between the guard column and the DB-5 and RT_X-200 columns. The injector and detector temperatures were set at 250 and 275°C, respectively. Helium was used as the carrier gas at measured linear velocities (methane injection, oven temperature 80°C) of 26.1, 28.4, and 27.8 cm/s, respectively, for the DB-5, DB-17, and RT_X-200 columns. The temperature program for

all columns, which was optimized according to the guidelines set forth by Krupcik et al.[27], was 80 to 300°C at 2°C/min, with no initial hold.

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. CI mass spectra were acquired with NH_3 as the reagent gas at a source temperature of 180°C, and NH_3 was introduced at a pressure of 4×10^{-4} torr as indicated on the source ionization gauge. For CI spectra, the instrument was scanned from m/z 60–650 at 1 s/decade. EI mass spectra were obtained at an ionization energy of 70 eV and at a source temperature of 200°C. For EI spectra, the instrument was scanned from m/z 20–650 at 1 s/decade. The accelerating voltage was 5 kV. Both CI and EI spectra were acquired at a resolution of 1000 (10% valley definition). Chromatography was conducted on a DB-5 column under the previously indicated conditions.

^1H NMR spectra were recorded on a Varian VXR-500S NMR spectrometer in $CDCl_3$ and were referenced to internal tetramethylsilane.

Partially methylated 1,5-anhydro-D-fucitol benzoates (**2b–8b**).—1,5-Anhydro-D-fucitol (60 mg), prepared by the method of Ness et al.[28], was dissolved in 3 mL of dry Me_2SO in a flame-dried 10-mL conical flask. Two 1-mL aliquots of this solution were removed and added separately to flame-dried 10-mL conical flasks. To each reaction was added 0.75, 1.5, and 2.5 equiv, respectively, of lithium methylsulfinyl carbanion, then, after stirring for 60 min, 0.2 mL of iodomethane was added to each reaction. After stirring for an additional 15 min, excess iodomethane was removed by venting the reaction vessel and bubbling through dry N_2. A portion (ca. 10%) of each mixture was saved, and to the remainder of each was added 0.5 mL of pyridine, 0.75 g of benzoic anhydride, and 0.2 mL of 1-methylimidazole. After stirring 30–45 min, 5 mL of aq $NaHCO_3$ and a larger stir bar were added, and the mixture was vigorously stirred overnight to ensure total hydrolysis of excess benzoic anhydride. The mixtures were combined, diluted with 75 mL of deionized water, and extracted (3 times) with 15-mL portions of $CHCl_3$. The organic extracts were combined and extracted 2 times each with 50-mL portions of satd aq $NaHCO_3$, 2 M H_2SO_4, and deionized water. The organic layer was dried (anhyd Na_2SO_4) then concentrated under vacuum to a syrup, and the residue was dissolved in MeCN, transferred to a 4-mL screwcap vial fitted with a Teflon liner, and concentrated to ~ 0.2 mL under vacuum.

Separation of the above mixture of benzoates (**2b–8b**) was accomplished by reversed-phase chromatography (see Fig. 1) using a semipreparative C_{18} column. Aliquots (20 μL) of the mixture were applied to the column, which was equilibrated in 40:60 MeCN–H_2O. The column was then eluted with a linear gradient to 95:5 MeCN–H_2O over 40 min. The individual components from eight or more applications were collected and combined and, after removal of solvent by evaporation under vacuum, were dissolved in $CDCl_3$ and identified by ^1H NMR spectroscopy.

Methylated 1,5-anhydro-D-fucitol acetates (**1, 2a–8a**).—Approximately one third to one half of each pure benzoate, obtained as described above, was transferred to a 4-mL screwcap vial, solvent was evaporated under a stream of dry N_2, and the product was redissolved in 1 mL of dry MeOH. To each vial was added 10–15 mg of freshly cleaned Na and after stirring for 4 h, excess Dowex-50 (H^+) cation-exchange resin was added to neutralize the methoxide anion. The solutions were stirred 30 min, filtered into a 4-mL screwcap vial, and carefully evaporated just to dryness under a gentle stream of dry N_2. The products were then acetylated by the addition of 1 mL of Ac_2O and 0.1 mL of 1-methyl-imidazole. After stirring for 15 min, ice chips were added, and the mixtures were stirred for an additional 30–60 min. The mixtures were then diluted with $CHCl_3$ (1–2 mL) and extracted sequentially with 2-mL portions of 2 M H_2SO_4 (2 times), satd aq $NaHCO_3$ (2 times), and water (2 times). The final $CHCl_3$ layers were concentrated to ~ 0.5 mL under a gentle stream of dry N_2 in 4-mL vials fitted with Teflon-lined caps. The pure standards were then chromatographed individually on the three aforementioned GLC columns under the conditions already described, except that the temperature of the columns was programmed from 80–250°C at 6°C/min. In this way, the relative orders of elution of the standards on each column were determined. In order to expedite acquisition of their mass spectra, further studies used mixtures of the standards prepared by acetylation of the portions of the partial methylation reactions that were saved. The latter mixtures were also found to contain the tri-*O*-methyl derivative (**1**), whose position of elution was verified by chromatography of an independently synthesized sample.

Determination of LTPGLCRI values of methylated 1,5-anhydro-D-fucitol acetates (**1, 2a–8a**).—In order to ensure that the mixture of standards contained only the title compounds, aliquots of the individual pure standards were combined such that the integral of the area (flame ionization detection) of each component was at least 5% of the area of the most abundant component. An aliquot of the stock solution of *n*-alkanes from $C_{11}H_{24}$ to $C_{26}H_{54}$ was diluted 20-fold with hexane, then amounts of the alkane standard solution and the methylated anhydroalditol acetate standard solution were chosen for injection such that their area responses were comparable. Typically, a 1.0-μL Hamilton syringe was loaded sequentially with 0.05 μL $CHCl_3$, 0.05 μL air, 0.5 μL methylated anhydroalditol acetate standard solution, 0.05 μL air, 0.1 μL alkane standard solution, and 0.1 μL air, then injected. The manner of loading the syringe is that recommended by the manufacturers of the GLC columns. Immediately after injection, the temperature program for the column was begun. LTPGLCRI values were determined in triplicate on each of the columns using the equation depicted in Table II.

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