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PARTIALLY ETHYLATED ALDITOL ACETATES AS DERIVATIVES FOR ELUCIDATION OF THE GLYCOSYL LINKAGE-COMPOSITION OF POLYSACCHARIDES*

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ABSTRACT

The use of partially ethylated alditol acetates for the analysis by gas–liquid chromatography of the components of polysaccharides, and the glycosidic linkages of these components, is described. The derivatives are prepared by procedures analogous to those for the synthesis of partially methylated alditol acetates. Derivatization requires two successive ethylations and more-strenuous conditions of hydrolysis and reduction than for the methyl analogs. The partially ethylated alditol acetates are formed in nearly quantitative yield and give single, sharp peaks on gas chromatography. Retention-time data, relative to two internal standards, are given for 79 glycosidic linkage-isomers of mannose, galactose, glucose, arabinose, xylose, rhamnose, and fucose, on four g.l.c. columns. One of these columns is a newly developed, highly polar, capillary column. Direct comparisons of these retention times to retention times of partially methylated alditol acetates are made. The ethyl analogs are eluted sooner that the corresponding methyl derivatives, and the amount of this shift in elution time is dependent upon the number of alkyl groups in the derivative. This change in elution time allows separation of many polysaccharide components by g.l.c. that are not separable as their partially methylated alditol acetates. Others, separated as their *O*-methyl derivatives, are coeluted as their partially ethylated alditol acetates. The two derivatives thus provide excellent complementary procedures because of their differential chromatographic separation and because of the similarity of their preparation.

INTRODUCTION

Many carbohydrate derivatives have been developed for gas–liquid chromatographic (g.l.c.) and combined gas chromatographic–mass spectrometric (g.l.c.–m.s.) analysis. Among these are the trimethylsilyl (Me_3Si) ethers[1–6], *O*-Me_3Si lactones[7–9], methyl ethers[10–13], acetates[14–18], trifluoroacetates[19–23], alkylboronates[24,25],

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O-Me_3Si *O*-methyloximes[26–28], *O*-methyl *O*-Me_3Si alditols and aldoses[29,30], permethylated aldonates[31], aldononitrile acetates[32,33], partially methylated aldononitrile acetates[34,35], and *O*-Me_3Si aldononitriles[36]. However, the derivatives used most widely for determining the position of glycosidic linkages in sugar residues in a polysaccharide are the partially methylated alditol acetates developed by Lindberg *et al.*[37]. These derivatives, for glycosidic linkage-determination, and alditol acetates[18], for the total aldose composition-analysis, have been used extensively in this laboratory for the determination of primary plant cell-wall structures[38–44].

Partially methylated alditol acetates have been widely accepted. They can be prepared nearly quantitatively and yield information on glycosidic linkages through retention time and mass-spectral data. These derivatives show only one g.l.c. peak for each linkage isomer of each sugar, because the anomeric center is removed by reduction. This elimination of the anomeric centers simplifies the chromatograms, but also leads to uncertainty in the identification of some of the derivatives because of the symmetry generated. This potential limitation has been overcome by the use of sodium borodeuteride for reduction of the aldoses[37,45,46]. The deuterium label allows mass-spectral identification, although it does not permit those partially methylated alditol acetates differing only in the position of the deuterium to be separated by g.l.c.

Partially methylated alditol acetates do have some limitations that are especially evident when they are used in analyzing complex polysaccharides in primary plant cell-walls. One disadvantage is their limitation to aldose and ketose constituents. Uronic acids, often present in complex polysaccharides, require alterations in the derivatization scheme[38]. Another major analytical problem is the complexity of the chromatograms; they often display two or more overlapping peaks, making quantitation of the individual components difficult without special integration techniques. This overlap may also obscure the mass-spectral data and impede identification. This uncertainty in mass-spectral identification is especially likely if three or more peaks overlap, as the same fragments are often obtained for many of the closely related derivatives. A quantitatively minor, partially methylated, alditol acetate, that coelutes with quantitatively major components, could be completely overlooked.

We have synthesized the partially ethylated alditol acetates in an attempt to resolve some of the polysaccharide components that are coeluted as their partially methylated alditol acetates. Examples of the separations, especially those beneficial to analysis of primary plant cell-walls, are presented. These derivatives are shown to be an excellent tool for the analysis of the glycosidic linkage-composition of polysaccharides.

EXPERIMENTAL

Materials. — Dimethyl sulfoxide (Fisher Scientific) was distilled over calcium hydride at 63° (6.5 torr) and stored under nitrogen in sealed bottles over molecular sieve (4 Å) until used. Ethyl iodide was used as obtained from Fisher Scientific. The

p-nitrophenyl and methyl glycosides used in synthesizing the partially ethylated alditol acetate standards were obtained from the following sources: *p*-nitrophenyl α-L-fucopyranoside, methyl α-L-rhamnopyranoside, methyl β-L-arabinopyranoside, methyl α-D-galactopyranoside, *p*-nitrophenyl β-D-xylopyranoside, and *p*-nitrophenyl α-D-mannopyranoside, from Koch–Light Laboratories, Ltd.; methyl β-D-xylopyranoside from Pierce Chemical Company; and *p*-nitrophenyl β-D-galactopyranoside from Sigma Chemical Company. The oligosaccharides and polysaccharides used in the synthesis of partially ethylated alditol acetate standards were obtained from the following sources: yeast mannan and arabinan from Koch–Light Laboratories, Ltd.; cellobiose, lactose, and dextran (mol. wt. = 200,000–275,000) from Sigma Chemical Company; and gentiobiose from Pierce Chemical Company. The galactan was isolated in this laboratory from citrus pectin by McNeil[47].

The liquid phases for the preparation of gas-chromatographic columns [ECNSS-M, Silar 10-C, Silar 5-CP, poly(ethylene glycol succinate) (PEGS), poly-(ethylene glycol adipate) (PEGA), and GE silicone XF-1150] were all obtained from Applied Science Laboratories. The stainless-steel No. 316 capillary tubing (1.59 mm o.d., 0.51 mm i.d.) was obtained from Handy & Harman Tube, Norristown, Pennsylvania.

Methods. — *Ethylation.* Ethylation of carbohydrate samples is performed according to the general methylation method of Hakomori[48] as adapted by Sandford and Conrad[49], but modified in order to ensure complete ethylation. The carbohydrate samples (1–6 mg) are dried over phosphorus pentaoxide for a minimum of 10 h at 60° *in vacuo*. After dissolution of the carbohydrate in 1.0 ml of dry dimethyl sulfoxide under nitrogen, 100 μl of 2.0M dimethylsulfinyl anion[50] (sodium "dimsyl") solution prepared according to Sandford and Conrad[49] is added at room temperature. Potassium "dimsyl" may also be used[51]. This solution is stirred for 15 min at room temperature if the carbohydrate is totally soluble, or for several h if the carbohydrate is only partially soluble. Ethyl iodide (22 μl, 1.35 equivs, based on the amount of "dimsyl") is then added at room temperature and the solution stirred for 2 h. A second ethylation is accomplished without isolation on the same sample by again adding 100 μl of 2.0M "dimsyl" followed by 22 μl of ethyl iodide in exactly the same manner.

Intentional underethylation. Model compounds for the synthesis of all partially ethylated alditol acetates for chromatographic and mass-spectral standards were not available, and so the various partially ethylated alditol acetates derived from the various glycosidic linkage-isomers of rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose were synthesized by intentionally underethylating known *p*-nitrophenyl and methyl glycosides and known oligo- and polysaccharides. This underethylation was accomplished by performing only one of the alkylation steps and by using smaller amounts of ethyl iodide. This intentional underethylation yields a mixture of partially ethylated derivatives of each aldose.

Isolation of ethylated oligo- and poly-saccharides and formation of alditol acetates. The partially ethylated carbohydrates are separated from dimethyl sulfoxide and other

reagents after ethylation by either (*a*) dialysis against distilled water overnight (for polysaccharides), or (*b*) by gel-permeation chromatography (for mono- and oligosaccharides). Gel-permeation chromatography is accomplished by adding 1 ml of 1:1 chloroform–methanol (v/v) to the ethylation mixture in dimethyl sulfoxide (1.0 ml) and eluting it through a 50 × 1.5 cm column of Sephadex LH-20 (Sigma Chemical Co.) equilibrated with 1:1 chloroform–methanol. Fractions (2.0 ml) of column effluent are collected and a portion of each fraction analyzed for carbohydrate by the anthrone procedure[52]. Fractions containing carbohydrate are pooled and the solvent removed by evaporation at 50° under a stream of filtered air.

Hydrolysis of the ethylated products is accomplished in a sealed tube (13 × 100-mm test tube) with 2.0M trifluoroacetic acid (normally 1.0 ml) for 2 h at 121°. The acid and water are subsequently removed by evaporation at 50° with a stream of filtered air. As with methylated derivatives, care must be taken to remove the samples from the water bath as soon as they become dry to avoid evaporation of the partially ethylated aldoses.

The partially ethylated aldoses released by hydrolysis are reduced to the alditols by the addition of 10 mg of sodium borodeuteride in 1.0 ml of M ammonia. This solution is stirred for 2 h at room temperature, and the excess reductant is decomposed by dropwise addition of acetic acid until evolution of hydrogen ceases.

After the reduction step, the solutions are again evaporated at 50° with filtered air to remove the ammonia and water. The borate remaining in the reaction vessel is removed as the volatile methyl borate by evaporating, from the sample, first 10% glacial acetic acid in methanol (twice) and then methanol (8 times). Sodium borate has been shown to interfere with the acetylation that follows[18].

The partially ethylated alditols are acetylated by heating them in a sealed tube with acetic anhydride (normally 0.5 ml) for 3 h at 121°.

Gas chromatography. The partially ethylated alditol acetates, dissolved in acetic anhydride, were injected directly into one of three gas chromatographs; a Hewlett–Packard model 7620A, or one of two F & M instruments (model 810). Liquid phases and columns used to determine standard relative retention-times were: (*a*) a 120 × 0.3-cm (o.d.) copper column containing 3% ECNSS-M on Gas Chrom Q (100–120 mesh); (*b*) a 120 × 0.3-cm (o.d.) copper column containing a mixture of 0.2% poly(ethylene glycol succinate) (PEGS), 0.2% poly(ethylene glycol adipate) (PEGA), and 0.4% GE silicone XF-1150 on Gas Chrom P (100–120 mesh); (*c*) a 120 × 0.3-cm (o.d.) copper column containing 2% Silar 10-C on Gas Chrom Q (100–120 mesh), and (*d*) a 15.2 m × 0.51-mm (i.d.) stainless-steel open tubular capillary column coated with PEGS, PEGA, and XF-1150 (the details of manufacturing this column are described later).

Columns (*a*) and (*b*) were temperature-programmed at 1°/min from 110 to 180° after a post-injection interval of 6 min and with a post-programming interval of 10 min. The helium flow-rate was approximately 60 ml/min on columns (*a*), (*b*), and (*c*). Column (*c*) was also temperature-programmed at 1°/min, but from 115 to 235° after a post-injection interval of 6 min. Column (*d*) was temperature-programmed at

1°/min from 135 to 205° following a post-injection interval of 6 min and with a post-programming interval of 20 min. The helium flow in column (*d*) was approximately 6 ml/min. A flow of 60 ml/min of helium make-up gas was added to the detector flow when using column (*d*) to increase the sensitivity. Balance columns in each case were identical to the sample columns except with column (*d*), when an uncoated 46 × 0.3-cm (o.d.) copper tube with a helium flow of 60 ml/min was used. Other chromatographic conditions for all four columns were: detector temperature, 270°; injector temperature, 260°; hydrogen flow, 50 ml/min; and air flow, 220 ml/min.

The capillary column (*d*) already described was coated by a dynamic coating procedure. The capillary tubing was cut to length (15.2 m) and the ends chemically milled with aqua regia according to Bambrick and Geoghegan[53] to remove burrs. The tubing was then cleaned according to Mon *et al.*[54] by using 200 lb. in^{-2} of nitrogen in a teflon reservoir to force through 50 ml of each of the following solvents: chloroform, acetone, water, concentrated nitric acid, water, concentrated ammonia, water, acetone, and chloroform. The teflon reservoir was a 1.75 m × 6.35-mm (o.d.) teflon tube connected by standard Swagelok fittings to the nitrogen pressure-regulator and the capillary tube. After drying with a flow of 40 ml/min of nitrogen for 30 min, the tubing was coated with 30 ml of chloroform containing 0.446 g PEGA (1%), 0.446 g PEGS (1%), and 1.871 g GE silicone XF-1150 (4%). This solution was placed in the teflon reservoir and sufficient nitrogen pressure applied (30–185 lb. in^{-2}) to maintain a flow of 0.2 ml/min. The flow rate was measured by collecting the column exit solution in a 10-ml graduated cylinder. This represents a linear velocity of approximately 2 cm/sec, which has been suggested[55] for optimum coating. This coating procedure is essentially the same as that recently described[56]. However, a porous layer, such as Silanox 101, was not incorporated in the column. As has been suggested[57], a 5-ft "pigtail" of the same stainless capillary tubing was attached to the column exit during coating to avoid "blowing out" the liquid phases from the last few feet of tubing. This is necessary to maintain an even layer of liquid phase. After coating, a nitrogen flow of 20 ml/min was continued for 10 h to remove residual chloroform, and the column was conditioned for 12 h at 190° with a flow of 9 ml/min of helium.

G.l.c. peak identity and retention-time data. The identity of each linkage isomer of each aldose as it emerged from the gas-chromatographic column was determined by combined gas chromatography–mass spectrometry. Structural assignments were made as described by Sweet *et al.*[58]. The mass-spectral fragmentation pathways are very similar to those previously described for methylated alditol acetates by Björndal *et al.*[37]. The use of known mono-, oligo- and poly-saccharides enables exact stereochemical identification to be made from these mass-spectral data.

Combined gas chromatography–mass spectrometry was performed on a Beckman GC45 gas chromatograph interfaced as described by Markey[59] to an Associated Electrical Industries MS-12 mass spectrometer that was modified for repetitive scanning. In this system, gas-chromatographic separations were conducted on column (*b*). Data handling of the combined gas chromatograph and mass spectro-

meter outputs was accomplished by a Digital Equipment Corporation PDP 8/I computer system as described by Plattner and Markey[60].

The identities of the gas-chromatographic peaks for any sample on the g.l.c. columns other than column (*b*) were assigned by comparing the elution profiles of the sample on these other columns to the profile on column (*b*). Normally, the profiles were so similar that identification was unambiguous; however, when there was uncertainty, the other columns were used in the g.l.c.–m.s. system, and the spectra of the g.l.c. peaks in question were identified.

Relative retention-times for the various partially ethylated alditol acetates were determined from the solvent front to the peak maximum. Some of the reported values are averages of several individual determinations, because of the presence of a particular partially ethylated alditol acetate in more than one sample. Two internal standards were used for the determination of relative retention-times, namely, 2,3,4,6-tetra-*O*-methylmannitol 1,5-diacetate (**1**), which is added to the samples after preparation of the partially ethylated alditol acetates and just before they are chromatographed, and *myo*-inositol hexaacetate (**2**), which is generated during acetylation from *myo*-inositol added with the trifluoroacetic acid prior to hydrolysis. These are ideal complementary internal standards as **1** is eluted relatively early in the chromatogram and **2** very late.

Estimation of the degree of underethylation. The degree of underethylation of a known sample (normally cellobiose) was estimated by summing the areas of all gas-chromatographic peaks not known to represent a partially ethylated alditol acetate expected from complete ethylation. For example, with cellobiose, all other gas-chromatographic peaks eluting after the peak representing terminal glucose except the peak representing 4-linked glucose are summed and compared with the sum of the areas of all g.l.c. peaks, including the two expected peaks. Peak areas were measured by electronic integration utilizing Infotronic Corporation equipment as previously described[18].

RESULTS

Perethylation of a polysaccharide. — Perethylation of a carbohydrate was accomplished by treatment with "dimsyl" anion followed by addition of ethyl iodide. Unlike methylation, however, a one-step conversion into the fully alkylated species is not possible. Underethylated products from cellobiose amount to 46% after one treatment, and 4–5% after both two and three treatments.

Previously, it has been reported that carbohydrates react slowly with "dimsyl" solutions[37,48]. We found this not to be the case if the carbohydrate is soluble in dimethyl sulfoxide. Cellobiose is underethylated in a two-step process to the same extent, about 12% of underethylated products, whether 15 min or 7 h is allowed for deprotonation of hydroxyl groups by the "dimsyl" solution before ethyl iodide is added. This phenomenon is also observed when ethylating dextran (mol. wt. = 200,000–275,000). The relative areas of the g.l.c. peaks attributable to the branched

glucose components (either naturally present in the polymer or arising through underalkylation) are constant, regardless of whether 15 min or 4 h is allowed for deprotonation by "dimsyl". In fact, the relative areas of these peaks are almost identical to the relative areas of the corresponding peaks obtained from methylation of the same dextran.

The addition of methyl iodide to a "dimsyl"-treated oligosaccharide must be accomplished slowly while maintaining the temperature around 20° to ensure complete methylation[49]. On the other hand, we found that the yield of perethylation products is scarcely altered by slow or fast addition of ethyl iodide and is independent of the temperature. It has also been found that the degree of underethylation of cellobiose is constant whether 2 or 16 h is allowed for the ethyl iodide (excess) to react at room temperature with the "dimsyl"-treated disaccharide. In fact, if the temperature of this mixture is raised to 121°, the degree of underethylation after 20 h remains the same as it was after 2 h at 23°.

Water and other proton sources must be carefully excluded whenever "dimsyl" treatment is used. The low yield of fully ethylated carbohydrate after a single ethylation reaction was initially attributed to one of these proton sources. However, extensive purification of the commercial ethyl iodide immediately prior to use (distillation from phosphorus pentaoxide or ethylmagnesium iodide) did not decrease the degree of underethylation. Also, the degree of underethylation was not increased by the addition to commercial ethyl iodide of 1% by volume of water, ethanol, or iodine, immediately prior to use.

Hydrolysis of ethylated oligosaccharides. — Optimum hydrolytic conditions with 2.0M trifluoroacetic acid were established by hydrolyzing samples of octa-*O*-ethylcellobiose at 121° for various intervals of time. After reduction and acetylation, peak-areas of the resultant partially ethylated alditol acetates were compared with the peak area of an internal standard (*myo*-inositol hexaacetate, **2**). The yield of 2,3,4,6-tetra-*O*-ethylglucitol 1,5-diacetate reaches a maximum after 2 h of hydrolysis and does not decrease upon further hydrolysis up to 8 h. A maximum yield of the 2,3,5-tri-*O*-ethylglucitol 1,4,5-triacetate is also obtained after 2 h of hydrolysis. In contrast, nonalkylated and permethylated polysaccharides are hydrolyzed under the same conditions but for only 1 h[18,39]. The validity of the use of hydrolysis for 2 h for large polymers, and for oligosaccharides containing sugars other than glucose, remains untested.

Reduction of partially ethylated aldoses. — The reduction of partially methylated aldoses to alditols previously has been assumed to be complete within minutes in a M ammoniacal solution of sodium borohydride, as the reduction of nonalkylated aldoses had been shown to be very fast[18]. However, the chromatogram of the partially ethylated alditol acetates of cellobiose showed two g.l.c. peaks both having short retention-times. The two carbohydrate components were identified by their mass spectra as 1-*O*-acetyl-2,3,4,6-tetra-*O*-ethyl-α- and β-D-glucopyranose. By integrating the g.l.c. peak-areas of the nonreduced glucose derivatives relative to the area of 2,3,4,6-tetra-*O*-ethylglucitol 1,5-diacetate, and by varying the reduction time of the

TABLE I

RELATIVE RETENTION-TIMES OF PARTIALLY ETHYLATED ALDITOL ACETATES ON FOUR DIFFERENT GAS CHROMATOGRAPHIC COLUMNS[a]

Sugar residue	*Glycosidic linkages (t = terminal residue)*	*Positions of O-ethyl groups*	*Relative retention-times*							
			T_a		T_b		T_c		T_d	
			2	1	2	1	2	1	2	1
Rhamnose	t	2,3,4	0.07	0.28	0.09	0.30	0.16	0.38	0.21	0.53
	2	3,4								
	3	2,4	0.22	0.77	0.25	0.81	0.33	0.81	0.36	0.90
	4	2,3	0.21	0.75	0.23	0.76	0.32	0.79	0.35	0.87
	2,3	4	0.36	1.25	0.38	1.24	0.43	1.06	0.45	1.14
	2,4	3	0.39	1.34	0.40	1.32	0.50	1.21	0.49	1.23
	3,4	2	0.34	1.18	0.36	1.18	0.44	1.06	0.45	1.13
	2,3,4		0.43	1.53	0.47	1.50	0.52	1.27	0.53	1.36
Fucose	t	2,3,4	0.11	0.40	0.14	0.46	0.22	0.53	0.26	0.64
	2	3,4	0.25	0.88	0.30	0.93	0.36	0.89	0.40	0.97
	3	2,4	0.25	0.88	0.29	0.91	0.36	0.89	0.39	0.95
	4	2,3	0.25	0.88	0.28	0.89	0.36	0.89	0.39	0.95
	2,3	4	0.38	1.35	0.43	1.37	0.50	1.22	0.50	1.24
	2,4	3	0.40	1.42	0.43	1.37	0.50	1.22	0.50	1.24
	3.4	2	0.35	1.24	0.39	1.23	0.45	1.11	0.46	1.15
	2,3,4		0.46	1.61	0.50	1.58	0.54	1.31	0.54	1.58
Arabinose	t-(*p*)	2,3,4	0.11	0.40	0.15	0.48	0.23	0.56	0.27	0.67
	2-(*p*)	3,4	0.26	1.00	0.30	1.00	0.41	1.01	0.41	1.01
	3-(*p*)	2,4	0.27	1.04	0.32	1.04	0.41	1.00	0.42	1.03
	4-(*p*) or 5-(*f*)	2,3	0.26	1.00	0.30	1.00	0.41	1.00	0.41	1.01
	t-(*f*)	2,3,5	0.09	0.32	0.11	0.36	0.20	0.49	0.24	0.59
	2-(*f*)	3,5	0.20	0.76	0.24	0.80	0.34	0.85	0.36	0.89
	3-(*f*)	2,5	0.23	0.88	0.27	0.89	0.37	0.92	0.38	0.94
	2,3-(*p*)	4	0.41	1.56	0.47	1.54	0.56	1.39	0.54	1.34
	2,3-(*f*)	5	0.34	1.31	0.38	1.29	0.47	1.17	0.47	1.17
	2,4-(*p*) or 2,5-(*f*)	3	0.45	1.73	0.49	1.62	0.57	1.42	0.55	1.36
	3,4-(*p*) or 3,5-(*f*)	2	0.43	1.64	0.47	1.57	0.55	1.36	0.53	1.32
	2,3,4-(*p*) or 2,3,5-(*f*)		0.56	2.11	0.61	1.98	0.66	1.62	0.63	1.60
Xylose	t	2,3,4	0.11	0.43	0.15	0.48	0.24	0.59	0.25	0.65
	2 or 4	3,4 or 2,3	0.29	1.09	0.33	1.06	0.43	1.07	0.43	1.08
	3	2,4	0.27	1.00	0.31	1.00	0.41	1.00	0.40	1.01
	2,3 or 3,4	4 or 2	0.48	1.83	0.51	1.68	0.60	1.49	0.57	1.45
	2,4	3	0.48	1.82	0.51	1.68	0.60	1.49	0.57	1.45
	2,3,4		0.69	2.57	0.70	2.24	0.76	1.87	0.71	1.80
Mannose	t	2,3,4,6	0.17	0.62	0.22	0.72	0.29	0.70	0.32	0.81
	2	3,4,6	0.34	1.30	0.39	1.26	0.46	1.13	0.47	1.20
	3	2,4,6	0.35	1.32	0.40	1.29	0.46	1.14	0.48	1.21
	4	2,3,6	0.36	1.34	0.40	1.31	0.47	1.15	0.49	1.22
	6	2,3,4	0.35	1.31	0.40	1.28	0.46	1.13	0.49	1.21
	2,3	4,6	0.49	1.85	0.53	1.77	0.59	1.44	0.59	1.53
	2,4	3,6	0.55	2.08	0.57	1.90	0.65	1.60	0.62	1.56

TABLE I (*continued*)

Sugar residue	*Glycosidic linkages (t = terminal residue)*	*Positions of O-ethyl groups*	*Relative retention-times*							
			T_a		T_b		T_c		T_d	
			2	1	2	1	2	1	2	1
	2,6	3,4	0.60	2.24	0.62	2.03	0.67	1.65	0.68	1.74
	3,4	2,6	0.50	1.88	0.54	1.78	0.59	1.45	0.59	1.50
	3,6	2,4	0.55	2.09	0.58	1.94			0.64	1.65
	4,6	2,3	0.55	2.09	0.58	1.94			0.64	1.66
	2,3,4	6	0.62	2.31	0.65	2.15	0.69	1.70	0.69	1.79
	2,3,6 & 2,4,6	4 or 3	0.79	2.95	0.80	2.60	0.83	2.02	0.82	2.11
	3,4,6	2	0.70	2.64	0.72	2.40	0.76	1.86	0.77	1.97
	2,3,4,6		0.84	3.06	0.86	2.79	0.87	2.12	0.87	2.24
Galactose	t	2,3,4,6	0.21	0.78	0.25	0.84	0.33	0.81	0.36	0.90
	2	3,4,6	0.40	1.43	0.42	1.42	0.50	1.22	0.50	1.24
	3	2,4,6	0.40	1.43	0.42	1.42	0.50	1.22	0.50	1.23
	4	2,3,6	0.39	1.41	0.42	1.43	0.49	1.21	0.50	1.24
	6	2,3,4	0.44	1.60	0.47	1.58	0.54	1.32	0.55	1.36
	2,3	4,6	0.53	1.92	0.56	1.90	0.62	1.50	0.60	1.48
	2,4	3,6	0.57	2.10	0.59	2.03	0.65	1.60	0.63	1.55
	2,6	3,4	0.63	2.32	0.67	2.26	0.71	1.72	0.68	1.68
	3,4	2,6	0.53	1.94	0.55	1.90	0.62	1.51	0.61	1.53
	3,6	2,4	0.63	2.32	0.67	2.29	0.70	1.70	0.68	1.67
	4,6	2,3	0.62	2.31	0.63	2.23	0.69	1.71	0.65	1.63
	2,3,4	6	0.66	2.34	0.68	2.35	0.71	1.73	0.69	1.69
	2,3,6	4	0.82	2.82	0.83	2.82	0.86	2.13		
	2,4,6	3	0.81	2.98	0.82	2.81	0.85	2.09	0.80	1.98
	3,4,6	2	0.75	2.79	0.76	2.67	0.79	1.94	0.74	1.85
	2,3,4,6		0.88	3.13	0.90	3.03	0.90	2.19	0.89	2.28
Glucose	t	2,3,4,6	0.19	0.69	0.22	0.75	0.30	0.75	0.35	0.85
	2	3,4,6	0.36	1.28	0.39	1.29	0.46	1.13	0.48	1.18
	3	2,4,6	0.36	1.28	0.39	1.29	0.45	1.11	0.48	1.18
	4	2,3,6	0.42	1.49	0.44	1.48	0.53	1.30	0.52	1.29
	6	2,3,4	0.40	1.38	0.41	1.39	0.48	1.20	0.50	1.24
	2,3	4,6	0.55	1.97	0.57	1.92	0.63	1.56	0.62	1.53
	2,4	3,6	0.58	2.04	0.59	1.98	0.66	1.63	0.63	1.57
	2,6	3,4	0.61	2.15	0.62	2.09	(0.68)	(1.69)	0.66	1.62
	3,4	2,6	0.55	1.94	0.57	1.90	0.63	1.56	0.61	1.52
	3,6	2,4	0.60	2.15	0.62	2.08	0.69	1.69	0.65	1.61
	4,6	2,3	0.61	2.17	0.62	2.09	0.69	1.69	0.65	1.62
	2,3,4	6	0.70	2.47	0.71	2.39	0.75	1.85	0.71	1.76
	2,3,6	4	0.82	2.94	0.82	2.76	0.87	2.14	0.81	2.02
	2,4,6	3	0.80	2.82	0.80	2.66	0.83	2.05	0.78	1.94
	3,4,6	2	0.76	2.68	0.77	2.58	0.81	1.99	0.76	1.88
	2,3,4,6		0.92	3.26	0.93	3.09	0.95	2.32	0.92	2.32

[a]The glycosidic linkages to each of the glycosyl residues from which the partially ethylated alditol acetates were synthesized are listed. Gas-chromatographic separations were performed on either a Hewlett–Packard Model 7620A or an F & M Model 810 gas chromatograph. Retention times are relative to both 2,3,4,6-tetra-*O*-methylmannitol 1,5-diacetate (**1**) and *myo*-inositol hexaacetate (**2**). Retention times (*T*) are given for four different column liquid phases as described in the text: column (*a*) 3% ECNSS-M, (*b*) 0.2% PEGS, 0.2% PEGA, and 0.4% XF-1150, (*c*) 2% Silar 10-C, and (*d*) PEGS, PEGA, and XF-1150 (1%, 1%, 4%) capillary.

partially ethylated aldoses from cellobiose, it was found that the reduction reaches a maximum (approximately 97% complete) in M ammonia (10 mg of sodium borodeuteride) after 2 h at room temperature.

G.l.c. retention-time data. — The retention-times relative to two internal standards, on four g.l.c. columns, of 79 possible linkage-isomers of the seven aldoses commonly found in primary plant cell-walls are presented in Table I. These retention times were determined from the known mono-, oligo-, and poly-saccharides, either fully ethylated or purposely underethylated to give mixtures of partially ethylated alditol acetates. The retention times have a precision of approximately ± 1–2% in the immediate vicinity of one of the internal standards, but the precision then decreases to approximately $\pm 10\%$ on either side of each internal standard. This is especially true with a given column over an extended period of time, as the column lifetime is fairly short (approximately 3 months), or when columns containing the same liquid phases but made at different times are compared. However, the relative elution-orders shown in Table I, together with the relative separations between any two peak maxima, are fairly reproducible as all of these determinations for Table I were made on the same column, without any change in conditions, during a single, continuous 36-h period. To use this table to identify an unknown peak in a chromatogram, it is best to use both internal standards but to rely more heavily on the retention time relative to the internal standard that is eluted closest to the unknown peak.

TABLE II

RELATIVE RETENTION-TIMES OF PARTIALLY METHYLATED ALDITOL ACETATES ON THREE DIFFERENT GAS-CHROMATOGRAPHIC COLUMNS[a]

Sugar residues	*Glycosidic linkages (t = terminal residue)*	*Positions of O-methyl groups*	*Relative retention-times*		
			T_a	T_b	T_c
Rhamnose	t	2,3,4	0.50	0.55	0.55
	2	3,4	0.92	0.92	0.90
	4	2,3	0.97	0.97	0.95
	2,4	3	1.38	1.33	1.33
	3,4	2	1.26	1.18	1.21
Fucose	t	2,3,4	0.69	0.71	0.69
	3	2,4	1.06		
	3,4	2	1.33		
Arabinose	t (*f*)	2,3,5	0.52	0.56	0.53
	t (*p*)	2,3,4	0.68	0.73	0.65
	2 (*f*)	3,5	0.91	0.92	0.85
	3 (*f*)	2,5	1.00	1.00	0.91
	5 (*f*) or 4 (*p*)	2,3	1.14	1.10	1.03
	2,5 (*f*), 2,4 (*p*)	3	1.60	1.51	1.44
	3,5 (*f*), 3,4 (*p*)	2	1.56	1.47	1.38
	2,3,5 (*f*) or 2,3,4 (*p*)		1.81	1.68	1.62

TABLE II (*continued*)

Sugar residues	*Glycosidic linkages (t = terminal residue)*	*Positions of O-methyl groups*	*Relative retention-times*		
			T_a	T_b	T_e
Xylose	t	2,3,4	0.68	0.71	0.66
	2 or 4	3,4 or 2,3	1.22	1.16	1.09
	3	2,4	1.16		1.02
	3,4 or 2,3	2 or 4	1.65[b]		1.50
	2,4	3	1.65	1.55	1.50
Mannose	t	2,3,4,6	1.00	1.00	1.00
	2	3,4,6	1.44	1.37	
	3	2,4,6	1.47	1.40	
	4	2,3,6	1.53	1.44	
	6	2,3,4	1.59	1.49	
	2,6	3,4		1.87	
	3,4	2,6		1.67	
	4,6	2,3	2.06		
Galactose	t	2,3,4,6	1.14	1.13	1.10
	2	3,4,6	1.61	1.49	1.52
	3	2,4,6	1.55	1.47	1.47
	4	2,3,6	1.59	1.48	1.52
	6	2,3,4	1.82	1.67	1.70
	2,4	3,6	1.99	1.81	1.87
	2,6	3,4	2.32	2.05	2.14
	3,4	2,6	1.88	1.72	1.77
	3,6	2,4	2.27	2.03	2.09
	4,6	2,3	2.19	1.95	2.04
Glucose	t	2,3,4,6	0.98	0.99	1.00
	3	2,4,6	1.44	1.38	1.39
	4	2,3,6	1.63	1.50	1.56
	6	2,3,4	1.58	1.47	1.52
	2,3	4,6	1.94	1.76	1.84
	2,4	3,6	1.96		1.88
	3,4	2,6	1.88	1.73	1.81
	3,6	2,4	2.10	1.87	1.98
	4,6	2,3	2.14	1.90	2.01

[a]The glycosidic linkages to each of the glycosyl residues from which the partially methylated alditol acetates were synthesized are listed. The gas-chromatographic separations were performed on a Hewlett–Packard Model 7620A gas chromatograph. The retention times are relative to 2,3,4,6-tetra-*O*-methylmannitol 1,5-diacetate (1). Retention times (*T*) are given for three different columns: (*a*) a 120 × 0.3-cm (o.d.) copper column containing 3% ECNSS-M on Gas Chrom Q (100–120 mesh); (*b*) a 120 × 0.3-cm (o.d.) copper column containing a mixture of 0.2% poly(ethylene glycol adipate), 0.2% poly(ethylene glycol succinate), and 0.4% silicone XF-1150 on Gas Chrom P (100–120 mesh); and (*e*) a stainless steel 15 m × 0.51-mm (i.d.) S.C.O.T. column (Perkin–Elmer Corp.) containing silicone OV-225. Chromatography on columns (*a*) and (*b*) was performed by temperature programming at 1° per min from 110 to 180° with a helium flow rate of approximately 60 ml per min. Chromatography on column (*e*) was performed by temperature programming at 0.5° per min from 150 to 190° with a helium flow-rate of approximately 6 ml per min. [b]Corrected from original published value of 1.75.

The relative retention-times of some of these same polysaccharide components, as their partially methylated alditol acetates as determined by Talmadge *et al.*[39] in this laboratory, are presented in Table II. They used two of the same columns used here, under the same conditions, but also included data for a commercial 15.2 m SCOT column coated with OV-225.

The relative retention-time reported for 1,2,3,4,5-penta-*O*-acetylarabinitol on column (*a*) demonstrates the nonreproducibility of the absolute numbers given in Tables I and II. As no alkyl groups are present in this derivative, it should give the same value in both Tables I and II. It does not (2.11 *vs.* 1.81 relative to **1**), because of the nonreproducibility of columns and conditions. The order of elution, however, is preserved with both tables, demonstrating that the relative order, and not retention times, should always be compared for identification purposes, except in the immediate vicinity of the internal standards.

PEGS, PEGA, and XF-1150 capillary column. — The capillary column coated with PEGA, PEGS, and XF-1150 used for determination of retention times for partially ethylated alditol acetates has been used during 20 months with little or no loss of theoretical-plate resolution even though it is routinely operated at temperatures up to 205°. Packed g.l.c. columns having the same liquid phases will not tolerate these high temperatures and have a much shorter lifetime, even at 185°. Our column has maintained approximately 5–6,000 theoretical plates, as measured with the *myo*-inositol hexaacetate peak at 200°. This represents a height equivalent per theoretical plate of 2.8 mm, which is comparable with values for other capillary columns, even though they are considerably less polar.

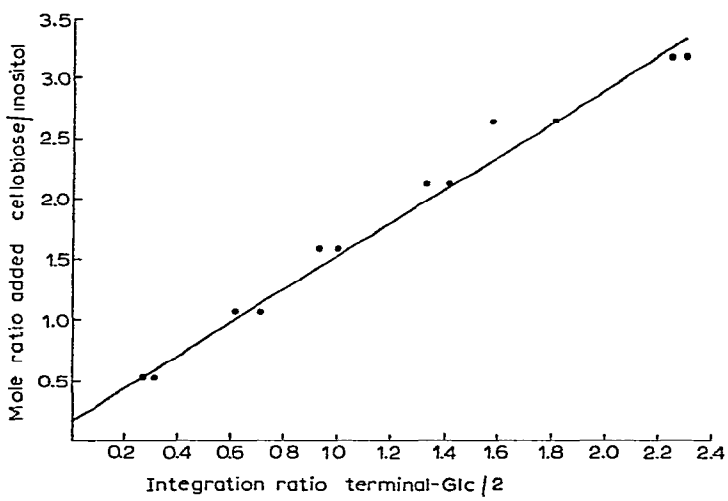

Fig. 1. Quantitation of a partially ethylated aditol acetate. The g.l.c. peak area of 2,3,4,6-tetra-*O*-ethylglucitol 1,5-diacetate [terminal-Glc, relative to *myo*-inositol hexaacetate (**2**)] is plotted as a function of the initial amount of cellobiose (1–6 mg) derivatized to its partially ethylated alditol acetates. A constant amount of *myo*-inositol was added prior to hydrolysis.

Quantitation of the derivatization. — It is imperative that any derivatization scheme for polysaccharide components be quantitative and linear in the range of concentrations that are normally encountered. To demonstrate the linearity of the response for partially ethylated alditol acetates, various amount of cellobiose (1.0–6.0 mg) were derivatized to the partially ethylated alditol acetates by the procedure described. The peak areas of the two derivatives (from terminal-Glc and 4-substituted-Glc) relative to the peak area of *myo*-inositol hexaacetate, formed by the addition of a known and constant amount of *myo*-inositol during the hydrolytic step, are compared to the relative amount of cellobiose added. The result is shown in Fig. 1 for terminal-Glc. A very similar plot, showing the same degree of data reproducibility, is obtained for 4-substituted Glc. The best straight line has been drawn through these points. If this straight line is used for calibration, the uncertainty error is approximately $\pm 10\%$. This uncertainty arises from losses caused by evaporation or degradation that occurs during derivatization. The losses due to evaporation can be especially significant for terminal aldose residues.

DISCUSSION

Synthesis of partially ethylated alditol acetates. — Cellobiose was selected as a model compound to determine the optimum conditions for preparation of partially ethylated alditol acetates. Generalization of these optimum conditions for all poly- and oligosaccharides is risky. However, ethylation of a large polysaccharide (dextran), under these conditions gives results very similar to those obtained by methylation of that same dextran.

The degree of underethylation has been used as an indication of extent of reaction during studies on the ethylation conditions. This degree of underethylation, as measured by summing the peak areas of unexpected chromatographic components, is probably greater than the actual degree of underethylation. The higher value results because much of the baseline noise included in this determination is not due to underethylation of carbohydrate, but more likely arises from impurities and degradation. This calculated value, however, does represent an upper limit for underethylation.

It was found necessary to perform two successive ethylations to obtain 90% perethylated oligo- or polysaccharide (equivalent to 97% ethylation); further treatments were considered unnecessary.

Uronic acid esters have been shown to undergo β-elimination when treated with strong base. For this reason, a second treatment of a polysaccharide with "dimsyl" anion after initial methylation has been objected to by Björndal *et al.*[37]. Nevertheless, in our laboratory, such complex polymers as are found in the primary cell-walls of plants are routinely remethylated to ensure complete etherification. This does lead to β-elimination of uronic acid esters; however, this β-elimination does not change the neutral-sugar analysis as the elimination does not degrade sugars that are glycosidically linked to a uronic acid. The uronic acids are then determined with a separate sample of polysaccharide by a procedure[38] not involving alkylation.

Although carbohydrates can be completely methylated by a single treatment with "dimsyl" anion and methyl iodide, complete ethylation is not achieved by a single treatment with the same concentration of "dimsyl" anion and ethyl iodide. A second treatment does complete the ethylation. This phenomenon can be explained by a deprotonation equilibrium and competing reactions consuming the alkyl iodide. The deprotonation of the carbohydrate reaches an equilibrium with the "dimsyl" anion in which part of the carbohydrate hydroxyl groups remain protonated. This equilibrium is reached very rapidly and before the alkyl iodide is added. When the alkyl iodide is added, it can be consumed by either the carbohydrate alkoxide ion or by the "dimsyl" anion to give either the carbohydrate ether or a substituted sulfoxide. These two reactions compete for the alkyl iodide that is added. When methyl iodide is used, the partially deprotonated carbohydrate apparently consumes the methyl iodide faster than does the "dimsyl" anion. This formation of an ether of the carbohydrate allows the deprotonation equilibrium to become reestablished by deprotonation of more hydroxyl groups. Thus, the excess "dimsyl" in solution does not consume methyl iodide very rapidly and acts only to deprotonate the carbohydrate as it becomes methylated to a continuously increasing degree. Complete methylation occurs by this continual shift of the equilibrium towards the alkylated species. This idea has been proposed by Björndal *et al.*[37].

However, when the alkyl iodide is ethyl iodide, the rate of consumption of ethyl iodide by the "dimsyl" anion appears to be significantly fast in comparison to the rate of consumption by the carbohydrate alkoxide ion, so that the deprotonation–protonation equilibrium is not continuously shifted to allow complete ethylation. This shift does not occur because of the consumption of the excess "dimsyl" by ethyl iodide. No doubt, both reactions that consume alkyl iodide, with carbohydrate alkoxide and with "dimsyl" anion, are slowed by the substitution of ethyl for methyl, but apparently the rate of reaction with carbohydrate alkoxide is decreased to a greater extent, so that the two reactions are more competitive.

The second treatment of the partially ethylated carbohydrate with "dimsyl" anion and ethyl iodide yields a fully ethylated species because the deprotonation equilibrium is then established with essentially all of the hydroxyl groups deprotonated. This is possible in the second treatment because the concentration of alkoxide ions on a given carbohydrate species is not nearly so large after the initial partial ethylation.

Other workers[37,48] have suggested a 3–4-h treatment of polysaccharides with "dimsyl" anion. This longer deprotonation time is not necessary for polysaccharides that are soluble in dimethyl sulfoxide because, as we have shown, the deprotonation equilibrium is established very quickly (in under 15 min). This longer deprotonation time, however, is probably beneficial for those polysaccharides that are only partially soluble in dimethyl sulfoxide. In these cases, the progressive deprotonation increases the solubility of the polysaccharide, probably by unfolding or uncoiling the polysaccharide structure and making more hydroxyl groups available for deprotonation.

Quantitative determinations. — It is important that any derivative used for

determination of polysaccharide structure be quantitatively formed and permit quantitation of the polysaccharide components. The conversion of various amounts of cellobiose into partially ethylated alditol acetates has shown that these derivatives can be used in quantitative determinations. The g.l.c. peak-area responses of terminal-Glc and 4-substituted Glc are linear over the range (1–6 mg) of cellobiose studied. The uncertainty of these determinations is approximately ±10%, probably because of evaporation losses and degradation of the derivatives, and should be considered the limits of the derivatization for quantitative determinations.

In another paper[61] we have presented the molar response-factors for various partially ethylated and partially methylated alditol acetates. These values will allow a more-accurate determination of the components of a polysaccharide.

Partially ethylated alditol acetates as derivatives for g.l.c. — Syntheses of specific monoethyl ethers of sugars have been described previously[62–65], but this study is the first application of perethylation of polysaccharides and the use of these polyethers with general g.l.c. and combined g.l.c.–m.s. The partially ethylated alditol acetates are formed in nearly quantitative yield, are eluted as a single, sharp peaks on g.l.c., exhibit readily interpretable mass spectra that fragment in a manner analogous to that of the methyl derivatives[58], and allow separation of many of the polysaccharide fragments that have not been resolved as their corresponding partially methylated alditol acetates. The ethyl derivatives are readily prepared by adaptation of the standard methylation method; some of the reaction parameters must be altered to attain maximum yields, and a second ethylation step is needed.

Accurate determination of the retention times of partially ethylated alditol acetates is important in identifying the components from an unknown polysaccharide because diastereomeric derivatives give essentially the same mass spectra; accurate determination of retention times in several chromatographic systems is essential.

The partially ethylated alditol acetates are eluted considerably sooner under the same chromatographic conditions than the corresponding partially methylated alditol acetates. This change in elution can be observed by comparing the retention times of compounds eluted near 2,3,4,6-tetra-*O*-methylmannitol 1,5-diacetate (**1**) in both Tables I and II. The most obvious example of this shift in elution time is terminal-Man, which as the methylated derivative is **1**. Its retention-time as the ethyl derivative, relative to **1**, is 0.62 on column (*a*). This very significant shift in retention time can be attributed to the increased hydrophobic nature of the ethyl derivative over the methyl analog. The resultant lowering of the interaction with the polar liquid phase on the column is more important than the increase in molecular weight in determining the shift in elution. This phenomenon is the basis for the value of the partially ethylated alditol acetate as a derivative for polysaccharide analysis.

Specific examples of the importance of the shift in elution order of partially ethylated alditol acetates relative to their methyl analogs are shown in Table III, which contains selected data from Tables I and II. The methyl derivatives of 3-substituted Ara*f*, terminal-Man, and terminal-Glc are eluted under a single peak;

TABLE III

SELECTED RETENTION-TIME DATA FROM TABLES I AND II[a]

Sugar residue	*Glycosidic linkages in compound*	*Relative retention-times*	
		Methyl	*Ethyl*
Ara*f*	3-	1.00	0.88
Man	terminal	1.00	0.62
Glc	terminal	0.98	0.69
Ara*f*	3,5-	1.56	1.64
Gal	4-	1.59	1.41
Man	6-	1.59	1.31
Ara*f*	2,5-	1.60	1.73
Glc	4-	1.63	1.49
Xyl	2,4-	1.65	1.82
Rha	2,4-	1.38	1.34
Man	4-	1.53	1.34

[a]These retention times allow direct comparison of the elution order of several carbohydrate linkage isomers as both their partially methylated and partially ethylated alditol acetates. Chromatography is on column (*a*) relative to 2,3,4,6-tetra-*O*-methylmannitol 1,5-diacetate (**1**) as the internal standard.

however, as ethyl derivatives, they do not. The separation between the two terminal sugars and 3-substituted-Ara*f* is especially large because both terminal sugars contain four alkyl groups to only two for 3-substituted Ara*f*. Thus, the terminal sugar derivatives are shifted to a much shorter elution-time by substitution of ethyl for methyl than is the 3-substituted Ara*f* derivative. Another example that demonstrates this same effect is also shown in Table III. Six different methylated sugars are eluted at very nearly the same retention times (1.56–1.65). If all were present in a single sample, one very broad, overlapping peak would be seen. However, the separations are much greater as their ethyl derivatives (1.31–1.82), the compounds containing the most alkyl groups being shifted to the shortest retention-times. In studies on the structure of primary plant cell-walls, this particular separation is exceedingly important, as four of these derivatives (3,5-substituted Ara, 4-substituted Gal, 4-substituted Glc, and 2,4-substituted Xyl) account for 36% of the primary cell-walls of dicotyledons[39]. This improvement in separation is even more significant because 4-substituted Glc (from cellulose) accounts for about 25% of the walls and its g.l.c. peak size masks the other partially methylated alditol acetates, making quantitation difficult. Many other examples of the change in chromatographic resolution may be found by examination of Tables I and II.

Ethylation instead of methylation can also cause two compounds to coelute that did not coelute as methyl derivatives. This is illustrated by the last example in Table III (2,4-substituted Rha and 4-substituted Man). For this reason, partially methylated and partially ethylated alditol acetates are best used as complementary derivatives.

In general, the effect of ethylation as opposed to methylation can be summarized as follows: if two or more compounds overlap as partially methylated alditol acetates and they contain a different number of alkyl ethers, ethylation instead of methylation will allow separation of the derivatives, with the compound containing the greater number of alkyl groups being eluted first. However, if the overlapping compounds contain the same number of alkyl groups, ethylation will generally not resolve the compounds but may increase their separation to a small extent.

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