Carbohydrate Research, 210 (1991) 311–317
Elsevier Science Publishers B.V., Amsterdam

Note

An improved method for the preparation of standards for glycosyl-linkage analysis of complex carbohydrates

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(Received July 16th, 1990; accepted September 18th, 1990)

Glycosyl-linkage analysis[1,2] (*i.e.*, methylation analysis) is a widely used procedure for determining the glycosyl-linkage composition of carbohydrates. Glycosyl linkages are deduced from the electron impact-mass spectra of partially *O*-methylated, partially *O*-acetylated alditol derivatives. While the various epimers of a given partially *O*-methylated, partially *O*-acetylated alditol (*e.g.*, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol *vs.* 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol) give nearly identical mass spectra, they can generally be separated by gas–liquid chromatography (g.l.c.). Thus, complete assignment of the identities of the peaks obtained during g.l.c.-m.s. of partially *O*-methylated, partially *O*-acetylated alditols obtained from an unknown sample relies on the availability of appropriate g.l.c. standards.

Procedures have been developed for the preparation of the required standards for glycosyl-linkage analysis. These protocols involve intentional undermethylation of methyl glycosides[3], alditols[4], or aldoses[5]. The mixture of partially methylated aldoses or methyl glycosides is then typically separated from other reactants and products by reversed-phase chromatography on C-18 cartridges[5,6] or by partitioning into chloroform–methanol[7]. The sample is then reduced with sodium borodeuteride (following acid-catalyzed hydrolysis in the case of methyl glycosides) and *O*-acetylated to form a mixture of partially methylated, partially acetylated alditols. These procedures work well for the tetra-*O*-methyl derivatives of hexoses and for the tri-*O*-methyl derivatives of deoxyhexoses and pentoses, but these methods produce poor yields of those derivatives carrying fewer *O*-methyl groups. We have found, for example, that the methyl glycosides in a mixture that are only partially *O*-methylated do not bind efficiently to a reversed-phase cartridge nor do they efficiently extract into chloroform–methanol, due to their more polar nature. Thus, those procedures are strongly biased towards the recovery of partially *O*-methylated, partially *O*-acetylated alditols corresponding to terminal residues, with limited recoveries of the derivatives corresponding to linear

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0008-6215/91/$ 03.50

glycosyl residues, and essentially no recovery of the derivatives corresponding to branched glycosyl residues.

We describe herein a simple procedure that yields a mixture of all possible partially *O*-methylated, partially *O*-acetylated alditols that can be formed from a given methyl glycoside. In this procedure, a partially methylated methyl glycoside is *O*-acetylated immediately following the undermethylation step to yield a mixture of partially *O*-methylated, partially *O*-acetylated methyl glycosides. The *O*-acetylation ensures that all components of the mixture are extracted into dichloromethane. The samples are extracted into dichloromethane, then hydrolyzed, deuterium-reduced, and re-acetylated. The resulting mixture contains roughly equivalent amounts of each of the possible partially *O*-methylated, partially *O*-acetylated alditols theoretically obtainable from the methyl glycoside.

EXPERIMENTAL

Reagents. — Methyl sulfoxide (anhydrous, packed under nitrogen), iodomethane (stabilized with copper), α-methyl-D-mannopyranoside, and β-methyl-D-xylopyranoside were obtained from Aldrich Chemical Co., Milwaukee, WI (U.S.A.); L-arabinose and L-fucose from Sigma Chemical Co., St. Louis, MO (U.S.A.); L-rhamnose from Calbiochem, La Jolla, CA (U.S.A.); α-methyl-D-glucopyranoside from United States Biochemical Corp., Cleveland, OH (U.S.A.); and α-methyl-D-galactopyranoside from Koch-Light Ltd., Colnbrook, Bucks, U.K. Methanolic HCl (M) was prepared by diluting 3M methanolic HCl [Supelco, Bellefonte, PA (U.S.A.)] into methanol. Potassium methylsulfinylmethanide (2.4–2.6M) was prepared from potassium hydride and dimethyl sulfoxide as has been described[6].

Gas–liquid chromatography. — The partially methylated alditol acetate mixtures were chromatographed on a 30 m × 0.25 mm (i.d.) SP2330 column (Supelco, Inc.) in a Hewlett–Packard 5880A gas chromatograph fitted with a flame-ionization detector and with the injection port operated in the split mode. Helium was used as the carrier gas at a flow rate of 4 mL/min. The oven was held at 80° for 2 min, then raised, first at 30°/min to 170°, then at 4°/min to 240°, where it was held for 10 min.

Gas–liquid chromatography–mass spectrometry. — E.i. spectra of the partially methylated alditol acetates eluting from a 30 m × 0.25 mm (i.d.) SP2330 column in a Hewlett–Packard 5890 gas chromatograph were recorded on a Hewlett–Packard 5970 Series mass-selective detector operated at an ionization potential of 70 eV. Helium was used as the carrier gas, and the temperature program was as described above.

Preparation of standards. — *(a) Starting materials.* A quantity of a methyl glycoside (dried overnight in a vacuum oven at 80°) containing 0.412 mmol of hydroxyl groups [*i.e.*, methyl D-glucopyranoside, methyl D-galactopyranoside, or methyl D-mannopyranoside (20 mg), or methyl D-xylopyranoside (22.6 mg)] was weighed into a 13 × 100 mm glass tube capped with a Teflon-faced, rubber-lined screw cap.

Methyl glycosides that were not commercially available were prepared as follows: L-arabinose (20.6 mg), L-fucose (22.5 mg), and L-rhamnose (22.5 mg) were weighed out

into separate 13 × 100 mm screw-capped tubes. M Methanolic HCl (2 mL) was added to each sample, and the tubes were capped tightly and placed in a heating block for 90 min at 80°. The samples were cooled to room temp, *tert*-butanol (200 μL) was added to each, and the samples were placed in a 40° water bath and concentrated to syrups in a stream of dry, filtered air. Each sample was dissolved in methanol (1 mL) and again concentrated to a syrup. The sample in each tube contained (assuming quantitative formation of the methyl glycosides) 0.412 mmol of hydroxyl groups.

(b) Partial methylation of methyl glycosides. Each methyl glycoside sample was dissolved in methyl sulfoxide (400 μL), and the tube was purged with argon. A quantity of potassium methylsulfinylmethanide sufficient to deprotonate two-thirds to three-fourths of the hydroxyl groups in each sample (*e.g.*, 2.5M potassium methylsulfinylmethanide (124 μL) to account for 75% of 0.412 mmol of hydroxyl groups) was added to each tube. The tubes were then purged with argon, capped, and mixed. Generally, each sample formed a yellowish gel. After 10 min, iodomethane (130 μL) was added to each sample and mixed. The samples turned clear and colorless almost immediately. After 10 min the tubes were placed in a 40° water bath, and the mixture was concentrated using a gentle stream of filtered air for 15 min to remove excess iodomethane.

(c) Acetylation of methyl glycosides and extraction of partially methylated, partially acetylated methyl glycosides. 1-Methylimidazole (200 μL), a catalyst for acetylation[8], was added to each sample and mixed. This procedure was followed by the addition of acetic anhydride (2 mL). The samples turned yellowish and frequently formed a granular precipitate (possibly potassium iodide). After 10 min at room temperature, water (5 mL) was added to each sample and mixed to decompose excess acetic anhydride (any precipitate disappeared at this point). After the samples had cooled to room temperature, each was extracted with dichloromethane (2 × 1 mL), centrifuging briefly as needed to aid in phase separation. The two dichloromethane extracts were then combined in a single clean tube. The dichloromethane extracts were extracted with water (5 × 4 mL) (samples were centrifuged as needed to separate phases), discarding the aqueous phase after the first four extractions. Following the fifth water extraction, a clean Pasteur pipet was used to carefully transfer the dichloromethane layer to a clean tube. The dichloromethane was then evaporated in a stream of filtered air while the tubes were standing in a 40° water bath.

(d) Hydrolysis of partially methylated, partially acetylated methyl glycosides. To each sample was added 2N trifluoroacetic acid (1 mL) containing 0.1 mg/mL of *myo*-inositol. The tubes were capped tightly, mixed, and placed in a heating block at 120° for 1 h. The samples were placed in a 40° water bath, and the solvent was evaporated in a stream of filtered air.

(e) Reduction of partially methylated glycoses. To each sample was added a 10 mg/mL solution of $NaBD_4$ in 2N NH_4OH (500 μL). The samples were capped, mixed, and left at room temperature for one to two hours. Excess $NaBD_4$ was decomposed by the addition of acetone (250 μL), and the solvent was evaporated in a stream of filtered air while the tubes were standing in a 40° water bath.

(f) Acetylation of the partially methylated alditols and extraction of the partially

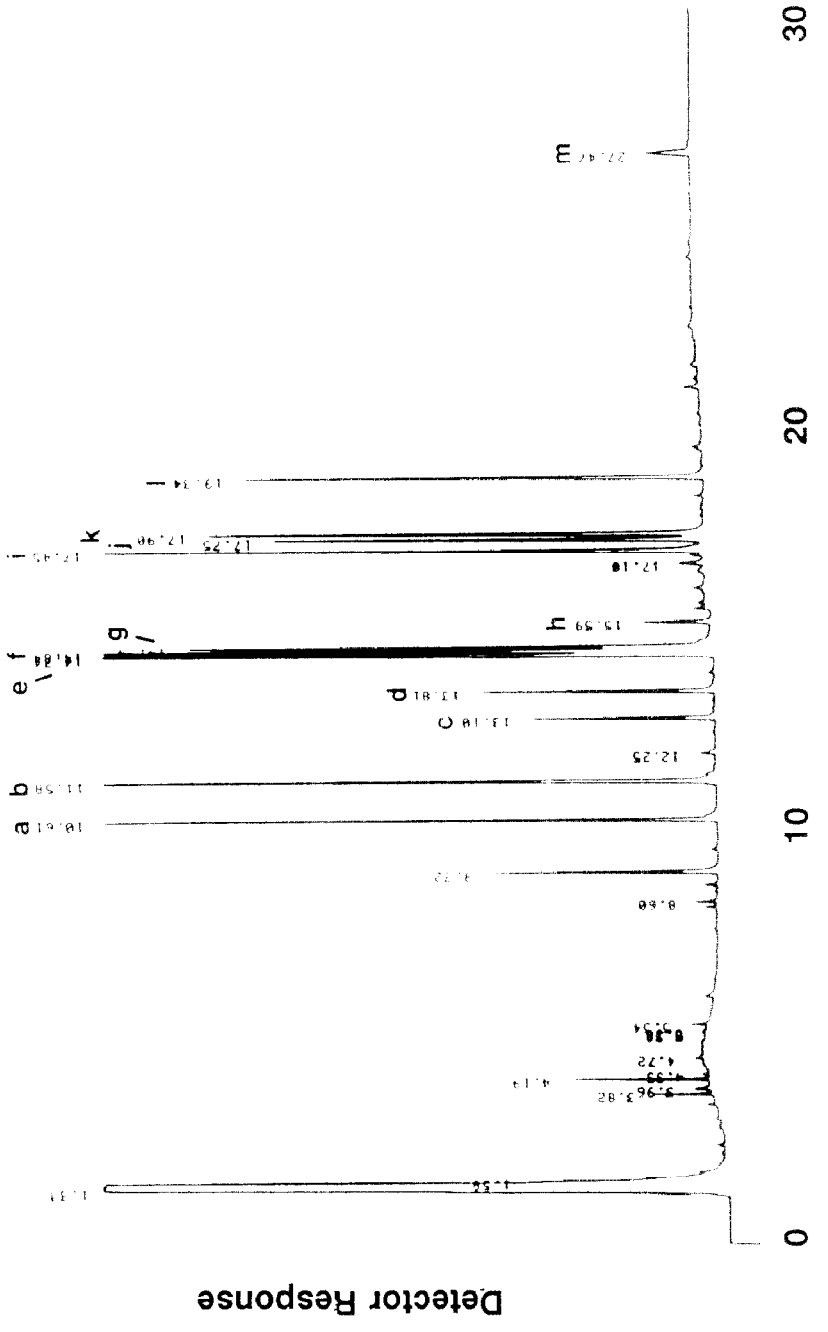

Fig. 1. Gas liquid chromatogram of alditol acetates of partially *O*-methylated, partially *O*-acetylated L-arabinose standard. Exact retention times are labeled on each peak. Deduced glycosyl linkages: a, T-Ara*f*; b, 1-Ara*p*; c, 2-Ara*f*; d, 3-Ara*f*; e, 4-Ara*p*; f, 5-Ara*f*; g, 2-Ara*p*; h, 2,3-Ara*f*; i, 3,4-Ara*p*; j, 3,5-Ara*f*; k, 2,4-Ara*p*; l, 2,5-Ara*f*; m, 2,3,4-Ara*p*. 2,3,5-Ara*f*; n, *myo*-inositol hexaacetate. Material eluting at 9.32 is a contaminant that was present in all samples.

methylated, partially acetylated alditols. Each sample was dissolved in glacial acetic acid (100 μL), and ethyl acetate (0.5 mL) and acetic anhydride (1.5 mL) were added to each and mixed. Acetylation of the partially methylated alditols was catalyzed[9] by the addition of 60% perchloric acid (58 μL). After five min at room temperature, water (5 mL) and 1-methylimidazole (100 μL) were added to each sample and mixed to decompose excess acetic anhydride[9]. After the tubes had cooled to room temperature, each sample was extracted twice with dichloromethane (1 mL). The dichloromethane extracts were combined in a clean tube and extracted with water (3 × 4 mL), centrifuging briefly as needed to aid in phase separation. Following the last extraction, the dichloromethane layer was carefully transferred to a clean tube. This material (1 μL) was injected for g.l.c. analysis, and 0.5 μL was used for g.l.c.–m.s. analysis.

RESULTS AND DISCUSSION

Tentative identification of the peaks obtained from g.l.c. of each partially *O*-methylated, partially *O*-acetylated standard mixture was made by comparison with a table of relative retention times of partially methylated alditol acetates[7] and by comparison with retention times determined previously in this laboratory (Jerry Thomas, unpublished results). Identities of the derivatives were confirmed by their e.i.-mass spectra (refs. 7, 10, and original data). A representative gas chromatogram of one of the standards (L-arabinose) is shown in Fig. 1. Partially methylated alditol acetates arising from both the furanose and pyranose ring forms of L-arabinose are identified, as a mixture of ring forms was formed during the methanolysis of L-arabinose. As expected, only derivatives of the pyranose ring form were produced from the commercially obtained methyl glycopyranosides. Likewise, no derivatives of the furanose forms of L-fucose and L-rhamnose were detected, as these 6-deoxyhexoses are known to form only low amounts of furanosides during methanolysis[11]. Retention times on a 30-m SP2330 g.l.c. column (relative to *myo*-inositol hexaacetate) of all the standards generated in this study are listed in Table I.

A significant amount (~1.5–10 mol%) of per-*O*-methylated methyl glycoside and smaller amounts of partially methylated, partially acetylated methyl glycosides were detected[12–14] in the L-fucose, L-rhamnose, D-mannose, and D-glucose samples, apparently due to incomplete hydrolysis. These compounds had retention times distinct from those of the partially methylated, partially acetylated alditols and thus did not interfere with analysis of the standards.

The described method is a simple, relatively fast procedure for making the standards necessary for glycosyl-linkage analysis of complex carbohydrates. In this procedure, the potassium methylsulfinylmethanide rather than iodomethane limits the methylation. Limiting the base relative to carbohydrate (with subsequent addition of excess iodomethane) is more straightforward to calculate than attempting to limit the amount of iodomethane relative to carbohydrate (in the presence of an excess of base). Acetylation of the partially methylated methyl glycosides facilitates sample clean-up and recovery of the mono-, di-, and tri-*O*-methyl derivatives as well as the unmethylated

TABLE I

Retention times of partially methylated alditol acetates on a 30-m SP2330 column[a]

Glycosyl linkage[b]	*Retention time*[c]	*Glycosyl linkage*	*Retention time*	*Glycosyl linkage*	*Retention time*
T-Rha	0.373	2-Man[f]	0.596	2,4-Man	0.728
T-Ara*f*	0.386	3-Man[f]	0.596	2,4-Glc	0.735
T-Fuc	0.416	2-Glc	0.601	2,4-Gal	0.738
T-Ara*p*	0.421	2,3,4-Rha	0.601	2,3,4-Man	0.738
T-Xyl	0.429	2,3-Fuc	0.605	4,6-Man	0.742
2-Rha	0.476	2,4-Fuc	0.610	3,6-Glc	0.759
2-Ara*f*	0.476	3-Gal	0.614	2,3,4-Gal	0.762
T-Man	0.482	4-Man	0.615	3,6-Man	0.765
3-Rha[d]	0.486	2,3,4-Fuc	0.619	2,6-Man	0.770
4-Rha[d]	0.486	6-Man	0.630	2,3,4-Xyl	0.776
T-Glc	0.487	2-Gal	0.633	4,6-Glc	0.777
3-Ara*f*	0.502	3,4-Ara*p* = 3,5-Ara*f*	0.634	2,6-Glc	0.777
3-Fuc	0.504	6-Glc	0.635	4,6-Gal	0.786
4-Fuc	0.518	4-Gal	0.639	2,3,4-Glc	0.791
T-Gal	0.519	2,3-Ara*p*	0.645	3,6-Gal	0.794
2-Fuc	0.528	4-Glc	0.649	2,6-Gal	0.812
3-Xyl	0.535	2,4-Ara*p* = 2,5-Ara*f*	0.651	3,4,6-Man	0.816
4-Ara*p* = 5-Ara*f*	0.536	2,4-Xyl[e]	0.677	3,4,6-Gal	0.840
3-Ara*p*	0.540	2,3-Xyl[e]	0.677	3,4,6-Glc	0.850
2-Ara*p*	0.543	3,4-Xyl[e]	0.677	2,3,6-Man = 2,4,6-Man	0.872
3,4-Rha	0.554	2,3-Man	0.680	2,4,6-Glc	0.878
2-Xyl[e]	0.562	3,4-Man	0.685	2,3,4,6-Man	0.884
4-Xyl[e]	0.562	6-Gal	0.687	2,3,6-Gal[g]	0.906
2,3-Ara*f*	0.567	2,3,4-Ara*p* = 2,3,5-Ara*f*	0.703	2,4,6-Gal	0.906
3,4-Fuc	0.569	3,4-Gal[g]	0.704	2,3,6-Glc	0.917
2,3-Rha	0.575	2,3-Gal[g]	0.704	2,3,4,6-Gal	0.918
3-Glc	0.591	3,4-Glc	0.715	2,3,4,6-Glc	0.956
2,4-Rha	0.593	2,3-Glc	0.723	*myo*-inositol-Ac$_6$	1.000

[a] Temperature program: 80° for 2 min, 30°/min to 170°, 4°/min to 240°, 10 min hold. [b] T-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl rhamnitol, *etc*. Unless otherwise indicated, all derivatives are assumed to arise from the pyranose form of the sugar. [c] Relative to *myo*-inositol hexaacetate. [d] Leading edge of the peak is primarily 3-Rha; trailing edge is primarily 4-Rha. [e] Enantiomeric mixture, inseparable by g.l.c. [f] Leading edge of the peak is primarily 2-Man, trailing edge is primarily 3-Man. [g] Leading edge of the peak is primarily 2,4-Xyl; trailing edge is primarily the enantiomeric mixture of 2,3- and 3,4-Xyl. [h] Leading edge of the peak is primarily 3,4-Gal; trailing edge is primarily 2,3-Gal. [i] Leading edge of the peak is primarily 4,6-Glc; trailing edge is primarily 2,6-Glc. [j] Enantiomeric mixture, inseparable by g.l.c.

derivatives by enabling all of the derivatives to partition into dichloromethane. Repeated evaporations to remove borate following the reduction step are avoided by using perchloric acid, a compound that catalyzes acetylation of partially methylated alditols even in the presence of borate[9]. Starting from approximately 20 mg of a methyl glycoside, enough standard mixture is produced to suffice for several hundred g.l.c. and g.l.c.–m.s. analyses.

ACKNOWLEDGMENTS

The authors thank Dr. Velupillai Puvanesarajah and Dr. Giuseppe Impallomeni for producing the potassium methylsulfinylmethanide used in this study, Dr. Puvanesarajah for helpful discussions of the e.i.-mass spectra of partially methylated, partially acetylated methyl glycosides, and William S. York for critical reading of the manuscript. This work was supported in part by the USDA/DOE/NSF Plant Science Centers Program, and by DOE grant No. DE-7G-09-87ER13810, and DOE grant No. DE-FG09-85ER13426.

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