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Methylation-GC-MS analysis of arabinofuranose- and galactofuranose-containing structures: rapid synthesis of partially *O*-methylated alditol acetate standards

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ABSTRACT

Arabinose and galactose were treated with MeOH containing traces of H_2SO_4 or HCl at 25°C to give mixtures of their methyl alpha- and beta-furanosides, as shown by 1D and 2D nuclear magnetic resonance (NMR). Oxidation of the Me alpha,beta-Ara*f* mixture with $NaIO_4$ preferentially oxidised the beta-isomer, to give pure Me alpha-Ara*f*. Each product was progressively *O*-methylated using the Purdie reagent (MeI/Ag_2O) at 25°C and resulting mixtures of partially methylated glycosides (PMGs) were rapidly assayed by thin layer chromatography (TLC) first to favour higher yields of mono-*O*-methyl derivatives and later for products with higher degrees of methylation. The products were converted to complex mixtures of partially *O*-methylated alditol acetate derivatives (PMAAs) by successive hydrolysis, reduction with $NaBD_4$, and acetylation. These can be used as gas chromatography-mass spectrometry (GC-MS) standards in methylation analysis of complex carbohydrates containing arabinofuranosyl and galactofuranosyl units. Of particular interest were the retention times and electron impact MS of the difficult to prepare alditol acetates of 5,6-Me_2Gal, 2,5-Me_2Gal, 2,5,6-Me_3Gal, 3,5,6-Me_3Gal, 5-MeAra, 2,5-Me_2Ara, and 3,5-Me_2Ara. The relative reactivities of hydroxyl groups for mixtures of Me alpha- and Me beta-Gal*f* were HO-2 > HO-3 > HO-6 > HO-5, that of Me alpha- and Me beta-Ara*f* HO-2 > HO-3 > HO-5, and that of Me alpha-Ara*f* HO-2 > HO-3 ≥ HO-5.

Key words: partially *O*-methylated alditol acetates, GC-MS standards, Purdie methylation, OH reactivity, NMR.

INTRODUCTION

Arabinose and galactose are widely found in complex carbohydrates and are present in pyranosyl and furanosyl rings in molecules encompassing a great variety of glycosidic linkages with α- and β-anomeric configurations. Units of Ara*f* are found in polysaccharides of plants, as in gums and free or protein-linked arabinogalactans: to the latter many functions have been assigned, including plant growth and development, as well as cell proliferation, expansion and death (Svetek et al. 1999). On the other hand, galactofuranosyl-containing molecules do not occur in green plants, but are widespread in glycolipids, proteoglycans, cell-wall polysaccharides, and exopolysaccharides (EPS) of fungi and yeasts (Barreto-Bergter and Gorin 1983, Levery et al. 1998, Sassaki et al. 2002, Jones et al. 2004). Gal*f* units are also present in macromolecules of bacteria and protozoans, but not in mammals, which

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are highly immunogenic to them. The Gal*f*-containing molecules of bacteria, protozoans, and fungi appear to play different roles, but their influence in the recognition and/or invasion parasite-host have been observed (Suzuki et al. 1997, Levery et al. 1998), probably by their β-Gal*f*-containing epitopes.

Methylation analysis of complex sugars provides information on the substitution position of their glycosidic linkages. In this procedure the molecule can be first completely *O*-methylated by the methods of Haworth 1915, Kuhn et al. 1955, and Hakomori 1964, but nowadays the most used frequently in our hands is that of Ciucanu and Kerek 1984. The products can be converted by successive hydrolysis to give partially *O*-methylated aldoses, which are reduced with $NaBH_4$, followed by acetylation to provide partially *O*-methylated alditol acetates (PMAAs), which on GC-MS, have typical retention times (R_t) and electron impact (e.i.) spectra (Jansson et al. 1970). Sometimes, the product suffers from a symmetry problem, for example 2,3-di-*O*- = 3,4-di-*O*-methylxylitol acetate, but this can be overcome by reduction with $NaBD_4$, which introduces deuterium at C-1 (Carpita and Shea 1989).

For the preparation of PMAA standards, there is an option of preparing each one individually or all at once by partial *O*-methylation of methyl aldosides. For the latter experiment, Haworth (Me_2SO_4/NaOH), Purdie (MeI/Ag_2O), Kuhn (MeI/DMF/Ag_2O), and Hakomori (MeI/methylsulfinylmethanide/Me_2SO) methylations have been carried out. The methylation of methyl α-mannopyranoside was performed out by Handa and Montgomery (1969), using the Haworth, Kuhn, and Hakomori procedures, and by Fournet and Montreuil (1973) with the Kuhn methylation, which formed all possible *O*-methyl derivatives, except for the mono-*O*-methyl ones. This was rectified by Fournet et al. (1974) who prepared and converted them to their mono-*O*-methyl alditol acetates, which were examined by GC-MS, sodium borodeuteride being used in the intermediate reduction step. A similar procedure was used for preparation of 15 *O*-methyl alditol acetates starting from methyl α-galactopyranoside (Fournet et al. 1978) and the MS data were presented in a tabular form.

Another of the early investigations directed to the preparation of methylation standards for carbohydrate analysis was by Elkin et al. (1975), who partially and fully *O*-methylated the methyl pyranosides of Rha, Fuc, Ara, Xyl, Man, Gal, and Glc, among others with the Purdie reagent (Purdie and Irvine 1903), but the resulting *O*-methyl derivatives were only poorly resolved by GLC and MS and not employed.

Although they did not contain the synthesis of PMAAs, the classic publications of Jansson et al. (1970) and Carpita and Shea (1989) contained the retention times (R_t's) and e.i. profiles on GC-MS, with few exceptions, of those that can arise from pyranosyl and furanosyl structures.

Lomax and Conchie (1982) used the Purdie reagent to partially and fully methylate the same methyl pyranosides and converted them to PMAAs, which were subjected to GC and although MS was mentioned, no details were included. Methyl arabinofuranoside but not methyl galactofuranoside were investigated.

Doares et al. (1991) carried out partial to complete methylation of methyl pyranosides of Rha, Fuc, Ara, Xyl, Man, Gal, and Glc with potassium methylsulphinylmethanide in Me_2SO/MeI to form PMGs. Their R_ts of resulting PMAAs, formed using an intermediate sodium borodeuteride reduction, were recorded and identification was accomplished by referring to the e.i. profiles of Carpita and Shea (1989). A study incorporating synthesis of partially to fully *O*-methylated PMG derivatives from the same methyl pyranosides, followed by their conversion to all possible PMAAs, with the exception of the 6-*O*-methyl derivative, and determination of their R_ts and e.i. breakdown profiles, was carried out by Sassaki et al. (2005) using the relatively easy to handle Purdie reagent.

The same approach has now been extended to the furanoside series, starting from synthesised methyl arabinofuranosides and methyl galactofura-

nosides, to finally form all the PMAAs necessary for GC-MS analysis of arabinofuranose- and galactofuranose-containing structures.

MATERIALS AND METHODS

Preparation of Methyl Glycofuranosides

Galactose and arabinose were obtained from Sigma-Aldrich, MO, U.S.A and each (500 mg) was stirred in 0.5% w/w MeOH-HCl (100 ml) or 0.5% w/w MeOH-H_2SO_4 (100 ml) at 25°C until complete dissolution (for Ara: 6 h, for Gal: 16 h). The solution was neutralised with excess pyridine, evaporated to a small volume, and acetylated with Ac_2O-pyridine (2 ml; 1:1, v/v) overnight at room temperature. The mixture was added to excess ice-water and after 1 h, it was extracted with $CHCl_3$, which was evaporated to dryness. The residue was dissolved in MeOH containing NaOMe (5 ml, 200 mM) and after 2 h, the solution was evaporated to dryness, and then treated with an aq. suspension of Dowex 50W-X8 H^+, providing after evaporation Me $\alpha\beta$-Ara*f* or Me $\alpha\beta$-Gal*f*.

Me $\alpha\beta$-Ara*f* (113 mg) with treated with 2 molar equivalents of $NaIO_4$ (295 mg) in H_2O (10 ml) for 30 min at 25°C. The solution was treated with a mixture of Amberlite IR-120 (H^+) and IR-400 (OH^-), the latter to remove dialdehyde, the suspension filtered, and the filtrate freeze dried to give Me α-Ara*f* (19 mg).

NMR Analysis of Methyl Arabinoside and Methyl Galactofuranoside Preparations

Each methyl glycofuranoside preparation was deuterium exchanged by repeated D_2O dissolution, followed by evaporation. 1D and 2D ^1H and coupled and decoupled ^{13}C NMR spectra were obtained using a Bruker Avance DRX-400 spectrometer with a 5 mm inverse probe. ^{13}C-NMR acquisitions were performed using the following parameters: FIDRES- 0.8466 Hz, AQ- 0.5906 s, DW 15.75 s, DE- 5.5 μs D1- 110 msec, D2- 3.4 msec, PL12-17dB (decoupler ^1H), waltz 16 pulse program. Coupled ^{13}C NMR spectrum was obtained under similar conditions using PL12 of 60db. The spectra were obtained in D_2O either at 30°C or 70°C, and chemical shifts measured in relation to Me_4Si ($\delta = 0$).

Purdie Methylation of Methyl Glycofuranosides

Each glycoside (10 mg) was submitted to vigorous stirring in MeI (1.5 ml), containing Ag_2O (250 mg) at room temperature over a period of 2 h. Each one gradually dissolved in the suspension and the degree of methylation was monitored at 30 min intervals using aliquots, which were removed and spotted on to TLC plates (solvent: $CHCl_3$-EtOH, 9:1 v/v), developed with orcinol-H_2SO_4 spray – 100°C for 5 min (Skipiski 1975), and the spot intensities measured by Scion Imaging.

Preparation of PMAAs

PMG mixtures were evaporated to dryness and the residue was hydrolysed with M H_2SO_4 for 8 h at 100°C. The solution was neutralised ($BaCO_3$), and the mixture containing partially *O*-methylated aldoses reduced with $NaBD_4$ (5 mg) for 4 h at room temperature. The solution was neutralised with 50 μl glacial HOAc, dried under reduced pressure, and co-distilled with 100 μl of MeOH at 50°C, this step being repeated thrice. The product was acetylated with Ac_2O-pyridine (500 μl; 1:1, v/v) overnight at room temperature. The PMAAs were extracted with $CHCl_3$ and washed with 2% aq. $CuSO_4$ and the organic layer containing PMAAs dried at room temperature, and the residue dissolved in acetone before GC-MS analysis.

GC-MS

Each PMAA mixture was dissolved in acetone and examined by GC-MS at a range from m/z 80 to 220, using a Varian GC, Model 3300 coupled to a Finnigan MS with ion trap detector (model ITD 800). The PMAA was applied to an OV-225 fused silica capillary column (Quadrex – 30 m × 0.25 mm i.d.), with He as carrier gas. Conditions: electron impact at 70 eV; injector temp.: 250°C; initial temp.: 50°C

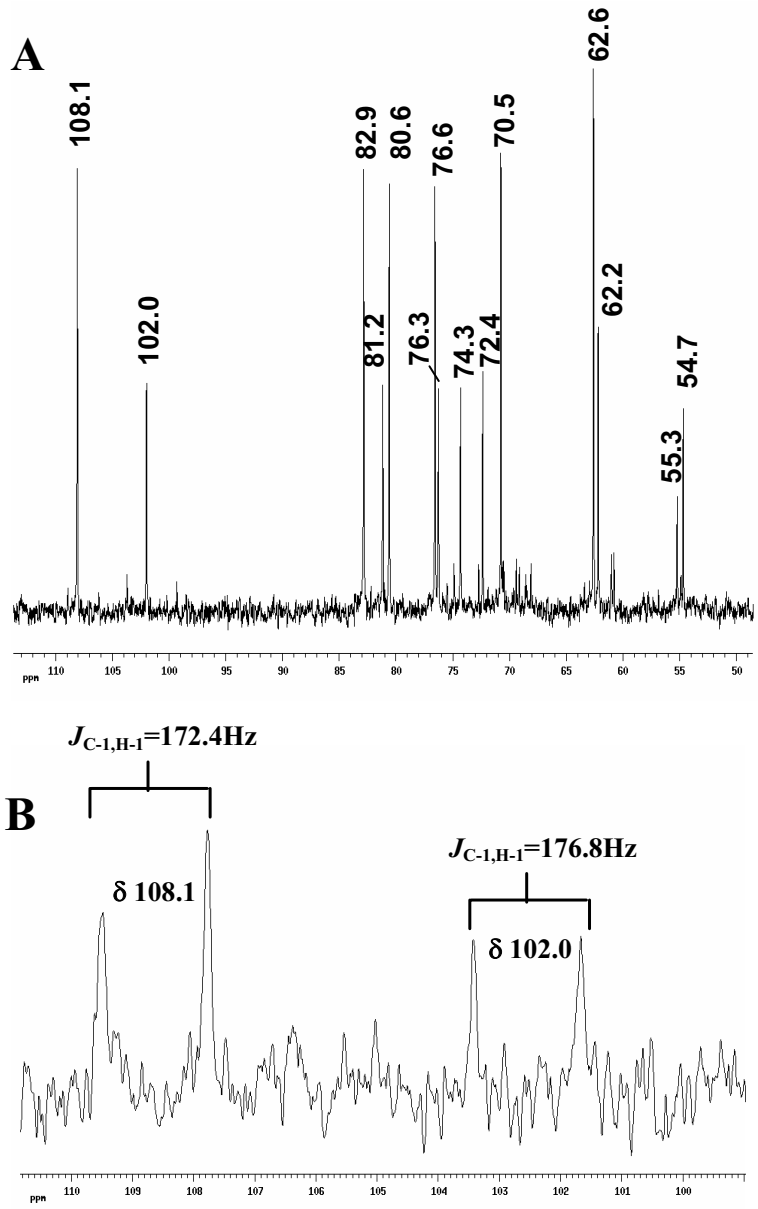


Fig. 1 – ^{13}C NMR spectrum of methyl $\alpha\beta$-galactofuranoside product (A) and its $^{13}C/^{1}H$-coupled anomeric region (B).

(1 min); temp. program: 40°C min^{-1} to 220°C, maintained for 25 min.

RESULTS AND DISCUSSION

Arabinose and galactose were each converted to mixtures of methyl glycofuranosides using MeOH containing traces of H_2SO_4 25°C. The acid was removed by neutralisation with pryridine, followed by acetylation and deacetylation, ^{13}C-NMR examination of the Me $\alpha\beta$-Gal product at 30°C in D_2O showed that they had furanosyl rings, as two typical low field signals at δ 108.1 and 102.0 (Fig. 1A) were present (Gorin and Mazurek 1975) in a ratio of $\sim$ 1.95 : 1. Its ^{13}C, ^{1}H-coupled spectrum contained C-1 doublets centred at δ 108.1 with J = 172.4 Hz) from Me β-Gal$f$, whereas that centred at δ 102.0 had J = 176.8 Hz (Fig. 1B). These values differed from those of a standard mixture of Me β-Gal$p$ of Me α-Gal$p$, which gave respective signals at δ 103.5 (Fig. 2A), J = 162.1 Hz (Fig. 2B) and δ 99.1 for Me

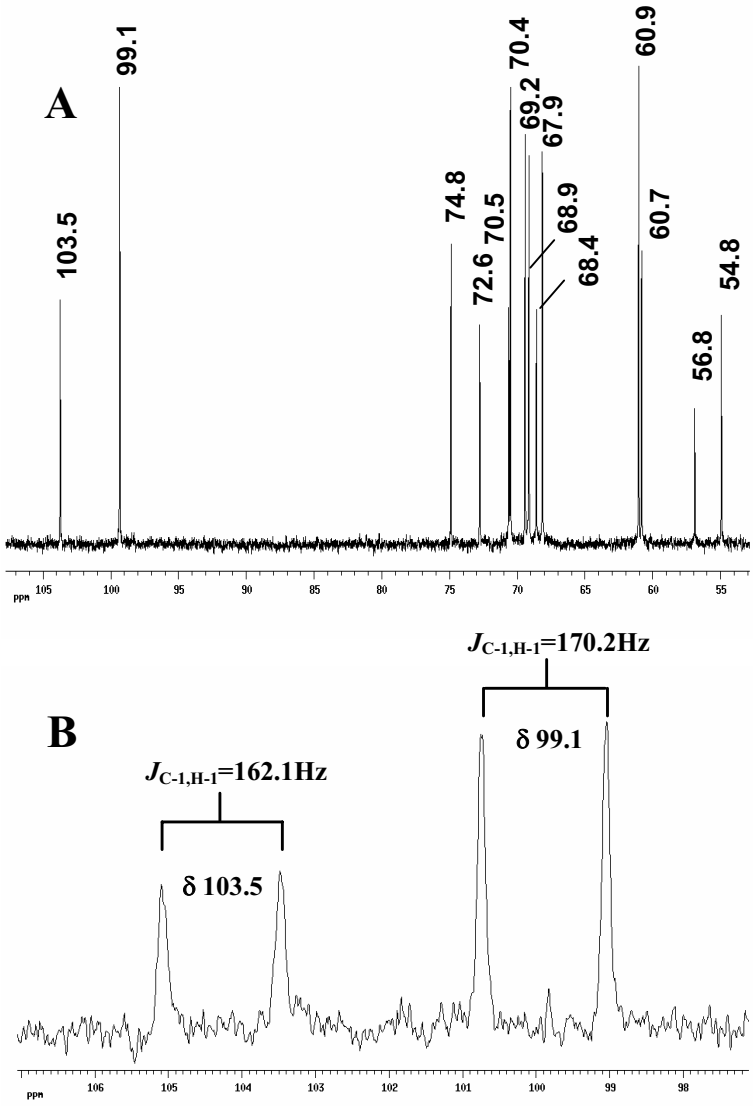


Fig. 2 – ^{13}C NMR spectrum of mixed methyl α- and methyl-β-galactopyranosides (A) and their $^{13}C/^{1}H$-coupled anomeric region (B).

β-Gal*p* (Fig. 2A) and J = 170.2 Hz (Fig. 2B) for Me α-Gal*p*. The J values are consistent with those of Chambat et al. (1978), who found C-1 of Me β-Gal*f* to have 172.5 Hz and C-1 of Me α-Gal*f* to have $\sim$ 175 Hz and Perlin and Casu (1969), who reported 169 Hz for α-Glc*p* and 160 Hz for β-Glc*p*.

Using a similar procedure, the decoupled and coupled ^{13}C-NMR spectra of the methyl glycosidic mixture formed from arabinose contained C-1 signals for Me α-Ara*f* and Me β-Ara*f* respectively at δ 108.7 (Fig. 3A) and J = 173.1 Hz (Fig. 3B) and 102.6 (Fig. 3A) and J = 173.1 Hz (Fig. 3B) in a ratio of $\sim$ 1.5 : 1 (Fig. 3).

When the Me $\alpha\beta$-Ara*f* mixture was treated with an excess of sodium periodate over a short period of time, the product after removal of dialdehyde with resin only gave the signals of Me α-Ara*f* at δ 108.7 (C-1), 84.2 (C-4), 81.1 (C-2), 76.9 (C-3), 61.7 (C-5), and 55.6 OCH_3, showing that the Me β-Ara*f* was preferentially oxidised.

Methylation of the methyl arabinofuranosides and galactofuranosides with the Purdie reagent over a period of time gave rise to PMGs with progressive increase in their degree of *O*-methylation. It

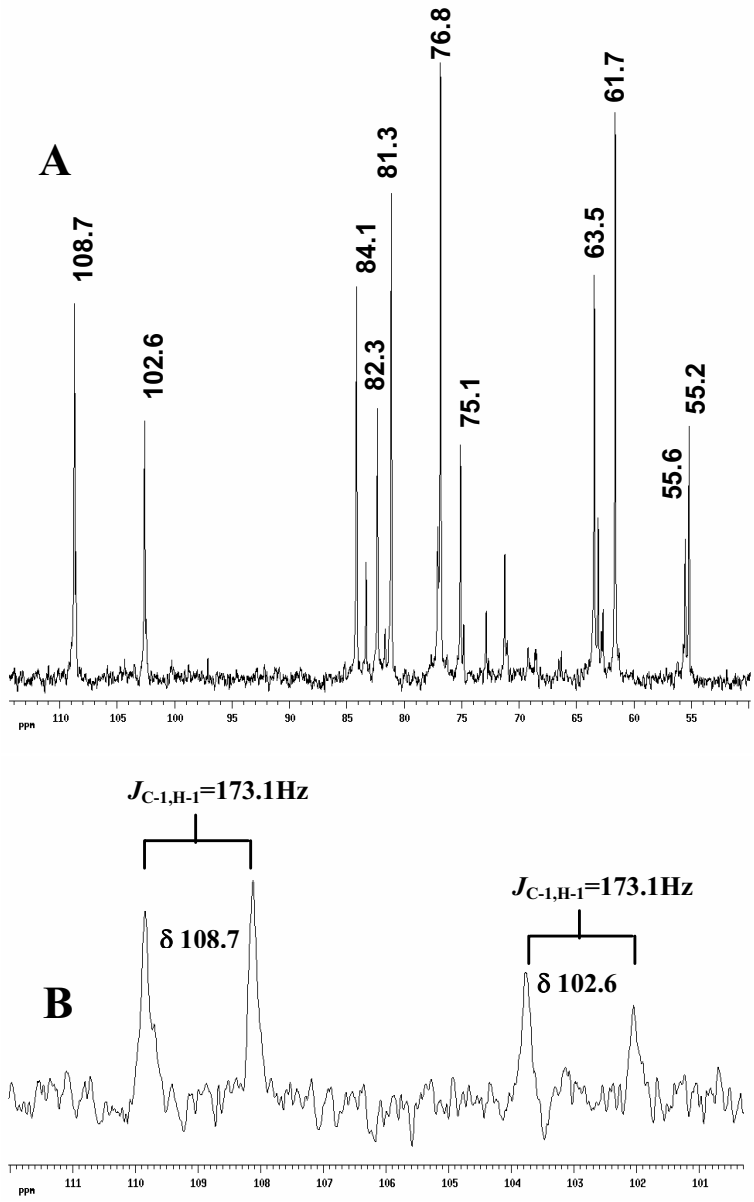


Fig. 3 – ^{13}C NMR spectrum of methyl $\alpha\beta$-arabinofuranoside product (A) and its ^{13}C/^{1}H-coupled anomeric region (B).

was found using TLC that after 1 hour Me $\alpha\beta$-Ara$f$ gave rise mainly to mono-O- (R_f 0.31-0.50) and di-O-methyl derivatives (R_f 0.52-0.62) and after two hours the tri-O-methyl derivative (R_f 0.75) was formed. The derivatives of Me $\alpha\beta$-Gal$f$ were mainly mono-O- (R_f 0.31-0.50) and di-O-methyl (R_f 0.52-0.62) after 1 hour and tri-O-methyl (R_f 0.64) and tetra-O-methyl (R_f 0.75) after two hours. The Purdie methylation has the advantage of being easier to handle than those of Kuhn et al. (1955) and Doares et al. (1991), since TLC examination could be carried out by directly spotting the reaction mixture at intervals of 30 minutes on to TLC plates, without prior removal of non-volatile solvents.

Each product obtained after 1 and 2 hours reaction time was converted to mixtures of PMAAs via successive hydrolysis, reduction with $NaBD_4$, and acetylation. The resulting PMAA mixtures contained the necessary components for analysis of all, with one exception, arabinofuranose- and galacto-

TABLE I

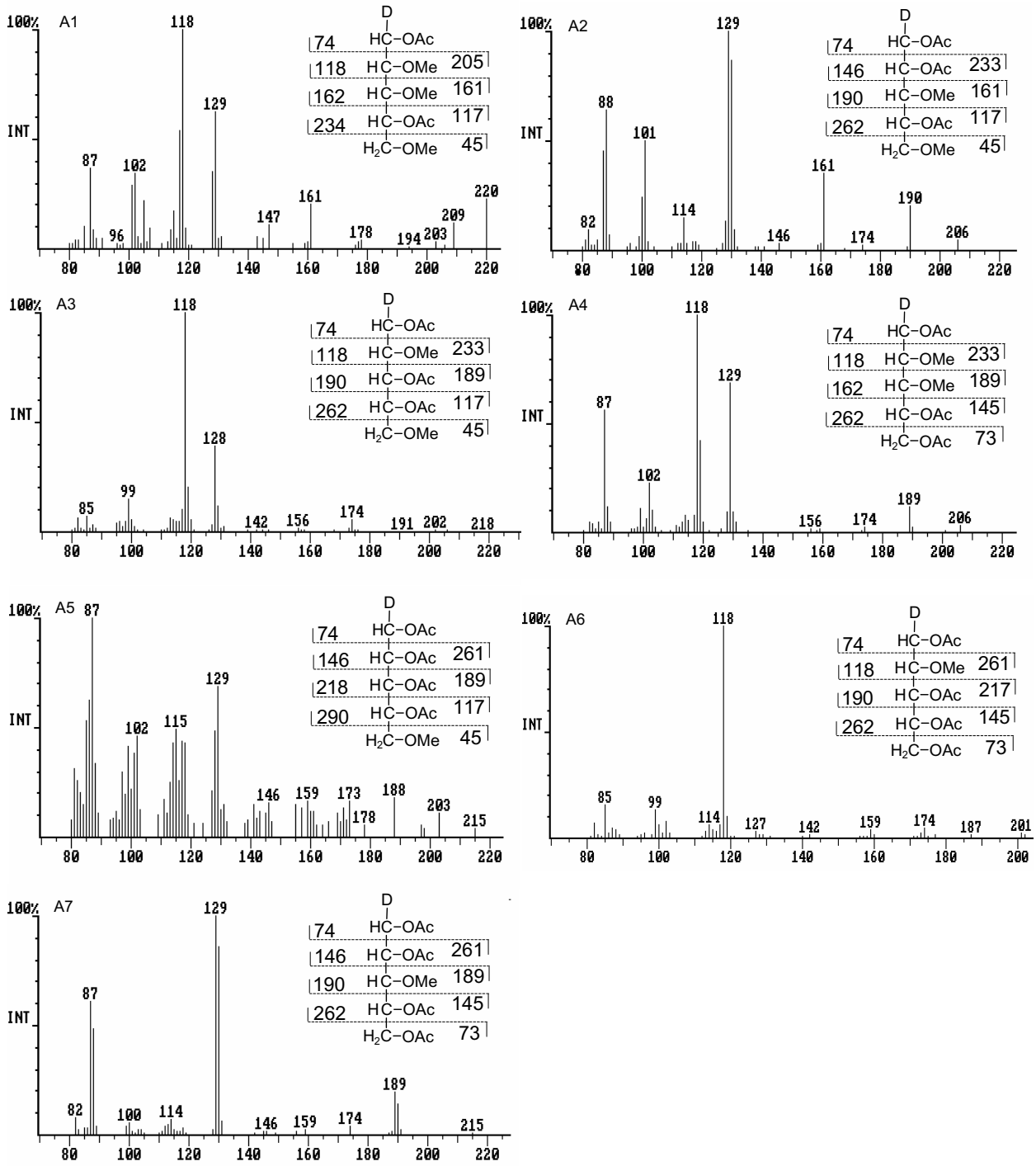
Partially *O*-methylated alditol acetates obtained following Purdie methylation synthesis after 2 h reaction with methyl furanosides of Ara and Gal.

OMe alditol acetate	R_t	From $\alpha\beta$-derivative[a]	From α-Ara*f*
2,3,5-Me_3Ara	7.3	1.0	1.7
3,5-Me_2Ara	8.3	0.7	1.3
2,5-Me_2Ara	8.5	11.1	18.0
2,3-Me_2Ara	9.1	25.0	24.5
5-MeAra	9.5	3.4	7.3
2-MeAra	10.5	46.1	39.5
3-MeAra	11.1	12.0	7.7
2,3,5,6-Me_4Gal	8.3	1.4	–
2,5,6-Me_3Gal	10.5	5.6	–
2,3,5-Me_3Gal	12.1	1.7	–
2,3,6-Me_3Gal	11.1	5.9	–
3,5,6-Me_3Gal	11.1	1.4	–
2,3-Me_2Gal	15.2	10.2	–
2,5-Me_2Gal	15.0	1.3	–
2,6-Me_2Gal	12.6	32.0	–
3,5-Me_2Gal	15.5	2.1	–
3,6-Me_2Gal	13.5	6.0	–
[b]5,6-Me_2Gal	12.0	5.3	–
2-MeGal	17.4	17.8	–
3-MeGal	20.1	8.7	–
[c]5-MeGal	17.4	0	–
6-MeGal	16.5	0.4	–

[a]Mixture of $\alpha\beta$-anomers obtained on methyl glycosidation; [b]5,6-Me_2Gal eluted before 2,3,5-Me_3Gal*f*; [c]Indentifiable in methylation analysis mixtures with identical R_t as that of 2-MeGal, but with a key ion at m/z 117 instead of 118.

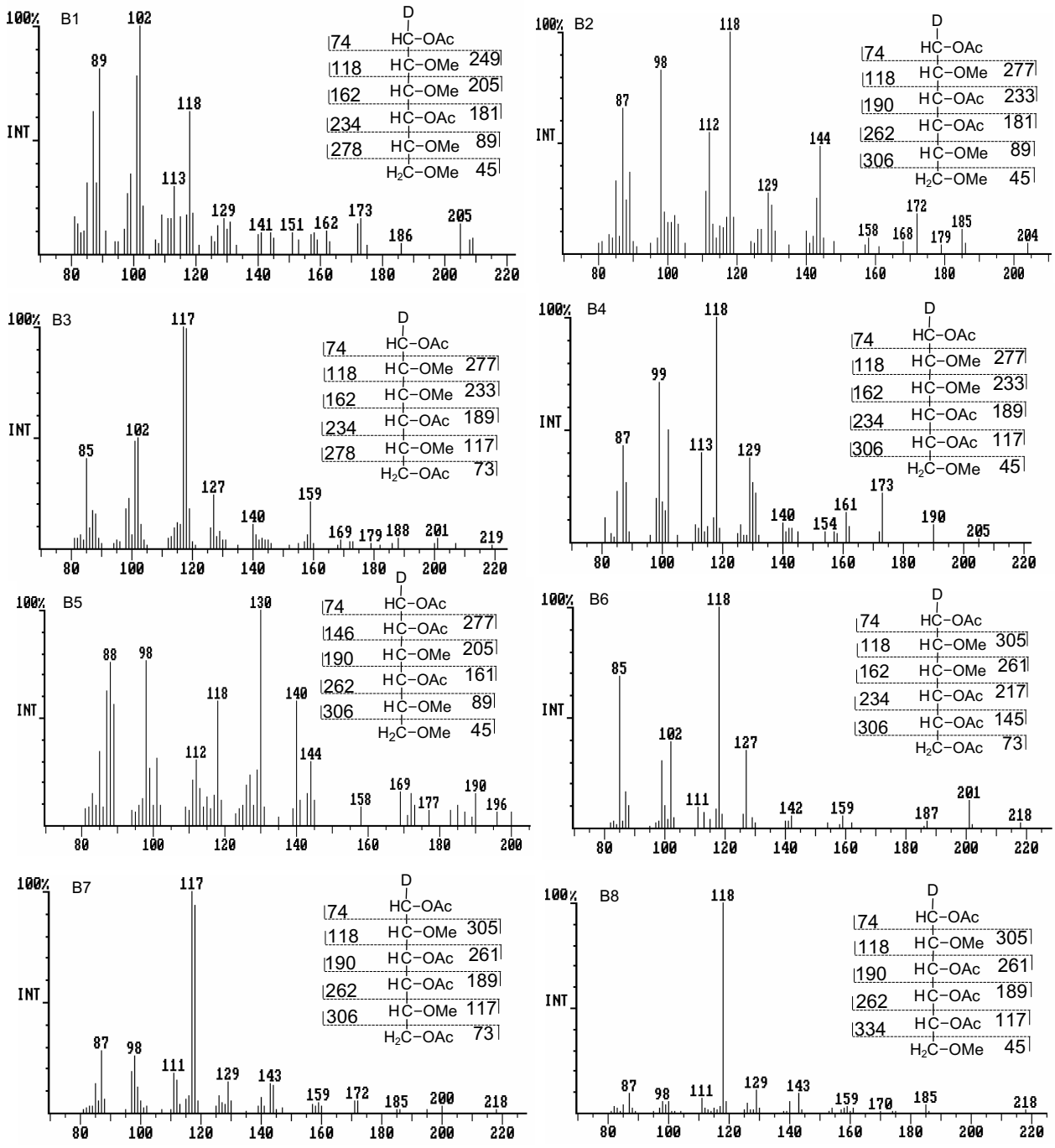
furanose-containing structures by GC-MS, by virtue of their typical R_ts and e.i. breakdown patterns (Table I and Fig. 4). The e.i. profile of each PMAA was recorded over a range of m/z 80 to 220 and its key ions are represented in Figure 4. Although some of the yields were rather low, the required derivatives of 3,5,6-Me_3Gal (1.4%), 2,5,6-Me_3Gal (5.6%), 2,5-Me_2Gal (1.3%), 3,5-Me_2Gal (2.1%), and 5,6-Me_2Gal (5.3%) were obtained, being were detectable on GC-MS. One exception in the rapid synthesis was the acetate of 5-*O*-methylgalactitol. However, its presence in any methylation analysis mixture could be detected since it has an R_t identical to that of the 2-*O*-methyl derivative and would give a similar EI-MS, but with a key ion at m/z 117 instead of 118 (Figure 4, B12).

For determination of the relative reactivities of hydroxyl groups in the Me $\alpha\beta$-Gal*f* mixture, attention was paid to the formation of mono-*O*-methyl derivatives, since further methylation of -

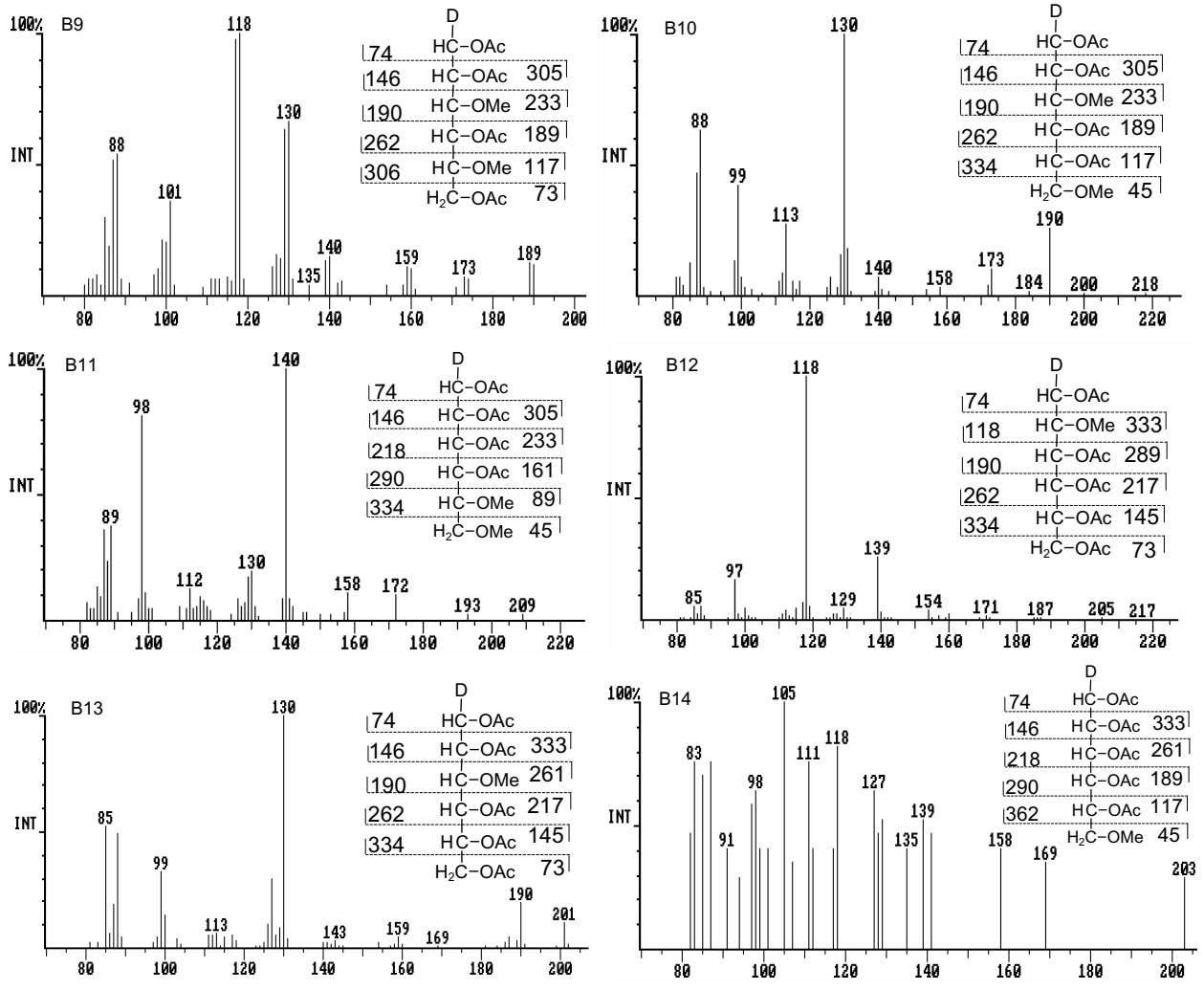


(4a)

Fig. 4 – EI-MS patterns at m/z 80 to 220 of PMAA standards deuterated at C-1: 2,3,5-Me_3Ara (A1), 3,5-Me_2Ara (A2), 2,5-Me_2Ara (A3), 2,3-Me_2Ara (A4), 5-MeAra (A5), 2-MeAra (A6), 3-MeAra (A7), 2,3,5,6-Me_4Gal (B1), 2,5,6-Me_3Gal (B2), 2,3,5-Me_3Gal (B3), 2,3,6-Me_3Gal (B4), 3,5,6-Me_3Gal (B5), 2,3-Me_2Gal (B6), 2,5-Me_2Gal (B7), 2,6-Me_2Gal (B8), 3,5-Me_2Gal (B9), 3,6-Me_2Gal (B10), 5,6-Me_2Gal (B11), 2-MeGal (B12), 3-MeGal (B13), and 6-MeGal (B14).


B1
74 HC–OAc
118 HC–OMe 249
162 HC–OMe 205
234 HC–OAc 181
278 HC–OMe 89
H2C–OMe 45
B2
74 HC–OAc
118 HC–OMe 277
190 HC–OAc 233
262 HC–OAc 181
306 HC–OMe 89
H2C–OMe 45
B3
74 HC–OAc
118 HC–OMe 277
162 HC–OMe 233
234 HC–OAc 189
278 HC–OMe 117
H2C–OAc 73
B4
74 HC–OAc
118 HC–OMe 277
162 HC–OMe 233
234 HC–OAc 189
306 HC–OAc 117
H2C–OMe 45
B5
74 HC–OAc
146 HC–OAc 277
190 HC–OMe 205
262 HC–OAc 161
306 HC–OMe 89
H2C–OMe 45
B6
74 HC–OAc
118 HC–OMe 305
162 HC–OMe 261
234 HC–OAc 217
306 HC–OAc 145
H2C–OAc 73
B7
74 HC–OAc
118 HC–OMe 305
190 HC–OAc 261
262 HC–OAc 189
306 HC–OMe 117
H2C–OAc 73
B8
74 HC–OAc
118 HC–OMe 305
190 HC–OAc 261
262 HC–OAc 189
334 HC–OAc 117
H2C–OMe 45

(4b)



(4c)

CHOH groups vicinal to those of -$CHOCH_3$ would be more rapid. Consequently we can interpret (Table I) and that in the case of the $\alpha\beta$-mixture of Me-Gal*f* the order is HO-2 > HO-3 > HO-6 > HO-5, which differs from those of Me α- and β-Gal*p*, which is HO-3 > HO-2 > HO-4 > HO-6 (Sassaki et al. 2005).

The relative reactivities for the Me $\alpha\beta$-Ara*f* mixture was HO-2 > HO-3 > HO-5, and for Me α-Ara*f*, they were not markedly different, with HO-2 > HO-3 $\geq$ HO-5 (Table I).

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RESUMO

Arabinose e galactose foram tratadas com metanol anidro contendo quantidades catalíticas de H_2SO_4 e HCl a 25°C visando a formação de suas respectivas misturas de alpha- e beta-metil- glicofuranose, as quais foram confirmadas por ressonância magnética nuclear (RMN) unidimensional e bidimensional. A mistura de alpha, beta-Me-Ara*f* foi oxidada com $NaIO_4$, dando origem somente à Me-alpha-Ara*f*, mostrando que o isômero beta é oxidado preferencialmente. Cada derivado furanosídico foi metilado pelo reagente de Purdie (Ag_2O/MeI) a 25°C, dando origem a misturas de metil glicofuranosídeos parcial-

mente metilados (Me-GFP), os quais tiveram seus respectivos graus de metilação monitorados por cromatografia em camada delgada (CCD), que variaram inicialmente em altos rendimentos de derivados mono metilados de Me-GFP a derivados com alto grau de metilação. Os produtos da reação foram convertidos a derivados alditol acetato parcialmente metilados (AAPM), após hidrólise, seguida de redução com $NaBD_4$ e posterior acetilação. Os derivados obtidos podem ser utilizados como padrões de AAPM em CG-EM para auxiliar na análise de metilação de carboidratos complexos que possam conter unidades de arabinofuranose e galactofuranose. Os derivados AAPM de 6-MeGal, 5,6-Me_2Gal, 2,5-Me_2Gal, 2,5,6-Me_3Gal, 3,5,6-Me_3Gal, 5-Me_2Ara, 2,5-Me_2Ara e 3,5-Me_2Ara mostraram espectros de eletroimpacto e tempos de retenção de maior interesse pois são de difícil síntese. A avaliação da reatividades dos grupos hidroxílicos nesta metilação mostrou que a mistura de alpha, beta-Me-Gal*f* reage preferencialmente em HO-2 > HO-3 > HO-6 > HO-5, a mistura de alpha, beta-Me-Ara*f* em HO-2 > HO-3 > HO-5, alpha-Me-Ara*f* em HO-2 > HO-3 $\geq$ HO-5.

Palavras-chave: alditol acetatos parcialmente metilados, padrões p/ CG-EM, metilação de Purdie, reatividade grupos hidroxílicos, RMN.

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