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Note

Oxidation of lactose with bromine

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Abstract—Oxidation of lactose by bromine in an aqueous buffered solution was conducted as a model experiment to examine the glycosidic linkage cleavage occurring during the oxidation of oligosaccharides and polysaccharides. The resulting oxidation products, after reduction with sodium borodeuteride, were characterized by GLC–MS analyses of the per-*O*-methyl or per-*O*-Me_3Si derivatives. Most of the products were carboxylic acids, of which lactobionic acid was major. Minor products, identified after partial fractionation on a BioGel P-2 column, comprised oxalic acid; glyceric acid; threonic and erythronic acids; tartaric acid; lyxonic, arabinonic, and xylonic acids; galactonic and gluconic acids; galactosylerythronic acid; galactosylarabinonic acid; galactosylarabinaric acid; galacturonosylarabinonic acid; and galactosylglucaric acid. No keto acids were identified. Galactose was detected as 1-deuteriogalactitol, the presence of which, together with the C_6 aldonic acids, supported a galactosidic bond cleavage. Galactosylarabinonic acid was the major constituent (7.5%) among minors, and others constituted 0.2–3.7% of the principal lactobionic acid. These products together comprised 29% of the lactobionic acid, more than half (17%) of which were accounted for by the galactosidic linkage cleavage, supporting the significant decrease in molecular weight seen earlier in the bromine-oxidized polysaccharides by glycosidic cleavage.

Keywords: Bromine oxidation; Lactose; Glycosidic bond cleavage

1. Introduction

The hydrodynamic properties of two well characterized extracellular polysaccharides (EPS9 and EPS6)[1,2] from *E. chrysanthemi* had been examined after the introduction of additional carboxylic acids by periodate oxidation of the EPSs and subsequent bromine oxidation. Bromine water in the presence of excess calcium carbonate converted the polyaldehyde into its polycarboxylic acid, resulting in the increased charge density of the EPS. However, extensive depolymerization of the periodate-oxidized polysaccharides occurred in the course of bromine oxidation, as shown by significant reduction in the molecular weight of the resulting products.[3] This depolymerization was also true with the native EPSs (Table 1).

Table 1. Molecular weight (M_w) of native polysaccharides (EPS9 and EPS6) and its periodate-oxidized polyaldehydes before and after bromine oxidation

Sample	M_w ($\times 10^6$)	
	Before Br_2 oxidation	After Br_2 oxidation
EPS9	4.11	0.01
EPS9A1.6[a]	3.72	0.05
EPS9A1.8[a]	3.80	0.06
EPS9A2.7[a]	4.33	0.04
EPS6	1.75	0.01
EPS6A1.2[a]	1.41	0.27
EPS61.7[a]	1.34	0.13

The samples were analyzed at 25 °C by SEC-LS in 0.15 M Na_2SO_4 containing 0.015 M EDTA.
[a] EPS9A1.6, Polyaldehyde derivative of EPS9 after 1.6 mol of periodate consumed per RU, and etc.; Yang et al.[3]

Abbreviations: EPS, extracellular polysaccharide; RU, repeating unit of EPS; EPS9, EPS from Ech9Sm6; EPS6, EPS from Ech6S+; GLC–FID, gas–liquid chromatography with flame-ionization detection; GLC–MS, gas chromatography with electron-impact (70 eV) mass-selective detector; HPAEC-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; SEC-LS, size exclusive chromatography with light scattering detection.

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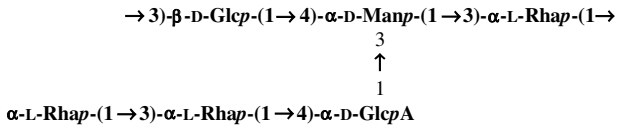
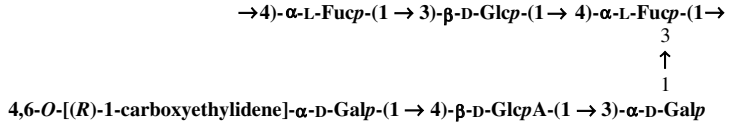


***Erwinia chrysanthemi* EPS9**



***Erwinia chrysanthemi* EPS6**

Oxidation of reducing sugars with bromine in buffered solution, such as barium benzoate, converts the sugars directly to the lactone[4] and provides a convenient preparation of aldonic acids. Such oxidation at neutral pH introduces keto groups into the non-reducing sugars, such as methyl glycosides[5] and trehalose,[6] in which steric hindrance brings in a selective oxidation of secondary hydroxyl group to the 2-, 3-, or 4-ulose. Reactions of polysaccharides with such reagents are more complex and selectivity of secondary hydroxyl groups is compromised, possibly as a result of conformational change in the polymer.[7] Also, prolonged reaction introduces carboxylic acid groups and depolymerization of the polysaccharide.[8] The acidic products introduced by such oxidation comprises dicarboxylic acids[9,10] as a result of the carbon ring cleavage, as seen from methyl glycosides and amylose, gluconic acid[11] from starch, and uronic acid[5] from methyl glycoside. This prompted a model experiment, the oxidation of lactose (1 mol equiv) by bromine (2 mol equiv), in which evidence of the galactosyl linkage cleavage was demonstrated. The resulting products, after reduction with borodeuteride and subsequent partial fractionation on BioGel P-2 column, were characterized by gas–liquid chromatography with flame-ionization detection (GLC–FID) or electron-impact mass-selective detection (GLC–MS) of per-*O*-methyl and per-*O*-Me_3Si derivatives.

2. Results and discussion

2.1. Identification of >C_6 acids

BioGel P-2 gel permeation chromatography (Fig. 1) of the oxidized products after reduction with $NaBD_4$ showed one peak (F1) by the phenol–H_2SO_4 method,[12] and three additional small peaks (F2, F3, and F4) by periodate-oxidation alditol assay.[13] Lactobionic acid,

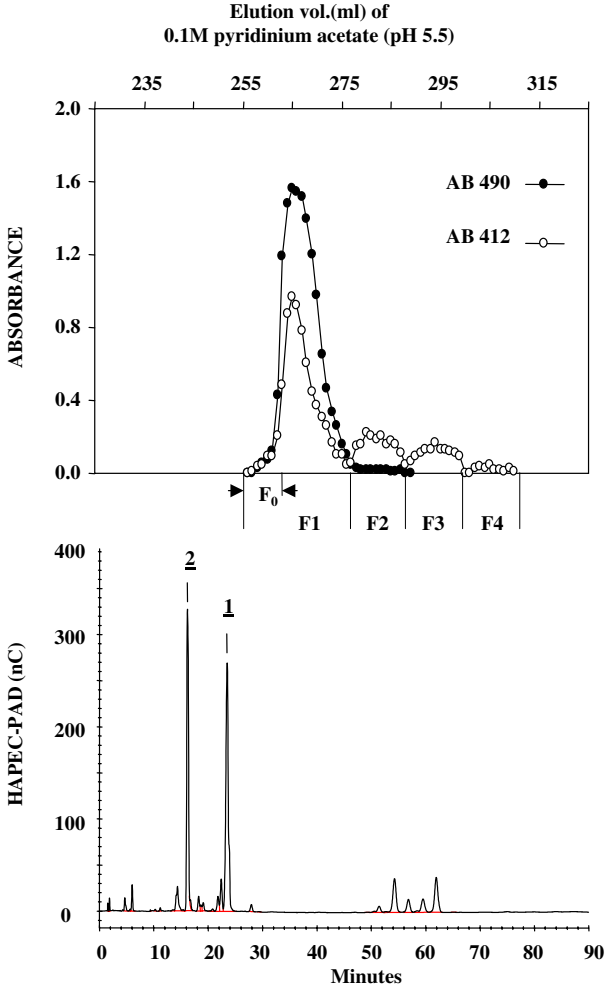


Figure 1. Purification of compounds, galactosylgluconic acid (**1**) and galactosylerythronic acid (**2**). Top: Fractionation of bromine-oxidized and reduced (with $NaBD_4$) products of lactose on a BioGel P-2 column (2.5 × 85 cm). Solid circle: phenol–H_2SO_4 assay; open circle: periodate-oxidation alditol assay. Bottom: Analysis of leading edge F_0 in the fraction F1 by HPAEC-PAD on a CarboPac PA1 column (4.0 × 250 mm) eluted with isocratic elution of 100 mM NaOH for the first 5 min, followed by linear gradient of 250 mM NaOAc in the presence of 100 mM NaOH for the next 85 min.

as determined by GLC–FID analysis of the per-*O*-methyl derivative, was the principal (>85%) component in the major peak F1 and no starting material was detected. Two principal constituents (**1** and **2**) present in the leading edge (F_0) to the major peak, as analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), were isolated preparatively (Fig. 1). Monosaccharide composition analyses (2 M TFA, 120 °C, 1 h) by GLC–MS of per-*O*-Me_3Si derivatives revealed that compound **1** was galactosylgluconic acid and compound **2** was galactosylerythronic acid.

The per-*O*-methyl derivative of galactosylgluconic acid (**1**) by GLC–MS analysis had a mass spectrum identical to that of reference lactobionic acid (Fig. 2). The spectrum comprised the most intense signals at *m*/*z* 75, 88, and 101, which were derived from the galactopyranosyl residue by carbon ring fissions, analogous to the respective fragment ions of J_1, H_1, and F_1 from a per-*O*-methyl hexopyranose.[14] Galactosyl residue (***a***) was identified by the detection of the ***a***A_1 ion at *m*/*z* 219; the subsequent loss of methanol afforded the fragment ions of ***a***A_2 (*m*/*z* 187) and ***a***A_3 (*m*/*z* 155). This series of fragmentations is characteristic of non-reducing glycosyl residues as seen in those of per-*O*-methyl disaccharides[14] or per-*O*-methyl aldobiuronic acids.[15]

The gluconic acid constituent (***b***) was identified by the detection of the ***ab***J_1 ion[14,15] at *m*/*z* 309, as a result of the carbon ring fission of the galactopyranosyl residue between the C-1 and C-2, followed by rearrangement of the methoxyl group at C-3 to C-1 (Scheme 1). The subsequent loss of methyl formate (CH_3O–CH=O, 60 amu) introduced another series, ***b***A ion, which was detected at *m*/*z* 249 (***b***A_1 ion), *m*/*z* 217 (***b***A_2) and *m*/*z* 185 (***b***A_3) after splitting off each methanol fragment.

The galactosylerythronic acid (**2**), as the per-*O*-methyl derivative, provided the same intense signals afforded by carbon ring fission as in compound **1** (Fig. 3). The agly-

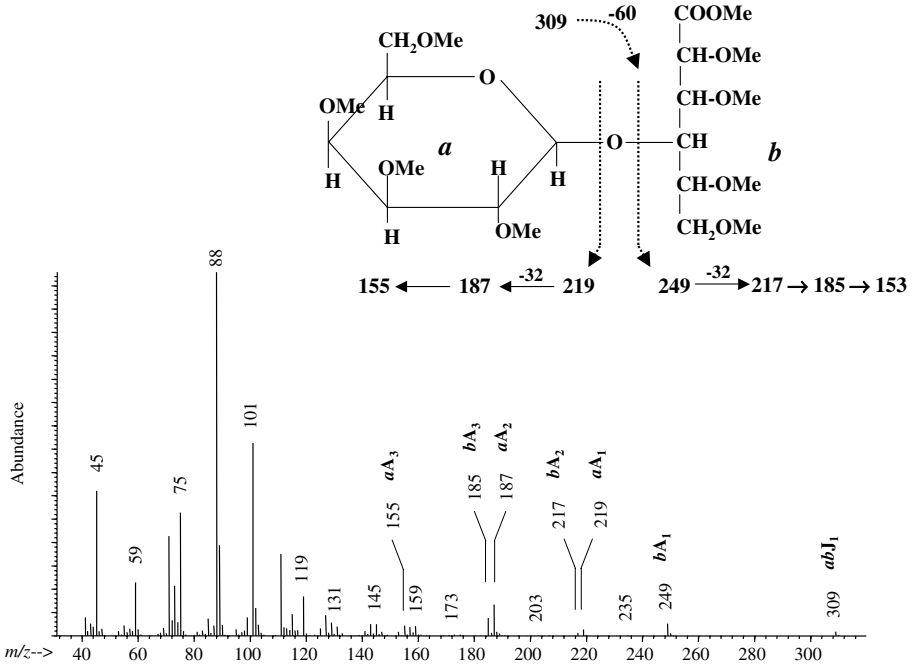
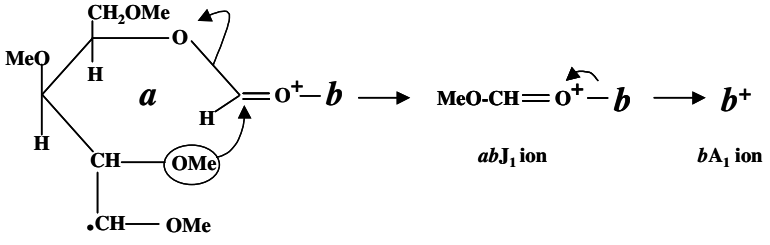


Figure 2. Mass spectrum of per-*O*-methyl galactosylgluconic acid (**1**) and its significant ions. The spectrum was acquired at 70 eV and mass scan range of 40–500 *m*/*z*; ***a*** and ***b*** denote galactose and gluconic acid moieties, respectively; A represents fragmentation pathway according to the nomenclature of fragmentation[14,15] and the subscript number represents the ion derived by the sequential loss of MeOH.



Scheme 1.

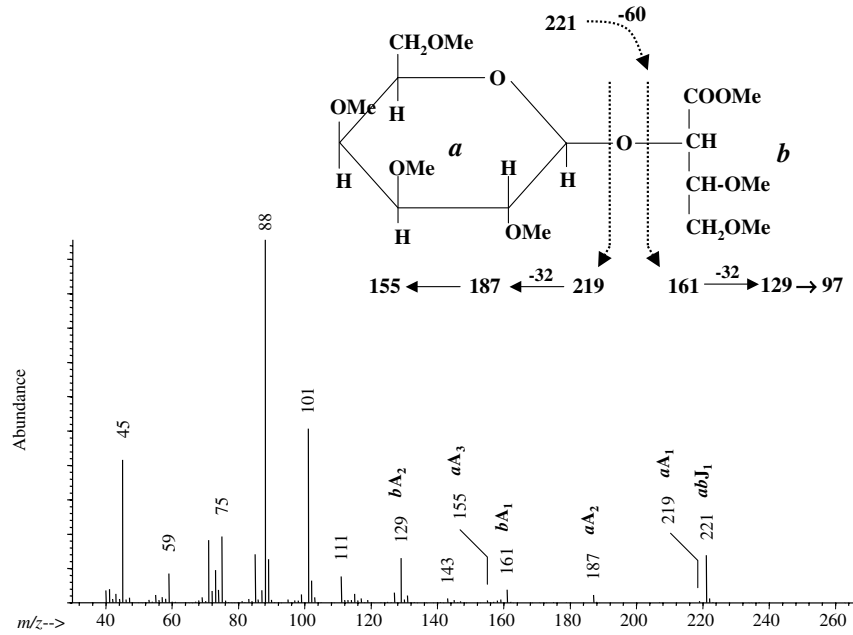


Figure 3. Mass spectrum of per-*O*-methyl galactosylerythronic acid (**2**) and its significant ions. The spectrum was acquired at 70 eV and mass scan range of 40–500 *m*/*z*; ***a*** and ***b*** denote galactose and erythronic acid moieties, respectively; A represents fragmentation pathway according to the nomenclature of fragmentation[14,15] and the subscript number represents the ion derived by the sequential loss of MeOH.

con residue, erythronic acid, was identified by the detection of the ***ab***J_1 ion at *m*/*z* 221, ***b***A_1 ion at *m*/*z* 161 and ***b***A_2 ion at *m*/*z* 129, being equivalent to those of gluconic acid with 88 mass units less and the loss of methanol limited to the ***b***A_2 ion.

Fragmentation pathways illustrated by these two aldobionic acids revealed the size of the glycon residue by detection of the ***a***A_1 ion and of the aglycon moiety by the ***b***A_1 ion signal. This pertinent fragmentation applied to other aldobionic acids, and the detection of such ions permitted identification of the constituents without further purification (Fig. 4). These aldobionic acids gave rise to much simpler mass spectra than disaccharides and the intense signal of the ***ab***J_1 ion affirmed their identification. Such products, as summarized in Table 2, comprised modified lactobionic acid, derived principally by oxidative decarboxylation of the gluconic acid residue with the galactosyl linkage retained.

The galacturonosyl residue, such as in compound **4**, was identified by a 14 amu increment of the ***a***A fragment series, due to a methyl ester group replacing a hydroxymethyl group in compound **1**. Additional increments of 44, 58, and 102 amu to the erythronic acid residue of compound **2** recognized the size of the aglycon to be arabinonic acid in **3**, arabinaric acid in **5**, and glucaric acid in **6**, respectively. Galactosylarabinaric acid (**5**) has the glycon and aglycon residues of the same mass, so that the resulting signal was additive, thus affording much stronger signals than those in other acids.

No inclusion of deuterium in these acids was detected, thus excluding keto acids being originally present in those fractions. The presence of erythronic acid in compound **2** and arabinonic acid in compound **3** implied the sequential oxidative degradation of C-1 and C-2 in the parent glucose residue. Also, oxidation of primary hydroxyl groups from both residues in the lactose occurred, as seen by the appearance of arabinaric, glucaric, and galacturonic acids.

Per-*O*-methyl derivatives were further reduced with Super Deuteride, $Li(Et)_3BD$, and then acetylated (pyridine–Ac_2O). The resulting partial *O*-acetyl and *O*-methyl derivatives gave signals of proportionally increased mass, such as 16 amu increment to the mono methyl ester present in the aldonic and galacturonic acid residues, and signals of 32 amu increment to dicarboxylic methyl esters in the aldaric acid residues. Thus, the extent of carboxyl groups in those acids was reaffirmed by the detection of such mass increases (data not shown).

Many aldobionic acids on a BioGel P-2 column, including lactobionic acid, eluted like aldonic acids of $\leqslant C_6$ (see below), which were present exclusively in fraction F2. This coelution is most likely a result of lactonization of such aldobionic acids, driven by cation-exchange treatment and drying during the work-up process. Thus, such uncharged lactones eluted like much smaller dynamic molecules during the gel diffusion process.

2.2. Identification of $\leqslant C_6$ acids and galactose

Aldonic acids of $\leqslant C_6$ present in fraction F2 were identified by GLC–MS analyses of the reference materials, as

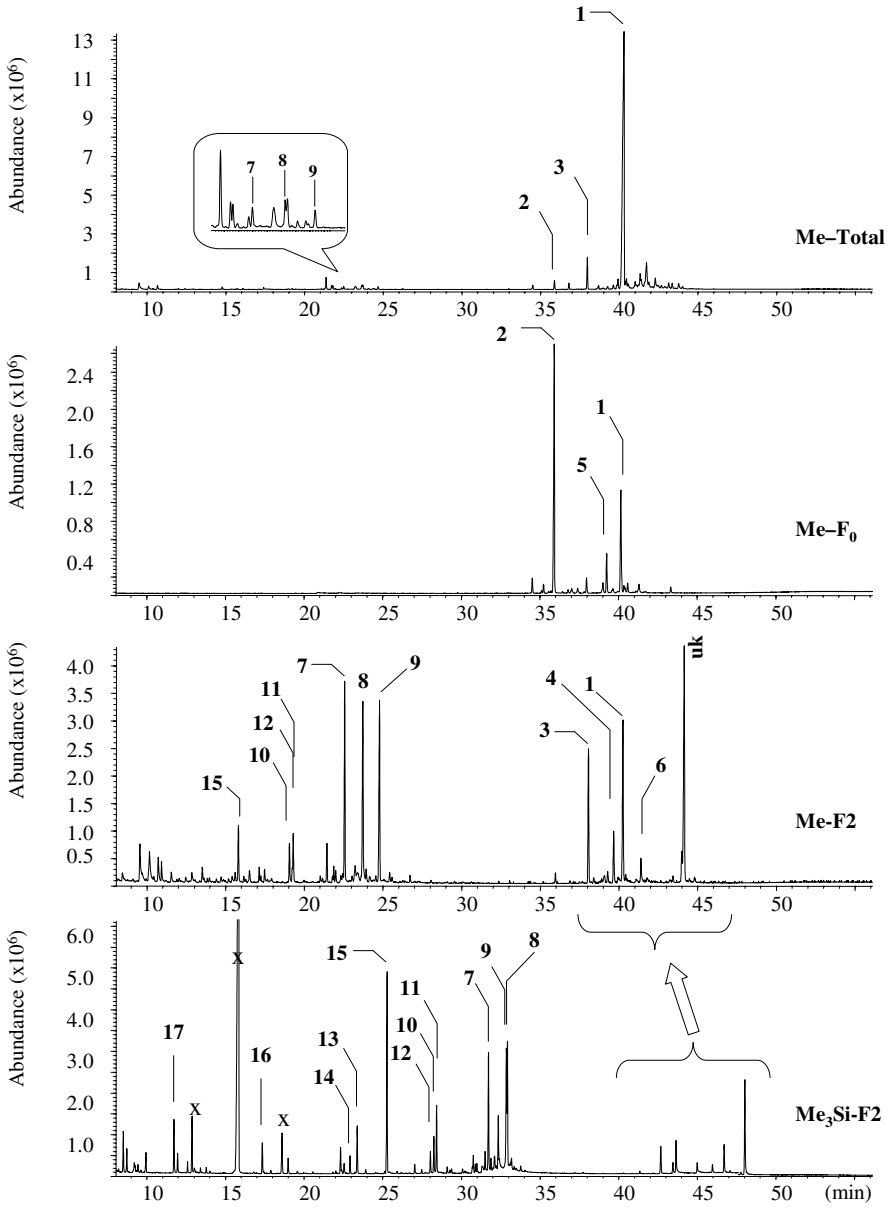


Figure 4. GLC–MS analyses of selected fractions, F_0, and F2, as per-*O*-methyl or per-*O*-Me_3Si derivatives. Total reaction product, as per-*O*-Me derivative, was included for comparison; x, reagent-related peaks; uk, unknown compound. Aldobionic acids present in the F2 were identified by their per-*O*-methyl derivative only, as depicted by arrow. Peak numbers refer to compounds in Table 3.

Table 2. Diagnostic fragment ions of galactosyl or galacturonosyl aldonic and aldaric acids as per-*O*-methyl derivatives

Compound (Table 3)	*m*/*z* ion[a] (%: relative intensity to the base peak, *m*/*z* 88)						
	***a*A$_1$**	***a*A$_2$**	***a*A$_3$**	***ab*J$_1$**	***b*A$_1$**	***b*A$_2$**	***b*A$_3$**
1	219 (1.9)	187 (9.2)	155 (2.8)	309 (1.3)	249 (3.9)	217 (0.9)	185 (5.3)
2	219 (0.5)	187 (2.1)	155 (0.7)	221 (13.1)	161 (3.6)	129 (12.3)	—
3	219 (0.7)	187 (2.8)	155 (0.8)	265 (4.7)	205 (3.3)	173 (2.5)	141 (3.3)
4	233 (3.5)	201 (6.8)	169 (1.5)	265 (4.3)	205 (3.5)	173 (5.0)	141 (12.7)
5	219 (11.1)	187 (4.1)	155 (1.0)	279 (3.2)	219 (11.1)	187 (4.1)	155 (1.0)
6	219 (1.1)	187 (7.0)	155 (2.0)	323 (0.7)	263 (4.9)	231 nd	199 (2.5)

Mass spectra of the compounds were acquired at 70 eV with mass scan range of 40–500 (*m*/*z*).

[a] ***a*** and ***b*** denote galactose and glucose residues in lactose, respectively; A represents fragmentation pathway (see Scheme 1); where the subscript number represents the ion derived by the sequential loss of MeOH; nd not detected.

Table 3. Distribution of reaction products as compound **1** equivalents, based on peak area in GLC–FID analyses

	Compound	Relative amount (%)
1	Galactosylgluconic acid	100.0
2	Galactosylerythronic acid	2.9
3	Galactosylarabinonic acid	7.5
4	Galacturonosylarabinonic acid	0.4
5	Galactosylarabinaric acid	0.5
6	Galactosylglucaric acid	0.2
7	Galactose	2.5
8	Gluconic acid	3.2
9	Galactonic acid	2.3
10	Lyxonic acid	0.9
11	Arabinonic acid	1.4
12	Xylonic acid	0.5
13	Threonic acid	1.0
14	Erythronic acid	0.4
15	Tartaric acid	3.7
16	Glyceric acid	0.6
17	Oxalic acid	0.6

per-*O*-methyl and per-*O*-Me_3Si derivatives (Table 3). Free galactose was also present, as detected by 1-deuteriogalactitol. Per-*O*-methyl derivatives of $<C_5$ aldonic acid are volatile. Their detection was low or completely absent in the GLC–MS analyses, as a result of losses during methylation work-up. Again, no deuterium was detected in those acids.

Mass spectra of reference pentonic and hexonic acids, as per-*O*-methyl and per-*O*-Me_3Si derivatives, were very similar within the isomers and differ only in the relative intensities of common fragment ions. Also, certain isomers were eluted identically or very closely under the condition used; such isomers included per-*O*-methyl gluconic and mannonic acids, per-*O*-methyl xylonic and arabinonic acids, and per-*O*-Me_3Si xylonic and ribonic acids. Thus, GLC–MS analyses of two complementary derivatives were necessary to identify those acids.

Per-*O*-methyl derivatives of fraction F2 in the GLC–MS contained one substantial, but unidentified compound, which eluted later than lactobionic acid, behaving as a larger molecule (Fig. 4). Its mass spectrum comprised the signals common to those of a galactosyl residue, as in lactobionic acid, but the aglycon residue was not identified. This compound might be an adduct of the aldobionic acid with an intermediate labile keto-compound[16] produced during oxidation, but its identification was not established.

The two fractions, F3 and F4, detected by a periodate-oxidation alditol assay, comprised <1% each of the reaction products by dry weight. They had an apparent elution volume larger than that of aldonic acid of $\leqslant C_6$, indicating that these materials should be smaller than such aldonic acids. However, nothing was detected by GLC–MS analyses of either per-*O*-methyl or per-*O*-Me_3Si derivatives, implying the absence of hydroxyl or carboxyl groups in these compounds, which again may be adducts of intermediate carbonyl compounds.

The presence of free galactose directly supported cleavage of the galactosidic linkage in lactose. The consecutive degradation of the resulting galactose is seen by the presence of galactonic, lyxonic, and threonic acids. Similar degradation of glucose released after galactosyl linkage cleavage afforded gluconic, arabinonic, and erythronic acids. Tartaric, glyceric, and oxalic acids were produced by further degradation of those acids from either sugar residue. Such acids can also be produced concomitantly by production of keto groups and their subsequent degradation to carboxylic acids. All those products supported evidence of galactosidic bond cleavage in the lactose.

Xylonic acid was also present and no detection of deuterium in this acid showed that it was not the result of reduction of a keto acid. The decarboxylation of C-6 in the glucaric acid, such as in the compound **6**, followed by galactosyl linkage cleavage might be a source of the xylonic acid.

Galactopyranosylarabinonic acid among the minor products was the most abundant compound (7.5% lactobionic acid equivalent), based on the peak area in GLC–FID, and other products constituted 0.2–3.7%. Those products, in total, comprised 29% of the lactobionic acid, more than half (17%) of which were derived from cleavage of the glycosidic linkage.

3. Experimental

The methods used for GLC–FID (HP5890 series II) or GLC–MS (HP 5971A), and for HPAEC-PAD have been described previously.[1–3] All the reagents used were of reagent grade unless noted.

3.1. Oxidation of lactose in bromine water

To an ice-chilled solution of lactose monohydrate (10 g; Aldrich Chemical, Milwaukee, WI, USA) and $CaCO_3$ (6.68 g; Fischer Scientific, Fair Lawn, NJ, USA) in water (550 mL) was added, with stirring, bromine (2.85 mL; EM Science Gibbstown, NJ, USA). The resulting solution was allowed to reach room temperature gradually and stirring continued until the bromine color disappeared (~30 h). The resulting white suspension (pH 5.9) was flushed with nitrogen gas to remove any trace of unreacted bromine. A portion (20% equivalent to the initial material) of the solution was taken, chilled in ice–water bath and mixed with cation-exchange resin (approximately 16 mequiv. of Rexyn 101, H-form), filtered, and washed. To the combined solution (pH 2.0) of filtrate and washings was added silver hydrogencarbonate[4] (freshly prepared from $AgNO_3$ of 3.0 g and $NaHCO_3$ of 1.5 g) in many portions. The resulting supernatant (pH 6) after centrifugation was treated with cation-exchange resin (Rexyn H-form,

8 mL) to remove any trace of $AgHCO_3$, adjusted to pH 5 with pyridine and freeze dried for further analyses.

The rest of the reaction solution (80% of the total reaction products) was adjusted to pH 3.1 by adding glacial acetic acid (~7 mL), and the resulting solution comprised small amounts of insoluble materials. The solution turned cloudy when concentrated to about 40 mL in a rotary evaporator at 37 °C. The resulting insoluble material, recovered by centrifugation, was freeze dried (35 mg), which is equivalent to 0.44% of the initial lactose.

3.2. Partial fractionation on BioGel P-2 gel filtration column

A portion (108 mg equivalent of the initial sugar) of the freeze-dried material in water (1 mL) was reduced with $NaBD_4$ (20 mg in 1 mL of 1 M NH_4OH) at room temperature for 4 h. Excess of borodeuteride was destroyed by adding AcOH and freeze dried. Borate was removed as methyl borate by coevaporation with absolute MeOH in a rotary evaporator at 40 °C. A portion of the resulting reduced material (54 mg equivalent of the initial 'sugar) was redissolved in elution buffer (2 mL; 0.1 M pyridinium acetate, pH 5.5) and was loaded on a BioGel P-2 column (2.5 × 85 cm; −400) equipped with flow adaptor. The elution was followed by periodate-oxidation alditol assay,[13] which was modified for off-line detection as follows: a portion (50 μL) of each fraction (2.1 mL) was diluted to 750 μL with water in a test tube (10 × 75 mm), mixed with 750 μL of 50 mM $NaIO_4$ and kept at room temperature for 2–3 min. To this solution was added the pre-mixed solution (750 μL) comprising $Na_2S_2O_3$ (150 g L^{-1}), NH_4OAc (57.6 g L^{-1}), and acetylacetone (20 mL L^{-1}) and heated in a boiling-water bath for 1 min. The resulting solution, when cooled to room temperature, was analyzed at 412 nm.

The resulting fractions were appropriately pooled and freeze dried for further analyses: F_0 of 5.5 mg (3.5%); F1 of 142 mg (86.7%); F2 of 14.3 mg (9.1%); F3 of 1.2 mg (0.8%); and F4 of 0.8 mg (0.5%).

The fractions were also analyzed by the phenol-H_2SO_4 method.[11]

3.3. GLC–FID and GLC–MS analyses of the reaction products

The reaction products were converted to per-*O*-Me and per-*O*-Me_3Si derivatives for GLC–MS analyses.

Per-*O*-methylation was achieved by a modified NaOH–Me_2SO–MeI system.[17] Briefly, a sample (containing 100–500 μg of sample) was evaporated to dryness by a nitrogen gas flow at 40 °C and dissolved in 100 μL of Me_2SO (dried over molecular sieve 4 Å; Burdick and Jackson high purity solvent, Muskegon, MI, USA). To this solution was added 50 μL of 1 M NaOH in Me_2SO (where 1 M NaOH in Me_2SO was prepared using 50% NaOH)[18] and briefly mixed before addition of 25 μL methyl iodide. The resulting solution was kept at room temperature for 15 min, with occasional stirring. Excess Me_2SO was removed by N_2 gas flow and its complete removal was effected by co-evaporation with $CHCl_3$. The resulting material in $CHCl_3$ (750 μL) was washed four times with water (750 μL). The $CHCl_3$ phase was carefully dried, redissolved in $CHCl_3$ (200 μL) and analyzed by GLC–FID and GLC–MS with splitless injection mode using a column of DB5 or DB5MS (B&W Scientific, Folsom, CA, USA) and temperature gradient (60 °C for the first 3 min, then increased to 300 °C at 5 °C min^{-1}).

For some experiments, in order to minimize loss of $\leqslant C_5$ aldonic acids due to their volatility, the resulting per-*O*-methyl derivative was mixed with $CHCl_3$ (2 mL) and Me_2SO was removed by washing with water (3 × 2 mL).

Per-*O*-Me_3Si derivatives of the reaction products were prepared. Reaction products and reference compounds were converted to sodium salts by adjusting to about pH 8 with NaOH. A portion (100–500 μg equivalent) of the resulting solution was evaporated to dryness by nitrogen gas flow and subsequent co-evaporation with CH_2Cl_2, and then 100 μL of the pre-mixed Me_3Si reagent[19,20] (bis(trimethylsilyl)trifluoroacetamide–chlorotrimethylsilane–pyridine = 5 g:50 μL:1.25 mL) was added and heated (80 °C, 1 h). The resulting solution (1–2 μL) was analyzed by GLC–FID and GLC–MS as above.

However, sodium salts of aldobionic acids were partially soluble, so approximately the same amount of glycerol was added to the samples before N_2 gas flow to avoid evaporation to complete dryness.

3.4. Preparation of standard aldonic acids

To an ice-cooled solution (6.7 mL) of 0.278 mmol monosaccharide (D-mannose, Sigma Chemical Co. St. Louis, MO, USA; L-arabinose, D-xylose, and D-lyxose, Pfanstiehl, Waukegan, IL, USA) and 0.67 mmol $CaCO_3$ was added dropwise 6.5 mL of 50 mM bromine water and kept for 2 h at room temperature. The bromine color disappeared within 1–2 h depending upon the nature of the monosaccharide. Bromide was removed by $AgHCO_3$ (3.2 mmol) as described above. The resulting product was analyzed as the per-*O*-methyl and per-*O*-Me_3Si derivatives by GLC–FID and GLC–MS. Per-*O*-methyl derivatives by GLC–FID analyses showed the corresponding aldonic acid comprising L-arabinonic and D-xylonic acids of >90% purity, D-lyxonic acid of 80% purity, and D-mannonic acid of 71% purity, based on the peak area of the principal component to those of the minor constituents including unreacted sugar.

Acknowledgements

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