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L-Altruronic acid formed by epimerization of D-galacturonic acid methyl esters during saponification of citrus pectin

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Abstract

While searching for oligosaccharides containing rhamnose residues in the endopolygalacturonase (EPG) digest of saponified citrus pectin, we found several oligomers containing, in addition to galacturonic acid, a sugar previously unreported in pectin. The 1- and 2-D ^{1}H NMR spectra of the oligosaccharides were consistent with the sugar being a uronic acid with its 2- and 3-hydroxyls being axial and 4-hydroxyl being equatorial. MALDI-TOF mass spectrometry indicated that the oligomers consisted solely of uronic acids. Reduction of the uronic acids in the oligosaccharides converted them to galactose and altrose. The altrose was found to be the L enantiomer by comparison of its trimethylsilyl (−)-2-butyl glycosides to those of authentic D-altrose and a racemic mixture. The sugar was not found in oligosaccharides prepared from EPG digestion of citrus pectin deesterified with pectin methylesterase rather than saponification. Thus, it appears that during saponification, a small proportion of the methylesterified galacturonic acid residues in pectins is epimerized at C-5 leading to formation of L-altruronic acid residues.

Keywords: Altruronic acid; Pectin; Epimerization; Alkali; Saponification; Galacturonic acid

1. Introduction

While searching for the putative single rhamnose 'kinks' in homogalacturonans, we treated commercial citrus pectin with sodium hydroxide to saponify esters prior to treatment with endopolygalacturonase (EPG), since the presence of methyl or acetyl esters inhibits EPG activity and its generation of oligosaccharides from pectin.[1] After enzymic digestion, the monomer, dimer, and trimer of galacturonic acid were the major products.[2] However, some fragments eluting at higher ionic strength than trigalacturonic acid were found on anion-exchange chromatography.[2] Some of these fragments contained an unsaturated galacturonic acid residue resulting from β-elimination of uronic acid.[3,4] But, in addition, three oligomers containing an unusual sugar residue, as indicated by their ^{1}H NMR spectra (see Table 2 in Ref. 2), were found.

In this paper, we describe the identification of this unusal sugar residue and propose a mechanism for its formation.

2. Results and discussion

Citrus pectin was saponified, digested with EPG, and fractionated by ultrafiltration and

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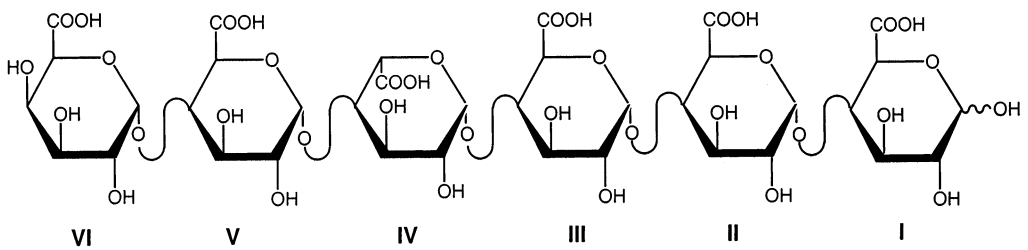
ion-exchange chromatography on a PA1 column, as described previously.[2] Several oligomers eluted from the PA1 column at higher ionic strength than trigalacturonic acid ($GalA_3$) (see Fig. 3 in Ref. 2).

GLC of the per-*O*-trimethylsilyl methyl glycosides generated from these oligomers (designated **10**, **11**, and **12**) showed them to contain predominantly galacturonic acid. However, two additional peaks, which did not correspond to known standards, were observed (peaks 1 and 3 in Fig. 1). The GLC–mass spectra of these two peaks were almost identical to those of the peaks from the galacturonic acid derivatives. Fragments were observed at $m/z = 423$, 391, 379, and 333, consistent with loss of a Me, Me + MeOH, Me + CO_2, and Me + Me_3SiOH, respectively, from a derivatized hexuronic acid.[5] Judging from the ratio of intensities of the ions at 204 and 217 in the spectra, the first peak is a furanose and the second a pyranose.[6] The presence of only one peak for each ring form indicates a dominance of one anomer over the other. This has been seen previously with both mannose and rhamnose,[6] reflecting the presence of an axial hydroxy group at C-2.



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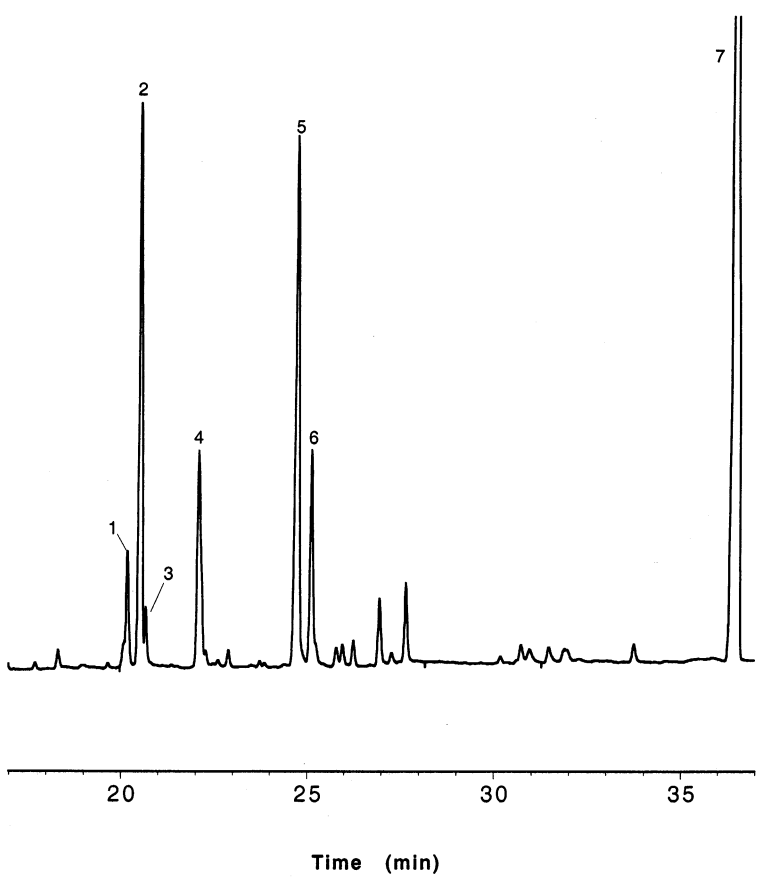


Fig. 1. Gas–liquid chromatogram of per-*O*-trimethylsilylated methyl glycosides of oligomer **12**. The numbered peaks are assigned as: 1 and 3, AltA; 2, 4, 5, and 6, GalA; 7, Inositol.

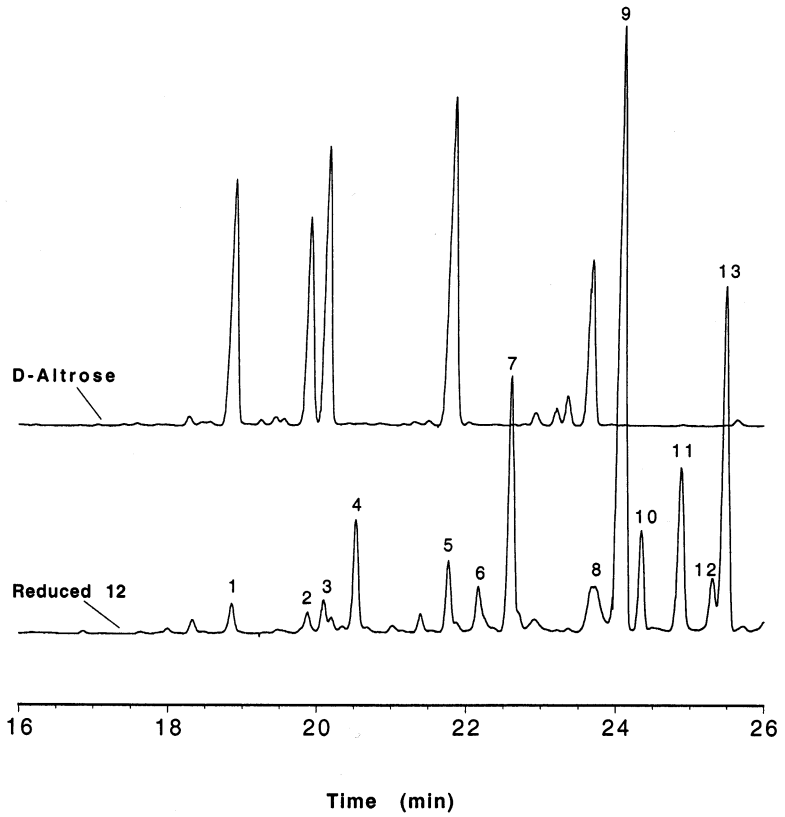


Fig. 2. Gas–liquid co-chromatogram of per-*O*-trimethylsilylated methyl glycosides of reduced oligomer **12** and authentic D-altrose. The numbered peaks are assigned as: 1, 2, 3, 5, and 8, Altrose; 4, 6, 11, and 12, GalA; 7, 9, 10, and 13, Gal.

The MALDI-TOF negative-ion mass spectrum of **10** was consistent with its being a mixture of [hexuronic acid tetramer with one 4,5-unsaturated residue $-$ H]$^-$ giving a peak at 702.6 (calculated 703.12) and [hexuronic acid pentamer $-$ H]$^-$ of mass 896.6 (calculated 897.16). Oligomers **11** and **12** had the masses expected for [hexamers and heptamers of uronic acids + Na $-$ 2H]$^-$, 1072.7 and 1271.78, respectively.

The ^{1}H NMR spectra of oligomers **10**, **11**, and **12** all contained a broad singlet at 4.95 ppm, a doublet at 4.21 ppm with 10-Hz splitting and a triplet at 4.17 ppm with 3.6-Hz splitting, in addition to the signals expected for GalA oligomers. The COSY and TOCSY spectra showed that the protons giving rise to these signals were in a one spin system, as were two others with signals at 4.01 and 3.82 ppm. The signals were identified as H-1–H-5 in the sequence 4.95, 3.82, 4.17, 4.01, 4.21 ppm. The 10-Hz splitting between H-5 and H-4 indicated that both were axial, whereas the 3.6-Hz splitting between H-3 and H-4 indicated that H-3 was equatiorial. Since H-1 was a broad singlet, it was likely that H-2 was also equatorial. This is the orientation of hydrogen atoms expected for L-altrose in the $^1C_4$ conformation, except for the lack of the two H-6 atoms. Thus, the sugar was tentatively identified as altruronic acid.

Authentic altruronic acid was not available, so a sample containing the putative altruronic acid was reduced to convert the uronic acid residues into their parent hexoses. Derivatives of these were compared with those of an altrose standard. The GLC trace of the products from reduction and derivatization of oligomer **12** along with the trace from authentic altrose is shown in Fig. 2. In addition to the major peaks from galactose, five peaks corresponding to authentic altrose were observed. It is clear from the presence of galacturonic acid peaks that the reduction was not complete. However, it was adequate to convert enough of the altruronic acid into a recognizable sugar derivative.

To determine which optical isomer of altruronic acid was present, the reduced, methanolyzed, oligomer was converted to trimethylsilyl (−)-2-butyl glycosides, and the products were compared to those formed from D-altrose with (−)-2-butanol and racemic 2-butanol.[7] The peaks arising from the putative altruronic acid co-chromatographed with the L-altrose derivatives (Fig. 3).

So far, only a few cases of naturally existing altruronic acid have been reported; e.g., Refs. 8, 9, and none has been previously reported in pectin. It is well known that β-elimination of methylesterified uronic acids occurs under alkaline conditions, but alkali also can promote epimerization of hexuronic acids at C-5.[10,11] In addition, enzymes are known in algae which epimerize the C-5 position of D-mannuronic acid in alginate to form L-guluronic acid.[12] Thus, it seems quite likely that altruronic acid was formed in the pectin either by enzymic- or alkali-induced epimerization. Avoidance of alkaline conditions during the de-esterification of the pectin necessary to make it susceptible to the EPG should eliminate generation of artifactual altruronic acid. When the same pectin sample was de-esterified by pectin methylesterase instead of alkali prior to EPG digestion, no altruronic acid-containing fragments were found. The de-esterification was not as complete as with the alkali, and more oligomers containing four or more uronic acid residues were obtained. However, the ^{1}H NMR spectra of these oligomers failed to show any altruronic acid signals. These results suggest the altruronic acid residues were created by alkaline epimerization rather than being a natural component of the pectin.

If the altruronic acid were formed by epimerization at C-5 of GalA residues in the pectin, one would expect it to be linked through its O-4 to the previous GalA residue, and to O-4 of the subsequent GalA residue in the oligomers. From the HMQC and TOCSY spectra of the oligomers designated **10** and **11**, we could identify all of the ^{1}H and ^{13}C chemical shifts of the altruronic acid residue and those of most of the GalA residues (see Table 1). In the HMBC spectrum there is a clear 3-bond correlation between H-1 of the al-

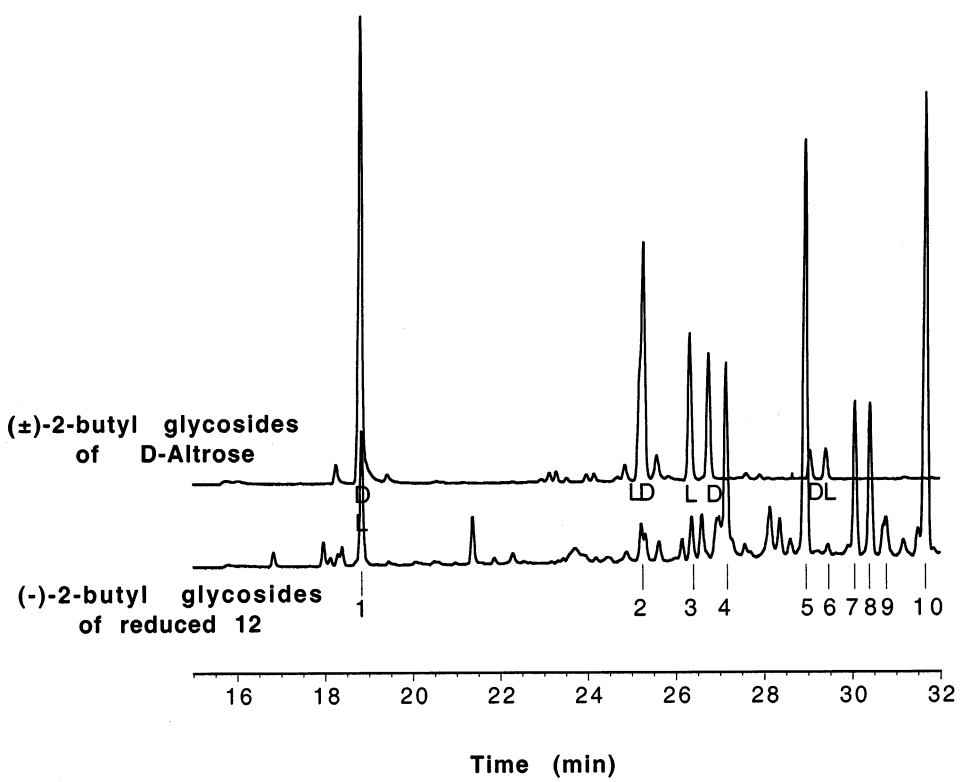
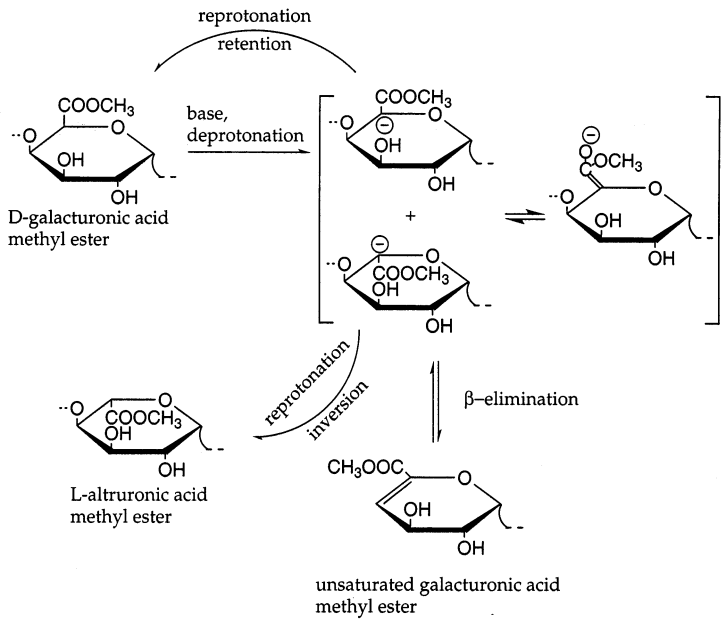


Fig. 3. Gas–liquid co-chromatogram of per-*O*-trimethylsilylated (−)-2-butyl glycosides of the reduced oligomer **12** and the trimethylsilylated (±)-2-butyl glycoside of authentic D-altrose. The numbered peaks are assigned as: 1, 2, 3, and 6, L-Altrose; 4, 5, 7, 8, and 9, D-Gal; 10, D-GalA. Two additional peaks appeared at 32.5 and 35.5 min for the GalA derivative.

Table 1
^{1}H and ^{13}C NMR chemical shifts (δ, ppm) for the altruronic acid-containing hexasaccharide **11**

Sugar Residue	H-1	H-2	H-3	H-4	H-5	C-1	C-2	C-3	C-4	C-5	C-6
I-α	5.34	3.87	4.01	4.45		92.6	68.5	70.6	77.9	70.5	175.5
I-β	4.62	3.53	3.78	4.41	4.07	96.8	71.7	72.7	77.6	74.4	174.9
II	5.20	3.86	4.04	4.45		99.3	69.4	69.4	77.9		175.5
III	5.20	3.86	4.04	4.48		99.3	69.4	69.4	81.5		175.5
IV	4.95	3.82	4.17	4.01	4.21	99.2	70.7	70.7	75.2	73.7	176.5
V	5.14	3.78	4.04	4.45		101.1	69.0	69.4	77.9		175.5
VI	5.10	3.79	3.94	4.40	4.80	99.3	69.4	69.6	71.2	71.9	175.5



Scheme 1. The reaction of base-promoted β-elimination and epimerization of polygalacturonan.

truronic acid (4.95 ppm) and C-4 of a GalA residue (81.5 ppm) showing that it is linked to O-4 of a GalA residue. The chemical shift of this C-4 is 3.6 ppm farther downfield than the C-4 resonances of the other 4-linked GalA residues. Since the chemical shift of C-1 of most of the GalA residues is almost identical to that of the AltA, the correlation between H-4 of the GalA residue to which the AltA is attached and C-1 of AltA is not resolvable from the GalA H-4:GalA C-1 correlations. There is a strong signal corresponding to an AltA H-4:GalA C-1 correlation showing that GalA is linked to O-4 of the AltA residue. The corresponding GalA H-1:AltA C-4 correlation is also present.

The formation of the altruronic acid residues can be explained by the reactions shown in Scheme 1. Under alkaline conditions, the proton attached to C-5 of the uronic acids is readily abstracted because of the carbonyl group's electron-withdrawing effect. The resulting carbanion could isomerize to the enolate. Unsaturated GalA is the product of β-elimination, which was found in oligomers **7** and **10** (see Fig. 3 in Ref. 2). If instead of elimination, reprotonation occurs, D-galacturonic acid or its epimer L-altruronic acid would be formed.

We estimate from integration of the peak areas of the CZE electropherogram of the entire mixture of low-molecular-weight fragments from the EPG digestion (see Fig. 4) and consideration of the sugar composition of the various oligomers, that about 2% of galacturonic acid in the commercial citrus pectin had epimerized into altruronic acid.

Although we have not rigorously shown the position of the altruronic acid within the oligomers, we can deduce where they are likely to be, from the known specificity of the EPG used to release them from the pectin. Our previous studies[1,13,14] and those of others[15] show that the EPG of *Aspergillus niger* needs four adjacent non-esterified GalA residues to be able to act, and that it cleaves between the third and fourth residues to which it is bound. We expect that altruronic acid cannot be accommodated in any of the four sugar-binding sites in the enzyme. Invoking the binding of four residues and cleavage between the third and fourth from the non-reducing end, one would predict three products for digestion of a pectin containing an AltA residue. These would be oligomers with one, two, or three GalA residues, followed by an AltA, followed by three more GalA residues: i.e., pentamers, hexamers, and heptamers, which are what was found.

3. Experimental

Alkaline de-esterification and EPG digestion of commercial pectin.—The methods for alkaline de-esterification, EPG digestion and subsequent isolation of oligosaccharides from citrus pectin have been described in a previous paper.[2] Since the conditions of the saponification may be critical to the degree of epimerization of the GalA residues, they are repeated here. Citrus pectin (10 g) was suspended in EtOH (30 mL), and water (300 mL) was added to dissolve the pectin. Saponification of the pectin was carried out at rt by adjusting the pH of the solution to pH 11 with 1 M NaOH and maintaining it there until there was no significant change after 15 min. The pH was then adjusted to pH 4 by addition of glacial AcOH.

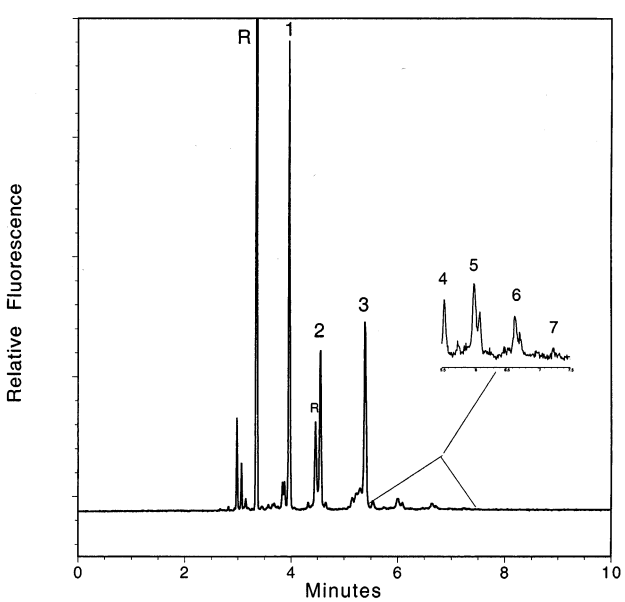


Fig. 4. Capillary zone electropherogram of ANTS-labeled oligomers from EPG-digested, saponified citrus pectin. Peaks 1, 2, and 3 are from the monomer, dimer, and trimer of GalA. Peaks 5, 6, and 7 resulted from the AltA-containing fragments. R = reagent peaks.

Pectin methylesterase de-esterification and EPG digestion of commercial pectin.—Citrus pectin (5 g) was suspended in MeOH (10 mL). Deionized water (100 mL) was added with stirring to dissolve the pectin, and the pH was adjusted to pH 7.0 by addition of 0.05 M NaOH. After addition of 10 μL of tomato pectin methylesterase (10 U/μL) (a gift from Dr Dean Della Penna, University of Reno, Nevada), the pH was maintained between pH 6.0 and 7.0 for 3 h (never allowing the pH to exceed pH 7 to avoid alkaline de-esterification) by adding 0.1 M NaOH dropwise. Subsequent EPG digestion and chromatographic separation of oligomers were the same as for the saponified sample.

Reduction of altruronic acid-containing fragment.—Water (50 μL) was added to dissolve a sample of oligomer **12** (100 μg) in a 4-mL glass vial; 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (20 mg) was added, and the pH of the solution kept at pH 4.5 for 1 h, as judged by pH paper, by addition of 0.1 M HCl. The sample was chilled on ice and 0.25 mL of 4 M imidazole·HCl pH 7 buffer and $NaBH_4$ (20 mg) were added. After reaction in an ice bath for 1 h, glacial AcOH was added to destroy the excess $NaBH_4$. This procedure is a combination of methods for reducing activated uronic acids to alcohols.[16–18] The reduced sample was desalted on a 15 × 0.9-cm Toyopearl HW40 gel-filtration column and lyophilized.

*Preparation of per-*O*-trimethylsilyl methyl and (−)-2-butyl glycosides and gas–liquid*

chromatography.—Oligomer **11** (10–100 μg) was methanolyzed for 12 h with 1.5 M HCl (200 μL) in dry MeOH. After cooling, one half of the methanolysis solution was evaporated under a stream of dry nitrogen gas to remove the HCl and derivatized with trimethylsilylation reagents for compositional analysis.[19] The other half of the solution was dried, and 1 M HCl (100 μL) in (−)-2-butanol was added and heated at 80 °C for 10 h and then cooled.[7] HCl was evaporated, and the sample was trimethylsilylated and analyzed using the same conditions as for the compositional analysis.

GLC–mass spectrometry.—The GLC–mass spectrometric studies were conducted with a Hewlett–Packard GLC–MS system consisting of a Gas–Liquid Chromatograph 5890 series 2 connected to an HP MS engine model 5989B. A DB-5-MS capillary column (30 m × 0.25 mm) was used with helium as the carrier gas.

Matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometry.—The freeze-dried samples were further desalted using a miniature cation-exchange cartridge fashioned from a DNA gel-loading pipet tip[20] prior to analysis. For the matrix, dihydroxybenzoic acid was dissolved in (70:30 v/v) 0.1% aq trifluoroacetic acid–MeCN to a concentration of 20 μg/μL. A sample of the matrix (0.5 μL) was mixed with 0.5 μL of the desalted sample on the MALDI target and dried in air. Spectra were obtained on a Perseptive Biosystems Voyager MALDI-TOF mass spectrometer in the negative-ion mode.

NMR spectroscopy.—All samples were dissolved in D_2O, and the spectra were recorded at 30 °C on Varian Inova 400 and 600 NMR spectrometers using the standard pulse sequences with water presaturation. The pH of the samples was pH 6.

Capillary electrophoresis.—Oligosaccharides were derivatized with 8-aminonaphthalene-1,3,6-trisulfonic acid for fluorescence detection as described previously[14] and electrophoresed on a custom-built apparatus using a He–Cd laser for excitation of fluorescence, and an intensified charge-coupled device camera for detection.[21]

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