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Analysis of *Macrocystis pyrifera* and *Pseudomonas aeruginosa* alginic acids by the reductive-cleavage method*

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ABSTRACT

Permethylated alginic acids comprised of 4-linked D-mannopyranosyluronic acid and 4-linked L-gulopyranosyluronic acid residues undergo reductive cleavage to yield, after acetylation, methyl 3-*O*-acetyl-2,6-anhydro-4,5-di-*O*-methyl-D-mannonate (**2b**) and methyl 3-*O*-acetyl-2,6-anhydro-4,5-di-*O*-methyl-D-gluconate (**3b**) as major products. Small amounts (*ca.* 13%) of ring-contracted products, namely methyl 2-*O*-acetyl-3,6-anhydro-4,5-di-*O*-methyl-D-mannonate (**9**) and methyl 2-*O*-acetyl-3,6-anhydro-4,5-di-*O*-methyl-D-gluconate (**10**), were also observed in these experiments. These results are in marked contrast to previous results on the reductive cleavage of 4-linked D-glucopyranosyluronic acid residues, wherein the ring-contracted product was formed exclusively. Formation of the ring-contracted products could be completely eliminated by reduction ($LiAlH_4$) of ester groups in the permethylated alginic acid prior to reductive cleavage. In the latter experiments, 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-D-mannitol (**5b**) and 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-L-gulitol (**6b**) were the sole products of reductive cleavage of the 4-linked ManA and 4-linked GulA residues, respectively. However, in the previous experiments it was noted that low yields of permethylated alginic acids were obtained and that extensive depolymerization occurred under methylation conditions. Depolymerization could be avoided and higher yields of permethylated polysaccharides could be obtained, by reduction of the carboxyl groups of the alginic acids prior to methylation. Reductive cleavage of the latter polysaccharides yielded the products expected from 4-linked D-mannopyranosyl and 4-linked L-gulopyranosyl residues, namely 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-mannitol (**13b**) and 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-L-gulitol (**14b**), respectively. Using the latter analytical strategy, it was established that the *Macrocystis pyrifera* alginate was comprised of 60% 4-linked ManA and 40% 4-linked GulA residues, whereas the *Pseudomonas aeruginosa* alginate was comprised of 80% 4-linked ManA and 20% 4-linked GulA residues.

INTRODUCTION

Although the reductive-cleavage technique has previously been used in the structural characterization of polysaccharides containing uronic acids, only those containing terminal[1] (nonreducing) and 4-linked[2] D-glucopyranosyluronic acid residues were examined. An interest in the mucoid exopolysaccharide of *Pseudomonas aeruginosa* (strain 2192), which was being investigated[3–6] in the context of its involvement in the complications of cystic fibrosis, prompted further study of the application of the reductive cleavage technique to polysaccharides of the alginic acid class.

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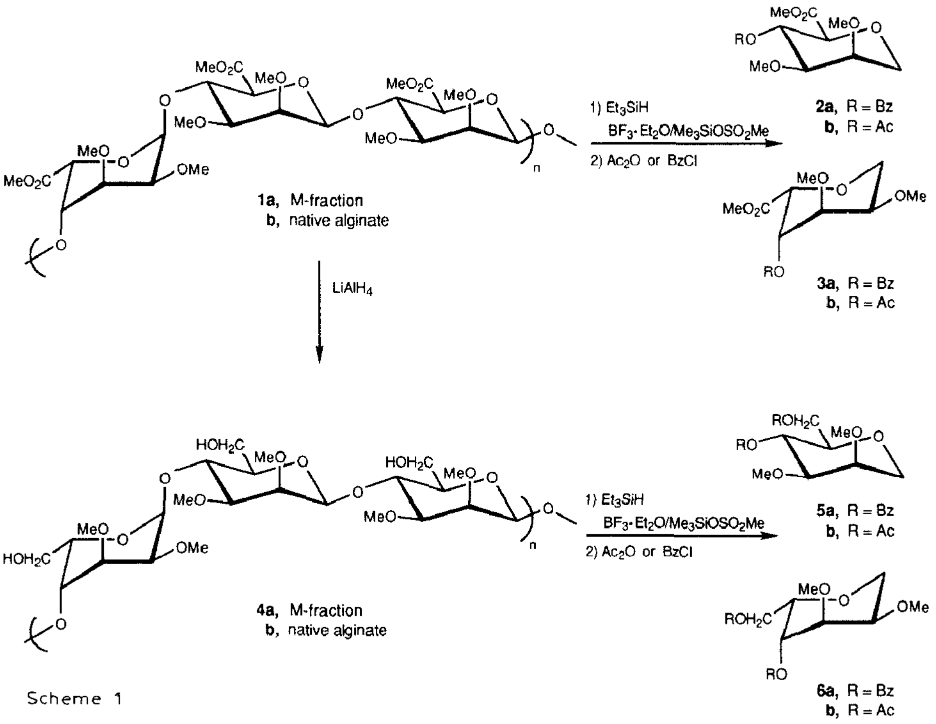
Alginates are linear 1,4-linked copolymers of β-D-mannopyranosyluronic acid (ManA) and its C-5 epimer α-L-gulopyranosyluronic acid (GulA). Based upon previous work[1,2], it was envisioned that reductive-cleavage analysis of alginic acid might potentially be accomplished directly after permethylation, after sequential permethylation and reduction of ester groups, or after sequential carboxyl reduction and permethylation. Prior to investigation of the *P. aeruginosa* alginate, a commercially available alginate from *Macrocystis pyrifera* (kelp) was used as a model to determine which method of analysis was most suitable. The commercial alginate was examined both directly and as a depolymerized sample (M-fraction) enriched in ManA residues[7].

RESULTS

Reductive cleavage of the permethylated M. pyrifera *alginate.* — The native alginate and M-fraction were permethylated under neutral conditions[8,9] and used in the following studies. Shown in Scheme 1 are the structures of the permethylated alginate and the expected products of total reductive cleavage. Reductive cleavage of the permethylated M-fraction (**1a**) was carried out in the presence of 5 equivalents of triethylsilane (Et_3SiH) as the reducing agent and a mixture consisting of 5 equivalents of trimethylsilyl methanesulfonate (Me_3SiOSO_2Me) and 1 equivalent of boron trifluoride etherate ($BF_3 \cdot Et_2O$) as the catalyst. A portion of the product was benzoylated, and the resulting benzoylated anhydroalditols were separated by reversed-phase h.p.l.c. (Fig.



Scheme 1

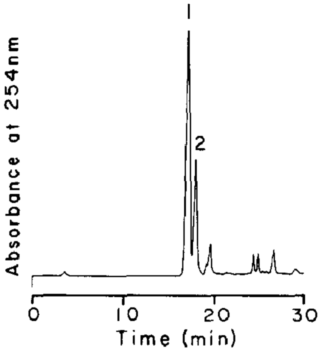


Fig. 1. High-performance liquid chromatogram of the anhydroalditol benzoates derived by reductive cleavage of the per-*O*-methylated M-fraction (**1a**) of *M. pyrifera* alginic acid. Chromatography was performed on a 0.92 × 25 cm column of DuPont Zorbax ODS, eluted with a concave, 20-min gradient [curve 5 (ref. 24), Beckman System Gold model 166 Programmable Detector Module] from 40:60 acetonitrile–water to 80:20 acetonitrile–water at a flow rate of 3 mL/min. The numbered peaks were identified as follows: 1, **2a**; 2, **3a**.

1). Peak 2 (Fig. 1) was identified as methyl 2,6-anhydro-3-*O*-benzoyl-4,5-di-*O*-methyl-D-gluconate (**3a**) by ^{1}H-n.m.r. spectroscopy. Its ^{1}H-n.m.r. spectrum contained characteristic resonances at δ 4.08 (dd, J 3.5, 9.6 Hz) for H-6e, 4.58 (d, J 1.8 Hz) for H-2, and 5.57 (dd, J 1.8, 3.9 Hz) for H-3 in addition to methoxyl and other ring-proton resonances. Peak 1 (Fig. 1) was identified as methyl 2,6-anhydro-3-*O*-benzoyl-4,5-di-*O*-methyl-D-mannonate (**2a**). The ^{1}H-n.m.r. spectrum of **2a** contained resonances at δ 4.17 (dd, J 8.0, 10.5 Hz) for H-6e, 4.35 (d, J 3.4 Hz) for H-2, and 5.84 (dd, J 3.4, 5.0 Hz) for H-3, in addition to methoxyl and other ring-proton resonances. The coupling constants of the ring protons, however, were not those expected for a *manno* configurational isomer existing in the $^3C_6$ conformation as shown (Scheme 1). In order to prove that compound **2a** possessed the structure as shown, it was subjected to reduction ($LiAlH_4$) and then to benzoylation to afford 1,5-anhydro-4,6-di-*O*-benzoyl-2,3-di-*O*-methyl-D-mannitol (**5a**). The ^{1}H-n.m.r. spectrum of the latter product was identical to that of an

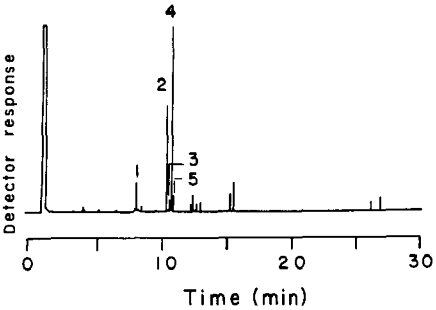
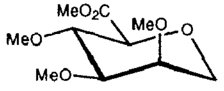


Fig. 2. Gas–liquid chromatogram of the anhydroalditol acetates derived by reductive cleavage of the per-*O*-methylated M-fraction (**1a**) of *M. pyrifera* alginic acid. The numbered peaks were identified as follows: 1, 7 or **8**; 2, **3b**; 3, **9** or **10**; 4, **2b**; 5, **10** or **9**.

authentic standard[10]. The unexpected coupling constants of the ring protons of **2a** may arise because the compound adopts a skew rather than a chair conformation.

The remaining portion of the product mixture from the reductive cleavage of **1a** was acetylated and analyzed by gas–liquid chromatography (g.l.c.) (Fig. 2) and g.l.c. combined with chemical-ionization mass spectrometry (c.i.-m.s.) and electron-ionization mass spectrometry (e.i.-m.s.). Peaks 2 and 4 (Fig. 2) were identified as methyl 3-*O*-acetyl-2,6-anhydro-4,5-di-*O*-methyl-D-gluconate (**3b**) and methyl 3-*O*-acetyl-2,6-anhydro-4,5-di-*O*-methyl-D-mannonate (**2b**), respectively. The c.i. mass spectra for both compounds indicated a molecular weight of 262, as expected, and their e.i. mass spectra displayed fragment ions at *m/z* 203, 171, 143, and 111, all consistent[11] with the pyran structures as indicated. In addition to the major components (**2b** and **3b**), several minor components of carbohydrate origin were also observed (Fig. 2). Peak 1 was found by c.i.-m.s. to have a molecular weight of 234, and its e.i. mass spectrum contained major fragment ions at *m/z* 175, 143, and 111. These data identify Peak 1 (Fig. 2) as either methyl 2,6-anhydro-3,4,5-tri-*O*-methyl-D-mannonate (**7**) or methyl 2,6-anhydro-3,4,5-tri-*O*-methyl-D-gluconate (**8**), arising from nonreducing terminal D-mannopyranosyluronic acid or L-gulopyranosyluronic acid groups, respectively. The two components with retention times of 11.0 min (Peak 3) and 11.4 min (Peak 5) appear to be ring-contracted products, namely methyl 2-*O*-acetyl-3,6-anhydro-4,5-di-*O*-methyl-D-mannonate (**9**) and methyl 2-*O*-acetyl-3,6-anhydro-4,5-di-*O*-methyl-D-gluconate (**10**). The c.i. mass spectra of both compounds indicated a molecular weight of 262, and their e.i. mass spectra were virtually identical to the mass spectrum of the related compound having the L-*gulo* configuration[2]. However, the data do not establish which peak (Peak 3 or 5, Fig. 2) is compound **9** and which is compound **10**.



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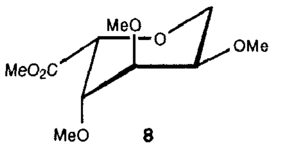

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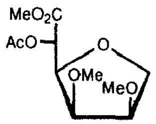

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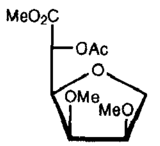

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Integration of the g.l.c. profile (Fig. 2) and correction for molar response[12,13] established that the M-fraction of *M. pyrifera* alginic acid was comprised of 76% ManA and 24% GulA residues, and possessed a degree of polymerization (d.p.) of 7.3. By the same method, native *M. pyrifera* alginic acid was found to be comprised of 61% ManA and 39% GulA residues, and to possess a d.p. of 13.

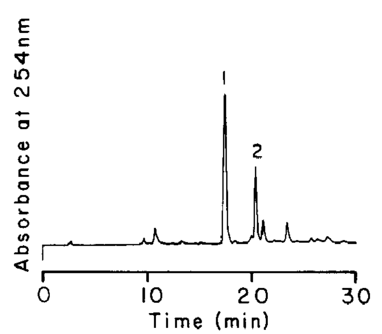


Fig. 3. High-performance liquid chromatogram of the anhydroalditol benzoates derived by reductive cleavage of the per-*O*-methylated and ester-reduced M-fraction (**4a**) of *M. pyrifera* alginic acid. Chromatography was conducted as described in Fig. 1 except that curve 0 (20 min; linear gradient of 40:60 acetonitrile–water to 20:80 acetonitrile–water) [see ref. 24] was used for the gradient. The numbered peaks were identified as follows: 1, **5a**; 2, **6a**.

Reductive cleavage of the permethylated and reduced M. pyrifera *alginate.* — Reductive cleavage of permethylated alginate was also accomplished after reduction of its ester groups with lithium aluminum hydride[14]. Sequential reductive cleavage of the ester-reduced M-fraction (**4a**, Scheme 1) and benzoylation produced two major components that were separated by h.p.l.c. (Fig. 3). Peak 1 was identified as 1,5-anhydro-4,6-di-*O*-benzoyl-2,3-di-*O*-methyl-D-mannitol (**5a**). The ^{1}H-n.m.r. spectrum for **5a** contained characteristic resonances at δ 3.76 (m) for H-2, 4.25 (dd, J 2.7, 12.8 Hz) for H-1e, 4.37 (dd, J 6.0, 12.1 Hz) for H-6, 4.55 (dd, J 3.0, 12.1 Hz) for H-6′, and 5.61 (t, J 9.3 Hz) for H-4. Peak 2 (Fig. 3) was identified as 1,5-anhydro-4,6-di-*O*-benzoyl-2,3-di-*O*-methyl-L-gulitol (**6a**). The ^{1}H-n.m.r. spectrum of **6a** displayed the expected downfield resonances for H-4 (δ 5.41, dd, J 1.3, 3.6 Hz), H-6 (δ 4.35, dd. J 5.2, 11.4 Hz) and H-6′ (δ 4.51, dd, J 7.3, 11.4 Hz), in addition to other ring-proton, methoxyl, and benzoyl resonances.

In a separate experiment, the permethylated and ester-reduced M-fraction (**4a**) was subjected to successive reductive cleavage and acetylation and the products were

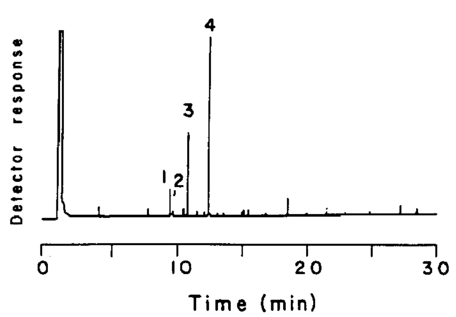
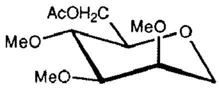


Fig. 4. Gas–liquid chromatogram of the anhydroalditol acetates derived by reductive cleavage of the per-*O*-methylated and reduced (LiAlH$_4$) M-fraction (**4a**) of *M. pyrifera* alginic acid. The numbered peaks were identified as follows: 1, **11**; 2, **12**; 3, **6b**; 4, **5b**.

analyzed by g.l.c. (Fig. 4) and g.l.c.–m.s. The c.i. mass spectra for both Peaks 3 and 4 (Fig. 4) indicated a molecular weight of 276, while their e.i. mass spectra displayed prominent fragment ions at *m/z* 203, 171, 143, and 111. These data are consistent for the proposed structures for 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-D-mannitol (**5b**, Peak 4) and 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-L-gulitol (**6b**, Peak 3), and indeed, the mass spectrum of the former was identical to that of an authentic standard[11]. In addition to the major components (**5b, 6b**), several minor components were observed in the g.l.c. profile (Fig. 4). Peaks 1 and 2 were identified as 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-mannitol (**11**) and 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-L-gulitol (**12**), respectively. These assignments were based upon g.l.c.-c.i.m.s. analysis, which indicated a molecular weight of 248 for both compounds, and g.l.c.–e.i.-m.s. analysis. The e.i. mass spectrum of Peak 1 (Fig. 4) was identical to that of an authentic sample[11] of **11**, and the e.i. mass spectrum of Peak 2 (Fig. 4) was very similar to that of Peak 1, displaying prominent fragment ions at *m/z* 175, 143, and 111. Integration of the g.l.c. profile (Fig. 4) and correction for molar response[12,13] gave the molar ratios of compounds **5b**, **6b**, **11**, and **12**. From these data it was determined that native alginate (**4b**) contained 72% ManA and 28% GulA and had a d.p. of 10, while the M-fraction (**4a**) contained 77% ManA and 23% GulA and had a d.p. of 9.



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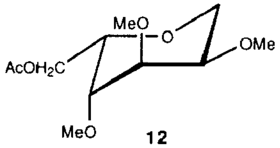

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Reductive cleavage of the carboxyl-reduced and permethylated M. pyrifera *and* P. aeruginosa *alginates*. — In a final strategy for analysis, the carboxyl groups of the native alginates were reduced with sodium borohydride in the presence of a water-soluble carbodiimide[15] prior to methylation. After reductive cleavage in the usual fashion, the products were benzoylated and separated by h.p.l.c. (Fig. 5). Analysis of the components by ^{1}H-n.m.r. spectroscopy revealed only two peaks of carbohydrate origin. Peak 1

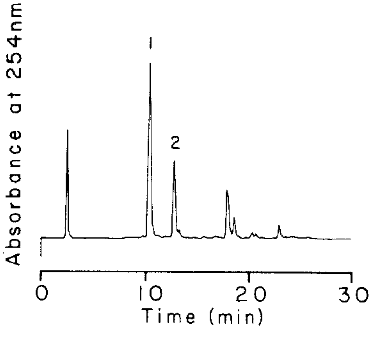
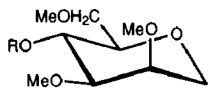
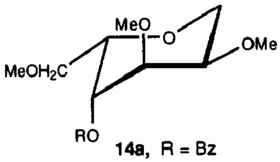


Fig. 5. High-performance liquid chromatogram of the anhydroalditol benzoates derived by reductive cleavage of carboxyl-reduced and per-*O*-methylated *M. pyrifera* alginic acid. Chromatography was conducted the same as in Fig. 3. The numbered peaks were identified as follows: 1, **13a**; 2, **14a**.

(Fig. 5) was identified as 1,5-anhydro-4-*O*-benzoyl-2,3,6-tri-*O*-methyl-D-mannitol (**13a**). The ^{1}H-n.m.r. spectrum of **13a** displayed characteristic signals for H-2 (δ 3.72, m), H-1e (δ 4.24, dd, J 2.5, 12.8 Hz), and H-4 (δ 5.37, t, J 9.4 Hz). Peak 2 (Fig. 5) was identified as 1,5-anhydro-4-*O*-benzoyl-2,3,6-tri-*O*-methyl-L-gulitol (**14a**) on the basis of its ^{1}H-n.m.r. spectrum which displayed characteristic resonances for H-3 (δ 3.82, m), H-1e (δ 3.97, dd, J 4.0, 10.4 Hz) and H-4 (δ 5.32, dd, J 1.5, 3.8 Hz).



13a, R = Bz
b, R = Ac



14a, R = Bz
b, R = Ac

The products of reductive cleavage of carboxyl-reduced and permethylated *M. pyrifera* alginate were also analyzed as their acetates by g.l.c. (Fig. 6). Peaks 1 and 2 (Fig. 6) were identified as 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-L-gulitol (**14b**) and 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-mannitol (**13b**), respectively. The c.i. mass spectra for both compounds (**13b** and **14b**) displayed ions at *m/z* 249 (M + H$^+$) and 266 (M + NH$_4$$^+$), indicating a molecular weight of 248, and their e.i. mass spectra were very similar, displaying fragment ions at *m/z* 203, 171, 143, and 111. Additionally, the e.i. mass spectrum of **13b** (Peak 2, Fig. 6) was identical to that of an authentic standard[11]. In addition to compounds **13b** and **14b**, several minor components were observed in the g.l.c. profile (Fig. 6). Two of these components were identified as compounds **3b** and **2b** (Peaks 3 and 4, respectively), arising from incomplete carboxyl reduction prior to permethylation and reductive cleavage. Integration of the g.l.c. profile (Fig. 6) and correction for molar response[12,13] established the percentages of ManA (60%) and GulA (40%) residues in the *M. pyrifera* alginate. From these integrals it was also established that 7 and 5% of the ManA and GulA residues, respectively, were not carboxyl reduced prior to methylation. It is important to note that products arising from terminal (nonreducing) ManA or GulA groups were not detected in this experiment (Fig. 6).

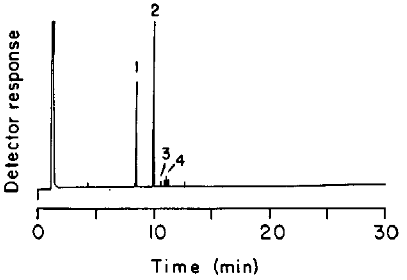


Fig. 6. Gas–liquid chromatogram of the anhydroalditol acetates derived by reductive cleavage of carboxyl-reduced and per-*O*-methylated *M. pyrifera* alginic acid. The numbered peaks were identified as follows: 1, **14b**; 2, **13b**; 3, **3b**; 4, **2b**.

The mucoid exopolysaccharide of *P. aeruginosa* was also subjected to the analysis as just described, and the products were analyzed as their acetates by g.l.c. Integration of the g.l.c. profile and correction for molar response[12,13] established that the polysaccharide was comprised of 80% 4-linked ManA residues and 20% 4-linked GulA residues. In this analysis, approximately 5% of the ManA residues were not reduced in the carboxyl-reduction step and, as in the case of the *M. pyrifera* alginate, products arising from terminal (nonreducing) ManA or GulA groups were not detected.

DISCUSSION

The most direct method of analysis of an alginic acid sample would appear to be direct reductive cleavage of the permethylated polymer. Indeed, total reductive cleavage of permethylated alginic acid followed by acetylation produced the two major products expected, namely, methyl 3-*O*-acetyl-2,6-anhydro-4,5-di-*O*-methyl-D-mannonate (**2b**) and methyl 3-*O*-acetyl-2,6-anhydro-4,5-di-*O*-methyl-D-gluconate (**3b**). That **2b** and **3b** were formed as the major products of reductive cleavage was actually quite surprising, considering the previous observation[2] that 4-linked D-glucopyranosyluronic acid residues undergo ring contraction exclusively to produce the isomeric 3,6-anhydroaldonate. Minor amounts (13% total) of 3,6-anhydroaldonates (**9** and **10**) were observed in the reductive cleavage of permethylated alginate but clearly, the ring isomerization required for their formation was not nearly as facile as was previously observed for 4-linked D-glucopyranosyluronic acid residues. In the absence of identifying each ring-contracted product (**9** and **10**), it was not possible to accurately determine the percent of ManA and GulA residues present in the copolymer. However, the ring isomerization problem was circumvented by reducing the ester groups in the permethylated polysaccharide prior to reductive cleavage. In the latter experiment, only the two major products that were expected, namely 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-D-mannitol (**5b**) and 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-L-gulitol (**6b**), were formed.

The major problem, however, with the direct analysis of alginates by sequential methylation and reductive cleavage is the difficulty of achieving total methylation without depolymerization. Because of the problems associated with depolymerization (*via* β-elimination) of polyuronic acids during base-assisted methylation[16], a neutral methylation was chosen. By using methyl trifluoromethanesulfonate and 2,6-di-*tert*-butylpyridine in trimethylphosphate[8,9], the depolymerized M-fraction could be methylated in yields of 83%; however, unsatisfactory results were obtained in the permethylation of native alginate due to low recovered yields (30%). Even more disturbing was the fact that native alginate (d.p. $>$900)[17,18] was degraded under these conditions of methylation to yield oligosaccharides with a d.p. of 13.

Because of the difficulties associated with direct methylation of alginic acid, methylation was performed after reduction of its carboxyl groups. Both the carboxyl reduction and permethylation proceeded in high yield and reductive cleavage yielded the expected products (**13b** and **14b**). Although traces of products (**2b** and **3b**) were present as a result of incomplete carboxyl reduction, accurate ManA:GulA ratios could easily be obtained by summing the amounts of **2b** and **13b** and the amounts of **3b** and **14b**. In this way, it was determined that the *M. pyrifera* alginate was comprised of 60% ManA and 40% GulA, whereas, the *P. aeruginosa* alginate was comprised of 80% ManA and 20% GulA. These values compared very favorably with the values obtained by integration of the anomeric proton resonances in the ^{1}H-n.m.r. spectra of the carboxyl reduced and permethylated polysaccharides, *i.e.*, 55% ManA and 45% GulA for the *M. pyrifera* alginate and 78% ManA and 22% GulA for the *P. aeruginosa* alginate. This strategy of analysis was also superior in that depolymerization during carboxyl reduction or methylation could not be detected; *i.e.*, products arising as a result of reductive cleavage of terminal (nonreducing) D-mannopyranosyl and L-gulopyranosyl groups were not detected.

Therefore, these studies demonstrate that alginic acids are best analyzed by reductive cleavage of the carboxyl-reduced and permethylated polymer. However, direct reductive cleavage of the fully methylated polymer might well be the method of choice if conditions for methylation of the alginate could be identified that gave high yields and did not result in depolymerization.

EXPERIMENTAL

General. — ^{1}H-N.m.r. spectra were recorded on an IBM NR-300 n.m.r. spectrometer in $CDCl_3$ as the solvent and were referenced to internal tetramethylsilane. ^{1}H-2D-COSY spectra were obtained in order to assign individual proton resonances. G.l.c.–m.s. analyses were performed using a VG Analytical Ltd. Model VG 7070E-HF high-resolution, double-focusing mass spectrometer. Column effluents were analyzed by chemical-ionization mass spectrometry (c.i.-m.s.) with ammonia as the reagent gas and by electron ionization mass spectrometry (e.i.-m.s.). For the acetylated anhydroalditol derivatives, the ammonium cluster ion (M + 18) and the protonated molecular ion (M + 1) are reported along with their percent intensity (in parenthesis) relative to the base peak. In e.i. mass spectra, *m/z* values are reported for ions below *m/z* 150 that comprise 10% or more of the intensity of the base peak and ions above *m/z* 150 that are prominent regardless of their absolute intensity. Analytical g.l.c. was performed on a Hewlett–Packard Model 5890A gas–liquid chromatograph equipped with a Hewlett–Packard 3392A integrator and a flame ionization detector. The column used was a J & W Scientific DB-5 fused silica capillary column (0.25mm × 30 m). The temperature of the column was held at 110° for 2 min, and then programmed to 300° at 6°/min. Medium-pressure liquid chromatography (m.p.l.c.) was carried out with an instrument consisting of a Rheodyne 7125 Injector, Eldex Model B-100-S4 pump, Scientific Systems Model LP-21 pulse dampener, and ChiraTech Scientific Instruments, Inc. ultraviolet absorption monitor. High-performance liquid chromatography (h.p.l.c.) was performed using a Beckman model 338 System Gold chromatograph.

Alginic acids were methylated with $MeOSO_2CF_3$ in trimethylphosphate with 2,6-di-*tert*-butylpyridine added as a proton scavenger[8,9]. Carboxyl-reduced[15] alginic acids were methylated by a modification[19] of the Hakomori[20] procedure. Reductive cleavages were carried out as previously described[21], and the products were either acetylated[13] or benzoylated[10].

Permethylated M-fraction of alginic acid from Macrocystis pyrifera (**1a**). — The lyophilized M-fraction (62 mg), derived by partial acid hydrolysis[7] of *M. pyrifera* alginic acid, was suspended in 6 mL of trimethyl phosphate and sonicated overnight in a small bath sonicator. To the turbid suspension was added 2,6-di-*tert*-butylpyridine (1.8 mL) and $MeOSO_2CF_3$ (1.2 mL), and the mixture was stirred for 3 h at 55° under N_2. After cooling to room temperature, the reaction was quenched by the addition of 10% aq. $NaHCO_3$ for 5 min (during which time slight gas evolution occurred). Dichloromethane was then added, and the mixture was partitioned into two layers. The aqueous layer was separated and extracted (5 ×) with CH_2Cl_2, and the organic layers were combined, reextracted (2 ×) with water, then dried over anhydr. Na_2SO_4, filtered, and concentrated. The residue was chromatographed on a column (2.5 × 30 cm) of Sephadex LH-20 in 2:1 (v/v) CH_2Cl_2–MeOH, and fractions which tested positive in the phenol–H_2SO_4 assay[22] were combined and evaporated to yield 63 mg (83%) of **1a**. ^{1}H-N.m.r.: δ 2.75–3.65 (complex, methoxyl, H-2,3,4), 3.65–3.82 (br s, MeO_2C), 3.92–4.22 (broad, H-5 ManA), 4.30–4.50 (broad, H-5 GulA), 4.50–4.72 (br, 0.8 H, H-1 ManA), and 4.92–5.21 (br, 0.2 H, H-1 GulA).

Permethylated alginic acid from Macrocystis pyrifera (**1b**). — The alginic acid (58 mg) from *M. pyrifera* was methylated as described for its M-fraction and, following purification, 15.8 mg (30%) of product (**1b**) was recovered. ^{1}H-N.m.r.: δ 3.20–3.68 (complex, methoxyl, H-2,3,4), 3.68–4.02 (br s, MeO_2C), 4.02–4.38 (br, H-5 ManA), 4.38–4.58 (br, H-5 GulA), 4.58–4.80 (br, 0.68 H, H-1 ManA), and 4.96–5.22 (br, 0.32 H, H-1 GulA).

*Methyl 2,6-anhydro-3-O-benzoyl-4,5-di-O-methyl-*D*-mannonate* (**2a**) *and methyl 3-O-acetyl-2,6-anhydro-4,5-di-O-methyl-*D*-mannonate* (**2b**). — A 39-mg sample of the permethylated alginic acid M-fraction (**1a**) was reductively cleaved for 3 h using 5 equiv. each of Et_3SiH and Me_3SiOSO_2Me and 1 equiv. of $BF_3 \cdot Et_2O$. Following workup, the products were benzoylated, then benzoylation byproducts were removed by m.p.l.c. using a column (0.8 × 25 cm) of silica gel (60–120 mesh) eluted with 3:1 (v/v) hexane–EtOAc. Benzoylated reductive cleavage products (**2a** and **3a**) were detected using a u.v. monitor set at 254 nm. Compounds **2a** and **3a** were separated by h.p.l.c. (Fig. 1), yielding **2a** as a syrup which crystallized upon standing. For **2a**: ^{1}H-n.m.r. ($CDCl_3$): δ 3.44, 3.50 (2 s, 6 H, 2 × MeO), 3.78 (s, 3 H, MeO_2C), 3.70–3.80 (complex, 3 H, H-4,5,6a), 4.17 (dd, 1 H, J 8.0, 10.5 Hz, H-6e), 4.35 (d, 1 H, J 3.4 Hz, H-2), 5.84 (dd, 1 H, J 3.4, 5.0 Hz, H-3), 7.44–7.51 (m, 2 H, *m*-Ar), 7.57–7.61 (m, 1 H, *p*-Ar), and 8.04–8.09 (m, 2 H, *o*-Ar).

A small portion of the reductive-cleavage product mixture was acetylated, and the products were separated by g.l.c. (Fig. 2) and analyzed by e.i.- and c.i.-m.s. For **2b**: g.l.c.–c.i.-m.s. (NH_3, positive): *m/z* 171 (100), 263 (23) and 280 (8); g.l.c.–e.i.-m.s.: *m/z* 43 (100), 45 (30), 58 (100), 59 (15), 71 (21), 73 (10), 74 (20), 75 (20), 85 (27), 87 (22), 88 (17), 97 (22), 101 (22), 103 (30), 111 (34), 117 (10), 129 (37), 143 (54), 145 (33), 159 (7), 171 (20), 202 (1), and 203 (13).

*Methyl 2,6-anhydro-3-*O*-benzoyl-4,5-di-*O*-methyl-*D*-gluconate* (**3a**) *and methyl 3-*O*-acetyl-2,6-anhydro-4,5-di-*O*-methyl-*D*-gluconate* (**3b**). — Compound **3a** was isolated as described for **2a** as a syrup which crystallized upon standing. For **3a**: ^{1}H-n.m.r. ($CDCl_3$): δ 3.40, 3.66 (2 s, 6 H, 2 × MeO), 3,70 (s, 3H, MeO_2C), 3.64–3.79 (complex, 2 H, H-5, 6a), 3.93 (m, 1 H, H-4), 4.08 (dd, 1 H, *J* 3.5, 9.6 Hz, H-6e), 4.58 (d, 1 H, *J* 1.8 Hz, H-2), 5.57 (dd, 1 H, *J* 1.8, 3.9 Hz, H-3), 7.42–7.49 (m, 2 H, *m*-Ar), 7.52–7.61 (m, 1 H, *p*-Ar), and 8.01–8.06 (m, 2 H, *o*-Ar).

Compound **3b** was separated by g.l.c. (Fig. 2) and analyzed as described for **2b**. For **3b**; g.l.c.–c.i.-m.s. (NH_3, positive): *m/z* 171 (97), 263 (100), and 280 (22); g.l.c.–e.i.-m.s.: *m/z* 43 (100), 45 (33), 58 (92), 59 (14), 71 (25), 73 (10), 74 (28), 75 (21), 85 (24), 87 (25), 88 (22), 97 (17), 101 (46), 103 (38), 111 (16), 117 (18), 129 (25), 143 (94), 144 (8), 145 (37), 171 (16), and 203 (20).

Permethylated and ester-reduced M-fraction of alginic acid (**4a**) *and native alginic acid* (**4b**) *from* Macrocystis pyrifera. — Permethylated M-fraction alginic acid (**1a**, 55 mg) was dissolved in 10 mL of 1:1 (v/v) CH_2Cl_2–Et_2O, 20 mg of $LiAlH_4$ was added, and the reaction was stirred for 5 h at room temperature. Excess $LiAlH_4$ was then decomposed by the slow addition of water, and solid material was removed by filtration. The filtrate was deionized using Dowex-AG501 X-8 mixed bed resin, filtered, and concentrated to afford a solid residue (70%). Reduction of ester groups in the permethylated native alginate (**1b**, 10.6 mg) was accomplished by the same procedure to yield **4b**. The ^{1}H-n.m.r. spectra of both products (**4a**, **4b**) indicated the absence of methyl ester resonances.

*1,5-Anhydro-4,6-di-*O*-benzoyl-2,3-di-*O*-methyl-*D*-mannitol* (**5a**) *and 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-*D*-mannitol* (**5b**). — A 30-mg sample of the permethylated and ester-reduced M-fraction (**4a**) was reductively cleaved for 2 h using 10 equiv. each of Et_3SiH and Me_3SiOSO_2Me and 2 equiv. of $BF_3 \cdot Et_2O$. Following workup, the products were benzoylated, and the benzoylation byproducts were removed as described for the isolation of **2a** and **3a**. Compounds **5a** and **6a** were separated by h.p.l.c. (Fig. 3), yielding **5a** as a syrup which crystallized upon standing. For **5a**: ^{1}H-n.m.r. ($CDCl_3$): δ 3.42, 3.50 (2 s, 6 H, 2 MeO), 3.43–3.51 (complex, 1 H, H-1a), 3.56 (dd, 1 H, *J* 3.2, 9.3 Hz, H-3), 3.76 (m, 1 H, H-2), 3.83 (ddd, 1 H, *J* 3.0, 6.0, 9.3 Hz, H-5), 4.25 (dd, 1 H, *J* 2.7, 12.8 Hz, H-1e), 4.37 (dd, 1 H, *J* 6.0, 12.1 Hz, H-6), 4.55 (dd, 1 H, *J* 3.0, 12.1 Hz, H-6′), 5.61 (t, 1 H, *J* 9.3 Hz, H-4), 7.32–7.56 (complex, 6 H, *m*-,*p*-Ar), and 7.99–8.05 (m, 4 H, *o*-Ar).

A small portion of the reductive-cleavage product mixture was acetylated, and the products were separated by g.l.c. (Fig. 4) and analyzed by e.i.- and c.i.-m.s. For **5b**: g.l.c.–c.i.-m.s. (NH_3, positive): *m/z* 277 (100) and 294 (55); g.l.c.–e.i.-m.s.: *m/z* 43 (62), 45 (11), 58 (100), 59 (15), 69 (17), 71 (18), 74 (26), 75 (25), 85 (37), 87 (29), 88 (11), 97 (32), 99 (10), 101 (25), 103 (32), 111 (17), 115 (21), 116 (22), 117 (25), 129 (33), 143 (49), 145 (30), 156 (38), 157 (8), 158 (11), 171 (28), 203 (10), 216 (0.3), and 217 (10).

*1,5-Anhydro-4,6-di-*O*-benzoyl-2,3-di-*O*-methyl-*L*-gulitol* (**6a**) *and 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-*L*-gulitol* (**6b**). — Compound **6a** was isolated as described for **5a** as a syrup which crystallized upon standing. For **6a**: ^{1}H-n.m.r. ($CDCl_3$): δ

3.40, 3.62 (2 s, 6 H, 2 × MeO), 3.63–3.68 (complex, 1 H, H-2), 3.73 (t, 1 H, *J* 10.4 Hz, H-1a), 3.88 (m, 1 H, H-3), 3.98 (dd, 1 H, *J* 4.6, 10.4 Hz, H-1e), 4.26 (ddd, 1 H, *J* 1.3, 5.2, 7.3 Hz, H-5), 4.35 (dd, 1 H, *J* 5.2, 11.4 Hz, H-6), 4.51 (dd, 1 H, *J* 7.3, 11.4 Hz, H-6′), 5.41 (dd, 1 H, *J* 1.3, 3.6 Hz, H-4), 7.40–7.60 (complex, 6 H, *m*-, *p*-Ar), and 7.98–8.09 (m, 4 H, *o*-Ar).

Compound **6b** was separated by g.l.c. (Fig. 4) and analyzed as described for **5b**. For **6b**; g.l.c.–c.i.m.s. (NH_3, positive): *m/z* 217 (100), 277 (16), and 294 (9); g.l.c.–e.i.m.s.: *m/z* 43 (98), 45 (17), 58 (100), 59 (15), 69 (18), 71 (21), 73 (12), 74 (44), 75 (23), 85 (44), 87 (42), 88 (19), 97 (13), 101 (28), 103 (37), 111 (16), 115 (14), 116 (23), 117 (39), 129 (17), 143 (99), 145 (26), 156 (62), 157 (6), 158 (9), 159 (5), 171 (7), 203 (5), and 216 (0.2).

Carboxyl-reduced and permethylated alginic acids from Macrocystis pyrifera *and* Pseudomonas aeruginosa *strain 2192.* — Alginic acid (40 mg) from *M. pyrifera* was dissolved in 20 mL of water and 0.84 g of solid 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate was added. The mixture was stirred for 2 h while the pH was maintained at 4.75 by the addition of 0.1N HCl. Sodium borohydride (60 mL, 2M) was then added in small portions while the pH was maintained at 7.0 by the addition of 4N HCl. After 2 h, the entire mixture was dialyzed against tap water (for 24 h) then lyophilized. A portion of the solid product was checked for uronate content by the carbazole assay[23] which indicated that total carboxyl reduction had not been achieved (quantitative measurements were not performed). Therefore, a second reduction was performed by the same procedure, except that 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride was used. Even though uronate was still present, as indicated by a qualitative carbazole assay, the product was lyophilized then methylated[19,20]. The carboxyl-reduced and methylated polysaccharide was then purified by chromatography on a column of Sephadex LH-20, as described for **1a**, yielding 27 mg (59%) of material. Integration of the H-1 signals for the D-mannopyranosyl (δ 4.60–4.74) and L-gulopyranosyl (δ 4.86–5.08) residues in the ^{1}H-n.m.r. spectrum of the product indicated that 55% Man*p* and 45% Gul*p* residues were present.

The alginic acid (5 mg) from *P. aeruginosa* strain 2192 was subjected to carboxyl reduction and permethylation by the same procedures, yielding 4.7 mg (81%) of the carboxyl-reduced and permethylated polysaccharide. Integration of the H-1 signals for the D-mannopyranosyl and L-gulopyranosyl residues in the ^{1}H-n.m.r. spectrum of the product indicated that 78% Man*p* and 22% Gul*p* residues were present.

*1,5-Anhydro-4-*O*-benzoyl-2,3,6-tri-*O*-methyl-*D*-mannitol* (**13a**) *and 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-*D*-mannitol* (**13b**). — Carboxyl-reduced and permethylated alginic acid (21 mg) from *M. pyrifera* was reductively cleaved for 2 h using 5 equiv. each of Et_3SiH and Me_3SiOSO_2Me and 1 equiv. of $BF_3 \cdot Et_2O$. Following workup, the products were benzoylated, and the benzoylation byproducts were removed by passing the mixture through a column of silica gel (60–120 mesh) contained in a Pasteur pipette. The column was eluted with 3:1 (v/v) hexane–EtOAc and fractions were collected and examined for benzoylated anhydroalditols by t.l.c. and g.l.c. Fractions containing **13a** and **14a** were combined, evaporated to dryness, then fractionated by h.p.l.c. (Fig. 5) yielding **13a** as a syrup. For **13a**; ^{1}H-n.m.r. ($CDCl_3$): δ 3.31, 3.40, 3.49 (3 s, 9 H, 3 × MeO), 3.42–3.58 (complex, 4 H, H-1a, 3, 6, 6′), 3.64 (ddd, 1 H, *J* 2.5, 7.4, 9.4 Hz, H-5), 3.71 (m, 1 H, H-2), 4.24 (dd, 1 H, *J* 2.5, 12.8 Hz, H-1e), 5.37 (t, 1 H, *J* 9.4 Hz, H-4), 7.42–7.47 (m, 2 H, *m*-Ar), 7.54–7.59 (m, 1 H, *p*-Ar), and 8.03–8.05 (m, 2 H, *o*-Ar).

A small portion of the reductive-cleavage product mixture was acetylated, and the products were separated by g.l.c. (Fig. 6) and analyzed by e.i.- and c.i.-m.s. For **13b**; g.l.c.–c.i.m.s. (NH_3, positive): *m/z* 249 (100) and 266 (24); g.l.c.–e.i.-m.s.: *m/z* 43 (69), 45 (30), 58 (29), 59 (21), 69 (18), 71 (19), 74 (13), 75 (16), 85 (26), 87 (45), 97 (100), 101 (16), 103 (22), 111 (23), 129 (53), 143 (26), 145 (6), 171 (72), and 203 (8).

1,5-Anhydro-4-O-benzoyl-2,3,6-tri-O-methyl-L-gulitol (**14a**) *and 4-O-acetyl-1,5-anhydro-2,3,6-tri-O-methyl-L-gulitol* (**14b**). — Compound **14a** was isolated as described for **13a** as a syrup. For **14a**; ^{1}H-n.m.r. ($CDCl_3$): δ 3.31, 3.39, 3.59 (3 s, 9 H, 3 × MeO), 3.44 (dd, 1 H, *J* 4.9, 10.0 Hz, H-6), 3.53 (dd, 1 H, *J* 7.1, 10.0 Hz, H-6′), 3.61–3.65 (complex, 1 H, H-2), 3.71 (t, 1 H, *J* 10.4 Hz, H-1a), 3.82 (m, 1 H, H-3), 3.97 (dd, 1 H, *J* 4.0, 10.4 Hz, H-1e), 4.08 (ddd, 1 H, *J* 1.5, 4.9, 7.1 Hz, H-5), 5.22 (dd, *J* 1.5, 3.8 Hz, H-4), 7.45–7.50 (m, 2 H, *m*-Ar), 7.58–7.63 (m, 1 H, *p*-Ar), and 8.06–8.09 (m, 2 H, *o*-Ar).

Compound **14b** was separated by g.l.c. (Fig. 6) and analyzed as described for **13b**. For **14b**; g.l.c.–c.i.-m.s. (NH_3, positive): *m/z* 249 (100) and 266 (29); g.l.c.–e.i.-m.s.: *m/z* 43 (54), 45 (22), 58 (22), 59 (16), 69 (10), 71 (14), 74 (20), 75 (21), 85 (12), 87 (40), 97 (31), 101 (14), 103 (34), 111 (13), 117 (10), 129 (23), 143 (100), 144 (9), 145 (10), 171 (12), and 203 (4).

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