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Note

The structural analysis of disaccharides from red algal galactans by methylation and reductive partial-hydrolysis

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Red algal galactans are generally characterised by a combination of chemical and spectroscopic techniques. Their constituent sugars, mainly galactose and 3,6-anhydrogalactose but sometimes xylose and others as well, can be determined by total hydrolysis, conversion to alditol acetate derivatives, and analysis by GLC and/or GLC–MS. The positions of glycosidic linkages and substituents on these sugar residues can be determined by 'methylation analysis' in which the polysaccharide is permethylated prior to hydrolysis and derivatisation. The positions of sulfate ester substituents are confirmed by analysis before and after solvolytic desulfation. These methods have been substantially improved by incorporating in situ reduction during hydrolysis, using the acid-stable reductant, 4-methylmorpholineborane (MMB) [1]. 3,6-Anhydrogalactose, which is rapidly lost during conventional total acid hydrolysis, is thereby protected as its stable alditol derivative.

In addition to information on the constituent sugars and their substituents, however, one also needs information on which sugar residue is connected to which. ^{13}C NMR spectroscopy has been particularly valuable in this regard for algal galactans with a significant degree of structural regularity [2]. Many red seaweeds, however, yield complex extracts with uninterpretable NMR spectra, and there is a clear need for improved methodology to help unravel their structures.

The preparation, separation and identification of oligosaccharides is a traditional method of determining carbohydrate sequence within a polysaccharide. This approach is

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particularly suitable when one of the glycosidic bonds is more susceptible to cleavage than the others. Most red algal galactans have a linear backbone comprised of alternating 3-linked β-D-galactopyranosyl and 4-linked α-D- or L-galactopyranosyl residues. In the case of the commercially important gel-forming agars and carrageenans, the 4-linked galactosyl residue is largely present as the 3,6-anhydride. The 3,6-anhydrogalactosidic bond is particularly labile to acid hydrolysis. Under relatively mild hydrolysis conditions virtually all the 3,6-anhydrogalactosidic bonds can be cleaved while most of the galactosidic bonds remain intact. In the presence of the borane reducing agent, this leads to the production of 'biitols', namely 3,6-anhydro-4-*O*-β-D-galactosylgalactitols [3]. (Strictly these should be named as 1,4-anhydrogalactitol derivatives, but we have retained the 3,6-anhydrogalactitol nomenclature throughout for clarity.)

In agars the 3,6-anhydrogalactosyl units are of the L-configuration whilst in carrageenans they are in the D-form. The derived agarobiitol and carrabiitol species, respectively, are thus diastereomers when they have the same substituents. After acetylation to improve volatility, they can be separated and identified by GLC–MS. This method has been developed and used by Usov and co-workers [4–6] to classify algal biomass into agarophytes or carrageenophytes. One limitation of this method is that, under the acetylation conditions used (acetic anhydride/pyridine, 100°C, 1 h), if the biitol contains a sulfate ester substituent, this will not necessarily be removed. Even if the resulting derivative is organic soluble and volatile enough to be eluted by GLC it will have a different retention time from the peracetylated product.

In this note we describe a modified *reductive partial-hydrolysis procedure* for preparing disaccharide alditol derivatives from naturally and chemically methylated red algal galactans containing 3,6-anhydrogalactosyl residues. Because the procedure incorporates an acetylative desulfation step, it is equally applicable to neutral or sulfated galactans. We have applied this procedure to chemically methylated samples, in particular, because GLC–MS analysis of the resulting partially methylated, partially acetylated biitols enables substitution patterns of adjacent residues to be determined.

A range of partially methylated, partially acetylated biitols has been produced from polysaccharides of known composition and characterised by GLC and GLC–CI(NH_3)MS. Each biitol derivative had a unique GLC retention time (Tables 1 and 2). Each species gave an $(M + NH_4)^+$ ion from which the total number of *O*-methyl groups could be determined, and a major fragment ion corresponding to the β-D-galac-

Table 1
GLC Retention times of partially methylated, partially acetylated carrabiitols

Retention time (min)	Position of *O*-methyl groups	Source
7.5	2,2′,4′,6′-	desulfated, methylated κ-carrageenan
7.9	2,2′,6′-	permethylated κ-carrageenan
8.1	2′,4′,6′-	base-treated, permethylated λ-carrageenan
8.6	2′,6′-	permethylated ι-carrageenan
9.0	4′,6′-	base-treated, permethylated λ-carrageenan
9.1	6′-	partially methylated ι-carrageenan
9.8	2′-	partially methylated ι-carrageenan
10.5	-	ι-/κ-carrageenan

Table 2
GLC Retention times of partially methylated, partially acetylated agarobiitols

Retention time (min)	Position of *O*-methyl groups	Source
7.2	2,2′,4′,6′-	permethylated *Pterocladia lucida* agar
7.7	2,2′,6′-	permethylated *Dasyclonium incisum* agar
8.2	2,6′-	native *Curdiea coriacea* agar
8.4	2′,6′-	partially methylated *Pterocladia lucida* agar
8.7	2,2′-	partially methylated *Pterocladia lucida* agar
8.9	6′-	native *Gracilaria chilensis* agar
9.3	2-	native *Dasyclonium incisum* agar
9.6	2′-	partially methylated *Pterocladia lucida* agar
10.3	-	native *Pterocladia lucida* agar

topyranosyl moiety from which the number *O*-methyl groups on this residue could be determined (Table 3).

When κ-carrageenan was methylated, subjected to reductive partial-hydrolysis (0.5 M aq CF_3CO_2H, MMB, 65°C, 7.5 h) and acetylated (Ac_2O–AcOH–$HClO_4$–EtOAc), it yielded 2,2′,6′-tri-*O*-methyl-carrabiitol tetraacetate, (2,2′,6′-*O*-Me-C). The prime denotes a position on the β-D-galactosyl moiety so that this biitol is methylated on O-2 of the 3,6-anhydro-D-galactitol moiety and on both O-2 and O-6 of the β-D-galactosyl moiety. The sulfate group present in κ-carrageenan (and other polysaccharides here examined) was efficiently cleaved during the acetylation step, so that a neutral derivative resulted. The diastereomeric 2,2′,6′-tri-*O*-methyl-agarobiitol tetraacetate (2,2′,6′-*O*-Me-A) was produced similarly from the polysaccharide (a 4-sulfated agar) from *Dasyclonium incisum* [7]. Both these biitol derivatives had an $(M + NH_4)^+$ ion at *m/z* 554 and a major fragment ion at *m/z* 275, but the retention time of the 2,2′,6′-tri-*O*-methyl-carrabiitol derivative was 7.9 min, while that of the corresponding agarobiitol derivative was 7.7 min (Fig. 1).

The polysaccharide from *Dasyclonium incisum* contains some native methylation on O-2 of the 3,6-anhydro-L-galactosyl residue, but this is masked by the introduction of

Table 3
Pseudomolecular and major fragment ions obtained from partially methylated, partially acetylated biitols (figures in brackets refer to trideuteromethylated species)

Number of -*O*-CH_3 (-*O*-CD_3) Groups		$(M+NH_4)^+$ ion	Gal^+ ion
Gal [a]	AnGal [b]		
0	0	638 (-)	331 (-)
0	1	610 (613)	331 (331)
1	0	610 (613)	303 (306)
1	1	582 (588)	303 (306)
2	0	582 (588)	275 (281)
2	1	554 (563)	275 (281)
3	0	554 (563)	247 (256)
3	1	526 (538)	247 (256)

[a] Gal refers to β-D-galactopyranosyl moiety.
[b] AnGal refers to 3,6-anhydrogalactitol moiety.

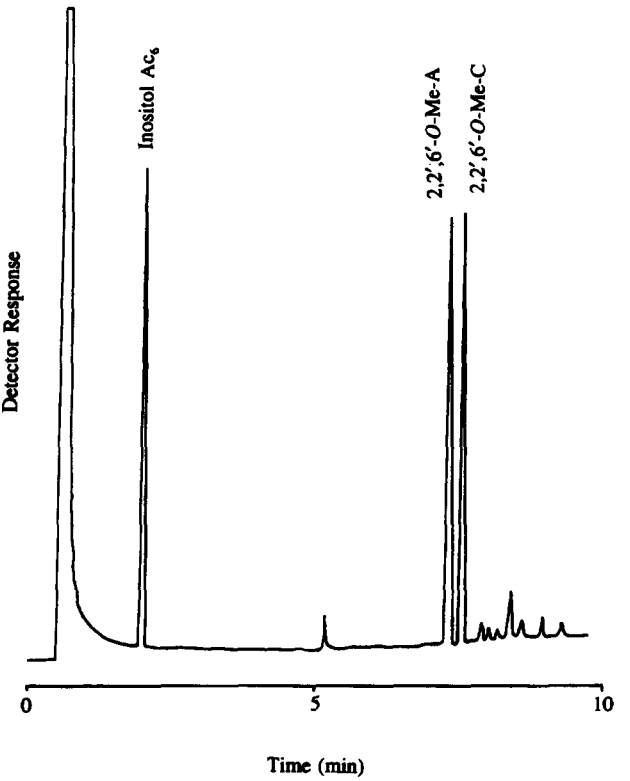

Fig. 1. Gas–liquid chromatogram of a mixture of partially methylated, partially acetylated biitols from permethylated κ-carrageenan and permethylated *Dasyclonium incisum* agar. (See text for description.)

additional *O*-methyl groups during the preparation of the permethylated sample. However, the position of native methylation can be determined if the chemical methylation is performed with trideuteroiodomethane. Thus, application of the reductive partial-hydrolysis procedure to a sample of the trideuteriomethylated polysaccharide from *D. incisum* produced one GLC peak which had two $(M + NH_4)^+$ ions at m/z 563 and 560. The presence of a single fragment ion at m/z 281 and the absence of one at m/z 275 indicates that the two methyl groups on the β-D-galactosyl moiety are trideuterated, while the methyl group on the 3,6-anhydrogalactosyl moiety is only partially trideuterated (Fig. 2). This confirms the presence of a native *O*-methyl group on O-2 of some of the 3,6-anhydro-L-galactosyl residues in this polymer as concluded from an earlier study [7].

Agar from *Pterocladia lucida* and ι-carrageenan were partially methylated then subjected to the reductive partial-hydrolysis procedure to obtain a range of partially methylated, partially acetylated biitols. Each of the components was separable by GLC, and their structures were determined as described above and, where possible, by comparison with species produced from polysaccharides of known composition. The

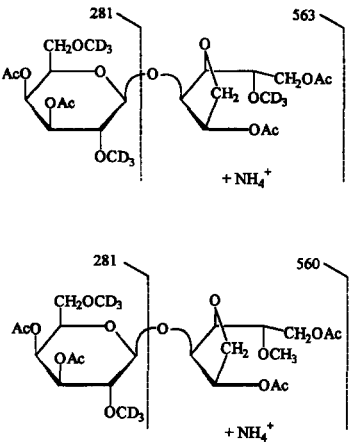

Fig. 2. Origin of the pseudomolecular ion and major fragment ion for the agarobiitol derivatives from per(trideutero)methylated *Dasyclonium incisum* polysaccharide.

results are summarised in Tables 1 and 2. For the five pairs of diastereomers, the agarobiitol derivative eluted ca. 0.2–0.3 min faster than the corresponding carrabiitol derivative in each case.

1. Conclusions

The reductive partial-hydrolysis procedure was applied to a range of red algal polysaccharides of known composition in order to produce a set of partially methylated, partially acetylated biitols. The components produced were identified by GLC retention time and from CIMS data.

The success of the reductive partial-hydrolysis procedure relies upon: (a) the presence of contiguous agarobiose or carrabiose residues in the polymer, and (b) *the removal of sulfate ester substituents during derivatisation*, since sulfated biitol derivatives would not elute on GLC.

The selection of the polysaccharides examined ensured that the first requirement was met, but the fact that *sulfate esters were effectively cleaved under the mild-acid catalysed acetylation / acetolysis conditions* was a pleasant surprise. Acetylative desulfation is a known reaction [8], but that it can effect desulfation under conditions wherein β-D-galactopyranosidic linkages remain intact was not. Studies on the quantitative aspects of the production of biitol derivatives will be published later. For the purpose of this Note, we report that sulfated and non-sulfated polysaccharides give roughly similar quantities of biitol derivatives as judged by GLC–MS total-ion intensities. The configuration of the 3,6-anhydrogalactitol moiety and the positions of introduced *O*-(trideutero)methyl groups on the adjacent residues provide valuable data on the structure of algal polysaccharides.

2. Experimental

Samples.—κ-Carrageenan was obtained from Coast Biologicals Ltd., Auckland, New Zealand. ι-Carrageenan was obtained from Sigma Chemical Co.

λ-Carrageenan from tetrasporophytic *Chondrus crispus* [9] was kindly supplied by Dr. J.S. Craigie, Institute for Marine Biosciences, NRCC, Canada and was base-treated according to the method of Craigie and Leigh [10]. Agars were obtained from *Pterocladia lucida* [1], *Gracilaria chilensis* [1], *Curdiea coriacea* [11], and *Dasyclonium incisum* [7]. Permethylations were performed according to Stevenson and Furneaux [1]. Partial methylations were performed according to the method of Miller et al. [12].

Reductive partial-hydrolysis procedure.—A sample of sulfated and/or methylated polysaccharide (1 mg) was placed in a screw-capped test tube (13 × 100 mm). The sample was dissolved, with gentle warming, in water (0.45 mL) containing 4-methylmorpholineborane (7.5 mg). Trifluoroacetic acid (2 M, 0.15 mL) was added, the tube was capped, and the mixture was heated at 65°C for 7.5 h. After cooling, EtOH (3 mL) was added, and the mixture was filtered through a 0.22-μm filter (Millipore). The filtrate was concentrated to dryness under a stream of dry air at 40°C. Acetonitrile (0.5 mL) was then added and evaporated. The resulting dry solid was acetylated using AcOH (0.04 mL), EtOAc (0.2 mL), Ac_2O (0.6 mL), and perchloric acid (60%, 0.023 mL) [13,14]. After 15 min at room temperature, water (2 mL) and 1-methylimidazole (0.04 mL) were added to decompose the Ac_2O. The mixture was extracted with CH_2Cl_2 (1 mL), and the organic layer was washed with water (4 × 3 mL). The phases were separated by brief, low-speed centrifugation. The aqueous phase was drawn off and discarded and the CH_2Cl_2 was then removed by evaporation at 40°C under a stream of dry air. The residue was then dried by addition and evaporation of MeCN (0.5 mL) at 40°C with a stream of dry air. The resulting partially methylated, partially acetylated biitols were dissolved in acetone (0.05 mL) and analysed by GLC and GLC–CIMS. GLC was conducted on a Hewlett–Packard 5890 Series II instrument fitted with an SGE fused silica BP-1 column (25 m × 0.22 mm i.d., 0.25-μm film thickness). The sample was introduced by split injection using H_2 as the carrier gas with a split ratio of 65:1 (column flow, 1.5 mL/min). Injector and FID detector temperatures were 320°C. The column temperature was held at 250°C for 1 min rising to 290°C at 5°C/min, held for 4 min, then rising to 310°C at 20°C/min and held for 4 min. GLC–CIMS was conducted as for GLC but using a Fisons Trio 1000 quadrapole mass spectrometer with ammonia as the reagent gas, an ion source temperature of 260°C, and He as carrier gas in the splitless mode.

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