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Avoiding oxidative degradation during sodium hydroxide/methyl iodide-mediated carbohydrate methylation in dimethyl sulfoxide

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ABSTRACT

A comparison of the methylation of cellobiitol by the Hakomori method (using potassium methylsulfinylmethanide – "potassium dimsyl" – as base) and by a published or a modified sodium hydroxide-mediated procedure is described. Evidence is presented that the modified sodium hydroxide-catalysed procedure is not prone to the oxidative deficiencies of the original and that, given its reduced tendency towards polysaccharide undermethylation, it should now become the method of choice for the methylation of these polymers.

INTRODUCTION

A method for the methylation of mono- and di-saccharides, due to Ciucanu and Kerek[1], has been widely adopted for the methylation of complex carbohydrates[2] and has been found to be straightforward and to give little undermethylation. It replaces the dimsyl (methylsulfinylmethanide) anion, used in the formerly prevalent Hakomori method[3] with powdered sodium hydroxide, whilst retaining dimethyl sulfoxide as solvent and methyl iodide as alkylating agent. Unfortunately, a disadvantage of the technique was subsequently noted by York et al.[4] during linkage analysis of oligoalditols from plant cell-wall xyloglucans[5]. When the oligoalditols were converted into partially methylated alditol acetates (PMAAs)[6], small proportions of unexpected PMAAs were detected. The authors[4] suggested that the results were consistent with oxidation of sugar hydroxyl groups by the reagent, and proposed a mechanism for this process. They also gave further evidence for the effect from earlier work on a xyloglucan oligosaccharide of known structure (independently evaluated by spectroscopic and enzymatic degradation)[7] where (1 → 4)-linked glucosyl residues were overestimated by the method. Oxidative degradation of terminal xylosyl residues attached to the 6-position of a (1 → 4,6)-linked glucosyl moiety accounted for their observations.

York et al.[4] tested their hypothesis in a model system by reducing cellobiose with sodium borodeuteride. The resulting cellobiitol-*1-d* was methylated by both the Hakomori method and the sodium hydroxide-mediated method of Ciucanu and Kerek[1]. They found evidence of oxidation products in the sodium hydroxide-mediated procedure. The mechanism of the oxidation was not investigated, but it was suggested[4] that a dimethylmethoxysulfonium salt formed by reaction of Me_2SO with methyl iodide could be responsible.

York et al.[4] concluded that the method was unsuitable for the detailed analysis of complex carbohydrates as it might give anomalous results, but suggested that its ease of use might outweigh its disadvantages in less rigorous applications. We now describe a modification of the method and give evidence that it avoids entirely the above disadvantages.

RESULTS AND DISCUSSION

York et al.[4] identified PMAA **1**, normally indicative of a 3-linked hexopyranosyl residue (Fig. 1), during their xyloglucan oligoalditol study[5]. The authors[4] suggested that PMAA **1** was formed from oxidation of C-6 of the terminal 4-linked glucitol of the oligoalditol during methylation by the method of Ciucanu and Kerek[1] and that this intermediate was protected from immediate β-eliminative degradation by ring closure and subsequent methylation to give a methyl glycopyranoside. (Fig. 1 also illustrates the possibility of cyclisation, in the general case, to a glycofuranoside and the subsequent conversion of this derivative into PMAA **2**. This was not reported by York et al.[4], but see below for cellobiitol-*1-d*.) In general, all oxidised terminal alditols able to cyclise to glycopyranosides or glycofuranosides would avoid immediate β-eliminative degradation in this way. These and other glycosidic rings would be prone to oxidation and β-eliminative cleavage; their loss would only be reflected indirectly by the detection of a PMAA corresponding to their aglycon, but now unsubstituted at its former linkage position. York et al.[4] examined the products of cellobiitol-*1-d* methylation (using the method of Ciucanu and Kerek[1]) by GLC–EIMS and noted minor components in the sodium hydroxide-promoted reaction which were absent in the Hakomori reaction. Selective ion monitoring revealed ion masses corresponding to a variety of methyl glycosides, all bearing terminal, non-reducing glucosyl substituents. Conversion of these products into PMAAs lent several minor components in the sodium hydroxide run. Examination of these compounds by GLC–EIMS suggested structures consistent with the mechanisms described above.

We agree with York et al.[4] that a dimethylmethoxysulfonium salt formed by reaction of Me_2SO with methyl iodide could be responsible. The reaction of primary alkyl iodides with Me_2SO to give this species initially (as the kinetic product; extended reflux gives the product of *S*-methylation, the trimethylsulfoxonium salt[8]) is known, although it is generally slow at room temperature[9]. Once formed, however, alkoxide exchange with a deprotonated carbohydrate hydroxyl

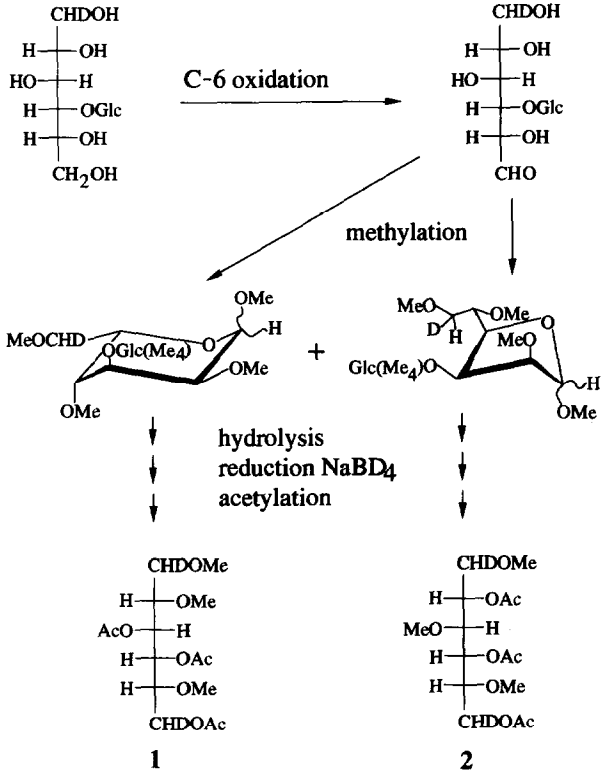

Fig. 1. Transformation of a 4-linked glucitol-*1-d* residue to PMAAs **1** and **2** by C-6 oxidation, conversion into a methyl pyranoside or furanoside by methylation, and subsequent derivatisation of the products.

group and the latter's subsequent oxidation to a carbonyl group (probably via an ylid) would be rapid[10] – see Fig. 2. Alkoxide exchange would be favoured over the base-catalysed breakdown of the dimethylmethoxysulfonium salt to formaldehyde and dimethyl sulfide[10], as the carbohydrate is present in solution at much higher concentration than the sparingly soluble[1] sodium hydroxide. The slowness of the initial reaction of Me_2SO with methyl iodide is no bar to this explanation; the speed of the subsequent reactions and the extremely small amounts of dimethylmethoxysulfonium salt required to account for the relatively minor levels of oxidation observed in the small amount of carbohydrate present make it a reasonable hypothesis, particularly given the carbohydrate's relatively high concentration.

Formation of the dimethylmethoxysulfonium salt is dependent on the simultaneous presence of Me_2SO and methyl iodide. These conditions only occur in the Hakomori procedure during the addition of methyl iodide. During this addition, deprotonated carbohydrate hydroxyl groups are methylated and dimsyl anion is converted into ethyl methyl sulfoxide. Both reactions are rapid[11]; thus if methyla-

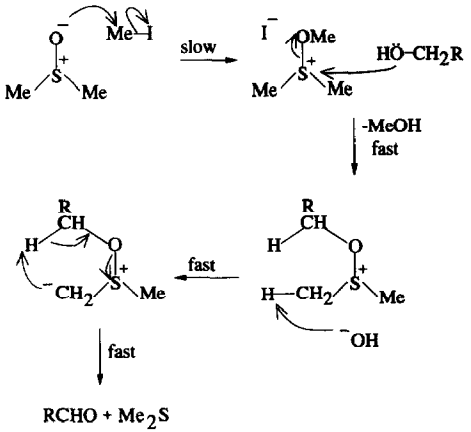

Fig. 2. Proposed mechanism of carbohydrate oxidation by a dimethylmethoxysulfonium salt formed from dimethyl sulfoxide and methyl iodide (the process is illustrated for a generalised primary alcohol, RCH_2OH).

tion of the carbohydrate is complete, its oxidation by any subsequently formed dimethylmethoxysulfonium salt is precluded. We realised that by simply adding the sodium hydroxide to the solution of carbohydrate sometime before the methyl iodide (sufficient to give complete deprotonation of the carbohydrate's hydroxyl groups) oxidation could also be avoided in this procedure if the dimethylmethoxysulfonium salt is indeed the oxidative species. In addition, if correct, this explanation indicates that extending the duration of treatment with base, methyl iodide, or both reagents simultaneously beyond that necessary for complete methylation should not affect the extent of oxidative degradation observed in any of the three procedures.

To test these points, cellobiitol-*1-d* was prepared as described by York et al.[4] and methylated by each of the three methods described in the experimental section. The duration of both base (10 min, Short; or 3 h, Long) and methyl iodide (10 min, Short; or 1 h, Long) treatment was varied in each case in order to investigate any dependency on this factor. The methylated alditols were first examined directly by GLC–EIMS[12] which revealed, in each case, permethylated cellobiitol-*1-d* as the major product (aA_1 *m/z* 219, aA_2 *m/z* 187, bJ_1 *m/z* 296, bJ_2 *m/z* 236). In addition, minor products of longer retention time were detected although their amount varied according to the procedure used. Like York et al.[4], non-reducing end fragment ions of these products (aA_1 *m/z* 219, aA_2 *m/z* 187) showed that they all contained a terminal tetra-*O*-methylhexitol residue. Reducing-end fragments indicated that they were methyl glycosides bearing either a single (bJ_1 *m/z* 280, bJ_2 *m/z* 220) or no (bJ_1 *m/z* 279, bJ_2 *m/z* 219) deuterium atom. Table I gives total ion current areas at *m/z* 280 and 279 expressed as a percentage of the total ion current area at *m/z* 187 for each experiment, obtained by selective ion monitoring across the entire product range. These data represent a measure of

TABLE I

Total ion current areas at m/z 279, 280 expressed as a percentage of that at m/z 187 for the product mixtures obtained from methylation of cellobiitol-*1-d* under a variety of regimes

m/z	SIM [a] S [b]	SIM L	SEQ SS	SEQ SL	SEQ LS	SEQ LL	DIM SS	DIM SL	DIM LS	DIM LL
279	8.1	5.1	0.3	0.5	0.09	0.3	0.04	0.5	0.05	0.05
280	15.4	12.9	1.2	1.8	0.4	1.0	0.2	3.2	0.2	0.2

[a] Key: SIM, methylated with sodium hydroxide and methyl iodide simultaneously by the method of Ciucanu and Kerek[1]; SEQ, methylated with sodium hydroxide and methyl iodide in a modified procedure where the base is added first; DIM, methylated by the modified method of Hakomori[10], using potassium dimsyl. [b] S, Short reaction time(s); L, Long reaction time(s) (see Experimental section for details). For SIM experiments, the total reaction time is given; for DIM and SEQ experiments, the first letter represents the duration of base treatment, and the second the duration of treatment with methyl iodide.

the degree of oxidation occurring in each case, although they are not absolutely specific. Unlike York et al.[4], we found these compounds even in the Hakomori-methylated material ("DIM"), although their levels were very small compared to the material methylated with sodium hydroxide and methyl iodide simultaneously ("SIM"); the material methylated by the new sequential procedure ("SEQ") compared favourably with the Hakomori-methylated material.

The methylated oligoalditols were then converted into PMAAs by the method of Harris et al.[11]. The yield of 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methyl-D-glucitol formed from the alditol end of the cellobiitol-*1-d* was low in all cases, typically less than 10% of the recovery of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol formed from the non-reducing end, and much lower than could be accounted for by oxidation of the alditol terminus. This loss has been shown to be due to the derivatisation procedure used[11] and is discussed in our following paper[13]. (The loss was subsequently corrected, allowing approximately equimolar recovery of the two derivatives. It was primarily a result of the relatively high volatility and water solubility of the pentamethyl derivative and thus did not affect the recoveries of the terminal glycosyl residue and the products of oxidation, which are tetra- and tri-methylated derivatives respectively). Other products obtained were identified by GLC–EIMS[4,14] and are listed in Table II; their molar percentage recovery relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol for each experiment is listed in Table III. As can be seen, the new sequential procedure produced very low levels of oxidation products, performing as well as the Hakomori procedure in this respect and far better than the simultaneous procedure of Ciucanu and Kerek[1].

An apparently high relative level of the product of C-1 oxidation, PMAA **8**, was observed in all the reactions (see Fig. 3). This could, in theory, reflect the susceptibility of this primary hydroxyl group to oxidation (by an unknown mechanism in the cases of the DIM and SEQ procedures) or it could indicate either undermethylation at O-4 of the terminal glucosyl residue (as noted by York et al.[4]) or initial under-reduction of the cellobiose used. The purity of the cellobiitol-*1-d* produced (the material for all subsequent experiments was prepared in a single

TABLE II

Anomalous partially methylated D-glucitol acetates formed from cellobiitol-*1-d* during permethylation reactions [a]

Compound	t_R [b]	Substituent sites [c]			Formation [d]
		O-Methyl	*O*-Acetyl	Deuterium	
3 [e]	1.026	2,3,5,6	1,4	1	UM(C-1, Glcol)
4 [f]	1.110	1,3,6	2,4,5	1,2	Ox(C-2, Glcol) [g]
5 [f]	1.130	1,3,6	2,4,5	1,2	Ox(C-2, Glcol) [g]
6	1.178	2,4,6	1,3,5	1	UM(C-3, T-Glc)
2	1.188	1,2,5	3,4,6	1,6	Ox(C-6, Glcol) [g]
1	1.218	1,3,5	2,4,6	1,6	Ox(C-6, Glcol) [h]
7	1.243	2,3,4	1,5,6	1	UM(C-6, T-Glc)
8	1.248	2,3,6	1,4,5	1/1,1 [i]	Ox(C-1, Glcol) [h]

[a] The list excludes the two major expected products 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methyl-D-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b] Retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a 30-m OV225 column (see Experimental). [c] For the purposes of nomenclature, the carbon atom bearing the deuterium atom introduced before methylation is defined as C-1. [d] UM indicates undermethylation at the indicated position; Ox indicates oxidation at the indicated position. [e] Other products of undermethylation were probably present in trace amounts but could not be quantified. [f] both D-*manno* and D-*gluco* derivatives were formed by borodeuteride reduction of the D-fructofuranosyl intermediate. [g] Produced by derivatisation of the methyl furanoside formed after oxidation at the site indicated. [h] Produced by derivatisation of the methyl pyranoside formed after oxidation at the site indicated. [i] A mixture of 1-deuterio- and 1,1-dideuterioalditols (see text).

TABLE III

PMAAs obtained from cellobiitol-*1-d* under a variety of methylation regimes, expressed as molar percentages of the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol produced

PMAA [a]	SIM [b] S [d]	SIM L	SEQ SS	SEQ SL	SEQ LS	SEQ LL	DIM SS	DIM SL	DIM LS	DIM LL	York SIM [c]
3	2.1 [e]			1.6	1.4	1.5	2.4		0.3	2.0	1.1
4	2.7	1.7									1.7
5	3.7	2.7	0.5			0.4					1.7
6	7.9	5.0		1.2	5.4	1.0	5.7	1.0	4.1	5.6	2.4
2	4.4	2.6									2.4
1	13.8	7.9			0.5		0.6		0.5		9.6
7	2.7	2.4	0.9	1.3	1.6	1.0	1.1	1.4	1.3	2.0	0.9
8	18.8	12.2	5.3	7.2	6.9	5.3	7.7	5.4	6.5	7.1	9.6
8 [f]	15.2	6.3	1.1	1.2	1.4	1.3	2.2	1.2	1.7	2.1	?

[a] See Table II. [b] Key: SIM, methylated with sodium hydroxide and methyl iodide simultaneously by the method of Ciucanu and Kerek[1]; SEQ, methylated with sodium hydroxide and methyl iodide in a modified procedure where the base is added first; DIM, methylated by the modified method of Hakomori[10], using potassium dimsyl. [c] Data obtained by York et al.[4], using the method of Ciucanu and Kerek[1]. [d] S, Short reaction time(s); L, Long reaction time(s) (see Experimental section for details). For SIM experiments, the total reaction time is given; for DIM and SEQ experiments, the first letter represents the duration of base treatment, and the second the duration of treatment with methyl iodide. [e] PMAAs produced by the method of Harris et al.[10]; the values given are uncorrected. [f] Approximate proportion of compound **8** formed by oxidation at C-1 (see text).

reaction) was checked by thin-layer chromatography, but it is possible that this was an insufficiently sensitive test. Although the oligoalditol was prepared by the method of York et al.[4], who assessed its purity by both nmr and GLC–EIMS, this possibility was considered the most plausible, as the levels of other products of undermethylation observed were low. In order to investigate this point, the incidence of deuterium labelling in the mass spectrum of PMAA **8** was examined more closely. PMAA **8**, if formed by under-reduction of cellobiose or by undermethylation of O-4 of the terminal glucosyl residue, will invariably bear a single deuterium atom at C-1. Thus, its mass spectrum will contain ions of m/z 118 and 162 (as a result of C-2–C-3 or C-3–C-4 bond cleavage, respectively[14]; see Fig. 3). If however, it arises from C-1 oxidation, it may bear either one or two deuterium atoms at C-1, giving rise to ions of m/z 118 and 162, or 119 and 163, respectively. Carbon–hydrogen bonds are weaker than carbon–deuterium bonds[15] and so more of the original deuterium atoms than the hydrogen atoms might be expected to remain after oxidation if this step were rate determining. In fact, as discussed above and illustrated in Fig. 2, it is not rate determining and so we may expect approximately equal amounts of the monodeuterio and dideuterio compounds by this mechanism. An excess of the singly deuterated compound would represent evidence of its formation by other means, i.e., presumably by cellobiose under-reduction. The ratios of the ion current areas at m/z 163 and 162 were determined by selective ion monitoring across the relevant PMAA peak (the higher mass pair was chosen for this comparison despite their lower intensity as they were less liable to interference from competing fragmentation pathways than the lower mass pair). From these values, the molar percentage of PMAA **8** formed by oxidation could be calculated; they are also given in Table III. Although the values so obtained for the SEQ and DIM experiments are still somewhat higher than those for the other products of oxidation, they are sufficient to show that factors other than oxidation were primarily responsible for the relatively high values indicated for these methods, and to reveal a higher level of oxidation for the SIM method. The inflation of the SIM and SEQ figures may be due to ion interference from competing fragmentation pathways or simply reflect the greater susceptibility of this primary hydroxyl group to oxidation. The observed lesser oxidation of the other primary hydroxyl group of the alditol might be due to its greater proximity to the terminal glucosyl residue at the 4-position and the resultant steric hindrance to oxidation by the bulky dimethylmethoxysulfonium salt. Kiefer et al.[7] showed that the terminal xylosyl residues of a xyloglucan oligoalditol were particularly prone to oxidation. This may have been due to their peripheral, relatively unhindered position in the molecule, which supports the argument that steric hindrance is important in the present case. Alternatively, this susceptibility might be intrinsic to, and/or a statistical outcome of, the relatively high degree of hydroxyl substitution of these residues.

No definite conclusions about the effect of varying the duration of treatment with either base of alkylating agent for the various methods could be drawn. In

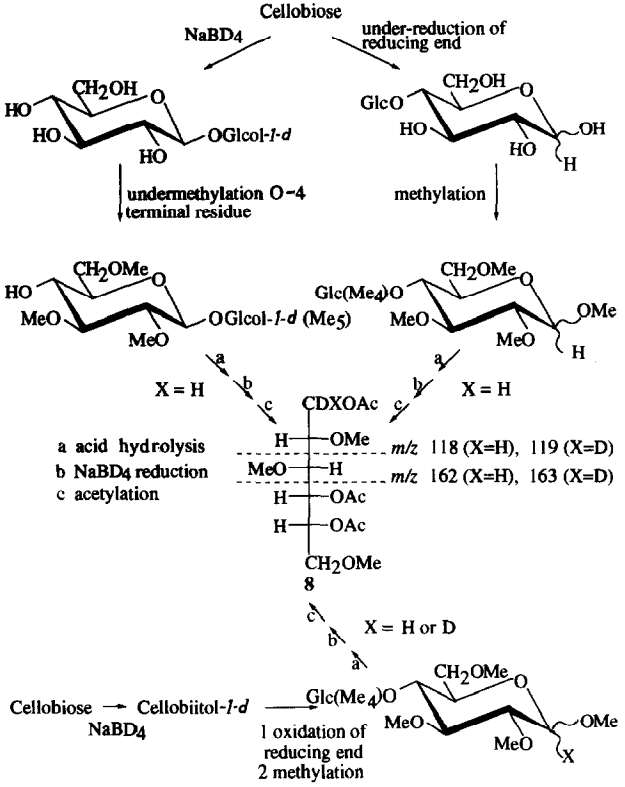

Fig. 3. Methylation analysis of cellobiose. Three possible pathways to PMAA 8.

particular, apparent (anomalous?) high or low levels of methyl glycosides in the oligoalditols of one variant of a particular methylation technique were not necessarily reflected in the eventual PMAA analysis of the sample; for example, see DIMSL, Tables I and III. Because of this, these data are best viewed as repeats of each of the three methylation techniques—the average level of oxidation in the SIM experiments was higher than that observed in the DIM and SEQ experiments. The absence of an obvious trend was taken as supportive of our theory of oxidation and its prevention.

CONCLUSION

The data presented show that the oxidative deficiencies (discovered by York et al.[4]) inherent in the NaOH/MeI/Me_2SO-mediated methylation procedure of Ciucanu and Kerek[1] can be overcome easily by treating the polysaccharide with sodium hydroxide before the addition of methyl iodide in a sequential manner, instead of adding the reagents simultaneously. The efficacy of the modification is readily explained by the proposed mechanism of the oxidation. Given the simplic-

ity and other advantages of the method over the widely employed Hakomori procedure (which we shall discuss in a future paper[16]), we propose that it is now the method of choice for the methylation of these materials.

EXPERIMENTAL

Reagents.—All reagents used were of the highest grade available. Me_2SO was stored over approximately one-quarter of its volume of 3A molecular sieves (which had been activated by heating at 300°C overnight) for at least 2 days before use.

Preparation of potassium methylsulfinylmethanide.—Potassium methylsulfinylmethanide (potassium dimsyl) was prepared by the method of Harris et al.[11].

Preparation of cellobiitol-1-d.—Cellobiitol-*1-d* (50 mg) was prepared by the method of York et al.[4]. The absence of starting material in the product was confirmed by thin layer chromatography on Analtec silica-G plates (5 × 20 cm), using 3:2:2 1-butanol–acetic acid–water as eluent. The plates were visualised by spraying with 50% H_2SO_4 and heating at 110°C for 5 min.

Samples for methylation.—Samples of cellobiitol-*1-d* (2 mg) were dried overnight at 40° in a vacuum oven and dissolved, under Ar, in Me_2SO (1 mL) at room temperature prior to their methylation by one of three methods.

Hakomori methylation.—Potassium dimsyl (1 mL) was added to the sample dissolved in Me_2SO under Ar and mixed. The mixture was sonicated at room temperature for either 10 min ("Short") or for 90 min and then left for a further 90 min ("Long"). Methyl iodide (0.75 mL) was added dropwise over 1 min to the vented, agitated frozen solution which was warmed to room temperature and sonicated either for 10 min ("Short") or for 30 min and then left a further 30 min ("Long").

"Simultaneous" methylation with sodium hydroxide and methyl iodide.—Methyl iodide (0.2 mL) followed by finely powdered dry NaOH (40 mg) was added to a stirred solution of the polysaccharide dissolved in Me_2SO under Ar. The mixture was left at room temperature for either 10 min ("Short") or 3 h ("Long").

"Sequential" methylation with sodium hydroxide and methyl iodide.—Finely powdered dry NaOH (100 mg) was added to a solution of the polysaccharide in Me_2SO under Ar. The mixture was sonicated at room temperature for either 10 min ("Short") or for 90 min and then left a further 90 min ("Long"). Methyl iodide (0.75 mL) was added dropwise over 1 min to the vented, agitated frozen solution which was warmed to room temperature and sonicated either for 10 min ("Short") or for 30 min and then left a further 30 min ("Long").

Isolation of methylated material.—In each case, the methylated alditols were isolated by adding water (1 mL) and CH_2Cl_2 (1 mL), vortexing the mixture, and separating the layers by centrifugation (200*g*, 5 min). The organic phase was washed with water (3 × 3 mL) and the permethylated oligosaccharide recovered by evaporation to dryness under vacuum at 40°C.

Preparation of PMAAs from methylated polysaccharides.—Methylated polysaccharides were converted into PMAAs using the procedure of Harris et al.[11].

Gas chromatography of permethylated oligoalditols.—Permethylated oligoalditols were monitored by GLC–EIMS, using a Vectographic Trio 1S mass spectrometer linked to a Hewlett–Packard model 5890 series 2 chromatograph. Injection, in the splitless mode, was onto a capillary column (30 m × 0.32 mm i.d., Restek OV225). High purity He was used as carrier. The oven temperature was maintained at 55°C for 2 min, raised at 10°C min^{-1} to 230°C and maintained at this temperature for 30 min.

Gas chromatography of PMAAs.—PMAAs were separated by cold column injection onto a capillary column (30 m × 0.32 mm i.d., Restek OV225) in a Carlo Erba model 5160 chromatograph equipped with a flame-ionisation detector. High purity He was used as carrier at a head pressure of 0.96 kg m^{-2}. Ten seconds after injection, the cooling was switched off, and the oven temperature maintained at 55°C for 1 min, raised at 45°C min^{-1} to 140°C and immediately raised at 2.5°C min^{-1} to 218°C. This temperature was maintained for 37 min. The detector temperature was 220°C. Integration was performed using a Spectra-Physics SP4400 integrator. The identity of the peaks was confirmed using a Vectographic Trio 1S mass spectrometer linked to a Hewlett–Packard model 5890 series 2 chromatograph fitted with a second OV225 column.

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