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Methylation analysis of cellulose sulphates

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Methylation analysis was applied to cellulose sulphates (CS). The degree of substitution (ds) ranged from 0.52 to 1.37. Problems arose under the basic conditions of the methylation step. Cellulose sulphates with a high degree of substitution at C-2 showed significant loss of sulphate; samples with predominantly C-6 substitution gave good results in agreement with ^{13}C NMR data and elemental analysis. In this paper reasons for this behaviour and the development of an appropriate procedure are presented. © 1997 Elsevier Science Ltd

INTRODUCTION

Naturally occurring sulphated polysaccharides are predominantly found in algae, e.g. agar, carrageenans or fucoidanes, or as glycoproteins in human tissue like heparin, heparan sulphate, chondroitin sulphate, dermatan sulphate or keratan sulphate. Some of the latter show anticoagulating or antithrombinic activity which can be increased by additional sulphation (Ludwig-Baxter *et al.*, 1991). Attempts have been made to prepare new sulphated polysaccharides, for example to produce water-soluble bioactive compounds (Williams *et al.*, 1992). Some groups have reported anti-HIV activity of cellulose sulphates (Yamamoto *et al.*, 1991) which is related to a certain substitution pattern. The polymers can be analysed by ^{13}C NMR, which is a rapid and nondestructive method to determine the partial ds values of the OH positions in the monomer unit (Hunt and Huckerby, 1980; Lahaye and Yaphe, 1985; Nehls *et al.*, 1985; Philipp *et al.*, 1987; Miyano *et al.*, 1992). However, by this method no differentiated monomer analysis is possible. In this paper methylation analysis is applied to cellulose sulphates. This method allows an exact description of the substitution pattern in the anhydroglucose unit (AGU) of cellulose sulphates. Stevenson and Furneaux (1991) analysed sulphated galactans from red algae. Kovensky *et al.* (1990) used methylation analysis for elucidation of the structure of heparin, heparan sulphate and some other sulphated (1→3)-β-D-glucans and found that some sulphate loss occurred under the strong basic conditions of the methylation step. Nevertheless, the authors thought this method appropriate for the estimation of sulphated positions in polysaccharides. In contrast, methylation analysis applied to cellulose sulphates poses more problems. The backbone consists of (1→4)-linked glucose units with adjacent, *trans*-hydroxyl groups. Basic methylation of a mono-2-sulphated or mono-3-sulphated AGU can form 2,3-epoxide structures (Percival, 1949). The optimization of methylation analysis is discussed in this paper.

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EXPERIMENTAL

General

Cellulose sulphates were prepared at the Fraunhofer Institut für Angewandte Polymerforschung (Teltow, Germany) (Philipp *et al.*, 1989; Wagenknecht *et al.*, 1993) under homogeneous conditions in N_2O_4/DMF with SO_3 (CS 1 and 3) or SO_2 (CS 2) as the sulphating agent.

All reagents and solvents (p.a. quality) were purchased from E. Merck (Darmstadt, Germany). Sephadex LH20 was obtained from Pharmacia (Uppsala, Sweden).

Ion exchange

An aqueous solution of cellulose sulphate sodium salt (20 mg/ml) was passed over an Amberlite IR 120 ion exchange column in the triethylammonium form. The eluted solution was freeze dried. Elemental analysis

showed a complete exchange of the sodium ions for triethylammonium ions. Depending on the ds of the material the products were flaky (ds 0.35–1) or glassy viscous (ds 1–1.8).

Permethylation (optimized conditions)

Cellulose sulphate triethylammonium salt (20 mg) was dissolved in a mixture of dry DMSO/1,1,3,3-tetramethylurea (TMU) (2 ml, 4:1, v/v). After addition of a molar sieve (150 mg, 3 Å) complete dissolution was achieved after 2 days. The solution was separated from the molar sieve under a stream of nitrogen. Deprotonation was achieved by addition of a 1.6 M solution (15 eq/AGU) of lithium methyl sulphinyl methanide (prepared from etheric 1.6 M methyl lithium solution and DMSO (1:1, v/v)) for 3 min. The reaction mixture was frozen in an ice bath, treated with methyl iodide (30 eq/AGU) and stirred at room temperature for 2 h. Excess methyl iodide was then removed *in vacuo*. The reaction steps of deprotonation and methylation were repeated again for CS with low partial ds at C-2. After evaporation of excess methyl iodide the methylated cellulose sulphate was obtained by gel chromatography on a Sephadex LH 20 column (40 g) with methanol as the eluent. All methylated samples were soluble in methanol and gave a clear film after evaporation of the solvent.

Hydrolysis, reduction and acetylation

Methylated cellulose sulphate (2 mg) was placed in a 1 ml reacti-vial (Wheaton) with a screw cap and hydrolysed with 2 M TFA (1 ml) for 2 h at 120°C. After removal of the aqueous acid in a stream of nitrogen, toluene was added (1 ml) and the remaining traces of acid were codistilled. Reduction at C-1 was achieved with 0.5 ml of an aqueous NH_3 solution (0.5 M) of $NaBD_4$ for 1 h at 60°C. Excess deuteride was removed by the addition of three drops of glacial acetic acid. Removal of borate was achieved by addition of methanolic acetic acid (15%) and evaporation to dryness five times in a stream of nitrogen. Acetylation of hydroxyl groups was accomplished with acetic anhydride (250 µl) and pyridine (20 µl) for 3 h at 95°C. The reaction mixture was carefully added to 10 ml of a saturated aqueous solution of $NaHCO_3$. When carbon dioxide production had ceased the solution was extracted five times with 2 ml dichloromethane. The organic phase was dried, concentrated to 2 ml and analysed by GLC and GLC-MS.

GLC AND GLC-MS

GLC was carried out on a Carlo Erba GC6000 Vega Series 2 gas chromatograph equipped with an on-column injection system, a Chrompack CP-Sil 8 CB capillary column (25 m×0.25 mm) and a 2 m retention gap, a flame ionization detector, and a Merck-Hitachi D-2500 Chromato-Integrator. Hydrogen was used as carrier gas (80 kPa). Temperature program: 70°C for 1 min, then increased at 20°C/min to 130°C, and at 4°C/min to 290°C. Peak areas were corrected using the effective carbon response concept according to Sweet *et al.* (1975). Mass spectra were obtained with a VG Analytical VG/70-250S instrument. For CI-MS, ammonia was used as the reactant gas.

RESULTS AND DISCUSSION

The application of methylation analysis on cellulose sulphates includes a step of solubilization of the sodium salts in DMSO before methylation. Stevenson and Furneaux (1991) achieved DMSO solubility of a sulphated galactan from red algae by cation exchange in the pyridinium or triethylammonium form. The latter method was successfully applied to cellulose sulphates by using an ion exchange column, which is superior to dialysis. Methylation was carried out by a modified Hakomori method with lithium methylsulphinylmethanide (lithium dimsyl) and methyl iodide (D'Ambra *et al.*, 1988). At the beginning of the study, methylation was carried out three times with a deprotonation time of 2 h. The cellulose samples with sulphate groups mainly at C-6 gave good and reproducible results in agreement with the ^{13}C NMR data (Figs 1 and 2 and Table 1). Figure 3 shows a gas chromatogram of a reaction mixture after permethylation, hydrolysis, reduction and acetylation. Problems arose from the sample CS 1 which had a high sulphate ds at C-2. The results of the analyses were not constant and showed a significant loss of sulphate at position C-2 (see Fig. 4). Percival (1949) described the

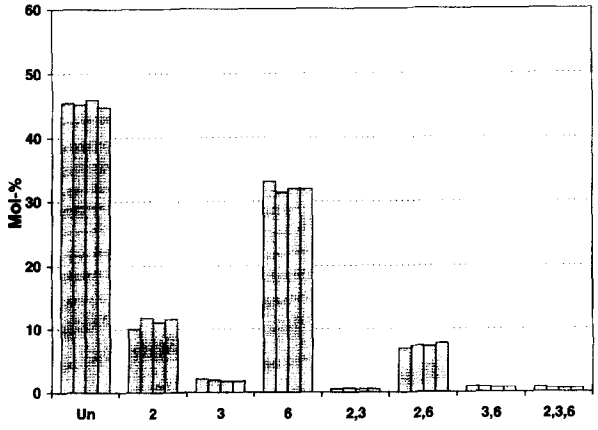


Fig. 1. Substitution pattern of CS 2 (ds 0.64). Two independent methylations and subsequent double analysis, performed with the optimized method.

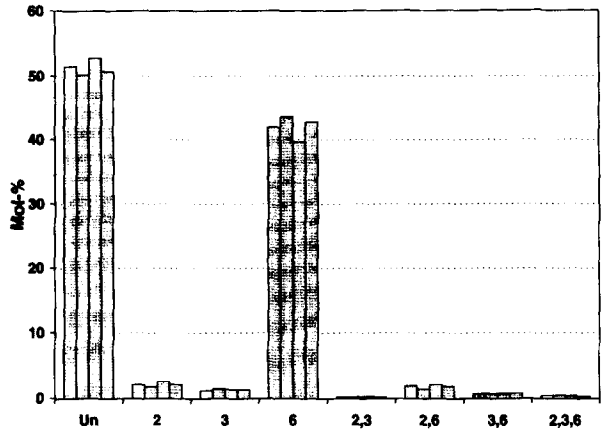


Fig. 2. Substitution pattern of CS 3 (ds 0.52). Two independent methylations and subsequent double analysis, performed with the optimized method.

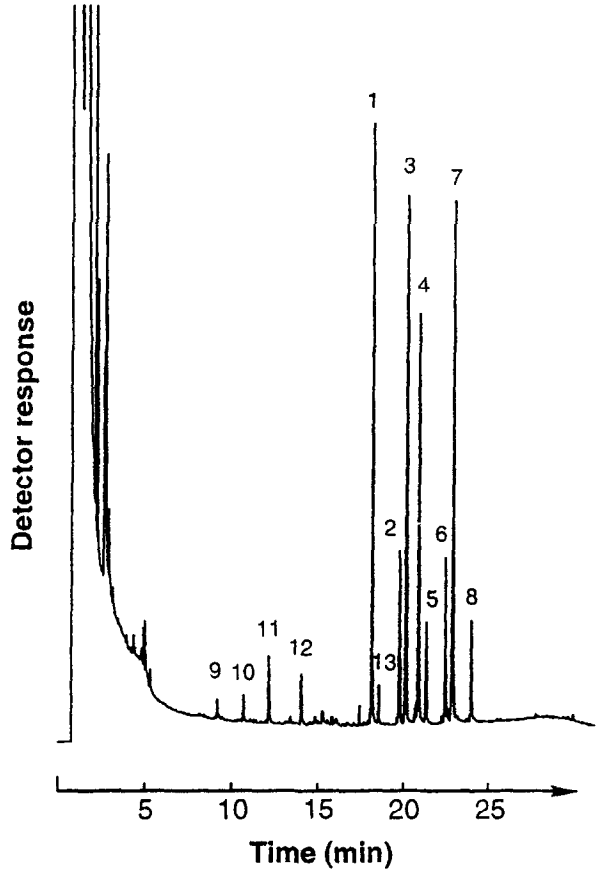


Fig. 3. Gas liquid chromatogram of the partially methylated glucitol acetates derived by standard methylation analysis of cellulose sulphates. The peaks were numbered according to the compound numbers in the footnote in Table 1. For GLC conditions see Experimental section.

intramolecular nucleophilic displacement of sulphate groups under formation of 2,3-oxirane residues from monosaccharides with a sulphate group adjacent to a *trans*-hydroxyl group under basic conditions.

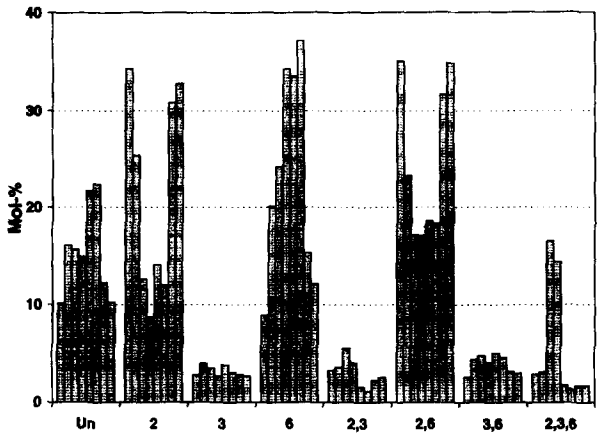


Fig. 4. Substitution pattern of CS 1 (ds 1.28). Four independent methylations and subsequent double analysis, performed without optimization of the methylation method.

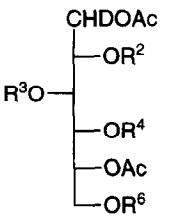
Obviously the constitution and stereochemistry of cellulose sulphates are responsible for this undesirable side reaction. The decrease of the ratio of 2-sulphation corresponds to an increase in the calculated amount of unsubstituted AGU, while the displacement of the 2-sulphate group from 2,6-disubstituted AGU results in the respective deviation of the apparent 6-sulphation. This result implies an epoxide ring opening during the methylation and subsequent methylation of both positions, C-2 and C-3, in the presence of hydroxide ions (see Scheme 1). The base mediated ring opening of oxiranes should lead to two epimeric sugars. However, Rej and Perlin (1990) observed that the 2,3-anhydro sugar formed from α-L-iduronic acid 2-sulphate during treatment with alkali was regioselectively opened back to the *ido* configuration with strong base at room temperature, while hot sodium carbonate gave the *galacto*-diastereomer. In the case of cellulose sulphate, this may explain the absense of *altro* derivatives in the hydrolysed products that showed significant sulphate loss. Lyophilization of the triethylammonium salt left up to 15% of water in the cellulose sulphate, which obviously favoured the loss of sulphate groups. Therefore, the solution in DMSO was dried before methylation. Another point of interest was the time of the deprotonating reaction. According to Narui *et al.* (1982) the intermolecular and intramolecular hydrogen bonds can be broken by addition of 1,1,3,3-tetramethylurea to the DMSO solution of the polysaccharide, which favours the methylation at the less reactive C-3 position. In this way complete methylation of mainly C-6 sulphated cellulose could be achieved after two short reaction steps without loss of sulphate groups. CS 1 with a high ds at C-2 showed sulphate loss at this position after double methylation, which may be a result of the formation of $-OSO_3Me$ and therefore a better leaving group for the side reaction described above. A fully alkylated product was obtained by one methylation, in contrast to CS 2

Table 1. Relative molar composition (mol %), ds values, ^{13}C NMR data and elementary analysis data (EA) of cellulose sulphates CS 1, CS 2 and CS 3. Average (A) and standard deviation (B) of two independent optimized methylations and subsequent double analysis (CS 1: four independent optimized methylations and double analysis)

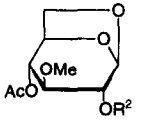
Sulphate position	Compound number	CS 1 (mol %)		CS 2 (mol %)		CS 3 (mol %)	
		A	B	A	B	A	B
—	**1**[a]	14.8	±1.9	45.4	±0.5	51.3	±1.1
2	**3**	23.7	±2.6	11.1	±0.9	2.2	±0.4
3	**2**	4.5	±0.7	2.0	±0.2	1.3	±0.1
6	**4+9+10+13**	17.7	±1.8	32.2	±0.8	42.0	±1.7
2,3	**5**	2.9	±0.3	0.5	±0.1	0.2	±0.0
2,6	**7+11+12**	27.7	±3.1	7.3	±0.3	1.8	±0.3
3,6	**6**	4.8	±0.7	0.8	±0.1	0.7	±0.0
2,3,6	**8**	3.8	±1.2	0.7	±0.1	0.4	±0.1
	Unsubst.	14.8	±1.9	45.4	±0.5	51.3	±1.1
	Monosubst.	45.9	±1.0	45.3	±0.3	45.5	±1.4
	Disubst.	35.4	±2.3	8.6	±0.3	2.8	±0.3
	Trisubst.	3.8	±1.2	0.7	±0.1	0.4	±0.1
	ds(2)	0.58	±0.05	0.20	±0.01	0.05	±0.01
	ds(3)	0.16	±0.02	0.04	±0.00	0.03	±0.00
	ds(6)	0.54	±0.01	0.41	±0.01	0.45	±0.01
	Σ ds	1.28	±0.03	0.64	±0.01	0.52	±0.01
	^{13}C NMR						
	ds(2)	0.73		0.19		0.12	
	ds(6)	0.56		0.39		0.43	
	Σ ds	1.29		0.58		0.55	
	EA						
	Σ ds	1.31		0.63		0.55	

[a]Structures:

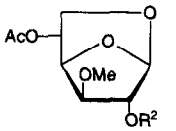
1 R^2, R^3, R^6 = Me, R^4 = Ac
2 R^2, R^6 = Me, R^3, R^4 = Ac
3 R^3, R^6 = Me, R^2, R^4 = Ac
4 R^2, R^3 = Me, R^4, R^6 = Ac
5 R^6 = Me, R^2, R^3, R^4 = Ac
6 R^2 = Me, R^3, R^4, R^6 = Ac
7 R^3 = Me, R^2, R^4, R^6 = Ac
8 R^2, R^3, R^4, R^6 = Ac
13 R^2, R^3, R^4 = Me, R^6 = Ac

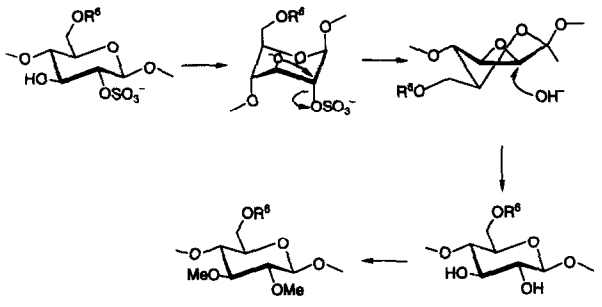


9 R^2 = Me
11 R^2 = Ac



10 R^2 = Me
12 R^2 = Ac





Scheme 1.

and CS 3, which required double methylation. The solubility of the methylated products in methanol after concentration by gel chromatography on Sephadex LH20 gives an advantage over dialysis against water, which led to completely insoluble material. This was due to an exchange of the triethylammonium ion for inorganic ions in tap water, as can be seen in the IR spectra of the dialysed products without the characteristic peaks of the triethylammonium ion. The ^{1}H NMR of the chromatographed product showed the presence of the triethylmethylammonium ion. Elementary analysis also proved the presence of nitrogen (N/S ratio 0.65). After acid hydrolysis of the glucosidic bonds and the sulphate esters the monomer mixture is reduced and acetylated to achieve partially methylated glucitol acetates for GLC- and GLC-MS analysis (see Fig. 3). This reaction pathway bears the information of the sulphate substitution pattern in an inverse way; all positions that had originally been sulphated were now acetylated. The mass spectra of the glucitol derivatives **1–8** (see footnote in Table 1) and **13** correspond to those in the literature (Björndal *et al.*, 1970; Jansson *et al.*, 1976). The compounds **9–12** are 1,6-anhydroglucopyranoses and -furanoses which are formed during acid hydrolysis of the polymer (Mischnick *et al.*, 1995). **9** and **11** are derived from the 6-sulphated AGU, **10** and **12** from the 2,6-disulphated AGU. The peak areas of these compounds are added to the corresponding glucitol derivatives when calculating the ds. Figure 5 shows the substitution pattern of CS 1 after optimization of the methylation. In fact, the hydrolysis step also causes deviations of the product composition (two adjacent columns refer to one methylation). This can be overcome by using the reductive hydrolysis of Stevenson and Furneaux (1991), especially for CS with high ds at C-2.

CONCLUSIONS

Methylation analysis is shown to be a convenient method for the elucidation of the substitution pattern of cellulose sulphates. DMSO-solubility of the sodium salts was achieved by ion exchange to the

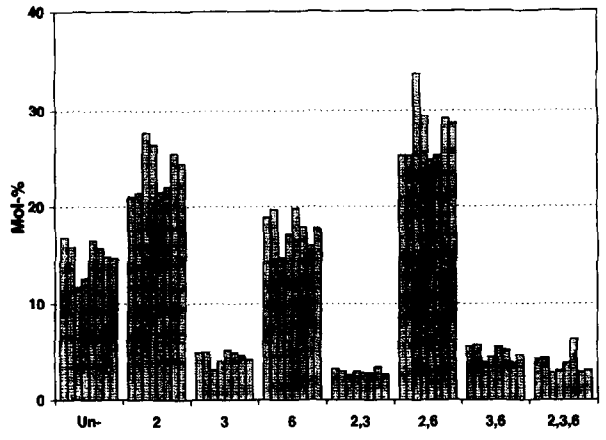


Fig. 5. Substitution pattern of CS 1 (ds 1.28). Four independent methylations and subsequent double analysis, performed with the optimized method.

triethylammonium salt. The base lability of a sulphate substituent adjacent to a free *trans* hydroxyl group required optimization of the deprotonation step, which could be reduced to 3 min in tetramethylurea/DMSO as the solvent. Drying of the polymer solution was also essential. Under the optimized conditions the monomer composition could be sufficiently determined. Results were in good agreement with ^{13}C NMR data and elemental analysis.

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