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# Note

## An n.m.r. study of the products of oxidation of cellulose and (1→4)-β-D-xylan with sodium nitrite in orthophosphoric acid

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Oxidation of polysaccharides with sodium nitrite in orthophosphoric acid converts a substantial portion of the monosaccharides having primary hydroxyl groups unsubstituted into uronic acids[1–7]. The extent of oxidation at secondary positions, particularly position 3 in dextran, was high when the concentration of the polysaccharide in the orthophosphoric acid was low[8]. Oxidation of a (1→4)-β-D-xylan with aqueous bromine yielded a product with a high proportion of ulose residues[9].

However, because of their sensitivity towards acid and alkali, determination of the quantity and type of ulose residues in oxidised polysaccharides is difficult and, hitherto, the resolution of n.m.r. spectra studies has not been adequate for this purpose[8–10]. We now report on the oxidation of cellulose and a (1→4)-β-D-xylan with sodium nitrite in orthophosphoric acid and the 2D 400-MHz $^1$H-n.m.r. spectroscopy of the products.

The xylan was obtained from retted jute sticks and contained D-xylose (89%), L-rhamnose (1%), D-galactose (trace), D-glucose (trace), and 4-*O*-methylglucuronic acid (10%) 2-linked to xylose[11–12]. The xylan and cellulose were each oxidised with sodium nitrite in orthophosphoric acid (20 mL/g of polysaccharide)[1] to give oxidised xylan and oxidised cellulose I, respectively. In an attempt to increase oxidation at secondary positions, cellulose was treated with sodium nitrite in orthophosphoric acid (30 mL/g of cellulose) which yielded oxidised cellulose II. Increase in the proportion of orthophosphoric acid caused an increase in the cleavage of the cellulose and xylan chains. Borohydride reduction of the oxidised polysaccharides, followed by sugar analysis[13], indicated the position of the keto groups. The formation of lyxitol (arabinitol) and ribitol from the oxidised xylan indicated that oxidation had occurred at positions 2 or 3 of the xylose residues, whereas the formation of mannitol from the

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oxidised cellulose indicated oxidation at position 2 of the glucose residues. Treatment of the oxidised xylan with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) followed by borohydride reduction[14] and sugar analysis yielded no 4-*O*-methylmannitol or 4-*O*-methylallitol, which indicated that no ulose derivatives of 4-*O*-methylglucuronic acid were present in the oxidised xylan. Treatment of the oxidised celluloses with EDC followed by reduction[14] yielded the same, or a slightly higher, ratio of mannose to glucose in the sugar analysis compared to the results obtained on borohydride reduction, which indicated that oxidation at position 6 did not prevent oxidation at position 2.

The proportion and positions of hydrated keto groups and other functional units were obtained by 1D and 2D $^{13}C$- and $^{1}H$-n.m.r. spectroscopy. The H-1 signals were assigned by correlation with H-2 in a 2D-COSY experiment. The lack of a correlation peak for H-1 indicated oxidation at position 2 or cleavage of the C-2 – C-3 bond. The relay COSY technique was used to identify residues that had been oxidised at position 3. Assignment of the $^{13}C$ signals was based on 2D heteronuclear correlated experiments, and $C(OH)_2$ groups were distinguished from C-1 by the multiplicity determined by a DEPT experiment. For purposes of comparison, the $^{1}H$-n.m.r. spectra of D-glucuronic acid, cellobiose, cellobiuronic acid, xylose, methyl α- and β-D-xylopyranoside, and the unoxidised xylan were recorded. The $^{1}H$- and $^{13}C$-n.m.r. data are recorded in Tables I and II, respectively.

In the region for anomeric protons, oxidised cellulose I had signals at 4.75 and 5.35 p.p.m. (weak) together with signals from α- and β-D-glucopyranose (or glucuronic acid) at the reducing ends. The signal at 4.75 p.p.m. was assigned to H-1 of a hydrated form of a 2-ulose residue. The signal at 5.35 p.p.m. had no correlation peak in a COSY experiment. The low chemical shift of this signal indicated a keto form of a 2-oxidised residue, although this form could not be detected in the corresponding $^{13}C$-n.m.r. spectrum (see below). A broad signal from H-1 of the main-chain residues, most of them oxidised to glucuronic acids, was also present in this region. Oxidised cellulose II gave no peak at 5.35 p.p.m. However, a peak corresponding to H-1 of another hydrated 2-ulose residue, probably in a different chemical environment, appeared at 4.77 p.p.m. (*cf.* the spectrum of the oxidised xylan below). The signals for H-1 of α- and β-D-glucopyranose (or glucuronic acid) residues were more intense in this product, which indicated more extensive degradation. Other weak H-1 signals, given only by oxidised cellulose II, possibly originated from ring-cleaved residues. No terminal gluconic acid residues could be detected in oxidised celluloses I and II.

In addition to the signal (103.2 p.p.m.) of C-1 of the main-chain residues, signals at 94.9 and 92.4 p.p.m. which corresponded to C-1 and C-2, respectively, of a hydrated 2-ulose residue were present in the $^{13}C$-n.m.r. spectrum of the oxidised cellulose I. No $^{13}C$ signals for COOH, other than that for uronic acid residues, were detected. No signals for a 2-keto residue could be detected. For oxidised cellulose II, $^{13}C$ signals corresponding to $C(OH)_2$ of two different hydrated 2-ulose residues were present. These signals were correlated with H-1 signals and probably originated from 2-ulose hydrates in different chemical environments. Weak $^{13}C$ signals for COOH indicated the presence of ring-cleaved residues, in agreement with the corresponding $^{1}H$-n.m.r. spectrum.

TABLE I

$^1$H-N.m.r. data ($\delta$, p.p.m.)

| | *H-1* | *H-2* | *H-3* | *H-4* | *H-5* | *H-5'* |
|---|---|---|---|---|---|---|
| *Oxidised cellulose I* | | | | | | |
| (1→4)-β-Glc | 4.51 | 3.32 | | | | |
| (1→4)-β-*ara*-2-Ulose | 4.75 | | | | | |
| | 5.35 | | | | | |
| (1→4)-β-GlcA | 4.55 | 3.38 | 3.63 | 3.71 | 3.87 | |
| β-D-Glc[a] (terminal) | 4.65 | 3.32 | | | | |
| α-D-Glc[a] (terminal) | 5.23 | 3.62 | | | | |
| [a] Probably uronic acids | | | | | | |
| *Oxidised cellulose II* | | | | | | |
| (1→4)-β-*ara*-2-Ulose | 4.77 | | | | | |
| D-*Glucuronic acid* | | | | | | |
| α | 5.23 | 3.57 | | | | |
| β | 4.62 | 3.28 | | | | |
| *Cellobiose* | | | | | | |
| α-D-Glc (terminal) | 5.23 | overlap | | | | |
| β-D-Glc (terminal) | 4.66 | 3.30 | | | | |
| β-D-Glc (internal) | 4.52 | 3.34 | | | | |
| *Cellobiuronic acid* | | | | | | |
| α-D-Glc (terminal) | 5.22 | overlap | | | | |
| β-D-Glc (terminal) | 4.66 | 3.29 | | | | |
| β-D-GlcA (internal) | 4.58 | 3.40 | | | | |
| *Oxidised jute xylan* | | | | | | |
| (1→4)-β-Xyl | 4.49 | 3.33 | 3.58 | 3.79 | 4.12 | 3.43 |
| (1→4)-β-*Xyl*-2-GlcA | 4.64 | 3.49 | | | | |
| 4-*O*-Me-β-GlcA | 5.26 | 3.59 | | | | |
| (1→4)-β-*threo*-2-Ulose | 4.74 | | | | | |
| | 4.96 | | | | | |
| α-Xyl (terminal) | 5.18 | 3.57 | | | | |
| β-Xyl (terminal | 4.59 | 3.27 | | | | |
| *Jute xylan* | | | | | | |
| (1→4)-β-Xyl | 4.49 | 3.33 | 3.59 | 3.80 | 4.13 | 3.41 |
| (1→4)-β-*Xyl*-2-*O*-GlcA | 4.65 | | | | | |
| 4-*O*-Me-β-GlcA | 5.26 | | | | | |
| *Xylose* | | | | | | |
| α-D-Xyl | 5.18 | | | | | |
| β-D-Xyl | 4.57 | 3.24 | 3.44 | 3.63 | 3.94 | 3.32 |
| α-D-Xyl-OMe | 4.79 | | | | | |
| β-D-Xyl-OMe | 4.32 | 3.29 | 3.47 | 3.64 (Me 3.55) | 3.98 | 3.33 |

TABLE II

$^{13}$C-N.m.r. data ($\delta$, p.p.m.)

| | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* |
|---|---|---|---|---|---|---|
| *Oxidised cellulose I* | | | | | | |
| (1→4)-*β*-Glc | 103.2 | | | | | 63.3 |
| (1→4)-*β-ara*-2-Ulose | 94.9 | 92.4 | | | | |
| (1→4)-*β*-GlcA | 103.0 | 73.5 | 74.9 | 81.4 | 76.1 | 175.7 |
| *β*-D-Glc[a] (terminal) | 96.6 | | | | | |
| α-D-Glc[a] (terminal) | 92.7 | | | | | |
| [a]Probably uronic acids | | | | | | |
| *Oxidised cellulose II* | | | | | | |
| (1→4)-*β-ara*-2-Ulose | 94.3 | 94.4 | | | | |
| | 94.9 | 92.4 | | | | |
| *Oxidised jute xylan* | | | | | | |
| (1→4)-*β*-Xyl | 102.2 | 73.2 | 74.2 | 76.9 | 63.5 | |
| (1→4)-*β-Xyl*-2-GlcA | 101.9 | 82.9 | | | | |
| 4-*O*-Me-*β*-GlcA | 98.1 | | | (Me 60.4) | | |
| (1→4)-*β-threo*-2-Ulose | 95.4 | 92.7 | | | | |
| | 94.6 | 92.8 | | | | |
| α-Xyl (terminal) | 92.5 | | | | | |
| *β*-Xyl (terminal) | 97.0 | | | | | |
| *Jute xylan* | | | | | | |
| (1→4)-*β*-Xyl | 102.3 | 73.3 | 74.2 | 76.9 | 63.6 | |
| (1→4)-*β-Xyl*-2-*O*-GlcA | 102.0 | 83.0 | | | | |
| 4-*O*-Me-*β*-GlcA | 98.1 | | | | | |

The signals for H-1 at 4.74 and 4.96 p.p.m. of the oxidised xylan (Fig. 1) were not present in the spectrum of unoxidised xylan and were assigned to H-1 of hydrated 2-ulose residues in different chemical environments. Other weak signals in this region probably originated from ring-cleaved residues. Signals close to that of H-1 in an unoxidised residue were assigned to other unoxidised residues in different chemical environments. No 3-oxidised residues could be detected by the relay COSY-technique.

The $^{13}$C-n.m.r. spectrum of the oxidised xylan contained signals that correspond to two $C(OH)_2$ groups and C-1 of 2-ulose residues (*cf.* oxidised cellulose II). These C-1 resonances were correlated with H-1 signals by a 2D heteronuclear correlation experiment. Other weak signals in this region probably originated from C-1 of other oxidised residues or ring-cleaved residues. There were several signals for C-1 of unoxidised residues, which probably reflected different chemical environments. A signal for C-6 of a 4-*O*-methylglucuronic acid residue was present also, but no signals from oxidised 4-*O*-methylglucuronic acid residues were found.

The proportions of ulose, glucuronic acid, and reducing end residues in the oxidised polysaccharides were calculated from the integrated $^1$H-n.m.r. spectra (Table III). Only small proportions of ulose residues were present in the products obtained by

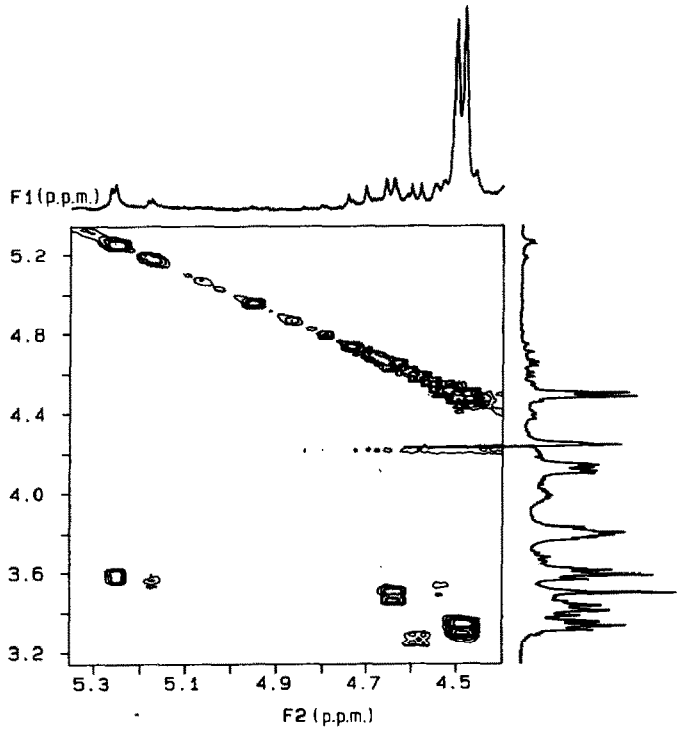


Fig. 1. $^1H$–$^1H$ COSY spectrum of oxidised xylan, showing the H-1 region versus the region 5.3–3.2 p.p.m. All cross-peaks are correlations between H-1 and H-2. A lack of cross-peak for H-1 indicated a 2-oxidised unit.

oxidation of xylan and cellulose with sodium nitrite/orthophosphoric acid. The polysaccharides were degraded to some extent when the proportion of phosphoric acid was high, which was reflected in an increased proportion of reducing end residues. This result contrasts with those for the oxidation of dextran with sodium nitrite/orthophosphoric acid, where high proportions of ulose residues were formed and there was negligible degradation.

In dextran, oxidation occurs preferentially at positions 3, probably because the steric interaction of H-3 and the axial aglycon is then removed. The preferential oxidation at positions 2 in $\beta$-glycans is probably a consequence of the greater ease of esterification of HO-2 compared to HO-3,4. The formation of ribitol from the borohydride-reduced oxidised xylan indicated that some oxidation had occurred at positions 3. However, this conclusion was not verified by the n.m.r. spectra. Either the ribose resulted from rearrangement of 2-ulose residues during the reduction, or the proportion of 3-ulose residues was too small to be detected by n.m.r. spectroscopy.

Thus, 2D-n.m.r. spectroscopy in the homo- and hetero-nuclear modes is a useful method in the study of ulose residues in polysaccharides, even though the spectra may be rather complex when similar residues have different chemical environments.

TABLE III

Proportions[a] (%) of the modified sugars in the oxidised polysaccharides

| | *Modified sugars* | | |
|---|---|---|---|
| | *2-Ulose* | *Glucuronic acid* | *Reducing end* |
| Oxidised cellulose I | 7 | 75 | <1 |
| Oxidised cellulose II | 10 | 79 | 11 |
| Oxidised xylan | 9 | | 12 |

[a] Determined by integration of the respective $^1$H-n.m.r. spectra.

## EXPERIMENTAL

*General methods.* — Solutions were concentrated under reduced pressure <40°. Optical rotations were measured with a Perkin–Elmer model 141 polarimeter. G.l.c. was performed on a Packard 427 instrument. Peak areas were measured with an Autolab Minigrator. Separations were performed on OV-275 quartz capillary columns (25 × 0.3 cm) at 160 → 200° (4°/min). For g.l.c.–m.s., Finnegan 4021 equipment was used. E.i. mass spectra were obtained at 70 eV and the helium flow rate was 25 mL/min. The proportions of carboxyl and carbonyl groups were measured by $^1$H- and $^{13}$C-n.m.r. spectroscopy (see below).

*N.m.r. methods.* — N.m.r. spectra were recorded with a Varian VXR 400 instrument; $^1$H at 400 MHz on solutions (3 mg/mL) in $D_2O$ at 85° with internal sodium 3-trimethylsilylpropanoate-$d_4$ (for some spectra, a 180°–$t_1$–90° pulse sequence was used to suppress the water-peak); $^{13}$C at 101 MHz for solutions (10 mg/mL) in $D_2O$ at room temperature with internal 1,4-dioxane (67.40 p.p.m.), using an inverse gated decoupling technique (45° r.f. pulse, 4.5-s pulse delay) in order to suppress n.O.e. (for quantification of uronic acids). 2D-N.m.r. spectroscopy was performed with standard COSY, relayed COSY, double-relayed COSY, and heteronuclear correlated C/H pulse sequences. The COSY experiments were performed with (90°–$t_1$–90°–$t_2$) for higher sensitivity.

*Oxidation of xylan.* — Jute xylan (500 mg) was suspended in aqueous 85% orthophosphoric acid (10 mL) at room temperature by grinding in a large mortar. Finely powdered sodium nitrite (1.0 g) was added with vigorous stirring during 10 min. The mixture was kept at room temperature without disturbing the foam that was formed. After 2 h, more (1.0 g) sodium nitrite was added with thorough mixing. After a further 2 h, the residue was extracted with ice-cold ether (4 × 25 mL). Water (20 mL) was added to the residue, the pH of the resulting solution was adjusted with M NaOH to 5.0, and the solution was dialysed against distilled water (4 × 4 L) and freeze-dried to give the oxidised xylan (0.39 g), $[\alpha]_{578}^{20}$ −36° (*c* 0.4, water). The carboxylic acid content was 0.1/sugar residue. The oxidised xylan (10 mg) was reduced conventionally with sodium borohydride (10 mg), and the product was dialysed against distilled water and freeze-dried. Sugar analysis of the product revealed lyxose, ribose, and xylose.

*Oxidation of cellulose.* — Cellulose (Whatman, 500 mg) was oxidised and processed, as described above for the xylan, to give oxidised cellulose I (0.47 g), $[\alpha]_{578}^{20}$ −35° (*c* 0.8, water). The proportion of uronic acid residues was 75%.

Cellulose (500 mg) was dissolved in aqueous 85% orthophosphoric acid (15 mL), oxidised with sodium nitrite (2 × 1.0 g), and processed as described above to give oxidised cellulose II (0.48 g), $[\alpha]_{578}^{20}$ −19° (*c* 0.9, water). The proportion of uronic acid residues was 79%.

Sugar analysis of the reduced oxidised celluloses revealed mannose and glucose.

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