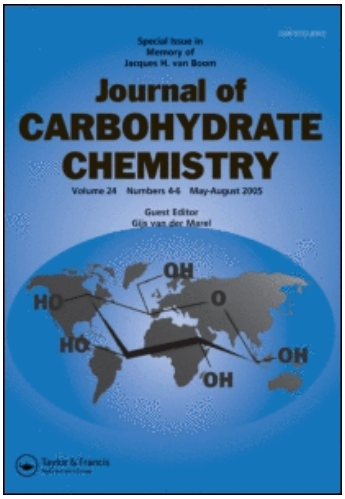

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4-*O*-β-D-GLUCOPYRANOSYL-D-GLUCONIC ACID (CELLOBIONIC ACID) PRODUCED BY OZONATION OF CELLOBIOSE: ISOLATION BY HPLC AND ASSIGNMENT OF NMR CHEMICAL SHIFTS

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ABSTRACT

Mild ozonation of cellobiose in acetic acid almost exclusively favors oxidation of the reducing end, thus yielding 4-*O*-β-D-glucopyranosyl-D-gluconic acid (''cellobionic acid''). This compound can be quantitatively separated from the substrate by preparative HPLC using acetonitrile/phosphate buffer on an aminopropyl silica column. The product was identified using one and two dimensional NMR techniques. ^{13}C and ^{1}H NMR chemical shifts are reported.

Key Words: Cellulose; Cellobiose; Glucose; Ozonation; Ozone; Bleaching; Pulp; Oxidation; Reducing end; Cellobionic acid; Aldonic acid; NMR spectroscopy; 2D-NMR; Chemical shift; HPLC; High pressure liquid chromatography

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INTRODUCTION

Due to its high oxidative power, ozone has gained widespread use as a powerful bleaching agent in the manufacture of bleached paper pulp by totally chlorine free bleaching sequences. Ozone is also used extensively in water treatment and purifcation. Mechanistic studies of reactions between ozone and cellulose have involved the use of several water soluble model compounds, e.g. glucose,[1,3–5] methyl glucoside,[1,6–11] 1,5-anhydrocellobiitol[10] and cellobiose.[1,3,4] Such studies have indicated a strong preference for oxidation at the reducing end under certain reaction conditions, but also chain cleavage and the introduction of carbonyl groups by oxidation of secondary hydroxyl groups have been reported.

In this paper, we report on the ozonation of cellobiose (4-*O*-β-D-glucopyranosyl-D-glucopyranose, CAS no. 528-50-7) under reaction conditions almost exclusively favoring oxidation of the reducing end, yielding 4-*O*-β-D-glucopyranosyl-D-gluconic acid (''cellobionic acid,'' CAS no. 534-41-8). The product is purified using preparative HPLC and subsequently identified by one and two dimensional NMR techniques.

RESULTS AND DISCUSSION

NMR Spectroscopic Analysis of the Reaction Mixture

The one dimensional ^{13}C NMR spectrum of ozonated cellobiose is shown in Figure 1. In addition to the 18 signals easily identifiable as cellobiose, a number of new signals were observed in the carbohydrate region, and one new signal was observed in the carboxyl region. The proximity of some of the new signals to signals arising from the non-reducing end of cellobiose indicated the presence of a C1-substituted glucose unit. Also, the new signal in the carboxyl region and the absence of non-carbohydrate type peaks indicated the presence of an acidic sugar. It has been reported in the literature that ozonation of cellobiose yields, among other products, 4-*O*-β-D-glucopyranosyl-D-gluconic acid (''cellobionic acid'')[1] and D-gluconic acid.[1–4] It was therefore assumed that the new peaks in the ^{13}C NMR spectrum of ozonated cellobiose arose from either D-gluconic acid or cellobionic acid. However, a purified reference cellobionic acid could not be acquired for reference purposes, and reference NMR spectra of cellobionic acid could not be found in the literature.

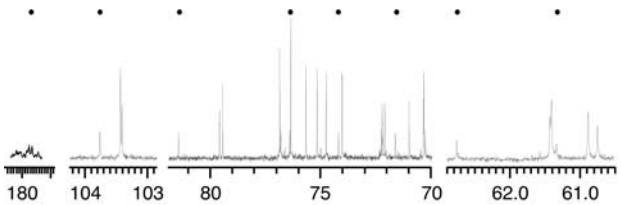

Figure 1. ^{13}C NMR spectrum of cellobiose after ozonation. The dots indicate signals not present in the spectrum of untreated cellobiose.

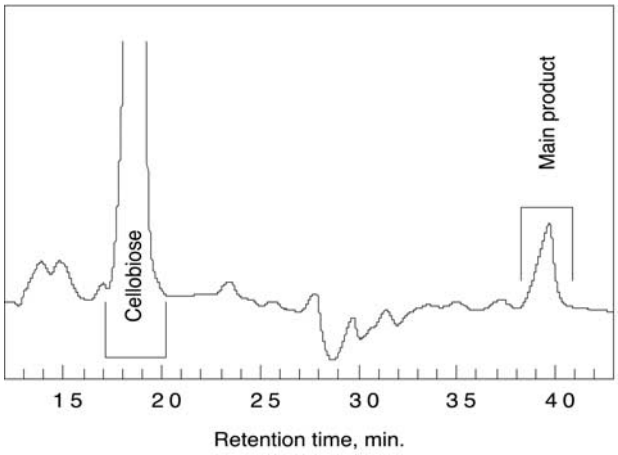

Figure 2. Preparative HPLC chromatogram of ozonated cellobiose.

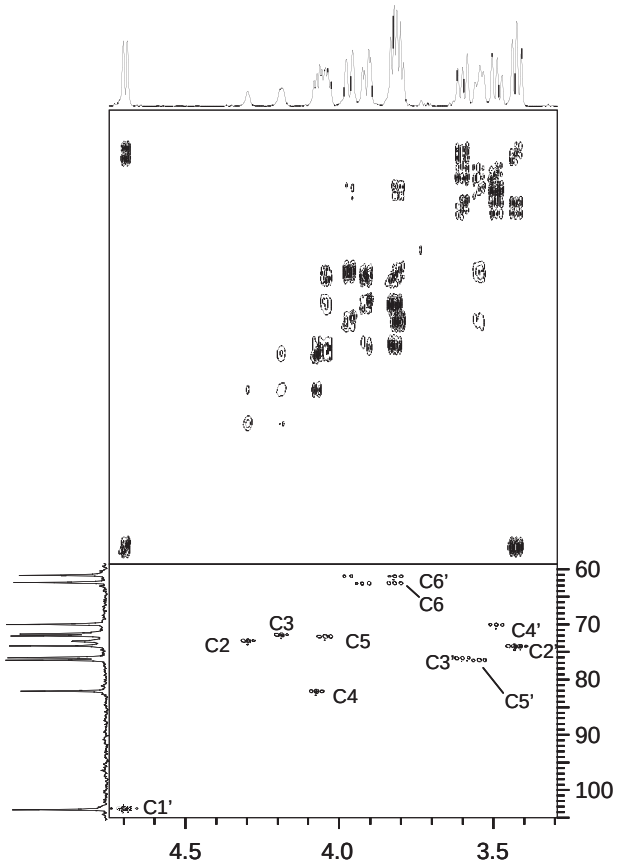

Figure 3. Two dimensional NMR spectra of cellobiose. Top: homonuclear shift-correlation (COSY) ^{1}H spectrum, bottom: heteronuclear shift-correlation (HSQC) $^{1}H-^{13}C$ spectrum.

Table 1. ^{1}H NMR Chemical Shifts[a] of Cellobionic Acid, pD 3.9

Dimer Unit	H1	H2	H3	H4	H5	H6	
Glucosidic unit	4.65	3.37	3.54	3.43	3.46	3.75	3.91
Aldonic acid end unit		4.29	4.15	3.99	3.97	3.75	3.86

[a]Recorded in D_2O with TSP as standard.

HPLC Separation of the Reaction Mixture

Analytical HPLC separation of the reaction mixture did not indicate the presence of significant amounts of D-gluconic acid. However, the main product eluted at a slightly higher retention time than D-gluconic acid, indicating, in this system, a dimeric acidic sugar,[12] supporting the hypothesis of cellobionic acid as the main reaction product (data not shown).

Due to the difficulty of unequivocal identification of the reaction products from the NMR spectra of the reaction mixtures, it was decided to separate the products by preparative HPLC for further analysis of the main product by two-dimensional NMR techniques. The preparative chromatogram from the separation of the product mixture is shown in Figure 2. The main product, with a retention time of approximately 40 min, was assumed to be cellobionic acid according to its retention time. This fraction was collected in four consecutive HPLC runs, freeze-dried and analyzed by two-dimensional NMR spectroscopic techniques.

NMR Spectroscopic Analysis of the Purified Product

The COSY-spectrum showed two independent coupling systems (Figure 3). One system contained 7 protons, corresponding to the glycosidic unit of cellobionic acid,

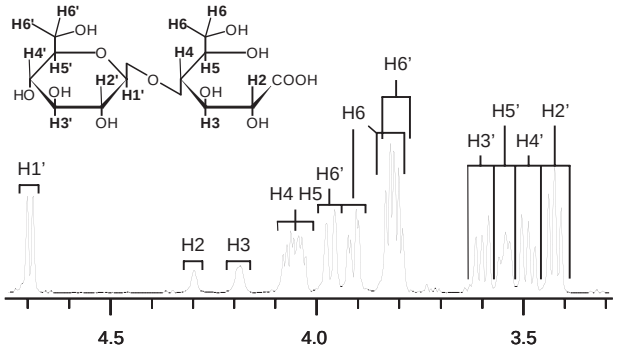

Figure 4. 600 MHz ^{1}H NMR spectrum of cellobionic acid, extracted from the COSY 2D NMR spectrum.

Table 2. ^{13}C NMR Chemical Shifts[a] of Cellobionic Acid

pD	Dimer Unit	C1	C2	C3	C4	C5	C6
pD 3.9	Glucosidic unit	103.5	74.0	76.1	70.1	76.6	61.3
	Aldonic acid end unit	178.9	73.1	71.8	82.2	72.2	62.6
pD 2.1	Glucosidic unit	103.6	74.1	76.3	70.1	76.6	61.3
	Aldonic acid end unit	176.6	72.2	71.4[b]	81.4	72.1[b]	62.7

[a]Recorded in D_2O with TSP as standard.
[b]Assignments may be interchanged.

and one system contained 6 protons. H1' of the glycosidic unit could be assigned to the doublet at 4.65 ppm, the only signal in the anomeric region. In the six-proton coupling system, H2 and H3 of the aldonic end could be assigned to the two signals downfield of the major area. Thus, it was concluded that the purified reaction product was cellobionic acid. The ^{1}H NMR chemical shifts for the cellobionic acid are given in Table 1. The 600 MHz 1D ^{1}H NMR spectrum of cellobionic acid, extracted from the COSY spectrum, is shown in Figure 4.

The ^{13}C NMR shifts were assigned from a proton-carbon shift correlated NMR spectrum (Figure 3). The two ^{13}C NMR signals assigned to C6 each correlated with two proton signals, assigned to H6 and H6' from the COSY spectrum. ^{13}C NMR chemical shifts of cellobionic acid are given in Table 2.

In order to ascertain whether the aldonic acid end group was present as the 1,5-lactone or the free aldonic acid, ^{13}C NMR data for cellobionic acid were compared to literature data for free gluconic acid and for glucono-1,5-lactone.[14] As can be seen from Table 3, the chemical shifts for the aldonic acid end group matches the chemical shift data for gluconic acid significantly better than those for the 1,5-lactone. It is therefore concluded that the cellobionic acid in this experiment is present as the free acid, as should also be expected from the thermodynamic stability for free aldonic acids compared to the 1,5-lactones.

Table 3. ^{13}C NMR Chemical Shifts[a] of the Aldonic Acid End Group, D-Gluconic Acid and D-Glucono-1-5-Lactone

		C1	C2	C3	C4	C5	C6
Aldonic acid end unit	pD 2.1	176.6	72.2	71.4[b]	81.4	72.1[b]	62.7
	pD 3.9	178.9	73.1	71.8	82.2	72.2	62.6
Reference data[14]	Gluconic acid	176.4	73.25	72.3	71.95	71.5	63.5
	Glucono-1, 5-lactone	175.2	82.24	75.7	72.0	67.95	60.8

[a]Recorded in D_2O with TSP as standard.
[b]Assignments may be interchanged.

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EXPERIMENTAL

Materials. Cellobiose: Merck, art. no. 2352; Acetonitrile (ACN): Baker HPLC-grade, art. no. 051860. Other chemicals were p.a. grade and used as received from Kebolab, Trondheim, Norway.

Ozone production. Ozone was produced from standard bottled oxygen gas using an ABB ozone reactor type LN103, and was delivered at a rate of 0.07 g/min. The ozone concentration was 9 % in oxygen and measured as absorbance at 258 nm[8] using a Shimadzu UV-160 A spectrometer.

Ozonation of cellobiose. Cellobiose (1 g) was dissolved in deionized water (300 mL, pH 2.7, acetic acid) and ozonated for 60 min using ozone gas (0.07 g/min) which was bubbled continuously through the solution. A portion (100 mL) of the reaction mixture was then concentrated under reduced pressure and freeze dried.

Separation by preparative HPLC. Preparative HPLC was performed according to a modification of a method described by Simms et al.[12] The instrument used was a Shimadzu HPLC equipped with a refractive index detector using an aminopropyl silica column (5 μ Hypersil APS-2, 20 mm × 25 cm). The mobile phase was acetonitrile:phosphate buffer pH 5 70:30 v/v.[13] The flow rate was 10 mL/min and the temperature was 30°C. Two mL of the mixture were injected in the HPLC after filtering through a 0.22 μm membrane filter. This method was not fully optimized, neither with respect to sample size nor to flow rate. Four consecutive HPLC runs were required to obtain a large enough sample size for NMR spectroscopic analysis. The isolated sample was concentrated under reduced pressure and freeze dried.

NMR analyses. Freeze dried samples were dissolved in D_2O (0.7 mL) and transferred to an NMR tube. Trimethylsilylpropionic acid 2,2,3,3-d_4 sodium salt (TSP) was added as a chemical shift standard. One dimensional ^{13}C NMR spectra were acquired on a Bruker Avance 400 instrument at 100 MHz detection frequency, using a pulse angle of 30°, acquisition time of 1.30 sec, a sweep width of 25125.6 Hz, and the relaxation delay was 1.8 sec. Two dimensional homonuclear shift-correlated (COSY) spectra were acquired on a Bruker Avance 600 instrument. The spectrum was acquired with presaturation of water signal during relaxation delay (phase sensitive spectrum using TPPI with double quantum filter). F2-acquisition parameters: Acquisition time was 0.42 sec, sweep width was 4401 Hz, time domain was 2048 k, observation frequency was 600.13 MHz, presaturation power level was 55 dB and relaxation delay was 3.0 sec. F1-acquisition parameters: Time domain was 256 k and the sweep width was 4.0 ppm. Two-dimensional heteronuclear shift correlated (HSQC) spectra were acquired on a Bruker Avance 600 instrument. F2-acquisition parameters: Acquisition time was 0.42 sec, sweep width was 2403 Hz, time domain was 2048 k, ^{1}H observation frequency was 600.13 MHz, ^{13}C observation frequency was 150.91 MHz, and the relaxation delay was 2 sec. F1-acquisition parameters: Time domain was 128 k, and sweep width was 60 ppm.

CONCLUSIONS

Mild ozonation of cellobiose in acetic acid solution yields 4-*O*-β-D-glucopyranosyl-D-gluconic acid (cellobionic acid) as almost the sole product. This compound can be purified in one stage by preparative HPLC using an aminopropyl silica column and an acetonitrile/phosphate buffer mobile phase. The product was successfully identified using two dimensional NMR techniques and NMR chemical shifts are reported.

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