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Assignment of structures to oligosaccharides produced by enzymic degradation of a β-D-glucan from barley by ^{1}H- and ^{13}C-n.m.r. spectroscopy

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ABSTRACT

The structures of one tri- (**1**), two tetra- (**2** and **3**), and one hexa-saccharide (**4**) produced by treatment of barley flour, after removal of the starch components, with a fungal β-D-glucanase (Finizyme) have been assigned on the basis of ^{1}H- and ^{13}C-n.m.r. data as follows: β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-D-Glc*p* (**1**), β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-D-Glc*p* (**2**), β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Glc*p* (**3**), and β-D-Xyl*p*-(1→4)-[α-L-Ara*f*-(1→3)]-[α-L-Ara*f*-(1→2)]-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl*p* (**4**).

INTRODUCTION

Barley β-D-glucan gives highly viscous aqueous solutions which can give rise to problems during the production of beer by lowering the rate of filtration, or by forming precipitates in the final product[1]. Therefore, the presence of endogenous β-D-glucanases is important but, unfortunately, these enzymes are destroyed by heat treatment during the production process. However, microbial β-D-glucanases, which are more heat stable, can be added.

We now describe the specificity of such a β-D-glucanase by the assignment by n.m.r. spectroscopy of structures to the oligosaccharides produced by degradation of barley β-D-glucan with a fungal enzyme (Finizyme, Novo-Nordisk).

RESULTS AND DISCUSSION

Barley β-D-glucan has been shown by enzymic and spectroscopic techniques[2–5] to be a linear polysaccharide of β-(1→4)- and β-(1→3) linkages in the ratio 2.3–2.7:1 (Fig. 1). The published[5] ^{13}C-n.m.r. data for a solution of the β-D-glucan in $(CD_3)_2SO$ have

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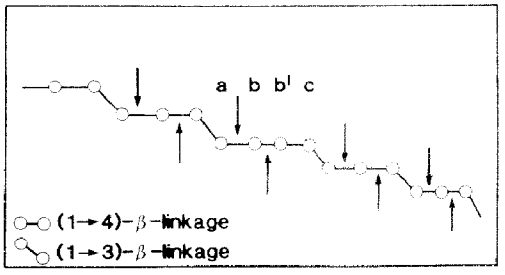


Fig. 1. Structure of barley β-D-glucan and the linkages hydrolysed by endo-[1→3(4)]-β-D-glucanase (EC 3.2.1.6) or *Bacillus subtilis* lichenase (EC 3.2.1.7) (↓) and cellulase (EC 3.2.1.4) (↑).

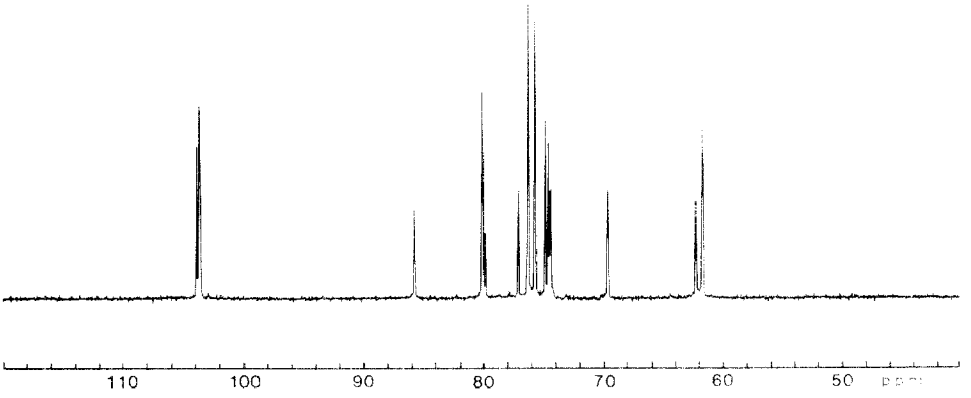
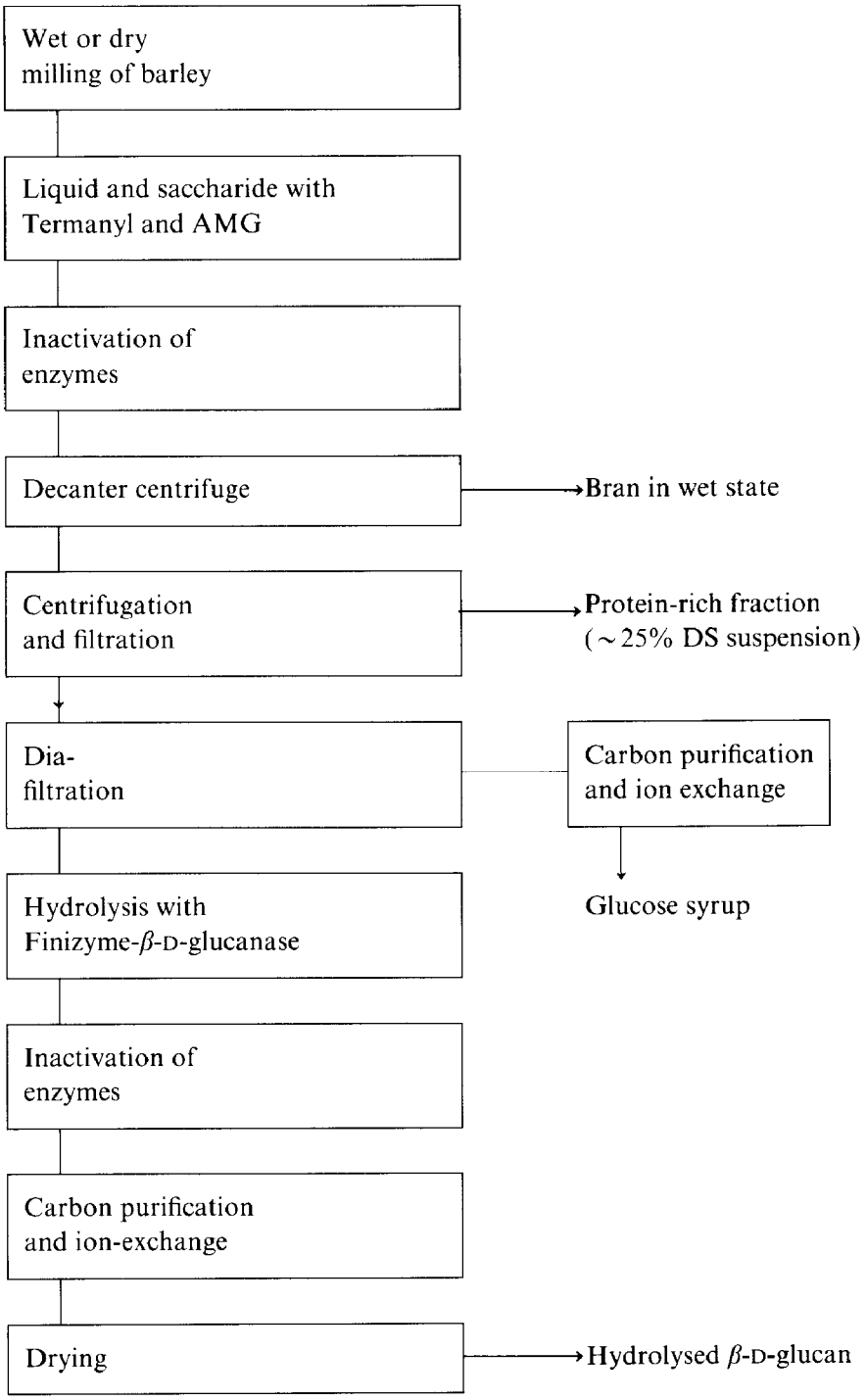


Fig. 2. ^{13}C-N.m.r. spectrum of a 1% solution of the β-D-glucan in D_2O at 125.77 MHz measured at 57°.

been confirmed by measurements on a solution in D_2O at 125.77 MHz (Fig. 2).

The β-D-glucan was obtained by treatment of barley flour with alpha-amylase and glucoamylase, and removal of the glucose produced by diafiltration as shown in Scheme 1. Treatment of the β-D-glucan fraction with a β-D-glucanase (Finizyme) gave a crude mixture of oligosaccharides, gel-permeation chromatography of which gave three major fractions with d.p. 3–5, respectively (Fig. 3). The first fraction was a trisaccharide (**1**), the second was a 3:1 mixture of two tetrasaccharides (**2** and **3**), which were further purified by h.p.l.c., and the third was a hexasaccharide (**4**) which did not originate from the β-D-glucan, but most likely from a cell-wall arabino-xylan. The assignment of structures to **1**–**4** was based solely on ^{1}H- and ^{13}C-n.m.r. data.

The ^{1}H-n.m.r. data obtained at 500 MHz for solutions in D_2O at 27° are given in Tables I and II. The assignments were based on COSY[6], relayed[7], and double-relayed COSY experiments, together with phase-sensitive double-quantum-filtered (DQF) COSY experiments[8]. Similarly, the ^{13}C-n.m.r. data (125.77 MHz) are given in Table III. The assignments were based on heteronuclear correlation spectroscopy[9] with the assigned proton signals and by comparison with data for model compounds. Rotating-frame n.O.e. spectroscopy (ROESY) experiments[10] confirmed the intra-residue interactions and assignments, and supported the structures assigned.



Scheme 1. NOVO process for the production of hydrolysed β-D-glucan.

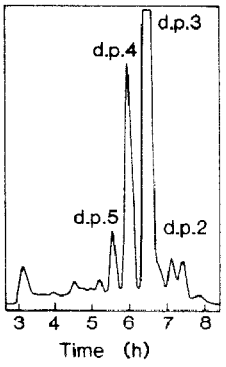


Fig. 3. Gel chromatogram of the products of degradation of the β-D-glucan from barley flour after treatment with Finizyme.

^{13}C-N.m.r. spectroscopy of the β-D-glucan. — A ^{13}C-n.m.r. spectrum, obtained for a 1% solution of the β-D-glucan in D_2O using a relaxation delay of 1.0 s at 27°, contained 12 rather broad lines, but a better spectrum (Fig. 2) was obtained at 57° and the 15 signals could be assigned on the basis of data for model compounds[11]. The data are presented in Table IV.

The region for signals of anomeric carbons contained resonances at 103.8 and 103.6 p.p.m., with the upfield signal of higher intensity, which were assigned to C-1 of unit **c** in Fig. 1 and C-1 of units, **a**, **b**, and **b'**, respectively. Integration of the signals indicated the proportions of β-(1→3)- and β-(1→4) linkages. Division of the sum of the integrated intensities of the signals for C-3 of unit **b** and C-4 of unit **c** by the intensity for C-3 of unit **a** gave a ratio of 1:2.3, but, if the last signal was replaced by that of C-2 of unit **a**, a ratio of 1:2.7 was obtained. The average ratio of 1:2.5 accords with results published by other methods[2-4] and earlier n.m.r. investigations[5].

Trisaccharide **1**. — The monosaccharide units in **1** are designated **a**–**c** starting from the reducing end. In the 1D ^1H-n.m.r. spectrum, only the signals for H-1 of units **a** and **b** could be observed, since that of H-1 of unit **c** was hidden under the HDO signal. The ^{13}C-n.m.r. spectrum showed **1** to be a trisaccharide, since it contained 24 signals from 3 C_6 units with the expected α,β-ratio of 2:3 for unit **a**.

Starting from the signal for H-1 of the α form of unit **a** at 5.23 p.p.m. ($J_{1,2}$ 3.7 Hz), that for H-2 was found from the cross-peak in the DQF-COSY spectrum at 3.58 p.p.m. ($J_{2,3}$ 10 Hz). The signal for H-3 could then be assigned from the cross-peak between H-2 and H-3 at 3.83 p.p.m. ($J_{3,4}$ 8.5 Hz) and that for H-4 at 3.65 p.p.m. ($J_{4,5}$ 10.3 Hz). These assignments were confirmed by comparing the results of the relayed and double-relayed COSY with normal COSY experiments. In the normal COSY spectrum, H-1 had a cross-peak only to H-2, but in the relayed COSY spectrum, a cross-peak to H-3 was observed also and, in the double-relayed COSY experiment, cross-peaks to H-2,3,4 were observed.

From the signal of H-4, the cross-peak in the DQF-COSY gave a chemical shift for the H-5 resonance of 3.95 p.p.m., which was connected to H-6A (3.88 p.p.m.). The

TABLE I

^{1}H-N.m.r. chemical shift data for **1–4**[a]

Unit		*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6A*	*H-6B*
Trisaccharide **1**								
β-D-Glc*p*	**c**	4.75	3.36	3.531	3.41	3.49	3.73	3.926
3-*O*-*β*-D-Glc*p*	**b**	4.54	3.528	3.77	3.53	3.52	3.76	3.932
4-*O*-D-Glc*p*	**aα**	5.23	3.58	3.83	3.65	3.95	3.88	3.91
	aβ	4.66	3.29	3.63	3.66	3.60	3.81	3.96
Tetrasaccharide **2**								
β-D-Glc*p*	**d**	4.49	3.30	3.487	3.40	3.47	3.70	3.90
4-*O*-*β*-D-Glc*p*	**c**	4.76	3.38	3.65	3.65	3.60	3.81	3.97
3-*O*-*β*-D-Glc*p*	**b**	4.52	3.515	3.75	3.52	3.485	3.71	3.91
			3.505					
4-*O*-D-Glc*p*	**aα**	5.21	3.56	3.81	3.63	3.93	3.79	3.86
	aβ	4.64	3.26	3.61	3.63	3.58	3.788	3.94
Tetrasaccharide **3**								
β-D-Glc*p*	**d**	4.73	3.348	3.51	3.39	3.47	3.70	3.90
3-*O*-*β*-D-Glc*p*	**c**	4.526	3.504	3.75	3.52	3.499	3.74	3.91
4-*O*-*β*-D-Glc*p*	**b**	4.520	3.351	3.641	3.65	3.60	3.810	3.97
		4.518	3.343	3.638				
4-*O*-D-Glc*p*	**aα**	5.20	3.56	3.809	3.63	3.93	3.82	3.85
	aβ	4.64	3.27	3.61	3.61	3.58	3.79	3.94

Unit		*H-1*	*H-2*	*H-3*	*H-4*	*H-5ax*	*H-5eq*
Hexasaccharide **4**							
β-D-Xyl*p*	**d**	4.46	3.27	3.427	3.61	3.29	3.93
α-L-Ara*f*-3-*O*	**e**	5.28	4.18	3.95	4.31	3.81	3.734
α-L-Ara*f*-2-*O*	**f**	5.23	4.158	3.97	4.14	3.83	3.730
4-*O*-*β*-D-Xyl*p*	**c**	4.66	3.58	3.85	3.88	3.44	4.157
4-*O*-*β*-D-Xyl*p*	**b**	4.483	3.303	3.567	3.80	3.428	4.154
		4.485	3.312	3.571			
4-*O*-D-Xyl*p*	**aα**	5.19	3.555	3.76	3.75		
	aβ	4.59	3.26	3.558	3.78	3.39	4.06

[a] Measured at 500 MHz on solutions in D_2O at 27° (internal acetone, 2.225 p.p.m.)

signal for H-6B was probably at 3.91 p.p.m., but the intensity was low due to the low proportion of the α form.

Likewise, the assignments for the *β* form of unit **a** started with the signal for H-1 (4.66 p.p.m., $J_{1,2}$ 7.7 Hz). Cross-peaks identified the signals for H-2 (3.29 p.p.m., $J_{2,3}$ 9.3 Hz) and H-3 (3.63 p.p.m., $J_{3,4}$ 9 Hz). There was no clear cross-peak between H-3 and H-4. A C–H correlation experiment indicated the resonances of H-6A and H-6B to be close to 3.81 and 3.95 p.p.m., respectively. In the DQF-COSY spectrum, signals at 3.81 and 3.96 p.p.m., respectively, were found for these protons, and that of H-5 was identified at 3.60 p.p.m. Therefore, in the ^{1}H-n.m.r. spectrum, the signals for H-4 must be close to those of H-3 and H-5, and therefore the cross-peaks were close to the diagonal in the DQF-COSY experiment and difficult to detect. However, the chemical

TABLE II

$J_{H,H}$ values (Hz)[a] for **1–4**

Unit		$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6A}$	$J_{5,6B}$	$J_{6A,6B}$
Trisaccharide **1**								
β-D-Glc*p*	**c**	7.8	9.8	8.8	10.1	5.4	3	12.5
3-*O*-β-D-Glc*p*	**b**	7.6	10			5	3.3	12.4
4-*O*-D-Glc*p*	**aα**	3.7	10	8.5	10.3	5		
	aβ	7.7	9.3	9	9.5	5	2.4	12.4
Tetrasaccharide **2**								
β-D-Glc*p*	**d**	8.0	10.2	9	9	5	2.5	12
4-*O*-β-D-Glc*p*	**c**	8.7	9.2			5	3	11.5
3-*O*-β-D-Glc*p*	**b**	8.5	9		9	4.5		
4-*O*-D-Glc*p*	**aα**	4.4	9.1	9	10.4		2.5	
	aβ	7.8	10.1		9.2	4.6	2.7	13
Tetrasaccharide **3**								
β-D-Glc*p*	**d**	7.7	10.3	8.9	10.0	5.8	2.2	12.0
3-*O*-β-D-Glc*p*	**c**	7.8	9.0	9.4		4.6	2.1	12.4
4-*O*-β-D-Glc*p*	**b**	7.5	8.9	9.0	9.3	4.8	2.2	12.3
		7.6	8.9					
4-*O*-D-Glc*p*	**aα**	3.7	9.6	8.9	10.0			11
	aβ	7.6	9.8			5.0	2.2	12.4

Unit		$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5A}$	$J_{4,5B}$	$J_{5A,5B}$
Hexasaccharide **4**							
β-D-Xyl*p*	**d**	7.7	9.7	9.8	9.5	5.0	12.9
α-L-Ara*f*-3-*O*-	**e**	~1	3	5.8	3.9	5.5	11.6
α-L-Ara*f*-2-*O*-	**f**	~2	3.8	6.2	3.5	6.0	12.0
4-*O*-β-D-Xyl*p*	**c**	7.5	9.5	9.3	8.9	5.0	12.6
4-*O*-β-D-Xyl*p*	**b**	7.9	9.9	9.5	9.5	5.4	12.8
4-*O*-D-Xyl*p*	**aα**	3.7	8				
	aβ	7.5	9.7	10.0	9.5	5.0	12.3

[a] Measured at 500 MHz on solutions in D_2O at 27° based on a first-order analysis (±0.2 Hz).

shift of the H-4 resonance could be assigned from the ^{13}C-n.m.r. spectrum, where C-4 gave the peak at 79.5 p.p.m. (correct intensity and close to that of C-4 of the α form of unit **a**). From the C–H correlation, this finding showed that H-4 resonated at 3.66 p.p.m. and therefore that cross-peak was difficult to observe.

For unit **b**, from the resonance of H-1 at 4.54 p.p.m., that for H-2 was found through the cross-peak in the DQF-COSY-spectrum to have a chemical shift of 3.528 p.p.m. ($J_{1,2}$ 7.6 Hz) indicative of the β form. From the signal for H-2, the chemical shift of the H-3 resonance was found to be 3.77 p.p.m. In the DQF-COSY-spectrum, the H-3/H-2 and H-3/H-4 cross-peaks were close together, but the chemical shift of the H-4 resonance was found at 3.53 p.p.m. For unit **b**, the resonances of H-4 and H-5 had similar chemical shifts so that the corresponding cross-peak was close to the diagonal, but a chemical shift for the H-5 resonance at 3.52 p.p.m. was measured and, from this value, those of the H-6A and H-6B resonances were found to be 3.76 and 3.932 p.p.m., respectively.

TABLE III

^{13}C-N.m.r. chemical shifts data for **1–4**[a]

Unit		*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Trisaccharide **1**							
β-D-Glc*p*	**c**	103.6	74.2	76.36	70.4	76.8	61.5
3-*O*-*β*-D-Glc*p*	**b**	103.1	73.8	84.9	68.8	76.39	61.4
4-*O*-D-Glc*p*	**aα**	92.6	72.0	72.2	79.6	70.9	60.8
	aβ	96.6	74.7	75.1	79.5	75.6	60.9
Tetrasaccharide **2**							
β-D-Glc*p*	**d**	103.39	73.97	76.4	70.3	76.8	61.4
4-*O*-*β*-D-Glc*p*	**c**	103.36	74.03	74.9	79.4	75.6	60.8
3-*O*-*β*-D-Glc*p*	**b**	103.1	73.9	84.7	68.8	76.3	61.4
4-*O*-D-Glc*p*	**aα**	92.6	72.0	72.2	79.6	70.9	60.8
	aβ	96.6	74.7	75.1	79.4	75.6	60.7
Tetrasaccharide **3**							
β-D-Glc*p*	**d**	103.6	74.3	76.4	70.4	76.8	61.5
3-*O*-*β*-D-Glc*p*	**c**	103.14	73.8	84.8	68.8	76.4	61.4
4-*O*-*β*-D-Glc*p*	**b**	103.14	73.8	74.9	79.2	75.6	60.7
4-*O*-D-Glc*p*	**aα**	92.6	72.05	72.12	79.5	70.9	60.8
	aβ	96.6	74.7	75.1	79.3	75.6	60.7

Unit		*C-1*	*C-2*	*C-3*	*C-4*	*C-5*
Hexasaccharide **4**						
β-D-Xyl*p*	**d**	101.74	73.4	76.0	69.6	65.5
α-L-Ara*f*-3-*O*	**e**	108.5	81.4	77.6	84.7	61.5
α-L-Ara*f*-2-*O*	**f**	109.1	81.6	77.1	84.8	61.6
4-*O*-*β*-D-Xyl*p*	**c**	100.3	79.0	77.9	74.1	62.9
4-*O*-*β*-D-Xyl*p*	**b**	102.1	73.1	74.1	76.1	63.4
4-*O*-D-Xyl*p*	**aα**	92.4	71.8	71.3	77.0	61.0
	aβ	96.9	74.4	74.3	76.8	63.4

[a] Measured at 125.77 MHz on solutions in D_2O at 27° (internal 1,4-dioxane, 67.4 p.p.m.).

TABLE IV

^{13}C-N.m.r. chemical shift data for the *β*-D-glucan[a]

Unit[b]		*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
3-*O*-*β*-D-Glc*p*	**a**	103.6	74.3	85.8	69.5	77.0	62.2
4-*O*-*β*-D-Glc*p*	**b**	103.6	74.5	75.6	80.0	76.2	61.6
(4-*O*-D-Glc*p*)	**b′**				79.9		
4-*O*-*β*-D-Glc*p*-3-*O*	**c**	103.8	74.8	75.6	80.0	76.2	61.6

[a] Measured at 125.77 MHz on a 1% solution in D_2O at 57° (internal 1,4-dioxane, 67.4 p.p.m.). [b] See Fig. 1.

Starting from the signal for H-1 of unit **c** at 4.75 p.p.m., which was hidden under the HDO signal in the 1D-^{1}H-n.m.r. spectrum, all protons in this sub-spectrum could be traced in the DQF-COSY spectrum: H-2 at 3.36 ($J_{1,2}$ 7.8 Hz, indicative of the *β* configuration), H-3 at 3.531, H-4 at 3.41, and H-5 at 3.49 p.p.m. The signals for H-6A,6B were detected by the shape of their cross-peaks at 3.73 and 3.926 p.p.m., respectively.

The ^{13}C-n.m.r. spectrum could then be assigned (Table III) from a C–H correlation experiment. The intensities of the signals in the 1D spectrum indicated those that belonged to the α and β forms of unit **a**.

Comparison with data for model compounds[11,12] and the large values of $^{3}J_{H,H}$ (8–10 Hz), which indicated vicinal diaxial protons[13], showed that units **a**–**e** each had the glucopyranose structure.

The structure **1** of the trisaccharide was determined from the data in Tables I–III by comparison with relevant model compounds: β-linked disaccharides of glucopyranose[13,14], particularly β-cellobiose [(1→4)-linked] and β-laminaribiose [(1→3)-linked]. A comparison of the chemical shifts assigned to the resonances of the reducing units with the corresponding data for β-cellobiose, and especially the chemical shifts of the C-4 resonance for unit **a**α (79.6 p.p.m.), **a**β (79.5 p.p.m.), and β-cellobiose (79.8 p.p.m.), indicated unit **a** to be 4-glycosylated. Furthermore, the chemical shift of the C-3 resonance of unit **b** at 84.9 p.p.m. corresponded well with that of the C-3 resonance for β-laminaribiose (86.0 p.p.m.) and indicated unit **b** to be 3-glycosylated. Thus, **1** was β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-D-Glc*p*.

This structure was confirmed by the fact that unit **c** did not have ^{13}C resonances at lower field than 76.8 p.p.m., other then that of C-1, and therefore could not have been glycosylated at a secondary position.

The structure **1** was supported further by a ROESY experiment[10]. In this spectrum, it was the ROE peaks, particularly from H-1, that were of value in the structural assignments (Table V). From these peaks, it was observed first that some of the earlier assignments of the chemical shifts were confirmed by intra-ring ROEs. However, the important cross-peaks were the inter-ring ROEs, which showed that unit **b** was 4-linked to unit **a** and that unit **c** was 3-linked to unit **b**.

Tetrasaccharides **2** *and* **3**. — The tetrasaccharides **2** and **3**, obtained in the ratio of ~3:1, were isolated by preparative h.p.l.c.

The ^{1}H and ^{13}C resonance for the major tetrasaccharide (**2**) were assigned using the techniques described above and the data are presented in Tables I–III. The units are represented by **a**–**d** starting from the reducing end.

The structure of **2** can be assigned solely on the basis of the data given in Tables I–III and by comparison with those for relevant model compounds and **1**. The chemical shifts of the resonances for unit **a** indicated 4-glycosylation, based on the downfield-shifted resonances of C-4 at 79.4 and 79.6 p.p.m. (α,β-ratio 2:3). Likewise, the resonance of C-3 at 84.7 p.p.m. indicates unit **b** to be 3-substituted, and that of C-4 of unit **c** at 79.4 p.p.m. indicated 4-substitution. Therefore, unit **d** must be at the non-reducing end, which fits well with the fact that there was no downfield shift of the C-2,3,4 resonances compared to those of methyl β-D-glucopyranoside[12]. Comparison of the chemical shifts of the H-1 resonances confirmed that **2** was β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-D-Glc*p*.

The structure **2** was confirmed by the ROESY experiment (Table V) based on the inter-unit ROE from the anomeric protons. A small ROE cross-peak for H-1 of unit **c** to the protons at positions next to the glycosylated carbon was observed.

TABLE V

ROESY cross-peaks[a] of **1–4**[a,b]

Trisaccharide **1**[a]

	H-2(**a**α)	H-4(**a**α)	H-3(**a**β)	H-4(**a**β)	H-5(**a**β)	H-2(**b**)	H-3(**b**)	H-4(**b**)	H-5(**b**)	H-2(**c**)	H-3(**c**)	H-4(**c**)	H-5(**c**)
H-1(**a**α)	w												
H-1(**a**β)			m		m								
H-1(**b**)		s[c]		s[c]		s[d]	m	s[d]	s[d]				
H-1(**c**)							s			m	s	m	s

Tetrasaccharide **2**[a]

	H-2(**a**α)	H-4(**a**α)	H-3(**a**β)	H-4(**a**β)	H-5(**a**β)	H-2(**b**)	H-3(**b**)	H-5(**b**)	H-2(**c**)	H-3(**c**)	H-4(**c**)	H-5(**c**)	H-2(**d**)	H-3(**d**)	H-4(**d**)	H-5(**d**)
H-1(**a**α)	w															
H-1(**a**β)			m		m											
H-1(**b**)		s[c]		s[c]		s[d]	s	s[d]								
H-1(**c**)							s		w	s		s				
H-1(**d**)											s		m	s	m	s

Tetrasaccharide **3**

	H-2(**a**α)	H-4(**a**α)	H-3(**a**β)	H-4(**a**β)	H-5(**a**β)	H-2(**b**)	H-3(**b**)	H-4(**b**)	H-5(**b**)	H-2(**c**)	H-3(**c**)	H-5(**c**)	H-2(**d**)	H-3(**d**)	H-4(**d**)	H-5(**d**)
H-1(**a**α)	w															
H-1(**a**β)			m		m											
H-1(**b**)[e]		s		s		m	s		s							
H-1(**c**)[e]								s			s	s				
H-1(**d**)											s		m	s	m	s

Hexasaccharide **4**

	H-2(**a**α)	H-4(**a**α)	H-2(**a**β)	H-3(**a**β)	H-4(**a**β)	H-5*eq*(**a**β)	H-5*ax*(**a**β)	H-2(**b**)	H-3(**b**)	H-4(**b**)	H-5*eq*(**b**)	H-5*ax*(**b**)
H-1(**a**α)	w											
H-1(**a**β)			w	m			s					
H-1(**b**)		s			m	m		m	s			s
H-1(**c**)										m	s	
H-1(**d**)												
H-1(**e**)												
H-1(**f**)												

	H-2(**c**)	H-3(**c**)	H-4(**c**)	H-5*eq*(**c**)	H-5*ax*(**c**)	H-2(**d**)	H-3(**d**)	H-4(**d**)	H-5*ax*(**d**)	H-2(**e**)	H-3(**e**)	H-2(**f**)	H-3(**f**)
H-1(**a**α)													
H-1(**a**β)													
H-1(**b**)													
H-1(**c**)	m	m			s								
H-1(**d**)			m	m		s	s	w	s				
H-1(**e**)	m	s								s	w		
H-1(**f**)	s	m										s	w

[a] Units designated in brackets. [b] w, weak; m, medium; s, strong. [c–d] Overlapping peaks. [e] H-1(**b**) and H-1(**c**) of **3** had similar chemical shifts and the peaks overlapped.

The n.m.r. data for the minor tetrasaccharide (**3**) were assigned as for **2** and are presented in Tables I–III.

However, it was not possible to perform a C–H correlation experiment for **3**, due to the small amount of pure compound available. Therefore, the ^{13}C resonances were assigned on the basis of the 1D-spectrum by comparison with data for **1** and **2**.

These ^{13}C-n.m.r. data showed that unit **a** was 4-glycosylated (resonances at 79.5 and 79.3 p.p.m. with intensities comparable to the α,β-ratio). Furthermore, the ^{1}H resonances of unit **a** had chemical shifts that were similar to those of unit **a** in **1** and **2**. Only one unit (**c**) was 3-glycosylated as seen from the ^{13}C-n.m.r. data that corresponded to the resonance of H-3 at 3.75 p.p.m. The chemical shift data indicated that unit **d** was unsubstituted and, from the resonance of H-1 at 4.73 p.p.m. (*cf.* 4.75 p.p.m. for H-1 of unit **c** in **1** and at 4.76 p.p.m. in **2**), it was 3-linked to unit **c**. Thus, **3** was β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Glc*p*.

This structure was confirmed by a ROESY spectrum which showed ROE cross-peaks from H-1 of unit **d** to H-3 of unit **c** (Table V). The ROE cross-peaks from H-1 of unit **c** to H-4 of unit **b** and from H-1 of unit **b** to H-4 of the α and β forms of unit **a** were seen less clearly, due to the almost identical chemical shifts of the H-1 resonances of units **b** and **c**.

The hexasaccharide **4**. — The ^{1}H- and ^{13}C-n.m.r. data showed that **4** was a hexasaccharide with four Xyl*p* and two Ara*f* units (D-xylose and L-arabinose). Arabinoxylans are present in cell walls from barley, especially those of the aleurone[15,16]. Therefore, **4** most likely originated from degradation of cell-wall fragments in the barley flour. The n.m.r. data were assigned as described above. The units are represented as **a–d** starting from the reducing end of the xylan chain, and **e** and **f** for the arabinofuranose side-chains.

Starting from the signal for H-1 at 5.19 p.p.m. ($J_{1,2}$ 3.7 Hz) for the α form of unit **a**, H-2 and H-3 could be assigned from the DQF-COSY experiment as resonating at 3.555 and 3.76 p.p.m., respectively. The signal for H-4 could be assigned only after the assignment of the rest of the spectral data and the determination of the structure had been completed from a C–H correlation experiment, and was shown to be at ~3.75 p.p.m. The signals for H-5*ax* and H-5*eq* could not be identified due to a low signal-to-noise ratio and overlap of signals.

From the signal of H-1 at 4.59 p.p.m. ($J_{1,2}$ 7.5 Hz), the resonance of the β form of unit **a** could be assigned from the DQF-COSY experiment: H-2, 3.26; H-3, 3.558; H-4, 3.78; H-5*ax*, 3.39; and H-5*eq*, 4.06 p.p.m.

As for **3**, the signals for H-1,2,3 in unit **b** showed small splittings due to the α and β forms of unit **a**, and established the sequence of units. Thus, H-1 resonated at 4.483 and 4.485 p.p.m. ($J_{1,2}$ 7.9 Hz) with the downfield signal corresponding to the α form. From these signals, the resonances of H-2 were identified at 3.303 and 3.312 p.p.m., and those of H-3 at 3.567 and 3.571 p.p.m.

The signals for H-4,5*eq*,5*ax* were not affected by the anomeric configuration of unit **a** and were assigned as follows: H-4, 3.80; H-5*eq*, 4.154; and H-5*ax*, 3.428 p.p.m.

Starting at the signal for H-1 at 4.66 p.p.m. ($J_{1,2}$ 7.5 Hz), the resonances of unit **c**

could be assigned from the DQF-COSY experiment: H-2, 3.58; H-3, 3.85; H-4, 3.88; H-5*eq*, 4.157; and H-5*ax*, 3.44 p.p.m. These chemical shifts are quite different from those for units **a** and **b**, due to the substitution with arabinose units.

Starting with the signal for H-1 at 4.46 p.p.m. ($J_{1,2}$ 7.7 Hz) in unit **d**, the remaining signals were assigned as follows: H-2, 3.27; H-3, 3.427; H-4, 3.61; H-5*eq*, 3.93; and H-5*ax*, 3.29 p.p.m.

Units **e** and **f** gave chemical shift data that were quite different from those for units **a**–**d** and the signals occurred generally towards lower field. The 1D ^{1}H-n.m.r. spectrum showed that the H-1 resonances at 5.28 and 5.23 p.p.m. had small J values in accord with an α-L-arabinofuranosidic structure[17].

From the signal for H-1 at 5.28 p.p.m. ($J_{1,2}$ 1 Hz) in unit **e**, the resonance of H-2 could be traced in the DQF-COSY spectrum to 4.18 p.p.m. Furthermore, H-3 was found to resonate at 3.95 p.p.m. and H-4 at 4.31 p.p.m., and H-4 was coupled to H-5A (3.81 p.p.m.) and H-5B (3.734 p.p.m.).

From the signal for H-1 at 5.23 p.p.m. ($J_{1,2}$ 2 Hz) in unit **f**, the following signals were assigned: H-2, 4.158; H-3, 3.97; H-4, 4.14; H-5A, 3.83; and H-5B, 3.730 p.p.m. Units **e** and **f** had similar chemical shift data, except for the H-4 resonances where a difference of 0.17 p.p.m. was observed, due most likely to a deshielding effect from the ring oxygen of unit **c** as inferred from inspection of a molecular model (HSEA calculation).

From the C–H correlation, almost all of the ^{13}C resonances could be assigned unambiguouly from the ^{1}H data. The only problem arose with the signals of unit **a**, where not all of the ^{1}H resonances could be assigned. Thus, C-5 of both the α and β forms of unit **a** were assigned on the basis of a comparison with data for model compounds[18,19].

The most important data for the structural assignment of units **a**–**d** were the $J_{H,H}$ values (Table II); $J_{1,2}$ (except for the α form of unit **a**) were in the range 7.5–7.9 Hz and proved the β-*xylo* configuration[12,13]. Similarly, the values of $J_{2,3}$, $J_{3,4}$, and $J_{4,5ax}$ were in the range 8–10 Hz, which indicated diaxial configurations, *i.e.*, xylopyranose[12].

From the above n.m.r. assignments, it was possible to propose a structure for **4**. Units **a**–**d** were Xyl*p* units, which was confirmed by comparison with published data (primarily ^{13}C) of such model compounds as xylopyranoses[18], xylobioses[18], and (1→4)-β-D-xylo-oligosaccharides[19].

Units **e** and **f** had ^{13}C resonances at low field, indicative of furanose rings[12]. This inference was supported by the $J_{C-1,H-1}$ values of 173 ± 1.5 (unit **e**) and 174 ± 1.5 Hz (unit **f**), which accorded with published values (171–174 Hz) for methyl aldopentofuranosides[20].

The ^{13}C chemical shifts also agreed with data for methyl α-D(L)-arabinofuranoside[21]. The α configuration was confirmed by the $J_{1,2}$ values of 1–2 Hz [*cf.* published data[17]: $J_{1,2}$ 1.2 (α) and 4.3 Hz (β)]. The other J values accorded with data for methyl α-D-arabinofuranoside[17].

The linkages in **4** could be assigned by the ROESY experiment, where the anomeric protons showed inter-unit ROE cross-peaks (Table V) that indicated unit **b** to be 4-linked to unit **a**, unit **c** to be linked to unit **b**, and unit **d** to be linked to unit **c**.

The positions of linkages from **e** and **f** were determined also by the ROESY experiment (Table V), which showed units **e** and **f** to be attached to the same unit. H-1 of unit **e** had a strong ROE to H-3 of unit **c** but also a medium ROE to H-2 of unit **c**, whereas H-1 of unit **f** had a strong ROE to H-2 of unit **c** and a medium ROE to H-3 of unit **c**. This symmetrical pattern can stem only from the attachment of the Ara*f* units to the same unit, and accords with the above results from HSEA calculations for **4** and fits with the fact that the ^{13}C resonances show that unit **c** is highly substituted.

The structure proposed for **4** is β-D-Xyl*p*-(1→4)-[α-L-Ara*f*-(1→3)-[α-L-Ara*f*-(1→2)]-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl*p*.

N.m.r. investigations of some feruloylated arabinoxylans such as [5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]-(1→3)β-D-Xyl*p*-(1→4)-D-Xyl*p*[22] and β-D-Xyl*p*-(1→4)-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-D-Xyl*p*[22] have been published, and the assignments for **4** accorded with those of comparable units in these feruloylated compounds. The ^{13}C resonances of β-D-Xyl*p*-(1→4)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-OMe have been assigned[23,24], as have those of 2- and 3- or 2,3-di-*O*-(α-L-Ara*f*)-β-D-Xyl derivatives[25,26], which accord with the present data.

The enzyme specifity for the hydrolysis of β-D-glucan with Finizyme can be determined on the basis of the structures of **1**–**3**. The enzyme has cellulase (EC 3.2.1.4) specificity, *i.e.*, hydrolysis of β-(1→4) linkages, except in structures where the "non-reducing" end unit is 3-substituted[2,27]. Hydrolysis of the latter type of linkage is observed[2,27] only when β-D-glucan is treated with lichenase (EC 3.2.1.73), but products of this type, namely, β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→3)-D-Glc*p* and β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→3)-D-Glc*p*, were not encountered in the present study.

EXPERIMENTAL

Fractionation of the oligosaccharides. — Preparative gel-permeation chromatography on a column (100 × 1.5 cm) of Bio-Rad P-2 (−400 mesh) at 65° was used to isolate the oligosaccharides. A Waters 510 dual-piston pump was used to deliver the solvent (double-deionised Milllipore-water) at 0.3 mL/min. The solvent was continuously degassed by bubbling a constant stream of He through the reservoir. A Waters Wisp 701 B autosampler was used to apply the sample to the column and the effluent was monitored with an LKB differential refractive index detector (Bromma 2142) maintained at 25°. Samples were collected with an LKB "Superroc" fraction collector, and the top fractions, which corresponded to d.p. 3–5, were each combined and freeze dried. A sample of 500 mg gave components of d.p. 3 (250 mg), 4 (120 mg), and 5 (25 mg).

H.p.l.c. of a part of fraction 2 (d.p. 4) was carried out on an LKB system, using a Novapak C_{18} 5 μ Radial-Pak column (5 mm i.d. × 10 cm), by elution with water at 0.5 mL/min and u.v. detection of fractions at 190 nm.

N.m.r. spectroscopy. — Solutions of 20 mg in 0.5 mL of D_2O were used. Spectra were recorded in 5-mm tubes at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C with a Bruker AM-500 spectrometer and at 27° except for the ^{13}C-n.m.r. spectra of the

β-D-glucan, which was recorded at 57°. The ^1H resonances were measured relative to internal acetone (2.225 p.p.m., DOH at 4.75 p.p.m. at 27°) and determined on a first-order basis. The values in Table I are given with two decimal points, except where this accuracy does not allow distinction between clearly separated and well characterised signals. The ^{13}C resonances are relative to internal 1,4-dioxane (67.4 p.p.m.).

Homonuclear 2D-n.m.r. spectroscopy was performed with Bruker DISNMRP software, except for the ROESY.

Relayed COSY experiments[7] were made with fixed delays of 30 ms and double-relayed COSY experiments with both delays of 30 ms in order to optimise coherence transfer for large couplings[28]. These experiments were performed with quadrature detection in the F_1 dimension, and a total of 256 t_1 increments of 16 scans each (32 for double-relayed) were recorded with a minimum delay between pulses of 0.1 s and a sweep width of 2500 Hz. The time-domain data matrix was zero-filled in the t_1 direction to 512 × 1024 points, treated with a non-shifted sine-bell function in both dimensions, and processed to give magnitude spectra.

The phase-sensitive COSY experiments were performed using double-quantum filtering[8,29] with the Bruker COSYPHDQ microprogram, using fixed delays of 30 ms. These experiments were performed using 512 t_1 increments and a sweep width of 2500 Hz, giving an aquisition time in t_1 of 0.205 s. In the F_2 dimension, 2048 data points were collected, giving an aquisition time of 0.819 s. The data matrix was zero-filled in the F_1 dimension to give a matrix of 2048 × 2048 points and was resolution-enhanced in both dimensions by a shifted sine-bell function before Fourier transformation.

The ^{13}C–^1H correlation experiments[9] were performed with the XHCORRD microprogram, using decoupling in the ^1H dimension; 128 t_1 increments of 1200 scans and a size of 2048 points were accumulated. The data matrix was zero-filled in the F_1 dimension to 256 × 2048 points before Fourier transform in the absolute mode, giving a digital resolution of 9.8 Hz in the ^{13}C dimension and 11.7 Hz in the ^1H dimension.

The ROESY experiments[10,30] were performed using the procedure of Griesinger *et al.*[31] with the spin-lock field surrounded by 2 hard 90° pulses in order to avoid frequency-dependent effects[32]. The transmitter was used for all hard pulses and the spin-lock field was delivered by the decoupler. The spin-lock field was placed in the middle of the spectrum. Quadrature in t_1 was obtained by the hypercomplex method of States *et al.*[33]. 512 t_1 values were recorded with 80 scans each and 2 dummy scans giving an aquisition time in t_1 of 0.223 s (sw 2300 Hz) and 0.890 s in t_2. The data sets were resolution-enhanced in the t_1 dimension by a shifted sine-bell function and zero-filled to 2048 × 2048 data points prior to Fourier transformation, thus giving a resolution of 1.1 Hz/point.

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