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LINKAGE OF *p*-COUMAROYL AND FERULOYL GROUPS TO CELL-WALL POLYSACCHARIDES OF BARLEY STRAW

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ABSTRACT

Treatment of cell walls of barley straw with *Oxyporus* "cellulase" (a mixture of polysaccharide hydrolases) released compounds containing *p*-coumaroyl and feruloyl groups bound to carbohydrates, two of which were identified as *O*-[5-*O*-(*trans*-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (PAXX) and *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX).

INTRODUCTION

Phenolic compounds, including the *trans* isomers of *p*-coumaric (4-hydroxycinnamic) and ferulic (4-hydroxy-3-methoxycinnamic) acids, are covalently bound to cell-wall polysaccharides of graminaceous plants[1–6]. They are thought to be present as esters because of their release from cell walls on treatment with alkali. Interest in these compounds arises from the finding that, when the walls are treated with alkali, the amount of phenols released is correlated with the amount of forage digested by ruminants[7–9]; increased digestibility is an important factor in improving rates of animal production.

The linkage of ferulic acid to cell-wall polysaccharides has been studied by determining the structure of water-soluble compounds released by treatment of sugar-cane bagasse[10] and wheat-bran[11] and maize[12] cell-walls with "cellulase". All of these products contained ferulic acid ester-linked to arabinose which in turn was linked to xylose, indicating that they were derived from feruloylated arabinoxylans.

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We have used similar methods to isolate water-soluble carbohydrates containing *p*-coumaroyl and feruloyl groups (PAXX and FAXX, respectively) from cell walls of barley straw and now report the characterisation of these compounds.

RESULTS

The yields of PAXX and FAXX released from finely ground cell-walls of barley straw by treatment with "cellulase" were 3.67 mg of PAXX/g of cell walls (d.s. 0.13) and 7.75 mg of FAXX/g of cell walls (d.s. 0.26). T.l.c. gave one spot for PAXX and one for FAXX, but h.p.l.c. gave two peaks for each compound representing the α and β anomers (Table I).

Hydrolysis of PAXX and FAXX with "cellulase" (*Aspergillus niger*) gave (t.l.c.) arabinose and xylose only. *trans-p*-Coumaric acid was the only phenolic acid released from PAXX, and *trans*-ferulic acid from FAXX. H.p.l.c. (method 4) gave a ratio for xylose and arabinose of ~2:1 for each hydrolysate. The sugar composition (mole %) of the carbohydrate portion of PAXX and FAXX, determined after hydrolysis with trifluoroacetic acid, was arabinose 33.5 (PAXX), 32.9 (FAXX); xylose 62.6 (PAXX), 65.8 (FAXX); galactose 0.9 (PAXX); glucose 3.0 (PAXX), 1.3 (FAXX). This represents molar ratios for xylose and arabinose of 1.9:1.0 (PAXX) and 2.0:1.0 (FAXX).

The positive ion spectrum on f.a.b.-m.s. of FAXX showed an intense ion at *m/z* 613 $(M + Na)^+$ and weak ions at 629 $(M + K)^+$ and 591 $(M + H)^+$. The negative ion spectrum showed an intense ion at *m/z* 589 $(M - H)^-$, indicating the molecular weight of FAXX to be 590 consistent with a molecule containing one *trans*-feruloyl, one arabinosyl, and two xylosyl residues. PAXX gave only a weak ion at *m/z* 583 $(M + Na)^+$ and no negative ions, indicating a molecular weight of 560 consistent with a molecule containing one *trans-p*-coumaroyl, one arabinosyl, and two xylosyl residues. Breakdown of PAXX during positive ion f.a.b.-m.s. occurred from the phenolic acid residue and was followed by two successive losses of pentose until only the third pentose remained, *i.e.*, *m/z*: 583 $(M + Na)^+ \rightarrow 437 \rightarrow 305 \rightarrow 173$ (pentose + $Na)^+$. In contrast, the breakdown of FAXX started at the sugar residue, *i.e.*, 613 $(M + Na)^+ \rightarrow 441 \rightarrow 309 \rightarrow 177$ (ferulic

TABLE I

H.P.L.C. AND T.L.C. OF PAXX AND FAXX

Compound	T *(min)* *(h.p.l.c., method 3)*	R_F *(t.l.c.)*			
		Method 1	*Method* 2	*Method* 3	*Method* 4
PAXX	25.6 and 35.2[a]	58	0	100	79
FAXX	37.8 and 53.3[a]	50	0	100	73

[a]Ratio of area of peak of lower *T* (β anomer) to that of higher *T* (α anomer) was ~2:1.

acid − OH)$^+$. The negative ion f.a.b. mass spectrum of FAXX revealed the sequence 589 (M − H)$^-$→457→325→193 (ferulic acid − H)$^-$.

The n.m.r. spectra of FAXX and PAXX are shown in Figs. 1–3 and the proposed structures are **1** and **2**.

The quantities of PAXX that were available were much smaller than for FAXX and it was not possible to remove impurities completely from PAXX. The ^{1}H signals at 1.93, 1.36, and 4.08 p.p.m. were believed to arise from acetic and lactic acid; another impurity (3.65–3.75 p.p.m.) could be removed by repeated h.p.l.c. as indicated by the insert in Fig. 1b (3.55–3.85 p.p.m.). The data and figures for PAXX are therefore presented together with those of FAXX (Fig. 1, and Tables II and III). The following discussion relates mainly to FAXX (by analogy, PAXX differs from FAXX in the replacement of the feruloyl group by the *p*-coumaroyl group).

The signals of the ferulic acid protons in FAXX were assigned as follows: δ 7.61 (d, $J_{7,8}$ 16 Hz, H-7), 7.16 (s, H-2), 7.10 (d, H-6), 6.84 (d, $J_{5,6}$ 8 Hz, H-5), and 6.33 (d, H-8). The *p*-coumaric acid protons were assigned as follows: δ 7.71 (d, $J_{7,8}$ 16 Hz, H-7), 7.53 (d, $J_{2,3}$ 9 Hz, H-2), 6.87 (d, H-3), and 6.33 (d, H-8). These assignments were based on a comparison with literature data[12] for ferulic and *p*-coumaric acid. The *J* values of 16 Hz indicated that the phenolic acids occurred as the *trans* isomers[13].

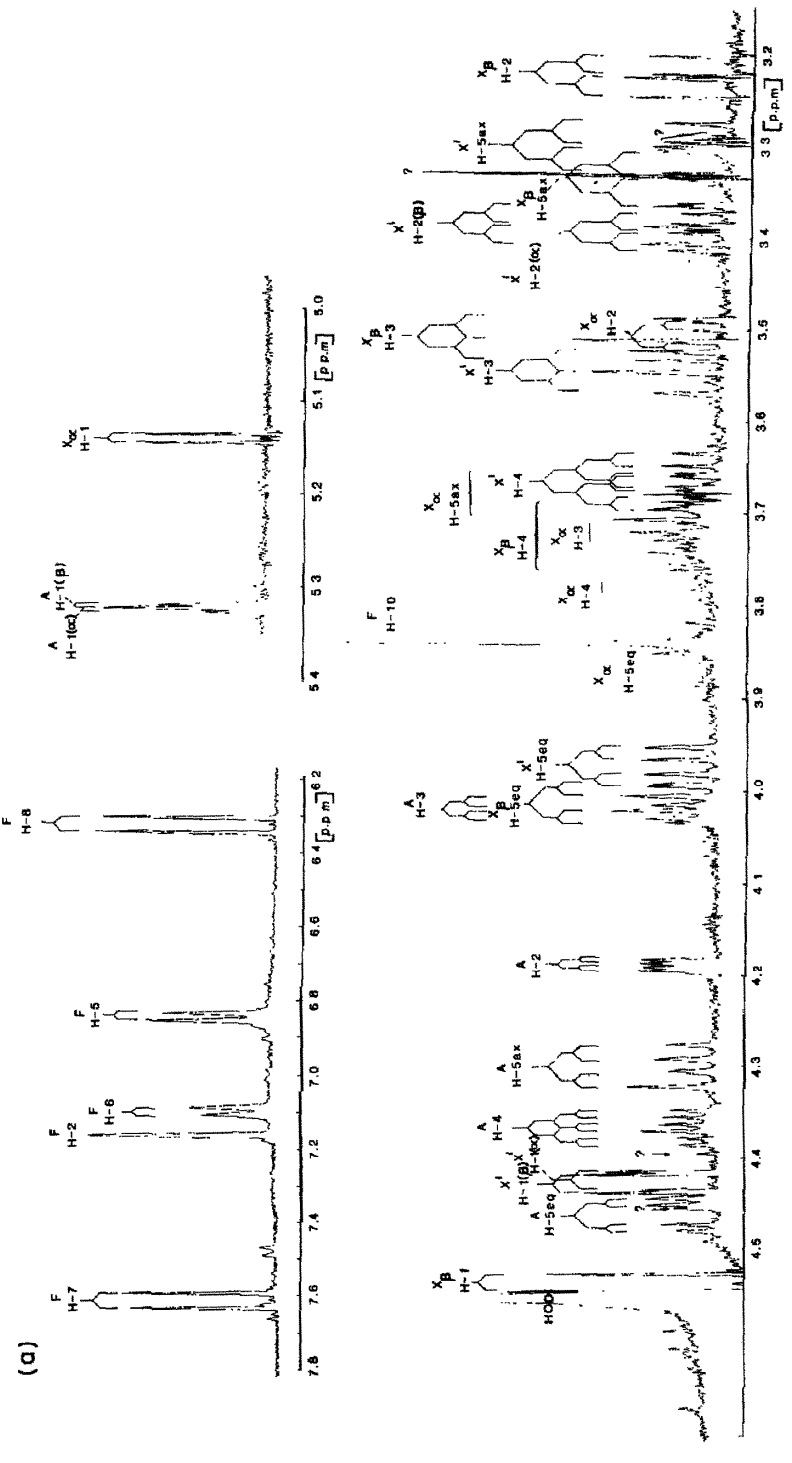
Two-dimensional, ^{1}H homonuclear, *J*-correlated spectroscopy (COSY) (Fig. 2) for FAXX revealed arabinose, β-xylose′, and α- and β-xylose (see **1**). Fig. 1 and Table II give the complete assignment of all sugar protons of FAXX and PAXX based on the 2D spectra. Peaks due to H-3,4,5 of α-xylose and H-4 of β-xylose could not be assigned because of marked second-order effects.

Integration of the signals for anomeric protons gave the ratios ~1.0:1.0:0.3:0.7 for α-L-arabinose, β-D-xylose′, α-D-xylose, and β-D-xylose. The αβ-ratio was 1:2 for the reducing xylose residue in FAXX compared with 3.6:1.0 for xylose, reflecting the effect of the rest of the FAXX molecule. Doubling of peaks was observed for H-1 and H-2 of xylose′ (the H-3 peaks were broadened) and H-1 of arabinose as described by Vliegenthart *et al.*[14].

A comparison of the anomeric shifts and coupling constants for the arabinose residue in FAXX and methyl α- and β-arabinofuranosides[15] indicated the L-arabinose to be α-linked to xylose′. Also, H-1 of arabinose was strongly deshielded (5.32 p.p.m., *cf.* 4.91 p.p.m. reported by Joseleau *et al.*[15]). The arabinose was esterified at O-5 as indicated by the low-field resonances of H-5 (4.30 and 4.46 p.p.m.); H-5 in arabinose would be expected[16] to resonate at ~3.8 p.p.m.

The reducing xylose residue was substituted at O-4; H-4β was deshielded relative to the corresponding proton in free xylose (0.14 p.p.m.), and H-3β and H-5β were also slightly deshielded (H-3, 0.10; H-5βax 0.10; H-5βeq 0.03 p.p.m.).

The assignment of the signals of β-xylose′ was as follows. Slight deshielding was observed for H-2 and H-3 (0.18 and 0.14 p.p.m., respectively), which suggested substitution at either O-2 or O-3. The position of this linkage was unambiguously


(a)
F H-7
F H-2
F H-6
F H-5
F H-8
A H-1(α)
A H-1(β)
Xα H-1
Xβ H-1
HOD
A H-5eq
X' H-1(β)
A H-4
A H-5ax
A H-2
A H-3
Xβ H-5eq
X' H-5eq
F H-10
Xα H-5ax
X' H-4
Xβ H-4
Xα H-3
Xα H-4
Xα H-5eq
Xβ H-3
X' H-3
Xα H-2
X' H-2(β)
X' H-2(α)
Xβ H-5ax
X' H-5ax
Xβ H-2
?
7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 [p.p.m.]
5.4 5.3 5.2 5.1 5.0 [p.p.m.]
4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 [p.p.m.]

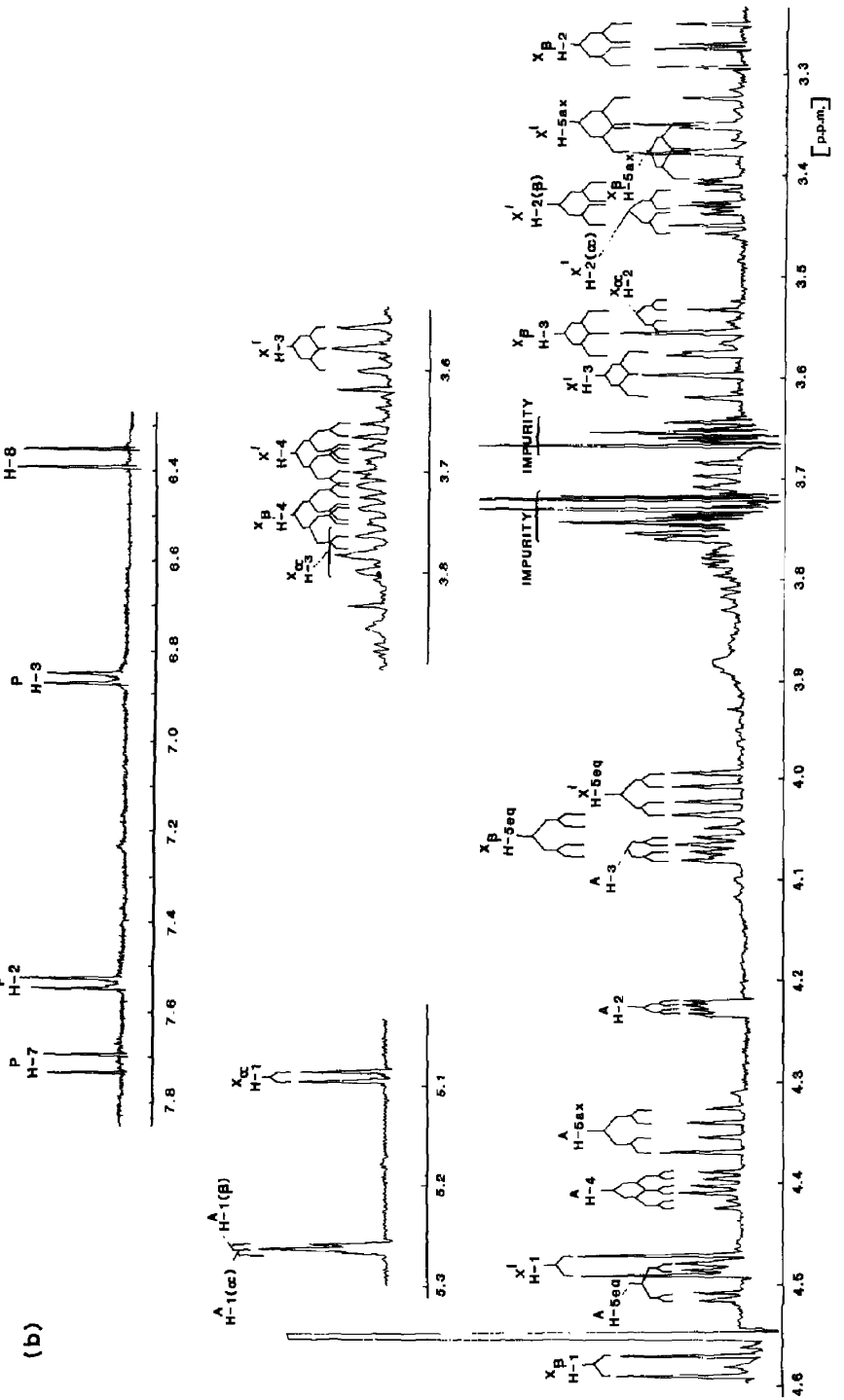


Fig. 1. ^{1}H-N.m.r. spectra of (*a*) FAXX, (*b*) PAXX.

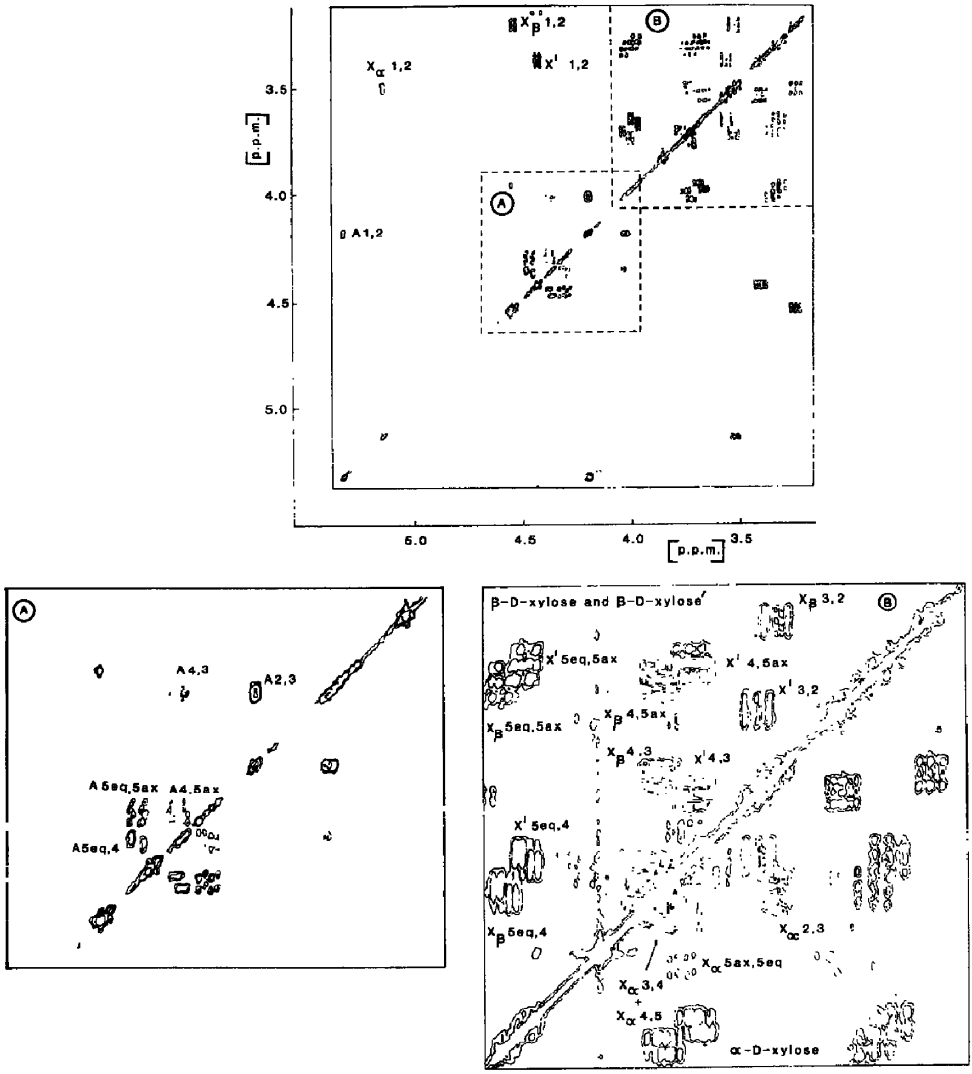


Fig. 2. 2D-COSY ^{1}H-N.m.r. spectrum of FAXX.

assigned by methylation analysis as described below. A shielding effect was noted for H-1 (0.13 p.p.m.).

^{13}C-N.m.r. spectroscopy confirmed the above assignments (Fig. 3, Table IV).

For FAXX, C-1 of the arabinosyl residue resonated 0.9 p.p.m. downfield from that of the corresponding signal of methyl α-L-arabinopyranoside[17]. The arabinosyl C-5 was deshielded by 2–5 p.p.m. in accord with literature data[18–21] (shifts due to ester linkage at CH_2OH and CHOH groups are −1.0 to +3.4 p.p.m.).

For the α- and β-xylosyl residues, the signals for C-4 were shifted by 8–9

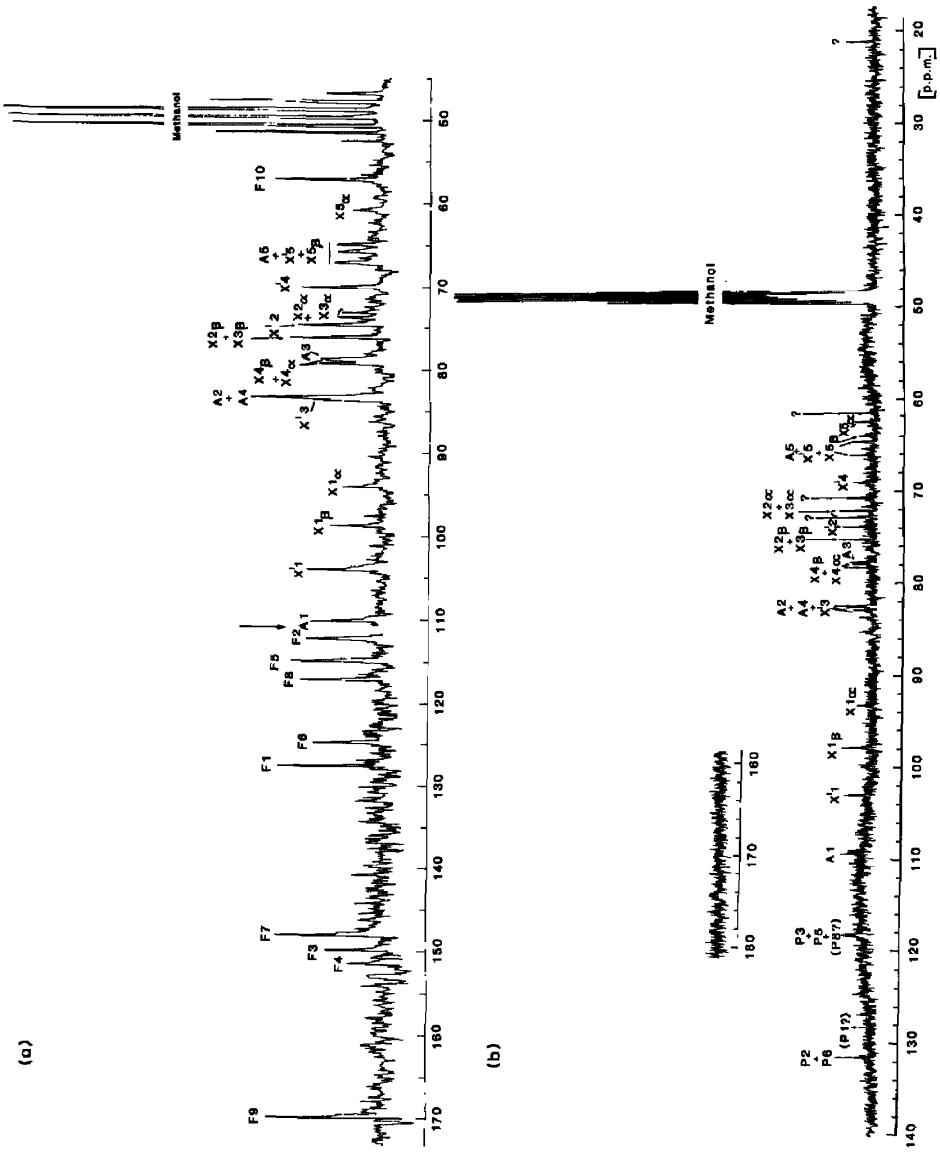
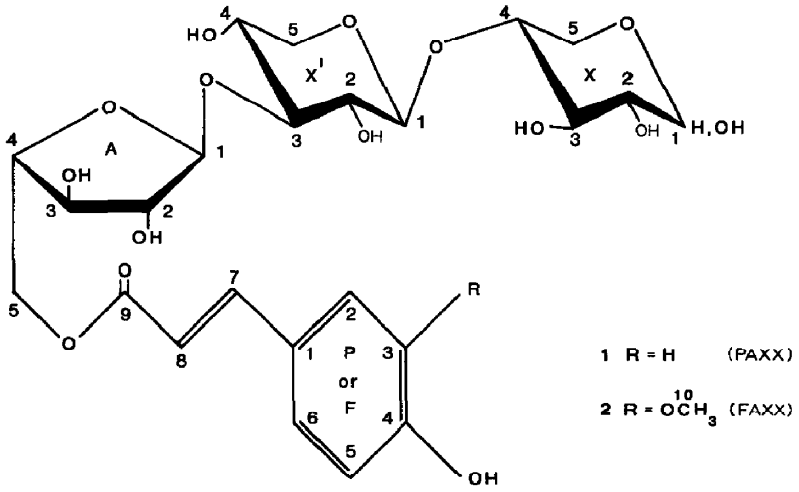


Fig. 3. ^{13}C-N.m.r. spectra of (*a*) FAXX, (*b*) PAXX.



p.p.m. downfield compared to that of the corresponding signal in xylose. These shifts agree with the data of Usui *et al.*[22], who found that the glycosidation shift of β-linked, in contrast to α-linked, glucobioses was similar to the methylation shift of methylated glucoses and was ~ +8 p.p.m. Hence, the linkage between the xylosyl residues in FAXX was β.

Usui *et al.*[22] also found that the glycosidation shift was smaller in α-linked glucobioses (3.2–7.4 p.p.m. downfield). Assuming that these shifts apply to other sugars, then the arabinosyl residue was α-linked to β-D-xylose'; this produced a

TABLE II

ASSIGNMENT OF ^{1}H-N.M.R. SPECTRA

FAXX[a]

	H-1	*H-2*	*H-3*	*H-4*	*H-5ax*	*H-5eq*
α-L-Arabinose	5.32	4.19	4.02	4.37	4.30	4.46
β-D-Xylose'	4.43	3.39	3.55	3.66	3.30	3.97
α-D-Xylose	5.14	3.51	3.72[c]	3.78[c]	3.68[c]	3.86[c]
β-D-Xylose	4.54	3.21	3.51	3.74[c]	3.33	4.01

PAXX[b]

	H-1	*H-2*	*H-3*	*H-4*	*H-5ax*	*H-5eq*
α-L-Arabinose	5.36	4.23	4.07	4.41	4.35	4.50
β-D-Xylose'	4.49	3.43	3.60	3.70	3.35	4.02
α-D-Xylose	5.19	3.54	3.78[c]	—	—	—
β-D-Xylose	4.59	3.27	3.55	3.74[c]	3.38	4.06

[a]In CD_3OD–D_2O at 25° (internal Me_4Si). [b]In D_2O at 25°. [c]Approximate shifts, determined by 2D-n.m.r. spectroscopy.

TABLE III

^{1}H COUPLING CONSTANTS (J, Hz)

FAXX

	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5ax}$	$J_{4,5eq}$	$J_{5,5}$
α-L-Arabinose	1.6	3.4	6.0	6.0	2.8	11.6
β-D-Xylose'	7.8	9.2	9.2	10.2	5.4	11.6
α-D-Xylose	3.6	8.8	6.4?	—	1.8	—
β-D-Xylose	8.0	9.4	9.4	10.4	5.4	11.6

PAXX

	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5ax}$	$J_{4,5eq}$	$J_{5,5}$
α-L-Arabinose	1.6	3.2	6.0	6.0	2.8	11.6
β-D-Xylose'	7.6	9.2	9.2	10.4	5.5	11.6
α-D-Xylose	3.6	8.4	—	—	—	—
β-D-Xylose	8.0	9.2	9.2	10.4	4.8	11.6

TABLE IV

^{13}C-N.M.R. DATA[a]

FAXX

	C-1	*C-2*	*C-3*	*C-4*	*C-5*
α-L-Arabinose	110.1	83.1	78.5	83.1	64.8[d]
β-D-Xylose'	103.8	74.5	83.5	69.8	66.9[d]
α-D-Xylose	93.9	73.5[b]	73.0[b]	79.1[c]	60.5
β-D-Xylose	98.6	76.0	76.0	78.6[c]	65.8[d]

PAXX

	C-1	*C-2*	*C-3*	*C-4*	*C-5*
α-L-Arabinose	109.5	82.7[b]	77.9	83.0[b]	64.2[c]
β-D-Xylose'	103.2	75.4	82.6[b]	69.2	66.2[c]
α-D-Xylose	93.3	72.8[d]	72.3[d]	78.4[e]	62.5
β-D-Xylose	98.0	74.0	75.4	78.0[e]	64.9[c]

[a]In CD_3OD–D_2O at 25° (internal Me_4Si). [b–c]These assignments may be interchanged.

downfield shift of 6.6 p.p.m. which is within the range found by Usui *et al.*[22].

Methylation analysis of PAXX and FAXX gave three major derivatives (Table V), namely, 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol derived from terminal arabinofuranosyl residues, 1,3-di-*O*-acetyl-2,4-di-*O*-methylxylitol derived from (1→3)-linked xylopyranosyl residues, and 1,4-di-*O*-acetyl-2,3-di-*O*-methylxylitol derived from (1→4)-linked xylopyranosyl residues in the ratios 1.0:1.4:1.1 (PAXX) and 1.0:1.1:0.9 (FAXX). Other partially methylated alditol acetates were

TABLE V

METHYLATION ANALYSIS OF PAXX AND FAXX

Glycosyl residue	*Methylated derivative*	*Linkage position(s)*	*Amount (mole %)*[a]	
			PAXX	*FAXX*
Arabinofuranosyl	2,3,5	Terminal	25	31
	2,5	3	1	tr[e]
	3,5	2	4[d]	2
Xylopyranosyl	2,3,4	Terminal	4	2
	2,3	4	28	28
	2,4	3	35	35
	2	3,4	2[b]	1[b]
	4	2,3	1[c]	1[c]
Hexopyranosyl	2,3,4,6	Terminal	1[d]	tr
	2,3,6	4	1	tr
	2,3,4	6	tr	tr

[a]Area divided by molecular weight of derivative. [b,c]Derivatives co-elute. Approximate quantification carried out based on unique ions in mass spectra. [d]Derivatives co-elute (quantification as in *b*,*c*). [e]Trace.

present in much smaller amounts. These results are consistent with the proposed structures of PAXX (**1**) and FAXX (**2**).

DISCUSSION

The results presented here show that cell walls isolated from barley straw yield *O*-[5-*O*-(*trans*-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (PAXX) and *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX) after treatment with "cellulase" (a mixture of polysaccharide hydrolases). The linkage of *trans*-*p*-coumaric acid to cell-wall polysaccharide has not been described in detail hitherto and this is the first report that *trans*-*p*-coumaric acid is linked in the same way to arabinoxylans as *trans*-ferulic acid.

Kato *et al.*[10,12] reported that treatment of sugar-cane bagasse and maize cell-walls with "cellulase" also released FAXX. They determined its structure by using methylation analysis and ^{13}C-n.m.r. spectroscopy; ^{1}H-n.m.r. was used only to detect the anomeric protons. We have also applied high-resolution ^{1}H-n.m.r. and two-dimensional (^{1}H–^{1}H) correlation experiments in the determination of the structures of PAXX and FAXX, which enabled an almost complete assignment of the ^{1}H-n.m.r. spectrum. F.a.b.-m.s. was used to confirm their molecular weights. It has also been reported recently that FAXX is released from isolated barley aleurone layers after treatment with gibberellic acid[23].

The isolation of 2-*O*-[5-*O*-(*trans*-feruloyl)-β-L-arabinofuranosyl]-D-xylopyranose (FAX) from wheat bran was reported by Smith and Hartley[11]. Their ^{1}H-n.m.r. spectrum shows a strong similarity to that presented here. Several proton

peaks that were not resolved at 360 MHz were resolved at 400 MHz and were assigned with the aid of 2D-n.m.r. spectroscopy, indicating the presence of a second xylose residue. The ratios of ferulic acid, arabinose, and xylose in FAX reported by Smith and Hartley[11] were 1:1:1 compared with 1:1:2 in the present work. It seems likely that FAX is identical to FAXX; hydrolysis misleadingly indicated the presence of only one xylose residue. It was suggested[11] that L-arabinose was glycosidically linked to O-2 of xylose, but our methylation analysis data and improved n.m.r. techniques have shown that the L-arabinose is linked to O-3 of xylose'; also, the anomeric configuration of the link is α, and not β as previously suggested. Furthermore, the oligosaccharide component of FAXX has been found in enzymic hydrolysates of several graminaceous arabinoxylans[24].

The calculated yield of FAXX from cell walls of barley straw was 7.75 mg/g of cell walls compared to 12.8–19.5 mg/g of cell walls from different sources of wheat bran[11], and it was estimated that 1 in every 150 pentose residues was feruloylated in wheat bran. For barley straw, we estimated that 1 in every 121 pentose residues was feruloylated and 1 in every 243 pentose residues was *p*-coumaroylated. One in every 31 arabinose residues was esterified with *p*-coumaric acid, and 1 in every 15 with ferulic acid. The quantities of PAXX and FAXX released from cell walls of barley straw by treatment with "cellulase" accounted for one sixth of the *p*-coumaric and half of the ferulic acid released by treatment with sodium hydroxide (calculations based on data from Graham and Åman[8]). The yield of PAXX depended on the extent of grinding before treatment with "cellulase", and PAXX was more difficult to release from cell walls than FAXX[6,25].

EXPERIMENTAL

All manipulations of solutions of phenolic compounds were carried out in "white" fluorescent light to prevent *trans–cis* isomerisation by u.v. radiation[26,27].

Isolation of PAXX and FAXX. — (a) Preparation of cell walls. Air-dried "OECD" barley straw[28] was ground to pass a 0.8-mm sieve. Cell walls were obtained by a modification[29] of the neutral detergent procedure[30]. The cell walls were stored over silica gel.

(*b*) *Fine grinding of cell walls.* Barley-straw cell walls (~10 g) were ground in liquid nitrogen in an Ultra Centrifugal Mill (Glen Creston, London) to pass a 0.08-mm sieve. The walls were re-ground twice and dried over silica gel. The particle size was further reduced by grinding the walls (2 g) in a mill (Sample Mixer Mill, M280, Glen Creston) fitted with a hardened tool-steel grinding cylinder for four 30-min periods. After each period, the mill was allowed to cool for 30 min. The sample was stored over silica gel.

(*c*) *Release of PAXX and FAXX from cell walls.* Finely ground cell-walls (150 mg) were shaken in the dark at 37° for 16 h with "cellulase" (Merck, from *Oxyporus* spp.; activity, 20 mU/mg; 56 mg in 10 mL of distilled water, pH 5.2). The suspension was filtered (glass porosity No. 1) and freeze-dried, and the residue was

stored at −20° before extraction. As a control, "cellulase" (11.25 mg in 2 mL) was incubated with L-arabinose (2 mg), D-xylose (5 mg), and *trans*-*p*-coumaric acid (2.0 mg) or *trans*-ferulic acid (2.0 mg) at 37° for 20 h. No PAXX or FAXX was detected by h.p.l.c. (method 3).

(*d*) *Purification of PAXX and FAXX.* Freeze-dried extracts from 8 batches were combined and water (0.5 mL) was added. The mixture was extracted with methanol (50 mL), and the suspension was filtered (cellulose acetate filter, pore size 0.2 μm; Schleicher & Schüll). This procedure was repeated 7 times. The filtrate was then concentrated at <35°, and a solution of the resulting oil in methanol–water (28:72, 25 mL) was filtered (cellulose acetate filter) and then subjected (1-mL aliquots) to h.p.l.c. using (method 1) a column (25 × 1.0 cm i.d.) of Spherisorb 5 ODS-1 (Phase Separations) and methanol–water (28:72) at 3 mL/min. The eluate was monitored at 250 nm with a Pye–Unicam LC3 u.v. detector. Compounds having peaks with retention times of 23–28 min (PAXX) and 28–39 min (FAXX) were collected. The eluates containing PAXX and FAXX (1 vol.) were diluted with water (3 vol.) and freeze-dried.

A solution of the foregoing product in the minimum volume of methanol–water (22:78) was filtered (cellulose acetate filter), and aliquots (~1 mL) were applied to a column (25 × 1.0 cm i.d.) of Spherisorb 5 ODS-1) and eluted with methanol–water (22:78) at 6 mL/min. The eluate was monitored at 280 nm (method 2). Compounds which eluted between 28–34 (PAXX) and 34–53 min (FAXX) were collected and freeze-dried.

H.p.l.c. (method 3) involved a column (25 × 0.5 cm i.d.) of Spherisorb 5 ODS-1, elution with methanol–water (22:78) at 2 mL/min, and monitoring at 325 nm.

T.l.c. was performed on cellulose plates (Schleicher & Schüll, F1440) with *1*, formic acid–water (4:96); *2*, toluene–formic acid–water (80:90:30, upper phase); *3*, ethyl acetate–pyridine–water (60:30:20); *4*, 1-propanol–ethyl acetate–water (60:10:30); and detection with u.v. radiation (364 nm) and diazotised *p*-nitroaniline to locate phenolic acids[31,32], or aniline hydrogenphthalate for sugars[33].

Characterisation of PAXX and FAXX. — (*b*) *Hydrolysis with acid.* PAXX (0.5 mg) or FAXX (0.5 mg) was hydrolysed with 2.5M trifluoroacetic acid (0.5 mL) for 2 h at 100° under argon. After cooling, the solution was concentrated to dryness with a stream of nitrogen. The residue was dissolved in H_2O (0.1 mL) and 13M ammonia (20 μL), and then a freshly prepared solution of borodeuteride in re-distilled diglyme (M, 0.25 mL) was added. Reduction was carried out for 90 min at 40°. Excess of sodium borodeuteride was then decomposed by adding 18M acetic acid (0.1 mL)[34,35]. The alditol acetates formed were analysed by g.l.c. and g.l.c.–m.s. G.l.c. was performed on a Hewlett–Packard 5890A gas chromatograph fitted with a flame-ionisation detector, dedicated cool on-column capillary inlet, and a Silar 10C support-coated open tubular (SCOT) glass-capillary column[34] (28 m × 0.5 mm i.d.). Ultra-high-purity helium was the carrier gas at 7 mL/min. Temperature programme: 38° 30 s, →190° at 70°/min, 190→230° at 3°/min, 230° for 10 min.

The detector temperature was held at 250°. Peak areas were recorded with a Hewlett–Packard model 3392A integrator. *myo*-Inositol hexa-acetate was the internal standard.

(*b*) *Hydrolysis with "cellulase"*. FAXX (1 mg) was dissolved in 0.1M sodium acetate buffer (pH 5.0, 1 mL) and "cellulase" (type I, 5 mg) from *Aspergillus niger* (Sigma) was added. The mixture was incubated at 37° for 22 h, filtered, and analysed for sugars by t.l.c. (solvent *3*) and h.p.l.c. (method 4), and for ferulic acid by t.l.c. (solvents *1* and *2*).

H.p.l.c. (method 4) involved a column (300 × 7.8 mm i.d.) of Aminex HPX 87P (BioRad) at 80° and elution with water at 0.6 mL/min, and detection with a refractive index detector, Refracto Monitor III (LDC).

(*c*) *Methylation analysis*. The procedure of Harris *et al.*[36] was used. PAXX (0.5 mg) or FAXX (0.5 mg) was dissolved in dimethyl sulphoxide (0.2 mL) and subjected to two rapid preliminary methylations. Sodium borodeuteride, instead of sodium borohydride, was used in the preparation of the partially methylated alditol acetates which were identified[37] by e.i.-m.s.

Alditol acetates and partially methylated alditol acetates were identified by their mass spectra and their g.l.c. retention times relative to that of *myo*-inositol hexa-acetate[38]. G.l.c.–m.s. was performed with a fully automated Finnigan MAT 1020B instrument. Alditol acetates and partially methylated alditol acetates were separated on a BP75, vitreous-silica, wall-coated, open tubular (WCOT) column[38] (25 m × 0.22 mm i.d.). Temperature programmes: alditol acetates, 170→250° at 5°/min, 250° for 10 min; partially methylated alditol acetates, 150→250° at 4°/min, 250° for 10 min. Compounds eluted from the column were detected using the total ion current [reconstructed ion chromatogram (r.i.c.)] by scanning from *m/z* 100 to 350 in 0.5 s for alditol acetates and 0.3 s for partially methylated alditol acetates. The amounts of partially methylated alditol acetates were determined as molar percentages calculated from the peak areas of the r.i.c.

(*d*) *N.m.r. spectroscopy*. ^{1}H-N.m.r. spectra (400 MHz) were recorded with a Bruker WH 400 spectrometer, PAXX (5 mg) was dissolved in 99.996 atom % D_2O (3 mL) and FAXX (~5 mg) was dissolved in 99.96 atom % CD_3OD–99.996 atom % D_2O (1:3, 3 mL). The diameter of the n.m.r. tube was 5 mm. All spectra were run with a field/frequency lock at 323 ±1 K. Spectral resolution was enhanced by a Lorentzian–Gaussian transformation with initial line-narrowing of typically −2.5 Hz. Chemical shifts are reported in p.p.m. relative to internal MeOH (a known impurity) at 3.35 p.p.m.

Homonuclear H,H, *J*-correlated spectroscopy was performed with the COSY-45 (N-type) programme[39], using standard Bruker software. The time domain matrix of 1024 t_2 points × 256 t_1 points was zero-filled to 1024 × 512 before transformation to give a digital resolution of 3.99 Hz/pt in both dimensions for PAXX (1.75 Hz/pt for FAXX). The total number of scans was 128 for PAXX (48 for FAXX) for each t_1 point with a waiting time of 4.0 s for PAXX (and 2.0 s for FAXX) between scans for relaxation. A sine-bell window function was applied

before each Fourier transformation and the resulting matrix was symmetrised across the diagonal only for PAXX before plotting the contour map.

For ^{13}C-n.m.r. spectra, PAXX (5 mg) or FAXX (10–20 mg) was dissolved in a minimum of 99.96 atom % CD_3OD–D_2O (1:3, 3–5 mL). The FAXX spectrum was recorded at room temperature with a Jeol FX 90Q n.m.r. spectrometer at 22.5 MHz. The probe diameter was 5 mm and the number of scans required was 49929 for FAXX. The PAXX spectrum was recorded at room temperature on a Bruker WH 400 spectrometer at 100 MHz. The probe diameter was 5 mm and the number of scans required was 70726.

(*e*) *Mass spectrometry.* F.a.b.-m.s. was performed on a MS 902 Kratos spectrometer. The accelerating potential was 8 kV, and a potential of 6–7 kV was applied to the xenon gun. Measurements were made with a Xe gas pressure of $\sim 1.3 \times 10^{-3}$ Pa, measured immediately above the source diffusion pump. Samples of PAXX or FAXX were introduced on a copper-probe tip at room temperature. Solutions of the samples in water or HCl were transferred to the probe tip and concentrated to dryness in the vacuum lock. The probe was removed and glycerol (1 μL) was added to dissolve the residue on the copper surface before re-insertion into the source.

Yields of PAXX and FAXX from cell walls. — An aqueous solution (2 mL) of "cellulase" (11.25 mg; Merck) was added to finely ground cell-walls (30 mg). The mixture was incubated in the dark at 37° for 16 h and then filtered, and 100 μL of the supernatant solution was analysed by h.p.l.c. (method 3). Quantitative measurements were made by comparing peak areas due to PAXX and FAXX with those of external standards (0.438 mg of PAXX in 2.0 mL of H_2O; 0.649 mg of FAXX in 2.0 mL of H_2O). Corrections were made for the overlapping of the α-PAXX and β-PAXX peaks.

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REFERENCES

1 S. Z. El-Basyouni, A. C. Neish, and G. H. N. Towers, *Phytochemistry*, 3 (1964) 627–639.
2 T. Higuchi, Y. Ito, and I. Kawamura, *Phytochemistry*, 6 (1967) 875–881.
3 R. D. Hartley, *J. Sci. Food Agric.*, 23 (1972) 1347–1354.
4 R. D. Hartley and E. C. Jones, *Phytochemistry*, 15 (1976) 1157–1160.
5 P. J. Harris and R. D. Hartley, *Nature (London)*, 259 (1976) 508–510.
6 R. D. Hartley and J. Haverkamp, *J. Sci. Food Agric.*, 35 (1984) 14–20.

7 X. ALIBES, F. MUÑOZ, AND R. FACI, *Anim. Feed Sci. Technol.*, 10 (1984) 239–246.
8 H. GRAHAM AND P. ÅMAN, *Anim. Feed Sci. Technol.*, 10 (1984) 199–211.
9 P. J. VAN SOEST, A. MASCARENHAS FERREIRA, AND R. D. HARTLEY, *Anim. Feed Sci. Technol.*, 10 (1984) 155–164.
10 A. KATO, J. AZUMA, AND T. KOSHIJIMA, *Chem. Lett.*, (1983) 137–140.
11 M. M. SMITH AND R. D. HARTLEY, *Carbohydr. Res.*, 118 (1983) 65–80.
12 Y. KATO AND D. J. NEVINS, *Carbohydr. Res.*, 137 (1985) 139–150.
13 L. M. JACKMAN AND S. STERNHELL, *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*, 2nd edn., Pergamon Press, Oxford, 1972, pp. 202 and 301.
14 J. F. G. VLIEGENTHART, L. DORLAND, AND H. VAN HALBEEK, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 209–374.
15 J.-P. JOSELEAU, G. CHAMBAT, M. VIGNON, AND F. BARNOUD, *Carbohydr. Res.*, 58 (1977) 165–175.
16 A. C. RICHARDSON, personal communication.
17 R. G. RITCHIE, N. CYR, B. HORSCH, H. J. KOCH, AND A. S. PERLIN, *Can. J. Chem.*, 53 (1975) 1424–1433.
18 D. R. BUNDLE, H. J. JENNINGS, AND T. C. P. SMITH, *Can. J. Chem.*, 51 (1973) 3812–3818.
19 E. D. DORMAN AND J. D. ROBERTS, *J. Am. Chem. Soc.*, 93 (1971) 4463–4472.
20 P. S. PASHKOV AND O. S. CHIZHOV, *Bioorg. Khim.*, 3 (1976) 437–497.
21 S. N. ROSENTHAL AND J. H. FENDLER, *Adv. Phys. Org. Chem.*, 13 (1976) 279–424.
22 T. USUI, N. YAMAOKA, K. MATSUDA, K. TUZIMURA, H. SUGIYAMA, AND S. SETO, *J. Chem. Soc., Perkin Trans. 1*, (1973) 2425–2432.
23 F. GUBLER, A. E. ASHFORD, A. BACIC, A. B. BLAKENEY, AND B. A. STONE, *Aust. J. Plant Physiol.*, 12 (1985) 307–317.
24 R. F. H. DEKKER AND G. N. RICHARDS, *Adv. Carbohydr. Chem. Biochem.*, 32 (1976) 277–352.
25 R. D. HARTLEY AND E. C. JONES, *J. Sci. Food Agric.*, 29 (1978) 92–98.
26. G. KAHNT, *Phytochemistry*, 6 (1967) 755–758.
27 R. D. HARTLEY AND E. C. JONES, *J. Chromatogr.*, 107 (1975) 213–218.
28 O. THEANDER, R. D. HARTLEY, AND C. S. STEWART, *Anim. Feed Sci. Technol.*, 10 (1984) 89–91.
29 R. D. HARTLEY, E. C. JONES, AND J. C. FENLON, *J. Sci. Food Agric.*, 25 (1974) 947–954.
30 P. J. VAN SOEST AND R. H. WINE, *J. Assoc. Off. Anal. Chem.*, 50 (1967) 50–55.
31 T. SWAIN, *Biochem. J.*, 53 (1953) 200–208.
32 P. RIBÉREAU-GAYON, in V. H. HEYWOOD (Ed.), *Plant Phenolics*, Oliver and Boyd, Edinburgh, 1972, p. 94.
33 K. G. KREBS, D. HEUSSER, AND H. WIMMER, in E. STAHL (Ed.), *Thin-layer Chromatography*, 2nd edn, Springer–Verlag, Berlin, 1969, p. 900.
34 A. B. BLAKENEY, P. J. HARRIS, R. J. HENRY, AND B. A. STONE, *Carbohydr. Res.*, 113 (1983) 291–299.
35 R. J. HENRY, P. J. HARRIS, A. B. BLAKENEY, AND B. A. STONE, *J. Chromatogr.*, 262 (1983) 249–256.
36 P. J. HARRIS, R. J. HENRY, A. B. BLAKENEY, AND B. A. STONE, *Carbohydr. Res.*, 127 (1984) 59–73.
37 H. BJÖRNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
38 A. BACIC, P. J. HARRIS, E. W. HAK, AND A. E. CLARKE, *J. Chromatogr.*, 315 (1984) 373–377.
39 A. BAX AND R. FREEMAN, *J. Magn. Reson.*, 44 (1981) 542–561.