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OCCURRENCE AND NATURE OF FERULIC ACID SUBSTITUTION OF CELL-WALL POLYSACCHARIDES IN GRAMINACEOUS PLANTS

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ABSTRACT

Cell walls of wheat bran were treated with *Oxyporus* "cellulase" (a mixture of polysaccharide hydrolases) in order to release compounds containing ferulic acid bound to carbohydrate. The major feruloyl compound released has been identified as 2-*O*-[5-*O*-(*trans*-feruloyl)-β-L-arabinofuranosyl]-D-xylopyranose. Similar treatment of the cell walls of other graminaceous plants also released this compound.

INTRODUCTION

Ferulic (4-hydroxy-3-methoxycinnamic) and *p*-coumaric (4-hydroxycinnamic) acids, mainly in the *trans* configuration, are covalently bound to cell walls of plants from several families, including the economically important Gramineae[1,2]. Small amounts (<1–2.5% walls) of these phenolic acids can be released from graminaceous cell-walls on treatment with alkali[3,4].

Ferulic and *p*-coumaric acids are thought to be esterified to cell-wall polysaccharides, because of their release from cell walls on treatment with alkali and because spectral changes accompanying the addition of alkali are diagnostic of ester bond-cleavage[5,6]. Ferulic acid is thought to be bound to pentosans, since a fraction of the water-soluble pentosans from wheat flour comprised xylose and arabinose residues together with a small proportion of ferulic acid[7,8]. Hartley[6] found that small molecules containing bound ferulic acid were released from cell walls of shoots of *Lolium multiflorum* Lam. on incubation with a commercial "cellulase" consisting of a mixture of polysaccharide hydrolases from *Oxyporus* spp. We have now treated cell walls of wheat bran and other graminaceous tissues with a different batch of "cellulase" from the same fungal source and, from the hydrolysates, have isolated and characterised the major oligosaccharide to which ferulic acid is esterified.

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RESULTS AND DISCUSSION

Previous work[9,10] indicated a high content of ferulic acid covalently bound to aleurone cell-walls of wheat and barley. The bran fraction is rich in aleurone, and a cell-wall fraction was therefore prepared from wheat bran. Ferulic acid is the preponderant phenolic acid (although only a trace component of the wall) released from bran cell-walls by treatment with NaOH (Table I). Enzymic hydrolysates ("cellulase" *Oxyporus* spp.) of these walls contained (l.c., systems *2* and *3*) a single component (FAX) that accounted for all of the ferulic acid esterified to the walls.

Walls from three different wheat-brans were examined, and those from cv. "Flanders" were isolated on a large scale because they contained the highest levels of bound ferulic acid (Table II).

A kinetic study of the degradation of cell walls of wheat bran showed that

TABLE I

AMOUNTS OF PHENOLIC ACIDS RELEASED FROM WHEAT BRAN CV. FLANDERS BY TREATMENT WITH NaOH

Phenolic acid	*Mg/g of cell wall*[a]	*Retention time*[b] *(min)*
trans-Ferulic acid	6.60	33.3
p-Hydroxybenzoic acid	0.034	10.0
trans-p-Coumaric acid	0.038	24.8
Vanillic acid	Trace	13.0
Unknown A	Trace	16.5
Unknown B	Trace	23.0

[a]Dry-weight basis. [b]L.c. (system 2), see Experimental. Phenolic acids identified by co-chromatography with the reference compound

TABLE II

PHENOLIC COMPOUNDS RELEASED FROM CELL WALLS FROM DIFFERENT SOURCES OF WHEAT BRAN BY TREATMENT WITH NaOH OR "CELLULASE"

Wheat bran	*Phenolic compounds released from cell wall (mg/g of cell wall)*			
	Filtrate from NaOH treatment[a]		*Filtrate from "cellulase" treatment*[b]	
	Absorbance method (trans-ferulic acid equiv.[c]*)*	*L.c. method (trans-ferulic acid content)*	*After 24 h (trans-ferulic acid equiv.*[d]*)*	*After 44 h*
cv. Flanders	12.8	6.6	5.4	6.4
cv. Huntsman	9.6	3.8	3.4	4.2
Durum	10.7	5.0	4.3	5.0

[a]Cell walls treated with M NaOH. [b]Cell walls treated with "cellulase" (*Oxyporus* spp.) solution. [c]Calc. from absorbance at 347 nm, by comparison with ε of sodium *trans*-ferulate at 347 nm. [d]Calc. from absorbance at 325 nm, with ε of *trans*-ferulic acid at the same wavelength. The peak area of FAX (A_{325}) (l.c., system *3*), relative to *trans*-ferulic acid, confirmed that FAX was the only phenolic compound present

rapid hydrolysis was practically complete after incubation for 24 h with the *Oxyporus* "cellulase". After prolonged enzymic hydrolysis, the carbohydrate–ferulic acid complexes accounted for all of the ferulic acid released from wheat-bran walls by treatment with NaOH (Table II; *cf.* l.c. assay of ferulic acid, and with that released by treatment with "cellulase").

FAX is soluble in water, methanol, ethanol, and 2-methoxyethanol, and slightly soluble in 1,4-dioxane and diethyl ether, and could be extracted with methanol from polymeric components (protein and polysaccharide) of the water-soluble fraction of the enzymic hydrolysate. The final step in the purification involved reverse-phase l.c. (system *3*), in which FAX was retarded relative to most of the more polar molecules by virtue of its aromatic function. This enabled the carbohydrate–ferulic ester to be separated from the large quantities of mono- and oligo-saccharides in the enzymic hydrolysates.

Reverse-phase l.c. of FAX consistently gave two peaks that could be fully resolved with methanol–water (system *3*). These two compounds were in the ratio of 2:1 (peak area), with retention times (*T*) of 19.3 and 23.9 min. When the ascending part of the first peak or the descending part of the second was collected separately and immediately re-chromatographed, the two peaks were again obtained in the same ratio. This indicated a rapidly equilibrating system and, in subsequent work, the fractions corresponding to both peaks were combined for chemical identification.

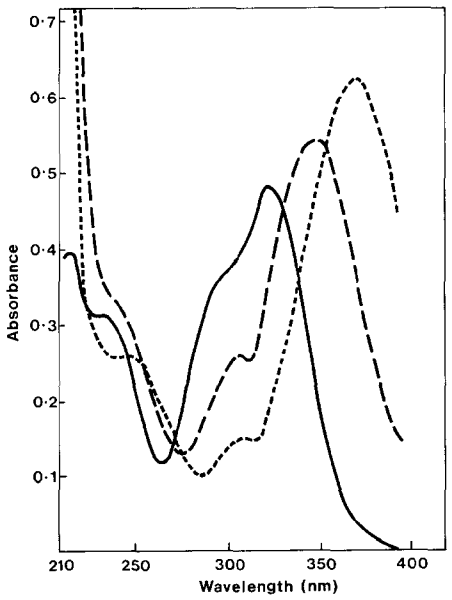


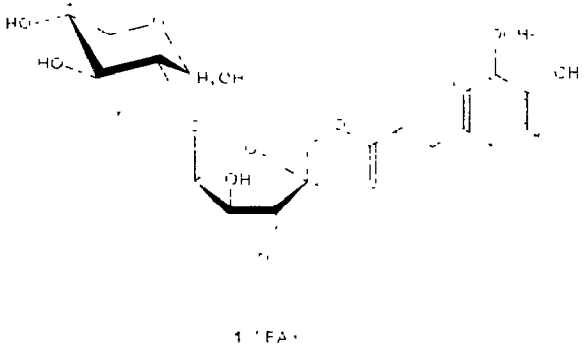
Fig. 1. Absorption spectra of FAX: ——, spectrum of FAX (0.02 mg) in water (1.0 mL), t = 0 min; -----, spectrum of FAX (0.02 mg) in 0.1M NaOH (1.0 mL), t = 30 s after addition of the alkali; — —, spectrum of FAX (0.02 mg) in 0.1M NaOH (1.0 mL), t = 75 min after addition of the alkali.

FAX behaved as one compound when examined by t.l.c. (systems *1*–*6*) and l.c. (systems *1* and *2*).

Identification of FAX as 2-O-[5-O-(trans-*feruloyl*)-β-L-*arabinofuranosyl*]-D-*xylopyranose* (**1**) — (*a*) *Complete hydrolysis.* FAX gave colour reactions typical of a pentose and of ferulic acid (aniline hydrogenphthalate, diazotised *p*-nitroaniline, u.v.-induced fluorescence), but its chromatographic mobility in t.l.c. and l.c. differed from those of a wide range of phenolic acids and mono- and oligo-saccharide reference-compounds. After alkaline or enzymic hydrolysis (*Aspergillus niger* "cellulase"), xylose, arabinose, and *trans*-ferulic acid were obtained in the molar ratios of 1.00:0.92:1.02. Xylose and arabinose were also identified as components of FAX hydrolysates by t.l.c. (solvent 5); *trans*-ferulic acid was identified by t.l.c. with solvents *1* and *2*.

(*b*) *U.v.-absorption spectra.* The spectral shifts of FAX (Fig. 1) on addition of alkali or sodium ethoxide are consistent with those of a compound containing ferulic acid ester-linked at its carboxyl group[5]. The initial shift of the absorption maximum from 325 to 370 nm immediately after the addition of dilute NaOH (0.1M final) probably represents the formation of the sodium salt of the phenolic hydroxyl-group. The subsequent hypsochromic shift to a maximum at 347 nm after 75 min represents the formation of sodium *trans*-ferulate.

The bathochromic shift in the absorption maximum from 327 to 388 nm on addition of sodium ethoxide[5] to a solution of FAX in ethanol confirms that the phenolic hydroxyl-group formed the sodium salt. The magnitude of the shift compared with that for ferulic acid indicates that the carboxyl group is esterified[5].



1 : FAX

(*c*) ^{1}*H-N.m.r. spectrum.* The 360-MHz spectrum in D_2O (see **1** for identification of protons) exhibited signals characteristic of a *trans*-feruloyl group. δ 6.31 (d, 1 H, $J_{3,2}$ 16.3 Hz, H-3), 6.87 (d, 1 H, $J_{8,9}$ 8.0 Hz, H-8), 7.09 (1 H, $J_{9,8}$ 8.0 Hz, H-9), 7.14 (s, 1 H, H-5), 7.61 (d, 1 H, $J_{2,3}$ 16.3 Hz, H-2), and methoxy protons at δ 3.85 (s, 3 H, H-10). The coupling constants of the side-chain double-bond protons indicated the *trans*-configuration[11].

The spectrum (Fig. 2) is also consistent with a pyranose sugar at the reducing

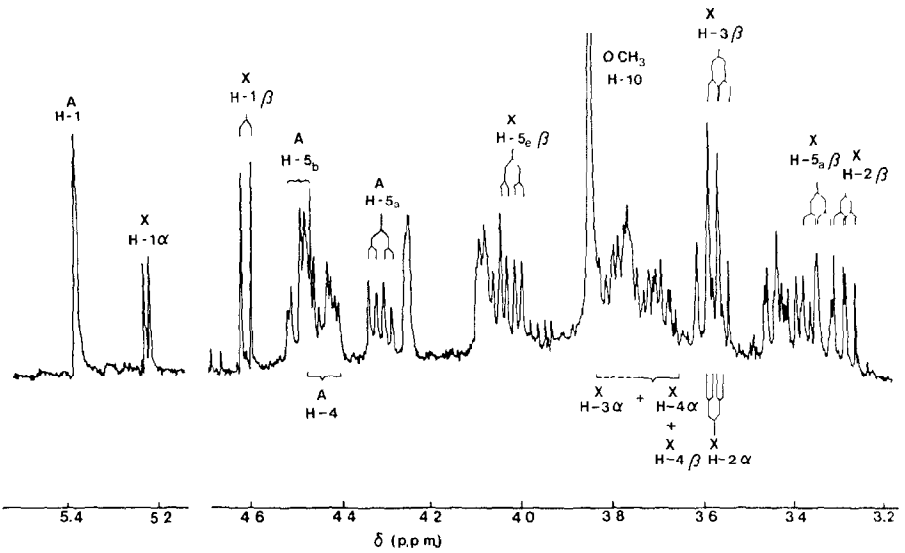


Fig. 2. 360-MHz ^{1}H-n.m.r. spectrum of FAX recorded in D_2O at 21° (X = xylose, A = arabinose).

end, the anomeric (X–H-1) α (δ 5.22, d, J 4.2 Hz) and β (δ 4.62, d, J 7.7 Hz) forms being in the ratio of ~1:2 and accounting for a total of one proton. The anomeric proton (A–H-1) of the furanose ring gave a single, asymmetric and broad peak (δ 5.38), consistent with a 1-*O*-linked furanose, but further downfield than expected (*cf.* δ 4.88 and 4.86 in refs. 12 and 17). The shift and 1,2-coupling do not allow assignment of the anomeric configuration[12].

Decoupling of the xylose H-1α and H-1β signals enabled identification of the signals for xylose H-2α (δ 3.57, dd, J 4.2, 9.0 Hz) and H-2β (δ 3.29, dd, J 9.2, 7.7 Hz). Esterification of HO-2 of xylose would produce a downfield shift of ~1 p.p.m. for the corresponding proton[13], indicating that the ferulic acid is not linked at this position. Similarly, by decoupling xylose H-2β, assignment of xylose H-3β (δ 3.56,

TABLE III

ASSIGNMENT OF SIGNALS IN ^{13}C-N.M.R. SPECTRUM OF FAX[a]

Residue	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*
β-L-Arabinofuranosyl	102.6	77.7	77.4	82.7	73.6
α-D-Xylopyranose	92.9	82.0	72.3	71.8	63.9
β-D-Xylopyranose	97.3	82.3	74.8	68.6	65.9

	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6,7*	*C-8*	*C-9*	*C-10*
trans-Feruloyl	~172	114.3	141.6	124.6	109.0	147.6	112.6	117.0	56.7

[a]In D_2O at 25°. Chemical shifts are expressed as p.p.m. relative to tetramethylsilane. For identification of C atoms, see **1**.

dd, J 7.7, 9.1 Hz) and tentative assignments of xylose H-3α, H-4α, and H-4β in the region of δ 3.65–3.83 suggested that the ferulic acid is not esterified to xylose.

The arabinose H-5a (δ 4.30, dd, J 12.0, 6.7 Hz) and b (δ ~4.5, complex multiplet) signals suggest that the ferulic acid is esterified to O-5 of arabinose. Esterification of a primary hydroxyl-group results in a downfield shift of ~0.5 p.p.m. for the corresponding protons, whereas unsubstituted arabinofuranose H-5 signals would normally occur in the region of 3.7–4.1 p.p.m.

Proton signals were not assigned for arabinose H-2 and H-3 and for xylose H-5$a\alpha$ and H-5$e\beta$.

(*d*) *^{13}C-N.m.r. spectrum.* Assignments of the signals in the 90-MHz ^{13}C-n.m.r. spectrum in D_2O are given in Table III, in p.p.m. relative to tetramethylsilane.

Signals for the C-H type of aromatic atom of the feruloyl group are stronger than, and upfield from, those for C-4, C-6, and C-7 (see **I** for numbering of carbon atoms). The C-1 signal may occur at ~172 p.p.m., but was very weak. The C-6 and C-7 signals could not be distinguished from one another at 147.6 p.p.m. The signal at 141.6 p.p.m. was assigned to C-3. Assignments for C-2, C-4, C-8, and C-9 are 114.3, 124.6, 112.6, and 117.0 p.p.m., respectively, and agree with published values for ferulic esters[14,15]. The signal at 109.0 p.p.m. was assigned to C-5 and that at 56.7 p.p.m. to the methoxyl carbon (C-10) of the feruloyl group.

Because the signal at 109.0 p.p.m. is more compatible[14,15] with that of feruloyl C-5, the arabinose C-1 signal must be that at 102.6 p.p.m.[12,16,17], which is consistent with a β-L-furanosyl residue at the non-reducing end[12,16,17]. The assignment of the arabinose C-5 signal at 73.6 p.p.m. is consistent with the feruloyl residue being esterified to this position, with a downfield shift of +9.3 p.p.m.

Assignments of the xylose signals are more compatible with 2- rather than 3-substitution. The β and α anomeric carbons were assigned the signals at 97.3 and 92.9 p.p.m., respectively, representing shifts of −0.3 and −0.4 p.p.m. compared with unsubstituted xylose[18]. The 2β and 2α positions were assigned the signals at 82.3 and 82.0 p.p.m., representing shifts of +7.2 and +9.5 p.p.m. compared with unsubstituted xylose[18]. If O-3 were substituted, a signal at ~84 p.p.m. would be expected for the β configuration; this was not observed. The results agree reasonably well with published data[19] for a 2-substituted, reducing xylose residue. The intensity of the signals at 97.3, 72.3, and 71.8 p.p.m. was much lower than expected and probably suggests that the C-1β and the C-3α and C-4α positions are sterically hindered. This is consistent with other data [see (*i*) below].

Some decomposition may have occurred or a small proportion of impurities may have been present, as unassigned signals were observed at 96.8, 59.9 (weak intensity), and 64.6 p.p.m. (moderate intensity).

(*e*) *G.l.c. of trimethylsilylated FAX (Me$_3$Si-FAX).* The T value of Me$_3$Si-FAX is consistent with that of a feruloyl disaccharide derivative[20] (Table IV). G.l.c. of Me$_3$Si-FAX gave two peaks in the ratio of 2:1 (as did reverse-phase l.c. of FAX). Formation of the oxime prior to trimethylsilylation resulted in only one

peak, indicating that the reducing end of the disaccharide component is free.

(f) G.l.c.–m.s. of Me_3Si-FAX. The mass spectrum of Me_3Si-FAX contained peaks at *m/z* (relative intensity): 349 (219), 348 (18), 260 (492), 259 (2224), 258 (61), 245 (44), 243 (176), 219 (668), 217 (7184), 204 (2088), 191 (1003), 169 (915), 147 (756), 145 (439), 133 (178), 129 (1422), 117 (375), 115 (464), 103 (1558), and 73 (4072). There were no ions that could be used to predict the molecular weight of FAX in the e.i. mass spectrum of Me_3Si-FAX or in the direct-insertion, c.i. mass spectrum of FAX. However, the mass spectrum of Me_3Si-FAX does indicate that the molecule contains feruloyl and pentose residues, including a pentofuranose at the non-reducing end. Ions of *m/z* 349, 348, 259, 258, 245, 243, and 169 are typical of the fragmentation of a trimethylsilylated, unsubstituted, pentofuranosyl residue at the non-reducing end[21].

The ions at *m/z* 259 and 260 could arise from a doubly substituted pentopyranose fragment. The fragment with *m/z* 467 is the pentose equivalent of the hexose *m/z* 569 peak present in the mass spectra of all disaccharides, which is said to come from the reducing-end (singly substituted) residue and to indicate the type of linkage or substitution[22]. Its presence could support a singly substituted xylose-residue. However, the negligible intensity of the ion is much less than predicted[22]. Thus, the m.s. evidence is consistent with a pentofuranose at the non-reducing end

TABLE IV

G L C OF TRIMETHYLSILYL DERIVATIVES[a]

Compound	*Column temperature (degrees)*	*Retention time (min)*
Monomer		
trans-Ferulic acid	150	6.3
	220	~1.0
Dimers		
1-*O*-(*trans*-*p*-Coumaroyl)-β-D-glucose	230	~0.5
Xylobiose	220	1.3
	230	~0.7
Cellobiose	150	[b]
	220	2.5, 2.9
	230	1.6
Oxime of cellobiose	230	1.4
Trimers		
2-*O*-[5-*O*-(*trans*-Feruloyl)-β-L-arabino-furanosyl]-D-xylopyranose (FAX)	150	[b]
	220	9.4, 10.2
	230	5.4, 5.8
Oxime of FAX	230	4.2
Cellotriose	220	30.0
	230	17.9, 21.9

[a]Column of 0.2% of OV-25 on Gas Chrom Q, 1.5 m × 4 mm i.d. [b]Was not eluted.

and perhaps a doubly substituted pentopyranose at the reducing end.

The two-carbon fragment *m/z* 204, originating from C-2–C-3 and C-3–C-4 of the pentose ring[21], is abundant. Other fragments of trimethylsilylated pentoses are those with *m/z* 217 (C-3–C-5, base peak) and 129 (abundant). Ions corresponding to CH_2-O^+-$SiMe_3$ (*m/z* 103) and Me_3Si^+ (*m/z* 73) are abundant. Ions derived from the trimethylsilylated feruloyl group are probably those with *m/z* 219, 191, and 117, as the spectrum of trimethylsilylated methyl *trans*-ferulate gave the following principal ions: *m/z* (relative intensity): 280 (M^+, 273), 250 ($M^+ - 30$, 963), 219 ($M^+ - 61$, 429), 191 ($M^+ - 89$, 284), 117 ($M^+ - 163$, 402), 89 ($M^+ - 191$, 524), 74 ($M^+ - 206$, 384), and 73 ($M^+ - 207$, 4288).

M.s. of Me_3Si-FAX suggests an unsubstituted, non-reducing pentosyl residue, but n.m.r. evidence indicates that the non-reducing arabinofuranosyl residue is *trans*-feruloylated at O-5. This apparent discrepancy could be caused by acyl migration of the feruloyl group under the conditions (pyridine–tetrahydrofuran, heat) used to prepare Me_3Si-FAX. The presence of the feruloyl group in FAX was confirmed by direct-insertion, c.i.-m.s. of underivatised FAX in comparison with ferulic acid.

(*g*) *I.r. spectrum.* FAX had major i.r. bands with ν_{max}^{KBr} 3700–3200 (OH), 2920 (CH), 1695 (C=O), 1633 and 1600 (aliphatic C=C, α,β conjugation to ester and aromatic C=C), 1517 (aromatic C=C), 890 and 844 cm^{-1} (anomeric configuration of sugars, since absent in i.r. spectrum of *trans*-ferulic acid and methyl ferulate). The C=O band at 1695 cm^{-1} is lower than expected for an ester (1730–1717 cm^{-1}), but H-bonding of C=O is possible.

(*h*) *Lability in acid and alkali.* FAX was labile in 0.1M acid and in 0.1M alkali. Disappearance of the arabinose, relative to the xylose, residue in 0.1M NaOH at 20° is probably caused by alkaline degradation reactions, although all solutions were rigorously purged with N_2. These data are consistent with 2- rather than 3-*O*-arabinosyl substitution of xylose, as 2-*O*-substitution inhibits alkaline degradation from the reducing end of the molecule[23]. Ferulic acid is also rapidly released from FAX in dilute alkali. The lability of FAX in 0.1M acid is consistent with an arabinofuranosylxylose structure. No arabinosylxylose disaccharide or feruloylarabinose was obtained by acid or alkaline hydrolysis.

(*i*) *Sodium borohydride reduction.* FAX was not reduced by sodium borohydride (60 molar excess for 24 h), although l.c. and g.l.c. showed that FAX has a free reducing-end. Furthermore, the low-intensity signal for β-xylose C-1 in the ^{13}C-n.m.r. spectrum also indicates steric hindrance.

(*j*) *O.r.d. spectra.* Aqueous solutions of FAX showed very intense absorption between 200 and 400 nm, because of the feruloyl chromophore, but had a relatively low, associated optical activity. The o.r.d. over the range 430–650 nm was a reasonably linear, one-term, Drude plot centred at ~280 nm. This analysis can be carried a stage further by using the Kronig–Kramer transformation and an iterative, least-squares, curve-fitting technique to estimate the intensity and width of the (inaccessible) transition giving rise to the o.r.d. spectrum. The results were consistent, with

a single optically active (laevorotatory) transition centred at ~280 nm with a bandwidth of ~25 nm, and a maximum molar ellipticity of ~ −5000 deg.cm^2.dmol^{-1}.

However, the u.v. spectrum of FAX indicated that there may be two optically active bands at ~240 and ~320 nm, which are too close together to be resolved by this method and are inaccessible by direct measurement of c.d. because of the feruloyl chromophore.

The observed moleular rotations, $[\phi]$ (deg.cm^2.dmol^{-1}), over the range 430–650 nm, are given in Fig. 3. From published data[24], it is possible to calculate the $[\phi]_D$ for β- and α-L-arabinosyl-D-xylose as ~ +24,110 and ~ −17,390, respectively. However, the $[\phi]_D$ value for FAX of −167 does not allow assignment of the L-furanosyl configuration. Goldschmid and Perlin[24] were also unable to assign, from rotational data, the configuration of the arabinosyl group of a trisaccharide, consisting of an L-arabinofuranosyl group (1→3)-linked to a xylobiose residue, that was isolated from an enzymic hydrolysate of wheat arabinoxylan.

(*k*) *Isomerisation induced by u.v. irradiation.* Reverse-phase l.c. (system *3*) of an aqueous solution of FAX (1 mg/mL) that had been exposed to u.v. irradiation[25]

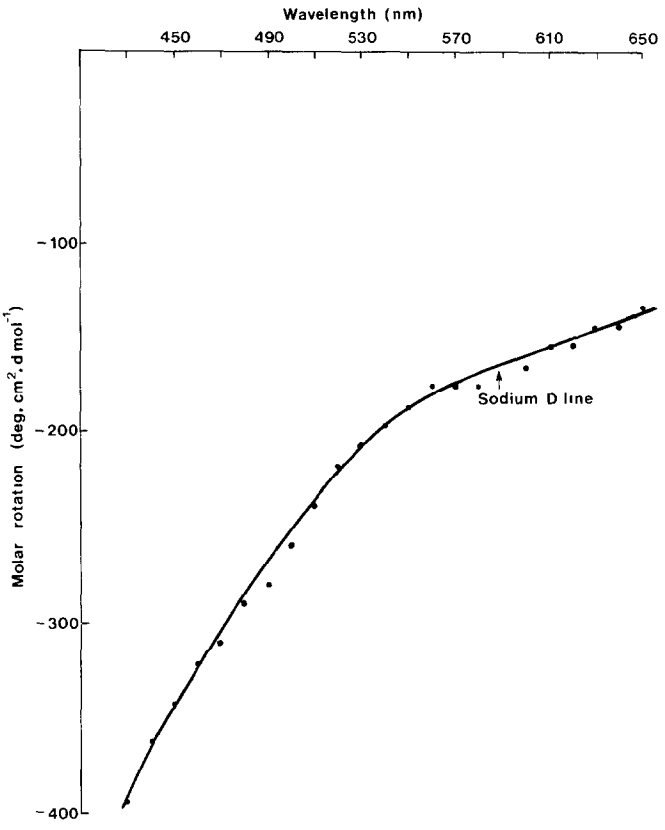


Fig. 3. Optical rotatory dispersion spectrum of FAX.

TABLE V

EFFECT OF U.V. IRRADIATION ON FAX

Compound irradiated	*Anomer*	*Before exposure to u.v. irradiation*		*After exposure to u.v. irradiation*	
		T^a *(min)*	*Amount*[b]	T^a *(min)*	*Amount*[b]
trans-FAX	β	19.1	1.00	19.3	1.00
	α	23.6	0.48	23.9	0.44
cis-FAX	β	Absent	—	13.8	0.14
	α	Absent	—	17.1	0.05

[a]L.c. retention time. [b]Relative to the peak area of the β anomer of *trans*-FAX.

for 1 h revealed two components in addition to FAX (Table V). The new components had retention times shorter than those of the original anomers and were probably the corresponding *cis*-isomers of FAX, as u.v. irradiation is known to cause *trans*→*cis* isomerisation in the side-chain double-bond of hydroxycinnamic acids[25,26]. Furthermore, in a similar l.c. system, *cis* isomers have shorter retention times than the corresponding *trans* isomers[27].

Action of Oxyporus "*cellulase*" *on phenolic acid–polysaccharide complexes in bran cell-walls.* — Alkaline treatment of cell-wall residues that were insoluble in water and resistant to attack by *Oxyporus* "cellulase" showed that ~70% of the feruloyl groups had been previously removed by enzymic hydrolysis (24 h; Table VI). Sequential treatment with *Oxyporus* "cellulase" and NaOH removed 59.8% of the cell-wall dry-matter. However, the cell-wall residues after treatment with *Oxyporus* "cellulase" still contained appreciable amounts of unidentified compounds that are soluble in alkali and absorb strongly in the 300–350 nm region. Significant differences were noted in the absorption maxima of alkaline extracts of bran cell-walls before and after treatment with "cellulase" (Table VI). These results emphasise the limitations of using u.v. absorption at 347 nm for determination of ferulic acid in alkaline extracts of cell walls, and help to explain earlier discrepancies between this and the l.c. methods (Table II, *cf.* determination of ferulic acid in the NaOH extract by absorbance and by l.c.). L.c. (systems *3* and *4*) failed to detect other aromatic compounds in the alkaline extract after neutralisation, or in an ether extract of the alkaline extract adjusted to pH 2.0.

Batches of *Oxyporus* "cellulase" are variable and ill-defined mixtures of polysaccharide hydrolases. The hydrolysate, after cell walls had been incubated with another batch of enzyme, contained a second ferulic ester-complex and much less FAX. When this batch of "cellulase" was incubated with isolated FAX, the bulk of the substrate remained unchanged even after 74 h. A small amount of the second feruloyl complex was present at 48 h, but had disappeared by 74 h with a concomitant appearance of free ferulic acid. Nevertheless, free ferulic acid was negligible in all *Oxyporus* "cellulase" hydrolysates of wheat-bran cell-walls.

TABLE VI

RELEASE OF *trans*-FERULIC ACID FROM CELL WALLS OF WHEAT BRAN (*cv.* FLANDERS) BY TREATMENT WITH SODIUM HYDROXIDE

	Proportion soluble in M *NaOH (% of cell wall)*[a]	*Filtrate from NaOH treatment*		
		Absorbance method		*L.c. method*
		λ_{max} *(nm)*	trans-*Ferulic acid equiv.*[b] *(mg/g of cell wall)*[a]	trans-*Ferulic acid content (mg/g of cell wall)*[a]
Cell walls	50.2	306		
		347	13.7	6.6
Residue from "cellulase" treatment of the walls[c]	15 5	298		
		330	7.1	1.9

[a]Dry-weight basis, as fraction of original cell-wall. [b]Calc from absorbance in 0.2M NaOH at 347 nm, by comparison with ε of sodium *trans*-ferulate at 347 nm. [c]Water-insoluble residue from the treatment of cell walls with "cellulase" (*Oxyporus* spp.). Yield of residue, 55.8% of original walls.

Presence of FAX in enzymic hydrolysates from other graminaceous cell-walls. — FAX was present in *Oxyporus* "cellulase" hydrolysates from other graminaceous cell-walls (barley straw, leaves of wheat and Italian ryegrass, and wheat endosperm containing <5% of aleurone cell-walls) and from water-soluble pentosans isolated from wheat flour. It is likely that the amount in cell walls is a function of genotype, cell type, component polysaccharides, and, possibly, conditions of growth.

Cell walls from wheat endosperm and bran, and a water-soluble pentosan isolated from wheat flour, were treated with NaOH or with *Oxyporus* "cellulase", and ferulic aid and FAX in the filtrates were determined by l.c. (systems *2* and *3*, respectively). From these results and from published analyses of the sugar residues present in wheat endosperm[28] and bran[29] cell-walls, it is possible to estimate the degree of feruloyl substitution of wheat pentosans. The estimates were as follows: water-soluble pentosans from flour, 1 in 20,000 pentose residues (0.4 μmol of feruloyl groups/g of polymer); endosperm cell-walls, 1 in 1140 (5.6 μmol/g of walls); and cell walls from bran (*cv.* Flanders), 1 in 150 (34.0 μmol/g of walls). The molar amounts of ferulic acid released from walls of polysaccharides by treatment with NaOH were the same as those released as FAX by treatment with *Oxyporus* "cellulase".

Role of FAX as a structural unit of cell-wall polymers. — Arabinoxylans from graminaceous cell-walls occur as linear backbones of (1→4)-linked β-D-xylopyranosyl residues, to which single arabinofuranosyl (and xylosyl, galactosyl, and glycosyluronic acid) groups are attached directly by (1→2) and/or (1→3) linkages[30]. Some internal xylosyl residues from the backbone of pentosans from "Beeswing" bran cell-walls[31] and from wheat and barley aleurone cell-walls[10] are

doubly substituted, at O-2 and O-3. Why then should ferulic esters specifically occur at 2-*O*- but not 3-*O*-arabinosyl branch-points of the xylan backbone, and what is the function of the feruloyl substituent? Feruloyl substituents of pentosans may act as precursors of more-complex aromatic molecules[32,33] or may modify the conformation of the pentosans, and both roles may be vital in determining and stabilising the quaternary structure of the cell-wall polymers.

EXPERIMENTAL

Isolation of cell walls. — (*a*) *Isolation of cell walls of wheat bran.* Wheat grain (*Triticum aestivum* L., *cv.* Flanders) was milled (Bühler Mill type MLU-202). The bran "stream" was further milled to pass through an 0.8-mm sieve (Christie and Norris Laboratory Mill, size 8), and "flour" was removed by sieving (Endocott, 0.15 mm). The retained bran (10 g) was shaken with neutral detergent solution[34] (100 mL, from which Na_2SO_3 and decahydronaphthalene were omitted) for 24 h at 25°. The cell walls from bran (50 g) were collected on a sinter (porosity No. 1), successively washed with cold water (1 L), hot water (1 L), acetone (300 mL), and diethyl ether (300 mL), and then dried *in vacuo* (yield of walls, 36% of dry, milled bran). The absence of cytoplasmic contaminants adhering to the cell walls was confirmed by microscopy, so that the origin of any ferulic esters could be reasonably attributed to cell-wall polysaccharides. Cell walls were also isolated from *cv.* Huntsman (yield: 39% of dry, milled bran) and from durum wheat (*T. durum*; yield: 27% of dry, milled bran).

(*b*) *Isolation of cell walls from other graminaceous sources.* Lyophilised leaf-blades of wheat (*T. aestivum* L.), Italian ryegrass (*L. multiflorum* Lam., *cv.* RVP), and barley straw (*Hordeum vulgare* L., *cv.* Julia) were treated with boiling, neutral detergent solution, and the cell walls were isolated as described above.

Isolation of FAX. — Bran cell-walls (1 g) from wheat (*cv.* Flanders) were suspended in a commercial "cellulase" [*Oxyporus* spp., Merck, 20 mU/mg; 450 mg/20 mL of 0.01M acetate buffer (pH 5.0)] and incubated in the dark for 24 h at 37° with stirring. In this and all other incubations, a few drops of toluene were added to prevent microbial growth. The suspension was filtered (porosity No. 1 sinter), and the residue was washed with water. The filtrate and washings were lyophilised, and then resuspended in methanol (10 mL). The insoluble material was collected by centrifugation, and re-extracted with methanol (5 × 10 mL); the combined methanolic extracts were concentrated *in vacuo* (30°), and FAX was isolated from the resulting oil by l.c. Samples of the oil (~60 μL) were fractionated on a column of Spherisorb 50DS (25 × 0.45 cm i.d.) with methanol–water (3:7) (1.0 mL/min, 8.96 × 10^6 Pa). The column effluent was monitored at 256 nm and 1.28 AUFS (Pye–Unicam LC3 detector with a 10-μL flow-cell). The fraction corresponding to FAX was collected manually, diluted with water, and lyophilised. FAX was stored in the dark *in vacuo* over silica gel. In all manipulations, care was taken to avoid u.v. irradiation, which causes *trans*→*cis* isomerisation of hydroxycinnamic acids.

Determination of the structure of FAX. — (*a*) *Complete hydrolysis.* Enzymic and alkaline treatments were used to achieve complete hydrolysis of FAX. Xylose and arabinose in the hydrolysates were measured by l.c. (system *1*), and ferulic acid by comparison with the absorbance of ferulic acid in 0.1M NaOH at λ_{max} 347 nm (ε 24,230) and by l.c. (system *2*).

FAX (1.0 mg) was incubated in a solution of type I "cellulase" (Sigma; 10 mg/mL, 0.01M acetate buffer, pH 5.0) from *Aspergillus niger* for 24 h at 37°. No transferase activity could be detected when the "cellulase" was incubated with amounts of ferulic acid and/or sugars equivalent to those present in the 24-h hydrolysate of bran cell-walls. Methanol (0.1 mL) was added to the enzymic hydrolysate before measurement of ferulic acid by l.c., and acetonitrile (1.0 mL) was added before measurement of sugars by l.c.

FAX (1.0 mg) was shaken in the dark with NaOH (0.1M, 50 mL) under N_2 (containing <5 p.p.m. of O_2) for 2 h at 20°, and the ferulic acid released was determined by u.v. absorption. No further increase in absorbance at 347 nm was detected after 75 min at 20°. For l.c. determination of the ferulic acid released, FAX (1.0 mg) was incubated with NaOH (0.1M, 5 mL), as described above, for 2 h, and the solution was then acidified (6M HCl, 0.1 mL). For l.c. determination of the sugars released, the alkaline solution of FAX was neutralised with Amberlite IR-120 (H^+) resin, the resin was removed by filtration and washed with water (5 × 1 mL), the filtrate and washings were evaporated to dryness *in vacuo*, and the residue was dissolved in acetonitrile–water (1:1, 100 μL).

(*b*) *Trimethylsilylation.* A suspension of FAX (1 mg) in "Tri-Sil Z" (100 μL, *N*-trimethylsilylimidazole reagent, Pierce) was shaken vigorously and incubated in the dark for 2 days at 37° before adding pyridine (100 μL) and tetrahydrofuran (100 μL), with heating for 30 min at 60° after the addition of each reagent. The suspension was concentrated to 100 μL in a stream of N_2, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (100 μL, Pierce) was added, and the suspension was incubated at 70–80° for 1 h. The following reference compounds were trimethylsilylated by the same method: (1→4)-β-xylobiose, cellobiose, cellotriose, *trans*-ferulic acid, and 1-*O*-(*trans*-*p*-coumaroyl)-β-D-glucose (gift of Dr. L. Nagels, Rijksuniversitair Centrum, Antwerp, Belgium).

(*c*) *Formation of the oxime.* FAX was suspended in a solution of hydroxylamine hydrochloride in pyridine (1 mg/100 μL) and heated for 30 min at 75°. The mixture was cooled, and trimethylsilylated as described above. Xylobiose and cellobiose were treated similarly.

(*d*) *G.l.c. of the trimethylsilyl derivative and reference compounds.* Trimethylsilyl derivatives were separated by using a glass column (1.5 m × 4 mm i.d.) packed with 0.2% of OV-25 on Gas Chrom Q (80/100 mesh); the N_2 flow-rate was 40 mL/min. The detector (FID) temperature was 50° above the column-oven temperature, as shown in Table IV; samples were injected directly into the column packing.

(*e*) *G.l.c.–m.s.* A Finnigan Automated Gas Chromatograph 4021 E_1/C_1 Mass Spectrometer was used. G.l.c. conditions: 0.2% OV-25 column (1.5 m × 4 mm

i.d.) at 230°; injector at 250°; separator at 250°; source at 270°; helium flow-rate, 20 mL/min. The scan was calibrated against tris(perfluoroheptyl)-1,3,5-triazine (mol.wt. 866). For the Me_3Si derivative of methyl *trans* ferulate the g.l.c. conditions were: 5% OV-25 column (1.5 m × 4 mm i.d.) at 205°, injector at 225°, separator at 225°; source at 250°; flow rate, 20 mL/min. The retention time was 6.3 min.

(*f*) *M.s.* Samples were directly inserted into the mass spectrometer (MS 902, Kratos); the temperature of the ion source was 200–300°. Spectra were compared with those of *trans*-ferulic acid, D-xylose, and xylobiose. For c.i.-m.s., samples were directly inserted into the Finnigan Mass Spectrometer, and isobutane was used as the ionising gas, source temperature, 270°; pressure, 2^{-7} Pa; and electron energy, 40 eV.

(*g*) *N.m.r. spectra.* ^{1}H-Spectra were recorded at 360 MHz in D_2O at 21° (relative to tetramethylsilane), and ^{13}C-spectra at 90.5 MHz in D_2O at 21° relative to tetramethylsilane, with 1,4-dioxane as internal standard.

(*h*) *I.r. spectra.* These were measured for KBr discs.

(*i*) *O.r.d. spectra.* Spectra of FAX (5 mg) in H_2O (1 mL) were recorded with a Jasco J20 spectropolarimeter (pathlength, 1 cm) at 25°. More-precise measurements were made at fixed wavelengths (Hg emission lines) with a Perkin–Elmer 241 polarimeter (same concentration and pathlength).

Partial hydrolysis of FAX with "cellulase". — FAX (37 μg) was incubated at 37° in the dark in acetate buffer (0.01M, 0.1 mL; pH 5.0) containing 30 mU of "cellulase" from *Oxyporus* spp. (20 mU/mg). Control incubations involved FAX plus buffer, and enzyme mixture plus buffer. Samples were removed periodically and examined by l.c. (system *3*).

Release of phenolic esters from cell walls and isolated polysaccharides by treatment with "cellulase". — Cell walls (50–100 mg) or polysaccharides (200 mg) were suspended in acetate buffer (0.01M, pH 5.0) containing "cellulase" from *Oxyporus* spp. (45 mg in 2 mL) and incubated in the dark for 24 h at 37° with shaking unless otherwise indicated. The suspension was filtered (porosity No. 1 sinter), the residue was washed with water, and the combined filtrate and washings were diluted to a standard volume. The residue was washed with acetone (20 mL) and diethyl ether (20 mL), dried *in vacuo*, and weighed.

Treatment of polysaccharides, cell walls, and cell-wall fractions with NaOH. — The sample (30–100 mg) was suspended in NaOH (M, 2 mL), purged with N_2, and incubated for 24 h at 20°. The suspension was filtered, the residue was washed with water (5 × 0.5 mL), and the filtrate and washings were diluted to a standard volume. The phenolic acids were measured spectrophotometrically (u.v. absorption), and by l.c. (system *2* below).

T.l.c. — Cellulose plates (F1400 Schleicher and Schüll) were used. Solvent systems for phenolic acids were *1*, formic acid–water (1:24); *2*, toluene–formic acid–water (8:9:3, upper phase); and, for sugars, *3*, 1-propanol–ethyl acetate–water (6:1:3); *4*, 1-propanol–ethyl acetate–water (4:1:2); *5*, pyridine–ethyl acetate–water (3:6:2); *6*, pyridine–ethyl acetate–water (4:1:2, upper phase). Plates

were examined by u.v. irradiation and then sprayed with aniline hydrogenphthalate (to detect sugars) or diazotized *p*-nitroaniline (to detect phenolic acids[35]). The R_F values for FAX were: solvent *1*, 0.43; *2*, zero; *3*, 0.74; *4*, 0.72; *5*, 0.89; and *6*, 0.82.

Liquid chromatography (l.c.). — (*a*) *System* 1 *for sugars.* A column of Spherisorb $5NH_2$ (25 × 0.45 cm i.d.) was used with acetonitrile–water (4:1) at 2.0 mL/min (5.65×10^6 Pa). The column and detector were maintained at 25°. The effluent was monitored with a refractive-index detector (Refracto Monitor Model 11074, Laboratory Data Control, Riviera Beach, Florida, U.S.A.). The limit of sensitivity was ~10 μg of pentose or hexose. Retention times were xylose, 7.4 min; arabinose, 8.5 min; glucose, 11.0 min; and xylobiose, 12.1 min.

(*b*) *System* 2 *for phenolic acids.* A reverse-phase column of Spherisorb 50DS (25 × 0.45 cm i.d.) was used[27] with 1-butanol–acetic acid–water (11:1:347) at 1.2 mL/min (7.65×10^6 Pa). The effluent was monitored at 280 nm and 0.04 AUFS.

(*c*) *System* 3 *for phenolic acid esters of sugars.* A reverse-phase column of Spherisorb 50DS (25 × 0.45 cm i.d.) was used with methanol–water (3:7) at 1.0 mL/min (13.79×10^6 Pa). The effluent was monitored at 280 and 325 nm and 0.04 AUFS.

(*d*) *System* 4 *for aromatic compounds of high molecular weight.* As for system *3*, except that the solvent was methanol–water (3:2).

Isolation of water-soluble pentosan from wheat flour. — Water-soluble pentosan was isolated from a commerial flour ("Sterling") according to the method of Medcalf *et al.*[36].

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REFERENCES

1 P. J. HARRIS AND R. D. HARTLEY, *Biochem. Syst. Ecol.*, 8 (1980) 153–160.
2 R. D. HARTLEY AND P. J. HARRIS, *Biochem. Syst. Ecol.*, 9 (1981) 189–203.
3 R. D. HARTLEY AND E. C. JONES, *Phytochemistry*, 16 (1977) 1531–1534.
4 R. D. HARTLEY AND E. C. JONES, *J. Sci. Food Agric.*, 29 (1978) 777–789.
5 L. JURD, *Arch. Biochem. Biophys.*, 66 (1957) 284–288.
6 R. D. HARTLEY, *Phytochemistry*, 12 (1973) 661–665.
7 H.-U. MARKWALDER, Ph. D. Thesis, Swiss Federal Institute of Technology, Zurich, No. 5497, 1975.

8 H. NEUKOM AND H.-U. MARKWALDER, *Cereal Foods World*, 22 (1977) 458
9 R. G. FULCHER, T. P O'BRIEN, AND J. W. LEE, *Aust. J. Biol Sci.*, 25 (1972) 23–24.
10 A. BACIC Ph. D. Thesis, La Trobe University, Melbourne, 1979
11 R D HARTLEY AND E. C JONES *Phytochemistry*, 15 (1976) 1157–1160
12 T. USUI S TSUSHIMA, N YAMAOKA K MATSUDA K TUZIMURA H SUGIYAMA S SETO K FUJIEDA, AND G. MIYAJIMA, *Agric. Biol Chem.*, 38 (1974) 1409–1410
13 R J. ABRAHAM, K. A. MCLAUCHLAN, L D. HALL, AND I HOUGH *Chem Ind (London)*, (1962) 213–214.
14 R COOPER, H E GOTTLIEB, AND D. LAVIE, *Phytochemistry*, 17 (1978) 1673–1675
15 D S HIMMELSBACH AND F. E FENTON *J Agric. Food Chem.*, 28 (1980) 1203–1208.
16 R. G S RITCHIE, N CYR B KORSCH H J. KOCH AND A S PERLIN *Can J Chem.*, 53 (1975) 1424–1433
17 J -P. JOSELEAU G CHAMBAT M VIGNON AND F BARNOUD *Carbohydr Res.*, 58 (1977) 165–175
18 P A J. GORIN AND M MAZUREK, *Can. J. Chem.*, 53 (1975) 1212–1223.
19 K SHIMUZU, *Carbohydr Res.*, 92 (1981) 219–224
20 K. M. BROBST *Methods Carbohydr. Chem.*, 6 (1972) 3–8
21 S HANESSIAN, *Methods Biochem Anal.*, 19 (1971) 105–228.
22 O S CHIZHOV N V MOLODTSOV, AND N K KOCHETKOV *Carbohydr Res.*, 4 (1967) 273–276
23 R L. WHISTLER AND J N. BEMILLER *Adv Carbohydr. Chem.*, 13 (1958) 289–329
24 H R GOLDSCHMID AND A S PERLIN *Can. J Chem.*, 41 (1963) 2272–2277
25 R D HARTLEY AND E C JONES, *J. Chromatogr.*, 107 (1975) 213–218.
26 T. W FENTON, M. M MUELLER, AND D R CLANDININ, *J Chromatogr.*, 152 (1978) 517–522
27 R D HARTLEY AND H BUCHAN, *J Chromatogr.*, 180 (1979) 139–143
28 D J. MARES AND B A STONE *Aust J Biol Sci.*, 26 (1973) 793–812
29 H NEUKOM H -U. MARKWALDER AND P SCHIBLI, *Lebensm -Wiss Technol.*, 10 (1977) 346–349
30 K C B. WILKIE *Adv Carbohydr Chem. Biochem.*, 36 (1979) 215–264. and references therein
31 S G RING AND R R SELVENDRAN, *Phytochemistry*, 19 (1980) 1723–1730
32 S Z EL-BASYOUNI A C NEISH AND G H. N TOWERS, *Phytochemistry*, 3 (1964) 627–639
33 S Z EL-BASYOUNI AND A C NEISH *Phytochemistry*, 5 (1966) 683–691
34 P J. VAN SOEST AND R H WINE *J Assoc Off Anal. Chem.*, 50 (1967) 50–55
35 P RIBEREAU-GAYON *Plant Phenolics*, Oliver and Boyd, Edinburgh, 1972, p 94
36 D G MEDCALF, B. L. D APPOLONIA, AND K A GILLES *Cereal Chem.*, 45 (1968) 539–549