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2-*O*-β-D-XYLOPYRANOSYL-(5-*O*-FERULOYL)-L-ARABINOSE, A WIDESPREAD COMPONENT OF GRASS CELL WALLS

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Key Word Index—*Festuca arundinacea*; Gramineae; tall fescue grass; feruloyl esters; oligosaccharides; arabinoxylans; plant cell walls.

Abstract—Graminaceous cell walls contain arabinoxylans in which some of the Ara*f* residues are 5-*O*-feruloylated. In the present study, mild acid hydrolysis of (pentosyl-^{3}H)-labelled *Festuca arundinacea* cell walls yielded the well-characterized 5-*O*-Fer-L-Ara (**1**) and at least seven new (*E*)-feruloylated oligosaccharides, **2**–**8**. Compound **2** was a feruloylated disaccharide, Fer-(D-Xyl*p*→L-Ara); electrophoresis in molybdate buffer, Smith degradation and methylation analysis showed a (1→2)-linkage. ^{1}H and ^{13}C NMR data and susceptibility to commercial β-D-xylosidase indicated a β-linkage. Graded alkaline hydrolysis of **2** showed no evidence for more than one ester group. Hydrolysis with commercial β-xylosidase yielded 5-*O*-feruloyl-L-arabinose. Compound **2** was thus 2-*O*-β-D-xylopyranosyl-(5-*O*-feruloyl)-L-arabinose. We propose that **2** was furanose-linked to a xylan backbone in the intact polymer. Compounds **3**–**8** also appeared to be *O*-feruloyl oligosaccharides with a former Ara*f* residue at the reducing terminus. Mild acid hydrolysis of cell walls from twenty other species of grass and one palm also yielded compounds **1**–**3**, and most or all of **4**–**8**. Therefore, these complex feruloylated side-chains of arabinoxylans are widespread or universal in the Gramineae. Their possible biological roles are discussed. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Hemicelluloses are the non-pectic polysaccharides that can be extracted from plant cell walls by cold aqueous alkali. In grasses, the major hemicelluloses of primary walls are xylans, which have a β-(1→4)-D-xylan backbone and short carbohydrate side-chains [1–3]. The major side-chains are single α-L-Ara*f* residues, which are attached to the β-D-Xyl*p* residues of the backbone, in grasses to *O*-3 and in dicotyledons mainly to *O*-2 [4]. The α-L-Ara*f* groups increase the solubility of xylans in water [5]. Some of the α-L-Ara*f* groups are further substituted with other sugar residues, including β-D-Xyl*p* and β-D-Gal*p* [1, 2]. Single residues of α-D-Glc*p*A and/or its 4-*O*-methyl ether are also attached to position *O*-2 of the β-D-Xyl*p* residues of the backbone [2].

Ester groups are also present. Xylans carry *O*-acetyl ester groups [6], which may decrease the digestibility of cell walls and limit hydrogen-bonding of the xylan to cellulose. A proportion of the α-L-Ara*f* residues are substituted at position 5 with *O*-feruloyl (Fer) ester groups [7–11]. Ishii [6] has shown that bamboo arabinoxylan carries *O*-Ac and *O*-Fer groups at positions 2 and 5, respectively, of α-L-Ara*f* residues.

Fer groups are incorporated into xylans as the (*E*)-isomer [11]. Dark-grown plants contain only the (*E*)-isomer, but exposure to light, and especially to UV-A, causes the formation of some (*Z*)-isomer. It has been suggested that photo-isomerization of cell wall bound Fer esters leads to changes in growth rate [12], such as those involved in phototropism.

Water-soluble arabinoxylans will gel in the presence of oxidizing agents such as peroxidase + H_2O_2, owing to the oxidative coupling of Fer groups, to yield diferuloyl and related phenolic dimers [13–16]. Peroxidase- or laccase-catalysed oxidative coupling may be a means of tightening the structure of the cell wall and, thus, restricting cell expansion [17–20]. Cross-linking by non-enzymic photo-dimerisation of Fer residues is also a possibility [21].

Another biological role of the Fer groups may be to protect the polysaccharide against enzymic hydrolysis. Correlations have been reported between Fer content and resistance of cell walls to microbial degradation [22], although Fer groups were found to

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confer only limited protection against digestion by rat caecal bacteria [23]; microbial feruloylesterases are widespread in nature [24].

It was reported [25] that *O*-Fer arabinoxylans in the walls of cultured cells of the grass, *Festuca arundinacea*, are relatively resistant to digestion by the fungal glycanase mixture 'Driselase', whereas this enzyme preparation can act successfully on other graminaceous cell walls to release *O*-Fer di- and trisaccharides. The main Driselase digestion products of maize shoot and barley aleurone cell walls (Fer-Ara-Xyl and Fer-Ara-Xyl_2 [8, 11]) differed from those (Fer-Ara_2 and Fer-Gal_2 [26, 27]) obtained from spinach, a dicotyledon. However, it cannot be assumed that all monocotyledons will have the same pattern of feruloylation in their wall polysaccharides. Therefore, we have now investigated the phenolic components of the primary cell walls of *F. arundinacea* and of several other grasses, in an attempt to discover unusual chemical features that might confer resistance to hydrolysis.

RESULTS AND DISCUSSION

Fluorescent oligosaccharides released from polysaccharides of several Gramineae by mild acid hydrolysis

Mild acid hydrolysis of alcohol-insoluble residue (AIR) prepared from cell cultures of *F. arundinacea* yielded at least eight fluorescent compounds (**1**–**8**), tentatively identified as *O*-Fer oligosaccharides. The fluorescence characteristics (blue, turning intense blue–green under NH_3 vapour) were identical with those of authentic *O*-Fer esters [26]. On PC in BAW, **1** co-migrated with 5-*O*-Fer-L-Ara, previously noted in studies of other Gramineae. However, **2**–**8** appeared to be new.

To determine whether **2**–**8** were limited to cell cultures, and to investigate their taxonomic distribution, we studied their occurrence in 22 species selected from a taxonomic tree [28]. Mild acid hydrolysis of AIR from leaves of each of the 22 species yielded **1**–**3** (Fig. 1 shows data for 14 species). In addition, **4**–**8** were detected in most of the species.

The mild acid hydrolysate from 100 mg of AIR from 14 of the spp. was subjected to PC in BAW. Compounds **1** and **2** were eluted, further purified by low-pressure reverse-phase chromatography (LP-RPC) followed by PC in BEW, then quantified by analytical HPLC (Table 1). The largest amounts of both **1** and **2** were obtained from *Anthoxanthum odoratum*, the smallest amount of **1** from *F. pratensis* and the smallest amount of **2** from *Avena sativa*. *F. pratensis* showed the highest ratio of **2**:**1**. The results show that **1** and **2** are widespread, if not universal, in the Gramineae and also occur in at least one member of the Palmae.

O-*Feruloylated*, ^{3}H-*labelled oligosaccharides from* Festuca

The efficient release of compounds **2**–**8** from AIR by mild acid hydrolysis suggested that they might be L-Ara derivatives whose reducing termini had been furanosidically linked (Ara*f*-) within the parent polymer. We therefore radiolabelled cells in their pentose residues. *Festuca arundinacea* cells readily incorporated L-[1-^{3}H]Ara into polysaccharides and yielded ^{3}H-labelled AIR of specific activity 0.79 MBq mg^{-1}. Complete acid hydrolysis of the AIR gave [^{3}H]Ara (1.676 MBq μmol^{-1}) and [^{3}H]Xyl (0.776 MBq μmol^{-1}) as the sole radioactive products, which, in view of the known metabolic fate of exogenous L-[1-^{3}H]Ara, L-Ara→L-Ara-1-P↔UDP-L-Ara↔UDP-D-Xyl [29], are assumed to be L- and D-, respectively.

To investigate the substituents attached to Ara*f* residues, we heated ^{3}H-AIR in mild acid to produce the eight fluorescent compounds (**1**–**8**; Fig. 2(a)). It has been shown that acyl migration within *O*-Fer sugars was negligible during mild acid hydrolysis [30]. The acid conditions used had been optimized for production of **2** (data not shown); **1**–**3** were each associated with a discrete peak of ^{3}H (Fig. 2(a)).

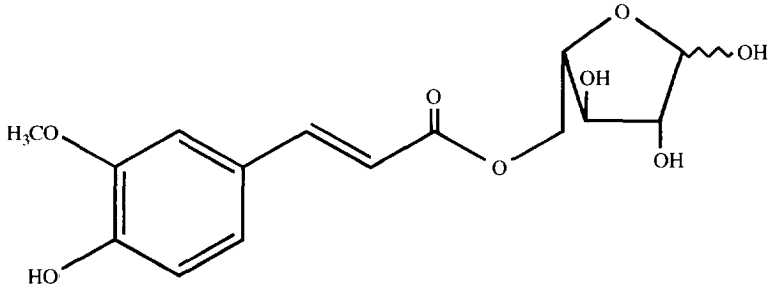
Compounds **1** and **2** were eluted from a preparative PC (BAW), freed of simple sugars by LP-RPC and further purified by PC (BEW). Each compound now gave a single peak in which fluorescence and ^{3}H exactly co-migrated (Fig. 2(b) and (c)).

Absorption spectra of purified **1** and **2** both showed λ_{max} 323 nm at pH ~5. When NaOH was added to ~50 mM, the λ_{max} immediately increased to 373 nm, then gradually (complete within 1 hr) decreased to 343 nm. These data are characteristic of *O*-Fer esters (immediate bathochromic shift due to ionisation of phenolic -OH group; gradual hydrolysis to sodium ferulate).

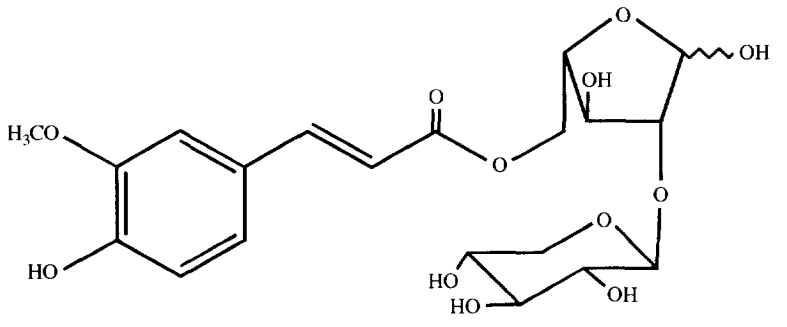
5-O-*Feruloyl*-L-*arabinose* (**2**)

Alkaline hydrolysis of ^{3}H-labelled **1** yielded a single radioactive product (**1a**), which co-migrated in five systems with authentic Ara (Table 2; Fig. 2(d)). To test for the presence of a second ester group (e.g. *O*-acetyl) in **1**, we examined samples by PC (BAW) after 0, 0.5, 1.0 and 2.0 min in 0.1 M NaOH (data not shown); only **1** and Ara were detected. Had an *O*-acetyl group also been present on the Ara moiety, at least one radioactive intermediate product would have been formed, which would have been resolved readily by PC. Had an acetyl group been attached to the phenolic hydroxyl group, the Fer group would not have exhibited its characteristic fluorescence. Thus, **1** was an *O*-Fer-L-Ara with no additional ester group.

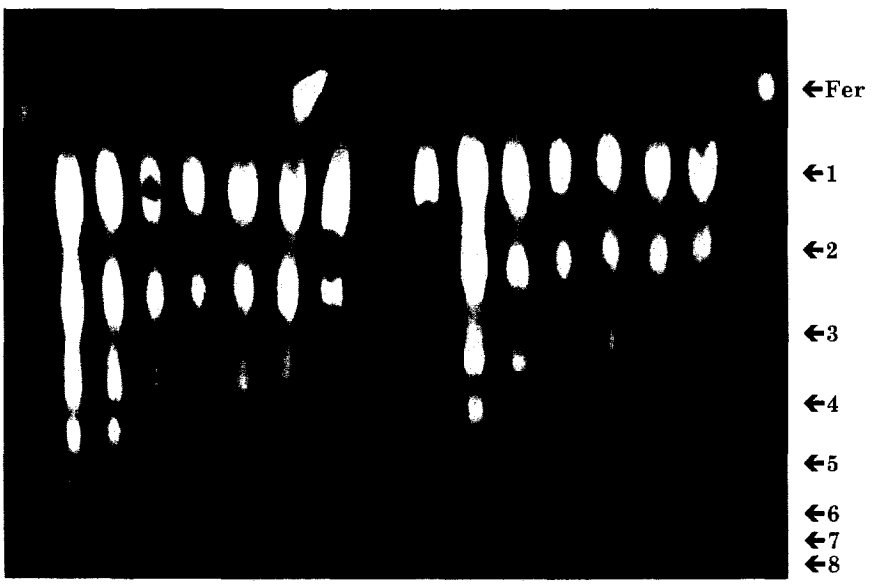
^{3}H-Labelled **1** exactly co-migrated with internal marker 5-*O*-Fer-L-Ara, from maize, on PC in several systems and on high-voltage paper electrophoresis (PE) in borate (data not shown). PC would be expected to separate 5-*O*-Fer-L-Ara from the cor-



1



2



Fer ***(a) (b) (c) (d) (e) (f) (g)*** **Fer** ***(h) (i) (j) (k) (l) (m) (n)*** **Fer**

Fig. 1. PC in BAW of mild acid hydrolysis products of AIR samples from 14 graminaceous species. The PCs were photographed under UV light in ammonia vapour: (a) *Festuca arundinacea*, (b) *F. gigantea*, (c) *F. pratensis*, (d) *F. rubra*, (e) *Agrostis tenuis*, (f) *Anthoxanthum odoratum*, (g) *Arrhenatherum elatius*, (h) *Avena sativa*, (i) *Bromus sterilis*, (j) *Dactylis glomerata*, (k) *Lolium multiflorum*, (l) *L. perenne*, (m) *Phleum pratense* and (n) *Triticum aestivum*. ←, Indicates approximate positions of ferulic acid (Fer) and compounds **1**–**8** (**8** = origin).

Table 1. Relative yields of compounds **1** and **2** from alcohol-insoluble residues from leaves of 14 species

Species*	Yield of AIR (g g^{-1} fr. wt)	Yield† **1**	Yield† **2**	Approx. ratio **2**:**1**
Festuca arundinacea	0.61	16.8	9.9	0.6
F. gigantea	0.35	12.4	7.1	0.6
F. pratensis	0.45	6.9	14.4	2.1
F. rubra	0.64	8.7	5.6	0.6
Lolium multiflorum	0.55	6.9	7.0	1.0
L. perenne	0.26	7.4	8.2	1.1
Dactylis glomerata	0.82	17.9	21.7	1.2
Bromus sterilis	0.87	18.3	17.2	0.9
Triticum aestivum	0.94	8.4	6.2	0.7
Avena sativa	0.79	9.7	5.3	0.6
Arrhenatherum elatius	0.82	16.1	10.7	0.7
Anthoxanthum odoratum	0.79	19.8	24.4	1.2
Agrostis tenuis	0.59	16.9	14.7	0.9
Phleum pratense	0.72	13.8	12.1	0.9

*Other species shown by PC to contain compounds **1** and **2** were *Hordeum vulgare*, *Secale sentinal*, *Sorghum verticilliflorum*, *Sorghum vulgare*, *Dichanthium sericeum*, *Bothriochloa ambigua*, *Zea mays*, *Trachycarpus fortunei*. All Gramineae, except *T. fortunei* (Palmae).

†Arbitrary units from HPLC traces of *O*-feruloyl oligosaccharides derived from 100 mg AIR.

responding esters of the secondary hydroxyl groups of Ara [30]. The results together confirm that **1** was 5-*O*-Fer-L-Ara, which is widespread in graminaceous cell walls [8–11].

2-O-*β*-D-*Xylopyranosyl*-(5-O-*feruloyl*)-L-*arabinose* (**2**)

Alkaline hydrolysis of ^{3}H-labelled **2** yielded a single radioactive product, **2a**, with $R_{Ara} < 1$ (Table 2). Partial alkaline hydrolysis revealed no intermediate products (Fig. 3); thus, Fer was the only ester-linked substituent in **2**.

Complete acid hydrolysis of **2a** yielded Ara and Xyl as sole radioactive products (Fig. 4(a)). The [^{3}H]Ara:[^{3}H]Xyl ratio (2.29:1) corresponded to a molar ratio of 1.06:1, assuming that the specific activities of the pentose moieties in **2a** were the same as in total AIR. On Bio-Gel P-2, ^{3}H-labelled **2a** co-eluted with the internal marker maltose. We conclude that it is a disaccharide of Ara and Xyl. Its stability to mild acid hydrolysis indicates that it had a pyranosyl linkage.

Compound **2a** was reduced with $NaBH_4$ (to yield **2b**), then subjected to complete acid hydrolysis. PC (BAB) revealed two ^{3}H-products, namely, Xyl and a pentitol (xylitol or arabinitol, not resolved; Fig. 4(b)). PE (borate) also revealed two ^{3}H-products, namely, arabinitol and a pentose (Xyl or Ara, not resolved; Fig. 4(c)). These data show that the reducing terminus of **2a** was Ara.

Compound **2b** was electrophoretically immobile in molybdate (Fig. 5(a)). Neutral sugars form anionic complexes with molybdate only if they have three OH groups in an appropriate configuration [31]. Xyl*p* residues do not bind molybdate (shown by the immobility of the marker methyl xylosides; Fig. 5(a)). Therefore, any electrophoretic mobility of **2b** would have been conferred by the arabinitol moiety. Blocking (either by substitution or by formation of the deoxy derivative) of arabinitol at *O*-5 has little effect on mobility [31], blocking at *O*-3 decreases it [shown by the marker Gal*p*-(1→3)-arabinitol], while blocking at *O*-2 prevents it altogether [shown by the marker 2-deoxy-D-*erythro*-pentitol ('2-deoxy-D-arabinitol'); allowance must be made for electro-endo-osmosis]. The immobility of **2b** shows that it was *O*-2-blocked and, therefore, a D-Xyl*p*-(1→2)-L-arabinitol. This was supported by Smith degradation of ^{3}H-labelled **2b**, which yielded [^{3}H]glycerol as the major, non-volatile, ^{3}H-product (Fig. 5(b) and (c)). The [1-^{3}H]Xyl moiety of **2b** will give [^{3}H]glycollaldehyde, which is lost as volatile products [26]. The [1-^{3}H]arabinitol moiety would yield [^{3}H]glycerol only if it were substituted at *O*-2. [1-^{3}H]Arabinitol substituted at *O*-3, *O*-4 or *O*-5 would have given [^{3}H]MeOH as the sole radioactive product. Thus, the data again show that compound **2b** is a D-Xyl*p*-(1→2)-L-arabinitol.

Non-radioactive **2** was purified by HPLC (data not shown). The alkaline hydrolysis product (**2a**) was then per-*O*-methylated, acid-hydrolysed, reduced with $NaBD_4$, per-*O*-acetylated and analysed by GC-mass spectrometry. The two major products had retention times and fragmentation patterns consistent with a 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-[1-^{2}H]pentitol and a 1,2,4-tri-*O*-acetyl-3,5-di-*O*-methyl-[1-^{2}H]pentitol. These data support the presence of the (1→2)-linkage demonstrated by PE and Smith degradation.

Commercial *β*-D-xylosidase converted ^{3}H-labelled **2** almost completely to [^{3}H]Ara and [^{3}H]Xyl (Fig. 6(a–e)). This shows that the enzyme contained esterase activity. Intermediate radioactive products included peaks attributable to all the ^{3}H-compounds expected for an *O*-feruloylated ^{3}H-disaccharide (Scheme 1).

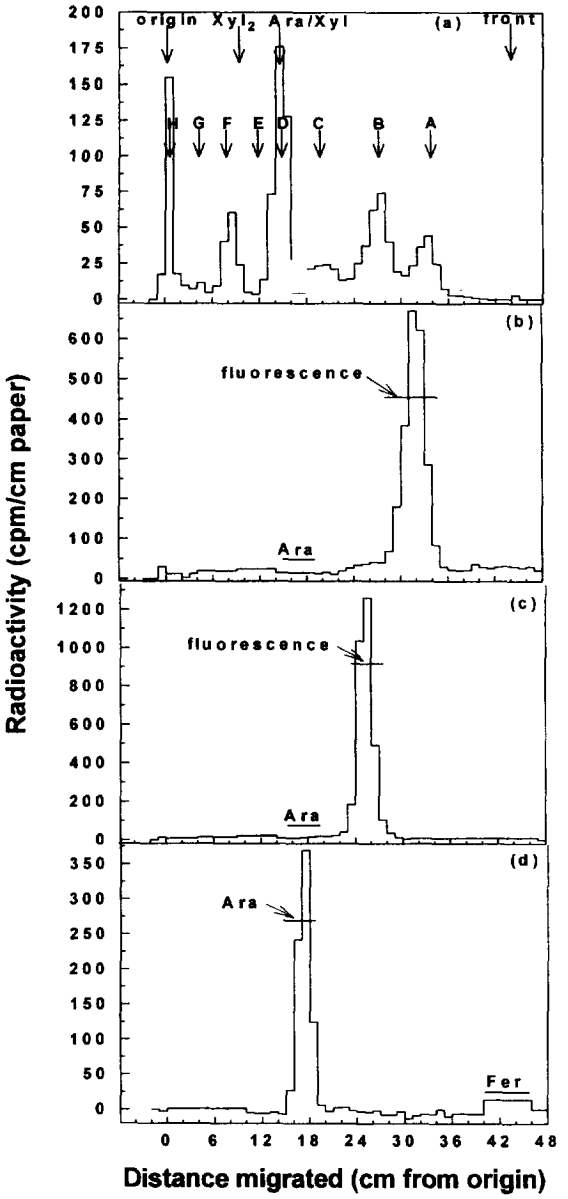
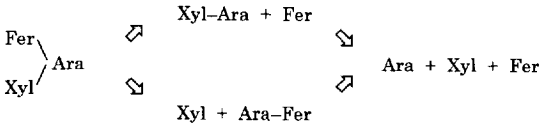


Fig. 2. PC separation of products of mild acid hydrolysis of (pentosyl-^{3}H)-labelled *Festuca* AIR. (a) PC in BAW of the crude hydrolysate. Eight blue fluorescent zones are marked A–H (= compounds **1**–**8**, respectively; ↓ = centre of zone). Units for this scale are kcpm/cm; some of the data (18–48 cm from origin) are reported on a 50-fold expanded *y*-axis. (b) PC in BEW of **1** after purification by LP-RPC. (c) PC in BEW of **2** after purification by LP-RPC. (d) PC in BAW of **1a** obtained by hydrolysis of **1** in 0.5 M NaOH for 7.5 min.



Scheme 1. Possible routes for the hydrolysis of compound **2**.

The '*β*-D-xylosidase' preparation also contained appreciable α-D-xylosidase activity, as shown by 26% conversion of the disaccharide α-D-[^{3}H]Xyl*p*-(1→6)-D-Glc to [^{3}H]Xyl within 50 min (data not shown). Thus, the enzymic data (Fig. 6(a–e)) suggested, but did not prove, that **2a** was *β*-D-Xyl*p*-(1→2)-L-Ara; NMR was used to resolve this question.

The ^{1}H NMR spectrum of **2a** (Fig. 7(a)) gave H-1 signals for α- and *β*-L-Ara*f* at *δ* 5.45 ($J_{1,2}$ = 1.8 Hz) and *δ* 5.40 ($J_{1,2}$ = 4.4 Hz), respectively. Two H-1 signals characteristic of *β*-D-Xyl*p* were also detected at *δ* 4.62 ($J_{1,2}$ = 7.7 Hz) and *δ* 4.58 ($J_{1,2}$ = 8.0 Hz). If the D-Xyl*p* residue had been α-linked, an H-1 signal would have been expected at *δ* 4.94–5.13 [32]. In the ^{13}C NMR spectrum (Fig. 7(b); Table 3), four signals were observed in the anomeric region: α- and *β*-L-Ara*f* at *δ* 101.4 and 96.8, respectively, and two signals for *β*-D-Xyl*p* at *δ* 104.5 and 103.8, attributable to long-range coupling associated with α ↔ *β* equilibration of the reducing terminal Ara. NMR data thus support evidence for a *β*-D-Xyl residue. The C-2 signals of α- and *β*-L-Ara*f* exhibited a characteristic glycosylation down-field shift to *δ* 90.2 and 84.6, respectively, in agreement with the proposed (1→2)-linkage.

A [^{14}C]Fer-monosaccharide, derived from **2** by digestion with commercial '*β*-xylosidase' (similar to Fig. 6c), co-migrated with the internal marker 5-*O*-Fer-L-Ara (from maize) on PC in BAW, BEW, EPW and ΦW and on PE in borate (data not shown). PC would separate 5-*O*-Fer-L-Ara from 2-, 3-, and 4-*O*-Fer-L-Ara [30]. We conclude that the ^{14}C-labelled product is 5-*O*-Fer-L-Ara and, thus, that **2** is 2-*O*-*β*-D-xylopyranosyl-(5-*O*-feruloyl)-L-arabinose.

Susceptibility to hydrolysis

ΔA_{380} showed that the half-lives ($t_{1/2}$) of **1** and **2** in 0.1 M NaOH were ~2 and ~14 min, respectively (Fig. 8). Thus, the ester bond in 5-*O*-Fer-L-Ara was appreciably more alkali-labile than that in the other *O*-Fer esters tested (Table 4).

Since **1**–**8** were released from AIR by mild acid hydrolysis, it is likely that their reducing termini had been Ara*f*-linked within the parent polymer. To test to what extent other linkages than Ara*f* would be cleaved by the mild acid used, we treated pure ^{3}H-labelled **2** with 0.1 M TFA at 100° for 1 hr. The released products (Fig. 6(f)) resembled those formed by commercial '*β*-xylosidase' (cf. Scheme 1). The Xyl*p* and *O*-Fer bonds were 60% and 10% hydrolysed, respectively. Thus, ~36% of **2** would survive the mild acid treatment. It is possible, however, that additional **2** was generated during the hydrolysis of AIR by the partial hydrolysis of compounds **3** to **8**.

CONCLUSIONS

In grasses, the major cell wall polysaccharides with Ara*f* units are arabinoxylans. The Ara*f* units are attached mainly to *O*-3 positions of a *β*-(1→4)-D-xylan backbone. It is well known that some of them possess *O*-Fer residues at position 5. The present paper reports the widespread occurrence, probably ubiquitous in the

Table 2. Chromatographic and electrophoretic data for compounds **1** and **2** and their de-esterified derivatives (**1a** and **2a**)

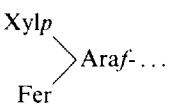
Compound	R_{Ara} (PC in BAW)	R_f (PC in BEW)	R_{Ara} (PC in EPW)	R_{Ara} (PC in BPW)	m_{Ara} (PE in borate buffer)
1	2.21	0.74	n.d.	n.d.	0.46
1a	1.00	n.d.	1.00	1.00	1.00
2	1.86	0.57	n.d.	n.d.	0.69
2a	0.64	n.d.	0.27	n.d.	n.d.

n.d., not determined.

m_{Ara}, electrophoretic mobility (corrected for electro-endo-osmosis) relative to that of arabinose.

Gramineae, of an additional Xyl*p* residue attached to the Fer-Ara group. The additional Xyl residue changed the molecular environment of the Fer group appreciably, as shown by its effect on susceptibility to alkaline hydrolysis (Table 4). It may also affect the susceptibility of the Fer-Ara residue to enzyme-catalysed reactions, such as attack by microbial hydrolases (in the rumen or caecum, in leaf litter, or secreted by phytopathogens) and oxidation by endogenous plant peroxidases involved in cell wall cross-linking [18].

Compound **2** was shown to be 2-*O*-*β*-D-Xyl*p*-(5-*O*-Fer)-L-Ara. This is a component of an *O*-Fer-tetrasaccharide isolated from shoots of *Cynodon dactylon* by enzymic hydrolysis of the xylan backbone [33]; **2** was also recently reported from maize bran [34]. The present work confirms its structure and demonstrates the wide taxonomic distribution of the branched side-chain,



Compounds **3** to **8**, which were also widespread in grass cell wall hydrolysates, also appeared to be Fer-oligosaccharides with a former Ara*f* residue at the reducing terminus. It is thus likely that 2-*O*-*β*-D-Xyl*p*-(5-*O*-Fer)-L-Ara is the core of a series of more complex Fer-oligosaccharides arranged along the xylan backbone. More detailed data on **3**–**8** are described in the following paper [35].

EXPERIMENTAL

Chemicals. *β*-D-Gal*p*-(1→3)-D-arabinitol and 2-deoxy-D-*erythro*-pentitol were synthesized from the corresponding reducing sugars by treatment with $NaBH_4$. Authentic 5-*O*-Fer-L-Ara was obtained by mild acid hydrolysis of *Zea* AIR [8] followed by prep. PC in BAW. L-[1-^{3}H]Ara (97 MBq μmol^{-1}) was from Amersham, and *α*-D-[1-^{3}H]Xyl*p*-(1→6)-D-Glc was prepd as described in ref. [36]. (*E*)-[U-^{14}C]Cinnamic acid (15 MBq μmol^{-1}) was prepd by treatment of L-[U-^{14}C]Phe with phenylalanine ammonia-lyase.

Plant material. Cell suspension cultures of *F. arundinacea* Schreber were incubated at 25° under white light and sub-cultured into fr. medium (Murashige and Skoog salts, 4.4 g l^{-1}; glucose, 20 g l^{-1}; no phytohormones; pH 5.7) every 14 days. For radiolabelling, a 4-day-old culture (100 ml) was supplied with 47 MBq of L-[1-^{3}H]Ara for 5 hr or (aseptically) with 2 MBq of (*E*)-[U-^{14}C]cinnamate for 7 days.

Seeds of *F. arundinacea*, *F. gigantea*, *F. pratensis*, *F. rubra*, *Lolium multiflorum*, *L. perenne*, *Dactylis glomerata*, *Bromus sterilis*, *Agrostis tenuis* and *Phleum pratense* were kindly donated by Dr K. J. Webb, IGER, Aberystwyth. *Sorghum verticilliflorum*, *Dichanthium sericeum* and *Bothriochloa ambigua* were kindly donated by Mr Moffat Pinkie Setshogo (The University of Edinburgh). Seeds of *Secale sentinal*, *Sorghum vulgare* and *Hordeum vulgare* were purchased from W. K. McNair, Portobello, Edinburgh. Seedlings of all the above species were grown in a greenhouse at Edinburgh and harvested after 6 weeks. Plant material of *Triticum aestivum*, *Avena sativa*, *Arrhenatherum elatius*, *Anthoxanthum odoratum and Trachycarpus fortunei* was field-grown near Edinburgh. *Zea mays* was field-grown near Santiago de Compostela, Spain.

Preparation of AIR. Leaves (5 g) were crushed in liquid N_2 by pestle and mortar. Sand (2 g) was added and homogenization continued with 10 ml 80% EtOH for 5 min. The supernatant was rejected. After two more treatments with 80% EtOH, the AIR was dried. For prepn of AIR from cell cultures, cells were packed into a column and washed for at least 24 hr with slowly flowing 80% EtOH.

Acid and alkaline hydrolysis. Mild acid hydrolysis of AIR was with 0.1 M TFA (50–100 μl mg^{-1} dry wt) at 100° for 1 hr. 'Complete' acid hydrolysis was with 2 M TFA at 120° for 1 hr; TFA was removed *in vacuo*. Esters were cleaved by treatment with 200 μl of 0.5 or 0.1 M NaOH for 1 hr at 25° under N_2 in the dark, then adjusted to pH 4.7 with HOAc; for unhydrolysed controls, HOAc was added before the NaOH.

Enzymic hydrolysis. *β*-D-Xylosidase (ex *Aspergillus niger*, Sigma) was pelleted from 3.5 M $(NH_4)_2SO_4$, redissolved at 10.4 nkat ml^{-1} buffer [0.25 M pyridine

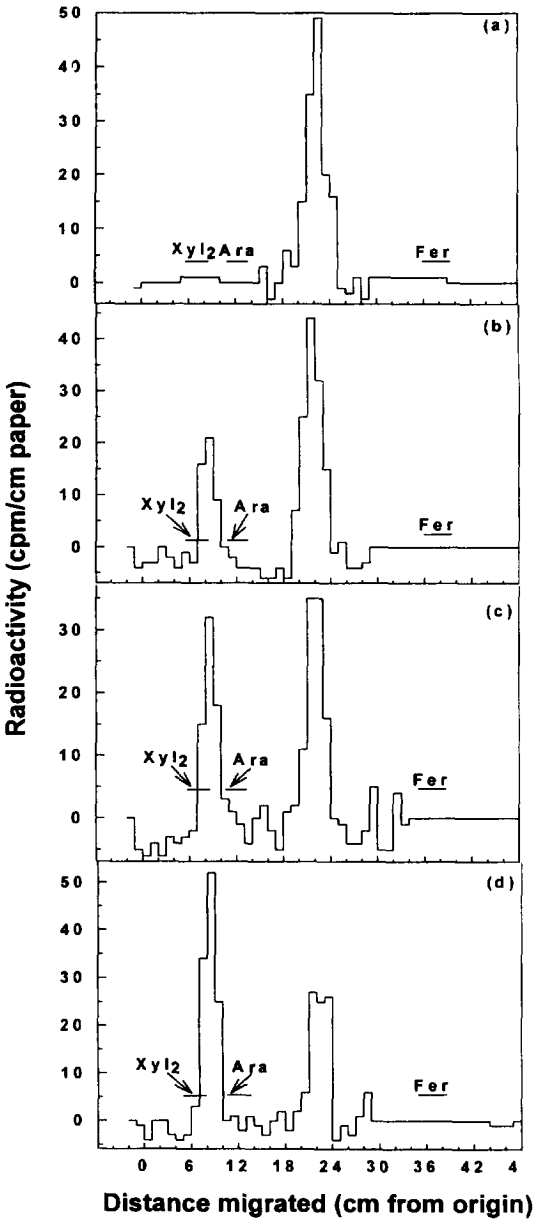


Fig. 3. PC in BAW of alkaline hydrolysis products of ^{3}H-labelled compound **2**. Hydrolysis (in 0.1 M NaOH) was terminated after (a) 0 min, (b) 1 min, (c) 2 min and (d) 4 min. Xyl_2, xylobiose.

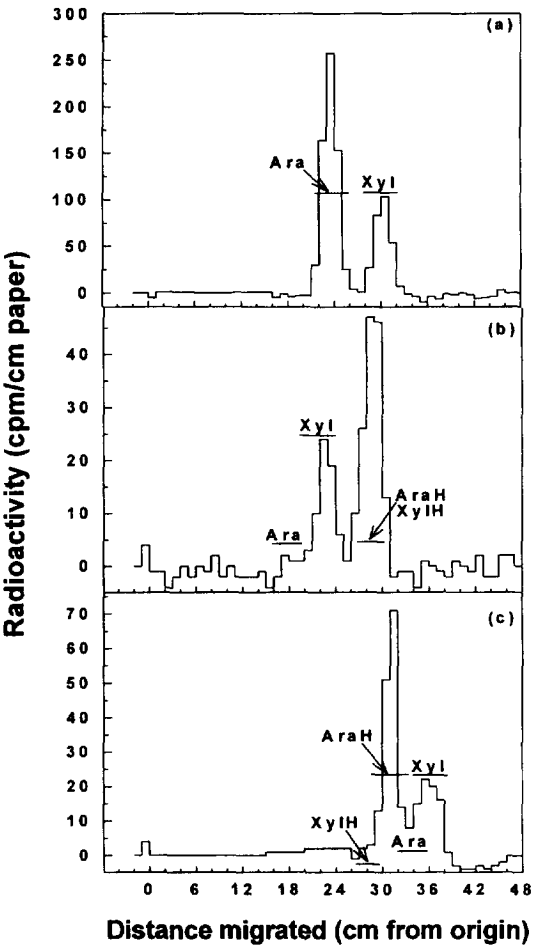


Fig. 4. Products of complete acid hydrolysis of **2** and **2b**. (a) PC in EPW of acid hydrolysis products of ^{3}H-labelled **2a**. Ara and Xyl as internal markers. (b) PC in BAB of acid hydrolysis products of ^{3}H-labelled **2b**. (c) PE in borate buffer of hydrolysis products of ^{3}H-labelled **2b**; AraH and Xyl were used as external and internal and Ara and XylH as external markers. AraH = arabinitol, XylH = xylitol.

(OAc$^-$), pH 5.0], added to a dried radioactive sample and incubated at 25°. After 10, 50, 250 and 1250 min, 100 μl was added to 10 μl HCO_2H to stop the reaction and the products analysed by PC (BAW). For prep. conversion of Fer-disaccharide (**2**) to Fer-monosaccharide, a portion of (feruloyl-^{14}C)-labelled **2** was treated with β-xylosidase for 40 min.

Paper chromatography and electrophoresis. PC was performed on Whatman 3MM paper by the descending method in the following solvents: BAW, *n*-BuOH–HOAc–H_2O (12:3:5); BEW, *n*-BuOH–EtOH–H_2O (20:5:11); BPW, *n*-BuOH–pyridine–H_2O (4:3:4); BAB, MeCOEt–HOAc–H_3BO_3-satd H_2O (9:1:1); EAW, EtOAc–HOAc–H_2O (10:5:6); EPW, EtOAc–pyridine–H_2O (8:2:1); ΦW, 80% (w/w) aq. PhOH. PE was performed on Whatman 3MM paper on a flat-bed, water-cooled system (20°), at 2 kV. Buffers used were molybdate (2% (w/v) $Na_2MoO_4 \cdot 2H_2O$, adjusted to pH 3.0 with HCO_2H; run time 5 hr) or borate (1.9% (w/v) $Na_2B_4O_7 \cdot 10H_2O$, pH 9.4; run time 1.7–2.5 hr). Bromophenol blue and picric acid were used as visible markers. Molybdate soln at pH 3.0, instead of pH 5.0 [31] was used because the lower pH maximized the difference in mobility between *O*-2- and *O*-3-blocked arabinitol derivatives.

Markers were 'external' (run alongside the radiolabelled sample) unless otherwise stated. 'Internal markers' [36] were pre-mixed with the radioactive sample to enable the demonstration of precise co-migration. After PC or PE and scintillation counting, paper strips were washed free of scintillant with PhMe, dried overnight and then either stained (to locate

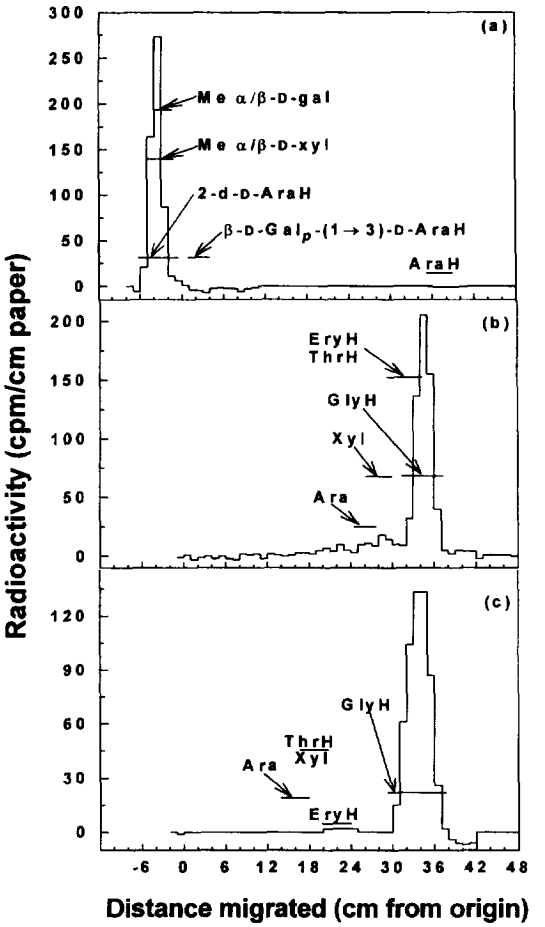


Fig. 5. Evidence for a Xyl-(1 → 2)-Ara linkage in compound **2**. (a) PE of ^{3}H-labelled **2b** in molybdate solution (pH 3.0). Internal markers used were 2-deoxy-D-*erythro*-pentitol (≡ '2-d-D-AraH'), and β-D-galactopyranosyl-(1→3)-D-ara-binitol. External markers were methyl α- and β-D-xyl-opyranosides, methyl α- and β-D-galactopyranosides and arabinitol. (b) PC in EAW of Smith degradation products obtained from ^{3}H-labelled **2b**. (c) PC in EPW of the major ^{3}H-labelled product shown in (b). Glycerol was an internal marker. EryH = erythritol, ThrH = threitol, GlyH = glycerol.

internal markers) or eluted with H_2O [37]. Reducing and non-reducing sugars were stained with aniline hydrogen-phthalate [38, modified as in ref. 36] and silver ($AgNO_3$–NaOH–$Na_2S_2O_3$ [39]), respectively. For silver-staining of papers containing borate, the NaOH dip was replaced by 2% NaOH and 4% pentaerythritol in 80% EtOH.

Low-pressure reverse-phase chromatography. C_{18}-substituted silica columns for LP-RPC (BondElut, 100

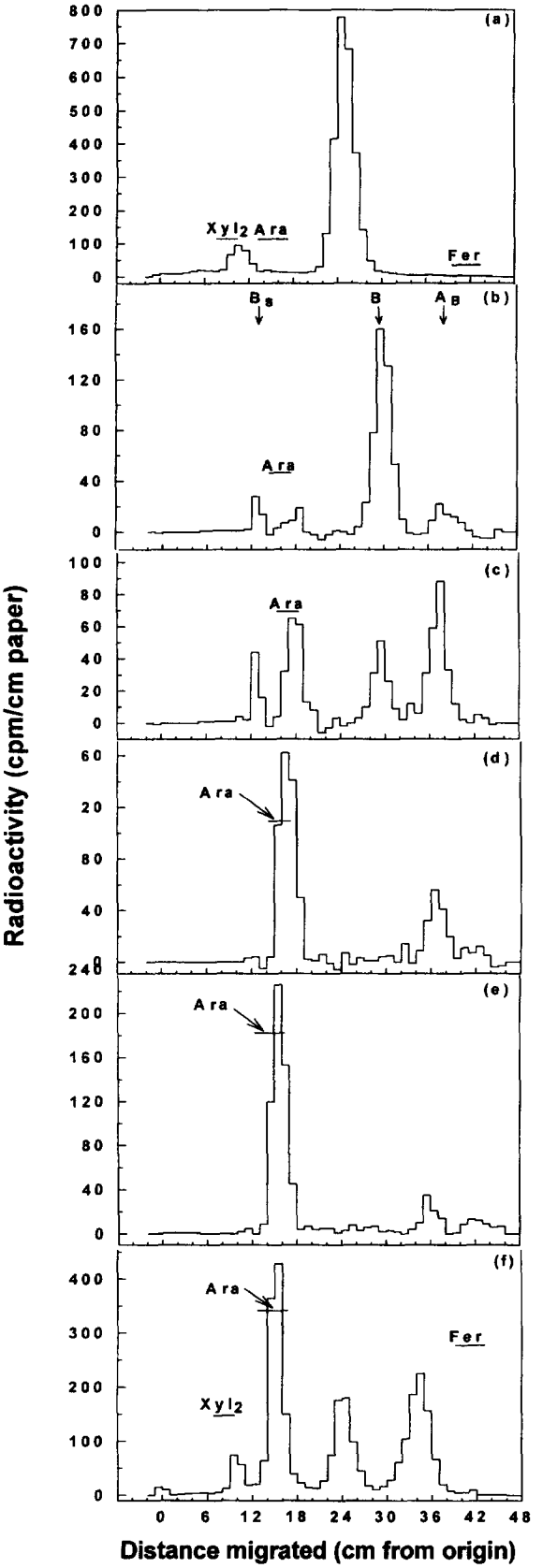


Fig. 6. PC in BAW of digestion products of ^{3}H-labelled **2**. (a) Untreated **2**. (b–e) Products of digestion with commercial β-D-xylosidase for 10 min (b), 50 min (c), 250 min (d) and 1250 min (e). A_B indicates the position of the putative 5-*O*-Fer-L-Ara. B and B_s indicate the positions of compounds **2** and **2a**, respectively. (f) Mild acid hydrolysis products of compound **2**. ↓, proposed identities; —, positions of external markers. Xyl_2, xylobiose.

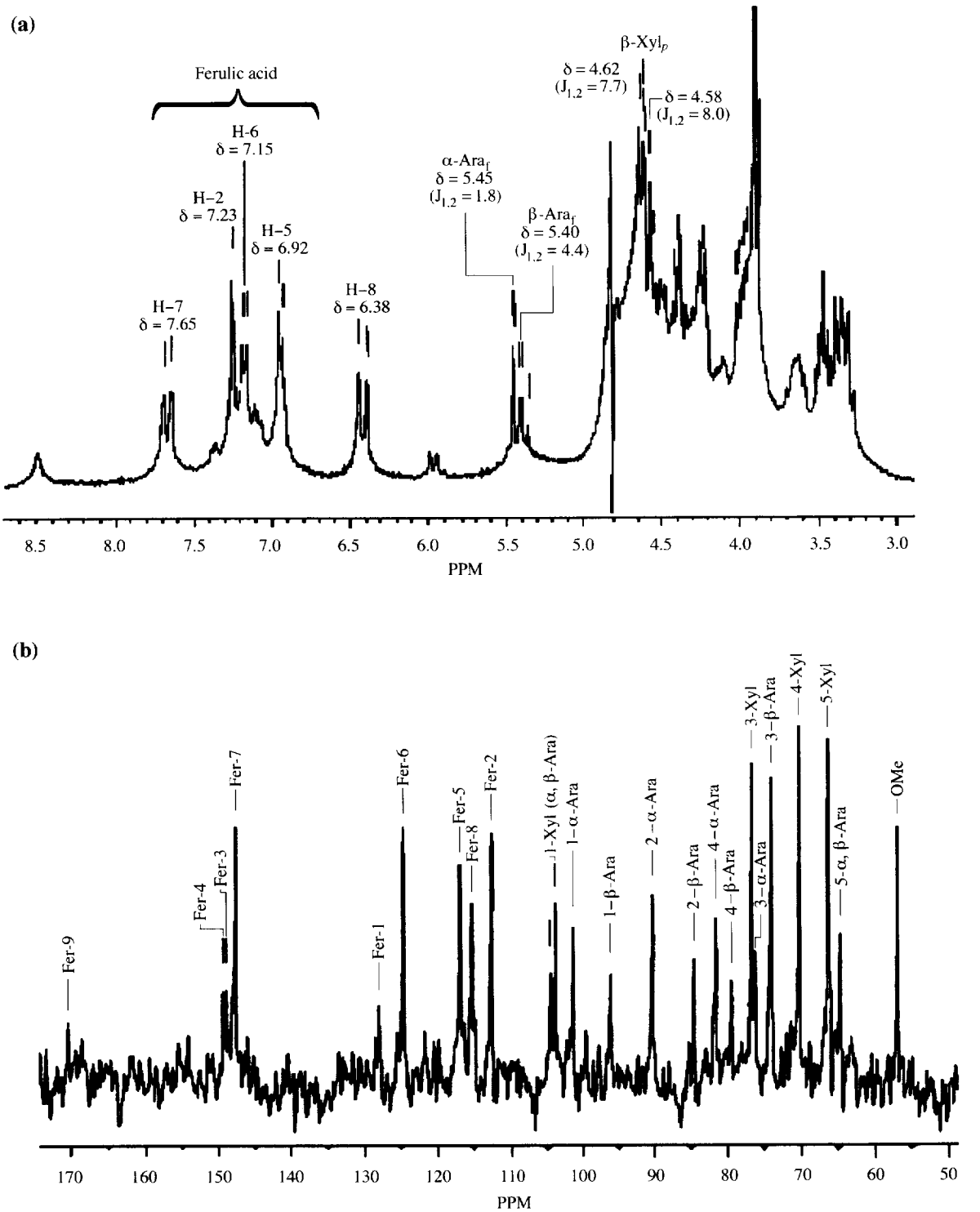


Fig. 7. NMR spectra of compound **2** in D_2O. (a) ^1H spectrum and (b) ^{13}C spectrum.

mg packing) were pre-treated with 3 ml MeOH followed by 3 ml H_2O. Aq. samples were passed through the column and rinsed with 5 ml H_2O to remove simple sugars; retained aromatic solutes were then eluted with 50% MeOH (10 ml) and dried *in vacuo*.

Gel-permeation chromatography. Samples were mixed with non-radioactive int. markers (Xyl, xylobiose, Glc, maltose and maltotriose; each 2 mg) and passed through a column of Bio-Gel P-2 (bed vol. 180 ml), in HOAc–pyridine–H_2O (1:1:23, pH ~4.7) at 6.8 ml hr^{-1}. Frs (2.6 ml) were collected; 100 μl was assayed for hexose [40] and 2 ml for ^3H, and 100 μl

Table 3. Assignments of signals in ^{13}C NMR spectrum of compound **2**

Residue	^{13}C chemical shifts (δ)*									
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	Me
α-L-Ara*f*	101.4	90.2	76.2	81.6	64.8	–	–	–	–	–
β-L-Ara*f*	96.2	84.6	74.1	79.5	64.8	–	–	–	–	–
β-D-Xyl*p*	104.2, 103.8	74.1	76.7	70.3	66.4	–	–	–	–	–
Ferulate	127.8	112.5	148.8	149.2	116.8	124.6	147.6	115.1	170.2	57.0

*MeOH was internal reference (δ 50.2 with respect to TMS).

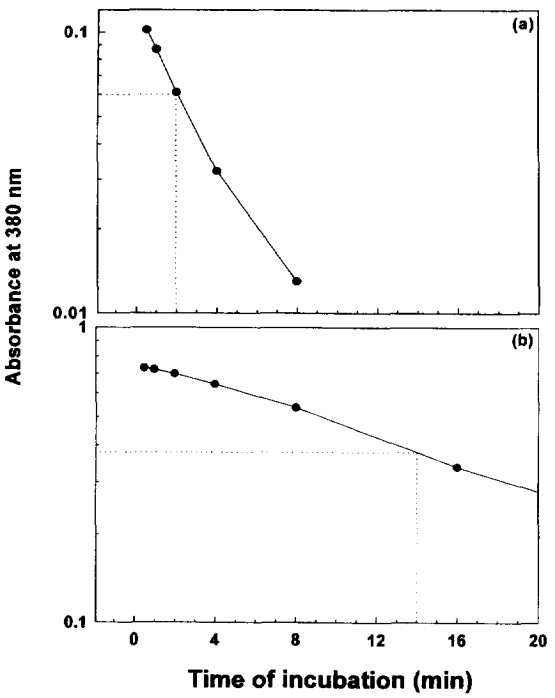


Fig. 8. Kinetics of alkaline hydrolysis of the ester bond in **1** and **2** in 0.1 M NaOH at 25°. (a) 5-*O*-Feruloyl-L-arabinose (compound **1**). (b) 2-*O*-β-D-Xylopyranosyl-(5-*O*-feruloyl)-L-arabinose (compound **2**). A_{380} is a measure of feruloyl ester remaining.

was subjected to PC (BAW) and stained to locate the markers.

HPLC. HPLC was performed on C_{18}-substituted silica (Spherisorb S5ODS2, Hichrom). The gradient was 15 → 25% MeCN in 40 min. Detection was by A_{280} to maximize detection of non-feruloyl aromatic impurities. Prep. HPLC was on a 25 × 1 cm column, flow-rate 4 ml min^{-1}. Analytical HPLC used a 25 × 0.46 cm column at 1 ml min^{-1}.

GC-MS and NMR. Amounts of **2** needed for NMR were prepd from 25 g of *F. arundinacea* cell culture AIR by mild acid hydrolysis, LP-RPC, PC in BAW, PC in BEW and prep. HPLC. Sample (3.5 mg) was dissolved in D_2O at 25° in a 5-mm sample tube. ^1H and ^{13}C NMR spectra were acquired by use of a 10-mm multinuclear NMR probe (^1H, 300 MHz; ^{13}C, 75 MHz). GC-MS was conducted on a spectrometer equipped with an SP 2330 column. We thank Dr V. Farkaš (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia) for performing these analyses.

Reduction with $NaBH_4$. Dried samples were treated with 0.5 M $NaBH_4$ in 1 M NH_3 (two 0.2-ml portions, each for 12 hr) at 25° in a capped tube. Excess $NaBH_4$ was destroyed with 80 μl HOAc and the solns were then passed through a 1.5-ml bed-vol. column of Dowex-50W (H^+) to bind NH_4^+ and Na^+. Samples were eluted with 8 ml H_2O, dried and freed of H_3BO_3 by re-drying × 10 from MeOH–HOAc (10:1).

Smith degradation. Compound **2b** was incubated in 0.1 ml of 50 mM $NaIO_4$ in 0.25 M HCO_2^- (Na^+) buffer, pH 3.7, for 6 days at 4° in the dark. Ethane-1,2-diol (20 μl) was then added and incubated for a further 1 hr. Oxidation products were reduced with $NaBH_4$, acidified with HOAc, treated with Dowex 50 and freed of H_3BO_3, as described above, re-dissolved in H_2O, passed through a 3-ml column of Dowex 1 (1X4-200; OAc^- form) to bind iodate, eluted with 8 ml H_2O, dried and treated with 2 M TFA at 100° for 10 min.

Methylation analysis. A portion (0.6 mg) of HPLC-purified **2** was methylated by the method of ref. [41], acid-hydrolysed, reduced with $NaBD_4$, acetylated and analysed by GC-MS. We thank Dr V. Farkaš for performing these analyses.

Assay of radioactivity. Strips of chromatography paper were assayed for radioactivity by scintillation-counting in PhMe containing 0.5% PPO and 0.05% POPOP. The counting efficiency for ^3H-labelled sugars was ~7%. For ^3H-samples of very low activity, the paper was soaked in 1 ml H_2O for 1 hr then mixed with 10 ml of 'Triton scintillant' (TS; 0.33% PPO and 0.033% POPOP in PhMe–Triton X-100 (2:1)). This increased the counting efficiency ~6-fold. Aq. solns were assayed for radioactivity after mixing with 10 vols of TS.

Determination of sp. act. of [^3H]*arabinose and* [^3H]*xylose residues in AIR.* Radiolabelled AIR (110 mg) was subjected to complete acid hydrolysis. The soln was dried and re-dissolved in 1 ml H_2O and 9 replicate 100-μl portions were subjected to PC in EPW for 48 hr. From each track, three 7-cm zones were cut out: (a) behind the origin (= blank), (b) Ara and (c) Xyl. Sugars were eluted from each zone and adjusted to 5 ml. Portions of each 5-ml sample were assayed

Table 4. Rate constants (k) for alkaline hydrolysis of O-feruloyl esters

Compound	$10^3 \times k$ ($mM^{-1} s^{-1}$)
Methyl ferulate*	15
Ethyl ferulate*	9
Hydroxyethyl ferulate*	15
4-O-(6-O-Feruloyl-β-D-Gal$p$)-D-Gal*	28
5-O-Feruloyl-L-Ara (**1**)†	58
2-O-β-D-Xyl$p$-(5-O-feruloyl)-L-Ara (**2**)†	8

*Calculated from data from ref. [26]; hydrolysis was conducted at 22°.
†Present work; hydrolysis was conducted at 25°.

for radioactivity (6×33 μl) and for reducing sugars by the PAHBAH-assay [42] (10×33 μl portions of a 10-fold diln), using L-Ara and D-Xyl (dried in a vacuum desiccator over P_2O_5) as standards. Readings from the eluates of zone (a) were subtracted from the values for Ara and Xyl.

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