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IN VITRO PEROXIDASE-CATALYSED OXIDATION OF FERULIC ACID ESTERS

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Key Word Index—Methyl ferulate; peroxidase; oxidation; plant cell walls.

Abstract—Methyl ferulate, a model feruloyl ester, was reacted with H_2O_2 and peroxidase in order to simulate the possible fate of such esters in the primary cell wall. The major dimeric product was identified as a cyclic dimer with a β-5 linkage and an α-*O*-4 linkage. This compound, which exceeded the yield of 5,5′-dehydrodiferulic acid dimethyl ester, could arise by nucleophilic substitution of an intermediary β-5 quinone methide with the neighbouring phenolic hydroxyl group. Inclusion in the reaction mixture of high concentrations of sugars as alternative nucleophiles, such as would occur in the plant cell wall, resulted in very little or no bonding of aromatic material to the sugar. In contrast to these *in vitro* findings, 5,5′-dehydrodiferulate is the only oxidatively-coupled dimer of ferulate to have been reported *in vivo*; the apparent formation of ether bonds between ferulate oxidation products and cell wall polysaccharides has been reported. Mechanisms are proposed which may facilitate bonding of ferulate-derived quinone methides to carbohydrates *in vivo*.

INTRODUCTION

Despite being structurally well characterized as esterified substituents of hemicellulosic and pectic polysaccharides [1–5], the precise role of feruloyl groups in the plant cell wall remains controversial. Recent work has strongly suggested that, in lignified secondary cell walls, such esters act predominantly as bridging units between lignin and hemicellulose [6, 7]. In the non-lignified actively growing primary wall, however, they presumably play another role. One hypothesis is that cross-linking of the feruloyl residues may play an important role in controlling cell wall extensibility [8, 9]. Such cross-linking may be achieved either photochemically or oxidatively. Photodimers of esterified phenolic acids are reported to be present in large amounts in the cell walls of many Graminaceous species [10, 11]. However, ferulic acid dimerizes much more slowly than *p*-coumaric acid [12] and photodimerized phenolic esters have, as yet, not been reported in primary cell walls.

The idea of oxidative coupling playing a role arose from the isolation of 5,5′-dehydrodiferulic acid from alkaline hydrolysates of wheat flour pentosans [13] and grass cell walls [14]. It was suggested that such coupling could be catalysed by cell wall-bound peroxidases via a reaction analogous to lignin synthesis [8, 14, 15]. The proposed reaction mechanism proceeds via an initial one electron oxidation in the presence of peroxidase–H_2O_2 to produce a series of mesomeric radicals, the most stable, in the case of coniferyl alcohol, being where the unpaired electron is at the β-position. These radicals will then spontaneously dimerize to form several possible structures, including the stable 5,5′-dehydrodiferulic acid. However, some of the dimers are quinone methides and susceptible to subsequent nucleophilic attack [16]. Within the cell wall, nucleophiles available for such reactions are likely to be water (to form the α-alcohol), organic acids, e.g. uronic acids (to form esters), and alcohols, e.g. carbohydrates and phenolic groups (to form ethers). The rate of this reaction is highly dependent on the nature of the nucleophile and pH [17–19]. At slightly acidic pH, i.e. that usually thought to be typical of the cell wall [20], the likely order of reactivity would be R-COOH > H_2O > ROH > PhOH [19].

The formation of ether linkages between cell wall polysaccharides and phenolics has long been hypothesized from model studies with coniferyl alcohol [16, 21, 22]. Indeed, evidence for their existence in primary walls of spinach has previously been reported [23]. It was found that after alkaline hydrolysis of cell wall preparations from [^{14}C]cinnamic acid-fed spinach cells, a large proportion of the label remained bound to the cell wall polysaccharides. Furthermore, it was found that when [^{14}C]feruloyl-[^{3}H]arabinobiose was added to suspension cultures of spinach, the ^{14}C became polymer-bound via alkali-stable linkages, although the [^{3}H]arabinobiose was released intact by alkaline hydrolysis [24]. The presence of etherified structures could have profound effects on the extensibility of growing cell walls.

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The purpose of the present work was to study the oxidative reactions of model feruloyl esters *in vitro* and to determine the structures likely to be found *in vivo*.

RESULTS

Preliminary oxidation experiments

An initial oxidation used the zutropf (gradual addition) technique in adding methyl ferulate and H_2O_2 to a highly concentrated glucose solution (75% w/w) containing peroxidase. After extraction into butanol, the products were passed through a column of Bio-Gel P-2. The UV absorbing material and the sugar were clearly resolved, and no evidence of any co-chromatography could be seen (data not shown). Little if any UV absorbance was found in the non-butanol-soluble fraction. A trace of material eluted in the void volume which gave a brown product with anthrone, quite distinct from the blue given by hexoses. The upper limit of the hexose yield in the V_0 was estimated at 1 μmol per 10.24 mmol methyl ferulate.

Figure 1 shows the HPLC separation of the precipitated oxidation products (CP). Fraction CW (solid phase extraction (SPE)-non-binding) contained negligible UV absorbing material. Comparison of these chromatograms with the controls lacking sugar showed no discernible qualitative difference (data not shown). Figure 2A shows the HPLC trace of the water-soluble plus water-insoluble, SPE-retained products of a small scale oxidation, in the presence of radioactive methyl D-glucosides; it shows a similar pattern to that for the non-radioactive experiment. Figure 2B shows the radioactivity of the eluate; the initial, large peak is unretained and is probably residual unreacted methyl D-glucosides. However, a second partially retained peak is clearly visible, and coincides with a UV-absorbing region. This would be compatible with some of the labelled sugar having become conjugated to a less polar substance, although it represents only 0.022% of the original label.

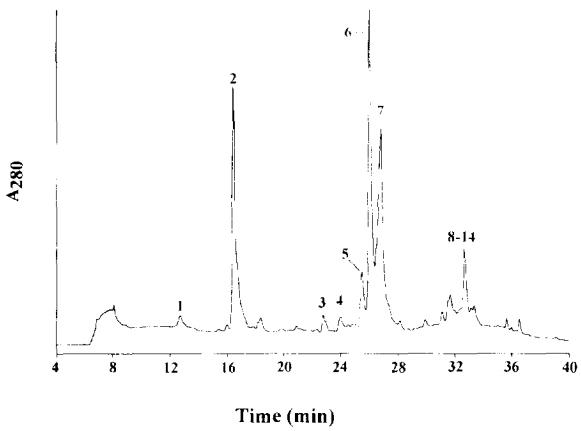


Fig. 1. HPLC trace of precipitated oxidation products formed by the action of peroxidase plus H_2O_2 on methyl ferulate in the absence of sugar.

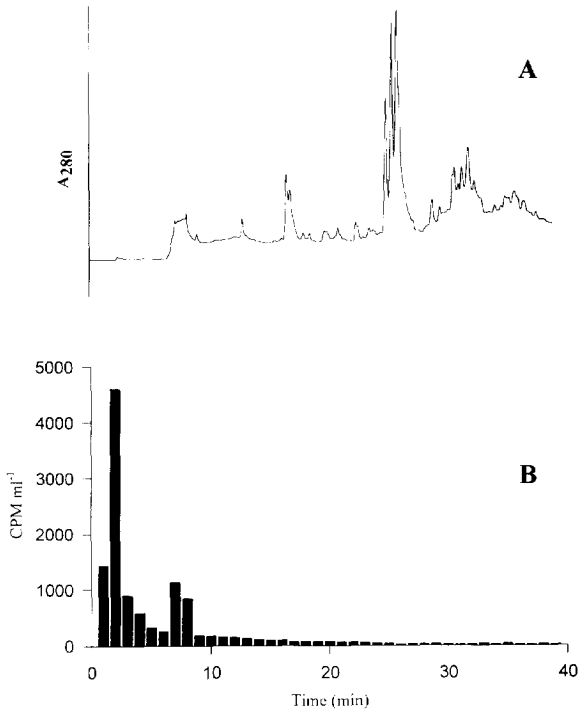


Fig. 2. HPLC trace of SPE-retained oxidation products of methyl ferulate formed in the presence of methyl [^{14}C]glucoside, (A) UV detection and (B) radioactivity.

Optimization of conditions

Conditions considered important in the reaction were rate of addition of substrate and H_2O_2, total reaction time and pH. Initial time-course experiments showed that after addition of substrate, H_2O_2 and peroxidase, oxidation was more or less complete after 2 hr and no significant difference was found even after a total of two days.

To try and optimize the addition rate of oxidant, H_2O_2 was added at rates ranging from 1×25 μl all at once to 16×1.56 μl at half hour intervals. Little qualitative difference between the products could be discerned (data not shown). The rate of addition of both methyl ferulate and H_2O_2 was also studied, again by comparison of one bulk addition and 16 additions at half hour intervals. Again, little qualitative difference was seen (data not shown).

The next important parameter considered was pH. Oxidation was performed at pH 3, 4, 5, 6, 7 and 8, with and without concentrated (55% w/w) solutions of methyl α-D-glucopyranoside. Methyl ferulate and H_2O_2 were added in five lots over 1 hr and the SPE-retained material was analysed. Again, relatively little qualitative difference could be discerned between the products formed at these pH values. However, very small peaks of relatively polar products were consistently present in the reactions containing the sugar (but not the controls) at pH values of 4, 5 and 6; the highest yield of these was at pH 5 (Fig. 3). Therefore, all further experiments were performed at pH 5.

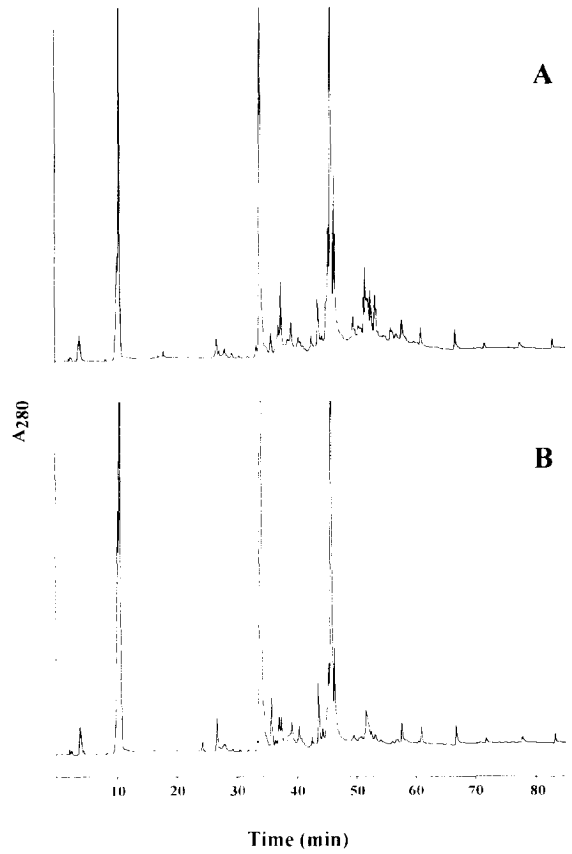
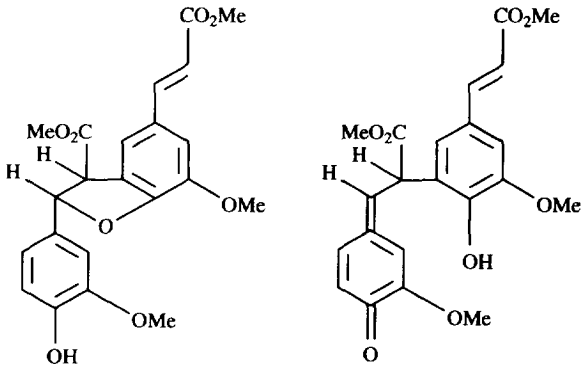


Fig. 3. HPLC analysis of SPE-retained oxidation products formed at pH 5, (A) with methyl α-glucoside and (B) without.

Product identification

In order to identify the reaction products, a large scale oxidation of methyl ferulate was performed in the absence of sugars and the products were isolated and purified by semi-preparative HPLC for structural analysis by NMR. In all, 14 peaks were collected in amounts necessary for ^{1}H NMR analysis; the pattern was very similar to that shown in Fig. 1, with which the following peak numbering corresponds. The UV spectrum of each peak was also recorded by diode-array detection. Peak 1, already shown to be an impurity in the methyl ferulate preparation, was identified as methyl α-methoxy-dihydroferulate and peak 2 was confirmed as unreacted methyl ferulate. The proposed structure for peak 6 ($[M]^+$ *m/z* 414) is shown in formula **1**. The signals at δ7.33 and 7.60 could not be assigned to the proposed structure and are thought to originate from a contaminant which may have been responsible for some complex noise in the aromatic region. The source of any contamination is uncertain as the sample was chromatographically pure.

Owing to the small sample size, the ^{1}H NMR spectrum for peak 5 was not easily assigned. However, the spectrum was very similar to that for peak 6 and was thought most likely to be a diastereoisomer. Peak 7 gave a spectrum similar to that expected for dimethyl 5,5′ dehydrodiferulate. NMR spectra of peaks 8–14 were highly



complex and lack of material made precise assignment difficult. The complexity of the aromatic region and their chromatographic behaviour suggested that they were tri- to tetrameric products.

Enzymic oxidation of methyl ferulate in presence of polysaccharides

Similar oxidation experiments were also performed using larch arabinogalactan instead of methyl D-glucopyranosides as the potential nucleophile. After the reaction, the mixture was centrifuged to remove the bulk of the free phenolic material. After washing, the supernatant was applied to a column of Sephacryl S-400 to remove residual, soluble, low M_r phenolics (Fig. 4). A large amount of UV-absorbing material eluted in the void volume and in another low K_{av} peak, but this may have included particulate material. The arabinogalactan eluted as a sharp peak at *ca* 500 ml and this co-eluted with a small peak of UV-absorbing material. The arabinogalactan peak was collected and re-chromatographed on a column of Sephacryl S-200, on which the polysaccharide eluted near the void volume. Approximately half of the UV absorbing material eluted in the totally included volume; however, some UV-absorbing material still ran in conjunction with the polysaccharide (data not shown). Untreated arabinogalactan did not contain any co-eluting UV-absorbing material. Thus, the results again provide evidence that some phenolic material had become cross-linked to the carbohydrate. However, it was evident that such cross-linking has occurred only to a very limited extent ($\leqslant 0.28\%$ of the initial methyl ferulate).

Non-enzymic oxidation

Oxidants which are not dependent on enzymic catalysis were also studied in an attempt to simulate peroxidase oxidation mechanisms in a low water system. Ammonium persulphate yielded no products under the conditions used. Manganese dioxide yielded small quantities of dimeric products, the yield of which approximately doubled in the presence of *p*-toluenesulphonic acid (*p*-TSA). Ferric chloride, interestingly, yielded only one

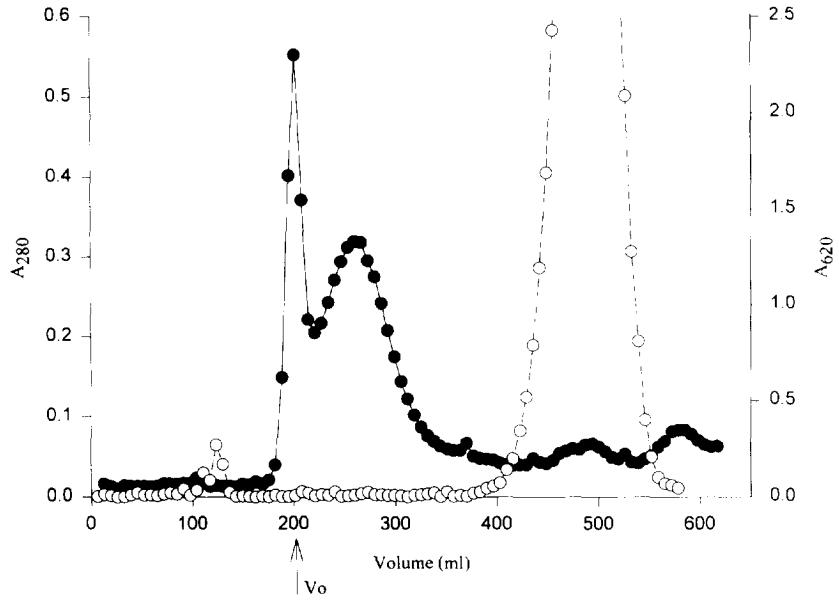


Fig. 4. GPC analysis on Sephacryl S-400 of oxidation products of methyl ferulate formed in the presence of arabinogalactan. ●—● A_{280}; ○---○ A_{620} (anthrone assay).

product, which, judging by retention time, was possibly the 5,5′ dimer. The presence of *p*-TSA made little difference. In none of the above cases was there any qualitative difference between the reaction in the presence or absence of glucose. Lead tetraacetate proved to be the most effective oxidant, giving the largest yield and the closest profile to the peroxidase-catalysed reaction. In the absence of *p*-TSA, there was no qualitative difference between the products formed with and without sugar (data not shown). However, with *p*-TSA (Fig. 5) a series of possibly five–six peaks can clearly be seen which only appear in the sugar reaction, eluting in the region where phenolic–sugar conjugates may be expected to run, based on their probable polarities.

DISCUSSION

Despite having been extensively studied *in vitro* [25–29], there still remains considerable uncertainty about the peroxidase-catalysed oxidation reactions of phenylpropanoids *in vivo* and the subsequent nucleophilic substitutions that may take place. To date, such studies have essentially concentrated on monolignols, i.e. coumaryl, coniferyl and sinapyl alcohols, as the substrates and either lignin biosynthesis or the possible formation of lignin–carbohydrate bonds via nucleophilic substitution of quinone methides. Recent work has concentrated on phenolic esters as the potential nucleophiles that react non-enzymically with lignol-based quinone methides [30]. Surprisingly little attention has been paid to phenolic esters as peroxidase substrates and their possible fate within the non-lignified primary cell wall.

Initial experiments were concerned with trying to extend previous coniferyl alcohol-based studies [22] to produce the methyl ferulate equivalent of guaiacylglycerol-α-(*O*-6-D-glucose)-β-coniferyl ether. However, using exactly the same conditions, the oxidation products of methyl ferulate apparently failed to react with sugars to nearly the same extent as did those of coniferyl alcohol. Further oxidation experiments using radioactive sugar confirmed that any cross-linking to sugars was slight. Attempts at increasing yields by altering conditions showed that the outcome of the reaction was little influenced by pH, incubation time or the rate of addition of H_2O_2 or methyl ferulate. It was thus reasoned that in all likelihood, if quinone methides were being formed, some other nucleophile was grossly out-competing the sugar.

In order to assess if the quinone methides were being produced, it was thus necessary to identify the products formed. It was shown that 5,5′-dehydrodiferulate was a relatively minor product of the reaction and from NMR analysis it would appear that the major product in all the oxidation experiments performed, is a quinone methide-derived cyclic dimer with a β-5 linkage and an α-*O*-4 linkage (**1**). This would be formed from the initially-formed β-5 quinone methide (**2**) by an intramolecular reaction involving the phenolic hydroxyl group as the nucleophile. If this is also true of the cell wall it would suggest that along with 5,5′-dehydrodiferulate, large amounts of this dimer should also be found in alkaline hydrolysates of primary cell walls. To date, no such structure has been reported from cell walls. Therefore, if the appropriate quinone methide is being formed, it may become bound to the wall via an alkali-stable linkage, possibly through the nucleophilic addition of a sugar residue, rather than the intra-molecular reaction with the phenolic group. Feruloyl esters did not appear to form products much larger than tetramers, whereas coniferyl

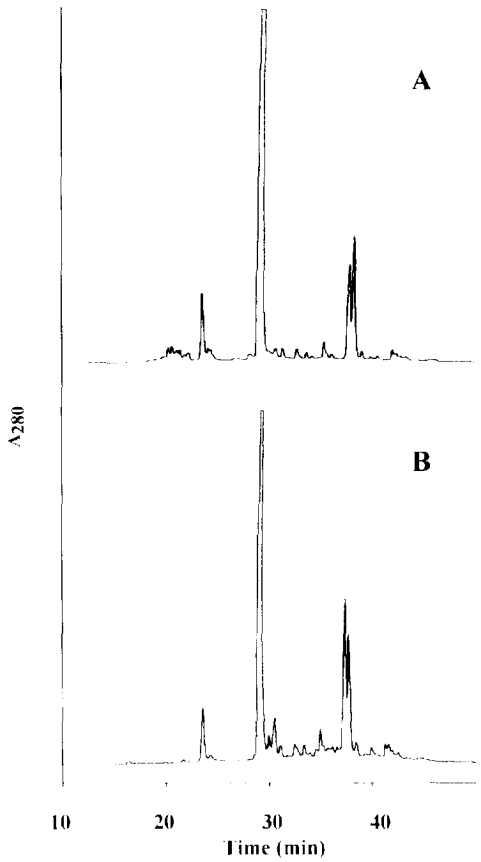


Fig. 5. HPLC analysis of lead tetraacetate oxidation products of methyl ferulate (with *p*-toluenesulphonic acid), (A) with glucose and (B) without glucose.

alcohol has been reported to polymerize up to M_rs of 3000 [31].

In a further attempt to mimic the cell wall more closely, polysaccharides were used as the potential nucleophile. Arabinogalactan (a backbone of β-(1 → 4) galactose with (1 → 5)-linked arabinan side-chains [32]) was chosen for its solubility (25% w/w was possible) and the large number of primary alcohol groups available. Again, however, whilst evidence compatible with cross-linking reactions was obtained, the yields were very low.

Non-enzymic oxidations were performed in an attempt to obtain model compounds, in order to identify products from enzymic reactions. The failure of ammonium persulphate and manganese dioxide to produce any apparent oxidation was surprising, as ammonium persulphate has previously been reported to be able to gel feruloylated pectic material and was thought to work in an analogous way to peroxidase-catalysed oxidation [33]; manganese dioxide has previously been used for synthetic lignin production from coniferyl alcohol [34]. The reaction of ferric chloride to yield only one product is interesting and may be of use in model synthesis. While the mechanism of lead tetraacetate oxidation of phenols is still not certain [35], it gave the closest reaction profile to that of peroxidase. Whilst it is known that lead tetraacetate will oxidize sugars to a variety of products, it is unlikely that any such products would absorb at 280 nm and be non-polar enough to bind to a C18 SPE column. Thus, lead tetraacetate may be valuable in the production of suitable phenolic–sugar models.

In our enzymic oxidation experiments, a major oxidation product was found to be a β-5/α-*O*-4 linked dimer (Scheme 1, **1**), confirming that quinone methides were being produced. It is thus of great interest to note that, *in vivo*, feruloylated derivatives apparently become extensively ether-bonded to primary cell wall polysaccharides [23, 24]. Possible mechanisms that direct reactions with polysaccharides *in vivo* include (a) exclusion of water by local hydrophobic domains within the cell wall, e.g., due to the clustering of feruloyl groups [36], (b) the existence of a novel lyase enzyme that would catalyse the reaction of quinone methides with carbohydrates, and (c) the possibility that a quinone methide that is ester-bonded to two wall polysaccharide chains is positioned with its reactive α-carbon adjacent to a suitable hydroxyl group on a neighbouring carbohydrate.

EXPERIMENTAL

Starting materials. For the synthesis of Me ferulate, ferulic acid (1 g, Sigma) was dissolved in 50 ml MeOH and stirred overnight at 50° with MeOH-washed Dowex-50W H^+ ion-exchange resin. The soln was then diluted to 250 ml with H_2O, buffered to pH 8 with $NaHCO_3$ and extracted with 3 × 50 ml *n*-BuOH. The *n*-BuOH phase was then dried-down and the Me ferulate recrystallized from EtOAc–hexane. The product showed a single UV-absorbing spot by TLC on silica gel with EtOAc–hexane (1:1, R_f 0.62). Yields were in excess of 90%. ^{1}H NMR (200 MHz $CDCl_3$): δ3.73 (3H, *s*, Ph-CH = *trans*) 3.8 (3H, *s*, CO_2–Me) 6.3 (1H, *d*, J = 16 Hz, = CH–CO_2 *trans*) 7.6 (1H, *d*, J = 16 Hz, Ph–CH = *trans*) 6.9–7.1 (3H, *m*, aromatic H).

Diferulic acid was synthesized according to refs [37, 38]. ^{1}H NMR (250 MHz, DMSO-d_6): δ3.89 (6H, *s*, methoxyl) 6.39 (2H, *d*, J = 15.9 Hz, = CH–CO_2 *trans*) 7.03 (2H, *s*, aromatic H) 7.32 (2H, *s*, aromatic H) 7.51 (2H, *d*, J = 15.9 Hz, Ph–CH = *trans*).

Me D-[6-^{14}C]glucopyranoside (α and β) was prepd by dissolving 10 μCi [U-^{14}C]glucose (Sigma, 318 mCi mmol^{-1}) in 2 ml MeOH and stirring for 24 hr at 65° with MeOH-washed Dowex-50W H^+. The mixt. was filtered, applied to Whatman 3MM paper and developed in *n*-BuOH–HOAc–H_2O (12:3:5). The chromatogram was analysed using a radio-TLC scanner. The appropriate band was cut out and and the methyl D-glucosides (α and β) were eluted with 50% MeOH.

Oxidation. Preliminary bulk experiment: this was performed according to ref. [22]. A soln of Me ferulate (9.5 mmol in 250 ml Me_2CO) and 6 ml of 30% (v/v) H_2O_2 (70 mmol) were added slowly, over 24 hr, to 200 g of a 75% (w/w) soln of glucose containing 2 mg peroxidase (Sigma Type VI). This was carried out in an open vessel, from which much of the Me_2CO evapd. After

a further 24 hr, the reaction mixt. was extracted with 5 × 50 ml *n*-BuOH which was dried and 150 mg applied to a 50 × 0.7 cm column of Bio-Gel P-2 (Biorad) and eluted with dioxane–H_2O (1:1). Non-radioactive experiment: Me ferulate (1 mmol in 1 ml MeOH) and 0.1 ml 30% (v/v) H_2O_2 (1.1 mmol) was slowly added (over 1 hr) to 5 ml of a 56% (w/w) soln of Me β-D-glucopyranoside (Sigma) in 50 mM sodium phosphate–citrate buffer (pH 4.2) containing 0.5 mg peroxidase (Sigma type VI) with continuous stirring at 25°. After a total reaction time of 2 hr, the mixt. was centrifuged at 27 000 *g* for 10 min, the supernatant collected and the pellet (CP) washed × 3 with H_2O. The collected supernatant and washings were passed through a C_{18} solid phase extraction (SPE) cartridge (Bond Elut, Varian), the unretained material and washings collected (CW) and the retained material (CO) eluted with 1 ml MeOH. Radioactive experiment: Me D-[^{14}C]glucoside (4 μCi) was added to 10 ml of the 56% soln of Me β-D-glucopyranoside mentioned above which was then reacted with 0.5 ml 30% H_2O_2 (5.5 mmol), 0.1 mg peroxidase (Sigma type VI) and Me ferulate (5 mmol), added all at once, in a 1 ml Reactivial and stirred for 2 hr. The reaction mixt. (including any insoluble matter) was washed out with 2 ml H_2O and passed through a C_{18} SPE cartridge. Unretained material and washings were collected and the retained material eluted with 1 ml MeOH.

Optimization experiments. Oxidations in presence of polysaccharide: to 125 g of a 25% (w/w) soln of larch arabinogalactan (Sigma) in 50 mM citrate–phosphate buffer (pH 5) was added 1 mg peroxidase (type II), 5 ml of a 4 mg ml^{-1} soln of Me ferulate (0.42 mmol) in Me_2CO and 25 ml of a 0.6% (v/v) soln of H_2O_2 (4.4 mmol) in buffer, dropwise over 8 hr. These additions were repeated on three consecutive days. The mixt. was then spun at 43 500 *g* for 1 hr and the supernatant collected and freeze-dried. Samples (100 mg) were then run on a 178 × 2.7 cm column of Sephacryl S-400 (Sigma) in H_2O. The void vol. peak was collected and re-chromatographed on a 72 × 1.6 cm column of Sephacryl S-200 (Sigma) also in H_2O. Non-enzymic oxidations: to 1.8 ml of dry DMSO or a 30% (w/w) glucose soln in dry DMSO, was added 0.1 ml of a 10 mg ml^{-1} soln of Me ferulate (4.8 μmol) in Me_2CO and 0.1 ml of a 100 mM soln of oxidant (10 μmol) in dry DMSO. Duplicate expts were also performed in the presence of a few crystals of *p*-TSA. The reaction mixt. was shaken, at room temp. for 2 hr, then diluted to 100 ml with H_2O and the whole sample, including any insoluble material, applied to a C18 SPE cartridge. The cartridge was washed with H_2O and the retained material then eluted with 1 ml MeOH and analysed by HPLC.

Analysis. HPLC analysis was performed with diode-array detection on a 25 × 0.46 cm column of Spherisorb S5ODS2 with a gradient of 100% H_2O–*n*-BuOH–HOAc (98.3:1.2:0.5) to 100% MeCN–*n*-BuOH–HOAc (98.3:1.2:0.5) over 1 hr at 1.2 ml min^{-1}, with UV detection at 280 nm. Semi-prep. HPLC was performed as described above on a 25 × 1.0 cm column at 3 ml min^{-1}. In the radioactive expt, 1-ml-frs were mixed with 10 ml scintillant [0.33% PPO + 0.033% POPOP in toluene-Triton X-100 (2:1 v/v)] and assayed for ^{14}C by liquid scintillation counting. Carbohydrates were determined by the anthrone assay [39]. Unless stated otherwise, ^{1}H NMR spectra were recorded at 300 MHz in CD_3OD, chemical shifts being calibrated to int. TMS.

Product identification. Peak 1: ^{1}H NMR (200 MHz $CDCl_3$): δ2.55–2.79 (2H, *m*, CH_2–CO_2), 3.20 (3H, *s*, C–O–Me), 3.68 (3H, *s*, Ph–O–Me), 3.88 (3H, *s*, CO_2Me), 4.54 (1H, *dd*, *J* = 9.1 and 4.7 Hz, C–H), 6.72–6.89 (3H, *m*, aromatic H). UV λ_{max} 278 nm. Peak 2: ^{1}H NMR (300 MHz CD_3OD): δ3.75 (3H, *s*, Ph–OMe), 3.87 (3H, *s*, CO_2–Me), 6.35 (1H, *d*, *J* = 16 Hz, =CH–CO_2 *trans*), 6.6–7.1 (3H, *m*, aromatic H), 7.6 (1H, *d*, *J* = 16 Hz, Ph–CH = *trans*). UV λ_{max} 323 nm (shoulder at 295 nm). Peak 5: EIMS (probe) 70 eV *m/z* (rel. int.): 43 (100), 382 (61), 415 (58), 59 (43), 167 (43), 152 (35), 151 (32), 350 (32). UV λ_{max} 321 nm (shoulder at 287 nm). Peak 6: EIMS (probe) 70 eV *m/z* (rel. int.): 416 (20), 415 (45), 414 (59), 383 (54), 382 (100), 351 (37), 350 (36), 59 (59). ^{1}H NMR (600 MHz CD_3OD): δ3.71 (1H, *s*, O–Me), 3.76 (2H, *s*, O–Me), 3.81 (1H, *s*, O–Me), 3.81 (2H, *s*, O–Me), 3.81 (2H, *s*, O–Me), 3.82 (1H, *s*, O–Me), 3.87 (1H, *s*, O–Me), 3.89 (2H, *s*, O–Me), 4.34 (0.3 H, *d*, *J* = 7.50 Hz, $MeCO_2$–CH–Ph), 4.37 (0.7 H, *d*, *J* = 7.5 Hz, $MeCO_2$–CH–Ph), 5.83 (0.3 H, *d*, *J* = 12.8 Hz, =CH–CO_2 *cis*), 6.00 (0.3 H, *d*, *J* = 7.3 Hz, Ph–CH–O–Ph), 6.0 (0.7 H, *d*, *J* = 7.3 Hz, Ph–CH–O–Ph), 6.40 (0.7 H, *d*, *J* = 15.9 Hz, =CH–CO_2 *trans*), 6.78 (1 H, *dd*, *J* = 8.24, 1.2 Hz, aromatic H), 6.82 (1H, *dq*, *J* = 8.1, 2.0, 0.6 Hz, aromatic H), 6.89 (0.3 H, *d*, *J* = 12.8 Hz Ph–CH = *cis*), 6.94 (1 H, *d*, *J* = 2.0 Hz, aromatic H), 7.19 (1H, *d*, *J* = 1.5 Hz, aromatic H), 7.21 (1H, *d*, *J* = 0.6 Hz, aromatic H), 7.33 (0.3H, *d*, *J* = 0.6 Hz,), 7.60 (0.3 H, *d*, *J* = 1.0 Hz), 7.63 (0.7H, *d*, *J* = 16.1 Hz, Ph–CH = *trans*). UV λ_{max} 324 nm (shoulder at 287 nm). Peak 7: EIMS (probe) 70 eV *m/z* (rel. int.): 415 (26), 414 $[M]^+$ (72), 354 (43), 295 (33), 167 (25), 164 (38), 133 (24), 43 (100). ^{1}H NMR (300 MHz CD_3OD): δ3.69 (6H, *s*, Ph–OMe), 3.95 (6H, *s*, CO_2–Me), 6.45 (2H, *d*, *J* = 16 Hz, =CH–CO_2 *trans*), 765 (2H, *d*, *J* = 16 Hz, Ph–CH = *trans*), 7.1 (2H, *m*, aromatic H), 7.4 (2H, *m*, aromatic H). UV λ_{max} 323 nm (shoulder at 287 nm).

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