Lignin–Feruloyl Ester Cross-links in Grasses. Part 1. Incorporation of Feruloyl Esters into Coniferyl Alcohol Dehydrogenation Polymers

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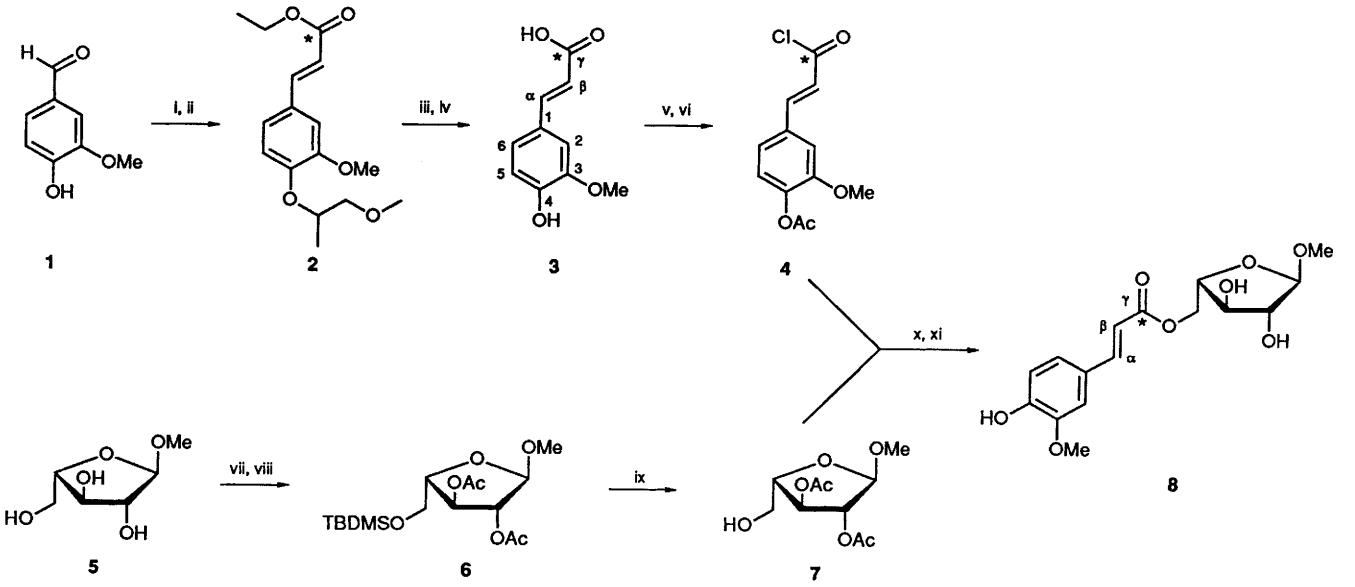
Methyl 5-*O*-(*E*)-[γ-^{13}C]feruloyl-α-L-arabinofuranoside (FA-Ara) has been synthesized and incorporated into a synthetic lignin dehydrogenation polymer (DHP) of coniferyl alcohol. Inverse-detected long-range C–H correlation NMR experiments on the DHP lignin gave correlation peaks indicative of the copolymerization of the FA-Ara and coniferyl alcohol into the DHP polymer. The bonding sites and modes, as determined by analysis of the carbonyl region of the long-range C–H correlated 2D NMR experiment, are predictable from free-radical coupling mechanisms. In addition to the abundant 4-*O*-α′ and 4-*O*-β′ ether couplings, structures involving the β-position of the feruloyl moiety of FA-Ara in β-ether, phenylcoumaran and pinoresinolide structures were present. The incorporation of feruloyl esters into a lignin DHP results in some structures which would not release ferulic acid by solvolytic schemes currently used for quantitation of ferulic acid in plant materials. Thus the degree to which hydroxycinnamic acids are involved in the lignification of forages may be significantly underestimated.

Ferulic acid [(*E*)-4-hydroxy-3-methoxycinnamic acid] in grasses is implicated in cross-linking cell-wall carbohydrates to lignins.[1] Whereas its attachment to carbohydrates, as esters, is relatively well defined,[1] the regiochemistry of its attachment to lignin is not well understood. The determination of the nature and scope of lignin–hydroxycinnamic acid–polysaccharide interactions in plant cell walls is critical towards our understanding of cell-wall biogenesis and degradation. Feruloyl esters can become 'opportunistically' involved[2] in cross-linking by trapping lignin quinone methide intermediates and/or can be directly involved in the free-radical polymerization process. In the former process, simple α-etherified structures would result, whereas copolymerization with lignin monomers would potentially result in a variety of structures, only some of which would be subsequently identifiable, by present solvolytic methods, as arising from ferulic acid.[2]

Direct elucidation of ferulic acid/lignin connectivity *via* NMR spectroscopy is a challenge because of sensitivity restrictions even with modern inverse-detection methodologies and because of the large number of potential structures that might be important. Our preliminary efforts have therefore been directed toward model approaches to determine what structures are possible from radical-coupling mechanisms, to obtain unambiguously the required chemical-shift and coupling-constant data for compounds of interest, and to optimize correlation experiments aimed at detecting these structures in plant cell-wall isolates. Consequently, carbohydrate moieties involved in cross-linking have been synthesized[3] and methodologies for the regiospecific attachment of hydroxycinnamic acids have been developed.[4] All possible ferulic acid esters and ethers of a common lignin model dimer, guaiacylglycerol-β-guaiacyl ether [1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol] have been synthesized and fully characterized by NMR spectroscopy.[5]

Lignin is formed from *p*-hydroxycinnamyl alcohol monomers, primarily coniferyl alcohol and sinapyl alcohol, by an enzyme-initiated dehydrogenative polymerization.[6,7] Resonance-stabilized phenoxyl radicals are produced from monomers and from the evolving lignin macromolecules and these couple in a variety of ways to build up the polymer. Intermediate quinone methides, which are produced from radical-coupling reactions involving the β-carbon, typically undergo nucleophilic attack at the α-position. Water would be the predominant nucleophile present in the plant cell wall and would afford an α-hydroxy group on β-ether structures. Free acids and alcohols could also attack the α-position of the quinone methides, leading to α-esters and α-ethers. In the case of feruloyl esters, the free phenol can trap quinone methides[8] or can become directly involved in the free-radical polymerization process (a mechanism alluded to by Fry,[9] and Bacic *et al.* and Yamamoto *et al.* in ref. 1).

It is generally thought that attack on the quinone methide is the mechanism by which feruloyl esters become bound to lignin. This is a reasonable and predictable occurrence, the validity of which can be demonstrated in model systems.[8] It has a number of frequently overlooked shortcomings, however. First, the feruloyl ester has to compete for the quinone methide with other nucleophiles including other phenols (from cinnamyl alcohol monomers and from the growing lignin polymer itself), acids present in other cell-wall components (*e.g.*, uronic acids), and water. In isolated lignins, at least 90% of the β-ether quinone methides formed result in α-hydroxy structures, indicating that water is the primary addition product.[6] In cases where acids are in competition with phenols, esters are produced almost exclusively.[5,8,10] A more philosophical question arises when feruloyl esters and lignification are considered in relation to the development of the plant cell wall. Feruloylated polysaccharides, typically arabinoxylans, have been proposed to cross-link to lignin in order to impart various properties to the plant cell wall.[1,11,12] Therefore it seems rather unlikely that the method of cross-link formation would involve an uncontrollable reaction, especially when considering the spatial problem of placing a quinone methide sufficiently close to the feruloylated polysaccharide to produce the α-ether. Finally, although some peroxidase specificity has been demonstrated,[13,14] it seems unlikely that these phenolic feruloyl esters would be available in the matrix for addition to quinone methods and yet not be amenable to H-abstraction and the radical-coupling process. Consequently, we suspect that feruloyl esters are also incorporated into the lignin structure through copolymerization with lignin monomers. An implication of this hypothesis is that feruloyl esters could become involved in a variety of structures, many of which would not solvolytically cleave to the parent



Scheme 1 Synthesis of [γ-^{13}C]ferulic acid **3**, and FA-Ara **8**. *Reagents:* i, $EtOCH{=}CH_2$, pyridinium toluene-*p*-sulfonate; ii, NaH, triethyl phosphono[1-^{13}C]acetate; iii, HCl; iv, 4 mol dm^{-3} NaOH; v, Ac_2O–pyridine; vi, $SOCl_2$; vii, Bu^tMe_2SiCl, pyridine; viii, Ac_2O; ix, 80% HOAc; x, pyridine; xi, pyrrolidine–95% EtOH

ferulic acid monomers by present analytical methods (acidolysis, thioacidolysis, or high-temperature base treatment).

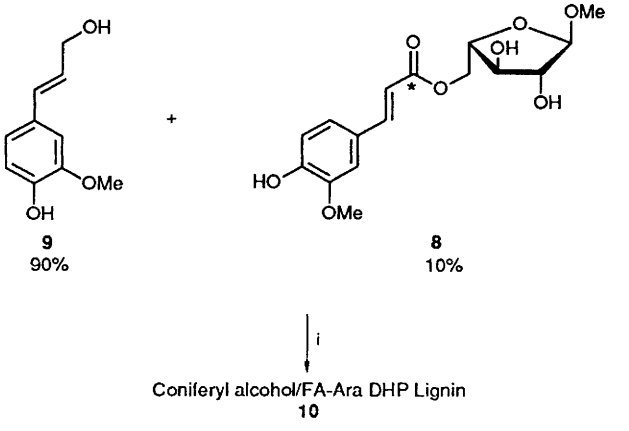
Reactions which follow the initial radical-production step are independent of enzymic control, and the lignification process can be reasonably mimicked *in vitro* with the use of purified enzymes and hydrogen peroxide,[15] providing synthetic dehydrogenation polymer (DHP) lignins. It should be emphasized (see Discussion below) that, although DHP lignins are not identical with lignins produced by the cell wall, the synthetic materials provide important information pertaining to the free radical-coupling products which are possible. The linkages present in the DHP lignins can then be used as a basis for investigating the regiochemistry in native tissues. Incorporation of a strategically ^{13}C-labelled feruloyl ester of methyl α-L-arabinofuranoside into a coniferyl alcohol DHP was seen as an effective way to determine the extent and detailed attachment regiochemistry of incorporation of feruloyl esters into lignin polymers. The preparation, and fruitful analysis of this polymer by long-range C–H correlation NMR methods, is the subject of this paper.

Results and Discussion

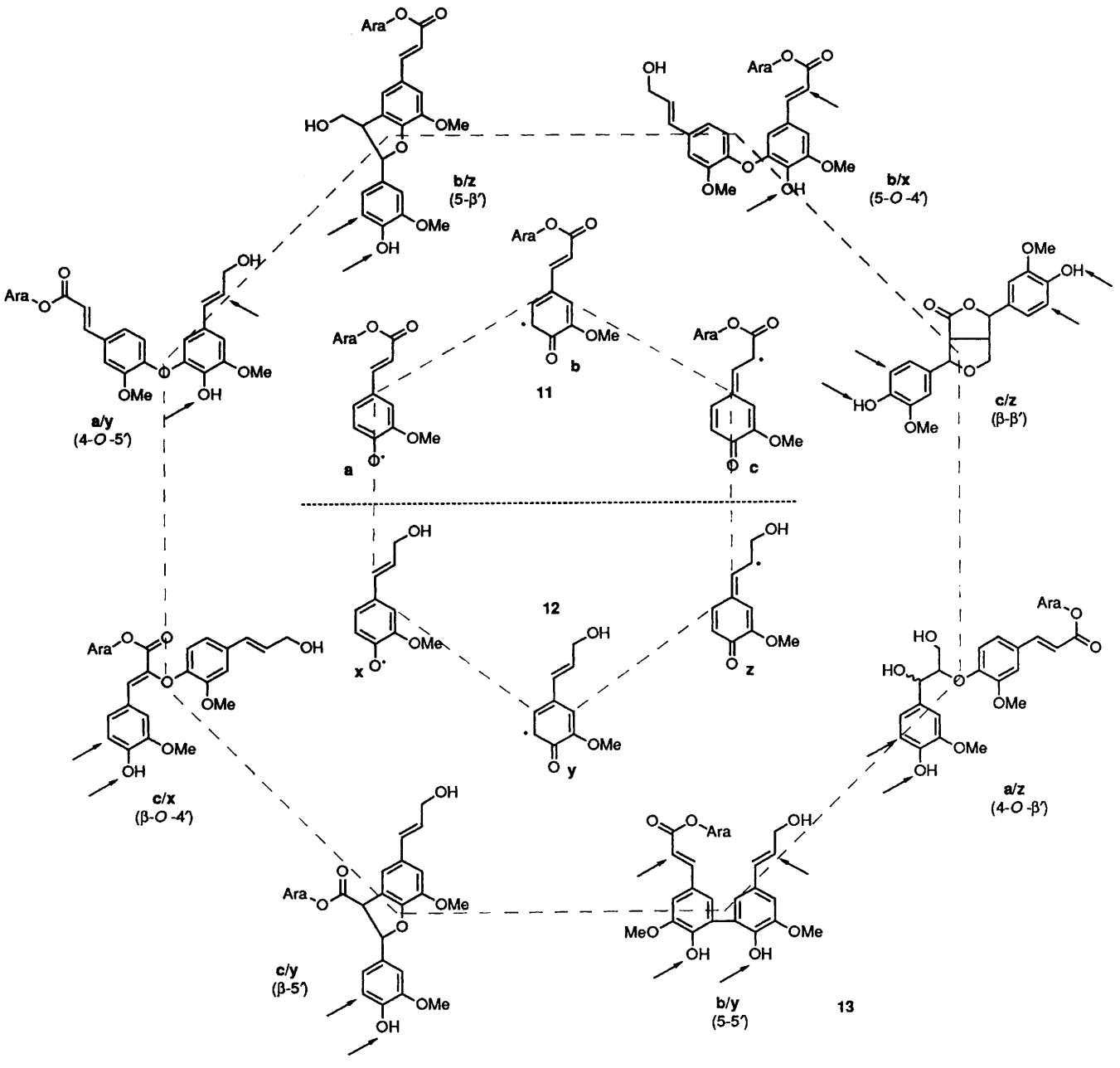
The synthetic scheme for the preparation of [γ-^{13}C]ferulic acid **3** and methyl 5-*O*-feruloyl-α-L-arabinofuranoside (FA-Ara, **8**) is shown in Scheme 1. The preparation of diacetate **7** from methyl α-L-arabinofuranoside **5**, as well as the subsequent coupling with ferulic acid derivative **4**, has been reported previously.[4] The modifications described here improve this methodology by exploitation of one-pot, multi-step reactions. Thus only the requisite amount of the more costly [γ-^{13}C]ferulic acid is required for coupling to glycoside **7**, and the loss of material associated with chromatographic purifications and crystallizations is avoided.

The synthesis of coniferyl alcohol **9** was also modified to simplify the procedure and improve the purity of the product, particularly with regard to 1,4-reduction products. A recent synthesis of coniferyl alcohol by metallation of eugenol with BuLi/KOBu^t followed by consecutive dimethoxyborylation and oxidation[16] provides a novel method, but the procedure and work-up are considerably more demanding than for simple reduction methods. Previously, ethyl or methyl ferulate had been reduced with lithium aluminium hydride[17] or sodium bis-(2-methoxyethoxy)aluminium hydride.[18,19] With each reductant we have observed small and varying amounts of 1,4-reduction products and the $LiAlH_4$ reaction is particularly capricious in this respect. Newman *et al.*[20] performed the ferulate reduction using the 'ATE'-complex produced by the reaction of diisobutylaluminium hydride (DIBAL-H) with butyllithium.[21] We have concluded that such strategies are unnecessary and that simple DIBAL-H reduction at 0 °C affords a very clean product (see Experimental section), and allows decagram-scale preparations.[22] The method works equally well for the preparation of the other lignin monomers, *p*-coumaryl and sinapyl alcohols.[22]

The DHP preparation procedure was carefully chosen. It was critical for this study to obtain significant polymerization (avoiding the accumulation of low molecular mass oligomers) while attaining a product that has good solubility characteristics for NMR spectroscopy. The 'Zutropfverfahren' method,[15] wherein buffered monomer and hydrogen peroxide solutions are added separately in a dropwise fashion to a buffered solution of peroxidase enzyme, provided satisfactory and consistent coniferyl alcohol polymers in repeated trials. These synthetic polymers differed from isolated lignins (as has been noted previously[23]) in having higher proportions of phenylcoumaran (β-5) and pinoresinol (β-β) structures, as well as coniferyl



Scheme 2 Preparation of FA-Ara copolymer DHP **10**. *Reagents:* i, H_2O_2, peroxidase



Scheme 3 Initial radical-coupling products for FA-Ara radical **11** (resonance forms **a**, **b** and **c** shown) and coniferyl alcohol radical **12** (resonance forms **x**, **y** and **z** shown). The conventions used for describing adduct compounds **13** is illustrated with an example: **13a/z** represents the final product resulting from formal coupling of the localized FA-Ara radical **11a** with the coniferyl alcohol radical **12z**, the FA-Ara radical being specified first; the descriptive nomenclature, *e.g.* β-*O*-4′, follows standard lignin conventions with the first term indicating the coupling site to FA-Ara, and the second (primed) site to the coniferyl alcohol or lignin moiety. The arrows indicate the primary sites for further radical coupling to generate the extended copolymer.

alcohol endgroups, and consequently lower proportions of β-*O*-4 structures and lower hydroxy-group content. All indications are that DHPs and isolated lignins are structurally analogous, and this increased proportion of the β-5 and β-β structures has aided in the assignment of these less prevalent units. If higher molecular mass (but less solvent-soluble) DHPs are required, peroxide-generation strategies using glucose oxidase and glucose,[24] or polymerization in dialysis membranes[25] can be used.

The FA-Ara DHP **10**, Scheme 2, was prepared according to Kirk and Brunow's[19] Zutropf-like method by separate dropwise addition of two buffered solutions (pH 6.5), one containing the FA-Ara **8** (40% γ-^{13}C-labelled) and coniferyl alcohol **9** monomers (1:9) and the peroxidase enzyme, and the other containing hydrogen peroxide, to a stirred volume of pH 6.5 buffer, in the dark at ambient temperature (see Experimental section). The level of substrate **8** was chosen at 10% to minimize statistical homo-condensation products, while giving a sufficient loading to determine products.

Scheme 3 shows the eight major initial products **13** that would be expected from cross-coupling of phenoxyl radicals **11** and **12** from FA-Ara **8** and from coniferyl alcohol **9**, respectively. These products can be regarded as arising from formal coupling of the localized radicals **11a–c** and **12x–z**. Arrows in Scheme 3 indicate where further radical-coupling can occur following subsequent phenolic H-abstraction. Products from preformed

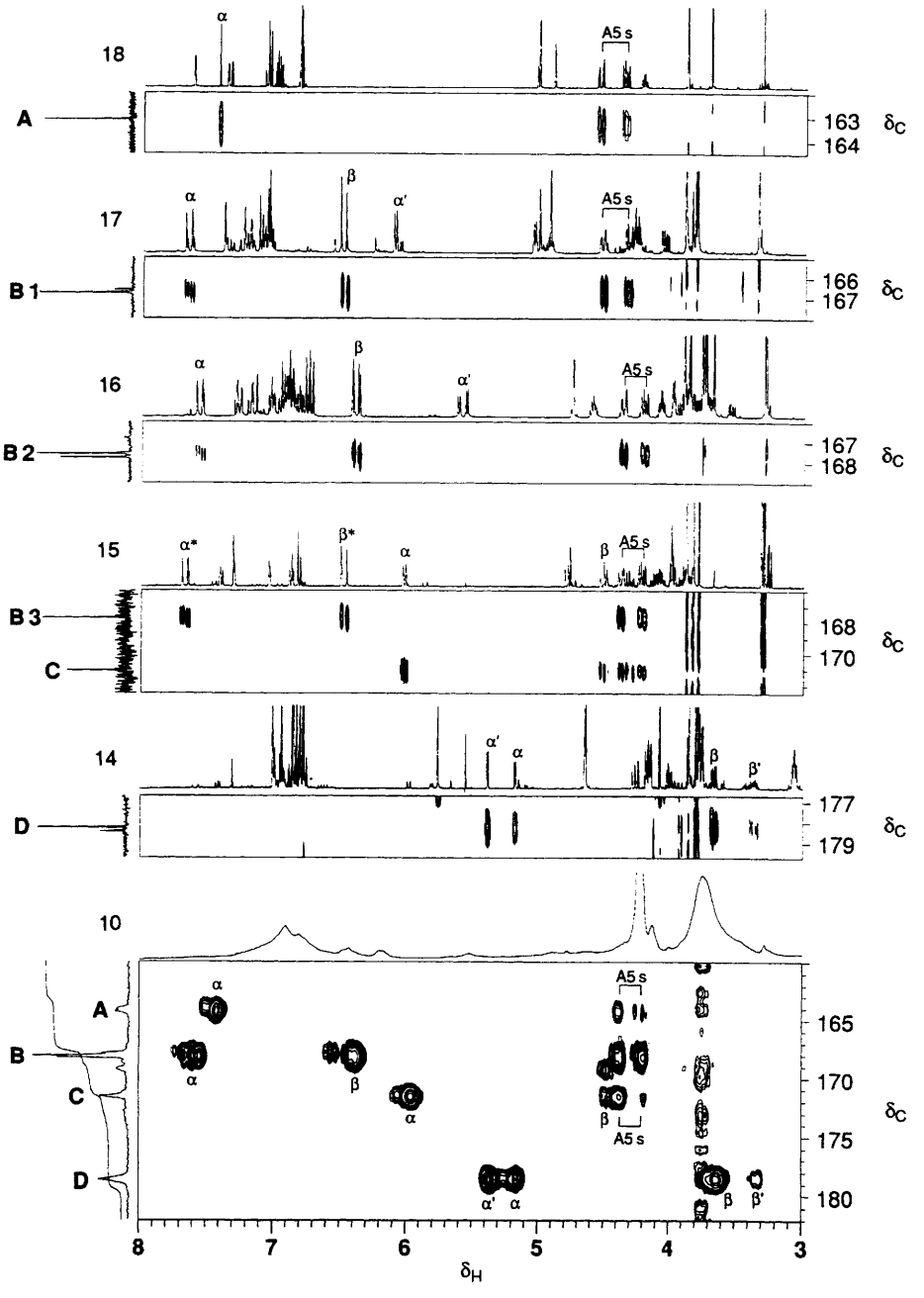
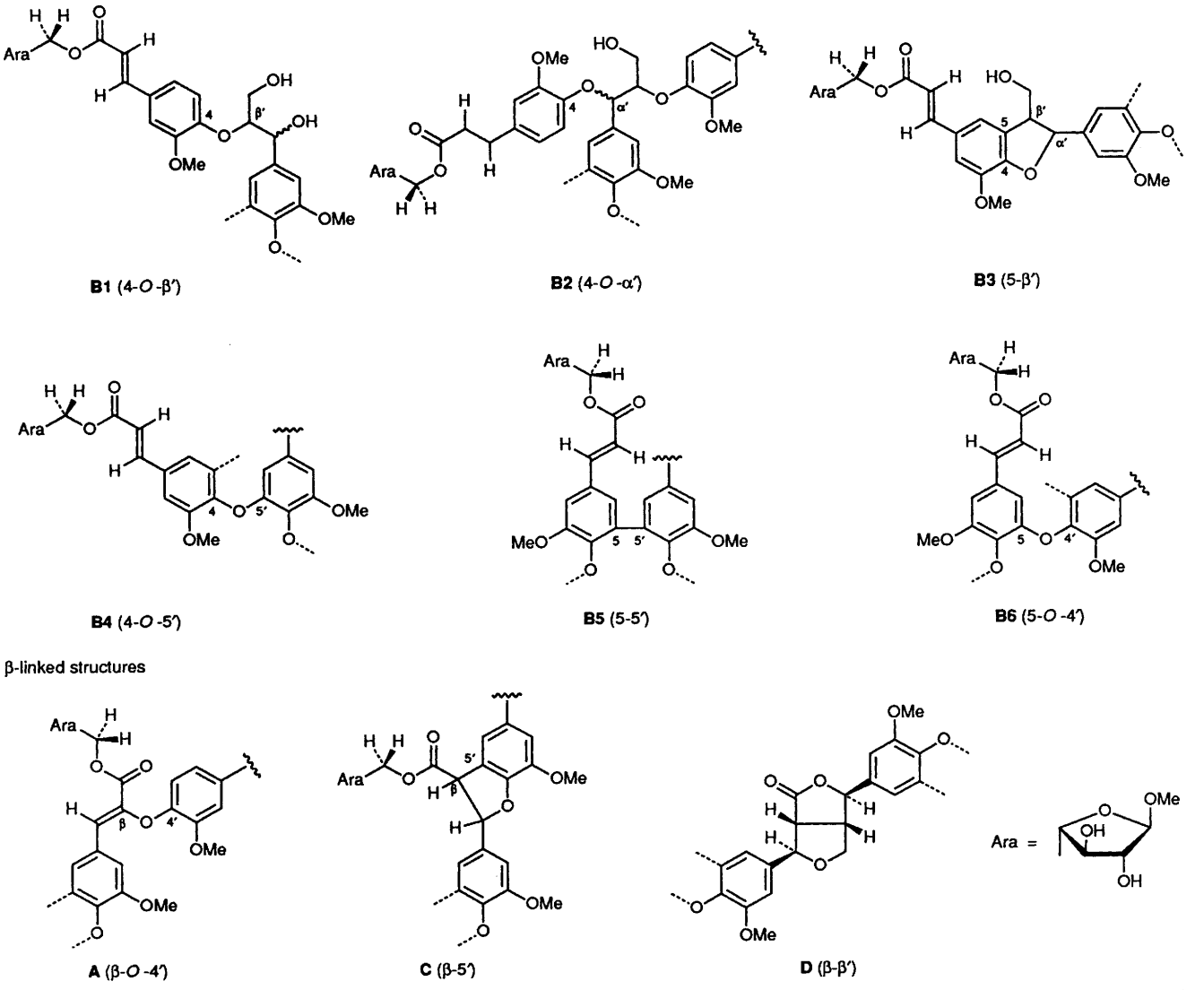


Fig. 1 Portion of inverse-detected long-range 2D C–H correlation spectrum of DHP **10** showing just the carbonyl carbon region. The 1D carbon and proton spectra along the axes are from quantitative 1D experiments. Peak groupings labelled **A**–**D** are assigned to the structures shown as such. Authentication of assignments is shown *via* similar regions of the 2D heteronuclear multiple bond coherence spectra of model compounds **14**–**18**. Regrettably, compounds **17** and **18** are fully acetylated and consequently don't model the parent compounds precisely, particularly with regard to the arabinosyl proton shifts. Compound **14** was not obtained in pure form (see caption to structures **14**–**18**) and the spectrum slice shown is from a mixture of the required compound **14** along with pinoresinol and the dilactone (see text).

oligomers and polymers can also arise, so the coniferyl alcohol endgroups would not necessarily be directly attached to the feruloyl moiety. Also, α-ether products can be formed by attack of compound **8** on intermediate quinone methides formed from radical-coupling involving the β-positions as described later.

Long-range C–H correlation experiments are particularly valuable for establishing connectivity. Fig. 1 shows a small subsection, incorporating only the carbonyl region, of an ^{1}H-detected long-range 2D C–H correlation spectrum of the DHP **10**, and the corresponding region of several dimers synthesized for proof of the assignments made. It is immediately clear from the ^{13}C spectrum, shown as the vertical projection in Fig. 1, that the single carbonyl in the precursor monomer FA-Ara **8** has generated a variety of carbonyl moieties in polymer **10** that are both conjugated (α,β-unsaturated) and unconjugated (groupings labelled **A**–**D**). The long-range correlations from the labelled carbonyl carbon to protons with two or three intervening bonds are particularly diagnostic and provide evidence for β-*O*-4′, β-5′, 4-*O*-β′ and β-β′ products of the types shown. [The convention used here, *e.g.* β-*O*-4′, follows standard lignin-labelling conventions with the first term indicating the coupling site to the FA-Ara and the last (primed) site to the coniferyl alcohol or lignin moiety].

The major peak cluster, **B** (Fig. 1), shows that the double bond is intact (α and β protons at normal chemical shifts), and the correlation with the 5-protons of the arabinosyl moiety

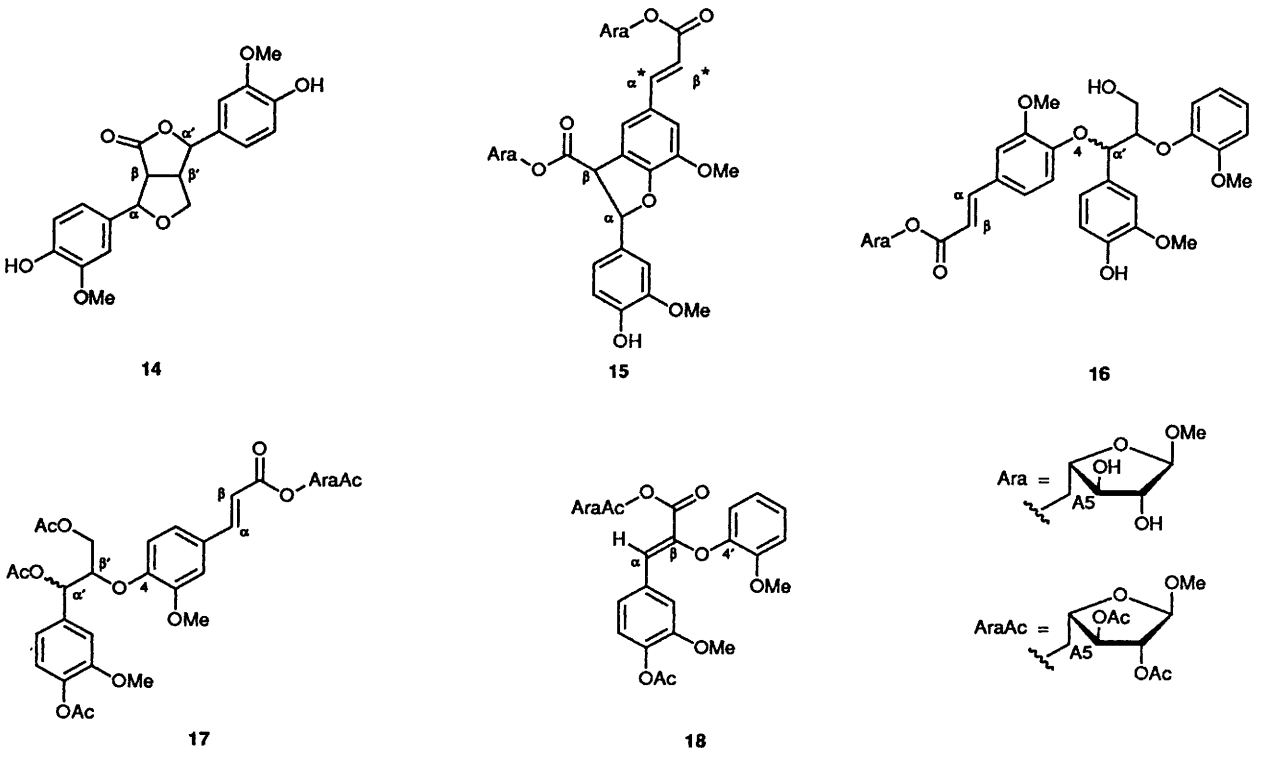


Structures proposed for peaks **A**, **B**, **C** and **D** of Fig. 1. The dotted lines signify additional attachment sites to the lignin polymer. Note that 'Ara' in these figures represents the arabinosyl moiety less the C-5, so that the C-5 protons can be shown explicitly, depicting their 3-bond relationship to the C-γ carbonyl, which results in the long-range correlations shown in Fig. 1.

indicates that the ferulic acid–arabinosyl ester linkage has remained intact. The multiplicity of peaks in this region is certainly due to the variety of substitutions possible on the aromatic ring, both through the phenol and/or the 5-position as shown in structures **B1**–**B6** and to remote stereochemistry (*e.g.*, the *threo* or *erythro* β-ether subunit attached at C-4 in **B1**) and/or regiochemistry [*e.g.*, 4-*O*-α′ (**B2**) *vs.* 4-*O*-β′ (**B1**) substitution]. The spectra (Fig. 1) from the 4-*O*-α′ models **16** and 4-*O*-β′ models **17** coincide with the major peaks in group **B**, suggesting that the two largest peaks in the DHP carbon spectrum are probably due to phenolic-etherified structures. It is important to recognize that structures **B1** and **B2** result from fundamentally different reaction pathways (Scheme 4): whereas 4-*O*-β′ structures **B1** are formed as a direct result of radical-coupling mechanisms and implicate FA-Ara directly in the enzymically initiated dehydrogenation, 4-*O*-α′ structures **B2** result from attack of the intact FA-Ara on quinone methide intermediates. Determination of the partitioning between these two pathways, which is not clear from these data, will be the subject of a future study using 4-labelled FA-Ara.

Peak cluster **A** (Fig. 1) also depicts structures with the arabinosyl moiety attached but retains only one (shifted) vinylic proton. It is quickly concluded that this represents the β-*O*-4′ structure shown for structure **A**, as formed *via* the mechanism illustrated in Scheme 4. The observation of this β-linked product demonstrates that feruloyl moieties will, like coniferyl alcohol radicals, couple efficiently at the β-position on the sidechain. The difference in this instance is that the resulting quinone methide simply eliminates the acidic β-proton to form the conjugated structure rather than trapping water or another nucleophile to form an α-oxy product (Scheme 4). This elimination mechanism was previously noted in the polymerization of coniferaldehyde.[26] Interestingly, it is only the *Z*-isomer that is evident in Fig. 1. This is in complete accord with observations made from the synthesis of compound **18**, a model for peaks **A**, by elimination of the β-proton from the quinone methide or the α-bromide; only the more extensively conjugated *Z*-isomer resulted from the quinone methide reaction (a reaction which parallels that in the DHP synthesis and lignification itself), whereas both geometrical isomers resulted from β-proton elimination *via* the more reactive carbonium ion.[27]

Peak cluster **C** again retains the arabinosyl moiety, but the conjugated double bond has disappeared. The correlations with protons at δ 5.95 and 4.48 indicate a phenylcoumaran (β-5′) structure, resulting from the mechanism in Scheme 4. Model **15**, a homo-dimer synthesized by silver(I) oxide-mediated radical coupling of FA-Ara **8**, shows similar correlations, confirming the assignment.

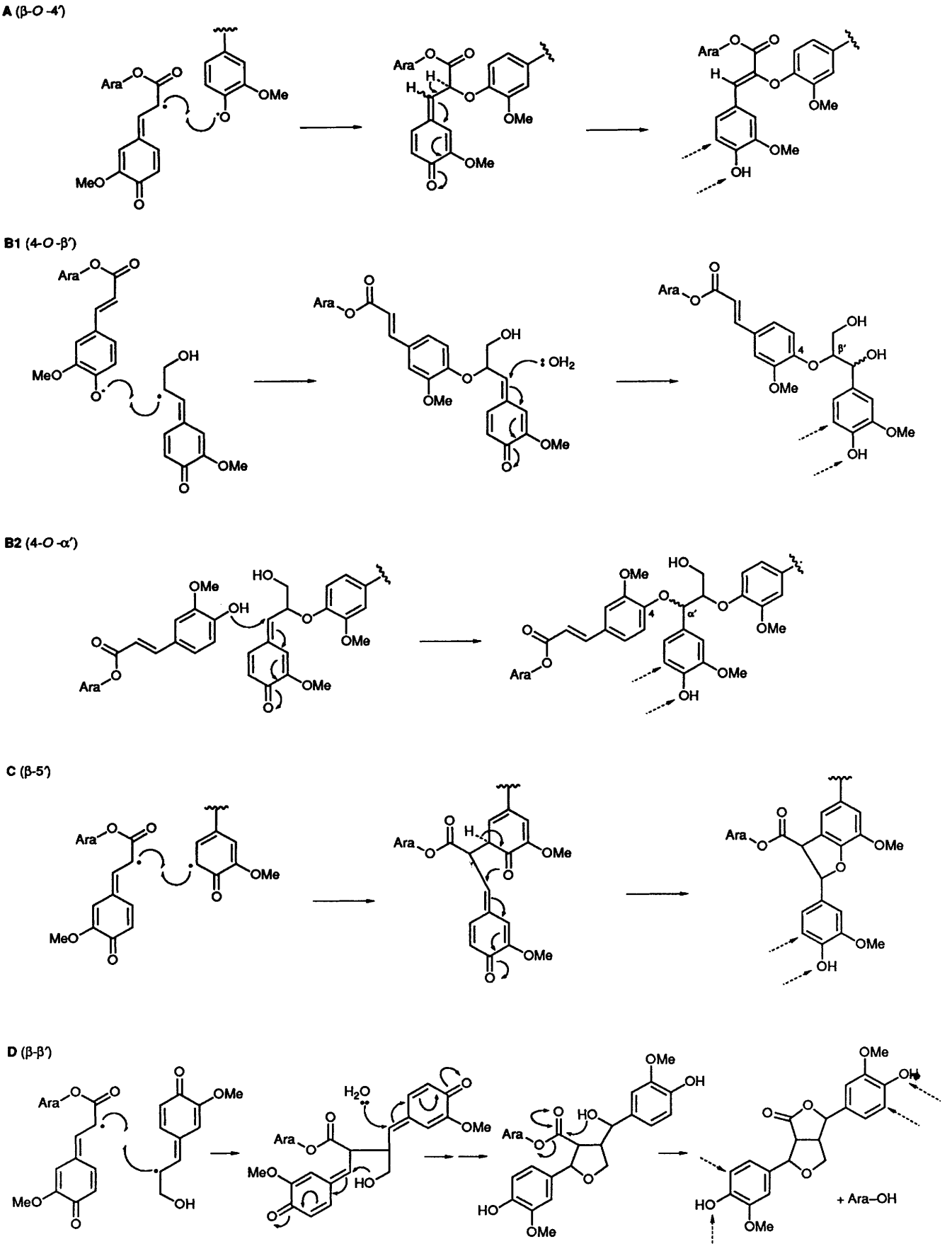


Synthesized compounds for authentication of the assignments made in Fig. 1. Syntheses and full characterization are reported in the companion paper.[27] Compound **14**, prepared from silver(I) oxide-mediated mixed radical coupling of ferulic acid and coniferyl alcohol, was not separated from the pinoresinol and dilactone also produced. Compounds **17** and **18** remain fully acetylated due to the difficulty of achieving selective deacetylation.

With peak cluster **D**, the arabinosyl moiety is no longer attached and the correlations point to the half-lactone/half cyclic ether pinoresinolide[28] product represented by structure **D** and derived from β-β′ coupling as shown mechanistically in Scheme 4. Similar β-β coupling has been observed for ferulic acid to give the dilactone[29] [4,8-bis-(4-hydroxy-3-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane-2,6-dione], and for coniferyl alcohol itself to give pinoresinol.[23] The deoxo dimer **14**, prepared[27] for authentication *via* silver(I) oxide-mediated mixed radical coupling of ferulic acid and coniferyl alcohol, could not be separated from the homo-β-β coupling products pinoresinol and the dilactone. Consequently, the spectrum of compound **14** in Fig. 1 contains these other components, but only the 2D slice containing the correlations to the C-γ carbonyl of structure **14** is shown. Efforts are underway to prepare the resinolide **14** by more traditional means as alluded to in a paper by Vande Velde *et al.*[30] Peak cluster **D** represents further evidence of β-coupling products. More importantly, the asymmetry in the product provides excellent proof that coupling is taking place between the feruloyl moiety and the more predominant coniferyl alcohol or evolving DHP polymer radicals. It is also the only mechanism at this point which involves cleavage of the ester to eliminate the carbohydrate moiety.

It is important to note that, of the products depicted in structures **A**–**D** only the 4-*O*-β′ product **B1** (corresponding to the **a**/**z** coupling product in Scheme 3), the 4-*O*-α′ product **B2** (formed by the addition mechanism in Scheme 4), and possibly the β-*O*-4′ product **A** (corresponding to the **c**/**x** product in Scheme 3) could regenerate ferulic acid (or logical derivatives) upon acidolysis, thioacidolysis, or high-temperature alkaline solvolysis. The carbon plot shown on Fig. 1 was from a high-resolution spectrum run under quantitative conditions. Examination of the integrals shows that cluster **A** represents 9% of the total carbonyl resonances (and may give ferulic acid products) and cluster **B** 37%. While the major peaks of cluster **B** probably arise from phenolic α- or β-ethers that are not further cross-linked, there are other structures in this region that may be (structures **B3**–**B6**, or the cross-linked structures indicated by the dashed bonds in structures **B1** and **B2**). We estimated from these spectra that less than 40% of the products from this DHP would be susceptible to hydrolytic cleavage. Using base hydrolysis at 170 °C, it was experimentally determined that only 10% of the ferulic acid could be recovered from DHP **10**, increasing speculation that, if hydroxycinnamoyl ester copolymerization is involved in plant lignification, the quantity of hydroxycinnamic acids measured by current procedures may severely underestimate their incorporation into the lignin/phenolic acid polymer complex.

Conclusions.—Feruloyl esters, if present in the lignifying matrix, are capable of entering into the free-radical polymerization process generally associated with lignification. Provided that peroxidases are sufficiently non-specific, H-abstraction from these free-phenolic feruloyl esters and copolymerization into the lignin polymer seems inevitable and must at least compete with the simple 'opportunistic' trapping of intermediate quinone methides that generates 4-*O*-α′ ethers. Among the implications is that ferulic acid is likely to be involved in structures from which it cannot be released and quantitated by current solvolytic methods. Consequently, the quantity and importance of ferulic acid in non-woody plant cell-wall complexes are probably being underestimated. Two fundamental questions need to be addressed: what is the partitioning between opportunistic attack on quinone methides (to give α-ethers) *vs.* radical incorporation, and which of the products identified in these studies are present in plant cell wall complexes? The former can be answered from analogous DHP studies using 4-^{13}C-labelled FA-Ara by quantitation of α-ether *vs.* the radical-derived β-ether and other products. The latter is evasive and requires careful labelling studies.



Scheme 4 Mechanisms for the formation of structures of types A–D

Experimental

M.p.s were determined on an Electrothermal Engineering Ltd digital melting point apparatus and are uncorrected. Evaporations were conducted under reduced pressure at temperatures less than 42 °C unless otherwise noted. Solutions in organic solvents were dried with sodium sulfate and filtered before evaporation. Further elimination of organic solvents as well as drying of the residues was accomplished under higher vacuum (10–14 N m^{-2}) at room temperature. Column chromatography was performed with silica gel 60 (230–400 mesh) and TLC was done on silica gel 60-F254 plates (Merck). NMR spectra of samples in [$^2H_6$]acetone were run at 300 K on a Bruker AMX-360 narrow-bore instrument fitted with a 5 mm 4-nucleus (QNP) probe with normal geometry (proton coil further from the sample). The central solvent signals were used as internal reference (^1H, δ 2.04; ^{13}C, δ_C 29.8). The inverse long-range C–H correlation spectrum in Fig. 1 was run, using 100 mg of DHP **10** in (9:1) [$^2H_6$]acetone–D_2O (0.3 cm^3), with Bruker's standard inv41plrnd sequence[31] incorporating a low-pass filter and no carbon decoupling, with 2K data points in the proton dimension and 256 increments in the carbon dimension, using 480 scans per increment. The 90 degree pulse angles were 10.7 μs and 4.7 μs for ^1H and ^{13}C respectively, and the long-range coupling delay was optimized at 110 ms (corresponding to a long-range C–H coupling constant of 4.5 Hz). Unshifted squared sine-bell (Q0) apodization was applied in each dimension and the matrix was zero-filled and Fourier transformed (using magnitude-mode phase correction) to give a final matrix of 2K by 1K real points, resulting in digital resolutions of 1.78 and 16.8 Hz/pt. Complementary spectra of model compounds were obtained in an analogous fashion, but with 64 scans per increment.

The synthesis and complete characterization of model compounds **14**–**18**, used to authenticate assignments in the 2D C–H correlation spectrum of Fig. 1, are reported in our companion paper.[27] Light petroleum refers to the fraction boiling in the range 40–60 °C.

Synthesis of [γ-^{13}C]*FA-Ara* [*Methyl* 5-O-(E)-*Feruloyl*-α-L-*arabinofuranoside*] **8**.—This synthetic scheme minimizes isolation of reaction intermediates and provides FA-Ara **8** in 60% overall yield based on ^{13}C-labelled ferulic acid **3**. Analytical data for all compounds are described elsewhere.[4]

(E)-[γ-^{13}C]*Ferulic Acid* **3**.—Ethyl 4-*O*-(ethoxyethyl)-[γ-^{13}C]ferulate **2** was prepared on a 3 mmolar scale from vanillin **1** and triethylphosphono-[1-^{13}C]acetate (Aldrich Chemical Co.) as described by Newman *et al.*[20] The ethoxyethyl group was removed by treatment of a chloroform solution of compound **2** with 3 mol dm^{-3} HCl. The ester was saponified by treatment of the product directly with 2 mol dm^{-3} degassed aq. NaOH, acidification with 2 mol dm^{-3} HCl, and extraction into EtOAc. The resulting ^{13}C-labelled ferulic acid **3** was crystallized from acetone–hexane in 75% overall yield (from vanillin).

4-O-*Acetyl*-[γ-^{13}C]*feruloyl Chloride* **4**.—[γ-^{13}C]Ferulic acid **3** (202 mg, 1 mmol) was dissolved in freshly distilled pyridine (3 cm^3). Acetic anhydride (0.5 cm^3) was added and the mixture was left in the dark overnight. The solution was diluted with toluene and evaporated to give a syrup at 50 °C. Three subsequent additions and evaporations of toluene eliminated all of the remaining pyridine, acetic acid, and acetic anhydride.[32] The crude 4-*O*-acetylferulic acid was dissolved in benzene (5 cm^3) and thionyl dichloride (0.35 cm^3) was added. The mixture was refluxed and stirred for 30 min. The solution was then evaporated to give an off-white solid, and two further additions and evaporations of toluene eliminated any remaining undesired volatiles.

[γ-^{13}C]*FA-Ara* **8**, *Coupling and Deprotection*.[4]—Methyl 2,3-di-*O*-acetyl-α-L-arabinofuranoside **7** (303 mg, 1.22 mmol) was dissolved in freshly distilled pyridine under nitrogen. The mixture was cooled in an ice–water-bath, and the crude acid chloride **4**, dissolved in toluene (5 cm^3), was added *via* a dropping funnel during ~5 min. After addition was complete, the mixture was removed from the ice–water-bath and was stirred in the dark for 2 h. The product was evaporated to give a syrup, which was diluted with methylene dichloride and washed successively with water, 3% HCl, and water again. The organic layer was dried over sodium sulfate and processed to afford crude, fully acetylated, γ-^{13}C-labelled FA-Ara. Deacetylation was accomplished by dissolution of the syrup in 95% ethanol (5 cm^3), addition of pyrrolidine (0.5 cm^3), and stirring of the mixture in the dark for 24 h (monitoring by TLC). The entire mixture was transferred to a column of ion-exchange resin [Amberlite IRA-120(H^+); 10 cm^3] in ethanol. The eluate was processed and submitted to silica gel chromatography [40 g; (2:1) chloroform–ethyl acetate]. Processing of the appropriate fractions afforded γ-^{13}C-labelled FA-Ara **8** (224 mg, 64%), which was crystallized from methylene dichloride–light petroleum. Two- or three-bond coupling constants from the γ-carbonyl carbon to H_β, H_α, and the two 5-Hs of the arabinosyl moiety were 2.4, 6.8, 2.8 and 2.8 Hz, respectively.

Synthesis of Coniferyl Alcohol **9**.—Ethyl ferulate was prepared from ferulic acid (Sigma) by being stirred overnight with EtOH/HCl [prepared by addition of acetyl chloride (10 cm^3) to ethanol (100 cm^3)],[33] evaporation and crystallization from ethyl acetate–light petroleum. A solution of ethyl ferulate (1.0 g, 4.5 mmol) in toluene (50 cm^3, freshly distilled), under nitrogen, was cooled in an ice–water-bath and DIBAL-H (12 cm^3 of a 1.5 mol dm^{-3} solution in toluene, 18.0 mmol) was added slowly *via* syringe during 10 min. After addition was complete, the mixture was stirred for 1 h. The reaction mixture was then carefully quenched with ethanol (10 cm^3). The solvents were partly removed at 40 °C. Water (50 cm^3) was added, and the aqueous layer, containing a gelatinous precipitate of aluminium salts, was extensively extracted with ethyl acetate (4 × 150 cm^3), dried, and evaporated to dryness at below 30 °C to give coniferyl alcohol **9** as a solid (795 mg, 98%). Crystallization from methylene dichloride–light petroleum gave crystalline compound **9** (608 mg, 75%), m.p. 77.9–78.6 °C (lit.,[16] 74–75 °C). A slightly modified method for large-scale preparation (10–20 g), along with the syntheses of the syringyl and *p*-coumaryl homologues, are reported elsewhere.[22]

Preparation of the Coniferyl Alcohol/[γ-^{13}*C*]*FA-Ara DHP* **10**, *Scheme* 2.—Coniferyl alcohol **9** (450 mg, 2.50 mmol) and methyl-5-*O*-feruloyl-α-L-arabinofuranoside **8** (50 mg, 0.15 mmol; 40% γ-^{13}C-labelled) were dissolved in acetone (10 cm^3) and added to stirred phosphate buffer (200 cm^3; 0.01 mol dm^{-3}; pH 6.5; degassed), containing horseradish peroxidase (330 units; EC 1.11.1.7, Type II). A second solution containing commercial hydrogen peroxide (0.3 cm^3 of 30% solution, 2.65 mmol) was prepared in phosphate buffer (210 cm^3). The two solutions were simultaneously added, at room temperature, to stirred phosphate buffer (100 cm^3). The additions were accomplished by using a double-channel Masterflex peristaltic pump at the rate of 8 cm^3/h. The reaction mixture was kept in the dark and, after additions were complete, was stirred for ~70 h. The resulting pinkish suspension was then filtered through 0.2 μm nylon membrane and thoroughly washed with distilled water. The insoluble DHP polymer was taken up in distilled water and freeze dried to give an amorphous light-beige powder (430 mg, 86%).

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