Lignin–ferulate cross-links in grasses. Part 4.[1–3] Incorporation of 5–5-coupled dehydrodiferulate into synthetic lignin

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Ferulates and dehydrodiferulates have a significant role in cross-linking polysaccharides to lignin in grass cell walls. Among the various ferulate dehydrodimers, the 5–5-coupled dehydrodimer (*E*,*E*)-4,4′-dihydroxy-3,3′-dimethoxy-5,5′-bicinnamate is capable of the most extensive cross-linking into lignin, forming major branch-points. However, current literature fails to recognise the role of radical cross-coupling reactions with lignin monomers/oligomers. Here we demonstrate that a synthetic model for 5–5-coupled dehydrodiferulate polysaccharide esters in grass cell walls biomimetically incorporates into synthetic lignins *via* radical coupling mechanisms to produce a range of cross-coupled structures. The incorporation profile is remarkably similar to that for ferulate. Importantly, significant coupling at the cinnamoyl 8-position readily occurs. Evidence for the expected incorporation of the 5–5-coupled dehydrodiferulate into the newly discovered dibenzodioxocine structures is readily apparent in HMQC or HSQC spectra. Since some of the structures that dehydrodiferulates are involved in cannot be hydrolytically cleaved, their current 'quantification' is a significant underestimation of the importance of these species. What is clear is that dehydrodiferulates can have a powerful role in effecting lignin–polysaccharide cross-linking.

Introduction

Ferulic acid is esterified to grass cell wall polysaccharides, notably to arabinoxylans at the C-5 position of α-L-arabinofuranoside moieties as has been reviewed.[4–12] Dimerizations of such ferulate esters provide pathways for cross-linking polysaccharide chains.[6,13–15] Phenol oxidative coupling, *via* the action of peroxidases and hydrogen peroxide for example, produces a whole range of dehydrodimers; synthesis, identification and quantitation of released dehydrodiferulic acids from grass cell walls has been reported.[13] Until that paper, the only *established* dehydrodiferulic acid, released in small amounts from grass cell walls by saponification was the 5–5-coupled isomer **1a**, Scheme 1, commonly referred to as 'diferulic acid.'[16,17] Importantly, the other dimers are being increasingly found and are universally present in higher quantities than **1a**.[12,18–23] It is likely that they are ubiquitous in gramineae. They have also been discovered in significant quantities in sugar beet[18,19] and water chestnuts,[24] and are even implicated in the cessation of cell extension in pine hypocotyls (the light response).[20]

Ferulate radicals cross-couple with lignin radicals to effect lignin–polysaccharide cross-linking mediated by ferulic acid.[1,3,25] There is further evidence that ferulate-polysaccharide esters function as nucleation sites for the lignification process in the plant cell wall; the observation of products resulting from coupling with lignin monomers and not with pre-formed oligomers is strongly suggestive of this role.[1] Dehydrodiferulates, already cross-linking wall polysaccharides, function similarly. That is, they also cross-couple with lignin moieties to produce a tightly cross-linked lignin–polysaccharide network. Although diagnostic NMR evidence for such dimer incorporation has not yet been presented, hydrolytic treatment of biomimetically lignified cell wall systems has revealed that dehydrodiferulates incorporate into structures from which the corresponding diacids can be released by high temperature base solvolysis.[21,26] The release of the range of dehydrodiferulate acids from such solvolysis of saponified plant materials confirms that their 4-O-lignin ethers are present in grasses.[21]

It has been recently shown that 5–5-coupled dehydrodiferulate **1e** incorporates into lignin to a significantly greater extent than the other dimers and is less releasable (~30%).[23] Although dehydrodiferulate **1e** is not the major diferulate isomer in grass walls,[13] it is one of the most intriguing in terms of cell wall cross-linking. It is unique among the dimers in having the potential to couple the lignin moieties at both sidechain and phenolic positions. Since coupling at the sidechain 8-position preserves the phenolic positions, 5–5-coupled dehydrodiferulates can conceivably couple to lignin molecule(s) at all four available positions, producing an extreme branching point and a very tightly coupled lignin–polysaccharide network. However, the favorable 8–β-coupling between **1e** and lignin monomers would furnish furofuranoid structures such as **11**, Scheme 1.[3] In this scenario the 'diferulic acid' (polysaccharide cross-linker) incorporates into the lignin polymer but loses its connection to the polysaccharide(s).

What is required therefore is to understand how 5–5-coupled dehydrodiferulate can incorporate into lignins and, in particular, whether it is possible to couple at the 8-positions. The best method to determine available coupling reaction pathways is to incorporate strategically ^{13}C-labeled 5–5-coupled dehydrodiferulate biomimetically into a synthetic lignin (DHP = dehydrogenation polymer). This approach was used successfully to determine the possible fates of ferulate monomers and provided the necessary database for structural authentication in plant cell walls.[1–3] Here we report on the successful incorporation of dehydrodiferulate **1d**, a good model compound for its cell wall counterpart **1e**, into a coniferyl alcohol DHP and on the structural identification of coupling products as revealed by NMR spectroscopy.

Results and discussion

^{13}C-Labeled dehydrodiferulate model compound

An appropriate model for dehydrodiferulates in grass cell walls

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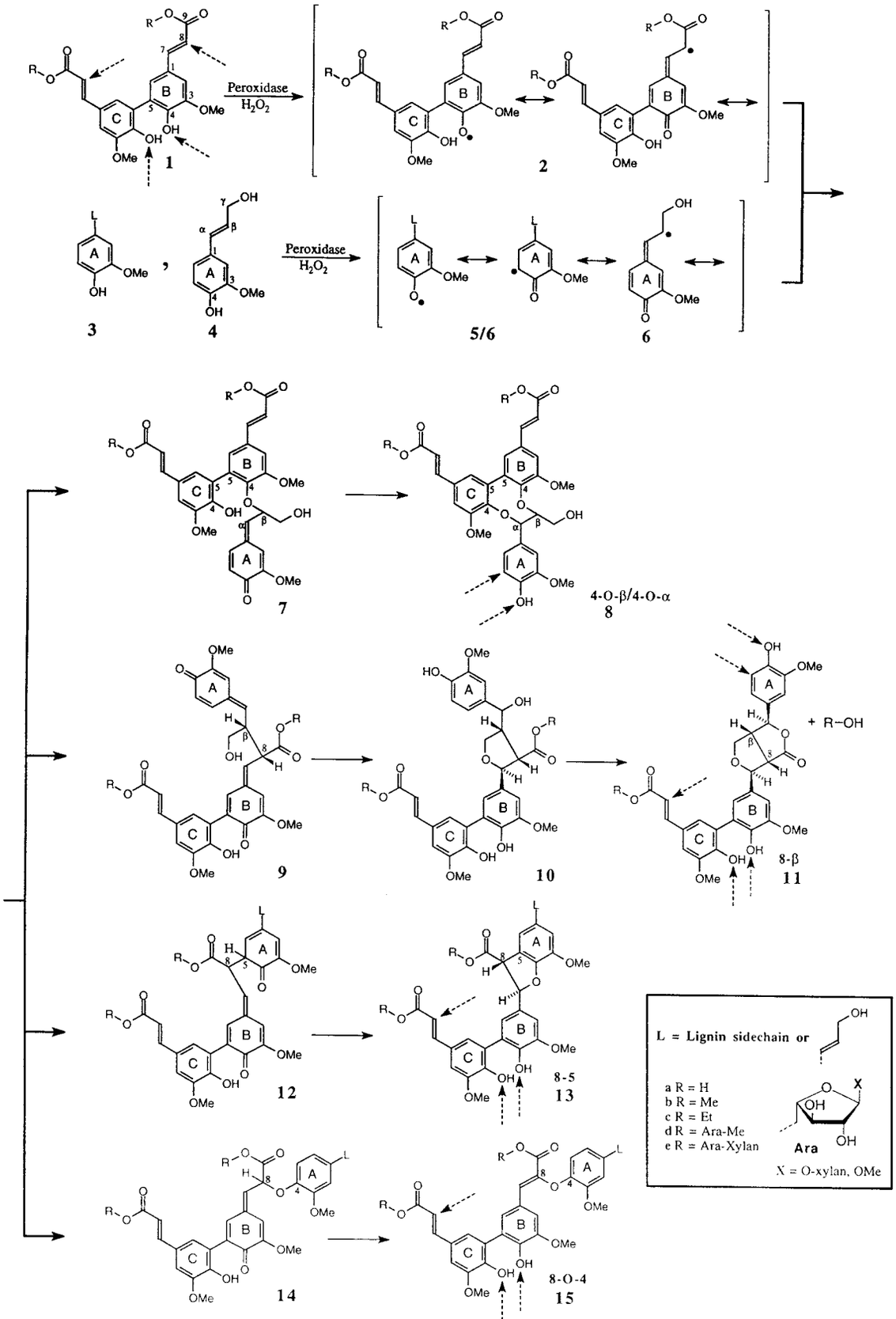
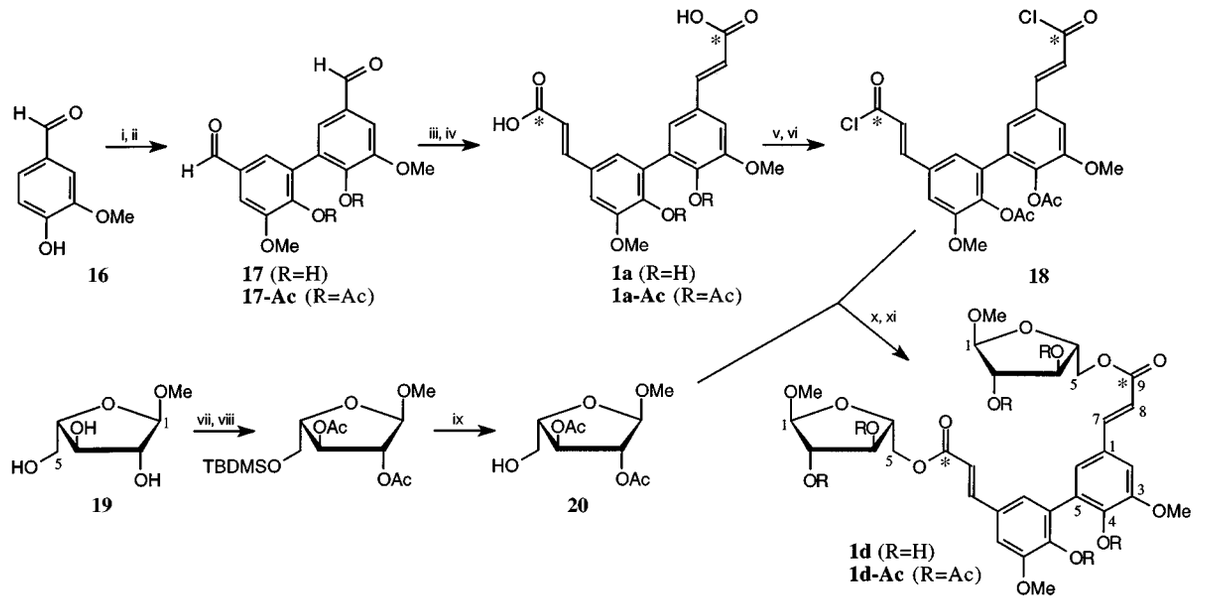


Scheme 1 Peroxidase–H_2O_2 produces radical **2** from 5–5-coupled dehydrodiferulate **1** and radicals **5**/**6** from coniferyl alcohol **4** and pre-formed lignin dimers or oligomers **3**. The dehydrodiferulate radical **2** cross-couples with a coniferyl alcohol radical, the latter always reacting at the β-position; initial 4–O–β-coupling leads *via* intermediate **7** to dibenzodioxocine **8**. Alternatively, 8–β-coupling can lead to the furofuranoid monoepoxylignanolide[2,54,55] **11** *via* intermediates **9** and **10**. Cross-coupling with a lignin oligomer can also afford products from 8-coupling of the ferulate moiety; 8–5-coupling leads to the phenylcoumaran **13** *via* **12** whilst 8–O–4-coupling leads to the styryl aryl ether **15** *via* **14**. Dashed arrows on the dehydrodiferulate **1** and final products **8**, **11**, **13** and **15**, indicate sites at which further radical coupling reactions are possible. The scheme shows only the initial coupling to ferulate moiety designated B. Except for the production of the 4–O–β/4–O–α product **8**, coupling of the other moiety, C, is essentially independent of the first coupling. The convention used here for naming compounds and cross-products is to designate the position on the ferulate moiety first and the position on the lignin or coniferyl alcohol last; *e.g.* an 8–β-compound implies that the ferulate moiety's 8-position has coupled to the β-position of coniferyl alcohol.



Scheme 2 Synthesis of ^{13}C-labeled compound **1d**, a model for 5–5-coupled dehydrodiferulate in grass walls. *Reagents and Conditions:* i, peroxidase–H_2O_2; ii, Ac_2O–pyridine; iii, NaH–triethylphosphono[1-^{13}C]acetate; iv, 4 M KOH–1,4-dioxane; v, Ac_2O–pyridine; vi, $SOCl_2$; vii, TBDMSCl–pyridine; viii, Ac_2O; ix, 80% HOAc; x, DMAP–CH_2Cl_2; xi, pyrrolidine–CH_2Cl_2.

is one in which the attachment to the polysaccharide is mimicked. Model **1d** ('diFA-Ara') has the ester linkage to the 5-position of an α-L-arabinofuranoside unit, just as in the natural arabinoxylan. The compound would arise in the plant system as one of the dimers resulting from coupling of two polysaccharide-ferulate esters. We cannot directly make **1d** synthetically by radical coupling of FA-Ara (the feruloylated arabinofuranoside) since *in vitro* coupling of such low molecular-mass ferulates by peroxidase–H_2O_2 or a variety of single-electron metal oxidants yields predominantly 8–5-coupling products; essentially no 5–5-coupled product is formed.[3,13,27–29] Instead vanillin **16** was radically coupled to 5–5-dehydrodivanillin **17**[30] and the sidechain extended *via* a Wittig–Horner reaction, Scheme 2.[3,31] Utilization of triethyl phosphono[1-^{13}C]acetate furnished the 9-labeling required for NMR studies. Acylation of the arabinofuranoside **19** was accomplished *via* methyl 2,3-di-*O*-acetyl-α-L-arabinofuranoside **20**[32] and the acyl chloride **18** as previously reported for the ferulate analogue,[3] Scheme 2. Deprotection afforded the required model **1d**.

Preparation of a dehydrodiferulate–synthetic lignin copolymer

The dehydrodiferulate model **1d**, 10% by weight, was radically copolymerized with coniferyl alcohol **4**[33] using the peroxidase–H_2O_2 system previously described.[3] The resulting DHP, obtained as a light pinkish powder in 84% yield, had significantly different properties than normal DHPs. In particular, it was not soluble in 9:1 acetone–water; dimethyl sulfoxide was required to effect solution for NMR spectroscopy.

NMR studies

^{13}C NMR spectroscopy of the diferulate–coniferyl alcohol DHP {in [$^2H_6$]Me_2SO (2 parts) diluted with [$^2H_6$]acetone (1 part) to lower the viscosity} revealed the incorporation of [9-^{13}C]dehydrodiferulate **1d** into some four different structure types [vertical projection on Fig. 1(*a*)] as was the case for ferulate monomers [vertical projection on Fig. 1(*b*)].[3] Long-range correlations from the labeled 9-carbons were totally diagnostic, Fig. 1(*a*), particularly when compared to the corresponding ferulate monomer experiment, Fig. 1(*b*). Although the solvent difference caused some significant proton shifts, it is clear that 8–O–4, 4–O–, 8–5, and 8–β coupling had occurred (see legend to Scheme 1 for naming conventions). The incorporation profile is remarkably similar to that of ferulate. The dehydrodiferulate reacts with the lignin monomer **4** to give 8–β and 4–O–β structures (lignin monomers **4** invariably react at their β-positions in cross-coupling reactions)[1] and with dimers or oligomers **3** to give 8–O–4 and 8–5 structures, Scheme 1. Interestingly, the release of 5–5-coupled dehydrodiferulic acid **1a** from biomimetically lignified walls is similar to ferulic acid's (~30%).[26] This is much lower than 8–8-, 8–O–4- and 8–5-coupled dehydrodiferulic acids' release (40–60%) suggesting that coupling of 5–5-dehydrodiferulate at the 8-position is indeed significant. Clearly, 5–5-coupled dehydrodiferulates **1b**–**e** are capable of radical coupling reactions with lignin monomers and dimers or oligomers to give the range of expected structures. How extensively each molecule is cross-coupled (*i.e.* how many of the four possible coupling sites on the dimer are occupied) is impossible to determine without model compounds for each of these possibilities.

Two other points need noting. Firstly, the 8–β structures **11**, just as in the ferulate 8–β products,[3] loose connectivity with their polysaccharide components which are displaced by transesterification. Therefore, while prominent, these 8–β products **11** do *not* ultimately result in lignin–polysaccharide cross-linking, just further lignin–lignin cross-linking. Secondly, if diferulates act similarly to ferulates in ryegrass,[1] reacting only with lignin monomer radicals (at their β-positions), 8–β and 4–O–β products, but not 8–5- and 8–O–4-products, may be formed *in vivo*. Such a determination will await very high field NMR examinations of ^{13}C-enriched lignins.

Dibenzodioxocine structures

Another unique feature of incorporation of 5–5-coupled dimers **1** into lignins is the ability to form dibenzodioxocines **8** *via* intermediate **7**. These structures have recently been identified in a variety of lignins and are strikingly predominant.[34–36] Normally, incorporation of dehydrodiferulate's two ferulate moieties into lignin would be considered to be independent events. This unique structure (**8**) allows a sequential incorporation of both moieties following a single radical coupling event, Scheme 1. The dehydrodiferulate–coniferyl alcohol product **8** is not the only dibenzodioxocine that can be formed; further reactions of any 8-coupled product (such as **11**, **13** or **15**) with additional coniferyl alcohol **4** can produce dibenzodioxocines. The 8–β (**11**) or 8–5 (**13**) moieties will produce dibenzodioxocines with one or more *saturated* sidechains and may be expected to have slightly different NMR shifts than **8**. There are

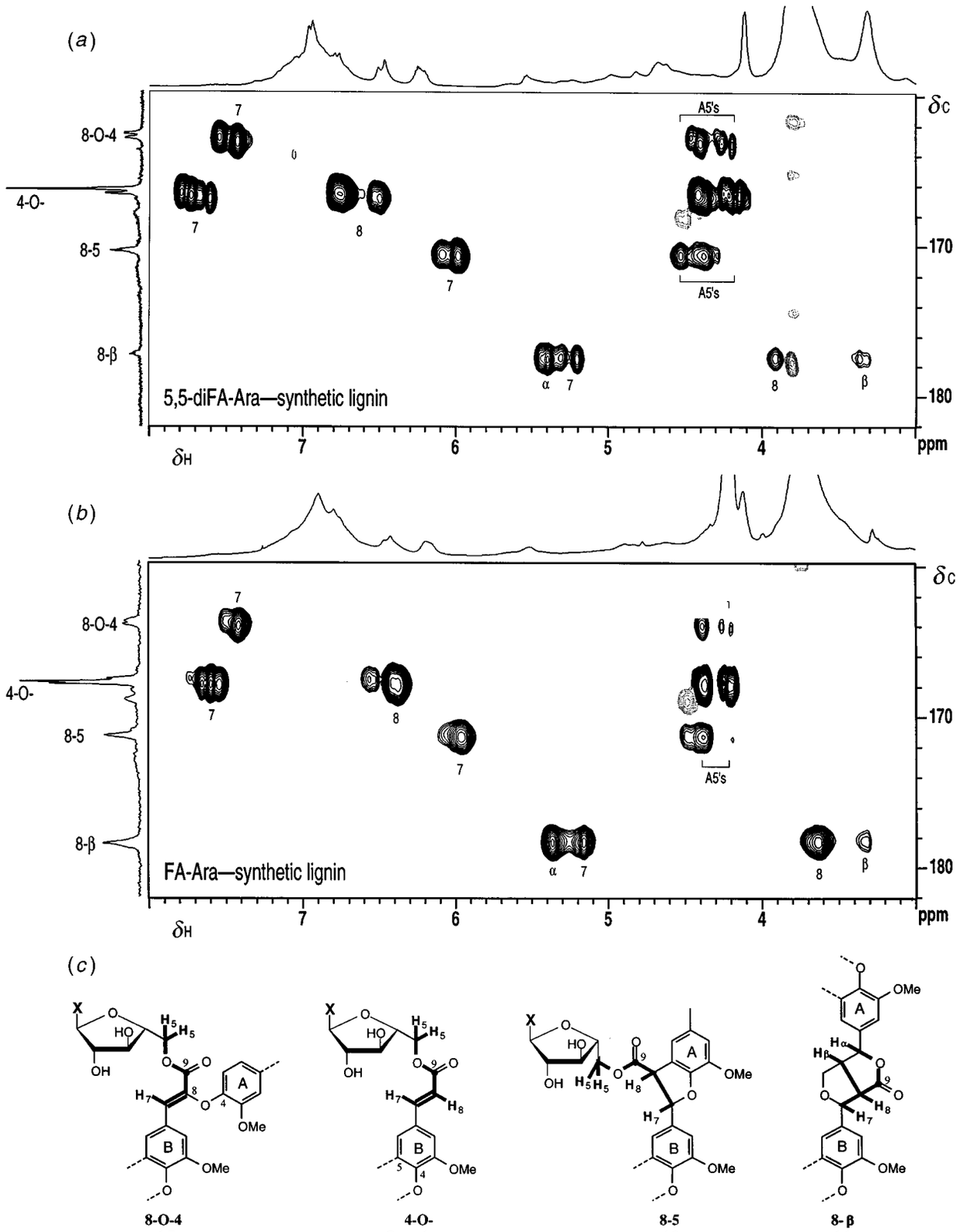


Fig. 1 Partial 360 MHz HMBC spectra (carbonyl carbon region only) showing correlations of C-9 with protons that are within three-bonds. The concurrent matching of several (up to five) carbon and proton chemical shift data makes the assignments essentially unambiguous. Note that, as occurs frequently in NMR spectra of such complex polymers, proton peaks for the minor components of interest are not readily discerned in the proton spectrum but such protons readily give correlation peaks in 2D NMR experiments, especially in heteronuclear experiments when correlating with the labeled carbon position. The 5,5-diFA-Ara DHP (*a*) has some solvent-induced shifts caused by the Me_2SO. Nevertheless, as in the FA-Ara DHP (*b*),[3] clear evidence is seen for 8–O–4, 4–O–, 8–5 and 8–β cross-coupling structures. In fact, the incorporation profiles are remarkably similar. NMR conditions were as described previously.[3] (*c*) The two- and three-bond couplings from C-9 giving rise to correlations in the NMR spectra. Grey (lighter) contours are from resonances not pertaining to this paper or from T_1-noise artifacts (in the methoxy region, δ_H ~3.8).

potential dibenzodioxocines where the β-attached or the α-attached or both moieties have saturated sidechains; hence considerable structural diversity is possible.

In an effort to determine if dibenzodioxocine structures such as **8** play important contributions, models were prepared to obtain the NMR data required to identify them in the DHP.

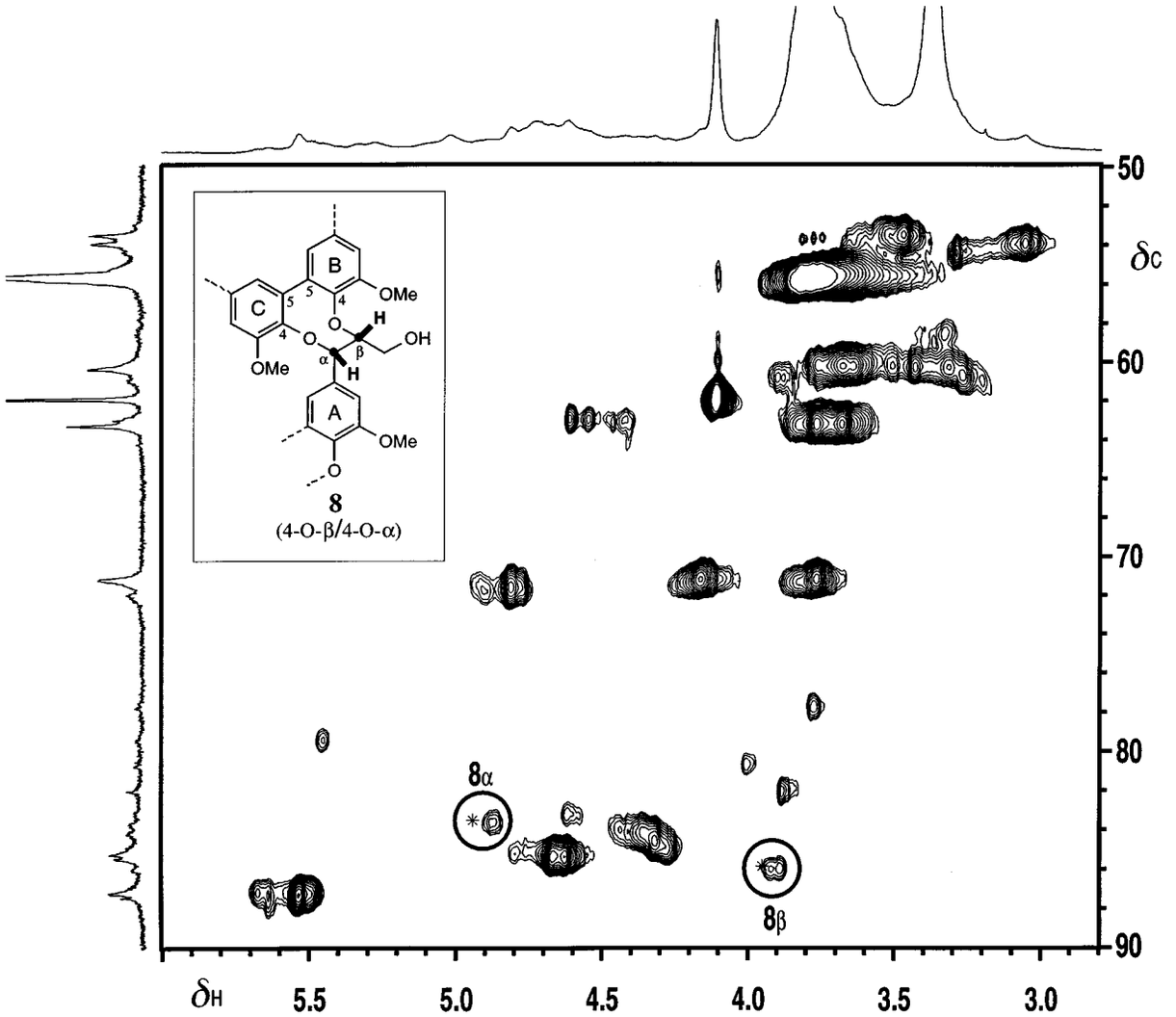


Fig. 2 Partial (sidechain region) 750 MHz gradient-selected HSQC spectrum showing clear evidence for the dibenzodioxocine structures **8**. Correlations from the parent model **8** are shown as (*). HMBC and HMQC–TOCSY spectra (not shown) provided further confirmatory evidence by correctly correlating other resonances. NMR conditions: 750 MHz; Bruker pulse program 'invietgs'—a 2D ^{1}H–^{13}C correlation *via* double INEPT transfer using trim pulses, phase sensitive using echo/antiecho TPPI, gradient selection, decoupling during the acquisition; 4K by 256 increments of 16 scans collected; sweep widths 11 ppm, 150 ppm giving acquisition FID resolutions of 2 and 110.5 Hz pt^{-1}. Processing used Gaussian multiplication (GB 0.01, LB −0.3 Hz) in F_2 and cosine-squared bell apodization in F_1.

The most direct way to synthesize these models was *via* radical-coupling methods.[36] Thus, the 5–5-coupled dehydrodiferulate **1b** or **1c** was reacted with an excess of coniferyl alcohol using Ag_2O as a single-electron oxidant.[29] The purified yields of the required products was low (*ca.* 15–20% based on dehydrodiferulate **1**) but the single-step preparation was significantly easier than a linear multi-step approach that did not exploit radical coupling. The data for these compounds agreed closely with those reported for similar compounds.[34,35] Spectra were fully consistent with the dibenzodioxocine structures; the HMQC,[37] HMBC[38] and HMQC–TOCSY[39] experiments were the most diagnostic. In particular, in the long-range ^{13}C–^{1}H correlation experiment (HMBC, not shown), H_α correlated nicely with the aromatic ring C-4 carbon. This three-bond coupling correlation establishes the α–O–4 bond formation. NMR data from appropriate models for all the dibenzodioxocine possibilities are not available to make more detailed assignments of the DHP spectra.

Data from models run in [$^2H_6$]acetone and from the DHP run in 2:1 [$^2H_6$]Me_2SO–[$^2H_6$]acetone initially seemed to have little in common. However, the solvent shifts afforded by the Me_2SO were remarkable (see Experimental section). When models were run in 2:1 [$^2H_6$]Me_2SO–[$^2H_6$]acetone, their chemical shifts coincided with significant shifts in the polymeric DHP. As seen in Fig. 2, the dibenzodioxocine structure is clearly identified in the aliphatic region of the HSQC[40,41] spectrum; it is also easily seen in lower field HMQC or HSQC spectra (*e.g.* at 360 MHz) without utilizing gradients. The HMBC and HMQC–TOCSY spectra of the DHP (not shown) are also consistent. In the HMBC spectrum H_α correlates with carbons β, A_1, A_2, A_6 and C_4, with the Hα–C_4 correlation proving the cyclic nature of the structure.

Conclusions

Until recently, literature on dehydrodiferulates and their cross-linking chemistry with lignin perpetuated two misconceptions. Firstly, the only dehydrodiferulate ever considered was the 5–5-coupled dehydrodimer **1**. This dimer is certainly not the major dehydrodimer in any plant sample recently analyzed but, fortuitously, has the potential to be one of the more important in effecting tight lignin–polysaccharide cross-linking due to the number of coupling sites available to it. Secondly, it has been assumed that the only cross-linking structure to lignin is an α-ether or benzyl aryl ether.[42,43] Such ethers can be produced by attack of the dehydrodiferulate phenols on lignin quinomethane intermediates, which themselves result from the coupling of a lignin monomer with another monomer or a lignin oligomer. Adherence solely to such a mechanism, as has been pointed out for ferulate,[1,3,9,25] has a number of significant draw-

backs. It denies the ferulate or dehydrodiferulate access to radical coupling mechanisms that are producing the very quinomethanes with which they can react. In the case of the 5–5-coupled dehydrodiferulate, nucleophilic attack by the sterically encumbered phenols is likely to be a particularly poor reaction. In practical terms, this mechanism has the unfortunate consequence that quantification of dehydrodiferulates by their hydrolytic release is assumed to quantify their total involvement in cross-linking. As demonstrated here, dehydrodiferulates are also capable of undergoing radical coupling reactions with lignin monomers and oligomers. Such radical mechanisms actively incorporating diferulates into lignins as integral components are likely to prove more important than the nucleophilic addition mechanism producing benzyl aryl ethers that is generally cited to date.

Now that the radical coupling mode has been unequivocally demonstrated *in vivo* for ferulates,[1] and it has been shown here that 5–5-coupled dehydrodiferulate will undergo the similar range of reactions *in vitro*, it would be remiss to continue to ignore such mechanisms for dehydrodiferulates. Since some of the structures in which dehydrodiferulates will be involved cannot be hydrolytically cleaved, their current 'quantification' is an underestimation of the importance of these species. Dehydrodiferulates, now more significant components of grass walls than previously recognized,[13] can play a powerful role in effecting lignin–polysaccharide cross-linking. The demonstration of the ability of 5–5-coupled dehydrodiferulates to form dibenzodioxocines during lignification is novel. Such structures lacking 8-attached units may fully release dehydrodiferulic acid **1a** upon high-temperature base treatment.[43] The understanding gained from this study of the chemistry by which dehydrodiferulates cross-couple into lignins provides a basis for understanding degradability of grass polysaccharides; such effects are currently being quantified.[23,44–47]

One caution relating to the extent of polysaccharide–polysaccharide–lignin cross-linking that may by occurring *via* the 5–5-coupled dehydrodiferulate comes from recent molecular modeling studies[48] which suggest that 5–5-coupling, unlike the other modes, could be from intramolecular reactions; *i.e.* coupling of two ferulates on the same arabinoxylan chain with an FA–Ara–Xyl–Xyl–Xyl–Ara–FA domain. Current studies are shedding light on further details of dehydrodiferulates' incorporation into lignins.

Experimental

General

Melting points are uncorrected. Evaporations were conducted under reduced pressure at temperatures less than 45 °C unless otherwise noted. Further elimination of organic solvents, as well as drying of the residues, was accomplished under moderate vacuum (40–120 mtorr) at room temperature. Column chromatography was performed on silica gel 60 (230–400 mesh) and TLC was with Alugram Sil-G/UV_{254} plates (Macherey–Nagel), with visualization by UV light. Acetone and methylene chloride (CH_2Cl_2) were dried by passage through a column of alumina. Tetrahydrofuran (THF) was distilled from sodium–benzophenone immediately before use. Light petroleum refers to the boiling range 40–60 °C. Coniferyl alcohol **4** was prepared from ethyl ferulate by diisobutylaluminium hydride reduction as previously described.[33]

NMR spectra of samples in [$^2H_6$]acetone or 2:1 [$^2H_6$]Me_2SO–[$^2H_6$]acetone were run at 300 K on a Bruker AMX-360 360 MHz 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 references (^{1}H, 2.04 ppm; ^{13}C, 29.8 ppm). One- and two-dimensional NMR spectra were obtained using standard Bruker pulse programs; ^{1}H–^{13}C correlation information was obtained with the usual combination of the inverse-detected one-bond and long-range ^{1}H–^{13}C correlation experiments, HMQC[37] and HMBC,[38] and by the HMQC–TOCSY[39] experiment. Full data for all title compounds and key intermediates appears in the NMR Database of Model Compounds for Lignin and Related Cell Wall Components.[49] The gradient-selected HSQC[40,41] spectrum of the diFA-Ara DHP in 2:1 [$^2H_6$]Me_2SO–[$^2H_6$]acetone (Fig. 2) was on a Bruker DMX-750 750 MHz narrow-bore instrument fitted with an inverse triple-resonance ^{13}C–^{15}N–^{1}H inverse probe with XYZ-gradients.

High resolution EI-MS data were collected on a Kratos MS-80RFA spectrometer. Percentage values in parentheses refer to the height relative to the spectrum base peak.

Synthesis of [9,9′-^{13}C]5,5′-dehydro-di-FA-Ara 1d {[9,9′-^{13}C]-1,1-Bis[(*E*)-3-oxo-3-α-L-arabinofuranosylprop-1-enyl]-3,3′-dimethoxy-4,4′-dihydroxy-5,5′-biphenyl‡}

4,4′-Di-*O*-acetyl-5,5′-bivanillin 17-Ac. Bivanillin **17** (1.01 g, 3.35 mmol), prepared from vanillin **16** using peroxidase and H_2O_2 as described by Baumgartner and Neukon,[30] was dissolved in Ac_2O–pyridine (1:1 v/v; 25 cm^3). The reaction mixture was stirred at ambient temperature for 24 h, after which time it was quenched by the addition of ethanol (95%; 10 cm^3). After stirring for 1 h, the mixture was diluted with toluene (25 cm^3) and evaporated to an oil. Two subsequent additions and evaporations of toluene were performed to eliminate any remaining undesired volatiles. The resulting brown syrup was dissolved in CH_2Cl_2 and successively washed with aq. 3% HCl, water and saturated aq. $NaHCO_3$. Drying ($MgSO_4$) and evaporation of the organic layer furnished crude **17-Ac** (1.07 g) as a brown foam. Purification by silica gel chromatography [$CHCl_3$–EtOAc (5:1)] gave a pale yellow foam (629 mg), which was crystallized from acetone–light petroleum to afford pure **17-Ac** (551 mg, 43%) as small white needles, mp 105–106 °C [lit.,[50,51] (MeOH, EtOH) 117–118 °C, lit.,[52] (H_2O–HOAc) 128–129 °C—melting points seem to vary with crystallizing solvent]; *m/z* 386 (M^+, 11%), 344 (70), 302 (100); δ_H 2.08 (6 H, s, 2 × OAc), 3.96 (6 H, s, 2 × OMe), 7.48 (2 H, d, *J* 1.8, A-6, A-6′), 7.64 (2 H, d, *J* 1.8, A-2, A-2′), 9.99 (2 H, s, 2 × *H*CO); δ_C 20.22 (2 × OAc), 56.81 (A3-OMe, A3′-OMe), 112.04 (A-2, A-2′), 125.91 (A-6, A-6′), 132.19 (A-5, A-5′), 135.86 (A-1, A-1′), 143.49 (A-4, A-4′), 153.41 (A-3, A-3′), 168.15 (2 × AcO), 191.69 (2 × H*C*O).

4,4′-Di-*O*-acetyl-[9,9′-^{13}C]-(*E*,*E*)-5–5′-dehydrodiferulic acid diethyl ester {diethyl [9,9′-^{13}C](*E*,*E*)-4,4′-dihydroxy-3,3′-dimethoxy-5,5′-bicinnamate‡} 1c-Ac. NaH (132 mg, 5.50 mmol) was suspended in anhydrous THF (2 cm^3). Triethyl phosphono-[1-^{13}C]acetate (Aldrich Chemical Co., 750 mm^3, 3.76 mmol) was added *via* syringe under N_2. The mixture was stirred at ambient temperature for 15 min, after which time a solution of the bivanillin derivative **17-Ac** (709 mg, 1.84 mmol, from two runs) in anhydrous THF (10 cm^3) was added. This reaction mixture was stirred at ambient temperature for 6 h, and subsequently hydrolyzed with water, extracted with Et_2O, washed with saturated aq. sodium hydrogen sulfite, dried and evaporated to dryness to afford relatively cleanly the diethyl dehydrodiferulate diacetate **1c-Ac** as an off-white solid (863.5 mg, 89%); δ_H 1.27 (6 H, t, *J* 7.1, 2 × C*H*$_3$CH$_2$OCO), 2.08 (6 H, s, 2 × AcO), 3.94 (6 H, s, 2 × OMe), 4.20 (4 H, qd, $^3J_{HH}$ 7.1, $^3J_{CH}$ 3.1, 2 × CH$_3$C*H*$_2$OCO), 6.58 (2 H, dd, $^3J_{HH}$ 16.0, $^2J_{CH}$ 2.4, F-8, F-8′), 7.15 (2 H, d, *J* 1.9, F-6, F-6′), 7.51 (2 H, d, *J* 1.9, F-2, F-2′), 7.66 (2 H, dd, $^3J_{HH}$ 16.0, $^3J_{CH}$ 6.8, F-7, F-7′); δ_C 14.57 (d, $^3J_{CC}$ 2.0, 2 × *C*H$_3$CH$_2$OCO), 20.29 (2 × AcO), 56.68 (F3-OMe, F3′-OMe), 60.79 (2 × CH$_3$*C*H$_2$OCO), 111.93 (F-2, F-2′), 119.81 (d, $^1J_{CC}$ 75.8, F-8, F-8′), 123.86 (F-6, F-6′), 132.33 (F-5, F-5′), 133.71 (d, $^3J_{CC}$ 7.2, F-1, F-1′), 140.29 (F-4, F-4′), 144.23 (F-7, F-7′), 152.93 (F-3, F-3′), 166.91 (m, F-9, F-9′), 168.40 (2 × AcO).

‡ The numbering system shown on structure **1** is used here rather than the systematic IUPAC numbering scheme.

[9,9′-^{13}C]5–5′-Dehydrodiferulic acid {[9,9′-^{13}C](*E*,*E*)-4,4′-dihydroxy-3,3′-dimethoxy-5,5′-bicinnamic acid‡} [9,9′-^{13}C]-1a. The ^{13}C-labeled diethyl dehydrodiferulate diacetate described above (**1c-Ac**) was directly saponified under N_2 by treatment with degassed aq. 40% KOH–1,4-dioxane (10:1, 50 cm^3) at ambient temperature for 72 h. The reaction mixture was then neutralized by addition of 2 mol dm^{-3} HCl, extracted with EtOAc and washed with saturated aq. NaCl. The organic layer was dried over $MgSO_4$ and evaporated to afford [9,9′-^{13}C]5–5′-dehydrodiferulic acid **1a** as an off-white solid (538.5 mg, 76%); δ_H([$^2H_6$]acetone + 2 drops [$^2H_6$]dimethyl sulfoxide) 3.96 (6 H, s, 2 × OMe), 6.39 (2 H, dd, $^3J_{HH}$ 15.9, $^2J_{CH}$ 2.6, F-8, F-8′), 7.18 (2 H, d, *J* 1.9, F-6, F-6′), 7.33 (2 H, d, *J* 1.9, F-2, F-2′), 7.61 (2 H, dd, $^3J_{HH}$ 15.9, $^3J_{CH}$ 6.8, F-7, F-7′); δ_C([$^2H_6$]acetone + 2 drops [$^2H_6$]dimethyl sulfoxide) 56.53 (F3-OMe, F3′-OMe), 109.96 (F-2, F-2′), 116.64 (d, $^1J_{CC}$ 73.6, F-8, F-8′), 125.79 (F-5, F-5′), 126.05 (F-6, F-6′), 126.62 (d, $^3J_{CC}$ 7.1, F-1, F-1′), 145.59 (F-7, F-7′), 147.44 (F-4, F-4′), 148.99 (F-3, F-3′), 168.54 (m, F-9, F-9′). Full characterization of the unlabeled compound has been previously reported.[13]

4,4′-Di-*O*-acetyl-[9,9′-^{13}C]5–5′-dehydrodiferulic acid 1a-Ac. Reacetylation of **1a** (210 mg, 0.54 mmol) was accomplished by treatment with Ac_2O–pyridine (1:1 v/v; 10 cm^3) at ambient temperature for 24 h, after which time the reaction mixture was diluted with toluene and evaporated to give a syrup. This syrup was dissolved in CH_2Cl_2, washed successively with aq. 3% HCl and water, dried over $MgSO_4$ and evaporated to dryness. The resulting solid residue was further treated with aq. 3% HCl–EtOAc (1:1 v/v; 100 cm^3); the mixture was vigorously stirred overnight at ambient temperature, then separated and the organic layer was washed with saturated aq. NaCl, dried and evaporated to furnish the desired acetylated ^{13}C-labeled dehydrodiferulic acid **1a-Ac** (231 mg, 90%) as a white solid; attempted mp, decomposed and darkened over a large range starting at ~340 °C, did not melt at 400 °C, (lit.,[53] > 350 °C); δ_H([$^2H_6$]-acetone + 2 drops [$^2H_6$]dimethyl sulfoxide) 2.08 (6 H, s, 2 × OAc), 3.93 (6 H, s, 2 × OMe), 6.54 (2 H, dd, $^3J_{HH}$ 16.0, $^2J_{CH}$ 2.5, F-8, F-8′), 7.13 (2 H, d, *J* 1.8, F-6, F-6′), 7.50 (2 H, d, *J* 1.8, F-2, F-2′), 7.62 (2 H, dd, $^3J_{HH}$ 16.0, $^3J_{CH}$ 6.8, F-7, F-7′); δ_C([$^2H_6$]acetone + 2 drops [$^2H_6$]dimethyl sulfoxide) 20.27 (2 × AcO), 56.66 (F3-OMe, F3′-OMe), 111.89 (F-2, F-2′), 120.60 (d, $^1J_{CC}$ 73.4, F-8, F-8′), 123.59 (F-6, F-6′), 132.18 (F-5, F-5′), 133.80 (d, $^3J_{CC}$ 7.2, F-1, F-1′), 139.98 (F-4, F-4′), 143.88 (F-7, F-7′), 152.79 (F-3, F-3′), 168.18 (m, F-9, F-9′), 168.40 (2 × AcO).

[9,9′-^{13}C]5,5′-dehydro-diFA-Ara {[9,9′-^{13}C]-1,1-Bis[(*E*)-3-oxo-3-α-L-arabinofuranosylprop-1-enyl]-3,3′-dimethoxy-4,4′-dihydroxy-5,5′-biphenyl‡} 1d. The [9,9′-^{13}C]dehydrodiferulic acid diacetate **1a-Ac** (223 mg, 0.47 mmol) was suspended in anhydrous benzene (5 cm^3) and thionyl chloride (0.60 cm^3, 8.22 mmol) was added. The mixture was refluxed for 45 min, after which time the solution was evaporated. Three subsequent additions and evaporations of toluene were followed by further drying of the residue under vacuum (*ca.* 1 h) to ensure complete elimination of any undesired volatiles. The resulting crude acyl chloride was then readily dissolved in CH_2Cl_2 (5 cm^3) and added to a 0 °C-cooled solution of methyl 2,3-di-*O*-acetyl-α-L-arabinofuranoside **20**[3,32] (246 mg, 0.99 mmol) in CH_2Cl_2 (5 cm^3) under N_2. DMAP [4-(dimethylamino)pyridine, 230 mg, 1.88 mmol] was added, and the reaction mixture was stirred at 0 °C for 30 min, then at ambient temperature for 12 h, after which time it was diluted with CH_2Cl_2 and washed successively with water, aq. 3% HCl and water. Drying of the organic layer over $MgSO_4$ and evaporation gave a brown foamy residue (473 mg). No purification of this ^{13}C-labeled material was performed at this stage of the synthesis. The residue was dissolved in CH_2Cl_2 (5 cm^3) and directly treated with pyrrolidine[3] (1.5 cm^3) for 48 h in the dark. The reaction mixture was then partially evaporated, diluted with EtOAc, washed with aq. 3% HCl (three times) and aq. saturated NaCl, dried over $MgSO_4$ and evaporated to afford an oily residue, which was submitted to preparative thin layer chromatography [EtOAc–AcOH (100:1)] to furnish **1d** (110 mg, 34%) as a pale yellow oil; δ_H 3.31 (6 H, s, 2 × 1′-OMe), 3.92 (2 H, dd, *J* 6.6, 3.9, 2 × H-3′), 3.94 (6 H, s, F3-OMe, F3′-OMe), 4.01 (2 H, dd, *J* 3.9, 1.8, 2 × H-2′), 4.09 (2 H, td, *J* 6.4, 3.5, 2 × H-4′), 4.24 (2 H, ddd, $^3J_{HH}$ 11.8, 6.2, $^3J_{CH}$ 2.8, 2 × H-5′a), 4.38 (2 H, dt, $^3J_{HH}$ 11.8, 2.9, $^3J_{CH}$ 2.9, 2 × H-5′b), 4.77 (2 H, d, *J* 1.7, 2 × H-1′), 6.45 (2 H, dd, $^3J_{HH}$ 15.9, $^2J_{CH}$ 2.4, F-8, F-8′), 7.25 (2 H, d, *J* 1.9, F-6, F-6′), 7.33 (2 H, d, *J* 1.9, F-2, F-2′), 7.68 (2 H, dd, $^3J_{HH}$ 15.9, $^3J_{CH}$ 6.9, F-7, F-7′), 8.01 (2 H, s, F4-OH, F4′-OH); δ_C 54.99 (2 × 1′-OMe), 56.51 (F3-OMe, F3′-OMe), 64.72 (2 × 5′), 79.05 (2 × 3′), 82.02 (2 × 4′), 83.17 (2 × 2′), 109.77 (F-2, F-2′), 110.25 (2 × 1′), 115.59 (d, $^1J_{CC}$ 76.8, F-8, F-8′), 125.45 (F-5, F-5′), 126.39 (F-6, F-6′), 126.43 (d, $^3J_{CC}$ 7.2, F-1, F-1′), 146.13 (F-7, F-7′), 147.48 (F-3, F-3′), 148.85 (F-4, F-4′), 167.37 (F-9, F-9′). NMR data of the non-labeled compound is in the NMR Database of Model Compounds of Lignin and Related Plant Cell Wall Components.[49]

Preparation of dibenzodioxocine models 8b, 8c by oxidative coupling

[(6*R,7*R**)-2,11-Bis(2-ethoxycarbonylvinyl)-7-(4-hydroxy-3-methoxyphenyl)-4,9-dimethoxy-6,7-dihydrodibenzo[*e*,*g*][1,4]-dioxocin-6-yl]methanol 8c.** Diethyl 5–5-coupled dehydrodiferulate **1c** (574 mg, 1.30 mmol) and coniferyl alcohol **4** (584 mg, 3.24 mmol, 2.5 equiv.) were dissolved in acetone. Ag_2O (1.51 g, 6.49 mmol) was added and the mixture stirred at room temperature for 5 h, monitoring starting material loss by TLC. The product was extracted into EtOAc and washed with saturated aq. NH_4Cl. The organic phase was dried over $MgSO_4$, filtered and the solvent evaporated under reduced pressure. Flash chromatography using 2:3 EtOAc–$CHCl_3$ eluted the required compound **8c** (187 mg, 23%) in the early fractions. The product **8c** was a white solid (Found: M^+, 620.2269. $C_{34}H_{36}O_{11}$ requires *M*, 620.2258); δ_H 4.92 (1 H, d, *J* 10.1, H-α), 4.15 (1 H, m, H-β), 3.82 (1 H, m, H-γ_2), 3.46 (1 H, m, H-γ_1); δ_C 85.0 (C-α), 87.4 (C-β), 62.4 (C-γ); δ_H(2:1 [$^2H_6$]Me_2SO–[$^2H_6$]acetone) 4.94 (1 H, d, *J* 10.0, H-α), 3.95 (1 H, m, H-β), 3.92 (1 H, m, H-γ_2), 3.26 (1 H, m, H-γ_1); δ_C(2:1 [$^2H_6$]Me_2SO–[$^2H_6$]acetone) 83.6 (C-α), 85.9 (C-β), 60.5 (C-γ).

Dimethyl [(6*R,7*R**)-2,11-Bis(2-methoxycarbonylvinyl)-7-(4-hydroxy-3-methoxyphenyl)-4,9-dimethoxy-6,7-dihydrodibenzo-[*e*,*g*][1,4]dioxocin-6-yl]methanol 8b.** Prepared as described for **8c** from dimethyl 5–5-coupled dehydrodiferulate **1b** and coniferyl alcohol **4**; δ_H 4.91 (1 H, d, *J* 10.1, H-α), 4.12 (1 H, m, H-β), 3.82 (1 H, m, H-γ_2), 3.46 (1 H, m, H-γ_1); δ_C 85.2 (C-α), 87.7 (C-β), 62.6 (C-γ).

Preparation of the coniferyl alcohol–[9,9′-^{13}C]5,5′-dehydro-diFA-Ara dehydrogenation polymer (DHP)

Coniferyl alcohol **4**[33] (454 mg, 2.52 mmol) and [9,9′-^{13}C]5,5′-dehydrodiFA-Ara **1b** (52 mg, 0.08 mmol) were dissolved in acetone (10 cm^3) and added to stirred phosphate buffer (200 cm^3; 0.01 mol dm^{-3}, pH 6.8; degassed), containing horseradish peroxidase (Sigma Co., EC 1.11.1.7, Type II, 2 mg). A second solution containing commercial hydrogen peroxide (273 mm^3 of aq. 30% solution, 2.60 mmol) was prepared in phosphate buffer (210 cm^3). The two solutions were added simultaneously at ambient temperature to stirred phosphate buffer (100 cm^3), containing 15 mg of vanillyl alcohol. The addition and work-up procedures were as described previously.[3] The DHP was isolated as a pale pinkish solid, 84% yield.

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