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p-COUMAROYLATED SYRINGYL UNITS IN MAIZE LIGNIN: IMPLICATIONS FOR β-ETHER CLEAVAGE BY THIOACIDOLYSIS

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Key Word Index—*Zea mays*; Gramineae; maize; lignin; *p*-coumaric acid; β-ether cleavage; thioacidolysis.

Abstract—Recent NMR studies of lignin isolated from maize demonstrated that *p*-coumarate esters are attached exclusively to the γ-position of phenylpropane side chains. Thioacidolysis/desulphuration experiments have revealed that *p*-coumarate units are attached primarily (*ca* 90%) to syringyl moieties in maize lignin. In model studies with guaiacylglycerol and syringylglycerol-β-guaiacyl ethers, cleavage of β-ether linkages by thioacidolysis was reduced 40% by γ-acylation of phenylpropane side chains with *p*-coumarate. Our results indicate that γ-*p*-coumarate esters significantly reduce the yields of syringyl products recovered after thioacidolysis of grass lignins.

INTRODUCTION

p-Coumaric acid is a major component of lignin in grasses, comprising up to 20% of lignins isolated from maize [1]. Recent NMR studies have demonstrated that *p*-coumaric acid is esterified exclusively to the γ-position of β-aryl ethers (Fig. 1) and other types of lignin substructures [1]. For reasons yet to be elucidated, the *p*-coumarate moiety does not incorporate into lignin and remains as a terminal unit with a free phenolic group and an unsaturated side chain [1]. Correlative evidence from NMR spectroscopy [1], radiotracer/microscopy [2] and solvolytic studies [3–5] suggest that *p*-coumarate esters are attached primarily to syringyl units in grass lignins.

Solvolytic cleavage of β-*O*-4 inter-unit linkages by thioacidolysis has proved to be an extremely useful tool for characterizing and identifying structural components in lignin [6, 7]. Since *p*-coumarate esters are only partially cleaved during thioacidolysis [8], dimeric thioacidolysis products from maize were analysed to determine if *p*-coumarate is esterified to syringyl units in lignin. The effect of *p*-coumarate esters on the cleavage of β-aryl ethers by thioacidolysis was also investigated.

RESULTS AND DISCUSSION

Maize lignin (24:1 dioxane–water soluble fraction isolated from ball-milled and cellulase-degraded cell walls [11]) was subjected to thioacidolysis under standard conditions [8] to release phenylpropane units acylated with *p*-coumaric acid. Thioethylated products were hydrogenated with Raney nickel and analysed by GC-mass spectrometry [9, 10]. Mass spectra of two previously unidentified peaks were consistent with compounds **3a** and **3b**, hydrogenated forms of coniferyl and sinapyl *p*-coumarate (Figs 1 and 2). The identity of these peaks was confirmed by comparing their GC retention times and mass spectra with those of authentic compounds. Recovery of conferyl and sinapyl *p*-coumarates was low (representing less than 10% of *p*-coumarate esters in maize lignin) and varied in tandem between thioacidolysis runs. A large proportion of the *p*-coumarate esters were cleaved during thioacidolysis; the concentration of *p*-coumarate monomers was similar to that of syringyl monomers.

Thioacidolysis/Raney nickel products from maize walls and maize lignin [1] were analysed by GC-FID to estimate the relative abundance of *p*-coumarate esters. The peak area of sinapyl *p*-coumarate (**3b**) was consistently eight to nine times greater than that of coniferyl *p*-coumarate (**3a**), indicating that *p*-coumarate moieties were primarily attached to syringyl units. A similar ratio of syringyl to guaiacyl *p*-coumaroylated products was also observed after acetyl bromide/Zn reductive cleavage of maize lignin (Lu, F., unpublished results). This new lignin characterization method specifically cleaves β-ether structures while leaving *p*-coumarate esters intact [11, 12]. The relatively high proportion of **3b** recovered after thioacidolysis is due in part to syringyl units being involved in a higher

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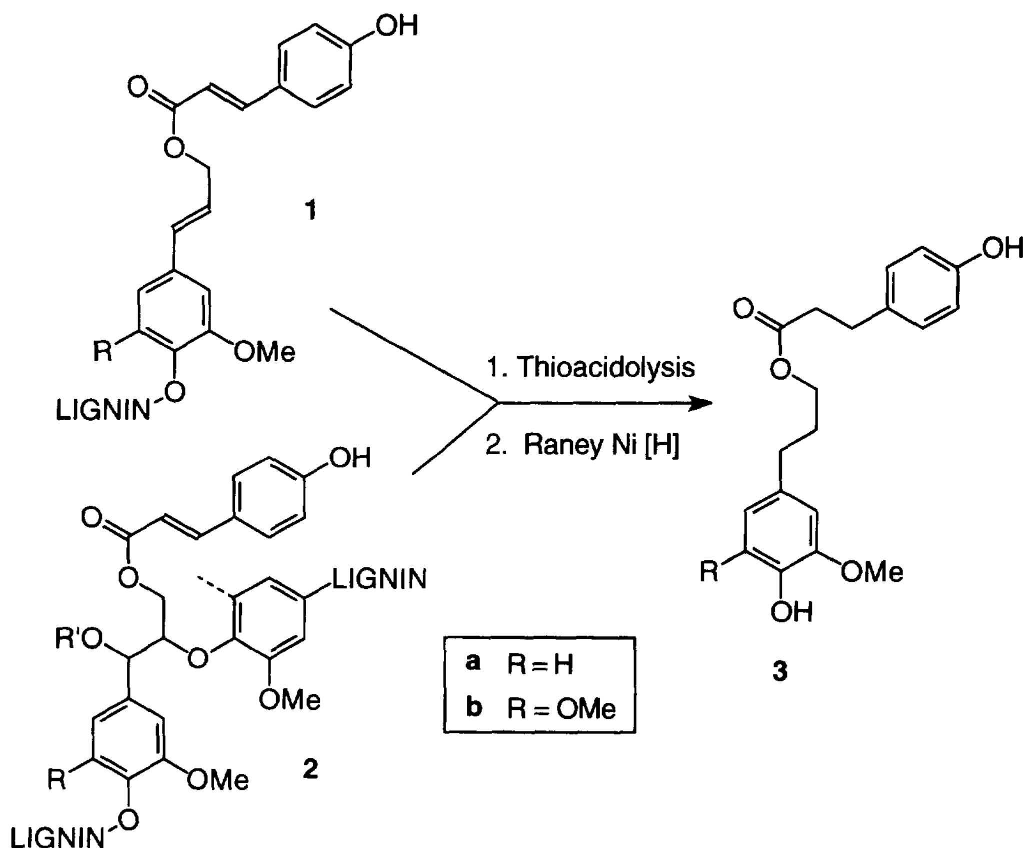

Fig. 1. *p*-Coumarate esters are incorporated into lignin to give, among other products, two types of β-ether structures: the hydroxycinnamyl *p*-coumarate end unit (**1**) and the β-ether (**2**). Thioacidolysis cleaves β-ethers while leaving a small proportion of the *p*-coumarate esters intact. After Raney nickel treatment, products **3a** and **3b** result, providing a distinction between *p*-coumarate esterification of guaiacyl and syringyl units in lignin.

proportion of labile β-ether structures than guaiacyl units. However, the overall ratio of syringyl to guaiacyl units of this lignin was only 1.4 as determined by thioacidolysis [13]. Since acylation did not differentially affect the release of syringyl or guaiacyl units from β-ether models (**4**, Fig. 3), our results demonstrate that *p*-coumarate esters are primarily attached to syringyl units in maize lignin. Preferential attachment of *p*-coumarate to syringyl lignins or lignin precursors (e.g. sinapyl *p*-coumarate) may involve enzyme specificity. However, temporal aspects of lignification are more likely involved since deposition of *p*-coumarate and syringyl-rich lignins occur concurrently at late stages of lignification [2–5].

The possibility of **3b** being an artefact formed during thioacidolysis was discounted by subjecting a mixture of coniferyl *p*-coumarate β-ether (**4a**, Fig. 3) and willow lignin (a syringyl-guaiacyl lignin with no *p*-coumarate esters [10]) to thioacidolysis/desulphurization. Although small quantities of **3a** were recovered, **3b** was not, confirming that **3b** was a component of maize lignin and not an artefact formed by transesterification reactions.

Model studies were done to identify methods for improving the recovery of *p*-hydroxycinnamyl *p*-coumarate products from lignins subjected to thioacidolysis. Recovery of **3b** was not improved when sinapyl *p*-coumarate β-ether (**4b**) was subjected to thioacidolysis under reduced duration or temperature. We then evaluated if saturation of phenylpropane sidechains by hydrogenation would allow stabilization and subsequent quantitation of *p*-hydroxycinnamyl *p*-coumarate end units (**1**) and β-ether structures (**2**, Fig. 1) by thioacidolysis. Although unsaturated sinapyl *p*-coumarate (**10b**, Fig. 4) was extensively degraded, saturated sinapyl *p*-coumarate (**3b**) was quantitatively recovered after thioacidolysis, indicating that *p*-coumarate end units would be quantified by this approach. These end units, however, comprise only a small proportion of the structures involving *p*-hydroxycinnamyl *p*-coumarates [1]. Unfortunately, attempts to release **3b** from a saturated form of **4b** failed due to γ-ester cleavage, apparently mediated by thioethyl addition to the syringyl sidechain. Since these β-ether structures are abundant in maize lignin [1] further efforts to quantify *p*-coumarate ester structures by thioacidolysis were abandoned. More recent work by our group demonstrated that β-ether cleavage allows

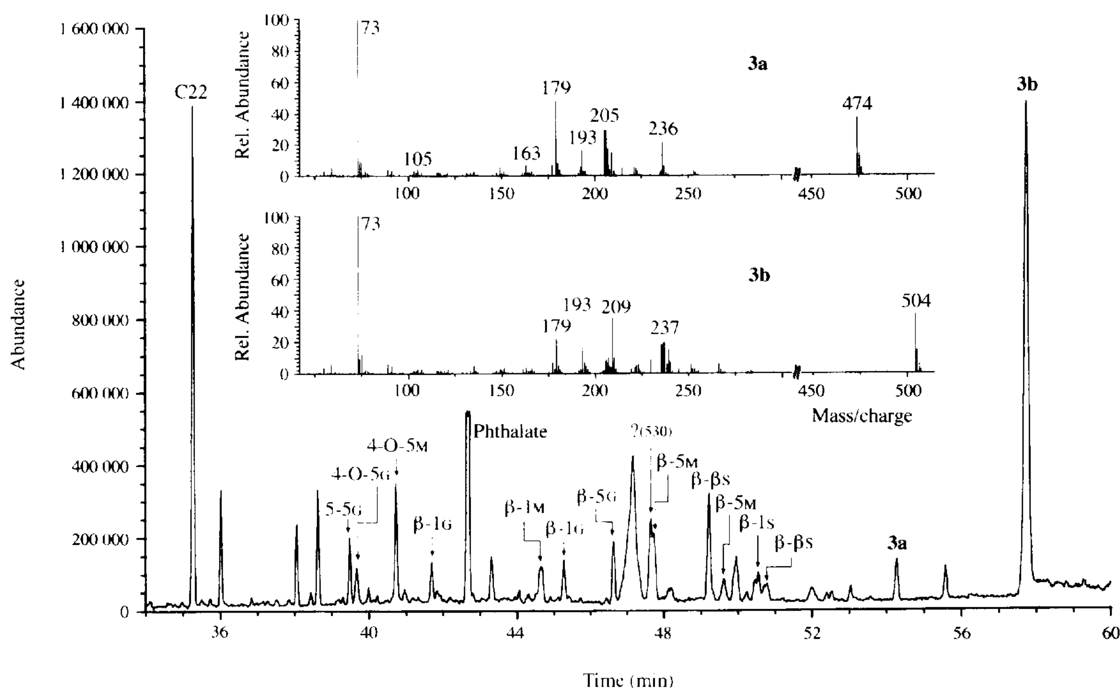

Fig. 2. GC-MS of dimers recovered after thioacidolysis of maize lignin. Dimers were trimethylsilylated prior to analysis. *p*-Coumarate ester products (**3a** and **3b**) are observed later in the total ion chromatogram than previously identified products [9, 23]. Peaks are identified by their linkage types (e.g. 5–5) and the aromatic nuclei involved (G = guaiacyl-guaiacyl, S = syringyl-syringyl, and M = guaiacyl-syringyl or syringyl-guaiacyl).

acetyl bromide/Zn reduction by quantitative recovering of *p*-coumaroylated structures from lignin [12].

In the course of our model studies, we observed that significant quantities of *p*-coumaroylated *β*-ethers resisted degradation during thioacidolysis. Under standard thioacidolysis conditions (100° for 5 hr), yields of syringyl or guaiacyl monomers recovered from *p*-coumaroylated *β*-ether models (**4**) were *ca* 45%. In contrast, yields of monomers from nonacylated syringylglycerol-*β*-guaiacyl and guaiacylglycerol-*β*-guaiacyl ether models were *ca* 75%, which is comparable to that reported by Lapierre *et al.* [14]. It has been observed that yields of thioacidolysis products from *p*-coumaroylated grass lignins are lower than non-acylated wood lignins [15]. Recently, Chesson *et al.* [5] found that yields of syringyl products from maize lignin were two-fold greater with NaOH–microwave digestion than with thioacidolysis. Both of these methods for cleaving of *β*-ethers gave comparable yields of guaiacyl products. When considered together, these results suggest that yields of syringyl products from thioacidolysis are significantly reduced by acylation of *β*-ether structures with *p*-coumaric acid.

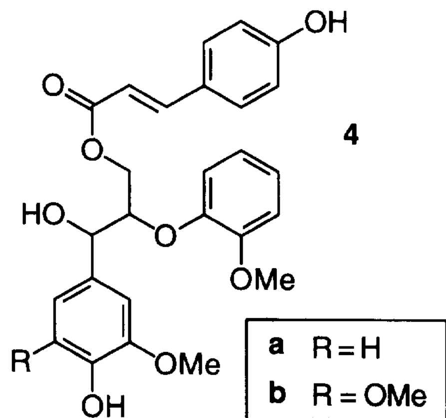

Fig. 3. Models (**4**) used to investigate the release of hydroxycinnamyl *p*-coumarates (**3**) from *β*-ether structures (**2**) during thioacidolysis.

CONCLUSIONS

Analysis of thioacidolysis/Raney nickel products has demonstrated that *p*-coumaric acid is esterified primarily to syringyl units in maize lignin. Recovery of *p*-hydroxycinnamyl *p*-coumarates was low due to ester cleavage and incomplete *β*-ether cleavage by thioacidolysis. Modifications of the analytical procedure did not signicantly improve yields. Since acylation of lignin by *p*-coumarate significantly reduces thioacidolysis yields, alternate methods of *β*-ether cleavage (e.g. NaOH–microwave digestion or acetyl bromide/Zn reductive cleavage) should be used for solvolytic analysis of grass lignins.

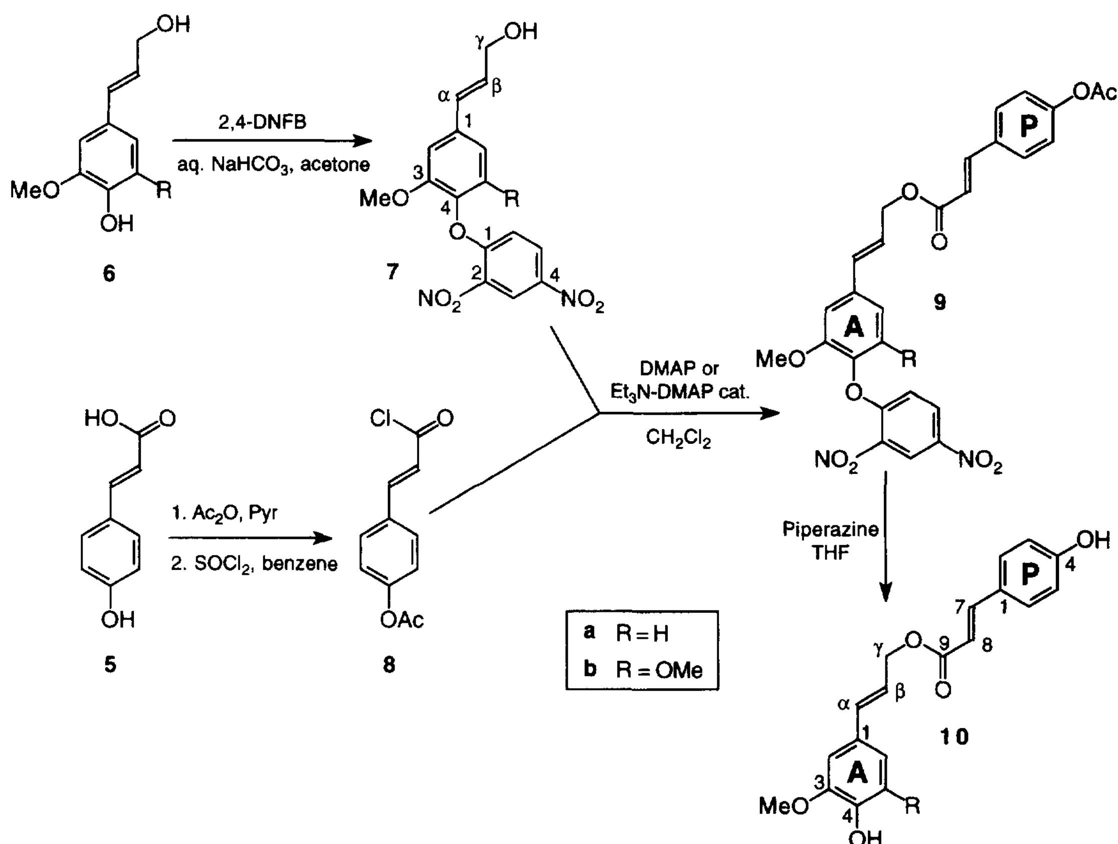

Fig. 4. Synthetic route to *p*-hydroxycinnamyl *p*-coumarates (**10**).

EXPERIMENTAL

General. Mps were measured on an electrothermal digital mp apparatus and were uncorr. Evapns were conducted under red. pres. at temps <42° unless otherwise noted. Further elimination of organic solvents, as well as drying of residues, was accomplished under high vacuum (90–120 mtorr) at room temp. CC was performed on silica gel 60 (230–400 mesh) and TLC was performed with Alugram Sil-G/UV_{254} plates (Macherey-Nagel), with visualization by UV light. NMR spectra of samples in Me_2CO-d_6 (unless otherwise noted) were run at 300 K on a Bruker AMX-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 int. reference ^{1}H, 2.04 ppm; ^{13}C, 29.8 ppm). All assignments were fully authenticated by the normal complement of 1D and 2D inverse-detected experiments. Full data for all title compounds and key intermediates recorded in Me_2CO-d_6, DMSO-d_6 and $CDCl_3$, are given in the recently released NMR database of plant cell wall model compounds [16]. 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. THF was distilled from Na–benzophenone immediately before use. Petrol refers to the boiling range 40–60°.

p-*Hydroxycinnamyl* p-*coumarates* (**10**). Modifications to the methods of Nakamura and Higuchi. [17] allowed simpler syntheses of compounds **10** with better reproducibility (Fig. 4). The synthesis of coniferyl *p*-coumarate (**10a**) using the original 2,4-dinitrophenyl (DNP) protected **5** was recently described [1]. The modification preparation of **10a** *via* the acetate **8** was analogous to that of sinapyl *p*-coumarate (**10b**) described below.

Sinapyl alcohol 2,4-dinitrophenyl ether [*4-(2,4-dinitrophenoxy)-3,5-dimethoxycinnamyl alcohol*] (**7b**). Sinapyl alcohol (**6b**, 352 mg, 1.67 mmol) was dissolved in Me_2CO (2 ml) and cooled to 0°. To this stirred sol was added $NaHCO_3$ (281 mg, 3.34 mmol) in H_2O (5 ml). 2,4-Dinitrofluorobenzene (342 mg, 1.84 mmol) in Me_2CO (2 ml) was then slowly added. The resulting orange soln was stirred in the dark at room temp. for 24 hr. A yellow solid pptd on hydrolysis with cold aq. 3% HCl. The mixt. was stirred for *ca* 2 hr, after which time the yellow ppt. was recovered by filtration and washed with H_2O and Et_2O. Recrystallization from hot Me_2CO afforded **7b** (571 mg, 91%) as fine yellow crystals, mp 146–148° (lit. 153–154°[18]); ^{1}H NMR δ: 3.82 (6H, *s*, A3/5-OMes), 3.96 (1H, *t*, $J = 5.5$ Hz, γ-OH), 4.26 (2H, *td*, $J = 5.3$, 1.6 Hz, γs), 6.50 (1H, *dt*,

J = 15.9, 4.9 Hz, β), 6.64 (1H, *dt*, J = 15.9, 1.6 Hz, α), 6.93 (2H, *s*, A2/6), 7.05 (1H, *d*, J = 9.3 Hz, DNP-6), 8.39 (1H, *dd*, J = 9.3, 2.8 Hz, DNP-5), 8.83 (1H, *d*, J = 2.8 Hz, DNP-3).

Coupling reactions with **9**. Coupling of 4-acetoxycinnamoyl chloride (**8**) [19] with the protected *p*-hydroxycinnamyl alcohols was efficiently carried out using 4-dimethylaminopyridine (DMAP) [20]. The dinitrophenyl ethers (**7a**) [17] (prepd as described for **7b**) or **7b** (1.09 mmol) were dissolved in dry CH_2Cl_2 (5 ml). The soln was cooled in an ice-water bath, and (**8**) (1.33 mmol) and DMAP (179 mg, 1.46 mmol) were successively added. (For large-scale reactions, DMAP was used catalytically and Et_3N added.) The cooling bath was removed and the reaction mixt. stirred for 1 hr, after which time TLC [$CHCl_3$–EtOAc (5:1)] indicated complete conversion into a faster moving material. The soln was diluted with CH_2Cl_2 and washed successfully with cold aq. 3% HCl and aq. NH_4Cl. Drying (Na_2SO_4), evapn and purification by silica gel chromatography [$CHCl_3$–EtOAc (1:1) gave **9a** and **9b** in 92–94% yield after crystallization. Compound **9a** (in aq. Me_2CO) and **9b** (in $CHCl_3$–petrol) crystallized as pale yellow spherulites; **9a**, mp 145–147°; ^{1}H NMR: δ 2.27 (3H, *s*, OAc), 3.82 (3H, *s*, A3-OMe), 4.88 (2H, *dd*, J = 6.2, 1.3 Hz, γs), 6.53 (1H, *dt*, J = 15.9, 6.1 Hz, β), 6.58 (1H, *d*, J = 16.0 Hz, P-8), 6.81 (1H, *dt*, J = 15.9, 1.3 Hz, α), 7.09 (1H, *d*, J = 9.3 Hz, DNP-6), 7.20 (3H, *m*, A-6, P-3/5), 7.28 (1H, *d*, J = 8.2 Hz, A-5), 7.40 (1H, *d*, J = 1.9 Hz, A-2), 7.73 (1H, *d*, J = 16.0 Hz, P-7), 7.74 (2H, *m*, P-2/6), 8.40 (1H, *dd*, J = 9.3, 2.8 Hz, DNP-5), 8.84 (1H, *d*, J = 2.8 Hz, DNP-3); **9b**, mp 177–179°; ^{1}H NMR: δ 2.27 (3H, *s*, OAc), 3.83 (3H, *s*, A3-OMe), 4.88 (2H, *dd*, J = 6.1, 1.3 Hz, γs), 6.56 (1H, *dt*, J = 15.9, 6.1 Hz, β), 6.58 (1H, *d*, J = 16.0 Hz, P-8), 6.79 (1H, *dt*, J = 15.9, 1.3 Hz, α), 7.01 (2H, *s*, A-2/6), 7.06 (1H, *d*, J = 9.3 Hz, DNP-6), 7.20 (2H, *m*, P-3/5), 7.73 (1H, *d*, J = 16.0 Hz, P-7), 7.75 (2H, *m*, P-2/6), 8.38 (1H, *dd*, J = 9.3, 2.8 Hz, DNP-5), 8.83 (1H, *d*, J = 2.8 Hz, DNP-3).

Deprotection. Deprotection was accomplished at room temp. using piperazine in THF, significantly milder conditions than those used in ref. [17]. Compound **9a** or **9b** (0.906 mmol) was dissolved in dry THF (10 ml), and piperazine (780 mg, 9.05 mmol) in dry THF (10 ml) was added dropwise at room temp. under N_2. The soln was stirred for 2 hr, after which time the reaction mixt. was diluted in EtOAc (30 ml) and washed thoroughly with aq. NH_4Cl (*ca* 10 × 15 ml) to ensure complete removal of excess of piperazine and 2,4-dinitrobenzene by-product from the organic layer. After drying ($MgSO_4$) and evap, the resulting syrup was submitted to silica gel chromatography [$CHCl_3$–EtOAc (1:1)] (silica gel was deactivated by treatment with 1% HOAc–EtOH) to afford **10a** and **10b** in 87–90% yield; **10a**, pale yellow solid; ^{1}H NMR: δ 3.86 (3H, *s*, A3-OMe), 4.78 (2H, *dd*, J = 6.5, 1.3 Hz, γ's), 6.25 (1H, *dt*, J = 15.8, 6.5 Hz, β), 6.37 (1H, *d*, J = 16.0 Hz, P-8), 6.65 (1H, *dt*, J = 15.8, 1.3 Hz, α), 6.79 (1H, *d*, J = 8.1 Hz, A-5), 6.88 (2H, *m*, P-3/5), 6.91 (1H, *dd*, J = 8.1, 2.0 Hz, A-6), 7.11 (1H, *d*, J = 2.0 Hz, A-2), 7.54 (2H, *m*, P-2/6), 7.63 (1H, *d*, J = 16.0 Hz, P-7); ^{13}C NMR δ: 56.20 (A3-OMe), 65.49 (γ), 110.20 (A-2), 115.45 (P-8), 115.83 (A-5), 116.68 (P-3/5), 121.19 (A-6), 121.72 (β), 126.90 (P-1), 129.39 (A-1), 130.91 (P-2/6), 134.90 (α), 145.49 (P-7), 147.80 (A-4), 148.53 (A-3), 160.65 (P-4), 167.27 (P-9); **10b**, pale yellow solid; ^{1}H NMR: δ 3.84 (6H, *s*, A3/5-OMes), 4.78 (2H, *dd*, J = 6.5, 1.3 *Hz*, γs), 6.28 (1H, *dt*, J = 15.8, 6.5 Hz, β), 6.38 (1H, *d*, J = 15.9 Hz, P-8), 6.64 (1H, *dt*, J = 15.8, 1.3 Hz, α), 6.79 (2H, *m*, A-2/6), 6.89 (2H, *m*, P-3/5), 7.55 (2H, *m*, P-2/6), 7.63 (1H, *d*, J = 15.9 Hz, P-7); ^{13}C NMR δ: 56.61 (A3/5-OMes), 65.42 (γ), 105.16 (A-2/6), 115.52 (P-8), 116.70 (P-3/5), 122.07 (β), 126.99 (P-1), 128.22 (A-1), 130.95 (P-2/6), 135.14 (α), 137.27 (A-4), 145.49 (P-7), 148.84 (A-3/5), 160.62 (P-4), 167.22 (P-9).

Hydrogenation to authentic compounds **3**. Compounds **3** were prepd from compounds **10** by hydrogenation. For example, **10b** (60 mg, 0.168 mmol) was dissolved in 0.5 ml CH_2Cl_2 and Pd/C (5% Pd on C, 15 mg) was added under H_2. After stirring for 5 hr, the Pd/C was filtered and washed (CH_2Cl_2). Evapn of the solvent left a pale yellow oil which was purified by silica gel chromatography [CH_2Cl_2–MeOH (20:1)] to give pure **3b** in 60% yield as an oil. Compound **3a** was obtained from **10a** according to the same procedure. **3a**, ^{1}H NMR: δ 1.86 (2H, *m*, β), 2.55 (2H, *t*, J = 7.4 Hz, α), 2.56 (2H, *t*, J = 7.6 Hz, P-8), 2.82 (2H, *t*, J = 7.6 Hz, P-7), 3.80 (3H, *s*, OMe), 4.03 (2H, *t*, J = 6.5 Hz, γ), 6.61 (1H, *dd*, J = 8.0, 2.0 Hz, A-6), 6.73 (1H, *d*, J = 7.9 Hz, A-5), 6.75 (2H, *m*, P-3/5), 6.79 (1H, *d*, J = 2.0 Hz, A-2), 7.06 (2H, *m*, P-2/6), 7.26 (1H, *s*, ArOH), 8.10 (1H, *s*, ArOH); ^{13}C NMR δ: 30.75 (P-7), 31.33 (β), 32.24 (α), 36.69 (*P*-8), 56.16 (OMe), 64.03 (γ), 112.73 (A-2), 115.62 (A-5), 116.00 (P3/5), 121.55 (A-6), 130.05 (P-2/6), 132.38 (P-1), 133.55 (A-1), 146.58 (A-4), 148.16 (A-3), 156.57 (P-4), 173.09 (P-9); MS: 330 $[M]^+$ (38), 164 (100), 149 (19), 137 (42), 107 (39); HR MS, found: $[M]^+$, 330.1442, $C_{19}H_{22}O_5$ requires M, 330.1467. **3b**, ^{1}H NMR: δ 1.87 (2H, *m*, β), 2.55 (2H, *t*, J = 7.6 Hz, α), 2.56 (2H, *t*, J = 7.5 Hz, P-8), 2.82 (2H, *t*, J = 7.6 Hz, P-7), 3.79 (6H, *s*, A3/5-OMes), 4.03 (2H, *t*, J = 6.5 Hz, γ), 6.48 (2H, *s*, A-2/6), 6.74 (2H, *m*, P-3/5), 6.89 (1H, *s*, A4-OH), 7.06 (2H, *m*, P-2/6), 8.08 (1H, *s*, P4-OH); ^{13}C NMR δ: 30.77 (P-7), 31.31 (β), 32.71 (α), 36.71 (P-8), 56.57 (OMes), 64.04 (γ), 106.69 (A-2/6), 116.01 (P-3/5), 130.07 (P-2/6), 132.40 (P-1), 132.59 (A-1), 135.07 (A-4), 148.62 (A-3/5), 156.61 (P-4), 173.08 (P-9); MS: 360 $[M]^+$ (92), 194 (100), 179 (20), 168 (45), 167 (76), 163 (43), 107 (69); HR MS, Found: $[M]^+$, 360.1576, $C_{20}H_{24}O_6$ requires M, 360.1573.

β-Ether models. Prepn of guaiacylglycerol and syringylglycerol-β-guaiacyl ethers, and **4a** have been previously reported [20]. Compound **4b** was prepd using the same synthetic methodology [20, 21]. Hydrogenation of **4b** was performed as described for **3b**. Data are deposited in the database [16].

Analytical methods. Thioacidolysis [8, 22] and Raney nickel desulphuration [9] were performed on 10 mg lignin, 40 mg cell walls and 2–5 mg model compounds. Desulphurized products were trimethylsilyated with BSTFA (25 μl) plus pyridine (5 μl) for 30 min at 60° and identified by GC-MS [10]. The relative abundance of **3a** and **3b** recovered after thioacidolysis was determined by GC using a 0.25 mm × 30 m DB-1 (J & W Scientific) column and FID with He as carrier gas (1.0 $cm^3 min^{-1}$). The column was held at 200° for 1 min, ramped at 5° min^{-1} to 300° and then ramped at 10° min^{-1} to 350°. The injector and detector were set at 350°. The injector vol. was 2–4 μl and the split ratio was 50:1. Under these conditions, peaks corresponding to the int. standard (docosane), **3a** and **3b** were observed at 11.4, 22.4 and 24.0 min, respectively.

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Mention of trade name, proprietary product or specific equipment does not constitute a guarantee of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

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