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Identification of the Structure and Origin of Thioacidolysis Marker Compounds for Cinnamyl Alcohol Dehydrogenase Deficiency in Angiosperms*[S]

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Molecular marker compounds, derived from lignin by the thioacidolysis degradative method, for cinnamyl alcohol dehydrogenase (CAD) deficiency in angiosperms have been structurally identified as indene derivatives. They are shown to derive from hydroxycinnamyl aldehydes that have undergone 8–O–4-cross-coupling during lignification. As such, they are valuable markers for ascertaining plant responses to various levels of CAD down-regulation. Their derivation illustrates that hydroxycinnamyl aldehydes incorporate into angiosperm lignins by endwise coupling reactions in much the same way as normal monolignols do, suggesting that the hydroxycinnamyl aldehydes should be considered authentic lignin precursors.

Perturbations of the lignin biosynthetic pathway have the potential to enhance the utilization of plant cell walls in various natural and industrial processes. In forages fed to ruminant animals, for example, lignins inhibit the rumen degradability of potentially digestible polysaccharides (1). In chemical pulping for the production of fine paper, the aim is to selectively remove the lignin from the cellulose fibers. Recently it has become evident that there is potential beyond down-regulating lignification to produce low lignin plants; inducing structural and compositional changes in the polymer may also be beneficial for many processes (2–8).

The definitive way to determine the response of a plant to up- or down-regulation of lignin biosynthetic pathway genes/enzymes and the impact on the lignification process itself is by lignin structural analysis. Molecular marker compounds that allow the degree of change to be elucidated are vital particularly when various levels of down-regulation need to be assessed.

CAD[1] (cinnamyl alcohol dehydrogenase) is the last enzyme on the pathway to the monolignols, primarily coniferyl and sinapyl alcohols **4** (Fig. 1), from which polymeric lignin is derived. One of the most prominent effects of CAD down-regulation is an increased incorporation of hydroxycinnamyl aldehydes **1** into the lignin polymer (7, 9–19). Such an incorporation is logical; the hydroxycinnamyl aldehyde **1** precursors to the hydroxycinnamyl alcohol monolignols **4** are anticipated to build up when the flux to the monolignols is reduced and can be used for polymerization in much the same way as the monolignols themselves (9, 13, 18–20).[2] What was not obvious *a priori* is that these aldehydes, products of incomplete monolignol synthesis, would necessarily be exported to the wall, but that appears to be the case.

In softwoods, in which their (guaiacyl) lignins are overwhelmingly derived from coniferyl alcohol **4G**, coniferyl aldehyde **1G** is not well incorporated (18, 19).[2] This is largely due to simple chemical cross-coupling propensities; coniferyl aldehyde **1G** does not 8–O–4-cross-couple efficiently with guaiacyl units **2G** in the evolving polymer in plants (18). The prime incorporation mechanism then is by dimerization, or by cross-coupling with a coniferyl alcohol monomer (Fig. 1*b*) (13, 19, 22). Coniferyl alcohol **4G** overwhelmingly prefers coupling at its β-position in cross-coupling reactions (23); coniferyl aldehyde **1G** does not favor coupling with guaiacyl moieties **2G** at its analogous 8-position except in pure dimerization reactions.[2] The products of the cross-coupling of coniferyl alcohol and coniferyl aldehyde are therefore β–5- or β–O–4-products **5** and **6** (Fig. 1*b*). The resulting products retain unsaturated cinnamyl aldehyde units in the polymer as end units. Coniferyl aldehyde **1G** might also 5–5-couple with guaiacyl lignin units **2G** (not shown). Coniferyl aldehyde monomers are only poorly incorporated into softwood lignins and degradation products only poorly released by any of the diagnostic degradative methods based on ether cleavage reactions, such as thioacidolysis (24) or derivatization followed by reductive cleavage, the "DFRC" method (25).

The intimate incorporation of hydroxycinnamyl aldehydes into angiosperm lignins is, in contrast, rather facile due to the different chemical cross-coupling propensities of the rad-

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[S] The on-line version of this article (available at http://www.jbc.org) contains full NMR data.

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[1] The abbreviations used are: CAD, cinnamyl alcohol dehydrogenase; GC-MS, gas chromatography-mass spectrometry; NOE, nuclear Overhauser effect.

[2] Kim, H., Ralph, J., Lu, F., Ralph, S. A., Boudet, A.-M., MacKay, J. J., Sederoff, R. R., Ito, T., Kawai, S., and Ohashi, H. (2003) *Org. Biomol. Chem.*, in press.

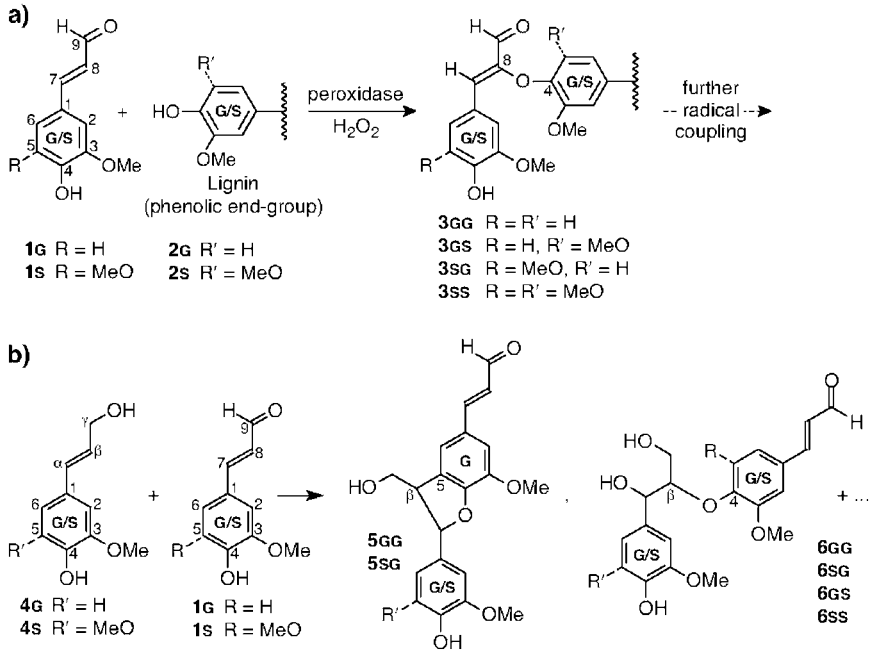

FIG. 1. **Possible modes of incorporation of coniferyl and sinapyl aldehydes 1 into the lignins of CAD-deficient plants.** *a*, the four possible products from 8–O–4-cross-coupling with lignin G and S units **2**; compound **3GG** is not observed *in vivo* in tobacco (18). *b*, possible products from coupling of hydroxycinnamyl alcohols **4** (at their favored β-positions) with hydroxycinnamyl aldehydes **1** (at their 4–O- or 5-positions), resulting in hydroxycinnamyl aldehyde end units (in structures **5** or **6**) in lignins. Both types of ethers, in units **6** and **3**, cleave under thioacidolysis conditions. Compounds are denoted with **G** or **S** descriptors (*e.g.* **2S**); for dimeric compounds, the bottom ring as drawn is the first descriptor (*e.g.* **3SG** is a lignin unit derived from coupling sinapyl aldehyde (at its 8-position) with a guaiacyl unit (at its 4–O-position).

icals involved (14, 18).[2] Sinapyl aldehyde **1S**, the precursor of sinapyl alcohol **4S** in angiosperms, readily 8–O–4-couples with both syringyl and guaiacyl lignin units **2**. Coniferyl aldehyde **1G** also 8–O–4-cross-couples with syringyl units **2S** (but again, not guaiacyl units **2G**). As a result, endwise incorporation of both coniferyl and sinapyl aldehydes **1** into angiosperm lignins is more substantial and, more importantly, occurs in very much the same way as the incorporation of the monolignols **4** themselves. A new hydroxycinnamyl aldehyde monomer (radical) cross-couples with the growing lignin oligomer/polymer **2** (radical), at a phenolic guaiacyl or syringyl end unit. Subsequently, a new monomer (radical), *e.g.* a normal monolignol **4**, may cross-couple with the new phenolic end unit (radical) resulting from the prior coupling reaction, and the hydroxycinnamyl aldehyde unit becomes etherified and intimately incorporated into the lignin. The only difference is that in "normal" lignification, the monomers are comprised largely of the normal monolignols **4**, coniferyl and sinapyl alcohols, whereas in CAD-deficient plants, the monomers additionally include the hydroxycinnamyl aldehydes **1**, coniferyl and sinapyl aldehydes.

Hydroxycinnamyl aldehydes either involved in 8–O–4-linkages or incorporated as 4–O–β-end groups will release monomers following ether cleaving reactions in thioacidolysis, which therefore serve as marker compounds for hydroxycinnamyl aldehyde incorporation. However, there are invariably hydroxycinnamyl aldehyde end-groups in normal lignins (26), in structures **6**, so their products are not *diagnostic* for CAD deficiency, and they do not qualify as molecular maker compounds for CAD deficiency.

Two new isomeric syringyl monomers have been detected following thioacidolysis of a range of CAD-deficient materials during extensive studies at the Institut National de la Recherche Agronomique in Paris-Grignon, France (27). The characteristic peaks, labeled **7S** and **8S** in Fig. 2, were suspected as products derived from the incorporation of sinapyl aldehyde into the transgenics' lignins. Here we detail the identification of thioacidolysis CAD marker compounds and provide evidence that 8–O–4-coupled hydroxycinnamyl aldehyde units **3** in lignin are their source. We also demonstrate how a common aldehyde staining method may fail to reveal the substantial incorporation of aldehydes into angiosperm lignins, thus emphasizing the importance of more diagnostic analytical methods.

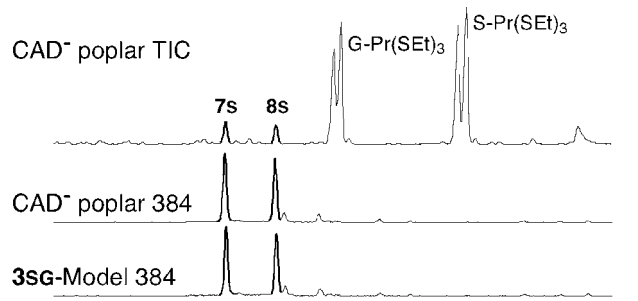

FIG. 2. **GC-MS traces showing the two (silylated) thioacidolysis CAD markers in transgenic poplar (total-ion chromatogram and selected-ion (*m/z* 384) chromatogram, and the analogous selected-ion chromatogram from thioacidolysis of an 8–O–4-model compound 3SG.**

MATERIALS AND METHODS

General

All chemicals were purchased from Aldrich unless otherwise noted. The Raney nickel preparation was Aldrich's 50-μm pore size, 80–100 m^2/g surface area, 50% slurry in water. The products of thioacidolysis from lignins and hydroxycinnamyl aldehyde 8–O–4-model compounds **3** were examined by GC-MS. GC (Thermoquest Trace GC 2000) conditions were as follows: DB1 column (25 m × 0.2 mm, 33-μm film thickness, J & W Scientific) was used; initial column temperature, 200 °C, held for 1 min, first ramped at rate of 4 °C/min to 248 °C, second ramped at rate of 30 °C/min to 300 °C for 25 min; inlet temperature was 250 °C. MS (Thermoquest GCQ/Polaris MS) conditions were as follows: ion source temperature, 220 °C, transfer line temperature, 300 °C. UV spectra were recorded on DU-50 Series Spectrophotometers (Beckman) at wavelengths between 400 and 700 nm.

NMR Spectroscopy

NMR spectra were acquired on a Bruker DRX-360 instrument fitted with a 5-mm ^{1}H/broadband gradient probe with inverse geometry (proton coils closest to the sample). Compounds (5–60 mg) were dissolved in 0.4 ml of acetone-d_6 (unless otherwise noted); the central acetone solvent peak was used as internal reference (δ_C 29.80, δ_H 2.04). We used the standard Bruker implementations of the traditional suite of one-dimensional and two-dimensional (gradient-selected, ^{1}H-detected, *e.g.* COSY, TOCSY, HMQC, or HSQC, HMQC-TOCSY, HMBC) NMR ex-

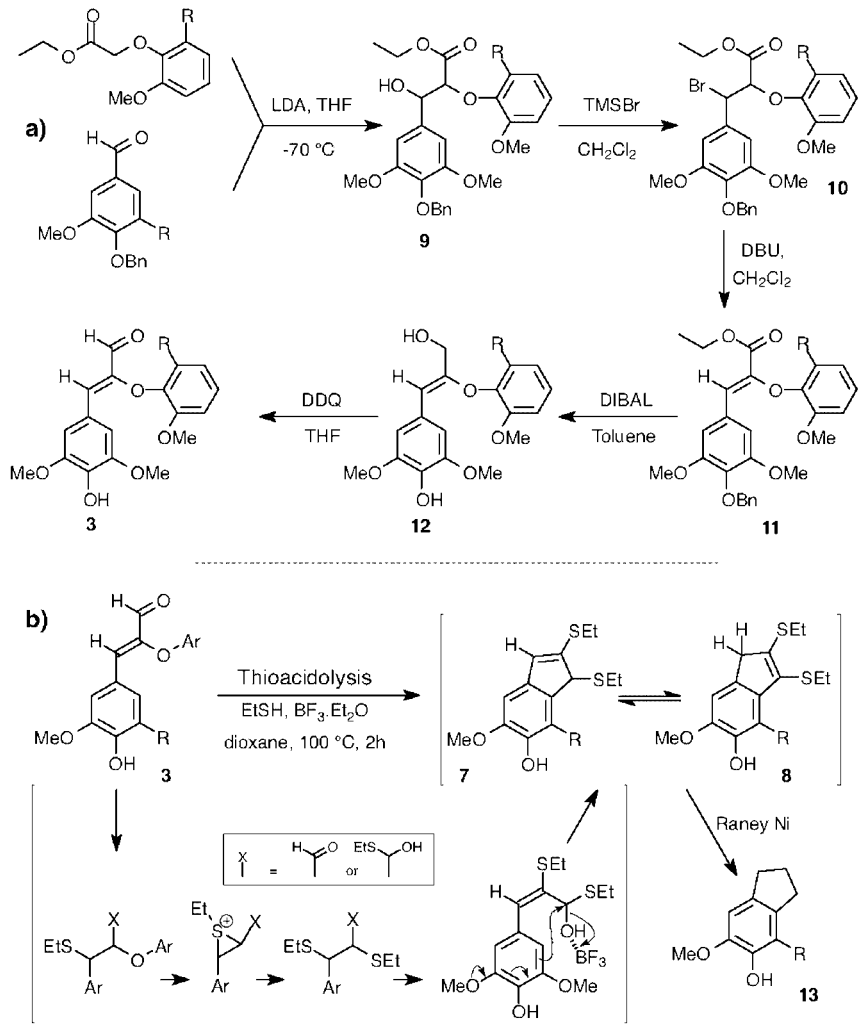

FIG. 3. **Synthetic scheme for hydroxycinnamyl aldehyde 8–O–4-guaiacyl/syringyl ether model compounds 3 (*a*) and their thioacidolysis reactions (*b*).** Note: thioacidolysis of etherified structures in lignin will cleave and release the free phenol at some point during the thioacidolysis process. The exact order of the various thioethylation and aromatic electrophilic addition reactions is unknown; thus –*X* can be $–CH(SEt)_2$ or –CHO throughout.

periments for structural elucidation and assignment authentication. The fully authenticated NMR data are also deposited in the "NMR data base of lignin and cell wall model compounds" available via the internet at www.dfrc.ars.usda.gov/software.html (28). Experimental data given here list the data base compound number (*e.g.* 3047). NOESY spectra were acquired with a 1.5-s mixing time (τ_m); TOCSY spectra used a 100-ms mixing time; HMBC spectra used 100-ms long range coupling delays.

Plant Materials and Lignin Isolation

Preparation of CAD-down-regulated transgenic poplars and isolation of the lignin were described previously (29). Using antisense and sense gene construction techniques (30), greenhouse-grown or field-grown poplars were produced. Main stems were harvested, and lignins were isolated.

Synthesis of Hydroxycinnamyl Aldehyde Cross-coupled 8–O–4-dimers

Four different model compounds **3**, representing the four cross-coupling possibilities of coniferyl and sinapyl aldehydes with guaiacyl and syringyl units in lignin, were synthesized as in Fig. 3. The following procedure for model **3SG** is illustrative. Full NMR data are provided as Supplementary Material and are deposited in our NMR data base (28).

Ethyl 2-Methoxyphenoxy Acetate—Ethyl 2-methoxyphenoxy acetate was prepared from guaiacol and ethyl chloroacetate as described previously (31).

Syringyl Aldehyde Benzyl Ether—Syringyl aldehyde (10 g, 54.9 mmol) was dissolved in dioxane (300 ml). Benzyl bromide (11.27 g, 65.48 mmol) was added, followed by anhydrous K_2CO_3 (15.2 g, 109.9 mmol). The mixture was refluxed overnight in an oil bath. After checking for reaction completion by TLC ($CHCl_3$-EtOAc, 10:1), the inorganic salts were filtered off. The filtrate was diluted with water (300 ml), 1 N NaOH (5 ml) was added to partition any non-benzylated phenols, and the solution extracted with EtOAc (3 × 100 ml), which was washed with saturated aqueous NaCl, dried over $MgSO_4$, and concentrated on a rotary evaporator. The red oil was subjected to flash column ($CHCl_3$-EtOAc, 10:1), and syringaldehyde benzyl ether was collected as a light yellow oil (7.8 g, 28.5 mmol, 52%).

*β-Hydroxy Ester **9SG***—Syringaldehyde benzyl ether and ethyl 2-methoxyphenoxy acetate were dried under vacuum overnight. All glassware and syringes were oven-dried at 50 °C for several days. Lithium diisopropylamide (11.33 ml, 17 mmol) was stirred in a dried round-bottom flask at −70 °C and kept 10 min. Ethyl 2-methoxyphenoxy acetate (2.39 g, 11 mmol) was dissolved in anhydrous tetrahydrofuran (15 ml) and added dropwise over 20 min to the lithium diisopropylamide solution. The mixture was stirred for 30 min. Syringaldehyde benzyl ether (3.11 g, 11 mmol) was dissolved in anhydrous tetrahydrofuran (15 ml) and added dropwise to the reaction mixture, which gradually became pale yellow. The stirring was continued for 30 min at −70 °C and the flask allowed to warm to room temperature over a period of 1 h. The reaction mixture was quenched by the addition of saturated aqueous NH_4Cl. The solution was extracted with EtOAc, washed with saturated aqueous NaCl, and dried over $MgSO_4$. The concentrated yellow oil was purified by flash chromatography ($CHCl_3$-EtOAc, 10:1). The β-hydroxy ester **9SG** was obtained as a mixture of *erythro- and threo*-isomers; 4.1 g, 9 mmol, 81%) and was confirmed by NMR. The isomers were separated by preparative TLC (cyclohexane:EtOAc:HOAc 100:50:1) and each isomer crystallized from EtOH/H_2O. For NMR data, see Supplementary Material.

*3-(4-Benzyloxy-3,5-dimethoxyphenyl)-2-(2-methoxyphenoxy)acrylic Acid Ethyl Ester **11SG***—Trimethylsilyl bromide (1.28 ml, 9.7 mmol) was added to a stirred solution of β-hydroxy ester **9SG** (*erythro:threo* mixture; 2.34 g, 4.85 mmol) in CH_2Cl_2 (30 ml). After the mixture had been stirred for 2 h, diazabicycloundecene (1.45 ml, 9.7 mmol) was added. The mixture was stirred overnight. The solution was diluted in EtOAc, washed with saturated aqueous NaCl, and dried over $MgSO_4$. The

concentrated red oil was purified by flash column ($CHCl_3$-EtOAc, 10:1) to give a red solid **11SG** (1.26 g, 2.71 mmol, 56%).

*4-[3-Hydroxy-2-(2-methoxyphenoxy)-propenyl]-2,6-dimethoxy-phenol **12SG***—Compound **11SG** (320 mg, 0.69 mmol) in toluene (30 ml), under nitrogen, was cooled in an ice water bath. Diisobutylaluminum hydride (Aldrich, 1.62 ml of 1.5 M solution, 2.42 mmol, 3.5 eq.) in toluene was slowly added via syringe over 10 min. The stirring was continued for 2 h. The reaction mixture was then carefully quenched with ethanol (5 ml). Water (50 ml) was added. The aqueous layer was extracted with ethyl acetate (4 × 200 ml). The extracted layer was dried over $MgSO_4$ and evaporated to give crude red oil. The mixture was separated on TLC ($CHCl_3$-EtOAc, 1:1) to give pure **12SG** as a yellow oily product (144 mg, 0.43 mmol, 63%). For NMR data, see Supplementary Material.

*3-[4-Hydroxy-3,5-dimethoxyphenyl-2-(2-methoxyphenoxy)]-propenal **3SG***—Compound **12SG** (144 mg, 0.43 mmol) was dissolved in tetrahydrofuran (20 ml). 2,3-Dichloro-5,6-dicyano-benzoquinone (DDQ; 50 mg, 0.22 mmol) was added and stirred overnight. The resulting yellow solution products were dried on an evaporator and separated from DDQ and the trace of starting material by TLC ($CHCl_3$-EtOAc, 1:1). Compound **3SG** (84 mg, 0.25 mmol, 59%) was obtained as pale yellow solid after drying. For NMR data, see Supplementary Material.

Thioacidolysis of Models **3**

Thioacidolysis was performed on two representative model compounds **3SG** and **3GS**; the major products, two isomeric indenes, **7** and **8** (Fig. 3), were isolated and their structures deduced from MS and NMR. Raney nickel desulfurization of the thioacidolysis products afforded the indane **13**.

*2,3-Bis-ethylsulfanyl-4,6-dimethoxy-3H-inden-5-ol **7S** and 2,3-bis-ethylsulfanyl-4,6-dimethoxy-1H-inden-5-ol **8S***—Compound **3SG** (30 mg, 0.091 mmol) was dried overnight, dissolved in 6 ml of dioxane, and transferred into a 9-ml screw-top vial. Ethanethiol (0.9 ml) and BF_3 etherate (0.225 ml) were added. The total volume was adjusted to 9 ml with dioxane. The vial was capped tightly with a Teflon-lined screwcap and put into an oil bath at 100 °C. The reaction was allowed to proceed for 4 h. The reaction tube was cooled in ice water. The vial was washed out with CH_2Cl_2 and water several times and all contents transferred to a separatory funnel. The pH of the aqueous phase was adjusted to 3–4 by addition of 0.4 M $NaHCO_3$, extracted with CH_2Cl_2 (3 × 30 ml), and dried over $MgSO_4$. The solution was concentrated on a rotary evaporator that was connected to bleach (sodium hypochlorite) trap. The final products (28 mg, 0.091 mmol) were separated by TLC ($CHCl_3$-EtOAc, 10:1). The two major products, **7S** and **8S**, were identified by NMR and GC-MS.

Compound **7S**: δ_H 1.00 (3H, t, J = 7.5, 9-S-CH_2-C*H*3), 1.33 (3H, t, J = 7.4 Hz, 8-S-CH_2-C*H*$_3$), 2.22(1H, dq, J = 12.1, 7.5 Hz, 9a-S-C*H*$_2$-CH_3), 2.04 (1H, dq, J = 12.1, 7.5 Hz, 9b-S-C*H*$_2$-CH_3), 2.94 (2H, q, J = 7.4 Hz, 8-S-C*H*$_2$-CH_3), 3.82 (3H, s, 5-OMe), 3.96 (3H, s, 3-OMe), 4.39 (1H, d, J = 1.2 Hz, 9), 6.38 (1H, d, J = 1.2 Hz, 7), 6.63 (1H, s, 6); δ_C 14.4 (9-S-CH_2*C*H$_3$), 14.5 (8-S-CH_2*C*H$_3$), 22.2 (9-S-*C*H$_2$CH$_3$), 26.8 (8-S-*C*H$_2$CH$_3$), 52.6 (9), 56.7 (5-OMe), 60.1 (3-OMe), 100.1 (6), 124.9 (7), 128.9 (2), 135.8 (1), 137.7 (4), 45.1 (8), 145.2 (3), 149.5 (5); MS 312 (M^+, 62), 251 (100), 223 (32), 218 (12). Compound trimethylsilyl-**7S**: MS 384 (M^+, 55), 354 (5), 323 (100), 295 (19), 293 (17).

Compound **8S**: δ_H 1.15 (3H, t, J = 7.4 Hz, 9-S-CH_2-C*H*$_3$), 1.30 (3H, t, J = 7.4 Hz, 8-S-CH_2-C*H*$_3$), 2.89 (2H, q, J = 7.37 Hz, 9-S-C*H*$_2$-CH_3), 2.95 (2H, q, J = 7.37 Hz, 8-S-C*H*$_2$-CH_3), 3.58 (2H, d, J = 0.9 Hz, 7), 3.82 (3H, s, 5-OMe), 3.85 (3H, s, 3-OMe), 6.87 (1H, t, J = 0.9 Hz, 6); δ_C 15.1 (9-S-CH_2*C*H$_3$), 15.4 (8-S-*C*H$_2$CH$_3$), 26.3 (8-S-*C*H$_2$CH$_3$), 29.1 (9-S-*C*H$_2$CH$_3$), 41.7 (7), 56.9 (5-OMe), 62.3 (3-OMe), 105.0 (6), 126.9 (9), 131.5 (2), 133.8 (1), 104.0 (3), 141.6 (4), 147.1 (5), 148.4 (8); MS 312 (M^+, 77), 283 (11), 251 (100), 236 (8), 223 (6), 218 (30). Compound trimethylsilyl-**8S**: MS 384 (M^+, 64), 355 (17), 323 (100), 293 (27), 290 (30), 260 (18).

*2,3-Bis-ethylsulfanyl-6-methoxy-1H-inden-5-ol **8G***—Thioacidolysis of compound **3GS** (39 mg, 0.091 mmol) was performed as for compound **3SG**. The final product (20 mg, 0.071 mmol) was separated by TLC (petroleum ether/EtOAc, 7:3). The major product **8G** was identified by NMR and GC-MS.

Compound **8G**: δ_H 1.67 (3H, t, J = 7.4 Hz, 9-S-CH_2-C*H*$_3$), 1.31 (3H, t, J = 7.4 Hz, 8-S-CH_2-C*H*$_3$), 2.81 (2H, q, J = 7.4 Hz, 9-S-C*H*$_2$-CH_3), 2.99 (2H, q, J = 7.4 Hz, 8-S-C*H*$_2$-CH_3), 3.58 (2H, d, J = 0.8 Hz, 7), 3.83 (3H, s, 5-OMe), 6.87 (1H, s, 3), 7.06 (1H, t, J = 0.8 Hz, 6); δ_C 15.5 (8-S-CH_2*C*H$_3$), 15.7 (9-S-CH_2*C*H$_3$), 26.5 (8-S-*C*H$_2$CH$_3$), 27.7 (9-S-*C*H$_2$CH$_3$), 41.6 (7), 56.8 (5-OMe), 106.4 (3), 109.0 (6), 128.6 (9), 133.3 (1), 140.1 (2), 146.1 (5), 146.9 (4), 148.2 (8). Compound trimethylsilyl-**8G**: MS 354 (M^+, 100), 325 (18), 293 (76), 260 (35), 230 (12).

*4,6-Dimethoxy-indan-5-ol **13S***—The mixture of compounds **7S** and **8S** (6 mg, 0.019 mmol) was dissolved in MeOH (5 ml) and stirred. Raney nickel was added as an aqueous slurry (2 ml), and the mixture was stirred overnight at room temperature. The pH was adjusted to 3–4 by addition of 3% HCl. The solution was filtered through a glass filter to remove the Raney nickel, and the filtrate was dried over $MgSO_4$. The final product (3 mg, 0.016 mmol, 81%) was purified by TLC ($CHCl_3$-EtOAc, 10:1).

Compound **13S**: δ_H 1.98 (2H, quint, J = 7.2 Hz, 8), 2.78 (2H, br t, J = 7.2 Hz, 7), 2.83 (2H, br t, J = 7.2 Hz, 9), 3.77 (3H, s, 5-OMe), 3.79 (3H, s, 3-OMe), 6.58 (1H, s, 6), δ_H (benzene-d_6): 1.86 (2H, quin, J = 7.4 Hz, 8), 2.71 (2H, td, J = 7.4, 0.7 Hz, 7), 2.86 (2H, t, J = 7.4 Hz, 9), 3.28 (3H, s, 5-OMe), 3.75 (3H, s, 3-OMe), 6.32 (1H, s, 6); δ_C 26.1 (8), 30.3 (9), 33.5 (7), 56.7 (5-OMe), 59.8 (3-OMe), 104.2 (6), 128.6 (2), 134.9 (1), 138.2 (4), 144.7 (3), 148.5 (5).

*6-Methoxy-indan-5-ol **13G***—Desulfurization of the compound **8G** (7 mg, 0.025 mmol) was same as for compounds **7S** and **8S**. The final product (1.5 mg, 0.009 mmol, 37%) was purified by TLC (petroleum ether/EtOAc, 7:3).

Compound **13G**: δ_H 1.99 (2H, quint, J = 7.6 Hz, 8), 2.74 (2H, t, J = 7.6 Hz, 7), 2.76 (2H, t, J = 7.6 Hz, 9), 3.78 (3H, s, 5-OMe), 6.66 (1H, s, 3), 6.78 (1H, s, 6); δ_C 26.5 (8), 33.1 (7), 33.3 (9), 56.5 (5-OMe), 108.7 (6), 111.6 (3), 135.0 (1), 136.7 (2), 146.1 (4), 147.0 (5).

Phloroglucinol-HCl Staining of Hydroxycinnamaldehyde 8–O–4-ether Units

Vanillin, coniferyl aldehyde, sinapyl aldehyde, and two different hydroxycinnamyl aldehyde-8–O–4-guaiacyl/syringyl model compounds, **3GS** and **3SG**, were selected to perform phloroglucinol-HCl staining reactions. All reactants were prepared at the same concentration (3.33 μM) in MeOH. Phloroglucinol (25 mg) was fully dissolved in MeOH (25 ml), and 25 ml of 37% HCl was added. Two drops of freshly prepared phloroglucinol HCl reagent was added 1 min before beginning UV-visible spectral acquisition (400–700 nm).

RESULTS

Model compounds for hydroxycinnamyl aldehyde 8–O–4-units **3**, *e.g.* **3SG** (Figs. 1 and 3) were synthesized following Nakatsubo's method (31) to compounds **9** (Fig. 3). Elimination to the unsaturated 8–O–4-ester **11** was via bromination with TMSBr (32) and HBr elimination using the strong non-nucleophilic base diazabicycloundecene, as previously used in for 8–O–4-coupled diferulate (33). Reduction of the ester to the alcohol **12** (without double-bond reduction) was via DIBAL (34). Finally DDQ oxidation (35) produced the required unsaturated aldehyde unit and model compounds **3** for thioacidolysis.

Recently, two unknown peaks in GC spectra after thioacidolysis of CAD-deficient angiosperms were detected as described in the introduction (Fig. 2). The compound levels varied systematically with the amount of CAD down-regulation (27); the importance of the new markers (**7S** + **8S**), relative to the conventional syringyl monomers (S–CHSEt–CHSEt–CH_2SEt), was found to increase concomitantly with the level of CAD deficiency. This signature was observed even before any wood phenotype (red coloration of the xylem) could be seen or before any other lignin structural alteration could be detected. These isomers had a nominal M_r of 312 and an apparent formula $C_{15}H_{20}O_3S_2$. Raney nickel desulfurization (36, 37) generated a single new monomer with nominal M_r of 194 and formula $C_{11}H_{14}O_3$. The latter was found by GC-MS to be not the commercially available syringyl prop-1-ene **14**, nor the pro-2-en **15**, Fig. 4, that could be prepared by Rh(III)-assisted isomerization (38), both of which had different GC retention times. Syringyl cyclopropane **16** appeared to be a possibility particularly since DFRC degradation of cinnamaldehydes produced cyclopropanes (39). However, synthesized **16** also had a GC retention time which differed from the thioacidolysis/Raney nickel product. The only possibility remaining (while retaining the syringyl moiety) was the indane **13**. However, its authentication was required and the exact structures of the precursor thiol

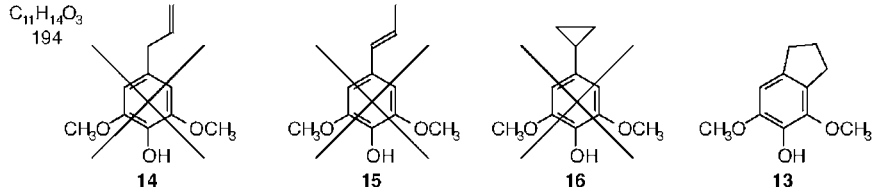

FIG. 4. **Possible structures for the MW 194 (silylated: 266) marker compound from thioacidolysis followed by Raney nickel desulfurization.** The crossed-out structures were eliminated by comparison with the authentic compounds. The marker structure **13** was elucidated by NMR.

products also remained unresolved. Rather than synthesize suspected compounds independently, we sought to elucidate the source of the thioacidolysis products using model compounds and to prepare sufficient products for isolation and full structural characterization, along with establishing their identity to the thioacidolysis products.

Thioacidolysis of the sinapyl aldehyde 8–O–4-dimer model compound **3sG** (Fig. 3) did indeed cleanly give the same two isomeric compounds as thioacidolysis of CAD-deficient angiosperms, in approximately equal amounts (Fig. 2). The isomers were separated by preparative TLC ($CHCl_3$-EtOAc, 10:1). The structural similarity of the two isomers, which had very close R_f values, however, impeded separation and only partially separated fractions were available. With almost identical mass spectra (Fig. 5), the structures could only be reliably elucidated by NMR. Each separated fraction, which was contaminated by the other isomer, was examined. Major compounds in each fraction were recognized by discrimination of the peaks in ^1H and ^{13}C NMR. Proton NMR showed that one of the isomers (the faster moving isomer labeled **7s**) had two aromatic or double-bond protons and one aliphatic proton (presumably with a thioethyl group on the same carbon). One peak in the aromatic region was a doublet and had the same coupling constant (1.05 Hz) as an aliphatic proton. The remaining peak in the aromatic region was a singlet. All three proton peaks had the same integral. The slower moving isomer labeled **8s** had only two diagnostic peaks: one aromatic (broad triplet) and one aliphatic (doublet; $J = 0.9$ Hz) proton peak. The integration showed one proton for the narrow triplet and two protons for the doublet. Full structural elucidation of compounds **7s** and **8s** by the usual complement of one-dimensional and two-dimensional NMR methods revealed them to be indene isomers related by a 1,3-sigmatropic proton shift by which they presumably equilibrate under the thioacidolysis conditions. These structures are logical from the anticipated thioacidolysis mechanisms (Fig. 3), at least in hindsight. To obtain concrete evidence for the structural assignment, NOESY experiments were performed; correlations are summarized in the usual way in Fig. 6.

For the isomer **7s**, only proton H6 (s, 6.63 ppm) on the aromatic ring exhibited NOE with H7 (d, 6.38 ppm), which was on the double bond. H7 showed a correlation with H_2C (H12, q, 2.94 ppm), which was on one of the thioethyl groups. H6 did not show a correlation with the thioethyl group. This series of NOE effects clearly ruled out other possible structures (Fig. 6, **i–iii**). The coupling constant (1.05 Hz) between H7 and H9 (d, 4.39 ppm) was also reasonable for the allylic long range (four-bond) coupling. The other isomer **8s** was much easier to identify, because two protons H7 (d, 3.58 ppm) were on the same carbon and coupled with H6 (narrow triplet, 6.87 ppm). The NOESY spectrum showed NOEs between H6-H7, H7-H12 (q, 2.95 ppm), and H7-H13 (t, 1.3 ppm) ruling out the regioisomer **iv**.

Raney nickel desulfurization of either (or a mixture) of the two isomers **7s/8s** gave the indane **13s** (Fig. 3*b*). This product had the same mass spectrum and co-chromatographed with the indane produced from thioacidolysis followed by Raney nickel desulfurization of CAD-deficient poplar samples. The structure was validated by conventional one-dimensioal and two-dimensional NMR experiments.

Coniferyl aldehyde 8–O–4-dimers gave mainly (>95%) one isomer of an analogous guaiacyl product (Fig. 5*d*). Structural elucidation showed it to be **8G**. The regiochemistry of the methoxyl (see Fig. 6) was readily established by observing the singlet aromatic proton (H3) at 6.87 ppm and a narrow triplet (H6, $J = 0.8$ Hz) at 7.06 ppm, which is coupled to the two 7-protons (Fig. 6); protons 5 and 6 would have been coupled 8 Hz doublets if it was the other regioisomer (with the methoxyl at carbon 3). A NOESY experiment showed an analogous series of NOEs as discussed for compound **8s**. An additional NOE correlation between H3 (s, 6.87 ppm) and H10 (q, 2.81 ppm) was also apparent. Small amounts of **7G** were detected by GC-MS along with the major isomer **8G** (Fig. 5*d*), but this isomer was not fully authenticated by NMR. The dominance of **8G** over **7G** appears to be explained by simple thermodynamics. Molecular modeling (MacSpartan *Pro*), using MMFF94 (Merck Pharmaceuticals) molecular mechanics calculations, indicates that isomer **8G** has a much lower energy (180 kJ/mol) than **7G** (364 kJ/mol). By comparison, the syringyl analogs **7S** (255 kJ/mol) and **8S** (297 kJ/mol), with more similar predicted energies, were present in nearly equal quantities, as seen by GC-MS analysis.

From the CAD-deficient poplars, the same guaiacyl isomer **8G** was found at low levels, together with the product G–CHSEt–CH_2–$CH(SEt)_2$ derived from coniferyl aldehyde end groups in structures **6** (Fig. 1) (27). The levels suggest that down-regulating CAD activity in poplar more specifically causes the incorporation of sinapyl aldehyde into the lignins.

DISCUSSION

Thioacidolysis Marker Compounds—Many research groups (10, 24, 40, 41) have reported low yields of monomers derived from aldehydes by thioacidolysis and hypothesized that aldehydes in lignin may be inefficiently released by thioacidolysis because they are incorporated into carbon-carbon (C–C) linked structures in lignin (11, 15, 42). However, NMR studies of tobacco CAD-down-regulated transgenic lignin revealed considerable amounts of 8–O–4-coupled hydroxycinnamyl aldehyde structures (14, 18, 19); a pine mutant lignin contained smaller amounts (19, 43).

The incorporation of sinapyl and coniferyl aldehydes into the lignins of CAD-deficient poplars (and other plants) suggested that the new thioacidolysis products resulted from a sinapyl aldehyde moiety incorporated into lignin. Since thioacidolysis rather specifically cleaves ether linkages, the only likely source of the compounds was β–O–4-units **6** or 8–O–4-units **3**. However, we already know that products R–CHSEt–CH_2–$CH(SEt)_2$ result from coniferyl and sinapyl aldehydes **1** and from their 4–O-ethers **6** (representing end units in lignin) (44). The likely source of the new compounds was therefore logically from the hydroxycinnamyl aldehydes more intimately incorporated into the lignin, the 8–O–4-coupled hydroxycinnamyl aldehyde units **3**. These units have been established in CAD-deficient angiosperms by NMR methods (14, 18, 19).[2]

Radical coupling of an hydroxycinnamyl aldehyde at its 8-po-

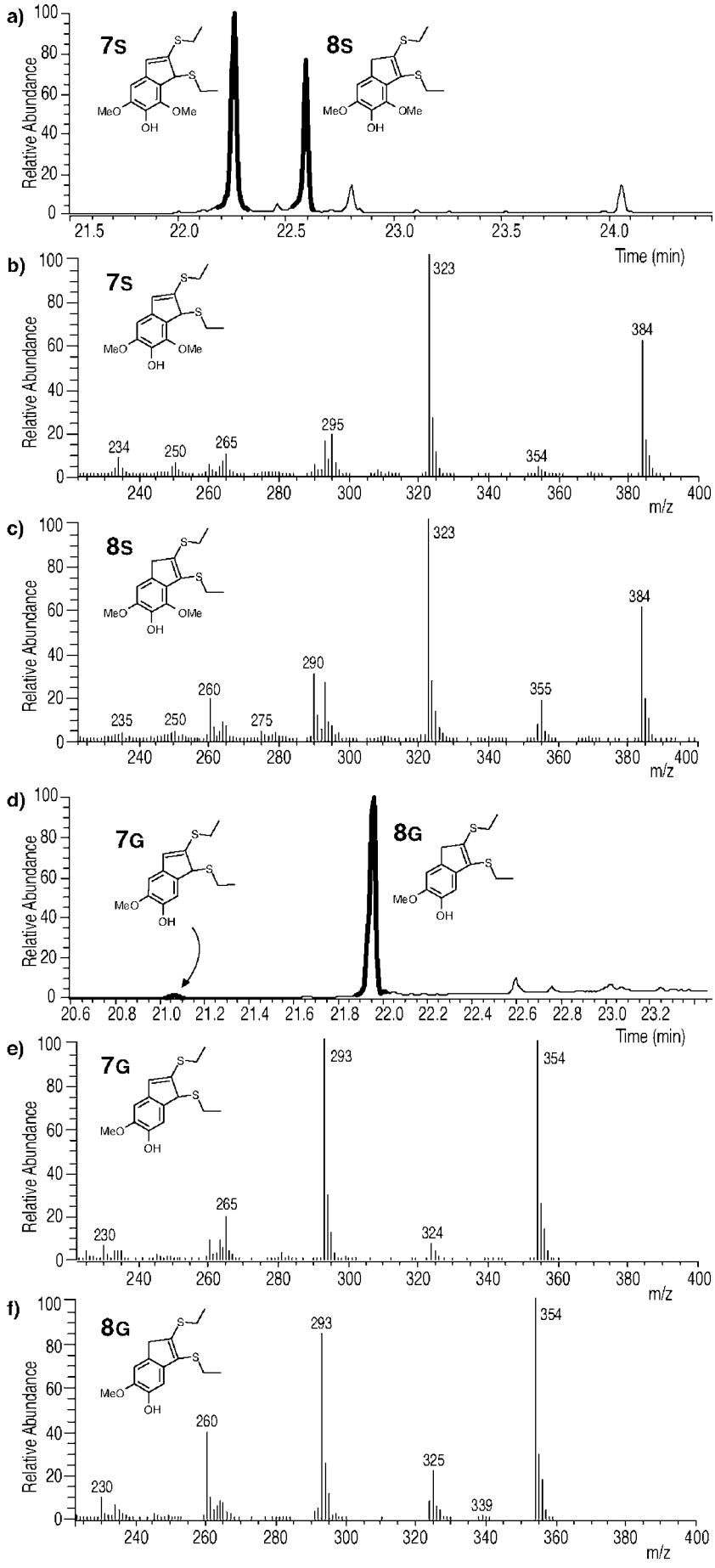

FIG. 5. **GC-MS total-ion chromatograms of thioacidolysis products from model 3SG (*a*) and model 3GS (*d*).** MS spectra of the isomeric pairs (*b*, *c* and *e*, *f*) are not sufficiently diagnostic, so NMR was required to deduce the exact structures shown.

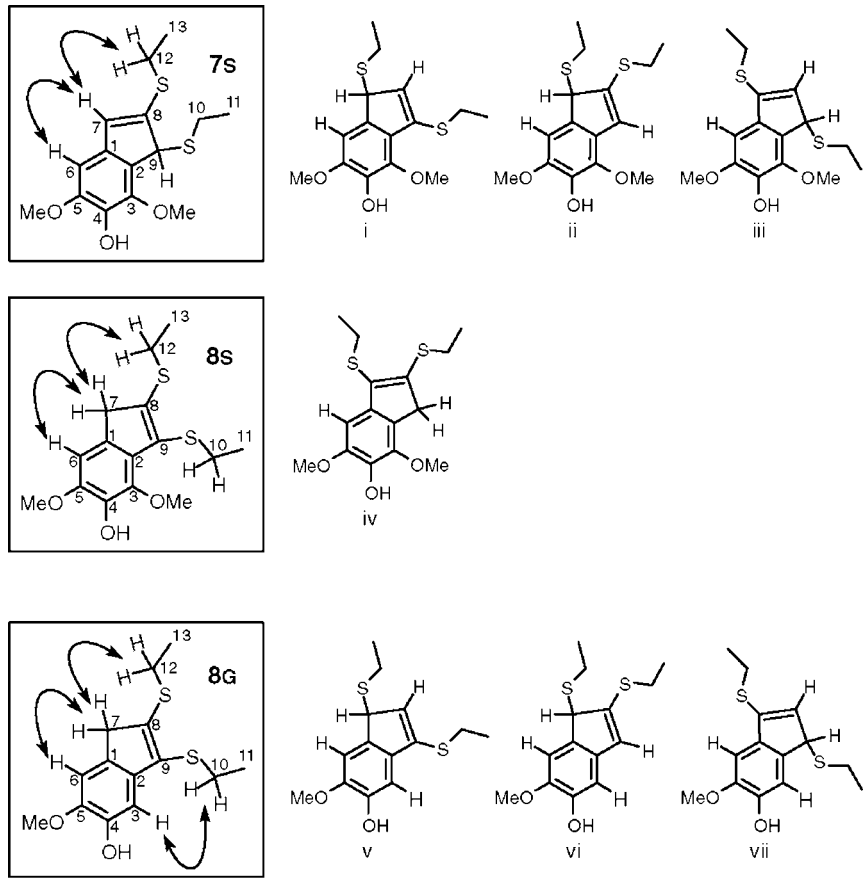

FIG. 6. **Possible structures for the marker compounds 7 and 8 showing NOESY correlations on the deduced structures (*boxed*).**

sition results initially in a quinone methide. Unlike in the *β*–O–4-coupling of hydroxycinnamyl alcohols, where re-aromatization is by nucleophilic addition of water to the quinone methide, the hydroxycinnamyl aldehyde quinone methide 8–O–4-coupling product re-aromatizes by elimination of the acidic 8-proton to give the unsaturated 8–O–4-product; this elimination was observed in the dimerization reactions of coniferyl aldehyde (20, 45)[2] and has recently been established for both coniferyl and sinapyl aldehydes in lignification as well (18).[2] Model compounds **3** (Fig. 1) were therefore chosen as thioacidolysis substrates expected to liberate the observed markers.

Thioacidolysis of compounds **3** did indeed give the same marker compounds as those derived from CAD-deficient angiosperms. Detailed NMR analysis reveals that they are indene derivatives **7s** and **8s**, isomers related by a 1,3-sigmatropic shift. Raney nickel desulfurization produced the indane **13s**, with its structure established by NMR, co-chromatographing with the CAD-deficient angiosperm product, and having the same mass spectrum. We have therefore not only deduced the structures of the marker compounds, but also demonstrated their derivation from hydroxycinnamyl aldehyde 8–O–4-structures **3**, *i.e.* from hydroxycinnamyl aldehydes that have undergone endwise polymerization into the growing lignin polymer.

Phloroglucinol-HCl Staining of Hydroxycinnamyl Aldehyde 8–O–4-Ether Units—Phloroglucinol staining has long been utilized for localizing lignins in plant tissue sections (46). It stains lignins a deep mauve color that was recognized to derive from reactions primarily of hydroxycinnamyl aldehyde endgroups in lignins. The origin of such groups was not clear at the time (47), as succinctly discussed recently (48); they were assumed to result from post-lignification oxidation reactions. Much of the work on CAD-down-regulated transgenics and deficient mutants, however, now suggests that hydroxycinnamyl aldehyde

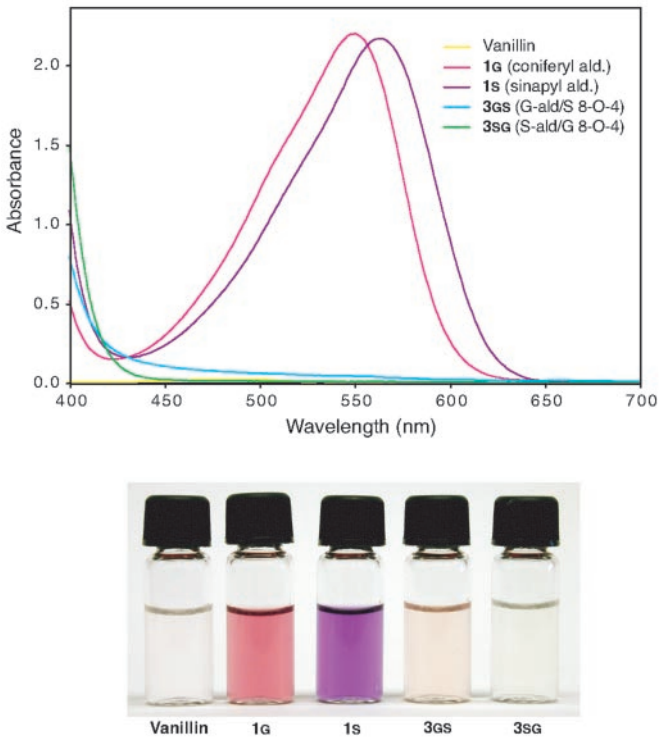

FIG. 7. **Picture of freshly prepared phloroglucinol-HCl-treated solutions of the hydroxybenzyl aldehyde vanillin, hydroxycinnamyl aldehydes 1G and 1S, and two hydroxycinnamyl aldehyde 8–O–4-coupled models, 3GS and 3SG.** The characteristic lignin staining is only achieved with the hydroxycinnamyl aldehydes **1**. UV spectra show that the characteristic peak at ~550 nm is not present in products from the hydroxycinnamyl aldehyde 8–O–4-coupled models **3**.

monomers always co-incorporate into lignins along with the traditional monolignols (19).

However, work on CAD-deficient mutants and transgenics has produced some differing results. No significant differences in aldehyde levels could be detected by NMR in maize *bm*1 mutant isolated lignins in Provan's study (12), even though colorimetric analysis by phloroglucinol staining and UV experiments indicated high aldehyde contents. Baucher *et al.* (30) produced transgenic poplar with ~70% reduced CAD activity and examined the lignin by thioacidolysis. The lignin content of the transgenics was similar to normal poplar lignin, as in tobacco and pine, and the xylem showed the diagnostic red coloration. They were convinced of the incorporation of aldehydes into poplar CAD-down-regulated transgenic lignins by performing phloroglucinol-HCl staining tests and UV spectral analysis (30). But phloroglucinol staining in some angiosperm samples suggests that levels are not significantly increased. Researchers have continued to use the phloroglucinol-HCl test to detect aldehydes components in lignin, including in mutants and transgenics (21, 30, 49). In view of what we now know about the incorporation of hydroxycinnamyl aldehydes into angiosperm lignins, it is logical to address some of these issues here.

As discussed above, hydroxycinnamyl aldehydes incorporate intimately into angiosperm lignins by endwise coupling reactions with the growing lignin polymer. Thus, the abundance of cinnamyl aldehyde endgroups (*e.g.* **5** and **6**), the ones known to stain characteristically with phloroglucinol-HCl, is not necessarily elevated in the CAD-deficient plants. The abundance of structures such as the 8–O–4-cross-coupled ether units **3**, however, can be strikingly increased. These units are also (substituted) hydroxycinnamyl aldehydes. But do they stain with phloroglucinol-HCl? In fact they do not. Fig. 7 shows a picture of the solutions and UV spectra of phloroglucinol-HCl products from vanillin, coniferyl, and sinapyl aldehydes, as well as from two 8–O–4-dimer models **3**, all at the same concentration (3.33 μM). Obviously, the 8–O–4-structures **3** do not give the same characteristic color as the hydroxycinnamyl end groups provide. Consequently, phloroglucinol-HCl staining cannot be assumed to be a reliable indicator of the levels of total aldehyde incorporation.

CONCLUSIONS

Molecular marker indene derivatives, derived from lignin degradation by the thioacidolysis method, for CAD deficiency in angiosperms have been structurally identified and shown to derive from hydroxycinnamyl aldehydes that have undergone 8–O–4-(cross-) coupling. As such, they are valuable for ascertaining plant responses to various levels of CAD-down-regulation. They further illustrate that hydroxycinnamyl aldehydes incorporate into angiosperm lignins by endwise coupling reactions in much the same way as normal monolignols do, suggesting that the hydroxycinnamyl aldehydes should be considered authentic lignin precursors. The phloroglucinol-HCl staining reaction, while a useful rapid test for lignin, does not stain many types of structures from hydroxycinnamyl aldehyde incorporation. More diagnostic methods, such as analytical thioacidolysis or detailed NMR studies, are therefore essential for characterizing CAD-deficient plants.

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