New Phytologist Supporting Information

Article title: A new approach to zip-lignin? 3,4-Dihydroxybenzoate is compatible with lignification

Authors: Faride Unda, Yaseen Mottiar, Elizabeth L. Mahon, Steven D. Karlen, Kwang Ho Kim, Dominique Loqué, Aymeric Eudes, John Ralph, Shawn D. Mansfield

Article acceptance date: 17 March 2022

The following Supporting Information is available for this article:

**Methods S1** Generation, selection, and cultivation of transgenic hybrid poplar. Cell wall composition. Wood density. Lignin histology. Synthesis of the benzodioxane model compound.

**Table S1** Phenotypic characterizations, wood density, and biomass of stem of five-month-old greenhouse-grown QsuB-poplar and wild-type (WT) trees.

**Fig. S1** Transcript abundance of the bacterial 3-dehydroshikimate dehydratase QsuB in the developing xylem of hybrid poplar.

**Fig. S2** Comparison of growth phenotypes of wild-type poplar and QsuB poplar lines 2, 10

**Fig. S3** Comparison of air-dried stem sections of wild-type poplar and QsuB-poplar line 1.

**Fig. S4** Representative transversal cross-sections of poplar xylem stained with phloroglucinol-HCl and toluidine-blue of QsuB-poplar line 1 and wild-type poplar.

**Fig. S5** UHPLC traces of alkaline hydrolysates of extractive-free wood from QsuB-poplar line 1 and wild-type poplar.

**Fig. S6** UV-Vis spectra of compounds released by alkaline hydrolysis of extractive-free stem tissue of QsuB-poplar line 1.

**Fig. S7** Two-dimensional heteronuclear single-quantum coherence (HSQC) NMR spectra profiling the isolated enzyme lignins from wild-type poplar and QsuB-poplar line 15.
**Methods S1** Generation, selection, and cultivation of transgenic hybrid poplar. Cell wall composition. Wood density. Lignin histology. Synthesis of the benzodioxane model compound.

*Generation, selection, and cultivation of transgenic hybrid poplar*

Agrobacterium-mediated transformation was used to generate transgenic hybrid poplar lines expressing a plastid-targeted 3-dehydroshikimate dehydratase gene from *Corynebacterium glutamicum* (GenBank Accession Number YP_001137362.1). The pTKan-pC4H::schl::qsuB expression construct described previously by Eudes *et al.* (2015) was transferred into *Agrobacterium tumefaciens* EHA105. Leaf discs of *Populus alba × grandidentata* (P39) were then harvested from four-week-old tissue culture-grown plants, and ten plates each containing 50 leaf discs (6 mm²) were co-cultivated with 30 mL of *Agrobacterium* culture (OD = 0.1–0.2) in 50-mL Falcon tubes for 30 min at 28 °C in a gyratory shaker at 100 rpm. Following co-cultivation, the explants were blotted dry on sterile filter paper and placed abaxial-side-up onto woody plant media (WPM) media containing 0.1 µM 1-naphthaleneacetic acid, 0.1 µM 6-benzylaminopurine, and 0.1 µM thidiazuron. The plates were incubated in the dark for two days at room temperature and, after the third day, residual *Agrobacterium* was eliminated by transferring the leaf discs to fresh WPM media as above but also containing 250 mg L⁻¹ cefotaxime and 500 mg L⁻¹ carbenicillin. The plates were kept in the dark for an additional two days and then explants were transferred to fresh WPM media as above but with 250 mg L⁻¹ cefotaxime, 500 mg L⁻¹ carbenicillin, and 30 mg L⁻¹ hygromycin, and incubated under subdued
lighting. After the emergence of shoots, one shoot per leaf disc was excised and placed onto fresh WPM media containing 0.1 µM 6-benzylaminopurine, 250 mg L⁻¹ cefotaxime, 500 mg L⁻¹ carbenicillin, and 30 mg L⁻¹ hygromycin. After 6 weeks of growth under normal lighting (16-h days), explants were transferred to fresh WPM media containing 0.1 µM 1-naphthaleneacetic acid, 250 mg L⁻¹ cefotaxime, 500 mg L⁻¹ carbenicillin, and 50 mg L⁻¹ kanamycin.

PCR-based screening was then used to identify transgenic plant lines. A CTAB-based extraction method was used to isolate DNA from poplar leaves. Transgene incorporation was confirmed by PCR using gene-specific primers: QsuB FW 5' -TGGAAACAGCTACCATAACG-3' and QsuB RV 5' -ACCTCATCAGTCTCCCATCC-3'.

Transgenic trees were propagated and multiplied on antibiotic-free WPM media until six plantlets of each transgenic line as well as non-transformed controls were of similar size. The trees were then moved to two-gallon pots containing perennial soil (50% peat, 25% fine bark, and 25% pumice; pH 6.0), in a greenhouse where they were maintained on flood-tables with supplemental lighting (16-h days) and watered daily with fertilized water.

Syleptic branches were counted for each tree after three months of growth. At five months, stem diameter was measured using digital calipers at 10 cm above the root collar. The trees were then harvested, the leaves and bark were removed, and the developing xylem was scraped from the stems. Xylem scrapings, bark and leaf tissue were stored at -80 °C, while the remaining stem was left to air dry.

RNA was isolated from developing xylem tissue using TRIzol reagent (ThermoFisher Scientific). Contaminating DNA was removed using a DNase I DIGEST kit (Ambion, ThermoFisher Scientific), and 1 µg of DNase-treated RNA was used to generate cDNA with the iScript cDNA synthesis kit (Bio-Rad Labs). The resulting cDNA was stored at -20 °C until use. Real-time quantitative PCR (RT-qPCR) reactions consisted of 10 µL of SsoFast Eva Green Supermix (Bio-Rad Labs), 20 pmol of primers, 1 µL of cDNA, and deionized water to a total volume of 20 µL. qPCR was performed using an CFX 96 System (Bio-Rad Labs) with the following primers: QsuB RT Fw 5' -TGGAAACAGCTACCATAACG-3' and QsuB RT Rv 5' -ACCTCATCAGTCTCCCATCC-3'. The following thermal cycler regime was used to amplify the 231-bp fragment: 30 s at 95 °C, 39 cycles of 95 °C for 5 s, and 58 °C for 30 s, followed by 95 °C for 30 s, and a melt curve cycle of 58 °C to 95 °C with an increment of 0.5 °C for 5 s.
**Cell wall composition**

Debarked and air-dried stem samples from greenhouse-grown trees were subjected to acid hydrolysis using a modified Klason lignin method (Cullis et al., 2004). Samples were first ground in a Wiley mill to pass a 40-mesh sieve, Soxhlet-extracted overnight with hot acetone, and then dried for 24 h at 105 °C. Approximately 200 mg of extractive-free wood powder was treated with 72% sulphuric acid for 2 h, diluted to ~3% with 112 mL deionized water, and autoclaved at 121 °C and 15 psi for 60 min. After cooling, the mixture was filtered through a medium-coarseness fritted-glass crucible and the retentate was dried at 105 °C. Acid-insoluble lignin was then determined gravimetrically, whereas the acid-soluble lignin fraction was measured spectrophotometrically by analysing the filtrate at 205 nm using an extinction coefficient of 110 L g⁻¹ cm⁻¹.

The composition of structural polysaccharides was evaluated by high-performance anion-exchange chromatography of acid hydrolysates (Huntley et al., 2005). Glucose, xylose, mannose, galactose, arabinose, and rhamnose were separated using a DX-600 HPLC (Dionex) fitted with a CarboPac PA1 column (Dionex) and a pulsed amperometric detector equipped with a gold electrode using water as an eluent at 1 mL min⁻¹ and post-column addition of 200 mM NaOH to enable electrochemical detection. Fucose was used as an internal standard for normalisation purposes, and sugar concentrations were determined from standard curves generated with calibration standards that were processed in parallel.

The lignin monomer composition was determined following a modified thioacidolysis procedure (Robinson & Mansfield, 2009). Gas chromatography was performed using a Thermo Trace 1310 instrument (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a flame ionization detector and a TG-5MS capillary column (30 m x 0.32 mm x 0.25 μm film thickness) and a flame-ionisation detector. Helium was used as the carrier gas at a flow rate of 1 mL min⁻¹, the injection port was heated to 250 °C, and the following program was used for the column oven: 130 °C for 3 min, followed by a ramp at 6.5 °C min⁻¹ to 230 °C, hold for 6 min, and a ramp at 2.5 °C min⁻¹ to 250 °C, hold for 10 min. Aryl 1,2,3-trithioethylpropane standards were used to identify the lignin monomers released by thioacidolysis, specifically p-hydroxyphenyl-trithioethylpropane, guaiacyl-trithioethylpropane, and syringyl-trithioethylpropane were employed to identify the corresponding H, G, and S monomers, respectively (Yue et al., 2012). Peaks were integrated using the Chromleon 7 software package (Thermo Scientific).
To liberate cell-wall-bound acetyl groups, 1 mL of 2 M NaOH was added to approximately 30 mg of extractive-free ground xylem tissue along with 100 µL of butyric acid (1:20 dilution in water) as an internal standard in screw-cap vials which were incubated at 30 °C for 24 h in a thermomixer set to 500 rpm. The mixture was then acidified by adding 100 µL of 72% sulphuric acid and allowed to cool for 5 min on ice. The vials were centrifuged at 15,900 rcf for 2 min, and the supernatants were filtered through 0.45-µm syringe filters. Quantification of acetic acid was achieved using a Summit HPLC (Dionex) equipped with a refractive index detector (Shodex RI-101) and an Aminex HPX-87H (300mm × 7.8 mm) column (Bio-Rad Laboratories, Hercules, CA, USA) using isocratic 5 mM sulphuric acid at 0.6 mL min\(^{-1}\) with the column maintained at 60 °C. The internal standard was used for normalisation purposes, and an external calibration curve enabled quantification of acetyl groups.

**Wood density**

Wood density was measured by X-ray diffraction (Quintex Measurement System Inc., TN, USA) using radial cross-sections cut to a thickness of 1.68 mm with a precision pneumatic saw. Prior to scanning, samples were Soxhlet-extracted with hot acetone for 24 h and then allowed to dry and acclimate to ambient humidity. The wood density was measured adjacent to the pith on opposite sites and an average value was taken for each sample. A calibration curve was prepared using poplar wood samples of known density, measured by volume and weight.

**Lignin histology**

Stem samples were cut into 40-µm cross sections with a Spencer AO860 sledge microtome (Spencer Lens Co., NY, USA), and treated with either 0.02% toluidine blue or with a 2:1 mixture of 3% phloroglucinol and concentrated HCl. The sections were mounted onto glass slides and visualized using a Reichert-Jung Polyvar microscope (Leica Microsystems, Wetzlar, Germany). Images were taken with an Olympus UC90 colour camera (Olympus Corp., Tokyo, Japan).

**Synthesis of benzodioxane model compound**

The model compound BD was synthesized by the cross-coupling of coniferyl alcohol (M, 50 mg) with methyl 3,4-dihydroxybenzoate (MDHB, 50 mg) using silver (I) oxide as the 1-electron oxidant (in an unoptimized synthesis; Figure S7), as has been described previously for similar
compounds with similar oxidants (Quideau and Ralph, 1994; Lu et al., 2010; Morreel et al., 2004; Ralph et al., 2001). The required benzodioxane cross-product BD (12 mg, ~12%) was separated by preparative TLC on a 2-mm silica-gel-60 preparative TLC plate (Macherey-Nagel, Deren, Germany) by elution with hexane:ethyl acetate 7:3 (v/v) twice, and then 1:1 once, with which the required compound had migrated about two thirds of the way up the plate; the scraped silica-gel was extracted with ethyl acetate.

NMR (500 MHz, DMSO-d₆): δH 7.47 (m, 2H, MDHB_2/6), 7.34 (d, J = 2.1 Hz, 1H, 2), 7.30 (dd, J = 8.3, 2.1 Hz, 1H, 6), 7.02 (d, J = 8.9 Hz, 1H, MDHB_5), 6.79 (d, J = 8.3 Hz, 1H, 5), 4.98 (d, J = 7.9 Hz, 1H, α), 4.22 (ddd, J = 7.9, 4.7, 2.5 Hz, 1H, β), 3.53 (dd, J = 12.4, 2.5 Hz, 1H, γ2), 3.33 (dd, J = 12.4, 4.6 Hz, 1H, γ2), 3.80 (s, 3H, OMe), 3.76 (s, 3H, OMe); δC 77.9 (β), 76.4 (α), 60.0 (γ). LC-QTOF-MS: m/z [M-H]⁻ calculated for C₁₈H₁₇O₇: 345.0974; found: 345.0986.
| Line 1 | Line 2 | Line 5 | Line 10 | Line 14 | Line 15 | WT       |
|-------|--------|--------|---------|---------|---------|----------|
| Diameter (mm) | 13.10 ± 0.65  | 12.28 ± 0.49  | 17.24 ± 0.04  | 12.04 ± 0.55  | 13.83 ± 0.51  | 15.83 ± 0.90  | 15.72 ± 0.45 |
| No. of sylleptic branches | 9.3 ± 2.17 | 6.5 ± 0.87 | 7.3 ± 2.75 | 6.0 ± 0.71 | 8.5 ± 1.44 | 9.0 ± 3.24 | 1.3 ± 0.95 |
| Wood density (kg m⁻³) | 616.1 ± 31.0 | 302.6 ± 13.6 | 387.5 ± 32.2 | 282.4 ± 7.1 |

**Table S1** Phenotypic characterizations. Diameter, number of sylleptic branches and wood density of poplar. Diameter at 10 cm from the base of stem of five-month-old greenhouse-grown QsuB poplar and wild-type (WT) trees. Values represent average of biological replicates +/- SE, values in bold are significantly different from the WT using Student’s t-test (P < 0.01) n= 3
Fig. S1 Transcript abundance of the bacterial 3-dehydroshikimate dehydratase *QsuB* in the developing xylem of five-month-old hybrid poplar. Transcripts were detected by RT-qPCR as relative expression $\Delta C_t$ to the translation initiation factor 5A using the formula $2^{(\Delta C_t)}$. Bars represent the standard error of the mean from three biological replicates ($n=3$).
**Fig. S2** *QsuB*-poplar phenotype. Five-month-old greenhouse-grown transgenic *QsuB*-poplar line 2 and 10, and wild-type trees showing no significant effects of the transgene on the height of the trees.
**Fig. S3** Air-dried stem sections for five-month-old poplar. a) Wild-type (WT) poplar, b) *QsuB*-poplar line 1, c) WT on the far left and all *QsuB*-poplar lines included on the study, d) Comparison between WT (left) and *QsuB*-poplar line 1 (right). Pictures show the darker colour and the cracking of the wood of the transgenic lines.
**Fig. S4** Representative transversal cross-sections of five-month old poplar xylem stained with phloroglucinol-HCl (a, b) and toluidine-blue (c, d) for wild-type poplar (a, c) and QsuB-poplar line 1 (b, d). Black arrow heads in b point to partially collapsed walls of vessel elements. Scale bars represent 50 µm.
**Fig. S5** UHPLC traces of alkaline hydrolysates of extractive-free wood from *QsuB*-poplar line 1 (blue) and WT (grey). The trace of an alkaline hydrolysate subjected to acid hydrolysis is also shown for *QsuB*-poplar line 1 (pink) at 295, 265 and 255 nm. The structures of compounds released by alkaline hydrolysis of *QsuB*-poplar line 1 are shown at the bottom.
Identification of peaks 1-10 of Fig. S5

Peak #1 DHBA 4-O-glucoside
RT: 10.693 min in HRMS run
HRMS (negative mode): C_{13}H_{15}O_9 [M-H]^- calculated. 315.0722 Da, found 315.0728 Da.
MS/MS (negative mode): \( m/z = 315.0718 \) Da fragments to \( m/z = 216.9093, 153.0193, 109.0296, 96.9601 \) Da
Observed loss of hexose (M-162.0529) to the DHBA fragment (M = 153.0193)
HRMS (positive mode): C_{13}H_{17}O_9 [M+H]^+ calculated 317.0867 Da, found 317.0828 Da.
MS/MS (positive mode): \( m/z = 317.0828 \) Da fragments to \( m/z = 155.0320 \) Da
Observed loss of hexose (M-162.0529) to the DHBA fragment (M = 155.0320)
NMR: Chemical shifts match literature (with slight shift from different solvent)
1H NMR [500 MHz, CD_3OD]: \( \delta_H \) ppm 3.40-3.55 (4H, \( m \), gluc_2,3,4,5), 3.72 (1H, dd, \( J = 12.3, 5.4 \) Hz, gluc_6a), 3.91 (1H, dd, \( J = 12.3, 2.2 \) Hz, gluc_6b), 4.93 (1H, d, \( J = 7.4 \) Hz, gluc_1), 7.23 (1H, d, \( J = 8.6 \) Hz, DHBA_5), 7.48 (1H, d, \( J = 1.8 \) Hz, DHBA_2), 7.51 (1H, dd, \( J = 8.6, 1.8 \) Hz, DHBA_6).

HSQC (\( ^1\text{H}-^{13}\text{C} \), 500.1 MHz/125.8 MHz): \( \delta_H/\delta_C \) ppm: 7.48/118.0 (DHBA_2), 7.23/116.7 (DHBA_5), 7.51/123.1 (DHBA_6), 4.91/102.9 (gluc_1), 3.52/74.5 (gluc_2), 3.47/78.2 (gluc_3), 3.42/71.0 (gluc_4), 3.49/77.3 (gluc_5), 3.72/62.1 (gluc_6a), 3.91/62.1 (gluc_6b).

Note: Peak lost following acid treatment.
Reference for comparison: Schuster et al 1986

Peak #2 Possible ID: 4-O-glucosyl-3-methoxybenzoate
RT: 13.267 min in HRMS run, from spectral deconvolution
HRMS (negative mode): C_{14}H_{17}O_9 [M-H]^- calc. 329.0878 Da, found 329.0881 Da
MS/MS (negative mode): \( m/z = 329.0881 \) fragments to \( m/z = 167.0352, 152.0115, 123.0452, 108.0216 \) Da
Observed loss of hexose (M-162.0529) to the vanillic acid fragment (M = 152.0115)
HRMS (positive mode): C_{14}H_{19}O_9 [M+H]^+ calc. 331.1024 Da, found 331.0985 Da.
MS/MS (positive mode): \( m/z = 331.0985 \) Da fragments to \( m/z = 169.0474 \) Da.
Observed loss of hexose (M-162.0529) to the vanillic acid fragment (M+H = 169.0474)
NMR: too weak
Note: Peak lost following acid treatment.

Peak #3 DHBA 3-O-glucoside
RT: 13.418 min in HRMS run
HRMS (negative mode): C_{13}H_{15}O_9 [M-H]^- calc. 315.0722 Da, found 315.0727 Da.
MS/MS (negative mode): \( m/z = 315.0718 \) Da fragments to \( m/z = 216.9097, 153.0193, 109.0299, 96.9606 \) Da
Observed loss of hexose (M-162.0529) DHBA fragment (M = 153.0193)
HRMS (positive mode): C_{13}H_{17}O_9 [M+H]^+ calc. 317.0867 Da, found 317.0833 Da.
MS/MS (positive mode): \( m/z = 317.0833 \) Da fragments to \( m/z = 155.0320 \) Da
Observed loss of hexose (M-162.0513) to the DHBA fragment (M = 155.0320)
NMR: Chemical shifts match literature (with slight differences due to different solvent)
1H NMR [500 MHz, CD_3OD]: \( \delta_H \) ppm 3.43-3.54 (4H, \( m \), gluc_2,3,4,5), 3.77 (1H, dd, \( J = 12.0 \) Hz, 4.4 Hz, gluc_6a), 3.78 (1H, dd, \( J = 12.0, 1.7 \) Hz, gluc_6b), 4.84 (1H, d, \( J = 7.4 \) Hz, gluc_1), 6.88 (1H, d, \( J = 8.4 \) Hz, H_5), 7.64 (1H, dd, \( J = 8.4, 1.0 \) Hz, H_6), 7.83 (1H, d, \( J = 1.0 \) Hz, H_2).
**HSQC** ($^1$H-$^{13}$C, 500.1 MHz/125.8 MHz): 7.83/115.4 ppm (DHBA$_2$), H-5 (6.88 ppm) – C-5 (118.8 ppm) (DHBA$_3$), 7.64/125.9 ppm (DHBA$_6$), 4.84/102.8 ppm (gluc$_1$), (3.53/73.4 ppm (gluc$_2$), (3.48/76.9 ppm (gluc$_3$), (3.48/69.6 ppm (gluc$_4$), (3.51/76.1 ppm (gluc$_5$), (3.77/60.8 ppm (gluc$_6a$),), 3.78/60.8 ppm (gluc$_6b$).

**HMBC** ($^1$H-$^{13}$C, 500.1 MHz/125.8 MHz): selected diagnostic correlations ($^1$H to $^{13}$C)

4.84/145.0 ppm (gluc$_1$-DHBA$_3$), 7.88/145.0 ppm (DHBA$_{H1-C3}$), 7.88/151.8 ppm (DHBA$_{H1-C4}$), 7.88/122.8 ppm (DHBA$_{H1-C1}$), 7.88/125.9 ppm (DHBA$_{H1-C6}$), 7.88/168.8 ppm (DHBA$_{H1-C7}$), 6.88/145.0 ppm (DHBA$_{H5-C3}$), 6.88/151.8 ppm (DHBA$_{H5-C4}$), 6.88/122.8 ppm (DHBA$_{H5-C1}$), 7.64/118.8 ppm (DHBA$_{H6-C5}$), 7.64/151.8 ppm (DHBA$_{H6-C4}$).

**Note:** Peak lost following acid treatment.

**Reference for comparison:** Yamanaka et al. 1995

---

**Peak #4 DHBA**

**RT:** 14.437 min in HRMS run

**HRMS (negative mode):** C$_7$H$_5$O$_4$ [M-H]$^-$ calc. 153.0193 Da, found 153.0189 Da

**MS/MS (negative mode):** $m/z$ = 153.0189 Da fragments to $m/z$ = 109.0291 Da

**HRMS (positive mode):** C$_7$H$_5$O$_4$ [M+H]$^+$ calc. 155.0339 Da, found 155.0321 Da.

**MS/MS (positive mode):** $m/z$ = 155.0321 Da fragments to $m/z$ = 137.0212, 125.0554, 111.0423 Da

**Note:** Validated with authentic standard. Peak increased following acid treatment.

---

**Peak #5 Possible ID: DHBA-O-pyranoside**

**RT:** 16.410 min in HRMS run

**HRMS (negative mode):** C$_{12}$H$_{13}$O$_8$ [M-H]$^-$ calc. 285.0616 Da, found 285.0625 Da

**MS/MS (negative mode):** $m/z$ = 285.0625 Da fragments to $m/z$ = 153.0193, 96.9603 Da

Observed loss of xylose (M-132.0432) to the DHBA fragment (M = 153.0193)

**HRMS (positive mode):** Not detected.

**NMR:** too weak

**Note:** Peak lost following acid treatment.

**Reference for comparison:** Liang et al. 2013

---

**Peak #6 Possible ID: 3-O-glucosyl-4-methoxybenzoate**

**RT:** 16.436 min in HRMS run

**HRMS (negative mode):** C$_{14}$H$_{17}$O$_9$ [M-H]$^-$ calc. 329.0878 Da, found 329.0884 Da

**MS/MS (negative mode):** $m/z$ = 329.0884 Da fragments to $m/z$ = 167.0353, 152.0122 Da

Observed loss of hexose (M-162.0529) to the vanillic acid fragment (M = 167.0353) and then down to vanillin (M-H = 152.0122)

**HRMS (positive mode):** C$_{14}$H$_{19}$O$_9$ [M+H]$^+$ calc. 331.1024 Da, found 331.0986 Da.

**MS/MS (positive mode):** $m/z$ = 331.0986 Da fragments to $m/z$ = 169.0476, 125.0577 Da

Observe loss of hexose (M-162.0529) to the vanillic acid fragment (M+H = 169.0476)

**NMR:** Similar to literature (with slight shift from different solvent: acetone vs. pyridine)
**1H NMR [500 MHz, acetone-d$_6$]**: δ ppm 3.48-3.65 (4H, m, gluc$_{2,3,4,5}$), 3.74 (1H, m, gluc$_{6a}$), 3.85 (1H, m, gluc$_{6b}$), 3.93 (3H, s, ivOMe), 5.06 (1H, d, J = 6.2 Hz, gluc$_1$), 7.11 (1H, d, J = 8.4 Hz, iv$_5$), 7.75 (1H, dd, J = 8.4, 1.0 Hz, iv$_6$), 7.79 (1H, d, J = 1.0 Hz, iv$_2$).

**Reference for comparison:** Wang et al. “Novel cyanoglucosides from the leaves of Hydrangea macrophylla” Helvetica Chimica Acta, 2011, 94, 847-852.

---

**Peak #7 pHBA**

**RT:** 19.219 min in HRMS run

**HRMS (negative mode):** C$_7$H$_5$O$_3$ [M-H]$^-$ calc. 137.0244 Da, found 137.0244 Da

**MS/MS (negative mode):** m/z = 137.0244 Da fragments to m/z = 93.0346 Da

**HRMS (positive mode):** C$_{14}$H$_{19}$O$_9$ [M+H]$^+$ calc. 139.0390 Da, found 139.0385 Da

**MS/MS (positive mode):** m/z = 139.0385 Da fragments to 121.0381 Da

**Note:** Validated with authentic standard. Peak retained following acid treatment.

---

**Peak #8 Possible ID: 4-O-xylosyl-3-methoxybenzoate**

**RT:** 19.523 min in HRMS run

**HRMS (negative mode):** C$_{13}$H$_{16}$O$_8$ [M-H]$^-$ calc. 299.0772 Da, found 299.0775 Da

**MS/MS (negative mode):** m/z = 299.0775 Da fragments to m/z = 255.2347, 167.0353, 152.0120 Da

**HRMS (positive mode):** C$_{13}$H$_{16}$O$_8$Na [M+Na]$^+$ calc. 323.0737 Da, found 323.0700 Da

**MS/MS (positive mode):** m/z = 323.0700 Da fragments too weak to resolve from background

**NMR:** Only a trace amount of this compound was isolated.

**1H NMR [500 MHz, CD$_3$OD]:** δ ppm Very weak, but did observe signals that fit for -OMe, the vanillic acid ACD (d, dd, d) substituted aromatic pattern, and signals that are reasonable for the anomeric proton and the methylene of pentopyranoside.

**Note:** Peak lost following acid treatment.

---

**Peak #9 4-hydroxy-3-methoxybenzoic acid**

**RT:** 21.607 min in HRMS run

**HRMS (negative mode):** C$_8$H$_7$O$_4$ [M-H]$^-$ calc. 167.0350 Da, found 167.0348 Da

**MS/MS (negative mode):** m/z = 167.0353 Da fragments to 152.0109, 138.9286, 123.0451, 108.0214, 121.0306 Da

**HRMS (positive mode):** C$_8$H$_9$O$_4$Na [M+Na]$^+$ calc. 169.0495 Da, found 169.0473 Da

**MS/MS (positive mode):** m/z = 169.0473 Da fragment too weak to resolve from background

**NMR:** δ ppm 3.92 (3H, s, ivOMe), 6.92 (1H, d, J = 8.2 Hz, V$_5$), 7.57 (1H, d, J = 1.9 Hz, V$_2$), 7.60 (1H, dd, J = 8.2, 1.9 Hz, V$_6$).

**Note:** Validated with authentic standard. Peak retained following acid treatment.
Peak #10  Possible ID: 3-hydroxy-4-methoxybenzoic acid
RT: 22.80 min in HRMS run
HRMS (negative mode): C₈H₇O₄ [M-H]⁻ calc. 167.0350 Da, found 167.0348 Da
MS/MS (negative mode): m/z = 167.0361 Da fragments to 152.0128, 123.0504, 108.0213, 121.0303 Da
Fig. S6 UV-Vis spectra of compounds released by alkaline hydrolysis of extractive-free stem tissue of \textit{QsuB}-poplar line 1.
Fig. S7 Two-dimensional heteronuclear single-quantum coherence (HSQC) NMR spectra profiling the isolated enzyme lignins from WT (a) and QsuB-poplar line 15 (b) (as in the main paper), and the benzodioxane model BD from the cross-coupling of the methyl ester of DHBA (MDHB) with coniferyl alcohol (See Figure S8). The spectra show, color-coded to the structures below, the S and G units typical of a hardwood lignin and, in the WT, the pendent p-hydroxybenzoate (pHB) units acylating the lignin sidechain that is typical in poplar and a few other species. The QsuB-poplar line 15 lignin spectrum (b) has two notable features, the drastic reduction in levels of pHB groups, and compelling evidence for the presence of DHB ester units. Peaks matching those from the model compound BD (as shown in red, and by the orange × for each sidechain C/H pair are apparent in (b); the full model data for the model is given (c). As is usual for NMR, the evidence is not absolute but quite compellingly diagnostic given that four items of simultaneously matching data are evident – the carbon and proton shifts for each of the α and β positions characteristic of the benzodioxane from DHB.
Fig. S8 Preparation of the radical cross-coupling benzodioxane product BD between methyl DHB MDHB and coniferyl alcohol M as a model to obtain chemical shifts for the analogous structure in lignin HSQC spectra (see Figure 4 and Figure S7).
Fig. S9 Pathway diagram depicting the biosynthesis of DHBA from 3-dehydroshikimate, an intermediate of the shikimate pathway, as well as the production of DHB–monolignol conjugates, 4-hydroxy-3-methoxybenzoate, 3-hydroxy-4-methoxybenzoate, and various related glycosides. Two hypothetical routes are shown for the incorporation of DHB into the lignin of *QsuB*-poplar as pendent groups and backbone-integrated benzodioxane units.
References

Cullis IF, Saddler JN, Mansfield SD. 2004. Effect of initial moisture content and chip size on the bioconversion efficiency of softwood lignocellulosics. Biotechnology and Bioengineering, 85(4), 413-421.

Huntley SK, Ellis D, Gilbert M, Chapple C, Mansfield SD. 2003. Significant increases in pulping efficiency in C4H-F5H-transformed poplars: improved chemical savings and reduced environmental toxins. J Agric Food Chem 51, 6178–6183

Lu F, Marita JM, Lapierre C, Jouanin L, Morreel K, Boerjan W, Ralph J. 2010. Sequencing around 5-hydroxyconiferyl alcohol-derived units in caffeic acid O-methyltransferase-deficient poplar lignins. Plant physiology, 153(2), 569-579.

Morreel K, Ralph J, Lu F, Goeminne G, Busson R, Herdewijn P, Goeman JL, Johan Van der Eycken, Boerjan W, Messens E. 2004. Phenolic profiling of caffeic acid O-methyltransferase-deficient poplar reveals novel benzodioxane oligolignols. Plant physiology (Bethesda), 136, 4023-4036.

Quideau S. & Ralph J. 1994. A biometric route to lignin model compounds via silver (I) oxide oxidation. Synthesis of dilignols and non-cyclic benzyl aryl ethers. Holzforschung, 48, 12-22.

Ralph J, Lapierre C, Lu F, Marita JM, Pilate G, Van Doorsselaere J, Boerjan W, Jouanin L. 2001. NMR evidence for benzodioxane structures resulting from incorporation of 5-hydroxyconiferyl alcohol into Lignins of O-methyltransferase-deficient poplars. J Agric Food Chem. 49(1):86-91. Erratum in: J Agric Food Chem. 49(7):3508.

Robinson AR, & Mansfield SD. 2009. Rapid analysis of poplar lignin monomer composition by a streamlined thioacidolysis procedure and near-infrared reflectance-based prediction modeling. The Plant Journal: For Cell and Molecular Biology, 58(4), 706-714.

Yue F, Lu F, Sun R-C, Ralph J. 2012. Synthesis of lignin-derived thioacidolysis monomers and their uses as quantitation standards. Journal of Agricultural and Food Chemistry, 60, 922-928.