

## Original Article

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# Synthesis of hydroxycinnamoyl shikimates and their role in monolignol biosynthesis

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**Abstract:** Hydroxycinnamoyl shikimates were reported in 2005 to be intermediates in monolignol biosynthesis. 3-Hydroxylation of *p*-coumarate, originally thought to occur via coumarate 3-hydroxylase (C3H) from *p*-coumaric acid or its CoA thioester, was revealed to be via the action of coumaroyl shikimate 3'-hydroxylase (C3'H) utilizing *p*-coumaroyl shikimate as the substrate, itself derived from *p*-coumaroyl-CoA via hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase (HCT). The same HCT was conjectured to convert the product, caffeoyl shikimate, to

caffeoyl-CoA to continue on the pathway starting with its 3-*O*-methylation. At least in some plants, however, a more recently discovered caffeoyl shikimate esterase (CSE) enzyme hydrolyzes caffeoyl shikimate to caffeic acid from which it must again produce its CoA thioester to continue on the monolignol biosynthetic pathway. HCT and CSE are therefore monolignol biosynthetic pathway enzymes that have provided new opportunities to misregulate lignification. To facilitate studies into the action and substrate specificity of C3H/C3'H, HCT, and CSE enzymes, as well as for metabolite authentication and for enzyme characterization, including kinetics, a source of authentic substrates and products was required. A synthetic scheme starting from commercially available shikimic acid and the four key hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, and sinapic acid) has been developed to provide this set of hydroxycinnamoyl shikimates for researchers.

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## 1 Introduction

Lignification is the process of polymerizing phenolic monomers into lignin (Freudenberg and Neish 1968), a cell wall polymer crucial for plant growth and development. Interest in lignins is escalating as lignin is established to be a particularly metabolically plastic polymer that Nature and humans can engineer for a variety of purposes (Boerjan et al. 2003; del Río et al. 2021; Mottiar et al. 2016; Ralph et al. 2004, 2019b, 2021; Vanholme et al. 2008, 2010, 2012, 2019). As society attempts to move away from fossil resources, lignins are also becoming recognized as the planet's largest biorenewable source of natural aromatics (Abu-Omar et al. 2021; Sun et al. 2018). Lignin-derived aromatic compounds have valuable substitution patterns and functionalization that may be 'exploited'; examples include synthesis of intermediates in the preparation of widely used anticancer drugs (Blondiaux et al. 2019), the synthesis of paracetamol (acetaminophen) from

*p*-hydroxybenzoate groups on certain lignins (Ralph et al. 2019a), and the conversion of a primary syringyl lignin hydrogenolysis monomer, syringyl propanol, to biologically active compounds (Elangovan et al. 2019). At the same time, it has become increasingly important to secure processes that convert cell wall polysaccharides via saccharification and fermentation to fuels and chemicals while preserving the lignin for optimal conversion to valuable products (Abu-Omar et al. 2021; Liao et al. 2020).

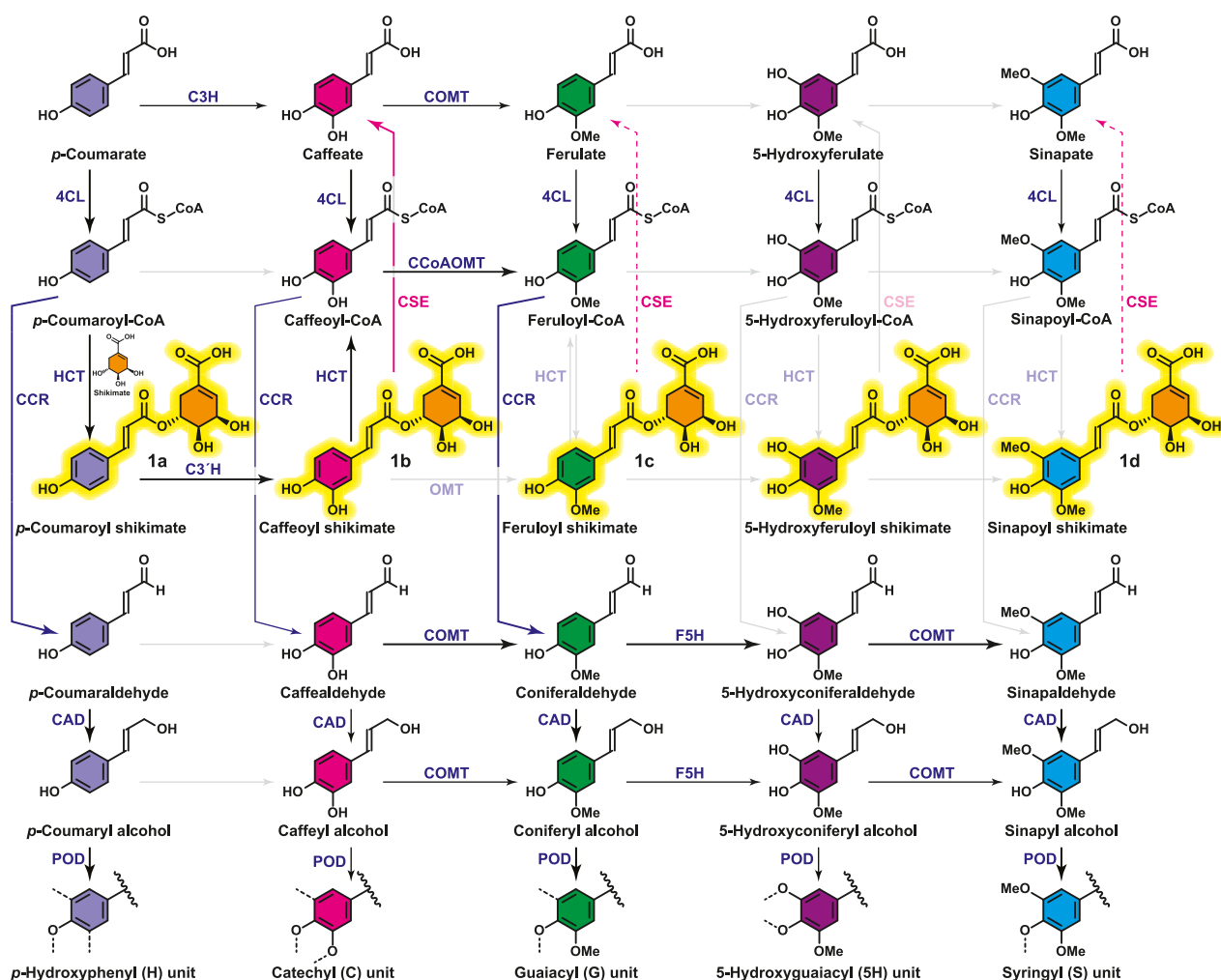
Key to studies in these areas is an improved knowledge of the genes, enzymes, and pathway processes involved in the synthesis of lignin monomers and the polymer itself. Aromatic hydroxylation steps are key flux-limiting steps in the pathway for which knowledge continues to advance. The 3-hydroxylation of the aromatic ring on the pathway to monolignols has undergone considerable refinement in the last decade or so. As is always the case, the discovery of new genes provides fresh ways of perturbing or influencing the process to ultimately produce, *in planta*, different lignins that may have beneficial properties for a biorefinery operation.

Hydroxycinnamoyl shikimate esters, particularly *p*-coumaroyl shikimate **1a** and caffeoyl shikimate **1b** (Figure 1), as enzyme substrate and product, respectively, became important in the monolignol biosynthetic pathway with the key discovery that these, and quinate esters, were the substrates for the 3-hydroxylation by C3'H (often denoted simply as C3H; enzyme abbreviations can be found in Figure 1 caption) (Hoffmann et al. 2005; Petersen et al. 2010). Previously it was thought that *p*-coumaric acid or its CoA thioester derivatives were the hydroxylase substrates; the recent revelation of a cytosolic ascorbate peroxidase also acting as a C3H on *p*-coumaric acid in the early steps of lignin biosynthesis brings this pathway back into contention (Barros et al. 2019).

The discovery of the intermediacy of the hydroxycinnamoyl shikimate esters (Hoffmann et al. 2005) also held interest for synthetic organic chemists. The CoA derivative was seen as the first step in activating the acid for its reduction. The CoA is not incompatible with the need to functionalize the aromatic ring and indeed a CoA binding site is common in various enzymes, and CoA derivatives have been demonstrated to undergo the hydroxylation and methylation reactions, even spawning the common name for an *O*-methyltransferase involved in monolignol biosynthesis (CCoAOMT, caffeoyl-CoA *O*-methyltransferase). However, the revelation that the common synthetic design of utilizing an ester to protect the acid as an intermediate step is Nature-inspired is somehow comforting; with the acid stabilized, functionalizing the aromatic ring, before proceeding to activate the acid (again) for reduction, is facilitated.

More importantly, the discovery of a hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase (HCT) enzyme in the monolignol biosynthetic pathway (Figure 1) provided another powerful way of manipulating the flux through the pathway. Downregulation of *HCT* genes, or knockouts, produced elevated levels of H-units in lignins (Bhattarai et al. 2018; Escamilla-Treviño et al. 2014; Eudes et al. 2016; Gallego-Giraldo et al. 2014; Legrand et al. 2016; Levsh et al. 2016; Li et al. 2010; Lin et al. 2015; Moglia et al. 2016; Pu et al. 2009; Shadle et al. 2007; Shi et al. 2010; Vanholme et al. 2013a; Wagner et al. 2007; Walker et al. 2013; Ziebell et al. 2010), as with similar perturbation of *C3H/C3'H* genes (Barrière et al. 2004; Chen et al. 2011; Coleman et al. 2008; Fornale et al. 2015; Franke et al. 2002a,b; Kim et al. 2014, 2020; Patten et al. 2007; Ralph et al. 2006, 2012; Robinson et al. 2018; Sundin et al. 2014). The HCT enzyme was hypothesized, although not firmly established, to also catalyze the return of the product, caffeoyl shikimate **1b**, back to the CoA level (Hoffmann et al. 2005). Boerjan's group investigated other genes that appeared to coexpress with genes of the monolignol biosynthetic pathway. This led to the discovery of yet another enzyme, CSE (caffeoyl shikimate esterase), that cleaves the ester in caffeoyl shikimate **1b** to produce caffeic acid **2b**, at least in some plant species (Ha et al. 2016; Saleme et al. 2017; Vanholme et al. 2013b, 2019). This pathway also then requires a further 4CL step to again produce the CoA ester, caffeoyl-CoA, for 3-*O*-methylation, reduction to the aldehyde, further hydroxylation and methylation if syringyl units are required, and the final reduction of the coniferaldehyde or sinapaldehyde to their monolignols, coniferyl and sinapyl alcohol, Figure 1. Researchers wondered whether the caffeoyl shikimate **1b** might be the substrate that is methylated by an *O*-methyltransferase to produce feruloyl shikimate **1c** (via the gray arrow and light generic OMT enzyme in Figure 1), before taking it back, directly via HCT or indirectly via CSE to ferulic acid and then via 4CL, to the feruloyl-CoA, and eventually down to coniferaldehyde via CCR, and finally coniferyl alcohol again via CAD. The availability of the synthetic compounds **1a–d** (Figure 2) may help furthering an understanding into whether these pathways, with gray arrows in Figure 1, occur *in planta*; the recent finding (de Vries et al. 2021) of the accumulation of the four hydroxycinnamoyl shikimates in CSE knock-out poplar mutants suggests the pathways designated with the dashed CSE arrows are operational.

For studies into the action of various enzymes, including C3'H (Kim et al. 2020; Ralph et al. 2006, 2012; Takeda et al. 2018; Weng et al. 2010), HCT (Vanholme et al. 2013a; Wagner et al. 2007), and CSE (Ha et al. 2016; Saleme et al. 2017; Vanholme et al. 2013b), but also CHS (Eloy et al. 2017), 4CL (Tsai et al. 2020), and general stress



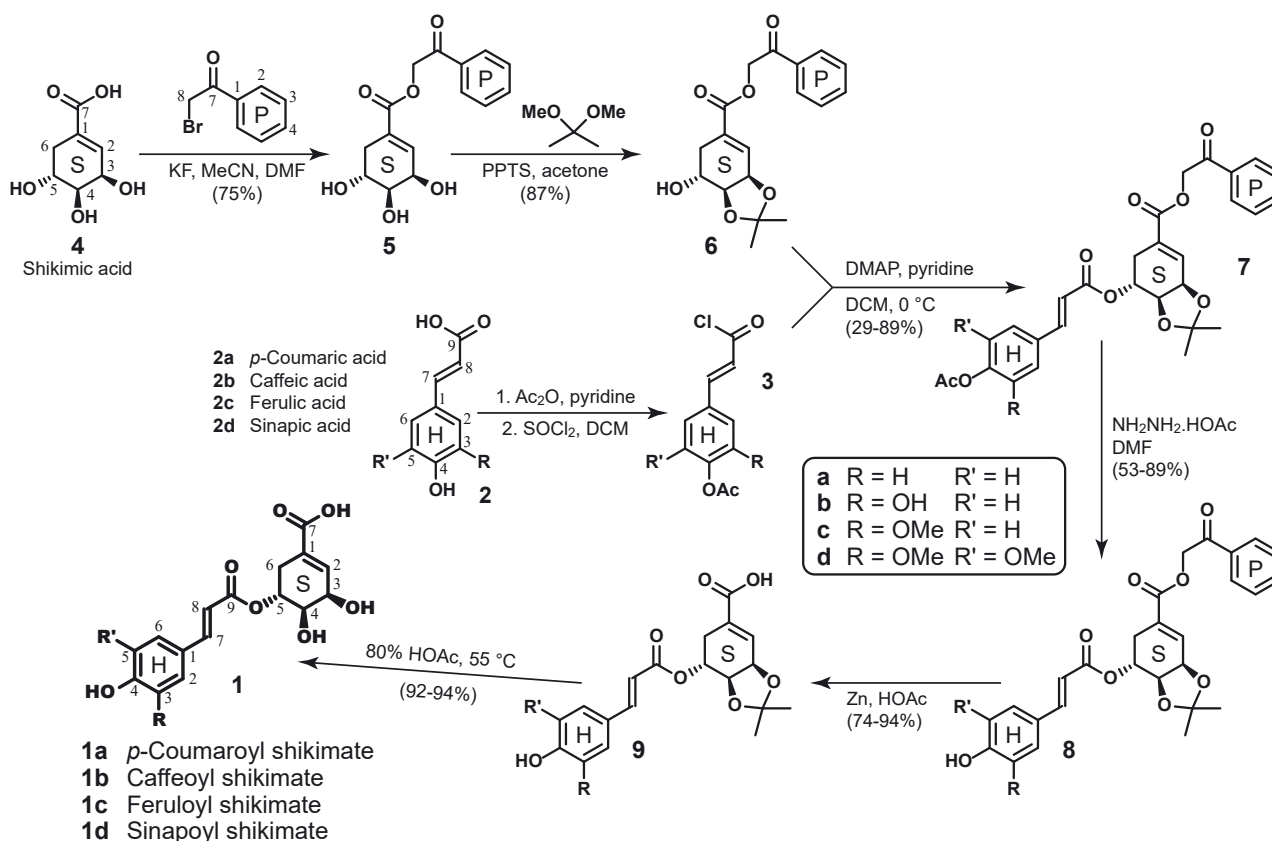
**Figure 1:** Monolignol biosynthetic pathway from *p*-coumaric acid through to polymeric lignin **H**, **G**, and **S** units, emphasizing the hydroxycinnamoyl shikimate intermediates *p*-coumaroyl shikimate **1a** and caffeoyl shikimate **1b** [based on Figure 1 in a recent opinion article (Vanholme et al. 2019)]. There is no current evidence for pathways in gray to be involved in monolignol biosynthesis *in planta*; the CSE pathways to ferulate and sinapate have been suggested recently based on the accumulation of the four hydroxycinnamoyl shikimates in CSE knock-out poplar mutants (de Vries et al. 2021); the major pathway to **H**, **G**, and **S** units is shown with bold arrows. 4CL, 4-coumarate: CoA ligase; HCT, hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase; C3'H, *p*-coumaroyl shikimate 3'-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; POD, peroxidase (as well as laccase).

mechanisms (Varbanova et al. 2011), we synthesized, and provided for the various research groups, the first compounds in this series, *p*-coumaroyl shikimate **1a** and caffeoyl shikimate **1b**. Most recently, feruloyl shikimate **1c** and sinapoyl shikimate **1d** were needed to elucidate kinetic parameters (de Vries et al. 2021); other studies by various groups utilizing these compounds await publication. To further study and engineer the role and activity of HCT, CSE, and other lignin biosynthetic pathway enzymes (such as the possibility of producing feruloyl shikimate by methylating caffeoyl shikimate via COMT or another OMT), and to use as standards in various

biological assays to study enzyme activities, we synthesized shikimate esters **1a–d**, for which chemical syntheses have not previously been reported (although bacterial synthesis has been described (Cha et al. 2014)).

## 2 Materials and methods

The full Materials and methods section, including NMR data for all intermediates **2–9**, is provided in the Supplementary Material; only the data for the final hydroxycinnamoyl shikimates **1a–d** are provided in the text here; proton NMR spectra of **1a–d** are in Figure 3, carbon spectra in Figure 4.



**Figure 2:** Synthetic scheme for the synthesis of hydroxycinnamoyl shikimates **1** starting from commercially available shikimic acid and the four hydroxycinnamic acids. Details are in the text, including the experimental section. For ease in labeling NMR data from the various units, S is used to designate the shikimate moiety, P for the phenacyl moiety, and H for the various hydroxycinnamate moieties; **a–d** are for *p*-coumarate, caffeate, ferulate, and sinapate, respectively. Yields or yield-ranges are given (for non-optimized reactions, in parentheses) for each step. DMF, dimethylformamide; PPTS, pyridinium *p*-toluenesulfonate; DMAP, 4-(dimethylamino)pyridine; DCM, dichloromethane (methylene chloride).

## 2.1 Materials and instrumentation

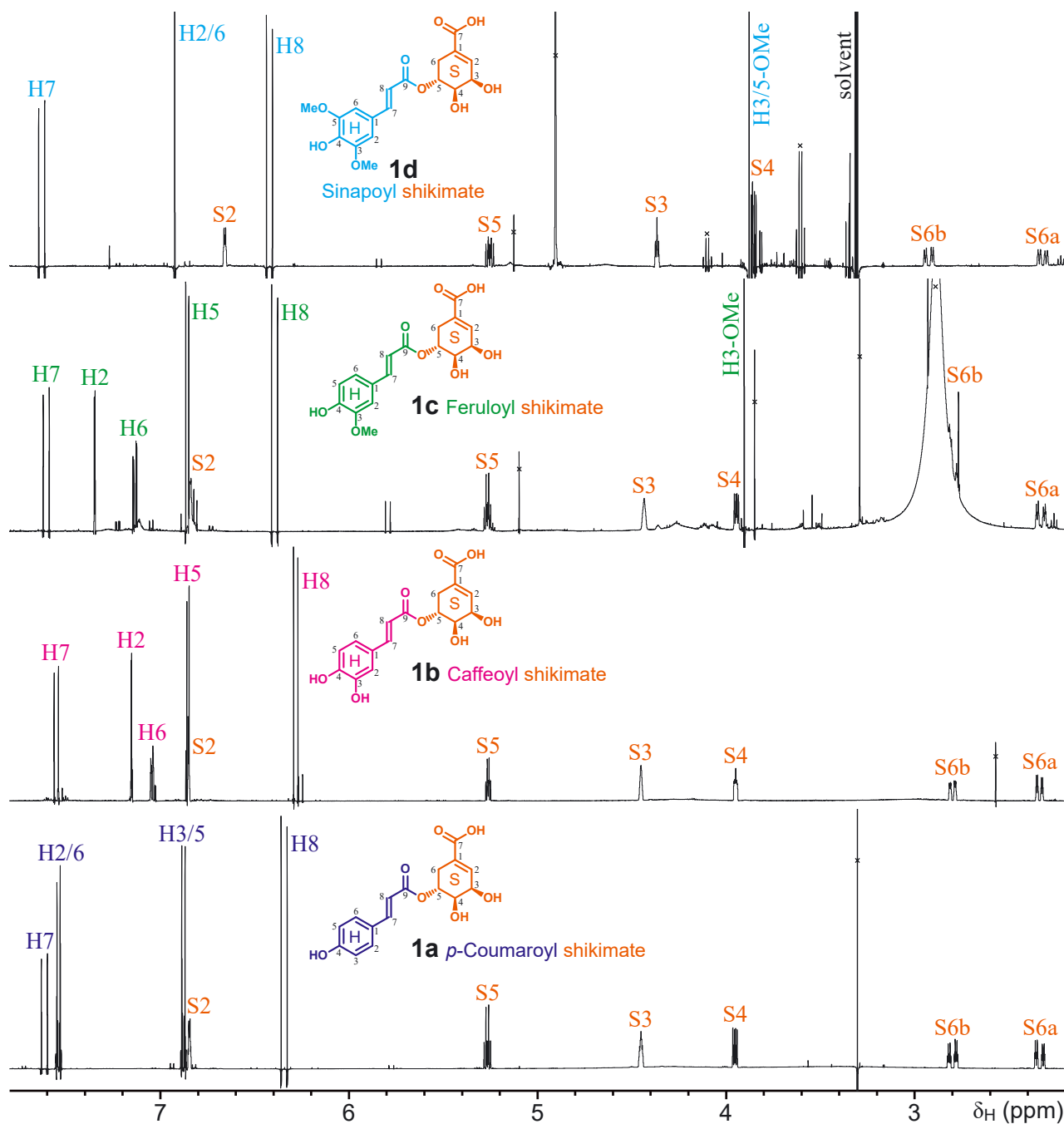
All commercially purchased reagents and solvents were used as received unless otherwise indicated. Flash column chromatography was performed on a Teledyne Isco flash chromatograph using RediSep® Rf cartridges, silica 60 M (0.04–0.063 mm) supplied by Macherey-Nagel. Thin-layer chromatographic analysis was conducted using pre-coated TLC sheets ALUGRAM® SIL G/UV<sub>254</sub> supplied by Macherey-Nagel. <sup>1</sup>H, <sup>13</sup>C, HSQC, HMBC, and COSY NMR experiments were all conducted on a Bruker Biospin (Billerica, MA, USA) AVANCE 500 or 700 MHz spectrometer (Billerica, MA, USA) fitted with cryogenically-cooled, <sup>1</sup>H-optimized gradient probes with inverse geometry (proton coils closest to the sample); residual or deuterated solvent peaks were used for internal reference (2.04/29.8 ppm for acetone-*d*<sub>6</sub>, 2.49/39.5 ppm for DMSO-*d*<sub>6</sub>, 7.24/77.0 for CDCl<sub>3</sub>, and 3.31/49.15 ppm for methanol-*d*<sub>4</sub>). The NMR data for hydroxycinnamoyl shikimates **1a–d** are summarized in Table 1; the textual data (including <sup>1</sup>H NMR coupling constants) are provided in the Materials and methods section for these final compounds. NMR data for intermediates are provided in the Supplementary Material.

High-resolution mass spectra (HRMS) were collected in the University of Wisconsin-Madison, Department of Chemistry Mass

Spectrometry Lab on a Thermo Q Exactive™ Plus, or in-house on a Bruker Impact II Ultra-high Resolution QqTOF mass spectrometer using electrospray ionization.

## 2.2 Synthesis of hydroxycinnamoyl shikimic acid esters 1a–1d from shikimic acid and hydroxycinnamic acids

The carboxylic group of shikimic acid **4** (Sigma-Aldrich) was protected via its phenacyl ester **5** (Figure 2) by reaction with 2-bromoacetophenone (Clark and Miller 1977). The 3- and 4-hydroxy groups of the shikimic acid moiety were then protected as an acetonide, producing compound **6**. This was then coupled with the four different hydroxycinnamic acids **2**, viz., *p*-coumaric acid **2a**, caffeic acid **2b**, ferulic acid **2c**, and sinapic acid **2d**, in which the phenolic groups were first protected as acetates. The hydroxycinnamic acids were either used as their acid chlorides (**3a–d**) or as their carbodiimide derivatives (generated *in situ*, not shown) to give the corresponding esters **7a–d** in extensively protected form. Compounds **7** were then deprotected stepwise, first removing the phenolic acetate using hydrazine hydrate (Zhu et al. 2013) to produce the free-phenolic compounds **8**. These products were then treated with Zn in



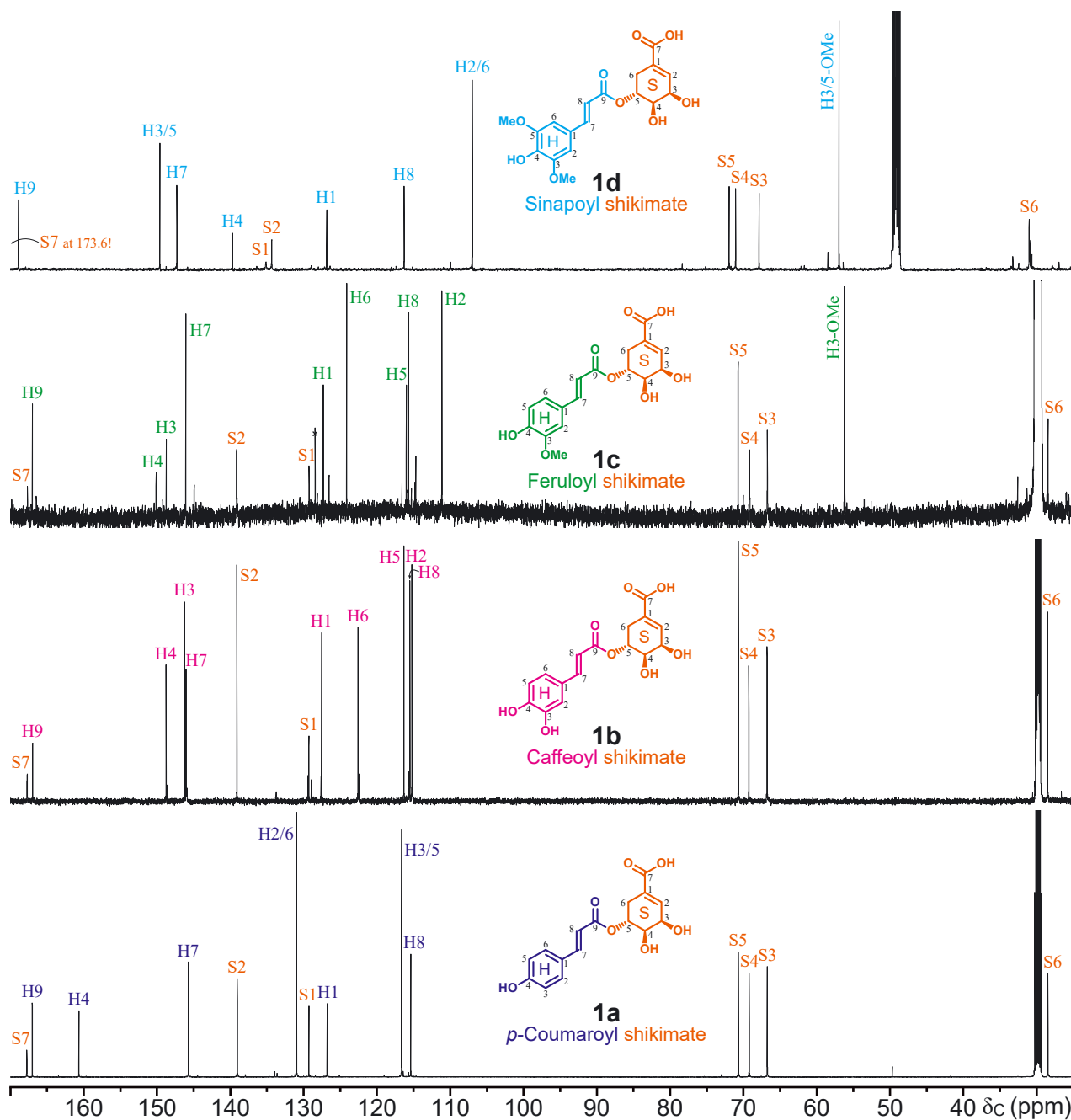
**Figure 3:** Proton NMRs of **1a–d**. **1a** is at 500 MHz in acetone- $d_6$ ; **1b** is at 700 MHz, in acetone- $d_6$ ; **1c** is at 500 MHz in acetone- $d_6$ ; **1d** is at 500 MHz in methanol- $d_4$ .

acetic acid (Hendrickson and Kandall 1970) to remove the phenacyl ester, producing compounds **9**. Finally, the acetonide was cleaved using aqueous acetic acid to give the targeted hydroxycinnamoyl shikimates **1**. Overall yields from shikimic acid **4** through to compounds **5** and then **6**, and coupling with acetylated hydroxycinnamoyl chlorides **3**, followed by the various deprotection steps to the final hydroxycinnamoyl shikimates **1** were 44% in the case of **1a**, although were as low as 9% for **1d**

because the crucial coupling step was run only once (at 29% yield) and no improvement was attempted. Methods and spectroscopic data are provided below or in the Supplementary Material, as noted.

## 2.2.1 Synthesis of acetate derivatives **3** of hydroxycinnamic acids **2**:

Acetyl *p*-coumaric acid, diacetyl caffeic acid, acetyl ferulic acid, and



**Figure 4:** Carbon NMRs of **1a–d** from the same samples and conditions as for proton NMR spectra in Figure 3. Note that protonated-carbon assignments are validated via HSQC correlations whereas non-protonated carbons were assigned from their HMBC correlations.

acetyl sinapic acid, were prepared according to published procedures (Hatfield et al. 1991; Helm et al. 1992).

**Example: Synthesis of diacetyl caffeic acid.** Caffeic acid **2b** (2 g, 11.10 mmol) was dissolved in acetic anhydride (4 mL) and pyridine (4 mL) and stirred overnight at room temperature. The reaction mixture was evaporated under reduced pressure to yield the desired diacetate in quantitative yield. See the Supplementary Material for NMR data.

**2.2.2 Synthesis of acid chlorides (3a–d) from the acetate-protected hydroxycinnamic acids:** The acetyl derivatives of the hydroxycinnamic acids were each then dissolved in dichloromethane (DCM, 5 mL) and thionyl chloride (3 mL) was added followed by a few drops of pyridine and the mixture was stirred at room temperature for 3 h. The reaction mixture was evaporated under reduced pressure azeotroping with toluene. The acid chlorides **3a–d** were used in the next step without purification.

**Table 1:** NMR data for compounds **1a–d**, the synthetic hydroxycinnamoyl shikimates.

| Compound | Position |         | <sup>13</sup> C |          | <sup>1</sup> H |         |         |          |
|----------|----------|---------|-----------------|----------|----------------|---------|---------|----------|
|          | 1a       | 1b      | 1c              | 1d       | 1a             | 1b      | 1c      | 1d       |
| Solvent  | Acetone  | Acetone | Acetone         | Methanol | Acetone        | Acetone | Acetone | Methanol |
| H1       | 126.8    | 127.5   | 127.3           | 126.8    |                |         |         |          |
| H2       | 131.0    | 115.2   | 111.1           | 107.0    | 7.54           | 7.15    | 7.35    | 6.92     |
| H3       | 116.6    | 146.2   | 148.7           | 149.6    | 6.88           | –       | –       | –        |
| H4       | 160.6    | 148.8   | 150.1           | 139.7    |                |         |         |          |
| H5       | 116.6    | 116.3   | 116.0           | 149.6    | 6.88           | 6.853   | 6.86    | –        |
| H6       | 131.0    | 122.6   | 124.1           | 107.0    | 7.54           | 7.04    | 7.14    | 6.92     |
| H7       | 145.7    | 146.0   | 146.1           | 147.3    | 7.61           | 7.55    | 7.60    | 7.63     |
| H8       | 115.4    | 115.5   | 115.7           | 116.3    | 6.34           | 6.28    | 6.39    | 6.42     |
| H9       | 167.0    | 167.0   | 167.0           | 168.9    |                |         |         |          |
| H-OMe    |          |         | 56.3            | 56.9     |                |         | 3.904   | 3.88     |
| S1       | 129.3    | 129.3   | 129.3           | 135.1    |                |         |         |          |
| S2       | 139.0    | 139.1   | 139.1           | 134.3    | 6.85           | 6.851   | 6.84    | 6.66     |
| S3       | 66.8     | 66.8    | 66.8            | 67.9     | 4.45           | 4.45    | 4.44    | 4.37     |
| S4       | 69.2     | 69.3    | 69.2            | 71.1     | 3.95           | 3.95    | 3.94    | 3.85     |
| S5       | 70.7     | 70.7    | 70.7            | 72.0     | 5.27           | 5.26    | 5.27    | 5.25     |
| S6       | 28.5     | 28.5    | 28.4            | 31.0     | 2.34           | 2.34    | 2.33    | 2.32     |
|          |          |         |                 |          | 2.80           | 2.80    | 2.79    | 2.93     |
| S7       | 167.8    | 167.7   | 167.7           | 173.6    |                |         |         |          |

NMR solvents are deuterated. Additional decimal places are used only to distinguish the order of close peaks.

**2.2.3 Synthesis of phenacyl shikimate 5:** Potassium fluoride (1.47 g, 25.27 mmol) and 2-bromoacetophenone (2.51 g, 12.63 mmol) were stirred together in a mixture of acetonitrile (13 mL) and *N,N*-dimethylformamide (DMF, 8 mL) at room temperature for 2 min. Shikimic acid **4** (2.0 g, 11.48 mmol) was then added, and the reaction mixture was stirred overnight. The solvent (acetonitrile) was evaporated from the reaction mixture under reduced pressure. The residue was dissolved in ethyl acetate and washed with water (20 mL, 4×) to remove any remaining DMF. The organics were collected and dried over sodium sulfate, filtered, and evaporated under reduced pressure. The crude product was purified using flash chromatography eluting with ethyl acetate and hexane (1:1) and then increasing the polarity to 100% ethyl acetate to give the phenacyl ester **5** (2.50 g, 8.55 mmol) in 74% yield. See the Supplementary Material for NMR and MS data for compound **5**.

**2.2.4 Synthesis of phenacyl shikimate acetone 6:** To a solution of the ester **5** (1.09 g, 3.73 mmol) in acetone (20 mL) was added 2,2-dimethoxypropane (2.75 mL, 22.35 mmol) and pyridinium *p*-toluenesulfonate (PPTS, 130 mg, 0.518 mmol) and the reaction mixture was stirred at room temperature overnight. The solvent was evaporated under reduced pressure. The resulting residue was purified using flash chromatography (SiO<sub>2</sub>, ethyl acetate:hexanes (1:1 to 4:1)) to afford product **6** (1.08 g, 3.24 mmol) in 87% yield as a white solid. See the Supplementary Material for NMR and MS data for compound **6**.

**2.2.5 Synthesis of compounds 7a–d: general procedure for coupling the acetyl cinnamoyl chlorides (3a–d) with shikimic acid derivative 6:** The protected shikimate derivative **6** was dissolved in DCM (15 mL), 4-(dimethylamino)pyridine (DMAP, 0.2 eq) was added, and the

mixture stirred for 2 min. The acid chloride **3** (**3a–d**, 2 eq) was then added into the stirring solution followed by pyridine (12 mL) and the reaction mixture was stirred for 5 h at room temperature. The reaction mixture was diluted with DCM and washed with acidified water (20 mL, 3×). The organics were collected and dried over sodium sulfate, filtered, and the solvent was evaporated under reduced pressure. The crude mixture was then purified using flash chromatography eluting with 35% ethyl acetate in hexane to yield the desired esters **7a–d**.

The product **7a** was prepared by the general procedure from **3a** and 1.46 g of **6** with a yield of 2.1 g (89%). Product **7b** was prepared in 76% yield by the general procedure (except that triethylamine was used instead of pyridine/DMAP) from **3b** and **6**. Product **7c** was prepared as for **7b** from **3c** and **6**. Product **7d** was prepared in just 29% yield by the general procedure from **3d** and 0.218 g of **6**; no attempt was made to improve the yield. See the Supplementary Material for NMR data of compounds **7a–d**.

**2.2.6 Synthesis of compounds 8a–d: deprotection of the acetyl group in hydroxycinnamoyl shikimate derivatives 7a–d:** Product **8a** was synthesized as follows. The ester **7a** (1.46 g, 2.80 mmol) was dissolved in DMF (9 mL) and hydrazine acetate (0.69 g, 3 eq) was added. The reaction mixture was stirred at room temperature for 70 min. The mixture was diluted with ethyl acetate and washed with water (6 × 25 mL). The organics were collected and dried over sodium sulfate, filtered, and the solvent was evaporated under reduced pressure to give the product **8a** as a white solid with a yield of 1.19 g (89%). Product **8b** was prepared by the procedure from **7b** (51.5 mg) as described for compound **8a** with a yield of 30 mg (68%). Product **8c** was prepared from **7c** in essentially quantitative yield by the procedure described for compound **8a**. Product **8d** was prepared from **7d**

(110 mg) by the procedure described for compound **8a** with a yield of 53.9 mg (53%). See the Supplementary Material for NMR data of **8a–d**.

### 2.2.7 Synthesis of compounds **9a–d**: deprotection of the phenacyl group on the shikimic acid moiety in compounds **8a–d**:

Product **9a** was synthesized as follows. Compound **8a** (1.19 g, 2.49 mmol) was dissolved in acetic acid (110 mL) and Zn dust (2.26 g) was added. The reaction mixture was stirred at room temperature overnight. The mixture was diluted with excess water and washed with hexanes (4 × 100 mL). The hexanes layer was discarded, and the aqueous layer was extracted with ethyl acetate (8 × 20 mL). The organics were collected and dried over sodium sulfate, filtered, and the solvent was evaporated under reduced pressure to give the solid product **9a** with a yield of 0.82 g (92%). Product **9b** was prepared from **8b** (29.7 mg, 0.060 mmol) by the procedure described for compound **9a** with a yield of 16.7 mg (74%). Product **9c** was prepared from **8c** by the procedure described for compound **9a**. Product **9d** was prepared from **8d** (53.9 mg, 0.100 mmol) by the procedure described for compound **9a** with a yield of 39.6 mg (94%). See the Supplementary Material for NMR data of compounds **9a–d**.

### 2.2.8 Synthesis of final products **1a–d**: deprotection of the acetal group in the shikimic acid moiety in compounds **9a–d**:

As an illustrative example, we use the synthesis of caffeoyl shikimate **1b**. Compound **9b** (0.82 g, 2.18 mmol) was dissolved in aqueous acetic acid (80%, 200 mL) and the reaction mixture was stirred at 55 °C overnight. The solvent was evaporated under reduced pressure to give the caffeoyl shikimate **1b** product as a colorless solid (0.68 g) in 93% yield. Products **1a**, **1c**, and **1d** were prepared in similarly high yields by the same procedure.

**2.2.8.1 p-Coumaroyl shikimate 1a:** <sup>1</sup>H NMR (500 MHz, acetone-*d*<sub>6</sub>, Figure 3): δ<sub>H</sub> = 2.34 (1H, ddt, *J* = 18.3, 5.1, 1.6 Hz, S6a), 2.80 (1H, ddt, *J* = 18.3, 5.0, 2.0 Hz, S6b), 3.95 (1H, dd, *J* = 7.1, 4.1 Hz, S4), 4.45 (1H, m, S3), 5.27 (1H, dt, *J* = 7.1, 5.0 Hz, S5), 6.34 (1H, d, *J* = 15.9 Hz, H8), 6.85 (1H, m, S2), 6.88 (2H, d+, *J* = 8.6 Hz, H3/5), 7.54 (2H, d+, *J* = 8.6 Hz, H2/6), 7.61 (1H, d, *J* = 15.9 Hz, H7). <sup>13</sup>C NMR (125 MHz, acetone-*d*<sub>6</sub>, Figure 4): δ<sub>C</sub> = 28.5 (S6), 66.8 (S3), 69.2 (S4), 70.7 (S5), 115.4 (H8), 116.6 (H3/5), 126.8 (H1), 129.3 (S1), 131.0 (H2/6), 139.0 (S2), 145.7 (H7), 160.6 (H4), 167.0 (H9), 167.8 (S7). HRMS (ESI): calcd for C<sub>16</sub>H<sub>16</sub>O<sub>7</sub> [M+H]<sup>+</sup> *m/z* 321.0969 found 321.0975. The major (~1.2%) 'impurity' in compound **1a** is the isomer in which the *p*-coumarate moiety is *cis*.

**2.2.8.2 Caffeoyl shikimate 1b:** <sup>1</sup>H NMR (700 MHz, acetone-*d*<sub>6</sub>, Figure 3): δ<sub>H</sub> = 2.34 (1H, ddt, *J* = 18.3, 5.1, 1.6 Hz, S6a), 2.80 (1H, ddt, *J* = 18.3, 5.0, 2.0 Hz, S6b), 3.95 (1H, dd, *J* = 7.2, 4.1 Hz, S4), 4.45 (1H, m, S3), 5.26 (1H, dt, *J* = 7.2, 5.0 Hz, S5), 6.28 (1H, d, *J* = 15.9 Hz, H8), 6.851 (1H, m, S2), 6.853 (1H, d, *J* = 8.2 Hz, H5), 7.04 (1H, dd, *J* = 8.2, 2.1 Hz, H6), 7.15 (1H, d, *J* = 2.1 Hz, H2), 7.55 (1H, d, *J* = 15.9 Hz, H7). <sup>13</sup>C NMR (175 MHz, acetone-*d*<sub>6</sub>, Figure 4): δ<sub>C</sub> = 28.5 (S6), 66.8 (S3), 69.3 (S4), 70.7 (S5), 115.2 (H2), 115.5 (H8), 116.3 (H5), 122.6 (H6), 127.5 (H1), 129.3 (S1), 139.1 (S2), 146.0 (H7), 146.2 (H3), 148.8 (H4), 167.0 (H9), 167.7 (S7). HRMS (ESI): calcd for C<sub>16</sub>H<sub>16</sub>O<sub>8</sub> [M+H]<sup>+</sup> *m/z* 337.0918 found 337.0905.

**2.2.8.3 Feruloyl shikimate 1c:** <sup>1</sup>H NMR (500 MHz, acetone-*d*<sub>6</sub>, Figure 3): δ<sub>H</sub> = 2.33 (1H, ddt, *J* = 18.4, 5.0, 1.5 Hz, S6a), 2.79 (1H, ddt, *J* = 18.4, 5.0, 2.0 Hz, S6), 3.90 (3H, s, H3-OMe), 3.94 (1H, dd, *J* = 7.0, 4.1 Hz, S4), 4.44 (1H, m, S3), 5.27 (1H, dt, *J* = 7.1, 5.0 Hz, S5), 6.39 (1H, d, *J* = 15.9 Hz, H8), 6.84 (1H, m, S2), 6.86 (1H, d, *J* = 8.2

Hz, H5), 7.14 (1H, dd, *J* = 8.2, 2.0 Hz, H6), 7.35 (1H, d, *J* = 2.0 Hz, H2), 7.60 (1H, d, *J* = 15.9 Hz, H7). <sup>13</sup>C NMR (125 MHz, acetone-*d*<sub>6</sub>, Figure 4): δ<sub>C</sub> = 28.4 (S6), 56.3 (H3-OMe), 66.8 (S3), 69.2 (S4), 70.7 (S5), 111.1 (H2), 115.7 (H8), 116.0 (H5), 124.1 (H6), 127.3 (H1), 129.3 (S1), 139.1 (S2), 146.1 (H7), 148.7 (H3), 150.1 (H4), 167.0 (H9), 167.7 (S7). HRMS (ESI): calcd for C<sub>17</sub>H<sub>18</sub>O<sub>8</sub> [M-H]<sup>−</sup> *m/z* 349.0929, found 349.0929.

**2.2.8.4 Sinapoyl shikimate 1d:** <sup>1</sup>H NMR (500 MHz, methanol-*d*<sub>4</sub>, Figure 3): δ<sub>H</sub> = 2.32 (1H, ddt, *J* = 18.3, 6.5, 1.5 Hz, S6a), 2.93 (1H, ddt, *J* = 18.3, 5.4, 1.3 Hz, S6b), 3.85 (1H, m, S4), 3.88 (6H, s, H3/5-OMe), 4.37 (1H, t, *J* = 4.1 Hz, S3), 5.25 (1H, ddd, *J* = 8.3, 6.4, 5.5 Hz, S5), 6.42 (1H, d, *J* = 15.9 Hz, H8), 6.66 (1H, m, S2), 6.92 (2H, s, H2/6), 7.63 (1H, d, *J* = 15.9 Hz, H7). <sup>13</sup>C NMR (125 MHz, methanol-*d*<sub>4</sub>, Figure 4): δ<sub>C</sub> = 31.0 (S6), 56.9 (H3/5-OMe), 67.9 (S3), 71.1 (S4), 72.0 (S5), 107.0 (H2/6), 116.3 (H8), 126.8 (H1), 134.3 (S2), 135.1 (S1), 139.7 (H4), 147.3 (H7), 149.6 (H3/5), 168.9 (H9), 173.6 (S7). HRMS (ESI): calcd for C<sub>18</sub>H<sub>20</sub>O<sub>9</sub> [M-H]<sup>−</sup> *m/z* 379.1035, found 379.1056.

## 3 Results and discussion

A synthetic method to flexibly provide the various, previously synthetically unreported, hydroxycinnamyl shikimates **1** for various studies by ourselves and collaborators was required. Other versions of a synthetic scheme to these compounds have been tested over the years, but we describe only our ultimately used method that provides the compounds with reasonable ease and flexibility.

In the synthetic scheme, Figure 2, the carboxylic acid in shikimic acid **4** was selectively protected as its phenacyl ester **5** (Hendrickson and Kandall 1970), which is stable through the other reaction steps and is ultimately removable by, for example, Zn/HOAc. The vicinal 3,4-diol of shikimic acid was protected as an acetonide (a 1,2-acetonide, or an isopropylidene), via reaction with 2,2-dimethoxypropane (Schmid and Bryant 1995), producing compound **6** in which the 5-hydroxyl remains free to allow its specific derivatization. The crucial acylation was achieved via a coupling reaction with each (acetylated) hydroxycinnamoyl chloride **3** of interest (Inanaga et al. 1979; Kawanami et al. 1981), from chlorination of the various hydroxycinnamic acids after phenolic groups had first been protected as their acetates. Deprotection steps were then required. The first was removal of the acetate(s) on the hydroxycinnamate moiety to produce compounds **8**, conveniently and cleanly accomplished using hydrazine acetate (Zhu et al. 2013). The next was removal of the phenacyl group protecting the acid to produce compounds **9**, using Zn/HOAc (Hendrickson and Kandall 1970). Finally, the acetal was removed to regenerate the shikimate moiety's 3,4-diol and produce the required hydroxycinnamoyl shikimates **1**, using 80% HOAc at 50 °C (Schmid and Bryant 1995).

From the NMR data for hydroxycinnamoyl shikimates **1a–d**, summarized in Table 1, it is evident that the shikimate moiety chemical shifts are relatively invariant and independent of the hydroxycinnamate attached, with the exception of **1d** in perdeutero-methanol; the differences may relate more to the solvent change.

## 4 Conclusions

The four hydroxycinnamoyl shikimates (*p*-coumaroyl shikimate **1a**, caffeoyl shikimate **1b**, feruloyl shikimate **1c**, and sinapoyl shikimate **1d**) are important compounds either demonstrated to be in the monolignol biosynthetic pathway (**1a–b**) or implicated or otherwise of interest in it (**1c–d**). As such, the synthetic compounds have long been in demand to facilitate studies into the action and substrate specificity of various enzymes, as well as for metabolite authentication and quantification. The syntheses for these compounds **1** are finally described herein. All four shikimate esters can be prepared from the same general route. This methodology should make the compounds more accessible for plant biochemistry researchers, and the NMR and MS data available here will help researchers authenticate their own synthetic or isolated compounds.

## Abbreviations

|         |                                                             |
|---------|-------------------------------------------------------------|
| 4CL     | Cinnamate CoA ligase                                        |
| C3H     | <i>p</i> -Coumarate 3-hydroxylase                           |
| C3'H    | <i>p</i> -Coumaroyl shikimate 3'-hydroxylase                |
| CAD     | Cinnamyl alcohol dehydrogenase                              |
| CoA     | Coenzyme A                                                  |
| CCoAOMT | Caffeoyl-CoA <i>O</i> -methyltransferase                    |
| CCR     | Cinnamoyl-CoA reductase                                     |
| CHS     | Chalcone synthase                                           |
| CSE     | Caffeoyl shikimate esterase                                 |
| DCM     | Dichloromethane (methylene chloride)                        |
| DMAP    | 4-(Dimethylamino)pyridine                                   |
| DMF     | Dimethylformamide                                           |
| G units | Guaiacyl units in lignin                                    |
| HCT     | Hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase |
| H units | <i>p</i> -Hydroxyphenyl units in lignin                     |
| HOAc    | Acetic acid                                                 |
| HMBC    | Heteronuclear multiple-bond correlation                     |
| HRMS    | High-resolution mass spectra (or spectrometry)              |
| HSQC    | Heteronuclear single-quantum coherence                      |
| NMR     | Nuclear magnetic resonance (spectroscopy)                   |
| PAL     | Phenylalanine ammonia lyase                                 |
| POD     | Peroxidase                                                  |

|                  |                                       |
|------------------|---------------------------------------|
| PPTS             | Pyridinium <i>p</i> -toluenesulfonate |
| S units          | Syringyl units in lignin              |
| SiO <sub>2</sub> | Silica-gel (from chromatography)      |
| TLC              | Thin-layer chromatography             |

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**Author contributions:** PFS performed initial syntheses of two primary products (by a slightly different route not described), producing the **1a** and **1b** needed for early studies. FL designed and tested the new synthetic approach. DP and VIT synthesized, authenticated, and characterized the compounds. JR, RV, FL, and WB conceived and oversaw the study (and utilized the materials in other studies, as cited). All authors wrote and revised the manuscript.

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- Supplementary Material:** The online version of this article offers supplementary material describing further Materials and methods details including NMR and MS data for intermediates 2–9. The full raw Bruker NMR data for compounds 1a–d, including spectral annotations/assignments, structures, and processing methods are also available here (<https://doi.org/10.1515/hf-2021-0149>).