A group of sequence-related sphingomonad enzymes catalyzes cleavage of β-aryl ether linkages in lignin β-guaiacyl and β-syringyl ether dimers

Daniel L. Gall,[1,2]* John Ralph,[2,3] Timothy J. Donohue,[2,4] and Daniel R. Noguera[1,2,5]

[1]Department of Civil & Environmental Engineering, University of Wisconsin, Madison, WI 53706

[2]U.S. Department of Energy Great Lakes Bioenergy Research Center, Wisconsin Energy Institute, University of Wisconsin, Madison WI 53726

[3]Department of Biochemistry, University of Wisconsin, Madison, WI 53706

[4]Department of Bacteriology, University of Wisconsin, Madison, WI 53706

[5]Environmental Chemistry and Technology Program, University of Wisconsin, Madison WI 53706

*To whom correspondence should be addressed: Daniel Gall, 5175 Wisconsin Energy Institute, 1552 University Ave., Madison, WI 53726-4084. Phone: 608-265-8465; e-mail address: dlgall@wisc.edu

SUPPORTING INFORMATION – GENE CLONING

Preparation of pVP302K. An 822-bp fragment was amplified from pVP202K using upstream and downstream primers. An upstream 95-bp primer containing (a) a 5′-end (N-terminal) 26-bp region encoding a partial $NHis_8$ tag (for subsequent cloning into vector pVP102K), (b) an internal 41-bp region containing an in-frame Tev protease cleavage site (ENLYFQS), and (c) a 3′-end (C-terminal) 28-bp region (annealing to pVP202K) was obtained commercially. Additionally, a 57-bp downstream primer containing (a) a 5′-end 24-bp region (for subsequent cloning into vector pVP102K) and (b) a 3′-end 33-bp region (annealing to vector pVP202K) was designed. The resulting 822-bp fragment containing the N-terminal $NHis_8$ tag and Tev protease site, as well as the C-terminal *Vibrio cholarae* RtxA protease-$CHis_8$ fusion was subsequently cloned into pVP102K by PCR overlap extension,[1-4] yielding pVP302K.

Preparation of the pVP102K-RpHypGST expression plasmid. A 786-bp fragment encoding the RpHypGST (UniProt ID Q6N1R4_RHOPA) ORF was amplified from *Rhodopseudomonas palustris* genomic DNA and was cloned into the NheI and AgeI restriction sites of pVP102K, yielding pVP102K-RpHypGST. Because the N-terminus of SsLigE and LigE homologs was inaccessible to Tev protease cleavage, pVP102K-RpHypGST was designed for expression of LigE with residual N-terminal amino acid translated between the Tev cleavage site and the native start codon. The resulting construct allowed for cleavage of the $NHis_8$ tag by Tev protease, affording 28.6 kDa recombinant enzyme RpHypGST (Fig. S1A) with residual N-terminal amino acids after cleavage with Tev protease (termed N-RpHypGST hereafter). The yield of N-RpHypGST after expression and protein purification was 19.6 mg L_{cells}^{-1}.

Preparation of the pVP202K-RpHypGST expression plasmid. A 728-bp fragment encoding the RpHypGST (UniProt ID Q6N1R4_RHOPA) ORF was amplified from *R. palustris* genomic DNA and cloned into pVP202K. The plasmid was cut with BspHI and BsaI restriction enzymes, and vector

pVP202K was cut with NcoI and BsaI. The two resulting fragments were ligated, producing pVP202K-RpHypGST, which was used for the expression of recombinant 25.5 kDa RpHypGST with a residual C-terminal leucine residue (termed C-RpHypGST hereafter). The yield of C-RpHypGST after expression and protein purification was 20.8 mg L_{cells}^{-1}.

Preparation of the pVP202K-NaLigE expression plasmid. A 904-bp fragment encoding the NaLigE (UniProt ID Q2G5N2_NOVAD) ORF was amplified from *N. aromaticivorans* DSM12444 genomic DNA and cloned into pVP202K by the PCR overlap method[1-4] to yield pVP202K-NaLigE, which was used for the expression of the 31.1 kDa enzyme NaLigE (Fig. S1B). The yield of NaLigE after expression and protein purification was 40.4 mg L_{cells}^{-1}.

Preparation of the pVP202K-NsLigE expression plasmid. An 842-bp fragment containing the NsLigE-encoding (UniProt ID F6IKY6_9SPHN) ORF was amplified from pMK1157550 (obtained from Invitrogen) and cloned into the NcoI and BsaI restriction enzyme sites of pVP202K, producing pVP202K-NsLigE, which was used for the expression of the 30.8 kDa enzyme NsLigE (Fig. S1C). The yield of NsLigE after expression and protein purification was 74.9 mg L_{cells}^{-1}.

Preparation of the pVP202K-SsLigE expression plasmid. As previously described,[5] the ORF encoding *Sphingobium* sp. strain SYK-6 SsLigE (UniProt ID G2IN93_9SPHN) was cloned into expression vector pVP202K by the PCR overlap method.[1-4] The first round of PCR yielded a 906-bp amplicon, which was used to prime plasmid pVP202K in the second round of PCR, affording the 5,470-bp plasmid pVP202K-SsLigE, which was used for expression of 32.1 kDa SsLigE (Fig. S1D). The yield of SsLigE after expression and protein purification was 65.0 mg L_{cells}^{-1}.

Preparation of the pVP202K-SsLigP expression plasmid. As previously described,[5] a 902-bp fragment containing the SsLigP-encoding (UniProt ID E1CJ68_9SPHN) ORF and was cloned into vector pVP202K by the PCR overlap method,[1-4] affording plasmid pVP202K-SsLigP, which was used for the expression of 31.0 kDa SsLigP (Fig. S1E). The yield of SsLigP after expression and protein purification was 48.8 mg L_{cells}^{-1}.

Preparation of the pVP302K-NaLigF1 expression plasmid. A 707-bp fragment containing the NaLigF1-encoding (UniProt ID Q2G6J3_NOVAD) ORF was amplified from *N. aromaticivorans* DSM12444 genomic DNA and cloned into pVP302K by PCR overlap extension,[1-4] yielding pVP302K-NaLigF1, which was used for the expression of the 28.9 kDa enzyme NaLigF1 (Fig. S1F). The yield of NaLigF1 after expression and protein purification was 46.3 mg L_{cells}^{-1}.

Preparation of the pVP202K-NaLigF2 expression plasmid. An 839-bp fragment encoding the NaLigF2 (UniProt ID Q2G4C2_NOVAD) ORF was amplified from *N. aromaticivorans* DSM12444 genomic DNA and cloned into pVP202K by PCR overlap[1-4] to produce pVP202K-NaLigF2, which was used for expression of the 29.3 kDa enzyme NaLigF2 (Fig. S1G). The yield of NaLigF2 after expression and protein purification was 70.6 mg L_{cells}^{-1}.

Preparation of the pVP102K-SsLigF expression plasmid. As previously described,[5] an 822-bp fragment containing the SsLigF-encoding (UniProt ID G2IN92_9SPHN) ORF was amplified and the amplicon was restricted with AsiSI and SacII. The resulting 809-bp fragment was inserted into the AsiI-SacII region of pVP102K, affording plasmid pVP102K-SsLigF, which was used for the expression of 30.0 kDa SsLigF (Fig. S1H). The yield of SsLigF after expression and protein purification was 50.2 mg L_{cells}^{-1}.

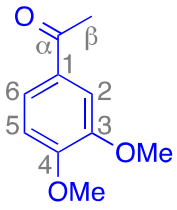
SUPPORTING INFORMATION – SYNTHETIC DETAILS AND NMR DATA

CHEMICAL SYNTHESES:

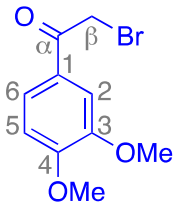
General. β-Ether-linked model compounds were synthesized according to the method of Adler and Eriksoo [6, 7]. R_f values were calculated as the ratio of elution volume to total solvent volume (v/v). Chromatographic elution volumes were calculated as the product of the mobile phase flow rate (in mL min^{-1}) and a given compound's retention time (t_R, in min).

Chemicals and reagents were purchased from Sigma-Aldrich. ^1H and ^{13}C NMR spectra were recorded on a Bruker Biospin (Billerica, MA) AVANCE 700 MHz spectrometer fitted with a cryogenically cooled 5-mm TXI gradient probe with inverse geometry (proton coils closest to the sample). Chemical shifts are reported in parts per million (ppm). The central NMR solvent peaks were used as internal references (δ_H: 2.05 ppm and δ_C: 29.8 ppm for acetone-d_6; δ_H: 4.79 ppm for (HDO in) D_2O [8, 9]. *J* values are recorded in Hz. Carbon and proton assignments for all compounds as labeled in the included ^1H and ^{13}C NMR spectra were determined by via the aid of 2D COSY, HSQC, and HMBC NMR spectra. Merck-EMD Millipore aluminum-backed Silica Gel 60 F_{254} normal-phase thin-layer chromatography plates were used for small-scale separation of organic compounds using a mixture of hexane and ethyl acetate as the mobile solvent. Biotage KP-Sil silica gel was used for preparative separations of organic compounds by flash chromatography using a CombiFlash R_f delivery module using a mixture of hexane and ethyl acetate as the mobile phase.

Synthesis of β-bromo-α-(4-O-methyl)-guaiacylethanone (Fig. S2A-B). A solution of ethyl acetate (200 mL), α-(4-O-methyl)-guaiacylethanone (7.24 g, 40.2 mmol), and pyridinium tribromide (13.5 g, 42.2 mmol) was prepared in a 500-mL round-bottom flask with magnetic stirring. After 30 min, the reaction mixture was washed three times with saturated Na_2CO_3, once with H_2O, and once with brine. The organic layer was then dried over $MgSO_4$ and the solvent was evaporated *in vacuo*. The resulting residue was then dissolved in hot methanol and allowed to cool, affording crystalline β-bromo-α-(4-O-methyl)-guaiacylethanone (6.1 g, 59% yield).

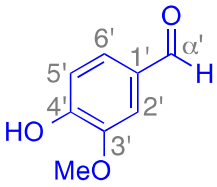


α-(4-O-methyl)-guaiacylethanone [from Sigma-Aldrich]:
^1H NMR (700 MHz, acetone-d_6) δ 7.61 (dd, 1H, *J* = 8.4, 2.1 Hz, H6); 7.49 (d, 1H, *J* = 2.1 Hz, H2); 7.01 (d, 1H, *J* = 8.4 Hz, H5); 3.88 (s, 3H, 4-OMe); 3.86 (s, 3H, 3-OMe); 2.51 (s, 3H, $H\beta_{a/b/c}$).
^{13}C NMR (176 MHz, acetone-d_6) δ 196.4 (Cα); 154.4 (C4); 150.0 (C3); 131.2 (C1); 123.8 (C6); 111.3 (C5); 111.1 (C2); 56.1 (4-OMe); 55.9 (3-OMe); 26.3 (Cβ).

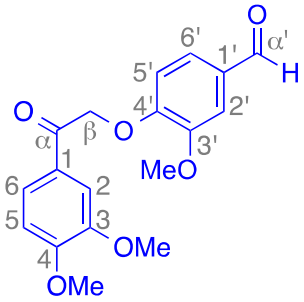


β-Bromo-α-(4-O-methyl)-guaiacylethanone [synthetic]:
^1H NMR (700 MHz, acetone-d_6) δ 7.72 (dd, 1H, *J* = 8.4, 2.1 Hz, H6); 7.55 (d, 1H, *J* = 2.1 Hz, H2); 7.08 (d, 1H, *J* = 8.4 Hz, H5); 4.69 (s, 2H, $H\beta_{a/b}$); 3.92 (s, 3H, 4-OMe); 3.88 (s, 3H, 3-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 190.5 (Cα); 155.1 (C4); 150.2 (C3); 127.9 (C1); 124.5 (C6); 111.8 (C5); 111.5 (C2); 56.2 (4-OMe); 56.0 (3-OMe); 32.5 (Cβ).

Synthesis of α-(4-O-methyl)-guaiacylethanone-β-(1′-formyl)-guaiacyl ether (Fig. S2A). To a 250-mL round-bottom flask containing a magnetically stirred solution of vanillin (2.4 g, 15.8 mmol) dissolved in acetone (60 mL), anhydrous K_2CO_3 (4.3 g, 31.0 mmol) was added and the reaction mixture was set to reflux for 10 min. β-bromo-α-(4-O-methyl)-guaiacylethanone (4.0 g, 15.5 mmol) dissolved in acetone (20 mL) was then added and the resulting mixture was incubated at refluxing temperature for 2 h. Carbonates were then removed by filtration and the filtrate was evaporated *in vacuo*. The resulting residue was taken up with ethyl acetate and washed twice with aqueous 1 N NaOH, twice with H_2O, and once with brine. The organic layer was then dried over $MgSO_4$ and the solvent was again evaporated *in vacuo*. The residue was then dissolved in hot ethanol and allowed to cool, affording crystalline α-(4-O-methyl)-guaiacylethanone-β-(1′-formyl)-guaiacyl ether (4.5 g, 87% yield).

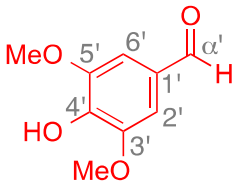


vanillin [from Sigma-Aldrich]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.83 (s, 1H, Hα′); 8.70 (s, 1H, 4′-OH); 7.46 (dd, 1H, J = 8.0, 1.8 Hz, H6′); 7.44 (d, 1H, J = 1.8 Hz, H2′); 7.01 (d, 1H, J = 8.0 Hz, H5′); 3.93 (s, 3H, 3′-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 191.0 (Cα′); 153.5 (C4′); 148.9 (C3′); 130.7 (C1′); 127.0 (C6′); 115.9 (C5′); 110.8 (C2′); 56.2 (3′-OMe).

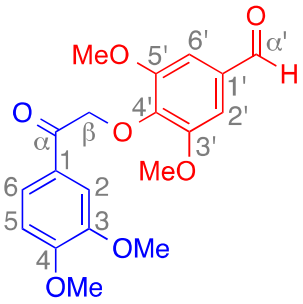


α-(4-O-methyl)-guaiacylethanone-β-(1′-formyl)-guaiacyl ether [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.86 (s, 1H, Hα′); 7.77 (dd, 1H, J = 8.4, 2.0 Hz, H6); 7.59 (d, 1H, J = 2.0 Hz, H2); 7.48 (dd, 1H, J = 8.7, 1.9 Hz, H6′); 7.46 (d, 1H, J = 1.9 Hz, H2′); 7.11 (d, 1H, J = 8.4 Hz, H5); 7.06 (d, 1H, J = 8.7 Hz, H5′); 5.63 (s, 2H, Hβ$_{a/b}$); 3.93 (s, 3H, 4-OMe); 3.92 (s, 3H, 3-OMe); 3.89 (s, 3H, 3′-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 192.6 (Cα); 191.2 (Cα′); 155.1 (C4); 154.3 (C4′); 150.8 (C3′); 150.3 (C3); 131.6 (C1′); 128.4 (C1); 126.2 (C6′); 123.5 (C6); 113.5 (C5′); 111.6 (C5); 111.2 (C2); 110.9 (C2′); 71.4 (Cβ); 56.2 (4-OMe); 56.2 (3-OMe); 56.1 (3′-OMe).

Synthesis of α-(4-O-methyl)-guaiacylethanone-β-(1′-formyl)-syringyl ether (Fig. S2B). The synthesis of α-(4-O-methyl)-guaiacylethanone-β-(1′-formyl)-syringyl ether was carried out via the same procedure that yielded α-(4-O-methyl)-guaiacylethanone-β-(1′-formyl)-guaiacyl ether. In this case, the starting materials were syringaldehyde (2.9 g, 15.8 mmol), acetone (total volume: 80 mL), anhydrous K_2CO_3 (4.3 g, 31.0 mmol), and β-bromo-α-(4-O-methyl)-guaiacylethanone (4.0 g, 15.5 mmol). From ethanol, α-(4-O-methyl)-guaiacylethanone-β-(1′-formyl)-syringyl ether was crystallized (4.7 g, 84% yield).

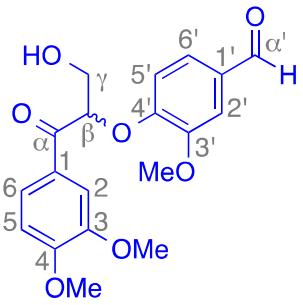


Syringaldehyde [from Sigma-Aldrich]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.82 (s, 1H, Hα′); 8.32 (s, 1H, 4′-OH); 7.24 (s, 2H, H2′/H6′); 3.92 (s, 6H, 3′-OMe/5′-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 191.1 (Cα′); 149.0 (C3′/ C5′); 142.9 (C4′); 129.0 (C1′); 107.7 (C2′/ C6′); 56.6 (3′-OMe/5′-OMe).



α-(4-O-methyl)-guaiacylethanone-β-(1′-formyl)-syringyl ether [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.91 (s, 1H, Hα′); 7.77 (dd, 1H, J = 8.4, 2.0 Hz, H6); 7.60 (d, 1H, J = 2.0 Hz, H2); 7.27 (s, 2H, H2′/H6′); 7.08 (d, 1H, J = 8.4 Hz, H5); 5.31 (s, 2H, Hβ$_{a/b}$); 3.91 (s, 3H, 4-OMe); 3.89 (s, 6H, 3′-OMe/5′-OMe); 3.88 (s, 3H, 3-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 193.2 (Cα); 191.6 (Cα′); 154.8 (C4); 154.2 (C3′/C5′); 150.1 (C3); 142.7 (C4′); 133.0 (C1′); 128.9 (C1); 123.7 (C6); 111.5 (C2); 111.4 (C5); 107.5 (C2′/ C6′); 75.2 (Cβ); 56.6 (3′-OMe/5′-OMe); 56.2 (4-OMe); 56.0 (3-OMe).

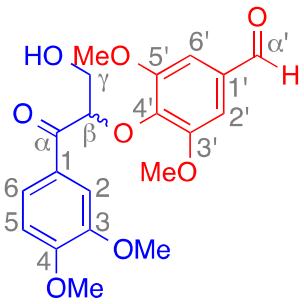
Synthesis of racemic α-(4-O-methyl)-guaiacylglycerone-β-(1′-formyl)-guaiacyl ether, *racem*-GβG (Fig. S2A). To a magnetically stirred solution of 1,4-dioxane (30 mL), α-(4-O-methyl)-guaiacylethanone-β-(1′-formyl)-guaiacyl ether (1.4 g, 4.3 mmol), and formaldehyde (0.13 g, 4.44 mmol, 0.34 mL of 37% formaldehyde in H_2O) in a 250-mL round-bottom flask, anhydrous K_2CO_3 (1.2 g, 8.7 mmol) was added and the reaction mixture was set to 40 °C. After 3 h, the reaction was cooled to room temperature, carbonates were removed by filtration, and 1,4-dioxane was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and washed three times with H_2O and once with brine. The organic layer was then dried over $MgSO_4$ and the solvent evaporated *in vacuo*. Racemic α-(4-O-methyl)-guaiacylglycerone-β-(1′-formyl)-guaiacyl ether (*racem*-GβG) was then crystallized from ethyl acetate and hexane (1.2 g, 79% yield). Enantiomers Gβ(*S*)G and Gβ(*R*)G were purified from crystalline *racem*-GβG (50 mg, 138.8 nmol) by repetitive (8–10 injections) preparative chiral chromatography (See experimental procedures in main text for details) to provide enantiopure starting material for the synthesis of MTPA(*R*)-esters.



α-(4-O-methyl)-guaiacylglycerone-β-(1′-formyl)-guaiacyl ether, *racem*-GβG [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.82 (s, 1H, Hα′); 7.86 (dd, 1H, J = 8.5, 2.0 Hz, H6); 7.64 (d, 1H, J = 2.0 Hz, H2); 7.44 (d, 1H, J = 1.9 Hz, H2′); 7.42 (dd, 1H, J = 8.2, 1.9 Hz, H6′); 7.08 (d, 1H, J = 8.5 Hz, H5); 6.98 (d, 1H, J = 8.2 Hz, H5′); 5.84 (t, 1H, J = 4.9 Hz, Hβ); 4.45 (t, 1H, J = 6.2 Hz, γ-OH); 4.15 – 4.10 (m, 2H, Hγ$_{a/b}$); 3.91 (s, 3H, 4-OMe); 3.90 (s, 3H, 3-OMe); 3.86 (s, 3H, 3′-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 194.7 (Cα); 191.2 (Cα′); 155.1 (C4); 153.6 (C3′); 150.9 (C3); 150.1 (C4′); 131.8 (C1′); 128.9 (C1); 126.2 (C6′); 124.2 (C6); 114.6 (C5′); 111.9 (C2); 111.5 (C5); 111.2 (C2′); 83.3 (Cβ); 63.9 (Cγ); 56.2 (4-OMe); 56.2 (3′-OMe); 56.0 (3-OMe).

Gβ(*S*)G:
Analytical HPLC (column AD-H, Hexane/EtOH = 3/2, flow rate = 1.0 mL min^{-1}) t_R = 16.6 min, R_f = 0.25. Preparative HPLC (column AY-H, Hexane/EtOH = 3/7–0/1 gradient, flow rate = 2.5 mL min^{-1}) t_R = 16.1 min, R_f = 0.49.

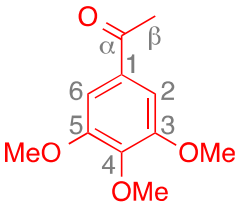
Gβ(*R*)G:
Analytical HPLC (column AD-H, Hexane/EtOH = 3/2, flow rate = 1.0 mL min^{-1}) t_R = 20.2 min, R_f = 0.21. Preparative HPLC (column AY-H, Hexane/EtOH = 3/7–0/1 gradient, flow rate = 2.5 mL min^{-1}) t_R = 27.7 min, R_f = 0.28.



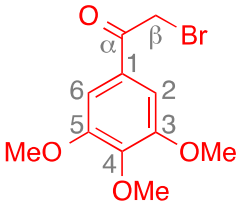
α-(4-O-methyl)-guaiacylglycerone-β-(1′-formyl)-guaiacyl ether, *racem*-GβS [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.90 (s, 1H, Hα′); 7.80 (dd, 1H, J = 8.4, 2.0 Hz, H6); 7.63 (d, 1H, J = 2.0 Hz, H2); 7.26 (s, 2H, H2′/H6′); 7.07 (d, 1H, J = 8.4 Hz, H5); 5.52 (t, 1H, J = 5.4 Hz, Hβ); 3.93 – 3.89 (m, 2H, $H\gamma_{a/b}$); 3.91 (s, 3H, 4-OMe); 3.88 (s, 3H, 3-OMe); 3.82 (s, 6H, 3′-OMe/5′-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 194.6 (Cα); 191.6 (Cα′); 154.6 (C4); 153.8 (C3′/C5′); 150.1 (C3); 142.5 (C4′); 133.0 (C1′); 129.6 (C1); 124.2 (C6); 111.9 (C2); 111.4 (C5); 107.4 (C2′/ C6′); 86.1 (Cβ); 63.9 (Cγ); 56.6 (3′-OMe/5′-OMe); 56.1 (4-OMe); 56.0 (3-OMe).

Gβ(*S*)S:
Analytical HPLC (column AD-H, Hexane/EtOH = 3/2, flow rate = 1.0 mL min^{-1}) t_R = 16.0 min, R_f = 0.26.
Gβ(*R*)S:
Analytical HPLC (column AD-H, Hexane/EtOH = 3/2, flow rate = 1.0 mL min^{-1}) t_R = 18.1 min, R_f = 0.23.

Synthesis of β-bromo-α-(4-O-methyl)-syringylethanone (Fig. S2C-D). The synthesis of β-bromo-α-(4-O-methyl)-syringylethanone was carried out via the same procedure that yielded β-bromo-α-(4-O-methyl)-guaiacylethanone. In this case, the starting materials were α-(4-O-methyl)-syringylethanone (3.0 g, 14.3 mmol) and pyridinium tribromide (4.8 g, 15.0 mmol) in ethyl acetate (50 mL). From methanol, β-bromo-α-(4-O-methyl)-syringylethanone was crystallized (2.7 g, 66% yield).

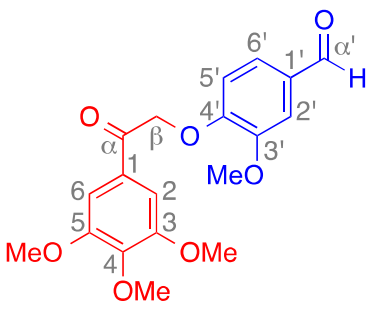


α-(4-O-methyl)-syringylethanone [from Sigma-Aldrich]:
^{1}H NMR (700 MHz, acetone-d_6) δ 7.30 (s, 2H, H2/H6); 3.90 (s, 6H, 3-OMe/5-OMe); 3.80 (s, 3H, 4-OMe); 2.56 (s, 3H, $H\beta_{a/b/c}$).
^{13}C NMR (176 MHz, acetone-d_6) δ 196.8 (Cα); 154.2 (C3/C5); 142.3 (C4); 133.5 (C1); 106.7 (C2/C6); 60.6 (4-OMe); 56.5 (3-OMe/5-OMe); 26.6 (Cβ).



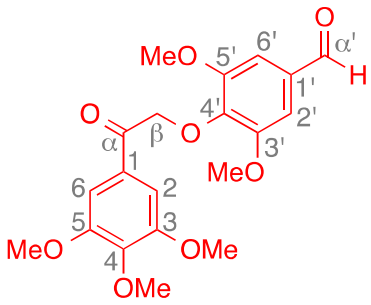
β-Bromo-α-(4-O-methyl)-syringylethanone [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 7.37 (s, 2H, H2/H6); 4.78 (s, 2H, $H\beta_{a/b}$); 3.91 (s, 6H, 3-OMe/5-OMe); 3.82 (s, 3H, 4-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 190.9 (Cα); 154.3 (C3/C5); 144.1 (C4); 130.3 (C1); 107.3 (C2/C6); 60.7 (4-OMe); 56.6 (3-OMe/5-OMe); 33.0 (Cβ).

Synthesis of α-(4-O-methyl)-syringylethanone-β-(1′-formyl)-guaiacyl ether (Fig. S2C). The synthesis of α-(4-O-methyl)-syringylethanone-β-(1′-formyl)-guaiacyl ether was carried out via the same procedure that yielded α-(4-O-methyl)-guaiacylethanone-β-(1′-formyl)-guaiacyl ether. In this case, the starting materials were vanillin (0.4 g, 2.7 mmol), acetone (total volume: 30 mL), anhydrous K_2CO_3 (0.7 g, 5.2 mmol), and β-bromo-α-(4-O-methyl)-syringylethanone (0.8 g, 2.6 mmol). From ethanol, α-(4-O-methyl)-syringylethanone-β-(1′-formyl)-guaiacyl ether was crystallized (0.9 g, 92% yield).



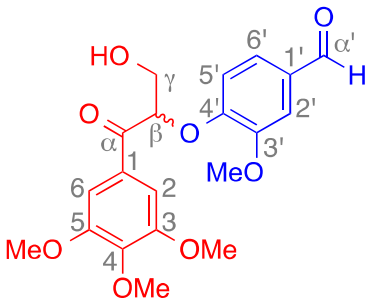
α-(4-O-methyl)-syringylethanone-β-(1′-formyl)-guaiacyl ether [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.86 (s, 1H, Hα′); 7.48 (dd, 1H, J = 8.7, 1.9 Hz, H6′); 7.47 (d, 1H, J = 1.9 Hz, H2′); 7.41 (s, 2H, H2/H6); 7.07 (d, 1H, J = 8.7 Hz, H5′); 5.68 (s, 2H, Hβ$_{a/b}$); 3.94 (s, 3H, 3′-OMe); 3.91 (s, 6H, 3-OMe/5-OMe); 3.83 (s, 3H, 4-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 193.1 (Cα); 191.2 (Cα′); 154.3 (C3/C5); 154.1 (C4′); 150.8 (C3′); 144.1 (C4); 131.6 (C1′); 130.7 (C1); 126.2 (C6′); 113.5 (C5′); 110.9 (C2′); 106.5 (C2/C6); 71.7 (Cβ); 60.7 (4-OMe); 56.6 (3-OMe/5-OMe); 56.2 (3′-OMe).

Synthesis of α-(4-O-methyl)-syringylethanone-β-(1′-formyl)-syringyl ether (Fig. S2D). The synthesis of α-(4-O-methyl)-syringylethanone-β-(1′-formyl)-syringyl ether was carried out via the same procedure that yielded α-(4-O-methyl)-guaiacylethanone-β-(1′-formyl)-guaiacyl ether. In this case, the starting materials were syringaldehyde (0.5 g, 2.7 mmol), acetone (total volume: 30 mL), anhydrous K_2CO_3 (0.7 g, 5.2 mmol), and β-bromo-α-(4-O-methyl)-syringylethanone (0.8 g, 2.6 mmol). From ethanol, α-(4-O-methyl)-syringylethanone-β-(1′-formyl)-syringyl ether was crystallized (0.9 g, 90% yield).



α-(4-O-methyl)-syringylethanone-β-(1′-formyl)-syringyl ether [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.91 (s, 1H, Hα′); 7.40 (s, 2H, H2/H6); 7.28 (s, 2H, H2′/H6′); 5.34 (s, 2H, Hβ$_{a/b}$); 3.90 (s, 6H, 3-OMe/5-OMe); 3.89 (s, 6H, 3′-OMe/5′-OMe); 3.82 (s, 3H, 4-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 193.7 (Cα); 191.6 (Cα′); 154.3 (C3/C5); 154.2 (C3′/C5′); 143.8 (C4); 142.6 (C4′); 133.1 (C1′); 131.1 (C1); 107.5 (C2′/C6′); 106.7 (C2/C6); 75.3 (Cβ); 60.6 (4-OMe); 56.6 (3′-OMe/5′-OMe); 56.5 (3-OMe/5-OMe).

Synthesis of racemic α-(4-O-methyl)-syringylglycerone-β-(1′-formyl)-guaiacyl ether, *racem*-SβG (Fig. S2C). The synthesis of racemic α-(4-O-methyl)-syringylglycerone-β-(1′-formyl)-guaiacyl ether (*racem*-SβG) was carried out via the same procedure that yielded *racem*-GβG. In this case, the starting materials were α-(4-O-methyl)-syringylethanone-β-(1′-formyl)-guaiacyl ether (0.8 g, 2.2 mmol), formaldehyde (0.07 g, 2.3 mmol, 0.17 mL of 37% formaldehyde in H_2O), 1,4-dioxane (20 mL), and anhydrous K_2CO_3 (0.6 g, 4.4 mmol). From ethyl acetate and hexane, *racem*-SβG was crystallized (0.7 g, 82% yield).

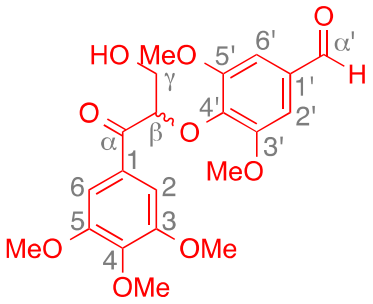


α-(4-O-methyl)-syringylglycerone-β-(1′-formyl)-guaiacyl ether, *racem*-SβG [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.83 (s, 1H, Hα′); 7.48 (s, 2H, H2/H6); 7.44 (d, 1H, J = 1.9 Hz, H2′); 7.43 (dd, 1H, J = 8.0, 1.9 Hz, H6′); 7.01 (d, 1H, J = 8.0 Hz, H5′); 5.86 (t, 1H, J = 4.8 Hz, Hβ); 4.47 (t, 1H, J = 6.2 Hz, γ-OH); 4.16 (dd, 2H, J = 6.2, 4.8 Hz, $H\gamma_{a/b}$); 3.91 (s, 3H, 3′-OMe); 3.89 (s, 6H, 3-OMe/5-OMe); 3.81 (s, 3H, 4-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 195.4 (Cα); 191.2 (Cα′); 154.2 (C3/C5); 153.5 (C4′); 150.9 (C3′); 145.5 (C4); 131.8 (C1′); 131.1 (C1); 126.2 (C6′); 114.6 (C5′); 111.2 (C2′); 107.2 (C2/C6); 83.5 (Cβ); 63.9 (Cγ); 60.6 (4-OMe); 56.5 (3-OMe/5-OMe); 56.2 (3′-OMe).

Sβ(*S*)G:
Analytical HPLC (column AY-H, Hexane/EtOH = 1/1, flow rate = 2.5 mL min^{-1}) t_R = 16.7 min, R_f = 0.47.

Sβ(*R*)G:
Analytical HPLC (column AY-H, Hexane/EtOH = 1/1, flow rate = 2.5 mL min^{-1}) t_R = 19.5 min, R_f = 0.40.

Synthesis of racemic α-(4-O-methyl)-syringylglycerone-β-(1′-formyl)-syringyl ether, *racem*-SβS (Fig. S2D). The synthesis of racemic α-(4-O-methyl)-syringylglycerone-β-(1′-formyl)-syringyl ether (*racem*-SβS) was carried out via the same procedure that yielded *racem*-GβG. In this case, the starting materials were α-(4-O-methyl)-syringylethanone-β-(1′-formyl)-syringyl ether (0.8 g, 2.1 mmol), formaldehyde (0.06 g, 2.1 mmol, 0.16 mL of 37% formaldehyde in H_2O), 1,4-dioxane (20 mL), and anhydrous K_2CO_3 (0.6 g, 4.1 mmol). From ethyl acetate and hexane, *racem*-SβS was crystallized (0.7 g, 78% yield). Enantiomers Sβ(*R*)S and Sβ(*S*)S were purified from crystalline *racem*-SβS (50 mg, 118.9 nmol) by repetitive (8–10 injections) preparative chiral chromatography (See experimental procedures in main text for details) to provide enantiopure starting material for the synthesis of SβS-MTPA(*R*) esters.



α-(4-O-methyl)-syringylglycerone-β-(1′-formyl)-syringyl ether, *racem*-SβS [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.90 (s, 1H, Hα′); 7.43 (s, 2H, H2/H6); 7.27 (s, 2H, H2′/H6′); 5.57 (t, 1H, J = 5.3 Hz, Hβ); 3.93 (d, 2H, J = 5.3 Hz, $H\gamma_{a/b}$); 3.89 (s, 6H, 3-OMe/5-OMe); 3.84 (s, 6H, 3′-OMe/5′-OMe); 3.82 (s, 3H, 4-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 195.2 (Cα); 191.6 (Cα′); 154.1 (C3/C5); 153.8 (C3′/C5′); 143.6 (C4); 142.4 (C4′); 133.0 (C1′); 132.0 (C1); 107.4 (C2′/C6′); 107.2 (C2/C6); 85.8 (Cβ); 63.7 (Cγ); 60.6 (4-OMe); 56.6 (3′-OMe/5′-OMe); 56.5 (3-OMe/5-OMe).

Sβ(*R*)S:
Analytical HPLC (column AY-H, Hexane/EtOH = 1/1, flow rate = 2.5 mL min^{-1}) t_R = 18.4 min, R_f = 0.43.
Preparative HPLC (column AY-H, Hexane/EtOH = 7/3, flow rate = 2.5 mL min^{-1}) t_R = 20.6 min, R_f = 0.38.

Sβ(*S*)S:
Analytical HPLC (column AY-H, Hexane/EtOH = 1/1, flow rate = 2.5 mL min^{-1}) t_R = 24.2 min, R_f = 0.32.
Preparative HPLC (column AY-H, Hexane/EtOH = 7/3, flow rate = 2.5 mL min^{-1}) t_R = 27.9 min, R_f = 0.28.

ASSIGNMENT OF ABSOLUTE CONFIGURATIONS TO Sβ(*S*)S AND Sβ(*R*)S:

General. To test whether Sβ(*R*)S elutes prior to the Sβ(*S*)S, or if the Lig β-etherases exhibited alternative stereospecificities for SβS enantiomers, we used a modification of Mosher's method to determine the absolute configuration of each isomer.[10] Using chiral chromatography, we purified a total of four enantiomers: two from *racem*-GβG with known absolute configurations, Gβ(*S*)G and Gβ(*R*)G,[11] as well as two from *racem*-SβS with unknown absolute β-configuration, low-t_R SβS, and high-t_R SβS.

Preparative separation of GβG enantiomers. To separate enantiomers Gβ(*S*)G and Gβ(*R*)G, crystalline *racem*-GβG (5.0 mg, 13.9 nmol) was dissolved in ethanol (5 mL) and injected into a CHIRALPAK AY-H column (10 by 250 mm). A mixture of ethanol and hexane was used as the mobile phase at a flow rate of 2.5 mL $min.^{-1}$ The ethanol fraction of the total flow (with hexane as the remainder) was adjusted over a gradient as follows: 0–8 min, 70% ethanol; 8–14 min, gradient from 70–100% ethanol; 14–35 min, 100% ethanol; 35–38 min, gradient from 100–70% ethanol; 38–45 min, 70% ethanol. Fractions containing Gβ(*S*)G (15–22 min) and Gβ(*R*)G (26–34 min) (Fig. S3A) were collected, pooled, and solvents were dried *in vacuo*. The aforementioned procedure was repeated four additional times in order to collect approximately 25 mg of each enantiomer. To aid in the assignment of absolute configuration to carbon β, enantiopure Gβ(*S*)G and Gβ(*R*)G were used as starting materials for syntheses of α(*R*)-methoxy-trifluoromethyl-phenylacetate (MTPA(*R*)) esters Gβ(*S*)G-MTPA(*R*) and Gβ(*R*)G-MTPA(*R*) (see below). β-Etherase assays with SsLigF1 confirmed that the low-t_R enantiomer (15–22 min) eluting from the AY-H column was Gβ(*S*)G whereas assays with SsLigE indicated that the high-t_R enantiomer (26–34 min) was Gβ(*R*)G (see Results for details).

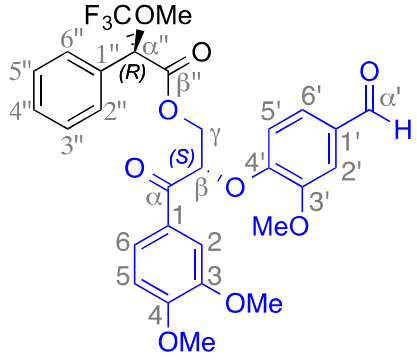
Preparative separation of SβS enantiomers. To separate enantiomers Sβ(*R*)S and Sβ(*S*)S, crystalline *racem*-SβS (5.0 mg, 11.9 nmol) was dissolved in ethanol (5 mL) and injected into a CHIRALPAK AY-H column (10 by 250 mm) using a 7/3 mixture of hexane/ethanol as the mobile phase at a flow rate of 2.5 mL $min.^{-1}$ Fractions containing Sβ(*R*)S (20-26 min) (Fig. S3B) and Sβ(*S*)S (28-36 min) were collected, pooled, and solvents were dried *in vacuo*. The aforementioned procedure was repeated four additional times in order to collect approximately 25 mg of each enantiomer for use in syntheses of SβS-MTPA(*R*) esters.

General procedure for syntheses of MTPA(*R*) esters. Enantiopure preparations of Gβ(*S*)G, Gβ(*R*)G, Sβ(*R*)S, and Sβ(*S*)S were obtained via preparative chiral chromatography (Fig. S3). In four parallel reactions, the Cγ-OH moieties of GβG enantiomers (10 mg, 27.8 nmol) and SβS enantiomers (10 mg, 23.8 nmol) were acylated using α(*S*)-methoxy-trifluoromethyl-phenyl-acetyl chloride (MTPACl(*S*)), producing four diastereometric MTPA(*R*) Cγ-esters. Phenylacetylation of Gβ(*S*)G (t_R = 15.1 min) and Gβ(*R*)G (t_R = 29.6 min) yielded Gβ(*S*)G-MTPA(*R*) and Gβ(*R*)G-MTPA(*R*), respectively. Phenylacetylation of Sβ(*R*)S (t_R = 27.6 min) and Sβ(*S*)S (t_R = 37.3 min) yielded Sβ(*R*)S-MTPA(*R*) and Sβ(*S*)S-MTPA(*R*), respectively.

General procedure for synthesis of GβG-propenone. Because both the Gβ(*S*)G-MTPA(*R*) and the Gβ(*R*)G-MTPA(*R*) preparations contained small amounts of contaminating α-(4-O-Me)-guaiacyl-β,γ-propenone-β-(1′-formyl)-guaiacyl ether (GβG-propenone), an additional reaction was carried out (over an extended reaction period) to synthesize pure GβG-propenone. Using *racem*-GβG (30 mg, 83.3 nmol) and MTPACl(*S*) as starting materials in a 2 h reaction, pure GβG-propenone was obtained and subsequently used as a standard for identifying the desired ^{1}H and ^{13}C resonances in the Gβ(*S*)G-MTPA(*R*) and Gβ(*R*)G-MTPA(*R*) NMR spectra.

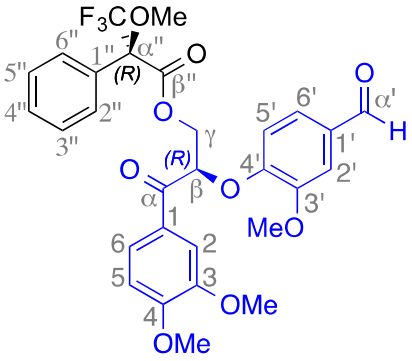
Synthesis of β(*S*)-(1′-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl α′′(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Gβ(*S*)G-MTPA(*R*) (Fig. S3). In a 50-mL conical flask with magnetic stirring at 0 °C, a solution of Gβ(*S*)G (10 mg, 28 nmol), diisopropylethylamine (7 mg, 55 nmol, 10 μL), and dimethylaminopyridine (2 mg, 14 nmol) dissolved in dichloromethane (2.0 mL), α′′(*S*)-methoxyl-

trifluoromethyl-phenyl acetyl chloride [MTPACl(*S*)] (28 mg, 111 nmol, 21 µL) was added dropwise. After 5 min, the reaction was collected in a Pasteur pipette and loaded onto an 8 g Redi*Sep*®R_f flash chromatography column, with which dichloromethane (20 mL) was used as the mobile phase. The column removed residual diisopropylethylamine, dimethylaminopyridine, and α′′(*S*)-methoxyl-trifluoromethyl-phenyl acetyl chloride, whereas the product MTPA(*R*) ester was collected from flow-through fractions. Dichloromethane was then evaporated *in vacuo* and the oil containing the desired product was analyzed by NMR spectroscopy. Integration of the ^{1}H NMR spectral regions for protons γH_a and γH_b revealed that the reaction products were a ~2:1 mixture β(*S*)-(1′-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl α′′(*R*)-methoxyl-trifluoromethyl-phenyl acetate, [Gβ(*S*)G-MTPA(*R*)] (6 mg, 38% yield) and a contaminant which was found to be a β,γ-unsaturated alkene, GβG-propenone (2 mg, 21% yield).



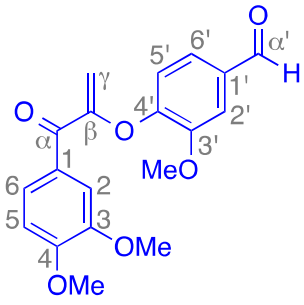
β(*S*)-(1′-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl α′′(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Gβ(*S*)G-MTPA(*R*) [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.85 (s, 1H, Hα′); 7.86 (dd, 1H, *J* = 8.5, 2.0 Hz, H6); 7.61 (d, 1H, *J* = 2.0 Hz, H2); 7.51 (d, 2H, *J* = 7.8 Hz, H3′′/H5′′); 7.45 (d, 1H, *J* = 1.8 Hz, H2′); 7.43 – 7.35 (m, 3H, H2′′/H6′′/H4′′); 7.41 (dd, 1H, *J* = 8.3, 1.8 Hz, H6′); 7.08 (d, 1H, *J* = 8.5 Hz, H5); 6.99 (d, 1H, *J* = 8.3 Hz, H5′); 6.26 (dd, 1H, *J* = 6.6, 3.2 Hz, Hβ); 4.99 (dd, 1H, *J* = 12.2, 3.2 Hz, Hγ_a); 4.91 (dd, 1H, *J* = 12.2, 6.6 Hz, Hγ_b); 3.92 (s, 3H, 4-OMe); 3.84 (s, 3H, 3-OMe); 3.83 (s, 3H, 3′-OMe); 3.51 (bs, 3H, α′′-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 192.6 (Cα); 191.2 (Cα′); 166.9 (Cβ′′); 155.4 (C4); 152.6 (C4′); 151.0 (C3′); 150.2 (C3); 132.8 (C1′′); 132.3 (C1′); 130.5 (C4′′); 129.2 (C2′′/C6′′); 128.3 (C1); 128.3 (C3′′/C5′′); 126.0 (C6′); 124.3 (C6); 124.2 (α′′-CF_3); 115.3 (C5′); 111.7 (C2); 111.6 (C2′); 111.5 (C5); 85.7 (α′′); 78.7 (Cβ); 66.5 (Cγ); 56.3 (4-OMe); 56.3 (3′-OMe); 56.0 (3-OMe); 55.9 (α′′-OMe).

Synthesis of β(*R*)-(1′-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl α′′(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Gβ(*R*)G-MTPA(*R*) (Fig. S3). The synthesis of β(*R*)-(1′-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl α′′(*R*)-methoxyl-trifluoromethyl-phenyl acetate [Gβ(*R*)G-MTPA(*R*)] was carried out via the same procedure that yielded Gβ(*S*)G-MTPA(*R*). However, since Gβ(*R*)G-MTPA(*R*) was the desired product, Gβ(*R*)G (10 mg, 28 nmol) was used as the starting material [instead of Gβ(*S*)G]. Integration of the ^{1}H NMR spectral regions for protons γH_a and γH_b revealed that the reaction products were a ~2:1 mixture Gβ(*R*)G-MTPA(*R*) (7 mg, 44% yield) and GβG-propenone (2 mg, 21% yield).



β(*R*)-(1′-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl α′′(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Gβ(*R*)G-MTPA(*R*) [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.85 (s, 1H, Hα′); 7.84 (dd, 1H, J = 8.5, 2.0 Hz, H6); 7.58 (d, 1H, J = 2.0 Hz, H2); 7.51 (d, 2H, J = 7.8 Hz, H3′′/H5′′); 7.47 (d, 1H, J = 1.8 Hz, H2′); 7.42 – 7.33 (m, 3H, H2′′/H6′′/H4′′); 7.42 (dd, 1H, J = 8.3, 1.8 Hz, H6′); 7.08 (d, 1H, J = 8.5 Hz, H5); 6.99 (d, 1H, J = 8.3 Hz, H5′); 6.29 (dd, 1H, J = 6.1, 3.1 Hz, Hβ); 5.10 (dd, 1H, J = 12.2, 3.1 Hz, Hγ_a); 4.86 (dd, 1H, J = 12.2, 6.1 Hz, Hγ_b); 3.92 (s, 3H, 4-OMe); 3.88 (s, 3H, 3-OMe); 3.84 (s, 3H, 3′-OMe); 3.57 (bs, 3H, α′′-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 192.3 (Cα); 191.2 (Cα′); 166.9 (Cβ′′); 155.4 (C4); 152.6 (C4′); 151.0 (C3′); 150.2 (C3); 132.8 (C1′′); 132.3 (C1′); 130.5 (C4′′); 129.2 (C2′′/C6′′); 128.2 (C1); 128.2 (C3′′/C5′′); 125.9 (C6′); 124.2 (C6); 124.2 (α′′-CF_3); 115.2 (C5′); 111.7 (C2); 111.6 (C2′); 111.5 (C5); 85.5 (α′′); 78.7 (Cβ); 66.5 (Cγ); 56.3 (4-OMe); 56.3 (3′-OMe); 56.1 (3-OMe); 56.0 (α′′-OMe).

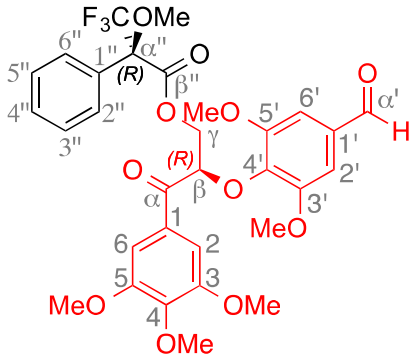
Synthesis of α-(4-O-methyl)-guaiacyl-β,γ-propenone-β-(1′-formyl)-guaiacyl ether, GβG-propenone. So that the ^{1}H and ^{13}C NMR spectral regions of the contaminating β,γ-unsaturated alkene could be identified in the Gβ(*S*)G-MTPA(*R*) and Gβ(*R*)G-MTPA(*R*) reaction products, we synthesized α-(4-O-methyl)-guaiacyl-β,γ-propenone-β-(1′-formyl)-guaiacyl ether (GβG-propenone). The synthesis of GβG-propenone was carried out via the same procedures that yielded Gβ(*S*)G-MTPA(*R*) and Gβ(*R*)G-MTPA(*R*). In this case, the starting materials were *racem*-GβG (30 mg, 83 nmol), diisopropylethylamine (21 mg, 165 nmol, 30 µL), and dimethylaminopyridine (6 mg, 42 nmol) dissolved in dichloromethane (4.0 mL), with dropwise addition of MTPACl(*S*) (83 mg, 333 nmol, 63 µL). Further, the reaction was incubated for a period of 2 h (rather than 5 min) after the addition of MTPACl(*S*) to enable MTPA(*R*) elimination and formation of GβG-propenone to reach completion. After the extended reaction period, neither Gβ(*S*)G-MTPA(*R*), nor Gβ(*R*)G-MTPA(*R*) was found in the products. ^{1}H and ^{13}C NMR revealed that the reaction had afforded pure GβG-propenone (20 mg, 70% yield).



α-(4-O-methyl)-guaiacyl-β,γ-propenone-β-(1′-formyl)-guaiacyl ether, GβG-propenone, [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.94 (s, 1H, Hα′); 7.77 (dd, 1H, J = 8.4, 2.0 Hz, H6); 7.58 (d, 1H, J = 1.8 Hz, H2′); 7.56 (d, 1H, J = 2.0 Hz, H2); 7.55 (dd, 1H, J = 8.0, 1.8 Hz, H6′); 7.25 (d, 1H, J = 8.0 Hz, H5′); 7.08 (d, 1H, J = 8.4 Hz, H5); 5.37 (d, 1H, J = 2.4 Hz, Hγ_a); 5.08 (d, 1H, J = 2.4 Hz, Hγ_b); 3.96 (s, 3H, 3′-OMe); 3.91 (s, 3H, 4-OMe); 3.88 (s, 3H, 3-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 191.5 (Cα′); 188.4 (Cα); 157.4 (Cβ); 154.8 (C4); 151.9 (C3′); 150.0 (C4′); 149.9 (C3); 134.7 (C1′); 129.3 (C1); 125.4 (C6); 125.4 (C6′); 120.6 (C5′); 112.8 (C2); 112.4 (C2′); 111.3 (C5); 104.0 (Cγ); 56.4 (3′-OMe); 56.2 (4-OMe); 56.0 (3-OMe).

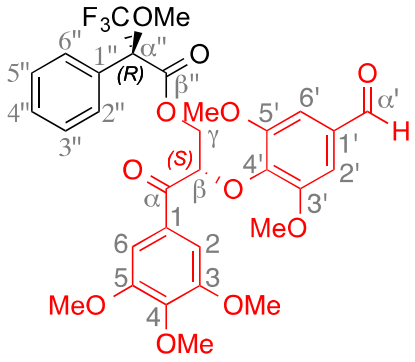
Synthesis of β(*R*)-(1′-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl α′′(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Sβ(*R*)S-MTPA(*R*) (Fig. S3). In a 50-mL conical flask with magnetic stirring at 0 °C, a solution of Sβ(*R*)S (12 mg, 28 nmol), diisopropylethylamine (7 mg, 55 nmol, 10 µL), and dimethylaminopyridine (2 mg, 14 nmol) dissolved in dichloromethane (2.0 mL), MTPACl(*S*) (28 mg, 111 nmol, 21 µL) was added dropwise. After 5 min, the reaction was collected in a Pasteur pipette and loaded onto an 8 g Redi*Sep*®R_f flash chromatography column, with which dichloromethane (20 mL) was used as the mobile phase. The column removed residual diisopropylethylamine, dimethylaminopyridine,

and α´´(*S*)-methoxyl-trifluoromethyl-phenyl acetyl chloride, whereas the product MTPA(*R*) ester was collected from flow-through fractions. Dichloromethane was then evaporated *in vacuo* and the oil containing the desired product was analyzed by NMR spectroscopy. ^{1}H and ^{13}C NMR revealed that the reaction had afforded essentially pure β(*R*)-(1´-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl α´´(*R*)-methoxyl-trifluoromethyl-phenyl acetate [Sβ(*R*)S-MTPA(*R*)] (10 mg, 65% yield).



β(*R*)-(1´-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl α´´(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Sβ(*R*)S-MTPA(*R*) [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.88 (s, 1H, Hα´); 7.46 – 7.36 (m, 5H, H2´´/H3´´/H4´´/H5´´/H6´´); 7.37 (s, 2H, H2/H6); 7.22 (s, 2H, H2´/H6´); 6.13 (dd, 1H, *J* = 5.3, 4.2 Hz, Hβ); 5.00 (dd, 1H, *J* = 11.9, 4.2 Hz, Hγ_a); 4.68 (dd, 1H, *J* = 11.9, 5.3 Hz, Hγ_b); 3.84 (s, 6H, 3-OMe/5-OMe); 3.82 (s, 3H, 4-OMe); 3.80 (s, 6H, 3´-OMe/5´-OMe); 3.51 (bs, 3H, α´´-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 193.4 (Cα); 191.6 (Cα´); 166.8 (Cβ´´); 154.1 (C3/C5); 153.7 (C3´/C5´); 143.9 (C4); 141.2 (C4´); 133.1 (C1´); 132.8 (C1); 131.3 (C1´´); 130.5 (C4´´); 129.2 (C2´´/C6´´); 128.2 (C3´´/C5´´); 124.2 (α´´-CF_3); 107.3 (C2´/C6´); 107.2 (C2/C6); 85.3 (α´´); 80.4 (Cβ); 66.9 (Cγ); 60.7 (4-OMe); 56.5 (3´-OMe/5´-OMe); 56.5 (3-OMe/5-OMe); 55.9 (α´´-OMe).

Synthesis of β(*S*)-(1´-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl α´´(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Sβ(*S*)S-MTPA(*R*) (Fig. S3). The synthesis of β(*S*)-(1´-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl α´´(*R*)-methoxyl-trifluoromethyl-phenyl acetate [Sβ(*S*)S-MTPA(*R*)] was carried out via the same procedure that yielded Sβ(*R*)S-MTPA(*R*). However, since Sβ(*S*)S-MTPA(*R*) was the desired product, Sβ(*S*)S (12 mg, 28 nmol) was used as the starting material [instead of Sβ(*R*)S]. ^{1}H and ^{13}C NMR revealed that the reaction had afforded essentially pure Sβ(*S*)S-MTPA(*R*) (9 mg, 59% yield).



β(*S*)-(1´-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl α´´(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Sβ(*S*)S-MTPA(*R*) [synthetic]:
^{1}H NMR (700 MHz, acetone-d_6) δ 9.88 (s, 1H, Hα´); 7.47 – 7.36 (m, 5H, H2´´/H3´´/H4´´/H5´´/H6´´); 7.40 (s, 2H, H2/H6); 7.21 (s, 2H, H2´/H6´); 6.08 (dd, 1H, *J* = 5.8, 4.2 Hz, Hβ); 4.92 (dd, 1H, *J* = 11.9, 4.2 Hz, Hγ_a); 4.77 (dd, 1H, *J* = 11.9, 5.8 Hz, Hγ_b); 3.85 (s, 6H, 3-OMe/5-OMe); 3.81 (s, 3H, 4-OMe); 3.76 (s, 6H, 3´-OMe/5´-OMe); 3.45 (bs, 3H, α´´-OMe).
^{13}C NMR (176 MHz, acetone-d_6) δ 193.6 (Cα); 191.6 (Cα´); 166.9 (Cβ´´); 154.1 (C3/C5); 153.6 (C3´/C5´); 143.9 (C4); 141.3 (C4´); 133.1 (C1´); 132.7 (C1); 131.3 (C1´´); 130.5 (C4´´); 129.2 (C2´´/C6´´); 128.2 (C3´´/C5´´); 124.2 (α´´-CF_3); 107.3 (C2´/C6´); 107.3 (C2/C6); 85.6 (α´´); 80.3 (Cβ); 66.9 (Cγ); 60.7 (4-OMe); 56.5 (3´-OMe/5´-OMe); 56.5 (3-OMe/5-OMe); 55.9 (α´´-OMe).

Analysis of 1H NMR spectra for assignment of absolute configurations to Sβ(*S*)S and Sβ(*R*)S. Given that (a) SsLigE and SsLigP each cleaves a diguaiacyl β(*R*)-ether bond and SsLigF1 degrades the β(*S*)-enantiomer,[5, 11-13] (b) the low-t_R GβG enantiomer was cleaved by SsLigF1 (Fig. 2H), and (c) the high-t_R GβG enantiomer was cleaved by both SsLigE (Fig. 2D) and SsLigP (Fig. 2E), we have assigned the absolute β(*S*)-configuration to the low-t_R isomer (Gβ(*S*)G) and the β(*R*)-configuration to the high-t_R enantiomer (Gβ(*R*)G). In parallel reactions (Fig. S3A), Cγ-MTPA(*R*) esters were synthesized by acylating (with MTPACl(*S*)) the Cγ-OH positions of enantiopure Gβ(*S*)G and Gβ(*R*)G, yielding two diastereometric derivatives, Gβ(*S*)G-MTPA(*R*) and Gβ(*R*)G-MTPA(*R*), and small quantities of an achiral β,γ-unsaturated alkene (GβG-propenone) that appeared in each preparation. We then compared the aligned ^{1}H NMR spectra of Gβ(*S*)G-MTPA(*R*) (Fig. S4A) and Gβ(*R*)G-MTPA(*R*) (Fig. S4B) and observed that splitting of the γH_a and γH_b proton spectral regions were unique to each diastereomer, as expected for an MTPA(*R*)-acylated primary alcohol with an adjacent chiral carbon.[14] We found that the difference in chemical shifts between the γH_a and γH_b spectral regions (termed $\Delta\delta_{ab}$ hereafter) was lesser for the Gβ(*S*)G-MTPA(*R*) diastereomer ($\Delta\delta_{ab}$ = 0.08 ppm, Fig. S4A) than for the Gβ(*R*)G-MTPA(*R*) ester ($\Delta\delta_{ab}$ = 0.24 ppm, Fig. S4B).

Based on the chromatogram alignment of β-etherase assays with substrate *racem*-SβS (Fig. 5), we hypothesized that the low-t_R SβS isomer was Sβ(*R*)S and that the high-t_R SβS enantiomer was Sβ(*S*)S. To test this, we again synthesized two Cγ-MTPA(*R*) esters (Fig. S3B) from enantiopure SβS isomers. We then analyzed the ^{1}H NMR spectra of the Cγ-MTPA(*R*) ester derived from the low-t_R SβS isomer (Fig. S4C) and of the Cγ-MTPA(*R*) ester derived from the high-t_R SβS enantiomer (Fig. S4D). We found that splitting of the γH_a and γH_b spectral regions was greater for the MTPA(*R*) ester of the low-t_R SβS isomer ($\Delta\delta_{ab}$ = 0.32 ppm, Fig. S4C) than for the MTPA(*R*) ester of the high-t_R SβS isomer ($\Delta\delta_{ab}$ = 0.15 ppm, Fig. S4D). Thus, based on the similarities between of the γH_a–γH_b splitting patterns of Gβ(*R*)G-MTPA(*R*) (Fig. S4B) and the low-t_R SβS isomer-derived MTPA(*R*) ester (Fig. S4C), we have confirmed the hypothesis and assigned an absolute configuration to the low-t_R SβS enantiomer and have concluded its identity to be Sβ(*R*)S. We have also assigned the high-t_R SβS enantiomer as Sβ(*S*)S due to the similarity of its MTPA(*R*) ester's ^{1}H NMR spectrum (Fig. S4D) with that of Gβ(*S*)G-MTPA(*R*) (Fig. S4A). Thus, as was the case with substrates GβG (Fig. 2), GβS (Fig. 3), and SβG (Fig. 4), we conclude that each LigE/LigP homolog exhibits β(*R*)-stereospecificity whereas each LigF homolog catalyzes β(*S*)-ether cleavage of SβS enantiomers (Fig. 5).

SUPPORTING INFORMATION REFERENCES

1. Shevchuk, N. A.; Bryksin, A. V.; Nusinovich, Y. A.; Cabello, F. C.; Sutherland, M.; Ladisch, S., Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. *Nucleic Acids Research* **2004,** *32*, (2).
2. Bryksin, A. V.; Matsumura, I., Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids. *Biotechniques* **2010,** *48*, (6), 463-465.
3. Horton, R. M.; Cai, Z.; Ho, S. N.; Pease, L. R., Gene splicing by overlap extension: Tailor-made genes using the polymerase chain reaction. *Biotechniques* **2013,** *54*, (3), 129-133.
4. Horton, R. M., *In vitro* recombination and mutagenesis of DNA : SOEing together tailor-made genes. *Methods in molecular biology (Clifton, N.J.)* **1993,** *15*, 251-61.
5. Gall, D. L.; Kim, H.; Lu, F.; Donohue, T. J.; Noguera, D. R.; Ralph, J., Stereochemical features of glutathione-dependent enzymes in the *Sphingobium* sp. strain SYK-6 β-aryl etherase pathway. *J Biol Chem* **2014,** *289*, (12), 8656-67.
6. Adler, E.; Eriksoo, E., Guaiacylglycerol and its β-guaiacyl ether. *Acta chemica Scandinavica* **1955,** *9*, 341-342.
7. Landucci, L. L.; Geddes, S. A.; Kirk, T. K., Synthesis of C-14-labeled 3-methoxy-4-hydroxy-α-(2-methoxyphenoxy)-β-hydroxypropiophenone, a lignin model-compound. *Holzforschung* **1981,** *35*, (2), 67-70.
8. Gottlieb, H. E.; Kotlyar, V.; Nudelman, A., NMR chemical shifts of common laboratory solvents as trace impurities. *Journal of Organic Chemistry* **1997,** *62*, (21), 7512-7515.
9. Fulmer, G. R.; Miller, A. J. M.; Sherden, N. H.; Gottlieb, H. E.; Nudelman, A.; Stoltz, B. M.; Bercaw, J. E.; Goldberg, K. I., NMR chemical shifts of trace impurities: Common laboratory solvents, organics, and gases in deuterated solvents relevant to the organometallic chemist. *Organometallics* **2010,** *29*, (9), 2176-2179.

10. Dale, J. A.; Mosher, H. S., Nuclear magnetic-resonance enantiomer reagents - configurational correlations via nuclear magnetic resonance chemical shifts of diastereomeric mandelate, *o*-methylmandelate, and α-methoxy-α-trifluoromethylphenylacetate (MTPA) esters. *Journal of the American Chemical Society* **1973,** *95*, (2), 512-519.
11. Hishiyama, S.; Otsuka, Y.; Nakamura, M.; Ohara, S.; Kajita, S.; Masai, E.; Katayama, Y., Convenient synthesis of chiral lignin model compounds via optical resolution: four stereoisomers of guaiacylglycerol-β-guaiacyl ether and both enantiomers of 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxy-phenoxy)-propan-1-one (erone). *Tetrahedron Letters* **2012,** *53*, 842-845.
12. Tanamura, K.; Abe, T.; Kamimura, N.; Kasai, D.; Hishiyama, S.; Otsuka, Y.; Nakamura, M.; Kajita, S.; Katayama, Y.; Fukuda, M.; Masai, E., Characterization of the third glutathione S-transferase gene involved in enantioselective cleavage of the β-aryl ether by *Sphingobium* sp. Strain SYK-6. *Bioscience, biotechnology, and biochemistry* **2011,** *75*, (12), 2404-7.
13. Masai, E.; Ichimura, A.; Sato, Y.; Miyauchi, K.; Katayama, Y.; Fukuda, M., Roles of the enantioselective glutathione S-transferases in cleavage of β-aryl ether. *Journal of Bacteriology* **2003,** *185*, (6), 1768-1775.
14. Tsuda, M.; Toriyabe, Y.; Endo, T.; Kobayashi, J., Application of modified Mosher's method for primary alcohols with a methyl group at C2 position. *Chemical & pharmaceutical bulletin* **2003,** *51*, (4), 448-451.

SUPPORTING INFORMATION FIGURES

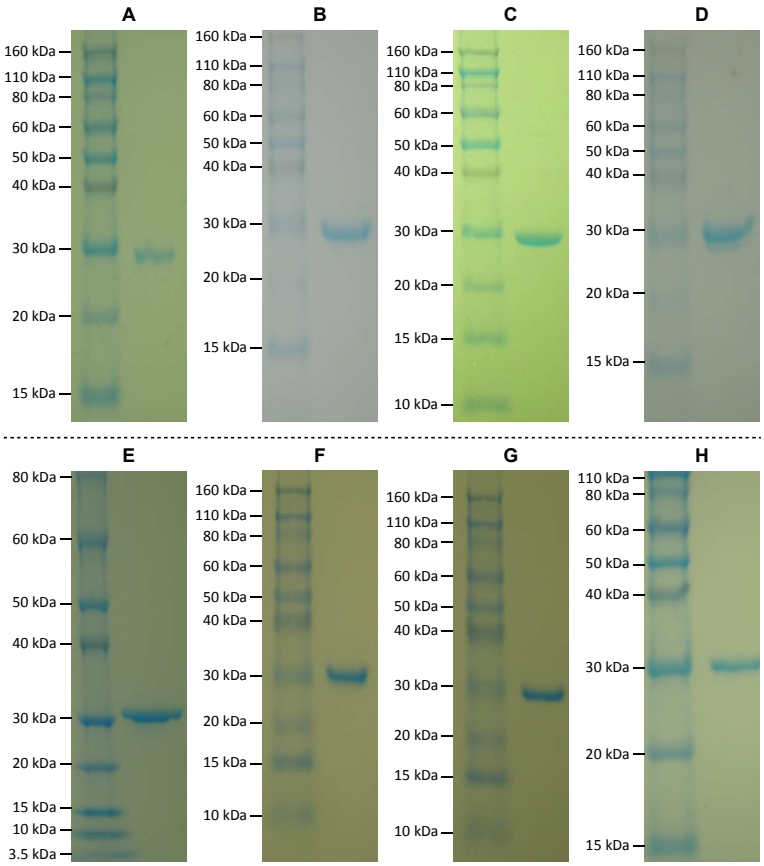


Fig. S1. Images of SDS-12% PAGE gels loaded with enzyme preparations (**A**), 28.6-kDa RpHypGST (**B**), 31.1-kDa NaLigE (**C**), 30.8-kDa NsLigE (**D**) 32.1-kDa SsLigE, (**E**) 31.0-kDa SsLigP, (**F**) 28.9-kDa NaLigF1, (**G**) 29.3-kDa NaLigF2, and (**H**) 30.0-kDa SsLigF.

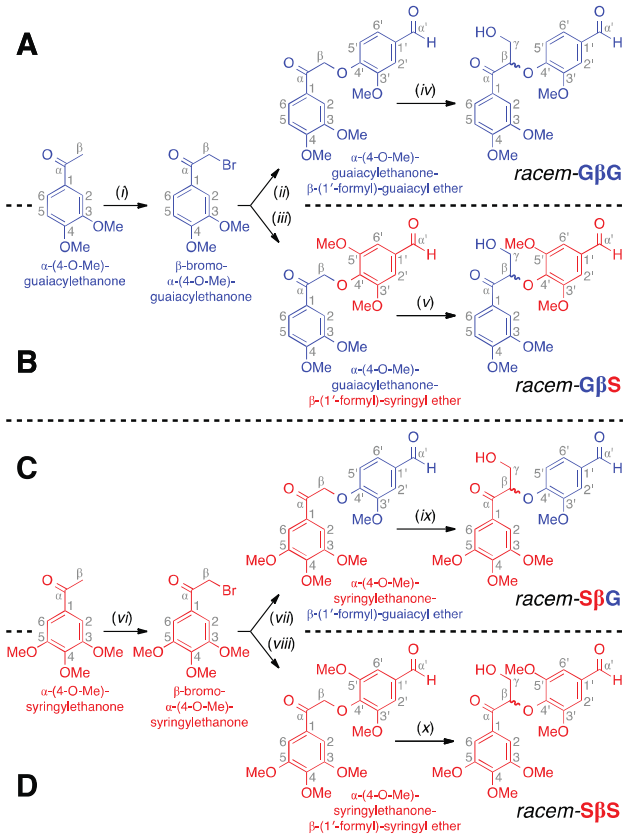


Fig. S2. Synthetic schemes for the preparation of β-etherase substrates (**A**) **GβG**, (**B**) **GβS**, (**C**) **SβG**, and (**D**) **SβS**. Reagents and conditions: (*i*) pyridinium tribromide, EtOAc, 30 min, 59%; (*ii*) vanillin, K_2CO_3, acetone, 1 h, 87%; (*iii*) syringaldehyde, K_2CO_3, acetone, 1 h, 84%; (*iv*) formaldehyde, K_2CO_3, 1,4-dioxane, 3 h, 79%; (*v*) formaldehyde, K_2CO_3, 1,4-dioxane, 3 h, 88%; (*vi*) pyridinium tribromide, EtOAc, 30 min, 66%; (*vii*) vanillin, K_2CO_3, acetone, 1 h, 92%; (*viii*) syringaldehyde, K_2CO_3, acetone, 1 h, 90%; (*ix*) formaldehyde, K_2CO_3, 1,4-dioxane, 3 h, 82%; (*x*) formaldehyde, K_2CO_3, 1,4-dioxane, 3 h, 78%.

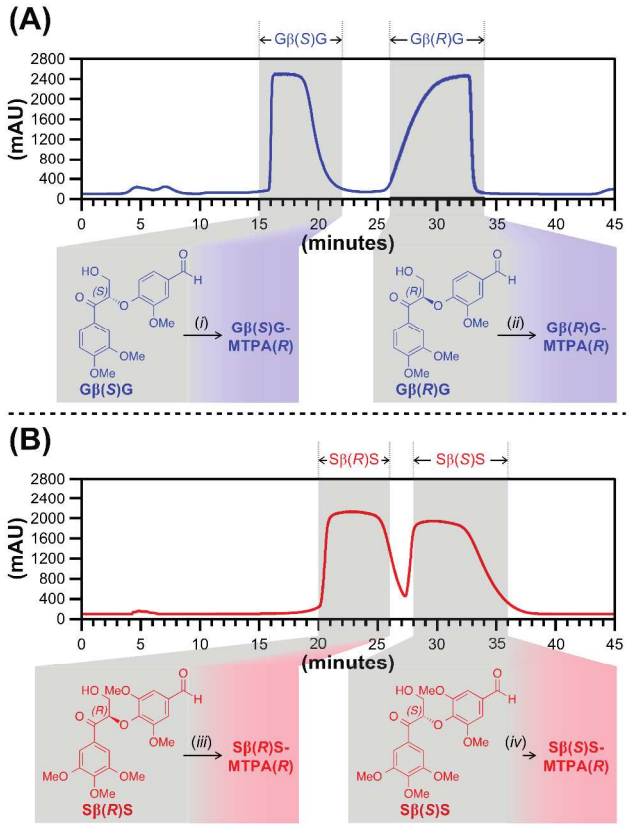


Fig. S3. Preparative chiral HPLC chromatographic separations (CHIRALPAK AY-H column, λ = 280 nm) of (**A**) *racem*-**GβG** starting material, yielding **Gβ(*S*)G** (15-22 min) and **Gβ(*R*)G** (26-34 min) and (**B**) *racem*-**SβS** starting material, yielding **Sβ(*R*)S** (20-26 min) and **Sβ(*R*)S** (28-36 min). Reagents and conditions: (*i-iv*) diisopropylethylamine, dimethylaminopyridine, MTPACl(*S*), dichloromethane, 5 min, flash chromatography. Reaction yields: (*i*) 38%, (*ii*) 44%, (*iii*) 65%, (*iv*) 59%. Product MTPA(*R*) esters: (*i*) **Gβ(*S*)G-MTPA(*R*)**; (*ii*) **Gβ(*R*)G-MTPA(*R*)**; (*iii*) **Sβ(*R*)S-MTPA(*R*)**; and (*iv*) **Sβ(*S*)S-MTPA(*R*)**. Chemical structures of MTPA(*R*) esters are shown in Fig. S4.

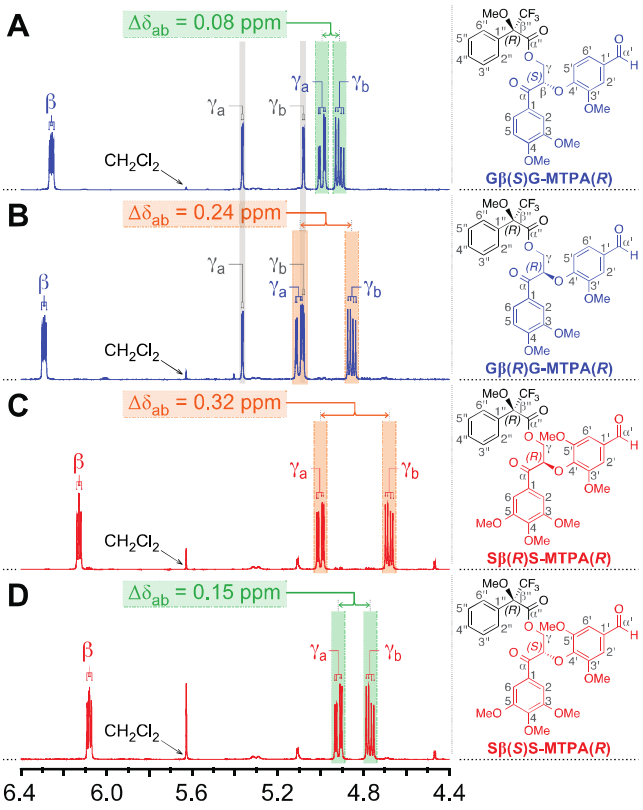


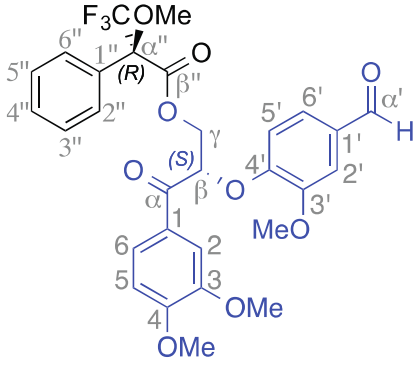
Fig. S4. Aligned ^{1}H NMR spectra (4.40–6.40 ppm) of β-(1′-formyl)-guaiacyl-α-(4-O-Me)-guaiacylglyceryl α(*R*)-methoxy-trifluoromethyl-phenyl-acetate (**GβG-MTPA(*R*)** esters; shown in **blue**) and β-(1′-formyl)-syringyl-α-(4-O-Me)-syringylglyceryl α(*R*)-methoxy-trifluoromethyl-phenyl-acetate (**SβS-MTPA(*R*)** esters; shown in **red**). Proton assignment labels correspond with the carbon to which the proton is bound. Alphabetical subscripts differentiate two non-identical geminal protons. γH_a and γH_b proton spectral regions are highlighted by shading for β(*R*)-configured (**orange**), β(*S*)-configured (**green**), and achiral GβG-propenone (**grey**). (**A**) **Gβ(*S*)G-MTPA(*R*)**, (**B**) **Gβ(*R*)G-MTPA(*R*)**, (**C**) **Sβ(*R*)S-MTPA(*R*)**, and (**D**) **Sβ(*S*)S-MTPA(*R*)**.

SUPPORTING INFORMATION - ^{1}H AND ^{13}C NMR SPECTRA OF MODEL COMPOUNDS AND REACTION PRODUCTS

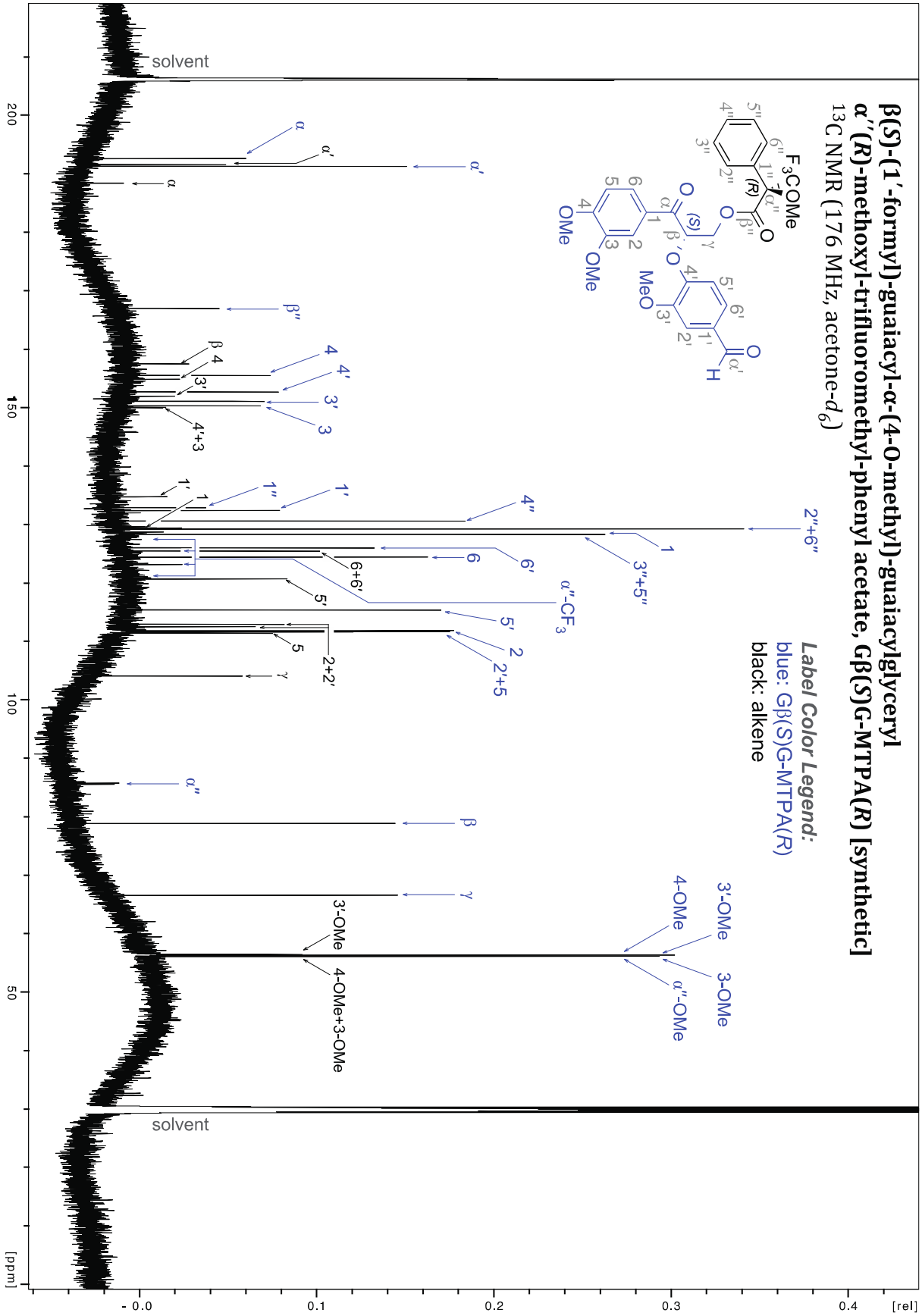
COMPOUND:	Spectra:	Page:
• β(*S*)-(1´-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl α´´(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Gβ(*S*)G-MTPA(*R*) [synthetic]	^{1}H	S - 21
	^{13}C	S - 22
• β(*R*)-(1´-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl α´´(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Gβ(*R*)G-MTPA(*R*) [synthetic]	^{1}H	S - 23
	^{13}C	S - 24
• α-(4-O-methyl)-guaiacyl-β,γ-propenone-β-(1´-formyl)-guaiacyl ether, GβG-propenone [synthetic]	^{1}H	S - 25
	^{13}C	S - 26
• β(*R*)-(1´-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl α´´(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Sβ(*R*)S-MTPA(*R*) [synthetic]	^{1}H	S - 27
	^{13}C	S - 28
• β(*S*)-(1´-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl α´´(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Sβ(*S*)S-MTPA(*R*) [synthetic]	^{1}H	S - 29
	^{13}C	S - 30

β(*S*)-(1′-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl α′′(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Gβ(*S*)G-MTPA(*R*) [synthetic]

^{1}H NMR (700 MHz, acetone-d_6)

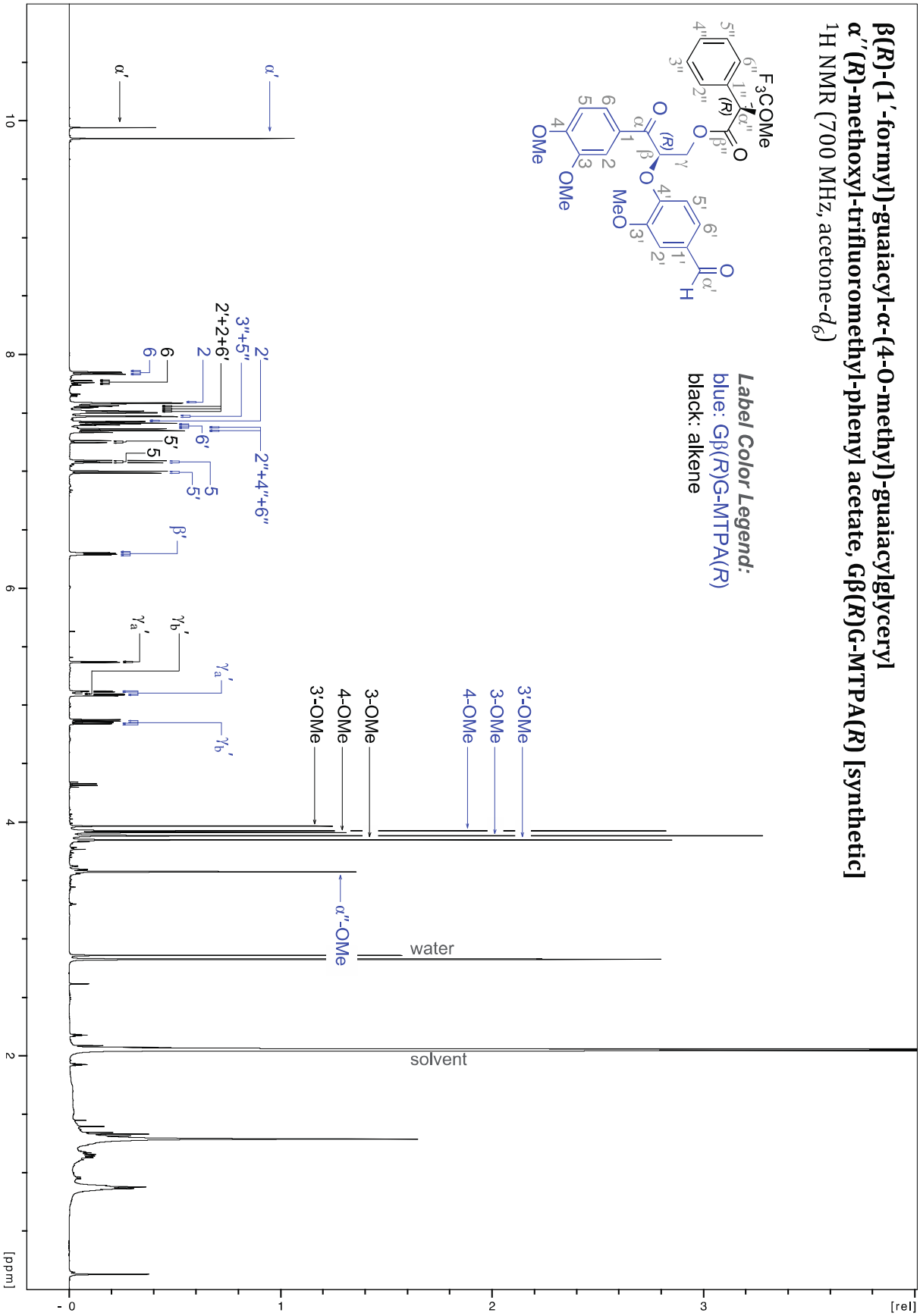


Label Color Legend:
blue: Gβ(*S*)G-MTPA(*R*)
black: alkene


β(S)-(1′-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl
α″(R)-methoxyl-trifluoromethyl-phenyl acetate, Gβ(S)G-MTPA(R) [synthetic]
13C NMR (176 MHz, acetone-d6)
Label Color Legend:
blue: Gβ(S)G-MTPA(R)
black: alkene
solvent
solvent
[ppm]
[rel]
2″+6″
3″+5″
α″-CF3
3′-OMe
4-OMe
α″-OMe
3-OMe
3′-OMe
4-OMe+3-OMe

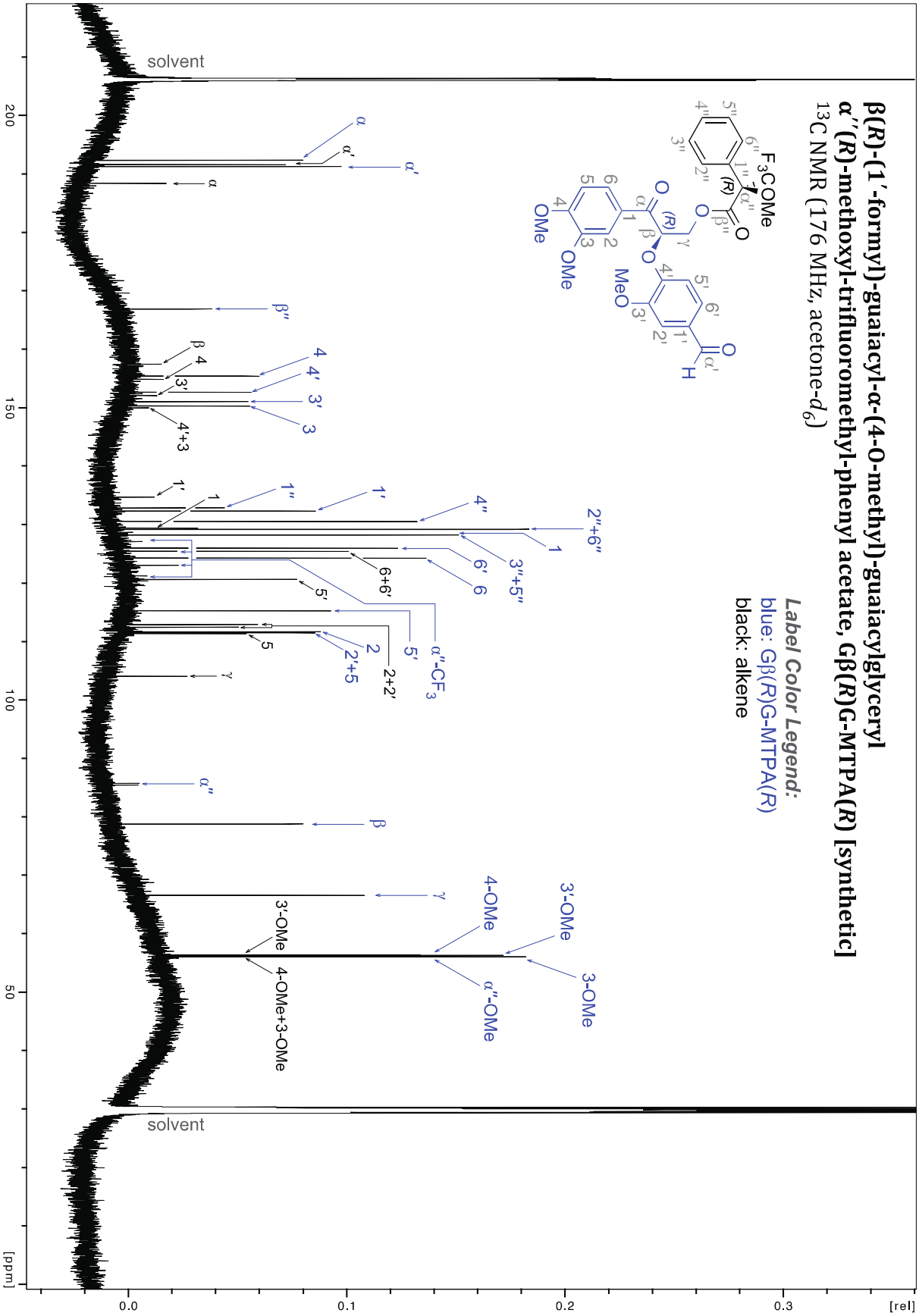
β(*R*)-(1′-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl α″(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Gβ(*R*)G-MTPA(*R*) [synthetic]

^{1}H NMR (700 MHz, acetone-d_6)



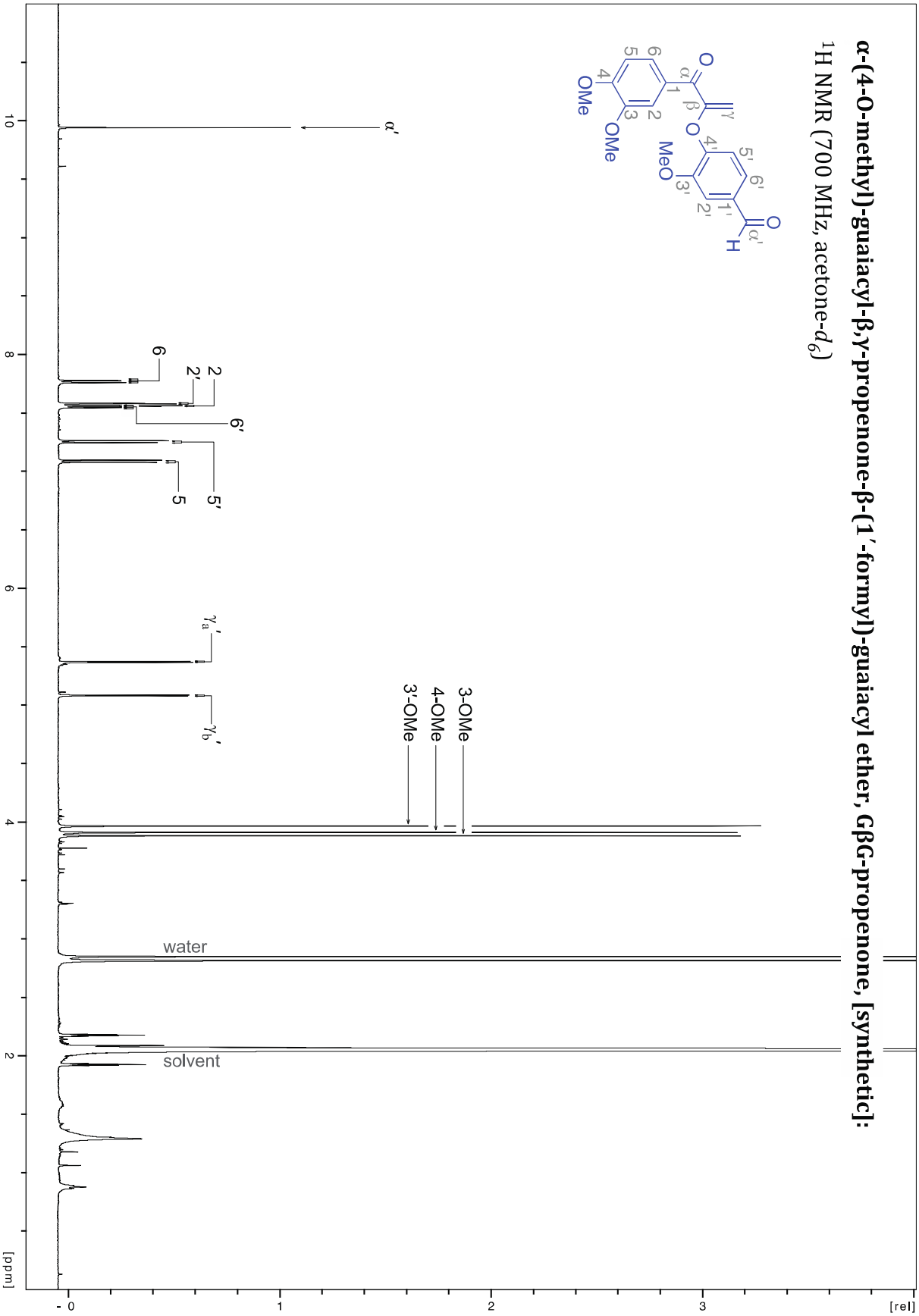
β(*R*)-(1′-formyl)-guaiacyl-α-(4-O-methyl)-guaiacylglyceryl α″(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Gβ(*R*)G-MTPA(*R*) [synthetic]

^{13}C NMR (176 MHz, acetone-d_6)



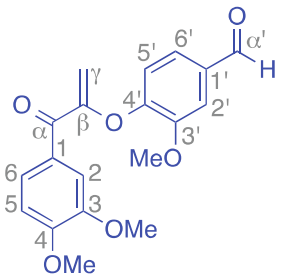
α-(4-O-methyl)-guaiacyl-β,γ-propenone-β-(1′-formyl)-guaiacyl ether, GβG-propenone, [synthetic]:

^{1}H NMR (700 MHz, acetone-d_6)



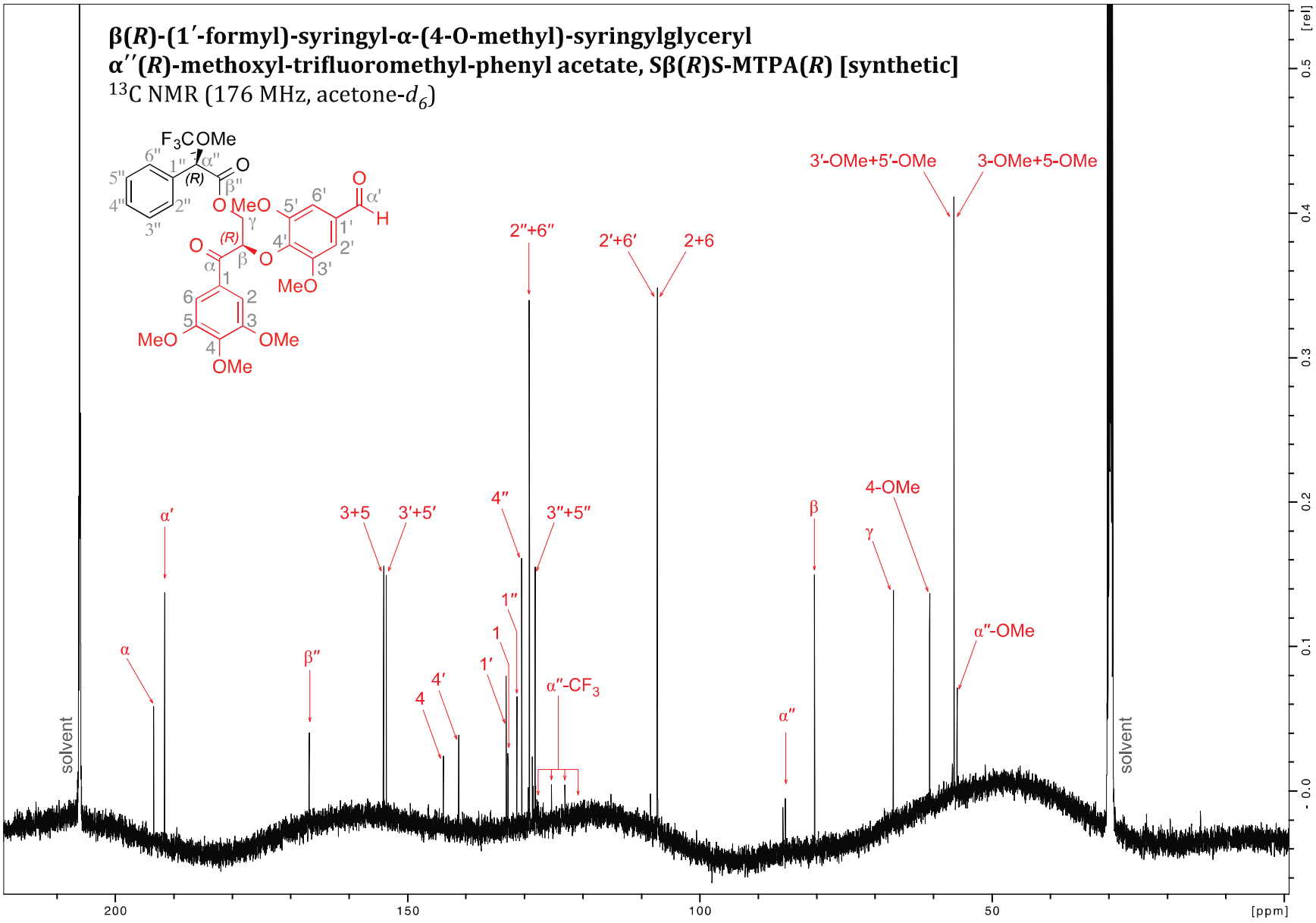
α-(4-O-methyl)-guaiacyl-β,γ-propenone-β-(1′-formyl)-guaiacyl ether, GβG-propenone, [synthetic]:

^{13}C NMR (176 MHz, acetone-d_6)



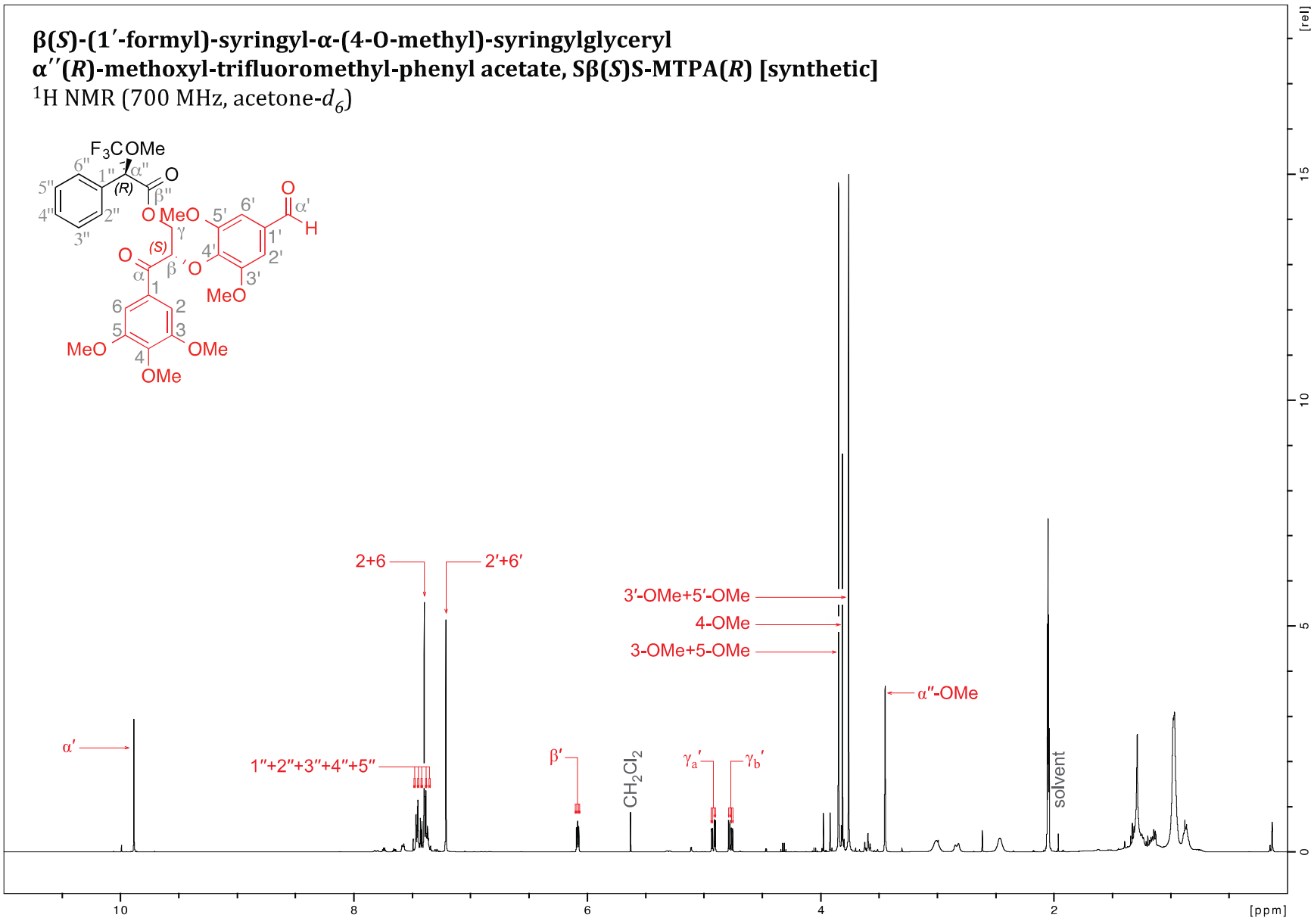
β(R)-(1′-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl α″(R)-methoxyl-trifluoromethyl-phenyl acetate, SβS(R)-MTPA(R) [synthetic]

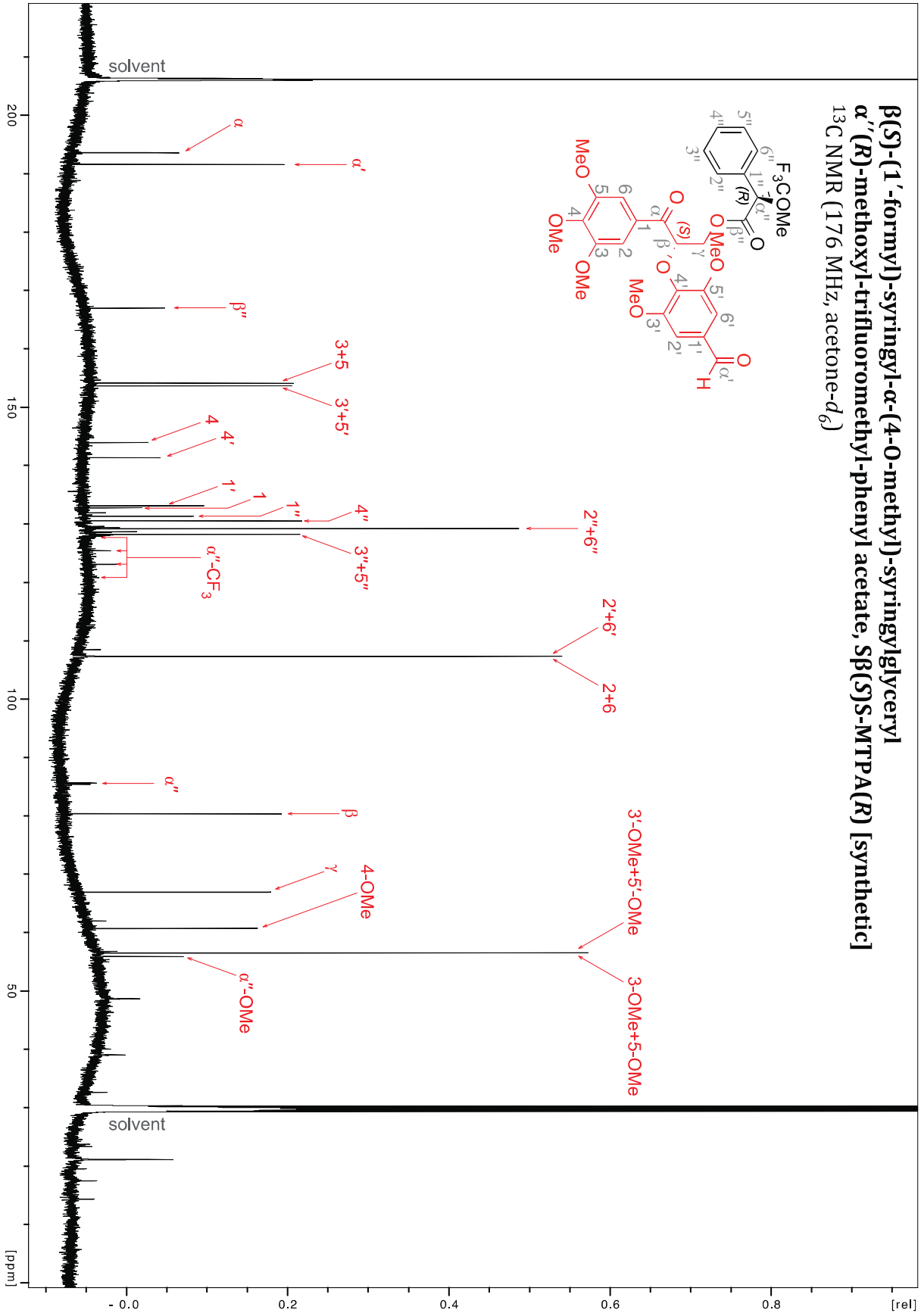
^{1}H NMR (700 MHz, acetone-d_6)


β(R)-(1′-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl
α′′(R)-methoxyl-trifluoromethyl-phenyl acetate, Sβ(R)S-MTPA(R) [synthetic]
13C NMR (176 MHz, acetone-d6)
F3COMe
MeO
MeO
OMe
OMe
MeO
H
3′-OMe+5′-OMe
3-OMe+5-OMe
2″+6″
2′+6′
2+6
α′
3+5
3′+5′
4″
3″+5″
β
4-OMe
γ
α
β″
1″
1
1′
4′
4
α″-CF3
α″
α″-OMe
solvent
solvent
200
150
100
50
[ppm]
0.5
0.4
0.3
0.2
0.1
0.0
[rel]

β(*S*)-(1′-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl α′′(*R*)-methoxyl-trifluoromethyl-phenyl acetate, Sβ(*S*)S-MTPA(*R*) [synthetic]

^{1}H NMR (700 MHz, acetone-d_6)




β(S)-(1′-formyl)-syringyl-α-(4-O-methyl)-syringylglyceryl α″(R)-methoxyl-trifluoromethyl-phenyl acetate, Sβ(S)S-MTPA(R) [synthetic]
13C NMR (176 MHz, acetone-d6)
solvent
α
α′
β″
3+5
3′+5′
4
4′
1′
1
1″
4″
3″+5″
α″-CF3
2″+6″
2′+6′
2+6
α″
β
γ
4-OMe
α″-OMe
3′-OMe+5′-OMe
3-OMe+5-OMe
solvent
200
150
100
50
[ppm]
- 0.0
0.2
0.4
0.6
0.8
[rel]