Cascade enzymatic cleavage of the β-O-4 linkage in a lignin model compound

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Synthesis of 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (1-4)

1-(4-Benzyloxy-3-methoxyphenyl)ethan-1-one (**11**):

Benzoyl chloride (30 mL) was added to a solution of 1-(4-hydroxy-3-methoxyphenyl)ethan-1-one **10** (200 mmoles, 33.2 g) in pyridine (300 mL) at 5 °C under magnetic stirring: the conversion was complete in 20 min. The largest amount of pyridine was evaporated at low pressure. The crude product was then diluted in ethyl acetate and the residual pyridine was removed through extraction in 1 M HCl. 1-(4-Benzyloxy-3-methoxyphenyl)ethan-1-one **11** was crystallized from ethyl acetate/hexane, with a 80% yield. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ_H 2.63 (3H, s, CH_3CO), 3.90 (3H, s, CH_3O), 7.27 (1H, d, H4''), 7.53 (2H, m, H3'' and H5''), 7.59-7.70 (3H, m, H2', H5' and H6'), 8.23 (2H, d, H2'' and H6''). MS (ESI) *m*/*z*: 293 $[M + Na]^+$.

1-(4-Benzyloxy-3-methoxyphenyl)-2-bromoethan-1-one (**12**):

To a solution of **11** (160 mmoles, 43 g) in 480 mL of $CHCl_3$ cooled at 5 °C were added under magnetic stirring 8.17 mL of bromine (160 mmoles). After 20 min the temperature was gradually raised to 40 °C. After 2 h the reaction mixture was washed with a solution of sodium metabisulfite to remove the excess of bromine. The organic solvent was dried on sodium sulfate, evaporated under reduced pressure and the crude product was crystallized from hexane/ethyl acetate affording **12** with a 77% yield. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ_H 3.91 (3H, s, CH_3O), 4.47 (2H, s, $BrCH_2CO$), 7.30 (1H, d, $C_{4''}H$), 7.54 (2H, dd, H3'' and H5''), 7.60-7.70 (3H, m, H2', H5' and H6'), 8.23 (2H, d, H2'' and H6''). MS (ESI) *m*/*z*: 349 and 351 $[M + H]^+$.

1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)ethan-1-one (**13**):

To a solution of 1-(4-benzyloxy-3-methoxyphenyl)-2-bromoethan-1-one **12** (123 mmoles, 42.9 g) and 2-methoxyphenol (123 mmoles, 13.5 mL) in 490 mL DMF under magnetic stirring, K_2CO_3 (177 mmoles, 24.5 g) was added at 25 °C. After 2 h the reaction mixture was poured into 2.5 L of

water. The mixture was extracted three times with AcOEt (3 x 800 mL). These organic phases were dried on sodium sulfate and the solvent removed by evaporation at low pressure. The product was crystallized from $CH_3OH/(iPr)_2O$/hexane to give **13** with a 63% yield. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ_H 3.90 (3H, s, CH_3O overlapping 3H, s, CH_3O), 5.32 (2H, s, OCH_2CO), 6.85-7.05 (4H, m, H3''', H4''', H5''' and H6'''), 7.29 (1H, d, H4''), 7.53 (2H, dd, H3'' and H5''), 7.6-7.8 (3H, m, H2', H5' and H6'), 8.23 (2H, d, H2'' and H6''). MS (ESI) *m*/*z*: 393 $[M + H]^+$.

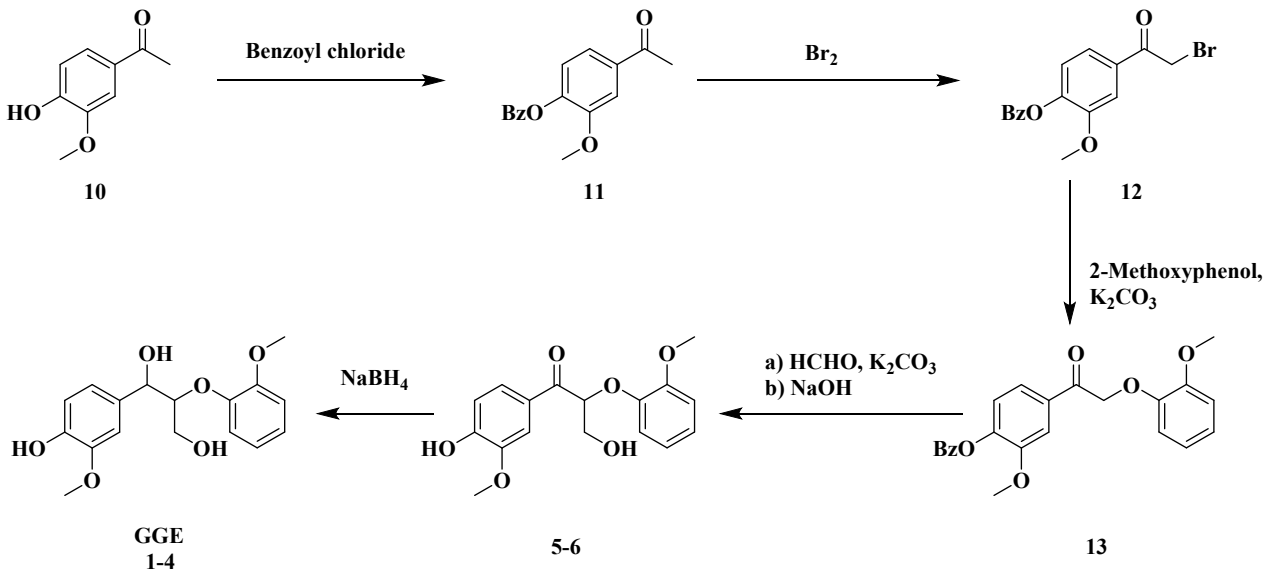
3-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propan-1-one (**5-6**):

To a solution of 1-(4-benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)ethan-1-one **13** (39 mmoles, 15.3 g) in DMSO (130 mL) and K_2CO_3 (6.5 g, 47 mmoles) was added drop-wise a solution of HCHO 37 % (855 mmoles, 63.6 mL) under magnetic stirring at 25 °C. The reaction was monitored by TLC (AcOEt/hexane, 1:1). At complete conversion of the substrate, the reaction mixture was quenched with 6 N HCl and the intermediate extracted in AcOEt. Following complete removal of the organic solvent, the intermediate was dissolved in the minimum amount of DMSO and poured into 250 mL of 1 N NaOH. A further amount of DMSO could be added to facilitate the dissolution of the intermediate. The reaction was kept at 25 °C for 2 h; then it was then acidified, extracted with ethyl acetate, dried on sodium sulfate and evaporated under reduced pressure. The product was purified by flash chromatography using n-hexane/ethyl acetate 1:1 as eluent to give **5-6** with a 40% yield. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ_H 3.90 (3H, s, CH_3O), 3.91 (3H, d, CH_3O), 4.08 (2H, d, *J* = 5.12 Hz, HO$\underline{CH_2}$CH), 5.40 (1H, t, *J* = 5.12 Hz, HO$CH_2\underline{CH}$), 6.08-6.23 (0.9H, br, $CH_2\underline{OH}$), 6.81-7.04 (5H, m, H5', H3''', H4''', H5''', H6'''), 7.27 (0.6H, s, Ph$\underline{OH}$), 7.65 (1H, d, *J* = 1.92 Hz, H2'), 7.72 (1H, dd, *J* = 8.32, 1.92 Hz, H6'). MS (ESI) *m*/*z*: 319 $[M + H]^+$.

1-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (GGE, **1-4**):

A solution of 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propan-1-one **5-6** (6.3 mmoles, 2 g) dissolved in 15 mL of ethanol was cooled at 5 °C and 1.57 g of $NaBH_4$ were

added under magnetic stirring. Then, the temperature was allowed to slowly raise to 25 °C. After 3 h the reaction mixture was cooled at 5 °C and the residual $NaBH_4$ quenched with water. The ethanol was evaporated under reduced pressure, the crude reaction mixture was extracted with ethyl acetate and evaporated. Purification by flash chromatography using ethyl acetate/dichloromethane 1:2 mixture as eluent afforded GGE with a 65% yield. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ_H 3.49-3.55 (0.5H, m, CH_2OH), 3.63-3.72 (2H, m, CH_2OH), 3.87 (3H, s, CH_3O), 3.95 (3H, d, J = 10.1 Hz, CH_3O), 3.91-3.96 (0.5H, m, CH_2OH), 4.03-4.07 (0.5H, m, CHCHOPh), 4.17-4.21 (0.5H, m, CHCHOPh), 4.98 (1H, m, HOCHCH), 5.25-6.00 (0.8H, br, CH_2OH), 6.8-7.15 (7H, m , H2', H5', H 6', H3''', H4''', H5''' and H6'''), 7.27 (0.8H, s, PhOH). MS (ESI) *m*/*z*: 343 $[M + Na]^+$.



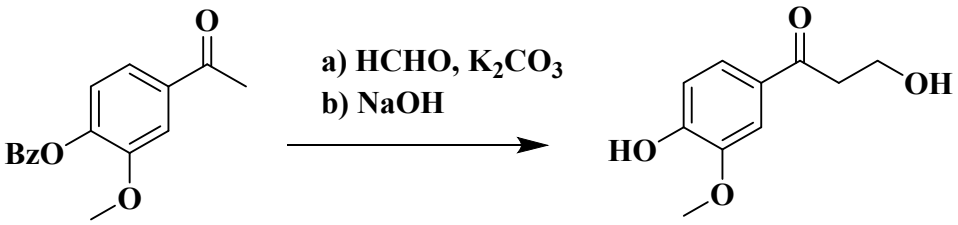
NMR and chiral HPLC analyses:

The ^{1}H NMR analysis of the GGE (see Fig. S4A) confirms the presence of four stereoisomers of the compound as indicated by: a) the presence of four couples of doublets of doublets as signals for the two hydrogens H_b [δ_H 3.49-3.55 (0.5H, H_b), 3.63-3.72 (1H, H_b) and 3.91-3.96 (0.5H, m, H_b)]; b) the signal of H_c which is a partially overlapping couple of doublets [δ_H 4.98 (1H, Hc)]; c) the signals of the hydrogen of the Cβ (H_a), which appear as two separated multiplets in ratio 57:43 calculated from the integration value of the two signals [δ_H 4.03-4.07 (0.57H, m) and 4.17-4.21 (0.43H, m)],

respectively. The ratio values correspond to the ratio between the couples of diastereoisomers (**1** + **4**) and (**2** + **3**).

Chiral HPLC analysis was performed on a Merck Hitachi apparatus L6200 equipped with a UV detector L4200 set at 280 nm and fitted with a Chiralpak IA column (4.6x250 mm, Daicel Chemical Industries). A mixture of hexane and 2-propanol (9/1) was used as the mobile phase at flow rate of 1.0 mL/min. The area of each peak was estimated by nonlinear curve-fitting of the elution profile using PeakFit software: a value of 19.3, 23, 24.5 and 33.2% was obtained for the four stereoisomers, with a ratio between the couples of diastereoisomers corresponding to 42.2:57.3, in agreement with the values obtained by NMR analysis.

Synthesis of 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (9)



11 **9**

To a solution of 1-(4-benzyloxy-3-methoxyphenyl)ethan-1-one **11** (1 g, 3.7 mmol) and K_2CO_3 (613 mg, 4.4 mmol) in DMSO (17 mL), 0.6 mL of 35% formaldehyde (8.8 mmol) was added dropwise at room temperature. The reaction was followed by TLC (ethyl acetate/hexane 6:4). After 60 min the reaction was quenched with a solution of aqueous 1 N HCl, extracted twice with ethyl acetate and dried under reduced pressure. The crude oil obtained was then dissolved in 10 mL of DMSO and 100 mL of NaOH was added. The reaction was left stirring for 1 h and then acidified to pH 3 with 1 N HCl, extracted three times with ethyl acetate. The organic phases were dried on sodium sulphate and evaporated under reduced pressure. The product **9** was purified by flash chromatography from ethyl acetate/hexane 3/7 to 4/6 (yield of 10 %). ^{1}H NMR (CD_3OD, 400 MHz): δ 3.15 (2H, t, *J* =

6.37, 6.23 Hz), 3.85 (3H, s), 4.1 (2H, t, *J* = 6.37, 6.23 Hz), 6.9 (1H, d, *J* = 8.08 Hz), 7.59 (1H, dd, *J* = 6.37, 1.9 Hz), 7.60 (1H, d, *J* = 2.2 Hz). MS (ESI) *m/z:* 219 $[M + Na]^+$, $[M-H]^-$= 194.9.

Fig. S1 SDS-PAGE analysis of the purified recombinant Lig enzymes. Gel was stained with Coomassie blue. (Lane 1) Molecular weight marker proteins; (lanes 2-6) purified LigD, L, E, F and G, respectively, after HiTrap Chelating chromatography and dialysis.

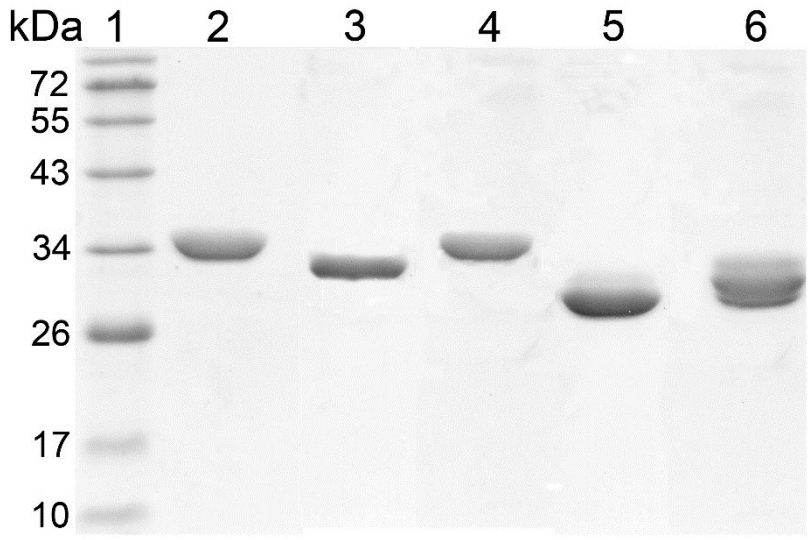
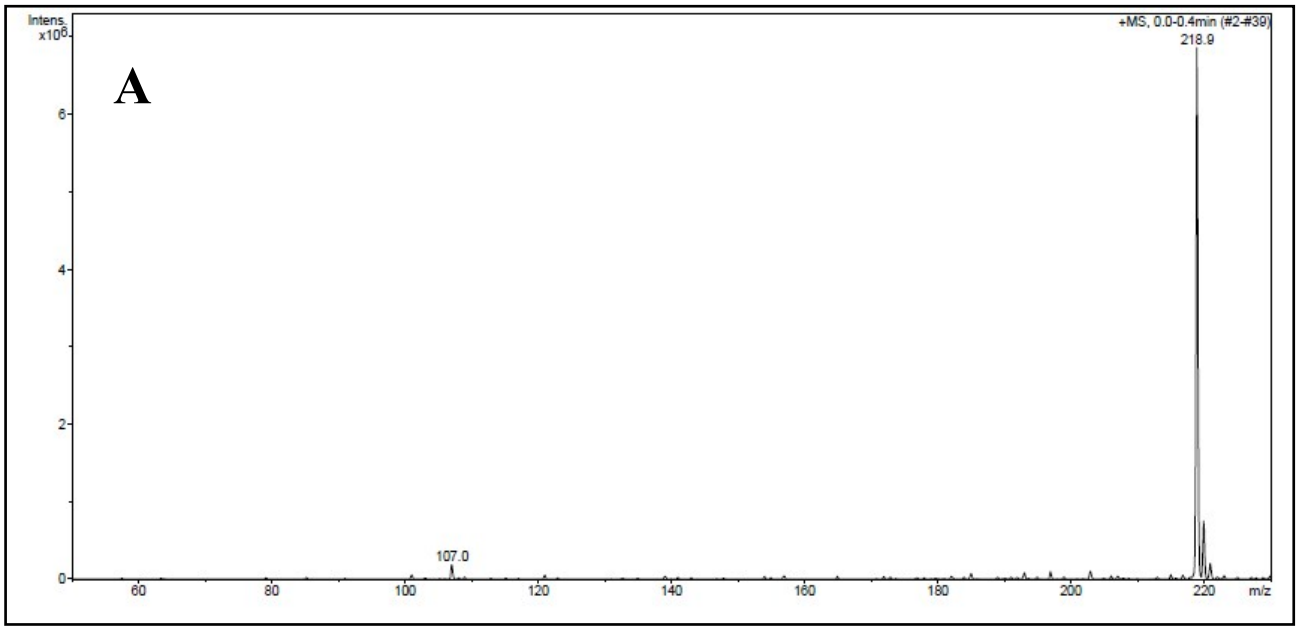


Fig. S2 (A) Mass spectra of 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (**9**) obtained by chemical synthesis and (B) by enzymatic degradation of GGE catalyzed by the Lig system.



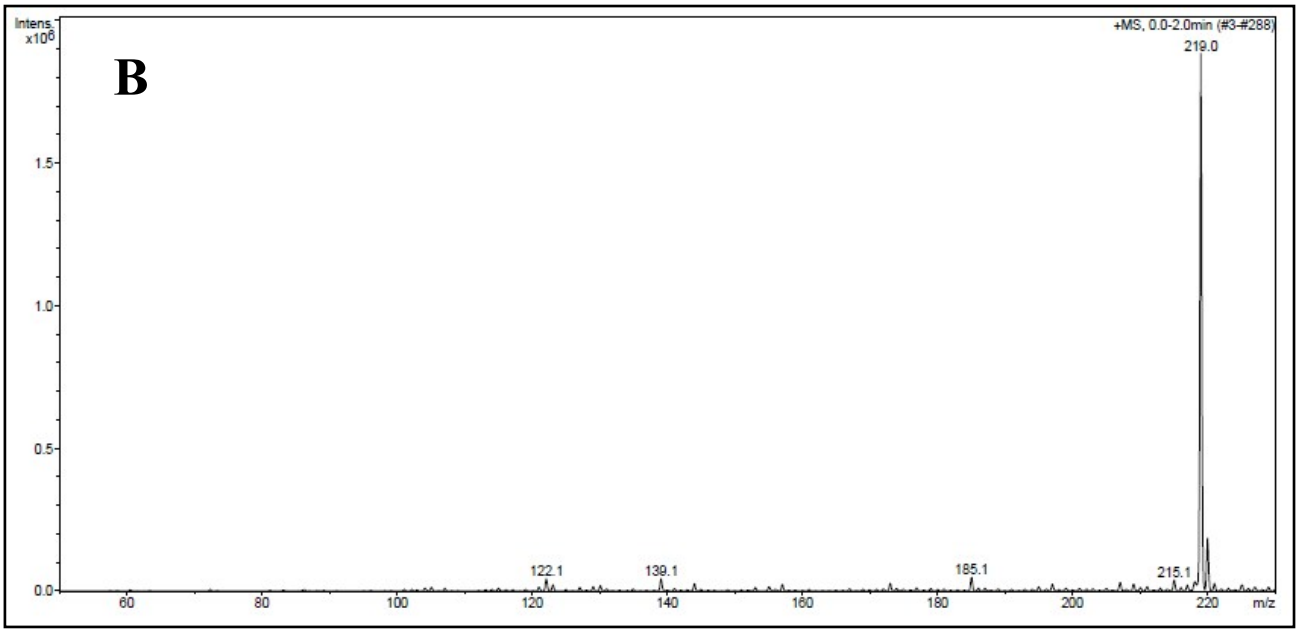


Fig. S3 HPLC calibration curves for (A) GGE (**1-4**), (B) 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propan-1-one (**5+6**), (C), guaiacol, and (D) 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (**9**).

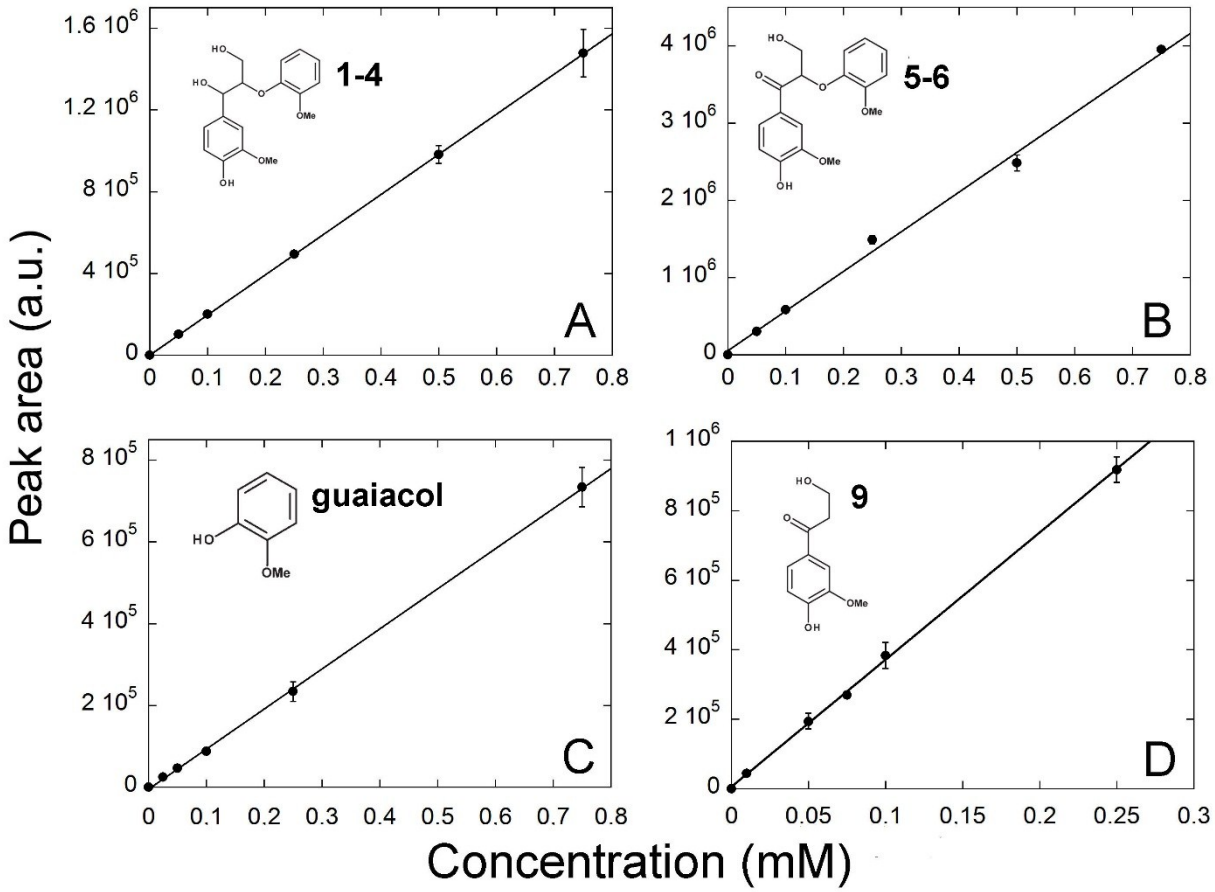


Fig. S4 (A) NMR analysis and (B) elution profile of chiral HPLC analysis of GGE (1-4) (black line). The colored lines show the deconvolution analysis of peaks by nonlinear curve fitting (using PeakFit software) corresponding to four stereoisomers of compound GGE.