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Controlling the polymerization of coniferyl alcohol with cyclodextrins.

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Supplementary Information

1. *Enzymes and chemicals*

The laccase LAC3 (from *Trametes* sp C30) was produced in *Aspergillus niger* and purified as described previously.[1] Protein concentration was estimated by UV-visible spectroscopy using an $\varepsilon_{600\text{ nm}} = 5 \times 10^3\ \mathrm{M^{-1}\cdot cm^{-1}}$ for the T1 copper. Laccase activity of the purified protein solution was assayed using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS). One unit (U) of laccase oxidizes one micromole of ABTS per minute.

Alpha-cyclodextrin (αCD), beta-cyclodextrin (βCD) and gamma-cyclodextrin (γCD) were obtained from Aldrich or TCI Europe. Beta-cyclodextrin (βCD) was recrystallized twice in 60 % ethanol. Dehydrodiconiferyl alcohols (**2**) and (±)-pinoresinols (**3**) were obtained from the bioconversion of coniferyl alcohol (**1**) (purchased from Sigma France) catalysed by the enzyme LAC3, purified by preparative chromatography (silica) and characterized by ^{1}H NMR and mass spectrometry. All other chemicals were purchased from Sigma France.

2. *Synthesis of (±)-guaiacylglycerol 8-O-4'-coniferyl alcohol ethers (**4**)*

4 was obtained by oxidation of **1** in the presence of silver oxide (Ag_2O) following a protocol described Kishimoto et al., 2015.[2] The product of interest was purified by preparative chromatography (silica) and characterized by ^{1}H NMR and mass spectrometry.

3. *Coniferyl alcohol oxidation in the presence of CD*

Oxidation of coniferyl alcohol was performed at 30°C in Britton-Robinson buffer adjusted at pH 5.0 and oxidation products were detected by reverse phase HPLC. A typical reaction mixture (1 mL) contained LAC3 (5 U/L) and coniferyl alcohol ($2.5\ 10^{-3}$ M) in the absence or presence of CD (up to $12\ 10^{-3}$ M). The reaction proceeded for 8-hours and was initiated by the addition of the enzyme. For each time-point, the reaction was stopped by mixing 100-µL aliquot to 100 µL acetonitrile containing 2.85 mM benzophenone. 30 µL was injected on C18 column (EC150/4 Nucleosil® 100-5-18 Macherey-Nagel™) and analyzed by HPLC (Waters™ Alliance® 2690/2690D Separations Module) with the following gradient: solvent A water containing 3% acetic acid, solvent B acetonitrile; t=0min 90%A 10%B, t=5min 90%A 10%B, t=25 min 50% A 50% B, t= 27 min 50% A 50% B, t=28 min 90%A 10% B, t=30 min 90%A 10%B. Coniferyl alcohol, dehydrodiconiferyl alcohol, pinoresinol and benzophenone were eluted at 4.0 min, 6.6 min, 8.2 min and 16.6 min, respectively. Quantity was normalized using benzophenone as internal reference.

Apparent K_M and k_{cat} values were obtained from the initial rate (v), enzyme concentration (E) and substrate concentration (S) according to the equation $v = k_{cat}\ E\ S/(K_M + S)$. Inhibition kinetics data were obtained using appropriate equations. All data were determined using non-linear regression fitting using Prism software, Graphpad, San Diego, (CA). Because laccase catalysis involves two substrates and the [O_2] was invariant and assumed to be saturating in this study, the measured K_M for the various substrates used should be considered apparent. Because of the assumption that 100% of the laccase participated in the catalysis as active enzyme, the measured k_{cat} should also be considered apparent.

4. *Fitting kinetics*

The oxidation of coniferyl alcohol (**1**) by the enzyme laccase is a mono-electronic process leading to the transient formation of a radical species (**1$^{\bullet}$**) (eq. 1). Radical molecules recombine to give dimers **2**, **3** and **4** according to eq. 2. Dimers are themselves subsequently oxidized by the enzyme (eq. 2, 4, 5) in a reaction similar to the oxidation of **1**.

$$\mathbf{1} \xrightarrow[-\mathrm{H}^+ - e^-]{k_1} \mathbf{1}^{\bullet} \qquad \text{(eq. 1)}$$

$$n\mathbf{1}^{\bullet} \xrightarrow{k_{dim}} m\mathbf{2} + k\mathbf{3} + l\mathbf{4} \qquad \text{(eq. 2)}$$

$$\mathbf{2} \xrightarrow[-\mathrm{H}^+ - e^-]{k_2} \mathbf{2}^{\bullet} \qquad \text{(eq. 3)}$$

$$\mathbf{3} \xrightarrow[-\mathrm{H}^+ - e^-]{k_3} \mathbf{3}^{\bullet} \qquad \text{(eq. 4)}$$

$$\mathbf{4} \xrightarrow[-\mathrm{H}^+ - e^-]{k_4} \mathbf{4}^{\bullet} \qquad \text{(eq. 5)}$$

Kinetic data presented in Figure 1 from the main article were simulated using the stochastic kinetics simulator Kinetiscope (freeware)[3]. In the simulation, we introduced:

- the initial concentration of coniferyl alcohol [**1**] = 2.5 10^{-3}M
- rate constants from (eq. 1, 3, 4, 5) initially derived from experimental data presented in Fig. 1 and Fig. 2 from the main article: $k_1 = 3.3\ h^{-1}$; $k_2 = 0.3\ h^{-1}$; $k_3 = 0.5\ h^{-1}$; $k_4 = 0.25\ h^{-1}$
- a bi-molecular constant k_{dim} from (eq. 2) arbitrary set to a value of >> k_1, k_2, k_3, k_4 (i.e.$10^6\ M^{-1}.\ h^{-1}$). With this *approximation* we recognize the recombination of radicals as a process considerably faster than oxidation steps and we do not distinguish the formation of each dimer **2**, **3** and **4** (eq. 2).

The model obtained (Fig. SI1) is compatible with experimental data (Fig. 1 main text).

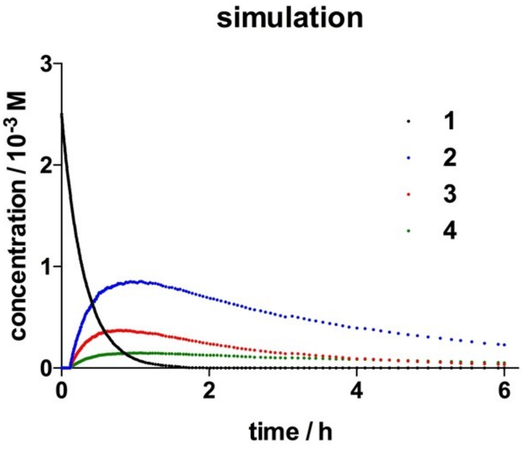

Figure SI1. Simulation of the formation of lignans from the oxidation of coniferyl alcool. Stochastic calculations performed with the freeware Kineticsope. Initial parameters: **[1]** = 2.5 10^{-3}M, $k_1 = 3.3\ h^{-1}$; $k_2 = 0.3\ h^{-1}$; $k_3 = 0.5\ h^{-1}$; $k_4 = 0.25\ h^{-1}$.

Eventually, kinetics of the two successive oxidations leading to the formation and to the disappearance of each dimer (see Fig. 1 panels 2 and 3 main text) can be fitted using the simple model:

$$\mathrm{A} \xrightarrow{k_1} \mathrm{B} \xrightarrow{k_2} \mathrm{C}$$

treated as a succession of order 1 kinetics in which the dimerization step is not influencing the rate (i.e. $k_{dim} >> k_1, k_2$).

$$Y=((a * k_1)/(k_2- k_1)) * (\exp(-k_1 * X)- \exp(-k_2*X))$$

with k_1 = rate constant for the oxidation of the alcohol and k_2 = rate constant for the oxidation of the dimer.

5. *Determination of the apparent association constant and complex structure by NMR*

All experiments were performed at 298K in D_2O on a Bruker Advance III 600MHz spectrometer, equipped with a 5mm triple resonance high resolution probe. All NMR datasets were processed in TOPSPIN 3.2 version (Bruker BioSpin, Germany). Proton NMR spectra were acquired with a spectral width of 6000 Hz and relaxation delay of 2s during which a water pre-saturation was applied.

Ka values were extracted from Scott plots ($[CD]_t/\Delta\delta_{obs} = ([CD]_t/\Delta\delta_c) + (1/Ka\Delta\delta_c)$).[4]

δ_l = chemical shift of a proton from the ligand or from the free CD.

δ_c = chemical shift of the same proton in the ligand:CD complex.

$\Delta\delta_{obs} = \delta_l - \delta_c$

Plots for βCD complexes are given in Figure SI2.

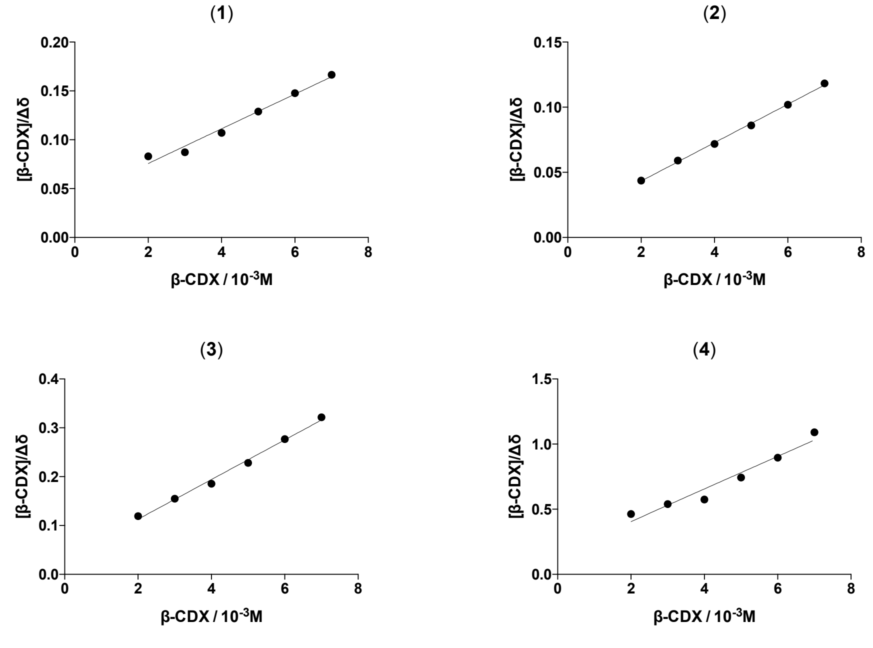

Figure SI2. Representation of the ligand: βCD interaction (Scott plots). [Ligand] = 0.5 10^{-3}M, [βCD] = 2-7 10^{-3}M.

The stoichiometry for all the CD:guest complexes was studied plotting the extend of the shifts as function of the evolution of the molar ratio between the CD (β or γCD) and the ligand (Job Plots). Plots for βCD complexes are given in Figure SI3.

For complex structure analysis, 2D phase-sensitive ROESY were acquired by pulse field gradient-selected methods, with 32 scans and 2048-time domain in F2, and 384 experiments in F1 by using the TPPI method and a mixing time (spin-lock) of 200 ms at a field of 6 kHz.

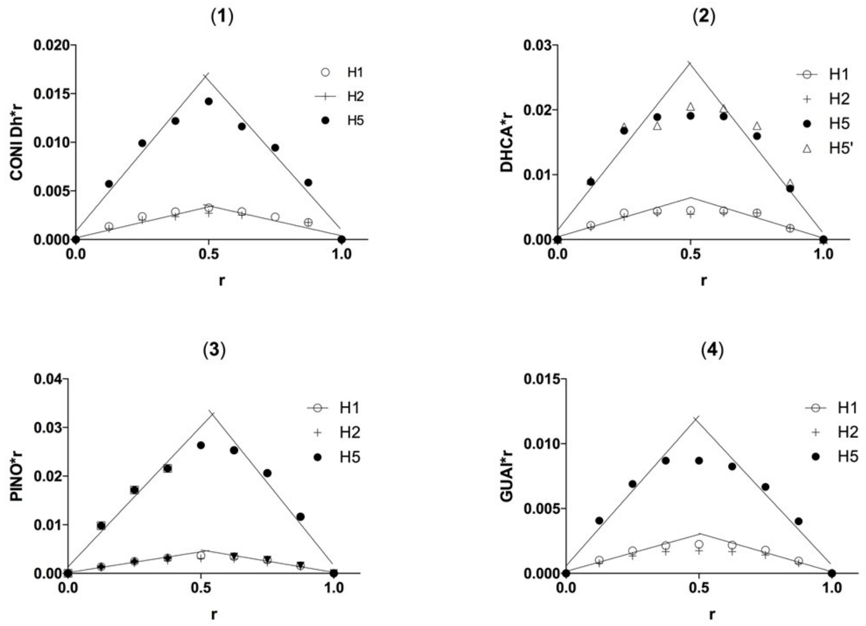

Figure SI3. Representation of the ligand: βCD interaction (Job plots). [Ligand] = 0-8 10^{-3}M, [βCD] = 8-0 10^{-3}M. Chemical shifts from βCD protons H1, H2 and H5.

βCD/pinoresinol complex deduced from the observed strong, medium, and low ROESY correlations effects between protons H_2, H_5, H_6 of (±)-pinoresinol and protons H'_3, H'_5, H'_6 of� βCD is presented in the main text (Fig. 3). The proposed model is consolidated by strong interactions between protons H_8 and H_9 of (±)-pinoresinol with protons H'_5 of ��CD (red double arrows).

It should be pointed out that only unambiguous correlations were considered. Those with overlapping patterns potentially involving intramolecular interactions were discarded as for example in the coniferyl alcohol 2D-ROESY map (see red rectangle in panel B of Fig. SI.4).

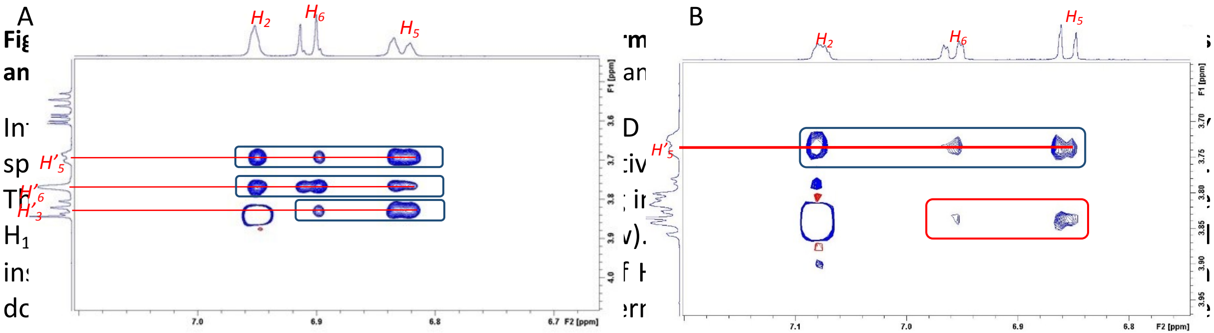

Fig m
an an
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other H_{15} presents lower cross peaks. Therefore, only the complex with the deepest inclusion is illustrated in Figure SI5.

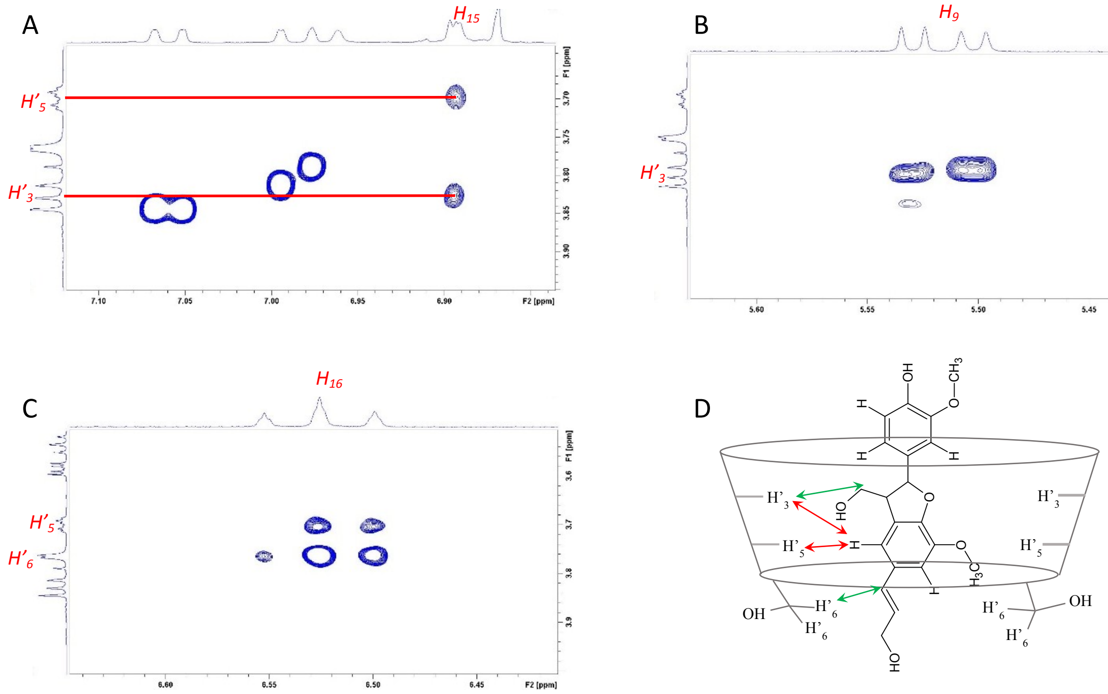

Figure SI5. Fragments of ROESY spectra showing the intermolecular interactions between protons from dehydro di-coniferyl alcohol and proton from βCD. Panel A, zoom in the interactions between the proton H15 of dehydro di-coniferyl alcohol and protons H'3 and H'5 of βCD; panel B, zoom in the interactions between the proton H9 of dehydro di-coniferyl alcohol and protons H'3 of βCD (figure panel D); panel C, zoom in the interactions between the proton H16 of dehydro di-coniferyl alcohol and protons H'5 and H'6 of βCD (figure panel D); panel D, scheme of the potential interactions; double arrows indicate spatial dipolar interactions classified from each CD proton: close (red), intermediate (green).

6. *Evolution of dimers production as function of the initial coniferyl alcohol concentration*

Oxidation of coniferyl alcohol was performed and analysed as described earlier in the text (see paragraph 2). A typical reaction mixture (1 mL) contained LAC3 (5 U/L) coniferyl alcohol (2.5, 5 or 10 10^{-3} M) in the presence (or absence) of 12 10^{-3} M of βCD.

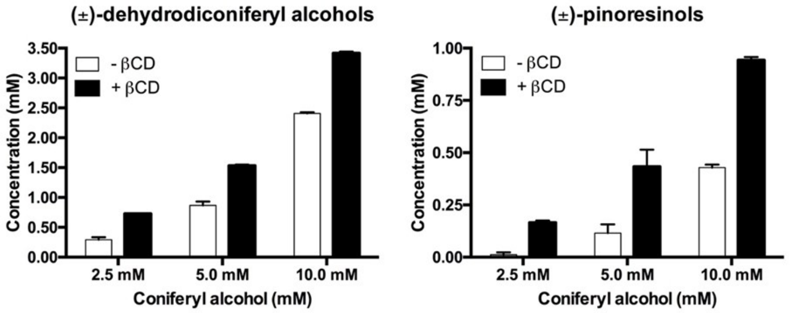

Figure SI6. Effects of a variable initial concentrations of coniferyl alcohol on the formation of dimers in the presence of βCD.

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