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Supramolecular Chirality and Crystallization from Biocatalytic Self-assembly in Lipidic Cubic Mesophases

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1. Materials and Methods

1.1 Materials

Dimodan, was a gift from Danisco, Denmark. Benzaldehyde, phosphate, K_2HPO_4, KH_2PO_4, $MgSO_4$, Thiamine-diphosphate (ThDP) were all purchased from Sigma without further purification. Phosphate buffer solution (pH= 7.0, 50 mM, with 2.5 mM $MgSO_4$, and 0.3 mM ThDP) is used in this work.

Protein expression and purification: The plasmid pKK-bznB encoding the enzyme benzaldehyde lyase (BAL) was kindly provided by Dr. Clément Dince (ETH Zurich, Switzerland). The plasmid pKK-bznB was transformed into *E. coli* BL21-gold (DE3). The cultures were grown at 37 °C in LB-medium until an OD_{600} of approximately 0.6 was reached. Expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. The cells were harvested after incubation at 20 °C overnight. The protein was purified by Ni-NTA chromatography and stored at -80 °C (long term) or 4 °C (short term).

1.2 Sample preparation

1.2.1 (*R*)-benzo synthesis in LCMs

Both enzyme and substrates were homogenized in LCMs using a setup composed of two connected Hamilton RN syringes.[1] Briefly, one syringe was loaded with 80 ul enzyme BAL buffer solution (10 mg/ml); the second syringe was loaded with 120 mg lipid and the desired amount of substrates required. The reaction is initiated only when mixing occurs. The lipidic and aqueous components were then mixed until the blend was completely homogeneous. It was then transferred to a sealed vial and kept at room temperature for 24 h.

1.2.2 Standard (*R*)-benzoin doped in LCMs

The Hamilton RN syringes were also used to dope standard (*R*)-benzoin in LCMs. Briefly, one syringe was loaded with 80 ul phosphate buffer solution; the second syringe was loaded with 120 mg lipid and the desired amount of (*R*)-benzoin required. (*R*)-benzoin was fully soluble in the lipid. The lipid and buffer solution were then mixed until the blend was completely homogeneous. It was then transferred to a sealed via and kept at room temperature for 24 h.

For standard (*R*)-benzoin in DMSO, just dissolve a certain amount of (*R*)-benzoin in 1 ml DMSO.

1.2.3 Synthesized (*R*)-benzoin in solution

0.02 mmol benzaldehyde was dissolved in 120 ul DMSO, and then 80ul BAL solution (10 mg/ml) was added. The mixture was stirred for 24 h, at room temperature.

1.3 NMR measurement

Internal standard method was used to quantify the concentration of standard (*R*)-benzoin in solution. Dimethylmalonic acid was chosed as internal standard substrate. 20 ul dimethylmalonic acid solution (0.1 mmol /ml) and 20 ul (*R*)-benzoin solution (0.01, 0.03, 0.05,

0.07, 0.1 mmol/ml) was added into NMR tubes and then 1 ml DMSO-d was added. The peak at 1.3 ppm comes from six proton of the two methyl group of dimethylmalonic acid. The peaks between 7 ppm and 8 ppm come from the ten protons of the two phenyl group of (*R*)-benzoin.

1.4 Small angle X-ray scattering (SAXS) measurement

Static SAXS patterns were acquired using a Bruker AXS Micro X-ray. The X-ray source was a well-collimated (2D-Kratky) beam of wavelength λ = 1.5418 Å from a microfocused source operating at 50 kV and 1000 μA. Diffracted X-rays were collected on a 2D Pilatus K100 detector. The scattering vector q = (4π/λ) sinθ, with 2θ, the scattering angle, was calibrated using silver behenate in the q-range from 0.03 to 0.6 $Å^{-1}$. Samples were placed inside a stainless steel cell between two thin, replaceable mica sheets and sealed by an O-ring, with a sample volume of 10μL with a thickness approximately 1 mm. Scattered intensity was collected over 30 min. Data were collected and azimuthally averaged using the Saxsgui software to yield 1D intensity versus scattering vector q. The lattice parameter for LCM was calculated using the following equation:

For *Pn3m* phase, $$a = \frac{2\pi}{q^*}\sqrt{2} \quad (1b)$$

, where q^* corresponds to the first peak in the SAXS spectra.

Using the geometrical features of the lipidic cubic mesophases, the lipid length (l_{lip}) within the bilayer can be obtained by solving the following equation[2]

$$\varphi_{lipid} = 2A_0\left(\frac{l_{lip}}{a}\right) + \frac{4}{3}\pi\chi\left(\frac{l_{lip}}{a}\right)^3 \quad (2)$$

, where φ_{lipid} is the lipid volume fraction, A_0 is the minimal surface in a unit cell to (unit cell colume)$^{2/3}$, χ is the Euler–Poincaré characteristic,

For the *Pn3m* phase, $A_0 = 1.919$, $\chi = -2$. The radius of water channel is estimated using the following equation[3]

$$r = 0.391a - l_{lip} \quad (3b)$$

l_{lip} was calculated to be equal to 1.75 nm and the diameter of water channel is 3.72 nm.

1.5 High Performance Liquid Chromatography HPLC measurement

HPLC analyses were performed on Agilent 1200. 10 mg sample (from Section 1.2) was dissolved in 990 ml ethanol. And then 5 μL solution were injected and eluted with the following conditions: 40 vol.% water, 60 vol.% acetonitrile, and 1% (v/v) phosphoric acid, flow rate 1.1 mL min^{-1}, detection at 263 nm. The amount of (*R*)-benzoin product was quantified from the peak areas using external standard methodology. The enantiomeric excess was determined by chiral HPLC phase, employing a column Chiralpak AD, with a mobile phase composed of hexane/2-propanol (95:5) at a flow rate of 1 mL min^{-1}.

1.6 Circular Dichroism (CD) measurement

Circular dichroism and optical rotatory dispersion data were recorded using a Jasco J-815 spectrometer, equipped with a Peltier controlled cell holder. Spectra at 25 °C were collected from 260 to 400 nm with a bandwidth of 5 nm and a scan speed of 100 nm min^{-1}. For all the CD and ORD measurements, samples were transferred to a set of detachable quartz cuvettes with 0.1 mm path length (Helma 106-QS for CD, Hellma 124-QS for ORD).

2. Supporting data

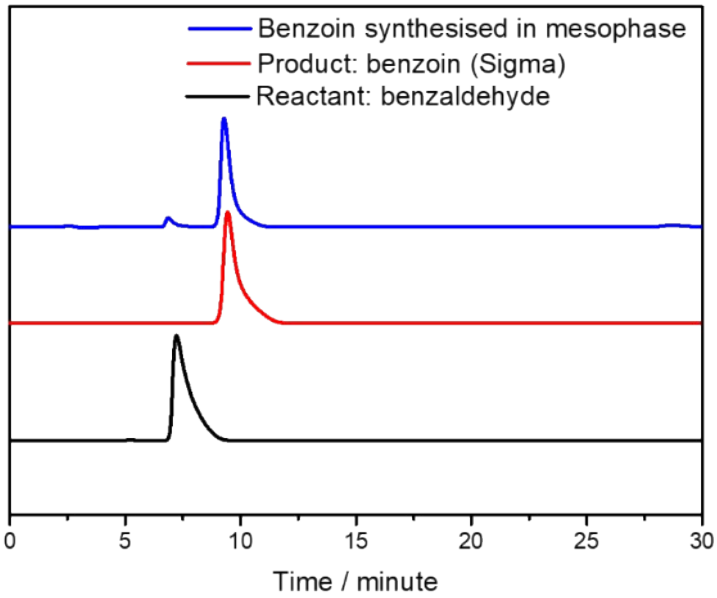


Figure S1. HPLC trace of benzaldehyde (black line), pure (*R*)-benzoin (red line) and synthesized (*R*)-benzoin in LCMs (blue line).

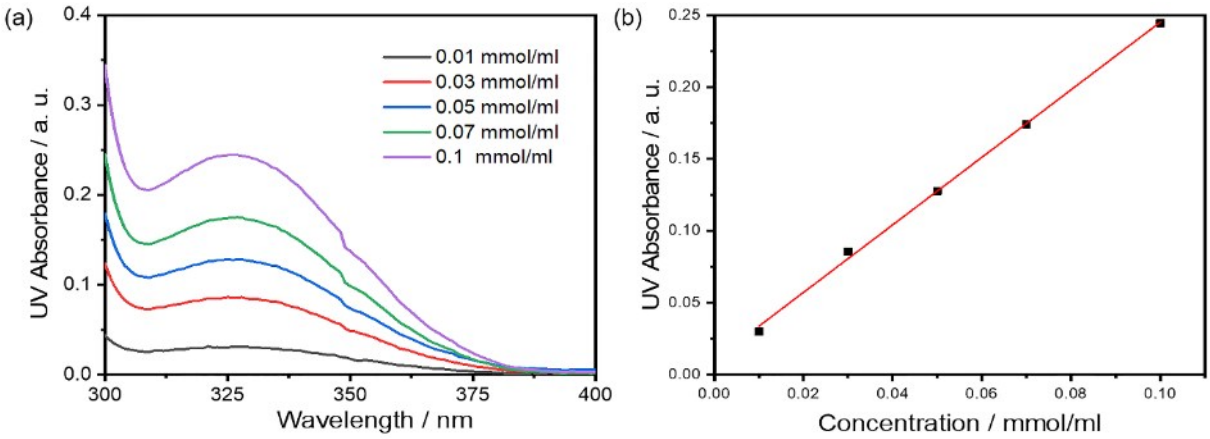


Figure S2. UV absorption of standard (*R*)-benzoin in DMSO with different concentrations, the UV absorption increases linearly with concentration of (*R*)-benzoin, proving the (*R*)-benzoin is fully soluble in DMSO.

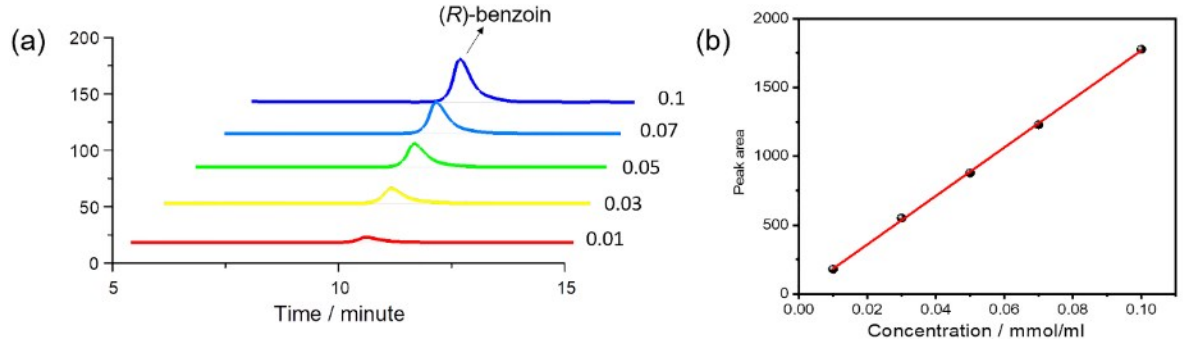
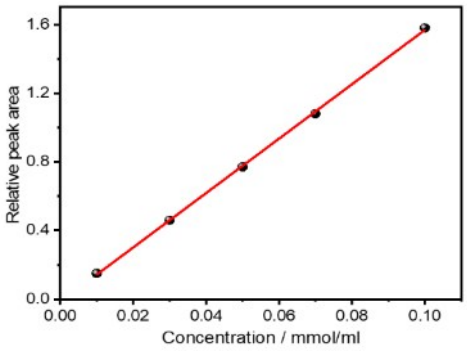


Figure S3. HPLC trace of standard (*R*)-benzoin in DMSO with different concentrations.



C (in theory) / mmol/ml	C (calculate by NMR results)) / mmol/ml
0.01	0.009
0.03	0.28
0.05	0.47
0.07	0.65
0.1	0.095

Figure S4. Relative peak area of peaks between 7 ppm and 8 ppm of (*R*)-benzoin with different concentration, the table shows the precise concentration of (R)-benzoin in solution calculated by NMR results.

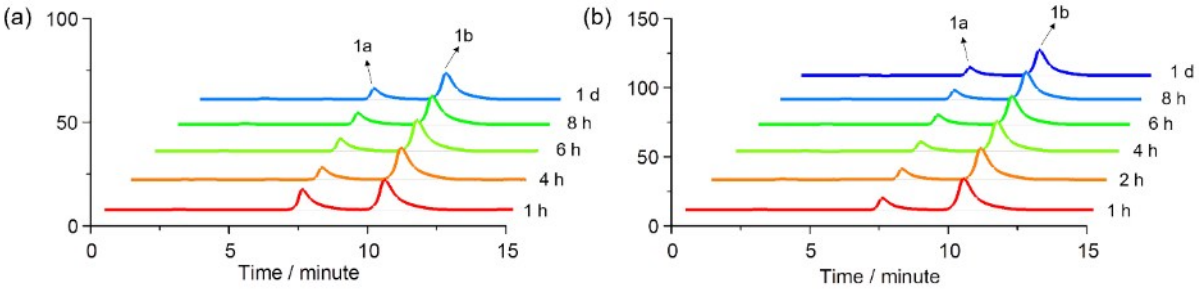


Figure S5. HPLC trace of synthesized (*R*)-benzoin in LCMs; (a) the concentration of substrate is 0.1 mmol/ml, (b) the concentration of substrate is 0.14 mmol/ml. 1a correspond to benzaldehyde. 1b correspond to (*R*)-benzoin.

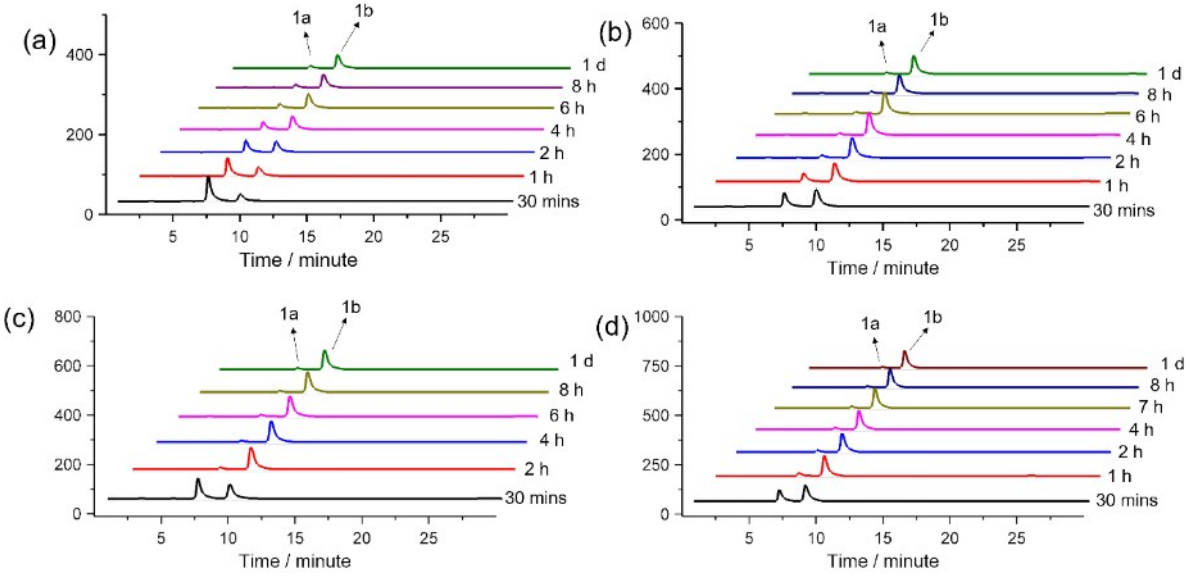


Figure S6. HPLC trace of synthesized (*R*)-benzoin in LCMs; the concentration of substrate is (a) 0.2 mmol/ml, (b) 0.3 mmol/ml, (c) 0.4 mmol/ml, (d) 0.45 mmol/ml.1a correspond to benzaldehyde. 1b correspond to (*R*)-benzoin.

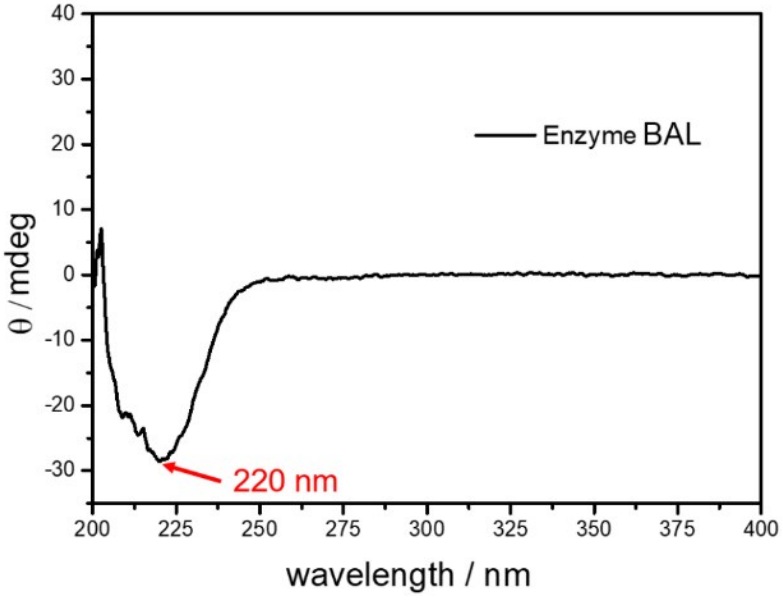


Figure S7. CD spectrum of enzyme BAL.

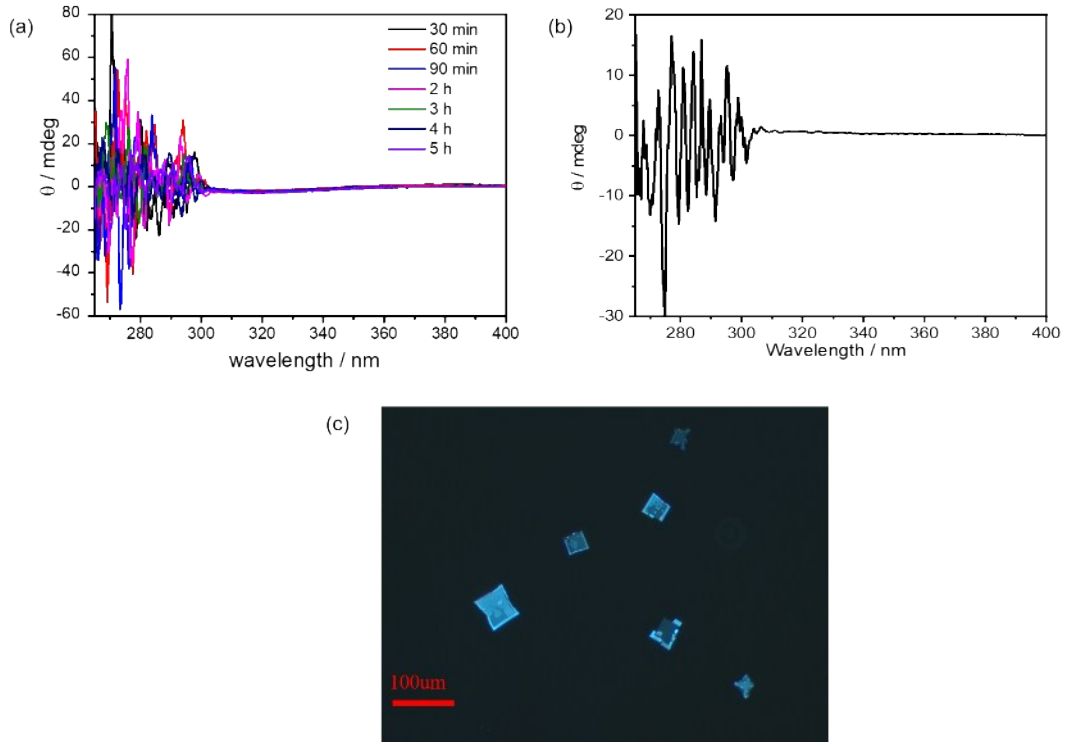


Figure S8. (a) CD spectrum of synthesized benzoin in solution; (b) CD spectrum of benzaldehyde in solution; (c) polarized microscopy image of plate like crystals of benzoin synthesized in solution.

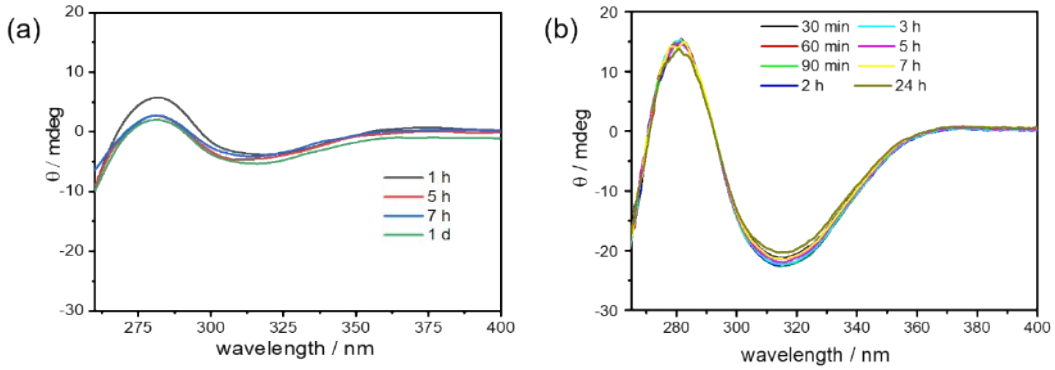


Figure S9. CD spectrum of synthesized (*R*)-benzoin in LCMs, the concentration of substrate is (a) 0.02 mmol/ml and (b) 0.06 mmol/ml.

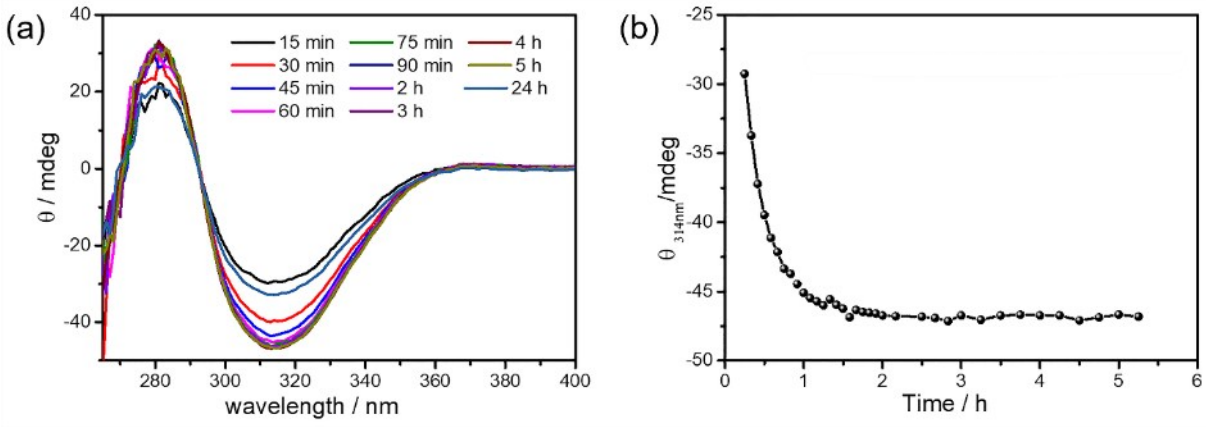


Figure S10. CD spectrum of synthesized (*R*)-benzoin in LCMs, the concentration of substrate is 0.1 mmol/ml.

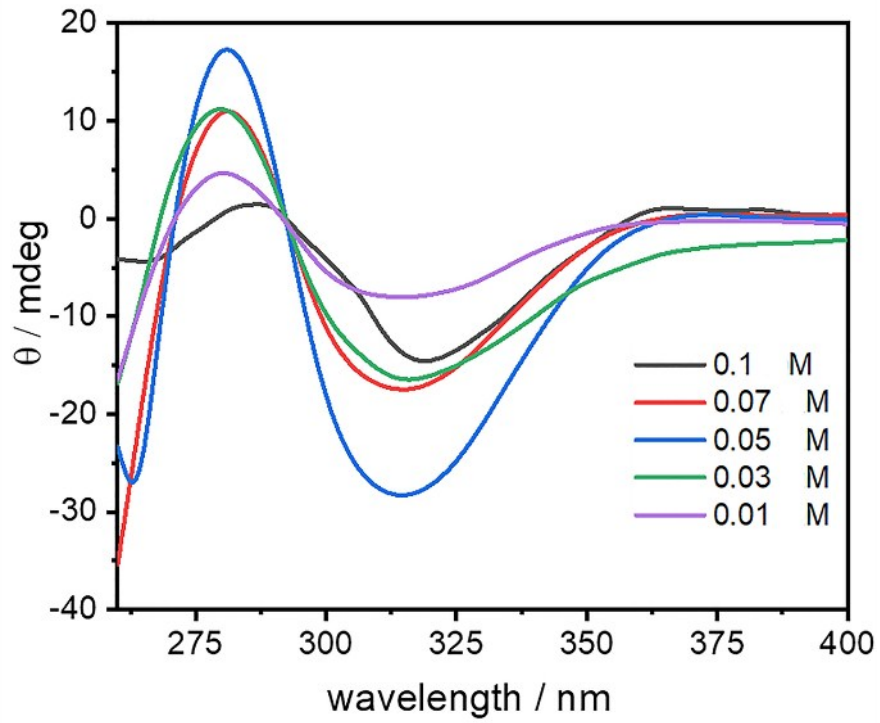


Figure S11. CD spectrum of doped (*R*)-benzoin in LCMs.

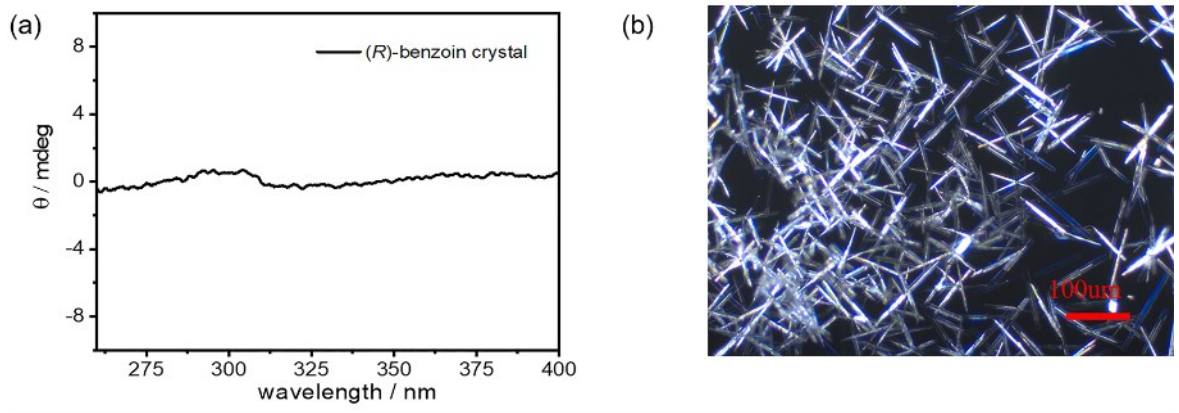


Figure S12. CD spectrum (a) and polarized optical microscopy image (b) of (*R*)-benzoin crystals. 20 mg standard (*R*)-benzoin is dissolved in 1 ml acetone and completely dried in the CD cuvette.

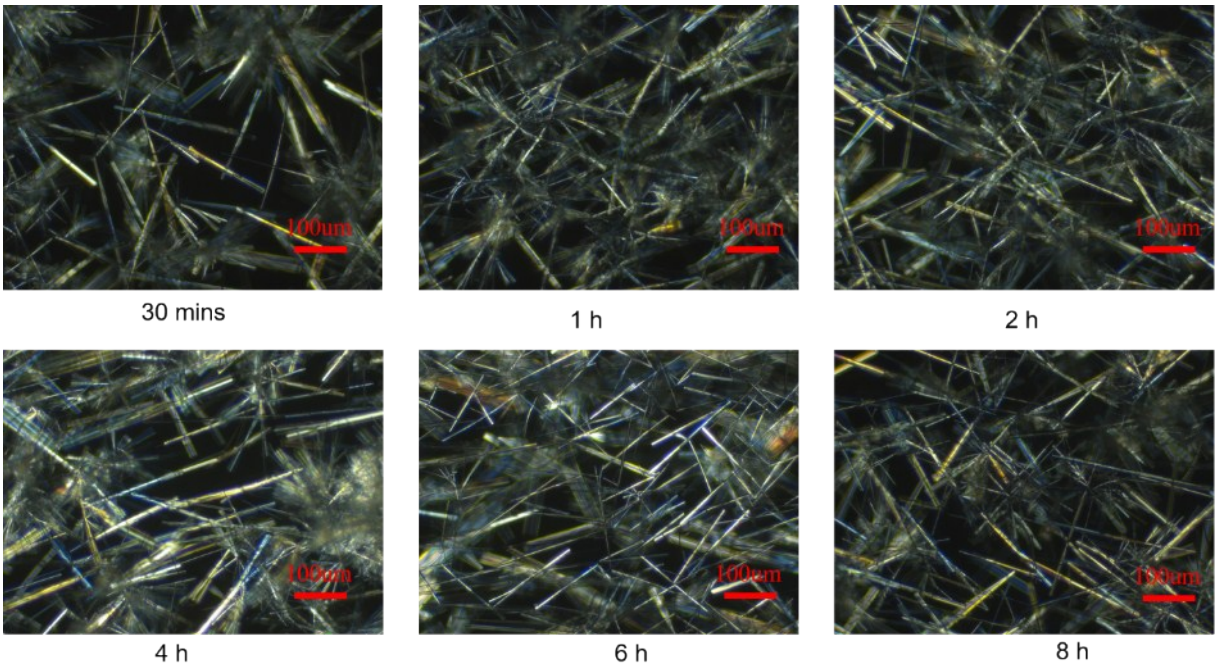


Figure S13. Polarized microscopy images of synthesized (*R*)-benzoin in LCMs after different reaction time, the concentration of substrate is 0.3 mmol/ml.

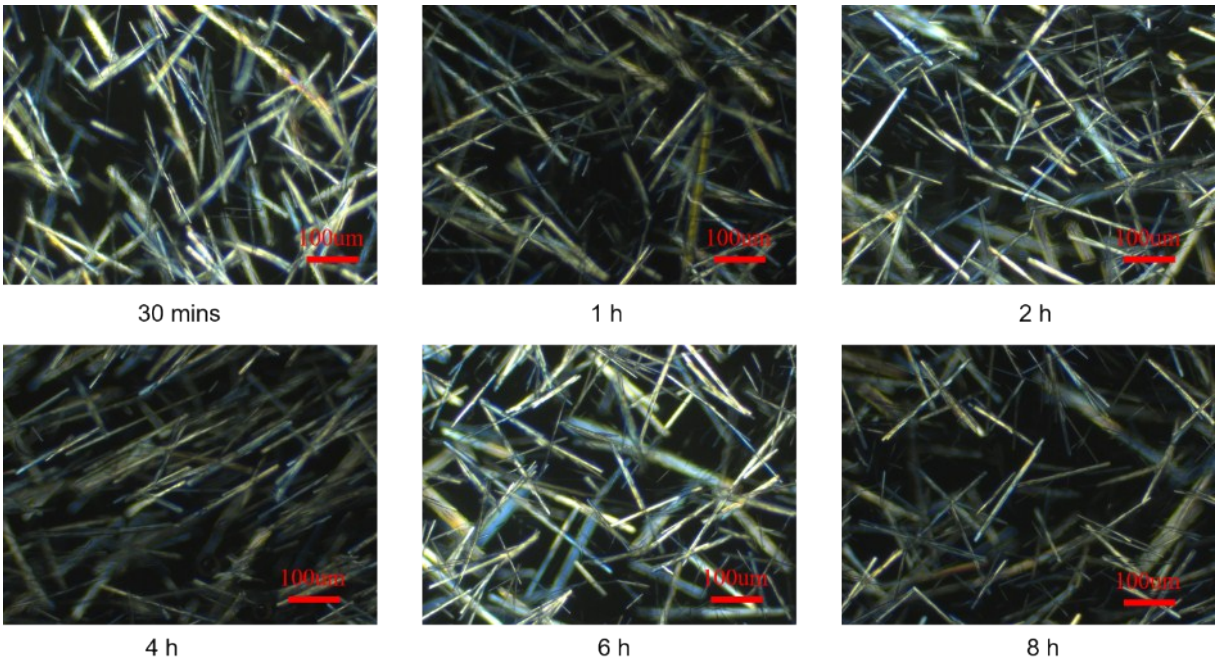


Figure S14. Polarized microscopy images of synthesized (*R*)-benzoin in LCMs after different reaction time, the concentration of substrate is 0.4 mmol/ml.

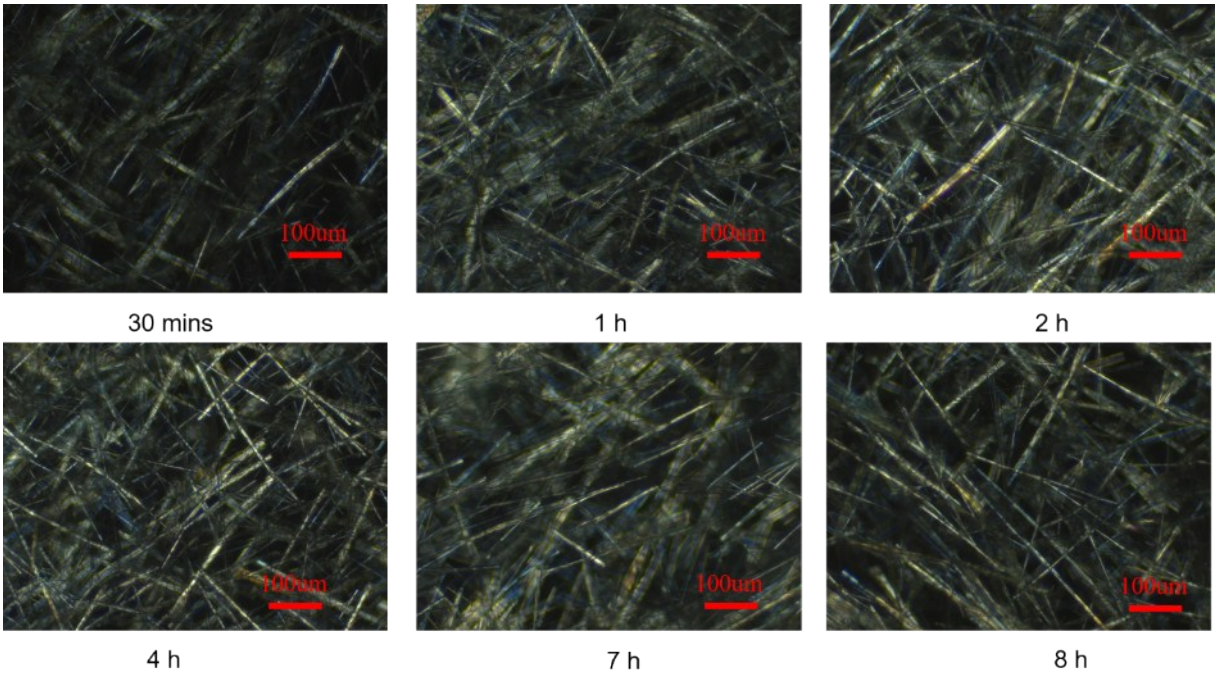


Figure S15. Polarized microscopy images of synthesized (*R*)-benzoin in LCMs after different reaction time, the concentration of substrate is 0.45 mmol/ml.

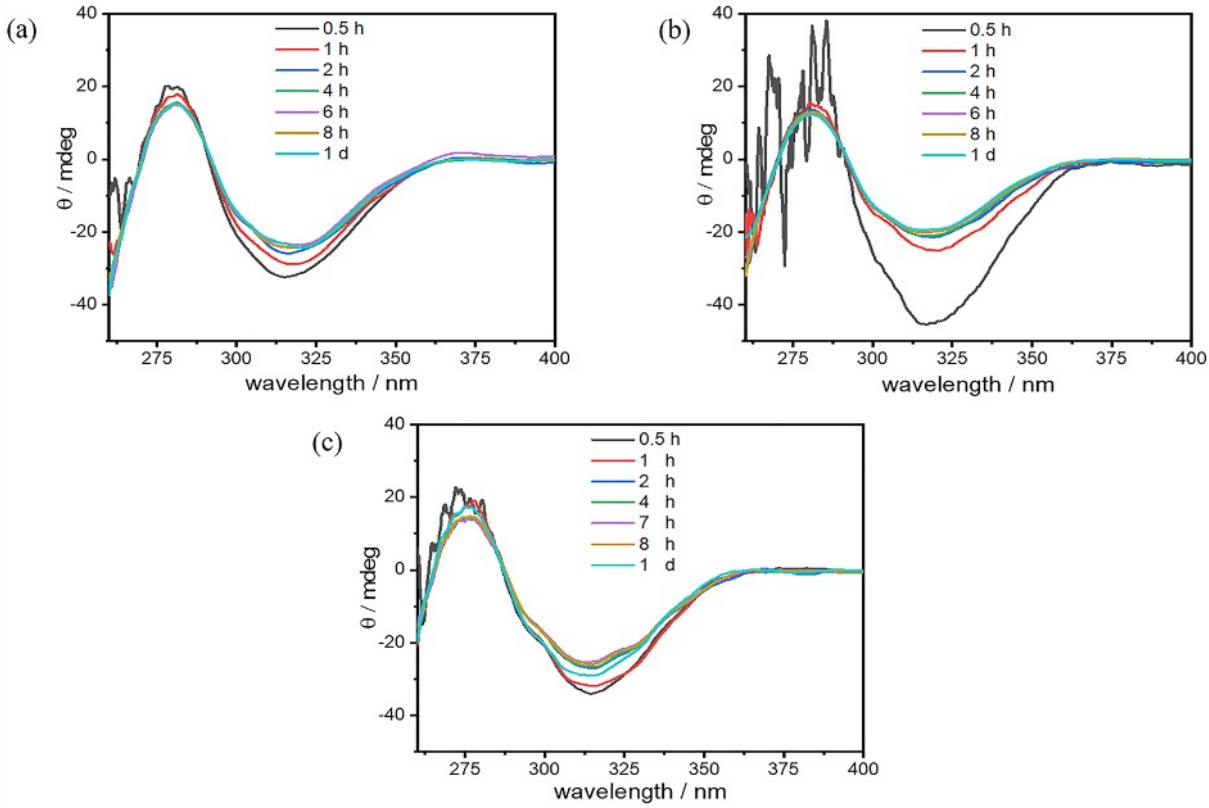


Figure S16. CD spectrum of synthesized (*R*)-benzoin in LCMs, the concentration of substrate is (a) 0.3 mmol/ml, (b) 0.4 mmol/ml, (c) 0.45 mmol/ml.

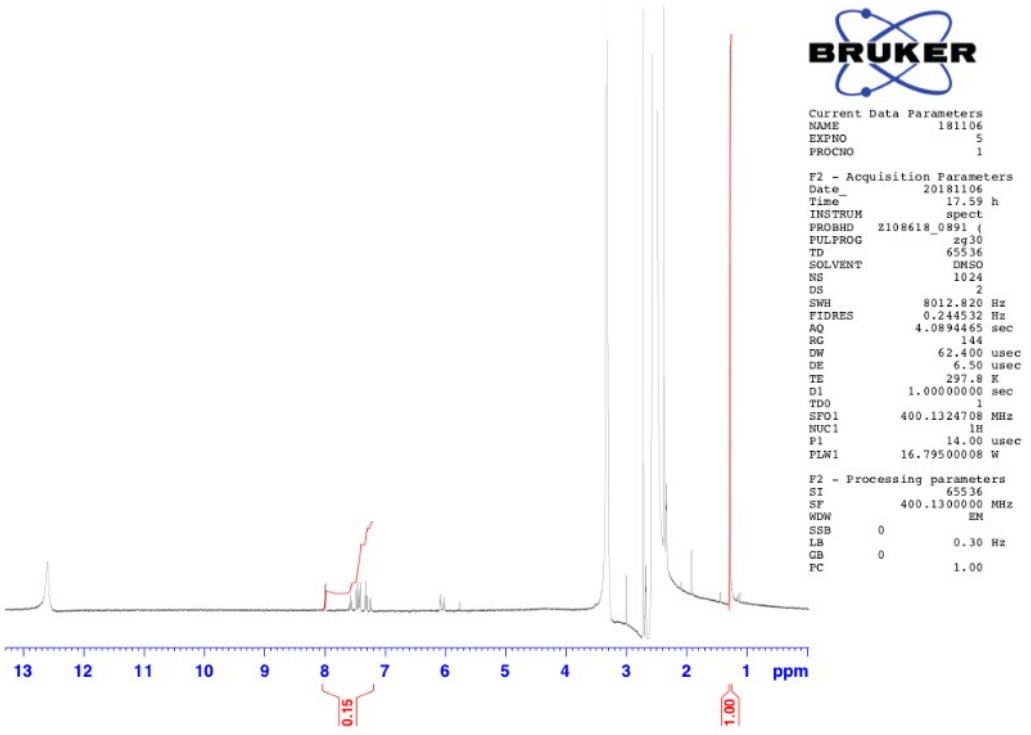


Figure S17 NMR spectra of 0.01 mmol/ml (R)-benzoin solution and 0.1 mmol/ml dimethylmalonic acid solution.

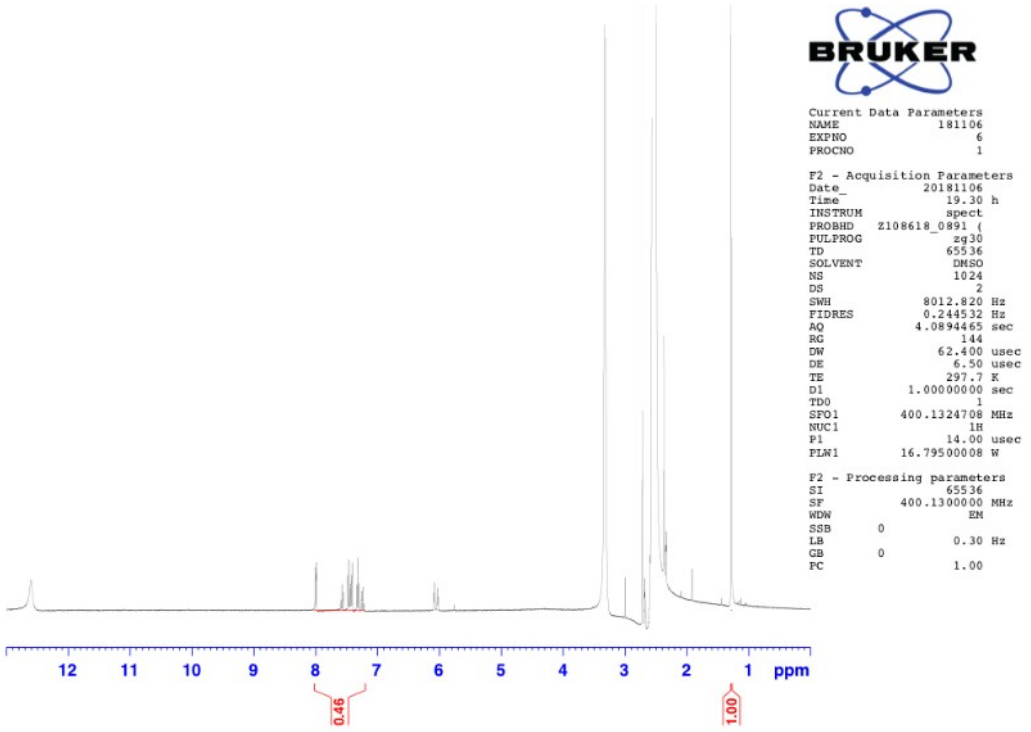


Figure S18 NMR spectra of 0.03 mmol/ml (R)-benzoin solution and 0.1 mmol/ml dimethylmalonic acid solution.

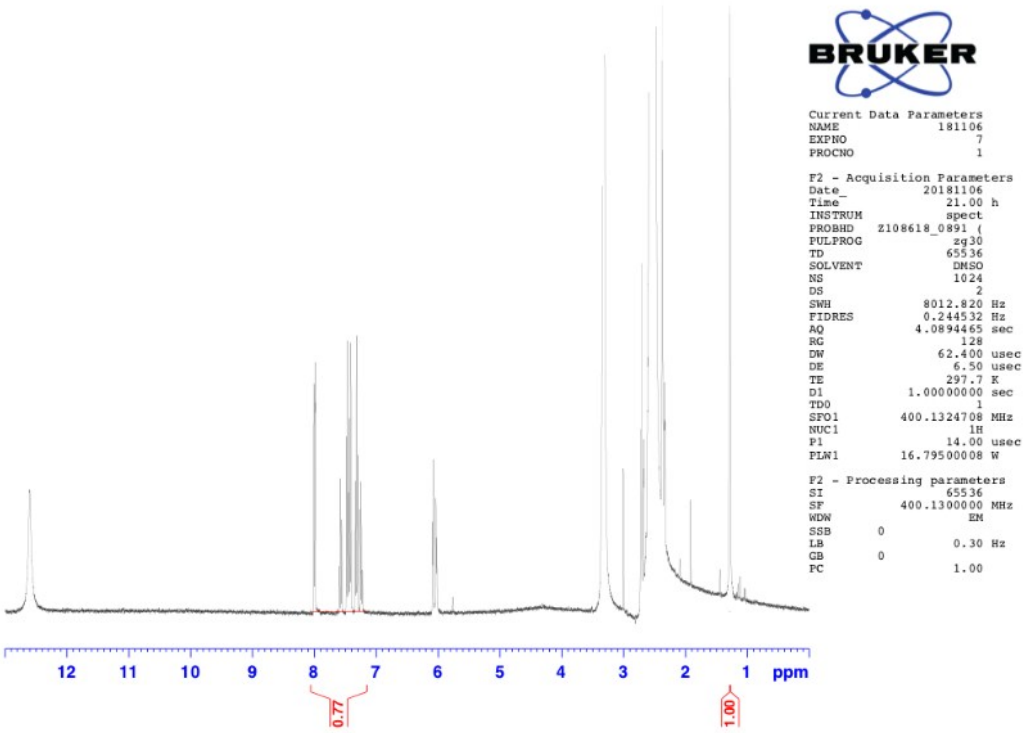


Figure S19 NMR spectra of 0.05 mmol/ml (R)-benzoin solution and 0.1 mmol/ml dimethylmalonic acid solution.

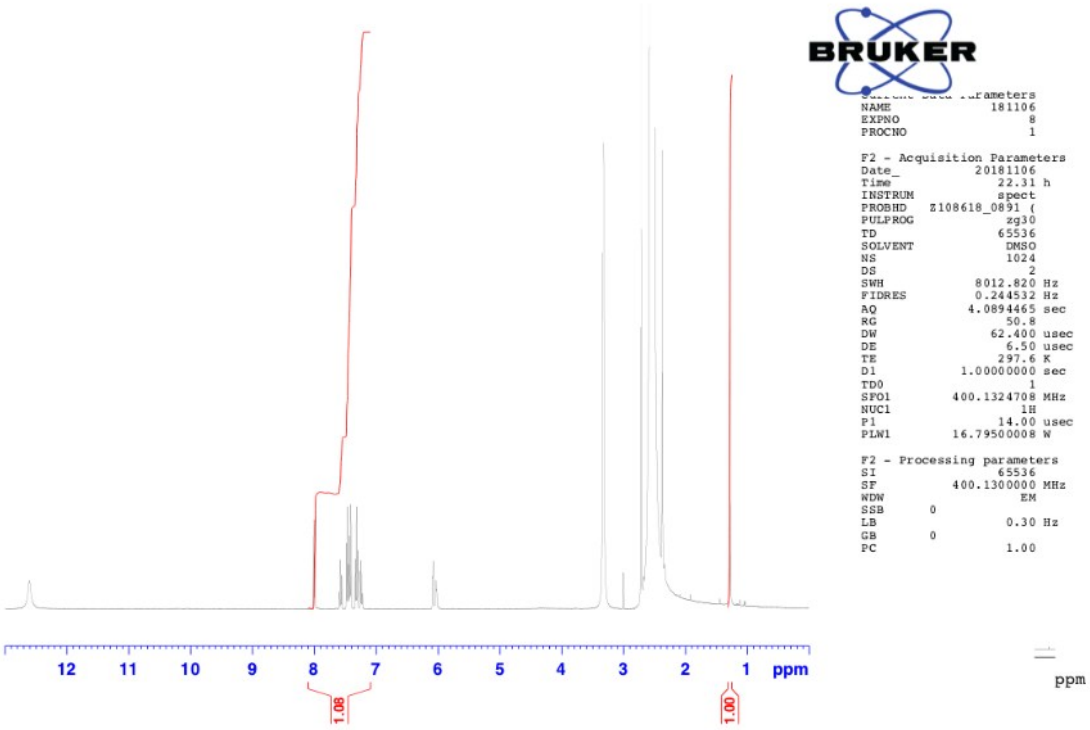


Figure S20 NMR spectra of 0.07 mmol/ml (R)-benzoin solution and 0.1 mmol/ml dimethylmalonic acid solution.

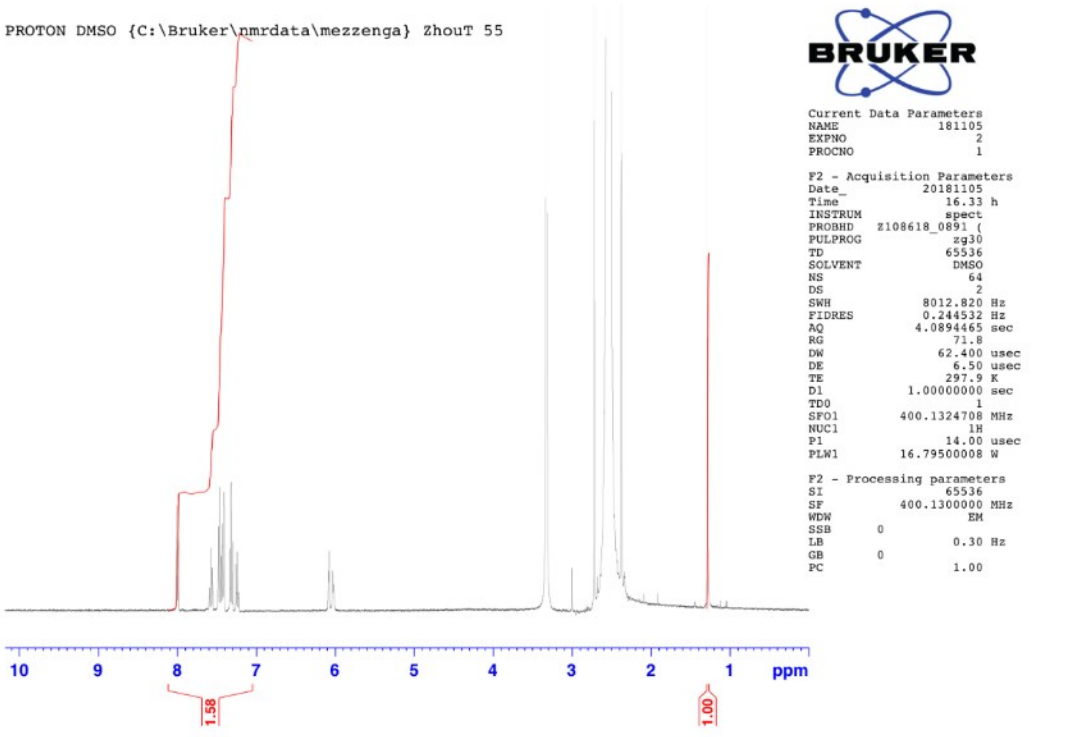


Figure S21 NMR spectra of 0.1 mmol/ml (R)-benzoin solution and 0.1 mmol/ml dimethylmalonic acid solution.

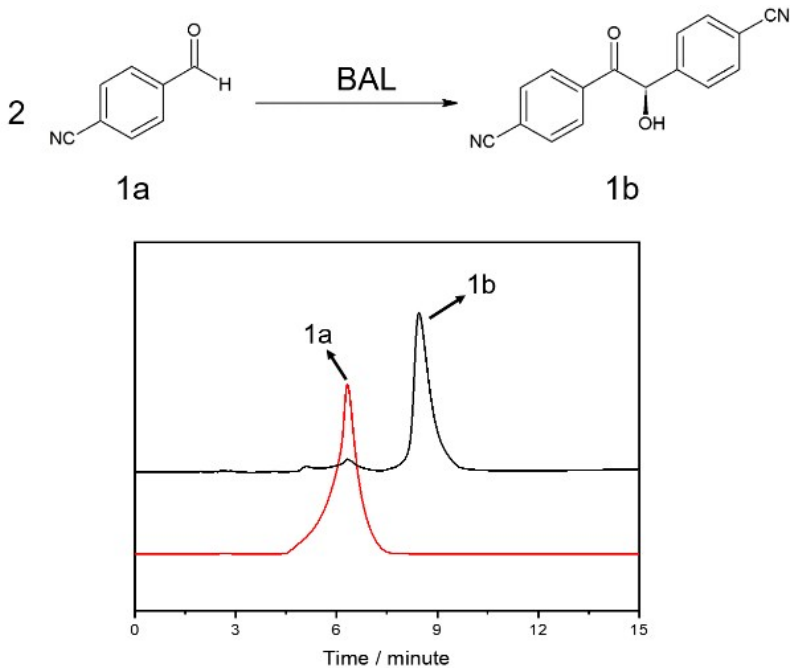


Figure S22. HPLC trace of 4-cyanbenzaldehyde in LCMs in absence (red line) and presence (black line) of enzyme BAL. The yield is over 95%.

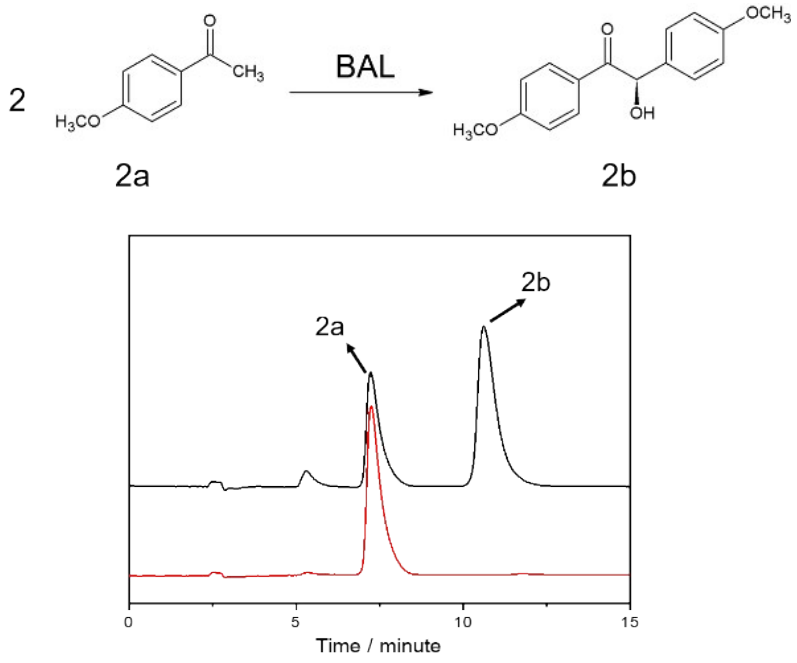


Figure S23. HPLC trace of 4-methoxybenzaldehyde in LCMs in absence (red line) and presence (black line) of enzyme BAL. The yield is 65%.

References:

1. W. J. Sun, J. J. Vallooran, W. K. Fong, R. Mezzenga, *J. Phys. Chem. Lett.* 2016, **7**, 1507 −1512.