Electronic Supplementary Material (ESI) for Reaction Chemistry & Engineering.

Supplementary Information

A chemo-enzymatic tandem reaction in a mixture of deep eutectic solvent and water in continuous flow

Bianca Grabner*[a], Anna K. Schweiger[b], Kristian Gavric[a], Robert Kourist*[b] and Heidrun Gruber-Woelfler*[a]

[a] Institute of Process and Particle Engineering, Graz University of Technology, Inffeldgasse 13/III, 8010 Graz (Austria)

[b] Institute for Molecular Biotechnology, Graz University of Technology, Petersgasse 14, 8010 Graz (Austria)

*Correnspondence to: Bianca Grabner, e-mail: b.grabner@tugraz.at; Robert Kourist, e-mail: kourist@tugraz.at; Heidrun Gruber-Woelfler, e-mail: woelfler@tugraz.at

General. All chemicals and starting materials were purchased from Sigma Aldrich and used as received.

Expression of *Bs*PAD and variants. The recombinant pET28a expression plasmid, containing the *padC* gene (Gene ID: 398579 encoding for PAD from *Bacillus subtilis*, was constructed as described elsewhere.[1] Both recombinant plasmids pET28a_*Bs*PAD_WT were provided by the authors. Chemo-competent *E. coli* BL21 (DE3) cells were transformed with the expression plasmids and single colonies were used to inoculate overnight cultures (5 mL LB-Kan, 40 µg/µL kanamycin), which were incubated at 37 °C and 130 rpm. The complete overnight culture was used to inoculate 200 mL TB-Kan medium in 1 L baffled flasks. Cultures were incubated at 37 °C and 130 rpm, until OD_{600} reached 0.5-0.7 and protein expression was induced by addition of IPTG to a final concentration of 0.1 mM. After incubation at 20 °C and 120 rpm for 20-24 h the cells were harvested by centrifugation (15 min, 4500 rpm, 4 °C) and the cell pellet was washed once with 50 mM KPi buffer (pH 6). Cells were either stored at -20 °C or directly used for the preparation of cell-free extract.

Preparation of cell-free extract and freeze-drying. Cell pellets were resuspended in 50 mM KPi buffer (pH 6) to a concentration of 100 mg_{CWW} mL^{-1} and lysed by sonication (Branson sonifier 250; 5 min, Duty cycle 5, Output control 50 %). The cell-free extract obtained after centrifugation (20 min, 11000 rpm, 4 °C) was sterilized by filtration, shock-frozen in liquid nitrogen and directly used for freeze-drying (AdVantage Pro Lyophilizer, SP Scientific). The lyophilized cell-free extract was stored at -20 °C until further use.

Enzyme immobilization. Cell-free extract (CFE) was dissolved in 2 % (w/v) sodium alginate solution in 50 mM potassium phosphate buffer (pH 6.0) in a concentration of 38.8 mg/mL. The mixture was then dropwise added to a 2 % (w/v) $BaCl_2$-solution in purified water using a syringe and a needle (0.8 mm ID) in order to form uniform beads of approximately 2-3 mm diameter. The beads were gently stirred in $BaCl_2$-solution for 1 h to solidify and turned from almost clear to opaque. The beads were washed with 0.9 % (w/v) NaCl-solution and dried in ambient conditions for 30 min in order to solidify the surface of the beads and make them more resistant to shear forces. In the case more enzyme needed to be immobilized, the amount of potassium phosphate buffer was adjusted to the amount of cell-free extract. All the other parameters and concentrations were kept constant.

Preparation of DES. For the preparation of the deep eutectic solvent (DES), the components choline chloride (ChCl) and glycerol were weighed out in a ratio of 1:2 (mol/mol) and mixed together. The mixture was heated to 80 °C and stirred for 1 h. After cooling down, the prepared clear viscous liquid could be used as reaction solvent.

Preparative synthesis of 4-Vinylphenol. *Para*-coumaric acid **1** (5 mmol) was mixed with 500 mL potassium phosphate buffer (50 mM, pH 6.0 (final concentration: 10 mM)) in a 1 L round bottom flask (Substrate does not completely dissolve in buffer!). 100 mg of lyophilized cell free extract (CFE) of PAD WT were added and the reaction mixture was stirred at 30 °C. The reaction was monitored by thin layer chromatography (TLC) on silica coated aluminium plates (Merck, silica gel 60, F254) and spots were visualized with UV light (254 nm) (cyclohexane/ethyl acetate = 1:1, R_f (**1**) = 0.36, R_f (**3**) = 0.78). When full conversion was detected by TLC, the reaction mixture was extracted with methyl-*tert*-butyl ether (MTBE) (2x 300 mL). The enzyme precipitating in this step was filtered off. To ensure quantitative extraction of the product, TLC analysis of the aqueous layer was performed after extraction. The combined organic layers were washed with brine (1 x 300 mL) and dried over anhydrous Na_2SO_4. Parts of the solvent were removed to obtain the product as a 50 mM solution in MTBE. The solution was stored at 4 °C until further use.

Decarboxylation Batch Experiments. The prepared alginate beads (38.8 mg CFE in 1 mL KPi buffer) were suspended in the solvent mixture of 5 mL DES (choline chloride/glycerol 1:2 (mol/mol)) and 5 mL potassium phosphate buffer (50 mM, pH 6.0) in a round bottom flask and heated to 30 °C. 115 mg (0.7 mmol) of *para*-coumaric acid were added (did not dissolve!) and samples were taken after 0, 15, 30, 60, 120 and 240 min. For each sample 100 µL of the reaction mixture were diluted with 1 mL of HPLC solvent (methanol: HPLC buffer 7:3, HPLC buffer = water:H_3PO_4 300:1)). The sample was filtered through cotton to remove particles from non-dissolved *para*-coumaric acid.

Hot-filtration test. A hot-filtration test was conducted to investigate the heterogeneity of the biocatalyst. The reaction was set up similar to the batch decarboxylation. Samples were taken after 0, 15, 30 and 45 min. After the 45 min sample the alginate beads were removed by filtering the reaction mixture through a preheated filter (30 °C). The remaining solution was further heated and stirred until the whole reaction time of 240 min was reached. Samples were taken after 60, 90, 120, 180 and 240 min of the overall reaction time.

Synthesis of the Pd-catalyst. The catalyst synthesis was adopted from the procedure described by Lichtenegger *et al.*[2] All the starting materials ($(NH_4)_2Ce(NO_3)_6$ (2.124 g), SnC_2O_4 (3.162 g), $PdCl_2$ (0.034 g) and glycine (3.345 g) were weight out in a mortar and well mixed with a pestle. The mixture is then dispersed in 3 mL of water in a 600 mL beaker. The dispersion was treated with ultrasound until a homogeneous solution is obtained. The redox mixture was heated to 350 °C in a furnace, where a self-propagating combustion reaction takes place. The product, a light yellow-brown porous solid, was ground and again heated in the furnace to 350 °C for another 5 h. The obtained yellow-brown powder was used as catalyst without further treatment.

Heck Batch Experiments. MTBE of the storage solution (avoid polymerization) needed to be removed from the starting material under reduced pressure prior the reaction because MTBE was found to inhibit the reaction. In order to avoid polymerization of the reactive styrene substrate the solution was cooled in an ice bath. The concentration of the starting solution was 50 mM. 14.0 mL of solution was evaporated to obtain 84.1 mg (0.7 mmol) of 4-vinylphenol. The remaining substrate (colorless oil) was dissolved in the reaction solvent consisting of 5 mL DES (choline chloride/glycerol 1:2 (mol/mol)), 5 mL potassium phosphate buffer (50 mM, pH 6.0), 5 mL ethanol and 5 mL purified water. The second substrate (iodobenzene) (1.05 mmol) and K_2CO_3 (1.05 mmol) were added and the mixture was heated to 85 °C. Afterwards the reaction was started by adding 5 mg of catalyst. Samples were taken after 0, 10, 20, 30, 60, 90, 120 and 240 min and analyzed by means of HPLC. For each sample 100 µL of the reaction mixture were diluted with 1 mL of HPLC solvent (methanol: HPLC buffer 7:3, HPLC buffer = water:H_3PO_4 300:1)). After 24 h ethanol was evaporated under reduced pressure and the product was extracted in MTBE (3x 30 mL). The organic phases were combined, washed with brine and MTBE was removed under reduced pressure. The crude product (yellow oil) was purified by column flash chromatography on silica gel using hexane:ethyl acetate 3:1 (v/v). Isolated yield: 35 %

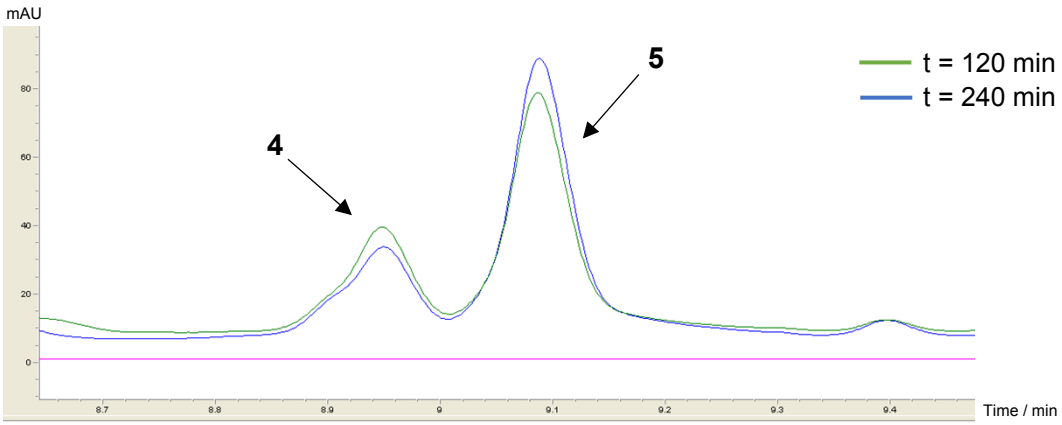


Figure S1. Isomerization of products in Heck reaction in batch over time. Comparison of HPLC peaks of product **5** and side-product **4** at two different time points during Heck coupling in batch, t = 120 min (green) and t = 240 min (blue)

One-pot experiment. For the one-pot reaction the enzymatic decarboxylation was conducted as described previously but with increased catalyst loading (80 mg). After 180 min the alginate beads with PAD was removed from the reaction mixture by filtration. 10 mL of solvent (5 mL ethanol and 5 mL water) were added containing the substrates iodobenzene (1.05 mmol, 1.5 mol-eq. with respect to *para*-coumaric acid, 214.2 mg) and K_2CO_3 (1.05 mmol, 1.5 mol-eq., 145.1 mg). After heating the solution to 85 °C the catalyst (25.2 mg) were added to start the reaction. Samples were taken over time and analyzed by means of HPLC.

Decarboxylation flow experiment. 160 mg of CFE PAD was immobilized as described previously in 4 mL final bead volume in 2 % (w/v) alginate beads. The immobilized biocatalyst was packed in a stainless steel column (120 mm x 8 mm ID). The remaining free volume of the column was filled with glass beads. The used capillaries, fittings and syringe adapters were standard HPLC equipment (1/16 inch OD. x 0.03 inch ID, PEEK). The column was heated to 30 °C in a water bath and flushed with solvent (DES:buffer 1:1 (v/v)) at a flow rate of 45.5 µL/min from a syringe pump (Lambda VIT-FIT, Syringe pump equipped with 20 mL stainless steel syringe, Lambda Instruments). After 1 of flushing the reaction was started by switched to the feed stock containing 20 mM *para*-coumaric acid **1**. Samples were collected at the outlet in 15 min intervals. From the product stream 50 µL were diluted with 500 µL of HLPC solvent (methanol:HPLC buffer 7:3, HPLC buffer = water:H_3PO_4 300:1)). To test enzyme leaching, samples were collected at the outlet of the reactor at t = 40, 80, 120, 190 and 250 min. Additional substrate was added and the samples were kept at ambient conditions overnight. The next day 50 µL of the sample were diluted and analyzed as usual. No further product formation was observed.

Heck flow experiment. The prepared catalyst powder was packed in a stainless steel column (40 mm x 8 mm ID) (2 g). The used capillaries, fittings and syringe adapters were standard HPLC equipment (1/16 inch OD. x 0.03 inch ID, PEEK). The packed-bed reactor was heated to 85 °C in a water bath and flushed with solvent (DES:buffer:ethanol:water 1:1:1:1 (v/v/v/v)) at a flow rate of 45.5 µL/min from a syringe pump (Lambda VIT-FIT, Syringe pump equipped with 20 mL stainless steel syringe, Lambda Instruments). After 1 of flushing the reaction was started by switched to the feed stock containing 10 mM 4-vinylphenol, 15 mM iodobenzene and 15 mM K_2CO_3. Samples were collected at the outlet in 15 min intervals. From the product stream 50 µL were diluted with 500 µL of HLPC solvent (methanol:HPLC buffer 7:3, HPLC buffer = water:H_3PO_4 300:1)).

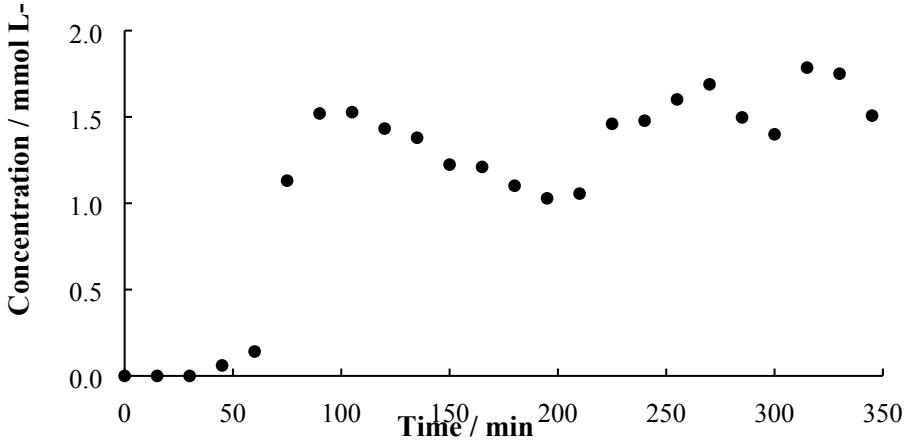


Figure S2. Concentration of 4-hydroxystilbene from 4-vinylphenol (10 mM) in a continuous flow Heck cross coupling with iodobenzene (1.5 mol-eq.) in the presents of K_2CO_3 (1.5 mol-eq.) using

$Sn_{0.79}Ce_{0.20}Pd_{0.01}O_{2-\delta}$ as heterogeneous catalyst. 85 °C, solvent: DES:buffer:ethanol:water 1:1:1:1 (v/v/v/v), flow rate: 91 µL min^{-1}

Two-step flow experiment. Two stock solutions were prepared. Stock A served as feed for the enzymatic decarboxylation consisting of DES:potassium phosphate buffer (50 mM, pH 6.0) in a ratio of 1:1 (v/v) and **1** (20 mM.) Stock B was the feed for the Heck coupling and was mixed with the outlet of the decarboxylation before entering the Pd-packed column. Stock B contained 30 mM (1.5 mol-eq. with respect to **1**) **3** and 30 mM (1.5 mol-eq.) K_2CO_3 dissolved in DES:ethanol:water in a ratio of 1:6.75:2.25. The used capillaries, fittings and syringe adapters were standard HPLC equipment (1/16 inch OD. x 0.03 inch ID, PEEK). A T-mixing element (Advantage™ Stainless Steel Tee, 0.25 mm Thru-hole for 1/16 inch OD tubing) was used to mix the product stream of the first reactor with the substrate feed for the second reactor. The packed columns were heated in water baths of 30 °C for the decarboxylation (160 mg PAD immobilized in alginate bead of a total volume of 4 mL in two stainless steel columns in a series (each 40 mm x 8 mm ID) were flushed with solvent with 45.5 µL/min overnight to remove loosely bound enzyme and non-linked alginate) and 85 °C for the Heck reaction (6 g of catalyst powder in a stainless steel column 120 mm x 8 mm ID). After flushing the system with solvent for 1 h, the feed was switched to the stock solutions (Lambda VIT-FIT, Syringe pump equipped with 20 mL stainless steel syringe, Lambda Instruments, flow rate: 45.5 µL/min, respectively). The pressure in the system was kept at 5.17 bar (IDEX BPR Cartridge 75 psi Gold Coat). Samples were collected at the outlet in 15 min intervals. From the product stream 50 µL were diluted with 500 µL of HLPC solvent (methanol: HPLC buffer 7:3, HPLC buffer = water:H_3PO_4 300:1)).

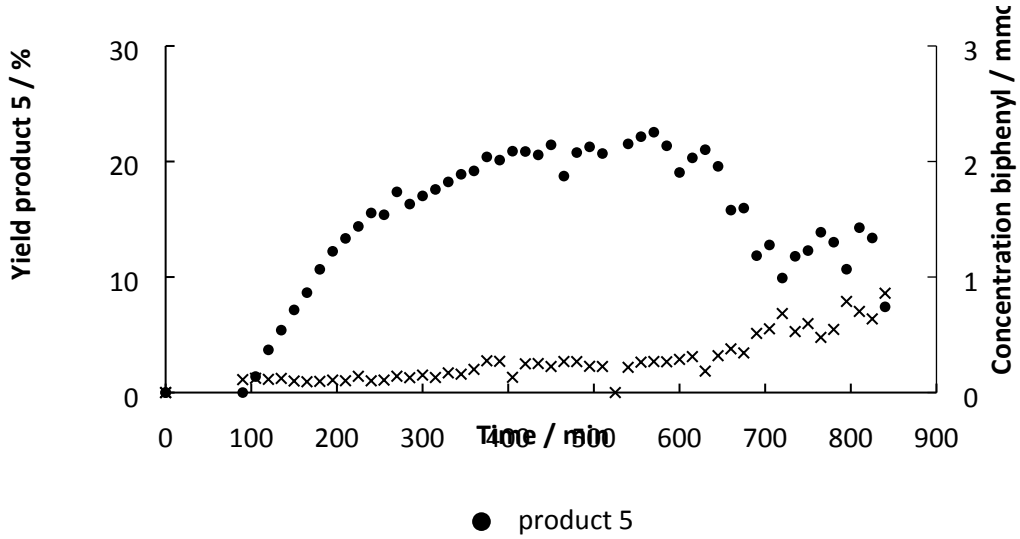


Figure S3. Yield‡ of **5** and concentration of the side-product biphenyl due to homo-coupling to **2** over time for the first attempt of continuous synthesis of **3** by enzymatic decarboxylation by *Bs*PAD and subsequent Pd-catalyzed Heck reaction using $Sn_{0.79}Ce_{0.20}Pd_{0.01}O_{2-\delta}$ to couple **3** and **2**, yield determined by HPLC.

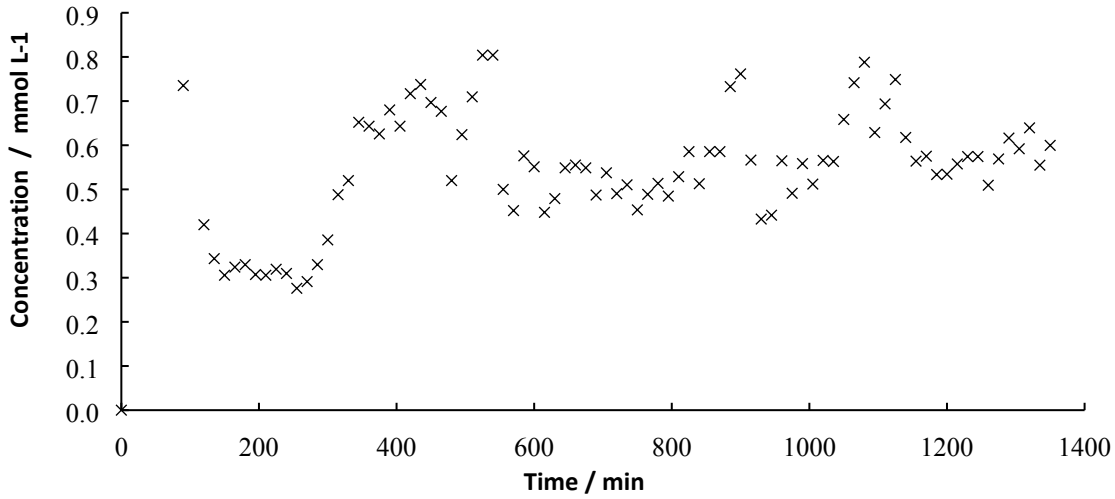


Figure S4. Concentration of side-product biphenyl due to homo-coupling of **2** over time for the flow process for continuous synthesis of **3** by enzymatic decarboxylation by *Bs*PAD and subsequent Pd-catalyzed Heck reaction to couple **3** and **2**, concentration determined by HPLC.

HPLC Analysis. Samples were collected and analysed in singlets (no error bars shown). However, repetition of the experiments led to similar outcomes and hence proved reproducibility of the results. The samples were analyzed by reversed phase high performance liquid chromatography (RP-HPLC) using an Agilent Instrument 1100 Series equipped with a ThermoFischer Scientific Accucore™ C18 reversed phase column (50 x 4.6 mm ID; 2.6 µm). 2.0 µL of the sample was injected. Eluent: 0-1 min 60 % H_2O:H_3PO_4 300:1, 40 % methanol; 1-12 min gradient to 10 % H_2O:H_3PO_4 300:1, 90 % methanol; 12-14 min gradient to 60% H_2O:H_3PO_4 300:1, 40 % methanol. Sample analysis lasted 16 min. Flow rate: 1 mL/min. Column temperature: 25 °C. For detection of the analyts, a UV detector was used. Retention times: *para*-coumaric acid **1** 1.1 min (282.4 nm), 4-vinylphenol **3** 3.4 min (237.4 nm), iodobenzene **2** 8.7 min (237.4 nm), *para*-hydroxy-1,1-diphenylethylene **4** 8.9 min (237.4 nm), 4-hydroxystilbene **5** 9.1 min (282.4 nm). Spectra of is shown in Figure S5 and Figure S6.

NMR. NMR-measurements were recorded using a Bruker Avance III 300 MHz spectrometer in $CDCl_3$. ^{1}H-NMR of product **5** and side-product **4** are shown in Figure S7 and S8.

***para*-Hydroxystilbene** (**5**)[3]**:** ^{1}H-NMR: 7.50 – 7-48 (d, 2H, Ar-H), 7.43 - 7.40 (d, 2H, Ar-H), 7.37 - 7.32 (t, 2H, Ar-H), 7.26 - 7.22 (t, 1H, Ar-H), 7.09 - 6.94 (dd, 2H, H-C=C-H), 6.85 – 6.82 (d, 2H, Ar-H), 4.77 (s, 1H, O-H) ppm.

***para*-Hydroxy-1,1-diphenylethylene** (**4**)**:** ^{1}H-NMR: 7.31 – 7.28 (m, 5H, Ar-H), 7.25 - 7.20 (d, 2H, Ar-H), 6.81 - 6.78 (d, 2H, Ar-H), 5.39 (s, 1H, C=C-H), 5.35 (s, 1H, C=C-H), 4.74 (s, 1H, O-H) ppm.

Figure S5. HPLC spectrum a sample (t = 10 min) of Heck coupling reaction in batch according to the description above (section Heck Batch Experiments).

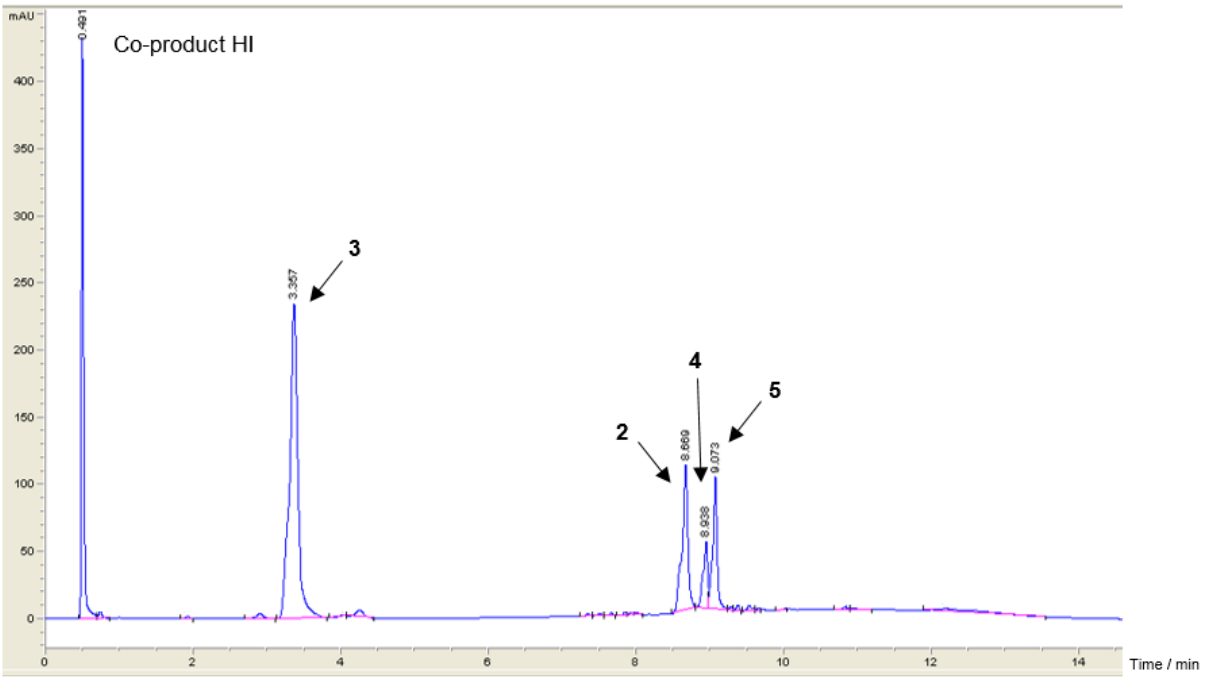
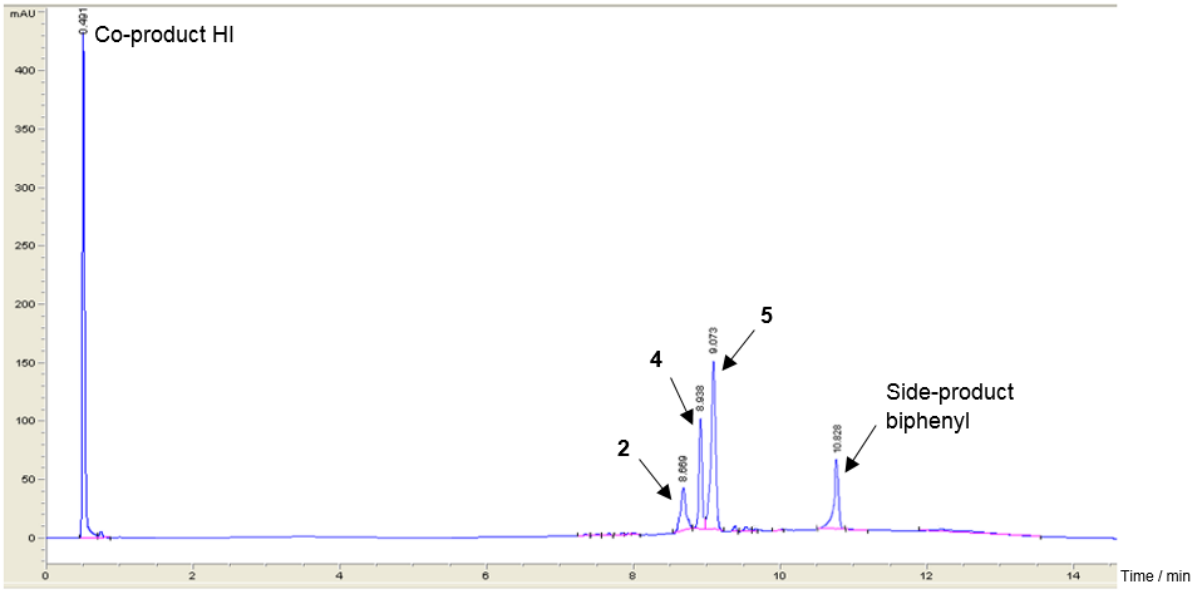


Figure S6. HLPC spectrum of product stream of the two-step flow experiment.



Time / min

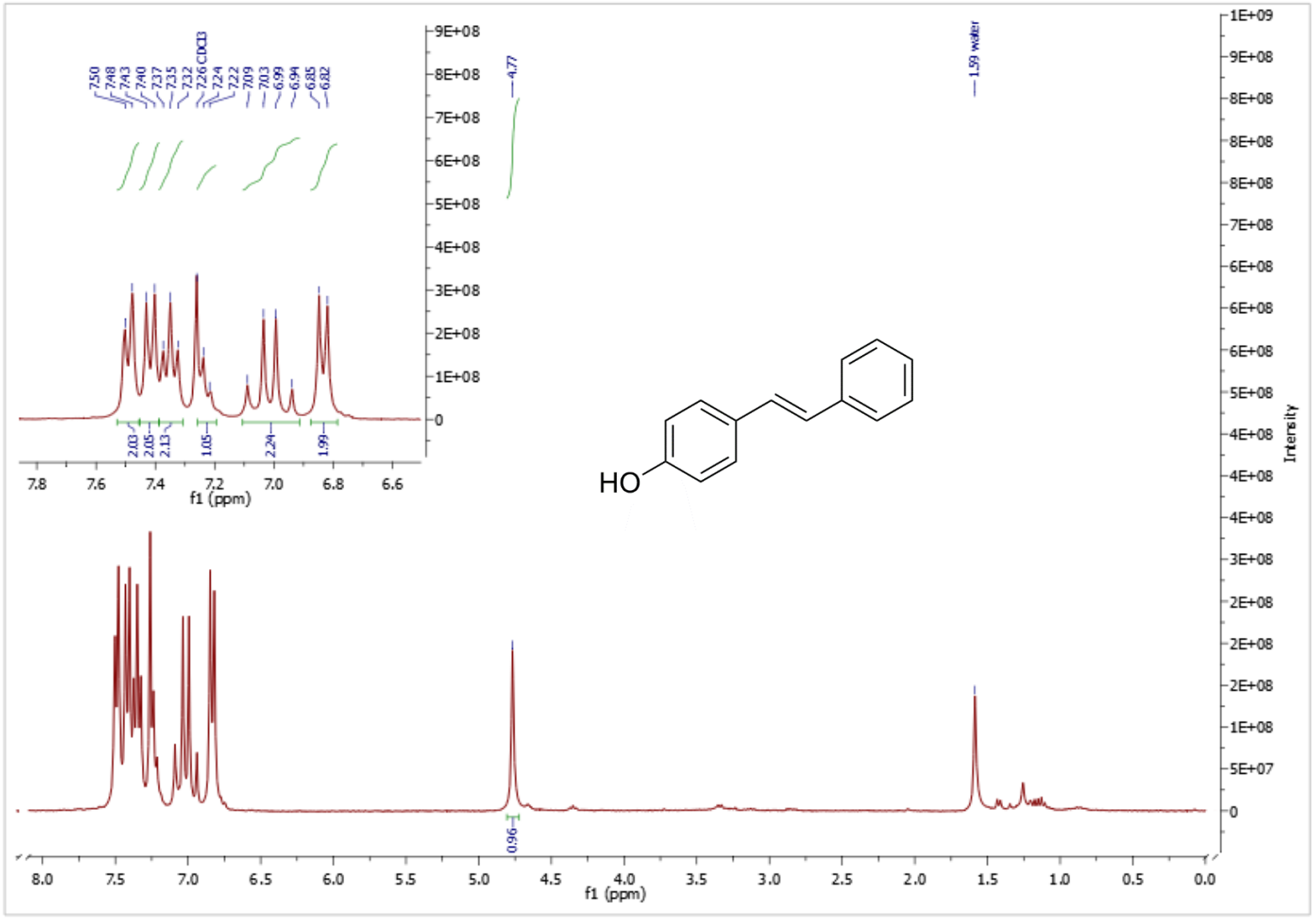


Figure S7. ^{1}H-NMR of product **5**

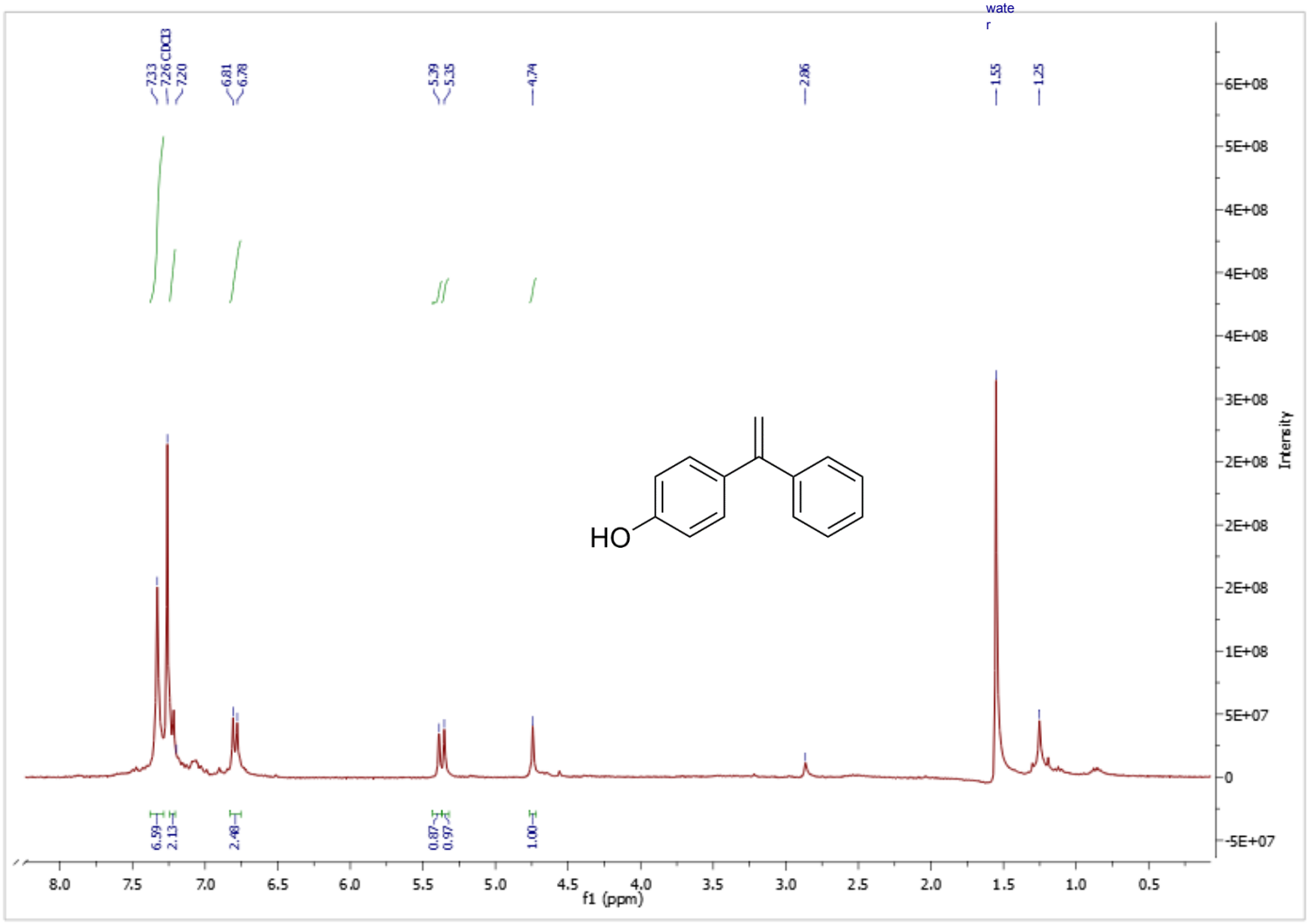


Figure S8. ^{1}H-NMR of side-product **4**

Notes and References.

‡ $A_{(limiting)}$ + B → P; Yield Y [%] = $(c_{P,t}/c_{A,0})$*100; Conversion X [%] = $(c_{A,0}-c_{A,t})/c_{A,0}$*100, Yield and conversion determined by HPLC after calibration

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