Green Chemistry

Electronic Supporting Information (ESI)

Design of a green chemoenzymatic cascade for scalable synthesis of bio-based styrene alternatives

Philipp Petermeier, [a] Jan Philipp Bittner,[b] Simon Müller,[b] Emil Byström,[c] and Selin Kara*[a,d]

[a] Biocatalysis and Bioprocessing Group, Department of Biological and Chemical Engineering, Aarhus University, 8000 Aarhus C, Denmark

[b] Institute of Thermal Separation Processes, Hamburg University of Technology, 21073 Hamburg, Germany

[c] SpinChem AB, Tvistevägen 48C, 90736 Umeå, Sweden

[d] Institute of Technical Chemistry, Leibniz University Hannover, 30167 Hannover, Germany

E-mail: selin.kara@bce.au.dk

Table of Contents

1. Expression of *Bs*PAD in *E. coli* BL21-Gold(DE3)

The plasmid encoding for the phenolic acid decarboxylase was provided by Prof. Robert Kourist from TU Graz. Competent cells of *E. coli* BL21-Gold(DE3) (Art. No. 230132, *Agilent Technologies*) were transformed with the plasmid according to the cell supplier's instructions. A single colony from an agar plate was used to inoculate LB medium (20 mL, 50 mg/L Kanamycin) and incubated at 37 °C and 150 rpm overnight. Glycerol stocks were prepared by diluting 700 µL of this overnight culture (ONC) with 300 µL of 60% (m/m) aqueous glycerol. The stocks were kept at room temperature for 15 min, then flash-frozen in liquid nitrogen and stored at -20 °C (work stock) and -80 °C (backup stock). For enzyme expression, LB medium (20 mL, 50 mg/L Kanamycin) was inoculated with 50 µL of cell suspension from a thawed glycerol stock and incubated at 37 °C and 150 rpm for 16-18 h in a 100 mL baffled Erlenmeyer flask. 10 mL of ONC were used to inoculate TB medium (1 L, 50 mg/L Kanamycin) and the resulting culture was incubated at 37 °C and 80 rpm in a 5 L baffled Erlenmeyer flask. Samples (1 mL) were taken at regular intervals and analysed for their optical density at 600 nm (OD_{600}). When the OD_{600} reached a value of about 1.5 (approx. after 3 h), protein expression was induced by addition of IPTG (1 mL, 1 mol/L in H_2O) and incubation was continued at 20 °C and 80 rpm for about 21 h. For harvesting, cultures were centrifuged (8,000 rpm, 4 °C, 20 min) to yield wet cell pellets. The supernatant was treated with Virkon® and discarded. The pelleted cells were resuspended in KPi buffer (50 mM, pH 6.0, 6 mL per gram wet cell pellet), cell suspensions (in fractions of 50 mL) transferred to round-bottom flask (250 mL), flash-frozen in liquid nitrogen and lyophilized overnight to obtained lyophilized whole-cell preparations (typically 2.3-3.2 g/L), which were stored at -20 °C until use. For the preparation of cell-free extracts (CFE), lyophilized whole-cells (1 g) were transferred to 50 mL plastic tubes and resuspended in MilliQ water (25 mL). The cell suspensions were cooled on wet ice for 1 h before ultrasonic cell disruption (MS73 probe, 20%, 2 s ON, 4 s OFF, 2 min per cycle, 4 cycles). Next, the suspensions were transferred to 50 mL centrifuge tubes and centrifuged (14,000 rpm, 4 °C, 20 min) to remove cell debris. The supernatants were transferred to round-bottom flask (250 mL), flash-frozen in liquid nitrogen and lyophilized overnight to obtain lyophilized cell-free extract preparations, which were stored at -20 °C until use.

2. HPLC-UV analytics

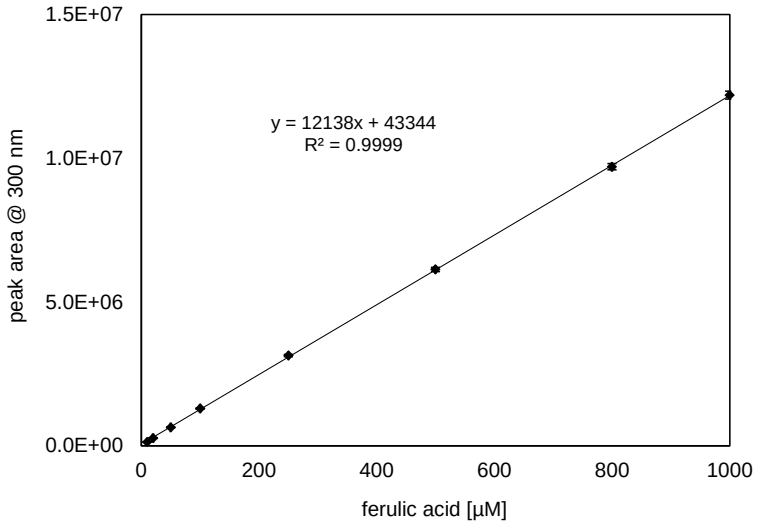

Figure S 1. Calibration for ferulic acid. (triplicates in water/acetonitrile 1/1; 10 µL injection volume; $\lambda_{detection}$ = 300 nm)

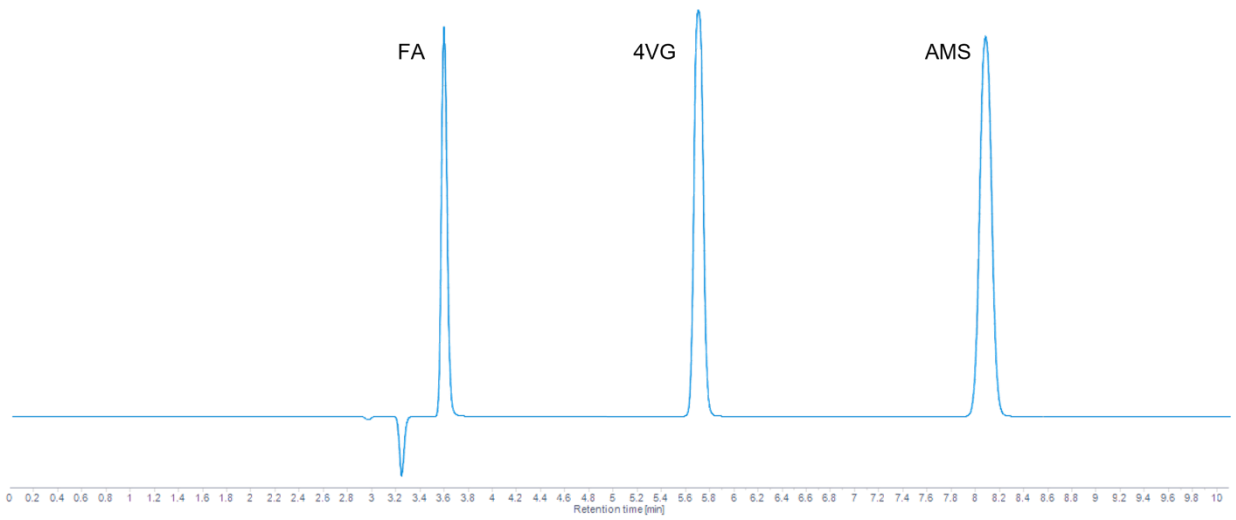

Figure S 2. Chromatographic separation of model substrate ferulic acid (FA, t_r 3.6 min), intermediate 4-vinylguaiacol (4VG, t_r 5.7 min), and product 4-acetoxy-3-methoxystyrene (AMS, t_r 8.1 min) using reported HPLC method.

3. COSMOtherm results

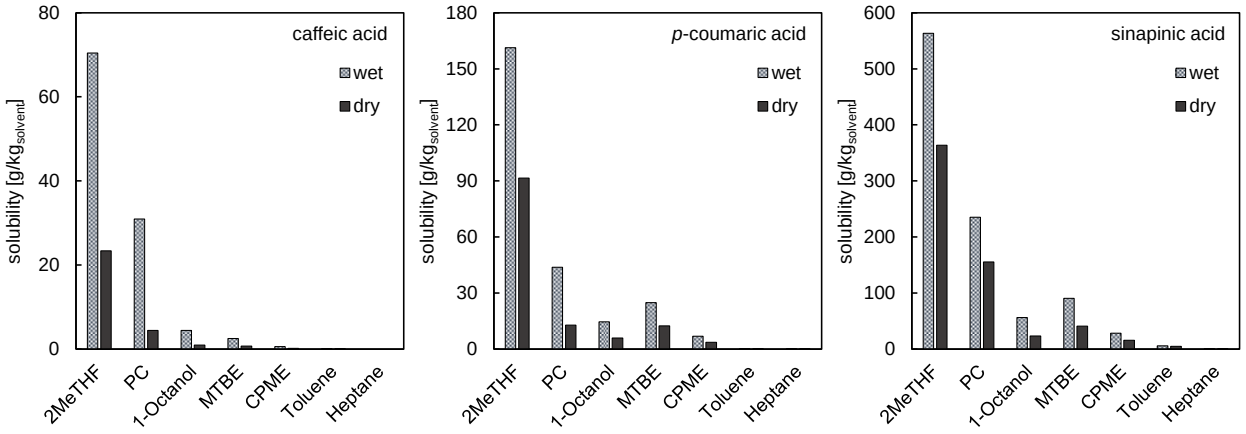

Figure S 3. Solubilities of caffeic acid, *p*-coumaric acid, and sinapinic acid as calculated in various organic solvents. The solubilities were calculated for dry, and water saturated (wet) solvents at +30 °C using BIOVIA COSMOtherm 2020 and reference solubilities in water.

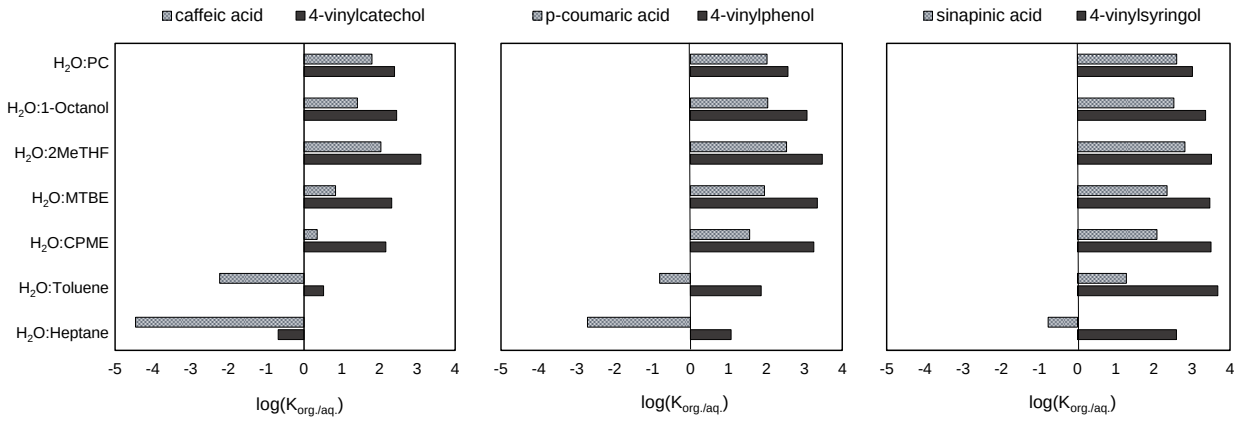

Figure S 4. Calculated partition coefficients at infinite dilution (log $K_{org./aq.}$) for three substrate-products pairs of interest between equilibrated organic solvent-water phases at +30 °C. software: BIOVIA COSMOtherm 2020.

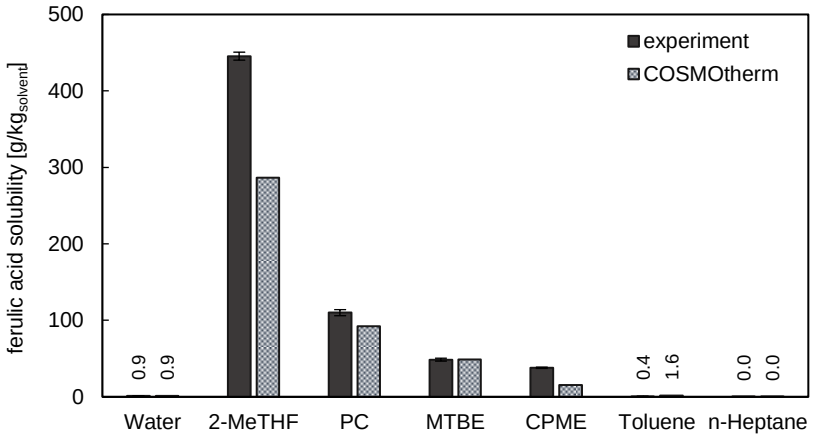

Figure S 5. Comparison of calculated and experimentally determined solubilities of the model compound ferulic acid in selected solvents. Organic solvents were equilibrated over water at room temperature (24 h) and the solubility of ferulic acid was determined for a temperature of +30 °C.

4. Substrate-specific activities of *Bs*PAD WT

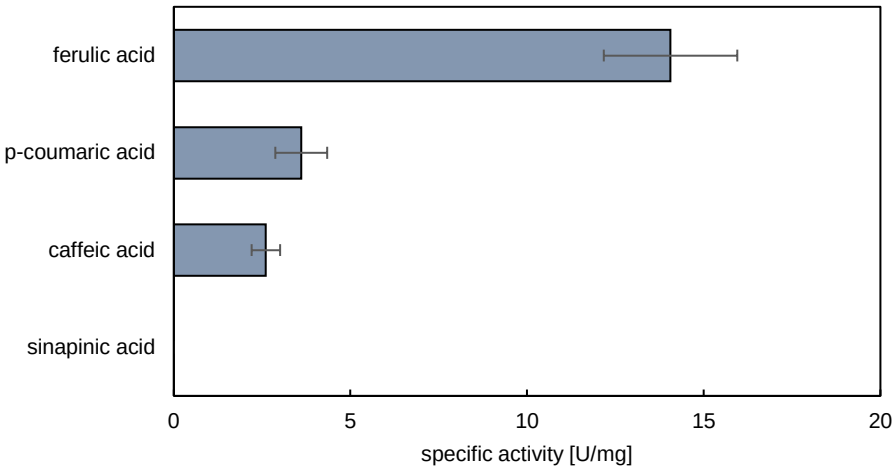

Figure S 6. Specific activities of *Bs*PAD WT whole-cell preparations towards different phenolic acid substrates: ferulic acid, *p*-coumaric acid, caffeic acid and sinapinic acid. Experimental conditions: 10 mM substrate, 50 mg/L lyophilized whole-cells, KPi buffer (50 mM, pH 6.0), 5 vol-% DMSO, +30 °C, 1,000 rpm.

5. Influence of CPME-water saturation on *Bs*PAD activity

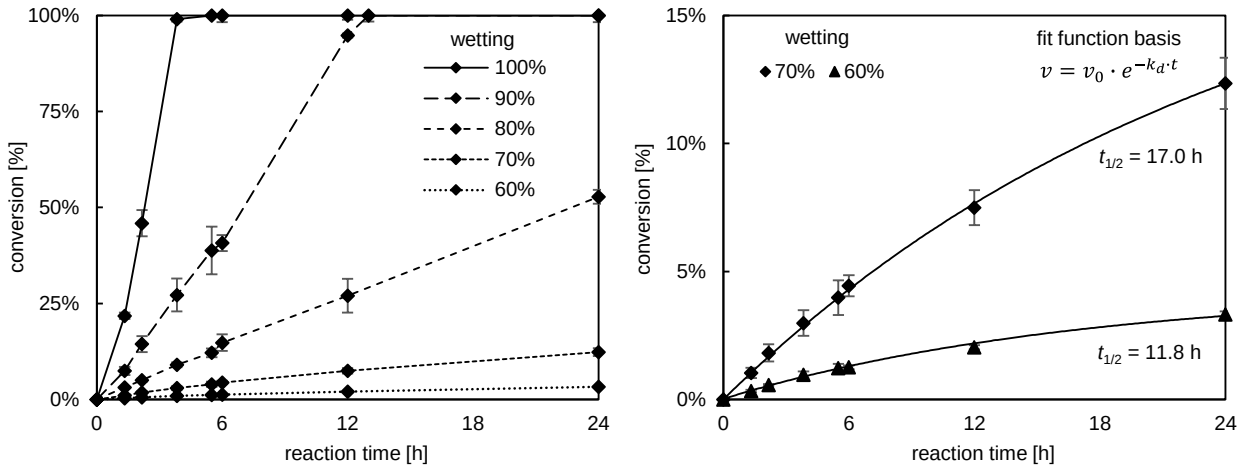

Figure S 7. Biocatalytic decarboxylation of ferulic acid in CPME at varying water saturation levels. Conditions: 100 mM FA, 100 mg/L lyophilized whole-cells, wetted CPME, 1,000 rpm, +30 °C, exp. duplicates. (left: visual aid lines; right: fit curves)

6. *Bs*PAD activity in re-wetted CPME

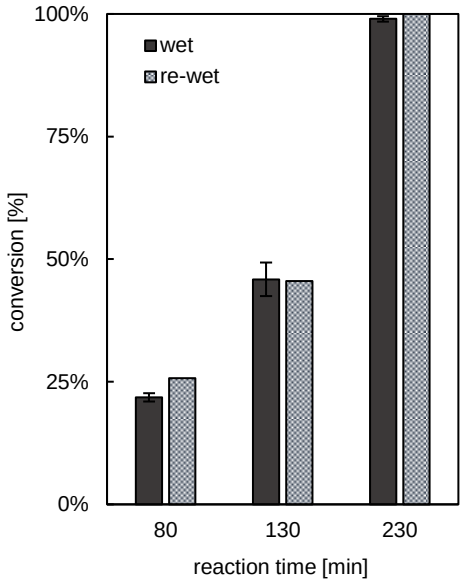

Figure S 8. Comparison of biocatalytic ferulic acid decarboxylation in wet and re-wetted CPME by lyophilized whole-cells harbouring *Bs*PAD. The wet CPME was equilibrated over H_2O (24 h, rt), whereas the re-wetted was first dried over molecular sieves (24 h, rt) and then mixed with a minor excess of water (1.1 wt-%) before the respective organic phases were used as reaction media for the biotransformation. The lack of significant differences indicates no adverse effects caused by the drying over molecular sieves. Full activity is recovered after re-wetting the solvent. Reaction conditions: 100 mM FA, 100 mg/L cells, wet CPME, 1,000 rpm, +30 °C.

7. Reusability of *Bs*PAD-8415F in consecutive batches

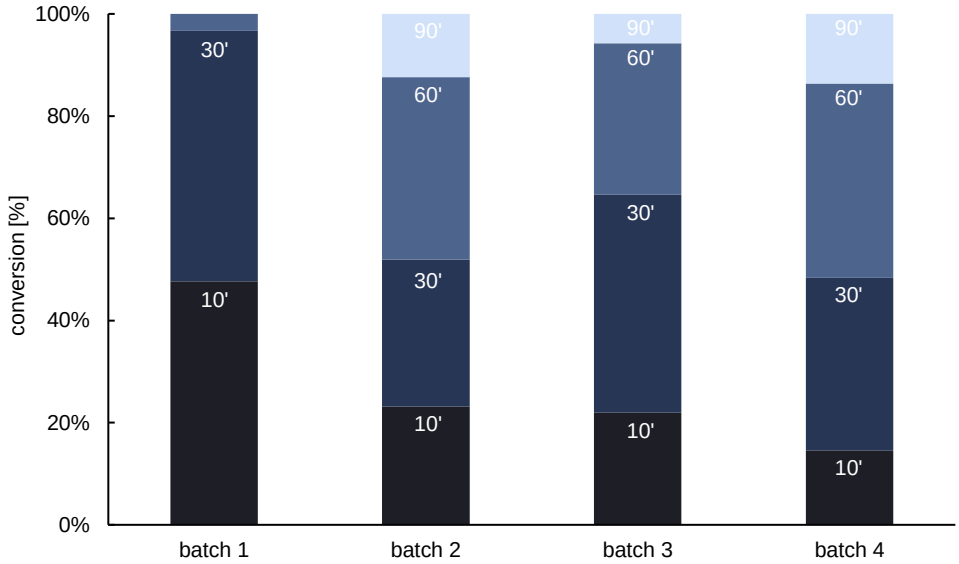

Figure S 9. Repetitive batch results for the biocatalytic decarboxylation of ferulic acid in wet CPME using *Bs*PAD-8415F in four consecutive batches. The differently accentuated bars show the reaction progress after 10, 30, 60 and 90 min. After the second batch beads were stored overnight in KPi buffer (50 mM, pH 6.0). As enzymatic activity strongly depends on the available water content, this exposure of enzyme beads to water and the resulting addition of pore water to the reaction system is hypothesized to have caused slightly increased reactivity in batch 3 compared to batch 2. In all cases full conversion was achieved after 90 min. Reaction conditions: 100 mM ferulic acid, 4 g/L *Bs*PAD-8415F, water saturated CPME, 1.5 mL reaction volume, 1,000 rpm, +30 °C, experimental duplicates.

8. Enzyme leaching

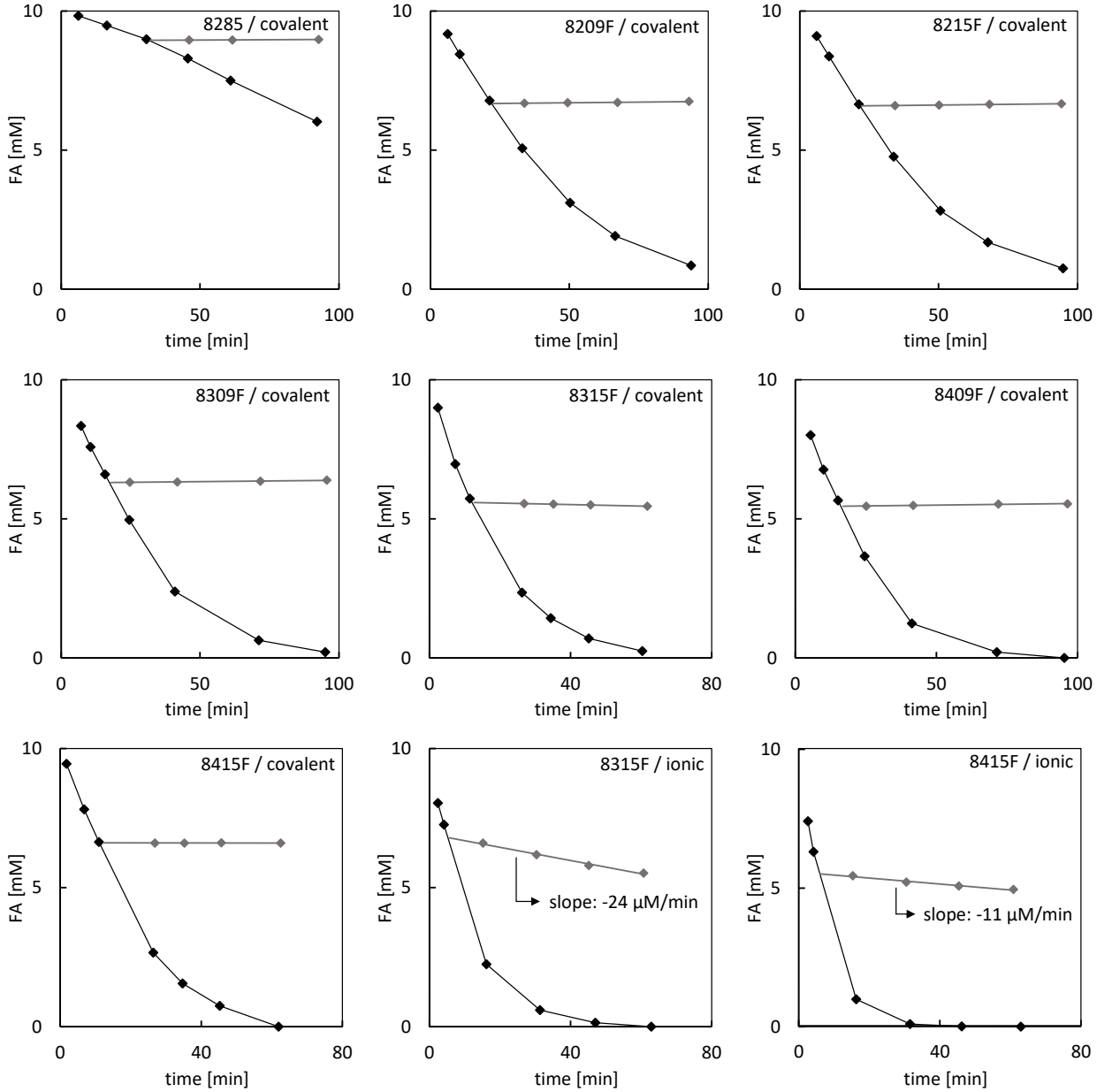

Figure S 10. Results of enzyme leaching assays in KPi buffer (50 mM, pH 6.0). For this, regular activity assays with immobilized enzyme were run and after an initial exposure time of 10 min, part of the homogeneous supernatant was transferred to another tempered reaction vessel. If no active enzyme has leached into the liquid phase, the reaction only continues in the vessel with heterogeneous biocatalyst (black), but not in the other (grey). As the data shows, no significant enzyme leaching was found for any of the covalent immobilized enzyme preparations. However, both ionic immobilized enzymes leached into the aqueous reaction system. Conditions: 10 mM FA, 4 g/L *Bs*PAD-beads, KPi buffer (50 mM, pH 6.0), 5 vol-% DMSO, 1,000 rpm, +30 °C.

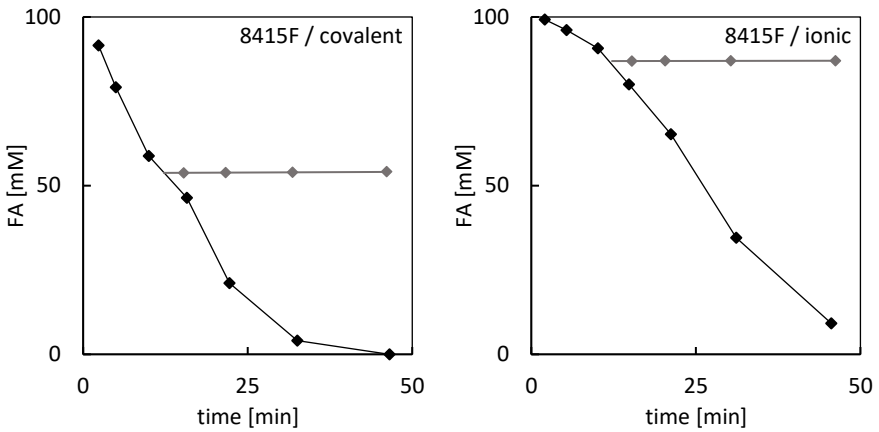

Figure S 11. Results of enzyme leaching assays in wet CPME. The same methodology as in Figure 10 applies. Neither covalent nor ionic immobilized enzyme showed leaching of active enzyme into the supernatant. Conditions: 100 mM FA, 4 g/L *Bs*PAD-beads, water saturated CPME, 1,000 rpm, 30 °C, initial leaching time ~ 10 min.

9. DoE results for the optimization of chemical acetylation

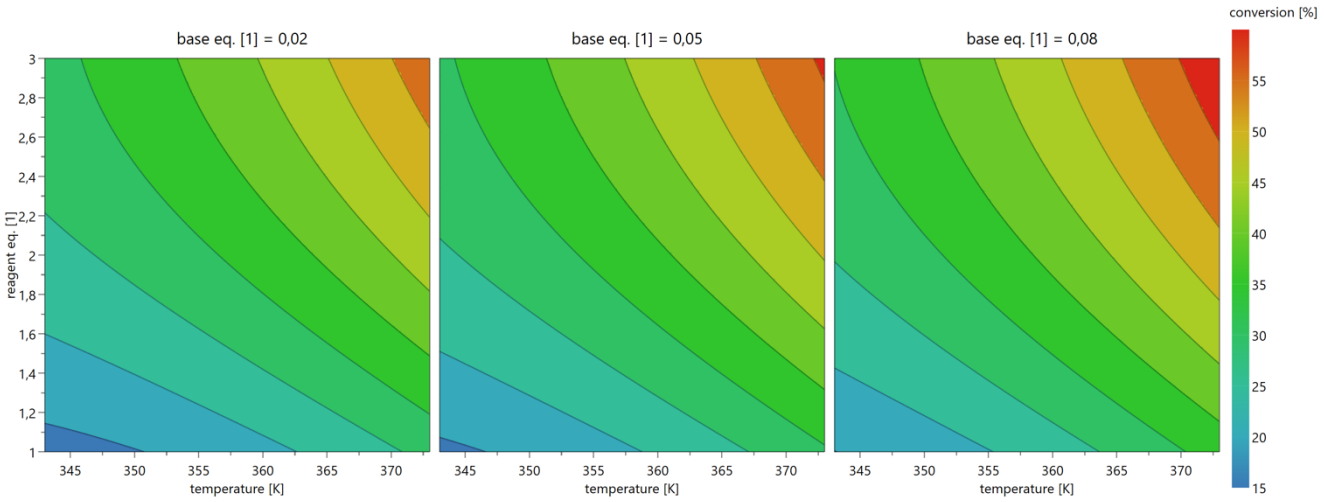

Figure S 12. Response 4D contour plot for conversion [%] of 4VG to AMS as a function of temperature [K], Ac_2O equivalents [1] (reagent), and NaOAc equivalents [1] (base) under following otherwise constant reaction conditions: 100 mM 4VG, wet CPME, 1,000 rpm, 10 min. DoE software used: MODDE® 13.0.1, Umetrics, SARTORIUS.

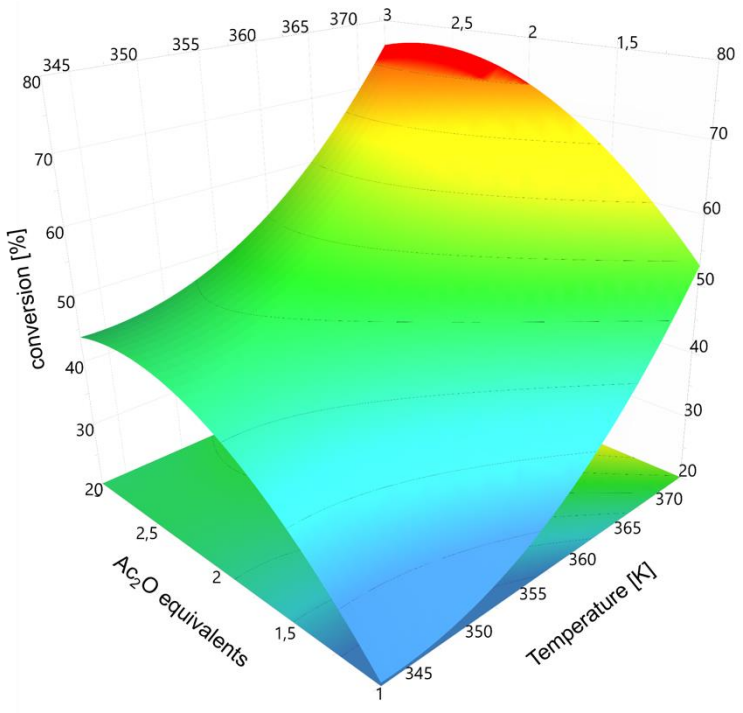

Figure S 13. Response surface plot for conversion [%] of 4VG to AMS as a function of temperature [K] and Ac_2O equivalents [1] und following otherwise constant reaction conditions: 100 mM 4VG, 0.08 eq. NaOAc, water saturated CPME, 1,000 rpm, 20 min. DoE software used: MODDE® 13.0.1, Umetrics, SARTORIUS.

10. NMR spectra of 4-acetoxy-3-methoxystyrene

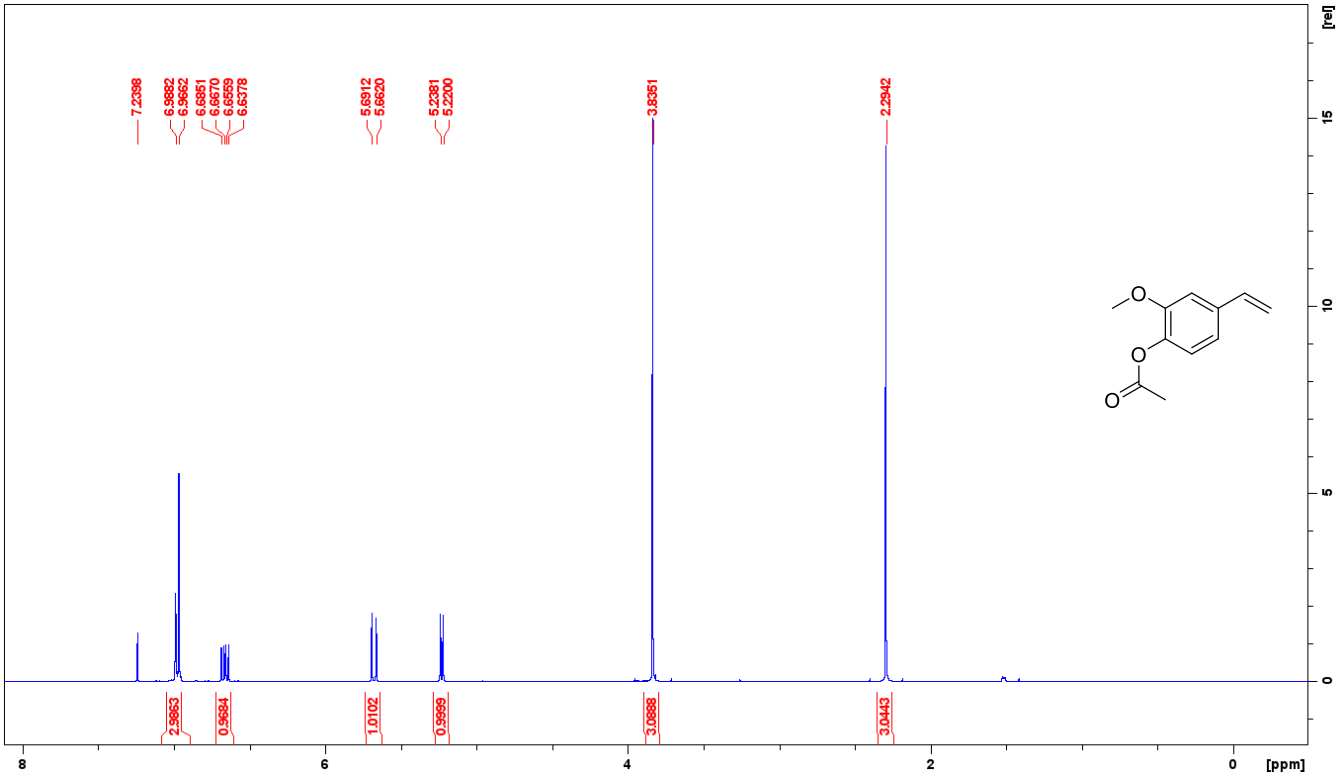

Figure S 14. ^{1}H-NMR spectrum of 4-acetoxy-3-methoxystyrene in $CDCl_3$ at +25 °C.

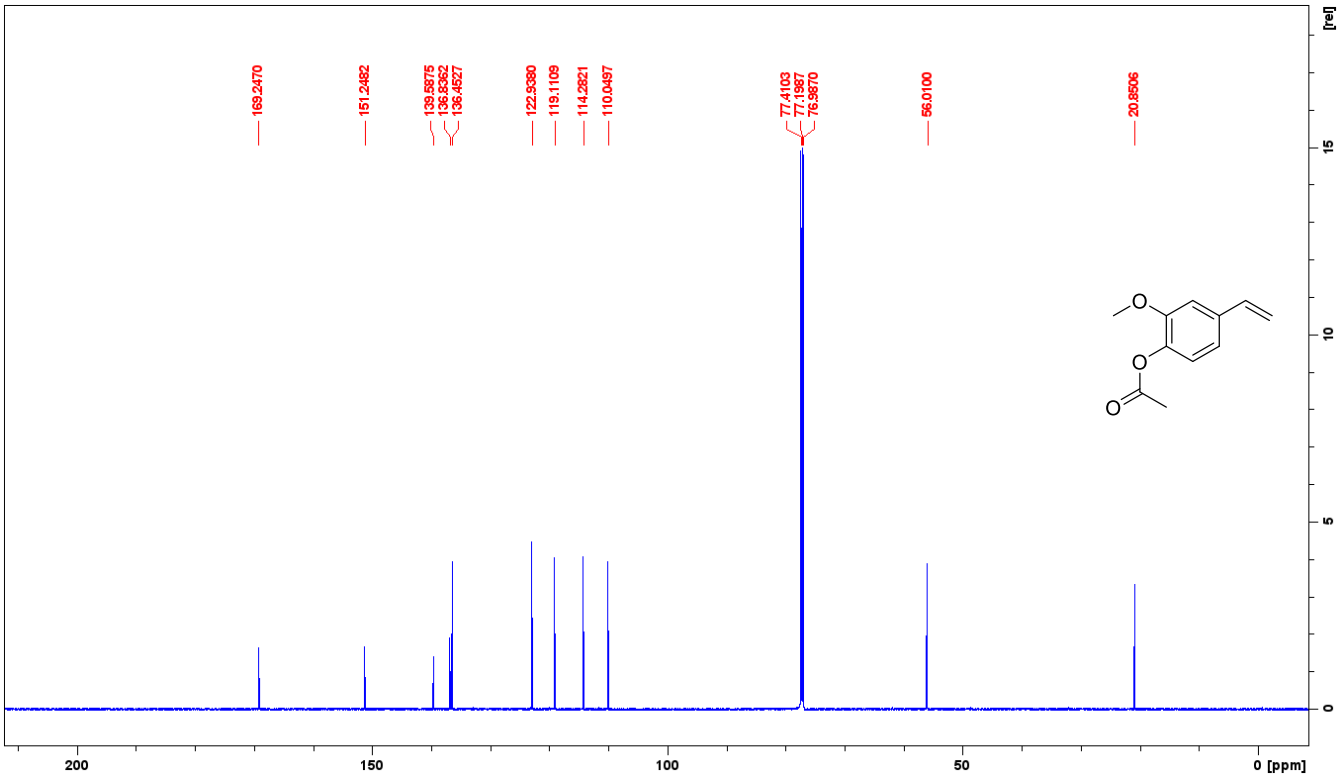

Figure S 15. ^{13}C-NMR spectrum of 4-acetoxy-3-methoxystyrene in $CDCl_3$ at +25 °C.

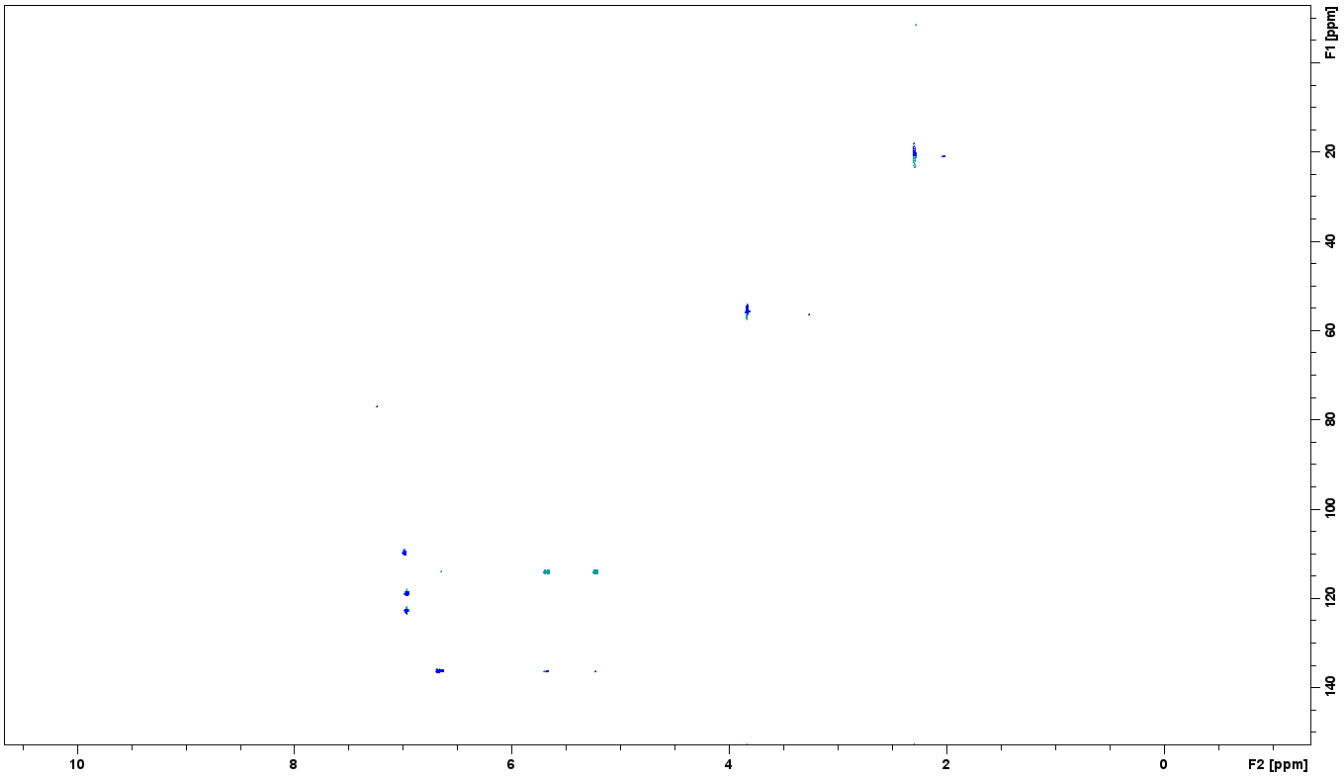

Figure S 16. HSQC-NMR spectrum of 4-acetoxy-3-methoxystyrene in $CDCl_3$ at +25 °C.

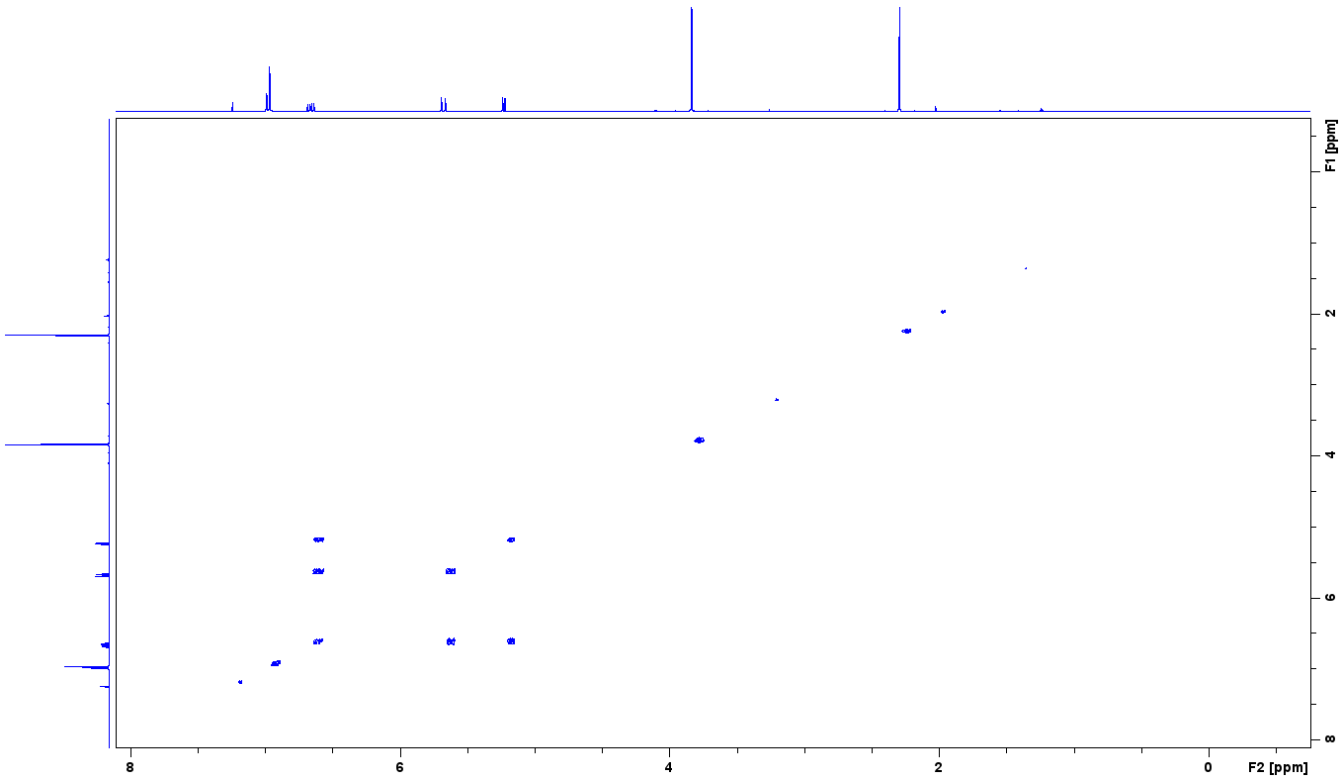

Figure S 17. COSY-NMR spectrum of 4-acetoxy-3-methoxystyrene in $CDCl_3$ at +25 °C.