Supporting information

Extractive *In-Situ* Product Removal for the application of L-alanine as amine donor in enzymatic metaraminol production

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1.) pH-dependent activity of *Cv*2025

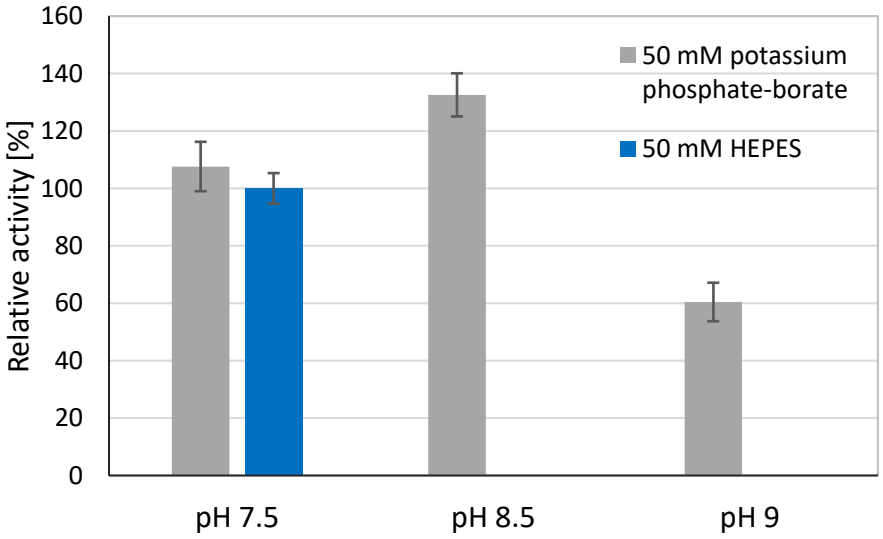

Figure S1.1: Enzyme activity of *Cv*2025 at different pH conditions. 50 mM HEPES buffer pH 7.5, 50 mM potassium-phosphate-borate buffer pH 7.5, 8 or 9, 0.1 mM PLP, 0.1 mg/mL *Cv*2025 (purified enzyme), 5 mM sodium pyruvate, 2.5 mM α-methylbenzylamine, 25 °C, optical assay measuring the absorbance at 300 nm for 5 min (Schätzle *et al.*, 2009). The activity in the standard reaction buffer (HEPES) was set to 100 %. Error bars show standard deviation of three technical replicates.

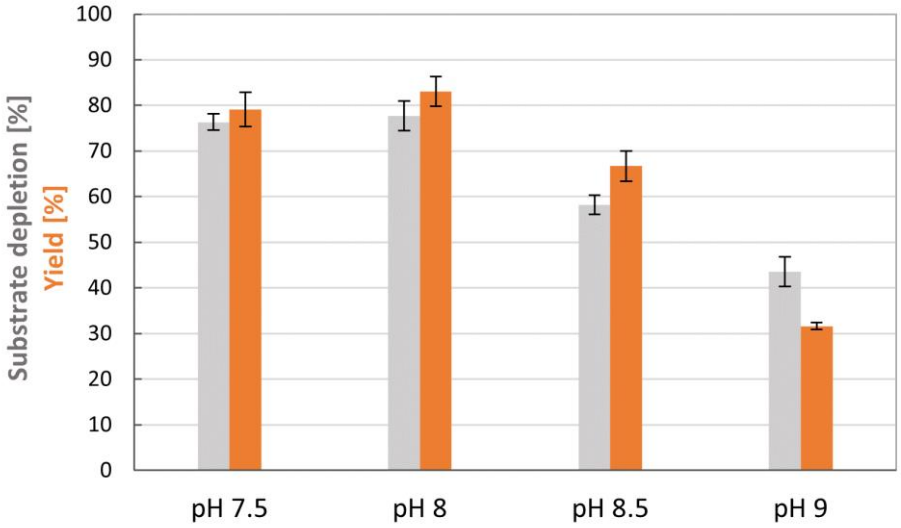

Figure S1.2: Transamination of (*R*)-3-OH-PAC to metaraminol using 250 mM isopropylamine (IPA) as amine donor under different pH values. 50 mM HEPES buffer pH 7.5 or 8, 50 mM borate buffer pH 8.5 or pH 9, 20 mM (*R*)-3-OH-PAC, 250 mM isopropylamine, 1 mM PLP, 10 mg/mL *Cv*2025 (lyophilized, whole cells), 1 mL, 30°C, 700 rpm, 24 h.

2.) Catalyst preparation

2.1) Transformation und cultivation procedure of *Escherichia coli* BL21 (DE3) with pET29a_*Cv*2025-His6

Chemically competent *Escherichia coli* BL21 (DE3) cells were transformed using a standard heat-shock protocol. The competent cells were thawed on ice before adding 1 µL of plasmid DNA (with a concentration of 170 ng/µL) to 50 µL cells. Subsequently, the mixture was incubated on ice for 30 minutes. To initiate the transformation, the mixture was heated for 90 seconds at 42°C. After the heat-shock, the cells were cooled on ice for 5 minutes, before 500 µL lysogeny broth (LB)-medium was added and the transformed cells were incubated at 37°C and 300 rpm for 1 hour. 50-100 µL of the transformation mixture was plated onto LB- agar plates containing 50 µg/mL kanamycin. The plates were incubated at 37°C overnight. 50 mL LB medium was inoculated with a single transformed colony and incubated at 37°C and 120 rpm in 250 mL shake flasks with baffles. The next day, 1 L auto-induction medium (12 g/L peptone, 24 g/L yeast extract, 91 mM potassium phosphate buffer, 2 g/L lactose, 6.3 g/L glycerol, 0.5 g/L glucose, 10 mg/L kanamycin) was inoculated in 5 L shake flasks with baffles in a ratio of 1:200 from the pre-culture. The main culture was incubated at 37°C and 90 rpm for 2-3 h before proceeding with the cultivation at 20°C and 90 rpm for 48 h. The production of *Cv*2025 was verified via SDS-PAGE (expected molecular weight 52 kDa).

2.2) Enzyme purification via immobilized metal-affinity chromatography (IMAC)

A 10 % (w/v) cell suspension in equilibration buffer (100 mM Tris buffer, 0.2 mM pyridoxal-5'-phosphate (PLP), 300 mM NaCl) was prepared for cell disruption by ultrasonification in a flow cell at 4°C (70 % amplitude, 0.5 cycle time, 30 min). Afterwards, the cell suspension was centrifuged (21500 rpm, 30 min, 4°C) to obtain the supernatant as cell-free crude extract. Protein purification was

achieved via affinity chromatography (ÄKTApurifier, GE Healthcare, Chicago, USA) using Ni-NTA superflow column material (Qiagen, Hilden, Germany). The crude extract was prepared in equilibration buffer (100 mM Tris buffer, 0.2 mM pyridoxal-5'-phosphate (PLP), 300 mM NaCl) as described in the materials and methods section. After a washing step (100 mM Tris, 0.2 mM PLP, 25 mM imidazole, 300 mM NaCl), the amine transaminase was eluted (100 mM Tris, 0.2 mM PLP, 300 mM imidazole). The elution fraction was desalted on a Sephadex G-25 column (GE Healthcare) with 10 mM Tris + 0.2 mM PLP and frozen in a crystallization dish at -20°C before lyophilization. The purification of the amine transaminase *Cv*2025 in the elution fraction was verified via SDS-PAGE with > 90 % purity. The protein content in the lyophilized powder was 50 % (w/w).

3.) Production and purification of (*R*)-3-OH-phenylacetylcarbinol (-PAC) as substrate for the transaminations towards metaraminol and for use as HPLC standard

For 50 mL batch reactions, 300 mM pyruvate and 100 mM 3-hydroxy benzaldehyde were provided in 100 mM potassium phosphate buffer pH 6.0 + 1 mM thiamine pyrophosphate (ThDP) and 2.5 mM $MgCl_2$. To aid in dissolving the substrate 3-hydroxy benzaldehyde, 2.5 % DMSO (v/v) were added. To start the reaction, 0.4 g crude cell extract containing a pyruvate decarboxylase from *Acetobacter pasteurianus*, with high stereoselectivity towards the (*R*)-product, were added and the reaction mixture was incubated rotating in a rotating shaker at 30°C for 24 h. The conversion was analyzed via UHPLC analysis as described in the method section (Agilent 1290 Infinity II, DAD 220 nm, Agilent Zorbax Eclipse plus C18, 2 x 100 mm, 1.8 mm, 20°C, 5 mL injection volume). For product purification, the reaction mixture was acidified with 37 % HCl to precipitate the protein and subsequently centrifuged at 1700 rcf for 10 min at 4°C. The supernatant was filtered twice. To extract the target compound from the aqueous phase, three extraction steps with 100 mL ethyl acetate were performed. Afterwards, the aqueous phase was adjusted to pH 11 and the extraction step was repeated three more times. The extraction efficiency was checked via HPLC samples from the aqueous raffinate as described before and thin-layer chromatography (TLC) using molybdenum stain (25 g/L phosphoric molybdic acid, 10 g/L $Ce(SO_4)_2$ dihydrate, 6 % (v/v) H_2SO_4). The organic phase was washed with $NaHCO_3$ and dried with $MgSO_4$. The drying agent was removed by filtration and the organic solvent was evaporated at 40°C and 100 mbar. The purity of the product was determined via 1H-NMR analysis using trimethylsilylpropanoic acid (TMSP) as reference (Spinsolve 60 NMR spectrometer, Magritek, Aachen, Germany).

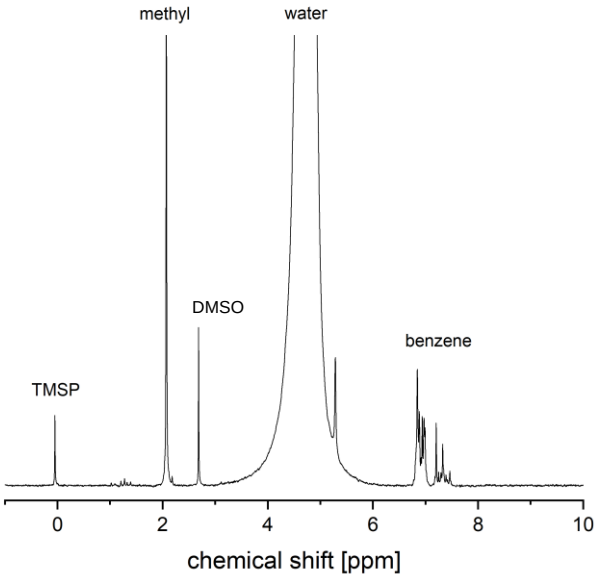

Figure S3.1: ^{1}H-NMR spectrum for (*R*)-3-OH-PAC. Chemical shift compared to the internal standard sodiumtrimethylsilylpropionate (TMSP). Besides the expected signals for the product, DMSO is evident as an impurity.

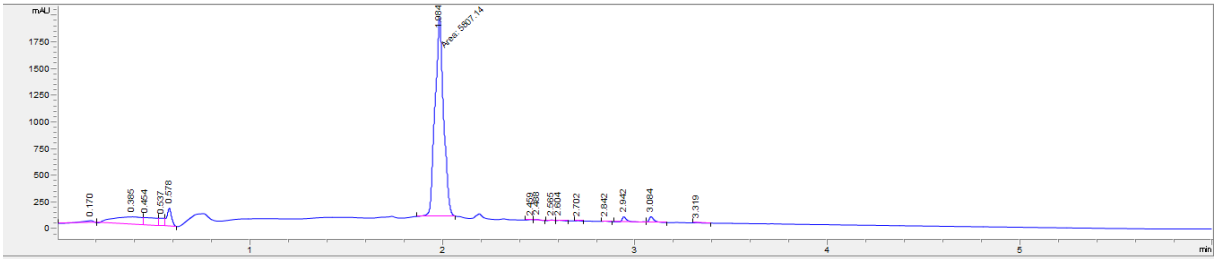

Figure S3.2: Chromatogram of purified (*R*)-3-OH-PAC (Agilent 1290 Infinity II, DAD 220 nm, Agilent Zorbax Eclipse plus C18 column, 2 x 100 mm, 1.8 µm particle size, 20 °C, 5 µL injection volume, 0.5 mL/min, 10-90 % acetonitrile in 5 min). The retention time of (*R*)-3-OH-PAC was 1.9 min.

4.) Plasmid information *Cv*2025-His6

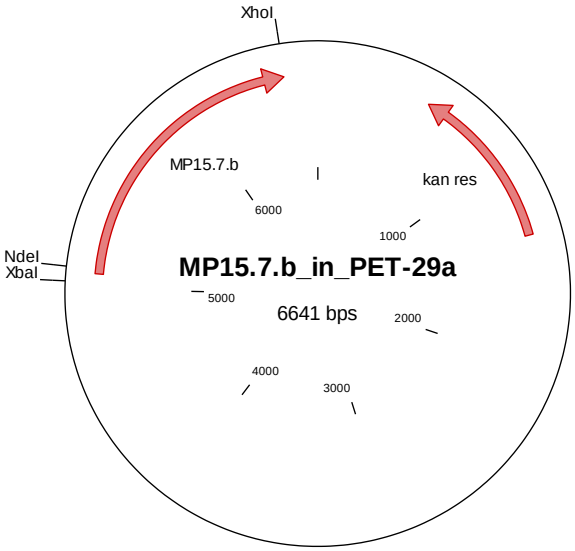

Figure S4.1: Plasmid of *Cv*2025-His6 in PET-29a.

Gene sequence of *Cv*2025-His6 in PET-29a (coding sequence for enzyme highlighted in red, coding sequence for the His_6-tag underlined):

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Protein sequence of *Cv*2025-His6:

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5.) Reaction analytics

5.1) HPLC analytics with online derivatization for determination of metaraminol in pure substance systems

The metaraminol in the pure substance system was quantified via HPLC measurement using the online derivatization method with orthophtalic aldehyde and mercaptoethanol as described in the tables 1 and 2. The calibration curve for metaraminol and an exemplary chromatogram are shown below as well (Agilent 1200, DAD 334nm, Machery Nagel Nucleodur C18ec column, 1 x 100 mm, 3 mm, 40°C)

Before measurement, pre-column online derivatization using orthophthalic (OPA) aldehyde solution was performed following the derivatization procedure given in table 1, where vial 99 contains distilled water and vial 100 contains 0.1 mL of OPA reagent and 0.9 mL of 0.4 M borate buffer titrated to pH 10.4.

Table 1: Online derivatization procedure for metaraminol quantification

Step	Derivatization Procedure
1	DRAW 4.5 µL from vial 100
2	DRAW 1.5 µL from sample
3	DRAW 0.5 µL from air
4	NEEDLE wash in vial 99
5	DRAW 4.5 µL from vial 100
6	MIX 11.0 µL in seat
7	WAIT 1.00 min
8	INJECT
9	DRAW and EJECT 100 mL from vial 99

The gradient elution is given in table 2. The retention time for metaraminol was 12.7 minutes.

Table 2: HPLC gradient elution given for the elution of the OPA metaraminol complex

Time [min]	% B	Flow [mL/min]
0.0	10.0	0.4
5.0	10.0	0.4
10.0	90.0	0.4
16.0	90.0	0.4
17.0	10.0	0.4

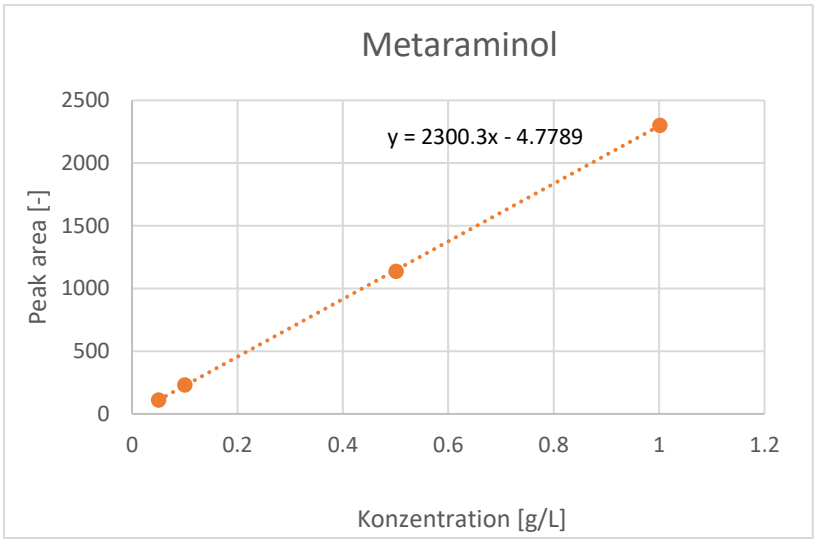

Figure S5.1: Calibrations for HPLC quantification of metaraminol.

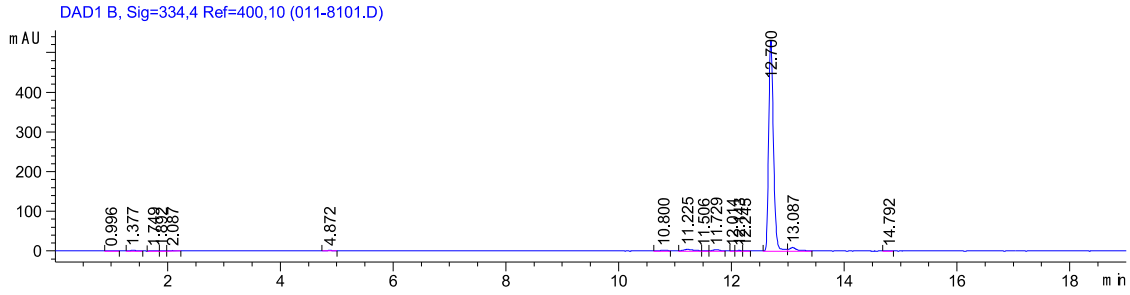

Figure S5.2: Typical HPLC chromatogram for the quantification of (1*R*,2*S*)-2-amino-1-(3-hydroxyphenyl)propan-1-ol (metaraminol) using online derivatization with orthophtalic aldehyde and mercaptoethanol. The retention time of metaraminol was 12.7 min.

5.2) UHPLC analytics for simultaneous determination of (*R*)-3-OH-PAC and metaraminol in the enzymatic reaction system

The reaction components were quantified using two different UHPLC methods as the retention and separation for the reaction product metaraminol was ideal in an isocratic elution, while the substrate (*R*)-3-OH-PAC was analyzed using a gradient method. Calibrations for both reaction components are shown below (Agilent 1290 Infinity II, DAD 220 nm, Agilent Zorbax Eclipse plus C18 column, 2 x 100 mm, 1.8 µm, 20 °C, 5 µL injection volume).

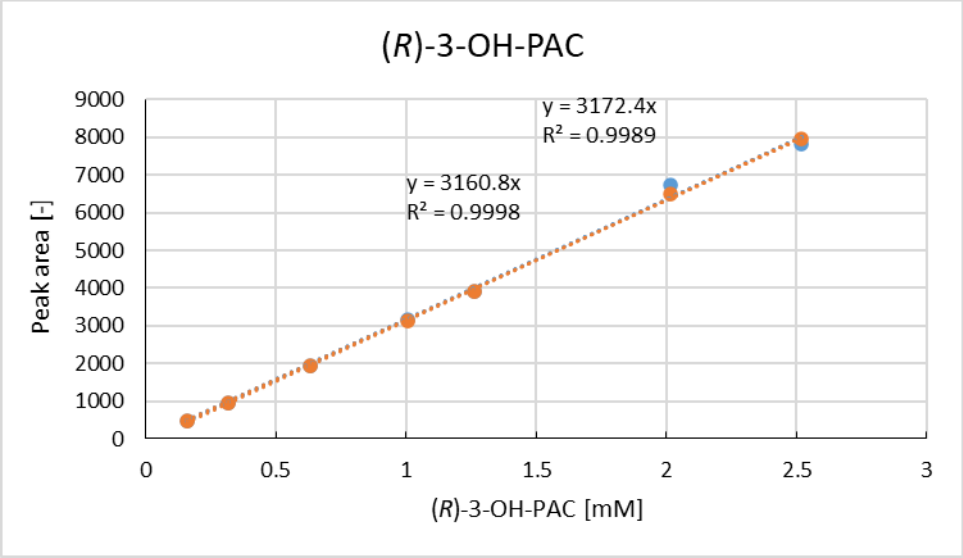

Figure S5.3: Calibrations for HPLC quantification of (*R*)-3-OH-PAC. Double determination between 0.125 mM and 2.5 mM in 50 % acetonitrile (v/v) in ultra-pure water. Gradient method (0.5 mL/min, 10-90 % acetonitrile in a linear gradient over 5 min, 90-10% H_2O + 0.1 % diethylamine + 0.075% trifluoroacetic acid).

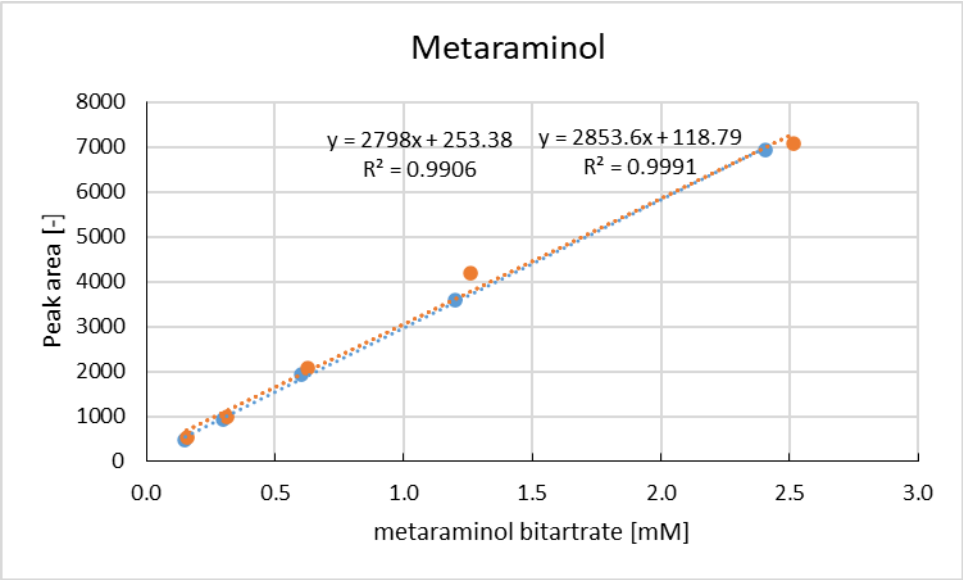

Figure S5.4: Calibrations for HPLC quantification of metaraminol. Double determination between 0.125 mM and 2.5 mM in 50 % acetonitrile (v/v) in ultra-pure water. Isocratic method (0.3 mL/min, 15 % acetonitrile + 85 % H_2O + 0.1 % diethylamine + 0.075% trifluoroacetic acid).

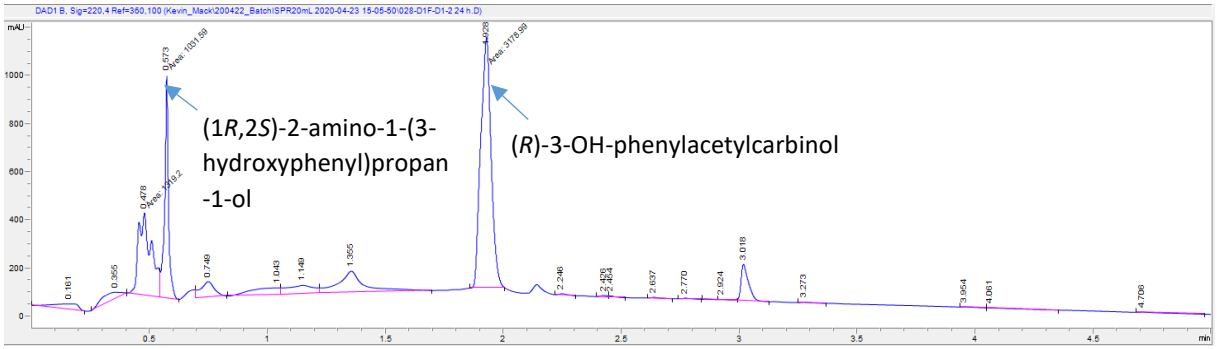

Figure S5.5: Typical HPLC chromatogram of the transamination of (*R*)-3-OH-phenylacetylcarbinol to (1*R*,2*S*)-2-amino-1-(3-hydroxyphenyl)propan-1-ol (metaraminol) using a gradient elution (0.5 mL/min, 10-90% acetonitrile in a linear gradient over 5 min, 90-10% H_2O + 0.1% diethylamine + 0.075% trifluoroacetic acid). The retention time of (*R*)-3-OH-phenylacetylcarbinol was 1.9 min. This method was used for (*R*)-3-OH-PAC detection in biotransformations.

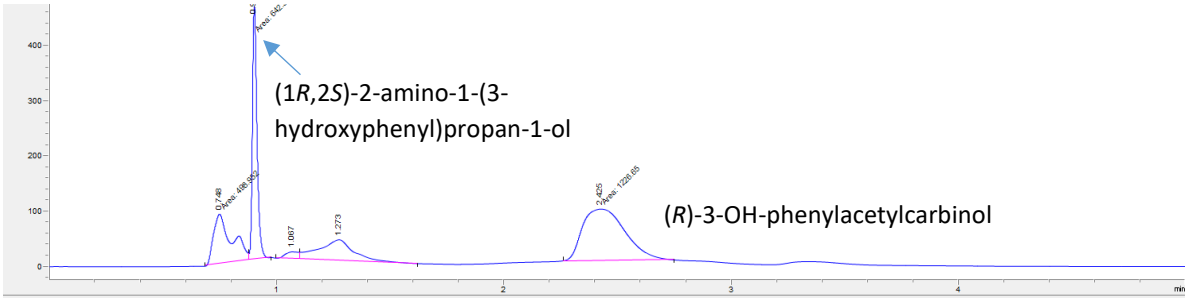

Figure S5.6: Typical HPLC chromatogram of the transamination of (*R*)-3-OH-phenylacetylcarbinol to (1R,2S)-2-amino-1-(3-hydroxyphenyl)propan-1-ol (metaraminol) using an isocratic elution (0.3 mL/min, 15% acetonitrile + 85% H_2O + 0.1% diethylamine + 0.075% trifluoroacetic acid). The retention time of metaraminol was 0.9 min. This method was used for metaraminol detection in biotransformations.

During alkaline pH condition in the reaction unidentified side components were observed, which resulted presumably from chemical background reactions or instabilities of the substrate (Figure S10).

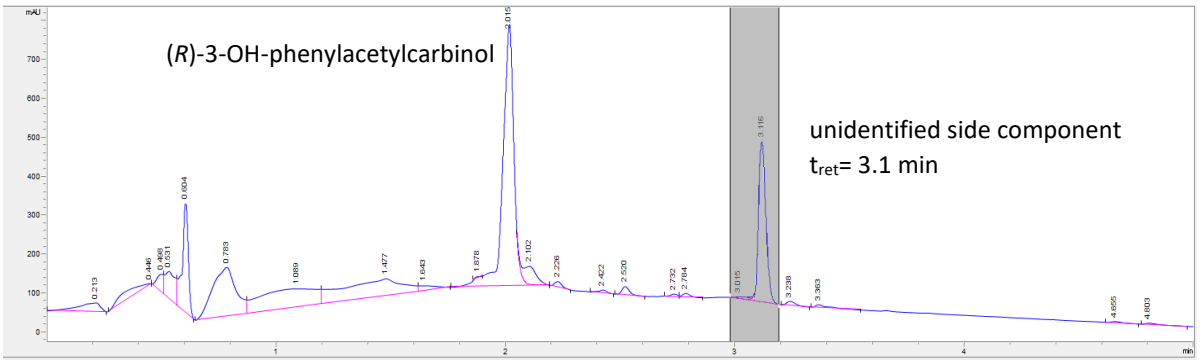

Figure S5.7: Side component formation after basification (pH 9) of aqueous reaction phase (grey). Gradient method (0.5 mL/min, 10-90% acetonitrile in a linear gradient over 5 min, 90-10% H_2O + 0.1 % diethylamine + 0.075% trifluoroacetic acid.