Regioselective Enzymatic Carboxylation of Phenols and Hydroxystyrene Derivatives

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SUPPORTING INFORMATION

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Materials

Salicylic acid (**1b**), 1-hydroxy-2-naphtoic acid (**2b)**, catechol (**4a**), 2,3-dihydroxybenzoic acid (**4b**), resorcinol (**5a**), 2,6-dihydroxybenzoic acid (**5b**) 2,4-dihydroxybenzoic acid (**5c**), olivetol (**6a**), *p*-coumaric acid (**7c**), 2-methoxy-4-vinylphenol (**8a**), ferulic acid (**8c**), sinapic acid (**9c**), as well as $KHCO_3$, KH_2PO_4 and Na_2PO_4*2 H_2O were from Sigma Aldrich. 1-Naphthol (**2a**) and *p*-vinylphenol (**7a**) were purchased from Alfa Aesar. Phenol (**1a**), 3-aminophenol (**3a**) and 4-aminosalicylic acid (**3b**) were obtained from Fluka.

Phenolic acid decarboxylase from *Lactobacillus plantarum* (PAD_Lp, GI: 300769086) and from *Bacillus amyloliquefaciens* (PAD_Ba*,* GI: 308175189)*,* subcloned in a pET 28a (+) vector, were kindly provided by Byung-Gee Kim (School of Chemical and Biological Engineering, Institute of Bioengineering, Seoul National University).

2,3-Dihydroxybenzoic acid decarboxylase from *Aspergillus oryzae* (2,3-DHBD_Ao), salicylic acid decarboxylase from *Trichosporon moniliiforme* (SAD_Tm) and 2,6-dihydroxybenzoic acid decarboxylase from *Rhizobium* species (2,6-DHBD_Rs) were synthesised and ligated into a pET 21a (+) vector at Geneart AG (Germany,Regensburg). Their DNA sequences were identified by sequence comparison in the NCBI Genebank (GI: 94730373 for 2,3-DHBD_Ao, GI: 225887918 for SAD_Tm and GI: 116667102 for 2,6-DHBD_Rs).

Cloning and Overexpression of (De)carboxylases

The plasmids containing the (de)carboxylase genes were transformed into chemically competent *Escherichia coli* BL21 (DE3) cells and heterologous overexpression was performed as follows:

For preculturing 500mL LB medium [Trypton (10g/L Oxoid L0042), yeast extract (5g/L Oxoid L21), NaCl (5g/L Roth 9265.1)] supplemented with the appropriate antibiotics [ampicillin (100µg/mL Sigma Aldrich) for 2,3-DHBD_Ao, SAD_Tm and 2,6-DHBD_Rs, kanamycin (50µg/mL Roth) for PAD_Lp and PAD_Ba] were inoculated with 3mL ONC (starter culture) and incubated at 37°C and 120rpm until an OD_{600} of 0.6–1.0 was reached. Then IPTG [450µg/mL, 2mM (for 2,3-DHBD_Ao) or 175µg/mL, 0.5mM (for the other decarboxylases) Peqlab] was added for induction and the cells left over night at 20°C and 120rpm. The next day the cells were harvested by centrifugation (20min, 5900rcf, 4°C), washed with phosphate buffer (5mL, 50mM, pH 7.5) and recentrifuged under the same conditions to obtain a cell pellet.

To obtain lyophilized cells the cell pellet was resuspended in phosphate buffer (5mL, 50mM, pH 7.5), shock frozen in liquid nitrogen followed by lyophilization.

SDS-Pages

Successful overexpression of soluble enzyme was obtained for all decarboxylases. In order to check whether the enzymes are soluble, cells were disrupted using ultrasonication and the separated supernatant and remaining pellet were analysed by SDS-PAGE (see Figures S1-S3)

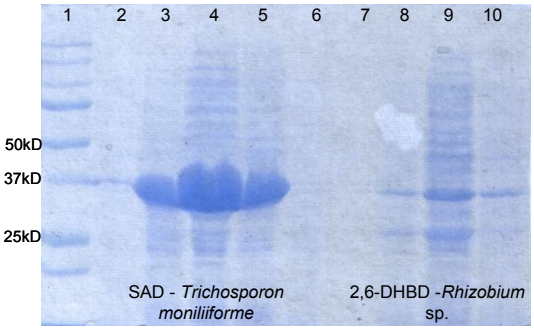


Figure S1: SDS-PAGE showing overexpression and solubility of SAD_Tm and 2,6-DHBD_Rs. Lane1: Precision Plus Protein Standard All Blue Standard (5µL, BIORAD), lane 2/7: before induction, lane 3/8: induced with IPTG (0.5mM), lane 4/9: supernatant, lane 5/10: pellet; 10 µL were applied for lanes 2-10 .

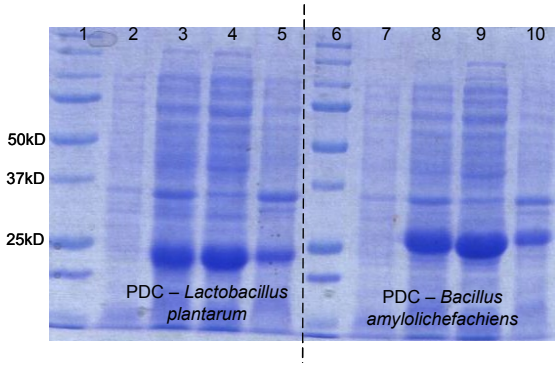


Figure S2: SDS-PAGE showing overexpression and solubility of PDC_Lp and PDC_Ba. Lane1/6: Precision Plus Protein Standard All Blue Standard (5µL, BIORAD), lane 2/7: before induction, lane 3/8: induced with IPTG (0.5mM), lane 4/9: supernatant, lane 5/10: pellet, 10 µL were applied for lanes 2-10 .

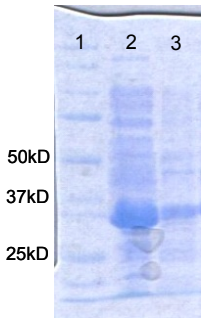


Figure S3: SDS-PAGE showing solubility of 2,3-DHBD_Ao. Lane1: Precision Plus Protein Standard All Blue Standard (5µL, BIORAD), lane 2: supernatant (10µL), lane 3: pellet (10µL).

General procedure for the carboxylation of substrates 1a-9a

All carboxylation reactions were performed in glass vials capped with septums. Lyophilized whole cells (30mg *E. coli* host cells containing the corresponding overexpressed enzyme) were resuspended in phosphate buffer (1mL or 900µL, pH 5.5, 100mM) and were rehydrated for 30min. The substrate was added either directly or as a stock solution (100µL) to yield a final concentration of 10mM, followed by addition of $KHCO_3$ (3M, 300mg). After the addition of potassium bicarbonate the vials were immediately tightly closed and the mixture was shaken at 30°C and 120rpm. After 24h the reaction mixture was centrifuged (15700rcf, 15min), an aliquot of 100µL of each sample was diluted with 1mL of an H_2O/acetonitrile mixture (v/v = 50%) supplemented with trifluoroacetic acid (3% v/v, 30µL). After incubation at room temperature for 5min, the samples were again centrifuged (15700rcf, 15min) and analysed on reverse-phase HPLC to determine the conversion. Products were identified by comparison with authentic reference material.
For biotransformations applying naphthol (**2a**) and olivetol (**6a**), respectively, the substrate stock solution was prepared in acetonitrile to overcome solubility problems, leading to a final acetonitrile concentration of 10% v/v in the reaction mixture. The work up was performed as described above.

Control experiments using empty host

In order to verify the absence of competing background reaction(s) in empty *E. coli* host cells, blank experiments were performed using the empty host (without plasmid encoding for the corresponding decarboxylase) for carboxylation reactions by maintining all other reaction parameters. An overlay of the HPLC chromatograms for the control experiment using phenol as substrate is shown in Figure S4: (i) substrate reference (grey line, phenol **1a**), (ii) product reference (green line, salicylic acid **1b**), (iii) empty *E. coli* whole cells without plasmid (blue line). Control experiments were performed for all substrates **1a-9a** using the standard screening procedure as described above.

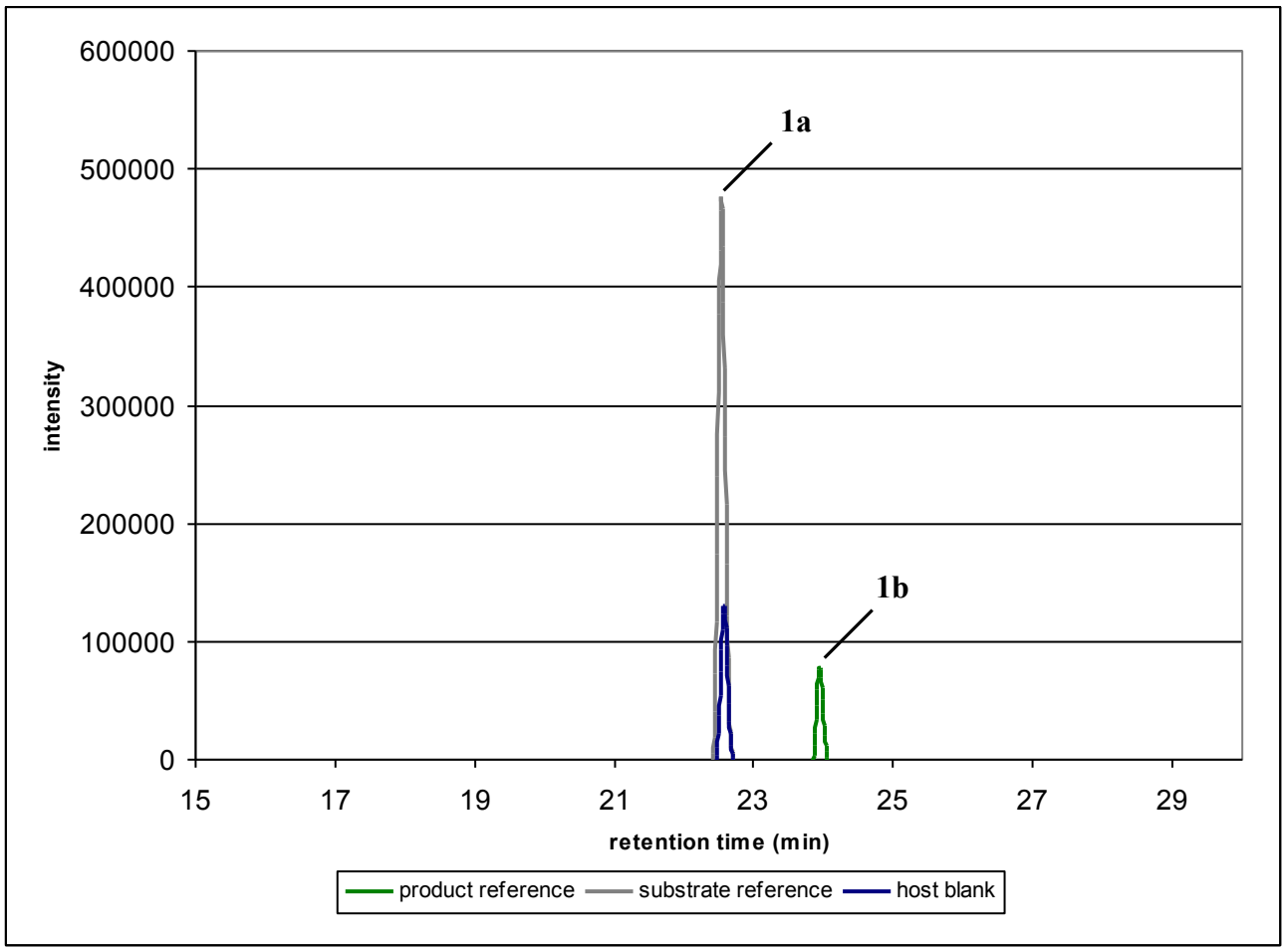


Figure S4: An overlay of the HPLC chromatograms for the substrate reference (grey line, phenol **1a**), product reference (green line, salicylic acid **1b**) and empty *E. coli* whole cells without plasmid encoding for the decarboxylase (blue line).

Codon optimised sequences of decarboxylases

PDC_Ba (GI: 308175189)

MENFIGSHMIYTYENGWEYEIYIKNDHTIDYRIHSGMVGGRWVRDQEVNIVKLTEGVYKV
SWTEPTGTDVSLNFMPNEKRMHGIIFFPKWVHEHPEITVCYQNDYIDVMKESREKYDTYPK
YVVPEFADITYLNNAGINNEALISEAPYEGMTDDIRAGKLK

PDC_Lp (GI: 300769086)

MTKTFKTLDDFLGTHFIYTYDNGWEYEWYAKNDHTVDYRIHGGMVAGRWVTDQKADIV
MLTEGIYKISWTEPTGTDVALDFMPNEKKLHGTIFFPKWVEEHPEITVTYQNEHIDLMEQSR
EKYATYPKLVVPEFANITYMGDAGQNNEDVISEAPYKEMPNDIRNGKYFDQNYHRLNK

2,3-DHBD_Ao (GI: 94730373)

MLGKIALEEAFALPRFEEKTRWWASLFSTDAETHVKEITDINKIRIEHADKHGVGYQILSYT
APGVQDIWDPVEAQALAVEINDYIAEQVRVNPDRFGAFATLSMHNPKEAADELRRCVEKY

GFKGALVNDTQRAGPDGDDMIFYDNADWDIFWQTCTELDVPFYMHPRNPTGTIYEKLWA
DRKWLVGPPLSFAHGVSLHVLGMVTNGVFDRHPKLQIIMGHLGEHVPFDMWRINHWFED
RKKLLGLAETCKKTIRDYFAENIWITTSGHFSTTTLNFCMAEVGSDRILFSIDYPFETFSDAC
EWFDNAELNGTDRLKIGRENAKKLFKLDSYKDSSA

SAD_Tm (GI: 225887918)
MRGKVSLEEAFELPKFAAQTKEKAELYIAPNNRDRYFEEILNPCGNRLELSNKHGIGYTIYS
IYSPGPQGWTERAECEEYARECNDYISGEIANHKDRMGAFAALSMHDPKQASEELTRCVK
ELGFLGALVNDVQHAGPEGETHIFYDQPEWDIFWQTCVDLDVPFYLHPEPPFGSYLRNQYE
GRKYLIGPPVSFANGVSLHVLGMIVNGVFDRFPKLKVILGHLGEHIPGDFWRIEHWFEHCS
RPLAKSRGDVFAEKPLLHYFRNNIWLTTSGNFSTETLKFCVEHVGAERILFSVDSPYEHIDV
GCGWYDDNAKAIMEAVGGEKAYKDIGRDNAKKLFKLGKFYDSEA

2,6-DHBD_Rs (GI: 116667102)
MQGKVALEEHFAIPETLQDSAGFVPGDYWKELQHRLLDIQDTRLKLMDAHGIETMILSLN
APAVQAIPDRRKAIEIARRANDVLAEECAKRPDRFLAFAALPLQDPDAATEELQRCVNDLG
FVGALVNGFSQEGDGQTPLYYDLPQYRPFWGEVEKLDVPFYLHPRNPLPQDSRIYDGHPW
LLGPTWAFAQETAVHALRLMASGLFDEHPRLNIILGHMGEGLPYMMWRIDHRNAWVKLP
PRYPAKRRFMDYFNENFHITTSGNFRTQTLIDAILEIGADRILFSTDWPFENIDHASDWFNAT
SIAEADRVKIGRTNARRLFKLDGA

Synthesis of substrates and reference material

2,6-Dimethoxy-4-vinylphenol (9a): Sodium bis(trimethylsilyl)amide (1.05g, 5.7mmol, Sigma Aldrich) was added under cooling to a stirred solution of methyltriphenylphosphonium bromide (1.85g, 5.2mmol, Sigma Aldrich) in freshly distilled THF (8mL). Immediately afterwards a yellow colour change could be observed. After 1.5h of stirring solid syringaldehyde (0.46g, 2.54mmol, Sigma Aldrich) was added to the solution and the stirring was continued for 4h. Afterwards the mixture was acidified using H_2SO_4 (0.1M, 5mL) and extracted with CH_2Cl_2. The combined organic phases were dried over Na_2SO_4, evaporated and purified by flash chromatography on silica (eluent: CH_2Cl_2) to yield 18% of **9a** (81.6mg, 0.45mmol).[1]
TLC: R_f = 0.38 (silica, CH_2Cl_2); GC-MS: *m/z* 180; ^{1}H-NMR ($CDCl_3$): δ 3.93 (6H, s); δ 5.15, 5.18, 5.19 (1H, dd, J = 0.61 and 10.83); δ 5.56 (1H, s); δ 5.59, 5.65 (1H, dd, J = 0.70 and 17.50); δ 6.59, 6.63, 6.65, 6.68 (1H, dd, J = 10.78 and 17.47); δ 6.67 (2H, s); ^{13}C-NMR ($CDCl_3$): δ 56.24, 56.27, 102.99, 111.84, 129.18, 134.77, 136.81, 147.05. NMR data corresponded to literature.[2]

2,6-Dihydroxy-4-pentylbenzoic acid (6b), 2-hydroxy-5-vinylbenzoic acid (7b) and 2-hydroxy-3-methoxy-5-vinylbenzoic acid (8b): Reference material for the carboxylation product of olivetol **(6a)**, *p*-vinylphenol **(7a)** and 2-methoxy-4-vinylphenol **(8a)** applying a benzoic acid (de)carboxylase was synthesised using standard carboxylation reactions (see above) and identified by HPLC-MS and NMR analyses.

2-Hydroxy-5-vinylbenzoic acid (7b) and 2-hydroxy-3-methoxy-5-vinylbenzoic acid (8b): After 24h hours the reaction mixture was extracted with EtOAc (3x500µL) for the removal of non-converted substrate. The aqueous phase was acidified with HCl (800µL, 3M) and products were extracted with EtOAc (3x500µL). The organic phases were combined and dried over Na_2SO_4. Thereafter the organic solvent was removed under air pressure and the obtained products were subjected to NMR analyses (HMBC for structure determination).

2-Hydroxy-5-vinylbenzoic acid (7b): HPLC-MS: M^{-1} 163, ^{1}H-NMR (acetone-d_6): δ 5.17, 5.20 (1H, d, J = 10.96); δ 5.70, 5.76 (1H, d, J =17.63); δ 6.70, 6.73, 6.76, 6.79 (1H, dd, J = 10.92 and 17.60); δ 6.95, 6.98 (1H, d, J = 8.65); δ 7.70, 7.71, 7.73, 7.74 (1H, dd, J = 2.31 and 8.66); δ 7.93, 7.94 (1H, d, J = 2.29). The aromatic substitution pattern of **7b** was confirmed by a 2D HMBC experiment. Three-bond H-C correlations were found between both aromatic signals at δ 7.70-7.74 and δ 7.93-7.94 and the vinyl CH carbon at δ ~136.

2-Hydroxy-3-methoxy-5-vinylbenzoic acid (8b): HPLC-MS: M^{-1} 193; ^{1}H-NMR (acetone-d_6): δ 3.77 (3H, s), δ 5.02, 5.06 (1H, d, J = 11.51); δ 5.58, 5.64 (1H, d, J = 17.60); δ 6.53, 6.57, 6.59, 6.63 (1H, dd, J = 10.90 and 17.59); δ 7.25 (1H, d, J = 2.01); δ 7.37 (1H, d, J = 1.98). The aromatic substitution pattern of **8b** was confirmed by a 2D HMBC experiment. Three-bond H-C correlations were found between both aromatic doublets at δ 7.25 and δ 7.37 ppm and the vinyl CH carbon at δ ~135.

2,6-Dihydroxy-4-pentylbenzoic acid (6b): After 24h substrate and product were extracted from the reaction mixtures with EtOAc (2x500µL). The aqueous phase was acidified with HCl (800µL, 3M) and again extracted with EtOAc (2x500µL). The organic phases were combined and the solvent removed under air pressure. The dried compounds were then dissolved in acetonitrile and separated on a preparative HPLC column. To obtain the carboxylated product in optimum yield and purity, fractions containing **6b** were combined and lyophilized prior to NMR measurements.

HPLC-MS: M^{-1} 223; ^{1}H-NMR (acetone-d_6): δ 0.86, 0.89, 0.91 (3H, t, J = 2 * 6.68); δ 1.32, 1.33 (4H, d, J = 3.30); δ 1.57, 1.60, 1.62, 1.65 (2H, m), δ 2.48, 2.51, 2.54 (2H, t, J = 2 * 7.65); δ 6.32 (2H, s).

Analytical procedures

General

TLCs were run on silica (Merck silica gel 60, F_{254}). NMR experiments were acquired either on a Bruker Avance III 300MHz spectrometer using a 5mm BBO probe or on a Bruker Avance III 500MHz spectrometer using a 5mm TXI probe with z-axis gradients at 300K. All GC-MS measurements were carried out on an Agilent 7890A GC system, equipped with an Agilent 5975C mass-selective detector (electron impact, 70eV) and a HP-5-MS column (30m x 0.25mm x 0.25µm) using helium as carrier gas at a flow of 0.55mL/min. Following temperature program was used in all GC-MS measurements: initial temperature: 100°C, hold for 0.5min, 10°C/min, to 300°C. HPLC-MS experiments were acquired on a HPLC system from Agilent equipped with an Agilent 6120 quadrupol mass-detector and a reversed phase Nucleodur column C18e 150*4 5µm, column temperature was 30°C. Method for **7b** and **8b** was run over 32min with H_2O + formic acid (0.1%) as the mobile phase (flow rate 0.5mL/min) and an acetonitrile gradient (0-2min 0%, 2-30min 0-100%, 30-32min 100%). Method for **6b** was run over 16min with H_2O + formic acid (0.1%) as the mobile phase (flow rate 1.0mL/min) and an acetonitrile gradient (0-15min 0-100%, 15–16min 100%). Preparative HPLC purification was performed on an HPLC system from JASCO equipped with a multiwavelength detector (JASCO MD-910) and a reversed-phase LiChroCART 250-10 LiChrospher column 100RP-18e 10µm, at room temperature. Method for **6b** was run over 60min with H_2O/MeCN (60/40 v/v) supplemented with trifluoroacetic acid (0.1%) as the mobile phase (flow rate 0.25mL/min).

Determination of conversion

All analyses were carried out on a HPLC system from Shimadzu equipped with a diode array detector (SPD-M20A) and a reversed-phase Phenomenex Luna column C18 (2) 100A 250*4.60mm 5µm, column temperature 25°C. Conversions were determined by comparison with calibration curves for products and substrates prepared with authentic reference material.

Method for **4a** and **4b** was run over 35min with H_2O + trifluoroactic acid (0.1%) as the mobile phase (flow rate 0.5mL/min) and an acetonitrile + trifluoroactic acid (0.1%) gradient (0-2min 0%, 2-15min 0-32%, 15-20min 32%, 20-35min 32-100%). The compounds were spectrophotometrically detected at 254nm. Retention times: **4a** 21.81min, **4b** 23.40min.

Method for **3a** and **3b** was run over 60min with P_i-buffer (0.12M, pH 2.4) as the mobile phase (flow rate 1.0mL/min). The compounds were spectrophotometrically detected at 270nm. Retention times: **3a** 5.10min, **3b** 42.02min.

Method for **1a**, **1b**, **2a**, **2b**, **5a-5c**, **7a-7c**, **8a-8c** and **9a-9c** was run over 32min with H_2O + trifluoroactic acid (0.1%) as the mobile phase (flow rate 0.5mL/min) and an acetonitrile +

trifluoroactic acid (0.1%) gradient (0-2min 0%, 2-30min 0-100%, 30-32min 100%). The compounds were spectrophotometrically detected at 254nm except for **2a**, **2b, 9a** and **9c,** which were detected at 280nm. Retention times: **1a** 23.02min, **1b** 24.33min, **2a** 28.13min, **2b** 29.19min, **5a** 17.96min, **5b** 20.75min, **5c** 20.18min, **7a** 26.30min**, 7b** 27.43min**, 7c** 20.42min, **8a** 26.78min, **8b** 25.71min, **8c** 20.87min, **9a** 26.20min, **9c** 20.78min.

Method for **6a** and **6b** was run over 37min with H_2O + trifluoroactic acid (0.1%) as the mobile phase (flow rate 0.5mL/min) and an acetonitrile + trifluoroactic acid (0.1%) gradient (0-2min 0%, 2-30min 0-100%, 30-37min 100%). The compounds were spectrophotometrically detected at 270nm. Retention times: **6a** 28.02min, **6b** 32.37min.

Optimisation of carbonate concentration

In order to elucidate the preferred cosubstrate of (de)carboxylases, i.e. bicarbonate or CO_2, experiments were performed under CO_2-pressure (2bar) as well as with increasing bicarbonate concentrations (0.1 – 4M). For this purpose, the carboxylation reaction of catechol employing the 2,3-DHBD from A*spergillus oryzae* was investigated in more detail.

Regarding the source of carbon dioxide, it turned out that increased CO_2 pressure (2bar) did not have any effects (data not shown). Increasing the amount of bicarbonate was going in hand with a continuous enhancement of the carboxylation activity up to 3M where it levelled off (Figure S5).

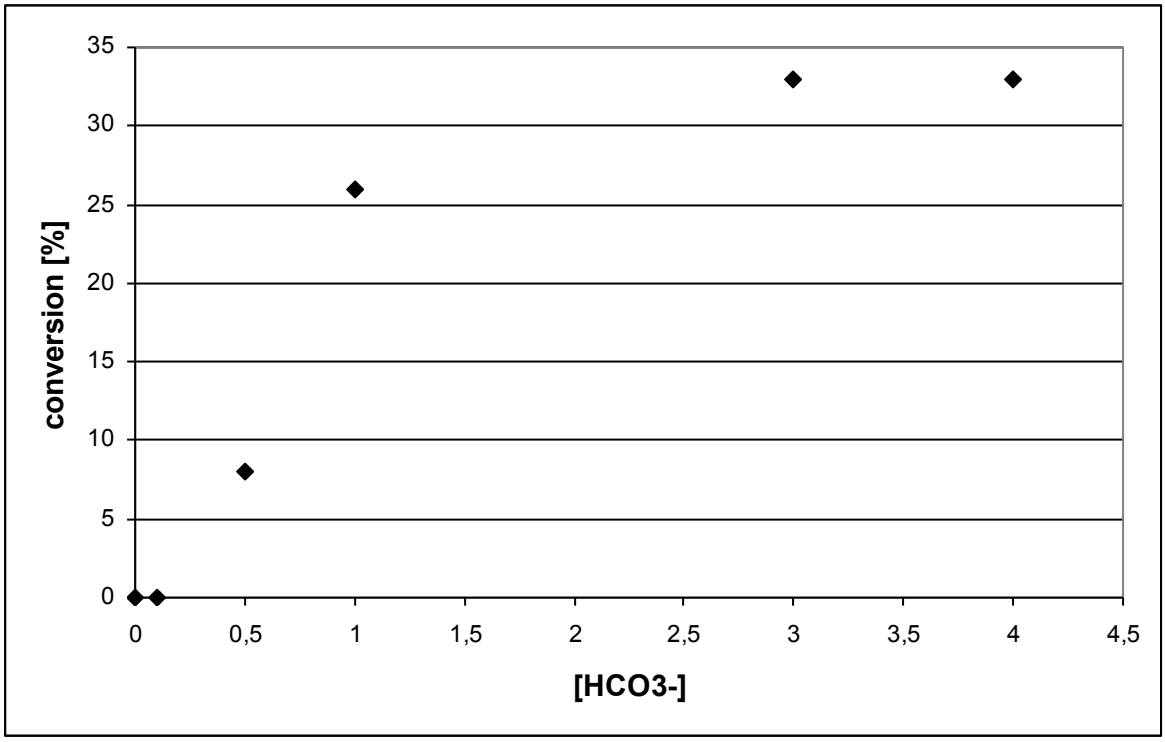


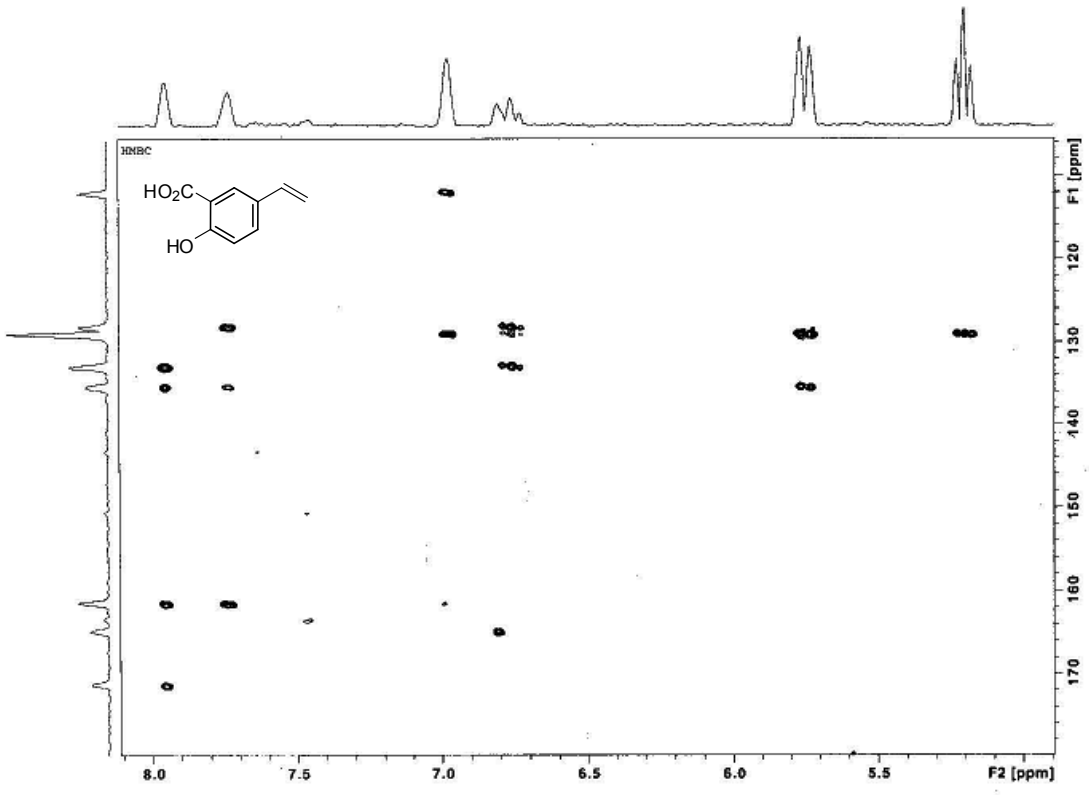
Figure S5. Conversion of catechol applying an increasing bicarbonate concentration.

CO_2 pressure experiments

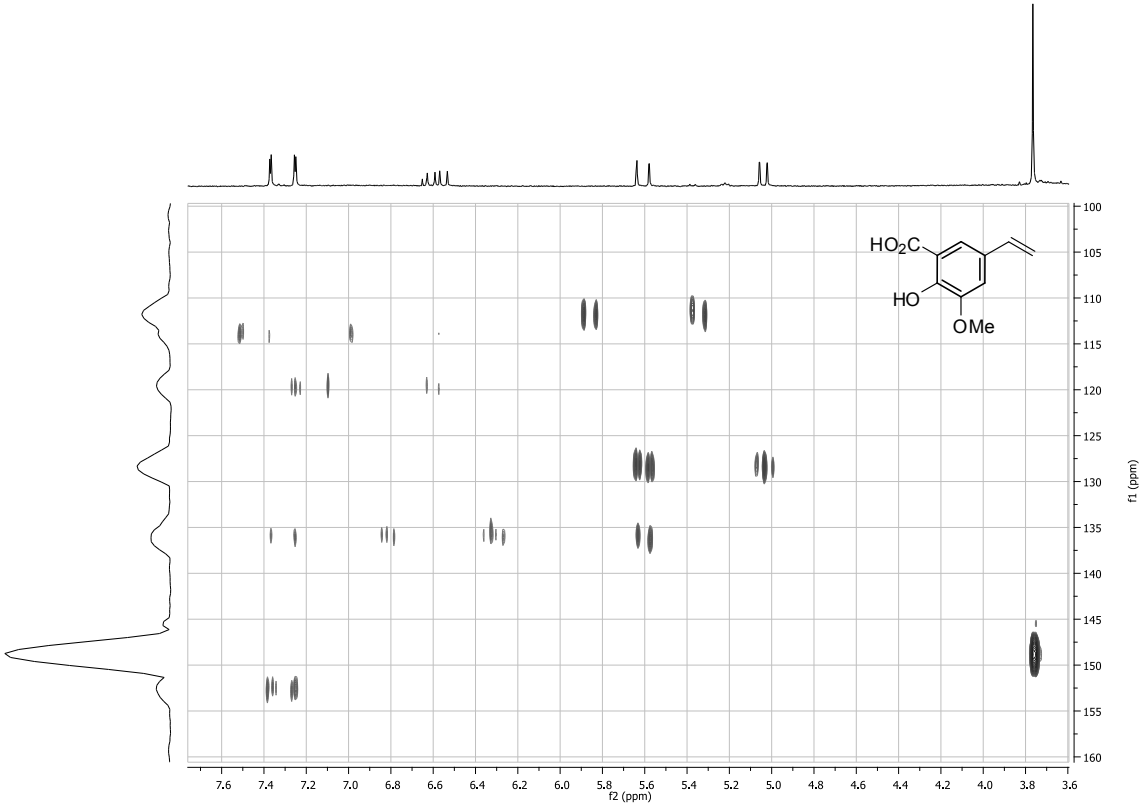
Lyophilized whole cells (30mg *E. coli* host cells containing the overexpressed 2,3-DHBD_Ao) were resuspended in phosphate buffer (900µL, pH 5.5, 100mM) and rehydrated for 30min. Thereafter the substrate catechol was added as a stock solution (100µL) to yield a final concentration of 10mM. The biotransformations were performed at room temperature in open reaction vessels, which were placed in a pressure apparatus at 2bar CO_2 with shaking.[3] After 24h the reaction mixture was centrifuged (15700rcf, 15min), an aliquot of 100µL of each sample was diluted in 1mL of an H_2O/acetonitrile mixture (v/v = 50%) supplemented with trifluoroacetic acid (3% v/v, 30µL). After incubation at room temperature for 5min, the samples were again centrifuged (15700rcf, 15min) and analysed on reverse-phase HPLC to determine the conversion. Products were identified by comparison with authentic reference material.

HMBC-NMR spectra

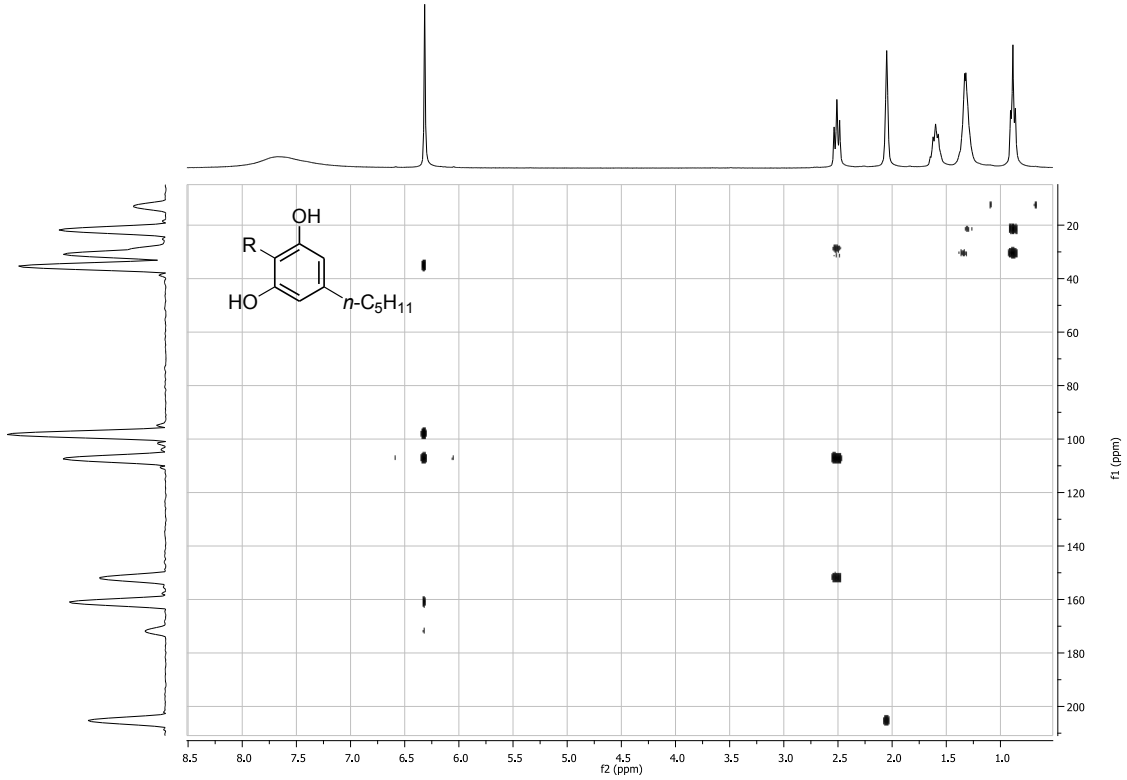
2-Hydroxy-5-vinylbenzoic acid (7b):



2-Hydroxy-3-methoxy-5-vinylbenzoic acid (8b):

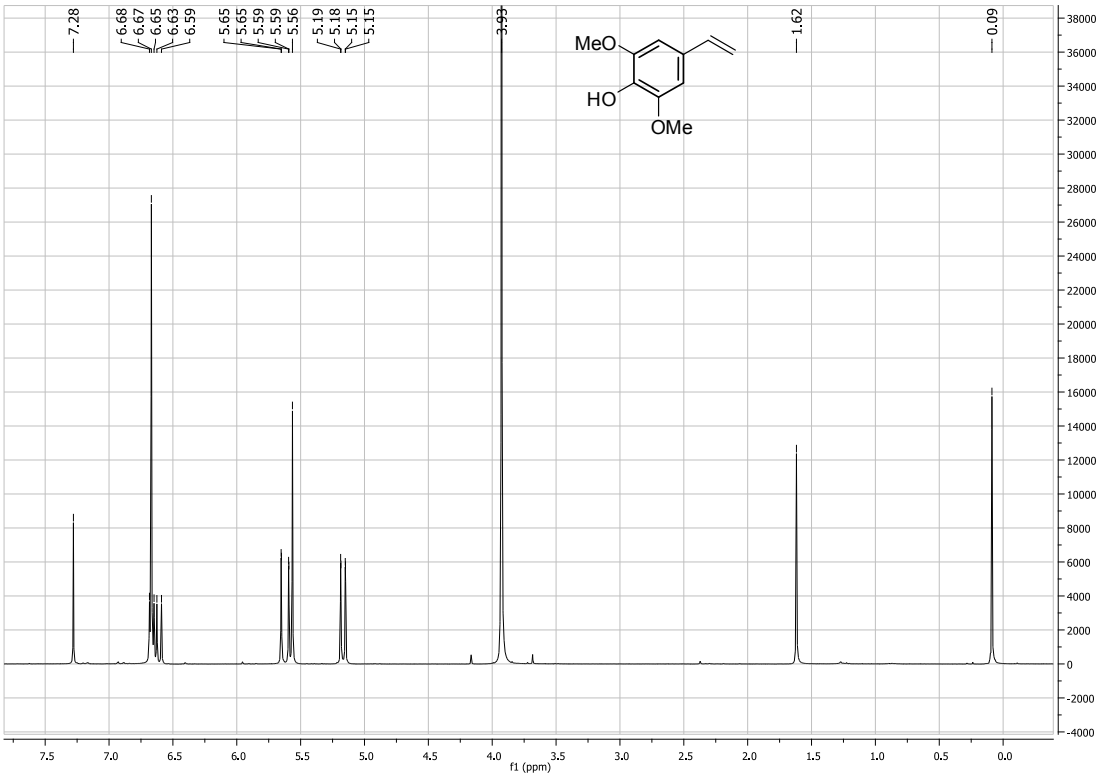


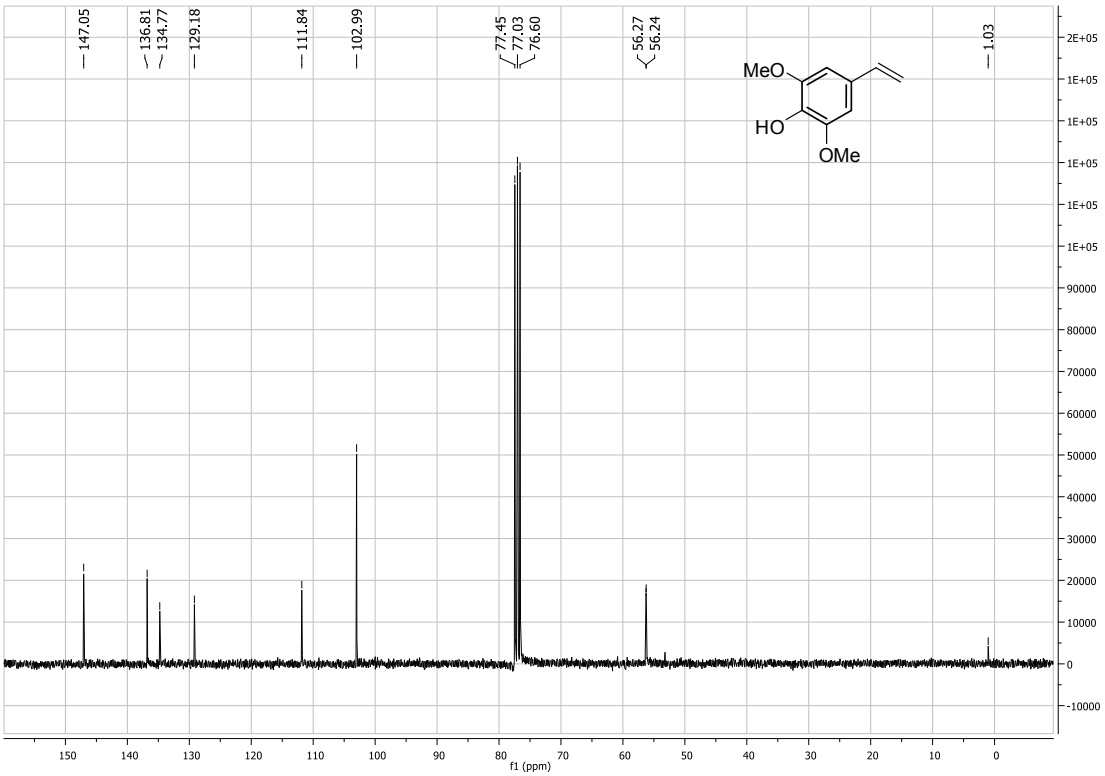
2,6-Dihydroxy-4-pentylbenzoic acid (6b):



^{1}H and ^{13}C-NMR spectra

2,6-Dimethoxy-4-vinylphenol (9a):





References and Notes

(1) M. Mure, S. X. Wang, J. P. Klinman, *J. Am. Chem. Soc.* **2003**, *125*, 6113-6125.

(2) A. Sharma, R. Kumar, N. Sharma, V. Kumar, A. K. Sinha, *Adv. Synth. Catal.* **2008**, *350*, 2910-2920.

(3) M. Fuchs, M. Schober, J. Pfeffer, W. Kroutil, R. Birner-Gruenberger, K. Faber, *Adv. Synth. Catal.* **2011**, *353*, 2354-2358; pictures of the pressure apparatus are available in the electronic supporting information.