Electronic Supplementary Material (ESI) for Catalysis Science & Technology

Stereoselective synthesis of bulky 1,2-diols with alcohol dehydrogenases

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Supplementary Information

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1. Cloning, expression and purification of recombinant ADHs

1.1. Strains and plasmids

Escherichia coli strains BL21(DE3) and DH5α, as well as the pET-22b(+) vector were purchased from Novagen (Madison, USA). The strain DH5α was used as a host organism for cloning, whereas BL21(DE3) was implemented for expression.
The cloning vectors p_RADH and p_SADH containing sequence optimised *radh* and *sadh* genes[1, 2] were subcloned as described below. The corresponding genes were synthesised by GeneArt. The *adht* gene was synthesised by Sloning BioTechnology GmbH, Germany. The HLADH was purchased from Evocatal (Düsseldorf, Germany). The CPCR-clone was obtained from the research group of M. Ansorge-Schumacher (Berlin, Germany). The LBADH-clone was obtained from W. Hummel (Jülich, Germany). The FADH-clone was kindly provided by T. Oikawa (Kansai University, Japan) and the clone with TADH was from A. Schmid (Dortmund, Germany). References concerning the cloning strategies of these respective genes can be found in the literature mentioned in Table 1.

Table 1. Details concerning vector harbouring the genes for the studied oxidoreductases and conditions of cell cultivation.

	Oxidoreductase							
	ADHT	CPCR	FADH	HLADH	LBADH	RADH	SADH	TADH
Vector	pET-22b(+)	pET-26b(+)	pET-3b	–	pET-21a(+)	pET-22b(+)	pET-22b(+)	pET-11b
Antibiotic	Amp	Kan	Amp	–	Amp	Amp	Amp	Amp
Antibiotic concentration [μg mL^{-1}]	100	50	100	–	100	100	100	100
Additive	$ZnCl_2$, 1 mM	–	–	–	–	–	–	–
Reference	this work	3	4	synthetic gene	5	this work	this work	6

Amp – ampicillin, Kan – kanamycin

1.2. Subcloning of radh and sadh

The *radh* and *sadh* genes were subcloned into the expression vectors pET-22b(+) by digestion with the restriction enzymes *Nde*I and *Xho*I (Figure 1). To obtain sufficient plasmid for sequencing and further studies, *E. coli* DH5α was transformed with the plasmids pET-22b(+)_RADH and pET-22b(+)_SADH, respectively.[7] Positive clones were selected on lysogeny broth (LB) agar plates using ampicillin sodium salt (100 μg mL^{-1}) for selection. Gene sequences were confirmed by sequencing. For enzyme production, the genes were overexpressed in *E. coli* BL21(DE3).

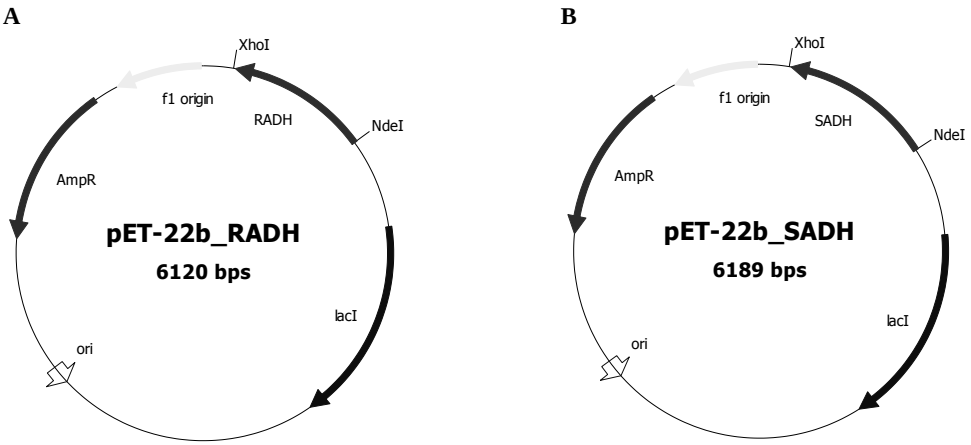


Figure 1. Vector maps of RADH (**A**) and SADH (**B**) cloned into the high-copy plasmid pET-22b(+).

1.3. Expression and purification of recombinant ADHs

1.3.1. Expression

Cultivation of *E. coli* cells carrying the respective *adh* genes were grown in shaking flasks (150 rpm) at 37 °C in 1 L of LB medium[8] supplemented with appropriate amounts of antibiotics (100 µg mL–1 of ampicillin or 50 µg mL–1 of kanamycin depending on the vector) and further required additives (Tab. 1). Protein expression was initiated by the addition of isopropyl-ß-D-1-thiogalactopyranoside (IPTG, 1 mM final concentration), when the optical density at 600 nm reached 0.7. The bacterial cultures were incubated for further 5 hours at 30 °C (in case of RADH and SADH at 20 °C). The cells were harvest by centrifugation (15,900 × g, 40 min, 4 °C, centrifuge Beckmann AV-J20XP) and stored at –20 °C until further use.

Cells containing the RADH plasmid were grown in a 9 L bioreactor (Infors, Switzerland) in LB medium supplemented with glucose (15 g L^{-1}) and ampicillin sodium salt (100 µg mL^{-1}) for selective growth. Cultivation was carried out without further optimisation. Growth was followed by measurements of the optical density at 600 nm. IPTG was added (0.7 mM, final concentration) when the optical density reached 0.8. Further growth was followed at 22 °C for 20 hours during the production phase. The cells were harvested by centrifugation (15,900 × g, 40 min, 4 °C), and stored at –20 °C. From a 9 L culture approximately 180 g wet weight cells were obtained.

1.3.2. Cell disruption

E. coli BL21(DE3) cells containing the respective ADH, were resuspended in TEA-HCl or Tris-HCl buffer (50 mM, pH 7.0) supplemented with magnesium and calcium chloride, if necessary (Tab. 2) and lysozyme (1 mg mL^{-1}). Cell disintegration was performed by ultrasonification (Hielscher, sonotrode S1, cycle 0.5, amplitude 40, about 1 mL of cell suspension, 20 % w v^{-1}) on ice in 20 seconds intervals for a total time of 3 minutes. Cell debris was removed by centrifugation (20,800 × g, 40 min, 4 °C). These crude cell extracts were used for the initial screening towards the reduction of various 2-hydroxy ketones.
To obtain larger quantities of RADH for enzyme purification, cells of E. coli BL21(DE3)-RADH were resuspended (about 50 mL of cell suspension, 20 % w v–1) as described above but at pH 7.5 and disrupted with the sonotrode S3 for 3 minutes at 3 minutes interval for a total of 15 minutes. Cell debris was removed by centrifugation (48,250 × g, 40 min, 4 °C).

Table 2. Buffer systems for cell disruption and activity assays for the different oxidoreductases.

	Oxidoreductase							
	ADHT	CPCR	FADH	HLADH	LBADH	RADH	SADH	TADH
Buffer	Tris-HCl	TEA-HCl	Tris-HCl	TEA-HCl	TEA-HCl	TEA-HCl	TEA-HCl	Tris-HCl
Concentration [mM]	50	50	50	50	50	50	50	50
pH	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Additive	–	–	–	–	$MgCl_2$	$CaCl_2$	–	–
Additive concentration [mM]	–	–	–	–	1.0	0.8	–	–

1.3.3. Purification of RADH

Gel filtration
The crude cell extract of RADH was first desalted using a Sephadex G-25 column (880 mL) (GE Healthcare, Sweden), which was previously equilibrated with TEA-HCl, 10 mM, pH 7.5. The desalting procedure was performed with a flow rate of 10 mL min^{-1} and the eluate was collected in 10

mL fractions. Protein-containing fractions were determined at 280 nm, pooled and applied to anion-exchange chromatography for further purification.

Anion-exchange chromatography

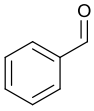
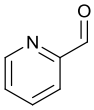
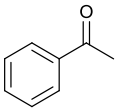
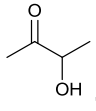
The desalted crude cell extract (130 mL) was loaded onto a Q-Sepharose Fast Flow column (diameter: 1.6 cm, gel bed volume: 28 mL) (GE Healthcare, Sweden), which was equilibrated with at least three column volumes of equilibration buffer (TEA-HCl, 50 mM, pH 7.5) prior to use. After removal of unbound proteins by washing with equilibration buffer, elution was started with a linear NaCl-gradient (0-200 mM) within 150 minutes. The flow rate was set to 1 mL min^{-1} and the eluate was collected in 10 mL fractions. RADH-containing fractions eluted with 150 mM NaCl. The collected fractions were subjected to an activity assay and SDS-PAGE electrophoresis. Fractions containing active RADH were pooled and again desalted using the Sephadex G-25 column with the same procedure as described above. Freeze-dried RADH was stored at –20 °C.

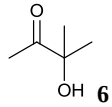
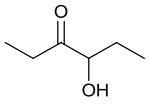
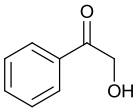
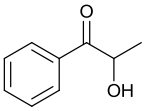
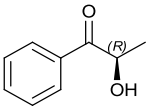
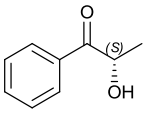
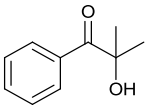
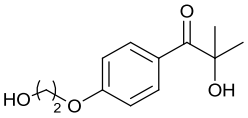
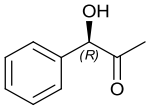
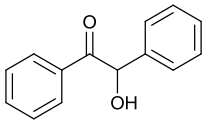
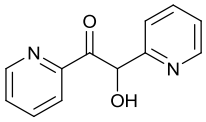
1.4. SDS electrophoresis

SDS-PAGE[9] was carried out on 4-12% NuPAGE® Novex Bis-Tris gels (Invitrogen). For the estimation of the molecular weight of proteins the PageRuler™ Plus Prestained Protein Ladder (10-250 kDa, Fermentas) was used. The gel was stained in SimplyBlue™ SafeStain (Invitrogen) or with the SilverXpress® Silver Staining Kit (Invitrogen).

2. Activity-screening of ADHs

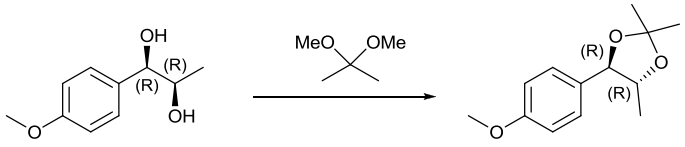
Table 3. Substrate specificity of crude cell extracts containing overexpressed alcohol dehydrogenases. The reduction activity towards aldehydes, ketones and 2-hydroxy ketones is measured in triplicate by consumption of NADPH (0.2 mM) at wavelengths of 340 nm at 30°C.
n.a. – activity not detectable; n.d. – activity not determined; CPCR – *Candida parapsilosis* carbonyl reductase, FADH – *Flavobacterium frigidimaris* alcohol dehydrogenase, HLADH – Horse liver alcohol dehydrogenase, SADH – *Sphingobium yanoikuyae* alcohol dehydrogenase, TADH – *Thermus* sp. alcohol dehydrogenase. HPP – 2-hydroxy propiophenone = 2-hydroxy-1-phenylpropan-1-one (**9**); PAC – phenylacetylcarbinol = 1-hydroxy-1-phenylpropan-2-one (**12**, here only (*R*)-enantiomer); Benzoin – 2-hydroxy-1,2-diphenylethanone (**13**); α-pyridoin – 2-hydroxy-1,2-di(pyridine-2-yl)ethanone (**14**).

Substrate	Concentration [mM]	Specific activity [U mg^{-1}]				
		CPCR	FADH	HLADH	SADH	TADH
Aldehydes						
 **1**	10	0.26±0.00	1.41±0.00	0.96±0.01	n.a.	0.68±0.00
 **2**	10	0.12±0.00	4.12±0.03	0.54±0.02	0.03±0.00	0.40±0.00
 **3**	10	n.a.	1.47±0.02	0.12±0.01	n.a.	0.01±0.00
Ketones						
 **4**	10	n.a.	n.a.	n.a.	n.a.	0.54±0.00
2-Hydroxy ketones: aliphatic						
 **5**	10	n.a.	n.a.	n.a.	n.a.	n.a.

Substrate	Concentration [mM]	Specific activity [U mg^{-1}] CPCR	FADH	HLADH	SADH	TADH
 **6**	10	n.a.	n.a.	n.a.	n.a.	n.a.
 **7**	10	n.a.	n.a.	n.a.	n.a.	n.a.
2-Hydroxy ketones: 2-HPP and derivatives						
 **8**	10	n.a.	n.a.	n.a.	n.a.	n.a.
 ***rac*-9**	10	n.d.	n.d.	n.d.	n.d.	n.d.
 **(*R*)-9**	10	n.d.	n.d.	n.d.	n.d.	n.d.
 **(*S*)-9**	10	n.d.	n.d.	n.d.	n.d.	n.d.
 **10**	10	n.a.	n.a.	n.a.	n.a.	n.a.
 **11**	5	n.a.	n.a.	n.a.	n.a.	n.a.
2-Hydroxy ketones: PAC						
 **(*R*)-12**	10	n.d.	n.d.	n.d.	n.d.	n.d.
2-Hydroxy ketones: Benzoin and α-pyridoin						
 **13**	saturated	n.d.	n.d.	n.d.	n.d.	n.d.
 **14**	saturated	n.a.	n.a.	n.a.	n.a.	n.a.

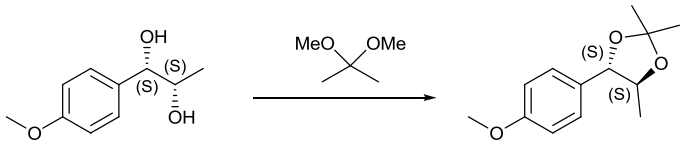
3. Determination of absolute configuration of (1*R*,2*R*)-38 and (1*S*,2*S*)-38

The diols (1*R*,2*R*)-**38** and (1*S*,2*S*)-**38** (10 mg, 0.05 mmol) were dissolved in dry dichloromethane (700 µL) and 2,2-dimethoxypropane (34µL, 0.27 mmol, 5 eq.) before catalytic amounts of *p*-toluenesulfonic acid (2 mg) were added at 0°C. The reaction mixtures were stirred at room temperature for 30 min, subsequently quenched with saturated aqueous $NaHCO_3$, and extracted with dichloromethane. Combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. Subsequent filter flash chromatography (EtOAc/cyclohexane 30/70) yielded (4*R*,5*R*)-**39** (9.9 mg, 81 %) and (4*S*,5*S*)-**39** (8.5 mg, 85 %). (4*R*,5*R*)-**39**: $[\alpha]_D^{20} = -0.13$ (*c* 0.5, CH_3CN); (4*S*,5*S*)-**39**: $[\alpha]_D^{20} = +0.15$ (*c* 0.5, CH_3CN).



(1*R*,2*R*)-**38** (4*R*,5*R*)-**39**

Scheme 1. Preparation of (4*R*,5*R*)-**39**



(1*S*,2*S*)-**38** (4*S*,5*S*)-**39**

Scheme 2. Preparation of (4*S*,5*S*)-**39**

^{1}H NMR ($CDCl_3$): δ (ppm) = 1.26 (d, J = 6.0 Hz, 3H, CH*C*H_3), 1.50, 1.55 (s, 6H, CH_3), 3.81 (s, 3H, OCH_3), 3.81-3.88 (m, 1H, *C*HCH_3), 4.42 (d, J = 8.6 Hz, 1H, *C*HCHCH_3), 6.88-6.92 (m, 2H, CH_{arom}), 7.28-7.32 (m, 2H, CH_{arom}).
^{13}C NMR ($CDCl_3$): δ (ppm) = 16.1 (CH*C*H_3), 27.1, 27.4 (CH_3), 55.3 (OCH_3), 79.1, 84.6 (*C*H-O), 108.2, 129.5 (C_{arom}), 113.9, 127.9 (CH_{arom}), 159.6 (*C*-OCH_3).

The absolute configurations of (4*R*,5*R*)-**39** and (4*S*,5*S*)-**39** were determined by vibrational circular dichroism (VCD) and quantum chemical calculations (GAUSSIAN 09). FT-VCD and FT-IR spectra were recorded on a Bruker Tensor 27 FTIR spectrometer equipped with a Bruker PMA 50 VCD side-bench module with a resolution of 4 cm^{-1} in a 100 µm BaF2 cell.

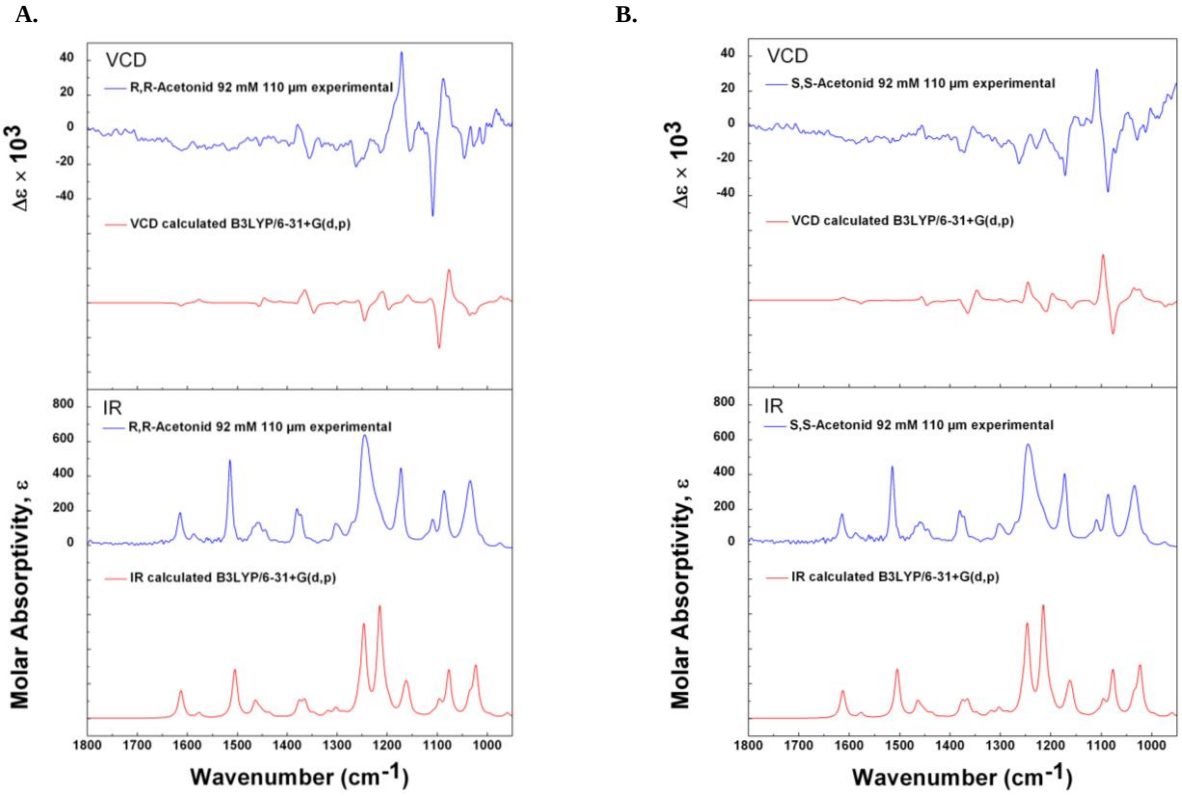


Figure 2. The experimental (blue) and the calculated (red) spectra, for the acetonides (4*R*,5*R*)-**39** (**A**) and (4*S*,5*S*)-**39** (**B**). Experimental IR and VCD spectra of (4*R*,5*R*)-**39** and (4*S*,5*S*)-**39** (92 mM in $CDCl_3$) and the Boltzmann-weighted average of theoretical IR and VCD spectra (B_3LYP/6-31G*) were calculated for four conformers of (4*R*,5*R*)-**39** and (4*S*,5*S*)-**39**.

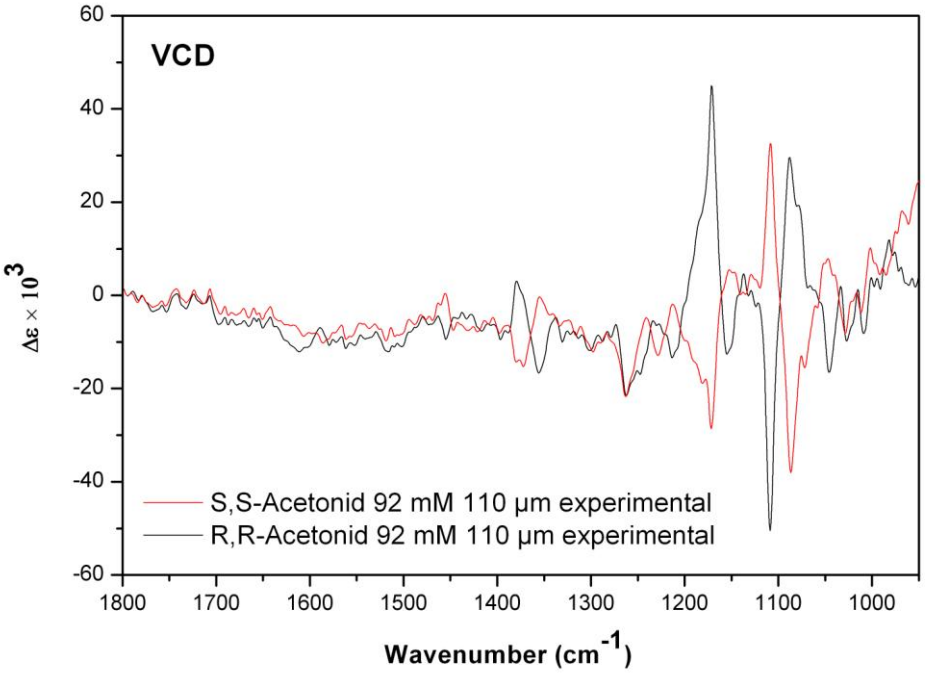


Figure 3. Superposed VCD-spectra of (4*R*,5*R*)-**39** and (4*S*,5*S*)-**39.**

4. References

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