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Asymmetric reductions in aqueous media: enzymatic synthesis in cyclodextrin containing buffers

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Abstract

The enzymatic reduction of hydrophobic ketones in cyclodextrin containing media is reported yielding the corresponding alcohols (*S*)-1-(2-naphthyl)-ethanol, (*S*)-(*E*)-4-phenyl-3-en-2-ol and 1,2,3,4-tetrahydro-2-(1-hydroxyethyl)-1-oxonaphthalene in good yield and high enantiomeric excess. The reaction was catalyzed by a carbonyl reductase from *Candida parapsilosis*, NADH regeneration was carried out using either 2-propanol in a substrate coupled mode or formate dehydrogenase from *Candida boidinii* and formate as hydrogen donor. In continuous experiments high stability of the enzymes in media containing heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin could be demonstrated. Space time yields of 120 g/(Ld) were obtained under not yet optimized conditions during the production of (*S*)-1-(2-naphthyl)-ethanol.

The enzymatic conversion of hydrophobic compounds, poorly soluble in water, presents a unique challenge in the production of fine chemicals.[1,2] Addition of water miscible solvents to increase the solubility of educts is problematic, as many enzymes are destabilized by changes in the dielectric constant and perturbation of the water structure.[3] Lipases in their natural environment act at aqueous–lipid interfaces and may be employed with advantage in biphasic reaction media in the presence of a water immiscible solvent.[4–6] For dehydrogenases, which often denature at such interfaces, a bi-membrane reactor[7–10] and an emulsion reactor[11] have been designed to facilitate the transfer of the educt into the aqueous phase and the removal of the product after conversion. Application of enzymes in nearly anhydrous media have been reported,[12–14] but the reaction rate is usually much reduced and problems arise if the pH needs to be adjusted in the course of the reaction or coupled reactions are desired. Another possibility to increase the apparent solubility of hydrophobic compounds is the application of cyclodextrins in homogeneous aqueous media.

Cyclodextrins are torus-shaped cyclic maltooligosaccharides of six (α), seven (β) or eight (γ) D-glucose units linked by α-1,4-glycosidic linkages.[15] By intramolecular hydrogen bonds between the glucose units an apolar cavity is formed, which accepts hydrophobic guest molecules.[16] The hydrophilic

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shell of the torus maintains solubility in water. The hydrolysis of deacetyl-lanatoside A to digitoxin by β-glucosidase[17] and the hydrolysis of *p*-nitrophenylbutyrate as well as triolein or olive oil by lipases[18] was enhanced by addition of cyclodextrins, especially heptakis-(2,6-di-*O*-methyl)-β-cyclo-dextrin (DIMEB). The enzymatic oxidation of steroids by cholesterol oxidase[19] or phenoloxidase[20] in the presence of cyclodextrins has been studied as well.

We recently analyzed, in detail, the enzymatic reduction of 2-acetylnaphthalene **1c** in cyclodextrin containing media using a carbonyl reductase from *Candida parapsilosis* (*CP*CR) and formate dehydrogenase (FDH) from *Candida boidinii* for in situ NADH regeneration.[21] DIMEB at 100 mM concentration enhanced the apparent solubility of **1c** about 150 fold. The reaction velocity of the coupled reaction was unaffected by the presence of DIMEB, indicating that the kinetics of dissociation of the preformed complex between **1c** and DIMEB is not rate limiting.

Since the production of chiral alcohols is of high interest we extended our studies to other substrates of *CP*CR and explored enzymatic reductions in continuous processes using different modes of NADH regeneration, as illustrated in Fig. 1.

*CP*CR was originally screened as an NADH dependent β-ketoester-reductase.[22] Later it was shown, that the enzyme has a very broad substrate range and accepts, among other carbonyl compounds, diketones and aromatic ketones as substrates.[23,24] Due to the alcohol dehydrogenase activity of the *CP*CR, NADH regeneration may be carried out in a substrate coupled reaction (line I, Fig. 1) using isopropanol as hydrogen donor.[25] Besides, the enzyme coupled mode (line II, Fig. 1) was employed with FDH as a second enzyme and formate as the ultimate hydrogen donor. Table 1 summarizes the results obtained for batch conversions in preparative scale.

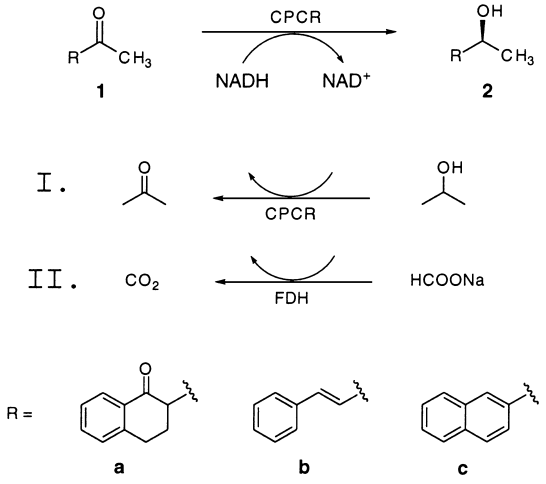

Figure 1. General reaction scheme

In all cases high conversions were reached after several days as judged by TLC. Only in the case of **2c**, two weeks were needed for the conversion due to the low enzyme concentrations applied. The corresponding alcohol was extracted with hexane and/or ether, respectively, and purified by column chromatography over silica before further analysis. On a preparative scale **2c** was obtained in high *ee* (>99%) and 70% isolated yield. The enzymatic reduction of the ketones **1a** and **1b** by *CP*CR is reported for the first time. The (*S*)-alcohol **2b** has been obtained before by enantioselective esterification of the corresponding racemate in 32% yield and *ee* >95% using *Pseudomonas* lipase.[26] High chemoselective catalytic hydrogenation (>99.9%) of the carbonyl group of **1b** was achieved by Noyori et al. using a $RuCl_2$ (binap)$(dmf)_n$-diamine–KOH system.[27] Stereoselectivity was only moderate, yielding 70% *ee* of

Table 1
Enzymatic reduction of aromatic ketones yielding *S*-alcohols

Compound	isolated yield [%]	enantiomeric excess [%]	diastereomeric excess
2 a	60	c.n.d.	2 : 1
2 b	77	> 99	-
2 c	70	> 99	-

c.n.d. = could not be determined, see text

the (*R*)-enantiomer. Using an oxazaborilidine catalyst, Corey and Helal[28] reported 97% *ee* and 90% yield for the (*R*)-enantiomer of **2b** performing the reaction at −78°C in toluene. Based on the known sign of the specific rotation the product obtained by reduction with *CP*CR is the (*S*)-enantiomer, which was isolated with an *ee* of >99% as determined by gas chromatography. By NMR analysis it was recognized that the isolated product **2b** contained about 5% of the saturated alcohol 4-phenyl-2-butanol. Analysis of the educt employed showed that **1b** contained about 5% of the corresponding saturated ketone, which evidently is also a substrate for *CP*CR. The *ee* of the saturated alcohol was not determined, but it is expected that this reduction by the *CP*CR will also be performed at a very high level of enantioselectivity.

To the best of our knowledge, regioselective reduction of **1a** in the side chain has never been achieved before. From NMR data it is evident that the cyclic ketone group remained intact. An earlier detailed kinetic study of the *CP*CR revealed a small and a large hydrophobic binding pocket of the enzyme differing in volume and affinity towards potential substrates. The small binding site accepts only alkyl groups (preferentially a methyl group) while the presence of an aromatic ring in the large binding pocket led to low K_M values and good reaction rates.[24] Based on these results we conclude that the high regioselectivity is a steric effect directing the positioning of the substrate **1a** in the binding site. We assume that the product of the enzymatic reduction has the (*S*)-configuration, in accordance with all other products obtained so far using *CP*CR. Definite proof is expected from structure determination by X-ray analysis. The product was isolated as an oil and could not be crystallized yet. The latter may be related to the fact that two diastereomers were obtained in the ratio of 2:1 as determined by NMR. During the regioselective reduction of the diketone a second stereogenic center at C2 is generated. The composition of the stereoisomers could not be determined, although the silylated, acetylated and non-derivatized products were analyzed on different GC phases.[†]

The reduction of ethyl-2-methyl-3-oxo-butanoate with *CP*CR led to the *syn*-product with (2*R*,3*S*)-configuration in diasteromeric excess of 98%.[29] With the diketone **1a** the diasteromeric discrimination is less pronounced, which may reflect a slower reracemization step at C2.

Continuous reductions of 2-acetylnaphthalene were performed to demonstrate the feasibility of operating a membrane reactor with DIMEB containing reaction media, to generate reliable data on enzyme stability during prolonged times and to test the different modes of coenzyme regeneration under these conditions. Fig. 2 illustrates the conversion–time course of the reaction over 120 h under various residence times using FDH for coenzyme regeneration. The reactor was charged with 4.6 U/mL *CP*CR and 4.4 U/mL FDH. After about 10 h a steady conversion of 52% was reached at a residence time τ of 1 h, representing a space time yield of 43 g/(Ld). Increasing τ to 2 h a new steady state was established at 79%

[†] Tested GC phases: Lipodex B and Lipodex E from Macherey & Nagel (Düren, Germany), Cyclodex β 1/P, α-2P, γ-1P, γ-Lactone, β-2P from Chromatographie Service (Langerwehe, Germany) and Chiraldex β-PH, GTA and BP-H from Astec (Whippany, NJ, USA).

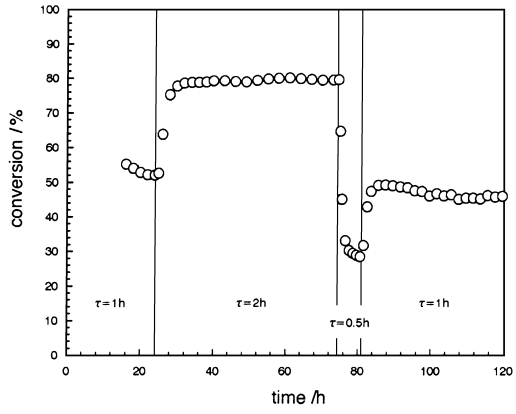

Figure 2. Continuous reduction of 2-acetylnaphthalene (20 mM) in 100 mM TEA buffer pH 7.0 containing 0.5 mM NAD^+, 1 M sodium formate, 2 mM DTT and 50 mM DIMEB. Initial catalyst/concentration 4.6 U/mL *CP*CR and 4.4 U/mL FDH

conversion and maintained at this level for 40 h. The space time yield dropped to 33 g/(Lh). Reducing τ to 0.5 h the conversion dropped to 29%. After 81 h of operation, start conditions were re-established and a new steady state at 46% conversion reached. From the drop in conversion a deactivation of the enzymes of 1.2% per day can be estimated demonstrating that both enzymes are very stable in DIMEB containing reaction media.

Increasing the ratio of *CP*CR activity to total enzyme activity should result in a higher conversion being reached,[23] which was not intended here, however a lower ratio was chosen in order to see the enzyme deactivation more clearly. The small decay in conversion does not distinguish whether *CP*CR, or FDH, or both deactivated slowly. In control experiments incubation of *CP*CR in the presence of 0.2 mM 2-acetylnaphthalene in 100 mM TEA buffer pH 7.0 resulted in 15% residual activity of the enzyme after 24 h. Between 25 and 30% loss of activity can be attributed to oxidation of *CP*CR in the absence of reducing agents. The observed high stability in the presence of DIMEB during prolonged operation in a continuously operated stirred tank reactor may be due to the low concentration of the uncomplexed ketone, which no longer affects the enzyme stability.

In a second continuous reduction (Fig. 3) the high stability of *CP*CR under operating conditions was confirmed. The conversion–time curve is very stable, from the difference in conversion after 20 and 90 h a deactivation of 0.2% per day was determined for *CP*CR. Using 260 mM 2-propanol (2% v/v) for NADH regeneration in the substrate coupled mode a maximal conversion of 82% was reached at τ=2 h. Reducing τ to 0.5 h, 71% conversion was obtained, corresponding to a space time yield of 118 g/(Ld). Under these conditions the productivity was about 2.5 fold higher as observed in Fig. 2 with NADH regeneration by FDH. Since conditions for both experiments are not yet optimized this result should not be over interpreted. Nevertheless, it could be demonstrated that in DIMEB containing media substrate coupled coenzyme regeneration is possible and space time yields well in excess of 120 g/(Ld) are expected under optimized reaction conditions, especially by increasing the enzyme concentration in the reactor.

1. Experimental

1.1. Chemicals

DIMEB, heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin was obtained from Wacker-Chemie (Düsseldorf, Germany), 2-acetylnaphthalene was purchased from Fluka (Buchs, Switzerland), benzylidenacetone

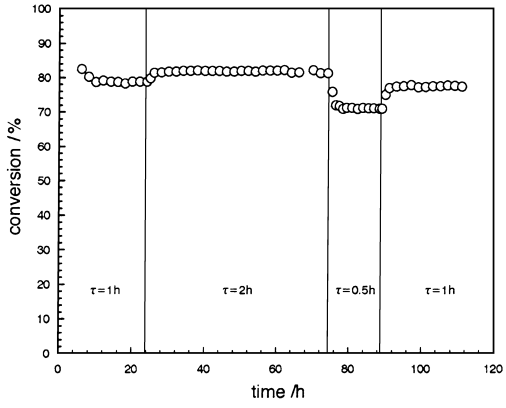

Figure 3. Continuous reduction of 2-acetylnaphthalene (20 mM) in 100 mM TEA buffer pH 7.0 containing 0.5 mM NAD^+, 260 mM 2-propanol, 2 mM DTT, 50 mM DIMEB and 4.6 U/mL *CP*CR

from Merck (Darmstadt, Germany), 2-acetyl-1-tetralone from Aldrich (Steinheim, Germany) and used as delivered. NAD^+ and NADH were products from Biomol or Oriental Yeast (Tokyo, Japan). All other chemicals, buffer salts and solvents were obtained in high quality from commercial sources.

1.2. Enzymes

Formate dehydrogenase (FDH) from *Candida boidinii* was supplied by Dipl.-Ing. Holger Gieren, Institute of Enzymetechnology (Jülich, Germany) and prepared as described by Weuster-Botz et al.[30] Carbonyl reductase (*CP*CR) from *Candida parapsilosis* was isolated as described by Peters et al.[23] and partially purified by fractional precipitation with poly(ethylene glycol) and anion exchange chromatography on Sepharose Q Fast Flow (Amersham Pharmacia, Freiburg, Germany) to a specific activity of ~16 U/mg in the standard assay system as described by Zelinski and Kula.[21] *CP*CR was stored in buffer solution containing 50% glycerol, 1 mM DTT at −20°C.

1.3. Analytical procedures

NMR spectra were obtained on a Varian VXR 300 at 300 MHz (^{1}H) and 75 MHz [^{13}C] shifts are reported as ppm relative to TMS as internal reference. Abbreviations: m=multiplet, d=doublet, s=singulet, br=broad Perkin Elmer model 241 MC polarimeter with a 10 cm path length cell was used to determine $[\alpha]_D$. Gas chromatographic analysis was carried out using equipment (GC9A) from Shimadzu (Düsseldorf, Germany) and flame ionization for detection. In general, separation was performed on a chiral Lipodex-*E*-γ-Cyclodextrin column (25 m×0.2 mm ID) from Macherey and Nagel (Düren, Germany) operated with helium as carrier gas and injecting 1 μL samples. The column was thermostated at 70°C, while injector and detector were kept at 250°C. Thin layer chromatography: Samples (up to 5 μL) were applied on silica plates 60 F_{254}, Merck (Darmstadt, Germany) and developed with *n*-hexane:ethyl acetate (3:1). After drying in a warm air current the plates were viewed under UV light. Capillary electrophoresis was performed as described by Zelinski and Kula.[21]

1.4. Batch conversions

Enzymatic transformations of 5.5 mmole **1a**, **1b** were carried out at room temperature with stirring (120 rpm) in volumes of 50–300 mL containing 100 mM triethanolamine (TEA) buffer pH 7, 2 mM

dithiotreitol (DTT), 250 mM sodium formate, 1 mM NAD^+, 100 mM DIMEB and 1 U/mL *CP*CR and 3 U/mL FDH. Reduction of **1c** was performed in the same buffer at 20 mM **1c**, 1 M sodium/formate, 0.5 mM NAD^+, 50 mM DIMEB, at low enzyme concentrations of 0.007 U/mL *CP*CR and 0.035 U/mL FDH for two weeks. At the end of the reaction the complete mixture was ultrafiltrated in an Amicon cell using a YM 10 membrane (Witten, Germany) and the retentate washed with TEA buffer to separate the enzymes. The filtrates were extracted several times with hexane and/or ether, the solvent dried over anhydrous sodium sulfate and removed by rotary evaporation. The resulting oils were purified by chromatography over silica gel 60 (Merck, Darmstadt, Germany) with *n*-hexane:ethylester (1:4) for development.

1.5. Continuous conversions

A 10 mL thermostated (25°C) enzyme membrane reactor was used as described by Wandrey and Kula.[31] The reactor was equipped with an ultrafiltration membrane YM-10, Amicon (Witten, Germany) cut of 10 kDa, and operated with a magnetic stirrer at about 150 rpm. The reactor was sterilized before use percolating a 0.1% solution of peracetic acid for 16 h and then flashed with sterile water before introducing the substrate solution containing 20 mM 2-acetylnaphthalene, 0.5 mM NAD^+, 1M sodium formate, 2 mM DTT and 50 mM DIMEB in 100 mM TEA buffer pH 7.0 through a microporous membrane (cellulose nitrate, 0.1 μm pore size, Sartorius AG, Göttingen, Germany). Enzymes were injected into the inlet stream before passage through the microporous filter. In the case of the substrate coupled NADH regeneration FDH was omitted and 260 mM 2-propanol added to the reaction medium. An alternating piston pump (Pharmacia P-500, Freiburg, Germany) was used for constant delivery of substrate solution to achieve the desired residence time. The microporous membrane in the inlet stream and the ultrafiltration membrane in the reactor defined and maintained the sterile area. The ultrafiltration membrane retains the enzymes in the reactor, while buffer and low molecular weight compounds of the reaction medium can pass. The reactor effluent was collected using a fraction collector. Single fractions were extracted manually with 0.5 volumes of toluene and educt and product were determined by analyzing a suitable aliquot by capillary electrophoresis and/or gas chromatography as described above.

1.6. NMR analysis: 1,2,3,4-tetrahydro-2-(1-hydroxyethyl)-1-oxonaphthalene

^{1}H NMR (300 MHz): major: δ 8.01 (d, *J*=7.7 Hz, 1H, H-8), 7.52–7.54 (m, 1H, H-6), 7.29–7.35 (m, 1H, H-7), 7.25 (d, J=7.7 Hz, 1H, H-5), 4.39 (m, 1H, H-2), 4.5–4.3 (br, 1H, OH), 3.04 (m, 2H, H-4), 2.67–2.59 (m, 1H, H-9), 2.14–2.28 (m, 1H, H-3a), 2.11–1.98 (m, 1H, H-3b), 1.29 (d, *J*=7.7 Hz, 3H, H-10), minor: δ 8.01 (d, *J*=7.7 Hz, 1H, H-8), 7.52–7.54 (m, 1H, H-6), 7.29–7.35 (m, 1H, H-7), 7.25 (d, *J*=7.7 Hz, 1H, H-5), 4.17 (m, 1H, H-2), 3.25–3.1 (br, 1H, OH), 3.04 (m, 2H, H-4), 2.41–2.50 (m, 1H, H-9), 2.14–2.28 (m, 1H, H-3a), 1.74–1.91 (m, 1H, H-3b), 1.29 (d, *J*=7.7 Hz, 3H, H-10). ^{13}C NMR (75 MHz): major: δ 200.8 (C-1), 144.3 (C-4a), 132.8 (C-8a), 132.8 (C-6), 128.8 (C-7), 127.2 (C-5), 126.6 (C-8), 66.9 (C-9), 53.4 (C-2), 29.0 (C-4), 23.8 (C-3), 19.3 (C-10), minor: δ 202.3 (C-1), 144.3 (C-4a), 133.6 (C-8a), 132.3 (C-6), 128.7 (C-7), 127.4 (C-5), 126.7 (C-8), 68.2 (C-9), 54.1 (C-2), 28.9 (C-4), 25.6 (C-3), 20.2 (C-10). Major to minor diastereomer observed in a ratio of 2:1. (*S*)-(*E*)-4-Phenylbut-3-en-2-ol [81176-43-4] ^{1}H NMR (300 MHz): δ 7.39–7.18 (m, 5H), 6.56 (d, *J*=16.0 Hz, 1H), 6.25 (dd, *J*=16.0 Hz, 8.0 Hz, 1H), 4.48 (m, 1H), 1.77 (s br, 1H), 1.37 (d, *J*=6.4 Hz, 3H). (*S*)-1-(2-Naphthyl)ethanol ^{1}H NMR (300 MHz $CDCl_3$): δ=7.87–7.46 (m, 7H), 5.09 (q, 1H-c-1′), 1.76 (brs OH), 1.60 (d, 3H-1C-2′) ppm, the data correspond to literature values.[32]

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