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Biocatalytic oxidation of benzyl alcohol to benzaldehyde via hydrogen transfer

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ABSTRACT

Various types of biocatalysts like oxidases, alcohol dehydrogenases, and microbial cells were tested for the oxidation of benzyl alcohol. Oxidases in combination with molecular oxygen led to low conversion. Alcohol dehydrogenases and microbial cells were tested in a hydrogen transfer reaction employing acetaldehyde, chloroacetone, and acetone as hydrogen acceptor. Excellent conversion (95%) could be achieved employing lyophilised cells of *Janibacter terrae* DSM 13953 at a substrate concentration of 97 mM.

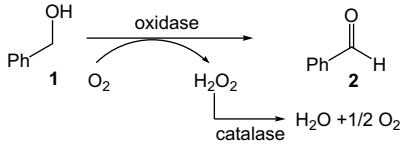
1. Introduction

Oxidation of alcohols to yield carbonyl compounds is one of the most fundamental and important processes in synthetic organic chemistry. In the search for alternatives driven by the immaturity of many organic oxidation reactions[1–5] a lot of emphasis has been put on the development of 'green' chemical processes.[6–11] For instance for the biocatalytic oxidation of primary alcohols laccases in combination with mediators[12–14] as well as various redox enzymes have been employed.[15–21] Here we describe the chemoselective bio-oxidation of benzyl alcohol to the corresponding aldehyde avoiding overoxidation to benzoic acid using oxidases, isolated alcohol dehydrogenases (ADHs), and microbial cells. For the last two options various formal hydrogen acceptors were studied such as acetaldehyde, chloroacetone as well as acetone.

2. Results and discussion

2.1. Oxidases

As a first approach we tested a library of fourteen commercial alcohol oxidases for the oxidation of benzyl alcohol to benzaldehyde. Alcohol oxidases[22–25] have been efficiently employed for the oxidation of *n*-alkanols requiring just molecular oxygen as oxidant (Scheme 1). Hydrogen peroxide is formed as side product, which can be disproportioned by a catalase yielding water and molecular oxygen.



Scheme 1. Oxidation employing an oxidase and a catalase.

Out of the fourteen oxidases tested only four showed low conversion of approximately 5%. The active oxidases originated from *Pichia pastoris*, *Candida boidinii*, *Hansenula* sp. as well as the galactose oxidase from *Dactylium dendroides*. Since this was below our expectations, we tested commercial alcohol dehydrogenases (ADHs) for the oxidation of benzyl alcohol.

2.2. Commercial alcohol dehydrogenases

A significant number (>200) of alcohol dehydrogenases are commercially available.[26] Out of these, a library of 33 selected ADHs were tested in a biocatalytic hydrogen transfer reaction employing acetaldehyde as hydrogen acceptor (Scheme 2). Acetaldehyde has rarely been employed as hydrogen acceptor,[27–29] probably due to its supposed inhibition effect on enzymes. However, employing it in excess in the testing allows identifying easily enzymes which are stable in the presence of the reactive aldehyde moiety. Additionally our aim was to identify a single biocatalyst/enzyme, which performed the desired oxidation as well as the recycling of the cofactor, which is in contrast to approaches where an additional enzyme is used for the recycling.[26]

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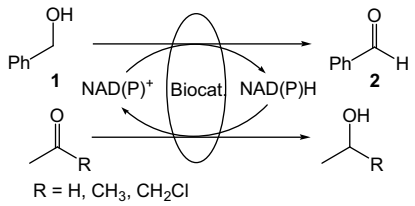


Scheme 2. Oxidation of benzyl alcohol via biocatalytic hydrogen transfer.

2.2.1. Testing of ADHs

For preparative significance substrate **1** was used at a concentration of 97 mM with an almost 10-fold excess of acetaldehyde (951 mM) as hydrogen acceptor to ensure shifting the equilibrium to benzaldehyde. Furthermore the elevated acetaldehyde concentration represents an additional selection parameter, namely to select for stability in the presence of the aldehyde. Although many new ADHs have become commercially available recently, the 'old' and frequently employed horse liver ADH (HLADH)[30] preparation displayed the highest activity (Table 1). The ADH-A from *Rhodococcus ruber* showed only low activity for primary alcohols which is in accordance with previous reports where *sec*-alcohols are preferentially transformed.[31]

Table 1
Oxidation of **1** via hydrogen transfer employing ADHs and acetaldehyde

ADH[a]	conv.[b,c]
Horse liver ADH	++++
R. ruber ADH-A	+
PADH 101	++
PADH 102	+
PADH 103	+
KRED 117	++
KRED 118	+
KRED 123	+
KRED 126	+

[a] PADHs and KREDs are commercially available from Codexis (PADH=primary ADH, KRED=keto reductases). *R. ruber* ADH-A is described in literature[29] and is also commercially available from Codexis.
[b] Reaction conditions: crude ADH preparation (7.5 mg), 1 mM NA(P)D$^+$, Pi buffer (600 μL, pH 7.5, 50 mM), **1** (97 mM), acetaldehyde (951 mM), 30 °C, 120 rpm, 23 h.
[c] ++++ indicates >50% conv., +++: 30–50%, ++: 10–30%, +: 5–10%.

2.2.2. Alternative hydrogen acceptors

Having identified HLADH as suitable ADH various alternative hydrogen acceptors were tested. From the three alternative acceptors (acetaldehyde, chloroacetone, acetone) studied (Fig. 1), acetaldehyde clearly worked best leading to 50% conversion and chloroacetone was second.

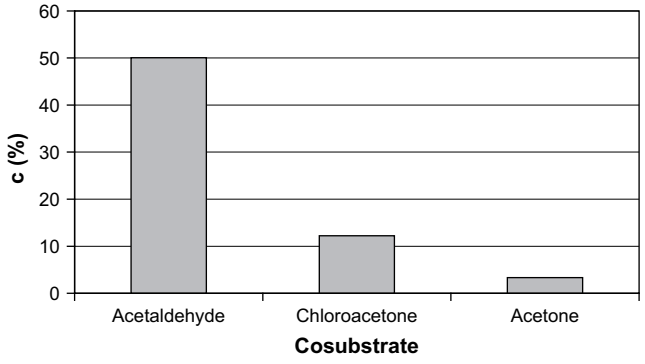


Figure 1. Alternative hydrogen acceptors employed in the hydrogen transfer process for the oxidation of **1**. Reaction conditions: HLADH (7 mg), NAD$^+$ (3 mM), Pi buffer (600 μL, pH 7.5, 100 mM), **1** (97 mM), acetaldehyde (951 mM), chloroacetone (669 mM), or acetone (728 mM), 30 °C, 120 rpm, 23 h.

The low conversion achieved employing acetone (3%) can be attributed to the non-favoured reaction equilibrium. Calculating the relative Gibbs free energies (MP2/cc-pVTZ//MP2/cc-pVDZ) for a model-reaction—acetone plus ethanol leading to 2-propanol and acetaldehyde—showed that the equilibrium is far on the left side, thus the formation of a primary alcohol is highly favoured over the formation of a secondary alcohol (ΔG ~4 kcal/mol). The related model reaction between ethanol and chloroacetone leading to 1-chloro-2-propanol and acetaldehyde has a ΔG ~−1 kcal/mol,[32] thus the equilibrium of the oxidation reaction employing chloroacetone is slightly on the side of the desired aldehyde. Therefore chloroacetone should actually work very nicely as oxidant with respect to thermodynamics, so either sterical hindrance (ketone versus aldehyde) leads to the observed much slower reaction or chloroacetone is too reactive leading to destruction/inhibition of the enzyme.

2.2.3. Concentration of acetaldehyde

Since acetaldehyde was the best hydrogen acceptor the conversion at varied concentrations of acetaldehyde was measured (Fig. 2). Without acetaldehyde no oxidation was found, which clearly indicated that it was required for the transformation.

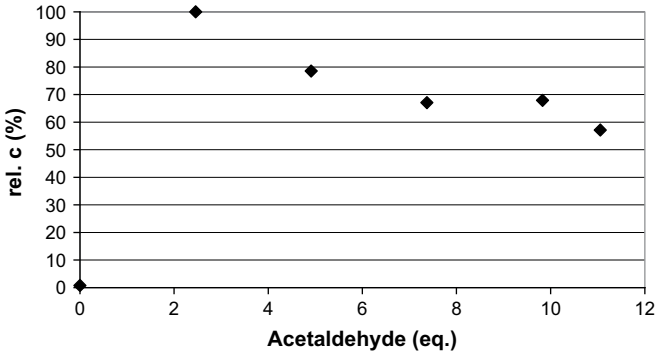
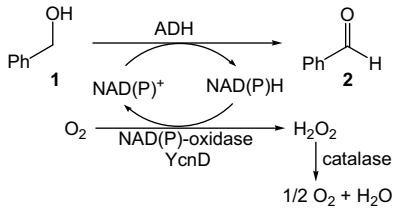


Figure 2. Biocatalytic oxidation of **1** at varied equivalents of hydrogen acceptor. Reaction conditions: HLADH (3 mg), NAD$^+$ (3 mM), Pi buffer (600 μL, pH 7.5, 100 mM), **1** (97 mM), acetaldehyde, 30 °C, 120 rpm, 23 h.

The highest conversion after 23 h was achieved employing 2.5 equiv of acetaldehyde, increasing the amount of acetaldehyde led to lower conversions. Although at higher concentrations of acetaldehyde the equilibrium should be shifted to higher conversions, the transformations slowed down most likely due to inhibition of the HLADH.

2.2.4. Oxidation employing molecular oxygen and an NADPH-oxidase

Molecular oxygen as oxidant would be highly favourable due to its high redox potential which would lead to improved conversion. For this purpose we coupled the HLADH-catalysed oxidation with the recycling of NAD$^+$ by the oxidase YcnD from *Bacillus subtilis*[33] (Scheme 3). Although YcnD shows a preference for NADPH, separate experiments showed that it also transforms NADH at slightly reduced rate.



Scheme 3. Recycling of NAD(P)$^+$ by an NAD(P)-oxidase.

Although the oxidation of primary alcohols to aldehydes and further on to acids has been previously shown to work successfully with NAD(P)-oxidases,[19] the oxidation of **1** to benzaldehyde **2** led only to low conversion (4%) employing HLADH (Table 2). Addition of catalase had no measurable effect for HLADH; however, testing the commercial primary ADH PADH103 the addition of catalase led to a clear improvement of conversion (11%, 6% without catalase). Another improvement was achieved by using larger reaction tubes providing larger gas volume and therefore a higher excess of oxygen. By doubling the gas volume an increase of conversion up to 21% for PADH103 was achieved, although the amount of oxygen was not limiting in any of the experiments.

Table 2
Oxidation of **1** employing ADHs and NADPH-oxidase YcnD

ADH	cofactor	pH	tube[a] (mL)	catalase	c (%)[b]
HLADH	NAD^+	7.5	1.2	—	4
HLADH	NAD^+	7.5	1.2	+	4
PADH101	NADPH	8.5	2	+	3
PADH102	NADPH	8.5	2	+	5
PADH103	NADPH	8.5	1.2	—	6
PADH103	NADPH	8.5	1.2	+	11
PADH103	NADPH	8.5	2	+	21

[a] Size of eppendorf tube.
[b] Reaction conditions: HLADH (3 mg)/PADH (1.8 mg), NAD^+ (3 mM), YcnD (10 μL, 13 μM), Pi buffer (600 μL, pH 7.5, 100 mM), **1** (97 mM), catalase (4.5 mg), 30 °C, 120 rpm, 22 h.

Since PADH103 was not sufficient active or stable (Table 1) and since we experienced severe limitations with the commercial supply of HLADH we started a screening of commercial microbial strains to identify a better biocatalyst.

2.3. Microbial cells

A total of 218 micro-organisms (bacteria, yeasts stored in lyophilised form) were tested for their ability to oxidise benzyl alcohol **1** at the expense of acetaldehyde in a hydrogen transfer like fashion. The library consists of pre-selected strains, which were chosen for their known ability to stand organic chemicals or catalyse chemical transformations of interest. Seventeen strains were active showing a conversion above 5% (Table 3).

Table 3
Oxidation of **1** via hydrogen transfer employing lyophilised microbial strains and acetaldehyde

Microbial strain[a]	c[b]
Gordonia alkanivorans DSM 44369	++
J. terrae DSM 13953	++++
Mycobacterium gilvum DSM 9487	++++
Norcardia corynebacterioides DSM 20151	+++
Norcardia nova DSM 43843	++++
Pseudomonas cichorii DSM 50259	+
Pseudomonas elodea ATCC 31461	++
Pseudomonas sp. DSM 6978	++
Pseudomonas syringae DSM 1241	+++
Ralstonia sp. DSM 6428	++
Ralstonia sp. DSM 9750	+
Arthrobacter sp. DSM 312	++
R. ruber DSM 44190	++
R. ruber DSM 44540	++
R. ruber DSM 43338	+++
R. ruber DSM 44541	+
R. ruber DSM 44539	++

[a] Strains are commercially available from the German culture collection (DSMZ) or the American type culture collection (ATCC).
[b] ++++ indicates >60% conv., +++: 30–60%, ++: 10–30%, +: 5–10%.

The most active strain was *Janibacter terrae* DSM 13953. The chemo-selective oxidation of a primary alcohol to yield exclusively the aldehyde without overoxidation to the corresponding carboxylic acid is a rather difficult task in organic chemistry. Even selected alcohol dehydrogenases have been shown to oxidise aldehydes to carboxylic acids.[34–36] Therefore, we tested the reaction by *J. terrae* specifically for the formation of benzoic acid. We could not detect any benzoic acid, which indicated us that *J. terrae* acts as a highly chemoselective catalyst. Therefore this strain was used further to investigate the optimum of the reaction conditions.

2.3.1. Optimisation of reaction conditions

As a first parameter the influence of the pH on conversion was investigated. The lyophilised cell preparation of *J. terrae* could be employed over a broad pH range with highest conversion from pH 7.5 even up to basic conditions (pH 10.5) (Fig. 3).

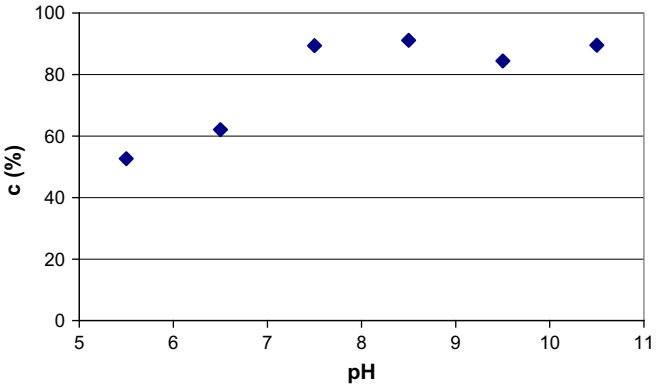


Figure 3. Biocatalytic oxidation of **1** at varied pH employing lyophilised cells of *J. terrae* and acetaldehyde. Reaction conditions: Cells (24 mg), acetaldehyde (951 mM), Pi buffer (600 μL, 100 mM), **1** (97 mM), 30 °C, 120 rpm, 23 h.

Testing the conversion at varied equivalents of acetaldehyde (Fig. 4) showed that the reaction reaches highest conversion quickest already with five or even 2.5 equiv of acetaldehyde. Although higher amounts of acetaldehyde should have led to a higher conversion due to a shift of the equilibrium, the results suggested that more than 5 equiv lead to inhibition or destruction of the catalyst as already observed for HLADH (see Section 2.2.3). Without acetaldehyde no oxidation was found, which clearly indicated that it was required for the transformation.

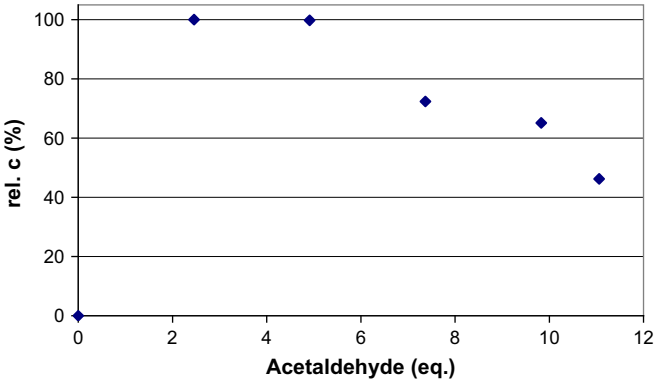


Figure 4. Variation of amount of hydrogen acceptor. Reaction conditions: Cells (24 mg), acetaldehyde, Pi buffer (600 μl, pH 8.5, 100 mM), **1** (97 mM), 30 °C, 120 rpm, 16 h.

From the time course of the reaction it was concluded that the oxidative hydrogen transfer of benzyl alcohol **1** already reached its highest value after 5-h reaction time (Fig. 5).

Therefore, to get a clearer picture for the optimum of the temperature the transformation of **1** was stopped already after 2 h at varied temperature (Fig. 6). The observed conversion increased

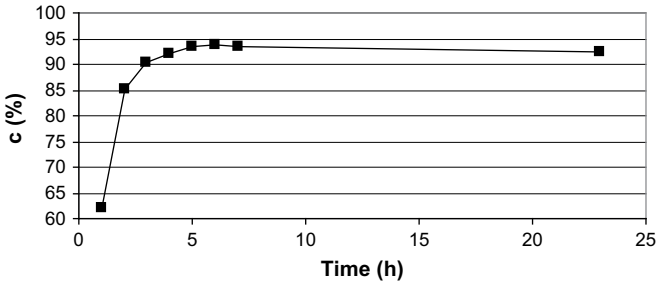


Figure 5. Time course of oxidation of **1** via hydrogen transfer. Reaction conditions: Cells (800 mg), acetaldehyde (951 mM), Pi buffer (24 mL, pH 7.5, 100 mM), **1** (97 mM), 30 °C, 120 rpm.

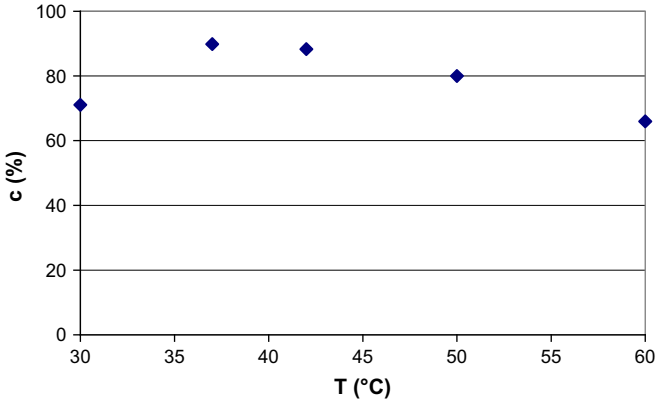


Figure 6. Dependency of conversion on temperature for the oxidation of **1** via hydrogen transfer. Reaction conditions: Cells (20 mg), acetaldehyde (951 mM), Pi buffer (600 μL, pH 7.5, 100 mM), **1** (97 mM), 2 h, 120 rpm.

from 71% at 30 °C to reach its highest value at 37 °C (90% conversion). Even at 60 °C the *J. terrae* preparation showed still reasonable conversion (66% conv.).

Chloroacetone and propanal were tested as alternative hydrogen acceptors. Chloroacetone reacted slower than acetaldehyde just leading to 65% conversion (95% for acetaldehyde). Propanal on the other hand was equally suitable as acetaldehyde leading to the same conversion (95%).

The oxidation of benzyl alcohol was also demonstrated on a 40-fold larger scale (250 mg) leading again to 95% conversion within 6 h.

2.3.2. Other benzyl alcohol derivatives

To test whether other benzyl alcohol derivatives were accepted as well, various *ortho*-, *meta*- and *para*-substituted derivatives were tested (Table 4). From the substrates tested the *meta*-derivatives were transformed fastest leading to highest conversions within the reaction time. The derivatives possessing a substituent in *ortho* position were the poorest substrates probably due to sterical hindrance.

Table 4
Oxidation of derivatives of benzyl alcohol via hydrogen transfer employing lyophilised cells of *J. terrae* DSM 13953 and acetaldehyde

Substituent	c (%)[a]
—	95[b]
p-F	72
p-Cl	8
p-CH_3	16
m-OCH_3	84
m-CH_3	96
m-I	88
o-CH_3	4
o-F	23

[a] Measured by GC–MS. Reaction conditions: Cells (100 mg), acetaldehyde (951 mM), Pi buffer (3 mL, 100 mM), substrate (97 mM), 30 °C, 120 rpm, 24 h.
[b] Product contained 5% of cinnamaldehyde.

In conclusion, we have identified *J. terrae* DSM 13953 as a suitable biocatalyst for the chemoselective oxidation of benzyl alcohol derivatives to the corresponding aldehydes avoiding the formation of the corresponding carboxylic acids via hydrogen transfer employing acetaldehyde as hydrogen acceptor.

3. Experimental part

3.1. General

Acetaldehyde, benzyl alcohol, NAD^+ as well as HLADH were purchased from Fluka (Sigma–Aldrich–Fluka, Vienna, Austria). Substituted benzyl alcohols were purchased from Sigma–Aldrich (Sigma–Aldrich–Fluka, Vienna, Austria). Enzymes named KRED and PADH as well as $NADP^+$ were obtained from Codexis (Redwood city, USA). *J. terrae* DSM 13953 obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DMSZ, Braunschweig, Germany, http://www.dmsz.de). ADH-A was employed as lyophilised *E. coli* powder and prepared as previously described.[30] Trypticase soy broth (Sigma T-8907) and yeast extract (Oxoid L21) were purchased from Thermo Fisher (Vienna, Austria).

3.1.1. Preparation of lyophilised cells of J. terrae DSM 13953

Cultivation medium M92: Trypticase soy yeast extract medium: 30 g/L Trypticase soy broth (Sigma T-8907), 3 g/L yeast extract (Oxoid L21), pH 7.0.

J. terrae was cultivated in M92 medium (330 mL) in baffled shake flasks (1 L) at 120 rpm for three days at 28 °C. The cells were harvested by centrifugation (18,000 g) and washed twice with sodium phosphate buffer (50 mM, pH 7.5) before the cells were suspended in a minimum amount of the above buffer, shock frozen with liquid nitrogen and freeze dried.

3.2. Experimental procedures

3.2.1. Biocatalytic oxidation employing HLADH and acetaldehyde

Alcohol dehydrogenase (3 mg) and NAD^+ (1.8 μmol) were suspended in phosphate buffer (0.6 mL, 100 mM, pH 7.5) in eppendorf tubes. The enzyme preparations were rehydrated by shaking at 30 °C, 120 rpm for 30 min. After addition of benzyl alcohol (6 μL, 6.24 mg, 58 μmol) the reaction mixture was shaken at 30 °C and 120 rpm for 23 h. The reaction was stopped by extraction with ethyl acetate (2 × 0.5 mL) and centrifugation (12,000 rpm, 2 min). The organic phase was dried (Na_2SO_4) prior to determination of conversion by GC.

3.2.2. Biocatalytic oxidation employing HLADH and YcnD

Alcohol dehydrogenase (3 mg), the cofactor (1.8 μmol), NAD(P)H-oxidase [YcnD from *B. subtilis*] (10 μL, 13 μM) were suspended in phosphate buffer (0.6 mL, 100 mM, pH 7.5) in eppendorf tubes with or without catalase (4.5 mg). The enzyme preparations were rehydrated by shaking at 30 °C, 120 rpm for 30 min. After addition of benzyl alcohol (6 μL, 6.24 mg, 58 μmol) the reaction mixture was shaken at 30 °C and 120 rpm for 22 h. The reaction was stopped by extraction with ethyl acetate (2×0.5 mL) and centrifugation (12,000 rpm, 2 min). The organic phase was dried (Na_2SO_4) prior to determination of conversion by GC.

3.2.3. Biocatalytic oxidation employing J. terrae

Typical optimised procedure: Lyophilised cells of *J. terrae* DSM 13953 (20 mg) were rehydrated in phosphate-buffer (0.6 mL, 100 mM, pH 7.5) in eppendorf tubes (2 mL) by shaking at 30 °C, 120 rpm for 30 min. Afterwards, acetaldehyde (8 μL, 6.3 mg,

0.14 mmol) and benzyl alcohol (6 μL, 6.25 mg, 58 μmol) were added. The reaction mixture was shaken at 30 °C and 120 rpm for 5 h. The reaction was stopped by extraction with ethyl acetate (2 × 0.5 mL) and centrifugation (12,000 rpm, 2 min). The organic phase was dried (Na_2SO_4) prior to determination of conversion by GC.

3.3. Analytics

Conversions for the oxidation of benzyl alcohol were determined on a Varian 3800 Gas Chromatograph using a CPWax 52 CB column (30 m×0.25 mm×0.25 μm) and the following programme: 120 °C for 2 min, temperature increase to 210 °C by 20 °C/min and finally 210 °C for 2 min [pressure 14.5 psi N_2].

Retention times: benzyl alcohol 5.0 min, benzaldehyde 3.1 min.

Conversions for the oxidation of benzyl alcohol derivatives were determined using an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass-selective detector (electron impact, 70 eV) and an Agilent HP-5ms [30 m×250 μm×0.25 μm, 5%-phenylmethylpolysiloxane phase column]. Helium (flow 2 mL/min) was used as carrier gas. The following programme was used: 100 °C during 0.5 min, temperature increases to 300 by 10 °C/min. Retention times: see Table 5, cinnamaldehyde 6.7 min.

Table 5
Retention times of benzaldyde and benzylalcohol derivatives on GC–MS

Substituent	Aldehyde (min)	Alcohol (min)
—	3.7	4.2
p-F	3.5	4.3
p-Cl	5.2	6.4
p-CH_3	4.8	5.2
m-OCH_3	6.5	6.8
m-CH_3	4.6	5.2
m-I	7.5	8.8
o-CH_3	4.6	5.3
o-F	3.6	4.3

3.4. Calculation of reaction enthalpies

All calculations were done with the Gaussian 03 suite[37] of programs using second-order Møller-Plesset (MP2)[38] optimizations with the double-zeta correlation consistent basis set, followed by MP2 triple-zeta single point energy calculations (MP2/cc-pVTZ//MP2/cc-pVDZ).[39] Zero-point energy and thermal corrections obtained by the standard rigid rotor-harmonic oscillator approximation are unscaled (Table 6).

Table 6
Relative Gibbs free energies (MP2/cc-pVTZ//MP2/cc-pVDZ) refer to the reaction ketone+H_2→alcohol

Carbonyl compound	Δ*G* (kcal/mol)
Acetone	−3.8
Acetaldehyde	−7.4

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