Pergamon

SCIENCE DIRECT®

Tetrahedron 59 (2003) 9517–9521

TETRAHEDRON

Efficient production of raspberry ketone via 'green' biocatalytic oxidation

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Received 2 September 2003; revised 7 October 2003; accepted 8 October 2003

Abstract—For the development of a 'green' oxidation method, the transformation of 4-(*p*-hydroxyphenyl)butan-2-ol (rhododendrol) into 4-(*p*-hydroxyphenyl)butan-2-one (raspberry ketone) was used as a model reaction. Different lyophilized cells of *Rhodococcus* spp. have been screened for their ability to perform the desired oxidation. *Rhodococcus equi* IFO 3730 and *R. ruber* DSM 44541 were able to use acetone as a hydrogen acceptor in a hydrogen transfer-like process. The oxidation can be performed at substrate concentrations up to 500 g/L.

1. Introduction

The availability of environmentally benign oxidation methods for the oxidation of *sec*-alcohols to the corresponding ketones still represents a significant problem for synthetic organic chemistry. Whereas, chemical protocols are still bounded to the use of toxic hypervalent metals or expensive reagents (e.g. Swern-oxidation), biocatalytic methods are hampered by the instability of alcohol dehydrogenases towards elevated concentrations of organic materials, such as substrate, product and co-solvents. As a consequence, large-scale bio-oxidations employing *sec*-alcohol dehydrogenases are restricted to the use of fermenting cells[1–6] and are thus limited by low substrate concentration.[7–9] Recently, we developed an asymmetric biocatalytic hydrogen transfer protocol employing lyophilized cells of *Rhodococcus ruber* DSM 44541 using 2-propanol as hydrogen donor (for ketone reduction) and acetone as hydrogen acceptor (for alcohol oxidation) as co-substrates.[10–12] Herein we report an extended screening for further strains and demonstrate the applicability of this system on a preparative-scale synthesis of 4-(*p*-hydroxyphenyl)butan-2-one (raspberry ketone, **1**), which represents the principal flavor component of raspberries. The latter compound was chosen as model substrate, since the microbial oxidation of the corresponding alcohol (rhododendrol **2**) obtained from natural sources, such as the bark of various birch spp., represents the key step in the synthesis of natural raspberry ketone **1**. According to regulations within the European Community, flavor substances labeled 'natural' may only be obtained either by physical processes (such as distillation and extraction of natural sources) or by enzymatic/microbial processes transforming precursors isolated from Nature.[13]

The main natural source of raspberry ketone is raspberry (*Rubus idaeus*, up to 3.7 mg/kg of fresh berries) although the compound is also present in other fruits, such as peach, grapes, apples, various berries, vegetables (e.g. rhubarb), and in the bark of trees (e.g. yew, maple, and pine).[14,15] However, the amount of raspberry ketone obtainable from these sources is to low and is too expensive to be of industrial significance. Due to the wide area of applications (e.g. in aroma formulations of kiwi, cherry, strawberry) as well as the high price for the natural flavour compound (EUR 600–2000 kg^{-1}) in comparison to the synthetic analogue (EUR 10 kg^{-1}),[16] the food industry shows a strong interest in economic processes for natural products.[17] The possible natural precursor of raspberry ketone is 4-(*p*-hydroxyphenyl)-2-butanol **2** (rhododendrol, betuligenol), which is most abundant as the corresponding 2-glycosides (glucoside, mannoside) in the bark of birch (*Betula* spp.), rhododendron (*Rhododendron* spp.), maple (*Acer* spp.), fir (*Abieta* spp.), and yews (*Taxus* spp.).[18–20] Deglycosilation is achieved using the β-glycosidase activity of various micro-organisms or plants[15] yielding alcohol **2**, which is subjected to bio-oxidation to obtain natural raspberry ketone **1**.

Besides its application in flavor and fragrance formulations, raspberry ketone **1** is used in an entirely new section of the food and cosmetics industry, called 'cosmaceutical industry', a combination of cosmetics and nutraceuticals. Therein, it is a constituent of skin-lightening cosmetic, and weight loss advancing dietary supplements (Vitarosso®), due to its capability to burn subcutaneous fat.[21–23] Since the preference of the consumer for 'natural' labelled

Keywords: biocatalysis; *Rhodococcus ruber*; oxidation; hydrogen transfer; raspberry ketone.
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0040–4020/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2003.10.019

food/cosmetics additives is constantly increasing, an economic biocatalytic process for raspberry ketone from natural sources is needed.

2. Results and discussion

A screening for strains capable of oxidizing rhododendrol *rac*-**2** was performed using rehydrated lyophilized cells in phosphate buffer, while the substrate was added directly as solid without organic co-solvent to give a final concentration of 20 g/L. All *Rhodococcus* spp. investigated oxidized the alcohol to the corresponding ketone (Table 1) at varying rates (conversion of 16 to 32% after 24 h).

Table 1. Screening of *Rhodococcus* spp. for bio-oxidation activity (without co-substrate)

Entry	Strain	Conversion (%)[a]
1	*Rhodococcus ruber* DSM 44539	16
2	*Rhodococcus ruber* DSM 44540	19
3	*Rhodococcus ruber* DSM 43338	27
4	*Rhodococcus equi* IFO 3730	30
5	*Rhodococcus ruber* DSM 44541	32

[a] Lyophilized cells in phosphate buffer (pH 8.0, 50 mM, 24°C) containing *rac*-**2** (20 g/L), 24 h.

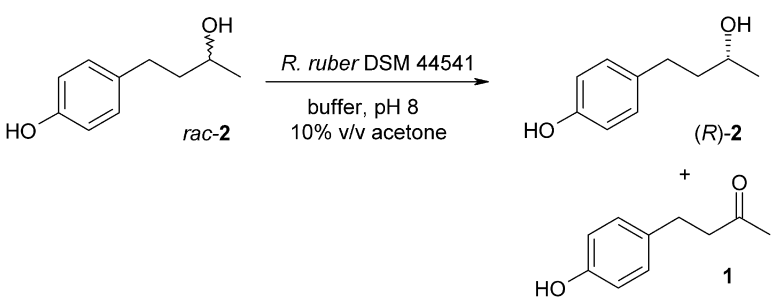
In a next step, the most active strains *Rhodococcus equi* IFO 3730 and *Rhodococcus ruber* DSM 44541 (entries 4 and 5) were tested for the ability of accept acetone as hydrogen acceptor (10% v/v). The tolerance of acetone as co-substrate at elevated concentrations is crucial to render an efficient oxidation processes, since it acts as hydrogen acceptor and increases the solubility of the lipophilic substrate. We were pleased to see that the conversion increased for both strains to 52% within 24 h under these conditions. The (*S*)-enantiomer was oxidized preferentially, while the (*R*)-enantiomer remained behind (Scheme 1). The enantiomeric excess (ee) of the remaining (*R*)-alcohol obtained by *R. ruber* DSM 44541 was 98% at 52% conversion, which corresponds to an enantioselectivity (expressed as the enantiomeric ratio) of $E=91$.

In order to investigate the influence of the cell-status on the catalytic activity, lyophilized cells (obtained by harvesting the cells in the stationary phase, washing, shock freezing followed by lyophilization) were compared with freshly harvested (resting) cells. Resting cells exhibited a 50% higher activity. For both cell preparations no significant by-products were found. In contrast, the use of fermenting cells proved to be inefficient. Thus, addition of rhododendrol (3.3 g/L) to a two days old fermentation broth caused the cells to agglomerate, and no conversion was observed. Likewise, attempts towards enzyme induction by substitution of glucose in the fermentation medium by rhododendrol was unsuccessful, indicating that rhododendrol cannot be utilized as a carbon source and/or acts as growth inhibitor. Due to these limitations, experiments with fermenting cells were discontinued. Since lyophilized cells can be stored for at least one year without significant loss of activity, most of the following experiments were performed using the lyophilized cell preparation.

For the assessment of the method for preparative applications, the maximum substrate concentration is of crucial importance. Thus, measuring the relative apparent initial activity of lyophilized cells at a constant level of acetone (10% v/v) with increasing substrate concentration, it was found that the concentration of *rac*-rhododendrol could be increased up to 500 g/L (3.0 mol/L) (Fig. 1). Interestingly, at 5% v/v acetone, the activity maximum was observed at 180 g/L of *rac*-**2**, while the reaction did not go to completion, which can be explained by the fact that the co-substrate was not in excess. The efficiency of comparable systems in terms of substrate concentration reported for the bio-oxidation of *rac*-rhododendrol using *Candida* and *Acetobacter* sp.[15] or *Pichia farinosa*[24] in aqueous buffer is much lower, namely 0.2 and 1.5 g/L, respectively.

In order to prove the viability of the method, rhododendrol *rac*-**2** was bio-oxidized to raspberry ketone applying an oxidative kinetic resolution on a preparative scale. Thus, *rac*-**2** (2.66 g, 160 mmol) was converted employing resting cells (0.44 g dry cell weight) in phosphate buffer (50 mM, pH 8.0) at 10% v/v initial acetone concentration, while the acetone concentration was stepwise increased to finally reach 20% v/v after 15 h. The reaction was stopped after 44 h to give 1.17 g of isolated ketone **1** after flash chromatography, which corresponds to a 89% yield with respect to the theoretical maximum of 50% of a kinetic resolution. This corresponds to a space-time yield of 10 mmol/L h and a productivity of 2.66 g product per one gram of cells.

In Nature, both enantiomers of rhododendrol **2** occur in various sources. For instance, in the bark of the silver birch *Betula nana* the (*R*)-enantiomer dominates (ee 94%) while for *Betula saposhnikovii* the (*S*)-enantiomer is in excess (ee 93%).[25] (*S*)-Rhododendrol is the aglycon part of rhododendroketoside, which is an ingredient of the stem bark of the



Scheme 1. Oxidative kinetic resolution of rhododendrol (*rac*-**2**).

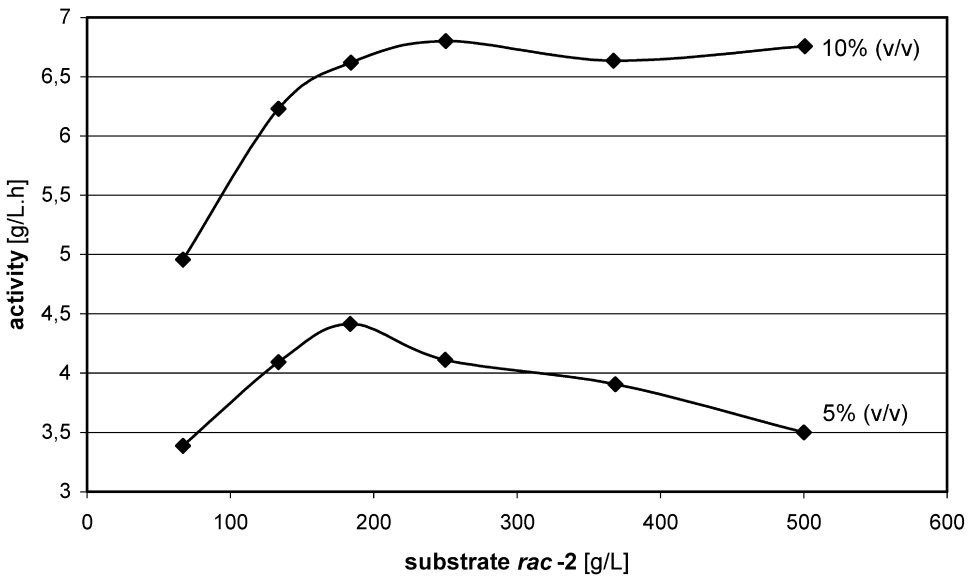


Figure 1. Apparent initial activities for the oxidation of *rac*-rhododendrol *rac*-**2** at different substrate concentrations.

Japanese maple tree *Acer nikoense* which has been used in traditional folk medicine for the treatment of hepatic disorders and eye disease in Japan.[26] Furthermore, (*R*)-rhododendrol is of interest for the pharmaceutical industry, because it possesses anti-inflammatory and hepatoprotective effects.[27,28] As shown in the screening, by oxidative kinetic resolution of *rac*-rhododendrol *rac*-**2**, the (*R*)-enantiomer (*R*)-**2** is accessible. In order to provide access to both enantiomers of **2**, we synthesized (*S*)-rhododendrol using lyophilized cells of *R. ruber* DSM 44541 via biocatalytic reduction of raspberry ketone **1** using 2-propanol (50% v/v) as hydrogen donor.[11] Enantiomerically pure rhododendrol (*S*)-**2** (ee >99%) was obtained in 87% (0.88 g) isolated yield and 92% conversion on a one gram scale within 22 h.

To simulate a possible oxidative production of 'natural' raspberry ketone, starting with enantiomeric pure (*S*)-alcohol (*S*)-**2** (which is present in nature), the biocatalytic oxidation of the obtained (*S*)-alcohol (*S*)-**2** using acetone as hydrogen acceptor (20% v/v) was performed giving ketone **1**, after 17 h, at 95% conversion and 83% yield.

We have reported the biocatalytic oxidative kinetic resolution of racemic rhododendrol **2** furnishing raspberry ketone **1** and (*R*)-**2** in 98% ee. In a complementary fashion, bioreduction of **1** gave access to enantiopure (*S*)-rhododendrol. Due to the exceptionally high substrate concentrations, performed in gram-scale reactions, this methodology is applicable to the large-scale synthesis of 'natural' ingredients for flavor- and fragrance-formulations.

3. Experimental

3.1. Raspberry ketone 1 is commercially available (Aldrich)

3.1.1. Racemic alcohol *rac*-2. Raspberry ketone **1** (700 g, 4.72 mol) was dissolved in methanol (2.5 L). While the reaction was kept at 20°C by ice cooling sodium borohydride (96%, 74 g, 1.888 mol) was added in portions within 3 h. After completion (16 h) ice (50 mL) was added and the mixture left stirring for 1 h. The mixture was carefully acidified using HCl (6 M). Part of the solvent (0.5 L) was evaporated under reduced pressure before the mixture was filtered and the precipitant was washed with diethyl ether. The solvent of the remaining mixture was evaporated under reduced pressure on a rotavapor at 90°C. The remaining pure yellowish oil was diluted with ethyl acetate (1:1) and purified by flash chromatography through silica gel (500 g) and eluted using ethyl acetate. The solvent was evaporated under reduced pressure and left standing for one hour to crystallise (white crystals) (650 g, 92%, mp 71–72°C). Spectroscopic data were consistent with those reported in the literature.[29]

^{1}H NMR (D_6-acetone): δ 6.93 (2H, d, J=8.4 Hz, Ar), 6.73 (2H, d, J=8.4 Hz, Ar), 3.70 (1H, m, C*H*–OH), 2.57 (2H, m, Ph–CH_2), 1.64 (2H, m, Ph–CH$_2$–CH_2), 1.12 (3H, t, J=6 Hz, CH_3).

All micro-organisms were obtained from culture collections and were cultivated as described previously.[30]

3.2. Screening procedure

Lyophilized cells (60 mg) were rehydrated in phosphate buffer (1 mL, 50 mM, pH 8.0) at room temperature on a rotary shaker (130 rpm) in an Eppendorf vial (30 min). *rac*-Rhododendrol *rac*-**2** (20 mg, 0.12 mmol) was added and the reaction mixture was shaken at 24°C for 24 h (130 rpm). The reaction was stopped by extraction with ethyl acetate (0.5 mL), the organic layer was separated from the cell debris using centrifugation (13,000 rpm, 5 min) and dried over Na_2SO_4. Conversion was determined by GC analysis. For the screening experiments with 10% v/v acetone: 0.9 mL buffer was used and the substrate was added dissolved in acetone (0.1 mL).

3.3. Variation of the substrate concentration

Rehydrated lyophilized cells (30 mg) in phosphate buffer (0.30 mL, 50 mM, pH 8.0) were mixed with a solution containing the appropriate amount of *rac*-rhododendrol *rac*-**2** (40 to 300 mg) in buffer (0.24–0.27 mL) and acetone (30–60 μL) to give a total volume of 0.60 mL, the vials were shaken (130 rpm) at 24°C for 3.6 h. The reaction was stopped and analysed as described above.

3.4. Preparative biocatalytic kinetic oxidative resolution of *rac*-rhododendrol *rac*-2

rac-Rhododendrol *rac*-**2** (2.66 g) was mixed with a cell suspension harvested during the stationary phase (corresponding to 440 mg dry cell weight) in phosphate buffer (12.0 mL, 50 mM, pH 8.0) and acetone (1.4 mL) in a 50 mL screw-capped test tube. The mixture was shaken at 30°C at 130 rpm (rotary shaker). After 4 and 15 h, acetone (0.80 and 1.0 mL, respectively) was added. The reaction was stopped after 44 h by extraction with ethyl acetate (3×50 mL). The combined organic layers were dried (Na_2SO_4) and purified by flash chromatography to give 1.17 g of the ketone **1** (44.5%).

3.5. Preparative biocatalytic reduction of raspberry ketone 1 to (*S*)-2

Lyophilized cells of *Rhodococcus ruber* DSM 44541 (1.2 g) were rehydrated in phosphate buffer (15 mL, 50 mM, pH 7.5, 140 rpm, rt) for 30 min in a 100 mL round-bottom flask. Raspberry ketone **1** (1.0 g, 6.1 mmol) dissolved in 2-propanol (15 mL) was added. The entire mixture was shaken at 140 rpm and room temperature for 22 h. The mixture was extracted with ethyl acetate (2×70 mL), the combined organic phases were dried ($MgSO_4$) and concentrated in vacuo. Purification by flash chromatography yielded 0.88 g (87%) of (*S*)-**2** (ee >99%).

3.6. Preparative biocatalytic oxidation of (*S*)-2

Lyophilised cells of *Rhodococcus ruber* DSM 44541 (1.0 g) were rehydrated in phosphate buffer (15 mL, 50 mM, pH 7.5, 140 rpm, rt) for 30 min. Enantiopure (*S*)-rhododendrol (*S*)-**2** (0.88 g, 5.3 mmol) dissolved in acetone (3.1 mL) was added and the mixture was shaken at room temperature (140 rpm). After 17 h a sample (500 μL) was analyzed by GC indicating 95% conversion. Ethyl acetate was added (60 mL) and worked up, as described above, gave 0.72 g (83%) of raspberry ketone **1**.

Conversions were determined using a Varian 3900 GC with a HP Innowax column (30 m×0.25 mm×0.25 μm, 1.0 bar N_2). Temperature program: 125°C/0 min—10°C/min–175°C/0 min–25°C/min–250°C/9.5 min. Ketone **1**: T_{ret}=11.6 min, *rac*-rhododendrol **2**: T_{ret}=12.3 min.

For the determination of the enantiomeric excess of **2**, samples were acetylated[31] and analysed on a Jasco HPLC with a Daicel-OD-H column (0.46×25 cm) at 20°C using an array detector. Flow: 0.6 mL/min, eluent: *n*-heptane/2-propanol=9:1, (*R*)-**2**: T_{ret}=10.4 min, (*S*)-**2**: T_{ret}=11.7 min, **1**: T_{ret}=17.4 min.

The absolute configuration of rhododendrol was confirmed by comparison of the optical rotation with literature data: (*S*)-**2**: $[\alpha]_D^{20}=-11.8$ (*c* 1.0, EtOH 96%, 77% ee), lit.[20] −13.6 (*S*) (*c* 1.0, EtOH).

Acknowledgements

This study was performed within the Spezialforschungsbereich 'Biokatalyse' and the Research Centre Applied Biocatalysis. Financial support by Ciba SC (Basel), Fonds zur Förderung der wissenschaftlichen Forschung (Vienna, project no. F115), TIG, SFG, Province of Styria and the City of Graz is gratefully acknowledged.

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