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Asymmetric whole-cell bioreduction of an α,β-unsaturated aldehyde (citral): competing *prim*-alcohol dehydrogenase and C–C lyase activities

Mélanie Hall,[a] Bernhard Hauer,[b] Rainer Stuermer,[b] Wolfgang Kroutil[a] and Kurt Faber[a,*]

[a]*Department of Chemistry, Organic and Bioorganic Chemistry, University of Graz, Heinrichstrasse 28, A-8010 Graz, Austria*
[b]*BASF AG, GVF/E-B9, D-67056 Ludwigshafen, Germany*

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Abstract—Asymmetric bioreduction of (*E*/*Z*)-3,7-dimethyl-2,6-octadienal (citral) using the enoate reductase activity of whole cells of yeasts, bacteria and fungi, gave the α,β-saturated aldehyde (*R*)-3,7-dimethyl-6-octenal (citronellal), which constitutes an important flavour component, in up to ⩾95% ee. Depending on the microorganism, various amounts of *prim*-alcohols (nerol/geraniol and citronellol) were formed due to the action of competing *prim*-alcohol dehydrogenases. Citral lyase activity—leading to the loss of a C_2-fragment (acetaldehyde) forming sulcatone—and oxidation of the aldehyde moiety yielding the carboxylic acid (geranic/neric acid) were detected as additional metabolic activities.

1. Introduction

Citral is an antimicrobial terpene, which exerts a typical 'lemon-scent', it is therefore widely used in the flavour and fragrance industry, the world consumption in 1996 was 1200 tons.[1] It was originally detected as a major constituent of lemon grass oil and was found to consist of a *cis*/*trans*-mixture of isomers, referred to as geranial and neral, respectively.[2] Furthermore, it has been used as a starting material for the synthesis of vitamins A, E and (methyl)ionone.[3] While citral (being nonchiral) is a relatively inexpensive commodity material, its chiral reduction product citronellal, which serves as an important ingredient in the perfumery industry and as the starting material for the synthesis of nonracemic menthol,[4,5] is highly valuable with a value increase of approx 1:20. Due to the presence of multiple C=C and C=O double bonds, the asymmetric reduction of citral in a chemo-, regio- and stereoselective fashion is a challenging task. Since the interconversion of the stereoisomers of citral—neral (*Z*) and geranial (*E*)—has been shown to be catalysed by amino acids and proteins, there is no use in applying pure stereoisomers as the substrates for biotransformations.[6] Nonbiological (chemo-catalytic) strategies for the reduction are still limited by insufficient chemo- and stereoselectivity.[7–11] The biocatalytic counterpart for the selective transformation of citral into citronellal would involve the use of enoate reductases [EC 1.3.1.31], members of the 'old yellow enzyme' family.[12,13] These flavin-dependent oxidoreductases catalyse the asymmetric reduction of electronically activated C=C bonds, possessing (at least one) electron-withdrawing substituent, such as a carbonyl- or nitro-group. The catalytic mechanism of enoate reductases has been studied in great detail[14] and it has been shown that a hydride (derived from the flavin-co-factor) is transferred stereoselectively onto Cβ, while a Tyr residue adds a proton, which is ultimately derived from the solvent, onto Cα from the opposite side (Scheme 1). Thus, the reduction is strictly *trans*-specific and resembles an asymmetric Michael-type addition of a chiral hydride onto an enone; a rare case of a reduction occurring in a *syn*-mode has been observed with plant cell cultures from *Nicotiana tabacum* (tobacco).[15] As a consequence of this mechanism, nonactivated C=C bonds, such as the terminal bond in citral, are therefore completely unreactive. The catalytic cycle of enoate reductases is completed by the reduction of the oxidised flavin at the expense of NAD(P)H, which is ultimately derived from an external H-donor via another redox-reaction.

* Corresponding author. Tel.: +43 316 380 5332; fax: +43 316 380 9840; e-mail: Kurt.Faber@Uni-Graz.at

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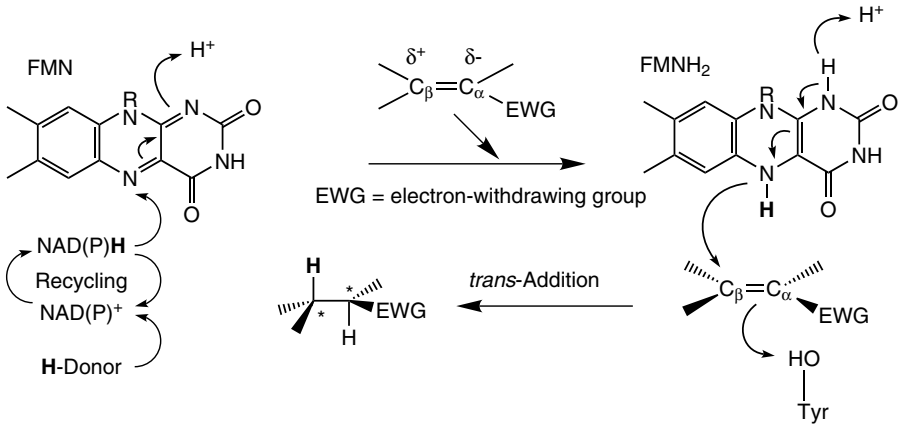


Scheme 1. Catalytic mechanism of enoate reductases.

Due to the complexity of co-factor recycling, the vast majority of biotransformations using enoate reductases has been performed using whole (fermenting) cells, the most prominent being baker's yeast[16–19] and (strict or facultative) anaerobic bacteria.[20–22] Although the stereoselectivities achieved were often excellent, the chemoselectivity of whole-cell bioreductions with respect to C=C- versus C=O-reduction are often poor, which is due to the presence of competing alcohol dehydrogenases. Since enoate reductases and alcohol dehydrogenases depend on the same nicotinamide co-factor, 'redox-decoupling' of both enzyme activities is hardly possible. Nicotinamide-independent enoate reductases have rarely been reported.[23] In search of a stable and durable enoate reductase for the preparative-scale bioreduction of enones and enals we initiated screening among yeasts, bacteria and fungi using citral as a substrate.[24]

2. Results and discussion

Our screening encompassed a selection of 28 strains of yeasts, bacteria and fungi, known to possess a broad secondary metabolism. The use of lyophilised cells allows simple long-term storage of biocatalysts and provides an enhanced penetration of substrate and products through partially permeabilised membranes. Half of the candidates showed reasonable to high activity for the biotransformation of citral.[†] Depending on the major type of activity, four different groups were identified among the positives (Fig. 1).

2.1. Enoate reductase group 1

Depending on the strain, the desired enoate reductase activity (ER) was more or less concealed by *prim*-alcohol dehydrogenase activities (ADH) by a relative factor of ER/ADH ~1:3, leading to the formation of citronellal and citronellol through the sequential bioreduction of the C=C- and the C=O-bond by enoate reductases and *prim*-alcohol dehydrogenases via pathways A→B or B→A, respectively (Scheme 2). The strongest enoate reductase activity was detected in *Candida parapsilosis* DSM 70125 at about an equal level (ER/ADH ratio ~1:1), which furnished (*R*)-citronellal and (*R*)-citronellol in >95% and 92% ee, respectively. The stereochemical identity of the latter products derived from enone reduction (step A) followed by aldehyde reduction (step B) and vice versa indicates that the stereoselectivity is entirely derived from the C=C-reduction step A and that the *prim*-alcohol dehydrogenase catalysed reduction of the aldehyde moiety has no measurable impact. Further strains, which showed reasonably strong ER activity, were *Pichia angusta* DSM 70277, *Rhodococcus ruber* DSM 44540, *Rhodococcus* sp. R 312 CBS 717.73 and *Botrytis cinerea* DSM 877.

2.2. *prim*-Alcohol dehydrogenase group 2

Predominant *prim*-alcohol dehydrogenase activity (ADH) was found in several strains, which led to the formation of nerol/geraniol and citronellol as the major product via the reduction of the aldehyde moiety. This *prim*-ADH-activity was especially strong in *Candida boidinii* FCC 070, *Yarrowia lipolytica* DSM 1345, *Mycobacterium gilvum* DSM 9487 and *Sphingomonas* sp. DSM 11094.

2.3. C–C Lyase group 3

A remarkably strong C–C lyase activity was detected in the red yeast *Rhodotorula mucilaginosa* DSM 70404 and the bacterium *R. ruber* DSM 44541, which led to the formation of 6-methyl-5-hepten-2-one (sulcatone) going in hand with the loss of a C_2-fragment. This type of microbial biodegradation has been attributed to oxidative pathways in *B. cinerea*,[25] *Penicillium italicum*[26] and *Pseudomonas incognita*.[27] However, due to the absence of the presumed (oxidised) intermediates, such as epoxides and/or *vic*-diols, the involvement of a C–C lyase, that is, 'citral lyase',[28] which catalyses the addition of H_2O across the

[†] *Bacillus subtilis* DSM 10, *Curvularia lunata* FCC 080, *Geotrichum candidum* DSM 6401, *Lactobacillus paracasei* DSM 20008, *Mortierella alpina* ATCC 8979, *Mucor plumbeus* CBS 110.16, *Mycobacterium paraffinicum* NCIMB 10420, *Penicillium simplicissimum* FCC 072, *Phanerochaete chrysorporium* ATCC 24725, *Pseudomonas putida* DSM 291, *Rhizopus arrhizus* DSM 906, *Syncephalastrum racemosum* ATCC 18192, *Trametes hirsuta G* FCC 047 showed very poor activities.

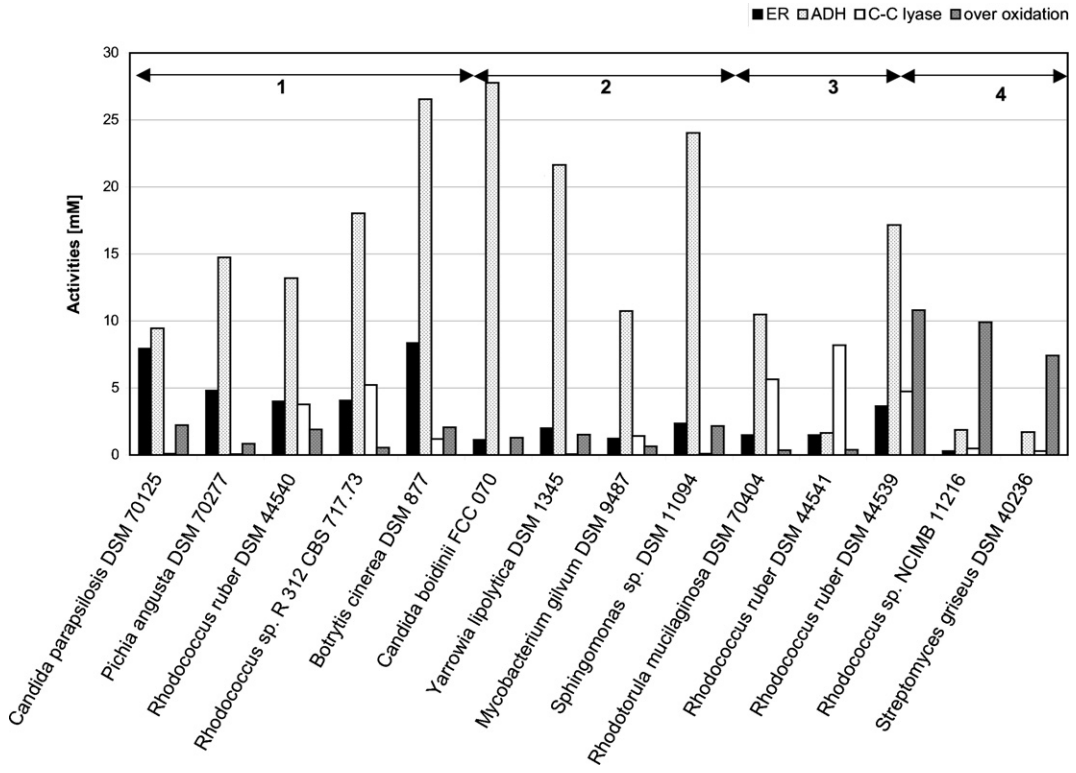
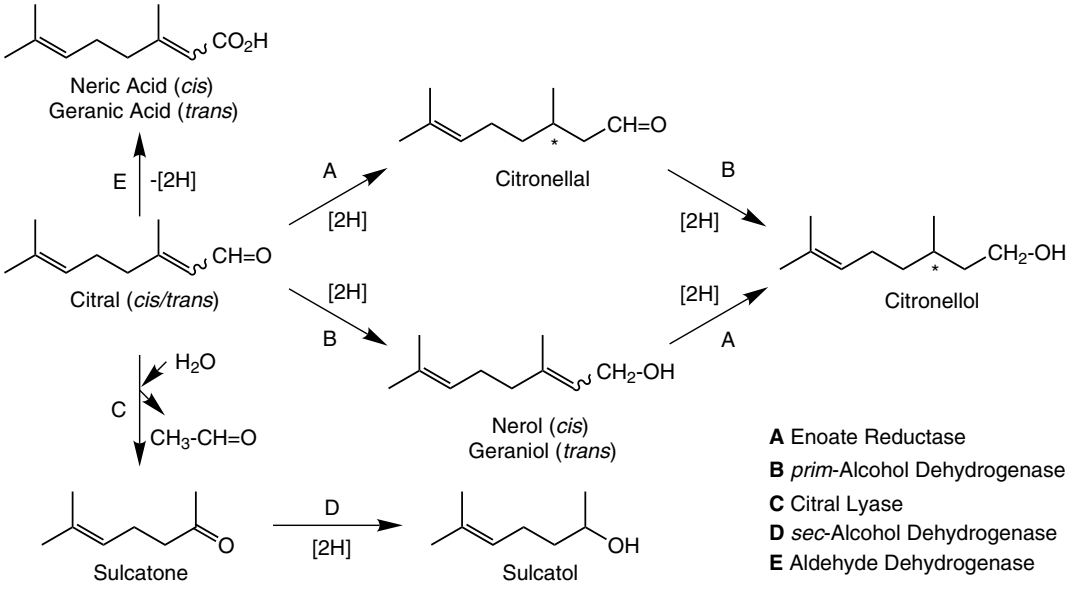


Figure 1. Biotransformation of citral using lyophilised microbial cells. Activities are denoted as product concentrations (mM) under standard conditions; enoate reductase activity (ER) is expressed as the sum of citronellal and citronellol; *prim*-alcohol dehydrogenase activity (ADH) as the sum of citronellol, geraniol and nerol; C–C lyase activity is calculated as the sum of sulcatone and sulcatol, over-oxidation represents the aldehyde dehydrogenase activity responsible for the formation of geranic/neric acid.



Scheme 2. Microbial biotransformation of citral.

C=C bond, followed by retro-aldol reaction[29] appears more plausible. In a follow-up reaction, the sulcatone thus formed was reduced stereoselectively to (*S*)-6-methyl-5-hepten-2-ol (sulcatol) by *sec*-alcohol dehydrogenase(s). This latter activity was predominantly expressed in bacteria and appeared to be (*S*)-selective independent of the strain (Table 1). The best selectivities were obtained with *R. mucilaginosa* DSM 70404 and *R. ruber* DSM 44539 (ee up to 95%).

Table 1. Reduction of sulcatone to sulcatol

Strain	(*S*)-Sulcatol ee (%)
Rhodotorula mucilaginosa DSM 70404	95
Mycobacterium gilvum DSM 9487	50
Rhodococcus ruber DSM 44540	80
Rhodococcus ruber DSM 44541	60
Rhodococcus ruber DSM 44539	94
Rhodococcus sp. R 312 CBS 717.73	67

2.4. Aldehyde dehydrogenase group 4

The oxidation of citral by an aldehyde dehydrogenase yielded carboxylic acid (geranic/neric acid) as the major product with *Rhodococcus* sp. NCIMB 11216 and *Streptomyces griseus* DSM 40236. Mixed activities at comparably high levels were expressed in *R. ruber* DSM 44539.

3. Conclusion

The results from our screening showed that enoate reductase activity for the chemo- and stereoselective asymmetric bioreduction of the α,β-unsaturated aldehyde citral is widely distributed among fungi, bacteria and yeasts, most notably in *C. parapsilosis* DSM 70125, *P. angusta* DSM 70277, *R. ruber* DSM 44540, *Rhodococcus* sp. R312 (CBS 717.73) and *B. cinerea* DSM 877. Although the stereoselectivities achieved with whole cells of *C. parapsilosis* DSM 70125 were excellent (up to 95% ee), chemo-selectivites with respect to reduction of the C=C- versus C=O-bond were partially concealed by (i) competing *prim*-alcohol dehydrogenases, which lead to the undesired reduction of the carbonyl group, and (ii) C–C lyases, which led to the loss of a C_2-fragment (presumably acetaldehyde via a retro-aldol reaction). In order to overcome these limitations, clones possessing the required enoate reductase together with a suitable metabolic set of enzymes for the recycling of the nicotinamid co-factor, but lacking the interfering *prim*-alcohol dehydrogenases and C–C lyases, respectively, are now being constructed in our laboratory for the efficient bioreduction of α,β-unsaturated carbonyl compounds on a large scale.

4. Materials and methods

4.1. General

(*R*)-Sulcatol was purchased from Alfa Aesarl; *rac*-sulcatol was from Acros; geraniol, nerol, (*R*)- and (*S*)-citronellal, (*R*)- and (*S*)-citronellol were from Aldrich; neral was obtained from BASF and sulcatone, citral and geranic acid were from Fluka. GC–MS analyses were performed on a HP 6890 Series GC system equipped with a 5973 Mass Selective Detector and a 7683 Series Injector using a (5%-phenyl)-methylpolysiloxane capillary column (HP-5Msi, 30 m, 0.25 mm ID, 0.25 μm film). GC–FID analyses were carried out on a Varian 3800 using H_2 as the carrier gas (14.5 psi).

4.2. Preparation of lyophilised cells

All strains were obtained from the American Type Culture Collection (ATCC), the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSM), the National Collection of Industrial, Marine and Food Bacteria (NCIMB), the Centraalbureau voor Schimmelcultures (CBS); FCC stands for our in-house Fab-Crew-Collection. Lyophilised cells were obtained by cultivating the organisms in 250 mL of a complex medium in 1 L baffled shake flasks at 120 rpm. After 3 days, the cells were harvested by centrifugation (8000 rpm, 20 min), washed, shock-frozen in liquid nitrogen and lyophilised. The cells were stored at +4 °C and used as such for biotransformations.

Media were prepared as recommended by the culture collection, except for *R. mucilaginosa* DSM 70404, *M. gilvum* DSM 9487, *R. ruber* DSM 44540, *R. ruber* DSM 44541, *R. ruber* DSM 44539, *Rhodococcus* sp. NCIMB 11216, *Rhodococcus* sp. R 312 CBS 717.73 and *Sphingomonas* sp. DSM 11094, which were grown in the following medium: yeast extract (10 g/L, Oxoid L21), bacteriological peptone (10 g/L, Oxoid L37), glucose (10 g/L, Fluka 49150), NaCl (2 g/L, Roth 9265.1), $MgSO_4{\cdot}7H_2O$ (0.15 g/L, Fluka 63140), NaH_2PO_4 (1.3 g/L, Fluka 71496), K_2HPO_4 (4.4 g/L, Merck 5101). *Botrytis cinerea* DSM 877 was grown in a medium made from glucose (30 g/L), L-asparagin-monohydrate (4.5 g/L, Fluka 11160), KH_2PO_4 (1.5 g/L, Fluka 60220), $MgSO_4{\cdot}7H_2O$ (1.03 g/L, Fluka 63140), trace element solution SL 4 (1 mL/L), yeast extract (3 g/L, Oxoid L21), the pH was adjusted to 6.0 with NaOH. Trace element solution (SL 4): $FeCl_3{\cdot}6H_2O$ (80 mg/L, Fluka 44944), $ZnSO_4{\cdot}7H_2O$ (90 mg/L, Aldrich 22,137-6), $MnSO_4{\cdot}H_2O$ (30 mg/L), $CuSO_4{\cdot}5H_2O$ (5 mg/L, Aldrich 46,913–0), EDTA (0.4 g/L).

4.3. General procedure for the screening

Lyophilised cells (40 mg) were rehydrated in a Tris buffer (0.8 mL, 10 mM, pH 7.5) containing 1% glucose (w/v) for 1 h at 30 °C on an orbital shaker at 140 rpm. Citral (2.2 mg, 14.5 μM, 18 mM) was added and the mixtures were shaken at 30 °C and 140 rpm. After 48 h, the samples were extracted three times with EtOAc (0.4 mL each) containing 0.1% (v/v) 1-octanol as the internal GC-standard. The combined organic phases were dried over Na_2SO_4 and the resulting samples analysed on achiral GC–FID. The products were identified by comparison with commercially available reference samples by co-injection on GC–MS and achiral GC–FID.

4.4. Analytical procedures

4.4.1. Determination of conversion. Products were analysed by GC–FID using a polyethylene glycol phase capillary column (Varian CP-Wax 52 CB, 30 m, 0.25 mm, 0.25 μm film) with a split ratio of 20:1. Programme: 100 °C, hold for 2 min, 15 °C/min to 240 °C, hold for 10 min. Retention times were as follows: Sulcatone 4.02 min, sulcatol 4.95 min, citronellal 5.21 min, 1-octanol 5.83 min, neral 7.10 min, geranial 7.53 min, citronellol

7.68 min, nerol 8.01 min, geraniol 8.38 min, neric and geranic acid 11.66 and 11.99 min, respectively.

4.4.2. Determination of enantiomeric excess and absolute configuration. The enantiomeric excess of citronellal and citronellol was determined using a modified β-cyclodextrin capillary column (Hydrodex-β-TBDAc, 25 m, 0.25 mm). Temperature programme for citronellal: 40 °C hold 2 min, 4 °C/min to 120 °C, hold 1 min, 20 °C/min to 180 °C, hold 3 min. Retention times: (*S*)- and (*R*)-citronellal 19.84 and 19.97 min, respectively. Temperature programme for citronellol: 90 °C hold 20 min, 5 °C/min to 160 °C, hold 1 min. Retention times: (*S*)- and (*R*)-citronellol 26.17 and 26.33 min, respectively. Sulcatol was analysed after derivatisation using acetic anhydride (15 μL) and cat. DMAP with shaking at room temperature for 5 h. The reaction was quenched by the addition of H_2O (300 μL), EtOAc (200 μL) was then added and the organic layer was dried over Na_2SO_4. Analyses were carried out using a β-cyclodextrin capillary column (CP-Chirasil-DEX CB, 25 m, 0.32 mm, 0.25 μm film). Temperature programme: 80 °C, hold 10 min, 3 °C/min 180 °C, hold 2 min. Retention times were as follows: (*S*)- and (*R*)-sulcatol (as acetate) 5.18 and 7.09 min, respectively. The absolute configuration of citronellal, citronellol and sulcatol was determined by co-injection with reference materials of a known absolute configuration.

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