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A biocatalytic route towards rose oxide using chloroperoxidase

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ABSTRACT

The chiral monoterpene alcohol citronellol was converted to the corresponding bromohydrin by the haem-thiolate enzyme chloroperoxidase (CPO) from *Caldariomyces fumago* in the presence of hydrogen peroxide and bromide ions. A conversion rate of 51% could be achieved under adapted reaction conditions, which easily yield product in the gramme per litre range while only needing catalytic amounts of enzyme. The bromohydroxylation was shown to be highly regioselective yielding 6-bromo-3,7-dimethyloctane-1,7-diol as the sole product. Product identity was confirmed by GC–MS, ^{1}H- and ^{13}C-NMR spectroscopy and the synthesis of reference compounds. However, the reaction was shown to be non-stereospecific because enantiopure (*R*)- and (*S*)-citronellol, respectively, gave 1:1-diasteromeric mixtures of the corresponding bromohydrins. A racemic mixture of (*R/S*)-citronellol was bromohydroxylated without any detectable enantiodiscrimination. The total lack of stereospecificity and enantiodiscrimination points to a reaction mechanism where the oxidised bromide intermediate is not a ligand to the Fe(III)-haem at the distal site but is released from the enzyme active site. The final bromide transfer occurs probably outside the active site via a diffusible oxidised bromide species and the demonstrated regioselectivity is purely chemically controlled. The generated bromohydrins can be straightforward converted via two reactions steps into rose oxide which is a highly valuable flavour and fragrance substance.

1. Introduction

Chloroperoxidase (CPO) is a 42-kDa haem-thiolate enzyme that is secreted by the fungus *Caldariomyces fumago*. It catalyses the hydrogen peroxide-dependent chlorination of the intermediate cyclopentanedione during the biosynthesis of the antibiotic caldariomycin (Kühnel, Blankenfeldt, Terner, & Schlichting, 2006). Additionally, CPO catalyses the halogenations and oxidations of a wide range of substances because it shows only low substrate specificity (Blasiak & Drennan, 2009; Butler & Sandy, 2009). This feature makes CPO an attractive catalyst for bio-oxidation reactions of a wide range of applications using low cost oxidising agents like hydrogen peroxide (Dembitsky, 2003). Surprisingly, despite the detailed investigation of the catalytic potential of CPO, only until recently monoterpenoids have been shown to be substrates for this enzyme (Aguila et al., 2008; Kaup, Piantini, Wüst, & Schrader, 2007). However, the chemistry of the catalysed reaction is highly dependent on the chemical structure of the substrate and the reaction conditions. In the presence of halide ions the bicyclic monoterpene carene yielded exclusively the corresponding halohydrins with high stereoselectivity (Kaup et al., 2007) while monocyclic limonene yielded exclusively the 1,2-diols with high regioselectivity but very low stereoselectivity (Aguila et al., 2008). This work describes the regioselective CPO catalysed bromohydroxylation of the chiral monoterpene alcohol citronellol **1** to the corresponding bromohydrins **2** (Fig. 1). This oxyfunctionalisation of citronellol is of large interest for the flavour and fragrance industry because the reaction is the key step of numerous synthetic routes to rose oxide **6** which is a valuable flavour and fragrance compound and is currently produced on >100 tons per year industrial scale by photooxidation of citronellol (Bicas, Dionisio, & Pastore, 2009). Although light can be regarded as a "green" reagent, the high energy demand of artificial light sources must not be disregarded (Bicas et al., 2009). The need of bulk amounts of organic solvents, such as methanol or acetonitrile, and of pigments used as triplet photosensitisers, which may interfere with product purification, are additional drawbacks (Dincalp & Icli, 2001). However, biotechnological attempts to develop a straightforward and environmentally friendly route from citronellol to rose oxide have been so far less successful. One of the first publications in this area by Onken and Berger (1999) reported the biotransformation of citronellol by the basidiomycete *Cystoderma carcharias* in an aerated membrane bioreactor yielding rose oxide in small amounts (up to 10 mg per day) from the exhaust air of the bioreactor. *Aspergillus* sp. and *Penicillium* sp. were screened for their ability to bioconvert

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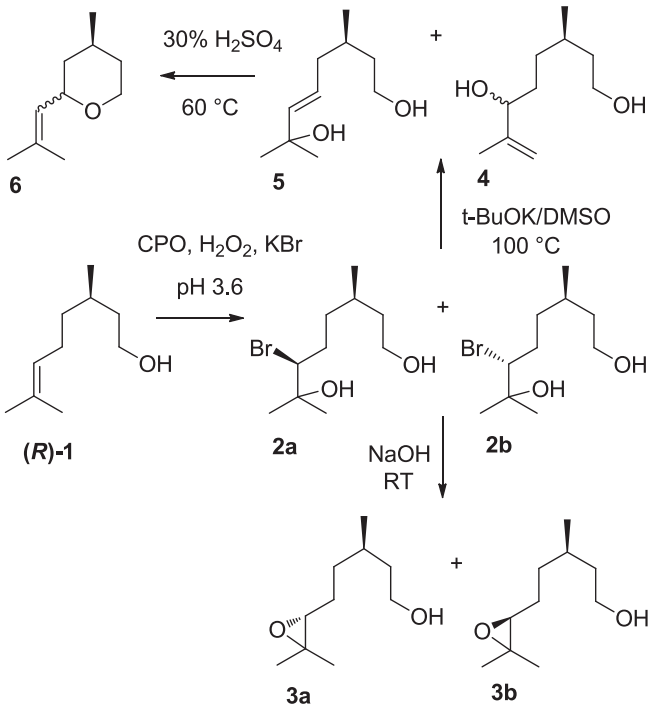

Fig. 1. Chloroperoxidase-catalyzed formation of the diastereomeric bromohydrins **2a**/**2b** from (*R*)-citronellol (***R***)-**1** and conversion of **2a**/**2b** to the corresponding epoxides **3a**/**3b** or to rose oxide **6** via the diols **4** and **5**; DMSO, dimethyl sulfoxide; t-BuOK: potassium tert-butylate.

citronellol to rose oxide (Demyttenaere, Vanoverschelde, & De Kimpe, 2004) but the yields were rather low (1–3% of rose oxide). More recently the group of Pastore was able to produce up to 100 mg/l of rose oxide using sporulated surface cultures of *Penicillium* sp. (Marostica, Macedo, & Pastore, 2005). In this study a novel biocatalytic approach for the synthesis of rose oxide is described by combining the CPO catalysed oxyfunctionalisation of citronellol with a chemical two step synthesis. The results are also discussed in the context of the reaction mechanism of the CPO-catalysed oxyhalogenation which remained up to now controversial, with two different hypotheses (Libby, Beachy, & Phipps, 1996; Reddy et al., 2002).

2. Materials and methods

2.1. Materials

(*R*/*S*)-Citronellol and enantiomers were purchased from Aldrich (Germany). Chloroperoxidase was purchased from Fluka (Germany). All other chemicals were analytical reagent grade. Silica gel 60 (0.040–0.063 mm) for flash chromatography was purchased from Merck (Germany). TLC plates Polygram Sil G/UV_{254}, 0.2 mm, 8 × 4 cm, were purchased from Macherey Nagel (Germany).

2.2. NMR

^{1}H- and ^{13}C-NMR spectra were recorded with a XP-400 spectrometer (Bruker, Rheinstetten, Germany) at 400 (^{1}H) and 100 MHz (^{13}C) at ambient temperature. All chemical shifts are given with respect to the solvent ($CDCl_3$ = 7.26 ppm for ^{1}H, and 77.0 ppm for ^{13}C). Hydrogen signals were assigned by ^{1}H/^{1}H-COSY techniques using standard pulse programs as provided by Bruker (Rheinstetten, Germany).

2.3. GC-FID

The bromohydrins were initially analysed with an Agilent (Böblingen, Germany) 6890 N gas chromatograph by split injection at 250 °C of 1 μl with a split ratio of 50:1. A DB-WAX capillary column (30 m, 0.25 mm i.d., 0.25 μm) from J&W Scientific (Waldbronn, Germany) was used with He at 0.8 ml/min (22 cm/s) as carrier gas. The oven was programmed from 50 to 240 °C at a rate of 5 °C/min. The detector was a FID at 300 °C; hydrogen flow 40 ml/min; air flow 450 ml/min.

2.4. GC–MS

Reaction products were analysed with a Varian (Darmstadt, Germany) Chrompack CP 3800 gas chromatograph by split injection at 240 °C of 1 μl with a split ratio of 50:1. A DB-225MS capillary column (30 m, 0.25 mm i.d., 0.25 μm) from J&W Scientific (Waldbronn, Germany) was used with He at 0.8 ml/min (22 cm/s) as carrier gas. The oven was programmed from 50 to 240 °C at a rate of 5 °C/min. The detector was a Varian Saturn 2000 ion trap. Transfer line temperature 220 °C; ion trap temperature 150 °C; scan range 40–650 amu; ionisation mode: EI with automatic gain control (AGC); emission current 10 μA; scan time 1.0 s; filament and multiplier delay 5 min.

2.5. Enzymatic conversions

For monoterpene conversions a previously established protocol was used (Kaup et al., 2007) and adapted (see Table 1). Briefly, approximately 100 μl of CPO (4 U) was incubated in 100 mM citric acid buffer, pH 3.6 with 30% (v/v) tert-butanol, 12 mM of the monterpene substrate and 10 mM sodium bromide. Hydrogen peroxide was added to a total concentration of 10 mM over a reaction time of 60 min in 50 μl portions every minute. As negative control either CPO or hydrogen peroxide was omitted from the assays described above. Reaction was monitored by TLC (*n*-pentane/ethyl acetate 5/4 v/v) using anisaldehyde/sulphuric acid as spray reagent. Samples were extracted with *n*-hexane, dried over sodium sulphate and stored at −20 °C until GC–MS analysis. Enzymatic conversions were conducted independently at least in triplicate. For NMR analysis the samples were purified by flash chromatography (*n*-pentane/ethyl acetate 5/4 v/v).

Table 1
Conversion rates of CPO catalyzed oxidation of (*R*/*S*)-citronellol (12 mM) using different reaction conditions.

Hydrogen peroxide [mM]	Bromide [mM]	pH	tert-butanol [vol.%]	Temperature [°C]	[a]Conversion rate [%]
10	10	2.1	10	25	0
10	10	2.7	10	25	30
10	10	3.6	10	25	36
10	10	4.6	10	25	26
10	10	3.6	30	25	51
10	10	3.6	40	25	48
10	10	3.6	46	25	19
10	10	3.6	10	45	11
–	10	3.6	10	25	0
10	–	3.6	10	25	0
10	10	3.6	10	25	[b]0

[a] Conversion rates were determined by GC–MS assuming equal response factors for citronellol and its bromohydrine and expressed as% peak area of bromohydrine with respect to the total peak area (bromohydrine + residual citronellol).
[b] No enzyme added.

2.6. Synthesis of reference compounds

All four stereoisomers of the citronellol bromohydrins (6-bromo-3,7-dimethyloctane-1,7-diol **2**) and the citronellol epoxides (5-(3,3-dimethyloxiran-2-yl)-3-methylpentan-1-ol **3**) were synthesised as previously published (Demuth, Xing, & Schaffner, 2001) using racemic citronellol as starting material. Spectral data (^{1}H/^{13}C-NMR) were identical to those previously published (Demuth et al., 2001). MS data (low resolution ion trap; EI 70 eV) for both diastereomers of 6-bromo-3,7-dimethyloctane-1,7-diol **2**: m/e (relative intensity): Diastereomer 1: 178 (31), 176 (31), 137 (16), 121 (16), 97 (60), 81 (21), 70 (35), 59 (100), 55 (46). Diastereomer 2: 178 (29), 176 (27), 137 (25), 121 (19), 97 (100), 81 (37), 70 (49), 59 (64), 55 (65).

2.7. Synthesis of rose oxide

Citronellol bromohydrins were converted into *cis*-/*trans*-rose oxide **6** using a previously published procedure (Demuth et al., 2001).

3. Results

Both enantiomers and a racemic mixture of citronellol **1** have been tested as substrates for the CPO-catalysed bio-oxidation in the presence of hydrogen peroxide and sodium bromide as halide source. In all cases a single product was obtained as indicated by thin layer chromatography on silica gel. The conversion was adapted with respect to pH and volume of added tert-butanol and conversion rates up to 51% could be achieved after 1 h reaction time at pH 3.6 (see Table 1). Isolated yields were in the gramme range after purification by flash chromatography. In a typical reaction 1.87 g citronellol gave 1.84 g of pure product. Analysis by GC–MS revealed the presence of two very closely eluting major products peaks of equal peak height with almost identical mass spectra indicating the presence of isomers (Fig. 2). The products could be identified as a 1:1 diastereomeric mixture of 6-bromo-3,7-dimethyloctane-1,7-diol **2**, the diastereomeric α,β-bromohydrins of citronellol (Fig. 1). The corresponding reference compounds were synthesised as a 1:1 diasteromeric mixture by regioselective formation of the bromohydrin using racemic citronellol **1** and *N*-bromosuccinimide. The reference compounds co-eluted with the CPO-generated products when co-injected in GC. Products and reference compounds showed identical mass spectra and identical ^{1}H- and ^{13}C-NMR spectra. The presence of a 1:1 diastereomeric mixture of the α,β-bromohydrins **2** is unequivocally proven by the splitting of the ^{13}C- and ^{1}H-NMR signals. An illustrative example is the signal of the H-6 proton which is attached to the chiral centre and shows two double doublets indicating the presence of a diastereomeric mixture (Fig. 3).

Two minor peaks were detected as well by GC–MS analysis of the generated CPO-catalysed reaction products (Fig. 2) and identified as citronellol epoxide diastereomers (5-(3,3-dimethyloxiran-2-yl)-3-methylpentan-1-ol **3**). The corresponding reference compounds were synthesised by epoxidation of racemic citronellol by *m*-chloroperbenzoic acid (Demuth et al., 2001). These epoxides were identified as artifacts and are generated in the capillary column during the gas chromatographic analysis by an intramolecular SN2 reaction of the bromohydrin. In support of this finding the epoxides are visible as well when pure, synthetically derived, bromohydrin is injected. When a carbowax stationary phase was used the bromohydrins were even completely converted into the epoxides in the capillary column. Nevertheless, the formation of the diastereomeric epoxides **3** when the CPO reaction product is treated with sodium hydroxide is a second independent proof of structure of the generated enzymatic products (Fig. 1).

The fact that both enantiomers of citronellol **1** gave a 1:1 mixture of the corresponding bromohydrin diastereomers **2** demonstrates that the CPO-catalysed bromohydroxylation is

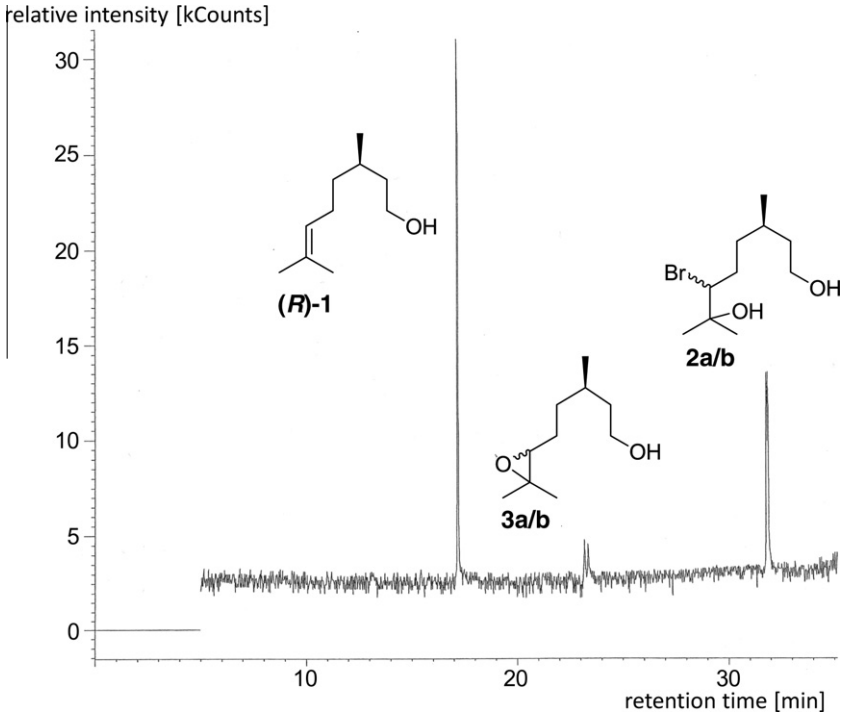

Fig. 2. GC–MS analysis of the reaction mixture after incubation of (*R*)-citronellol (***R*)-1** with chloroperoxidase. Enlargement of the peak which is attributed to the bromohydrin reveals the presence of two closely eluting diastereomers **2a**/**2b** (enlargement not shown). The presence of two diastereomers is visible for the epoxides **3a**/**3b** which are artifacts that are generated in the in the capillary column of the GC system.

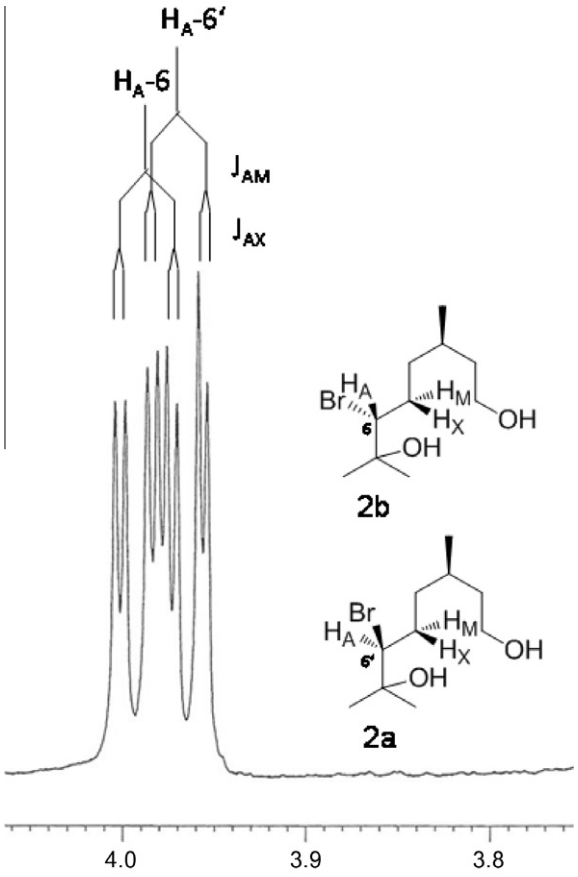

Fig. 3. Section of the ^{1}H-NMR spectrum of the diastereomeric bromohydrins **2a**/**2b** generated from (*R*)-citronellol (***R***)-**1** by chloroperoxidase. The section shows the splitting of the H-6 proton of the respective diastereomers. The signal assignments of the H-6 and H-6′ protons of the different diastereomers were arbitrarily chosen and are interchangeable.

non-stereospecific. The same result was obtained with racemic citronellol demonstrating the complete lack of enantiodiscrimination.

To illustrate the synthetic usefulness of the CPO-catalysed bromohydroxylation of citronellol the bromohydrins were converted into rose oxide **6** via the diols **4** and **5** in two reaction steps with yields of 77 and 60%, respectively (Fig. 1).

4. Discussion

The complete lack of stereospecificity and enantiodiscrimination, both usually features of enzymatic reactions, is in agreement with the results of previous studies on CPO-catalysed bromohydroxylations that were carried out with smaller substrates like *cis*-/*trans*-propenylphosphonic acid, propylene and styrene (Kollonitsch, Marburg, & Perkins, 1970). This phenomenon has ultimately led to the proposition of a reaction mechanism that includes the involvement of a freely diffusible oxidised halide species that is not a ligand to the Fe(III)-haem at the distal site but is released from the enzyme active site (Butler & Sandy, 2009; Manoj, 2006). Hypohalous acid or molecular halogen, which react as X^+ equivalents with electron rich substrates are thought to be candidates for these diffusible species. The reaction is then presumed to proceed via a halonium intermediate, similarly to the chemical formation of halohydrins by using for example *N*-bromosuccinimide. The exact nature of the halogenating species continuous to be a subject of much discussion (Blasiak & Drennan, 2009; Libby et al., 1996; Reddy et al., 2002). Regardless of the exact species responsible for halogenations, our results corroborate reports on non-stereospecific CPO-catalysed bromohydroxylation (Kollonitsch et al., 1970) and show that large substrates probably are unable to access haem intermediates in the active sites and likely react with diffusible oxidised bromide species outside the active site. For efficient CPO-catalysed epoxidation of short-chain alkenes with double bonds close to the chain terminus the critical chain length was C_9 and demonstrates the rather small active site of CPO (vanDeurzen, vanRantwijk, & Sheldon, 1997). The observed regioselectivity in the CPO-catalysed formation of the bromohydrins is probably purely chemically controlled and in agreement with a reaction that takes place via a distorted bromonium ion that reacts with a hydroxyl ion via a *trans* addition at the tertiary C atom.

The generated bromhydrins of citronellol **2** are interesting intermediates for the synthesis of valuable flavour and fragrance substances like rose oxide **6** which can be obtained after treatment of the bromohydrins with potassium tert-butylate followed by acid treatment (Demuth et al., 2001). This reaction sequence yields a high percentage of *cis*-rose oxide which is the most valuable and appreciated diastereomer in the flavour and fragrance industry. Noteworthy, mimicking such monoterpene bio-halohydroxylation in the presence of halide salts by chemical means would need a sixfold stoichiometric concentration of fluoroboric acid to activate the halide ion instead of a nanomolar enzyme preparation only, as shown for the chemical conversion of 3-carene (Barluenga, Marco-Arias, Gonzáles-Bobes, Ballesteros, & Gonzáles, 2004). Moreover, the CPO-catalysed oxyfunctionalisation of citronellol is a green chemistry approach that avoids the high energy demand of the artificial light driven, dye sensitised photooxidation of citronellol which is currently performed industrially on >100 tons per year scale for the synthesis of *cis*- and *trans*-rose oxide (Bicas et al., 2009).

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