Epoxidation of conjugated C=C-bonds and sulfur-oxidation of thioethers mediated by NADH:FMN-dependent oxidoreductases

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Three FMN-dependent oxidoreductases, YcnD and YhdA from *Bacillus subtilis* and Lot6p from *Saccharomyces cerevisiae*, oxidised α,β-unsaturated carbonyl compounds and a thioether, respectively, to furnish the corresponding racemic epoxides or sulfoxide, respectively. The mechanism of this enzyme-mediated (rather than enzyme-catalysed) oxidation was shown to proceed *via* the NADH-dependent reduction of O_2, forming H_2O_2, which acted as oxidant in a spontaneous (non-enzymatic) fashion.

Introduction

During the course of our studies on the asymmetric bioreduction of activated alkenes,[1] we sought to extend the set of readily available enzymes beyond the typical enoate reductases from the old-yellow-enzyme (OYE) family, such as OYEs 1–3 from *Saccharomyces* and *Zymomonas mobilis* NCR-reductase[2] or OPR-isoenzymes from tomato and YqjM from *Bacillus subtilis*.[3] Our candidates of interest were oxidoreductases which utilize NAD(P)H as hydride source to reduce the flavin-cofactor FMN in their active site. The flavoprotein YcnD from *Bacillus subtilis* was assumed to be responsible for the delivery of reduced FMN to enzymes that require a reduced cofactor for activity, such as luciferase.[4] From the same organism, thermostable YhdA was recently shown to reduce nitroaromatics, chromate and the –N=N– double bond of various azo-dyes.[5] The structural homolog Lot6p from *Saccharomyces cerevisiae* catalyses a two-electron reduction of various quinones.[6] These three enzymes react *via* a ping-pong bi-bi mechanism, where the nicotinamide cofactor reduces first the FMN, followed by the subsequent reduction of the substrate. This mechanistic similarity with enoate reductases[7] together with the undefined physiological role of these enzymes led us to investigate their substrate-spectrum in the bioreduction of activated C=C bonds.[8]

Results and discussion

Citral (**1a**) is a non-chiral terpene which occurs as *E*/*Z*-mixture of isomers, denoted as geranial and neral, respectively.[9] While non-chiral **1a** is an inexpensive commodity material, its α,β-reduction product 'citronellal' is highly valuable since it serves as important ingredient of perfumes. The latter can be obtained *via* asymmetric bioreduction using enoate reductases.[10,2c] When we tested YcnD, Lot6p and YhdA for this transformation in the presence of NADH as hydride source, we were surprised to find that the α,β-C=C bond of **1a** was not *reduced*, but *epoxidised* in up to 36% yield (Scheme 1, Table 1).

This unexpected outcome of the reaction was verified with cyclohexenone (**2a**), which gave 2,3-epoxycyclohexanone **2b** in 20–60%. In a similar fashion, ketoisophorone (**3a**) was not reduced to levodione (2,2,6-trimethylcyclohexane-1,4-dione) as expected,[2a,3b,c] but furnished epoxydione **3b** (28–75%). Finally, menadione (**4a**, vitamin K3[11]) gave epoxymenadione **4b** in up to 69% yield. In nature, the latter is formed as oxidation product from vitamin K3 during the post-translational γ-carboxylation of glutamyl residues of vitamin K-dependent proteins, which play a key role in signal transduction, growth control, and haemostasis.[12] Overall, YcnD and YhdA showed superior activities than Lot6p. The short-chain aldehyde **5a** could not be epoxidised due to decomposition of its presumed epoxy-product **5b**. Epoxidation of **5a** using chemical methods failed for the same reason.

The requirement for an electron-withdrawing (activating) group, such as an aldehyde or ketone moiety, for epoxidation seemed to be rather strict, since epoxidation of **1a** occurred only at the α,β-C=C bond, whereas the terminal (non-activated) olefin remained intact. In line with this observation, styrene **8a** and α-methylstyrene **9a** proved to be unreactive (Scheme 2).

Since alkene-epoxidation and thioether-sulfoxidation are often catalysed by the same class of (flavin-dependent) enzyme,[13] we tested YcnD, YhdA and Lot6p for the oxidation of thioanisol **6a**. In particular, YcnD furnished sulfoxide **6b** in 80% yield, without formation of sulfone resulting from over-oxidation. However, thioether **7a**, which serves as synthetic precursor for the anti-ulcer

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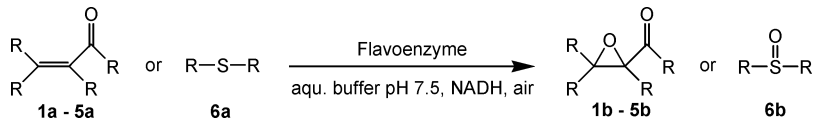
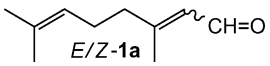
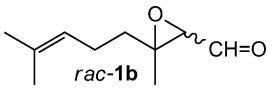
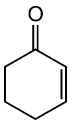
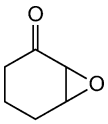
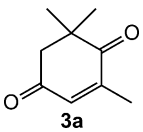
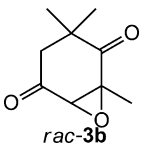
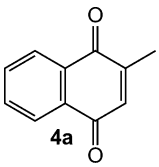
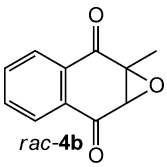
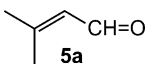
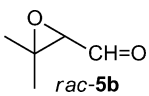
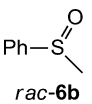
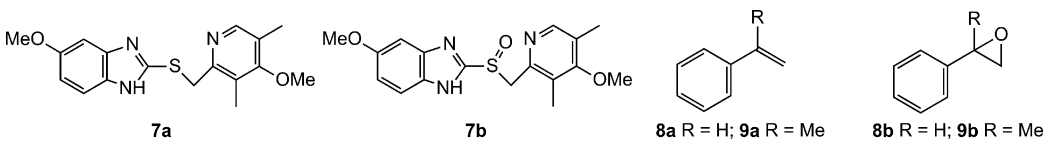


Scheme 1 Enzyme-mediated alkene epoxidation and sulfoxidation of thioether.

Table 1 Enzyme-mediated epoxidation of alkenes and thioether oxidation

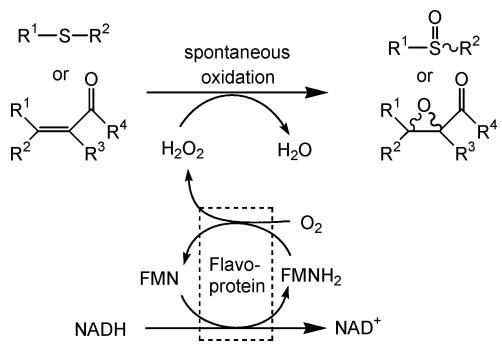
Substrate	Product	Enzyme	Conditions	Conversion [%]
 *E/Z*-**1a**	 *rac*-**1b**	YcnD	NADH	30
		Lot6p	NADH	22
		YhdA	NADH	36
		—	H_2O_2	42
		Lot6p	H_2O_2	43
		YhdA	H_2O_2	41
 **2a**	 *rac*-**2b**	YcnD	NADH	60
		Lot6p	NADH	20
		YhdA	NADH	35
		—	H_2O_2	24
		Lot6p	H_2O_2	26
		YhdA	H_2O_2	25
 **3a**	 *rac*-**3b**	YcnD	NADH	75
		Lot6p	NADH	28
		YhdA	NADH	42
		—	H_2O_2	33
 **4a**	 *rac*-**4b**	YcnD	NADH	22
		Lot6p	NADH	24
		YhdA	NADH	69
		—	H_2O_2	77
 **5a**	 *rac*-**5b**	YcnD	NADH	decomposition
		Lot6p	NADH	
		YhdA	NADH	
 **6a**	 *rac*-**6b**	YcnD	NADH	80
		Lot6p	NADH	25
		YhdA	NADH	25
		—	H_2O_2	36



Scheme 2 Non-substrates for enzyme-mediated epoxidation and sulfoxidation.

agent omeprazol (**7b**) was unreactive (<1%), which is presumably due to the electron-withdrawing effect of the imidazole moiety.

The fact that all of the oxidation products were formed in racemic (or near racemic) form[14] led us to the assumption, that the oxidation reaction was proceeding without direct enzyme catalysis, similar to enzymatic halogenation catalysed by heme- or vanadium-depending haloperoxidases.[15] The most plausible oxidant would be H_2O_2, which could arise through enzyme-catalysed reduction of molecular oxygen at the expense of NADH, an activity which is common for flavin-dependent NAD(P)H-oxidases[16] (Scheme 3). In order to verify this hypothesis, oxidation of **1a** and **2a** was performed using YcnD in presence of (H_2O_2-degrading) catalase, and secondly, by exclusion of O_2 under an atmosphere of Ar. No epoxides were detected in either case. In a complementary fashion, spontaneous epoxidation occurred at comparable rates in presence of H_2O_2 in the absence of enzymes. Finally, blank-experiments in the absence of NADH (with external H_2O_2 added) showed no significant rate acceleration in the epoxidation of substrates **1a** and **2a** using Lot6p and YhdA. These data exclude the possibility that both flavoproteins could catalyse



Scheme 3 Mechanism of enzyme-mediated epoxidation of alkenes and sulfoxidation of thioethers.

the direct alkene-epoxidation using H_2O_2 as oxidant. These data prove that this process is 'enzyme-mediated' rather than 'enzyme-catalysed'.

In this context it should be mentioned that the enzymes used in this study (YcnD, YhdA and Lot6p) play an essential role in oxidative stress response by two-electron-reduction of quinones to furnish the corresponding hydroquinones.[4,5a,6] In other words, in vivo the electrons are passed onto a quinone substrate (rather than oxygen) and hence alkene epoxidation and sulfoxidation observed here are adventitious and most probably do not occur in a cellular environment.

Overall, this epoxidation- and sulfoxidation-reaction bears some resemblance to the lipase-catalysed formation of peroxycarboxylic acids (from H_2O_2 and fatty acids), which in turn act as oxidants in the epoxidation of alkenes, sulfoxidation of thioethers and the Baeyer–Villiger reaction in a non-enzymatic and thus non-stereoselective fashion.[17]

Experimental section

General

Citral (**1a**), menadione (**4a**), prenal (**5a**), omeprazole sulfide (**7a**) and omeprazole (**7b**) were provided by BASF (Ludwigshafen), cyclohexenone (**2a**) was from Fluka, ketoisophorone (**3a**) was purchased from ABCR, thioanisole (**6a**) and styrene (**8a**) were from Aldrich, α-methylstyrene (**9a**) was purchased from Lactan, NADH and NADPH were purchased from Biocatalytics/Codexis, sodium metaperiodate was from Lancaster and catalase from bovine liver was from Sigma.

Bacillus subtilis YcnD and YhdA and *Saccharomyces cerevisiae* Lot6p were expressed and purified as recently reported.[4–6]

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 carrier gas (14.5 psi). HPLC analyses were performed using a Shimadzu system equipped with a Chiralcel AD column (25 cm, 0.46 cm). NMR spectra were measured on a Bruker AMX spectrometer at 360 MHz.

Synthesis of reference material

***diastereomeric-rac*-2,3-Epoxy-3,7-dimethyl-6-octenal (1b).** To a stirred mixture of citral **1a** (380 mg, 2.5 mmol), *sec*-butylamine (8 μl, 3 %mol) and methanol (5 ml) an aqueous 30% H_2O_2 (1.25 g, 11 mmol) solution was slowly added. The mixture was stirred at room temperature for 12 h. Diethyl ether (10 ml), H_2O (1.5 ml) and brine (3 ml) were added. The organic layer was separated, washed with brine, dried (Na_2SO_4) and concentrated by evaporation at atmospheric pressure and room temperature. Silica gel chromatography followed (eluent petroleum ether/ethyl acetate, 10:1) and afforded **1b** in 36% yield (151 mg, 0.9 mmol).[18]

^{1}H-NMR ($CDCl_3$): δ 1.41 (s, 3H), 1.43 (s, 3H), 1.50–1.76 (m, 16H), 2.06–3.18 (m, 4H), 3.13 (d, 1H, $J = 5.1$ Hz), 3.17 (d, 1H, $J = 5.0$ Hz), 5.03–5.06 (m, 2H), 9.41 (d, 1H, $J = 5.1$ Hz), 9.44 (d, 1H, $J = 5.0$ Hz). ^{13}C-NMR ($CDCl_3$): δ 17.2, 17.6, 17.6, 22.1, 23.4, 24.2, 25.6, 33.4, 38.3, 63.5, 64.1, 64.6, 122.4, 122.6, 132.7, 133.3, 198.8, 199.5.

***rac*-2,3-Epoxy-1-cyclohexanone (2b).** To a stirred mixture of cyclohexenone (**2a**) (240 mg, 2.5 mmol), *sec*-butylamine (8 μl, 3%mol) and methanol (5 ml) an aqueous 30% H_2O_2 (1.25 g, 11 mmol) solution was slowly added. The mixture was stirred at room temperature for 12 h. Diethyl ether (10 ml), H_2O (1.5 ml) and brine (3 ml) were added. The organic layer was separated, washed with brine, dried (Na_2SO_4) and concentrated by evaporation at atmospheric pressure and room temperature. Silica gel chromatography followed (eluent pentane/diethyl ether, 10:1) and afforded **2b** in 57% yield (160 mg, 1.43 mmol).[19]

^{1}H-NMR ($CDCl_3$): δ 1.26–2.58 (m, 6H) 3.59–3.60 (m, 1H), 3.23 (d, 1H, $J = 3.9$ Hz). ^{13}C-NMR ($CDCl_3$): δ 17.0, 22.9, 36.4, 55.1, 55.9, 205.9.

***rac*-2,3-Epoxy-3,5,5-trimethyl-1,4-cyclohexanedione (3b).** To a stirred mixture of ketoisopherone (**3a**) (380 mg, 2.5 mmol), *sec*-butylamine (8 μl, 3%mol) and methanol (5 ml) an aqueous 30% H_2O_2 (1.25 g, 11 mmol) was slowly added. The mixture was stirred at room temperature for 12 h. Diethyl ether (10 ml), H_2O (1.5 ml) and brine (3 ml) were added. The organic layer was separated, washed with brine, dried (Na_2SO_4) and concentrated by evaporation at atmospheric pressure and room temperature. Silica gel chromatography followed (eluent pentane/diethyl ether, 25:1) and afforded **3b** in 45% yield (190 mg, 1.13 mmol).

^{1}H-NMR ($CDCl_3$): δ 1.05 (s, 3H), 1.26 (s, 3H), 1.53 (s, 3H), 2.13 (d, 1H, $J = 13.4$ Hz), 3.13 (d, 1H, $J = 13.4$ Hz), 3.47 (s, 1H). ^{13}C-NMR ($CDCl_3$): δ 16.0, 26.1, 27.0, 45.5, 47.1, 62.9, 64.8, 204.2, 205.6.

***rac*-2,3-Epoxy-2-methyl-1,4-naphthoquinone (4b).** A solution of menadione (**4a**) (200 mg, 1.16 mmol) and sodium percarbonate (365 mg, 1.16 mmol) in ethanol (4 ml) and H_2O (1.2 ml) was stirred at room temperature for 10 min. H_2O (20 ml) was added, the suspension filtered, washed and dried in vacuo. White crystalline product in 76% yield (166 mg, 0.88 mmol) was obtained.[20]

^{1}H-NMR (DMSO): δ 1.61 (s, 3H), 4.11 (s, 1H), 7.84–7.96 (m, 4H). ^{13}C-NMR (DMSO): δ 14.8, 61.5, 62.0, 126.7, 127.2, 132.2, 132.2, 134.9, 135.1, 192.1.

***rac*-Methyl phenyl sulfoxide (6b).** A mixture of thioanisole **6a** (248 mg, 2 mmol) and aqueous 30% H_2O_2 (227 mg, 2 mmol) was stirred at 35 °C for 18 h. The solution was saturated with NaCl and extracted with ethyl acetate (3 × 5 ml). The combined organic phases were washed with saturated aqueous $Na_2S_2O_3$ (5 ml), dried (Na_2SO_4) and evaporated to yield **6b** in 85% yield (238 mg, 1.7 mmol).[21]

^{1}H-NMR ($CDCl_3$): δ 2.67 (s, 3H), 7.42–7.50 (m, 3H), 7.59–7.61 (m, 2H). ^{13}C-NMR ($CDCl_3$): δ 43.9, 123.5, 129.3, 131.0, 145.6.

General procedure for the enzymatic oxidation

An aliquot of the isolated enzyme YcnD, YhdA or Lot6p (protein purity >90%, protein content 90–110 μg/mL) was added to a Tris-HCl buffer solution (0.8 mL, 50 mM, pH 7.5) containing the substrate (5 mM) and the cofactor NADH (10 mM). The mixture was shaken at 30 °C and 120 rpm for 48 h and the products were extracted with EtOAc (2 × 0.5 mL). The combined organic phases were dried (Na_2SO_4) and the resulting samples were

analyzed on achiral GC. Products were identified by comparison with authentic reference materials which were either commercially available or were independently synthesized as described above, *via* co-injection on GC-MS and achiral GC.

General procedure for reaction with the YcnD/catalase system. To the reaction mixture was added catalase from bovine liver (20–25 U) and the reaction was carried out as described above.

General procedure for reaction under Ar with YcnD. The medium was flushed with argon for 10 min and after addition of YcnD, the reaction mixture was stirred under protective atmosphere for 24 h and worked up as described above.

General procedure for the blank-reaction in presence of H_2O_2. Blank-reaction in presence of H_2O_2 was performed in a Tris-HCl buffer solution (0.8 mL, 50 mM, pH 7.5) containing the substrate (5 mM) and H_2O_2 (10 mM). The reaction and workup was carried out as described in the general procedure.

General procedure for the blank-reaction in presence of H_2O_2 and enzyme. An aliquot of the isolated enzyme YcnD, YhdA or Lot6p (8–10 μl, protein purity >90%, protein content 90–110 μg/mL) was added to a Tris-HCl buffer solution (0.8 mL, 50 mM, pH 7.5) containing the substrate (5 mM) and H_2O_2 (10 mM). The reaction and workup was carried out as described in the general procedure.

Analytical procedures

Determination of conversion. Conversions for citral (**1a**) and cyclohexenone (**2a**) were determined by GC-FID using a 14% cyanopropyl-phenyl phase capillary column (J & W Scientific DB-1701, 30 m, 0.25 mm, 0.25 μm), detector temperature 250 °C, split ratio 30:1. Temperature program for citral (**1a**) and cyclohexenone (**2a**): 110 °C, hold for 5 min, 10 °C/min to 200 °C, hold for 2 min. Retention times were as follows: *diastereomeric-rac*-2,3-epoxy-3,7-dimethyl-6-octenal (**1b**) 9.10 min and 9.31 min, citral (*E*/*Z*) (**1a**) 9.54 min and 10.10 min, cyclohexenone (**2a**) 4.13 min and *rac*-2,3-epoxy-1-cyclohexanone (**2b**) 5.97 min.

Conversions for ketoisopherone (**3a**), menadione (**4a**) and thioanisole (**6a**) were analysed by GC-FID using a 6% cyanopropyl-phenyl phase capillary column (Varian CP-1301, 30 m, 0.25 mm, 0.25 μm), detector temperature 250 °C, split ratio 30:1. Temperature program for ketoisopherone (**3a**) and thioanisole (**6a**): 110 °C, hold for 5 min, 30 °C/min to 200 °C, hold for 2 min. Temperature program for menadione (**4a**): 160 °C, 5 °C/min to 180 °C, 20 °C/min to 250 °C. Retention times were as follows: *rac*-2,3-epoxy-3,5,5-trimethyl-1,4-cyclohexanedione (**3b**) 6.00 min, ketoisopherone (**3a**) 6.19 min, menadione (**4a**) 5.55 min, *rac*-2,3-epoxy-2-methyl-1,4-naphthoquinone (**4b**) 5.78 min, thioanisole (**6a**) 4.98 min and *rac*-methyl phenyl sulfoxide (**6b**) 8.51 min. The conversion of omeprazole sulfide (**7a**) was analysed by HPLC using a Chiralcel AD column (25 cm, 0.46 cm). Eluent *n*-heptane/isopropanol 65:35, flow 0.3 ml/min, temperature 18 °C (60 min isocratic). Retention times were as follows: omeprazole sulfide (**7a**) 20.13 min and (*R,S*)-omeprazole (**7b**) 26.50 min and 33.43 min.

Determination of enantiomeric excess. The enantiomeric excess of *diastereomeric-rac*-2,3-epoxy-3,7-dimethyl-6-octenal (**1b**) was analysed by GC-FID using a modified γ-cyclodextrin capillary column (Varian Chiraldex G-PN, 25 m, 0.32 mm). Detector temperature 200 °C, injector temperature 180 °C, split ratio 20:1. Temperature program: 80 °C, hold for 2 min, 5 °C/min to 120 °C, hold for 2.50 min, 10 °C/min to 160 °C, hold for 2 min. Retention times were as follows: *diastereomeric-rac*-2,3-epoxy-3,7-dimethyl-6-octenal (**1b**) 7.78 min and 8.08 min (diastereomers) and (*E*/*Z*)-citral (**1a**) 8.43 min and 9.14 min.

The enantiomeric excess of *rac*-2,3-epoxy-1-cyclohexanone (**2b**) was analysed by GC-FID using a modified β-cyclodextrin capillary column (Hydrodex-β-TBDAc, 25 m, 0.25 mm). Detector temperature 200 °C, injector temperature 180 °C, split ratio 20:1. Temperature program: 65 °C, hold for 10 min, 30 °C/min to 180 °C, hold for 2 min. Retention times were as follows: cyclohexenone (**2a**) 13.63 min and *rac*-2,3-epoxy-1-cyclohexanone (**2b**) 13.95 min and 14.33 min.

The enantiomeric excesses of *rac*-2,3-epoxy-3,5,5-trimethyl-1,4-cyclohexanedione (**3b**) and *rac*-2,3-epoxy-2-methyl-1,4-naphthoquinone (**4b**) were analysed by GC-FID using a β-cyclodextrin capillary column (CP-Chirasil-DEX-CB, 25 m, 0.32 mm, 0.25 μm). Detector temperature 200 °C, injector temperature 180 °C, split ratio 20:1. Temperature program for *rac*-2,3-epoxy-3,5,5-trimethyl-1,4-cyclohexanedione (**3b**): 90 °C, hold for 2 min, 4 °C/min to 115 °C, 20 °C/min to 180 °C, hold for 2 min. Temperature program for *rac*-2,3-epoxy-2-methyl-1,4-naphthoquinone (**4b**): 80 °C, hold for 2 min, 5 °C/min to 140 °C, hold for 2 min, 20 °C/min to 160 °C, hold for 2 min. Retention times were as follows: *rac*-2,3-epoxy-3,5,5-trimethyl-1,4-cyclohexanedione (**3b**) 7.29 min and 8.98 min, ketoisopherone (**3a**) 8.65 min, menadione (**4a**) 16.80 min and *rac*-2,3-epoxy-2-methyl-1,4-naphthoquinone (**4b**) 17.11 min and 17.44 min.

The enantiomeric excess of *rac*-methyl phenyl sulfoxide (**6b**) was analysed by HPLC using a Chiralcel AD column (25 cm, 0.46 cm). Eluent *n*-heptane/ethanol 95:5, flow 1 ml/min, temperature 24 °C (40 min isocratic). Retention times were as follows: thioanisole (**6a**) 3.74 min and *rac*-methyl phenyl sulfoxide (**6b**) 23.92 min and 25.58 min.

The enantiomeric excess of omeprazole (**7b**) was analysed by HPLC using a Chiralcel AD column (25 cm, 0.46 cm). Eluent *n*-heptane/isopropanol 65:35, flow 0.3 ml/min, temperature 18 °C (60 min isocratic). Retention times were as follows: omeprazole sulfide (**7a**) 20.13 min and omeprazole (**7b**) 26.50 min and 33.43 min.

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