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Studies towards the synthetic applicability of biocatalytic allylic oxidations with the lyophilisate of *Pleurotus sapidus*

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ABSTRACT

The edible fungus *Pleurotus sapidus* (*PSA*) is a particularly interesting biocatalytic system for allylic oxidation and has a remarkably broad substrate range from terpenoids to fatty acids. The oxidations are most likely catalyzed by a lipoxygenase and involve the formation of peroxides *via* radical intermediates in the first rate-limiting step. We provide herein a rationalization of the observed regioselectivity of these conversions by means of computational determination of bond dissociation enthalpies of a set of tailor-made spirocyclic terpenoids. It was found that only strongly activated allylic positions (BDH_{298} of <80 kcal/mol) with neighboring heteroatoms or with activating alkyl groups are oxidized to the corresponding unsaturated lactones or enones, respectively. With the synthesis and purification of allylic hydroperoxide intermediates, we have been able to characterize the putative direct precursors of enones in *PSA* oxidations. Our results suggest a two-step oxidation mechanism involving hydroperoxide intermediates which are rapidly converted to the observed enones by an enzymatic reaction.

1. Introduction

Regioselective oxidations of CH-bonds are attractive synthetic transformations with a broad spectrum of applications in academia and a high impact on the industrial chemical value chain as they convert relatively cheap precursors into value-added products [1,2]. Among these transformations, allylic oxidations are of high interest, because the olefinic starting materials are readily available as cheap bulk chemicals and many interesting derivatives, such as terpenes are available from renewable sources [3,4]. In addition, the resulting allyl alcohols [5–10] or α,β-unsaturated carbonyl compounds are attractive synthetic targets of high economic and scientific interest [11–17]. Allylic oxidations of olefins to enones have classically been performed with strong oxidants, such as chromium or other metal-based reagents [18,19]. In addition, metal-free and biocatalytic methods have been reported [3]. Several of these biocatalytic protocols have been applied to the synthesis of fine chemicals [20–22], drugs [23] and food ingredients [24–26]. A particularly interesting biocatalytic system for allylic oxidation is the edible fungus *Pleurotus sapidus* (*PSA*), which has a remarkably broad substrate range from terpenoids to fatty acids [27–31]. We have recently shown that the lyophilisate of *PSA* is able to catalyze allylic and benzylic oxidations in a broad range of olefinic substrates including simple cyclohexene derivatives and several functionalized terpenoids with preparatively useful yields [32]. Biocatalytic allylic oxidations with *PSA* may be performed with the lyophilisate of commercially available fungal fruiting bodies or with mycelium from submerged cultures. A *PSA*-derived dioxygenase has been shown to be responsible for the allylic oxidation of valencene to nootkatone and the same enzyme oxidizes unsaturated fatty acids [29,31,33–35]. It is thus likely that this dioxygenase is the major oxidant in other allylic oxidations with *PSA*-lyophilisate, too. However, since the lyophilisate is a mixture of enzymes, alternative oxidation pathways cannot be ruled out for other substrates. The reaction mechanism was proposed to involve the initial formation of allyl radicals, which would subsequently be converted to allylic hydroperoxides **2** (Scheme 1) [24,29]. These peroxides were found to be the major products of *PSA*-catalyzed oxidations of fatty acids and were proposed as intermediates in the allylic oxidation of valencene to nootkatone [29,31]. The abstraction of an allylic hydrogen and the resulting formation of radical intermediates is plausible and is reflected by our recent finding that

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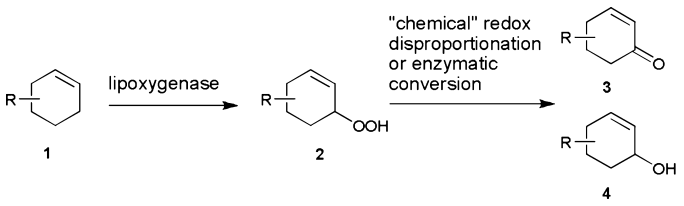

Scheme 1. Proposed pathways for allylic oxidations of alkenes **1** with the lyophilisate of *Pleurotus sapidus* (*PSA*).

the reactivity of substrates towards *PSA* oxidation is largely determined by bond-dissociation energies for the participating allylic CH-bonds with a threshold of about 80 kcal/mol [36–40]. Intermediate allylic peroxides were assumed to be unstable and have not been thoroughly characterized so far. It is furthermore unclear if they are converted by "chemical" redox disproportionation or an enzymatic transformation to the typical reaction products (enones and/or allylic alcohols). In addition, olefinic terpenoids and unsaturated fatty acids are substrates for autoxidations which may be alternative non-enzymatic reaction pathways.

In this paper, we report our attempts towards a mechanistic understanding of allylic oxidations with the lyophilisate of *PSA*. Rationalization of the observed regioselectivity is provided by computational determination of bond dissociation enthalpies and correlation with structural and electronic features of selected tailor-made spirocyclic terpenoids. In addition, we investigate the role of alternative autoxidations and peroxide intermediates through synthesis of representative examples and their use as substrates for *PSA*.

2. Experimental

2.1. General methods

TLC was performed on silica gel aluminum sheets (Macherey and Nagel). The reagent used for developing TLC plates was phosphomolybdic acid (5 g phosphomolybdic acid, 100 mL EtOH). Flash column chromatography was performed on silica gel (Macherey and Nagel, 40–60 μm).

^{1}H NMR chemical shifts are calibrated to residual non-deuterated solvent ($CDCl_3$, δ_H = 7.26 ppm). ^{13}C NMR chemical shifts are referenced to the solvent signal ($CDCl_3$, δ = 77.16 ppm). NMR spectra were recorded at 300 (75), 400 (100), 500 (125) or 600 (150) MHz on Bruker Avance instruments. The coupling constant (*J*) is given in Hz. The chemical shifts δ are reported in ppm and the signal patterns are indicated as s (singlet), d (doublet), t (triplet), q (quartet), sext (sextet), m (multiplet), and br. (broad). NMR-signals have been assigned on the basis of 2D-NMR (HH-COSY, HMBC, and HSQC) experiments. Relative stereoinformation has been assigned on the basis of 1D or 2D NOE-experiments.

The atom numbers used for NMR peak assignment do not refer to IUPAC nomenclature and are available from the structures provided on the spectra in the supporting information. ESI and APCI mass spectra were recorded with a Bruker MicroTOF-Q instrument operating in positive mode. Samples were dissolved in CH_3CN—H_2O mixtures or pure MeOH and directly injected using a syringe. All of the reagents were reagent grade and used without further purification unless otherwise specified. Solvents for the reactions were distilled prior to use. All air- or moisture-sensitive reactions were conducted under nitrogen or argon in flame- or oven-dried glassware and were magnetically stirred.

Compounds **20** [41], **24** [42], **26** [42,43], and **28** [44], **32a** [40], were synthesized according to the literature.

The filamentous fungus *P. sapidus* (*PSA*) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ 8266), Braunschweig, Germany. Production of biomass and lyophilisate were described previously by Fraatz et al. [24] For all new oxidations with *PSA*, a parallel positive control experiment with the known substrate theaspirane and the same batch of *PSA*-lyophilisate was performed, confirming enzymatic activity of the lyophilisate used. Protein concentrations were determined by Lowry assay using bovine serum albumin as standard, and peroxidase activity was quantified as previously described in the presence of 0.2 mM H_2O_2 [45]:

Peroxidase activity: 16.7 U/g lyophilisate (253 mU/mg protein)
Solubilized protein: 66 mg/g lyophilisate

2.2. Experimental procedures and analytical data

Valencene peroxides 29 and 30. A sample of valencene (**9**) (204 mg, 1.0 mmol) and 1 mL of a $CDCl_3$ stock solution, of the photosensitizer *meso*-tetraphenyl-porphyrine (TPP, 5×10^{-4} m) was filled into a NMR tube. During the irradiation with a 50 W white LED lamp at room temperature, oxygen was bubbled through the solution. The reaction was followed by TLC and NMR spectroscopy. After complete conversion, the solvent was evaporated under reduced pressure at 5 °C which resulted in a mixture of hydroperoxides **29/30** 4:1 (230 mg, 0.97 mmol, 97%) as a green, highly viscous oil. Column chromatography (pentane/Et_2O 100:1.5 → 100:2.5) gave hydroperoxide **29** (141 mg, 0.6 mmol, 60%) and rearranged hydroperoxide **30** (36 mg, 0.15 mmol, 15%) as orange oils.

(4*R*,4*aS*,6*R*)-2-Hydroperoxy-4,4*a*-dimethyl-6-(prop-1-en-2-yl)-2,3,4,4*a*,5,6,7,8-octahydronaphthalene29 R_f = 0.35 (pentane/Et_2O 8.5:1.5, phosphomolybdic acid). **HRMS** (ESI): calculated for $C_{15}H_{24}O_2 + Na^+$ = 259.1669, found = 259.1676. **^{1}HNMR** (400 MHz, $CDCl_3$): δ (ppm) = 7.86 (s, 1H, OOH), 5.42–5.39 (m, 1H, 1-H), 4.69–4.67 (m, 1H, 13-H), 4.37–4.34 (m, 1H, 2-H), 2.34 (ttt, $^3J_{H,H}$ = 14.1 Hz, $J_{H,H}$ = 2.5 Hz, $J_{H,H}$ = 2.5 Hz, 1H, 8-H), 2.24 (tt, $^3J_{H,H}$ = 12.5 Hz, $^3J_{H,H}$ = 3.0 Hz, 1H, 6-H), 2.17 (ddd, $^2J_{H,H}$ = 14.1 Hz, $^3J_{H,H}$ = 4.2 Hz, $^3J_{H,H}$ = 2.6 Hz, 1H, 8-H), 1.93 (ttt, $J_{H,H}$ = 14.7 Hz, $J_{H,H}$ = 1.5 Hz, $J_{H,H}$ = 1.5 Hz, 1H, 3-H), 1.87 (td, $^2J_{H,H}$ = 12.9 Hz, $^3J_{H,H}$ = 2.8 Hz, 1H, 5-H), 1.82–1.76 (m, 1H, 7-H), 1.76–1.72 (m, 1H, 4-H), 1.70 (s, 3H, 12-H), 1.56–1.47 (m, 1H, 3-H), 1.31–1.19 (m, 1H, 7-H), 1.05–0.98 (m, 1H, 5-H), 0.92 (s, 3H, 10-H), 0.90 (d, $^3J_{H,H}$ = 7.0 Hz, 3H, 9-H). **^{13}C NMR** (100 MHz, $CDCl_3$): δ (ppm) = 153.5 (C8a), 150.2 (C11), 115.9 (C1), 108.8 (C13), 78.5 (C2), 44.5 (C5), 40.8 (C6), 38.6 (C4a), 35.2 (C4), 32.8 (C8), 32.6 (C7), 30.9 (C3), 20.9 (C12), 17.1 (C10), 15.2 (C9).

(4*R*,4*aS*,6*R*)-1-Hydroperoxy-4,4*a*-dimethyl-6-(prop-1-en-2-yl)-1,2,3,4,4*a*,5,6,7-octahydronaphthalene30 R_f = 0.40 (pentane/Et_2O 8.5:1.5, phosphomolybdic acid). **HRMS** (ESI): calculated for $C_{15}H_{24}O_2 + Na^+$ = 259.1669, found = 259.1659. **^{1}H NMR** (400 MHz, $CDCl_3$): δ (ppm) = 7.41 (s, 1H, OOH), 5.76 (dd, $^3J_{H,H}$ = 5.2 Hz, $^3J_{H,H}$ = 2.1 Hz, 1H, 8-H), 4.75–4.72 (m, 13H, 13-H), 4.35–4.33 (m, 1H, 1-H), 2.39–2.31 (m, 1H, 6-H), 2.20 (dtd, $^2J_{H,H}$ = 17.9 Hz, $^3J_{H,H}$ = 5.2 Hz, $^3J_{H,H}$ = 1.8 Hz, 1H, 7-H), 2.11–2.06 (m, 1H, 2-H), 1.92 (ddd, $^2J_{H,H}$ = 17.9 Hz, $^3J_{H,H}$ = 11.5 Hz, $^3J_{H,H}$ = 2.1 Hz, 1H, 7-H), 1.79 (td, $^2J_{H,H}$ = 12.6 Hz, $^3J_{H,H}$ = 2.1 Hz, 1H, 5-H), 1.75 (s, 3H, 12-H), 1.62-1.57 (m, 2H, 2-H/3-H), 1.34–1.27 (m, 2H, 3-H/4-H), 1.18 (t, $^3J_{H,H}$ = 12.6 Hz, 1H, 5-H), 1.09 (s, 3H, 10-H), 0.88 (d, $^3J_{H,H}$ = 6.2 Hz, 3H, 9-H). **^{13}C NMR** (100 MHz, $CDCl_3$): δ (ppm) = 150.0 (C11), 140.1 (C8a), 130.1 (C8), 108.9 (C13), 87.8 (C1), 43.7/43.6 (C4/C5), 38.1 (C4a), 37.4 (C6), 31.6 (C7), 30.1 (C2), 26.1 (C3), 20.9 (C12), 19.9 (C10), 15.7 (C9).

(2*R,5*R**)-7-Hydroperoxy-2,10,10-trimethyl-6-methylene-1-oxaspiro[4.5]decane 31**. A sample of theaspirane (**5**) (194 mg, 1.0 mmol) and 1 mL of a $CDCl_3$ stock solution, of the photosensitizer *meso*-tetraphenyl-porphyrine (TPP, 5×10^{-4} m) was filled into a NMR tube. During the irradiation with a 50 W white LED lamp at room temperature, oxygen was bubbled through the solution. The reaction was followed by TLC and NMR spec-