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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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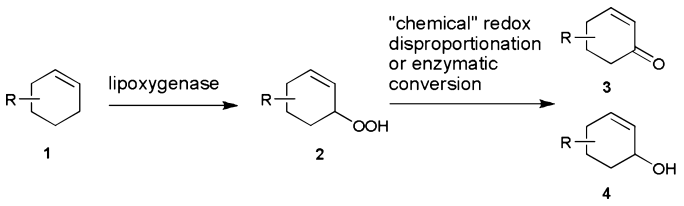


Scheme 1. Proposed pathways for allylic oxidations of alkenes **1** with the lyophilisate of *Pleurotus sapidus* (*PSA*).

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-

troscopy. After complete conversion, the solvent was evaporated under reduced pressure at 5 °C which gave hydroperoxide **31** (215 mg, 0.95 mmol, 95%) as a green, highly viscous oil. R_f = 0.35 (pentane/Et_2O 8:2, phosphomolybdic acid). **HRMS** (ESI): calculated for $C_{13}H_{22}O_3 + Na^+$ = 249.1461, found = 249.1461. **^{1}H NMR** (400 MHz, $CDCl_3$): δ (ppm) = 7.87 (br. s, 1H, OOH), 5.21 (d, $^2J_{H,H}$ = 1.7 Hz, 1H, 11-H), 5.13–5.12 (m, 1H, 11-H), 4.62 (t, $^3J_{H,H}$ = 4.8 Hz, 1H, 7-H), 4.10–4.03 (m, 1H, 2-H), 2.02–1.93 (m, 3H, 3-H/4-H/4-H), 1.89–1.81 (m, 1H, 8-H), 1.76–1.69 (m, 1H, 8-H), 1.61–1.54 (m, 1H, 9-H), 1.46–1.37 (m, 2H, 3-H/9-H), 1.22 (d, $^3J_{H,H}$ = 6.1 Hz, 3H, 14-H), 0.90/0.87 (2*s, 2*3H, 12-H/13-H). **^{13}C NMR** (100 MHz, $CDCl_3$): δ (ppm) = 148.1 (C6), 112.0 (C11), 90.0 (C5), 86.4 (C7), 74.4 (C2), 38.0 (C10), 34.3 (C9), 33.8 (C3), 31.2 (C4), 26.3 (C8), 24.1/22.7 (C12/C13), 21.1 (C14).

(2*R,5*R**)-2,10,10-Trimethyl-6-methylene-1-oxaspiro[4.5]decane 33**. Allylalcohol **32a** (1.00 g, 4.75 mmol) was converted to the corresponding acetate by treatment with pyridine (50 mL) and Ac_2O (250 mL) at rt for 24 h. The reaction mixture containing **32b** was concentrated to dryness under reduced pressure and the residue was dissolved in dry THF (15 mL). The solution was treated with formic acid (570 μL, 14.25 mmol), Et_3N (2.04 mL, 14.25 mmol), $(PPh_3)_2PdCl_2$ (70 mg, 0.1 mmol) and PPh_3 (162 mg, 0.61 mmol). The reaction was heated to reflux for 24 h under a nitrogen atmosphere. The mixture was cooled to rt, diluted with Et_2O (20 mL) and washed with water (20 mL), 5% HCl solution (10 mL) saturated aqueous $NaHCO_3$ solution (15 mL) and brine (20 mL), dried with Na_2SO_4 and evaporated. The residue was purified by column chromatography (pentane/Et_2O 100:1.5 → 100:2.5) to give spiroether **33** (497 mg, 2.56 mmol, 54%) as a colorless oil. R_f = 0.77 (pentane/Et_2O 100:1, phosphomolybdic acid). **HRMS** (APCI): calculated for $C_{13}H_{22}O + H^+$ = 195.1743, found = 195.1750. **^{1}H NMR** (400 MHz, $CDCl_3$): δ (ppm) = 4.86 (dd, $^4J_{H,H}$ = 2.5 Hz, $^4J_{H,H}$ = 1.7 Hz, 1H, 11-H), 4.69 (dd, $^4J_{H,H}$ = 2.5 Hz, $^4J_{H,H}$ = 1.7 Hz, 1H, 11-H), 4.08–4.00 (m, 1H, 2-H), 2.31 (td, $^2J_{H,H}$ = 13.4 Hz, $^3J_{H,H}$ = 4.1 Hz, 1H, 7-H), 2.10–1.99 (m, 2H, 4-H/7-H), 1.95–1.87 (m, 1H, 3-H), 1.71 (td, $^2J_{H,H}$ = 12.7 Hz, $^3J_{H,H}$ = 8.2 Hz, 1H, 4-H), 1.54–1.47 (m, 2H, 8-H), 1.45–1.40 (m, 2H, 9-H), 1.39–1.31 (m, 1H, 3-H), 1.23 (d, $^3J_{H,H}$ = 6.1 Hz, 3H, 14-H), 0.90/0.88 (2*s, 2*3H, 12-H/13-H). **^{13}C NMR** (100 MHz, $CDCl_3$): δ (ppm) = 151.7 (C6), 105.5 (C11), 90.4 (C5), 74.3 (C2), 38.9 (C9), 38.4 (C10), 34.2 (C3), 33.8 (C7), 31.9 (C4), 24.3 (C12), 23.0 (C8), 22.5 (C13), 20.9 (C14).

General procedure for the biotransformation: 600 mg *PSA* lyophilisate were dissolved in 30 mL Tris–HCl buffer (20 mm, pH 7.5). The dried biomass was rehydrated by stirring at 900 rpm for 10 min. The substrate was added and the solution was stirred at 900 rpm at rt. The reaction progress was controlled by GC-FID. After 24 h another 400 mg of *PSA* lyophilisate and 20 mL buffer were added. After 48 h the reaction was stopped by adding 50 mL of Et_2O and further stirring of the reaction mixture for 30 min. The solution was filtered and the aqueous phase was extracted with Et_2O three times. The combined organic phases were dried with $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was analyzed by NMR and if required purified by column chromatography.

Biotransformation of theaspiran hydroperoxide 31. According to the general procedure hydroperoxide **31** (180 mg, 0.8 mmol) was treated with 1.3 g lyophilisate for 72 h. The crude product was purified by column chromatography (pentane/Et_2O 9:1 → 8:2) to give the enone **34** (15.1 mg, 0.07 mmol, 9%) and the allylic alcohol **32a** (21.6 mg, 0.1 mmol, 13%) as a colorless oils. The NMR spectra of **32a** matched those reported in the literature [40]. **(2*R**,5*R**)-2,10,10-Trimethyl-6-methylene-1-oxaspiro[4.5]decan-4-one 34**. R_f = 0.35 (pentane/Et_2O 8:2, phosphomolybdic acid). **HRMS** (ESI): calculated for $C_{13}H_{20}O_2 + H^+$ = 209.1536, found = 209.1540. **^{1}HNMR** (400 MHz, $CDCl_3$): δ (ppm) = 5.71 (d, $^2J_{H,H}$ = 1.8 Hz, 1H, 11-H), 5.29 (d, $^2J_{H,H}$ = 1.8 Hz, 1H, 11-H), 4.13–4.20 (m, 1H, 2-H), 2.52–2.38 (m, 2H, 8-H), 2.06–1.95 (m, 2H, 3-H/4-H), 1.78–1.71 (m, 1H, 9-H), 1.68–1.54 (m, 2H, 4-H/9-H), 1.50–1.41 (m, 1H, 3-H), 1.20 (s, 3H, 14-H), 1.03/1.02 (2*s, 2*3H, 12-H/13-H). **^{13}C NMR** (100 MHz, $CDCl_3$): δ (ppm) = 203.4 (C7), 152.3 (C6), 116.9 (C11), 90.4 (C5), 75.3 (C2), 37.0 (C8), 36.8 (C10), 33.8 (C9), 32.6 (C3), 30.6 (C4), 23.7/22.5 (C12/C13), 21.6 (C14).

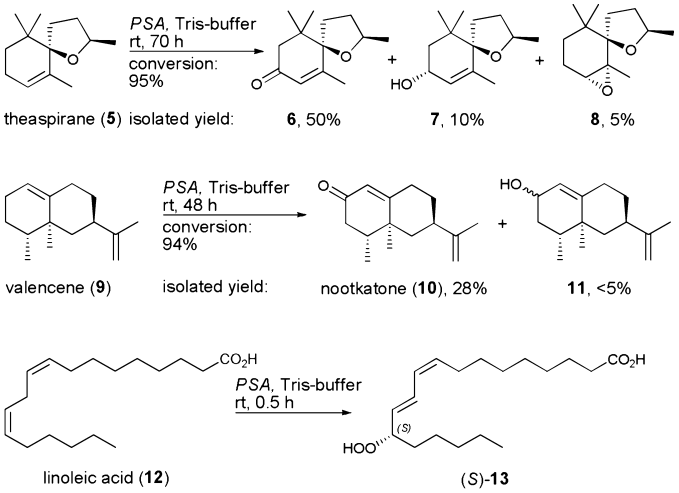
Biotransformation of valencene hydroperoxide 29. According to the general procedure hydroperoxide **29** (324 mg, 1.4 mmol) was treated with 800 mg lyophilisate for 48 h. The crude product was purified by column chromatography (pentane/Et_2O 9:1 → 8:2) to give the enone **10** (47.5 mg, 0.22 mmol, 16%) and the allylic alcohol **11** (20.4 mg, 0.1 mmol, 7%) as colorless oils. The NMR spectra of **10** and **11** matched those reported in the literature [32].

Biotransformation of spiroether 33. According to the general procedure spiroether **33** (50 mg, 0.26 mmol) was treated with 600 mg lyophilisate for 96 h. The crude product was analyzed by ^{1}H and ^{13}C NMR and the pure substrate was identified. No conversion was observed.

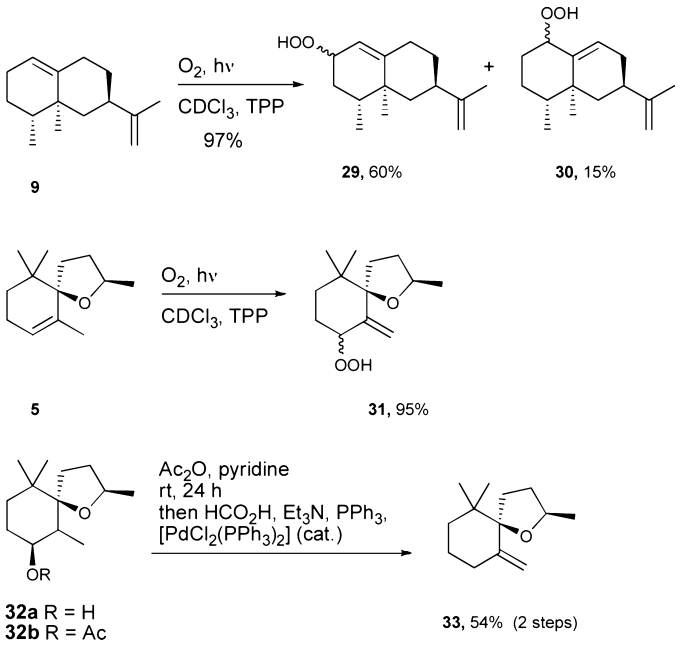
Biotransformation of spiroether 20. According to the general procedure spiroether **20** (382 mg, 2.5 mmol) was treated with 800 mg lyophilisate for 72 h. The crude product was purified by column chromatography (pentane/Et_2O 10:1 → 7:1) to give lactone **21** (46 mg, 0.28 mmol, 11%), enone **22** (39 mg, 0.24 mmol, 10%) and aldehyde **23** (53 mg, 0.32 mmol, 13%) as colorless oils. **1-Oxaspiro[5.5]undec-3-en-2-one21**. R_f = 0.52 (pentane/Et_2O 1:1, phosphomolybdic acid). **^{1}HNMR** (400 MHz, $CDCl_3$): δ (ppm) = 6.72 (td, $^3J_{H,H}$ = 9.8 Hz, $^3J_{H,H}$ = 4.2 Hz, 1H, 4-H), 5.98 (td, $^3J_{H,H}$ = 9.8 Hz, $^4J_{H,H}$ = 2.0 Hz, 1H, 3-H), 2.39 (dd, $^3J_{H,H}$ = 4.2 Hz, $^4J_{H,H}$ = 2.0 Hz, 2H, 5-H), 1.98–1.92 (m, 2H, 7-H), 1.78–1.69 (m, 2H, 8-H), 1.58–1.41 (m, 5H, 7-H/8-H/9-H), 1.37-1.29 (m, 1H, 9-H). **^{13}C NMR** (100 MHz, $CDCl_3$): δ (ppm) = 163.9 (C2), 143.2 (C4), 121.1 (C3), 81.3 (C6), 36.4 (C7), 34.6 (C5), 25.4 (C9), 21.6 (C8). **1-Oxaspiro[5.5]undec-2-en-4-one 22**. R_f = 0.65 (pentane/Et_2O 1:1, phosphomolybdic acid). **^{1}HNMR** (400 MHz, $CDCl_3$): δ (ppm) = 7.23 (d, $^3J_{H,H}$ = 6.1 Hz, 1H, 2-H), 5.33 (d, $^3J_{H,H}$ = 6.1 Hz, 1H, 3-H), 2.48 (s, 2H, 5-H), 2.03–1.98 (m, 2H, 7-H), 1.64–1.40 (m, 10H, 7-H/8-H/9-H). **^{13}C NMR** (100 MHz, $CDCl_3$): δ (ppm) = 192.5 (C4), 161.3 (C2), 105.8 (C3), 82.4 (C6), 47.4 (C5), 34.4 (C7), 25.2 (C9), 21.5 (C8). **(*E*)-4-(1-Hydroxycyclohexyl)-but-2-enal 23**. R_f = 0.25 (pentane/Et_2O 1:1, phosphomolybdic acid). **^{1}H NMR** (400 MHz, $CDCl_3$): δ (ppm) = 9.52 (d, $^3J_{H,H}$ = 7.9 Hz, 1H, 1-H), 6.98 (td, $^3J_{H,H}$ = 15.6 Hz, $^3J_{H,H}$ = 7.5 Hz, 1H, 3-H), 6.15 (tdd, $^3J_{H,H}$ = 15.6 Hz, $^3J_{H,H}$ = 7.9 Hz, $^4J_{H,H}$ = 1.2 Hz, 1H, 2-H), 2.48 (dd, $^3J_{H,H}$ = 7.5 Hz, $^4J_{H,H}$ = 1.2 Hz, 2H, 4-H), 1.61–1.43 (m, 10H, 2′-H/3′-H/4′-H). **^{13}C NMR** (100 MHz, $CDCl_3$): δ (ppm) = 194.0 (C1), 154.4 (C3), 135.7 (C2), 71.7 (C1′), 45.6 (C4), 37.9 (C2′), 25.6 (C4′), 22.2 (C3′).

Biotransformation of spiroether 24. According to the general procedure spiroether **24** (400 mg, 2.2 mmol) was treated with 800 mg lyophilisate for 48 h. The crude product was purified by column chromatography (pentane/Et_2O 1:1) to give lactone **25** (135 mg, 0.7 mmol, 32%) as a colorless oil. **4,7-Dimethyl-1-oxaspiro[5.5]undec-3,7-dien-2-one25**. R_f = 0.20 (pentane/Et_2O 2:1, phosphomolybdic acid). **HRMS** (ESI): calculated for $C_{12}H_{16}O_2 + Na^+$ = 215.1043, found = 215.1048. **^{1}HNMR** (600 MHz, $CDCl_3$): δ (ppm) = 5.82 (s, 1H, 3-H), 5.64–5.62 (m, 1H, 8-H), 2.71 (d, $^2J_{H,H}$ = 18.3 Hz, 1H, 5-H), 2.17 (d, $^2J_{H,H}$ = 18.3 Hz, 1H, 5-H), 2.10–2.04 (m, 1H, 11-H), 1.98–1.92 (m, 3H, 10-H/10-H/11-H), 1.96 (s, 3H, 12-H), 1.81–1.76 (m, 1H, 9-H), 1.75–1.74 (m, 3H, 13-H), 1.55–1.48 (m, 1H, 9-H). **^{13}C NMR** (150 MHz, $CDCl_3$): δ (ppm) = 164.8 (C2), 155.4 (C4), 134.1 (C7), 128.4 (C8), 116.0 (C3), 81.6 (C6), 36.6 (C5), 33.9 (10), 25.3 (C11), 23.4 (C12), 19.6 (C9), 18.5 (C13).

Biotransformation of spiroether 26. According to the general procedure spiroether **26** (80 mg, 0.45 mmol) was treated with 1.2 g lyophilisate for 96 h. The crude product was purified by column chromatography (pentane/Et_2O 2:1 → 1:1) to give enone **27** (10 mg, 0.06 mmol, 14%) as a colorless oil. **4,7-Dimethyl-1-oxaspiro[5.5]undec-4,7-dien-9-one27**. R_f = 0.10



Scheme 2. Biocatalytic oxidation of theaspirane (**5**), valencene (**9**) and linoleic acid (**12**). 12, [31,32,40].



Scheme 3. Preparation hydroperoxides **29**–**31** from valencene and theaspirane with *meso*-tetraphenyl-porphyrine (TPP) as photosensitizer and synthesis of an alkene precursor **33** for biotransformation with *PSA*.

(pentane/Et_2O 5:1, phosphomolybdic acid). **HRMS** (APCI): calculated for $C_{12}H_{16}O_2 + H^+ = 193.1216$, found = 193.1197. ^{1}**HNMR** (400 MHz, $CDCl_3$): δ (ppm) = 5.84 (q, $^4J_{H,H}$ = 1.3 Hz, 1H, 8-H), 5.34 (s, 1H, 5-H), 3.92 (ddd, $^2J_{H,H}$ = 11.4 Hz, $^3J_{H,H}$ = 5.9 Hz, $^3J_{H,H}$ = 1.7 Hz, 1H, 2-H), 3.77 (ddd, $^2J_{H,H}$ = 11.4 Hz, $^3J_{H,H}$ = 11.0 Hz, $^3J_{H,H}$ = 3.4 Hz, 1H, 2-H), 2.58 (ddd, $^2J_{H,H}$ = 17.2 Hz, $^3J_{H,H}$ = 7.0 Hz, $^3J_{H,H}$ = 5.2 Hz, 1H, 10-H), 2.38 (ddd, $^2J_{H,H}$ = 17.2 Hz, $^3J_{H,H}$ = 8.8 Hz, $^3J_{H,H}$ = 5.9 Hz, 1H, 10-H), 2.32–2.26 (m, 1H, 3-H), 2.16–2.13 (m, 2H, 11-H), 1.86 (s, 3H, 13-H), 1.82–1.78 (m, 1H, 3-H), 1.77 (s, 3H, 12-H). ^{13}**C NMR** (100 MHz, $CDCl_3$): δ (ppm) = 199.0 (C9), 164.3 (C7), 135.7 (C4), 127.6 (C8), 123.0 (C5), 74.1 (C6), 60.1 (C2), 34.2 (C10), 33.1 (C11), 29.8 (C3), 23.6 (C12), 19.3 (C13).

Biotransformation of spiroether 28. According to the general procedure spiroether **28** (200 mg, 1.33 mmol) was treated with 800 mg lyophilisate for 96 h. The crude product was analyzed by ^{1}H and ^{13}C NMR and the pure substrate was identified.

3. Results and discussion

We have previously shown that allylic spiroethers, such as theaspirane (**5**) (Scheme 2) are suitable substrates for allylic oxidations with *PSA*. The conversion of theaspirane (**5**) according to Scheme 2 was used as a positive control experiment for each new batch of *PSA* lyophilisate used in the following conversions to confirm activity of the biocatalyst. In this context, it is of note that attempts to convert **5** with thermally inactivated lyophilisate failed and no conversion was observed for 72 h. The same is true for attempted conversions of **5** with active lyophilisate under exclusion of oxygen or the addition of excess H_2O_2. A table with a summary of these experiments may be found in the supporting information. The results suggest that (1) a non-enzymatic autocatalytic formation of oxidation products like **6**–**8** is slow under the conditions employed, (2) oxygen is a necessary cooxidant as expected for a lipoxygenase catalyzed conversion and (3) addition of hydrogen peroxide is not favoring the oxidation of **5**.

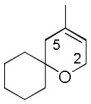
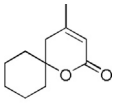
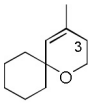
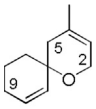
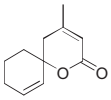
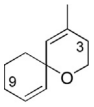
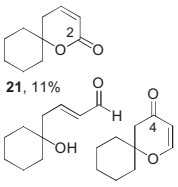
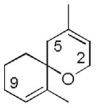
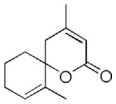
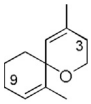
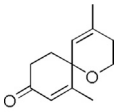
To evaluate the first step of *PSA* oxidations, we have synthesized a number of unsaturated spiroethers (Table 1) by the intramolecular silyl-modified Sakurai reaction [46]. These spiroethers contain allylic positions with only slightly differing stereoelectronic properties and are thus useful model compounds to investigate the first step of biocatalytic oxidations with *PSA*, which is the abstraction of allylic hydrogen and the formation of allyl radicals. All spiroethers **14**, **16**, **17**, **19**, **20**, **24**, **26**, and **28** were treated with *PSA* lyophilisate according to the protocol for theaspirane (**5**) depicted in Scheme 2.

The results of all biotransformations are summarized in Table 1 together with selected bond dissociation energies which have been calculated by DFT under implicit consideration of water as solvent (see Supplementary information for detailed information).

The experimental results from Table 1 confirm our earlier finding of a limiting bond dissociation enthalpy of about 80 kcal/mol for successful conversion of alkenes with *PSA* [40]. In consequence, only strongly activated allylic positions with neighboring heteroatoms (entries 1, 3, 5, and 6) or with activating alkyl groups (entry 7) are oxidized to the corresponding unsaturated lactones or enones, respectively. Allyl ethers like **14**, **17**, **20**, **24**, and **26** are all good substrates for *PSA* oxidations and in case of the methyl substituted derivatives **14**, **17**, and **24** (entries 1, 3, and 6) a single lactone isomer is the major product of oxidation, which might be a consequence of steric hindrance upon the attack of oxygen towards the intermediate allyl radical or a rearrangement [47,48] of the tertiary alkyl peroxides formed after the attack of oxygen. In contrast, the unsubstituted allyl ether **20** gave a mixture of three oxidation products **21**–**23**. Position 9 of spiroethers **24** and **26** seems to be right at the borderline for reactivity with *PSA*. In spiroether **24** only the more reactive position 2 is attacked and lactone **25** is formed (entry 6). In spiroether **26** position 9 with a slightly lower BDH is oxidized to the enone **27** (entry 7) although conversion is relatively low in this case.

In previous studies it was suggested that a lipoxygenase is responsible for allylic oxidations by *PSA* [29]. The reaction mechanism should therefore include allylic hydroperoxides as intermediates. In fact, hydroperoxides have been found to be the major products of allylic oxidation of linoleic acid using a recombinant lipoxygenase from *PSA* [29,31]. For other substrates such as valencene, hydroperoxide intermediates have been found and analyzed by chromatography and MS [29]. However, a plausible explanation has not been provided for their spontaneous conversion to enones, which are the major products obtained in this case. Therefore we synthesized allylic hydroperoxides derived from valencene and theaspirane *via* photooxygenation using singlet oxygen ($^1O_2$) as reactive species (Scheme 3) [49]. For valencene (**9**), this led to a mixture of two isomeric hydroperoxides **29** and **30**.

Table 1
Yields and conversions for biotransformations of spirocyclic model compounds **14**, **16**, **17**, **19**, **20**, **24**, **26** and **28** with *PSA* lyophylisate.

Entry	Starting material	BDH_{298} (kcal/mol)[a]		Conv[b]	Products[c]
1 [40]	 **14**	75.7 (2-H)	83.1 (5-H)	77%	 **15**, 45% yield
2 [40]	 **16**	83.4 (3-H)		<5%	–
3 [40]	 **17**	73.5 (2-H)	82.9 (5-H)80.7 (9-H)	>95%	 **18**, 32% yield
4 [40]	 **19**	83.5 (3-H)	80.3 (9-H)	<5%	–
5	 **20**	74.9 (2-H)	80.7 (5-H)	>95%	 **21**, 11% **23**, 13% **22**, 10%
6	 **24**	74.5 (2-H)	80.2 (5-H)80.7 (9-H)	>95%	 **25**, 32%
7	 **26**	83.8 (3-H)	79.1 (9-H)	50%	 **27**, 13%
8	 **28**	80.2 (2-H)	80.2 (5-H)	<5%	–

[a] B3LYP/6-31 + G** SCRF = (PCM, solvent = H_2O).
[b] Reactions were performed with 0.6–1.2 g *PSA* lyophilisate in TRIS-buffer at room temperature for 48–96 h. Conversion was determined after work up by GC-FID.
[c] Isolated yields are given after column chromatography.

Hydroperoxide **29** is the rearrangement product of one of the initial formed singlet oxygen ene products whereas the second hydroperoxide **30** did not rearrange [48]. These compounds are remarkably stable and were separated by standard column chromatography. Peroxide **29** is the putative direct precursor of nootkatone (**10**) upon biocatalytic oxidation of valencene (**9**) and thus a particularly interesting compound for the study of *PSA* oxidations. On the other hand the photochemical oxidation of theaspirane (**5**) gave hydroperoxide **31**, which is not a direct precursor of theaspiranone (**6**) and cannot be formed easily by biocatalytic oxidation of theaspirane (**5**). It was thus interesting to check whether **31** would be converted to the corresponding enone by *PSA*. This prompted us to synthesize alkene **33** as a reference substrate for oxidation with *PSA* according to a procedure reported by Serra et al. starting from allylic alcohol **32a** *via* elimination of the intermediate allylic acetate (Scheme 3) [50].

With analytically pure peroxides **29** and **30** in hand we were able to check the presence of peroxide intermediates in *PSA* oxidations of valencene. For this purpose, an oxidation of valencene with *PSA* lyophilisate was extracted with Et_2O after 50% conversion. The resulting extract was evaporated *in vacuo* and analyzed by NMR. A part of the corresponding crude ^1H NMR is shown in Fig. 1 along with the spectra of pure valencene (**9**), nootkatone (**10**) and both peroxides **29** and **30**. The methine protons of peroxides **29** and **30** at 4.3–4.4 ppm are well separated from other signals and may thus be used as markers. In the crude spectrum of a valencene oxidation (trace A), neither of the peroxides **29** and **30** was observed.

The absence of peroxides in the biocatalytic oxidation of valencene (**9**) suggested two possible routes for the generation of nootkatone (**10**). The first route involves a rapid enzymatic transformation of intermediate peroxides, most likely by the same enzyme responsible for the formation of the peroxides. This combined lipoxygenase/peroxidase functionality has been described for other lipoxygenases before [34,51]. The second route involves a rapid chemical transformation of intermediate peroxides most likely by redox disproportionation. Given the reasonable stability of peroxides **29** and **30**, a rapid chemical conversion to nootkatone (**10**) seemed unlikely to us. However, it cannot be ruled out due to possible (non-enzymatic) catalytic effects of metal ions present in the

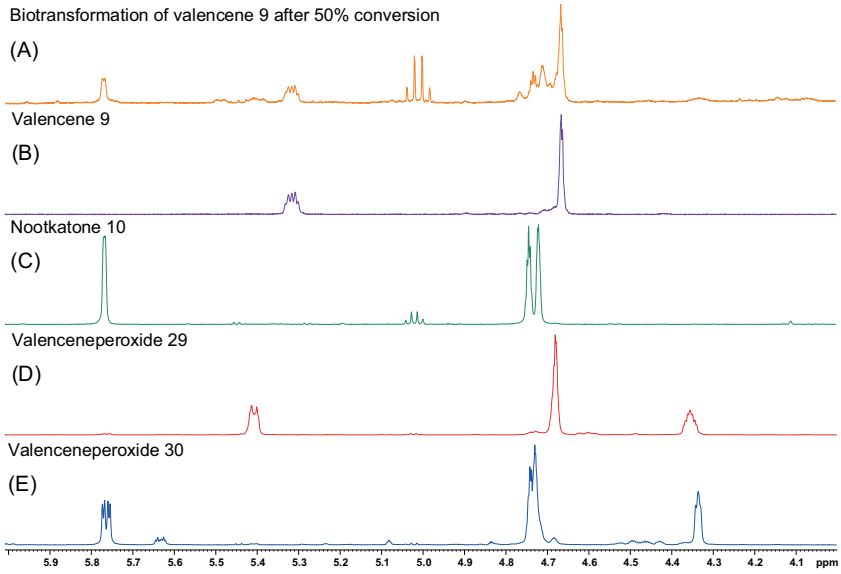
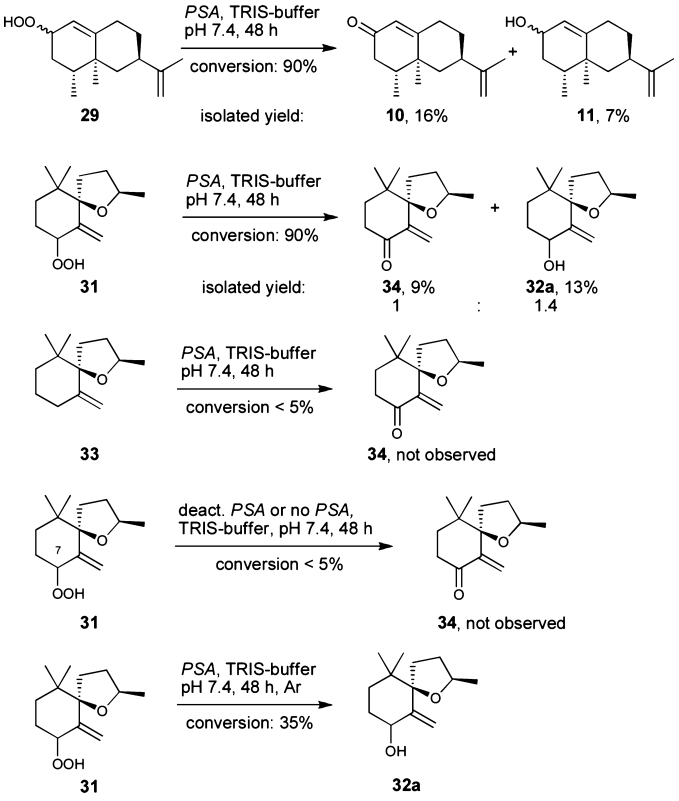


Fig. 1. Stretches of the ^{1}H NMR spectra of the biotransformation of valencene stopped after 50% conversion (A) with reference NMR spectra of valencene (**9**) (B), nootkatone (**10**) (C) and valencene peroxides **29** (D) and **30** (E).



Scheme 4. Evaluation of peroxides **29**, **31**, and alkene **33** as substrates for *PSA* oxidation.

reaction mixture. Peroxides **29** and **31** were therefore treated with *PSA* lyophilisate according to our standard protocol (Scheme 4). As expected, valencene-derived peroxide **29** was rapidly converted to nootkatone (**10**) as a major product along with stereoisomeric nootkatols. Interestingly, theaspirane-derived peroxide **31** was also converted to the corresponding enone **34** and alcohol **32a**. This conversion is remarkable, because peroxide **31** cannot be formed from alkene **33** with *PSA* ($BDH_{7\text{-}H}$ = 81.7 kcal/mol at B3LYP/6-31 + G** SCRF = PCM). Once a peroxide is formed it is thus obviously non-selectively converted to the corresponding enone. This finding raised again the question for a possible non-enzymatic conversion of peroxides like **29** and **31**. However, neither reaction of **31** in TRIS buffer without *PSA* lyophilisate nor with thermally deactivated *PSA* lyophilisate furnished any of the target enone **34** and peroxide **32a** was left unchanged (conversion < 5%). It is worth noting the effect of oxygen on this reaction. If the conversion of **31** with *PSA* was performed under an argon atmosphere, low conversion of **31** (35%) and the formation of alcohol **32a** as single product was observed.

4. Conclusions

Biocatalytic oxidations with *PSA* show a remarkable broad substrate scope and a number of isoprenoids and fatty acids have been successfully converted to enones and alcohols as major products. 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 **14**, **16**, **17**, **19**, **20**, **24**, **26**, and **28**. 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.

Hydroperoxide intermediates have been proposed for *PSA* oxidations in previous studies, but they have never been characterized nor has a plausible explanation for their spontaneous conversion to enones been provided. With the synthesis and purification of allylic hydroperoxide **29** derived from valencene we have been able to characterize the putative direct precursor of nootkatone in *PSA* oxidations of valencene. We have not been able to observe allylic peroxide **29** during the biocatalytic oxidation of valencene. However, using pure peroxide **29** as a substrate, we found that it is converted to nootkatone by an enzymatic reaction. The non-enzymatic redox disproportionation of **29** was found to be slow under the conditions employed.

In summary our results suggests a two-step oxidation mechanism involving hydroperoxide intermediates which are rapidly converted to the observed enones. The initial radical reaction determines the regioselectivity of the conversions. Both steps of the

oxidation may be catalyzed by the same enzyme with lipoxygenase and peroxidase activity.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.07.008

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