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PAPER

Enzymatic allylic oxidations with a lyophilisate of the edible fungus *Pleurotus sapidus*†

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Allylic oxidations belong to the most attractive synthetic transformations because they convert readily available and cheap starting materials into value-added products. In this study, we describe oxidative conversions of terpenoids and a number of related cycloalkenes with a lyophilisate of the edible fungus *Pleurotus sapidus*. The biocatalytic protocol is simple and the biocatalyst is readily available. The conversions of various cycloalkenes proceed cleanly in most cases to the corresponding enones. The substrate scope is remarkable and includes a number of mono- and sequiterpenes, functionalized terpenoids as well as simple cyclohexenes and benzylic substrates. Enzymatic allylic oxidations by *Pleurotus sapidus* are thus an excellent non-toxic alternative to metal-mediated oxidation procedures in academic labs and for industrial application in food technology, cosmetics or pharmaceutical research.

Introduction

Enzymatic reactions are valuable tools for organic synthesis. Applications range from industrial ton-scale productions of bulk chemicals[1] to sophisticated transformations in complex natural product syntheses in academia.[2–3] Today, the enzymatic toolbox covers a wide range of chemical reactions and the repertoire of enzymatic reactions is continuously expanded by screening efforts,[4] directed evolution,[5] and protein[6] as well as genetic engineering.[7] The advantages of enzymes in organic synthesis are manifold and include "green" arguments such as toxicity issues and mild reaction conditions as well as frequently high levels of chemoselectivity, regioselectivity and stereoselectivity.[8–9] On the other hand, selectivity is often also regarded a disadvantage, because the substrate scope of many enzymatic conversions is limited. In consequence, it is often hard to predict for synthetic organic chemists, whether a given enzymatic protocol will have a favourable outcome for an individual synthetic problem. An additional frequently encountered problem for many synthetic chemists is the availability of the required biocatalysts. These factors account for a somewhat reserved attitude of many chemists towards enzymatic operations and result in a relatively low number of enzymatic transformations in the synthetic organic literature compared to non-biocatalytic methods.

Selective oxidations of C–H bonds are particularly attractive transformations with a broad application spectrum and a high impact on the industrial chemical value chain as they convert relatively cheap molecules into value-added products.[10] Among these transformations, allylic oxidations are of high interest, because the required olefinic educts are readily available and the resulting allyl alcohols or α,β-unsaturated carbonyl compounds are attractive synthetic targets. Traditionally, chromium based oxidants have been used for conversions of olefins to α,β-unsaturated carbonyl compounds and excellent protocols have been developed by Muzard,[11–12] Müller[13] and Dauben.[14] These stoichiometric protocols have later been replaced by catalytic metal based variants (Fig. 1).[15–18]

However, from an economical and ecological point of view it is still desirable to compliment the metal catalysed variants with

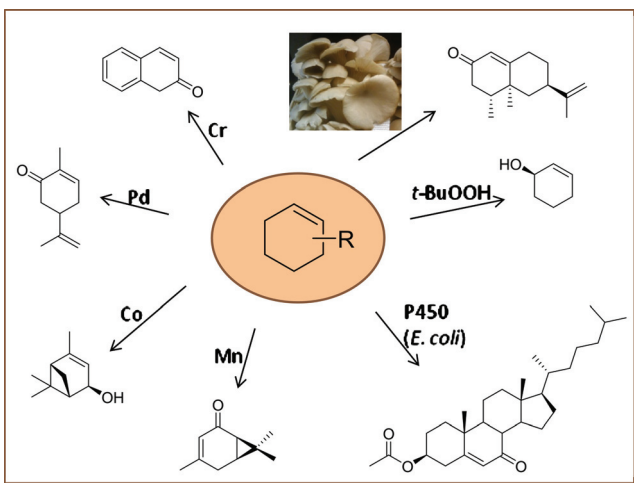

Fig. 1 Selected methods for the allylic oxidation of alkenes.

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Fig. 2 Submerged culture of *Pleurotus sapidus*.

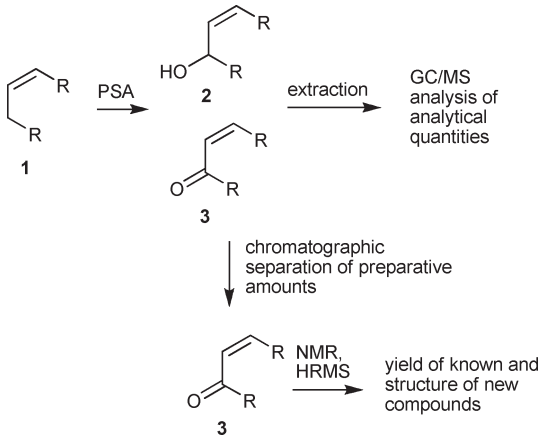
biocatalytic approaches. In fact, a number of biocatalytic methods for the conversion of olefins into α,β-unsaturated carbonyl compounds have been reported and applied to the synthesis of fine chemicals,[19–21] drugs[22] and food ingredients.[23–25]

In this context, a number of microorganisms or fungi are able to oxidize terpenoids, which are attractive substrates for many flavour compounds. A good example is the selective and efficient allylic oxidation of the sesquiterpene (+)-valencene **4** to the grapefruit flavour compound (+)-nootkatone **5** that was achieved with the edible fungus *Pleurotus sapidus* (*PSA*).[23,26] Particularly toxicity issues make the use of edible fungi attractive compared to other biocatalytic systems like bacteria or yeasts: they are nontoxic and for this reason their application in food, pharmaceutical and cosmetic industry is simple and safe. It should be noted that biocatalytic oxidations with *PSA* may be performed with the lyophilisate of the fungus. The required biocatalytic systems are thus readily available even for synthetic laboratories without microbiological expertise. The catalytically active lyophilisate may be obtained in any desired amount by freeze drying of the fungal fruiting bodies, which are commercially available and may be grown in submerged cultures (Fig. 2).

In this paper we describe the scope of the enzymatic allylic oxidation with *PSA* for synthetic organic chemistry. A special focus is set on the practicality of the method for the synthetic community, and we attempt to provide an easy-to-use protocol for preparatively useful enzymatic allylic oxidations using lyophilisates of the fungus.

Results and discussion

The standard work flow of the following study is depicted in Scheme 1. In typical experiments with alkenes **1**, the biotransformations were run on a small scale, and the outcome of the reaction (in most cases mixtures of allyl alcohols **2** and enones **3**) was evaluated by GC-MS analysis of an ether extract of the reaction. The products **2** and **3** were identified by their Kováts-indices and a comparison of MS-spectra with literature data. This method allows the fast evaluation of test conversions, but is restricted to known reaction products **2** and **3**. To evaluate the chemical yields of the biotransformation and to identify new compounds, we have performed a number of conversions on a preparative scale and have purified the resulting mixtures by column chromatography, HPLC or preparative GC. The resulting products (*e.g.* **3**) were thus obtained in sufficient quantities to analyze their structure by NMR and HRMS. Each transformation



Scheme 1 Workflow for the analysis of biotransformations.

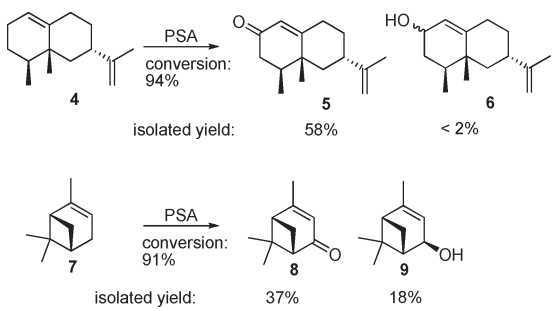
has also been performed under otherwise identical conditions with temperature deactivated lyophilisate and without lyophilisate but 5 equivalents of H_2O_2. No conversion of starting materials was detected in these blind probes after 48 h, confirming enzymatic transformations of alkenes with *PSA*.

We started our investigation with two well-known allylic oxidations of valencene **4** and pinene **7**.[23,26–28]

It should be noted that the oxidation of (+)-valencene **4** to (+)-nootkatone **5** has also been reported by Willershausen *et al.* with supernatants of the white rot fungus *Phanerochaete chrysosporium*,[29] whereas in another approach fungal laccases were used.[30] In addition, (+)-nootkatone **5** was produced by Kaspera *et al.* with submerged cultures of the ascomycete *Chatomium globosum*.[31–32]

On a preparative scale, both conversions proceeded smoothly and gave the expected α,β-unsaturated ketones **5** and **8** along with varying quantities of the corresponding alcohols **6** and **9** as the major byproducts. The regioselectivity of these conversions matches the expectations for oxidations *via* a radical mechanism.[10,23]

The biotransformation of α-pinene **7** gave verbenone **8** and *trans*-verbenol **9** (Scheme 2). The relative stereochemistry of the latter was verified unambiguously by 2D-NOESY-NMR. The reactions depicted in Scheme 2 were both followed by GC-MS indicating almost complete conversion of starting materials after 48 h. The transfer of these high conversion rates into



Scheme 2 Biocatalytic oxidations of valencene **4** and α-pinene **7** using *PSA*-lyophilisates.

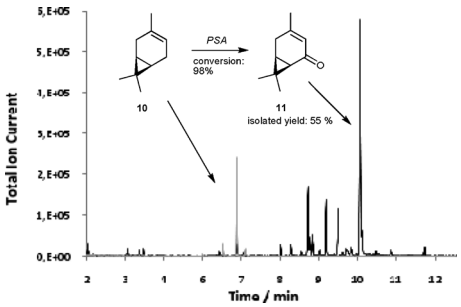


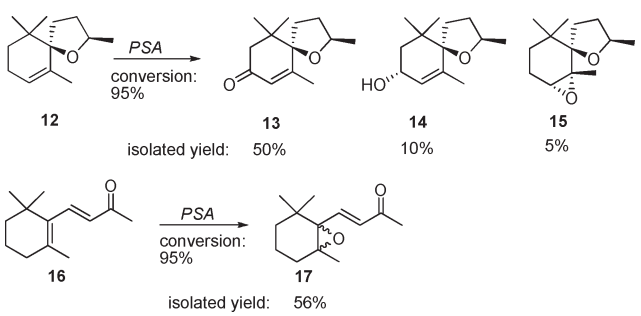
Fig. 3 GC trace for the crude product obtained by oxidation of careen **10**.

preparatively useful isolated chemical yields was verified by standard chromatographic purification of both ketones **5** and **8**. It should be noted, that the isolated yields are affected by the high volatility of the target ketones leading to significant losses of the products during work-up.

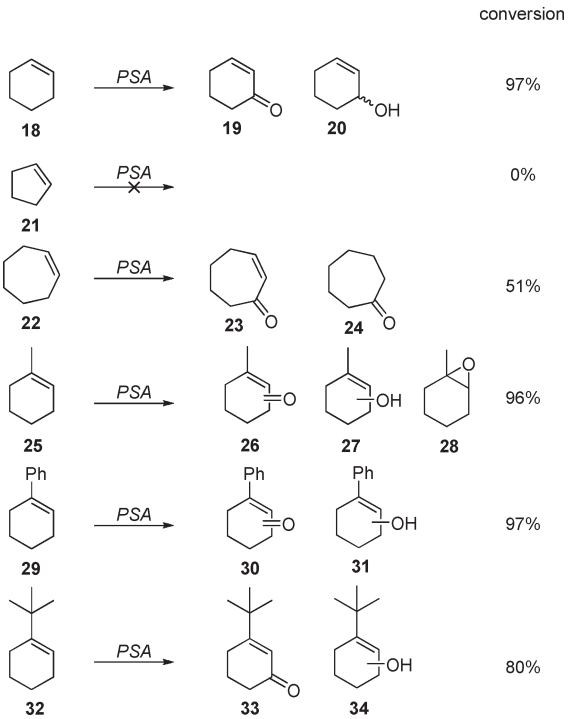
To evaluate the substrate scope of the oxidation with *PSA*, we selected further mono- and sequiterpenes containing cyclohexenyl moieties and submitted them to our standard oxidation conditions. We followed the reactions again by GC-MS and identified most of the products by their Kováts-indices and a comparison of MS-spectra with literature data. In some cases we have not been able to identify the products unambiguously based on GC-MS parameters. These substances were purified and identified by NMR-analyses.

The biotransformation of 3-carene **10** resulted in only one main product (Fig. 3) that was identified as 3-caren-5-one **11** by NMR after chromatographic purification. The reaction is quite clean and proceeds with high regio- and chemoselectivity: neither the regioisomeric 3-caren-2-one nor 3-caren-5-ol or 3-caren-2-ol were detectable.

Particularly interesting is the oxidation of functionalized terpenoids like theaspirane **12** and β-ionone **16** (Scheme 3). The conversion of theaspirane proceeded smoothly to give the expected enone **13** as the major product, along with some allyl alcohol **14** and epoxide **15**. Theaspirane and its derivatives have various biological activities and are used as flavors and lead structures in pharmaceutical research.[33–36] The three oxidation products from theaspirane are useful precursors for the synthesis of vitispirane.[35,37] Interestingly, the oxidation of β-ionone **16** did not give the expected dienone, but the epoxide **17** as an enantiomeric mixture (4% ee) in a quite clean conversion. Compound



Scheme 3 Biocatalytic oxidation of theaspirane **12** and β-ionone **16**.



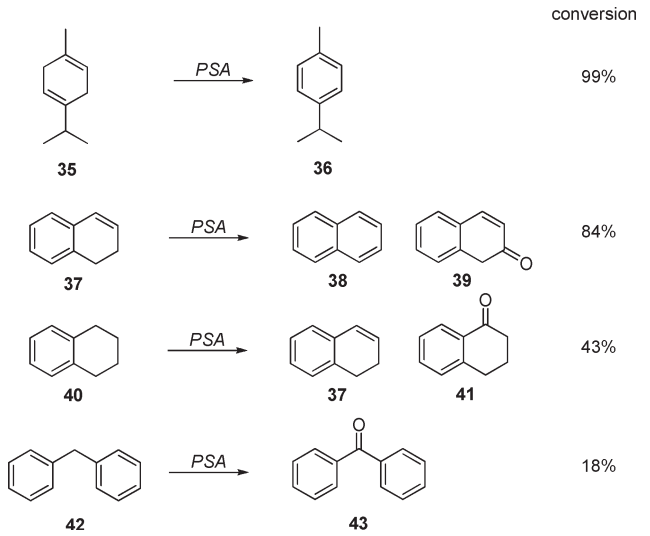
Scheme 4 Biotransformations of non-terpenoide starting materials.

17 has been used as a model metabolite in medicinal chemistry[38] and is also an important precursor in the synthesis of flavour compounds. A prominent example is the synthesis of dihydroactinidiolide, a major component of black tea aroma.[39] Encouraged by these positive results with terpenoids, we submitted a number of non-terpenoid substrates to the enzymatic oxidation protocol and varied the core structure of the alkene.

As depicted in Scheme 4, cyclopentene **21** is not recognized as a substrate at all, and cycloheptene **22** is oxidized very slowly to the corresponding enone **23** and cycloheptanone **24**. Cyclohexene **18** in turn was completely converted to a mixture of cyclohexenone **19** and an isomeric mixture (3% ee) of cyclohexenol **20**. Remarkable is the fact, that *PSA* oxidized cyclohexene **18** at only one allylic position. Several other cyclohexene derivatives like methylcyclohexene **25** and phenylcyclohexene **29** were also converted successfully to the expected allylic alcohols and enones, but with limited regioselectivity. Of the simple cyclohexene derivatives depicted in Scheme 4, only *tert*-butyl cyclohexene **32** was converted to the enone **33** with good regioselectivity.

As depicted in Scheme 5, terpinene **35** was oxidized completely to cymene **36**. In contrast, the oxidation of dihydronaphthalene **37** gave a 1 : 1-mixture of naphthalene **38** and the target ketone **39**. Benzylic substrates like **40** and **42** were also converted by *PSA* although significantly slower than alkenes. However, the conversions were again quite clean giving the expected ketones **41** and **43**.

In conclusion, a number of new conversions with the lyophilisate of *PSA* have been described. The substrate scope of the reaction is relatively broad and derivatives of cyclohexene are generally accepted as substrates of these biocatalytic oxidations (although with varying regioselectivities). Several sesqui- and monoterpenes are oxidized cleanly to the corresponding enones



Scheme 5 Allylic and benzylic oxidations.

with high regioselectivity. In addition, benzylic oxidations may be performed with *PSA* to give exclusively ketones as oxidation products. The experimental procedures are extremely simple as is the preparation of the catalyst making this method an attractive protocol for the oxidation of terpenoids to valuable oxidation products.

Experimental section

The filamentous fungus *Pleurotus sapidus* was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ 8266), Braunschweig, Germany.

Production of biomass and lyophilisation were described previously by Fraatz *et al.*[23]

General procedure for the bioconversion

400 mg of *PSA* lyophilisate were dissolved in 20 mL TRIS HCl buffer. The dried cell mass was rehydrated by stirring at 900 rpm for 10 min. Afterwards 0.25 mmol of the substrate were added. The reaction mixture was stirred at 25 °C and 900 rpm for 24 h. Subsequently, additional 400 mg lyophilisate and 20 mL TRIS-HCl-buffer were added. After stirring of the reaction mixture for the next 24 h, 50 mL Et_2O were added and the resulting mixture was stirred for further 30 min. Solids were removed by filtration, and the aqueous solution was extracted three times each with 100 mL Et_2O. The organic layer was dried over Na_2SO_4, concentrated to 1 mL and analysed without further treatment by gas chromatography (GC-FID) and mass spectrometry (GC-MS). If required, the product was purified by column chromatography.

Physical and spectral data of the products

Biotransformation of (+)-valencene **4**; according to the general procedure 55 μL (+)-valencene **4** (0.25 mmol) were treated with 800 mg lyophilisate for 48 h. The crude product was purified by column chromatography (Pentane/Et_2O 1 : 1) to give 15 mg of (+)-nootkatone **5** (28% yield). **(+)-Nootkatone 5**. ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 5.76 (s, 1H), 4.75–4.72 (m, 2H), 2.56–2.47 (m, 1H), 2.40–2.20 (m, 4H), 2.05–1.88 (m, 3H), 1.73 (s, 3H), 1.40–1.30 (m, 2H), 1.11 (s, 3H), 0.96 (d, 3H, ^3J = 6.9 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$, δ): 200.0, 170.7, 149.3, 124.8, 109.4, 44.1, 42.2, 40.6, 40.5, 39.5, 33.2, 31.8, 21.0, 17.0, 15.1; GC-MS *m/z* (%): 218 (12, M^+), 203 (27, M − Me), 200 (20, M − H_2O), 190 (31, M − C_2H_4), 176 (20, M − C_2H_2O), 175 (25, M − C_3H_7), 162 (17, M − C_4H_8), 147 (64, M − C_4H_8 + Me), 146 (41, M − 72), 133 (52, M − 85), 121 (61, M − 97), 108 (47, M − 110), 107 (37, M − 111), 105 (50, M − 113), 93 (61, M − 125), 91 (83, M − 127), 80 (44, M − 138), 79 (82, M − 139), 77 (57, M − 141).

Biotransformation of α-pinene **7**: according to the general procedure 39 μL α-pinene **7** (0.25 mmol) were treated with 800 mg lyophilisate for 48 h. The crude product was purified by column chromatography (pentane/Et_2O 9 : 1) to give 33 mg of verbenone **8** (37% yield) and 16 mg of verbenol **9** (18% yield). **verbenone 8**. GC-MS *m/z* (%): 150 (34, M^+), 135 (61, M − Me), 107 (100, M − C_3H_7), 91 (72, M − 59), 80 (53, M − 70), 79 (46, M − 71), 67 (23, M − 83), 55 (25, M − 95). **Verbenol 9**. ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 5.34–5.35 (m, 1H), 4.26–4.27 (m, 1H), 2.23–2.28 (m, 1H), 2.15–2.19 (m, 1H), 2.01–2.04 (m, 1H), 1.72 (t, 3H, ^3J = 1.5 Hz), 1.32 (s, 3H), 1.25–1.30 (m, 1H) 0.87 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$, δ): 149.1, 118.9, 70.7, 48.3, 47.3, 46.4, 28.8, 26.8, 22.8, 20.6; GC-MS *m/z* (%): 152 (M^+), 134 (M−H_2O), 119 (M−33), 91 (M−61), 77 (M−75), 65 (M −87), 51 (M−101). GC-MS *m/z* (%): 134 (15, M − H_2O), 119 (45, M − Me − H_2O), 91 (100, M − C_3H_7 + H_2O), 77 (25, M − 75).

Biotransformation of 3-carene **10**: according to the general procedure 39 μL 3-carene **10** (0.25 mmol) were treated with 600 mg lyophilisate for 48 h. The crude product was purified by column chromatography (pentane/Et_2O 10 : 1) to give 28 mg of 3-carene-5one x (25% yield). **3-Carene-5-one 11**. ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 5.82 (s, 1H), 2.63 (dd, 1H, ^2J = 20.4 Hz, ^3J = 8.2 Hz), 2.32 (d, 1H, ^3J = 20.8 Hz), 1.86 (s, 3H), 1.55 (d, 1H, ^3J = 7.4 Hz), 1.44 (t, 1H, ^3J = 8.0 Hz), 1.18 (s, 3H), 1.03 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$, δ): 196.8, 159.1, 126.5, 33.0, 28.6, 28.0, 26.0, 23.9, 22.7, 14.5; GC-MS *m/z* (%): 150 (48, M+), 135 (20, M − Me), 107 (100, M − C_3H_7), 91 (81, M − CH_3O + H_2O), 79 (60, M − C_4H_8 + Me).

Biotransformation of β-ionone **16**: according to the general procedure 51 μL β-ionone **16** (0.25 mmol) were treated with 1000 mg lyophilisate for 120 h. The crude product was purified by column chromatography (pentane/Et_2O 10 : 1) to give 30 mg of β-ionone-epoxide **17** (56% yield). **β-Ionone-4,5-epoxide 17**. ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 7.01 (d, 1H, ^3J = 15.9), 6.28 (d, 1H, ^3J = 15.9), 2.26 (s, 3H), 1.90–1.85 (m, 1H), 1.77–1.71 (m, 1H), 1.43–1.39 (m, 3H, 1-H), 1.12 (s, 6H), 1.09–1.05 (m, 1H), 0.91 (s, 3H);^{13}C-NMR (100 MHz, $CDCl_3$, δ): 197.8, 142.9, 132.6, 70.8, 66.0, 35.6, 33.7, 30.0, 28.4, 26.0, 25.9, 20.9, 17.0; GC-MS *m/z* (%): 208 (3, M^+), 190 (18, M − H_2O), 175 (50, M − Me − H_2O), 123 (95, M − 85), 69 (84, M − 139).

Biotransformation of *cis*-theaspirane **12**: according to the general procedure 125 mg *cis*-theaspirane **12** (0.64 mmol) were treated with 1000 mg lyophilisate for 70 h. The crude product

was purified by column chromatography (pentane/Et_2O, 95 : 5, 6 : 4) to give 66 mg of theaspirone **13** (50% yield) and 13 mg of theaspirol **14** (10% yield). **Theaspirone 13**. ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 5.72 (s, 1H), 4.24–4.16 (m, 1H), 2.40 (m, 1H), 2.35–2.30 (m, 1H), 2.20 (m, 1H), 2.05–2.01 (m, 1H), 1.83–1.75 (m, 1H), 1.55–1.44 (m, 1H), 1.98–1.97 (m, 3H), 1.30 (d, ^3J = 6.0 Hz, 3H), 1.02 (2 s, 3H), 0.97 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$, δ): 198.6, 168.5, 125.0, 88.7, 77.9, 50.4, 40.9, 34.5, 32.8, 24.6/23.1, 20.6, 19.1; GC-MS *m*/*z* (%): 208 (M^+), 153 (10, M − 55), 152 (100, M − 56), 111 (22, M − 97), 110 (85, M − 98), 96 (15, M − 112), 82 (15, M − 126), 69 (15, M − 139), 55 (12, M − 153). **Theaspirol 14**. ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 5.33–5.32 (m, 1H), 4.25–4.20 (m, 1H), 4.16–4.08 (m, 1H), 2.10–1.37 (m, 6H), 1.75–1.74 (m, 3H), 1.27–1.25 (m, 3H), 0.95 (s, 3H), 0.90 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$, δ): 143.7, 124.9, 88.7, 77.3, 66.3, 45.4, 39.4, 35.0/34.6, 25.1/22.3, 20.8, 18.2; GC-MS *m*/*z* (%): 210 (M^+), 192 (96, M − H_2O), 177 (48, M − 33), 154 (100, M − 56), 149 (24, M − 61), 135 (44, M − 75), 125 (22, M − 85), 121 (66, M − 89), 119 (32, M − 91), 107 (38, M − 103), 98 (35, M − 112), 93 (98, M − 117), 119 (68, M − 101), 133 (51, M − H_2O), 141 (35, M − 33), 145 (29, M − 61), 155 (50, M − 75).

Biotransformation of cyclohexene **18**: according to the general procedure 101 µL cyclohexene **18** (0.25 mmol) were treated with 600 mg lyophilisate for 48 h. **Cyclohexenone 19**. GC-MS *m*/*z* (%): 96 (24, M^+), 68 (100, M − C_2H_4). **Cyclohexenol 20**. GC-MS *m*/*z* (%): 98 (22, M^+), 97 (30, M − 1), 83 (40, M − Me), 79 (77, M − 19), 77 (40, M − 21), 70 (100, M − 28).

Biotransformation of **cycloheptene 22**: according to the general procedure 58 µL cycloheptene **22** (0.25 mmol) were treated with 800 mg lyophilisate for 120 h. **Cycloheptanone 24**. GC-MS *m*/*z* (%): 112 (22, M^+), 84 (20, M − C_2H_4), 68 (63, M − 44), 55 (100, M − 57). **Cyclohepten-1-one 23**. GC-MS *m*/*z* (%): 110 (30, M^+),81 (98, M − 29), 68 (59, M − 42), 67 (71, M − 43), 66 (68, M − 44), 54 (100, M − 56).

Biotransformation of *tert*-butyl cyclohexene **32**: according to the general procedure 42 µL *tert*-butyl cyclohexene **32** (0.25 mmol) were treated with 600 mg lyophilisate for 23 h. ***tert*-Butyl cyclohexenone 33**. GC-MS *m*/*z* (%): 152 (26, M+), 137 (8, M − CH_3), 124 (27, M − C_2H_4), 109 (100, M − C_3H_7), 96 (94, M − 56), 81 (40, M − 71), 67 (58, M − 85), 57 (55, M − 95), 55 (31, M − 97), 51 (27, M − 101).

Biotransformation of terpinene **35**: according to the general procedure 40 µL terpinene **35** (0.25 mmol) were treated with 800 mg lyophilisate for 96 h. **Cymene 36**. ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 7.14–7.10 (m, 4H), 2.89–2.85 (m, 1H), 2.31(s, 3H), 1.24 (d, 6H, ^3J = 7.0 Hz);^{13}C-NMR (100 MHz, $CDCl_3$, δ): 146.0, 135.3, 129.1, 126.4, 33.8, 24.2, 2.1; GC-MS *m*/*z* (%): 134 (24, M^+), 119 (100, M − Me), 117 (16, M − 17), 91 (34, M − C_3H_7), 77 (13, M − 57), 65 (13, M − 69).

Biotransformation of 1,2-dihydronaphthalene **37**: according to the general procedure 45 µL 1,2-dihydronaphthalene **37** (0.25 mmol) were treated with 800 mg lyophilisate for 48 h. **Naphthalene 38**. GC-MS *m*/*z* (%): 128 (100, M^+), 102 (10, M − C_2H_2). **Enone 39**. GC-MS *m*/*z*: 146 (46, M^+), 115 (62, M − CH_3O), 104 (100, M − C_2H_2O).

Biotransformation of tetrahydronaphthalene **40**: according to the general procedure 34 µL tetrahydronaphthalene **40** (0.25 mmol) were treated with 800 mg lyophilisate for 74 h. **1,2-Dihydronaphthalene 37**. GC-MS *m*/*z* (%): 130 (100, M^+), 128 (81, M − 2), 115 (52, M − CH_3), 102 (16, M − 28), 91 (10, M − 39), 77 (24, M − 53), 63 (40, M − 67), 51 (64, M − 79). **Ketone 41**. GC-MS *m*/*z* (%): 146 (36, M^+), 131 (16, M − CH_3), 118 (100, M − 28), 115 (17, M − 31), 90 (88, M − 56), 77 (11, M − 69), 63 (41, M − 83), 51 (32, M − 95).

Biotransformation of diphenylmethane **42**: according to the general procedure 42 µL diphenylmethane **42** (0.25 mmol) were treated with 1000 mg lyophilisate for 48 h. **Benzophenone 43**. GC-MS *m*/*z* (%): 182 (44, M^+), 105 (100, M − 77), 77 (65, M − 105), 51 (35, M − 131).

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