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Microbial monooxygenases applied to fragrance compounds

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Abstract

Biotransformation is an important tool in organic syntheses, especially for the production of chiral molecules and whenever chemical reactions are inefficient. Fragrance compounds were biotransformed by *Trichosporum cutaneum* CCT 1903 whole cells. Batch reactions with *cis*-jasmone (**1**), (*R*)-(−)-carvone (**3**), α- and β-ionones (**4**, **5**) and (*R*)-(+)-limonene (**10**) produced 4-hydroxyjasmone (**1a**), 7,8-epoxyjasmone (**1b**), 7,8-dihydroxyjasmone (**1c**), (1*S*,2*R*,4*R*)-neoisodihydrocarveol (**3a**), (6*R*)-isoprenyl-(3*R*)-methyl-2-oxo-oxepanone (**3b**), (3*R*)-isopropenyl-6-oxoheptanoic acid (**3c**), 2,3-epoxy-(5*R*)-isopropenyl-2-methylcyclohexenol (**3d**), α-homo-cyclogeraniol (**4a**), 4-oxo-7,8-dihydro-β-ionone (**5a**), uroterpenol (**10a**) and (*R*)-(+)-limonene-1,2-diol (**10b**) as pure samples for spectroscopic identification (^{1}H and ^{13}C NMR, ^{1}H,^{1}H-gCOSY, HSQC, gHMBC). The stereochemical consequences of the transformations are also discussed.
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Keywords: Biotransformation; Monoterpenes; Baeyer–Villiger monooxygenase; Epoxidation; Hydroxylation

1. Introduction

Biocatalytic processes, using whole cells or isolated enzymes as catalytic agents, have found widespread applications [1]. This is particularly true in the pharmaceutical and agrochemical industries where optically pure molecules are particularly critical [2]. The application of oxidoreductases for the production of enantiomerically pure compounds is an attractive synthetic strategy in organic chemistry due to the high chemo-, regio- and stereo-specificity that can be achieved [3].

Oxygenases are oxidoreductases capable of incorporating molecular oxygen into organic compounds [4]. The oxygen transfer can be intermediated by three different enzymes: monooxygenases, dioxygenases and oxidases. These reactions are particularly fascinating since direct oxyfunctionalization of unactivated organic molecules is a challenge to synthetic chemists [5].

For several reasons, industrial-scale processes that make use of oxygenases are preferably based on whole-cell biocatalysts. First, the *in vitro* cofactor regeneration, though feasible, is relatively expensive. Second, oxygenases often consist of multiple components frequently located in the cell membrane [6].

Bioprospecting oxygenases and hydrolases in commercially available microorganisms (CCT, Coleção de Culturas Tosello [7] and CBMAI, Coleção Brasileira de Microrganismos de Ambiente e Indústria) to transform sulfides into sulfoxides [8,9], ketones into esters/lactones and epoxides into diols [10], we found that *Trichosporum cutaneum* CCT 1903 could preferentially oxidize the C_7–C_8 *cis*-jasmone double bond [11]. Aware that non-oxygenated terpenes are responsible for the fatty unpleasant notes in fragrant mixtures while oxygenated terpenes are responsible for the floral and woody notes of essential oils [12], the aim of the present research was to transform fragrant compounds by *T. cutaneum* CCT 1903 whole cells exploiting its oxygenase activity. The stereochemical consequences of the transformations are also discussed.

2. Experimental

2.1. General methods

Cis-jasmone (**1**), 6-methyl-5-hepten-2-one (**2**), (*R*)-(−)-carvone (**3**), α- and β-ionones (**4**, **5**), linalool (**6**), β-citronellol (**7**), geraniol (**8**), α-bisabolol (**9**), (*R*)-(+)-limonene (**10**), valencene (**12**), α- and β-pinene (**13**, **14**) are commercially available. 1-Isopropenyl-2,5-dimethoxy-4-methylbenzene (**11**) and jinkoh-eremol (**15**) were isolated from Brazilian plants

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[13]. Thin-layer chromatography (TLC) was performed using precoated plates (Aluminum foil, silica gel 60 F_{254} Merck, 0.25 mm), with visualization done using *p*-anisaldehyde/sulfuric acid followed by heating (approximately 120 °C). Merck 60 silica gel (230–400 mesh) was used for column chromatography. Enzymatic reactions were monitored by GC (FID) with a HP 5890 chromatograph, using hydrogen as the carrier gas, or by GC–MS using a HP 5970-MS detector and helium as the carrier gas. The fused silica capillary columns used were either a J and W Scientific DB-5 (30 m × 0.25 mm × 0.25 μm), a chiral column heptakis-(2,6-di-*O*-methyl-*O*-pentyl-3)-β-cyclodextrin (25 m × 0.25 mm × 0.25 μm) kindly provided by A. Morel (Laboratory of Organic Chemistry, Santa Maria Federal University, Brazil) and a Chirasil-β-cyclodextrin (25 m × 0.25 mm × 0.25 μm) from Chrompack (The Netherlands), the latters for determination of the enantiomeric excesses. ^{1}H NMR spectra were recorded with an Inova 500 (499.88 MHz) or a Varian Gemini 300 (300.07 MHz) spectrometers. ^{13}C NMR spectra were obtained with an Inova 500 (125.70 MHz) or a Varian Gemini 300 (75.45 MHz) spectrometers. $CDCl_3$ was used as solvent, with Me_4Si (TMS) as internal standard.

2.2. Microorganism

The yeast *T. cutaneum* CCT 1903 was purchased from the Culture Collection of the André Tosello Foundation.

2.3. Preservation of the microorganism

The microorganism was stored (at 4 °C) in slants containing 20 mL of yeast and malt extract medium [glucose (10 g L^{-1}, synth), peptone (5 g L^{-1}, oxoid), yeast extract (3 g L^{-1}, oxoid), malt extract (3 g L^{-1}, oxoid)] and agar (20 g L^{-1}, oxoid).

2.4. General procedure for microorganism culture

The *T. cutaneum* CCT 1903 cells from slants were cultivated for 96 h in an orbital shaker (150 rpm) at 28 °C in Erlenmeyer flasks (500 mL) containing 100 mL of the yeast and malt extract medium prepared as described in Section 2.3. Sterile materials were used to perform the experiments and the microorganism was manipulated in a laminar flow cabinet.

2.5. General procedure for biotransformation

The substrate (20 μL) and wet *T. cutaneum* CCT 1903 biomass (0.5–2.0 g) were added to an Erlenmeyer flask (125 mL) containing phosphate buffer solution (50 mL, Na_2HPO_4 and KH_2PO_4, 72 mM, pH 6.5). The mixture was stirred in an orbital shaker (28 °C, 150 rpm). Upon reaching the appropriate degree of conversion, the cells were separated by centrifugation, the aqueous solution was saturated with sodium chloride and extracted with ethyl acetate and the organic layer was dried with anhydrous Na_2SO_4. After filtration, the organic solvent was then evaporated. The progress of the biotransformation was monitored by GC–MS.

2.6. General procedure for multibioreaction

To four flasks (125 mL,) containing 50.0 mL of phosphate buffer (Na_2HPO_4 and KH_2PO_4, 72 mM, pH 6.5) were added 2 g each of *T. cutaneum* CCT 1903 wet cells, obtained as described in 2.4 and 10.0 mg of each compound to be tested. Flask 1: 6-methyl-5-hepten-2-one (**2**), (*R*)-(−)-carvone (**3**), α- and β-ionones (**4**, **5**); flask 2: linalool (**6**), β-citronellol (**7**), geraniol (**8**), α-bisabolol (**9**); flask 3: (*R*)-(+)-limonene (**10**), 1-isopropenyl-2,5-dimethoxy-4-methylbenzene (**11**), valencene (**12**); flask 4: α- and β-pinene (**13**, **14**) and jinkoh-eremol (**15**). The bioreactions were monitored for 96–120 h. The samples (1 mL) were saturated with sodium chloride and extracted with EtOAc (3× 800 μL). The organic solvents of the four reactions were evaporated and the residue were diluted in 100.0 μL of diethyl acetate containing 0.2 mg·mL^{-1} of pentadecane (internal reference). The GC–MS analyses were conducted as follows: 50–290 °C with a 20 °C·min^{-1} ramp, T_{inj} = 200 °C, 1 mL min^{-1} flow rate.

2.7. Synthesis of the cis-jasmone (*1*) metabolites

Scaling up a bioreaction for *cis*-jasmone (**1**, 1.22 mmol) resulted 300 mg crude biotransformation product. Purification by silica gel column chromatography eluted with hexane–ethyl acetate–NH_4OH (starting from 93:2:5 and increasing the polarity of the final eluting mixture to 9.5:9.5:1) furnished pure 4-hydroxyjasmone (0.062 mmol, 3.4%), 7,8-epoxyjasmone (0.242 mmol, 13%), and 7,8-dihydroxyjasmone (0.112 mmol, 6.2%). 4-Hydroxyjasmone (**1a**) MS (EI, 70 eV) *m*/*z* (rel. inten.): 180 ($M^{\bullet+}$, 100), 151 (83), 137 (39), 133 (47), 111 (40), 109 (82), 105 (49), 95 (48), 79 (54), 77 (51), 67 (38), 53 (30), 43 (81). ^{1}H NMR (499.88 MHz, $CDCl_3$) δ: 5.41 (1H, dtt, *J* = 17.8, 7.5 and 1.5 Hz, H-8), 5.23 (1H, dtt, *J* = 17.8, 7.3 and 1.5 Hz, H-7), 4.71 (1H, d_l, H-4), 2.94 (2H, d_l, H-6), 2.78 (1H, dd, *J* = 18.4 and 6.3 Hz, H-8), 2.28 (1H, dd, *J* = 18.4 and 2.2 Hz, H-5), 2.16 (2H, ddd, *J* = 7.5, 7.3 and 1.5 Hz, H-9), 2.10 (3H, s, H-11), 0.99 (3H, t, *J* = 7.5 Hz, H-10). ^{13}C NMR (125.69 MHz, $CDCl_3$) δ: 204.9 (C-1), 168.7 (C-3), 140.9 (C-2), 132.9 (C-8), 124.1 (C-7), 71.6 (C-4), 44.2 (C-5), 21.1 (C-6), 20.5 (C-9), 13.7 (C-11), 14.1 (C-10). 7,8-Epoxyjasmone (**1b**) MS (EI, 70 eV) *m*/*z* (rel. inten.): 180 ($M^{\bullet+}$, 8), 165 (26), 122 (46), 121 (26), 110 (64), 109 (32), 95 (26), 79 (100), 67 (31), 41 (28). ^{1}H NMR (499.88 MHz, $CDCl_3$) δ: 3.01 (1H, dd, *J* = 6.5 and 4.5 Hz, H-7), 2.84 (1H, td, *J* = 6.5 and 4.5 Hz, H-8), 2.53 (2H, m, H-5), 2.49 and 2.26 (2H, dd, *J* = 14.0 and 7.5 Hz, H-6), 2.37 (2H, m, H-4), 2.09 (3H, s, H-11), 1.60 (2H, m, H-9), 1.03 (3H, t, *J* = 7.5 Hz, H-10). ^{13}C NMR (125.69 MHz, $CDCl_3$) δ: 209.1 (C-1), 172.5 (C-3), 136.7 (C-2), 58.5 (C-8), 55.6 (C-7), 34.1 (C-4), 31.8 (C-5), 21.9 (C-6), 21.1 (C-9), 17.5 (C-11), 10.5 (C-10). 7,8-Dihydroxyjasmone (**1c**) MS (EI, 70 eV) *m*/*z* (rel. inten.): 198 ($M^{\bullet+}$, absent), 180 (13), 140 (20), 139 (100), 111 (23), 110 (86), 95 (18), 79 (17), 67 (20), 41 (15). ^{1}H NMR (499.88 MHz, $CDCl_3$) δ: 3.46 (1H, m, H-7), 3.19 (1H, m, H-8), 2.60–2.56 (2H, m, H-8), 2.45–2.41 (4H, m, H-6 and H-4), 2.09 (3H, s, H-11), 1.52 (2H, m, H-9), 0.94 (3H, t, *J* = 7.5 Hz, H-10). ^{13}C NMR (125.69 MHz, $CDCl_3$) δ: 212.1 (C-1), 174.4 (C-3), 137.3 (C-2), 74.1 (C-8), 72.6 (C-7), 34.1 (C-4), 32.1 (C-5), 28.3 (C-6), 26.1 (C-9), 17.4 (C-11), 10.2 (C-10).

*2.7.1. Synthesis of the (S) and (R)-MTPA esters derivatives of 4-hydroxyjasmone (**1a**)*

To a solution of 4-hydroxyjasmone (10.0 mg, 0.055 mmol) in CH_2Cl_2 (0.2 mL), were sequentially added (*R*)-methoxy-(trifluoromethyl)phenylacetic acid (MTPA) (19.43 mg, 0.082 mmol), 4-dimethylaminopyridine (13.15 mg, 0.108 mmol) and 1.3-dicyclohexylcarbodiimide (17.08 mg, 0.082 mmol) at 0 °C. The mixture was stirred for 24 h under N_2 and then passed through a silica gel column eluting with CH_2Cl_2 and CH_2Cl_2:MeOH 19:1. The solvent was removed in vacuum to give the (*R*)-Mosher ester **1.1a** (12.7 mg, 0.032 mmol, 58%). MS (EI, 70 eV) *m/z* (rel. inten.): 396 ($M^{\bullet+}$, absent), 190 (10), 189 (100), 162 (55), 133 (46), 105 (28), 77 (22), 55 (14), 41 (8). ^{1}H NMR (300.07 MHz, $CDCl_3$) δ: 7.53–7.51 (2H, m, Ph), 7.43–7.41 (3H, m, Ph), 5.98 (1H, d, J = 6.0 Hz, H-4), 5.41 (1H, m, H-8), 5.20 (1H, m, H-7), 3.54 (3H, s, $-OCH_3$), 2.98–2.63 (3H, m, H-5 and H-6), 2.33 (1H, dd, J = 18.7 and 2.23 Hz, H-5), 2.11 (2H, m, H-9), 1.88 (3H, s, H-11), 0.97 (3H, t, J = 7.5 Hz, H-10). Treatment with (*S*)-(−)-methoxy-(trifluoromethyl)phenylacetic acid as described above yielded the (*S*)-Mosher ester **1.2a** (11.9 mg, 0.03 mmol, 62%). MS (EI, 70 eV) *m/z* (rel. inten.): 396 ($M^{\bullet+}$, absent), 190 (11), 189 (100), 162 (59), 133 (50), 105 (31), 91 (25), 77 (23), 55 (14), 41 (9). ^{1}H NMR (300.07 MHz, $CDCl_3$) δ: 7.52–7.49 (2H, m, Ph), 7.43–7.40 (3H, m, Ph), 5.85 (1H, d, J = 6.3 Hz, H-4), 5.42 (1H, m, H-8), 5.20 (1H, m, H-7), 3.57 (3H, s, $-OCH_3$), 2.96 (2H, d_l, J = 7.5 Hz, H-6), 2.93 (1H, dd, J = 19.3 and 6.3 Hz, H-5), 2.19 (1H, dd, J = 19.3 and 2.1 Hz, H-5), 2.13 (2H, m, H-9), 2.03 (2H, s, H-11), 0.97 (3H, t, J = 7.6 Hz, H-10).

*2.8. Synthesis of the (R)-(−)-carvone (**3**) metabolites*

Scaling up the bioreaction with (*R*)-(−)-carvone (**3**) and following the same procedure described above resulted in the isolation of (1*S*,2*R*,4*R*)-neoisodihydrocarveol (3.2 mg, 0.021 mmol, 3.8%), (6*R*)-isoprenyl-(3*R*)-methyl-2-oxo-oxepanone (28.6 mg, 0.170 mmol, 31%), (3*R*)-isopropenyl-6-oxoheptanoic acid (5.0 mg, 0.027 mmol, 5.0%) and 2,3-epoxy-(5*R*)-isopropenyl-2-methylcyclohexenol (2.0 mg, 0.012 mmol, 2.2%) after purification with silica gel chromatography, eluting with Hex:EtOAc, 9.5:0.5–1:1. (1*S*,2*R*,4*R*)-Neoisodihydrocarveol (**3a**) $[\alpha]_D^{23}$ = −13.6 (*ca.* 0.1, EtOH), Ref. [14] $[\alpha]_D^{25}$ = −20 (*ca.* 0.2, EtOH). MS (EI, 70 eV) *m/z* (rel. inten.): 154 ($M^{\bullet+}$, 2), 136 (80), 121 (100), 107 (98), 93 (94), 79 (91), 67 (66), 55 (41), 41 (75). ^{1}H NMR (499.88 MHz, $CDCl_3$) δ: 4.70 (2H, s_l, H-9), 3.89 (1H, s_l, H-2), 2.27 (1H, ddd, J = 12.5, 3.3 and 3.2 Hz, H-4), 1.91 (1H, ddd, J = 13.3, 3.3 and 3.2 Hz, H-3), 1.76 (1H, dddd, J = 12.5, 3.5, 3.3 and 3.2 Hz, H-5), 1.72 (3H, s, H-10), 1.56 (1H, m, H-1), 1.46 (2H, m, H-6), 1.42 (1H, m, H-3), 1.25 (1H, dddd, J = 12.5, 12.4, 4.5 and 3.2 Hz, H-5), 0.97 (3H, d, J = 6.0 Hz, H-7). ^{13}C NMR (125.69 MHz, $CDCl_3$) δ: 150.3 (C-8), 108.4 (C-9), 71.0 (C-2), 38.6 (C-3), 37.8 (C-4), 36.0 (C-1), 31.4 (C-5), 28.1 (C-6), 21.0 (C-7), 18.3 (C-10). (6*R*)-Isoprenyl-(3*R*)-methyl-2-oxo-oxepanone (**3b**) MS (EI, 70 eV) *m/z* (rel. inten.): 168 ($M^{\bullet+}$, 13), 156 (21), 138 (19), 123 (33), 109 (40), 95 (64), 83 (91), 67 (100), 55 (66), 41 (98). ^{1}H NMR (499.88 MHz, $CDCl_3$) δ: 4.94, 4.83 (2H, m, H-10), 3.53 (2H, m, H-7), 2.44 (1H, m, H-6), 2.26 (1H, m, H-3), 1.67 (3H, s, H-11), 1.61 (1H, m, H-5), 1.45–1.35 (3H, m, H-4, H-5), 1.18 (3H, d, J = 7.0 Hz, CH_3-C-3). ^{13}C NMR (125.69 MHz, $CDCl_3$) δ: 182.3 (C-2), 144.5 (C-9), 114.1 (C-10), 64.0 (C-7), 49.8 (C-6), 39.3 (C-3), 31.0 (C-5), 26.6 (C-4), 18.7 (C-11), 16.7 (CH_3-C-10). (3*R*)-Isopropenyl-6-oxoheptanoic acid MS (**3c**) (EI, 70 eV) *m/z* (rel. inten.): 184 ($M^{\bullet+}$, absent), 166 ($M^{\bullet+} - H_2O$, 28), 151 (8), 123 (26), 107 (25), 95 (25), 79 (25), 67 (23), 53 (22), 43 (100). ^{1}H NMR (499.88 MHz, $CDCl_3$) δ: 4.82 and 4.77 (2H, m, H-9), 2.57 (1H, m, H-3), 2.41 (4H, m, H-2 and H-5), 2.13 (3H, s, H-7), 1.74 (2H, m, H-4), 1.66 (3H, d, J = 5.1 Hz, H-10). ^{13}C NMR (125.69 MHz, $CDCl_3$) δ: 208.6 (C-6), 178.0 (C-1), 145.0 (C-8), 113.1 (C-9), 42.8 (C-3), 41.1 (C-5), 38.7 (C-2), 30.0 (C-7), 26.2 (C-4), 18.4 (C-10). 2,3-Epoxy-(5*R*)-isopropenyl-2-methylcyclohexenol (**3d**) MS (EI, 70 eV) *m/z* (rel. inten.): 168 ($M^{\bullet+}$, 4), 150 (13), 135 (19), 109 (67), 91 (100), 79 (60), 67 (60), 53 (37), 43 (77). ^{1}H NMR (300.06 MHz, $CDCl_3$) δ: 4.70 (2H, m, H-9), 4.18 (1H, t, H-2), 3.11 (1H, d, J = 5.1 Hz, H-6), 2.45–1.20 (5H, m, H-3, H-4 and H-5), 1.68 (3H, s, H-10), 1.42 (3H, s, H-7). ^{13}C NMR (75.50 MHz, $CDCl_3$) δ: 148.4 (C-8), 109.6 (C-9), 77.0 (C-2), 69.6 (C-6), 60.0 (C-1), 33.0 (C-4), 32.9 (C-3), 28.9 (C-5), 20.2 (C-10), 19.5 (C-7).

*2.9. Synthesis of the α- and β-ionones (**4**, **5**) metabolites*

Scaling up the bioreaction of α- and β-ionones (**4**, **5**) and following the same procedure described above resulted in the isolation of the α-homo-cyclogeraniol (6.0 mg, 0.036 mmol, 5%) and 4-oxo-7,8-dihydro-β-ionone (8.0 mg, 0.038 mmol, 5.3%) after purification by silica gel chromatography, eluting with Hex:EtOEt, 9:1–1:1. α-Homo-cyclogeraniol (**4a**) MS (EI, 70 eV) *m/z* (rel. inten.): 168 ($M^{\bullet+}$, 2), 124 (53), 109 (41), 107 (35), 94 (35), 91 (30), 81 (100), 79 (54), 67 (31), 41 (34). ^{1}H NMR (300.07 MHz, $CDCl_3$) δ: 5.32 (1H, s_l, H-4), 3.69 (2H, t, J = 7.5 Hz, H-8), 2.36 (2H, t, J = 8.0 Hz, H-2), 1.97 (2H, m, H-3), 1.77 and 1.65 (2H, m, H-7), 1.71 (3H, d, J = 2.0 Hz, H-11), 1.51 (1H, m, H-6), 0.92 (3H, s, H-10), 0.89 (3H, s, H-9). ^{13}C NMR (75.50 MHz, $CDCl_3$) δ: 136.4 (C-5), 120.4 (C-4), 63.5 (C-8), 45.9 (C-6), 34.4 (C-7), 32.4 (C-1), 31.3 (C-2), 27.3 (C-9), 27.2 (C-10), 23.4 (C-11), 23.0 (C-3). 4-Oxo-7,8-dihydro-β-ionone (**5a**) MS (EI, 70 eV) *m/z* (rel. inten.): 209 ($M+1$, 5), 208 ($M^{\bullet+}$, 41), 165 (100), 137 (56), 135 (42), 123 (37), 109 (37), 107 (36), 43 (47). ^{1}H NMR (300.07 MHz, $CDCl_3$) δ: 2.59 (2H, m, H-8), 2.47 (4H, m, H-3 and H-7), 2.19 (3H, s, H-10), 1.81 (2H, t, J = 6.9 Hz, H-2), 1.73 (3H, s, H-13), 1.15 (6H, s, H-11 and H-12). ^{13}C NMR (75.50 MHz, $CDCl_3$) δ: 207.0 (C-9), 198.8 (C-4), 163.4 (C-6), 131.3 (C-5), 42.2 (C-8), 37.2 (C-2), 36.4 (C-1), 34.1 (C-3), 29.8 (C-10), 26.7 (C-11 and C-12), 24.0 (C-7), 11.4 (C-13).

*2.10. Synthesis of the (R)-(+)-limonene (**10**) metabolites*

Scaling up the bioreaction of (*R*)-(+)-limonene (**10**) and following the same procedure described above resulted in the isolation of the (+)-(4*R*)-*p*-1-menth-1-ene-8,9-diol (3.2 mg, 0.019 mmol, 6.1%) and (*R*)-(+)-limonene-1,2-diol (2.6 mg, 0.015 mmol, 5%) after purification by silica gel chromatography,

eluting with Hex:EtOAc, 9:1–1:1. (+)-(4*R*)-*p*-1-Menth-1-ene-8,9-diol (uroterpenol, **10a**) MS (EI, 70 eV) *m*/*z* (rel. inten.): 170 ($M^{\bullet+}$, absent), 152 ($M^{\bullet+} - H_2O$, 37), 139 (41), 121 (100), 95 (49), 79 (31), 71 (33), 57 (27), 43 (74). ^{1}H NMR (499.88 MHz, $CDCl_3$) δ: 6:5 mixture of (4*R*,8*R*)- and (4*R*,8*S*)-forms: 5.41 (*R*), 5.35 (*S*), (1H, m, H-2), 3.58, 3.44 (*R*), 3.55, 3.40 (*S*), (2H, qAB, *J* = 10.8 Hz, H-9), 1.98 (2H, m, 2× OH), 1.64 (3H, s, CH_3-7), 1.13 (*R*), 1.09 (*S*), (3H, s, CH_3-10). ^{13}C NMR (125.69 MHz, $CDCl_3$) δ: 6:5 mixture of (4*R*,8*R*)-form: 134.3 (C-1), 120.0 (C-2), 74.6 (C-8), 68.6 (C-9), 40.8 (C-4), 30.8 (C-6), 26.9 (C-3), 23.0 (C-5), 23.4 (C-7), 19.4 (C-10) and (4*R*,8*S*)-form: 133.9 (C-1), 120.4 (C-2), 74.6 (C-8), 68.3 (C-9), 40.6 (C-4), 31.0 (C-6), 25.8 (C-3), 24.3 (C-5), 23.3 (C-7), 20.5 (C-10). (*R*)-(+)-Limonene-1,2-diol (**10b**) IE/EM *m*/*z* (rel. inten.): 170 ($M^{\bullet+}$,

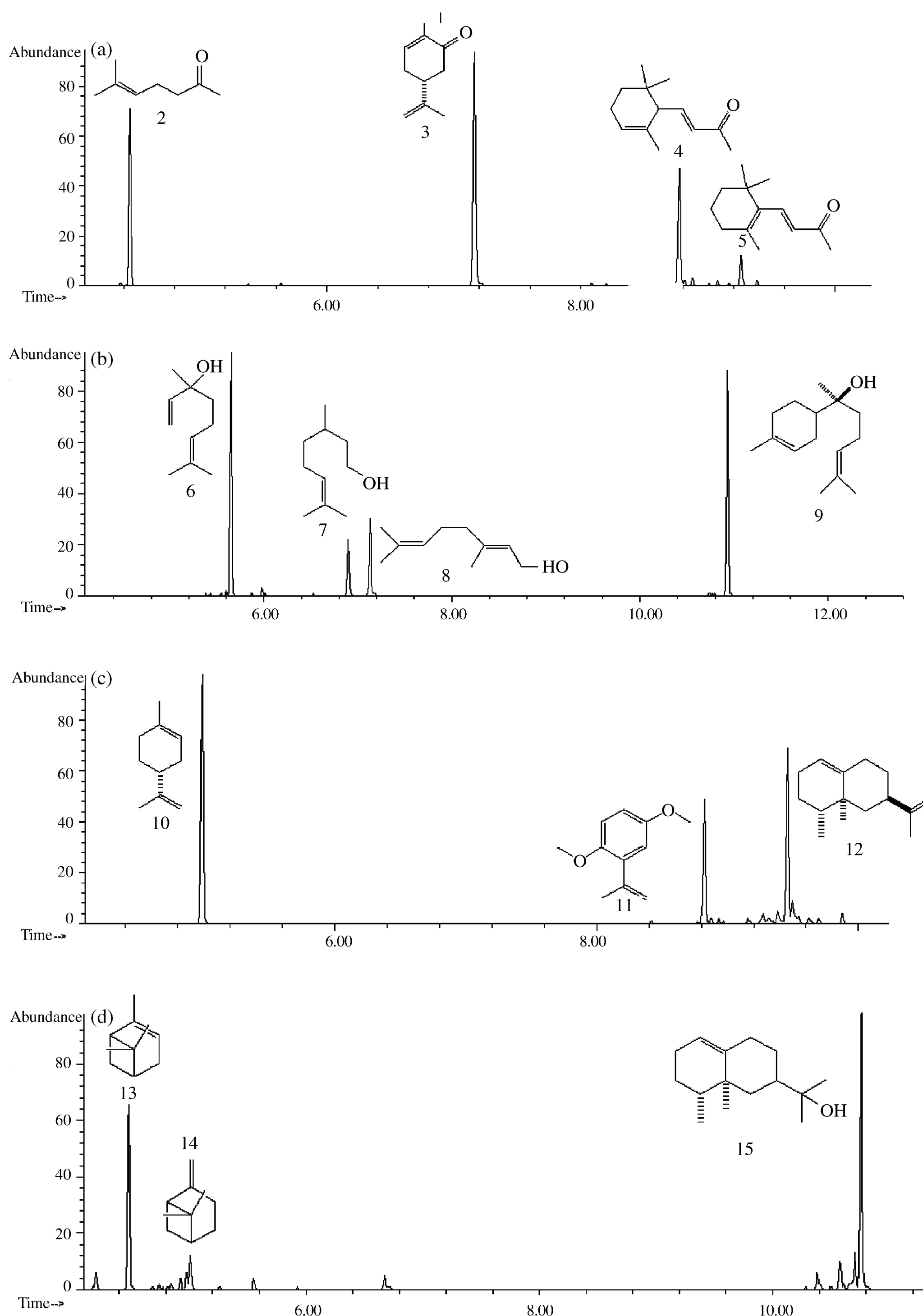
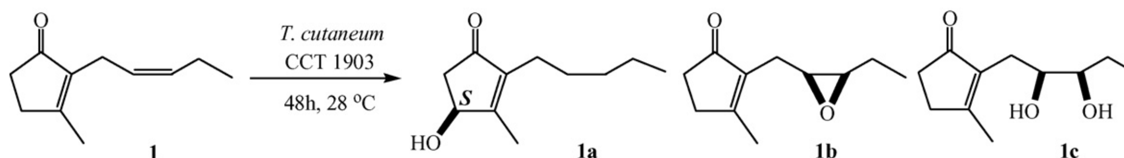


Fig. 1. Total ion chromatograms (GC–MS) of: (a) 6-methyl-5-hepten-2-one (**2**), (*R*)-(−)-carvone (**3**), α- and β-ionones (**4**, **5**); (b) linalool (**6**),(β-citronelol (**7**), geraniol (**8**), α-bisabolol (**9**); (c) (*R*)-(+)-limonene (**10**), 1-isopropenyl-2,5-dimethoxy-4-methylbenzene (**11**), valencene (**12**); (d) α- and β-pinene (**13**, **14**), jinkoh-eremol (**15**). Column: HP5 (30 m × 0.25 mm × 0.25 μm). Conditions: 50–290 °C at 20 °C min^{-1}; T_{inj} = 200 °C; 1 mL min^{-1} flow rate; split 50:1.



Scheme 1. *Cis*-jasmone (**1**) biotransformation products **1a**, **1b** and **1c** using *Trichosporum cutaneum* CCT 1903 whole-cell.

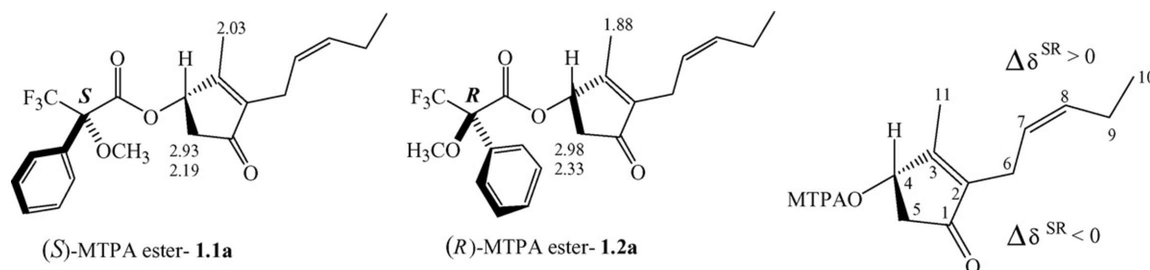


Fig. 2. Conformational models of MTPA esters derivatives of **1a** and ^{1}H NMR chemical shifts undergoing phenyl group anisotropy and the measured $\Delta\delta^{SR}$.

absent), 152 ($M^{\bullet+} - H_2O$, 20), 137 (23), 123 (17), 108 (36), 79 (41), 71 (70), 67 (56), 55 (28), 43 (100). ^{1}H NMR (499.88 MHz, $CDCl_3$) δ: 4.71 (2H, m, H-9), 3.67 (1H, s_l, H-2), 2.40–2.23 (1H, m, H-4), 1.95–1.50 (6H, m, H-3, H-5 and H-6), 1.73 (3H, s, H-10), 1.26 (3H, s, H-7). ^{13}C NMR (125.69 MHz, $CDCl_3$) δ: 149.1 (C-8), 108.9 (C-9), 73.7 (C-2), 71.9 (C-1), 37.5 (C-4), 34.4 (C-6), 34.1 (C-3), 28.2 (C-5), 24.5 (C-7), 21.2 (C-10).

3. Results and discussion

Based on our monooxygenase screening assays using fluorescent probes and applied to microorganism whole cells we selected *T. cutaneum* CCT 1903, *Aspergillus oryzae* CCT 0975 and *Geotrichum candidum* CCT 1205 as good candidates for further investigation [15]. To evaluate which monooxygenases were present we have monitored several bioreactions in one pot named “multibioreaction methodology” [11]. We have thus observed epoxidation and hydroxylation of *cis*-jasmone (**1**) by *T. cutaneum* CCT 1903, revealing the presence of monooxygenase. To determine the enantioselectivity and substrate specificity of this enzyme we have selected 14 substrates [6-methyl-5-hepten-2-one (**2**), (*R*)-(−)-carvone (**3**), α- and β-ionones (**4**, **5**), linalool (**6**), β-citronellol (**7**), geraniol (**8**), α-bisabolol (**9**), (*R*)-(+)-limonene (**10**), 1-isopropenyl-2,5-dimethoxy-4-methylbenzene (**11**), valencene (**12**), α- and β-pinene (**13**, **14**), jinkoh-eremol (**15**)] which were evaluated in four multibioreactions: Fig. 1a–d. Of these (*R*)-(−)-carvone, α- and β-ionones and (*R*)-(+)-limonene were oxidized. Batch reactions with *cis*-jasmone (**1**), (*R*)-(−)-carvone (**3**), α- and β-ionones (**4**, **5**) and (*R*)-(+)-limonene (**10**) produced **1a–c**, **3a–d**, **4a**, **5a**, **10a–b** as pure samples for further spectroscopic identification and characterization (^{1}H and ^{13}C NMR, ^{1}H,^{1}H-gCOSY, HSQC and gHMBC).

3.1. Biotransformation of cis-jasmone (1)

The incubation of **1** with *T. cutaneum* whole cells for 48 h gave 4-hydroxyjasmone (**1a**), 7,8-epoxyjasmone (**1b**) and 7,8-dihydroxyjasmone (**1c**), Scheme 1.

To determine the absolute configuration of the 4-hydroxyjasmone (**1a**) asymmetric center we applied Mosher’s methodology [16,17]. Esterifications of compound **1a** with (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) in the presence of 4-dimethylaminopyridine and 1,3-dicyclohexylcarbodiimide produced **1.1a** and **1.2a** (Fig. 2). From the chemical shift differences $\Delta\delta^{SR}$ ($\{\Delta\delta = \delta[(S)\text{-MTPA ester}] - \delta[(R)\text{-MTPA ester}]\}$; Table 1) we concluded that the configuration is (4*S*) in **1a**. Enantiomeric excess determined by GC was 86%, therefore hydroxylation of *cis*-jasmone was regio- and stereo-selective.

Cis-jasmone epoxidation was performed with *m*-chloroperoxybenzoic acid furnishing the desired racemic standard [(±)-**1b**] for chromatographic monitoring (Fig. 3a). The GC-FID chromatogram of **1b** revealed that the *trans*-epoxide was present in 6% and derived from the *trans*-jasmone present in the commercial sample, however, the major epoxide depicted a H_7–H_8 coupling constant of 4.5 Hz attesting to the *cis* configuration of the epoxide **1b** (94%) [18]. The enantiomeric excess of **1b** (e.e. 92%.) was evaluated by chiral GC analyses (Fig. 3b).

The relative stereochemistry of the diol **1c** arising from ring opening of the *cis* epoxide **1b** was not easily accessed. After several failures under different conditions and with different chiral columns the diol (±)-**1c** was derivatized to (±)-**1d** (Scheme 2). Three out of four stereoisomers of (±)-**1d** eluted with different times from a cyclodextrin column (Chirasil) under specific chromatographic conditions. The two major constituents (retention

Table 1
Partial ^{1}H NMR (300.06 MHz, $CDCl_3$) data of the (*S*)- and (*R*)-Mosher esters derivatives of compound **1a**[a]

Hydrogen	δ_H		$\Delta\delta^{SR}$
	1.1a	**1.2a**	
5	2.93	2.98	−0.05
5	2.19	2.33	−0.14
11	2.03	1.88	0.15

[a] Chemical shifts are referenced to TMS (δ 0.00).

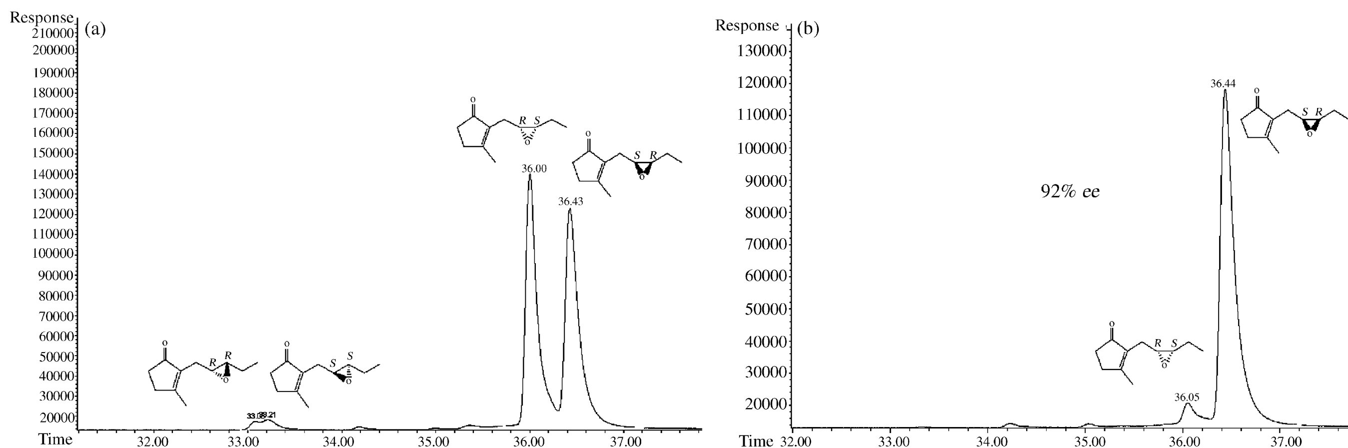
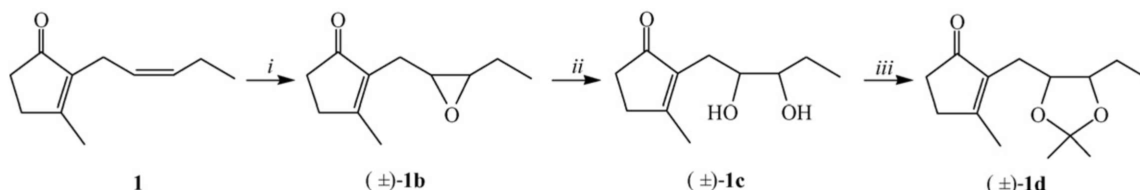


Fig. 3. GC-FID chromatogram of: (a) (±)-7,8-epoxyjasmone [(±)-**1b**]; (b) 7,8-epoxyjasmone **(1b)** obtained with *Trichosporum cutaneum* CCT 1903 whole-cell. Chiral column: heptakis (2,6-di-*O*-methyl-*O*-pentyl-3)-β-cyclodextrin. Conditions: 50–180 °C at 2 °C min^{-1}; T_{inj} = 220 °C and T_{det} = 280 °C; *P* = 10 psi.



Scheme 2. Synthesis of acetonide (±)-**1d**: (i) *m*-chloroperoxybenzoic acid, CH_2Cl_2, 0 °C; (ii) H_2SO_4, 1,4-dioxane, H_2O; (iii) $(CH_3)CO/H^+$, 0 °C, 78%.

time 23.24 and 23.37 min) of the synthetic mixture (Fig. 4a) were identified as the (+) and (−) *trans*-acetonide (±)-**1d** by applying Rychnovsky's methodology [19] in which the ^{13}C NMR chemical shift similarities of the acetal methyl groups of the acetonide (±)-**1d** (δ_c 27.3 and 27.1) is related to *trans* configuration. Thus major isomers are derived from the *syn* diol (±)-**1c**.

The diol **1c** obtained by biocatalysis was derivatized to **1d** and analyzed by GC-FID revealing that the first eluting (23.24 min) enantiomer of the *trans*-acetonide was present in e.e. 53% (Fig. 4b). The low enantiomeric excess of the diol in comparison with the epoxide (e.e. 92%) was in part assigned to spontaneous ring opening that was also observed in a parallel experiment.

3.2. Biotransformation of (R)-(−)-carvone (3)

(*R*)-(−)-Carvone (**3**) was biotransformed by *T. cutaneum* CCT 1903 cells yielding (1*S*,2*R*,4*R*)-neoisodihydrocarveol (**3a**). The configuration at C_1 and C_2 of **3a** were determined by comparison of the specific optical rotation of the isolated compounds with the values reported for several stereoisomers [20] of known configuration at C_4. Hydrogen is stereoselectively introduced onto the C–C double bond adjacent to the carbonyl group and onto the carbonyl group. However, in the transformation of (*R*)-(−)-carvone oxidation prevailed over reduction and the following products were identified: (6*R*)-isoprenyl-(3*R*)-methyl-2-oxo-oxepanone (**3b**),

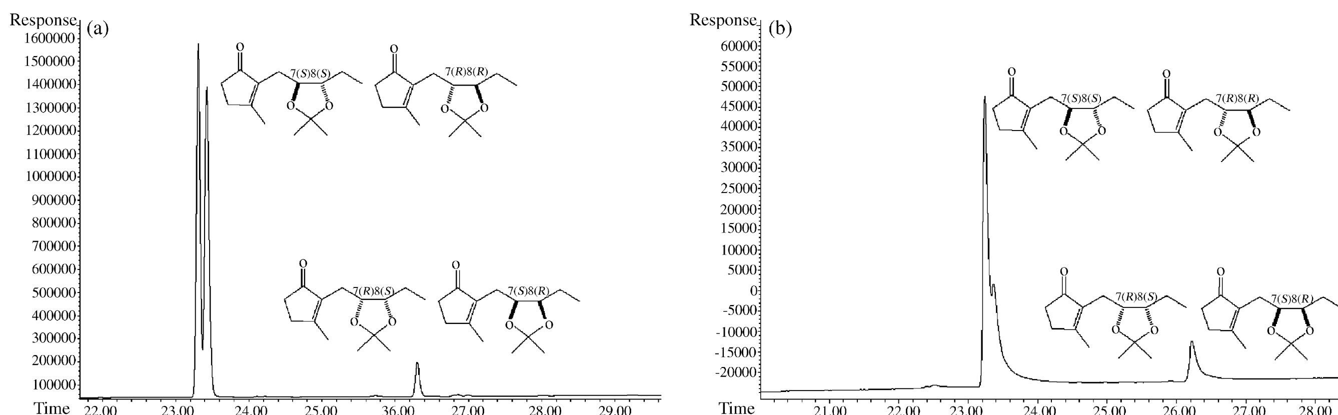
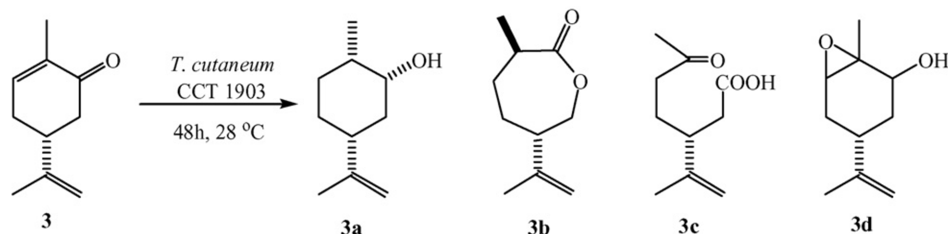
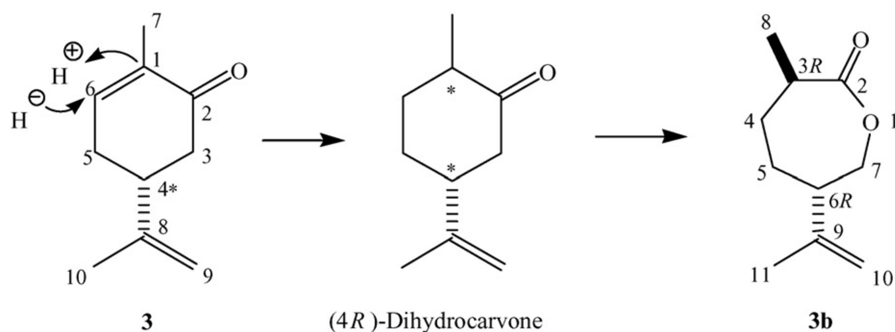


Fig. 4. CG-FID chromatogram of: (a) acetonide (±)-**1d** and (b) acetonide **1d**. Chiral column: Chirasil-β-cyclodextrin. Conditions: 70–180 °C at 3 °C min^{-1}; T_{inj} = 220 °C and T_{det} = 250 °C; *P* = 15 psi.



Scheme 3. (*R*)-(−)-Carvone (**3**) biotransformation products (**3a–d**) using *T. cutaneum* CCT 1903 whole-cell.



Scheme 4. Baeyer–Villiger oxidation of (*R*)-(−)-carvone using *T. cutaneum* CCT 1903 whole-cell.

(3*R*)-isopropenyl-6-oxoheptanoic acid (**3c**) and 2,3-epoxy-(5*R*)-isopropenyl-2-methylcyclohexenol (**3d**), Scheme 3.

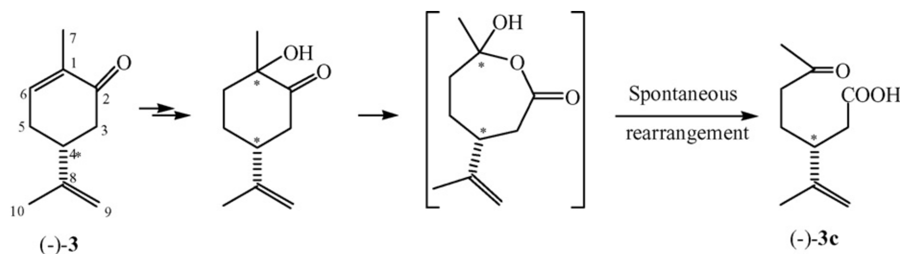
Lactone (6*R*)-isoprenyl-(3*R*)-methyl-2-oxo-oxepanone (**3b**) formation was rationalized from (*R*)-(−)-carvone regioselective partial reduction producing dihydrocarvone that was further biotransformed by a Baeyer–Villiger monooxygenase (BVMO), Scheme 4. It should be pointed out that the apparent unusual regioselectivity of oxygen insertion (between the carbonyl group and the least substituted carbon atom) is not so unusual in enzyme catalyzed reations. **3b** SPECTROSCOPIC data were identical to those previously reported [21]. Also, it is interesting to note that, contrary to BV oxidations with the most common peracids, the 9,10-double bond was not oxidized. Carbon-4 is not affected by the transformation of **3** into **3b**, therefore the (−)-carvone (*R*)-C_4 absolute configuration becomes the **3b** (*R*)-C_6 upon biotransformation. The 3,6-*trans* relationship in **3b**, was obtained by observing C_8 methyl signal enhancement (2.21% at 1.18 ppm) in the ^{1}H NMR NOE difference spectrum as a consequence of H_6 excitation, thus the configuration at C_3 is (*R*).

The formation of the (3*R*)-isopropenyl-6-oxoheptanoic acid (**3c**) is in agreement with the degradation mechanism of alicyclic monoterpenes proposed by Trudgill [22] and confirmed by van der Werf [23], which involve lactone-forming monooxygenase activity as the first step towards ring opening (Scheme 5). These BVMO catalyze the insertion of one atom of oxygen next to an alicyclic keto group. The lactone thus formed is subsequently hydrolyzed by a lactone hydrolase, or spontaneously rearranges into the corresponding oxo-acid when the oxygen atom is inserted between a hydroxyl-substituted carbon and a keto group.

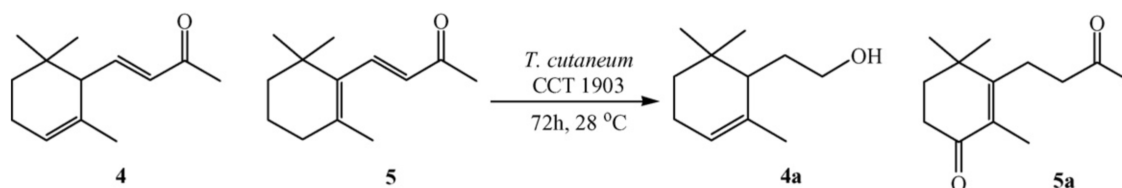
The carvone simultaneous regioselective reduction and epoxidation produced the 2,3-epoxy-(5*R*)-isopropenyl-2-methylcyclohexenol (**3d**) in 2.18%. The spectral data (MS, ^{1}H and ^{13}C NMR) of **3d** were compared with those in the literature, confirming the proposed structure of epoxy-alcohols and their derivatives, which are useful synthetic intermediates [24].

3.3. Formation of α- and β-ionones (4, 5) metabolites

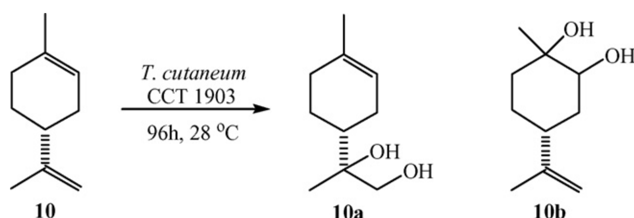
T. cutaneum CCT 1903 promoted the hydrogenation and concomitant oxygenation at C_4 of β-ionone yielding the 4-oxo-7,8-dihydro-β-ionone (**4a**), Scheme 6 [25]. Product **4a** gave EIMS $M+1$, 209.236 ($C_{13}H_{20}O_2$) and the ^{13}C NMR spectrum revealed four tertiary methyl, five quaternary and four methylene carbon signals. On the other hand, the formation of α-homocyclogeraniol (**5a**) suggested that a Baeyer–Villiger reaction had



Scheme 5. Microbial biotransformation pathway for (*R*)-(−)-carvone.



Scheme 6. α- and β-ionones biotransformation products using *T. cutaneum* CCT 1903 whole-cell.



Scheme 7. Biotransformation of (*R*)-(+)-limonene (**10**) by whole-cell of *T. cutaneum* CCT 1903.

occurred in the first step of biotransformation, as reported by Krasnobajew et al. [26]. An oxygenase-type enzyme system is proposed to be responsible for the degradation of the ionone side chain. In fact such conversions are well documented in microbial steroid transformations. Several research groups [27,28] have already described the side chain degradation of C_{21}-steroids by fungi, leading ultimately to lactones or esters, where the oxygen atom of the ester group derives from molecular oxygen.

3.4. Biotransformation of (R)-(+)-limonene (10)

In the biotransformation of (+)-**10** by *T. cutaneum* CCT 1903 the two metabolites isolated were identified as (+)-(4*R*)-*p*-1-menth-1-ene-8,9-diol (**10a**) and (+)-limonene-1,2-diol (**10b**), Scheme 7. Metabolite **10a** was produced as a (4*R*,8*R*)- and (4*R*,8*S*)-diastereomeric mixture in 6:5 ratio [29], by oxidation of the 8,9-double bond of (+)-**10**. Interestingly, these compounds constitute key chiral building blocks allowing for the two-step synthesis of the four stereoisomers of bisabolol [30]. All bisabolol stereoisomers are known natural compounds and (−)-α-bisabolol is known to be of high industrial value in the cosmetic industry [31].

Metabolite (+)-**10b** was produced by oxidation of the 1,2-double bond of (+)-**10**, and a search in the literature revealed that (+)-**10b** has been obtained by biotransformations applying several different microorganisms [32,33].

In general, microbial oxidations of **10** are at the 1,2-double bonds and at the C_7 and C_6 positions [34]. On the other hand, mammals oxidize the 8,9-double bond and C_7 position. In the present case *T. cutaneum* CCT 1903 yeast has oxidized both 1,2- and 8,9-double bonds, therefore displaying a metabolic pathway which is characteristic of both mammals and microbes.

4. Conclusion

In conclusion the present paper demonstrates that *T. cutaneum* CCT 1903, a commercially available microorganism, has an outstanding oxygenase activity. Further investigation will reveal whether the hydroxylation, epoxidation and Baeyer–Villiger reactions are catalyzed by cytochrome P450 dependent monooxygenases and/or flavin dependent Baeyer–Villigerases.

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