ORIGINAL ARTICLE

Antimicrobial activity of new bicyclic lactones with three or four methyl groups obtained both synthetically and biosynthetically

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Abstract Ten new derivatives of isophorone were obtained through a five-step synthesis. Among the products were several unsaturated, bicyclic lactones with three or four methyl groups. These lactones were used as the substrates for biotransformation mediated by selected fungal strains (*Fusarium* species, *Syncephalastrum racemosum*, *Cunninghamella japonica*, *Penicillium* species, *Absidia* species, *and Pleurotus ostreatus*). Four new hydroxylactones were obtained as a result of biotransformation. Because the unsaturated lactone with four methyl groups was a diastereoisomeric mixture, a structural analysis was conducted. The hydroxylactones were also included in this analysis. Both the unsaturated lactones and hydroxylactones were examined for their antimicrobial activity. It was found that some of these compounds exhibited growth inhibition against pathogenic strains of bacteria (*Staphylococcus aureus*, *Pseudomonas fluorescens*), yeasts (*Candida albicans*) and filamentous fungi (*Alternaria* sp., *Penicillium* sp.). All obtained compounds were also subjected to scent analysis.

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1. Introduction

Modern medicine continues to search for new drugs and preparations to combat diseases caused by ubiquitous bacteria, viruses and fungi. Infections caused by these pathogens decrease the quality of life of the average inhabitant of the earth. Environmental pollution, poor nutrition and abuse of all kinds of pharmaceuticals leave us particularly susceptible to infections of all kinds of pathogens. Among the bacteria,

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the main group of unicellular prokaryotic organisms, *Staphylococcus aureus* is extremely dangerous. This Gram-positive bacterium can cause necrotizing pneumonia, endocarditis or toxic shock syndrome, among others. Strains of this microorganism that are resistant to methicillin cause the majority of hospital-acquired infections in the world [1]. Another commonly occurring drug-resistant bacterium, *Pseudomonas fluorescens*, may cause infections of the urinary tract, meninges, bones, joints, and others [2].

In addition, long-term use of antimicrobial therapy or chemotherapeutic agents can cause an imbalance of bacteria normally present in the colon. This results in weakened immunity, leaving the patient particularly susceptible to *Candida* fungal infections [3]. Populations particularly vulnerable to candidiasis include patients struggling with chronic illnesses such as diabetes, eating disorders and absorption, cancer and blood disorders [4]. Filamentous fungi, such as those of the genus *Alternaria* and *Penicillium* are equally dangerous, and their spores can cause inhaled allergies [5].

More and more mutating pathogens have become resistant to currently available drugs. For this reason, it is very important to search for new chemical compounds able to replace those compounds that are no longer active. Especially noteworthy to this search are plant extracts used in traditional folk medicine. Very often, the compounds of these isolates with specific biological properties are molecules containing a lactone ring. Particular attention should be paid to hydroxylactones, many of which exert anticancer [6–9], anti-inflammatory [10–12], antiviral [13,14], antifungal [15–17], or antibacterial [18,19] activities. Such compounds are not only derived solely from plant material; they may also be produced by chemical synthesis [20–22] or biotransformation [23–27].

For more than ten years our team has been obtaining new hydroxylactones from the chemically derived halolactones [23–27]. For this purpose we use filamentous fungi capable of performing hydrolytic dehalogenation. This method allows us to obtain compounds whose acquisition by standard chemical synthesis would be either impossible, or too complex to be viable. In the presented work, we used unsaturated lactones as substrates, hoping to obtain new derivatives with interesting biological properties.

2. Experimental

2.1. Chemistry

Analytical TLC was performed on silica gel-coated aluminium plates (DC-Alufolien Kieselgel 60 F254, Merck, Darmstadt, Germany) with a mixture of hexane, acetone and diethyl ether in various ratios. Compounds were detected by spraying the plates with 1% $Ce(SO_4)_2$, 2% $H_3[P(Mo_3O_{10})_4]$ in 10% H_2SO_4 or 20% ethanolic H_2SO_4, containing 0.1% of anisaldehyde, followed by heating to 120 °C. Preparative column chromatography was performed on silica gel (Kieselgel 60, 230–400 mesh ASTM, Merck, Dramstadt, Germany) with a mixture of hexane, acetone and diethyl ether (in various ratios) as eluents. GC analysis was carried out on an Agilent Technologies 6890N (Varian, Agilent Technologies, Santa Clara, CA, USA) instrument using a DB-17 column (cross-linked methyl silicone gum, 30 m × 0.32 mm × 0.25 μm) or on a Varian CP3380 (Varian, Agilent Technologies, Santa Clara, CA,

USA) instrument using the Thermo TR-5 (30 m × 0.32 mm × 1.0 μm) capillary columns:. The molar masses of the obtained compounds were confirmed by a high resolution mass spectrometry analysis using a Waters GCT Premier instrument (*ESI* ionization) (Waters Division, Milford, Massachusetts). An enantiomeric excess of the products obtained during biotransformation was determined by GC analysis using the chiral column CP-cyclodextrin-B-325 (30 m × 0.25 mm × 0.25 μm) under the following conditions: injector 200 °C, detector (FID) 220 °C, column temperature: 140 °C (hold 45 min), 140–200 °C (rate 20 °C/min), 200 °C (hold 1 min). Optical rotations were determined on a P-2000 polarimeter (Jasco Easton, PA, USA) in chloroform solutions, whose concentrations are denoted in g/100 mL. The melting points were determined on a Boetius apparatus. The refractive index was measured on a Carl Zeiss Abbe and Pulfrich refractometer (Jena, Germany).

NMR spectra were recorded in $CDCl_3$ solution on a Bruker Avance DRX 300 MHz spectrometer (Bruker, Billerica, MA, USA) or on a Bruker Avance 600 MHz spectrometer (Bruker, Billerica, MA, USA). IR spectra were recorded on a Thermo-Nicolet IR 300 FT-IR spectrometer (Waltham, MA, USA).

2.1.1. Synthesis

The commercially available ketone, isophorone (**1**), was purchased from Fluka. The known allyl alcohol **2** was obtained according to the method described by Magnusson and Thoren [28]. All of the subsequent products were obtained according to procedures that are described below:

2.1.1.1. General procedure for the synthesis of ethyl esters (3a-b). Ester **3a** (1.14 g, 5.42 mmol, yield 95%) was obtained as a product of the Claisen rearrangement with orthoacetate modification [29] and ester **3b** (two diastereoisomers) (1.44 g, 6.44 mmol, yield 80%) was obtained in the same manner as the first one, but with orthopropionate modification characterized by the data given below.

2.1.1.1.1. Ethyl (3,5,5-trimethylcyclohex-2-en-1-yl)acetate (3a). **3a**: n_D = 1.4618; IR (KBr): 2954, 1736, 1456, 1368, 1269, 1180, 1033 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$): δ 0.87 (s, 3H, CH$_3$-10), 0.95 (s, 3H, CH$_3$-11), 1.26 (t, J = 7.1 Hz, 3H, CH$_3$-13), 1.52 (m, 2H, CH$_2$-6), 1.58 (d, J = 17.3 Hz, 1H, one of CH$_2$-4), 1.63 (s, 3H, CH$_3$-9), 1.80 (dm, J = 17.3 Hz, 1H, one of CH$_2$-4), 2.23 (dd, J = 9.6 and 9.6 Hz, 2H, CH$_2$-7), 2.60 (m, 1H, H-1), 4.14 (q, J = 7.1 Hz, 2H, CH$_2$-12), 5.20 (s, 1H, H-2) ppm; ^{13}C NMR (300 MHz, CDCl$_3$): 14.33 (C-13), 23.90 (C-9), 25.36 (C-10), 29.98 (C-5), 31.12 (C-3), 31.84 (C-11), 41.28 (C-7), 42.31 (C-4), 44.08 (C-6), 60.22 (C-12), 122.77 (C-2), 133.95 (C-1), 173.10 (C-8) ppm; *ESIHRMS*: calcd for C$_{13}$H$_{22}$O$_2$, m/z 211.1692 (M + H)$^+$, found 211.1698.

2.1.1.1.2. Ethyl 2-(3,5,5-trimethylcyclohex-2-en-1-yl)propanoate (3b). **3b**: n_D = 1.4542; IR (KBr): 2956, 1735, 1458, 1365, 1250, 1187, 1075 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$): δ 0.86 (s, 3H, CH$_3$-10), 0.95 (s, 3H, CH$_3$-11), 1.00 (m, 1H, one of CH$_2$-6), 1.08 (d, J = 6.9 Hz, 3H, CH$_3$-12), 1.26 (t, J = 7.1 Hz, 3H, CH$_3$-14), 1.28 (m, 1H, one of CH$_2$-6), 1.49 (m, 1H, one of CH$_2$-4), 1.63 (s, 3H, CH$_3$-9), 1.81 (m, 1H, one of CH$_2$-4), 2.32 (m, 1H, CH$_2$-7), 2.42 (m, 1H, H-1), 4.15 (q, J = 7.1 Hz, 2H, CH$_2$-13), 5.12 (s, 1H, H-2) ppm; ^{13}C NMR (300 MHz, CDCl$_3$): 13.50 (C-12), 14.35 (C-14), 24.04 (C-9), 25.26 (C-10), 29.90 (C-5), 31.98 (C-11), 36.77 (C-4),

38.42 (C-3), 44.08 (C-6), 44.53 (C-7), 60.08 (C-13), 122.04 (C-2), 134.23 (C-1), 176.25 (C-8) ppm; *ESIHRMS*: calcd for $C_{14}H_{24}O_2$, m/z 225.1849 $(M + H)^+$, found 225.1814.

2.1.1.2. General procedure for the synthesis of acids (4a-b). Basic hydrolysis of esters **3a** and **3b** (two diastereoisomers) according to the procedure described previously [30] gave 1.00 g (5.49 mmol, yield 70%) of acid **4a** and 1.00 g (5.09 mmol, yield 79%) of acid **4b** (two diastereoisomers) with the following physical and spectral data:

2.1.1.2.1. (3,5,5-Trimethylcyclohex-2-en-1-yl)acetic acid (4a). **4a**: n_D = 1.4788; IR (KBr): 2950, 1706, 1459, 1364, 1213, 1166, 946 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$): δ 0.88 (s, 3H, CH$_3$-10), 0.96 (s, 3H, CH$_3$-11), 1.48 (ddd, J = 12.6, 5.3 and 1.3 Hz, 2H, CH$_2$-6), 1.56 (d, J = 17.4 Hz, 1H, one of CH$_2$-4), 1.64 (s, 3H, CH$_3$-9), 1.80 (d, J = 17.4 Hz, 1H, one of CH$_2$-4), 2.30 (dd, J = 11.3 and 7.3 Hz, 2H, CH$_2$-7), 2.59 (m, 1H, H-1), 5.23 (s, 1H, H-2); ^{13}C NMR (300 MHz, CDCl$_3$): 23.88 (C-9), 25.33 (C-10), 29.98 (C-5), 30.90 (C-3), 31.81 (C-11), 40.79 (C-7), 42.24 (C-4), 44.06 (C-6), 122.43 (C-2), 134.36 (C-1), 178.17 (C-8), ppm; *ESIHRMS*: calcd for $C_{11}H_{18}O_2$ m/z 183.1380 $(M + H)^+$, found 183.1385.

2.1.1.2.2. 2-(3,5,5-Trimethylcyclohex-2-en-1-yl)propanoic acid (4b). **4b**: m.p. = 69–70 °C; IR (KBr): 2950, 1706, 1458, 1365, 1262, 1213, 1165 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$): δ 0.87 (s, 3H, CH$_3$-10), 0.97 (s, 3H, CH$_3$-11), 1.00 (m, 1H, one of CH$_2$-6), 1.12 (d, J = 6.9 Hz, 3H, CH$_3$-12), 1.34 (m, 1H, one of CH$_2$-6), 1.55 (d, J = 17.1 Hz, 1H, one of CH$_2$-4), 1.65 (s, 3H, CH$_3$-9), 1.80 (d, J = 17.1 Hz, 1H, one of CH$_2$-4), 2.40 (m, 1H, CH$_2$-7), 2.49 (m, 1H, H-1), 5.18 (s, 1H, H-2) ppm; ^{13}C NMR (300 MHz, CDCl$_3$): 13.15 (C-12), 24.00 (C-9), 24.10 (C-9), 25.23 (C-10), 29.92 (C-5), 31.97 (C-11), 32.06 (C-11), 36.50 (C-3), 36.60 (C-3), 38.01 (C-4), 40.04 (C-4), 43.79 (C-7), 44.05 (C-7), 44.17 (C-6), 120.04 (C-2), 121.80 (C-2), 134.71 (C-1), 135.18 (C-1), 181.82 (C-8), 182.77 (C-8) ppm; *ESIHRMS*: calcd for $C_{12}H_{20}O_2$, m/z 197.1536 $(M + H)^+$, found 197.1542.

2.1.1.3. Procedure for synthesis of bromolactone (5a) and lactone (6a). According to the known procedure [23] we obtained 0.25 g (yield 23%) of bromolactone **5a** and 0.69 g (yield 70%) unsaturated lactone **6a** from 1 g of acid **4a**. Dehydrodehalogenation of this mixture, according to the known procedure [30], gave 0.81 g (yield 82%) of unsaturated lactone **6a** characterized by the data presented below:

2.1.1.3.1. 2-Bromo-2,4,4-trimethyl-9-oxabicyclo[4.3.0]nonan-8-one (5a). **5a**: oil; IR (KBr): 2956, 1781, 1454, 1364, 1212, 1166, 975 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$): δ 0.93 (s, 3H, CH$_3$-10), 1.02 (dd, J = 13.2 Hz, 1H, one of CH$_2$-5), 1.24 (s, 3H, CH$_3$-11), 1.45 (ddd, J = 10.4, 5.4 and 2.3 Hz, 1H, one of CH$_2$-5), 1.68 (d, J = 15.8 Hz, 1H, one of CH$_2$-3), 1.93 (s, 3H, CH$_3$-9), 1.98 (dm, J = 15.8 Hz, 1H, one of CH$_2$-3), 2.25 (d, J = 16.6 Hz, 1H, one of CH$_2$-7), 2.75 (dd, J = 16.6 and 6.1 Hz, 2H, CH$_2$-7), 2.87 (m, 1H, H-6), 4.64 (d, J = 3.4 Hz, 1H, H-1) ppm; ^{13}C NMR (300 MHz, CDCl$_3$): 26.34 (C-9), 30.51 (C-4), 31.54 (C-6), 33.28 (C-10), 34.30 (C-11), 38.76 (C-7), 38.91 (C-5), 47.90 (C-3), 61.43 (C-2), 84.50 (C-1), 176.04 (C-8) ppm; *ESIHRMS*: calcd for $C_{11}H_{17}BrO_2$, m/z 261.0485 $(M + H)^+$, found 261.0490.

2.1.1.3.2. 2,4,4-Trimethyl-9-oxabicyclo[4.3.0]non-2-en-8-one (6a). **6a**: n_D = 1.4932; IR (KBr): 2958, 1783, 1452, 1358, 1210, 1165, 972 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$):

δ = 0.97 (s, 3H, CH$_3$-10), 1.01 (s, 3H, CH$_3$-11), 1.22 (dd, J = 12.4 and 13.3 Hz, 1H, one of CH$_2$-5), 1.45 (ddd, J = 13.2, 12.8 and 4.7 Hz, 1H, one of CH$_2$-5), 1.82 (s, 3H, CH$_3$-9), 2.28 (d, J = 17.4 Hz, 1H, one of CH$_2$-7), 2.65 (m, 1H, H-6), 2.87 (dd, J = 17.4 and 8.0 Hz, 2H, CH$_2$-7), 4.54 (d, J = 5.4 Hz, 1H, H-1), 5.55 (s, 1H, H-3) ppm; ^{13}C NMR (300 MHz, CDCl$_3$): 21.15 (C-9), 26.92 (C-10), 30.61 (C-11), 31.37 (C-4), 36.81 (C-7), 38.82 (C-5), 79.23 (C-1), 44.06 (C-6), 32.33 (C-4), 127.17 (C-2), 139.45 (C-3), 176.80 (C-8) ppm; *ESIHRMS*: calcd for $C_{11}H_{16}O_2$, m/z 181.1223 $(M + H)^+$, found 181.1228.

2.1.1.4. Procedure for synthesis of bromolactone (5b) and lactone (6b). Using the same conditions described above and 1.00 g of acid **4b** (two diastereoisomers) we obtained the mixture of bromolactone **5b** (two diastereoisomers) (0.83 g, 59%) and unsaturated lactone **6b** (two diastereoisomers) (0.30 g, 30%). Dehydrodehalogenation of this mixture gave 0.77 g (78%) of unsaturated lactone **6b** as a diastereoisomeric mixture (40% A, 60% B) with the following physical and spectral data:

2.1.1.4.1. 2-Bromo-2,4,4,7-tetramethyl-9-oxabicyclo[4.3.0] nonan-8-one (5b). **5b**: m.p. = 54–55 °C; IR (KBr): 2955, 1772, 1454, 1197, 1166, 970 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$): δ 0.92 (s, 3H, CH$_3$-10 B), 0.94 (s, 3H, CH$_3$-10 A), 1.02 (m, 2H, CH$_2$-5 B), 1.20 (d, J = 7.0 Hz, 3H, CH$_3$-12 B), 1.22 (s, 3H, CH$_3$-11 A), 1.23 (s, 3H, CH$_3$-11 B), 1.33 (dd, J = 7.5 Hz, 3H, CH$_2$-12 A), 1.46 (ddd, J = 13.8, 5.4 and 2.1 Hz, 2H, CH$_2$-5 A), 1.68 (dd, J = 15.6 and 8.4 Hz, 2H, CH$_2$-3 A), 1.93 (s, 3H, CH$_3$-9 B), 1.94 (s, 3H, CH$_3$-9 A), 2.00 (m, 2H, CH$_2$-3 B), 2.38 (q, J = 7.6 Hz, 1H, H-7 A), 2.52 (ddd, J = 12.8, 5.4 and 3.7 Hz, 1H, H-6 A), 2.81 (m, 1H, H-6 B), 2.87 (m, 1H, H-7 B), 4.54 (d, J = 2.5 Hz, 1H, H-1 B), 4.77 (d, J = 3.7 Hz, 1H, H-1 A) ppm; ^{13}C NMR (300 MHz, CDCl$_3$): 9.16 (C-12 B), 13.67 (C-12 A), 26.37 (C-11 A), 27.22 (C-11 B), 30.43 (C-5 B), 33.37 (C-9 B), 34.28 (C-9 A), 34.43 (C-10 A), 34.53 (C-10 B), 35.85 (C-4 B), 35.88 (C-4 A), 37.94 (C-6 A), 39.53 (C-5 A), 42.57 (C-3 B), 43.23 (C-6 B), 45.11 (C-7 A), 48.15 (C-3 A), 61.65 (C-7 B), 82.65 (C-1 A), 82.95 (C-1 B), 139.21 (C-2 A), 139.35 (C-2 B), 178.46 (C-8 B), 179.26 (C-8 A) ppm; *ESIHRMS*: calcd for $C_{12}H_{19}BrO_2$ m/z 275.0641 $(M + H)^+$, found 275.0653.

2.1.1.4.2. 2,4,4,7-Tetramethyl-9-oxabicyclo[4.3.0]non-2-en-8-one (6b). **6b**: n_D = 1.4871; IR (KBr): 2968, 1772, 1458, 1169, 974 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$): δ 0.95 (s, 3H, CH$_3$-10 B), 0.96 (s, 3H, CH$_3$-10 A), 1.00 (s, 3H, CH$_3$-11 B), 1.02 (s, 3H, CH$_3$-11 A), 1.04 (d, J = 14.3 Hz, 1H, one of CH$_2$-5 B), 1.18 (d, J = 7.3 Hz, 3H, CH$_3$-12 B),), 1.25 (d, J = 13.2 Hz, 1H, one of CH$_2$-5 A), 1.39 (d, J = 7.7 Hz, 3H, CH$_3$-12 A), 1.40 (ddd, J = 14.3, 4.5 and 1.2 Hz, 1H, one of CH$_2$-5 B), 1.48 (ddd, J = 13.2, 4.8 and 1.2 Hz, 1H, one of CH$_2$-5 A), 1.82 (s, 6H, CH$_3$-9 A, CH$_3$-9 B), 2.28 (m, 1H, H-6 A), 2.39 (qd, J = 7.7 and 1.2 Hz, 1H, H-7 A), 2.64 (m, 1H, H-6 B), 2.94 (m, 1H, H-7 B), 4.23 (d, J = 5.0 Hz, 1H, H-1 B), 4.62 (d, J = 5.7 Hz, 1H, H-1 A), 5.53 (s, 2H, H-3 A, H-3 B) ppm; ^{13}C NMR (300 MHz, CDCl$_3$): 9.35 (C-12 B), 15.62 (C-12 A), 21.06 (C-9 A), 26.94 (C-10 B), 26.94 (C-10 B), 27.22 (C-10 A), 28.48 (C-11 B), 30.55 (C-11 A), 32.06 (C-4 B), 32.20 (C-4 A), 33.61 (C-5 B), 35.85 (C-6 B), 38.71 (C-5 A), 39.03 (C-6 A), 39.96 (C-7 B), 43.26 (C-7 A), 77.40 (C-1 A), 77.44 (C-1 B), 127.17 (C-2 B), 127.18 (C-2 A), 139.22 (C-3 A), 139.37 (C-3 B), 175.39 (C-8 B), 179.97

(C-8 A) ppm; *ESIHRMS*: calcd for $C_{12}H_{18}O_2$, *m/z* 195.1380 $(M + H)^+$, found 195.1385.

2.2. Biotransformations

2.2.1. Microorganisms

All of the fungal strains that were used in the biotransformations came from the collection of the Institute of Biology and Botany, Medical University, Wrocław (*Fusarium culmorum* AM10, *Fusarium avenaceum* AM11, *Fusarium oxysporum* AM13, *Fusarium tricinctum* AM16, *Fusarium semitectum* AM20, *Fusarium equiseti* AM22, *Fusarium scirpi* AM199, *F. culmorum* AM196, *Fusarium solani* AM203, *Syncephalastrum racemosum* AM105, *Cunninghamella japonica* AM472, *Penicillium vermiculatum* AM30, *Penicillium chermesinum* AM113, *Penicillium frequentans* AM351, *Absidia coerulea* AM93, *Absidia cylindrospora* AM336, *Pleurotus ostreatus* AM600). These strains were cultivated on Sabouraud's agar containing 0.5% aminobac, 0.5% peptone, 4% glucose and 1.5% agar dissolved in distilled water at 28 °C and stored in a refrigerator at 4 °C.

2.2.1.1. Screening biotransformation. The fungal strains used for the biotransformations were cultivated at 25 °C in two Erlenmeyer flasks (300 mL). Each flask contained 100 mL of medium consisting of 3% glucose and 1% bacteriological peptone (Biocorp). After three days, 10 mg of the substrate dissolved in 1 mL of acetone was added to each flask with the grown culture. Incubation of the shaken cultures with substrate was continued for 14 days. After 5, 9 and 14 days of incubation, the mixture of unreacted substrate, products and mycelium were extracted with dichloromethane (15 mL) and analyzed by GC (DB-17 column).

2.2.1.2. Preparative biotransformation. A total of 100 mg of halolactone **3–5** was dissolved in 10 mL acetone and distributed between 10 Erlenmeyer flasks (300 mL) with the 3-day fungal strains cultures that were prepared as described above. Microorganisms that were able to form any product were incubated with substrate for 14 days and then the product mixture was extracted with dichloromethane (3 × 40 mL). Combined organic fractions were dried over anhydrous magnesium sulfate and the solvent was evaporated *in vacuo* and subsequently, pure product was separated from the unreacted substrate and the metabolites of the fungi by column chromatography (silica gel, hexane:acetone 3:1).

The physical and spectral data of the hydroxylactones obtained during biotransformation are presented below:

2.2.1.2.1. 9-Hydroxy-2,4,4-trimethyl-9-oxabicyclo[4.3.0] nonan-8-one (7a). Oil; IR (KBr): 3438, 2957, 1751, 1471, 1213, 1170 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$): δ 1.00 (s, 3H, CH$_3$-10), 1.05 (s, 3H, CH$_3$-11), 1.24 (dd, *J* = 13.7 and 13.7 Hz, 1H, one of CH$_2$-5), 1.50 (ddd, *J* = 13.2, 4.7 and 1.2 Hz, 1H, one of CH$_2$-5), 1.88 (m, 1H, OH), 2.25 (d, *J* = 17.5 Hz, 1H, one of CH$_2$-7), 2.68 (m, 1H, H-6), 2.89 (dd, *J* = 17.5 and 8.0 Hz, 1H, one of CH$_2$-7), 4.20 (dd, *J* = 4.3 and 0.9 Hz, 2H, CH$_2$-9), 4.81 (d, *J* = 5.4 Hz, 1H, H-1), 5.86 (s, 1H, H-3) ppm; ^{13}C NMR (300 MHz, CDCl$_3$): 26.76 (C-10), 30.29 (C-11), 31.24 (C-6), 32.23 (C-4), 36.57 (C-7), 38.84 (C-5), 64.76 (C-9), 75.48 (C-1), 130.83 (C-2),

140.82 (C-3), 176.60 (C-8) ppm; *ESIHRMS*: calcd for $C_{11}H_{16}O_2$, *m/z* 181.1223 $(M + H)^+$, found 181.1228.

2.2.1.2.2. 9-Hydroxy-2,4,4,7-tetramethyl-9-oxabicyclo [4.3.0]nonan-8-one (7b). Oil; IR (KBr): 3444, 2958, 1766, 1457, 1198, 962 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$): δ 1.03 (s, 3H, CH$_3$-10), 1.08 (s, 3H, CH$_3$-11), 1.30 (dd, *J* = 13.4 and 13.3 Hz, 1H, one of CH$_2$-5), 1.41 (d, *J* = 7.6 Hz, 3H, CH$_3$-12), 1.56 (dd, *J* = 13.4 and 4.8 Hz, 1H, one of CH$_2$-5), 1.72 (m, 1H, OH), 2.34 (m, 1H, H-6), 2.41 (m, 1H, H-7), 4.24 (d, *J* = 16.2 Hz, 2H, CH$_2$-9), 4.94 (d, *J* = 5.6 Hz, 1H, H-1), 5.87 (s, 1H, H-3) ppm; ^{13}C NMR (300 MHz, CDCl$_3$): 15.54 (C-12), 27.00 (C-10), 30.24 (C-11), 32.11 (C-4), 38.75 (C-5), 38.81 (C-6), 43.06 (C-7), 64.85 (C-9), 73.68 (C-1), 130.82 (C-2), 140.66 (C-3), 179.62 (C-8) ppm; *ESIHRMS*: calcd for $C_{12}H_{18}O_3$, *m/z* 211.1329 $(M + H)^+$, found 211.1334.

2.2.1.2.3. 11-Hydroxy-2,4,4,7-tetramethyl-9-oxabicyclo [4.3.0]nonan-8-one (8b). Oil; IR (KBr): 3420, 2958, 1767, 1457, 1197, 963 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$): δ 1.03 (s, 3H, CH$_3$-10), 1.22 (dd, *J* = 7.2 and 5.1 Hz, 1H, one of CH$_2$-5), 1.37 (d, *J* = 7.6 Hz, 3H, CH$_3$-12), 1.65 (m, 1H, OH), 1.84 (dd, *J* = 13.5 and 5.1 Hz, 1H, one of CH$_2$-5), 2.42 (qd, *J* = 7.6 and 1.6 Hz, 1H, H-7), 2.48 (m, 1H, H-6), 3.85 (d, *J* = 1.8 Hz, 2H, CH$_2$-11), 4.69 (d, *J* = 5.7 Hz, 1H, H-1), 5.49 (s, 1H, H-3) ppm; ^{13}C NMR (300 MHz, CDCl$_3$): 15.48 (C-12), 21.28 (C-9), 25.10 (C-10), 30.60 (C-5), 37.61 (C-4), 39.00 (C-6), 43.13 (C-7), 69.38 (C-11), 77.07 (C-1), 131.73 (C-2), 134.14 (C-3), 179.75 (C-8) ppm; *ESIHRMS*: calcd for $C_{12}H_{18}O_3Na$, *m/z* 233.1215 $(M + Na)^+$, found 233.1154.

2.2.1.2.4. 7-Hydroxy-2,4,4,7-tetramethyl-9-oxabicyclo[4.3.0] nonan-8-one (9b). Oil; IR (KBr): 3444, 2959, 1775, 1451, 1204 cm^{-1}, ^{1}H NMR (300 MHz, CDCl$_3$): δ 0.94 (d, *J* = 13.6 Hz, 1H, one of CH$_2$-5), 0.98 (s, 3H, CH$_3$-10), 1.03 (s, 3H, CH$_3$-11), 1.37 (m, 1H, one of CH$_2$-5), 1.41 (s, 3H, CH$_3$-12), 1.60 (m, 1H, OH), 1.84 (s, 3H, CH$_3$-9), 2.48 (dt, *J* = 14.5 and 4.2 Hz, 1H, H-6), 4.83 (d, *J* = 4.8 Hz, 1H, H-1), 5.54 (s, 1H, H-3) ppm; ^{13}C NMR (300 MHz, CDCl$_3$): 19.36 (C-12), 21.00 (C-9), 26.90 (C-10), 30.74 (C-11), 32.27 (C-4), 34.11 (C-5), 42.52 (C-6), 77.63 (C-1), 127.07 (C-2), 139.19 (C-3), 177.05 (C-7), 177.10 (C-8) ppm; *ESIHRMS*: calcd for $C_{12}H_{18}O_3$, *m/z* 211.1329 $(M + H)^+$, found 211.1334.

2.2.1.3. Bioassay. Tests were made using the following strains of bacteria: *Escherichia coli* C1, *S. aureus*, *Bacillus subtilis* B5, *P. fluorescens* W1; yeast: *Candida albicans* KL-1, *Yarrowia lipolytica* ATCC 20460, *Saccharomyces cerevisiae* SV30; and filamentous fungal: *Alternaria* sp., *Fusarium linii* A3, *Aspergillus niger* XP, *Penicillium* sp. These strains came from the collection at the Department of Biotechnology and Food Microbiology, Wroclaw University of Environmental and Life Sciences. The tests were performed on the automated Bioscreen C system (Automated Growth Curve Analysis System, Lab Systems, Finland) following the procedure below. The bacterial cultures were carried out for 48 h in a liquid broth containing 15 g of dry bullion (Biocorp) and 10 g of glucose dissolved in 1 L of distilled water. Yeast and fungi were cultured in YPG medium containing 10 g of yeast extract, 10 g of bacteriological peptone and 10 g of glucose dissolved in 1 L of distilled water for 48 and 96 h, respectively. The working volume in the wells of the Bioscreen plate was 300 μL, comprising 280 μL of culture medium, and 10 μL of cell or spore solution (final density 1×10^6 cells mL^{-1}). Unsaturated lactones and hydroxylactones were dissolved in 10 μL dimethyl

sulfoxide and used at a final concentration of 0.1% (w/v). The temperatures were controlled to 30 °C (bacteria, yeasts) and 25 °C (filamentous fungi). The optical densities of the cell suspensions were measured automatically at 560 nm at regular intervals of 30 min., for 2–4 days. The cell cultures were placed on a continuous shaker. Each culture was performed in three replicates.

The data were analyzed using spreadsheet software (Excel 97) and the means for the triplicates of each culture medium type were calculated. The mean values were used to generate the growth curves for each investigated strain, constituting a function of the incubation time and the culture medium absorbency. The resulting microbial growth curves were compared to control cultures in the medium supplemented with dimethyl sulfoxide.

2.3. Odour evaluation

The odour evaluation was performed for ethanolic solutions (10%) of samples with the use of a strip blotter. The samples were judged by the five experienced panellists who evaluated the odour characteristics of new compounds according to literature [27,31,32].

3. Results and discussion

3.1. Chemistry

Two racemic unsaturated lactones **6a** and **6b** were synthesised from the commercially available ketone isophorone **1**. The first known allyl alcohol **2** was synthesised using the procedure described earlier [28]. Obtained alcohol **2** was subjected to Claisen rearrangement using triethyl orthoacetate [29] and triethyl orthopropionate, which led to the two esters **3a** and **3b**. Basic hydrolysis of these esters led us to obtain two unsaturated acids (**4a,b**) of which the second was a pair of diastereomers (we didn't separate them). During the next step, we tried to obtain bromolactones using a known procedure [23]. Unfortunately, during these reactions a mixture of bromolactones (**5a,b**) and unsaturated lactones (**6a,b**) were obtained with the latter predominating instead of the expected products. An attempt to separate the products by column chromatography allowed us only to increase the amount of unsaturated lactone in the mixture. We were able to isolate only a small amount of the bromolactones **5a** and **5b**. Therefore we decided to perform a dehydrodehalogenation reaction using DBU in toluene according to the known method [30]. As a result of these reactions, the unsaturated lactones **6a** and **6b**, of which the second was a mixture of two diastereoisomers, were obtained. After isolation (in a small amount) of one of these diastereoisomers (named **A**), the appropriate structures of the two compounds (named **A** and **B**) were assigned (Scheme 1).

The analysis of the NMR spectra allowed us to determine the structures of particular compounds. In the case of ester **3a**, a wide multiplet of proton H-1 suggested its axial position, which proves that the carboetoxy group occupied an equatorial position. We could observe the same situation for acid **4a**. Looking at spectra of bromolactone **5a** and unsaturated lactone **6a** we could observe that proton H-6 had retained its axial position (wide multiplet) and proton H-1 was in equato-
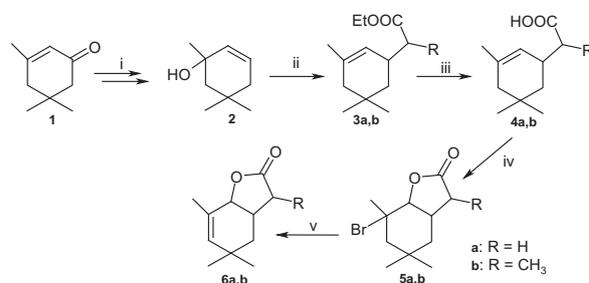

Scheme 1 Synthesis of unsaturated lactones **6a**, **6b** (i) (1) H_2O_2, OH^-, (2) N_2H_4, AcOH, MeOH; (ii) $CH_3C(OC_2H_5)_3$, CH_3CH_2-COOH or $CH_3CH_2C(OC_2H_5)_3$, CH_3CH_2COOH, 137 °C; (iii) KOH, EtOH, 100 °C; (iv) NBS, THF, rt; (v) DBU, toluene, 110 °C.

rial position (Scheme 2). This observation suggested that the cyclohexane ring was in the chair conformation. The presence of the γ-lactone ring in the molecules of lactones **5a** and **6a** was confirmed by the IR spectra absorption bands at 1776 cm^{-1} and 1781 cm^{-1} for **5a** and **6a**, respectively.

Analysis of the NMR spectra of ester **3b** and acid **4b** proved that proton H-1 occupied an axial position and was in an equatorial position, similar to the previous case. Adding an additional methyl group on carbon C-7 led to obtaining these compounds as pairs of diastereoisomers. However, this was confirmed only by GC analysis. In the NMR spectra of lactone **6b**, an almost equimolar mixture of two diastereoisomers was observed. The analysis of these spectra proved that the cyclohexane ring was in the chair conformation. The wide multiplet of proton H-6 indicated its axial position, while the shape of the signal of proton H-1 demonstrated its equatorial position. An additional methyl group at carbon C-7 was located across the plane of the lactone ring (case **A**) or in the plane of lactone ring (case **B**) (Scheme 3). This observation was proved by the fact that in case **A** this signal was a multiplet which looked like a quintet, while in case **B** the signal from H-7 was a quartet of doublets. The IR spectra absorption bands at 1772 cm^{-1} for both of the lactones confirmed the presence of the γ-lactone ring.

3.2. Biotransformations

Research carried out by our team confirmed that most filamentous fungi biotransformed unsaturated lactones to their oxygen derivatives. A regioselective enzymatic hydroxylation or epoxidation is catalyzed by oxygenases and specifically cytochrome P-450 monooxygenase [33]. In the experiments conducted earlier on similar systems, we observed the best results for a strain of *A. cylindrospora* AM336. The unsaturated lactone containing two methyl groups in cyclohexene ring was hydroxylated by this strain with a good yield [34]. Also unsaturated bicyclic lactones with p-menthane system are also transformed by this strain to hydroxyderivative.
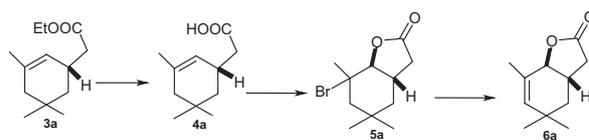
Scheme 2 Structures of compounds **3a**–**6a**.
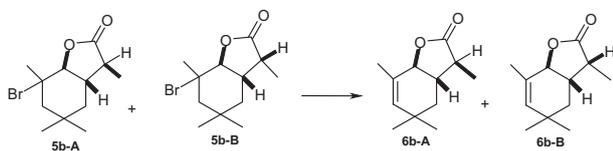
Scheme 3   Structures of compounds **3b**–**6b**.

Transformation was carried out using not only *A. cylindrospora* but also *Absidia glauca* and *S. racemosum* [35]. On the other hand tricyclic unsaturated lactone, for example isodrimenin was hydroxylated in allylic position by the strain *Rhizopus arrhizus* [36].

In our previous research, we observed that the strain of *A. cylindrospora* was able also to oxidize the double bond to the oxirane ring in case of bicyclic unsaturated lactones with two or three methyl groups in the cyclohexene ring. This effect was due to the fact that allyl position was blocked by *gem*-dimethyl-group [37] and it was impossible to obtain allyl hydroxy derivative. Furthermore in bicyclic sesquiterpene lactones with germacrene or guaiac system epoxidation of the double bond by *Cunninghamella echinulata* was observed [38]. Epoxidation of the double bond of tricyclic dehydrocostus lactone was also observed by Xiao-Ci Ma [39]. This reaction was catalyzed by fungus *Aspergillus candidus* and *Mucor polymorphosporus*.

Both unsaturated lactones **6a** and **6b** (as a mixture of **A** and **B**) obtained during synthesis were subjected to a screening biotransformation. We intended to determine which fungal strains are able to introduce a hydroxy group into these lactones. We chose the following strains: *F. culmorum* AM10, *F. avenaceum* AM11, *F. oxysporum* AM13, *F. tricinctum* AM16, *F. semitectum* AM20, *F. equiseti* AM22, *F. scirpi* AM199, *F. culmorum* AM196, *F. solani* AM203, *S. racemosum* AM105, *C. japonica* AM472, *P. vermiculatum* AM30, *P. chermesinum* AM113, *P. frequentans* AM351, *A. coerulea* AM93, *A. cylindrospora* AM336, and *P. ostreateus* AM600. The progress of each of the screening transformations was monitored by means of standard techniques (TLC and GC). Results of this step are shown in Tables 1 and 2.

During the screening biotransformation, 17 different fungal strains were tested. Only three or four were able to perform hydroxylation of substrates **6a** or **6b** respectively. In the case

Table 1   Hydroxylation of unsaturated lactone **6a** during screening biotransformation.

Entry	Strain	Days	Substrate 6a (%)	Product 7a (%)
1	*Penicillium vermiculatum* AM30	5	27.1	72.9
		9	16.0	84.0
		14	6.2	**93.8**
2	*Absidia cylindrospora* AM336	5	82.9	–
		9	40.7	16.1
		14	14.4	21.2
3	*Pleurotus ostreatus* AM600	5	100	–
		9	62.8	37.2
		14	34.4	**65.6**

The best results are highlighted in bold.

of lactone **6a** only one product (**7a**) was present in every transformation. When lactone **6b** was used as a substrate, the formation of three different products (**7b**, **8b** and **9b**) was observed (Schemes 4 and 5).

In the next step, that is the preparative biotransformation, we used only the fungal strains that were able to transform substrates with yields exceeding 50%. During the preparative scale biotransformation of unsaturated lactone **6a**, one and the same hydroxylactone **7a**, which was the result of the substitution of a hydroxy group in an allylic position, was obtained. It is worth noting that of the two possible allylic positions (C-1 and C-9) only one was selected (at C-9) and it is probable that this one was more accessible. Use of *P. vermiculatum* strain allows to obtain the product 7a with 87.4% yield (according to GC). In the case of *P. ostreateus* strain this lactone was created with 61.9% yield. Isolated yields for these reactions were 23.6% (0.026 g) and 14.5% (0.016 g) respectively. Extremely interesting for us is the fact that in the available literature we did not find positive examples of hydroxylation using a strain of *Penicillium vermiculatum*.

In the case of unsaturated lactone **6b**, the formation of hydroxylactone **7b**, which was analogous to product **7a** mentioned above, was also observed. However, in this case, two other unexpected hydroxylactones were created: the first with a hydroxy group substituted at methyl group C-11 (**8b**), and

Table 2   Hydroxylation of unsaturated lactone **6b** during screening biotransformation.

Entry	Strain	Days	Substrate 6b (%)	Product 7b (%)	Product 8b (%)	Product 9b (%)
1	*Penicillium vermiculatum* AM30	5	100		–	–
		9	–	**44.3**	**55.7**	–
		14	–	44.3	55.7	–
2	*Syncephalastrum racemosum* AM 105	5	75.2	–	–	24.8
		9	57.3	–	–	42.7
		14	48.7	–	–	**52.2**
3	*Cunninghamella japonica* AM472	5	100	–	–	–
		9	81.8	4.2	5.8	8.2
		14	66.3	11.7	12.0	10.0
4	*Pleurotus ostreatus* AM600	5	79.9	6.9	8.0	5.2
		9	70.3	10.4	12.0	7.3
		14	57.0	16.0	18.8	8.2
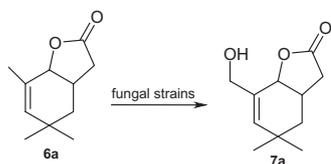
Scheme 4 Biotransformation of lactone **6a**.
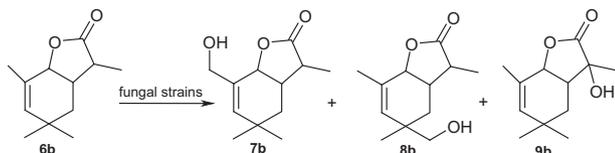
Scheme 5 Biotransformation of lactone **6b**.

the second in which carbon C-7 was attacked by a hydroxy group (**9b**). The formation of the last product reveals a large stereoselectivity bias of the microorganism that was able to conduct this transformation. As was mentioned earlier, unsaturated lactone **6b**, which was used as the substrate for biotransformation, was a mixture of two diastereoisomers (**A**, **B**). Such situation caused that during the biotransformation, catalyzed by *P. vermiculatum* AM30, the mixture of the two hydroxylactones **7b** (44.1%) and **8b** (55.9 %) was created. The separation of this mixture of lactones led to obtain pure products: **7b** (0.031 g, yield 28.2%) and **8b** (0.041 g, yield 37.3%). We were able to isolate these two products and determine their structures. Product **7b** was created from the minor

substrate **6b-A**, while the major substrate **6b-B** was transformed into product **8b**. In the case of lactone **6b-A**, the signal from proton H-7 was a multiplet that looked like a quintet, the same as for hydroxylactone **7b** (multiplet). In the case of unsaturated lactone **6b-B** and hydroxylactone **8b**, the signal from proton H-7 was a quartet of doublets with coupling constants 7.6 and 1.6 Hz for both of them. This shows that even a small change in the structure of the substrate has a large impact on the structure of resulting product. The unexpected product **9b** was obtained when *S. racemosum* AM105 was used for biotransformation. This product was created with yield 48.5% according to GC. Pure compound was obtained with yield 11.8% (0.013 g).

We were able to evaluate an enantiomeric excess and optical rotation only for one of the obtained products of biotransformation: hydroxylactone **9b**. These values are as follows: ee = 47.3% and $[\alpha]_D^{20} = -4.953, c = 0.17\%$.

3.3. Biological activity

Because of the fact that of the several tested microorganisms only a few proved to be capable of performing changes to the structure of lactones **6a** and **6b**, we decided to check their antimicrobial properties. We tested these lactones on different bacteria, yeast and fungi strains for their ability to inhibit or reduce the growth of the selected microorganisms. Hydroxylactones **7a**, **7b** and **8b** obtained during biotransformation were also subjected to these biological tests. Hydroxylactone **9b** was obtained only in small quantities, which were insufficient to perform biological tests with this compound. The results of these tests are shown in Table 3.

Table 3 Assessment of the effects of unsaturated lactones **6a**, **6b** and hydroxylactones **7a**, **7b** and **8b** on the growth of bacteria, yeast and filamentous fungi.

Strain	Control		Compound									
			6a		6b		7a		7b		8b	
	Lag phase [h]	ΔOD	Lag phase [h]	ΔOD	Lag phase [h]	ΔOD	Lag phase [h]	ΔOD	Lag phase [h]	ΔOD	Lag phase [h]	ΔOD
Bacteria												
E. coli	2	1.29	4	1.26	6.5	1.16	4	1.16	7.5	0.84	3.5	0.95
S. aureus	6	1.20	22.5	0.97	15	0.78	17	0.49	–	0	15	0.65
B. subtilis	8	1.18	28.5	1.07	10.5	0.66	–	0	–	0	–	0
P. fluorescens	4	1.28	-	0	3.33	1.22	–	0	–	0	–	0
Yeast												
C. albicans	2	0.78	3	0.70	–	0	2.5	0.75	2	0.66	2.5	0.69
Y. lipolytica	3	1.21	3	1.03	–	0	3	0.97	7	0.96	3.5	0.96
S. cerevisiae	4	1.14	6	0.98	12	0.8	9	0.97	7.5	1.38	10.5	0.63
Filamentous fungi												
Alternaria sp.	10.5	1.34	22	0.99	–	0	21	1.00	17.5	0.46	30	0.99
F. linii	19.5	1.40	–	0	–	0	–	0	–	0	32	1.09
A. niger	3.5	1.60	3.0	1.44	12	0.8	5.0	1.18	7.50	0.63	4.5	1.29
Penicillium sp.	9.5	2.07	9.5	2.01	–	0	15.5	1.61	16.5	1.34	17	1.43

Grey colour indicated complete inhibition of growth of the microorganism by the compound.

The biological tests performed on unsaturated lactones showed that lactone **6a** inhibited growth of only one bacterial strain (*P. fluorescens*) and one filamentous fungal strain (*F. linii*). In the case of lactone **6b** the results were better. This compound inhibited growth of two yeast strains (*C. albicans* and *Y. lipolytica*) and three filamentous fungal strains (*Alternaria* sp., *F. linii* and *Penicillium* sp.). It was very interesting that both compounds showed activity against the *F. linii,* which explains why it was impossible for the different *Fusarium* species chosen by us to carry out the biotransformation of lactones **6a** and **6b**.

Bioassay tests performed on the hydroxylactones obtained during biotransformation showed that the best results were obtained for compounds **7a**, **7b** and **8a**, when they were used against bacteria strains (except *E. coli*). All three hydroxylactones inhibited growth of *B. subtilis* and *P. fluorescens*. Additionally, compound **7b** was active against *S. aureus*. All three of the tested lactones were inactive against yeast strains and most of the fungal strains. Only in the case of lactones **7a** and **7b**, growth inhibition of *F. linii* was observed.

3.4. Odour characteristic

An olfactory analysis of the new compounds was also performed. The most interesting compounds in regard to their scent were the two esters **3a** and **3b**. These compounds were characterized by a fruity smell, with a distinct hint of pear in the case of ester **3b**. A fruity note was observed also for hydroxylactone **7b**, but opposite to esters there were dry woody notes. Hydroxylactone **9b** was characterized by other interesting smells associated with the forest (notes of dry needles and resins). Interestingly, all of the hydroxylactones were characterized by a dry, sometimes woody, scent. Unfortunately, unsaturated lactones **7a** and **7b** were almost odourless.

For many years we've been obtaining the compounds with interesting odouriferous properties by chemical synthesis [40,31,41]. Our previous research made us suppose that molecules such as ethyl esters **3a** and **3b** are characterized by a fruity odour. The smell of hydroxylactones turned out to be in line with our previous results [42].

4. Conclusions

Two unsaturated lactones (**6a** and **6b**) were obtained through a five-step synthesis. The second compound (**6b**) was created as a pair of diastereoisomers, named **6b-A** and **6b-B**. These lactones (**6a** and **6b**) were subjected to a screening biotransformation using 17 fungal strains. It appears that only three of these strains were able to transform the unsaturated lactones into hydroxy derivatives. The chosen microorganism showed very high regiospecificity. When lactone **6a** was used as the substrate, generation of only the hydroxylactone was observed. The two fungal strains *P. vermiculatum* AM30 and *P. ostreateus* AM600 were able to perform hydroxylation of lactone **6a** in one of the two possible allylic positions (methyl group CH$_3$-9). In the case of the diastereoisomeric mixture of lactone **6b-A** and **6b-B**, creation of three hydroxylactones was observed. Biotransformation with the *P. vermiculatum* AM30 strain led to the generation of two different hydroxylactones. Each of these was the result of hydroxylation of two possible allylic positions. In the case of isomer **6b-A**, methyl group

CH$_3$-9 was hydroxylated, in the case of isomer **6b-B** methyl group CH$_3$-11 was hydroxylated. The second strain (*S. racemosum* AM105) was able to transform lactone **6b** to an unexpected product, in which the hydroxy group attacked the tertiary carbon C-7. This compound was obtained with enantiomeric excess equal to 47.3% and with a predominance of the (−) isomer. The biological tests performed on unsaturated lactone **6a** and hydroxylactones **7a**, **7b** and **8a** showed that these lactones inhibited the growth of the pathogenic bacterial strains (*S. aureus, P. fluorescens*) and only one of the filamentous fungal strains (*F. linii*). Unsaturated lactone **6b** inhibited the growth of pathogenic yeast strain (*C. albicans*) and strains of filamentous fungi (*Alternaria* sp., *Penicillium* sp.). The fact that all of the lactones inhibited growth of the *F. linii* strain could explain the earlier observation that the unsaturated lactones **6a** and **6b** could not be biotransformed by different *Fusarium* strains. Olfactory analysis carried out on all of the new products proved that the most interesting smell, a predominantly fruity aroma, characterized esters **3a** and **3b** and hydroxylactone **7b**. The scent of hydroxylactone **9b** was also interesting, as it was endowed with a forest scent.

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