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Lactones 27 [1]: Transformations of γ-lactones fused to a dimethylcyclohexane ring in *Absidia cylindrospora* cultures

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Abstract

Two saturated (**4a**,**b**) and one unsaturated (**5**) bicyclic γ-lactones containing a dimethylcyclohexane ring were subjected to biotransformation using the fungal strain *Absidia cylindrospora*. Six new compounds (**6–11**) and one known (**12**) [K.W. Rosenmund, H. Herzberg, H. Schutt, Chem. Ber. 87 (1954) 1258] [2] were isolated. All substrates were stereoselectively hydroxylated by the microorganism at either the C-4 (in the case of **4a** and **5**) or C-2 position (in case of **4a** and **4b**) giving the corresponding hydroxylactones with tertiary (**6** and **9**) or secondary (**8** and **10**) hydroxy groups, respectively.

The hydroxy group was also introduced into C-3 (in the case of **4a**) and C-6 (in the case of **4b**) positions. The structures of all obtained products were established on the basis of their spectral data. In the case of lactones **8–10** these structures were undoubtedly confirmed by their X-ray analysis.
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1. Introduction

Microbial hydroxylation of non-activated carbon atoms is one of the most used biotransformation reactions employed in organic synthesis [3,4]. This reaction catalyzed by cytochrome P-450 monooxygenases [5] is a very valuable method of functionalization of organic molecules. A variety of organic compounds have been subjected to hydroxylation by fungi [6–11]. The introduction of this functional group into the molecule leads to intensification of the biological properties of the compounds isolated from natural sources [12], or increases their solubility in water which is very important for their use as potential drugs [13]. Additionally, microbial transformation can be a good way to predict metabolic pathways in mammals [14,15].

Terpenes and terpenoids, both natural and synthetic, are one of the most studied substrates in this research area [16–23]. Among these chemicals, the ones with a lactone moiety have attracted particular attention because of their wide spectrum of biological activity [24–26]. Encouraged by the literature reports about hydroxylation of naturally occurring molecules with a lactone ring, [27–30] we applied some fungal strains to regioselective functionalization of some synthetic saturated [31] and unsaturated [1] bicyclic γ-lactones, possessing an alkyl substituted cyclohexane ring. The results obtained with the fungus *Absidia cylindrospora* have prompted us to use this microorganism for microbial transformation of three additional lactones containing the dimethylcyclohexane ring system. In this paper we would like to present the results of biotransformations of these three lactone substrates.

2. Materials and methods

2.1. Analysis

The progress of chemical reactions and biotransformations as well as the purity of isolated intermediates and products were monitored by TLC on silica gel coated aluminium plates (DC, Alufolien Kieselgel 60 F_{254}, purchased from Merck) and by GC analysis carried out on a Varian CP-3380 instrument with an HP-1 column (crosslinked methyl silicone gum, 25 m × 0.32 mm × 0.25 μm) and HP-5 column (crosslinked methyl silicone gum, 30 m × 0.32 mm × 0.25 μm). The enantiomeric compositions of the products obtained were

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determined by GC analysis using a CP-cyclodextrin-B-2,3,6-M-19, 25 m × 0.25 × mm × 0.25 μm chiral column. All the GC columns were purchased from Hewlett-Packard.

Preparative flash chromatography was performed on silica gel (Kieselgel 60, 230–400 mesh, purchased from Merck).

^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions on a Bruker Avance DRX 300 spectrometer. The assignments of ^{13}C chemical shifts were made by means of distortionless enhancement by polarization transfer method (DEPT 135) and C/H correlation heteronuclear multiple quantum coherence (HMQC). IR spectra were determined using an FTIR Thermo-Mattson IR 300 Spectrometer. Optical rotations were measured on a Autopol IV automatic polarimeter (Rudolph). All the melting points are uncorrected. They were determined on a Boetius apparatus.

2.2. X-Ray crystallographic data

Crystallographic measurements were performed at 100 K using an Oxford Cryosystem device on a Kuma KM4CCD κ-axis diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). Crystals were positioned at 65 mm from the CCD camera, 612 frames were measured at 0.75° intervals with a counting time of 5–10 s. The data were corrected for Lorentz and polarization effects. No absorption correction was applied. Data reduction and analysis were carried out with the CrysAlis CCD and CrysAlis Red programs [32]. Structures were solved by direct methods (program SHELXS97) and refined by the full matrix least-squares method on all F^2 data using the SHELXL97 programs [33]. Non-hydrogen atoms were refined with anisotropic displacement parameters; hydrogen atoms were placed in calculated positions or found in $\Delta\rho$ maps. Before the last cycle of refinement all H atoms were fixed and were allowed to ride on their parent atoms. The Friedel pairs were merged before the final refinement.

Crystallographic data for crystals **8**, **9** and **10** in this paper, have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 276160–276162, respectively. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB12 1EZ, UK (fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

2.3. Substrates for biotransformation

Racemic lactones: 4,6-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**4a**), and 4,6-dimethyl-9-oxabicyclo[4.3.0]non-2-en-8-one (**5**) were synthesized from ester **1a** according to the procedure described earlier [34].

Here we present the spectral data of these compounds in order to compare them with the spectra of corresponding biotransformation products and notice the changes caused by the microorganism applied.

2.3.1. 4,6-Dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (*4a*)

n_D = 1.4765; ^{1}H NMR ($CDCl_3$), δ: 0.86 (d, J = 6.1 Hz, 3H, CH_3-4), 1.07 (m, 2H, CH_2-5), 1.13 (s, 3H, CH_3-6), 1.20–1.49 (three m, 3H, CH_2-3 and H-4), 1.60 (dddd, J = 15.1, 13.8, 3.9 and 3.6 Hz, 1H, H-2, axial), 2.10 (ddt, J = 15.1, 5.5 and 2.6 Hz, 1H, H-2, equatorial), 2.21 and 2.36 (two d, J = 16.5 Hz, 2H, CH_2-7, AB system), 4.13 (m, 1H, H-1); ^{13}C NMR ($CDCl_3$); δ: 21.39(C-10), 22.18(C-11), 24.97(C-2), 27.14(C-4), 27.92(C-3), 39.10(C-6), 41.78(C-5), 46.33(C-7), 83.40(C-1), 176.66(C-8); IR (film, cm^{-1}): 2940(s), 1796(s), 1208(s), 1133(s).

2.3.2. 4,6-Dimethyl-9-oxabicyclo[4.3.0]non-2-en-8-one (*5*)

n_D = 1.5057; ^{1}H NMR ($CDCl_3$), δ: 1.01 (d, J = 7.1 Hz, 3H, CH_3-4), 1.11 (s, 3H, CH_3-6), 1.17 (dd, J = 13.1 and 11.4 Hz, 1H, one of CH_2-5), 1.49 (dd, J = 13.1 and 5.0 Hz, 1H, one of CH_2-5), 2.22 (m, 1H, H-4), 2.30 and 2.50 (two d, J = 17.1 Hz, 2H, CH_2-7, AB system), 4.28 (d, J = 4.3 Hz, 1H, H-1), 5.80 (ddd, J = 10.0, 4.3 and 2.4 Hz, 1H, H-2), 5.90 (d, J = 10.0 Hz, 1H, H-3); ^{13}C NMR ($CDCl_3$); δ: 20.55(C-10), 21.35(C-11), 27.16(C-4), 37.47(C-6), 38.36(C-5), 45.02(C-7), 79.96(C-1), 120.53(C-2), 140.25(C-3), 175.77(C-8); IR (film, cm^{-1}): 2961(s), 1764(s), 1210(m), 997(m), 952(m).

2.4. Synthesis of 3,3-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (*4b*)

Lactone **4b** was synthesized in four step synthesis from 6,6-dimethylcyclohex-2-en-1-ol (Scheme 1).

2.4.1. Ethyl (4,4-dimethylcyclohex-2-en-1-yl) acetate (*1b*)

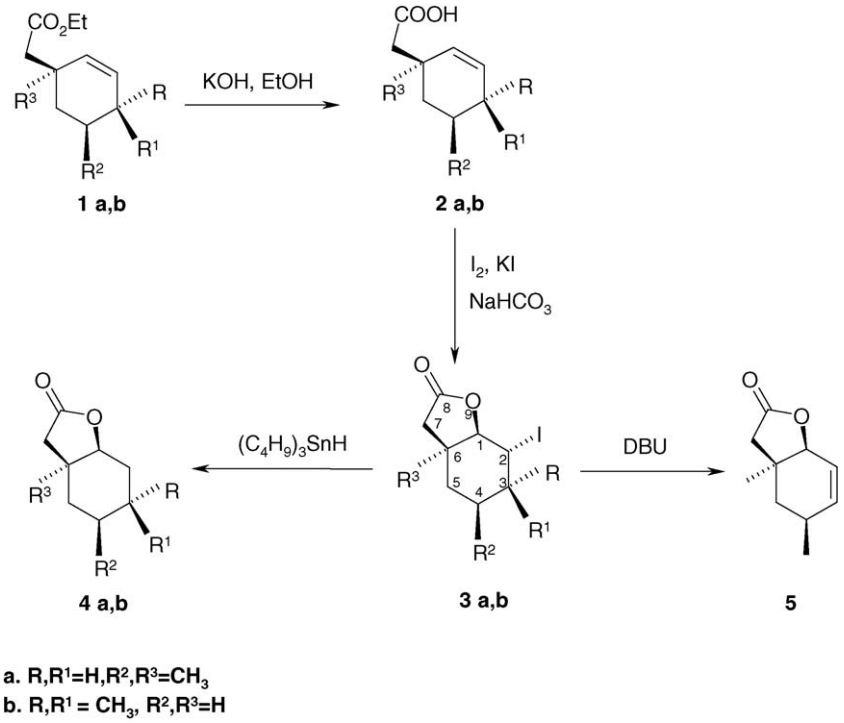
A mixture of 6,6-dimethylcyclohex-2-en-1-ol (8.2 g, 0.084 mol), triethyl orthoacetate (77 ml, 0.422 mol) and a catalytic amount (0.1 ml) of propionic acid was heated at 138 °C for 30 h with continuous distilling off the ethanol. When the reaction was complete (TLC, GC), the rest of ethyl orthoacetate was removed by distillation and the crude product was purified by means of column chromatography (silica gel, hexane:acetone 15:1) yielding 7.2 g (51%) of ester **1b** with the following physical and spectral data:

n_D = 1.4472; ^{1}H NMR ($CDCl_3$), δ: 0.93 and 0.94 (two s, 6H, $(CH_3)_2$C<), 1.23 (t, J = 7.1 Hz, 3H, –OCH$_2$CH_3), 1.33–1.47 (two m, 3H, CH_2-6 and one of CH_2-5), 1.75 (m, 1H, one of CH_2-5), 2.20 (dd, J = 14.8 and 8.2 Hz, 1H, one of CH_2-7), 2.27 (dd, J = 14.8 and 6.8 Hz, 1H, one of CH_2-7), 2.48 (m, 1H, H-1), 4.11 (q, J = 7.1 Hz, 2H, −OCH_2CH$_3$), 5.35–5.44 (two m, 2H, H-2 and H-3); IR (film, cm^{-1}): 2955(m), 1736(s), 1161(m).

2.4.2. (4,4-Dimethylcyclohex-2-en-1-yl) acetic acid (*2b*)

Ester **1b** (7.2 g, 0.043 mol) was dissolved in a 2.5% ethanol solution of KOH (20 ml) and heated under reflux for 3 h. Then the ethanol was evaporated and the residue was diluted with water. Organic impurities were extracted twice with diethyl ether. The water layer was acidified with 1 M HCl adjusting pH to 4 and extracted with diethyl ether. The ethereal fractions were combined, washed with brine and dried over magnesium sulfate. After evaporating of solvent under vacuum 5.8 g (98%) of pure acid **2b** was afforded:

oily liquid, ^{1}H NMR ($CDCl_3$), δ: 0.94 and 0.95 (two s, 6H, $(CH_3)_2$C<), 1.28–1.41 (two m, 2H, CH_2-6), 1.48 (ddd, J = 15.4, 9.8 and 2.7 Hz, 1H, one of CH_2-5), 1.79 (m, 1H, one



Scheme 1.

of CH_2-5), 2.26 (dd, $J = 15.2$ and 8.1 Hz, 1H, one of CH_2-7), 2.34 (dd, $J = 15.2$ and 6.7 Hz, 1H, one of CH_2-7), 2.51 (m, 1H, H-1), 5.37–5.46 (two m, 2H, H-2 and H-3); IR (film, cm^{-1}): 2880–3220(s,b), 1709(s), 1293(m), 746(w).

2.4.3. 2-Iodo-3,3-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (*3b*)

A mixture of acid **2b** (5.8 g, 0.042 mol) in 25 ml of diethyl ether and 70 ml of 0.5 M $NaHCO_3$ solution was stirred 1 h at room temperature. Then a solution of I_2 (20.5 g) and KI (41.1 g) in 170 ml water was added dropwise. The mixture was stirred until a stable brown colour was obtained and after diluting with ether it was washed with $Na_2S_2O_3$. The separated organic layer was washed with saturated $NaHCO_3$ solution, brine and dried with anhydrous magnesium sulfate. The crude product was chromatographed on silica gel (hexane:acetone, 9:1) and 8.25 g (74%) of iodolactone **3b** was obtained. The product is characterized by the data given below:

mp = 100–120 °C; ^{1}H NMR ($CDCl_3$), δ: 1.04 and 1.07 (two s, 6H, $(CH_3)_2C<$), 1.52 (m,1H, one of CH_2-4), 1.62–1.71 (two m, 2H, one of CH_2-4 and one of CH_2-5), 1.88 (m, 1H, one of CH_2-5), 2.34 (dd, $J = 17.2$ and 8.4 Hz, 1H, one of CH_2-7), 2.49 (dd, $J = 17.2$ and 11.6 Hz, 1H, one of CH_2-7), 2.73 (m, 1H, H-6), 3.93 (d, $J = 9.3$ Hz, 1H, H-2), 4.83 (dd, $J = 9.3$ and 7.1 Hz, 1H, H-1); IR (KBr, cm^{-1}): 2964(s), 1776(s), 1169(s), 1130(s), 1089(s).

2.4.4. 3,3-Dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (*4b*)

Tributyltin hydride (2.32 g, 0.008 mol) was added to a solution of iodolactone **3b** (1.22 g, 0.004 mol) in benzene (10 ml) and the reaction mixture was stirred at room temperature until all iodolactone has reacted (TLC, 2 days). Then the benzene was partially evaporated in vacuo and the crude product was purified by column chromatography (silica gel, hexane:acetone, 6:1) to furnish the desired product **4b** (0.5 g, 83%) with the following physical and spectral data:

oily liquid; ^{1}H NMR ($CDCl_3$), δ: 0.89 and 0.94 (two s, 6H, $(CH_3)_2C<$), 1.19 (ddd, $J = 13.6$, 9.4 and 4.2 Hz, 1H, H-4, axial), 1.35 (dddd, $J = 13.6$, 7.4, 3.9 and 1.2 Hz, 1H, H-4, equatorial), 1.47 (m, 1H, one of CH_2-5), 1.53 (dd, $J = 15.0$ and 5.3 Hz, 1H, one of CH_2-2), 1.62–1.72 (two m, 2H, one of CH_2-2 and one of CH_2-5), 2.30 (dd, $J = 16.0$ and 5.1 Hz, one of CH_2-7), 2.42 (m, 1H, H-6), 2.51 (dd, $J = 16.0$ and 7.0 Hz, 1H, one of CH_2-7), 4.55 (m, 1H, H-1); ^{13}C NMR ($CDCl_3$), δ: 22.87(C-2), 28.42(C-10), 28.60(C-3), 29.41(C-11), 34.11(C-6), 34.92(C-4), 35.31(C-7), 39.92(C-5), 79.28(C-1), 177.17(C-8); IR (film, cm^{-1}): 2947(s), 1775(s), 1167(s), 1001(m), 943(m).

2.5. Microorganism and growth media

The chemicals used for the preparation of the growth media were purchased from BTL in Poland, except glucose which was bought in POCh (Poland).

The fungal strain *A. cylindrospora* came from the collection of the Institute of Biology and Botany, Medical University in Wroclaw. It was maintained at 4 °C in the refrigerator on Sabouraud agar slants of the following composition: aminobac (catalogue no. S-0002) 5 g, peptone K (S-0011) 5 g, glucose (459560117) 40 g and agar (S-0001) 15 g in distilled water (1 l) at pH 5.7.

2.6. Biotransformation

In all experiments the strain was cultivated at 25 °C in 16 Erlenmeyer flasks (250 ml capacity) containing medium which consisted of 3 g glucose (459560117) and 1 g peptobac (S-0009) in water (100 ml). After 72 h of shaking (25 °C, 140 rpm) the appropriate substrate was dissolved in acetone (10 mg in 1 ml for each flask) and added to the dense suspension of cells. After 4, 7, 10 and 14 days the aliquots of product mixtures were taken, extracted twice with methylene chloride and analyzed by TLC and GC to determine the degree of transformation of substrates. When the amount of substrate was lower than 20% in the reaction mixture the biotransformation was stopped and the products were extracted three times with methylene chloride after centrifugation of the biomass. The organic extracts were pooled, dried over anhydrous $MgSO_4$ and concentrated in vacuo. The crude product mixtures were chromatographed on silica gel to afford pure products.

2.6.1. Biotransformation of 4,6-dimethyl-9-oxabicyclo [4.3.0]nonan-8-one (**4a**)

The biotransformation of 160 mg of **4a** was continued for 14 days to obtain a mixture of products which were separated on a silica gel (hexane:acetone, 2:1) to give 55 mg (30% isolated yield) of 4-hydroxy-4,6-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**6**), 11 mg (6%) of 3-hydroxy-4,6-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**7**) and 15 mg (8%) of 2-hydroxy-4,6-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**8**).

*2.6.1.1. 4-Hydroxy-4,6-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**6**).* oily liquid; ^{1}H NMR, δ: 1.18 (s, 3H, CH_3-4), 1.29 (s, 3H, CH_3-6), 1.32 (m, 1H, H-5, axial), 1.42 (ddd, J = 14.0, 5.0 and 2.5 Hz, 1H, H-3, equatorial), 1.48–1.59 (two m, 2H, H-3, axial and H-5, equatorial), 1.94 (ddd, J = 15.1, 7.0 and 3.2 Hz, 1H, H-2, equatorial), 2.05 (m, 1H, H-2, axial), 2.20 and 2.31 (two d, J = 16.5 Hz, 2H, CH_2-7, AB system), 4.20 (m, 1H, H-1); ^{13}C NMR ($CDCl_3$), δ: 20.69(C-2), 23.40(C-11), 31.28(C-3), 32.22(C-10), 38.80(C-6), 43.88(C-5), 47.27(C-7), 68.93(C-4), 83.50(C-1), 176.82(C-8); IR (film, cm^{-1}): 3458 (s), 2929(s), 1759(s), 1216(m), 1026(m), 967(s).

*2.6.1.2. 3-Hydroxy-4,6-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**7**).* mp = 93–110 °C; $\alpha_D^{20} = -12.0°$ (CH_2Cl_2, c = 0.15), ee = 30%; ^{1}H NMR ($CDCl_3$), δ: 0.99 (d, J = 6.2 Hz, 3H, CH_3-4), 1.20 (s, 3H, CH_3-6), 1.24 (m, 1H, one of CH_2-5), 1.46–1.55 (m, 2H, one of CH_2-5 and H-4), 1.61 (ddd, J = 14.7, 10.4 and 3.4 Hz, 1H, H-2, axial), 2.36–2.42 (m, 1H, H-2, equatorial) 2.26 and 2.38 (two d, J = 16.6 Hz, 2H, CH_2-7, AB system) 3.41 (td, J = 10.4 and 3.7 Hz, 1H, H-3), 4.27 (m, 1H, H-1); ^{13}C NMR ($CDCl_3$), δ: 17.79(C-10), 21.32(C-11), 33.76(C-2), 34.53(C-4), 38.83(C-6), 40.57(C-5), 45.59(C-7), 71.13(C-3), 85.45(C-1), 173.24(C-8); IR (KBr, cm^{-1}): 3460(s), 2923(s), 1753(s), 1055(s).

*2.6.1.3. 2-Hydroxy-4,6-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**8**).* mp = 141–143 °C; $\alpha_D^{20} = -16.4°$ (CH_2Cl_2, c = 0.53), ee = 100%; ^{1}H NMR ($CDCl_3$), δ: 0.89 (d, J = 6.5 Hz, 3H, CH_3-4), 1.03 (m, 1H, H-5, axial) 1.28 (s, 3H, CH_3-6), 1.34 (ddd, J = 14.4, 12.3 and 3.0 Hz, 1H, H-3, axial), 1.47 (m, 1H, H-5, equatorial), 1.69 (m, 1H, H-3, equatorial) 1.93 (m, 1H, H-4), 2.24 and 2.41 (two d, J = 16.7 Hz, 2H, CH_2-7, AB system), 4.02 (d, J = 2.5 Hz, 1H, H-1), 4.27 (m, 1H, H-2); ^{13}C NMR ($CDCl_3$), δ: 20.93(C-4), 21.84(C-10), 22.88(C-11), 36.41(C-3), 38.69(C-6), 41.93(C-5), 46.27(C-7), 66.69(C-2), 84.33(C-1), 175.96(C-8); IR (KBr, cm^{-1}): 3351(s), 2921(s), 1790(s), 1212(m), 1069(m).

Crystal data for (**8**): $C_{10}H_{16}O_3$, M_w = 184.23, colourless needle, crystal size 0.30 mm × 0.10 mm × 0.10 mm, hexagonal, space group $P3_2$, a = 20.2085(7), c = 6.2259(3) Å, α = 90, β = 90, γ = 120 Å, V = 2201.92(15) Å^3, Z = 9, D_c = 1.250 Mg m^{-3}, T = 100(2) K, R = 0.046, wR = 0.070 (for 3990 reflections with I > 2_(I)) for 362 variables. CCDC No. 276160.

2.6.2. Biotransformation of 4,6-dimethyl-9-oxabicyclo[4.3.0]non-2-en-8-one (**5**)

The biotransformation process was carried out for 8 days starting from 160 mg of **5**. The product **9** (49 mg, 27% isolated yield) was purified by means of column chromatography using hexane:acetone 3:1 as a developing system.

*2.6.2.1. 4-Hydroxy-4,6-dimethyl-9-oxabicyclo[4.3.0]non-2-en-8-one (**9**).* mp = 85–86 °C; $\alpha_D^{20} = +3.98°$ (CH_2Cl_2, c = 1.98), ee = 28%; ^{1}H NMR ($CDCl_3$), δ: 1.33 and 1.34 (two s, 6H, CH_3-6 and CH_3-4), 1.44 (s, 1H, –OH), 1.69 and 1.76 (two d, J = 14.5 Hz, 2H, CH_2-5, AB system), 2.38 and 2.44 (two d, 2H, J = 17.2 Hz, CH_2-7, AB system), 4.43 (d, J = 4.4 Hz, 1H, H-1), 5.87 (dd, J = 10.0 and 4.4 Hz, 1H, H-2), 5.96 (d, J = 10.0 Hz, 1H, H-3); ^{13}C NMR ($CDCl_3$), δ: 24.32(C-10), 31.22(C-11), 37.29(C-6), 43.41(C-5), 44.83(C-7), 67.07(C-4), 79.78(C-1), 121.94(C-2), 138.44(C-3), 175.78(C-8); IR (KBr, cm^{-1}): 3457(s), 2922 (m), 1738(s), 1231(s), 1142(m), 947(m).

Crystal data for (**9**): $C_{10}H_{14}O_3$, M_w = 182.21, colourless plate, crystal size 0.20 mm × 0.20 mm × 0.10 mm, orthorhombic, space group $P2_12_12_1$, a = 6.6422(5), b = 8.6488(7), c = 16.6140(13) Å, V = 954.43(13) Å^3, Z = 4, D_c = 1.268 Mg m^{-3}, T = 100(2) K, R = 0.036, wR = 0.058 (for 914 reflections with I > 2_(I)) for 115 variables. CCDC No. 276161.

2.6.3. Biotransformation of 3,3-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**4b**)

The fungus *A. cylindrospora* was incubated with 160 mg of substrate for 10 days. The isolated products of this transformation were separated on silica gel (ether diethyl:benzene, 4:1) to give: 35 mg (19% isolated yield) of 2-hydroxy-3,3-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**10**), 20 mg (11%) of 6-hydroxy-3,3-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**11**) and 5 mg (3%) of 3,3-dimethyl-9-oxabicyclo [4.3.0] nonan-5,8-dion (**12**). The physical and spectral data of compounds isolated are given below.

*2.6.3.1. 2-Hydroxy-3,3-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**10**).* mp = 87–90 °C; $\alpha_D^{20} = +38.6°$ (CH_2Cl_2, c = 0.3), ee = 98%; ^{1}H NMR ($CDCl_3$), δ: 0.89 and 1.02 (two s, 6H,

$(CH_3)_2C<$), 1.36–1.46 (m, 2H, CH_2-4), 1.60 (ddd, $J = 14.9$, 6.4 and 3.8 Hz, 1H, H-5, equatorial) 1.80 (m, 1H, H-5, axial), 2.37–2.40 (m, 2H, CH_2-7), 2.84 (m, 1H, H-6), 3.30 (d, $J = 8.3$ Hz, 1H, H-2), 4.36 (dd, $J = 8.3$ and 8.0 Hz, 1H, H-1); ^{13}C NMR ($CDCl_3$), δ: 18.04(C-10), 20.96(C-5), 28.05(C-11), 32.26(C-7), 32.98(C-4), 34.97(C-6), 35.11(C-3), 77.69(C-2), 83.83(C-1), 175.25(C-8); IR (film, cm^{-1}): 3430(s), 2922(s), 1763(s), 1169(m), 1002(m).

Crystal data for (**10**): $C_{10}H_{16}O_3$, $M_w = 184.23$, colourless needle, crystal size 0.30 mm × 0.10 mm × 0.10 mm, orthorhombic, space group $P2_12_12_1$, $a = 6.6208(4)$, $b = 19.3336(10)$, $c = 22.9492(12)$ Å, $V = 2937.6(3)$ Å^3, $Z = 4$, $D_c = 1.250$ Mg m^{-3}, $T = 100(2)$ K, $R = 0.039$, wR = 0.061 (for 2412 reflections with I > 2_(I)) for 349 variables. CCDC No. 276162.

*2.6.3.2. 6-Hydroxy-3,3-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one (**11**).* oily liquid; $\alpha_D^{20} = -10.7^\circ$ (CH_2Cl_2, $c = 0.26$), ee = 31%; ^{1}H NMR ($CDCl_3$), δ: 0.96 (s, 6H, $(CH_3)_2C<$), 1.19 (m, 1H, H-5, axial), 1.26 (ddd, $J = 14.0$, 11.6 and 4.5 Hz, 1H, H-4, axial), 1.46 (dtd, $J = 14.0$, 4.5 and 2.1 Hz, 1H, H-4, equatorial), 1.78 (ddd, $J = 14.5$, 11.6 and 4.5 Hz, 1H, H-2, axial), 1.92–2.00 (m, 2H, H-5, equatorial and H-2, equatorial), 2.38 and 2.70 (two d, $J = 17.2$ Hz, 2H, CH_2-7, AB system), 4.39 (dd, $J = 9.3$ and 6.1 Hz, 1H, H-1); ^{13}C NMR ($CDCl_3$), δ: 25.57(C-10), 30.44(C-3), 30.55(C-2), 30.77(C-11), 34.95(C-4), 40.34(C-7), 42.21(C-5), 75.32(C-6), 84.29(C-1), 175.03(C-8); IR (film, cm^{-1}): 3428(m), 2948(s), 1776(s), 1170(m), 1067(m), 994(m).

*2.6.3.3. 3,3-Dimethyl-9-oxabicyclo[4.3.0]nonan-5,8-dion (**12**).* oily liquid; ee = 100% (according to GC); ^{1}H NMR ($CDCl_3$), δ: 1.13 and 1.18 (two s, 6H, $(CH_3)_2C<$), 2.11 (dd, $J = 14.6$ and 7.8 Hz, 1H, one of CH_2-2), 2.19 (dd, $J = 14.6$ and 5.8 Hz, 1H, one of CH_2-2), 2.34 (dd, $J = 17.9$ and 4.0 Hz, 1H, one of CH_2-7), 2.49 (dd, $J = 15.4$ and 0.9 Hz, 1H, one of CH_2-4), 2.54 (d, $J = 15.4$ Hz, 1H, one of CH_2-4), 2.86 (dd, $J = 17.9$ and 9.1 Hz, 1H, one of CH_2-7), 2.99 (m, 1H, H-6), 4.86 (m, 1H, H-1).

3. Results and discussion

The substrates of biotransformations were two saturated (**4a**,**b**) and one unsaturated (**5**) racemic bicyclic γ-lactones containing a dimethylcyclohexane ring**.** One known (**1a**) [34] and one new (**1b**) racemic γ,δ-unsaturated esters were the starting materials for the synthesis of the lactones mentioned (Scheme 1).

The ester **1b** was obtained as the product of Claisen rearrangement (orthoacetate modification) of the known [35] alcohol, 6,6-dimethylcyclohex-2-en-1-ol. The ester formed was hydrolyzed in an ethanolic solution of KOH to give acid **2b** (broad absorption band at 2880–3220 cm^{-1} in the IR spectrum) which was a substrate for iodolactonization described by Mori and Nakazono [36]. The formation of only one iodolactone was indicated by both TLC and GC analyses and the structure of δ-iodo γ-lactone **2c**, not described previously in the literature, was confirmed with the aid of spectral methods. The absorption band found in the IR spectrum (1776 cm^{-1}) is characteristic for the stretching vibration of the C=O bond in the γ-lactone ring. The coupling constant between the H-1 and H-2 protons ($J = 9.3$ Hz) showed *trans* diaxial orientation of these protons and indirectly proved that the iodine atom as well as the C–O bond of the γ-lactone ring are *trans* oriented, both bonds being in equatorial positions. The last step of the synthesis, the reduction of iodolactone **3b** with tributyltin hydride, gave the saturated lactone **4b**, which conformation was changed during this reaction. The shape of signal from H-1 proton indicated its equatorial position as well as the axial position of C–O bond. The presence of a γ-lactone ring in the molecule was confirmed by IR spectrum (absorption band at 1775 cm^{-1}).

Table 1
Composition (in percent according to GC) of product mixtures of biotransformation of lactones **4a**, **4b** and **5** by *A. cylindrospora*

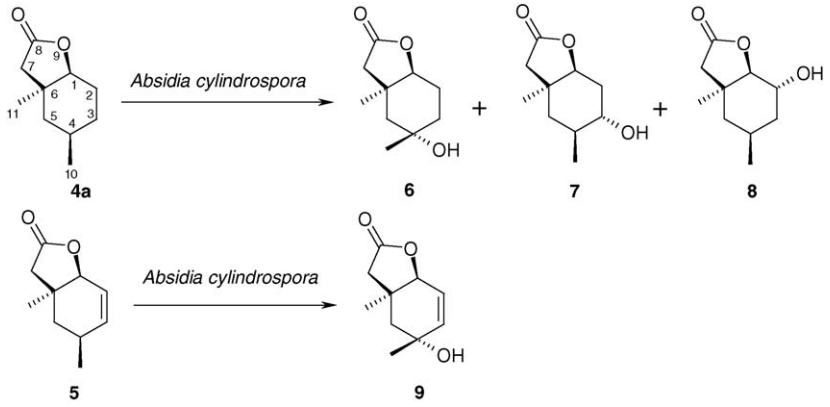
Entry	Substrate (%)		Products of transformation (%)	
			6	48
1	**4a**	37	**7**	6
			8	9
			10	22
2[a]	**4b**	10	**11**	18
			12	5
3	**5**	20	**9**	80

[a] Unidentified products were also present in the product mixture.

On the basis of our earlier results of the biotransformation of saturated and unsaturated lactones containing a cyclohexane system, we found that *A. cylindrospora* is one of the most effective fungal strains for the transformation of this class of compounds [1,31]. So we decided to apply this strain in our current studies hoping to obtain some hydroxy- or epoxylactones.

The results of biotransformation of lactones **4a**,**b** and **5** are summarized in Table 1. The composition of product mixtures shows that the lactone **4b** was transformed to the highest extent whereas lactone **4a** was less readily accepted as a substrate. In contrast to previously described transformations, the saturated substrates **4a** and **4b** were converted into a mixture of products. Three products were isolated in the experiment with **4a**—one major (**6**, 48%) and two minor ones (**7** and **8**, 6 and 9%, respectively). The incubation of substrate **4b** with the employed fungus afforded also three major derivatives (**10**, **11** and **12**) with the predominance of **10** (22%) although GC analyses showed also the presence of other compounds. However, their contents were less than 4%. So they have not been isolated and identified. The compound **9** was the only product of conversion of the unsaturated lactone **5** (80%).

The structures of all obtained products were established on the basis of their spectral data. The IR spectra of all three products of transformation of **4a** (Scheme 2) showed that the γ-lactone ring has been retained during the biotransformation in all cases (absorption band at 1759 cm^{-1} for **6**, 1753 cm^{-1} for **7** and 1790 cm^{-1} for **8**). Strong bands found at 3458, 3460 and 3351 cm^{-1} in the IR spectra of **6**, **7** and **8**, respectively, suggested the presence of a hydroxy group in each molecule. Hydroxylation was additionally confirmed by the signals present in the ^{13}C NMR spectra at: 68.93 ppm (for **6**), 71.13 ppm (for



Scheme 2.

7) and 66.69 ppm (for **8**) from the carbon atoms directly connected to the hydroxy group. The locations of hydroxy groups were deduced from comparative analysis of ^{1}H NMR spectra of substrate **4a** and the respective products. In the case of major product (**6**) the hydroxy group was introduced in the C-4 position. The important factors which confirmed hydroxylation of this tertiary carbon atom were: disappearance of the multiplet from H-4 and the lack of new signals between 3 and 4 ppm. Moreover, instead of the doublet ($J = 6.1$ Hz) from the methyl group at C-4 (present in the spectrum of **4a**) a singlet significantly shifted downfield by 0.35 ppm appeared in the spectrum of **6**. It is worth to notice that the similar hydroxylation at a tertiary carbon atom was observed in our earlier studies with a substrate with a cyclohexane ring substituted with one methyl group at C-4 [31].

Contrary to the product **6**, in the ^{1}H NMR spectrum of **7** one of the signals of CH_2-3 protons disappeared whereas the other one was shifted downfield to 3.41 ppm. This fact unequivocally indicates the presence of a hydroxy group at the C-3 position. The values of the coupling constants of the H-3 proton with two neighbouring axial protons H-2 and H-4 ($J = 10.4$ Hz) and with equatorial proton H-2 ($J = 3.7$ Hz) proved the equatorial orientation of the hydroxy group introduced into the molecule.

Similarly, the second minor product of transformation (**8**) was also identified as a hydroxylactone with a secondary hydroxy group. Analyzing the ^{1}H NMR spectrum, we drew the conclusion that this time the biohydroxylation affected the C-2 position. The shape of the multiplet of H-2 at 4.27 ppm which looks like a quartet together with the doublet of H-1 with only one small coupling constant ($J = 2.5$ Hz) convinced us that, in opposite to lactone **7**, the OH group is located in the axial position. Our assignments were fully confirmed by X-ray crystallography.

The crystal structure (Fig. 1) showed that the product **8** possesses the axial hydroxy group at C-2 *trans* situated to the γ-lactone moiety as well as to the equatorial methyl group at C-4. The value of torsion angle between H-1 and H-2 calculated from X-ray analysis (71.9°) is compatible with the corresponding coupling constant found in the ^{1}H NMR spectrum ($J = 2.5$ Hz). The cyclohexane ring exists in the molecule in an only slightly deformed chair conformation and three symmetrically independent molecules were found in the crystals of the described compound.

Among all the products of the biotransformation of the lactone **4a** only the product of the hydroxylation at C-2 (lactone **8**) was obtained as the pure (−)-enantiomer. The enantiomeric excess of hydoxylactone **7** was low (30%) and the hydroxylactone **6** was obtained as a racemic mixture.

Biotransformation of unsaturated lactone **5**, in contrast to its saturated analogue **4a**, furnished only one product (**9**) (Scheme 2). The IR spectrum showed the presence of both a lactone ring (1738 cm^{-1}) and a hydroxy group in the molecule (3457 cm^{-1}). The peak at 67.07 ppm in the ^{13}C NMR spectrum confirmed the presence of a hydroxy group and the peaks at 121.94 and 138.44 ppm showed that the double bond remained unaffected. In the ^{1}H NMR spectrum the remarkable shifting downfield of the signal from the C-10 methyl group by 0.32 ppm could be observed. Moreover, this signal appeared as a singlet in comparison with the doublet in the spectrum of substrate **5**. The other important clues which let us assign the place of bio-

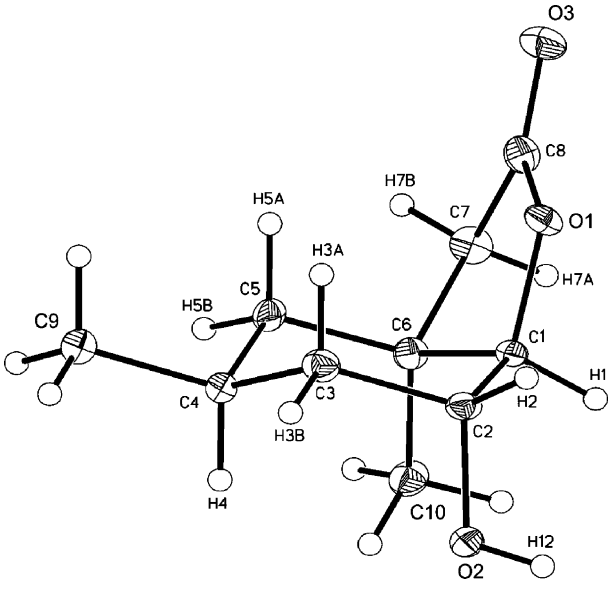


Fig. 1. Molecular structure of **8** with crystallographic numbering.

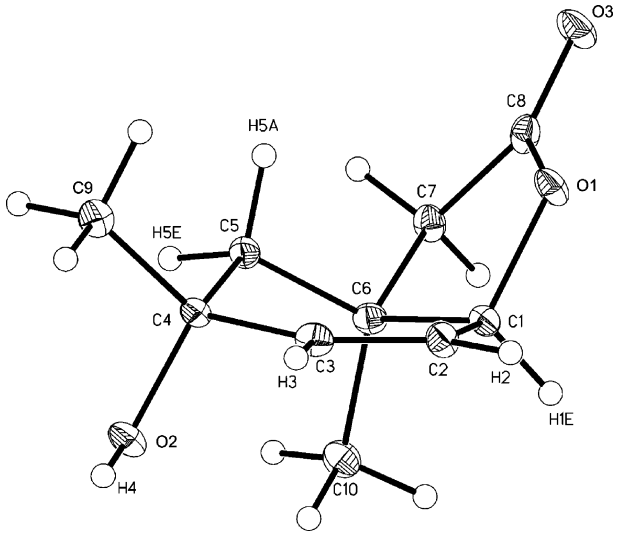


Fig. 2. Molecular structure of **9** with crystallographic numbering.

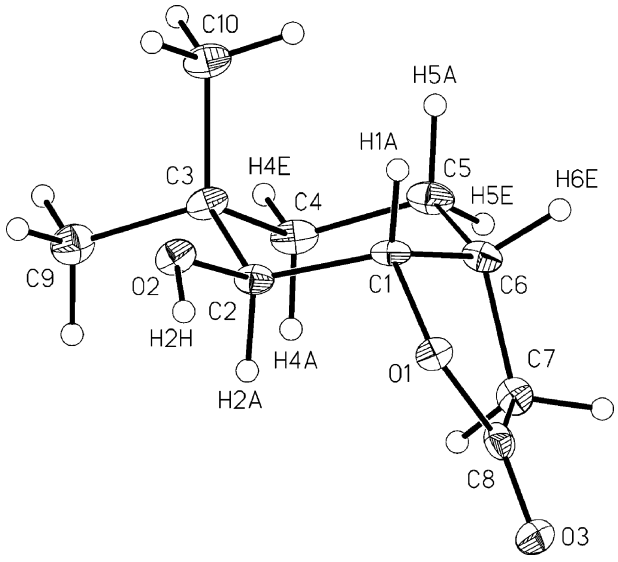


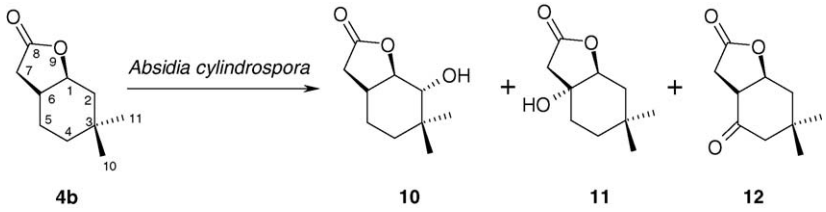
Fig. 3. Molecular structure of **10** with crystallographic numbering.

hydroxylation were the lack of any new signal between 3 and 4 ppm, the disappearing of the multiplet from H-4 and finally, the simplifying of the multiplicity of the signal from the olefinic proton H-2. This proton in the spectrum of **5** was coupled to three protons: H-2 ($J = 10$ Hz), H-1 ($J = 4.3$ Hz) and H-4 ($J = 2.4$ Hz). The last one, a long distance coupling constant, was eliminated in the spectrum of product **9**. All these data, suggesting the biohydroxylation in allylic position C-4, were fully supported by the crystal structure as shown in Fig. 2.

It can be seen that the conformation of cyclohexane ring in the molecule may be described as a half-chair. The flattening of the molecule is caused by the double bond C-2–C-3. The hydroxy group is situated *trans* to the γ-lactone functionality in the pseudoaxial position at C-4. The C–O bond is also pseudoaxial and the value of the dihedral angle between the H-1 and H-2 (49.1°) is consistent with their coupling constant ($J = 4.4$ Hz). The observed allylic hydroxylation was analoguous to the one achieved in the case of a 4-monosubstituted unsaturated lactone [1]. The hydroxylactone **9** was optically active (ee = 28%) with the predominance of (+)-enantiomer.

Bioconversion of saturated lactone **4b** with a gem-dimethylcyclohexane system proceeded in a different way (Scheme 3). The substrate was converted in few products but only three of them predominated in the product mixture and could be isolated and identified. On the basis of their spectral data two major compounds **10** and **11** were found to be the products of biohydroxylation at C-2 (compound **10**) and, surprisingly for us, at C-6 (compound **11**) whereas the third, the minor compound **12**, was a ketolactone having a carbonyl group at C-5. In the IR spectra of the hydroxylactones **10** and **11** we observed strong absorption bands at 1763 and 1776 cm^{-1}, respectively, indicating that no reaction on the γ-lactone ring occurred. The presence of a hydroxy group was indicated by both IR spectra (bands at 3430 cm^{-1} (for **10**) and 3428 cm^{-1}(for **11**)) and ^{13}C NMR spectra (signals at 77.69 and 75.32, respectively). The analysis of their ^{1}H NMR spectra delivered very interesting results. In case of product **10**, the doublet at 4.36 ppm from H-2 with a high coupling constant ($J = 8.0$ Hz) undoubtedly proved the location of OH group in the C-2 equatorial position. However, the signal from H-1 proton differed significantly in comparison with the one observed in the spectrum of substrate **4b**. It was described as a doublet of doublets with two high coupling constants with similar values ($J = 8.3$ and 8.0 Hz). Such shape of this multiplet indicated its axial orientation as well as the equatorial orientation of the C–O bond in the γ-lactone ring. All these data suggested a change of conformation of the molecule in the course of biotransformation performed and were clearly confirmed by the crystal structure obtained for **10** (Fig. 3).

It can be noticed that the cyclohexane ring occurs in the slightly twisted chair conformation and the OH group and C–O bond of γ-lactone ring are situated in *trans* diequatorial positions. The values of dihedral torsion angles between the relevant protons: H-1–C-1–C-2–H-2 (−165.0°) and H-1–C-1-C-6–H-6 (35.5°), being the subject of our special interest, correspond with the coupling constants found in ^{1}H NMR spectrum ($J = 8.3$



Scheme 3.

and 8.0 Hz, respectively). The X-ray analysis of **10** indicates the presence of three symmetrically independent molecules in the crystal.

A similar situation took place in the case of product **11**. This time the H-1 proton is coupled with two neighbouring protons at C-2: with the bigger coupling constant ($J = 9.3$ Hz) with the axial proton and the smaller one ($J = 6.1$ Hz) with the equatorial proton. Such a shape of the observed multiplet is the result of the axial orientation of this hydrogen atom as it was stated in the case of compound **10**. The lack of signal from H-6 proton is a clear evidence of the introduction of a OH group by the microorganism in the C-6 position and the formation of a hydroxylactone with a tertiary hydroxy group.

In our opinion changes of the chair conformation in products **10** and **11** are caused by the preference of the molecules to exist in more energetically stable conformations, with the newly introduced bulky hydroxy group in equatorial positions instead of axial ones.

The measurements of the optical rotations showed that both hydroxylactone **10** as well as **11** were optically active. The product **10** found to be dextrorotatory whereas the lactone **11** was laevorotatory. The enantiomeric excess determined for the compound **10** was much more higher (98%) than in case of lactone **11** (31%).

Unfortunately, the third identified product of this transformation (**12**) was isolated in a very small amount (3% isolated yield), so that only a partial spectral analysis could be performed. Nevertheless, concluding from the available data (^{1}H NMR) we could propose its structure. The presence of a new carbonyl group at C-5 considerably shifted the signals of H-6 and CH_2-4 to 2.49, 2.54 and 2.99 ppm, respectively. The shapes of the named multiplets, especially those coming from CH_2-4 methylene protons, were remarkably simplified. The formation of this product can be explained by the hydroxylation at C-5 and subsequent oxidation of the OH group to the carbonyl in the course of the biotransformation.

It is worth to mention that the genus *A. cylindrospora* was applied earlier to the hydroxylation of adamantane and its derivatives [37,38] as well as Trimegestone® (RU27987), a 3-keto-$\Delta^{4,9(10)}$-19-norsteroid drug used in the therapy of osteoporosis [39]. During these transformations no further oxidation of alcohols by this genus was observed, so the formation of the ketolactone **12** is the first example of such activity among the strains of this genus.

4. Conclusions

The fungal strain *A. cylindrospora* hydroxylates lactones fused to a 4,6-dimethylcyclohexane ring mainly at the C-4 position whereas in the case of lactone fused to 3,3-dimethylcyclohexane ring, the hydroxylation at C-2 predominates.

In all cases the hydroxy group is introduced *trans* to the γ-lactone moiety.

The equatorial orientations of OH groups in the compounds **10** and **11** are the result of hydroxylation at axial positions C-2 and C-6, respectively, followed by a change of chair conformation. Such change was not observed in the case of lactone **8**, where the equatorial orientation of methyl group at C-4 makes the OH group occupy the axial position at C-2. Such orientation is apparently more energetically favourable for the molecule.

The products of biotransformation (except **6**) are optically active with the predominance of the (−) enantiomer (in the case of **7**, **8** and **11**) or the (+) isomer (in the case of **9** and **10**). The highest enantioselectivity was observed in the case of hydroxylation at C-2 (100% and 98% ee for the products **8** and **10**, respectively).

Acknowledgements

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