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Lactones 26 [1]: Stereoselective microbial epoxidation of unsaturated bicyclic γ-lactones with the alkylsubstituted cyclohexane system

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The paper dedicated to Prof. A. Siewiński on the occasion of his 80th birthday.

Abstract

The strain *Absidia cylindrospora* was chosen among eight fungal strains for the biotransformation of unsaturated lactones **1a**–**c**. The processes were carried out by means of shaken cultures. The compounds **1a** and **1b** were efficiently converted into the corresponding *trans*-epoxylactones (**2a** and **2b**), whereas the transformation of **1c** gave the unsaturated hydroxylactone **3**, with the tertiary hydroxy group introduced in the allylic position. The compound **2b** was obtained with 100% ee. The structures of compounds **2a** and **2b** were fully confirmed by the X-ray analysis, which showed the half boat and half chair conformation of cyclohexane ring in these molecules, respectively. All the products were not reported previously in the literature.

Keywords: *Absidia cylindrospora*; Bioepoxidation; Hydroxylation; Lactones

1. Introduction

Terpenoid and sesquiterpenoid lactones belong to the most numerous group of natural compounds with biological activity isolated from natural sources. They can be found in plants [2–6], microorganisms [7–9], insects [10–12] or marine organisms [13–15]. Natural lactones exhibit many interesting activities like antimicrobial [16–18], cytostatic [19–21] or antifeedant [22–24].

Analyzing the structures of biologically active terpenoid lactones isolated from natural sources it was found that the lactone ring is often accompanied by other functions containing oxygene atom like hydroxy, acetoxy, carbonyl or epoxide ring [25–27]. Microbial transformations of lactones proceeding regio- and stereoselectively are the good way of their additional functionalization, which often results in increasing their biological activities [21].

For the lactones possessing the double bonds two main reactions are observed during the incubation of these substrates with microorganisms. One of them is the reduction of exocyclic double bond, especially in the α-methylene-γ-lactones [28], the other one is the formation of oxirane ring [29,30]. The epoxidation is catalyzed by the same cytochrome P-450 systems, which are responsible for the hydroxylation of unactivated carbon atoms [31]. However, during the literature studies we found that the lactones with the epoxide ring are very unstable and undergo further reactions in the biological systems. As a result it is difficult to isolate epoxylactones from the reaction mixtures.

The biotransformations of lactones is mainly focused on the compounds of natural origin, especially the isoprenoid ones. There are only a few reports about the application of microorganisms to the transformation of the synthetic analogues of naturally occurring compounds. The examples can be the hydroxylation of campholene lactone by *Fusarium culmorum* [32], dehydrohalogenation and hydroxylation of iodolactones by *Botritis cinerea* [33] and the enzymatic Diels–Alder reaction catalyzed by solanopyrane A synthase, isolated from *Alternaria solani* [34].

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As a part of our research programme directed towards the synthesis of biologically active lactones we developed the convenient synthetic path, which leads from γ,δ-unsaturated esters to saturated and unsaturated bicyclic γ-lactones [35]. Being interested in regioselective and stereoselective functionalization of the lactones obtained, we were prompted to use the biotransformation methods. In one of our last reports we described an efficient and stereospecific hydroxylation of three saturated lactones with the alkylsubstituted cyclohexane ring by means of *F. culmorum* and *Absidia cylindrospora* cultures as whole cells biocatalysts, yielding the hydroxylactones with the secondary or tertiary hydroxy group [36]. As we were also interested in the products of transformations of the unsaturated analogues of these lactones we carried out their biotransformation using some fungal strains to obtain the functionalized compounds with the epoxide ring. Here we would like to present the results of these studies.

2. Materials and methods

2.1. Analysis

The progress of biotransformations as well as the purity of isolated products were monitored by TLC technique on silica gel-coated aluminium plates (DC–Alufolien Kieselgel 60 F_{254}, Merck) and by GC analysis carried out on a Varian CP-3380 instrument using HP-5 column (cross-linked methyl silicone gum, 30 m × 0.32 mm × 0.25 μm). The temperatures during GC analysis were as follows: injector 250 °C, detector (FID) 300 °C, column temperature: 180 °C (hold 1 min), 180–220 °C (rate 6 °C/min), 220–300 (rate 30 °C/min), 300 °C (hold 1 min). The enantiomeric excesses were determined on the basis of GC analysis using the chiral column CP-cyclodextrin-B-2,3,6-M-19, 25 m × 0.25 mm × 0.25 μm at the following conditions: injector 200 °C, detector (FID) 250 °C, column temperature: 135 °C (hold 1 min), 135–200 °C (rate 0.5 °C/min), 200 °C (hold 2 min).

The products were purified by means of preparative column chromatography on silica gel (Kieselgel 60, 230–400 mesh).

^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ solution 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 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 measured on the 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 a 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 [37]. 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 [38]. 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.

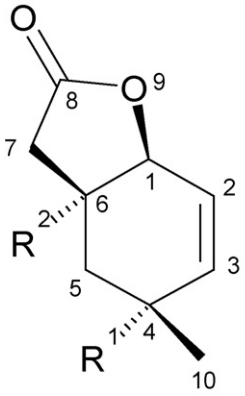
Crystallographic data for crystals **2a** and **2b** in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 602648-602649, respectively. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB12 1EZ, UK (fax +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

2.3. Substrates

The substrates for biotransformations were racemic unsaturated lactones **1a**–**c** (Fig. 1), synthesized from corresponding γ,δ-unsaturated esters, according to the procedure described earlier [35]. Here we present only their spectral (IR, ^{1}H NMR and ^{13}C NMR) data. Comparative analysis of spectral data of transformed substrates (**1a**–**c**) with the ones belonging to the respective products (**2a**,**b** and **3**) will facilitate to study the changes in the molecules of substrates caused by microorganisms employed.

2.3.1. 4,4,6-Trimethyl-9-oxabicyclo[4.3.0]non-2-en-8-one (1a)

mp 42–43 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.04 (s, 6H, $(CH_3)_2C$), 1.21 (s, 3H, CH_3-6), 1.47 and 1.58 (two d, J = 14.4 Hz,



1 a-c

1a: R^1,R^2=CH_3
1b: R^1=CH_3, R^2=H
1c: R^1,R^2=H

Fig. 1. Racemic unsaturated lactones – substrates for biotransformation.

AB system, 2H, CH_2-5), 2.28 and 2.53 (two d, $J = 17.3$ Hz, AB system, 2H, CH_2-7), 4.40 (d, $J = 3.9$ Hz, 1H, H-1), 5.64 (dd, $J = 10.1$ and 3.9 Hz, 1H, H-2), 5.78 (d, $J = 10.1$ Hz, 1H, H-3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 25.9$ (C-10), 30.0 (C-11), 31.4 (C-12), 31.8 (C-6), 37.4 (C-4), 43.3 (C-5), 43.9 (C-7), 80.6 (C-1), 119.0 (C-2), 142.8 (C-3), 176.1 (C-8); IR (KBr, cm^{-1}): 2976 (s), 1796 (s), 1468 (s), 1380 (m), 1140 (m), 1028 (m).

2.3.2. 4,4-Dimethyl-9-oxabicyclo[4.3.0]non-2-en-8-one (**1b**)

mp 54–55 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.00$ and 1.05 (two s, 6H, $(CH_3)_2$C), 1.28 (dd, $J = 13.4$ and 13.2 Hz, 1H, one of CH_2-5), 1.50 (ddd, $J = 13.4$, 4.7 and 1.0 Hz, 1H, one of CH_2-5), 2.25 (d, $J = 17.3$ Hz, 1H, one of CH_2-7), 2.66 (dddd, $J = 13.2$, 8.0, 5.2 and 4.7 Hz, 1H, H-6), 2.86 (dd, $J = 17.3$ and 8.0 Hz, 1H, one of CH_2-7), 4.70 (dd, $J = 5.2$ and 4.3 Hz, 1H, H-1), 5.80 (dd, $J = 10.0$ and 4.3 Hz, 1H, H-2), 5.92 (d, $J = 10.0$ Hz, 1H, H-3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 26.6$ (C-10), 30.0 (C-11), 30.7 (C-6), 31.9 (C-4), 36.7 (C-5), 38.5 (C-7), 74.8 (C-1), 119.6 (C-2), 145.1 (C-3), 176.4 (C-8); IR (KBr, cm^{-1}): 2976 (s), 1772 (s), 1180 (m), 972 (m), 944 (m).

2.3.3. 4-Methyl-9-oxabicyclo[4.3.0]non-2-en-8-one (**1c**)

$n_D = 1.4895$; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.94$ (m, 1H, one of CH_2-5), 1.02 (d, $J = 7.1$ Hz, 3H, CH_3-4), 1.68 (dt, $J = 12.8$ and 4.4 Hz, 1H, one of CH_2-5), 2.15 (m, 1H, H-4), 2.24 (d, $J = 17.4$ Hz, 1H, one of CH_2-7), 2.46 (m, 1H, H-6), 2.82 (dd, $J = 17.4$ and 8.0 Hz, 1H, one of CH_2-7), 4.69 (m, 1H, H-1), 5.88 (dm, $J = 10.4$ Hz, 1H, H-2), 5.94 (d, $J = 10.4$ Hz, 1H, H-3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 20.7$ (C-10), 29.5 (C-6), 33.2 (C-5), 33.7 (C-4), 36.9 (C-7), 75.1 (C-1), 121.6 (C-2), 141.3 (C-3), 176.4 (C-8); IR (film, cm^{-1}): 2944 (s), 1792 (s), 1172 (m), 972 (m).

2.4. General procedure for chemical epoxidation of lactones **1a** and **b**

To the solution of **1a** (0.36 g) or **1b** (0.3 g) in methylene chloride (6 ml) the anhydrous m-CPBA (1.4 g) was added and the mixture was stirred in room temperature for 5 h. Then it was successively washed with saturated solutions of sodium sulfite, sodium bicarbonate and brine. The organic layer was dried over anhydrous $MgSO_4$ and the solvent was evaporated in vacuum. The products of epoxidation were separated and purified using column chromatography (silica gel, hexane:acetone 4:1) to afford pure diastereoisomeric epoxylactones. Here we present their physical and spectral (IR and ^{1}H NMR) data.

2.4.1. The products of epoxidation of lactone **1a**

(±)-*Trans*-epoxylactone (first eluted, GC retention time 5.296): mp 71–72 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.05$ and 1.12 (two s, 6H, $(CH_3)_2$C), 1.18 (s, 3H, CH_3-6), 1.28 and 1.31 (two d, $J = 14.6$ Hz, AB system, 2H, CH_2-5), 2.22 and 2.61 (two d, $J = 17.9$ Hz, AB system, 2H, CH_2-7), 2.93 (d, $J = 3.4$ Hz, 1H, H-3), 3.36 (dd, $J = 3.4$ and 1.2 Hz, 1H, H-2), 4.38 (d, $J = 1.2$ Hz, 1H, H-1); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 27.2$ (C-10), 28.8 (C-11), 29.4 (C-12), 29.7 (C-6), 36.3 (C-4), 40.9 (C-5), 42.4 (C-7), 53.3 (C-2), 60.4 (C-3), 80.1 (C-1), 175.5 (C-8). IR (KBr, cm^{-1}): 2923 (s), 1793 (s), 1207 (m), 1021 (m).

(±)-*Cis*-epoxylactone (second eluted, GC retention time 6.242): mp 42–45 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.03$ and 1.08 (two s, 6H, $(CH_3)_2$C) 1.19 (s, 3H, CH_3-6), 1.29 and 1.70 (two d, $J = 14.1$ Hz, 2H, CH_2-5), 2.24 and 2.66 (two d, $J = 17.3$ Hz, AB system, 2H, CH_2-7), 3.02 (d, $J = 4.5$ Hz, 1H, H-3), 3.39 (dd, $J = 4.5$ and 1.6 Hz, 1H, H-2),4.49 (d, $J = 1.6$ Hz, 1H, H-1) ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 25.8$ (C-10), 28.6 (C-11), 30.2 (C-12), 31.9 (C-6), 36.1 (C-4), 45.2 (C-5), 45.6 (C-7), 53.2 (C-2), 61.9 (C-3), 84.0 (C-1), 175.9 (C-8) IR (KBr, cm^{-1}): 2934 (s), 1786 (s), 1211 (m), 1044 (m).

2.4.2. The products of epoxidation of lactone **1b**

(±)-*Trans*-epoxylactone (first eluted, GC retention time 5.212): mp 70–73 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.01$ and 1.11 (two s, 6H, $(CH_3)_2$C), 1.07 (dd, $J = 13.0$ and 12.0 Hz, 1H, one of CH_2-5), 1.22 (dd, $J = 13.0$ and 4.9 Hz, 1H, one of CH_2-5), 2.17 (d, $J = 17.3$ Hz, 1H, one of CH_2-7), 2.46 (dddd, $J = 12.0$, 7.6, 4.9 and 4.8 Hz, 1H, H-6), 2.72 (dd, $J = 17.3$ and 7.6 Hz, 1H, one of CH_2-7), 2.99 (d, $J = 3.3$ Hz, 1H, H-3), 3.46 (d, $J = 3.3$ Hz, 1H, H-2), 4.73 (d, $J = 4.8$ Hz, 1H, H-1); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 23.8$ (C-10), 29.3 (C-11), 29.6 (C-6), 29.7 (C-4), 36.3 (C-5), 37.4 (C-7), 52.2 (C-2), 60.8 (C-3), 75.5 (C-1), 177.2 (C-8); IR (KBr, cm^{-1}): 2924 (s), 1787 (s), 1457 (s), 1164 (m), 1018 (m).

(±)-*Cis*-epoxylactone (second eluted, GC retention time 6.432): mp 60–65 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.00$ and 1.09 (two s, 6H, $(CH_3)_2$C), 1.15 (ddd, $J = 13.5$, 6.1 and 1.1 Hz, 1H, one of CH_2-5), 1.48 (dd, $J = 13.5$ and 13.2 Hz, 1H, one of CH_2-5), 2.15 (dd, $J = 17.8$ and 2.2 Hz, 1H, one of CH_2-7), 2.46 (ddddd, $J = 13.2$, 9.4, 7.5, 6.0 and 2.2 Hz, 1H, H-6), 2.73 (dd, $J = 17.8$ and 9.4 Hz, 1H, one of CH_2-7), 2.95 (dd, $J = 4.0$ and 1.1 Hz, 1H, H-3), 3.43 (dd, $J = 4.0$ and 3.8 Hz, 1H, H-2), 4.81 (dd, $J = 7.5$ and 3.8 Hz, 1H, H-1).^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 22.7$(C-10), 27.8 (C-11), 29.2 (C-6), 30.7 (C-4), 35.1 (C-7), 36.0 (C-5), 51.1 (C-2), 61.5 (C-3), 75.8 (C-1), 176.1 (C-8); IR (KBr, cm^{-1}): 2934 (s), 1780 (s), 1460 (s), 1154 (m), 1052 (m).

2.5. Biotransformations

2.5.1. Microorganisms

The microorganisms we used in our research were obtained from either the Collection of the Institute of Biology and Botany, Medical University, Wrocław [AM] or the Collection of The Institute of Microbiology and Food Biotechnology, University of Environmental and Life Sciences, Wrocław [AR]. Microorganisms were maintained on Sabouraud dextrose agar slopes at 4 °C. The following fungal strains were used for preliminary screening:*A. cylindrospora* (AM 336), *Beauveria bassiana* (AM 446), *Fusarium culmorum* (AM 3/1), *Fusarium avenaceum* (AM 12), *Fusarium equiseti* (AM 22), *Nigrospora oryzae* (AM 8), *Rhodotorula rubra* (AM 82) and *Yarrovia lipolityca* (AR 101).

2.5.2. Growing media

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

The microorganisms were cultivated on Sabouraud agar containing 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 28 °C and stored in refrigerator at 4 °C.

2.5.3. Screening procedure

In all transformation experiments the strains were cultivated by shaking at 25 °C in Erlenmeyer flasks which contained 100 ml of medium consisting of glucose (459560117)-3 g and peptobac (S-0009)-1 g in water (100 ml). After 5 days, 10 mg of respective substrate dissolved in 1 ml of acetone was added to the grown cultures in each flask. The incubation of the shaken cultures with substrate was being continued for 12 days on a rotary shaker at 150 cpm. After 4, 7 and 12 days of incubation the products of biotransformation were extracted with methylene chloride and analyzed by TLC (silica gel, hexane:acetone 3:1) and GC (HP-5 column). The results of GC analyses for lactone **1a–c** are presented in Table 1.

2.6. Preparative biotransformation

2.6.1. General procedure

The substrates studied **1a–c** (160 mg) were dissolved in acetone (16 ml) and distributed between 16 Erlenmeyer flasks with the 5 day cultures of *A. cylindrospora* prepared as described in the screening procedure. The incubation of these microorganisms with the substrates was continued for 14 days and the conversion of substrates as well as rate of products were monitored by TLC and GC.

After that time the processes were stopped and the cells were removed by centrifugation or filtration. The water-liquids residues were extracted three times with methylene chloride. The organic solutions were centrifugated, dried with anhydrous $MgSO_4$ and evaporated under vacuum to give mixtures of products as well as metabolites produced by the fungi employed. The mixtures were separated on a silica gel column chromatography to obtain pure biotranformations products.

*2.6.2. Preparative biotransformation of 4,4,6-trimethyl-9-oxabicyclo[4.3.0]non-2-en-8-one (**1a**)*

The biotransformation proceeded 14 days to give 80 mg of brown products mixture. Its purification by the column chromatography with hexane:acetone 3:1 as a developing system allowed to isolate 78 mg (45% isolated yield) of pure product **2a**, identified as 2,3-epoxy-4,4,6-trimethyl-9-oxabicyclo[4.3.0]nonan-8-one with the following physical and spectral data.

mp 71–72 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.05$ and 1.12 (two s, 6H, $(CH_3)_2C$), 1.18 (s, 3H, CH_3-6), 1.28 and 1.31 (two d, $J = 14.6$ Hz, AB system, 2H, CH_2-5), 2.22 and 2.61 (two d, $J = 17.9$ Hz, AB system, 2H, CH_2-7), 2.93 (d, $J = 3.4$ Hz, 1H, H-3), 3.36 (dd, $J = 3.4$ and 1.2 Hz, 1H, H-2), 4.38 (d, $J = 1.2$ Hz, 1H, H-1); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 27.2$ (C-10), 28.8 (C-11), 29.4 (C-12), 29.7 (C-6), 36.3 (C-4), 40.9 (C-5), 42.4 (C-7), 53.3 (C-2), 60.4 (C-3), 80.1 (C-1), 175.5 (C-8); IR (KBr,

Table 1
The composition of the product mixtures of biotransformation of lactones **1a–c**

Entry	Strain	Time of transformation (days)	Products of transformations (%)[a]					
			1a		**1b**		**1c**	
			1a	**2a**	**1b**	**2b**	**1c**	**3**
1	*Absidia cylindrospora 336*	2	96.5	3.5	90.0	10.0	96.6	3.4
		4	54.0	46.0	81.5	18.5	87.3	12.7
		6	17.8	82.2	19.0	81.0	28.8	71.2
		8	8.2	91.8	8.5	91.5	10.3	89.7
2	*Fusarium culmorum 7*	2	92.0	8.0	100	0.0	95.4	4.6
		4	76.8	23.2	95.6	4.4	89.9	10.1
		6	33.5	66.5	93	7.0	84.8	15.2
		8	28.1	71.9	90	10.0	83.7	16.3
3	*Fusarium avenaceum 12*	2	97.7	2.3	100	0.0	97.3	2.7
		4	88.8	11.2	100	0.0	95.4	4.6
		6	70.0	30.0	98	2.0	94.0	6.0
		8	64.6	35.4	96	4.0	93.1	6.9
4	*Beauveria bassiana 446*	2	100.0	0.0	100.0	0.0	100.0	0.0
		4	97.0	3.0	94.0	6.0	99.0	1.0
		6	94.4	5.6	93.7	6.3	95.5	4.5
		8	93.2	6.8	92.4	7.6	93.0	7.0
5	*Yarrowia lipolytica 71*	2	100.0	0.0	99.0	1.0	100.0	0.0
		4	100.0	0.0	98.2	1.8	100.0	0.0
		6	94.7	5.3	98.0	2.0	97.8	2.2
		8	91.0	9.0	97.3	2.7	96.6	3.4

[a] According to GC.

cm^{-1}): 2923 (s), 1793 (s), 1207 (m), 1021(m). Anal. Calcd. for $C_{11}H_{16}O_3$: C, 67.32; H, 8.22. Found: C, 67.26; H, 8.29.

Crystal data for **2a**: $C_{11}H_{16}O_3$, $M_w = 196.24$, colourless block, crystal size 0.10 mm × 0.10 mm × 0.10 mm, orthorhombic, space group $P2_12_12_1$, $a = 10.7693(12)$ Å, $b = 23.007(2)$ Å, $c = 8.2461(9)$ Å, $V = 2043.1(4)$ Å^3, $Z = 8$ Å, $D_c = 1.276$ Mg m^{-3}, $T = 100(2)$ K, $R = 0.056$, w$R = 0.083$ (for 1153 reflections with $I > 2\sigma(I)$)for 127 variables. CCDC No. 602649.

2.6.3. Preparative biotransformation of 4,4-dimethyl-9-oxabicyclo[4.3.0]non-2-en-8-one (*1b*)

The biotransformation was continued for 14 days to obtain 70 mg of products mixture as a brown dense liquid. After the column chromatography with hexane:acetone:methylene chloride 4:1:2 as a developing system 43.5 mg (25% isolated yield) of pure product **2b** (2,3-epoxy-4,6-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one) were isolated with the physical and spectral data given below:

mp 70–73 °C; $[\alpha]_{589}^{20} = +37.36$ ($CHCl_3$, $c = 1.1$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.01$ and 1.11 (two s, 6H, $(CH_3)_2C$), 1.07 (dd, $J = 13.0$ and 12.0 Hz, 1H, one of CH_2-5), 1.22 (dd, $J = 13.0$ and 4.9 Hz, 1H, one of CH_2-5), 2.17 (d, $J = 17.3$ Hz, 1H, one of CH_2-7), 2.46 (dddd, $J = 12.0$, 7.6, 4.9 and 4.8 Hz, 1H, H-6), 2.72 (dd, $J = 17.3$ and 7.6 Hz, 1H, one of CH_2-7), 2.99 (d, $J = 3.3$ Hz, 1H, H-3), 3.46 (d, $J = 3.3$ Hz, 1H, H-2), 4.73 (d, $J = 4.8$ Hz, 1H, H-1); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 23.8$ (C-10), 29.3 (C-11), 29.6 (C-6), 29.7 (C-4), 36.3 (C-5), 37.4 (C-7), 52.2 (C-2), 60.8 (C-3), 75.5 (C-1), 177.2 (C-8); IR (KBr, cm^{-1}): 2924 (s), 1787 (s), 1457 (s), 1164 (m), 1018 (m). Anal. Calcd. for $C_{10}H_{14}O_3$: C, 65.91; H, 7.74. Found: C, 65.85; H, 7.69.

Crystal data for **2b**: $C_{10}H_{14}O_3$, $M_w = 182.21$, colourless plate, crystal size 0.20 mm × 0.10 mm × 0.10 mm, orthorhombic, space group $P2_12_12_1$, $a = 5.8814(6)$ Å, $b = 8.8009(7)$ Å, $c = 17.4476(14)$ Å, $V = 903.12(14)$ Å^3, $Z = 4$ Å, $D_c = 1.340$ Mg m^{-3}, $T = 100(2)$ K, $R = 0.028$, w$R = 0.055$ (for 1362 reflections with $I > 2\sigma$(I)) for 174 variables. CCDC No. 602648.

2.6.4. Preparative biotransformation of 4-methyl-9-oxabicyclo[4.3.0]non-2-en-8-one (*1c*)

The biotransformation was carried out for 14 days to furnish 85 mg of crude brownish residue. It was separated by silica gel column chromatography (developed with hexane:acetone:methylene chloride 4:1:2) to yield 60.9 mg of product **3** (35% isolated yield), identified as 4-hydroxy-4-methyl-9-oxabicyclo[4.3.0]non-2-en-8-one. On the basis of GC analysis on the chiral column it turned out to be the racemic mixture. Its physical and spectral data are as follows:

Oily liquid; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.35$ (s, 3H, CH_3-4), 1.42 (m, 1H, one of CH_2-5), 1.57 (s, 1H, OH), 1.79 (ddd, $J = 13.8$, 4.2 and 1.1 Hz, 1H, one of CH_2-5), 2.27 (m, 1H, one of CH_2-7), 2.88 (m, 2H, H-6 and one of CH_2-7), 4.75 (m, 1H, H-1), 5.98 (dd, $J = 9.9$ and 4.0 Hz, 1H, H-2), 6.05 (dd, $J = 9.9$ and 1.1 Hz, 1H, H-3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 29.7$ (C-10), 29.8 (C-6), 36.3 (C-5), 38.4 (C-7), 66.4 (C-4), 74.3 (C-1), 123.8 (C-2), 139.2 (C-3), 176.3 (C-8); IR (film, cm^{-1}): 3441 (s), 2969 (s), 1762 (s), 1175 (m). Anal. calcd. for $C_9H_{12}O_3$: C, 64.27; H, 7.19. Found C, 64.20; H, 7.28.

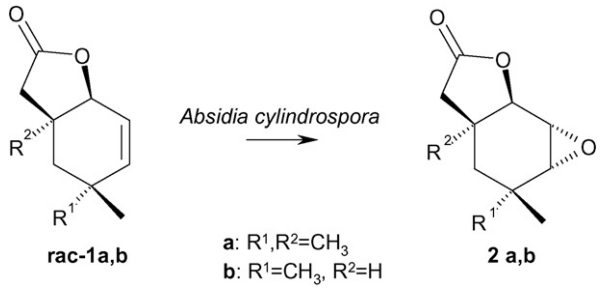
3. Results and discussion

The substrates used for the biotransformations, the racemic mixtures of δ,ε-unsaturated bicyclic γ-lactones (**1a**–**c**) were synthesized as described earlier [35]. The cyclohexane ring present in these molecules was substituted with three (**1a**), two (**1b**) and one (**1c**) methyl group. Two of them (**1a** and **b**) possessed the *gem*-dimethylcyclohexane system with two methyl groups located at C-4 position (Fig. 1). Such range of substrates let us establish the relationship between the structure and the course of transformation of these compounds by the fungal strains applied.

At the beginning of our work we performed the screening-scale experiments using eight fungal strains being at our disposal. We applied the whole-cells systems, incubating the grown cultures with appropriate substrates at 25 °C and monitoring the progress of biotransformation by means of standard techniques (TLC and GC). The results for the five most efficient strains are presented in Table 1.

In case of all three lactones the formation of only one product was observed. The most effective biocatalyst turned out to be *A. cylindrospora* (entry 1), which transformed all three lactones to a high extent (about 90%). The most consumed substrate in case of *F. culmorum* was **1a** (about 70%). The average extend was also achieved for the conversion of **1a** by *F. avenaceum.* In all other cases the contents of products in the mixtures were very low, not exceeding 10%.

The preparative scale-transformations were conducted by means of *A. cylindrospora*. The incubation of lactones **1a** and **1b** with this strain gave 78 and 43.5 mg of the corresponding products **2a** and **2b**, respectively. On the basis of GC and TLC analysis they were found to be the same as those detected during the screening experiments. A lot of information about their structures was obtained from the spectral data (^{1}H NMR, ^{13}C NMR and IR). The absorption bands in the IR spectra at 1793 and 1787 cm^{-1}, respectively, confirmed that in both molecules the γ-lactone ring was not touched by the microorganisms. The lack of absorption bands over 3000 cm^{-1} as well as the analysis of ^{1}H NMR and ^{13}C NMR spectra showed that no hydroxylation reactions occurred. However, the molecular peak at MS-spectra suggested the introduction of oxygene atom into both molecules. Comparing the ^{1}H NMR spectra of both substrates and products it is easy to notice that the signals from the protons H-2 and H-3 were shifted upfield from over 5 ppm (which is characteristic for the olefine protons) to 3.36 and 2.93 ppm, respectively (in case of **2a**) and to 3.46 and 2.99 ppm, respectively (in case of **2b**). Such chemical shifts are typical for the oxirane protons. Similarly, the positions of signals belonging to the C-2 and C-3 atoms in the ^{13}C NMR spectra changed significantly. They appeared at 53.3 and 60.4 ppm, respectively (in the spectrum of compound **2a**) and similarly at 52.2 and 60.8 ppm, respectively (in the spectrum of compound **2b**). In the spectra of substrates **1a** and **b** they were located at 119.0 (C-2) and 142.8 ppm (C-3) (in case of compound **1a**) and at 119.6 (C-2) and 145.1 ppm (C-3) (in case of compound **1b**). The above data led us to the



Scheme 1.

conclusion that the double bond C-2–C-3 in both unsaturated substrates was epoxidized yielding the corresponding epoxylactones (Scheme 1). It is worth noting that in both cases no diol products of epoxide hydrolysis were detected.

Moreover, the small coupling constant of H-2 with H-1 ($J = 1.2$ Hz) in case of product **2a** or lack of coupling between these protons observed in case of product **2b** indicated *trans* orientation of the epoxide moieties to the γ-lactone rings in both molecules. The coupling constants between H-2 and H-3 protons in the epoxylactones **2a** and **2b** ($J = 3.4$ and 3.3, respectively) are consistent with literature date connecting the vicinal coupling constant of oxirane protons.

All those assignments were fully confirmed by the X-ray analysis. The values of dihedral angles between the bonds: H-1–C-1–C-2–H-2 in both compounds (−69.2° in compound **2a** and 73.9° in compound **2b**) were compatible with the coupling constants ($J = 1.2$ Hz and 0 Hz, respectively) between these protons found in the ^{1}H NMR spectra. Both crystallic structures confirmed *trans* orientation of the oxirane rings to the γ-lactone rings. They also showed the pseudoaxial orientations of C–O bonds in the lactone moieties. It can be pointed out that in the lactone **2a** the cyclohexane ring exists in the relatively uncommon half boat conformation with the atoms C-1, C-2, C-3, C-4 and C-5 situated nearly in the same plane and the C-6 carbon placed above this plane (Fig. 2).

On the contrary, in the product **2b** the half chair conformation was found. In this molecule one can see that the plane of cyclohexane ring includes only the atoms C-1, C-2, C-3 and C-4, whereas C-5 is situated below and C-6, above this plane (Fig. 3).

Very interesting results were obtained from the GC analysis of compound **2b** using the chiral column (Fig. 4). It turned out that the microbial epoxidation was highly stereoselective (formation of only *trans*-epoxylactone among two possible diastereoisomeric epoxides). Besides, the transformation of **1b** gave surprisingly pure (+) enantiomer of epoxylactone **2b**, which was univocally proven by the comparison of GC analysis of the products of chemical and microbial epoxidation of **1b**.

Taking into consideration the fact that after biotransformation only 8.5% of substrate remained unreacted, during the process the microbial hydrolysis of the γ-lactone ring of the other one enantiomer probably occurs leading to hydroxyacid. This compound was not isolated from the mixture because at high pH value it existed as a salt, insoluble in the methylene chloride. On the other hand, in the case of lactone **1a** the microbial hydrolysis

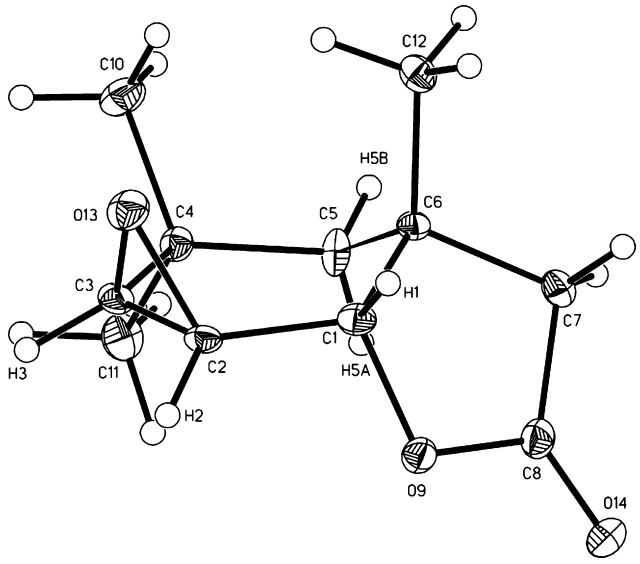


Fig. 2. Crystal structure of the epoxylactone **2a** with crystallographic numbering (one of the possible enantiomers).

took place to a less extent because, at the comparative degrees of conversion, the enantiomeric excess of compound **2a** was much more lower (9%) than in the case of product **2b** (Table 2). However, high (100%), "*trans*" stereoselectivity of epoxidation of **1a** was also observed. Unfortunately, in the method of crystallographic analysis applied in our studies the anomal dispersion for the O, C and H atoms is not sufficient for the exact determination of the absolute configuration of the chiral centers of the crystal and the entire molecule.

Obtaining of the stable epoxylactones by microbial stereoselective epoxidation is very uncommon. Formation of epoxides is often the first reaction during the microbial transformation of unsaturated sesquiterpene lactones. The epoxylactones formed are usually not isolated because the oxirane ring is opened and the various further reactions are observed. The epoxida-

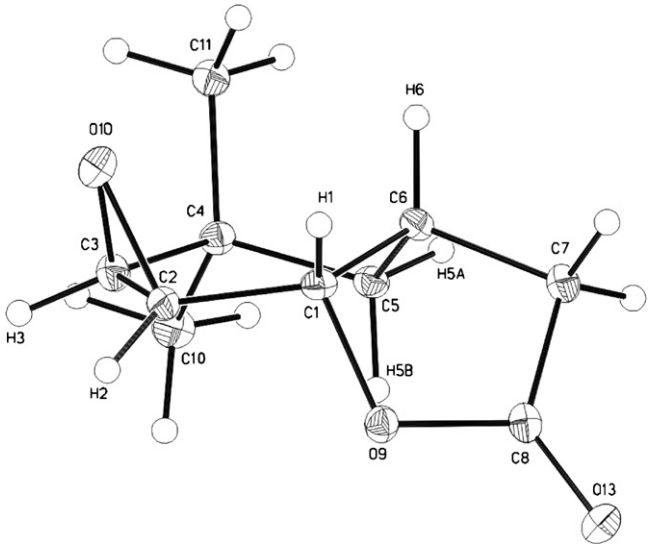


Fig. 3. Crystal structure of the epoxylactone **2b** with crystallographic numbering (one of the possible enantiomers).

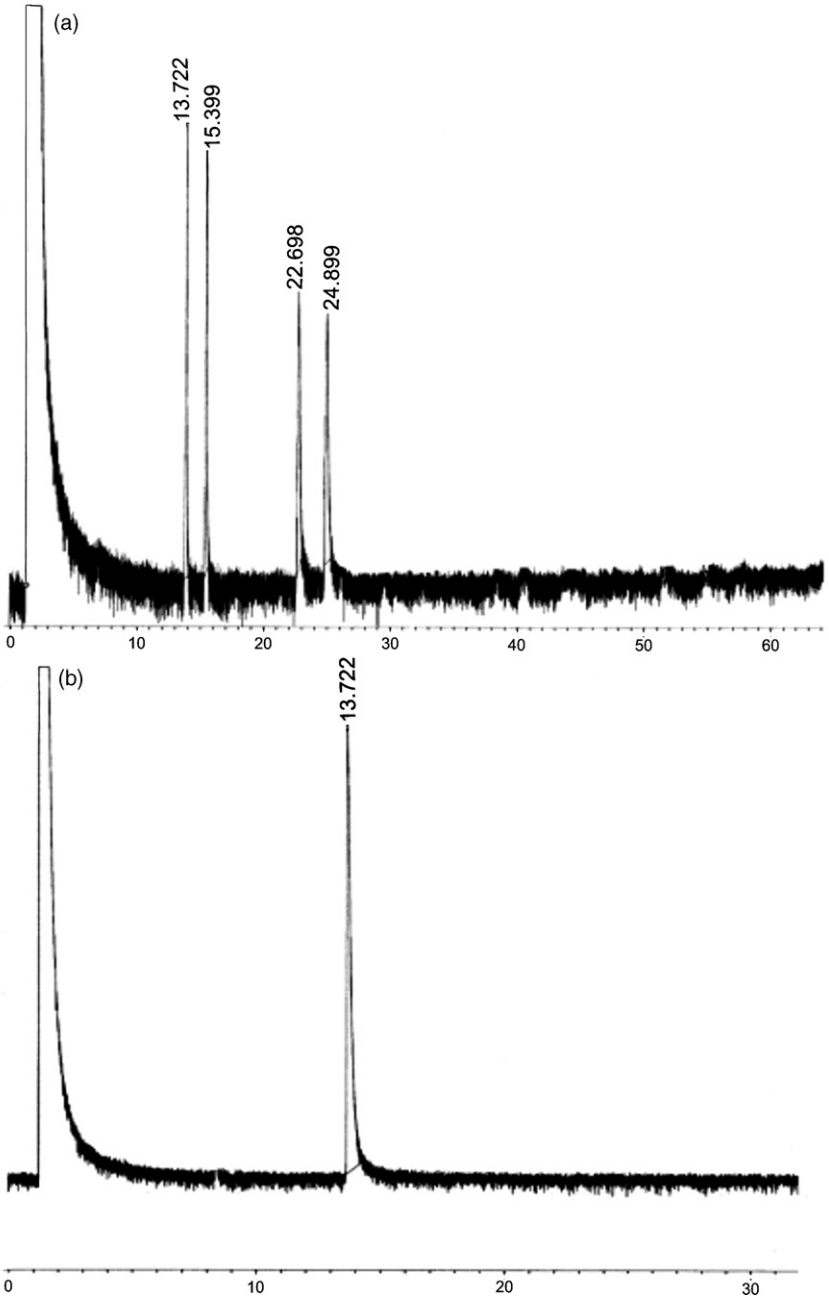


Fig. 4. The GC chiral column chromatogram of the products of chemical (a) and microbial (b) epoxidation of the unsaturated lactone **1b**.

tion of costunolide by *Cunninghamella echinulata* is followed by the intramolecular cyclization of germacrane skeleton to the eudesmane one [29]. The products of this rearrangement are hydroxylactone and two lactones with the double bond. In other process, deoxyvulgarin is first epoxidized by *Rhizopus nigricans* and then the products with hydroxy group in allylic position are formed [30]. The only one stable product of regioselective epoxidation was the epoxylactone formed from the dehydrocostuslactone by means of *R. oryzae* strain [29].

Table 2
Results of preparative biotransformations of lactones **1a**–**c**

Product	Content in the reaction mixture (%)[a]	Isolated yield (%)	ee of product (%)	ee of unreacted substrate (%)
2a	92.3	45	9	10 (for **1a**)
2b	91.5	25	100	99 (for **1b**)
3	89.6	35	0	7 (for **1c**)

[a] According to GC.

Scheme 2.

The preparative transformation of the lactone **1c** carried out with *A. cylindrospora* proceeded in a different way. The isolated product **3** (30 mg, 35% isolated yield) was identical to the one observed during the screening experiment (TLC and GC). Its spectral data let us identify it as the product of biohydroxylation. In the IR spectrum, besides the absorption band at 1762 cm^{-1} confirming the presence of the γ-lactone ring, the strong band at 3441 cm^{-1} indicated that the hydroxy group was introduced into the molecule. It was additionally proved by the signal present at 66 ppm in the ^{13}C NMR spectrum. The signals at 123.8 (C-2) ppm and 139.2 (C-3) ppm in the ^{13}C NMR spectrum as well as the multiplets of H-2 and H-3 protons at 5.98 and 6.05, respectively in ^{1}H NMR spectrum showed that, in opposite to the compounds **2a** and **2b**, the double bond remained unaffected. The comparative analysis of the ^{1}H NMR spectra of **1c** and **3** let us assign the position of the hydroxy group. The important features of the spectrum of **3** were: lack of new signal between the 3 and 4 ppm, the simplifying the shapes of multiplets belonging to the olefine protons and disappearing the signal from H-4 proton. Those data together with the fact that singlet from the methyl protons was shifted downfield by 0.33 ppm in comparison with doublet ($J = 7.1$ Hz) present in the spectrum of substrate **1c** pointed to the conclusion that the hydroxy group was regioselectively introduced in the allylic, pseudoaxial position C-4 (Scheme 2). Although we did not have any standard of this compound, two peaks in ratio 50:50 detected on the GC chromatogram obtained from the analysis on the chiral column were the undoubted proof that the hydroxylactone obtained was a racemic mixture. It is worth to notice that the hydroxylation of the tertiary carbon atom C-4 was also observed during our earlier studies over the transformation of saturated analogue of lactone **1c** by the same strain [36].

4. Conclusions

The functionalization of unsaturated γ-lactones fused to the alkylsubstituted cyclohexane system by means of *A. cylindrospora* strongly depended on the number and location of methyl groups present in the molecule. The compounds with 4,4-*gem*-dimethyl system were stereoselectively epoxidized and the corresponding epoxylactones were isolated. In both cases the microbial epoxidation was highly stereoselective yielding only the isomer with *trans* orientation of the oxirane ring to the lactone moiety, whereas the chemical oxidation of the substrates led to the racemic mixtures of *cis*- and *trans*-epoxylactones.

When the cyclohexane was substituted by only one methyl group at C-4 no epoxidation was observed. Instead of that the product of hydroxylation of the allylic position at the tertiary carbon atom C-4 was identified. These results suggest that the P450 monooxygenase present in these fungi prefers the hydroxylation of allylic position over the epoxidation reaction.

All the products obtained were new chemical compounds, not described previously in the literature. Product **2b** was obtained as a single enantiomer.

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