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Influence of structure of lactones with the methylcyclohexene and dimethylcyclohexene ring on their biotransformation and antimicrobial activity

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Abstract: The aim of this article is influence of the structure of lactones with the methylcyclohexene and dimethylcyclohexene ring on their biotransformation and antimicrobial activity. This work was based on the general remark that even the smallest change in the structure of a compound can affect its biological properties. The results of the biotransformation of four bicyclic unsaturated lactones with one or two methyl groups in the cyclohexene ring was tested using fifteen fungal strains (*Fusarium* species, *Penicillium* species, *Absidia* species, *Cunninghamella japonica*, and *Pleurotus ostreatus*) and five yeast strains (*Yarrowia lipolytica*, *Rhodorula marina, Rhodorula rubra*, *Candida viswanathii*, and *Saccharomyces cerevisiae*). During these transformations, new epoxylactone and hydroxylactone were obtained. The relationship between the substrate structure and the ability of the microorganisms to transform them were analysed. Only compounds with C–O bond of lactone ring in the equatorial position were transformed by fungus. All presented here lactones were examined also for their antimicrobial activity. It turned out that these compounds exhibited growth inhibition of bacteria and fungi, mainly *Bacillus subtilis*, *Candida albicans*, *Aspergillus niger, and Penicillium expansum*.

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1 Introduction

Among naturally occurring terpenoid lactones, it is possible to find those in which an exo or endocyclic double bond is present in the molecule [1–4]. Sometimes, these compounds have different interesting biological properties such as antimicrobial [5], antiproliferative [6], cytotoxic [7, 8], or antifeedant effects [9]. Lactones with a double bond are sometimes characterized by an interesting odor such as coconut [10, 11], butter cake, milky almond [12], green tea [13], or celery leaves or stem [14].

Biotransformed unsaturated terpenoid lactones [4, 15–17] as well as mono-[18] or sesquiterpenoid [19, 20] compounds can undergo several different reactions: hydrogenation of the double bond [15, 16], epoxidation [15, 21], or hydroxylation [15, 17–20]. The hydroxy group can be positioned at different places in the molecule. The first possibility is to obtain the diol by using one of two possible methods: opening the epoxide ring [15] or directly by dihydroxylation of the double bond [15, 18–20]. The second possibility is to introduce the hydroxy group in an allylic position [15, 17, 18, 20, 21].

As we know from the literature mentioned above, biotransformation of unsaturated terpenoid compounds may yield different, interesting derivatives such as epoxylactones or hydroxylactones. For this reason, we have chosen to carry out biotransformation of some unsaturated bicyclic lactones. Our goal was to determine the influence of the number (one or two) and position of methyl groups present in the molecule on the type of products formed during biotransformation. We hypothesized that the unsaturated lactones we chose as well as the products of their biotransformation would be characterized by interesting antimicrobial activities. Our previous experience had shown that the hydroxylactones obtained by

biotransformation were able to inhibit the growth of pathogenic bacteria and fungi [22–24].

2 Materials and methods

2.1 Chemical analysis

The progress of chemical reactions and biotransformations as well as the purity of isolated products were monitored by TLC-technique on silica gel-coated aluminum plates (DC-AlufolienKieselgel 60 F254, Merck, Darmstadt, Germany). Chromatograms were developed with a mixture of hexane, acetone, and diethyl ether in various ratios (for unsaturated lactones hexane : diethyl ether 4 : 1, for products biotransformation hexane : acetone 3:1). 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, Darmstadt, Germany) with a mixture of hexane and acetone (for unsaturated lactones hexane : acetone 4 : 1, for products biotransformation hexane : acetone 3:1) as eluents. GC analysis was carried out on an Agilent Technologies 6890N instrument(Varian, Agilent Technologies, Santa Clara, CA, USA) using a DB-17 column (cross-linked methyl silicone gum, 30 m × 0.32 mm × 0.25 μm) or on a Varian CP3380 instrument (Varian, Agilent Technologies, Santa Clara, CA, USA) using Thermo TR-5 (30 m × 0.32 mm × 1.0 μm) capillary columns. The enantiomeric compositions of the products obtained during biotransformation were 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) 250 °C, column temperature 140 °C, hold 45 min; ramp 140–200 °C at rate 20 °C/min and hold 1 min at 200 °C. The molar masses of the obtained compounds were confirmed by high-resolution mass spectrometry analysis using a Waters LCT Premier XE instrument (*ESI* ionization) (Waters Division, Milford, Massachusetts).

^{1}H NMR and ^{13}C NMR spectra were recorded in a $CDCl_3$ solution on a BrukerAvance DRX 300 MHz spectrometer (Bruker, Billerica, MA, USA) or on a BrukerAvance™ 600 MHz spectrometer (Bruker, Billerica, MA, USA). Chemical shifts are reported in reference to the residual solvent signal ($\delta_H = 7.26$). IR spectra were recorded on a Thermo-Nicolet IR300 FT-IR spectrometer (Waltham, MA, USA). Optical rotations were determined on a P-2000 polarimeter (Jasco Easton, PA, USA) in chloroform solutions, with concentrations denoted in g/100 mL. The melting points were determined on a Boetius apparatus. The refractive index was measured on Carl Zeiss Abbe and Pulfrichrefractometer (Jena, Germany).

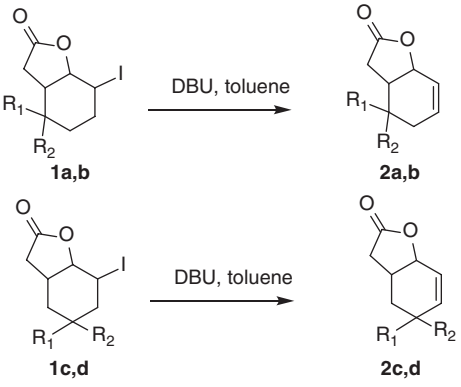
2.2 Synthesis of substrates

Two new racemic unsaturated lactones **2a** and **2b** were synthesized from the δ-iodo-γ-lactones **1a** [25] and **1b** [26] according to the procedure described earlier [27]. Two unsaturated lactones **2c** and **2d** were obtained in an analogous manner [27] (Scheme 1).

In this article, we present the synthesis and spectral data of new compounds **2a** and **2b** and the spectral data of compounds **2c** and **2d** (according to literature) [27]. This data will be useful in studying the relationship between the structure of the substrate and its interaction with microorganisms.

2.2.1 *5,5-Dimethyl-9-oxabicyclo[4.3.0]non-2-en-8-one* 2a

The mixture of 2.5 g (8.5 mmol) of iodolactone **1a** and 2 mL 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) dissolved in 40 mL of toluene was subjected to processing according to a known procedure [27]. The product of this reaction was a colorless liquid (1.3 g, 92% yield).The spectral data of product **2a** are as follows: n_D: 1.4850. IR (KBr, cm^{-1}) ν 2959, 1779, 1168, 1003. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96 (s, 3H, CH_3-9), 1.00 (s, 3H, CH_3-10), 1.83 (ddd, *J* 18.4, 4.8, 0.7 Hz, 1H, one of CH_2-4), 2.03 (dd, *J* 18.4, 2.6 Hz, 1H, one of CH_2-4), 2.30 (m, 1H, one of CH_2-7), 2.36 (m, 1H, one of CH_2-7), 2.43 (m, 1H, H-6), 4.96 (m, 1H, H-1), 5.71 (dd, *J* 10.2, 1.2 Hz,



Scheme 1: Synthesis of unsaturated lactones **2a–d.**

1H, H-2), 5.88 (ddd, *J* 10.2, 4.8, 2.6 Hz, 1H, H-3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 26.9 (C-10), 28.1 (C-9), 30.3 (C-7), 31.5 (C-5), 32.0 (C-5), 34.5 (C-4), 44.6 (C-6), 77.0 (C-1), 123.2 (C-2), 130.2 (C-3), 176.9 (C-8). *ESIHRMS m/z* 167.1068 $[M+H]^+$ (calculated for $C_{10}H_{14}O_2$ $[M+H]^+$, 167.1067).

2.2.2 *5-Methyl-9-oxabicyclo[4.3.0]non-2-en-8-one* 2b

Iodolactone **1b** (2.3 g, 8.2 mmol) and 1 mL 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were mixed, and the reaction was carried out in an analogous manner as described above. A colorless liquid was obtained (1.1 g, 78% yield). The spectral data of product **2b** are given below: n_D: 1.4935. IR (KBr, cm^{-1}) ν 2958, 1779, 1171, 948. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00 (d, *J* 6.6 Hz, 3H, CH_3-9), 1.53 (m, 1H, H-5), 1.74 (m, 1H, one of CH_2-4), 2.16 (m, 1H, one of CH_2-4), 2.23 (m, 1H, H-6), 2.51 (dd, *J* 17.6, 2.3 Hz, 1H, one of CH_2-7), 2.73 (dd, *J* 17.6, 8.2 Hz, 1H, one of CH_2-7), 4.82 (m, 1H, H-1), 5.90 (m, 1H, H-2), 6.11 (m, 1H, H-3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 18.9 (C-9), 28.3 (C-5), 31.7 (C-4), 34.1 (C-7), 40.8 (C-6), 44.6 (C-6), 76.2 (C-1), 122.7 (C-2), 133.7 (C-3), 176.5 (C-8). *ESIHRMS m/z* 153.0916 $[M+H]^+$ (calculated for $C_9H_{12}O_2$ $[M+H]^+$, 153.0910).

The spectral data of 4.4-dimethyl-9-oxabicyclo[4.3.0] non-2-en-8-one **2c** are as follows: IR (KBr, cm^{-1}) ν 2976, 1772, 1180, 972, 944. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00 (s, 3H, CH_3-9), 1.05 (s, 3H, CH_3-10), 1.28 (dd, *J* 13.4, 13.2 Hz, 1H, one of CH_2-5), 1.50 (ddd, *J* 13.4, 4.7, 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, 4.7, 1H, H-6), 2.86 (dd, *J* 17.3, 8.0 Hz, 1H, one of CH_2-7), 4.70 (dd, *J* 5.2, 4.3 Hz, 1H, H-1), 5.80 (dd, *J* 10.0, 4.3 Hz, 1H, H-2), 5.92 (d, 1H, *J* 10.0, H-3).

The spectral data of 4-methyl-9-oxabicyclo[4.3.0]non-2-en-8-one**2d** are given below: IR (KBr, cm^{-1}) ν 2957, 1789, 1173, 967, 948. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00 (m, 1H, one of CH_2-5), 1.06 (d, *J* 7.2 Hz, 3H, CH_3-9), 1.73 (ddd, *J* 12.6, 4.3, 4.3 Hz, 1H, one of CH_2-5), 2.19 (m, 1H, H-4), 2.28 (d, *J* 17.4 Hz, 1H, one of CH_2-7), 2.51 (m, 1H, H-6), 2.86 (dd, *J* 17.4, 8.1 Hz, 1H, one of CH_2-7), 4.73 (m, 1H, H-1), 5.91 (dm, *J* 10.0 Hz, 1H, H-3), 5.98 (d, *J* 10.0 Hz, 1H, H-2). ^{13}C NMR (600 MHz, $CDCl_3$) δ 20.8 (C-9), 29.7 (C-4), 33.3 (C-5), 33.8 (C-6), 37.0 (C-7), 75.1 (C-1), 121.7 (C-2), 141.4 (C-3), 176.4 (C-8).

2.3 Microorganisms

The fungal and yeast strains used in all biotransformation came from the collection of the Institute of Biology and Botany, Medical University, Wrocław (*Fusarium culmorum* AM10, *Fusarium culmorum* AM196, *Fusarium avenaceum* AM11,*Fusarium oxysporum* AM13, *Fusarium semitectum* AM20, *Fusarium solani* AM203, *Penicillium vermiculatum* AM30, *Penicillium chermesinum* AM113, *Penicillium frequentas* AM351, *Absidia coerulea* AM93, *Absidia glauca* AM177, *Absidia cylindrospora* AM336, *Absidia glauca* AM524, *Cunninghamella japonica* AM472, *Pleurotus ostreatus* AM482, *Yarrowia lipolytica* AM71, *Rhodorula marina* AM77, *Rhodorula rubra* AM82, *Candida viswanathii* AM120, *Saccharomyces cerevisiae* AM464). All of these strains were cultivated on Sabouraud's agar containing 0.5% of aminobac, 0.5% of peptone, 4% of glucose, and 1.5% of agar dissolved in distilled water at 28 °C and stored in refrigerator at 4 °C.

2.4 Screening procedure

Biotransformation was performed in two 300 mL Erlenmeyer flasks containing 100 mL of medium consisting of 3% glucose and 1% peptone. When the culture was grown (3 days), 10 mg of the substrate dissolved in 1 mL of acetone was added to each flask. Incubation of shaken cultures with substrate was continued for 14 days. After 5, 9, and 14 days of incubation, the medium contained unreacted substrate, product and mycelium were extracted with dichloromethane (15 mL) and analyzed by GC (DB-17 column).

2.5 Preparative biotransformation

This step was similar to this described above, with the difference that we used 10 Erlenmeyer flasks (300 mL) containing 3-day cultures of fungal strains to which was added 100 mg of substrate dissolved in 10 mL acetone. After 14 days, the postreaction mixture was extracted with dichloromethane (3×40 mL). The combined organic fractions were dried over anhydrous magnesium sulfate and the solvent was evaporated in vacuo. Pure product was separated from the unreacted substrate and the metabolites of the fungi by using column chromatography (silica gel, hexane : acetone 3:1).

Epoxylactone(*2,3-epoxy-5,5-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one*) (**3a**) was obtained as a white solid. The spectral data are given below:

mp: 57–58 °C. IR (KBr, cm^{-1}) ν 2918, 1771, 1159, 1018. ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.91 (s, 3H, CH_3-9), 1.03 (s, 3H, CH_3-10), 1.78 (dd, *J* 15.8, 2.0 Hz, 1H, one of CH_2-4), 1.85 (dd, *J* 15.8, 3.2 Hz, 1H, one of CH_2-4), 2.33-2.47 (m, 3H, H-6 and CH_2-7), 3.26 (d, *J* 3.5 Hz, 1H, H-2), 3.33 (m, 1H, H-3), 4.83(d, *J* 6.3 Hz, 1H, H-1). ^{13}C NMR (151 MHz, $CDCl_3$) δ 27.8 (C-9), 28.6 (C-10), 29.5 (C-5), 30.0 (C-7), 33.7 (C-4), 42.9 (C-6), 50.4 (C-2),

52.9 (C-3), 74.9 (C-1), 176.0 (C-8). *ESIHRMS m/z* 183.1021 $[M+H]^+$ (calculated for $C_{10}H_{14}O_3$ $[M+H]^+$, 183.1016).

Hydroxylactone(*4-hydroxy-5,5-dimethyl-9-oxabicyclo[4.3.0]nonan-8-one*) **4a** was obtained as a white solid. The spectral data are as follows:

IR (KBr, cm^{-1}) ν3453, 2965, 1772, 1477, 1171, 1001. ^1H NMR (600 MHz, $CDCl_3$) δ 0.97 (s, 3H, CH_3-9 A), 0.99 (s, 3H, CH_3-10 A), 1.02 (s, 3H, CH_3-9 B), 1.09 (s, 3H, CH_3-10 B), 1.27 (m, 1H, OH A), 2.19 (m, 1H, OH B), 2.36 (dd, *J* 17.4, 10.9 Hz, 1H, one of CH_2-7 B), 2.42 (m, H-6 A), 2.48 (dd, *J* 17.4, 8.7 Hz, 1H, one of CH_2-7 B), 2.60 (dd, *J* 17.8, 8.9 Hz, 1H, one of CH_2-7 A), 2.69 (m, H-6 B), 2.89 (dd, *J* 17.8, 6.2 Hz, 1H, one of CH_2-7 A), 3.90 (s, 1H, H-4 A), 4.09 (s, 1H, H-4 B), 4.87 (m, 1H, H-1 A), 5.00 (m, 1H, H-1 B), 5.83 (m, 1H, H-2 B), 5.92 (m, 2H, H-3 A and H-3 B), 6.00 (m, 1H, H-2 A). ^{13}C NMR (151 MHz, $CDCl_3$) δ 18.7 (C-9 A), 20.8 (C-9 B), 24.7 (C-10 B), 25.7 (C-10 A), 29.5 (C-7 B), 31.4 (C-7 A), 35.3 (C-5 A), 37.0 (C-5 B), 42.5 (C-6 A), 44.7 (C-6 B), 70.7 (C-4 B), 72.7 (C-4 A), 75.7 (C-1 A), 76.5 (C-1 B), 123.6 (C-2 B), 124.8 (C-3 A), 133.8 (C-3 B), 133.98 (C-2 A), 176.3 (C-8 B), 176.8 (C-8 A). *ESIHRMS m/z* 183.1021 $[M+H]^+$ (calculated for $C_{10}H_{14}O_3$ $[M+H]^+$, 183.1016).

2.6 Bioassay

The tests have been made by using following bacteria strains: *Escherichia coli* ATCC 10536, *Staphylococcus aureus* DSM 799, *Bacillus subtilis* PCM 2850, yeast strains: *Candida albicans* CBS 767 and filamentous fungi strains: *Aspergillus niger* KKBMZ X1, *Fusarium linii* KKBMZ 3A, *Penicillium expansum* CBS 664. *Alternaria alternata* CBS 1526 . The test was performed in the automated Bioscreen C system (Automated Growth Curve Analysis System, Lab Systems, Finland) with procedure analogous to described before [24].

2.7 Statistical analysis

Results of microbiological experiments are shown as mean ± S.E. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparison test.

3 Results and discussion

During our previous studies, we noticed that some of the tested compounds were not converted by microorganisms. That prompted us to compare the properties of selected compounds with their spatial structure. In order for this, four unsaturated structurally related lactones with difference in position and number of methyl groups in cyclohexene ring **2a–d** were chosen. These substrates were subjected to a screening biotransformation in order to verify the ability of microorganisms to convert these compounds into other products. Fifteen fungal strains and five yeast strains were used for this step. It turned out that only one of the substrates was transformed. Positive results of these tests are presented in Table 1.

As it is shown in Table 1, of the 20 tested microorganisms, only some were capable of converting one of the chosen substrates. Lactone **2a**, with cyclohexene ring containing two methyl groups, was transformed by seven microorganisms into the same product – epoxylactone **3a**. The best biotransformation results were obtained for strains of *F. culmorum* AM10 (entry 1) and AM196 (entry 2). In other cases, the degree of conversion did not exceeded 20% except for *P. vermiculatum* AM30 (entry 6), which created 33% of product. The other compounds, lactones **2b**, **2c**, and **2d**, were not converted at all. Knowing from past experiences [21] that the *A. cylindrospora* AM336 strain was able to transform lactones **2c** and **2d**, the ability of this strain to convert lactones **2a** and **2b**, which were not tested earlier, was also checked. In the case of lactone **2a** instead of epoxylactone **3a**, a *cis–trans* mixture of hydroxylactone **4a** was obtained (entry 8). Lactone **2b** was also not transformed by this strain.

The obtained results were a complete surprise. Firstly, biotransformation of lactones **2c** and **2d** has been described in the literature [21]: *A. cylindrospora* strain was able to catalyze the epoxidation of these substrates. Secondly, *Fusariumoxy sporum* catalyzed hydroxylation most often, but epoxidation very seldomly [21, 22]. For this reactions, such enzymes as chloroperoxidase, unspecific peroxygenase (UPO, EC 1.11.2.1.), or intracellular P450 monooxygenases [28] may be responsible.

Table 1: Positive results of screening biotransformation of lactone (**2a**) after 14 days of incubation (in % according to GC).

Entry	Microorganism	Screening biotransformation/%		
		(2a)	(3a)	(4a)
1	*Fusarium culmorum*AM10	14.8	85.2	–
2	*Fusarium culmorum* AM196	41.5	58.5	–
3	*Fusarium avenaceum*AM11	89.0	11.0	–
4	*Fusarium oxysporum*AM13	86.5	13.5	–
5	*Fusarium solani* AM203	86.4	13.6	–
6	*Penicillium vermiculatum* AM30	66.6	33.4	–
7	*Absidia coerulea* AM93	87.0	13.0	–
8	*Absidia cylindrospora* AM336	54.0	–	29.0 + 17.0

Hitherto chloroperoxidase and peroxygenase have not been detected in *Fusarium* strains. It seems to be probable that monooxygenases were responsible for the biotransformation described in this article. Some P450 monooxygenases have been identified in *Fusarium*. CYP55A1 (EC 1.7.1.14, class IX P450 enzymes), which is specific for NADH and participates in denitrification. CYP58 (class II P450 enzymes) catalyses epoxidation in mycotoxin synthesis. In the detoxification/degradation of xenobiotics, the first published and best studied P450 is pisatin demethylase (PDA, CYP57A1). Hydroxylation of fatty acids is catalyzed by $P450_{foxy}$ (CYP505A1, EC 1.14.14.1) – a catalytically self-sufficient flavocytochrome, which belongs to class VIII of the P450 enzymes. It is important to note that $P450_{foxy}$ (CYP505A1) shares several characteristics (e.g., primary structure organization, strong preference for NADPH over NADH as the electron donor, function, catalytic rate) with $P450_{BM3}$ (CYP102A1) from *Bacillus megaterium*. The main difference between these two enzymes is intracellular localization. CYP505A1 was recovered from the membrane fraction, whereas CYP102A1 is a soluble protein like other bacterial P450s [29].

Very recently, a diastereoselective epoxidation of *cis*-1,2,3,6-tetrahydrophthalate was described, which was catalyzed by *Bacillus megaterium* $P450_{BM3}$ [30]. Based on this information, it seems to be probable that CYP505A1 could catalyze the epoxidation described in our article.

Strains of *Absidia* are known for their ability to hydroxylate structurally different substrates, such as steroids [31] and lactones [32] among others. Despite this fact, there are no reports in the scientific literature on the specific enzymes responsible for hydroxylation, which were isolated from the genus *Absidia*. Hydroxylation in the allylic position of the substrate may be catalyzed by an enzyme homologous to monooxygenases $P450_{BM3}$. Recently, hydroxylation of other compounds in this position catalyzed by CYP_{BM3} has been described [33].

In the next step, three strains: *F. culmorum* AM10 (entry 1), *F. culmorum* AM196 (entry 2), and *A. cylindrospora* AM336 (entry 8) were used to conduct biotransformation of **2a** on the preparative scale. The obtained results are shown in Figure 1.

During both preparative biotransformations carried on lactone **2a** using *Fusarium* strains as a biocatalyst, the same product – a new epoxylactone **3a**, was obtained. *F. culmorum* AM10 gave this product with a high degree of conversion (86.1%); after purification, 25.8 mg (23.5%) of pure epoxylactone **3a** was obtained. *F. culmorum* AM196 was a less efficient biocatalyst, giving only 51.2% of epoxylactone **3a** and consequently 11.0 mg (10.0%) of pure compound **3a**. The transformation conducted by *A. cylindrospora* AM336 strain provided another product–hydroxylactone **4a**. This product was created as a *cis*–*trans* mixture in a ratio of 67:33; named B and A, respectively. Hydroxylactone **4a** was obtained with a yield of 7.6 mg (6.9%) (Scheme 2).

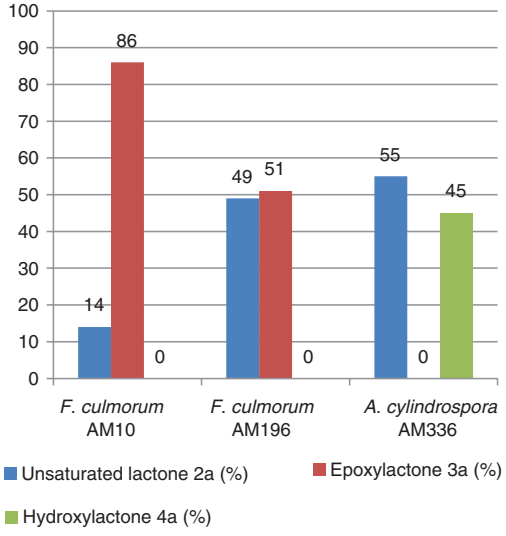
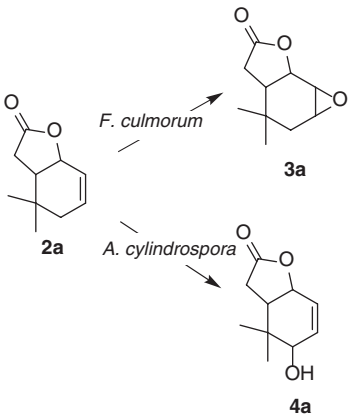


Figure 1: Results of preparative biotransformations (by GC-FID) of lactone (**2a**).

Since one of our goals was to obtain product characterized by high optical purity, we examined enantiomeric excess and also optical rotation for both of the epoxylactones obtained from the biotransformation. Results of these measurements are as follows: **3a** from *F. culmorum* AM10 ee3.7%, $[\alpha]_D^{20}$ 1.6 (*c* 0.995, $CHCl_3$), **3a** from *F. culmorum* AM196 ee16.9%, and $[\alpha]_D^{20}$ 4.0 (*c* 0.45, $CHCl_3$). In both cases, the microorganisms preferred the formation of the (+)-enantiomer of epoxylactone **3a**.

Hydroxylactone **4a** was obtained in a small amount (almost 8 mg) and also as a mixture of two isomers, so



Scheme 2: Biotransformation of unsaturated lactone (**2a**).

we were not able to separate them during the purification. Therefore, we focused only on determining its structure.

In order to find the correlation between the structure and its ability to biotransformation, we compared spectral data (^{1}H NMR, ^{13}C NMR, COSY, HMQC, and IR) of substrates and products. Analysis of the ^{1}H NMR spectra of these lactones indicated some differences in the structures of these molecules. In all of them, the cyclohexene ring took the form of a deformed chair. In the case of **2a**, proton H-1 was in the axial position and proton H-6 was in the equatorial position, which was confirmed by their shapes – wide and narrow multiplets, respectively, whereas in molecules of lactone **2b**, the proton H-1 was in the equatorial position (narrow multiplet) and proton H-6 was in the axial position (wide multiplet) (Figure 2).

Looking at ^{1}H NMR spectra of unsaturated lactones **2c** and **2d**, we could see that the shapes of their molecules were similar to the shape of lactone **2b**. In these cases, the signals coming from proton H-6 were doublet of doublet of doublet of doublet with coupling constants equal to 13.2, 8.0, 5.2, and 4.7 Hz [for **2c**] and wide multiplet [for **2d**], which indicated their axial position. In the other case, the signals of proton H-1 were doublet of doublet with a small coupling constant (5.2 and 4.3 Hz) and narrow multiplet for **2c** and **2d**, respectively, suggesting their equatorial position (Figure 3).

Differences and similarities in the structures of our four lactones could bring about the differences observed during the biotransformation. Lactone **2a** was transformed into epoxylactone **3a** by several strains and into a *cis-trans* mixture of hydroxylactone **4a** by one microorganism. In the case of lactones **2b**, **2c**, and **2d**, we did not observe even a trace of product.

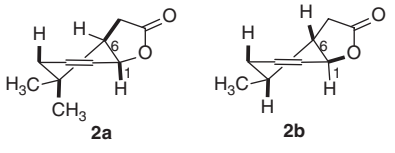


Figure 2: Structures of lactones (**2a**) and (**2b**).

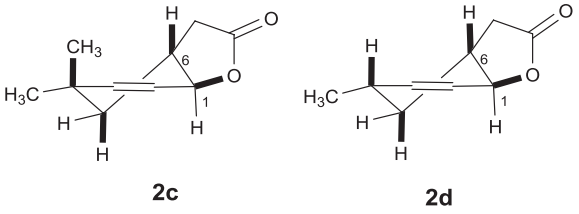


Figure 3: Structures of lactones (**2c**) and (**2d**).

As a result of biotransformation, we obtained epoxylactone **3a** in which the γ-lactone ring was retained (absorption band at 1771 cm^{-1} on IR spectra). Analysis of the NMR spectra indicated that the cyclohexene ring was in the chair conformation. The signal of proton H-1 as a doublet with coupling constant 6.3 Hz and the wide multiplet of proton H-6 suggested their axial and equatorial positions, respectively (similar to the substrate). The signal of proton H-2 as a doublet with a coupling constant of 3.5 Hz indicated its equatorial position. Two doublets from CH_2-4 with coupling constants equal to 15.8, 3.2 Hz and 15.8, 2.0 Hz implied an axial orientation of the oxirane ring (Figure 4).

The second product obtained during biotransformation of unsaturated lactone **2a** was hydroxylactone **4a**. This compound was obtained as a mixture of *cis–trans* isomers. GC analysis proved that these two isomers were formed in the ratio of 67% : 33%, which allowed a precise assignment of the signals in the NMR spectra. Analysis of the NMR spectra indicated that the deformed chair conformation of the cyclohexene ring was retained. The hydroxy group was introduced in an allylic position. The predominant position for the hydroxy group was the *cis* location (Figure 5).

Analyzing the influence of the substrate's structure on the degree of biotransformation revealed that the amount of methyl groups in the cyclohexene ring (one or two) does not matter. However, the spatial orientation of the lactone ring relative to the cyclohexene ring was very important. In cases where the C–O bond of the lactone ring was in the equatorial position (compound **2a**), two strains of filamentous fungi were capable of epoxidation of the double bond and one strain introduced the hydroxy group in allylic position. In contrast, when the C–O bond of the lactone ring was in the axial position (compounds

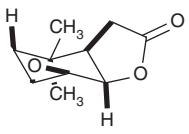


Figure 4: Structure of epoxylactone (**3a**).

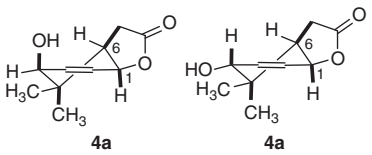


Figure 5: Structure of hydroxylactone (**4a**).

2b–**d**), the tested microorganisms were completely unable to convert the substrates.

Based on the results of biotransformation were examined whether the structure of lactones affects on their toxic properties. Synthetically unsaturated lactones **2a**–**2d** and microbiologically obtained epoxylactone **3a** were examined for their antimicrobial activity. This biological test was chosen to determine the ability of these lactones to inhibit the growth of some bacteria, yeast, and fungal strains. Statistical analysis was performed for the results of microbiological tests. Calculations were made for each of the strains separately. Control and further compounds were treated as test variability. The underlying assumption was the null hypothesis – the average values for the control and substances do not differ. The results for a strain of Fusarium were statistically insignificant because it showed no differences between the mean values that were obtained after analysis of variance. For the other strains, the hypothesis was verified by an F-Senedecora test at the significance level of $p < 0.05$. It has been shown that for each strain averages are significantly different. Then Dunnett's test was checked, in which means are significantly different from the average for the control. Results have been presented in Table 2.

Results presented in Table 2 indicate that the tested compounds affected growth of the selected bacteria strains. The strongest inhibitory effect was observed in the case of lactone **2a** against the *B. subtilis* strain. In this case, the optical density (ΔOD) increase was equal to 0.60, preceded by a 7.5-h duration of the lag-phase, while in the control culture, these values was equal to 1.13 and 1 h, respectively.

The yeast strain *C. albicans* was relatively sensitive to the effects of the tested compounds. The lowest increase in biomass, compared to a control culture, was ΔOD of 0.46 and 0.52 and were found in the presence of lactones **2a** and **2b**. It was preceded by a long-lasting lag-phase of 8.5 and 18.5 h, respectively. Despite the short adaptive phase, the growth of the *C. albicans* strain also was inhibited significantly by lactone **2d**.

The tested compounds restricted or inhibited strongly the growth of filamentous fungi. Among the cultures tested, the highest sensitivity was shown by the *P.expansum* strain, whose growth was inhibited by lactone **2a** and was significantly reduced in the presence of the other compounds, demonstrated by a decrease in optical density (ΔOD) in the range 0.55–0.68 as compared with the control culture at 1.74.

A strain of *A. niger* was also highly sensitive; its growth was completely inhibited by lactones **2a** and **2b**. Although similar in the control culture lag-phase, lactone

Table 2: The assessment of the effects of the unsaturated lactones **2a–2d** and epoxylactone **3a** on the growth of bacteria, yeast and fungi.

Strain	Control			2a		2b		2c		2d		Compound 3a	
	Lag-phase/h	ΔOD±SD	Lag-phase/h	ΔOD±SD	Lag-phase/h	ΔOD±SD	Lag-phase/h	ΔOD±SD	Lag-phase/h	ΔOD±SD	Lag-phase/h	ΔOD±SD
Bacteria												
E. coli	1	1.11±0.05	1	0.95±0.05	1	0.79±0.02	1	0.8±0.07	1	0.77±0.05	1	0.8±0.01
S. aureus	2	1.40±0.09	3.5	1.15±0.03	3	1.19±0.09	3.5	1.09±0.09	3	1.25±0.04	3	1.18±0.06
B. subtilis	1	1.13±0.06	7.5	0.60±0.11	6.5	0.7±0.08	7.5	0.83±0.03	2	0.79±0.03	3	1.09±0.01
Fungi												
C. albicans	2	1.44±0.03	8.5	0.46±0.09	18.5	0.52±0.09	3	1.0±0.11	2	0.79±0.08	4	1.01±0.11
A.alternata	7.5	0.92±0.06	10.0	1.0±0.11	8.0	0.73±0.08	8.0	0.83±0.04	8.0	0.8±0.06	9	0.51±0.11
F. linii	15	1.31±0.05	32.5	1.30±0.13	15.5	1.28±0.11	15.0	1.52±0.05	16.5	1.10±0.15	20.5	1.48±0.16
A. niger	17	1.13±0.06	–	0	–	0	17	1.02±0.09	17	0.52±0.04	17	1.04±0.05
P. expansum	21	1.74±0.12	–	0	29.5	0.55±0.03	22	0.68±0.05	21	0.59±0.04	21.5	0.67±0.06

Statistics on a completely randomized design were determined using the one-way analysis of variance (ANOVA) procedure at a level of significance set at $p < 0.050$. A comparison of average growth OD microorganisms relative to the control by Dunnett's test. Underlined number means the average significantly different from control. Notes: OD (optical density) was measured for λ 550 nm. SD, Standard deviation.

2d caused significantly reduced growth of this strain. For comparison, the ΔOD of this strain was 0.52, whereas in the control culture, this parameter was equal to 1.13. In the case of the fungi *A. alternata*, the strongest inhibitory effect was observed in the presence of epoxylactone**3a**, in which the ΔOD biomass growth was equal to 0.51 in comparison to the control culture at 0.92.

It is worth noting that lactone **2a**, although it caused the longest adaptive phase in culture of this strain, it allowed the highest level of biomass ΔOD at 1.0. The tested compounds generally did not exert inhibitory effects on the growth of the strain *F. linii*. Only in the presence of lactone **2a**, was there a prolongation of the lag-phase to 32 h and a slowdown of growth; yet, the final biomass growth was even higher than for the control culture.

4 Conclusions

Four unsaturated lactones **2a–d** were tested on their biotransformation ability by 20 fungal strains. Only one from four substrates, lactone **2a** was transformed into epoxylactone **3a** and hydroxylactone **4a**. Analysis of the NMR spectra of these unsaturated lactones proved that lactone **2a** was characterized by a different structure than lactones **2b**–**2d**. The differences in structure may explain why lactone **2a** was transformed into two products, and why lactones **2b**–**2d** were not. The biological tests proved that lactone **2a** was the best inhibitor for the growth of *Aspergillus niger* and *Penicillium expansum*. According to these tests, the presence of one or two methyl groups at carbon C-5 caused the stronger growth inhibition of bacteria and fungi strains. Introduction of the epoxide ring in the place of double bond resulted in reduction of growth inhibition of the tested strains.

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