Journal of Bioscience and Bioengineering
VOL. 119 No. 1, 72–76, 2015

www.elsevier.com/locate/jbiosc

Microbial transformations of halolactones with *p*-menthane system

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Received 17 March 2014; accepted 13 June 2014
Available online 12 July 2014

Biologically active piperitone-derived racemic iodo-, bromo- and chlorolactones (1–3) were transformed with the use of microbial enzymatic systems. Four strains of filamentous fungi *Absidia glauca* AM254, *Absidia cylindrospora* AM336, *Mortierella vinaceae* AM149 and *Nigrospora oryzae* AM8 transformed halolactones (1–3) to four new halohydroxylactones (4–7). In all biotransformations the hydroxy group was incorporated in inactivated methine carbon atom at isopropyl substituent. In *N. oryzae* AM8 culture the bromolactone with additional hydroxy group in α-position, relative to C=O bond in γ-lactone ring, was also formed as a product. The structures of new compounds were established on the basis of spectral data.

[**Key words:** Microbial hydroxylation; Halolactones; Fungal transformation; Piperytone derivatives; Terpenoid lactones]

Isoprenoid lactones are a large group of compounds isolated from plants. There exhibited antibacterial (1,2), antifungal (3), anti-inflammatory (4,5) and anticancer (6–8) properties. Our interest is focused on monoterpenoid lactones with *p*-menthane system. These compounds are well described due to their attractive odor properties (9–11). Over the last decade the deterrent activity of lactones with *p*-menthane system was also reported (12–14). The presented iodo- (**1**), bromo- (**2**) and chlorolactones (**3**) exhibited deterrent activity against *Myzus persicae* (15). Microbial transformations of halolactones serves as the model processes to investigate the possible metabolic pathways of those compounds in living organisms. It is well-known that microbial transformation of organic compounds can be crucial in metabolism investigations. Fungal strains are very good model of drug metabolism (16–18). The most commonly encountered pathways are oxidative reactions which led to formation of easily excreted polar substances. Taking into consideration the previous biotransformation experiments carried out in our research group we were expected mainly hydroxylation, eventually dehalogenation reaction products (11,19–21). The fungal transformations of bicyclic γ-lactones with alkyl substituted cyclohexane ring lead to obtain the products of regioselective or even stereoselective hydroxylations. One of the well-known fungi strain which poses the ability to oxidative metabolism is *Absidia cylindrospora*. This biocatalyst is able to convert the saturated, unsaturated and halolactones to hydroxy and epoxy derivatives (11,19,22–24).

In this paper we present the transformations of biologically active halolactones (**1**–**3**) with *p*-menthane system in filamentous fungi culture to evaluate possible metabolic pathways of those substances in living organisms.

MATERIALS AND METHODS

General procedures The progress of transformations as well as the purity of isolated products were checked by TLC (silica gel coated aluminium plates, DC-Alufolien Kieselgel 60 F_{254}, Merck) and GC analysis performed on a Hewlett Packard HP 5890 A2 instrument using Agilent DB-17 capillary column ((50%-phenyl)-methylpolysiloxane 30 m × 0.25 mm × 0.25 μm) and the hydrogen as the carrier gas (Agilent, Warszawa, Poland). The temperature programme was as follows: injector 250°C, detector (FID) 300°C, column temperature: 80°C (1 min), 80–200°C (rate 20°C min^{-1}), 200–300°C (rate 30°C min^{-1}), 300°C (2 min). The enantiomeric excesses of biotransformation products were calculated on the basis of GC analysis using CP Chirasil-Dex CB column (25 m × 0.25 mm × 0.25 μm) at the following conditions: injector 230°C, detector (FID) 250°C, column temperature: 85°C (hold 1 min), 85–200°C (rate 0.5°C min^{-1}), 200°C (hold 15 min).

Column chromatography (silica gel Kieselgel 60, 230–400 mesh, Merck) was applied to the purification of the biotransformation products.

The NMR spectra (^1H, ^{13}C NMR and correlation spectra: ^1H–^1H COSY, ^1H–^{13}C HMQC, ^1H–^{13}C HMBC) were recorded in a $CDCl_3$ solution on a Brüker Avance DRX 300 and Brüker Avance II 600 MHz spectrometers (Brüker, Karlsruhe, Germany).

IR spectra were determined using Mattson IR 300 Thermo Nicolet spectrophotometer. The melting points (uncorrected) were determined on a Boetius apparatus. Optical rotations were measured on Jasco P-2000 digital polarimeter (version with iRM controller).

Substrates for biotransformations Racemic lactones: *c*-5-iodo-*c*-4-isopropyl-*r*-1-methyl-7-oxa-*cis*-bicyclo[4.3.0]nonan-8-one (**1**), *c*-5-bromo-*c*-4-isopropyl-*r*-1-methyl-7-oxa-*cis*-bicyclo[4.3.0]nonan-8-one (**2**), *c*-5-chloro-*c*-4-isopropyl-*r*-1-methyl-7-oxa-*cis*-bicyclo[4.3.0]nonan-8-one (**3**) were synthesized from piperitone according to the procedure described earlier (15).

Here we present the spectral data of those compounds in order to compare them with the spectra of corresponding biotransformation products. They will be useful in studying the structure changes in the substrates caused by microorganisms.

c-5-Iodo-*c*-4-isopropyl-*r*-1-methyl-7-oxa-*cis*-bicyclo[4.3.0]nonan-8-one (**1**). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.28 (m, 1H, H-4), 0.86 and 0.96 (two d, J = 6.6 Hz, 6H, $(CH_3)_2CH$–), 1.24–1.37 (m, 2H, $(CH_3)_2CH$– and one of CH_2-3), 1.42 (m, 1H, one of CH_2-2), 1.46 (s, 3H, CH_3-1), 1.61–1.67 (m, 2H, one of CH_2-3 and one of CH_2-2), 2.25 and 2.43 (two d, J = 16.7 Hz, 2H, CH_2-9), 4.63 (m, 1H, H-5), 4.69 (m, 1H, H-6); ^{13}C NMR (151 MHz, $CDCl_3$) δ: 19.46 and 20.22 (CH_3)$_2$CH–), 21.74 (C-3), 25.26 (CH_3-1),

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.06.007

TABLE 1. The composition (in % according to GC) of the product mixtures of screening biotransformations of lactone **1**.

Time of incubation (days)	*N. oryzae* AM8		*M. vinaceae* AM149		*A. glauca* AM254		*A. cylindrospora* AM336	
	1	**4**	**1**	**4**	**1**	**4**	**1**	**4**
3	67	33	61	39	43	57	60	40
6	45	55	38	62	32	68	43	57
9	18	82	15	85	10	90	29	71
12	10	90	11	89	8	92	11	89
15	4	96	8	92	3	97	0	100

33.54 (CH_3)$_2$**C**H−), 34.21 (C-2), 34.24 (C-5), 38.22 (C-1), 43.19 (C-4), 46.42 (C-9), 87.88 (C-6), 174.00 (C-8); IR (KBr, cm^{-1}): 2960 (m), 1775 (s), 1373 (w), 1229 (m), 633 (m).

c-5-Bromo-*c*-4-isopropyl-*r*-1-methyl-7-oxa-*cis*-bicyclo[4.3.0]nonan-8-one (**2**). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.94 and 0.97 (two d, J = 6.6 Hz, 6H, (C**H**$_3$)$_2$CH−), 1.18 (m, 1H, H-4), 1.41 (s, 3H, CH_3-1), 1.43−1.49 (m, 2H, one of CH_2-3 and one of CH_2-2), 1.57 (m, 1H, (CH_3)$_2$**C**H−), 1.63−1.68 (m, 2H, one of CH_2-3 and one of CH_2-2), 2.28 and 2.47 (two d, J = 16.7 Hz, 2H, CH_2-9), 4.54 (d, J = 2.3 Hz, 1H, H-6), 4.60 (m, 1H, H-5); ^{13}C NMR (151 MHz, $CDCl_3$) δ: 19.55 and 20.55 (**C**H$_3$)$_2$CH−), 20.06 (C-3), 23.87 (CH_3-1), 30.69 (CH_3)$_2$**C**H−), 33.96 (C-2), 37.99 (C-1), 43.26 (C-4), 46.30 (C-9), 52.10 (C-5), 86.28 (C-6), 174.52 (C-8); IR (KBr, cm^{-1}): 2967 (m), 1780 (s), 1375 (w), 1268 (m), 653 (m).

c-5-Chloro-*c*-4-isopropyl-*r*-1-methyl-7-oxa-*cis*-bicyclo[4.3.0]nonan-8-one (**3**). ^{1}H NMR (600 MHz, $CDCl_3$)δ: 0.95 and 0.96 (two d, J = 6.8 Hz, 6H, (C**H**$_3$)$_2$CH−), 1.35 (s, 3H, CH_3-1), 1.36−1.52 (m, 3H, H-4, one of CH_2-3 and one of CH_2-2), 1.60−1.66 (m, 3H, (CH_3)$_2$**C**H−, one of CH_2-3 and one of CH_2-2), 2.27 and 2.46 (two d, J = 16.7 Hz, 2H, CH_2-9), 4.38 (d, J = 2.3 Hz, 1H, H-6), 4.54 (m, 1H, H-5); ^{13}C NMR (151 MHz, $CDCl_3$)δ: 19.14 (C-3), 19.62 and 20.68 (**C**H$_3$)$_2$CH−), 23.07 (CH_3-1), 29.19 (CH_3)$_2$**C**H−), 33.75 (C-2), 37.76 (C-1), 43.11 (C-4), 46.14 (C-9), 58.02 (C-5), 85.85 (C-6), 174.76 (C-8); IR (KBr, cm^{-1}): 2968 (m), 1785 (s) 1376 (m), 1344 (w), 1012 (s) 687 (m).

Microbial transformations Cultivation of microorganisms was carried out on Sabouraud agar slants of following composition: aminobac (5.0 g), peptone K (5.0 g), glucose (40.0 g) in distilled water (1 L). The microorganisms were cultivated at 28°C and stored in refrigerator at 4°C. Thirty strains of filamentous fungi and yeasts used in this work came from the collection of the Institute of Biology and Botany, Wrocław Medical University (*Absidia coerulea* AM93, *Absidia glauca* AM254, *A.* AM336, *Armillaria mellea* AM296, *Beauveria bassiana* AM278, *Chaetomium* sp. AM432, *Fusarium avenaceum* AM12, *Fusarium culmorum* AM10, *F. culmorum* AM196, *Fusarium scirpi* AM199A, *Fusarium solani* AM203, *Fusarium tricinctum* AM16, *Laetiporus sulphurens* AM524, *Mortierella isabellina* AM212, *Mortierella vinaceae* AM149, *Mucor circinelloides* AM385, *Nigrospora oryzae* AM8, *Penicillium camembertii* AM83, *Penicillium chermesinum* AM113, *Penicillium frequentans* AM351, *Penicillium lilacinum* AM111, *Penicillium vermiculatum* AM81, *Pholiota aurivella* AM522, *Rhodotorula marina* AM77, *Rhodotorula rubra* AM82, *Saccharomyces cerevisiae* AM464, *Spicaria fusispora* AM136, *Syncephalastrum racemosum* AM105, *Trametes versicolor* AM536) and from the collection of the Department of Phytopathology, Kraków Agricultural University (*Cenangium ferruginosum* AR56).

The strains were cultivated at 25°C in 300 mL Erlenmeyer flasks containing 50 mL of medium (30.0 g glucose, 5.0 g peptone, 5.0 g aminobac in distilled water (1 L)). After 4 days of growth 10 mg of iodolactone (**1**) in 1 mL of acetone were added to the shaken cultures (150 rmp). The incubation was carried on for 15 days. After 3, 6, 9, 12 and 15 days of incubation, the products were extracted with methylene chloride. Additionally, for all microorganisms the metabolite standard was prepared. The strains were cultivated in the same condition without substrate and extracted simultaneously with biotransformations samples. Those two types of extracts were analyzed by TLC and GC. The transformation products were identified by comparison of biotransformation and metabolites samples. Four strains of fungi (*A. glauca* AM254, *A. cylindrospora* AM336, *M. vinaceae* AM149, *N. oryzae* AM8) were able to transform iodolactone (**1**). For selected strains the screening experiments were carried out also with bromo- (**2**) and chlorolactones (**3**) as substrates.

The screening experiments allowed us to choose four strains of fungi capable to transform iodolactone (**1**): *A. glauca* AM254, *A. cylindrospora* AM336, *M. vinaceae* AM149, *N. oryzae* AM8. The strains which were chosen in screening experiment cultivated in 12 Erlenmeyer flasks and 10 mg of substrate dissolved in 1 mL of acetone was added to the shaken culture in each flask (condition the same as described in screening procedure). After the optimum time for each biotransformation process (Tables 1–3) the products were extracted three times with methylene chloride (50 mL for each flask). The organic layers were pooled, dried over anhydrous $MgSO_4$ and the solvent was evaporated *in vacuo*. The products of biotransformations were separated and purified by column chromatography. Obtained compounds were analyzed by GC with chiral column to establish the enantiomeric excess and by NMR and IR spectroscopy to establish the structure.

For biotransformations of **1**, **2** and **3** in *N. oryzae* AM8 culture the special conditions have to be applied. After 4 days of growth the biomass was transferred to 12 Erlenmeyer flasks (300 mL) containing 50 mL of phosphate buffer (pH = 7.2). Then 10 mg of substrate dissolved in 1 mL of acetone were added to each flask. Finally the products were extracted three times with methylene chloride. The procedure of purification was the same as described earlier. The physical and spectral data of obtained products are given below.

c-5-Iodo-*c*-4-(1'-hydroxy-1'-methyl)ethyl-*r*-1-methyl-7-oxa-*cis*-bicyclo[4.3.0] nonan-8-one (**4**). Colorless crystals (m.p. = 77−81°C); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.22 and 1.33 (two s, 6H, (C**H**$_3$)$_2$C−OH), 1.28 (m, 1H, H-4), 1.52 (s, 3H, CH_3-1), 1.53−1.80 (m, 4H, CH_2-2 and CH_2-3); 2.31 and 2.48 (two d, J = 16.8 Hz, 2H, CH_2-9), 4.63 (m, 1H, H-5), 4.67 (m, 1H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 19.71 (C-3), 25.41 (CH_3-1), 26.94 and 29.29 ((**C**H$_3$)$_2$C−OH), 27.68 (C-5), 34.82 (C-2), 38.58 (C-1), 44.77 (C-4), 46.43 (C-9), 71.59 ((CH_3)$_2$**C**−OH), 88.71 (C-6), 173.77 (C-8); IR (KBr, cm^{-1}): 3466 (s), 2974 (s), 1763 (s), 1218 (s).

c-5-Bromo-*c*-4-(1'-hydroxy-1'-methyl)ethyl-*r*-1-methyl-7-oxa-*cis*-bicyclo [4.3.0]nonan-8-one (**5**). Colorless crystals (m.p. = 63−66°C); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.25 and 1.35 (2 s, 6H, (C**H**$_3$)$_2$C−OH), 1.43 (s, 3H, CH_3-1), 1.52 (m, 1H, one of CH_2-2), 1.67-1.82 (m, 4H, one of CH_2-2, H-4 and CH_2-3), 2.30 and 2.49 (two d, J = 16.8 Hz, 2H, CH_2-9), 4.49 (d, J = 2.4 Hz, 1H, H-6), 4.67 (m, 1H, H-5); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 17.64 (C-3), 23.87 (CH_3-1), 27.43 and 28.59 ((**C**H$_3$)$_2$C−OH), 34.35 (C-2), 38.21 (C-1), 45.20 (C-4), 46.20 (C-9), 49.46 (C-5), 71.82 ((CH_3)$_2$**C**−OH), 86.73 (C-6) 174.21 (C-8); IR (KBr, cm^{-1}): 3481 (s), 2972 (s), 1780 (s), 1214 (s), 736 (m).

c-5-Bromo-*c*-4-(1'-hydroxy-1'-methyl)ethyl-9-hydroxy-*r*-1-methyl-7-oxa-*cis*-bicyclo[4.3.0]nonan-8-one (**6**). Oily liquid; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.25 and 1.35 (two s, 6H, (C**H**$_3$)$_2$C−OH), 1.30 (m, 1H, one of CH_2-2), 1.42 (s, 3H, CH_3-1), 1.57−1.85 (m, 4H, one of CH_2-2, CH_2-3 and H-4), 3.71 (s, 1H, H-9), 4.65 (m, 1H, H-5), 4.81 (d, J = 2.5 Hz, 1H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 17.35 (C-3), 18.16 (CH_3-1), 27.39 and 28.57 ((**C**H$_3$)$_2$C−OH), 31.52 (C-2), 36.35 (C-1), 44.82 (C-4), 48.66 (C-5), 77.92 (C-9), 85.51 (C-6), 174.53 (C-8); IR (KBr, cm^{-1}): 3414 (s), 2973 (S), 1767 (s) 1214 (m), 737 (w).

c-5-Chloro-*c*-4-(1'-hydroxy-1'-methyl)ethyl-*r*-1-methyl-7-oxa-*cis*-bicyclo [4.3.0]nonan-8-one (**7**). Colorless crystals (m.p. = 48−52°C); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.27 and 1.34 (two s, 6H, (C**H**$_3$)$_2$C−OH), 1.38 (s, 3H, CH_3-1), 1.50 (m, 1H, one of CH_2-2), 1.63−1.88 (m, 4H, H-4, CH_2-3 and one of CH_2-2), 2.30 and 2.46 (two d, J = 16.8 Hz, 2H, CH_2-9), 4.33 (d, J = 2.5 Hz, 1H, H-6), 4.67 (m, 1H, H-5); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 16.63 (C-3), 23.16 (CH_3-1), 27.84 and 28.37 ((**C**H$_3$)$_2$C−OH), 34.13 (C-2), 38.05 (C-1), 45.35 (C-4), 46.12 (C-9), 57.03 (C-5), 71.90 ((CH_3)$_2$**C**−OH), 86.26 (C-6), 174.54 (C-8); IR (KBr, cm^{-1}): 3462 (s), 2930 (m), 1788 (s), 1189 (m), 757 (m).

TABLE 2. The composition (in % according to GC) of the product mixtures of screening biotransformations of lactone **2**.

Time of incubation (days)	*N. oryzae* AM8			Time of incubation (days)	*M. vinaceae* AM149		*A. glauca* AM254		*A. cylindrospora* AM336	
	2	**5**	**6**		**2**	**5**	**2**	**5**	**2**	**5**
1[a]	77	23	0	1	46	54	81	19	89	11
6[a]	41	42	18	2	22	78	65	35	75	25
1	7	75	18	3	4	96	44	56	66	34
2	3	80	17	6	0	100	17	83	52	48
3	2	83	15	9			14	86	23	77
				12			7	93	9	91
				15			0	100	0	100

[a] Time of incubation in h.