Bioorganic & Medicinal Chemistry 19 (2011) 2464–2469

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Microbial transformation of (+)-nootkatone and the antiproliferative activity of its metabolites

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ARTICLE INFO

Article history:
Received 6 October 2010
Revised 28 October 2010
Accepted 28 January 2011
Available online 4 March 2011

Keywords:
Biotransformation
Sesquiterpenes
(+)-Nootkatone
Botrytis cinerea
Didymosphaeria igniaria
Aspergillus ochraceus
Aspergillus niger MB
Chaetomium sp.
Fusarium culmorum
Cancer cell lines: A549 (human lung adenocarcinoma) and HL-60 (human promyelocytic leukemia)
Antiproliferative activity

ABSTRACT

Six metabolites were obtained as a result of microbial transformation of (+)-nootkatone **(1)** by the fungal strains: *Botrytis, Didymosphaeria, Aspergillus, Chaetomium* and *Fusarium*. Their structure were established as (+)-(4*R*,5*S*,7*R*,9*R*)-9α-hydroxynootkatone **(2)**, (+)-(4*R*,5*S*,7*R*)-13-hydroxynootkatone **(3)** and (+)-(4*R*,5*S*,7*R*,9*R*,11*S*)-11,12-epoxy-9α-hydroxynootkatone **(4)**, (+)-(4*R*,5*S*,7*R*,11*S*)-11,12-epoksynootkatone **(5)**, (+)-(4*R*,5*S*,7*R*)-11,12-dihydroxynootkatone **(6)** and (+)-(4*R*,5*S*,7*R*)-7,11,12-trihydroxynootkatone **(7)** on the basis of their spectral data. Two products: **(4)** and **(7)** were not previously reported in the literature. The antiproliferative activity of (+)-nootkatone **(1)** and isolated metabolites **(2–7)** of its biotransformation has been evaluated.

1. Introduction

Bicyclic sesquiterpene (+)-nootkatone is a natural compound which was first isolated from the heartwood of Alaska yellow cedar (*Chamaecyparis nootkatensis*) and was found in trace amount in grapefruit (*Citrus paradise*).[1,2] This sesquiterpene ketone possesses pleasant grapefruit flavor and occurs in orange and grapefruit juice and in peel oils obtained from orange, grapefruit, lemon, mandarin and lime.[3] The literature presents wide range of biological activity of nootkatone and describes potential fields of its application. Nootkatone inhibits the gastric ulcer formation in rats as well as the activity of acetylcholinesterases and of human cytochrome P450 monooxygenases CYP450.[4–7] It is known as the potential insecticide against larvae of *Drosophila melanogaster* and a strong repellent to termites (*Coptotermes formosanus*).[8,9] As the carries of specific odor with interesting activity, (+)-nootkatone **(1)**, is industrially valuable product in the fragrance, food, cosmetics and pharmaceutical applications.

The objective of our research project is the synthesis of new active derivatives of natural compounds with enhanced biological activity. Recently we have published the synthesis of oxiderivatives of farnesol by fungal strains.[10] Here we report results of functionalization of (+)-nootkatone **(1)** with the fungal cultures: *Botrytis cinerea*, *Didymosphaeria igniaria*, *Aspergillus ochraceus*, *Aspergillus niger* MB, *Chaetomium* sp. and *Fusarium culmorum.*

Nootkatone and products of its microbial transformations were subjected to the biological tests. The antiproliferative activity towards the cancer cell lines: A549 (human lung adenocarcinoma) and HL-60 (human promyelocytic leukemia) was investigated.

2. Results and discussion

Biotransformation of (+)-nootkatone **(1)** was carried out by fungal cultures: *B. cinerea* AM235 and *D. igniaria* KCh6670, *A. ochraceus* AM456, *A. niger* MB, *Chaetomium* sp. KCh6651 and *F. culmorum* AM10 which were selected in the screening procedure of 20 fungal strains.

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doi:10.1016/j.bmc.2011.01.062

In the culture of *B. cinerea* AM235 two products: (+)-(4*R*,5*S*,7*R*,9*R*)-9α-hydroxynootkatone **(2)** and (+)-(4*R*,5*S*,7*R*)-13-hydroxynootkatone **(3)** were formed (Fig. 1). After eight days of incubation they were isolated in 32% (61 mg) and 28% (54 mg) yields, respectively. The reaction course was monitored by means of TLC and GC. The GC analysis afforded the information about changes in quantitative ratio of products in the course of the process (Table 1). In the first 24 h only substrate was observed in the reaction mixture. In the second day (+)-(4*R*,5*S*,7*R*,9*R*)-9α-hydroxynootkatone **(2)** was detected and since then its content increased proportionally. The second product (+)-(4*R*,5*S*,7*R*)-13-hydroxynootkatone **(3)** was formed in fourth day of biotransformation, independently from product **(2)**. Both products are the result of hydroxylation at allylic positions C-9 **(2)** and C-13 **(3)** in the nootkatone **(1)**.

The product **2** was obtained as a single isomer. Its structure was established on the basis of spectral data. The complete ^{1}H and ^{13}C NMR analyzes with COSY and HMQC let to confirm the structure of (+)-(4*R*,5*S*,7*R*,9*R*)-9α-hydroxynootkatone **(2)**. In the ^{1}H NMR spectrum of product **(2)** triplet at $\delta = 4.45$ ppm ($J = 2.9$ Hz) of the proton H-9 was observed. Hydroxylation at C-9 was confirmed by signal of carbon atom C-9 in the ^{13}C NMR spectrum shifted downfield to 73.3 ppm as well as by the absorption band at 3405 cm^{-1} in IR spectrum. The relative stereochemistry at C-9 was confirmed by the analyzes of the coupling constants of related proton signals and by comparison with the literature spectral data of this compound.[11] The coupling constants of 2.9 Hz between H-9 ($\delta = 4.45$) and both of H-8 indicated that H-9 is equatorial orientation. The orientation of hydroxy group at C-9 is then axial and it is *trans* situated to the propenyl group at C-7.

(+)-(4*R*,5*S*,7*R*,9*R*)-9α-Hydroxynootkatone **(2)** was obtained previously in very small yield (8%) as the product of biotransformation of (+)-nootkatone **(1)** with cultured plant cells of *Marchantia polymorpha*.[11] 9α-Hydroxynootkatone **(2)** was also isolated from whole-cell biotransformation reaction of (+)-valencene carried out with P450 mutants.[12] 9β-Isomer of product **(2)**, was obtained from the microbial conversion of (+)-nootkatone **(1)** by *F. culmorum* (14.9%).[13]

Hydroxylation at C-13 in the isopropenyl group of (+)-(4*R*,5*S*,7*R*)-13-hydroxynootkatone **(3)** was confirmed by both ^{1}H NMR and ^{13}C NMR data. In the ^{1}H NMR spectrum of this product two multiplets at $\delta = 4.90$ ppm and $\delta = 5.06$ ppm from protons of double bond C11–C12 and characteristic singlet from two protons of isolated methylene group was observed. The chemical shift of this singlet ($\delta = 4.14$ ppm) confirmed the introduction the hydroxy group at the position C-13. The presence of hydroxy group in product **3** is confirmed by the signal of carbon atom C-13 at 65.2 ppm in the ^{13}C NMR spectrum (Table 3). In the IR spectrum absorption bands at 3399 cm^{-1} and 1047 cm^{-1} characteristic for stretching vibrations O–H and C–O in alcohols, respectively are visible.

(+)-Nootkatone **(1)** transformed in the culture of *Chaetomium* sp. KCh6651 gave after two days (+)-(4*R*,5*S*,7*R*,11*S*)-11,12-epoxynootkatone **(5)**, which was isolated in 44% yield (51 mg) as the mixture of epimers with 50% diastereoisomeric excess (de) with predominance of isomer with configuration 11*S*. The configuration at C-11 was determined by comparison of spectral (^{1}H NMR) data of product with the literature ^{1}H NMR data.[12] The biotransformation of **1** in the culture *Chaetomium* sp. KCh6651 proceeded very fast. After 24 h of incubation the conversion of substrate was on the level 43% and after 48 h nootkatone **(1)** was completely transformed to the product **5**. The structure of **5** was confirmed by spectroscopic data. In the ^{1}H NMR spectrum of **5** two doublets ($J = 4.7$ Hz) at $\delta = 2.59$ and $\delta = 2.66$ ppm of protons CH_2-12. The signals at $\delta = 53.7$ and $\delta = 58.9$ ppm in the ^{13}C NMR spectrum indicate the presence of oxirane ring. The characteristic absorption bands at 1288 cm^{-1} and 840 cm^{-1} in the IR spectrum also confirm the epoxidation of nootkatone.

In the culture of *D. igniaria* KCh6670 the process of biotransformation of (+)-nootkatone **(1)** was enantioselective and led to the

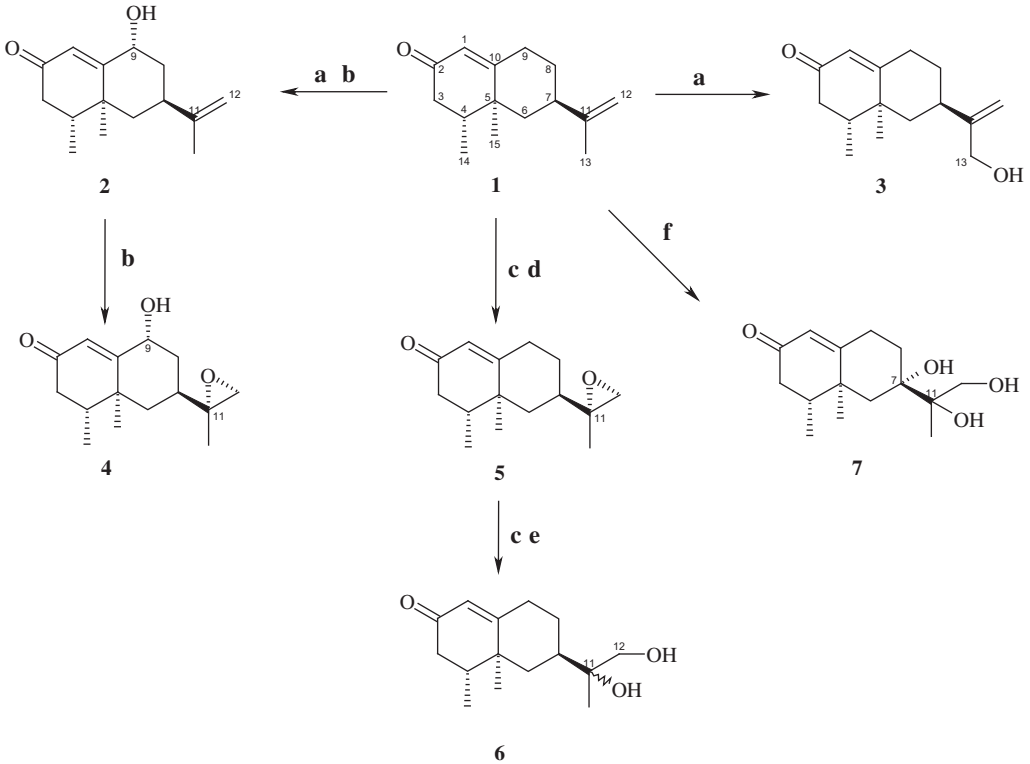


Figure 1. Metabolic pathway of (+)-nootkatone (**1**) in the culture: *B. cinerea* AM235 (a), *D. igniaria* KCh6670 (b), *A. ochraceus* AM456 (c), *Chaetomium* sp. KCh6651 (d), *F. culmorum* AM10 (e), *A. niger* MB (f).

Table 1
The compositions (according to GC) of products mixture in the biotransformations of (+)-nootkatone by fungal strains

Microorganism	Time of incubations (days)	Biotransformation products (%)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
B. cinerea AM235	1	100	–	–	–	–	–	–
	2	89	11	–	–	–	–	–
	4	63	30	7	–	–	–	–
	6	48	36	16	–	–	–	–
	8	0	60	40	–	–	–	–
D. igniaria KCh6670	0.5	74	26	–	–	–	–	–
	1	3	76	–	21	–	–	–
	2	0	68	–	32	–	–	–
A. ochraceus AM456	1	53	–	–	–	41	6	–
	2	15	–	–	–	33	52	–
	3	0	–	–	–	36	64	–
F. culmorum AM10	2	100	–	–	–	–	0	–
	4	83	–	–	–	–	17	–
	6	71	–	–	–	–	29	–
	8	18	–	–	–	–	82	–
	10	0	–	–	–	–	100	–
Chaetomium sp. KCh6651	1	57	–	–	–	43	–	–
	2	0	–	–	–	100	–	–
	3	0	–	–	–	100	–	–
A. niger MB	2	72	–	–	–	–	–	18
	3	63	–	–	–	–	–	37
	4	4	–	–	–	–	–	96

mixture of two products **2** and **4** (Fig. 1). The biotransformation of (+)-nootkatone **(1)** with *D. igniaria* KCh6670 proceeded very fast. The progress of the reaction monitored by TLC and GC showed that after 12 h 26% of (+)-9α-hydroxynootkatone **(2)** in the reaction mixture was observed. After one day of incubation the mixture of products contained only 3% of unreacted substrate, 76% of (+)-9α-hydroxynootkatone **(2)** and 21% of (+)-(*4R,5S,7R,9R,11S*)-11,12-epoxy-9α-hydroxy-nootkatone **(4)**. In the second day of the biotransformation the amount of **2** gradually decreased, whereas the amount of **4** increased proportionally. This observation led to the conclusion that product **4** is formed in two step of biotransformation: first the hydroxylation at C-9 takes place and then double bond C11–C12 is epoxydized. Products **2** and **4** were isolated in high yields: 70% (82 mg) and 19% (24 mg), respectively.

The structure of (+)-(*4R,5S,7R,9R,11S*)-11,12-epoxy-9α-hydroxynootkatone **(4)** was confirmed by spectroscopic data. In the ^{1}H NMR spectrum of product **(4)** the characteristic triplet at δ = 4.45 ppm (J = 2.9 Hz) of the proton H-9 could be detected. The chemical shift of this signal confirms the presence of the hydroxy group at the C-9. The presence of oxirane ring in the structure of product **4** was confirmed by presence two doublets (J = 4.7 Hz) at δ = 2.58 and δ = 2.70 ppm of protons CH_2-12. The signals from C-11 and C-12 in the ^{13}C NMR spectrum were shifted from 149.0 and 109.2 ppm in nootkatone to 59.0 and 53.0 ppm, respectively in compound **(4)**.

The relative stereochemistry at C-9 was the same like in the product **(2)**. The orientation of hydroxy group at C-9 as axial and *trans* for the propenyl group at C-7 was compared with the literature spectral data of this compound.[11] The configuration at C-11 was also determined by comparison of its spectral ^{1}H NMR data with the literature ^{1}H NMR data.[12]

(+)-(*4R,5S,7R,9R,11S*)-11,12-Epoxy-9α-hydroxynootkatone **(4)** was not reported previously. In the literature epoxynootkatone with oxirane ring at C11–C12 is known as well as the product of hydroxylation reaction of nootkatone at C-9 but it is the first report about the formation of **(4)** as the results both of these reactions.[11–13]

In the reaction catalyzed by the strain *A. ochraceus* AM456 two products were formed: (+)-(*4R,5S,7R,11S*)-11,12-epoxynootkatone **(5)** with 78% de and the 11,12-dihydroxynootkatone **(6)**. The latter was obtained as the mixture of epimers (54:46) at C-11. The process of biotransformation was carried out three days to the final conversion of substrate **1**. The products **5** and **6** were isolated in the 20% (26 mg) and 12% (17 mg) yields, respectively. In the ^{1}H NMR spectrum of **6** the replacement of two doublets of the protons of double bond in **1** by the new signals at δ = 3.44 and δ = 3.59 ppm was observed. There are two doublets (J = 10.9 Hz) from the diastereotopic protons of the methylene group at C-12 and they indicate the presence of hydroxy groups at the C-11 and C-12. The shift of the singlet of methyl group at C-11 from δ = 1.73 in **1** to δ = 1.07 ppm in **6** also confirms the transformation of double bond into 11,12-dihydroxymoiety. Two signals at δ = 68.5 ppm and 74.4 ppm (^{13}C NMR of **6**) instead of the signals from the vinyl carbons in the ^{13}C NMR spectrum of **1** also confirm this transformations. The characteristic strong absorption band for stretching vibrations of O–H in the IR spectrum at 3412 cm^{-1} also indicates the hydroxy groups in the product **6**.

The product **6** was formed also in the biotransformation of nootkatone **(1)** in the culture *F. culmorum* AM10. After 10 days incubation of nootkatone **(1)** the (+)-(*4R,5S,7R*)-11,12-dihydroxynootkatone **(6)** was isolated in 71% yield (142 mg) as the mixture of epimers at C-11 (55:45). The process of transformation was very effective. The epoxynootkatone, which is certain the intermediate compound, was not observed in the reaction mixture.

Both products (+)-(*4R,5S,7R,11S*)-11,12-epoxynootkatone **(5)** and (+)-(*4R,5S,7R*)-11,12-dihydroxynootkatone **(6)** are known in the literature. The first one, was obtained in the biotransformation of **1** with fungal strains: *A. niger, Botryosphaeria dothidea* and *F. culmorum*.[13] Epoxy derivative **(5)** of nootkatone to that time was obtained as a racemic mixture in the chemical oxidation of nootkatone with *m*-CPBA and in the process of transformation of valencen using genetically modified cytochrome $P450_{BM-3}$ of bacteria *Bacillus megaterium* as the catalyst.[12]

A. niger MB oxidized nootkatone **(1)** at the double bond as well as at the C-7 to form the triol **7**. The product **7** was identified as (+)-(+)-(*4R,5S,7R*)-7,11,12-trihydroxynootkatone. The time course for the biotransformation of (+)-nootkatone **(1)** by *A. niger* MB are shown in Table 1. The formation of **7** reached the maximum (96%) after four days and then was isolated in 33% yield (74 mg) with 20% de. The transformation of **1** to triol **7** seems to be closely related to the formation of **6**. However the intermediate products **5** and **6** which was not detected in the reaction mixture. The structure of **7** was determined by spectroscopic (^{1}H and ^{13}C

Table 2
^{1}H NMR chemical shifts (δ) of compounds **1–7** (solvent $CDCl_3$)

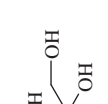
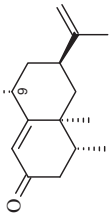
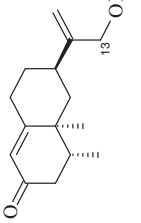
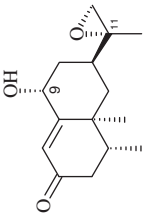
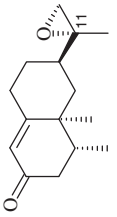
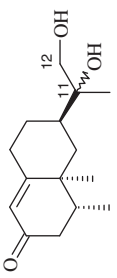
Proton	**1** 	**2** 	**3** 	**4** 	**5** 	**6** 	**7**
H-1	5.77, m	5.86, s	5.77, m	5.87, s	5.76, m	5.73, m	5.73, s
H-3	1.24–1.42, m	2.31, m	1.83–2.58, m	2.28, dd, J = 17.4 and 4.0 Hz	2.24–2.52, m	1.82–2.53, m	1.71–2.51, m
H-4	2.23–2.57, m	1.93–2.11, m	1.83–2.58, m	1.92–2.25, m	2.24–2.52, m	1.82–2.53, m	1.71–2.51, m
H-6	1.24 and 1.88, 2m	1.47–1.57, m	1.83–2.58, m	1.92–2.25, m	1.55–1.70, m	1.82–2.53, m	1.36–1.39, m
H-7	2.23–2.57, m	2.78, tt, J = 12.6 and 2.7 Hz	1.83–2.58, m	1.92–2.25, m	2.24–2.52, m	1.82–2.53, m	1.71–2.51, m
H-8	1.24 and 1.88, 2m	1.93–2.11, m	1.83–2.58, m	1.47 and 1.92, 2m	1.84–2.08, m	1.82–2.53, m	1.36–1.39, m
H-9	2.23–2.57, m	4.45, t, J = 2.9 Hz	1.83–2.58, m	4.47, t, J = 2.9 Hz	1.84–2.08, m	1.82–2.53, m	1.71–2.51, m
H-12	4.72–4.74, m	4.77, 2m	4.90–5.06, 2m	2.58, d, J = 4.7 Hz	2.59, d, J = 4.7 Hz	3.44, d, J = 10.9 Hz	3.66, d, J = 8.5 Hz
				2.70, d, J = 4.7 Hz	2.65, d, J = 4.7 Hz	3.59, d, J = 10.9 Hz	3.89, d, J = 8.5 Hz
H-13	1.73, s	1.75, s	4.14, s	1.28, s	1.25, s	1.07, s	1.20, s
H-14	0.96, d, J = 6.8 Hz	0.94, d, J = 6.8 Hz	0.95, d, J = 6.8 Hz	0.96, d, J = 6.8 Hz	0.97, d, J = 6.8 Hz	0.96, d, J = 6.7 Hz	0.97, d, J = 6.8 Hz
H-15	1.11, s	1.31, s	1.11, s	1.29, s	1.07, s	1.06, s	1.07, s

Table 3
^{13}C NMR chemical shifts (δ) of compounds **1–7**

Carbon	Compounds						
	1	**2**	**3**	**4**	**5**	**6**	**7**
C-1	124.63	127.12	124.7	127.43	124.94	124.50	124.65
C-2	199.63	200.62	199.68	200.18	199.47	199.97	199.47
C-3	43.89	43.62	44.34	42.36	42.09	42.08	42.04
C-4	40.28	41.14	40.35	41.28	39.57	40.61	40.77
C-5	39.29	38.76	39.39	38.53	39.04	39.67	39.11
C-6	42.03	42.27	41.97	40.35	40.41	38.69	39.29
C-7	40.42	33.94	35.94	33.00	40.56	39.25	82.85
C-8	31.58	37.88	33.08	34.97	28.65	27.39	26.96
C-9	32.99	73.27	32.04	73.00	32.50	32.99	32.77
C-10	170.53	168.40	170.37	167.49	168.83	171.07	170.31
C-11	149.03	148.86	152.51	58.96	58.94	74.30	72.72
C-12	109.20	109.33	109.03	53.05	53.49	68.36	73.00
C-13	20.77	20.91	65.17	18.65	17.82	20.12	21.65
C-14	14.86	14.47	14.85	14.59	14.94	15.03	14.92
C-15	16.81	18.06	16.74	18.09	16.80	16.86	16.79

NMR) data. In the ^{1}H NMR spectra of **7** two doublets (J = 8.5 Hz) at δ = 3.67 and 3.88 ppm of protons at C-12 were observed. The signals for C-11 and C-12 in the spectrum of ^{13}C NMR of **7** were found at δ = 72.7 ppm and 73.0 ppm. In this spectrum the signal from C-7 at δ = 82.85 ppm was also observed. In the literature microbial hydroxylation at the C-7 position of nootkatone **(1)** was described and diol of nootkatone **(6)** is also known.[13] However the formation of metabolite **7** have not been previously reported.

The results of biotransformation of (+)-nootkatone **(1)** presented here indicate that two metabolic pathways in the cultures of fungal strains studied could be considered (Fig. 1). The major pathway is the epoxidation of double bond between C11–C12 in the culture of *A. ochraceus* AM456 and *Chaetomium* sp. 6651 and possibly in *A. niger* MB and *F. coulmorum* AM10. In the culture of strains of *B. cinerea* AM235 and *D. igniaria* KCha6670 hydroxylation of allylic positions at C-13 and C-9, respectively took place. *D. igniaria* KCha6670 additionally oxidized the double bond of isopropenyl group in 9-hydroxynootkatone **(2)**.

In the next step of our studies the proliferation of the cancer cells lines A549 (human lung adenocarcinoma) and HL-60 (human promyelocytic leukemia) exposed either to (+)-nootkatone **(1)** and its derivatives **(2–7)** was investigated. The compounds **(1–7)** were tested in SRB or MTT (for cell lines A549 and HL-60, respectively) assay. The results of cytotoxic activity in vitro were expressed as an ID_{50} (μg/ml) that is the concentration of compound which inhibits the proliferation of 50% of tumor cells. The results of the cytotoxicity studied are summarized in Table 4.

In the series of compounds **1–7** tested against A549 cells the most potent activity showed 13-hydroxynootkatone **(3)** with the ID_{50} value (36 μg/ml) lower than that of nootkatone **(1)**. The presence of hydroxy group at C-13 led to increase of activity towards tested cancer cell line. The other compounds studied in this series did not showed the interesting activity.

The antiproliferative activity of compounds studied against HL-60 leukemia cells was higher than this observed for A549 cells. The proliferation of HL-60 cells was strongly inhibited by substrate **1** and the metabolites **2**, **3**, **5** and **7** with ID_{50} values 4.27–27.6 μg/ml (Table 4) while epoxyalcohol **(4)** and diol **(6)** did not show any significant activity against leukemia cells. These results may suggest that compounds **1–3**, **5** and **7** can be considered as good candidates for further studies.

Considering the overall activities of **1–7** it can be postulated that the presence of an additional hydroxy groups in the cyclohexane ring increases the activity. However it looks that the isopropenyl group is the most important moiety in the structure

Table 4
The antiproliferative activity in vitro against the human cancer cell lines

Cell line	Compounds ID_{50} (μg/ml)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
A549	58.4 ± 6.6	62.7 ± 20	36 ± 3	288.6 ± 34.4	167 ± 9	325 ± 210	290 ± 19.6
HL-60	4.27 ± 1.61	25.7 ± 8.9	4.4 ± 2.3	345 ± 108	27.6 ± 11.3	284 ± 143	25.8 ± 10.2

of studied compounds which showed antiproliferative activity. On the other hand, the leukemia cells (HL-60) appear to be more sensitive to the cytotoxic effects of compounds studied **(1–7)**.

3. Experimental

3.1. Analysis

The course of microbial transformations as well as the purity of isolated products were checked by TLC technique (SiO_2, DC-Alufolien Kieselgel 60 F_{254}, Merck). Chromatograms were developed using the following developing systems: hexane/acetone 2:1. Visualization was made using a solution of 10 g $Ce(SO_4)_2$ and 20 g phosphoromolybdenic acid in 1 dm^3 H_2SO_4, followed by heating. Column chromatography (SiO_2, Kieselgel 60, 230–400 mesh, 40–63 μm, Merck) was performed using the same eluents (hexane/acetone, 2:1) as in TLC technique. Gas chromatography (GC) analysis was carried out on Agilent Technologies 6890N (Network GC System) instrument with DB-17 column (cross-linked methyl silicone, 30 m × 0.25 mm × 0.25 μm). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance DRX 300 spectrometer. IR spectra were determined on a Mattson IR 300 Thermo Nicolet spectrometer. Optical rotation were measured on an Autopol IV automatic polarimeter (Rudolph). Elemental analysis were performed on the Vario EL III CHNS (Elementor).

3.2. Materials

The substrate used for the biotransformation experiments, (+)-nootkatone **(1),** was purchased from Fluka.

3.2.1. (+)-Nootkatone (1)
Melting point 32 °C, boiling point 125 °C at 0.5 mm Hg, ^{1}H NMR see Table 1, ^{13}C NMR see Table 2.

3.2.2. Microorganisms
The fungal strains: *B. cinerea* AM235 and *D. igniaria* KCh6670 *A. ochraceus* AM456, *A. niger* MB, *Chaetomium* sp. KCh6651 and *F. culmorum* AM10 were cultivated on a Sabouraud agar consisting of: aminobac 5 g, peptone K 5 g, glucose 40 g and agar 15 g in distilled water 1 l at 28 °C and pH 5.5 and stored in refrigerator at 4 °C.

3.3. Biotransformation

3.3.1. Screening procedure
The fungal strains were transferred from the slants to 300-ml Erlenmeyer flask containing 100 ml of medium (3% glucose, 1% aminobac in water). Pre-incubation was performed at 25 °C for 48 h. After this time the portions of 1 ml of the culture solution were transferred to inoculate 300-ml flasks, each containing 100 ml of the medium. After cultivation at 25 °C for four days on a rotary shaker, 10 mg of substrate, dissolved in 0.5 ml of acetone was added to the grown cultures. In control experiments, the substrates were incubated in the medium without fungi. For the time-course analysis after 0.5, 1, 2, 4, 6, 8 and 10 days 10 ml of the transformation mixture were taken out and extracted with dichloromethane. The extracts were dried over $MgSO_4$ and concentrated in vacuo. Residues were dissolved in 1 ml of acetone and analyzed by TLC and GC.

Screening procedure was carried out for 20 fungal strains which were from the Institute of Biology and Botany, Medical Academy of Wrocław: *Rhodotorula rubra* AM4, *Nigrospora oryzae* AM8, *F. culmorum* AM10, *Fusarium avenaceum* AM12, *Fusarium oxysporum* AM13, *Fusarium equiseti* AM15, *Pezicula cinnamomea* AR53, *Rhodotorula marina* AM77, *Penicillium chermesinum* AM113, *B. cinerea* AM235, *Beauveria bassiana* AM278, *Acremonium roseum* AM346, *A. ochraceus* AM456, *Pleurotus ostreatus* AM482, *Laetiporus sulphurens* AM525, *Trametes versicolor* AM536, and *A. niger* MB, *Penicillium digitatum* KCh904, *Chaetomium* sp. KCh6651, *D. igniaria* KCh6670 were from Department of Chemistry at Wroclaw University of Environmental and Life Sciences (Poland). The screening of fungal strains led to the selection of six microorganisms that had ability to biotransformation of (+)-nootkatone **(1)**.

3.3.2. Preparative biotransformation
In order to isolate and identify the products, preparative-scale biotransformation were performed. Portion of 1 ml of the pre-incubation culture solution were used to inoculate 11–18 flasks (300-ml Erlenmeyer) containing 100 ml of medium in each flask. The cultures were incubated at 25 °C for four days on rotary shaker and then the substrate (10 mg (+)-nootkatone on the 100 ml of medium) dissolved in acetone was added to each flask. After one and seven days of incubation the mixtures were extracted three times with dichloromethane, dried ($MgSO_4$) and concentrated in vacuo. The crude product mixtures were separated by column chromatography (silica gel, hexane/acetone 2:1). Pure biotransformation products were identified by means of spectral analyzes (TLC, ^{1}H NMR and ^{13}C NMR) and optical rotation measurements.

3.3.3. Products of biotransformations
3.3.3.1. (+)-(4*R*,5*S*,7*R*,9*R*)-9α-Hydroxynootkatone (2). ^{1}H NMR (see Table 2), ^{13}C NMR (see Table 3), IR (film, cm^{-1}): 3405(s), 2966(s), 1663(s),1443(m), 1375(s), 1296(s), 1049(m), 888(s); $[\alpha]_D^{20}$ +42.4 (*c* 1.22, CH_2Cl_2).

3.3.3.2. (+)-(4*R*,5*S*,7*R*)-13-Hydroxynootkatone (3). ^{1}H NMR (see Table 2), ^{13}C NMR (see Table 3), IR (film, cm^{-1}): 3340(s), 2935(s), 1656(s),1454(m), 1357(s), 1298(s), 1047(s), 902(w); $[\alpha]_D^{20}$ +114.2 (*c* 0.93, CH_2Cl_2).

3.3.3.3. (+)-(4*R*,5*S*,7*R*,9*R*,11*S*)-11,12-Epoxy-9α-hydroxynootkatone (4). ^{1}H NMR (see Table 2), ^{13}C NMR (see Table 3), IR (film, cm^{-1}): 3416(m), 2934(m), 1664(s), 1289(m); $[\alpha]_D^{20}$ +48.6 (*c* 0.90, CH_2Cl_2). Anal. Calcd for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86. Found: C, 71.86; H, 8.98.

3.3.3.4. (+)-(4*R*,5*S*,7*R*,11*S*)-11,12-epoksynootkatone (5). ^{1}H NMR (see Table 2), ^{13}C NMR (see Table 3), IR (film, cm^{-1}): 2932(m), 1667(s), 1456(w), 1288(m), 840(w), 798(w); $[\alpha]_D^{20}$ +157.1 (*c* 1.30, CH_2Cl_2).

3.3.3.5. (+)-(4*R*,5*S*,7*R*)-11,12-Dihydroxynootkatone (6). ^{1}H NMR (see Table 2), ^{13}C NMR (see Table 3), IR (film, cm^{-1}): 3412(s), 2969(s), 1655(s), 1300(s), 1046(s), 942(w), 736(s); $[\alpha]_D^{20}$ +151.9 (*c* 1.00, CH_2Cl_2) Lit. $[\alpha]_D^{20}$ +130.5 (*c* 0.81, $CHCl_3$).[13]

3.3.3.6. (+)-(4*R*,5*S*,7*R*)-7,11,12-Trihydroxynootkatone (7). ^{1}H NMR (see Table 2), ^{13}C NMR (see Table 3), IR (film, cm^{-1}): 3318(s), 2977(s), 1668(s), 1378 (s), 1207(s), 1057(s), 733(s); $[\alpha]_D^{20}$ +126.4 (*c* 1.12, CH_2Cl_2). Anal. Calcd for $C_{15}H_{24}O_4$: C, 67.14; H, 9.01. Found: C, 67.03; H, 9.08.

3.4. Antiproliferative assay in vitro

3.4.1. Cells

The following established in vitro cancer cell lines were applied: A549 (human lung adenocarcinoma) and HL-60 (human promyelocytic leukemia). Both cancer cell lines were obtained from American Type Culture Collection (Rockville, Maryland, USA) and are being maintained in the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

A-549 cells were cultured in RPMI 1640+Opti-MEM (1:1) (both from Gibco, Scotland, UK), HL-60 cells in RPMI 1640 medium (Gibco, Scotland, UK) supplemented with 2 mM L-glutamine and 1.0 mM sodium pyruvate, 10% fetal bovine serum (all from Sigma–Aldrich Chemie GmbH, Steinheim, Germany). All culture media were supplemented with 100 units/ml penicillin, and 100 μg/ml streptomycin (both from Polfa Tarchomin S.A., Warsaw, Poland). All cell lines were grown at 37 °C with 5% CO_2 humidified atmosphere.

Twenty-four hours before addition of tested agents, the cells were plated in 96-well plates (Sarstedt, Germany) at a density of 10^4 cells per well in 100 μl of culture medium. An assay was performed after 72 h of exposure to varying concentrations of the tested agents (from 0.1 to 100 μg/ml). The results were calculated as the ID_{50} (inhibitory does 50%), the dose of tested agent which inhibits 50% of the proliferation of the cancer cell population. ID_{50} values were calculated for each experiment separately and mean values ± SD are presented in the tables. Each compound at each concentration was tested in triplicate in a single experiment, which was repeated 3–5 times.

Ethanol, which was used as a solvent (in a dilution corresponding to its highest concentration applied to the tested compounds), did not exert any inhibitory effect on cell proliferation. In the antiproliferative assays to evaluate cytostatic effect, the SRB or MTT methods were applied

Human lung adenocarcinoma A549 cells were routinely grown at 37 °C in RPMI 1640+OptiMEM medium supplemented with 5% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine. Human promyelocytic leukemia HL-60 cells were maintained in a suspension culture in an RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 units/ml penicillin, 2 mM glutamine, 1 mM sodium pyruvate, 4.5 g/dl glucose. The cultures were maintained at 37IC in humid atmosphere saturated with 5% CO_2.

3.4.2. SRB assay

The details of this technique were described by Skehan.[14] The cells attached to the plastic were fixed by gently layering cold 50% TCA on the top of the culture medium in each well. The plates were incubated at 4 °C for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B dissolved in 1% acetic acid for 30 min. Unbound dye was removed by rinsing (4×) with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Multiskan RC photometer.

3.4.3. MTT assay

This technique was applied for the cytotoxicity screening against human promyelocytic leukemia cells growing in suspension culture. For the last 3–4 h of incubation, 20 μl of MTT solution was added to each well (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; stock solution: 5 mg/ml). The mitochondria of viable cells reduce the pale yellow MTT to a navy blue formazan: the more viable cells are present in well, the more MTT will be reduced to formazan. When incubation time was completed, 80 μl of the lysing mixture was added to each well (lysing mixture: 225 ml dimethylformamide, 67.5 g sodium dodecyl sulfate and 275 ml of distilled water). After 24 h, when formazan crystals had been dissolved, the optical densities of the samples were read on an Multiskan RC photometer at 570 nm wavelength.

3.4.4. Statistical evaluation

One-way analysis of variance (ANOVA) followed by a Mann–Whitney U Test was applied. *P*-values <0.05 were considered significant.

Acknowledgements

This Project was financed by European Union from the European Social Fund, the State budget and Silesia Region budget. Project No. Grant/I/15/2009P.

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