PERGAMON

Phytochemistry 54 (2000) 455–460

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Microbial transformation of dehydropinguisenol by *Aspergillus* sp.

El Hassane Lahlou[a], Yoshiaki Noma[b], Toshihiro Hashimoto[a], Yoshinori Asakawa[a,*]

[a]*Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, 770-8514 Tokushima, Japan*
[b]*Faculty of Domestic Sciences, Tokushima Bunri University, Yamashiro-cho, 770-8514 Tokushima, Japan*

Received 9 December 1999; received in revised form 8 March 2000

Abstract

Two metabolites were obtained by microbial transformation of a furanosesquiterpene alcohol, dehydropinguisenol, using *Aspergillus niger* and *Aspergillus cellulosae*. Their structures were established as 10-oxo-lejeuneapinguisenol and lejeuneapinguisenol on the basis of their spectroscopic data. The latter compound was obtained after 4 and 9 days of incubation with *A. cellulosae* at 30°C and 25°C, respectively. *Aspergillus niger* produced both metabolites after 3 and 5 days incubation at 30°C, respectively. A possible pathway for the formation of these compounds is discussed here together with their antimicrobial activity against *A. niger* and *A. cellulosae*. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Biotransformation; *Aspergillus niger*; *Aspergillus cellulosae*; Dehydropinguisenol; Antimicrobial activity

1. Introduction

Microorganisms are able to transform a huge variety of organic compounds, such as terpene hydrocarbons, alkaloids, steroids, antibiotics and amino acids (Kieslich, 1976). Many compounds with properties of therapeutic and/or industrial interest are obtained by microbial transformation, such as the production of L-aspartic acid from fumaric acid and L-malic acid from the same substrate (Vanek et al., 1999).

Recently we reported the isolation and the elucidation of structures of several pinguisane-type sesquiterpenes, including two new ones, and aromatic compounds from the liverwort *Trocholejeunea sandvicensis* (Lahlou et al., 2000). The large quantity of dehydropinguisenol (**1**) (1.80 g) isolated from this plant permitted us to examine biotransformations of the compound using two types of fungus, *Aspergillus niger* and *Aspergillus cellulosae*. These fungi are known for their ability to introduce oxygenated functional groups to terpene substrates (Noma and Asakawa, 1995). Dehydropinguisenol is a pinguisane-type sesquiterpenoid, and the carbon skeleton of this class of compound does not appear to obey the biogenetic isoprene rule. Sesquiterpenoids of this type have thus far not been found in higher plants and are apparently limited to liverworts (Asakawa, 1995). Compounds of this family have been reported to show superoxide release inhibitory and insect antifeedant activities (Asakawa, 1998; Wada and Munakata, 1971).

In this paper we describe the microbial transformation of dehydropinguisenol (**1**) by *Aspergillus* species. Two new metabolites were produced and identified as 10-oxo-lejeuneapinguisenol (**2**) and lejeuneapinguisenol (**3**). A possible pathway for the formation of these metabolites by *A. niger* is proposed. The antimicrobial activity of compounds (**1**–**3**) is also discussed.

2. Results and discussion

Incubation of **1** with *A. cellulosae* for 4 days at 30°C led to the formation of compound **3** (10%) together with a mixture of degradation products of the substrate. The mass spectrum of **3** exhibited a molecular

* Corresponding author. Tel.: +81-88-622-9611; fax: +81-88-655-3051.
E-mail address: asakawa@ph.bunri-ac.jp (Y. Asakawa).

PII: S0031-9422(00)00154-0

Table 1
^{1}H NMR spectral data of compounds (**1**–**3**) (600 MHz, $CDCl_3$)

H	1	2	3
1	1.67 *m*	2.04 *m*	2.00 *m*
2a	1.16 *m*	1.30 *m*	1.25 *m*
2b	1.83 *m*	1.79 *m*	1.72 *m*
3a	1.78 *m*	1.72 *m*	1.63 *m*
3b	2.10 *m*	2.13 *m*	2.09 *m*
7a	–	2.36 *d* ($J = 16.8$ Hz)	2.32 *d* ($J = 16.8$ Hz)
7b	4.52 *s*	2.40 *d* ($J = 16.8$ Hz)	2.38 *d* ($J = 16.8$ Hz)
10	6.48 *d* ($J = 1.8$ Hz)	–	2.55 *m*
11	7.36 *d* ($J = 1.8$ Hz)	4.37 *m*	3.61 *m*
12	1.04 *s*	0.85 *s*	0.81 *s*
13	0.81 *d* ($J = 7.2$ Hz)	0.87 *d* ($J = 7.2$ Hz)	0.84 *d* ($J = 7.2$ Hz)
14	1.22 *s*	1.19 *s*	1.13 *s*
15a	5.15 *s*	1.93 *s*	1.96 *s*
15b	5.29 *s*	–	–

ion at m/z 236 corresponding to the molecular formula $C_{15}H_{24}O_2$, which was confirmed by analysis of the high resolution mass spectrum. The ^{1}H NMR spectrum of **3** (Table 1) displayed signals for a secondary methyl group and for three tertiary methyl groups, one of which was vinylic. Signals corresponding to the exo-methylene group, to the furane system and to methine proton H-7, which were present in the spectrum of **1** were not observed in the spectrum of **3** thus indicating a hydrogenation and an elimination of these functional groups by the fungus. The presence of an α,β-conjugated ketone group, a tetra-substituted double bond and a primary alcohol group in **3** was suggested by ^{13}C NMR spectral signals at δ 199.8, 163.0, 131.6 and 62.0 ppm, respectively (Table 2). The presence of these groups was substantiated by observation of bands at 1620, 1580 and 3440 cm^{-1} in the IR spectrum of **3**. The positions of the functional groups cited above were established by analysis of the HMQC and HMBC spectra of **3**.

Table 2
^{13}C NMR spectral data for compounds (**1**–**3**) (150 MHz, $CDCl_3$)

C	1	2	3
1	38.4	38.2	38.1
2	29.0	29.4	29.7
3	34.3	33.8	34.3
4	145.8	168.1	163.0
5	118.4	135.0	131.6
6	149.6	196.2	199.8
7	66.4	43.2	43.7
8	50.0	46.4	46.1
9	50.8	52.3	52.3
10	106.8	205.9	29.5
11	143.2	69.7	62.0
12	12.9	17.9	18.0
13	15.0	14.6	14.8
14	25.4	20.8	20.9
15	107.8	18.1	17.5

In the HMBC spectrum the proton signal at δ 1.13 ppm, corresponding to H-14, showed a three-bond correlation with the carbon signal at δ 163.0 ppm ascribed to C-4. This carbon was two bonds away from the vinylic methyl signal, which also displayed a three-bond correlation with a carbon signal at δ 131.6 ppm attributed to C-5. These correlations located the double bond between C-4 and C-5. The methyl proton H-12 at δ 0.81 ppm exhibited a three-bond correlation with a carbon signal at δ 43.7 ppm in the HMBC spectrum. This carbon was linked to the methylene proton at δ 2.35 ppm in the HMQC spectrum, which was assigned to proton H-7. The H-7 proton exhibited two- and three-bond correlations, respectively, with C-5 and with a carbon signal at δ 199.8 ppm, corresponding to the carbonyl group at C-6. Both C-6 and C-4 were three bonds away from the proton signal at δ 2.55 ppm, which was attributed to H-10. Carbon C-5 was correlated with H-10 and with a methylene proton signal at δ 3.61 ppm through two and three bonds, respectively. This signal was confirmed to be related to a carbinol carbon at δ 62.0 ppm in the HMQC spectrum that was assigned to C-11. The correlations cited above indicated that the ketone and hydroxyl groups were located at C-6 and C-11, respectively. Other important correlations are shown in Fig. 1. These data are in accordance with the structure **3** proposed for lejeuneapinguisenol.

In an attempt to improve the yield of lejeuneapinguisenol **3** by *A. cellulosae*, the next incubation was performed at 25°C for 9 days. However, the same yield was obtained as from the first experiment carried out at 30°C. In the 30°C incubation no substrate **1** remained in the culture medium, but in the 25°C incubation, about 15% of the substrate **1** was recovered.

Incubation of **1** with *A. niger* for three days at 30°C led to the formation of compound **2** (5%). When the incubation was extended to 5 days, compound **2** was converted to **3** (5%), and no **2** was detected in the medium. The peak corresponding to metabolite **2** in the GCMS chromatogram overlapped with the degradation

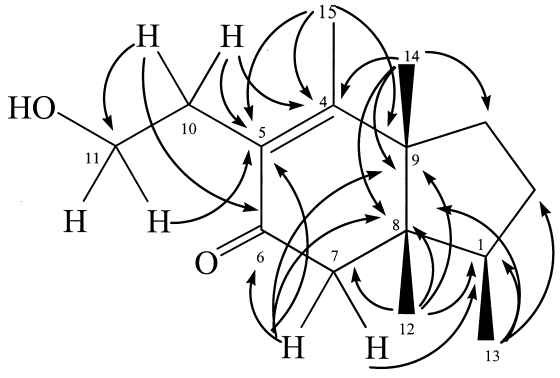


Fig. 1. Two- and three-bond HMBC correlations for compound **3**.

products of the substrate, making it difficult to calculate the exact area of this peak. We were, therefore, unable to discern the time-course for this biotransformation. However, the conversion of metabolite **2** to **3** was confirmed by TLC, in which the spot corresponding to **2** completely disappeared after 5 days incubation. These results indicated that compound **2** is the precursor of **3**. The mass spectrum of **2** exhibited a $[M - 18]^+$ at m/z 232 and its high resolution mass spectrum established a molecular formula $C_{15}H_{22}O_3$. The ^1H NMR spectrum of **2** (Table 1) was similar to that of **3** and presented signals for four methyl groups, two of which were assigned to the vinylic and secondary methyl groups. The ^{13}C NMR spectrum of **2** (Table 2) indicated the presence of the same functional groups as found in **3**, displaying carbon signals at δ 196.2, 168.1, 135.0 and 69.7 ppm, but with an additional ketone group at δ 205.9 ppm. A combination of HMQC and HMBC spectra of **2** led to the determination of the positions of the functional groups mentioned above. The H-7, -11, -12, -13, -14 and H-15 protons presented HMBC correlations similar to those observed for **3** (Fig. 2), revealing that the double bond, one of the carbonyl groups and the hydroxyl group were located at C-4 and C-5, and C-6 and C-11, respectively. The position of the second carbonyl group at C-10 was deduced from the two-bond correlation observed between H-11 and the carbon signal at δ 205.9 ppm in the HMBC spectrum. From these data, the structure of **2** was elucidated as 10-oxo-lejeuneapinguisenol.

Compound **2** was not detected in the medium of *A. cellulosae*, suggesting that the pathway for the transformation of dehydropinguisenol **1** by *A. niger* is different from that of *A. cellulosae*. The conversion of **1** to **2** and **3** by *A. niger* could be explained through the pathway shown in Fig. 3, in which enzymatic hydrogenation and oxidation play a key role.

Furanopinguisenol (**4**), a sesquiterpene which has a similar structure to that of **1** but with a β methyl group in the C-4 position instead of the exomethylene group, was incubated with *A. niger* and *A. cellulosae* for three and four days, respectively, at 30°C; however, no metabolites were produced. These results indicated the substrate specificity for the fungus, and also that the methyl at C-4 in intermediate **1a**, which was implicated in the hydrogenation process, is probably in an α position (Fig. 3). Protonation of the putative intermediate **1a** followed by removal of a molecule of water leads to the formation of oxonium ion, which is attacked by a molecule of water present in the medium to give the hemiacetal intermediate. This latter compound is hydrolyzed to its aldehydic and enolic derivative, with reduction of the aldehyde group affording the putative intermediate **1b**. Previously we have reported that the microbial hydroxylation and oxidation of a sesquiterpene alcohol, hinesol, by *A. niger*, was dependent upon a cytochrome P-450 enzyme (Hashimoto et al., 1999a, 1999b). Therefore, it is likely that a cytochorome P-450 enzyme is also involved in the oxidation step of the transformation of **1b** to **1c** (Fig. 3). Oxidation of the putative intermediate **1c** would give metabolite **2**, which could subsequently be reduced to yield metabolite **3**.

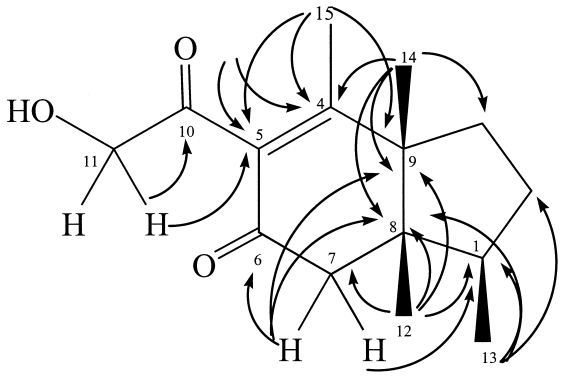
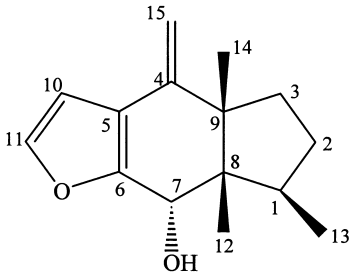


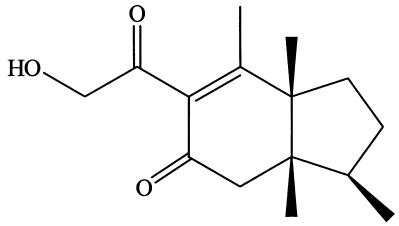
Fig. 2. Two- and three-bond HMBC correlations for compound **2**.

Incubation of **1** and 1-aminobenzotriazole (an inhibitor of cytochrome P-450) (Hashimoto et al., 1999a, 1999b) with *A. niger* for three days at 30°C apparently led to direct formation of **3** without production of **2**, which was not detected in the medium. This suggested that 1-aminobenzotriazole inhibited the pathway proposed in Fig. 3 for the formation of **3** via **2**, triggering an alternate pathway which may be similar to that used by *A. cellulosae* to form **3**. *A. niger* may, therefore, have the enzymatic capability to tranform **1** to **3** by different pathways.

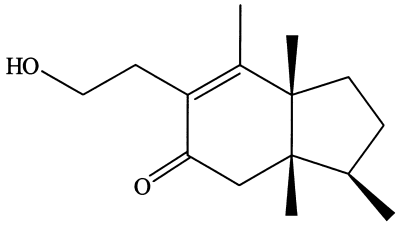
The antifungal activity of compounds **1**–**3** was assayed against *A. niger* and *A. cellulosae* using bioautography with a TLC plate. Compound **3** showed antifungal activity against *A. niger* at 10 μg in bioautography while **1** revealed weak activity against this fungus at the same concentration. Compound **2** was not active against *A. niger* or *A. cellulosae*. Compounds **1** and **3** both showed antifungal activity against *A. cellulosae* at 10 μg in bioautography, with compound **1** showing higher activity than compound **3**. These results suggest that *A. cellulosae* cannot tolerate the furanic structure of substrate **1**, and transforms **1** to **3**, thereby reducing the antimicrobial activity. The transformation of **1** to **3** in order to reduce its biological activity is presumably a detoxification process of this fungus (Sandrock and VanEtten, 1998; Dixon and Harrison, 1994).



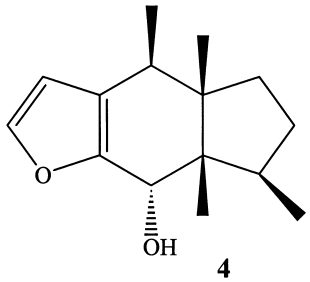
1



2



3



4

3. Experimental

3.1. General

^{1}H and ^{13}C NMR, HMQC and HMBC: TMS as international standard using Varian UNITY 600 (600 MHz); MS: JOEL JMS-AX 500; IR: JASCO FT/IR-5300; UV: HITACHI U-3000 spectrophotometer; Optical Rotation: JASCO DIP-1000 polarimeter.

3.2. Microorganism, media and culture conditions

Aspergillus cellulosae IFO 4040 was obtained from the Department of Microbiology, Osaka University, Osaka prefecture, Japan. *Aspergillus niger* was isolated in our laboratories from soil in Osaka prefecture, Japan, and was identified according to its physiological and morphological characters. A Czapek-peptone medium was used for the biotransformation [1.5% sucrose, 1.5% glucose, 0.5% polypeptone, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl and 0.001% $FeSO_4{\cdot}7H_2O$ in distilled water (pH 7)]. Erlenmeyer flasks (500 ml) containing 200 ml of medium were inoculated with a suspension of *A. niger* or *A. cellulosae* and then incubated at 30°C for 4–6 days in a rotary shaker operating at 100 rpm. After full growth of the microorganisms, solution of substrate **1** in EtOH were added to the media culture of *A. cellulosae* (100 mg/ml) and to the media culture of *A. niger* (200 mg/ml). The incubations were then continued for a further 3 or 5 days at 30°C and for 9 days at 25°C.

3.3. Extraction and isolation

After the completion of the incubation time, the cultures were filtered and the broths were extracted with ether (3 × 70 ml). The extracts were dried over Na_2SO_4 and the solvent was removed in vacuo.

The crude extract of the culture broth of *A. cellulosae* was subjected to a silica gel column (40 g) eluted with a mixture of *n*-hexane–EtOAc. Compound **3** (10%, weight of the isolated metabolite/weight of the substrate used for the experiment) was obtained from the material eluted with 30% EtOAc. Compound **2** was isolated from the crude extract of the culture broth of *A. niger* which was incubated for 3 days. The cited crude extract was submitted to a silica gel column eluted with gradients of *n*-hexane–EtOAc. The material eluted with 30% EtOAc was subjected to prep. TLC developed with benzene–EtOAc (95:5) to give **2** (5%) as a colourless oil. Compound **3** was also isolated from the broth culture of *A. niger* after incubation for 5 days. The crude extract of this culture was applied to a silica gel column eluted with gradients of *n*-hexane–EtOAc. The material eluted with 30% EtOAc yielded **3** (5%).

3.4. Bioautography

An aliquot of the ether solution of each compound (**1**–**3**) corresponding to 3 and 10 μg, respectively, was applied to a TLC plate, which was then developed with *n*-hexane–EtOAc (65:35) up to 11 cm. After evaporating the solvent, conidia of *A. niger* or *A. cellulosae* suspended in a Czapek-Dox medium were sprayed on the thin layer plate, which was then incubated in a

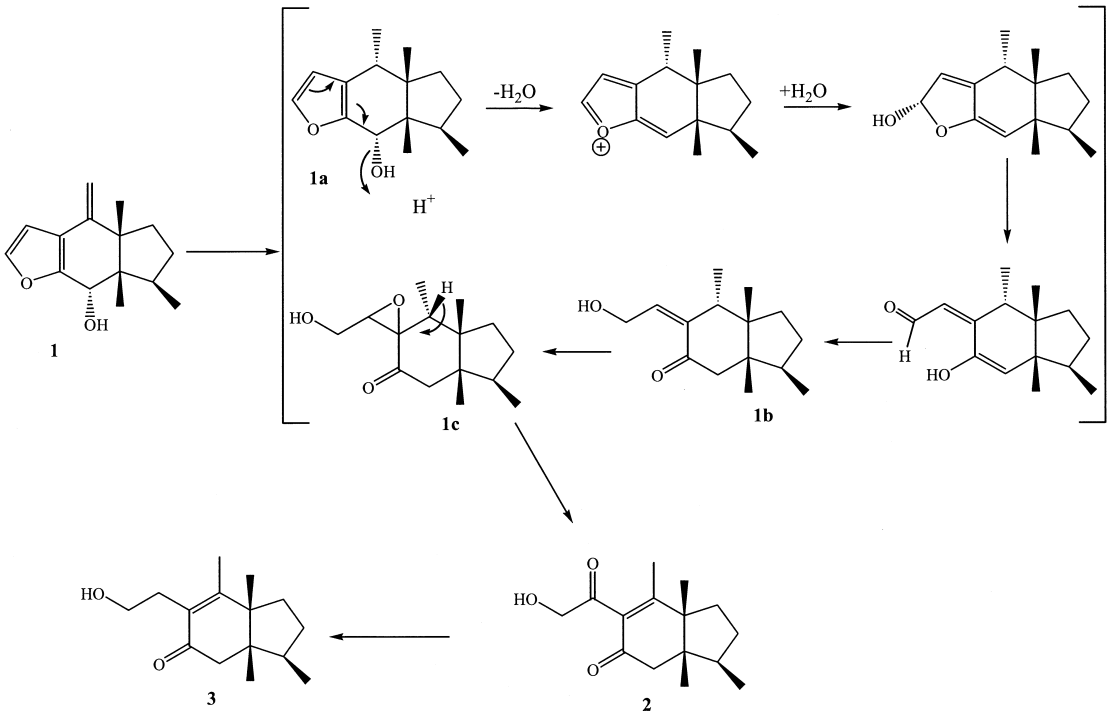


Fig. 3. A possible pathway for the biotransformation of compound **1** by *A. niger*.

moist chamber at 25°C in darkness. Antifungal zones lacking aerial mycelia were detected in the absence of hyphae turning brown on exposure of the plate to iodine vapor.

3.5. Dehydropinguisenol (*1*)

$[\alpha]_D^{18} + 84.2°$ (*c* 0.74 g/100 ml, MeOH); FTIR ν_{max} cm^{-1}: 3400, 1645, 1590, 1505, 1000; in ^1H and ^{13}C NMR spectra: see Tables 1 and 2; EIMS *m/z* (rel. int:): 232 $[M]^+$ (64), 217 (34), 121 (45), 109 (100), 66 (29).

3.6. 10-Oxo-lejeuneapinguisenol (*2*)

$[\alpha]_D^{20}$ −65.0° (*c* 0.35 g/100 ml, $CHCl_3$); FTIR ν_{max} cm^{-1}: 3440, 1620, 1580; UV λ_{max}^{EtOH} nm (log ε): 206 (3.63), 242 (3.86) ($c = 0.50 \times 10^{-3}$ M, EtOH); for ^1H and ^{13}C NMR spectra: see Tables 1 and 2; HR-EIMS: found 250.1599, $C_{15}H_{22}O_3$ requires 250.1569; EIMS: 232 $[M - 18]^+$, 219 (100), 163 (38), 135 (40), 95 (29).

3.7. Lejeuneapinguisenol (*3*)

$[\alpha]_D^{20}$ −2.0° (*c* 0.15 g/100 ml, $CHCl_3$); FTIR ν_{max} cm^{-1}: 3460, 1710, 1670, 1610; UV λ_{max}^{EtOH} nm (log ε): 205 (3.31), 244 (3.57) ($c = 1.06 \times 10^{-3}$ M, EtOH); for ^1H and ^{13}C NMR spectra: see Tables 1 and 2; HR-EIMS: found 236.1748, $C_{15}H_{24}O_2$ requires 236.1776; EIMS *m/z* (rel. int.): 236 $[M]^+$ (20), 218 (11), 162 (23), 127 (45), 110 (100), 95 (66).

Acknowledgements

We thank Dr. Tanaka (TBU) and Miss Y. Okamoto (TBU) for providing 600 MHz NMR spectra and mass spectra. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 09771933) from the Ministry of Education, Science, Sport and Culture of Japan.

References

Asakawa, Y., 1995. Herz, W., Kirby, G.W., Moore, R.E., Steglich, W., Tamm, Ch. (Eds.), Progress in the Chemistry of Organic Natural Products, vol. 65. Springer, Vienna, p. 1.

Asakawa, Y., 1998. Romeo, J.T. (Ed.), Phytochemicals in Human Health Protection, Nutrition, and Plant Defense, vol. 33. Kluwer Academic Publishers/Plenum Press, p. 319.

Dixon, R.A., Harrison, M.J., 1994. Relevance of phytoalexin — a critical review. Annual Review of Phytopathology 32, 479.

Hashimoto, T, Noma, Y, Akamatsu, Y, Tanaka, M, Asakawa, Y., 1999a. Biotransformation of sesquiterpenoid by microorganisms (5): biotransformation of dehydrocostuslactone. In: 43rd Symposium of Chemistry on Terpenes, Essential Oils and Aromatics, Symposium papers, Oita, Japan, 202.

Hashimoto, T., Noma, Y., Kato, M., Takaoka, S., Asakawa, Y., 1999b. Biotransformation of hinesol isolated from the crude drug *Atractylodes lancea* by *Aspergillus niger* and *Aspergillus cellilosae*. Chemical Pharmaceutical Bulletin 47, 716.

Kieslich, K., 1976. Microbial Transformation of Non-Steroid Cyclic Compounds. Georg Thieme Verlag, Stuttgart, Germany.

Lahlou, E.H., Hashimoto, T., Asakawa, Y., 2000. Highly oxygenated sesquiterpenes from the liverwort *Trocholejeunea sandvicensis*. Phytochemistry 53, 271.

Noma, Y., Asakawa, Y., 1995. Bajaj, Y.P.S. (Ed.), Biotechnology in Agriculture and Forestry Medicinal and Aromatic Plants VIII, vol. 33. Springer-Verlag, Berlin, Heidelberg, New York, p. 62.

Sandrock, R.W., VanEtten, H.D., 1998. Fungal sensitivity to and enzymatic degradation of the phytoanticipin α-tomatine. Phytopathology 88, 137.

Vanek, T., Valterovia, I., Vaisar, T., 1999. Biotransformation of (S)-(−) and (R)-(+)-limonene using *Solanum aviculare* and *Diocorea* deltoidea plant cells. Phytochemistry 50, 1347.

Wada, K., Munakata, K., 1971. Insect feeding activity in plants, part III. Agricultural Biological Chemistry 35, 115.