Microbial transformation of the diterpene mulin-11,13-dien-20-oic acid by *Mucor plumbeus*

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Two new mulinane-type derivatives: 16-hydroxy mulin-11,13-dien-20-oic (2) acid and 7α,16-dihydroxy mulin-11,13-dien-20-oic (3) acid were obtained by microbial transformation of mulin-11,13-dien-20-oic acid (1), along with tyrosol (4) using liquid cultures of *Mucor plumbeus*. The latter compound has not been previously identified in the genus *Mucor*. Structural elucidation of these metabolites was achieved using 1D- and 2D-NMR spectroscopy.

Keywords: NMR; ^{1}H NMR; ^{13}C NMR; 2D NMR; *Azorella compacta*; biotransformation; diterpenoids; *Mucor plumbeus*; tyrosol

Introduction

Some mulinane-type diterpenoids have been reported from some *Azorella* species as well as yaretane-, azorellane- and madreporane-type diterpenoids.[1–4] Mulin-11,13-dien-20-oic acid is the major diterpene isolated from *Azorella compacta*.[5] It has been shown to display trichomonicidal effects and to have trypanocidal activity too.[4,6]

Microbial transformation is now becoming a useful tool for structural modifications of bioactive natural products and to allow the production of regio- and stereoselective compounds under mild conditions, especially the inactived sites of hydrocarbons.[7] Some filamentous fungi have been used for biotransformation of diterpenes including *Aspergillus niger*,[8] *Cunningamella elegans*,[9] *Gibberella fujikuroi*,[10] *Fusarium* species,[11] and *Mucor plumbeus*.[12–18]

The aim of this work was to assess the biotransformation of mulin-11,13-dien-20-oic acid by filamentous fungi. We isolated two new derivatives by microbial transformation using liquid cultures of *M. plumbeus*. Here, we report a complete assignment of ^{1}H and ^{13}C NMR of these two compounds.

Materials and Methods

Chemicals

Silica gel 60 (63–200 μm) was used for column chromatography; *n*-hexane with increasing amounts of ethyl acetate was used as eluent. Thin layer chromatography (TLC) was performed on Kieselgel 60 GF254 using *n*-hexane/ethyl acetate (7 : 3, v/v). Detection was achieved by spraying with H_2SO_4–MeOH (5 : 95, v/v) followed by heating at 120 °C.

Instrumentation

Measurements of NMR spectra of compounds were made on a Bruker Avance AM-400 spectrometer equipped with 5-mm probes. The compounds were dissolved in 0.8 ml of chloroform-*d* ($CDCl_3$) containing tetramethylsilane (TMS) as internal standard. Chemical shifts (δ) were reported in parts per million and coupling constants (*J*) in Hertz. The pulse conditions were as follows: for the ^{1}H-NMR spectrum, spectrometer frequency (SF) = 400.13 MHz, acquisition time (AQ) = 1.979 s, relaxation delay (RD) = 1.0 s, 30° pulse width = 2 μs, spectral width (SW) = 8278.1 Hz, line broadening (LB) = 0.3 Hz, Fourier Transform (FT) size = 32 K; for the ^{13}C-NMR spectrum, SF = 100.61 MHz, AQ = 1.2 s, RD = 2.0 s, 30° pulse width = 2 μs, SW = 27 173.9 Hz, LB = 1.0 Hz, FT size = 32 K; for the H–H COSY spectrum, AQ = 0.19 s, RD = 1.5 s, SW = 5341.8 Hz, FT size = 1024 × 1024; for the HMBC spectrum, AQ = 0.19 s, RD = 1.5 s, SW = 5341.8 (^{1}H) and 30 191.03 (^{13}C) Hz, FT size = 1024 × 1024, and 7.7 Hz long-range coupling constant. The NOESY spectrum of compound **3** was performed with eight scans, AQ = 0.09 s, RD = 1.0 s, SW = 5995.2 Hz, FT size = 1024 × 1024, and LB = 0.0 Hz.

The infrared (IR) spectra were recorded on a Vector 22 FT-IR spectrometer. The mass spectra were recorded using a Thermo Finnigan MAT 95XP model (using EIMS for low- and high-resolution analysis) spectrometer.

Isolation of substrate

The mulin-11,13-dien-20-oic acid (**1**) was isolated from the leaves of *A. compacta* as previously reported.[5]

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Microorganisms and media

All microorganisms were obtained from the American Type Culture Collection (ATCC). They belonged to the following species: *G. fujikuroi* IMI 58 289, *M. plumbeus* IMI 116 688, *A. niger* ATCC 16 404, and *C. elegans* ATCC 868. The spores of fungi maintained on Czapeck agar slants at 8 °C, were inoculated into the autoclaved Czapeck liquid medium.[19] This medium was comprised of KNO_3 (2 g/l), KCl (0.5 g/l), $FeSO_4$ (0.01 g/l), $MgSO_4$ (0.5 g/l), $KHPO_4$ (1 g/l), yeast extract (5 g/l), and glucose (30 g/l) and was adjusted to pH 5.5. A standard two-stage fermentation protocol was employed in all experiments by liquid culture.[20]

Microbial transformation

Biotransformation by liquid culture

Screening procedure. The strains were cultivated at 28 °C in Erlenmeyer flasks (100 ml) that contained 25 ml of Czapeck liquid medium. After 3 days, 50 mg of substrate in 1 ml of Tween 80 were added to the grown cultures to obtain a final concentration of 0.5 mg/ml. The control consisted of culturing the fungus under the same conditions but without the substrate. The cultures were shaken at 200 rpm for 15 days. Biotransformation was monitored everyday by TLC (silica gel, *n*-hexane: ethyl acetate 7:3).

Preliminary screening studies using different cultures of microorganisms were done to determine whether these were capable of transforming mulin-11,13-dien-20-oic acid. Four strains of filamentous fungi were cultivated and tested in the Czapeck liquid medium and only *M. plumbeus* degraded **1** to form more polar products. Then, *M. plumbeus* was chosen for the preparative scale biotransformation in the liquid culture.

Preparative biotransformation. The substrate (800 mg dissolved in 6 ml Tween 80) was added to the 3-day cultures of *M. plumbeus* prepared as described in the screening procedure. The cultures were shaken in three Erlenmeyer flasks (3000 ml) with 600 ml of medium in each flask. After 15 days of shaking, the products were extracted with ethyl acetate (3 × 500 ml). The organic solutions were dried over $MgSO_4$ and the solvent was evaporated under reduced pressure to give an oily residue (950 mg). The products were separated by column chromatography on silica gel (200 g) in a column (70-cm length, 3.5-cm internal diameter). The column chromatography was eluted with mixtures of *n*-hexane-ethyl acetate (0 up to 60%). Elution with *n*-hexane-ethyl acetate (30% v/v) afforded 7 mg of **2** (0.8% isolated yield). However, elution with *n*-hexane-ethyl acetate (50% v/v) gave **3** and **4** as a mixture. These products were purified by Sephadex LH-20 (eluted with MeOH) to give 6 mg of **3** (0.75%) and 8 mg of **4** (1.3%).

The ^{1}H- and ^{13}C-NMR data of compounds **2**–**3** are presented in Table 1, while the structures of compounds are given in Fig. 1.

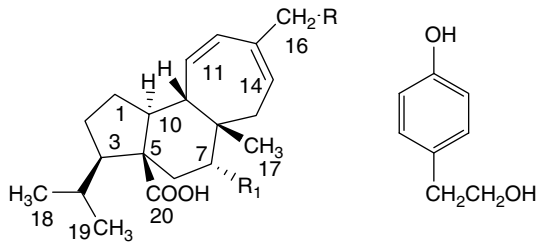
16-hydroxy mulin-11,13-dien-20-oic acid. Compound **2**: pale yellow oil. HREIMS: calcd. for $C_{20}H_{30}O_3$ ($M^{+\bullet}$): 318.2194, found: 318.2189. EI-MS: *m/z* (rel. int. %): 318 [$M^{+\bullet}$] (11), 279 (22), 257 (7), 255 (10), 229 (7), 209 (14), 167 (38), 163 (29), 149 (100), 105 (16), 91 (18), 71 (22), 57 (30), 55 (20). FT-IR ν_{max}: 3300–2500 br, 2950, 1700, 1480, 1270, 1190 cm^{-1}.

7α,16-hydroxy mulin-11,13-dien-20-oic acid. Compound **3**: pale yellow oil. HREIMS: calcd. for $C_{20}H_{30}O_4$ ($M^{+\bullet}$): 334.2144, found: 334.2128. EI-MS: *m/z* (rel. int. %): 334 [$M^{+\bullet}$] (8), 316, (100), 298 (38), 271 (58), 255 (53), 253 (51), 229 (25), 227 (45), 171 (37), 147 (50), 146 (46), 145 (44), 132 (50), 121 (95), 119 (74), 108 (74), 106 (61), 105 (86), 95 (51), 93 (54), 91 (81), 81 (46), 79 (55), 77 (36), 69 (44), 67 (35), 57 (29), 55 (50), 53 (17). FT-IR ν_{max}: 3422, 2958, 2873, 1697, 1450, 1385, 1365, 1240, 1180 cm^{-1}.

Table 1. The full assignments of proton and carbon signals of **2** and **3** ($CDCl_3$)

	^{1}H NMR		^{13}C NMR	
C	**2**	**3**	**2**	**3**
1	1.70	1.50[a] m	24.6 t	24.3 t
	2.04 dd (2.5; 11.3)	1.99 m	–	–
2	1.55[a]	1.51[a] m	28.8 t	28.8 t
	1.95 m	2.02 m		
3	1.54[a]	1.52[a]	57.7 d	57.5 d
4	1.54[a]	1.52[a]	31.8 d	31.9 d
5	–	–	58.6 s	56.7 s
6	1.40[a]	α: 1.48 dd (11.9; 12.1) β: 2.67 dd (3.8; 12.1)	32.7 t	40.6 t
	2.43 dt (3.5; 13.0)	–	–	–
7	2.73 brd (17.0)	β: 3.68 dd (3.8; 11.9)	36.3 t	76.5 d
	1.73[a]	–	–	–
8	–	–	34.8 s	39.6 s
9	2.22 dd (6.2; 9.4)	2.19 dd (7.1; 9.1)	50.4 d	48.6 d
10	1.71 m	1.69 m	55.1 d	54.7 d
11	5.67 brd (12.7)	5.77 dd (7.1; 12.6)	134.6 d	133.1 d
12	5.77 d (12.7)	5.88 d (12.6)	127.5 d	124.8 d
13	–	–	135.9 s	136.5 s
14	5.66 brs	5.83 d (7.6)	124.2 d	127.5 d
15	1.40 brd (14.0)	2.31 brd (17.1)	41.1 t	39.6 t
	1.60 dd (7.4; 14.0)	2.44 dd (7.6; 17.1)	–	–
16	4.05 brs	4.13 brs	69.2 t	69.1 t
17	0.84 s	0.96 s	27.1 q	27.4 q
18	0.84 d (5.9)	0.90 d (5.6)	22.7[a] q	22.7[a] q
19	1.01 d (5.9)	1.07 d (5.6)	22.4[a] q	22.3[a] q
20	–	–	186.3 s	178.5 s

[a] Assignments may be interchanged between values of same column.



R R$_1$

1 H H

2 OH H

3 OH OH

4

Figure 1. The structure of compound **1** and its biotransformation products **2**–**3**. Compound **4**: Tyrosol.

Results and Discussion

After incubation in liquid culture for 15 days *M. plumbeus* was able to convert **1** into **2** and **3**, along with other known microbial metabolite **4**, identified as tyrosol. The structure of the known

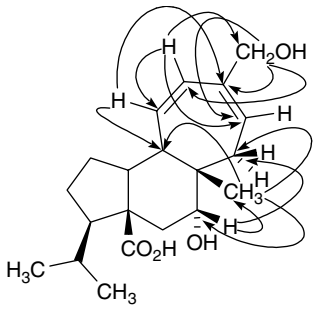


Figure 2. The main correlations in the HMBC spectrum of compound **3**.

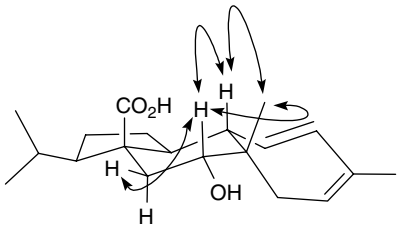


Figure 3. The key NOESY of **3**.

compound was done on the basis of their spectroscopic analysis (1D and 2D NMR) and physical properties and was compared with literature data.[21]

Compound **2** was deduced as follows: ^{1}H-NMR spectrum showed the proton signals at δ 5.77 (1H, d, J = 12.7 Hz, H-12), 5.67 (1H, brd, J = 12.7 Hz, H-11), 5.66 (1H, brs, H-14), 1.01 (3H, d, J = 5.9 Hz, H-19), 0.84 (3H, d, J = 5.9 Hz, H-18), 0.84 (3H, s, H-17), which confirmed its mulinane skeleton with a diene system. In addition, one signal at 4.05 (2H, brs, H-16) revealed the hydroxylation of the methyl group C-16. The structure of **2** was determined as 16-hydroxy mulin-11,13-dien-20-oic acid. 1D and 2D NMR permitted the correct assignments of all signals of **2**. The molecular formula was proved to be $C_{20}H_{30}O_3$ (m/z = 318.2189) based on HREIMS and ^{13}C-NMR data. In the HMBC spectrum, the proton signal at δ 5.66 (H-14) showed long-range correlations with the carbon signals at δ 127.5 (C-12), δ 69.2 (C-16), and δ 34.8 (C-8), and the proton signal at δ 5.77 (H-12) had cross peaks with δ 124.2 (C-14), δ 69.2 (C-16), and δ 50.4 (C-9) indicating that the hydroxylation was at C-16. In addition, the proton signal at δ 5.67 (H-11) correlated with δ 135.9 (C-13) and δ 34.8 (C-8), confirming the diene system. The other known signals were deduced by comparison with the spectra of compound **1**.[5]

Compound **3** was obtained as yellow oil. Analysis of the ^{1}H- and ^{13}C-NMR spectra (Table 1) plus the H–H COSY and HMBC experiments allowed us to assign unequivocally all ^{1}H and ^{13}C signals of this compound. The molecular formula of compound **3** was deduced as $C_{20}H_{30}O_4$ (m/z = 334.2128) by HREIMS and ^{13}C-NMR data. The ^{1}H-NMR spectrum showed the proton signal at 5.88 (1H, d, J = 12.6 Hz, H-12), 5.83 (1H, d, J = 7.6 Hz, H-14), 5.77 (1H, dd, J = 12.6; 7.1 Hz H-11), 1.07 (3H, d, J = 5.6 Hz, H-19), 0.90 (3H, d, J = 5.6 Hz, H-18), 0.96 (3H, s, H-17) that confirmed its mulinane structure. In addition, in the ^{1}H-NMR spectrum of **3**, the signals were similar to those of **2**, except that one more proton signal at δ 3.68 (1H, dd) could be observed on **3**, suggesting the presence of one hydroxyl proton more than **2**. In the HMBC spectrum (Fig. 2), the proton signal at δ 2.44 (H-15) showed long-range correlations with the carbon signals at δ 48.6 (C-9), δ 76.5 (C-7), δ 127.5 (C-14), and δ 136.5 (C-13); the proton signal at δ 3.68 (H-7) had peaks correlated with the carbon signal at δ 27.4 (C-17) and δ 39.6 (C-15), and the proton signal at δ 0.96 (H-17) had peaks correlated with δ 39.6, δ 76.5, and δ 48.6 indicating δ 76.5 to be the C-7 signal. Similarly δ 48.6 belongs to C-9 and δ 39.6 to C-15. These data determined the position from the second hydroxyl group at C-7. The relative stereochemistry of C-7 was assigned on the basis of a 2D NOESY experiment and coupling constants. In the NOESY spectrum, correlation between δ 3.68 (H-7) and 0.96 (3H-17), 2.19 (H-9) and 2.67 (H-6β) confirmed which 7-OH had the α-configuration (Fig. 3). It was consistent with dihedral angles for H-6α/H-7β (165.3°) and H-6β/H-7β (45.9°) calculated by the program[22] for this compound. According to these data, the theoretical coupling constants (J = 12.1; 5.2 Hz) were obtained by the program,[23] which are in good agreement with the measured J-values (J = 11.9; 3.8 Hz). This compound was identified as 7α,16-dihydroxy mulin-11,13-dien-20-oic acid.

M. plumbeus is known to biotransform *ent*-kaurene diterpenes such as epicandicandiol and candicandiol,[15] as well as the abietane diterpenoids known as dehydroabietanol and teideadol.[16] In the same manner, for the diterpene (s): labdanes,[12] stemodanes,[17,24] stemarin,[24] and ribenone.[25]

Tyrosol has been reported from cultures of *G. fujikuroi, Ceratocystis sp*., and from a marine strain of *Bacillus subtilis* KMM3427 associated with sea sponge *Verongia sp*. Also, the compound was isolated from *Ligustrum ovalifolium, Fraxinus excelsior* and from peanut.[26] To the best of our knowledge, this is the first report of tyrosol production by *M. plumbeus*. Tyrosol was reported to prevent NF-κB, IRF-1 (Interferon regulatory factor-1) and STAT-1α (Signal transducer and activator of transcription-1α) activation and pro-inflammatory genes,[27] not to exert cytotoxic effects on cells derived from the buccal mucosa[28] and act as a hydroxyl radical scavenger and antioxidant.[29,30]

The diterpenes described in Apiaceae have very unusual skeletons that have been found only in *Azorella, Mulinum,* and *Laretia* genus. Until now, efforts have not been made to modify their structures with microorganisms. In addition, further work to optimize their yield (compounds **2** and **3**) is being achieved due to the important biological activities from the Apiaceae diterpenoids.

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