Microbial Hydroxylation of Bufalin by *Cunninghamella blakesleana* and *Mucor spinosus*

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The microbial transformation of a cytotoxic bufadienolide, bufalin (**1**), was carried out using two strains of filamentous fungi. *Cunninghamella blakesleana* catalyzed the specific 12α-hydroxylation of bufalin and produced 12α-hydroxybufalin (**2**) and 7*β*,12α-dihydroxybufalin (**3**) as the major metabolites, together with 7*β*-hydroxybufalin (**4**) and 12*β*-hydroxybufalin (**5**) in low yields. Two minor products were isolated from the culture broth of *Mucor spinosus* and were identified as 7*β*,15α-dihydroxybufalin (**6**) and 5*β*,7*β*-dihydroxybufalin (**7**), respectively. Metabolites **2**, **3**, **6**, and **7** are new compounds, and their structures were fully characterized by NMR and MS spectroscopy.

Bufalin, cinobufagin, and resibufogenin are the major bufadienolides isolated from the traditional Chinese drug ChanSu.[1,2] They are steroids with a characteristic α-pyrone ring at C-17 and possess strong antitumor activity. Previous reports showed that they exhibit significant inhibitory effects against human myeloid leukemia cells, human hepatoma cells, and prostate cancer cells with IC_{50} values of 10^{-9}−10^{-10} mol/L. These activities are mediated by the induction of cell apoptosis and cell differentiation.[3−6] For several years we have focused on the biotransformation of these bufadienolides to obtain new analogues with improved cytotoxicity and increased water solubility, which are of essential importance for the development as new drugs. More than 40 biotransformed products have been obtained, so far, including 18 new compounds.[7−10] The major biotransformation reactions involved hydroxylation at the 1*β*-, 5*β*-, 7*β*-, 12*β*-, and 16α-positions. In this paper, we report the specific 12α-hydroxylation of bufalin (**1**) by *Cunninghamella blakesleana* AS 3.970. Two new minor products from the culture broth of *Mucor spinosus* AS 3.3450 are also described.

A screen of 20 fungal strains for the biotransformation of bufalin had been carried out as described in our previous paper.[10] Two new significant peaks were observed in the HPLC chromatogram of the biotransformed products from *C. blakesleana* AS 3.970. This biotransformation was scaled up in the present study, and the two target products were obtained by silica gel column chromatography and preparative liquid chromatography. Their structures were identified as 12α-hydroxybufalin (**2**) and 7*β*,12α-dihydroxybufalin (**3**). Two minor products were also purified and characterized as the known 7*β*-hydroxybufalin (**4**) and 12*β*-hydroxybufalin (**5**),[10] respectively. The biotransformation of bufalin by *M. spinosus* AS 3.3450 to afford 12 metabolites was reported previously, mainly involving the hydroxylation at C-7*β*, C-12*β*, and C-16α.[10] In this study, two new minor products were obtained by preparative liquid chromatography and identified as 7*β*,15α-dihydroxybufalin (**6**) and 5*β*,7*β*-dihydroxybufalin (**7**). The structures (Figure 1) were characterized by mass spectrometry and extensive

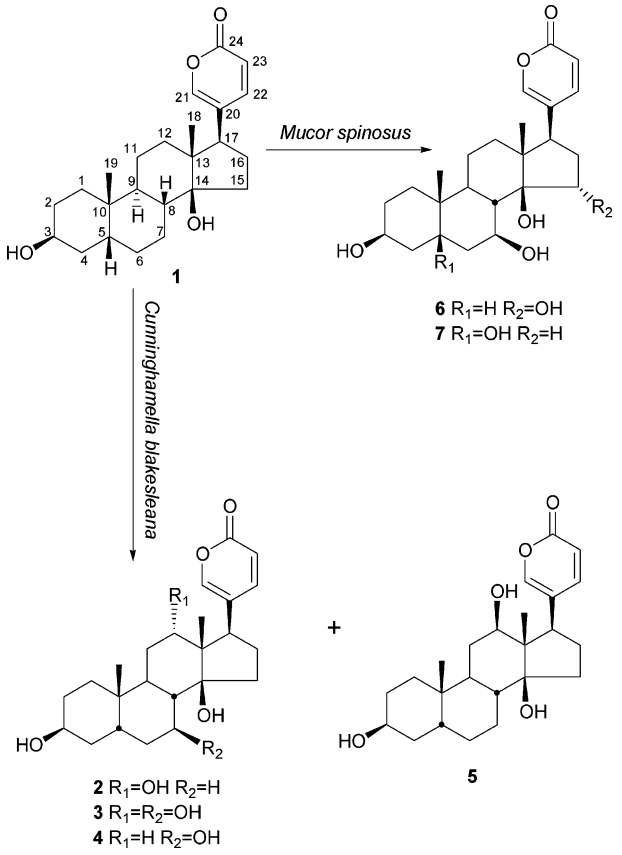

Figure 1. Biotransformation of bufalin (**1**) by *Cunninghamella blakesleana* and *Mucor spinosus*.

NMR techniques, including ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, HMBC, and NOESY.

The atmospheric pressure chemical ionization mass spectrum (APCI-MS) of **2** gave a $[M + H]^+$ ion at *m/z* 403, suggesting the molecular formula $C_{24}H_{34}O_5$. When compared to bufalin, an additional oxygen-bearing tertiary carbon appeared at *δ* 74.0. According to the HMBC spectrum, it showed long-range couplings with 18-CH_3 (*δ* 0.52) and H-17 (*δ* 3.13), indicating the introduction of a hydroxyl group at C-12. In contrast to 12*β*-hydroxylated bufadienolides, where C-18 appeared at remarkably higher fields (∼*δ* 10.5),[10] the C-18 of **2** resonated at *δ* 18.0,

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indicating α-configuration of the C-12 hydroxyl group. There was little effect on the chemical shift of C-18 due to their *γ-trans* positions. In accordance, C-9 (δ 27.9) and C-17 (δ 44.1) shifted upfield by 6.9 and 6.0 ppm when compared to bufalin, respectively, since they are in the *γ-gauche* positions of 12α-OH. On the basis of the above evidence, compound **2** was identified as 12α-hydroxybufalin.

The APCI mass spectrum of **3** gave a $[M + H]^+$ ion at *m/z* 419, suggesting the molecular formula $C_{24}H_{34}O_6$. The ^{13}C NMR spectrum showed two additional oxygenated methine signals at δ 68.9 and 73.7, suggesting that **3** was a dihydroxylated product of bufalin. Similar to **2**, the signal at δ 73.7 showed HMBC correlations with 18-CH_3 (δ 0.54) and H-17 (δ 3.15), and C-18 appeared at δ 18.1. Thus metabolite **3** was also hydroxylated at C-12α. The other signal (δ 68.9) corresponded to an unusually broad peak at δ 3.80 in the HMQC spectrum, and C-6 (δ 36.5) and C-8 (δ 46.3) were shifted downfield by 10.0 and 5.1 ppm when compared to bufalin, respectively. This evidence supported the hydroxylation at C-7. The broad signal of H-7 (δ 3.80) resulted from its axial−axial couplings with H-6β and H-8, indicating that 7-OH should be in the β-configuration. Accordingly, the signal of 14-OH (δ 5.34) appeared at a rather low field, as a result of the intramolecular hydrogen bonding of 7β-OH with 14-OH. Therefore, compound **3** was identified as 7β,12α-dihydroxybufalin.

Compounds **6** and **7** were obtained as minor products from *M. spinosus*. The APCI mass spectrum of **6** gave a $[M + H]^+$ ion at *m/z* 419, suggesting the molecular formula $C_{24}H_{34}O_6$. The ^{13}C NMR spectrum showed two additional oxygen-bearing signals at δ 68.5 and 79.0, suggesting that **6** was a dihydroxylated product of bufalin. Similar to **3**, C-8 of **6** shifted downfield to δ 46.5, and 14β-OH to δ 5.45, suggesting that a 7β-OH had been introduced. In addition, C-16 (δ 40.6) shifted downfield by 12.2 ppm when compared to bufalin, indicating an additional hydroxylation at C-15. The NOE enhancement of H-15 (δ 4.21) with 14β-OH (δ 5.45) supported the α-configuration of 15-OH. Thus, compound **6** was identified as 7β,15α-dihydroxybufalin.

The APCI mass spectrum of **7** gave a $[M + H]^+$ ion at *m/z* 419, suggesting the molecular formula $C_{24}H_{34}O_6$. An oxygenated quaternary carbon appeared at δ 73.2, which showed HMBC correlation with 19-CH_3 (δ 0.82), suggesting hydroxylation at C-5. In accordance, C-10 shifted downfield to δ 39.6, while C-1 shifted upfield to δ 24.8 due to a *γ-gauche* effect. The other new signal appeared at δ 69.9. Its corresponding proton signal (δ 3.63) appeared as a very broad peak, and 14β-OH shifted downfield to δ 5.42. This evidence indicated the presence of a 7β-hydroxyl group. Therefore, compound **7** was identified as 5β,7β-dihydroxybufalin.

The two major products **2** and **3** from *C. blakesleana* were both hydroxylated at C-12α, and they constituted more than 90% of the total metabolites. This reaction could thus be considered as a specific one. Indeed, 7β-hydroxybufalin (**4**) was completely converted into 7β,12α-dihydroxybufalin (**3**) as the only product, within 2 days when it was added to the cultures of *C. blakesleana*. Although a number of products hydroxylated at the 1β-, 5β-, 7β-, 11β-, 12β-, and 16α-positions had been reported before, this is the first report of 12α-hydroxylation of bufadienolides. Interestingly, we had discovered the specific 12β-hydroxylation of bufadienolides by *Alternaria alternata* AS 3.4578.[9] These two reactions allowed the directed and stereoselective synthesis of 12-OH bufadienolides.

In vitro cytotoxic activities of the biotransformed products were determined with three human cancer cell lines by the MTT method.[12] The results are given in Table 1. 12α-Hydroxybufalin (**2**) showed significant inhibitory effects against all three tested cancer cell lines. The IC_{50} values ranged from 4.2×10^{-1} to 6.0×10^{-2} μmol/L. However, its effects were apparently weaker than 12β-hydroxybufalin (**5**), which exhibited almost comparable activities to bufalin.[10] Compound **3** was further hydroxylated at C-7β, and its activities were about 10-fold weaker than **2**. 5β,7β-Dihydroxybufalin (**7**) was significantly inhibitory to HeLa cells, and the IC_{50} value was 0.022 μmol/L. Most dihydroxylated bufadienolides we obtained previously showed weak cytotoxicities, and compound **7** appeared to be an exception. Its activities against Bel-7402 and BGC-823 cells, together with those of compound **6**, however, were not determined due to the limited amounts of pure compounds. Their potential as drug candidates still needs further evaluation.

Table 1. Cytotoxic Activities of the Biotransformed Products against Human Cancer Cell Lines ($n = 3$)[a]

compound	IC_{50} (μmol/L)		
	Bel-7402[b]	BGC-823[c]	HeLa[d]
1	7.0×10^{-3}	4.5×10^{-2}	2.8×10^{-2}
2	4.1×10^{-1}	4.2×10^{-1}	6.0×10^{-2}
3	2.9	5.3	4.7×10^{-1}
5	2.6×10^{-2}	3.1×10^{-2}	7.6×10^{-3}
7	n.d.[e]	n.d.	0.022

[a] The data for compounds **1** and **5** were adopted from the literature.[10] [b] Human hepatoma cells. [c] Human gastric cancer cells. [d] Human cervical carcinoma cells. [e] n.d., not determined.

Experimental Section

General Experimental Procedures. Melting points were determined with an XT4A apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 243B polarimeter. UV spectra were recorded with a TU-1901 UV−vis spectrophotometer. IR spectra were determined in KBr with an Avatar 360 FT-IR spectrophotometer. ^1H and ^{13}C NMR spectra were obtained on a Bruker DRX-500 spectrometer (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR) in DMSO-d_6 at ambient temperature with tetramethylsilane (TMS) as the internal standard. The chemical shifts (δ values) are given in parts per million (ppm) relative to TMS at 0 ppm. The coupling constants (*J* values) are reported in hertz (Hz). APCI mass spectra were measured on a Finnigan LCQ Advantage mass spectrometer in the positive ion mode. High-resolution mass spectra (HR-MS) were obtained on a Bruker APEX II Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. A SpectraSERIES HPLC apparatus (Thermo Quest) with a 100 μL loop was used for preparative liquid chromatography. Samples were separated on a YMC ODS-A column (5 μm, φ 10 × 250 mm). The flow rate was 2.0 mL/min, and the detection wavelength was 296 nm. Bufalin (**1**) was isolated from the Chinese drug ChanSu and unambiguously identified by NMR and MS techniques.[11] The purity was determined to be >99.5% by HPLC analysis. Silica gel (200−300 mesh) for column chromatography was purchased from Qingdao Marine Chemical Corporation, Qingdao, China. All chemical solvents used for product isolation were of analytical grade.

Microorganisms and Culture Media. *Cunninghamella blakesleana* AS 3.970 and *Mucor spinosus* AS 3.3450 were purchased from China General Microbiological Culture Collection Center, Beijing, China, and were maintained on potato agar slants at 4 °C. Fermentations were carried out in a potato medium consisting of 20 g of potato extract, 20 g of glucose, and 1000 mL of distilled H_2O. The media were sterilized in an autoclave at 121 °C and 1.06 kg/cm^2 for 30 min.

Biotransformation of Bufalin (1) by *C. blakesleana* AS 3.970. Mycelia of *C. blakesleana* from agar slants were aseptically transferred to 250 mL Erlenmeyer flasks contain-

ing 80 mL of liquid potato medium. The fungus was incubated at 25 °C on a rotary shaker (180 rpm) in the dark for 24 h to make a stock inoculum. Then 5 mL of the inoculum was added to each of the 10 1-L flasks containing 350 mL of potato medium. After 36 h incubation, a total amount of 200 mg of bufalin dissolved in 10 mL of EtOH was distributed equally among the 10 flasks. The incubation was allowed to continue for four additional days on the shaker. The cultures were then pooled and filtered through Whatman no. 1 filter paper. The filtrate was extracted with 3 L of EtOAc three times. The organic extract was concentrated and evaporated to dryness in a rotary evaporator under reduced pressure at 60 °C to yield 0.43 g of a brownish solid. The extract was subjected to silica gel column chromatography (55 g, 200−300 mesh, ϕ 2 × 30 cm) and eluted in 100 mL fractions with petroleum ether (60−90 °C) and acetone (4:1 to 1:1, v/v), gradually increasing the proportion of acetone. Fraction 2 was purified to yield **4** (3.2 mg). Fractions 3−7 were subjected to preparative liquid chromatography and eluted with MeOH−H_2O (45:55, v/v) to give **2** (52.7 mg), **3** (34.6 mg), and **5** (2.9 mg). Their purities were above 95%, as determined by HPLC/UV determination.

12α-Hydroxybufalin (2): white powder; $C_{24}H_{34}O_5$; mp 150−153 °C; $[\alpha]^{25}_D$ +11.7° (*c* 1.27, MeOH); UV λ_{max} (MeOH) 208.0, 300.0 nm; IR ν_{max} (KBr) 3452, 2937, 2870, 1709, 1628, 1536, 1450, 1376, 1242, 1134, 1032, 947 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.94 (1H, dd, J = 10.0 and 2.0 Hz, H-22), 7.43 (1H, d, J = 2.0 Hz, H-21), 6.28 (1H, d, J = 10.0 Hz, H-23), 4.61 (1H, d, J = 3.5 Hz, 12-OH), 4.16 (1H, d, J = 2.5 Hz, 3-OH), 4.03 (1H, s, 14-OH), 3.88 (1H, br s, H-3), 3.40 (1H, br s, H-12), 3.13 (1H, dd, J = 9.5 and 6.0 Hz, H-17), 0.83 (3H, s, H-19), 0.52 (3H, s, H-18); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 161.4 (s, C-24), 148.8 (d, C-21), 147.9 (d, C-22), 123.5 (s, C-20), 114.2 (d, C-23), 83.4 (s, C-14), 74.0 (d, C-12), 64.6 (d, C-3), 51.5 (s, C-13), 44.1 (d, C-17), 41.4 (d, C-8), 35.7 (d, C-5), 34.5 (s, C-10), 33.5 (t, C-15), 33.1 (t, C-4), 30.4 (t, C-16), 29.5 (t, C-1), 29.0 (t, C-11), 27.9 (d, C-9), 27.6 (t, C-2), 26.4 (t, C-6), 23.7 (q, C-19), 20.9 (t, C-7), 18.0 (q, C-18); APCI-MS *m/z* 403 [M + H]$^+$; HR-FT-ICRMS *m/z* 403.2474 (calcd for $C_{24}H_{35}O_5$, 403.2479).

7β,12α-Dihydroxybufalin (3): white powder; $C_{24}H_{34}O_6$; mp 165−167 °C; $[\alpha]^{25}_D$ +13.1° (*c* 1.14, MeOH); UV λ_{max} (MeOH) 206.0, 298.0 nm; IR ν_{max} (KBr) 3415, 2935, 1706, 1627, 1537, 1448, 1245, 1139, 1031, 953 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.95 (1H, dd, J = 10.0 and 2.0 Hz, H-22), 7.43 (1H, d, J = 2.0 Hz, H-21), 6.27 (1H, d, J = 10.0 Hz, H-23), 5.47 (1H, d, J = 4.5 Hz, 7-OH), 5.34 (1H, s, 14-OH), 4.70 (1H, d, J = 4.0 Hz, 12-OH), 4.19 (1H, d, J = 3.0 Hz, 3-OH), 3.85 (1H, s, H-3), 3.80 (1H, br s, H-7), 3.41 (1H, s, H-12), 3.15 (1H, br s, H-17), 0.86 (3H, s, H-19), 0.54 (3H, s, H-18); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 161.4 (s, C-24), 148.7 (d, C-21), 148.0 (d, C-22), 123.3 (s, C-20), 114.3 (d, C-23), 84.4 (s, C-14), 73.7 (d, C-12), 68.9 (d, C-7), 64.2 (d, C-3), 51.0 (s, C-13), 46.3 (d, C-8), 44.0 (d, C-17), 36.6 (d, C-5), 36.5 (t, C-6), 34.8 (t, C-15), 34.2 (t, C-4), 34.2 (s, C-10), 30.3 (t, C-16), 29.1 (t, C-1, C-11), 27.4 (t, C-2), 27.2 (d, C-9), 23.6 (q, C-19), 18.1 (q, C-18); APCI-MS *m/z* 419 [M + H]$^+$; HR-FT-ICRMS *m/z* 419.2422 (calcd for $C_{24}H_{35}O_6$, 419.2428).

Biotransformation of Bufalin (1) by *M. spinosus* AS 3.3450. The biotransformation procedure was the same as described in our previous paper.[10] A total amount of 800 mg of bufalin was used. The biotransformation products were separated on a silica gel column chromatography (135 g, 200−300 mesh, ϕ 3 × 45 cm) and eluted in 100 mL fractions with petroleum ether (60−90 °C) and acetone (4:1 to 1:1, v/v). Fractions 38−55 [petroleum ether−acetone (2:1)] were subjected to preparative liquid chromatography and eluted with MeOH−H_2O (50:50, v/v) to afford **6** (2.1 mg) and **7** (2.4 mg).

7β,15α-Dihydroxybufalin (6): white powder; $C_{24}H_{34}O_6$; mp 251−253 °C; $[\alpha]^{25}_D$ −7.4° (*c* 0.14, MeOH); UV λ_{max}(MeOH) 204.0, 298.0 nm; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.69 (1H, d, J = 10.0 Hz, H-22), 7.52 (1H, s, H-21), 6.27 (1H, d, J = 10.0 Hz, H-23), 5.51 (1H, d, J = 4.0 Hz, 7-OH), 5.45 (1H, s, 14-OH), 4.86 (1H, d, J = 3.0 Hz, 15-OH), 4.21 (2H, br s, H-7, H-15), 4.18 (1H, br s, 3-OH), 3.82 (1H, br s, H-3), 2.68 (1H, t, J = 8.5 Hz, H-17), 0.83 (3H, s, H-19), 0.58 (3H, s, H-18); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 161.3 (s, C-24), 148.9 (d, C-21), 147.2 (d, C-22), 122.0 (s, C-20), 114.4 (d, C-23), 84.1 (s, C-14), 79.0 (d, C-15), 68.5 (d, C-7), 64.4 (d, C-3), 48.9 (d, C-17), 46.7 (s, C-13), 46.5 (d, C-8), 40.6 (t, C-16), 39.0 (t, C-12), 37.1 (t, C-6), 36.7 (d, C-5), 34.7 (s, C-10), 34.3 (t, C-4), 32.7 (d, C-9), 29.3 (t, C-1), 27.5 (t, C-2), 22.5 (q, C-19), 20.8 (t, C-11), 18.2 (q, C-18); APCI-MS *m/z* 419 [M + H]$^+$; HR-FT-ICRMS *m/z* 419.2432 (calcd for $C_{24}H_{35}O_6$, 419.2428).

5β,7β-Dihydroxylbufalin (7): white powder; $C_{24}H_{34}O_6$; mp 162−165 °C; $[\alpha]^{25}_D$ −14.1° (*c* 0.19, MeOH); UV λ_{max}(MeOH) 205.0, 301.0 nm; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.93 (1H, dd, J = 10.0 and 2.0 Hz, H-22), 7.51 (1H, d, J = 2.0 Hz, H-21), 6.26 (1H, d, J = 10.0 Hz, H-23), 5.67 (1H, br s, 7-OH), 5.42 (1H, s, 14-OH), 5.20 (1H, br s, 3-OH), 4.84 (1H, br s, 5-OH), 3.95 (1H, br s, H-3), 3.63 (1H, br s, H-7), 0.82 (3H, s, H-19), 0.60 (3H, s, H-18); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 161.3 (s, C-24), 149.2 (d, C-21), 147.5 (d, C-22), 122.5 (s, C-20), 114.3 (d, C-23), 84.4 (s, C-14), 73.2 (s, C-5), 69.9 (d, C-7), 66.3 (d, C-3), 49.9 (d, C-17), 47.3 (s, C-13), 45.3 (d, C-8), 44.6 (t, C-6), 40.2 (t, C-12), 39.6 (s, C-10), 37.9 (t, C-4), 36.3 (d, C-9), 33.2 (t, C-15), 28.4 (t, C-16), 27.0 (t, C-2), 24.8 (t, C-1), 21.4 (t, C-11), 16.8 (q, C-19), 16.7 (q, C-18); APCI-MS *m/z* 419 [M + H]$^+$; HR-FT-ICRMS *m/z* 419.2425 (calcd for $C_{24}H_{35}O_6$, 419.2428).

Bioassay. Human hepatoma Bel-7402 cells, human gastric cancer BGC-823 cells, and human cervical carcinoma HeLa cells were maintained in RPMI 1640 medium (GIBCO/BRL, Rockville, MD) supplemented with 10% (v/v) fetal bovine serum and cultured in 96-well microtiter plates for the assay. Appropriate dilutions (10^{-3}−10^2 μmol/L) of the test compounds were added to the cultures. After incubation at 37 °C, 5% CO_2 for 72 h, the survival rates of the cancer cells were evaluated by the MTT method.[12] The activity was shown as IC_{50} value, which is the concentration (μmol/L) of test compound to give 50% inhibition of cell growth. Results are expressed as the mean value of triplicate determinations.

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