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Microbial Transformations of the Antimelanoma Agent Betulinic Acid

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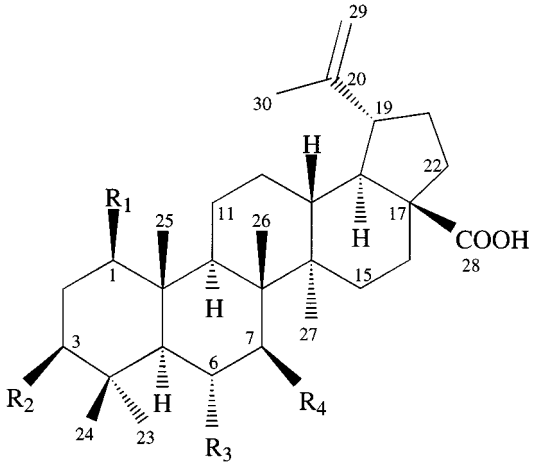
Microbial transformation studies of the antimelanoma agent betulinic acid (**1**) were conducted. Screening experiments showed a number of microorganisms capable of biotransforming **1**. Three of these cultures, *Bacillus megaterium* ATCC 14581, *Cunninghamella elegans* ATCC 9244, and *Mucor mucedo* UI-4605, were selected for preparative scale transformation. Bioconversion of **1** with resting-cell suspensions of phenobarbital-induced *B. megaterium* ATCC 14581 resulted in the production of the known betulonic acid (**2**) and two new metabolites: 3*β*,7*β*-dihydroxy-lup-20(29)-en-28-oic acid (**3**) and 3*β*,6α,7*β*-trihydroxy-lup-20(29)-en-28-oic acid (**4**). Biotransformation of **1** with growing cultures of *C. elegans* ATCC 9244 produced one new metabolite characterized as 1*β*,3*β*,7*β*-trihydroxy-lup-20(29)-en-28-oic acid (**5**). Incubation of **1** with growing cultures of *M. mucedo* UI-4605 afforded metabolite **3**. Structure elucidation of all metabolites was based on NMR and HRMS analyses. In addition, the antimelanoma activity of metabolites **2**−**5** was evaluated against two human melanoma cell lines, Mel-1 (lymph node) and Mel-2 (pleural fluid).

Betulinic acid (**1**), 3*β*-hydroxy-lup-20(29)-en-28-oic acid, is a widely distributed pentacyclic lupane-type triterpene in the plant kingdom.[1,2] Betulinic acid has been shown to exhibit a variety of biological activities, including inhibition of Human Immunodeficiency Virus (HIV) replication in H9 lymphocyte cells,[3] blockage of HIV-1 entry into cells,[4] and inhibition of DNA polymerase *β*.[5] Synthetic derivatives of **1** have also been investigated as specific inhibitors of HIV-1.[6,7] In addition, betulinic acid has been reported as a melanoma-specific cytotoxic agent[8] in both in vitro cell culture and in vivo studies. The antitumor activity of **1** is mediated by the induction of apoptosis,[8] as demonstrated by a variety of cellular responses. Due to its high antitumor activity and lack of toxicity, betulinic acid is an attractive and promising new lead compound against human melanoma.

An essential component of preclinical development of a drug is the elucidation of its mammalian metabolism. Because there have been no reports on the mammalian metabolism of betulinic acid (**1**), a prospective approach was undertaken to study the metabolism of **1** utilizing microorganisms as in vitro model systems. Selected microorganisms have been utilized successfully as in vitro models to mimic and predict the metabolic fate of drugs and other xenobiotics in mammalian systems.[9−12] In an earlier study, we described the structure elucidation and biological activity of a fungal metabolite of **1**, 28-*O*-*β*-D-glucopyranosyl 3*β*-hydroxy-lup-20(29)-en-28-oate, which was isolated from resting-cell suspensions of *Cunninghamella* species NRRL 5695.[13] In the present study, we report the isolation, structure elucidation, and in vitro antimelanoma activity of four new metabolites of **1**. Incubation of **1** with resting-cell suspensions of phenobarbital-induced *Bacillus megaterium* ATCC 14581 resulted in the production of three metabolites: 3-oxo-lup-20(29)-en-28-oic acid (**2**), 3*β*,7*β*-dihydroxy-lup-20(29)-en-28-oic acid (**3**), and 3*β*,6α,7*β*-trihydroxy-lup-20(29)-en-28-oic acid (**4**). Metabolite **5**, 1*β*,3*β*,7*β*-trihydroxy-lup-20(29)-en-28-oic acid, was isolated from incubations with *Cunninghamella elegans* ATCC 9244. In addition, *Mucor mucedo* UI-4605 was able to biotransform **1** to metabolite **3**. Metabolites **2**−**5** were characterized based on 1D and 2D NMR experiments and HRMS analyses.



Compound	R_1	R_2	R_3	R_4
1	H	OH	H	H
2	H	=O	H	H
3	H	OH	H	OH
4	H	OH	OH	OH
5	OH	OH	H	OH

Results and Discussion

Three cultures, *B. megaterium* ATCC 14581, *C. elegans* ATCC 9244, and *M. mucedo* UI-4605, were selected for preparative scale transformation of betulinic acid (**1**). These

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cultures were capable of reproducibly biotransforming **1** to several metabolites. In addition, the monooxygenase systems in the three cultures have demonstrated a high degree of similarity with mammalian microsomal monooxygenases and the potential to serve as in vitro models of mammalian drug metabolism.[10,17−20] Preliminary screening of **1** using resting-cell suspensions of *B. megaterium* revealed that **4** was the only biotransformation product of **1**. However, incubation of **1** with phenobarbital-induced *B. megaterium* yielded two additional biotransformation products, metabolites **2** and **3**. A preparative scale biotransformation of **1** using resting-cell suspensions of phenobarbital-induced *B. megaterium* in a phosphate buffer afforded metabolites **2**−**4** in 0.44%, 0.085%, and 0.45% yield, respectively. Incubation of **1** with growing cells of *M. mucedo* UI-4605 also resulted in the production of metabolite **3** in 1.3% yield.

The physical and spectral data of **2** were in agreement with the reported data for betulonic acid.[14] Betulonic acid (**2**) is an intermediate in the synthesis of **1** from betulin[15] and has previously been isolated from a medicinal herb.[16] This is the first report of **2** as a microbial transformation product of **1**.

The HRMS of **3** showed a $[M + Na]^+$ ion at *m/z* 495.3457. The IR spectrum of metabolite **3** revealed absorptions at 3436 (OH) and 1701 cm^{-1} (C=O, carboxylic acid). Compared to **1**, the ^{13}C NMR spectrum of **3** exhibited a new signal at δ 75.5, while the ^1H NMR spectrum of **3** showed a new double doublet at δ 4.13 (1H, J = 4.7, 10.3 Hz). The DEPT experiment showed the presence of a new CH signal at δ 75.5 and the disappearance of a CH_2 signal, confirming that **3** was a monohydroxylated metabolite of **1**. The new CH signal was unambiguously assigned to C-7 based on one-bond and long-range (two- and three-bond) $^1H-^{13}C$ correlation experiments. In these experiments, the new proton signal at δ 4.13 showed correlations with C-8, C-14, C-26, and the new carbon signal at δ 75.5, confirmimg C-7 as the site of hydroxylation. Furthermore, C-4 and C-10 were unambiguously assigned based on these long-range C−H correlation experiments. Two-bond correlations were observed between C-4 and the protons of CH_3-23 and CH_3-24, and between C-10 and the protons of CH_3-25. The relative stereochemistry of the hydroxyl group at C-7 in **3** was established as β(equatorial) based on NOESY data. The NOESY spectrum of **3** revealed enhancements between H-7 and H-5, H-9, and CH_3-27, thereby indicating that H-7 must be α(axial). Based on all the evidence, metabolite **3** was characterized as 3β,7β-dihydroxy-lup-20(29)-en-28-oic acid.

Metabolite **4** (0.45% yield) was the major metabolite of **1** from *B. megaterium* ATCC 14581 resting-cell suspensions. The HRFABMS of metabolite **4** showed a $[M + Na]^+$ ion at *m/z* 511.3405, indicating that two oxygen atoms were added to the structure of **1**. Compared to **1**, the ^1H NMR spectrum of **4** revealed two new proton signals at δ 4.67 (1H, dd, J = 4.8, 11.5 Hz) and 4.18 (1H, dd, J = 5.1, 10.8 Hz). The DEPT spectrum showed the presence of two new CH signals at δ 73.1 and 68.9 and the disappearance of two CH_2 signals, confirming that **4** was a dihydroxylated metabolite of **1**. The two new carbon signals at δ 68.9 and 73.1 in **4** were assigned to C-6 and C-7, respectively, based on one-bond and long-range (two- and three-bond) $^1H-^{13}C$ correlation experiments. A one-bond correlation was observed between the new proton signal at δ 4.18, which was assigned to H-7 based on NOESY data, and C-7. In addition, a three-bond correlation was observed between C-7 and H-9. Furthermore, the new proton signal at δ 4.67, which was assigned to H-6 based on NOESY data, showed correlations with C-6 and C-10.

Table 1. Cytotoxicity of Compounds **1**−**5**

	human melanoma cell lines	
compound	Mel-1	Mel-2
1	3.3[a]	1.0
2	16.0	0.1
3	17.1	7.2
4	10.9	16.8
5	>20	>20

[a] Results are shown as ED_{50} values (μg/mL).

The relative stereochemistry of the two hydroxyl groups at C-6 and C-7 in **4** was assigned as α(equatorial) and β(equatorial), respectively, based on NOESY experiments. The NOESY spectrum of **4** showed NOE enhancements between H-6 and protons H-11 and CH_3-26, indicating that H-6 must be β(axial). In addition, NOE enhancements were observed between H-7 and protons H-5, H-9, and CH_3-27, confirming that H-7 is α(axial). Based on all these observations, metabolite **4** was characterized as 3β,6α,7β-trihydroxy-lup-20(29)-en-28-oic acid.

Preparative scale biotransformation of **1** using growing cultures of *C. elegans* ATCC 9244 afforded metabolite **5** in 0.37% yield. Similar to **4**, the HRESIMS of **5** showed a $[M + Na]^+$ ion at *m/z* 511.3403. Compared to **1**, the ^1H NMR spectrum of **5** revealed two new proton signals at δ 4.15 (1H, dd, J = 4.8, 10.8 Hz) and 3.75 (1H, dd, J = 4.4, 11.0 Hz). The DEPT spectrum showed the presence of two new CH signals at δ 79.9 and 73.8, and the disappearance of two CH_2 signals, indicating that **5** was a dihydroxylated metabolite of **1**. The two new carbon signals at δ 79.9 and 73.8 in **5** were assigned to C-1 and C-7, respectively, based on HMQC and HMBC data. Two- and three-bond correlations were observed between H-7 and C-8, C-9, and C-26. In addition, a one-bond correlation was observed between H-7 (δ 4.15) and C-7. Furthermore, H-1 (δ 3.75) was correlated with C-1, C-5, C-10, and C-25.

The relative stereochemistry of the two hydroxyl groups at C-1 and C-7 in **5** was established as β(equatorial) based on NOESY experiments. The NOESY spectrum of **5** showed NOE enhancements between H-1 and H-9, indicating that H-1 must be α(axial). In addition, NOE enhancements were observed between H-7 and protons H-9 and CH_3-27, confirming that H-7 is α(axial). Based on all the evidence, metabolite **5** was characterized as 1β,3β,7β-trihydroxy-lup-20(29)-en-28-oic acid. ^{13}C and ^1H NMR chemical shift assignments of metabolites **3**−**5** were based on $^1H-^1H$ and $^1H-^{13}C$ chemical shift correlation experiments and comparisons to the assignments of **1**.[21,22] The in vitro cytotoxicity of **1**−**5** was evaluated against two human melanoma cell lines, Mel-1 (lymph node) and Mel-2 (pleural fluid). The ED_{50} values (μg/mL) for compounds **1**−**5** are listed in Table 1. Compared to **1**, metabolite **5** showed no activity (ED_{50} >20 μg/mL), whereas metabolites **3** and **4** were less active against both Mel-1 and Mel-2. Metabolite **2** was also less active than the parent compound **1** against Mel-1. However, metabolite **2** was more active than **1** against Mel-2. In addition, metabolite **2** exhibited a high degree of selectivity against Mel-2 in comparison to **1** and the other tested metabolites. The cytotoxicity results indicate that the in vitro antimelanoma activity of **1** was significantly altered by oxidation at different sites of the molecule. The results also demonstrate that the structural requirements for cytotoxicity in the tested molecules differ from one melanoma cell line to the other.

The main objective of our research efforts is to utilize microorganisms as in vitro models to predict and prepare

potential mammalian metabolites of **1**. The potential value of microbiological transformations as a powerful tool in facilitating drug metabolism studies has been extensively reviewed.[9,10,12] In the present report, three cultures were selected for preparative scale transformation of **1**, *B. megaterium* ATCC 14581, *C. elegans* ATCC 9244, and *M. mucedo* UI-4605, yielding four metabolites of **1**, **2**−**5**. The generated microbial metabolites of **1** were evaluated for antimelanoma activity (Table 1) and will serve as reference standards in establishing analytical procedures for the identification of the metabolites of **1** in mammalian systems.

Microbial cytochrome P450 systems display properties remarkably similar to known adrenal mitochondrial and hepatic microsomal systems. Due to their high similarity with mammalian cytochrome P450 systems, several eukaryotic and prokaryotic monooxygenase systems have been studied extensively as models of human and other mammalian P450 systems. One of these models is CYP 102 (BM-3), which is produced by *B. megaterium* ATCC 14581. CYP102, which exhibits selectivity for compounds containing a carboxylic acid moiety, is a multifunctional enzyme that has been categorized as a class II (microsomal) cytochrome P450 system due to its structural similarity with mammalian cytochrome P450 enzymes.[17−19] The crystal structure of CYP102 has been used to construct templates for homology modeling of human microsomal P450 systems.[20] In addition, monooxygenase systems in eukaryotic organisms, such as *M. mucedo* and *C. elegans*, have demonstrated a high degree of parallelism to hepatic microsomal monooxygenase systems.[10] These studies, which strongly indicated the potential for these cultures to serve as models for mammalian drug metabolism, constituted the basis for our investigation of the biotransformation of betulinic acid (**1**) by *B. megaterium*, *C. elegans*, and *M. mucedo*.

CYP 102, the P450 system produced by *B. megaterium* ATCC 14581, is a barbiturate-inducible cytochrome P450. In an effort to optimize conditions for the biotransformation of **1** by CYP 102 and other P450 systems in *B. megaterium*, all incubations of **1** with *B. megaterium* in this study were conducted following P450 induction with phenobarbital sodium. Three metabolites of **1**, **2**−**4**, were isolated from resting-cell suspensions of phenobarbital-induced *B. megaterium*, whereas only one metabolite of **1**, metabolite **4**, was recovered from incubations with uninduced *B. megaterium*.

Preliminary screening studies indicated that the biotransformation of **1** to metabolites **2**−**4** by growing cells of *B. megaterium* was low yielding, which necessitated the optimization of incubation, extraction, and chromatographic procedures for the isolation and purification of metabolites. In an attempt to improve the yield of metabolites **2**−**4**, the use of resting-cell suspensions of *B. megaterium* was investigated. In resting-cell incubations, the cells of the biocatalyst, suspended in a phosphate buffer, are alive but not growing. The buffer affords a much cleaner incubation medium when compared to the complex culture medium used for growing cultures, eliminating any interference from the incubation medium with the bioavailability of the substrate and/or extraction of metabolites. Incubation of **1** with resting-cell suspensions of *B. megaterium* resulted only in a modest (4−7%) increase in the yields of metabolites **2**−**4**. Incubation of **1** with resting-cell suspensions of *C. elegans* and *M. mucedo*, however, did not result in a significant increase in the yields of metabolites **5** and **3**, respectively.

Experimental Section

General Experimental Procedures. Melting points were determined in open capillary tubes with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 digital polarimeter. IR spectra were recorded in KBr using a Nicolet Impact 400D infrared spectrophotometer. The term *in vacuo* refers to removal of solvent with a rotary evaporator under water aspirator vacuum (15−30 mmHg). Centrifugation was carried out using a Heraeus Megafuge 2.0R centrifuge at 4° C and 2800*g*. ^{1}H and ^{13}C NMR spectra were obtained in C_5D_5N on a JEOL-Eclipse 400 FT-NMR spectrometer operating at 400 and 100 MHz, respectively. The chemical shifts are reported in ppm (δ), and the coupling constants (J values) are in Hz. Standard pulse sequences were used for APT, COSY, DEPT, FLOCK, HETCOR, HMBC, HMQC, and NOESY experiments. HRMS were obtained using Mariner Electrospray TOF (PE Biosystems, Foster City, CA) and VG 70-SEQ (VG Analytical, Manchester, England) mass spectrometers at the Mass Spectrometry Facility, Department of Medicinal Chemistry, University of Washington, Seattle, WA 98195.

Chromatographic Conditions. TLC analyses were carried out on precoated Si gel 60 UV_{254} (E. Merck, Darmstadt) and C_{18} Si gel UV_{254} (Macherey-Nagel) plates. The adsorbents used for column chromatography were Si gel 60 Å, 70−230 mesh (Aldrich Chemical Co., Milwaukee, WI), lipophilic Sephadex LH-20 (Sigma Chemical Co., MO), and C_{18} Si gel (Aldrich, Milwaukee, WI). The solvent systems used for TLC were $CHCl_3$−MeOH (9:1, v/v) and CH_3CN−0.1% TFA (8:2, v/v). The visualization of TLC plates was performed using anisaldehyde−H_2SO_4 spray reagent. The spots were visualized by spraying the plate and then heating it at 110 °C for 3 min in an oven. All solvents were of reagent-grade quality or better. Dimethyl formamide (DMF) and pyridine (C_5H_5N) were stored over 4-Å molecular sieves.

Microorganisms and Media. *B. megaterium* ATCC 14581 and *C. elegans* ATCC 9244 were obtained from the American Type Culture Collection, Manassas, VA. *M. mucedo* UI-4605 was obtained from the Department of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA. All preliminary screening experiments were carried out in a complex medium consisting of dextrose, 20 g; yeast extract, 5 g; peptone, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; and distilled H_2O, 1000 mL. Stock cultures of *B. megaterium* ATCC 14581 were maintained on slants of Nutrient Agar (Difco), while stock cultures of *C. elegans* ATCC 9244 and *M. mucedo* UI-4605 were maintained on Mycological Agar (Difco) at 4 °C. The 0.1 M phosphate buffer (pH 7.2) used for resting-cell suspensions of *B. megaterium* consisted of K_2HPO_4, 10.6 g; KH_2PO_4, 4.08 g; and distilled H_2O, 1000 mL.

Fermentation Procedures. Microbial metabolism studies were carried out by incubating the cultures with shaking on a Innova 5000 Digital Tier Shaker (New Brunswick Scientific Co., NJ), operating at 150 rpm and 25 °C. Preliminary screening experiments were conducted in 125-mL foam-plugged culture flasks, each containing 25 mL of medium. The media were sterilized at 121 °C and 18 psi for 15 min. Fermentations were carried out according to a standard two-stage protocol. In general, the substrate was added to the incubation media 24 h after inoculation of stage II cultures as a 5% solution in DMF at a concentration of 0.1 mg/mL of stage II medium. Cultures were sampled at 24-h intervals by extracting 3 mL of the broth with 3 mL of EtOAc. The extracts were concentrated and chromatographed on TLC plates. Substrate controls were composed of sterile medium to which the substrate was added and incubated without the microorganism. Culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions without the addition of the substrate.

Biotransformation of Betulinic Acid (1) to Metabolites 2−4 by *B. megaterium* ATCC 14581. Betulinic acid (**1**) was obtained from Indofine Chemical Co. (Somerville, NJ). The physical and spectral data of **1** have previously been reported

in the literature.[21,22] A standard two-stage fermentation protocol was adopted for the bioconversion of **1**. Ten 125-mL flasks, each containing 0.1 mL of *B. megaterium* ATCC 14581 inoculum in 25 mL of a sterile complex medium supplemented with 3 g/L of beef extract, were incubated on the shaker for 3 days at room temperature. Phenobarbital sodium (50 mg) in sterile water was added to each 125-mL flask at 24, 48, and 72 h following inoculation. After the 72-h incubation period, stage I cultures were transferred to 1-L flasks containing 200 mL of beef-extract-enriched complex medium and then incubated on the shaker for 24 h. At the end of 24 h, the cells were harvested from each flask by centrifugation at 2800 × *g* and 4° C, washed with sterile distilled H_2O, and resuspended in 60 2-L flasks, each containing 400 mL of sterile 0.1 M phosphate buffer (pH 7.2). A total of 2.4 g of **1** was then dissolved in 2.4 mL of DMF and distributed equally among the 60 2-L resting-cell suspension flasks. After 6 days of incubation on the shaker, the suspensions were pooled together and extracted with 3 × 8 L of EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated in vacuo to afford a yellowish residue. The residue was chromatographed on a Si gel column using $CHCl_3$−MeOH (90:10, v/v) as an eluent to yield two fractions, A and B.

Fraction A was subjected to repeated Si gel column chromatography with a gradient of ethyl acetate−hexane (0:100 to 15:85, v/v), followed by a final purification step on a Sephadex LH-20 column with $CHCl_3$, which afforded homogeneous fractions exhibiting a single spot on TLC (R_f 0.78). The homogeneous fractions were combined and evaporated in vacuo to give metabolite **2**. Crystallization from EtOAc−MeOH (90:10, v/v) afforded white needles of metabolite **2** (6 mg, 0.44% yield). The physical and spectral data of **2** were consistent with the reported data for betulonic acid.[16]

Fraction B was subjected to a Sephadex LH-20 column using $CHCl_3$ as an eluent, which afforded two fractions, B1 and B2. Fraction B1 was loaded on a Si gel TLC plate and chromatographed using ethyl acetate−hexane (40:60, v/v), followed by a final purification step on a Sephadex LH-20 column with $CHCl_3$ to afford metabolite **3** (R_f 0.47, 2.2 mg, 0.085% yield). Fraction B2 was subjected to repeated Si gel column chromatography with a gradient of ethyl acetate−hexane (20:80 to 100:0, v/v), followed by a gradient of MeOH−EtOAc (1:99 to 10:90, v/v), which afforded metabolite **4** (R_f 0.32). Crystallization from MeOH−EtOAc (1:9, v/v) yielded white needles of metabolite **4** (9 mg, 0.45% yield).

Biotransformation of Betulinic Acid (1) to Metabolite 5 by *C. elegans* ATCC 9244. Twenty 1-L flasks, each containing 0.5 mL of *C. elegans* ATCC 9244 inoculum in 200 mL of sterile complex medium, were incubated on the shaker for 3 days at room temperature. Following the 72-h incubation period, stage I cultures were transferred to 40 1-L flasks, each containing 200 mL of sterile complex medium, and then incubated on the shaker for 24 h. At the end of 24 h, a total of 800 mg of **1** was dissolved in 0.8 mL of DMF and distributed equally among the 40 1-L flasks. After 15 days of incubation on the shaker, the cells were filtered, and the filtrate was extracted with 3 × 1.3 L of EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated in vacuo to afford 2.2 g of a brownish residue. The residue was chromatographed on a C_{18} Si gel column using CH_3CN−0.1% TFA (80:20, v/v) as an eluent to yield fraction A.

Fraction A was subjected to repeated Si gel column chromatography with a gradient of ethyl acetate−hexane (0:100 to 40:60, v/v), followed by a purification step on a Sephadex LH-20 column with MeOH to yield metabolite **5**. Crystallization from EtOAc−MeOH (90:10, v/v) afforded white needles of metabolite **5** (R_f 0.5, 3.2 mg, 0.37% yield).

Biotransformation of Betulinic Acid (1) to Metabolite 3 by *M. mucedo* UI-4605. Twenty 1-L flasks, each containing 0.1 mL of *M. mucedo* UI-4605 inoculum in 200 mL of sterile complex medium, were incubated on the shaker for 3 days at room temperature. Following the 72-h incubation period, stage I cultures were transferred to 55 1-L flasks, each containing 200 mL of sterile complex medium, and then incubated on the shaker for 24 h. At the end of 24 h, a total of 1.1 g of **1** was dissolved in 2.2 mL of DMF and distributed equally among the 55 stage II culture flasks. After 12 days of incubation on the shaker, the cells were filtered, and the filtrate was extracted with 3 × 4.3 L of EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated in vacuo to afford 1.6 g of a brownish residue. The residue was chromatographed on a Si gel column using ethyl acetate−hexane (80:20, v/v) as an eluent to yield fraction A.

Fraction A was subjected to C_{18} Si gel column chromatography, with a gradient of CH_3CN−0.1% TFA (100:0 to 75:25, v/v), followed by a final purification step on a Si gel column with $CHCl_3$−MeOH (97:3, v/v). Crystallization from ethyl acetate−hexane (10:90, v/v) afforded white flakes of metabolite **3** (9.2 mg, 1.3% yield).

Metabolite 3: mp 265−267 °C; $[\alpha]^{25}_D$ −14.63° (*c* 0.68 g/100 mL, C_5H_5N); IR (KBr) ν_{max} 3436, 2944, 1701 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) δ 4.96 (1H, s, H-29), 4.78 (1H, s, H-29), 4.13 (1H, dd, J = 4.7, 10.3 Hz, H-7), 3.56−3.47 (1H, m, H-18), 3.45 (1H, t, J = 8.0 Hz, H-3), 2.84 (1H, m, H-13), 2.70−2.52 (1H, m, H-16), 2.48−2.42 (1H, m, H-6), 2.35−2.32 (1H, m, H-6), 2.29−2.25 (2H, m, H-15, H-22), 1.99−1.97 (2H, m, H-12, H-21), 1.81−1.78 (3H, m, H-2, H-19), 1.76 (3H, s, Me-30), 1.76−1.74 (1H, m, H-21), 1.69−1.66 (2H, m, H-1, H-16), 1.57−1.54 (1H, m, H-22), 1.51−1.48 (2H, m, H-11, H-15), 1.38 (3H, s, Me-26), 1.38−1.36 (2H, m, H-9, H-11) 1.27 (3H, s, Me-27), 1.20 (3H, s, Me-23), 0.99(3H, s, Me-24), 0.99−0.97 (2H, m, H-1, H-5), 0.88 (3H, s, Me-25); ^{13}C NMR (C_5D_5N,100 MHz) δ 39.8 (t, C-1), 28.9 (t, C-2), 78.5 (d, C-3), 39.8 (s, C-4), 53.7 (d, C-5), 34.6 (t, C-6), 75.5 (d, C-7), 47.8 (s, C-8), 51.7 (d, C-9), 38.2 (s, C-10), 21.9 (t, C-11), 26.9 (t, C-12), 39.9 (d, C-13), 44.3 (s, C-14), 31.7 (t, C-15), 33.8 (t, C-16), 57.1 (s, C-17), 48.5 (d, C-18), 50.2 (d, C-19), 152.1 (s, C-20), 30.8 (t, C-21), 38.2 (t, C-22), 29.1 (q, C-23), 16.8 (q, C-24 and C-25), 11.5 (q, C-26), 15.7 (q, C-27), 179.5 (s, C-28), 110.3 (t, C-29), 20.1 (q, C-30); HRESIMS *m/z* 495.3457 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_4Na$, 495.3450).

Metabolite 4: mp 280−282 °C; $[\alpha]^{25}_D$ −30.05° (*c* 1.21 g/100 mL, C_5H_5N); IR (KBr) ν_{max} 3448, 2958, 1690 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) δ 4.94 (1H, s, H-29), 4.78 (1H, s, H-29), 4.67 (1H, dd, J = 4.8, 11.5 Hz, H-6), 4.18 (1H, dd, J = 5.1, 10.8 Hz, H-7), 3.54−3.48 (1H, m, H-18), 3.45 (1H, t, J = 8.4 Hz, H-3), 2.70−2.65 (1H, m, H-13), 2.36−2.33 (1H, m, H-16), 2.22−2.18 (1H, m, H-22), 2.12−2.08 (2H, m, H-21), 1.98−1.87 (1H, m, H-12), 1.86−1.84 (2H, m, H-2, H-19),1.81 (3H, s, Me-30), 1.67−1.57 (3H, m, H-1, H-16, H-22), 1.54−1.51 (2H, m, H-11, H-16), 1.41 (3H, s, Me-26), 1.40−1.32 (3H, m, H-9, H-11, H-12), 1.39 (3H, s, Me-27), 1.29−1.24 (3H, m, H-2, H-15), 1.25 (3H, s, Me-23), 1.04 (3H, s, Me-24), 0.99−0.91 (2H, m, H-1, H-5), 0.89 (3H, s, Me-25); ^{13}C NMR (C_5D_5N,100 MHz) δ 39.1 (t, C-1), 28.1 (t, C-2), 77.9 (d, C-3), 39.2 (s, C-4), 52.7 (d, C-5), 68.9 (d, C-6), 73.1 (d, C-7), 48.3 (s, C-8), 51.1 (d, C-9), 38.1 (s, C-10), 20.7 (t, C-11), 26.1 (t, C-12), 37.9 (d, C-13), 42.5 (s, C-14), 29.9 (t, C-15), 31.8 (t, C-16), 55.1 (s, C-17), 47.6 (d, C-18), 49.7 (d, C-19), 150.7 (s, C-20), 28.9 (t, C-21), 37.5 (t, C-22), 28.3 (q, C-23), 16.2 (q, C-24 or C-25), 15.8 (q, C-25 or C-24), 9.1 (q, C-26), 11.3 (q, C-27), 179.1 (s, C-28), 109.9 (t, C-29), 19.4 (q, C-30); FABMS *m/z* 511 $[M + Na]^+$; HRFABMS *m/z* 511.3405 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_5Na$, 511.3399).

Metabolite 5: mp 262−265 °C; $[\alpha]^{25}_D$ −10.9° (*c* 0.28 g/100 mL, C_5H_5N); IR (KBr) ν_{max} 3460, 2940, 1689 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) δ 4.92 (1H, s, H-29), 4.74 (1H, s, H-29), 4.15 (1H, dd, J = 4.8, 10.8 Hz, H-7), 3.75 (1H, dd, J = 4.4, 11.0 Hz, H-1), 3.60−3.50 (2H, m, H-3, H-18), 3.19−3.16 (1H, m, H-11), 2.92−2.87 (1H, m, H-13), 2.72−2.68 (1H, m, H-6), 2.43−2.41 (1H, m, H-6), 2.37−2.34 (1H, m, H-2), 2.28−2.22 (3H, m, H-2, H-16, H-22), 2.10−2.00 (2H, m, H-12, H-15), 1.97−1.94 (1H, m, H-15), 1.86 (1H, br s, H-19), 1.83 (1H, br s, H-9), 1.81 (3H, s, Me-30), 1.79−1.70 (1H, m, H-11), 1.62−1.52 (2H, m, H-16, H-22), 1.45 (3H, s, Me-26), 1.37−1.35 (1H, m, H-12), 1.33 (3H, s, Me-27), 1.31−1.27 (1H, m, H-21), 1.22 (3H, s, Me-25), 1.20 (3H, s, Me-23), 1.04 (3H, s, Me-24), 1.00−0.98 (1H, m, H-5); ^{13}C NMR (C_5D_5N,100 MHz) δ 79.9 (d, C-1), 39.2 (t, C-2), 78.5 (d, C-3), 38.9 (s, C-4), 52.4 (d, C-5), 33.7 (t, C-6), 73.8 (d, C-7), 47.6 (s, C-8), 50.5 (d, C-9), 47.6 (s, C-10), 23.5 (t, C-11), 26.3 (t, C-12), 38.8 (d, C-13), 44.0 (s, C-14), 30.8 (t, C-15), 33.0 (t, C-16), 56.0 (s, C-17), 47.6 (d, C-18), 49.4 (d, C-19), 151.0 (s,

C-20), 29.5 (t, C-21), 37.2 (t, C-22), 28.0 (q, C-23), 15.5 (q, C-24), 10.7 (q, C-25), 12.3 (q, C-26), 14.8 (q, C-27), 178.7 (s, C-28), 109.2 (t, C-29), 19.0 (q, C-30); HRESIMS *m/z* 511.3403 [M + Na]$^+$ (calcd for $C_{30}H_{48}O_5Na$, 511.3399).

In Vitro Cytotoxicity Assay. The antimelanoma activity of **1**−**5** was determined against two cultured human melanoma cell lines, Mel-1 (lymph node) and Mel-2 (pleural fluid), as described previously.[8]

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