Glucosidation of Betulinic Acid by *Cunninghamella* Species

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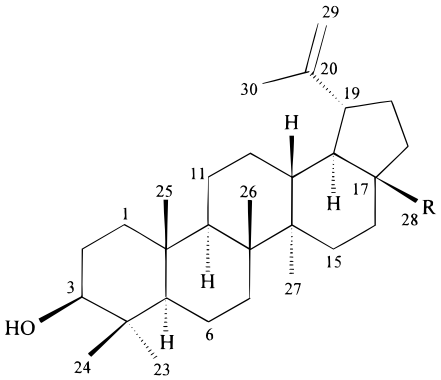
Microbial transformation of the antimelanoma agent betulinic acid (**1**) was studied. Preparative scale biotransformation with resting-cell suspensions of *Cunninghamella* species NRRL 5695 resulted in the production of a fungal metabolite of **1**, which has been characterized as 28-*O*-*β*-D-glucopyranosyl 3*β*-hydroxy-lup-20(29)-en-28-oate (**2**) based on spectral and enzymic data. The in vitro cytotoxicity assay of metabolite **2** revealed no activity against several human melanoma cell lines.

The lupane-type pentacyclic triterpene betulinic acid (**1**), 3*β*-hydroxy-lup-20(29)-en-28-oic acid, is widely distributed in nature. Considerable amounts of betulinic acid (up to 2.5%) are available in the outer bark of a variety of tree species that are valuable for timber purposes.[1] A closely related compound, betulin (**3**), is a major constituent of white-barked birch trees (*Betula* species) with yields up to 22% (dry wt).[2,3] Compound **3** can easily be converted to **1** in high yield.[4] The triterpene **1** has been shown to exhibit a variety of biological activites,[5−11] including inhibition of human immunodeficiency virus (HIV) replication in H9 lymphocyte cells,[9] blockage of HIV-1 entry into cells,[10] and cytotoxicity against a variety of cultured human tumor cells.[11] Synthetic derivatives of **1** have also been investigated as specific inhibitors of HIV-1.[12,13] Recently, betulinic acid (**1**) was identified as a melanoma-specific cytotoxic agent in both in vitro cell culture and in vivo studies.[14] The antitumor activity of **1** was mediated by the induction of apoptosis, as demonstrated by a variety of cellular responses.[14] Due to its favorable therapeutic index in these studies, betulinic acid (**1**) is currently undergoing preclinical development for treatment or prevention of malignant melanoma.

An important factor in the evaluation of the efficacy and safety of a drug is the study 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, particularly fungi, have been utilized successfully as in vitro models to mimic and predict the metabolic fate of drugs and other xenobiotics in mammalian systems.[15−17] In this study we report the isolation and structure elucidation of 28-*O*-*β*-D-glucopyranosyl 3*β*-hydroxy-lup-20(29)-en-28-oate (**2**), a conjugated fungal metabolite of betulinic acid (**1**) from resting-cell suspensions of *Cunninghamella* species NRRL 5695. This is the first report describing the biotransformation of the triterpene **1** and the isolation of the new glucopyranosyl ester **2**.

A total of 13 fungal cultures was screened for the ability to catalyze the bioconversion of betulinic acid (**1**). *Cunninghamella* species NRRL 5695 was the only culture capable of reproducibly bioconverting **1** to a more polar metabolite (**2**). Preparative biotransformation of **1** using



1 R = COOH
2 R = COO-*β*-D-Glucose
3 R = CH_2OH

resting-cell suspensions of *Cunninghamella* species afforded metabolite **2** in 0.77% yield. The parent compound (**1**) was recovered unchanged from the incubations in 92% yield. Preliminary screening studies revealed that **1** is somehow being trapped inside the cells or at the cell wall, which may explain the low yield of its bioconversion to **2**. In addition, our studies showed that metabolite **2** is not excreted extracellularly by *Cunninghamella* species. These observations were based on the fact that we were not able to recover any detectable quantities of **1** and **2** after extraction of the aqueous buffer. As a result, recovery of **1** and **2** was accomplished by homogenizing the cells with an ultrasonic cell disruptor at 20 kHz, followed by extraction of the cell homogenate with ethyl acetate.

Metabolite **2** was isolated and purified by repeated column chromatography. The HRFABMS of metabolite **2** showed a molecular ion at *m*/*z* 641.3992 (calcd for $C_{36}H_{58}O_8$ Na, 641.4029). The IR spectrum of **2** showed absorptions at 3400 (OH) and 1734 cm^{-1} (ester). The ^{13}C and ^{1}H NMR spectra of **2** showed new proton and carbon signals with chemical shifts that are characteristic of *β*-D-glucose. The anomeric proton H-1′ of the glucose moiety in **2** resonated as a doublet at 6.45 ppm. A coupling constant of 8.42 Hz for H-1′ indicated that the stereochemistry of the glucosidic linkage at C-1′ of D-glucose is *β*. The *β* stereochemistry at C-1′ was further confirmed by enzymic hydrolysis of the glucosidic linkage in **2** using *β*-glucosidase enzyme. Comparisons of the ^{13}C NMR spectra of **1** and **2** indicated that **2** is a glucopyranosyl ester of **1** at C-28, based on the upfield

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Table 1. ^{13}C NMR Data for Compounds **1** and **2** in C_5D_5N[a]

carbon	1	2
C-1	39.1 t	39.1 t
C-2	28.1 t	28.1 t
C-3	78.0 d	78.0 d
C-4	39.4 s	39.4 s
C-5	55.7 d	55.7 d
C-6	18.6 t	18.6 t
C-7	34.7 t	34.5 t
C-8	40.9 s	41.0 s
C-9	50.8 d	50.8 d
C-10	37.3 s	36.8 s
C-11	21.0 t	20.9 t
C-12	25.9 t	25.8 t
C-13	38.4 d	38.2 d
C-14	42.4 s	42.6 s
C-15	31.0 t	30.7 t
C-16	32.7 t	32.1 t
C-17	56.3 s	56.8 s
C-18	47.6 d	47.3 d
C-19	49.5 d	49.6 d
C-20	150.7 s	150.7 s
C-21	30.1 t	30.0 t
C-22	37.5 t	37.3 t
C-23	28.5 q	28.5 q
C-24	16.3 q[b]	16.3 q[b]
C-25	16.3 q[b]	16.2 q[b]
C-26	16.2 q[b]	16.2 q[b]
C-27	14.8 q	14.8 q
C-28	178.7 s	175.2 s
C-29	110.3 t	110.0 t
C-30	19.4 q	19.2 q
C-1′ (D-glucose)		95.3 d
C-2′		74.2 d
C-3′		79.4 d
C-4′		70.8 d
C-5′		78.7 d
C-6′		62.0 t

[a] δ in ppm; multiplicity determined by DEPT experiments; assignments are based on $^1H-^1H$ and $^1H-^{13}C$ chemical shift correlation experiments and comparisons to the assignments of **1**. [b] Interchangeable within column.

shift of C-28 from 178.7 to 175.2 ppm (Table 1). The DEPT experiment of **2** showed the appearance of a new CH_2 group at 62.0 ppm and five new CH groups at 95.3, 79.4, 78.7, 74.2, and 70.8 ppm (Table 1). Based on all the evidence, metabolite **2** was characterized as 28-*O*-β-D-glucopyranosyl 3β-hydroxy-lup-20(29)-en-28-oate. ^{13}C and ^1H NMR chemical shift assignments of metabolite **2** were based on $^1H-^1H$ and $^1H-^{13}C$ correlation experiments and comparisons to the assignments of **1**. The proton[18,19] and carbon[20] assignments of **1** have been reported in the literature. The ^{13}C NMR assignments of **1** are listed in Table 1 for comparative purposes.

The in vitro cytotoxicity of betulinic acid (**1**) and its metabolite **2** was evaluated against four human melanoma cell lines: MEL-1 (lymph node), MEL-2 (pleural fluid), MEL-3 (liver), and MEL-7 (metastatic melanoma from growing area). The ED_{50} values of **2** (>20 μg mL^{-1} against all four cell lines) indicated that metabolite **2** is not active against the tested melanoma cell lines when compared to **1**. The ED_{50} values of **1** against MEL-1, -2, -3, and -7 were 1.4, 1.2, 1.8, and 4.4 μg mL^{-1}, respectively. The results strongly suggest that the free carboxylic acid group in **1** is essential for cytotoxic activity against melanoma. The antimelanoma activity was lost when compound **1** was converted to the glucopyranosyl ester **2**. These findings are consistent with the reported in vitro antitumor activity of derivatives of **1** at C-28.[11,21] The in vitro antimelanoma activity of **1** was considerably attenuated when the carboxylic acid at C-28 was converted to the methyl ester, the aldehyde, or a methyl group. In addition, betulin (**3**), which has a primary alcohol instead of the carboxylic acid at C-28, showed a remarkably reduced antimelanoma activity compared to **1**. Further microbial transformation studies of **1** are in progress.

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 IR spectrophotometer. Microbial cells were homogenized using a Tekmar Sonic Disruptor TMX 600 at 20 kHz. ^1H 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 parts per million, and the coupling constants (*J* values) are in Hertz. Standard pulse sequences were utilized for COSY, HETCOR, DEPT, and APT experiments. HRMS and LRMS were obtained using a VG 70-SEQ mass spectrometer (VG Analytical, Manchester, England) at the Mass Spectrometry Facility, Department of Medicinal Chemistry, University of Washington, Seattle, WA 98195.

Chromatographic Conditions. TLC analyses were carried out on precoated Silica G-25 UV_{254} plates (E. Merck, Darmstadt). The adsorbent used for column chromatography was Si gel 70−230 mesh 60 Å (Aldrich Chemical Co., Milwaukee, WI). The solvent system used for TLC was $CHCl_3$−MeOH (9:1v/v) solution, and 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.

Microorganisms and Media. The microbial cultures were obtained from the culture collection of the School of Pharmacy, Northeast Louisiana University, and were originally from the American Type Culture Collection (ATCC), Manassas, VA, or the USDA Northern Regional Research Laboratories (NRRL), Peoria, IL. UI cultures were 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 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 fungi and bacteria were stored on slants of Mycophil and Eugon (Difco, Detroit, Michigan) agar, respectively, at 4 °C. The 0.1 M phosphate buffer (pH 7.2) used for resting-cell suspensions of *Cunninghamella* species consists of K_2HPO_4, 10.6 g; KH_2PO_4, 4.08 g; and distilled H_2O, 1000 mL. The 0.07 M phosphate buffer (pH 5) used for the enzymic hydrolysis of metabolite **2** consists of K_2HPO_4, 7.0 g; KH_2PO_4, 4.08 g; and distilled H_2O, 1000 mL.

Reagents. Dimethylformamide (DMF) and pyridine were stored over 4 Å molecular sieves. β-Glucosidase (one enzyme unit liberates 1.0 μmol of β-D-glucose from salicin per min at pH 5.0 at 37° C) was purchased from Sigma Chemical Co., St. Louis, Missouri.

Fermentation Procedures. Microbial metabolism studies were carried out by incubating the cultures with shaking on an Innova 5000 Gyrotory Tier Shaker (New Brunswick Scientific Co., NJ), operating at 150 rpm, at 25 °C. Preliminary screening experiments were carried out in 125-mL stainless steel-capped DeLong culture flasks 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 5 days 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 evaluated by TLC. Substrate controls were prepared by adding the substrate to sterile medium and then incubated without microorganisms. Culture controls consisted of fermentation blanks in which the micro-

organisms were grown under identical conditions without the addition of the substrate. Substrate-autoclaved culture controls consisted of microbial cultures that were grown to maturity (5−7 days), autoclaved for 30 min, and then incubated after the substrate was added. Culture controls of the resting-cell incubations of *Cunninghamella* species were prepared by suspending the mycelium in a sterile 0.1 M phosphate buffer (pH 7.2) containing 2% dextrose, and then incubated without the addition of the substrate.

Preparative Biotransformation of 1 to 2. Betulinic acid (**1**) was obtained from Aldrich Chemical Co. (Milwaukee, WI). The physical and spectral data of **1** have been reported in the literature.[18−20] Twenty-four 2-L flasks, each containing 400 mL of *Cunninghamella* species NRRL 5695 stage II culture, were incubated on the shaker for 5 days to get as much cell mass as possible. The mycelium from each flask was filtered under vacuum through a Büchner funnel, washed several times with sterile distilled H_2O, and suspended in a 1-L flask containing dextrose (2% w/v) in 200 mL of sterile 0.1 M phosphate buffer (pH 7.2). A total of 0.96 g of **1** was then dissolved in 0.96 mL of DMF and distributed equally among 24 flasks containing resting-cell suspensions of *Cunninghamella* species. After 9 days of incubation on the shaker, the suspensions were filtered and the cells washed with distilled H_2O. The cells were then frozen before homogenization using a Tekmar sonic disruptor at 20 kHz. The cell homogenate was extracted with 3 × 2.0 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 EtOAc−hexane and EtOAc−MeOH as eluting systems, and 10-mL fractions were collected. Repeated column chromatography with a gradient of EtOAc−hexane (20:80 to 100:0), followed by EtOAc−MeOH (100:1 to 100:10) afforded homogeneous fractions that showed a single spot on TLC (R_f 0.32). The homogeneous fractions were combined and evaporated in vacuo to give metabolite **2**.

Compound 2: Crystallization from EtOAc−MeOH (9:1) afforded white needles (0.01 g, 0.77% yield); mp 203−205 °C; $[\alpha]^{25}_D$ −6.0°(*c* 0.5 g/100 mL, C_5H_5N); IR (KBr) ν_{max} 3483, 2961, 1735 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) δ 6.45 (1H, d, J = 8.42 Hz, H-1′), 4.87 (1H, br s, H-29), 4.72 (1H, s, H-29), 4.45−4.36 (3H, m, H-4′, H-6′), 4.30 (1H, t, J = 8.42 Hz, H-5′), 4.19 (1H, t, J = 8.79 Hz, H-2′), 4.06−4.04 (1H, m, H-3′), 3.45−3.41(2H, m, H-3, H-18), 2.69−2.65 (3H, m, H-13, H-16), 2.20−2.15 (2H, m, H-22), 2.09−2.06 (2H, m, H-15), 1.93−1.87 (4H, m, H-2, H-12), 1.83−1.76 (1H, m, H-19), 1.72 (3H, s, Me-30), 1.65−1.59 (2H, m, H-1), 1.50−1.48 (2H, m, H-6), 1.35−1.32 (5H, m, H-7, H-9, H-11), 1.19 (3H, s, Me-23), 1.12 (3H, s, Me-24, 25 or 26), 1.01 (3H, s, Me-27), 0.98 (3H, s, Me-24, 25 or 26), 0.78 (3H, s, Me-24, 25 or 26), 0.82−0.76 (1H, m, H-5); ^{13}C NMR (C_5D_5N, 100 MHz), see Table 1; FABMS *m*/*z* 641 [M + Na]$^+$; HRFABMS *m*/*z* 641. 3992 (calcd for $C_{36}H_{58}O_8$ Na, 641.4029).

Enzymic Hydrolysis of 2 by β-Glucosidase Enzyme. A total of 3 mg (0.0048 mmol) of metabolite **2** was dissolved in 30 μL of DMF and added to 5 mL of 0.07 M phosphate buffer (pH 5). The enzyme β-glucosidase (3.4 mg, 30 units/mg) was then added to the solution, and the mixture was incubated in a Dubnoff Metabolic Shaker Incubator (Precision Scientific, Winchester, VA) at 100 rpm and 37 °C for 3 days. Substrate controls consisted of the phosphate buffer solution to which metabolite **2** was added in DMF and incubated at 37 °C without the enzyme. After incubation, the aqueous solution was extracted with 3 × 5 mL of *n*-BuOH. The organic solvent was washed with distilled H_2O, dried over anhydrous Na_2SO_4, and evaporated in vacuo. TLC analysis of the residue showed a single spot identical to an authentic sample of betulinic acid (**1**).

In Vitro Cytotoxicity Assay. Cytotoxicity of **1** and **2** was determined against four cultured human melanoma cell lines: MEL-1 (lymph node), MEL-2 (pleural fluid), MEL-3 (liver), and MEL-7 (metastatic melanoma from growing area) as described previously.[14]

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