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Anticancer Activity of 3-*O*-Acylated Betulinic Acid Derivatives Obtained by Enzymatic Synthesis

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An easy and efficient strategy to prepare betulinic acid esters with various anhydrides was used by the enzymatic synthesis method. It involves lipase-catalyzed acylation of betulinic acid with anhydrides as acylating agents in organic solvent. Lipase from *Candida antarctica* immobilized on an acrylic resin (Novozym 435) was employed as a biocatalyst. Several 3-*O*-acyl-betulinic acid derivatives were successfully obtained by this procedure. The anticancer activity of betulinic acid and its 3-*O*-acylated derivatives were then evaluated *in vitro* against human lung carcinoma (A549) and human ovarian (CAOV3) cancer cell lines. 3-*O*-glutaryl-betulinic acid, 3-*O*-acetyl-betulinic acid, and 3-*O*-succinyl-betulinic acid showed $IC_{50} < 10\,\mu g/ml$ against A549 cancer cell line tested and showed better cytotoxicity than betulinic acid. In an ovarian cancer cell line, all betulinic acid derivatives prepared showed weaker cytotoxicity than betulinic acid.

Key words: enzymatic synthesis; Novozym 435; 3-*O*-acyl-betulinic acid; betulinic acid; anticancer agents

Betulinic acid (**1**) (3β-hydroxy-lup-20(29)-en-28-oic acid), pentacyclic lupane triterpene, is a known natural product which possess several pharmacological activities, including inhibition of human immunodeficiency virus (HIV), anti-bacterial, anti-malarial, anti-inflammatory, anthelmintic, antioxidant, and anticancer properties.[1)] It has been identified as a highly selective growth inhibitor against human melanoma,[2,3)] neuroectodermal,[4)] and malignant[5)] tumor cells and was reported to induce apoptosis in these cells.[1)] Nevertheless, further clinical development of betulinic acid in the pharmaceutical industry is strongly hampered because of its poor hydrosolubility and pharmacokinetic properties (absorption, distribution, metabolism, and elimination).[6)] Thus, much work has been focused on modification of betulinic acid at the C-3 and/or C-28 positions in order to increase its hydrosolubility and biological activity.[7–10)] Methods for the synthesis of 3-*O*-acyl-betulinic acid based on chemical catalytic esterification have been described.[10–13)] However, the application of enzymes in organic synthesis provides advantages, since it can be carried out under mild reaction conditions, high selectivity, and product purity.[14,15)] Study of the enzymatic acylation of betulinic acid was initiated in our laboratory for the synthesis of 3-*O*-acetyl-betulinic acid using Novozym 435 (*Candida antarctica* lipase), giving the expected product in 85% yield.[16)] Recently, the synthesis of 3-*O*-benzoyl-betulinic acid using *Candida antarctica* lipase as biocatalyst in an organic solvent was reported by Yasin *et al.*[17)] giving betulinic acid ester at 48.5% yield using benzoyl chloride as acylating agent.

We herein report the enzymatic synthesis of several 3-*O*-acyl-betulinic acid derivatives (**2–11**, Scheme 1) using various anhydrides and *Candida antarctica* lipase (Novozym 435) as the catalyst in organic solvent. The cytotoxicity of the synthesized compounds was evaluated on human lung carcinoma (A549) and human ovarian (CAOV3) cancer cell lines.

Materials and Methods

Enzyme. Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3; Novozym 435, 10000 PLU/g) from *Candida antarctica*, supported on a macroporous acrylic resin with a water content of 3% (w/w) was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark).

Chemicals. Chloroform and *n*-hexane (Fisher Chemical, Loughborough, UK) were used as the organic solvents. Betulinic acid was isolated from Malaysian *Callistemon speciosus* by a previous method.[18)] Phthalic anhydride, 3-methyl phthalic anhydride, succinic anhydride, maleic anhydride, glutaric anhydride, 3,3-dimethyl glutaric anhydride, acetic anhydride, butyric anhydride, isobutyric anhydride, and valeric anhydride were purchased from Acros Organics (Geel, Belgium). Ethyl acetate, dimethyl sulfoxide (DMSO), celite®545, Na_2SO_4, K_2CO_3, and HCl were from Merck (Darmstadt, Germany). MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and RPMI 1640 medium were from Sigma (St. Louis, MO). Fetal bovine serum was from BioWhittaker Inc. (Walkersville, USA). All chemicals were of analytical reagent grade.

Cell lines. Cell lines A549 and CAOV3 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C, 5% CO_2, and 90% humidity.

Analytical methods. The progress of the reactions for the synthesis of 3-*O*-acyl-betulinic acid derivatives was monitored by thin layer

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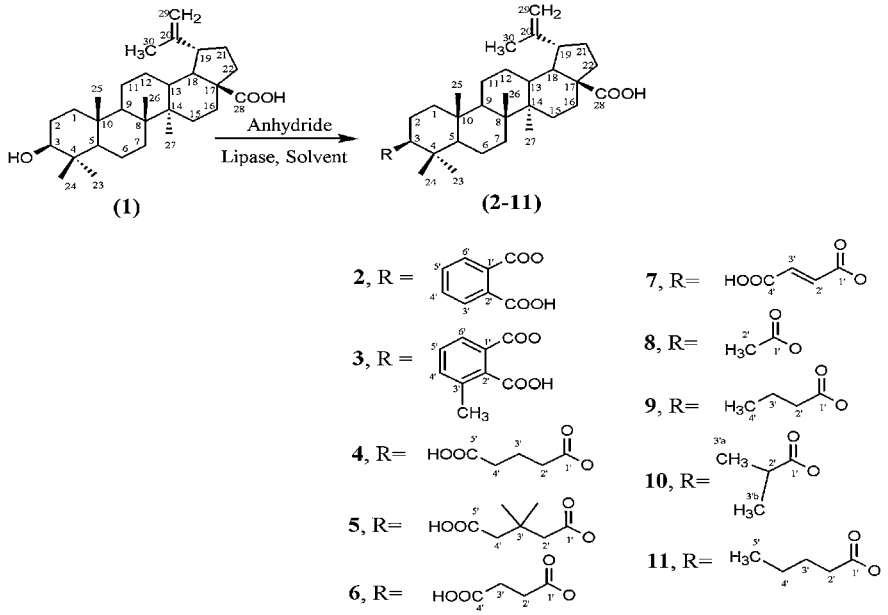

Scheme 1. Esterification of Betulinic Acid with Various Anhydrides by Lipase as Biocatalyst in Organic Solvent.

chromatography (TLC) on silica gel plates eluted with a mixture of *n*-hexane/ethyl acetate (9:1, v/v). The plates were visualized under a UV lamp and/or iodine vapor. NMR spectra were recorded with a Varian Unity Inova 500 NMR spectrometer operating at a resonance frequency of 499.89 MHz for the ^{1}H-NMR spectra and 125.71 MHz for the ^{13}C-NMR spectra. The products were also analyzed by Fourier Transform Infrared (FT-IR) spectroscopy (Perkin Elmer, Spectrum 100 FTIR, MA, USA). The mass spectra of the products were recorded using a gas chromatograph-mass spectrometer (Thermo Finnigan, Trace GC Polaris Q, MA, USA). The products were also analyzed using CHN analyzer (Leco, CHNS-932, MI, USA). The melting points of purified products were determined using the melting point equipment (Barnstead Electrothermal, ESSEX, UK).

General procedure for enzymatic synthesis of 3-O*-acyl-betulinic acid derivatives.* Ester synthesis was performed in a 150 ml glass-stoppered round bottom flask. To a magnetically stirred solution of betulinic acid (150 mg, 0.328 mmol), acid anhydride (0.364 mmol), celite®545 (1.0 g), K_2CO_3 (36.0 mg, 0.260 mmol), chloroform (50 ml) and *n*-hexane (50 ml) was added Novozym 435 (0.873 g). The reaction mixture was magnetically stirred at 54 °C for 20 h at 150 rpm. After 20 h of reaction, the enzyme was removed by filtration and washed twice with chloroform. The filtrate was evaporated and ethyl acetate was then added and the mixture was washed twice with aqueous solution of HCl and twice with water. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was chromatographed on silica gel 60 (*n*-hexane/ethyl acetate, 9:1–5:1, v/v) as eluent. The structures of the products were analyzed using ^{1}H-NMR, ^{13}C-NMR, FT-IR, CHN analyzer and MS spectroscopy.

3-O-*Phthalyl-betulinic acid* (***2***). Starting with phthalic anhydride (53.9 mg); after crystallization from CH_3CN–H_2O gave colorless needles (128 mg, 64.7%); mp 256–258 °C. IR ν_{max} ($CHCl_3$) cm^{-1}: 3474 (OH), 1761 (C=O ester), 1696 (C=O acid), 1653 (C=C), 1600 and 1582 (C=C aromatic), 1292 (C–O). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.76, 0.83, 0.94, 0.97, 0.98, 1.69 (each 3H, s, 6 × CH_3), 3.00 (1H, m), 4.40 (1H, dd, J = 4.5, 12.0 Hz), 4.61 and 4.74 (each 1H, br s), 7.55–7.61 (2H, m), 7.72 (1H, d, J = 7.0 Hz), 7.92 (1H, d, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.83 (C-27), 16.16 (C-24), 16.38 (C-26), 16.72 (C-25), 18.39 (C-6), 19.52 (C-30), 23.83 (C-11), 25.68 (C-12), 28.15 (C-2), 28.31 (C-23), 29.85 (C-21), 30.75 (C-15), 32.31 (C-16), 34.47 (C-7), 37.34 (C-10), 38.10 (C-22), 38.19 (C-13), 38.56 (C-4), 39.50 (C-1), 40.99 (C-8), 42.65 (C-14), 47.12 (C-18), 48.42 (C-19), 50.66 (C-9), 55.65 (C-5), 57.97 (C-17), 80.83 (C-3), 109.95 (C-29), 128.76 (C-6′), 129.18 (C-3′), 130.57 (C-2′), 131.06 (C-1′), 133.10 (C-4′ and C-5′), 150.59 (C-20), 168.32 (C=O ester), 169.24 (2′-COOH), 181.11 (C-28, COOH). MS m/z: 604 (M$^+$), 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for $C_{38}H_{52}O_6$: C, 75.39; H, 8.60. Found: C, 75.69; H, 8.61.

3-O-*(3′-Methyl phthalyl)-betulinic acid* (***3***). Starting with 3-methyl phthalic anhydride (59.0 mg); after crystallization from CH_3CN–H_2O gave colorless needles (101 mg, 49.7%); mp 238–241 °C. IR ν_{max} ($CHCl_3$) cm^{-1}: 3468 (OH of COOH), 1722 (C=O ester), 1688 (C=O acid), 1644 (C=C), 1600 and 1580 (C=C aromatic), 1290 (C–O). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.76, 0.83, 0.94, 0.97, 0.98, 1.69, 2.46 (each 3H, s, 7 × CH_3), 3.00 (1H, m), 4.50 (1H, dd, J = 4.5, 12.0 Hz), 4.61 and 4.75 (each 1H, br s), 7.47 (1H, d, J = 7.5 Hz), 7.41 (1H, t, J = 8.0 Hz), 7.91 (1H, d, J = 8.0 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.46 (C-27), 15.56 (C-24), 16.36 (C-25), 16.41 (C-26), 17.96 (C-6), 18.53 (C-30), 19.50 (3′-CH_3), 21.10 (C-11), 25.75 (C-12), 27.63 (C-2), 28.22 (C-23), 29.66 (C-21), 30.78 (C-15), 32.38 (C-16), 34.57 (C-7), 37.27 (C-10), 37.44 (C-22), 38.61 (C-13), 38.96 (C-4), 39.06 (C-1), 40.94 (C-8), 42.66 (C-14), 47.10 (C-18), 49.50 (C-19), 50.77 (C-9), 55.58 (C-5), 56.70 (C-17), 79.25 (C-3), 109.96 (C-29), 123.51 (C-4′), 123.76 (C-6′), 131.87 (C-2′), 135.68 (C-5′), 138.11 (C-1′), 140.69 (C-3′), 150.62 (C-20), 165.35 (C=O ester), 169.30 (2′-COOH), 181.55 (C-28, COOH). MS m/z: 618 (M$^+$), 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for $C_{39}H_{54}O_6$: C, 75.62; H, 8.73. Found: C, 75.89; H, 8.90.

3-O-*Glutaryl-betulinic acid* (***4***). Starting with glutaric anhydride (41.5 mg); after crystallization from MeOH–H_2O gave colorless needles (87 mg, 46.4%); mp 273–275 °C. IR ν_{max} ($CHCl_3$) cm^{-1}: 2500–300 (OH of COOH), 1726 (C=O ester), 1687 (C=O acid), 1644 (C=C), 1237, 1190 and 1142 (C–O). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.76, 0.83, 0.94, 0.98, 0.97, 1.65 (each 3H, s, 6 × CH_3), 2.99 (1H, m), 4.50 (1H, dd, J = 4.0, 11.5 Hz), 4.61 and 4.74 (each 1H, br s), 2.37 (2H, d, J = 7.0 Hz), 2.53 (2H, t, J = 7.5 Hz), 2.57 (2H, t, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.88 (C-27), 16.26 (C-26), 16.56 (C-24), 16.69 (C-25), 18.52 (C-6), 19.92 (C-3′), 19.92 (C-30), 21.04 (C-11), 25.68 (C-12), 27.59 (C-2), 28.17 (C23), 29.90 (C-21), 30.67 (C-15), 32.26 (C-16), 32.26 (C-4′), 33.18 (C-2′), 34.57 (C-7), 37.43 (C-10), 38.54 (C-22), 38.95 (C-13), 38.95 (C-4), 39.08 (C-1), 40.94 (C-8), 42.66 (C-14), 47.12 (C-18), 49.39 (C-19), 50.89 (C-9), 55.58 (C-5), 56.59 (C-17), 81.26 (C-3), 109.97 (C-29), 150.97 (C-20), 172.51 (C-1′, C=O ester), 178.68 (C-5′, COOH), 181.53 (C-28, COOH). MS m/z: 570 (M$^+$), 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for $C_{35}H_{54}O_6$: C, 73.58; H, 9.46. Found: C, 73.31; H, 9.55

3-O-*(3′,3′-Dimethyl glutaryl)-betulinic acid* (***5***). Starting with 3,3-dimethylglutaric anhydride (51.7 mg); after crystallization from MeOH–H_2O gave colorless needles (79 mg, 40.2%); mp 217–220 °C. IR ν_{max} ($CHCl_3$) cm^{-1}: 2500–300 (OH of COOH), 1722 (C=O ester),

1642 (C=C), 1237 and 1150 (C–O). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.76, 0.82, 0.93, 0.96, 0.97, 1.15, 1.17, 1.69 (each 3H, s, 8 × CH_3), 2.60 (2H, s), 2.51 (2H, s), 2.98 (1H, m), 4.41 (1H, dd, J = 5.0, 11.0 Hz), 4.60 and 4.73 (each 1H, br s). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.57 (C-27), 15.56 (C-24), 16.21 (C-26), 16.76 (C-25), 18.52 (C-6), 19.54 (C-30), 21.11 (C-11), 25.69 (C-12), 27.43 (C-2), 27.79 (3′-CH_3), 28.07 (3′-CH_3), 28.16 (C-23), 29.89 (C-21), 30.77 (C-15), 32.34 (C-16), 32.54 (C-3′), 34.55 (C-7), 37.42 (C-10), 38.18 (C-22), 38.58 (C-13), 38.93 (C-4), 38.98 (C-1), 40.98 (C-8), 42.67 (C-14), 44.04 (C-2′), 44.83 (C-4′), 47.14 (C-18), 49.45 (C-19), 50.77 (C-9), 55.55 (C-5), 56.65 (C-17), 80.63 (C-3), 109.96 (C-29), 150.62 (C-20), 183.21 (C-28, COOH), 174.86 (C-1′, C=O ester), 177.52 (C-5′, COOH). MS m/z: 598 (M^+), 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd for $C_{37}H_{58}O_6$: C, 74.14; H, 9.69. Found: C, 74.68; H, 9.58.

3-O-*Succinyl-betulinic acid* (***6***). Starting with succinic anhydride (36.4 mg); after crystallization from MeOH–H_2O gave colorless needles (132 mg, 72.4%); mp 276–279 °C. IR ν_{max} ($CHCl_3$) cm^{-1}: 3463 (OH of COOH), 1727 (C=O ester), 1686 (C=O acid), 1640 (C=C), 1237 and 1157 (C–O). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.76, 0.83, 0.94, 0.97, 0.98, 1.69 (each 3H, s, 6 × CH_3), 3.00 (1H, m), 2.75 (4H, s), 4.50 (1H, dd, J = 4.5, 12.0 Hz), 4.61 and 4.74 (each 1H, br s). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.62 (C-27), 15.58 (C-24), 18.52 (C-26), 18.70 (C-25), 18.89 (C-6), 19.60 (C-30), 21.08 (C-11), 25.73 (C-12), 27.66 (C-2), 28.22 (C-23), 28.59 (C-2′ and C-3′), 29.94 (C-21), 30.78 (C-15), 32.38 (C-16), 34.57 (C-7), 37.44 (C-10), 38.10 (C-13), 38.27 (C-4), 38.92 (C-22), 39.45 (C-1), 40.93 (C-8), 42.67 (C-14), 47.11 (C-18), 49.06 (C-19), 50.75 (C-9), 55.52 (C-5), 56.50 (C-17), 81.23 (C-3), 109.73 (C-29), 150.43 (C-20), 172.76 (C-1′, C=O ester), 174.33 (C-4′, COOH), 180.73 (C-28, COOH). MS m/z: 557 (M^+), 511, 483, 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for $C_{34}H_{52}O_6$: C, 73.19; H, 9.33. Found: C, 73.11; H, 9.36.

3-O-*Maleyl-betulinic acid* (***7***). Starting with maleic anhydride (35.6 mg); after crystallization from MeOH–H_2O gave colorless needles (45 mg, 24.7%); mp 258–260 °C. IR ν_{max} ($CHCl_3$) cm^{-1}: 3467 (OH of COOH), 1729 (C=O ester), 1685 (C=O acid), 1642 (C=C), 1272, 1237, 1191 and 1147 (C–O). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.76, 0.82, 0.88, 0.94, 0.97, 1.69 (each 3H, s, 6 × CH_3), 3.00 (1H, m), 6.86 (1H, d, J = 5.5 Hz), 7.23 (1H, d, J = 5.0 Hz), 4.50 (1H, dd, J = 4.5, 11.5 Hz), 4.61 and 4.74 (each 1H, br s). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.32 (C-27), 15.57 (C-24), 18.51 (C-26), 18.76 (C-25), 18.85 (C-6), 19.52 (C-30), 21.16 (C-11), 25.75 (C-12), 27.55 (C-2), 28.21 (C-23), 29.98 (C-21), 30.76 (C-15), 32.34 (C-16), 34.57 (C-7), 37.41 (C-10), 38.14 (C-4), 38.84 (C-22), 39.10 (C-13), 39.45 (C-1), 40.94 (C-8), 42.63 (C-14), 47.21 (C-18), 49.13 (C-19), 50.77 (C-9), 55.53 (C-5), 56.52 (C-17), 80.25 (C-3), 110.21 (C-29), 136.36 (C-2′), 137.24 (C-3′), 150.42 (C-20), 173.29 (C-1′, C=O ester), 174.23 (C-4′, COOH), 181.52 (C-28, COOH). MS m/z: 555 (M^+), 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for $C_{34}H_{50}O_6$: C, 73.54; H, 9.01. Found: C, 73.24; H, 9.36.

3-O-*Acetyl-betulinic acid* (***8***). Starting with acetic anhydride (37.1 mg); after crystallization from MeOH–H_2O gave colorless needles (130 mg, 79.3%); mp 288–290 °C. IR ν_{max} ($CHCl_3$) cm^{-1}: 2500–3500 (OH of COOH), 1729 (C=O ester), 1698 (C=O acid), 1644 (C=C), 1247 (C–O). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.84, 0.85, 0.86, 0.94, 0.98, 1.70, 2.05 (each 3H, s, 7 × CH_3), 3.00 (1H, m), 4.48 (1H, dd, J = 5.5, 10.5 Hz), 4.62 and 4.75 (each 1H, br s). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.89 (C-27), 16.28 (C-24), 16.40 (C-25), 16.70 (C-26), 18.39 (C-2), 19.58 (C-30), 21.08 (C-6), 21.55 (C-2′), 23.93 (C-11), 25.67 (C-12), 28.18 (C-23), 29.93 (C-21), 30.80 (C-15), 32.39 (C-16), 34.47 (C-7), 37.29 (C-22), 37.35 (C-10), 38.03 (C-4), 38.61 (C-13), 38.66 (C-1), 40.93 (C-8), 42.65 (C-14), 47.17 (C-19), 49.50 (C-18), 50.62 (C-9), 55.65 (C-5), 56.65 (C-17), 81.20 (C-3), 109.98 (C-29), 150.60 (C-20), 171.31 (C-1′, C=O ester), 182.56 (C-28, COOH). MS m/z: 498 (M^+), 483, 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for $C_{32}H_{50}O_4$: C, 76.99; H, 10.02. Found: C, 77.03; H, 10.12.

3-O-*Butyryl-betulinic acid* (***9***). Starting with butyric anhydride (57.5 mg); after crystallization from MeOH–H_2O gave colorless needles (81.8 mg, 47.6%); mp 265–269 °C. IR ν_{max} ($CHCl_3$) cm^{-1}: 3462 (OH of COOH), 1728 (C=O ester), 1685 (C=O acid), 1642 (C=C), 1272, 1236, 1189 and 1145 (C–O). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.76, 0.84, 0.85, 0.94, 0.95, 1.69 (each 3H, s, 6 × CH_3), 0.98 (3H, t, J = 7.0 Hz), 2.99 (1H, m), 4.49 (1H, dd, J = 5.0, 11.0 Hz), 4.61 and 4.74 (each 1H, br s), 2.34 (2H, t, J = 7.5 Hz), 1.62–1.69 (2H, m). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 13.85 (C-4′), 14.85 (C-27), 16.18 (C-24), 16.35 (C-26), 16.73 (C-25), 18.39 (C-2), 18.86 (C-3′), 19.55 (C-30), 21.08 (C-6), 23.97 (C-11), 25.58 (C-12), 28.15 (C-23), 29.87 (C-21), 30.77 (C-15), 32.35 (C-16), 34.48 (C-7), 36.18 (C-2′), 37.00 (C-22), 37.34 (C-10), 38.06 (C-4), 38.59 (C-13), 39.07 (C-1), 40.94 (C-8), 42.66 (C-14), 47.15 (C-19), 49.45 (C-18), 50.65 (C-9), 55.64 (C-5), 56.64 (C-17), 80.88 (C-3), 109.94 (C-29), 150.62 (C-20), 173.85 (C-1′, C=O ester), 180.08 (C-28, COOH). MS m/z: 526 (M^+), 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for $C_{34}H_{54}O_4$: C, 77.45; H, 10.25. Found: C, 77.70; H, 10.21.

3-O-*Isobutyryl-betulinic acid* (***10***). Starting with isobutyric anhydride (57.5 mg); after crystallization from MeOH–H_2O gave colorless needles (80 mg, 46.2%); mp 260–262 °C. ν_{max} ($CHCl_3$) cm^{-1}: 3463 (OH of COOH), 1726 (C=O ester), 1685 (C=O acid), 1642 (C=C), 1237 and 1148 (C–O). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.76, 0.85, 0.94, 0.98, 1.17, 1.69 (each 3H, s, 6 × CH_3), 1.20 and 1.21 (each 3H, d, J = 7.0 Hz), 2.51–2.69 (1H, m), 3.00 (1H, m), 4.46 (1H, dd, J = 4.5, 11.0 Hz), 4.61 and 4.73 (each 1H, br s). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.90 (C-27), 16.15 (C-3′b), 16.22 (C-26), 16.38 (C-24), 16.77 (C-25), 16.98 (C-3′a), 18.39 (C-6), 19.42 (C-30), 21.10 (C-11), 25.69 (C-12), 27.52 (C-2), 28.16 (C-23), 29.88 (C-21), 30.78 (C-15), 32.35 (C-16), 34.08 (C-2′), 34.71 (C-7), 37.34 (C-10), 38.18 (C-22), 38.59 (C-13), 38.94 (C-4), 39.06 (C-1), 40.95 (C-8), 42.66 (C-14), 47.14 (C-18), 49.45 (C-19), 50.66 (C-9), 55.58 (C-5), 56.65 (C-17), 80.64 (C-3), 109.96 (C-29), 150.62 (C-20), 177.17 (C-1′, C=O ester), 183.76 (C-28, COOH). MS m/z: 526 (M^+), 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for $C_{34}H_{54}O_4$: C, 77.45; H, 10.25. Found: C, 77.50; H, 10.11.

3-O-*Valeryl-betulinic acid* (***11***). Starting with valeric anhydride (67.7 mg); after crystallization from MeOH–H_2O gave colorless needles (77 mg, 43.8%); mp 259–261 °C. IR ν_{max} ($CHCl_3$) cm^{-1}: 3466 (OH of COOH), 1728 (C=O ester), 1685 (C=O acid), 1642 (C=C), 1272, 1236, 1189 and 1144 (C–O). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.76, 0.84, 0.85, 0.90, 0.97, 1.69 (each 3H, s, 6 × CH_3), 0.93 (3H, t, J = 7.5 Hz), 2.99 (1H, m), 4.48 (1H, dd, J = 5.0, 11.0 Hz), 4.61 and 4.73 (each 1H, br s), 2.36 (2H, t, J = 7.5 Hz), 1.34–1.42 (2H, m), 1.60–1.66 (2H, m). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 13.89 (C-5′), 14.80 (C-27), 16.20 (C-26), 16.38 (C-24), 16.76 (C-25), 18.40 (C-6), 19.54 (C-30), 22.40 (C-4′), 23.96 (C-11), 25.69 (C-12), 26.98 (C-3′), 27.46 (C-2), 28.17 (C-23), 29.87 (C-21), 30.77 (C-15), 32.34 (C-16), 34.03 (C-2′), 34.81 (C-7), 37.33 (C-10), 38.06 (C-22), 38.58 (C-13), 38.94 (C-4), 39.06 (C-1), 40.95 (C-8), 42.66 (C-14), 47.13 (C-18), 49.44 (C-19), 50.65(C-9), 55.65 (C-5), 56.64 (C-17), 80.88 (C-3), 109.96 (C-29), 150.61 (C-20), 174.04 (C-1′, C=O ester), 180.39 (C-28, COOH). MS m/z: 540 (M^+), 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for $C_{35}H_{56}O_4$: C, 77.66; H, 10.35. Found: C, 77.70; H, 10.41.

MTT cytotoxic assay. In vitro cytotoxic activity of betulinic acid (**1**) and its 3-*O*-acylated derivatives (**2–11**) was done against human lung carcinoma (A549) and human ovarian (CAOV3) cancer cell lines by Microculture Tetrazolium Salt (MTT) assay. The assay was carried out in 96-well microtiter plates. Various concentrations of the compound were added into the 96-well microtiter plates before the cells were seeded. The control contained only untreated cells, included for each sample. The assay was performed in duplicate and the culture plates were incubated for 72 h at 37 °C in a 5% CO_2 humidified incubator. After 72 h of incubation, the fractions of surviving cells were measured relative to the untreated cell population by colorimetric MTT assay. A volume of 20 µl of MTT (5 mg/ml) in phosphate buffer solution was added to each microtiter well and incubated for 3 to 4 h. After this incubation period, 100 µl of Dimethyl sulfoxide (DMSO) was added to each well to dissolve the resulting MTT formazan crystals by pipetting up and down 10–20 times. The plate was left at room temperature for 15–30 min. Then the optical density (OD) was measured on an ELIZA microplate reader at 570 nm. The percentage of cell viability was

Table 1. Cytotoxicity Assay of Betulinic Acid (**1**) and 3-*O*-Acyl-betulinic Acid Derivatives (**2–11**) against Human Lung Carcinoma (A549) and Human Ovarian (CAVO3) Cancer Cell Lines

Compounds	IC_{50} (μg/ml)	
	A549	CAOV3
1	8.4	9.4
2	>30	>30
3	18.4	>30
4	6.4	>30
5	>30	>30
6	7.4	15
7	26.6	>30
8	6.8	>30
9	11.4	>30
10	12.1	>30
11	>30	>30

calculated using the following equation: % Viability = (OD sample/OD control) × 100. A plot of percentage of cell viability against the concentration of the drug gives a measure of cytotoxicity. The cytotoxic index used was IC_{50}, the drug concentration lethal to 50% of the tumor cells as calculated from the plot.

Results and Discussion

In initial work in our laboratory, the enzymatic synthesis of 3-*O*-phthalyl-betulinic acid (**2**) was chosen as a reaction model for optimization the reaction parameters using response surface mythology (RSM). Several betulinic acid esters (**2–11**) were synthesized under the optimal operation conditions, obtained by the RSM technique. Betulinic acid (**1**) and its derivatives (compounds **2–11**) were then screened for cytotoxicity *in vitro* against human lung carcinoma (A549) and human ovarian (CAOV3) cancer cell lines by MTT assay. The results presented in Table 1 are expressed as the concentration inhibiting 50% of the cell growth (IC_{50}).

Based on the IC_{50}values, compounds with $IC_{50} < 10$ μg/ml were considered strongly active, those with IC_{50} ranging from 10 to 30 μg/ml were considered moderately active, and those with $IC_{50} > 30$ μg/ml were weakly active. Betulinic acid (**1**), 3-*O*-glutaryl-betulinic acid (**4**), 3-*O*-succinyl-betulinic acid (**6**), and 3-*O*-acetyl-betulinic acid (**8**) showed high activity against the lung A549 cell line ($IC_{50} < 10$ μg/ml), while 3-*O*-(3′-methyl phthalyl)-betulinic acid (**3**), 3-*O*-maleyl-betulinic acid (**7**), 3-*O*-butyryl-betulinic acid (**9**), and 3-*O*-isobutyryl-betulinic acid (**10**) showed moderate activity against the A549 (10 μg/ml < IC_{50} < 30 μg/ml). In contrast, 3-*O*-phthalyl-betulinic acid (**2**), 3-*O*-(3′,3′-dimethyl glutaryl)-betulinic acid (**5**), and 3-*O*-valeryl-betulinic acid (**11**) showed weakly cytotoxicity against the A549 cell line ($IC_{50} > 30$ μg/ml). Betulinic acid (**1**) and compounds **2–11** are arranged in order of decreasing activity for the lung A549 cell line: **4** > **8** > **6** > **1** > **9** > **10** > **3** > **7** > **2** (about **5** and **11**).

Compared to betulinic acid (**1**), 3-*O*-glutaryl-betulinic acid (**4**), 3-*O*-succinyl-betulinic acid (**6**), and 3-*O*-acetyl-betulinic acid (**8**) exhibited stronger cytotoxicity against the lung A549 cell line. All the betulinic acid derivatives (compounds **2–11**) reported here showed weaker cytotoxicity than betulinic acid (**1**) against the ovarian CAVO3 cancer cell line. 3-*O*-succinyl-betulinic acid (**6**) exhibited moderate cytotoxicity towards CAVO3 cell line ($IC_{50} = 15.0$ μg/ml).

By comparing the cytotoxic activities of compounds **8–11**, it was found that the cytotoxic potency may be dependent on the length of the alkyl chain on the acyl group at the C-3 position. The compounds having a shorter alkyl chain on the acyl group at the C-3 position were found to be more toxic on the cancer cell line. A similar trend was observed for compounds **4** and **5**. These results suggest that increasing the bulkiness or the chain length of the alkyl on acyl group at C-3 position may decrease cytotoxicity against the cancer cell line.

3-*O*-Phthalyl-betulinic acid (**2**) had weak cytotoxicity on the A549 cell line ($IC_{50} > 30$ μg/ml). Incorporation of a methyl group on the aromatic ring (compound **3**) led to a further increase in cytotoxic potency, suggesting that the presence of an electron-donating group might change the electrostatic properties. The presence of a double bond on the acyl group at the C-3 position may not be critical for the cytotoxic activity; the saturated form in compound **6** was observed to be more cytotoxic than its unsaturated form in compound **7**.

Conclusions

This is the first report on the enzymatic synthesis of 3-*O*-acyl-betulinic acid derivatives using acid anhydrides. It was found that *Candida antarctica* lipase performed esterification of the betulinic acid with anhydrides. On the basis of our *in vitro* cytotoxic results and the structure-activity relationship (SAR), we concluded that (i) 3-*O*-glutaryl-betulinic acid (**4**), 3-*O*-succinyl-betulinic acid (**6**), and 3-*O*-acetyl-betulinic acid (**8**) were the most active compounds as compared to betulinic acid (**1**) against human lung carcinoma (A549), (ii) 3-*O*-glutaryl-betulinic acid (**4**) exhibited the best cytotoxicity against A549 cell line, (iii) all the betulinic acid derivatives (compounds **2–11**) showed weaker cytotoxicity than betulinic acid (**1**) against a human ovarian cell line (CAVO3).

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