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 O-acyl-betulinic acid derivatives were successfully obtained by this procedure. The anticancer activity of betulinic acid and its 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 IC50 < 10 μg/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.1–3 It has been identified as a highly selective growth inhibitor against human melanoma,2–3 neuroectodermal,4 and malignant5 tumor cells and was reported to induce apoptosis in these cells.3,5 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).3,4,5 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-acyt-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 benzoic 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.13 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, NaN3, K2CO3, 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% CO2, 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
7.61 (2H, m), 7.72 (1H, d, 0 J 4.40 (1H, dd, 10.2 Hz), 7.92 (1H, d, J = 7.6 Hz), 7.92 (1H, d, J = 7.5 Hz).

\[ 1^1 C \text{ NMR (CDCl}_3, 125 \text{ MHz}: \delta 14.83 (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'), 35.47 (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), 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'), 130.64 (C-1'), 133.10 (C-4'), 135.09 (C-20), 168.32 (C=O ester), 169.24 (2-C=O, COOH), 181.11 (C-28, COOH). MS m/z: 570 (M^+), 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for C_{36}H_{34}O_7: C, 75.39; H, 8.60. Found: C, 75.69; H, 8.61.

3-O-(3'-Methyl phthalal)-betulinic acid (3). Starting with 3-methyl phthalic anhydride (59.0 mg); after crystallization from CH\textsubscript{3}CN-H\textsubscript{2}O gave colorless needles (101 mg, 49.7%); mp 238–241 \textdegree C. IR \nu_{max} (CHCl\textsubscript{3}) cm\textsuperscript{-1}: 3468 (OH of COOH), 1722 (C=O ester), 1688 (C=O acid), 1644 (C=C), 1600 and 1550 (C=C aromatic), 1290 (C=O). \[ 1^1 C \text{ NMR (CDCl}_3, 150 \text{ MHz}: \delta 0.76, 0.83, 0.94, 0.97, 0.98, 1.69 (each 3H, s, 6 x CH\textsubscript{3}), 2.99 (1H, m), 4.50 (1H, d, 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), 7.81 (1H, d, J = 8.0 Hz). \[ 1^3 C \text{ NMR (CDCl}_3, 125 \text{ MHz}: \delta 14.16 (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\textsubscript{3}), 21.10 (C-11), 25.75 (C-12), 27.63 (C-23), 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), 39.86 (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-11), 140.69 (C-3'), 150.62 (C-20), 165.35 (C=O ester), 169.30 (2'-COOH), 181.55 (C=O, COOH). MS m/z: 618 (M^+), 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for C_{36}H_{34}O_7: C, 75.62; H, 8.73. Found: C, 75.89; H, 8.90.

3-O- Glutaral-betulinic acid (4). Starting with glutaric anhydride (41.5 mg); after crystallization from MeOH-H\textsubscript{2}O gave colorless needles (87 mg, 46.4%); mp 273–275 \textdegree C. IR \nu_{max} (CHCl\textsubscript{3}) cm\textsuperscript{-1}: 2500–300 (OH of COOH), 1726 (C=O ester), 1687 (C=O acid), 1644 (C=C), 1237, 1190 and 1142 (C=O). \[ 1^1 N \text{ NMR (CDCl}_3, 500 \text{ MHz}: \delta 0.76, 0.83, 0.94, 0.98, 0.97, 1.65 (each 3H, s, 6 x CH\textsubscript{3}), 2.99 (1H, m), 4.50 (1H, d, 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). \[ 1^3 C \text{ NMR (CDCl}_3, 125 \text{ MHz}: \delta 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'), 35.47 (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), 120.97 (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_{36}H_{34}O_7: C, 73.58; H, 9.46. Found: C, 73.31; H, 9.55.

3-O-(3',5'-Dimethyl glutaral)-betulinic acid (5). Starting with 3,3-dimethylglutaric anhydride (51.7 mg); after crystallization from MeOH-H\textsubscript{2}O gave colorless needles (79 mg, 40.2%); mp 217–220 \textdegree C. IR \nu_{max} (CHCl\textsubscript{3}) cm\textsuperscript{-1}: 2500–300 (OH of COOH), 1722 (C=O ester),
3-O-Butyryl-betulinic acid (9). Starting with butyric anhydride (57.5 mg); after crystallization from MeOH–H2O gave colorless needles (80 mg, 46.2%); mp 260–262 °C. IR ν(CH) (cm−1): 3463 (OH of COOH), 1729 (C=O ester), 1685 (C=O acid), 1642 (C=O). 1H NMR (CDCl3, 500 MHz): δ 0.76, 0.84, 0.88, 0.89, 0.97, 0.99 (each 3H, s, 6 × CH3), 1.00 (1H, m), 1.60–1.66 (2H, m). 13C NMR (CDCl3, 125 MHz): δ 14.89 (C-27), 15.77 (C-24), 18.51 (C-28), 21.25 (C-32), 22.55 (C-26), 31.37 (C-13), 33.24 (C-2), 35.45 (C-3), 37.22 (C-23), 39.40 (C-30), 40.98 (C-9), 43.44 (C-14), 47.39 (C-16), 50.02 (C-5), 57.25 (C-6), 80.64 (C-3), 109.54 (C-29), 150.16 (C-20), 173.79 (C=O). MS m/z: 557 (M+H)+, 543, 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for C35H40O5: 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–H2O gave colorless needles (77 mg, 43.8%); mp 259–261 °C. IR ν(CH) (cm−1): 3466 (OH of COOH), 1728 (C=O ester), 1685 (C=O acid), 1642 (C=O). 1H NMR (CDCl3, 500 MHz): δ 0.76, 0.84, 0.88, 0.89, 0.97, 0.99 (each 3H, s, 6 × CH3), 1.00 (1H, m), 1.60–1.66 (2H, m). 13C NMR (CDCl3, 125 MHz): δ 14.89 (C-27), 15.77 (C-24), 18.51 (C-28), 21.25 (C-32), 22.55 (C-26), 31.37 (C-13), 33.24 (C-2), 35.45 (C-3), 37.22 (C-23), 39.40 (C-30), 40.98 (C-9), 43.44 (C-14), 47.39 (C-16), 50.02 (C-5), 57.25 (C-6), 80.64 (C-3), 109.54 (C-29), 150.16 (C-20), 173.79 (C=O). MS m/z: 557 (M+H)+, 543, 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for C35H40O5: C, 77.45; H, 10.25. Found: C, 77.70; H, 10.11.

3-O-Valeryl-betulinic acid (11). Starting with valeric anhydride (67.7 mg); after crystallization from MeOH–H2O gave colorless needles (77 mg, 43.8%); mp 259–261 °C. IR ν(CH) (cm−1): 3466 (OH of COOH), 1728 (C=O ester), 1685 (C=O acid), 1642 (C=O). 1H NMR (CDCl3, 500 MHz): δ 0.76, 0.84, 0.88, 0.89, 0.97, 0.99 (each 3H, s, 6 × CH3), 1.00 (1H, m), 1.60–1.66 (2H, m). 13C NMR (CDCl3, 125 MHz): δ 14.89 (C-27), 15.77 (C-24), 18.51 (C-28), 21.25 (C-32), 22.55 (C-26), 31.37 (C-13), 33.24 (C-2), 35.45 (C-3), 37.22 (C-23), 39.40 (C-30), 40.98 (C-9), 43.44 (C-14), 47.39 (C-16), 50.02 (C-5), 57.25 (C-6), 80.64 (C-3), 109.54 (C-29), 150.16 (C-20), 173.79 (C=O). MS m/z: 557 (M+H)+, 543, 456, 438, 423, 395, 248, 220, 203, 189. Anal. Calcd. for C35H40O5: C, 77.45; H, 10.25. Found: C, 77.70; H, 10.11.

**MTT cytotoxic assay. In vitro cytotoxic activity of betulinic acid (1) and its 3-O-acetylated 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 at 72h at 37 °C in a 5% CO2 humidified incubator. After 72h of incubation, the fractions of surviving cells were measured relative to the untreated cell population by colorimetric MTT assay. A volume of 20μl 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, 10μl 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

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀ (µg/ml)</th>
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<tr>
<td></td>
<td>A549</td>
</tr>
<tr>
<td>1</td>
<td>8.4</td>
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<tr>
<td>2</td>
<td>&gt;30</td>
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<td>3</td>
<td>18.4</td>
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<td>7.4</td>
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<td>10</td>
<td>12.1</td>
</tr>
<tr>
<td>11</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

calculated using the following equation: % Viability = (OD sample/ OD control) x 100. A plot of percentage of cell viability against the concentration of the drug gives a measure of cytotoxicity. The cytotoxic index used was IC₅₀, 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 methodology (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 (CAVO3) cancer cell lines by MTT assay. The results presented in Table 1 are expressed as the concentration inhibiting 50% of the cell growth (IC₅₀).

Based on the IC₅₀ values, compounds with IC₅₀ < 10 µg/ml were considered strongly active, those with IC₅₀ ranging from 10 to 30 µg/ml were considered moderately active, and those with IC₅₀ > 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 (IC₅₀ < 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 < IC₅₀ < 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 (IC₅₀ > 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 (IC₅₀ = 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 (IC₅₀ > 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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References


