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SYNTHESIS AND ANTITUMOR ACTIVITY OF C-3(*R*) HYDROXY MODIFIED BETULINIC ACID DERIVATIVES

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Four novel betulinic acid (BA) derivatives modified at the C-3 position were prepared by the Mitsunobu reaction and subsequently evaluated for antitumor activity against four common cancer cell lines in vitro*. The results from the MTT assay revealed that the four tested compounds showed remarkable cytotoxic activity against four cancer cell lines, much better than BA. Western blotting results indicated that the four compounds, especially compound* ***2c****, caused a significant up-regulation expression of the caspase-12 in CT26 cell. Our results showed that configuration inversions of chiral C-3 of BA could also produce BA derivatives with potent antitumor activity.*

Keywords: betulinic acid, antitumor activity, cinnamic acids, MTT assay.

Betulinic acid (**1**, BA) is a pentacyclic triterpene of the lupane group. Due to its potential biological activities, especially antitumor activity, hundreds of BA derivatives were synthesized for clinical trial [1–7]. Modifications of BA have focused mainly on the C-3 hydroxyl, C-20 allylic, C-28 carboxylic acid, A-ring, D-ring, and E-ring positions [8, 9]. Among these target sites, the 3β-hydroxy moiety is a primary location for chemical modifications, including esterification, amination, etherification, oxidation, and so on [10]. Recently, a series of C-3 modified BA derivatives was synthesized and exhibited higher antitumor ability [11–15]. Several structure activity relationship studies [16–18] have indicated that a moderately bulky and electron-withdrawing group at or near the C-3 site would improve the antitumor activity regardless of the configuration of 3-OH (whether the configuration of the hydroxy group at C-3 is *R* or *S*). However, it was noted that most of the C-3 modified BA derivatives had (*S*)-configuration, and there are very few reports on the antitumor activities of compounds with (*R*)-configuration at this site. Thus, the synthesis of C-3(*R*) BA derivatives is of great interest and, based on it, we developed novel BA derivatives by introducing key substituting groups into the 3β-hydroxy group of BA. Cinnamic acid possesses potential antitumor activity [19, 20] and could be widely employed for the construction of new molecules as basic blocks in the field of medicinal chemistry. We selected four frequently used cinnamic acids, including three cinnamic acids with the electron-withdrawing groups as substituents for synthesizing novel BA derivatives with the (*R*)-configuration of the C-3 OH site by the classical Mitsunobu reaction. Biological evaluation of these BA analogues against four cancer cell lines was carried out, and the cytotoxicity of C-3 modified BA derivatives against 4T1 cell was studied for the first time.

Four new BA derivatives **1a–d** were synthesized according to the procedure described in Scheme 1. The BA esters **2a–d** were synthesized by interaction with the corresponding cinnamic acids in the presence of DEAD, PPh_3, and dry THF at 0°C under an inert (N_2) atmosphere according to the Mitsunobu reaction [21].

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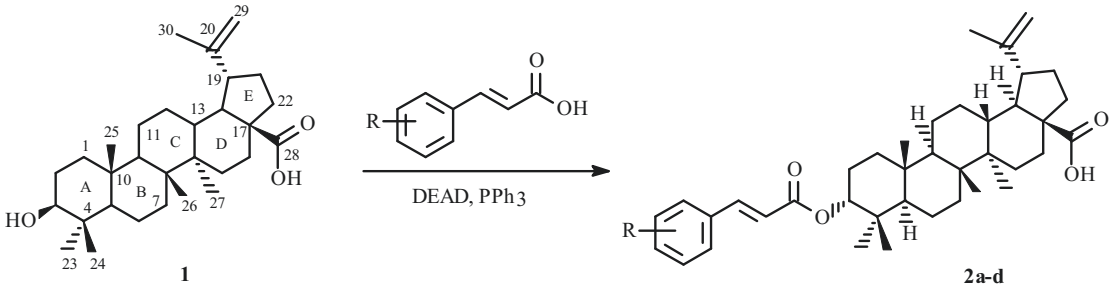
TABLE 1. Cytotoxicity Profile of **1**, **2a–d** against Selected Cancer Cell Lines (IC_{50}, μM ± SD)

Compound	7721	A549	4T1	CT26
1	30.33 ± 1.52	45.31 ± 2.26	41.06 ± 2.05	50.11 ± 2.51
2a	17.48 ± 0.87	23.11 ± 1.15	38.99 ± 1.95	26.92 ± 1.35
2b	19.98 ± 0.10	23.81 ± 1.19	33.50 ± 1.65	28.84 ± 1.44
2c	17.53 ± 0.88	20.14 ± 1.01	22.34 ± 1.12	16.98 ± 0.85
2d	21.42 ± 1.07	26.90 ± 1.35	27.61 ± 1.38	33.88 ± 1.69



Scheme 1. Synthesis of the BA derivatives **2a–d**. Reagents and conditions: diethyl azodicarboxylate (DEAD), triphenylphosphine (PPh_3), dry tetrahydrofuran (THF), –30°C, 15 min, 0°C, 2–4h.

The cytotoxic activity of BA and its derivatives **2a–d** *in vitro* was determined by using the MTT assay, and the result of testing against four cancer cell lines, human hepatocellular carcinoma cell (7721), human lung adenocarcinoma cell (A549), mouse breast cancer cell (4T1), and human colon cancer cell (CT26), is summarized in Table 1. The up-regulation effects of compounds **2a–d** was studied by Western blot analysis. As shown in Fig. 1, pretreatment with 25 μM of the four compounds led to the increase in the caspase-12 protein level. Among them, compound **2c** was the most potent derivative, showing the strongest cytotoxic effect on caspase-12 protein expression. Different concentrations (10, 30, and 100 μM) of compound **2c** were subsequently selected to study its effect through the caspase signaling pathway to up-regulate the proteinase with only cell sap added and BA (50 μM) as the reference. These results indicated that compounds **2a–d** can accelerate cell apoptosis in CT26 cell through up-regulation of caspase-12 expression. The antitumor activity of compounds **2a–d** might be attributed to the caspase signaling pathway. Thus, the studied BA derivatives displayed good antitumor activity, and compound **2c** is a lead compound.

In human hepatocellular carcinoma cell (7721), compounds **2a–d** had better cytotoxicity than BA. Compounds **2a** and **2c** were almost 1.8-fold more potent than BA. In human lung carcinoma cell (A549), compounds **2a–d** also showed higher potency than BA. Compound **2c** was the most active compound, which displayed 2.3-fold better cytotoxicity than BA. In mouse breast cancer cell (4T1), although compounds **2a** and **2b** showed comparable activity with BA, compounds **2c** and **2d** exhibited higher cytotoxicity than BA. In human colon cancer cell (CT26), all the compounds tested showed stronger cytotoxic activities than BA. Compound **2c** was the most potent, possessing an almost threefold higher inhibitory activity than BA. It is a noteworthy that all four BA derivatives studied had the C-3(*R*) OH configuration. Thus, compared to the C-3(*S*) BA derivatives reported [11–15], the antitumor activity of the four C-3(*R*) BA derivatives are as follows: (1) compound **2c** exhibited similar potency to compound **3** against human colon cancer cell lines CT26 and DLD-1; (2) the cytotoxicity of C-3(*R*) BA derivatives was close to C-3(*S*) BA derivatives against human lung carcinoma cell, such as A549 cell; (3) the four C-3(*R*) BA derivatives **2a–d** showed stronger cytotoxic activity than BA against human hepatocellular carcinoma cell (HepG2 and 7721).

As discussed above, modifications at the C-3 position in BA seemed to be critical to antitumor activity, particularly for the C-3(*R*) structure. However, the synthesis and antitumor activities of C-3(*R*) BA derivatives have rarely been reported so far. In this study, we show that the introduction of electron-withdrawing groups in the C-3(*R*) site of BA ring also significantly elevated cytotoxicity, and the obtained assay results showed that the introduction of an aromatic lipophilic side chain and electron-withdrawing groups such as the nitro group (NO_2) could obviously increase cytotoxic activity.

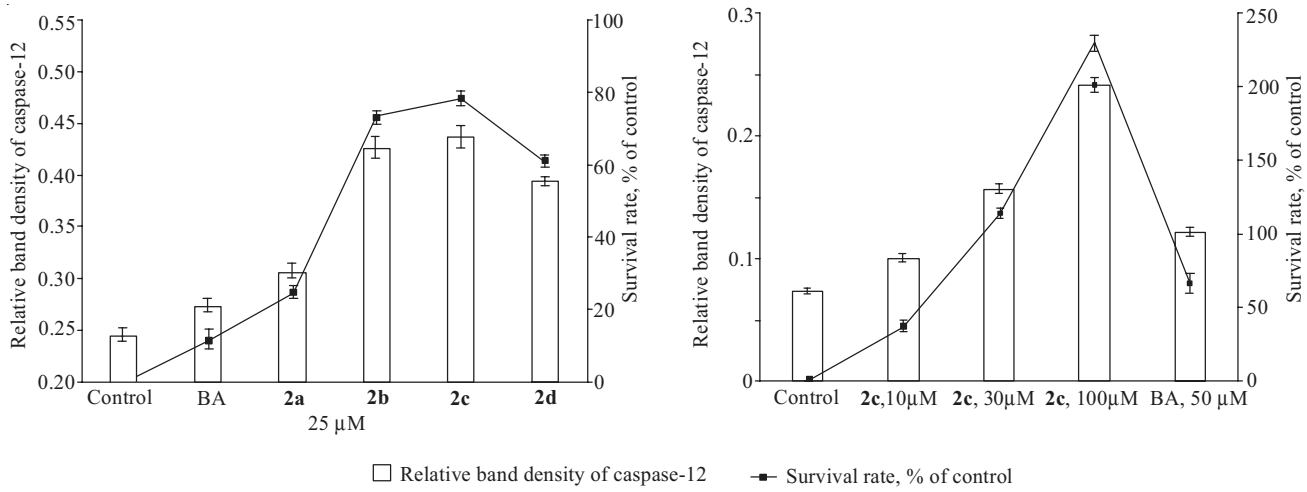


Fig. 1. Effects of compounds **2a–d** on cytotoxic activity in CT26 cell. Image J software was used to analyze the levels of caspase-12 with GAPDH as the reference. Data represent mean ±SD of three independent experiments.

EXPERIMENTAL

General. ^{1}H NMR spectra were recorded on Bruker AM 600 MHz spectrometers with tetramethylsilane (TMS) as internal standard. Melting points (mp) were recorded on an SRS OptiMelt-100 fully automatic micro melting point instrument. Electrospray ionization mass spectra (ESI-MS) were recorded on an Agilent 6520B Q-TOF system. FT-IR spectra were recorded on a Nicolet 380 fourier transform infrared spectrometer using KBr pellets in the 400–4000 cm^{-1} range. Column chromatography (CC) was performed on silica gel (200–300 mesh; Qingdao Makall Group Co., Ltd.; Qingdao, China). All reactions were monitored using thin-layer chromatography (TLC) on silica gel plates. Reagents were analytical reagent grade and purchased from Aladdin.

General Procedure for the Preparation of Compounds 2a–d. A mixture of betulinic acid (0.31 g, 0.67 mmol), PPh_3 (0.21 g, 0.8 mmol), and cinnamic acid (0.8 mmol) in dry THF (10 mL) was stirred at –30°C under an inert (N_2) atmosphere; then a solution of DEAD (130 μL, 0.8 mmol) in dry THF (10 mL) was added dropwise over 15 min while the temperature was maintained at –30°C. When the addition was complete, the mixture was allowed to warm gradually to 0°C and was vigorously stirred for 2–4 h. After completion of the reaction as indicated by TLC, saturated aqueous $NaHCO_3$ (20 mL) was added. The aqueous phase was separated and extracted with ether (3 × 10 mL). The organic extracts were combined, dried, and concentrated. To the residue was added ether (3 × 10 mL) and hexane (3 × 10 mL) whereupon the bulk of the triphenylphosphine oxide was filtered off. Concentration of the residue gave a viscous oil, which was purified by CC using ethyl acetate–petroleum ether (3:1) to give **2a–d** as white solids.

(1*R*,3a*S*,5a*R*,5b*R*,7a*R*,9*R*,11a*R*,11b*R*,13a*R*,13b*R*)-9-(Cinnamoyloxy)-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)icosahydro-3a*H*-cyclopenta[a]chrysene-3a-carboxylic Acid (2a). White solid, yield 65%, mp 241–243°C. ESI-MS *m/z* 586.7. IR (KBr, ν, cm^{-1}): 3473, 3238, 2940, 2863 (C–H), 1747 (COOH), 1682 (C=O), 1640, 1533, 1482, 1435 (C=C). PMR (600 MHz, DMSO-d_6, δ, ppm, J/Hz): 7.62 (3H, dd, J = 12.4, 7.5), 7.55 (2H, dd, J = 7.3, 2.7), 4.68 (1H, d, J = 1.8), 4.56 (1H, s), 4.03 (1H, q, J = 7.1), 2.98–2.91 (2H, m), 2.22 (1H, td, J = 12.7, 3.4), 2.14–2.09 (1H, m), 1.83–1.77 (2H, m), 1.64 (3H, s), 1.61–1.52 (2H, m), 1.50 (1H, d, J = 11.3), 1.43 (4H, dd, J = 8.7, 4.4), 1.40 (1H, d, J = 4.3), 1.39–1.33 (3H, m), 1.33–1.27 (4H, m), 1.24 (1H, dd, J = 12.7, 2.2), 1.17 (2H, t, J = 7.1), 1.09 (1H, d, J = 12.4), 0.97 (1H, dd, J = 13.0, 4.5), 0.92 (3H, s), 0.86 (6H, d, J = 2.9), 0.85–0.80 (1H, m), 0.76 (3H, s), 0.65 (3H, s).

(1*R*,3a*S*,5a*R*,5b*R*,7a*R*,9*R*,11a*R*,11b*R*,13a*R*,13b*R*)-9-(((*E*)-3-(4-Bromophenyl)acryloyl)oxy)-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)icosahydro-3a*H*-cyclopenta[a]chrysene-3a-carboxylic Acid (2b). White solid, yield 63%. mp 240–242°C. ESI-MS *m/z* 666.6. IR (KBr, ν, cm^{-1}): 3423, 3244, 2993, 2861 (C–H), 1747 (COOH), 1692 (C=O), 1626, 1583, 1530, 1482 (C=C), 1070 (C–Br). PMR (600 MHz, DMSO-d_6, δ, ppm, J/Hz): 12.36 (1H, s), 7.61 (3H, d, J = 8.6), 7.58 (1H, s), 7.55 (1H, s), 6.57 (1H, d, J = 16.0), 4.69 (1H, d, J = 1.8), 4.56 (1H, s), 4.27 (1H, d, J = 5.1), 4.03 (1H, dd, J = 14.1, 7.0), 2.96 (2H, ddd, J = 16.0, 10.6, 5.4), 2.22 (1H, td, J = 12.8, 3.4), 2.11 (1H, d, J = 9.7), 1.83–1.76 (2H, m), 1.64 (3H, s), 1.56 (2H, dd, J = 28.5, 15.4), 1.50 (1H, d, J = 11.3), 1.45–1.41 (3H, m), 1.35 (3H, dd, J = 12.3, 10.0), 1.33–1.29 (3H, m), 1.25 (2H, d,

J = 13.8), 1.17 (2H, t, J = 7.1), 1.09 (1H, d, J = 10.3), 0.97 (1H, dd, J = 13.0, 4.5), 0.93 (3H, s), 0.86 (6H, d, J = 2.6), 0.76 (3H, s), 0.65 (3H, s).

(1*R*,3a*S*,5a*R*,5b*R*,7a*R*,9*R*,11a*R*,11b*R*,13a*R*,13b*R*)-5a,5b,8,8,11a-Pentamethyl-9-(((*E*)-3-(4-nitrophenyl) acryloyl)oxy)-1-(prop-1-en-2-yl)icosahydro-3a*H*-cyclopenta[a]chrysene-3a-carboxylic Acid (2c). White solid, yield 58%, mp 254–256°C. ESI-MS *m/z* 632.7. IR (KBr, ν, cm^{-1}): 3441, 3116, 2934, 2863 (C–H), 1746 (COOH), 1685 (C=O), 1626, 1599, 1524, 1423 (C=C), 1343 (C-NO_2). PMR (600 MHz, DMSO-d_6, δ, ppm, J/Hz): 12.08 (1H, s), 7.56 (2H, d, J = 3.0), 7.54 (2H, d, J = 2.7), 4.69 (1H, d, J = 1.8), 4.56 (1H, s), 4.27 (1H, d, J = 5.0), 4.03 (2H, q, J = 7.1), 3.00–2.93 (2H, m), 2.22 (1H, td, J = 12.7, 3.4), 2.13–2.09 (1H, m), 1.80 (2H, dd, J = 8.7, 4.8), 1.64 (3H, s), 1.53 (1H, d, J = 6.4), 1.50 (1H, d, J = 11.3), 1.46–1.41 (4H, m), 1.39–1.34 (3H, m), 1.33–1.29 (3H, m), 1.26–1.22 (1H, m), 1.20 (1H, dd, J = 10.0, 4.1), 1.17 (3H, t, J = 7.1), 1.09 (1H, d, J = 12.4), 0.97 (1H, dd, J = 13.0, 4.6), 0.92 (3H, s), 0.86 (6H, d, J = 3.2), 0.76 (3H, s), 0.65 (3H, s).

(1*R*,3a*S*,5a*R*,5b*R*,7a*R*,9*R*,11a*R*,11b*R*,13a*R*,13b*R*)-9-(((*E*)-3-(4-Chlorophenyl)acryloyl)oxy)-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)icosahydro-3a*H*-cyclopenta[a]chrysene-3a-carboxylic Acid (2d). White solid, yield 62%, mp 253–255°C. ESI-MS *m/z* 622.2. IR (KBr, ν, cm^{-1}): 3449, 3238, 2940, 2866 (C–H), 1746 (COOH), 1682 (C=O), 1622, 1587, 1488, 1399 (C=C), 1230 (C–Cl). PMR (600 MHz, DMSO-d_6, δ, ppm, J/Hz): 12.25 (1H, s), 7.73 (2H, d, J = 8.5), 7.58 (1H, d, J = 16.0), 7.51–7.44 (2H, m), 6.56 (1H, d, J = 16.0), 4.69 (1H, d, J = 2.0), 4.56 (1H, s), 4.27 (1H, d, J = 5.1), 4.03 (1H, q, J = 7.1), 2.95 (2H, qd, J = 10.7, 5.4), 2.22 (1H, td, J = 12.7, 3.5), 2.15–2.08 (1H, m), 1.84–1.76 (2H, m), 1.64 (3H, s), 1.54 (1H, d, J = 6.8), 1.50 (1H, d, J = 11.3), 1.43 (4H, dd, J = 12.0, 7.3), 1.41–1.37 (2H, m), 1.36–1.33 (2H, m), 1.31 (2H, d, J = 6.1), 1.25 (2H, dd, J = 12.9, 2.3), 1.16 (3H, dt, J = 12.8, 5.7), 1.09 (1H, d, J = 10.4), 0.93 (3H, s), 0.87 (6H, d, J = 2.6), 0.76 (3H, s), 0.65 (3H, s).

Reagents and Cell Culture. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) and 96-well plates (Beyotime Biotechnology) were used for studies. All cells were purchased from the American Tissue Culture Collection (ATTC). Cells were grown in high glucose Dulbecco′s modified Eagle′s medium (DMEM) containing 10% (v/v) fetal calf serum and 100 mM penicillin and 100 mM streptomycin. All cells were propagated at 37°C in a humidified atmosphere containing 5% CO_2 in air. Compounds **2a–d** were dissolved in dimethyl sulfoxide to make stock solutions and kept at –20°C. The final concentration of the vehicle in the solution never exceeded 0.1% and had no effects on cell viability.

Cell Proliferative Assay. Cell proliferative assays were carried out using 96-well plate cultures and MTT staining. Briefly, test compounds were added from stock solutions at 10 mM in the incubation mixture in a small volume of dimethyl sulfoxide (DMSO). Selected cancer cell lines (3000 cells/well) were treated with serial dilutions (100, 80, 40, 30, 20, and 10 μM) of BA and derivatives in 96-well culture plates for 72 h. Then 20 μL of MTT solution was added to each well, and the microplates were further incubated at 37°C for 4 h. The medium was aspirated, and the insoluble formazan product was dissolved in DMSO for at least 2 h in the dark. Then the extent of MTT reduction was quantified by measuring the absorbance at 540 nm. IC_{50} values were calculated using the formula $IC_{50} = 1 – [(C_{experimental\ group} – C_{blank})/(C_{control} – C_{blank})]$ through nonlinear fitting in Origin software. $C_{experimental\ group}$, $C_{control}$, and C_{blank} are mean the concentrations of the experimental group, the only cell sap added group, and the no-additions (NA) group, respectively. The results were determined from replicates of 96 wells from at least three independent experiments

Protein Extraction and Western Blotting Analysis. The operation was conducted as follows [22]. The CT26 cells on 6-well plates were rinsed twice with cold PBS and lysed in 100 μL RIPA lysis buffer (10 mM HEPES, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, and 1 mM PMSF) on ice for 30 min. Insoluble components of cell lysates were removed by centrifugation at 12000 g for 5 min at 4°C, and protein concentrations were measured using the BCATM protein quantification kit. The caspase-12 protein level was determined by western blotting analysis of 50 μg of cell extract using antibody against caspase-12 (2202). Briefly, the protein samples were centrifuged (4°C, 12000g, 10 min) and boiled for 10 min, then subjected to 10% SDS-polyacrylamide gel electrophoresis at a constant 20 mA current for 1 h. The resolved proteins were transferred to a PVDF membrane by wet rotation at 70 mV and blocked with a blocking buffer (2% fat-free milk, 10 mM Tris-Cl, 50 mM NaCl, 0.1% Tween 20, pH 7.4) at room temperature for 1 h. The membrane was incubated with primary antibodies against caspase-12 and GAPDH overnight on the converter at low temperature (4°C). The next day, the membrane was washed using the washing buffer (10 mM Tris-Cl, 50 mM NaCl, 0.1% Tween 20, pH 7.4) three times, twice for 5 min and once for 10 min, to remove any nonspecific primary antibody binding. Then the membrane was incubated with an appropriate dilution of secondary antibody at room temperature for 2 h. After washing three times with washing buffer, the membrane was illuminated with ECL reagent according to the manufacturer′s instructions. A photograph of the gel was taken, and the relative band density was analyzed by optical densitometry using Image J. Survival rate values were calculated using the formula $SR = (D_{experimental\ group} – D_{control})/(D_{control})$ through nonlinear fitting in Origin software. $D_{experimental\ group}$, $D_{control}$, and D_{blank} are the mean band density of the

experimental group, the only cell sap added group, and the no-additions (NA) group, respectively. The results were determined from replicates of six wells from at least three independent experiments.

In summary, compounds **2a–d** exhibited more potent cytotoxic activity than BA in four cancer cell lines. Especially, compound **2c** was the most active in A549, 4T1 with CT26 cell lines and may be chosen for further studies. The electron-withdrawing groups on the aromatic ring at the C-3 position showed stronger cytotoxicity. Moreover, the stronger the electron-withdrawing ability, the better the cytotoxicity when comparing compound **2c** with **2a**, **2b**, and **2d**. This brief SAR study should not be overlooked for a better understanding of the structural requirements at the C-3 position of BA for antitumor effects.

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