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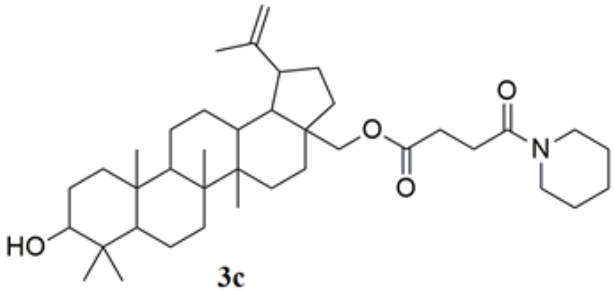
Graphical abstract

Novel betulin acid derivatives were synthesized and investigated for their antitumor activity.



3c

Cells	IC_{50} (μM)
MGC-803	4.3
PC3	4.5
Bcap-37	5.2
A375	7.5
MCF-7	5.2

Synthesis and in vitro antitumor evaluation of betulin acid ester derivatives as novel apoptosis inducers

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Conflicts of interest: None.

Author's contributions:

Sheng-Jie Yang and Ming-Chuan Liu synthesized the compounds and carried out most of the bioassay experiments. Hong-Mei Xiang took part in the compound structural elucidation and bioassay experiments. Qi Zhao carried out some structure elucidation experiments. Wei Xue assisted in structural elucidation experiments. Prof. Song Yang is the co-corresponding author for this work.

Abbreviation list:

ADM: adriamycin

AO/EB: acridine orange/ethidium bromide

BE: betulin

^{13}C NMR: ^{13}C Nuclear Magnetic Resonance

DMF: N, N-dimethylformamide

DMSO: dimethyl sulfoxide

HCPT: 10-hydroxyl camptothecine

^{1}H NMR: Proton Nuclear Magnetic Resonance

IR: Infra-red

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

TUNEL: terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling

Abstract

Nineteen betulin derivatives modified at the C-3 and C-28 positions were synthesized and assessed for antitumor activities against the MGC-803, PC3, Bcap-37, A375, and MCF-7 human cancer cell lines in vitro by MTT assay. Some derivatives (compounds **3a–3d** and **5**) displayed strong antitumor properties, with IC_{50} values between 4 and 18 μM. Compound **3c**, containing piperidine group at C-28 positon, had IC_{50} values of 4.3, 4.5, 5.2, 7.5, and 5.2 μM on the five cancer cell lines, respectively. Subsequent fluorescence staining and flow cytometric analysis indicated that compound **3c** induced apoptosis in MGC-803 cell line, with an apoptosis ratio of 31.11% after 36 h of treatment at 10 μM **3c**.

Keywords: Betulin derivatives; Synthesis; Antitumor; Cancer cells; Apoptosis

1. Introduction

Betulin (BE; 1; lup-20(29)-ene-3β, 28-diol), is an abundant and naturally occurring pentacyclic triterpenoid [1,2]. This compound is a major effective component of many traditional Chinese medicines that is widely distributed in plants [3-5], and forms up to 30% of the dry weight of the extractive (e.g., the bark of birch trees) [6-8]. BE possesses various biological and pharmacological activities [9], such as anti-HIV [10], anti-inflammatory [11,12], , antiretroviral [13], antibacterial properties [14], and anticancer properties [15,16]. Recently, some studies have shown that BE had marked antitumor activities against various types of cancer cell lines [17], such as colorectal (DLD-1), prostate (PC3), breast (MCF-7), and lung (A549) cancer cell lines, and could induce A549 cell-line apoptosis [18-21]. Furthermore, a recent report found BE to be more active against other melanoma lines (G361, SK-MEL-28), neuroblastoma cell lines (GOTO, NB-1), and leukemia lines (HL-60, U937, K562) [22]. However, compared to other pentacyclic triterpenoids, BE is inactive against melanoma cell line (MEL-2) [23]. Researchers had demonstrated that keeping a polar substituent at the C-3 position was essential for the pharmacological activities of pentacyclic triterpenes [24]. According to the structure-activity relationship, a hydrogen donor group at either C-3 or C-28 position can improve cell proliferation inhibition significantly [25]. In recent years, the drug, with introduced amino alkyl groups has attracted considerable attention. Recent studies have shown that the introduction of amino alky groups had unexpected improvements in the anti-HIV or antitumor activities of compounds [25]. In 2010, Kommera et al. found that the antitumor activities of BE derivatives with amino acyl groups introduced at C-28 position were significantly improved, and had the therapeutic potential in the treatment of gastric carcinoma [26]. Furthermore, previous work already indicated that the introduction of aromatic carboxyl groups to pentacyclic triterpenoids could result in good cytotoxicity [27,28].

Thus, in view of the previous rationale and in continuation of an ongoing program aiming at developing more potential anticancer drugs, in the present study nineteen BE derivatives modified at C-3 and C-28 positions were designed and synthesized. The antitumor activities of these derivatives were screened in vitro by MTT assay using five human cancer cell lines MGC-803 (human gastric carcinoma cell line), PC3 (human prostate carcinoma cell line), Bcap-37 (human breast carcinoma cell line), A375 (human malignant melanoma cell line), and MCF-7 (human

breast carcinoma cell line). Additionally, the antitumor mechanism of the BE derivatives was investigated through acridine orange/ethidium bromide staining (AO/EB), Hoechst 33258 staining, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, and flow cytometric analysis.

2. Results and discussion

2.1. Chemistry

The syntheses of BE derivatives are summarized in Scheme 1. The structure of BE was modified at C-3 and C-28 positions. Succinic anhydride and maleic anhydride were introduced at the C-28 position in dry dichloromethane (DCM) in the presence of pyridine to obtain compounds **2** and **4**. Compound **2** was treated with corresponding amines in dry DCM, in the presence of PyBOP to afford compounds **3a–3d**. The 28-OH and 3-OH of BE were acetylated with aromatic carboxyl group in the presence of DCC and DMAP in dry DCM at room temperature to gain the compounds **6a–6d**. Finally, only the 28-OH of BE was acetylated using EDCI instead of DCC to obtain the compounds **7a–7g** in high yields. All the compounds were fully characterized by various spectroscopic methods.

Scheme 1

2.2. Evaluation of antitumor activities

In vitro inhibitory activities of the synthesized BE derivatives were evaluated against MGC-803, PC3, Bcap-37, A375, and MCF-7 cell lines by MTT assay. Hydroxycamptothecine (HCPT) and Adriamycin (ADM) were used as positive controls. A culture medium containing 0.1% DMSO served as negative control. All BE derivatives including ADM and HCPT were dissolved in DMSO. Each experiment was repeated thrice. The IC_{50} values are summarized in Table 1.

Table 1

Table 1 presents the modified compounds at the C-28 position with selected amino groups displaying potent antitumor activities against the five cancer cell lines. These compounds had IC_{50} values between 4 and 18 μM. Furthermore, the most active compound among the synthesized BE derivatives was compound **3c**. This compound had approximately four times more activity than that of BE, and the IC_{50} values were 4.3, 4.5, 5.2, 7.5, and 5.2 μM on the five cancer cell lines,

respectively. Further experiments indicated that proliferation of the five cancer cells were significantly inhibited by compound **3c** in a concentration-dependent manner (Figure 1).

Figure 1

Compounds **2** and **4**, with succinic anhydride and maleic anhydride introduced at the C-28 position, had more activity than that of the parent BE. This result may be attributed to the introduced carboxyl group, which was more hydrophilic than the hydroxyl group. This phenomena was similar to Drag-Zalesinska observations, wherein the most active compounds were those with the highest solubility in water [29].

Recently, some researchers studied the structure-activity relationships of other pentacyclic triterpenes including Betulinic acid, Ursolic acid, and Oleanolic acid. These pentacyclic triterpenes had similar structure-activity relationships despite the different structures.[30] Previous studies showed that the introduction of amino alkyl groups at the C-28 position further enhanced the antitumor activities. In this study, the compounds modified at the C-28 position with selected amino groups displayed potent antitumor activities (IC_{50} values, between 4 and 18 μM), identical with the previous reports [30].

Remarkably, when 28-OH was esterified with aromatic acid (**7a–7g)**, the modification led to a decline in activity. In addition, when 28-OH and 3-OH were esterified at the same time (**6a–6d**), the activity was reduced or even disappeared. The low activity can be explained by the observation of Kommera et al. [26]: "One reason for low activity can be due to the straight and rigid phenyl groups and the possible interactions with the π-system of the phenyl ring lead to a much bulky molecule, thereby with a lower ability to penetrate the cell membrane."

In addition, the active compounds **2**, **3a–3d**, **4**, and **5** were also tested on normal cell line NIH3T3. As shown in Table 2, compounds **2** and **3a–3d** were less toxic on normal cell lines than that on cancer cell lines, and more selective to cancer cell lines than compounds **4** and **5**. Compounds **4** and **5** were selective to cancer cells, and were, to some extent, cytotoxic on normal cells.

Table 2

*2.3. Preliminary investigation of the apoptosis-inducing effect of title compound **3c***

BE and its derivatives have been reported to induce apoptosis in certain cancer cell lines [31]. In the present study, compound **3c** was selected to analyze the mechanism of growth inhibition of

MGC-803 cells. To determine whether or not the growth inhibitory activity of compound **3c** were related to the induction of apoptosis, the morphological changes of MGC-803 cells were investigated by AO/EB staining, Hoechst 33258 staining, and TUNEL assay.

The cell morphological changes brought by compound **3c** were assessed using fluorescence microscopy after AO/EB staining. AO permeates all the cells and stains the nuclei green, whereas EB only penetrates the cells when cytoplasmic membrane integrity is lost, and stains the nucleus red, dominating over AO. The stained cells revealed four different types under a fluorescence microscope: the chromatin of living cells was green with normal structure; the chromatin of non-apoptotic dead cells was red with normal structure; yellow coloration of early apoptotic cells with pyknotic morphology; and orange coloration of late apoptotic cells with pyknotic morphology. With HCPT as positive control, compound **3c** at 5 μM against MGC-803 cells from 12 h to 48 h was detected *via* AO/EB staining. The HCPT concentration was 10 μM against MGC-803 cells for 48 h. The results are shown in Figure 2**A**. Cells treated with compound **3c** from 12 to 48 h had morphological changes. The nuclei stained as yellow green or orange, and the morphology showed pyknosis, membrane blebbing, and cell budding. These phenomena were associated with cell apoptosis.

The morphological changes during cell apoptosis were also assayed using Hoechst 33258 staining. Live cells with uniformly light blue nuclei were treated with Hoechst 33258 and observed under a fluorescence microscope. Four types of stained cells were observed, characterized by cytoplasmic and nuclear shrinkage, chromatin condensation, and apoptotic body. With HCPT (at 10 μM for 48 h) as positive control, compound **3c** at 5 μM was tested against MGC-803 cells from 12 h to 48 h *via* Hoechst 33258 staining. Results are shown in Figure 2**B**, which shows that the cells of the negative group were stained blue, indicating normal condition. However, the cells of HCPT group appeared compact and condensed, with strong blue fluorescence, signifying typical apoptosis characteristics. The cells treated with compound **3c** from 12 to 48 h had morphological changes. The blue emission light in apoptotic cells was brighter than the negative group. Condensed chromatin could also be found in many treated cells and some cells formed apoptotic bodies, which is a characteristic of apoptotic cells. These results were similar with the previous AO/EB dual staining results.

TUNEL, one of the popular methods to investigate the apoptosis induction, relies on the presence

of nicks in the DNA. DNA nicks can be identified by terminal deoxynucleotidyl transferase, an enzyme that will catalyze the addition of dUTPs, which are secondarily labeled with a marker. Cells with brown precipitate are positive to apoptosis. With HCPT used as positive control at 10 μM for 48 h, compound **3c** at 5 μM was tested against MGC-803 cells from 12 to 48 h and apoptosis was detected using TUNEL assay. Results are shown in Figure 2**C**. Cells of the negative group did not appear as brown precipitates, whereas HCPT group appeared as brown precipitates. After 48 h, most of the cells treated with compound **3c** also appeared as brown precipitates. Therefore, compound **3c** induced apoptosis against MGC-803 cells. The results were identical with the previous experiments.

Figure 2

The apoptosis ratios induced by compound **3c** in cancer cells were quantitatively assessed by FCM. In early apoptotic cells, phosphatidylserine (PS), which is distributed inside the lipid bilayer of normal cells, was transferred from the inside of the cell membrane to the outside. Annexin V, a Ca^{2+} dependent phospholipid-binding protein with a high affinity for PS, was used to detect early apoptosis. Propidine Iodide (PI) is a red fluorescent dye, which stains cells that have lost membrane integrity. When the cells are dual stained with annexin V-FITC and PI, four different periods of apoptotic cells can be observed. Cells that are not stained with either annexin V or PI are viable and reside in the lower lift quadrant. Cells that are only stained with annexin V are in the stage of early apoptosis and reside in the lower right quadrant. Cells that are stained by both chemicals are nonviable apoptotic/necrotic cells and scatter in the upper right quadrant, while the upper left quadrant represents the nuclear fragments/necrotic cells stained only with PI. As shown in Figure 3**A**, with HCPT as positive control, compound **3c** (10 μM) could induce apoptosis of MGC-803 cells. The highest apoptosis ratio, 31.11% was obtained after 36 h of treatment at a concentration of 10 μM. Furthermore, as shown in Figure 3**B,** the apoptosis of MGC-803 cells increased gradually in a time-dependent manner.

Figure 3

3. Conclusions

In summary, nineteen BE derivatives were designed and synthesized. The growth inhibitory activities were evaluated against MGC-803, PC3, Bcap-37, A375, and MCF-7 human cell lines in

vitro for all derivatives. Among the BE derivatives synthesized, compound **2**, **3a–3c**, and **5** displayed strong antitumor properties showing IC_{50} values between 4 and 18 μM. Compound **3c** possessed the most potent activities, with IC_{50} values of 4.3, 4.5, 5.2, 7.5, and 5.2 μM for the five cancer cell lines, respectively. However, after introduction of aromatic acid in BE, the activity was reduced or even disappeared. Structure-activity relationship was also investigated in this study. Moreover, compound **3c** was selected to analyze the mechanism of growth inhibition of MGC-803 cell lines by AO/EB staining, Hoechst 33258 staining, TUNEL assay, and flow cytometric analysis. These mechanistic studies demonstrated that compound **3c** inhibited cell growths by inducing cell apoptosis. Apoptosis ratio reached 31.11% after 36 h of treatment at 10 μM, which is higher than the ratio observed for the positive control HCPT. These findings provide a powerful incentive for further research on the chemical modification and structure-activity relationships of BE and other triterpenoid acids.

4. Experimental section

4.1. General

Betulin with more than 98% purity was purchased from Zhejiang Tiancao Biotech Co., Ltd. Reagents of analytical grade were obtained from Yuda Chemistry Co., Ltd., and used without further purification unless otherwise noted. Silica gel (200–300 mesh) used in column chromatography was provided by Tsingtao Marine Chemistry Co., Ltd. IR spectra were recorded on a Bruker VECTOR22 spectrophotometer in KBr disks. ^{1}H NMR and ^{13}C NMR were performed on a JEOL-ECX500 spectrometer at 22 °C, with TMS as the internal standard.

4.2. Synthesis

*4.2.1. General procedure for compounds **2, 3a-3d, and 4-5**.*

Betulin reacted with succinic anhydride or maleic anhydride (0.50 g, 5.00 mmol) in dry DCM (35 mL) containing DMAP (0.06 g, 0.50 mmol) and the reaction mixture was reflux for 5 h, then the mixture was poured into the 100 mL distilled water which containing 1N HCl (10 mL) and partitioned with ethyl acetate that containing 20% butanol (3×30 mL), The organic layer was dried over Na_2SO_4, and purified via silica gel column chromatography (Flash column) with chloroform/methanol (20:1, v/v) to obtain the compounds **2** and **4**. And then, compounds **2** or **4** (1

mmol), amines (1.2 mmol), and PyBOP (1.2 mmol) were added to DCM (10 mL) containing Et_3N (0.5 mmol); the mixture was then stirred at room temperature for 6–12 h. After the reaction was completed, the mixture was poured onto 100 mL of distilled water and partitioned with ethyl acetate (3×50 mL). The target compounds were purified on a flash column with chloroform/methanol (20:1, v/v) to yield compounds **3a** and **5**.

Compound (**2**): Yield: 73.3%; white power, mp: 223-225 ^oC; IR (KBr, cm^{-1}): ν_{max} 3445, 3071, 2930, 2873, 1730, 1640, 882; MS (EI): 542 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 4.81 (1H, s, Hb-29), 4.67 (1H, s, Ha-29), 4.50 (1H, d, *J* = 8.5 Hz, Hb-28), 4.09 (1H, d, *J* = 10.2 Hz, Ha-28), 3.15~3.17 (1H, m, H-3), 2.85~2.89 (4H, m, CH_2), 1.66 (3H, s, H-30), 1.16 (3H, s, CH_3), 0.96 (3H, s, CH_3), 0.92 (3H, s, CH_3), 0.87 (3H, s, CH_3), 0.78 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 174.8 (C=O), 173.2 (C=O), 150.4 (C-20), 110.1 (C-29), 77.9 (C-3), 62.8 (C-28), 55.6 (C-5), 50.5 (C-9), 48.9 (C-19), 47.7 (C-17), 46.7 (C-18), 42.7 (C-14), 40.9 (C-8), 39.3 (C-4), 39.0 (C-1), 37.6 (C-13), 37.2 (C-10), 34.7 (C-7), 34.3 (C-22), 29.9 (CH_2), 29.9 (C-21), 29.8 (CH_2), 29.8 (C-16), 28.4 (C-23), 28.1 (C-2), 27.2 (C-15), 25.4 (C-12), 20.7 (C-11), 19.1 (C-30), 18.6 (C-6), 16.2 (C-25), 16.1 (C-26), 15.9 (C-24), 14.7 (C-27).

*4.2.2. General procedure for compounds **6a-6d**.*

Betulin (1 mmol), aromatic acids (2 mmol), DCC (2 mmol), and DMAP (1 mmol) were added to DCM (25 mL); the mixture was then stirred at room temperature for 6–12 h. After the reaction was completed, the mixture was poured onto 100 mL of distilled water and partitioned with ethyl acetate (3×50 mL). The target compounds were purified on a flash column with petroleum ether/EtOAc (20:1, v/v) to yield compounds **6a-6d**.

Compound (**6a**): Yield: 73.9%; colorless oil; IR (KBr, cm^{-1}): ν_{max} 3438, 1715, 2945, 1640, 1377, 1030; MS (EI): 650 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 8.04~8.06 (4H, m, PhH), 7.53~7.55 (2H, m, PhH), 7.41~7.45 (4H, m, PhH), 4.72~4.74 (1H, m, H-3), 4.70 (1H, s, Hb-29), 4.69 (1H, s, Ha-29), 4.54 (1H, d, *J* = 10 Hz, Hb-28), 4.10 (1H, d, *J* = 10.8 Hz, Ha-28), 1.74 (3H, s, H-30), 1.07 (3H, s, CH_3), 1.01 (3H, s, CH_3), 0.99 (3H, s, CH_3), 0.92 (3H, s, CH_3), 0.86(3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 167.1 (C=O), 166.4 (C=O), 150.2 (C-20), 132.9 (C), 132.8 (C), 131.0 (CH), 130.6 (CH), 129.7 (CH), 129.6 (CH), 128.5 (CH), 128.4 (CH), 110.0 (C-29), 81.7 (C-3), 63.4 (C-28), 55.4 (C-5), 50.4 (C-9), 48.9 (C-19), 47.8 (C-17), 46.8 (C-18), 42.9 (C-14), 41.0 (C-8), 38.5 (C-4), 38.3 (C-1), 37.8 (C-13), 37.2 (C-10), 34.8 (C-7), 34.2 (C-22), 30.0 (C-21), 29.8 (C-16), 28.2 (C-23), 27.2 (C-2), 25.3 (C-15),

23.9 (C-12), 20.9 (C-11), 19.2 (C-30), 18.3 (C-6), 16.9 (C-25), 16.3 (C-26), 16.2 (C-24), 14.9 (C-27).

*4.2.3. General procedure for compounds **7a-7g**.*

Betulin (0.45 g, 1.00 mmol), aromatic acids (1.20 mmol), EDCI (0.23 g, 1.20 mmol), and DMAP (0.06 g, 0.50 mmol) were added to DCM (10 mL) containing Et_3N (0.05 g, 0.50 mmol); the mixture was then stirred at room temperature for 6-12 h. After the reaction was completed, the mixture was poured onto 100 mL of distilled water and partitioned with ethyl acetate (3×50 mL). The target compounds were purified on a flash column with petroleum ether/EtOAc (10:1, v/v) to yield compounds **7a-7g**.

Compound (**7a**): Yield: 72.2%; colorless oil; IR (KBr, cm^{-1}): ν_{max} 2944, 2873, 1711, 1640, 1012, 880, 754, 703; MS (EI): 546 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 8.05 (2H, d, *J* = 7.3 Hz, PhH), 7.56 (2H, t, *J* = 11.4 Hz, PhH), 7.45 (2H, t, *J* = 14.4 Hz, PhH), 4.71 (1H, s, Hb-29), 4.60 (1H, s, Ha-29), 4.50 (1H, d, *J* = 9.5 Hz, Hb-28), 4.09 (1H, d, *J* = 10.3 Hz, Ha-28), 3.14~3.17 (1H, m, H-3), 1.69 (3H, s, H-30), 1.05 (3H, s, CH_3), 1.01 (3H, s, CH_3), 0.95 (3H, s, CH_3), 0.83 (3H, s, CH_3), 0.75 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 167.0 (C=O), 150.2 (C-20), 132.9 (C), 130.6 (CH), 129.6 (CH), 128.4 (CH), 109.9 (C-29), 77.1 (C-3), 63.4 (C-28), 55.4 (C-5), 50.5 (C-9), 48.9 (C-19), 47.8 (C-17), 46.8 (C-18), 42.8 (C-14), 40.9 (C-8), 38.9 (C-4), 38.8 (C-1), 37.7 (C-13), 37.2 (C-10), 34.8 (C-7), 34.3 (C-22), 30.1 (C-21), 29.7 (C-16), 28.1 (C-23), 27.5 (C-2), 27.2 (C-15), 25.3 (C-12), 20.9 (C-11), 19.3 (C-30), 18.4 (C-6), 16.2 (C-25), 16.1 (C-26), 15.4 (C-24), 14.9 (C-27).

4.3. Cell lines and culture

MGC-803, PC3, Bcap-37, A375, MCF-7, and NIH3T3 cell lines were obtained from the Institute of Biochemistry and Cell Biology, China Academy of Science. MGC-803 is gastric cancer cell line, PC3 is prostate cancer cell line, A375 is malignant melanoma cell line, Bcap-37 and MCF-7 are breast cancer cell lines. The entire cancer cell lines were maintained in the RPMI 1640 medium, whereas the NIH3T3 cell line was maintained in DMEM medium. They were supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37 °C. All cell lines were maintained at 37 °C in a humidified 5% carbon dioxide and 95% air incubator.

4.4. MTT Assays

All tested compounds were dissolved in DMSO and subsequently diluted in the culture medium before treatment of the cultured cells. When the cells were 80-90% confluent, they were harvested by treatment with a solution containing 0.25% trypsin, thoroughly washed and resuspended in supplemented growth medium. Cells (2×10^3/well) were plated in 100 μL of medium/well in 96-well plate. After incubations overnight, the cells were treated with different concentrations of extracts in RPMI 1640 with 10% FBS for 72 h. In parallel, the cells treated with 0.1% DMSO served as negative control and ADM (Adriamycin) as positive control. Finally, 100 μL of MTT was added, and the cells were incubated for 4 h. The MTT-formazan formed by metabolically viable cells was dissolved in 100 μL of SDS for 12 h. The absorbance was then measured at 595 nm with a microplate reader, which is directly proportional to the number of living cells in culture.

4.5. AO/EB Staining

The cells were seeded at a concentration of 5×10^4 cell/mL in a volume of 0.6 mL on a sterile cover slip in 6-well tissue culture plates. Following incubation, the medium was removed and replaced with fresh medium plus 10% FBS and then supplemented with compounds. After the treatment period, the cover slip with monolayer cells was inverted on the glass slide with 20 μL of AO/EB stain (100 μg/mL). The fluorescence was read using an IX71SIF-3 fluorescence microscope.

4.6. Hoechst 33258 Staining

The cells grown on the sterile cover slip in 6-well tissue culture plates were treated with compounds for a certain range of treatment time. The culture medium containing compounds was removed, and the cells were fixed in 4% paraformaldehyde for 10 min. The cells were washed twice with PBS, and were consequently stained with 0.5 mL of Hoechst 33258 staining for 5 min. The stained nuclei were washed twice with PBS, and were consequently observed under an IX71SIF-3 fluorescence microscope at 350 nm excitation and 460 nm emissions.

4.7. TUNEL Assay

TUNEL assays were performed using a colorimetric TUNEL apoptosis assay kit according to the manufacturer's instructions. The cells grown in 6-well culture clusters were treated as mentioned in mitochondrial depolarization assay. The MGC-803 grown in 6-well tissue culture plates were

washed with PBS and fixed in 4% paraformaldehyde for 40 min. The cells were washed once with PBS, and were consequently permeabilized with immunol staining wash buffer for 2 min on ice. The cells were rewashed once with PBS, and were consequently incubated in 0.3% H_2O_2 in methanol at room temperature for 20 min to inactivate the endogenous peroxidases, after which the cells were washed thrice with PBS. Thereafter, the cells were incubated with 2 μL of TdT-enzyme and 48 μL of Biotin-dUTP per specimen for 60 min at 37 °C. The cells were terminated for 10 min, and were consequently incubated with streptavidin-HRP (50 μL per specimen) conjugate diluted at 1:50 in sample diluent for 30 min. The cells were washed three times with PBS, and were consequently incubated with diaminobenzidine solution (200 μL per specimen) for 10 min. Thereafter, the cells were rewashed twice with PBS, and were consequently imaged under an XDS-1B inverted biological microscope.

4.8. Flow Cytometry Analysis

Prepared MGC-803 cells (1×10^6/mL) were washed twice with cold PBS and then re-suspended gently in 500 μL binding buffer. Thereafter, cells were stained in 5 μL Annexin V-FITC and shaked well. Finally, 5 μL PI was added to these cells and incubated for 20 min in a dark place, analyzed by FACS Calibur, Becton Dickinson.

4.9. Statistical Analysis

All statistical analyses were performed using SPSS 10.0, and the data were analyzed using one-way ANOVA. The mean separations were performed using the least significant difference method. Each experiment was performed in triplicate, and all experiments were run thrice and yielded similar results. Measurements from all the replicates were combined, and the treatment effects were analyzed.

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Captions for Figures and Tables

Figure 1. Effect of compound **3c** on cancer cell proliferation.

Figure 2. Apoptosis induction studies of compound **3c**. (A) AO\EB staining. (B) Hoechst 33258 staining. (C) TUNEL assay.

Figure 3. Annexin V/PI dual staining of MGC-803 cell lines. (A) Apoptosis ratios. (B) A: negative control; B–D: cells treated with HCPT (10 μM) as positive control; E–G: cells treated with compound **3c** (10 μM).

Table 1 IC_{50} (μM) for 72 h of antitumor activities of BE derivatives on human cancer cell lines in vitro

Table 2 Selectivity index [a] of **2**, **3a–3d**, **4**, and **5** toward cancer cells

[a] selectivity index (IC_{50} on mouse embryo fibroblast cell line/IC_{50} on corresponding cancer cell line)

Scheme 1. Reagents and conditions: (a) Succinic anhydride, DMAP, pyridine, DCM, r.t., 8 h; (b) R_1H, PyBOP, DCM, r.t., 12 h; (c) Maleic anhydride, DMAP, pyridine, DCM, r.t., 24 h; (d) Piperidine, PyBOP, DCM, r.t., 12 h; (e) R_2COOH, DCC, DMAP, DCM, r.t., 24 h; (f) R_3COOH, EDCI, DMAP, DCM, r.t., 24 h.

Table 1 IC_{50} (μM) for 72 h of antitumor activities of BE derivatives on human cancer cell lines *in vitro*

Compound	MGC-803	PC3	Bcap-37	A375	MCF-7
1	30.2 ± 0.8	22.4 ± 0.9	25.5 ± 0.5	28.0 ± 0.3	19.3 ± 0.5
2	11.3 ± 0.2	12.5 ± 0.3	15.7 ± 0.9	17.9 ± 0.5	14.5 ± 0.6
3a	8.5 ± 0.2	9.6 ± 0.4	6.7 ± 0.3	10.3 ± 0.8	9.2 ± 0.6
3b	8.9 ± 0.3	8.3 ± 0.2	7.9 ± 0.1	12.6 ± 0.6	10.1 ± 0.3
3c	4.3 ± 0.4	4.5 ± 0.2	5.2 ± 0.4	7.5 ± 0.6	5.2 ± 0.7
3d	9.2 ± 0.6	11.2 ± 0.7	6.4 ± 0.3	18.5 ± 0.9	11.5 ± 0.4
4	21.3 ± 0.5	25.8 ± 0.4	18.3 ± 0.1	23.2 ± 0.2	17.1 ± 0.1
5	5.3 ± 0.2	9.6 ± 0.2	7.4 ± 0.5	14.4 ± 0.4	6.3 ± 0.4
6a-6d	>100	>100	>100	>100	>100
7a-7g	>100	>100	>100	>100	>100
HCPT	29.1 ± 2.6	34.5 ± 1.5	28.1 ± 1.0	27.8 ± 1.2	48.2 ± 0.4
ADM	0.7 ± 0.2	0.6 ± 0.1	1.2 ± 0.2	1.0 ± 0.6	1.35 ± 0.1

Table 2 Selectivity index [a] of **2**, **3a-3d**, **4**, and **5** toward cancer cells

Compound	MGC-803	PC3	Bcap-37	A375	MCF-7
2	6.45	5.83	4.64	4.07	5.02
3a	7.29	6.45	9.25	6.01	6.73
3b	4.97	5.33	5.60	3.52	4.38
3c	8.23	7.86	6.81	4.72	6.80
3d	6.23	5.12	8.97	3.10	4.99
4	< 3	< 3	< 3	< 3	< 3
5	< 3	< 3	< 3	< 3	< 3

[a] selectivity index (IC_{50} on mouse embryo fibroblast cell line/IC_{50} on corresponding cancer cell line)

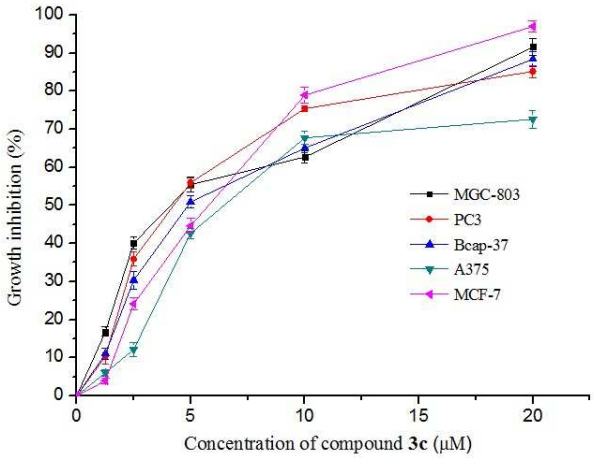


Figure 1. Effect of compound **3c** on cancer cell proliferation.

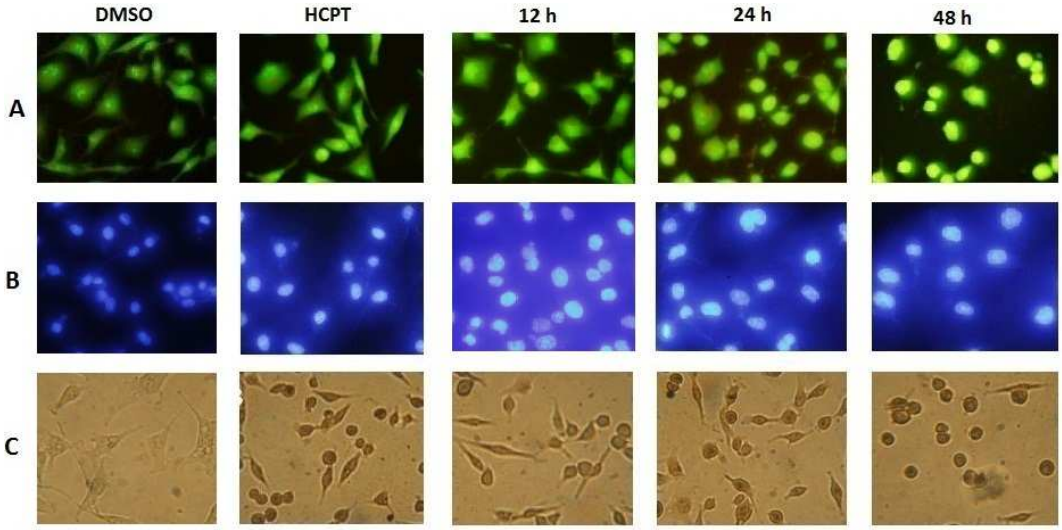


Figure 2. Apoptosis induction studies of compound **3c**. (A) AO\EB staining. (B) Hoechst 33258 staining. (C) TUNEL assay.

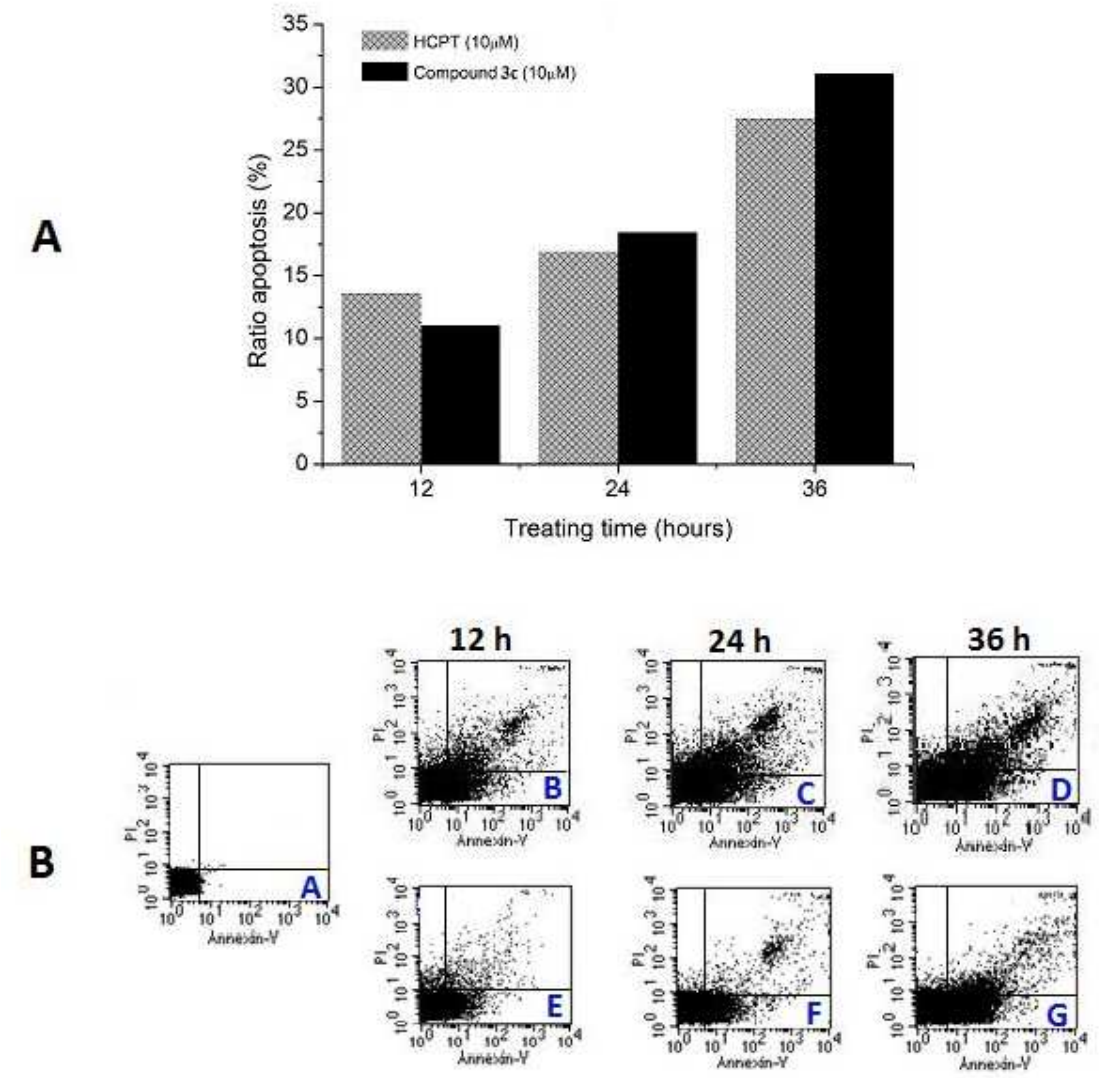
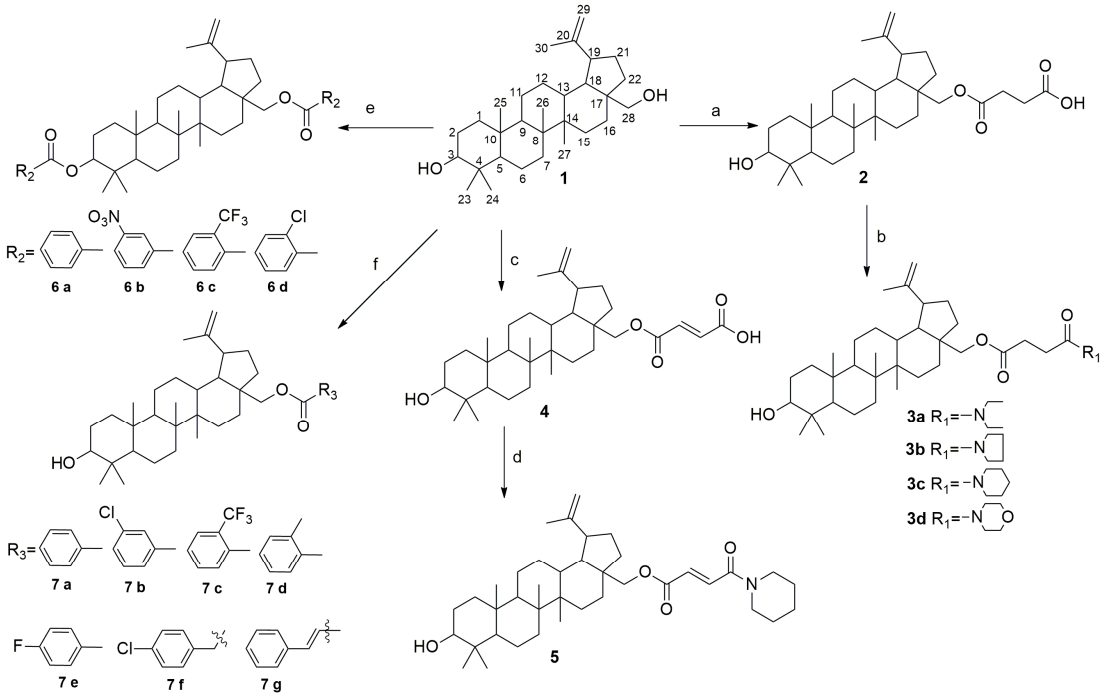


Figure 3. Annexin V/PI dual staining of MGC-803 cell lines. (A) Apoptosis ratios. (B) A: negative control; B–D: cells treated with HCPT (10 μM) as positive control; E–G: cells treated with compound **3c** (10 μM).



Scheme 1. Reagents and conditions: (a) Succinic anhydride, DMAP, pyridine, DCM, r.t., 8 h; (b) R_1H, PyBOP, DCM, r.t., 12 h; (c) Maleic anhydride, DMAP, pyridine, DCM, r.t., 24 h; (d) Piperidine, PyBOP, DCM, r.t., 12 h; (e) R_2COOH, DCC, DMAP, DCM, r.t., 24 h; (f) R_3COOH, EDCI, DMAP, DCM, r.t., 24 h.

Highlights

1. Nineteen betulin derivatives modified at the C-3 and C-28 positions were synthesized
2. The antitumor activities were investigated against various cancer cell lines.
3. Compound **3c** displayed the most potent antitumor activity.
4. Further mechanism study indicated compound **3c** could induce apoptosis of cancer cells.

Supporting Information

Synthesis and in vitro antitumor evaluation of betulin acid ester derivatives as novel apoptosis inducers

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Data of other compounds

Compound (**3a**): Yield: 61.1%; pale yellow oil; IR (KBr, cm^{-1}): ν_{max} 3445, 2947, 2870, 1728, 1033, 882; MS (EI): 597 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 4.63 (1H, s, Hb-29), 4.53 (1H, s, Ha-29), 4.23 (1H, d, *J* = 9.2 Hz, Hb-28), 3.84 (1H, d, *J* = 10.8 Hz, Ha-28), 3.30~3.33 (4H, m, CH_2), 3.15~3.17 (1H, m, H-3), 2.62~2.64 (4H, m, CH_2), 1.63 (3H, s, H-30), 1.15 (3H, s, CH_3), 1.05 (3H, s, CH_3), 0.97 (3H, s, CH_3), 0.92 (3H, s, CH_3), 0.87 (3H, s, CH_3), 0.78 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 173.7 (C=O), 170.3 (C=O), 150.3 (C-20), 109.8 (C-29), 78.9 (C-3), 62.8 (C-28), 55.3 (C-5), 50.4 (C-9), 48.9 (C-19), 47.7 (C-17), 46.7 (C-18), 42.7 (C-14), 41.9 (CH_2), 40.9 (C-8), 40.4 (C-4), 38.9 (C-1), 38.7 (C-13), 37.6 (CH_2), 37.1 (C-10), 34.6 (C-7), 34.2 (C-22), 29.8 (CH_2), 29.6 (C-21), 28.0 (CH_2), 27.9 (C-16), 27.8 (C-23), 27.4 (C-2), 27.1 (C-15), 25.2 (C-12), 20.8 (C-11), 19.2 (C-30), 18.6 (C-6), 16.2 (C-25), 16.1 (C-26), 15.5 (C-24), 14.8 (C-27), 14.2 (CH_3), 13.1 (CH_3).

Compound (**3b**): Yield: 62.8%; pale yellow oil; IR (KBr, cm^{-1}): ν_{max} 3444. 2942, 2860, 1728, 1641459, 882; MS (EI): 595 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 4.63 (1H, s, Hb-29), 4.53 (1H, s,

Ha-29), 4.22 (1H, d, *J* = 11.5 Hz, Hb-28), 3.84 (1H, d, *J* = 10.3 Hz, Ha-28), 3.14~3.16 (1H, m, H-3), 2.63~2.66 (4H, m, CH_2), 2.52 (4H, t, *J* = 12.5 Hz, CH_2), 1.79~1.82 (m, CH_2), 1.63 (3H, s, H-30), 1.01 (3H, s, CH_3), 0.96 (3H, s, CH_3), 0.81 (3H, s, CH_3), 0.71 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 173.4 (C=O), 169.7 (C=O), 150.2 (C-20), 109.9 (C-29), 77.4 (C-3), 62.8 (C-28), 55.3 (C-5), 50.4 (C-9), 48.9 (C-19), 47.7 (C-17), 46.5 (CH_2), 46.4 (C-18), 45.8 (CH_2), 42.7 (C-14), 40.9 (C-8), 39.0 (C-4), 38.7 (C-1), 37.6 (C-13), 37.2 (C-10), 34.5 (C-7), 34.2 (C-22), 29.8 (CH_2), 29.7 (C-21), 29.4 (CH_2), 29.2 (C-16), 28.1 (C-23), 27.4 (C-2), 27.1 (C-15), 26.1 (CH_2), 25.2 (C-12), 24.5 (CH_2), 20.7 (C-11), 19.1 (C-30), 18.6 (C-6), 16.2 (C-25), 16.1 (C-26), 15.9 (C-24), 14.7 (C-27).

Compound (**3c**): Yield: 63.6%; pale yellow oil; IR (KBr, cm^{-1}): ν_{max} 3439, 2943, 2868, 1733, 1453, 1030, 880; MS (EI): 609 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 4.64 (1H, s, Hb-29), 4.54 (1H, s, Ha-29), 4.25 (1H, d, *J* = 6.5 Hz, Hb-28), 3.84 (1H, d, *J* = 11.8 Hz, Ha-28), 3.48~3.50 (4H, m, CH_2), 3.14~3.16 (1H, m, H-3), 2.62~2.64 (4H, m, CH_2), 1.64 (3H, s ,H-30), 1.57~1.59 (m, CH_2), 1.06 (3H, s, CH_3), 0.96 (3H, s, CH_3), 0.92 (3H, s, CH_3), 0.78 (3H, s, CH_3), 0.72 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 173.7 (C=O), 169.4 (C=O), 150.3 (C-20), 109.9 (C-29), 78.9 (C-3), 62.8 (C-28), 55.3 (C-5), 50.4 (C-9), 48.8 (C-19), 47.7 (C-17), 46.5 (CH_2), 46.4 (C-18), 42.9 (CH_2), 42.7 (C-14), 40.9 (C-8), 39.0 (C-4), 38.7 (C-1), 37.6 (C-13), 37.2 (C-10), 34.6 (C-7), 34.2 (C-22), 29.8 (CH_2), 29.7 (C-21), 29.4 (CH_2), 29.6 (C-16), 28.1 (C-23), 27.4 (C-2), 27.1 (C-15), 26.4 (CH_2), 25.5 (CH_2), 25.2 (C-12), 24.6 (CH_2), 20.8 (C-11), 19.2 (C-30), 18.4 (C-6), 16.2 (C-25), 16.1 (C-26), 15.5 (C-24), 14.8 (C-27).

Compound (**3d**): Yield: 64.4%; pale yellow oil; IR (KBr, cm^{-1}): ν_{max} 3446, 2945, 2860, 1730, 1647, 1028, 881; MS (EI): 611 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 4.64 (1H, s, Hb-29), 4.54 (1H, s, Ha-29), 4.25 (1H, d, *J* = 13.3 Hz, Hb-28), 3.85 (1H, d, *J* = 8.4 Hz, Ha-28), 3.63~3.65 (4H, m, CH_2), 3.47 (4H, t, *J* = 5.6 Hz, CH_3), 3.14~3.17 (1H, m, H-3), 2.62~2.65 (4H, m, CH_2), 1.66 (3H, s, H-30), 1.01 (3H, s, CH_3), 0.96 (3H, s, CH_3), 0.92 (3H, s, CH_3), 0.77 (3H, s, CH_3), 0.71 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 173.4 (C=O), 169.9 (C=O), 150.2 (C-20), 109.9 (C-29), 78.9 (C-3), 66.9 (CH_2), 66.5 (CH_2), 62.9 (C-28), 55.3 (C-5), 51.9(CH_2), 50.4 (C-9), 48.8 (C-19), 47.7 (C-17), 46.5 (C-18), 45.7 (CH_2), 42.2 (C-14), 40.9 (C-8), 38.9 (C-4), 38.7 (C-1), 37.6 (C-13), 37.2 (C-10), 34.6 (C-7), 34.2 (C-22), 29.8 (CH_2), 29.3 (C-21), 29.0 (CH_2), 28.0 (C-16), 27.8 (C-23), 27.4 (C-2), 27.1 (C-15), 25.2 (C-12), 20.8 (C-11), 19.2 (C-30), 18.4 (C-6), 16.2 (C-25), 16.1 (C-26), 15.5 (C-24), 14.8 (C-27).

Compound (**4**): Yield: 65.8%; white power, mp: 232-234 ^{o}C; IR (KBr, cm^{-1}): ν_{max} 3435, 2942,

1728, 1375, 1030, 881; MS (EI): 540 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 6.35 (1H, d, *J* = 10.4 Hz, CH), 6.23 (1H, d, *J* = 14.8 Hz, CH), 4.66 (1H, s, Hb-29), 4.56 (1H, s, Ha-29), 4.37 (1H, d, *J* = 11.3 Hz, Hb-28), 3.97 (1H, d, *J* = 7.9 Hz, Ha-28), 3.14~3.16 (1H, m, H-3), 1.64 (3H, s, H-30), 0.99 (3H, s, CH_3), 0.94 (3H, s, CH_3), 0.92 (3H, s, CH_3), 0.79 (3H, s, CH_3), 0.72 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 167.2 (C=O), 167.2 (C=O), 149.9 (C-20), 133.9 (CH), 128.6(CH), 110.1 (C-29), 79.0 (C-3), 64.8 (C-28), 55.3 (C-5), 50.4 (C-9), 48.9 (C-19), 47.7 (C-17), 46.5 (C-18), 42.7 (C-14), 40.9 (C-8), 38.9 (C-4), 38.7 (C-1), 37.8 (C-13), 37.2 (C-10), 34.5 (C-7), 34.2 (C-22), 29.7 (C-21), 29.5 (C-16), 28.0 (C-23), 27.2 (C-2), 27.0 (C-15), 25.2 (C-12), 20.8 (C-11), 19.1 (C-30), 18.3 (C-6), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.8 (C-27).

Compound (**5**): Yield: 60.7%; pale yellow oil; IR (KBr, cm^{-1}): ν_{max} 3240, 2930, 1734, 1640, 1455, 883; MS (EI): 607 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 6.50 (1H, d, *J* = 10.2 Hz, CH), 5.95 (1H, d, *J* = 11.5 Hz, CH), 4.62 (1H, s, Hb-29), 4.52 (1H, s, Ha-29), 4.23 (1H, d, *J* = 9.1 Hz, Hb-28), 3.87 (1H, d, *J* = 12.6 Hz, Ha-28), 3.55~3.58 (4H, m, CH_2), 3.12~3.14 (1H, m, H-3), 1.61 (3H, s, H-30), 1.48~1.51 (m, CH_2), 0.95 (3H, s, CH_3), 0.92 (3H, s, CH_3), 0.89 (3H, s, CH_3), 0.75 (3H, s, CH_3), 0.69 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 165.3 (C=O), 165.2 (C=O), 150.1 (C-20), 138.4 (CH), 122.8 (CH), 110.0 (C-29), 77.4 (C-3), 63.5 (C-28), 55.3 (C-5), 50.4 (C-9), 48.9 (C-19), 47.7 (C-17), 47.4 (CH_2), 46.3 (C-18), 42.7 (C-14), 42.1 (CH_2), 40.9 (C-8), 38.9 (C-4), 38.7 (C-1), 37.6 (C-13), 37.2 (C-10), 34.6 (C-7), 34.2 (C-22), 29.7 (C-21), 29.5 (C-16), 28.1 (C-23), 27.4 (C-2), 27.1 (C-15), 26.1 (CH_2), 25.2 (C-12), 24.6 (CH_2), 20.8 (C-11), 19.2 (C-30), 18.3 (C-6), 16.2 (C-25), 16.1 (C-26), 15.4 (C-24), 14.8 (C-27).

Compound (**6b**): Yield: 75.2%; white solid; mp: 222-225 ^{o}C;IR (KBr, cm^{-1}): ν_{max} 3456, 1698, 1638, 1377, 1032, 882, 744; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 8.83 (1H, s, PhH), 8.81 (1H, s, PhH), 8.37~8.41 (m, PhH), 7.62~7.67 (2H, m, PhH), 4.75~4.77 (1H, m, H-3), 4.76 (1H, s, Hb-29), 4.61 (1H, s, Ha-29), 4.58 (1H, d, *J* = 10.5 Hz, Hb-28), 4.15 (1H, d, *J* = 10.8 Hz, Ha-28), 1.72 (3H, s, H-30), 1.08 (3H, s, CH_3), 1.01 (3H, s, CH_3), 1.00 (3H, s, CH_3), 0.90 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 164.9 (C=O), 164.2 (C=O), 150.0 (C-20), 148.4 (C), 148.4 (C), 135.3 (C), 135.2 (C), 132.7 (CH), 132.3 (CH), 129.8 (CH), 129.7 (CH), 127.5 (CH), 127.3 (CH), 124.6 (CH), 124.5 (CH), 110.2 (C-29), 82.9 (C-3), 64.4 (C-28), 55.5 (C-5), 50.4 (C-9), 48.9 (C-19), 47.8 (C-17), 46.8 (C-18), 42.9 (C-14), 41.0 (C-8), 38.4 (C-4), 38.3 (C-1), 37.8 (C-13), 37.2 (C-10), 34.8 (C-7), 34.2 (C-22), 30.0 (C-21), 29.6 (C-16), 28.2 (C-23), 27.1 (C-2), 25.2 (C-15), 23.8 (C-12), 20.9 (C-11), 19.2 (C-30), 18.3 (C-6), 16.9

(C-25), 16.3 (C-26), 16.2 (C-24), 14.9 (C-27).

Compound (**6c**): Yield: 65.4%; white solid; mp: 234-236 ^{o}C; IR (KBr, cm^{-1}): ν_{max} 3446, 2945, 2870, 1711, 1642, 1033; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.76~7.78 (m, PhH), 7.59~7.61 (m, PhH), 4.75~4.77 (1H, m, H-3), 4.74 (1H, s, Hb-29), 4.60 (1H, s, Ha-29), 4.55 (1H, d, *J* = 10.8 Hz, Hb-28), 4.12 (1H, d, *J* = 10.4 Hz, Ha-28), 1.66 (3H, s, H-30), 1.12 (3H, s, CH_3), 1.03 (3H, s, CH_3), 1.00 (3H, s, CH_3), 0.93 (3H, s, CH_3), 0.88 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 167.5 (C=O), 166.8 (C=O), 150.1 (C-20), 132.3 (C), 131.8 (C), 131.7(C), 131.6 (C), 131.1 (CH), 130.8 (CH), 130.2 (CH), 129.9 (CH), 128.7 (CH), 128.3 (CH), 126.8 (CH), 126.7 (CH), 124.6 (CF_3), 122.4 (CF_3), 110.0 (C-29), 83.2 (C-3), 64.7 (C-28), 55.6 (C-5), 50.3 (C-9), 49.0 (C-19), 47.8 (C-17), 46.5 (C-18), 42.8 (C-14), 40.9 (C-8), 38.5 (C-4), 38.0(C-1), 37.7 (C-13), 37.2 (C-10), 34.5 (C-7), 34.1(C-22), 29.7 (C-21), 29.6 (C-16), 28.0 (C-23), 27.0 (C-2), 25.2 (C-15), 23.3 (C-12), 20.9 (C-11), 19.2 (C-30), 18.2 (C-6), 16.6 (C-25), 16.2 (C-26), 14.8 (C-24), 14.3 (C-27).

Compound (**6d**): Yield: 72.5%; white solid; mp: 212-214 ^{o}C; IR (KBr, cm^{-1}): ν_{max} 3433, 2942, 2899, 1718, 1640, 882, 734; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.84 (1H, d, *J* = 7.9 Hz, PhH), 7.80 (1H, d, *J* = 7.4 Hz, PhH), 7.40~7.42 (m, PhH), 7.26~7.28 (m, PhH), 4.73~4.76 (1H, m, H-3), 4.71 (1H, s, Hb-29), 4.60 (1H, s, Ha-29), 4.53 (1H, d, *J* = 10.8 Hz, Hb-28), 4.12 (1H, d, *J* = 11.1 Hz, Ha-28), 1.70 (3H, s, H-30), 1.08 (3H, s, CH_3), 1.06 (3H, s, CH_3), 0.95 (3H, s, CH_3), 0.93 (3H, s, CH_3), 0.88 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 166.3 (C=O), 165.7(C=O), 150.1 (C-20), 133.7 (C), 133.5 (C), 132.6 (C), 132.3 (C), 131.6 (CH), 131.3 (CH), 131.2 (CH), 131.1 (CH), 131.0 (CH), 130.5 (CH), 126.7 (CH), 126.6 (CH), 110.0 (C-29), 82.7 (C-3), 64.2 (C-28), 55.6 (C-5), 50.4 (C-9), 48.9 (C-19), 47.8 (C-17), 46.7 (C-18), 42.8 (C-14), 41.0 (C-8), 38.5 (C-4), 38.1 (C-1), 37.8 (C-13), 37.2 (C-10), 34.8 (C-7), 34.2 (C-22), 29.9 (C-21), 29.7 (C-16), 29.6 (C-23), 28.2 (C-2), 27.2 (C-15), 25.3 (C-12), 20.9 (C-11), 19.2 (C-30), 18.3 (C-6), 16.9 (C-25), 16.3 (C-26), 16.1 (C-24), 14.9 (C-27).

Compound (**7b**): Yield: 69.4%; colorless oil; IR (KBr, cm^{-1}): ν_{max} 3444, 2930, 2866, 1712, 1455, 881, 739; MS (EI): 581 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.99 (1H, s, PhH), 7.93 (1H, d, *J* = 7.9 Hz, PhH), 7.52 (1H, d, *J* = 8.2 Hz, PhH), 7.39 (1H, t, *J* = 15.1 Hz, PhH), 4.70 (1H, s, Hb-29), 4.59 (1H, s, Ha-29), 4.52 (1H, d, *J* = 10.4 Hz, Hb-28), 4.09 (1H, d, *J* = 12.3 Hz, Ha-28), 3.15~3.18 (1H, m, H-3), 1.73 (3H, s, H-30), 1.08 (3H, s, CH_3), 0.98 (3H, s, CH_3), 0.95 (3H, s, CH_3), 0.81 (3H, s, CH_3), 0.74 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 165.8 (C=O), 150.1 (C-20), 134.6 (C), 133.0 (C), 132.3 (CH), 129.8 (CH), 129.7 (CH), 127.8 (CH), 110.0 (C-29), 79.0 (C-3), 63.8 (C-28), 55.4 (C-5), 50.5 (C-9),

48.9 (C-19), 47.8 (C-17), 46.7 (C-18), 42.8 (C-14), 40.9 (C-8), 38.9 (C-4), 38.8 (C-1), 37.7 (C-13), 37.2 (C-10), 34.8 (C-7), 34.3 (C-22), 30.0 (C-21), 29.7 (C-16), 28.1 (C-23), 27.5 (C-2), 27.2 (C-15), 25.3 (C-12), 20.9 (C-11), 19.3 (C-30), 18.4 (C-6), 16.2 (C-25), 16.1 (C-26), 15.5 (C-24), 14.9 (C-27).

Compound (**7c**): Yield: 67.6%; colorless oil; IR (KBr, cm^{-1}): ν_{max} 3433, 2951, 2931, 1701, 1022, 882, 735; MS (EI): 615 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.76 (1H, d, *J* = 12.3 Hz, PhH), 7.71 (1H, d, *J* = 14.6 Hz, PhH), 7.58~7.60 (2H, m, PhH), 4.69 (1H, s, Hb-29), 4.58 (1H, s, Ha-29), 4.51 (1H, d, *J* = 10.5 Hz, Hb-28), 4.13 (1H, d, *J* = 9.7 Hz, Ha-28), 3.15~3.18 (1H, m, H-3), 1.71 (3H, s, H-30), 1.07 (3H, s, CH_3), 0.97 (3H, s, CH_3), 0.92 (3H, s, CH_3), 0.81 (3H, s, CH_3), 0.75 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 167.5 (C=O), 150.1 (C-20), 131.8 (C), 131.1 (CH), 130.2 (CH), 128.5 (CH), 126.8 (CH), 126.7 (C), 121.7(CF_3), 110.0 (C-29), 79.0 (C-3), 64.9 (C-28), 55.4 (C-5), 50.5 (C-9), 49.0 (C-19), 47.7 (C-17), 46.5 (C-18), 42.7 (C-14), 40.9 (C-8), 38.9 (C-4), 38.8 (C-1), 37.7 (C-13), 37.2 (C-10), 34.5 (C-7), 34.3 (C-22), 29.7 (C-21), 29.6 (C-16), 28.1 (C-23), 27.5 (C-2), 27.1 (C-15), 25.3 (C-12), 20.9 (C-11), 19.3 (C-30), 18.4 (C-6), 16.2 (C-25), 16.1 (C-26), 15.4 (C-24), 14.9 (C-27).

Compound (**7d**): Yield: 65.2%; colorless oil; IR (KBr, cm^{-1}): ν_{max} 3448, 2927, 1698, 1457, 880, 730; MS (EI): 560 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.92 (1H, d, *J* = 8.2 Hz, PhH), 7.04 (1H, d, *J* = 8.3 Hz, PhH), 7.25~7.27 (2H, m, PhH), 4.70 (1H, s, Hb-29), 4.59 (1H, s, Ha-29), 4.46 (1H, d, *J* = 10.8 Hz, Hb-28), 4.09 (1H, d, *J* = 11.5 Hz, Ha-28), 3.16~3.18 (1H, m, H-3), 2.61 (3H, s, CH_3), 1.69 (3H, s, H-30), 1.05 (3H, s, CH_3), 1.03 (3H, s, CH_3), 0.96 (3H, s, CH_3), 0.82 (3H, s, CH_3), 0.75 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 168.1 (C=O), 150.2 (C-20), 140.2 (C), 132.0 (C), 131.8 (CH), 129.9 (CH), 129.9 (CH), 125.7 (CH), 110.0 (C-29), 79.0 (C-3), 63.2 (C-28), 55.4 (C-5), 50.5 (C-9), 49.0 (C-19), 47.8 (C-17), 46.7 (C-18), 42.8 (C-14), 40.9 (C-8), 38.9 (C-4), 38.8 (C-1), 37.7 (C-13), 37.2 (C-10), 34.9 (C-7), 34.3 (C-22), 30.0 (C-21), 29.7 (C-16), 28.0 (C-23), 27.5 (C-2), 27.2 (C-15), 25.3 (C-12), 22.1 (CH_3), 20.9 (C-11), 19.3 (C-30), 18.4 (C-6), 16.1(C-25), 16.1 (C-26), 15.5 (C-24), 14.9 (C-27).

Compound (**7e**): Yield: 68.1%; colorless oil; IR (KBr, cm^{-1}): ν_{max} 2947, 2866, 1688, 1455, 882, 744; MS (EI): 565 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 8.03~8.06 (2H, m, PhH), 7.11 (2H, t, *J* = 14.5 Hz, PhH), 4.70 (1H, s, Hb-29), 4.59 (1H, s, Ha-29), 4.50 (1H, d, *J* = 10.1 Hz, Hb-28), 4.07 (1H, d, *J* = 8.4 Hz, Ha-28), 3.16~3.18 (1H, m, H-3), 1.68 (3H, s, H-30), 1.04 (3H, s, CH_3), 0.99 (3H, s, CH_3), 0.95 (3H, s, CH_3), 0.81 (3H, s, CH_3), 0.75 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 166.0 (C=O), 150.1 (C-20), 132.1 (C), 126.8 (C), 115.7 (CH), 115.5 (CH), 110.0 (C-29), 79.0 (C-3), 63.5 (C-28),

55.4 (C-5), 50.5 (C-9), 48.9 (C-19), 47.8 (C-17), 46.8 (C-18), 42.8 (C-14), 40.9 (C-8), 38.9 (C-4), 38.8 (C-1), 37.7 (C-13), 37.2 (C-10), 34.8 (C-7), 34.3 (C-22), 30.0 (C-21), 29.7 (C-16), 28.1 (C-23), 27.5 (C-2), 27.2 (C-15), 25.3 (C-12), 20.9 (C-11), 19.2 (C-30), 18.4 (C-6), 16.2 (C-25), 16.1 (C-26), 15.4 (C-24), 14.9 (C-27).

Compound (**7f**): Yield: 66.5%; colorless oil; IR (KBr, cm^{-1}): ν_{max} 3446, 2935, 1712, 1640, 1033, 882, 722; MS (EI): 581 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.29 (2H, d, *J* = 8.2 Hz, PhH), 7.20 (2H, d, *J* = 8.4 Hz, PhH), 4.66 (1H, s, Hb-29), 4.56 (1H, s, Ha-29), 4.29 (1H, d, *J* = 10.3 Hz, Hb-28), 3.85 (1H, d, *J* = 10.5 Hz, Ha-28), 3.59 (1H, s, CH_2), 3.15~3.18 (1H, m, H-3), 1.65 (3H, s, H-30), 1.02 (3H, s, CH_3), 1.01 (3H, s, CH_3), 0.95 (3H, s, CH_3), 0.80 (3H, s, CH_3), 0.75 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 171.6 (C=O), 150.1 (C-20), 133.1 (C), 132.7 (CH), 130.7 (CH), 128.7 (CH), 110.0 (C-29), 79.0 (C-3), 63.5 (C-28), 55.4 (C-5), 50.4 (C-9), 48.9 (C-19), 47.8 (C-17), 46.5 (C-18), 42.8 (C-14), 40.9 (C-8), 40.9 (CH_2), 38.9 (C-4), 38.8 (C-1), 37.7 (C-13), 37.2 (C-10), 34.6 (C-7), 34.2 (C-22), 29.8 (C-21), 29.6 (C-16), 28.1 (C-23), 27.5 (C-2), 27.1 (C-15), 25.3 (C-12), 20.9 (C-11), 19.2 (C-30), 18.4 (C-6), 16.2 (C-25), 16.1 (C-26), 15.4 (C-24), 14.8 (C-27).

Compound (**7g**): Yield: 73.9%; colorless oil; IR (KBr, cm^{-1}): ν_{max} 3442, 2941, 1706, 1640, 880, 722; MS (EI): 572 (M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.69 (1H, d, *J* = 14.7 Hz, PhH), 7.49~7.52 (2H, m, PhH), 7.36~7.38 (2H, m, PhH), 6.46 (1H, d, *J* = 14.2 Hz, CH), 5.01 (1H, d, *J* = 5.4 Hz, CH), 4.70 (1H, s, Hb-29), 4.59 (1H, s, Ha-29), 4.41 (1H, d, *J* = 9.8 Hz, Hb-28), 3.99 (1H, d, *J* = 10.5 Hz, Ha-28), 3.14~3.16 (1H, m, H-3), 1.69 (3H, s, H-30), 1.05 (3H, s, CH_3), 1.02 (3H, s, CH_3), 0.95 (3H, s, CH_3), 0.82 (3H, s, CH_3), 0.77 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 167.6 (C=O), 150.3 (C-20), 144.7 (CH), 134.5 (C), 130.3 (CH), 128.9 (CH), 128.2 (CH), 118.3 (CH), 110.0 (C-29), 79.0 (C-3), 62.9 (C-28), 55.4 (C-5), 50.5 (C-9), 48.9 (C-19), 47.8 (C-17), 46.7 (C-18), 42.8 (C-14), 40.9 (C-8), 38.9 (C-4), 38.8 (C-1), 37.7 (C-13), 37.2 (C-10), 34.8 (C-7), 34.2 (C-22), 30.0 (C-21), 29.7 (C-16), 28.1 (C-23), 27.5 (C-2), 27.2 (C-15), 25.3 (C-12), 20.9 (C-11), 19.3 (C-30), 18.4 (C-6), 16.2 (C-25), 16.1 (C-26), 15.5 (C-24), 14.9 (C-27).