



Synthesis, Characterization and Cytotoxic Activity of Betulinic Acid and *Sec*-Betulinic Acid Derivatives against Human Colorectal Carcinoma

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ABSTRACT

Different derivatives based on the betulinic acid triterpenoidal scaffold were synthesized and characterized spectroscopically. The derivatives including synthetic intermediates were evaluated for their cytotoxic activity against the human colorectal carcinoma cell line (HCTT111) using a colorimetric sulforhodamine assay (SRB). Among the synthesized compounds, compound **6** featuring a 20-keto *seco*-betulinic acid conjugated with a lactone benzyl ester through an amide linkage at C-28, exhibited the lowest IC₅₀ value.

Keywords: Betulinic acid, *Seco*-betulinic acid, Lactone derivatives,
Ester derivatives, cytotoxicity, cancer.

INTRODUCTION

The pentacyclic lupane triterpenoid betulinic acid, **1** (3 β , hydroxy-lup-20(29)-en-28-oic acid) is one of the first natural products identified

from plants in 1788¹. It exhibits a number of biological activities including antitumor properties versus a panel of cancer cell lines. While previous studies suggest that betulinic acid is selectively cytotoxic against melanoma cell lines², it was also reported

to exhibit activity against other types of human cancers including neuroblastoma, glioblastoma, medulloblastoma, Ewing tumor, leukemia as well as several carcinoma, i.e. head and neck, colon, breast, hepatocellular, lung, prostate, renal cell, ovarian or cervix carcinoma. Mechanistic studies indicate that it triggers the mitochondrial path to apoptosis³, inhibits aminopeptidase N, an enzyme that is involved in the regulation of angiogenesis and overexpressed in several cancers⁴⁻⁶, modulate activity of the transcription factor nuclear factor- κ B (NF- κ B), a key regulator of stress-induced transcriptional activation. Because it exhibits relative selective cytotoxicity against malignant over normal cells, betulinic acid is a promising anti-cancer agent.

Several derivatization studies have been reported for betulinic acid leading to the synthesis of a library of betulinic acid congeners. Structurally, the triterpenoidal skeleton possesses three sites amenable for modification, including the C-3 hydroxyl, C-20 alkene, and C-28 carboxylic acid positions. While it holds a great potential to be developed as anti-tumor agent, it suffers from poor solubility in aqueous solutions and thus limiting its activity *in vivo*^{7, 8}.

In the light of the purported potential of betulinic acid and its derivatives as cancer chemopreventive agents, there exists a niche to develop new betulinic acid-inspired natural product analogs that may present better cytotoxic activity. In this paper we disclose the synthesis and evaluation of cytotoxicity versus human colorectal cancer cells of new betulinic acid and C-20 *seco* derivatives *in vitro*.

EXPERIMENTAL

Material

General. Infrared (IR) spectra (KBr) were recorded on a Thermo Nicolet Impact 6700 instrument (KBr pellet). ¹H NMR spectra were recorded with a Bruker Ultra Shield 300 MHz spectrometer at 300 K, using TMS as an internal standard. Chemical shifts are expressed in parts per million (ppm, units). Coupling constants are in units of hertz (Hz). The spin multiplicities are indicated by the symbols s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q

(quartet), m (multiplet), and br (broad). MS spectra were recorded on a Agilent GC-7890A MS 5975 and Agilent LCMS QToF 6520. TLCs and preparative thin-layer chromatography were performed on silica gel GF/UV 254, and the chromatograms were performed on silica gel (100-200 mesh) visualized under UV light at 254 nm and 365 nm. All solvents were of reagent grade and, when necessary, were purified and dried by standard methods. Concentration of solutions after reactions and extractions involved the use of a rotary evaporator operating at a reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulfate.

Chemistry

In this work, a series of the new betulinic acid and C-20 *seco* derivatives were synthesized and characterized spectroscopically. Subsequently, the cytotoxicity of the derivatives including intermediates against human colorectal carcinoma cell line were explored, and the results are described below.

Synthesis of compounds 4 and 8

To a solution of betulinic acid 1 (30.0 mg, 0.07 mmol) in dry THF (2 mL) was added HOBt (11.2 mg, 1.1 equiv.), HBTU (30.0 mg, 1.0 equiv.) at 0°C and trimethylsilyloxycyclohexene amine TFA salt 3 or benzyl-D-alanine salt (1.1 equiv.) was added next. The ice bath was removed and the reaction mixture was stirred for 24 h at room temperature. The solvent was removed by rotary evaporator and the reaction mixture was redissolved in EtOAc (5 mL), and washed with 1M HCl (5 mL), 5% NaHCO₃ (5 mL) and brine (5 mL). The organic layer was dried over by Na₂SO₄ and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (hexane: ethyl acetate, 15:1) to give products 4 as white solid or 8 (as white solid) in 69% or 85% yields, respectively.

(1R,3aS,5aR,5bR,9S,11aR)-9-hydroxy-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)-N-((1R,6R)-6-trimethylsilyloxy)cyclohex-3-enyl)icosahydro-1H-cyclopenta[a]chrysene-3a-carboxamide (4).

R_f = 0.66 (SiO₂, hexane/ethylacetate 4:1); Yields: 69%; m.p. 124- 126°C. ¹H-NMR (300 MHz CDCl₃); δ = 5.23-5.35 (m, 2H, CH-Olefin), 4.73

(brs,1H, H-29), 4.61 (brs,1H, H-29), 3.66-3.75 (m, 1H, CHO), 3.53-3.65 (m, 1H, CHN), 3.31-3.37 (m,1H, C-OH), 3.17-3.20 (m, 1H, CH, H-19)), 2.26-2.55 (m, 2H, CH₂), 1.96-2.11(m, 2H, CH₂), 1.69 (s,3H, CH₃), 0.97 (s,3H, CH₃), 0.96 (s,3H, CH₃), 0.93 (s,3H, CH₃), 0.82 (s,3H, CH₃), 0.75 (s,3H, CH₃), 0.0 (s,9H, CH₃-OTMS)

¹³C-NMR (75.5 MHz, CDCl₃): δ = 172.0 (C=O), 150.12 (C=C, C-20), 124.1- 124.6 (C=C, Olefin), 107.42 (C=C, C-29), 83.71 (C-3), 78.8 (C_{quart}, cyclohexene), 78.9 (C_{quart}, cyclohexene), 57.34 (C-17), 55.48 (C-5), 51.16 (C-9), 50.6 (CH, Olefin), 50.41 (C-18), 46.1 (C-H, Olefin), 45.40 (C-19), 42.49 (C-14), 41.76 (C-8), 39.81 (C-1), 39.62 (C-4), 38.2 (C-13), 36.41 (C-10), 36.39 (C-22), 34.24 (C-7), 31.80 (C-16), 30.72 (C-15), 32.9 (CH₂-Olefin), 29.9 (CH₂-Olefin), 29.68 (C-21), 28.01 (C-23), 24.26 (C-12), 23.55 (C-11), 22.61 (C-6), 19.7 (C-30), 19.31 (C-2), 16.71 (C-26), 16.45 (C-25), 16.23 (C-24), 15.05 (C-27), 0.00 (OTMS), MS [Cl, NH₃]: m/z (%) 624.1 [M + H⁺], **MS-ESI** (Cl, NH₃): Calculated for [C₃₉H₆₅NO₃Si]: 623.6473, found 623.6460.

(2R)-benzyl 2-1R,3aS,5aR,5bR,9S,11aR)-9-hydroxy-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)icosahydro-1H- cyclopenta[a]chrysene-3a-carboxamido propanoate (8).
Rf = 0.3 (SiO₂, hexane/ethylacetate 4:1); m.p. 117-119°C.

¹H-NMR (300 MHz CDCl₃): δ= 8.11-8.21 (m,1H,NH), 7.31-7.48 (m, 5H, Bn), 5.01-5.28 (d, 2H, J = 4 Hz, CH₂Bn), 4.62 (brs, 1H, H-29), 4.75 (brs,1H, H-29), 4.11-4.48 (m, 1H, CHN), 2.96-3.01 (m, 1H, CH-OH), 3.15-3.31 (m,1H,CHCH₃), 1.70 (m,3H, CH₃), 1.42 (s,3H, CH₃), 1.21 (s,3H, CH₃), 1.10 (CH₃), 0.97 (s,3H, CH₃), 0.81 (s,3H, CH₃), 0.69 (s,3H, CH₃),

¹³C-NMR (75.5 MHz, CDCl₃): δ = 175.8 (C=O), 172.0 (C=O), 150.9 (C=C, C-20), 136.4 (C_{quart}), 128.7- 128.5 (Ph), 109.5 (C=C, C-29), 78.9 (C-3), 65.7 (CH₂Ph), 57.03 (C-17), 56.2 (CHN-ala), 55.42 (C-5), 51.05 (C-9), 50.48 (C-18), 45.40 (C-19), 42.40 (C-14), 41.60 (C-8), 39.81 (C-1), 39.88 (C-4), 38.5 (C-13), 35.41 (C-10), 36.30 (C-22), 34.24 (C-7), 31.80 (C-16), 30.62 (C-15), 29.38 (C-21), 28.2 (CH₃-ala), 28.21 (C-23), 24.46 (C-12), 23.54 (C-11), 22.61 (C-6), 19.6 (C-30), 19.31 (C-2), 16.77 (C-26), 16.48

(C-25), 16.27 (C-24), 15.04 (C-27). **IR** (film) ν_{max} cm⁻¹ = 3410.4, 2924.01, 1690.3, 1166.3, 1591.1, 1241.7, **MS-ESI** (Cl, NH₃): m/z. Calculated for [C₄₀H₅₉NO₄]: 617.4444, found 618.3316 [M + H⁺].

Synthesis of compounds 5

To a solution of compound 4 (30 mg, 1 equiv.) in 4 ml of biphasic solution of CCl₄:MeCN:H₂O (1:1:2) in 50 ml round bottom flask was added 1.8 mg RuCl₃·3H₂O (8.3 % mol) at 0°C and the mixture was stirred vigorously for 5 minutes. NaIO₄ (70 mg, 4.1 equiv.) was added portion wise and the reaction mixture was stirred for 8 h at 0-5°C. After completion of the reaction, the mixture was diluted with 10 ml of distilled water and extracted with 15 ml DCM (3x). The combined organic layer was dried over MgSO₄ and the solvent removed using rotary evaporator. The crude product was purified by column chromatography on silica gel (ethyl acetate: methanol, 9:1) to furnish 5 as brown solid in 55% yield.

2-((2S,3R)-3-((1R,3aS,5aR,9S,11aR)-1-acetyl-9-hydroxy-5a,5b,8,8,11a-pentamethylcosahydro-1H-cyclopenta[a]chrysene-3a-carboxamido)-5-oxotetrahydrofuran-2-yl)acetic acid (5).

Rf = 0.60 (SiO₂, hexane/ethylacetate 4:1); m.p. 151-153°C.

¹H-NMR (300 MHz, CDCl₃): ¹H-NMR (300 MHz CDCl₃): δ= 4.93-5.03 (m,1H,CHN), 4.60-4.68 (m, 1H, CHCH₂COOH), 3.30-3.36 (m,1H,CH-OH), 3.14-3.36 (m,1H,CH, H-19), 2.48-2.76 (m, 2H, CH₂CN), 2.30- 2.49 (m, 2H, CH₂-COO), 1.65 (s,3H,CH₃), 1.40 (s,3H, CH₃), 1.25 (s, 3H, CH₃), 1.12 (s, 3H, CH₃), 0.97 (s,3H, CH₃), 0.81 (s,3H, CH₃)

¹³C-NMR (75.5 MHz, CDCl₃): δ = 171.9 (C=O), 169.6 (C=O), 149.71 (C=C, C-20)), 107.81 (C=C, C-29), 83.53 (C-3), 78.8 (C-OH, lactone), 66.8 (CH₂, lactone), 56.95 (C-17), 55.35 (C-5), 50.51 (C-9), 49.65 (C-18), 45.88 (C-19), 42.36 (C-14), 40.78 (C-8), 38.7 (C-13), 38.88 (C-1), 38.7 (CH₂-CO, lactone), 36.73 (C-10), 36.53 (C-22), 34.25 (CH₂-lactone), 34.20 (C-7), 31.82 (C-16), 30.23 (C-15), 29.68 (C-21), 28.09 (C-23), 26.73 (C-12), 23.54 (C-11), 22.67 (C-6), 19.5 (C-30), 18.27 (C-2), 16.13 (C-26), 16.08 (C-25), 15.35 (C-24), 14.78 (C-27).

IR (film) ν_{\max} cm^{-1} = 2978.69-2933, 3420.82, 1714.86, 1255.07, 1499, 1367.79, 1738.28. MS [Cl, NH_3]: m/z (%) 600.1 [M + H⁺], MS-ESI (Cl, NH_3): m/z , Calculated for [C₃₅H₅₃NO₇]: 599.3822, found 599.3460.38 [M⁺].

Synthesis of compounds 6

To a stirred solution of **5** (20 mg, 1 equiv.) in 4 ml dry DMF was added NaHCO₃ (28 mg, 6 equiv.), followed by benzyl bromide (0.1 ml, 1.6 equiv.) sequentially at room temperature. The reaction mixture was stirred for 24 hours at room temperature. Then, the mixture was diluted with 4 ml EtOAc : H₂O (1:1) solution and the solution was extracted. The aqueous layer extracted with EtOAc and the combine organic layer was dried over by anhydrous MgSO₄ and concentrated reduced pressure to give yellow crude oil which was purified by column chromatography on silica gel (hexane: ethyl acetate, 6:4) to afford **6** as yellowish oil.

Benzyl 2-((2S,3R)-3-((1R,3aS,5aR,5bR,9S,11aR)-1-acetyl-9-hydroxy-5a,5b,8,8,11a-penamethylcosahydro-

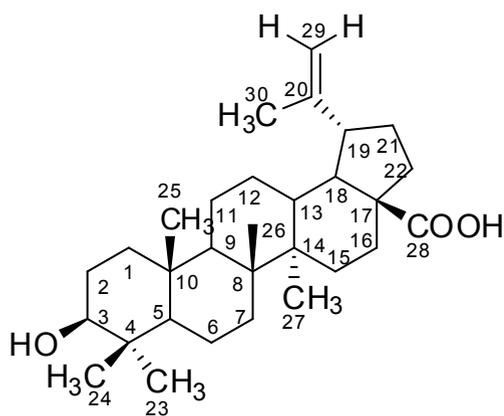


Fig. 1: Structure of betulinic acid

1H-cyclopenta[a]chrysene-3a-carboxamido}-5-oxotetrahydrofuran-2-yl)acetate (**6**).

R_f = 0.57 (SiO₂, hexane/ethylacetate 4:1);

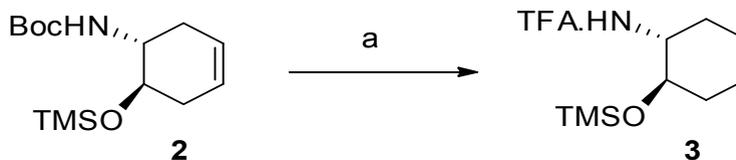
¹H-NMR (300 MHz CDCl₃): δ = 8.11-8.21 (d, J = 4.2 Hz), 1H, NH), 7.27-7.33 (m, 5H, Bn), 5.15-5.18 (d, 2H, J = 4 Hz, CH₂Bn), 4.90-4.95 (m, 1H, CHN), 4.69 (s, 1H), 3.32-3.38 (m, 1H, CH-OH), 3.07-3.11 (m, 1H, CH, H-19), 2.57-2.66 (m, 2H, CH₂CN), 2.44-2.56 (m, 2H, CH₂-COO) 1.75 (s, 3H, CH₃), 1.17 (s, 3H, CH₃), 1.11 (s, 3H, CH₃), 0.98 (s, 3H, CH₃), 0.85 (s, 3H, CH₃), 0.66 (m, 3H, CH₃).

¹³C-NMR (75.5 MHz, CDCl₃): δ = 177.2 (C=O), 173.3 (C=O), 149.78 (C=C, C-20), 132.4 (C_{quar}), 128.3-128.5 (Ph), 107.21 (C=C, C-29), 81.22 (C-3), 78.5 (C-OH lactone), 68.2 (CH₂Ph), 56.67 (C-17), 55.60 (C-5), 53.7 (CHN- lactone), 52.34 (C-9), 50.58 (C-18), 45.93 (C-19), 43.21 (C-14), 42.86 (C-8), 38.61 (C-1), 38.9 (C-13), 38.81 (C-4), 37.09 (C-10), 36.48 (C-H lactone), 36.41 (C-22), 34.44 (C-7), 31.91 (C-16), 29.68 (C-15), 29.11 (C-21), 28.95 (C-23), 25.70 (C-12), 23.64 (C-11), 22.66 (C-6), 19.65 (C-2), 19.5 (C-30), 16.66 (C-26), 16.42 (C-25), 16.21 (C-24), 14.07 (C-27).

IR (film) ν_{\max} cm^{-1} = 2978.69-2933, 3420.82, 1714.86, 1255.07, 1499, 1367.79, 1738.28. MS-ESI (Cl, NH_3): m/z Calculated for [C₄₂H₅₉NO₇]: 689.4292, found 689.2452.

Synthesis of compounds 7

To a stirred solution of **1** (100 mg, 1 equiv.) in 6 ml dry DMF was added NaHCO₃ (110 mg, 6 equiv.), followed by benzyl bromide (60.1 mg, 1.6 equiv.) sequentially at room temperature. The reaction mixture was stirred for 24 hours at room temperature. Then, the mixture was diluted with 4 ml EtOAc-H₂O (1:1) solution and the solution was extracted. The aqueous layer extracted with EtOAc and the combine organic layer was dried over by anhydrous MgSO₄ and concentrated reduced pressure to give crude



Reagents and conditions (a) TFA, DCM, 0°C, 3 hrs, quant

Scheme 1: Synthesis of trimethylsiloxy cyclohexene amine salt

white solid product which was purified by column chromatography on silica gel (*n*-hex–EtOAc, 6:4) to afford 68% of a white solid 7.

(1R,3aS,5aR,5bR,9S,11aR)-benzyl 9-hydroxy-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)icosahydro-1H-cyclopenta[a]chrysene-3a-carboxylate (7)

R_f = 0.5 (SiO₂, hexane/ ethyl acetate), m.p. 162-164°C. ¹H NMR (300 MHz, CDCl₃) : δ = 7.30-7.50 (m, 5H, BnH), 5.12 (d, *J*= 2.2 Hz, CH₂Ph), 4.73(brs,1H, H-29), 4.61(brs,1H, H-29), 3.26-3.32 (m, 1H, CHO), 3.03-3.15 (m, 1H, CH, H-19), 2.26-2.55 (m, 2H, CH₂), 1.69 (s,3H, CH₃), 0.97 (s,3H, CH₃), 0.96 (s,3H, CH₃), 0.93 (s,3H, CH₃), 0.82 (s,3H, CH₃), 0.75 (s,3H, CH₃)

¹³C NMR (75.5MHz, CDCl₃) : δ= 175.91 (C=O), 150.20 (C=C, C-20), 136.51 (C_{quart}), 128.7-128.2 (Ph), 107.94 (C-29), 78.96 (C-3), 70.10 (CH₂Ph), 57.04 (C-17), 55.40 (C-5), 50.59 (C-9), 49.99 (C-18), 46.58 (C-19), 42.50 (C-14), 40.82 (C-8), 38.88 (C-1), 38.7 (C-13), 38.60 (C-4), 37.22 (C-10), 36.32 (C-22), 34.92 (C-7), 31.53 (C-16), 29.69 (C-15), 28.36 (C-21), 27.99 (C-23), 25.71

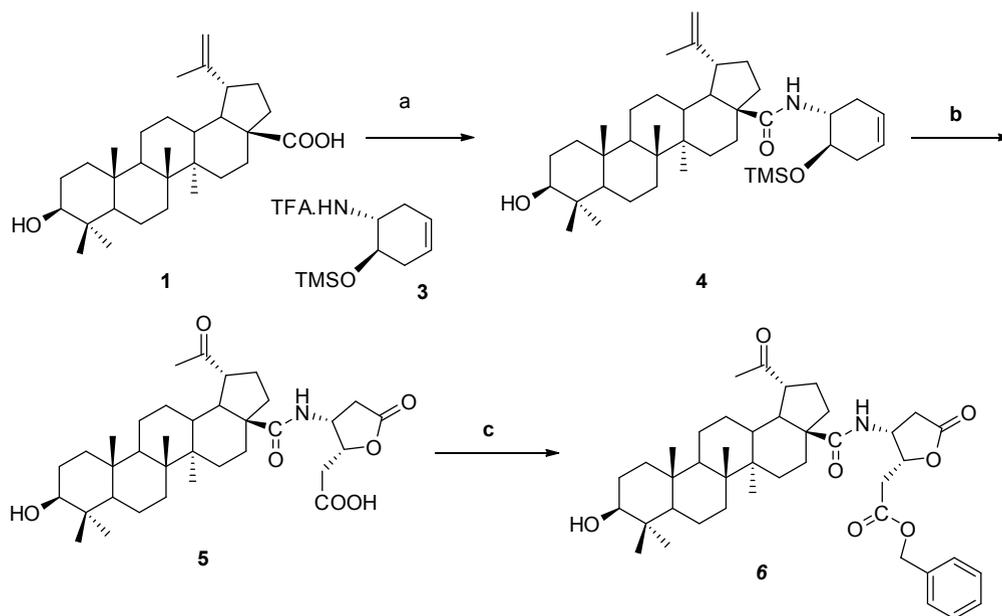
(C-12), 23.66 (C-11), 21.94 (C-6), 19.66 (C-2), 19.6 (C-30), 16.15 (C-26), 16.11 (C-25), 15.35 (C-24), 14.84 (C-27). MS [Cl, NH₃]: *m/z* (%) 547.3 [M + H⁺]. MS-ESI (Cl, NH₃): *m/z* Calculated for [C₃₇H₅₄NO₃] : 546.4073, found 547.2991 [M + H]⁺

Cytotoxicity Assay

HCT116 were seeded at 2,500 cells/ well (96 well plate) and exposed to respective compounds for 72 h before being subjected to fixation with 10% cold TCA and staining with 0.4% sulforhodamine. Plates were left to air-dry at room temperature and bound SRB was solubilised in 10 mM Tris base solution. The plate was read at 570nm using a spectrophotometer. A dose-response curve was plotted from which the IC₅₀ value was determined. IC₅₀ was defined as concentration of compound that corresponded to 50% cell viability when compared to control.

RESULTS AND DISCUSSION

Scheme 1 shows the key starting materials for the synthesis of betulinic-γ-butyrolactone derivatives. NBoc trimethyl siloxycyclohexene 2



Reagents and conditions: a) dry DMF, BnBr, NaHCO₃, 24 hrs, rt, 68% (b) DCM, HOBT (1.1 equiv.), HBTU, (1 equiv.) DiPEA (1.1 equiv, 0°C), 85%

Scheme 2: Synthesis of betulinic acid-γ-butyrolactone analogs

underwent deprotection Boc reaction by using trifluoro acetic acid (TFA, 30%) in DCM at 0°C to generate trimethyl siloxycyclohexene ammonium salt **3** in quantitative yield. The target compound, betulinic- γ -butyrolactone ester **6**, was synthesized in three steps reaction starting from compound **3**¹⁰. The ammonium salt was incorporated with betulinic acid using standard peptide coupling procedure to afford betulinic-trimethylsiloxycyclohexene peptide **4** in 69% yield. Betulinic acid was preactivated by coupling reagent, HOBt/HBTU and in the presence of strong base DIPEA.

Subsequently, peptide **4** underwent lactonization by ruthenium tetroxide, RuO₄ in the presence of sodium periodate as an oxidant to give the corresponding betulinic- γ -butyrolactone carboxylic acid **5**. The RuO₄ catalytic oxidative system was generated in situ from 8.3 % of RuCl₃·3H₂O and 4.1 equivalent of NaIO₄ in biphasic solution of CCl₄:MeCN:H₂O with ratio of (1:1:2). The conversion of cyclohexene silyl ether with co-solvent system was completed in 8 hours at 0°C.

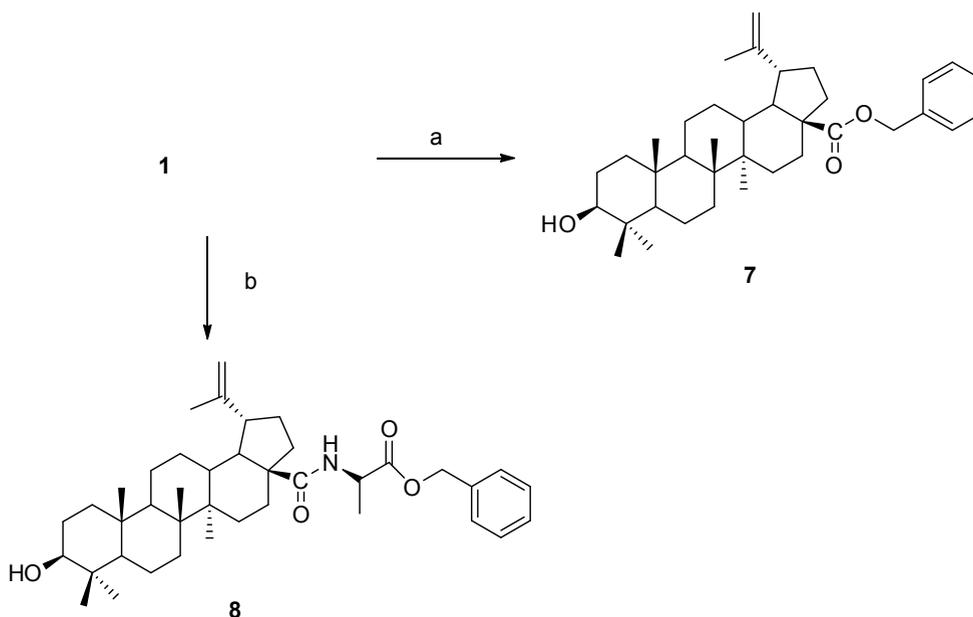
In accordance to previous study on lactonisation reaction involving oxidative cleavage

reaction of trimethyl siloxycyclohexene scaffold, the configuration of both asymmetric centre on the butyrolactone skeleton is *cis* configuration⁹. The possibility of *cis* confirmation may be due to the presence of ruthenium tetroxide which could create interactions on the similar face between nitrogen lone pair to metal and chelation of metal and carboxylate oxygen atom.

Benzylic protection of carboxylic acid **5** was envisioned by treating **5** with benzyl bromide in the present of sodium hydrogen carbonate as base to give betulinic- γ -butyrolactone ester **6**. In addition, betulinic acid underwent benzylation reaction after being treated with benzyl bromide, BnBr to afford **7**. Betulinic-D-alanine peptide could be synthesized by applying peptide synthesis procedure furnished peptide **8** in a good yield¹⁰.

Cytotoxic Activity

The inhibitory activity of the synthesized compounds against HCT116 was evaluated by cytotoxic assay. In general, compound **1** had emerged as the potent anticancer agent, with an IC₅₀ of 3.0 μ g/mL. This was followed by compound **7**, **8**, and **4**, with IC₅₀ value 25.33, 36.67 and 50.00



Reagents and conditions (a) DCM, HOBt (1.1 equiv.), HBTU, (1 equiv.) DIPEA (1.1 equiv, 0°C), 69%. (b) RuCl₃·3H₂O, NaIO₄, CCl₄:MeCN:H₂O, 8 hrs, 0°C, 55%. (c) dry DMF, BnBr, NaHCO₃, 24 hrs, rt, 72%

Scheme 3: Synthesis of betulinic acid ester and betulinic acid peptide

$\mu\text{g/mL}$ respectively. The IC_{50} of compound 5 and 7 were beyond the range of concentration tested. Betulinic-g-butyrolactone carboxylic acid, 5 yielded an $\text{IC}_{50} > 100 \mu\text{g/mL}$. Its benzyl ester derivative 6, however, yielded IC_{50} at least four times lower (IC_{50} value = $25.3 \mu\text{g/mL}$). Eventhough none of the compounds exhibited comparable activity to that of positive control (5-FU), the results showed that incorporation of betulinic acid with g-butyrolactone ester moiety contributed to the inhibition of HCT116.

CONCLUSION

In summary, several novel of betulinic- γ -butyrolactone and betulinic acid derivatives were synthesized and tested for their in vitro cytotoxic

activities against a human colorectal carcinoma cell lines (HCT116). The results of preliminary biological activity showed that two synthesized compounds (**6** and **8**) possessed potential cytotoxicities. Further structure exploration especially on the aromatic butyrolactone ester side chains are in progress in our lab.

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