Synthesis, Characterization and Cytotoxic Activity of Betulinic Acid Glucosides against Human Myeloid Leukemia and Human T-4 Lymphoblastoid Cell Lines

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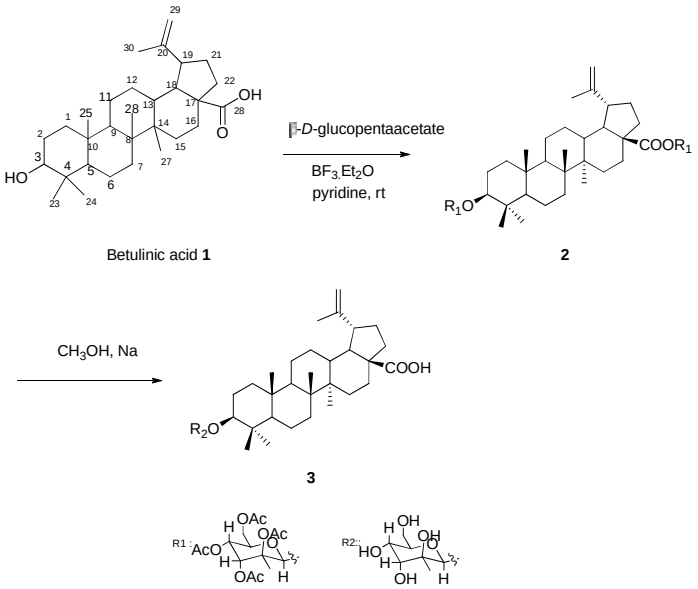
Abstract— **Glycosylation of betulinic acid with -*D*-glucose-pentaacetate was successfully synthesized in the presence of boron trifluoro etherate ($BF_3.Et_2O$) in pyridine to give betulinic acid bisglucoside in 92.8% yield. Further treatment of bisglucosides with methanol and sodium metal produced high polarity betulinic acid glucoside with 45% yield. Evaluation of human myeloid leukemia cell (HL60) and human T4-lymphoblastoid cell (CEMSS) activities with both synthesized compounds revealed that both compounds were less potent compared to betulinic acid itself but their water solubility were slightly increase.**

***Index Terms*— Anti-leukemia, Betulinic acid, Bisglycoside Glycosylation**

I. INTRODUCTION

The lupane type pentacyclic triterpene betulinic acid (1), 3-β-hydroxy-lup-20(29)-ene-28-oic, was isolated from higher plants (*Melaleuca cajuputi sp)* [1]. It has a molecular formula of $C_{30}H_{48}O_3$ with three active sites at C-3, C-20 and C-28 . Betulinic acid has been reported to possess antitumor activity toward cultured human melanoma cell in vitro and vivo model [2], [3]. It also has potential to block the function of DNA polymerase β selectively and able to act as antitumor agent [4]. The drawback of betulinic acid especially in the medical application or as a drug agents in pharmaceutical industry has been influenced by its lower solubility in water as well as in organic solvents. The chemical modification of betulinic acid especially at C-3 or C-28 by incorporating with glucose moieties is expected to increase the betulinic acid solubility [5].

Hence, studies on modification of betulinic acid scaffold are still wanting and significant. Herein we embark on a new procedure to synthesis betulinic acid-glycosides [6]. The present study describes the synthesis of 2,3,4,6-Tetra-O-acetyl-*β*-D-glucopyranosyl-3*β*-(2,3,4,6-tetra -O-acetyl-*β*–D-glucopyranosyloxy)-lup-20(29)-en-28-oate (2) by treating betulinic acid with *β*-D-glucose-pentaacetate in the presence of a lewis acid as catalyst (boron trifluoride etherate) in anhydrous pyridine and preparation of polar 3*β*–(*β*-D-glucopyranosyloxy-lup-20(29)-en-28-oic acid (3) by deacetylation method (Scheme 1).



Scheme 1 Synthesis of betulinic acid-glucosides

II. MATERIALS AND METHOD

A. Chemicals and raw materials

All the chemicals used were of analytical grade and purchased from Sigma Chemical Co.
The ^{1}H-NMR and ^{13}C-NMR data were obtained by analyzing the samples with high resolution NMR (model:Varian Unity Inova 500) in deuterated chloroform, CDCl3and deuterated methanol CD_3OD.

B. Spectroscopic instrumentation

Mass spectroscopy of the samples were analyzed using a LC-MS (Thermo Finnigan model). The samples were also analyzed using Fourier-transform infrared spectroscopy (Perkin Elmer, Spectrum BX, Germany).
The NMR data were obtained by analyzing the samples with high resolution NMR (model:Varian Unity Inova 500).Elemental analysis was performed using CHNS analyzer at UiTM.
Results are reported in ppm relative to trimethylamine (TMS)= 0.0 (δ). Melting point of purified products were

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determined using melting point equipment (Mettler Toledo model .

C. Synthesis of Betulinic Acid Glucoside 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl-3β-(2,3,4,6-tetra-O-acetyl-β–D-glucopyranosyloxy)-lup-20(29)-en-28-oate (2)

Boron trifluoride etherate (0.06 ml, 42 mmol) was added *via* syringe to a stirred solution of betulinic acid (0.192 x 10^{-3}mg, 0.42 mmole) and *β*-D-glucose-pentaacetate (0.117 x 10^{-3}mg , 0.3mmole) in anhydrous pyridine (5ml) under nitrogen atmosphere. The solution was stirred for 3 hours at room temperature. The mixture was then diluted with chloroform, dried over with magnesium sulfate, filtered and the solvent was then evaporated to give the crude product. The crude product was then purified using column chromatography using chloroform and ethyl acetate at 1:1 ratio as solvent system.

The crude product was isolated using column chromatography using eluting system of chloroform and ethyl acetate at 1:1 ratio to afford the desired product (2) from fractions 20-53 (0.178 x 10^{-3} mg, 92.8%. Melting point : 228-232 ^{o}C, CHN analysis : Found C, 61.6; H, 7.2; O, 31.2%, requires C, 62.4; H, 7.5; O, 30.1%.

D. Synthesis of Betulinic Acid Glucoside 3β–(β-D-glucopyranosyloxy-lup-20(29)-en-28-oak acid (3)

To a stirred solution of compound (2), (40 mg, 0.037 x 10^{-3} mmol) in dry methanol (5 ml) was added catalytic amount of sodium metal until base condition was achieved (pH > 7). The reaction mixture was stirred for 5 hours. The progress of the reaction was monitored using TLC.
After completion of the reaction, the solution was then neutralized with Amberlite ion-exchange resin (H^{+}) and filtered, concentrated under pressure to afford the required product as brown colored amorphous (3), 18 x10^{-3} mg, 45.0 %. CHN analysis : Found C, 68.4; H, 9.1; O, 21.5%, requires C, 69.9; H, 9.4; O, 20.7%.

E. Cytotoxic test

The cytotoxic effect of synthesized compounds were assessed by MTT Assay on human myeloid leukemia cell (HL60) showed the IC_{50} value was 2.4 µg/ml and inhibition on human T4-lymphoblastoid cell (CEMSS) conducted at Biotechnology and Biomolecular Sciences Department, Universiti Putra Malaysia

III. RESULTS AND DISCUSSION

A. Chemistry

Bisglucosides ,3,4,6-tetra-O-acetyl-*β*-D-glucopyranosyl-3*β*-2,3,4,6-tetra-O-acetyl-*β*–D-glucopyranosyloxy)-lup-20(29)-en-28-oate) (2) could be synthesized in the presence of boron trifluoro etherate at room temperature. The role played by Lewis acid catalyst, BF_3 was possibly to form a weak interaction between the oxygen atom on the pyranoside ring or anomeric acetyl functionality, resulting the glycosidic bond is weakened [7]. Through these complexation, an oxonium intermediate is generated and will be attacked on the most electrophilic carbon by betulinic acid carboxylate nucleophile to give compound (2). This study also demonstrated that 3*β*–(*β*-D-glucopyranosyloxy-lup-20(29)-en-28-oak (3) could be prepare using deacetylation method using sodium metal in methanol.The IR data of betulinic acid and its glucosides are summarized in Table 1.

Table 1 The IR data of betulinic acid and its glucosides

Functional group	Stretching frequencies (ν_{max} cm^{-1})		
	Betulinic acid	**(2)**	**(3)**
O-H	3450.0	-	3684.5
-C=O	1696.0	1753.3	1757.2
-C-O	1236.0	1221.6	1216.2
-C=C	886.0	756.9	758.4

The ^{1}H-NMR and ^{13}C-NMR data for compound 2 and compound 3 are showed on Table 2 and Table 3 respectively.

Table 2 The ^{1}H-NMR signals for 2 and 3

Protons	Group	Betulinic Acid	Compound 2	Compound 3
CH_3(C-4)	(3H,s)	0.75	0.74	0.77
CH_3(C-4)	(3H,s)	0.82	0.81	0.88
CH_3(C-8)	(3H,s)	0.93	0.92	0.92
CH_3(C-10)	(3H,s)	0.99	0.95	0.95

CH_3(C-14)	(3H,s)	1.20	0.96	1.01
CH_3(C-20)	(3H,s)	1.70	1.68	1.71
H-19	1H,m	3.00	3.00	3.20
H-3	1H,dd	3.20	3.20	3.60
H-29	1H,s	4.61	4.61	4.49
H-29	1H,s	4.75	4.75	4.51
		carbohydrate part		
H_1 (^{3})			5.72 d (*J* =	4.59
H_1 (^{28})			9.0)	3.50
H_2 (^{3})			4.92 d (	
H_2 (^{28})			*J* =	3.70 -
H_3 (^{3})			5.2)	3.85
H_3 (^{28})			5.12 m	3.70 -
H_4 (^{3})			5.16 m	3.85
H_4 (^{28})			5.21 d (*J* =	4.00
H_5 (^{3})			5.6)	
H_5 (^{28})			5.25 m	
$2H_6$(^{3})$2H_6$ (^{28})			5.09 m	
			5.13 m	
OAc (^{3}, ^{28})			3.82 m	3.72 -
			3.85 m	3.80
			4.12 dd (*J*= 11.1, 6.2 Hz),4. 08 dd, (*J*= 6.1, 6.2 Hz)	
			4.31 dd (*J*= 6.1, 6.2 Hz)	
			4.27 dd (*J*= 6.0, 6.7 Hz)	
			2.00 s	
			2.03 s	
			2.08 s	
			2.11 s	

^{3} : 3-*O*- glucose-pentaacetate
^{28} : 28-*O*- glucose-pentaacetat

Table 3 The ^{1}H-NMR signals for 2 and 3

Carbon	Betulinic Acid	Compound 2	Comp.3
C-1	38.8	39.1	39.1
C-2	27.4	27.6	27.6
C-3	79.0	79.2	78.1
C-4	38.7	38.9	39.2
C-5	55.3	55.6	55.5
C-6	18.3	18.5	18.4
C-7	34.3	34.6	34.6
C-8	40.7	40.9	40.6
C-9	50.5	50.7	50.1
C-10	37.2	37.4	37.7
C-11	20.8	20.8	20.5
C-12	25.4	25.7	25.9
C-13	38.4	38.6	38.7
C-14	42.4	42.7	42.3
C-15	29.7	29.9	29.5
C-16	32.1	32.4	32.3
C-17	56.3	56.5	56.5
C-18	49.2	49.5	49.3
C-19	46.7	47.1	46.7
C-20	150.4	150.2	152.0
C-21	30.5	30.8	30.5
C-22	37.0	37.1	37.2
C-23	28.2	28.2	28.2
C-24	15.3	15.6	15.2
C-25	16.1	16.3	16.4
C-26	16.0	16.1	16.3
C-27	14.7	14.9	15.1
C-28	180.8	170.9	174.7
C-29	108.3	109.9	108.7
C-30	19.4	19.6	19.5

Carbohydrate Part

Carbon	Compound 2 (^{3} , ^{28})	Compound 3
C-1	91.9, 91.9	92.8
C-2	72.9 , 72.9	71.8
C-3	70.5 , 70.5	75.3
C-4	68.0 , 68.0	68.4
C-5	73.0 , 73.0	76.9
C-6	61.7 , 61.7	61.7
OAc	170.3 , 170.3	
	169.6 , 169.6	
	169.5 , 169.5	
	169.2 , 169.2	
	21.0 , 21.0	
	20.9 , 20.9	
	20.8 , 20.8	
	20.6 , 20.6	

Cytoxic effect ^{3} : 3-*O*- glucose-pentaacetate
^{28} : 28-*O*- glucose-pentaacetate

Inhibition of betulinic acid on human myeloid leukemia cell (HL60) showed the IC_{50} value was 2.4 µg/ml and inhibition on human T4-lymphoblastoid cell (CEMSS) demonstrated the IC_{50} value of 1.8 µg/ml. A comparison of the HL60 activity of (2) and (3) indicated that both compounds are less potent than betulinic acid with IC_{50} values of 3.6 µg/ml and 2.80 µg/ml, respectively (Table 4) but their water solubility are increase.

The study of human T-4 lymphoblastoid cell (CEMSS) activity of 2 and 3 revealed that both compounds also less potent than betulinic acid with IC_{50} values of 11.0 µg/ml and 19.2 µg/ml respectively. These findings showed that the replacement of hydroxyl group at C-3 of betulinic acid with *β-D*-glucosepentaacetate or *β-D*-glucose will lowering the anti-leukemia activities. Therefore, the hydroxyl group at C-3 appeared to be an essential functional group for the selective anti-leukemia activities.

Table 4 Cytotoxic effects (µg/ml) of betulinic acid, compound 2 and 3
[a] Concentration which inhibits human myeloid leukemia cell by 50%.

Compound	IC_{50}[a]	IC_{50}[b]
Betulinic acid	2.4	1.8
Compound 2	3.6	11.0
Compound 3	2.8	19.2

[b] Concentration which inhibits human T4-lymphoblastoid cell by 50%.

IV. CONCLUSIONS

We have successfully develop a new preparative method for synthesizing glucosides of betulinic acid by using a Lewis acid

and □-*D*-glucosepentaacetate to afford bisglucosides (2,3,4,6-tetra-O-acetyl-*β*-D-glucopyranosyl-3*β*-2,3,4,6-tetra-O-acetyl-*β*–D-glucopyranosyloxy)-lup-20(29)-en-28-oate) (2). This study also demonstrated that 3*β*–(*β*-D-glucopyranosyloxy-lup-20(29)-en-28-oak (3) could be prepare using deacetylation method.

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