Enzymatic Synthesis and Anti-Cancer Activity of 3-*O*-B-D-Arabinopyranoside-Betulinic Acid

Hamisu Abdu

***Abstract*----**The weak hydro solubility of betulinic acid (1) hampers the clinical development of its natural anticancer agent. In order to circumvent this problem and to enhance the pharmacological properties of betulinic acid, 3-O-β-D-arabinopyranoside betulinic acid was successfully synthesized via the reaction between betulinic acid and arabinose using Novozyme 435 as biocatalyst in organic solvent which gave 86.5 % yield. The structure of the product obtained was elucidated using spectroscopic methods. The hydro solubility of was greatly enhanced upon the addition of sugar moiety on the C-3 of the betulinic acid. The anti cancer activity of 3-O-β-D-arabinopyranoside betulinic acid was also evaluated against cultured human breast cancer (MCF-7), human T-promyelocytic leukaemia (HL-60), mouse embryonic fibroblast normal cell line (3T3) and human cervical carcinoma cancer (HeLa) cell lines. Interestingly, 3-O-β-D-arabinopyranoside betulinic acid showed strong activity against Human T-promyelocytic leukemia (HL-60) and Mouse embryonic fibroblast cancer (3T3) with IC_{50} values of 6.1 and 5.4μg/ml respectively. However, it was found to have moderate activity against cultured human breast cancer (MCF-7) with IC_{50} values 15.9 μg/ml and weak activity against human cervical carcinoma cancer (HeLa) cell line with IC_{50} value 23.4 μg/ml respectively.

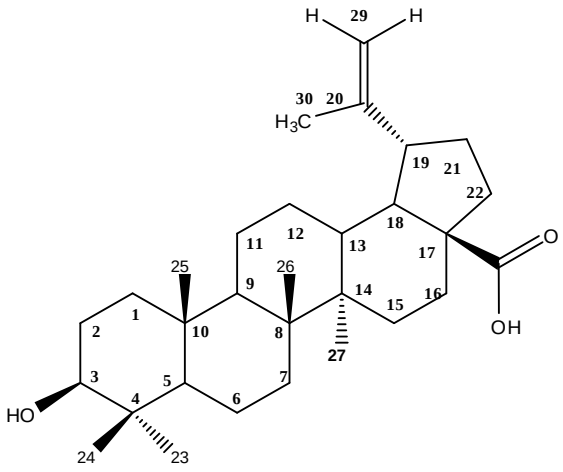
***Keywords*----**Enzymatic synthesis, 3-*O*-β-D-arabinopyranoside betulinic acid, Novozyme-435, cancer cells.

I. Introduction

BETULINIC acid (1) is a natural agent isolated from the bark of white birches and it was first described to induce apoptosis in neuro ectodermal tumour cells [1]. These natural products have been used for combating human diseases for thousands of years since they exhibit biological properties which can be exploited for medical applications [2]. Synthetic transformations of these natural compounds for the developing biologically active agents have become the basis of the actively advancing scientific direction of perfect organic synthesis and medicinal chemistry [3]. The greatest attention of researchers is attracted by native compounds with reliably established biological activity.

Betulinic acid belongs to the naturally occurring pentacyclic lupane-type triterpenoids which was reported to posseses various pharmacological activities which include anti-cancer, anti-HIV, anti-malarial, anti-inflammatory and anti-fungal [4, 5, 6 and7]. However, the major hinderance for the future clinical development of betulinic acid and its analogs reside in its weak hydrosolubility in aqueous media like serum, blood and non-polar solvents like water used for bioassays [8].

If one of the strategies to increase betulinic acid hydrosolubility is by the synthesis of its glycoside derivatives. Furthermore, the bioactivity of betulinic acid, in some cases can be improved upon the addition of sugar moiety at either C-3 or C-28 or both. Some natural and synthetic betulinic acid glycosides were also reported in the literature [9]. For instance, 3-*O*-β-D-arabinopyranoside-betulinic acid (2) was synthesised by chemical reactions [8] and shown some bioactivities. The chemical synthesis however seems to be difficult in their purification procedure. Thus, in connection with my continious efforts the enzymatic synthesis of 3-*O*-β-D-arabinopyranoside-betulinic acid (2) is now reported by reaction between betulinic acid (1) and arabinose using Novozyme® 435 in organic solvent. Interestingly, it was observed that the reaction was clean and simple, and gave high yield of product. The evaluation of this compound toward some cancer cell lines was also reported herein. The structures of compound (1) and (2) were shown in Figure 1.



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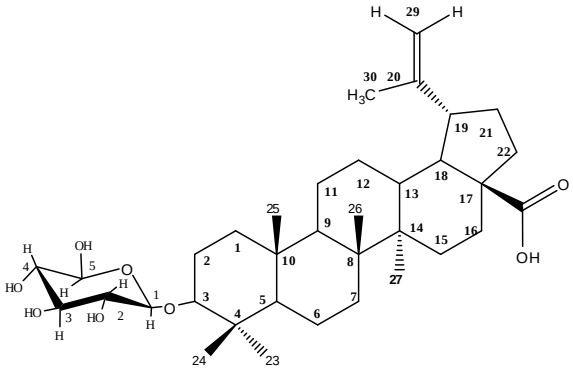

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Fig.1 Structures of compound 1 and 2

II. Experiment and Materials

Betulinic acid was donated by Prof.(Dr) Faujan B.H.A. (Chemistry Department University Putra Malaysia), arabinose was purchased from Merck, Germany, Novozyme® 435 was purchased from Novo Nordisk A/S (Denmark), *t*-butanol, hexane, ethylethanoate, Dimethylsulfoxide (DMSO) and Microculture Tetrazolium salt (MTT) were purchased from Merck, Germany. It is important to note that all chemicals used in this work were of analytical reagents grade. Nevertheless, they are also pure and distilled. The cancer cell lines HL-60, MCF-7, HeLa; and 3T3 were supplied by Institute of Bioscience (IBS), UPM and were purchased from American Type Cell Culture Collection (ATCC), USA.

Preparation of 3-O-β-D-Glucopyranoside-betulinic acid (2)

This compound was prepared using betulinic acid (22.8mg, $0.5x10^{-1}$ mmol) and arabinose (18.0mg, $1.0x10^{-1}$ mmol) dissolved in *t*-butanol (10 ml). Novozyme® 435 (180mg) was then added and the reaction mixture was incubated on a water bath shaker (Memmert WB 14, Germany) for 30 h, at 55.0^{O}C and 150 rpm. The progress of the reaction was monitored using thin layer chromatography (TLC) with hexane and ethylacetate as the eluent (8:2 v/v). The enzymes was then removed by filtration. Removal of the solvent under reduce pressure gave a yellowish solid. The product was then purified through celite flash column chromatography followed by crystallized from *t*-butanol to give the pure material of the desired product as light yellow crystal, 0.0265g (85.6%). It melted at of 201.5-203.5^{o}C (uncorrected) (literature >200 ^{o}C [8]

The NMR spectra of the compound was recorded with Varian Unity Inova 400 NMR spectrometer operating at a resonance frequency of 499.89 MHz for ^{1}HNMR spectra and 125.71MHz for ^{13}C-NMR spectra. The mass spectrum was recorded using Shimadzu, QP5050, Japan.

I. Cytotoxic activity of the product was evaluated against HL-60, Cancer cell line), MCF-7 Cancer cell lines (Human breast cancer), HeLa Cancer cell line (Human cervical carcinoma cancer) and 3T3 cell line (Mouse embryonic fibroblast cancer) respectively. All these cell lines were supplied by Institute BioScience (IBS) University Putra Malaysia, and were purchased from American Type Cell Culture Collection (ATCC), USA. Culture were maintained according to [10] as monolayers in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/steptomycin at 37^{o}C in a humidified atmosphere using 5% carbon(iv)oxide (CO_2).

II. The cytotoxic activity of the product was evaluated using calorimetric Microculture Tetrazolium salt assay (MTT). Exponentially the growing cells were plated in 96-well microplates (Coster Corning Inc.) at a density of $5x10^{3}$ cells per well in 100µL of the culture medium and these were allowed to adhere for 72 h before treatment in order to prevent confluence [10]. After 72 h of incubation, the fractions of the surviving cells were measured relative to the untreated cell population by MTT assay. A volume of 20ml of MTT salt (5mg/ml) in phosphate buffer solution was added to each microtiter well and incubated again for 3-4 h. 100µl of Dimethyl sulfoxide (DMSO) was then added to dissolve the remaining MTT formazan crystal by pippeting up and down 10-20 times. The plates was left at room temperature for 15-30 minutes. The optical density (OD) was measured on an ELIZA microplate reader at 570 nm and the percentage of cell viability was calculated using the equation:

% viability = (OD sample / OD control) x100%

III. A plot of percentage cell viability against the concentration of the drug gives a measure of the cytotoxicity. The cytotoxic index used was IC_{50}, the drug concentration lethal to 50% of the tumor cells as calculated from the plate.

IV. Results and Discussion

The product obtained after purification appeared to be a light yellow crystal (87.5%) with a melting point of 201.5-203.5^{o}C (literature >200^{o}C [8].

The ^{1}H-NMR spectra of 3-*O*-β-D-arabinopyranoside-betulinic acid indicates the presence of 6 methyl groups (each singlets) at δ 0.84, 1.03, 1.25, 1.08, 1.34 and 1.82 respectively (each 3H, assigned for 23-CH_3, 24-CH_3, 25-CH_3, 26-CH_3, 27-CH_3 and 30-CH_3 respectively). The signal at δ 3.49 (1H, dd) was due to the hydrogen proton assigned at C-3 position. The presence of two hydrogens at C-29 position was confirmed by the presence of proton signals at δ 4.80 (1H,s) and δ 4.97 (1H,s). The doublet proton signal at δ 4.69 (1H, d) was assigned as the proton attached to the carbon bearing at position C-1' (δ 102.8) . The signal at δ 3.48 (1H, m) was due to the hydrogen at C-19 position. The ^{13}C-NMR of of 3-*O*-β-D-arabinopyranoside-betulinic acid showed the presence of signal at δ 82.9, which was assigned to C-3 of the compound. The signal at 149.9 ppm and 108.6 ppm was due to the carbon double bond between C-20 and C-29 respectively. The ^{13}C-NMR of the compound also shows a carboxyl carbon signal at δ 177.5 ppm assigned as C-28 (COOH).

The selected HMBC (Figure 2) shows that proton signal H-30 has a correlation with C-20, C-29, and C-1 respectively. Furthermore, proton signal H-29 was also correlated to C-30.

There is also a correlation between proton signal H-24 with C-30 and C-25. The selected HSQC (Figure 3) shows that an olefinic hydrogens H-29 were correlated with C-29 and C-3' has a corelation with H-3'. Furthermore, there is a correlation between H-1' with C-3'. The H-H COSY data (Figure 4) indicated a correlation between protons H-19 and H-30, H-29 and H-30 respectively. The complete data assignment of the ^{13}C-NMR, ^{1}H-NMR, HMBC and HSQC strongly agreed with the structure of the product.

The crude product was analysed using hexane and ethylacetatae (8:2, v/v) as the eluent. The crude product was then purified by using flash column chromatography. Evaporation of the solvent gave pure product 28.6mg (76.8 %). mp 202 ^{o}C; *Rf* = 0.26; *m/z* = 588. ^{1}H NMR (C_5D_5N) δ: 0.84, 1.03, 1.25, 1.08, 1.34 (all s, each 3H, H-23, H 24, H-25, H-26, H-27), 1.82 (s, 3H, H-30), 3.49 (dd, 1H, J = 11.5 Hz, J = 4.0 Hz, H-3), 3.55 (m, 1H, H-19), 4.18 (m, 1H, H'- 4), 4.29 (m, 1H, H'-5), 4.18 (m, 1H, H'-4), 4.40 (m, 1H, H'-3), 4.61 (br s, 1H, H'-2), 4.73 (br s, 1H, H-29α), 4.92 (br s, 1H, H-29β), 5.44 (br s, 1H, H'-1). ^{13}C NMR (C_5D_5N) δ: 38.1, 29.8, 82.9, 37.8, 84.5, 18.1, 36.1, 39.7, 49.5, 37.8, 21.5, 26.8, 37.2, 41.4, 30.7, 33.4, 55.2, 46.3, 48.4, 149.9, 31.5, 36.2, 28.6, 17.4, 15.0, 13.5, 13.0, 177.5, 108.6, 19.7 (C-1 to C-30), 67.9 (C'-5), 72.5 (C'-2), 75.5 (C'-3), 68.4 (C'-4), 102.8 (C'-1).

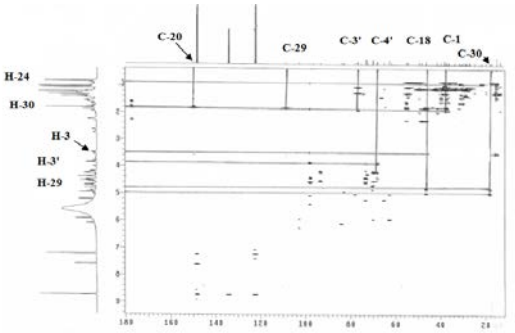

Fig. 2 HMBC spectrum of 3-O-β-D- Arabinopyranoside-betulinic acid

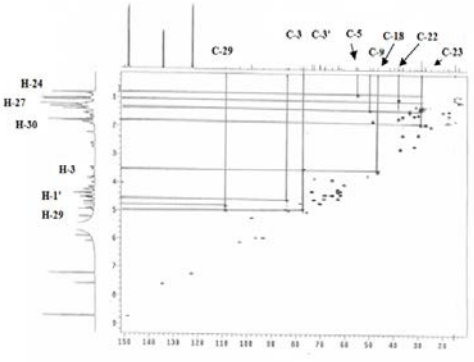

Fig. 3 HSQC spectrum of 3-O-β-D-Arabinopyranoside-betulinic acid

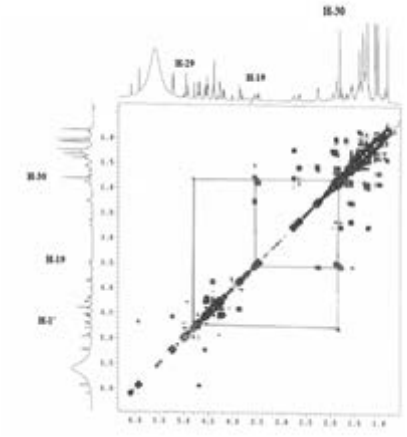

Fig. 4 1H-1H COSY spectrum of 3-O-β-D-Arabinopyranoside-betulinic acid

V. Bioactivity Studies

Based on IC_{50} values, compounds with IC_{50}< 10 µg/ml were considered to be strongly active, those with IC_{50} ranging from 10-30 µg/ml were considered to be moderately active while compounds with IC_{50}> 30 µg/ml were considered as weakly active [11,12]. Our bioactivity results conducted, betulinic acid (1) showed high activity against cultured human breast cancer (MCF-7), human T-promyeloctic leukaemia (HL-60), and human cervical carcinoma cancer (HeLa) cell lines with IC_{50} values 0.8, 4.4 and 4.8 µg/ml respectively, however, betulinic acid was shown to be highly inactive against mouse embryonic fibroblast normal cell line (3T3) with IC_{50} value > 30 µg/ml. In contrast, 3-*O*-β-D-arabinopyranoside betulinic acid showed strong activity against human T-promyeloctic leukaemia (HL-60) and mouse embryonic fibroblast normal cell line (3T3) with IC_{50} values 5.4 and 6.1 µg/ml, on the other hand, this compound possesses moderate activity against cultured human breast cancer (MCF-7) with IC_{50} value 15.9 and and weak activity against human cervical carcinoma cancer (HeLa) cell line with IC_{50} value 23.4 µg/ml. Since the introduction of glucose in betulinic acid reduced their cytotoxic activity, it may due to the fact that the glycoside is having higher molecular weight thus may not pass through the cell membrane completely, although the introduction of sugar in betulinic acid molecule was expected to increases its hydrosolubility properties.

VI. Conclusion

In conclusion, 3-*O*-*β*-D-arabinopyranoside-betulinic acid was prepared and characterized using spectroscopic data. The anticancer activity was evaluated against cancer cell lines. It was shown that betulinic acid (1) showed high activity against MCF-7, HL-60, and HeLa cell lines with IC_{50} values 0.8, 4.4 and 4.8µg/ml respectively. Interestingly, 3-*O*-*β*-D-arabinopyranoside-betulinic acid also showed strong activity

against cultured MCF-7, HL-60 and 3T3 with IC_{50} values 8.5, 8.4 and 2.75µg/ml, respectively, and moderately activity against HeLa cell line with IC_{50} value 12.0 µg/ml. In general, the 3-*O*-*β*-D-arabinopyranoside-betulinic acid showed less activity against tested cell lines as compered to betulinic acid itself. It may due to the higher molecular weight of the 3-*O*-*β*-D-arabinopyranoside-betulinic acid as compared to betulinic acid.

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