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ENZYMATIC SYNTHESIS AND ANTI CANCER ACTIVITY OF 3-O-A-D ARABINOPYRANOSIDE METHYL BETULINATE

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KEYWORDS:
Enzymatic synthesis, 3-O-α-D-Arabinopyranoside methyl betulinate, Novozyme-435, cancer cells.

Abstract

The weak hydro solubility of lupane-type triterpenes lupeol (1), betulin (2), and methyl betulinate (3), hampers the clinical development of its natural anticancer agent. In order to circumvent this problem and to enhance the pharmacological properties of betulinic acid and its analogues, 3-O-α-D-Arabinopyranoside of methyl betulinate was successfully synthesized via the reaction between methyl betulinate and D-arabinose using Novozyme 435 as biocatalyst in organic solvent which gave 94.21 % yield. The structure of the product obtained was elucidated using spectroscopic methods. The hydro solubility of 3-O-α-D-Arabinopyranoside methyl betulinate was greatly enhanced upon the addition of sugar moiety on the C-3 of the betulinic acid. The anti cancer activity of 3-O-α-D-Arabinopyranoside methyl betulinate 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-α-D-Arabinopyranoside methyl betulinate showed strong activity against Human T-promyelocytic leukemia (HL-60), Mouse embryonic fibroblast cancer (3T3) and cultured human breast cancer (MCF-7) with IC_{50} values of 4.7, 8.2 and 7.1 µg/ml respectively. However, it was found to have weak activity against human cervical carcinoma cancer (HeLa) cell line with IC_{50} value IC_{50} > 30 µg/ml respectively. .

INTRODUCTION

Lupeol, betulin, and betulinic acid are found in the external bark of yellow (Betula alleghaniensis) and white (Betula papyrifera) birches in the boreal forest of North America [1]. These pentacyclic triterpenes of the lupane-type were recently been investigated by the scientific community due to their various pharmacological and medicinal properties. Lupeol known for its in vivo anti-inflammatory activity exhibited in vitro cytotoxicity against human epidermoid carcinoma (A-431) and human hepatocellular carcinoma (Hep-G2), while it did not affect the growth of tumor cell lines such as human melanoma (MEL-2), human lung carcinoma (A-549), and murine melanoma (B16-F10). Moreover, lupeol was found to exhibit a significant anti-angiogenic activity on in vitro tube formation of human umbilical vein endothelial cells (HUVEC). In addition, induction of apoptosis (programmed cell death) by lupeol was observed in human leukemia HL-60 cells [2].

Methyl-betulinate and betulinic acid are natural agent isolated from the bark of white birches and and they were first described to induce apoptosis in neuro ectodermal tumour cells [3]. 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 [4]. 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 [5]. The greatest attention of researchers is attracted by native compounds with reliably established biological activity.

Pentacyclic lupane-type triterpenoids was reported to posseses various pharmacological activities which include anti-cancer, anti-HIV, anti-malarial,

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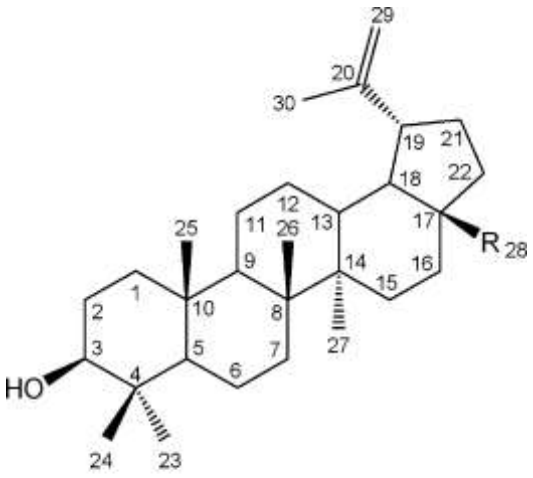
anti-inflammatory and anti-fungal [6-9]. However, the major hinderance for the future clinical development of betulinic acid and its analogs reside in its weak hydrosolubility in aqueous media like serum, blood and non-polar solvents like water used for bioassays [10].

One of the strategies to increase lupane-type triterpenes hydrosolubility is by the synthesis of its glycoside derivatives. Furthermore, the bioactivity of lupane-type triterpenes, 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 [11]. For instance, 3-*O*-α-D-Arabinopyranoside methyl betulinate was synthesized by chemical reactions [10] and shown some bioactivities. The chemical synthesis however seems to be difficult in their purification procedure. Thus, in connection with our continious efforts, the enzymatic synthesis of 3-*O*-α-D-Arabinopyranoside methyl betulinate is now reported by reaction between methyl betulinate and glucose using Novozyme® 435 in organic solvent. To the best of our knowledge, this is the first report published for the enzymatic synthesis of 3-*O*-α-D-Arabinopyranoside methyl betulinate. 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.



Lupeol **1**: R = CH_3
Betulin **2**: R = CH_2OH
Betulinic acid **3**: R = COOH

Figure 1: Structures of Lupane-Type Triterpenoids

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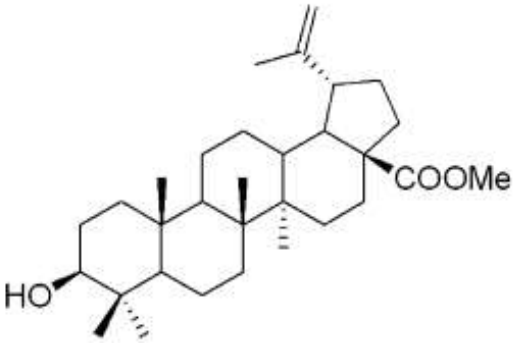
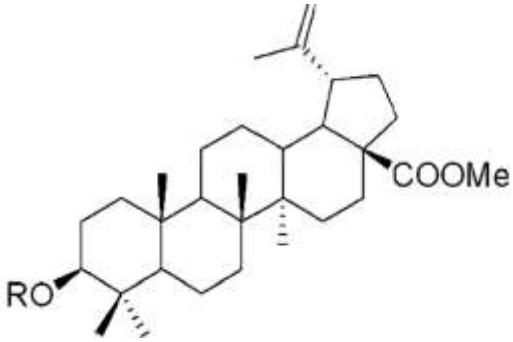


Figure 2: Structure of methyl betulinate



R = D-Ara

Figure 3: Structure of 3-*O*-α-D-Arabinopyranoside methyl betulinate

Experiment and Materials

Methyl betulinate was donated by Prof.(Dr) Faujan B.H.A. (Chemistry Department University Putra Malaysia), glucose 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), University Putra Malaysia (UPM) and were purchased from American Type Cell Culture Collection (ATCC), USA.

Preparation of 3-*O*-α-D-Arabinopyranoside methyl betulinate

This compound was prepared using methyl betulinate (22.8mg, $0.5x10^{-1}$ mmol) and D-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 ethyl acetate 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 (94.21%). It melted at about 199°C (literature > 200°C) [10]

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.

Cytotoxic activity of the product was also 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 Bio Science (IBS) University Putra Malaysia, and were purchased from American Type Cell Culture Collection (ATCC), USA. Culture were maintained according to [12] as monolayers in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/steptomycin at 37°C in a humidified atmosphere using 5% carbon(iv)oxide (CO_2).

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 [12]. 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:

$$\% \text{ viability} = (\text{OD sample} / \text{OD control}) \times 100\%$$

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.

Results and Discussion

The product obtained after purification appeared to be a light yellow crystal (94.21%, R_f = 0.24), with a melting point of 199°C (literature > 200°C) [10]. The ^{1}H-NMR spectra of 3-*O*-α-D-Arabinopyranoside of methyl betulinate indicates the presence of 6 methyl groups (each singlets) at δ 0.75, 0.83, 0.90, 0.93, 0.99 and 1.66 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 3.23 (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 4.59 (1H,s) and δ 4.75 (1H,s). The doublet proton signal at δ 4.32 (1H, d) was assigned as the proton attached to the carbon bearing at position C-1' (δ 106.93) . The signal at δ 3.01 (1H, m) was due to the hydrogen at C-19 position. The ^{13}C-NMR of of 3-*O*-α-D-Arabinopyranoside of methyl betulinate acid showed the presence of signal at δ 88.82, which was assigned to C-3 of the compound. The signal at 150.75 ppm and 109.72 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 57.45 ppm assigned as C-28 ($COOCH_3$).

The selected HMBC 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 shows that an olefinic hydrogens H-29 were correlated with C-29 and C-3' has a correlation with H-3'. Furthermore, there is a correlation between H-1' with C-3'. The H-H COSY data indicated a correlation between protons H-19 and H-30, H-29 and H-30 respectively. The characteristics of the chemical shifts and the vicinal coupling constants of the anomeric protons obtained correspond to that of α-D-arabinosides (d = 4.06–4.70 ppm, *J* = 5.6– 7.1 Hz) [13]. The complete data assignment of the ^{13}C-NMR, ^{1}H-NMR, HMBC and HSQC strongly agreed with the structure of the product.

Table 1: ^{1}H NMR, ^{13}C NMR, HMBC and ^{1}H-^{1}H COSY Data of the 3-*O*-α-D-Arabinopyranoside methyl betulinate

Carbon	**δ_C (ppm) in C_5D_5N**	**δ_H (ppm) in C_5D_5N**	**HMBC**	**COSY**	**δ_C (ppm) Lit. in (C_5D_5N)**	**δ_H(ppm) Lit. in (C_5D_5N) (Gauthier *etal.*, 2006)**
1	39.24				39.28	
2	26.63				25.63	
3	84.82	3.23(1H,dd)*J* = 11. 7 Hz, *J* = 3.81 Hz			84.81	3.23 (1H,dd) *J* = 11. 8 Hz, *J* = 3.8 Hz
4	38.33				38.34	
5	55.88				55.87	
6	18.42				18.42	
7	34.43				34.44	
8	40.86				40.85	
9	50.65				50.63	
10	37.18				37.18	
11	19.53				19.51	
12	23.15				23.15	
13	38.55				38.54	
14	42.52				42.51	
15	29.77				29.78	

16	32.30				32.29	
17	56.69				56.69	
18	47.11				47.10	
19	49.59	3.01 (m, 1H)	H-29	H-30	49.59	3.00 (m, 1H)
20	150.75				150.74	
21	30.73				30.73	
22	37.14				37.11	
23	28.33	0.75 (3H,s)			28.32	0.75 (3H, s)
24	16.54	0.83 (3H,s)			16.54	0.81 (3H, s)
25	16.24	0.90 (3H,s)			16.23	0.90 (3H, s)
26	16.10	0.93 (3H,s)			16.09	0.93 (3H, s)
27	14.77	0.99 (3H,s)			14.76	0.98 (3H, s)
28	176.81		H-30		176.81	
29(α,β)	109.72	4.59, 4.75 (1H,s)	H-19, H-30	H-30	109.72	4.59, 4.73 (1H,s)
30	21.05	1.66 (3H,s)	H-29	H-29, H-30	21.04	1.68 (3H, s)
1′	106.93	4.32 (1H, d), *J* = 6.2 Hz			106.92	4.31 (1H, d), *J* = 6.1 Hz
2′	75.83	3.71(m, 1H)			75.82	3.70 (m, 1H)
3′	78.77	3.68 (m, 1H)			78.79	3.66 (m, 1H)
4′	71.84	3.93(m, 1H)			71.84	3.93(m, 1H)
5′ (α,β)	78.35	3.53, 3.95, *J* = 9.5 Hz			78.35	3.52, 3.95 (*J* = 9.4 Hz

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 [14, 15]. From our bioactivity results conducted, methyl

betulinate 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 2.3, 5.1 and 9.2 µg/ml respectively, however, methyl betulinate 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*-α-D-Arabinopyranoside methyl betulinate showed strong activity against human T-promyeloctic leukaemia (HL-60) and mouse embryonic fibroblast normal cell line (3T3) and cultured human breast cancer (MCF-7) with IC_{50} values 4.7, 8.2 and 7.1 µg/ml respectively, on the other hand, this compound possesses weak activity against human cervical carcinoma cancer (HeLa) cell line with IC_{50} value > 30 µg/ml. Because the introduction of arabinose in methyl betulinate reduced their cytotoxic activity, it may due to the fact that the glycoside is having higher molecular weight thus cannot pass through the cell membrane completely, even though the introduction of sugar in methyl betulinate molecule was expected to increases its hydrosolubility properties.

Conclusion

In conclusion, 3-*O*-α-D-Arabinopyranoside methyl betulinate was prepared and characterized using spectroscopic data. The anticancer activity was these compounds was evaluated against cancer cell lines. It was shown that methyl betulinate showed high activity against MCF-7, HL-60, and HeLa cell lines with IC_{50} values 2.3, 5.1 and 9.2 µg/ml respectively. Interestingly, 3-*O*-α-D-Arabinopyranoside methyl betulinate also showed strong activity against cultured MCF-7, HL-60 and 3T3 with IC_{50} values 4.7, 8.2 and 7.1 µg/ml, respectively, and moderately activity against HeLa cell line with IC_{50} value > 30 µg/ml. In general, the 3-*O*-α-D-Arabinopyranoside methyl betulinate showed less activity against tested cell lines as compered to methyl betulinate itself. It may due to the higher molecular weight of the 3-*O*-α-D-Arabinopyranoside methyl betulinate as compared to methyl betulinate.

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