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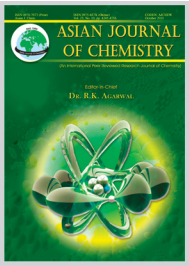

Lipase Catalyzed Synthesis of Anticancer Compound: 3β-(3-Methylphthalyl)-lup-20(29)-ene-28-oic

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Lipase catalyzed synthesis reaction of betulinic acid with 3-methylphthalic anhydride in presence of Novozyme 435 as biocatalyst gave 3β-(3-methylphthalyl)-lup-20(29)-ene-28-oic acid, which is a new candidate for anticancer agent. The reaction was carried out in chloroform at 37 ºC for 24 h. 3β-(3-methylphthalyl)-lup-20(29)-ene-28-oic acid showed a higher cytotoxicity as compare to parent compound.

Key Words: Betulinic acid, Betulinic ester, Enzyme, Cytotoxicity.

INTRODUCTION

Betulinic acid (3-β-hydroxy-lup-20(29)-ene-28-oic) is a naturally occurring pentacyclic triterpenoid. It is found in the bark of several species of plants, including the Ber tree (*Ziziphus mauritiana*), the white birch (*Betula pubescens*)[1] and other plants[2]. Betulinic acid was reported as a selective inhibitor of human melanoma[3], posesses antiviral[4], antimalarial and antiinflammatory properties[5] and discovered as potential anticancer agent by inhibition of topoiomerase[6]. It was demonstrated, that betulinic acid induces apoptosis in human melanoma *in vitro* and *in vivo* model systems[7]. Moreover betulinic acid was also found to be active against neuroectodermal, (neuroblastoma, medulloblastoma, Ewing's sarcoma)[8], malignant brain tumors and ovarian carcinoma[7].

For the past few years, many researches have been carried out to synthesize betulinic acid derivatives. In particular, Hashimoto *et al.*[9] have reported on the synthesis of some betulinic acid derivatives and its cytotoxic. They noted that the substituents at C-28 play an important role in the demonstration of anti-HIV activity. Replacement of COOH group at C-28 with a CH_2OH group as in betulin or with an ester group results in the reduction of the anti-HIV activity. They suggested that the carboxylic acid group at C-28 appears to be essential for the selective HIV inhibitory effect[10]. Although betulinic acid itself can be considered as an effective medical remedy[11]. However Kvasnica *et al.*[1] reported that some derivatives of betulinic acid are much more effective than the parent betulinic acid itself. Furthermore, modification of betulinic acid structure by carrying esterification reactions at C-3 showed a higher cytotoxic activity[12] against broader spectrum of tumors from different histogenetic origin, such as, mesenchymal (CEM, K562, K562-tax), epithelial (HT29, PC-3) and neuroektodermal (SK-MEL2) tumors.

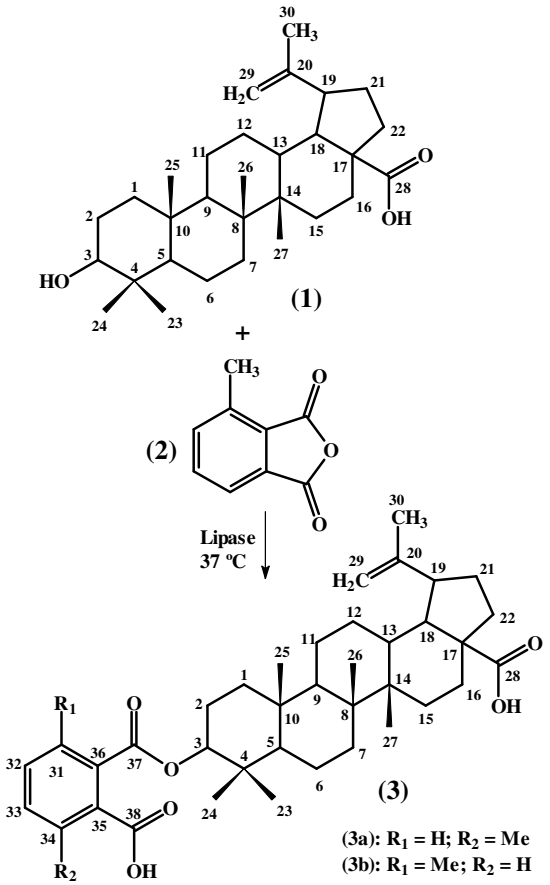
It is well documented that lipase catalyzed many potentially important reactions including hydrolysis, esterifications and transesterifications either in water or organic solvent systems and it is presently regarded as a routine procedure in synthesis organic chemistry[13]. This enzyme has been used successfully in the synthesis of esters and amides[14] and also for the medium chain glycerides[15]. This enzyme has a broad substrate specificity and a strong regioselective acylation properties which may eliminate the protection/deprotection steps needed in the usual organic synthetic route. Thus, in continuation to our interest on the use of enzyme as catalyst for the synthesis of betulinic acid ester, the present communication reports the preparation of a new anticancer candidate of betulinic acid ester, 3β-(3-methylphthalyl)-lup-20(29)-ene-28-oic acid using Novozyme 435. Its detail spectroscopy data and cytotoxicity against human lung carcinoma (A549) and human ovarian (CAOV3) cancer cell lines are also presented.

EXPERIMENTAL

Novozyme 435 lipase (*Candida antartica* lipase) was obtained from Novo Nordisk Industry A/S (Bagsvaerd, Denmark). It was used as received without pretreatment. Other chemicals were of analytical grade. The FT-IR spectrum was recorded on Perkin-Elmer fourier transform infrared model

1650, while mass spectrum was recorded on Shimadzu gas chromatograph mass spectometry model QP5050 and NMR spectrum was recorded on JOEL NMR model ECA-400. Compounds cytotoxicity was analyzed using MTT cytotoxicity assay.

To a solution of betulinic acid (**1**) (300 mg, 6.58×10^{-4} mmol) and 3-methylphthalic anhydride (**2**) (110 mg, 6.78×10^{-4} mmol) in chloroform (20 mL) was added Novozyme 435 (1 g). The reaction mixture was stirred at 150 rpm for 24 h at temperature approximately 37 ºC in a water bath apparatus. The reaction mixture was then filtered off to remove the excess of enzyme and solvent evaporation gave the crude product as white crystalline material. To the best of our knowledge, this is the first report on the preparation of the derivative (**3**) using 3-methylphthalic anhydride through enzymatic reaction. The synthesis pathway is shown in **Scheme-I**. The product (**3a**) was formed due to the inductive effect of methyl group of the phthalic moisture and none of its isomer was isolated. Its cytotoxicity against human lung carcinoma (A-549) and human ovarian (CAOV3) cancer cell lines are shown ion Table-1.



Scheme-I: Synthesis of 3β-(3-methylphthalyl)-lup-20(29)-ene-28-oic

TABLE-1
CYTOTOXIC ACTIVITY OF 3β-(3-METHYLPHTHALYL)-LUP-20(29)-ENE-28-OIC AND BETULINIC ACID AGAINST A549 AND CAOV3 CELLS

Compound	IC_{50} (μg/mL)	
	A549	CAOV3
Betulinic Acid	18.4	> 30
3β-(3-Methylphthalyl)-lup-20(29)-ene-28-oic	4.4	3.6

RESULTS AND DISCUSSION

The ^{1}H NMR signals in d-chloroform of the product resonated at δ 0.76 (s, 3H), 0.83 (s, 3H), 0.94 (s, 3H), 0.94 (s, 3H), 0.98 (s, 3H), 1.42 (s, 3H) and 1.70 (s, 3H) confirmed for the present of seven methyl groups. A triplet signal at δ 4.48 was due to the proton at C-3 position and signal at δ 4.61-4.75 was assigned vinylic for proton at C-29. A doublet signal at δ 7.58 was assigned for aromatic protons at C-32 and C-34 position and a triplet signal at δ 7.73 was assigned for aromatic proton at C-33. All the spectroscopic data are very similar with that reported in the literature for the analogous compound.

The ^{13}C NMR data of 3β-(3-methylphthalyl)-lup-20(29)-ene-28-oic acid was closely related to that of betulinic acid, with an addition of carbonyl carbon at d 150.6 ppm. For comparison with ^{13}C NMR data of betulinic acid, its ^{13}C NMR data in d-chloroform are summarized in Table-2.

TABLE-2
^{13}C NMR DATA OF BETULINIC ACID AND 3β-(3-METHYLPHTHALYL)-LUP-20(29)-ENE-28-OIC ACID (PRODUCT) IN d-CHLOROFORM AND HMBC/ HSQC DATA OF PRODUCT

Carbon number	Betulinic acid (δ) (**1**)	Product (δ) (**3a**)	HSQC next carbon, ^{2}J	HMBC long carbon, ^{3}J
1.	38.7	39.1	H-2	H-3, 9
2.	18.2	18.5	H-1, 3	H-9
3.	81.2	79.3	H-2	H-1, 5, 23, 24
4.	38.5	38.9	-	-
5.	55.2	55.6	H-6	H-1, 7, 9
6.	21.0	18.5	H-5, 7	-
7.	34.7	34.6	H-6	-
8.	41.3	41.0	-	-
9.	50.5	50.7	H-11	H-1, 5, 7, 12
10.	37.2	37.3	-	-
11.	21.6	21.1	H-9, 12	-
12.	25.7	25.7	H-11,13	-
13.	38.3	38.6	H-12,18	H-11, 15, 19
14.	42.4	42.7	-	-
15.	30.2	31.7	H-15,16	-
16.	32.9	32.4	-	-
17.	57.1	56.6	-	-
18.	47.7	47.1	H-13, 19	H-12, 14, 16, 20,21,28
19.	49.4	49.5	H-18, 21	H-13, 17,22
20.	150.3	131.1	-	H-17
21.	30.6	30.8	H-19, 22	-
22.	37.0	37.4	H-21	-
23.	28.6	28.2	-	H-2, 6, 10
24.	16.5	16.4	-	H-2, 6, 10
25.	16.4	16.3	-	H-2, 4,6,8,11
26.	16.4	15.6	H-6.7,12,18	H-6,10,11,15
27.	15.0	14.9	H-	H-7, 12, 16,
28.	182.3	181.5	7.12,16,18	18
29.	109.3	109.9	H-22, 26	H-22, 26
30.	19.4	19.6	-	-
31.		129.0	H-19, 29	H-29, 19
32.		131.1	-	-
33.		131.1	H-31, 34	H-35
34.		129.0	H-31, 34	H-35
35.		132.1	H-32, 36,	H-37
36.		132.1	38	-
37.		150.6	-	-
38.		150.6	-	H-1, 5
39.		27.6	H-3	-
			-	H-37
			H-32, 36	

The mass spectrum of the 3β-(3-methylphthalyl)-lup-20(29)-ene-28-oic acid showed molecular ion at *m/z* 618 ($C_{39}H_{54}O_6$), as expected. The fragments appeared at *m/z* 592, 568, 410, 302, 248 and 220, which are in agreement with that of betulinic acid ester or betulinic acid fragmentation.

Purification of the product was carried on silica gel (0.063-0.200 mm) column chromatography (3 cm, i.d. 45 cm length) using a mixture of hexane and ethyl acetate (9:1, v/v) as the eluent to elute white crystals (109 mg, 36.0 %), m.p. 286-287 ºC). (Found: C, 75.89; H, 8.90. $C_{39}H_{54}O_6$ requires C, 75.62; H, 8.73; γ_{max} (film, cm^{-1}) 1690 (C=O ester), 1236 (C-O), 1452 and 1376 (C=C) cm^{-1}; δ H NMR ($CDCl_3$, ppm) 0.76 (s, 3Me), 0.83 (s, 3Me), 0.94 (s, 3Me), 0.94 (s, 3Me), 0.98 (s, 3Me), 1.42 (s, 3Me), 1.70 (s, 3Me) (for seven methyl groups), 4.48 (t, Me), 4.68 (s, Me); ^{13}C NMR, C-1 (δ 39.90), C-2 (δ 28.22), C-3 (δ 79.27), C-4 (δ 38.95), C-5 (δ 55.58), C-6 (δ 18.52), C-7 (δ 34.56), C-8 (δ 40.93), C-9 (δ 50.74), C-10 (δ 37.29), C-11 (δ 21.08), C-12 (δ 25.73), C-13 (δ 38.63), C-14 (δ 42.67), C-15 (δ 31.67), C-16 (δ 32.38), C-17 (δ 56.58), C-18 (δ 47.13), C-19 (δ 49.50), C-20 (δ 131.14), C-21 (δ 30.79), C-22 (δ 37.44), C-23 (δ 27.61), C-24 (δ 16.35), C-25 (δ 16.27), C-26 (δ 15.58), C-27 (δ 14.92), C-28 (δ 181.49), C-29 (δ 109.95), C-30 (δ 19.60), C-31 (δ 129.04), C-32 (δ 131.14), C-33 (δ 131.14), C-34 (δ 129.04), C-35 (δ 132.07), C-36 (δ 132.07), C-37 (δ 150.63), C-38 (δ 150.63) and C-39 (δ 18.52). It had *m/z* 618 and fragments at *m/e* 592, 568, 410, 302, 248 and 220. The COSY analysis showed a direct coupling between H-29 (δ 4.61 - 4.75) with H-30 (δ 1.70). The proton at H-13 (δ 1.34-1.38) also showed a direct coupling with H-19 (δ 1.34-δ 1.38) while H-25 (δ 0.98) showed a direct coupling with H-1 (δ 2.17 - 2.29). This indicated that the reaction gave the desired product of the betulinic acid ester. The aromatic protons observed at δ 7.73 (H-34) showed direct coupling to a proton at δ 129.04 in the HSQC spectrum while long range HMBC was correlated to carbons at δ 131.14 (2J), d 132.07 (^2J) and the carbonyl group at δ 150.63 (^3J). For proton H-32 and H-33 (δ 7.58), the HSQC value of ^2J was correlated to carbon at δ 129.04 while the HMBC ^3J value was correlated to carbon at δ 132.07. The carbonyl group of ester (O=C-O) C-37 showed direct coupling to a carbon at δ 79.27 (^2J) while long range HMBC was correlated to carbons at δ 39.09 (^3J) and δ 55.58 (^3J).

Conclusion

Esterification of hydroxyl group at C-3 position of betulinic acid was carried out successfully by stirring betulinic acid with 3-methyl phthalic anhydride in the presence of Novozyme 435 as catalyst, in chloroform. The expected product, 3β-(3-methylphthalyl)-lup-20(29)-ene-28-oic acid was isolated in 40 % yield, as white crystals. Cytotoxicity of 3β-(3-methylphthalyl)-lup-20(29)-ene-28-oic acid was shown to be higher and better than parent compound, betulinic acid.

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