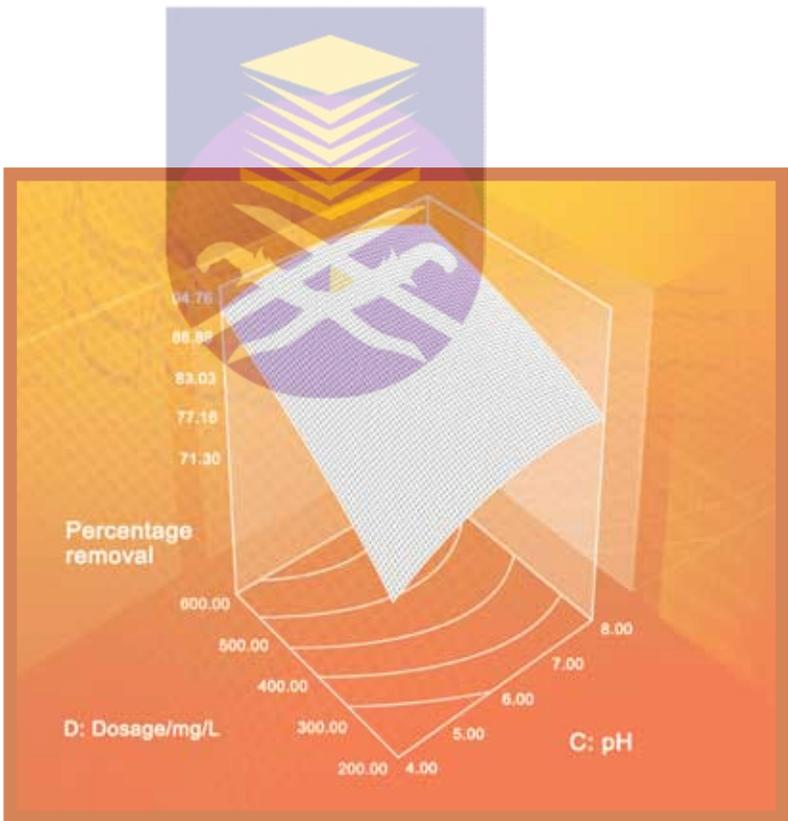


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Lipase Catalyzed Betulinic Acid Derivatives and its Cytotoxic Activity

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ABSTRACT

Betulinic acid, a triterpenoid found in many plant species, has attracted attention due to its important pharmacological properties, such as anti-cancer and anti-HIV activities. In order to obtain derivatives potentially useful for detailed pharmacological studies, derivatives were synthesized through the reaction of betulinic acid with benzoyl chloride and acetic anhydride using a lipase catalyst. Enzyme-catalyzed reaction of betulinic acid with benzoyl chloride yielded 3 β -benzoyl-lup-20(29)-ene-28-oic acid ester (BCL), whereas the acetic anhydride reaction yielded 3 β -acetoxy-lup-20(29)-ene-28-oic acid ester (BAA). The BAA underwent further reaction with 1-decanol to produce 3 β -acetoxy-lup-20(29)-ene-28 decanoate (BAAD). The prepared betulinic acid derivatives were tested for cytotoxic activity on three cancer cell lines in vitro: all tested compounds showed greater activity than betulinic acid.

Keywords: *Betulinic acid, betulinic acid derivatives, enzymatic synthesis, cytotoxic activity*

Introduction

Betulinic acid (Figure 1) is a naturally sourced pentacyclic triterpene isolated from various plants, which are prevalent throughout the tropics. Betulinic acid is a valuable compound, because it exhibits important

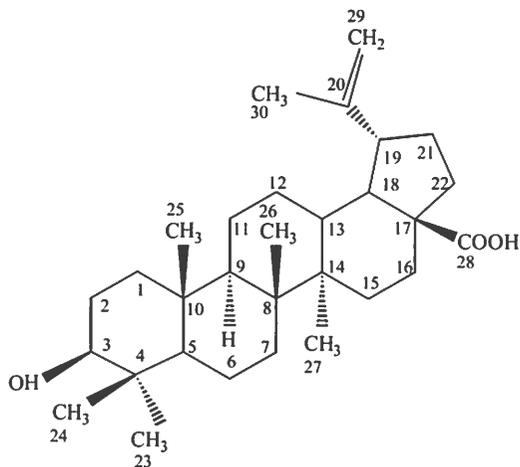


Figure 1: Structure of Betulinic Acid

biological activity. Betulinic acid has been reported to have anti-melanoma, anti-neuroblastoma, anti-leukemia, anti-HIV and anti-malaria properties [1-3]. However, betulinic acid is sparingly soluble in water and various organic solvents, which poses absorption problems by the body. Betulinic acid derivatives however may have improved solubility and consequently enhance its property for specific uses. The inherent properties exhibited by betulinic acid have attracted attention regarding the preparation of its derivatives and structural–activity relationships, for which the target positions are primarily the hydroxyl moiety at C-3 and the carboxyl group [3, 4].

There are three positions (C3, C20 and C28) on the betulinic acid molecule where modifications can be made to yield derivatives. Chemical synthesis of betulinic acid derivatives, for example esterification in the presence of an acid or alkaline catalyst, usually results in a complex mixture. However, the use of chemical catalysts leads to several problems including the generation of corrosive acids, which are not reusable; neutralization of the resulting reaction mass generates large quantities of dilute dissolved salts, and reduced conversion yields and selectivity. Using enzymes in an organic media alleviate the disadvantages of conventional reaction routes and have yielded new methods to produce many valuable products under milder conditions. The specificity of lipase as a biocatalyst to form ester bonds enables better reaction control thus increasing the yield.

Biological transformation of organic compounds is useful during drug development. The derivatives may have improved properties compared to the parent compound, or they may be identical to the metabolites of those compounds when administered to mammals [5]. In this case, biological transformation is a viable alternative to chemical synthesis for the preparation of drug metabolites [6]. Within literature there exist reports on the general biotransformation of triterpenes, and of lupanes in particular [7]. The objective of this work is to obtain new betulinic acid derivatives using lipase as a catalyst and evaluate the cytotoxicity of the derivatives with respect to cancer cell lines.

Materials and Methods

Materials

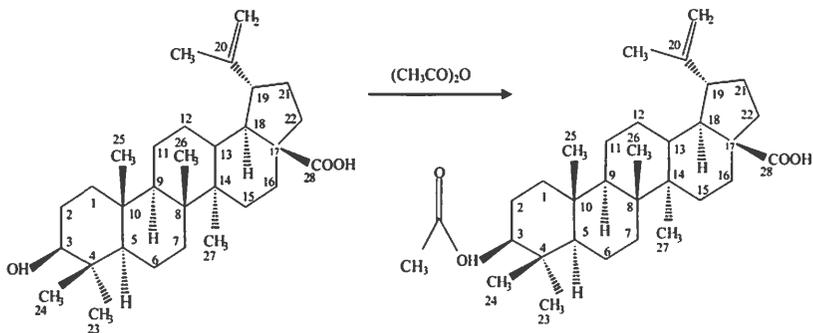
Immobilized Lipases from *Candida Antarctica* (Novozyme) and *Mucor Miehei* (Lipozyme) were purchased from Novo Nordisk Inc. (Danbury, CT). Lipases from Amano species were purchased from Amano International Enzyme Co. (Nagoya, Japan). Benzoyl chloride (purity > 95 %) was obtained from Sigma (St Louis, MO, USA). All solvents were supplied by JT Baker (Phillipsburg, NJ, USA). Betulinic acid was extracted from Malaysian *Callistemon speciosus* using the method reported by Ahmad *et al.* [8]. All other chemicals used were of analytical grade.

Screening of Enzymes

The specific activities of the selected five commercial immobilized lipases, (Novozyme, Lipozyme, Amano 20, Amano LV and Amano LMU) for the esterification reaction were determined. The specific activity of the enzymes is expressed as mmole/min/g protein according to the assay method described by Hazra *et al.* [9]. The enzyme with the highest catalytic efficiency and specificity was chosen for the optimization study.

Enzymatic Synthesis of 3 β -acetoxy-lup-20(29)-ene-28-oic Acid Ester (BAA)

BAA was prepared by reacting betulinic acid (30 mg) with acetate anhydride (5 ml) and Novozyme (50 mg). The mixture was incubated at 37 °C and 150 rpm for 24 hours with continuous agitation. The brownish

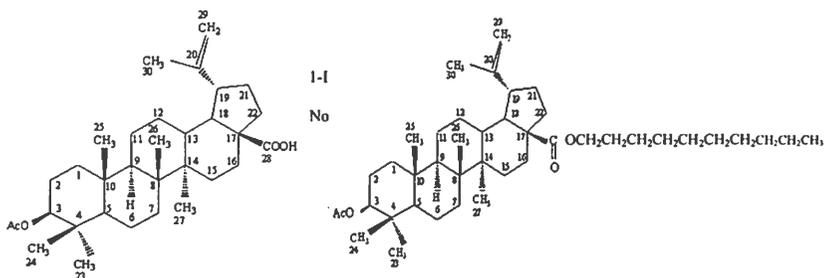


Scheme 1: Catalyzed Reaction of Betulinic Acid and Acetate Anhydride Using Novozyme

crystals obtained were filtered using a Buchner funnel and dried in a vacuum desiccator until they attained a constant weight. The product was then used in the synthesis of 3 β -acetoxy-lup-20(29)-ene-28 decanoate (BAAD).

Enzymatic Synthesis of 3 β -acetoxy-lup-20(29)-ene-28 Decanoate (BAAD)

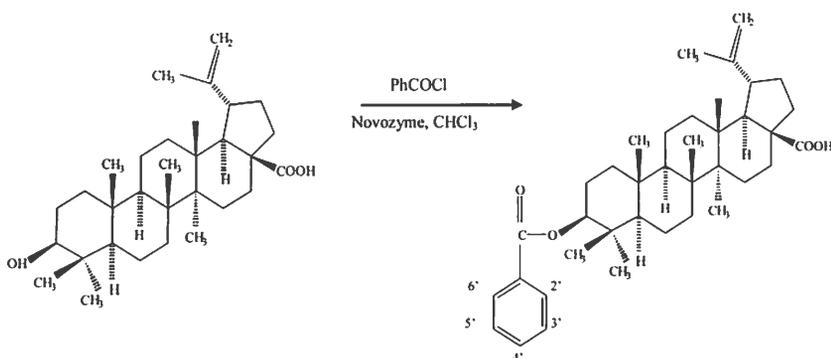
Enzymatic esterification of BAAD was performed by mixing the desired amounts of BAA (20 mg, 4.02×10^{-4} mmole), alcohols (4.82×10^{-4} mmole) and Novozyme (50 mg) in chloroform. The reaction mixture was incubated for 24 hours at 150 rpm and 37 °C. 1-decanol was subsequently chosen for further study in the synthesis of BAAD.



Scheme 2: Catalyzed Reaction of BAA with 1-decanol Using Novozyme as Catalyst

Enzymatic Synthesis of β -benzoyl-lup-20(29)-ene-28-oic Acid Ester (BCL)

The reaction mixture consists of betulinic acid (0.10 g), benzoyl chloride (0.12 g), chloroform (15 ml) and Novozyme lipase (0.5 g). The mixture was incubated for 24 hours with continuous agitation at 150 rpm. The product was separated and detected using thin layer chromatography using a mobile phase of chloroform/ethyl acetate (9.5/0.5) v/v. The mixture was chromatographed using a silica gel column (3 cm, i.d. and 30 cm length) using chloroform/ethyl acetate (9.5/0.5) v/v as the eluting system.



Scheme 3: Catalyzed Reaction of Betulinic Acid and Benzoyl Chloride Using Novozyme

Analysis of the Product

The products of the enzymatic esterification were separated and analyzed using thin layer chromatography (TLC) on a pre-coated silica gel plate (60 F₂₅₄, Merck, Darmstadt, Germany) and developed using a mobile phase of chloroform/ethyl acetate (9.5:0.5) v/v. Qualitative analysis was performed using a gas chromatograph (G-3000, Hitachi Japan), which was equipped with a polar capillary column, DB-17HT. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The injector and detector temperature were set at 300 °C. Quantification of each sample was achieved by comparison with internal response factor standards using heptadecane (internal standard). The conversion factor was determined using mixtures containing known quantities of betulinic acid ester, substrates and internal standards. Functional group analysis of the sample

was performed using a Perkin-Elmer Fourier Transform Infrared Spectrometer (model 1650) and a mass spectrum of the product was obtained using a Hewlett Packard gas chromatograph mass spectrometer. The structure of the product was determined using a JOEL nuclear magnetic resonance machine, model ECA-400.

Procedures for Cytotoxicity Evaluation

The samples were screened for cytotoxic activity using the Microculture Tetrazolium Salt (MTT) assay reported by Mosmann [10]. The cell line used was obtained from RIKEN cell bank, Tsukuba, Japan. Cells were grown and maintained in a RPMI 1640 (Sigma, USA) medium supplemented with 10 % fetal bovine serum (Flow Lab, Australia) at 37 °C, 5 % CO₂ and 90 % humidity. Screening was performed using 96-flat-bottomed microwell plates (Nunc, Denmark) in which various sample concentrations has been established prior to cell seeding. Control samples contained only untreated cells and were included in each sample set. The assay was performed in triplicate whereby the culture plates were incubated for three days at 37 °C in a 5 % CO₂ humidified incubator. After three days of incubation, the fractions of surviving cells were determined relative to the untreated cell populations using colorimetric MTT assay. 20 µl of MTT solution was added to each well followed by 4 hours of incubation at 37 °C. 150 µl of the resulting medium was removed from each well and the formed formazan crystals were solubilized with 100 µl of dimethyl sulfoxide (DMSO) added to each well. The plate was left at room temperature for 15-30 minutes and absorbance values at 550 nm were measured with a microplate reader (BIO TEK EL 340, USA). Cytotoxicity was expressed as CD₅₀, which is the concentration required to reduce the absorbance of treated cells by 50 % with respect to the control samples (untreated cells).

Results and Discussion

Screening of Enzymes

Of the five commercial lipases tested, Novozyme exhibited the best catalytic efficiency for the enzymatic synthesis of BAAD and BCL and was consequently chosen for the optimization study (Table 1). The Novozyme catalyzed yields of BAAD and BCL were 38 % and 45 %, respectively.

Table 1: Screening of Lipases from Different Sources

Source	Yields (%) (BAA)	Yields (%) (BAAD)	Yields (%) (BCL)
Novozyme	85 %	38 %	45 %
Amano 20	-	-	38 %
Amano LV	-	-	25 %
Amano LMU	-	-	12 %
Lipozyme	73 %	29 %	-

* Specific activity was measured by one unit of enzyme is defined as the amount of enzyme which produced 1 μmol of betulinic acid derivative produced/min/g protein

Analysis of the Product

Synthesis of BAA

The BAA structure was identified with respect to the following spectral data: BAA /infrared/absorption; the product gives adsorption at ν_{max} 3435 cm^{-1} (O-H), ν_{max} 2945 cm^{-1} (CH_2), ν_{max} 1694 cm^{-1} (C=O ester), ν_{max} 1642 cm^{-1} (C=O acid) and ν_{max} 1247 cm^{-1} (C-O). The $^1\text{H-NMR}$ for betulinic acid ester acetate resonates at δ 0.79 (s, 3H), δ 0.84 (s, 3H), 0.95 (s, 3H), δ 0.99 (s, 3H), δ 1.46 (s, 3H), δ 1.71 (s, 3H) for six methyl groups, δ 2.06 (s, 3H for methyl acetate), δ 1.99 (m, 2H) and δ 2.27 (m, 2H), δ 3.03 (t, 1H at C-19), δ 4.48 (t, 1H at C3), δ 4.76 (s, 1H, C-29, α/β) and δ 4.48 (t, 1H, C-29 β/α). The $^{13}\text{C-NMR}$ of betulinic acid ester acetate has the following signals: δ 38.8 (C-1), 18.0 (C-2), 81.4 (C-3), 38.2 (C-4), 55.8 (C-5), 21.2 (C-6), 34.6 (C-7), 41.1 (C-8), 50.8 (C-9), 37.5 (C-10), 21.7 (C-11), 25.8 (C-12), 38.7 (C-13), 42.8 (C-14), 30.0 (C-15), 32.5 (C-16), 56.8 (C-17), 49.7 (C-18), 47.3 (C-19), 150.8 (C-20), 30.9 (C-21), 37.4 (C-22), 28.3 (C-23), 16.8 (C-24), 16.4 (C-25), 16.5 (C-26), 15.1 (C-27), 182.6 (C-28), 109.7 (C-29), 19.7 (30), 24.0 (CH_3 for acetate) and 171.5 (C=O for acetate). Similar results reported by Ahmad *et al.* [8]. Table 2 summarizes the $^{13}\text{C-NMR}$ data for BAA.

Synthesis of BAAD

The BAAD structure was identified according to the following spectral data: BAAD /infrared/absorption; CH_2 (2927 cm^{-1}), C=O ester (1744 cm^{-1}), C-O (1241 cm^{-1}) and C=C (1042 cm^{-1}). The infrared absorption data showed no absorption for the OH and COOH functional groups,

which indicates the attachment of substrates at the C3 and C28 positions. The $^1\text{H-NMR}$ signals for BAAD resonate at δ 0.843 (s, 3H), δ 0.86 (s, 3H), δ 0.90 (t, 3H), δ 0.95 (s, 3H), δ 0.99 (s, 3H), δ 1.71 (s, 3H) and δ 2.06 (s, 3H). The signal at δ 0.90 appears as a triplet due to the CH_3 group present in residual 1-decanol. The triplet signal at δ 3.03 is due to the proton at C-19 and d 4.48 is due to the proton at the C-3 position. Signals at δ 4.79 and δ 4.62 were assigned for the two protons at the C-29 position, β/α , attached to a double bond carbon-carbon. A broad signal at δ 1.28 was assigned to a CH_2 of the 1-decanol chain and a triplet signal at δ 4.07 was assigned to the OCH_2 ester group at the C-28 position. The BAAD $^{13}\text{C-NMR}$ data is similar to that for BAA with an additional ester peak at δ 171.30 ppm and others for CH_2 and CH_3 signals due to residual 1-decanol. The BAAD $^{13}\text{C-NMR}$ data is summarized in Table 2 along with that for BAA. The BAAD mass spectrum shows a molecular mass ion at m/z 638, which corresponds to the parent molecule ion mass. The fragments at m/z 248 and 189 correspond to of the two primary components of betulinic acid ester.

Synthesis of BCL

The BCL structure was proven with respect to its NMR spectra. Typically, the proton magnetic resonance of the product indicates the presence of six methyl groups resonating as singlets at δ 0.92, 0.95, 0.99, 1.02, 1.08 and 1.73. The presence of two hydrogens at the C-29 position is observed by resonance at δ 4.78 (s, 1H, C-29 β/α) and 4.65 (s, 1H, C-29 α/β). A doublet of triplets structure at δ 3.05 is assigned to the hydrogen at C-19. The signals due to the five aromatic protons observed at δ 7.46, 7.58 and 8.06 respectively, which confirms the presence of the benzene ring in the desired product. The ^{13}C spectrum of the product exhibits a downfield shift for C-3 at δ 81.76, as summarized in Table 2, which is due to increased deshielding caused by close proximity to the ester group. The infrared spectrum for the purified product absorbs at ν_{max} 1788 cm^{-1} (C=O stretching for ester carbonyl) and ν_{max} 1245 cm^{-1} (O=C-O stretching). The mass spectrum of the desired product has a molecular mass ion at m/z 560, which corresponds to the BCL structure, which has lost the PhCO_2 unit to give the betulinic acid. The remaining fragments follow the normal fragmentation pattern of the betulinic acid molecule.

Cytotoxicity Evaluation

The cytotoxic activity of betulinic acid and its derivatives *in vitro* was determined by performing an MTT cytotoxicity assay on cultured leukemia cell lines (HL60, PN6 and WEHI3) and cultured brain cancer cells (MCF-7), the results for which are summarized in Table 3. The cytotoxicity evaluation of these compounds with respect to cancer cells indicates that 3 β -benzoyl-lup-20(29)-ene-28-oic acid ester (BCL) is the most active compound compared to betulinic acid (BA), 3 β -acetoxy-lup-20(29)-ene-28-oic acid ester (BAA) and 3 β -acetoxy-lup-20(29)-ene-28 decanoate (BAAD). These results suggest that the cytotoxicity profile of betulinic acid derivatives may be sensitive to the size of the substituents at the C-3 position in addition to electrostatic sensitivities.

Table 2: The ^{13}C -NMR Data of BCL, BAA and BAAD

C /H	δ (BCL)	δ (BAA)	δ (BAAD)
C-1	38.56	38.80	38.6
C-2	28.29	18.00	18.4
C-3	81.76	81.40	81.2
C-4	38.37	38.2	37.4
C-5	55.74	55.8	55.7
C-6	18.36	21.2	21.2
C-7	34.50	34.6	34.7
C-8	40.88	41.1	40.9
C-9	50.57	50.8	50.9
C-10	38.10	37.5	37.3
C-11	21.04	21.7	21.2
C-12	25.62	25.8	25.7
C-13	37.33	38.7	38.0
C-14	42.61	42.8	42.7
C-15	30.68	30.0	29.5
C-16	32.31	32.5	32.1
C-17	56.63	56.8	56.1
C-18	49.39	47.7	47.7
C-19	47.20	49.4	49.4
C-20	150.6	150.8	150.2
C-21	31.50	30.9	30.2
C-22	23.92	37.4	34.5
C-23	38.66	28.3	28.1
C-24	17.03	16.8	16.7
C-25	16.31	16.4	16.3
C-26	28.38	16.5	16.4

(continued)

Table 2 (continued)

C /H	δ (BCL)	δ (BAA)	δ (BAAD)
C-27	29.96	15.1	14.9
C-28	181.2	182.6	171.3
C-29	109.5	109.7	109.9
C-30	19.30	19.7	19.6
CH ₃ for acetate		24.0	23.9
C=O for acetate		171.5	171.5
C=O esters	166.5		171.3
C-1'	131.2		
C-2'/6'	129.70		
C-3'/5'	128.50		
C-4'	132.91		
1'			14.3
2'			21.5
3'			22.9
4'			23.9
5'			26.1
6'			28.8
7'			29.4
8'			29.3
9'			29.7
10'			29.9

Table 3: Cytotoxicity Assay of Betulinic Acid (BA) and its Derivatives Against Various Cancer Cell

Compound	HL60 (IC ₅₀)	MCF7 (IC ₅₀)	PN6 (IC ₅₀)	WEHI3 (IC ₅₀)
BA	7.5	16.5	7.8	3.2
BCL	2.4	2.3	4.3	1.8
BAA	4.2	>50	>50	>50
BAAD	2.6	>50	>50	>50

IC₅₀ (μg/ml)

BA = Betulinic acid

BAA = 3β-acetoxy-lup-20(29)-ene-28-oic acid ester

BAAD = synthesis of 3β-acetoxy-lup-20(29)-ene-28 decanoate

BCL = 3β-benzoil-lup-20(29)-ene-28-oic acid ester

As shown in Table 3, all activities using BCL have IC₅₀ less than 50, which indicates significant cytotoxicity activity. Introducing an acetate group at position C-3 results in a loss in cytotoxicity with respect to cancer cells. However, further transformation of BAA to produce BAAD results

in strong cytotoxic activity against the HL60 cell line. These results suggest that the cytotoxicity profile of betulinic acid derivatives may be sensitive to the size of the substituents. Young *et al.* [10] reported a similar cytotoxicity profile trend with respect to the activity of betulinic acid derivatives modified at the C-3 position. It was suggested that the presence of a carboxylic acid group at the C-28 position directly effects cytotoxic activity. Kim *et al.* [11] suggested that the cytotoxicity profile of betulinic acid derivatives may be sensitive to the size of the substituents, which is corroborated by Darrick *et al.* [12] who reported that replacing the C-3 hydroxyl group with an acetyl group decreases cytotoxic activity. The study presented demonstrates that simple modification of the parent structure of betulinic acid produces a number of potentially important derivatives, which have improved selective toxicity profiles or introduce general toxic effects.

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