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ENZYMATIC SYNTHESIS OF BETULINIC ACID ESTER

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BY

CHEW WON YIN

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Abstract of the thesis presented to the Senate of the Universiti Putra Malaysia in the fulfilment of the requirement for the degree of Master of Science

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May 2001

Chairman: Associate Professor Dr. Mahiran Basri

Faculty: Science and Environmental Studies

Enzymatic synthesis of betulinic acid ester (3-β-hydroxyl-oley-lup-20(29)-en-28-oic acid) from betulinic acid and oleic acid in chloroform were investigated. Five commercial lipases (*Candida rugosa*, *Aspergillus niger*, *Penicillium roquerti*, Novozyme 435 and Lipozyme) were tested for their suitability for the reaction. Among the lipase tested, Novozyme 435 and Lipozyme were chosen for optimization studies because of their higher specific activity. The effect of various reaction parameters such as time course, temperature, organic solvent, amount of enzyme, mole ratio of substrates, initial water activity (a_w) and continuous water activity (a_w) were studied to determine optimal condition of betulinic acid ester.

The optimal condition for betulinic acid ester synthesis using Novozyme 435 were obtained at incubation period of 13 h; temperature, 40^0C; mole ratio of substrates, 6.0; amount of lipase, 120 mg; organic solvent, chloroform, initial water activity (a_w), 0.12 and continuous water activity (a_w), 0.59. Optimal condition using Lipozyme were obtained at incubation period of 13 h; temperature, 50^0C; mole ratio of substrates, 5.0; amount of lipase, 80 mg; organic solvent, chloroform; initial water activity (a_w), 0.75 and continuous water activity (a_w), 0.59. The maximum conversion for Novozyme 435 and Lipozyme at optimal condition were 95.15% and 64.55% respectively without removal of water in the reaction medium. This result clearly demonstrated that Novozyme 435 was well suited for the preparation of betulinic acid ester in organic media (chloroform).

For scale up reaction, betulinic acid ester was easily isolated and purified using column chromatography with solvent system; anhydrous ether: hexane (20:8.0, v/v). The percentage conversion obtained was 75.68% when the reaction was scale up to thirteen folds. This study indicated that enzymatic reaction might be easily scaled up while maintaining the process selectivity as well as produced high yield of betulinic acid ester.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Master Sains

SINTESIS ESTER ASID BETULINIK DENGAN MENGGUNAKAN ENZIM

Oleh

CHEW WON YIN

Mei 2001

Pengerusi: Profesor Madya Dr. Mahiran Basri

Fakulti: Sains dan Pengajian Alam Sekitar

Ester asid betulinik (asid 3-β-hidroksi-olei-lup-20(29)-en-28-oik) boleh disintesis terus daripada asid betulink dan asid oleik dalam kloroform. Lima jenis lipase (*Candida rugosa*, *Aspergillus niger*, *Penicilium roquerti*, Novozyme 435 dan Lipozyme) telah diuji untuk memenuhi kesesuaian dalam tindak balas. Di antara lipase yang diuji, Novozyme 435 dan Lipozyme dipilih untuk kajian optimum disebabkan oleh aktivitinya yang tinggi. Kesan-kesan pelbagai parameter tindak balas seperti masa tindak balas, suhu, pelarut organik, kuantiti lipase, pecahan mol reaktan (mmol asid betulinik/mol asid oleik), aktiviti air awal (a_w) dan aktiviti

dikaji untuk menentukan keadaan tindak balas maksimum bagi sintesis tersebut.

Sintesis ini diperoleh dengan menggunakan Novozyme 435 pada masa tindak balas 13 jam; suhu, 40^0C; pelarut, kloroform; kuantiti lipase, 120 mg; pecahan mol reaktan, 6.0; aktiviti air awal (a_w), 0.12 dan aktiviti air berterusan (a_w), 0.59. Keadaan maksimum diperolehi dengan menggunakan Lipozyme ialah masa tindak balas, 13 jam; suhu 50^0C; pelarut organik, kloroform; kuantiti lipase, 120 mg; pecahan mol reaktan, 6.0; aktiviti air awal (a_w), 0.75 dan aktiviti air berterusan (a_w), 0.59. Penghasilan maksimum untuk Novozyme 435 dan Lipozyme pada keadaan maksimum ialah 95.15% dan 64.55% tanpa menyingkirkan air dari media tindak balas. Keputusan ini dengan jelas menunjukkan Novozyme 435 adalah enzim yang paling sesuai untuk penyediaan ester asid betulinik dalam pelarut organik.

Bagi tindak balas skala besar, ester asid betulinik boleh diasing dan ditulenkan dengan menggunakan kromatografi turus. Pelarut organik yang digunakan ialah campuran dietil eter: heksana (20:80, v/v) dan penghasilan yang diperoleh adalah 75.66% apabila tindak balas dalam skala yang besar sehingga tiga belas kali ganda. Kajian ini menunjukkan tindak balas enzimatik adalah mudah, berskala serta boleh menghasilkan peratusan tinggi.

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I declare that this thesis has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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(CHEW WON YIN)

Date:

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LIST OF ABBREVIATIONS

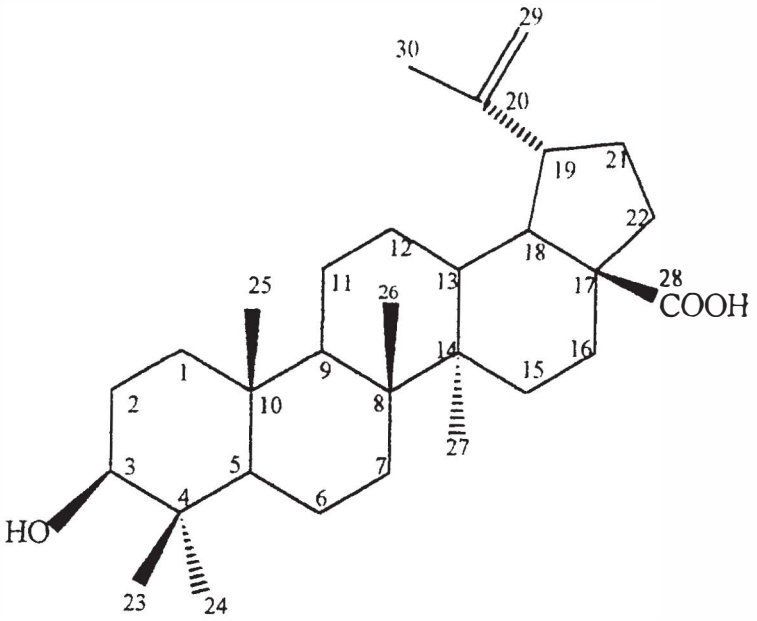
Log P	logarithm of the partition coefficient
a_w	water activity
TLC	thin layer chromatography
FT-IR	Fourier transform infra red
GC	gas chromatography
NMR	nuclear magnetic
TNBS	trinitrobenzene sulfonate
BSA	bovine serum albumin
PPTS	Pyridium *p*-toluene sulfonic acid salt
THP	Tetrahydropyridin
THF	Tetrahydrofuran
DHP	Dihydropyran
TAG	Triacylglycerol

CHAPTER I

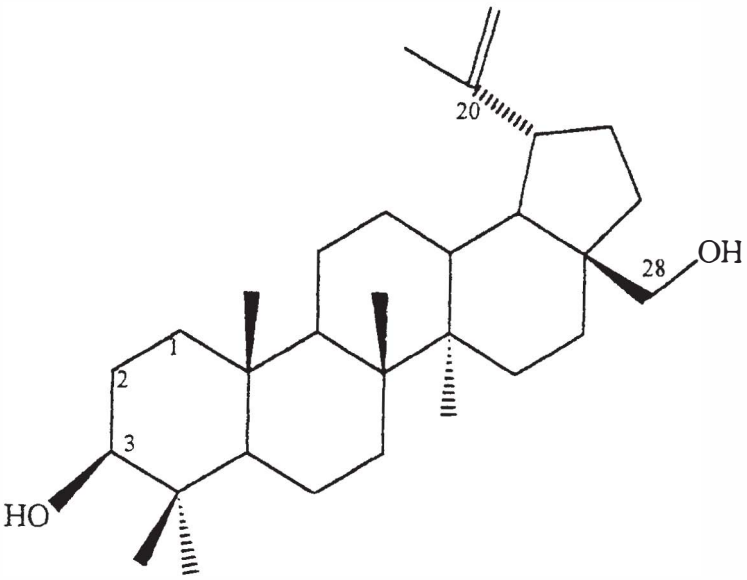
INTRODUCTION

The lupane type pentacyclic triterpene betulinic acid (1), 3-β-hydroxy-lup-20(29)-ene-28-oic, is widely distributed in nature. Considerable amounts of betulinic acid (up to 2.5%) are available in the outer bark of a variety of tree species that are valuable for timber purposes (O'Connell *et al.*, 1988). A closely related compound, betulin (2), lup-20(29)-ene-3β,28-diol, is a major constituent of white barked birch tree with yield up to about 25%. Betulin can be easily converted chemically to betulinic acid in a high yield (Kim *et al.*, 1997). Betulinic acid has been shown to exhibit a variety of biological activities, including inhibition of human immunodeficiency virus (HIV) replication in lymphocyte cells, blockage of HIV-1 entry into cells and cytotoxicity against a variety of cultured human tumor cells (Bringmann *et al.*, 1997). In addition, betulinic acid was identified as a melanoma specific cytotoxic agent in both *in vitro* cell cultures and *in vivo* studies (Pisha *et al.*, 1995).

Betulinic acid derivatives can be used more efficiently in a topically applied composition to selectively treat or prevent or inhibit a melanoma (Pezzuto *et al.*, 1999). It could be absorbed more efficiently as compared to their acid counterpart and thus are more desirable. Derivatives of betulinic acid also have been investigated as specific inhibitors of HIV-1 and as potential of anti-HIV drug candidates (Kashiwada *et al.*, 1998). Furthermore, the lower water activity of the betulinic acid can be overcome by providing an appropriate derivative of betulinic acid. Modifying the parent structure of


29
30
20
19
21
22
12
18
25
11
26
13
17
28
COOH
1
9
14
16
15
2
10
8
27
3
5
7
4
6
HO
23
24

(1)


20
28
OH
1
2
3
HO

(2)

betulinic acid also can further improve antitumor activity against various cancer cells (Pezzuto *et al.*, 1999).

There are several studies which reported on the preparation of betulinic acid derivatives using chemical catalysis. The use of solids acid, clay minerals or inorganic catalysts was reported (Bringmann *et al.*, 1997, Li *et al.*, 1998). However, the process usually carried out at higher temperature (> 100^0C) and a produced some impurities which may caused coloration or toxicity to the product. The chemical reaction is also tedious and nonselective. Moreover, the products obtained need further purification either by alkaline washing, stream refining, ultrafiltration or activated carbon treatment. The isolation of the end product may not be economical.

The increasing emphasis on the use of biocatalysts for their favourable properties may offer an improvement over these conventional methods. Enzymatic reaction is carried out at ambient pressure and temperature (40-60^0C). The overall cost is also brought down by the fact that the reaction need not be highly corrosive-resistant (minerals acids used as catalyst in conventional procedure are very corrosive) (Grandhi *et al.*, 1997). The products of such bioprocesses are usually pure (Anonymous, 1981). Furthermore, the lower temperature reaction employed ensured minimal thermal degradation (Linfield *et al.*, 1984, Kosugi *et al.*, 1988).

In this study, an alternative synthesis of betulinic acid derivatives using lipases has been investigated. The modification at C-3 position was chosen to yield more potent

In this study, an alternative synthesis of betulinic acid derivatives using lipases has been investigated. The modification at C-3 position was chosen to yield more potent of betulinic acid derivatives. To date, there are no published reports on the enzymatic esterification of betulinic acid at the modification of C-3 position. Therefore, the objectives of this study are to synthesize betulinic acid ester from betulinic acid and oleic acid using lipase and to optimize the reaction condition with respect to the effect of different reaction time, temperature, mole ratio, amount of enzyme, organic solvents, initial water activity (a_w) and continuous water activity (a_w). The product was charaterized using Fourier Transform-Infrared Spectroscopy (FT-IR), stet chromatography (TLC), gas chromatography (GC) and Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR).

CHAPTER II

LITERATURE REVIEW

Natural Products as Drugs

The search for new pharmacologically active agents obtained by screening natural sources such as microbial fermentation and plants extracts has led to the discovery of many clinically useful drugs that play a major role in the treatment of human diseases (Shu, 1999). Analysis of the number and source of anticancer and antiinfective agent, reported mainly in *Annual Reports of Medicinal Chemistry* from 1984 to 1995, indicates that over 60% of the approved drugs (for the period 1989-1995) developed in these disease areas are of natural origin. Of the world's 25 best selling pharmaceutical agents, 12 are natural product origin (O'Neill *et al.*, 1993).

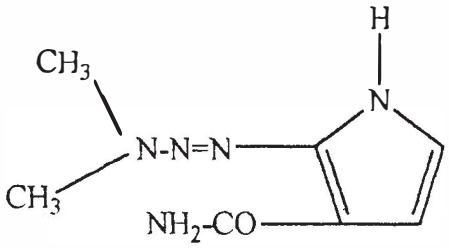
The use of traditional medicine is widespread and plants still present a large source of structurally novel compounds that might serve as leads for the development of novel drugs (Heras *et al.*, 1998). The role played by plants in the provision of novel agents have potential in the treatment and prevention of many diseases such as cancer, acquired immunodeficiency syndrome (AIDS) and related infections, and malaria has been reviewed in an American Chemical Society Series volume (Baker *et al.*, 1995). Cragg *et al.*, (1997) also reported that at least 119 compounds derived from the 90 plant species can be considered as important drugs and currently was used in one or more countries. Further evidence of the importance of natural products is provided by the fact

that close to half of the best selling pharmaceuticals in 1991 were either plant drugs or their derivatives (O'Neill *et al.*, 1993).

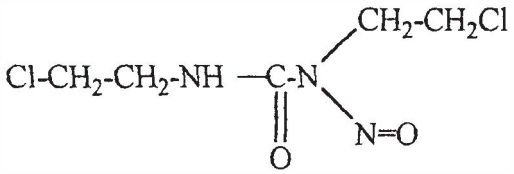
Background for the Invention of Betulinic Acid as Plant-Derived Anticancer Agents

The incidence of melanoma has been increasing at a rate higher than that of any type of cancer for the past four decades. It is now anticipated that as many as 1 in 90 Caucasian Americans will develop maglinant melanoma in their lifetime (Ries *et al.*, 1990, Brozena *et al.*, 1993). Although an increasing proportion of melanomas is diagnosed at a stage that is early enough to be responsive to surgical treatment (10 year survival rate greater than 90%), it has been estimated that over 7,000 patients will die with metastatic melanoma in the United States in 1995.

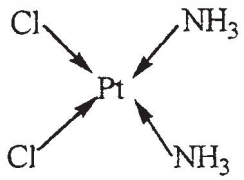
Patients with metastatic melanoma which is not amenable to surgical extirpation, treatment options are limited. Since DTIC (5-(3,3-dimethyl-1-triazenyl)-1-*H*-imidazole-4-carboxamide) (3), also known as dacarbazine is the most efficacious single chemotherapeutic agent for melanoma having an overall response rate of 24%. But, the duration of response to DTIC is generally quite poor (Comis et al., 1976). Combination therapy with other synthetic and recombinant agents, including BCNU (*N*, *N*'-bis(2-chloroethyl)-*N*-nitrosourea (4), also known as carmustine, cisplatin (5), tamoxifen (6), interferon-α (INF-α) and interleukin-2 (IL-2), has a higher response rate (example: 30-50%) in some trials, but a durable complete response rate is uncommon and toxicity is increased (Mc Clay *et al.*, 1994).


H
CH3
N
N-N=N
CH3
NH2-CO

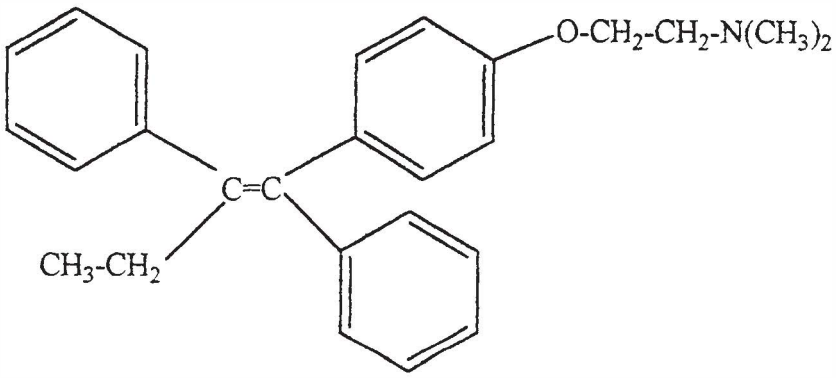
(3)


CH2-CH2Cl
Cl-CH2-CH2-NH
C-N
N=O
O

(4)


Cl
NH3
Pt
Cl
NH3

(5)


O-CH2-CH2-N(CH3)2
C=C
CH3-CH2

(6)

Various drugs derived from natural products, such as adriamycin (7) (doxorubicin) derivatives, bleomycin (8), etoposide (9), and vincristine (10), and their derivatives have been tested for efficacy against melanoma either as single agents or in combination therapy. However, similar to the synthetic and recombinant compounds, these compounds exhibited low response rates, transient complete responses, and high toxicity (Thompson, *et al.*, 1992).

Under the auspices of National Co-operative Natural Product Drug Discovery supported by the National Cancer Institute in United States, the potential antitumor activity of approximately 2,500 extracts derived from globally collected plants was evaluated. As a result of their bioassay-guided fractionation studies, compounds isolated from the stem bark of *Ziziphus mauritiana* displayed selective cytotoxicity against human melanoma cells. This led to the isolation of a pentacyclic triterpene, betulinic acid (1), as the active principle. (Pisha *et al.*, 1995).

Betulinic Acid

Betulinic acid, 3β-hydroxy-lup-20(29)-ene-28-oic acid, is a natural product isolated from several genus of higher plants (Table 1 as example). The isolated active compound has a molecular formula of $C_{30}H_{48}O_3$, as determined by high-resolution mass spectral analysis (Pezzuto *et al.*, 1999). Betulinic acid may be crystallised from chloroform-methanol as shining needles with a melting point 294-296^0 (Fukunaga *et al.*, 1985). The optical rotation of the compound was measured as + 7.3^0 (c=1.2; pyridine). Betulinic