# Betulinic Acid and Its Derivatives: A Review on their Biological Properties

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**Abstract:** Betulinic acid is a naturally occurring pentacyclic triterpenoid and has been shown to exhibit a variety of biological activities including inhibition of human immunodeficiency virus (HIV), antibacterial, antimalarial, antiinflammatory, anthelmintic and antioxidant properties. This article reports a survey of the literature dealing with betulinic acid related biological properties that has appeared from the 1990's to the beginning of 2003. A broad range of medical and pharmaceutical disciplines are covered, including a brief introduction about discovery, phytochemical aspects, organic synthesis, anti-HIV and cytotoxic mechanisms of action. Various structural modifications carried out and their biological and pharmacokinetic profiles are also incorporated.

**Keywords:** Betulinic acid**,** Anti-HIV derivatives, Anti-HIV mechanism, Anticancer derivatives, Anticancer mechanism; Antiinflammatory, Antimalarial, biotransformation.

## INTRODUCTION

Natural products are the organic molecules which are elaborated by living tissues derived from higher plants, fungi, microbes, marine organisms and animals and exhibit a remarkably wide range of chemical diversity and a multiplicity of biological properties. Thousands of year's natural resources have been in use for combating human ailments. Over the last fifteen years interest in drugs of plant origin has been reviving and growing steadily, and the drug researchers are exploring the potential of natural products for the cures of still unsurmountable diseases like cancer and AIDS.

Betulinic acid (**1**), 3β-hydroxy-lup-20(29)-en-28-oicacid, is a widely distributed pentacyclic lupane-type triterpene in the plant kingdom. 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 [1]. A closely related compound, betulin (**1a**), is a major constituent of white-barked birch trees (*Betula* species) with yields up to 22% (dry weight) [2, 3]. Compound **1a** can be easily converted to **1** in high yields synthetically [4].

White birch bark, *Betula alba* (which contains betulinic acid) has been used by Native Americans as a folk remedy. They used it in tea and other beverages to treat stomach and intestinal problems such as diarrhea and dysentry. In Russia, it has been reportedly used since 1834. In 1994, scientists at the University of North Carolina reported that chemicals found in white birch bark slowed the growth of human immunodeficiency virus (HIV) [5]. The following year, a researcher at the University of Illinois reported that betulinic acid killed melanoma cells in mice [6]. Since then, a number of researchers have conducted laboratory tests on betulinic acid to determine antitumor properties, especially with respect to melanoma cells with some promising results which may warrant future study. Betulinic acid has recently been selected by the National Cancer Institute for addition into the RAID (Rapid Access to Intervention in Development) program.

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## PHYTOCHEMICAL ASPECTS

Betulinic acid is a triterpene of natural origin isolated from various plants. It can be isolated from the methanolic extract of *Quisqualis fructus* [7], the dichloromethane-methanol extract of the twigs of *Coussarea paniculata* [8], the dichloromethane-methanol (1:1 v/v) extract of Argentinean legume *Caesalpinia paraguariensis* [9], the methanolic extract of the leaves of *Vitex negundo* [10], twigs of *Ilex macropoda* [11], the ethanolic extract of the roots of *Anemone raddeana* [12], leaves and wood of *Doliocarpus schottianus* [13], ethanolic extract of *Tovomita krukovii* [14], fruits of *Chaenomeles lagenaria* [15], methanol, hexane and ethyl acetate extracts of stem bark of *Berlinia grandiflora* [16], methanolic extract of the aerial parts of Vietnamese *Orthosiphon stamineus* [17], leaves of *Eucalyptus camaldulensis* [18], stem barks of *Physocarpus intermedium* [19] and *Tetracentron sinense* [20], chloroform extract of barks of *Syncarpa glomulifera* [21], methanolic extract of leaves of *Combretum quadrangulare* [22], methyl ethyl ketone extract of *Tetracera boiviniana* [23], dichloromethane extract of stem bark of Brazilian medicinal plant *Zizyphus joazeiro* [24], ethanolic extract of the root barks of the Tanzanian tree *Uapaca nidida* [25], *Ipomea pescaprae* [26], *Ancistrocladus heyneanus* [27], *Diospyros leucomelas* [28], and from the leaves of *Syzrgium claviforum* [5].

During the isolation of betulinic acid from the above mentioned plant sources, a closely related compound betulin (**1a**) was also isolated from many plants in high yield (up to 22%). Son *et al*. [29] developed a method for obtaining betulinic acid from betulin. Betulin was oxidized with chromium oxide (VI) into betulonic acid (**2**) which was reduced with sodium borohydride to yield a mixture of 3-hydroxy epimers containing 85% of natural beta-epimer.

## ANTI-HIV ACTIVITY OF BETULINIC ACID DERIVATIVES

Betulinic acid has been shown to inhibit HIV-1 replication [30]. Based on its chemical structure, betulinic acid derivatives have been reported as inhibitors of HIV-1 entry [31], HIV-protease [32] or of reverse transcriptase (RT)

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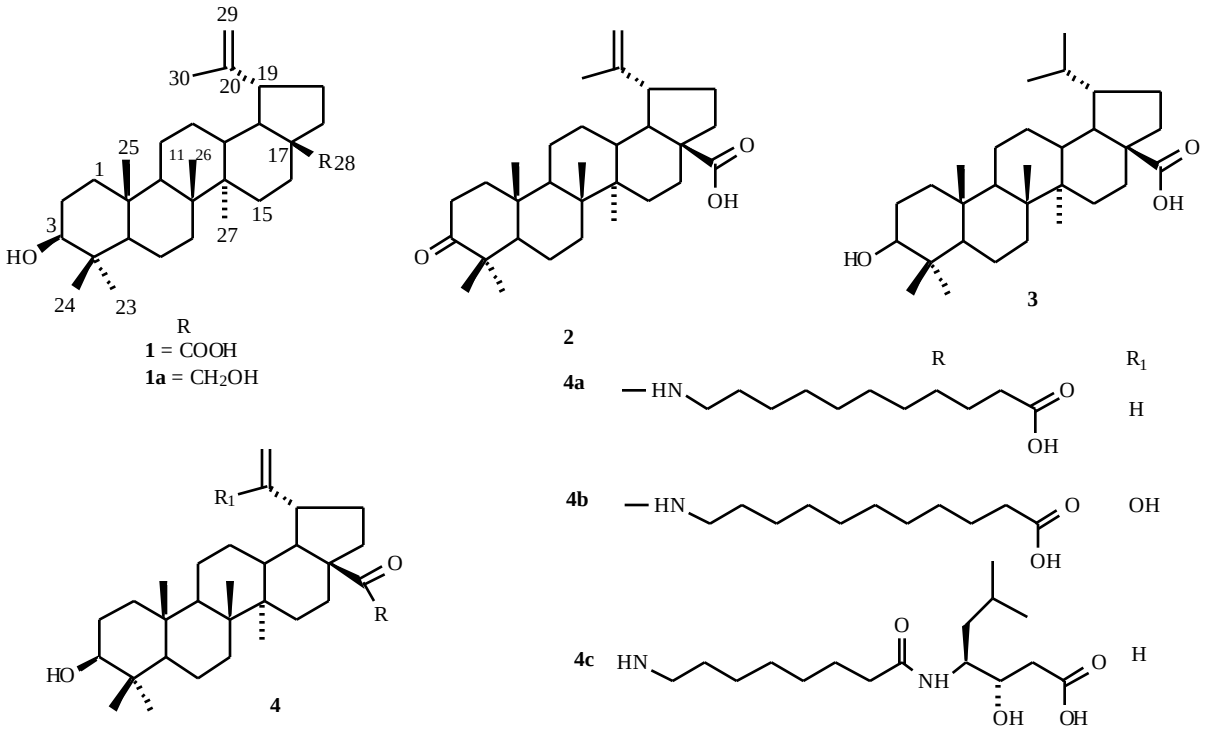



**Fig. (1).** Structures of betulinic acid, betulin, dihydrobetuline acid and amide derivatives of betulink acid.

[33]. Since a number of betulinic acid derivatives have been shown to inhibit HIV-1 at a very early stage of the viral life cycle, these compounds have the potential to become useful additions to current anti-HIV therapy, which relies primarily on combination of RT and protease inhibitors.

## STRUCTURE-ACTIVITY RELATIONSHIP STUDIES

Fujoka *et al*. [5] isolated betulinic acid from the leaves of *Syzrgium claviflorum* and on random screening it was found that betulinic acid exhibited inhibitory activity against HIV-1 replication in H9 lymphocyte cells with an $EC_{50}$ value of 1.4 μM and a selectivity index (SI) value of 9.8. Hydrogenation of betulinic acid yielded dihydrobetulinic acid (**3**) which showed slightly more potent anti-HIV activity with an $EC_{50}$ of 0.9 μM and a SI of 14.

Mayaux *et al*. [31] synthesized certain amide derivatives of betulinic acid (**4a-c**) by a five step procedure starting from betulinic acid, with an overall yield of 31% [44]. Among them, the compoundN'-{N-[36-hydroxyl-20(29)ene-28-oyl]-8-amino octanoyl}-1-statin (**4c**) was found to be the most potent anti-HIV compound particularly against HIV-1 strain IIIB/LAI with a SI of 200. The compounds were not active or were much less active against two isolates of Zairian origin NDK and ELI. However, no significant activity could be found against HIV-2 (ROD and EHO isolates). Compound **4c** was also examined for a possible inhibition of the *in vitro* activity of several purified HIV-1 enzymes. No inhibition of RT, integrase, or protease was detected at a concentration of the derivatives compatible with cellular activity, suggesting that the corresponding steps were not involved in the inhibitory mechanism of these compounds.

Kashiwada *et al*. [34] reported syntheses and anti-HIV activities of some derivatives by modifying the C-3 hydroxyl group in betulinic acid and dihydro betulinic acid. **1** and **3** were treated with 3, 3-dimethylglutaric anhydride and

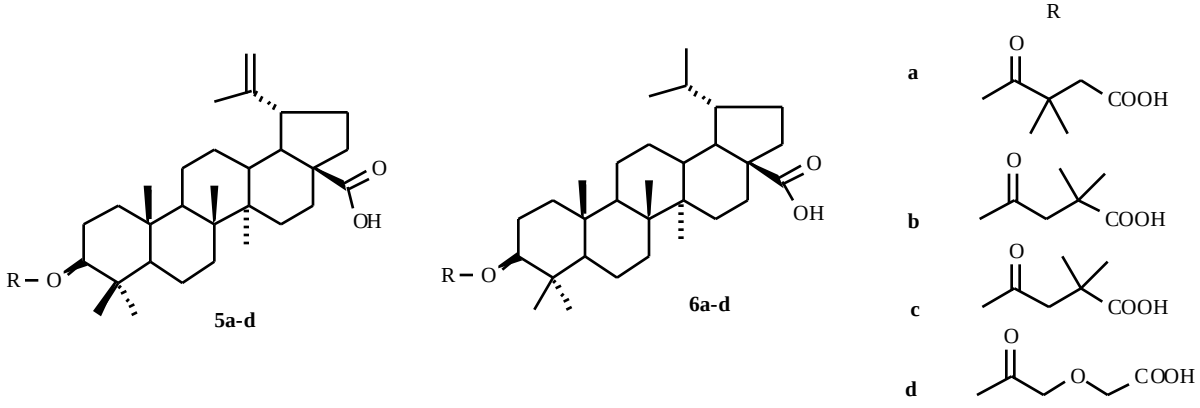


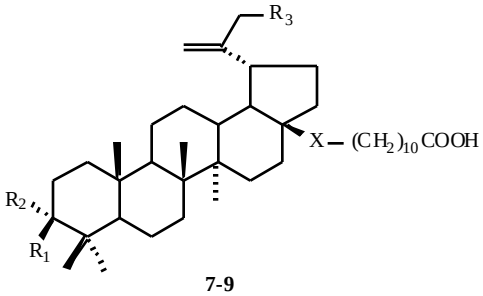

**Fig. (2).** $C_3$ modified derivatives of betullinic acid and dihydrobetulinic acid.

diglycolic anhydride in pyridine in the presence of 4-(dimethylamino) pyridine to furnish the corresponding 3-O-acyl derivatives (**5c**, **5d**, **6c** and **6d**). In contrast, similar treatment of **1** and **3** with dimethylsuccinic anhydride afforded a mixture of 3-O-(2', 2'-dimethylsuccinyl) and 3-O-(3', 3'-dimethylsuccinyl) betulinic acid derivatives (**5a** and **5b**) and dihydro betulinic acid derivatives (**6a** and **6b**), respectively. The anti-HIV assay indicated that compounds **5b** and **6b** were extremely potent in acutely infected H9 lymphocytes with $EC_{50}$ values less than 3.5 x $10^{-4}$ μM and SI values > 20,000 and > 14,000, respectively. In contrast, compounds **5a** and **5c** showed anti-HIV activities with $EC_{50}$ values of 2.7 and 0.56 μM, respectively, and SI values of 5.9 and 13.8, respectively. Compounds **5c**, **5d**, **6c** and **6d** also exhibited anti-HIV activities with $EC_{50}$ values ranging from 0.01 to 2.3 x $10^{-3}$ μM and SI values from 1017 to 2344. None these compounds inhibited the HIV-RT in the concentration range of 167-219 μM. In the HIV-induced membrane fusion inhibition assay compounds **5a-d** and **6a-d** inhibited syncytia formation in the concentration range of 33-70 μM.

Evers *et al*. [35] synthesized a series of ω-undecanoic acid and amides of lup-20(29)-en-28-oic acid derivatives by modifying the $C_{17}$-COOH and $C_{30}$-methyl groups of betulinic acid and evaluated for activity in CEM4 and MT-4 cell cultures against HIV-1 strain IIIB/LAI. Structural variations in ring A of the triterpene highlighted the importance of the 3β-hydroxy substituent. Epimerization of the hydroxyl group at C-3 from 3β (**7a**) to 3α (**7b**) led to a 10-fold drop in activity. The 3-keto derivative (**7c**) was found to show intermediate activity, whereas the 3-deoxy derivative (**7g**) displayed no activity at all. These point to a critical hydrogen bond interaction involving the oxygen at the 3 position, preferentially occurs in the 3β-position. The 3β-methoxy (**7d**) and 3-amino (**7h**) derivatives were found inactive. The inactivity of **7d** might be due to a steric hindrance by the methyl group. The introduction of a second hydroxyl group led to a complete loss of activity. Thus, almost all chemical modifications in ring A led to considerable loss in activity.

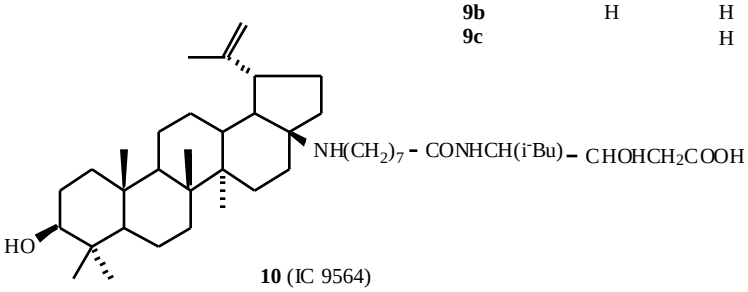
The antiviral properties of 30-(hydroxyethyl)thio- (**8a**), 30-[2-(diethylamino)ethyl]thio- (**8b**), 30-(1-pyrrolidinyl)- (**8e**), and 3, 30-dihydroxy- (**8g**) derivatives remained high, but were not better than that of the unsubstituted derivative **7a**. This illustrated a lack of steric requirements by the HIV-1 molecular target for groups at position $C_{30}$. However, an acidic substitution such as 30-(carboxymethyl) thio (**8c**) was clearly detrimental to potency. A similar drop in activity was observed when a secondary nitrogen was directly attached at position $C_{30}$ (**8d** and **8i**). The combination of a free carboxyl and a secondary amine as well as an unsubstituted amino moiety (**8f** and **8h**) led to a significant drop in activity. In conclusion, the isopropylidene group seems important for optimal activity, probably due to binding to a hydrophobic pocket. A certain lack of steric hindrance accommodates a variety of substituents without improvement of activity. However, favorable interactions were observed for primary and secondary amines as well as for the free carboxylic acid moiety.

Variations at the $C_{28}$ carboxyl position such as N-methylation of the amide moiety in **7f**, replacement of the amide by an ester in **7e,** and replacement of the carbonyl by a methylene as in **9c** led to a complete loss of activity. The importance of the hydrogen donating NH group was highlighted by the fact that the corresponding ester **7e** was completely inactive. This was further corroborated by the




**7-9**

| | $R_2$ | $R_3$ | X |
|---|---|---|---|
| **7a** | H | H | CONH |
| **7b** | OH | H | CONH |
| **7c** | = O | H | CONH |
| **7d** | H | H | CONH |
| **7e** | H | H | COO |
| **7f** | H | H | $CONCH_3$ |
| **7g** | H | H | CONH |
| **7h** | H | H | CONH |
| **8a** | H | $SCH_2CH_2OH$ | CONH |
| **8b** | H | $SCH_2CH_2N(Et)_2$ | CONH |
| **8c** | H | $SCH_2COOH$ | CONH |
| **8d** | H | $NHCH_2CH_2OH$ | CONH |
| **8e** | H | l-pyrrolidinyl | CONH |
| **8f** | H | $NHCH_2COOH$ | CONH |
| **8g** | H | OH | CONH |
| **8h** | H | $NH_2$ | CONH |
| **8i** | H | $NHCOCH_3$ | CONH |
| **9a** | H | H | NHCO |
| **9b** | H | H | NHCONH |
| **9c** | | H | $CH_2NH$ |




**10** (IC 9564)

**Fig. (3).** $C_{17}$ modified betulinic acid derivatives.

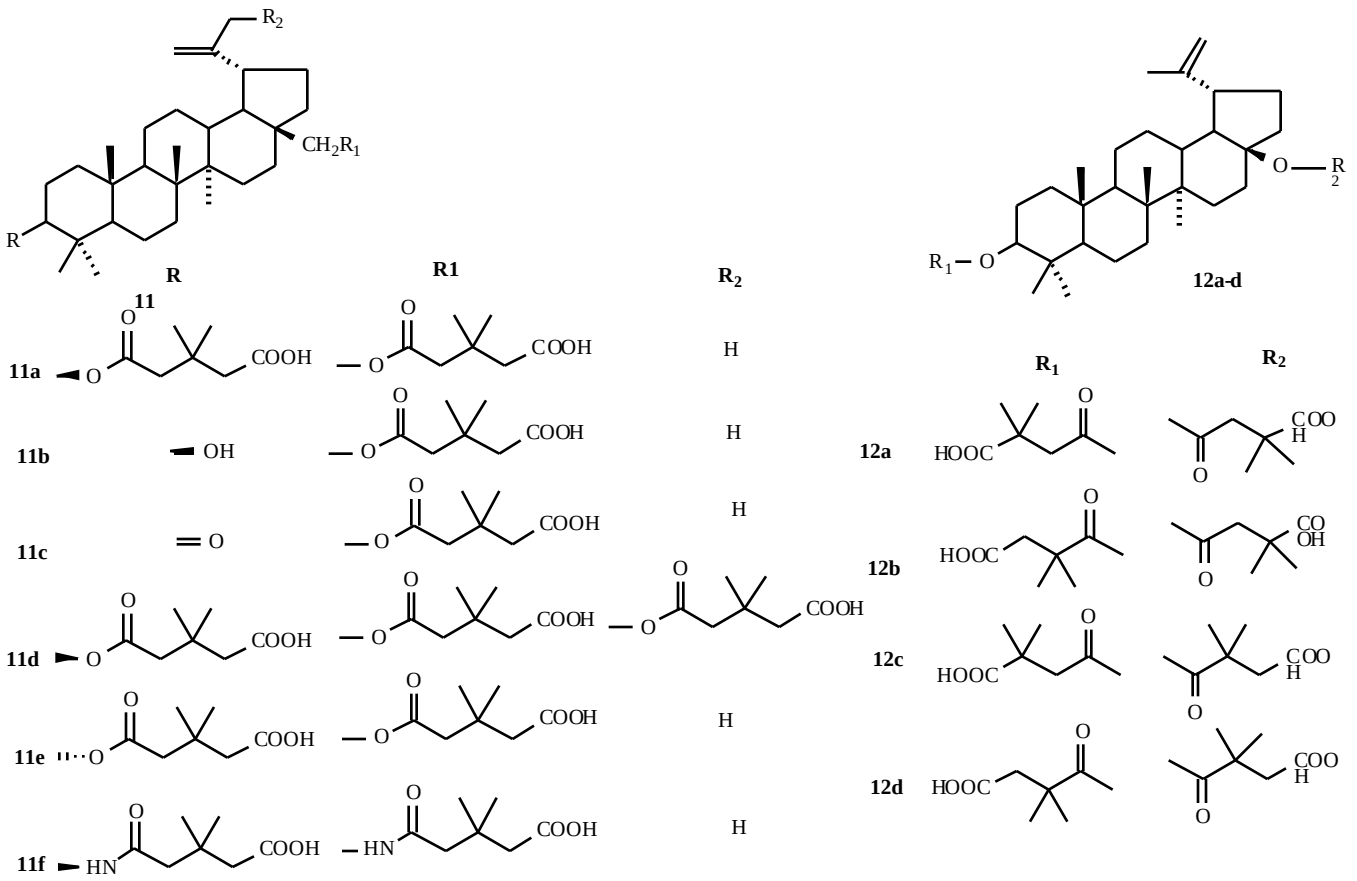



**Fig. (4).** O-acyl derivattives of betulinic acid and dihydrobetulinic acid.

lack of biological activity of the reversed amide **9a** and the urea derivative **9b** in which the NH group occupied a different special position. The dramatic loss of activity for most of the modification on the triterpene skeleton suggests a stringent specificity for the compounds. All the compounds were inactive against HIV-2 ROD. All these compounds were found to interfere with HIV-1 entry in the cells at a post-binding step.

Soler *et al*. [36] synthesized a novel series of ω-aminoalkanoic acid derivatives of betulinic acid and evaluated for their activity against HIV-1. The anti-HIV-1 activities of several members of this new series were found to be in the nanomolar range in CEM-4 and MT-4 cell cultures. Among them, compound **10** was found to display the best overall activity with an $EC_{50}$ of 50 ± 26 nM (CEM4) and 40 ±19 nM (MT-4) with a SI of >100. Nevirapine tested under the same conditions, displayed an $IC_{50}$ value of 84 ± 21 nM (CEM4). No inhibition was observed with **10** against RT, integrase or protease at concentrations well above the $EC_{50}$ concentration in cellular assays.

Sun *et al*. [37] synthesized various O-acyl betulinic and dihydro betulin derivatives. Among them, the most potent compound **11a** with two 3', 3'-dimethylglutaryl groups displayed anti-HIV activity with an $EC_{50}$ value of 0. 66 nM and SI of 21,515. The dihydro betulin derivative of **11a** showed a SI of 2253. Monoacylbetulin (**11b**), containing a substituted glutaryl group only at $C_{28}$ position, had an $EC_{50}$ value of 3.6 μM and a SI of 7.8. Conversion of the 3β-hydroxy group of **11b** to the monoketo derivative led to **11c**, which showed slightly lesser activity ($EC_{50}$ = 29.2 μM; SI = 2.9) than the corresponding nonketone derivative **11b**. The triacylated compound **11d** displayed potent anti-HIV activity with an $EC_{50}$ value of 0.045 μM and a SI of 389. The 3-epi derivative of **11a** (**11e**), which displayed lower inhibition of HIV. Bioisosteric replacement of **11a** to an amide derivative (**11f**) resulted in reduction of anti-HIV activity ($EC_{50}$ = 0.5 μM; SI = 36.6). The above results showed that acylation only at the $C_{28}$ position did not result in significant increase or decrease of activity. However, compounds with acyl side chains at both $C_3$ and $C_{28}$ positions reached optimal activity. An addition of a third chain at $C_{30}$ led to increased potency. Activity was affected by the type of side chain linkage (ester) and the 3β configuration resulted in the most impressive $EC_{50}$ as well as SI values.

Kashiwada *et al*. [38] prepared four isomeric 3, 28-di-O-(dimethylsuccinyl) betulin derivatives and evaluated their anti-HIV potency. Among these derivatives, **12c** demonstrated the highest activity in acutely infected H9 cells with an $EC_{50}$ value of 0.87 nM and inhibited uninfected H9 cell growth with an $IC_{50}$ value of 36.9 μM. Its calculated SI value (42,400) was comparable to that of zidovudine (41,622). Compound **12a** was also extremely potent with an $EC_{50}$ value of 0.02 μM and SI of 1680. Compound **12b** displayed fair activity ($EC_{50}$ = 0.4 βM; SI = 96.5), while **12d** was toxic.

Sun *et al*. [39] synthesized compound **10** analogues and compounds **13a** and **13b** were the most promising compounds against HIV infection with $EC_{50}$ values of 0.33

and 0.46 μM respectively. Both compounds inhibited syncytium formation with $EC_{50}$ value of 0.40 and 0.33 μM, respectively. The structure activity relationship data also indicated that the double bond in **10** can be eliminated and the statin moiety can be replaced with L-leucine while retaining anti-HIV activity.

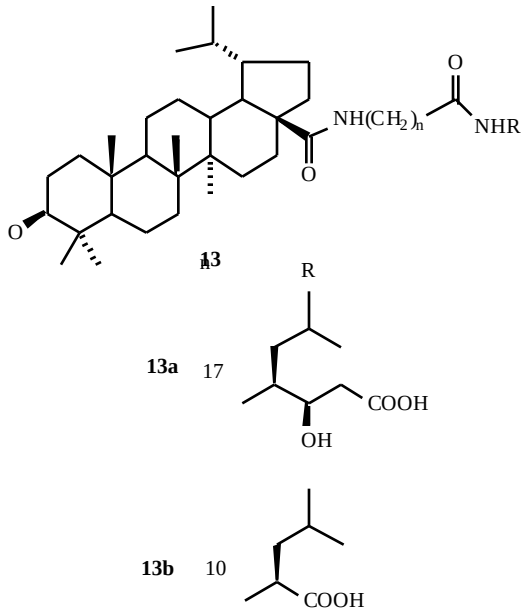



**Fig. (5).** $C_{17}$ derivatives of betulinic acid with potent anti-HIV activity.

## ANTI-HIV MECHANISM OF ACTION

The HIV-1 and HIV-2 envelope glycoproteins (Env) consist of noncovalent complexes of surface (gp120) and transmembrane (gp41) subunits, both derived from a gp160 precursor which is oligomerized and cleaved during its transport to the cell surface [40]. The function of these proteins is to mediate virus entry by allowing binding of virions to the cell surface and fusion of their lipidic envelopes with the cell membrane.

The initial step of virus entry (binding) is mediated by gp120, while gp41 is responsible for the membrane fusion process itself. These events seem to be usually triggered by the interaction of gp120 with two classes of cell surface molecules, CD4 and chemokine receptors, in particular CCR5 or CXCR4, often viewed as HIV co-receptors [41]. *In vivo*, strains using CXCR4 (termed X4 strains) or both CXCR4 and CCR5 (R5X4) are isolated at later stages of infection, while strains using CCR5 (R5) are predominant at the early stages. The X4 strains, in particular, when adapted to replication in T-cell lines, are characterized by a relatively labile gp120-gp41 association, evidenced by the shedding of gp120 spontaneously or upon contact with soluble CD4 or anti-gp120 antibodies [42], while the gp120-gp41 complex of R5 strains seems comparatively stable [43].

Like other retroviral transmembrane proteins, gp41 comprises an N-terminal extracellular domain (ectodomain), a membrane-spanning domain, and C-terminal cytoplasmic domain, apparently dispensable for the fusion process [40]. The main features of the ectodomain are a hydrophobic N-terminal sequence ("fusion peptide"), thought to insert in the target cell membrane, and two domains with a predicted α-helix conformation separated by a region containing a conserved dicystein motif, representing a highly immunogenic determinant [44]. Several residues in the proximal helix and the loop region of gp41 seem to be involved in interaction with gp120 [45]. Peptides corresponding to the proximal (N) and distal α-helix domains of HIV-1 gp41 spontaneously from highly stable coiled-coil structures with an inner core of three parallel N helices on which are stacked three C helices placed in an antiparallel orientation [46]. Structural analysis of the gp41 ectodomain of the HIV-2 related similar immunodeficiency virus revealed the same organization [47]. Whether the formation of this structure is the motive force driving the viral and target membranes into a closer position [46], or whether this structure is already present in the native form of gp41 is not known [47]. Very few compounds targeting gp41 have been described to date. Among them, betulinic acid derivatives were found to block cell-cell fusion and HIV-1 infection at a post binding step [31] by preventing gp41 from attaining its fusion-active conformation [46]. In fact, evidence was obtained from two different experiments that **4c** acts at an early stage in the infection cycle. First, scientists observed a clear dose-dependent inhibitory effect of compounds at micromolar concentration on the synthesis of proviral cytoplasmic DNA, as measured by PCR, only 2h after the onset of infection. Secondly, they studied the influence by adding the compounds at various times soon after the exposure of MT-4 cells to HIV-1. Such an experiment showed that postponing the addition of **4c** for 1h was enough to cancel the inhibitory potency of this compound on the subsequent production of viral antigens. Compound **4c** blocked virus infection at a post binding step necessary for virus membrane fusion and that target of this compound is contained within, or interacts with the HIV-1 envelop gp120/gp41. This was the first report of a nonpeptidic compound having this potential, since until now only monoclonal antibodies or peptides have been shown to selectively affect the HIV-1 membrane fusion step [48, 49]. "Time of addition" experiments suggested that compound **10** interacted with an early step of HIV-1 replication. As syncytium formation but not virus-cell binding, seem to be affected, these derivatives were assumed to interact with the post binding virus-cell fusion process [36].

Kanamoto *et al*. [50] examined the mechanism of anti-HIV action of the novel compound YK-FH312 (**5b**). To determine the step(s) of HIV replication affected by **5b**, a syncytium formation inhibition assay in MOLT-4/HIV-1 IIB and MOLT-4 co-culture [51], a multinuclear activation of galactosidase indicator (MAGI) assay in MAGI-CCR5 cells [52], an electron microscopic observation [53] and a time of addition assay [54] were performed. In neither the syncytium formation inhibition assay nor in the MAGI assay for de nova infection, did the compound show inhibitory effects against HIV replication. Conversely, no virions were detected in HIV-1 infected cell cultures treated with YK-FH312 either by electron microscopic observation or by viral yield in the supernatant. In accordance with a p24 enzyme linked immunosorbant assay of culture supernatant in the time of addition assay, 1/K-FH312 inhibited virus

expression in the supernatant when it was added 18h post infection. However, western blot analysis of the cells in the time of addition assay revealed that the production of viral proteins in the cell was not inhibited completely by YK-FH312. These results suggest that YF-FH312 might affect HIV at the step(s) of virion assembly and/or budding of virions.

Holz-Smith *et al*. [55] studied the role of HIV-1 envelope in the anti-HIV activity of the derivative IC9564 (**10**). Compound **10** inhibited replication of both HIV-1 primary isolates (DH 012, 89.6 and QZ 4734) and laboratory-adapted strains (NL4-3). DH 012 and 89.6 are dualtropic viruses that can use both CCR5 and CXCR4. In the virus infectivity reduction assay, the $IC_{90}$ of **10** for NL4-3 was 0.22 ± 0.05 μM. The $IC_{90}$ for the known HIV-1 RT inhibitor Zidovudine against NL4-3 in the same assay is 0.045 μM. The $IC_{90}$s for DH012, QZ4734, and HIV-1 89.6 are >5, 2.65 and 1.84 μM, respectively. To test the antifusion activity, **10** was tested in a MOLT4/CEM-IIB fusion assay system. The concentration of **10** required to completely inhibit syncytium formation was 0.33 μM. The compound **10** did not significantly affect simian immunodeficiency virus (SIV) or respiratory syncytial virus (RSV) replication at concentrations up to 30 μM. The lack of activity against both SIV and RSV suggests that compound **10** specifically disrupts HIV-1 entry rather than a nonspecific charge-charge interaction or hydrophobic binding. Analysis of a chimeric virus derived from exchanging envelope regions between compound **10**-sensitive and compound **10**-resistant viruses indicated that regions within gp120 and the 25-amino acids at the N-terminus (fusion domain) of gp41 are key determinants for the drug sensitivity. By developing a drug resistant mutant from the NL4-3 virus, two mutations were found within the gp120 region (G237R and R252K) and one was found within the fusion domain of gp41 region (R533A). The mutations were reintroduced into the NL4-3 envelope and analyzed for their role in the resistance of compound **10**. Both of the gp120 mutations contributed to the drug sensitivity. On the contrary, the gp41 mutation (RS33A) did not appear to affect the compound **10** sensitivity. These results suggest that HIV-1 gp120 plays a key role in the anti-HIV-1 activity of compound **10**.

## ANTICANCER ACTIVITY

Betulinic acid was identified as a highly selective growth inhibitor of human melanoma, neuroectodermal and malignant tumor cells and was reported to induce apoptosis in these cells. Anticancer agents with different modes of action have been reported to trigger apoptosis in chemoselective cells [56]. Alterations of mitochondrial functions such as permeability transition (PT) have been found to play a major role in the apoptosis process including cell death induced by chemotherapeutic agents [57, 58].

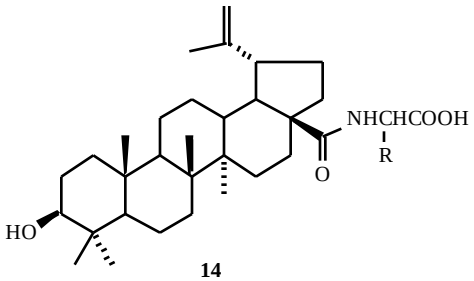
Selzer *et al*. [59] studied the effect of betulinic acid alone and in combination with irradiation in human melanoma cells. Betulinic acid strongly and consistently suppressed the growth and colony forming ability of all human melanoma cell lines. In combination with ionizing radiation, the effect of betulinic acid on growth inhibition was additive in colony-forming assays. Betulinic acid also induced apoptosis in human melanoma cells as demonstrated by AnnexinV binding and by the emergence of cells with apoptotic characteristics and was more pronounced in human melanoma cell lines than in normal human melanocytes.

Zuco *et al*. [60] studied the *in vitro* cytotoxicity of betulinic acid in melanoma and non-melanoma tumor cell lines and compared with that of doxorubicin. It was also tested on cell lines expressing a different p53 status. Betulinic acid proved active *in vitro* against a panel of neoplastic cell lines, including melanomas, small and non-small cell lung carcinomas, ovarian and cervical carcinomas. It exerted its antiproliferative activity on all the tested lines in a very narrow range of doses (1.5-4.5 μg/ml), and was effective against wild-type p53 and mutant p53 neoplastic cell lines derived from cancers clinically resistant to conventional antineoplastic drugs. In contrast, doxorubicin showed its cytotoxic activity in a larger range of concentrations (0.014-0.34 μg/ml). The growing colony inhibition assay indicated that betulinic acid exerted a cytotoxic effect on two wild-type p53 cells (IGROV-1 and A2780) and one mutant p53 cell (ME665/2/60/), while it had a cytostatic effect on the mutant p53 cell line ME665/2/21. In the *in vivo* experiments on IGROV-1 ovarian carcinoma xenografts, survival times of mice receiving betulinic acid (100mg/kg intraperitoneally (i.p.) every 3-4 days for a total of six treatments) were significantly higher ($p<0.01$) than those of controls; the survival time increased from 16 ± 1.03 days in control mice to 22 ± 2.59 days in animals receiving betulinic acid.

Fulda *et al*. [61, 62] identified betulinic acid as a new cytotoxic agent against neuroectodermal tumor cells including neuroblastoma, medulloblastoma, glioblastoma and Ewing's sarcoma cells, which represent the most common solid tumors of childhood. Neuroblastoma cells resistant to CD95- or doxorubicin-triggered apoptosis remained sensitive to treatment with betulinic acid, and betulinic acid exhibited potent antitumor activity on primary tumor cell cultures from all neuroblastoma (4/4), all medulloblastoma (4/4) with an $ED_{50}$ of 3-15 μg/ml and most glioblastoma patients (20/24) with an $ED_{50}$ of 5-16 μg/ml *ex vivo*. These findings suggest that betulinic acid may be a promising new lead in the treatment of neuroectodermal tumors *in vivo*.

Jeong *et al*. [63] coupled the betulinic acid with a series of amino acids at the C-28 carboxylic acid portion and evaluated the cytotoxicity of the derivatives against cultured human melanoma (MEL-2) and human epidermal carcinoma of the mouth (KB) cell lines. Among the derivatives, the free acid of the alanine (**14b**) and valine (**14c**) analogues showed toxicity against KB ($ED_{50}$ = 4.6 and 9 μg/ml, respectively). Methyl ester of **14b** and **14c** conjugates and the free acid of the glycine (**14a**) conjugate showed toxicity against MEL-2 comparable to betulinic acid ($ED_{50}$ = 3.5, 2.1, 4.2 and 4.2 μg/ml respectively). The free acid of the **14b** conjugate showed the best toxicity profile ($ED_{50}$ = 1.5 μg/ml) against MEL-2; however it also showed toxicity against KB ($ED_{50}$ = 4.6 μg/ml). Meanwhile the methyl ester of **14a**, and methionine (**14f**), tryptophan (**14h**), and alanine (**14b**) analogues showed improved cytotoxicity against MEL-2 when converted to the corresponding free acid conjugates ($ED_{50}$ 4.2-10.2 μg/ml, 9-12.9 μg/ml, 8.6->20 μg/ml and

1.5-3.5 μg/ml, respectively). However, the methyl ester of the phenyl alanine (**14g**), leucine (**14d**), glutamic acid (**14e**) and **14c** analogues showed the loss of cytotoxicity against MEL-2 when converted to the corresponding free acid conjugates ($ED_{50}$ = 9-20 μg/ml, 6.2-9 μg/ml, 15.3->20 μg/ml and 2.1-9 μg/ml, respectively).

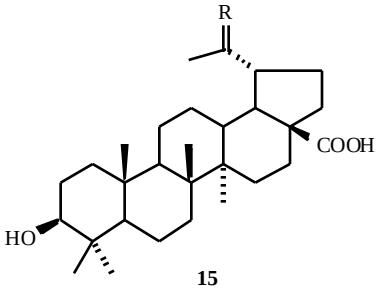



**14**

| | R | | R |
|---|---|---|---|
| **14a** | H | **14e** | $CH(CH_3)CH_2CH_3$ |
| **14b** | $CH_3$ | **14f** | $CH_2CH_2SCH_3$ |
| **14c** | $CH(CH_3)_2$ | **14g** | $CH_2C_6H_5$ |
| **14d** | $CH_2CH(CH_3)_2$ | **14h** | $CH_2$(3-indote) |

**Fig. (6).** $C_{28}$ peptide derivatives of betulinic acid.

Kim *et al*. [64] modified the C-20 alkene functional group of betulinic acid. The chemical modification at this position was initiated by converting the double bond to a ketone (**15a**) using a $OsO_4/NaIO_4$ system. The ketone functionality was readily transformed to oximes (**15b** and **15c**). The compounds were evaluated for their cytotoxicity against the human colon carcinoma cell line HCY-116, and human melanoma cell lines M14-MEL, SK-MEL-2, and UACC-257. The results showed that when the double bond was oxidized to a ketone (**15a**), loss of cytotoxicity was observed, suggesting that the presence of highly electronegative oxygen atom may change the electrostatic property of betulinic acid, rendering it less toxic. Converting to oximes (**15b** and **15c**) also appeared to result in the loss of cytotoxicity, probably due to the same reason described above. These results suggest that the cytotoxicity profile of betulinic acid derivatives may be sensitive to both the size of substituent at the C-20 position and its electrostatic properties.




**15**

| | R |
|---|---|
| **15a** | O |
| **15b** | NOH |
| **15c** | $NOCH_3$ |

**Fig. (7).** $C_{20}$ modified betulinic acid derivatives.

## ANTICANCER MECHANISM

Betulinic acid is a novel anticancer drug and induces apoptosis and hence differs from "Classical" anticancer agents such as doxorubicin [65]. Betulinic acid is a prototype cytotoxic agent that triggers apoptosis by a direct effect on mitochondria [66]. In isolated mitochondria, betulinic acid directly induces a loss of transmembrane potential independent of a benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone inhibitable caspase. This is inhibited by bongkrekic acid, an agent that stabilizes the PT pore complex. Mitochondria undergo betulinic acid induced PT mediated cleavage of caspase-8 and caspase-3 in a cell-free system. Soluble factors such as cytochromeC or AIF (apoptosis-inducing factor) released from betulinic acid treated mitochondria are sufficient for cleavage of caspases and nuclear fragmentation. Addition of cytochrome C to cytosolic extracts results in the cleavage of caspase-3, but not of caspase-8. However, supernatants of mitochondria, which have undergone PT, as well as partially purified AIF, activate both caspase-8 and caspase-3 in cytosolic extracts and suffice to activate recombinant caspase-8. These finding show that the induction of mitochondrial PT alone is sufficient to trigger the full apoptosis program and that betulinic acid may induce apoptosis *via* a direct effect on mitochondria.

## ANTIBACTERIAL ACTIVITY

Betulinic acid extracted from the leaves of *Vitex negundo* demonstrated antibacterial activity against *Bacillus subtilis* at a concentration of 1000 μg/disc with a zone of inhibition of 18.8 $mm^2$ [10]. Similarly betulinic acid and its three new derivatives namely 7β-(4-hydroxybenzoyloxy) betulinic acid, 7β-(4-hydroxy-3'-methoxybenzoyloxy) betulinic acid and 27-(4-hydroxy-3'-methylbenzoyloxy) betulinic acid, which were isolated from the stem bark of Brazilian medicinal plant *Zizyphus jaazerio* [24], showed considerable activity against Gram-positive bacteria.

## ANTIMALARIAL ACTIVITY

The *in vitro* antiplasmodial activity ($IC_{50}$) of betulinic acid isolated from the root bark of the Tanzanian tree against Chloroquine-resistant (K1) and –sensitive (T9-96) *Plasmodium falciparum* were found to be 19.6 μg/ml and 25.9 μg/ml, respectively. The *in vitro* activity of the related triterpene betulin demonstrated no activity up to 500 mg/ml for both K1 and T9-96 strains. When betulinic acid was tested for *in vivo* activity in a murine malaria model (*P. berghi*), the top dosage employed (250 mg/kg/day) was ineffective at reducing parasitemia and exhibited some toxicity [25].

Betulinic acid isolated from *Triphyophyllum peltatum* and *Ancistrocladus heyneanus* also exhibited moderate to good *in vitro* antimalarial activity against asexual erythrocytic stages of the human malaria parasite *P.falciparum* [27].

## ANALGESIC AND ANTI-INFLAMMATORY ACTIVITY

Betulinic acid isolated from *Diospyros leucomelas* showed anti-inflammatory activity in the Carrageenan and serotonin paw edema tests and TPA and EPP ear edema tests [28]. Betulinic acid isolated form *Ipomoea pes-caprae*

showed pronounced antinociceptive properties in the writhing test and formalin test in mice [26].

## ANTHELMINTIC ACTIVITY

Enwerem *et al*. [16] examined the anthelmintic activity of methanol, hexane and ethyl acetate extracts of *Berlina grandiflora*, which contain betulinic acid as the major component. *Caenorhabditis elegans*, a free living soil nematode, was used as an *in vitro* model in the study. A suspension of worms was treated with the extracts. After seven days of incubation, activity was assessed in terms of number of worms exhibiting motility. The results showed that the crude extracts (500 ppm) showed antihelmintic activity in the order ethyl acetate > methanol > hexane. Betulinic acid isolated from the ethyl acetate fraction showed strong anthelmintic activity at 100 ppm comparable to piperazine.

## MISCELLANEOUS PROPERTIES

Yamashita *et al*. [12] investigated the effect of betulinic acid and betulin on the stimulus induced superoxide generation and tyrosyl phosphorylation of proteins in human neutrophils. The various stimuli employed were N-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbal-12-myristate-13-acetate (PMA) and arachidonic acid (AA). The fMLP-induced superoxide generation was remarkably suppressed by betulin, while betulinic acid showed no effect. The PMA-induced superoxide generation was suppressed by betulin in a concentration-dependent manner, while the efficiency was lower than that of the fMLP-induced superoxide generation. The AA-induced superoxide generation was weakly enhanced by betulinic acid. The effect of these triterpenoids on the tyrosyl phosphorylation of protein in fMLP-treated human neutrophils showed that betulin suppressed the tyrosyl phosphorylation, but it was not affected by betulinic acid.

Chou *et al*. [67] examined the effect of betulinic acid on intracellular free $Ca^{2+}$ levels in Madin Darby canine kidney cells (MDCK). It caused significant increase in $[Ca^{2+}]i$ concentration dependently between 25 and 500 nM with an $EC_{50}$ of 100 nM. The $[Ca^{2+}]i$ signal was composed of an initial gradual rise and a plateau. The response was decreased by removal of extracellular $Ca^{2+}$ by 45% ± 10%. In a $Ca^{2+}$-free medium, pretreatment with 1 μM thapsigargin (an endoplasmic reticulum $Ca^{2+}$ pump inhibitor) abolished the betulinic acid –induced (250 μM) $[Ca^{2+}]i$ increase. Conversely, pretreatment with betulinic acid only partly inhibited the thapsigargin-induced $[Ca^{2+}]i$ increase. Addition of 3mM $Ca^{2+}$ induced a $[Ca^{2+}]i$ increase after pretreatment with 250 nM betulinic acid in a $Ca^{2+}$-free medium for 5 min. This $[Ca^{2+}]i$ increase was not altered by the addition of 20 μM of SKF96365 and 10 μM econazole, two drugs that have been shown to inhibit capatative $Ca^{2+}$ entry. Inhibiting inositol 1,4,5-triphosphate formation with a phospholipase C inhibitor U73122 (2 μM) abolished the betulinic acid-induced (250 nM) $Ca^{2+}$ release. Pretreatment with 10 μM $La^{3+}$, inhibited betulinic acid-induced (250 nM) $[Ca^{2+}]i$ increases by 85%, whereas 10 μM of verapamil, nifedipine and diltriazem had no effect. Tryphan blue exclusion revealed that acute exposure to betulinic acid (250 nM) for 2-3 min decreased cell viability by 6%, which could be prevented by pretreatment with 2 μM U73122. Together, the results suggest that betulinic acid induced significant $[Ca^{2+}]i$ increases in MDCK cells in a concentration-dependent manner, and also induced mild cell death.

Betulin and betulinic acid modified at the C-3 and C-28 positions have been evaluated *in vitro* for antiviral activity. It was found that simple modifications of the parent structure of lupane triterpenes produced highly effective agents against influenza A and herpes simplex type 1 viruses [68, 69]. Betulinic acid isolated from the ethanol extract of *Tovomita krukovii* was studied for its effect on aspartic proteases by Zhang *et al*. [14]. Specifically, it showed inhibitory effects against *Candida albicans* secreted aspartic protease with an $IC_{50}$ value of 6.5 μg/ml.

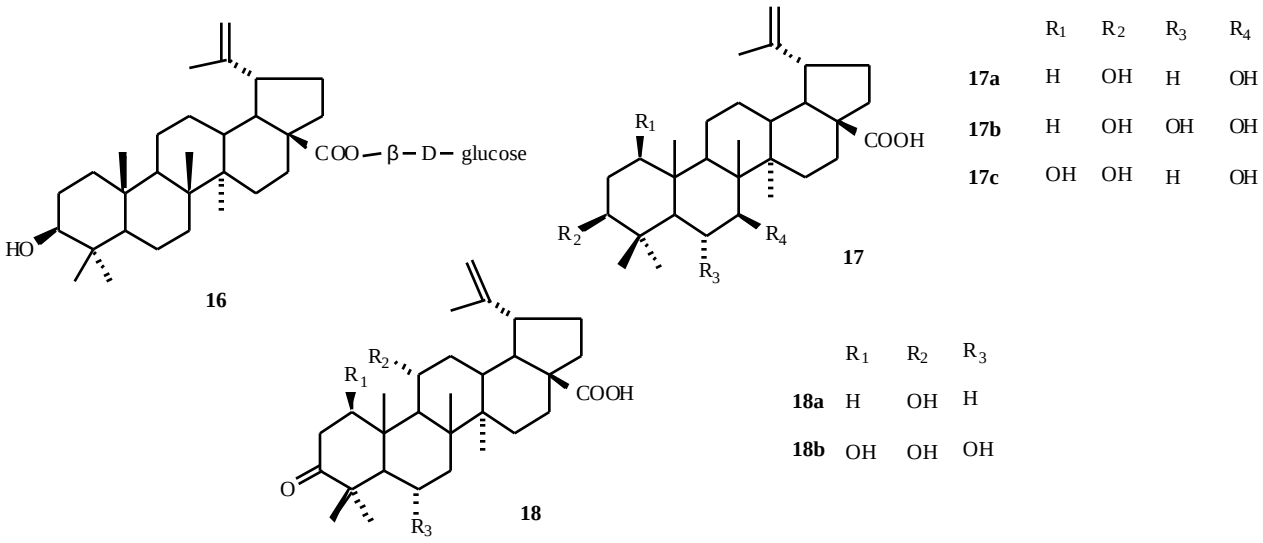
## METABOLIC TRANSFORMATION OF BETULINIC ACID

Betulinic acid is currently undergoing preclinical development for the treatment or prevention of malignant melanomas. An important factor in the evaluation of the efficacy and safety of a drug is the study of its mammalian metabolism. A prospective approach was undertaken to study the metabolism of betulinic acid utilizing microorganisms (particularly fungi) as *in vitro* model systems to mimic and predict the metabolic fate and other xenobiotics in mammalian systems [70].

Chatterjee *et al*. [71] reported the isolation and structural elucidation of 28-O-β-D-glucopyranosy-3β-hydroxy-lup-20(29)-en-28-oate (**16**), a conjugate fungal metabolite of betulinic acid, from resting cell suspensions of *Cunninghamella* species NRRL5695. A total of 13 fungal cultures were screened for the ability to catalyze the bioconversion of betulinic acid. *Cunninghamella* species NRRL569T was the only culture capable of reproducibly bioconverting **1** to the more polar metabolite **16**. The *in vitro* cytotoxicity assay of **16** revealed no activity against several human melanoma cell lines.

Kouzi *et al*. [72] studied the microbial transformation of betulinic acid utilizing three microorganisms. Bioconversion of betulinic acid (**1**) with resting-cell suspensions of Phenobarbital-induced *Bacillus megaterium* ATCC14581 resulted in the production of the known betulinic acid (**2**) and two new metabolites: 3β, 7β-dihydroxy-lup-20(29)-en-28-oic acid (**17a**) and 3β, 6α, 7β-trihydroxy-lup-20(29)-en-28-oic acid (**17b**). Biotransformation of **1** with the growing culture of *Cunninghamella elegans* ATCC9244 produced one new metabolite characterized as 1β, 3β, 7β-trihydroxy-lup-20(29)-en-28-oic acid (**17c**). Incubation of **1** with growing cultures of *Mucor mucedo* UI-4605 afforded metabolite **17a**. The *in vitro* cytotoxicity of all the compounds was evaluated against two human melanoma cell lines, Mel-1 (lymph node) and Mel-2 (pleural fluid). Compared to **1** ($ED_{50}$ = 3.3 and 1.0 μg/mL), metabolite **17c** showed no activity ($ED_{50}$ = >20 μg/mL), while metabolites **17a** ($ED_{50}$ = 17.1 and 7.2 μg/mL) and **17b** ($ED_{50}$ = 10.9, and 16.8 μg/mL) were less active against both Mel1 and Mel2.

Chatterjee *et al*. [73] studied the biotransformation of betulinic acid with a resting cell suspension of *Bacillus*




| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| **17a** | H | OH | H | OH |
| **17b** | H | OH | OH | OH |
| **17c** | OH | OH | H | OH |

| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| **18a** | H | OH | H |
| **18b** | OH | OH | OH |

**Fig. (8).** Microbial biotransformation products of betulinic acid.

*megaterium* ATCC13368, which resulted in the production of four metabolites, which were identified as 3-oxo-lup-20(29)-en-28-oic acid (**2**), 3-oxo-11α-hydroxy-lup-20(29)-en-28-oic acid (**18a**), and 3β, 7β, 15α-trihydroxy-lup-20(29)-en-28-oic acid (**18b**). Metabolites **2**, **18a** and **18b** were more cytotoxic than betulinic acid against Mel-2 cell line.

## PHARMACOKINETICS AND TISSUE DISTRIBUTION OF BETULINIC ACID

Udeani *et al*. [74] studied the pharmacokinetics and tissue distribution of betulinic acid in CD-1 mice. The results showed that after ip 250 and 500 mg/Kg dose, the serum concentrations reached peaks at 0.15 and 0.23h, respectively. The 250 and 500 mg/Kg betulinic acid i.p. doses were found to have elimination half-lives of 11.5 and 11.8h and total clearances of 13.6 and 13.5 l/Kg/h, respectively. The pharmacokinetic parameters observed for i.p. betulinic acid 500 μg/Kg in the skin of mice were as follows: $K_a$ ($h^{-1}$) 0.257, $K_{10}$ ($h^{-1}$) 0.234, $t_{1/2}$ (α) (h) 2.63, $t_{1/2}$ (β) (h) 20.2, V (l/Kg) 0.61, AUC (μg/mL) 3504, $T_{max}$ (h) 3.90 and $C_{max}$ (μg/mL) 300.9. The distribution of betulinic acid in tissues at 24h post i.p. administration in a descending order was as follows: perirenal fat (2260 μg/g), ovary (1998 μg/g), spleen (1287 μg/g), mammary gland (1184 μg), uterus (980 μg/g), and bladder, lymph node, liver, small intestine, caecum, lung, thymus, colon, kidney, skin, heart and brain (1 μg/g).

Recently, Cheng *et al*. [75] developed a robust assay based on liquid chromatography/mass spectrometry to conduct a quantitative analysis of betulinic acid in mouse, rat and dog plasma. At 15 and 25 μg/mL in mouse, rat or dog plasma, betulinic acid was 99.99% bound to serum proteins, and, at 5 μg/mL, betulinic acid was > or =99.97% bound following i.p.l or intravenous administration *in vivo*.

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