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Review

# Lupane-Type Triterpenoids Biotransformation

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**Abstract:** A lot of scientific material has been accumulated in recent years about the biotransformation processes of lupine-type triterpenoids. The novelty of this review is the summary of these works, the analysis and identification of the most suitable biocatalysts for future researchers, and the presentation of development trends for new derivatives. The known biotransformation examples of the most common lupine-type triterpenoid representatives are considered. Analysis of their biosynthesis pathways starting with squalene is included also. Various approaches in this study are discussed to biotransformation using fungi, bacteria, and plant cell cultures. The conversion of betulin to betulinic acid is a process of special, even extreme interest. Cytochromes P450 are responsible for the catalysis of oxidation reactions while air oxygen is the oxidizer. The expression and activity of these enzymes are crucial factors for product yield. Basically, any given lupine-type triterpenoid can be transformed with P450 monooxygenases. Sadly, P450 catalysts are heme and NAD (P) H-dependent thus using isolated enzymes is not an option for biotransformation. So the whole-cell catalytic processes are completed by the formation of acids, ketones, or other oxidized products. Fungi cell cultures especially *Cunninghamella blakesleeana*, *Armillaria luteo-virens*, and *Rhodotorula mucilaginosa* are characterized by one of the highest conversion rates. Also, fungi cells are tolerant to the antibacterial activity of lupine-type triterpenoids. Thus fungi are the most successful biocatalysts for biotransformation. Applications of lupane-type triterpenoids such as pro-drugs and cosmetics are addressed in the final part of this study. It has clearly indicated development prospects for obtaining new useful derivatives.

**Keywords:** Bioconversion, Biocatalysis, Biosynthesis, Pentacyclic Triterpenoids, Lupeol, Betulin, Betulinic Acid, Betulon, Betulonic Acid

## Introduction

### *What are the Terpenoids*

Plants' secondary metabolites have always been the most significant resources of biologically active substances. They can be divided into three groups: Phenols, alkaloids, and terpenes. Terpenes are a group of hydrocarbon-based organic compounds and they often have a strong odor. Terpenes play a critical role as one of the key building blocks in plant cells. The basic unit of terpenes is isoprene 1 (Fig. 1). Isoprene's derivatives are often referred to as terpenoids. They are obtained when a terpene undergoes oxidation, reduction, or rearrangement.

Terpenoids aren't strictly located in some specific plant organ. The majority of them are located in the surface tissues. They protect a plant body from water loss, mechanical damage, and any biological or chemical attacks. Terpenoids act as a "bulwark" to the environment. There is continuous research interest in pentacyclic triterpenoids. This is primarily due to their high and diverse biological activity. Among them, the most attractive are lupane 2 derivatives (Fig. 2).

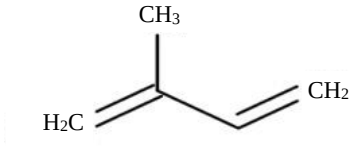


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**Fig. 1:** Isoprene 1 structure

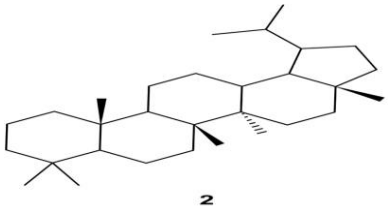

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**Fig. 2:** Lupane 2 structure

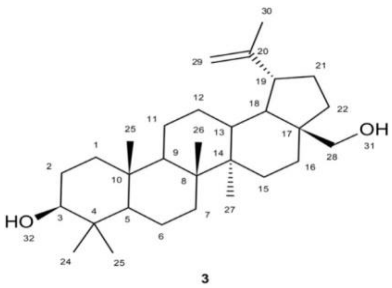


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**Fig. 3:** Betulin 3 structure

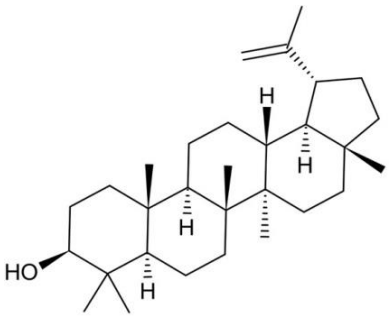


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**Fig. 4:** Lupeol 4 structure

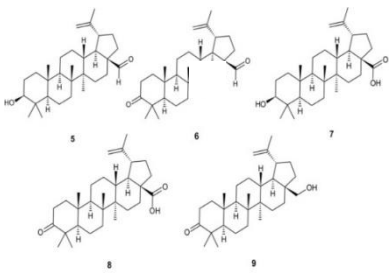

**Fig. 5:** The structures of betulinic aldehyde 5, betulonic aldehyde 6, betulinic acid 7, betulonic acid 8 and betulon 9

## *Structure and Properties of the Terpenoids*

The quantity of lupine-type triterpenoid research has been a steadily growing trend in recent decades. These molecules have anti-inflammatory, antibacterial, antiviral, hepatoprotective, and antitumor activities (Hordyjewska *et al.*, 2018; 2019; Amiri *et al.*, 2020). Plants containing such triterpenoids are of extreme interest in pharmaceutical practice as a source of potentially effective new drugs.

One of the most environmentally friendly, renewable, and relatively cheap resources of lupine-type triterpenoids are plants of the *Betulaceae* family. They are widespread tree species located in the northern hemisphere of the Earth. Triterpenoids are crystals accumulated in large cells of the bark. Their containment varies from 10-45% in dry weight. Such a wide range of triterpenoid content is the result of various factors mainly tree age, weather conditions, and location (Wani *et al.*, 2020). It's obvious that numerous species of *Betula* are promising raw materials for obtaining triterpenoids thanks to the availability and renewability of birch tree base (Singh *et al*., 2012).

The basis of lupine-type triterpenoids is a structure containing a complex polycyclic system with a cyclopentanoperhydrophenanthrene core. That structure consists of four cyclohexane and one saturated cyclopentane ring, all of them fused together. The most common product of the extraction process is betulin 3 (lup-20 (29)-ene-3β, 28-diol) (Fig. 3) (Meng *et al.*, 2018).

Betulin 3 is an extremely accessible reagent due to developed efficient extraction methods from birch bark (Alexandr *et al.*, 2018; Bachořík and Urban, 2021). Betulin 3 is a constant "companion" with its biosynthetic precursor lupeol 4 (Fig. 4).

Along with compound 3 birch bark extracts also contain a small number of betulin's oxidized derivatives: Betulinic aldehyde 5, betulonic aldehyde 6, betulinic acid 7, betulonic acid 8, and betulon 9 (Fig. 5).

## *Bioavailability of the Terpenoids*

It's necessary to increase the solubility of lupane-type triterpenoids considering their high hydrophobicity. Modifications need to be done in order to improve their bioavailability. That can be achieved by introducing hydrophilic groups into their chemical structure, e.g. by oxidizing. Betulinic acid 7 is a more valuable pharmaceutical substance than betulin 3 due to this precise reason. Most researchers are aimed specifically at the transformation of betulin 3 into betulinic acid 7 (Saneja *et al.*, 2018).

Modifications at C-3 and C-28 and other positions of 3 make it possible to obtain not only 7 and 8 respectively but other derivatives too (Boparai *et al.*, 2017; Jonnalagadda *et al.*, 2017; Cunha *et al.*, 2021). Thus the

work of Kruszniewska-Rajs *et al.* (2022) has shown the antitumor potential of 28-propionic 10 and 29-diethyl phosphonate 11 derivatives of 3 (Fig. 6). The presence of free for substitution groups in other positions open up opportunities for further modifications (Kruszniewska-Rajs *et al.*, 2022).

Drąg-Zalesińska *et al*. (2019) have demonstrated significant activity of betulin-diamino butanoic ester 12 (Fig. 7) stimulating collagen synthesis in human fibroblasts (Drąg-Zalesińska *et al.*, 2019).

## *Modifications of the Terpenoids*

The chemical synthesis of betulinic acid 7 (Fig. 8) is usually carried out by Jones oxidation (Boparai *et al.*, 2017).

Such a process is characterized by high energy costs, environmentally harmful reagents, and a lot of by-products/ Chemical oxidation also require complex and expensive catalytic systems such as gold nanoparticles or graphite nitride materials (Kolobova *et al.*, 2019; Shcherban *et al.*, 2019).

Biotransformation methods are the alternative to obtain new betulin 3 derivatives. This approach allows the process to be carried out with greater stereo-and regioselectivity, under milder reaction conditions, with relatively low cost and less environmental impact (Milner and Maguire, 2012).

There is even an advantage that some reactions that are extremely difficult to carry out in chemical synthesis can be easily carried out using enzymatic systems. Microorganisms or plants are often the only possible tool to achieve that goal. The mentioned advantage was clearly demonstrated in Jan Bachořík and Milan Urban's review. Authors have summarized all possible positions in molecule 7 that can be modified enzymatically (Fig. 9) (Bachořík and Urban, 2021).

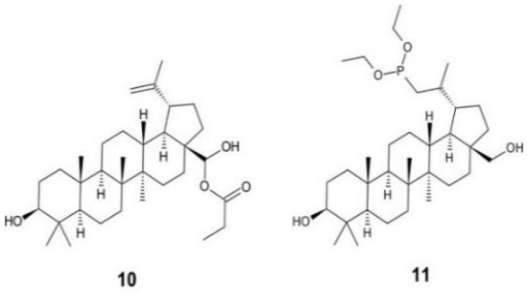


**Fig. 6:** Derivatives 10-11 structures

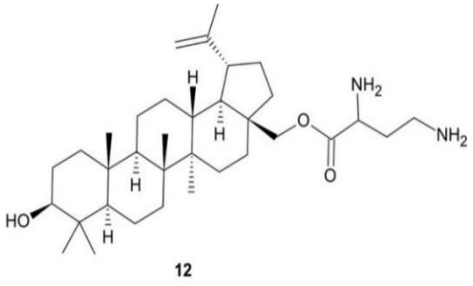


**Fig. 7:** Betulin ester of diaminobutanoic acid 12 structure

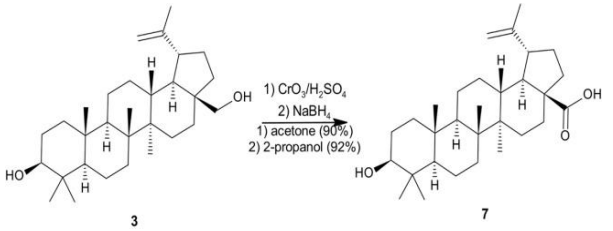


**Fig. 8:** Scheme of the direct oxidation of 3 according to jones and obtaining of 7

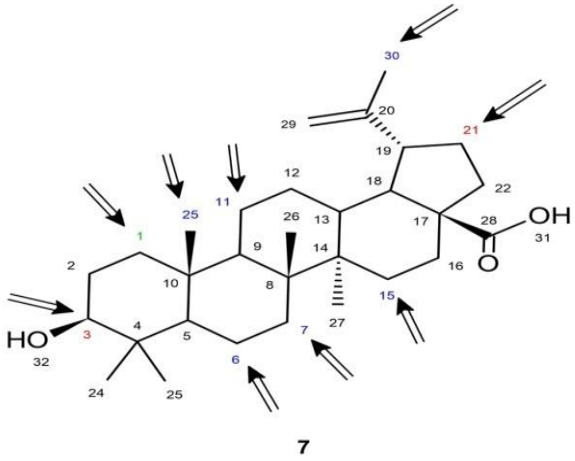


**Fig. 9:** Positions in compound 7 that are subject to biotransformation by microorganisms: Acetylation-1; oxidation-3, 21; hydroxylation-6, 7, 11, 15, 25, 30

## *Biosynthesis of Lupane-Type Triterpenoids*

It's necessary to understand the bioconversion mechanisms of betulin 3. Biotransformations in a laboratory flask or fermenter are only simulations of biosynthetic pathways in the cells. Efficient oxidation requires appropriate enzymes. Modern sequencing technologies and other molecular biology methods make it possible to find both the genes and the proteins involved in these processes.

A generally accepted model for the biosynthesis of lupine-type triterpenoids was developed. The common precursor compound 2,3-oxidosqualene 13 (Fig. 10) undergoes cyclization by specific enzymes of the Oxidosqualene Cyclase (OSC) group.

Subsequently, these structures are saturated with oxygen by various cytochromes P450. Plant genomes contain more than ten OSCs and about two hundred and fifty P450. Both of them are involved in different pathways of functionalization. The OSC stage represents a key branch point leading to the biosynthesis of either steroidal saponins or non-steroidal triterpenoid molecules. The biosynthesis of triterpenoids occurs through the dammarenyl cation 14 in the chair conformation (Fig. 11).

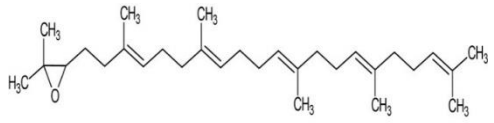

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**Fig. 10:** 2,3-oxidosqualene 13 structure

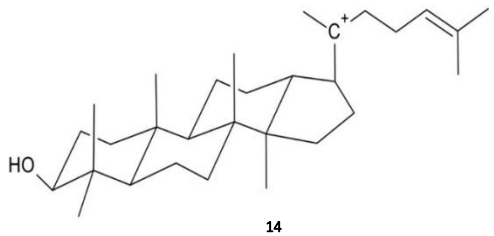

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**Fig. 11:** Structure of dammar enyl cation 14 in the chair conformation

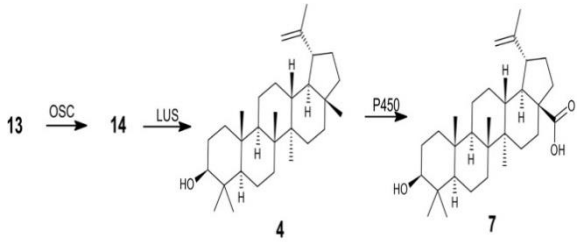

**Fig. 12:** Simplified scheme of the biosynthesis of 4 from 13 and the obtaining of 7

More than a hundred different triterpene backbones can be obtained from 2,3-oxidosqualene 13. Lupeol 4 is only one of the most common and studied (Cárdenas *et al*., 2019). 14 becomes 4 with the help of Lupeol Synthases (LUS) through cyclization and rearrangement. Then the C-28 position of lupeol 4 is subsequently oxidized to a carboxyl group by cytochrome P450 enzymes. All of the above leads to the production of betulinic acid 7 (Fig. 12) (An *et al.*, 2020).

The oxidation of 4 and the preparation of these derivatives occur with the participation of P450 enzymes. Therefore, the high enzymatic activity of P450 cytochromes and proper enzyme engineering is extremely important in the bioconversion of 3-7 (Luo *et al.*, 2022).

The obvious approach would be genetic modification and the production of transgenic plants. That is aimed at achieving overexpression of the necessary enzymes inside the cell (Bachořík and Urban, 2021). However, one should take into account the existing effective techniques for the extraction of betulin 3 from the incredibly rich and renewable base of *Betulaceae* trees. Thus the approach of bioconversion of extracted organic substances is more advantageous.

## *Biotransformation of Lupane-Type Triterpenoids Using Fungi Cells*

### *Cunninghamella Spp.*

At the beginning of the XXI century, the bioconversion of triterpenoids using *Cunninghamella Blakesleeana* fungi cells was sufficiently studied. There is an experimental work by Feng *et al.* (2013) where the authors have offered an innovative one-step approach to the biotransformation of betulin 3-betulinic acid 7. The biotransformation proceeded at 28°C and a shaker speed of 180 rpm for two days. Structures of products were confirmed by reverse phase High-Performance Liquid Chromatography (HPLC) after centrifugation and extraction using ethyl acetate. The average yield of betulinic acid 7 was up to 2.67%. 7 is undoubtedly the most important and desirable product. Four other derivatives have also been isolated, the exact structures of which, however, haven't been determined (Feng *et al.*, 2013).

Apparently, the unidentified molecules may be the products of further oxidation of betulinic acid 7. The basis for this assumption is the confirmed biotransformation of 7 by growing *Cunninghamella elegans* cells ATCC 9244. The hydroxylation of C-1 and C-7 positions has occurred thus completing the synthesis of 1,3,7-trihydroxy-lup-20 (29)-en-28-oic acid 15 (Fig. 13) (Kouzi *et al*., 2000).

### *Saccharomyces Cerevisiae*

Czarnotta *et al.* (2017) have obtained betulinic acid 7 with a titer of 182 mg/L using *Saccharomyces cerevisia* cells. They have combined metabolic and technological engineering. The engineered yeast strains expressed heterologous Lupeol synthase (LUP1) and P450 reductase (CPR) from *Arabidopsis thaliana* and P450 monooxygenase (CYP) from *Catharanthus roseus* along with homologous squalene monooxygenase (ERG1). The authors also found that an excess of ethanol plays a critical role in the fermentation process (Czarnotta *et al.*, 2017).

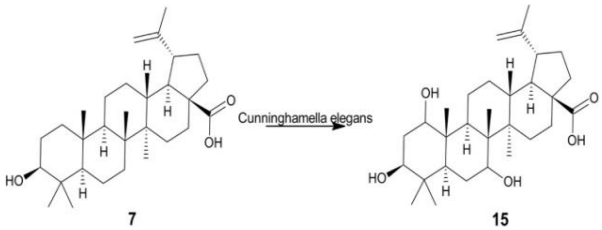

**Fig. 13:** Scheme of biotransformation of 7 into 15 using *Cunninghamella elegans*

### *Yarrowia Lipolytica*

There is the paper of Jin *et al.* (2019) where the authors have used *Yarrowia lipolytica* fungi cells. The titer of betulinic acid 7 was 51.87 mg/L during fermentation. In order to maximize the production of 7 the authors divided the betulinic acid synthesis route into four distinct modules similar to the biosynthetic pathway in plant cells: CYP/CPR, mevalonate, Acetyl-CoA, and redox cofactor generation. This titer was obtained from flask fermentation so it is highly likely that *Yarrowia Lipolytica* production can be further improved with proper scaling (Jin *et al.*, 2019).

### *Armillaria Luteo-Virens*

The microorganism proposed by Liu *et al.* (2011) in order to optimize the biotransformation of betulin 3 to betulinic acid 7 is *Armillaria luteo-virens* Sacc ZJUQH100-6. Biotransformation was carried out with shaking at 120 rpm, temperature 28°C, and absence of light. The authors have shown an increase in a yield of 7-9.32% varying the conditions. For example, the presence of 0.57% polysorbate 80 in the mixture, pH 6, and the 3-day-long inoculation stage were the optimal parameters (Liu *et al.*, 2011).

All of the methods using fungi mentioned are presented in (Fig. 14).

### *Rhodotorula Mucilaginosa*

Mao *et al*. (2012) have performed biotransformation 3 using yeast extracted from the soil. Primary screening for the most tolerant strain to high concentrations of betulin 3 was carried out on 47 samples. *Rhodotorula mucilaginosa* turned out to be the most promising species. The main product of the process isolated by HPLC and identified by Mass Spectrometry (MS) was betulon 9 (Fig. 15).

This yeast strain allowed conversion of 52.65% betulin 3 under optimal conditions such as a temperature of 30°C, shaking speed is 150 rpm, pH 6, and fermentation for 24 h (Mao *et al.*, 2012).

## *Biotransformation of Lupane-Type Triterpenoids Using Bacteria Cells*

### *Bacillus Spp.*

Kumar and Dubey (2017) have carried out biotransformation of betulin 3 to betulinic acid 7 with *Bacillus megaterium* strain (Fig. 16). The working biocatalyst train KD235 which has shown maximum tolerance to 3 and high conversion values during preliminary screening experiments.

They have achieved the conversion of 2% at optimal conditions such as temperature at 30°C, pH 6.5, and betulin 3 concentration of 3 g/L. The authors also proved an 11% increase in conversion with proper scaling by replacing the laboratory flask with a fermenter (Kumar and Dubey, 2017).

*Bacillus megaterium* was used in the further conversion of betulinic acid 7 into betulonic acid 8. For example, *B. megaterium* ATCC 14581 made it possible to obtain 8 and as well as new 7-hydroxy 16 and 6,7-dihydroxy derivatives 17 (Fig. 17).

However, *B. megaterium* ATCC 13368 cells further hydroxylated betulonic acid 8 at positions C-1 and helped to obtain 18, and at positions, It helped to obtain 19 (Fig. 18) (Kouzi *et al.*, 2000).

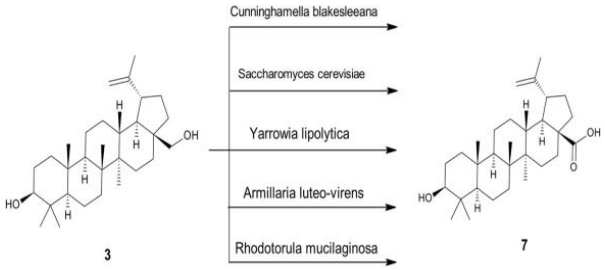


**Fig. 14:** Scheme of biotransformation of 3 into 7 using fungi

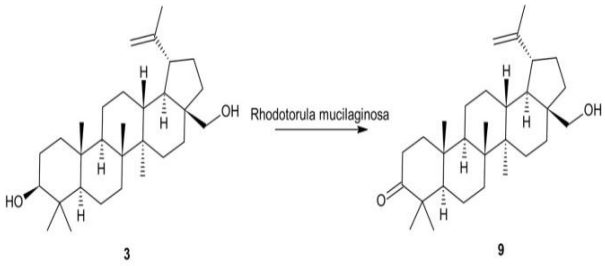


**Fig. 15:** Scheme of biotransformation of 3 into 9 using *Rhodotorula mucilaginosa*

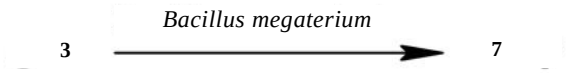


**Fig. 16:** Scheme of biotransformation of 3 into 7 with *Bacillus megaterium*

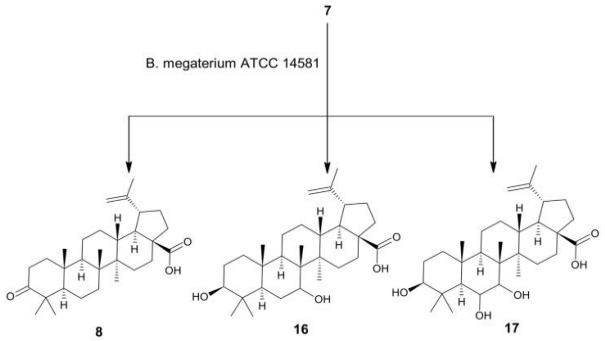


**Fig. 17:** Scheme of biotransformation of 7 into 8, 16, and 17 using *Bacillus megaterium*

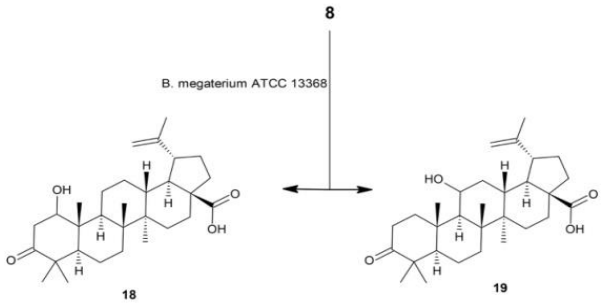


**Fig. 18:** Scheme of biotransformation of 8 into 18 and 19 using *Bacillus megaterium*

## *Rhodococcus Spp*.

Grishko *et al.* (2013) have shown the ability of actinobacteria *Rhodococcus* to biotransformation of betulin 3 into betulon 9-a promising intermediate (Fig. 19). After screening about 104 strains the authors identified *Rhodococcus rhodochrous* IEGM 66 as the most active biocatalyst.

Bacteria were grown in Erlenmeyer flasks and then added to the nutrient medium. Betulin 3 was added to the culture medium in dimethyl sulfoxide solution 48 h after the start of *Rhodococcus* cultivation. Actinobacteria catalyzed the conversion of 3 at a concentration of 0.5 g/L into 9 with an average conversion of 60% up to 75% in 96 h. The dependence of the conversion of betulin 3 and yield of betulon 9 from the composition of the medium was studied. So the highest yield of 9 at 44.9% is achieved on the medium with the addition of 1% n-hexadecane. The authors suggested that a high degree of bioconversion of betulin in the presence of n-hexadecane is due to an increase in the hydrophobicity of the *Rhodococcus* cell wall. Thus it promoted the interaction of bacterial cells with triterpenoid. Also, the high conversion rates of betulin 3 were shown in the range from 39.9% up to 72.2% using *R. Erythropolis, R. longus,* and *R. ruber* (Grishko *et al.*, 2013).

Despite their widespread use as the main tool in biotechnological processes, there is no data on the use of *E. coli* strains for the biotransformation of betulin 3. This may be associated with the proven high antibacterial activity of the latter (Oloyede *et al.*, 2017).

## *Biotransformation of Lupane-Type Triterpenoids Using Plant Cells*

An interesting case is that (Häkkinen *et al*., 2018) used suspension cultures of *Nicotiana tabacum* L. and *Catharanthus roseus* (L.) *G. don.* for the biotransformation of betulonic acid 8 derivatives namely 20(29)-dihydrolup-2-ene[2,3-d] isoxazol-28-oic acid 20, 1-betulonoylpyrrolidine 21 and lupa-2,20(29)-dieno [2,3-b] pyrazine-28-oic acid 22 (Fig. 20).

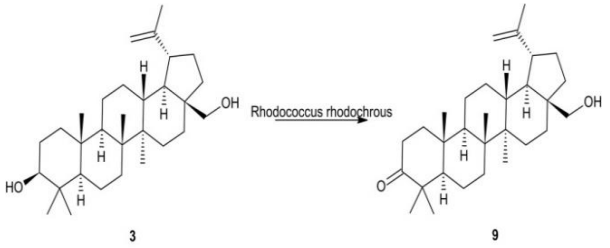


**Fig. 19:** Scheme of biotransformation of 3-9 using *Rhodochrous rhodochrous*

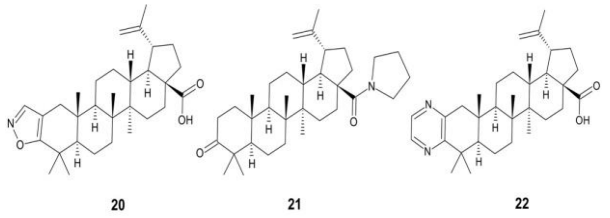


**Fig. 20:** Structures of 20, 21 and 22

Cyclodextrins were also used in the process in order to increase the solubility of reagents in water through the formation of supramolecular complexes. The corresponding products were not found during isolation and purification although the authors presumably observed oxidation of the reagents. Biotransformation of 22 ended with the formation of four products, pentose, hexose, and hexose conjugates. However, their exact structure has remained unconfirmed. The highest relative substrate uptake was seen in the suspension of *Catharanthus roseus* (L.) *G. don.* The maximum conversion was up to 1.8%. The authors concluded that all biotransformation products are more hydrophilic than original compounds which is critical for bioavailability in therapeutic applications (Häkkinen *et al*., 2018).

## *Summarizing Remarks*

Summarizing biotransformation examples of lupine-type triterpenoids should be noted that the authors give preference to fungi cell cultures as a biocatalyst. That is probably connected not only to the technological features of their genetic engineering but also to the bactericidal activity of biotransformation objects to the majority of bacteria microorganisms. The limiting factor in the use of plant suspension cultures is the low solubility of substances and as a result, their low ability to penetrate into plant cells. Favor for the use of fungi is also evidenced by the highest conversion of the substrate among other biocatalysts up to 99%. (Table 1) summarizes the initial reagents and products relative to the biocatalyst used.

**Table 1:** Reviewed biotransformation methods of lupine-type triterpenoids

| Biocatalysts | Type of bioproducent | Initial reagent | Major product | References |
|---|---|---|---|---|
| Fungi | *Cunninghamella blakesleeana* | 3 | 7 | Feng *et al*. (2013) |
| | *Cunninghamella elegans* | 7 | 15 | Kouzi *et al*. (2000) |
| | *Saccharomyces cerevisiae* | 3 | 7 | Czarnotta *et al*. (2017) |
| | *Yarrowia lipolytica* | 3 | 7 | Jin *et al*. (2019) |
| | *Armillaria luteo-virens* | 3 | 7 | Liu *et al*. (2011) |
| | *Rhodotorula mucilaginosa* | 3 | 9 | Mao *et al*. (2012) |
| Bacteria | *Bacillus megaterium* | 3 | 7 | Kumar and Dubey (2017) |
| | | 7 | 8, 16, 17 | Kouzi *et al*. (2000) |
| | | 8 | 18, 19 | Kouzi *et al*. (2000) |
| | *Rhodococcus rhodochrous* | 3 | 9 | Grishko *et al*. (2013) |
| Plants | *Nicotiana tabacum;* | 20, 21, 22 | Conjugates | Häkkinen *et al*. (2018) |
| | *Catharanthus roseus* (L.) *G. don* | | | |

### *Lupane-Type Triterpenoids Applications and Development Prospects*

#### *Use in Cosmetics*

In the modern market, lupine-type triterpenoids confidently have occupied the niche of dietary supplements. A wide range of betulin phytomedicines can be noted from Birch World (Russia), *Betula* Farm (Russia), and Incoda (India).

Lupanes are also presented in cosmetics like creams “Berkana Birch” from Einhorncreme (Germany), “Birch Moisturizing Cream with Betulin” from Sylveco (Poland), “Birkencreme” from Birkenstock (Germany), “Youth Surge SPF 15” from Clinique (USA), “Gel-en-huilesolaire corps" from Clarins (France). Also, examples may serve extracts of 3, 4, and 7 from “Terra Aromatica” (Russia).

In the USA "Hoof Doctor-omega-3" from Mineral Medix Corp. is commercially available. It's a veterinary drug that has passed the necessary tests but has not received FDA approval yet.

#### *Pharmaceutical Limitations*

As mentioned above in the review lupine-type triterpenoids and in particular botulin 3, lupeol 4, and betulinic acid 7 have numerous functional activities. However, their use as full-fledged drugs is not observed despite the extensive number of experimental proofs of their properties.

Betulin 3 and its derivatives haven’t yet been fully annotated in the Drug Bank. These substances are still in the “investigational” category. This is probably due to the low commercial availability which therefore depends on the lack of effective methods of their preparation, isolation, and purification. The solution to these issues should lead to the intensification of the use of pharmaceutical substances based on lupine-type triterpenoids. The future is the obtaining of new terpene agents in the fight against cancer and AIDS.

## Conclusion

### *Biotransformation Advantages*

Pentacyclic lypane-type triterpenoids are important natural sources of pro-drugs with a variety of biological activities. Often their bioavailability needs to be optimized. For example, to increase the solubility of the original molecule, it usually requires adding hydrophilic functional groups. These modifications can be achieved by organic synthesis but they have several drawbacks: Harsh reaction conditions and hazardous toxic reagents. Biotransformation proves to be a successful alternative in this case. Enzymes though operate under mild conditions and can produce pure enantiomers.

### *Future Research Directions*

At the present day, a relatively small number of biotransformation techniques are developed to obtain and modify lupine-type triterpenoids. However, the same systems are used for biotransformation of other triterpenoids (Luchnikova *et al.*, 2020). There is a huge space for carrying out oxidation reactions catalyzed by various cytochromes P450 using whole-cell cultures of fungi, bacteria, and plants. The future is obtaining active oxidized derivatives with greater bioavailability for the treatment of cancer and other diseases. The full potential of lupine-type triterpenoid biotransformation has not been discovered to date.

We hope that this review has summarized the accumulated material about the biotransformation of lupane-type triterpenoids highlighting that the most promising biocatalysts are fungi and the most desirable products are acids. Thus attracting new researchers to work in the field of biotransformation.

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## Author's Contributions

**Zhumadilov Sayat Sagatovich:** Conducted data analysis and authored the section on bacterial biotransformation of lupine-type triterpenoids.

**Bakibaev Abdigali Abdimanapovich:** Performed comprehensive analysis and compiled a summary regarding the chemical structure of lupine-type triterpenoids.

**Zhadan Konstantin Vasilevich:** Analyzed data and contributed to the section detailing lupane-type triterpenoids.

**Kartbayeva Gulnaz Tolymbekovna:** Executed data analysis and developed the section on the biotransformation of lupine-type triterpenoids using plant suspension cultures.

**Kassenov Rymchan Zeinollaevich:** Analyzed data and formulated the section on the biosynthesis of lupine-type triterpenoids.

**Ramazanov Alibek Kairidenovich:** Managed the text layout and prepared the article in accordance with the author's instructions.

**Yerniyazova Bibizhan Bakytzhanovna:** Responsible for the creation and editing of figures, diagrams and tables.

**Kusherbayev Sultan Asanbaevich:** Analyzed data and crafted the section on the dissemination of lupine-type triterpenoids.

## Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

### *Conflict of Interest Statement*

The authors declare no conflicts of interest.

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