Harnessing Pivotal Advances for Production and Structural Derivation of the Promising Molecule Ursolic Acid

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Abstract

Ursolic acid (UA) is a ursane-type pentacyclic triterpenoid compound, naturally produced in plants via specialized metabolism and exhibits vast range of remarkable physiological activities and pharmacological manifestations. Owing to significant safety and efficacy in different medical conditions, UA may serve as a backbone to produce its derivatives with novel therapeutic functions. This review systematically provides an overview of the pharmacological activities, acquisition methods and structural modification methods of UA. In addition, we focused on the synthetic modifications of UA to yield its valuable derivatives with enhanced therapeutic potential. Furthermore, harnessing the essential advances for green synthesis of UA and its derivatives by advent of metabolic engineering and synthetic biology are highlighted. In combination with the advantages of UA biosynthesis and transformation strategy, large-scale production and applications of UA is a promising platform for further exploration.

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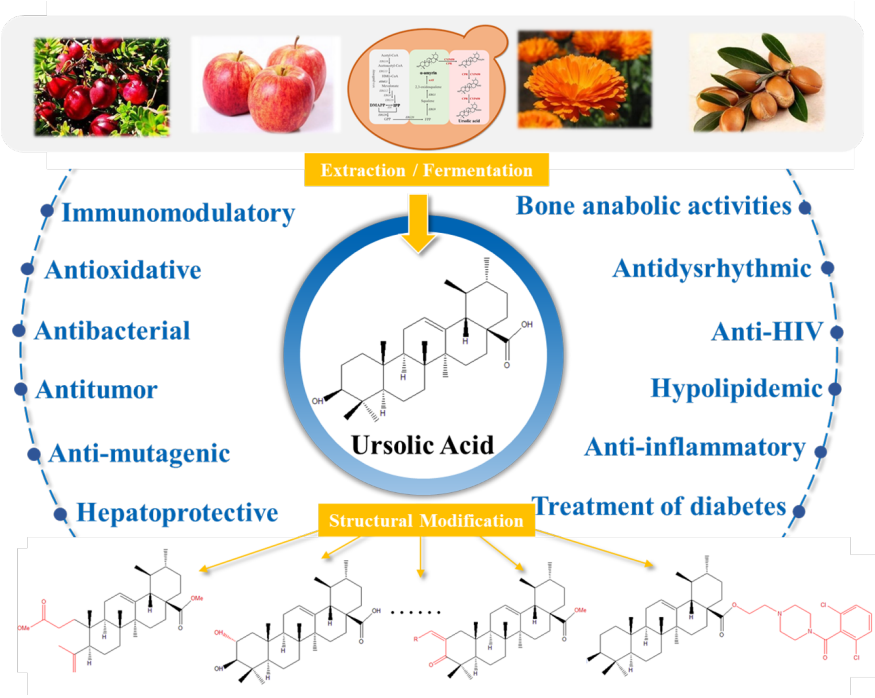
Keywords: Ursolic Acid; Ursolic Acid derivatives; Biosynthesis; Metabolic engineering

Graphical abstract



Introduction

Ursolic acid (UA, 3β-hydroxy-urs-12-en-28-oic acid) is a kind of pentacyclic triterpenoids existing naturally in plants, which plays a significant role as a hormone or regulatory factor in plant growth and insect resistance[1,2]. Structurally, it is a ursane-type pentacyclic triterpenoid with C-3 hydroxy and C-28 carboxylic acid group (*Fig. 1*). Apart from its significance in plants, UA and its derivatives also exhibit remarkable physiological and pharmacological manifestations. Up to now, it has shown a wide range of clinical manifestations, including anti-inflammatory [3], anti-oxidation[4], diabetes treatment [5], antibacterial [6], anti-tumor metastasis[7], anti-tumor activity [8], anti-angiogenesis [9], skin care[10], and superior liver protection function[11].

Fig. 1 *Chemical structure of Ursolic acid (UA)*

The main way to obtain UA on a large scale currently relies on extraction of plants rich in it. However, it encounters several limitations, such as high energy consumption and low yield by plant extraction, which make it difficult to meet commercial needs. In addition, UA endures obstacles in therapeutic applications and drug development due to fast metabolism, poor water solubility and low bioavailability [12,13].

In view of the excellent potential of UA as therapeutic drug, its structural modification is an attractive step to synthesize the potentially active derivatives in order to expand the pharmacological properties for

further exploration [14-16]. More importantly, with the advent of synthetic biology and metabolic engineering, transition towards the synthesis of natural product by employing these approaches is of great concern. Cell factories such as*Saccharomyces cerevisiae* (*S. cerevisiae*) have been genetically engineered for green production of terpenoids, such as artemisinic acid [17], ginsenoside [18], oleanolic acid [19] and lycopene [20], which provide new thoughts for industrial production of UA. In this article, we systematically reviewed the significance of production and modification of UA to explore more applications in pharmacy and pharmaceutical chemistry. Particularly, we focused on the de novo synthesis of UA and its derivatives in engineered microbial cell factories in advancements for green production and green chemistry.

Physiological and pharmacological profile of UA

Studies have shown that UA has broad application prospects, which is employed in the management of various chronic diseases by mediating and regulating the pharmacological processes and related signaling pathways [21,22].

UA plays an anti-inflammatory role in inflammatory cascade. It inhibits the activation, proliferation and cytokine secretion of T cells, B cells and macrophages of mouse lymphocytes. By inhibiting phosphorylation of Extracellular Regulated protein Kinases (ERK) and c-Jun N-terminal Kinase (JNK) induced by mitogen, it limits the activation of immunomodulatory transcription factors NF-κB, NF-AT and AP-1 in lymphocytes [23].

In another study, UA has been revealed to exhibit hepatoprotective properties as it significantly inhibits the HCV genotype 1b replicon and HCV genotype 2aJFH1 virus [24]. It is partly due to the inhibition of HCV-NS5BRdRp activity as a noncompetitive inhibitor. Researchers explored the expression of HPVE6/E7 by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and found that UA down-regulate it [25].

UA also shown the promising results in the management of hepatitis A and B with the cure rate of 89.3% as it reduces the liver weight, ALT/AST level, hepatic steatosis, and palmitic acid induced fat accumulation in L02 cells[11,26]. It exerts hepatoprotective effects by increasing β-oxidation of fat and inhibiting endoplasmic reticulum pressure.

In addition, UA exhibits significant therapeutic potential in cancer treatment due to its high activity and low toxicity. Studies have shown that UA decreases the activity of extracellular phosphorylated signal regulated kinase and depolarizes the mitochondrial membrane[27]. It has also been found that UA promotes the apoptosis of human bladder cancer cell line T24 by activating the ASK1-JNK signaling pathway and induction of the endoplasmic reticulum stress response[28]. Bioactivities of UA and its relevant mechanism of action have been summarized in *Table 1* . On the basis of understanding pharmacological and physiological activities, UA will be better developed to meet more daily and medical needs.

Table 1. Bioactivities of UA and related mechanisms of action

Bioactivities	**Mechanism of Action**
Antibacterial	effective on the biofilm of Gram-positive bacteria such as *Staphylococcus aureus* to prevent persistent
Anti-Angiogenesis	interference with Signal Transducer and Activator of Transcription 3 (STAT3), Mitogen-Activated Pr
Antihepatodamage	increase the β-oxidation of fat and inhibiting endoplasmic reticulum pressure.
Anti-Inflammatory	inhibits the phosphorylation of ERK and JNK, limits the activation of NF-κB, NF-AT and AP-1 in l
Antiviral	down-regulate HPVE6/E7
Anti-Tumor	regulate transcription factors expression and protein kinases related to tumorigenesis.
Anti-Oxidation	regulates the changes of reactive oxygen species levels induced by ultraviolet B.
Diabetes Treatment	inhibits the activity of α-amylase; reduces blood glucose levels and body mass index.

How to obtain UA and what's challenges for application

UA is widely distributed as triterpene saponin aglycone or free acid in various medicinal plants as well as sev-

eral fruits, such as*Ligustrum lucidum* [33], *Centella asiatica*[34], *Lysimachia clethroides*[35], *Crataegus pinnatifida* [36]and *Malus domestica* [37]. However, its percentage varies from species to species due to related biosynthetic enzymes and metabolism [38]. Currently, industrial production of UA mainly depends on extraction of natural plants. A variety of extraction methods have been developed to extract UA from different plants.

Table 2. Selected Methods for Isolation from Available Plant Sources for UA

Method	**Extraction and purification**
Microwave Method	Weigh the plant powder and mix with the appropriate amount of extractant. After soaking
Rapid Solvent Extraction	The fast solvent method can be implemented using systems introduced by related compani
Impregnation	Wash the fruit and leaves with distilled water, then air dry for a week to remove all water.
Soxhlet Extraction	Degrease 4 parts of 2.00g aliquots in Soxhlet extraction unit for 4 hours. After drying, 80%
Hot Reflux Method	The dried plant material was mixed with 100 mL of acetone. Connect with cooling water a
Ultrasonic Method	First, the ground material and solvent are mixed into the flask, and the flask is placed in a
Supercritical Fluid Extraction	First, the liquid carbon dioxide is pressurized to the required pressure in the coolant pump

However, plant cultures occupy a large area of cultivated land and plant growth may be affected by climate change and growth cycle, which subsequently reduces the adequate supply of raw materials. The challenges for UA's application also do include current production methods which consume a lot of organic reagents, and eventually produce waste liquids to cause serious ecological pressure to surrounding environment. So the traditional extractions of UA make it difficult to meet the commercial needs and environmental protection goals.

Structure modification to improve the efficacy of UA

Although UA exhibits remarkable physiological and pharmacological activities with effective and safe therapeutic profile, its poor water solubility, short plasma half-life and poor bioavailability limit further clinical applications [44]. In this regard, structural modifications of UA to cope with these limitations have been an attractive area of research to explore its valuable derivatives with enhanced pharmacological profile. In recent years, structural modification of UA is achieved by means of chemical transformation and microbial transformation to yield valuable derivatives.

4.1Chemical modification

UA structure is characterized by a hydroxyl group and carboxylic group which can be involved in the transfer of lone pair electrons to metal atoms [45]. According to the structural properties of reported derivatives, UA usually undergoes structural modifications on C-3/C-28 positions or the ring A of UA skeleton.

4.1.1Modifications on C-3/C-28

Researchers isolated UA and five triterpenoids from apple peel and synthesized a series of UA analogs. Structural modification of UA at C-3 indicated significant anti-proliferative activity [46]. In another study, acetyl group at the C-3 and an alkylamino and/or piperidine moiety at the C-28 enhanced the anticancer activity of UA derivative [46] (*Fig. 2A*). In addition, structural modification of UA by addition of piperazine moieties may enhance its anti-cancer properties. Addition of acyl piperazine motif at C-28 position while C-3 retains the polar group significantly enhanced the anticancer activity against breast cancer and gastric cancer cell lines [47] (*Fig. 2B*).

In order to clear on the mechanism of chemical modification at C-3 and C-28 in detail, researchers synthesized a series of UA derivatives by employing different electronic chemical modification at the two sites. Derivatives showed stronger cytotoxicity due to presence of positive charge (*Fig. 2C*), indicating that the increased lipophilicity may enhance the therapeutic potential of gastric carcinoma[48]. These findings of UA derivatives led the ways to structurally modify the UA skeleton to yield its valuable derivatives with diverse pharmacological properties.

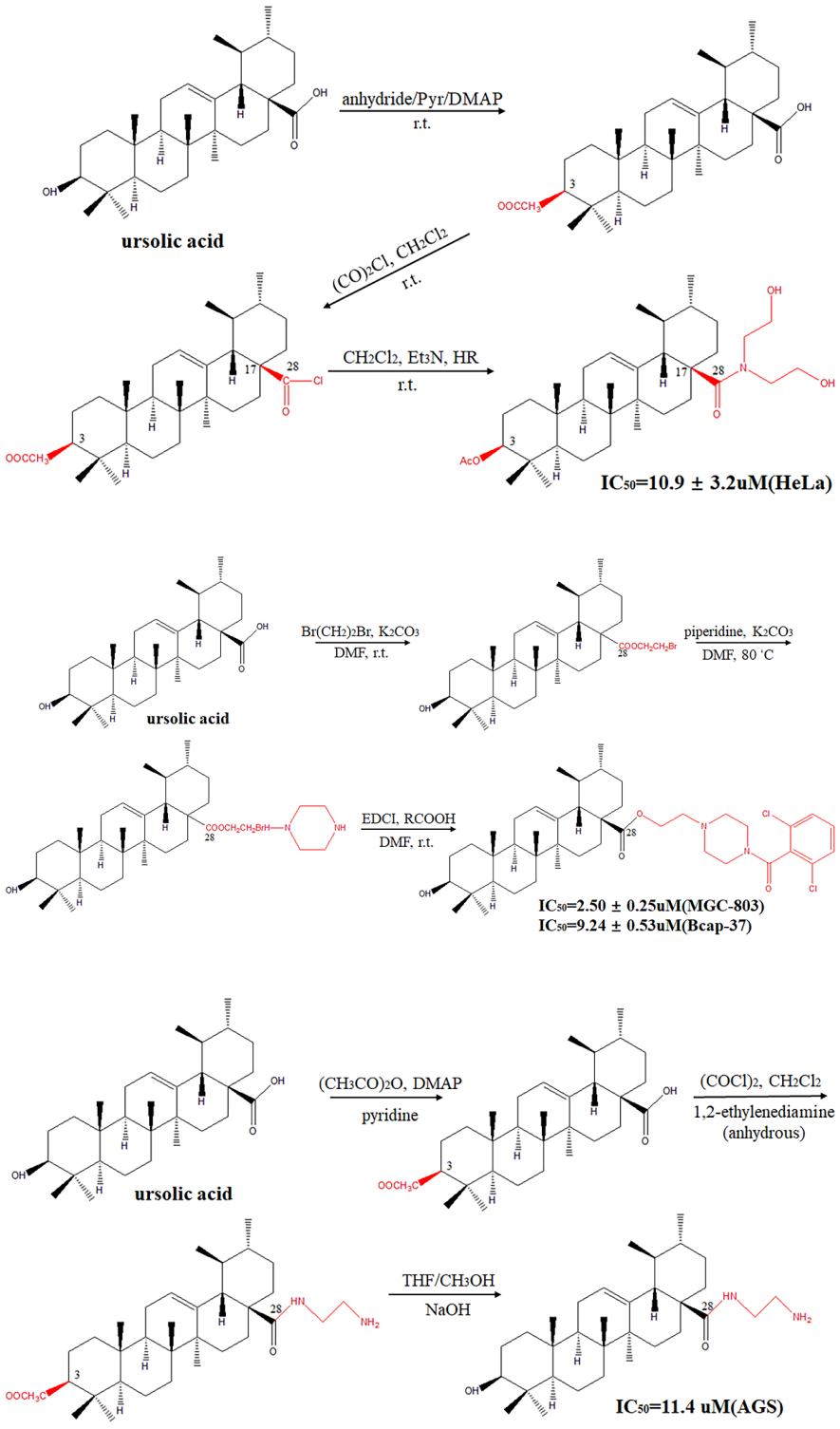


Fig. *2 Modification on C-3/C-28*

4.1.2 Modifications on ring A

UA and Oleanolic acid (OA) are isomers, the difference between which is only at the position of Me29(*Fig. 3*). Derivatives of OA such as 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) exhibit significant anti-inflammatory and anti-tumor activity due to the modification of the ring A [49,50]. Considering that both UA and OA are characterized by similar triterpene templates, similar chemical modifications have been applied to UA in recent years.

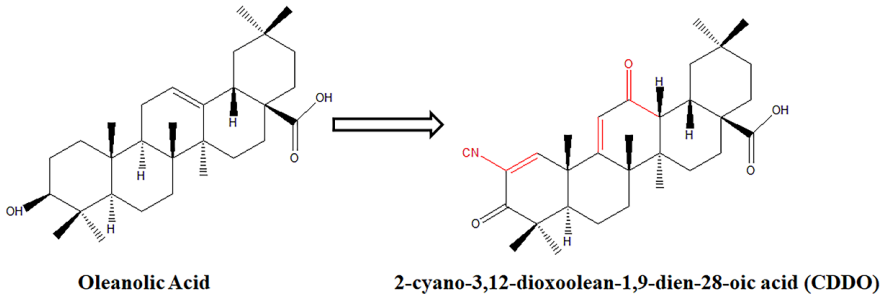
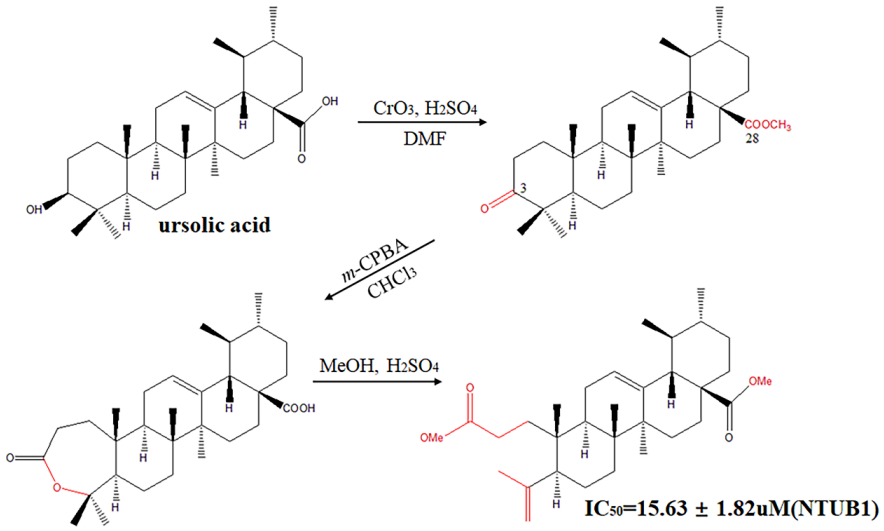


Fig. 3 *Oleanolic Acid and CDDO*

UA derivatives have been synthesized after oxidation, lactoseization and ring-opening reaction with increased cytotoxicity to NTUB1 cells compared to UA by 2-fold [51] (*Fig.4A*), providing new insights for the further development of its derivatives. Moreover, UA derivatives containing N-acyl imidazole or N-alkyl imidazole derivatives with the help of α,β-unsaturated ketone have also been characterized as significant anticancer activity [52](*Fig.4B*). Introduction of N-alkyl heterocyclic motifs bound to α,β-unsaturated ketones in ring A can provide better Michael receptors, which allows UA derivatives to interact with certain target proteins interaction potentially.



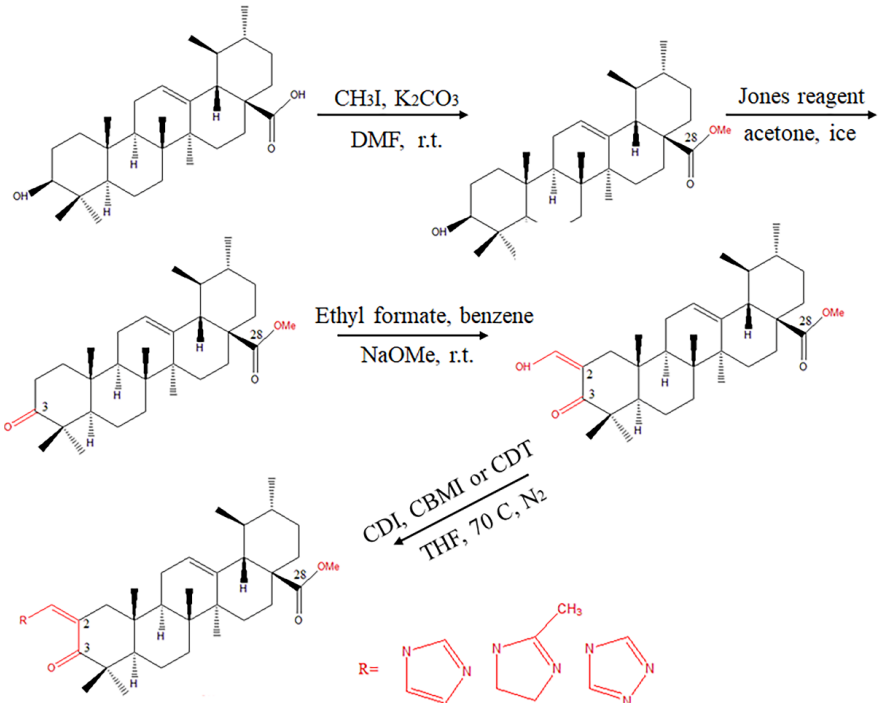


Fig. 4 *UA derivatives modified on ring A*

4.2Directed transformation of UA by microorganisms

Biosynthesis of valuable products have advantages over chemical synthsis in terms of milder reaction conditions and presence of abundant enzymes along with high stereo-, regio- and chemo- selectivities[53]. Therefore, microbial transformation is increasingly adopted as an alternative way to modify the structure of natural and synthetic compounds [54,55]. In particular, it is easy to achieve the glycosylation of pentacyclic triterpenes that is difficult during chemical synthesis [56]. Generally, UA biotransformation is carried out by dissolving the appropriate quantity of UA in strain suspended in fresh sterile broth under sterile conditions. After co-cultivation with strains, the transformed products of UA are eventually separated through suction filtration and extraction.

In recent years, various UA derivatives have been synthesized through the transformation of numerous microbial strains. It is reported that*Nocardia sp.* NRRL 5646 converts UA into oleanolic acid methyl ester (*Fig.5A*), in which two intermediates are formed by participation in the "reverse biosynthesis" involving methyl migration from C-19 to C-20 [57]. A single component is produced by this biotransformation followed by hydrolysis to yield the corresponding triterpenoid acid. At the same time, UA has been found to be transformed into different derivatives by the same strain (*Fig. 5B*). Researchers speculate that 3β-hydroxysteroid dehydrogenase and 3-ketosteroid-Δ1-dehydrogenase are involved in the transformation of steroid [58].

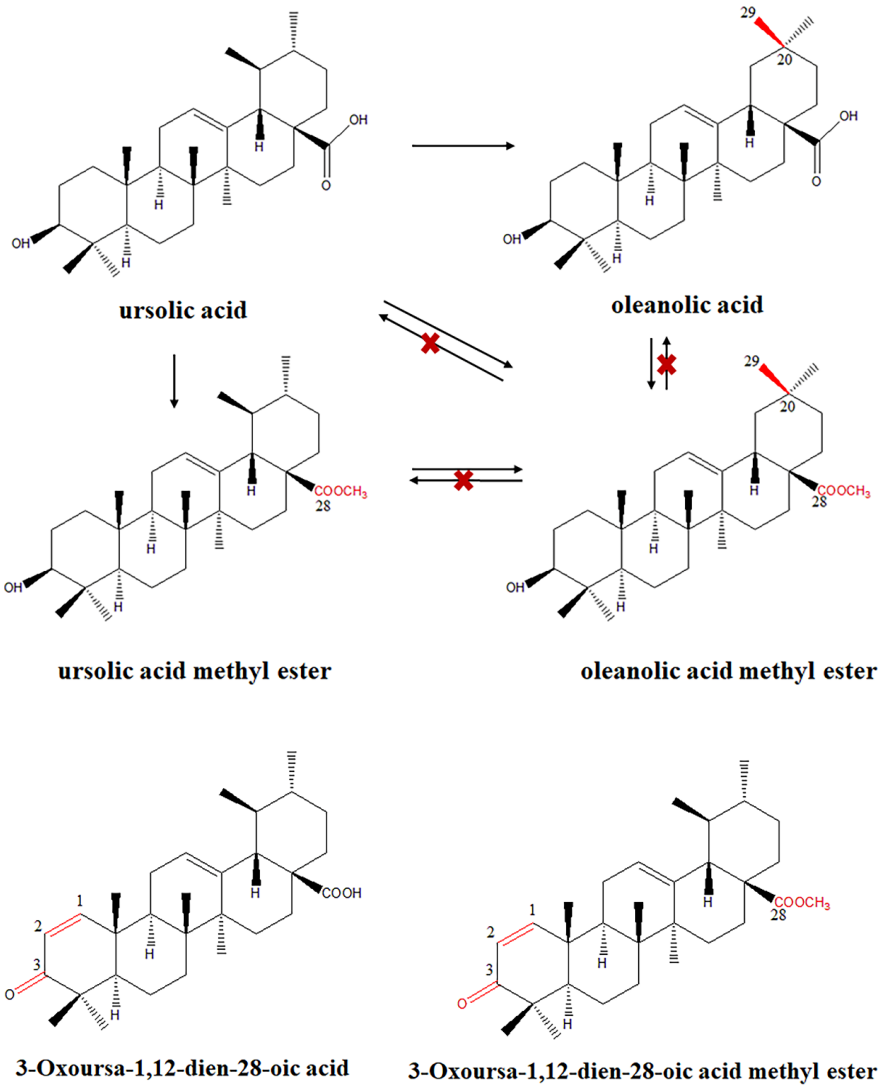
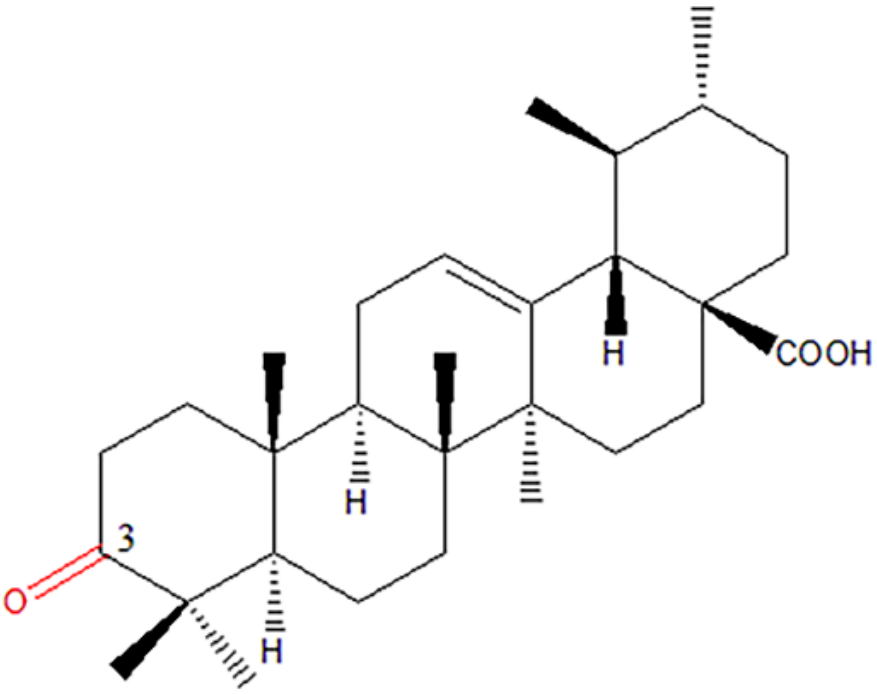
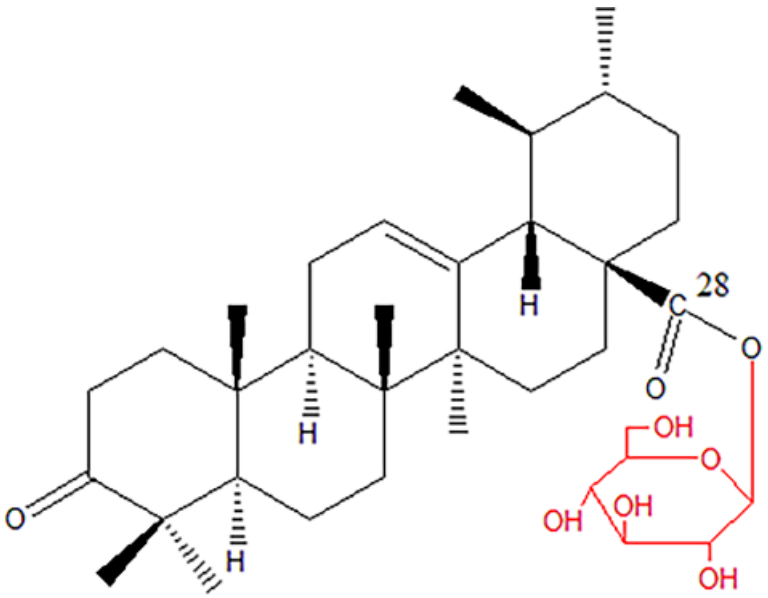


Fig. 5 *Transformation of UA by Nocardia sp. NRRL 5646*

Moreover, UA has been well modified by various fungi[59]. *Aspergillus flavus* (ATCC 9170) was able to convert UA into the new natural product 3-oxo-UA derivative with C-3 hydroxylation (*Fig. 6A*) [60]. In addition, four UA derivatives have been produced by *Syncephalastrum racemosum*(Cohn) Schroter AS3.264 [61], indicating the inhibitory effect against protein tyrosine phosphatase 1B (*Fig. 6C*). UA was also biotransformed into six glycosylated and hydroxylated products by an endophytic fungi *Alternaria longipes* AS3.2875[62]. One of the resultant new compounds, 28-o-β-d-glucopyranosyl-3-oxo-UA, showed stronger antibacterial activity against *Staphylococcus aureus* and methicillin-resistant*Staphylococcus aureus* (*Fig. 6B*). Liu *et al.*obtained three new derivatives transformed by *Mucor spinosus*AS3.3450 [63](*Fig.6D*). Among them, 3β,7β,21β-trihydroxy-UA showed stronger cytotoxicity against HeLa cells, K562 cells and KB cells, enlightening its potential therapeutic role in anticancer therapy.



3-oxo derivative of ursolic acid



28-o-β-d-glucopyranosyl-3-oxo-ursolic acid

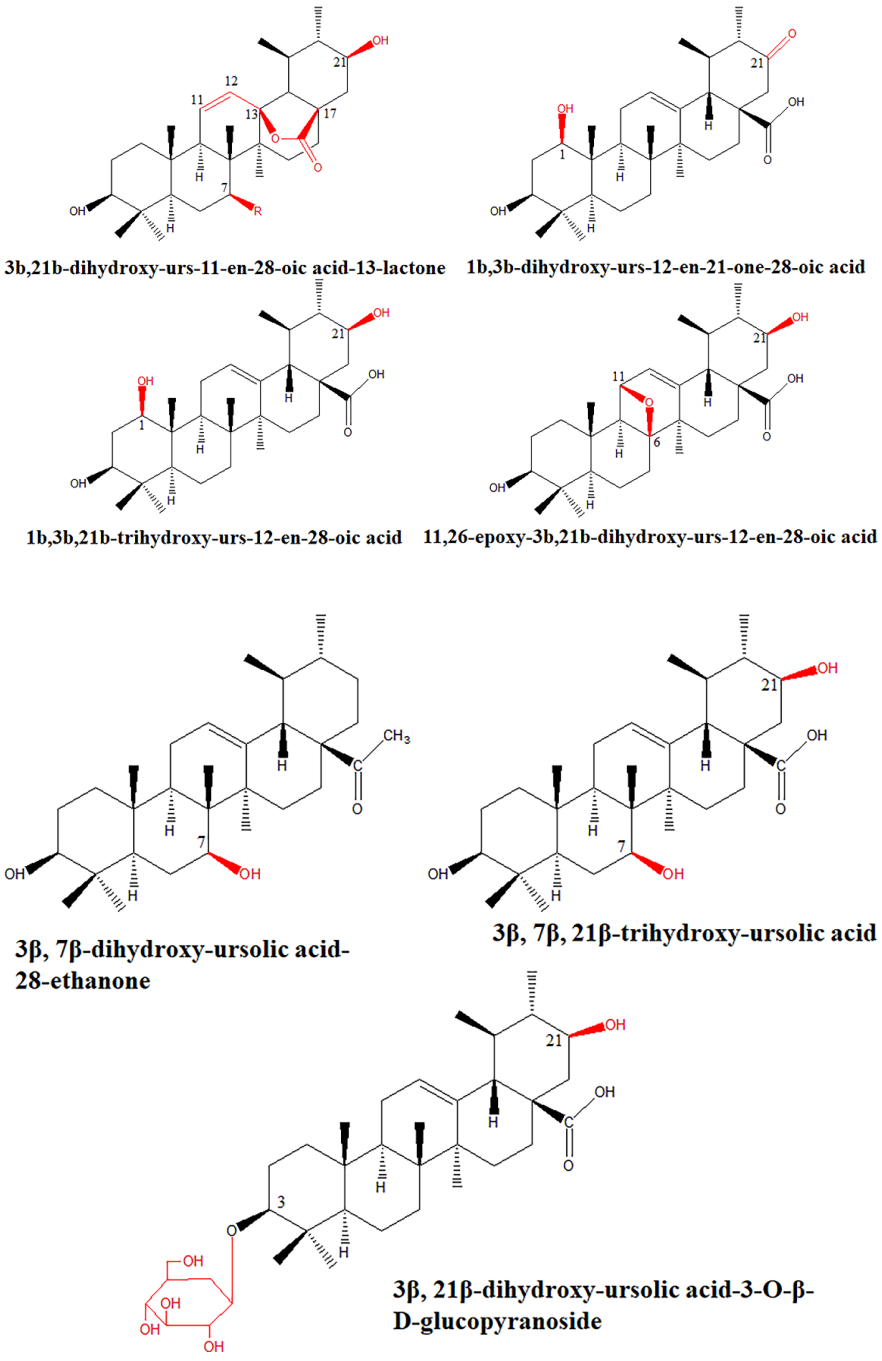


Fig.6 *UA derivatives biotransformed by fungi*

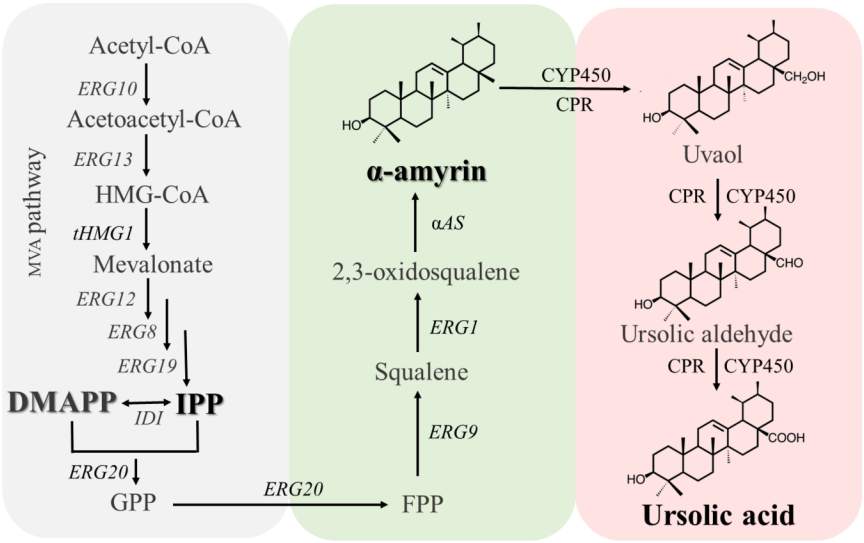
Efficient synthesis of UA and its derivatives in engineered microbial cell factories

As mentioned above, traditional extraction methods are increasingly unfavorable for large-scale production of UA. Furthermore, complex structure and oxidation position, as well as the optical purity of the final product all increase the difficulty for its chemical synthesis. Green biosynthesis of valuable compounds by microbial cell factories may reduce the limitations of traditional extraction methods from plants as it is not restricted by the natural climate and is suitable for large-scale industrial production under high density

fermentation. More importantly, some terpenoids are synthesized efficiently in microorganisms with various regulatory strategies, such as excavation and modification of key enzymes [18,64], optimization of endogenous metabolic pathways [65-67], inhibition of branching pathways [68-70]. Therefore, microbial engineering also has a wide application prospect in the green synthesis of UA and its derivatives.

5.1 The biosynthesis pathway of UA

In recent years, synthetic pathway of UA has been well explored in plants, which is roughly divided into three modules: synthesis of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), synthesis of α-amyrin and oxidation of α-amyrin. In plant organelles, IPP and DMAPP are synthesized by methyl erythritol-4-phosphate (MEP) pathway in plastids and mevalonate (MVA) pathway in cytoplasm. Afterwards, two IPP molecules are combined with one molecule of DMAPP end-to-end, and condense to farnesyl diphosphate (FPP). Two FPP molecules bind together to form the triterpenoid precursor, squalene followed by oxidiation to form 2,3-oxidosqualene, which subsequently undergoes cyclization into α-amyrin, the direct precursor of UA. Eventually, α-amyrin is oxidized at C-28 to form UA through a three-step oxidation reaction by employing the action of Cytochrome P450 (CYP450) monooxygenase and its redox chaperone Cytochrome P450 Reductase (CPR). In addition to the common rate-limiting enzymes for triterpenoid, α-amyrin synthase (αAS) and CYP450 greatly affect the synthesis of UA (*Fig. 7A*).



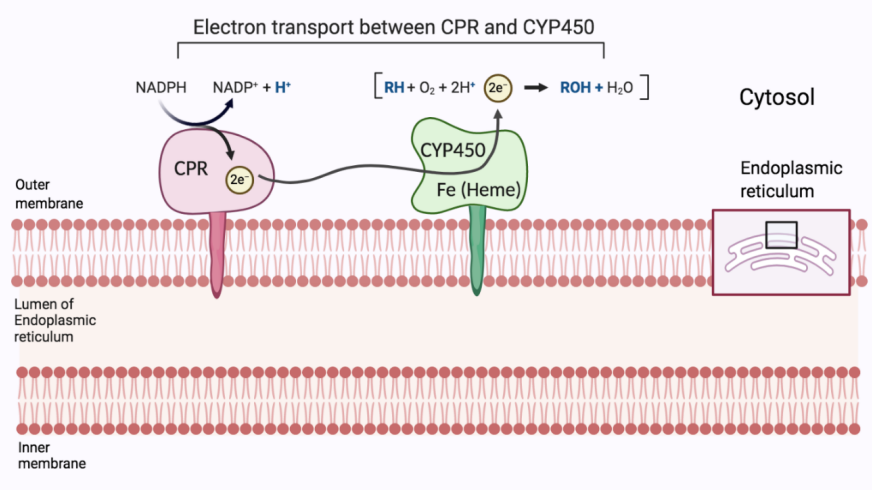


Fig. 7 *Biosynthesis of UA (A) Biosynthesis pathway; (B) The electron transfer between CYP450 and CPR*

It is generally believed that cyclization of 2,3-oxidosqualene is carried out by the Oxidosqualene Cyclase (OSC) by employing protonation, cascade cyclization of polyene addition, hydride and/or methyl translocation, and unwinding [71]. αASs are multifunctional enzymes, which catalyze 2,3-oxidosqualene to several pentacyclic triterpenoids like α-amyrin, β-amyrin and lupeol etc[72,73]. However, product isomerization limits their catalytic ability and ultimately affects the downstream synthesis of UA. CYP450s are most versatile proteases found in nature, the first one of which was discovered in the rat liver microsomes in 1958[74]. CYP450s contain the heme, which primarily binds to the cytoplasmic surface of Endoplasmic Reticulum (ER) and catalyze a series of oxidation reactions like oxidation, hydroxylation, dealkylation and breakage of carbon-carbon bonds [75]. Mainly, CYP450s catalysis involves the introduction of oxygen into inactive carbon-hydrogen bonds [76], while the corresponding reduction process is assisted by CPR. CYP450s utilize two electrons from NADH or NADPH transferred by CPR to the heme center of iron to activate the oxygen molecules and further catalyze the substrates to form functional groups.

5.2 De novo synthesis of UA in engineered microbial cell factories

With strong resistance to low pH, osmotic stress and environmental factors, engineered microbial cell factories such as *S. cerevisiae* is widely used to biosynthesize the valuable natural products on industrial scale. What's more, *S. cerevisiae* has proven to be an enormously suitable candidate for synthesis of complex terpenoids due to its endogenous MVA pathway, which provides sufficient precursors to synthesize the terpenoids to meet subsequent demand[65]. Terpene skeleton is modified by CYP450 in a better way due to presence of protein modification system, biofilm system and redox system in *S. cerevisiae* [18]. With clear genetic background and suitable biological safety, various gene manipulation and genetic modification methods have been widely employed in *S. cerevisiae*for the green synthesis of terpenoids.

As early as 2012, α-amyrin has been synthesized in microbial cell factories [77], but the UA synthesis was inadequate. It is generally accepted that UA biosynthesis in *S. cerevisiae* is limited due to the accumulation of α-amyrin and the oxidation ability of CYP450 [78]. These limitations led researchers to adopt metabolic engineering and synthetic biology approaches to synthesize UA more efficiently in *S. cerevisiae* . Dai *et al.* introduced CYP716A15 and CPR from *Vitis vinifera* and αAS from*Eriobotrya japonica* into *S. cerevisiae* . With ERG1, ERG 9 and ERG 20 overexpressed to enhance the precursor production, they subsequently got a final UA yield of 1.76 mg/L/OD_{600}[75]. In another study, Lu *et al.* heterologously expressed CYP716A12 from *Medicago Sativa* , αAS from*Catharanthus roseus* , ERG1 from *Candida albicans* , and CPR from *Arabidopsis thaliana* in *Saccharomyces cerevisiae* . By optimization of fermentation conditions and overexpression of tHMG1, ERG9, ERG20 to strengthen MVA pathway, the yield of UA in shake flask culture

reached 25.85mg/L [79].

5.3 Optimization strategies to enhance the microbial synthesis of UA

Biosynthesis of UA in microbial cell factories provides an environment friendly platform for synthesis of specialized terpenoids, while the bioproduction of UA still needs to be improved as compared to its counterpart triterpenes. Therefore, multiple optimization strategies are still needed to further develop the production potential of *S. cerevisiae.* On the basis of UA's synthetic pathway, we propose three strategies to enhance the green biosynthesis of UA at industrial scale.

5.3.1Strengthening precursors supplementation

Microbial biosynthesis of natural products is improved by adopting effective metabolic engineering approaches to enhance the precursors supply and reducing their unnecessary consumption. Common strategy includes the overexpression of MVA-related (e.g. ERG9, ERG1and ERG20) genes and tHMG1 (the truncated version of HMG1) gene. Lu *et al.*enhanced the supply of 2,3-oxidosqualene as mentioned above, resulting in 25.99 mg/L α-amyrin in shake flask culture [79].

Enhancement of precursor supply is also achieved by adopting the traditional metabolic engineering approaches to overexpress the key genes, utilization of balanced pathways and downregulation of competitive pathways [80]. In another study, researchers balanced the metabolic pathway and achieved transcriptional regulation of aligned oleanane-type triterpenoids by overexpression of UPC2-1, a global transcription factor for ergosterol synthesis in yeast[81]. In addition, they reconstructed the promoter at the binding site of UPC2 and the galactose regulatory network to promote gene expression, resulting in a 65 and 6.8-fold increase in β-amyrin and oleanolic acid, respectively [82]. This also provides a new idea for the synthesis and regulation of both α-amyrin and UA in yeast.

Furthermore, the catalytic potential of αAS also plays a decisive role in the synthesis of α-amyrin and UA. A highly active αAS, *Md* OSC1 from *Malus domestica* , have been identified through bioinformatics screening and phylogenetic analysis [37]. *Md* OSC1 expression combined with overexpression of genes related to the MVA pathway in *S. cerevisiae* yielded an α-amyrin titer of 11.97±0.61 mg/L [83]. Furthermore, the yield of α-amyrin was increased to 11-fold higher than that of the control with the triple mutant *Md* OSC1$^{N11T/P250H/P373A}$ by remodeling *Md* OSC1 [84]. Owing to these strategy, UA biosynthesis will be guaranteed by enhancing the supply of its precursors.

5.3.2Enhancing the coupling efficiency of CPR with CYP450

Lower microbial production of UA as compared to α-amyrin depicts the inefficient oxidation process which in turn depends upon both the catalytic efficiency of CYP450 as well as electron transfer between CYP450 and CPR [80]. In this regard, it is essential to excavate and characterize CYP450 and CPR with high catalytic efficiency. By means of gene linkage, genome sequencing and transcriptome sequencing, many CYP450s with the ability to oxidize C-28 of α-amyrin have been identified from *Arabidopsis thaliana* , *Medicago truncatula* , *Barbarea vulgaris* and other plants. Combining different sources of CYP with CPR and finding the best combination is a common way to improve its oxidation ability (*Table 3*).

Ταβλε 3. Οξιδατιον οφ ̃-28 ποσιτιον οφ α-αμψριν

CYP450	Source	Function
CYP716A1	*Arabidopsis thaliana*	C-28 oxidation of α-amyrin and β-amyrin; C-28 hydroxylation of lupeol
CYP716A2	*Arabidopsis thaliana*	C-28/C-22α hydroxylation of α-amyrin; C-16/C-22α/C-28 hydroxylation of β-a
CYP716A12	*Medicago truncatula*	C-28 oxidation of α-amyrin, β-amyrin and lupeol
CYP716A15	*Vitis vinifera.*	C-28 oxidation of α-amyrin, β-amyrin and lupeol
CYP716A44	*Lycopersicum esculentum*	C-28 oxidation of α-amyrin, β-amyrin
CYP716A46	*Lycopersicum esculentum*	C-28 oxidation of α-amyrin, β-amyrin
CYP716A48	*Oleaeuropaea.*	C-28 oxidation of α-amyrin, β-amyrin and lupeol

CYP450	Source	Function
CYP716A49	*Beta vulgaris.*	C-28 oxidation of α-amyrin, β-amyrin and lupeol
CYP716A50	*Coffea arabica.*	C-28 oxidation of α-amyrin, β-amyrin and lupeol
CYP716A52V2	*Panax schinseng*	C-28 oxidation of α-amyrin, β-amyrin and lupeol
CYP716A80	*Barbarea vulgaris*	C-28 oxidation of α-amyrin, β-amyrin and lupeol
CYP716A81	*Barbarea vulgaris*	C-28 oxidation of α-amyrin, β-amyrin and lupeol
CYP716A83	*Centella asiatica*	C-28 oxidation of α-amyrin, β-amyrin
CYP716A175	*Malus domestica*	C-28 oxidation of α-amyrin, β-amyrin, lupeol and germanicol
CYP716A179	*Glycyrrhiza uralensis*	C-28 oxidation of α-amyrin, β-amyrin and lupeol
CYP716A252	*Ocimum basilicum*	C-28 oxidation of α-amyrin, β-amyrin
CYP716A253	*Ocimum basilicum*	C-28 oxidation of α-amyrin, β-amyrin
CYP716A265	*Lagerstroemia speciosa.*	C-28 oxidation of α-amyrin, β-amyrin and lupeol
CYP716A266	*Lagerstroemia speciosa.*	C-28 oxidation of α-amyrin, β-amyrin and lupeol
CYP716AL1	*Catharanthus roseus*	C-28 oxidation of α-amyrin, β-amyrin and lupeol

In addition, exact mechanism of action of CYP450 and CPR is not clear yet and there is a need to explore the ultimate cause that affects the electron transfer efficiency between them. Analysis of the protein structure of CYP450 helps to elucidate the underlying causes of electron transfer from CPR to CYP450. In addition, with the advent of protein engineering along with high throughput screening, rational design and modification of CYP450 and CPR have attracted much attention, which with optimized structures have also become common means to solve the problem of insufficient oxidation [80].

5.3.3Optimization of subcellular structure

S. cerevisiae harbors diverse organelles with different structures and specialized functions. Rational design and harnessing of these organelles to produce valuable compounds is of great application prospect in terpenoid synthesis. Heterologous FPP synthase and sesquiterpene synthase to mitochondria by fusing the mitochondrial targeting signal peptide of yeast COX4, resulting in a 20-fold increase in sophoradiene production [98]. ER can also be subjected to morphological modifications such as increasing the membrane area to harbor more enzymes to enhance the catalysis processes. As many endogenous or exogenous proteins are located in ER membrane, like CYP450, the area of ER membrane have been significantly increased by knocking out the PAH1 by CRISPR/cas9 in *S. cerevisiae* to enhance the activity of enzymes related to terpene synthesis for efficient biosynthesis of terpenoids [99]. This engineering strategy increased the yield of β-amyrin, artemisinic acid, alfalfa acid and its glycosylated derivatives by 8-fold, 2-fold, 6-fold and 16-fold respectively, which showed great potential of microbial cell factories to enhance the yield of terpenoids.

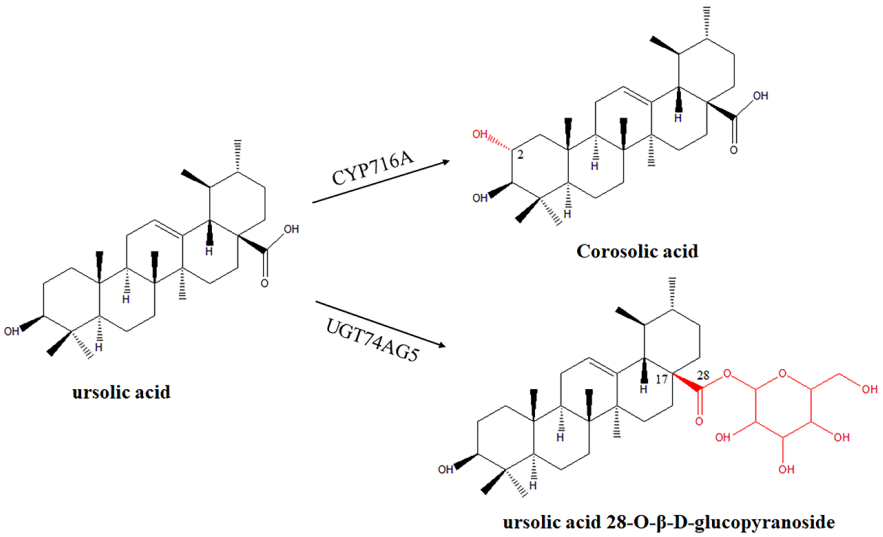
In addition, mass transfer efficiency can significantly boost the synthetic capacity of microorganisms [65]. The highest yield of α-amyrin was obtained in engineered *S. cerevisiae* by expanding the storage pool, where DGA1 (Diacylglycerol acyltransferase) was overexpressed to enhance the intracellular storage capacity[84]. Draw lessons from it, UA biosynthesis can also be improved by modifying chemical mass transfer such as the translocation of its synthesis and the condition of transportation.

By combining all these strategies including the precursor supplementation, enhancing the coupling efficiency of CPR with CYP450 and optimization of subcellular structure, de novo biosynthesis of UA will be significantly enhanced in microbial cell factories. These studies fully demonstrated the potential of utilizing *S. cerevisiae* cell factories to synthesize UA efficiently, providing an effective means to replace traditional extraction and chemical synthesis.

5.4UA derivatives decorated by engineered microorganisms

Biosynthesis of UA derivatives by construction of metabolic pathways in engineered microbial cell factories have been promoted along with the development of de novo microbial synthesis of UA. In recent years, biosynthesis of UA derivatives mainly involved in hydroxylation on C-2α and glycosylation.

For C-2α hydroxylation of UA, sequence analysis of RNA of *Avicennia marina* leaves revealed the functional CYP716C53, which catalyzed the C-2α hydroxylation of UA to yield Corosolic acid (*Fig. 8*) [100]. It's a triterpenoid compound which has attracted commercial and research interest for unique anti-diabetic properties [101]. In addition, the heterologous expression of *Lagerstroemia speciose* -CYP716C55 in *S. cerevisiae* also led to C-2α hydroxylation of UA [96]. Moreover, CYP716C49 was identified in *Crataegus pinnatifida* and its expression along with the αAS from *E. japonica* as well as CYP716A15 and CPR from *V. vinifera* in microbial cell factories increased the production titer of Corosolic acid to 141.0 mg/L [75].



Φιγ. 8 *Στρυστυρες οφ ὂροσολις Αχιδ ανδ Υρσολις Αχιδ 28-O-B-Δ-Γλυχοπψρανοσιδε*

Glycosylation involves the attachment of one or more sugar moieties to the parent compound and contributes for diverse physicochemical properties, such as water solubility, structural integrity and pharmacological efficacy [80]. To achieve the glycosylated derivatives, heterologous expression of UDP-glycosyltransferases (UGTs) is less frequent due to their lower in vivo catalytic activity. In relation to glycyrrhetinic acid, structural diversification of UA by glycosylation is still in infancy. In one study, transcriptomic data analysis of *Ilex Asprella* revealed UGT74AG5 and its expression in UA-producing *S. cerevisiae* strain confirmed its glycosylation activity against UA to synthesize its glycosylated derivative, UA-28-O-β-D-glucopyranoside[102] (*Fig. 8*). Although the synthesis of UA derivatives in cell factories is still in its infancy, the de novo synthesis of UA in *S. cerevisiae* provides strong support for its development.

Perspective

UA exhibits significant therapeutic properties to be used in disease management and drug development. UA derivatives with different structural modifications have been designed and synthesized to explore more effective therapeutic products with higher oral bioavailability and efficacy. Particularly, many structurally modified UA derivatives by chemical means seem to be effective against variety of cancer cell lines in vitro. Due to the great value potential and pharmacological manifestations of UA and its derivatives, there is a need to develop an economical and feasible approach to produce the high yield of UA and its varied derivatives.

Although considerable efforts are currently being made to develop effective approaches for sequestering UA from various medicinal plants, its biosynthesis in microbial cell factories is a more attractive strategy. In this regard, *S. cerevisiae* presents a potential microbial host to synthesize terpenoids efficiently. However, bioengineering strategies for bioproduction of either UA or its derivatives have not been developed effectively. With the advent in synthetic biology and metabolic engineering, improved metabolic pathways and key enzyme-protein engineering lead to efficient biosynthesis of valuable products, which provides strong support

for better utilizing*S. cerevisiae* as a cell factory. Increased production of terpene derivatives in *S. cerevisiae* can be achieved by optimization of endogenous pathways, employing CRISPR gene editing technology, modification of key enzymes and utilization of synthetic *S. cerevisiae* chromosome system (SCRaMbLE). More importantly, there are powerful tools such as computational biology, genomics and transcriptomics accompanied by synthetic biology to identify and manipulate novel terpenoid synthesis pathways in microbial cell factories for their green production. Advances in synthetic biology technology will promote to develop a fully automated robotic screening platform for high-throughput screening of engineered strains. Combination of different regulation strategies to design and improve the metabolic pathways in *S. cerevisiae* is expected to be an efficient approach over the traditional methods to sequester UA and its derivatives in future for further exploration. Although there is a long way to go to develop an economically feasible and easy method for UA, its rich pharmacological activity and great value potential are worthy of extensive exploration in many scientific fields.

List of abbreviations

S. cerevisiae = *Saccharomyces cerevisiae*

UA = Ursolic acid

IPP = Isopentenyl diphosphate

DMAPP = Dimethylallyl diphosphate

FPP = Farnesyl diphosphate

CYP450 = Cytochrome P450

UGTs = UDP-glycosyltransferases

αAS = α-amyrin synthase

OSCs = Oxidosqualene Cyclases

ER = Endoplasmic reticulum

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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