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Review

Biotransformation of triterpenes

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ABSTRACT

Triterpenes are a versatile group of biologically active ingredients present in several phytoextracts. They often occur as glycoconjugates with a distinct bioactivity from their aglycone counterparts. Deglycation of triterpenes can be performed by enzymatic and chemical reactions, whereas biotransformation is generally more specific and occurs without unwanted alterations to the molecule. Further structural modification of the triterpenes to enhance their pharmaceutical relevance can be efficiently carried out by the application of biotransformational processes using microorganisms or isolated enzymes. In particular, the whole-cell process is favorable if cofactor-dependent modifications are preferred, such as hydroxylations/oxidations of the lead compound. However, the isolation of triterpenes from domestic plant material has some disadvantages such as extract variability and instability. These problems can be overcome through the use of cell culture technology, where the *ex vivo* cultivated cells are used for the reproducible production of the target compound.

The relevant topics that should be addressed to establish a (biotransformational) production process of pharmaceutically relevant triterpenes are covered in this review. In particular, we focus on the production process using tissue engineering, the corresponding analytical techniques, and the biotransformation reaction required to obtain bioactive compounds from precursor molecules.

Contents

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1. Introduction

Triterpenes belong to the biologically versatile group of terpenes, which are composed of isoprene subunits and consist of approximately 30,000 identified compounds [1]. In relation to the number of isoprenoid units, terpenes are subdivided into monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterpenes (C_{25}), triterpenes (C_{30}), and tetraterpenes (C_{40}), where the carbon skeleton is acyclic, or contains mono-, bi-, tri-, tetra-, and pentacyclic structures, respectively.

The terms triterpenes and triterpenoids are often used to describe the same C_{30}-terpene compound, but differentiation between both expressions is required. Whereas triterpene is used as a collective term to describe natural occurring terpenes, the broader expression triterpenoid also covers natural degradation products, for example, ionones as well as natural and synthetic derivatives such as terpene alcohols, aldehydes, ketones, acids, esters, epoxides, and hydrogenation products [2]. Due to the relatively complex structures of terpenoid compounds, they are usually referred to by their trivial names instead of systematic IUPAC nomenclature. Due to the methyl-group transfer reaction via *S*-adenosylmethionine, some tetracyclic triterpenes are composed of 31 carbon atoms.

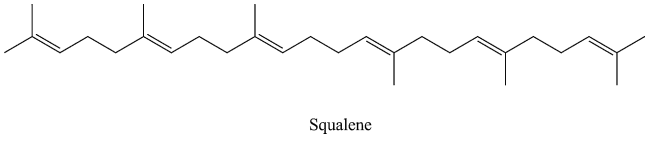
Terpenes have a widespread occurrence and can be found in all organisms, i.e., in prokaryotic as well as eukaryotic systems. However, most of the bioactive terpenes have been detected in higher plants. Whereas mono- and sesquiterpenes are predominantly found in essential oils of plant raw material, the higher terpenes, such as triterpenes, are mainly found in balsams and resins [3,4].

The simplest triterpene with respect to the complexity of its carbon skeleton is the compound squalene (Fig. 1); this compound first isolated from fish liver oils but is also found in plant oils and mammalian fats [5]. Because of its unsaturated bonds, the squalene structure features reactivity and thermolability [6]. Squalene is the precursor of the diverse group of polycyclic triterpenes and is itself produced via head-to-head condensation of two C_{15} units of farnesyl diphosphate [2]. Although cyclization occurs in the presence of specific enzymes, the so-called cyclases, the cyclization products depend on the folding of squalene prior to cyclization.

From a biological perspective, it is assumed that the most important triterpenoid structures are the oleanane, ursane, lupane, and dammarane–euphane carbon skeletons [1] (see Fig. 2). These polycyclic structures can occur as free triterpenoids or triterpenic glycosides as well as their precursors. The corresponding biological effects of such terpenoids are very diverse and can be summarized as follows: anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory, and tonic effects [1]. Further information about isolation and bioactivity of manifold triterpene derivatives can be found in recently published reviews [7,8].

The global market for plant-derived drugs has been estimated to be approximately 30.69 billion USD for the last decade, with phytochemicals such as terpenes and steroids representing the most significant fraction with estimated annual sales of 12.4 billion USD [9]. Because biological raw materials must be derived from well-known plant sources with reproducible content levels of the drug or its precursors, domestic cultivation of the plant sources has several advantages compared to harvesting from the wild. These advantages include a decrease in misidentification, genetic and phenotypic variability, extract variability and instability, toxic compounds, and contaminants [10]. However, two-thirds of the 50,000 different medicinal plant species in use are collected from the wild, whereas in Europe, only approximately 10% of medicinal species used commercially are cultivated [10]. To fulfill the pharmaceutical guidelines, the production and extraction of the corresponding triterpene/triterpenoid should be as reproducible as possible. Therefore, the use of tissue engineering and cell culture techniques may enable an expansion of the cultivation of medicinal plant ingredients. Such an *ex vivo* production of triterpenes offers the opportunity to optimize the yield of the target compound and to obtain a uniform, high quality product. As previously mentioned, many triterpenes exhibit significant biological activity, but several triterpenoids possess hemolytic and cytostatic properties that restrict their pharmaceutical use. To overcome these limitations and to expand the range of usable triterpenes, a transformation of the compound by means of chemical or biotechnological techniques is possible. However, biotransformation is preferred to achieve a modification with an optimal regio- and enantioselectivity.

In this review, we provide an overview of the relevant topics that must be addressed to establish the processes for the production of pharmaceutically relevant triterpenes. Thus, triterpene production by tissue engineering, the corresponding analytical techniques, and the necessary biotransformation reactions to obtain bioactive compounds from precursor molecules are covered here.



Squalene

Fig. 1. Molecular structure of squalene.

2. Analysis of triterpenes

Triterpenes are found in various plants such as flowers, leaves, bark, cork, and wood. This material has to be pre-treated prior to isolation of the corresponding compound. First, the plant material is usually dried (air-dried at room temperature [11], at 40 °C [12] or using a microwave oven [13]), then ground into a powder (e.g., by mills with rotating blades or in a mortar [11] under N_2 liquid, respectively) and sieved [12]. Extractions are carried out with ethanol [14], methanol [15,11,13,16], chloroform or ethyl acetate by maceration [16], using a Soxhlet extractor [15,12] or by ultrasonic extraction [12,13]. The samples can be subjected to a solid phase extraction (SPE) clean-up procedure [12]. The resulting plant extracts can be directly subjected to chromatography or dried (under reduced pressure [14,12,16], by lyophilization [15]) and further extracted with other solvents.

Sample preparation for analytical steps (e.g., for triterpene analysis after biotransformation) is done by removing an aliquot of the culture broth. Subsequently, the fraction can be extracted with ethyl acetate [17–19] or ethyl acetate/2-propanol (8:2) [20]. The organic phase can then be separated by centrifugation and concentrated under reduced pressure. The resulting extract can be further dried over anhydrous Na_2SO_4 [20].

For isolation of a specific triterpene, the plant extract can be sequentially fractionated by silica gel columns used with various solvents [12,20].

A very simple analytical technique to identify triterpenes is thin layer chromatography (TLC). TLC is commonly carried out on silica gel plates. The extracts are separated in different solvent systems (dichloromethane–petroleum-ether [11], dichloromethane–methanol–acetic acid [11] chloroform–methanol [17,20]). Detection is performed by treating the plate with an anisaldehyde reagent [11,20], an acetic anhydride reagent [11] or 10% sulfanilic acid in ethanol [17], followed by heating. This procedure colorizes the corresponding analyte.

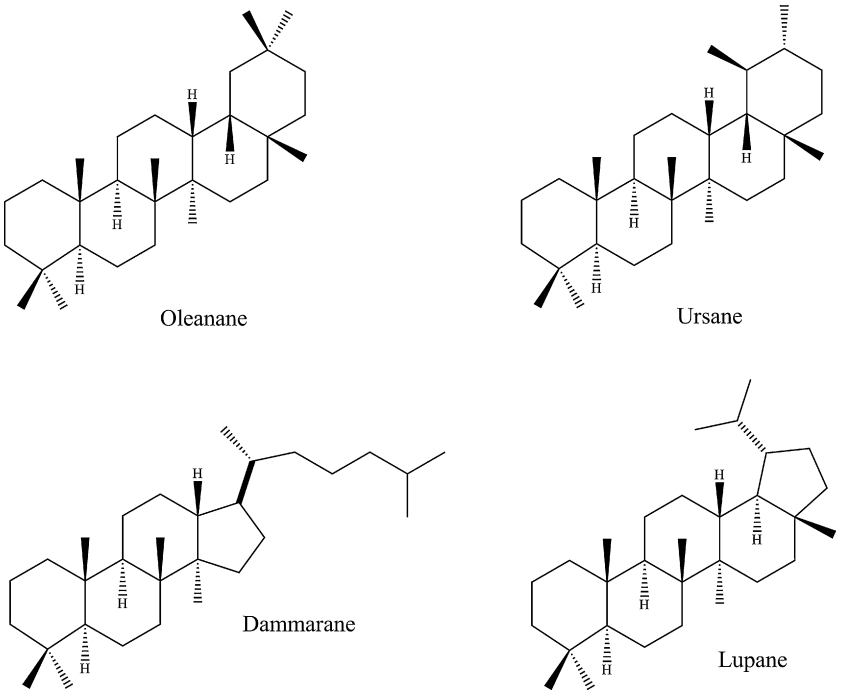


Fig. 2. Molecular structures of oleanane, ursane, lupane and dammarane skeletons.

Gas chromatography (GC) can be applied to determine the concentration of the target triterpenes. GC analysis requires a derivatisation reaction such as silylation, acylation or alkylation to make the analytes volatile. Silylation is the most commonly used method for triterpenes and is described below. For derivatisation, extracts are dried in a vacuum drier. After addition of the silylation mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane (50:40:10), the samples are heated to 75 °C for 20 min. Separation of the compounds is then achieved on a HP5 column (30 m × 320 μm i.d.; 0.25 μm liquid phase) with helium as the carrier gas. A flame ionization detector is used to analyse the fractions [11].

A much more sophisticated approach utilizes a mass spectrometer that is coupled to the GC system. For GC/MS analysis electrospray ionization (ESI-MS) is used in the negative mode. Silylation is achieved by adding pyridine–hexamethyldisilazane–trimethylchlorosilane (50:40:10) to the sample and is then heated to 75 °C for 20 min. A HP5 column (30 m × 320 μm i.d.; 0.25 μm liquid phase) is used for the separation process with helium as the carrier gas [11]. For GC-EI/MS, the dried dichloromethane-soluble plant extracts are treated with an excess of diazomethane in sealed vials. The samples are sonicated to produce methylated extract samples. After the removal of the solvents under reduced pressure, the resulting residues are re-dissolved in dichloromethane. A fused silica capillary column is then used with helium as a carrier gas [16].

A very common approach for analysis of biotransformation experiments makes use of liquid chromatography, in particular HPLC analysis. Prior to analysis, the residue is usually dissolved in methanol and filtered through a syringe filter to remove suspended particles that can destroy the column. Reversed-phase columns (RP) and isocratic methods with UV-detection are commonly used to analyse the samples. The mobile phase can be acetonitrile–water (91:9 v/v) [18], acetonitrile-1.25% H_3PO_4 (86:14 v/v) [14], acetonitrile–water acidified to pH 3 with phosphoric acid (9:1) [12], methanol-0.1% formic acid (92:8) [19], methanol-0.03 mol/L phosphate buffer pH 2.8 (88:12 v/v) [13], or methanol–water–tetrahydrofuran (94:5:1 v/v) [15]. The compounds are detected at 206 nm [14], 210 nm [12,13,18], 212 nm [19] and 220 nm [15], respectively. Furthermore, HPLC methods with evaporative light scattering detection (ESLD) have been used. The measurement can be carried out on a RP column using water (containing 5% methanol and 0.05% TFA) and methanol (containing 0.05% TFA) as the mobile phase with gradient conditions [21]. A choice of mobile phases (e.g., acetonitrile–chloroform) is proposed for an analytical column packed with porous graphitic carbon (PGC) [22].

If the HPLC is coupled to a mass spectrometer, then more detailed information concerning the mass and structure of the corresponding fraction can be obtained. The HPLC-MS approach requires a volatile mobile phase. Neutral triterpenes (which possess only one hydroxyl group in the molecule) cannot be easily ionized by electrospray mass spectrometry (ESI). However, atmospheric-pressure chemical ionization (APCI) or atmospheric-pressure photoionization (APPI) are suitable for the ionization of the triterpenes [23]. HPLC-$APCI_{pos}$-MS/MS analyses can be carried out with a RP C18 column with methanol-0.1% formic acid (92:8 v/v) as the mobile phase applying an isocratic method [19].

More structural information concerning the carbon skeleton itself can be gained by the use of HPLC-NMR. For NMR analysis, it is necessary to use deuterated solvents. ^{1}H NMR spectra can be recorded by HPLC-NMR in the “stopped-flow” mode, where the HPLC pump stops when the compound is in the flow probe of the coupled NMR spectrometer to perform the spectra. A mobile phase with acetonitrile-d_3 and D_2O (92:8 v/v) can be applied using an isocratic method with a RP C18 column [19].

3. Biotransformation of triterpenes

3.1. Microbial biotransformation of triterpenoids

Microbial transformation of xenobiotics is a very useful approach to expand the chemical diversity of natural products [24]. Advantages often associated with biocatalysis are the increased regio- and stereoselectivities and the environmentally friendly reaction conditions. Moreover, microbial transformation is often the only rational way to convert a precursor molecule to a desired

Table 1
Examples of triterpenoid biotransformations. For each triterpenoid, the transforming microorganism together with the type and site of the reaction catalyzed are given.

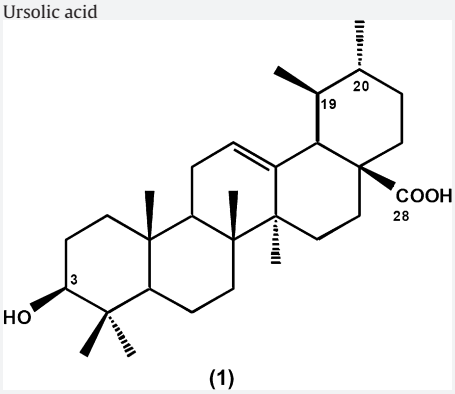
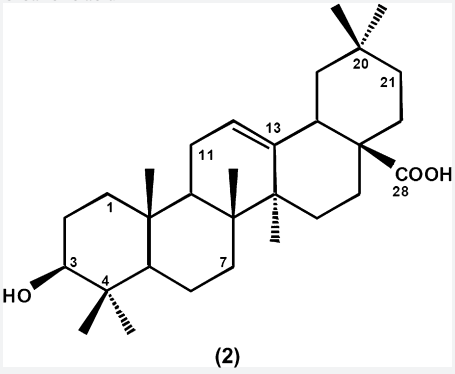
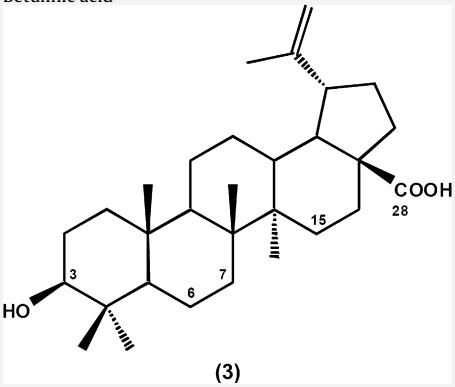
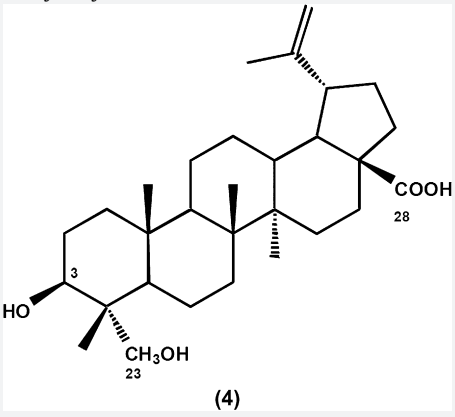
Triterpenoid substrate	Microorganism	Reaction	Ref.
Ursolic acid  (1)	*Nocardia* sp. NRRL 5646	Methyl esterification of the C-28 carboxyl group.	[27]
		Conversion from the ursane to the oleanane skeleton by a methyl group migration from C-19 to C-20 site.	
	Aspergillus flavus ATCC 9170	Dehydrogenation of the C-3 secondary alcohol group.	[30]
Oleanolic acid  (2)	*Nocardia* sp. NRRL 5646	Methyl esterification of the C-28 carboxyl group.	[27]
	Cunninghamella blakesleena	Diverse hydroxylations at the C-1β, C-7β, C-13β sites. Dehydrogenation of the C-3 secondary alcohol group. Lactone formation.	[8]
	Chaetomium longirostre RF-1095	Oxidative ring-A cleavage, hydroxylation at the C-21β site.	[42]
Betulinic acid  (3)	*Nocardia* sp. NRRL 5646	Methyl esterification of the C-28 carboxyl group.	[27]
	Bacillus megaterium ATCC 14581	Dehydrogenation of the C-3 secondary alcohol group. Hydroxylation at the C-6α and C-7β sites.	[28]
	Bacillus megaterium ATCC 13368	Dehydrogenation of the C-3 secondary alcohol group. Hydroxylation at the C-7β and C-15α sites.	[29]
	Cunninghamella elegans ATCC 9244	Hydroxylation at the C-1β and C-7β sites.	[28]
	Cunninghamella sp. NRRL 5695	Introduction of a β-D-glycopyranosyl at the C-28 carboxylic acid group.	[41]
23-Hydroxybetulinic acid  (4)	*Nocardia* sp. NRRL 5646	Methyl esterification of the C-28 carboxyl group.	[27]

Table 1 (*Continued*)

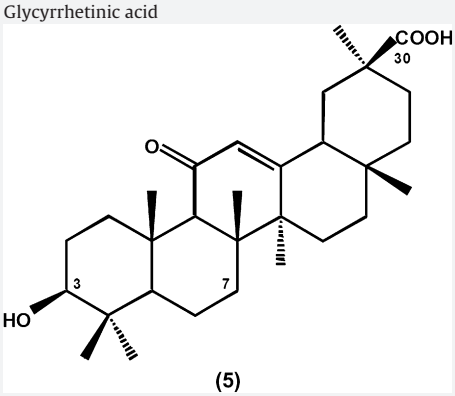
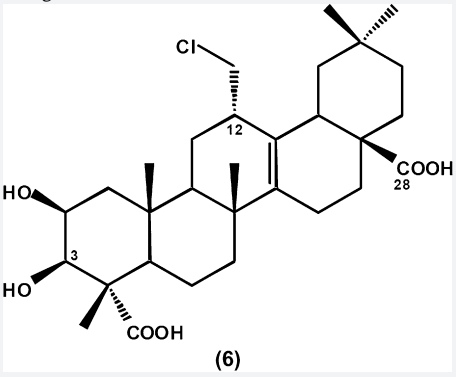
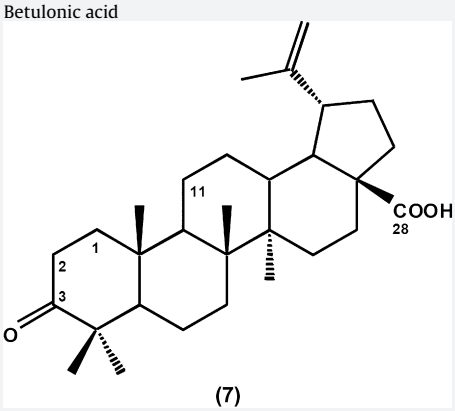
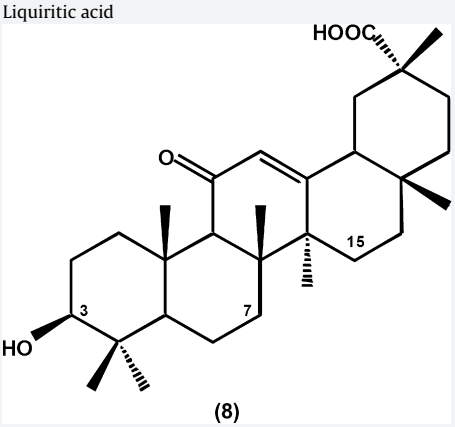
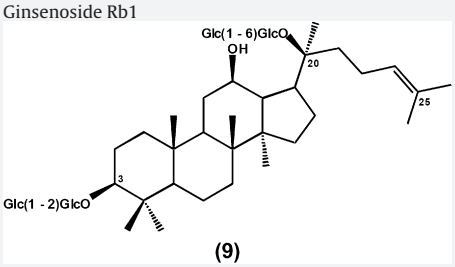
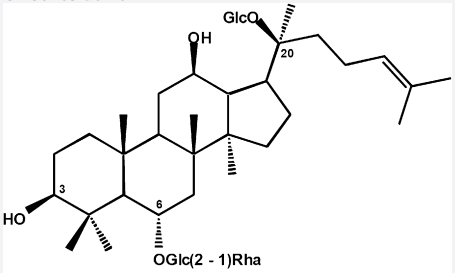
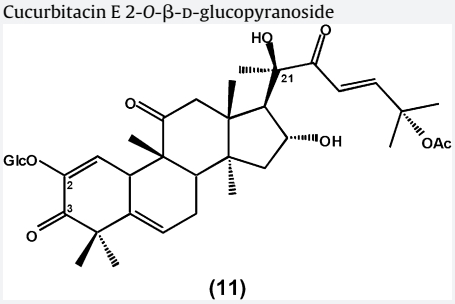
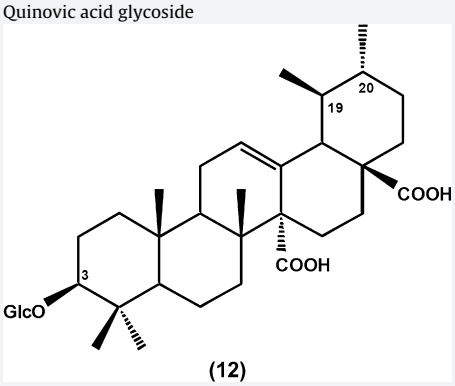
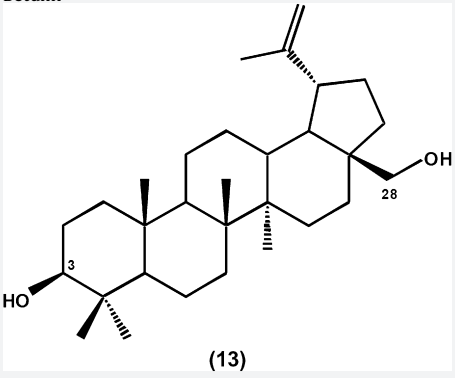
Triterpenoid substrate	Microorganism	Reaction	Ref.
Glycyrrhetinic acid  (5)	*Nocardia* sp. NRRL 5646	Methyl esterification of the C-30 carboxyl group.	[27]
	Curvularia lunata ATCC 13432	Hydroxylation at the C-7β site as main reaction.	[33]
	Mucor spinosus AS 3. 3450	Hydroxylation at the C-7β site as main reaction.	[8]
	Mucor polymorphosporus	Hydroxylation at the C-7β site as main reaction.	[8]
Senegenin  (6)	*Nocardia* sp. NRRL 5646	Methyl esterification of the C-28 carboxyl group.	[27]
		C–C bond cleavage at the C-12 site.	
Betulonic acid  (7)	*Nocardia* sp. NRRL 5646	Methyl esterification of the C-28 carboxyl group.	[26]
		Ketone α-hydroxylation at the C-2 site.	
	Bacillus megaterium ATCC 13368	Hydroxylation at the C-1 or C-11 site.	[29]
	Chaetomium longirostre RF-1095	Oxidative ring-A cleavage, hydroxylation, decarboxylation.	[43]
Liquiritic acid  (8)	*Curvularia lunata* ATCC 13432	Hydroxylations at the C-7β and(or) C-15β sites.	[8]
	Cunninghamella ATCC 3229	Hydroxylations at the C-7β and(or) C-15β sites.	[8]
	Mucor griseo-cyanus ATCC 1207-a	Hydroxylations at the C-7β and(or) C-15β sites.	[8]

Table 1 (*Continued*)

Triterpenoid substrate	Microorganism	Reaction	Ref.
Ginsenoside Rb1  (9)	*Aspergillus niger* KTC 6909	Deglycosylations at the C-3 and C-20 sites.	[36]
	Aspergillus niger AS 3.1858	Deglycosylations at the C-3 and C-20 sites.	[36]
	Aspergillus usamii KTC 6956	Deglycosylations at the C-3 and C-20 sites.	[36]
	Fusarium sacchari	Deglycosylations at the C-3 and C-20 sites.	[38]
	Bacillus megaterium GP27	Deglycosylations at the C-20 site.	[39]
	Curvularia lunata AS 3.1109	Deglycosylations at the C-20 site. Addition of a tertiary hydroxyl group at the C-25 site by hydration of the double bond.	[37]
	Rhizopus stolonifer AS 3.822	Deglycosylations at the C-3 and C-20 sites.	[37]
Ginsenoside Re  (10)	*Aspergillus niger* KTC 6909	Deglycosylations at the C-6 and C-20 sites.	[36]
	Aspergillus niger AS 3.1858	Deglycosylations at the C-6 and C-20 sites.	[36]
	Aspergillus usamii KTC 6956	Deglycosylations at the C-6 and C-20 sites.	[36]
Cucurbitacin E 2-*O*-β-D-glucopyranoside  (11)	*Curvularia lunata* NRRL 2178	Deglycosylations at the C-3 site. Oxidation reactions on the side chain.	[40]
Quinovic acid glycoside  (12)	*Nocardia* sp. NRRL 5646	Deglycosylations at the C-3 site. Methyl group migration from C-19 to C-20.	[24]
Betulin  (13)	*Aspergillus foetidus* ZU-G1	Transformation of the C-28 carboxyl group into a C-28 hydroxyl group.	[25]
	Aspergillus oryzae AS 3.498	Transformation of the C-28 carboxyl group into a C-28 hydroxyl.	[25]

product [25], whereas chemical conversion, especially of complex or multiply functionalized substrates usually suffers from low specificities. In the past decades, several studies have demonstrated that microbial transformation is a versatile tool to enlarge the structural diversity of triterpenoids [26]. A great challenge for the realization of a desired biotransformation reaction is finding the appropriate microorganism. Thus, classical screening of a series of microbial strains is still the most widely used technique. Very recently, a comprehensive review on microbial transformation of triterpenoids was published [8]. This chapter of the current review focuses on a selection of microbial strains frequently cited in the respective literature and the corresponding biotransformation reactions exemplified with tetra- and pentacyclic triterpenoids. The microbial transformations discussed are summarized in Table 1. For each substrate, the type and the site of the functionalization reaction carried out by the transforming strain are provided.

Several natural product biotransformations with *Nocardia* sp. NRRL 5646 have been described in the past [24]. However, biotransformation of triterpenoid acids by this strain has only been reported recently [27]. This bacterium metabolized six different pentacyclic triterpene acids such as ursolic acid (**1**), oleanolic acid (**2**), betulinic acid (**3**), 23-hydroxybetulinic acid (**4**), glycyrrhetinic acid (**5**), and senegenin (**6**) to selectively yield their corresponding 28-methyl esters. Among these transformations, senegenin (**6**) was selectively converted to senegenic acid 28-methyl ester, i.e., in addition to the esterification reaction at C-28, a C–C bond cleavage at C-12 position was catalyzed by *Nocardia* sp. NRRL 5646. Two skeleton-rearrangement metabolites, oleanolic acid (**2**) and oleanolic acid methyl ester were also obtained from ursolic acid (**1**) and ursolic acid methyl ester, respectively, illustrating the unique ability of this *Nocardia* strain to catalyze a "retro-biosynthetic" conversion from the ursane to the oleanane skeleton by methyl migration from C-19 to C-20. With betulonic acid (**7**), the same microorganism was used again to produce the corresponding 28-methyl ester, 3-oxo-lup-20(29)-en-28-oate [26]. However, an unexpected metabolite, 2α-acetoxy-3-oxo-lup-20(29)-en-28-oate, was also isolated. This novel product was synthesized via the introduction of an asymmetric hydroxyl group at C-2 of betulonic acid (**7**) followed by the acetylation of the hydroxyl group. It was the first successful microbial transformation of a lupane-type pentacyclic triterpenoid involving a ketone α-hydroxylation activity.

In another biotransformation, betulonic acid (**7**) was produced from betulinic acid (**3**) using two *Bacillus megaterium* strains [28]. *B. megaterium* ATCC 14581 yielded betulonic acid (**7**) without additional modifications and the new 7β-hydroxy, 6α,7β-dihydroxy derivatives. In contrast, resting cells of *B. megaterium* ATCC 13368 further hydroxylated the 3-oxo-derivative product (betulonic acid, **7**) at the C-1 or C-11 position. The same strain also produced a betulinic acid derivative bearing additional hydroxyl groups at C-7β and C-15α sites [29]. Conversion of ursolic acid (**1**) by the fungus *A. flavus* ATCC 9170 yielded 3-oxo-ursolic acid [30]. The formation of this less polar metabolite, known as ursonic acid, was catalyzed via dehydrogenation of the secondary alcohol group at the C-3 position.

The biotransformation of betulinic acid (**3**) with growing cultures of the fungus *Cunninghamella elegans* ATCC 9244 produced a more polar metabolite via the introduction of two additional hydroxyl groups at the C-1 and C-7 positions resulting in 1β,3β,7β-trihydroxy-lup-20(29)-en-28-oic acid [28]. Another *Cunninghamella* species, *C. blakesleena*, also performed mostly hydroxylations on oleanolic acid (**2**) resulting in the formation of six metabolites. Some of these metabolites possessed additional hydroxyl groups at the C-1β, C-7β or C-13β positions, together with a 3β-hydroxy-11-oxo derivative and a lactone [8].

A recent study showed that fungi of the genera *Arthrobotrys*, *Chaetophoma*, *Dematium* and *Colletrotrichum* are also useful for mild, selective oxidations of lupane substrates such as betulinic (**3**) and betulonic acids (**7**) [31].

Curvularia lunata, a well-known fungus used for 11β-hydroxylation of steroids [32], also performed triterpenoid hydroxylations. The major product of the microbial transformation of another oleanane-type triterpenoid, glycyrrhetinic acid (**5**), also known as 18β-glycyrrhetinic acid, by *C. lunata* ATCC 13432 was identified as 7β-hydroxyglycyrrhetinic acid [33]. Epimeres of glycyrrhetinic acid (**5**), such as liquiritic acid (**8**), were partially converted by the same strain as well as by the fungi *Cunninghamella* sp. ATCC 3229 and *Mucor griseo cyanus* ATCC 1207-a to their 7β-hydroxy, 15α-hydroxy, and (or) 7β-15α-dihydroxy derivatives [8]. The fungal strains *Mucor polymorphosporus* AS 3.3443 and *Mucor spinosus* AS 3.3450 also produced 7β-hydroxyglycyrrhetinic acid as a major metabolite from glycyrrhetinic acid.

In another study, the same *Mucor* strain showed also a strong biocatalytic activity to selectively oxidize triterpenoids such as ginsenosides [34,35].

Several studies illustrated the potential of bacteria and filamentous fungi to selectively hydrolyze triterpenoid glucosides, thereby releasing the less polar free natural compounds. With ginsenosides Rb_1 (**9**) and Re (**10**) as biotransformation precursors, *A. niger* KTC 6909, *A. niger* AS 3.1858 and *A. usamii* KTC 6956 were able to selectively hydrolyze the sugar moieties attached to the C-3/C-6 and C-20 hydroxyl groups found in ginsenosides Rb_1 and Re (**9,10**) to produce deglycosylated ginsenosides [36,37]. The antitumor ginsenoside C-K was formed as main conversion product by *Fusarium sacchari* via successive deglycosylations at the C-3 and C-20 sites of ginsenoside Rb1 (**9**) [38]. β-glucosidase activity was also detected for *Bacillus megaterium* GP27. This bacterium almost completely converted ginsenoside Rb_1 (**9**) via a regioselective deglycosylation at the C20 position to the pharmaceutically active minor ginsenoside Rd [39]. The incubation of ginsenoside Rb_1 (**9**) with *C. lunata* AS 3.1109 and *Rhizopus stolonifer* AS 3.822 yielded a less polar metabolite, as a result of a hydrolysis reaction of one glucose moiety at the C-20 position. After two additional hydrolyses of glucose moieties at the C-3 and C-20 positions, *Rhizopus stolonifer* AS 3.822 yielded the ginsenosides Rg_3 and RH_2. In contrast, *C. lunata* AS 3.1109 catalyzed the addition of a tertiary hydroxyl group at the C-25 position by hydration of the side chain double bond yielding a novel ginsenoside metabolite [37]. A similar reaction pathway was observed during the microbial transformation of the tetracyclic triterpenoid cucurbitacin E 2-*O*-β-D-glucopyranoside (**11**) by *C. lunata* NRRL 2178, where the first step was glycolysis at the C-2 site followed by oxidation reactions specific to the side-chain of the cucurbitacin skeleton [40].

The ursane-type pentacyclic triterpenoid, quinovic acid glycoside (**12**), was also deglycosylated at the C-3 position by *Nocardia* sp. NRRL 5646 [24]. Furthermore, the bacterium catalyzed a carbon skeleton rearrangement involving a methyl group migration from C-19 to C-20 resulting in the formation of cincholic acid, the biogenetic counterpart to aglycon quinovic acid.

The first report on the biotransformation of betulinic acid (**3**) described the production of a novel glycosylated pentacyclic triterpenoid. In this report, *Cunninghamella* sp. NRRL 5695 introduced a β-D-glycopyranosyl at the C-28 carboxylic acid group of the substrate [41]. Very recently, various filamentous fungi were screened for the biotransformation of betulin (**13**) to betulinic acid (**3**) [25]. Among the fungi tested, two *Aspergillus* strains, *A. foetidus* ZU-G1 and *A. oryzae* AS 3.498, showed a significant ability to oxidize the C-28 hydroxyl group of the lupane-type triterpenoid substrate to the corresponding carboxylic acid (**3**). Nevertheless, the enzymatic mechanism of this regiospecific reaction has yet to be elucidated.

The filamentous fungus *Chaetomium longirostre* RF-1095 has been used to obtain oxygenated derivatives from pentacyclic triter-

penoids [42]. This strain efficiently transformed oleanolic acid (**2**) to hydroxylated- and ring-A cleaved-compounds. The detection of a seven-membered lactone derivative among the metabolites suggested that the formation of the 4-hydroxy-3,4-*seco*-3-oic acid derivatives arose from a Baeyer–Villiger oxidation of the 3-ketone followed by hydrolysis of the resulting lactone. The hydroxylation at C-21β probably occurred after the ring-A cleavage. Fig. 3 shows the transformation pathway of oleanolic acid (**2**) by *Chaetomium longirostre* RF-1095. With the same microorganism, the substrate betulonic acid (**7**) underwent several transformations such as oxidative ring cleavage, hydroxylation and decarboxylation [43]. Analogous ring-A cleavage oxidation reactions have been observed in the biotransformation of triterpenoid substrates with the fungi *Septomyxa affinis* ATCC 6737 and *Glomerella fusarioides* ATCC 9552 [8,44].

The short overview given in this chapter reveals that many microorganisms are versatile whole-cell biocatalysts to perform interesting functionalizations of triterpenoid compounds. A number of microorganisms have been shown to be able to hydroxylate non-activated positions of the triterpene skeleton, to oxidize the hydroxyl group of 3β-hydroxylated triterpenes, to regioselectively hydrolyze triterpene glycosides or for carrying out ring-A oxidative cleavage.

Most of the studies on triterpene biotransformation thus far have focused on the discovery of new biologically active compounds or have been set up to mimic and predict the metabolism of biological compounds in mammalian systems [29]. Consequently, reactions are usually performed on a small scale and the setup of the reaction system, for example, how to deliver the hydrophobic substrate to the microbial cells, is mainly driven by considerations to meet analytical requirements rather than to optimize biotransformation and space–time yields. Nevertheless, in a very similar field, namely the biotechnology of steroids, industrial processes were introduced in the middle of the last century and have been continuously improved and diversified during the subsequent decades [45,46]. Currently, microbial transformations of steroids, such as the controlled side chain degradation of phytosterols to yield androstendion and androsta-dien-dione with mutants of *Mycobacterium* sp. or regio- and stereoselective steroid hydroxylations with *Curvularia* sp., are carried out on a large scale with market volumes of 1000 t/a and <100 t/a, respectively [47]. Thus, for industrially relevant triterpenoid target compounds, a straightforward bioprocess development and scale-up of the underlying microbial transformation processes could be expected. Several relevant bioactive target compounds are presented in Table 1.

3.2. Enzymatic transformation

Compared with the whole-cell-approach, the application of isolated enzymes for modification of the triterpene molecules is reported rarely. Most relevant applications of isolated enzymes can be summarized as follows: cyclizations, deglycosylations and oxidation/hydroxylations. However, some of the representative literature is presented within this subchapter.

The cyclisation of squalene and 2,3-oxidosqualene generating polycyclic triterpenes has been an interesting topic for many years because of the high stereoselectivity of this process. Many papers have been published considering the synthetic pathways used by enzymes performing these cyclizations, such as squalene hopene cyclase (SHC) and 2,3-oxidosqualene cyclase (OSC) [48]. The cyclization is performed via defined steps. First, the enzyme binds to the substrate, embracing a conformation specific for this particular substrate. A cation formation is triggered by the protonation either of a double bond or an epoxide followed by successive ring closure and rearrangement reactions [49]. Abe et al. [50] described a pathway for the cyclization of (3S)2,3-oxidosqualene

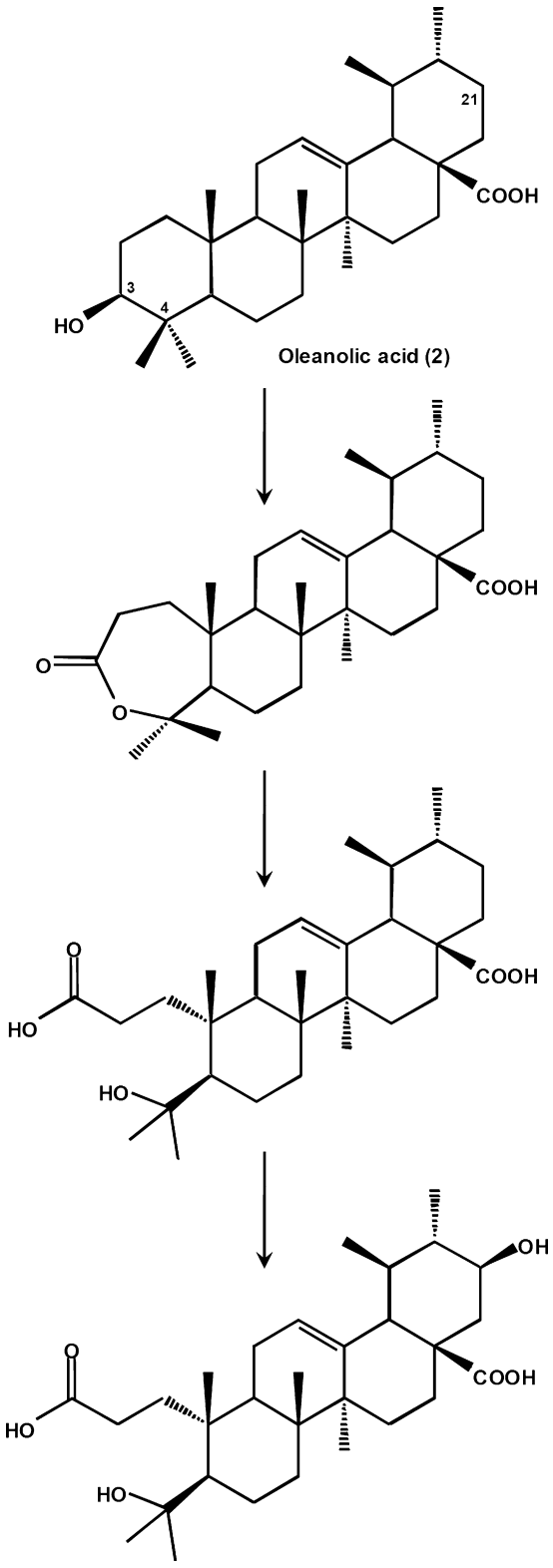


Fig. 3. Pathway of oleanolic acid biotransformation by *Chaetomium longinostre*. Adapted from Shirane et al. [42].

and proposed one for 22,23-dihydro-2,3-oxidosqualene. In both approaches, β-amyrin synthase is the enzyme performing the transformation. β-amyrin synthase was purified from several plants including *Pisum sativum* so that its cDNA could be cloned and expressed afterwards by *Saccharomyces cerevisiae*. It is reported to bind the substrate ((3S)2,3-oxidosqualene) in the

chair–chair–chair–boat-conformation. The D-ring is formed by the closure of a five-membered ring generating a Markovnikov tertiary cation followed by an expansion of the ring yielding a tetracyclic secondary cation. The baccarenyl cation is built by creating a six-membered D-ring, and β-amyrin is finally synthesized via the intermediates lupanyl cation and oleanyl cation. The cyclization of 22,23-Dihydro-2,3-oxidosqualene was performed by incubation with recombinant β-amyrin synthase from *Pisum sativum*. Two main products resulted from this preparation: euph-7-en-3β-ol and bacchar-12-en-3β-ol. As a result, Abe et al. [50] suggested that even a tiny modification of the active site of the enzyme would elicit different characteristics in cyclizations. In a subsequent publication, Abe et al. [51] described the cyclization of 24,30-Bisnor-2,3-oxidosqualene using the same recombinant β-amyrin synthase from yeast as mentioned previously. In addition to β-amyrin, a mixture of three substances within the product was present: 29,30-bisnor-β-amyrin, 29,30-Bisnorgermanicol and 29,30-bisnor-δ-amyrin. Therefore, they assumed that the occurrence of these three regioisomers resulted from a deficit in the final rearrangement reactions. When observing the substrate specificity, Dang and Prestwich [49] examined two regions of SHC because of their reputed responsibility in initiation and termination of cyclization. They found that the triple mutation of the DDTAVV motif of SHC (pUC19-derived plasmid encoding His6-tagged SHC from *A. acidocaldarius*) into the DCTAEA motif of eukaryotic OSC immensely affected the substrate converted by mutant SHC. There was no detectible transformation of squalene, but a cyclization of 2,3-oxidosqualene occurred, resulting in the production of mono- and pentacyclic triterpenes. Even different mutations of one residue (Glu45) posited to be involved in the termination of the squalene cyclization process led to different catalytic activities; thus, these investigated regions in the enzymes have a significant impact on their usability. Hoshino et al. [52] proposed that the substrate specificity of SHC is exceedingly wide in general when using (3S)2,3-oxidosqualene. Compared to that, OSC only uses (3S)2,3-oxidosqualene for cyclization. Also, using *Alicyclobacillus acidocaldarius* as a donor of SHC, they demonstrated that the deletion of Gly600, a residue appearing in SHC but not in OSC, changed the substrates used for enzymatic cyclization. No cyclization product could be detected using squalene. The insertion of a racemic mixture of (3S)2,3-oxidosqualene and (3R)2,3-oxidosqualene led to six major products. The substrate specificity of a prokaryotic cyclase could be changed into that of an eukaryotic cyclase; this finding is interesting regarding the evolutionary development of these cyclases. Quite similar results are presented in the work of Kushiro et al. [53]; however, they studied the product specificity instead of the substrate specificity as well as the modification of the product by executing a site-directed mutation that impacts the relevant amino acid residues. The two triterpene synthases, β-amyrin (PNY) and lupeol (OEW) synthase, were expressed in several mutant forms. Based on this mutational analysis, lupeol synthase was found to be a β-amyrin synthase originating only from β-amyrin instead of lupeol. These are only a few examples of investigations concerning the mutation of triterpene synthases changing the specificities of enzymes.

Another group of enzymes are important in the transformation complex of triterpenes: glycosidases and deglycosidases. Shafiee et al. [54] used a recombinant β-D-glucosidase to deglycosilate enfumafungin, a triterpene glycoside, acetylated at the C-2 position and bearing a cyclic hemi-acetal and a carboxylic acid functionality. The remaining functionalities after the glycosylation indicate that the antifungal activity of the starting substance were still present, representing a starting point for further investigations regarding the production of more extensive derivates.

A large enzyme group that appears in almost every organism is the group of cytochrome P450 enzymes. These enzymes are able to perform oxidations as well as reductions and peroxidations, and they can be used for several tasks, for example, in the synthesis of fine chemicals. The main problem in using cell-free extracts for enzymatic transformation is the dependence of the P450-systems on discrete protein partners, which significantly lowers their applicability in biotechnology. Fujii et al. [55] used a recombinant cytochrome P450 (moxA) from *Nonomuraea recticatena* for a hydroxylation of oleanolic acid to obtain queretaroic acid, which is a substance reported to possess physiological activity. *Escherichia coli* was used to express the enzyme and harbored a plasmid carrying the genes for P450 as well as genes to express its redox partners needed for the hydroxylation. In order to improve the results of the conversion reaction, cell-free lysates were used that contained the expressed P450. Among other compounds, oleanolic acid was added as a substrate as well as NADH and NADPH when the redox partners were expressed by the *E. coli*. P450 moxA showed its ability to convert oleanolic acid into queretaroic acid via hydroxylation with a conversion ratio of about 17%, implying an increased yield compared to the whole-cell approach. This finding may be an indicator of the minimal permeability of the cell to oleanolic acid such that it is not able to reach the P450 before cell disruption. They stated that the cell-free approach could be a powerful tool to identify molecules undetectable in whole cells because of a lack of permeability of the cell membrane. Mouri et al. [56] found a cytochrome P450 from *Bacillus megaterium*, P450BM3, which could carry out oxygenation in a cell-free system. The reaction system was linked to a two-step cofactor regeneration. NADH and NADPH represent the redox cofactors. Their regeneration was performed by a bacterial glycerol dehydrogenase (GLD) that is dependent on NAD^+ and a bacterial soluble transhydrogenase (STH). In a cascade, cofactors, such as NADH, are generated by GLD out of glycerol that works as a sacrificial cosubstrate and is consumed in the process. The alteration between generating and using the cofactors makes this P450 effective, even in a cell-free preparation. Through a 10-fold rise in the contingent of STH, a nine-fold increase in the reaction rate could be detected, indicating that the step requiring STH was also the rate-determining step. Another approach using cofactors for P450 systems was investigated by Chefson, Zhao and Auclair [57]. Instead of using other enzymes to do the preliminary work, they utilized hydrogen peroxide donors and optionally organic peroxides to provide the cofactors used for the transformation of the precursor. Based on these conditions, they performed catalytic reactions in aqueous media without mutating the P450 system. Compared to natural cofactors, the initial and product verified formation rates were twice as high, retaining regioselectivity as well as stereoselectivity. The best cofactor reported was cumene hydroperoxide, yielding 210% of the naturally obtained amount of product within 1 h. Even differences in stability compared to the wild type turned out to be smaller than suggested, presumably because of the low quantity of peroxides used. Given the improved properties and the small amount of cofactors needed, this approach represents an interesting approach for further use of P450 systems.

4. Plant cell and tissue cultures for the production of triterpenes

4.1. Plant cell and tissue cultures

The following sections consider plant cell and tissue cultures as production systems for pharmaceutically relevant triterpenes. These sections focus mainly on undifferentiated calli, which require exogenous plant growth regulators (natural or synthetic hormones) for maintenance, and *Agrobacterium rhizogenus*-transformed hairy root cultures, which can grow in hormone-free media.

The first permanent callus culture was developed from carrot in 1937 by two French scientists, Gautheret and Nobecourt [58], following previous experiments by Haberlandt and White [59]. The mechanism whereby *Agrobacterium rhizogenes* transforms plants was largely elucidated at a molecular level in the 1970s and 1980s [60–63], and hairy roots were subsequently applied for secondary metabolite production [64]. Other important advances that greatly facilitated the investigation and application of plant cell and tissue cultures included the development of viable cultivation systems (described in more detail in Section 4.2.2) and appropriate media, such as MS Murashige and Skoog [133], GB5 Gamborg [134] and LS Linsmaier and Skoog [135]. The first and most highly publicized commercial application of plant *in vitro* cultures was shikonin production using *Lithospermum erythrorhizon* [60,65]. Several companies are now using plant cell and tissue cultures industrially; for example, *Phyton Biotech, Inc.* is producing the anticancer drug taxol from plant cell cultures, and the Swiss company *ROOTec bioactives GmbH* is producing high value plant-derived compounds from hairy roots. However, this technology is not widely used yet, raising obvious questions about the advantages and disadvantages of such cultures.

Plant *in vitro* cultures provide interesting options to traditional crops and chemical syntheses for the production of biologically active metabolites. They have several advantages because the target substances are produced in cells, tissues or organs that are cultivated in bioreactors under sterile conditions and hence are completely insulated from adverse environmental factors, both biotic (e.g., diseases and herbivores) and abiotic, for example, unfavorable climatic and soil conditions [66–68]. These cultures also have a high potential for genetic optimization because *in vitro* cultures do not interact with the environment in the same manner as genetically modified crops. In addition, continuous production is possible, with consistent quality and quantities.

Furthermore, unlike collection from the wild, plant *in vitro* culture-use does not increase the risk of extinction of rare medicinal plant species [66,68]. Unfortunately, the amounts of desired metabolites produced by plant *in vitro* cultures are often lower than the contents in intact plants [69]. Therefore, intense optimization efforts are required. Another drawback is that high yields and market prices for the products are crucial for the financial viability of *in vitro* cultures, but prices depend on the costs of competing production processes, notably traditional cultivation of intact plants and chemical synthesis of the biologically active substances [66]. According to calculations based on 1988 prices, if secondary metabolites are produced by suspension cultures, then the product concentration should be more than 1 g/L and the price per kg should be at least 500 USD [66].

Therefore, it is essential to increase the productivity of cell cultures to ensure their economic viability. Strategies to enhance the yields of secondary metabolites in plant *in vitro* cultures include media and hormone optimization, improvement of bioreactor designs and operation modes [70,71] and the use of various techniques such as elicitation [72,73], immobilization [74,75], and genetic modification [76].

4.2. In vitro production of triterpenoids with cell and tissue cultures

4.2.1. Plant in vitro cultures producing triterpenoids

Substantial efforts have been made to produce triterpenoids using plant *in vitro* cultures from different plants because of their high pharmaceutical interest. Early studies included experiments with *Glycyrrhiza glabra* (liquorice) callus and cell suspension cultures. Suspension cultures in 300-mL shake flasks were found to contain betulinic acid (a lupane-type triterpene), its precursor lupeol, and amyrin, a possible precursor of oleanane-type triterpenes [77]. In 1989, Fujioka et al. [78] identified a large number of dammarane and oleanane saponins by ^{13}C NMR in callus tissue of *Panax japonicus*. *Panax* spp. are known to produce a wide-spectrum of triterpenes [79], but mostly triterpenes bonded to glycosides in forms called saponins [80] or ginsenosides in ginseng species [81]. Callus cultures of the *Panax* species *P. sikkimensis*, *P. pseudoginseng* and *P. quinquefolium* have been found to contain 0.95%, 1.10% and 1.2% ginsenosides, respectively, on a fresh weight basis [82]. Significant differences among species were observed in the amounts of Rg and Rb (dammarane-type) ginsenosides detected in the calli examined in the cited study, but their complements were found to be quite stable over the tested cultivation time (6-20 months) [82]. However, in similar experiments with four callus lines of *P. ginseng*, both the numbers of ginsenosides produced and their amounts decreased after prolonged cultivation [79]. One year after induction, nine different ginsenosides were found in the callus lines; however, on average, only five were found four years later, and the amounts declined in most cases by ca. 70% [79]. This reduction of metabolite amounts and spectra is a frequently reported problem with callus cultures [83,84]. In addition, undifferentiated calli are not as genetically stable as organ cultures, and growth regulators such as 2,4-D (2,4-Dichlorophenoxy acetic acid) are known to influence the endopolyploidy and metabolic activity of callus cells particularly strongly [85,86].

The effects of growth regulators on callus physiology and ginsenoside production have been tested in both organogenic and non-organogenic calli of *P. ginseng* [84]. Callus was grown for six months on 0.1 mg/L kinetin and 1 mg/L 2,4-D to obtain homogenous, undifferentiated callus. The medium was then supplemented with kinetin and 2 mg/L of one of the following auxins: 2,4-D, NAA (1-naphthalene acetic acid) or IBA (3-indolebutyric acid). After just five weeks, strong differences in the amounts of several dammarane-type ginsenosides were detected together with changes in morphology [84]. The highest metabolomic change detected was a ca. 356% increase in Re ginsenoside content in non-organogenic callus. The morphology and physiology also changed where each callus formed non-organogenic, root and bud forming callus. Investigations of the synthesis of oleanolic acid (OA) saponins (glucosides and glucuronides) in *Calendula officinalis* cell suspension cultures also highlight the influence of plant hormones on triterpene production. Cell suspension cultures grown with 0.1 mg/L 2,4-D and 0.5 mg/L 2iP (6-(γ,γ-dimethylallylamino) purine) have been found to synthesize and secrete more OA glucosides than cultures grown with 0.4 mg/L 2,4-D and 0.4 mg/L kinetin [80,87]. Interestingly, the secretion of OA glucosides was independent of light conditions in the 2,4-D/kinetin culture, but light promoted their secretion in the 2,4-D/2iP cultures [87]. This finding highlights the strong effects of growth regulators and the challenges researchers face in optimizing plant *in vitro* cultures with respect to morphology, growth behavior, and metabolic activity; i.e., to generate homogenous, friable, fast-growing calli that consistently produce high quantities of target substances. However, callus and hairy root cultures producing triterpenoids – mainly OA, ursolic acid (UA), and different ginsenosides – have been successfully created from various plants (Table 2) [80,84,88–97].

4.2.2. Larger scale triterpenoid production

An important step in the development of biotechnological processes for producing biologically-active metabolites is the cultivation of plant *in vitro* systems in bioreactors [98] and at larger scales. Cell cultures mostly form aggregates, are heterogeneous and sensitive to shear stress. However, they have similarities with bacterial and yeast cultures and can be cultivated in stirred tank reactors. In some early investigations [99], it was concluded that the best systems for culturing plant *in vitro* cells are airlift bioreactors, with either internal or external loops. The advantages of such sys-

Table 2
In vitro cultures producing triterpenoids at callus stage or in small-scale cell suspension cultures and in hairy root cultures (c, callus; s, suspension; hr, hairy roots).

Plant	Culture type	Substances and amounts	Author
Salvia officinalis	c	UA max. 258 μg/g Dry Weight (DW) (friable) UA max. 110 μg/g DW (compact)	Bolta et al. [88]
	s	UA max. 392 μg/g DW (friable) UA max. 12 μg/g DW (compact)	
Camptotheca acuminata	c + s	Eight triterpenic acids (OA and UA methylesters, maslinic, asiatic, corosolic acids) % triterpenes in extract: Callus 1.5%; Suspension 4.6%; Media 3.8%	Pasqua et al. [89]
Actinidiaceous plants	c	Eight ursane and three oleanane type triterpenes (UA, OA, maslinic acid)	Takazawa et al. [90]
Actinidia arguta		ca. 0.4% UA and OA mix in callus	
Actinidia chinensis		ca. 0.2% UA and OA mix in callus	
Actinidia polygama		ca. 0.1% UA and OA mix in callus	
Centella asiatica	c	max. 0.24% (DW) madecassic acid, 0.19% asiatic acid	James et al. [91]
	s	max. 0.28% madecassic acid, 0.16% asiatic acid	
Tripterygium wilfordii	hr	triptocallic acids, (pentacyclic triterpenes) no amounts given	Nakano et al. [92]
Perilla frutescens	s	16 mg/L OA, 25 mg/L UA, 14 mg/L tormentic acid	Wang et al. [93]
Scutellaria baicalensis	s	max triterpenoid conc. in media after elication: 13.7 mg/L	Yoon et al. [94]
Calendula officinalis	s	max. 0.84 mg/g DW OA (72 h after 100 μM jasmonic acid (JA) elicitation)	Wiktorowska et al. [95]
Taraxacum officinale	c	major triterpenoids UA and OA, total triterpenoids 0.11% per DW	Akashi et al. [96]
Panax ginseng	c	Rb and Rg Ginsenoside mostly between 20 and 40 mg/g DW	Bonfill et al. [84]
Panax ginseng	hr	max. ginsenoside content 10.5 mg/g DW	Yu et al. [97]
Panax ginseng	s	L-2 suspension culture: max. ginsenoside content 4.05 mg/g DW (0.41%)	Reshetnyak et al. [79]

tems are the reduced stress levels due to the absence of mechanical agitation, but ensuring there is sufficient mixing [65] and oxygen transfer at high cell densities can be difficult [100]. These may be the main reasons why bioreactors with mechanical agitation systems have been used much more frequently recently. In attempts to reduce mechanical stress levels inside bioreactors, diverse types of impellers have been developed and tested, for example, rushton turbine impeller and propeller impeller [101]. Other types of bioreactors, such as column, drum and diffusion bioreactors have also been used but have not been used in any commercial applications to date.

Hairy root cultures can be favorable as production systems because they are differentiated, require lower inoculum sizes than other types of culture, and can be reused, similar to immobilized cells [64]. They can also be easily separated from their media because of the root networks they form [65]. Due to their atypical morphology, hairy roots require reactors with special features, but they can be cultivated in stirred tank reactors with modifications such as meshes to ensure that the roots do not come into direct contact with the stirrer [64], or in airlift reactors, bubble columns or mist reactors that generate sufficiently low shear stresses [60,100,102]. There are also some recently developed reactor systems, notably wave bag reactors, in which the cells grow in plastic bags rocking on a platform [100,103]. These bags are disposable and thus reduce preparation time and contamination risks. Furthermore, shear stress and foaming are strongly reduced [100], and both plant cell suspension cultures and hairy roots have been successfully cultivated in these systems. Thus, many different cultivation systems can be efficiently used for plant cell and tissue cultures, but the morphology and shear sensitivity of the cultures as well as the oxygen transfer and mixing limitations of the reactors should be carefully considered [102,100].

Most investigations of triterpenoid production by *in vitro* cultures have focused on calli or cell suspensions in shake flasks with small working volumes, mostly ca. 100 mL [82,84,93,94,90]. However, to use plant *in vitro* cultures as production systems, large scales and high yields are required. Ginseng has been used in most attempts to scale-up production to date. Notably, hairy roots of *P. ginseng* have been cultivated in a bubble column with a 4-L working volume, and the effects of light and temperature have been tested on their ginsenoside production [97]. Substantial differences in both biomass growth and ginsenoside production were observed among cultures grown with 16 h/8 h day/night temperature regimes of 13/20 °C, 20/13 °C, 25/25 °C and 30/25 °C. The highest biomass and ginsenoside accumulation was found at 25/25 °C, which is within the range of most common cultivation temperatures of plants (24-28 °C) [97]. Light was another tested variable. More specifically, the effects of illuminating the cultures with fluorescent, metal halide, blue, red or red plus blue light on their growth and ginsenoside production, relative to dark controls, were evaluated. It was found that dark or red light conditions induced the highest growth rate, whereas irradiation with fluorescent light increased ginsenoside production [97]. In addition, Zhong et al. (2005) [71] described high density cultivations with *P. notoginseng* suspension cultures in specially developed centrifugal impeller bioreactors (CIB) at the 3-L and 30-L scale, in which biomasses of 22 and 26 g DW/L, with 4.02 and 4.12 mg/g DW of Rg1 ginsenosid accumulated by day 13 of cultivation following addition of methyl jasmonate (MJ). These studies provide important preliminary information regarding cultivation parameters and reactor design for process optimization and further application of *in vitro* cultures for triterpenoid production.

4.2.3. Elicitation as a strategy to enhance triterpene yields

As mentioned above, another essential step in bioprocess development is to enhance the production of the desired metabolites by an appropriate treatment, such as elicitation, which has been applied to many types of cultures [59,68,104,105]. As often reported for secondary metabolite production in cell cultures [66,69], triterpenoid concentrations are generally lower than those in intact plants [95,91]. However, Takazawa et al. [90] described callus cultures from *Actinidia arguta* that reportedly produced 20-times more oleanolic and ursolic acid than stems of the corresponding plant, and *Camptotheca acuminata* callus produces higher amounts of triterpenoids than the leaves or roots of the intact plant [89].

Elicitation can be used to enhance the production of many metabolites that participate in some way in plant defense responses. Generally, either the level of a signal molecule involved in plant defense signal cascades is artificially enhanced, or the defense system is activated by imitating stress or an attack [83]. Key molecules in the signaling cascade include jasmonic acid (JA), salicylic acid (SA) and ethylene. These substances and their derivatives (e.g., methyl jasmonate) play important roles in the defense against attacks by microbes and herbivores, as well as abiotic stresses, such as wounding and ozone exposure [106]. Therefore, they are

often used as elicitors in plant *in vitro* cultures. Another elicitation strategy is to imitate pathogenic attacks or abiotic stresses by challenging plant *in vitro* cultures with constituents of fungi, bacteria or the plants themselves (e.g., polysaccharides, chitosan or glycoproteins) or with heavy metal ions, other stress-inducing chemicals or UV light [68,59,83].

Several investigations of possible ways to elicit the production of triterpenes in plant *in vitro* cultures have been presented in the literature. Notably, cell suspension cultures from *Scutellaria baicalensis* were treated with a yeast elicitor preparation (50 μg/ml) and methyl jasmonate (100 μM) by Yoon et al. [94], who found that both substances induced increases in triterpene contents, but the peak levels occurred after 35–40 h and 15–20 h, respectively. More oleanolic acid and ursolic acid were also secreted into the culture medium when the yeast elicitor was used (up to 9 mg/L). This result confirms previous findings that elicitors influence not only the productivity of cells but also the release of products into the medium [59]. Furthermore, downstream processing of substances of interest released into the culture medium can be easier and cheaper than extraction of biomass, and biomass can be reused after adding fresh medium, thus allowing continuous production and saving time [105]. These are important considerations for any industrial application of plant cell and tissue cultures.

Similar results have been observed in investigations of the effects of jasmonic acid, chitosan, yeast extract, and fungal pectin on the production and secretion of oleanolic acid from *Calendula officinalis* suspension cultures [95]. Cultures elicited with yeast extract released high amounts of oleanolic acid into the medium (0.43 mg/L after 48 h treatment with 200 mg/L yeast extract), whereas jasmonic acid (100 μM) induced the highest increases in intracellular levels of OA (to 0.84 mg/g DW, 72 h after treatment; 9.4-fold higher than in controls). However, elicitation can also have negative effects on cell growth and viability. For instance, reductions in biomass production were observed, especially following JA and chitosan treatment of the *Calendula officinalis* suspension cultures [95]. Elicitation experiments with methyl jasmonate (200 μM on the eighth day of cultivation) have also been reported, using *P. notoginseng* in the 3-L and 30-L centrifugal impeller bioreactors described mentioned above. No adverse effects on growth were detected, but a significant rise in ginsenoside production by the cells was observed [71]. Elicitation experiments have also been done with several other kinds of cultures, for example, suspension cultures from *P. notoginseng* [107], *Scutellaria baicalensis* [94] and *Perilla frutescens* [93].

4.3. Transgenic plants as tools for the production of valuable compounds

4.3.1. Generation of transgenic plants

Plants synthesize large numbers of valuable metabolic (end) products, including compounds such as starch, sugars and triacylglycerols, all of which occur at high levels in typical storage organs [108]. In addition, diverse compounds of low abundance, including most solutes derived from secondary metabolism, are present in plants. Many of these compounds have high economic value, for example, vitamins, pigments, alkaloids, phenolics, glycolipids and terpenoids [109]. Moreover, transgenic plants have also been recently used for the production of compounds with pharmacological relevance. For example, genes encoding antibodies, antigens and scarce enzymes have been successfully introduced into the plant genome, leading to high levels of fully functional and naturally processed (modified) proteins [110,111].

The identification and selection of as yet uncharacterized plant species and breeding of suitable species are typical approaches to modify the content and quality of plant ingredients, but this is a prolonged, costly and imprecise process. In contrast to classical breeding approaches, modern molecular techniques allow precisely targeted modifications of the plant genome. A frequently used approach to directly modify metabolic pathways is to introduce either antisense- or sense mutations. Generally, sense mutations are used to overexpress the corresponding mRNA, and a heterologous structural gene is introduced into the host cell genome to prevent so-called co-suppression [112]. In contrast, when an antisense approach is adopted a substantial part of an endogenous gene is inserted in an antisense orientation, leading to hybridization of the endogenous sense and the introduced antisense mRNAs. Such hybridized mRNA fragments are degraded and thereby reduce the levels of functional mRNA, ultimately decreasing the amount of the corresponding protein.

Accordingly, for both approaches, it is necessary to introduce novel DNA into the nuclear genome of the plant to be modified. In general, three experimental approaches can be used to introduce additional DNA: *Agrobacterium tumefaciens*-mediated, biolistic transfer of DNA or electroporation. In recent years, *Agrobacterium*-mediated DNA transfer has become the most frequently used approach, and highly virulent laboratory *Agrobacterium* strains have been identified. These strains are suitable for transforming nearly all monocotyledonous and dicotyledonous crop plants, laboratory model plants and even rare plant species [113–115].

Agrobacterium contains a T-DNA (*transfer DNA*) located in the Ti (*tumor inducing*) plasmid. Bacterial T-DNA genes are required for infection and the subsequent exploitation of the host plant. Using molecular techniques, this T-DNA can be easily modified by inserting the selected DNA, comprising a selection marker gene (usually conferring resistance to an antibiotic or herbicide, e.g., kanamycin or Basta®, respectively) and the gene of interest, which is to be expressed in the host plant. In this context, the choice of a suitable promoter segment governing the expression strength as well as the cell type and tissue specificity of the selected gene in the host plant is critical.

After transferring the novel genes into the nuclear host cell genome, the transformed cells are selected by exploiting the resistance conferred by the selection marker gene, i.e., only transformed cells will have resistance to the corresponding antibiotic or herbicide. Due to the totipotency of all plants, the transformed cells will differentiate into complete new plants [116] or can be used as precursors for transgenic cell suspension cultures. Independent mutant lines can be screened on the basis of mRNA accumulation (via Northern blot analysis), protein abundance, protein activity, and/or accumulation of the desired cellular product.

4.3.2. Examples of transgenic plants with altered levels of valuable compounds

Today, there are numerous examples of genetically modified (GM) plants. Most GM plants used for agronomic production have herbicide or pathogen resistance but are beyond the scope of this review. However, there are also many examples of transgenic plants that have modified contents of even the most abundant storage products such as starch, sugars, lipids, and other valuable compounds that are essential for plant nutrient quality. Starch, for instance, is a highly abundant plant storage product that is important both for its high calorific value and as a precursor for industrial processing. In general, starch consists of two types of molecules, amylopectin and amylose [117], and from an industrial perspective, it is desirable to have starch consisting of only one of these molecules. By expressing DNA encoding mRNA for a specific starch synthase isoform (starch grain-bound starch synthase, GBSS) in an antisense orientation, the company BASF Plant Science was able to generate a potato mutant that is apparently free of amylose (http://www.basf.com/group/corporate/en/content/products-and-industries/biotechnology/plant-biotechnology/index) but still

produces high yields in the field. Applications are currently being considered to use this mutant for public field production in Europe.

Moreover, total starch yields in potatoes have been increased by several genetic strategies to modify tuber energy status (i.e., adenylate metabolism). Examples include the following: (i) the introduction of a bacterial ADP pyrophosphorylase to potato-tubers, taken from glycogen-storing *E. coli* that is not subject to regulatory inactivation by inorganic phosphate [118]; (ii) the alteration of cytosolic adenylate kinase activity [119]; and (iii) increased provision of ATP in starch-synthesizing amyloplasts [120]. However, no mutant lines carrying these modifications are currently available on the market.

Sugars are highly abundant soluble carbohydrates (including sucrose, glucose, fructose and the fructose polymer fructane) that are frequently transported from photosynthetic tissues to storage organs in plants and, subsequently, to developing tissues [121]. Today, there are numerous examples of crop plants with modified sugar metabolism and consequently altered yields of storage organs. For instance, an apoplastic (cell-wall-located) invertase has been used to increase levels of monosaccharides and thus the size of tubers and tuber yields in potato [122]. In addition, sugar beet plants with high fructan contents have been generated via the expression of 1-sucrose:sucrose fructosyl transferase in their roots [123]. Interestingly, potato tubers have also been used for the production of novel carbohydrates that are not usually present in plants, for example, the structural sucrose isomer palatinose (isomaltulose, 6-*O*-alpha-D-glucopyranosyl-D-fructose), which is non-cariogenic and has low calorific value. This compound is produced on an industrial scale from sucrose by an enzymatic rearrangement using immobilized bacterial cells. Expression of a chimeric sucrose isomerase gene from the bacterium *Erwinia rhapontici* in potato led to a nearly quantitative conversion of sucrose into palatinose [124].

Storage lipids are extremely energy-rich plant ingredients. Partly for this reason the lipid and fatty acid composition of plants has been modified in several cases to optimize the content of industrially valuable compounds or to optimize their nutrient quality [125]. In particular, the degree of desaturation of fatty acids in storage lipids of edible fruits has frequently been improved by genetic alteration of the corresponding desaturase enzymes [126,127].

4.3.3. Plants with altered triterpene metabolism

Many plants possess triterpenes, for example, saponins, presumably due to strong selective pressures because many of these compounds have defense activities against pathogens and herbivores (see e.g., Agrelli et al. [128]). They also have high pharmaceutical value (see the Introduction); thus, researchers have expended considerable effort to optimize triterpene composition and triterpene levels in plant tissues [129]. Plants of medical importance, such as ginseng (*P. ginseng*), "Siberian ginseng" (*Eleutherococcus senticosus*), lavender (*Lavandula officinalis*) and sage (*Salvia officinalis*), contain remarkably high levels of endogenous triterpenes, and they are used as raw materials for industrial processing [130]. It has proved possible to increase triterpene levels in both Siberian ginseng [131], and (real) ginseng [76] by overexpression of the enzyme squalene synthase. These experimental results indicate that squalene synthase, which catalyzes the first committed step in sterol-, as well as triterpene biosynthesis [121], is rate-limiting in these species.

Saponins represent a subclass of triterpenes that have high pharmacological relevance. Overexpression of β-amyrin synthase, a key enzyme in saponin biosynthesis, in the leguminosae species *Medicago truncatula*, has been found to induce increases not only in saponin but also in bayogenin, medicagenic acid and zanhic acid contents compared to wild-type levels [132]. Together, these observations demonstrate the possibility to increase triterpene levels and modify their profiles in cell suspension cultures from various plants by molecular approaches.

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