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Artemisinic acid: A promising molecule potentially suitable for the semi-synthesis of artemisinin

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Artemisinic acid, an amorphane sesquiterpene, is isolated from *Artemisia annua* L. Although having less efficacy than artemisinin, artemisinic acid has a variety of pharmacological activity, such as antimalarial activity, anti-tumor activity, antipyretic effect, antibacterial activity, allelopathy effect and anti-adipogenesis effect. This development has drastically increased artemisinic acid demand worldwide. Although many approaches, namely extraction of artemisinic acid from *A.annua* L, *in vitro* production of artemisinic acid by cell and tissue culture, total chemical synthesis and fermentation production by use of synthetic biology technology can improve artemisinic acid production, *A.annua* L. is currently the only commercial source for the artemisinic acid supply in the international market. Recently tremendous advances, however, demonstrate that the production of artemisinic acid in microorganisms and further semi-synthesis to artemisinin is a feasible complementary strategy that would help reduce artemisinin cost in the future. The key genes encoding for enzymes regulating the biosynthesis of artemisnic acid *in planta* are fully understood to enable metabolic engineering of the pathway, and results from pilot genetic engineering studies in microbial strains thus far are very inspiring. This review, therefore, covers the recent developments related to the physico-chemical properties of artemisinic acid, bioactivity of this important molecular, solvent extraction strategies and chemical analysis, and highlights a scale production of artemisinic acid by synthetic biology and the relevant enzymes and genes. In the end the status of artemisinic acid in the biosynthesis pathway of artemisinin is discussed in detail. Together these results provide a synopsis of a more global view of artemisinic acid than previously available.

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1 Introduction

Artemisinin, a sesquiterpene lactone endoperoxide derived from the sweet wormwood plant *Artemisia annua* L, provides the basis for the most effective treatments of malaria, particularly in the form of artemisinin-based combination therapies (ACTs).[1–5] In addition to being currently the best therapeutic against both drug-resistant and cerebral malaria-causing strains of *Plasmodium falciparum*,[6] artemisinin and its derivatives have also been demonstrated to be effective against a variety of other diseases as well, such as hepatitis B,[7–9] schistosomiasis,[10–12] and a range of cancer cell lines, including breast cancer,[13–15] human leukemia,[16–18] colon,[19–21] small-cell lung carcinomas[22–24] and drug-resistant cancers.[25–27] The drug also plays an important role in allelopathic activity with potential as a natural herbicide.[28–30] However, artemisinin and its derivatives are not available to the millions of the world's poorest people because of the labor-intensive and time-consuming production. It is of great importance to develop unconventional and alternate strategies for the commercial production of artemisinin which can lower prices and stabilize supply for the very often impoverished population of the countries where malaria occurs. These strategies include chemical synthesis,[31–33] plant tissue and cell cultures,[34–36] breeding boost[37–39] and designed fermentation production of artemisinin using synthetic biology technology.[40–42]

Synthetic biology is the design and construction of new biological components, such as enzymes, genetic circuits, and cells, or the redesign of existing biological systems.[43–45] In terms of pharmaceutical industries applications, synthetic biology has focused on engineering organisms for high-value products, and be considered as the next-generation biorefining approach. The underlying idea of this strategy encompasses transfer of a complete plant terpenoid pathway to microorganisms or plants to obtain artemisinin or its precursors in large-scale production. This approach requires sound knowledge of artemisinin biosynthesis and identification of the genes involved. Because of no completely elucidated artemisinin biosynthetic pathway post-artemisinic acid or dihydroartemisinic acid production, artemisinin production by synthetic biology is a semi-synthetic route in combination with additional genes, that is, the first synthesis of simple precursor such as amorpha-4,11-diene,[42,46,47] arte-

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misinic acid[40,41,48] and dihydroartemisinic acid[46,49,50] by use of metabolic engineering, and then the use of a semi-synthetic route for artemisinin from these precursors.[46,51] Among these precursors, artemisinic acid with a much-less complexity has a related chemical structure to that of artemisinin and can easily be transformed to artemisinin in an economical way, which provides an alternative and cheap way to improve artemisinin production from biomass. Moreover, the level of artemisinic acid was 8- to 10-fold higher than that of artemisinin in chemotype II species of *A.annua*, which implies that artemisinin production could be potentially improved 8- to 10-fold by converting artemisinic acid to artemisinin.[49,52,53] Also, different engineered hosts producing artemisinic acid, potentially suitable for the semi-synthesis of artemisinin, have also been developped.[40,41,48] All of these properties make artemisinic acid an attractive and ideal starting point for artemisinin production in large quantities by synthetic biology techniques. Up to date, various aspect of this promising molecule undergoing intense study includes its properties, biological actions, production by use of different approaches, chemical analysis, total synthesis and biotransformation.

2 Physico-chemical properties of artemisinic acid

Artemisinic acid, an amorphane type and bicyclic sesquiterpene (Fig. 1),[54] is also referred to as arteannuic acid,[55–69,70*a–d*] arteannuinic acid,[70–74] artemisic acid,[57,70*a*,75–77] qinghao acid[54,57,61,70*a*,78–80] and qinghaosuan.[81] Artemisinic acid was isolated from *Artemisia annua* L. for the first time in 1981 by two Chinese groups.[76,82] Artemisinic acid is a colorless, non-volatile compound, which is purified as white crystals with a melting point of 131 °C. Its molecular weight is 234 in accordance to the empirical molecular formula $C_{15}H_{22}O_2$. Moreover, there is a carboxyl group in artemisinic acid, which indicated to be a weak acid solution when artemisinic acid was dissolved in water. Artemisinic acid was dissolved in $NaHCO_3$ solution, petroleum ether, acetone, alcohol and other organic solvents.[49,76,80–83] Since first isolation, the purification and structural characterization of artemisinic acid was performed by different laboratories.[62,83,84] In 1985, the A and B ring configuration of artemisinic acid was determined using circular dichroism (CD).[68] The total synthesis of artemisinic acid, starting from R-(+)-citronellal, was completed in 1989.[72] Misra et al. studied the crystals of hydrogen-bonded dimer of artemisinic acid by X-ray and the results showed the cyclization during intermolecular hydrogen bonding occurs by the opposite orientation of the α,β-methylene group in each molecule.[80] The 400 MHz ^{1}H NMR spectrum of data of artemisinic acid was published in 1996.[85] The absolute configuration of artemisinic acid was determined by the X-ray crystallographic analysis of its ρ-bromophenacyl derivative of artemisinic acid by Pang *et al.* in 1997.[81] So far, although artemisinic acid analogues were found in other plants, artemisinic acid was only found in *A.annua* and other *Artemisia* species were devoid of this compound, at most, contained traces.[86]

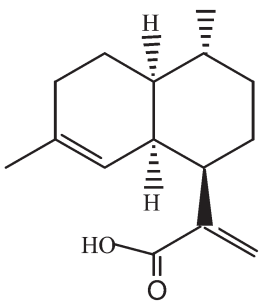

Fig. 1 Structure of artemisinic acid.

Considerable difference in artemisinic acid content was found between *A.annua* plants of different geographical origin,[49,52,87–92] which depends on the chemical type of *A.annua*. More and more evidences indicated the existence of at least two chemotypes within the species *A.annua* on the basis of the variation between the levels of artemisinin, artemisinic acid, and dihydroartemisinic acid.[52,87–91,93] Much higher levels of dihydroartemisinic acid and artemisinin were found in chemotype I plants. Conversely, notably lower levels of artemisinin and dihydroartemisinic acid but higher levels of artemisinic acid were detected in chemotype II plant.[52,87,88] Also the accumulation of the artemisinic acid-derived compounds arteannuin B and *epi*-deoxyarteannuin B was present in the chemotype with high content of artemisinic acid and the dihydroartemisinic acid-derived compound dihydro-*epi*-deoxyarteannuin B was accumulated in the dihydroartemisinic acid accumulating chemotype.[52,87] These two chemotypes showed differential metabolic response to different hormones elicitation.[92] Maes *et al.* investigated the effect of jasmonate, gibberellin and cytokinin on the two *A.annua* lines. The results indicated the size of glandular trichomes were markedly distinct in the two chemotypes under different phytohormons elicitation.[92]

The production variation of artemisinic acid was present in different development stage of *A.annua*.[49,94–97] At the vegetative stage of *A.annua*, artemisinic acid was more abundant, then the artemisinic acid content decreased with the plant development to flowering.[49,94–95,97]

Concentration of artemisinic acid has been found to vary in different parts of *A.annua* L.[73] Huang *et al.* found that artemisinic acid content in the top leave was higher level than other parts of *A.annua*.[73]

Different external conditions, such as phytohormones,[88,92,95,98–99] sugar,[93,100] drying,[101] DMSO,[102] and so on,[100] affect artemisinic acid content in *A.annua* L. Exogenous GA3[92,95] or methyl jasmonate[88] treatment resulted in the content decline of artemisinic acid. On the contrary, exogenous application of salicylic acid[98] or jasmonic acid[92] can induce pronounced increases in artemisinic acid accumulation. Metabolic response to phytohormones elucidation, however, was present in chemotype-dependent fashion in

different *A.annua* species.[88] Exogenous application of MeJA resulted in an accumulation of dihydroartemisinic acid and artemisinin in Type I plants. In Type II plants, however, artemisinic acid and artemisinin level decreased dramatically under MeJA elucidation. Squalene and other sesquiterpenes, (*e.g.*, caryophyllene, germacrene D), were stimulated by MeJA in both chemotypes.[88] Besides the intact plant, different factors can also affect the artemisinic acid production in transformed tissues. Weathers *et al.* notes that artemisinic acid was undetectable where phosphate was greater than 0.5 mM and inoculums culture age was 14 d in transformed roots culture.[100]

3 Bioactivity of artemisinic acid

3.1 Antimalarial activity

Although having less efficacy than artemisinin, artemisinic acid had some antimalarial activities on *P. berghei*.[79] The combination of artemisinic acid, arteannuin B, scopoletin and artemisinin with the same ratio has the similar antimalaria activity on *P. berghei* with artemisinin. The similar therapeutic effect of the combination with 25% content of artemisinin revealed different ingredients of *A.annua* had synergistic effect with artemisinin.[79]

3.2 Anti-tumor activity

Artemisinic acid derivatives with α-methylene lactone ring structure were synthesized from artemisinic acid and showed antitumor activity against leukemia P388 cell *in vitro*, which is the first case of artemisinic acid derivatives with antitumor activity.[75] More recently, Sun *et al.* also confirmed that artemisinic acid derivatives with alpha-methylene lactone ring structure can inhibit the growth of cancer cells in the further experiments.[77] Compared with those derivatives, the inhibitory activity of artemisinic acid on the tumor cells was significantly reduced.[75,103,104] These results indicated that alpha-methylene lactone ring structure played an important role in the artemisinic acid structure–activity relationship, which provided a clue that preparation of low toxicity and efficient anti-cancer new drugs from artemisinic acid.

3.3 Antipyretic effect

A.annua is a traditional Qingre drugs, and the mechanism of antipyretic action of the *A.annua* was extensively studied.[74,105] The results showed that artemisinic acid was of antipyretic effects, which was characterized as strong action, rapid onset and short duration.[74] Further experiments indicated that in addition to artemisinic acid, other compounds from *A.annua*, like artemisinin B and scopoletin, were also of antipyretic effects with different degrees. The above results revealed that antipyretic action of *A.annua* depended on the integration effect of more than one active compound.[74,105]

3.4 Antibacterial activity

Although no antifungal activity on *Candida albicans* and *Cryptoccocus neoformans*,[106] artemisinic acid has shown antibacterial activity on Gram-positive bacteria such as *Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus* with different inhibition degree.[70a,105] The minimal inhibitory concentration of artemisinic acid against *Staphylococcus aureus* 20, 109, Alcaligenes and nitrate-negative bacilli is 0.125 mg mL^{-1}, while the effective concentration against *E.coli*, *Salmonellatyphi* and *Shigella sonnei* is 0.5 mg mL^{-1}.[107]

3.5 Allelopathy effect

Allelopathy is a biological phenomenon by which an organism produces one or more allelochemicals that inhibit or stimulate the growth, survival, and reproduction of nearby growing organisms.[108,109] A variety of allelochemicals have been identified and the allelochemicals are considered as resources for developing herbicides, plant growth stimulators and pharmaceuticals.[110–112] These allelochemicals can have beneficial (positive allelopathy) or detrimental (negative allelopathy) effects on the target organisms. Artemisinic acid is one of the allelochemicals, and it has phytotoxicity. Although less effect than arteether, arteannuin B and artemisinin,[60,63,113] artemisinic acid can inhibit seedling growth of the dicotyledons and the monocotyledons.[113] The inhibitory effect of artemisinic acid on root was more pronounced than on the shoot.[113] Deng *et al.* reported that ethanol extract of *A.annua* can inhibit the germination and seedling growth of the investigated plants. Six compounds were isolated from the ethanol extract, and five of them were identified as artemisinic acid, artemisinin B, coumarin, palmitic acid and stigmasterol. The results indicated that artemisinic acid may play important role in allelopathy of *A.annua*.[114]

3.6 Anti-adipogenesis effect

Most recent, Lee *et al.* performed a screening for candidate anti-obesity agent using *A.annua* L and the active compound was determined to be artemisinic acid. Further study demonstrated that artemisinic acid can inhibit adipogenic differentiation of hAMSCs (human adipose tissue-derived mesenchymal stem cells) by reducing the transcripts level of C/EBP δ(CCAAT/enhancer binding protein), which was mediated by the inhibition of JNK(Jun N-terminal kinase).[115a] Taken together, these findings suggested that artemisinic acid may be used as a complementary treatment option for obesity associated with metabolic syndrome.[115a] Just a few days ago, the melanogenesis inhibition mechanism of artemisinic acid was also elucidated by the same laboratory.[115b]

4 Production of artemisinic acid

Despite its efficacy against malaria and its potential for use against certain cancers and viral diseases, artemisinin is in short supply mainly because of low production *in planta*, which make artemisinin be too expensive for many people in developing countries where malaria frequently occurs. In recent years, besides selection for high-producing lines and traditional breeding,[37,39,116] many other efforts have been made to improve artemisinin production. One of the alternate and promising approaches for improving the artemisinin supply is to synthesize artemisinin from its simple precursors

such as artemisinic acid *via* semi-synthetic route. In this approach, production of artemisinic acid in large scale is the prerequisite of improving artemisinin supply. There are many approaches can be performed to produce artemisinic acid, including direct extraction from plants,[49,62,76,82,117a,117b] tissue and cell culture,[100,116,133–137] total chemical synthesis[66,72] and fermentation preparation by use of synthetic biology technology.[40,41,53,197,203,204]

4.1 Extraction of artemisinic acid from plants

A.annua plant to date is likely to remain the only consistent commercial source of artemisinic acid.[49,62,76,82,117a,117b] In terms of artemisinic acid originated from plant, current initiateives to increase the usually low artemisinic acid content in plant may include (i) plantation expansion of *A.annua*,[118–120] (ii) breeding of high-yield cultivars,[49,117b] (iii) cultivation of transgenic plants,[121] and (iv) screening potential new sources of artemisinic acid.[122–127] The increased cultivation of *A.annua* in plantations is now an important strategy to enhance the yield of artemisinin, artemisinic acid and other sesquiterpenoid.[118–120] A large-scale plantation of *A.annua*, however, will result in vicious competition with grain crops in the land. Moreover, it will take relatively long growing cycles of *ca.* 12–15 months to produce appreciate yields of artemisinic acid.[119] Although the main aim of enhancement in plantation of *A.annua* is production improvement of artemisinin, the strategy can be using for reference in increasing artemisinic acid content.

The second approach for improving the economics of production of artemisinic acid is the selection of high-producing clones.[49,117b] The concentration of artemisinic acid in the different accessions of *A.annua* has been observed to vary between 0.06 to 0.53%.[49] Gupta has identified a variant of *A.annua* containing high amounts of artemisinic acid (0.8%), which has been cloned by micropropagation in tissue cultures.[49] More recently, a breeding program to improve artemisin and artemisinic acid production in plants was performed by use of identified artemisinin yield QTL (quantitative trait loci).[117b]

In order to get high-artemisinic acid-yield transgenic plants, two basic strategies, that is overexpressing the related genes of artemisinic acid biosynthesis and limiting the expression of key enzymes competing for precursors of artemisinic acid. Artemisinic acid is synthesized *via* the isoprenoid pathway in *A.annua*. In this pathway, FPP serves as the last common precursor of different terpenoids synthase, including amorpha-4,11-diene synthase, which is the first diverting enzyme in artemisinic acid biosynthesis. The conversion step catalyzed by terpenoids synthases represents a metabolic shunting point at which the cellular carbon flux is directed towards artemisinic acid or other sesquiterpenes, depending on their competition with the available FPP pool. To redirect more FPP to artemisinic acid biosynthetic pathway, expression upregulation of genes responsible for FPP synthesis[99] or level down-regulation of genes responsible for FPP conversion to the competing pathway[121] are usually two strategies for increased artemisinic acid production in transgenic *A.annua*.

In addition to *A.annua*, other species of *Artemisia* have been screened as potential new sources for agricultural production of artemisinic acid.[122–127] Although no detection of artemisinic acid, some of these species yield comparable amounts of artemisinin to that of *A.annua*.[122–127] In view of artemisinic acid is a precursor of artemisinin, together with amplification of sequences fragments essential for ADS enzyme function,[128] it is reasonable to postulate that other *Artemisia* species producing artemisinin may contained artemisinic acid.

4.2 *In vitro* production of artemisinic acid by cell and tissue culture

Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation. It is a fascinating and useful tool which allows the rapid production of many genetically identical plants using relatively small amounts of space, supplies and time, irrespective of the hindrances in natural conditions. As an alternative to field-grown crops or to chemical synthesis, the technique was also used to produce artemisinic acid for semi-synthetic artemisinin. Many investigators have successfully micropropagated *A.annua*, with the leaf[129–131] and stem[132–134] as explants.

Although no artemisinic acid was detected in callus of *A.annua*.[116] Several groups, however, had found artemisinic acid in transformed *A.annua* shoot and root cultures,[100,116,133–137] even in plantlets regenerated from hairy root clones.[136] Moreover, artemisinic acid content in *in vitro* hairy root cultures was affected by many factors including medium composition,[100,135,136] and the exogenous application of plant growth regulators.[134,137] Weathers *et al.* reported the effects of four factors, phosphate and nitrate salts, sucrose, and culture inoculums age on both root biomass and terpenoid production in transformed *A.annua* root cultures.[100] They found artemisinic acid was undetectable in experiments where phosphate was greater than 0.5 mM and for nearly all culture inoculums ages of 14 d.[100] Also, there is a report that gibberellic acid (GA3) can affect the growth and the secondary metabolite production of hairy roots of *A.annua*. The data showed that lower concentrations of GA3 appeared to improve the higher level of artemisinic acid.[134] The oligosaccharide elicitor (OE) from the fungal can stimulate conversion from artemisinic acid to artemisinin in hairy roots.[137] Exogenous H_2O_2 and O_2 can also reduce artemisinic acid content in hair root cultures. Ferreira *et al.* later reported artemisinic acid in plant from shoot cultures that were grown and harvested in the field or greenhouse, however, they found no artemisinic acid in root cultures differentiated from shoot culture.[135] Although no actual amount of artemisinic acid measured, artemisinic acid production by tissue culture of *A.annua* is feasible.

4.3 Chemical synthesis

The first total synthesis of artemisinic acid was achieved in 1989 by Zhou *et al.* who began their construction of the molecule with R-(+)-citronellal (Fig. 2).[72] Also, efficient synthesis of [15-^{2}H]- and [15-^{3}H]-artemisinic acid was accomplished by Xia *et al.* starting from methyl arteannuate in 1991 (Fig. 3–

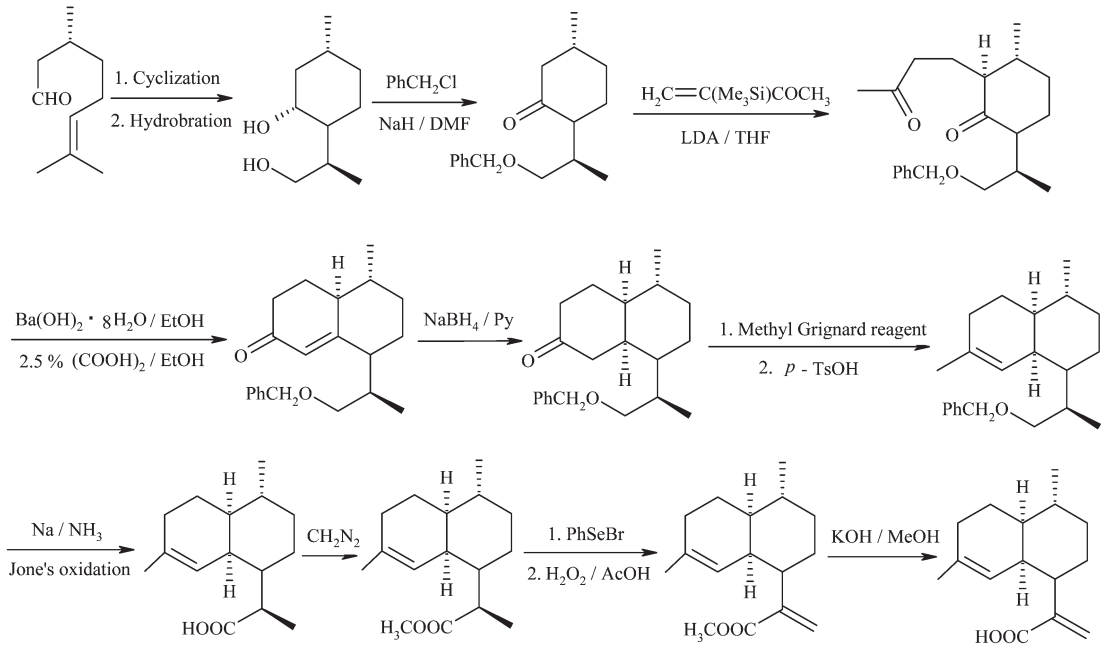

Fig. 2 Total synthesis of artemisinic acid.

4).[66] The synthetic artemisinic acid is commercially unaffordable due of low yield and complexity.

4.4 Scale production of artemisinic acid by synthetic biology technology

Currently, artemisinin is the only available drug that is globally effective against malarial parasite.[1–5] Due to extreme low concentration in the plant and low yield of its chemical synthesis,[31–33] the application of artemisinin is hampered by its availability. Such limitations cause supply problems, which increase the price of artemisinin.

Over the years, significant strides made in synthetic biology provide a chance for large-scale production of artemisinin, which is needed to satisfy global demand.[40–42,46–50] Synthetic biology of natural products is a very recent field of research. The underlying idea encompasses reconstruction of a complete plant terpenoid pathway in chassis cells such as *E.coli*[40,42,47] and *S.cerevisiae*.[41,46] The strategy is considered a novel avenue for the production of artemisinin, which means supply improvement because of higher yields of artemisinin by use of metabolic engineering. Therefore, a better understanding of the biosynthetic pathway leading to artemisinin in growing plants of *A.annua*, that is availability of well-characterized genes expression, is required for this approach.

Although the transformation of artemisinin acid and/or dihydroartemisinic acid into artemisinin is incompletely understood,[61,138] it is widely accepted that the complete pathway to artemisinic acid can be divided into three steps: (1) biosynthesis of FPP from isopentenylpyrophosphate (IPP) provided by both the mevalonic acid (MVA) and non-MVA pathways;[139–143] (2) cyclization of FPP to amorpha-4,11-diene catalyzed by amorpha-4,11-diene synthase for which corresponding cDNAs have been cloned independently by several groups;[144–146] and (3) biosynthesis of artemisinic acid from amorpha-4,11-diene, *via* artemisinic alcohol and artemisinicaldehyde intermediates (Fig. 5). Most genes encoding enzymes that are believed to be involved in artemisinic acid biosynthesis have been cloned recently.[152–217] With the aim of improving artemisinic acid production, its biosynthetic pathway has been reconstituted in different hosts such as *E.coli*,[40] *S.cerevisiae*[41,147–149] and *in planta*.[48,150,151]

4.4.1 Cloning of genes related to artemisinic acid biosynthesis. *Related genes of MEP pathway in A.annua.* A number of related genes of MEP pathway, such as 1-deoxy-D-xylulose

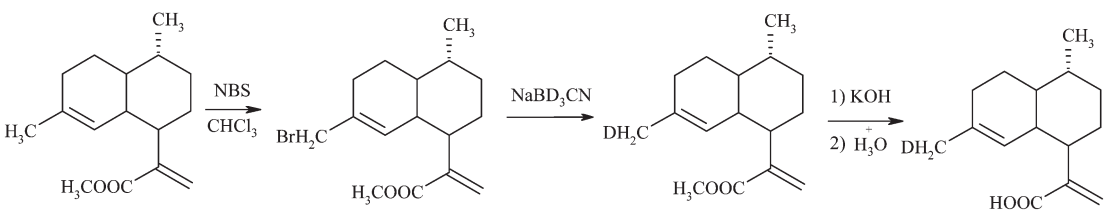

Fig. 3 Synthesis of [15-2H] –artemisinic acid.

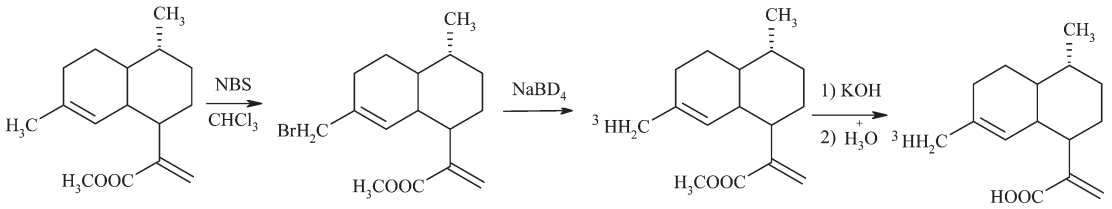

Fig. 4 Synthesis of [15-3H] –artemisinic acid.

5-phosphate synthase (DXPS),[152,154] 1-deoxyxylulouse 5-phosphate reductoisomerase (DXPR),[152,153] 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (HDS)[155] and hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR),[156] have been isolated and functionally characterized by different groups. DXPS,[152,157] DXPR[152,157,158] and HDR,[157] were expressed in different tissues of *A.annua*, even in hairy roots lacking functional chloroplasts.[152,157] Furthermore, as a committed precursors pathway of artemisinic acid, the expression of MEP pathway genes were regulated by growth conditions and other environmental factors, such as culture age of the transformed roots,[152] light,[152] sugars[93] and exogous elicitors.[159,160]

HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase. HMGR is the rate-limiting enzyme in artemisinin synthesis, which can catalyze HMG-CoA to yield mevalonic acid (MVA).[142] The HMGR encoding gene is the first sequence that was registered in GenBank in all of the genes related to artemisinin biosynthesis. So far, three gene versions of HMGR were cloned from *A.annua* L. (GenBank accession no.U14624, U14625 and AF142473), which were submitted to GenBank directly, and the papers related to the gene cloning and functional characterization were not published.[161,162] HMGR can be a target gene of *A.annua* miRNA by computational analysis.[163] In addition, as a rate-limiting enzyme of the terpenoid pathway,[164,165] HMGR was found to limit artemisinin formation in *A.annua*.[166] The regulation by HMGR was reflected that more expression of HMGR was seen in highly biosynthetically active tissues, such as flower buds and young leaves, in which artemisin was produced.[157] Moreover, overexpression of HMGR and other related genes in artemisinin pathway led to significant increase in the artemisinin or its precursors yield from the transgenic *A.annua*[129,130] or engineered microbes.[41,46,167–172]

HMGR is considered to be an important regulator enzyme in plant terpenoid biosynthesis. More and more investigations revealed the expression of HMGR gene was regulated when *A.annua* was exposed to different treatments, such as sugar,[93] radiation,[173] exogous elicitors,[159] regulators[99,174a] and heavy metal.[174b]

FPPS, farnesyl pyrophosphate synthase. FPPS is a l′-4 prenyltransferase which catalyzes two consecutive condensations of isopentenyl diphosphate (IPP) with the allylic diphosphates, dimethylallyl diphosphate (DMAPP) and the resultant geranyl pyrophosphate (GPP).[175] The gene cloning and functional analysis of FPPS in *A.annua* was first reported by Matsushita *et al.* in 1996.[175] The FPPS cDNA was 1032 bp long and encoded a protein of 343 aa residues with a calculated molecular weight of 39.42 kDa. The deduced amino acid sequence of *A.annua* FPPS was highly similar to counterpart from other organisms, and contained the two conserved domains found in polyprenyl synthases including FPPS, geranylgeranyl diphosphate synthases and hexaprenyl diphosphate synthases.[175] The catalytic activity of FPPS was confirmed by heterologous *E.coli* expression.[175] Besides the study of Matsushita *et al.*, there were two papers that reported the FPPS gene cloning and functional characterization in different *A.annual* strains.[176,177] FPPS is a key enzyme of the isoprenoid pathway, and overexpression of heterologous FPPS is an effective way to improve the artemisinin content in *A.annua*.[178,179] Meanwhile, the expression of FPPS was regulated by different exogous stresses, like sugar,[93] radiation,[173] heavy metal[174b] and biotic regulators.[99,121] Compared to other genes responsible to artemisinic acid biosynthesis, the expression pattern of FPPS gene is clearly distinct. FPP serves as a common precursor of GPP and different sesquiterpenes, which are lied intensively in different tissue. FPPS showed similar levels of transcript in all tissues analysed. Furthermore, FPP is also required for biosynthesis of sterols, components of cellular membranes, expression of FPPS is found in different cell types.

ADS, amorpha-4,11-diene synthase. Amorpha-4,11-diene (ADS) is a sesquiterpene synthase, which can catalyze the cyclization of farnesyl pyrophosphate (FPP) to produce (1*S*,6*R*,7*R*,10*R*)-amorpha-4,11-diene.[144–146,180,181] Although the presence of an unidentified enzyme-bound sesquiterpene-like intermediate from FPP in the biosynthesis of artemisinin was firstly determinated by Akhila *et al.* more than ten years ago,[182,183] the first detection of amorpha-4,11-diene and partial purification of amorpha-4,11-diene synthase from the plant was reported by Bouwmeester *et al.* in 1999.[55] The low level of the volatile amorpha-4,11-diene in the plant and the high amorpha-4,11-diene synthase activity were considered to be strong evidence that amorpha-4,11-diene is an intermediate in the biosynthesis of artemisinin. The cDNA gene encoding for amorpha-4,11-diene synthase had been cloned and functionally characterized by several groups.[144–146,184–187] Codon-optimized variants of ADS were also synthesized and introduced into *E.coli* and *S.cerevisiae* for high production of amorpha-4,11-diene, respectively.[42,188] ADS cDNA has a 1641-bp open reading frame coding for 546 amino acids with a calculated molecular mass of 63.9 kDa.[144–146] Although the ADS genomic sequences were also isolated and submitted

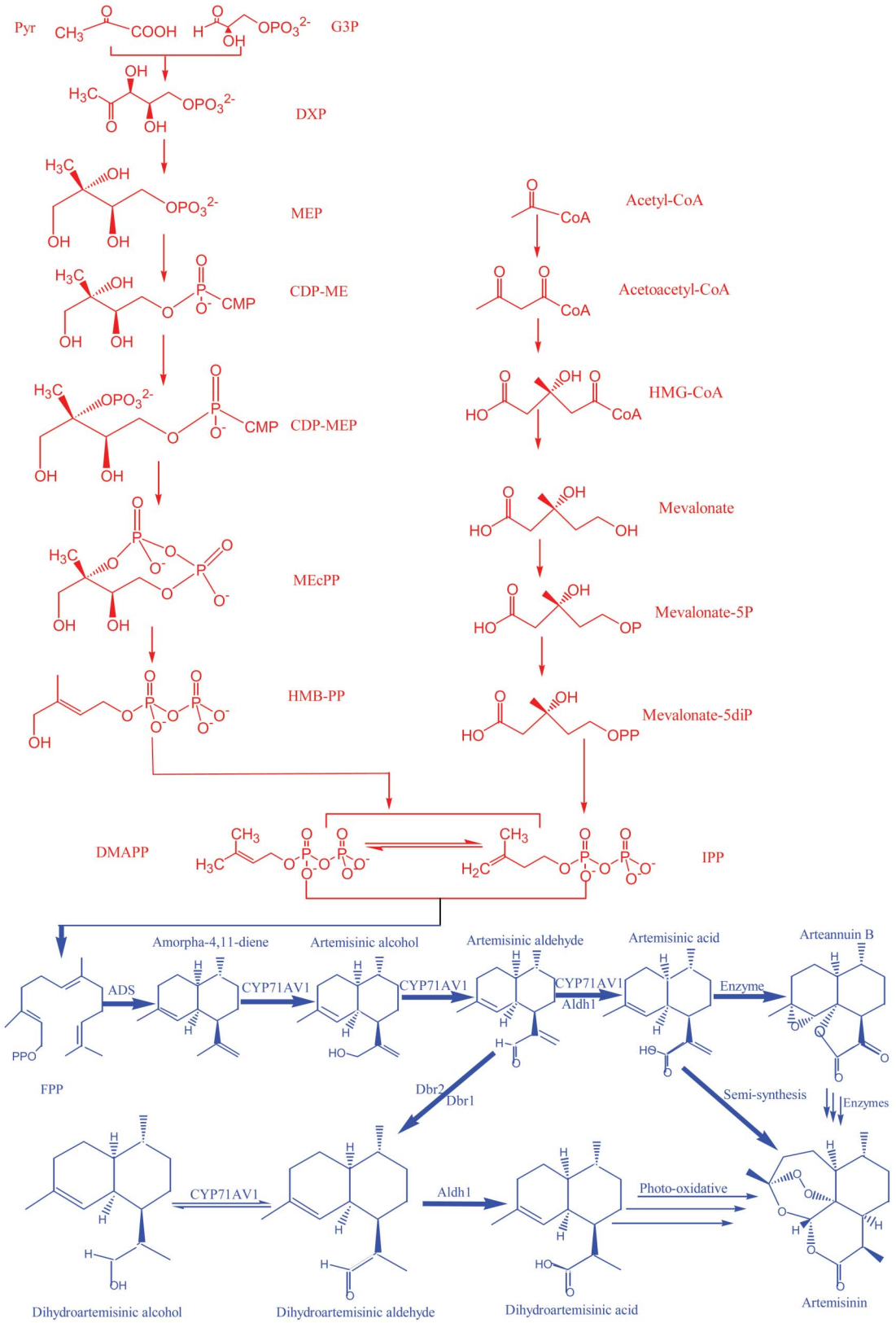

Fig. 5 Biosynthetic pathway of artemisinic acid

directly to GenBank with accession number of AF327527 in 2001, the related paper was published until 2006.[184] The genomic ADS has a complex organization including seven exons and six introns, and belongs to class III terpene synthase.[184,185] The ADS promoter sequence was isolated and submitted to GenBank with accession number of AY528931 by Ye's group in 2004. Two years later, ADS promoter sequence was used to explore the tissue specificity and developmental pattern of amorpha-4,11-diene synthase.[189] To date, most sequences related to ADS were all isolated from *A.annua*. It is opined that transcription of ADS has tissue[209] and cell-specificity.[153]

The recombinant ADS has a broad pH optimum.[55,144,184,190] As most sesquiterpene synthases, ADS is characterized by the generation of one dominant product (91% amorpha-4,11-diene) along with traces of a varying number of by-products.[144,146,187,190,191] No monoterpene synthase activity, however, could be detected in recombinant ADS.[144]

Amorpha-4,11-diene synthase is located at a branching point of terpene metabolism and it is considered a key enzyme in the biosynthesis of artemisinin. ADS was introduced into different hosts such as *E.coli*,[40,42,47,167–171,192–196] *S.cerevisiae*,[41,46,72,197–204] *Aspergillus nidulans*,[205] *Nicotiana tabacum L*,[146,206] and *Arabidopsis thaliana*[189] alone or together with other genes related to artemisinin biosynthesis. Detailed characterization of ADS structure and function facilitated heterologous production improvement of amorpha-4,11-diene in engineered hosts by new tools in synthetic biology. The high titers of amorpha-4,11-diene was achieved to the g L^{-1} level by strain improvement and fermentation optimization.

Amorpha-4,11-diene synthase is considered to be a key regulatory enzyme in the biosynthesis of artemisinin. There are several elicitor and hormone-responsive elements present in ADS promoter sequence.[189] The transcripts of ADS gene was dramatically fluctuated when *A.annua* was exposed to different stresses such as phytohormones,[88,95,99] irradiation,[173,207,208] environmental stresses,[146,208] sugars,[93] DMSO,[102] heavy metal[174b] and so on.[209] Ultraviolet irradiation[173] and glucose[99] pretreatment triggered ADS overexpression, while exogenous application of methyl jasmonate can result in differential expression of ADS gene in a chemotype-dependent fashion.[88] marginal induction. The chemotype-dependent metabolic responses to phytohormones were present in *A.annua* pre-treated with phytohormones. As shown in the study of Wu *et al.*, MeJ elicitation had quite different effect on ADS transcript in the two chemotypes. For type I plants, ADS gene showed upregulation in response to MeJ induction. For type II plants, however, expression of ADS decreased dramatically.[88]

AMO, amorpha-4,11-diene monooxygenase. Amorpha-4,11-diene monooxygenase refers to the enzyme that can oxidase amorpha-4,11-diene at C-12 position to form more oxygenated products *in vivo*. The genes encoding amorpha-4,11-diene monooxygenase were recovered from *A.annua*[41,210–213] and other plants.[149,213,214a,214b]

Taking into consideration the high *in vitro* amorpha-4,11-diene synthase activity and minute quantities in the leaf essential oil of *A.annua*, Bouwmeester *et al.* hypothesized the presence of further P450 dependent hydroxylation modification of amorpha-4,11-diene *in planta*.[55] In the latter work, Bertea *et al.* determined that a cytochrome P450 monooxygenase (P450) catalyses the first region-specific hydroxylation of amorpha-4,11-diene in *A.annua*.[213] The cDNA encoding amorpha-4,11-diene monoxygenase CYP71AV1 was first cloned and functionally characterized by two laboratories.[41,210a] Further cDNA analysis of the two sequences showed the presence of the length difference of deduced ORF of amorpha-4,11-diene monoxygenase. The full-length CYP71AV1 cDNA of Keasling group's encoded an ORF of 495aa protein,[41] whereas full-length ORF was deduced to encode a 488aa polypeptide in the Covello laboratory, with a 7aa deleting in N-terminus of CYP71AV1.[210a] The fact that the two enzymes can catalyze a three-step oxidation of amorpha-4,11-diend to artemisinic acid indicated the amino acids sequence of N-terminus of CYP71AV1 has no effect on its activity. From then on, CYP71AV1 gene was cloned from different strains of *A.annua* by many researchers.[211,212] So far, dozens of CYP71AV1 genes had been registered in GenBank library. CYP71AV1 is a multifunctional enzyme and has substrate selection. CYP71AV1 can act on amorpha-4,11-diene, without activity on other monoterpenes and sesquiterpenes, such as limonene, α-pinene, beta-pinene, pinocarveol, (−)-alloisolongifolene caryophyllene, (−)-α-gurjunene, (+)-γ-gurjunene, (+)-ledene, (+)-β-selinene and (+)-valencene.[210a] CYP71AV1 can also convert dihydroartemisinic alcohol to form the corresponding aldehyde at about 50% of the rate for artemisinic alcohol, but it has almost no activity on the dihydroartemisinic aldehyde.[216] Expression of CYP71AV1 by RT-PCR analysis is tissue specific, with the highest expression levels in trichomes, followed by flower bud (flower bud), very low levels of expression in leaves and roots.[158,209,210a,210b,210c] Further experiments revealed the expression of CYP71AV1 was cell specific, with sole expression in the apical cells of glandular secretory trichomes.[153]

Besides *A.annua*, other CYP71AV1 homologues were isolated from other plants outside *A.annua*.[149,214] Kraker *et al.* first reported an alternate C-12 oxidative activity in chicory extracts and the microsomal enzyme preparation of chicory roots can catalyze the hydroxylation of amorpha-4,11-diene in the presence of NADH.[215] The recombinant germacrene A oxidase isolated from five plants species including lettuce (*Lactuca sativa*) can selectively oxidize at C-12 position of both germacrene A and amorpha-4,11-diene.[214] It is the first P450 enzyme that has been demonstrated to be functional in catalyzing the three-step conversion of non-natural substrate amorpha-4,11-diene to artemisinic acid.

In addition to the cDNA isolation of germacrene A oxidase described by Nguiyen *et al.*, Cankar *et al.* also recovered another cDNA encoding (+)-valencene oxidase designated CYP71AV8 from chicory (*Cichorium intybus* L.).[149] CYP71AV8 have a variety of terpene-hydroxylation activities, with C-2 position oxidation of (+)-valencene and C-12 position hydroxylation of both amorpha-4,11-diene and germacre A. When amorpha-4,11-diene was supplied as substrate of CYP71AV1, not only artemisinic alcohol and artemisinic aldehyde, but also small amounts of artemisinic acid and dihydroartemisinic aldehyde were detected in the products, as was previously shown for CYP71AV1.[41,210a,216] More than one mono-oxygenase can function as amorpha-4,11-diene oxidative activity,

which showed the catalytic plasticity is embedded in P450 enzymes. In this respect, the versatility of amorpha-4,11-diene monoxygenases could serve as ideal molecular templates to create a catalytically more efficient amorpha-4,11-diene oxidase through *in vitro* evolution and engineering methods.[217a]

The expression pattern of CYP71AV1 was studied extensively due to its involvement of artemisinin biosynthesis. Lines of evidence suggest that the transcription of CYP71AV1 gene was significantly affected by biotic treatment like phytohormones,[88] more availability of FPP,[121] and abiotic stresses such as chilling,[217b] which deepened our understanding for its application in semi-synthetic artemisinin by synthetic biology techniques.

CPR, NADPH:cytochrome P450 oxidoreductase. The native redox partner of CYP71AV1, NADPH:cytochrome P450 oxidoreductase (CPR), was also isolated from *A.annua*, and its biochemical function was confirmed *in vitro*.[41]

Dbr2, artemisinic aldehyde double bond reductase. Through a combination of partial protein purification, mass spectrometry, and expressed sequence tag analysis, a cDNA clone corresponding to the artemisinic aldehyde-11(13) reductase (Dbr2) was isolated. Dbr2, a 415-amino acid protein with a predicted molecular mass of 45.6 kDa, showed high amino acid sequence identity to plant 12-oxophytodienoate reductases (OPRs) and related enzymes. The recombinant Dbr2 can catalysis artemisinic aldehyde to form (11*R*)-dihydroartemisinic aldehyde as the major product. In addition to artemisinic aldehyde, Dbr2 also has activity on 2-cyclohexen-1-one, (+)-carvone and 2*E*-nonenal. No activity was detected with arteannuin B, artemisinic acid, artemisinic alcohol, artemisitene, coniferyl aldehyde, 2*E*-nonenal, (+)-α-pinene, (+)-pulegone, and sabinone as substrates. The pH optimum of Dbr2 was determined to be pH 7.5. Both NADPH and NADH can serve as cofactor of recombinant Dbr2. The high expression of Dbr2 was found in glandular trichomes.[50]

In addition to Dbr2, another cDNA encoding a distinct artemisinic aldehyde double bond reductase was also isolated from *A.annua.* The resultant cDNA encoded for a 347 amino acid protein with a calculated molecular weight of 38.5 kDa. Based on the sequence comparisons and phylogenetic analysis, *A.annua* Dbr1 appears to be a member of the medium-chain dehydrogenase/reductase (MDR) superfamily. The 11-epimers of dihydroartemsinic aldehyde, (11*R*)-and (11*S*)-dihydroartemisinic aldehyde with an S : R ratio of approximately 7, was detected when artemisinic aldehyde was supplied as a substrate in *in vitro* assay of recombinant Dbr1. Recombinant Dbr1 was also active on other 2,3-unsaturated aldehydes, such as 2*E*-hexenal and 2*E*-nonenal. No activity, however, was detected, no activity was detected with artemisinic acid, artemisinic alcohol, coniferyl aldehyde nonanal, hexanal, (+)-α-pinene, 2-cyclohexen-1-one, (*R*)-(−)-carvone and (+)-pulegone. The pH optimum of Dbr1 was determined to be 7.0. No activity was detected with NADH as cofactor. *Artemisia annua* Dbr1 expression is readily detectable in aerial parts of the plant, but not in the roots. The expression is somewhat stronger in flower buds and leaves than in glandular trichomes where artemisinin is synthesized.[218]

ALDH1, aldehyde dehydrogenase. An aldehyde dehydrogenase gene, named Aldh1, was isolated from *Artemisia annua*. The *A.annua* aldehyde dehydrogenase 1 gene has an open reading frame (ORF) that encodes a 499 amino-acid protein with a calculated molecular weight of 53.8 kDa. The recombinant ALDH1 can catalyze the NAD(P)-dependent oxidation of artemisinic aldehyde and dihydroartemisinic aldehyde to generate corresponding acid products. In addition to artemisinic aldehyde and dihydro artemisinic aldehyde, the short chain alkyl aldehydes, like 2-nonenal and octanal, and sinapaldehyde and 2-phenylpropanal can also serve as substrates of purified ALDH1. With dihydroartemisinic aldehyde as the substrate, recombinant Aldh1 can use NAD and NADP as cofactors effectively. Aldh1 gene is expressed in the aerial parts of the plants (highest expression in the glandular trichomes, moderate expression in the flower buds and low expression in the leaves), but not in the roots. This expression pattern matches that of CYP71AV1 and is consistent with a role for the gene product in the metabolism of secondary metabolites in glandular trichomes.[216]

4.4.2 Engineering *E. coli* to produce a high level of artemisinic acid. Although the drawback of limited expression of cytochrome P450 in *E.coli*, the successful reconstruction of artemisinic acid pathway in *E.coli* was first conducted by Chang *et al.*[40] In this report, Chang *et al.* improved the production of artemisinic acid up to 105 ± 10 mg L^{-1} by combinational use of codon optimization, N-terminal transmembrane engineering, replacement of expression vector and selection of appropriate host.[40] This is the first example of *in vivo* production of functionalized terpenoids in *E. coli* at high titer using native plant P450s, which may offer a new opportunity for engineering *E. coli* for production of complex natural products or natural product intermediates.

4.4.3 Metabolic engineering of *S.cerevisiae* for artemisinic acid production. Yeast platform offers many of the same advantages that prokaryotic systems do for terpene hydrocarbon production, plus may provide the biosynthetic machinery necessary for the proper functioning of the downstream modifying enzymes like cytochrome P450 hydroxylases. The yeast system, like *S.cerevisiae*, was intensively used as a host of drug production in synthetic biology. The first artemisinic acid production in *S.cerevisiae* was reported by Ro *et al.* in 2006.[41] In the engineered yeast constructed by Ro *et al.*, the production of artemisinic acid reached up to 100 mg L^{-1} when all *A.annua*-derived genes (ADS, CYP71AV1, and CPR) were expressed on a single host. The transgenic yeast produced more artemisinic acid in contrast to relative plant biomass (4.5% dry weight in yeast compared to 1.9% artemisinic acid, and 0.16% artemisinin in *A.annua*) and in a shorter time period (4–5 days for yeast *versus* several months for *A.annua*.[41] The efficient conversion of amorpha-4,11-diene to artemisinic acid by *S. cerevisiae* expressing the ER-bound AMO demonstrated it to be an ideal microbial platform to functionally express genes encoding membrane-bound enzymes.

In order to develop an industrially competent yeast strain, many biological engineering efforts have been applied to significantly improve artemisinic acid production in the

engineered microorganism. Artemisinic acid production was improved to 250 μg mL^{-1} in shake-flask cultures and 1 g L^{-1} in bioreactors by modulating the selection marker of the expression plasmid and the composition of the culture medium.[203] Subsequent development of a high-density fed-batch fermentation process with a dissolved oxygen-stat algorithm that controlled carbon delivery and agitation simultaneously, allowed production of 2.5 g L^{-1} artemisinic acid,[204] 25-fold over previously reported titers.[41] These results validated heterologous production of artemisinic acid in bioreactors as a potential supply route for inexpensive artemisinin.

Because of substrate competence of artemisinic acid and dihydroartemisinic acid, further incorporation of Dbr2 gene (artemisinic aldehyde reductase) in the artemisinic acid producing yeast resulted in 15.7 ($\pm$1.4) mg L^{-1} dihydroartemisinic acid and production decline of artemisinic acid to 11.8 ($\pm$2.8) mg L^{-1}.[53]

In addition to CYP71AV1, other genes encoding for enzymes catalyzing C-12 oxidation of amorpha-4,11-diene was also used for reconstruction of engineered yeasts producing artemisinic acid. A chicory cytochrome P450 was used for co-expression with amorpha-4,11-diene synthase in yeast to produce artemisinic acid.[197]

4.4.4 Metabolic engineering of plants for artemisinic acid production. In search of alternative production system, agriculture crops with rapid accumulation of biomass at low costs were often used for heterogous hosts for artemisinic acid production. Biosynthetic genes leading to artemisinic acid, like HMGR, FPPS, ADS and AMO, were combined and expressed in plants for high artemisinic acid yield. These attempts to produce artemisinic acid in heterologous plants, however, did not lead to its accumulation due to internal glycosylation[49] and insufficient oxidation toward artemisinic acid.[205] Based on previous research, Farhi *et al.* successfully produced artemisinin in engineered tobacco by conditions optimization, such as concomitant expression of CPR and CYP71AV1, light intensity enhancement during transgenic tobacco cultivation, targeting to appropriate cellular compartment and excessive gene expression of rate-limiting enzyme conducive to enhance precursor availability, single-vector-based transformation and employing different promoters to avoid gene silencing.[151] This is the first case of artemisinin production in heterologous host by metabolic engineering. Although low level, this research paves the way for further development of a sustainable production platform of artemisinin.[151]

5 The separation and purification of artemisinic acid from *A.annua* L

From the above descriptions, the amount of artemisinic acid is about 8–10 times more than that of artemisinin in the plant *A.annua*.[243] Moreover, artemisinic acid has a related cadinane-type chemical structure to that of artemisinin and can easily be transformed to artemisinin *in vitro*. The utilization of artemisinic acid as a starting material for the synthesis of artemisinin, therefore, has a practical importance. Presently, all the commercial artemisinic acid was from *A.annua*. Consequently, an efficient means of extracting artemisinic acid from *A.annua* is required, which could be potentially improved artemisinin production 8-fold by semi-synthetic route derived from artemisinic acid.

The extraction of artemisinic acid from *A.annua* is widely reported in the literatures.[41,49,50,62,64,67,73,76,83,84,95,99,101,107,115*a*,117*a*,134,145,183,210*a*,219–230] The liquid extraction with petroleum ether,[101,183,219–221] acetone,[67,73,76,221] methanol,[95,99,117*a*,145,224] toluene,[64,134,225,226] ethanol,[62,107,221,222] dichlormethane,[50,94,210*a*] n-hexane[41,49,64,84,115*a*,219,221,227–229] and supercritical fluid extraction with carbon dioxide[225,226,230] are the most currently applied isolation methods.

6 Chemical analysis of artemisinic acid

Up to now, a number of techniques are available for the estimation of artemisinic acid including the high performance liquid chromatography (HPLC) with ultraviolet detection (UV),[46,49,64,73,90,95,99,101,133,134,136,220,231–233] or evaporative light scattering detection (ELSD),[231,232] HPLC coupled with mass spectrometry (HPLC-MS),[219,234,235] supercritical fluid chromatography (SFC),[225,226,236] thin-layer chromatography (TLC),[229] gas chromatography analysis(GC),[94,121,216] GC coupled with mass spectrometry (GC-MS)[41,65,94,115*a*,135,149,150,210*a*,213,216,221,230,237] and nuclear magnetic resonance spectroscopy (NMR).[54,80–82,115*a*,224,229]

7 Chemical synthesis and biotransformation using artemisinic acid as starting material

Artemisinic acid has a wide activity mentioned above, and its content is relatively abundant. Thus, many groups search for a route from artemisinic acid to its derivatives through chemical synthesis or biotransformation. These researches provide many kinds of artemisinic acid analogs, and in some degree can help to elucidate the biosynthesis pathway of artemisinin.

7.1 Chemical synthesis from artemisinic acid

Up to now, many compounds such as artemisinin (**2**)[51] and its analogs (**3–10**),[56,238–244] (+)-deoxoartemisitene (**11**)[33,245] deoxoartemisitone (**12**),[245] demethyldeoxoartemisininylic acid (**15**),[33] 13-bromodeoxoartemisinin (**16**),[33] arteannuin A (**17**),[246] (−)-arteannuin B (**18**),[247–250] arteannuin D (**19**),[251] desoxyartemisinin (**20**),[53] deoxodeoxyartemisinin (**21**),[252] (+)-deoxoartelinic acid (**22**),[253] (−)-deoxoartemisinin (**23**),[250,254] homodeoxoartemisinin (**24**),[250] *epi*-deoxyarteannuin B (**25**),[255*a*] amorpha-4,11-diene (**26**),[55,150,210*a*] artemisilactone (**27**, **28**, **29**, **30**)[255*b*] have been synthesised through the chemical method from the initiator artemisinic acid (Fig. 6). Among these compounds, some molecules had antimalarial activity comparable to artemisinin.[250,253,254] However, the chemosynthesis

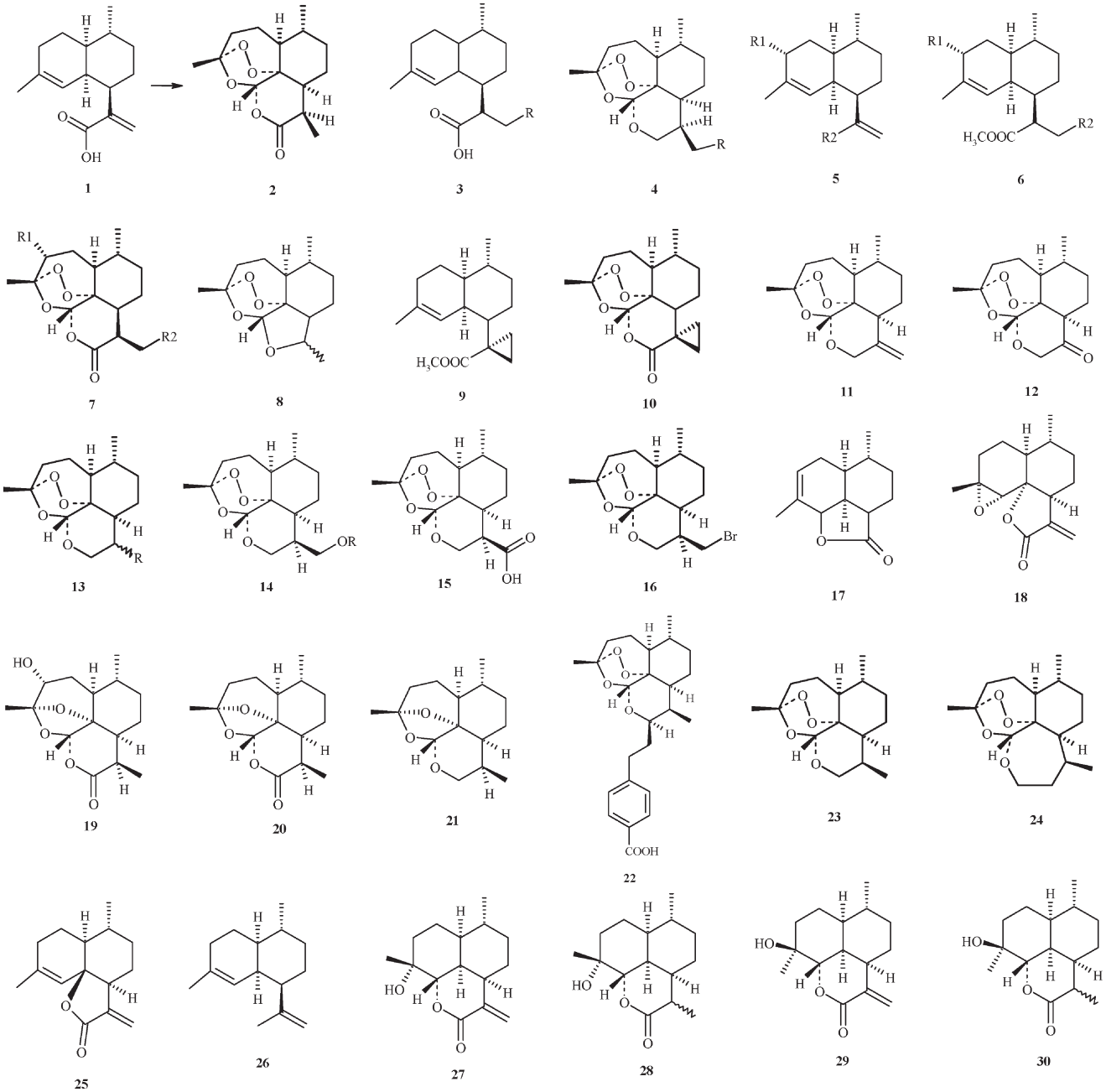

Fig. 6 The chemical conversion of artemisinic acid into ''artemisinin'' components.

method cost tedious procedures and serious pollution, and no industrialized production has been reported.

7.2 Biotransformation of artemisinic acid

Biotransformations are chemical reactions catalyzed by cells, organs or isolated enzymes.[256–258] Biotransformation is an effective tool for the preparation of compounds, which may be otherwise difficult to prepare by conventional synthetic methods. Biotransformation offers the advantages of high stereo-specificity, operation at non-extreme pH and near room temperature. Hence, biotransformation of artemisinic acid can extend its diversity and improve its biological availability.

Up to date, many artemisinic acid derivatives had been obtained through the method of biotransformation (Fig. 7 and Table 1). A couple of stereoisomers, 3-α-hydroxyartemisinic acid (**31**) and 3-β-hydroxyartemisinic acid (**32**) were obtained when artemisinic acid was biotransformed by *Aspergillus flavipes* and *Mucor mucedo*, respectively.[259] Plant biotransformation of artemisinic acid produced end products in a similar stereo-specificity with microbiological bioconversion of artemisinic acid. 3-α-hydroxyartemisinic acid (**31**), was also obtained after two days of artemisinic acid administration to the suspension cells of *Cephalotaxus fortunei* and *A.annua*.[260] Besides 3-α-hydroxyartemisinic acid (**31**), three more products

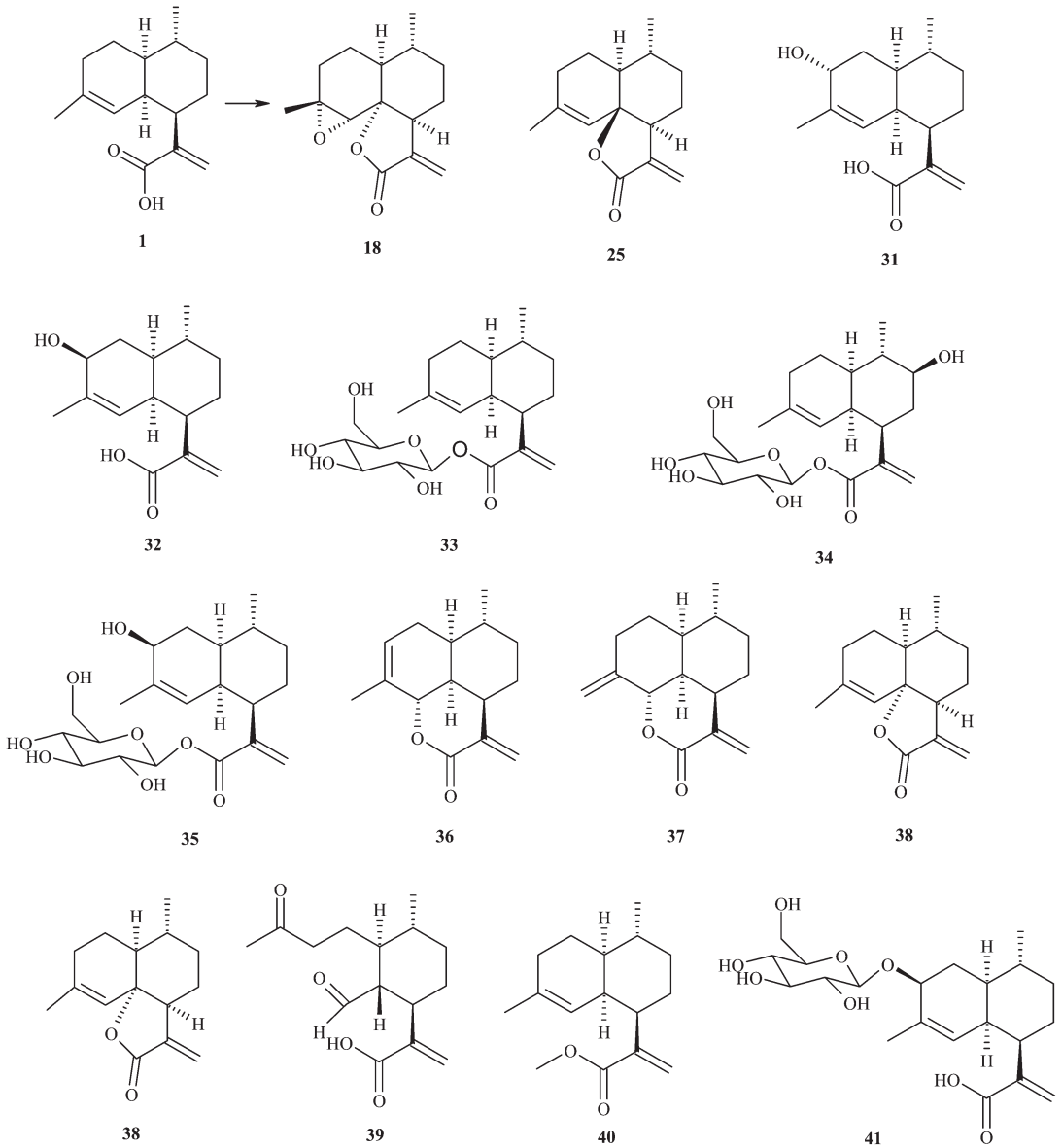

Fig. 7 The biotransformation of artemisinic acid into ''artemisinin'' components.

were also produced by *A.annua* biotransformation of artemisinic acid. Kawamoto *et al.* added artemisinic acid into the calli of *A.annua* induced from young stems to conduct biotransformation. After two days of culture, products artemisinic acid β-D-glucopyranosyl ester (**33**) was isolated from the medium, while products 9-β-hydroxyartemisinic acid β-D-glucopyranosyl ester (**34**) and 3-β-hydroxyartemisinic acid β-D-glucopyranosyl ester (**35**) were isolated from the cells. These three products were not detected on HPLC analyses of the culture to which no substrate was added, which proved these three compounds transformed from the artemisinic acid.[261] In addition to *A.annua*, other plant species such as *Averrhoa carambola*[262] and *Catharanthus roseus*[263] were also used as biocatalyst systems to biotransform artemisinic acid. No corresponding biotransformation product, however, was obtained when crown galls of *Panax quinquefolium* was used in the biocatalytic cell of artemisinic acid.[263] Artemisinic acid was added to the suspension of transgenic hairy roots of *P. multiflorum* which had been pre-cultured for 7 d and co-cultured for another 2 d. The biotransformation products were identified as isoannulide (**36**), annulide (**37**), 3-β-hydroxyartemisinic acid (**32**) and deoxyarteannuin B (**38**).[264] As mentioned above, biotransformation of artemisinic acid was catalyzed not only by cells, but also by isolated enzyme. Caputi *et al.* used a platform of 107 recombinant glycosyltransferases to catalysis artemisinic acid, and results revealed that some glycosyltransferases could biotransform artemisinic acid into corresponding glycoside.[265] Some of these biotransformed products were determined to have anticancer activity, which showed an alternative to chemical synthesis to acquire anticancer agents.[262,263]

Table 1 Biotransformation of artemisinic acid

Substrate	Products	Biocatalyst system	References
Artemisinic acid	3-α-Hydroxyarteminisic acid (**31**)	*Aspergillus flavipes*	259
Artemisinic acid	3-β-Hydroxyartemisinic acid (**32**)	*Mucor mucedo*	259
Artemisinic acid	3-α-Hydroxyarteminisic acid (**31**)	*Cephalotaxus fortunei*	260
Artemisinic acid	3-α-Hydroxyarteminisic acid (**31**)	*A.annua*	260
	①Artemisinic acid β-D-glucopyranosyl ester (**33**)		261
	②9-β-Hydroxyartemisinic acid		
Artemisinic acid	β-D -glucopyranosyl ester (**34**)	*A.annua*	
	③3-β-Hydroxyartemisinic acid		
	β-D-glucopyranosyl ester (**35**)		
	①3-β-Hydroxyartemisinic acid (**32**)		262
Artemisinic acid	②Artemisinic acid 3-β-O-β-D-glucopyranoside (**41**)	*Averrhoa carambola*	
	③3-β-Hydroxyartemisinic acid		
	β-D- glucopyranosyl ester (**35**)		
Artemisinic acid	①3-α-Hydroxyarteminisic acid (**31**)	*Catharanthus roseus*	263
	②3-β-Hydroxyartemisinic acid (**32**)		
Artemisinic acid	Artemisinic acid glucose ester	①Recombinant glycosyltransferase (GTs) ②Whole-cell biocatalysis of engineered *E.coli* expressing recombinant GTs	265

The *in vivo* transformations of artemisinic acid have been observed in *A.annua* by GD Brown *et al.*[61] Arteannuin B (**18**) is the major metabolite from the artemisinic acid, together with *epi*-deoxyarteannuin B (**25**), isoannulide(**36**), annulide (**37**), deoxyarteannuin B (**38**), a *seco*-cadinanne (**39**), and the methyl ester of artemisinic acid (**40**).

8 The status of artemisinic acid to artemisinin biosynthesis

8.1 Early work: artemisinic acid is a biogenetic precursor of artemisinin biosynthesis

Although many reports described the stereospecific synthesis of arteannuin B from artemisinic acid,[247,249,266] the first discussion about the biosynthetic implications of artemisinic acid in relation to artemisinin was first reported by El-Feraly in 1986.[78] Formation of arteannuin B was observed when artemisinic acid was subjected to single oxygen ($^1O_2$) generated by dye-sensitized photo-oxygenation. In view of the similar conversion of artemisinic acid to arteannuin B could take place *in vivo*, plus conversion identification of arteannuin B to artemisinin in cell-free system of *A.annua*,[78] El-Feraly *et al.* suggested that artemisinic acid may be the precursor of artemisinin.[78] Wang *et al.* converted artemisinic acid ′H-labeled at C-15 to both arteannuin B and artemisinin in *A.annua* homogenate system, which indicated artemisinic acid is the common precursor of arteannuin B and artemisinin.[69] The notion is in accordance with the subsequent investigations.[59,70c,73] Their results, however, did not show whether artemisinic acid is converted to artemisinin *via* the intermediacy of arteannuin B. In 1992, Nair *et al.* reported results which clearly indicate that arteannuin B is an intermediate in the bioconversion of artemisinic acid to artemisinin.[271] Sangwan *et al.* found that [14C]-artemisinic acid was incorporated into arteannuin B as well as artemisinin, both *in vivo* and in a cell free system. This is the first report of *in vivo* and *in vitro* transformation of artemisinic acid by the plant system.[59] In the cell-free system homogenized from leaves of *A.annua*, Bharel *et al.* further confirmed that artemisinic acid and arteannuin B can be enzymatically converted into artemisinin.[267] Moreover, there were several reports that deoxyarteannuin B was regarded as an alternative intermediate in the biosynthesis of artemisinin from artemisinic acid.[61,70b]

There are different conclusions drawn from similar experiments in early literature. Some experimental studies considered arteannuin B be a precursor to artemisinin,[182,248,270–272] which is contradicted to results of Wang *et al.*[70c] In addition, contrary to previous suggestions, Brown *et al.* observed the absence of artemisinin when labeled artemisinic acid was fed to cut *A.annua* plants.[61] Although all of the confusing suggestions, most early investigations (pre-1993) supported that artemisinic acid is a biogenetic precursor of artemisinin.

8.2 Later work: artemisinic acid is a "byproduct " in artemisinin biosynthesis

The notion of artemisinic acid conversion to artemisinin had been varied since 1999. In a paper published in 1999, no acquisition of detectable oxidation products when artemisinic acid was added to chlorophyll a-catalyzed photooxidation system, which mimic photooxidation conditions that may also be present in the living plant.[91] In another investigation performed *in vivo* with a stable isotope label, Brown *et al.* detected the absence of any incorporation of label into artemisinin.[61] Both the results were at variance with the earlier literature,[59,69,70c,73,78] which indicated artemisinic acid was not converted directly into artemisinin. This conclusion was in agreement with the more recent literature, in which there was a gathering consensus that dihydroartemisinic acid,

rather than artemisinic acid, was the true late-stage precursor to artemisinin.[70c,91] Wang *et al.* firstly described a conversion of dihydroartemisinic acid to artemisin by the homogenate of *A.annua*.[70c] The chemical conversion of dihydroartemisinic acid to artemisinin was verified under conditions that may also be present in the living plant. The results suggest that the conversion of dihydroartemisinic acid into artemisinin in the living plant might be a non-enzymatic conversion.[91] These authors postulated a new conversion pathway of dihydroartemisinic acid to artemisinin, with dihydroartemisinic acid hydroperoxide as an intermediate.[91,273] The subsequent isolation and characterization of dihydroartemisinic acid hydroperoxide provided a strong evidence for an *in vivo* non-enzymatic conversion of dihydroartemisinic acid to artemisinin.[268] In addition, dihydroartemisinic acid can undergo slow spontaneous auto-oxidation to artemisinin in the absence of photosensitizer,which was required for the generation of singlet oxygen ($^1O_2$).[269] They also proposed that tertiary allylic hydroperoxide of dihydroartemisinic acid present in the pathway of spontaneous dihydroartemisinic acid transformation of artemisinin. Bioconversion of dihydroartemisinic acid to artemisinin under both conditions of photosensitizer absence and presence revealed that dihydroartemisinic acid may involved in the biogenesis of artemisinin.[269] Taking into consideration the presence of a series of oxygenated amorpha-4,11-diene compounds that are putative intermediates in artemisinin biosynthesis, and a number of related enzymes, Berta *et al.* hypothesized a biosynthetic pathway of artemisinin, namely, FPP → amorpha-4,11-diene → artemisinic alcohol → artemisinic aldehyde → dihydroartemisinic aldehyde → dihydroartemisinic acid → artemisinin. This is the first time to point out that dihydroartemisinic acid, but not artemisinic acid is the immediate precursor of artemisinin.[213] From then on, the genes encoding for enzymes responsible for artemisinin biosynthesis pathway postulated by Berta *et al.* were cloned and functionally characterized. The first cloning was CYP71AV1 gene, which encoded for a cytochrome P450 catalyzing amorpha-4,11-diene to form artemisinic aldehyde *via* artemisinic alcohol.[41,210a] Soon after, the gene encoding for a reductase was also cloned from *A.annua*. The resultant protein, Dbrr2, was responsible for the reduction of artemisinic aldehyde to dihydroartemisinic aldehyde.[50] Most recently, the expression of a specific aldehyde dehydrogenase (ALDH1) cDNA in *E.coli* and the characterization of purified recombinant enzyme demonstrated that ALDH1 was involved in the oxidation of artemisinic acid to form dihydroartemisinic acid.[216] The genes isolation and functional identification of these proteins provided strong evidence supporting the pathway which Bertea *et al.* postuated.[213]

As mentioned above, the early findings contradicted the conclusion of the late papers.

Ferreira *et al.* reported postharvest drying can affect the concentration of artemisinin, dihydroartemisinic acid and artemisinic acid in dryed *A.annua*. The results showed postharvest drying can increase artemisinin production with simultaneous and significant decrease of dihydroartemisinic acid and artemisinic acid. Considering the reduction of dihydroartemisinic acid is in a proportion with increase of artemisinin, the authors reiterated the hypothesis that dihydroartemisinic acid is the main biosynthetic precursor of artemisinin.[101]

8.3 Our opinion: artemisinic acid is related to artemisinin biosynthesis with chemo-type dependent fashion

Although contradicted results presented in some data, the notion that both artemisinic acid and dihydroartemisinic acid were the precursor of artemisinin can be drawn from previous literature. *In vitro* biochemical studies strongly supported the suggestion of both artemisinic acid and dihydroartemisinic acid yielding artemisinin.[59,69,70c,91] Based on the available literature data, two pathways to artemisinin from both artemisinic acid and dihydroartemisinic acid, respectively, were proposed. In the branch of artemisinic acid yielding artemisinin, the following biosynthetic sequence was suggested: artemisinic acid → arteannuin B → artemisinin. Both the bioconversion of artemisinic acid to arteannuin B and arteannuin B to artemisinin were strongly supported by *in vitro* experiments and *in vivo* feeding trials. Although the exact genes regulating the conversion were not isolated from *A.annua* yet, the enzymes controlling arteannuin B to artemisinin were purified from *A.annua*[274,275] and microorganisms.[276,277] The successful isolation and characterization of the enzyme catalyzing the conversion of arteannuin B to artemisinin in *A.annua* indicated the indeed existence of conversion of arteannuin B to artemisinin *in vivo*. In another branch to artemisinin *via* dihydroartemisinic acid, the detailed pathway was listed below: dihydroartemisinic acid → dihydroartemisinic acid hydroxide → artemisinin. Most biochemical evidences strongly supported the notion of dihydroartemisinic acid as a precursor. In conclusion, although the status of artemisinic acid in artemisinin biosynthetic pathway has not been characterized very well, the semi-synthetic artemisinin production from artemisinic acid has made great progress, which is closer to the industry requirement.

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