Identification of Intermediates and Enzymes Involved in the Early Steps of Artemisinin Biosynthesis in *Artemisia annua*

**Abstract**

An important group of antimalarial drugs consists of the endoperoxide sesquiterpene lactone artemisinin and its derivatives. Only little is known about the biosynthesis of artemisinin in *Artemisia annua* L., particularly about the early enzymatic steps between amorph-4,11-diene and dihydroartemisinic acid. Analyses of the terpenoids from *A. annua* leaves and gland secretory cells revealed the presence of the oxygenated amorph-4,11-diene derivatives artemisinic alcohol, dihydroartemisinic alcohol, artesunic acid, dihydroartesunic acid and dihydroartemisinic acid. We also demonstrated the presence of a number of biosynthetic enzymes such as the amorph-4,11-diene synthase and the – so far unknown – amorph-4,11-diene hydroxylase as well as artemisinic alcohol and dihydroartemisinic aldehyde dehydrogenase activities in both leaves and glandular trichomes. From these results, we hypothesise that the early steps in artemisinin biosynthesis involve amorph-4,11-diene hydroxylation to artemisinic alcohol, followed by oxidation to artesunic aldehyde, reduction of the C11-C13 double bond to dihydroartesunic aldehyde and oxidation to dihydroartemisinic acid.

**Key words**
*Artemisia annua* · Asteraceae · artemisinin · glandular trichomes · biosynthetic pathway · sesquiterpenoids

**Introduction**

*Artemisia annua* L. (sweet wormwood), a member of the Asteraceae family has been used for many years in the treatment of malaria. The active compound responsible for its pharmacological action is the sesquiterpene lactone endoperoxide artesimin (Fig. 1). Based on this secondary plant metabolite, several synthetic derivatives such as artemether, arteether, artesunic acid and artelenic acid have been produced, which are effective against multidrug-resistant *Plasmodium falciparum* strains, the organism responsible for malaria [1, 2]. Because chemical synthesis of artemisinin is an expensive multi-step process, the plant remains the only commercial source of the drug. However, this compound is present in the leaves and the flowers in only small amounts ranging from 0.01% to 0.8% of dry weight [3].

In spite of the commercial value of artemisinin, its biosynthetic pathway is not yet completely elucidated. Knowledge of the exact biosynthesis of artemisinin may enable us to influence its formation in a direct way, for example, by metabolic engineering. Several authors have demonstrated that artesunic acid and/or dihydroartesunic acid are intermediates in the formation of artemisinin [1, 3, 4]. In addition, it is clear that the first dedicat-

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ed step in the biosynthesis of artemisinin is the cyclisation of farnesyl diposphate (FDP) to amorphoa-4,11-diene, the first specific precursor of artemisinin (Fig 1) [5]. The enzyme responsible for this reaction has been characterised [5] and the corresponding cDNA has been cloned [6]. Recently, Keasling and co-workers were able to produce amorphoa-4,11-diene in an E. coli strain engineered with the yeast mevalonate pathway and a synthetic amorphoa-4,11-diene synthase cDNA [7].

However, nothing is known about the enzymes involved in the conversion of amorphoa-4,11-diene to (dihydro)artemisinic acid, nor have any of the putative intermediate products so far been described. Modifications of the amorphoa-4,11-diene skeleton to produce artemisinic acid most likely involve a cytochrome P450 enzyme leading to the production of artemisinic alcohol (Fig 1) [5]. Subsequently, the alcohol could be oxidised twice by either cytochrome P450 enzymes or dehydrogenases yielding artemisic acid (Fig 1). Recently, a similar sequence of enzymatic reactions has been reported for the formation of germacrene acid in sesquiterpene lactone biosynthesis in chicory [8]. The C11-C13 double bond of the artemisinic acid could be reduced yielding dihydroartemisinic acid which was shown to be convertible non-enzymatically to artemisinin [1, 3, 4, 9]. Alternatively, the C11-C13 double bond reduction may occur in artemisinic alcohol or artemisinic aldehyde yielding dihydroartemisinic alcohol or dihydroartemisinic aldehyde as intermediates, respectively. To study this unknown part of the pathway, we have analysed the presence of putative intermediates and enzymes involved in the conversion of these intermediates in leaves and glandular trichome extracts of A. annua.

Materials and Methods

GC-MS, radio-GC and NMR analyses
GC-MS analysis was done on an HP 5890 series II gas chromatograph and HP 5972A mass selective detector (70 eV), equipped with a capillary HP-5MS or HP-INNOWax column (both 30 m x 0.25 mm, film thickness of 0.25 µm). The inlet temperature was 250 °C and the detection temperature 290 °C for all samples. Using a constant helium flow of 1 mL min⁻¹ the oven programme was as follows. HP-5MS: initial temperature 55 °C for 4 min followed by a ramp of 5 °C min⁻¹ to 280 °C, and final time of 10 min. HP-INNOWax: initial temperature 80 °C for 2 min followed by a ramp of 5 °C min⁻¹ to 260 °C, and final time of 10 min. Radio-GC analysis was performed using a Carlo Erba 4160 series GC equipped with a RAGA-93 radioactivity detector (Raytest) and FID as described before [5] but with the following temperature programme: 1 min at 70 °C followed by a ramp of 5 °C min⁻¹ to 240 °C and a final time of 10 min.

NMR spectra were recorded at 200 MHz (¹H) or 50 MHz (¹C) with CDCl₃ as solvent and TMS as internal standard.

Plant material
Seeds of Artemisia annua L. (Asteraceae) of Vietnamese origin were obtained from Dr. Charles Lutg of Artece BV (Maarsen, The Netherlands). Taxonomically verified specimens are deposited at the Department of Pharmaceutical Biology, Groningen University, the Netherlands and at the Institute of Materia Medicca, Hanoi, Vietnam. Plants were grown in a greenhouse in 5 litre plastic pots containing potting compost at 21/18 °C (16/8 h). Natural daylight was supplemented with artificial light (SON-T AGRO) during the high-temperature period, and fertiliser was applied as required. When the plants were about 1.5 m in height, leaves or young shoot tips were collected and used fresh or were frozen in liquid N₂ and stored at −80 °C for later use.

Synthesis of reference compounds
Artemisinic alcohol: Artemisinic acid (100 mg, isolated from A. annua leaves) was dissolved in 6 mL of distilled ether and esterified with CH₃N₂ during 1 hour. Part of the ester obtained was used in the next reaction without purification. After removal of the ether 8.6 mg of the ester was dissolved in 20 mL of a mixture of ether/THF (1:1), cooled to −30 °C under a flow of dry nitrogen gas and stirred for 1 hour at −30 °C with an excess of LiAlH₄. The reaction was stopped by the addition of Na₂SO₄·10 H₂O. The mixture was allowed to warm to room temperature, stirred for another 30 min and dried with MgSO₄. After filtration and removal of the solvent, the desired alcohol was isolated and purified by preparative TLC performed on silica gel plates using a mixture of petroleum ether/ethyl acetate (3:1) as the eluent, with an Rf value of 0.7. Artemisinic alcohol was obtained in an overall yield of 14% as an inseparable mixture with dihydroartemisinic alcohol (9:1). The structure of artemisinic alcohol was confirmed by 1H-NMR and GC-MS. 1H-NMR: δ = 5.18 (br s, 1H, C = CH), 5.04 (br s, 1H, H₃C = C), 4.83 (br s, 1H, H₂C = C), 4.10 (s, 2H, CH₂OH), 2.47 (m, 1H, CH₃), 2.12 (s, 3H, CH₃), 2.00 (m, 1H, CH₂OH), 1.82 – 2.00 (m, 1H), 1.50 (br s, 3H, CH₃). Mass spectrum: m/z = 220 (M⁺, 18% rel. int.), 202 (54), 189 (80), 187 (60), 184 (46), 145 (40), 132 (47), 121 (100), 119 (71), 93 (60), 79 (51). Retention index on HP5-MS column: 1761.

Dihydroartemisinic alcohol: A small amount (170 mg) of an impure sample (40% pure) of dihydroartemisinic acid isolated from A. annua [1] was dissolved in hexane-ether (4:1), and the compounds were separated by column chromatography on a silica gel column (20 x 1.8 cm). The fractions with a dihydroartemisinic acid purity higher than 57% (GC-MS) were evaporated and used for the next steps. Dihydroartemisinic acid (25 mg) was dissolved in 25 mL of distilled ether and esterified with CH₃N₂ (1 h), reduced with LiAlH₄ (two spautula tips, 1 h) and worked up as described above. The alcohol was purified by column chromatography on silica gel using a mixture of hexane-ether (3:1) as the eluent. Dihydroartemisinic alcohol was obtained in an overall yield of 43% and the structure was confirmed by 1H-NMR and GC-MS. 1H-NMR: δ = 5.19 (br s, 1H, C = CH), 3.72 (dd, 1H, J = 10.6 and 3.2 Hz, CH₂OH), 3.62 (t, 1H, J = 6.6 Hz, OH), 3.50 (dd, 1H, J = 6.2 and 10.6 Hz, CH₂OH), 2.45 (br s, CH₃CH₂OH), 0.90 – 2.0 (m, 12H), 1.64 (m, 3H, CH₃C = C), 0.98 (d, 3H, J = 6.8 Hz, CH₃CH₂OH), 0.84 (d, 3H, J = 6.6 Hz, CH₃CH₂). Mass spectrum: m/z = 222 (M⁺, 12% rel. int.), 204 (1), 191 (13), 164 (28), 163 (100), 162 (17), 135 (9), 121 (16), 107 (20), 93 (17), 81 (21), 55 (12). Retention index on HP5-MS column: 1755.

Dihydroartemisinic aldehyde: This compound was obtained from dihydroartemisinic alcohol by Swern oxidation [10]. To a solution of oxalyl chloride (2 equivs.) in dichloromethane (0.2 M) at −78 °C, DMSO (4 equivs., dried on KOH) was added. The mixture was stirred for 5 min and the alcohol was added as a solution in
CH₂Cl₂ (0.2 M). After 20 min stirring, the reaction mixture was treated with Et₃N (6 equivs.) and allowed to warm to -40°C for 30 min. Cold demineralised water was added and the layers were separated. The aqueous layer was extracted twice with CH₂Cl₂, the combined organic layers were dried with MgSO₄, and filtered. The reaction afforded a diastereomeric mixture of dihydroartemisinic aldehyde. The solvent was removed and the aldehydes were purified using column chromatography on silica gel using a hexane-ether (3:1) as the eluent, and used as a mixture of epimers during the subsequent experiments. This product was obtained in a yield of 67% referred to the alcohol and in an overall yield of 54%. ¹H-NMR: δ = 9.55 (d, 1H, J = 3.8 Hz, CHO), 5.11 (br s, 1H, C = CH), 1.0 - 2.5 (m, 13H), 1.63 (s, 3H, CH₃C = C), 1.05 (d, 3H, J = 7.0 Hz, CH₃CHO), (d, 3H, J = 6.2 Hz, CH₃CH₂CHO). Mass spectrum: m/z = 220 (M⁺, 0.1% rel. int.), 205 (0.5), 187 (1), 162 (100), 147 (34), 121 (11), 105 (15), 91 (20), 79 (19), 55 (15), 41 (16). Retention index on HP5-MS column: 1673.

**Isolation of artemisinic aldehyde**

Artemisinic aldehyde was isolated from 446 g of flowers and 714 g of leaves of *A. annua*. The volatile compounds were extracted from the plant material through hydrodistillation. The oil obtained was analysed by GC-MS. The aldehydes and ketones were extracted from the oil with 2 g of Girard's reagent P as described by Maurer and Grieder and were separated by column chromatography [11]. This extraction yielded a small amount of artemisinic aldehyde (76% pure) derived from flowers, whereas no arte-

In order to obtain more artemisinic aldehyde, the remaining fractions from the oil treated with Girard's reagent P (which contain artemisinic alcohol according to GC-MS), and the fractions originating from the leaves and the flowers were combined and purified on a silica column using 18% EtOAc in petroleum-ether (40 - 65°C) as the eluent. The fractions were analysed by TLC [eluent: petroleum ether (40 - 65°C)−EtOAc, 5:1], and only the fractions containing artemisinic alcohol were combined. The eluent was removed, the remaining oil was dissolved in 8 mL of pentane and 2 mL EtO. This mixture was vigorously shaken for 2 days at room temperature with 0.8 g of MnO₂ (Merck, precipitated active, for synthesis). The mixture was filtered, evaporated and purified by column chromatography on silica using a mixture of 3% EtOAc in petroleum ether (40 - 65°C) as the eluent. The fractions containing artemisinic aldehyde in a percentage higher than 82% were combined with the artemisinic aldehyde obtained from flowers (76% pure). The resulting mixture was further purified by column chromatography on 5% (w/w) AgNO₃/silica using a mixture of 3% EtOAc in hexane as the eluent. Artemisinic aldehyde was obtained in a yield of approximately 2 mg and the structure was confirmed by ¹H-NMR, ¹³C-NMR, and MS. ¹H-NMR: δ = 9.51 (s, 1H, CHO), 6.17 (s, 1H, CH₂=CH), 6.12 (s, 1H, CH₂=CH), 4.87 (s, 1H, C = CH₂), 2.7 (m, 1H), 2.51 (m, 1H), 0.85 - 2.0 (m, 10H), 1.57 (s, 3H, CH₃C = Cⁱ⁻ⁿ), 0.88 (d, 3H, CH₃CH=CH₂); ¹³C-NMR: δ = 19.77 (q, C₁₅), 23.4 (q, C₁₅), 25.43 (m, 1H), 25.44, 25.45, 32.6, 35.11 (t, C₃, C₄, C₅, C₆, C₇), 27.59, 37.38, 39.51, 41.23 (d, C₈, C₉, C₁₀, C₁₁), 120.08 (d, C₁₂), 134.75 (t, C₁₃), 135.14 (q, C₁₄), 152.47 (q, C₁₅), 194.76 (d, C揭开). Mass spectrum: m/z = 218 (M⁺, 57% rel. int.), 203 (42), 162 (46), 147 (64), 119 (88), 105 (66), 91 (100), 79 (82), 77 (69), 55 (41), 41 (63). Retention index on HP5-MS column: 1675.

**Leaf terpenoids and enzyme assays**

*A. annua* terpenoids were extracted from young shoots of greenhouse-grown plants according to [12] as described before [5]. Enzyme assays were carried out in duplicate and repeated at least once. For this two, grams of fresh young leaves of *A. annua* were ground in liquid nitrogen in the presence of 1 g insoluble polysilicopolypropyridolone (PVP) and some sand, using a precooled mortar and pestle. The fine powder was transferred to a beaker on ice containing 2 g Amberlite XAD-4 polystyrene resin beads. Twelve mL of extraction buffer containing 100 mM Tris (pH 7.5), 50 mM sodium metabisulphite, 50 mM ascorbic acid, 2.5 mM EDTA, 2.5 mM EGTA, 5 mM DTT, 5 mM FAD, 5 mM FMN, 0.5 mM glutathione (reduced form), 1 µg/mL catalase, 5 µg/mL leupeptin, 0.2% (w/v) BSA, and 20% (v/v) glycerol were added to the mixture. The slurry was gently stirred and then filtered through cheesecloth pre-moistened with extraction buffer. The filtrate was centrifuged for 20 min at 20,000 g at 4°C and the resulting supernatant was centrifuged for 90 min at 150,000 g at

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4 °C. The microsomal pellet was used for hydroxylase assays and the supernatant for reductase and dehydrogenase assays. For hydroxylase assays, the microsomal pellet was homogenised in hydroxylase buffer containing 25 mM Tris (pH 7.5), 1 mM ascorbic acid, 2.5 mM EDTA, 5 mM DTT, 5 μM FAD, 5 μM FMN, 1 μg/ml leupeptin, and 10% (v/v) glycerol, using a glass rod and teflon potter, in such a way that 1 ml of suspension contained the microsomes originating from 0.3 g of plant material. One ml of this suspension was incubated in a 9 ml teflon-lined screw cap vial with 60 μM amorpha-4,11-diene for 90 min at 30 °C in the presence or absence of an NADPH-regenerating system. The NADPH-regenerating system consisted of 1 mM NADPH, 5 mM glucose 6-phosphate, and 1 IU glucose 6-phosphate dehydrogenase (all from Sigma) [8]. After incubation, 10 nmol cis-nerolidol were added as an internal standard to each assay to allow quantification of the GC-MS results. Each assay was then extracted twice with 1 ml ether/pentane (1:4) and the organic phase was filtered through a small glass column containing 0.90 g of silica and some anhydrous MgSO₄. The column was washed with 1.5 ml of ether and the extract was carefully concentrated to approximately 50 μl under a stream of nitrogen and analysed by GC-MS.

For reductase and dehydrogenase assays, the 150,000 g supernatant was desalted with an Econo-Pac 10DG column (Bio-Rad, Hercules, CA) to an assay buffer containing 25 mM Tris (pH 7.5) or 25 mM glycine (pH 9), respectively, and 2 mM DTT and 10% (v/v) glycerol. One ml of this enzyme preparation was incubated in a 9 ml teflon-lined screw cap vial at 30 °C with 100 μM of substrate, with or without 1 mM NADH/NADPH or NAD⁺/NAD⁺, respectively. After 60 min, 2.5 nmol cis-nerolidol were added as an internal standard and the sample was acidified with 20 μL of 5 M HCl [8]. Subsequently the assay was extracted and analysed by GC-MS as described above.

**Glandular trichome terpenoids and enzyme assays**

Glandular trichomes were isolated according to Lange et al. [13] with minor modifications. The essential oil content of the glandular trichomes was analysed for the presence of the putative intermediates in the artemisinin biosynthetic pathway. One hundred mg of glands were ground in 1 ml of dichloromethane containing 1 mg/ml cis-nerolidol as an internal standard. The extract was loaded on a small glass column containing MgSO₄. The column was washed twice with 1 ml ether/pentane (1:4), concentrated and analysed by GC-MS as described above. Several enzyme assays were performed with intact glandular trichomes from A. annua. About 15 mg of glands were re-suspended in 1 ml two-steps assay buffer containing 25 mM Tris (pH 7.5), 10% (v/v) glycerol, 10 mM MgCl₂, 6 mM Na₂VO₃, 2 mM DTT, 5 mM FAD/FMN and 1 mM NADPH, supplemented with an NADPH-regenerating system as described above for the hydroxylation assay. As substrate 20 μM unlabelled FDP was added to the assay, along with 2.2 × 10⁶ dpm [³H]-FDP. The samples were incubated for 2 hours at 30 °C in a water bath with gentle shaking. Preparation of the samples for radio-GC analysis was identical to the preparation for GC-MS analysis. To extract enzymes, 100 mg of trichomes were used to prepare a 150,000 g supernatant as described above for leaves but PVPP and XAD-4 were added in an...
amount of 100 mg and the extraction buffer contained 100 mM NaH₂PO₄/Na₂HPO₄ (pH 7.4), 250 mM sucrose, 1 mM EDTA, 1 mM DTT, and 5 μg/mL leupeptin. The supernatant was desalted and enzymes assayed as described for leaves.

Results

Based on the biosynthetic pathway postulated by us in Fig. 1, we have extracted and analysed the essential oil of *A. annua* leaves and glandular trichomes to look for the putative intermediates and search for the enzymes involved in the conversion of these intermediates, in order to elucidate the early steps of artemisinin biosynthesis. First, a number of reference compounds such as artemisinic alcohol, dihydroartemisinic alcohol, artemisinic aldehyde and dihydroartemisinic aldehyde were synthesised – using artemisinic acid and dihydroartemisinic acid as starting materials – or isolated (artemisinic aldehyde). The structures of all isolated or synthesised compounds were confirmed using NMR and MS. GC-MS analyses of the synthesised dihydroartemisinic aldehyde resulted in two peaks (on both HP-5MS and HP-INNOWax), which showed similar retention times and essentially the same mass spectra; it is assumed that they are stereoisomers. Presumably, these compounds are C11 epimers that are formed during synthesis or chromatography due to enolisation.

The GC-MS chromatogram of glandular trichome secretory cell extracts was similar to that obtained from leaves (Fig. 2). In both cases, artemisinic alcohol, artemisinic aldehyde, dihydroartemisinic alcohol, and dihydroartemisinic aldehyde were detected. Artemisinic acid, dihydroartemisinic acid and the sesquiterpene olefins that were detected have been reported before as constituents of *A. annua* essential oil [1], [5].

Conversion of farnesyl diprophosphate into amorpha-4,11-diene

Fig. 3 shows the results obtained by feeding intact glands of *A. annua* with [³H]-FDP in the presence of an NADPH regenerating system. The assays showed a large radioactive amorpha-4,11-diene peak, indicating that the conversion from FDP to amorpha-4,11-diene occurred. A number of other peaks were observed, corresponding to other sesquiterpenoids, including germacrene A (peak 2). Peak 10 was identified as artemisinic alcohol on the basis of co-elution with a non-labelled standard (Fig. 3B). Other intermediates in the pathway such as dihydroartemisinic aldehyde (peak 5) and dihydroartemisinic alcohol (peak 9) were tentatively identified. Peaks that could not be identified were probably other sesquiterpene alcohols and/or aldehydes since they originated from the radioactive labelled farnesyl diprophosphate.

Conversion of amorpha-4,11-diene into artemisinic alcohol

In enzyme assays with microsomal pellets of *A. annua* leaf extracts with amorpha-4,11-diene as substrate and in the presence of NADPH we found a small, but consistent increase in artemisinic alcohol peak area on top of the endogenous artemisinic alcohol that was inevitably introduced into the enzyme assay together with the microsomal pellet (Fig. 4). There was no change in dihydroartemisinic alcohol peak area. This amorpha-4,11-diene hydroxylase activity was not found in the soluble protein fraction (150,000 g supernatant). The same assay was conducted on microsomal pellets of an *A. annua* glandular trichome extract, but no hydroxylase activity could be detected.

Conversion of artemisinic alcohol into dihydroartemisinic acid via artemisinic aldehyde and dihydroartemisinic aldehyde

Fig. 5 shows the results of the incubation of a mixture of artemisinic alcohol and dihydroartemisinic alcohol with the 150 g young leaf supernatant in the presence of NAD⁺/NADPH at pH 9.0. In the presence of cofactors (Fig. 5B), the ratio between arte-
Fig. 5  Identification by GC-MS of the products formed in: (A, B) (HP-5MS column) a 150,000 g supernatant from A. annua leaves incubated with artemisinic alcohol/dihydroartemisinic alcohol at pH 9.0 in the absence of cofactors (A) or in the presence of NAD+ and NADP+ (B). (C, D) (HP-INNOWax column) a 150,000 g supernatant from A. annua glandular trichomes incubated with artemisinic alcohol/dihydroartemisinic alcohol at pH 9.0 in the absence of cofactors (C) or in the presence of NAD+ and NADP+ (D). (E, F) (HP-5MS column) a 150,000 g supernatant from A. annua glandular trichomes incubated with dihydroartemisinic aldehyde at pH 9.0 in the absence of cofactors (E) or in the presence of NAD+ and NADP+ (F). (G, H) (HP-5MS column) a 150,000 g supernatant from A. annua glandular trichomes incubated with artemisinic acid in the absence of cofactors (G) or in the presence of NADH and NADPH (H). AAOH: artemisinic alcohol; DHAAOH: dihydroartemisinic alcohol; AAA: artemisinic aldehyde; DHAAA: dihydroartemisinic aldehyde; DHAA: dihydroartemisinic acid; AA: artemisinic acid.

Artemisinic alcohol and dihydroartemisinic acid strongly decreased showing that artemisinic alcohol was converted to artemisinic aldehyde, dihydroartemisinic aldehyde and dihydroartemisinic acid. None of these intermediates were formed in the absence of cofactors (Fig. 5A). Artemisinic acid was not detected in any of these experiments. The same experiment was done using a 150,000 g glandular trichome secretory cell supernatant (Figs. 5C,D). Although the enzyme activity was lower (the ratio artemisinic alcohol/dihydroartemisinic alcohol decreased less than in Figs. 5A,B), also here artemisinic aldehyde and dihydroartemisinic aldehyde were formed, but dihydroartemisinic acid was not detected (Fig. 5D). No products were formed in the absence of cofactors (Fig. 5C). Again, only artemisinic alcohol was converted into the aldehydes; there was no change in the dihydroartemisinic alcohol amount. To test whether the conversion of dihydroartemisinic aldehyde to dihydroartemisinic acid that was observed in leaf extracts also occurred in trichomes, we incubated the 150,000 g supernatant of the glandular trichomes with dihydroartemisinic aldehyde in the presence of NAD+/ NADP+ (Figs. 5E,F). Under these conditions we detected conversion of dihydroartemisinic aldehyde into dihydroartemisinic acid, as can be concluded from the decrease in dihydroartemisin-
nic aldehyde peak area and the appearance of dihydroartemisinic acid in the reaction mixture (Fig. 5F). No conversion was detected in the absence of cofactors (Fig. 5E).

In a series of other experiments, the 150,000 g supernatant from leaf and secretory cell extracts was also incubated with the other intermediates of the putative pathway. The enzymatic oxidation, with NAD'/NADP+ as cofactor, of dihydroartemisinic alcohol to dihydroartemisinic aldehyde did not occur, but the reverse reaction, obtained by incubating the assay with dihydroartemisinic aldehyde in the presence of NADH and NADPH, led to the production of a small peak of dihydroartemisinic acid (data not shown). The enzymatic reduction of artemisinic acid to dihydroartemisinic acid did not occur. Surprisingly, in this assay we did detect a peak of dihydroartemisinic aldehyde. This double reduction occurred only in the presence of NADH/NADPH (Fig. 5H).

No dihydroartemisinic aldehyde was detected in the absence of these cofactors (Fig. 5G). In enzyme assays with artemisinic aldehyde as substrate and either NAD/NADP or NADH/NADPH as cofactors (dehydrogenase and reductase assays, respectively) we could not detect any product.

Discussion

So far, only small parts of the artemisinin pathway have been elucidated [5], [9]. In this paper we describe for the first time in A. annua the identification of a series of oxygenated amorphoa-4,11-diene compounds that are putative intermediates in artemisinin biosynthesis, and a number of enzymes involved in their biosynthesis.

The glandular trichomes and leaf extracts were shown to contain a number of mono- and sesquiterpene olefins and oxidised terpenoids including artemisinic alcohol, dihydroartemisinic alcohol, artemisinic aldehyde, dihydroartemisinic alcohol and dihydroartemisinic acid. In a series of enzyme assays on trichome and leaf extracts we could subsequently demonstrate the enzymatic conversion of a number of these putative intermediates. [3H]-FDP was converted to amorpha-4,11-diene and to germacrene A in intact A. annua glandular trichomes (Fig. 1, step 1; Fig. 3) and amorpha-4,11-diene was converted by a putative cytochrome P450 dependent hydroxylase to artemisinic alcohol (Fig. 1, step 2; Figs. 3 and 4). The presence of enzymatic activity in the glandular trichomes is analogous to results with glandular structures from mint [13] and basil [14] that have also been shown to contain all the enzymatic machinery for essential oil production. The next steps in the biosynthetic pathway are the oxidation of artemisinic alcohol at C12 by a dehydrogenase yielding artemisinic aldehyde (Fig. 1, step 3; Figs. 5A – D) and the subsequent reduction of the C11-C13 carbon-carbon double bond in artemisinic alcohol yielding dihydroartemisinic aldehyde (Fig. 1, step 4). In the experiments performed with leaf extracts we also detected dihydroartemisinic acid as a product (Figs. 5A, B). This suggests that dihydroartemisinic aldehyde is further oxidised to dihydroartemisinic acid in a second dehydrogenase reaction (Fig. 1, step 5) and this was confirmed using a glandular trichome 150,000 g supernatant (Figs. 5E, F).

Recent findings demonstrate that dihydroartemisinic acid is indeed the precursor of some highly oxygenated compounds, including artemisinin, in A. annua plants [4]. The in vivo accumulation of oxygenated sesquiterpenoids is probably related to the autoxidation of dihydroartemisinic acid in glandular trichomes, where the lipophilic environment can contribute to the stability of the hydroperoxide intermediates [4]. The authors also showed that there is no significant desaturation of dihydroartemisinic acid at C11-C13 to yield artemisinic acid and claim that the reverse reaction, the reduction of artemisinic acid at the C11-C13 position to yield dihydroartemisinic acid has never been demonstrated [4]. Our biochemical experiments also show that there is no direct enzymatic conversion of artemisinic acid into dihydroartemisinic acid (Figs. 5G,H).

Taking into consideration the presence of artemisinic alcohol in both leaf and glandular trichome extracts and the nature of the conversion of amorpha-4,11-diene into artemisinic alcohol, it would be logical to assume that in this reaction a cytochrome P450 dependent hydroxylase is involved [5]. The conversion of amorpha-4,11-diene into artemisinic alcohol was not efficient and its detection hindered by endogenous artemisinic alcohol present in the enzyme preparation but a consistent difference between assays with or without NADPH was found (Fig. 4). As a result of the limited amount of material, the assay on microsomal pellets from secretory cells did not show conversion of the amorpha-4,11-diene into artemisinic alcohol. It is known that the P450 enzymes are present at low levels in plant cells and that in vitro activities of cytochrome P450 enzymes are often underestimated as a result of inefficient extraction and poor stability [15]. However, we have demonstrated that amorpha-4,11-diene can also be efficiently converted into artemisinic alcohol by a cytochrome P450 enzyme preparation from chyrcy roots (Cichorium intybus L.) capable of the hydroxylation of various sesquiterpene olefins in the presence of NADPH [16]. The proposed pathway also further resembles in many aspects the biosynthetic pathway of sesquiterpene lactones in chyrcy, since also there the olefinic precursor – germacrene A – is first hydroxylated to a germacrene alcohol and further oxidised by dehydrogenases via the corresponding aldehyde to germacra-1(10),4,11(13)-trien-12-oic acid [8], [17]. Our enzyme assays with artemisinic aldehyde as substrate were unsuccessful, likely as a result of the high reactivity of isoprenoidal side chains to nucleophilic attack as has been reported and discussed by de Kraker et al. also for other aldehydes [8].

In order to evaluate all other possible conversions, we have also incubated the enzyme preparations with the other putative intermediates and appropriate cofactors. Our results showed that there are biosynthetic links between most of the compounds depicted in Fig. 1, also in reverse directions. However, the results of our enzyme assays and the chemical reactivity of the intermediates suggest that the pathway proceeds according to the bold arrows in Fig. 1. Indeed, the oxidation of allylic alcohols (like artemisinic alcohol) is much easier than the oxidation of aliphatic alcohols (like dihydroartemisinic alcohol) and the reduction of the carbon-carbon double bond is chemically most feasible in an α,β-unsaturated aldehyde (like artemisinic aldehyde) because of Michael-type chemistry [18]. A reduction is also possible in the corresponding α,β-unsaturated carboxylic acid (like artemisinic
acid), but this is not likely to occur in water because the carboxylic acid group is charged at physiological pH, thereby reducing the reactivity of the carbon-carbon double bond. Michael-type additions are not possible in aliphatic alcohols, such as artemisinic alcohol, so this compound is the least likely to be reduced. For these reasons, we conclude that the route — as shown in bold arrows in Fig. 1 — amorph-4,11-diene → artemisinic alcohol → artemisinic aldehyde → dihydroartemisinic aldehyde → dihydroartemisinic acid → artemisinin is the most likely one to occur in vivo in A. annua.

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