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Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis

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Abstract

The endoperoxide sesquiterpene lactone artemisinin and its derivatives are a promising new group of drugs against malaria. Artemisinin is a constituent of the annual herb *Artemisia annua* L. So far only the later steps in artemisinin biosynthesis — from artemisinic acid — have been elucidated and the expected olefinic sesquiterpene intermediate has never been demonstrated. In pentane extracts of *A. annua* leaves we detected a sesquiterpene with the mass spectrum of amorpha-4,11-diene. Synthesis of amorpha-4,11-diene from artemisinic acid confirmed the identity. In addition we identified several sesquiterpene synthases of which one of the major activities catalysed the formation of amorpha-4,11-diene from farnesyl diphosphate. This enzyme was partially purified and shows the typical characteristics of sesquiterpene synthases, such as a broad pH optimum around 6.5–7.0, a molecular mass of 56 kDa, and a K_m of 0.6 μM. The structure and configuration of amorpha-4,11-diene, its low content in *A. annua* and the high activity of amorpha-4,11-diene synthase all support that amorpha-4,11-diene is the likely olefinic sesquiterpene intermediate in the biosynthesis of artemisinin.

Keywords: *Artemisia annua*; Asteraceae; Biosynthesis; Sesquiterpenoids; Artemisinin; Amorpha-4,11-diene

1. Introduction

Malaria is an infectious disease caused by protozoa of the genus *Plasmodium*, which are carried by mosquitoes of the genus *Anopheles*. The most severe form of malaria, *Malaria tropica*, is responsible for 300–500 million clinical cases each year of which about 90% occur in Africa. It is estimated that malaria causes between 1.5 and 3 million deaths per year, mainly African children (Butler, 1997). In the past, the fight against malaria was based on two strategies: (i) The extermination of the mosquito vector with pesticides such as DDT. This approach was stopped because of the emergence of mosquito resistance against DDT and the undesirable side effects such as pollution of rivers. As a consequence the occurrence of malaria is now spreading. (ii) The large scale use of quinine and chloroquine for the treatment of patients with malaria and as prophylaxe. This has evoked resistance of the protozoa against these drugs making the fight against malaria more difficult, and malaria now forms an increasing problem in the (sub)tropics. Moreover, it is anticipated that due to global warming, malaria will spread to countries, such as Southern Europe and the USA. Therefore, there is a continuous search for new remedies against malaria.

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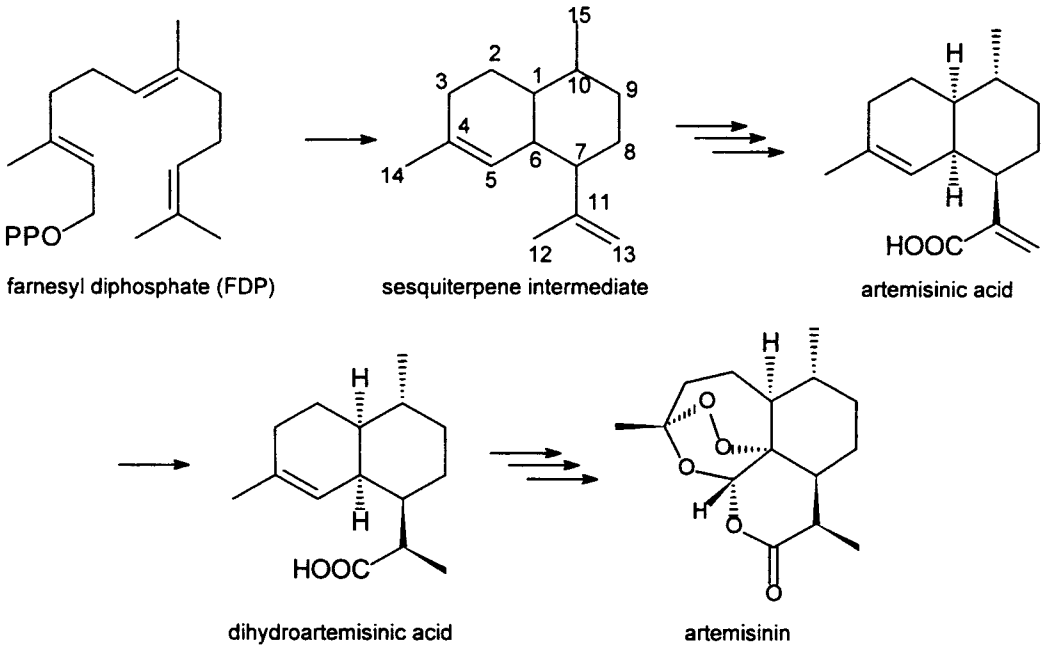


Fig. 1. Proposed biosynthetic pathway of artemisinin in *Artemisia annua*.

A very promising new group of drugs against malaria are the endoperoxide sesquiterpene lactone artemisinin and its derivatives (Fig. 1). Trials with artemisinin and derivatives were all very promising, and artemisinin derivatives are now being marketed (Van Geldre, Vergauwe, & Van den Eeckhout, 1997). Artemisinin, also known as qinghaosu, is a constituent of the traditional Chinese medicinal herb *Artemisia annua* L. (Asteraceae). The earliest mention of this herb dates back to 168 BC in the ancient recipes found in the tomb of the Mawangdui Han dynasty (Klayman, 1985). Its antimalarial activity was described as early as 1596 by Li Shizhen in his Ben Cao Gang Mu (Compendium of Materia Medica) (Klayman, 1985). The active principle was isolated and identified as artemisinin in 1972. Since then there has been a tremendous scientific and commercial interest in this rediscovered antimalarial compound.

Artemisinin is a structurally complex compound and so far the plant *A. annua* is the only commercially feasible source of artemisinin for drug formulations (Van Geldre et al., 1997). *A. annua* is a cosmopolitan species, growing wild in many countries, e.g. in China and Vietnam, the Balkan, the former Soviet Union, Argentina and Southern Europe (Van Geldre et al., 1997), and large differences exist in artemisinin content between different strains of *A. annua* (Delabays, Benakis & Collet, 1993; Woerdenbag, Bos, Salomons, Hendriks, Pras & Malingré, 1993). A substantial increase in the content of artemisinin would be required to make artemisinin available on a large scale also to the people in The Third World. Selection for high producing lines and traditional breeding, and research on the effects of environmental conditions and cultural practices could perhaps lead to an improvement of artemisinin content (Delabays et al., 1993; Ferreira, Simon & Janick, 1995; Gupta et al., 1996; Laughlin, 1994). A biotechnological approach could be another way to increase artemisinin content in *A. annua*. However, the biosynthetic pathway of artemisinin has not been completely resolved. Several authors have demonstrated that *A. annua* converts artemisinic (also named arteannuic) acid and dihydroartemisinic (also named dihydroarteannuic) acid to artemisinin (Kim & Kim, 1993; Sangwan, Agarwal, Luthra, Thakur & Singh-Sangwan, 1993a; Wallaart, Van Uden, Lubberink, Woerdenbag, Pras & Quax, 1999) (Fig. 1). Akhila, Thakur and Popli (1987) and Akhila, Rani and Takur (1990) hypothesised a pathway in which the formation from farnesyl diphosphate of an unidentified enzyme-bound sesquiterpene-like intermediate represents the first committed step in the biosynthesis of artemisinin.

In addition, many authors have analysed extracts of *A. annua* to search for possible intermediates in the biosynthesis of artemisinin. Artemisinic and dihydroartemisinic acid were reported by many authors, as well as many olefinic mono- and sesquiterpenes and putative intermediates en route from dihydroartemisinic acid to artemisinin (Ahmad & Misra, 1994; Brown, 1994; Charles, Cebert & Simon, 1991; Jung, ElSohly, Croom & McPhail, 1986; Jung, ElSohly & McChesney, 1990; Ranasinghe, Sweatlock & Cooks, 1993; Woerdenbag et al., 1993; Wallaart, T. E., Pras,

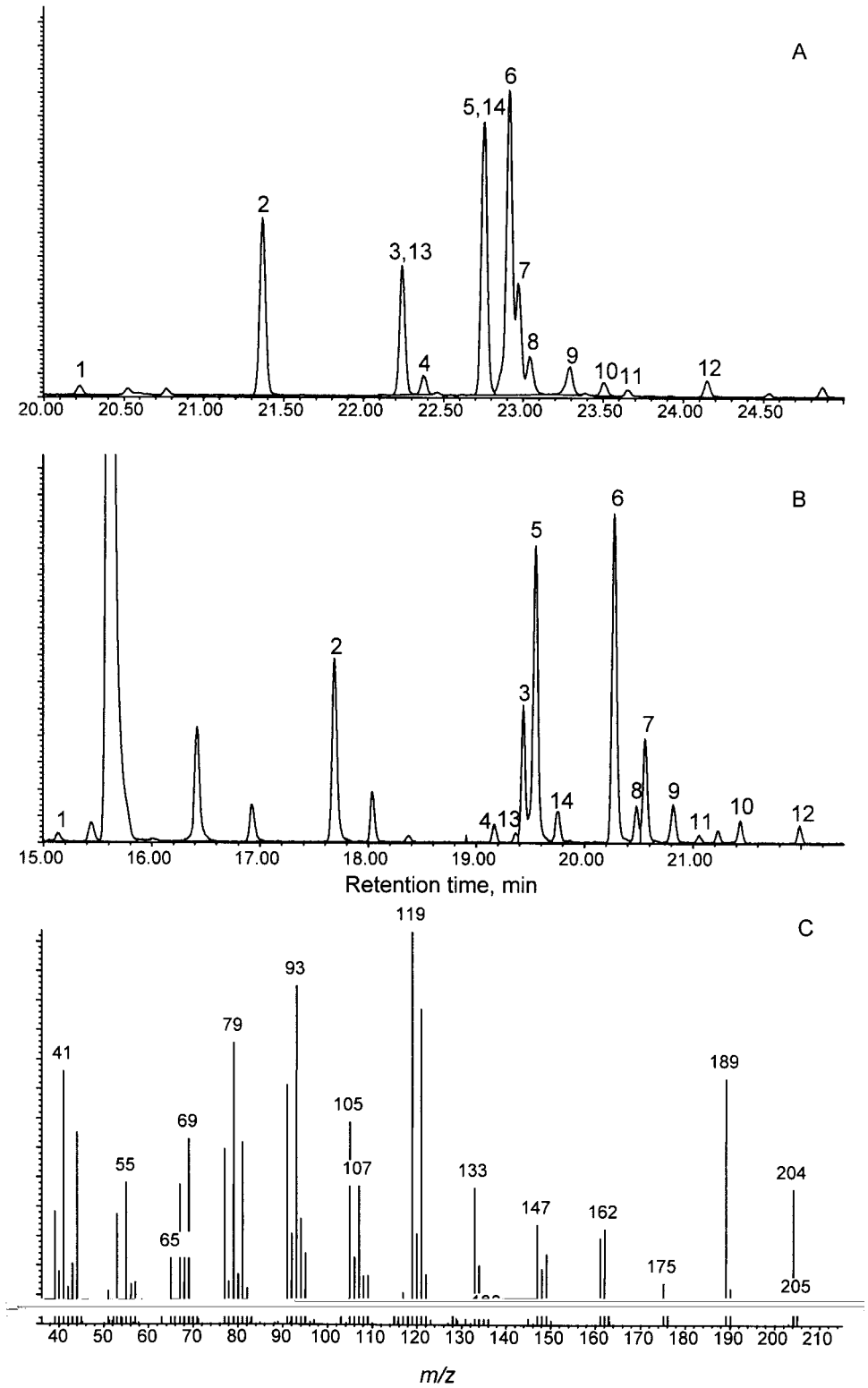


Fig. 2. Sesquiterpene olefins in solvent extracts of greenhouse-grown *Artemisia annua* leaves. Extracts were analysed by GC–MS using a HP 5890 series II gas chromatograph and HP 5972A Mass Selective Detector equipped with (A) an HP-5MS or (B) an HP-INNOWax column (both 30 m × 0.25 mm i.d., 0.25 μm film thickness). (C) Mass spectrum of compound 14. Compounds (numbers between brackets are retention indices on HP-5MS and HP-INNOwax columns, respectively): 1, α-copaene (1381, 1481); 2, β-caryophyllene (1427, 1582); 3, *trans*-β-farnesene (1462, 1658); 4, muurola-4,11-diene (1467, 1646); 5, selina-4,11-diene (1483, 1663); 6, germacrene D (1490, 1819); 7, unknown 1 (1491, 1706); 8, β-selinene (1494, 1702); 9, bicyclogermacrene (1504, 1718); 10, germacrene A (1513, 1747); 11, γ-cadinene (1519, 1729); 12, unknown 2 (1541, 1772); 13, α-humulene (1462, 1654); 14, amorpha-4,11-diene (1483, 1672). For further details, see Experimental.

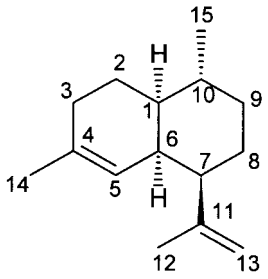
N. & Quax, W. J., personal communication). However, none of the reported olefinic sesquiterpenes seemed to fit in the biosynthetic pathway, nor was a possible intermediate between the sesquiterpene olefin and artemisinic acid ever detected, with the exception of artemisinic alcohol which was tentatively identified in the roots of *A. annua* by Woerdenbag et al. (1993).

It has been postulated that the cyclisation of the ubiquitous precursors geranyl diphosphate, farnesyl diphosphate (FDP) and geranylgeranyl diphosphate to the respective olefinic mono-, sesqui- and diterpene skeletons represents the regulatory step in the biosynthesis of terpenoids (Gershenzon & Croteau, 1990; McCarvey & Croteau, 1995). The accumulation of artemisinic and dihydroartemisinic acid and the absence of any intermediates en route from FDP to these two compounds support that the first step(s) in the biosynthetic pathway of artemisinin (and again some step(s) from (dihydro)artemisinic acid to artemisinin) are indeed regulatory/rate-limiting. In the present work we studied the identity of the probable olefinic sesquiterpene intermediate in the biosynthesis of artemisinin, and isolated and characterised the sesquiterpene synthase that catalyses its formation from FDP (Fig. 1).

2. Results

2.1. Identification of olefinic sesquiterpenes in A. annua leaves

Several authors have analysed hydrocarbon sesquiterpenes in *A. annua* tissues (Ahmad & Misra, 1994; Charles et al., 1991; Woerdenbag et al., 1993), but a compound that could be the olefinic sesquiterpene intermediate in the biosynthesis of artemisinin was never identified. Fig. 2 shows the results of our analyses of leaf extracts of greenhouse-grown *A. annua* on two different GC-columns. Results with field-grown plants were similar (data not shown). Of the 14 sesquiterpene olefins that were found, ten were identified using GC–MS libraries (Wiley and personal library of W.A. König), retention indices (Adams, 1995) and authentic standards. Compound 4 was shown to be identical to an authentic sample of muurola-4,11-diene isolated from *Amyris balsamifera* essential oil (W.A. König, unpublished data). Retention indices of identified compounds on the HP-5MS column correlated well with the retention indices given by Adams (1995) for a DB-5 column ($R^2=0.998$). Two of the 14 compounds could not be identified (Fig. 2, compound 7: *m/z* 204 M^+ (30), 189 $[M\text{-}Me]^+$ (21), 93 (100), 91 (94), 79 (71), 105 (66), 77 (64), 133 (61), 69 (56), 161 (53), 55 (51), 67 (48) and compound 12: *m/z* 204 M^+ (9), 189 $[M\text{-}Me]^+$ (14), 93 (100), 161 (71), 107 (59), 121 (55), 79 (53), 91 (47), 68 (44), 67 (43), 105 (40), 119 (40)). The presence of compound 14 could only be demonstrated by using two different GC-columns as it co-eluted with the major product 5 (selina-4,11-diene) on the HP-5MS column. The mass spectrum of compound 14 (Fig. 2C) closely resembles the mass spectrum of cadina-4,11-diene (Bohlmann, Gerke, Jakupovic, King & Robinson, 1984; *m/z* 204 M^+ (?), 189 $[M\text{-}Me]^+$ (56), 119 (100), 121 (86), 93 (79), 105 (51), 91 (46), 162 (31), 161 (30)) which was later renamed amorpha-4,11-diene (**1**) (Scheme 1) (Connolly & Hill, 1991). Considering the structure and configuration, amorpha-4,11-diene is a suitable candidate for the olefinic first intermediate in the biosynthesis of artemisinin (Fig. 1). The identity of compound 14 was unambiguously proven by semi-synthesis of **1** from artemisinic acid. On the HP-INNOwax column compound 14 had the same retention time and mass spectrum as the synthesised **1** (Fig. 3). On the HP-5MS column the synthesised **1** co-eluted with compounds 5 (selina-4,11-diene) and 14 (data not shown), but had a clearly different mass spectrum than selina-4,11-diene.



1 amorpha-4,11-diene

Scheme 1.

2.2. Enzyme isolation and identification

When a crude enzyme extract of the field-grown *A. annua* was assayed for sesquiterpene synthase activity with [^{3}H]FDP as substrate, a range of labelled sesquiterpenes was detected by radio-GC (Fig. 4). In addition to the olefinic sesquiterpenes, labelled farnesol (produced by aspecific phosphohydrolases) was detected in the Et_2O phase of the assay extract (data not shown). Co-injection of an *A. annua* pentane extract, which had been analysed using GC–MS enabled the identification of β-caryophyllene (compound 2) and germacrene A (compound 10) and probably α-copaene (compound 1). However, due to the low resolution in both the FID- and radio-trace, the major peaks in the radio-trace could not be attributed to one single peak in the FID-trace. Radio-peak "a" probably belongs to *trans*-β-farnesene (compound 3),

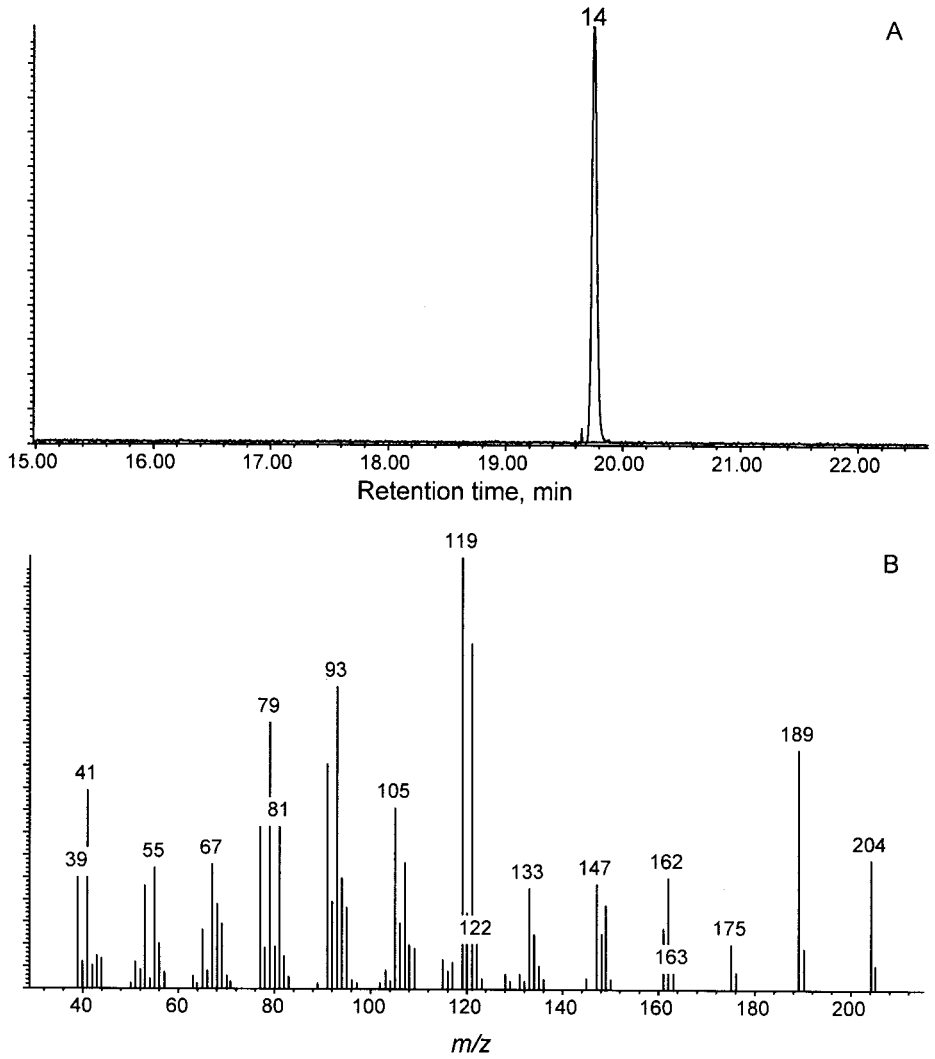


Fig. 3. GC–MS analysis of semi-synthetic standard of amorpha-4,11-diene using a HP 5890 series II gas chromatograph and HP 5972A Mass Selective Detector equipped with an HP-INNOWax column (30 m × 0.25 mm i.d., 0.25 μm film thickness). (A) Chromatogram; (B) mass spectrum.

but could also belong to compounds 4 and 13 (also see Fig. 2B), the major radio-peak "b" could belong to compounds 5 and 14, and radio-peak "c" could be germacrene D (compound 6), but also compounds 7, 8, 9, 11. Radio-GC analysis of the radiolabelled products of the enzyme preparation after fractional $(NH_4)_2SO_4$ precipitation showed the same pattern of products as for the crude extract. GC–MS analysis showed that the $(NH_4)_2SO_4$ precipitation had removed the endogenous sesquiterpenes from the enzyme preparation. GC–MS analysis of the sesquiterpenes produced by this enzyme preparation from unlabelled FDP (Fig. 5A) showed the presence of the sesquiterpenes α-copaene (1), β-caryophyllene (2), *trans*-β-farnesene (3), selina-4,11-diene (5), germacrene D (6), bicyclogermacrene (9), germacrene A (10) and as one of the major products, amorpha-4,11-diene (14). Identification was based on retention times/retention indices on two columns (HP-5MS and HP-INNOwax) and mass spectra. The mass spectrum of compound 14 (amorpha-4,11-diene) is shown in Fig. 5B. Comparison of the results in Fig. 5A with the results in Fig. 4 showed that the radiolabelled product-peak "a" in Fig. 4 belongs to *trans*-β-farnesene, "b" to amorpha-4,11-diene (and not selina-4,11-diene), and "c" to germacrene D.

2.3. Purification and characterisation of amorpha-4,11-diene synthase

Although the presence of amorpha-4,11-diene synthase in plants of *A. annua* had now been demonstrated, the presence of many other sesquiterpene synthases obstructed a straightforward purification. In an attempt to find a more suitable starting material, the young shoot tips of greenhouse-grown *A. annua*

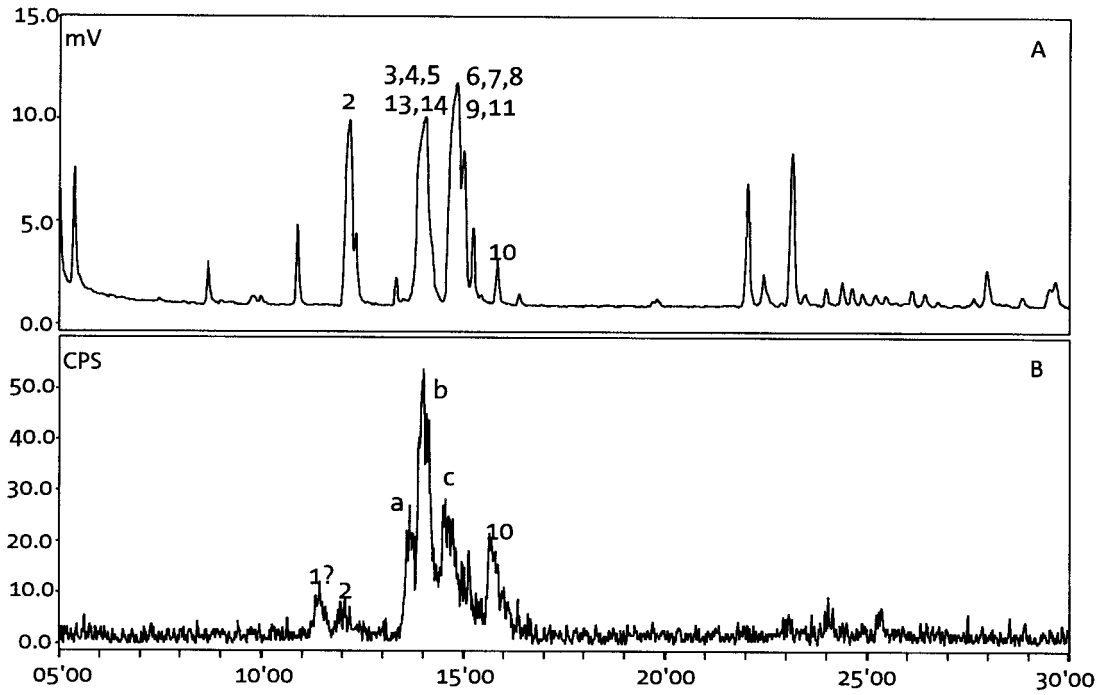


Fig. 4. Radio-GC analysis of the labelled products formed from [^{3}H]farnesyl diphosphate in an assay with a crude enzyme preparation of field-grown *Artemisia annua*. (A) FID-signal of a co-injected solvent extract of *A. annua* leaves (as in Fig. 2). (B) Radio-trace showing radiolabelled products of the assay. The gaschromatograph was equipped with an EconoCap EC-WAX capillary column. Compounds: 1, α-copaene; 2, β-caryophyllene; 3, *trans*-β-farnesene; 4, muurola-4,11-diene; 5, selina-4,11-diene; 6, germacrene D; 7, unknown 1; 8, β-selinene; 9, bicyclogermacrene; 10, germacrene A; 11, γ-cadinene; 12, unknown 2; 13, α-humulene; 14, amorpha-4,11-diene; a, probably 3 possibly 4 and/or 13; b, 5 and/or 14; c, 6, 7, 8, 9 and/or 11.

plants were assayed for sesquiterpene synthase activity (Fig. 6). This plant material contained much less different sesquiterpene synthase activities, consisting almost exclusively of amorpha-4,11-diene synthase (This was confirmed by GC–MS analysis.) Using DEAE anion exchanger batch incubation, $(NH_4)_2SO_4$ precipitation and Mono-Q anion exchange chromatography (FPLC), amorpha-4,11-diene synthase was separated from the aspecific phosphohydrolase activity and the small amounts of other sesquiterpene synthases present in the crude extract such as germacrene A synthase (Fig. 6C). The enzyme preparation thus obtained was used for characterisation. Enzyme assays with this preparation were linear for over 30 min, even at 0.2 μM FDP (Fig. 7A). The enzyme had a K_m of about 0.6 μM (Fig. 7B), a molecular mass of 56 kDa (Fig. 7C) and a broad pH optimum around 6.5–7.0.

3. Discussion

For the first time the presence of amorpha-4,11-diene (**1**) in an *A. annua* solvent extract was demonstrated. The structure and configuration of this compound make it the likely olefinic sesquiterpene intermediate in the biosynthesis of artemisinin (Fig. 1). Moreover, the enzyme activity that catalyses the formation of **1** from FDP is the major sesquiterpene synthase activity in *A. annua* leaf extracts. The enzyme catalysing the formation of **1** from FDP has the typical characteristics of sesquiterpene synthases (Croteau & Cane, 1985; De Kraker, Franssen, De Groot, König & Bouwmeester, 1998; Salin, Pauly, Charon & Gleizes, 1995; Schmidt, Bouwmeester, De Kraker & König, 1998). It has a broad pH optimum around 6.5–7.0 and a molecular mass of 56 kDa. The affinity for the substrate FDP is high (but in the range expected) with a K_m of 0.6 μM.

Of the 12 sesquiterpene olefins we have identified in *A. annua* leaf extracts (Fig. 2), five (α-copaene, β-caryophyllene, α-humulene, *trans*-β-farnesene and germacrene D) have been reported before as constituents of *A. annua* essential oil (Ahmad & Misra, 1994; Charles et al., 1991; Woerdenbag et al., 1993). The presence in *A. annua* of amorpha-4,11-diene, muurola-4,11-diene, bicyclogermacrene and germacrene A is reported for the first time, the latter probably because of its labile character (De Kraker et al., 1998). Selina-4,11-diene and β-selinene are proton-induced rearrangement products of germacrene A, and β-elemene (which has been reported to be a constituent of *A. annua* oil (Woerdenbag et al., 1993)) is a temperature-induced

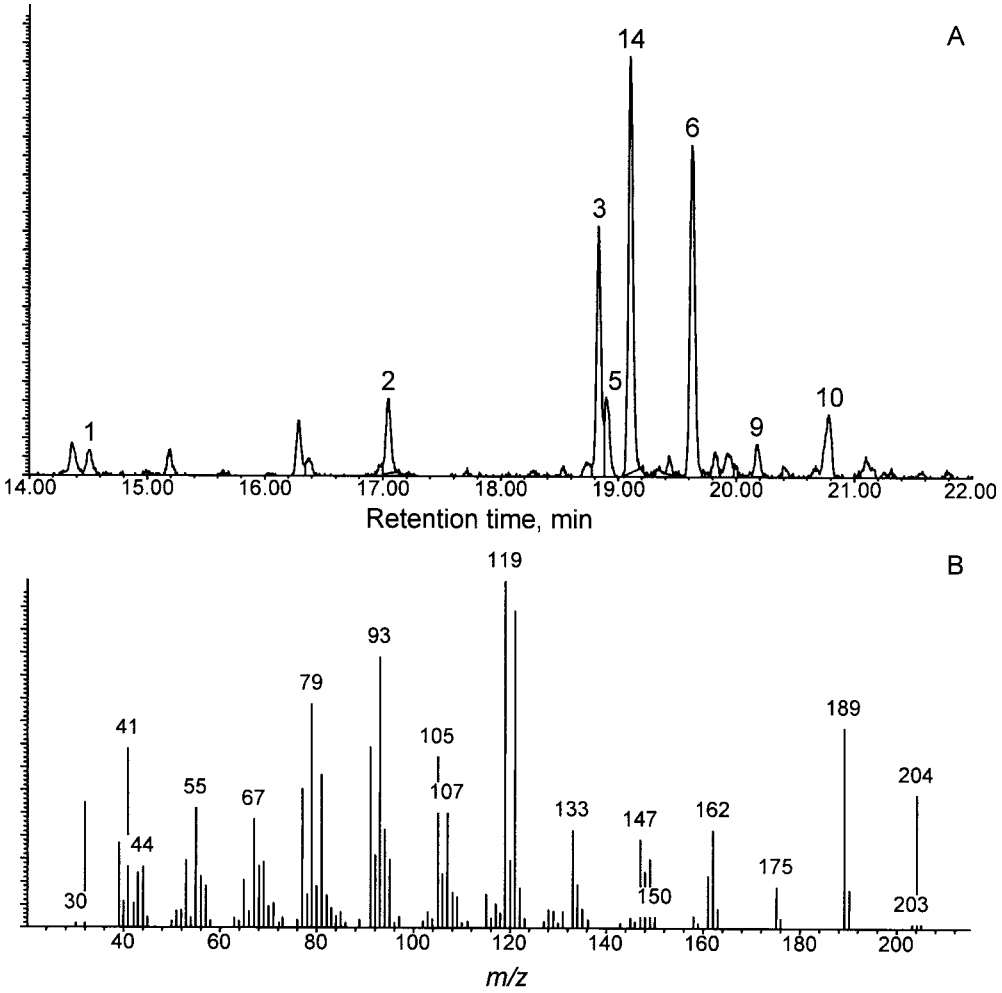


Fig. 5. GC–MS analysis of the products formed from farnesyl diphosphate by the $(NH_4)_2SO_4$-precipitated enzyme preparation of field-grown *Artemisia annua* using a HP 5890 series II gas chromatograph and HP 5972A Mass Selective Detector equipped with an HP-INNOWax column (30 m × 0.25 mm i.d., 0.25 μm film thickness). (A) Chromatogram of selected ions m/z 93 + 119 + 161 + 189 + 204; (B) mass spectrum of compound 14. Compounds: 1, α-copaene; 2, β-caryophyllene; 3, *trans*-β-farnesene; 5, selina-4,11-diene; 6, germacrene D; 9, bicyclogermacrene; 10, germacrene A; 14, amorpha-4,11-diene.

rearrangement product of germacrene A (De Kraker et al., 1998; Teisseire, 1994). In our analysis, rearrangement of germacrene A to β-elemene was prevented by using a low injection port temperature of 150°C as described by De Kraker et al. (1998). γ-Cadinene may be a proton-induced rearrangement product of germacrene D (Teisseire, 1994). The results of the enzyme assays generally confirm these conclusions: after $(NH_4)_2SO_4$ precipitation we could demonstrate α-copaene, β-caryophyllene, *trans*-β-farnesene, amorpha-4,11-diene, germacrene D, bicyclogermacrene and germacrene A synthase activities (Fig. 5A). The trace of selina-4,11-diene present in Fig. 5A was probably due to proton-induced rearrangement of germacrene A. We could not detect biosynthesis of the other sesquiterpenes perhaps because the enzyme activities were too low (muurola-4,11-diene, unknown 1?, unknown 2?, α-humulene) or because they represent rearrangement products which were not formed during the enzyme assay but only in planta or during extraction from the plant (unknown 1?, β-selinene, γ-cadinene, unknown 2?).

The differences in sesquiterpene synthase activities between the two plant materials (Figs. 4B and 6B) are large. Although the plants were grown from the same batch of seeds the differences could be due to the presence of chemotypes, to the environmental conditions the plants were exposed to or to differences in developmental stage. Naturally, the field-grown plants were exposed to harsher conditions than the greenhouse-grown and several authors have described the induction of terpene synthases as a result of wounding (Funk, Lewinsohn, Vogel, Steele & Croteau, 1994), or fungal elicitors (Chappell, 1995; Vögeli & Chappell, 1988). In addition, several authors have demonstrated changes in terpenoid accumulation patterns during

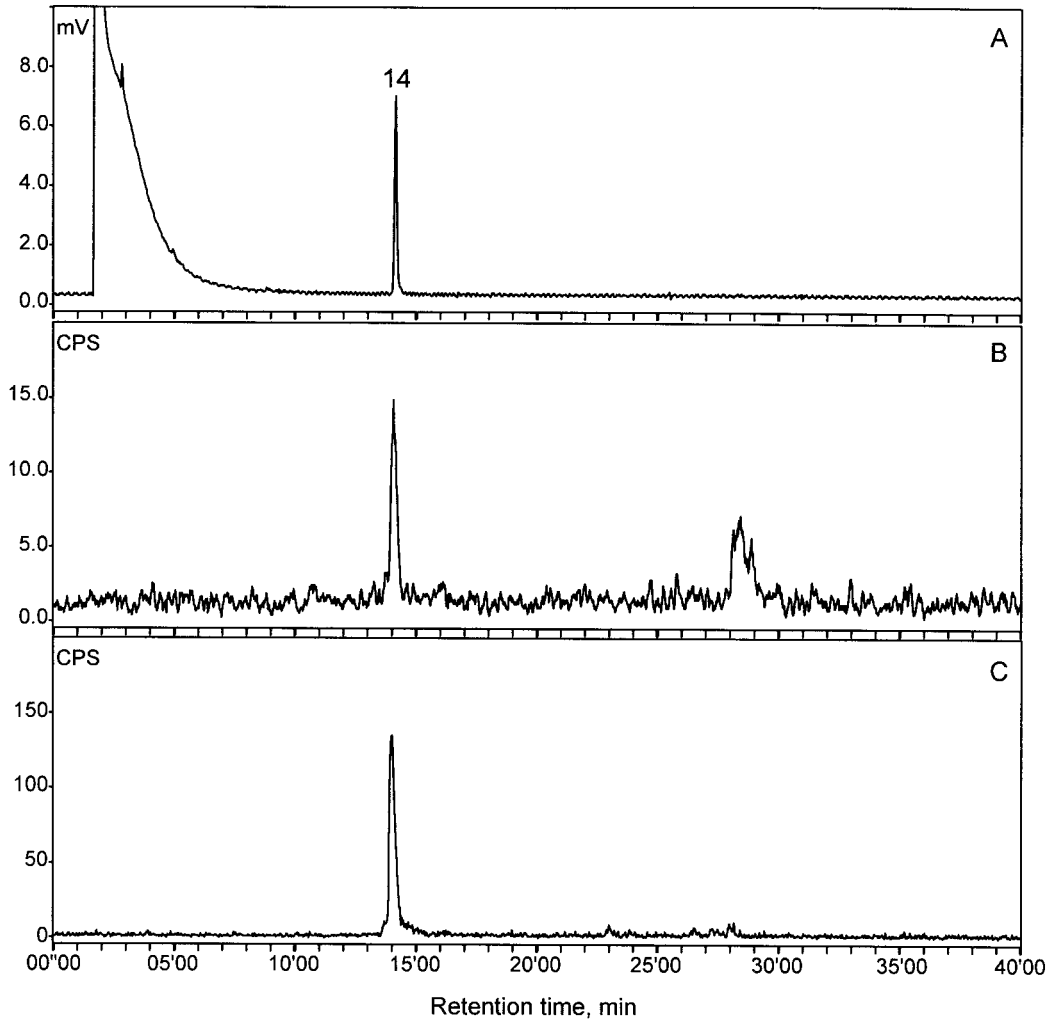


Fig. 6. Radio-GC analysis of the labelled products formed from [^{3}H]farnesyl diphosphate in an assay with a crude enzyme preparation of greenhouse-grown *Artemisia annua*. (A) FID-signal of a co-injected standard of amorpha-4,11-diene (14). (B) and (C) Radio-traces showing radiolabelled products of assays on (B) a crude enzyme preparation and (C) after partial purification of amorpha-4,11-diene synthase using DEAE anion exchanger batch incubation, $(NH_4)_2SO_4$ precipitation and Mono-Q anion exchange chromatography (FPLC). The second peak in (B) represents farnesol, produced from farnesyl diphosphate by phosphohydrolase activity. The gaschromatograph was equipped with an Alltech EC-WAX capillary column. For further details, see Experimental.

development — also in *A. annua* (Woerdenbag et al., 1994) — which correlated with changes in the activities of the enzymes involved (Bouwmeester, Gershenzon, Konings & Croteau, 1998; Croteau, Felton, Karp & Kjonaas, 1981). The latter explanation is favoured by the fact that the sesquiterpene profiles of field- and greenhouse-grown plants are essentially similar (data not shown).

The fact that amorpha-4,11-diene (**1**), in spite of the high in vitro amorpha-4,11-diene synthase activity, can only be detected in minute quantities in the leaf essential oil, strongly suggests that in planta further functionalisation of the amorpha-4,11-diene (**1**) skeleton occurs. Further modifications of the amorpha-4,11-diene skeleton to produce the next known intermediate in artemisinin biosynthesis, artemisinic acid (Sangwan et al., 1993a), can easily be envisaged to occur analogously to the formation of kaurenic and abietic acid from kaurene and abietadiene, respectively (Funk & Croteau, 1994; West, 1980). The first step likely is the cytochrome P-450 catalysed hydroxylation at C12 of **1** yielding artemisinic alcohol. This compound has not been reported in *A. annua* shoot essential oil extracts, but was tentatively identified in the roots (Woerdenbag et al., 1993). Subsequently, the alcohol could be oxidised two additional times at C12 by either cytochrome P-450 enzymes or dehydrogenases yielding the corresponding acid, artemisinic acid (Funk & Croteau, 1994; Sangwan, Singh-Sangwan & Luthra, 1993b; West, 1980). Research to demonstrate incorporation of labelled amorpha-4,11-diene into artemisinin or its

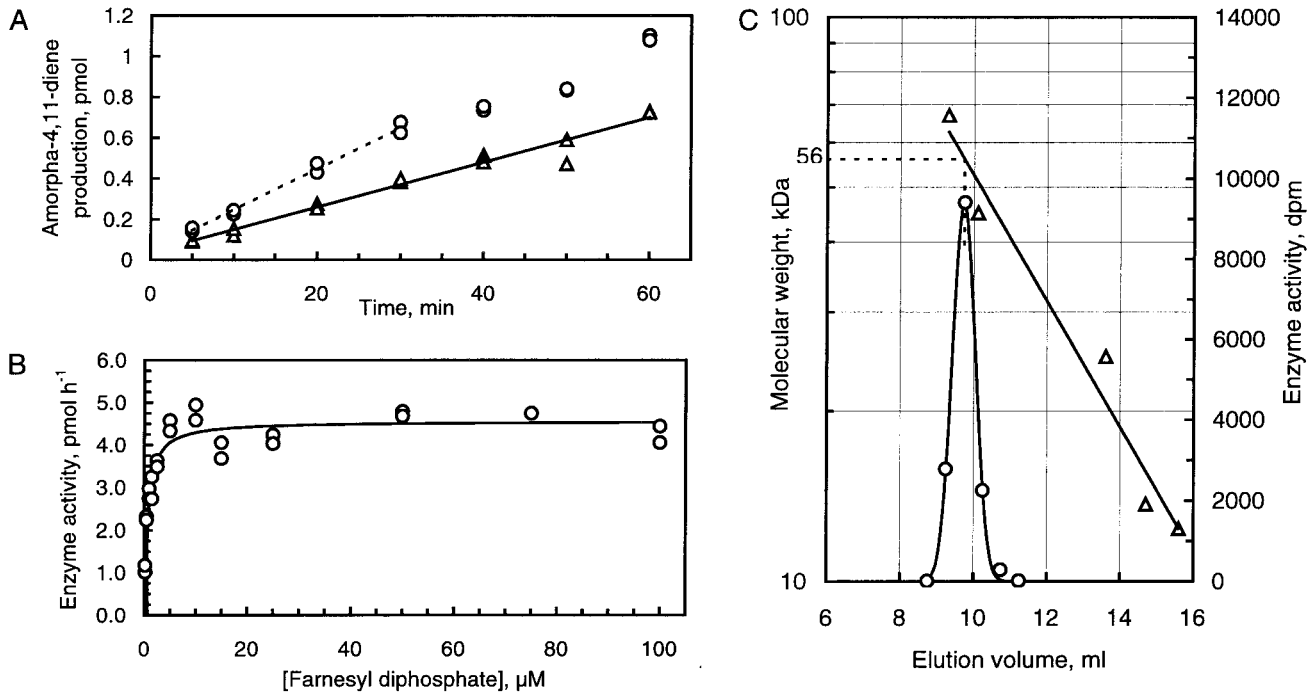


Fig. 7. Characteristics of amorpha-4,11-diene synthase, which was partially purified using DEAE anion exchanger batch incubation, $(NH_4)_2SO_4$ precipitation and Mono-Q anion exchange chromatography (FPLC). (A) At 0.2 μM farnesyl diphosphate the enzyme assay was linear for over 30 min for undiluted enzyme (○) and up to 60 min for 2-fold diluted enzyme (△). (B) Michaelis–Menten kinetics: $V = 4.56[S]/(0.61 + [S])$, $R^2 = 0.91$, $K_m = 0.61$ μM. (C) Enzyme activity (○) as function of elution volume on a Superdex 75 FPLC column (HR10/30), indicating an estimated M_r of 56 kDa. The column was calibrated using cytochrome C, ribonuclease A, α-chymotrypsinogen, ovalbumin and BSA (△).

known precursors artemisinic or dihydroartemisinic acid is underway.

The absence in *A. annua* of any intermediates en route from FDP to (dihydro)artemisinic acid suggests that these steps are non-rate limiting. Also in other species there is evidence that the regulation of terpenoid metabolism resides at the level of the first dedicated steps in the biosynthetic pathway (McCarvey & Croteau, 1995). For example, in *Clarkia breweri* there was a close correlation between linalool synthase activity and the production of linalool and linalool oxides (Pichersky, Raguso, Lewinsohn & Croteau, 1994), and in *Carum carvi* there was a close correlation between limonene synthase activity and the accumulation rate of limonene and carvone (Bouwmeester et al., 1998).

If the enzymatic formation of amorpha-4,11-diene is indeed the rate-determining step in artemisinin biosynthesis, the isolation of the gene encoding this enzyme would enable a molecular approach to develop a new variety of *A. annua* containing higher amounts of dihydroartemisinic acid and/or artemisinin. This could make artemisinin available at lower costs so that also people in The Third World, who suffer most from malaria, can benefit from this valuable and effective drug against malaria.

4. Experimental

4.1. Plant material

Seeds of *Artemisia annua* L. (Asteraceae) of Vietnamese origin were obtained from Artecef BV (Maarssen, The Netherlands). Taxonomically verified specimens are deposited at the Department of Pharmaceutical Biology, Groningen University, The Netherlands and at the Institute of Materia Medica, Hanoi, Vietnam. Plants were grown on an experimental field in Buitenpost, The Netherlands. A second batch of plants was grown in a greenhouse in 5-l plastic pots containing potting compost at 21/18°C (16/8 h). Natural daylight was supplemented with artificial light (SON-T AGRO) during the first 6 and last 5 h of the high temperature period. Fertiliser was applied as required. When the plants were about 1.5 m in height, young shoot tips were collected, frozen in liq N_2, and stored at −80°C.

4.2. Substrates, chemicals and reference compounds

A standard of germacrene D was obtained from *Solidago canadensis*, standards of germacrene A, β-selinene and selina-4,11-diene from *Frullania tamarisci*. γ-

Cadinene was prepared by acid catalysed rearrangement of germacrene D (from *Solidago canadensis*) and identified by NMR techniques (Bülow, 1998). Pentane and Et_2O were redistilled before use.

Amorpha-4,11-diene (**1**) was synthesised from artemisinic acid isolated from *A. annua* (Wallaart et al., 1999). Starting from artemisinic acid it is obvious to convert the carboxylic functionality into a methyl group via reduction. However, several efforts showed it was impossible to obtain a reasonable yield. Therefore artemisinic acid was first esterified with CH_2N_2 in Et_2O and than reduced using $NaBH_4$ in the presence of $NiCl_2$, yielding an epimeric 7:1 mixture of 11*R*/*S* methyl dihydroartemisinate which was used without purification in the next reaction (Xu, Zhu, Huang & Zhou, 1986). Again a straightforward reduction proved troublesome and therefore a stepwise procedure was established. Reduction with $LiAlH_4$ in THF (Ye & Wu, 1989) converted the ester group into a hydroxy function which was mesylated using mesylchloride (CH_3SO_2Cl) in pyridine to afford a 7:1 epimeric 11*R*/*S* mixture purified by column chromatography on silicagel with a mixture of EtOAc in hexane (7:3) as eluent. Because elimination of methanesulfonic acid resulted in a complex mixture the mesylate was first converted into a iodide by stirring it with NaI in acetone at room temperature and now a dehydrohalogenation was easily performed with potassium *tert*-butoxide in *tert*-butyl alcohol (Kesselmans, 1992). Amorpha-4,11-diene was isolated and purified by column chromatography on silicagel using pentane as eluent in an overall yield of 25%. Spectral data [^{1}H NMR (200 and 400 MHz) and MS] were in complete agreement with reported data except the (erroneously?) reported absorption of H-5 at δ 5.50 instead of δ 5.05 ppm (Bohlmann et al., 1984). ^{13}C-NMR (100 MHz, DEPT, $CDCl_3$): δ 19.8 (q), 22.6 (q), 23.6 (q), 25.8 (t), 26.1 (t), 26.5 (t), 27.9 (d), 35.4 (t), 37.6 (d), 41.8 (d), 47.7 (d), 109.8 (t), 120.9 (d), 134.6 (s), 150.0 (s).

4.3. Isolation and identification of olefinic sesquiterpenes from A. annua

Sesquiterpenes of *A. annua* were extracted from young shoots of field-grown plants or full-grown leaves of greenhouse-grown plants essentially as described by Zänglein (1993). Two grams of leaves were homogenised in 20 ml MeOH in a cooled mortar and pestle. The MeOH extract was filtered over glasswool and applied to a Sep-Pak C18 cartridge pre-equilibrated with MeOH. The eluent was collected in a 20 ml pentane/200 ml water two-phase system. After mixing, the two phases were separated and the pentane phase decolorised with activated carbon and washed with 2 ml of water. The pentane phase was passed over a short column of aluminium oxide overlaid with anhydrous $MgSO_4$, and analysed by GC–MS using a HP 5890 series II gas chromatograph and HP 5972A Mass Selective Detector equipped with an HP-5MS or HP-INNOWax column (both 30 m × 0.25 mm i.d., 0.25 μm film thickness). The oven was programmed at an initial temperature of 70°C for 1 min, with a ramp of 5°C min^{-1} to 210°C and final time of 5 min. The injection port (splitless mode), interface and MS source temperatures were 150, 290 and 180°C, respectively, and the He inlet pressure was controlled by electronic pressure control to achieve a constant column flow of 1.0 ml min^{-1}. Spectra were obtained at 70 eV, and scanning was performed from 30 to 250 atomic mass units.

4.4. Isolation and identification of sesquiterpene synthases from A. annua

For the comparison of enzyme activities in field-grown and greenhouse-grown plants of *A. annua*, 10 g of frozen young leaves were ground in a pre-chilled mortar and pestle in 40 ml of pre-chilled buffer containing 50 mM Mopso (pH 7.0), 20% (v/v) glycerol, 50 mM sodium ascorbate, 50 mM $NaHSO_3$, 10 mM $MgCl_2$ and 5 mM DTT slurried with 1 g polyvinylpolypyrrolidone (PVPP) and a spatula tip of purified sea sand. Ten grams of polystyrene resin (Amberlite XAD-4) were added and the slurry was stirred carefully for 10 min and then filtered through cheesecloth. The filtrate was centrifuged at 20,000*g* for 20 min (pellet discarded), and then at 100,000*g* for 90 min. A 3-ml sample of the supernatant was desalted to a buffer containing 15 mM Mopso (pH 7.0), 10% (v/v) glycerol, 1 mM sodium ascorbate, 10 mM $MgCl_2$ and 2 mM DTT (buffer A) and used for enzyme assays/product identification.

For the identification of the enzymatic products, 20 μM [^{3}H]FDP (50 Ci mol^{-1}; for radio-GC analysis) or 50 μM unlabelled FDP (for GC–MS analysis) were added to 1 ml of the enzyme preparations. After the addition of a 1 ml pentane overlay, the tubes were carefully mixed and incubated for 1 h at 30°C. Controls that had been boiled for 5 min showed no enzymatic activity. Following the assay, the tubes were vigorously mixed and briefly centrifuged. The organic layer was removed and passed over a short column of aluminium oxide (grade III) overlaid with anhydrous $MgSO_4$. The assay was extracted once more with 1 ml of Et_2O, which was also passed over the aluminium oxide column, and the column washed with 1.5 ml of Et_2O. Pentane and Et_2O extracts were analysed either separately or combined. Before radio-GC analysis unlabelled reference compounds were added to the extract, which was then slowly concentrated under a stream of N_2.

Radio-GC was performed on a Carlo-Erba 4160

Series gas chromatograph equipped with a RAGA-90 radioactivity detector. Samples of 1 μl were injected in the cold on-column mode. The GC was equipped with an EconoCap EC-WAX column (30 m × 0.32 mm i.d., film thickness 0.25 μm) and operated with a He flow of 1.2 ml min^{-1}. The oven temperature was programmed to 70°C for 5 min, followed by a ramp of 5° min^{-1} to 210°C and a final time of 5 min. To determine retention times and peak identities (by co-elution of radioactivity with reference standards), about 20% of the column effluent was split with an adjustable splitter to an FID (temperature 270°C). The remainder was directed to the conversion reactor and radio detector. Components were quantitatively reduced before radioactivity measurement by passage through a conversion reactor filled with platinum chips at 800°C. H_2 was added prior to the reactor at 3 ml min^{-1}, and CH_4 as a quench gas prior to the radioactivity detector (5 ml counting tube) to give a total flow of 36 ml min^{-1}.

GC–MS analysis of the enzymatic products was carried out as described above. In assays on the crude enzyme preparation it was impossible to discriminate between (in vitro) enzymatically produced sesquiterpenes and sesquiterpenes which were co-extracted with the enzymes from the leaf material. However, endogenous sesquiterpenes were effectively removed using fractional $(NH_4)_2SO_4$ precipitation at 20 and 60% (giving over 90% recovery of sesquiterpene synthase activity) enabling GC–MS identification of the enzymatically produced sesquiterpenes.

4.5. Partial purification and characterisation of amorpha-4,11-diene synthase

Ten gram of young leaves of greenhouse-grown plants were extracted essentially as described above but now the buffer contained 25 mM Mes (pH 5.5), 20% (v/v) glycerol, 25 mM sodium ascorbate, 25 mM $NaHSO_3$, 10 mM $MgCl_2$ and 5 mM DTT (buffer B). The 100,000*g* supernatant was added to 12.5 g DEAE anion exchanger (Whatman DE-52), which had been rinsed several times with buffer B, and stirred carefully for 10 min. After centrifugation at 18,000*g* for 20 min, the supernatant was decanted and the DE-52 pellet discarded. Proteins in the supernatant were precipitated by adding $(NH_4)_2SO_4$ to a final concentration of 70%, careful stirring for 30 min, and centrifugation at 20,000*g* for 10 min. The resulting pellet was resuspended in 6 ml buffer B and desalted to buffer A. After addition of glycerol up to 30% (v/v) this enzyme preparation could be frozen in liq N_2 and stored at −80°C without loss of activity. 0.5 ml of this enzyme preparation was applied to a Mono-Q FPLC column (HR5/5), previously equilibrated with buffer A without sodium ascorbate, with 0.1% Tween-20. The enzyme was eluted with a gradient of 0–2.0 M KCl in the same buffer. For determination of enzyme activities, 10 μl of the 0.75-ml fractions were diluted 10-fold in an Eppendorf tube with buffer A and 20 μM [^{3}H]FDP was added. The reaction mixture was overlaid with 1 ml of hexane to trap volatile products. After incubation for 30 min at 30°C, the vials were vigorously mixed, and centrifuged briefly to separate phases. A portion of the hexane phase (750 μl) was transferred to a new Eppendorf tube containing 40 mg of silica gel to bind farnesol produced by phosphohydrolases, and, after mixing and centrifugation, 500 μl of the hexane layer was removed for liquid scintillation counting in 4.5 ml of scintillation cocktail. The active fractions were combined, and an assay carried out to determine product identity (see above). Anion exchange chromatography on Mono-Q separated amorpha-4,11-diene synthase from all other FDP-converting activities, and this enzyme preparation was used for enzyme characterisation. The pH optimum was determined with duplicates of 5-fold diluted enzyme preparation in buffer A at pH 6.5, 6.75, 7.0, 7.25, 7.5, or buffer A containing 15 mM NaAc (pH 4.0, 4.5, 5.0, 5.5), 15 mM Mes (pH 5.5, 6.0, 6.5), or 15 mM Tris–HCl (pH 7.5, 7.75, 8.0, 8.5, 9.0) instead of Mopso. Enzyme kinetics were determined using duplicates of 5- and 10-fold diluted enzyme preparation (in buffer A) and [^{3}H]FDP concentrations ranging from 0.25 to 100 μM. The molecular mass was determined using size-exclusion chromatography. 200 μl of the Mono-Q eluent was loaded on a Superdex 75 FPLC column (HR10/30) and eluted in 0.5-ml fractions in the same buffer as used for Mono-Q. Enzyme activity was determined as described for Mono-Q, but using undiluted eluent. The column was calibrated using cytochrome C, ribonuclease A, α-chymotrypsinogen, ovalbumin and BSA.

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