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BIOSYNTHESIS OF ARTEMISININ IN *ARTEMISIA ANNUA*

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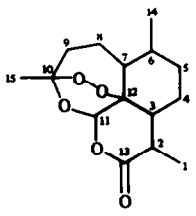
Key Word Index—*Artemisia annua*; Compositae; biosynthesis; sesquiterpene lactones; artemisinin; arteannuin B.

Abstract—The isotope ratios ($^3H:^{14}C$) in arteannuin B and artemisinin biosynthesized in *Artemisia annua* from [4*R*-$^3H_1$, 2-^{14}C]-, [5-$^3H_2$, 2-^{14}C]- and [2-$^3H_2$, 2-^{14}C](3*RS*)- mevalonate have revealed that two specific 1,2-hydride shifts take place during the oxidation and lactonization of the germacrane skeleton to yield dihydrocostunolide. The *gem*-methyls of DMAPP retain their identity until the final steps of artemisinin biosynthesis. Arteannuin B is considered to be a late precursor of artemisinin and the following biosynthetic sequence is suggested: farnesylpyrophosphate → germacrane skeleton → dihydrocostunolide → cadinanolide → arteannuin B → artemisinin.

INTRODUCTION

Artemisia annua L. is a herb from the Compositae family which has been used for many centuries in Chinese traditional medicine for the treatment of fever and malaria. Artemisinin (**1**), a sesquiterpene lactone, has been held responsible for the therapeutic effects of the plant [1–5]. This unusual compound has a peroxide grouping but lacks a nitrogen containing heterocyclic ring which is found in most antimalarial compounds. It attracted the attention of synthetic and natural product chemists [6, 7] and the unique structure initiated us to investigate the biosynthetic pathway in the plant. It is established that farnesyl pyrophosphate (FPP) is the precursor for the sesquiterpenes [8, 9]. The 2-*trans*,6-*trans* isomer (**4**) hypothetically gives rise to certain known skeletons but the 2-*cis*,6-*trans* isomer (**5**) has been thought to form those compounds for which **4** seems to be stereochemically unsuitable. The most characteristic feature of sesquiterpene biosynthesis is an initial intramolecular attack by an electrophilic centre at C-12 on either the central or the distal double bond of the precursor and this addition is governed by either electronic or steric influences [10].



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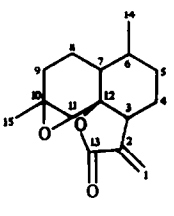

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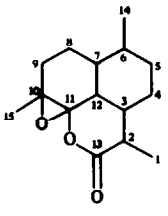

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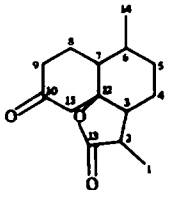

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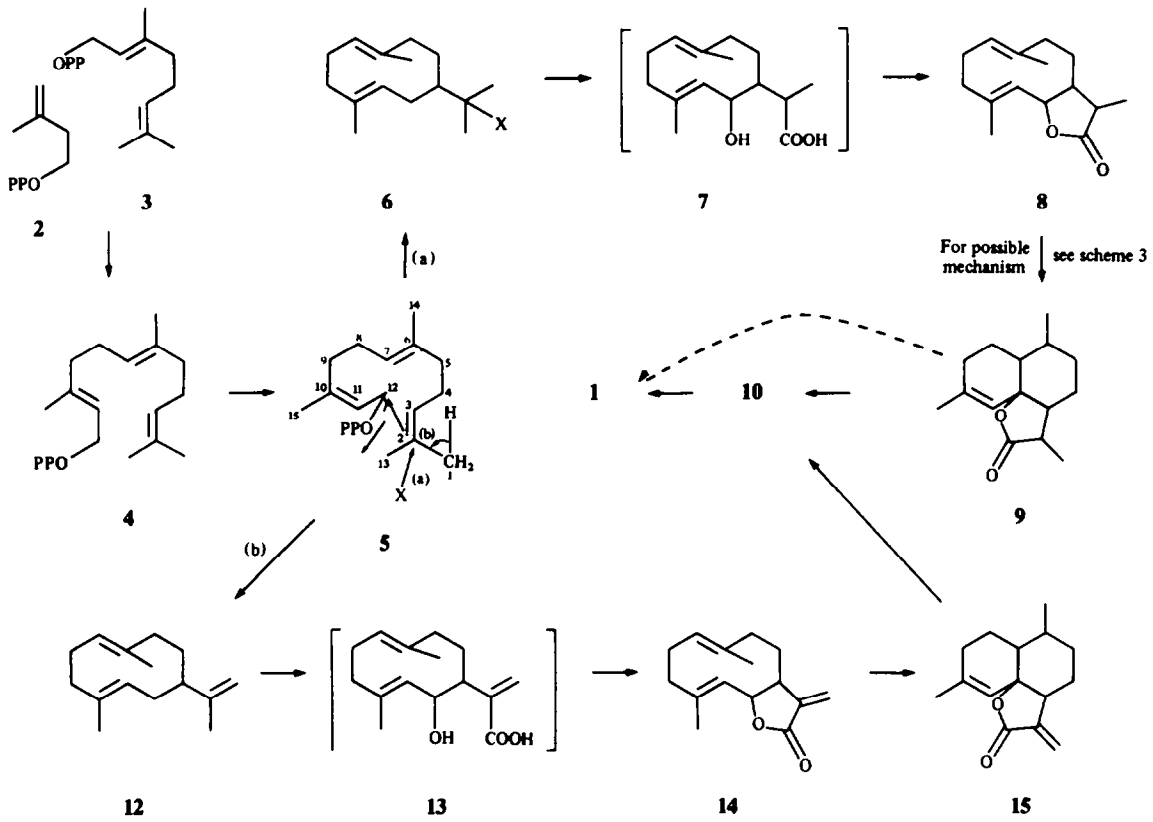
Two different schemes can be proposed for the biosynthesis of artemisinin in *A. annua*. The *cis*-isomer of FPP (**5**) may cyclise to **6** which then enters the pathway **6** → **7** → dihydrocostunolide (**8**) → cadinanolide (**9**) → arteannuin B (**10**) → artemisinin (**1**). The alternative squence is **5** → germacrane (**12**) → (**13**) → costunolide (**14**) → **15** → **10** → **1** (Scheme 1). In order to find out which of these biosynthetic sequences is operating within the plant system, we have fed the plant with appropriately labelled ($^3H/^{14}C$) precursors. The labelled arteannuin B (**10**) and artemisinin (**1**) were isolated and their isotope ratio ($^3H:^{14}C$) determined in order to draw some conclusion regarding the biosynthetic pathway.

RESULTS AND DISCUSSION

Mevalonic acid (MVA) is incorporated into artemisinin in 0.008–0.01% yield. Based on earlier work on sesquiterpene biosynthesis [11] the expected position of tracer ^3H in FPP, artemisinin (**1**) and arteannuin B (**10**) from doubly labelled MVA is shown in Table 1. The results from experiment 1 indicate that **1** and **10**, isolated from *A. annua* after being fed with [2-$^3H_2$,2-^{14}C]MVA, lost at least two ^3H atoms during the process of biosynthesis and these two ^3H are obviously lost from C-13 of **5** (Scheme 1) during the cyclization, oxidation and lactonization process; C-5 and C-9 do not seem to be involved in any biogenetic step. This suggests that the C-1 and C-13 *gem*-methyls originating from DMAPP retain their identity until the final steps of artemisinin biosynthesis.

The isotope ratios ($^3H:^{14}C$) in **1** and **10** biosynthesized from [4*R*-$^3H_1$,2-^{14}C]MVA (Table 1, experiment 2) suggest that at least one ^3H is lost from the C-3, C-7 or C-11 position of FPP (**5**) while it is being converted into **1** (Scheme 1). In order to determine which ^3H was lost during the biogenetic process compound **1** ($^3H:^{14}C$ = 2:3) was converted to **18** ($^3H:^{14}C$ = 1:3) and C-11, together with the associated ^3H or H was lost as formic acid. This suggests that the ^3H originally present at C-11 in FPP was intact also in **1** thus eliminating the possibility



Scheme 1. Postulated biosynthetic pathways to artemisinin and arteannuin B from farnesyl pyrophosphate or its biogenetic equivalent.

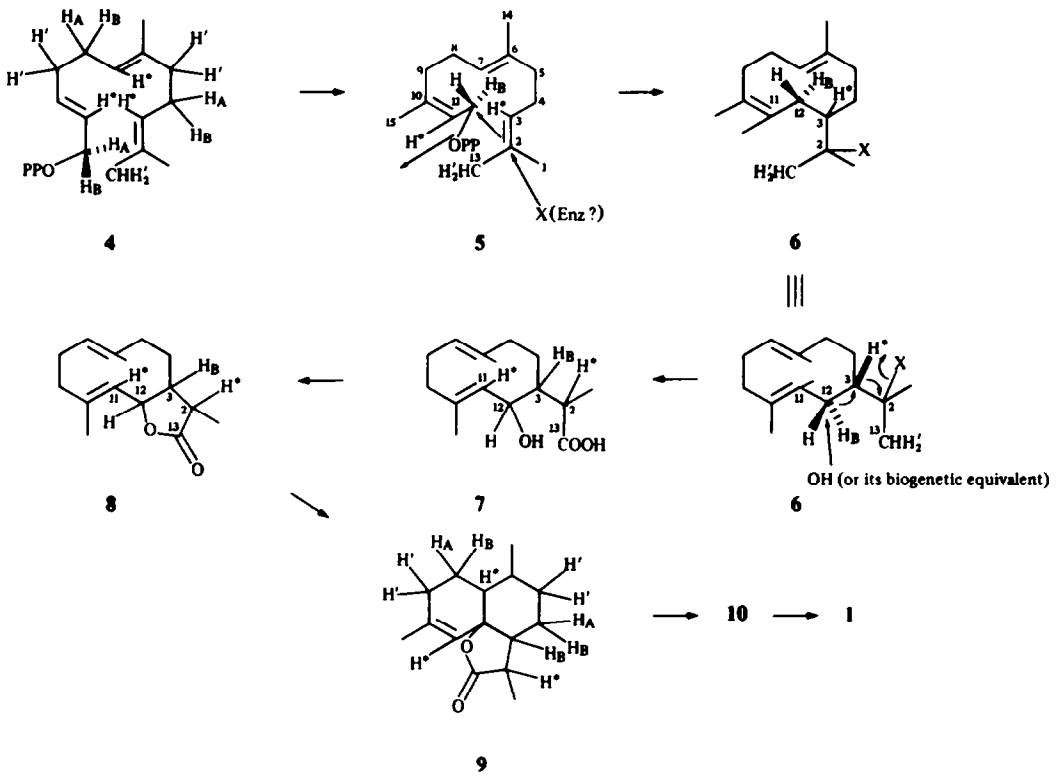
of **11** as an intermediate between arteannuin B (**10**) and artemisinin (**1**) because all the hydrogens are lost from C-11 in **11**. The isotope ratio ($^3H:^{14}C = 1:3$) in **18** suggests that either the ^3H at C-7 or C-3 was lost during the biosynthesis of **1**. Theoretically ^3H at C-7 in **5** should not be lost. However, the ^3H at C-3 may migrate to C-2 (Scheme 2, **6** → **7** → **8**- - - → **9**) and be eliminated during the formation of the exomethylene group in arteannuin B (**10**). This also indicates that **10** is the precursor of **1**. If compound **1** was formed directly (**8**- - → **9**- - → **1**), it would have retained all the ^3H atoms from [4*R*-$^3H_1$]MVA. This isotope ratio ($^3H:^{14}C = 2:3$) for **1** also eliminates the possibility of the biosynthetic sequence **5** → **12** → **13** → **14** → **15** → **10** → **1** (Scheme 1) because in this case the ^3H at C-3 will not shift to C-2 and hence it should be retained until the final steps of biosynthesis.

The results from [5-$^3H_2$,2-^{14}C]MVA feeding experiments (Table 1, experiment 3) indicated that only one ^3H out of six was lost. This is an interesting observation. This ^3H (H_A) is expected to be lost and exchanged with H during the redox *trans–cis* interconversion of **4** to **5** [12, 13] by analogy to the geraniol ↔ nerol interconversion in higher plants [14]. Although various redox schemes for double bond isomerization have received experimental support from a variety of sources, several examples of isomerization without compulsory loss of hydrogen in the biosynthesis of sesquiterpenes have been reported [15–18]. In the present case it is not certain that

Table 1. Isotope ratios ($^3H:^{14}C$) in artemisinin and arteannuin B isolated from *A. annua* after being fed with doubly labelled mevalonate

Experiment	Precursor	Isotope ratio in the precursor ($^3H:^{14}C$)	Expected carbon atom to be labelled with ^3H in FPP, artemisinin and arteannuin B*	Isotope ratio ($^3H:^{14}C$) Artemisinin	Arteannuin B
1	[2-$^3H_2$,2-^{14}C]MVA	1:1	C-5, C-9, C-13	2:3	2:3
2	[4*R*-$^3H_1$,2-^{14}C]MVA	1:1	C-3, C-7, C-11	2:3	2:3
3	[5-$^3H_2$,2-^{14}C]MVA	1:1	C-4, C-8, C-12	0.82:1	0.82:1

*For numbering see Schemes 1 and 2.



4 5 6 8 7 6 9

Scheme 2. Suggested mechanism for the cyclization of farnesyl pyrophosphate and its further oxidation and lactonization to arteannuin B and artemisinin. H* is derived from [4*R*-$^3H_1$]MVA; H_A and H_B are derived from [5-$^3H_2$]MVA and H' from [2-$^3H_2$]MVA. It has been shown that H_A is lost during *trans–cis* isomerization of farnesyl pyrophosphate. Only those hydrogens are shown which are actively involved in migrations and only those carbons are numbered which are involved in this discussion.

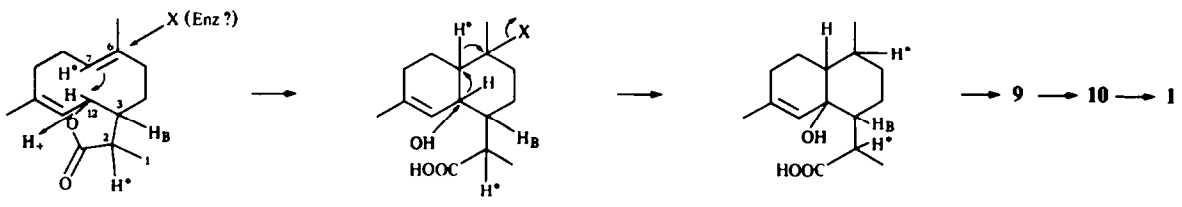
^3H (H_A) has lost during isomerization; it could well have been lost during cyclization and formation of **9** from **8** (Scheme 2). However, ^3H (H_B) at C-12 in **4** is retained in **5** and **6** and then migrates from C-12 to C-3 with a concomitant migration of the H at C-3 to C-2. However, it can be postulated that instead of ^3H (H_B) the normal isotope H may have migrated to C-3 leaving the ^3H (H_B) at C-12. In this case an X-group mechanism is hypothesized for the formation of **9** from **8** (Scheme 3). The H or ^3H at C-12 in **7** may shift to C-7 and the H at C-7 moves to C-6 releasing the enzyme (X). However, it has not been proved yet which of the protons (H_R or H_S) is shifted to C-3.

At present our results suggest that the biosynthetic route to artemisinin (**1**) proceeds as follows: **2** + **3** → **4** → **5** → **6** → **7** → **8** → **9** → **10** → **1** (Scheme 2).

EXPERIMENTAL

Materials. Specimens of *A. annua* were grown in the experimental farms of CIMAP, Lucknow, India. [2-^{14}C]-, [4*R*-$^3H_1$]-, [4*S*-$^3H_1$]-, [2-$^3H_2$]- and [5-$^3H_2$]MVA were purchased from the Radiochemical Centre, Amersham, U.K. and Bhabha Atomic Research Centre, Bombay, India.

Feeding methods and isolation of products. Labelled MVA (K salt, 5 μCi/g of plant material, sp. act. [2-$^3H_2$]MVA 1.28 Ci/mmole; [2-^{14}C]MVA 53 mCi/mmole; [4*R*, or 4*S*-4-$^3H_1$]MVA 1.36 Ci/mmole; [5-$^3H_2$]MVA 2.52 Ci/mmole) was fed to cut stems of *A. annua* (3-month-old specimens) over 1 hr, and the foliage was then maintained on nutrient medium for 36–48 hr before collection [3, 19]. Twigs of *A. annua* that were fed with radiolabelled precursor were extracted with boiling petrol (40–60°) for 48 hr. The solvent was removed *in vacuo* and



8 16 17

Scheme 3. An X-group mechanism for the formation of cadinanolide (**9**) from dihydrocostunolide (**8**).

the brown syrup thus obtained was dissolved in $CHCl_3$ (10 ml) and to this was added CH_3CN (100 ml). The insoluble material was filtered and the filtrate evapd under red. pres. Carrier artemisinin (**1**) and arteannuin B (**10**) (25–50 mg) were added at this stage to the residue which was chromatographed on a silica gel column using EtOAc–$CHCl_3$ (7.5:100) as eluent. The fractions containing **1** and **10** were subjected to PLC (silica gel plates, 7.5% EtOAc in $CHCl_3$). Compounds **1** and **10** (bands at R_f 0.66 and 0.48, respectively) were eluted and crystallized from cyclohexane. Radiochromatographic scanning of a variety of TLC separations showed that the products were at least 99% radiochemically pure. ^{1}H NMR, IR and mass spectra of the products were consistent with the data available in the literature [4, 7].

Degradation of **1** *to* **18**. To a soln of **1** (2 g) in MeOH (40 ml) was added with stirring 10% K_2CO_3 soln (20 ml). After 1 hr, H_2O (50 ml) was added and the whole mixture extracted with Et_2O. The aq. layer was acidified with dil. HCl to pH 2 and extracted exhaustively with Et_2O. The Et_2O extracts were washed with saturated NaCl soln and dried (Na_2SO_4). Evapn gave an oily residue which was taken up with some MeOH and refrigerated for several days to give white crystals. TLC of the mother liquor on silica gel ($CHCl_3$–MeOH; 4:0.3, I_2 vapour detection) showed three major spots at R_f 0.8, 0.6 and 0.3, along with a number of minor ones. Preparative TLC was performed on silica GF with $CHCl_3$–MeOH (100:5) the component of R_f 0.8 was eluted first. Gradient elution was then used and $CHCl_3$–MeOH (100:20) gave a few fractions which contained the pure compound of R_f 0.3 and yielded 26 mg of oil **18**.

Radiochemical methods. These have been described previously [14, 20]. The samples for assay by liquid scintillation spectrometry contained 2000–3000 dpm as ^{14}C and up to 20000 dpm as ^{3}H. 40 000 disintegrations were accumulated to ensure that 2σ was $\pm 1\%$. Radioactive compounds were purified by recrystallization (of appropriate solid derivatives, if necessary) to constant sp. radioactivity. All experiments were carried out in duplicate.

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