Notes

Enzymatic Synthesis of Artemisinin from Natural and Synthetic Precursors

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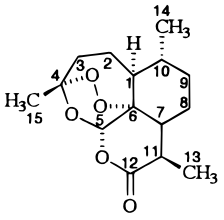
To investigate the biosynthetic pathway of artemisinin, an assay system for the determination of activity of the enzymes involved in its synthesis has been developed. Results from these experiments have shown that HEPES provides a better buffer system than Tris−HCl. The enzyme(s) requires Mg^{2+} and/or Mn^{2+}, and the addition of ATP and NADPH+H^+ significantly enhances the enzyme activity. A new substrate, dihydroarteannuin B, has been synthesized that can easily be radiolabeled with high specific activity. It is utilized by the enzyme system and is converted to artemisinin with the same efficiency as the natural substrates. This can be conveniently used as a precursor for elucidation of the pathway for artemisinin biosynthesis.

Malaria continues to be a major health concern in tropical countries. Various chemotherapeutic agents currently in use have become ineffective, as *Plasmodium falciparum* has developed resistance to these drugs. It is, therefore, quite imperative to look for new antimalarial drugs. Artemisinin (Qinghaosu) (**1**), a unique sesquiterpene lactone with an endoperoxide bridge, obtained from the above-ground parts of *Artemisia annua* L. (Asteraceae), has been found to be active against both, the chloroquine-resistant as well chloroquine-sensitive strains of *Plasmodium falciparum* and also against cerebral malaria.[1−5]

Currently, the primary method of obtaining artemisinin is by extraction of intact plants. However, endogenous bioproduction of artemisinin is relatively low (ranging from 0.01 to 0.5%), thereby limiting its clinical utilization.[6] To enhance its bioproduction, it is pertinent to understand the complete biosynthetic pathway, which is only partially known as yet.[7] As reported by Nair et al.,[8] arteannuin B is one of the immediate precursors of artemisinin. There have been several reports suggesting that artemisinic acid is converted to arteannuin B and to artemisinin.[9,10] However, none of these reports provide direct proof whether arteannuin B is the precursor of artemisinin or not. To study the biosynthetic pathway of artemisinin, we have chemically synthesized dihydroarteannuin B by the reduction of arteannuin B and have used it as an alternate precursor in our assay system. In this report, we present data to show that dihydroarteannuin B is enzymatically converted to artemisinin and can be used as a convenient precursor for the biosynthesis of artemisinin in an in vitro system.

A number of different buffers have been reported for the preparation of leaf homogenate. We used a number of these systems for standardization of the enzyme assay. The results with two systems, namely HEPES (buffer 1) and Tris−HCl (buffer 2), which were most effective, have been shown. It has been found that buffer 2 (see the Experimental Section) is a better system as far as the rate of conversion of arteannuin B to artemisinin is concerned. However, the preference for substrates is different between the two buffers. The substrate specficity of the enzyme in buffer 1 was dihydroarteannuin B > artemisinic acid > arteannuin B, whereas in buffer 2 it was on the order of artemisinic acid > arteannuin B > dihydroarteannuin B (Table 1). This is probably because Tris−HCl is an ionic buffer that might affect the coupling of enzyme and substrate. On the other hand, HEPES has better buffering capacity and is less prone to variation in pH with the change in temperature. The overall rate of



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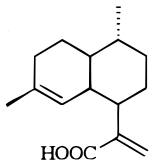

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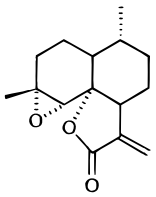

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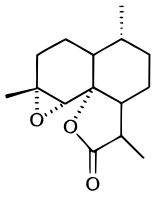

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Table 1. Effect of Various Buffers and Substrates on in Vitro Biosynthesis of Artemisinin

substrate	artemisinic acid	arteannuin B	dihydroarteannuin B
Buffer	Artemisinin biosynthesized (μmol/mg of protein)		
Tris	0.003	0.002	0.010
HEPES	0.017	0.014	0.011

[a] Leaves of *A. annua* (80−100 days old) were homogenized in buffer 1 or 2 (see Experimental Section for details) and incubated with various substrates for 150 min. The amount of artemisinin produced was estimated as described in the text. The control values representing endogenous levels were deducted, and the amount of artemisinin produced has been expressed as μmol/mg protein.

conversion for all three substrates was higher in HEPES buffer than in Tris−HCl buffer. For conversion of dihydroarteannuin B to artemisinin, both buffer 1 and buffer 2 were equally effective.

The time course studies show that conversion of artemisinic acid or arteannuin B to artemisinin is at a maximum after 150 min of incubation (Figure 1). All the enzyme assays were therefore, carried out for 150 min at 30 °C. The decline in the level of artemisinin at longer incubations may probably be due to its chemical instability in the aqueous environment of the cell free system. This is in concordance with the findings of Kudakessril et al.,[11] who reported that artemisinin is relatively unstable in aqueous environment. The possibility that upon longer incubations the artemisinin is getting converted to its metabolites, however, cannot be ruled out.

The highest enzyme activity was found during the full vegetative growth, i.e., 80−100 days after sowing (data not shown). All the analyses reported here were carried out by harvesting the leaf material from the plants of this age. In initial experiments, no cofactors were added, and the rate of conversion was found to be very low. A number of cofactors in different combinations and in different concentrations were added, and their effect on the conversion of various precursors to artemisinin was observed. As shown in Table 2, it was found that a combination of ATP (0.1 mM), $NADPH+H^+$ (0.1 mM), $MgSO_4$ (1 mM) and $MnSO_4$ (1 mM) added together resulted in the maximum conversion. It is well established that divalent cations ions such as Mg^{2+} and Mn^{2+} are essential for the terpene biosynthesis.[12] We have, therefore, included both Mg^{2+} and Mn^{2+} in our assay system.

The HPLC analysis does not show the presence of any other intermediate compound during the conversion of arteannuin B to artemisinin, suggesting that arteannuin B is the immediate precursor of artemisinin, which is in agreement with Nair et al.[8] It has been reported previously as well as detected by us through HPLC that the relative yield of arteannuin B is high in plants. Arteannuin B can serve as a suitable precursor for in vitro bioproduction of artemisinin using an immobilized enzyme system.

To study the biosynthetic pathway of artemisinin the radiolabeled precursors are essential, so that their conversion can be followed conveniently. It is tedious to synthesize labeled precursors by chemical means, and their biosynthesis in vivo results in poor incorporation of radioactivity leading to very low specific activity. We used a novel approach to synthesize various radiolabeled derivatives of arteannuin B that could act as suitable precursors for the biosynthetic pathway studies. We have found that the reduction of arteannuin B at C-13 with tritiated sodium borohydrate ($NaBT_4$) formed its tritiated dihydro derivative with high specific activity. The compound maintains the structural integrity of arteannuin B and is used by the enzyme(s) as substrate for its efficient conversion to artemisinin. Radiolabeled dihydroarteannuin B (3.2×10^5 cpm, specific activity -1.29×10^9cpm/mmol) was used as the substrate, and its conversion to artemisinin was followed. The artemisinin purified through preparative TLC gave 1.0×10^4 cpm/mg of protein. The authenticity of the radiolabeled artemisinin obtained was established by cocrystallizing it with cold artemisinin as carrier. Though a number of phytochemical studies have been done on *A. annua*, dihydroarteannuin B has not been isolated as a naturally occurring chemical constituent of the plants. It suggests that probably dihydroarteannuin B does not exist in vivo. Alternatively, it might be getting converted to artemisinin very fast.

As shown in Table 1, the rate of conversion of dihydroarteannuin B to artemisinin is high. It can therefore, serve as an alternate substrate for assaying the enzyme activity in an in vitro system. It can also serve as a convenient radiolabeled precursor for study of the metabolic pathway of artemisinin and its breakdown to the products such as artemisitene.

To purify the enzyme(s), the leaf homogenate was separated into different fractions by ammonium sulfate precipitation, and each fraction was assayed for enzyme activity. The majority of the proteins were precipitated in the 0−25% fraction, which included most of the cellular proteins and the pigments. The highest enzyme activity, however, was obtained in the 75−100% $(NH_4)_2SO_4$ fraction. This fraction had 3.1% of the total leaf proteins but ~25% of the total enzyme activity. There was, thus, an 8-fold enrichment of the enzyme activity (Table 3). The SDS−PAGE of the 75−100% fraction showed two prominent bands at molecular range of 12.5 and 14 kD (data not shown). Experiments are in progress to further purify these enzymes.

Experimental Section

General Experimental Procedures. Analytical HPLC was performed on a Perkin-Elmer instument using a C18 column (4 i.d. × 125 mm) (mobile phase, 100 mM PO_4 buffer/MeOH 60:40) and UV detection at 260 nm. Electrospray mass spectra were obtained using a VG platform (Fisons Instruments, UK quadrapole mass spectrometer equipped with Masslynx data system, Dynolite detector system and pneumatic nebulizer-assisted electrospray LC/MS interface). The molecular ions were detected as total ion current in ES^+ mode. The ^1H, ^{13}C, and 2D-NMR spectra were obtained with a Bruker-DRX (300 MHz) spectrometer operating at 300 MHz for ^1H and 75 MHz for ^{13}C, respectively; chemical shifts are reported on (scale and coupling constants are in Hz). HMQC (J_{CH} = 145 Hz) and HMBC ($^HJ_{CH}$-optimized for 8 Hz) experiments were performed using standard Bruker UXNMR pulse sequences.

Plant Material. *A. annua* L. plants were raised from the seeds obtained from Walter Reed Army Institute of Research, Washington, D.C. The plants were main-

Table 2. Effect of Various Cofactors on in Vitro Biosynthesis of Artemisinin[a]

cofactors	Mg^{2+} (1 mM)	Mn^{2+} (1 mM)	ATP (0.1 mM)	$NADPH^{+}$ (0.1 mM)	FAD (0.1 mM)	Fe^{2+} (1 mM)	α-ketoglutarate (2.5 mM)	artemisinin biosynthesized (μmol/mg protein)
1	−	−	−	−	−	−	−	0.003
2	+	+	+	−	+	+	−	0.012
3	+	+	+	−	−	−	+	0.012
4	+	+	+	−	−	+	−	0.009
5	+	+	+	+	−	+	+	0.020
6	+	+	+	+	−	−	−	0.026

[a] The leaf material was homogenized in buffer 2 and incubated with artemisinic acid for 150 min in the presence of various cofactors. Optimum concentration of the each cofactor was used. The amount of artemisinin was estimated as described in the text.

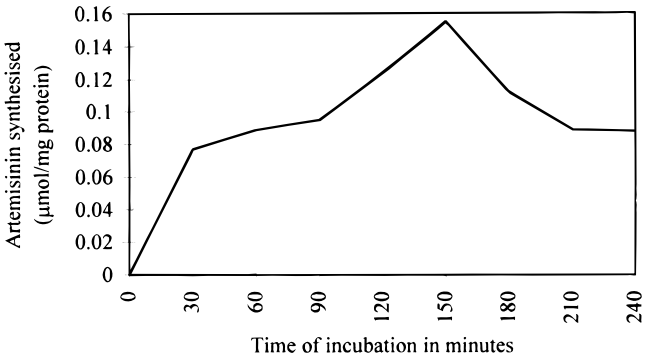

Figure 1. Time kinetics for enzymatic conversion of artemisinic acid to artemisinin. Leaf material (80−100 days old) was homogenized in HEPES-β-mercaptoethanol buffer and incubated with 100 μg artemisinic acid for various time intervals along with the required cofactors. Artemisinin synthesized was quantitated by HPLC. See text for details.

Table 3. Distribution of the Enzyme Activity and Proteins in Various Ammonium Sulfate-Precipitated Fractions (Enzyme Unit: 1 μg/min of Artemisinin Produced)[a]

fraction (%)	protein (mg/mL)	enzyme activity (unit/mL)	specific activity
crude homogenate	1.310	0.0270	0.0207
0−25	1.000	0.0002	0.0002
25−50	0.205	0.0002	0.0010
50−75	0.065	0.0004	0.0059
75−100	0.040	0.0064	0.1620

[a] Leaf homogenate was fractionated by precipitation with various concentrations of ammonium sulfate and desalted, and the related enzyme activity and protein content were measured.

tained in the herbal garden of Jamia Hamdard following the standard agronomic practices. The leaf material for enzyme assay was harvested at preflowering stages of plant growth starting from the age of 80−100 days.

Enzyme Assay. A cell-free system obtained from the leaf homogenate was used as enzyme source. To prepare the cell-free system, two different homogenizing buffers were used: (1) Tris−HCl (50 mM) containing EDTA (3 mM), ascorbic acid (5 mM), DTT (2 mM), sucrose (300 mM), PVP (0.02 g/mL); final pH 7.5; (2) HEPES (50 mM) containing β-mercaptoethanol (2 mM), final pH 7.2. Young leaves were washed with deionized water and ground with sand in a prechilled mortar and pestle with 2 volumes of chilled buffer. The ratio of biological material to buffer was kept as 1:2. The homogenate was centrifuged at 20000*g* for 15 min, and the resulting supernatant was used as the enzyme source.

Substrate. Artemisinic acid (**2**), arteannuin B (**3**), and dihydroarteannuin B (**4**) were used as substrates for the enzyme assay. Artemisinic acid was dissolved in 10% Triton X 100, and arteannuin B and dihydroarteannuin B were dissolved in 10% DMSO. The concentration of all the stock solutions was 1 mg/mL.

Incubation. Leaf homogenate (equivalent to 1 g fresh leaf material) was incubated with 100 μL of substrate stock (100 μg, and the following cofactors were added: 0.1 mM ATP + 0.1 mM NADPH + H^+ + 1 mM $MgSO_4$ + 1 mM $MnSO_4$. Controls received all the constituents except the substrate. The controls thus show an endogenous level of artemisinin. These values were deducted from the values obtained that have been expressed as μmol artemisinin produced per mg of protein. The reaction vials were stoppered and incubated at 30 °C for 150 min in a shaking water bath.

Extraction and Detection of End Products. After incubation, the reaction mixture was extracted with diethyl ether (3×) and dried over Na_2SO_4. Artemisinin was detected on TLC plates by cochromatography with standard artemisinin using benzene/EtOAc (9:1) as the solvent system. Artemisinin gave a characteristic yellow spot when the plates were sprayed with anisaldehyde reagent.

Quantitation. The synthesized artemisinin was quantitated using the method of Zhao et al.[13] with certain modifications. Radiolabeled artemisinin was purified through preparative TLC and was monitored in a liquid scintillation counter (Pharmacia LKB).

Synthesis of Dihydroarteannuin B. Dihydroarteannuin B was synthesized by the reduction of arteannuin B at the C-13 position using the catalyst-based reduction protocol described for other compounds by Zhou.[14] For this, arteannuin B (0.04 mmol) was dissolved in 10 mL of MeOH and cooled to −15 °C, and $NiCl_2 \cdot 6H_2O$ (0.42 mmol) was added. It was followed by addition of $NaBH_4$ (3.9 mmol), added slowly with constant stirring). The reaction mixture was incubated for 30 min in an ice bath with constant stirring. Thereafter, the reaction was stopped by diluting it with 10 mL of H_2O and its acidification to pH 6.0 with 3 N HCl. It was later extracted with EtOAc, the solvent was evaporated, and the residue was crystallized with EtOAc and hexane. The complete conversion of arteannuin B to its dihydro derivative was determined by the analysis on TLC (40% EtOAc in hexane was used as the solvent system), followed by spraying with vanillin/H_2SO_4 reagent. Arteannuin B gave a blue color while dihydroarteannuin B gave a turquoise blue color (R_f 0.8). The reduction was further confirmed by MS and NMR studies. Dihydroarteannuin B was obtained as crystals (EtOH and hexane): mp 168−170 °C; ^1H NMR ($CDCl_3$, 300 MHz) (2D-COSY and HMQC) assignments δ 2.82 (1H, s, H-5), 2.64 (1H, m, J = 7.5 Hz, H-11), 1.79 (2H, m, H-3), 1.69 (2H, m, H-2), 1.80 (2H, m, H-9), 1.78 (2H, m, H-7),1.34 (3H, s, Me-15), 1.37 (1H,

m, H-10), 1.32 (1H, m, H-1), 0.94 (3H, d, J = 7.5 Hz, Me-14), 1.20 (3H, d, J = 7.5 Hz, Me = 13). ^{13}C NMR ($CDCl_3$); assignment from HMQC experiment δ 56.47 (C-5), 53.02 (C-7), 42.96 (C-1), 36.06 (C-11), 33.19 (C-9), 33.19 (C-10), 20.83 (C-3), 21.69 (C-15),16.52 (C-14), 14.51 (C-2), 11.06 (C-13), 22.84 (C-8). Electrospray MS (CH_3CN/H_2O 50:50) m/z M + H^+ 251.0 (43.72), M + H_2O 268.0 (100), M + Na^+ 273.0 (48.91), M + K^+ 289.0 (5.19). Similarly, ^{3}H-labeled dihydroarteannuin B (specific activity 1.29 × 10^9 cpm/mmol) was synthesized when $NaBT_4$ was used in place of $NaBH_4$.

Partial Purification of Enzymes. Efforts were made to partially purify the enzymes. In the first set of experiments, 5 g of leaf material was homogenized in HEPES−β-mercaptoethanol buffer, and then the proteins were precipitated using ammonium sulfate. A total of five fractions of 0−25%, 25−50%, 50−75%, and 75−100% $(NH_4)_2SO_4$ saturation and the final supernatant were obtained and then desalted through a Sephadex G −25 column. The protein concentration of each desalted fraction was estimated by using the Bradford method[15] using bovine serum albumin as the standard. The enzyme assay was carried out using 1 mL of each of the fraction with dihydroarteannuin B as the substrate.

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