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Biosynthesis of artemisinin from 11,12-dihydroarteannuic acid

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Abstract : To investigate the biosynthetic pathway leading to artemisinin, the putative precursors, arteannuic acid and 11,12-dihydroarteannuic acid, were incubated in a cell-free system. For the experiment with dihydroarteannuic acid, artemisinin was generated in tumor homogenate. These results showed that dihydroarteannuic acid could be converted to artemisinin enzymatically. However, the experimental condition failed to convert arteannuic acid into 11,12-dihydroarteannuic acid(Received February 20, 1992, accepted March 31, 1992).

The possibility that artemisinin{**1**}, an antimalarial principle isolated from *Artemisia annua* and arteannuin B{**2**}, was produced independently from arteannuic acid{**3**} has been suggested.[1,2] Another possibility that arteannuin B and artemisinin{**1**} are generated sequentially from arteannuic acid *in vivo* has also been reported.[3-5] Artemisinin was demonstrated to be generated spontaneously from the organic solvent-dissolved 11,12-dihydroarteannuic acid{**4**} through photochemical reaction without any apparent photosensitizer(unpublished result in this lab).

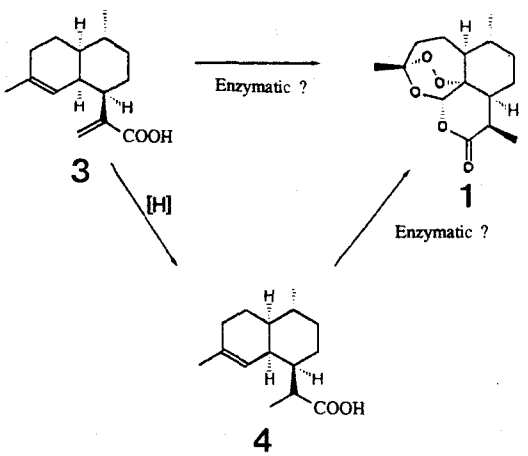
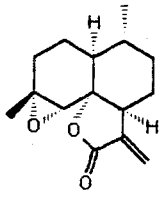


Fig. 1. Proposed biosynthetic pathway of artemisinin {**1**}.

The enzyme system for the reduction of arteannuic acid into the dihydro acid *in vivo* should exist in the plant since the dihydro acid as well as arteannuic acid are the plant constituents. Artemisinin could be then generated spontaneously *in vivo* via nonenzymatic reaction(Fig. 1). However, possibility still remains that the enzymic machinery for the conversion of (dihydro)arteannuic acid into artemisinin{**1**} exists in the plant along with the nonenzymatic mechanism. These reasonings prompted us to study the biosynthesis of artemisinin{**1**} from its putative immediate precursors, arteanuic acid{**3**} and 11,12-dihydroarteannuic acid{**4**}.



2

Key words : Biosynthesis, *Artemisia annua* L., artemisinin, arteannuic acid, 11,12-dihydroarteannuic acid
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Materials and Methods

Synthesis of 11,12-dihydroarteannuic acid{4}[5]

Arteannuic acid(100 mg), isolated from *Artemisia annua* through the procedure described by Kim and Lim,[6] and $NiCl_2 \cdot 6H_2O$(0.49 eq, 51.4 mg) were dissolved in 5 ml of MeOH and chilled to −15 ℃. $NaBH_4$ (5.3 eq, 88.4 mg) was added to the mixture in small portions. The solution was stirred for 3 hours at room temperature and was then acidified with 10% of HCl till the black precipitate dissolved. This was neutralized with 5% Na_2CO_3 and extracted with chloroform. After washing with brine, the extract was dried with $MgSO_4$ and evaporated. The product was stored *in vacuo*.

Cell-free incubation

The cell-free extracts of various tissues were prepared by the method of Kudakasseril *et al.*[7] with modification as follows. The fresh leaves, calli, or tumors[8] of *A. annua* were homogenized with the borate-phosphate buffer(0.05 M, pH 7.5) containing 5 mM of ascorbic acid, 2 mM of dithiothreitol, 2% of polyvinylpyrrolidone, and 0.2 M of sucrose in a mortar. The homogenate was mixed with XAD-4 (two times the weight of the plant material) and 2.5g of polyvinylpolypyrrolidone, and stirred for 5 minutes in a refrigerator. The solution was then centrifuged at 10,000 rpm for 10 min in a JA-20 rotor(Beckman) at 4 ℃. The supernatant was incubated with arteannuic acid or dihydroarteannuic acid for 24 hours at 30 ℃. In the arteannuic acid incubation, NADPH regeneration system(5 μM of NADPH, 5 μM of glucose-6-phosphate, and 0.02 units of glucose-6-phosphate dehydrogenase) was included.

Table 1. Formation of artemisinin in various combination of incubation mixtures of *Artemisia annua* tissues

Tissue	NADPH and {3} Added	{4} Added	{4} Detected	{1} Detected
Normal Calli	Yes	−	No	No
	−	Yes	−	No
Tumor	Yes	−	No	No
	−	Yes	−	Yes

For the detailed experimental condition, see the Material and Method section. Formation of artemisinin{1} and 11,12-dihydroarteannuic acid{4} was verified against boiled cell-free system and a blank experiments.
− : Not applicable

Analysis of the incubation produts

Detection of artemisinin{1} was done with HPLC after derivatizing it into Q260 as described in the literature.[9] Artemisinin sample was prepared as follows : The reaction mixture was made basic by the addition of 1 N NaOH and extracted with ethyl acetate. The extract was concentrated and used in the artemisinin{1} detection. The remaining basic aqueous layer was acidified with 1 N HCl and again extracted with ethyl acetate. The second extract was dried under the stream of N_2, treated with ethereal diazomethane, and was subsequently analyzed for the dihydro acid with GC/MS.

Chromatographic conditions

HPLC and GC/MS conditions were described in the accompanying paper in this issue.[9]

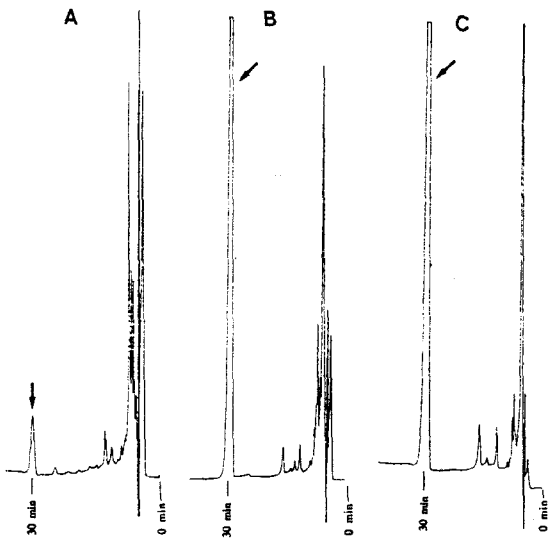

Fig. 2. HPLC analysis of artemisinin{1} prepared from (A) crown gall, (B) authentic Q260 plus sampleA, (C) authentic artemisinin{1}, 80 μg.
For analysis conditions, see the text.

Results and Discussion

In the cell-free experiment of the dihydroarteannuic acid{**4**}, the acid was prepared immediately before use as it was found to be considerably labile under light(unpublished result from this lab). XAD-4 was added to the plant homogenate to remove the endogeneous terpenes,[10] and indeed the resin was found very effective in removing the endogeneous terpenes : HPLC and GC/MS analysis indicated no detectable residual terpenes of interest.

Among various cell-free experiments, artemisinin {**1**} was found in the tumor extract incubation with the dihydroateannuic acid(Fig. 2). In contrast, the incubation with the boiled homogenate system did not generate artemisinin{**1**}. In all other incubations with the plant leaves or normal calli, artemisinin{**1**} was not found(Table 1). This signified that the enzymatic system which converts the dihydroateannuic acid into artemisinin{**1**} exists in the plant, though the activity had been detected only with the transformed calli.

Since normal plant leaves[1] and tissue cultures[8] contain the dihydro acid, a reductase responsible for the generation of the dihydro acid{**4**} from arteannuic acid{**3**} must be present in these tissues. However, the cell-free system failed to convert arteannuic acid into 11,12-dihydroarteannuic acid{**4**}. The whole cell-free system contained NADPH, the usual reductant of the terpenoid redutase system such as (+)-pulegone reductase,[12] which catalyzes $\Delta^{8,9}$ reduction of (+)-pulegone. The instability of the reductase or the insufficient detection sensitivity could have attributed to the result. Efforts to detect the reductase activity is ongoing in this lab.

Acknowledgement

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11,12-Dihydroarteannuic acid에서 artemisinin의 생합성

김남철 · 김수언(서울대학교 농화학과 및 농업생물신소재연구센터)

초록 : 국화과의 일종인 개똥쑥(*Artemisia annua* L., 青蒿)에는 항말라리아 물질인 artemisinin이 함유되어 있다. 11,12-Dihydroarteanuic acid는 cell-free 방법으로 수행한 실험에서, *Agrobacterium tumefaciens*로 형질 전환된 crown gall의 homogenate와의 incubation에서 artemisinin으로 전환되었다. 그러나 arteannuic acid는 NADPH와 함께 incubation하였을 때 dihydro acid로 전환되지 않았다. 따라서 이 결과는 식물체의 조건에 따라서, dihydro acid가 자발적으로 뿐만 아니라, 생체내에서 효소 반응에 의해서도 artemisinin으로 전환될 수 있음을 나타낸다.