Isolation and Identification of Dihydroartemisinic Acid from *Artemisia annua* and Its Possible Role in the Biosynthesis of Artemisinin

T. Eelco Wallaart,* Wim van Uden, Heidi G. M. Lubberink, Herman J. Woerdenbag, Niesko Pras, and Wim J. Quax

Department of Pharmaceutical Biology, University Centre for Pharmacy, Groningen Institute for Drug Studies, University of Groningen, A. Deusinglaan 1, NL-9713 AV Groningen, The Netherlands

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Dihydroartemisinic acid (**2**) was isolated as a natural product from *Artemisia annua* in a 66% yield, and its structure was confirmed by ^{1}H and ^{13}C NMR spectroscopy. Compound **2** could be chemically converted to artemisinin (**4**) under conditions that may also be present in the living plant. The results suggest that the conversion of **2** into **4** in the living plant might be a nonenzymatic conversion.

In the tropical world human malaria is a widespread and commonly occurring infectious disease, mainly caused by *Plasmodium falciparum*. In large parts of South-East Asia, West Africa, and the Amazon area, resistance of this malaria parasite to the currently used antimalarial drugs is common, increasing, and life threatening.[1] New antimalarial drugs, also effective against these resistant parasites, are urgently needed. Artemisinin (**4**), a sesquiterpene lactone endoperoxide isolated from the aerial parts of the plant *Artemisia annua* L. (Asteraceae), and a series of semisynthetic derivatives have recently been developed as a new class of antimalarial drugs.[2] Total chemical synthesis of artemisinin is possible, but very complicated, resulting in very poor yields.[3,4] Therefore, the plant appears to be the only economic source for production purposes. The aerial parts of wild stands of *A. annua* contain between 0.01 and 0.5% (w/w) artemisinin.[5,6] Higher contents are desirable to make artemisinin available as a relatively cheap antimalarial drug[7] for the very often impoverished population of the countries where malaria occurs. Elucidation and knowledge of the biosynthetic pathway of artemisinin may enable us to influence its formation in a direct way, by metabolic engineering, for example. An inventory of the sesquiterpenes present in *A. annua* should yield possible precursors of artemisinin.

One of the most abundant sesquiterpenes in *A. annua* is artemisinic acid[8] (**1**), which was shown by labeling experiments[9] to be a precursor of **4**. Several hypothetical pathways for the biosynthesis of **4**, with **1** as an intermediate, were suggested only on the basis of the structural relationship of compounds isolated from *A. annua* or hypothetical intermediates.[10−12] The reactions in which **4** was obtained from **1**, and the intermediates are known, are through organic chemistry.[13−15] The chemical reaction described by Acton and Roth[15] is very straightforward, consisting of only three steps (Scheme 1).

Singlet oxygen, ($^1O_2$) and triplet oxygen, ($^3O_2$), which are the reactants for the chemical, nonenzymatic conversion of dihydroartemisinic acid (**2**) into **4** are known to be present in the living plant.[16] The aim of this study was to convert **2**, isolated from *A. annua*, into **4** by approaching the conditions that may also be present at the site in the plant where **4** is formed. We compared the chemical reaction products with the constituents of *A. annua*, which can eventually lead to the elucidation of a novel biosynthetic pathway for **4**.

Results and Discussion

Dihydroartemisinic acid (**2**) was isolated by an acid alkaline separation followed by column chromatography, from dried leaves of the Vietnamese *A. annua* strain, containing 0.17% **2** on a dry weight basis (DW) as one of the main components. The yield of the isolation was 66%. This finding contradicted the experiences of Haynes and Vonwiller,[17] who reported in 1991 that **2** was never found among the chemical constituents of *A. annua*, although Huang et al.[18] had already reported the isolation and identification of **2** from *A. annua* in 1987. An explanation for the absence of **2** in *A. annua* plant material may be the instability of **2**, leading to its rapid disappearance after harvest.[19] Another explanation may be the chemical differences between the Vietnamese *A. annua* variety used in this study and the varieties used in other studies. To make sure that **2** was not an artifact of the isolation, extracts with EtOH, toluene, and $CHCl_3$ of fresh leaf material were analyzed by HPLC, and the $CHCl_3$ extract was also analyzed by GC−MS. In all these HPLC analyses a peak was present, identical with respect to shape and retention time to **2**, obtained by isolation. GC−MS analyses of the $CHCl_3$ extract confirmed the presence of **2** in this extract.

The conversion of **2** into **4**, as done by Acton and Roth,[14] was performed in CH_2Cl_2, methylene blue-catalyzed at −78 °C, and irradiated with a Westinghouse Ceramalux high-intensity C400S51 electric discharge street lamp. These conditions will never occur in the living plant. In our approach, the conditions used to convert **2** into **4**, photooxidatively, mimicked the conditions that may occur at the site in the plant where **4** is biosynthesized and/or sequestered. Duke et al.[20] demonstrated the presence of **4** in biseriate glandular trichomes by extracting **4** from leaves by a 5-s dip in $CHCl_3$, without observable damage to other leaf epidermal cells, and its absence in extracts of a glandless biotype. The oil-containing glandular trichromes have been proven to be the sites where many terpenoids are biosynthesized.[21,22]

To mimic the conditions in the oil-producing trichomes, **2** was dissolved in mineral oil and exposed to light and air. The potential of photosensitizing reactions among secondary plant products is a widespread phenomenon.[16] Secondary plant substances of diverse biogenetic origin are capable of the photogeneration of $^1O_2$.[16] Because such

* To whom correspondence should be addressed. Tel.: +31 50 5280681 or +31 50 3633363. Fax: +31 50 3637570. E-mail: T.E.Wallaart@farm.RUG.NL.

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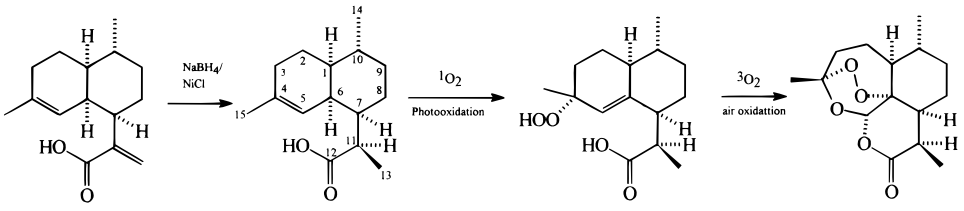
Scheme 1[a]



1 **2** **3** **4**

[a] Three-step partial synthesis of **4**, starting with **reduction** of the exocyclic double bond of **1**, yields a racemic mixture of dihydroartemisinic acid (**2**), followed by a **photooxidation** (singlet oxygen, $^1O_2$), which proceeds through a classical ene reaction with rearrangement of the endocyclic double bond, yields dihydroartemisinic acid hydroperoxide (**3**). Ring cleavage induced by **air oxidation** (triplet oxygen, $^3O_2$), and reformation then completes the ring structure with **4** as end-product.[15]

Table 1. Conversion (%) of **2** into **4** Catalyzed by Chlorophyll a in Mineral Oil, Light, and Air Exposed

nmol Chlorophyll a/μmol **2**	time (h)					
	0	8	24	48	72	120
0	0	0	0	0	0.7	0.8
0.049	0	0.6	2.4	4.8	5.4	12.0
0.12	0	0.8	3.8	6.2	6.9	6.9
0.24	0	0.9	6.7	8.0	8.2	12.0
0.49	0	1.5	8.1	8.8	8.8	6.4
1.2	0	3.3	10.0	9.8	13.0	9.3
2.4	0	5.1	10.2[a]	9.6	8.8	9.0
4.9	0	6.1	10.8	9.6	9.9	6.8

[a] No duplicate data available.

Table 2. Conversion (%) of **2** into **4** Catalyzed by Chlorophyll a, Light, and Air Exposed, in the Absence of Mineral Oil

nmol Chlorophyll a/μmol **2**	time (h)					
	0	8	24	48	72	120
0	0	0	0	0.9	1.5	1.2
0.049	0	1.2	7.3	15.9	10.0	12.1
0.12	0	2.0	10.5	15.0	17.5	12.4
0.24	0	3.6	12.5	16.5	19.7	26.8
0.49	0	6.5	11.6	15.6	14.1	N.D.[a]
1.2	0	9.3	15.8	12.9	15.3	16.0
2.4	0	9.2	11.5	11.8	12.3	11.4
4.9	0	8.9	10.2	9.5	10.6	10.0

[a] N.D., measurement not done.

secondary plant products may be present in the trichomes, a reaction with chlorophyll a, a photogenerator of $^1O_2$, was also carried out under the same conditions (Table 1). Chlorophyll a was used in this experiment because of its photosensitizing capacities and its stability and not because we expected chlorophyll a to be present in the trichomes.

Besides the cytoplasm and the endoplasmic reticulum, as the organelles in which sesquiterpenes are produced,[23] chloroplasts from young leaves were also found to be organelles in which various terpenoid end-products are synthesized.[24] Because high levels of **4** are also found in young *A. annua* leaves, biosynthesis of **4** in chloroplasts cannot be excluded. Excited chlorophyll and an oxygen-generating system is a potential source of $^1O_2$ production,[16,25] which is the essential molecule for the conversion of **2** into **4**. To mimic the $^1O_2$-producing conditions in the chloroplasts, chlorophyll a was added to **2** and exposed to light and air, in the absence of mineral oil, (Table 2). *A. annua* as used in our laboratory was grown under greenhouse conditions with an average temperature of 23 °C and a photo period of 16 h, 3600 lux. Under these conditions *A. annua* was growing very well and producing **4** at a content of approximately 0.4% (DW). The in vitro photooxidation reactions of **2** were also carried out under these conditions but permanently illuminated. Incubation of **2** dissolved in mineral oil yielded 0.79% **4** after 120 h of incubation (Table 1). Much higher yields were obtained when chlorophyll a was added. Because the optimal ratio between chlorophyll a and **2** was not known, a concentration series was made. A conversion of nearly 12% was obtained after 120 h incubation at the concentrations of 0.049 and 0.24 nmol chlorophyll a/μmol of **2**. This indicates that **4** can be produced under conditions that may also be present in the trichomes, even without addition of compounds with the capacity of photogeneration of $^1O_2$, such as chlorophyll a.

The conversion was, however, more efficient in the absence of mineral oil (Table 2). Taking into account that **2** dissolved in mineral oil causes a decrease of the surface (exposed to the air)-to-volume ratio, slower reactions can be expected. A conversion of 1.23% after 120 h of incubation was obtained by exposing **2** to light and air only. Nearly 27% conversion was obtained after 120 h of incubation at the optimal chlorophyll a concentration of 0.24 nmol chlorophyll a/μmol of **2** (Table 2). We expected this chlorophyll a-catalyzed reaction to be identical to the reaction as shown in Figure 1. In that case the intermediate dihydroartemisinic acid hydroperoxide (**3**) must be detectable in this chlorophyll a-catalyzed conversion of **2** into **4**. Using LC−MS we confirmed the presence of **3** in the reaction mixtures. Compound **3**, produced in a way analogous to the method as described by Roth and Acton,[14] was used as a reference compound. Therefore, it is very likely that the reaction in our experiments is identical to the reaction as described by Acton and Roth[15] (Scheme 1). Besides oxygen, higher plants are producing $^1O_2$;[16] the conditions for the conversion of **2** into **4** seem to be present in the living plant.[17]

In analogy to the chlorophyll a-catalyzed photooxidation of **2** yielding **4**, we tried to photooxidize the unsaturated equivalent of **2**, artemisinic acid (**1**). Artemisitene, the unsaturated equivalent of **4**, can then be expected as the oxidation product. No oxidation products of **1** were obtained under conditions, as described in this study, for the photooxidation of **2**.

The function of **2** in *A. annua* seems to be that of an antioxidant. Compound **2** is quenching $^1O_2$, resulting in the hydroperoxide **3**. It is known from literature that these peroxides are likely to be contributors to damage and dysfunction in cell and organelle membranes.[16,27] By the conversion of **3** into **4**, in the plant, this problem is solved. In this way **4** can be regarded as a compound in which reactive oxygen is stored as a stable product.

More evidence for a $^1O_2$-mediated biosynthetic pathway of **4** can be obtained if **3** can be detected in *A. annua*. Further research will concentrate on the isolation and identification of **3** from *A. annua* to further prove the biosynthetic pathway of **4**.

Experimental Section

General Experimental Procedures. The melting point was determined with an Electrothermo IA 9100. Optical rotations were measured on a Perkin−Elmer 241 polarimeter. UV spectra were obtained on a Pharmacia Ultrospec II spectrophotometer. IR spectra were recorded on a ATI Mattson Genesis Ref I using Win FIRST software. ^{1}H and ^{13}C NMR data were recorded using a Varian VXR 300 NMR apparatus (300 MHz). HSQC spectra were recorded using a Varian Unity 500 NMR apparatus (500 MHz). All spectra were recorded in $CDCl_3$ at room temperature. The chemical shifts are denoted in δ units (ppm) relative to $CDCl_3$, δ 7.26 ppm for ^{1}H and δ 76.91 for ^{13}C. Coupling constants are given in Hertz (Hz).

GC−MS (EI) was performed on a Unicam 610/Automass 150 system. The GC conditions were: column, DB-1 fused-silica (15 m × 0.25 mm i.d., film thickness 0.10 μm; J & W scientific, Folsom, CA); oven temperature program, 150−320 °C at 10 °C/min; injector temperature, 250 °C; carrier gas, helium; inlet pressure, 5 psi; linear gas velocity, 29 cm/s; split ratio, 1:20; injected volume, 1.0 μL. MS conditions: ionization energy, 70 eV; ion source temperature, 160 °C; interface temperature, 260 °C; scan speed, 1 scan/s; mass range, 34−500 u. HPLC was performed using an Isco HPLC pump 2350, an Isco V^4 absorbance detector (ISCO Inc., Lincoln, NE), a Kontron autosampler 360, and a Kontron PC Integration packet (Kontron Instruments SpA, Milan, Italy). The HPLC conditions for **2** were: analytical column, Lichrosorb 7 RP 18, 100 mm × 3 mm, cat. no. 28297, Chrompack (Middelburg, The Netherlands); mobile phase, $H_2O-H_3PO_4$ (0.1M)−CH_3CN = 49:1:50, (v/v); flow rate, 0.75 mL/min; wavelength, 210 nm; detector sensitivity, 0.1; injected volume, 10 μL; temperature, room temperature.

Liquid chromatography−mass spectrometry (LC−MS): the chromatographic equipment consisted of two Spectroflow 400 HPLC pumps (ABI Analytical Kratos Division, Ramsey, New Jersey), coupled to an ABI Analytical Kratos Division Spectroflow 450 solvent programmer (isocratically used) with a Rheodyne 7125 manual injector (20-μL loop) and a Spectroflow 757 UV absorbance detector (ABI Analytical Kratos Division). The detection wavelength was 210 nm and the column was a Lichrosorb 7 RP 18, 100 × 3 mm i.d., cat. no. 28297, Chrompack (Middelburg, The Netherlands). The mobile phase consisted of H_2O−HCOOH (0.5M)−CH_3CN = 55:1:44, (v/v). The HPLC column was connected to the ion spray interface by means of a pneumatically controlled flow splitter (JEOL application note MS49), providing a flow of approximately 5 μL/min to the mass spectrometer.

Mass spectra were obtained on a Nermag R 30−10 mass spectrometer (Delsi-Nermag, Argenteuil, France) equipped with a custom-built atmospheric pressure ionization (API) source. During the LC−MS operation the quadrupole was typically scanned from *m*/*z* 100 to *m*/*z* 800 at 2 s/scan. The multiplier was 700 V in negative mode. Nozzle voltage was 150 V to obtain the fragmentation. Nitrogen (99.9%) was used as a curtain gas (75−100 L/h). A voltage of −3 kV was applied to the ion spray interface.[26] Nitrogen (99.9%) was used as a nebulizing gas, and the pressure was 3 bar.

Plant Material. *Artemisia annua* L. seeds of Vietnamese origin were obtained from ARTECEF BV (Maarssen, The Netherlands). Taxonomically verified specimens are deposited at our institute and at the Institute of Materia Medica Hanoi (Vietnam). After a 4-week greenhouse period, the seedlings were allowed to grow freely in the open at an experimental field belonging to our research institute in Groningen (The Netherlands). The plants were harvested in October 1995, after a 6-month growing period.

Isolation Procedure for 2. Leaves of *A. annua* were dried through lyophilization and ground to powder. This leaf material (100 g) was extracted with 200 mL of 96% EtOH in an ultrasonic bath over 30 min. After suction filtration, the plant material was boiled in 200 mL of 96% EtOH for 10 min and filtered. The EtOH extracts were pooled. A mixture of 20 g of Norit and 30 g Celite was added, stirred for 15 min, filtered, and the filter cake washed with 50 mL of additional 96% EtOH. The light yellow solution was concentrated by rotary evaporation to 100 mL and diluted with 150 mL H_2O. The aqueous EtOH mixture was extracted with Et_2O (3 × 50 mL). The Et_2O solution was extracted with 5% $Na_2CO_{3\ (aq)}$ (2 × 40 mL). The H_2O phase was adjusted to pH 1 with HCl (fuming, 37%) and extracted with Et_2O (3 × 50 mL). After drying with $MgSO_4$ and filtration, the solvent was removed by rotary evaporation, resulting in a yellow oil. To separate the polyunsaturated fatty acids from **1** and **2** the yellow oil was chromatographed using petroleum ether (40−60)−Et_2O (6:4) as eluent on a (20 × 1.8 cm) column of Si gel 40 (#70240 J. T. Baker, Deventer, The Netherlands) impregnated with 5% $AgNO_3$. The chromatographic procedure was monitored by argentation-TLC (#105719 Merck Si gel 60 F_{254}, petroleum ether (40−60)−Et_2O, 1:1). Fractions exhibiting the first single, but tailing, spot on TLC were pooled and yielded 151 mg of a colorless oil. Compounds **1** and **2** were separated by dissolving the colorless oil in an equal volume of hexane and passing it through a (20 × 1.8 cm) Si gel 40 column (4:1 hexane−Et_2O). Compound **1**, >95% pure (GC), was present in the 10-mL fractions 12 to 17, while compound **2**, >95% pure (GC), was present in fractions 19 to 26. The fractions containing **1** and **2** were pooled separately, and the eluent was removed by rotary evaporation, resulting in a colorless oil for both compounds. Crystallization of these compounds occurred spontaneously within 6 h. The concentration of **2** in the same batch of dried *A. annua* leaves was determined by HPLC, by using a concentration series of **2**, and yielded a concentration of 0.17% (DW).

Identification of 2: dried *A. annua* leaves (100 g) yielded 112 mg of **2** as a white fluffy powder, >95% pure (GC) corresponding with 66% yield of isolation; mp 136−137 °C; $[\alpha]^{23}_D$ −10.6° (*c* 0.017, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 220 (2.58) nm, IR (KBr) ν_{max} 3220 (OH), 1731 (C=O), 1698 (C=C) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.12 (1H, s, H-5), 2.5 (1H, m, H-11), 2.5 (1H, m, H-6), 1.95 and 1.56 (2H, m, H-9), 1.92 and 1.80 (2H, m, H-8), 1.64 (3H, s, CH_3-15), 1.64 (1H, m, H-1), 1.60 and 0.96 (2H, m, H-2), 1.43 (1H, m, H-10), 1.37 (1H, m, H-7), 1.19 (3H, d, $^3J_{11,13}$ = 6.95, CH_3-13), 1.11 (1H, q of d, H-3), 0.87 (3H, d, $^3J_{10,14}$ = 6.23, CH_3-14) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 183.0 (s, COOH), 135.9 (s, C-4), 119.2 (d, C-5), 43.6 (d, C-1), 42.1 (d, C-6), 41.7 (d, C-7), 36.3 (d, C-11), 35.2 (t, C-2), 27.6 (d, C-10), 27.3 (t, C-3), 26.5 (t, C-8), 25.7 (t, C-9), 23.7 (d, CH_3-15), 19.6 (d, CH_3-14), 15.0 (d, CH_3-13) ppm; EIMS *m*/*z* (rel int.) 236 [M]$^+$ (2), 163 (28), 162 (100), 148 (3), 147 (20), 135 (2), 134 (2), 133 (4), 121 (8), 119 (5), 107 (14), 106 (6), 105 (12), 95 (9), 94 (7), 93 (17), 91 (20), 81 (16), 79 (20), 77 (12), 69 (3), 67 (7), 65 (4), 55 (20), 53 (7), 45 (4), 43 (7), 41 (22), 39 (8).

Identification of 2 in Fresh Plant Material. Immediately after harvesting, fresh leaves of *A. annua* (ca. 0.2 g) were thoroughly ground with 200 mg of fine granular quartz (Merck, Darmstadt, Germany) and 2 mL of an organic solvent in a mortar for 2 min. The extracts were separated from the plant pulp by centrifugation for 1 min in a microcentrifuge (13 000 rpm). The obtained extract was used for GC−MS and HPLC analyses. Retention time of **2** on GC−MS was approximately 1.5 min, and on HPLC, approximately 9 min.

Photooxidation of 2. To a concentration series of chlorophyll a (Fluka) 0.14, 0.35, 0.70, 1.4, 3.5, 7.0, and 14 nmol dissolved in $CHCL_3$ was added 2.86 μmol of **2**, also dissolved in a small volume of $CHCl_3$. Seven of these chlorophyll-a concentration series were made in 2.5-mL glass vials. After removal of $CHCl_3$ under a gentle stream of nitrogen, the samples were exposed to 3600 lux (Philips HPI-T 400W lamp) and to air at 23 °C. The same experiment was also performed with chlorophyll a and **2** dissolved in 10 μL of mineral oil (light white oil, Sigma). By adding 200 μL of MeOH, the reactions were stopped at t = 0, 8, 24, 48, 72, and 120 h. HPLC analyses of **4** were performed with the hydrolyzed product of **4** Q260 as described by Pras *et al.*[27] Compound **4** was identified in the reaction mixtures by GC−MS in comparison with a reference sample, kindly provided by ARTECEF BV (Maarssen, The Netherlands).

Identification of **3** in a 24-h light-exposed 0.24 nmol chlorophyll a/μmol of **2** reaction mixture was done by LC−MS analyses. The retention time of **3** was ca. 3 min. The most

important fragment ions of **3** obtained by LC−MS were at *m*/*z* (rel int.) 267 (100), 249 (14), 235 (42), 191 (24), and 166 (14), corresponding to $[M - H]^-$, $[M - H - H_2O]^-$, $[M - H - O_2]^-$, $[M - H - O_2 - CO_2]^-$, and probably $[M - H - O_2-(CH_2-CH-CH-CH_2) - CH_3]^-$.

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