Isolation and Identification of Dihydroartemisinic Acid Hydroperoxide from *Artemisia annua*: A Novel Biosynthetic Precursor of Artemisinin

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Dihydroartemisinic acid hydroperoxide (**2**) was isolated for the first time as a natural product from the plant *Artemisia annua* in a 29% yield. Its structure was identified by ^{1}H and ^{13}C NMR spectroscopy. Compound **2** is known as an intermediate of the photochemical oxidation of dihydroartemisinic acid (**1**) leading to artemisinin (**3**). The presence of **1** and **2** in the plant and the conditions under which **1** can be converted into **2**, which can very easily oxidize to **3**, provide evidence for a nonenzymatic, photochemical conversion of **1** into **3**, in vivo, in the plant.

More than 40% of the world's population is at risk of being infected with malaria.[1] This disease, caused by protozoa of the genus *Plasmodium* and spread by mosquitoes of the genus *Anopheles*, is responsible for over 100 million infections each year. More than 85% of all malaria infections are related to Malaria tropica caused by *Plasmodium falciparum*. Malaria tropica is also the most severe form of malaria, causing annually the death of more than 1 million persons, mainly African children (estimations of the W. H. O.). Traditionally, malaria is cured with antimalarial drugs such as the chloroquine derivatives. Unfortunately, most *P. falciparum* strains have become resistant to these traditionally used drugs.[2]

A new class of antimalarial drugs that are also effective against these multidrug-resistant strains are the artemisinin derivatives.[3] Artemisinin (**3**) is a very potent antimalarial drug isolated from the aerial parts of the plant *Artemisia annua* L. (Asteraceae).[4] Because chemical synthesis of **3** results only in very low yields, the plant remains the only profitable source for production.[5] However, the plant produces this secondary metabolite in relatively small amounts, up to a maximum of 0.85% on a dry-weight basis.[6] Higher levels of **3** in the plant are desirable for commercial production purposes. Knowledge of the exact biosynthesis of this sesquiterpene lactone endoperoxide may enable us to influence its formation in a direct way, such as by metabolic pathway engineering.

Recently, we have reported the isolation of dihydroartemisinic acid (**1**) from *A. annua* and its photochemical conversion into **3**, with dihydroartemisinic acid hydroperoxide (**2**) as an intermediate, under conditions that may also be present in the living plant.[7] The suggestion was made that this nonenzymatic conversion probably can also take place in vivo in the plant. By establishing the presence of **2** in *A. annua*, strong evidence is obtained for an in vivo nonenzymatic (photochemical) conversion of **1** into **3**.

Dihydroartemisinic acid (tertiary)hydroperoxide (**2**), > 92% pure (HPLC), was isolated from the Vietnamese *A. annua* strain in 28% yield. Exposure of **2** to air for 24 h at room temperature yielded **3** (verified by TLC, ^{1}H NMR, and GC−MS) and dihydro-*epi*-deoxyarteannuin B (**4**)[8] (verified by TLC and GC−MS). Because **3** and **4** were already clearly visible by TLC after only 4 h, it can be concluded that **2** is quite reactive and easily converted into **3**. Therefore, during isolation and analytical procedures it was very important to avoid oxidation of **2** and photooxidation of **1** because both oxidation processes will interfere with the measurement of the actual concentration of **2** in the plant material. To be sure that **2** was not an artifact of isolation, a chloroform extract of fresh leaf material was prepared in the absence of light and oxygen, and because of the thermolability of **2**, this compound was analyzed by DAD and LC−MS. In this extract a compound was found with retention time, absorption maximum, and mass spectrum identical to **2**, as was obtained by photooxidation of **1**.[9]

The type of reaction in which **1** is attacked by singlet oxygen ($^1O_2$), yielding **2**, is also known in the photooxidation of polyunsaturated fatty acids (**5**) yielding lipid hydroperoxides (**6**) (Scheme 1). This photooxidation of **5** is known in the literature as a reaction in which no enzymes are involved.[10] In this reaction, the reactant $^1O_2$ is produced by energy transfer of a photon to triplet oxygen ($^3O_2$) mediated by a UV-absorbing molecule (chromophore). In analogy with the photooxidation of **5**, it seems to be very likely that the conversion of **1** into **2** in *A. annua* may also take place in a nonenzymatic way, as we have previously suggested.[7]

The question that remains is how $^1O_2$ is produced in *A. annua*. Although $^1O_2$ can be formed in various ways, including enzymatic catalysis, $^1O_2$ is generally produced in biological systems by the mechanism of energy transfer from photoexcited compounds.[11] Certain secondary plant products (chromophores) are capable of photosensitizing reactions that involve the transfer of light energy to oxygen.[11,12] Secondary plant products of diverse biogenetic origin, such as certain quinones, furanocoumarins, polyacetylenes, thiophenes, benzofurans, and possibly chromenes, are capable of the photogeneration of singlet oxygen.[11] The polyacetylenes, annuadiepoxide and ponticaepoxide,[13] and the chromenes, 2,2-dihydroxy-6-methoxychromene and 2,2,6-trihydroxychromene ponticaepoxide, are known to be present in the aerial parts of *A. annua*.[14] Because the presence of compounds with the potential of generating $^1O_2$ is a very widespread and common phenomenon among the plant kingdom,[11] it is not unlikely that even more compounds with this capacity are present in *A. annua*. The large number of compounds with potential for photosensitizing reactions among secondary plant products and the diversity of their biogenetic origin suggest the widespread use of $^1O_2$ as a protective agent against

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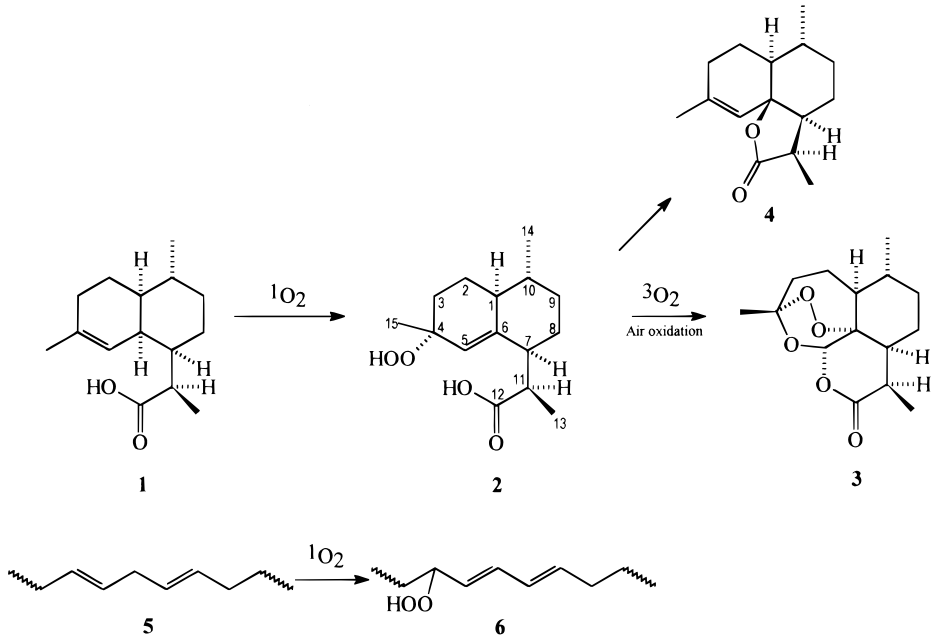
Scheme 1. Partial Biosynthesis of **3** Starting with the Attack of **1** by Singlet Oxygen ($^1O_2$)[a]



[a] This reaction proceeds through an ene reaction with rearrangement of the endocyclic double bond yielding **2**. By air oxidation (triplet oxygen, $^3O_2$) **2** can be converted into **3** and **4**. The reaction type in which **1** is converted into **2** is identical to the photooxidation of polyunsaturated fatty acids (**5**), yielding lipid hydroperoxides (**6**).

pathogens and predators.[11] Other secondary metabolites of higher plants have been shown to protect the plant against damaging photodynamic reactions by quenching the excited singlet state of oxygen, like the flavonoid quercetin also found among the constituents of *A. annua*[14] and known to be a potent quencher of $^1O_2$.[10,15] Besides quercetin, **1** may also be an interesting candidate here. That $^1O_2$ is produced in *A. annua* by the transfer of light energy to oxygen and that **1** is acting as a quencher of $^1O_2$ were demonstrated by a 35% higher yield of **3** in leaves dried in the presence of daylight compared with those dried in the dark. The *A. annua* plants used in this experiment contained 0.5−0.8% artemisinin (dry wt). By drying the leaf material in the presence of light, substantially higher yields of artemisinin can be obtained. Harvested plant material can be dried in the open to reach this goal.

In conclusion, the presence of **2** in *A. annua* confirms the formation of $^1O_2$ at the place where **1** is produced and/or sequestered, probably the glandular trichomes.[16,17] Photogeneration of $^1O_2$, at this location, mediated by a secondary product (chromophore-like compound) seems to be very likely, although enzymatic production of $^1O_2$ at this place cannot be completely excluded. Nevertheless, the existence of a nonenzymatic pathway for the formation of **3** starting with **1**, in *A. annua*, seems very plausible.

Experimental Section

General Experimental Procedures. HPLC, GC−MS, and LC−MS were performed as described previously.[7] Optical rotations were measured on a Perkin−Elmer 241 polarimeter. Diode array detection (DAD) analyses were performed on a Shimadzu SPD-M6A photodiode array detector. The DAD conditions were: column, Lichrosorb 7 RP 18, 100 × 3 mm i.d., cat. no. 28297 (Chrompack, Middelburg, The Netherlands); mobile phase, $H_2O-H_3PO_4$ (0.1M)−CH_3CN = 55:1:44 (v/v); flow rate, 0.75 mL/min; wavelength, scanning from 195 to 600 nm; detector sensitivity, 400 mAbs; injected volume, 10 μL; temperature, room temperature.

^1H 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 for ^1H and δ 76.91 for ^{13}C. Coupling constants are given in Hertz (Hz).

IR spectra were recorded on a ATI Mattson Genesis Ref I, using Win FIRST software.

Thin-Layer Chromatography (TLC). Chromatograms were run on precoated Si gel 60 F_{254} plates (Merck art. 5715). Aliquots of 2 μL of the eluted fractions were applied to the plates by means of micro capillaries. The TLC plates were developed in closed chambers with petroleum ether (40−60)−EtOAc = 1:1 (v/v) as the eluent. Spots were visualized by dipping the plates for 10 s in a mixture of H_2SO_4 (96%)−CH_3COOH (100%)−*m*-anisaldehyde (4-methoxybenzaldehyde) = 2:100:1 (v/v) followed by drying for 6 min at 120 °C.

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. Germination of *A. annua* seeds took place in potting compost and at climate-room conditions (23 °C and 16 h per day ca. 3000 lux) after ca. 3 days. After a period of four weeks total, under climate-room conditions, the seedlings were allowed to grow freely in the open at an experimental field belonging to our research institute in Groningen, The Netherlands. Young leaves (sprouts) were harvested at the middle of the day in the first week of August 1996, after a 4-month growing period.

Isolation Procedure for Compound 2. Freshly harvested young leaves from 4-month-old plants of *A. annua* were immediately frozen in liquid nitrogen and dried (in the absence of light) through lyophilization overnight. To minimize air exposure, 100 g of the dried and ground leaves were immediately extracted with 200 mL of 96% EtOH in an ultrasonic bath for 20 min. After sonification the EtOH extract was separated from the plant pulp by suction filtration and the plant material extracted for a second time under the same conditions. The EtOH extracts were pooled, and a mixture of 20 g Norit and 30 g Celite was added, stirred for 15 min, filtered, and washed with an additional 50 mL of 96% EtOH. The EtOH was removed under vacuum to yield a dark yellow, oily substance. This oily substance was directly dissolved in a small volume of petroleum ether (40−60)−Et_2O = 1:1, and

chromatographed on a Si gel column (20 × 1.8 cm, J. T. Baker, Deventer, The Netherlands) using the solvent described above as the mobile phase. The column effluent was monitored by TLC using **2** obtained by photooxidation of **1** as a reference.[9] Crude **2** (68 mg, 79% pure, HPLC) was further purified on a Si gel column using petroleum ether(40−60)−EtOAc = 3:1 as the eluting solvent. The concentration of **2** in the same batch of lyophilized *A. annua* leaves was determined by HPLC, by using a concentration series of **2**, and yielded a concentration of 0.14% (dry wt).

Identification of 2. Dried *A. annua* leaves (100 g) yielded 40 mg of **2** as a clear, colorless oil, >92% pure (HPLC), corresponding to a 29% yield: $[\alpha]^{23}_{D}$ −8.8° (*c* 0.037, MeOH), IR (KBr) ν_{max} 3380−3020 (OH, COOH), 1705 (C=O), 1657 (C=C) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.26 (1H, s, H-5); 2.74 (1H, m, H-11); 2.15 (1H, m, H-7); 1.92 and 1.48 (4H, m, H-2 and H-3); 1.83 and 1.21 (2H, m, H-9); 1.77 and 1.22 (2H, m, H-8); 1.58 (1H, m, H-1); 1.30 (3H, s, CH_3-15); 1.28 (3H, d, $^3J_{11,13}$= 6.6, CH_3-13); 1.24 (1H, m, H-10) 0.93 (3H, d, $^3J_{10,14}$= 5.8, CH_3-14) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 180.7 (s, COOH); 146.3 (s, C-6); 120.1 (d, C-5); 80.6 (s, C-4); 47.1 (d, C-7); 44.9 (d, C-1); 41.1 (d, C-11); 38.5 (d, C-10); 35.4 (t, C-8); 32.7 (t, C-9); 28.7/22.6 (t, C-2/C-3); 24.4 (d, CH_3-15); 19.9 (d, CH_3-14); 15.6 (d, CH_3-13) ppm; LC−MS analyses yielded a retention time of ca. 3 min, and the most important fragment ions obtained were at ***m/z*** (rel int.) 267 (100), 249 (14), 235 (42), 191 (24), and 166 (14), corresponding to $[M - H]^-$, $[M - H - H_2O]^-$, $[M - H - H_2O]^-$, $[M - H - O_2 - CO_2]^-$, and probably $[M - H - O_2 - (CH_2{-}CH{-}CH{-}CH_2) - CH_3]^-$; DAD analyses yielded a retention time of ca. 3 min and an absorption maximum at 200 nm.

Identification of 2 in Fresh Plant Material. During all steps of the entire identification process of **2** in *A. annua* leaves, care was taken to prevent exposure of the extracts to air and light. Fresh leaves (ca. 0.2 g) were thoroughly ground with ca. 200 mg fine granular quartz (Merck, Darmstadt, Germany) and 2 mL of $CHCl_3$ in a mortar for about 2 min under a blanket of nitrogen and in the absence of light. The $CHCl_3$ extract was separated from the plant pulp by centrifugation for 1 min in a microcentrifuge (13 000 rpm). The obtained extract was transferred to a 2-mL amber-colored vial, and the $CHCl_3$ was evaporated under a flow of nitrogen. The residue was redissolved in 1 mL MeOH and directly used for DAD and LC−MS analyses.

Leaves of *A. annua* Dried in the Presence versus Absence of Light. Fresh leaves (20 g) were randomly collected from a single plant and divided after mixing into two equal portions of 10 g. One portion was dried in an incubator in the absence of light at 23 °C. The other portion was dried in the presence of 9 h light (ca. 5000 lux) per day over 3 days, also at 23 °C. After 72 h, the two portions were powdered, and from each portion three samples of ca. 35 mg were accurately weighed. HPLC analyses of **3** were performed with the hydrolyzed product of **3** Q260 as described by Pras et al.[18]

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