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Artemisinin Story from the Balkans

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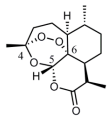
Dedicated to our late Professors Milutin Stefanović and Dragoslav Jeremić

The isolation is reported of artemisinin (qinghaosu), a drug remarkably effective against malaria, from the aerial parts of *Artemisia annua* L. (sweet wormwood) at the Department of Chemistry, University of Belgrade (now Faculty of Chemistry), Serbia by the end of 1970, almost two years before the isolation of the same compound in China.

Keywords: Artemisinin, Arteannuin B, *Artemisia annua* L.

In 2015 the Nobel Prize in Physiology or Medicine was awarded to Tu Youyou (Chinese) for her discovery of a novel therapy against malaria, as well as to Satoshi Ōmura (Japanese) and William C. Campbell (Irish-American) for their discovery of a novel therapy against infections caused by roundworm parasites.



1 Artemisinin

Tu Youyou searched ancient Chinese literature on herbal medicine in her quest to develop novel malaria therapies in the course of a secret national *"Project 523"*, starting in 1967 in P. R. China. The origin of the project was a request from the North Vietnamese Government to Mao Zedong in China, for assistance in managing chloroquine drug-resistant malaria affecting their military forces during the Vietnam War [1]. In 1969 Tu Youyou from the Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine, was appointed head of an antimalarial research group involving both phytochemical and pharmacological researchers. She started testing more than 2,000 Chinese herb preparations known from traditional Chinese medicine on antimalarial effects. Finally, her choice was narrowed down to a single species - *Artemisia annua* L. (Asteraceae), *qinghao* (the "blue green herb"). This herb, featured prominently in the Chinese Pharmacopoeia in relation to decoctions used to treat intermittent fevers (recorded use of *qinghao* spans over 2000 years), turned out to be the most interesting candidate. Tu Youyou developed a purification procedure, which rendered the active agent from the aerial parts of *A. annua*, named *artemisinin* (**1**) (or *qinghaosu*), a drug that is remarkably effective against malaria [1,2]. This happened at the end of 1972. According to Chinese sources [1a], the chemical structure of *qinghaosu* (**1**), with a 4,5-seco-cadinane skeleton, was elucidated *ca* 3 years after isolation, at the end of 1975 as a result of a collaborative effort of three Chinese institutes: (*i*) Beijing Institute of Traditional Chinese Materia Medica, Academy of Traditional Chinese Medicine, (*ii*) Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences and (*iii*) Beijing Institute of Biophysics, Chinese Academy of Sciences. According to [1b, pp. 41-44] the Chinese scientists working in those days on structure determination of **1** also took into account the experiments performed in (former) Yugoslavia where novel cadinane sesquiterpene lactone structures were assigned to two compounds extracted from the same plant a couple of years before (see below, compounds **1A** and **2**) [3,4]. These experiments commenced in former Yugoslavia in 1970, at the Faculty of Science, Department of Chemistry (now Faculty of Chemistry), University of Belgrade under the leadership of Professors Milutin Stefanović and Dragoslav Jeremić. M. Stefanović was an organic chemist, working mostly in the steroid field, whereas D. Jeremić, a physico-chemist, was a head of the Center for Instrumental Analysis (CIA), founded in 1966, and equipped with sophisticated analytical instruments (^{1}H NMR, MS, IR, UV-Vis and GC). A group of M. Stefanović, three chemists and one botanist, was in charge of plant collection, extraction and isolation of constituents of the extracts. One of the authors of this manuscript (A. J.) was a member of this group. A group of D. Jeremić measured spectra of the isolated compounds and carried out their interpretation. The remaining authors of this MS (V. V. and S. M.) belonged to this group, running spectra on a service base for internal and external users. By pure chance this phytochemical work commenced with species of *Artemisia* such as *A. annua* L., *A. vulgaris* L. *A. campestris* L., *A. absinthium* L. and *A. scoparia* W. et K. In those days nobody from the Belgrade group was aware of the healing properties of *A. annua*, known from Chinese traditional medicine. They also did not know what was going on in China at the same time (Project 523).

A. annua and *A. vulgaris* L., together with *Ambrosia artemisifolia* L. were the subject of the Ph.D. thesis [5] of Abdulaziz Behbud, a graduate student from Afganistan, under the supervision of M. Stefanović. The aerial parts of *A. annua* were collected in October 1970 at a locality close to the center of Belgrade, named Staro sajmište (Old Fairgrounds) on the left bank of the river Sava. From the $CHCl_3$ extract, worked up by the usual procedure for the isolation of sesquiterpene lactones, *i.e.* treatment with $Pb(OAc)_2$ in

Prof. Dragoslav Jeremić 1929-2011

Prof. Milutin Stefanović 1924-2009

order to remove tars and plant pigments [6], followed by silica gel CC, two crystalline substances denoted as "**A**" (**1A**) and "**B**" (**2**) were isolated. A detailed isolation procedure is described in ref [5]. ^{1}H 60 MHz NMR and IR spectra of substance **1A** (both measured on 10 December, 1970) are shown in Figures 1 and 2, respectively. As shown later, these were probably the first spectra of artemisinin ever recorded. Both compounds contained strong IR bands at 1760 (**1A**) and 1775 (**2**) cm^{-1} typical of a lactone ring. The electron-impact mass spectrum of **1A** contained a weak molecular ion at *m/z* 282, as well as a fragment ion at *m/z* 250, due to the loss of 32 amu corresponding to a molecule of oxygen [3b,5]. At the same time, compound **2** exhibited molecular ion at *m/z* 248 [3a,5]. Precise mass measurement of the molecular ions of both compounds, using peak matching technique (with PFK as a standard) revealed molecular weights of 282.1470 (**1A**) and 248.1420 (**2**), corresponding to molecular formulas of $C_{15}H_{22}O_5$ and $C_{15}H_{20}O_3$, respectively [3a,3b]. The precise mass of **1A** was almost identical to that (282.1472) reported after *ca.* 5 years for artemisinin (qinghaosu) (**1**) by Chinese authors [7]. Molecular formulas of **1A** and **2**, obtained by MS were also compatible with the elemental C,H-analysis. The IR and MS evidence indicated sesquiterpene lactone structures for both compounds. This was expected since the majority of sesquiterpene lactones identified before mainly originated from species of the family Asteraceae. However, determination of structures **1A** and **2** appeared to be rather complicated. The available spectral data of **1A** and **2** did not fit any of the common sesquiterpene lactone skeletons reported before. Structural fragments identified in **1A** (**a**,**b**,**c**,**d**) and **2** (**e**,**f**,**g**) are presented in Figures 3 and 4. The major problem in structure elucidation of **1A** was the disposition of five oxygens, two of them belonging to the lactone ring (Figure 3, fragment **c**). A low-field singlet in the ^{1}H NMR spectrum (δ~5.8, Figures 1 and 3) indicated an isolated proton α-positioned to two oxygens (Figure 3, fragment **a**). At the same time, the remaining oxygen bonding sites were carbons bearing no protons. This structural problem could have probably been solved more efficiently by ^{13}C NMR spectral data (not to mention recent 2D NMR techniques), but unfortunately in those days (beginning of 70s) ^{13}C NMR measurement was not available in Belgrade. However, the overall pattern of the ^{1}H NMR spectra indicated the same type of skeleton for **1A** and **2** (see Figures 3 and 4). In order to obtain more information regarding the structures of **1A** and **2** some chemical reactions were performed on the isolated compounds (e.g. reduction with NBH_4, catalytic hydrogenation, hydrolysis). A breakthrough in structure elucidation occurred after the acid hydrolysis (HCl) of **2**, yielding quantitatively a single hydrolysis product **3** (Scheme 1), identified by means of ^{1}H 60 MHz NMR and MS. The identification of δ-lactone **3**, in combination with the available ^{1}H NMR, IR, MS, ORD and CD data of its precursor **2**, led to the assignment of structure of **2** as the first member of a new class of sesquiterpene lactones - *cadinanolides* (scheme 1). Formation of **3** could be rationalized in terms of acid-catalyzed 4,5-epoxide opening, together with formation of a Δ^3-double bond by elimination of H-3. This is followed by isomerization of the γ-lactone in **2** into the more stable δ-lactone (intramolecular acid-catalyzed transesterification) in **3** [8].

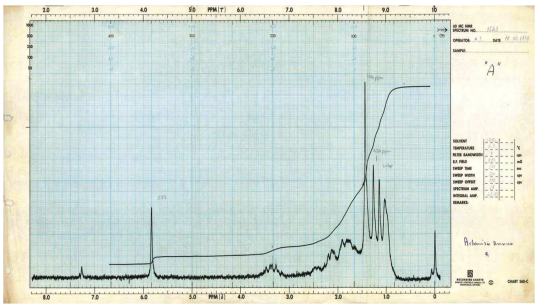


Figure 1: ^{1}H 60 MHz ($CDCl_3$) NMR spectrum of substance "**A**" measured on a Varian A60A CW NMR spectrometer on 10/12/1970 at CIA (Belgrade).

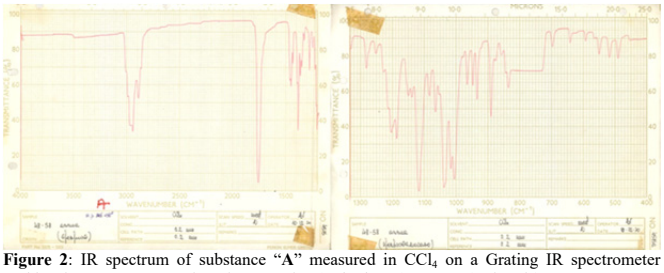


Figure 2: IR spectrum of substance "**A**" measured in CCl_4 on a Grating IR spectrometer Perkin Elmer 337 measured on the same day (10/12/1970) at CIA, (Belgrade).

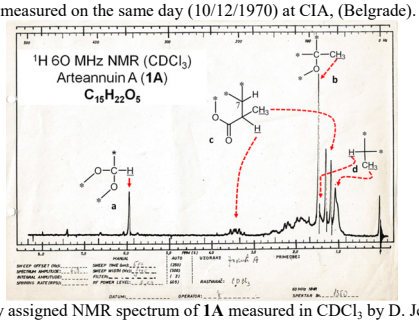


Figure 3: Partially assigned NMR spectrum of **1A** measured in $CDCl_3$ by D. Jeremić on a CW instrument Varian A60A.

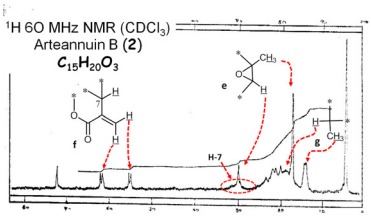
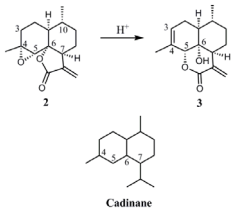
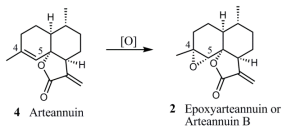


Figure 4: Partially assigned NMR spectrum of **2** measured in $CDCl_3$ on a CW instrument Varian A60A (reproduced from Ph.D. thesis of A. Behbud [5]).



Scheme 1: Acid catalysed hydrolysis of **2**

The attempts of D. Jeremić to gain additional proof regarding the (cadinane) structure **2** are also illustrated by his notes on the proposed EI MS fragmentation scheme of this compound (Figure 5). According to the above-mentioned evidence, lactone **2** was assigned as an epoxide of a hypothetical Δ^4-cadinanolide precursor **4**, named *arteannuin* (scheme 2) [3a]. Although subsequent biosynthetic investigations [9] showed that **4** was not the precursor of **2**, this assumption prompted structure elucidation of the co-occurring lactone **1A**, as the ozonide of a similar hypothetical Δ^4-cadinanolide **5**, named *dihydroarteannuin* (Scheme 3).



4 Arteannuin **2** Epoxyarteannuin or Arteannuin B

Scheme 2: Proposed formation of **2** by epoxidation of **4**

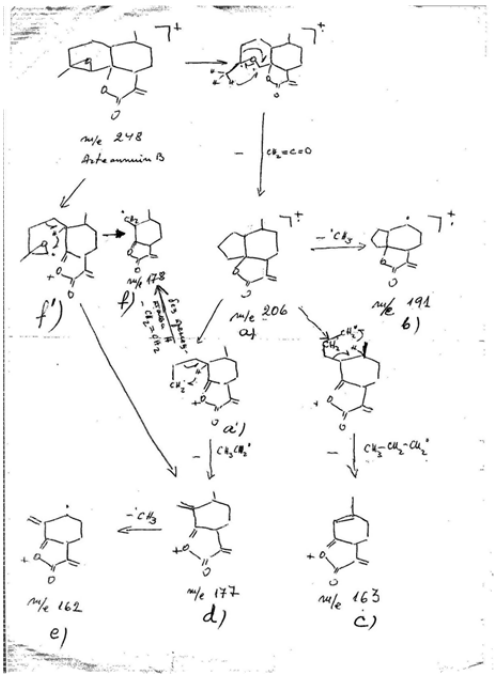
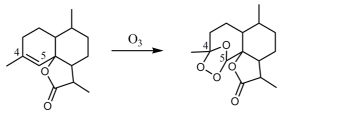


Figure 5: Notes of D. Jeremić regarding his assumptions about EI MS fragmentation of **2**.



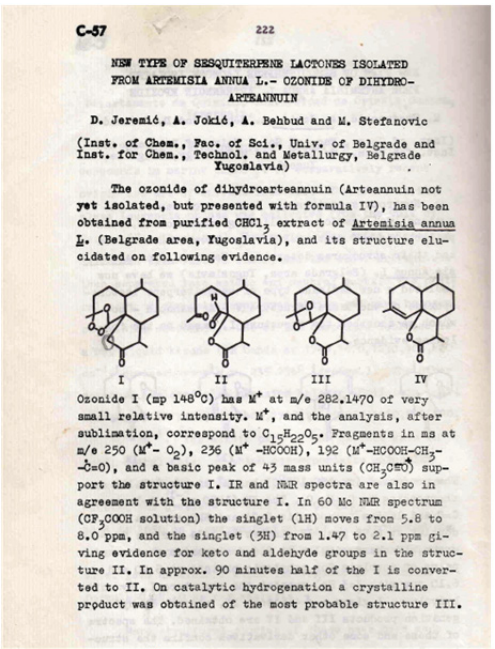
5 Dihydroarteannuin **1A** Dihydroarteannuin ozonide or Arteannuin A

Scheme 3: Proposed formation of **1A** by ozonisation of **5**.

The ozonide structure of **1A** was quite compatible with the available spectral data. Both structures **1A** and **2** were reported at the 8th International Symposium on the Chemistry of Natural Products, New Delhi [3a,3b] by one of the co-authors of this manuscript (A. J.) as "*arteannuin epoxide"* and "*ozonide of dihydroarteannuin"*, respectively (see Figure 6).

A detailed structure elucidation of **2** and its hydrolysis product **3** was published by the Belgrade group in 1973 [4]. In this publication epoxy-lactone **2** was called "*arteannuin B*" and this name is still being used today. The structure of **2** was also confirmed a year later by the spectroscopic and X-ray evidence (on the samples of **2** and **3** originating from Belgrade) independently in two laboratories, one from USA [10] and another from Switzerland [11]. Since that time, **2** was found in many extracts of *A. annua* from different localities around the world [12]. In China this lactone was isolated by the end of 1972 [1b, p. 38], together with artemisinin. It was shown recently [13], that **2**, in combination with arteannuinic acid and scopoletin (both co-occuring in *A. annua*), can function synergistically with artemisinin to increase its antimalarial activity.

It is interesting to note that hypothetical compounds **4** and **5,** named arteannuin and dihydro-arteannuin, respectively, by the Belgrade group [3a,3b] were isolated subsequently from *A. annua* and reported under the names of *deoxyarteannuin B* and *dihydro-deoxyarteannuin B* [14-16], each of them occurring as 6α- and 6β-*O* epimers.



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NEW TYPE OF SESQUITERPENE LACTONES ISOLATED FROM ARTEMISIA ANNUA L.- OZONIDE OF DIHYDRO-ARTEANNUIN

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(Inst. of Chem., Fac. of Sci., Univ. of Belgrade and Inst. for Chem., Technol. and Metallurgy, Belgrade Yugoslavia)

The ozonide of dihydroarteannuin (Arteannuin not yet isolated, but presented with formula IV), has been obtained from purified $CHCl_3$ extract of Artemisia annua L. (Belgrade area, Yugoslavia), and its structure elucidated on following evidence.

I II III IV

Ozonide I (mp 148°C) has M^+ at m/e 282.1470 of very small relative intensity. M^+, and the analysis, after sublimation, correspond to $C_{15}H_{22}O_5$. Fragments in ms at m/e 250 ($M^+ - O_2$), 236 (M^+ -HCOOH), 192 (M^+-HCOOH-CH_3-C=O), and a basic peak of 43 mass units (CH_3CO^+) support the structure I. IR and NMR spectra are also in agreement with the structure I. In 60 Mc NMR spectrum (CF_3COOH solution) the singlet (1H) moves from 5.8 to 8.0 ppm, and the singlet (3H) from 1.47 to 2.1 ppm giving evidence for keto and aldehyde groups in the structure II. In approx. 90 minutes half of the I is converted to II. On catalytic hydrogenation a crystalline product was obtained of the most probable structure III.

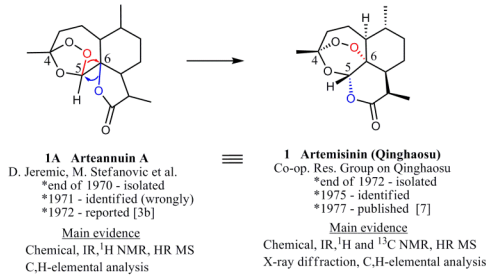
Figure 6: The abstract from 8th International Symposium on the Chemistry of Natural Products, held in New Delhi in 1972 about lactone **1A** [3b] (first report regarding artemisinin)

After the New Delhi Symposium [3] lactone **1A** was only mentioned once more by M. Stefanović *et al*. under the name "*arteannuin A*" in a note published in *Phytochemistry* in 1975 [17], which was about a novel flavonol isolated from *A. annua*, not about **1A**. In this note, arteannuin A was listed together with compounds co-occurring in *A. annua* from Serbia, and the reference attached to arteannuin A in this paper cites the New Delhi abstract [3b], followed by the statement: "the full experimental details will be published soon". Unfortunately, Stefanović and Jeremić never published these data. Arteannuin A quoted in this paper was actually lactone **1A** and should not be confused with a bisnor-cadinanolide isolated later from *A. annua* and reported under this name [18]. The information regarding arteannuin A mentioned in ref. [17] probably reached China, which in 1976 started its emergence from isolation caused by the Cultural Revolution. Thus, in reference [1a, p. 63] about the history of the Chinese Project 523, one can find the following:

"In 1976 information reached China that a phytochemist in Yugoslavia was isolating a substance similar to qinghaosu *from other species of the* Artemisia *genus. The National Academy of Chinese Traditional Medicine recommended to the Ministry of Health that Chinese data should be published as soon as possible, before publication from Yugoslavia*. Qinghaosu's *chemical structure was published (in Chinese) in the Chinese Science Bulletin 1977 under a collective authorship, the "Qinghaosu Structure Collaborative Research Group"* (see ref. [7])

After publication of this paper [7], a couple of Chinese publications regarding the same topic appeared by the end of the '70s [19-21]. Among them, reference [20] was rather important because it was the first full account published in the *Chinese Medical Journal* (English edition) of the pharmacology of artemisinin as an antimalarial drug, highly efficient against *Plasmodium falciparum*. It took *ca*. two years after this announcement that the news about this discovery spread all over the world.

It was obvious (by the similarity of spectral data of **1A** and **1**) that arteannuin A (**1A**), as named in ref. [17], and artemisinin (**1**) are the



Scheme 4: Arteannuin A (**1A**) vs. artemisinin (**1**); interchanging positions of oxygens at C(5)-C(6) fragment in **1A** leads to the (correct) structure **1** of artemisinin.

same compound. A comparison of the ozonide and endoperoxide structure, **1A** and **1**, respectively, is shown in Scheme 5. As can be seen, both structures exhibit the same 4,5-*seco*-cadinanolide skeleton and the only (small) difference is in the disposition of oxygens at C-5 and C-6.

Beyond any doubt, as based on the presented evidence, such as the original spectra of **1A**, and published papers regarding **2** [4,10,11], as well as the New Delhi reports [3a,3b], the Belgrade group was the first to isolate these compounds. Unfortunately, they did not realize the importance of this discovery.

The same conclusion about the question "who was first to isolate artemisinin?" was reached by William Burns from the UK, whose comprehensive inquiry about the discovery of artemisinin was presented in 2008 as a blog under the title "Qinghaosu Project" [22].

Two groups working on this topic in the early 70s (Chinese and Yugoslavian) followed substantially different approaches. Whereas the Belgrade group carried out phytochemical investigations aimed at examining local plant species not studied before, and trying to isolate as many compounds as possible, primarily focused on the new structures, the Chinese approach was typical for the method nowadays known as *"bioactivity guided fractionation"*.

Artemisinin is probably among the first peroxide compounds isolated from natural sources. Since the discovery of artemisinin, over 600 phytochemicals (including about fifty amorphane and cadinane sesquiterpenes) have been identified in *A. annua* [9] originating from different localities.

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