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FEATURE ARTICLE

A golden phoenix arising from the herbal nest — A review and reflection on the study of antimalarial drug Qinghaosu

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Qinghaosu (QHS) and its derivatives are a new generation of antimalarial drugs characterized by fast action, high efficacy, and good tolerance. This feature article states the discovery of QHS from traditional Chinese medicine qinghao (*Artemisia annua* L.) and reviews the progress during the past four decades in the research of phytochemistry of *A. annua*, chemical reactions and biotransformation of QHS, chemical synthesis and biosynthesis of QHS, synthesis and antimalarial activity of QHS derivatives and analogs, pharmacological studies, clinical application, and the antimalarial mechanism. Undoubtedly, QHS is an example of the value of traditional Chinese medicine in modern medicinal research.

1 Background

Malaria has been one of the most serious parasitic diseases around the world. In China, the falciparum malaria, known as miasma to ancient Chinese, was once widely spread in Southern China; while in the other areas of China, vivax malaria was quite common. For many years, the disease has been a major harm to human health and has claimed numerous victims' lives. In 1950s and 1960s, there were still about ten millions of malaria patients in China, where chloroquine and other antimalarial drugs were being produced and widely used. In 1970, according to the statistics released by the Ministry of Health of China, there was even a record-breaking number of 30 million of patients. According to the World Health Organization (WHO), Malaria is also endemic in other tropical and subtropical areas of the world; it affects approximately 200–500 million people each year [1].

Ancient people learned a lot from the disease. For example, aboriginal Indians in Southern America discovered that cinchona tree bark could cure malaria. In 1820, European chemists Caventou and Pelletier extracted quinine from it. Since then quinine became the cure for malaria. Despite of its shortcomings such as high toxicity, quinine is still in active use in modern clinics. In China, about 2000 years ago, Chang Shan (*Dichroa febrifuga* Lour) was found to be able to treat malaria [2]. In 1940s, Chou et al. extracted antimalarial ingredient febrifugine from it [3]. Chemists from abroad followed up with structure determination, total synthesis, and structure modification. However, it has never been approved as an antimalarial drug due to its strong vomit-inducing side effect.

There are also a lot of therapies for malaria recorded in many traditional Chinese medicine classics. However, there were no in-depth studies. One reason is that a great number of malaria patients have been cured since 1940s due to the mass-production of cost-effective synthetic medicines such as chloroquine, primaquine, and pyrimethamine (Figure 1). The public believed that the problem had been solved once and for all. However, plasmodium fought for its own survival with gene mutation. Since the early 1960s, drug-resistant malaria started to spread in the South-east Asia and other areas of the world. The battle against malaria continued. Even today, over one million victims die due to malaria each year, mostly children in Africa [1].

In early 1960s, the Vietnam War broke out. In the tropical forests, military forces of both sides suffered severe casualties caused by drug-resistant *falciparum* malaria, which far exceeded casualties in the combats by a great margin. To deal with the urgent situation, the US government organized

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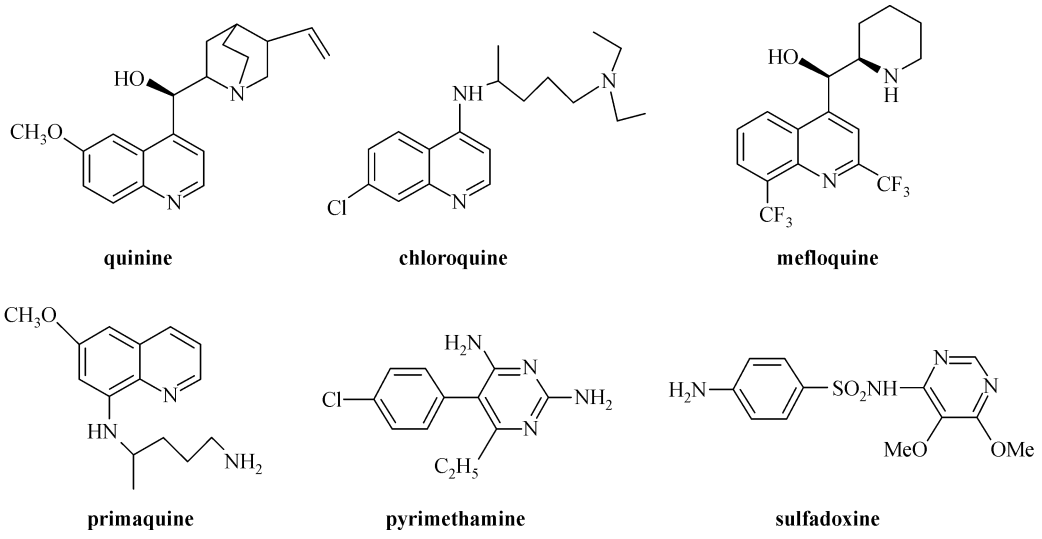


Figure 1 Chemical structures of known antimalarial drugs before 1986.

taskforces responsible for the research and development of new drugs treating drug-resistant malaria. During the following 12 years, over 250,000 compounds had been screened in primary tests using mice infected with *Plasmodium berghei* (*P. berghei*). As the result of this research program, four new chemical groups emerged as potentially new antimalarial [4], in which a derivative of a 4-quinolinemethanol (WR 142490) was marketed in 1985 under the generic name of mefloquine (Figure 1).

In the meantime, the Vietnam government sought support from China. The Chinese government agreed to help because the same disease was also hunting China at that time. However, the environment for scientific researches was hard in China especially during the difficult years of the so-called Cultural Revolution. In 1967, the National Steering Group was established. More than 60 research units joined force, with more than 500 researchers from institutions, colleges, pharmaceutical factories, and hospitals over the nation. Their goals included researches of new mosquito repellants, malaria prevention drug, treatment drugs, radical medicine, and mosquito killer equipment [5].

2 Discovery of qinghaosu

Many Chinese researchers were convinced that the best solution on drug-resistant malaria was to find active compounds with new chemical structures. The problem was how to find such compounds. Researchers thought about traditional Chinese medicine (TCM). Chinese ancestors gathered rich experience after thousands years of battles against malaria and recorded it in various medical books. Modern researchers could follow and screen these leads for ideal compounds. Therefore, researchers combed traditional medical books, learned from the folks in malaria endemic areas about their experience, and collected Chinese medicinal herbs that were claimed to treat malaria.

Consequently, some thousands of TCM had been studied [5]. A large number of crude extracts from raw materials soaked with different solvents and under different temperature were screened in mice infected *P. berghei*, and then, the active extracts were selected for advanced tests. By the 1970s, several new active principles were isolated, including qinghaosu (artemisinin) from Qinghao (*Artemisia annua* L.) [6,7], yingzhaosu A from Yingzhao (*Artabotrys hexapetalu* (LF) Bhand) [8], agrimols from Xianhecao (*Agrimonia pilosa* L.) [9,10], robustanol from Daye'an (*Eucalyptus robusta* Sm) [11], protopine from Nantianzu (*Nandina domestica* T.) [12], bruceine D and E from Yadanzhi (*Brucea javanica* (L) Merr) [13], and anluosu from Lingshui'anluo (*Polyalthia nemoralis* A. (DC)) [14]. Afterwards, their structure determination and structure modification were conducted (their structures are shown in Figure 2). Based on their chemical stability, antimalarial activity, toxicity, and availability, qinghaosu became the most promising drug candidate.

The history of Qinghao as a medicine could be traced back to 2000 years ago. The *Recipes for Fifty-two Kinds of Diseases* (Wu Shi Er Bing Fang) among archeological findings in the Mawangdui Tomb in Changsha mentioned that Qinghao could be used to treat hemorrhoids.

Ge Hong (283–343/363) first recorded Qinghao as an antimalarial drug in his *The Handbook of Prescriptions for Emergency Treatment* (Zhou Hou Bei Ji Fang), "take a handful of Qinghao, soak in 2 liters of water, wring out the liquid and drink" (Figure 3). The *Compendium of Materia*

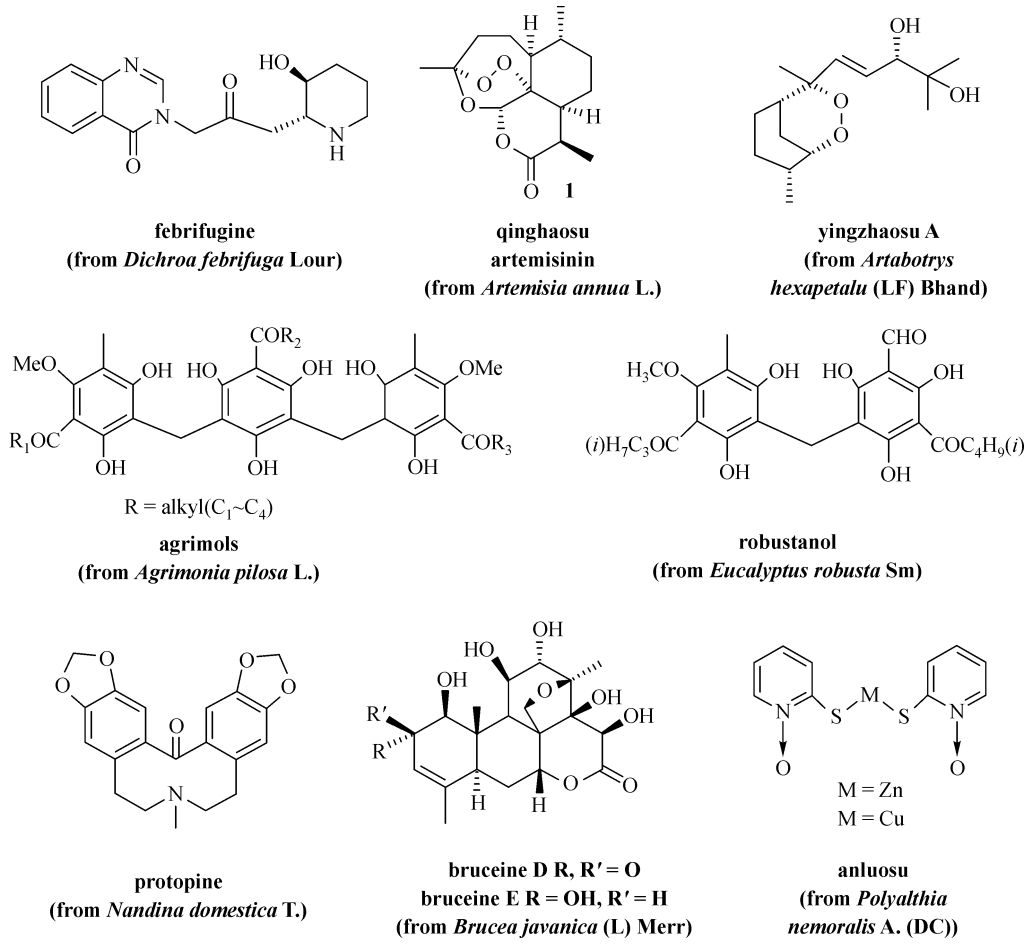


Figure 2 Antimalarial natural products.

Medica (Ben Cao Gang Mu) written by Li Shizhen in the Ming Dynasty and other traditional medical books also recorded many therapies on malaria treatment by Qinghao or Qinghao mixing with other herbal medicines.

In 1950s and 1960s, there were quite a few clinical reports on treatment of malaria by Qinghao published on medical journals in Jiangsu, Hunan, Guangxi, and Sichuan Provinces. The usual preparation methods included pressing for juice, boiling in water, or grinding to powder. It was noticed that in some areas of Gaoyou County, Jiangsu Province, residents were asked to drink an aqueous decoction of Qinghao, and malaria was under effective control. However, some research groups found that Qinghao extracts gathered by heating raw materials in water, ethanol, or benzene did not show antimalarial activity on animal models. In the second half of 1971, researchers from the Institute of Chinese Materia Medica, Academy of Traditional Chinese Medicine, were inspired by the "wring out the liquid" method recorded in *The Handbook of Prescriptions for Emergency Treatment* and extracted Qinghao with diethyl ether at low temperature. The neutral crude extract had noticeable effects against *P. berghei* in mice. Thus, in the summer of 1972, after the tests of animal toxicity and trials by a few subjects, the neutral crude extract was put under small-scale clinical observation in Hainan Island and Beijing, and the clinic results were satisfactory. Thereafter, Chinese herb Qinghao became the focus of the project. However, "Qinghaosu II" isolated by the Institute of Chinese Materia Medica did not show expected results in the clinical trial of 1973.

At the same time, the Yunnan Institute of Materia Medica and Shandong Institute of Traditional Medicine and Materia Medica successfully extracted the crystalline principle from *Artemisia annua* by petroleum ether or ethyl acetate and gained exciting results from their clinical trials for the treatment of *P. vivax* and *P. falciparum* cases. They named the antimalarial principle as huanghaosu or huanghuahaosu, respectively, which was laterly renamed qinghaosu (QHS), with artemisinin as its English name.

For a long time, Chinese herb Qinghao was just a common name for *Artemisia annua* and *Artemisia apiacea* [15,16]. Hence, in the 1977 edition of *Chinese Pharmacopoeia*, Qinghao was referred to both *Artemisia annua* and *Artemisia*

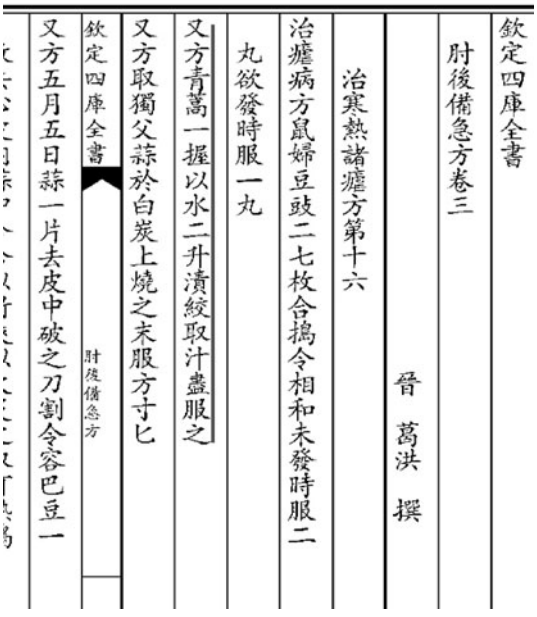


Figure 3 A page showing Qinghao for the treatment of malaria from *The Handbook of Prescriptions for Emergency Treatment*.

apiacea. Since QHS was isolated from *Artemisia annua,* the later editions of *Chinese Pharmacopoeia* referred Qinghao to *Artemisia annua* only.

After 30 years, the new generation of antimalarial drug QHS was hailed as the greatest medical discovery in the latter half of the 20th century [17]. Discovery of QHS demonstrate strongly that traditional Chinese medicine is an important resource for discovery of new drugs.

3 Components of *Artemisia annua* L

Since the discovery of QHS, systematic phytochemical work on *A. annua* has been conducted over the past two decades. Different *A. annua* materials, such as leaves, stems/flowers, roots, and seeds, as well as endophytes inside *A. annua*, have been employed for phytochemical investigations, and more than 150 natural products have been reported in the last century [18].

3.1 Terpenoids from *A. annua*

According to the research results, QHS is identified in all *A. annua* plants from different geographical origins, while its content varied drastically based upon growing areas and stages of plant development. QHS is present in the leaves and flowers of *A. annua* in ~ 0.01%–1.1% of air-dry weight [19–24]. Production of QHS (**1**) from *A. annua* rarely exceeds 1.0% of the air-dry weight, with the highest content from plants just before flowering.

From indigenous *A. annua*, continuous phytochemical studies by Chinese researches in the early 1980s led to the excavation of other nine sesquiterpenes including artemisinin A (**2**) [25], arteannuin B (**3**) [25], deoxyartemisinin (**4**) [25], artemisinin D (**6**) [26], artemisinin E (**7**) [26], artemisinin F (**8**) [27], artemisinic acid (**11**) [28–30], artemisinic acid, methyl ester (**13**) [31], and epoxyarteannuinic acid (**20**) [32] (Figures 3 and 4). Among them, arteannuin B (**3**) was reported early in the 1970s [33,34]. They are all closely related to the amorphene series of sesquiterpene characterized by the presence of a *cis*-decalin skeleton with the isopropyl group *trans* to the hydrogen on the ring juncture. From a biogenetic viewpoint, artemisinic acid (**11**) or its 11, 13-dihydro analog, dihydroartemisinic acid (**12**), which was isolated later from *A. annua* in less content, is the precursor in the biogenesis of QHS [35,36].

Some close compounds of artemisinic acid, artemisinic alcohol (**14**), dihydroartemisinic alcohol (**15**) [37,38], artemisinic aldehyde (**16**), and dihydroartemisinic aldehyde (**17**) were recently detected from glandular trichomes and leaf extracts of *A. annua* [38]. Other related compounds, cadin-4, 7 (11)-dien-12-al (**18**) [39], 6, 7-dehydroartemisinic acid (**19**) [40], and dihydroartemisinic acid hydroperoxide (**21**) [41] were isolated from leaves of *A. annua*. The allylic hydroperoxide (**21**) was once proposed as an intermediate for biosynthesis [42].

Another sesquiterpene bearing peroxy group is artemisitene (**10**), its content and antimalarial activity are greatly lower than that of QHS [43].

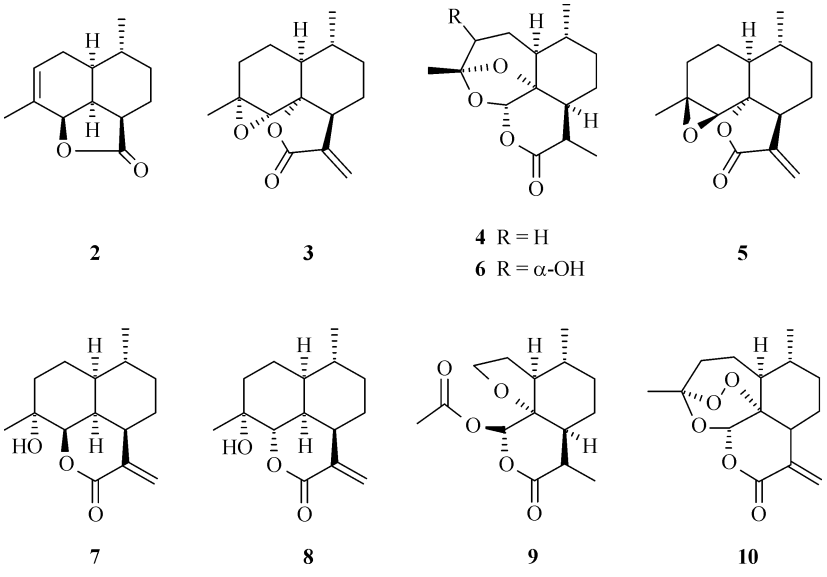


Figure 4 Structures of sesquiterpenes from *Artemisia annua* (2–10).

A new sesquiterpene named artemisinin G (**9**) was isolated from *A. annua* by Chinese scientists in 1992 [44]. It was reported that **9** was a decomposition product of QHS by heating at 190°C for 10 min or by refluxing xylene for 22 h [45,46].

Artemisinin G (**9**), deoxyartemisinin (**4**), and 3α-hydroxy-deoxyartemisinin (**6**), these three natural compounds were found to be the metabolites of QHS *in vivo* or the reaction products of QHS and ferrous ion (*vide infra*) in further research.

There are six structural relatives of arteannuin B in *A. annua*: artemisinin C (**5,** the β-epoxy isomer of arteannuin B) [47], dihydroarteannuin B (**23) [**48**]**, deoxyarteannuin B (**24)** [49], dihydro-deoxyarteannuin B (**25)** [49], *epi*-deoxyarteannuin B **(26)** [40,50], and dihydro-*epi*-deoxyarteannuin B **(27)** [51] (Figure 5).

Two new members of the unusual cadinanolide series of sesquiterpenes, annulide (**28**) and isoannulide, (**29**) were characterized by NMR spectrum [52]. 6α-Hydroxy isoannulide (**30**) was reported in 1994 [53] (Figure 5).

A bisnor-sesquiterpene, norannuic acid (**31**) [54], and further two new cadinane sesquiterpenes (**32**, **33**) were isolated and reported [39]. Soon after, compound **34** (a pair of isomers) and **35** were described [55]. Another new

Table 1 Sesquiterpenes isolated from *Artemisia annua*

No. of Comp.	Trivial Name (s)	Plant Part	Ref.
1	qinghaosu, artemisinin, arteannuin	Aerial part	[6,7,25]
2	qinghaosu I, artemisinin A, arteannuin A	Aerial part	[25]
3	qinghaosu II, arteannuin B, artemisinin B	Aerial part	[25,33,34]
4	qinghaosu III, deoxyartemisinin, deoxyarteannuin	Aerial part	[25]
5	artemisinin C, arteannuin C	Aerial part	[47]
6	qinghaosu IV, arteannuin D, 3α-OH-deoxyartemisinin	Aerial part	[26]
7	qinghaosu V, artemisinin E, arteannuin E	Aerial part	[26]
8	artemisinin F, artemisilactone	Aerial part	[27]
9	artemisinin G	Leaf	[44]
10	artemisitene	Aerial part	[43]
11	artemisinic acid, qinghao acid, arteannuinic acid	Aerial part	[28,29,30]
12	dihydroartemisinic acid	Aerial part	[35]
13	artemisinic acid methyl ester	Aerial part	[31]
14	artemisinic alcohol	Aerial part	[37,38]
15	dihydroartemisinic alcohol	Aerial part	[38]

(*Continued*)

No. of Comp.	Trivial Name (s)	Plant Part	Ref.
16	artemisinic aldehyde	Aerial part	[38]
17	dihydroartemisinic aldehyde	Aerial part	[38]
18	cadin-4, 7(11)-dien-12-al	Aerial part	[39]
19	6,7-dehydroartemisinic acid	Aerial part	[40]
20	epoxyarteannuinic acid	Aerial part	[32]
21	dihydroartemisinic acid hydroperoxide	Leaf	[41]
22	amorpha-4,11-diene		[42]
23	dihydroarteannuin B		[48]
24	deoxyarteannuin B	Aerial part	[49]
25	dihydro-deoxyarteannuin B	Aerial part	[50]
26	(+)-deoxyisoartemisinin B, *epi*-deoxyarteannuin B	Aerial part	[40,50]
27	dihydro-*epi*-deoxyarteannuin B	Aerial part	[51]
28	annulide	Aerial part	[52]
29	isoannulide	Aerial part	[49]
30	6α-hydroxyisoannulide		[53]
31	norannuic acid	Aerial part	[54]
32	cadin-4(15),11-dien-9-one	Aerial part	[39]
33	3-isobutyryloxy-cadin-4-en-11-ol	Aerial part	[39]
34		Aerial part	[55]
35		Aerial part	[55]
36		Leaf	[56]
37		Leaf	[56]
38	Arteannuin H	Leaf	[57]
39	Arteannuin I	Leaf	[57]
40	Arteannuin J	Leaf	[57]
41	Arteannuin N	Leaf	[57]
42	Arteannuin K	Leaf	[57,58]
43	Arteannuin L	Leaf	[57,58]
44	Arteannuin M	Leaf	[57,58]
45	Arteannuin O	Leaf	[58]
46		Seed	[59]
47		Seed	[59]
48		Seed	[59]
49		Seed	[59]
50		Seed	[59]
51		Seed	[59]
52		Seed	[59]
53		Seed	[59]
54		Seed	[59]
55		Seed	[59]
56		Seed	[59]
57		Seed	[59]
58		Seed	[59]
59		Seed	[59]
60	Nortaylorione	Essential oil	[60]
61	3α, 5β-dihydroxy-4α, 11-epoxybisnorcadinane	Aerial part	[61]
62	Abscisic acid	Aerial part	[62]
63	Abscisic acid, methyl ester	Aerial part	[62]

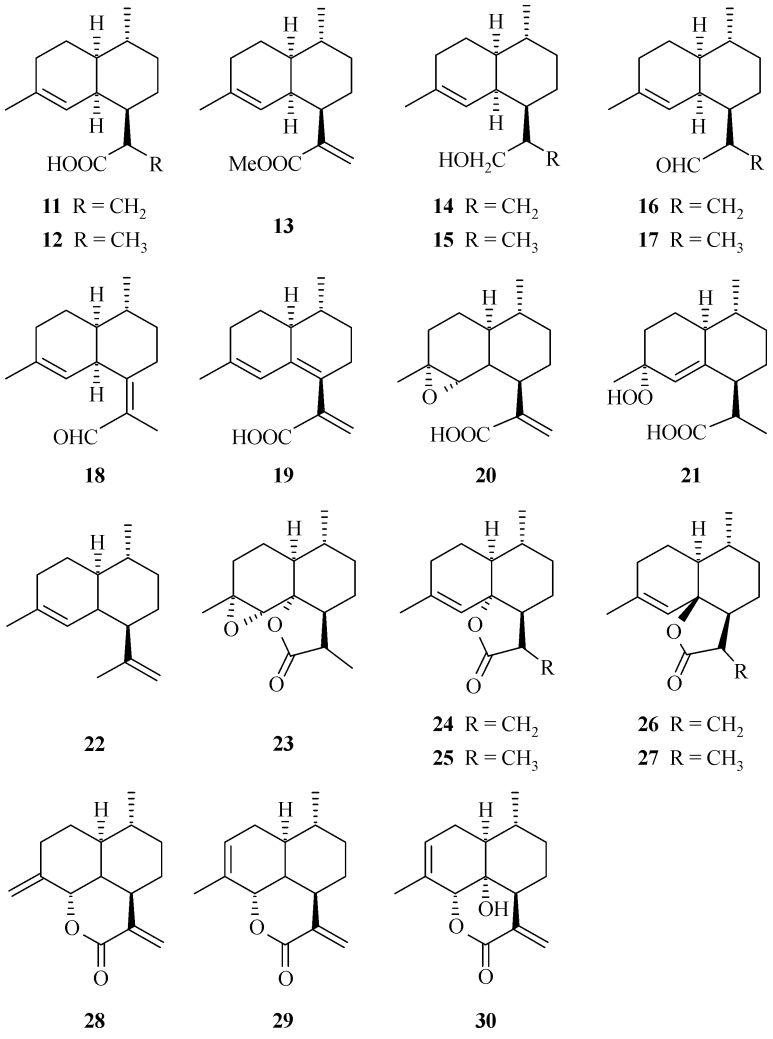


Figure 5 Structures of sesquiterpenes from *Artemisia annua* (11–30).

cadinane sesquiterpene (**36**) and a new eudesmane sesquiterpene (**37**) from the aerial parts of *A. annua* were reported in 1998 [56]. Seven new sesquiterpenes including a peroxylactone arteannuin H (**38**) and arteannuin I–M (**39**–**44**) were isolated [57,58]. These compounds were proposed to be biogenetically related to dihydroartemisinic acid (**11**) via some intermediate allylic hydroperoxides. A re-investigation of *A. annua* gave a novel cadinane diol, arteannuin O (**45**), the structure of which was established by 2D NMR and X-ray crystallography [58]. Synthesis of arteannuin O (**45**) from dihydro-*epi*-deoxyarteannuin B (**27**) led the authors to propose a structure revision of the stereochemistry claimed for the 5-OH group in arteannuins K (**42**), L (**43**) and M (**44**), as shown in their structures [58] (Figure 6).

In 2003, the first phytochemical investigation of natural products from the seeds of *A. annua* [59] led to the discovery of 14 new sesquiterpenes (**46**–**59**). The structures of all of these compounds were elucidated mainly from the results of 2D NMR spectroscopic studies including HMQC, HMBC, ^{1}H-^{1}H COSY, and NOESY. (+)-Nortaylorione (**60**), a norsesquiterpene, was described as a new natural product from *A. annua*. The structure elucidation including its relative and absolute configuration of compound **60** was not based on the real isolation from essential oil extract but was determined with the help of organic synthesis [60]. The new bisnor cadinane sesquiterpene **61** was lately isolated from *A. annua* [61]. In addition, two sesquiterpene plant hormones, abscisic acid (**62**), and its methyl ester (**63**) were found in an Indian growing *A. annua* [62] (Figure 7, Table 1).

Besides sesquiterpenes, several mono- (**64**–**69**), di- (**70**), and triterpenoids (**71**–**74**) have been obtained from *A. annua* [30,59,63–66] (Figure 8). Several common triterpenes were also found in *A. annua*, such as α-amyrenone, α-amyrin, β-amyrin, taraxasterone, oleanolic acid, and baurenol [39].

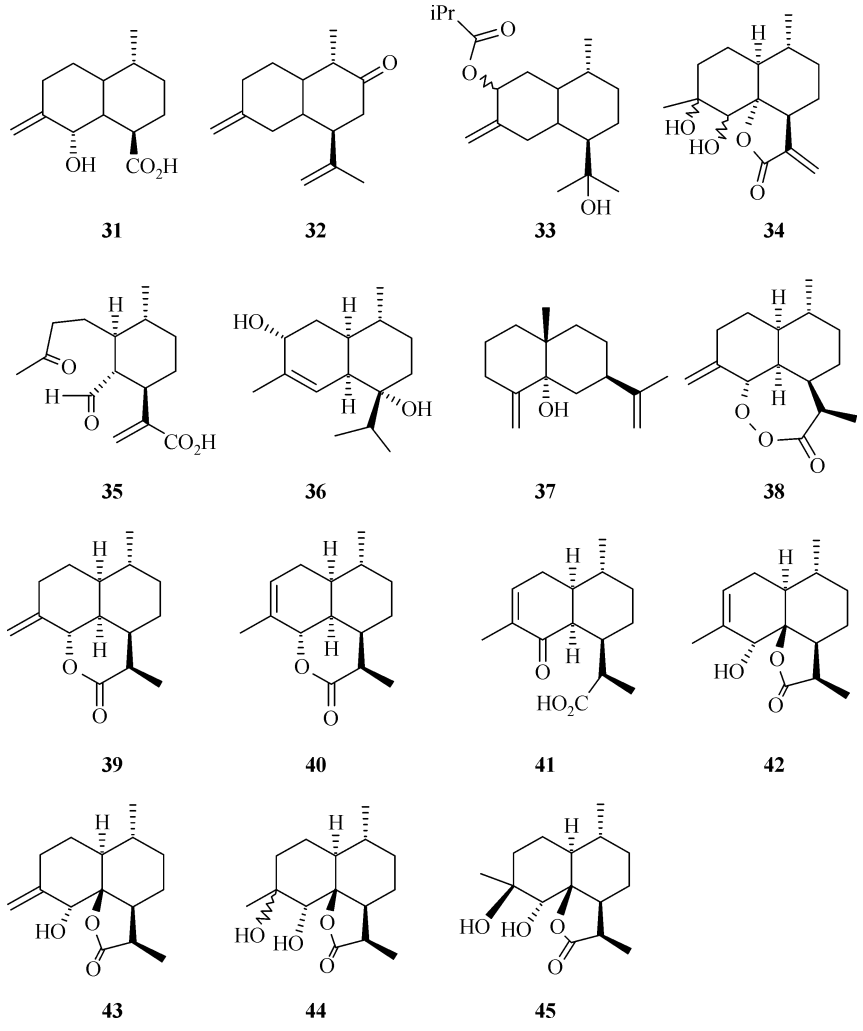


Figure 6 Structures of sesquiterpenes from *Artemisia annua* (31–45).

3.2 Essential oils from *A. annua*

Essential oil from *A. annua* was another active research interest as it could be potentially used in perfumery, cosmetics, and aromatherapy. Depending on its geographical origin, the oil yield in *A. annua* ranges between 0.02% and 0.49% on fresh weight basis and 0.04%–1.9% on dry weight basis [67]. Major components in the oil were reported to be artemisia ketone (**75**), isoartemisia ketone (**76**), 1, 8-cineole (**77**), and camphor (**78**) (Figure 9). GC/MS was employed to analyze the chemical composition in the essential oil, and more than 70 constituents have been identified. Germacrene-D and *trans*-β-farnesene were also found [37]. For more detailed information on the oil composition of essential oil from *A. annua*, the readers may refer to related references [26,37,64,68–77].

3.3 Flavonoids and coumarins from *A. annua*

Up to date, 46 flavonoids have been isolated from *A. annua* [26,62,65,74,78–80]. They are as follows: apigenin, artemetin, astragalin, axillarin, casticin, chrysoeriol, chrysosplenetin, chrysosplenol, chrysosplenol D, 3′-methoxy chrysosplenol, cirsilineol, cirsiliol, cirsimaritin, cynaroside, eupatorin, 2′, 4′, 5-trihydroxy-5′, 6, 7-trimethoxy flavone, 3′, 5, 7, 8-tetrahydroxy-3, 4′-dimethoxy flavone, 3, 3′, 5-trihydroxy-4′, 6, 7-trimethoxy flavone, 3, 5-dihydroxy-3′, 4′, 6, 7-tetramethoxy flavone, 4, 5, 5′-trihydroxy-3, 5, 6, 7-tetramethoxy flavone, 5-hydroxy-3, 4′, 6, 7-tetramethoxy flavone, 5-hydroxy-3, 4′, 6, 7-tetramethoxy flavone, isokaempferide, kaempferol, kaempferol-6-methoxy-3-*O*-β-D-glucoside, luteolin, luteolin-7-methyl ether, pachypodol, patuletin, patuletin-3-*O*-β-D-glucoside, penduletin, quercetagetin-3′, 4′, 6, 7-tetramethyl ether, quercetagetin-3, 4′-dimethyl ether, quercetagetin-3, 6-dimethyl ether, quercetagetin-4, 6′, 7-trimethyl ether, quercetagetin-4′-methyl ether, quercetin, quercetin-3′-*O*-β-D-glucoside, quercetin-3-methyl ether, quercimeritrin, isoquercitrin, retusin, rhamnentin, rutin, and tamarixetin.

About seven commonly occurring coumarins were found in

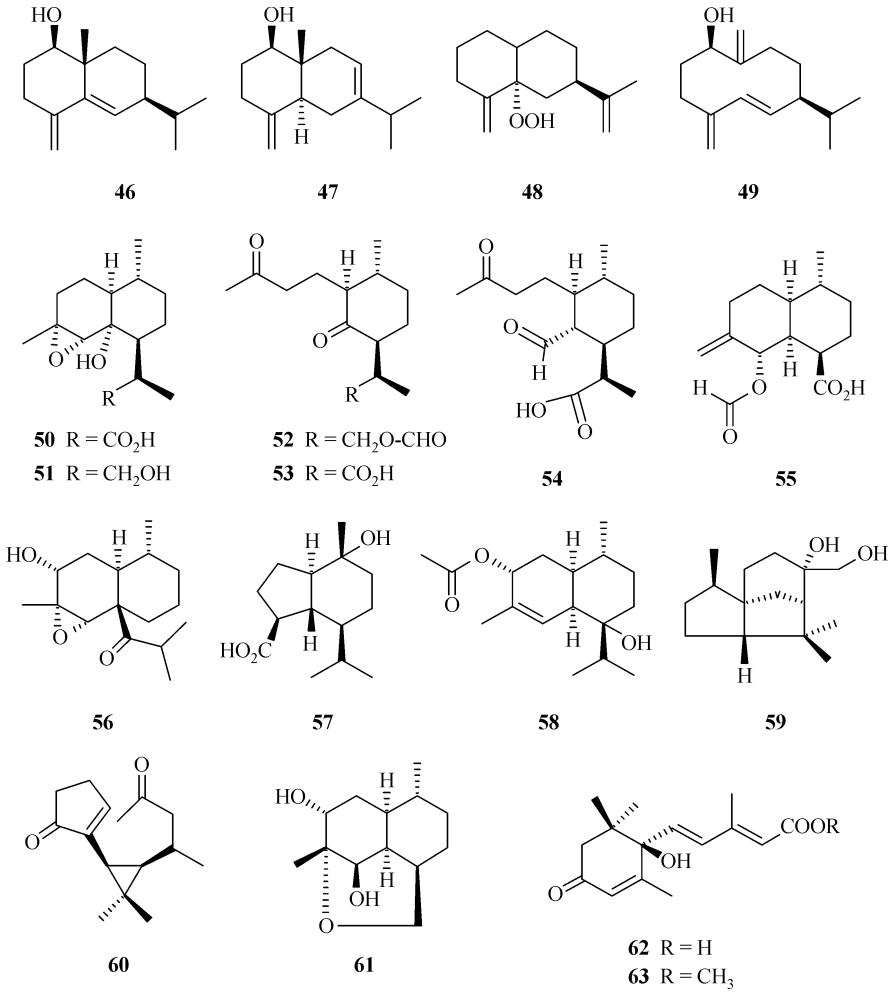


Figure 7 Structures of sesquiterpenes from *Artemisia annua* (46–63).

A. annua, namely, scopoletin, scopolin, aesculetin, 6, 8-dimethoxy-7-hydroxy coumarin, 5, 6-dimethoxy-7-hydroxy coumarin, tomentin, and coumarin [62,78–80].

3.4 Miscellaneous components and natural products from endophytes in *A. annua*

Two new chromene derivatives have been isolated from the aerial parts of *A. annua.* Their structures were resolved through normal NMR spectra as 2, 2-dihydroxy-6-methoxychromene (**79**) and 2, 2, 6-trihydroxychromene (**80**), respectively [80].

A novel cytokinin, 6-(3-methylbutylamino)-2-hydroxy-8, 9-dihydropurine (**81**) was obtained from a methanolic extract of the aerial part of Indian grown *A. annua* [81].

Two phenolic compounds have been described as new natural products from *A. annua.* The water-soluble part of an ethanol extract of the aerial parts afforded annphenone (**82**), a phenolic acetophenone. Column chromatography followed by HPLC of an Et_2O extract of the aerial parts yielded the new compound 5-nonadecylresorcinol-3-*O*-methyl ether (**83**), and its structure was deduced from NMR spectroscopy and confirmed by chemical synthesis [51,82] (Figure 10).

A new highly unstable polyacetylene (**84**) and the known polyacetylene ponticaepoxide (**85**) were obtained after repeated chromatographic purification of the crude petroleum ether extract of *A. annua* [83]. The new polyacetylene was named annuadiepoxide (**84**).

A new lipid constituent methyl 11, 12, 15-trihydroxy-13 (14)-octadecenoate (**86**) was recently isolated from the leaves of *A. annua* [61]. A lipophilic fraction of *A. annua* was found to contain aurantiamide acetate, a dipeptide (**87**) [84] (Figure 10).

It is interesting to note that the well known *A. annua* is seldom attacked by any of phytopathogenic fungi, which could be partially associated with the presence of endophytes [85]. Two endophytic fungi in *A. annua* have been phytochemically explored. From *Colletotrichum* sp., an

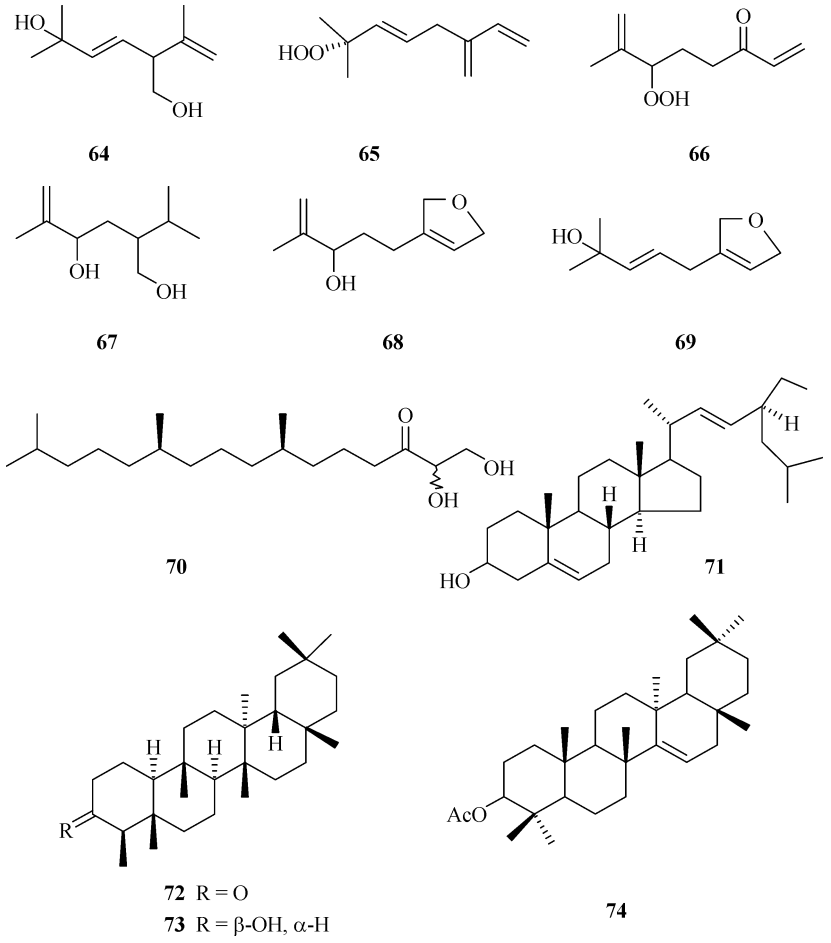


Figure 8 Structures of several mono-, di-, and tri-terpenoids from *Artemisia annua* (64–74).

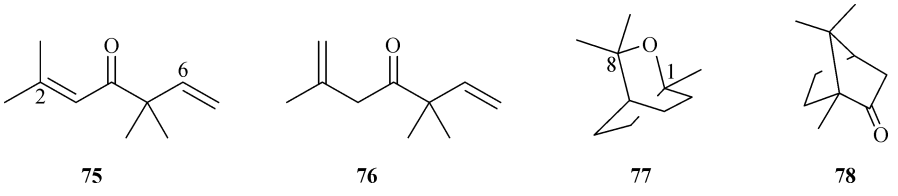


Figure 9 Structures of some constituents in the essential oil from *Artemisia annua* (75–78).

endophyte isolated from inside the stem of *A. annua*, 11 chemical constituents were isolated including three new antimicrobial metabolites [86]. Several known steroids were recorded as stigmasterol (**74**) [30,85,87], ergosterol [29], 3β, 5α, 6β-trihydroxyergosta-7, 22-diene, 3β-hydroxy-ergost-5-ene, 3-oxo-ergosta-4, 6, 8(14), 22-tetraene, 3β-hydroxy-5α, 8α-epidioxy-ergosta-6, 22-diene, 3β-hydroxy-5α, 8α-epidioxy-ergosta-6, 9(11), 22-triene, 3-oxo-ergost-4-ene, and plant hormone indole-3-acetic acid. Chemical structures of three new metabolites were elucidated by a combination of spectroscopic methods (IR, MS and NMR) as 6-isoprenylindole-3-carboxylic acid (**88**), 3β, 5α-dihydroxy-6β-acetoxy-ergosta-7, 22-diene (**89**), and 3β, 5α-dihydroxy-6β-phenylacetyloxy-ergosta-7, 22-diene (**90**), respectively. Two new metabolites with novel carbon skeletons, leptosphaerone (**91**), and leptosphaeric acid (**92**) were discovered from the AcOEt extract of endophytic fungus *Leptosphaeria* so. Iv 403 [88,89] (Figure 11).

To summarize, over 220 natural compounds, including QHS and other 62 sesquiterpenes, were identified in *A. annua*.

4 Structure determination of QHS [6,90]

The chemical structure of QHS posed a challenge to the researchers in the Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, and the Institute of Chinese

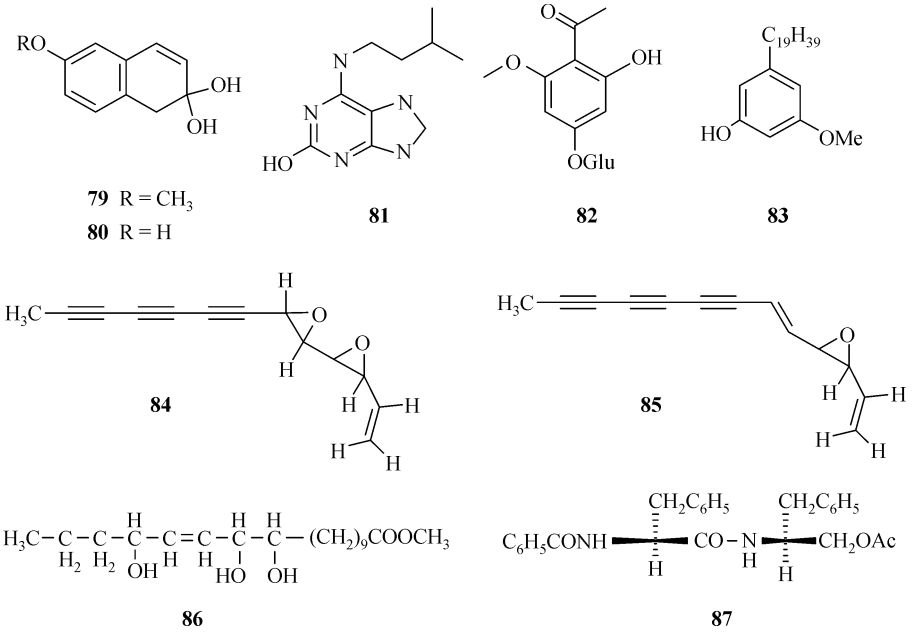


Figure 10 Structures of some constituents from *Artemisia annua* (79–87).

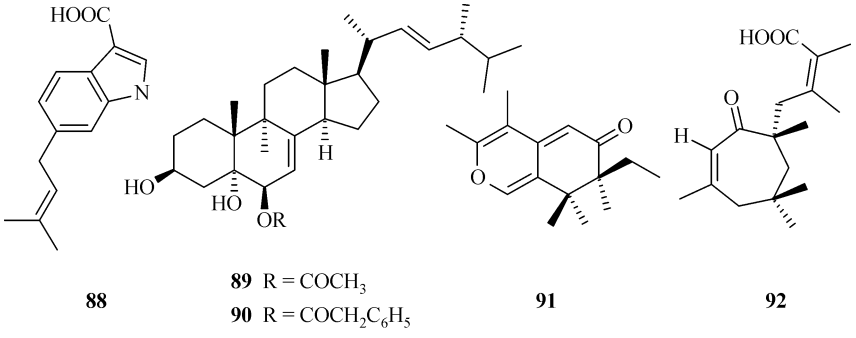


Figure 11 Structures of some constituents from *Artemisia annua* (88–92).

Materia Medica, China Academy of Chinese Medical Sciences, for hundreds days and nights in the period from 1973 to 1975, when QHS already showed its excellent antimalarial activity even in the clinic experience. With nationwide collaboration, the researchers collected all the important physical data (such as ^{1}H NMR, ^{13}C NMR, and HRMS) measured in the most advanced instruments available in China at that time. These data reveal that its molecular formula is $C_{15}H_{22}O_5$ with molecular weight 282, and the molecule type definitely does not belong to the alkaloid as quinine but likely to the sesquiterpene. However, it is not so easy to assign its exact structure. The major difficulty is how to arrange these five oxygen atoms in this 15-carbon-molecule skeleton with only one proton attached at the carbon bearing oxygen (5.68 in singlet) as appearing in the ^{1}H NMR spectrum. Considering the unstable property of usual peroxide compound, the researchers have negated for a time the idea that the stable multioxygenated QHS contains a peroxy group, until the peroxide structure of yingzhaosu A, another antimalarial natural product, was reported in a workshop in the early 1975 [8]. By the way, all the stereochemistry of yingzhaosu A was finally determined by total synthesis [91]. The peroxide group in QHS molecule was confirmed by simple qualitative analysis (NaI-AcOH) and quantitative analysis (PPh_3) soon after the researchers became aware of this probability. It was also recognized that the fragment 250 in the mass spectrum comes from the loss of molecular oxygen from QHS instead of the loss of a methanol as believed before. At the same time, the researchers also performed a series of chemical reaction studies for QHS. Based on all the physical data and the chemical properties, some fragments of molecule were deduced (Figure 12).

Afterward, referring to the structure of arteannuin B (**3**) isolated earlier also from *A. annua,* three possible structures

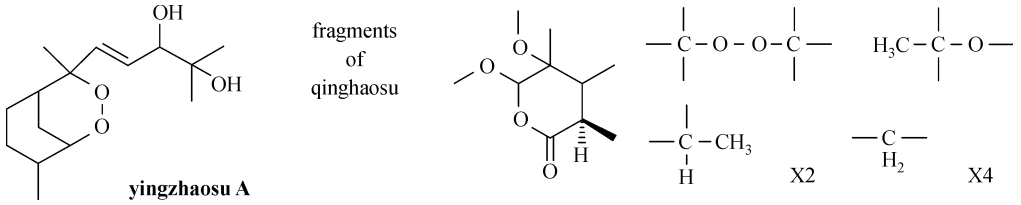


Figure 12 Yingzhaosu A and fragments of QHS.

(**93**, **94**, **95**) were suggested for QHS. As some peroxy lactones had been reported in the literature at that time, structure **93** was considered preferable (Figure 13).

The real structure **95** and the relative configuration of QHS were at last proved by X-ray crystal analysis. Finally, the absolute configuration was obtained by anomalous diffraction X-ray crystal analysis [92]. Thus, QHS has really an unprecedented unique structure with a 1, 2, 4-trioxane ring and an inter peroxyl ketal-actal-lactone consisting of a rare –O–O–C–O–C–O–C=O atomic chain (Figure 14). Up to now, none of the natural products with similar structure characteristics has been found in other plant species.

5 Chemical reactions and biotransformation of QHS

Since the discovery of QHS, its chemical reactivity and transformation have been exploited enthusiastically, which are very important not only for the structure determination of qinghaosu but also for the modification of QHS and development of new medicinal applications. Among them, the reduction and acidic degradation have received much more attention.

5.1 Reduction of QHS

The tandem peroxy, ketal, acetal, and lactone groups in the QHS molecule are all reducible under different reaction conditions, while usually, the peroxy group or lactone could be selectively reduced first.

The peroxy group can be reduced by hydrogenation in the presence of palladium/charcoal to afford a dihydroxy intermediate, which in turn converts to a stable product deoxyqinghaosu (**4**) under stand or by treatment with a catalytic amount of acid [90]. This one-oxygen-less product could also be obtained by reduction with zinc dust-acetic acid [93]. Recently, it was found that the reagent NaI-AcOH of qualitative analysis for peroxy group mentioned above could convert QHS to deoxyqinghaosu, too, but the conversion had only 27% yield. Bromide, unlike iodide, could not reduce qinghaosu under the same reaction condition. The reaction of triphenyl phosphine (PPh_3) with QHS applied to the

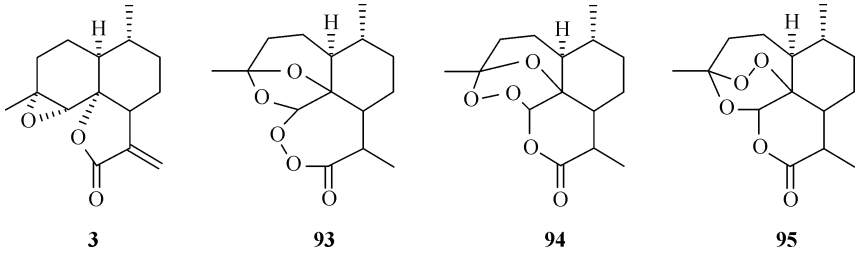


Figure 13 Arteannuin B and three proposed possible structures for QHS.

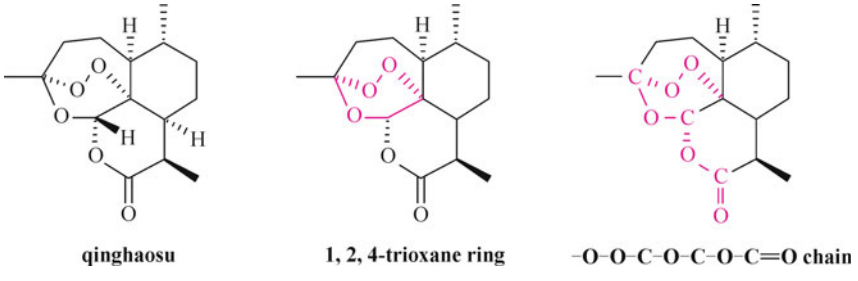
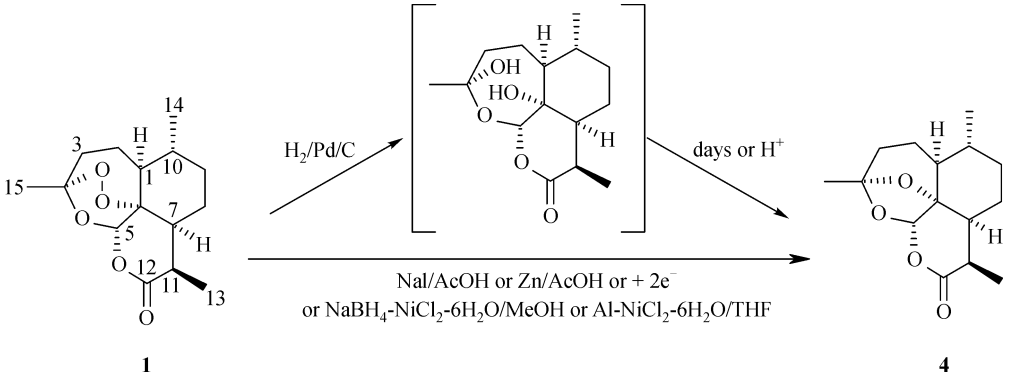


Figure 14 QHS and its structure characters.

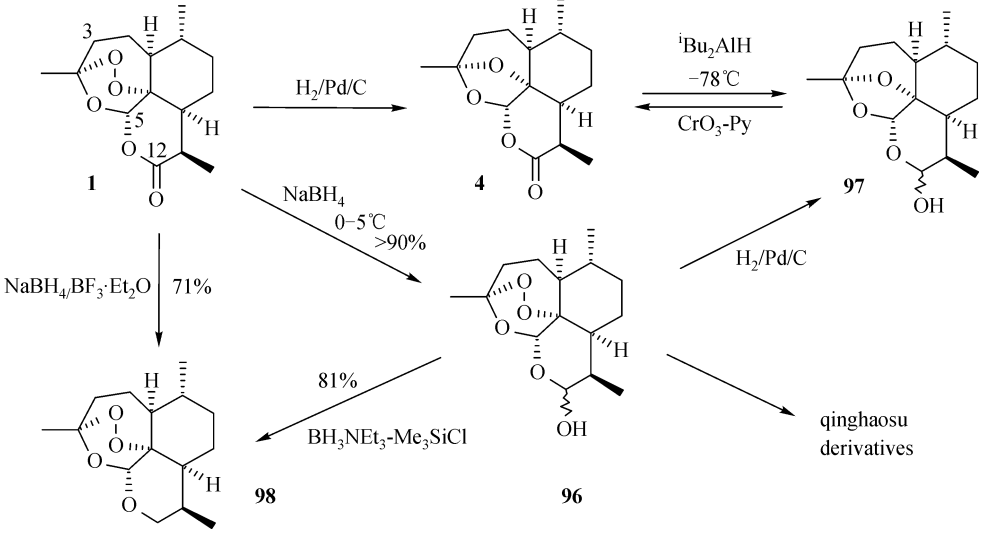
quantitative analysis of peroxy group is quite complicated; however deoxyqinghaosu still could be separated from the product mixture in 23% yield [94]. The electro-chemical reduction of QHS has been reported recently from several research groups, and a two-electron irreversible reduction process was observed [95–100]. When we repeated this slow electro-chemical reduction, deoxyqinghaosu (**4**) was found to be the only product [94]. In 2007, Bhattacharya reported that treatment of QHS with $NaBH_4$-$NiCl_2 \cdot 6H_2O$ in MeOH or Al-$NiCl_2 \cdot 6H_2O$ in THF all yielded deoxyqinghaosu (**4**) in nearly quantitative yields, instead of the desired lactone reduced product (Scheme 1) [101]. Another important reaction of the peroxy group is the single-electron reduction with ferrous ion, copper (I) ion, *etc*., which is related to the antimalarial mechanism and will be discussed in the later section in detail.

The lactone of QHS could be selectively reduced with mild hydride reducing agents (such as sodium borohydride, potassium borohydride, lithium borohydride, *etc*.) to lactol, called dihydroqinghaosu or dihydroartemisinin (DHA) (**96**), in over 90% yields [90]. It is an unusual reduction, because normal lactone could not be reduced with sodium borohydride under the same reaction condition (0°C–5°C, in methanol). It was the first time to be observed that the lactone might be reduced, but the peroxy group in the same molecule survived. However, the lactone of deoxyqinghaosu (**4**) resisted reduction with sodium borohydride and could only be reduced with isobutyl aluminum hydride to lactol, deoxydihydroqinghaosu (**97**) [90], which was identified with the product from hydrogenation of **96** (Scheme 2). These results show that the peroxy group assists the reduction of lactone with sodium borohydride to lactol but not to the over reduction product alcohols. Up to now, there is still no clear explanation for this reduction process. The easy availability of DHA makes the derivation of QHS possible, on which there will be a detailed discussion in the following section (*vide infra*). QHS can be further reduced with sodium borohydride in the presence of boron trifluoride to deoxoqinghaosu (**98**) [102]. Deoxoqinghaosu can also be obtained by reduction of **96** with BH_3NEt_3 and Me_3SiCl in DME (Scheme 2) [103].

The more powerful reducing agent lithium aluminum



Scheme 1 Reduction of peroxy group in QHS.



Scheme 2 Reductions of QHS.

hydride reduces not only lactone and peroxy group but also acetal and ketal to yield the exhaustively reduced product **99** and partially reduced products (Scheme 3) [104,105].

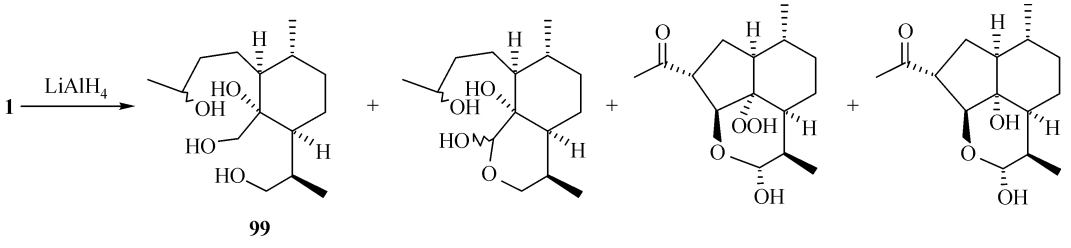
5.2 Acidic degradation of QHS

Treatment of QHS in a mixture of glacial acetic acid and concentrated sulfuric acid (10∶1) at room temperature yields a mixture of one-carbon-less products, from which a number of ketone-lactone or α, β-unsaturated ketone derivatives can be isolated [106]. X-ray crystal analysis of the major component **101** shows that its C-7 configuration is inverted in comparison with that of QHS [107]. An intermediate **100** for the formation of these products has been proposed (Scheme 4).

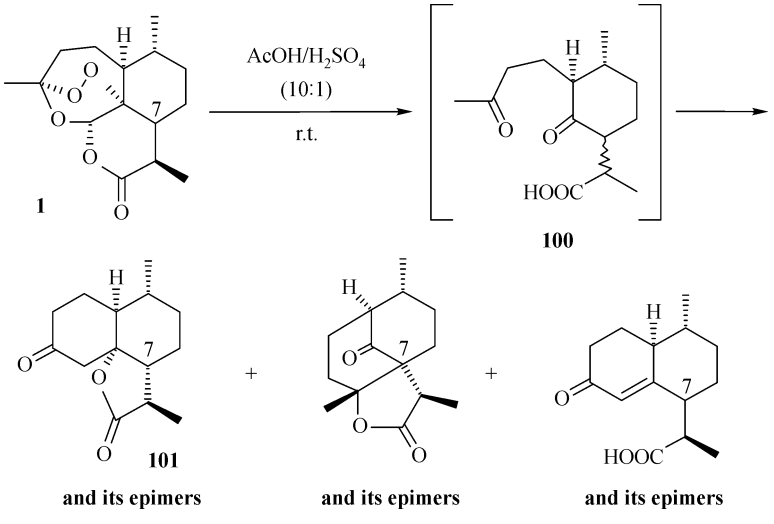
In a continued investigation, it was found that refluxing of a solution of QHS and a catalytic amount of acid in methanol afforded a mixture of methyl esters **102**, the treatment of which with glacial acetic acid and concentrated sulfuric acid (10∶1) in 0°C–5°C, gave in turn a C-7 configuration reserved diketone ester **103** and small amount of recovered QHS. The overall yield based on the recovered starting material could be over 90%. Intermediate **102** could be purified and identified. It can be reduced to deoxyqinghaosu (**4**) or peroxy reserved lactone **104** with different reagents. In the presence of a catalytic amount of acid, the isolated sample of **102** could be ring-closed and regenerated to QHS (Scheme 5) [93]. Diketone ester (**103**) is a useful relay intermediate for the synthesis of QHS and its derivatives and will be mentioned in the following sections. Later, Lee also reported that the treatment of QHS in methanol or ethanol with TsOH or 14% hydrochloric acid afforded the methyl or ethyl esters of **102** and **103** but in quite low yields [108]. Twenty years later, Singh reported the same ring open reaction once again, and the synthesis of tricyclic derivatives of qinghaosu from **102** [109].

5.3 Degradation in alkaline medium

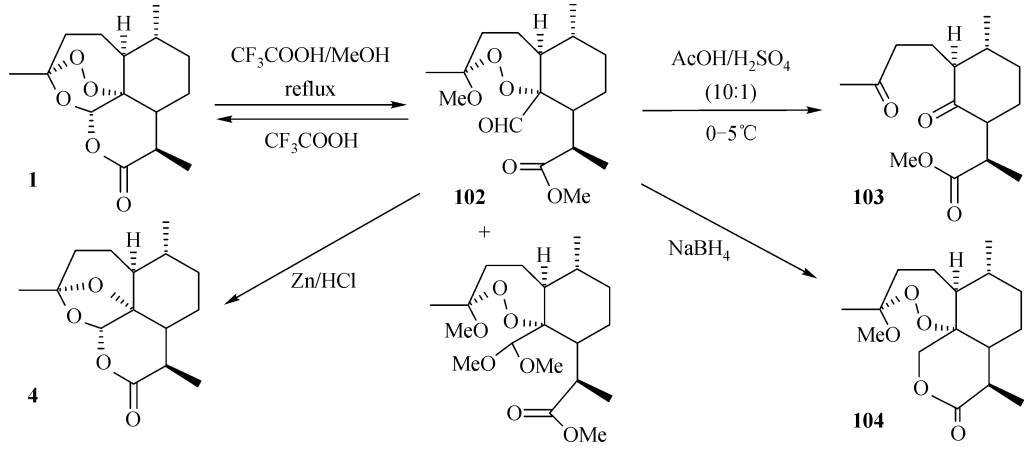
QHS is unstable in alkaline medium, and it may rapidly decompose in potassium carbonate-methanol-water at room temperature to complicate products, from which octahydroindene **105** can be isolated in 10% yield [90]. In 1983, Liang *et al.* repeated this reaction and suggested that the *trans* stereochemistry of lactone in **105** should be changed to *cis* as in **106** [110]. In the mean time, they also reported that the treatment of QHS with dilute sodium hydroxide in EtOH-H_2O might produce a cyclic enolate product **107** with a UV peak at 292 nm in 88% conversion and 15% isolated yield. This



Scheme 3 Reduction of QHS with $LiAlH_4$.



Scheme 4 Acidic degradation of QHS.



Scheme 5 Other products from acidic degradation of QHS.

reaction has been accepted as the pretreatment procedure for the quantitative analysis of QHS in the *Chinese Pharmacopoeia*. Further treatment of compound **107** in refluxing 5% NaOH gave a mixture, from which a small quantity of compound **101**, the same as an acidic degradation product (*vide supra*), could be identified [110] (Scheme 6).

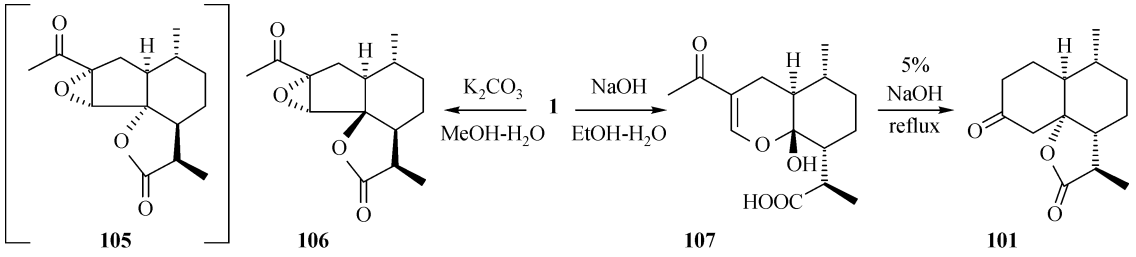
5.4 Pyrolysis

QHS is a quite stable compound versus those common peroxides; no decomposition is observed even at its melting point 156°C–157°C. However, pyrolysis takes place at 190°C for 10 min, giving a product mixture, from which compounds **108** (4%), **9** (12%), and **6** (10%) could be separated (Scheme 7) [45,46]. It is interesting that the later two compounds are also the components of Qinghao (*A. annua*) [26,44], the metabolites of qinghaosu *in vivo* [111,112], and the reaction products of qinghaosu with ferrous ion (*vide infra*).

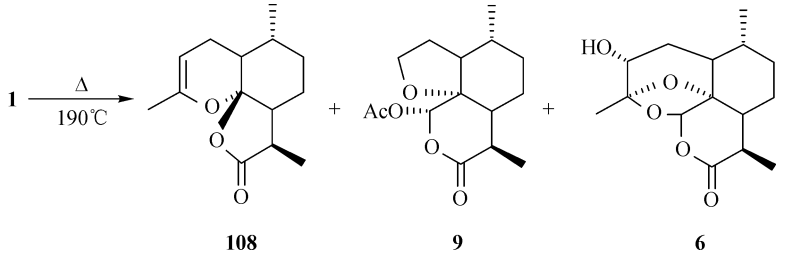
5.5 Biotransformation

Microbial transformation study can be served as a model for the study of QHS metabolism in the mammalian and can also blaze the new pathway to QHS derivatives. Thus, a number of research groups have endeavored to the transformation of QHS with different microbes and found that a hydroxy group can be introduced in some chemically inactive carbon positions of QHS.

It was reported in 1989 that QHS could be transferred to deoxyqinghaosu (**4**) by *Nocardia corallinaz* and 3α-hydroxy-deoxyqinghaosu (**6**) by *Penicilliam chrysogenum* in low yields [113], whereas in 2002, the biotransformation products



Scheme 6 Alkaline degradation of QHS.



Scheme 7 Pyrolysis of QHS.

9β-hydroxy-qinghaosu (**109**), 3β-hydroxy-qinghaosu (**110**), **4** and **6** with *Mucor polymorphosporus*, and **6** and 1α-hydroxydeoxyqinghaosu (**111**) with *Aspergillus niger* were identified by Guo's group [114]. In another report, 10β-hydroxyqinghaosu (50%) (**112**) was obtained with *Cunninghamella echinulata* [115]. In 2004, Williamson's group reported a rational and economical bioconversion of QHS to 9β-hydroxy-qinghaosu (**109**) in 78.6% yield by *Cunninghamella elegans*, and in the meantime, three other bioconversion products: 9β-hydroxy-11α-qinghaosu (**113**) (6.0%), **6** (5.4%), and **112** (6.5%) were also isolated [116]. In 2005, they reported a similar bioconversion of qinghaosu to 9β-hydroxy-qinghaosu (**109**) and 10β-hydroxyqinghaosu (**112**) by *Mucor ramannianus* strains [117]. One year later, the same group performed the transformation by the fungi *Eurotium amstelodami* and *Aspergillus niger* and isolated 2β-hydroxyqinghaosu (**114**) (63%) and 9β-hydroxyqinhaosu (**109**) (32%) from the extract of *E. amstelodami*, in 80% and 19% yields, respectively, from the extract of *A. niger* [118] (Figure 15). The later result was quite different in comparison with Guo's reports [114,115], where only derivatives of deoxyqinghaosu were obtained.

Microorganism *Streptomyces griseus* ATCC 13273 was recently also applied to the biotransformation of QHS, and artemisitone-9 (**115**) (12.5%), 9α-hydroxyqinghaosu (**116**) (16.5%), 9β-hydroxyqinghaosu (**109**) (16.1%), and 3α-hydroxydeoxyqinghaosu (**6**) (9.5%) were isolated [119] (Figure 15). It is worthy to notice that compound **6** might be the reaction product of QHS with ferrous ion, a reducing agent, in the incubating medium.

It is interesting that in a recent report, the biotransformation product **112** also could be selectively obtained from QHS by H_2O_2 oxidation in the presence of an iron (Fe)-based small molecule catalyst [120].

In the meantime, there were several reports about the microbial transformation of artemether, arteether, artemisitene, and deoxoqinghaosu, from which several 1α, 2β, 9α, 9β, 14-hydroxy derivatives and the products derived probably from the reaction with ferrous ion in the incubating medium were identified [121–127].

6 Study on the chemical synthesis and biosynthesis of QHS

6.1 Partial synthesis and total synthesis of QHS

Due to the exceptional antimalarial activity and unique structure, QHS has become an attractive target of organic synthesis since its discovery in 1970s. The first two syntheses published in 1983 were simultaneously utilizing [2 + 2] addition of enol ether with singlet oxygen followed by treatment of acid as the key step to induce the essential peroxy group. In the first report, starting from commercially available (-)-isopulegol (**117**), Hoffmann-La Roche's group synthesized the substrate (**118**) of photooxidation as shown in route **A** [128], whereas in the SIOC group, another component artemisinic acid (**11**) in *A. annua* was used as the starting material to synthesize **118** through **119** and dihydroartemisinic acid (**12**), which was then synthesized from another monoterpene citronellal (**120**) via **117**, as depicted in route **B** [129–132]. Based on these pioneering researches on the conversion of **118** to QHS (**1**), route **C** used a degradation product **103** as the relay intermediate to conveniently prepare the key intermediate **119** and finished a formal synthesis of **1** [133].

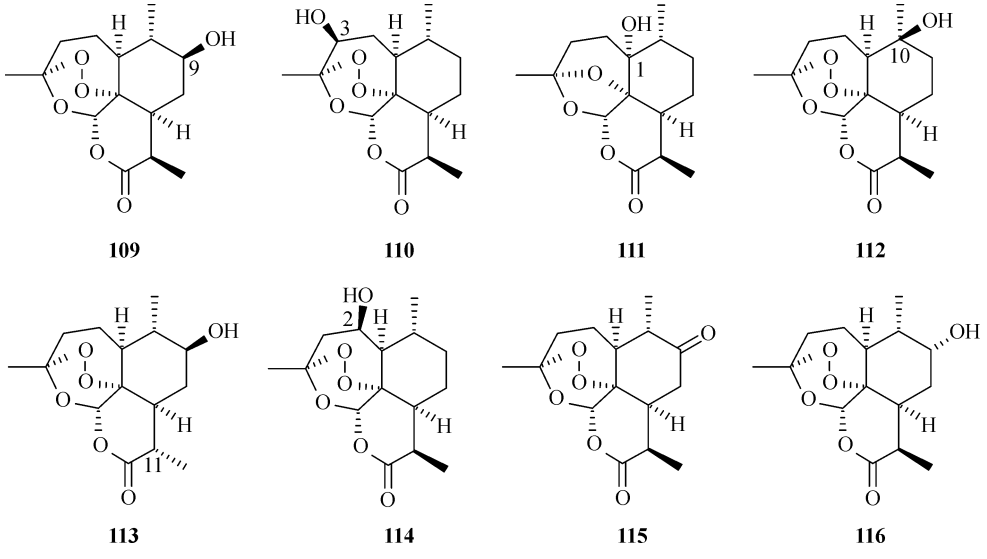


Figure 15 Products from microbial transformation of QHS.

Intermediate **103** can also be synthesized from **120** [134]. Following this protocol, both route **D** [135] and route **E** [136,137] published in 1990 and 2003, respectively, were using (+)-isolimonene (**121**) as the common starting material and the intramolecular Diels-Alder reaction or iodolactonization followed by Michael addition, and the Wittig reaction was respectively taken as the key steps to afford the key intermediates **12** and **118**. Hence, formal synthesis of QHS occurred. In another route **F** reported in 1992 and 1998, arteannuin B (**3**) in *A. annua* was taken as the starting material, and the deoxygenation followed by ozonization gave the intermediate **118** and finally QHS and deoxoqinghaosu (**98**) [138,139]. Starting also from dihydroartemisinic acid (**12**), as shown in route **B**, but using cyclo-enol ether **122** as the substrate of [2 + 2] addition with singlet oxygen, QHS (**1**) could be selectively synthesized in better overall yield in route **G** [140,141]. Besides this advantage, a more active compound deoxoqinghaosu (**98**) could be also synthesized as the penultimate product in route **G** (Scheme 8).

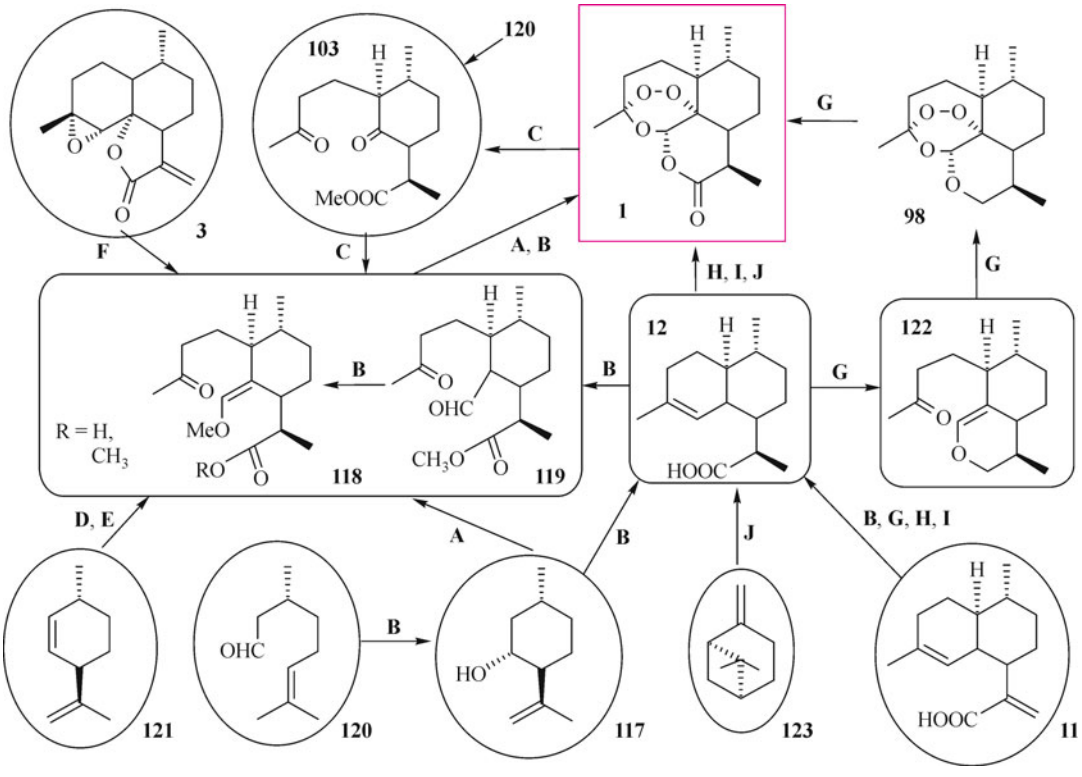
The so-called biomimetic synthesis of **1** from **12** in a manner of direct photooxidation in the route **H** [142,143] and route **I** [144] was reported in 1989, 1992, and 1990. The route **J** reported in 1993 was first the synthesis of **12** from α-pinene (**123**) and then to QHS (**1**) according to the route **H** [145] (Scheme 8).

The exceptional synthesis of QHS (**1**) is the utilization of the ozonization of vinylsilane to build the peroxy group instead of the photooxidation methods [146,147] (Scheme 9).

All of the above chemically synthetic routes to QHS have demonstrated their academic excellence; however, the yield of the key step for the formation of peroxy group is not always so satisfactory and is difficult to repeat. Therefore, new methodology for the synthesis of QHS is yet desired.

6.2 Biogenesis of QHS

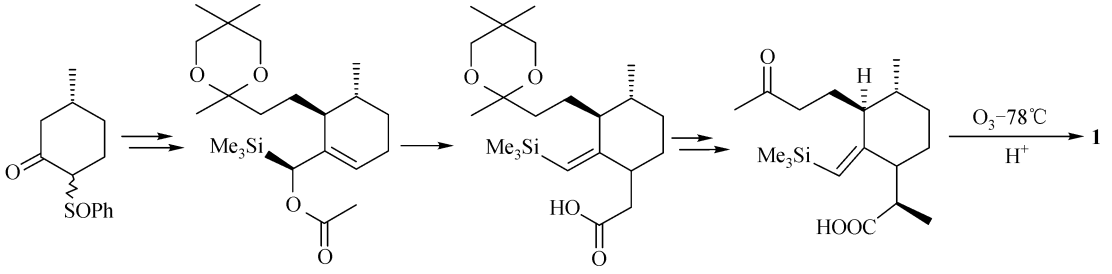
Since the 1980s, several laboratories have paid attention to the biogenesis of QHS. From the biogenesis point of view, QHS as a sesquiterpene seems to be synthesized from mavelonic acid lactone (MVA, **124**), therefore, three laboratories have found that QHS could be synthesized indeed from MVA or isopentenyl pyrophosphate (IPP, **125**), as they were incubated with the homogenate prepared from the leaves of Qinghao (*A. annua*) [148–151]. Based on the consideration that there are some plausible biogenetic relationships among three major compositions **1**, **11**, and **3** in Qinghao, Wang-Huang succeeded in the incorporation of MVA into **11** in Qinghao and then realized the biotransformation of **11** into **1** and **3** in the homogenate [148,149]. These experiments confirmed the previous proposal that **11** is the biosynthesis precursor of **1**. Further studies on the biotransformation of **11** also found that epoxide **20** was the precursor of **3**, but not the precursor of **1,**



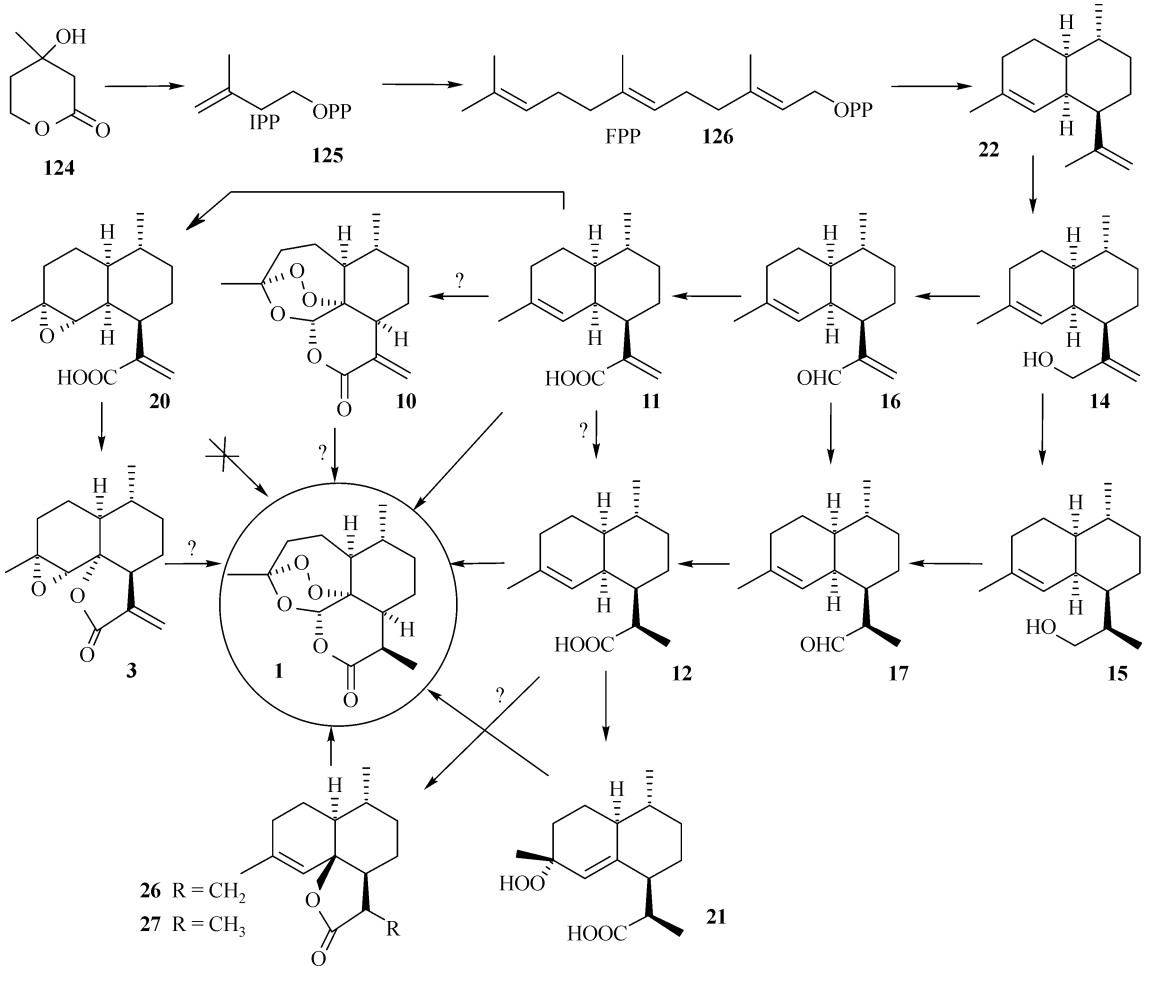
Scheme 8 Routes of total synthesis of QHS.

and **3** was not the precursor of **1**. Another Qinghao component **12** also can be biogenetically transferred into **1**, but it is not clear whether artemisinic acid (**11**) converts to **1** through dihydroartemisinic acid (**12**) or artemisitene (**10**) [152] (Scheme10).

Since then several laboratories also reported their research progress that artemisinic acid (**11**) was confirmed to be the biogenetic intermediate of QHS, and dihydroartemisinic acid (**12**) could be biotransferred to QHS too [153]. Furthermore, on the incubation with the cell free extracts of Qinghao leaves, artemisinic acid (**11**) could also be transferred into **3**, and in turn, **3** as well as dihydroarteannuin B (**23**) could be transferred to QHS. Therefore, they concluded that **3** was the biogenesis precursor of QHS, but it has not been mentioned whether **3** is the necessary intermediate for the biosynthesis of QHS [154–156]. It is unclear whether the different conclusions about the role of arteannuin B (**3**) came from the different experimental conditions, isotopically labeled precursor in the homogenate [152] versus unlabeled precursor in a cell free system [155]. The detailed process of



Scheme 9 Synthesis of QHS by ozonization of vinylsilane.



Scheme 10 Biogenesis of QHS.

conversion of artemisinic acid (**11**) or artemisinin B (**3**) into QHS has not been understood yet, though Wang *et al*. have suggested that deoxyisoartemisinin B (**26**) and dihydrodeoxyisoartemisinin B (**27**) should be the intermediates in the conversion of **11** and **12** to QHS [157]. It has been shown that photo-irradiation will accelerate this conversion. However, it is still uncertain whether there are some enzymes to catalyze a photooxidation in the biosynthesis of QHS. Recently, Brown *et al*. have performed an *in vivo* transformation with 15-labeled dihydroartemisinic acid by feeding it via the root to intact *A. annua* and concluded that allylic hydroperoxide (**21**) was the key intermediate in the conversion of **12** to **1** and its other congeners, and this conversion maybe did not need to invoke the participation of enzymes [158]. This biogenetic synthesis has been employed to prepare isotopically labeled QHS [159].

According to the general biogenetic pathway of terpenoid, sesquiterpenes of cadinane type, such as artemisinic acid, might be formed from farnesyl pyrophosphate (FPP, **126**), which is at first catalytically cyclized by amorpha-4, 11-diene synthase to this bicyclic diene **22**. In 1999, Bouwmeester's group detected and confirmed for the first time the presence of this diene (**22**), albeit in minute quantities, in leaf extracts of greenhouse-grown *A. annua*, and they also isolated the partially purified amorpha-4, 11-diene synthase. These results reveal that amorpha-4, 11-diene (**22**) is the likely intermediate in the biosynthesis of artemisinic acid (**11**) and then QHS and other cardinane sesquiterpenes. In addition, the low content of **22** in *A. annua* exposes that its enzymatic formation is likely the rate-determining step in QHS biosynthesis [160]. The stereospecific mechanism for cyclization of FPP (**126**) into amorpha-4, 11-diene (**22**) by amorpha-4, 11-diene synthase has been recently explored, too [161,162]. The next biosynthetic step of artemisinic acid (**11**) from amorpha-4, 11-diene (**22**) was reinvestigated by the isolation of putative intermediates and the enzymes involved [163]. Oxygenated amorpha-4, 11-diene derivatives artemisinic alcohol (**14**), dihydroartemisinol (**15**), artemisinic aldehyde (**16**), dihydroartemisnic aldehyde (**17**), and dihydroartemisinic acid (**12**) were isolated from *A. annua* leaves and gland secretory cells. Moreover, a series of enzyme assays with these alcohols or aldehydes as substrate and with or without NAD/NADP or NADH/NADPH as cofactors have been performed. In conclusion, it was suggested that the route, amorpha-4, 11-diene (**22**) → artemisinol (**14**) → artemisinic aldehyde (**16**) → dihydroartemisinic aldehyde (**17**) → dihydroartemisinic acid (**12**) → QHS (**1**), is the one most likely to occur in *A. annua*. As mentioned before, the conversion of artemisinic acid to QHS is confirmed. Therefore, this suggested that the route might just be the one of several biogenetic pathways. Meanwhile, a series of papers have reported that CYP71AV1, a cytochrome P450 monooxygenase from *A. annua* is involved in the hydroxylation of amorpha-4, 11-diene (**22**) and the oxidation of artemisinol (**14**) to artemisinic aldehyde (**16**) and then to artemisinic acid (**11**) [164,165], so there are probably several routes from amorpha-4, 11-diene (**22**) to QHS through artemisinic acid (**11**) or dihydroartemisinic acid (**12**) (Scheme 10).

Since the 1990s, several attempts have been made to enhance QHS production in the cell and tissue culture by omittance or addition of medium components, precursor feedings, light irradiation, temperature and modulating the biosynthesis route, *etc*. [153,166–170]. However, at present time, the biosynthesis approach is still a research project in the laboratory.

On the other hand, the progress in the production of QHS is also made from the selection and breeding of high yielding cultivars [171,172]. In this respect, a hybrid line containing up to 1.4% QHS on dry leaf basis has been obtained by selection and crossing, in wild populations, of genotypes with high QHS concentration.

The genetic engineering of Qinghao (*A. annua*) has also received great attention recently, and a number of preliminary results about the early stage of QHS biosynthesis have been reported [164,173]. For example, amorpha-4, 11-diene synthase, an enzyme responsible for the cyclization of farnesyl diphosphate to cyclic sesquiterpene, has been expressed in *Escherichia coli*, and the production of amorpha-4, 11-diene (**22**) identified [174,175]. Shortly thereafter, it was reported that the concentration of **22** produced by engineered *E. coli* in culture medium could reach 0.5 g/L [176]. In this respect, the most successful result is the engineering of *Saccharomyces cerevisiae* to produce high titers (up to 100 mg/L) of artemisinic acid using an engineered mevalonate pathway, amorphadiene synthase, and cytochrome P450 CYP71AV1 [177].

Recently, it has also been reported that farnesyl diphosphate synthase (FPS) was overexpressed in high-yield *A. annua*. The highest QHS content in transgenic *A. annua* was approximately 0.9% (dry weight), which was 34.4% higher than that in nontransgenic *A. annua* [178]. A preliminary report about abiotic stress-induced (chilling, heat shock, or UV light irradiation) expression of amorpha-4, 11-diene synthase gene (ADS) and cytochrome P450 monooxygenase gene (CYP71AV1) in *A. annua* was published in 2008 [179]. They declared that the transcription levels of ADS and CYP71AV1 were upregulated, and the QHS content in the prechilled plants was enhanced by 95.6%. In this respect, they also reported the cloning of QHS biosynthetic cDNAs and novel expressed sequence tags and the quantification of low temperature-induced gene overexpression in details [180].

In summary, all the studies on chemical synthesis or

biosynthesis of QHS, especially the introduction of the peroxy group into the molecular skeleton, are very impressive, but the aim of commercially supplying QHS through these approaches is still a long way to go, as the present cost of natural QHS is only 2000–3000 RMB (300–400 USD) per kilogram.

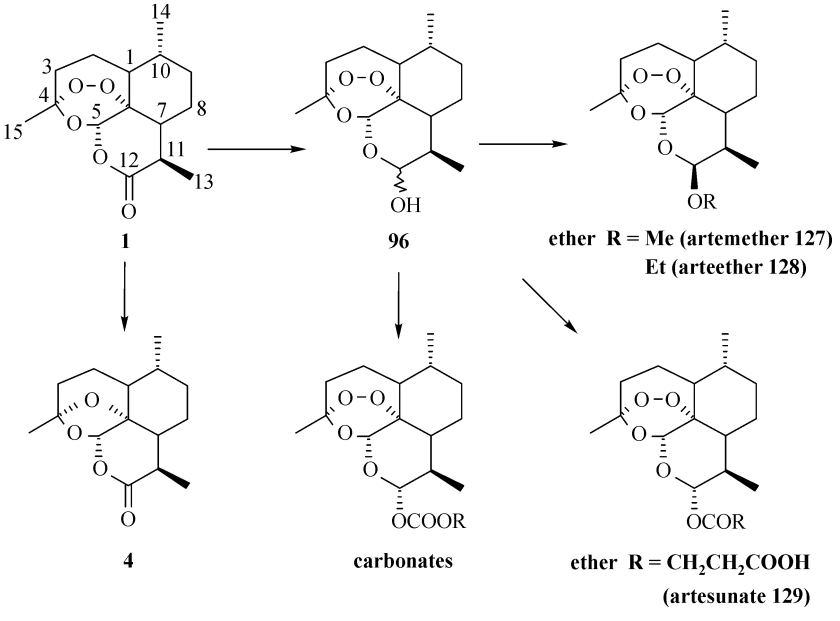
7 Synthesis and antimalarial activity of QHS derivatives and analogs

Early pharmacological and clinic studies showed that QHS possessed fast action, low toxicity, and high activity on both drug-resistant and drug-sensitive malaria. However, the formulation problem caused by its poor solubility in water or oil and high rate of parasite recrudescence were also observed. Therefore, the structure modification of QHS for the improvement of its antimalarial efficacy and solubility was in need.

It has been noted that QHS has a special structure segment of O–O–C–O–C–O–C–O, being quite different from those of all known antimalarial drugs. To determine the antimalarial activity of the primary chemical structure, the function of peroxy group for antimalarial activity was first examined [181,182]. The negative result of deoxyqinghaosu (**4**) against *P. berghei* in mice showed that the peroxy group was essential. Soon afterward, it was found that some other simple peroxides including monoterpene ascaridol had no antimalarial activity. These facts demonstrated that a peroxy group is an essential but not a sufficient factor. Thus, it was concluded that the O–O–C–O–C–O–C–O segment or whole molecular skeleton might play an important role in antimalarial activity at that time.

When DHA (**96**) was found to be more active than QHS and the introduction of a hydroxy group into the molecular nucleus could not improve its solubility in water, a plan of preparing its three types of derivatives (ethers, carboxylic esters, and carbonates) emerged. During 1976–1977, over 50 derivatives of DHA (Scheme 11) were synthesized and evaluated in China [182].

The first 25 compounds (in oil solution) were tested in mice infected with chloroquine-resistant *P. berghei* by administration of intramuscular injection [183]. Most of these derivatives showed higher activity than QHS (SD_{50} 6.50 mg/kg) and DHA (SD_{50} 3.65 mg/kg). In the ether series, SM 224 (R = CH_3, SD_{50} 1.02 mg/kg) is more active than SM 227 (R = C_2H_5, SD_{50} 1.95 mg/kg) and others. Then, SM 224, SM 108 (ester, R = CH_3, SD_{50} 0.66 mg/kg), and SM 242 (carbonate, R = *n*-C_3H_7, SD_{50} 0.50 mg/kg) were selected and compared with regard to their activity, stability, toxicity, and production cost. Because SM 224 was very soluble in oil and more stable than others, it became the candidate drug and was named artemether by the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. At the same time, QHS suppository and water-soluble sodium artesunate [184] were developed by the Institute of Chinese Materia Medica, the Academia of Chinese Traditional Medicine, and Gulin Pharmaceutical Factory, respectively. After long period of efforts [185–189], QHS suppository, sodium artesunate injection, and artemether oil injection were approved as new antimalarial drugs by the Chinese authority in 1986–1987. Since then, almost all patients with falciparum malaria were treated with QHS derivatives in China. However, QHS



Scheme 11 Synthesis of QHS derivatives.

suppository has not been manufactured and used in clinic officially, probably due to some problems in its clinical application and storage.

In the recent years, QHS was produced on the scale of more than 100 tons each year, and the yield of artesunate and artemether has continuously increased in China, some Asian and European countries, mainly for Artemisinin Combination Therapies (ACT). For instance, Kunming Pharmaceutical Corporation has produced doses of artemether oil injection for 8.5 million persons and oral preparations of artemether for 9.5 million persons and also supplied Novartis with 75 tons of artemether for the manufacturing of Coartem (artemether/benflumetol) since 2005.

In 2007, the technical know-hows in the manufacturing of artemether and benflumetol were opened [190], including the reduction of QHS with potassium borohydride and replacement of methylene dichloride by methyl acetate in the manufacture of artemether. These effective measures will reduce production cost.

Since Chinese researchers revealed their important discovery in 1977 [6], QHS and its derivatives immediately received high attention from WHO and research groups in many countries. To search for more active, highly bioavailable, or cheaper antimalarial drugs, QHS derivatives and analogs numbering in the thousands were synthesized and evaluated. Up to date, some reviews [191–195] have been published, and here, we summarize the major research work about medicinal chemistry of QHS, with emphasis on the work done in China.

7.1 Modification on C-12 of QHS

DHA can be recognized as a relative of a pyranose sugar with a free anomeric hydroxyl group, so these C-12 derivatives may be divided into three types: O-glycosides, N-glycosides, and C-glycosides according to the technical terms in carbohydrate chemistry.

7.1.1 O-glycosides

The ethers and esters of DHA prepared mainly in China may be considered as its O-glycosides (Figure 16).

Because of comparatively high content (0.6%–1.1%) of QHS in *A. annua* in some area of China and an efficient process of extraction, a large quantity of QHS is available in China. Based on our early work, it was known that little changes of C-12 substituent always led to quite difference in the antimalarial activity; hence, more C-12 esters, ethers, and thioethers of DHA were synthesized [196–198]. These derivatives were tested against *P. berghei* in mice, and some compounds were found to be more active than QHS but less than artemether.

In 2002, it was reported that a series of new esters and ethers of DHA were prepared. When the hydroxyl group of DHA was activated for displacement by nucleophiles, as in the Schmidt or Mitsunobu procedures, β-esters and β-ethers were obtained. Thus, the treatment of DHA with triphenylphosphane-diethyl azodicarboxylate and aromatic carboxylic acids in THF produced β-derivatives. DHA in dichloromethane containing trichloroacetonitrile was treated with a catalytic amount of 1, 8-diazabicyclo [5.4.0] undecane (DBU), and the intermediate trichloroacetimidate formed in situ was treated directly with succinic acid to yield β-artesunate in 45% yield [199].

In view of halogen atoms (especially, F) appearing in many existing antimalarial drugs, a series of ethers and esters of DHA containing halogen atoms were prepared [200–202]. Recently, some 12-CF_3, 12-OR derivatives, and 12-gem-difluoroethylene deoxoartemisinin or deoxoartemisitene were synthesized [203,204]. The presence of the CF_3 group at C-12 of QHS may increase the chemical stability under simulated stomach acid condition. It was reported that CF_3 analogs of artemether and arteether exhibited a high *in vivo* antimalarial activity in mice infected with *P. berghei* (Figure 17). Preclinical data of lead compounds and evidence for their strong and prolonged antimalarial activity were reported [205].

Another type of O-glycosides, 12β aryl ethers of DHA, was synthesized by reaction of acetyl DHA or trifluoroacetyl DHA with various substituted phenols in the presence of trifluoroacetic acid (Scheme 12). Most of these compounds were proved to have higher artimalarial activity against *P. berghei* in mice than QHS but less active than artemether [206].

Later, it was also reported that under the TMSOTf and

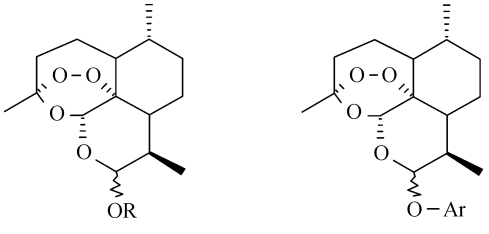


Figure 16 O-glycosides of DHA.

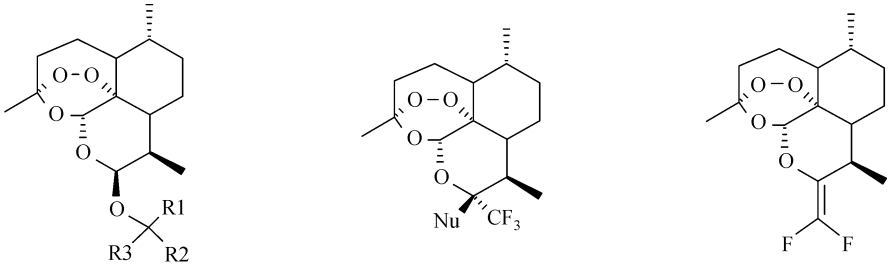
$AgClO_4$ promotion at −78°C, this kind of derivatives with both 12β and 12α isomers were formed from DHA and phenols with substituents (such as F, CF_3, Cl, Me, OMe, and naphthyl). 12β-*p*-Trifluoromethylphenyl ether exhibited excellent *in vivo* antimalarial potency with an ED_{50} of 2.12 mg/kg versus *P. berghei* [207].

7.1.2 N-glycosides (Figure 18)

11-Bromo-12-anilino derivatives were synthesized from 11, 12-dibromo deoxoqinghaosu and aromatic amines. However, no significant antimalarial activity against *P. berghei in vivo* was observed [208]. A Chinese group reported that DHA or trifluroacetyl DHA reacted with aromatic amines or heterocycles, such as triazole and benzotriazole, in the presence of acidic catalyst to afford N-glycosides (Scheme 13). All these compounds in oil solution by po administration were tested *in vivo* against *P. berghei* and were more active than QHS [209]. A paper published in 2005 described that the conversion of DHA into 12-arylamino-artemisinins was successful in a two-phase system of an organic solvent and dilute aqueous mineral acid with a phase-transfer catalyst [210].

In the series of N-glycosides, artemisone (**130**) synthesized by another Chinese group may be the most promising candidate [211,212]. 12β-Bromodeoxoqinghaosu reacted with thiomorpholine gave an intermediate, which was oxidized to artemisone (Scheme 14). It was reported that artemisone was 10 times more potent than artesunate *in vitro* against a panel of 12 *P. falciparum* strains, and consistently, 4 to 10 times more potent than artesunate in rodent models against *P. falciparum* lines and chloroquine- or QHS-resistant lines of *P. yoelii*.

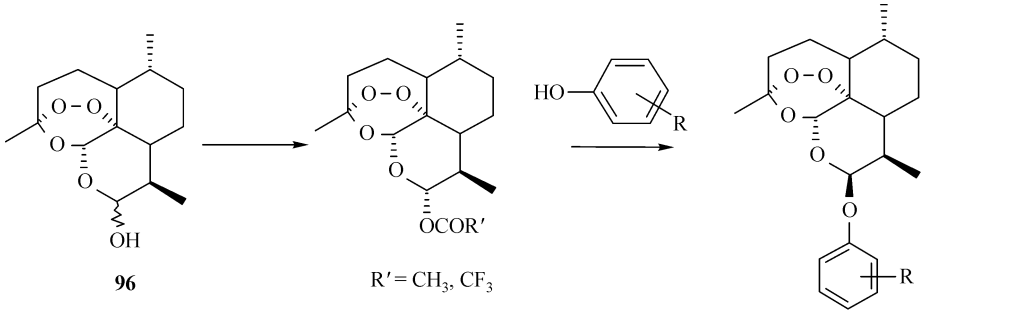
In preclinical studies, artemisone (BAY 44-9585) was shown to possess enhanced efficacy over artesunate. After the phase I program of artemisone was finished, the result was reported in 2008 [213]. Artemisone was well tolerated, with no serious adverse events and no clinically relevant changes in laboratory and vital parameters. The pharmacokinetics of artemisone over the 10- to 80-mg range demonstrated a short elimination half-life *t* (1/2) of 2.79 h (range, 1.56 to 4.88). Hence, artemisone may be used as artemisinin-based combination therapy for the treatment of falciparum malaria. However, its higher production cost will be a big obstacle for artemisone to be a new antimalarial drug.



R_1 = CF_3, CF_2CF_3, CF_2-CHF-CF_3, C_6F_5
R_2 = H, CH_3, C_6H_5, COOEt
R_3 = H, C_6H_5, C_6F_5

Nu = OH, OMe, OEt, $OCH_2C_6H_4$(COOMe)-*p*, $OCH_2CH=CH_2$, OCH_2CH_2OH, OCH_2CF_3, OOH, OCH_2CH_2COOH

Figure 17 Structures of fluoroartemisinins.



96

$R' = CH_3$, CF_3

R = H, OH, OCH_3, COOH, $COOCH_3$, Br, NO_2, $NHCOCH_3$

Scheme 12 Synthesis of 12-β aryl ethers of DHA.

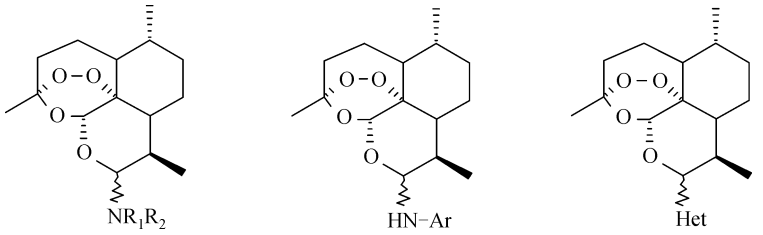
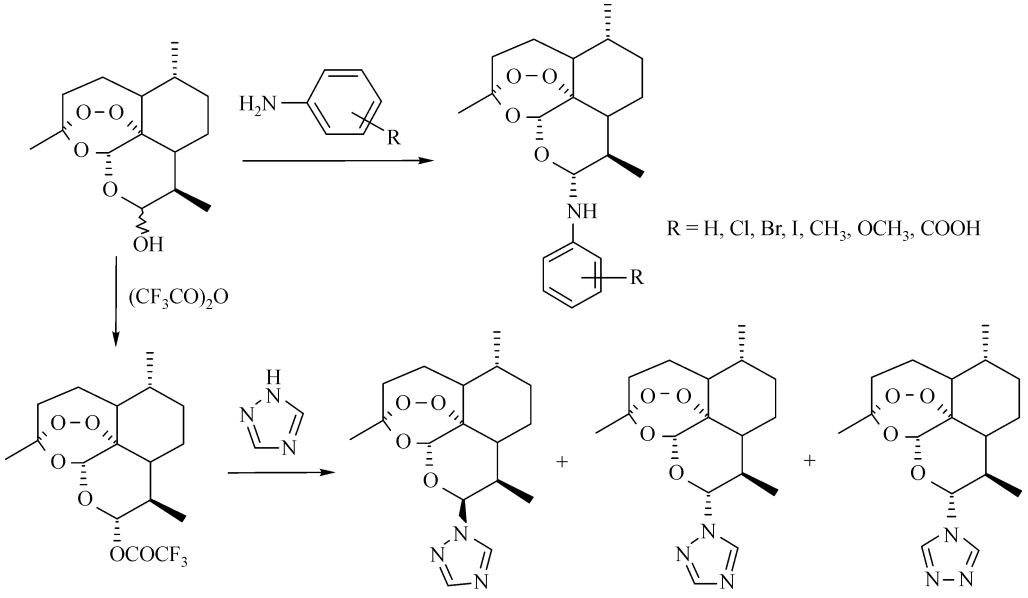
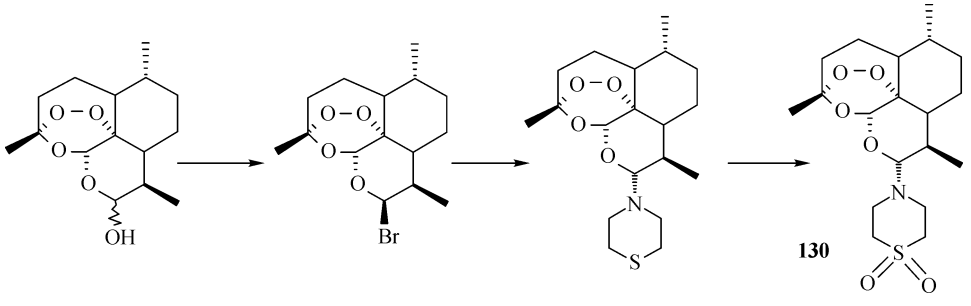


Figure 18 N-glycosides of DHA.



Scheme 13 Synthesis of some N-glycosides.



Scheme 14 Synthesis of artemisone.

7.1.3 C-glycosides (Figure 19)

Because the C-glycosides could not be converted into DHA *in vivo*, and their half-life might be significantly longer than that of the O-glycosides of DHA, a large number of C-glycosides have been synthesized. At first, some 12-alkyl-deoxoartemisinins from artemisinic acid were prepared in 5–7 steps [214,215]. However, the scarcity of artemisinic acid, low overall yield, and production of both 12α-isomer and 12β-isomer indicated the need for another approach. Hence, a new route employing DHA or its acetate **131** as the starting material was reported (Scheme 15). In the presence of an acid catalyst (boron trifluoride etherate or titanium tetrachloride), DHA, or **131** was reacted with allyltrimethylsilane to prepare 12β-allyldeoxoartemisinin **132** (R: $CH_2CH = CH_2$) and related compounds in high stereoselectivity [216,217]. The most active compound **132,** 12β-*n*-propyl-deoxoartemisinin, proved to be as active and toxic as arteether [218].

A series of C-12 carbon-substituted 12-deoxoartemisinin compounds (Scheme 16) were synthesized by using 12β-F-deoxoqinghaosu as an intermediate [219]. Some compounds have high antimalarial potencies *in vitro* against *P. falciparum.*

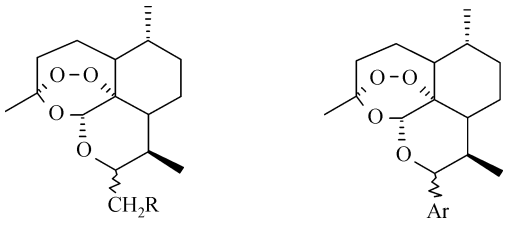
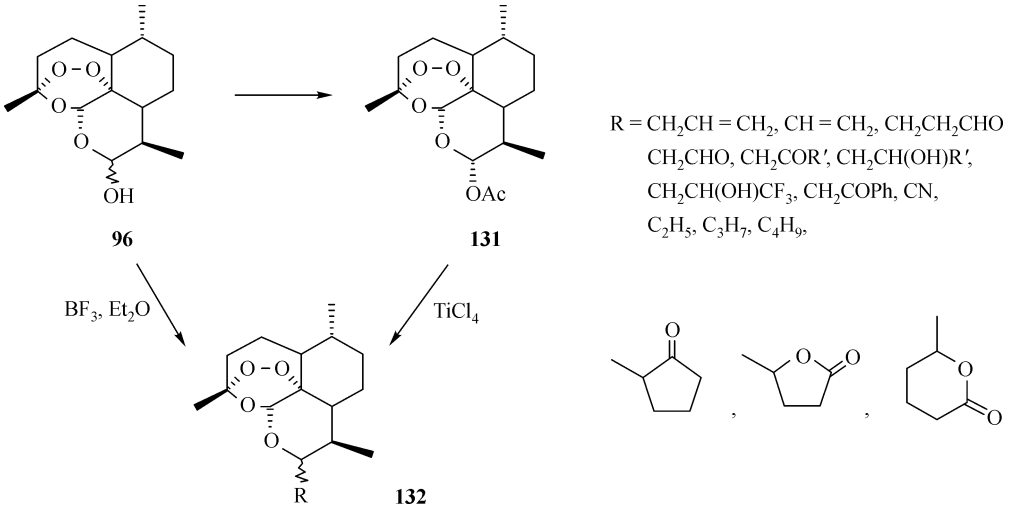
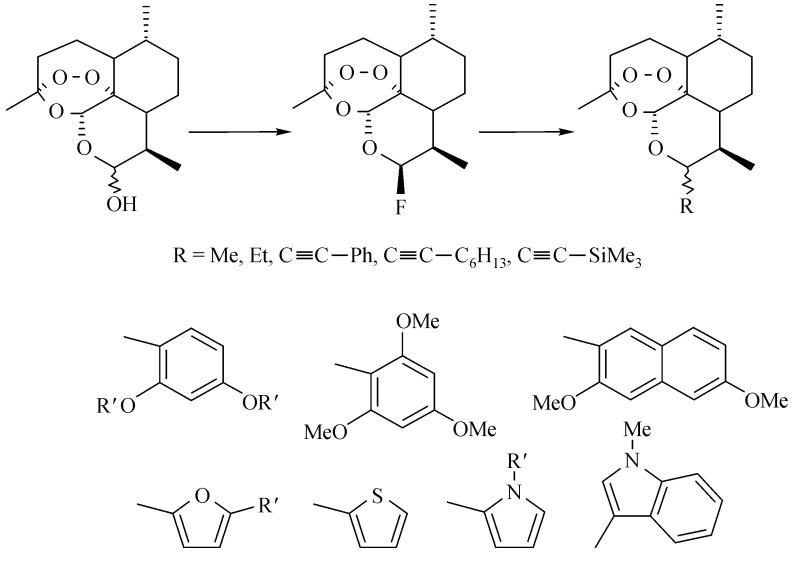


Figure 19 C-glycosides of DHA.



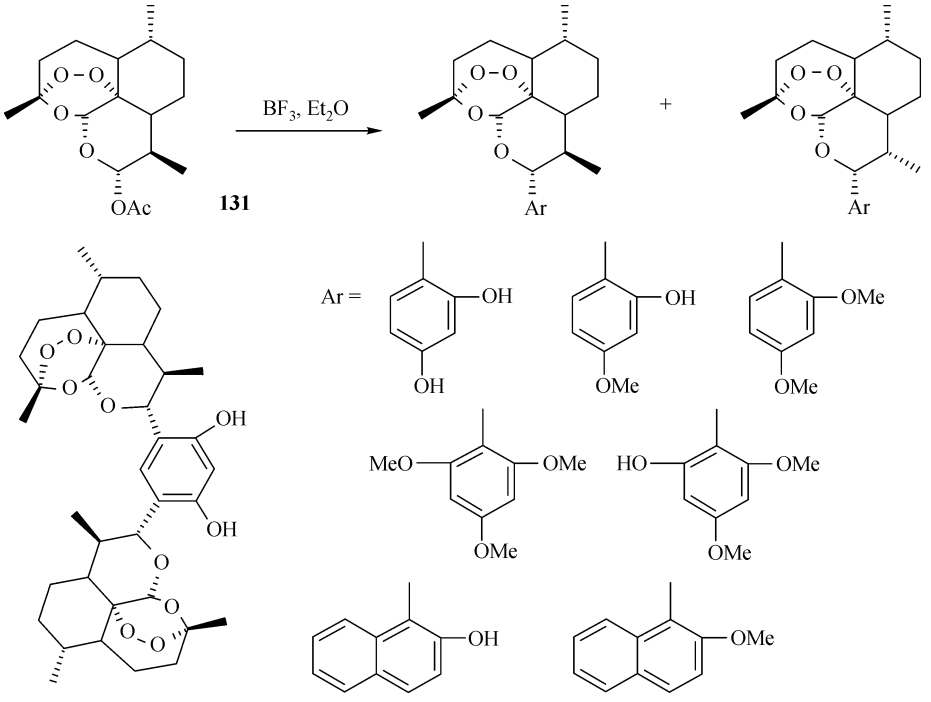
Scheme 15 Synthesis of some C-glycosides.



Scheme 16 Synthesis of C-glycosides through 12β-F-deoxoqinghaosu.

C-12 dihydroartemisinin furan, methylfuran, and *N*-methylpyrrole are more potent *in vivo* than QHS and chloroquine when administered subcutaneously. *N*-Methylpyrrole derivative is comparable to arteether in terms of oral potency.

At the beginning of the 1990s, Chinese researchers reported that DHA acetate **131** reacted with aromatic substrates in the presence of boron trifluoride-etherate to yield 12α aryl derivatives and a by-product 11α epimer (Scheme 17). Their antimalarial activity was higher than QHS, and some compounds were even higher than artemether. Besides, some compounds showed antitumor and other bioactivities [220,221].



Scheme 17 Synthesis of 12α aryl derivatives.

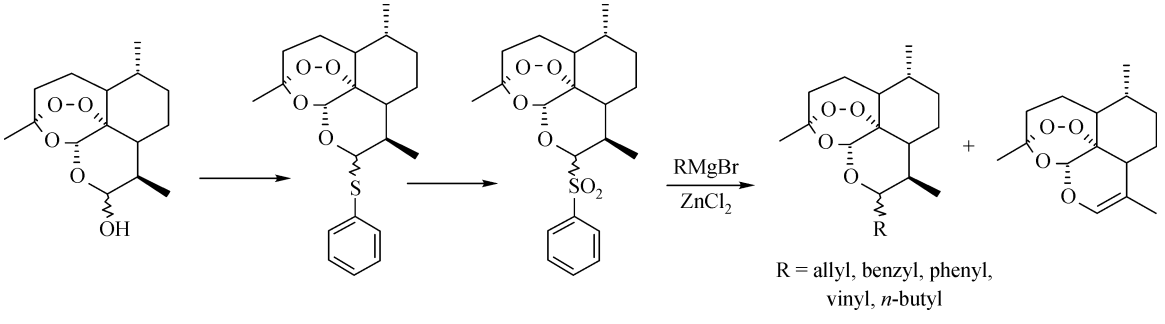
A new approach for synthesis of C-glycosides (Scheme 18**)** was reported in 2002 [222]. A direct substitution reaction of 12-benzenesulfonyl deoxoartemisinin, with organozinc reagents derived from allyl, benzyl, phenyl, vinyl, and *n*-butyl Grignard reagents stereoselectively produced 12-substituted deoxoartemisinins in good to moderate yields.

In view of that artesunate only has half-life of 20–30 min and artelinic acid (see Section 7.2.1) is still an *O*-glycoside, the synthesis of their *C*-glycoside analogs is of significance. Deoxoartelinic acid (**133**) was prepared from artemisinic acid by using sulfur ylide and photooxygenative cyclization in seven steps (Scheme 19). This compound showed superior *in vitro* antimalarial activity against the chloroquine-resistant K1 strain of *P. falciparum* and higher suppression (98.7%) than arteether *in vivo* against *P. chabaudi* infected mice. Deoxoartelinic acid also showed remarkable stability with a half-life of 258.66 h and improved solubility, which is four times more soluble than artemisinin in water [223].

Besides, 5′-carba-4′-deoxoartesunic acid (**134**) was synthesized [215]. No data of its half-life and antimalarial activity were mentioned.

Although some *C*-glucoses have higher activity *in vivo* or longer half-life, no further research data were reported.

Some researchers believe that the synthesis of QHS derivatives with longer half-life will be an ideal solution for high recrudescence in QHS treatment. From our viewpoint, "short half-life" might be considered a character of QHS rather than a weakness. With its tender chemical structure, QHS is foreordained to have a short life. Some other measures, such as combination therapy, could be taken as alternative solutions.



Scheme 18 Another route for C-glycosides.

7.2 Water soluble QHS derivatives

Sodium artesunate is the first water-soluble QHS derivative and used for treatment of the severe malaria patients by intravenous or intramuscular administration. However, the aqueous solution is instable, and its hydrolysis product, DHA, quickly subsides. Hence, the synthesis of stable and water-soluble QHS derivatives is an important research program.

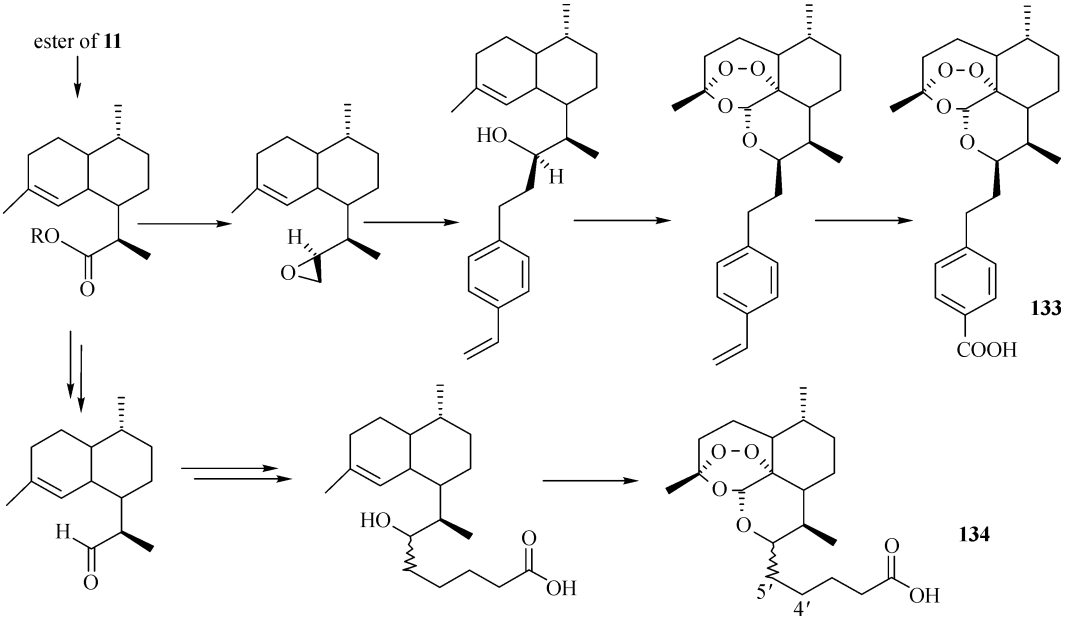
7.2.1 QHS derivatives containing carboxyl group

Because artemether has greater stability than artesunate, it was supposed that replacement of ester linkage by ether linkage in the artesunate molecule would enable it to be more stable. In fact, the sodium salts of compounds **135** were much less active and poorly water-soluble than sodium artesunate [224]. Compounds **136** and artelinic acid (**137**) were prepared (Figure 20) in the Walter Reed Army Institute of Research [225–227]. Artelinic acid has longer half-life of 1.5–3 h., and high activity *in vivo* and its sodium salt is relatively stable in aqueous solution. Its transdermal formulation is effective in mice [228]. Some data of preclinical study of artelinic acid were reported [229].

7.2.2 QHS derivatives containing basic substituent

In view of the known basic antimalarial drugs (such as chloroquine and quinine) being used in their salts for injection or oral administration, it was proposed that introducing an amino group into the QHS molecule may lead to water-soluble derivatives. Thus, four types of basic QHS derivatives were synthesized [224,230] (Figure 21).

These basic compounds combined with organic acid (such as oxalic acid and maleic acid) to yield the corresponding salts. Generally, they had good water-solubility and stability. Some compounds were much more active against *P. berghei* in mice than artesunate. However, their efficacies were less than that of artesunate against *P. knowlesi* in rhesus monkeys



Scheme 19 Synthesis of deoxoartelinic acid and 5′-carba-4′-deoxoartesunic acid.

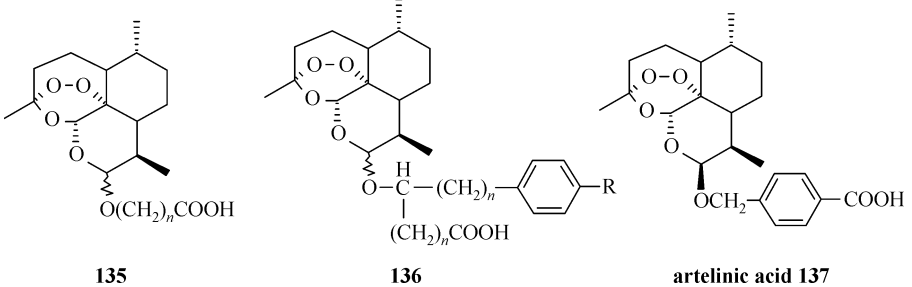


Figure 20 QHS derivatives containing carboxyl group.

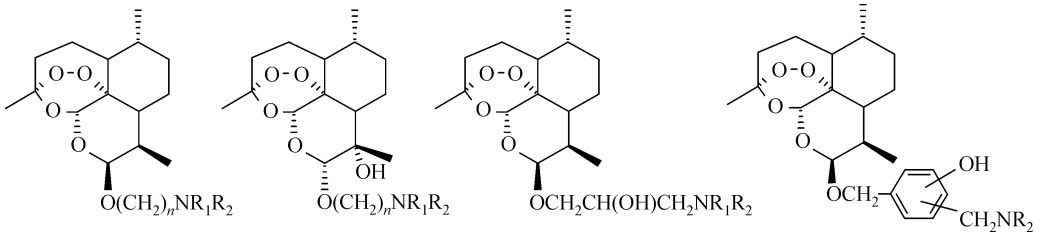


Figure 21 Structures of basic QHS derivatives.

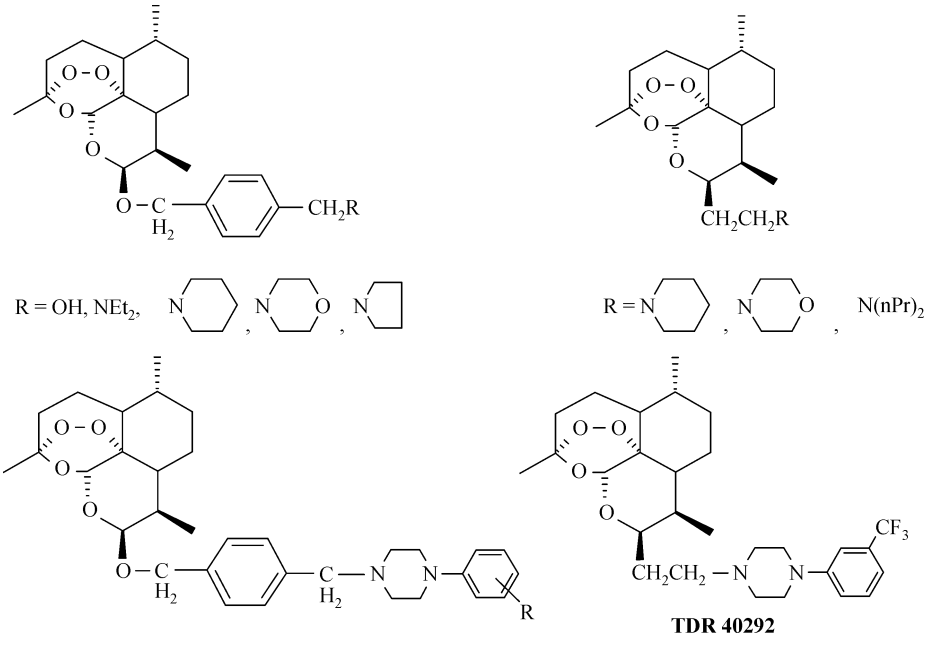


Figure 22 Other basic QHS derivatives.

at a dose of 3.16 mg/kg [224,230].

More QHS derivatives containing an amino group (Figure 22) were prepared [231–233]. TDR 40292 appeared to be superior to artemether and artesunate *in vitro* and *in vivo* antimalarial assessment [195].

7.3 Modification at C-4, C-11 and/or C-12

In QHS molecule, the 4-methyl is located near the peroxy group, so the modification on C-4 may offer some important information about the structure-activity relationship. Some compounds (Figure 23) were therefore synthesized [234,235], which were more active than QHS.

It was noteworthy that deoxoqinghaosu (**98**) itself was also more active than QHS *in vitro* and *in vivo* [236–238].

4-Alkyl-, 4-(arylalkyl)- and 4-(carboxyalkyl)-qinghaosu and-deoxoqinghaosu were prepared (Figure 24). The analogs were tested *in vitro* against W-2 and D-6 strains of *P. falciparum* and found to be, in some cases, much more active than the natural product QHS [239–241].

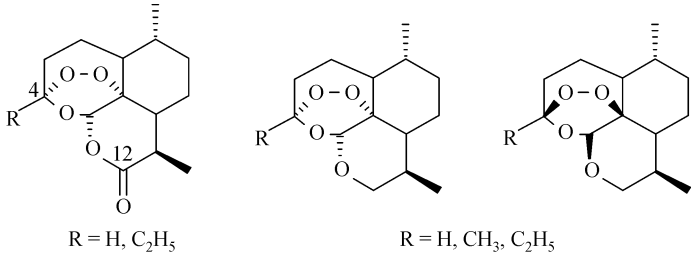


Figure 23 QHS analogs modified at C-4 and/or C-12.

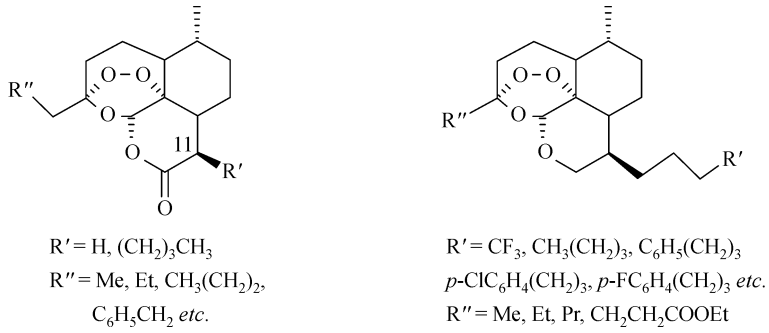


Figure 24 QHS analogs modified at C-4, C-11 and/or C-12.

7.4 Modification at C-3 and/or C-13

A series of QHS analogs of C-3 and/or C-13 modification were prepared from artemisinic acid (Figure 25). Among the analogs, only 13-nitromethylartemisinin had antimalarial activity comparable to QHS (**1**) [242,243].

A series of 13-substituted-artemisinin and 11-*epi* artemisinin derivatives were prepared by a titanium-tetrachloride catalyzed addition of trimethylsilyl enol ethers to artemisitene **(**Scheme 20**)**. Several compounds were 4 to 7 times more active *in vitro* than QHS against *P. falciparum* [244]. In addition, under non- or base-catalyzed conditions, artemisitene (**10**) reacted with triazole, benzotriazole, or benzimidazole to yield a series of Michael addition products (Scheme 20), which had antimalarial activity *in vivo* [245].

7.5 Modification at C-11 and/or C-12

A number of C-11 substituted QHS and its derivatives were prepared (Figure 26) and tested against *P. berghei* in mice

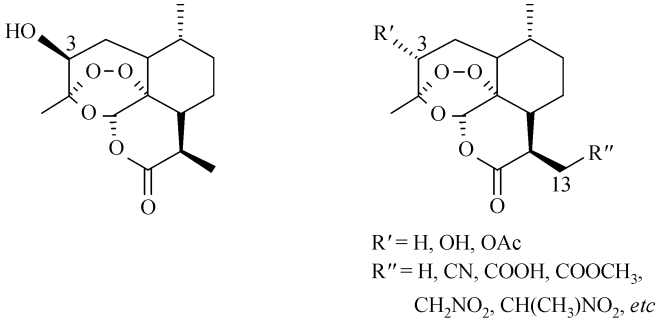
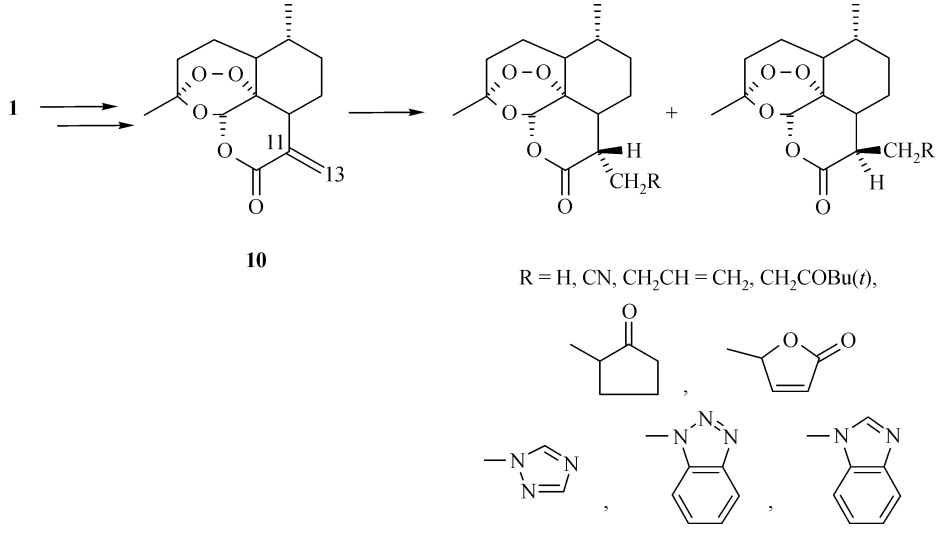


Figure 25 QHS analogs modified at C-3 and/or C-13.



Scheme 20 Synthesis of 13-substituted artemisinin.

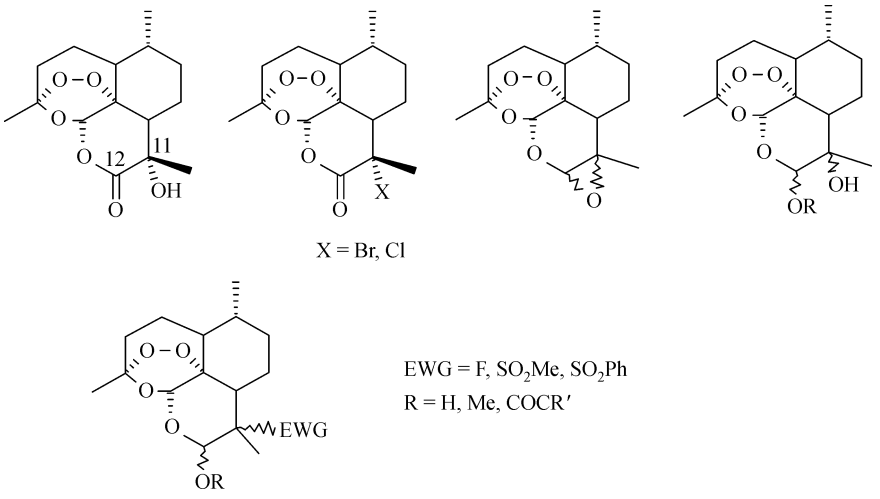


Figure 26 QHS analogs modified at C-11 and/or C-12.

[246–253]. Their obviously low antimalarial activity may be attributed to the introduction of 11α substituent (see Section 9).

Several 11-oxygenated QHS derivatives (Figure 27**)** were prepared from artemisitene and showed a wide variation *in vitro* antimalarial activity [247].

11-Substituted deoxoartemisinin **138** was produced from artemisinic acid using photooxidation as the key step (Scheme 21). Compound **138** (R = CH_2OH) was more active than QHS and artesunate *in vitro* [254,255].

7.6 Azaartemisinin

Reaction of QHS with methanolic ammonia or primary alkyl- and heteroaromatic amines yielded azaartemisinin or *N*-substituted-azaartemisinin (**139**) and *N*-substituted azadesoxyartemisinin (**140**) as by-products (Scheme 22). Some *N*-substituted-azaartemisinin had good antimalarial activity, such as compound **139** (R = CH_2CHO), which was 26 times more active *in vitro* and 4 times more active *in vivo* than QHS [256].

More *N*-alkyl derivatives **141** were prepared (Scheme 23) by means of Michael additions of azaartemisinin to electron-deficient alkenes [257].

A recent paper reported that some *N*-sulfonyl- and *N*-carbonyl-azaartemisinins were prepared (Figure 28) from azaartemisinin [258]. Several of the *N*-sulfonylazaartemisinins have melting points above 200°C and possess substantially greater thermal stabilities than the artemisinins in current clinical use.

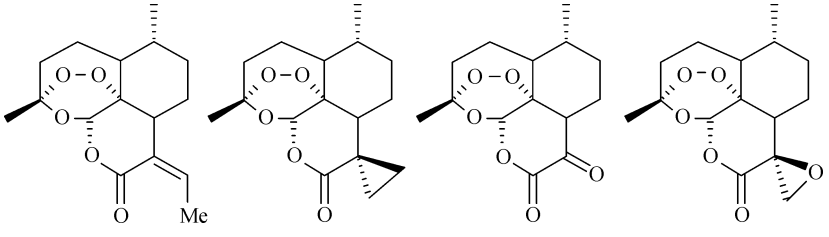
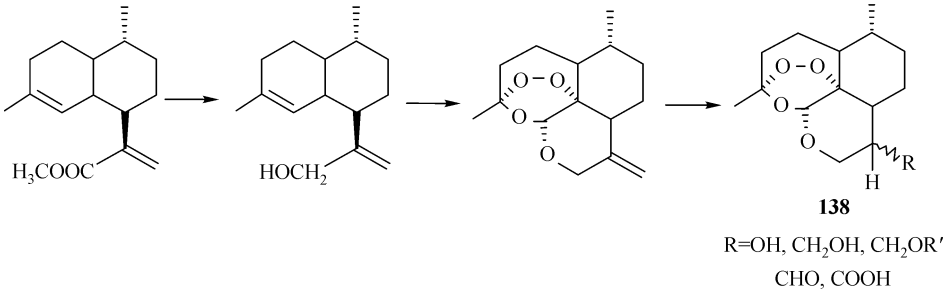
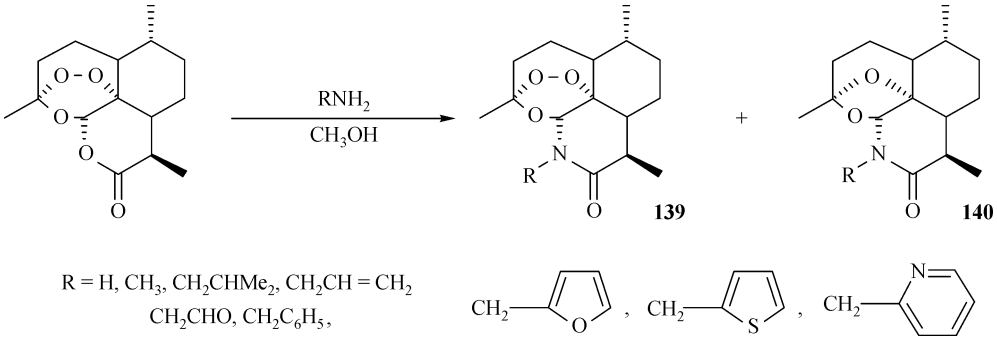


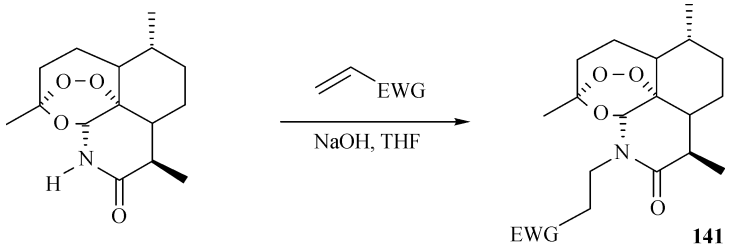
Figure 27 QHS analogs modified at C-11.



Scheme 21 Synthesis of deoxoartemisitene and its C-11 derivatives.



Scheme 22 Synthesis of azaartemisinin or *N*-substituted azaartemisinin.



Scheme 23 A new route to *N*-substituted-azaartemisinins.

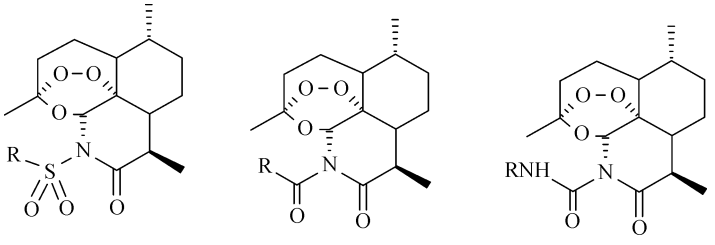


Figure 28 *N*-Sulfonyl- and *N*-carbonyl-azaartemisinins.

7.7 Carbaartemisinin

In order to inspect the effect of the segment of O–O–C–O–C–O–C=O in the QHS molecule, 4a-carba-artemisinin **142** and its analogs **143**–**145** (Figure 29) were synthesized and evaluated. These compounds displayed much lower antimalarial activity *in vitro* than QHS [259].

Compound **122** reacted with paraformaldehyde under the catalysis of Lewis acid $BF_3 \cdot OEt_2$ to give **146**, which was oxidized into **147** and **148** by RuO_4 (Figure 30). Their poor antimalarial activity has demonstrated once again that the peroxy group is essential for antimalarial activity [260].

7.8 Steroidal QHS derivatives

Some research groups synthesized steroidal QHS derivatives

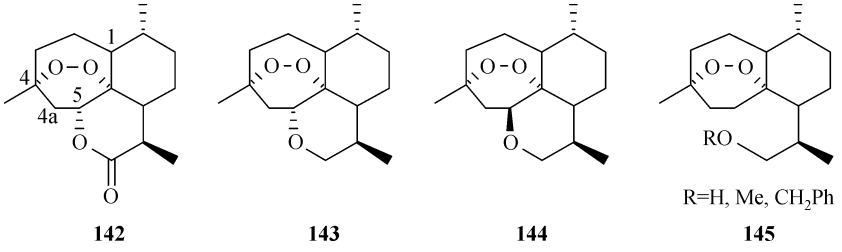


Figure 29 4a-Carba-artemisinin and its analogs.

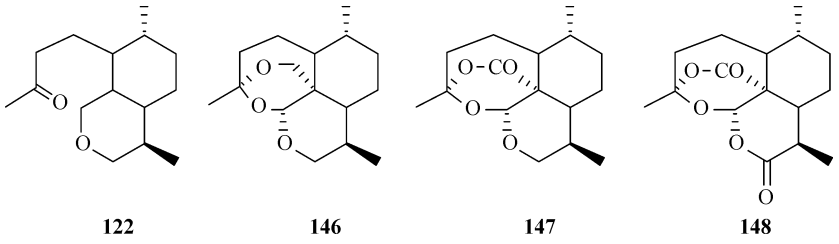


Figure 30 Carba-analogs of QHS.

in which QHS nucleus or trioxane combined with a steroidal skeleton in different styles [261] (Figure 31). These compounds showed better antimalarial activity than QHS.

7.9 Dimers and trimers

In medicine study, coupling two pharmacophores in one molecule is a common strategy to enhance the activity. Therefore, the dimers of QHS were extensively studied. In the 1980s, Chinese researchers synthesized some di-ethers and di-esters of DHA (Figure 32). These compounds were more active than QHS but less than artemether *in vivo* [185,262,263]. Later, two unsymmetric dimers were prepared and found to be comparable to arteether when tested in K-173-infected mice [264] (Figure 32).

Since the end of the 20th century, a number of new types of deoxoartemisinin dimers (Figure 33) have been developed [265–272]. Most compounds had both antimalarial and antitumor activities. Some compounds exhibited more potent antimalarial activity than natural QHS, even more than artesunate and artemether. It was encouraging that several of these dimers were especially potent and selective to inhibiting the growth of some human cancer cell lines in vitro. Some compounds were selected as candidates for further research.

Besides, Thai researchers synthesized artemisinin monomers, dimers, and trimers (Figure 34) by means of nucleophilic additions of artemisitene [273]. These compounds were evaluated *in vitro* antimalarial and cytotoxic activities (KB, BC cells). Most compounds had higher antimalarial activity than QHS.

7.10 1, 2, 4-Trioxanes, 1, 2, 4, 5-tetraoxanes, peroxides, and ozonides

Generally, medicines are required to be effective, nontoxic, and cheap, especially for antimalarials used in the developing countries. Artemisinins have several advantages over existing antimalarial drugs; however, they cost much more than chloroquine.

To find new generations of antimalarials being highly active and inexpensive, some research groups have synthesized a number of 1, 2, 4-trioxanes, 1, 2, 4, 5-tetraoxanes, peroxides, and ozonides as qinghaosu analogs or simplifiers since 1990s. These efforts have led to several promising compounds. Some reviews [195,274–278] well summarized the progress in the field. Here is only a brief introduction to these compounds.

1, 2, 4-Trioxanes with the essential structural characteristic of QHS (Figure 35) showed antimalarial activity *in vitro* and

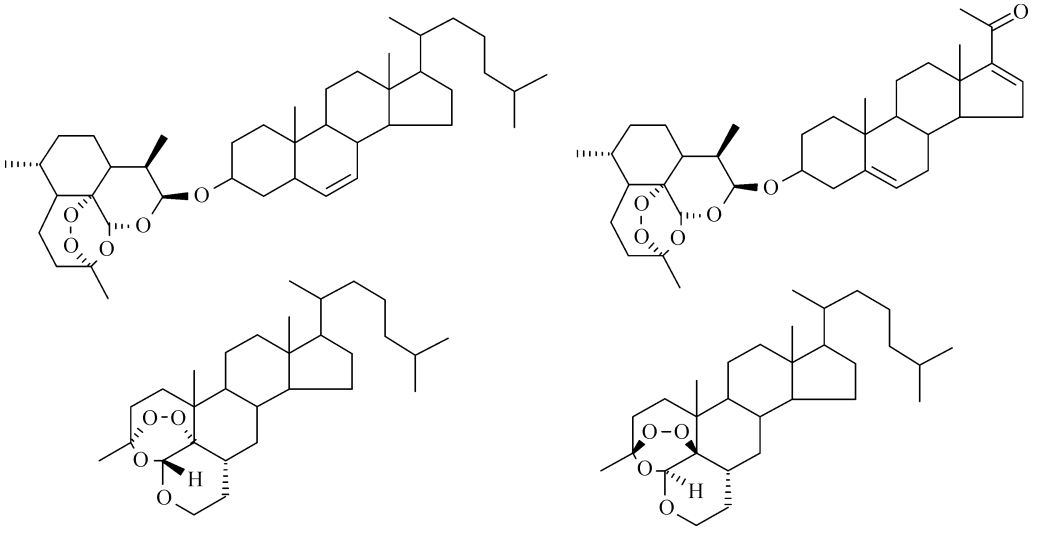


Figure 31 Steroidal QHS derivatives.

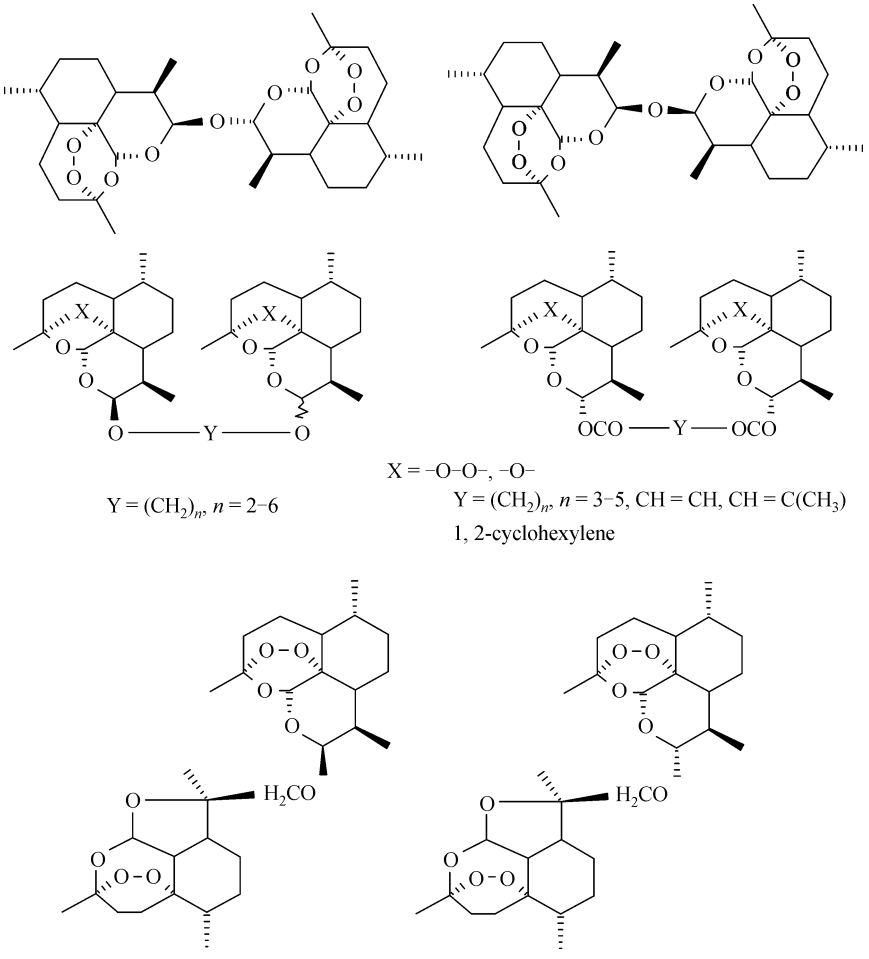


Figure 32 Some dimers of QHS.

in vivo. Among them, Fenozan B07 was first studied exhaustively, which showed potent blood schizontocidal activity against drug-sensitive and drug-resistant rodent malaria parasites. Besides, it could inhibit the development of parasites at all asexual stages except preschizonts, as well as gametocytes [279–280]. Fenozan B07 was not developed into antimalarial drug due to its poor bioavailability.

Recently, French researchers developed a new type of 1, 2, 4-trioxane containing an aminoquinoline entity, named trioxaquines [281–283]. Among these compounds, DU1302 was found to be the most active, its inhibitory concentration (IC_{50}) being as low as 6 nM for the highly chloroquine-resistant *P. falciparum* strain FcM29-Cameroon. Thus, DU1302 was selected for *in vivo* evaluation. The doses required to decrease parasitemia by 50% (ED_{50}) were 5 and 18 mg/kg/day for intraperitoneal and oral administration, respectively. Parasitemia clearance was complete without recrudescence at an intraperitoneal dose of 20 mg/kg/day. In addition, DU1302 did not induce any toxic effects in mice treated by the oral administration of 120 mg/kg/day over four consecutive days. Moreover, DU1302 exhibits potent activity against gametocytes [284]. Therefore, DU1302 can be considered as a promising antimalarial drug candidate (Figure 36).

1, 2, 4-Trioxanes were also a synthetic target in China. Compound **149** was prepared from (-)-menthol through a key step of photooxidation of cyclic enol ether (Scheme 24) [285].

When two cyclic enol ethers bearing a benzyl group (**150**, **152**) were taken as phytooxygenation precursors, their phytooxygenation products (**151**, **153**) were not the target compounds. The structures of **151** and **153** were elucidated by X-ray analysis (Scheme 25) [286–287].

Although tetraoxanes quite differ in structure from QHS, some simple analogs have been proven to be effective when given orally in mice infected with *P. berghei* with no observable toxic side effects [288–290]. Dispiro tetraoxane (Wr 148999) was found more active than QHS (Figure 37).

Recently, achiral spiro-1, 2, 4, 5-tetraoxanes (Figure 38) with high antimalarial activity, low toxicity, and high-stability profiles were reported [291,292].

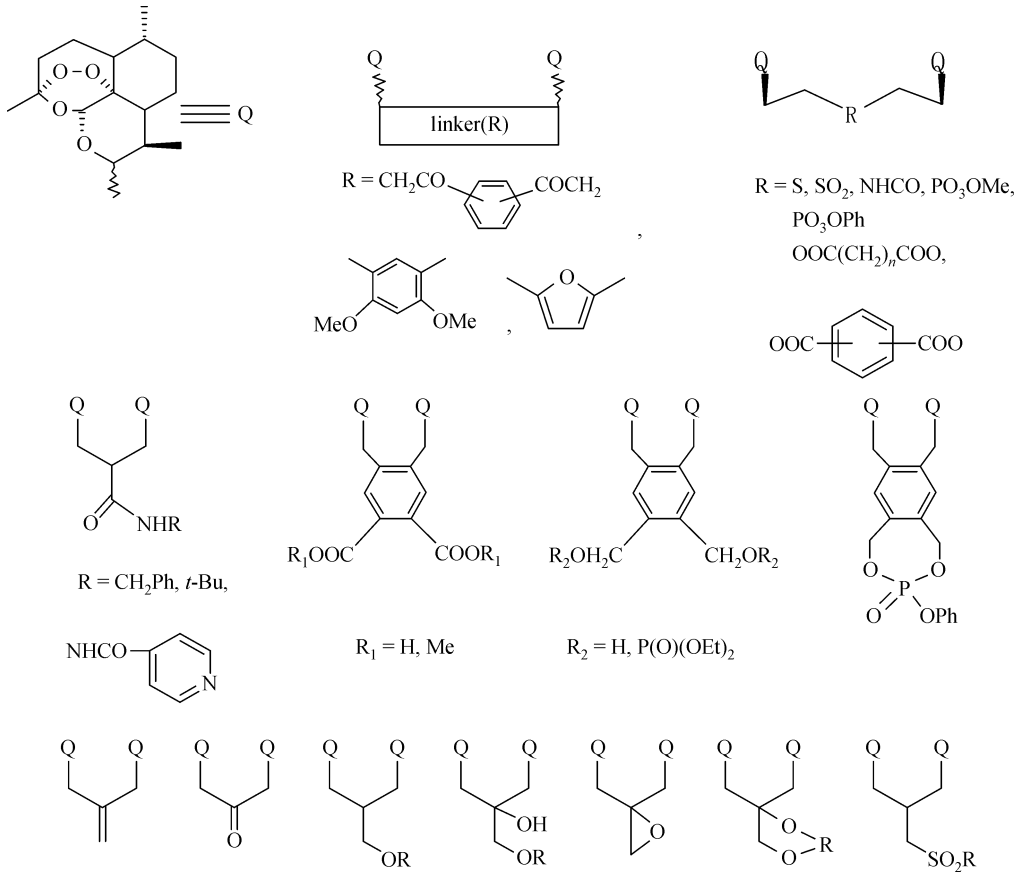


Figure 33 New dimers of QHS.

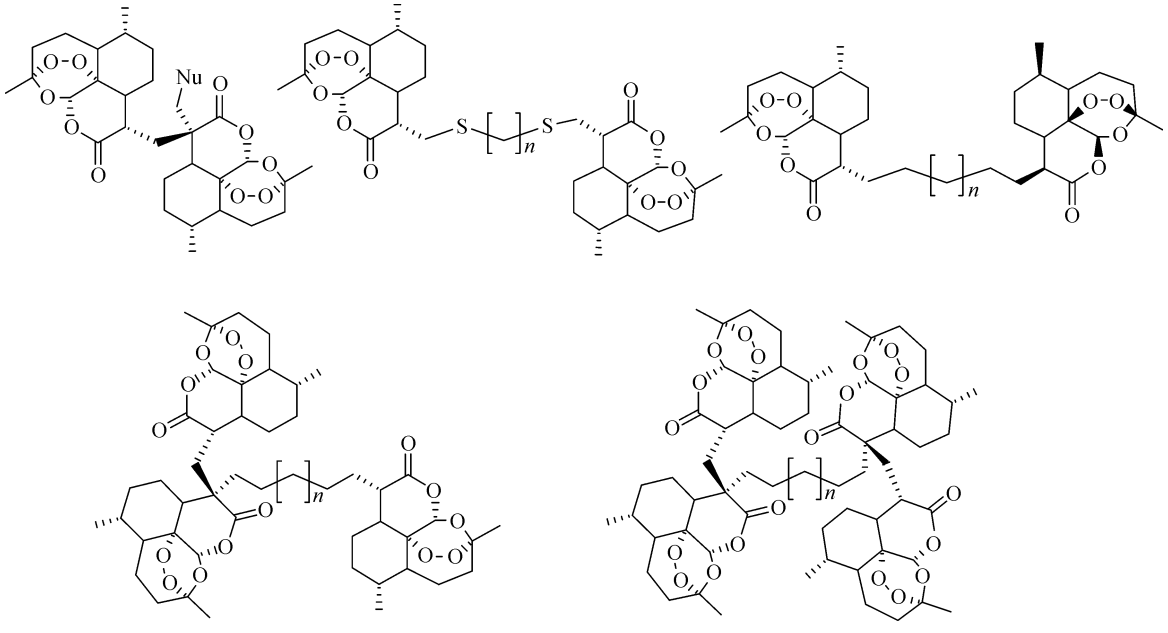


Figure 34 Artemisinin dimers, trimers, and tetramers.

In addition, more 1, 2, 4, 5-tetraoxepanes, 1, 2, 4, 5-tetraoxocanes, and spiro-1, 2, 4, 5-tetraoxacycloalkanes (Figure 39) were synthesized by Japanese researchers [293–296]. 1, 2, 4, 5, 7-Pentaoxocanes with alkyl and aryl substituents were also synthesized (Figure 39). They have similar structural characteristics (peroxide group linking

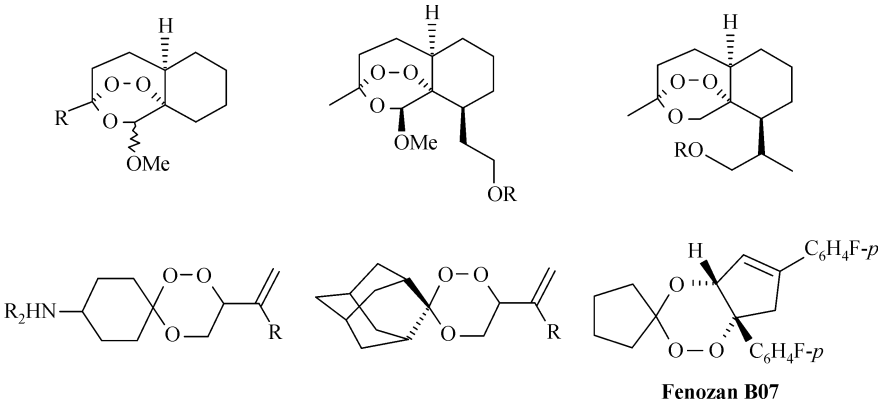


Figure 35 Some 1, 2, 4-trioxanes.

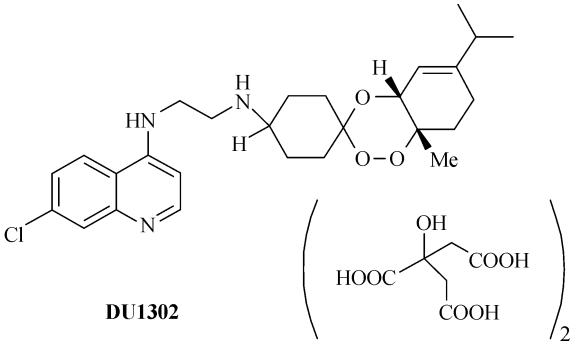
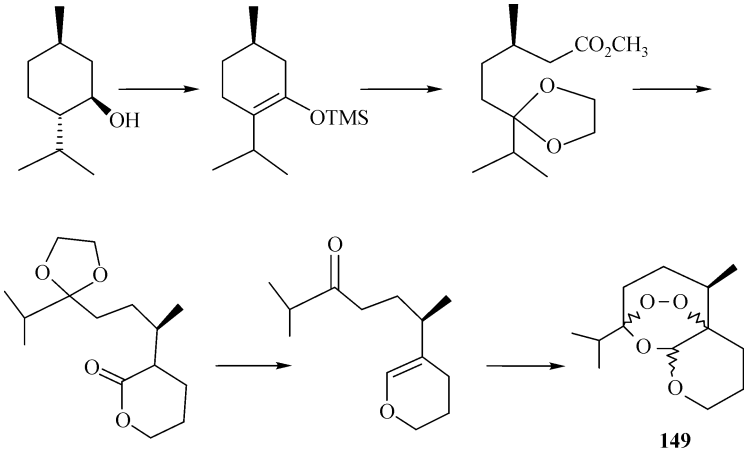


Figure 36 Structure of trioxaquine DU1302.

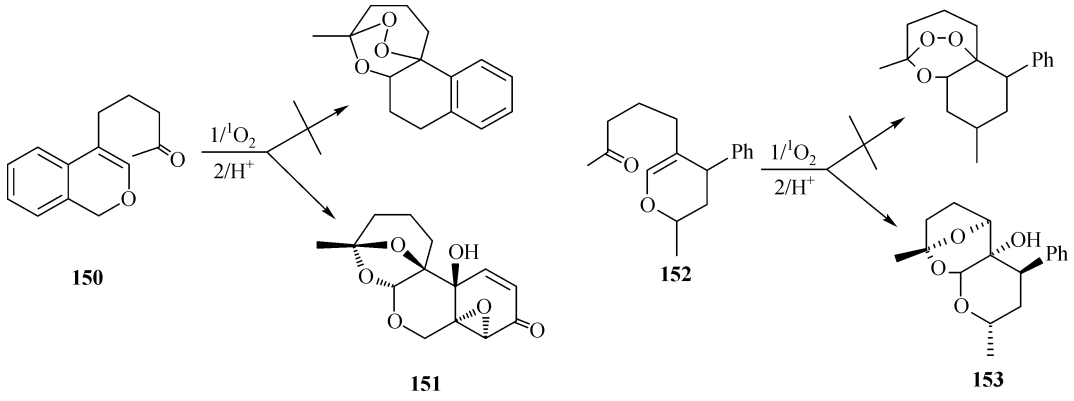


Scheme 24 1, 2, 4-Trioxane synthesized from (-)-menthol.

ketal) in QHS molecule. Some compounds showed antimalarial activity in mice closed to that of QHS.

Since antimalarial natural products QHS and yingzhaosu A were identified to be peroxide compound, a number of peroxides were synthesized and tested on their antimalarial activity *in vitro* and *in vivo*. Arteflene (Ro-42-1611) is an outstanding example in this class. It is quite active *in vitro* and *in vivo*. However, arteflene (Figure 40) has been found to have low activity in simian models and clinical trials because of its poor absolute bioavailability [297,298].

The new simplified analogs of QHS (Figure 41) have also been synthesized in China through simple routes without recourse to the commonly employed photosensitized oxidation [299–305]. The peroxy bonds in the target molecules



Scheme 25 Two unexpected phytooxygenation products (151 and 153).

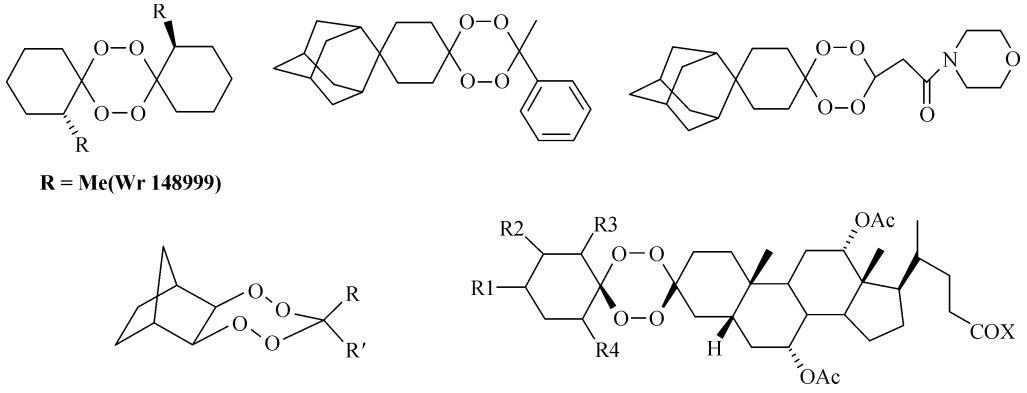


Figure 37 Structures of some tetraoxanes.

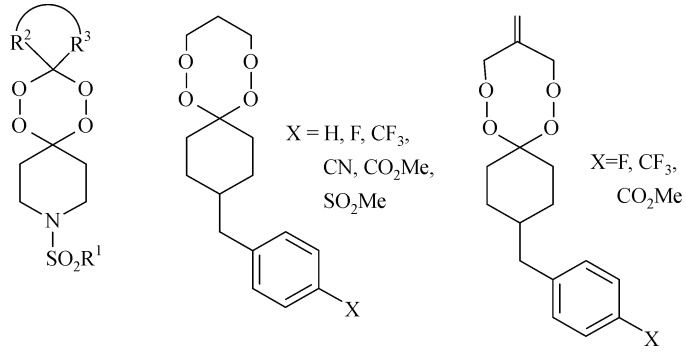


Figure 38 Achiral spiro-1, 2, 4, 5-tetraoxanes.

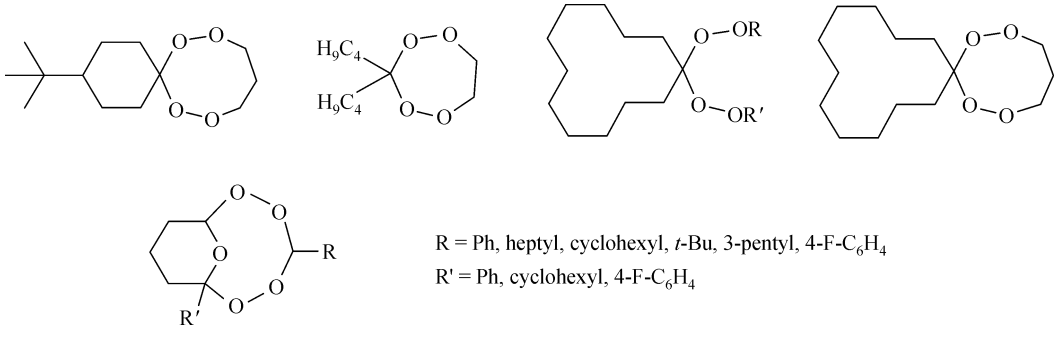
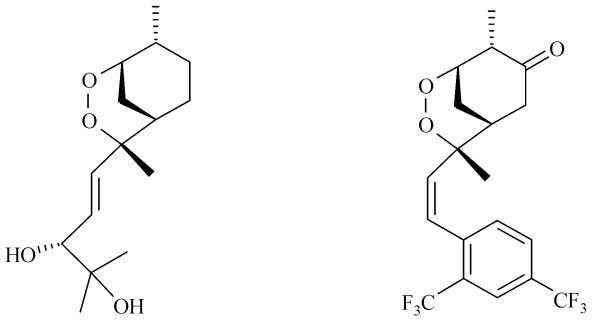


Figure 39 1, 2, 4, 5-Tetraoxepanes and 1, 2, 4, 5, 7-pentaoxocanes.



yingzhaosu A **arteflene**

Figure 40 Structures of yingzhaosu A and arteflene.

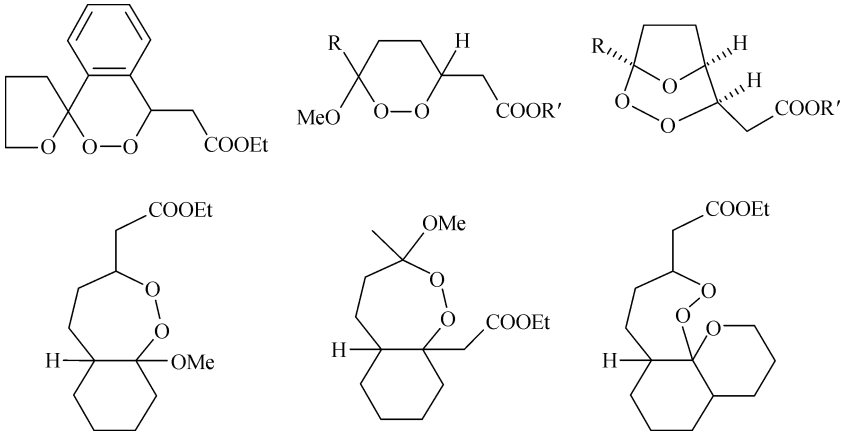


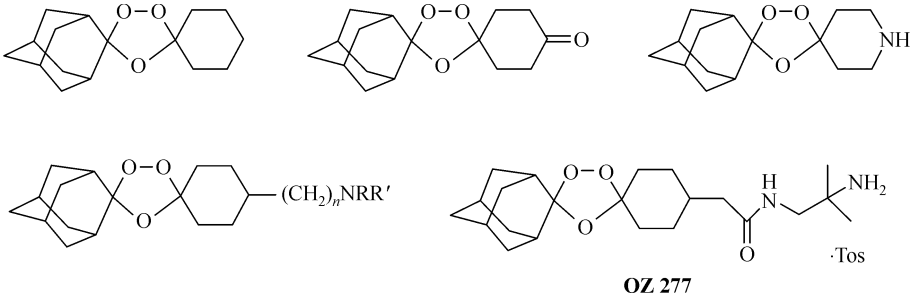
Figure 41 Some peroxides synthesized using a new route.

were taken from UHP (urea-hydrogen peroxide complex).

Since the end of the 20th century, some hundreds of ozonides (spiro 1, 2, 4-trioxolanes) have been synthesized by an American group. The most significant discovery is ozonides with an adamantane ring (Scheme 26). OZ 277 (RBx11160) was selected as a candidate due to its oral activity in mice and prolonged duration of action [306]. This research was supported by the Medicines for Malaria Venture (MMV). In 2006, two pharmacokinetic studies with RBx11160 were conducted. Preclinical studies of the combination of RBx11160 and piperaquine were completed. However, MMV has decided to stop funding the project [307]. New ozonides with better biopharmaceutical and physicochemical properties will be the next target.

In China, four types of ozonides (Figure 42) were prepared. Some compounds were found to have antischistosomal or antileishmanial activities [308,309].

In summary, these active simpler peroxides described above have more or less antimalarial activity and similar mechanism, *i.e.*, the peroxide bridge undergoes Fe(II)-mediated reduction to yield a carbon-centered radical, which can kill the malaria parasites by alkylation [310].



OZ 277

Scheme 26 Ozonides with an adamantane ring.

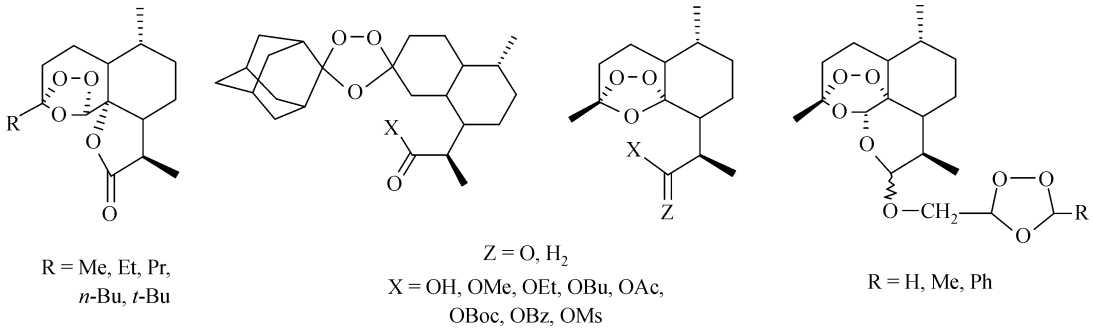


Figure 42 Other types of ozonides.

8 Bioactivities and medicinal applications of QHS and its derivatives

8.1 Against malarial

8.1.1 Antimalarial property

In the earlier researches, QHS and its derivatives showed excellent antimalarial action against the asexual forms of *P. berghei* and *P. cynomolgi.* The SD_{90} of QHS oil suspension, artemether oil solution, and sodium artesunate water solution administered by i. m. were 2.15, 0.53, and 1.77 mg/kg/day [186]. In the same time, they were found ineffective in treating the exoerythrocytic forms of *P. gallinaceum, P. cynomolgi*, and *P. yoelii yoelii*. However, subsequent studies confirmed their antitransmission properties and also proved that they could reduce gametocyte carriage rate [311–314]. A recent paper reported transmission-blocking activities of quinine, primaquine, and artesunate. The experimental result indicated that artesunate had higher transmission-blocking activity than quinine and primaquine, prevented the maturation of immature *P. falciparum* gametocytes, and reduced the transmission potential of mature gametocytes [315]. Six antimalarial trials conducted in The Gambia and Kenya demonstrated that artemisinin combination therapies (ACT) are more effective against sexual stage parasites (gametocytes) than previous first-line antimalarials and therefore have the potential to reduce parasite transmission [316].

To objectively evaluate antimalarial activity of QHS and its derivatives in different preparations, a Chinese group conducted an experiment against the asexual forms of erythrocytic stage of *P. berghei* (normal strain) in mice under the same experimental condition. The conclusion was drawn as follows: the therapeutic effect (ED_{90}) of QHS water suspension (po) was lowest; QHS water suspension (im), QHS oil suspension (im), artemether oil solution (im), sodium artesunate water solution (im), and sodium artesunate water solution (iv) were 2.5, 12, 52, 15, and 8 times higher, respectively, than that of QHS water suspension (po). Obviously, artemether oil solution (im) is most active; and taking the regression coefficients (b) of the minimum total of parasite clearance as standard, sodium artesunate water solution (iv) took effect in the shortest time [317].

8.1.2 Teratogenic effect

In the early studies of general and special toxicological experiments on QHS and its derivatives in China, the main toxic effects were manifested on the hemopoietic system, especially the erythroid series, and the myocardium was somewhat involved. However, these toxic reactions were reversible. The teratogenicity experiments in mice and rats showed both QHS and artemether to be embryo-toxic. The fetuses were absorbed, but some survived and developed well without deformity [188].

In view of the embryo-toxicity, the treatment of pregnant women with falciparum malaria by administration of QHS and its derivatives should be careful. In China, Vietnam, and Thailand, the follow-up observation of the therapeutic effect and remote reactions of QHS and its derivatives in treating malaria in pregnant woman has been made. Researchers were satisfied with the results: mothers and their babies or children (> 7 y) were in good health [318,319]. In Thailand, hundreds of pregnant women infected with multidrug-resistant *P. falciparum* were cured by administration of artemisinins. Birth outcomes did not differ significantly to community rates for abortion, stillbirth, congenital abnormality, and mean gestration at delivery [320]. To date, there has never been a report of a fetal abnormality following the use of QHS or its derivatives in pregnancy [321].

Furthermore, some ACTs, such as artesunate/mefloquine, artesunate/pyrimethamine-sulfadoxine, and dihydroartemisinin-piperaquine (DHA-PPQ), versus quinine were administrated for the treatment of multidrug-resistant falciparum malaria in pregnancy. These rescue treatments were effective

and well tolerated, and there was no evidence of toxicity for the mothers or the fetuses [322–324].

Though the clinic results were encouraging, the WHO recommendation was that artemisinin compounds cannot be recommended for the treatment of malaria in the first trimester. However, they should not be withheld if treatment is considered lifesaving for the mother, and other antimalarials are considered unsuitable. Because of the limited safety data, artemisinin compounds should only be used in the second and third trimesters when other treatments are considered unsuitable [325].

8.1.3 Neurotoxicity

In 1985, under the support of UNDP/World Bank/WHO-TDR, Walter Reed Army Institute of Research, and ACF Beheer BV, the Netherlands Ministry of Development Cooperation began the program of arteether. In 1994, during a study of the pharmacokinetics of arteether in animal, pathological evidence was reported of the neurotoxicity of arteether following parenteral administration of very high doses to dogs [326]. Afterward, the neurotoxicity of other related compounds including QHS, artemether, artesunate, and DHA was studied in rat, dog, or rhesus monkey. Similar pathological evidences of artemether and QHS, DHA in the brains of animals were observed. However, such effects have not been observed with oral administration of any QHS derivative or with intravenous artesunate [327–332]. There is no clinical evidence to date on serious neurotoxicity resulting from the use of any artemisinin drug in humans in prospective studies of more than 10,000 patients or in more than 2 million persons who have received these drugs in China, Myanmar, Thailand, and Vietnam [333,334]. Likewise, in a recent randomized trial in South-west Ethiopia, there was no detrimental effect of a standard oral regimen of Coartem (artemether/benflumeto) on peripheral hearing or brainstem auditory pathways in patients with uncomplicated falciparum malaria [335].

To our knowledge, animal models are important tool in the pharmacological research. The data from animal models will be useful for further researches. For example, the antimalarial activity of artemisinins against *P. berghei* in mice is comparable to the therapeutic effect in clinic, *i.e.*, artemether (ED_{90} 1.02 mg/kg) and arteether (ED_{90} 1.95 mg/kg) on *P. berghei* in mice [183] matching with artemether oil injection (80 mg in 1 mL-ampoule) and arteether oil injection (150 mg in 2 mL-ampoule) in clinic [336,337]. Hence, the animal model for screening antimalarial effect should be considered reliable. However, the test for neurotoxicity of artemisinins in animals is an opposite example. This fact reminds us that if we excessively count on data from animal experiments, good medicines may be missed.

8.1.4 Clinical studies

Since 1978, artemether oil injection and sodium artesunate aqueous injection were used in clinical trials. Their antimalarial effect was proved to be higher, faster, and more reliable than qinghaosu for the treatment of severe malarial patients. In 1987, artemether oil injection (made in Kunming Pharmaceutical Plant, 80 mg in 1-mL ampule) and sodium artesunate aqueous injection (made in Guilin Pharmaceutical Work, 60 mg of artesunic acid with a separate ampoule of 5% sodium bicarbonate solution) were registered as new antimalarial drugs in China. Then, other preparations (such as tablet, capsule, and suppository) were successively developed. After massive clinic trials in other countries, artemether oil injection (80 mg in 1-mL ampoule) was enrolled in the Ninth *List of Essential Medicines* for the treatment of severe malaria and artesunate tablet (50 mg/tablet) in the 11th *List* for treating uncomplicated malaria.

In 2007, artesunate injection (60 mg of artesunic acid with a separate ampoule of 5% sodium bicarbonate solution) was enrolled in the 15th *List* for use in the management of severe malaria, and as an update, artesunate tablet (50 mg/tablet) was used in combination with amodiaquine, mefloquine, or sulfadoxine-pyrimethamine.

In 1992, dihydroartemisinin tablet (60 mg/tablet) and Coartem (artemether-benflumetol, 20 + 120 mg/tablet) were registered in China. In the early 1980s, Chinese researchers synthesized benflumetol, which acts much longer than artemether and has a different target. Successive researches led to new antimalarial drug Coartem (artemether-benflumetol), the first Artemisinin Combination Therapy (ACT) in the world. It showed more effective and delay-resistance property in mice and good effect in clinic trails [338–346]. Since 1994, Novartis developed Coartem according to the international registration line and registered in 79 countries in 1999. Coartem was enrolled in the 12th *List of Essential Medicines* for treating uncomplicated malaria.

From the 1980s to the end of the 20th century, several million malaria patients in the world (mainly in China and South-East Asia) have been cured by administration of QHS, QHS derivatives and their combinations in various compositions, formulations, and doses. For the rational use of this new kind of antimalarials, the international Laveran Association held a symposium in Annecy, France, in April 1998. The following recommendations and opinions were generally agreed by participants at the meeting [347]:

1. QHS and its currently available derivatives (artemether

and artesunate) are the most rapidly acting antimalarial drugs, effective in adults and children against plasmodium malaria including multidrug resistant *P. falciparum*. They are effective in all areas of the world for the treatment of both severe and uncomplicated malaria.

2. Resistance to the drugs has not been identified so far, but this should not cause complacency in their use.

3. All the QHS drugs investigated have comparable efficacy. However, there may be a risk of therapeutic failure following use of substandard and inadequately labeled preparations now appearing on the international market. When available, Good Manufacturing Practices preparations must always be preferred.

4. QHS drugs are very well tolerated, and there is no evidence so far of serious clinical toxicity in man, although further surveillance is required. The neurotoxicity seen in animals after high doses of certain compounds has not been reported in man.

5. Whenever possible, these drugs should only be used in combination with a second, usually longer acting, antimalarial to protect both drugs against the risk of developing resistance.

6. These drugs should not be used for chemoprophylaxis.

Because 90% of malaria patients recovered within 48 h, after monotheraphy mentioned above, it is difficult for most poor malarial patients to take medicine of 5–7 d' dosage for an effective radical cure. To shorten the period of administration and delay the development of resistance to artemisinins, Artemisinin Combination Therapy (ATC) is needed.

In addition to artemisinins, other new antimalarial drugs, including benflumetol, piperaquine, naphthoquine, and pyronaridine (Figure 43), have also been developed in China since 1967. Having longer half-time and different targets, they are considered as the best partners for ACT. Thus, the studies of ACT (artemisinins with them) in various compositions and doses have been conducted in China since the 1980s.

Up to date, four ACT drugs have been elaborated in China by the Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, and the Tropical Medicine Institute, Guangzhou University of Traditional Chinese Medicine and other Institutes:

1. Coartem (artemether-benflumetol, 20 + 120 mg/tablet, 6 doses, 24 tablets/3-d course) registered in 1992;

2. Artekin (DHA-piperaquine diphosphate, 40 + 320 mg/tablet, 3 doses, 9 tablets/3-d course) registered in 2003;

3. Artequick (QHS-piperaquine, 80 + 400 mg/tablet, 2 doses, 10 tablets/2-d course) registered in 2006;

4. Arco (QHS-naphthoquine diphosphate, 125 + 50 mg/tablet, 1 or 2 doses, 8 tablets/1-d course), registered also in 2006.

Piperaquine diphosphate was proved to be more active than chloroquine *in vivo* by Chinese researchers and used as the first-line drug for malaria in China in 1970s'. As a result of intensive use, the resistance of *P. falciparum* to piperaquine emerged in the mid-1980s. After studies on DHA combination with piperaquine, pyrimethamine, and other drugs (trimethoprim and/or primaquine) in various compositions and doses,

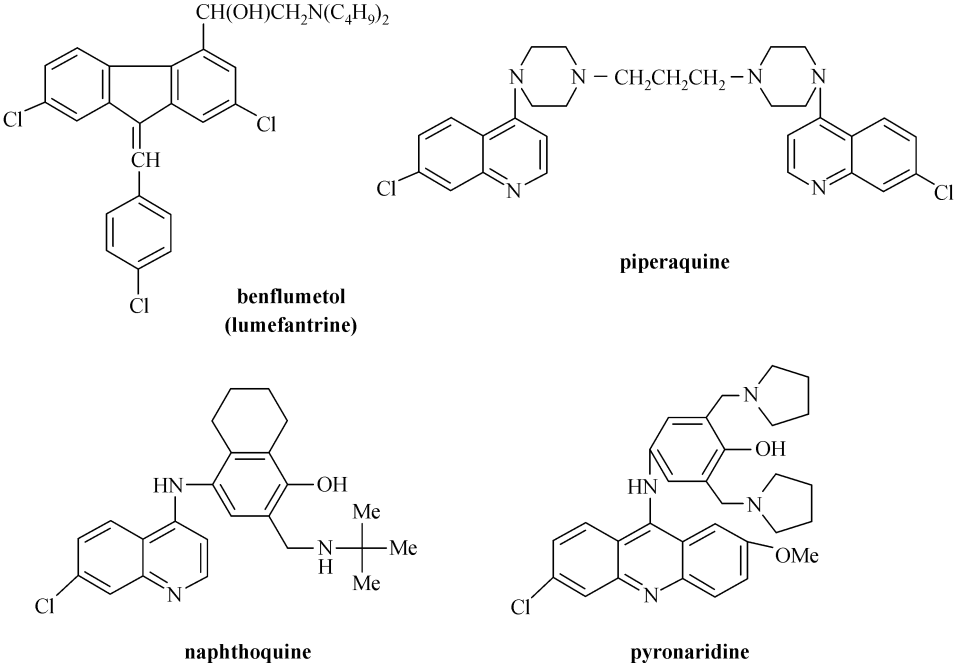


Figure 43 Structures of benflumetol, piperaquine, naphthoquine, and pyronaridine.

Artekin (DHA-piperaquine diphosphate) was at last approved in China.

Many randomized clinical trials of Artekin in China, Cambodia, Vietnam, Thailand, and some countries in Africa indicated its excellent tolerability, high cure rates against multidrug resistant falciparum malaria and cheaper cost [348–361]. Artekin is being used increasingly in South-east Asia and is already part of national treatment recommendations in Cambodia and Vietnam.

Considering the price of ACT drug, QHS is the cheapest one of artemisinins. Thus, the clinical trials of Artequick (QHS-piperaquine) were conducted in China, Vietnam, Cambodia, Indonesia, and Thailand. The results of clinical studies showed that Artequick controlled symptoms and signs of *falciparum* and *vivax* malaria rapidly, with Fever Clearance Time (FCT) 16–30 h and Parasite Clearance Time (PCT) 36–60 h. A 28-d follow-up demonstrated a cure rate of 97% and a recrudescence rate of 3%. This combination also is highly effective in *vivax* malaria with a relapse rate of 2% after one month follow-up [362,363]. To determine the optimum dose of Artequick combination therapies for acute uncomplicated plasmodium falciparum malaria, Thai doctors compared 3-d courses of artesunate-mefloquine, Coartem and Artekin as reference antimalarial treatments, with candidate regimens using (2–3)-d courses of Artequick. The 28-d cure rates were $< 80\%$ for the 2-d treatments with Artequick at 2.4 and 14.4 mg/kg, respectively, in the first study period, and Artequick at 3.2 and 16.0 mg/kg, respectively, but $> 98\%$ for the 3-d regimens. The results suggest that a 3-d course of Artequick at 3.2 mg/kg and 16.0 mg/kg, respectively, deserve further evaluation as an alternative treatment for multidrug-resistant falciparum malaria [356].

Here, we shall introduce the recent clinical advance of Artequick. In the period of 2004–2005, a pilot project entitled "Eliminating Malaria by Eradicating Source" was conducted among 21,343 inhabitants of 62 natural villages in the highly malaria endemic area of Kampong Speu Province and its peripheral areas in Cambodia. Three methods were adopted for eliminating source: 1) All confirmed malaria-infected patients were treated with Artequick regimen and fevered patients also treated with Artequick under problem treatment. 2) A mass treatment of Artequick was conducted among 17 villages where parasite carriage rates in children were $\geqslant 20\%$. 3) Every 10 d for 6 months, mass medications of a low dosage of primaquine were conducted among 27 villages where parasite carriage rates in children were $\geqslant 6\%$. All these measures were implemented by village malaria volunteers. The population parasite carriage rates in 17 malaria source villages were intensively monitored every 6 months. Two years after, the source elimination measures were initiated, the parasite carriage rate in children declined from 55.8% to 5.3%. Of that, the *P. falciparum* carriage rate declined from 37.0% to 2.3% while the carriage rate of *P. vivax* plus *P. malariae* dropped from 18.9% to 3.0%. The gametocyte carriage rate in children had dropped from 13.1% to 1.2%. Moreover, among eight villages the *P. falciparum* carriage rate in children had declined from 46.5% to zero averagely since the measures were adopted. The changes of parasite carriage rate in adults were similar to those in the children, *i.e.*, the parasite carriage rate declined from 46.5% to 6.3%, and *P. falciparum* carriage rate declined from 34.2% to 2.5%, and the carriage rate of *P. vivax* plus *P. malariae* dropped from 12.3% to 3.8%. Malaria with high prevalence and intensity could be reduced substantially, and malaria transmission sources could be eradicated within a short period of time through adopting this method, which is different from the traditional method of eliminating malaria by controlling mosquitoes. Based on these results, the method of Eliminating Malaria by Eradicating Source is considered effective and economic. It will be a promising strategy to be promoted in highly malaria endemic regions [364]. Another similar clinic trial is underway in Comoros, Africa.

Another ACT drug containing QHS is Arco (Co-naphthoquine). In clinic trials, Arco also showed rapid onset of action, high cure rate and good tolerance [365–368]. Its short administration (1-d course) may be easily accepted by most malaria patients.

Other ACTs, such as artemisinins-pyronaridine, have been studied in China. The clinical trials of the combination of pyronaridine with artesunate or artemether (pyronaridine 400 mg + artesunate 100 mg or artemether 150 mg, 2-d course) were conducted in Hainan Province, China [369,370]. Other clinical trials of combination of pyronaridine and DHA (400 mg + 100 mg/1st day, 200 mg + 100 mg/2nd, 3rd day) were conducted in Hainan and Yunnan Provinces, China, and Africa [371–372].

In other countries, new ACT drugs have also been developed [373–376]. Most researches focused on the combination of artesunate with known antimalarial drugs, such as mefloquine, amodiaquine, sulfadoxine/pyrimethamine, or chloroquine. There are evidences that combinations improve efficacy without increasing toxicity. However, the absolute cure rates achieved by combinations vary widely and are dependent on the level of resistance of the partner. In 2006, the WHO recommended four ACTs (artemether-lumefantrine, artesunate-mefloquine, artesunate-amodiaquine, and artesunate-sulfadoxine-pyrimethamine) for oral treatment of uncomplicated falciparum malaria. Generally, they are rapidly and reliably effective. The adverse effect of the ACT drugs is determined by the partner drug. If a partner has seen resistance in some endemic regions, the ACT drugs with the partner should not be used there anymore.

8.1.5 Drug Resistance

Resistance to antimalarial chemotherapy is a major concern for malaria control in the malaria-epidemic countries. Once QHS was introduced as a new antimalarial drug, naturally, the development of QHS-resistance is under close study immediately. In 1980s, QHS-resistant lines of *P. falciparum* and *P. yoelii* were successfully developed in some laboratories in China and other countries, but when administration was stopped, the resistance disappeared at once [377–381]. Since artemisinins (mainly monotherapy) were widely used in China and South-East Asia, continuous *in vitro* and *in vivo* surveillances have been made, especially in the area where resistance developed seriously in the history, such as Hainan and Yunnan provinces in China and the Thai/Cambodian and Thai/Myanmar borders. According to the results of surveillances, the sensitivity of *P. falciparum* to artemisinins dropped somewhat in some areas, but no QHS-resistant line of *P. falciparum* arose [382–396].

To prevent the onset and spread of drug resistance, WHO declared that oral administration of QHS monodrugs should be forbidden in 2004. However, some Chinese researchers hold a different viewpoint, after observing that the emergence of drug resistance seems to be closely related to the half-life of these antimalarials, *i.e.*, the longer a drug's half-life is, the faster its resistance is developed. For instance, chloroquine has a half-life of 6–50 d [397], and its resistance arose after being used for 15 y. Mefloquine has a half-life of 6.5–33 d [397], and its resistance arose after several years. Quinine has a half-life of only 6–12 h [397], and it has been used for nearly 100 y with resistance just arising now. Therefore, it is deduced that artemisinins (in various preparations including oil injection) with a half-life of 0.5–24 h [398] may have much longer lifespan. In fact, QHS and its derivatives (mainly monotherapy) have been used for nearly 30 y in China, and no resistant line has ever been found. A recent study was completed on whether mutation has occurred in field isolates in China [399]. 95 *P. falciparum* field isolates were collected in 2006–2007 from Hainan and Yunnan Provinces, and the results indicate that the S769N mutation in the PfATPase6 gene is not present in China, suggesting that artemisinin resistance has not yet developed [399].

Recently, a research group collected 530 *P. falciparum* isolates from three countries (Cambodia, French Guiana, and Senegal) with different artemisinin use and determined their *in vitro* susceptibility with an isotopic microtest. Artemether IC_{50} up to 117 and 45 nmol/L was seen in French Guiana and Senegal, respectively [400]. Another paper reported the development of ACT (artesunate-mefloquine) drug resistance on the Cambodia-Thai border [401].

These two reports reveal the shocking fact that, not long after ACTs were used as the first-line drugs in the epidemic regions with high resistance, QHS and its derivatives experienced reduction of effectiveness. We are seriously concerned whether this fact indicates a resistant line is emerging. On the other hand, artemisinin has been used for nearly 30 y as monotherapy in China without the development of resistant line. All these facts force us to scrutinize the ACT recommended by WHO in 2004, such as artesunate-mefloquine, artesunate-amodiaquine, and artesunate-sulfadoxine-pyrimethamine, whose partners have been widely used in the 20th century with resistant line emerging at varying degrees. It is important to study how these partners affect the development of artemisinin resistant line and whether the impact is adverse. We should ask ourselves whether artemisinins could really be protected by these old antimalarial drugs. These reports confirmed that the selection of partner for ATC is very important. We believe that the best choice for the partner of ACT should be new antimalarial drugs with longer half-life or different targets. Artemisinins are in a precarious situation, which demands urgent and in-depth laboratory and clinical researches.

8.2 Against other parasites

The outstanding antimalarial action of QHS drives researchers to conduct more studies in more medical applications. A lot of experimental and clinical studies done in China showed that artemisinin and its derivatives were not only the potent antimalarial drugs but also the useful agents for other diseases, especially as antiparasitic agents (such as against *Schistosoma japonicum*, C*lonorchis Sinensis*, *Theileria annulatan*, *Toxoplasma gondii*, *etc*.).

Because schistosomiasis has been a major infectious disease in China, the antischistosomal activity of QHS was soon assessed after antimalarial experiment of QHS [402]. In the end of 1970s, artemether and artesunate were confirmed to have higher antischistosomal activity than QHS in animal models [403–405]. Later, it was found that QHS derivatives could effectively kill the juvenile-stage parasites living in mice, but praziquantel could not. Their prevention of development of the mature female worms was also proved in other animal models (rat, rabbit, and dog) [406–409]. Since 1993, artemether and artesunate were studied in randomized, double-blind, placebo-controlled trials in Jiangxi, Hunan, Anhui, and Yunnan Provinces, China [410–421]. Repeated oral administration of artemether or artesunate (6 mg/kg, one dose every 1 or 2 weeks) was safe and efficacious in the prevention of *S. japonicum* infections, and then, artemether and artesunate were approved as the prevention drugs for schistosomiasis in 1996 in China. Afterward, artemether and artesunate showed the similar activity against *S. mansoni* and

S. hematobium in the laboratory studies and clinical trials in other countries [422–427].

Praziquantel has been used in Asia, Africa, and South America for treatment of Schistosomiasis for nearly 40 y. To address the concern about the development of tolerance and/or resistance to praziquantel, there is a need for research and development of novel drugs for the prevention and cure of schistosomiasis. Now, the combination of artemether and praziquantel is being studied [428–431].

Interestingly, a small study in the eastern Sudan observed the effects of the treatment of uncomplicated, falciparum malaria with artesunate-sulfamethoxypyrazine-pyrimethamine (AS-SMP), and artemether-lumefantrine (Coartem) on coinfections with *S. mansoni*. These combinations are currently very effective treatments not only for uncomplicated falciparum malaria but also for *S. mansoni* infections [432].

As for the study of artemisinins against other parasites (such as *Leishmaniasis*, *Clonorchis sinensis*, *Toxoplasma gondii*, and *Pneumocystis carnii*), a number of reports about *in vitro* and *in vivo* effect of artemisinins have been published, for example, high efficacy of artemether against *P. carnii* pneumonia in monkey model [433], activity of artemether and artesunate against *Clonorchis sinensis in vivo* [434], and activity of artemisinin derivatives against *Toxoplasma gondii in vitro* [435]. More related information may be found in a book about QHS research in China published last summer [436].

8.3 Antitumor activity

In China, *in vitro* antitumor activity of artemisinic acid and arteannuinB was first performed [437–439]. In the West, some natural components of *A. annua* (such as QHS, arteannuinB, artemisinic acid, artemisitene, flavonoids, and other terpenoids) proved their antitumor activities at varying concentrations against EN2, L-1210, P-388, A-549, HT-29, MCF-7, and KB *in vitro* [440–442].

In the assay of cytotoxicity of QHS and related compounds against *Ehrlich Ascites* tumor cells, QHS, artemether, arteether, and artesunate exhibited cytotoxicity (IC_{50} 12.2–19.9 μM), while artemisitene was more active (IC_{50} 6.8 μM), and the dimer of dihydroartemisinin was most potent (IC_{50} 1.4 μM) [443].

Later, artesunate and DHA, two antimalarial drugs, attracted general attention. The antitumor effect of artesunate was tested *in vitro* and *in vivo* in China [444–446] It exhibited cytotoxicity for six cell lines (IC_{50} 1–100 μg/mL) and antitumor effects on human nasopharyngeal cancer (CNE2, SUNE-1) and human liver cancer (BEL-7402) in nude mice. At the same time, artesunate was analyzed for its antitumor activity against 55 cell lines [447]. Artesunate was most active against leukemia and colon cancer cell lines. It is notable that none of CEM leukemia sublines, which are resistant to doxorubicin, vincristine, methotrexate, or hydroxyurea, showed cross-resistance to artesunate [447]. The anticancer activity of artesunate has also been shown in human xenograft tumors in mice [448].

In a study of antitumor activity of DHA, it was found that DHA could selectively kill cancer cells in the presence of ferrous sulfate or holotransferrin. Holotransferrin can increase intracellular iron concentration; nevertheless, normal breast cells (HTB 125) and lymphocytes had nonsignificant changes. According to reports, there are more transferring receptors on tumor cell surface than normal human cells, so there is more iron in the tumor cells. It is proposed that the free radical generated from artemisinins reacts with iron and kills the tumor cells [449–452].

Afterward, it was reported that artesunate and DHA inhibit angiogenesis and induce apoptosis [453–457].

To achieve higher antitumor activity, many *N*-glycoside, *O*-glycoside, and some dimers have been tested *in vitro* and *in vivo*, as mentioned above. Some new kinds of artemisinin derivatives are discussed below.

When compound **154** was found active (Figure 44), a study was conducted on the relationship between chemical structure and antitumor activity of the type of QHS derivative. This study led to the most active compound **155** (R = *p*-Br, IC_{50} = 11 nM, and 27 nM against P 388 and A 549 cell lines, respectively), comparable to VCR, a potent cytotoxic agent used in clinic. The inactive analog **156** indicates that the peroxy group is essential for both antitumor and antimalarial activities [458,459]. Compound **157**, yielded by coupling the cyanoarylmethyl group with artesunate, did not show higher antitumor activity than **155** [460]. Flow cytometry data show that these active compounds cause an accumulation of L1210 and P388 cells in the G1-phase of the cell cycle and apoptosis in P388 cells [459,460].

Recently, some amide-linked dimer, sulfide-linked dimer, and sulfone-linked dimer and trimer were synthesized, and these compounds showed potent and selective inhibition on the growth of certain human cancer cell lines (Figure 45). Trimer **158** was especially comparable to clinically used anticancer drugs [461].

To date, hundreds of papers about antitumor artemisinins appeared in the Chinese and international journals. These studies disclosed that the inhibitory activity of QHS and its derivatives (specially, DHA and artesunate) toward human cancer cells was in the nano- to micro-molar range. The abundant information on their antitumor activities and mechanisms (including the regulation of proliferation, apoptosis, angiogenesis, targets, and candidate genes) has been summarized in a review published in 2007 [462]. Soon

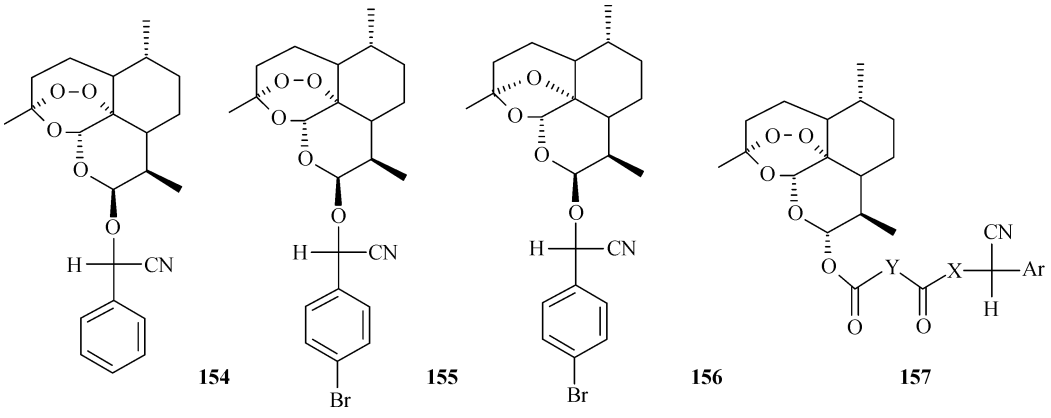


Figure 44 Dihydroartemisinin ethers containing cyanoaryl methyl group.

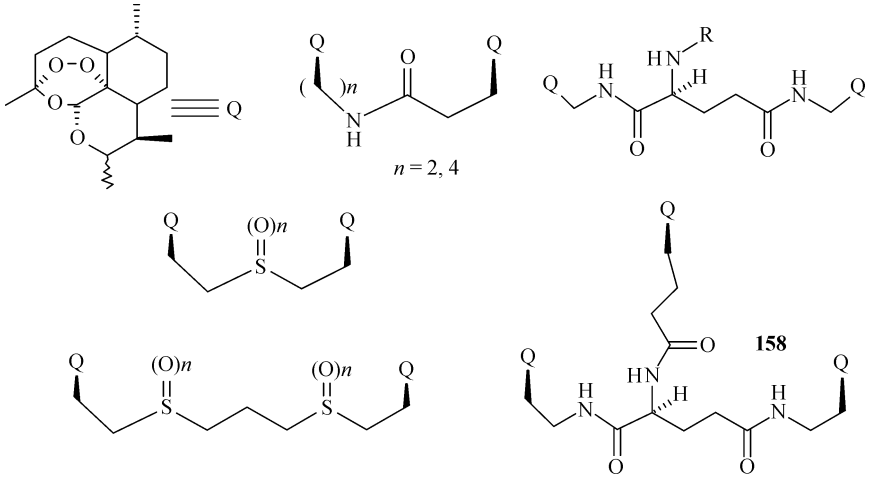


Figure 45 Dimers and trimers with anticancer activity.

afterward, some additional papers about antitumor mechanism of DHA were published [463–470].

The clinical trials of artesunate and artemether have been conducted, and a few papers reported the result of treatment of metastatic uveal melanoma, pituitary macroadenoma, or lung cancer, with artesunate and artemether either being used alone or in combination with conventional chemotherapy [471–473].

8.4 Immunosuppressive activity

Generally, antimalarial drugs possess immunosuppressive action and were often used for the treatment of dermatoses, such as chloroquine and hydroxychloroquine for lupus erythematosus and multiple solar dermatitis. Therefore, the research of immunological activity of QHS and its derivatives became an attractive program in China in the 1980s. Up to date, great progress has been made *in vitro* and *in vivo* tests, even in clinical trials.

At first, new antimalarial drugs (QHS, artesunate, and artemether) were assessed mainly in Chinese laboratories. The experimental results indicated that they possessed immunosuppressive activities [474–481]. Especially, artesunate and DHA showed potent effects on BXSB mice and also on SLE-like, psoriasis-like, or I–IV types of hypersensitivity mouse models [482–487].

The clinical trials of artemisinins (mainly artesunate) for the treatment of DLE, SLE, rheumatic arthritis, polymorphous light eruption, and chronic actinic dermatitis have been reported [488–495]. Some clinical results were promising, for example, 56 patients with lupus erythematosus (DLE 16, SCLE 10, and SLE 30) were treated by sodium artesunate (i.v. 60 mg, once a day, 15 d a course, 2–4 courses), with effect rate at 94%, 90%, and 80%, respectively [492].

To search for highly potent, low toxic, specific-selective, and tolerance-inducing immunosuppressive agents, a series of novel artemisinin derivatives (Figure 46) were synthesized and assessed for their cytotoxicity of lymphocyte, inhibition activity on ConA-induced T cell proliferation, and LPS-induced B cell proliferation in comparison with QHS,

artesunate, artemether, and cyclosporin A (CsA) *in vitro* [496–498]. Furthermore, SM 735, SM 905, SM 933, and SM 934 have been selected and tested in the animal models for 2,4-dinitrofluorobenzene (DNFB)-induced delayed-type hypersensitivity (DTH) reaction, sheep red blood cell (SRBC)-induced antibody production, and experimental autoimmune encephalomyelitis (EAE) [499–501]. Preclinical research of a lead compound is in progress.

Some papers about mechanism of anti-inflammatory properties of artemisinins involving NFκB, TNF-α, nitric oxide synthase, the Rig-G/JAB1 signaling pathways, and the Rig-G/JAB1 pathway have been published [502–514].

9 Exploration on the antimalarial mechanism of QHS and its derivatives based on their free radical reaction with Fe(II)

QHS is a sesquiterpene molecule containing carbon, hydrogen, and oxygen, but no nitrogen atoms, and can be used for the treatment of multidrug-resistant strains of *P. falciparum*. It is obvious that its antimalarial mechanism is quite different from that of previous antimalarial drugs, such as quinine, chloroquine, *etc*. Since the discovery of QHS, its action mode at the molecular level has attracted wide interest, although it is a very difficult task to get the answer. Actually, up to now, the action mode of quinine and other synthetic antimalarial drugs is still not so clearly understood [515].

QHS acts upon parasite at its intra-erythrocytic asexual stages. At that stage, the parasite takes hemoglobin as its nutritional resource, digests hemoglobin, and leaves free heme, which is then polymerized to parasite safety poly-heme (hemozoin). Two other points worth mentioning are that over 95% iron in the human body exists as heme in the red cell and that the peroxide segment of QHS and its derivatives is essentially responsible for its activity.

Being aware of the DNA cleavage with Fenton reagent [516,517] and the situation of qinghaosu-parasite-red cell mentioned above, this laboratory studied the reaction of QHS and its derivatives with ferrous ion in aqueous acetonitrile since the early 1990s. At first, the reaction of QHS and ferrous sulfate (1∶1 in mole) was run in H_2O-CH_3CN (1∶1 in volume, pH 4) at room temperature. It was interesting to find that the two major products were tetrahydrofuran compound **9** and 3α-hydroxy deoxyqinghaosu (**6**), which have been identified as the natural products of Qinghao, pyrolysis products, and the metabolites of QHS *in vivo* mentioned above. After careful chromatography, a minor product epoxide **159** was identified. In addition, acetylation of the remaining high polarity products yielded an acetyl tetrahydrofuran compound **160**. Based on the analysis of these products, a reaction mechanism of an oxygen-centered free radical followed by single-electron rearrangement was suggested in 1995–1996 [518] (Scheme 27).

Since then, a number of QHS derivatives (**161**) have been treated with ferrous sulfate in the same reaction condition [519]. Except for some hydrolysis products, similar derivatives of tetrahydrofuran compound **9** and 3α-hydroxy deoxyqinghaosu **6** were also isolated as the two major

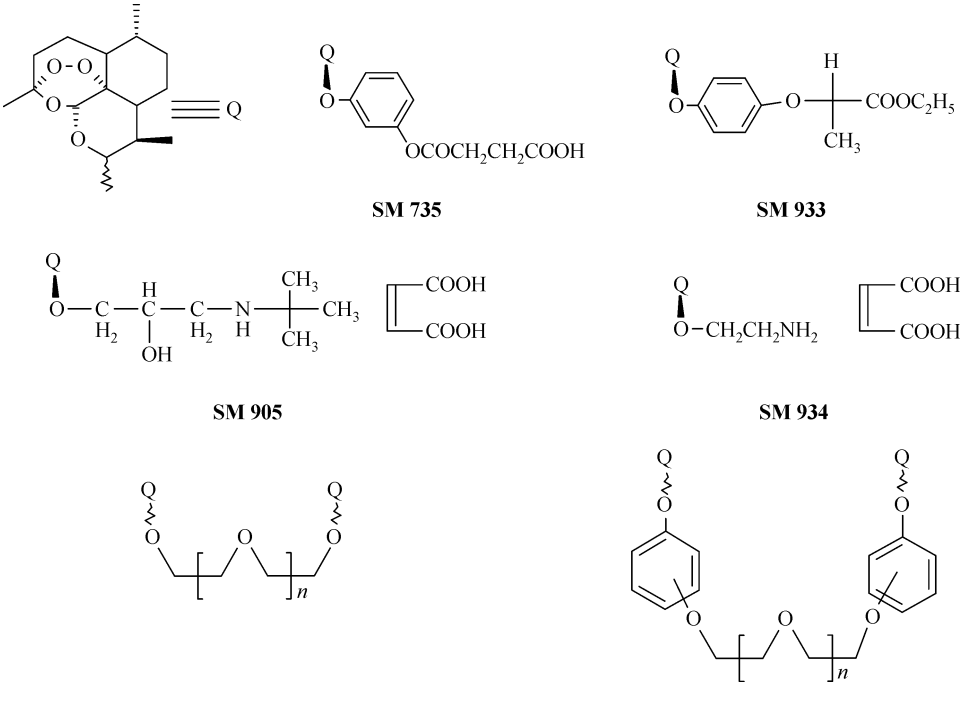
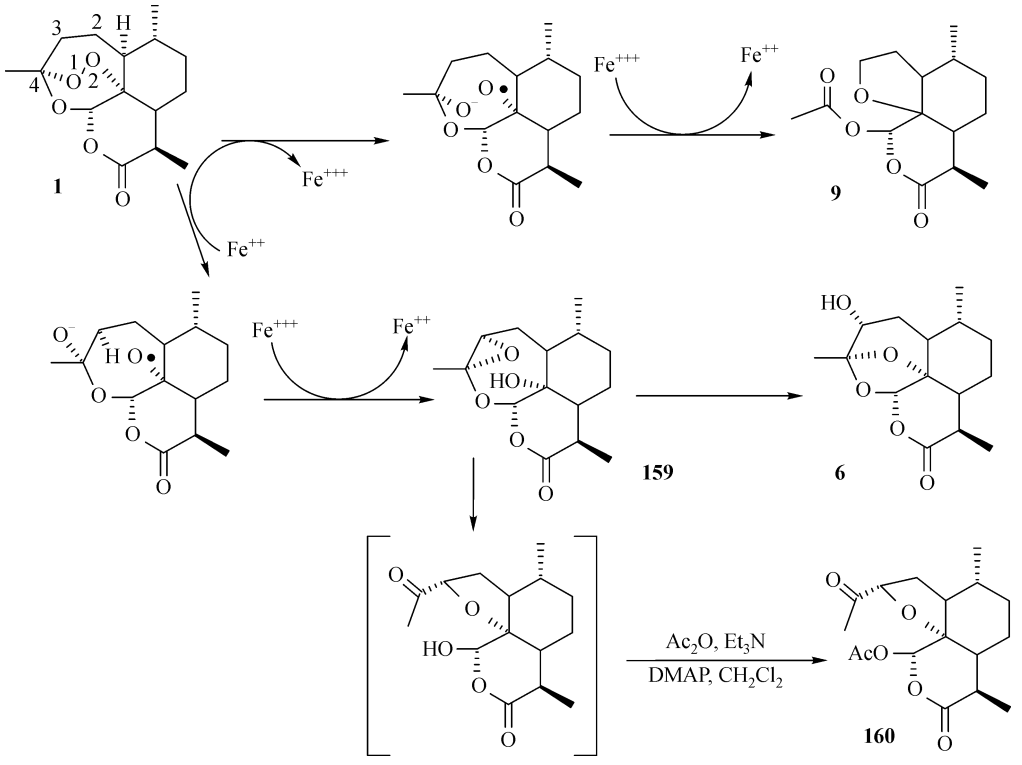


Figure 46 New types of QHS derivatives with immunosuppressive action.

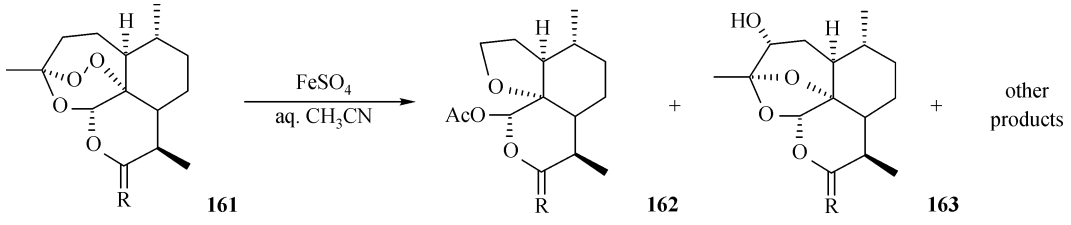


Scheme 27 Reaction of QHS with ferrous ion.

products but in somewhat different ratios (Scheme 28, Table 2). Usually, these derivatives shown in the Table 2 produced tetrahydrofuran compounds (**162b**–**f**) in higher ratios than **162a (9)** from QHS, and these derivatives also showed higher antimalarial activity than QHS. However, it is hard to say that there is a correlation between this reaction and activity at this stage.

While this project progressed, an ESR signal of secondary carbon-centered free radical (**164**) was detected in the reaction of QHS and equivalent ferrous sulfate in aqueous acetonitrile with MNP as trapping agent [519]. In the same year, Butler *et al*. detected the ESR signals of both primary (**165**) and secondary free radicals (**164**) with DMPO and DBNBS as trapping agent [520]. Figures 47 and 48 show the ESR spectra with MNP and DBNBS as trapping agents, respectively.

Based on these new evidences and results published by other laboratories, the reaction mechanism of QHS and ferrous ion was revised to one that proceeded through short-lived oxygen-centered free radicals and then carbon-centered free radicals [519,521]. There were two kinds of carbon-centered free radicals: primary C-centered free radical **165** and secondary one **164**. Both carbon-centered free radicals were then confirmed by the isolation of their hydrogen-abstracted products **166** and **4** too. The proposed mechanism is concisely depicted in Scheme 29. Thus, tetrahydrofuran compound **9** is derived from the primary C-centered free radical **165** and 3α-hydroxy deoxyqinghaosu (**6**) from the secondary C-centered free radical **164** via **159**. This free radical mechanism also may explain why these similar products were obtained from the reaction of QHS derivatives.



Scheme 28 Reaction of QHS derivatives with ferrous ion.

Table 2 Results of cleavage of **1** and its derivatives with $FeSO_4$ in aqueous CH_3CN

Entry	Compound	Product (yield)		
1	**161 a(1)**	**162 a (9)** (25%)	**163 a (6)** (67%)	Others ($<$ 10%)
2	**161 b (120)**	**162 b** (37%)	**163 b** (45%)	**163 e** (4%, $\alpha + \beta$)
3	**161 c (122)**	**162 c** (45%)	**163 c** (23%)	**163 e** (25%)
4	**161 d**	**162 d*** (39%)	**163 d** (56%)	
5	**161 e (91)**	**162 e** (46%)	**163 e** (25%)	
6	**161 f**	**162 f** (59%)	**163 f** (25%)	

Note: Hydrolysis of **162 d** led to a dialdehyde.

It has been found that QHS (**1**) can be decomposed by less than 1 equivalent of ferrous ion to its free radical degradation products, though in longer reaction time. However, in the presence of excess other reducing agents, such as mercaptans, cysteine, ascorbic acid, and QHS can be degraded by as little as 10^{-3} equivalent of ferrous sulfate to give compounds **9** and **6**. In the presence of 2 equivalents of cysteine, besides **9** and **6**, compounds **166**, **4** and, after treatment with acetic anhydride, compound **167** also could be separated. **166** and **4** were derived from abstracting a proton of the proposed free radicals **165** and **164**, respectively. However, compound **167** was supposed to be derived from **168**, an adduct of primary free radical **165** and cysteine [521] (Scheme 30). This deduction was then confirmed by the separation of an adduct **169** of cysteine and a QHS derivative from their Fe catalyzed reaction mixture [221] (Scheme 30).

Recently, the degradation reaction with artemether and a catalytic amount of Fe(II/III) in the presence of cysteine was also performed, giving not only the adduct (**170**) of primary radical but also the adduct (**171**) of secondary radical for the first time [522] (Scheme 31).

In 1990s, several laboratories were engaged in the study on the reaction of ferrous ion and QHS compounds and proposed that it was a free radical reaction [523–528]. Posner proposed that a high-valent iron-oxo was also intermediated during this reaction, but this viewpoint has not been generally accepted [523]. At the same time, Meunier also identified the adduct of radical **165** and tetraphenylporphyrin or heme and hence confirmed the intermediacy of carbon-centered free radical [527]. In line with all above data, it can be concluded that the reaction of QHS and its derivatives with ferrous ion is definitely a free radical reaction through a short-lived O-radical-anion and subsequent primary and secondary C-centered radicals.

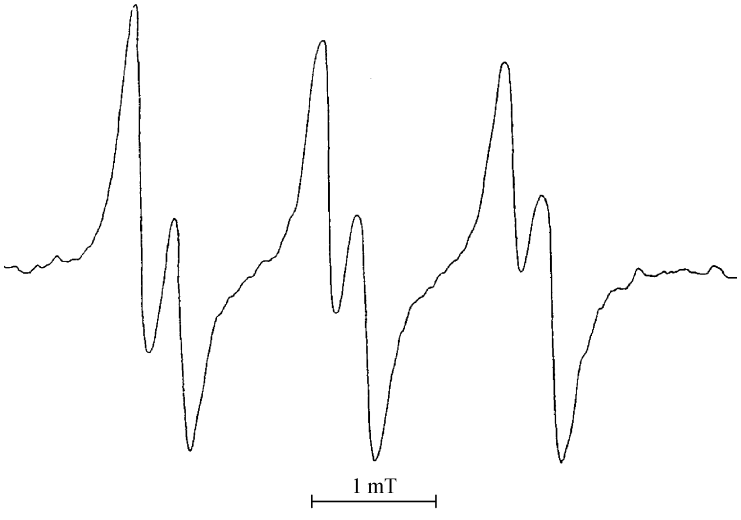


Figure 47 ESR signal recorded in a run in aqueous CH_3CN with QHS as substrate in the presence of 1 equiv. of $FeSO_4$ with MNP as trapping agent.

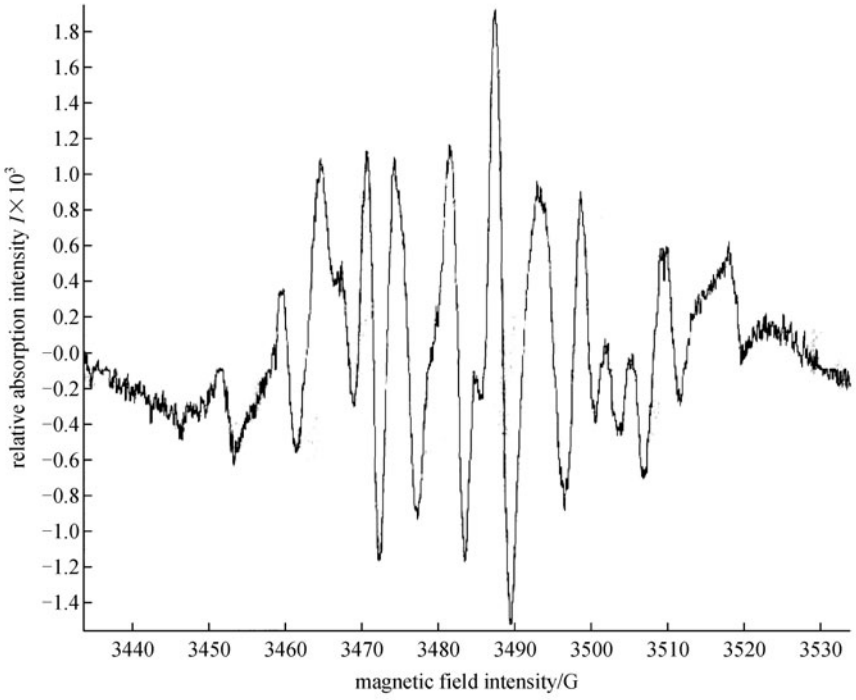
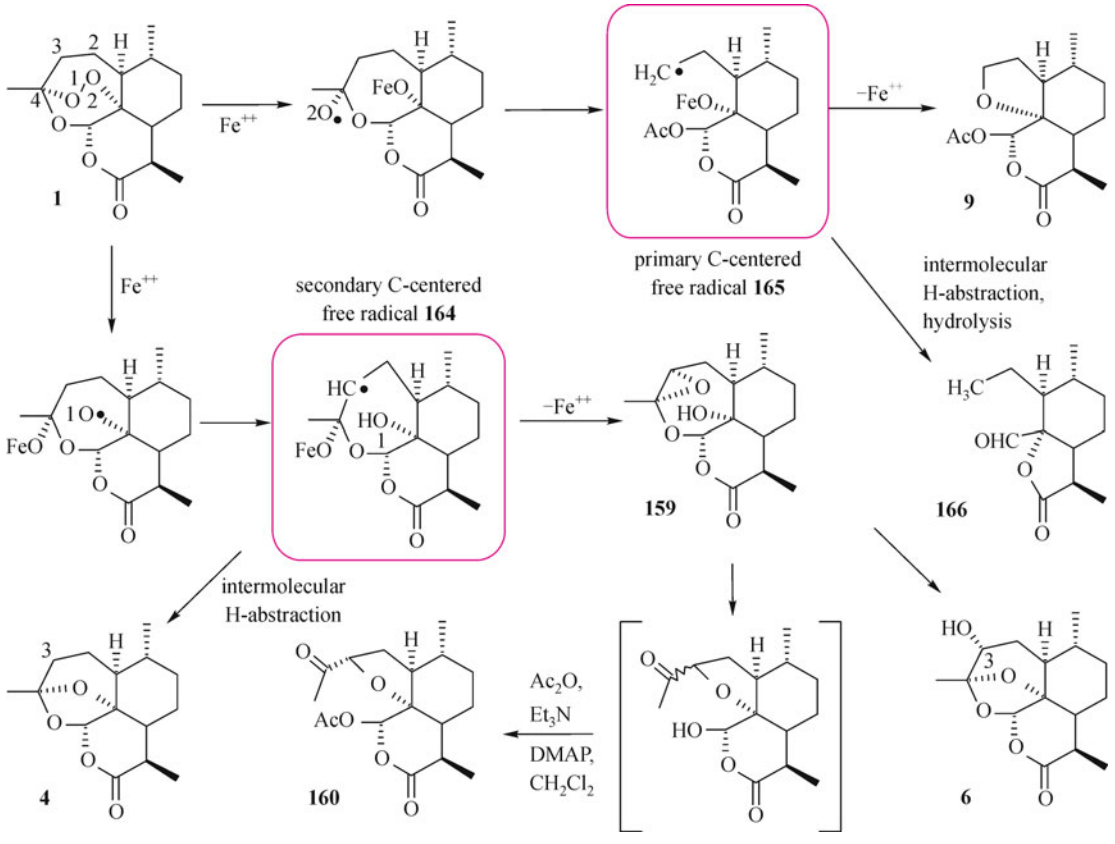
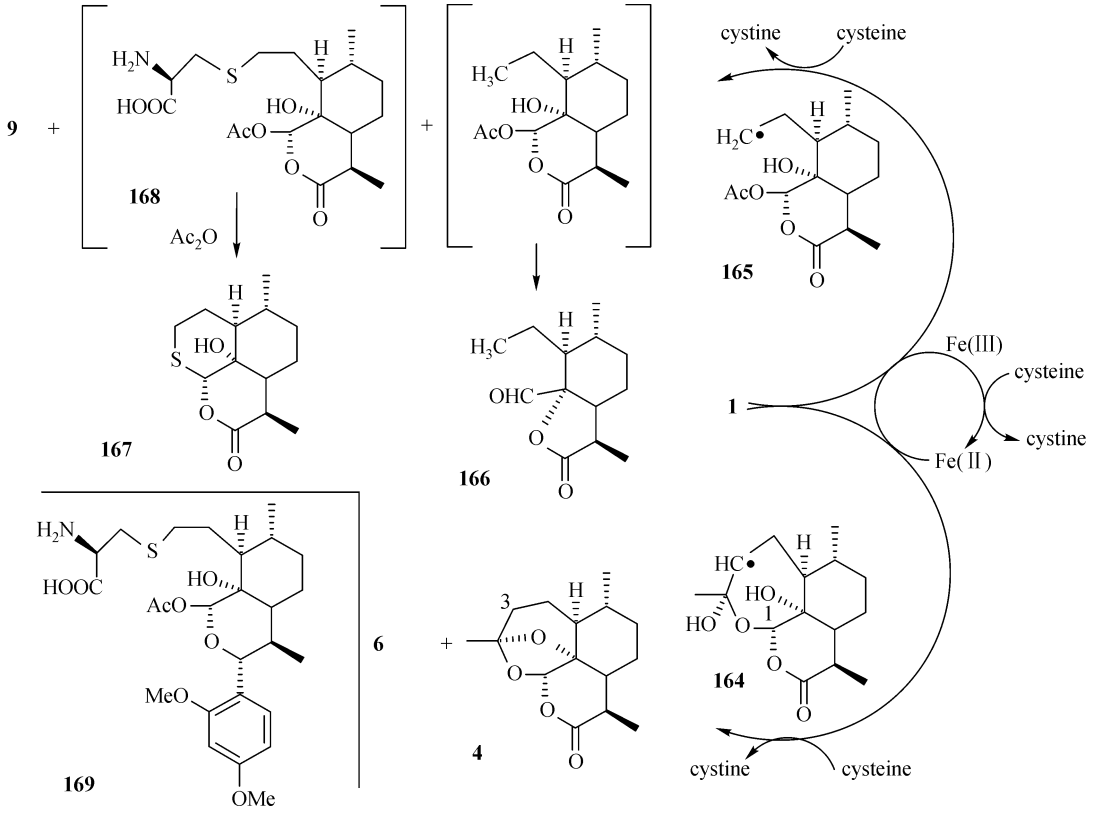


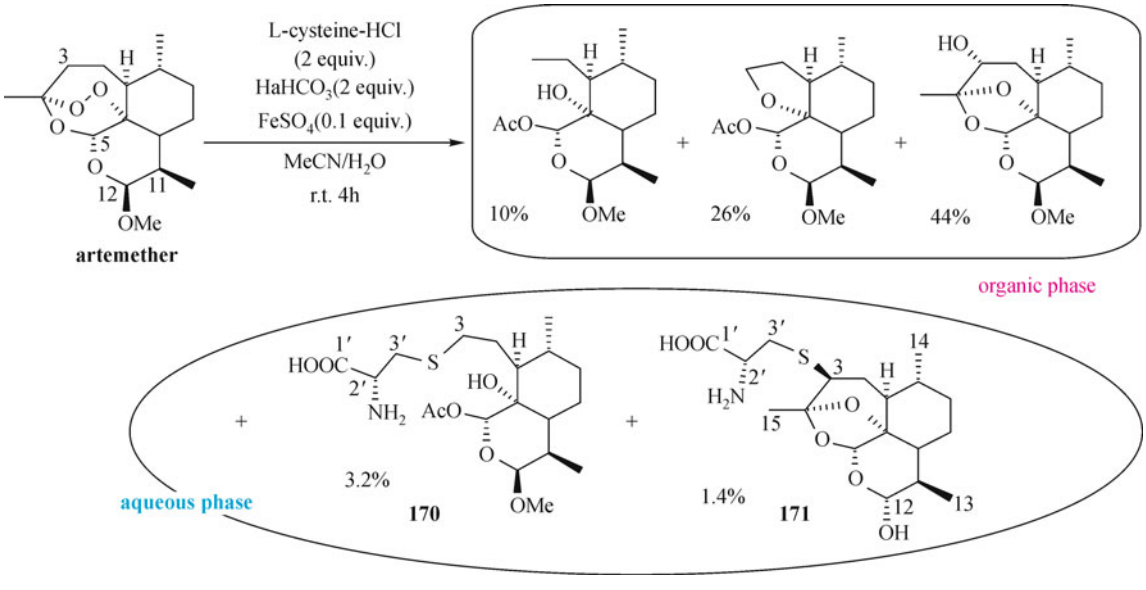
Figure 48 ESR signal recorded in a run in aqueous CH_3CN with QHS as substrate in the presence of 1 equiv. of $FeSO_4$ with DBNBS as trapping agent.



Scheme 29 Formation of carbon-center free radicals.



Scheme 30 Additive reaction of QHS and its derivative with cysteine.



Scheme 31 Reaction of artemether and cysteine in the presence of $FeSO_4$.

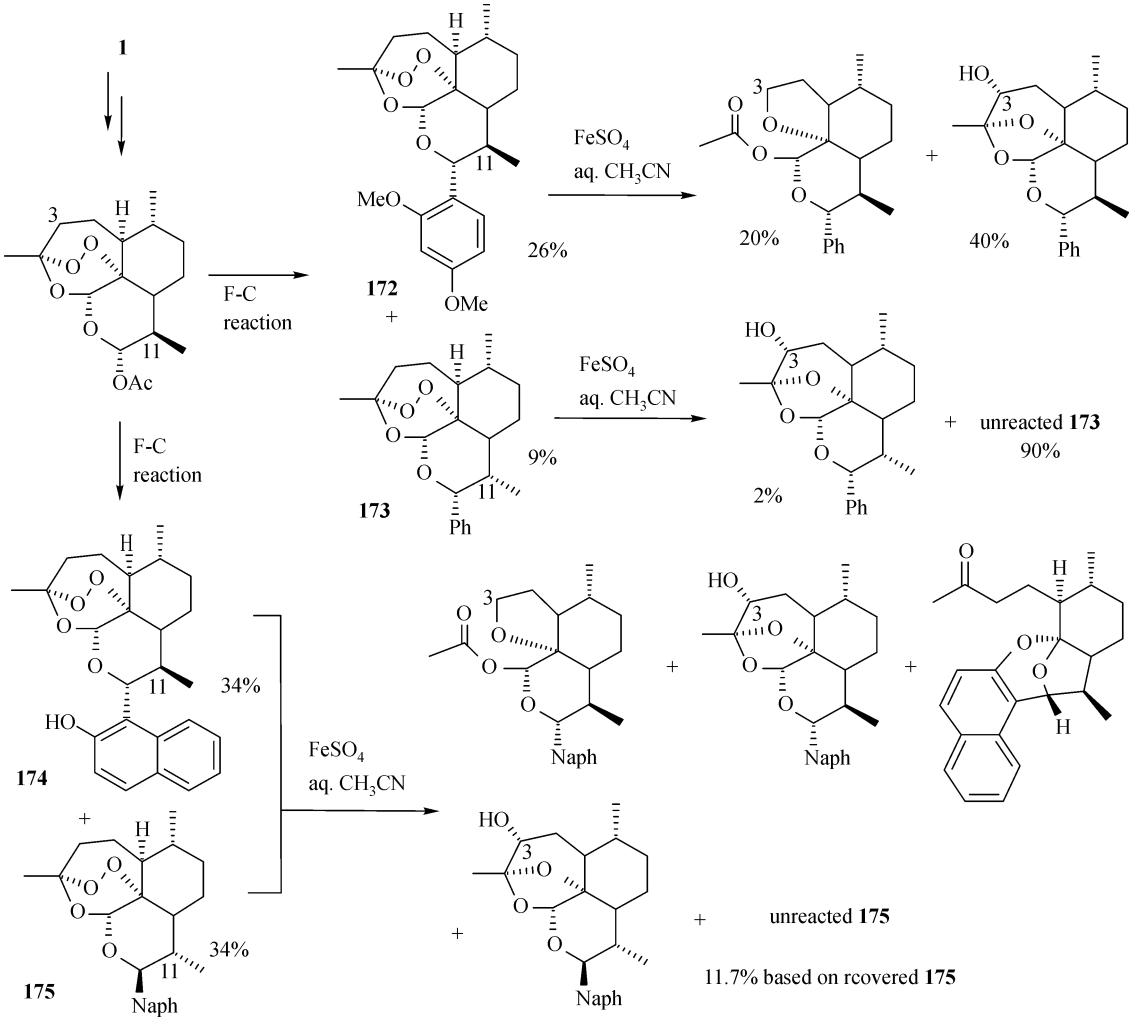
9.1 Antimalarial activity and the free radical reaction of QHS and its derivatives

After the clarification of the reaction mechanism of QHS and ferrous ion participated by C-centered radicals, researchers moved on to study whether this free radical mechanism was related to its antimalarial activity. For the study of the mode of action, several stable and UV-detectable C-12 aromatic substituted derivatives of QHS were synthesized [220,221]. Using the usual Lewis acid as the catalyst, the Friedel-Crafts alkylation gave the desired product **172** or **174** and also 11-methyl epimer **173** or **175** as the by-product. These products

were separated and subjected to the bioassay and chemical reaction with ferrous ion, respectively. It is interesting to find that these derivatives with normal configuration at C-11 showed higher bioactivity and also higher chemical reactivity in the reaction with ferrous ion. However, their C-11 epimers were obviously less active for antimalarial and almost inert to the reaction with ferrous ion [220,221] (Scheme 32, Table 3).

The 11α-epimers **173** and **175** are much less reactive than their corresponding 11β-epimers. The unfavorable influence from the 11α-substituents can also be found in other examples [246,250,251,528]. This lower reactivity may be attributed to the steric hindrance around O-1 atom in 11α-epimers, which blocks the way for Fe(II) to attack O-1 (Figure 49). These experimental results show that the cleavage of peroxide with Fe(II), and then, the formation of C-centered free radical, especially primary C-centered radical, are essential for the antimalarial activity.

Posner *et al.* have synthesized the 3-methyl-derivatives of QHS and found that the antimalarial activity of 3β-methyl derivative *in vitro* was about the same as QHS; however, the



Scheme 32 Reactions of QHS derivatives and $FeSO_4$.

Table 3 ED_{50}- and ED_{90}-values against *Plasmodium berghei* K173 strain (administered orally to mice as suspensions in Tween 80)

Compound	ED_{50} /(mg·kg^{-1})	ED_{90} /(mg·kg^{-1})
Artemether	1	3.1
172	1.27	5.27
173	4.18	76.27
174	0.58	1.73
175	7.08	60.99

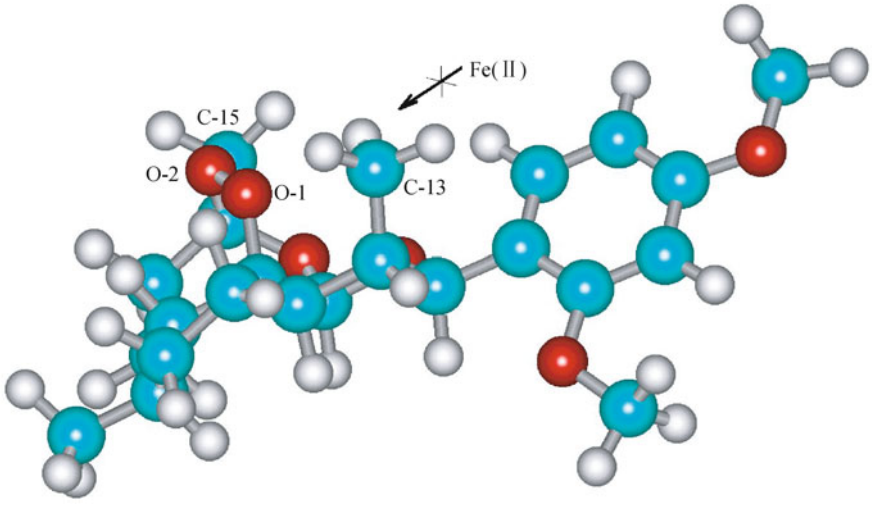


Figure 49 11α-Methyl group of compound 173 blocks the attack of Fe(II) on O-1.

activity of 3α-methyl- or 3, 3-dimethyl-derivative was at least two orders less than that of QHS. This difference was supposedly caused by the availability of C-3 free radical for the later derivatives, but it was not mentioned whether these bio-inactive compounds were also inert in the chemical reaction with ferrous ion [529].

9.2 Interaction of biomolecules with carbon-centered free radical

The C-centered free radical is the active species, but it is still a puzzle that target in the biosystem will be attacked by these radicals derived from QHS and its derivatives. This is an intriguing topic in the QHS research area.

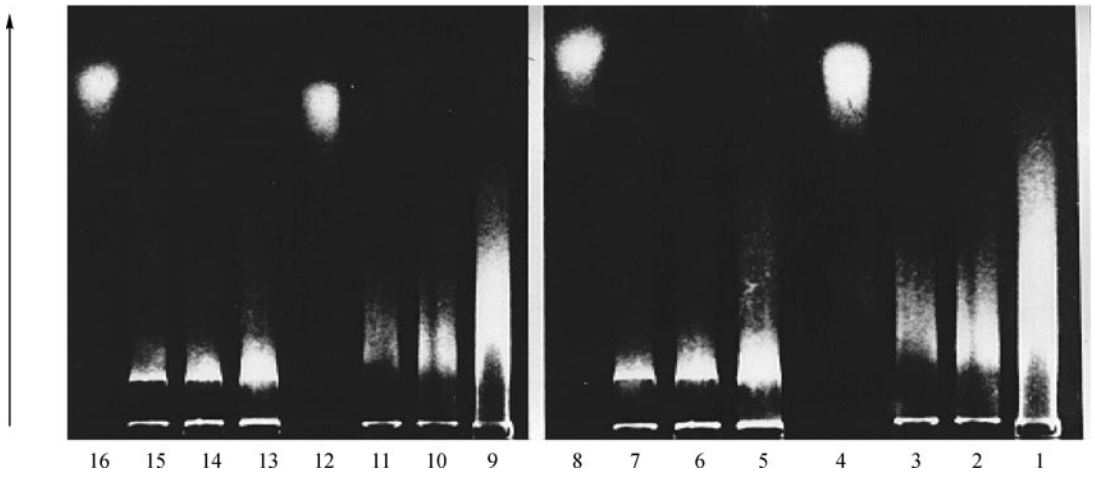
9.2.1 Interaction with DNA

Knowing that DNA could be cleaved with Fenton reagent, researchers were curious whether QHS-ferrous ion could also cleave DNA, though there are two different kinds of free radicals: the oxygen radical from Fenton reaction and carbon free radical from reaction of QHS-ferrous ion. It is known that malaria parasite is mainly living in a red blood cell. The mature red blood cell has no nucleus, while the parasite has. If QHS can permeate the membrane, reach the nucleus of parasite, and react with Fe(II), it will possibly interact with DNA. This may explain why QHS is only toxic to parasite but not to the normal red blood cell.

Given that the pH in blood serum is about 7.35–7.45 and that the ferrous ion will precipitate above pH 7, the DNA damage experiments with QHS and stoichiometric ferrous ion were performed in aqueous acetonitrile (1∶1), at 37°C and at pH 6.5 adjusted with a phosphate buffer. It was interesting to find that the cleavage of calf thymus, salmon, and a supercoiled DNA pUC 18 was observed, yielding a DNA fragment with about 100 base-pair (the marks not shown in the figure) (Figures 50 and 51) [518,530]. The phosphate buffer is important; otherwise, the calf thymus DNA was totally cleaved when the concentration of $FeSO_4$ was 200–2000 μM even in the absence of QHS. Changed forms of supercoiled DNA pUC 18 and unchanged form were only observed at the concentration of $FeSO_4$ being 10–25 μM and below 10 μM in the absence of phosphate buffer and QHS.

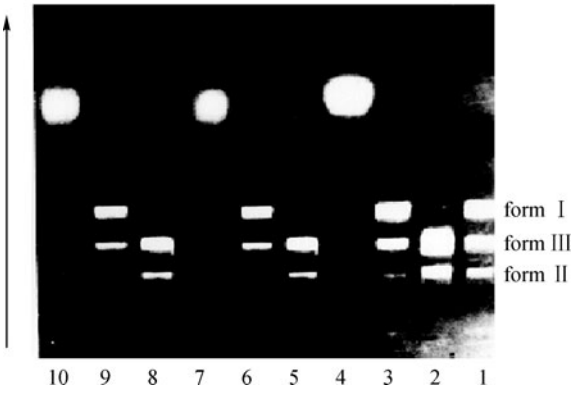
It was known that QHS and its derivatives could react with a catalytic amount of ferrous or ferric ions in the presence of excess reducing agents including cysteine or glutathione (GSH) to afford the free radical reaction products (*vide ante*). When the concentrations of pUC18 and QHS in aqueous acetonitrile were 0.040 μg/μL and 2 mM, the combination of 10 μM of $FeSO_4$ and 400 μM of L-cysteine/$NaHCO_3$ would totally cleave the supercoiled DNA pUC18. The absence of anyone of QHS, $FeSO_4$, and L-cysteine/$NaHCO_3$ would make it unable to cleave the DNA. The results were similar when the calf thymus DNA was used instead of pUC18 as the substrate, except that a fragment of 100 BP could be detected as the cleavage product (Figure 52). No difference was observed when reducing glutathione instead of cysteine was used as the reducing agent [531].

These experimental results confirm that these QHS-produced free radicals can cleave the DNA. At this stage, it is hard to say that the preconditions for the parasite DNA damage could be fulfilled, *i.e.*, QHS could permeate the membrane, reach the nucleus of parasite, and react with Fe(II). However, it is possible that the DNA damage may be



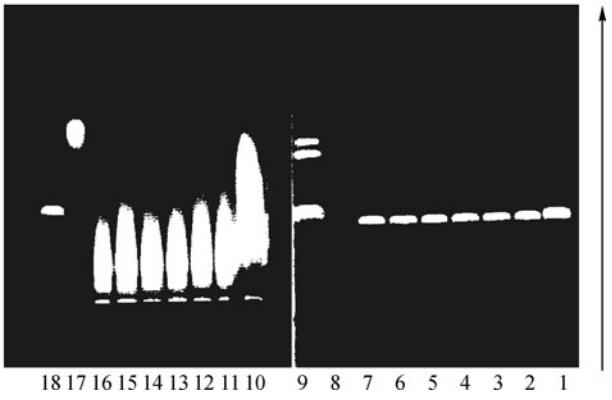
	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	
$FeSO_4$	+		+		+		+		+		+		+		+		FeSO4
Artemether	+	+			+	+			+	+			+	+			QHS
CT-DNA					+	+	+	+					+	+	+	+	CT-DNA
Salmon DNA	+	+	+	+					+	+	+	+					Salmon-DNA

Figure 50 Agarose gel electrophoresis illustrating the cleavage reactions of calf thymus DNA and salmon DNA by QHS or artemether and ferrous ion at 37°C for 12 h in a phosphate buffer solution.



10	9	8	7	6	5	4	3	2	1	
+		+	+		+	+		+		Fe^{++} (2 or 1 mM)
			+	+		+	+			Qinghaosu (2 mM)
+	+									Artemether (1 mM)

Figure 51 Agarose gel electrophoresis illustrating the cleavage reactions of DNA pUC18 by QHS or artemether and ferrous ion at 37°C for 12 h in a phosphate buffer solution.



	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
QHS (2 mM)		+		+	+		+	+			+		+	+			+	
$FeSO_4$ (10 μM)		+	+		+		+				+	+		+		+		
L-cys/$NaHCO_3$ (400 μM)		+	+	+		+					+	+	+		+			

Figure 52 Agarose gel electrophoresis illustrating the cleavage reactions of DNA pUC18 (40 ng/μL, lines 1–8) or calf thymus DNA (1 μg/μL, lines 10–17) by qinghaosu, a catalytic amount of ferrous ion, and cysteine at 37°C for 12 h. Lines 9 and 18 mark DNA (pUC18 DNA + Hinf I, 65, 75, 214, 396, 517, and 1419 BP sequentially from the top).

responsible for the toxicity of QHS and its derivatives on the tumor cell line, just as in the case of some other antitumor compounds, such as enediyne compounds. The positions of DNA attacked by the QHS-produced free radical are being studied, and it was observed that deoxygunosine (dG), perhaps deoxyadenosine (dA) in some cases, was attacked.

9.2.2 Interaction with amino acid, peptide, and protein

As early as 1980s, it has been indicated that some proteins, such as cytochrome oxidase in the membranes and mitochondria, were the target for the action of QHS [532]. Meshnick *et al.* have performed a series of experiments about the interaction between QHS and proteins in the presence of heme and concluded that the binding between QHS and albumin probably involved thiol and amino groups via both iron-dependent and -independent reactions. However, they have not isolated and confirmed such covalent adducts and also presented the question [533–536], 'how does protein alkylation lead to parasite death?'

Recent studies of the chemistry of digestive vacuole (pH 5.0–5.4) within *P. falciparum* have revealed a defined metabolic pathway for the degradation of hemoglobin. *Plasmodium* has a limited capacity for *de novo* amino acid synthesis, so hemoglobin proteolysis may be essential for their survival. However, hemoglobin degradation alone seems insufficient for the parasite's metabolic needs, since it is a poor source of methionine, cysteine, glutamine glutamate, and contains no isoleucine. On the other hand, as pointed out by Fracis *et al*., a number of experiments show that cysteine protease plays a key role in the hemoglobin degradation pathway. It has even been hypothesized that the plasmepsins generate hemoglobin fragments and cannot be further catabolized without cysteine protease action [537].

At the same time, malaria-parasite-infected red blood cells have a high concentration of the reducing glutathione (GSH, the main reducing agent in physiologic systems) [538]. It was also reported that excess GSH in parasite might be responsible for protecting the parasite from the toxicity of heme [539,540]. In general, GSH takes part in many biologic functions, including the detoxification of cytosolic hydrogen peroxide and organic peroxides, and then protects cells from being damaged by oxidative stress. Therefore, depletion of GSH or inhibiting glutathione reductase in parasite cells will induce oxidative stress and then kill these cells [541].

Accordingly, as the first step, the interaction of QHS and cysteine in the presence of a catalytic amount of Fe(II/III) was studied. From the reaction mixture, a water-soluble compound was isolated, which could be visualized with ninhydrin on TLC, and showed a formula of $C_{16}H_{27}NO_6S \cdot H_2O$. The treatment of this compound with acetic anhydride yielded a cyclic thioether **167**, which in turn undoubtedly showed the

formation of adduct **168** of QHS and cysteine through σ bond between C-3 and sulfur [521]. A stable adduct **169** from cysteine and **172** was then isolated in 33% yield using the same reaction protocol [221]. As mentioned above, both adducts (**170, 171**) of cysteine with the primary and secondary free radicals derived from artemether were also identified recently, albeit in low yields [522] (Figure 53). More recently, using heme as the Fe(III) resource, adduct of heme with **163** still could be identified [542]. These results convincingly show that free radicals derived from QHS and its peroxide derivatives can attack cysteine, but at this stage, it is still not known whether these adducts might be the inhibitor of cysteine protease and/or other enzymes.

The successful identification of cysteine adducts encouraged us to study the reaction of **172** and GSH-cat. Fe(III). After careful isolation, an adduct **176**, similar to **169**, was obtained in 1% yield from the aqueous layer, which was easily rearranged to compound **177** in acidic medium [543]. Thereafter, a similar adduct **178** between GS and the primary C-radical derived from QHS was isolated and structurally confirmed by NMR and other spectroscopy [543] (Figure 54).

As mentioned above, GSH plays an important role in protecting the parasite from oxidative stress. Therefore, these results are significant for understanding the action mode of QHS and its derivatives. The formation of GSH-QHS adduct reduces the amount of GSH, and the adduct itself might also cause inhibition of the glutathione reductase and other enzymes in the parasite. On the other hand, it is instructive that those C-centered radicals derived from QHS and its derivatives could attack free cysteine and cysteine residue not only in peptide but also probably in proteins. The formation of covalent bond adducts between parasite proteins and QHS and its derivatives mentioned in the literature are therefore mostly possible. In 2003, Krishna *et al.* reported that the malarial calcium-dependent ATPase (PfATP6) might be the molecular target for QHS [544]. PfATP6 is located in the sarco/endoplasmic reticulum situated outside the food vacuole of parasite. This inhibition of PfATP6 was iron-dependent, that is, the free radical derived from qinghaosu-iron ion was the active species. However, there was no definitive evidence that

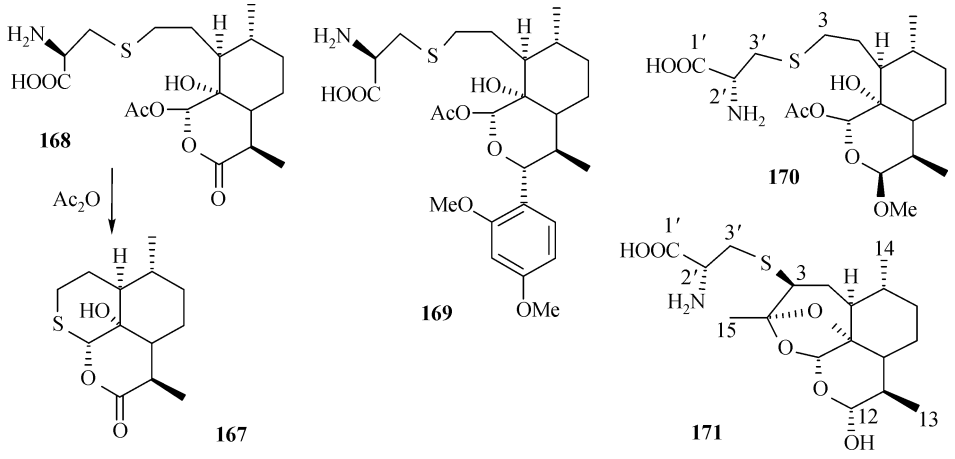


Figure 53 Adducts of QHS and its derivatives with cysteine.

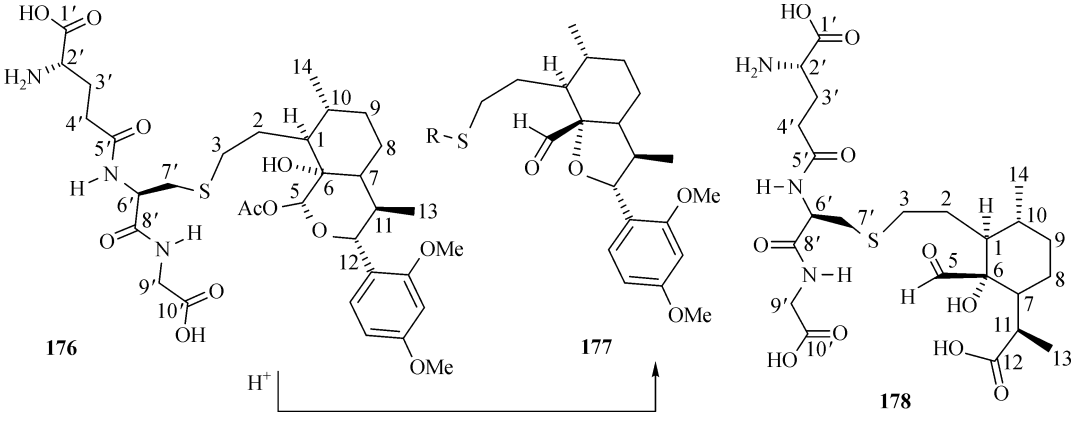


Figure 54 Adducts of QHS and its derivatives with GSH.

the free radicals from QHS bind to PfATP6 in several sites. In this respect, our experimental results that an adduct may be formed between the cysteine residue and the free radicals from QHS support Krishna's discovery.

9.2.3 Interaction with heme

Recently, Robert and Meunier reviewed their mechanistic study on QHS derivatives [526,527,545,546]. They identified some adducts of heme and the primary C-centered free radical **165** through the mesoposition from the reaction of QHS with Fe(III)-heme and 2, 3-dimethylhydroquinone in methylene chloride [547] or Fe(III)-heme and glutathione in dimethyl sulfoxide [548]. Figure 55 shows the major QHS-heme adduct after demetallation. However, they did not clearly point out how these adducts were related with the action mode of antimalarial or inhibition of the hemozoin formation. Also, in 2002, a heme-artemisinin named hemart was reported, which stalls all mechanisms of heme polymerization, resulting in the death of the malarial parasite [549]. However, their hemart was synthesized just by mixing equivalent heme and QHS in dimethyl acetamide (DMA) at 37°C for 24 h. It is not clear whether hemart is a complex or a covalent adduct. The primary C-centered free radical **165** is only formed by reaction of QHS and heme in the presence of reducing agents but not in the absence of reductants. In 2003, Haynes *et al*. pointed out that QHS antimalarials did not inhibit hemozoin formation and hence ruled out the role of the QHS-heme adduct as an inhibitor [550].

9.3 Other points of view and summary

In the early 1980s, Wu and Ji performed the Hansch analysis of antimalarial activity and distribution coefficient between oil and water of QHS derivatives and found out that the more lipophilic, the more active the derivative [551]. It is understandable that the lipophilic property of QHS derivative is related to its permeating ability across the membrane of cells, but it is just the first step for the mode of action. Recently, several laboratories have performed the cyclic voltammetry study of QHS and its derivatives. A correlation of the activities of QHS derivatives with their reduction potentials was reported [100]. However, it was also indicated that the electrochemical reduction of QHS and its derivatives was a two-electron transfer [96], which produced deoxyqinghaosu and its derivatives but not free radical reduction products. This was confirmed by isolation and identification of the electrochemical reduction product [94]. The major metabolites of QHS *in vivo* are the same as free radical reduction products, so it is debatable whether there is a correlation between the electrochemical reduction and the cleavage of peroxy bridge of QHS *in vivo*.

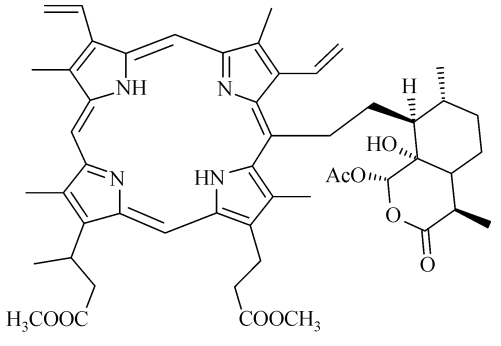


Figure 55 Major QHS-heme adduct after demetallation.

Recently, Jefford has concluded that the killing of the parasite by alkylation with carbon radical is logical and convincing, but the death of the parasite also may be caused by oxygen atom transfer or by the action of an oxy-electrophilic species [525]. However, Haynes has argued totally against the C-centered radical proposal based on the consideration of redox chemistry and structure-activity relationships [552,553]. In their recent paper, they concluded that the antimalarial activity did not correlate with the chemical reactivity of QHS derivatives against Fe(II) based on an experimental observation of a special C-12 nitrogen-substituted derivative [554,555]. However, Robert reported [556] that artemisone, a C-12 nitrogen-substituted derivative synthesized by Haynes, actually was an efficient heme alkylating agent, which is to the contrary of what Haynes claimed. Another totally different viewpoint was presented in 2001 that the antimalarial activity of QHS may be caused by 1) the interaction of the intact compound without chemical reaction, 2) the chemical reaction of QHS and/or its degradation products with the parasite biomolecules, or 3) the oxygen free radical occurring during redox reaction [557]. Their consideration is based on an undefined experimental result that DHA is the product from the reaction of QHS and ferrous sulfate in aqueous buffer. The formation of DHA determined only by TLC has no precedent, and the reduction of lactone to lactol with ferrous ion is also unbelievable in chemistry.

In recent accounts, Posner [558] has again mentioned that a high-valent iron-oxo intermediate is important for high antimalarial activity, but this opinion is not widely accepted [559]. In this context, a revision of mechanism about this high-valent iron-oxo intermediate in an example of QHS analog was reported from Posner's laboratory in 2008 [560]. Another very interesting observation was also reported from Posner's and O'Neill's laboratories. They synthesized a pair of enantiomers of 1, 2, 4-trioxane analog of QHS and found

that these isomers showed the same level of *in vitro* antiparasitic activity. Considering this result, they doubted the hypothesis that QHS derivatives interact with a specific protein-target site [561].

In summary, the mode of antimalarial action of QHS has been a noticeable research object since its discovery. Several theories have been postulated so far, but their further development has been very difficult as mentioned in Wu's comment [562]. Right now, from several experimental results and proposed theories, the postulation that the carbon centered free radical is the key active species killing the parasite might be the most convincing one. The next key point is which genuine target is attacked by this catalytic amount of radicals leading to collapse of the whole biosystem of the parasite. It also needs to be emphasized that the carbon-centered free radicals might attack multitargets, instead of just a special protein or other microbiomolecule.

10 Prospect

Just like the mythical firebird phoenix, which is reborn from the ashes of its nest every 500 y, QHS is brought to life from the nest of traditional Chinese herbs and becomes a wonder of the world. This rare natural compound ushered in a new era of malaria chemotherapy. Since 1979, thousands of scientific papers have recorded the important scientific achievements of QHS in a wide range of disciplines, including biology, chemistry, pharmacology, and medicine. Researches on QHS will continue in depth and breadth. We hope that more progress will be made on mechanisms and medical application of anticancer and immunosuppressive activities in the near future. At the same time, we sincerely hope the story of QHS will inspire more researchers to explore the treasures of traditional Chinese medicine, and more immortal firebirds will arise from the herbal nest.

Addendum

A work to create the genetic map of the Artemisia annua plant was performed by the Centre for Novel Agricultural Products at the University of York, UK and supported by the Bill and Melinda Gates Foundation, and the results have recently been published in Science [563]. The genetic map would be benefic to the plant breeders throughout the world to identify beneficial genetic markers in their own A. annua stock and tailor high qinghaosu yielding plants to local conditions.

Acknowledgements We sincerely thank Prof. Huang Hao for her contribution in the preparation of phytochemistry section about *Artemisia annua* L.

Ying LI graduated from the graduate school of Shanghai Institute of Materia Medica, Chinese Academy of Sciences in 1966. Right after her graduation, she was engaged in the exploration of new antimalarials. She and her colleagues conducted structure determination and synthesis of some active principles of traditional Chinese medicines. Since 1976, she has been working in the area of qinghaosu, especially the structure modification and structure-activity relationship of it. She synthesized a number of derivatives and analogs of qinghaosu, including artemether and arteether. Artemether has been approved to be a new antimalarial drug and prevention drug for Schistosomiasis. Her recent research interest is focused on qinghaosu derivatives in the application to anticancer and immunopression. She has published more than 60 papers and book chapters.

Yu-Lin WU graduated from the graduate school of Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. Since his graduation, Mr. Wu has been continuously engaged in chemical researches of natural products and organic synthesis mainly in the same Institute. He was promoted to full professorship in 1989, and was then appointed to several senior positions, including the Director of the State Key Laboratory of Bio-organic and Natural Products Chemistry, SIOC, CAS from 1990 to 1999. He has served in several academic societies, such as the Chairman of the Division of Organic Chemistry, Chinese Chemical Society (1994–2006). His major research interest focuses on the natural products, especially the active principles of Chinese traditional herbs and their structure-activity relationship. His research areas in organic chemistry and medicinal chemistry include antimalarial drug qinghaosu, antitumor natural product annonaceous acetogenins, insect antifeedent tonghaosu, and the syntheses of sialic acid and its analogs. His research group has published over 240 papers, book chapters, and 2 monographs in the area of organic chemistry.

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