Reactions of Artemisinin and Arteether with Acid: Implications for Stability and Mode of Antimalarial Action

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The currently accepted mechanism of trioxane antimalarial action involves generation of free radicals within or near susceptible sites probably arising from the production of distonic radical anions. An alternative mechanistic proposal involving the ionic scission of the peroxide group and consequent generation of a carbocation at C-4 has been suggested to account for antimalarial activity. We have investigated this latter mechanism using DFT (B3LYP/6-31+G* level) and established the preferred Lewis acid protonation sites (artemisinin O5a ≫ O4a ≈ O3a > O2a > O1a; arteether O4a ≥ O3a > O5b ≫ O2a >O1a; Figure 3) and the consequent decomposition pathways and hydrolysis sites. In neither molecule is protonation likely to occur on the peroxide bond O1−O2 and therefore lead to scission. Therefore, the alternative radical pathway remains the likeliest explanation for antimalarial action.

Introduction

Malaria remains the foremost cause of mortality and morbidity from infectious disease in the developing world. Current projections suggest that if global warming remains unchecked, it could re-establish itself in Europe and North America.[1] The trioxane artemisinin (**1**) (Figure 1), extracted from either *Artemisia annua* or *A. apiacea*, has been shown to be the active antimalarial ingredient in ancient antimalarial preparations of the herbal drug qing hao (blue-green hao[2a] or more correctly named huanghuahao)[2b] and has the capacity to address the problem of chloroquine drug resistant *Plasmodia*, especially of the lethal *falciparum* strain.[2c]

Identification of a rare 1,2,4-trioxane structural motif within this natural product has led to intensive synthetic endeavor including the construction of truncated analogues in order to identify the pharmacophore.[3] Antimalarial action of trioxanes has been studied in silico,[4] using model systems,[5a−l] against cloned[6] and wild type *Plasmodia* in vitro[7] and in rodent,[8] primate,[9] and human malarial infections in vivo.[10] In recent times, paradigms implicit in industrial programs have been adopted by academics in order to meet the necessary safety standards to develop safe antimalarials, especially the use of animal models to select the most potent and safest compounds incorporating the trioxane ring system.[11] Initial mistrust toward developing pharmaceuticals containing peroxides arose from perceived stability problems by Western industry, a belief not shared by Far Eastern pharmaceutical companies.[12] Artemisinin, unlike its peroxide counterparts, is so thermally stable[13] that it survives brief melting and sublimation[14] provided that exposure to transition metal ions[15,5h−j,l] and selected reducing agents are avoided.[16] However, following recent folklore practices, extracts employing boiling water were devoid of antimalarial efficacy, causing repeated setbacks in isolating the active principle.[2a]

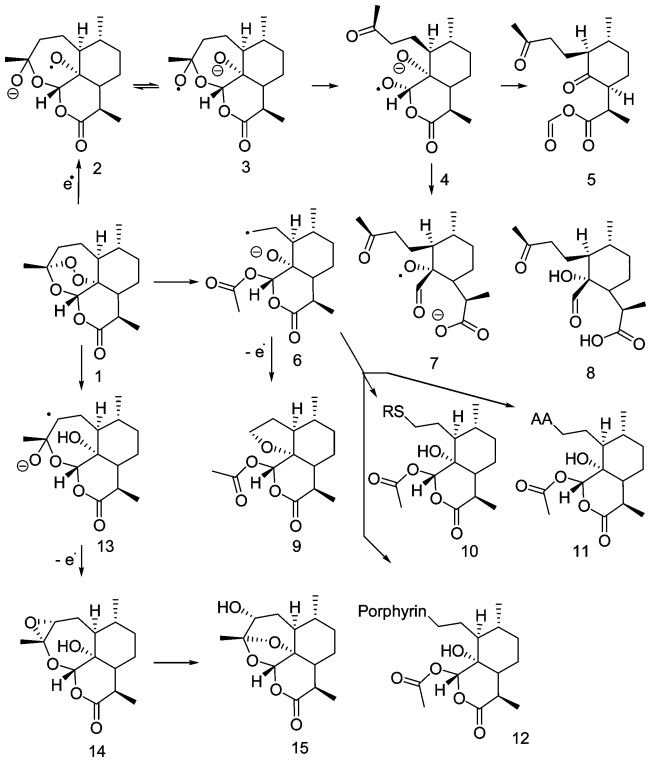

Figure 1. Putative routes involving reductive decomposition and subsequent fragmentation, rearrangement, and termination reactions of artemisinin and subsequent rearrangement. Some intermediates have been detected spectrometrically[18c] or by single-crystal X-ray diffractions methods: **2** and **3**, refs 18c, 26b; **8** as the methyl ester, refs 5g, 34c,d; **9**, refs 5h−k, 14c; **10**: where RS represents thiol attack; **11**: where AA represents free amino acid, peptide or enzyme target; **14**, refs 5h,l; **15**, arteannuin D (qinghaosu IV), ref 46; **15**, artemisinin G, ref 46.

Understanding the mechanism(s) of action of antimalarial trioxanes and peroxides[5a−l,17] has become even more important since their acceptance by the pharmaceutical and regulatory authorities as being suitable for use in human medicine. Consequently, rigorous testing in vivo is required to eliminate those compounds that are potentially (neuro) toxic and it is

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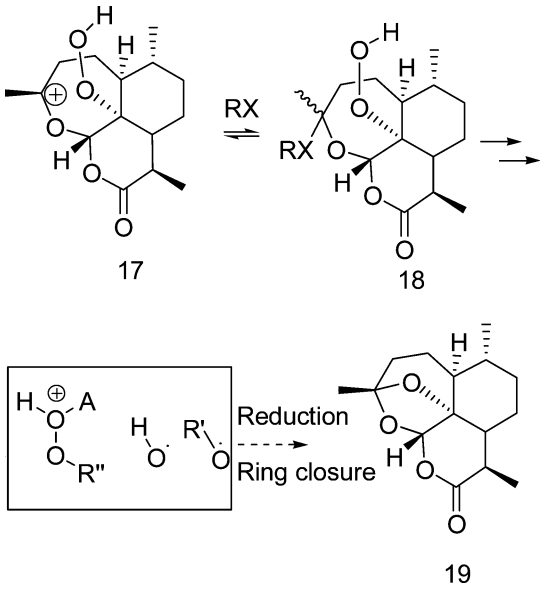

Figure 2. Nucleophile is denoted by R′XH, where X = O, NH, S; R′ = H, acyl, alkyl, etc. A is proton or Lewis acid. R″ represents an artemisinyl residue. Adapted from Haynes et al.[25] Some intermediates have been detected spectrometrically or by single-crystal X-ray diffractions methods: desoxyartemsinin **19**, refs 14a, 25, 28a,b.

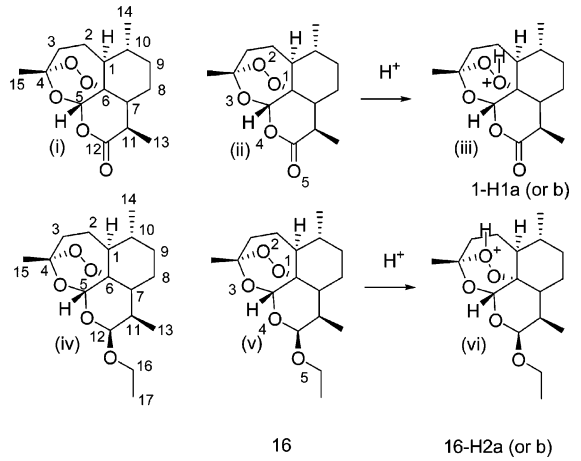

Figure 3. Structures of artemisinin (**1**) and the ethyl ether analogue arteether (**16**): (i) numbering system used for artemisinin carbon skeleton was according to Sy and Brown;[45] (ii) numbering system used for artemisinin oxygen skeleton; (iii) diagram indicating artemsinin protonated at O1; (iv) numbering system used for arteether carbon skeleton; (v) numbering system used for arteether oxygen skeleton; (vi) diagram indicating arteeether protonated at O2.[25,28a,b]

imprudent to rely on simple monoparametric indicators such as log *P* values.[11b] The principal mode of antimalarial action is currently believed to involve reductive, single electron transfer scission of the peroxide bond[18a,b] which forms distonic radical anions (Figures 1−3)[18c] capable of internally abstracting hydrogen atoms (via a 1,5 shift) to form a more reactive carbon-centered radical[19] (cf. Figure 1, **13**). Attack of this carbon-centered radical against susceptible targets, for instance, heme (Figure 1, **12**)[8b] or SERCA pump[20a−c] may initiate the central parasite killing event. Importantly, it has been suggested that a hydrophobic interaction with PfATP6 exposes the peroxide to the outside of the binding pocket, allowing activation by Fe^{2+} species,[20c] if indeed this is the only critical macromolecular target[20d−f] to form distonic radical anions.[17a,b] Alternatively, depending on the partial pressure of oxygen within the parasite, carbon-centered radicals undergo a propagation reaction with oxygen to form peroxyl radicals, which in turn may react with the aforementioned species. Artemisinin radicals generated by whatever mechanism,[21] including Hock cleavage (Figure 1, **6**), can initiate chain-branching free radical reactions[22] that may cause oxidative stress to parasites.[23] Parasites exhibit evolutionary adaptations, including retention of the apicoplast, to prevent damage from endogenous and exogenous radical flux(es).[24]

An alternative mechanistic pathway has been proposed by the Haynes group[5f,20d−f,25] that does not invoke distonic fragmentation of the peroxyl bond found in artemisinin-type compounds[18,26] but rather invokes ionic intermediates generated by exposure to hydronium ions (i.e. protons) (Figure 2).

Despite their potent antimalarial activity in vivo, poor formulation characteristics associated with artemisinin dosage forms have led to the development of semisynthetic derivatives that are superior in terms of efficacy or resistance to metabolism or solubility in aqueous media, permitting oral, parental, and rectal delivery.[27]

Early investigations involving oral dosage formulations highlighted the instability of artemisinin to both strong[14a] and, more importantly, weakly acidic conditions akin to those found in the stomachs of patients.[28a−c] Considering their propensity to hydrolysis, such concerns are especially important in the hemiacetal derivatives of artemisinin. To our knowledge, the interaction of trioxanes with protons has principally been studied from a dosage form viewpoint. Considering that the acidic food vacuole is associated with trioxane antimalarial action and that this compartment creates species that offer an alternative, ionic mode of antimalarial action merits further investigation using quantum mechanical methods.

Specifically, we wish to ascertain the energetics and sequence of ring opening in either the lactone or the trioxane pharmacophore or warhead in both artemisinin (**1**) and arteether (**16**) (Figure 3). Accurate prediction of sites amenable to protonation and successive ring opening/rearrangement reactions, and reconciliation with known decomposition products, may allow assessment of the relative importance of various ionic pathways (Figures 5 and 7−9) and, therefore, will facilitate comparison and contrast with the currently accepted radical modes of antimalarial activation. Subsequently, by establishing the most favorable free radical pathway formed from preprotonated artemisinin, it may be possible to ascertain if the radical or Lewis acid pathways best explain antimalarial activity. This study is complementary to the work of Taranto et al.,[29] who studied the effects of adding an electron to artemisinin to produce the distonic radical anion (Figures 1−3) and then protonating the resulting radical to give a neutral species. By contrast, in the current study, the interaction of the hydronium ion (proton) with every oxygen within the artemisinin framework was investigated to identify the relative ease of protonation and whether this process would initiate bond scission. We compared these results with those found for the ethyl ether analogue, arteether (**16**).[30]

Calculation Methods

The crystal structure of artemisinin has been obtained several times with no significant variations,[31] and the common structure was used as the initial model for **1** in our calculations. Structures of other molecules (Figure 1) were built using the Cerius[2] software package,[32a] and approximate structures were obtained using molecular mechanics minimization with the default universal force field. The structures were then geometry-optimized using the Gaussian 03 program.[32b] There are five oxygen atoms in the artemisinin molecule, which are numbered as 1, 2, 3, 4, and 5 as shown in Figure 3.

Table 1. Relative Energies of the Protonated Artemisinins (**1**) and Arteethers (**16**)

	relative energy,[b] kcal mol^{-1}	
proton position[a]	**1**	**16**
1a	31.41	16.70
1b	=1a[c]	18.52
2a	17.83	10.40
2b	26.33	11.78
3a	13.11	1.79
3b	=3a[c]	=3a[c]
4a	11.96	0.00
4b	20.33	5.02
5a	0.00	2.39
5b	4.00	=5a[c]

[a] The number refers to the oxygen to which the hydrogen is attached, and the letter a or b refers to the two different tetrahedral lone pair sites. [b] The protonation energies, defined as the energy of the protonated structure (the energy of **1** or **16**), can be calculated from this table by adding 220.59 and 219.58 kcal mol^{-1} to the two columns for **1** and **16**, respectively. [c] These structures, although the starting models a and b were different, converged to identical geometries.

The carbonyl −C═O involving O5 was then changed to an ethyl ether group −O−CH_2−CH_3 to give arteether (**16**). Each oxygen atom in artemisinin (**1**) (and the corresponding ether analogue **16**) was then protonated in turn for each calculation. For both artemisinin and arteether, both tetrahedral lone pair positions (denoted a and b) were considered in turn for the hydronium ion attached to the oxygen atoms 1−4 inclusive (Figure 3). For the carbonyl O5 in **1**, the two possible trigonal sites were investigated, while for the ether oxygen in arteether (**16**), the two tetrahedral sites were considered. The structures were optimized till convergence at the B3LYP/6-31+G* level using the Gaussian 03 program, and the resulting relative energies for artemisinin (**1**) and arteether (**16**) are shown in Table 1.

Results and Discussion

Selections of the converged protonated structures for artemisinin are given in Figure 4 together with their energies relative to **1-H5a**, which has the lowest energy. In **1-H1a** (see Figure 3iii) the O1−C6 bond length is 1.625 Å and O1−O2 is 1.505 Å. Structure **1-H1b** has converged to the same structure as **1-H1a** with relative energies of 31.41 kcal mol^{-1}; therefore, protonation at the O1 position is most unlikely (see Figure 3i for oxygen numbering scheme). In **1-H2a**, the O2−C4 distance is well and truly broken at 3.023 Å. The O1−O2 distance is 1.466 Å. Note that in **1-H2b**, the O2−C4 bond remains intact, which accounts for the much higher energy of that compound. In **1-H3a**, the O3−C5 bond is broken, the O2═C4 bond is shortened to 1.274 Å, whereas the O1−O2 bond stays approximately the same at 1.474 Å. **1-H3b** has converged to **1-H3a**. In **1-H4a**, the O4−C5 bond is cleaved to a distance of 2.421 Å. The O3−C5 bond length is 1.263 Å, thus tending toward a double bond. Since this protonated structure has the lowest energy of all, it suggests that this lactone ring is susceptible to hydrolysis more so than ring opening at the peroxidic oxygen atom 2, a result in accord with experimental evidence.[28] Similarly, in **1-H5a** the O4−C5 bond length is 1.648 Å so is tending toward breaking leaving O4═C12, which is 1.265 Å, thus corresponding to an acid group involving O4 and O5. In summary for artemisinin, **1-H2a** leads to O2−C4 breaking, **1-H3a** to O3−C5, and **1-H4a** to O4−C12 breaking, while protonation at O1 and O5 leads to bond lengthening but does not involve clear breaking of a bond. Figure 5 depicts the preferred pathway of artemisinin decomposition if either O1 or O2 is involved. The absence of products corresponding to **28** and **29**, in mechanistic investigations, supports the likelihood that protonation at O1 does not occur. In contrast, **23** has long been known to occur in the urine of patients dosed with **1**.[28]

The protonated structures of artemisinin (see Figure 3 (iv−vi)) can be usefully compared with those obtained for arteether, **16**, a selection of which are shown in Figure 6 (cf. refs 28a and 28b) together with their energies relative to **16-4aH**, which has the lowest energy (see Table 1).

In **16-H1a**, no bonds are broken but the C6−O1 bond length is increased to 1.645 Å while the O1−O2 bond length is increased to 1.503 Å. In **16-H2a** (and **16-H2b**) the C4−O2 bond is broken at 3.051 Å. In **16-H3a** (and **16-H3b**, which converged to the identical structure) the C4−O3 bond is broken to 2.831 Å. In **16-H4a**, which has the lowest energy (and **16-H4b**), no bonds are broken but the two bonds from O4 to the two neighboring carbon atoms C5 and C12 are lengthened to 1.562 and 1.628 Å, respectively. In **16-H5a** (and **16-H5b**, which converged to the identical structure) the O5−C12 bond is broken and C_2H_5OH is broken off, leaving a carbon radical on C12. Intracellular repair of structures **6**−**10** either with thiols (such as glutathione or cysteine) or with amines, including amino acids or polyamines, would provide analogues of artemisone in situ.[11b]

These results are significant when predicting the identity of the products generated during acid hydrolysis of artemisinins and their derivatives in the stomach following oral dosing of patients.[28] After a proportion of drug is successfully absorbed into the patient's blood stream, subsequent importation into the parasite may concentrate trioxanes into the acidic food vacuoles engaged in deconstruction of hemoglobin.[33]

The relative energies of the protonated species from artemisinin and arteether shown in Table 1 reveal some interesting trends that may correlate with observed decomposition products. The relative energies are significantly different for the two molecules. In particular for **1**, the most likely position for the proton is on the carbonyl oxygen O5, by more than 11.9 kcal mol^{-1}. By contrast for **16**, protonation on O3, O4, and O5 seems equally likely with energies differing by less than 3 kcal mol^{-1}. The subsequent reactions after protonation at O3 for artemisinin and arteether are shown in Figures 7 and 8, respectively.[28d]

The possibility of a Lewis acid mediated mode of trioxane antimalarial action[25] seems remote considering the high relative energy associated with the protonation of O2 for both molecules (Figure 3).

When protonation involves H5a/b, the products most commonly associated with the C12 carbocation (**49**) (Figure 9) induce decomposition that initially leaves the trioxane intact but unravels the lactone moiety.

Since collapse to cyclic products occurs quite readily and is thermodynamically favored, the open-chain forms can be studied by conversion to corresponding ethers and esters.[34] Biological testing of such hemiacetals indicates little or no antimalarial activity in vivo.[35]

To rationalize formation of the urinary metabolite or artemisinin, Haynes[25] has suggested Fenton reactions involving **22** as the critical intermediate. Also, it is conceivable that compounds analogous to **37** can arise (i.e., those that arise from artemisitene **38** to give **39** whose crystal structure is known[38]). This involves unraveling of the seven-membered ring, a rearrangement that will decrease the capacity for engaging in molecular recognition of the target receptor and corresponding loss of antimalarial activity (Figure 6).[28a] An analogous pathway involving arteether **16-H3a/b** and loss of ethanol suggests that arteether and artemisinin can decompose to a common peroxidic product **37** (Figures 7 and 8). However, the pharmacokinetics

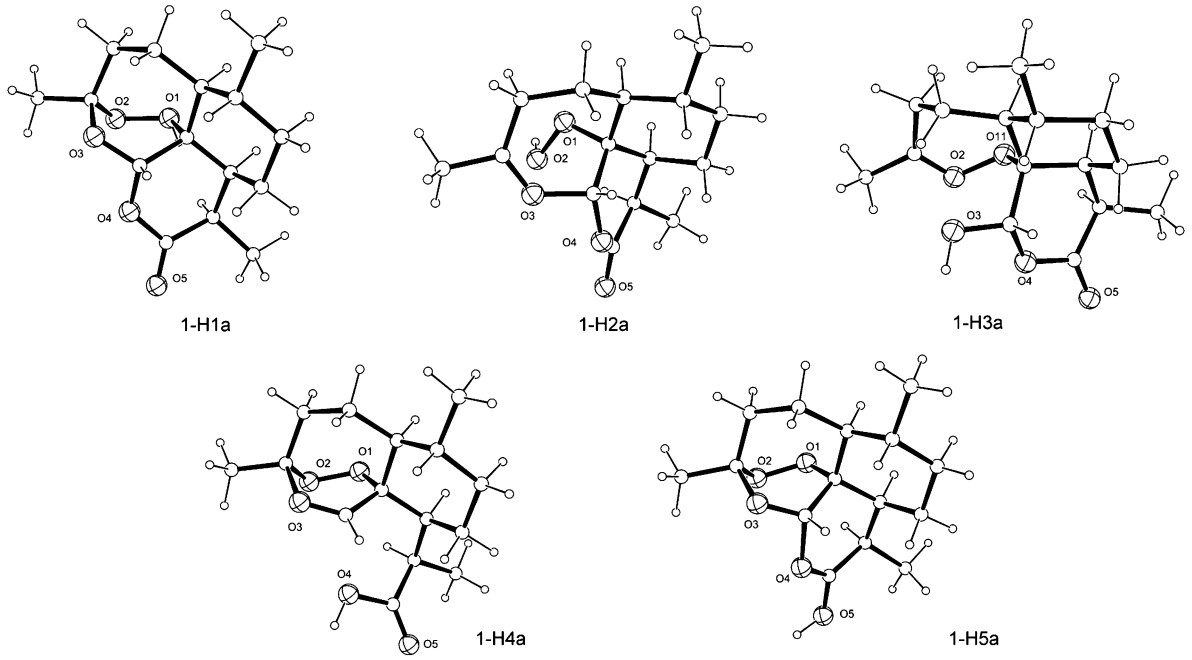

Figure 4. Selected structures of **1** after protonation. Relative energies (kcal mol^{-1}) are as follows: **1-H1a**, 31.41; **1-H2a**, 17.83; **1-H3a**, 13.11; **1-H4a**, 11.96; **1-H5a**, 0.00.

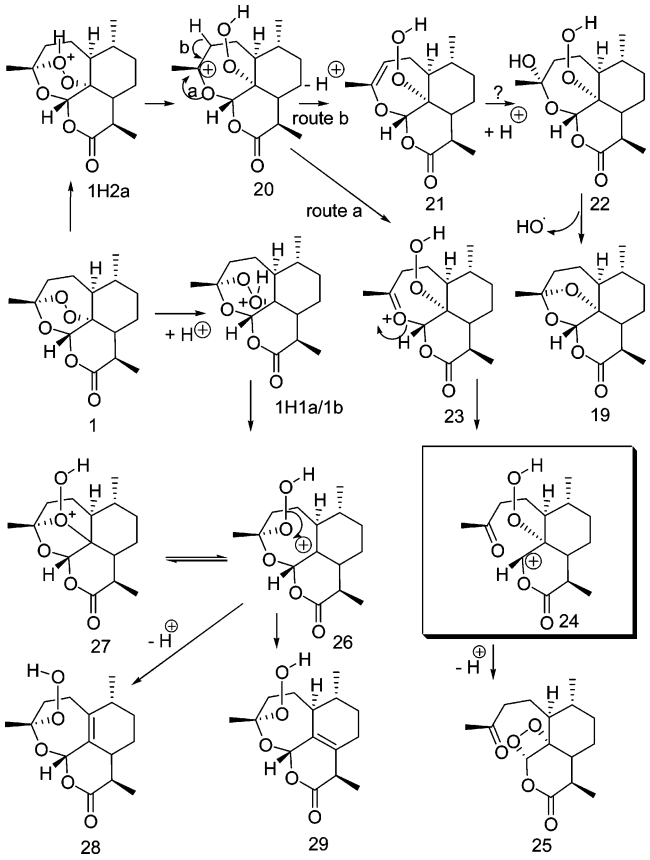

Figure 5. Proton induced decomposition pathways of artemisinin. Note than **21** can epoxidize to **14** (Figure 1). Hydration of **21** can form **22**, which putatively participates in further Fenton chemistry and generation of the highly damaging hydroxyl radical, thereby affording a route to the urinary metabolite **19** O2.[14a,25,28a,b] Compounds such as **25** are known to be stable but rearrange under acidic conditions back to the parent compound **1**.[34e]

of **37** and therefore its contribution toward overall antimalarial activity remain to be quantified in vivo.[28a]

Reduction of Protonated Forms of Artemisinins

It is interesting to compare these results with those found when artemisinin is reduced by the addition of an electron. In the calculations of Taranto et al.,[29] the electron was first added to the molecule. The most commonly accepted mechanism for the reductive decomposition of **1** starts with a single electron transfer from heme or free Fe(II) ion to the endoperoxide bond. We have predicted the various products that can arise from such reductions.[22] In previous work we have shown[17b] that when a bond is formed between Fe(II) in various environments and either O1 or O2, then in all cases, geometry optimization with Gaussian 03 at the B3LYP/6-31+G* level led to scission of the peroxide bond. By contrast, Taranto et al.[29] added just an electron to artemisinin, without specifying its site, and on geometry optimization at the B3LYP/6-31G* level found that scission of the O1−O2 bond occurred without any activation barrier to generate an O1−O2 distance of 2.185 Å. These investigators also found that the net atomic charge and spin density were distributed almost evenly between the two oxygen atoms O1 and O2 but with slightly higher charge on O1 (−0.561 on O1, −0.489 on O2) and slightly higher spin density on O2 (0.448 on O1, 0.509 on O2). This result demonstrates that the characterization of one oxygen as a radical and the other as negatively charged, i.e., a distonic species, is not clear-cut. However, when O1 is considered a radical and O2 as an anion, then the next step is a 1,5 hydrogen shift from C3−H to O1, but when O1 is an anion and O2 a radical, cleavage of the C3−C4 bond occurs to give a primary carbon centered radical. Taranto et al.[29] found the relative energy of the transition states for these two processes to be 19.82 and 21.92 kcal mol^{-1}. These authors then considered the likelihood that these reactions would occur in acid medium in the food vacuole and so added a proton to the anionic oxygen to make a neutral species. Consequently the energy barriers for the two processes were reduced to 7.11 and 3.21 kcal mol^{-1}, respectively.

In vivo experiments indicate that as the parasite deconstructs hemoglobin within an acidic parasite vacuole of the parasite, heme can be detected by conjugation to artemisinin[8,17a,b] (Figure 1, **12**). Protonation at the lactone ring (O4/O5) and subsequent

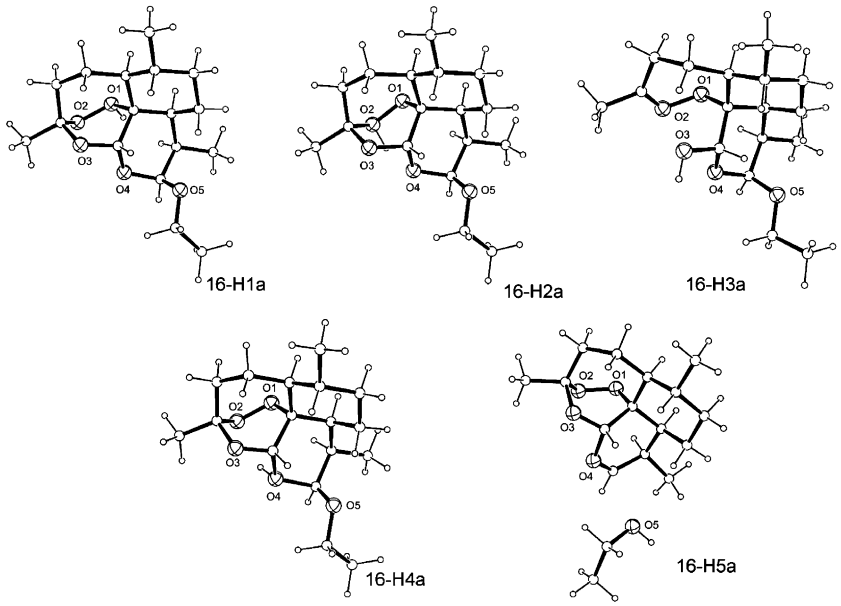

Figure 6. Selected structures of arteether after protonation. Relative energies (kcal mol^{-1}) are as follows: **16-H1a**, 16.70; **16-H2a**, 10.40; **16-H3a**, 1.79; **16-H4a**, 0.00; **16-H5a**, 2.39.

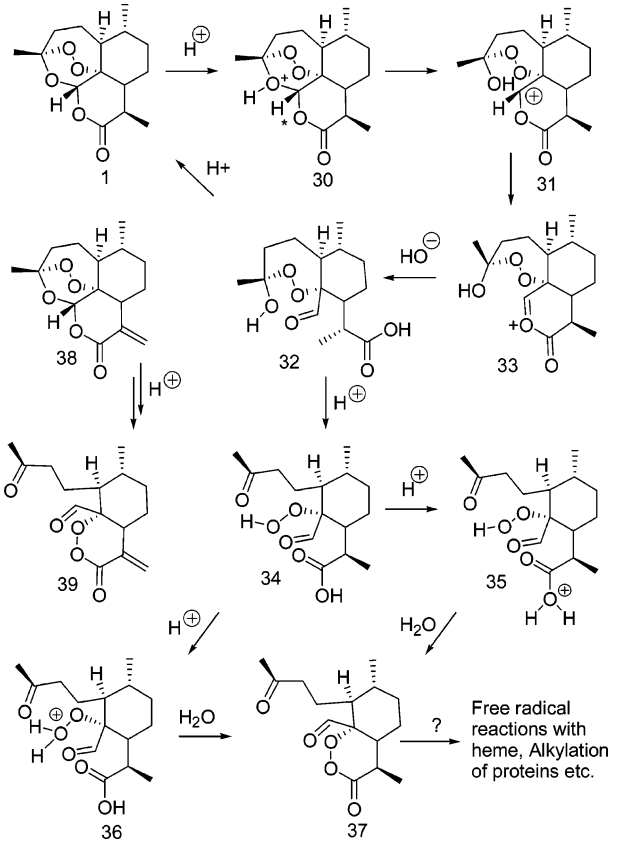

Figure 7. Postulated decomposition pathways of artemisinin involving **1-H3a** and **1-H3b** by analogy to arteether and artemistene.[25,28a,b] Note formation of a common intermediate **37**, which is less active in vitro than the parent compounds.

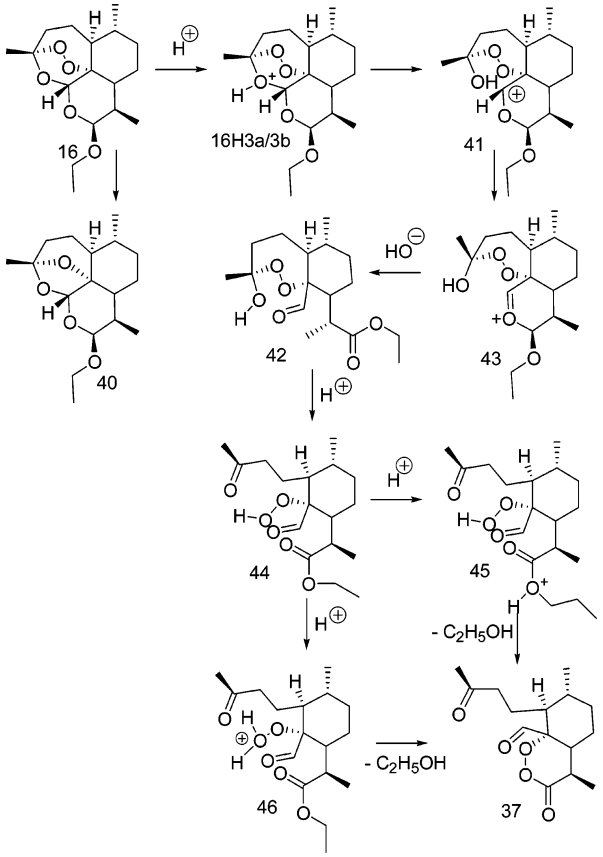

Figure 8. Decomposition pathway of arteether involving **16-H3a** and **16-H3b**.[28a,b]

unraveling of the acetal functionality appears to be the preferred pathway, and it is interesting to note that the reverse of this process was exploited in the total synthesis of artemisinin.[36] These results also suggest that the proposed ring opening of artemisinin and dihydroartemisinin that Haynes et al.[25] suggest as the preferred chemical model for antimalarial mode of action is not the lowest energy pathway (Figure 2). Haynes et al. state that "... in a biological system, the key to the general activity of the 1,2,4-trioxane in **1** is in the unique electronic ability of the trioxane to provide hydroperoxide through generation of an oxo-stabilized cation upon heterolysis of the C4−O2 bond (Figure 2)".

The absence of the additional trioxane oxygen in highly active antimalarial compounds such as arteflene[17b] suggests that if the mechanisms of both the trioxane and the peroxide involve the formation of a distonic radical anion, it is not necessary to invoke ring opening of trioxanes to the hydroperoxide. Krishna

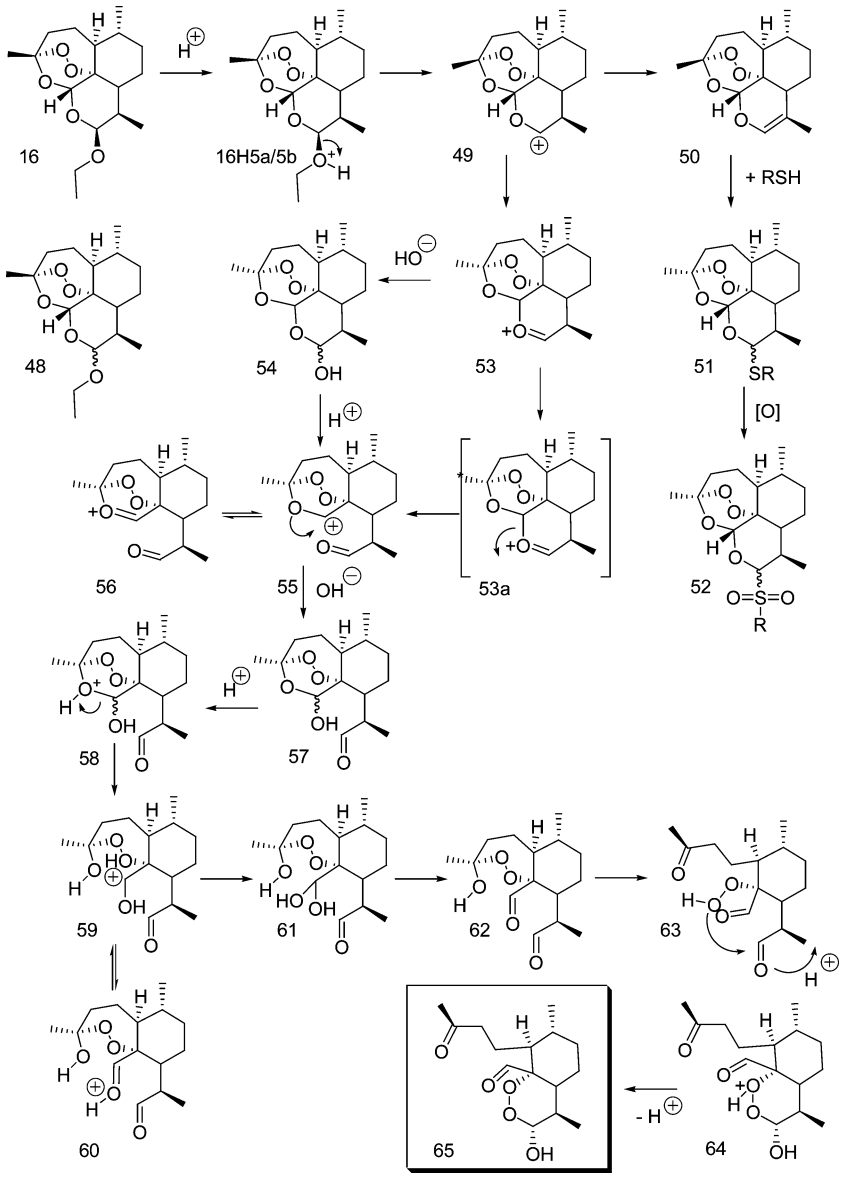

Figure 9. Decomposition pathway of arteether involving **16-H5a** and **16-H5b**.[28a,b] R represents suitable thiols present in amino acids, peptides, or proteins.

et al. argue that the locus of artemisinin action may be reinterpreted and suggest that heme may not be the target because accumulation of artemisinin is seen in structures other than the acidic food vacuole.[37] It can be noted that the Haynes mechanism also fails to explain why epiartemisinin, which possesses an inversion of stereochemistry at C-11 compared to **1**, is a remarkably poor antimalarial.[5e] However, the D-ring-contracted analogues of artemisinin indicate a reversion of antimalarial activity in vitro when comparing the respective isomers (inverted at the same methyl group as epiartemsinin).[5g] In this case, the authors ascribe the reduction in activity to increased rigidity rather than unfavorable steric interactions with the receptor. Clearly, further experiments are warranted to clarify these observations. Irrespective of the location of the heme, whether bound to a protein target (within the electron transport chain) or monomeric heme (but not hemozoin), the central cell killing event requires only small concentrations of metal ions to initiate chain initiation, propagation, and branching. This may be one reason parasites undertake de novo synthesis of heme cofactors; despite its relative abundance within the food vacuole,[38] it allows tight regulation of metal ions that would otherwise cause oxidative stress to the parasite. It should be noted that cultivation of parasites was impeded until they were grown with a concentration of oxygen lower than that found in the atmosphere,[39] and this suggests that this largely fermentative anaerobe is indeed susceptible to oxidants generated near or within sensitive structures. Recent evidence reveals that *Plasmodia*, in addition to known defenses (such as thiols), actively export copper, thereby minimizing Fenton induced radical damage to parasite architecture.[15a] Similarly, the host, like most aerobic organisms, constructs various molecules, including the protein hemopexin, to sequester both iron and heme, especially within the liver.[40]

Our results suggest that protonation is preferred in the acetal region and especially at C-12 (Figure 9, **49**). Formation of this species in compartments that accumulate chloride ions[41] and in the presence of various thiols, including glutathione and cysteine, suggests that artemisinin could be attacked at this site to generate prodrugs of artemisone-like compounds (Figure 9, **51**, **52**). The formation of adducts with proteins could then also occur in thiol-rich proteins and upon exposure to suitable agents could lead

to the formation of free radicals near or within structures incapable of resisting or repairing this type of oxidant damage.

Medicinal chemists have constructed a second generation of molecules[42] including fluorinated derivatives, which have reduced susceptibility to acid hydrolysis by eliminating susceptible sites.[43] Currently, none of these fluorinated analogues have achieved clinical usage; one factor may be the prohibitive construction costs of such compounds.[44]

In summary, the protonation order of preferences for artemisinin is O5a ≫ O4a ∼ O3a > O2a > O1a and for arteether O4a ≥ O3a > O5b ≫ O2a >O1a (Figure 3). Therefore, it seems likely that in arteether protonation can occur in all three sites at O3, O4, and O5 with different results. It is interesting to compare the effect of protonation at O4 in **1** and **16** in that in **1** the O−C bond is broken but the relative energy remains high, while in **16** the O−C bonds are not broken but the relative energy is low. Such results have consequences for the mechanism of antimalarial action in the two compounds. Electron-transfer to the peroxide bond results in dissociation of the O−O bond, producing an anion and a free radical. In an argon-purged solution, artemisinin reacts with solvated electrons (e_s^-) with $k = 4.4 \times 10^9$ dm^3 mol^{-1} s^{-1}, generating an absorption band rising in the ultraviolet region similar to the spectrum of the $CH_3^{\bullet}$-CHOH radical.[26b] However, most of the products isolated from reactions involving artemisinin and various oxidants indicate the involvement of transformation at C3−C4 loci. Results of this protonation study suggest that one reason that the artemsinin class of compounds is still clinically useful against chloroquine-resistant *Plasmodia* is their relative insensitivity to the (acidic) environment of the digestive food vacuole of *Plasmodia*. Consequently, a radical-based mechanism of antimalarial action is thermodynamically favored in comparison to one involving Lewis acid protonation, and distonic ring scission of the peroxide bond offers the best explanation for antimalarial activity when examining the currently available evidence.

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