Knowledge of the Proposed Chemical Mechanism of Action and Cytochrome P450 Metabolism of Antimalarial Trioxanes Like Artemisinin Allows Rational Design of New Antimalarial Peroxides

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Received November 21, 2003

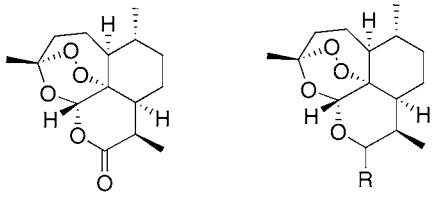
ABSTRACT

Evidence is reviewed elucidating the mechanism of iron-induced triggering of antimalarial trioxanes. As prodrugs, trioxanes undergo homolytic, inner-sphere, reductive cleavage by ferrous iron to form sequentially oxy radicals, carbon radicals, high-valent iron−oxo species, epoxides, aldehydes, and dicarbonyl compounds. One or more of these reactive intermediates and neutral alkylating agents likely kill the malaria parasites. Several new, orally active antimalarial peroxides have been designed rationally based on this fundamental mechanistic paradigm. Incorporating metabolism-blocking substituents also provides some new, potent, semisynthetic artemisinin derivatives.

Introduction

Currently, 300−500 million people worldwide have malaria. Each year, 1−2 million people, mostly young children, die of this infectious disease.[1] Vaccines to prevent people from contracting malaria are being developed, but no successful vaccine has yet been reported. Even when an effective vaccine does become available, however, it will probably not be 100% effective. Therefore, curing people who have malaria still will be required. Chemotherapeutic treatment of current malaria patients is becoming more and more difficult because the malaria *Plasmodium falciparum* parasites causing this disease have developed widespread resistance to such standard antimalarial drugs as quinine and especially chloroquine.[2,3] Therefore, the search for new classes of natural and manmade antimalarial drugs has become urgent.

In the early 1970s, Chinese chemists reported isolation and structure elucidation of the sesquiterpene 1,2,4-trioxane artemisinin (qinghaosu, **1**), the highly active antimalarial component of the ancient *Artemisia annua* (sweet wormwood) Chinese herbal remedy for fevers.[4] This important discovery represented a breakthrough in finding an effective antimalarial that was not quinoline-based and, therefore, that was effective against multidrug-resistant malaria parasites. Chemical synthesis of a mono-deoxygenated version of artemisinin (**1**) established that the peroxide unit in this natural trioxane was essential for its high antimalarial potency.[5] Sodium artesunate (**2a**) is a succinic acid half ester of the reduced lactol form of artemisinin (**1**) that, although prone to hydrolysis, is fast-acting, water-soluble, effective, and widely used in areas of the world where malaria is endemic.[6] No resistance to such trioxanes has been seen in the field or in the research laboratory. In combination with other antimalarial drugs, sodium artesunate (**2a**) is rapidly becoming the drug of choice in most third-world cases of malaria.[7]



Artemisinin (qinghaosu, (**1**))

Sodium artesunate, **2a**
R = α-OC(O)$CH_2CH_2CO_2$Na
Artemether, **2b**, R = β–OMe

Understanding the chemical mechanism of action and the metabolism of artemisinin (**1**) and related endoperoxides is essential to guide rational design of new antimalarial trioxanes. This short account will focus on our research into trioxane mechanism and metabolism and into application of this fundamental understanding to rational design of some efficacious and safe new antimalarials for chemotherapeutic treatment of people infected with malaria.

Carbon-Centered Radical Intermediates

The Meshnick group was the first to propose that ferrous iron [in the form of heme or iron(II) salts] triggers

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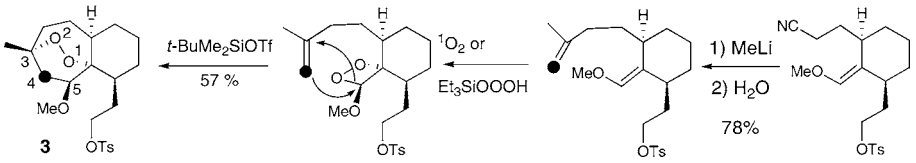
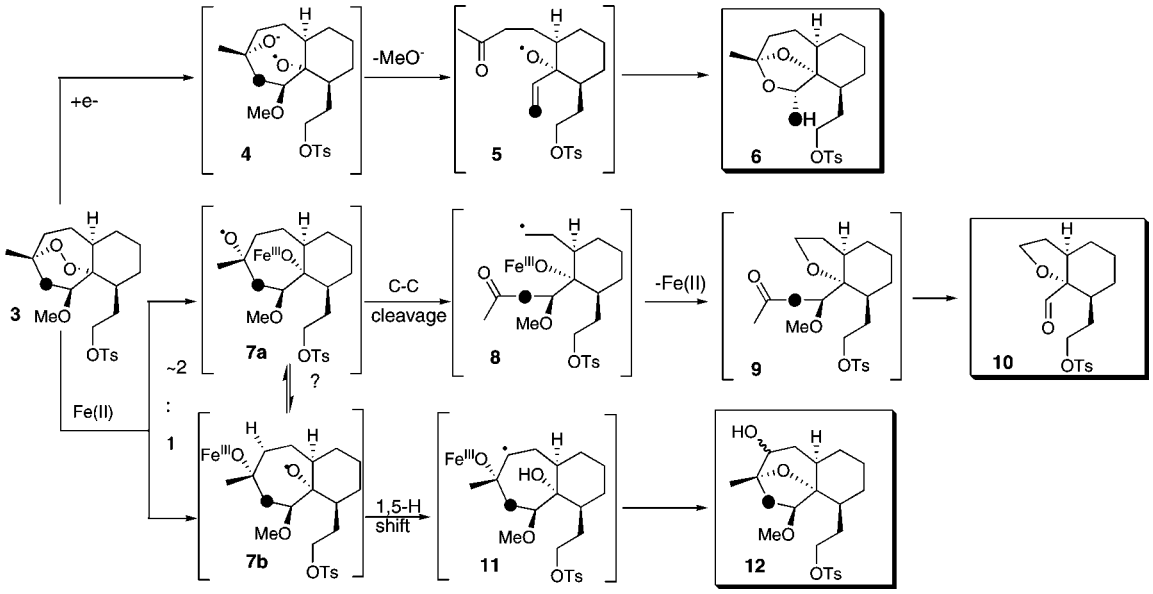
10.1021/ar020227u CCC: $27.50
Published on Web 04/24/2004

Scheme 1



Scheme 2

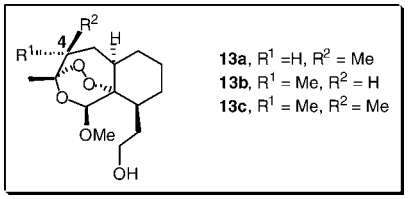


reductive cleavage of the peroxide bond in artemisinin to form oxygen-centered radicals.[8] The Posner group was the first to show that such oxy radicals then form carbon-centered radicals.[9] Carbon radicals are now internationally accepted as key initial intermediates in a cascade of chemical reactions leading from antimalarial trioxane to various intermediates, one or more of which kill the malaria parasites. The evidence for initial formation of carbon radicals and subsequent formation of other potentially cytotoxic species was generated as follows.

Isotopic (oxygen-18) labeling of oxygen-4 in antimalarially active simplified 1,2,4-trioxane **3**, as shown in Scheme 1, allowed the course of reductive cleavage of the peroxide bond to be followed.[9] Outer-sphere reducing agents (e.g., samarium diiodide, zinc metal, tritylllithium) all produced dioxolane **6**, involving extrusion of methoxide anion (Scheme 2). Carbon-13 NMR spectroscopy confirmed that the oxygen-18 label was located exclusively at the exocyclic hemiacetal position of dioxolane **6**. Formation of dioxolane **6** by deoxygenation of its parent trioxane has physiological relevance because malaria parasites convert trioxanes such as artemisinin into the corresponding dioxolanes. As an indication of the robustness of polycyclic trioxanes such as **3**, no reaction occurred when trioxane **3** was treated with typical peroxide-cleaving reagents such as dimethyl sulfide, triphenylphosphine, or sodium dithionite.

Inner-sphere reductive cleavage of the peroxide bond in simplified trioxane **3** by ferrous salts and by heme [generated in situ by benzyl mercaptan reduction of the iron(III) in hemin] formed neutral products **9**, **10,** and **12**. Ring-contracted tetrahydrofuran acetal **9**, aldehyde **10**, and hydroxy dioxolane **12** are typical of the metabolites formed from trioxanes such as artemisinin in the presence of rat liver microsomes;[10] indeed, when artemisinin was treated with ferrous bromide, an acetal like **9** and an hydroxy dioxolane like **12** were formed along with a dioxolane like **6**. When hemin/benzyl mercaptan reacted with trioxane **3**, dibenzyl disulfide was formed, analogous to the protein thiol oxidation products (i.e., disulfides) observed when artemisinin itself and hemin interact in the presence of red cell membranes. The radical mechanism in Scheme 2, similar to the mechanism proposed earlier for high-temperature (190 °C) pyrolysis of artemisinin,[11] accounts for these room-temperature ferrous-induced results. As shown in Scheme 2, iron(II)-induced cleavage of the peroxide bond in trioxane **3** leads to oxy radical intermediates **7a** and **7b** (possibly interconverting) in about a 2:l ratio. Then C−C bond cleavage of oxy radical **7a** initially produces primary carbon radical **8** and then labile ring-contracted tetrahydrofuran acetal **9** (characterized by ^{1}H and ^{13}C NMR) with oxygen-18 located in the acetoxy group as shown in Scheme 2 (mass spectrum, M − CH_3COO-18) and then produces stable electrophilic tetrahydrofuran aldehyde **10** lacking O-18. Intramolecular 1,5-hydrogen-atom abstraction within oxy radical intermediate **7b** produces the thermodynamically more stable secondary C-4 carbon radical **11** and ultimately leads to stable dioxolane alcohol **12** as a mixture of two diastereomers with O-18 not located in the methoxyl group (mass

Table 1. In Vitro Antimalarial Activity



	IC50 (ng/mL)	
compd	W-2 Indochina clone	D-6 African clone
13a	4.5	3.5
13b	>500	>500
13c	>500	>500
artemisinin (**1**)	8	8

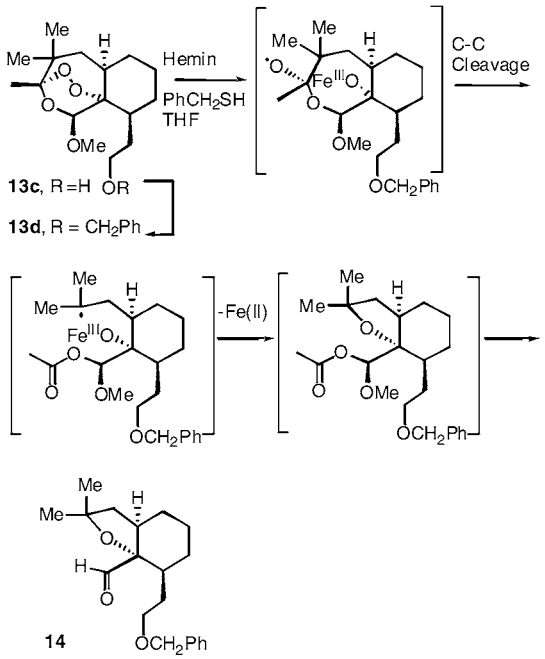
spectrum M − CH_3O). The overall yields of isolated aldehyde **10** plus hydroxy dioxolane **12** range from 60% to 70%.

The importance of secondary carbon radical intermediates such as **11** for the high antimalarial activity of some simplified trioxanes was demonstrated by study of some 4-methylated analogues.[12] 4-Monomethyl trioxanes **13a** and **13b** and 4,4-dimethyl trioxane **13c** as well as its more lipohilic *O*-benzyl ether **13d** were prepared and evaluated in vitro against both chloroquine-susceptible and chloroquine-resistant strains of *Plasmodium falciparum* malaria parasites. The results are shown in Table 1.

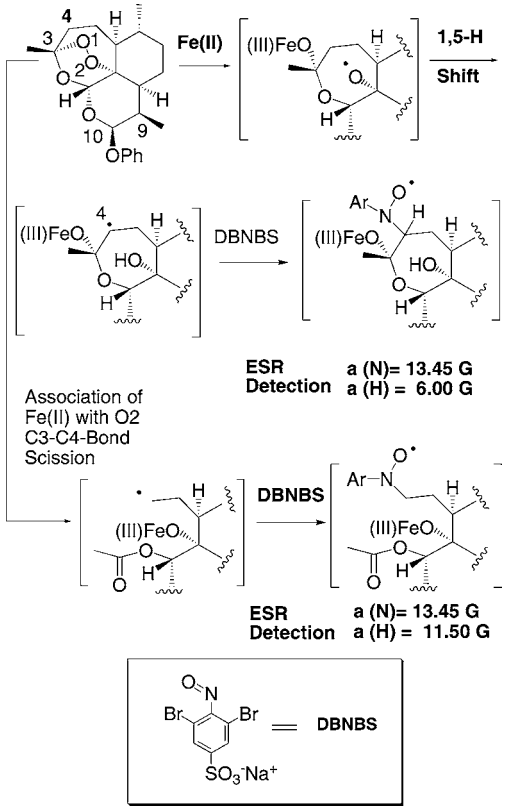
The antimalarial activities in Table 1 support the following conclusions: (1) 4-β-methyl trioxane **13a** that can undergo a 1,5-hydrogen-atom transfer is at least 100 times more potent than 4-α-methyl trioxane **13b** that cannot undergo such a hydrogen-atom transfer; (2) likewise, 4-β-methyl trioxane **13a** is at least 100 times more potent also than 4,4-dimethyl trioxanes **13c** and **13d** that cannot undergo such a hydrogen-atom transfer; and (3) impressively, simple 4-β-methyl trioxane **13a** is even more potent than complex natural artemisinin. Even though 4,4-dimethyl trioxane benzyl ether **13d** has very low antimalarial activity and cannot undergo the crucial intramolecular 1,5-hydrogen-atom shift shown in Scheme 2, it is reduced by heme to form fragmented aldehyde **14** in 38% yield (Scheme 3). This is a very important observation because it shows, in sharp contrast to some subsequent claims,[13] that even trioxanes carrying an α-oriented methyl substituent still can dock effectively with heme and can then undergo an inner-sphere reductive cleavage of their peroxide bond!

Definitive evidence for the generation of carbon radical intermediates during ferrous-mediated endoperoxide triggering of both artemisinin[14,15] and arteflene[16] has been provided by EPR spin-trapping techniques. For artemisinin, both the primary and secondary carbon-centered radicals have been efficiently spin-trapped post iron-mediated activation.[14] More recently, O'Neill and coworkers were able to spin trap both the primary and secondary carbon radicals obtained from a C-10 phenoxy analogue of artemether (Scheme 4).[17] The Meunier group also trapped the proposed C-4 primary carbon radicals as porphyrin adducts formed via heme activation of some trioxanes.[18] The Wu group, however, questioned the

Scheme 3



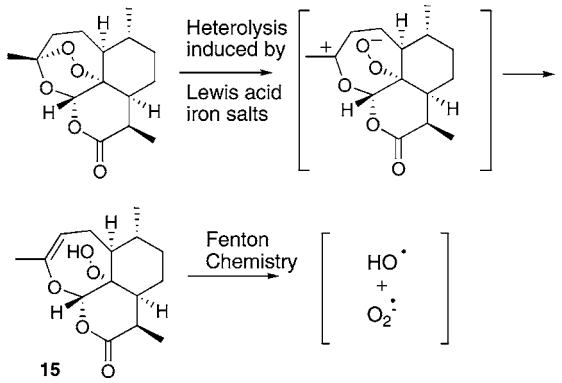
Scheme 4



central importance and the biological relevance of intermediate carbon radicals in the mechanism of antimalarial action of trioxanes.[19]

The Haynes group proposed that iron acts as a Lewis acid to facilitate initial ionic (rather than radical) activation of antimalarial trioxanes (Scheme 5).[20] In this scheme, heterolysis produces an oxygen-stabilized carbocation that then forms an unsaturated alkyl hydroperoxide (e.g., **15**). Such alkyl hydroperoxides would be expected to undergo

Scheme 5



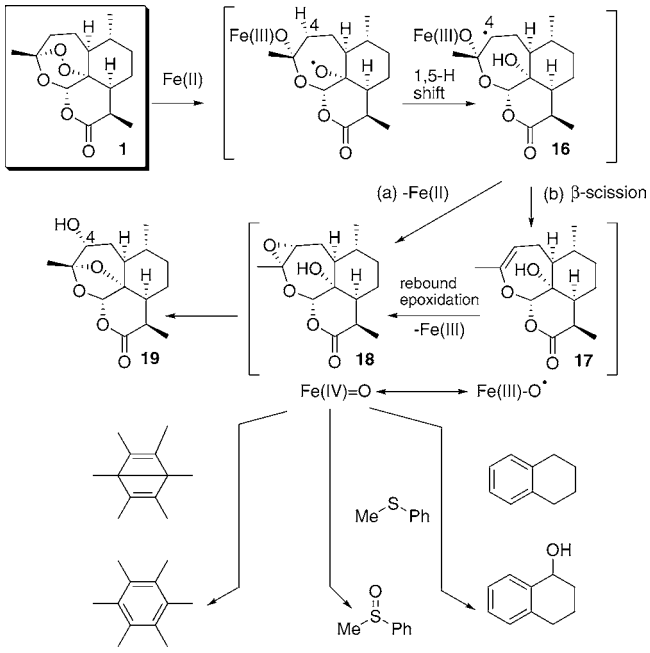
Fenton chemistry leading to formation of cytotoxic reactive oxygen species. Although this proposal is not widely accepted because of the absence of strong evidence supporting it, initial carbon radicals formed via homolytic cleavage of peroxides can be oxidized into carbocations by ferric species. The Bachi group has shown that such carbon radical-to-carbocation oxidation does occur in the presence of some antimalarial bicyclic endoperoxides and iron salts.[21a] In our opinion, the work of the Avery group on seco-artemisinin analogues argues strongly against an initial heterolytic mechanism; the two extremely similar analogues 5-nor-4,5-secoartemisinin and 5-nor-6-desmethyl-4,5-secoartemisinin, which can form virtually the same carbocations and, therefore, are predicted by the heterolysis mechanism to have similar antimalarial activities, in experimental fact have very different antimalarial activities.[21b]

Electrophilic Epoxide and High-Valent Iron−Oxo Intermediates

To account for formation of the observed 4-hydroxylated products such as dioxolane **12** in Scheme 2, the Posner group was the first to propose formation of an intermediate hydoxy−epoxide (e.g., **18** in Scheme 6).[22] After heme-promoted homolytic reductive cleavage of the peroxide bond in artemisinin and conversion of the corresponding oxy radical into the secondary carbon radical **16**, hydroxy−epoxide **18** can be formed directly by intramolecular closure (path a) and/or β-scission of Fe(IV)═O from secondary carbon radical **16** and then rebound epoxidation of intermediate vinyl ether **17** (Path b). After this proposal, the Wu group isolated hydroxy−epoxide **18** in low yield by exposure of artemisinin to ferrous salts.[15] Like 1,2-anhydro sugar epoxides,[23] highly electrophilic epoxide **18** is a potent alkylating agent and, like aflatoxin epoxide, may be cytotoxic. Hydroxy−epoxide **18** would be expected to rearrange very easily via a favorable SN1-type intramolecular opening of its epoxide by its free hydroxyl group, thereby forming the observed hydroxy−dioxolane **19**. Of physiological relevance, hydroxy−dioxolane **19** is one of the metabolites of artemisinin formed in the presence of rat liver microsomes.[10]

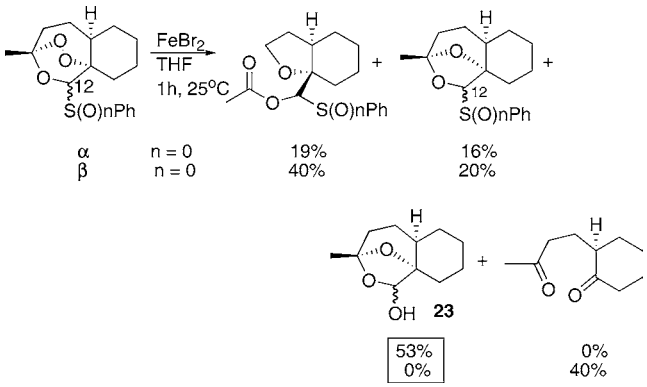
Evidence supporting the rebound epoxidation path b and the intermediacy of a high-valent iron−oxo species in Scheme 6, even using carefully deoxygenated solvents,

Scheme 6



is provided by the results when Scheme 6 is performed in the presence of the following "reporter" compounds: (1) methyl phenyl sulfide is oxygenated into the corresponding sulfoxide; (2) the benzylic position in tetralin is hydroxylated; and (3) hexamethyl Dewar benzene is rearranged into hexamethylbenzene.[22] All three of these reactions are reported to be characteristic of high-valent iron−oxo oxidants. Further direct Raman spectroscopy evidence for intermediate high-valent iron−oxo species has been provided by the Varotsis group.[24]

Further indirect evidence supporting the intermediacy and importance of high-valent iron−oxo species is provided by study of the antimalarial activities of some simplified phenylthio-substituted trioxanes.[25] A diastereomeric pair of simplified trioxanes each carrying a C-12 phenylthio group was prepared and shown to have dramatically different in vitro antimalarial activities; whereas the 12-α-oriented sulfide was inactive, the 12-β-oriented sulfide was quite active (IC50 = 56 nM). To understand the fundamental basis for this sharp difference in antimalarial activity as a function of only the sulfide's stereochemistry, ferrous iron-induced triggering of each one of these two diastereomeric trioxanes was studied. The product distribution from each diastereomer is shown in eq 1; a major difference between the two diastereomers is in the relative amounts of lactol **23** formed. Scheme 7, involving a high-valent iron−oxo intermediate, was proposed to rationalize these chemical and antimalarial results. Secondary carbon radical **20** carrying the 12-α-oriented phenylthio group can release a high-valent iron−oxo species in close proximity to the phenylthio group. This reactive iron−oxo species would then be intercepted and detoxified immediately by causing sulfide-to-sulfoxide oxidation, producing hydroxy−olefinic α-oriented sulfoxide **21** and finally, after hydrolysis, the observed lactol **23**. In contrast, the 12-β-oriented phenylthio diastereomer would release the high-valent iron−oxo species on the α



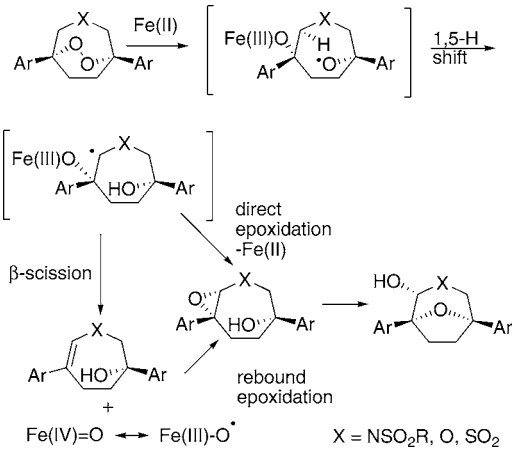
side, far away from the β-oriented phenylthio group, thereby allowing this iron−oxo species to migrate away and oxidize biomolecles vital to the malaria parasites. Because only the 12-β-phenylthio diastereomer is strongly antimalarial, it would seem that a high-valent iron−oxo intermediate is important for high antimalarial activity. Noteworthy also, both of the corresponding 12-phenylsulfone diastereomers, which cannot intercept and detoxify (i.e., reduce) a high-valent iron−oxo intermediate, are strongly antimalarial (IC50 = 33−59 nM).[25]

The intermediacy and importance of high-valent iron−oxo species after iron triggering of trioxanes are not widely accepted. In particular, the Meunier group argued against high-valent iron−oxo intermediates being formed from trioxanes.[26,27]

Mechanism-Based Design of New Antimalarials

The Posner group applied the fundamental mechanistic insights outlined in Scheme 6 to rational design of some symmetrical,[28] easily prepared, simple endoperoxides that would be reduced by iron(II) to form first an oxy radical and then, via a 1,5-hydrogen shift, a carbon-centered radical; via β-scission, a high-valent iron−oxo species would be then formed, capable of rebound expoxidation to produce a highly electrophilic (i.e., alkylating) epoxide (Scheme 8). Several seven-membered carbocyclic and heterocyclic endoperoxides were prepared. The carbocyclic endoperoxide in Scheme 8 with Ar = Ph was triggered by iron(II) to form a significant amount of the corresponding expected hydroxylated bicyclic ether shown in

Scheme 8



Scheme 8. As predicted based on chemical mechanism, the structurally simple parent diphenyl endoperoxide does indeed have considerable antimalarial activity (IC50 = 89 vs 9−11 nM for artemisinin)! This predicted result strongly increases confidence in the fundamental mechanism of antimalarial action shown in Schemes 2 and 6 and, therefore, also strongly supports the importance of carbon radicals, electrophilic epoxides, and high-valent iron−oxo species for high antimalarial acivity. One alone or a combination of these reactive species seems necessary (but not always sufficient) to kill the parasites in malaria-infected human red blood cells. There is no evidence, however, for a quantitative direct correlation between a trioxane's antimalarial potency and its ease of generating these reactive intermediates or the number of different reactive intermediates formed; we have not suggested such a correlation previously, and we do not suggest such a correlation now.[29]

Also, on the basis of the chemical mechanism outlined in Scheme 6, the Posner group introduced an aryl substituent at C-3 of some simplified trioxanes to facilitate β-scission of a strongly oxidizing and possibly cytotoxic high-valent iron−oxo species and, at the same time, to form an arene-conjugated (i.e., styrene-type) new carbon−carbon double bond that could be epoxidized to form a highly electrophilic and possibly cytotoxic epoxide.[30] Table 2 lists the various 3-aryl trioxanes prepared as well as their in vitro antimalarial potencies. Several of these rationally

Scheme 7

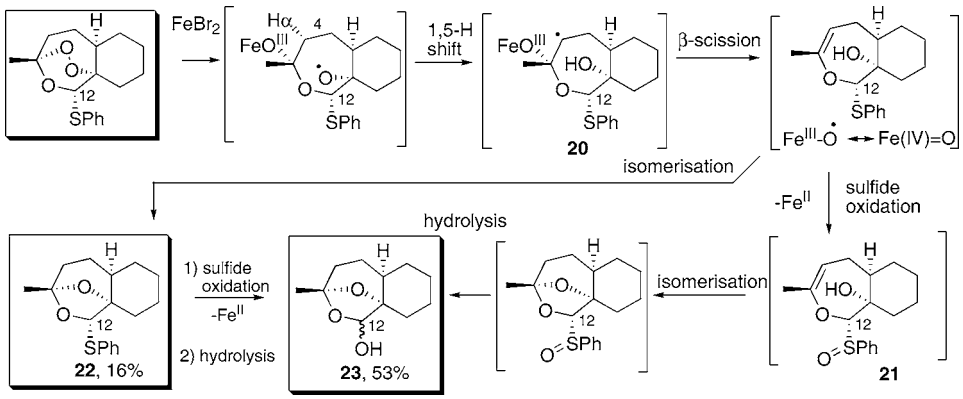
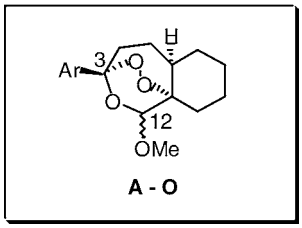


Table 2. In Vitro Antimalarial Activities[a]



A - O

trioxane	Ar or CH_3	C12-OMe	IC50 (nM)
A	Ph	α	100
		β	38
B	*p*-PhPh	α	76
		β	68
C	1-naphthyl	α	170
		β	44
D	*p*-ClPh	α	49
		β	55
E	*p*-MeO		>2500[b]
F	2-furyl	α	600
G	*p*-$HOCH_2$Ph	α	78
		β	15
H	*p*-$MeOCH_2$Ph	α	39
		β	
I	*p*-$MeOC(O)OCH_2$Ph	α	79
J	*p*-$MeOC(O)OCH_2$Ph		51
K	*p*-(*p*′-$FPhCH_2OCH_2$)Ph	α	42
		β	30
L	*p*-FPh	α	65
		β	30
M	*p*-F-*o*-MePh	α	99
		β	34
N	*p*-CF_3Ph	α	39
		β	53
O	CH_3	*β*	960[c]
artemisinin			9.2

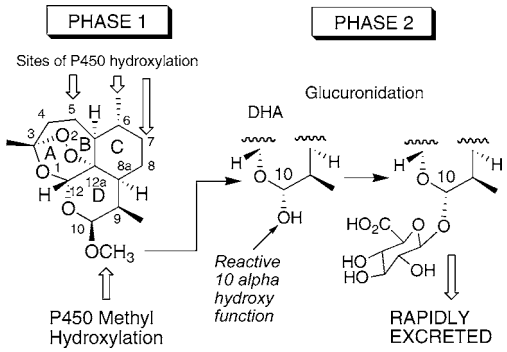
[a] The standard deviation for each set of quadruplicates was an average of 10% (≤59%) of the mean. R^2 values for the fitted curves were ≥0.989. [b] 12-Methoxy stereochemistry not unambiguously determined. [c] Assay may underestimate the potency of these volatile compounds.

designed and simplified trioxanes have high antimalarial activity not only in vitro, as shown, but also in vivo when administered to rodents either subcutaneously or orally. 3-*p*-Fluorophenyl trioxane **L** (12-α-OMe) is safer in rodents (e.g., normal animal weight gain during a 14-day iv administration of 160 *μ*mol/kg/day) than is the antimalarial artemether (about 20% lower animal weight gain at the same dose as **L**, unpublished results).

Metabolism-Based Design of New Antimalarials

Human plasma concentrations of oral, intravenous, or intrarectal artemisinin and its derivatives artemether, arteether, and artesunate reach a maximum within 1−3 h, when the concentration of the metabolite DHA is included.[31] Time-dependent first-pass metabolism in the gut and liver is observed for artemisinin derivatives. In addition to metabolic degradation, artesunate (**2a**) has also been shown to hydrolyze to dihydroartemisinin in vitro.[32] Thus, its half-life is several minutes, whereas that of DHA, at 40−60 min, is similar to that of artemether. As a hemiester, artesunate is intrinsically unstable, and it seems likely that its hydrolysis in vivo may not be enzyme

Scheme 9



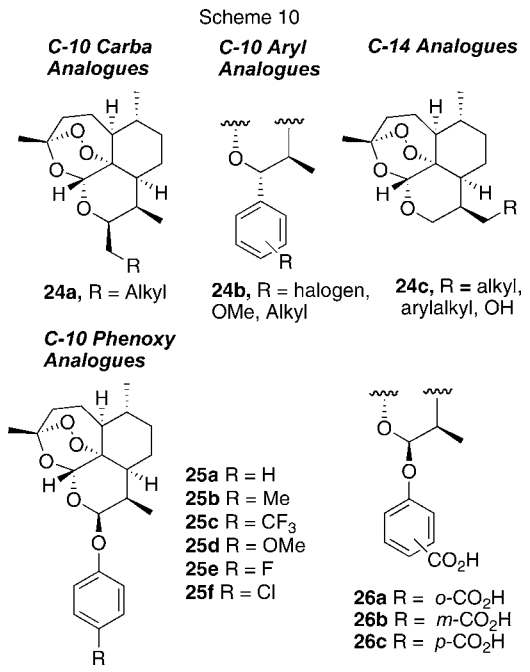
mediated. Artemisinin undergoes phase-I metabolism by CYP2B6, CYP2C19, CYP3A4, and at least one other member of the P-450 enzyme superfamily.[33]

CYP3A4 is primarily involved in artemether metabolism, and apart from oxidative dealkylation (i.e., hydroxylation of the methyl group of atremether) to DHA, the prime pathways are of hydroxylation in the liver and intestine.[34] Some but not all of the B,C-ring-hydroxylated metabolites derived from artemisinin derivatives are active antimalarials, although these may be eliminated through phase-II metabolism via generation of water-soluble conjugates such as glucuronides (Scheme 9). For example, Maggs and co-workers demonstrated that the major biliary metabolite of artemether in rats is the 7-α hydroxy glucuronide.[35] Hydroxylation by P-450 involves substrate, reducing cofactor, and heme iron which proceeds through the Fe(II) and Fe(III) oxidation states. Oxygen transfer occurs from a perferryl Fe(V)═O species generated by a reaction between dioxygen (O_2) and the Fe(II) complex. The sites of hydroxylation derive from presentation of the B,C ring end of the artemisinin tetracycle to the reactive perferryl center of the P450 enzyme active site (Scheme 9). It is remarkable to note that in all mammalian drug metabolism studies to date, there is no evidence for artemisinin analogues acting as irreversible inhibitors of the cytochrome P450 subfamily. In other words, heme-based P450 iron does not reductively activate artemisinins to radical species that subsequently alkylate the protein cavity surrounding the active site of the enzyme.

The basis for the very minor reductive isomerization that has been observed in drug metabolism studies on artemisinin or artemether may be a result of prostacyclin and thromboxane synthase heme thiolate enzymes [compare with the reductive isomerizations of 1,3-endoperoxide PGH_2 in the prostacyclin (PGI_2) and thromboxane (TXA_2) biosynthetic pathways[36]] or trace amounts of nontransferrin-bound iron. Overall, it is clear that cytochrome P450 catalyzes extensive hydroxylation and *O*-dealkylation without obtaining effective access to the shielded endoperoxide bridge.

From a medicinal chemistry perspective, the coupled phase-I and phase-II conjugation reactions of **2b** and the chemical instability of **2a** are the principal means through which these derivatives have a short half-life. As a result, a huge amount of work has been carried out to improve the potency and stability of these first-generation ana-

Scheme 10



logues. Metabolically more robust C-10 carba analogues **24a** (Scheme 10) and C-10-aryl analogues **24b** of DHA have been the focus of medicinal chemists for 10 years. Of note are the C-10 alkyl deoxo analogues prepared by Haynes,[37] Posner,[38,39] O'Neill,[40] Jung,[41,42] and Ziffer[43,44] and the C-10 aryl or heteroaromatic derivatives prepared by the groups of Haynes[37,45] and Posner.[46] In terms of antimalarial efficacy and ease of synthesis, the C-14 modified analogues **24c** prepared by the Avery[47] and Jung groups[48] are attractive alternatives to the C-10 carba analogues discussed above.[46] An alternative approach to increasing the metabolic stability of artemisinin derivatives involves incorporation of a phenyl group in place of the alkyl group (in the ether linkage) of first-generation analogues, e.g., **2a** and **2b**, in order to avoid the presence of any oxidizable O-alkyl groups as in artemether. O'Neill recently prepared in one step from DHA several C-10 phenoxy analogues **25a**−**f**, some of which are not only resistant to metabolism to DHA in animal models but are also superior antimalarials to artesunate in vivo in the mouse model of malaria.[17] For example, oil-soluble analogue **25c** had an ED90 value of 4 mg/kg versus *Plasmodium berghei* (oral) versus sodium artesunate (ED90 = 15 mg/kg). Furthermore, additional recent studies in this area have produced water-soluble C-10 phenoxy analogues **26a**−**c** in just two steps from DHA. These compounds are undergoing evaluation as metabolically more robust alternatives to **2a** and **2b** (unpublished results).

Conclusions

Unlike quinoline-based antimalarials (e.g., chloroquine) which have only one mechanism of action, trioxanes kill malaria parasites probably by generating more than one type of cytotoxic intermediate. Thus, trioxanes[49−52] are versatile prodrugs, triggered by ferrous iron, to produce several different types of highly reactive intermediates (e.g., oxy radicals, carbon radicals, high-valent iron-oxo species) as well as several different kinds of longer-lived neutral electrophiles (e.g., epoxides, aldehydes, and dicarbonyl compounds). This fundamental characteristic of trioxanes to act as prodrugs for generating such a wide variety of different harmful species suggests that it will be difficult for malaria parasites to develop resistance to this promising class of new antimalarial agents.

Whether or not the fundamental mechanistic and metabolism insights described here ultimately turn out to be a small or large part of the artemisinin complicated story,[53] these insights have guided and are continuing to guide rational design of valuable, new, orally active antimalarial peroxides.

G.H.P. thanks the NIH (AI 34885) and the Johns Hopkins Malaria Research Institute for financial support and the Department of Chemistry at The University of Liverpool for kind hospitality during August and September 2003. P.O.N. would like to thank Pfizer, Sandwich, Kent, U.K., Ultrafine Chemicals, Manchester, U.K., and the BMS Committee of the BBSRC (26/B13581) for financial support of some of the work described in this review. P.O.N. would also like to thank the Drug Discovery Committee of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) (Grant ID 980176 and A10043).

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AR020227U