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Reactions of Antimalarial Peroxides with Each of Leucomethylene Blue and Dihydroflavins: Flavin Reductase and the Cofactor Model Exemplified

Richard K. Haynes,*[a] Kwan-Wing Cheu,[a] Maggie Mei-Ki Tang,[a] Min-Jiao Chen,[a] Zu-Feng Guo,[a] Zhi-Hong Guo,*[a] Paolo Coghi,[b] and Diego Monti*[b]

Flavin adenine dinucleotide (FAD) is reduced by NADPH–*E. coli* flavin reductase (Fre) to $FADH_2$ in aqueous buffer at pH 7.4 under argon. Under the same conditions, $FADH_2$ in turn cleanly reduces the antimalarial drug methylene blue (MB) to leucomethylene blue. The latter is rapidly re-oxidized by artemisinins, thus supporting the proposal that MB exerts its antimalarial activity, and synergizes the antimalarial action of artemisinins, by interfering with redox cycling involving NADPH reduction of flavin cofactors in parasite flavin disulfide reductases. Direct treatment of the $FADH_2$ generated from NADPH–Fre–FAD by artemisinins and antimalaria-active tetraoxane and trioxolane structural analogues under physiological conditions at pH 7.4 results in rapid reduction of the artemisinins, and efficient conversion of the peroxide structural analogues into ketone products. Comparison of the relative rates of $FADH_2$ oxidation indicate optimal activity for the trioxolane. Therefore, the rate of intraparastic redox perturbation will be greatest for the trioxolane, and this may be significant in relation to its enhanced in vitro antimalarial activities. ^{1}H NMR spectroscopic studies using the BNAH–riboflavin (RF) model system indicate that the tetraoxane is capable of using both peroxide units in oxidizing the RFH_2 generated in situ. Use of the NADPH–Fre–FAD catalytic system in the presence of artemisinin or tetraoxane confirms that the latter, in contrast to artemisinin, consumes two reducing equivalents of NADPH. None of the processes described herein requires the presence of ferrous iron. Ferric iron, given its propensity to oxidize reduced flavin cofactors, may play a role in enhancing oxidative stress within the malaria parasite, without requiring interaction with artemisinins or peroxide analogues. The NADPH–Fre–FAD system serves as a convenient mimic of flavin disulfide reductases that maintain redox homeostasis in the malaria parasite.

Introduction

Artemisinin **1**, artesunate **2**, artemisone **3**,[1] and other derivatives are transformed, through catalytic processes mediated by the potent redox-active antimalarial drug methylene blue (MB) **7**[2,3,4] in the presence of excess ascorbate or the NAD(P)H model *N*-benzyl-1,4-dihydronicotinamide (BNAH) **13** in aqueous buffer at physiological pH, into products that arise via single-electron transfer or two-electron reduction.[5] The two-electron reduction involves the in situ generation of leucomethylene blue (LMB) **9**, which is re-oxidized to MB by the artemisinins, whilst the latter are reduced to deoxy products. The observation explains the ability of artemisinins to synergize the antimalarial activity of MB. MB acts as a subversive substrate for the malaria parasite *Plasmodium falciparum* flavoenzymes glutathione reductase (GR) and thioredoxin reductase (TrxR), among others, thereby resulting in the sequestration of NADPH away from its role of reducing the essential cofactor flavin adenine dinucleotide (FAD) required for ultimate maintenance of reduced glutathione levels in the parasite. Oxidation of LMB by artemisinins must speed up the cycle, thus enhancing sequestration of NADPH.

The two-electron reduction products are efficiently obtained from the artemisinins and reduced flavins when these are generated in situ from catalytic amounts of the antimalaria-active riboflavin (RF)[6,7] **9** or cofactors flavin mononucleotide (FMN) and FAD **11** by reduction with BNAH **13** or NAD(P)H. Thereby, the reduced conjugates RFH_2 **10**, $FMNH_2$, and $FADH_2$ **12** are rapidly oxidized by the artemisinins. If yeast GR, used as a surrogate for parasite GR, is treated with artemisinin in the presence of its substrate, oxidized glutathione (GSSG), accelerated consumption of NADPH takes place, especially under aerobic conditions.[5] Thus, incipient oxidation of $FADH_2$ by artemisinin results in formation of FAD, re-reduction by NADPH, and ensuing autoxidation of the $FADH_2$ by oxygen. This leads to enhanced consumption of NADPH; generation of reactive oxygen species (ROS) must also occur, although no attempt has been made to demonstrate this. Therefore, like MB, artemisinins per-

[a] *Prof. Dr. R. K. Haynes, K.-W. Cheu, M. M.-K. Tang, M.-J. Chen, Dr. Z.-F. Guo, Prof. Dr. Z.-H. Guo*
Department of Chemistry
Institute of Molecular Technology for Drug Discovery and Synthesis
The Hong Kong University of Science and Technology
Clear Water Bay, Kowloon, Hong Kong (P.R. China)
Fax: (+852) 2358-1594
E-mail: haynes@ust.hk
chguo@ust.hk

[b] *Dr. P. Coghi, Prof. Dr. D. Monti*
Department of Organic and Industrial Chemistry
CNR-ISTM, Via G. Venezian 21, 20133 Milano (Italy)
E-mail: diego.monti@istm.cnr.it

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turb redox homeostasis in the malaria parasite by interfering with redox cycling involving the flavin cofactor, and hence NADPH, in the functioning of GR. They likely also affect TrxR, lipoamide dehydrogenase, and others in the same way.[4]

This 'cofactor model' of artemisinin-induced cytotoxicity differs fundamentally from the 'ferrous iron-reductive bio-activation' model which posits a reaction of artemisinins with Fe^{II} to provide carbon radicals as cytotoxic agents.[8] Model studies indicate that both the Fe^{II}-mediated generation of carbon radicals from artemisinins and their trapping under biologically plausible conditions is difficult, as the radicals undergo facile internal quenching.[9,10] Artemisinins susceptible to decomposition by hemoglobin– (Hb) or heme–Fe^{II} display enhanced activities against malaria parasites cultured under carbon monoxide, a reagent that blocks the reaction of Hb–Fe^{II} and heme–Fe^{II} with the artemisinins through formation of stable Fe^{II}–CO adducts. Thus, the much-publicized chemical reactions of artemisinins with heme represents an attrition pathway for artemisinins in the intraparasitic environment.[11,12]

1,2,4,5-Tetraoxanes such as **4** and **5**[13,14] and 1,2,4-trioxolanes such as **6**,[15,16] have potent antimalarial activities (Figure 1). Although the reactivity of **6** with Fe^{II} was not assessed, other trioxolanes are readily decomposed.[14,16,17] The arylsulfonamide tetraoxane **5** is remarkably resistant to Fe^{II} under anhydrous conditions (1.0 equiv $FeBr_2$ in THF to 48 h).[14] Nevertheless, 'ferrous iron-reductive bio-activation' for the tetraoxanes is maintained by those that made this striking observation;[18] this requires Fe^{II}, relatively transient under normoxic aqueous conditions and likely bearing oxygen ligands within the intraparasitic labile iron pool,[19] to be appreciably more active in vivo in generating carbon radicals than a pumped-up anhydrous Fe^{II} halide, present at much higher concentrations in an organic solvent in an inert atmosphere in a laboratory flask. As implicit recognition of this problem, it is countered that 'the parasite death that ensues in the presence of artemisinin is more likely to involve specific radicals and targets rather than nonspecific cell damage caused by freely diffusing oxygen- and carbon-centered radical species'.[20] This begs the questions as to the nature of the radicals, the targets, and the putative role played by Fe^{III} chelators such as desferrioxamine (DFO).[10,21]

We now extend the scope of the cofactor model by examining the behavior of the artemisinins and peroxides toward LMB **8**, RFH_2 **10**, and $FADH_2$ **12** (Figure 1). Whilst previously LMB, RFH_2, and $FADH_2$ were generated with sodium dithionite,[5] we introduce *E. coli* flavin reductase (Fre)[22] for this purpose. This has the special benefit of permitting both the generation of the reduced conjugates of cofactors under biomimetic conditions, and an examination of their reactivity with peroxides without potential interference from excess dithionite. With dithionite, the reduced conjugate, in the case of LMB, has to be extracted into an organic solvent prior to examining its reaction with the artemisinin. Alternatively, in an aqueous medium the dithionite may have to be decomposed by decreasing the pH, and then re-establishing neutral pH prior to conducting the reactions of the of the artemisinins with the reduced conjugates.[5] NAD(P)H–flavin oxidoreductases do not contain a flavin cofactor, but reduce endogenous flavins that

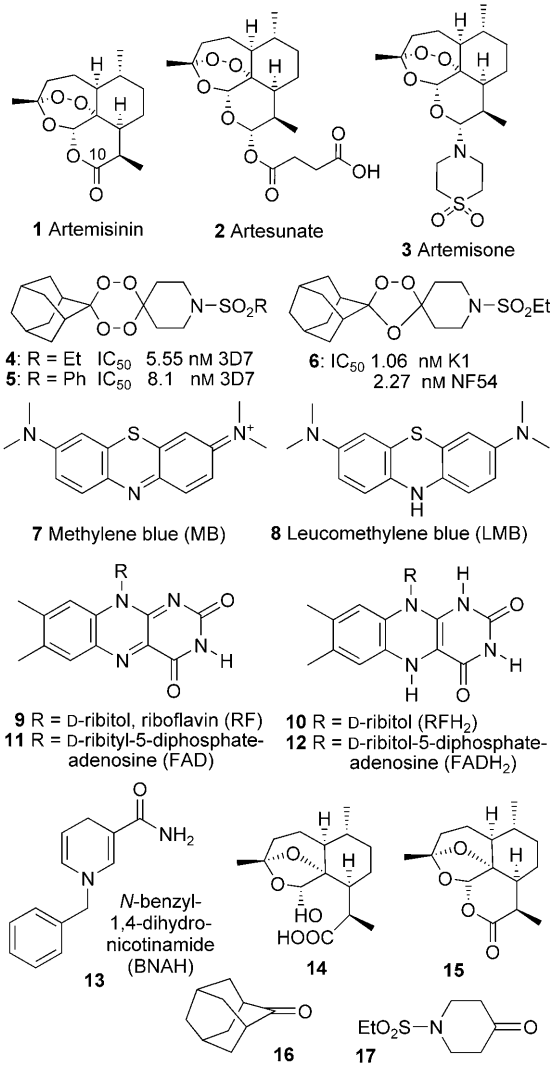


Figure 1. Artemisinins, peroxides, redox-active substrates, and ketone products discussed in this study. Antimalarial data for tetraoxanes **4** and **5** from Ref. [18], trioxolane **6** from Ref. [16].

bind to an acceptor site by transferring hydride from NAD(P)H.[23–27] Structures of isoforms that include the acceptor binding site have been elucidated, and the manner in which NAD(P)H reduces the bound acceptor has been discussed.[25,27] Of interest is their ability to use the acceptors MB or RF administered during treatment of methemoglobinemia.[23,25] In human erythrocytes, MB and RF are reduced by NADPH–flavin reductase to LMB and RFH_2, which in turn reduce methemoglobin to Hb. The MB and RF thereby regenerated are reduced by the NADPH–flavin reductase in continuation of the cycle.[23,25] In a malaria-infected erythrocyte, it is not known if the parasite uses flavin reductase for maintenance of redox homeostasis. A functionally related ferredoxin–$NADP^+$ reductase occurs in the parasite apicoplast,[28] and a chorismate synthase possessing flavin reductase activity has been detected in the parasite cytosol.[29] MB is a subversive substrate for each of parasite GR, TrxR, and lipoamide dehydrogenase in which the bound MB is reduced by the $FADH_2$ generated by reduction of

the FAD cofactor by NADPH.[4,30] Therefore, irrespective of the direct use of flavin reductase as such by the parasite, it will serve as a mimic of parasite flavin disulfide reductases under biologically plausible conditions.

Results

Peroxides, NADPH–Fre–MB and NADPH–Fre–FAD–MB

Addition of MB to NADPH in the presence of *E. coli* flavin reductase (Fre)[22] in aqueous buffer (pH 7.4) under argon resulted in rapid disappearance of the absorption at λ_{max} 660 nm as LMB was produced (Figure 2). Without Fre, partial reduction of MB by NADPH to a steady-state concentration consisting of ~25% LMB took place; the extent of reduction is about the same as that observed with NADH.[5] Thus, as previously reported,[23,25] MB serves as a substrate for Fre. Treatment of the NADPH–Fre–LMB solution with an excess (10 equiv) of each of artemisinin **1** and tetraoxane **4** resulted in rapid reappearance of the absorption at λ_{max} 660 nm as LMB was oxidized to MB (Figure 2 and figure S2 Supporting Information).

The suitability of Fre to serve as an in vitro mimic of the reduction of MB by flavoenzyme disulfide reductases was assessed. A mixture of excess NADPH with Fre was treated with FAD **11**, resulting in rapid disappearance of FAD absorptions at λ 370 and 445 nm as $FADH_2$ **12** was generated (Figure 3 and figure S3 Supporting Information). Treatment of this solution with MB rapidly produced LMB and FAD. This confirms that $FADH_2$ in GR or TrxR is capable of reducing MB.[4] The LMB solution obtained from NADPH–Fre–MB was treated with an excess of each of artemisinin **1** and tetraoxane **4**, resulting in reappearance of the absorptions due to MB. Thus, both rapidly oxidize LMB under physiological conditions. These experiments are more easily performed than experiments that involve the use of sodium dithionite to generate LMB by reduction of MB.

Peroxides, NADPH–Fre–FAD and BNAH–RF

$FADH_2$ was generated from NADPH–Fre–FAD in aqueous buffer at pH 7.4 under argon. The mixture was then treated directly with an excess of each of artemisinin **1**, artemisone **3**, tetraoxane **4**, and trioxolane **6** in pH 7.4 aqueous buffer/acetonitrile or THF, and with artesunate **2** in the aqueous buffer alone. In all cases, rapid re-oxidation of the $FADH_2$ to FAD occurred (Figure 4 and figure S4 Supporting Information). Whilst the rates of oxidation of $FADH_2$ by artemisinin and the tetraoxane were similar, it is apparent that the trioxolane **6** more rapidly oxidizes the reduced flavin than does artemisinin under the same conditions. The significance of this observation is discussed below.

The tetraoxane **4** and trioxolane **6** were converted, by catalytic RF **9** (0.2 equiv) and excess BNAH **13** in 1:1 aqueous pH 7.4 buffer/CH_3CN or buffer/THF under argon[5] for 3 h, into the ketones **16** and **17** (Figure 1) from which they were prepared. No other products were isolated. A 1:1 mixture of artemisinin **1** and tetraoxane **4** (1 equiv each, 2 equiv total) with catalytic RF (0.4 equiv)–BNAH (4.0 equiv) provided the two-electron reduction products **14** and **15** from **1**[5] and the ketones **16** and **17** from **4** (Figure 1). The relative rates of these reactions according to ^{1}H NMR spectroscopic examination of the reaction[5] are similar (Figure 5).

The tetraoxane **4** possesses two peroxide units, and therefore its oxidizing capacity should be twice that of artemisinin **1** or trioxolane **6**. A 1:1 mixture of **1** and **4** (2 equiv total) was treated with catalytic RF (0.2 equiv)–BNAH (2.0 equiv). If the two peroxide units in the tetraoxane react with the RFH_2 produced by the reduction of RF in situ by the BNAH, this will result overall in the consumption of 2 equiv of BNAH, and at least half of the te-

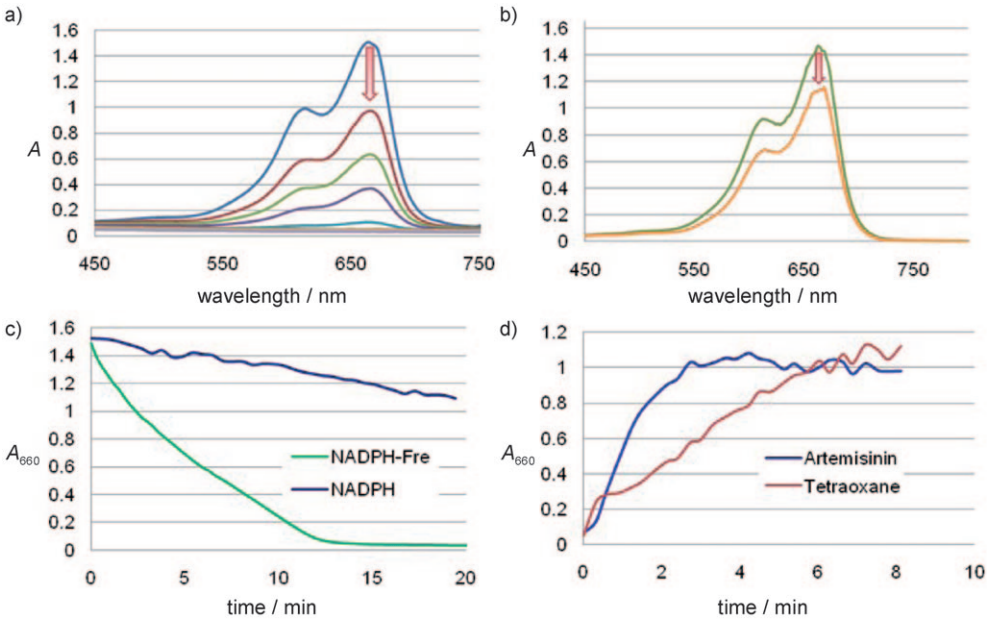


Figure 2. a) Reduction of MB by NADPH–Fre under Ar. MB (44 nmol) in pH 7.4 aqueous buffer at 22 °C was treated with Fre (4 nmol) and NADPH (230 nmol). Absorptions at λ_{max} 660 nm were monitored every 18 s until reduction to LMB had taken place (within 27 min). b) Partial reduction of MB by NADPH in the absence of Fre under Ar: MB (44 nmol) in pH 7.4 buffer at 22 °C was treated with NADPH (230 nmol). Absorptions at λ_{max} 660 nm were monitored every 18 s until no further reduction was observed to occur (within 19 min) (initial and last traces shown). c) Comparison of relative rates of reduction of MB by NADPH–Fre and NADPH under Ar under the foregoing conditions obtained by monitoring decrease in absorbance of MB at λ_{max} 660 nm. d) LMB solutions obtained according to panel a) above from NADPH (110 nmol), Fre (8 nmol), and MB (64 nmol) are respectively treated with each of artemisinin **1** (600 nmol, ~10 equiv) and tetraoxane **4** (601 nmol, ~10 equiv) in degassed CH_3CN. Absorptions at λ_{max} 660 nm were monitored every 1.8 s until complete oxidation had taken place (within 16 min); rate of increase of λ_{max} at 660 nm is plotted vs. time.

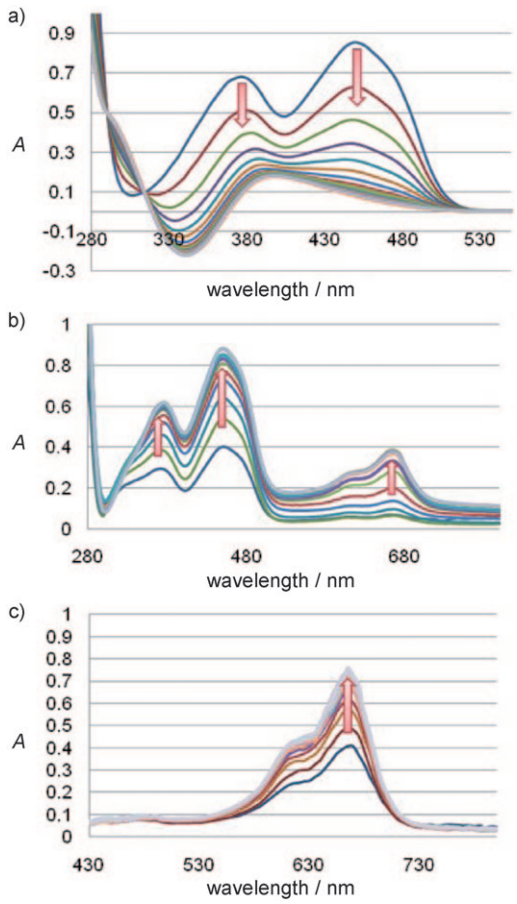


Figure 3. a) Reduction of FAD by NADPH–Fre under Ar. A mixture of NADPH (314.2 nmol) and Fre (4 nmol) in aqueous buffer at pH 7.4 at 22 °C was treated with FAD (205 nmol) and absorptions at λ_{max} 450 nm were monitored every 18 s until reduction to $FADH_2$ had taken place (within 28 min). b) Oxidation of $FADH_2$ by MB: the baseline was set to zero, and the foregoing solution was treated with MB (154 nmol). Absorptions at 450 nm were monitored every 30 s until maximum absorption was reached (within 9 min). Sufficient MB was added to ensure that residual MB was apparent, thus verifying that all NADPH had been consumed. c) The baseline was set to zero, and the preceding mixture was treated with artemisinin (599 nmol) in CH_3CN. There resulted a rapid initial oxidation of the LMB; absorptions at λ_{max} 660 nm were monitored every 18 s until the absorbance reached the maximum (within 28 min).

troxane will remain unconsumed. This turns out to be the case; the final mixture contains approximately 45% unreacted **1** and 64% unreacted **4** (Figure 5b). However, it was not possible to obtain reproducible rates of consumption of BNAH through monitoring reactions by NMR spectroscopy. Furthermore, an examination of diluted reaction mixtures of BNAH–RF–peroxide by UV spectroscopy was not possible because of the very slow reaction. Therefore, the enzyme-catalyzed reactions of each of artemisinin **1** and tetraoxane **4** in the presence NADPH–Fre–FAD were examined. Mixtures of NADPH (5 equiv), FAD (0.2 equiv), and **1** and **4** (each 1 equiv) in pH 7.4 buffer were treated with Fre, and the decrease in absorption due to NADPH at λ 340 nm was monitored. The results are shown in Figure 5c. It is apparent that the tetraoxane consumes approximately twice the amount of NADPH.

Discussion

The NADPH–Fre–FAD system may be used as a mimic of the effect of artemisinin on NADPH consumption in the yeast GR process.[5] Its use here greatly facilitates the generation of reduced flavin cofactors, and the results described herein confirm and extend those previously obtained by using sodium dithionite, and the model ascorbate–MB, BNAH–MB and –flavin systems.[5] Reduction of MB by NADPH alone is likely to be too slow to contribute effectively to redox cycling of MB (cf. Figure 2), as was previously assumed.[5] As gauged by the ease of reduction of MB to LMB by NADPH–Fre and by $FADH_2$ generated from NADPH–Fre–FAD (Figure 2), MB will undergo rapid redox cycling mediated by the flavoenzyme disulfide reductases GR, TrxR, and others. This is in accord with the demonstration that MB acts as a subversive substrate for these reductases, resulting in enhanced consumption of NADPH.[4,30] As artemisinins rapidly oxidize LMB, they will act in synergy with MB by accelerating NADPH consumption.

Reductive cleavage via hydride transfer from the reduced flavin to the peroxide bridge in the artemisinins has been discussed previously.[5] For the tetraoxane **4**, initial hydride transfer from the reduced flavin to the less-hindered oxygen of one of the two peroxide units occurs (Scheme 1). As tetraoxane **4** is capable of using the two peroxide units, it is proposed that intermediate **18** undergoes a second hydride-mediated cleavage. The resulting ketone hydrates then provide the ketones. Hydride transfer to the peroxide in trioxolane **6**, more facile than in the case of tetraoxane **4** (see below), generates hemiacetal **19**, which decomposes to the ketones. There is evidence adduced from ^{1}H NMR spectroscopy that the formation of piperidone **17** is not synchronous with the formation of adamantanone **16**. The latter forms more slowly, presumably due to slow collapse of the hydrate **16h** under the neutral reaction conditions. The alternative for all peroxides is that incipient formation of an adduct takes place via reaction through C4a of $FADH_2$, which collapses to FAD and the reduction products. This has been discussed for artemisinins, and the possibility should be borne in mind when the peroxides act as antimalarial agents in vivo.[5] The catalytic cycling of reduced flavin, either RFH_2 in the BNAH–RF or $FADH_2$ in the NADPH–Fre–FAD experiments, results in net consumption of BNAH or NADPH as the peroxide is reduced. Overall, the smooth reduction of tetraoxane **4** by LMB or $FADH_2$ stands in notable contrast to the inertness of tetraoxane **5** toward Fe^{II}.

Conclusions

The results described herein and in our previous work suggest that an appreciation of the mechanism of action of antimalarial peroxides should realign with their logical abilities to oxidize susceptible biomolecules. In spite of intensive investigations, the identity of putative targets associated with the presumed ferrous iron activation of artemisinins remains elusive. Iron in its own right contributes to oxidative stress, and this is exacerbated within the malaria parasite with its catabolism of Hb.[31] However, there arises the possibility that ferric iron oxidizes re-

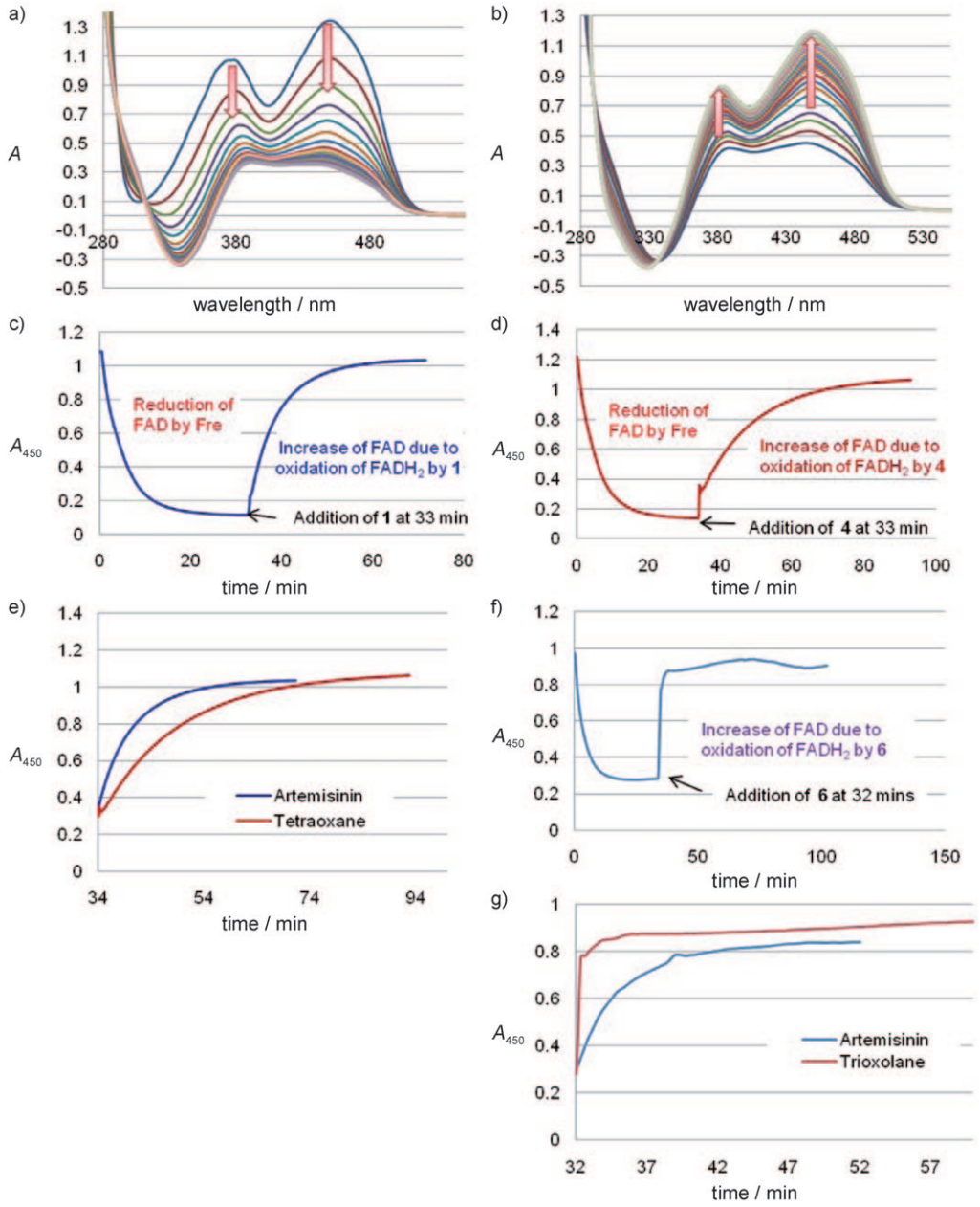


Figure 4. Oxidation of $FADH_2$ generated from NADPH–Fre–FAD by each of artemisinin **1**, tetraoxane **4**, and trioxolane **6**. a) NADPH (397.5 nmol, 1.90 equiv)–Fre (4 nmol) in aqueous buffer at pH 7.4 at 22 °C under Ar was treated with FAD (200 nmol) and absorptions at λ_{max} 450 nm were monitored every 3 s until absorptions due to FAD had decreased to a minimum. b) Artemisone **3** (600 nmol, 3 equiv) in CH_3CN under Ar was added, resulting in rapid oxidation of the $FADH_2$ to FAD; absorptions at λ_{max} 339 and 450 nm were monitored to convergence (~30 min). c) Oxidation of $FADH_2$ by artemisinin **1**: FAD (209 nmol) was added to NADPH (397.5 nmol)–Fre (4 nmol) in pH 7.4 aqueous buffer and decay of FAD was followed by monitoring decrease in absorption at λ_{max} 450 nm every 3 s until the FAD absorption no longer decreased (0-33 min). The $FADH_2$ solution was treated at 33 min with artemisinin **1** (599 nmol, 3 equiv) in CH_3CN. The rate of oxidation of $FADH_2$ to FAD was monitored at λ_{max} 450 nm until constant (~38 min). d) Oxidation of $FADH_2$ by tetraoxane **4**: the solution in panel c) was treated at 33 min with tetraoxane (601 nmol, 3 equiv) in CH_3CN and increase of absorption at λ_{max} 450 nm was monitored until constant (~38 min). e) Comparison of relative rates of oxidation of $FADH_2$ by artemisinin **1** and tetraoxane **4** (each 3 equiv with respect to FAD). f) Oxidation of $FADH_2$ by trioxolane **6**: FAD (200 nmol) was added to NADPH (258 nmol)–Fre (4 nmol) in pH 7.4 aqueous buffer and decay of FAD was followed by monitoring decrease in absorption at λ_{max} 450 nm every 3 s until the FAD absorption no longer decreased (0–32 min). The $FADH_2$ solution was treated at 32 min with artemisinin **1** (600 nmol, 3 equiv) or trioxolane **6** (600 nmol) in THF. The rate of oxidation of $FADH_2$ to FAD was monitored at λ_{max} 450 nm until constant (~38 min); the curve for trioxolane **6** is shown. g) Comparison of relative rates of oxidation of $FADH_2$ by artemisinin **1** and trioxolane **6** (each 3 equiv with respect to FAD) described in panel f).

duced flavin cofactors such as $FMNH_2$ and $FADH_2$ generated by Fre, GR, or other flavoenzymes in vivo.[23,32] The rate greatly exceeds that of Fe^{III} oxidation of thiols, including reduced glutathione GSH, in a biological medium.[23,32] Therefore, a link to the peroxide cofactor model may be provided by Fe^{III} through its assistance of the antimalarial action of peroxides, *not* by reacting with them, but rather by accelerating turnover of flavin cofactors which enhances the consumption of NADPH. Thus, in

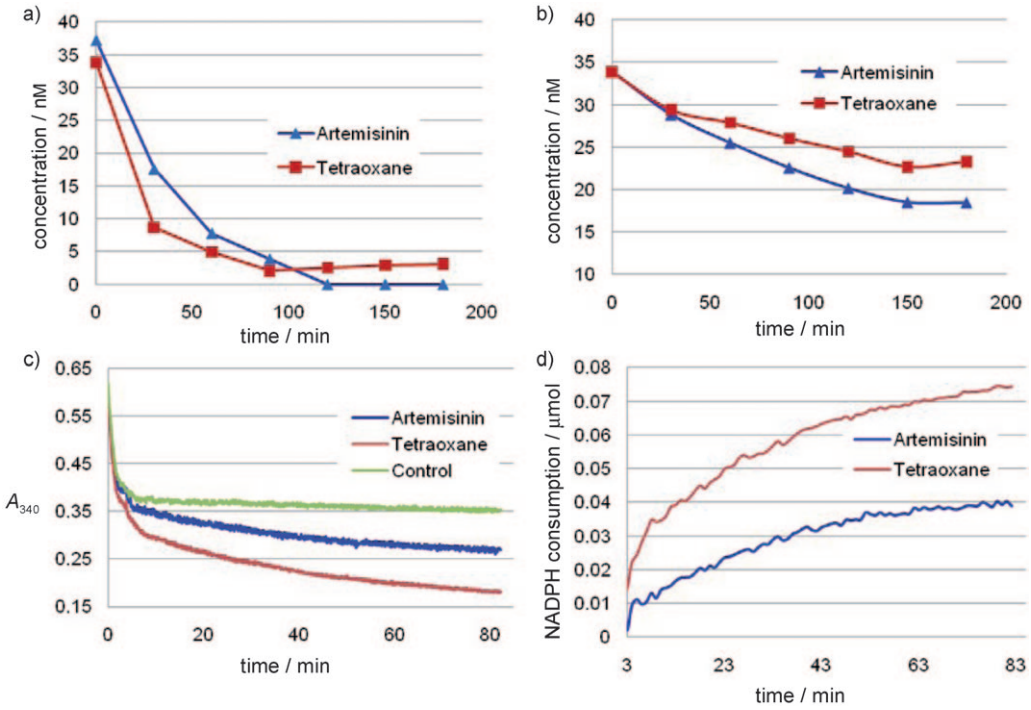
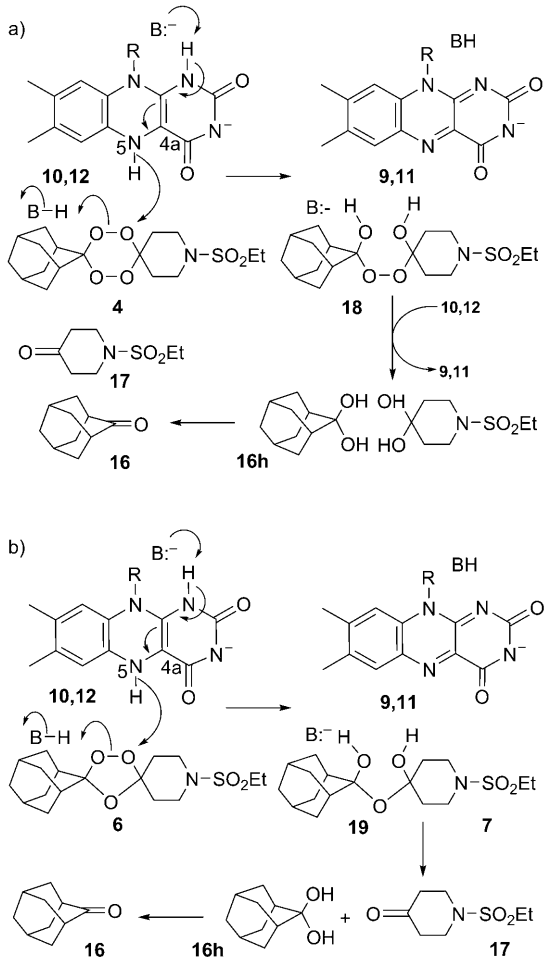


Figure 5. Comparison of reactions of artemisinin **1** and tetraoxane **4**. a) **1** and **4** and BNAH–RF: aliquots were withdrawn at 1 min, then at 30 min, and at 30 min intervals thereafter from 1:1 CH_3CN/buffer solution (pH 7.4) containing **1** (186 μmol) and **4** (186 μmol), RF **9** (74.4 μmol, 0.4 equiv), BNAH **13** (745 μmol, 4 equiv), and the internal standard 1,3,5-trimethoxybenzene (25.6 μmol) and quenched with 1 M $NaHSO_4$ prior to analysis by ^{1}H NMR spectroscopy. In the ^{1}H NMR spectrum signals at $\delta = 5.86$ (H-12, **1**), 2.96 (Me of -NSO_2Et in **4**) and 6.09 (standard) were used for calculating conversion. b) Experiment as in panel a) was repeated with **1** (252 μmol), **4** (252 μmol), RF (50.4 μmol, 0.2 equiv), BNAH (504 μmol, 2 equiv), and 1,3,5-trimethoxybenzene (33 μmol). Unreacted **1** and **4** at 180 min are 45 and 64%, respectively. c) NADPH consumption by **1** and **4** in the NADPH–Fre–FAD system under Ar: a solution of Fre (4 nmol) and FAD (41 nmol) in aqueous buffer at pH 7.4 was treated with NADPH (238.4 nmol) and then treated with **1** (40.4 nmol) and decay of absorption due to NADPH at λ 340 nm monitored each 3.9 s for 83 min. The experiment was also conducted with NADPH (236.3 nmol), Fre (4 nmol), and FAD (41 nmol) with tetraoxane **4** (40.4 nmol). The control experiment involved the use of Fre (4 nmol)–FAD (41 nmol)–NADPH (238.4 nmol) in aqueous buffer at pH 7.4. An initially rapid consumption of NADPH (0–6 min) is followed by a slower decay. A slow background consumption of NADPH occurs for the control, and at a slightly faster rate for experiments involving **1** and **4**. Although this may be due to slow leakage of air into the cuvette throughout the experiment resulting in oxidation of $FADH_2$ and reduction of the resulting FAD by NADPH–Fre, it is notable that NADPH attrition in the NADPH–Fre system is greater after treatment with each of **1** and **4** (cf. Ref. [5]). d) Relative amounts of NADPH consumed by each of **1** and **4** in the foregoing experiment.

the summary of the cofactor model as presented in Scheme 2, oxidation of reduced flavin cofactors by Fe^{III} is presented for consideration. Whilst there is debate over the origin of the effect of labile-Fe^{III} scavengers such as DFO in antagonizing antimalarial action of artemisinins in vitro,[10,21] the proposal is reconcilable with such data. The summary in Scheme 2 is based largely on incisive commentaries on effects of redox-active antimalarial drugs such as MB on parasite redox homeostasis.[4] Although it is not yet known if critical parasite redox flavoenzymes other than GR, especially TrxR and lipoamide dehydrogenase, interact with antimalarial peroxides, these, as gauged by interaction of with MB,[4] are likely to be affected.[5] Given its functional relationship with Fre described here, it is also apparent that the flavoenzyme ferredoxin–$NADP^+$ reductase, which is present in the parasite apicoplast,[28] must also be susceptible. Such susceptibility may underpin the enhanced in vitro biological activities of artemisinins usually observed toward apicomplexan parasites, such as *Plasmodium*, *Toxoplasma*, and *Babesia* species as compared with activities toward non-apicomplexan parasites.[33]

Clearly in an intraparasitic environment, trioxolanes are capable of oxidizing the reduced cofactor $FADH_2$ of flavoenzyme disulfide reductases more rapidly than tetraoxanes or artemisinins. Thus, the resulting rapid incipient perturbation of redox homeostasis may translate into a greater parasiticidal activity. Although differences in permeability and competing, but futile, decomposition pathways (such as with heme), may tend to mask intrinsic differences in antimalarial activities between artemisinins and trioxolanes, it is clear that the latter have more pronounced in vitro activities than structurally related tetraoxanes (cf. Figure 1). Nevertheless, the second peroxide unit in the tetraoxane has the potential to confer an extra dimension to biological activity. However, the peroxide units are incorporated within a six-membered ring, and may be less reactive, because of lower ring strain and greater steric shielding, than the peroxide within the five-membered trioxolane.[34] By flanking the tetraoxane nucleus by carbocyclic ring systems that are more strained and less hindered than those in **4**, and at the same time by using functional groups that improve permeability, it may be possible to enhance the ability of tetraoxanes to perturb redox homeostasis and permit application to other targets[35] as well.



Scheme 1. Reduction of peroxides by reduced flavins at pH 7.4. a) Tetraoxane **4** is proposed to undergo incipient cleavage by hydride transfer from reduced flavin to provide peroxy-hemiacetal **18**, which undergoes a second hydride-mediated cleavage. The alternative is for **18** to collapse via extrusion of a ketone to a hydroperoxy-hemiacetal that may likewise be reduced. b) Hydride cleavage of trioxolane **6** is proposed to generate hemiacetal **19**, which decomposes to the ketones. The latter reaction may proceed via hydrate **16h** of 2-adamantanone. The oxidation of two equivalents of reduced flavin by the tetroxane requires consumption of two reducing equivalents of BNAH or NADPH.

Experimental Section

Materials and Methods

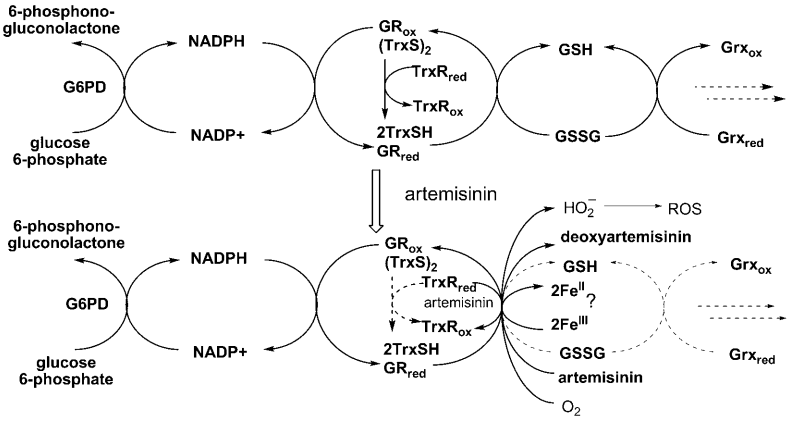
Artemisinin **1** was supplied by the Kunming Pharmaceutical Corporation (China), artesunate **2** was supplied by Dr. Robert Carter (Abbott–Knoll AG, Basel, Switzerland), and artemisone **3** was prepared according to a published procedure.[1] 2-Adamantanone (99%, Aldrich), methylene blue (certified, BSC, Sigma–Aldrich), nicotinamide (Aldrich, $\geq$99%), riboflavin (98%, Acros), FAD ($\geq$95%, Sigma), NADPH (~90%, Fluka) were used as received. *N*-Benzyl-1,4-dihydronicotinamide (BNAH) was prepared as previously described.[5] All reactions were carried out under an atmosphere of air or Ar as indicated. Pentane, CH_2Cl_2, EtOAc, and hexane were distilled and dried prior to use. TLC was performed with Merck Kieselgel 60 F_{254} plates and visualized with UV light (λ 254 nm) and/or heating after treatment with 5% ammonium molybdate in 10% concentrated H_2SO_4. Column chromatography was performed with Merck silica gel 60 (0.04–0.063 mm). UV/Vis spectra were recorded on an Agilent 8453 instrument, and ^{1}H and ^{13}C NMR spectra were obtained as solutions in $CDCl_3$ on a Bruker AV 400 spectrometer operating at 400 and 100 MHz, respectively. $CDCl_3$ was used as solvent unless otherwise stated. Melting points were carried out on a Leica Hot Stage DME E compound Microscope and are corrected. MS data were obtained on API QSTAR high-performance triple quadrupole time-of-flight mass spectrometer with electrospray ionization, and on a Waters Micromass GCT premier ToF high-resolution mass spectrometer (CI+, methane).

Tetraoxane 4: *1-(Ethanesulfonyl)piperidin-4-one* ***17***. Ethanesulfonyl chloride (1.7 mL, 18.6 mmol, 1.5 equiv) was added dropwise to a stirred solution of 4-piperidone monohydrate HCl (1.9 g, 12.4 mmol) and K_2CO_3 (4.45 g, 32 mmol) in acetone (20 mL) and H_2O (10 mL) at 0 °C. The mixture was stirred overnight at room temperature. A saturated solution of $NaHCO_3$ (10 mL) was added to quench the reaction. The mixture was extracted with CH_2Cl_2 (3× 15 mL) and dried over $MgSO_4$. After filtration, the solvent was removed by evaporation under reduced pressure to leave a colorless crystalline residue that was recrystallized from CH_2Cl_2/hexanes to give the product as colorless plates (2.0 g, 89%); mp: 68–69 °C; ^{1}H NMR (400 MHz): δ = 1.39 (3H, t, J = 7.6 Hz), 2.57 (4H, dd, J = 6.4, 6.6 Hz), 3.05 (2H, q, J = 7.6 Hz), 3.64 (4H, dd, J = 6.0. 6.0 Hz).[36]

Ethereal H_2O_2. An aqueous solution of H_2O_2 (30%, 62 mL) was saturated with NaCl and extracted with Et_2O (25 mL) in four portions (4×15 mL). The organic layer was separated followed by drying over $MgSO_4$ and filtered. The ethereal H_2O_2 was stored in a refrigerator at +4 °C.[37]

Tetraoxane **4**. According to a published procedure for preparing *gem*-dihydroperoxides,[37] ethereal H_2O_2 (1.44 M, 7 mL, 10 equiv) was added to a stirred mixture of 1-(ethylsulfonyl)piperidin-4-one (383 mg, 2 mmol) and phosphomolybdic acid (73 mg, 2 mol%), and the reaction mixture was stirred for 4 h at room temperature. Et_2O (15 mL) and H_2O (5 mL) were added to dilute the reaction mixture. The organic layer was separated and the aqueous solution was extracted with EtOAc (3×10 mL). The combined organic layer was washed with brine (3×8 mL) and then dried over $MgSO_4$. The solution was filtered and concentrated by evaporation under reduced pressure. The residue consisting of the crude *gem*-dihydroperoxide was dissolved in CH_2Cl_2 (2 mL) and CH_3CN (1 mL) and then added to a stirred solution of 2-adamantanone **16** (502 mg, 3.34 mmol) and rhenium(VII) oxide (32.4 mg, 0.0669 mmol, 3 mol%) in CH_2Cl_2 (3 mL).[38] The reaction mixture was stirred overnight at room temperature. The solution was concentrated by evaporation under reduced pressure, and H_2O (10 mL) was added. The mixture was extracted with CH_2Cl_2 (3×8 mL). The combined organic layer was washed with brine (3×8 mL) followed by drying over $MgSO_4$. After filtration, the solution was concentrated by evaporation under reduced pressure to leave the crude product as a gum. This was submitted to column chromatography with EtOAc/hexane 1:4 to give the tetraoxane as a fine white microcrystalline solid (376 mg, 50%); mp: 123–124 °C; ^{1}H NMR (400 MHz): δ = 1.37 (3H, t, J = 7.6 Hz), 1.56–2.04 (14H, m), 2.54 (2H, s), 2.96 (2H, q, J = 7.6 Hz), 3.40 (4H, s); MS (ESI) calcd for $C_{17}H_{27}NO_6SNa^+$ 396.1457, found 396.1482. The data are in agreement with those reported in the literature.[14]

Trioxolane 6: *O-methyl-2-adamantanone oxime*. A mixture of 2-adamantanone **16** (2.03 g, 13.52 mmol) and methoxylamine HCl (1.23 g, 14.71 mmol) in EtOH (30 mL) and pyridine (1.18 mL, 14.66 mmol) was heated at gentle reflux at 90 °C (bath temperature) under N_2 for 1.5 h. The mixture was cooled to room tempera-



Scheme 2. Proposed basis for oxidative stress cascade mediated by peroxidic antimalarials, as derived from commentary of redox drug action in Ref. [4] (GR = glutathione reductase, ox = FAD cofactor, red = $FADH_2$ cofactor; Trx = thioredoxin, SH = reduced, S_2 = oxidized; TrxR = thioredoxin reductase, ox = FAD, red = $FADH_2$; Grx = glutaredoxin): Maintenance of GSH and reduced thioredoxin (Trx) requires NADPH provided by the hexose monophosphate shunt (HMS), the rate-limiting enzyme of which is glucose-6-phosphate dehydrogenase (G6PD). HMS activity in *P. falciparum*-infected erythrocytes (IE) is up to 78-fold higher than that of uninfected erythrocytes; HMS activity of the parasite contributes 80% of the total observed in the intact IE, and HMS activity of the host cell is increased ~24-fold when compared with uninfected erythrocytes. The major portion of parasite HMS activity and the increased HMS activity of the host cell counteract oxidative stress. Treatment of yeast GR, a surrogate for parasite GR, with artemisinin **1** under aerobic conditions results in enhanced consumption of NADPH and restricted reduction of GSSG. That is, $FADH_2$ becomes susceptible to autoxidation after exposure of GR to the artemisinin. The putative effect of Fe^{III} is proposed as it rapidly oxidizes $FMNH_2$ and $FADH_2$ generated by NADPH–Fre, NADPH–GR, or NADPH–flavin diaphorases (Refs. [23, 32]). Possibly, exposure to artemisinin may render reduced flavin cofactor in GR amenable to oxidation by Fe^{III}. It is not yet known if other critical parasite redox flavoenzymes such as TrxR, lipoamide dehydrogenase, and the ferredoxin–$NADP^+$ reductase occurring in the parasite apicoplast (Ref. [28]), interact with antimalarial peroxides, but these are predicted to be affected.

ture, treated with brine (20 mL), and then extracted with CH_2Cl_2 (3×15 mL). The extracts were combined, and the solution was dried over $MgSO_4$ and then filtered. The solvent was evaporated under reduced pressure to leave the *O*-methyl oxime as a white solid; mp: 71.3–71.9 °C (lit.[39] 70–71 °C) (2.227 g, 92%); ^{1}H NMR (400 MHz): δ = 1.79–1.98 (12H, m), 2.54 (1H, s), 3.46 (1H, s), 3.81 (3H, s). The sample was dried thoroughly under high vacuum.

Trioxolane ***6***. For the following preparation to succeed, all reagents and solvents had to be scrupulously dry. Ozone was produced by using a Triogen Lab 2B Laboratory O_3 generator at a flow rate of 0.6 L min^{-1} O_2 at a discharge voltage of 50 V and bubbled through a stirred solution of dried *O*-methyl-2-adamantanone oxime (1.08 g, 6.0 mmol) and dried 1-(ethylsulfonyl)piperidin-4-one (1.15 g, 6.0 mmol) in dry distilled pentane (30 mL) and dry distilled CH_2Cl_2 (15 mL) at −78 °C for 2.5 h. After completion of the reaction, the solution was flushed with O_2 for 10 min and then warmed to room temperature. It was then concentrated by evaporation under reduced pressure at room temperature. The crude product was submitted to column chromatography with EtOAc/hexane 1:6 to give the product (1.223 g, 57.5%), as colorless rods; mp: 108.3–108.4 °C (lit.[16] 110–112 °C); ^{1}H NMR (400 MHz): δ = 1.37 (3H, t, J = 7.6), 1.69–2.00 (18H, m), 2.93–2.30 (2H, q, J = 7.6), 3.34–3.40 (2H, m), 3.45–3.51 (2H, m); MS (ESI) calcd for $C_{17}H_{27}NO_6SNa^+$ 380.1508, found 380.1528.

Preparation of *E. coli* flavin reductase Fre: The *E. coli* flavin reductase Fre was overexpressed with a C-terminal hexahistidine tag and purified to homogeneity as described previously.[40] Briefly, the Fre gene was amplified by Phusion High-Fidelity DNA Polymerase (Finnzymes) from the genomic DNA of *E. coli* K12 using 3′-ACA TGC CAT GGG Aat gac aac ctt aag ctg taa ag-5′ and 3′-ACC GCT CGA Gga taa atg caa acg cat cgc c-5′ as primers. The PCR product was digested by restriction enzymes and inserted into the pET-28a(+) vector (Novagen) between the NcoI and XhoI sites. The Fre gene was confirmed not to contain any spurious mutations by full-length sequencing of the cloned DNA insert. The gene product was expressed in Luria broth containing 0.2 mM IPTG at 16 °C for 24 h. Fre was purified from the crude extract prepared from the harvested cells, first by metal chelating chromatography using a 5 mL HiTrap Chelating HP column (GE Healthcare) and then by gel filtration using a Sephacryl S-100 column (GE Healthcare). It was established by SDS-PAGE that the tagged protein so obtained was purified to >95%. The purified protein was quantified by a Coomassie Blue protein assay kit (Pierce) and stored in phosphate-buffered saline (pH 7.4) containing 10% glycerol at −20 °C until use.

UV/Vis absorption, ^{1}H NMR spectroscopy, and decomposition experiments

Reduction of MB by NADPH–Fre, and reaction of LMB with artemisinin 1 and tetraoxane 6 (Figure 2 and figure S2 Supporting Information): NADPH (83.9 mg, 0.1007 mmol) was dissolved in degassed pH 7.4 buffer (5 mL) to generate a solution containing 0.0201 M. The NADPH solution was purged with Ar and was then stored at +4 °C for use. The NADPH solution (10 μL) was treated with pH 7.4 degassed buffer (1.6 mL) in an Ar-purged UV cuvette (d = 1 cm) at 22 °C. The absorbance of NADPH measured at λ_{max} 339 nm was 0.4949 AU. As the absorption coefficient of NADPH at λ 339 nm is 6220 M^{-1} cm^{-1},[41] the actual concentration of NADPH solution was 0.0128 M (~0.064 mmol). MB (12.8 mg, 0.0342 mmol) was dissolved in degassed pH 7.4 buffer (5 mL) to generate a solution containing 0.00685 M; the MB solution was purged under Ar and was stored at +4 °C for use.

a. *Reduction of MB by NADPH–Fre*: The MB solution (10 μL) was treated with pH 7.4 degassed buffer (1.9 mL) in an Ar-purged UV cuvette (d = 1 cm) at 22 °C. The absorbance of MB at λ_{max} 660 nm was 1.63 AU. The absorption coefficient of MB at λ_{max} 660 nm is 71 547 M^{-1} cm^{-1};[42] therefore, the actual concentration of the MB solution is 0.0044 M (~0.022 mmol). Fre (200 μL, 400 μM) was diluted to 1 mL by additional of degassed pH 7.4 aqueous buffer to generate a solution containing 80 μM. The purged MB solution (10 μL, 0.044 μmol) and degassed pH 7.4 buffer (1.85 mL) were added to an Ar-purged UV cuvette (d = 1 cm) at 22 °C with monitoring of the absorption at λ_{max} 660 nm until maximized. The MB solution in the cuvette was treated with Fre (50 μL, 4 nmol) and the NADPH solution (18 μL, 230.4 nmol). The reduction of MB was

followed by monitoring decrease of absorption at λ_{max} 660 nm until complete reduction to LMB had taken place.

b. *Control: reduction of MB by NADPH*: The MB solution in the cuvette was treated with the NADPH solution (18 μL, 230.4 nmol) only. The reduction of MB was followed by monitoring decrease of absorption at λ_{max} 660 nm until no further reduction was observed to occur.

c. *Comparison of NADPH–Fre and NADPH control reductions of MB*: The relative rates of reduction of MB in the presence of NADPH–Fre and in the absence of Fre under the foregoing conditions are compared by monitoring relative rates of decrease of λ_{max} at 660 nm.

d. *Comparison of rates of oxidation of LMB by each of artemisinin **1** and tetraoxane **4***: For the following experiment, the stock solutions were prepared as follows: Artemisinin stock solution (10 mL in CH_3CN), $M_r = 282.33$ Da, 0.5637 g, 1.997×10^{-3} mol, concentration: 1.997×10^{-3} mol per 0.01 L = 0.1997 M; tetraoxane stock solution (5 mL in CH_3CN), $M_r = 373.44$ Da, 0.1869 g, 5.005×10^{-4} mol, concentration: 5.005×10^{-4} mol per 0.005 L = 0.1001 M; NADPH stock solution (1 mL in pH 7.4 phosphate buffer), absorbance: 0.445, $\varepsilon = 6220\ M^{-1}cm^{-1}$ at λ 339 nm, concentration in cuvette (10 μL made up to 1.91 mL in buffer) = 0.445/(1 cm × 6220 $M^{-1}cm^{-1}$) = 0.0715 mM, concentration 7.15×10^{-5} M × 0.00191 L/10×10^{-6} L = 0.0137 M; MB stock solution (5 mL in pH 7.4 phosphate buffer), absorbance: 1.57, $\varepsilon = 71\,547\ M^{-1}cm^{-1}$ at λ 660 nm, concentration in cuvette (20 μL made up to 2.92 mL in buffer) = 1.57/(1 cm × 71 547 $M^{-1}cm^{-1}$) = 0.0193 mM, concentration: 1.93×10^{-5} M × 0.00161 L/5×10^{-6} L = 0.0032 M, Fre: 80 μM in pH 7.4 buffer. Artemisinin **1** (0.5637 g, 1.997×10^{-3} mol) was dissolved in degassed CH_3CN (10 mL) to give a final concentration of 0.1997 M. The MB solution was prepared from MB (7.2 mg, 0.0193 mmol) and degassed pH 7.4 aqueous buffer (5 mL) to give a final concentration of 0.00385 M. The tetraoxane **4** (186.9 mg, 0.5005 mmol) and degassed CH_3CN (5 mL) were mixed to give a final concentration of 0.1001 M. By measuring UV absorption at λ 660 nm, the actual concentration was calculated to be 0.0032 M as described above. NADPH (17.2 mg, 0.0206 mmol) was dissolved in degassed pH 7.4 aqueous buffer (1 mL) to give a final concentration of 0.0206 M; as above, the actual concentration by measurement of UV absorption at λ 339 nm was determined to be 0.0137 M. The Fre was prepared in pH 7 buffer to give a final concentration of 400 μM. The degassed pH 7.4 buffer (2900 μL) and MB solution (20 μL, 0.064 μmol) were added to an Ar-purged UV cuvette ($d = 1$ cm) at 22 °C. Once the solution had equilibrated to maximum absorbance at λ 660 nm, the solution was then treated with Fre (20 μL, 8 nmol) and the baseline was set to zero. The NADPH solution (8 μL, 110 nmol, 1.7 equiv) was added with mixing. Three experiments were carried out: 1. Control, which measured rate of decrease of MB until the reduction was complete. 2. Measuring the effect of addition of artemisinin (3 μL, 0.6000 μmol, 10 equiv) to the same cuvette on the rate of increase of MB absorbance; scans were recorded at intervals of 1.8 s until convergence. 3. Measuring the effect of addition of tetraoxane (6 μL, 600.6 nmol, 10 equiv) to the LMB solution, and scanning every 1.8 s until convergence was reached. In both cases inhomogeneity of the mixture, probably due to formation of fine particles of MB, took place after addition of artemisinin or tetraoxane in the CH_3CN, and this affected the quality of the spectra recorded. In Figure 2 d, the relative differences in rates oxidation of the LMB by each of artemisinin **1** and tetraoxane **4** are recorded.

Reduction of MB by NADPH–Fre–FAD, and reaction of LMB with artemisinin 1 and tetraoxane 4 (Figure 3 and figure S3): NADPH (17.2 mg, 0.0206 mmol) was dissolved in degassed pH 7.4 buffer (1 mL) to generate a solution containing 0.0206 M. The solution was purged with Ar and was stored at +4 °C for use. The NADPH solution (10 μL) was treated with pH 7.4 degassed buffer (1.9 mL) in an Ar-purged UV cuvette ($d = 1$ cm) at 22 °C. The absorbance of the NADPH solution at λ_{max} 339 nm was measured as 0.445 AU. The absorption coefficient of NADPH at λ 339 nm of 6220 $M^{-1}cm^{-1}$[41] gives an actual concentration of 0.0137 M (~0.01366 mmol). MB (12.8 mg, 0.0342 mmol) was dissolved in degassed pH 7.4 buffer (5 mL) to generate a solution containing 0.00685 M. The MB solution was purged with Ar and was stored at +4 °C for use. The MB solution (10 μL) was treated with pH 7.4 degassed buffer (1.9 mL) in an Ar-purged UV cuvette ($d = 1$ cm) at 22 °C. The absorbance of the MB solution at λ_{max} 660 was measured as 1.63 AU. The absorption coefficient of MB at λ 660 nm is 71 547 $M^{-1}cm^{-1}$;[42] therefore, the actual concentration of MB solution is 0.0044 M (~0.022 mmol). Fre (400 μL, 1000 μM) was diluted to 1 mL by addition of degassed pH 7.4 aqueous buffer to generate a solution containing 400 μM (0.4 μmol). FAD (17.4 mg, 0.0205 mmol) was dissolved in degassed pH 7.4 buffer (1 mL) to generate a solution containing 0.0205 M. The FAD solution was purged under Ar and was stored at +4 °C for use.

a. *Reduction of FAD by NADPH–Fre*: The purged NADPH solution (23 μL, 314.2 nmol), Fre (10 μL, 4 nmol) and degassed pH 7.4 buffer (1.9 mL) were added to an Ar-purged UV cuvette ($d = 1$ cm) at 22 °C and treated with the FAD solution (10 μL, 205 nmol). The reduction of FAD was followed by monitoring the decrease of absorption at λ_{max} 450 nm until reduction had taken place.

b. *Reduction of MB by $FADH_2$*: The above mixture was treated with the purged MB solution (30 μL, 131 nmol) by monitoring the increase of absorption at λ_{max} 450 nm until the oxidation of FAD had taken place; the appearance of an absorption at λ_{max} 660 nm indicated that there was no residual NADPH capable of initiating Fre-catalyzed reduction of MB.

c. *Oxidation of LMB by artemisinin*: The baseline was recorded, and the reaction mixture in the cuvette was treated with the purged artemisinin solution (3 μL, 0.599 μmol) prepared from artemisinin (563.7 mg, 1.9973 mmol) and degassed CH_3CN (10 mL) under Ar. The initial oxidation of the LMB was very fast, and it was not possible to record incipient absorption changes. Recording of spectra was continued until the maximum was reached (~28 min). It was noted that inhomogeneity of the mixture, probably due to formation of fine particles of MB, resulted after addition of artemisinin in the CH_3CN, and this affected the quality of the spectra recorded.

Figure S3: The tetraoxane solution was prepared from tetraoxane **4** (186.9 mg, 0.5005 mmol) and degassed CH_3CN (5 mL) to give a final concentration of 0.1001 M. The methylene blue (MB) solution was prepared from MB (12.8 mg, 0.0342 mmol) and degassed pH 7.4 aqueous buffer (5 mL) to give a final concentration of 0.00385 M. The concentration of MB as 0.00435 M was re-determined by UV absorption at λ 660 nm as described above. The NADPH solution was prepared from NADPH (17.2 mg, 0.0206 mmol) and degassed pH 7.4 aqueous buffer (1 mL) to give a final concentration of 0.0206 M. The concentration of NADPH as 0.0137 M was re-determined by UV absorption at λ 339 nm as described above. Fre was prepared in pH 7 buffer to give a final concentration of 400 μM. The degassed pH 7.4 buffer (2900 μL), the NADPH solution (23 μL, 0.315 μmol, 1.51 equiv), were added to a UV cuvette ($d = 1$ cm) at 22 °C and treated with Fre (10 μL, 4 nmol).

A background was scanned from λ 200–800 nm. The FAD solution (10 μL, 0.2088 μmol) was added with mixing. Three experiments were carried out: 1. Control, which measured rate of decrease of FAD until the reaction was finished. 2. Effect of immediate addition of MB solution (30 μL, 0.131 μmol, 0.623 equiv) to the same cuvette on the rate of increase of FAD. 3. Effect of addition of tetraoxane (6 μL, 601 nmol, 4.58 equiv) to the same cuvette on the rate of increase of MB. Scanning was carried out every 18 s until the convergence was reached.

Reduction of FAD by NADPH–Fre, and reaction of $FADH_2$ with artemisinins 1 and 3, tetraoxane 4, and trioxolane 6 (Figure 4 and figure S4): The NADPH solution was prepared from NADPH (83.9 mg, 1.007×10^{-4} mol) and degassed pH 7.4 aqueous buffer (5 mL) to give a final concentration of 0.02014 M. Concentration of NADPH 0.0128 M was re-determined by UV absorption at λ 339 nm as described above. The FAD solution was prepared from FAD (17.7 mg, 2.088×10^{-5} mol) and degassed pH 7.4 aqueous buffer (1 mL) to give a final concentration of 0.0200 M. The Fre solution was prepared in pH 7 buffer to give a final concentration of 400 μM. The degassed pH 7.4 buffer (2000 μL), the NADPH solution (25 μL, 0.3975 μmol, 1.90 equiv) were added to a UV cuvette ($d=$ 1 cm) at ambient temperature and treated with the Fre (10 μL, 4 nmol). A background was scanned from λ 220 to 600 nm.

a. *Reduction of FAD by Fre*: The FAD solution (10 μL, 0.200 μmol) was added to the cuvette with mixing. Decrease of absorptions due to FAD was monitored by scanning at λ_{max} 450 nm at 3 s intervals until reduction was completed.

b. *Oxidation of $FADH_2$ by artemisone **3***: The artemisone solution was prepared from artemisone (0.1607 g, 4.002×10^{-4} mol) and degassed CH_3CN (10 mL) to give a final concentration of 0.0400 M. The effect of addition of the artemisone solution (15 μL, 0.600 μmol, 3 equiv) to the same cuvette on the rate of oxidation of $FADH_2$ was monitored λ_{max} 450 nm over 30 min.

c. *Oxidation of $FADH_2$ by artemisinin **1***: A solution was prepared from **1** (563.7 mg, 1.997×10^{-3} mol) and degassed CH_3CN (10 mL) to give a final concentration of 1.997 M. The NADPH solution was prepared from NADPH (83.9 mg, 1.007×10^{-4} mol) and degassed pH 7.4 aqueous buffer (5 mL) to give a final concentration of 0.02014 M. The concentration of NADPH as 0.0128 M was re-determined from the UV absorption at λ 339 nm, as described above. The FAD solution was prepared from FAD (17.7 mg, 2.088×10^{-5} mol) and degassed pH 7.4 aqueous buffer (1 mL) to give a final concentration of 0.0200 M. The Fre was prepared in pH 7.4 buffer to give a final concentration of 400 μM. The degassed pH 7.4 buffer (2000 μL), the NADPH solution (25 μL, 0.3975 μmol, 1.90 equiv) were added to an Ar-purged UV cuvette ($d=$1 cm) at ambient temperature and treated with Fre (10 μL, 4 nmol). A background scan was taken from λ 220 to 600 nm. The FAD solution (10 μL, 0.209 μmol) was added with mixing, and the decrease of FAD absorption at λ_{max} 450 nm were recorded at 3 s intervals until the absorption was constant (33 min). Then artemisinin solution (3 μL, 0.5991 μmol, 3 equiv) was added, and the rate of increase of FAD by monitoring absorption at λ_{max} 450 nm was carried out over 38 min.

d and e. *Comparison of oxidation of $FADH_2$ by artemisinin **1** and tetraoxane **4**:* Compound **4** (186.7 mg, 4.999×10^{-4} mol) was dissolved in degassed CH_3CN (5 mL) to give a final concentration of 1.997 M. The NADPH solution was prepared from NADPH (83.9 mg, 1.007×10^{-4} mol) and degassed pH 7.4 aqueous buffer (5 mL) to give a final concentration of 0.02014 M. The concentration of NADPH as 0.0128 M was re-determined by measurement of the UV absorption at λ 339 nm. The FAD solution was prepared from FAD (17.7 mg, 2.088×10^{-5} mol) and degassed pH 7.4 aqueous buffer (1 mL) to give a final concentration of 0.0200 M. The Fre was prepared in pH 7 buffer to give a final concentration of 400 μM. The degassed pH 7.4 buffer (2000 μL), the NADPH solution (25 μL, 0.3975 μmol, 1.90 equiv), were added to a UV cuvette ($d=$1 cm) at ambient temperature and treated with the Fre (10 μL, 4 nmol). A background was scanned from λ 220 to 600 nm. The FAD solution (10 μL, 0.200 μmol) was added with mixing, and the decrease of FAD at λ_{max} 450 nm were recorded at 3 s intervals until the absorption was constant (33 min). The tetraoxane solution (6 μL, 0.6006 μmol, 3 equiv) was added, and the rate of increase of FAD absorption at λ_{max} 450 nm was monitored for 38 min. Panel e) plots the relative rates of oxidation of $FADH_2$ by artemisinin **1** and tetraoxane **4** by measuring increase absorption at λ_{max} 450 nm.

f and g. *Comparison of oxidation of $FADH_2$ by artemisinin **1** and trioxolane **6***: Artemisinin **1** (141.2 mg, 5.001×10^{-4} mol) was dissolved in degassed THF (5 mL) to give a final concentration of 1.997 M. The NADPH solution was prepared from NADPH (83.9 mg, 1.007×10^{-4} mol) and degassed pH 7.4 aqueous buffer (5 mL) to give a final concentration of 0.02014 M. The concentration of NADPH as 0.0172 M was re-determined by measurement of the UV absorption at λ 339 nm. FAD (17.4 mg, 2.05×10^{-5} mol) was dissolved in pH 7.4 aqueous buffer (1 mL) to give a final concentration of 0.0205 M. The Fre was prepared in pH 7 buffer to give a final concentration of 400 μM. The degassed pH 7.4 buffer (2000 μL), the NADPH solution (15 μL, 0.258 μmol, 1.26 equiv), were each added to a UV cuvette ($d=$1 cm) at ambient temperature and treated with Fre (10 μL, 4 nmol). A background was scanned from λ 220 to 600 nm. The FAD solution (10 μL, 0.205 μmol, 1.0 equiv) was added with mixing. Two experiments were carried out: 1. Control, which measured rate of decrease of FAD until the reaction was finished; scanning at λ_{max} 450 nm was conducted at 3 s intervals until absorption due to FAD no longer decreased. 2. Addition of artemisinin solution (6 μL, 0.60 μmol, 3 equiv) to the same cuvette on the rate of increase of FAD. The rate of turnover of FAD was followed through monitoring increase of absorption at λ_{max} 450 nm over 38 min.

The trioxolane solution was prepared from **6** (0.0357 g, 9.987×10^{-5} mol) and degassed THF (1 mL) to give a final concentration of 0.100 M. The NADPH solution was prepared from NADPH (0.0839 g, 1.007×10^{-4} mol) and degassed pH 7.4 aqueous buffer (5 mL) to give a final concentration of 0.02014 M. The concentration of NADPH as 0.0172 M was re-determined by measurement of the UV absorption at λ 339 nm. FAD (0.0174 g, 2.05×10^{-5} mol) was dissolved in degassed pH 7.4 aqueous buffer (1 mL) to give a final concentration of 0.0205 M. Fre was prepared in pH 7 buffer to give a final concentration of 400 μM. The degassed pH 7.4 buffer (2000 μL), the NADPH solution (15 μL, 0.258 μmol, 1.26 equiv), were each added to a UV cuvette ($d=$1 cm) at ambient temperature and treated with Fre (10 μL, 4 nmol). A background was scanned from λ 220 to 600 nm. The FAD solution (10 μL, 0.205 μmol, 1.0 equiv) was added with mixing. Two experiments were carried out: 1. Control, which measured rate of decrease of FAD until the reaction was finished; scanning at λ_{max} 450 nm was conducted at 3 s intervals until the absorption due to FAD no longer decreased. 2. Addition of trioxolane solution (6 μL, 0.60 μmol, 3 equiv) and examining effect on the rate of increase of FAD. The rate of turnover of FAD was followed by monitoring increase of absorption at λ_{max} 450 nm over 70 min.

Reaction of artemisinin 1 and tetraoxane 4 with BNAH and NADPH–Fre–FAD (Figure 5): **a.** 1. *BNAH–RF catalytic system*. Artemisinin **1** (52.6 mg, 0.186 mmol), tetraoxane **4** (69.5 mg, 0.186 mmol),

riboflavin **9** (28.0 mg, 0.0744 mmol, 0.20 equiv with respect to total peroxide), BNAH **13** (159.7 mg, 0.745 mmol, 2.0 equiv with respect to total peroxide), and 1,3,5-trimethoxybenzene (4.3 mg, 0.0256 mmol) were stirred in a 1:1 mixture of CH_3CN/phosphate buffer (5.5 mL) under Ar at room temperature. At the first minute, 0.5 mL of the reaction mixture was withdrawn and added to 1 M $NaHSO_4$ solution (1 mL) immediately. Et_2O (1 mL) was added to dilute the reaction mixture. The organic layer was separated and the aqueous layer was extracted with EtOAc (3×1 mL). The combined organic layer was washed with 1 M $NaHSO_4$ solution (2 mL) followed by brine (1 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was taken into $CDCl_3$, and the ^{1}H NMR spectrum was obtained. Steps were repeated at 30, 60, 90, 120, 150, and 180 min. Peak of internal standard taken for calibration: 6.09 ppm, integral calibrated to 3; peak of **1** taken for calculation $\delta = 5.864$ ppm (singlet, 1H); peak of tetraoxane taken for calculation: $\delta = 2.96$ ppm (quartet, 2H). Results were calculated as follows: no. of mol **1** added = 0.0526 g/282.33 g mol^{-1} = 1.863×10^{-4} mol; no. of mol of **4** added = 0.0695 g/373.44 g mol^{-1} = 1.861×10^{-4} mol;[1] = [no. of mol of **1** calculated/no. of mol of **1** at 0 min × 1.863×10^{-4} mol]/0.0055 L;[4] = [no. of mol of **4** calculated/no. of mol of **4** at 0 min × 1.861×10^{-4} mol]/0.0055 L. Data are plotted in Figure 5.

b. *Competitive reaction of artemisinin **1** and tetraoxane **4** with RFH_2 generated in the RF–BNAH catalytic system*. Artemisinin (71.1 mg, 252 μmol), tetraoxane (94.1 mg, 252 μmol), riboflavin (19 mg, 50.4 μmol, 0.2 equiv), BNAH (108 mg, 504 μmol, 2 equiv) and 1,3,5-trimethoxybenzene (5.5 mg, 32.7 μmol) were stirred in a 1:1 mixture of CH_3CN/phosphate buffer (7.5 mL) under Ar at room temperature. At the first minute, 0.7 mL reaction mixture was withdrawn and added to 1 M $NaHSO_4$ solution (1 mL) immediately. Et_2O (1 mL) was added to dilute the reaction mixture. The organic layer was separated and aqueous layer was extracted with EtOAc (3×1 mL). The combined organic layer was washed with 1 M $NaHSO_4$ solution (2 mL) followed by brine (1 mL), dried over $MgSO_4$, filtered and concentrated by evaporation under reduced pressure. The residue was taken into $CDCl_3$ and the ^{1}H NMR spectrum was obtained. Steps were repeated at 30, 60, 90, 120, 150, and 180 min. The signals used were at $\delta = 6.09$ ppm (ArH, standard, integral calibrated to 30, artemisinin **1** 5.864 ppm (singlet, 1H), 2-deoxyartemisinin **15** 5.698 ppm (singlet, 1H), ring-opened acid **14** 5.462 ppm (singlet, 1H), tetraoxane **4** 2.96 ppm (quartet, $J = 7.6$ Hz, 2H), and 1-(ethylsulfonyl)piperidin-4-one **17** 3.05 ppm (quartet, $J = 7.6$ Hz, 2H). The concentrations were adjusted as follows: no. of mol. of **1** added = 0.0711 g/282.33 g mol^{-1} = 2.518×10^{-4} mol; no. of mol of **4** added = 0.0955 g/373.44 g mol^{-1} = 2.557×10^{-4} mol;[1] = [no. of mol of **1** calculated × 2.518×10^{-4} mol/(no. of mol of **1** + no. of mol of **14** + no. of mol of **15**)]/0.0075 L;[4] = [no. of mol of **4** calculated × 2.557×10^{-4} mol/(no. of mol of **4** + no. of mol of **17**)]/0.0075 L.

c and d. *Consumption of NADPH in the NADPH–Fre–FAD system by artemisinin **1** and tetraoxane **4***. NADPH (17.2 mg, 20.6 μmol) was dissolved in degassed pH 7.4 buffer (1 mL) to generate a solution containing 0.0206 M; the NADPH solution was stored under Ar at +4 °C prior to use. The NADPH solution (13 μL) was treated with pH 7.4 degassed buffer (2.0 mL) in an Ar-purged UV cuvette (d = 1 cm) at 22 °C. The absorbance of NADPH at λ_{max} 339 nm was 0.56 AU. The absorption coefficient of NADPH at λ 339 nm is 6220 M^{-1} cm^{-1}; therefore, the actual concentration of NADPH solution was 0.01394 M (~13.941 μmol). FAD (17.4 mg, 20.5 μmol) was dissolved in degassed pH 7.4 buffer (1 mL) to generate a solution containing 0.0205 M. Fre (10 μL, 400 μM) was diluted to 100 μL by addition of degassed pH 7.4 aqueous buffer, to generate a solution containing 40 μM. The purged FAD solution (10 μL, 41 nmol), Fre (10 μL, 4 nmol) and degassed pH 7.4 buffer (2 mL) were added to an Ar-purged UV cuvette (d = 1 cm) at 22 °C. The reaction mixture was treated with NADPH (17 μL, 236.3 nmol). The oxidation of NADPH was followed by monitoring decrease of absorption at λ_{max} 340 nm until oxidation had taken place. 100 μL of a solution prepared from tetraoxane **4** (186.9 mg, 0.5005 mmol) in degassed CH_3CN (5 mL) was diluted to 5 mL with degassed CH_3CN in a 5 mL volumetric flask. 2 μL of this solution, containing 40.44 nmol tetraoxane **4** was added to the cuvette, and the decrease in concentration of the NADPH was followed by monitoring decrease of absorption at λ_{max} 340 nm. For the control experiment, pH 7.4 buffer (2 μL) was added to the cuvette, and absorption at λ_{max} 340 nm was monitored. For the comparison with artemisinin, NADPH (17.2 mg, 20.6 μmol) was dissolved in degassed pH 7.4 buffer (1 mL) to generate a solution containing 0.0206 M. The solution was stored under Ar at +4 °C prior to use. The NADPH solution (10 μL) was treated with pH 7.4 degassed buffer (2.0 mL) in an Ar-purged UV cuvette (d = 1 cm) at 22 °C. The absorbance of NADPH at λ_{max} 339 was 0.459 AU. The absorption coefficient of NADPH at λ 339 nm is 6220 M^{-1} cm^{-1}; therefore, the actual concentration of the NADPH solution was 0.0148 M (~0.0148 mmol). FAD (17.4 mg, 20.5 μmol) was dissolved in degassed pH 7.4 buffer (1 mL) to generate a solution containing 0.0205 M. Fre (10 μL, 400 μM) was diluted to 100 μL by the addition of degassed pH 7.4 aqueous buffer to generate a solution containing 40 μM. The purged FAD solution (10 μL, 41 nmol), Fre (10 μL, 4 nmol) and degassed pH 7.4 buffer (2 mL) were added to an Ar-purged UV cuvette (d = 1 cm) at 22 °C. The mixture was treated with NADPH (17 μL, 236.3 μmol). The oxidation of NADPH was followed by monitoring decrease of absorption at λ_{max} 340 nm. Artemisinin (57 mg, 202 μmol) was dissolved in degassed CH_3CN (10 mL). 2 μL of this solution, containing 40.4 nmol artemisinin was added to the cuvette, and the decrease of absorption due to NADPH at λ_{max} 340 nm was monitored.

Decomposition of tetraoxane 4 and trioxolane 6: *Tetroxane **4***. A mixture of tetraoxane (67.7 mg, 0.181 mmol), riboflavin (13.7 mg, 0.0364 mmol, 0.2 equiv), BNAH (116.8 mg, 0.543 mmol, 3 equiv) in a 1:1 mixture of CH_3CN/phosphate buffer (pH 7.4, 5 mL) was stirred under Ar for 3 h at room temperature. Solid $NaHSO_4{\cdot}H_2O$ (1.09 g) was added to quench the reaction, followed by 1,3,5-trimethoxybenzene (6.2 mg, 0.0369 mmol) as an internal standard. The resulting mixture was diluted with Et_2O (10 mL) and the organic layer was separated. The aqueous layer was extracted with EtOAc (3× 5 mL). The combined organic layer was washed with aqueous $NaHSO_4$ (1 M, 8 mL) followed by brine (8 mL) and then dried over $MgSO_4$. After filtration, the solution was concentrated by evaporation under reduced pressure to leave the crude product mixture that was analyzed by ^{1}H NMR spectroscopy.[5] This showed the presence of each of unreacted tetraoxane (15%), 2-adamantanone (89%, based on reacting tetraoxane) and piperidone (82%, based on reacting tetraoxane). The ketones were isolated from product mixtures from several reactions by chromatography with EtOAc/hexane (1:4) to give firstly the standard, then unreacted tetraoxane and adamantanone, MS (ToF) calcd for $C_{10}H_{15}O^+$ 151.1117, found 151.0629. Next EtOAc/hexane (2:1) was used to elute 1-(ethanesulfonyl)piperidin-4-one, MS (ToF) calcd for $C_7H_{14}NO_3SH^+$ 192.0689, found 191.0624. Samples were matched with authentic samples.

*Trioxolane **6***. A mixture of trioxolane (64.8 mg, 0.181 mmol), riboflavin (13.8 mg, 0.0366 mmol, 0.2 equiv), BNAH (77.6 mg, 0.362 mmol, 2 equiv) in a 1:1 mixture of THF/phosphate buffer (pH 7.4, 5 mL) was stirred under Ar for 3 h at room temperature. Solid $NaHSO_4{\cdot}H_2O$ (0.73 g) was added to quench the reaction, followed by

1,3,5-trimethoxybenzene (5.3 mg, 0.0315 mmol), which was added as an internal standard. The resulting mixture was diluted with Et_2O (10 mL) and the organic layer was separated. The aqueous layer was extracted with EtOAc (3×5 mL). The combined organic layer was washed with aqueous $NaHSO_4$ (1 M, 8 mL) followed by brine (8 mL) and then dried over $MgSO_4$. After filtration, the solution was concentrated by evaporation under reduced pressure to leave the crude product mixture that was analyzed by ^{1}H NMR spectroscopy.[5] This showed the presence of unreacted trioxolane (11%), 2-adamantanone (56%), and piperidone (86%). Products from several reactions were isolated by chromatography, and matched with authentic samples of the ketones as described above.

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