Biomimetic Fe(II)-Mediated Degradation of Arteflene (Ro-42-1611). The First EPR Spin-Trapping Evidence for the Previously Postulated Secondary Carbon-Centered Cyclohexyl Radical

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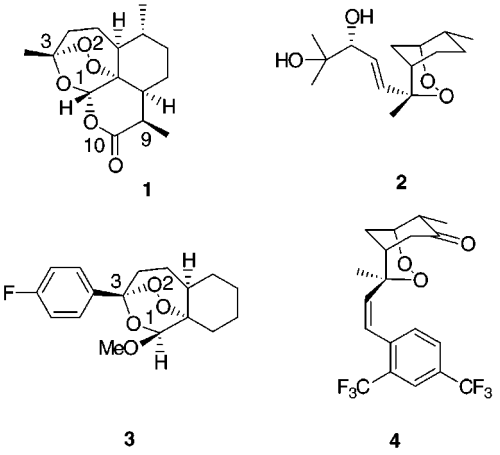
Received October 12, 1999

The discovery that the 1,2,4-trioxane artemisinin (qinghaosu (**1**)) and the endoperoxide yingzhaosu A (**2**) possess potent antimalarial activity against chloroquine-resistant *Plasmodium falciparum* has led to the development of synthetically more accessible compounds of enhanced activity such as the 3-aryl-1,2,4-trioxanes **3**[1] and arteflene (**4**) (Scheme 1).[2] Other simple endoperoxides possess antimalarial activity, at least in vitro, and it has been proposed that the peroxide class of drugs share a common mode of action.[3] Artemisinin has a unique carbon framework different from that of all previous antimalarials. Structure−activity studies have shown that the peroxy group is essential for antimalarial activity, the corresponding deoxy derivative being completely devoid of biological activity. These observations have stimulated studies on the role of the peroxy group in the biological activity of artemisinin and other peroxide-containing antimalarials.

The mode of action of peroxides against *P. falciparum* malaria has been suggested to be at the intraerythrocytic stage of parasite development. At this stage the parasite digests hemoglobin, which on proteolysis results in the generation of ferriprotoporphyrin IX (heme). Since "free heme" is toxic to the parasite, it is normally removed by oxidative polymerization to hemozoin, an insoluble pigment. In studies with artemisinin, Meshnick and co-workers have isolated a "heme−artemisinin adduct", which has led to the proposal that peroxide antimalarials may act by inhibiting this pathway.[4] Alternatively, heme in the presence of thiols has been shown to reductively cleave the peroxide bridge of artemisinin, resulting in the formation of several, potentially toxic, oxygen- and carbon-centered radical intermediates.[5] A number of model studies utilizing iron(II) salts and heme iron(II) have been reported for artemisinin, with several intermediates being proposed as the ultimate cytotoxic species.[6] Using a regiospecifically oxygen labeled antimalarial 1,2,4-trioxane, Posner et al. demonstrated for the first time that the 1,2,4-trioxane−peroxide bond was reduced to oxyl radicals which rearrange to the corresponding C-centered radicals.[7] Further work, with stereochemical probes, indicated that the formation of a secondary C-centered radical was of importance for high antimalarial potency.[8] Subsequently, on the basis of these observations, other workers using Fe(II)-based decompositions have suggested that similar radical pathways operate in Fe(II) isomerization of the parent drug artemisinin.[9,10] Meunier, following work with Mn(II)-based TPP porphyrin systems, has suggested that a primary C-centered radical may also have a role to play in the biological activity of artemisinin, since he was able to demonstrate porphyrin alkylation by this species.[10]

Scheme 1. Chemical Structures of Artemisinin (1), Yingzhaozu A (2), *C*-3-Aryl-1,2,4-trioxane Analogue (3), and Arteflene (Ro-42-1611) (4)



The most recent evidence for the formation of a secondary C-centered radical during Fe(II) cleavage of artemisinin comes from work carried out by Wu and Li.[11] Using ferrous sulfate mediated degradation in the presence of the spin-trapping agent 2-methyl-2-nitrosopropane (MNP), these workers observed an EPR signal indicating the presence of the spin-trapped secondary C-centered radical **6** derived from the C-4-radical species **5** (Scheme 2). This work, taken together with the original work of Posner, underlines the potential importance of secondary carbon radicals, not only as intermediates generated under Fe(II) conditions, but also as potential mediators of antiparasitic activity.

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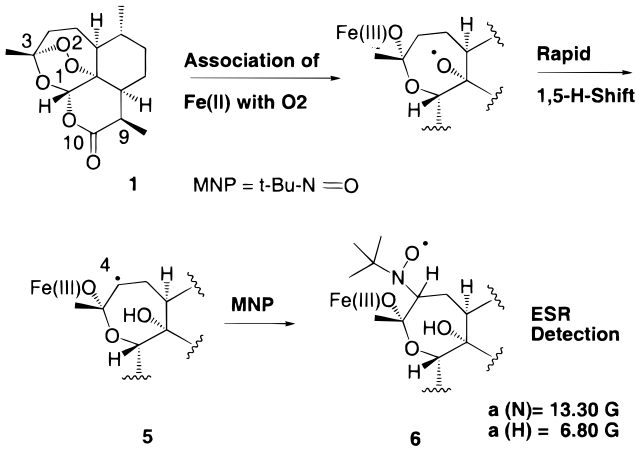
10.1021/jo991585m CCC: $19.00
Published on Web 02/15/2000

Scheme 2. Formation of a Spin-Trapped Secondary C-Centered Radical from Artemisinin



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Since these studies indicated the potential importance of the formation of a C-centered radical for biological activity in the 1,2,4-trioxane class of antimalarials, we recently investigated the Fe(II) reactions of the most successful of the semisynthetic endoperoxide antimalarials, arteflene (**4**). On the basis of the biomimetic Fe(II) reactions of arteflene, we proposed that the mechanism of antimalarial activity of **4** involves the formation of a secondary C-centered radical (**7a**, Scheme 3).[12] In this process, the intermediate oxyl radical **7**, formed by one-electron reduction of the peroxide bridge by association of O2 with Fe(II), fragments to produce an enone **7e** and the secondary C-centered radical **7a**. The other main product obtained following exposure of **4** to Fe(II) was the diol **7c**. This product is obtained by association of Fe(II) with O1 followed by a further one-electron reduction of **7b**.

The main aim of the present work was to provide direct evidence for the existence of radical **7a** during physiologically relevant reductive activation of **4** by ferrous ions and to investigate its ability to alkylate heme. The formation of heme adducts from this drug could in theory provide a potential mechanism of action for **4** since these adducts may not be substrates for the vital heme polymerization process that takes place in the food vacuole of the malaria parasite. This would result in a toxic buildup of iron-containing porphyrin species. Alternatively, the proposed radical intermediate could abstract hydrogens from vital parasitic protein molecules. To simulate this process, we aimed to facilitate H-transfer to the secondary carbon radical from a large excess of 1,4-cyclohexadiene (50:1) during the ferrous-mediated decomposition reaction. In our previous studies, we had proposed a third alternative potential mechanism of action for arteflene, which involves intraparasitic generation of the potentially protein reactive enone species **7e**. In this study, using a double bond reduced analogue, we have set out to determine whether, in fact, enone formation is required for high antimalarial drug potency.

To obtain evidence for the formation of the radical intermediate **7a**, we decided to investigate the electron-transfer reactions with EPR spin-trapping techniques. Since the radical intermediate we had proposed is

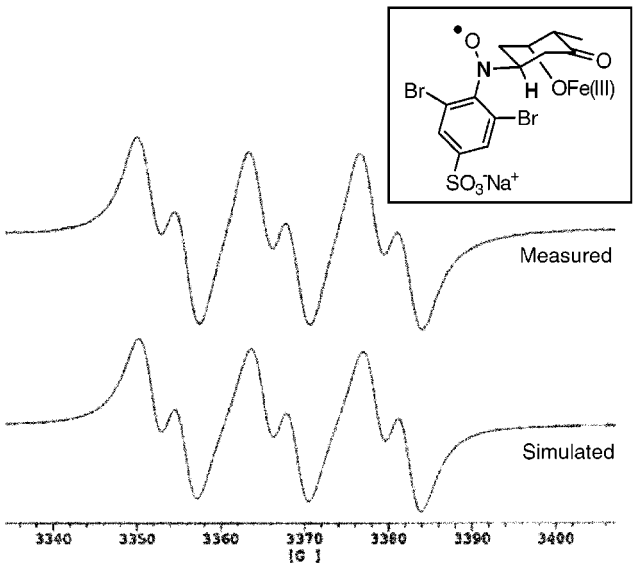

Figure 1. EPR signal for spin-trapped DBNBS radical derived from arteflene during ferrous-mediated reductive cleavage.

secondary in nature, we expected to obtain an EPR spectrum similar to that observed by Wu for the spin-trapped product **6**.[11] Two spin traps were employed in our study, namely, the water-soluble trap DBNBS (sodium 3,5-dibromo-4-nitrosobenzenesulfonate) and DMPO (5,5-dimethyl-1-pyrroline *N*-oxide), a nitrone derivative that forms a comparatively stable C−C bond with carbon radicals (Scheme 4).

Reaction of Fe(II) (as $FeCl_2 \cdot 4H_2O$, 2 mM) with arteflene (2 mM) in the presence of DBNBS in aqueous acetonitrile (1:1) at pH 7 gave a strong EPR signal after 10 min (Figure 1). The signal showed additional splitting besides that caused by the coupling to nitrogen; each line of the triplet was further split into a doublet, indicating that the observed species was $Ar-(R_1R_2CH)N-O^{\bullet}$ **9**. Thus, the radical was a secondary in agreement with the postulated cyclohexyl radical fragment **7a** (Scheme 3).

This result provides us with the first direct evidence for the formation of a potentially cytotoxic secondary C-centered radical following *biomimetic ferrous-mediated reduction of a yingzhaozu A analogue*. The fact that two, structurally unrelated endoperoxides, **1** and **4**, smoothly form the same type of radical is significant. The relevant hyperfine splitting information is shown in Table 1, and for the purpose of comparison, the values obtained for arteflene are compared with those for artemisinin. Attempts to trap the intermediate oxyl radical by increasing the concentration of the spin trap failed. Only a signal obtained from a C-centered radical was obtained. Presumably, the spin-trapped product is formed with the new, bulky substituent in the equatorial position as shown in Figure 1.

Studies using DMPO also led to a spectrum consistent with the formation of spin-trapped product **10** (Figure 2). Again, as in the previous example, increasing the concentration of DMPO gave only signals for a carbon radical with hyperfine coupling constants shown in Table 1.

The spectrum obtained using DMPO was similar to that obtained by Butler et al. for **1**.[13] On the basis of the

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Scheme 3. Ferrous-Mediated Degradation of Arteflene

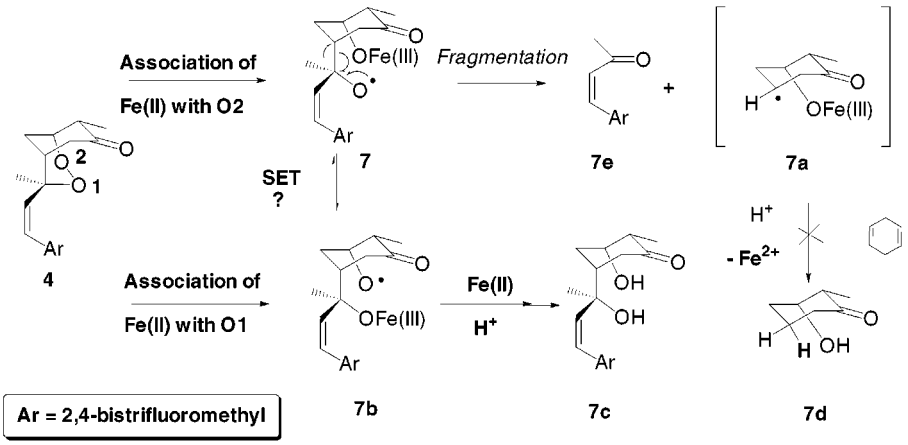

Table 1. EPR Parameters of the Radicals Formed on Reaction of Arteflene with Fe(II) in the Presence of DBNBS and DMPO[a]

endoperoxide	radical	spin trap	*a*(N)/G	*a*(H)/G	line width/G
arteflene (**4**)	**7a**	DBNBS	13.25	3.90	3.00
arteflene (**4**)	**7a**	DMPO	15.5	22.0	1.70
artemisinin[11]	**5**	MNP	14.81	3.01	
artemisinin[13]	**5**	DBNBS	13.30	6.80	
artemisinin[13]	**5**	DMPO	15.70	22.5	
dihydroarteflene (**11**)	**7a**	DBNBS	13.35	3.99	3.20

[a] The values obtained here are compared with values obtained previously for artemisinin.

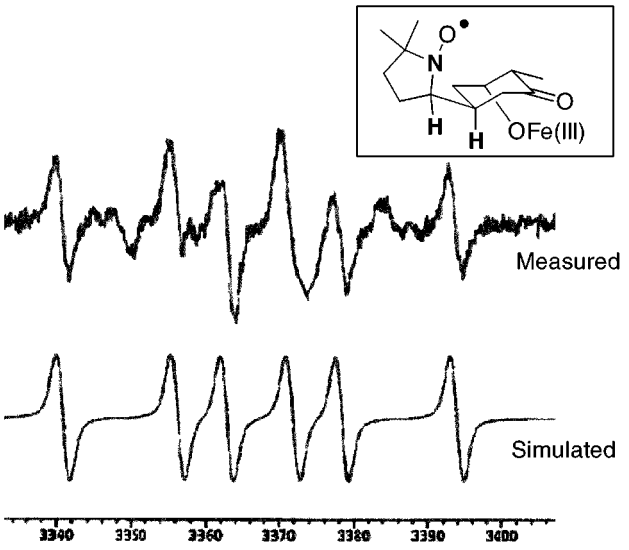

Figure 2. EPR signal for spin-trapped DMPO radical derived from arteflene during ferrous-mediated reductive cleavage.

assumption that an alkoxy radical reacts with DMPO with the same rate constant as tBuO• under these conditions (ca. 1×10^6 dm^3 mol^{-1} s^{-1}), the lack of oxygen-conjugated adducts with DMPO indicates the rate constants for peroxide bond cleavage and fragmentation to **7a** are greater than 1×10^7 dm^3 mol^{-1} s^{-1}.[14] The rates involved in these radical processes are therefore similar to those for **1**.

Not only do the attempts to trap the oxyl radical **7** indicate that the resultant cleavage to the enone and C-radical are rapid processes, but the fact that oxyl radical **7b** (formed by association with O1) is not trapped indicates one of two possible fates for this radical

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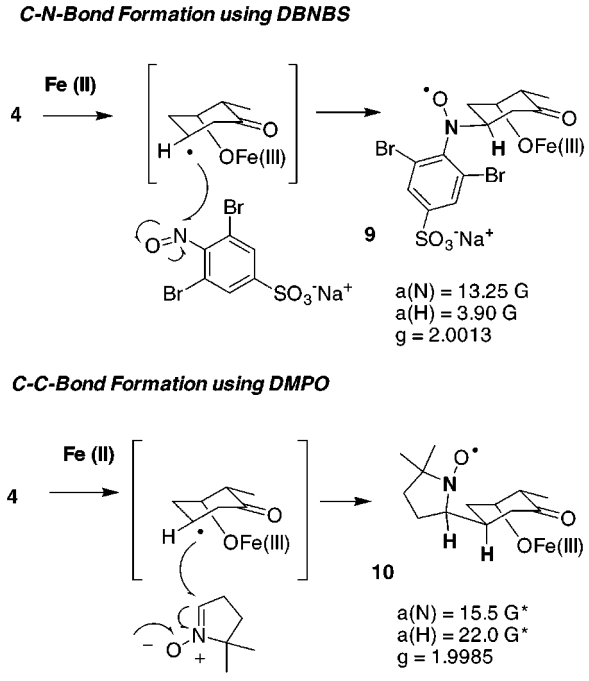
Scheme 4. Spin-Trapping of a Secondary C-Centered Radical Obtained from Arteflene Following Ferrous-Mediated Activation



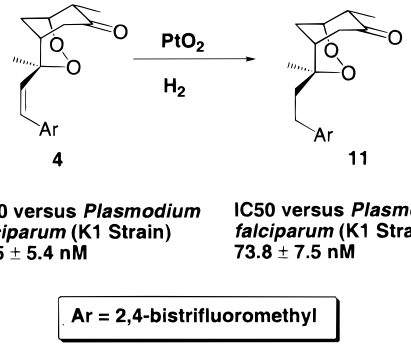
intermediate. Radical **7b** can undergo a further one-electron reduction to give the diol **7c**, a product identified and fully characterized in our previous study. Alternatively, this oxyl radical may be in equilibrium with oxyl radical **7** by single-electron transfer (SET), a situation recently proposed by Wu for the ferrous ion induced cleavage of **1**. In contrast to the proposal of Wu et al.,[11] we do not believe that the initial reductive cleavage of the peroxide bridge is a reversible process, since all starting material is consumed in these reactions.

Recently, Wu and co-workers have investigated near physiological conditions for the Fe(II) catalytic mediated degradation of **1**. From this work, a plausible mechanism for the known ferrous-mediated artemisinin parasite protein alkylation was proposed since these workers were able to show for the first time that artemisinin (0.05 M) is degraded by 10^{-3} equiv of ferrous iron in the presence of cysteine.[14] Furthermore, a cysteine adduct was isolated which accounted for up to half the starting material. Using similar methodology, the activation of arteflene and spin-trapping of the resultant radical were investigated using arteflene (2 mM), cysteine (4 mM), and $FeSO_4$ (0.002 mM). Arteflene was completely consumed in this process in under 1 h. In addition, a spin-trapped adduct was detected by EPR after 30 min with the same characteristics as that obtained using 1 equiv of ferrous ion as reducing agent. Thus, significantly, catalytic quantities of Fe(II) can mediate free radical formation from **4**.

Having obtained evidence for the existence of the secondary radical, we then investigated the potential of this radical to alkylate heme. A range of different conditions were investigated, but all attempts to produce a heme-alkylated porphyrin adduct failed. Thus, it would appear that the mechanism of action of arteflene may not involve alkylation of heme as has been postulated previously for artemisinin. Attempts to facilitate intermolecular hydrogen abstraction by the addition of a large excess of 1,4-cyclohexadiene (50 equiv) during ferrous-mediated activation also failed to produce the expected reduction product **7d** (Scheme 3). Analysis of reaction mixtures involved careful TLC and LC−MS. Although no products of intermolecular abstraction were obtained from **7a** with 1,4-cyclohexadiene, we cannot, at the present time, rule out that this process may be pharmacologically relevant, and more work is required to determine the importance of the intermolecular hydrogen abstraction reactions of endoperoxide-derived carbon radicals both in vitro and in vivo.

We propose that the antimalarial activity of arteflene is a result of heme iron or ferrous Fe(II) ion mediated one-electron reduction of the peroxide bond and subsequent formation of an α,β-unsaturated ketone (enone) and C-centered radical **7a**. This radical presumably kills the parasite either by alkylation of vital cellular macromolecules, other than heme, or possibly by hydrogen abstraction from proteins. In addition, it is feasible that the enone (Scheme 2) may exert toxicity by alkylation of protein thiol groups. In studies conducted with radiolabeled arteflene it was noted by Meshnick that *several specific malarial proteins were alkylated by arteflene*.[15] Since the radiolabel was in the vinyl function of arteflene, it seemed reasonable that the protein−arteflene adducts described in this work are in fact derived from reaction of the enone with protein thiol groups. To determine whether the formation of the enone is a prerequisite for high antiparasitic activity, the double bond of arteflene was reduced carefully with PtO_2 to give dihydroarteflene (**11**) (Scheme 5). The effect of this chemical modification on (i) the ability to form the secondary C-radical **7a** and (ii) on antiparasitic activity was determined. Using the same conditions as described earlier, the reaction of **11** with $FeCl_2.4H_2O$/DBNBS gave a strong EPR signal with

Scheme 5. Synthesis and Antimalarial Potency of Dihydroarteflene



the similar characteristic hyperfine coupling constants obtained for arteflene (Table 1). Thus, **11** can still form the cytotoxic cyclohexyl radical, presumably in this case by ferrous-mediated fragmentation to a saturated ketone. Although there was a decrease in the measured biological activity of dihydroarteflene (**11**), Scheme 5, this compound still has antimalarial activity in the nanomolar range. Therefore, enone formation intracellularly within the parasite food vacuole is not a prerequsite for high antimalarial activity.

During the preparation of this paper Meunier reported details of a Mn(II) TPP reductive activation of arteflene. These studies back up our observations that the cyclohexyl radical **7a** does not form a covalent adduct with a porphyrin macrocyle and also, in part, confirm our original proposal for the formation of **7a**.[16] However, it is not clear from their "model studies", whether the reactivity of a *manganese-containing TPP analogue in the presence of* $NaBH_4$ is relevant to the true picture inside the "iron-rich" malarial parasite food vacuole. We consider that our model studies, which also include the use of low concentrations of free ferrous Fe(II) ions in the presence of cysteine, are significantly closer to the actual conditions inside the parasite food vacuole.

In summary, we have now provided the first direct evidence for the formation of our previously postulated secondary C-centered radical intermediate **7a**, obtained during physiologically relevant ferrous-mediated activation, and have compared the results obtained with those of the prototype antimalarial peroxide artemisinin. On the basis of this mechanism of antimalarial action, it may now be possible to structurally modify arteflene and its analogues to produce compounds with even greater antimalarial activity. Results of the in vitro studies, EPR and biomimetic Fe(II) degradation studies, on 25 analogues of **4** will be reported shortly.

Experimental Procedures

General Methods. Arteflene was a gift from F. Hoffmann la Roche, Basel, Switzerland. The spin traps DBNBS and DMPO were obtained from Sigma-Aldrich.

$FeCl_2$·4H_2O-Mediated Degradation of Arteflene. Freshly purchased $FeCl_2$·4H_2O (77 mg, 0.39 mmol) was added to arteflene (163 mg, 0.40 mmol) in aqueous acetonitrile (10 mL, 1:1). Light was excluded from the reaction. The mixture was stirred for 1 h at room temperature and then filtered through Celite. The latter was washed with ethyl acetate (4 × 5 mL), and the organic extracts were dried with $MgSO_4$. The solvent was

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removed on a rotary evaporator and the resultant oil subjected to flash column chromatography on silica gel using hexane/ethyl acetate (4:1) as the eluent to give the enone 4-(2,4-bis(trifluoromethyl)phenyl)-*cis*-but-3-en-2-one (**7e**) (45 mg, 40% yield) and the diol **7c** (82 mg, 50% yield). Data for 4-(2,4-bis(trifluoromethyl)phenyl)-*cis*-but-3-en-2-one: oil; R_f = 0.80 (25% EtOAc/hexane, silica); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.11 (3H, s, $COCH_3$), 6.45 (1H, d, J = 12.36 Hz, vinyl-H), 7.11 (1H, d, J = 12.36 Hz, vinyl-H), 7.51 (1H, d, J = 8.10 Hz, Ar-H), 7.77 (1H, d, J = 8.10 Hz, Ar-H), 7.93 (1H, s, Ar-H); ^{13}C NMR δ 191.0, 139.2, 135.7, 131.8, 131.5, 130.0, 128.5, 128.3, 125.1, 123.1, 122.0, 30.9; EIMS 283 (M + 1, 100), 267 (47), 263 (M + 1 − HF), 80), 219 (M + 1 − C_2H_4O, 52); CIMS 565 (2M + 1, 56), 283 (M + 1, 100), 263 (M + 1 − HF, 80). Anal. Calcd for $C_{12}H_8F_6O$: C, 51.08; H, 2.86. Found: C, 51.40; H, 2.98. Data for diol **5**: oil; R_f = 0.20 (25% EtOAc/hexane, silica); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.15 (3H, d, J = 7.15 Hz, CH_3−CH), 1.47 (3H, s, CH_3), 1.68−1.80 (1H, m, CH), 2.10−2.41 (3H, m), 2.45−2.70 (2H, m, CH_2), 4.21 (1H, d, J = 4.40 Hz, *CH*OH), 6.23 (1H, d, J = 15.40 Hz, vinyl-H), 7.03 (1H, d, J = 15.40 Hz, vinyl-H), 7.88 (1H, s, Ar-H), 7.60−7.80 (2H, m, Ar-H), EIMS 392 (M^+ − 18, 100), 375 (49), 333 (36), 283 (62), 263 (69), 219 (33), 153 (34), 110 (82); CIMS 411 (M^+ + 1, 4), 393 (M^+ − 18, 100), 375 (10). Anal. Calcd for $C_{19}H_{20}F_6O_3$: C, 55.61; H, 4.91. Found: C, 55.50; H, 4.85.

Dihydroarteflene. Arteflene (100 mg, 0.24 mmol) was dissolved in ethyl acetate (5 mL) in a two-neck round-bottom flask. Platinum(IV) oxide (catalytic) was added in ethyl acetate (5 mL). A balloon containing hydrogen was connected to the reaction vessel, which was then sealed with a rubber septum. The reaction was allowed to stir for 15 min, after which standard workup gave an oil which was purified by silica gel chromatography to give the product as a foam (18 mg, 18%). This product was accompanied by the diol **7c** and tetrareduced (double bond and peroxide) byproducts. Data for dihydroarteflene: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.22 (3H, d, J = 6.87 Hz, CH_3−CH), 1.60 (3H, s, CH3), 1.45−1.70 (2H, m, CH), 1.95 (1H, m), 2.10 (1H, m), 2.40−2.50 (2H, m) 2.60−2.95 (2H, m), 4.41 (1H, s, *CH*O), 7.42 (1H, m, Ar−H), 7.70 (1H, m, Ar-H), 7.90 (1H, s, Ar-H); LC−MS (MeOH (40−70%), ammonium acetate (0.1 M), cone voltage 30 V), 428 (M + NH_4^+, 100), 411 (M + H^+, 28). Anal. Calcd for $C_{19}H_{20}F_6O_3$: C, 55.61; H, 4.91. Found: C, 55.58; H, 4.87.

EPR Spectroscopy. All spectra were recorded at ambient temperatures. Typical spectrometer conditions were center field 3368 G; field sweep 70 G; field modulation amplitude 1 G; time constant 320 ms; microwave power 10 mW; microwave frequency 9.45 GHz. Experiments involved the mixing of solutions of arteflene or dihydroarteflene (typically 2 mM, concentrations throughout being after mixing), the spin trap (typically 40 mM for DBNBS and 200 mM for DMPO), and finally the metal ion (typically 2 mM) in unbuffered deoxygenated aqueous acetonitrile (1:1) at pH 7. In a separate experiment with 2 mM arteflene, the amount of ferrous ion was reduced to 0.002 mM, and 2 equiv (4 mM) of cysteine was added to the reaction. Following filtration of the excess cysteine, the same spectrum was obtained as in the previous example.

Antimalarial Activity. Parasites were maintained in continuous culture using the method of Trager and Jensen.[17] Cultures were grown in culture flasks containing human erythrocytes (2−5%) with parasitemia in the range of 1−10% suspended in RPMI 1640 medium supplemented with 25 mM HEPES, 32 mM $NaHCO_3$, and 10% human serum (complete medium). Cultures were gassed with a mixture of 3% O_2, 4% CO_2, and 93% N_2. Antimalarial activity was assessed using an adaptation of the 48 h sensitivity assay of Desjardins et al.[18] using [^{3}H]hypoxanthine incorporation as an assessment of parasite growth. Stock drug solutions were prepared in 100% dimethyl sulfoxide (DMSO) and diluted to the appropriate concentration using the complete medium. Assays were performed in sterile 96-well microtiter plates, and each plate contained 200 μL of parasite culture (2% parasitemia, 0.5% haematocrit) with or without 10 μL drug dilutions. Each drug was tested in triplicate and parasite growth compared to that of control wells (which consituted 100% parasite growth). After 24 h of incubation at 37 °C, 0.5 μCi [^{3}H] hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto filter mats, dried for 1 h at 55 °C, and counted using a Wallac 1450 Microbeta Trilux liquid scintillation and luminescence counter. IC_{50} values were calculated by interpolation of the probit transformation of the log dose−response curve.

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