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EPR Evidence for the Involvement of Free Radicals in the Iron-Catalysed Decomposition of Qinghaosu (Artemisinin) and Some Derivatives; Antimalarial Action of Some Polycyclic Endoperoxides

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EPR experiments confirm that reaction of qinghaosu and some related endoperoxides with Fe^{2+} in aqueous acetonitrile leads to the production of carbon-centred radicals derived by rapid rearrangement of first-formed cyclic alkoxyl radicals. Signals obtained from qinghaosu itself with spin-traps DMPO and DBNBS are assigned to the adducts **(15)** and **(16)**, a finding which accounts for the formation of the major products **(11)** and **(14)**.

Keywords: Alkoxyl antimalarial, artemisinin, electron paramagnetic resonance, peroxides, peroxygen, qinghaosu, radical-rearrangement, radical-fragmentation, spin-trapping

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DBNBS, sodium 3,5-dibromo-4-nitrosobenzene-sulphonate; EPR, electron paramagnetic resonance

INTRODUCTION

Qinghaosu (artemisinin, **1**), isolated from the chinese herb qinghau (*Artemisia annua L.*), has been shown to have high antimalarial activity, a property shared by several related endoperoxides including deoxoqinghaosu **(2)**, dihydroqinghaosu **(3)**, arte-mether **(4a)** and arte-ether **(4b)**.[1–3] It has been suggested that the toxicity towards Plasmodium is brought about by electron transfer to the peroxide bridge from Fe(II), present at high concentration in red blood cells infected by the parasite. For example, Jefford and co-workers[4] conclude that Fe(II)-haem gives a radical-anion which decomposes to a toxic ferryl iron–oxene intermediate, and Posner and co-workers[5,6] propose the involvement of high-valent Fe=O species in the mechanism of action

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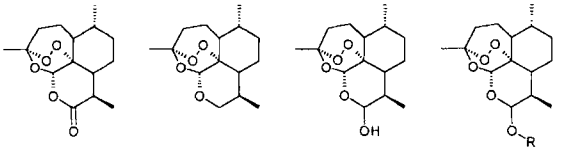
of some related antimalarial trioxane analogues; in contrast, Wu *et al.* propose[7] electron-transfer from Fe(II) to give free radicals (via a Fenton-like reaction) which may be responsible for the DNA damage detected in model systems. Antioxidants such as α-tocopherol, dithiothreitol[8] and glutathione[9] block antimalarial activity, as does catalase;[8] there is no reported activity in related compounds lacking the peroxide bridge.



STRUCTURES (1)–(4)

In order to establish the mechanism of reaction of qinghaosu with Fe(II) in aqueous solution, we have studied the potential electron-transfer reactions with EPR spin-trapping techniques.

MATERIALS AND METHODS

(a) Materials

All chemicals used were purchased from either Aldrich or Sigma and were used as supplied except for the following: DMPO was purified by treatment of an aqueous solution with charcoal and filtered prior to use; qinghaosu was a gift from Professor Yulin Wu of the Shanghai Institute of Organic Chemistry; DBNBS was prepared using the standard method of oxidation of sodium 3,5-dibromo-4-amino-benzenesulphonate with hydrogen peroxide in the presence of a sodium tungstate catalyst.[10]

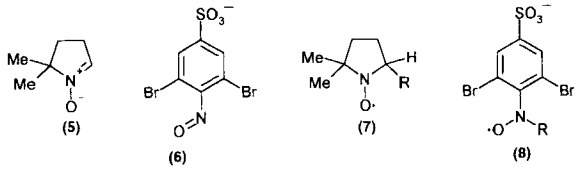
(b) ESR Spectroscopy

ESR spectra were recorded on Bruker ESP 300 X-band or JEOL JES-RE1-X spectrometers equipped with X-band microwave bridges and 100 kHz modulation. The hyperfine splittings and g-values were determined directly from the spectrometer field scan. All spectra were recorded at ambient temperature using a quartz aqueous-sample cell. Typical spectrometer conditions were: centre field 336.5 mT; field sweep 10 mT; field modulation amplitude 0.1 mT; time constant 320 ms; scan time 300 s; microwave power 10 mW; microwave frequency 9.45 GHz.

Experiments involved the mixing of solutions of qinghaosu or related compounds (typically 2 mM, concentrations throughout being after mixing), the spin-trap (typically, 40 mM for DBNBS, 200 mM for DMPO) and, finally, the metal ion (typically 2 mM with 1.1 equivalents of EDTA as chelating agent in some cases) in unbuffered deoxygenated aqueous acetonitrile (1 : 1) at pH *ca.* 7. Control experiments in which *either* the metal ion *or* the endo peroxide were omitted gave no detectable signals.

RESULTS

Reagents were mixed in the presence of one or other of the spin-traps DMPO **(5)** and DBNBS **(6)**, which react readily with short-lived radicals to produce longer-lived nitroxides (see e.g. Ref. [10]). For the former, the size of the resultant β-proton splitting in **(7)** distinguishes heteroatom (e.g. oxygen) and carbon adducts (R); for **(6)**, the addition of alkyl radicals to nitrogen, to give **(8)**, gives information about the radical precursor (from the β-H splitting pattern).



STRUCTURES (5)–(8)

(a) Reaction of Qinghaosu with Fe(II)

Reaction of Fe(II) (as ferrous sulfate, 2 mM) with qinghaosu (2 mM) in the presence of DBNBS

(40 mM) (all concentrations after mixing) in unbuffered deoxygenated aqueous acetonitrile (1 : 1) at pH *ca.* 7, gave a weak EPR signal which grew with time (over a period of several hours). The spectrum (Figure 1) is from a mixture of two nitroxides, with characteristic nitrogen hyperfine splittings a(N); the minor signal has a further triplet splitting (1 : 2 : 1) from two β-protons (characteristic of the trapping of a primary radical $\bullet CH_2$–R) and the dominant signal has a doublet splitting (characteristic of the trapping of a secondary carbon-centred radical) (for parameters, see Table I). The ratio of the (doubly integrated) intensities of these radicals was typically 1 : 4. After a considerable period of time (*ca.* 24 h) the primary radical adduct's signal had decayed. The signals were generally weaker when the concentrations of Fe(II), **(1)** or spin-trap were reduced; increase of concentrations led to little or no improvement in the signal-to-noise ratio and the relative ratios of adducts.

Very similar results were obtained in experiments in which EDTA was added (2.2 mM) though particular care had to be taken to deoxygenate solutions prior to mixing (to minimise the rapid reaction of Fe(II)–EDTA with oxygen). No signals were observed in the reaction between Fe(II) [or Fe(II)–EDTA] and $^{t}BuOO^{t}Bu$ under similar conditions.

These results suggest that electron-transfer from Fe(II) to the peroxide bond in **(1)** brings about fission to give an alkoxyl radical or radicals [see reaction (1)] whose further rapid reactions lead to carbon-centred radicals.

$$Fe(II) + RO{-}OR \longrightarrow Fe(III) + RO^{\bullet} + {}^{-}OR \quad (1)$$

In an attempt to trap the intermediate oxygen-centred radicals, solutions of Fe(II) and **(1)** (both at concentrations of 2 mM) were mixed in the presence of DMPO (in the concentration range 10–100 mM). Only signals from *carbon*-centred radicals were obtained (see Figure 2 and Table II).

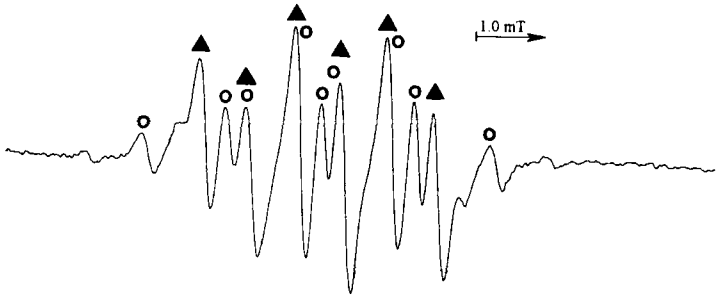


FIGURE 1 EPR spectrum of a reaction mixture containing qinghaosu (10 mM), Fe(II) (4 mM) and DBNBS (5 mM) in aqueous-acetonitrile (1 : 1); the spectrum was recorded $1\frac{1}{2}$ h after mixing. Signals are assigned to the primary carbon-centred adduct (**o**, **15**) and the secondary carbon-centred adduct (▲, **16**), see text.

TABLE I ESR parameters of the radicals formed on reaction of qinghaosu and derivatives with Fe(II), Ti(III) or Cu(I) in the presence of DBNBS in aqueous acetonitrile (1 : 1)

Endoperoxide	Radical adduct	a(N)/mT[a]	a(H)/mT[a]
Qinghaosu **(1)**	Primary carbon-centred **(15)**	1.36	1.18 (2H)
	Secondary carbon-centred **(16)**	1.33	0.68 (1H)
Arte-ether **(4a)**	Primary carbon-centred	1.36	1.20 (2H)
	Secondary carbon-centred	1.34	0.78 (1H)
Arte-mether **(4b)**	Primary carbon-centred	1.36	1.22 (2H)
	Secondary carbon-centred	1.35	0.75 (1H)

[a] ± 0.01 mT.

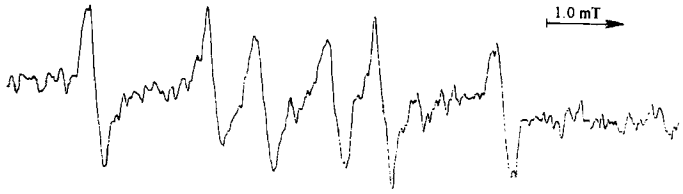


FIGURE 2 EPR spectrum of a reaction mixture containing qinghaosu (10 mM), Fe(II), (5 mM) and DMPO (100 mM) in aqueous-acetonitrile (1 : 1); the spectrum was recorded $1\frac{1}{2}$ h after mixing. Signals are assigned to a carbon-centred adduct, see text.

TABLE II ESR parameters of the radicals formed on reaction of qinghaosu and derivatives with Fe(II), Ti(III) or Cu(I) in the presence of DMPO in aqueous acetonitrile (1 : 1)

Endoperoxide	Radical adduct	a(N)/mT[a]	a(H)/mT[a]
Qinghaosu **(1)**	Carbon-centred	1.57	22.5
Arte-ether **(4a)**	Carbon-centred	1.61	22.5

[a] ± 0.01 mT.

This suggests that conversion of intermediate oxygen-centred radicals to carbon-centred radicals (fragmentation or rearrangement) must proceed extremely rapidly, with a rate constant greater than *ca.* $1 \times 10^7\ s^{-1}$, given that the rate constant for reaction of a typical alkoxyl radical with DMPO is $1 \times 10^6\ dm^3\ mol^{-1}\ s^{-1}$.[11]

In a separate experiment, we attempted to trap any intermediate oxygen-centred radicals via reaction with an excess of a potential hydrogen atom donor (ethanol, at concentrations up to 1 M) in the presence of DBNBS and DMPO: signals were unaffected, again lending support to the suggestion that *intramolecular* alkoxyl-radical reaction is extremely rapid.

(b) Reaction of Qinghaosu Derivatives with Fe(II)

Essentially similar results were obtained in experiments with the derivatives **(4a)**, the so called arte-ether, and **(4b)**, arte-mether (both of which have been found to be more active as antimalarial agents *in vivo*) with Fe(II) and Fe(II)–EDTA, both in terms of the hyperfine splittings from nitroxides formed as adducts and the ratio of radicals detected (the primary to secondary radical adduct ratio was again *ca.* 1 : 4). There was a significant enhancement in signal intensity for the arte-ether **(4a)** (by a factor of *ca.* 4); signals from the arte-mether **(4b)** were marginally weaker than the corresponding signals from qinghaosu.

(c) Reaction of Qinghaosu and its derivatives with Other Potential One-Electron Reductants

To gain further insight into the possibility that qinghaosu **(1)** and its derivatives react with Fe(II) by one-electron transfer, experiments were carried out with aqueous acetonitrile solutions of Ti(III) (2 mM; pH 3–6) in the presence and absence of EDTA. We have previously shown via EPR experiments that both Fe(II)– and Ti(III)–H_2O_2 reactions in water proceed to give •OH.[12] Reaction of **(1)** and Ti(III) in the presence of DBNBS and DMPO gave spectra closely similar to those obtained with Fe(II), with identical hyperfine splittings and ratios of adducts. Similar behaviour was noted in experiments with Cu(I) [2 mM], generated *in situ* by addition of ascorbate [1 mM] to Cu(II) in the presence of **(1)**, **(4a)** or **(4b)**.[13] No signals were obtained in the absence of Cu(II), which indicates that one-electron reduction of **(1)** and its derivatives by ascorbate is not rapid, at least as judged by EPR spin-trapping experiments. Weak signals were also obtained on reaction of qinghaosu with the Fe^{III}–porphyrin hemin which indicates that a related electron-transfer reaction (presumably to give Fe(IV)) can also occur.[14]

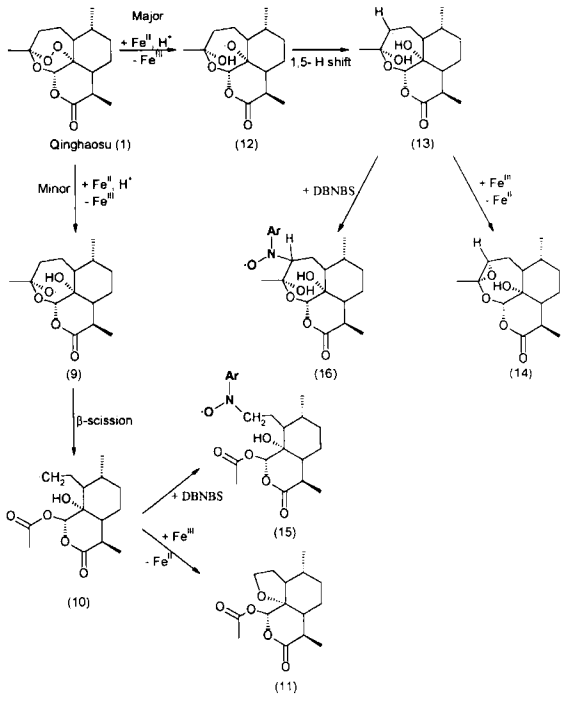
DISCUSSION

Our results establish the apparent efficiency of free-radical formation in the reaction between Fe(II) and other one-electron reductants with qinghaosu. Given the general lack of reactivity of Fe(II) towards dialkyl peroxides in aqueous solution (in contrast to H_2O_2 and ROOH) this in itself is notable. The structure has a relatively unhindered peroxide bond, with an O–O bond length (0.147 nm)[15] close to that of $^tBuOO^tBu$ (0.148 nm)[16] and H_2O_2 (0.146 nm).[17] The rapid reaction *may* reflect the importance of a greater release of strain in the endoperoxide bond, although it should be noted that qinghaosu itself is relatively resistant to homolytic fission (it may be heated to 150°C without significant decomposition).[18]

If we assume that electron transfer occurs to give a short-lived radical-anion, which readily fragments to give alkoxyl radical and alkoxide intermediates, then two subsequent pathways of cleavage are suggested by our detection of primary and secondary alkyl radicals. These can be closely linked to the mechanism proposed by Wu[7] to account for the major products found in the reaction of Fe(II) with qinghaosu (Scheme 1).

The primary radical trapped is believed to involve formation of the alkoxyl radical **(9)** which would be expected to undergo rapid fragmentation to give **(10)**, rather than the methyl radical. Subsequent oxidation of **(10)** by Fe(III), and trapping of the incipient carbenium ion by the nucleophilic hydroxyl groups would lead to the minor product **(11)**.

The alternative, and evidently preferred, mode of reaction in qinghaosu itself leads to the species **(12)** with hemiacetal and alkoxyl radical function, which is ideally placed to abstract a hydrogen via a cyclic transition state (1,5-shift). This radical **(13)** is believed to be responsible for the strong signals of a secondary alkyl adduct **(16)**. Oxidation of this radical by Fe(III) would be expected to lead to the epoxide **(14)** whose



SCHEME 1

derivatives account for the major products (see Ref. [7]).

On the assumption that the alkoxyl radicals generated react with DMPO with the same rate constant as $^tBuO^\bullet$ under these conditions (*ca.* $1 \times 10^6\ dm^3\ mol^{-1}\ s^{-1}$),[10] we estimate that, from the lack of observation of oxygen-conjugated adducts with DMPO (even at [trap] 0.1 mol dm^{-3}) the rate constants for fragmentation of **(9)** and the 1,5-hydrogen shift by **(12)** are greater than $1 \times 10^7\ dm^3\ mol^{-1}\ s^{-1}$. This is not unexpected on the basis of reported rate constants of related processes.[19,20] The radical oxidation steps noted above, which serve to recycle the iron and render the system catalytic, must also be rapid.[11]

The ready generation of carbon-centred radicals from qinghaosu by interaction with Fe(II) provides strong support to Wu's[7] proposed mechanism for the involvement of oxygen- and carbon-centred free radicals in the Fe(II) catalysed decomposition of qinghaosu.

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