Interaction of Qinghaosu (Artemisinin) with Cysteine Sulfhydryl Mediated by Traces of Non-Heme Iron**

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Although it was once considered to be nearly eradicated in the 1950s and 1960s, malaria, an infectious disease known since ancient times, still poses a serious threat to the health of mankind. It claims 1–3 million lives[1] annually owing to the emergence of multidrug-resistant strains. Urged by this situation, the scientific community has been making great efforts to develop novel antimalarial compounds since the 1960s. Qinghaosu (QHS, **1**, also called artemisinin; see Scheme 1), a natural 1,2,4-trioxane of herbal origin discovered by Chinese scientists in the 1970s, appears to be one of the most promising leads to such agents. While it is still unclear how QHS kills malaria parasites, the active species resulting from cleavage of the peroxy bond in QHS are now generally believed[2–5] to be responsible for the parasiticidal activity, and the intraerythrocytic heme iron is regarded as the trigger for the in vivo cleavage reactions by nearly all workers in this field. However, the commonly accepted opinions on an issue that is not fully understood are not necessarily objective descriptions of the genuine situation. Here we report that traces of non-heme iron in the presence of cysteine can also cleave QHS efficiently, and the transient carbon-centered radical formed can covalently bond to the ligand at iron through a sulfur atom. The implications of these new findings may substantially change our current understanding and thus greatly facilitate identification of the vital intracellular target molecules of QHS.

The mode of action is a long-pending problem in the chemotherapy of malaria with QHS-type agents. The current working theory[2c, 4f] is that the 1,2,4-trioxane, on contact with heme iron, is cleaved at the peroxy bond into a radical anion, which evolves further to give carbon-centered radicals, high valent iron, and other reactive intermediates; all these may then interact with so far unidentified vital intracellular target molecules through undefined mechanisms and finally lead to the death of the parasite. Following the pioneering work of Meshnick et al.[2a] and Posner and Oh,[4a] the cleavage process has been intensively investigated[3–5] in various model systems with near stoichiometric amounts of free Fe^{II} ion. Substantially reduced amounts of non-heme iron, such as in $FeSO_4$, fail to drive the reaction to completion. Along with the fact that heme is accumulated in the food vacuole[6] and the extraordinarily low toxicity of QHS with respect to uninfected cells (where there is practically no free heme), this leads to the presently well accepted notion[2c, 4f] that the in vivo cleavage of QHS (or 1,2,4-trioxanes in general) is exclusively induced by the heme iron. As a consequence, essentially all the subsequent biological as well as mechanistic studies aimed at identifying the target molecules of the 1,2,4-trioxanes are confined to those events either involving the heme (or its oxidized and polymerized form, hemozoin) directly[7] or closely associated[2c, 8] with the heme iron. However, further development of the theory has been rather difficult, because there seem to be no logical links between the parasite's death and the damage occurring to, for example, the free heme or hemozoin (none of which is essential for the parasite's life).

Now we have found that heme is not the only iron-containing species that can trigger the degradation of QHS in vivo. Although they play only a negligible role under near physiological conditions, free Fe^{II} ions may form one or more complexes with, for example, amino acids and thus be greatly activated. In the presence of cysteine, for instance, QHS can be degraded very rapidly. In aqueous acetonitrile at 38 °C, QHS is fully consumed instantly if one equivalent each (with respect to QHS) of $FeSO_4$ and cysteine is present. Reducing the amount of added iron slows down the reaction accordingly. However, even with as little as 10^{-4} equivalents of the added iron, the cleavage can be practically finished in about one day. In a typical run at 38 °C under N_2 in deaerated aqueous acetonitrile (1/1) containing two equivalents of cysteine (0.1 M), QHS (0.05 M) is completely degraded by 10^{-3} equivalents of $FeSO_4$ (5×10^{-5} M) in 3–4 h, giving a product mixture (recovered from the organic phase after workup) of compounds **2**, **3**, **4**, and **5** (Scheme 1) in an approximate molar ratio of 29:9:1:6, as shown by the ^1H NMR spectrum (300 MHz).

Apart from the tremendous rate acceleration, a striking consequence of the presence of cysteine is that a previously unknown aldehyde **5**[9] is formed in a significant quantity (Scheme 1; see also reference [3b]). It appears that this new product can be formed only from radical **7** by abstraction of a hydrogen atom from, for example, the SH group in cysteine followed by elimination of an acetate as shown in Scheme 2. The formation of **5** provides unequivocal evidence for the involvement of the highly reactive primary radical **7**, which was not trapped in our earlier work,[3b] in the degradation process. (However, Butler et al.[10] have reported concurrent trapping of both **6** and **7** using different trapping agents.)

The concentration of free amino acids (probably due to the catabolism of hemoglobin) is greater in infected than in uninfected erythrocytes,[11] and malaria parasite has a high concentration of the reduced glutathione (the main sulfurhydryl-containing reducing agent in physiological systems).[12] Therefore, the non-heme iron mediated degradation mechanism demonstrated in our model system may well be in operation in physiological systems too. It should be noted that this is the first piece of established evidence for intermolecular interactions—abstraction of a hydrogen atom from another molecule, a process that may cause damage to the

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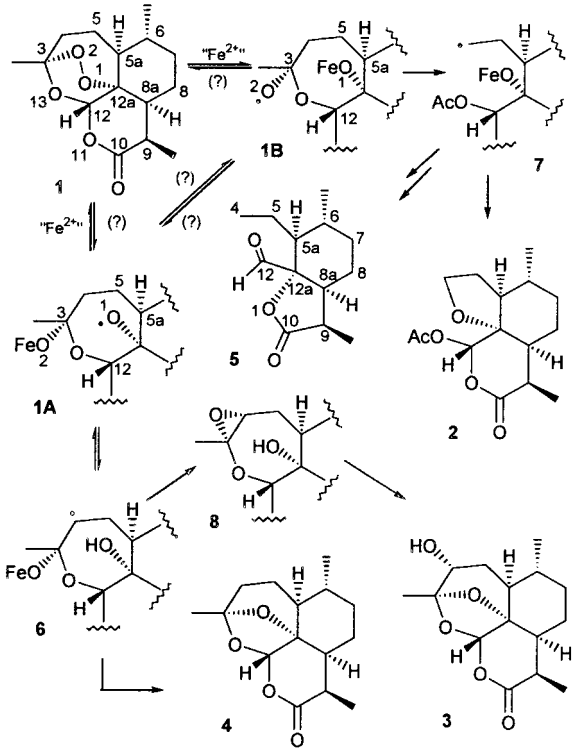
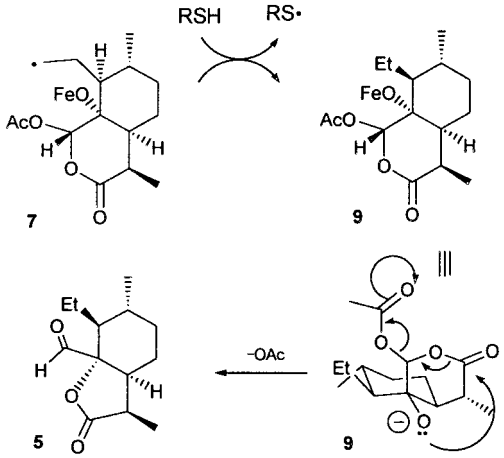


Scheme 1.

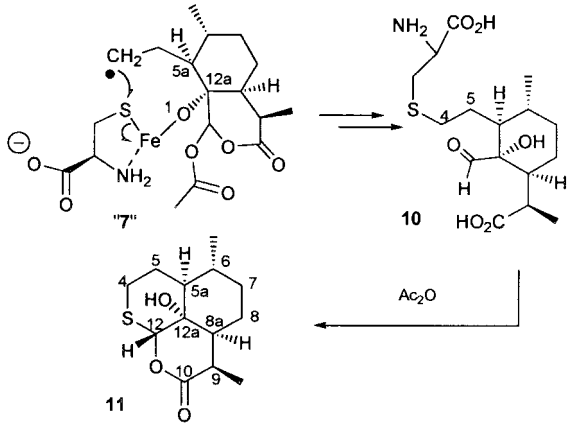


Scheme 2. RSH = cysteine.

hydrogen-donor molecule—of the intermediate carbon-centered radicals involved in the degradation of QHS. Apart from its evident importance to antimalarial studies, such a mechanism might play a critical role in the understanding of many other[13] bioactivities of QHS that do not seem to be related to the intraerythrocytic heme iron.

An even more exciting finding from this work is that a highly polar, water-soluble compound is detected in the reaction mixture that may amount up to nearly half of the starting QHS. With thin-layer chromatography (TLC) this compound can be clearly differentiated from the starting materials and all products soluble in organic solvents by the use of *n*BuOH/AcOH/H_2O (3/1/1) as eluent. Furthermore, it can be visualized with ninhydrin, revealing the presence of an NH_2 group. Isolation of this component from the reaction mixture by column chromatography on reverse-phase silica gel eluting with water containing an increasing amount of methanol gives a white solid, whose elementary analysis suggests a formula of $C_{16}H_{27}NO_6S \cdot 2H_2O$ (the presence of waters of crystallization is confirmed by the observations made in the determination of the melting point).

Based on our mechanistic knowledge, we have envisaged that the primary C-4 radical **7** may "intramolecularly" attack the cysteine sulfur atom complexed to the Fe^{III} ion and release an Fe^{II} ion (Scheme 3). Because the conformation of the C-5a/C-12a/O-1 linkage is relatively fixed in the *trans* arrangement—which, owing to the strain developed in the five-membered transition state, is expected to slow down the



Scheme 3.

otherwise very facile radical substitution at O-1 leading to **2**—the seven-membered ring in the transition state is expected to be much easier to attain than otherwise (e.g., in an unrestricted linear chain). The product resulting from such an "intramolecular" radical substitution at the sulfur atom followed by a series of degradation steps at the QHS moiety would lead to structure **10**, which fits the formula $C_{16}H_{27}NO_6S$. The ^1H NMR spectrum also shows a clear ABX system from the cysteine residue and two distinct Me doublets from QHS, but no sign of a Me triplet (which ensures that C-4 and C-5 do not exist as an ethyl group). The remaining signals, however, are seriously broadened, suggesting that we are dealing with an exchanging system.

Nonetheless, up to this point the presence of a sulfur and a nitrogen atom in **10**, which can only come from cysteine under the given conditions, and of the carbon framework from QHS are proven beyond all doubt. To demonstrate that the sulfur atom of the cysteine residue is indeed covalently bonded to C-4 of the QHS moiety and to get around all the difficulties caused by the high polarity and the exchangeable protons, we treated **10** with acetic anhydride. The result is very gratifying: The product of "acetylation"—acetic anhydride acts here as an activating agent of the carboxylic acid moiety in QHS and initiates cyclic acetal formation, which consequently generates a cyclic sulfonium ion as an excellent leaving group at the β-carbon atom of the cysteine residue—is easily characterized as compound **11** based on the IR, ^1H and ^{13}C NMR, DEPT, DQF-COSY, NOESY, HMQC, MS, and HR-MS data,[14]

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which undoubtedly show that a σ bond is present between the sulfur and C-4 in **11** (and therefore **10**).

The significance of the concatenation of QHS and cysteine observed in this simple chemical model system lies in the precise mechanistic basis it may offer at the molecular level for the radical-mediated alkylation of, for example, proteins[5a, 7b, 8] (especially in the absence of heme iron). Such a basis has been missing in all documented investigations in spite of its evident indispensability. To date, only addition of QHS to the porphyrin in a substantially simplified heme model has been elucidated,[7a] which unfortunately cannot explain how QHS can possibly be irreversibly connected to any of the biomolecules reported in the literature for a radical reaction associated with cleavage induced by an Fe^{II} ion.

Sulfur–iron bonds (or more generally S–M bonds, where M is a transition metal with low-lying redox states suitable for cleaving peroxides) similar to that studied in this work are known to exist in many enzymes and functional proteins. The potential structural difference between S–Fe bonds in malaria parasites and those in host cells might be responsible for the extraordinarily high selective cytotoxicity of QHS. By presenting the first model for the irreversible denaturing/disabling of the redox center in these species—which are liable to be overlooked in, for example, radioactive isotope labeling studies because of their low abundance—the present work may prompt further investigations into this so far largely overlooked dimension of non-heme proteins. Furthermore, the results along this line might practically reestablish our understanding of the parasiticidal action of QHS.

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[1] F. H. Collins, S. M. Pasewitz, *Annu. Rev. Entomol.* **1995**, *40*, 195–219.
[2] a) S. R. Meshnick, A. Thomas, A. Ranz, C. M. Xu, H. P. Pan, *Mol. Biochem. Parasitol.* **1991**, *49*, 181–190; b) S. R. Meshnick, Y.-Z. Yang, V. Lima, F. Kuypers, S. Kamchonwongpaisan, Y. Yuthavong, *Antimicrob. Agents Chemother.* **1993**, *37*, 1108–1114; c) J. Bhisutthibhan, X.-Q. Pan, P. A. Hossler, D. J. Walker, C. A. Yowell, J. Carlton, J. B. Dame, S. R. Meshnick, *J. Biol. Chem.* **1998**, *273*, 16192–16198.
[3] a) W.-M. Wu, Z.-J. Yao, Y.-L. Wu, K. Jiang, Y.-F. Wang, H.-B. Chen, F. Shan, Y. Li, *Chem. Commun.* **1996**, 2213–2214; b) W.-M. Wu, Y.-K. Wu, Y.-L. Wu, Z.-J. Yao, C.-M. Zhou, Y. Li, F. Shan, *J. Am. Chem. Soc.* **1998**, *120*, 3316–3325.
[4] a) G. H. Posner, C. H. Oh, *J. Am. Chem. Soc.* **1992**, *114*, 8328–8329; b) G. H. Posner, C. H. Oh, D.-S. Wang, L. Gerena, W. K. Milhous, S. Meshnick, W. Asawamahasadka, *J. Med. Chem.* **1994**, *37*, 1256–1258; c) G. H. Posner, J. N. Cumming, P. Ploypradith, C. H. Oh, *J. Am. Chem. Soc.* **1995**, *117*, 5885–5886; d) G. H. Posner, S. B. Park, L. Gonzalez, D.-S. Wang, J. N. Cumming, D. Klinedinst, T. A. Shapiro, M. D. Balci, *J. Am. Chem. Soc.* **1996**, *118*, 3537–3538; e) J. N. Cumming, P. Ploypradith, G. H. Posner, *Adv. Pharmacol.* **1997**, *37*, 253–297; f) G. H. Posner, M. H. Parker, J. Northrop, F. S. Elias, P. Ploypradith, S. Xie, T. A. Shapiro *J. Med. Chem.* **1999**, *42*, 300–304.
[5] a) M. A. Avery, P. C. Fan, J. M. Karle, J. D. Bonk, R. Miller, D. K. Goins, *J. Med. Chem.* **1996**, *39*, 1885–1897; b) C. W. Jefford, F. Favarger, M. G. H. Vicente, Y. Jacruier, *Helv. Chim. Acta* **1995**, *78*, 452–458; c) C. W. Jefford, M. G. H. Vicente, Y. Jacquier, F. Favarger, J. Mareda, P. Millason-Schmidt, G. Brunner, U. Burger, *Helv. Chim. Acta* **1996**, *79*, 1475–1487; d) A. J. Bloodworth, A. Shah, *Tetrahedron Lett.* **1995**, *36*, 7551–7554; e) R. K. Haynes, S. C. Vonwiller, *Tetrahedron Lett.* **1996**, *37*, 253–256; f) R. K. Haynes, S. C. Vonwiller, *Tetrahedron Lett.* **1996**, *37*, 257–260.
[6] A. F. G. Slater, A. Cerami, *Nature* **1992**, *355*, 167–169.
[7] a) A. Robert, B. Meunier, *J. Am. Chem. Soc.* **1997**, *119*, 5968–5969; b) S. Bharel, R. A. Vishwakarma, S. K. Jain, *J. Chem. Soc. Perkin Trans. 1* **1998**, 2163–2166.
[8] a) W. Asawamahasakda, I. Ittarat, Y.-M. Pu, H. Ziffer, S. R. Meshnick, *Antimicrob. Agents Chemother.* **1994**, *38*, 1854–1858; b) Y.-Z. Yang, B. Little, S. R. Meshnick, *Biochem. Pharmacol.* **1994**, *48*, 569–573; c) Y.-Z. Yang, W. Asawahasakda, S. R. Meshnick, *Biochem. Pharmacol.* **1993**, *46*, 336–339.
[9] Physical and spectroscopic data for **5** (^{1}H and ^{13}C NMR signals assigned based on the DQF-COSY and HMQC spectra): M.p. 45.5–46.5 °C; $[\alpha]_D^9 = +54.5$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 10.10$ (s, 1H), 2.68 (quint., 1H, $J = 8.0$ Hz; H-9), 2.41 (m, 1H; H-8a), 2.07 (m, 1H; H-6), 2.04 (m, 1H; H-7), 1.84 (dq, $J = 3.4$, 13.0 Hz, 1H; H-8β), 1.68 (br d, $J = 13.2$ Hz, 1H; H-8α), 1.63 (m, 1H; H-5), 1.52 (m, 1H; H-5a), 1.35 (m, 1H; H-5), 1.33 (m, 1H; H-7), 1.03 (d, $J = 6.4$ Hz, 3H; Me at C-6), 1.02 (d, $J = 6.5$ Hz, 3H; Me at C-9), 0.99 (t, $J = 7.3$ Hz, 3H, H-4); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 204.09$ (C-12), 179.17 (C-10), 91,73 (C-12a), 53.96 (C-5a), 52.84 (C-8a), 37.56 (C-9), 35.31 (C-7), 33.68 (C-6), 22.19 (C-5), 20.65 (C-8), 20.22 (Me at C-6), 13.34 (C-4), 11.42 (Me at C-9); IR (film): $\tilde{\nu} = 1795$, 1735 cm^{-1}; MS m/z (%): 225 ($[M+1]^+$, 56.3), 207 (58.3), 195 (100), 179 (12.7), 167 (0.4), 151 (17.3), 121 (81.3), 97 (29), 83 (91), 69 (65), 55 (91), 43 (18); elemental analysis calcd for $C_{13}H_{20}O_3$: C 69.60, H 8.99; found: C 69.68, H 9.14.
[10] A. R. Butler, B. C. Gilbert, P. Hulme, L. Irvine, L. Renton, A. C. Whitwood, *Free Radicals Res.* **1998**, *28*, 471–476.
[11] P. J. Rosenthal, S. R. Meshnick, *Mol. Biochem. Parasitol.* **1996**, *83*, 131–139
[12] H. Atamna, H. Ginsburg, *Eur. J. Biochem.* **1997**, *250*, 670–679.
[13] See for example a) A. C. Beekman, H. J. Woerdenbag, W. van Uden, N. Pras, A. W. T. Konings, H. V. Wikstrom, *J. Pharm. Pharmacol.* **1997**, *49*, 1254–1258; b) Q. Y. Zheng, L. G. Darbie (Hauser Chemical Research), WO-A 9701548 A1, **1997** [*Chem. Abstr.* **1997**, *126*, 157663]; c) H. C.-Y. Lai, N. P. Singh (University of Washington), WO-A 9634602 A1, **1996** [*Chem. Abstr.* **1997**, *126*, 26821]; d) S. O. Duke, F. E. Dayan, A. Hernandez, M. V. Duke, H. K. Abbas, *Brighton Crop Prot. Conf. Weeds* **1997**, *2*, 579–586 [*Chem. Abstr.* **1998**, *128*, 98854]; e) M. Jung, R. F. Schinazi, *Bioorg. Med. Chem. Lett.* **1994**, *4*, 931–4; f) R. L. Berens, E. C. Krug, P. B. Nash, T. J. Curiel, *J. Infect. Dis.* **1998**, *177*, 1128–1131.
[14] Physical and spectroscopic data for **11** (^{1}H and ^{13}C NMR signals assigned based on the DQF-COSY and HMQC spectra): M.p. 148.5–149.5 °C; $[\alpha]_D^9 = -64.9$ ($c = 0.53$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta =$ 5.95 (s, 1H; H-12), 3.46 (dq, $J = 1.2$, 7.2 Hz, 1H; H-9), 2.84 (dt, $J = 3.0$, 14 Hz, 1H; H-4α), 2.39 (dt, $J = 14$, 3.0 Hz, 1H; H-4β), 2.12 (tt, $J = 15$, 4.2 Hz, 1H; H-5β), 2.02 (m, 1H; H-5α), 2.00 (m, 1H; H-8a), 1.92 (m, 1H; H-7β), 1.89 (m, 1H; H-8α), 1.73 (m, 1H; H-6), 1.47 (dt, $J = 12$, 2.4 Hz, 1H; H-5a), 1.24 (m, 1H; H-8β), 1.19 (t, $J = 6.6$ Hz, 3H; Me at C-9), 1.12 (dq, $J = 3$, 13 Hz, 1H; 7α), 0.91 (d, $J = 6.0$ Hz, 3H; Me at C-6); ^{13}H NMR ($CDCl_3$): $\delta = 172.33$ (C-10), 80.60 (C-12), 67.76 (C-12a), 50.58 (C-5a), 44.70 (C-8a), 35.27 (C-9), 34.38 (C-7), 29.91 (C-6), 24.15 (C-4), 23.74 (C-8), 23.62 (C-5), 20.08 (Me at C-9), 12.78 (Me at C-6); IR (KBr): $\tilde{\nu} = 3501$, 1735 cm^{-1}; MS m/z (%) 256 (M^+, 35), 238 (100); HR-MS calcd for $C_{13}H_{20}O_3S$: 256.1133; found: 256.1132.

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