Heme Alkylation by Artesunic Acid and Trioxaquine DU1301, Two Antimalarial Trioxanes

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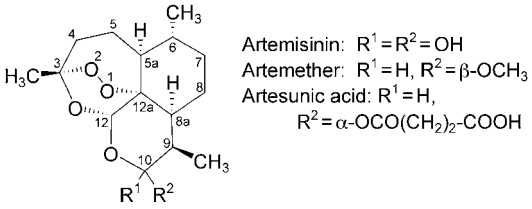
The sesquiterpene Artemisinin, an antimalarial drug that is effective against multidrug-resistant Plasmodium falciparum *strains, contains a 1,2,4-trioxane, and the endoperoxide function plays a key role in its biological activity. However, its poor solubility means that hemisynthetic derivatives, such as artesunic acid, are preferred for drugs.*

The reductive activation of the peroxide function of artemisinin by iron(II)-heme produces heme derivatives that are alkylated at meso positions by a C-centered radical derived from artemisinin. We checked if the alkylating ability of trioxane-based drugs toward heme, which might be related to its parasiticidal activity, is a general feature by comparing the chemical reactivity toward heme of the clinically relevant derivative artesunic acid and DU1301, a drug of the trioxaquine family, that is active against P. falciparum. *Both artesunic acid and trioxaquine DU1301 efficiently alkylated the heme macrocycle after activation of their peroxide function by the iron(II) of heme itself and thus gave rise to covalently coupled heme–drug products. This heme–drug adduct formation might be related to the high antimalarial activity of DU1301.*

Introduction

Artemisinin is an antimalarial drug, extracted from *Artemisia annua*, that is effective against multidrug-resistant *Plasmodium falciparum* strains.[1] This sesquiterpene exhibits a 1,2,4-trioxane, and the endoperoxide function plays a key role in its biological activity. However, artemisinin is poorly soluble in both water and oil, which are commonly used as vehicles for drug administration. For this reason, hemisynthetic derivatives obtained by reduction and functionalization of the lactone function of artemisinin are usually preferred. These compounds have been used for more than 20 years without any serious side effects or reported cases of resistance.[1a] Among them, artesunic acid, which is the hemiester of succinic acid and dihydroartemisinin (or its sodium salt, artesunate), is actually the most widely used.[1b]



The reductive activation of the peroxide function of artemisinin by iron(II)-heme produces heme derivatives that are alkylated at *meso* positions by a C-centered radical derived from artemisinin. The resulting heme–artemisinin covalent adducts have recently been characterized,[2] and the alkylating ability of artemisinin toward heme or parasite proteins might be related to its parasiticidal activity.[3]

Recently, we designed new antimalarial drugs named trioxaquines. These modular hybrid molecules contain two pharmacologically active moieties, a trioxane (as in artemisinin) and a 4-aminoquinoline (as in chloroquine), a motif known to efficiently accumulate within the parasites.[4]

We therefore checked if the alkylating ability of antimalarial trioxane-based drugs toward heme is a general feature, specially i) with the clinically relevant derivative artesunic acid and ii) with one drug of the trioxaquine family, DU1301, that is active against *Plasmodium falciparum* in vitro and in vivo.

Here, we report that both drugs, artesunic acid and the trioxaquine DU1301, were able to efficiently alkylate the heme macrocycle after activation of their peroxide function by the iron(II) of heme itself and thus give rise in high yield to covalently coupled heme–drug products, which have been characterized.

Results and Discussion

Alkylation of heme by artesunic acid

In the presence of artesunic acid, iron(II)-heme, generated in situ by a reducing agent, was quickly converted in high yield into heme–artesunic acid covalent adducts Fe-**2**, resulting from the alkylation of porphyrin *meso*-positions by a drug-derived alkyl radical centered at C4 (Scheme 1). The reaction was moni-

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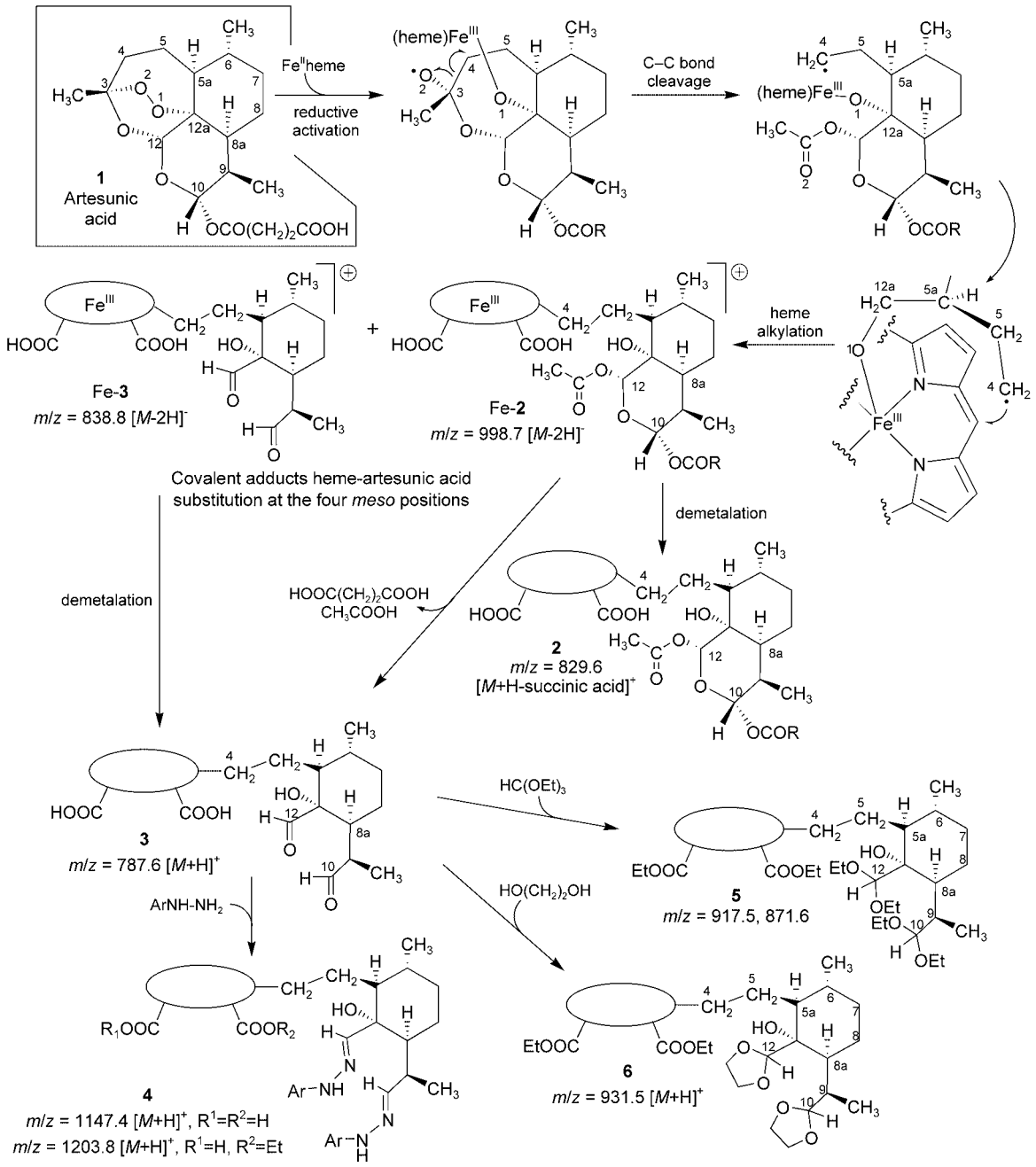


Scheme 1. *Alkylation of heme by artesunic acid. Alkylation occurred at four* meso *positions, only alkylation at the β position is depicted. The oval stands for the protoporphyrin-IX macrocycle, R = -(CH$_2$)$_2$-COOH, and Ar = 2,4-NO$_2$-C$_6$H$_3$-.*

tored by HPLC. In 40 minutes of reaction at room temperature, 96% of heme (λ_{max} = 398 nm) was converted to heme-alkylated derivatives (three different peaks, λ_{max} = 416 nm). These compounds were identified by their molecular peaks in ES$^-$-MS at *m/z* = 998.7 corresponding to $[M-2H]^-$. However, these heme–artesunic acid covalent adducts are more fragile that those resulting from the reaction of heme with artemisinin.[2] Consequently, the cleavage of the 6-membered C12-C10-C8a ring gave rise to the adduct Fe-**3**, bearing two aldehyde functions as a minor product (15%).

Strongly acidic conditions are needed for the demetalation of the porphyrin moiety. Consequently, this step provided the demetalated H_2PPIX–artesunic acid adduct **3** as the main product (Scheme 1). Compound **3** was detected in positive electrospray mass spectrometry (*m/z* = 787.6, $[M+H]^+$). However, a significant amount of ligand **2**, with an intact C12-C10-C8a cycle, was still present (32%), as evidenced by the signal at *m/z* = 829.6, which corresponded to $[M-\text{succinic acid}+H]^+$. The purification of these heme–artesunic acid adducts was difficult due to their poor solubility in organic solvents. In order to get a complete characterization, we therefore functionalized

the aldehyde and acid functions of compound **3** in several ways, namely i) treatment of the aldehydes with a phenylhydrazine derivative to get the *bis*-phenylhydrazone **4**, ii) esterification of the propionic side chains of the porphyrin and protection of the aldehydes by linear or cyclic acetals giving rise to **5** and **6**, respectively (Scheme 1).

The bis-dinitrophenylhydrazone derivative **4** was obtained in the presence of ethanol and sulfuric acid. Esterification of the propionate residues of the protoporphyrin-IX (PPIX) moiety then occurred, along with formation of the hydrazones. Compound **4** was characterized by ES^+-MS analysis: peaks were detected at $m/z = 1147.4$, 1175.4, and 1203.8, which corresponded to **4** with no, one, or two ethyl ester functions, respectively. The poor solubility of these compounds did not allow NMR analysis.

The reaction of **3** with triethyl orthoformate provided derivative **5**, which has two diethyl acetal functions at C10 and C12, and two ethyl esters on the propionate groups of the PPIX residue. Compound **5** has been characterized by ^1H NMR and is actually a mixture of four regioisomers due to the alkylation of the four *meso* carbons of the porphyrin macrocycle. The resonance of *meso* protons appeared as twelve peaks between 10.2 and 9.8 ppm, three for each of the four regioisomers. The complex patterns of vinyl protons confirmed the presence of regioisomers. The resonance of H_2C4, evidence of the covalent coupling between the heme and drug moieties, was detected at 5.45–5.00 ppm, along with the resonance of protons H10 and H12. As expected, compound **5** was not very stable under acidic conditions (for instance, in four days in solution in $CDCl_3$) and returned to the dialdehyde at C10 and C12 along with protonation of the intracyclic NH of the porphyrin.

The PPIX–artesunic acid covalent adduct **6**, with two ethylene acetals, at C10 and C12, and concomitant esterification of propionates was also obtained and characterized by ES-MS.

Alkylation of heme by trioxaquine DU1301

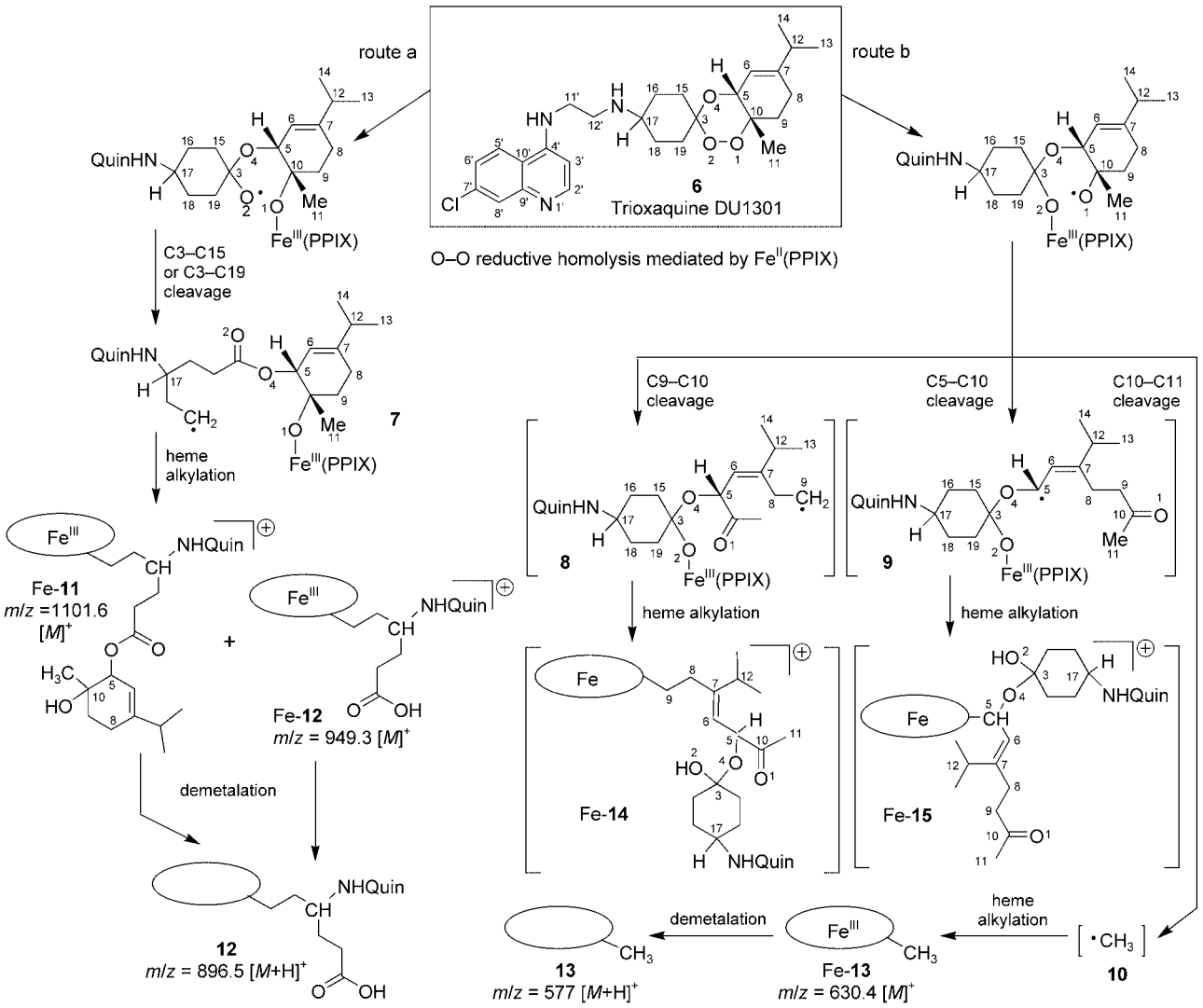
By treatment of trioxaquine DU1301 (**6**, Scheme 2) with heme or heme dimethyl ester in the presence of a reducing agent (ascorbic acid or 2,3-dimethylhydroquinone), a reductive activation of the peroxide occurred, as observed with artemisinin derivatives. After 30 min, HPLC analysis indicated that 60% of starting heme was converted to heme–drug adducts. This reaction produced two different alkoxy radicals, either on O2 (route a, Scheme 2) or on O1 (route b). The subsequent homolytic cleavage of an adjacent C–C bond can give rise to different alkyl radicals, namely **7** from route a, and **8**, **9**, and **10** from route b. Alkylation of *meso* carbons of heme by a trioxaquine-derived radical provided a covalent adduct heme–trioxaquine with retention of the complete structure of trioxaquine, identified by ES^+-MS at $m/z = 1101.8$ $[M+H]^+$. In principle, three structures can be proposed for this adduct: Fe-**11**, Fe-**14**, and Fe-**15**, formed by alkylation of heme by the radicals **7**, **8** and **9**, respectively. These three compounds have the same mass, corresponding to M(heme)+M(trioxaquine)−H. Adduct Fe-**12** was also detected [$m/z = 949.6$ $[M]^+$)]. This compound may be formed from Fe-**11** by protonation of O4 and loss of the cyclohexene ring in the mass spectrometer. Consequently, the more probable structure for the main heme–trioxaquine adduct is Fe-**11** (yield ca. 50% with respect to heme). Furthermore, both Fe-**14** and Fe-**15** have a hemiacetal structure at C3, which is supposed to be less stable than Fe-**11**, and no fragment coming from the putative Fe-**14** or Fe-**15** could be characterized. The yield of the alkylation reaction by HPLC was 50–60%.

In addition, Fe-**13** ($m/z = 630.4$ $[M]^+$; Scheme 2) was identified as a minor product (< 10% with respect to starting heme). The cleavage of the C10–C11 bond of the alkoxy radical on O1 produced a methyl radical **10**, which alkylated heme to yield Fe-**13**. This adduct was confirmed by detection, after demetalation of the heme–trioxaquine pool of adducts, of the *meso*-methylated protoporphyrin-IX derivative **13** ($m/z = 577$ $[M+H]^+$).

It should be noted that when heme reacted with an artemisinin derivative, its alkylation by a drug radical arising from the alkoxy radical on O1 has never been characterized. The absence of formation of an O1-radical in the heme activation of artemisinin derivatives should be attributed to a preferred interaction between heme and artemisinin derivatives, rather than to a particular intrinsic reactivity of trioxane entities.

The demetalation of the heme–trioxaquine covalent adducts yielded **12** as the main compound (Scheme 2). In fact, the covalent adduct Fe-**11**, containing the complete heme and trioxaquine moieties, was fragile under the strongly acidic demetalation conditions. The ester function was therefore hydrolyzed, and the α-terpinene-derived cyclohexene C5-C8-C10 cycle was lost, giving rise to compound **12** [$m/z = 896.5$ $[M+H]^+$].

This covalent adduct **12** was poorly soluble due to strong stacking interactions between the heme and aminoquinoline entities, and was difficult to purify for a complete characterization by NMR. We therefore repeated the alkylation reaction using the dimethyl ester of heme instead of heme itself (Scheme 3).[2c] In this case, the major covalent adduct **16**, resulting from the alkylation of the *meso* positions of the protoporphyrin-IX dimethyl ester by a trioxaquine derived alkyl radical, was characterized by ^1H NMR. The use of deuterated pyridine as solvent and heating to 353 K were necessary to avoid the stacking of the porphyrin and the quinoline cycles that considerably broaden the signals in other solvents. The *meso*-H resonances appeared at 10.2–10.6 ppm as a pattern of three sets of three singlets each when the spectrum was recorded at 293–323 K (total integral consistent with three protons). This clearly indicates the presence of three regioisomers of **16** (ratio: 50:30:20). As in the case of the alkylation of heme by artemisinin, there was no clear selectivity in the alkylation of the *meso* positions. The intracyclic NHs were detected at −2.96 ppm. One vinyl system was clearly identified at 6.48 (H_a), 6.23 (H_b), and 8.45 ppm (H_c). The other one was broad and partly overlapped with quinoline. The protons of the quinoline were detected at 7.70 ppm (H8′), 7.0–7.4 (H2′, H3′, H5′, and H6′), and 5.70 (HN-C4′). The covalent coupling between the trioxaquine and heme moieties was supported by the resonance of H_2C15 at 5.25 and 5.36 ppm.



Scheme 2. *Alkylation of heme by trioxaquine DU1301. Alkylation occurred at four* meso *positions, only alkylation at the β position is depicted. The structures in square brackets have not been characterized.*

Conclusion

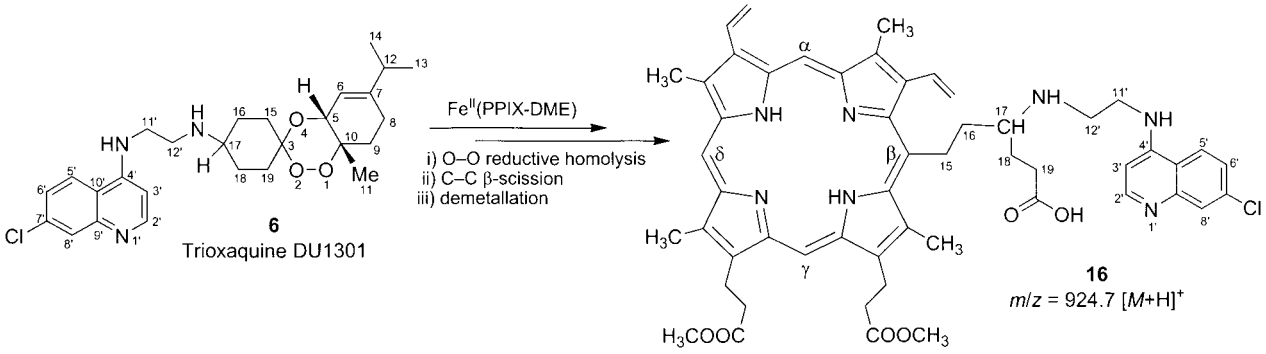
Trioxaquines with a 1,2,4-trioxane cycle have been designed in order to mimic the alkylating ability of artemisinin, which might be related to its biological activity. We therefore compared the chemical reactivity toward heme of artesunic acid, a widely used derivative of artemisinin, and the reactivity of a trioxaquine, DU1301, that is highly active against *Plasmodium* in vitro and in vivo.

Both artesunic acid and trioxaquine DU1301 were able to efficiently alkylate the heme macrocycle, after activation of their peroxide function by the iron(II) of heme, and give rise to covalent heme–drug adducts. In the case of artesunic acid, activation of the peroxide produced an alkoxy radical at O2 that, after isomerization into the primary alkyl radical **7**, reacted with heme. As previously reported for artemisinin in the presence of heme, no product arising from a putative radical on O1 was evidenced. However, in the case of DU1301, the two different routes produced alkoxy radicals either at O1 or at O2, giving rise to the different heme–trioxaquine covalent adducts Fe-**11** and Fe-**13**. This heme–drug adduct formation might be related to the high antimalarial activity of DU1301.

Experimental Section

Material and methods: NMR spectra were recorded on Brucker spectrometers at 250 and 500 MHz (^{1}H). Chemical shifts are given with respect to external TMS. LC-MS analyses and ES mass spectra were acquired on a API 365 Sciex Perkin–Elmer instrument. Chromatography columns were performed on silica gel 60 ACC Chromagel, 70–230 μm granulometry. Artesunic acid was a gift from Sanofi–Aventis, Gentilly, France.

HPLC conditions for the heme–artesunic acid adducts: The alkylation of heme was monitored on a 10 μm C18 Nucleosil column. The eluent solution was methanol/water/acetic acid (6.6:3.3:1), elution rate: 0.6 mL min^{-1}. Detection was at 406 nm. Retention times: 13.3 min (heme), 16.3, 19.9, 22.2 min (adducts).



Scheme 3. *Alkylation of heme dimethyl ester by trioxaquine DU1301. NMR characterization of the covalent adduct* ***16****. Alkylation occurred mainly at three* meso *positions, only alkylation at the β position is depicted.*

HPLC conditions for the heme–trioxaquine adducts: The alkylation of heme and heme dimethyl ester by trioxaquine was monitored on a 10 μm C18 nucleosil column. The eluants solutions were A: methanol/water/trifluoroacetic acid (70:30:0.1) and B: methanol/trifluoroacetic acid 100:0.1. The elution was a linear gradient from A/B = 100:0 to A/B = 0:100 over 25 min, followed by 10 min at A/B = 0:100. The flow rate was 1 mL min^{-1}. A diode-array detector was used for detection of the products and monitoring of UV/Vis spectra. Only the products that retain the quinoline nucleus (λ_{max} = 350 nm) and porphyrin moiety (λ_{max} = 400 nm), with $\varepsilon_{400}/\varepsilon_{350} = 2.6 \pm 0.2$, were considered. Retention times: 10.6 min (heme), 11.5–19.0 min (heme-trioxaquine adducts).

HPLC conditions for the (protoporphyrin-IX)–trioxaquine adducts: The analytical conditions were the same as described above for the metalated heme–trioxaquine adducts. The (protoporphyrin-IX)–trioxaquine adducts were characterized by $\varepsilon_{410}/\varepsilon_{350} = 3.3 \pm 0.2$ and retention times of 6.1, 8.5, 16.5, 17.7 min (H_2PPIX–trioxaquine adducts), 19.4 min (H_2PPIX).

HPLC conditions for the heme dimethyl ester–trioxaquine adducts: The analytical conditions were as described above for the heme–trioxaquine adducts. Retention times: 13.0 min (heme-DME), 14.5–18.0 min (heme-DME–trioxaquine adducts).

HPLC conditions for the protoporphyrin-IX dimethyl ester–trioxaquine adducts: The analytic conditions were the same as described above for the heme-trioxaquine adducts. Retention time: 21.0, 22.3 min [(H_2PPIX-DME)-trioxaquine adducts], 27.2 min (H_2PPIX-DME).

LC-MS conditions for the heme–trioxaquine adducts, the heme-dimethyl ester–trioxaquine adducts, and the protoporphyrin-IX–trioxaquine adducts: The LC conditions were as described above for the heme–trioxaquine adducts, except that the trifluoroacetic acid concentration in the eluent was 0.01%, and the flow rate was 0.5 mL min^{-1}. MS detection was in ES$^+$ mode.

Heme-artesunic acid adducts: Fe^{III}(PPIX)Cl (hemin, 65 mg, 100 μmol) and artesunic acid (38 mg, 100 μmol) were dissolved in DMSO (3.5 mL). A solution of sodium dithionite (85 wt%, 17.4 mg, 100 μmol) in water (0.4 mL) was added. The reaction was monitored by HPLC. After 40 min at room temperature, the reaction mixture was precipitated by addition of water. The precipitate was filtered, washed with water, dissolved in acetone, and dried under vacuum. Yield: 92% of a mixture of Fe-**2** and Fe-**3**, containing 4% of heme. UV/Vis (DMSO): λ_{max} (ε) = 416 nm (60×10^3 L mol^{-1} cm^{-1}); ES$^-$-MS: *m/z* (%): 998.7 (100) [*M*−2H]$^-$ for Fe-**2**, 838.8 (15) [*M*−2H]$^-$ for Fe-**3**

Demetalation of heme–artesunic acid adducts: A mixture of adducts Fe-**2** + Fe-**3** (100 μmol) was dissolved in glacial acetic acid (83 mL). A mixture of ferrous sulfate (380 mg, 2.5 mmol) and 12 M hydrochloric acid (2.5 mL) was then added. After 5 min, water (200 mL) was added, and the demetalated adducts **2** + **3** were extracted with ethyl acetate (4×75 mL). The organic phase was washed with water, dried over sodium sulfate, and concentrated. The product was precipitated by addition of hexane. Yield 85%; UV/Vis (ethyl acetate): λ_{max} (ε) = 426 (100), 570 nm (7); ES$^+$-MS: *m/z* (%): 787.6 (100) [*M*+H]$^+$ for **3**, 809.7 (48) [*M*−H+Na]$^+$ for **3**, 829.6 (71) [*M*+H−succinic acid]$^+$ for **2**.

Derivatization of heme–artesunic acid adductsi) *Dinitrophenylhydrazone* ***4***: Compound **3** (42 μmol) was dissolved in 95% ethanol (0.8 mL). A solution of 2,4-dinitrophenylhydrazine (84 μmol) in concentrated H_2SO_4/water/95% ethanol (1:1:3, *v/v/v*, 0.5 mL) was then added. After 20 min at room temperature, the mixture was cooled to 0 °C and diluted with 1 M H_2SO_4 (3 mL). The precipitate of *bis*-dinitrophenylhydrazone **4** was centrifuged, washed with water, and dried under vacuum. UV/Vis (acetone): λ_{max} (ε): 362 (49), 426 (100), 570 nm (7); ES$^+$-MS: *m/z* (%): 1147.4 ($R^1 = R^2 = H$, [*M*+H]$^+$), 1175.4 ($R^1 = H$, $R^2 = Et$, [*M*+H]$^+$), 1203.8 ($R^1 = R^2 = Et$, [*M*+H]$^+$).

ii) *Diethyl acetal* ***5***: Compound **3** (42 μmol) was dissolved in absolute ethanol (1.2 mL). Under argon, triethylorthoformate (84 μmol) and HCl (0.15 mL, 5 M in propan-2-ol) were added. After 24 h at room temperature, adducts **5** were precipitated by addition of water. The precipitate was centrifuged, washed with water, dried under vacuum, and purified by column chromatography: SiO_2, eluants: i) dichloromethane/methanol (98:2, *v/v*); ii) hexane/diethyl ether (50:50, *v/v*). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 10.21–9.79 (3H; *meso*-H), 8.33–7.98 (2H; vinyl-H_c), 6.36–5.89 (4H; vinyl H_a and H_b), 5.45–5.00 (4H; H_2C4, H10 and H12), 4.35 (4H; β-pyrrolic -CH_2-), 4.17 (4H; -COO-CH_2-CH_3, 4H; -CH_2-O-C10 or -CH_2-O-C12), 3.74–3.56 (12H; β-pyrrolic -CH_3, 2H; -CH_2-O-C10 or -CH_2-O-C12), 3.29–3.14 (4H; -CH_2-COOEt, 2H; -CH_2-O-C10 or -CH_2-O-C12), 2.00–0.65 (-CH_2-CH_3, H_2C5, H5a, H6, H_3C-C6, H_2C7, H_2C8, H8a, H9, and H_3C-C9), −2.60 to −3.10 ppm (2H; NH); ES$^+$-MS: *m/z*: 917.5 ([*M*+H]$^+$ for -CHO at C10 or C12, and diethylacetal at C12 or C10), 871.5 ([*M*]$^+$ for -CHO at C10 or C12, and -CH(=O$^+$Et) at C12 or C10).

iii) *Ethylene acetal* ***6***: Compound **3** (32 μmol) was dissolved in triethylorthoformate (0.5 mL). *p*-Toluene sulfonic acid (20 μmol) and ethylene glycol (680 μmol) were then added. After 8 h at 80 °C, water (10 mL) was added, and the solution was neutralized by aqueous NaOH saturated with NaCl and extracted with dichloromethane (5×20 mL). The organic phase was washed with water, dried over sodium sulfate, dried under vacuum, and purified by

column chromatography: SiO_2, eluants: i) dichloromethane/methanol (96:4, *v/v*); ii) hexane/diethyl ether (60:40, *v/v*). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 10.23–9.78$ (3H; *meso*-H), 8.35–7.80 (2H; vinyl-H_c), 6.38–5.80 (4H; vinyl H_a and H_b), 5.34–4.70 (4H; H_2C4, H10 and H12), 4.67, 4.50 and 4.37 (12H; β-pyrrolic -CH_2-, -CH_2-O-C10, and -CH_2-O-C12), 3.85–3.50 (16H; β-pyrrolic -CH_3 and -COO-CH_2-CH_3,), 3.30–3.05 (4H; -CH_2-COOEt), 2.00–0.7 (CH_2-CH_3, H_2C5, H5a, H6, H_3C-C6, H_2C7, H_2C8, H8a, H9, and H_3C-C9), −2.7 to −2.8 ppm (2H; NH); ES$^+$-MS: *m/z*: 931.5 $[M+H]^+$.

Heme–trioxaquine adducts: Fe^{III}(PPIX)Cl (hemin, 3.2 mg, 4,9 μmol), L-ascorbic acid sodium salt (3 mg, 15 μmol, 3 equiv), and the dicitrate salt of trioxaquine DU1301 **6** (*trans*,*cis*-diastereoisomer,[4c] 5.1 mg, 5.9 μmol, 1.2 equiv) were dissolved in DMSO (500 μL). The solution was stirred 30 min at 37 °C. HPLC monitoring indicated 60% conversion of heme and 100% conversion of trioxaquine. H_2O (4 mL) was added, and the mixture was separated in a centrifuge. The precipitate was washed twice with water and dried under vacuum. ES$^+$-MS: *m/z*: 630.4 ($[M]^+$ for Fe-**13**), 949.6 ($[M]^+$ for Fe-**12**), 1101.8 ($[M]^+$ for Fe-**11**); LC-MS: retention time (*m/z*): 22.9 min (616.4, heme, $[M]^+$), 24–26 min (1101.4, Fe-**11**, $[M]^+$; 475.2, Fe-**12**, $[M+H]^{2+}$).

Demetalation for the heme–trioxaquine adducts: The crude product was dissolved in pyridine (100 μL) under nitrogen, and the solution was diluted with glacial acetic acid (5 mL). A suspension of $FeSO_4$ (20 mg, 131 μmol, 27 equiv) in hydrochloric acid (37 wt%, 400 μL) was added. The solution was stirred for 10 min at 40 °C, then poured into water (20 mL). This acidic solution was neutralized by addition of 1 M aqueous NaOH. The organic products were extracted with dichloromethane. The organic layer was washed with water, dried over sodium sulfate, and evaporated to dryness. LC-MS: retention times (*m/z*): 24.3–25.3 min (577.4, **13**, $[M+H]^+$), 26–27 (896.5, **12**, $[M+H]^+$), 29.3 (563.3, H_2PPIX, $[M+H]^+$).

Heme dimethyl ester–trioxaquine adducts: Hemin dimethyl ester (heme-DME, 40 mg, 59 μmol), trioxaquine DU1301 **6** (*trans*,*cis*-diastereoisomer,[4c] 34.3 mg, 70.5 μmol, 1.2 equiv), and 2,3-dimethylhydroquinone (81.5 mg, 590 μmol, 10 equiv) were dissolved in degassed dichloromethane under nitrogen. This solution was stirred for 30 min at room temperature and evaporated to dryness. HPLC monitoring indicated 60% conversion of heme-dimethyl ester and 100% conversion of trioxaquine. The crude product was dissolved in glacial acetic acid (30 mL) under nitrogen. A suspension of $FeSO_4$ (180 mg, 1.2 mmol, 17 equiv) in hydrochloric acid (37 wt%, 1.2 mL) was added. The solution was stirred for 10 min at room temperature. Dichloromethane (100 mL) was then added under air. The organic layer was washed with aqueous saturated NaCl (3× 100 mL), aqueous sodium hydroxide (0.1 M, 2×100 mL), and water (2×100 mL), dried over sodium sulfate, and evaporated to dryness. The powder was separated by chromatography on silica gel. H_2PPIX-DME was first eluted with dichloromethane/ethanol (99:1, *v/v*), compound **16** was then eluted with dichloromethane/methanol/triethylamine (84:15:1, *v/v/v*). Further purification on a C18 column (CH_3OH/H_2O/HCOOH 73:18:9, *v/v/v*) afforded **16** with purity higher than 85% (HPLC). The yield of **16** from heme was 15% (alkylation, demetalation, and purification steps). ^{1}H NMR (500 MHz, C_5D_5, 353 K): $\delta = 10.53$, 10.52, 10.47, 10.45, 10.38, 10.35, 10.26 (3H; *meso*-H), 10.8 (1H; -COOH), 8.45 (1H; vinyl-H_c), 7.75 (1H; vinyl-H_c), 7.70 (H8′), 7.4–7.0 (H2′, H3′, H5′, and H6′), 6.48 (1H; vinyl-H_a), 6.23 (1H; vinyl-H_b), 5.97 (1H; vinyl), 5.70 (1H; HNC4′), 5.36–5.25 (2H; H_2C15), 4.58 (4H; β-pyrrolic -CH_2-), 3.8–3.5 (β-pyrrolic -CH_3, -CH_2-COOCH_3), 3.20 (H_2C11′), 3.10 (H_2C12′), 2.55 (HC17), 2.52 (H_2C19), 2.40 (HNC12′), 2.30–1.60 (H_2C16), 1.80 (H_2C18), −2.96 ppm (2H; NH); LC-MS: retention times (*m/z*): 30–35 min (924.7, **16**, $[M+H]^+$).

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Keywords: alkylation · artemisinin · heme · malaria · synthesis · trioxaquine

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