Artemisinin Derivatives

C10-Modified Artemisinin Derivatives: Efficient Heme-Alkylating Agents**

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Stichwörter:
alkylation · antiviral agents · heme proteins · iron · porphyrinoids

Artemisinin (**1**, Figure 1) is a sesquiterpene extracted from the leaves of *Artemisia annua* which contains a 1,2,4-trioxane structure that is essential for its activity against malaria-infected erythrocytes. The mechanism of action of artemisinin has been the center of intense debate over the last two decades.[1–4] Among the different possible mechanisms, one suggestion is that reductive activation of its endoperoxide function by the iron(II) center of heme generates a C4-centered radical that alkylates the *meso* positions of protoporphyrin-IX (PPIX).[5] We found that many antimalarial trioxanes related to artemisinin are also able to interact with heme or heme-like models and consequently alkylate the porphyrin ring.[4c,6] We were working on the identification of heme–artesunate and heme–trioxaquine adducts[7] when Haynes et al. reported the preparation and antimalarial activities of several C10-substituted artemisinin derivatives.[8] As all of these compounds are based on different substituents at the C10 position, no structural modification in terms of steric effects or reactivity is expected for the untouched trioxane entity of these derivatives. Consequently, one can expect that the heme-alkylating capacities of these molecules should be very similar to that of artemisinin, artemether, or artesunate. In Table 2 of the article by Haynes et al.,[8] the authors claimed that some of these efficient antimalarial C10-modified derivatives were unable to alkylate free heme. For example, the highly active 10-deoxoartemisinin derivative **2** (labeled **23** in Ref. [8]; see Figure 1) with a methylene group at C10 was totally inert to Fe^{II}-heme. On the other hand, the 10-(4-benzyl)-piperazinyl derivative **3** (labeled **18** in Ref. [8]) reacted with heme, however, the major reaction products, presumably heme–drug adducts, were not characterized.

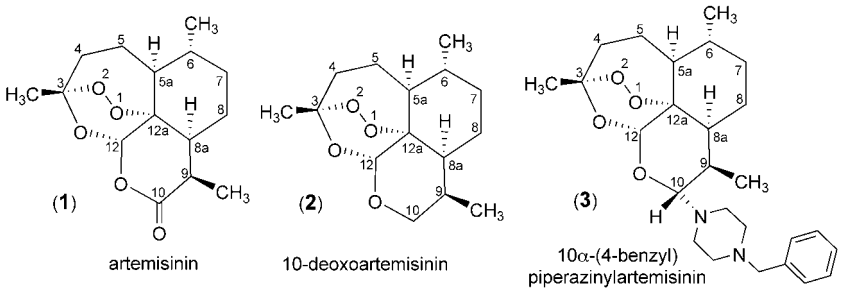

Figure 1. Structures of artemisinin (**1**), 10-deoxoartemisinin (**2**), and 10α-(4-benzyl)piperazinyl-10-deoxoartemisinin (**3**).

Herein we report that both compounds **2** and **3** are indeed potent heme-alkylating agents; both heme–drug adducts have been obtained in high yields. In fact, heme is poorly soluble in the mixture of acetonitrile and phosphate buffer (pH 7.4) used by Haynes et al. The saturation concentration of heme under these conditions at room temperature is not higher than 100 μM, and such a low solubility probably does not allow heme to react with artemisinin. For this reason of solubility, we used a homogeneous solution in dimethylsulfoxide (DMSO) containing 2–4 vol% of water in which heme was significantly more soluble (6 mM). Artemisinin itself and its derivatives **2** and **3** are not very soluble in aqueous solution. However, many drugs are insoluble in water, yet they are highly active, as hydrophobicity is required for them to cross cell membranes (e.g. the anticancer drug taxol is insoluble in water). Furthermore, solutions that contain some volume of DMSO are usually used in biologically relevant studies to evaluate the activity of artemisinin or related compounds in vitro and in vivo.[9,10]

Compound **3** was obtained from the corresponding 10-trimethylsilyl ether

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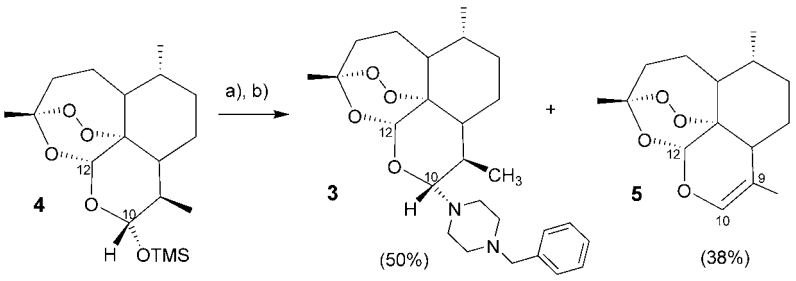
4[11,12] after modification of the procedure published by Haynes et al.[8] In our hands, the original procedure provided essentially the elimination product **5** with a double bond between C9 and C10 (Scheme 1). However, when agents other than dithionite can be used. The use of glutathione (which is a "biological" reductant present at approximately 5-mM concentration in red blood cells) to form heme– or hemoglobin–artemisinin adducts has already been reported.[5b,c] This confirms that this alkylation process is not dependent on the reductant used, provided that its redox potential is consistent with the reduction of iron(III)-heme to iron(II)-heme. As previously described for artemisinin,[2b,5] the reductive activation of the peroxide bond of 10-deoxoartemisinin leads to an alkoxy radical on O2 which, after β scission, gives a C4-centered radical. This species efficiently alkylates the *meso* carbon atoms of heme and leads to the four regioisomeric covalent adducts **6**-Fe. All of the porphyrinic material was precipitated from the reaction mixture and analyzed by mass spectrometry. The major compound was the adduct **6**-Fe, characterized by its molecular mass peak (m/z = 884.4 [M^+]) with the parent peak (m/z = 824.4 [$M-CH_3COOH]^+$) resulting from the loss of a molecule of acetic acid at C12. A small amount of unconverted heme (m/z = 616.3) was also present. A ratio of 89:11 of **6**-Fe to heme in the crude reaction mixture was indicated by mass spectrometry.



Scheme 1. Synthesis of 10α-(4-benzyl)piperazinyl-10-deoxoartemisinin (**3**): a) TMSBr, 25 min, 0°C; b) 1-benzylpiperazine, HFIP, 45 min, 0°C. TMS = trimethylsilyl.

1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) was used as an additive[10] in the amination step of the 10-brominated intermediate, the expected compound **3** was obtained in 50% yield (see Supporting Information). Compound **2** was prepared by using a modification of the described procedure,[13] and then the two compounds **2** and **3** were tested for their alkylation of heme.

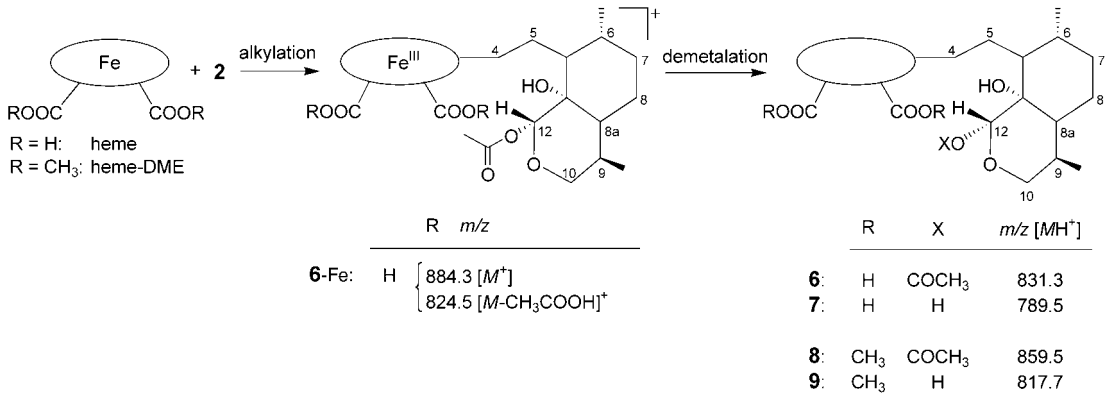
Alkylation of Heme by 2

Iron(II) protoporphyrin-IX, generated in situ from its iron(III) analogue and sodium dithionite, readily reacted with **2** at room temperature to provide the covalent heme–drug adduct **6**-Fe (1:1:1 molar ratio of [Fe^{III}(PPIX)Cl]/**2**/dithionite; Scheme 2). In fact, several reducing

The mixture of heme and **6**-Fe was demetalated to provide the free-base covalent adduct **6**, which was characterized by MS (m/z = 831.3 [MH]$^+$). However, owing to the strongly acidic conditions required for demetalation, the parent peak detected at m/z = 789.5 corresponded to compound **7**, which has a hemiacetal structure. The ratio of **6**/**7** was 3:10. As expected, a small amount of H_2PPIX was detected at m/z = 563.3 (ratio **6**+**7**/H_2PPIX = 81:19).

Owing to the low solubility of heme derivatives, the purification and characterization by NMR spectroscopy of these heme–drug adducts was not possible. We therefore repeated these reactions using heme-dimethylester (heme-DME) instead of heme. In this case, the alkylation reaction was immediately followed by demetalation to afford, as expected, a mixture of adducts **8** and **9**, which are the dimethylester analogues of compounds **6** and **7**. Adducts **8** and **9** were detected at m/z = 859.5 and 817.7, respectively (MH$^+$), with a ratio of **8**/**9** of 34:100, along with H_2PPIX-DME (m/z = 591.3). The yield of alkylation calculated from this analysis was 69% (for a 1:1 molar ratio of heme-DME/drug).

Column chromatography of this mixture yielded pure adduct **9**, which was characterized by 1D and 2D NMR spectroscopy. The ^{1}H NMR spectrum exhibited three signals in the range δ =



Scheme 2. Alkylation of heme and heme-dimethylester by 10-deoxoartemisinin (**2**), and demetalation of the obtained adducts. The configuration at C12 of adducts **7** and **9** has not been verified.

10.63–9.76 ppm for the protons in the *meso* positions. The resonance of the methylene group at C4 was detected as a broad pattern at $\delta = 5.42$–5.05 ppm, a proof of the covalent coupling between the porphyrin and drug moieties. The hemiacetal proton H12 gave a signal at $\delta = 4.63$ ppm. The signals for the two methylene protons at C10 at $\delta = 3.68$ and 3.27 ppm overlapped with those of the protons of the β-pyrrolic CH_3 and CH_2COOCH_3 groups, respectively. These assignments were attested by ^1H–^{13}C correlation spectroscopy. Both protons H10 correlated with C10, which was detected at $\delta = 61$ ppm, and proton H12 correlated with the resonance found at $\delta = 93$–95 ppm for C12. These NMR data allow to rule out the possible hydrolysis of the cycle C8a-C10-C12 with formation of an alcohol at C10 and an aldehyde at C12; in fact, such a product is expected to exhibit the same mass spectrum as **9**, but with different NMR characteristics.

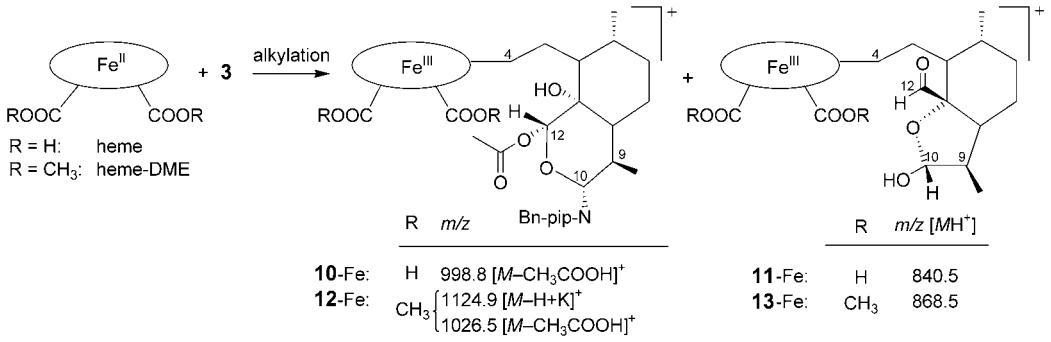
Alkylation of Heme by 3

Iron(II) protoporphyrin-IX was also efficiently alkylated by the C10-benzylpiperazinyl-substituted deoxoartemisinin derivative. Mass spectrometry of the crude porphyrinic residue revealed the presence of the adduct **10**-Fe, which corresponds to alkylation of heme at the *meso* positions by the drug **3** ($m/z =$ 998.8, $[M^{III}-CH_3COOH]^+$), along with a minor amount of unconverted heme (7% measured by HPLC; Scheme 3). The structure of **10**-Fe was confirmed by the presence of peaks at $m/z = 999.7$ and 499.9 which could be assigned to $[M^{II}-CH_3COO^-]^+$ and $[M^{III}-CH_3COO^-]^{2+}$, respectively (see Supporting Information). The reduction of iron(III) porphyrins and the loss of acetate at C12 in artemisinin-derived adducts under the analytical conditions of mass spectrometry are well-known phenomena.[15]

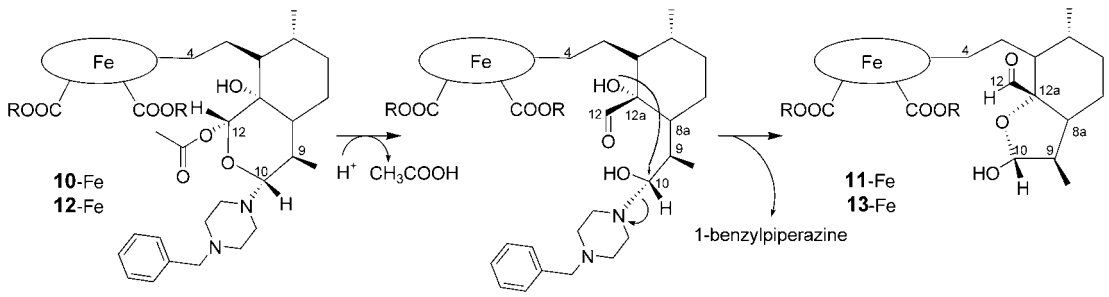
Covalent adduct **11**-Fe ($m/z = 840.3$, MH^+) was also produced during the alkylation of heme by **3** by hydrolysis of the C8a-C10-C12 cycle of adduct **10**-Fe, followed by nucleophilic attack of HO-C12a at C10, and loss of 1-benzylpiperazine (Scheme 4). This structure, which contains an exocyclic aldehyde, was confirmed by ^1H–^{13}C NMR correlation spectroscopy after demetalation of the heme-dimethylester derivative (see below). Minor peaks were also detected at $m/z = 858.7$ (hydration of the aldehyde function: +18 amu) and 872.5 (hydration of the aldehyde and methylation of the hydroxyl function: +32 amu). The ratio **10**-Fe/**11**-Fe was 43:57, as determined from mass spectrometry, while the ratio of **10**-Fe + **11**-Fe/[Fe(PPIX)] was 80:20.

Demetalation of the mixture of **10**-Fe and **11**-Fe was performed and gave rise to the respective free-base adducts **10** and **11**. Adduct **11** was characterized by the presence of its molecular peak at $m/z = 787.7$ (MH^+) and a small signal at $m/z = 805.8$ owing to hydration of the aldehyde function. Adduct **10** was characterized by the presence of a peak at $m/z = 829.7$ owing to the loss of the benzylpiperazinyl substituent (see Supporting Information).

The identification of the covalent adducts **10**-Fe and **11**-Fe was confirmed by using heme-dimethylester instead of heme as the starting material. Under these circumstances, the analogous adducts **12**-Fe ($m/z = 1124.9$ $[M-H+K]^+$, 1026.5 $[M-CH_3COOH]^+$) and **13**-Fe ($m/z = 868.5$ $[M^+]$) were observed (Scheme 4; yield = 77% from HPLC analysis). Demetalation of the mixture

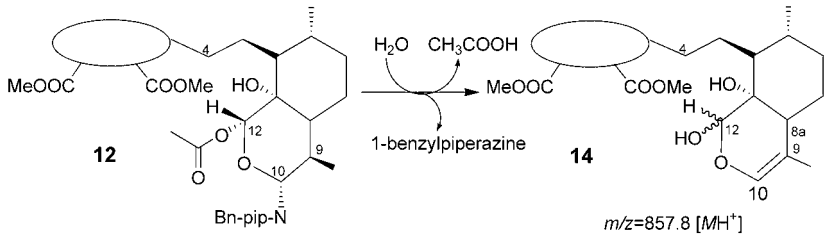


Scheme 3. Alkylation of heme and heme-dimethylester by 10α-(4-benzyl)piperazinylartemisinin (**3**). Bn = benzyl, pip = piperazinyl.



Scheme 4. Modification of the covalent adducts **10**-Fe and **12**-Fe to give **11**-Fe and **13**-Fe, respectively. The configuration at C10 is more probable, but has not been verified.

of **12**-Fe and **13**-Fe yielded **13** as the main adduct ($m/z = 815.6$ [MH^+]), while a significant peak at $m/z = 857.8$ was also detected that probably corresponds to the molecular ion [M^+] of compound **14** (see Scheme 5). The presence of this



Scheme 5. Structure of adduct **14**.

adduct, formed by the elimination of 9,10-piperazine from **12**, indicates that the C8a-C10-C12 cycle may withstand to some extent the harsh conditions required for the demetalation step. It also confirms the high propensity of the artemisinin derivatives that are substituted at C10 by an amine to produce the C9-C10 unsaturated derivative, as observed during the synthesis of **3**.

The structure of **13** was unambiguously determined by ^1H–^{13}C NMR correlation spectroscopy. The protons of the *meso* positions and the aldehyde (H12) appeared at $\delta = 9.82$–10.24 ppm (4H) and correlated with the *meso* carbon atoms and the carbonyl center C12 at $\delta = 95$–99 and 209 ppm, respectively. A broad pattern at $\delta = 5.10$–5.54 ppm accounted for three protons, namely from H_2C4 and H10, which correlated with C4 and C10 that were detected at $\delta = 31$–33 ppm and 107 ppm, respectively. These data confirm the presence of both the exocyclic aldehyde and the 5-membered cyclic hemiacetal. The NMR spectra were complex owing to the presence of four regioisomeric adducts. The confirmation of the stereochemistry at C10 could therefore not be obtained. However, the intramolecular reaction makes the *S* configuration probable.

Mass spectrometry is not, in principle, a quantitative method. However, heme derivatives are supposed to have similar volatilities under given conditions. Furthermore, the consistency between the results of HPLC and MS analyses confirms that the yields of the alkylation reactions of heme by **2** or **3** ranged from 70 to 90% under the reported conditions.

In conclusion, it is clear that compounds **2** and **3** are very efficient alkylating agents toward iron(II)-heme to provide covalent heme–drug adducts in high yields. The bulky amine substituent in the α position at C10 of compound **3** (on the same side as the peroxide bond) did not prevent the interaction with the iron center of heme that mediates the reductive activation. This is consistent with previous results obtained with model trioxanes which suggest that hindrance at C10 has no influence on the reactivity of the peroxide toward heme (active artesunate has an α configuration at C10). In fact, reduction of the carbonyl function at C10 to give an sp^3-hybridized carbon center renders the C8a-C10-C12 cycle relatively labile, as previously reported for artemether.[16] This feature somewhat complicates the characterization of the heme–drug adducts, but does not change the reactivity of the peroxide bond and, thus, does not alter the alkylating ability of these powerful antimalarial drugs.

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