Artemisinin mediated alteration of haemin to a δ-*meso* oxidation product: relevance to mechanism of action

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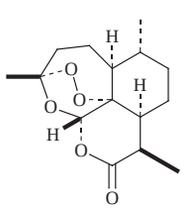
Antimalarial drug artemisinin and haemin react *in vitro*, to give a δ-*meso* hydroxy porphyrin isolated as a stable dimethyl esterified zinc complex and characterised as (20-hydroxyprotoporphyrinato dimethyl ester)zinc(II) by NOESY, visible absorption and ESI-MS; the structure sheds light on the mechanism of action of endoperoxide antimalarials.

Introduction

Artemisinin (qinghaosu), a sesquiterpene endoperoxide isolated from *Artemisia annua* is a remarkable life saving antimalarial compound, effective against drug-resistant falciparum and cerebral malaria.[1–4] Artemisinin has a unique structure (**1**) bearing a stable endoperoxide lactone (1,2,4-trioxane) totally different from previous antimalarials in its structure and mode of action. This has led to tremendous interest in the mechanism of action,[1] chemistry,[2] and drug development[3] of this novel class of antimalarials. The peroxide group is essential for antimalarial activity[4] and is mediated by activated oxygen (superoxide, H_2O_2 and/or hydroxyl radicals) or carbon free-radicals.[1,5] The high selectivity in the killing of parasites by **1** may be due



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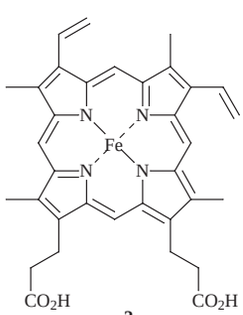

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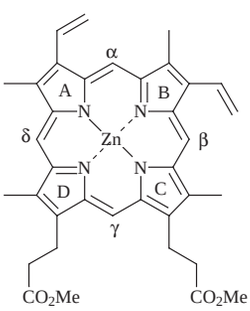

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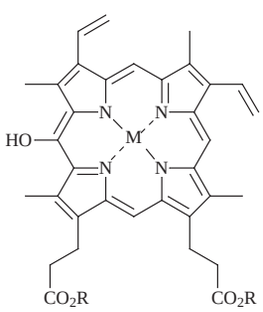

4, M = Fe, R = H
5, M = Zn, R = Me

to its interaction[6] with haem which accumulates in high quantities in parasitised red blood cells as a by-product of haemoglobin lysis by the malarial parasite.[7] Since free haem is toxic to the parasite, it is sequestered by oxidative polymerisation by the parasite to a non-toxic and insoluble material called haemozoin which accumulates as a crystalline pallet in the cytosol of the erythrocytes.[7] From studies with model systems, Jefford and co-workers suggested[8] that 1,2,4-trioxanes structurally related to **1** form a complex with Fe^{II} of haem and generate oxyl radicals, whereas Posner and co-workers proposed[9] that Fe-catalyzed decomposition of **1** leads to reactive carbon centred free-radicals, high valent iron–oxo species, and electrophiles. The chemical behaviour of artemisinin in the presence of haem and non-haem iron(II) and iron(III) has been studied, and artemisinin decomposition products of such reactions have been identified by Haynes and Vonwiller.[10] It has also been shown that **1** alkylates proteins,[11] cleaves DNA[12] and enhances haem-mediated oxidation of membrane lipids, binding irreversibly to haem in the parasite food vacuole.[13] Meshnick and co-workers reported[1b,6,14] that **1** forms covalent adducts with haemin and haem and binds to haemozoin *in vitro*, and identified[14] two such haemin–artemisinin adducts by electrospray mass spectrometry and radiolabelling. However the structure of the adducts were not determined due to difficulty in obtaining NMR spectra of the paramagnetic Fe^{III} porphyrins. Further experiments showed[14] that [^{14}C] artemisinin reacted with isolated haemozoin but incorporated less label than in intact parasites; this diminished efficiency of incorporation could be due to Fe^{II} being more reactive and abundant in cells than Fe^{III}. Since artemisinin loses its endoperoxide bridge in these interactions with haemin and haemozoin, the porphyrins formed are of considerable interest. In a significant recent study using haem model system, Robert and Meunier reported[15] a reaction of artemisinin with manganese(II)-*meso*-tetraphenylporphyrin (3 : 1 mol equiv. respectively) which led to a chlorin type covalent adduct in 25% yield, which was formed by C-alkylation of the tetraphenylporphyrin side chain mediated by reductive homolytic cleavage of the peroxide bridge of **1**. However the Mn^{II}-porphyrin model that was used for this reaction was tetra-substituted at all four *meso*-positions with phenyl groups.

Results and discussion

Here we report our results on the artemisinin–haemin interaction which leads to the identification and characterisation of a new δ-*meso*-hydroxyporphyrin product. Since in earlier studies[14] on the haemin–artemisinin interaction, reaction products could not be characterised due to the paramagnetism of the iron-containing porphyrins formed, we decided to use an alternative experimental strategy to replace iron with zinc during work-up of the incubation mixture. This strategy has been successfully used[16] for NMR analysis of porphyrins. The incubation of artemisinin and haemin chloride in degassed Tris-HCl buffer (pH 8.5) at 37 °C in the dark for 24 h was carried out. The mixture was extracted with diethyl ether and work-up

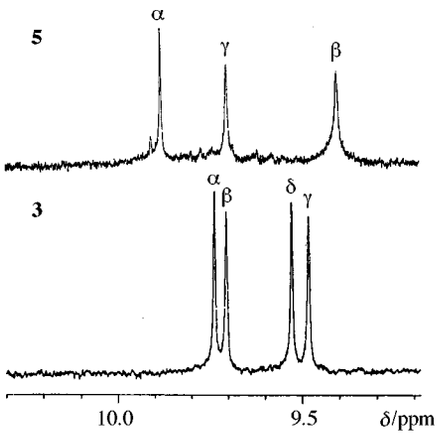

Fig. 1 ^{1}H NMR (300 MHz) spectral region for the *meso*-protons of **3** and **5**

of the aqueous fraction led to two major compounds, unreacted **2** and a new modified porphyrin product **4**, which were isolated as dimethyl esterified zinc complex **3** and **5** respectively, by esterification (Meerwein's salt Me_3OBF_4 or with MeOH/H$^+$) followed by removal of iron and formation of the zinc complex by the procedure of Smith and Fuhrhop,[16] to enable NMR analysis. Final purification of **3** and **5** was performed by silica column followed by semi-preparative reversed-phase high performance liquid chromatography.

To ensure that porphyrin product **4** (isolated as **5**) was being formed during reaction of artemisinin **1** with haemin **2** and not by oxidation of haemin during esterification and zinc metal transfer, we carried out simultaneously a control incubation experiment with haemin but *without* artemisinin. The work-up, esterification, metal-transfer and purification steps were carried out essentially as used for the haemin–artemisinin reaction. The control experiment led to unreacted **2** which was isolated and characterised as zinc protoporphyrin **3**. Keeping in view the previous reports[17] on the susceptibility of zinc(II) complexes of octaethyloxoporphyrins to oxygen, we carried out all incubations, work-up, esterification and metal-transfer steps with solvents purged with nitrogen.

The structural characterisation of **3** was relatively straightforward as determined by ^{1}H–^{1}H 2D-COSY and 2D-NOE(SY) NMR, visible absorption (soret band at λ_{max} 409 nm) and electrospray mass spectrometry [*m*/*z* 591.20 (M + H$^+$ without Zn]. All four characteristic *meso*-protons of the porphyrin ring (α, β, γ, δ) were clearly visible in the ^{1}H NMR spectrum (Fig. 1), their regiospecific assignments made by NOESY [mixing time 350 milliseconds; δ_H 9.74 (α-H), NOE with ring B-Me 3.48; 9.73 (β-H), NOE with ring C-Me 3.54; 9.50 (γ-H), NOE with C*H*$_2$CH$_2$CO$_2$Me of ring C and D at 4.24; 9.56 (δ-H), NOE with ring A-Me 3.44 and ring D-Me 3.55].

The modified δ-hydroxy-haem product **4** formed by incubation of haemin with artemisinin was isolated as dimethyl esterified zinc complex **5** and characterised as (20-hydroxyprotoporphyrinato dimethyl ester)zinc(II). Interestingly the ^{1}H NMR spectrum of **5** showed three rather than four *meso*-protons (Fig. 1), and the other resonances for four methyl, two vinyl, two propionate and two methoxy groups were at the usual chemical shifts (COSY and NOESY) when the spectra of **5** were compared to that of standard **3**. The one *meso*-proton that was missing in **5** was found to be the δ-*meso* proton as determined by NOE(SY) interactions of the α, β and γ-*meso* protons and the peripheral methyl, vinyl and propionate groups [δ_H 9.88 (α-H), NOE with ring B-Me 3.41 and vinyl α ring A 8.10; 9.40 (β-H), NOE with ring C-Me 3.57 and vinyl α ring B 8.05; 9.69 (γ-H), NOE with C*H*$_2$CH$_2$CO$_2$Me of ring C and D at 4.24; ring A-Me 3.47 and ring D-Me 3.60, no NOES with *meso*-protons]. The electrospray mass spectrum showed a molecular ion at *m*/*z* 606.60 (without Zn) corresponding to the addition of oxygen to haem. The Soret band shift and pattern in the UV–visible spectra was consistent with a *meso*-substituted porphyrin.[18]

The formation of δ-oxidised haem **4** by reaction of artemisinin and haemin is interesting in view of the proposed mechanisms (reviewed in Ref. 1) for the antimalarial action of **1** and its role in the inhibition of parasite-induced sequestering of free haem by polymerisation to haemozoin (malaria pigment). The reaction of **1** and **2** may be mediated by generation of a haem-iron–O-O–artemisinin adduct followed by transfer of oxygen by a free radical or electrophilic mechanism.[18] This is also interesting in view of previous studies by Ortiz de Montellano[18] on formation of δ-, γ- and α-*meso*-substituted haem products by reactions of haemoenzymes with phenyl- and alkyl-hydrazines and alkylhydroperoxide respectively, and also recent reports by Sugiyama *et al.*[19] on reaction of haem with H_2O_2–$H_2{}^{18}O$ to give C-18 hydroxylated iron-chlorin product, and Robert and Meunier[15] on reaction of artemisinin with a Mn^{II}-tetraphenylporphyrin haem model to give a C-alkylated chlorin adduct. The *meso*-hydroxyporphyrins have also been proposed[20] as intermediates in haem-oxygenase mediated haem metabolism in biological systems. The structure of **4** is different from haemozoin[7] which is an iron-carboxylate polymer of adjoining haem subunits. Further studies on the inhibition of parasite-induced haem polymerisation by **4** and the origin of added oxygen to the δ-*meso* position of the porphyrin ring are in progress.

Experimental

General Procedures

Artemisinin **1** was isolated from the leaves of *Artemisia annua* using a published method.[21] Haemin chloride (Bovine) and trimethyloxonium tetrafluoroborate (Meerwein's reagent) were purchased from Sigma and Aldrich respectively. The organic solvents used were of analytical purity. The NMR spectra (^{1}H, ^{13}C, two-dimensional ^{1}H–^{1}H DQF–COSY, TOCSY and NOESY) were obtained in $CDCl_3$ on a Bruker NMR spectrometer (Avance-DRX, 300 MHz); phase sensitive NOESY spectra were obtained with the use of a 1024 × 1024 data matrix and a mixing time of 350 ms, and collection of 256 t_1 increments with 32 FIDs each. Chemical shifts are expressed in δ (ppm) relative to $SiMe_4$ as internal standard; the coupling constants *J*, are expressed in Hz. Electrospray ionisation mass spectral data were obtained on a VG Platform-II (VG-BioTech, Fisons Instruments Co., UK) quadruple mass spectrometer equipped with MassLynx™ software and a pneumatic nebulizer-assisted electrospray LC–MS interface. A 1:1 mixture of acetonitrile–water was used as carrier solvent and the analyte was infused into the mass spectrometer at a flow rate of 10 mm^3 min^{-1}. FAB Mass spectra were recorded on a JEOL SX 102/DA-6000 spectrometer using xenon as carrier gas. Absorption spectra were recorded on a Beckman 640B UV spectrophotometer in 200–700 nm scan mode. Analytical and semi-preparative HPLC was carried out on a Rainin Dynamax instrument using reversed-phase columns (Microsorb MV, C-18, 5 μm, 4.6 × 250 mm; Waters' Delta Pak, C-18, 5 μm, 19 × 300 mm respectively), compounds eluted were detected at 400 nm. The purity of products was established by TLC on Kieselgel 60 F_{254} (Merck) glass plates developed in 50% ethyl acetate in chloroform, and spots were visualised by a UV lamp (Spectroline Model ENF-260C/F, Spectronics Corporation, USA) and analytical HPLC. Column chromatography was carried out with silica gel (60–120 mesh). The solvents and buffer solutions used for incubation and work-up were thoroughly purged with nitrogen gas and incubations were carried out in the dark. Solvent evaporations were carried out on a Büchi Rotavapor under reduced pressure.

Reaction of artemisinin with haemin

Haemin chloride (160 mg, 0.25 mmol) was dissolved in

degassed 1 M NaOH solution (1 cm^3) and diluted with 9 cm^3 of Tris-HCl buffer (50 mM, pH 8.5) and the final pH of the solution was adjusted to pH 8.5. The solution was treated with artemisinin (70 mg, 0.25 mmol, dissolved in 10 cm^3 of MeOH). The reaction mixture was incubated for 24 h at 37 °C in the dark with constant stirring and under a nitrogen atmosphere. After incubation, the methanol was removed under reduced pressure and the residual aqueous phase was extracted with diethyl ether (3 × 100 cm^3); the ether layer was then concentrated to dryness.

The incubation product was esterified by two different methods, one under acidic conditions and one under basic conditions both of which were successful. In the first esterification procedure the ethereal extract of the incubation mixture was allowed to stand overnight at 4 °C in 200 cm^3 of 5% H_2SO_4 in anhydrous MeOH (v/v). The mixture was then treated with ice-cold water (200 cm^3), extracted with CH_2Cl_2 (3 × 300 cm^3), and the organic layer was washed with water and concentrated under reduced pressure to give methyl esterified products. In the second esterification procedure, the MeOH of the incubation mixture was removed and CH_2Cl_2 (15 cm^3) was added to the residual aqueous phase. This biphasic mixture was cooled in ice and triethylamine (35 mm^3), followed by trimethyloxonium tetrafluoroborate (Meerwein reagent, 37 mg, 0.25 mmol), were added with vigorous stirring. The mixture was stirred for a further 1 h at the same temperature. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (3 × 15 cm^3). The combined CH_2Cl_2 fractions were washed with H_2O, dried with anhydrous Na_2SO_4 and concentrated under reduced pressure.

The esterified residue (obtained from either of the above esterification procedures) was then demetallated by dissolving it in a few drops of dry pyridine followed by addition of glacial AcOH (15 cm^3). The solution was stirred at room temperature under a stream of nitrogen for 15 min before addition of a saturated solution of $FeSO_4$ in hydrochloric acid (0.5 cm^3). The mixture was further stirred for 10 min and poured into a mixture of diethyl ether and saturated NaOAc (15 cm^3 each). The ether layer was washed with water, dried with anhydrous $NaSO_4$ and concentrated. The zinc complex of the methylated porphyrin was prepared by addition of a zinc acetate solution (500 mg in 1 cm^3 MeOH). The mixture was concentrated and the excess zinc acetate was removed by precipitation in $CHCl_3$ followed by filtration. The mixture was purified using a small silica gel column with 20% EtOH in $CHCl_3$ eluent to give, after four steps overall (incubation, esterification, iron-removal and zinc-insertion), unaltered (control) haemin product **3** (34 mg) and δ-hydroxylated porphyrin product **5** (32 mg). Compounds **3** and **5** were the only products which could be resolved on TLC and reversed-phase HPLC using a UV detector. A number of 24 h incubations of haemin chloride with different molar concentrations of artemisinin were also carried out and significant decomposition was observed at higher concentrations (10 mol equiv.) of artemisinin. Products **3** and **5** were further purified by semi-preparative reversed-phase HPLC using a MeOH–H_2O (96 : 4) mobile phase at a flow rate of 9 cm^3 min^{-1}. The purified haem product **3** eluted at retention time t_R 28 min [control (protoporphyrinato dimethyl ester)zinc(II)] and the altered haem product **5** at t_R 41 min [(20-hydroxyprotoporphyrinato dimethyl ester)zinc(II)]. Repeated HPLC runs were carried out to accumulate enough quantity of **5** for spectroscopic work. The HPLC fractions were pooled and concentrated under reduced pressure followed by freeze-drying to provide pure (20-hydroxyprotoporphyrinato dimethyl ester)zinc(II) **5**. ^{1}H NMR Assignments were made by two-dimensional DQF–COSY, TOCSY and NOESY experiments. δ_H(300 MHz, $CDCl_3$) 9.88 (1H, s, *meso*-α-H), 9.40 (1H, s, *meso*-β-H), 9.69 (1H, s, *meso*-γ-H), 8.10 (1H, dd, *J* 17.5, 11.3, vinyl α ring A), 8.05 (1H, dd, *J* 17.5, 11.3, vinyl α ring B), 6.05 (1H, dd, *J* 17.5, 2.0, vinyl β-*trans* ring A), 6.02 (1H, dd, *J* 17.5, 2.0, vinyl β-*trans* ring B), 5.95 (1H, dd, *J* 11.6, 2.0, vinyl β-*cis* ring A), 5.87 (1H, dd, *J* 11.8, 2.0, vinyl β-*cis* ring B), 4.24 (4H, m, 2 × $CH_2$$CH_2$-$CO_2$Me), 3.15 (4H, m, 2 × $CH_2$$CH_2$$CO_2$Me), 3.47 (3H, s, CH_3 ring A), 3.41 (3H, s, CH_3 ring B), 3.57 (3H, s, CH_3 ring C), 3.60 (3H, s, CH_3 ring D), 3.66 (6H, s, 2 × OMe); ES–MS (positive ion) *m*/*z* 606.60 $[M + H]^+$ without zinc; λ_{max}($CHCl_3$)/nm 432 (100, soret band, ε/dm^3 mol^{-1} cm^{-1} 10 390), 522 (7.4), 563 (8.5).

Preparation of (protoporphyrinato dimethyl ester)zinc(II) from haemin (control experiment)

A control experiment was carried out starting from haemin chloride (160 mg, 0.25 mmol) and *without* artemisinin. The incubation, esterification, iron removal and zinc metal insertion steps were carried out exactly as described above except that artemisinin was omitted. From the usual work-up and silica column chromatography, control haem product zinc protoporphyrin **3** could be isolated in an overall 40% yield (68 mg) after four steps. Finally, semi-prep HPLC purification of control product **3** was carried out to obtain enough material for spectroscopic studies which led to its characterisation as the zinc protoporphyrin **3**. ^{1}H NMR Assignments were made by 2-dimensional DQF–COSY, TOCSY and NOESY experiments. δ_H(300 MHz, $CDCl_3$) 9.74 (1H, s, *meso*-α-H), 9.73 (1H, s, *meso*-β-H), 9.56 (1H, s, *meso*-δ-H), 9.50 (1H, s, *meso*-γ-H), 8.20 (1H, dd, *J* 17.5, 11.3, vinyl α ring A), 8.12 (1H, dd, *J* 17.5, 11.3, vinyl α ring B), 6.35 (1H, dd, *J* 17.5, 2.0, vinyl β-*trans* ring A), 6.30 (1H, dd, *J* 17.5, 2.0 vinyl β-*trans* ring B), 6.15 (1H, dd, *J* 11.6, 1.9, vinyl β-*cis* ring A), 6.10 (1H, dd, *J* 11.5, 1.9, vinyl β-*cis* ring B), 4.24 (4H, m, 2 × $CH_2$$CH_2$$CO_2$Me), 3.15 (4H, m, 2 × $CH_2$$CH_2$$CO_2$$CO_2$Me), 3.44 (3H, s, CH_3 ring A), 3.48 (3H, s, CH_3 ring B), 3.54 (3H, s, CH_3 ring C), 3.55 (3H, s, CH_3 ring D), 3.61 (6H, s, 2 × OMe); ES–MS (positive ion) *m*/*z* 591.20 $[M + H]^+$ without zinc; λ_{max}($CHCl_3$)/nm: 411 (100, soret band, ε/dm^3 mol^{-1} cm^{-1} 22 977), 540 (14.6), 575 (11.9).

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