Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 1174–1183
Bioorganic & Medicinal Chemistry

Synthesis and heme-binding correlation with antimalarial activity of 3,6-bis-(ω-*N*,*N*-diethylaminoamyloxy)-4,5-difluoroxanthone

Rozalia A. Dodean,[a,b] Jane X. Kelly,[b] David Peyton,[a] Gary L. Gard,[a] Michael K. Riscoe[b] and Rolf W. Winter[a,b,*]

[a]*Department of Chemistry, Portland State University, Portland, OR 97207, USA*
[b]*Medical Research Service, RD-33, VA Medical Center, Portland, OR 97239, USA*

Received 7 July 2007; revised 19 October 2007; accepted 23 October 2007
Available online 26 November 2007

Abstract—In this study, the effect of fluorine upon the heme-binding ability of the xanthone nucleus was investigated for 3,6-bis-(ω-*N*,*N*-diethylaminoamyloxy)-4,5-difluoroxanthone (F2C5). 2-Fluoro-1,3-dimethoxybenzene was prepared by a new, improved method and used to build up the xanthone nucleus. The interaction of F2C5 with heme was investigated by UV–vis, ^{1}H NMR, and ^{19}F NMR spectroscopy. For the first time, NMR studies for the heme–drug interactions are carried out at pH 5.0, physiological for the acidic food vacuole of the malaria parasite.

1. Introduction

Malaria is the most prevalent insect-transmitted disease, causing illness and death in people in most of the tropical countries. The effectiveness of currently used antimalarial drugs is diminished because of the emergence of multidrug-resistant strains of *Plasmodium falciparum*, the causative agent of malaria.[5,9] The development of new drugs is therefore needed, with the current strategy of drug development aimed at inhibition of heme biocrystallization as a means to kill the parasite.

The malaria parasite digests hemoglobin within an acidic food vacuole, releasing a large amount of unbound heme which is highly reactive and toxic to the parasite. The liberated heme is detoxified by aggregation into an inert crystalline substance called hemozoin, which is nontoxic to the parasite due to its extreme insolubility. Formation of soluble drug–heme complexes inhibit hemozoin nucleation, leading to the accumulation of toxic heme and ultimately to the rupture of the parasite's acidic organelle.[23,13]

In the present study, the synthesis of a fluorine-containing xanthone is described and the ability of the new compound to bind heme is investigated. The fluorinated xanthone was designed with the assumption that introduction of fluorine into the xanthone nucleus increases binding affinity to heme, leading to the disruption of the heme aggregation process. The incorporation of fluorine has the additional advantage of detectability by ^{19}F NMR, providing a probe for the observation, uptake, and accumulation of the drug in viable parasite infected red blood cells.

1.1. Xanthones as antimalarial compounds

Xanthones were identified as a novel class of antimalarial compounds that prevent aggregation of heme into hemozoin by forming soluble complexes with heme.[6] A study of hydroxyxanthones showed that successive introduction of hydroxyl groups on the aromatic ring increased in vitro antimalarial potency. It was found that xanthones bearing hydroxyl groups at the 4- and 5-positions, especially when paired with neighboring 3- and 6-position hydroxyl groups, have the highest potency in the hydroxyxanthone series.[7,8]

It is also known that incorporation of amine-terminated *n*-alkoxy side chains at the 3- and 6-positions of the xanthone enhances ionic interaction with the propionate groups of the heme, with 3,6-bis-(ω-diethylaminoamyloxy) xanthone (C5) being the most effective.[8,16] In C5,

Keywords: Malaria; Heme-binding; Fluoroxanthone; Spectroscopic investigation of binding.
* Corresponding author. Address: Department of Chemistry, Portland State University, Portland, OR 97207, USA. Tel.: +1 503 220 8262; fax: +1 503 721 1084.; e-mail: winterr@ohsu.edu

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.10.083

the heme and xanthone can accommodate one another with an unstrained xanthone side chain-heme propionate interaction, concomitant to the interaction of the overlying aromatic porphyrin and xanthone cores.

1.2. A fluorinated xanthone

The difluorinated C5 (F2C5) was synthesized taking into account the high-potency character of C5 and the prior observation of enhancement of antimalarial activity when positions 4 and 5 of the xanthone are substituted by the electronegative oxygen atom. Docking models constructed for a prototypical polyhydroxyxanthone–heme complex suggested that positions 4 and 5 of the drug drug lie above the peripheral positions of negative charge density of heme.[7] Since the synthesis of 4,5-dioxygenated C5 could not be achieved, fluorine substitution was used instead. Incorporation of the highly electronegative fluorine in positions 4 and 5 of the xanthone was assumed to increase the partial positive charge of these positions, which should lead to an enhanced electrostatic interaction with the negative ring charge of heme (Fig. 1).[14] The stronger interaction would stabilize the drug–heme complex, thereby preventing further hemozoin formation.[10,15]

The heme–drug interaction was investigated by UV-titration, ^{1}H and ^{19}F NMR spectroscopy. NMR spectroscopic investigation of drug–heme binding was conducted for the first time at pH 5.0, relevant to the pH of the acidic vacuole of the parasite, where hemoglobin degradation and drug accumulation takes place. The results confirm that F2C5 complexes heme under the conditions existent in the acidic food vacuole with an antimalarial potency that is slightly larger than that of C5.

2. Results and discussion

2.1. Synthesis of F2C5 (*13*)

F2C5 (**13**) was prepared from 2-fluoro-1,3-dimethoxybenzene (**2**), which was the basic building block of both halves of the xanthone, as shown in Scheme 1. **2** was made by a new method, first involving fluorination of 1,3-cyclohexanedione with Selectfluor® in a quantitative reaction, in exact analogy to the synthesis of 2-chlororesorcinol.[20,17] A shorter route to **13** by direct fluorination of 3,6-dimethoxyxanthone with Selectfluor® led to a mixture of inseparable products, making this approach impractical.

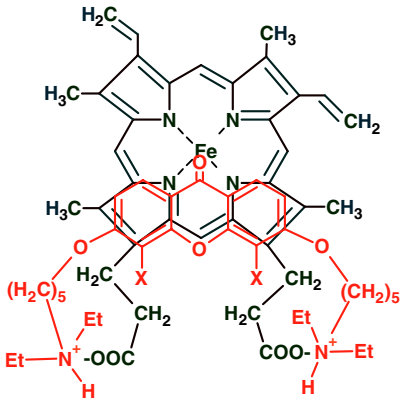


Figure 1. Model proposed for the possible docking orientation of F2C5 (shown in red) and heme.

The synthesis of 2,2-difluoro-1,3-hexanedione was previously achieved by treatment of 1,3-cyclohexanedione with fluorine in formic acid, when a mixture of mono- and di-substituted compounds was obtained.[1] The general interest for fluorinated 1,3-dicarbonyl compounds arises from their potential use as building blocks of molecules in the pharmaceutical and agrochemical industries.

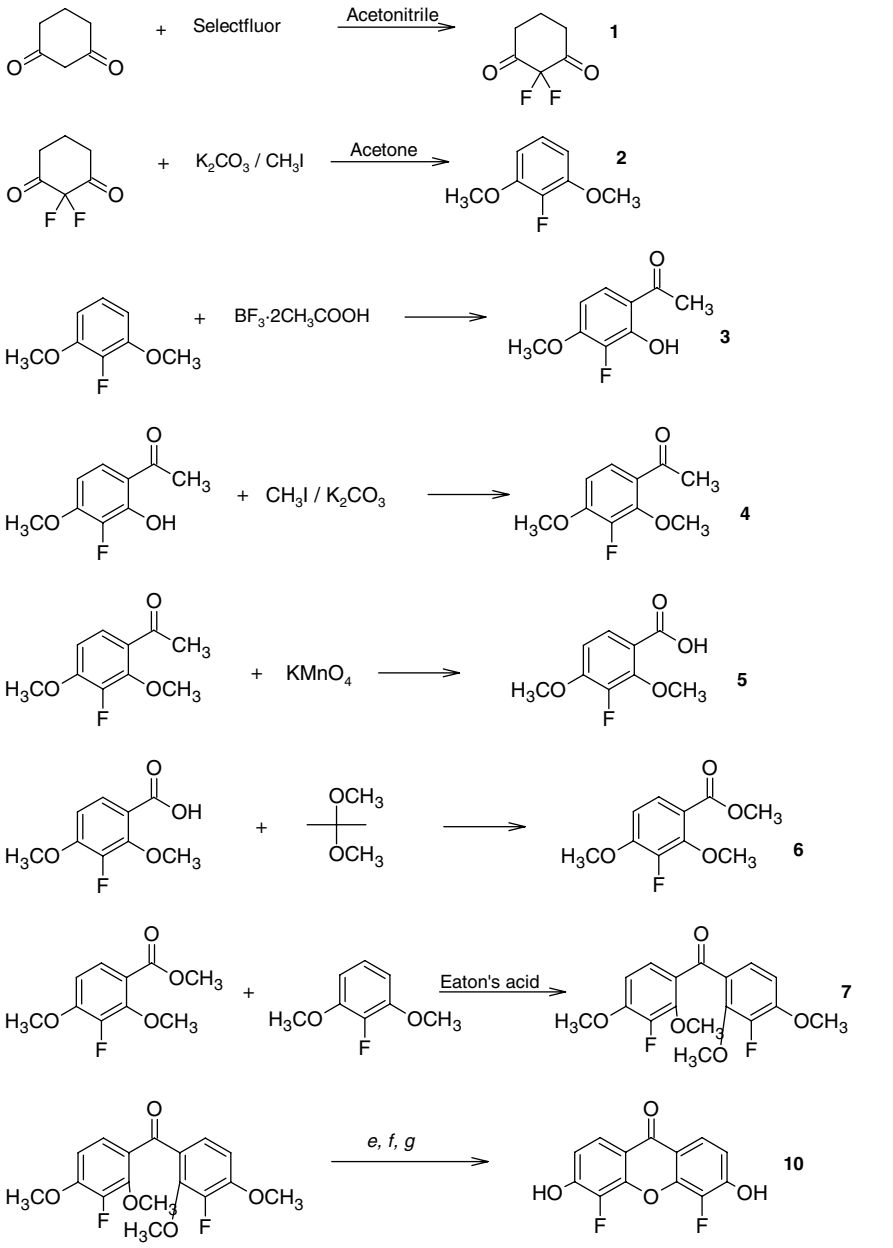
Conversion of **1** to **2** was accomplished in a single step with a maximum yield of 35%; we attribute this low yield to the reactivity of **1**, similar to that of 2,2-dichloro-1,3-hexanedione, where several products were obtained.[17] Preparation of the intermediate **2** by a multi-step procedure was previously reported, where direct fluorination of 1,3-dimethoxybenzene was carried out using Selectfluor®, leading to a mixture of 2,4 and 2,6 dimethoxyfluorobenzene.[19] Compound **2**, along with several other fluororesorcinols, is of interest as intermediate in the synthesis of fluorinated fluoresceins.[19]

Since the acetylation of **2** with the boron trifluoride–acetic acid complex leads to concomitant o-demethylation (product **3**), remethylation was necessary in order to obtain the desired product **4**. This way any difficulties due to the presence of the OH group were avoided in the subsequent reactions. **4** was oxidized with potassium permanganate to the benzoic acid **5**, which was converted to its methyl ester to facilitate purification. **5** and **2** were coupled in Eaton's acid, and the resulting benzophenone **7** was mono-ortho-demethylated with boron trichloride. Ring closure was effected by heating the *o*-hydroxy-*o*′-methoxy-benzophenone **8** to produce xanthone **9**, which has the required substitution pattern. Further chemical transformations to lead to the final F2C5 (**13**) are shown in Scheme 1.

2.2. Heme–drug interaction investigated by NMR spectroscopy

NMR spectroscopic studies were performed in order to confirm the interaction of heme with F2C5. Perturbation of the NMR spectrum of F2C5 induced by addition of aqueous heme was measured.[22] The observed values for the induced proton shifts were compared with the induced shifts for chloroquine and quinine, which were previously shown to interact with heme by close stacking.[12,11] The titrations of heme in aqueous drug solutions were conducted at pH 5, the pH of the digestive vacuole of the parasite.[13]

The addition of as much as 12% mol ratio of heme to the F2C5 solution did not cause heme precipitation. When a 24% mol ratio was added, the solution became slightly cloudy and the titration was stopped. The pH of the



Scheme 1. Synthesis of F2C5.

solution after the addition of 24% molar equivalent of heme was 5.5. The effect of heme addition on F2C5 proton resonance peaks is shown in Figure 2.

Addition of small amounts of heme solution resulted in broadening of all resonance lines and a change in chemical shifts of the aromatic and α-methylene protons (O–CH_2). The broadening of the resonance lines may indicate the formation of molecular complexes. An analogous lack of selectivity of the induced broadening was also observed upon addition of heme to an aqueous solution of quinine.[12] The aromatic and α-methylene

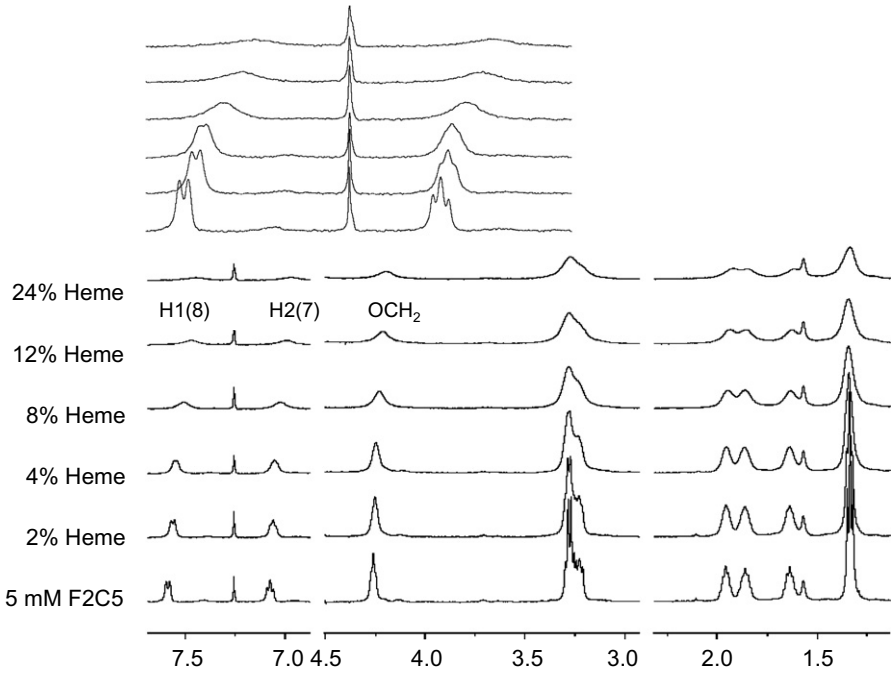


Figure 2. 500 MHz ^{1}H NMR spectra of 5 mM F2C5 aqueous solution at pH 5 as a function of increasing concentration of heme. Linewidth broadening and induced shifts for xanthone aromatic protons are shown in the top insert.

protons (near 4.2 ppm) were shifted to high field, while the shift, if any, of the other protons was not measurable. The plot of induced $\Delta\delta$ of the shifted protons versus the mol fraction of added heme is shown in Figure 3.

The magnitude of these shifts is similar to those reported by Moreau et al. for aromatic protons of chloroquine and quinine upon titration with heme.[12,11] However, these shifts are larger than those found for C5, giving support to our initial hypothesis that fluorine substitution in positions 4 and 5 of C5 would give an increased interaction with heme.[22] These porphyrin-induced shifts are a result of pseudocontact (also called dipolar) interaction due to the paramagnetic iron III atom of heme and to the ring current effect of the porphyrin.[22] The more shifted protons indicate stronger interaction between the drug and heme aromatic systems.

The same titration experiment of F2C5 with heme solution was followed by ^{19}F NMR, and the effect of heme addition on the fluorine resonance line is shown in Figure 4.

Addition of heme to the F2C5 solution resulted in the linewidth broadening and a downfield shift of the fluorine resonance peak. The plot of induced shift $\Delta\delta$ for the F2C5 fluorine versus the mol fraction of added heme is shown in Figure 5.

F2C5 has two chemically equivalent fluorines that shift to low field upon addition of heme. The magnitude of the shift is smaller than that of the aromatic protons.

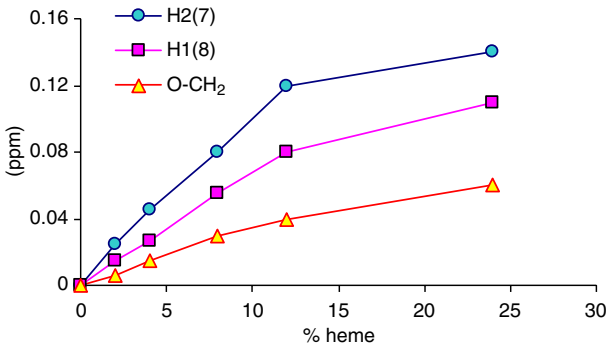


Figure 3. The dependence of induced shifts* of F2C5 selected protons on the concentration of heme upon titration of a solution of 5 mM F2C5 with heme at pH 5. *Upfield shifts are positive, downfield shifts are negative.[3]

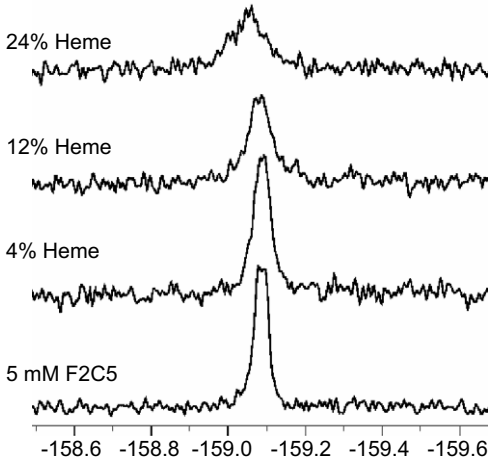


Figure 4. 376.5-MHz ^{19}F NMR spectra of 5 mM F2C5 aqueous solution of pH 5 as a function of increasing concentration of heme.

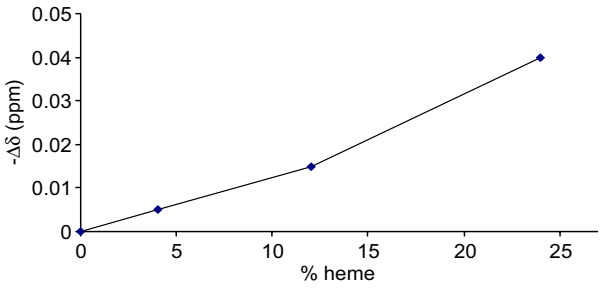


Figure 5. The dependence of induced shift of the fluorine of F2C5 on the concentration of heme upon titration of a pH 5 solution of F2C5 with heme.

This suggests that the fluorine atoms are not in the same close proximity range as the xanthone protons from the porphyrin ring, since fluorine chemical shifts are very sensitive to changes of the chemical environment. However, the induced shift of the fluorine atoms confirms the presence of an interaction between F2C5 and heme.

2.3. Binding affinity of F2C5 with heme investigated by UV–vis spectroscopy

The complexation of F2C5 with heme was followed by spectrophotometric titration. Binding affinity of F2C5 for heme was determined following the procedure described by Kelly,[8] and compared to the binding affinity of C5 and other known antimalarials.

Titration of heme with F2C5 caused a reduction of intensity and shift to longer wavelengths of the Soret band. The absorbance intensity at 386 nm as a function of drug concentration was plotted and fit to mathematical expressions representing the dependence of absorbance on free drug concentration.[8] Five models were examined in order to determine the stoichiometry of F2C5–heme complex, similarly to the previously published methods used to evaluate the binding of C5 to heme.[8] Equations for each model are given below, where Hm_2 represents the concentration of heme dimer, and C represents the concentration of F2C5.

Model 1.
A single drug molecule binds a single heme dimer (Eq. 1):

$$Hm_2 + C \overset{K_a}{\rightleftharpoons} Hm_2C. \quad (1)$$

Model 2.
Two drug molecules bind simultaneously to a single heme dimer (Eq. 2):

$$Hm_2 + 2C \overset{K_a}{\rightleftharpoons} Hm_2C_2. \quad (2)$$

Model 3.
Two drug molecules bind stepwise to a single heme dimer (Eqs. 3 and 4):

$$Hm_2 + C \overset{K_{a1}}{\rightleftharpoons} Hm_2C \quad (3)$$

$$Hm_2C + C \overset{K_{a2}}{\rightleftharpoons} Hm_2C_2. \quad (4)$$

Model 4.
A single drug molecule binds simultaneously to two heme dimer units (Eq. 5):

$$2Hm_2 + C \overset{K_a}{\rightleftharpoons} (Hm_2)_2C. \quad (5)$$

Model 5.
A single drug molecule binds sequentially to two heme dimer units (Eqs. 6 and 7):

$$Hm_2 + C \overset{K_{a1}}{\rightleftharpoons} Hm_2C \quad (6)$$

$$Hm_2C + Hm_2 \overset{K_{a2}}{\rightleftharpoons} (Hm_2)_2C. \quad (7)$$

Plots of all absorption curves produced spectra with several slightly scattered isosbestic points in the Soret range (data not shown). The plot of the first few spectral scans gave a well-defined isosbestic point at 401 nm, as shown in Figure 6.

The plot of the absorbance intensity changes at 386 nm as a function of F2C5 concentration is shown in Figure 7. None of the models fit the data well across the entire range of drug concentrations. When data for only the initial eight spectral scans were fitted, it was found that models 1, 3, 4, and 5 gave good fits, as is shown by their superimposition in Figure 7.

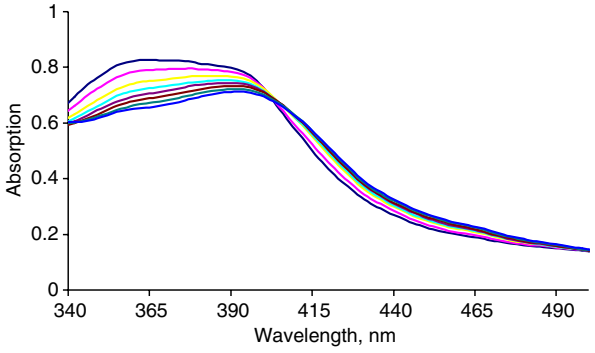


Figure 6. Spectroscopic changes observed in the Soret region of heme upon titration with F2C5. The first eight spectral scans are shown.

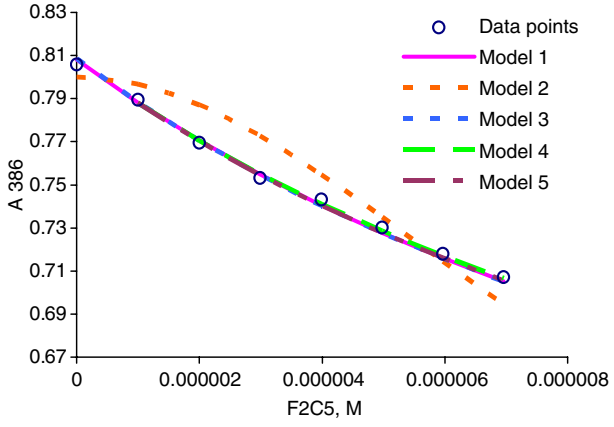


Figure 7. Absorbance of heme–drug complex as a function of F2C5 concentration. The best fits are represented by the solid pink line for model 1 and the dotted lines for models 3, 4, and 5. The orange dotted line represents a fit for model 2.

Table 1. Apparent binding affinity of F2C5 for heme in aqueous solution

Binding model	K_{a1}	K_{a2}
1	6.2×10^4 M^{-1}	—
3	6.8×10^4 M^{-1}	1.0 M^{-1}
4	4.7×10^{11} M^{-2}	—
5	6.5×10^4 M^{-1}	1.0 M^{-1}

The calculated binding constants derived from the fitting of the experimental absorbance as a function of drug concentration to the best-fitting models are given in Table 1. Model 2 did not provide a good fit to the experimental data and it was excluded.

Since the binding constant derived for model 4 diverged by a factor of 10^7 from the values found for the rest of the best-fitting models, it was considered implausible. The binding constants derived for models 1, 3, and 5 were similar, suggesting one-to-one binding as a starting point, followed by higher association. Model 1 assumes that a single F2C5 molecule binds to one heme dimer. Model 3 assumes sequential binding of two F2C5 molecules to one heme dimer, while model 5 suggests stepwise binding of two heme dimer molecules to one F2C5 molecule. This may be interpreted as sequential binding of F2C5 and heme with alternating drug and heme molecules, with a one to one complex as the basic association unit.

The binding affinity of F2C5 was compared to those of the structurally similar C5 and other known antimalarial compounds (Table 2).[8,4] The results show that F2C5 binds heme with a lower affinity than C5, but similarly as the commonly used antimalarials.

2.4. Antimalarial activity

The IC_{50} values for F2C5 against the multidrug-resistant strains W2 and D6 of *P. falciparum* were compared to those of other known antimalarials, and are shown in Table 3.[21,16] The fluorescence based MSF assay introduced by Smilkstein et al. was used for IC_{50} determinations.[18] The results show a slight increase in antimalarial potency of F2C5 relative to C5.

Table 2. Binding affinity (K_a) of selected compounds for heme in aqueous solution

Compound	K_a (10^5 M^{-1})
Chloroquine	4.0
Quinine	0.2
Mefloquine	0.1
C5	8.2
F2C5	0.6

Table 3. In vitro antimalarial activity against D6 and W2 strains of *Plasmodium falciparum*

Compound	IC_{50}, nM, D6	IC_{50}, nM, W2
Quinine	1.1×10^1	6.0×10^1
Chloroquine	7.8×10^0	2.9×10^2
Mefloquine	5.9×10^0	0.7×10^0
C5	1.8×10^2	1.2×10^2
F2C5	9.3×10^1	1.5×10^2

3. Conclusions

This work consisted of the design, synthesis, and evaluation of heme affinity of a fluorinated xanthone, F2C5. The ^1H NMR spectroscopic study confirmed the interaction of F2C5 with heme, as the protons attached directly to the aromatic rings and to the oxygen atoms adjacent to the aromatic rings showed an induced shift upon addition of increasing amounts of heme. The ^{19}F NMR study also confirmed heme–drug interaction, with the possibility of using the outcome of this study for monitoring drug uptake and complexation with heme in situ in the digestive vacuole of a viable intact parasitized red cell.

The UV–vis spectroscopic study showed a reduction and shift of the Soret band upon addition of drug to heme, indicating the formation of molecular complexes. The presence of several slightly scattered isosbestic points in the Soret region suggested the possible formation of one or more complexes with non-identical yet very similar absorption properties. This possibility was supported by the results obtained for the stoichiometry of the drug–heme complex, suggesting one-to-one binding as a starting point, followed by sequential binding of alternating drug and heme molecules.

Although the structure of the new fluorinated xanthone was designed based on previous observations of the structural features of similar xanthones, no obvious relationship could be established between this structure and antimalarial activity. Introduction of fluorine in the C5 molecule did not improve binding affinity as expected, although it slightly increased the antimalarial potency. Generally, there is a dual requirement that heme biocrystallization inhibitors not only disrupt the process of hemozoin formation, but also concentrate within the parasite's digestive food vacuole.[13] Some factors such as biotransportation, effectiveness of accumulation in the digestive food vacuole, and solubility also play a role in the potency of an antimalarial compound.

This work demonstrated that the new fluorinated xanthone forms soluble complexes with heme and inhibits heme aggregation. The fluorinated xanthone F2C5 was shown to have a slightly improved antimalarial activity when compared to that of its non-fluorinated analog, C5. The binding affinity for heme was lower than that of C5, with the conclusion that the precise structure–activity relationship for optimal antimalarial activity in the xanthone series warrants further investigation.

4. Materials and methods

4.1. Compounds

The reagents 1,3-cyclohexanedione and 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo-[2.2.2]octane-bis(tetrafluo-

roborate) (Selectfluor®) were purchased from Aldrich Chemical Company. Heme chloride was purchased from Sigma Chemical Company. DMSO-d_6, $CDCl_3$, D_2O, NaOD, and DCl were obtained from Alfa Aesar.

4.2. Gas chromatography–mass spectroscopy

Mass spectra were obtained using a Hewlett–Packard HP5890 series II gas chromatograph (25 m, DB5 column) with a HP5970 mass-selective detector operating at 70 eV. The injection port temperature was 250 °C. The programs were set such that the initial temperature, ranging between 50 and 150 °C, as indicated for the individual compounds, was maintained for 2 min and then raised to 280 °C at a rate of 11 °C/min. Mass spectra are reported following this order: *m*/*z*, molecular ion, relative %.

The precise molecular weight values (HRMS) were determined on a Kratos MS 50TC; chemical ionization with methane by Jeff Morré, Mass Spectrometry Laboratory, Oregon State University, (Corvallis, Oregon).

4.3. NMR spectroscopy

Proton NMR spectra were obtained on a Tecmag-Libra modified Nicolet NT-500 spectrometer with the water residual HOD peak suppressed as needed by a low-power presaturation pulse during the 3 s relaxation delay. Fluorine NMR spectra for compound characterization were obtained on EFT-90 spectrometer operating at 84.7 MHz. For the ^{19}F NMR spectroscopic investigation of drug–heme binding a Bruker AMX spectrometer operating at 376.47 MHz with a 'QNP' probe was used. Proton chemical shifts were referenced to $(CH_3)_3Si$ directly or through the residual water signal at 4.76 ppm, and ^{19}F chemical shifts were referenced to $CFCl_3$. For the heme–drug interaction study, proton chemical shifts were referenced to $CDCl_3$ contained within a sealed capillary tube. Deuterated solvents were used according to the solubility of compounds. All spectra were acquired at room temperature and were processed with MacNMR (Tecmag, Houston, TX) or SwanNMR.

An investigation using ^1H NMR and ^{19}F NMR spectroscopy of the binding interaction between F2C5 and heme was performed by titration of a 5 mM drug solution with aliquots of a 10 mM heme stock solution. All dilutions were made with a 20 mM NaD_2PO_4 buffer solution prepared from NaH_2PO_4 and D_2O. The 10 mM heme stock solution was prepared by addition of NaOD to a suspension of heme chloride until complete dissolution of the heme was observed. The pH of the solution was then adjusted to 9.0 using a concentrated DCl solution. For the consistency of experimental conditions, the heme stock solution was allowed to sit for 2 h before use. The pH of the 5 mM F2C5 was adjusted to 5.0 by addition of concentrated DCl solution. Titration of a blank 20 mM NaD_2PO_4 buffer solution was performed with the heme stock solution to observe heme self-aggregation. The solution became cloudy after the first addition of heme solution (2% molar equivalent), showing that in the absence of drug the heme aggregates instantaneously at pH 5.

4.4. UV–vis spectroscopy

For UV–vis absorbance spectroscopy, a Varian-Cary 3E spectrophotometer was used with 1 cm quartz cuvettes and a thermostatted cell holder set to 25 °C. To determine the binding affinity of the fluorinated xanthone to heme, aliquots of a solution of 1 mM of fluorinated xanthone were successively added to 2 ml of 20 μM heme and absorbance of the heme–drug solution was measured. The 20 μM heme solution was prepared from a fresh stock solution of 10 mM heme in 0.01 N NaOH. All dilutions were made with 20 mM phosphate buffer with the final pH of the solutions adjusted to 7.1. The absorption of the phosphate buffer solution was used as a baseline and the collected data were converted to Excel (Microsoft) format with Gram 386 software (Galactic Industries). The titration curves were plotted and the parameters were calculated using non-linear curve-fitting models with Excel software (Microsoft).[2] The concentrations of the fluorinated xanthone solutions were corrected for dilution effects.

4.5. Experimental details

4.5.1. 2,2-Difluoro-1,3-hexanedione hydrate (1). To a slurry of 50.0 g (0.141 mol) of Selectfluor® in 200 ml acetonitrile, 7.86 g (0.070 mol) of 1,3-cyclohexanedione was added over 20 min. The mixture was stirred for 45 min, when the reaction was complete (GC–MS). After filtration of the Selectfluor® residue and removal of the solvent, 100 ml water was added. The solution was acidified to pH 3 and extracted continuously for 8 h with ethyl ether using a Kutscher–Steudel liquid–liquid extractor. After removal of the ethyl ether, 10.0 g of a white-yellow solid **1** was obtained, in 86% yield, with a melting point of 109.5–110.5 °C. GC–MS: initial temp. = 50 °C, R_f = 6.27 min; 148 (M^+, 8%); 120 ($M - CO^+$, 27%); 101 ($M - CO - F^+$, 62%); 78 ($M - 2O - 2F^+$, 18%); 55 (C_3F^+ or $C_3H_3O^+$, 100%); 42 ($C_2H_2O^+$, 64%); 27 ($C_2H_3^+$, 10%). ^{19}F NMR ($CDCl_3$) t, φ = −129.7 ppm (s). ^1H NMR ($CDCl_3$): $\delta_{4,4'} = 1.90$ ppm (m, 2H), $\delta_{6,6'} = 2.14$ ppm (m, 2H), $\delta_{5,5'} = 2.64$ ppm (m, 2H), $\delta_{OH} = 2.95$ ppm (s, 2H).

4.5.2. 2-Fluoro-1,3-dimethoxybenzene (2). A solution of 10.00 g (0.068 mol) of 2,2-difluoro-1,3-hexanedione hydrate in 50 ml acetone was added over 1 h through a drip funnel to a slurry of 60.0 g (0.435 mol) finely ground potassium carbonate and 76.2 g (0.537 mol) methyl iodide in 300 ml of acetone, while vigorously stirring and heating to reflux. The mixture was refluxed for two more hours, when the reaction was complete (GC–MS). After suction filtration and removal of the solvent, a yellow-orange viscous liquid was obtained. Distillation under dynamic vacuum at 58 °C gave 3.40 g of **2** (light yellow viscous liquid), in 35% yield. GC–MS: initial temp. = 50 °C, R_f = 9.26 min; 156 (M^+, 100%); 141 ($M - CH_3^+$, 10%); 127 ($M - C_2H_5^+$, 23%); 113 ($C_5H_2O_2F^+$, 48%); 98 ($C_5H_3OF^+$, 15%); 83 ($C_5H_4F^+$, 15%);

70 ($C_4H_3F^+$, 23%); 51 ($C_4H_3^+$, 8%). ^{19}F NMR ($CDCl_3$) $\varphi = -161.2$ ppm (d-d), $J_{F4} = J_{F6} = 7.1$ Hz, $J_{F5} = 2.1$ Hz. ^{1}H NMR ($CDCl_3$): $\delta_4 = \delta_6 = 6.61$ ppm (d-d, 2H), $J_{45} = J_{65} = 8.55$ Hz, $J_{F4} = J_{F6} = 7.33$ Hz; $\delta_5 = 6.98$ ppm (t-d, 1H), $J_{45} = J_{65} = 8.55$ Hz, $J_{F5} = 2.45$ Hz; $\delta_{OCH_3} = 3.89 ppm (s, 6H)$.

4.5.3. 3-Fluoro-2-hydroxy-4-methoxyacetophenone (3). 2.0 g (0.013 mol) of 2-fluoro-1,3-dimethoxybenzene with 8.5 ml of boron trifluoride–acetic acid complex was heated at 80 °C for 4 h, when the end of reaction was confirmed by GC–MS analysis. The reaction mixture was poured into 120 ml of water and the product was extracted with 5×20 ml ethyl acetate. After passing through a short silica gel column with a mixture of hexanes–ethyl acetate (1:1), 2.3 g of a light yellow solid **3** was obtained, in 98% yield, with a melting point of 103 °C. GC–MS: initial temp. = 100 °C, $R_f = 8.54$ min; 184 (M^+, 34%); 169 ($M - CH_3^+$, 100%); 154 ($M - 2CH_3^+$, 6%); 126 ($M - C_3H_6O^+$, 7%); 43 ($C_2H_3O^+$, 8%). ^{19}F NMR ($CDCl_3$) $\varphi = -161.2$ ppm (d-d), $J_{F5} = 7.1$ Hz, $J_{F6} = 2.1$ Hz. ^{1}H NMR ($CDCl_3$): $\delta_6 = 7.50$ ppm (d-d, 1H), $J_{56} = 9.1$ Hz, $J_{F6} = 2.1$ Hz; $\delta_5 = 6.52$ ppm (d-d, 1H), $J_{56} = 9.1$ Hz, $J_{F5} = 7.1$ Hz; $\delta_{4(OCH_3)} = 3.96$ ppm (s, 3H); $\delta_{2(OH)} = 12.43$ ppm (s, 1H); $\delta_{1(COCH_3)} = 2.58$ ppm (s, 3H).

4.5.4. 3-Fluoro-2,4-dimethoxyacetophenone (4). A mixture of 2.30 g (0.013 mol) of 3-fluoro-2-hydroxy-4-methoxyacetophenone, 100 ml acetone, 11.00 g (0.080 mol) potassium carbonate, and 5 ml (0.080 mol) of iodomethane were refluxed for 1.5 h, when analysis by GC–MS confirmed the end of the reaction. After suction filtration and removal of the solvent, 2.3 g of **4** was obtained as a white-yellow solid, in 93% yield, with a melting point of 65 °C. GC–MS: initial temp. = 100 °C, $R_f = 8.65$ min; 198 (M^+, 21%); 183 ($M - CH_3^+$, 100%); 168 ($M - 2CH_3^+$, 25%); 125 ($M - C_4H_9O^+$, 12%); 43 ($C_2H_3O^+$, 10%). IR (cm^{-1}): 1676 (C=O), 1302, 1275, 1093 (C–F). ^{19}F NMR ($CDCl_3$): $\varphi = -152.83$ ppm (d-d), $J_{F5} = 7.3$ Hz. $J_{F6} = 2.1$ Hz. ^{1}H NMR ($CDCl_3$): $\delta_6 = 7.63$ ppm (d-d, 1H), $J_{56} = 9.0$ Hz, $J_{F6} = 2.0$ Hz; $\delta_5 = 6.72$ ppm (d-d, 1H), $J_{56} = 9.0$ Hz, $J_{F5} = 7.1$ Hz; $\delta_{4(OCH_3)} = 3.93$ ppm (s, 3H); $\delta_{2(OCH_3)} = 4.03$ ppm, (d, 3H), $J_{F2} = 2.20$ Hz; $\delta_{1(COCH_3)} = 2.56$ ppm (s, 3H). HRMS: $^{12}C_{10}{}^1H_{11}{}^{16}O_3{}^{19}F$: Calcd 198.06923, measured 198.06976.

4.5.5. 3-Fluoro-2,4-dimethoxybenzoic acid (5). Into a slurry of 2.00 g (0.010 mol) of 3-fluoro-2,4-dimethoxyacetophenone in 50 ml water, a solution of 6.00 g (0.038 mol) of potassium permanganate in 300 ml water was added over 3 h while vigorously stirring and heating to reflux. The mixture was filtered while hot, and the solid residue was washed with hot water. The filtrate was acidified to pH 2 and extracted with 4×50 ml ethyl acetate. 1.61 g of a white-yellow solid was obtained consisting of two products (GC–MS), the major one being 3-fluoro-2,4-dimethoxy benzoic acid (**5**). Purification by column chromatography gave a very small amount of pure **5**, with a melting point of 172 °C; the impurity was difficult to separate. The acid was converted into its ester for easier purification. GC–MS: initial temp = 75 °C, $R_f = 12.40$ min; 200 (M^+, 67%); 183 ($M - CH_3 - 2H^+$, 46%); 168 ($M - 2CH_3 - 2H^+$, 59%); 153 ($M - C_3H_{11}^+$, 100%); 125 ($M - C_4H_{11}O^+$, 54%); 97 ($M - C_5H_{11}O_2^+$, 27%); 44 (CO_2^+, 22%). IR (cm^{-1}): 1681 (C=O), 1297, 1099 (C–F). ^{19}F NMR ($CDCl_3$): $\varphi = -152.11$ ppm (multiplet, unresolved). ^{1}H NMR ($CDCl_3$): $\delta_6 = 7.91$ ppm (d-d, 1H), $J_{56} = 8.85$ Hz, $J_{F6} = 2.1$ Hz; $\delta_5 = 7.72$ ppm (d-d, 1H), $J_{56} = 8.85$ Hz, $J_{F5} = 7.32$ Hz; $\delta_{4(OCH_3)} = 3.97$ ppm (s, 3H); $\delta_{2(OCH_3)} = 4.18$ ppm (d, 3H), $J_{F2} = 2.44$ Hz; $\delta_{1(COOH)} = 2.12$ ppm (s, 1H). HRMS: $^{12}C_9{}^1H_9{}^{16}O_4{}^{19}F$: Calcd 200.04849, measured 200.04865.

4.5.6. Methyl 3-fluoro-2,4-dimethoxybenzoate (6). 1.61 g (0.008 mol) of impure 3-fluoro-2,4-dimethoxy benzoic acid in 200 ml methanol was mixed with 50 ml (0.403 mol) of 2,2-dimethoxypropane and 200 mg (0.001 mol) of toluene sulfonic acid and heated to reflux for 40 h. The mixture was poured into 400 ml water and extracted with 3×150 ml ethyl acetate. Purification by column chromatography on silica gel with a mixture of hexane–ethyl acetate (4:1) gave 0.65 g of light yellow crystals (**6**), in 30% yield from **4**. GC–MS: 214 (M^+, 33%); 183 ($M - OCH_3^+$, 100%); 168 ($M - OCH_3 - CH_3^+$, 25%); 153 ($M - OCH_3 - 2CH_3^+$, 18%); 125 ($M - OCH_3 - 2CH_3 - CO^+$, 18%); 97 ($M - OCH_3 - 2CH_3 - 2CO^+$, 10%); 44 ($CO_2^+$, 5%). ^{19}F NMR ($CDCl_3$): $\varphi = -152.87$ ppm (d-m) $J_{F5} = 7.3$ Hz. ^{1}H NMR ($CDCl_3$) $\delta_6 = 7.63$ ppm (d-d, 1H), $J_{56} = 8.85$ Hz, $J_{F6} = 2.1$ Hz; $\delta_5 = 6.73$ ppm (d-d, 1H), $J_{56} = 8.55$ Hz, $J_{F5} = 7.63$ Hz; $\delta_{4(OCH_3)} = 3.94$ ppm (s, 3H); $\delta_{2(OCH_3)} = 3.99$ ppm (d, 3H), $J_{F2} = 0.92$ Hz; $\delta_{1(COOCH_3)} = 3.90$ ppm (s, 3H).

4.5.7. 2,2′,4,4′-Tetramethoxy-3,3′-difluorobenzophenone (7). 0.650 g (0.003 mol) of methyl 3-fluoro-2,4-dimethoxybenzoate, 0.470 g (0.003 mol) of 2-fluoro-1,3-dimethoxybenzene, and 20 ml of Eaton's acid were mixed and heated to 80 °C for 3 h. The mixture was poured into 150 ml ice water and extracted with 5×50 ml ethyl acetate. Removal of solvent gave a thick red liquid that was passed through a short column of silica gel with methylene chloride. 1.00 g of **7** as a yellow-orange liquid was obtained in 97% yield. GC–MS: initial temp. = 100 °C, $R_f = 16.35$ min; 338 (M^+, 28%); 321 ($M - CH_3 - 2H^+$, 37%); 307 ($M - CH_3O^+$, 36%); 277 ($M - CH_3O - 2CH_3^+$, 10%); 183 ($C_6H_2(OCH_3)_2FCO^+$, 100%); 169 ($C_6H_2(OCH_3OH)FCO^+$, 36%); 155 ($C_6H_2(OCH_3)_2F^+$, 18%); 125 ($C_6H_2(OCH_3)F^+$, 20%); 28 (CO^+, 5%). ^{19}F NMR ($CDCl_3$): $\varphi = -153.71$ ppm (multiplet, unresolved). ^{1}H NMR ($CDCl_3$) $\delta_6 = 7.29$ ppm (d-d, 2H), $J_{56} = 8.70$ Hz, $J_{F6} = 2.13$ Hz; $\delta_5 = 6.73$ ppm (d-d, 2H), $J_{56} = 8.70$ Hz, $J_{F5} = 7.32$ Hz; $\delta_{4(OCH_3)} = 3.95$ ppm (s, 6H); $\delta_{2(OCH_3)} = 3.75$ ppm (d, 6H), $J_{F2} = 1.83$ Hz.

4.5.8. 2-Hydroxy-2′,4,4′-trimethoxy-3,3′-difluorobenzophenone (8). 1.00 g (0.003 mol) of 2,2′,4,4′-tetramethoxy-3,3′-difluorobenzophenone was dissolved in 5 ml of CH_2Cl_2 and cooled to 0 °C. 9.0 ml of a saturated BCl_3/CH_2Cl_2 solution was then added dropwise with continuous stirring. The formation of only one product was confirmed by TLC after one hour and the mixture was poured into 50 ml of ice water. The product **8** was

not isolated, but used in the next step to prepare **9**. GC–MS: initial temp. = 100 °C, R_f = 16.81 min; 324 (M^+, 10%); 293 ($M - CH_3O^+$, 100%); 183 ($C_6H_2(OCH_3)_2F$-CO^+, 17%); 168 ($C_6H_2(OCH_3O)FCO^+$, 47%); 156 ($C_6H_3(OCH_3)_2F^+$, 48%); 125 ($C_6H_2(OCH_3)F^+$, 14%); 28 (CO^+, 2%).

4.5.9. 3,6-Dimethoxy-4,5-difluoroxanthone (9). To the quenched mixture of 2-hydroxy-2′,4,4′-trimethoxy-3,3′-difluorobenzophenone obtained in the previous step, 100 ml of 2 N NaOH solution was added. The mixture was boiled until all of the CH_2Cl_2 had evaporated, and then refluxed for 1 h. A white solid (**9**) precipitated in pure form and was filtered, washed with water, and air-dried; 440 mg was obtained, in 51% yield. GC–MS: initial temp. = 100 °C, R_f = 17.06 min; 292 (M^+, 100%); 249 ($M - C_2H_3O^+$, 52%); 234 ($M - C_3H_6O^+$, 11%); 206 ($M - C_4H_6O^+$, 15%); 150 ($M - C_4H_8O_3F_2^+$, 5%); 28 (CO^+, 8%). ^{19}F NMR ($CDCl_3$) $\varphi = -157.6$ ppm (multiplet, unresolved). ^{1}H NMR ($CDCl_3$): $\delta_1 = \delta_8 = 8.07$ ppm (d-d, 2H), $J_{12} = 9.00$ Hz, $J_{F1} = 2.14$ Hz; $\delta_2 = \delta_7 = 7.05$ ppm (d-d, 2H), $J_{12} = 9.00$ Hz, $J_{F2} = 6.87$ Hz; $\delta_{3(OCH_3)} = \delta_{5(OCH_3)} = 4.05$ ppm (s, 6H). HRMS: $^{12}C_{15}{}^1H_{10}{}^{16}O_4{}^{19}F_2$: Calcd 292.05472, measured 292.05393.

4.5.10. 3,6-Dihydroxy-4,5-difluoroxanthone (10). 0.22 g (0.0007 mol) of 3,6-dimethoxy-4,5-difluoroxanthone, 2.0 g phenol, and 8.3 ml of 57% hydriodic acid were heated to reflux for one half hour, when the reaction was complete (TLC). The mixture was poured in 100 ml water, extracted with 4×50 ml ethyl acetate, and washed with 3×20 ml sodium bisulfite solution. After extraction of the solvent, the residual phenol was removed by boiling the product in 100 ml water for 1 h. Suction filtration gave 120 mg of **10** as a white-gray solid, in 60% yield. Melting point: decomposes at about 325 °C. IR: (cm^{-1}): 1634 (C=O), 1246, 1082 (C–F). ^{19}F NMR ($CDCl_3$): $\varphi = -160.39$ ppm (multiplet, unresolved). ^{1}H NMR ($CDCl_3$): $\delta_1 = \delta_8 = 7.92$ ppm (d-d, 2H), $J_{12} = 8.85$ Hz, $J_{F1} = 1.92$ Hz; $\delta_2 = \delta_7 = 7.07$ ppm (d-d, 2H), $J_{12} = 8.85$ Hz, $J_{F2} = 7.17$ Hz; $\delta_{(OH)} = 9.91$ ppm (s, 2H). HRMS: $^{12}C_{13}{}^1H_6{}^{16}O_4{}^{19}F_2$: Calcd 264.02342, measured 264.02308.

4.5.11. 3,6-Bis-(ε-bromoamyloxy)-4,5-difluoroxanthone (11). 0.140 g (0.0005 mol) of 3,6-dihydroxy-4,5-difluoroxanthone dissolved in 25 ml acetone, 0.39 g (0.0028 mol) of potassium carbonate, and 1.74 g (0.0076 mol) of 1,5-dibromopentane was refluxed for 20 h, when the reaction was complete (TLC). The solid residue was removed by suction filtration, and the product was purified by column chromatography using a mixture of hexane, ethyl acetate, and acetone (6:2.5:1.5). One hundred and twenty milligrams of **11** as white-yellow solid was obtained, in 47% yield. ^{19}F NMR (DMSO): $\varphi = -156.15$ ppm (d-m), $J_{2F} = 6.9$ Hz. ^{1}H NMR (DMSO): $\delta_1 = \delta_8 = 7.92$ ppm (d-d, 2H), $J_{12} = 9.15$ Hz, $J_{F1} = 1.83$ Hz; $\delta_2 = \delta_7 = 7.36$ ppm (d-d, 2H), $J_{12} = 9.15$Hz, $J_{F2} = 7.33$ Hz; $\delta_{(OCH_2)} = 4.26$ ppm (t, 4H), $J = 6.71$Hz; $\delta_{(BrCH_2)} = 3.58$ ppm (t, 4H), $J = 6.71$ Hz; $\delta_{(CH_2)} = 1.90$ ppm (m, 4H); $\delta_{(CH_2)} = 1.83$ ppm (m, 4H); $\delta_{(CH_2)} = 1.57$ ppm (m, 4H).

4.5.12. 3,6-Bis-(ε-*N*,*N*-diethylaminoamyloxy)-4,5-difluoroxanthone (12). Seventy-five milligrams (0.00013 mol) of 3,6-bis-(ε-bromoamyloxy)-4,5-difluoroxanthone dissolved in 5 ml DMSO with 2 ml of diethylamine was stirred at room temperature for 24 h. The end of the reaction was confirmed by TLC, with the formation of only one product (hexane, ethyl acetate, acetone, 6:2.5:1.5). The mixture was poured in 50 ml water and extracted with 3×50 ml ethyl acetate. Drying under high vacuum at room temperature for one half hour gave a light yellow oil (**12**) that was further converted into **13** without purification.

4.5.13. Hydrochloride of 3,6-bis-(ε-*N*,*N*-diethylaminoamyloxy)-4,5-difluoroxanthone (13). The 3,6-bis-(ε-*N*,*N*-diethylaminoamyloxy)-4,5-difluoroxanthone was dissolved in a few drops of methanol and acidified to pH 3 with dilute HCl solution. Drying under high vacuum with heating gave 30 mg of a light yellow solid that was confirmed by NMR to be the hydrochloride of 3,6-bis-ε-*N*,*N*-diethylaminoamyloxy-4,5-difluoroxanthone; yield 36% (from **11**). Melting point: 115 °C. IR (cm^{-1}): 1668 (C=O), 1295, 1102 (C–F). ^{19}F NMR (D_2O): $\varphi = -157.8$ ppm (br m, unresolved). ^{1}H NMR (D_2O): $\delta_1 = \delta_8 = 7.49$ ppm (d-d, 2H), $J_{12} = 9.15$ Hz, $J_{F1} = 1.83$ Hz; $\delta_2 = \delta_7 = 6.98$ ppm (d-d, 2H), $J_{12} = 9.15$ Hz, $J_{F2} = 7.35$ Hz; $\delta_{(OCH_2)} = 4.17$ ppm (t, 4H), $J = 6.11$ Hz;$\delta_{(NCH_2)} = 3.14$ ppm (t,4H), $J = 8.24$ Hz; $\delta_{(CH_2)} = 1.87$ ppm (m,4H); $\delta_{(CH_2)} = 1.77$ ppm (m, 4H); $\delta_{(CH_2)} = 1.55$ ppm (m, 4H); $\delta_{(CH_2\text{-ethyl})} = 3.18$ ppm (q, 8H), $J = 7.32$ Hz; $\delta_{(CH_3\text{-ethyl})} = 1.24$ ppm (t, 12H), $J = 7.32$ Hz. HRMS: $^{12}C_{31}{}^1H_{46}{}^{14}N_2{}^{16}O_4{}^{19}F_2$–2Cl: Calcd 547.3347, measured 547.3358.

References and notes

1. Chambers, R. D.; Hutchinson, J.; Batsanov, A. S.; Lehmann, C. W.; Naumov, D. Y. *J. Chem. Soc., Perkin Trans. 1: Org. Bioorg. Chem.* **1996**, 2271–2275.
2. Connors, K. A. *Binding Constants—The measurement of Molecular Complex Stability*: New York, NY, 1987.
3. Dolphin, D. *The Porphyrins. Physical Chemistry, Part B*: New York, NY, 1979; Vol. IV.
4. Dorn, A.; Vippagunta, S. R.; Matile, H.; Jaquet, C.; Vennerstrom, J. L.; Ridley, R. G.; Pharma Division, P.R.F.H.-L.R.L.B.S. *Biochem. Pharmacol.* **1998**, *55(6)*, 727–736.
5. Greenwood, B.; Mutabingwa, T. **2002**, *415*, 670–672.
6. Ignatushchenko, M.; Winter, R.; Riscoe, M. *Am. J. Trop. Med. Hyg.* **2000**, *62*, 77–81.
7. Ignatushchenko, M. V.; Winter, R. W.; Bachinger, H. P.; Hinrichs, D. J.; Riscoe, M. K. *FEBS Lett.* **1997**, *409*, 67–73.
8. Kelly, J. X.; Winter, R.; Peyton, D. H.; Hinrichs, D. J.; Riscoe, M. *Antimicrob. Agents Chemother.* **2002**, *46*, 144–150.
9. Knell, A. J. *Malaria: A Publication of the Tropical Programme of the Welcome Trust*: New York, NY, 1991.
10. Leed, A.; DuBay, K.; Ursos, L. M.; Sears, D.; De Dios, A. C.; Roepe, P. D. *Biochemistry* **2002**, *4132*, 10245–10255.
11. Moreau, S.; Perly, B.; Biguet, J. *Biochimie* **1982**, *64*, 1015–1025.
12. Moreau, S.; Perly, B.; Chachaty, C.; Deleuze, C. *Biochim. Biophys. Acta* **1985**, *840*, 107–116.
13. Olliaro, P. L.; Goldberg, D. E. *Parasitol. Today* **1995**, *11*, 294–297.

14. Portela, C.; Afonso, C. M.; Pinto, M. M.; Ramos, M. J. *Bioorg. Med. Chem.* **2004**, *12*, 3313–3321.
15. Portela, C.; Afonso, C. M. M.; Pinto, M. M. M.; Ramos, M. J. *FEBS Lett.* **2003**, *547*, 217–222.
16. Riscoe, M.; Kelly, J. X.; Winter, R. *Curr. Med. Chem.* **2005**, *12*, 2539–2549.
17. Schamp, N.; Verzele, M. *Bull. Soc. Chim. Belg.* **1964**, *73*, 38–43.
18. Smilkstein, M.; Sriwilaijaroen, N.; Kelly, J. X.; Wilairat, P.; Riscoe, M. *Antimicrob. Agents Chemother.* **2004**, *48*, 1803–1806.
19. Sun, W.-C.; Gee, K. R.; Klaubert, D. H.; Haugland, R. P. *J. Org. Chem.* **1997**, *62*, 6469–6479.
20. Wanzlick, H. W.; Mohrmann, S. *Chem. Ber.* **1963**, *968*, 2257–2258.
21. Winter, R. W.; Kelly, J. X.; Smilkstein, M. J.; Dodean, R.; Bagby, G. C.; Rathbun, R. K.; Levin, J. I.; Hinrichs, D.; Riscoe, M. K. *Exp. Parasitol.* **2006**, *114*, 47–56.
22. Xu Kelly, J.; Winter, R.; Riscoe, M.; Peyton, D. H. *J. Inorg. Biochem.* **2001**, *86*, 617–625.
23. Ziegler, J.; Linck, R.; Wright, D. W. *Curr. Med. Chem.* **2001**, *8*(2), 171–189.