A Nature-Inspired Betalainic Probe for Live-Cell Imaging of Plasmodium-Infected Erythrocytes

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Abstract

A model betalainic dye was semisynthesized from betanin, the magenta pigment of the red beet, and was effective for live-cell imaging of Plasmodium-infected red blood cells. This water-soluble fluorescent probe is photostable, excitable in the visible region and cell membrane-permeable, and its photophysical properties are not notably pH-sensitive. Fluorescence imaging microscopy of erythrocytes infected with Plasmodium falciparum, a causative agent of malaria in humans, showed that only the parasite was stained. Z-stacking analysis suggested that the probe accumulates proximal to the nucleus of the parasite. Indicaxanthin, one of the natural fluorescent betalains found in the petals of certain flowers, did not stain the parasite or the red blood cell.

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Introduction

Betalains are water-soluble pigments responsible for the visible fluorescence of flowering succulent plants. [1] The biosynthesis of betalains is based on the oxidative cleavage of 3,4-dihydroxyphenylalanine (DOPA) in the presence of DOPA 4,5-dioxygenase, which is encoded by the BtDOD1 gene [2], followed by a spontaneous aldime coupling between the resulting betalamic acid (HBt) and amines or amino acids. [3] Natural betalains are non-toxic antioxidants known to bind to biological membranes and interact with human lipoproteins, most likely due to their conjugated amphiphilic chemical structures (Scheme S1). [4] Extracts of plants pigmented with betalains have been used in popular medicine for the treatment of a variety of diseases including malaria, [5] a severe infectious disease responsible for millions of deaths each year worldwide. [6].

Plasmodium species are the causative agents of malaria. These obligate, intracellular organisms have a complex life cycle and switch between a mosquito vector and a vertebrate host. [7] Plasmodium spp. merozoites infect erythrocytes and undergo a differentiation process that starts with the ring stage, followed by the trophozoite stage and finally the schizont stage. Differentiation inside the erythrocyte occurs within the parasitophorous vacuole (PV), a vacuolar membrane that surrounds the intracellular parasite. The sequential pathway for the entry of exogenous nutrients required for parasite survival and multiplication includes crossing the red blood cell membrane (RBCM), the parasitophorous vacuole membrane and the parasite plasma membrane. [8] An essential aspect of the infection process is the remodeling of the RBCM and its protein constituents to permit a higher flux of nutrients and waste products into or away from the intracellular parasite. [9] A single type of broad-specificity channel, variously called the new permeation pathway (NPP), the nutrient channel, or the plasmodial surface anion channel, is responsible for the increased permeability of low molecular weight solutes, both charged and uncharged, with a strong preference for anions. [10].

Inspired by the biological role of betalains, we sought to create a new small, tunable and water-soluble molecular framework for dyes suitable for live-cell fluorescence imaging. Due to the presence of at least two carboxyl groups in the betalain framework, such dyes are negatively charged at near-neutral pH and favor cell permeation through anion channels, such as the NPP. The functional group attached to the betalainic moiety modulates the amphiphilic character of the dye and influences its electronic properties. Therefore, we prepared the artificial coumarinic dye BtC via aldime coupling of the fluorescent hydrophobic chromophore 7-amino-4-methylcoumarin (Cl20) to HBt. This dye was used for the identification of erythrocytes infected by Plasmodium spp. using fluorescence imaging.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations of the Council for International Organizations of Medical Sciences (International Guiding Principles for Biomedical Research). The authors have declared that no competing interests exist.
I. Semisynthesis

1. General. All chemicals were purchased from Sigma-Aldrich and used without further purification, except where noted. The NMR spectra were recorded using a Bruker Avance (9.4 T, 400 MHz) or Bruker Avance III (14.1 T, 600 MHz) spectrometer. Chemical shifts are reported as δ values (ppm) referenced to the solvent residual signals: D2O, δH = 4.63 (s) ppm, TFE, δH = 3.38 (m) and 5.02 (d) ppm. High-resolution mass spectra were obtained using a Bruker MicroTOF-QII mass spectrometer. Flash column chromatography was performed using 70–230 mesh silica gel. Absorption spectra were recorded in the visible region of the electromagnetic spectra (300–780 nm) at 25 ± 1 °C using a Varian Cary 50 Bio spectrophotometer equipped with a Peltier thermostated cell holder. Fluorescence spectroscopy studies were performed using a Varian Cary Eclipse spectrophotometer connected to a thermostated bath. The excitation and emission spectra were recorded at 25 °C.

1.2 Purification of betanin. Beet roots (Beta vulgaris subsp. vulgaris var. vulgaris, 0.5 kg) were peeled, sliced and homogenized in a centrifugal juice extractor (Phillips-Walita, RI1858) at the maximum speed. The juice was centrifuged (1570g, 30 min, 25 °C) and the supernatant was stored at −20 °C and used within 5 days. The betain/isobetain mixture was purified from beetroot juice by reversed-phase column chromatography (silica gel 90 C18 RP; 20 g; conditioned and eluted with deionized water at a flow rate of 0.3 mL min⁻¹). The concentration of betanin was determined by assuming a molar absorption coefficient (ε) of 6.5 × 10⁴ L mol⁻¹ cm⁻¹ at 536 nm. [11].

1.3 Betalamic acid. Betalamic acid was obtained by the alkaline hydrolysis of betanin, as previously described. [12].

UV-Vis: λmax 430 nm (water, pH 11), ε²⁻⁰⁻⁰ = 2.65 × 10⁴ L mol⁻¹ cm⁻¹ (water, pH 11); Fl: λmax 500 nm (water, pH 11), λexc 420 nm.

1.4 Indoxanthenin (BtP). L-Proline (100 equiv.) was added to an aqueous solution of betalamic acid (0.5–1.0 mL, pH 10), and the resulting solution was stirred at room temperature (RT). The reaction was stopped by ultrasonic irradiation for 5 min and stirred at RT for an additional 30 min. The reaction was spectrophotometrically monitored for the appearance of the BtP band at 520 nm. After completion, the solution was cooled (0 °C), and HCl (conc.) was slowly added until the solution reached pH 3. The resulting solution was then stirred at RT in the dark for 2 hours. The product was purified via gel-permeation chromatography using lipophilic Sephadex LH-20 with water as the eluent. The fractions containing BtP were combined and lyophilized. The resulting purple powder was stored at −20 °C, and the purity of the product was verified by RP-HPLC analysis prior to use. In a typical run, 0.7 mg (3.2 µmol) of betalamic acid yielded 0.4 mg (1 µmol, 31%) of BtP.

1-H-NMR (600 MHz, D2O/TMSP-d4, Scheme S3): δ 8.46 (s, 1H, N-H), 8.08 (d, 3J13,14 = 12.4 Hz, 1H, H-10), 7.73 (d, 3J11,14 = 8.4 Hz, 1H, H-14), 7.17 (d, 3J13,14 = 8.4 Hz, 1H, H-13), 7.12 (bs, 1H, H-17), 6.27 (s, 1H, H-5), 6.23 (s, 1H, H-20), 6.07 (d, 3J10a,10b = 12.4 Hz, 1H, H-9), 4.40 (bt, 3J9,10 = 8.3 Hz, 1H, H-2), 3.50 (dd, 3J5a,5b = 17.5 Hz, 3J5b,8 = 8.3 Hz, 1H, H-3a), 3.10 (dd, 3J9,10 = 17.5 Hz, 3J13,17 = 8.3 Hz, 1H, H-3b), 2.43 (s, 3H, H-22). [13]

1-C-NMR (125 MHz, D2O/TMSP-d4, Scheme S3): δ 147.7 (C-10), 129.4 (C-11), 116.2 (C-12), 114.1 (C-20), 110.4 (C-9), 108.4 (C-5), 105.7 (C-17), 56.6 (C-2), 20.7 (C-22).

1-C-NMR (125 MHz, TFE-capillary D2O/TMSP-d4, Scheme S3): δ (ppm) 148.3 (C-10), 129.6 (C-11), 116.4 (C-12), 114.6 (C-20), 111.2 (C-17), 109.6 (C-5), 107.4 (C-17), 20.1 (C-22).

LC-ESI(+)-MS: Rf = 5.6 min, [M+H]+ observed: 369.1. 

HRMS (m/z): [M+H]+ calculated for C19H17N2O6+, 369.1078; observed, 369.1082 (dift: ±0.08 ppm).

UV/Vis: λmax 520 nm (water), ε²⁻⁰⁻⁰ = 6.6 × 10⁴ L mol⁻¹ cm⁻¹ (water).

Color: CIE Lab D65/10: L* = 90.4, a* = 25.9, b* = 3.9, C* = 26.2, and h* = 331.4.

Fluorescence: λmax 570 nm (water, λexc 510 nm), ε²⁻⁰⁻⁰ = 4.3 × 10⁴ L mol⁻¹ cm⁻¹ (water).

II. Spectrophotometric Methods

The molar absorption coefficient (ε) of BtC was determined using an end-point method by performing a set of betalin degradation experiments, as previously described. [16]

Brieﬂy, the alkaline hydrolysis of BtC (Britton-Robinson buffer, pH 9) was spectrophotometrically monitored for 30 min at 1-min intervals. The initial pigment concentration and ε of BtC were determined using an end-point method by performing a set of betalin degradation solutions with known
concentrations. The betalamic acid solutions were stable under the experimental conditions, and no appreciable change in the spectral properties could be detected after 30 min.

**Color analysis** was performed using the CIE L*a*b* color space employing the Color software (v.3.1, Startek Technologies). L* indicates the lightness, and its value ranges from 0 (an ideal black object) to 100 (an ideal white object). In the CIE L*a*b* system, a* and b* are the chromaticity coordinates. A positive a* value indicates the red direction, and a negative a* value is the green direction; a positive b* value is the yellow direction, and a negative b* value is the blue direction. Two derived color parameters, the hue angle (\( h^u = \arctan2(b^*, a^*) \times 180/\pi \)) and the Chroma value (\( C^* = (a^2 + b^2)^{1/2} \)), were calculated.

The fluorescence quantum yield (\( \Phi_{FL} \)) was measured relative to fluorescein (NaOH(aq) 0.1 mol L–1, \( \Phi_{FL} = 0.79 \), EX 470–500 nm, EM 500–600 nm) with excitation at 475 nm. Fluorescence spectra of the standard and BtC were obtained under identical spectrometer conditions in triplicate and averaged (excitation at 475 nm, slits 2.5 nm (EX) and 5.0 nm (EM), photomultiplier power 600 V). The optical density at the peak was maintained below 0.1 to avoid inner-filter effects, and the integrated intensities of the emission spectra, corrected for differences in the indexes of refraction and concentration, were used to calculate the \( \Phi_{FL} \) using Eq. 1,

\[
\Phi_{FL}^X = \Phi_{FL}^P \frac{A^P}{A^X} \frac{S^X}{S^P} \left( \frac{n^X}{n^P} \right)^2
\]

where \( A \) is the absorbance at 475 nm, \( n \) is the refractive index of the solvent, \( S \) is the integrated area under the fluorescence

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**Figure 1.** Labeling of live *P. falciparum*-infected erythrocytes with BtC. BtP was used as the betalain control. Panels show representative wide-field images of three independent experiments. The arrow indicates the non-fluorescent hemoglobin crystal inside the DV of a mature trophozoite. \([\text{BtC}] = 28 \mu\text{mol L}^{-1}; [\text{BtP}] = 170 \mu\text{mol L}^{-1}; \) incubation = 2 min at 25°C; bar = 10 μm.

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spectrum and $\Phi_L$ are the absolute quantum yields; the superscripts $X$ and $P$ refer to the sample and standard, respectively.

III. Analytical Methods

**Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis.** Analytical RP-HPLC separations and analyses were performed using a Waters 2695 Alliance system instrument with a UV-Vis detector (dual-wavelength, Waters 2489) and a Supelcosil LC-18 (5 μm particle size, L×I.D.: 150×46 mm, Supelco) C18 column. The LC analysis conditions were as follows: Solvent A, water with 0.1% v/v TFA; Solvent B, 60% v/v MeCN/water with 0.1% v/v TFA, linear gradient from 5% to 95% B in 20 min at 25°C, flow rate: 1 mL min$^{-1}$, injection volume: 10 μL, spectrophotometric detection set at 254/536 nm for Bn, 484 nm for BtP and 520 nm for BtC. The analysis of BtC was performed in the absence of acid.

**HPLC-DAD-ESI(+)-MS/MS analysis** was performed using a Bruker Daltonics Esquire HCT ion trap mass spectrometer equipped with an electrospray source and coupled to a Shimadzu Prominence liquid chromatograph. The chromatograph was equipped with a Luna C18 column (3 μm particle size, L×I.D.: 150×2.0 mm, Phenomenex) maintained at 30°C and a PDA SPD-M20A detector. Nitrogen was used as the nebulizing (45 psi) and drying gas (6 L min$^{-1}$), 300°C), and helium was used as the buffer gas (4×10$^{-6}$ mbar). The capillary high voltage was set to 5,000 V. To avoid space-charge effects, the smart ion charge control (ICC) was set to the arbitrary value of 50,000.

**High-resolution mass spectrometric (HRMS) analysis** was performed using a Bruker MicroTOF-QII spectrometer with an electro spray ionization source with a capillary potential of 350/470 nm) and the exposure band-pass 525/50) with a Zeiss Axio Observer Z1 epifluorescence microscope (Carl Zeiss MicroImaging, 38 HE GFP filter set, excitation band-pass 470/40, beam splitter FT 495, emission band-pass 525/50) with a 63×/1.44 oil-immersion objective, image acquisition time was 10,000 ms. Differential interference contrast (DIC) images were obtained using transmitted light and recorded using the Axio Vision software.

**Confocal fluorescence microscopy.** Cells were treated as described for the epifluorescence imaging and incubated for 2 min in the dark at 4°C with BtC (35 μmol L$^{-1}$) and washed three times with PBS. The cells were subsequently stained with Hoechst 33258 dye (3.6 μmol L$^{-1}$) for 2 min. The coverslips were mounted on slides using 4 μL of ProLong Gold (Invitrogen) and observed in a Leica SP5 TS confocal microscope with a 100×/1.44 oil-immersion objective; the Z series was acquired according to the sampling criteria built into the software for sequential imaging of the Hoechst 33258 dye, which stains the nucleus (blue, EX/EM = 350/470 nm) and the BtC (green, at EX/EM = 520/570 nm). All images (8-bit grayscale) were acquired with the same exposure time and sensitivity. The raw images were processed with the ImageJ software.

IV. Experiments with Parasites

**Parasite cultivation.** *Plasmodium chabaudi* (clone AS) was maintained in BALB/C mice by weekly transfers from previously infected mice. Animals infected at the trophozoite stage (parasitaemia approximately 60%) were sacrificed, the blood was collected and leukocytes and platelets were removed from the whole blood by filtration through a powdered cellulose column (Whatman CF11). The trophozoite-infected erythrocytes were then washed three times by centrifugation (1,500×g, 5 min) in PBS. The parasites (10$^7$ cells mL$^{-1}$) were isolated by the lysis of the erythrocyte membranes with saponin in PBS (10 mg mL$^{-1}$). After pelleting to remove the red blood cell membranes, the parasites were washed twice in PBS by centrifugation (2,000×g, 4°C). *Plasmodium falciparum* (3D7 strain) was cultured as previously described [17] in RPMI 1640 medium supplemented with 10% human serum in an atmosphere of 5% O$_2$, 5% CO$_2$ and 90% N$_2$.

**Epifluorescence microscopy.** The parasitized erythrocytes were mounted wet on a glass-bottom petri dish (MatTek Corporation) treated with poly-L-lysine, incubated with BtC for 2 min, washed once with PBS and imaged within 10 min at 20°C using a Zeiss Axio Observer Z1 epifluorescence microscope (Carl Zeiss MicroImaging, 38 HE GFP filter set, excitation band-pass 470/40, beam splitter FT 495, emission band-pass 525/550) with a 63×/1.44 oil-immersion objective, image acquisition time was 10,000 ms. Differential interference contrast (DIC) images were obtained using transmitted light and recorded using the Axio Vision software.

V. Computational Methods

Gaussian09 was used for all of the quantum-chemical calculations. [18] All of the structures were optimized in the gas phase at the cam-B3LYP/6-31+G(d,p) level. [19] Stationary points were characterized as minima based on vibrational analysis, and the
coordinates of BtC in seven different protonation states are provided. The SMD parameterization of the IEPPCM was employed to model the effect of the solvent (water). Molecular descriptors of the betalains were estimated as the means of the Marvin Sketch 5.10.3 prediction program (ChemAxon, Budapest, Hungary). The LogD was predicted according to the method of Viswanadhan and collaborators. [20] The pKa values of the carboxyl portions of BtC were obtained based on the partial charges, [21] and the values were compared to those determined by quantum mechanics using a thermodynamic cycle [22].

Results and Discussion

Betalamic acid was prepared via the alkaline hydrolysis of betanin Ba, purified from red beet juice [23] and then coupled with C120 in water to yield BtC. The dye was purified by gel-permeation chromatography on lipophilic Sephadex LH-20 and subsequently characterized (File S1). The absorption and fluorescence spectra of BtC (λabs, W = 520 nm, λflu, W = 570 nm, h = 351 (D65/10–3), εW = 66,000 L mol–1 cm–1) are red-shifted relative to that of C120 (λabs, MeOH = 343 nm, λflu, MeOH = 445 nm, εMeOH = 16,180 L mol–1 cm–1) [24] due to the extended conjugation of BtC. Furthermore, the Stokes shift of the betalin is smaller than that of C120 (i.e., ΔC120 = 6685 cm–1 (102 nm), ΔC120 = 1687 cm–1 (50 nm), Figure S1). The betalin BtC is water-soluble and excitable with visible light, two relevant improvements over the majority of coumarins used as fluorescent probes. However, BtC is several orders of magnitude less fluorescent in solution than is C120 (fluorescence quantum yield (ΦFL) of BtC in water = 4.3 × 10–3 vs. ΦFL of C120, MeOH = 0.51). The lack of a fluorescence signal from the uninfected erythrocytes is likely a result of the low quantum yield of BtC. However, a weak fluorescence signal is convenient for the quantification of the probe uptake and accumulation inside the parasite. [25] Finally, BtC is reasonably stable in aqueous media [half-life: 118 ± 10 min at pH 7.0, (kabs = 6.4 ± 1.0 × 10–3 min–1 (HPLC); 5.4 ± 0.8 × 10–3 min–1 (UV-Vis) (Figure S2), pKa (hydrolysis constant) = 9.0 (Figure S3)], half-life: 2 days in 60% v/v aqueous 2,2,2-trifluoroethanol [26] and only weakly prone to photobleaching, as indicated by the emission profile under pulsed light irradiation at 520 nm in the presence or absence of infected erythrocytes (Figure S4).

To test the ability of BtC to label living Plasmodium spp.-infected red blood cells, parasitized erythrocytes were incubated with BtC and examined by fluorescence microscopy. Tests with P. berghei, P. chabaudi and P. falciparum and P. berghei resulted in similar results; therefore, only the results for the labeling of P. falciparum are presented. As shown in Figure 1, BtC selectively accumulates within the infected cells at the point of parasite localization, even in doubly infected erythrocytes. Importantly, no stain was observed in uninfected cells or in infected-erythrocytes incubated with a control probe, indicaxanthin BtP, ΦFL = 4.6 × 10–3 at pH 6.0. In more mature stages of the intraerythrocystic developmental cycle, the accumulation of BtC is even more evident because fluorescence was commonly observed throughout almost the entire parasite (excluding the hemoglobin crystals, which are most likely accumulated in the digestive vacuole (DV)). Furthermore, the fluorescence profile of the probe within the parasite is nearly identical to that in aqueous solution, indicating that the dye is not chemically modified upon uptake (Figures S4 and S5).

Confocal Z-stack sections of P. falciparum-infected erythrocytes revealed granular fluorescence in an unidentified compartment within the cytoplasm of the parasite (Figure 2 and Video S1). These results indicate that BtC may be taken up from the medium into the erythrocyte and then transferred to the parasite, where it accumulates within this intraparasitic compartment. Interestingly, the fixation of infected cell cultures with methanol, acetone or paraformaldehyde prior to or after incubation with BtC resulted in a loss of fluorescence (data not shown). The increase in cell membrane permeability caused by most fixing agents may favor the passive diffusion of the fluorescent probe and compromise the accumulation of the dye. [27].

Although the mechanism responsible for the vacuolar accumulation of BtC within Plasmodium remains undetermined, it is known that Ba and BtP are both incorporated into intact red blood cells. [28] The uptake of secondary metabolites is often coupled to a pH gradient between the vacuoles and the cytosol. The intracellular pH (pHi) of red blood cells, 7.33 [29], is higher than that of the parasite’s cytosol (pH = 7.1) [30], cytosomal vesicles (pH = 7) [31] or DV (pHi = 5.4–5.5) [32]. BtC is expected to be fully protonated at pH < 3 and has a double negative charge at pH 7.4 (Scheme S2). The experimental pKa of the imine moiety was determined to be 5.0 ± 0.2, although the ΦFL of BtC does not depend on the pH of the medium from pH 3 to 8 (Figure S3). Although BtC occurs in a more negative form in the cytoplasm than inside the acidic vacuoles, dye accumulation cannot be easily explained exclusively on this basis. Lipophilicity may also play a fundamental role in the vacuolar uptake of the betalamic dye. The theoretical distribution coefficient logD values of BtP are –5.5, –8.3 and –9.7 at pH 5.0, 6.5 and 7.4, respectively, which agrees with the reported experimental value of –7.25 at pH 6.0 [4]. For BtC, the values of logD are less negative, i.e., –1.8 (pH 5.0), –3.2 (pH 6.5) and –3.9 (pH 7.4), which suggests that the increased lipophilicity of the coumarin betalain may favor the crossing of cellular membranes. However, active transport cannot be ruled out because the uptake of betaxanthins into the vacuoles of an artificial mini-protoplast system has been reported to be MgATP-dependent. [33].

The lack of genetic material in red blood cells allows the detection of malaria parasites in blood samples by light or fluorescence microscopy by using DNA/RNA staining methods. [34] Giemsa staining is frequently used to examine blood films and smears due to its high sensitivity and low cost. The drawbacks of this method include extensive cell manipulation, long-term requirement (45 min or even longer), and the occurrence of either artifacts and pigment dots. [35,36] Staining with the BtC probe requires less than 5 min of incubation and allows live-cell imaging with minimal cell manipulation. Acidine orange is widely used for the fluorescent staining of parasitic DNA/RNA because it also circumvents some problems of the Giemsa method. However, despite the speed and versatility of the acidine orange staining, [35] this dye was found to be carcinogenic. [37,38] Other organic fluorophores are available for functional parasite imaging, such as the calcium-sensitive dye Fura Red, which has been used to determine the accumulation of calcium ions in the parasite food vacuole. [39] Malaria parasites have also been selectively imaged with wide-field fluorescence microscopy using fluorescent proteins or quantum dots. [34] However, most of these methods are designed for the study of specific cellular processes and are often more complex and expensive than the BtC staining.

In conclusion, BtC belongs to a new class of fluorescent dyes that can be easily semisynthesized from an abundant, non-toxic and inexpensive natural pigment. These betalamic conjugates have tunable properties through variations of the imine substituent and improved water solubility and affinity to biomolecules relative to the precursor amines. Finally, the betalamic acid scaffold may be useful for the rational design of selective probes for other biological imaging applications, including fluorescent protein tagging.
Supporting Information

Figure S1 Normalized absorption (--) and fluorescence emission (---) spectra of C120 (black) and BtG (green) in PBS. (TIFF)

Figure S2 Hydrolysis of BtC monitored by UV-Vis absorption spectroscopy or HPLC-DAD analysis. (A) Changes in the absorption spectrum of BtC in aqueous buffer (pH = 7.0) over 66 h and (B) the corresponding kinetic profile; (C) Changes in the chromatographic profile of BtC over 19 h and (D) the corresponding kinetic profile of HBt (red, t_R = 1.2 min). BtG (black, t_R = 5.5 min), C120 (blue, t_R = 11.6 min) and an unknown decomposition product (green, t_R = 2.4 min). (TIFF)

Figure S3 (A) Effect of pH on the absorption and fluorescence spectra of BtC, and (B) dependence of the second-order absorption and emission maxima on the pH. (TIFF)

Figure S4 Fluorescence decay of BtC in water, in isolated P. chabaudi and in P. chabaudi-infected red blood cells. Experiments with parasites were performed with an incubation – centrifugation – washing – resuspension sequence. (BtC) = 50 μmol L⁻¹; EM 570 nm. (TIFF)

Figure S5 Fluorescence profiles of BtC in aqueous solution (control) and in suspension containing P. chabaudi-infected erythrocytes. [BtC] = 50 μmol L⁻¹, 5 min incubation and washing (5 ×), EX 520 nm. (TIFF)

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