A Redox-Active Fluorescent pH Indicator for Detecting *P. falciparum* Strains with Reduced Responsiveness to Quinoline Antimalarial Drugs

Mouhamad Jida,[#a] Cecilia P. Sanchez, [#b] Karène Urgin,[a] Katharina Ehrhardt,[ab] Saravanan Mounien,[a] Aurelia Geyer,[a] Mourad Elhabiri,[a] Michael Lanzer,[*b] Elisabeth Davioud-Charvet*[a]

[a]UMR 7509 Centre National de la Recherche Scientifique and University of Strasbourg, European School of Chemistry, Polymers and Materials (ECPM), 25, rue Becquerel, F-67087 Strasbourg, France.

[b]Zentrum für Infektiologie, Parasitologie, Universität Heidelberg, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany.

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I. GENERAL INFORMATION

Solvents and reagents: Commercially available starting materials were purchased from Sigma-Aldrich, ABCR GmbH & Co. KG, Alfa Aesar, and Apollo Scientific and were used without further purification. Solvents were obtained from Sigma-Aldrich and Carlos Erba; unless noticed reagent grade was used for reactions and column chromatography (analytical grade) was used for recrystallizations. When specified, anhydrous solvents were required; dichloromethane (DCM) was distilled over CaH_2 under argon. Tetrahydrofuran (THF) was distilled over sodium/benzophenone under argon or dried by passage through an activated alumina column under argon. 1,4-Dioxane and dimethylformamide (DMF) were purchased anhydrous over molecular sieves from Sigma-Aldrich. All reactions were performed in standard glassware. Thin Layer Chromatography (TLC) were used to monitor reactions (vide infra). Crude mixtures were purified either by recrystallization or by flash column chromatography. The latter were performed using silica gel 60 (230-400 mesh, 0.040-0.063 mm) purchased from E. Merck. Automatic flash chromatographies were carried out in a Biotage Puriflash apparatus with UV-Vis detection at 254 nm (unless otherwise specified). Flash chromatography was performed using silica gel G60 (230–400 mesh) from Macherey Nagel. Monitoring and primary characterization of products were achieved by Thin Layer Chromatography on aluminium sheets coated with silica gel 60 F254 purchased from E. Merck. Eluted TLC's were revealed under UV (325 nm and 254 nm) and with chemicals.

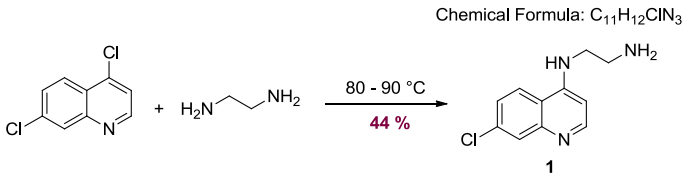
Nuclear Magnetic Resonance (NMR) The Nuclear Magnetic Resonance (NMR) spectra were registered in a *Bruker (Bruker DRX-300) avance 300* apparatus (^{1}H NMR 300 MHz, ^{13}C NMR 75 MHz) at the ECPM. *Bruker avance 400* apparatus was used (^{1}H NMR 400 MHz, ^{13}C NMR 100 MHz) for more complex spectra at the ECPM. All chemical shifts (δ) are quoted in parts per million (ppm) relative to TMS. The chemical shifts are referred to the used partial deuterated NMR solvent (for $CDCl_3$: ^{1}H NMR, 7.27 ppm and ^{13}C NMR, 77.16 ppm). The coupling constants (J) and the non-equivalence ($\Delta\nu$) are given in Hertz (Hz). Resonance patterns are reported with the following notations: br (broad), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets). In addition, the following acronyms will be used: C_q: quaternary carbon; CH_2: secondary carbon; CH_3: methyl group.

Microanalyses : Microanalyses were obtained at *"Service de Microanalyses" at the institut de chimie de Strasbourg.* ***Mass spectrometry:*** Mass spectra (ESI-MS) were obtained on a microTOF LC spectrometer (Bruker Daltonics, Bremen). High Resolution Mass (HRM) spectra were measured and fitted with calculated data. ***Melting point*** Melting points were determined on a Büchi melting point apparatus and were not corrected. ***Infrared spectroscopy.*** Infra red (IR) spectra were recorded on a diamond ATR spectrometer using neat samples. Infrared frequencies are reported in wave-

numbers (cm^{-1}), intensities were determined qualitatively and are indicated as vs (very strong), s (strong), m (medium), w (weak), b (broad).

II. GENERAL PROCEDURE FOR THE TOTAL SYNTHESIS OF FLUO-CQ (6)

1. Synthesis of N^1-(7-chloroquinolin-4-yl)ethane-1,2-diamine (1)



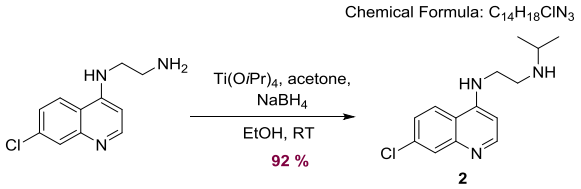
A mixture of 4,7-dichloroquinoline (15 g, 75.74 mmol, 1 eq.), and ethylenediamine (20.3 mL, 303 mmol, 4 eq.) was stirred at 80-90°C for 3.5 hours under Argon and then cooled to room temperature. A solution of 1N NaOH (150 mL) was added and the mixture was extracted with a solution of DCM/MeOH (8/2) (4x100 mL), dried over $MgSO_4$ and concentrated under vacuum (Crude: 11.2 g). The crude was recrystallized from ethanol to afford the desired compound (**1**) as a White solid (7.47 g) Yield 44 %.

Rf = 0.06 (eluent: DCM/MeOH, 8/2 at 10% in Triethylamine)

^{1}H NMR (400 MHz, CD_3OD): δ 8.36 (d, *J* = 5.7 Hz, 1H, Qn*H*), 8.13 (d, *J* = 9.1 Hz, 1H, Qn*H*), 7.78 (d, *J* = 2.2 Hz, 1H, Qn*H*), 7.40 (dd, *J* = 2.2, 9.1 Hz, 1H, Qn*H*), 6.57 (d, *J* = 5.7 Hz, 1H, Qn*H*), 3.46 (t, *J* = 6.4 Hz, 2H, CH_2), 2.98 (t, *J* = 6.4 Hz, 2H, CH_2).

^{13}C NMR (100 MHz, CD_3OD): δ 152.8 (C_q), 152.5 (C_q), 149.7 (*C*H), 136.4 (C_q), 127.6 (*C*H), 126.0 (*C*H), 124.3 (*C*H), 118.8 (C_q), 99.7 (*C*H), 46.3 (CH_2), 40.9 (CH_2).

2. Synthesis of N^1-(7-chloroquinolin-4-yl)-N^2-isopropylethane-1,2-diamine (3)



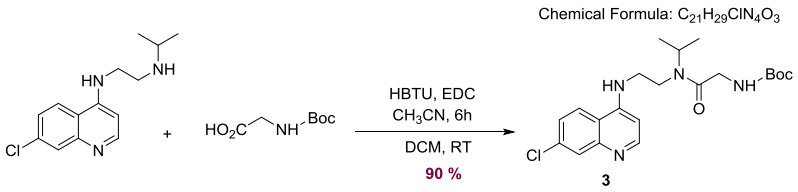
A mixture of acetone (0.796 mL, 10.8 mmol, 2.4 eq.), titanium tetraisopropoxide (2.7 mL, 9.02 mmol, 2 eq) and primary amine **(1)** (1 g, 4.51 mmol, 1 eq.) in absolute EtOH (7 mL) was stirred for 7 h at room temperature under argon. Then $NaBH_4$ (0.256 g, 6.77 mmol, 1.5 eq.) was added and the resulting mixture was stirred for 17 h. The mixture was slowly poured into 13.5 mL of a 2M NH_4OH solution. The resulting white inorganic precipitate was filtered and washed with DCM (20 mL). Phases were separated and the water layer was extracted with DCM (20 mL). The combined organic layers were extracted with 10 mL of a 1N HCl solution. The acidic layer was washed with DCM (20 mL) then treated with 2N NaOH solution to reach pH 10-12 and extracted with DCM (3x20 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under vacuum to afford the product (**2**) as colorless oil (1.09 g). Yield 92 %.

Rf = 0.5 (eluent: DCM/MeOH, 8/2 at 5% in triethylamine)

^{1}H NMR (400 MHz, CD_3OD): δ 8.35 (d, *J* = 5.6 Hz, 1H Qn*H*), 8.08 (d, *J* = 9.0 Hz, 1H, Qn*H*), 7.77 (d, *J* = 2.1 Hz, 1H, Qn*H*), 7.39 (dd, *J* = 2.1, 9.0 Hz, 1H, Qn*H*), 6.56 (d, *J* = 5.6 Hz, 1H, Qn*H*), 3.48 (t, *J* = 6.6 Hz, 2H, CH_2), 2.91 (t, *J* = 6.6 Hz, 2H, C*H*), 2.86 (sept, *J* = 6.0 Hz, 1H, C*H*), 1.09 (d, *J* = 6.0 Hz, 6H, CH_3)

^{13}C NMR (100 MHz, CD_3OD): δ 152.7 (C_q), 152.5 (C_q), 149.7 (*C*H), 136.3 (C_q), 127.6 (*C*H), 126.1 (*C*H), 124.3 (*C*H), 118.8 (C_q), 99.7 (*C*H), 49.8 (*C*H), 46.0 (*C*H_2), 43.6 (*C*H_2), 22.5 (*C*H_3).

3. Synthesis of tert-butyl (2-((2-((7-chloroquinolin-4-yl)amino)ethyl)(isopropyl)amino)-2-oxoethyl)carbamate (2)



To a solution of Boc-Glycine (1 g, 5.68 mmol, 1.5 eq.) and amine (**2**) (1 g, 3.79 mmol, 1 eq.) in DCM (20 mL) were added HBTU (1.44 g, 3.79 mmol, 1 eq.) and EDC (0.69 mL, 3.79 mmol, 1 eq.). The mixture was stirred for 3 days. Then, 1 eq. of Boc-Glycine was added. The mixture was stirred for 1 day. Then water (10 mL) and 10 mL of 1N NaOH solution were added. Phases were separated and the water layer was extracted with DCM (3x15 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under vacuum (crude: 3 g). The crude was purified with a silica gel column chromatography (eluent: AcOEt/MeOH, 9/1). The product (**3**) was obtained as a White solid (1.29 g). Yield 90 %.

Rf = 0.61 (eluent: DCM/MeOH, 9/1)

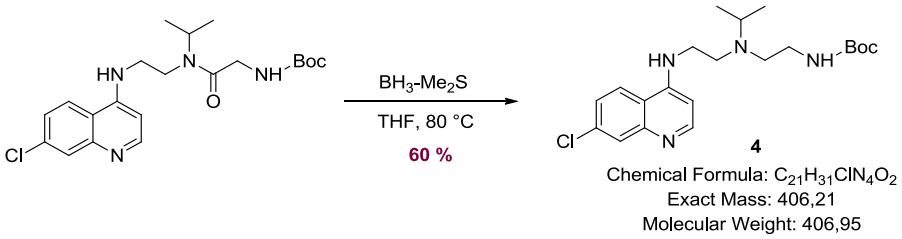
Mp = 178-180°C

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.48 (d, *J* = 5.43 Hz, 1H, Qn*H*), 7.93 (d, *J* = 2.20 Hz, 1H, Qn*H*), 7.76 (d, *J* = 9.02Hz, 1H, Qn*H*), 7.38 (dd, *J* = 8.88 Hz and *J* = 2.21 Hz, 1H, Qn*H*), 6.29 (d, *J* = 5.49 Hz, 1H, Qn*H*), 5.56 (bs, 1H, CH_2), 4.10-4.03 (m, 2H, CH_2 + N*H*), 3.99 (sept, 1H, C*H*), 3.75-3.71 (m, 2H, CH_2), 3.45-3.40 (m, 2H, CH_2), 2.53 (bs, 1H, N*H*), 1.46 (s, 9H, CH_3), 1.27 (d, *J* = 6.74 Hz, 6H, CH_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ 171.08 (C_q), 155.89 (C_q), 151.67 (*C*H), 150.32 (C_q), 148.74 (C_q), 135.17 (C_q), 128.25 (*C*H), 125.25(*C*H), 122.18(*C*H), 117.30 (C_q), 98.17 (*C*H), 80.11 (C_q), 48.19 (*C*H), 45.71 (*C*H_2), 42.86 (*C*H_2), 39.82 (*C*H_2), 28.51 (*C*H_3), 21.25 (*C*H_3).

IR : 3370 (w), 2972 (w), 1697 (m), 1662 (s), 1580 (vs), 1537 (m), 1427 (m), 1370 (m), 1290 (s), 1161 (s), 1051 (m), 854 (m), 812 (m), 766 (m).

4. Synthesis of tert-butyl (2-((2-((7-chloroquinolin-4-yl)amino)ethyl)(isopropyl)amino)ethyl) carbamate (4)



To a solution of the protected amine (**3**) (1.9 g, 4.51 mmol, 1 eq.) in anhydrous THF (10 mL) under reflux was added dropwise a 2M borane-methyl sulfide complex solution in THF (5.64 mL, 11.3 mmol, 2.5 eq.). The mixture was stirred for 35 minutes under reflux. The mixture was poured into 50 mL of a 3M HCl solution and 75 mL of a 3M NaOH solution were added. The mixture was extracted with DCM (3x100mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under vacuum (crude: 1.95 g). The crude was purified on an automatic silica column chromatography (eluent: $CHCl_3$/MeOH, gradient: (100/0 -> 90/10). The product was obtained as colorless oil (500 mg). The product (**4**) was precipitated from petroleum ether. The product was obtained as colorless oil (487 mg). Yield 60 %.

Rf = 0.49 (eluent: DCM/MeOH, 9/1)

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.52 (d, J = 5.32 Hz, 1H, Qn*H*), 7.94 (d, J = 2.21 Hz, 1H, Qn*H*), 7.71 (d, J = 9.01 Hz, 1H, Qn*H*), 7.35 (dd, J = 9.07 Hz and J = 2.44 Hz, 1H, Qn*H*), 6.35 (d, J = 5.33 Hz, 1H, Qn*H*), 5.94 (bs, 1H, N*H*), 4.76 (bs, 1H, N*H*), 3.28-3.23 (m, 2H, CH_2), 3.19-3.13 (m, 2H, CH_2), 3.01 (sept, 1H, C*H*), 2.83-2.79 (m, 2H, CH_2), 2.61-2.57 (m, 2H, CH_2), 1.30 (s, 9H, CH_3), 1.05 (d, J = 6.65 Hz, 6H, CH_3).

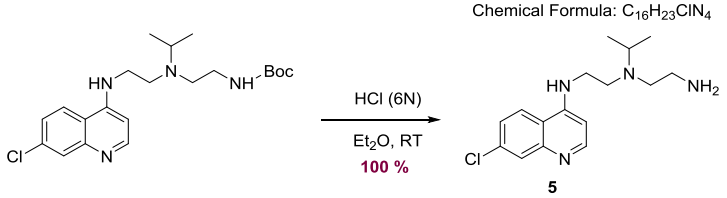
^{13}C NMR (75 MHz, $CDCl_3$): δ 156.18 (C_q), 152.24 (*C*H), 149.90 (C_q), 149.34 (C_q), 134.88 (C_q), 128.91 (*C*H), 125.50 (*C*H), 121.31 (*C*H), 117.63 (C_q), 99.48 (*C*H), 79.43 (C_q), 50.38 (*C*H), 49.56 (*C*H_2), 47.88 (*C*H_2), 40.57 (*C*H_2), 39.99 (*C*H_2), 28.40 (*C*H_3), 18.31 (*C*H_3).

IR: 3349 (w), 2967 (w), 2928 (w), 2852 (w), 1693 (m), 1579 (vs), 1522 (m), 1364 (m), 1280 (m), 1248 (m), 1167 (s), 1137 (m), 1080 (m), 875 (w), 805 (w).

Elemental analysis: calcd (%) for $C_{21}H_{31}ClN_4O_2$: N, 13.63; C, 62.11; H, 7.62; found N, 13.77; C, 61.98; H, 7.68.

HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{21}H_{32}ClN_4O_2$ 407.2184; found 407.2202.

5. Synthesis of *N*^{1}-(2-aminoethyl)-*N*^{2}-(7-chloroquinolin-4-yl)-*N*^{1}-isopropylethane-1,2-diamine (5)



To a solution of the protected amine (**4**) (100 mg, 0.246 mmol, 1 eq.) in ether (5 mL) was added HCl 6N (2 mL, 12 mmol, 48.8 eq.). The mixture was stirred for 30 min at room temperature. A 1N NaOH solution was added until a white precipitate appears. The mixture was extracted with DCM (3 x 10mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under vacuum to afford pure product (**5**) quantitatively as colorless oil compound (75.4 mg). Yield 100 %

The compound was involved directly in the next step (peptide coupling reaction) without further purification.

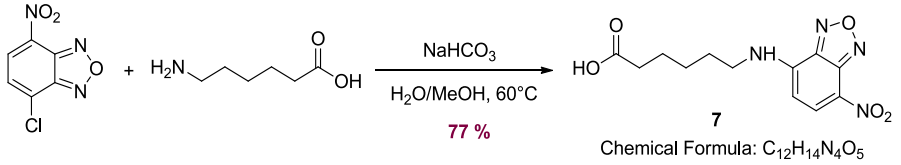
^{1}H NMR (300 MHz, $CDCl_3$): δ 8.40 (d, *J* = 5.65 Hz, 1H, Qn*H*), 7.90 (d, *J* = 8.97 Hz, 1H, Qn*H*), 7.85 (d, *J* = 2.45 Hz, 1H, Qn*H*), 7.29 (dd, *J* = 9.03 Hz and *J* = 2.17 Hz, 1H, Qn*H*), 6.32 (d, *J* = 5.98 Hz, 1H, Qn*H*), 3.32-3.28 (m, 2H, CH_2), 2.99 (sept, 1H, CH), 2.85-2.77 (m, 4H, CH_2), 2.64-2.60 (m, 2H, CH_2), 1.03 (d, *J* = 6.56 Hz, 6H, CH_3).

^{13}C NMR (300 MHz, $CDCl_3$): δ 152.1 (*C*H), 150.2 (C_q), 149.3 (C_q), 134.8 (C_q), 128.7 (*C*H), 125.2 (*C*H), 121.8 (*C*H), 117.7 (C_q), 99.3 (*C*H), 52.1 (*C*H_2), 50.3 (*C*H_2), 47.7 (*C*H_2), 41.0 (*C*H_2), 40.9 (*C*H_2), 18.2 (*C*H_3).

IR: 3279 (w), 2963 (w), 2920 (w), 2850 (w), 1578 (vs), 1450 (m), 1366 (m), 1329 (m), 1137 (m), 1079 (m), 875 (m), 804 (m), 767 (m).

6. Synthesis of the Key intermediate: 6-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)hexanoic acid (7)[1]

As described by Elliot and Prestwich,[2] NBD-6-aminohexanoic acid (**7**) was obtained by an economical nucleophilic substitution between 6-aminohexanoic acid and NBD-chloride in the presence of excess of sodium bicarbonate (3 equiv., eq. 38) in 77% of yield.



Sodium bicarbonate (2.52 g, 30.0 mmol, 3 eq.) and 6-aminohexanoic acid (2.64 g, 20.0 mmol, 2 eq.) were added to H_2O (20 mL). 4-chloro-7-nitrobenzofurazan (2.0 g, 10.0 mmol, 1 eq.) dissolved in MeOH (80 mL) was added and the mixture was stirred at 60 °C for 30 min. The solution was cooled in ice, acidified to pH 2.0 with concentrated HCl, and the MeOH was removed by rotary evaporation. Excess water (200 mL) was added and the suspension was homogeneously dispersed by bath sonication. A fine black powder was collected by filtration, washed with water, and dried under vacuum to give the desired compound as a white solid product. The crude NBD-6-aminohexanoic acid (**7**) was isolated with 2.26 g as a black solid (77% yield) in good purity.

Mp: 159 – 161°C

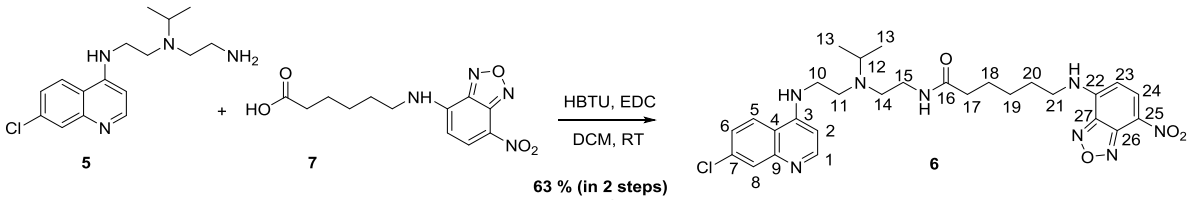
^{1}H NMR (300 MHz, DMSO-d6): δ 11.99(s, 1H), 9.53 (s, 1H), 8.49 (d, *J* = 9.5 Hz, 1H), 6.39 (d, *J* = 9.5 Hz, 1H), 3.45 (s, 2H), 2.22 (t, *J* = 7.25 Hz, 2H), 1.75 - 1.62 (m, 2H), 1.60 - 1.50 (m, 2H), 1.42 – 1.32 (m, 2H).

(1) NBD : 7-nitrobenzo-2-oxa-1,3-diazole

(2) Elliott, J. T., and Prestwich, G. D. (2000) Maleimide-Functionalized Lipids that Anchor Polypeptides to Lipid Bilayers and Membranes. *Bioconjugate Chem. 11*, 832-841.

IR: 2903 (w), 1698 (m), 1584 (s), 1495 (m), 1333 (m), 1298 (vs), 1168 (m), 1118 (m), 995 (m) 905 (w), 835 (m), 738 (w).

7. *N*-(2-((2-((7-chloroquinolin-4-yl)amino)ethyl)(isopropyl)amino)ethyl)-6-((7-nitrobenzo-[*c*][1,2,5]oxadiazol-4-yl)amino)hexanamide (6)



To a solution of molecule (**4**) (100 mg, 0.246 mmol, 1 eq.) in ether (5 mL) was added HCl (6 M, 2 mL). The mixture was stirred for 30 min. A 1N NaOH solution was added until a white precipitate appears. The mixture was extracted with DCM (2x10 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under vacuum. The residue (**5**) was dissolved in DCM (5 ml) then EDC (0.067 mL, 0.37 mmol, 1.5 eq.), HBTU (93.2 mg, 0.25 mmol, 1 eq.) and compound (**7**) (72.3 mg, 0.25 mmol, 1 eq.) were added. The mixture was stirred for 4 hours. Water (5 mL) and 5 mL of 1N NaOH solution were added. The mixture was extracted with DCM (3x10 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under vacuum. The crude was purified with automatic silica gel column chromatography (eluent: $CHCl_3$/MeOH, gradient: (100/0 -> 90/10). The product (**6)** was obtained as an orange solid (90 mg). Yield 63 %.

Rf = 0.45 (eluent: DCM/MeOH, 9/1)

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.56 (d, *J* = 5.4 Hz, 1H, Qn*H*), 8.48 (d, *J* = 8.7 Hz, 1H, Qn*H*), 7.99 (d, *J* = 2.1Hz, 1H, Qn*H*), 7.73 (d, *J* = 8.7 Hz, 1H, Qn*H*), 7.40 (dd, *J* = 9.0 Hz and J = 2.1 Hz, 1H, Qn*H*), 6.42 (d, *J* = 5.4 Hz, 1H, Qn*H*), 6.13 (d, *J* = 9.9 Hz, 1H, Qn*H*), 5.81 (bs, 1H, N*H*), 5.59 (bs, 1H, N*H*), 3.42-3.34 (m, 2H, CH_2), 3.32-3.23 (m, 4H, CH_2), 3.08 (sept, 1H, C*H*), 2.86-2.82 (m, 2H, CH_2), 2.65-2.61 (m, 2H, CH_2), 1.69 (bs, 3H, CH_2 and 1 N*H*), 1.59-1.54 (m, 2H, CH_2), 1.51-1.46 (m, 2H, CH_2), 1.37-1.27 (m, 2H, CH_2), 1.12 (d, *J* = 6.6 Hz, 6H, CH_3).

^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 8.51 (d, *J* = 5.4 Hz, 1H, H_1), 8.42 (d, *J* = 8.6 Hz, 1H, H_{23}), 7.94 (d, *J* = 2.2 Hz, 1H, H_8), 7.71 (d, *J* = 8.9 Hz, 1H, H_5), 7.35 (dd, *J* = 8.9, 2.2 Hz, 1H, H_6), 6.38 (d, *J*= 5.4 Hz, 1H, H_2), 6.09 (d, *J* = 8.6 Hz, 1H, H_{24}), 5.85 (br s, 1H, H_{HNCO}), 5.74 (br s, 1H, H_{NH}), 3.37 (m, 2H, H_{21}), 3.28 (m, 4H, H_{10}+H_{15}), 3.06 (sept, *J* = 6.6 Hz, 1H, H_{12}), 2.83 (t, *J* = 5.5 Hz, 2H, H_{14}), 2.62 (t, *J* = 5.6 Hz, 2H, H_{11}), 1.64 (t, *J* = 7.8 Hz, 2H, H_{17}), 1.50 (m, 2H, H_{20}), 1.35 (m, 2H, H_{18}), 1.15 (m, 2H, H_{19}), 1.10 (d, *J* = 6.6 Hz, 6H, H_{13}).

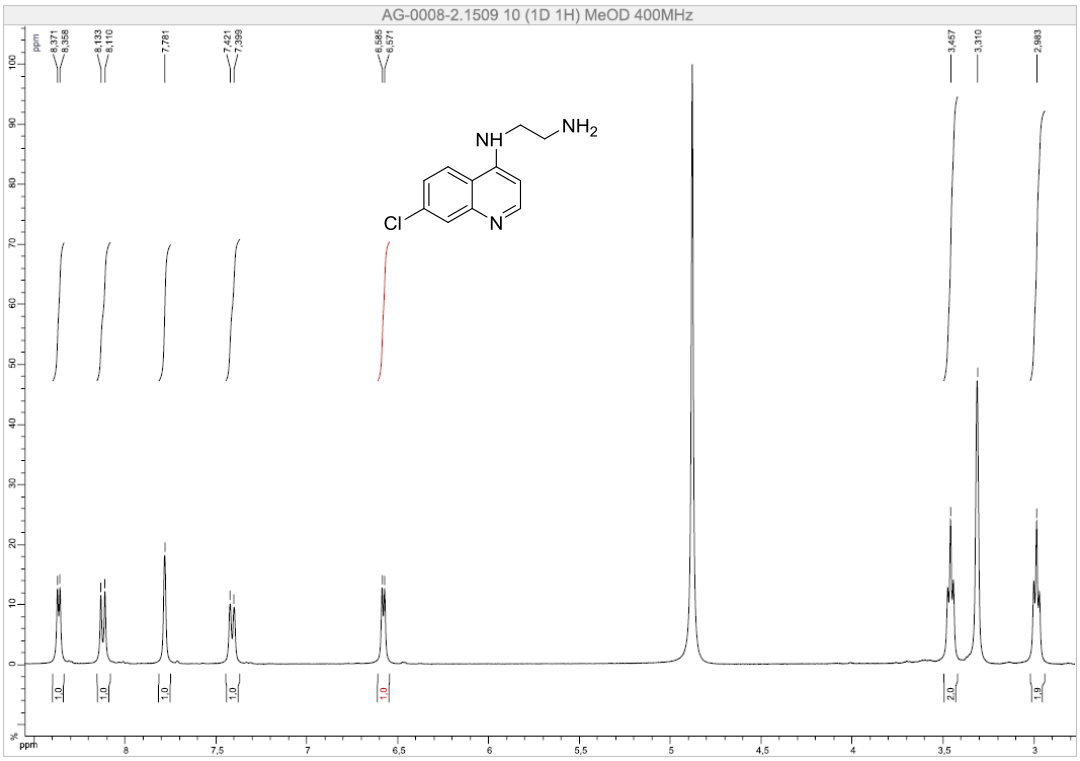
^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 172.96 (C_{16}), 152.07 (C_1), 150.23 (C_3), 148.96 (C_9), 144.64 (C_{26}), 144.46(C_{27}), 144.19 (C_{22}), 136.76 (C_{23}), 135.25 (C_7), 128.47 (C_8), 125.68 (C_6), 123.26 (C_{25}), 121.39 (C_5), 117.47 (C_4), 99.56 (C_2), 98.78 (C_{24}), 50.38 (C_{12}), 49.44 (C_{11}), 47.66 (C_{14}), 43.95 (C_{21}), 40.81 (C_{15}), 38.48 (C_{10}), 35.94 (C_{17}), 27.90 (C_{20}), 25.45(C_{19}), 24.72 (C_{18}), 18.36 (C_{13}).

IR: 3380 (w), 2967 (w), 2929 (w), 1694 (m), 1579 (vs), 1531 (m), 1365 (m), 1282 (s), 1250 (s), 1168 (s), 1136 (m), 1080 (m), 877 (w), 806 (w).

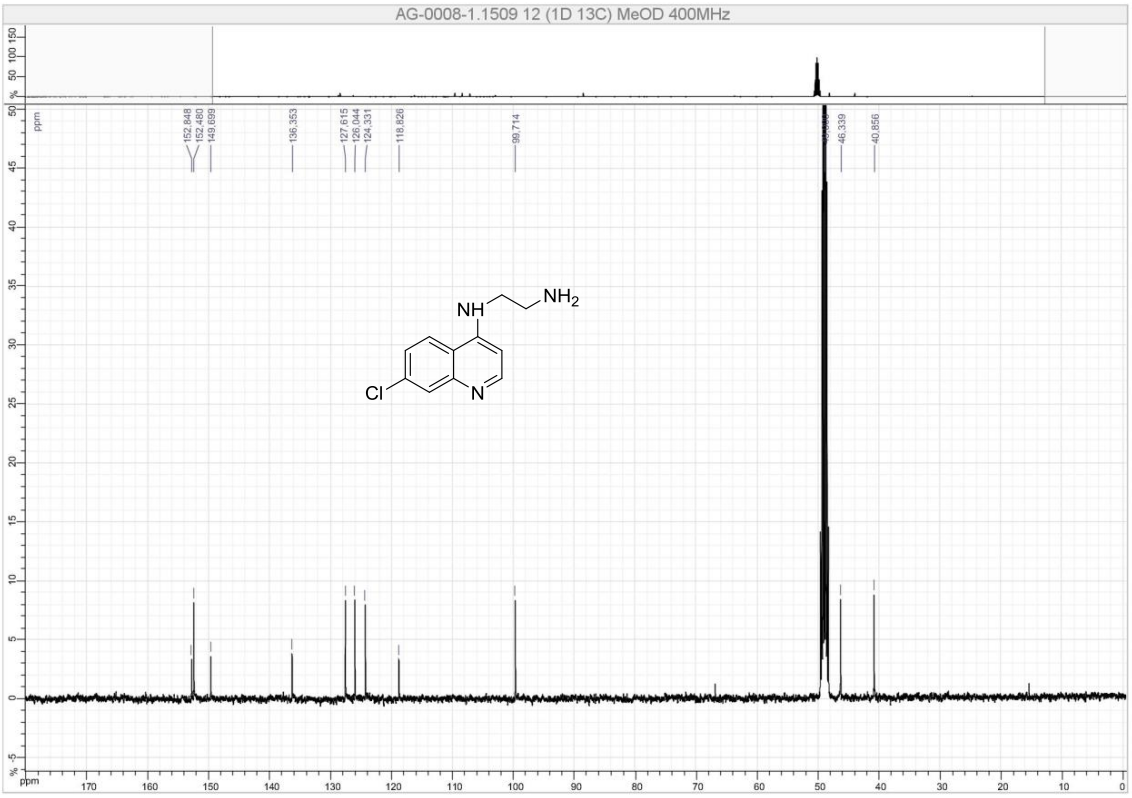
HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{28}H_{36}ClN_8O_4$ 583.2519; found 583.2529.

III. NMR Spectra

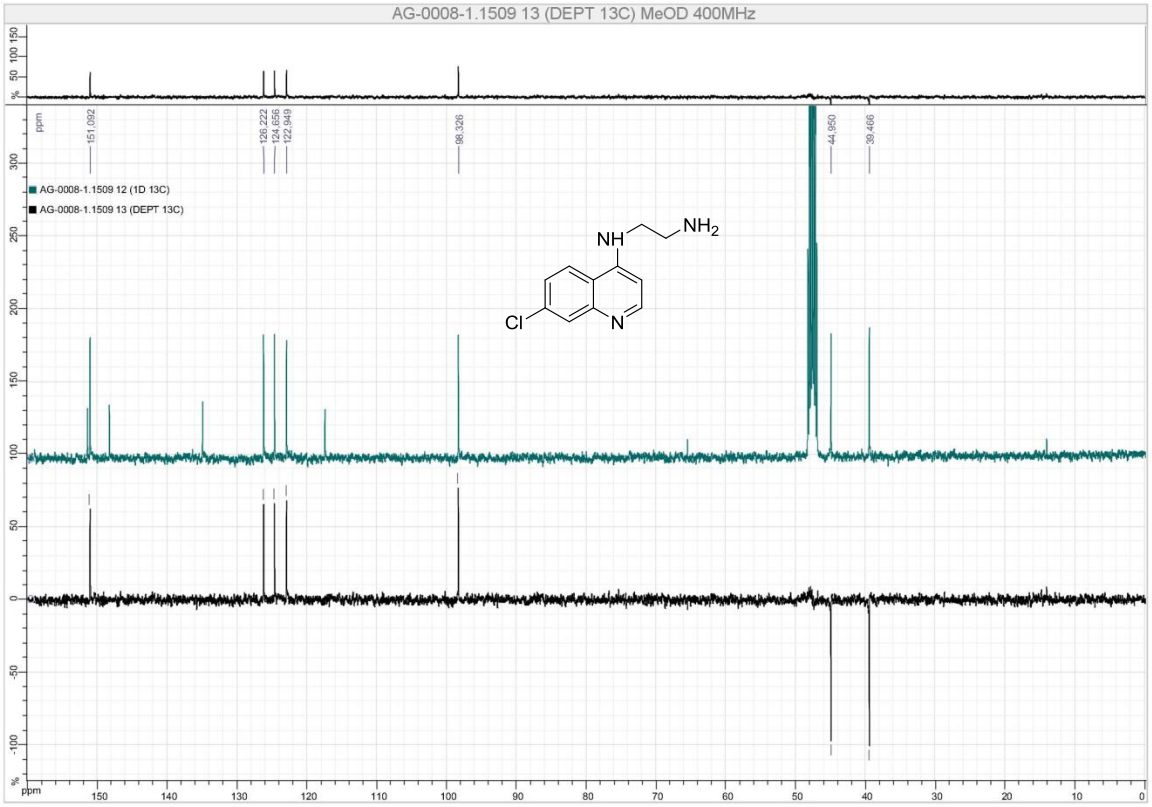
(1) ^{1}H NMR (400 MHz, MeOD)



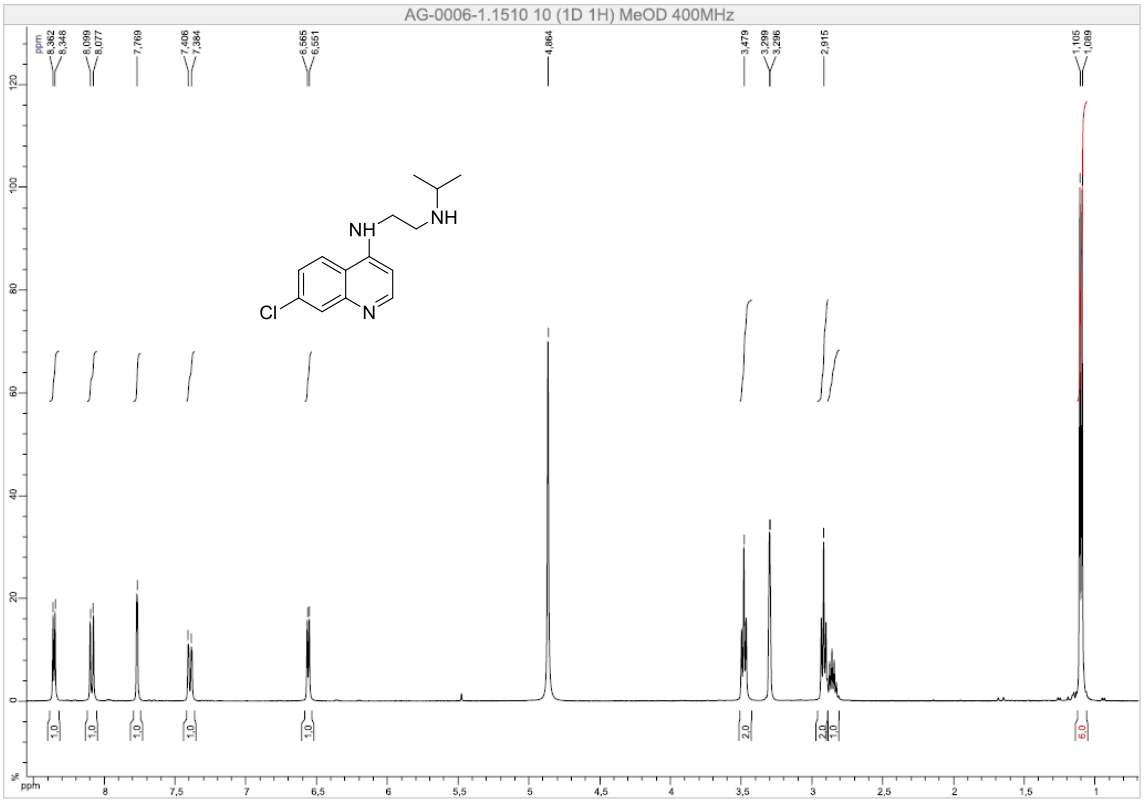
(1) ^{13}C NMR (100 MHz, MeOD)



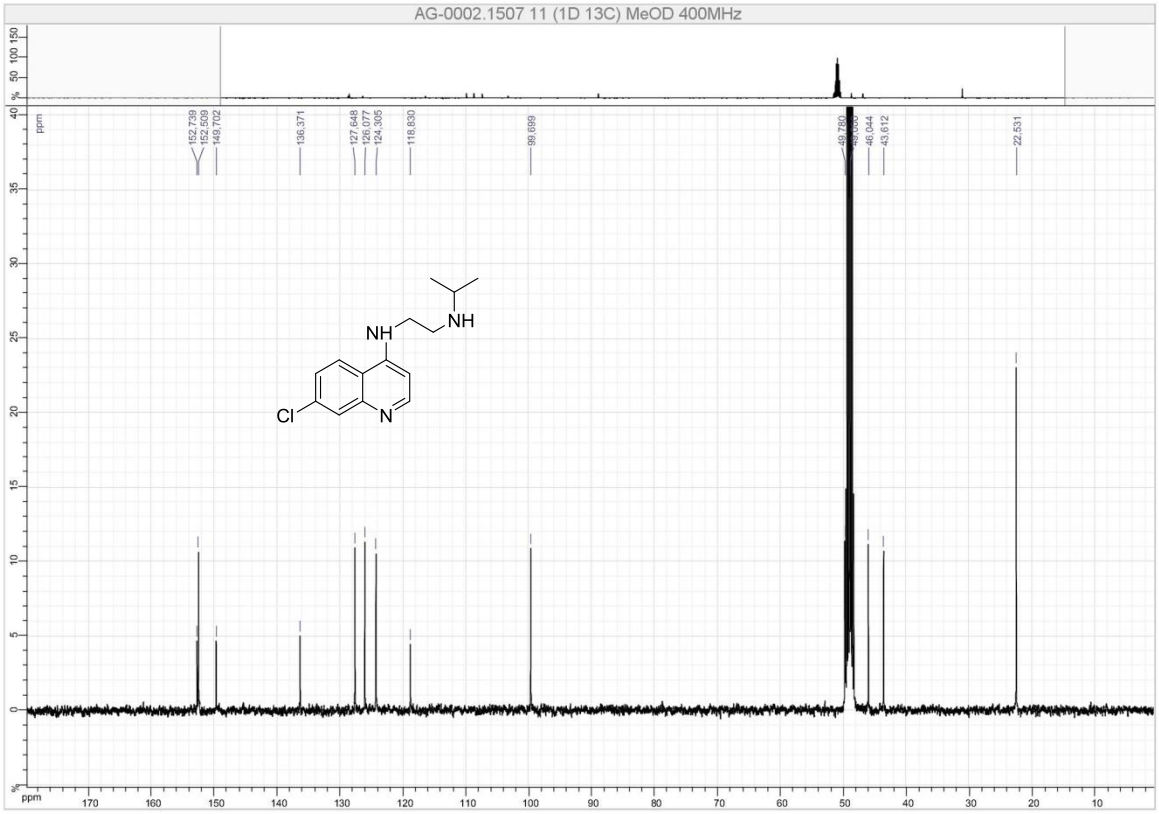
(1) ^{13}C DEPT NMR (100 MHz, MeOD)



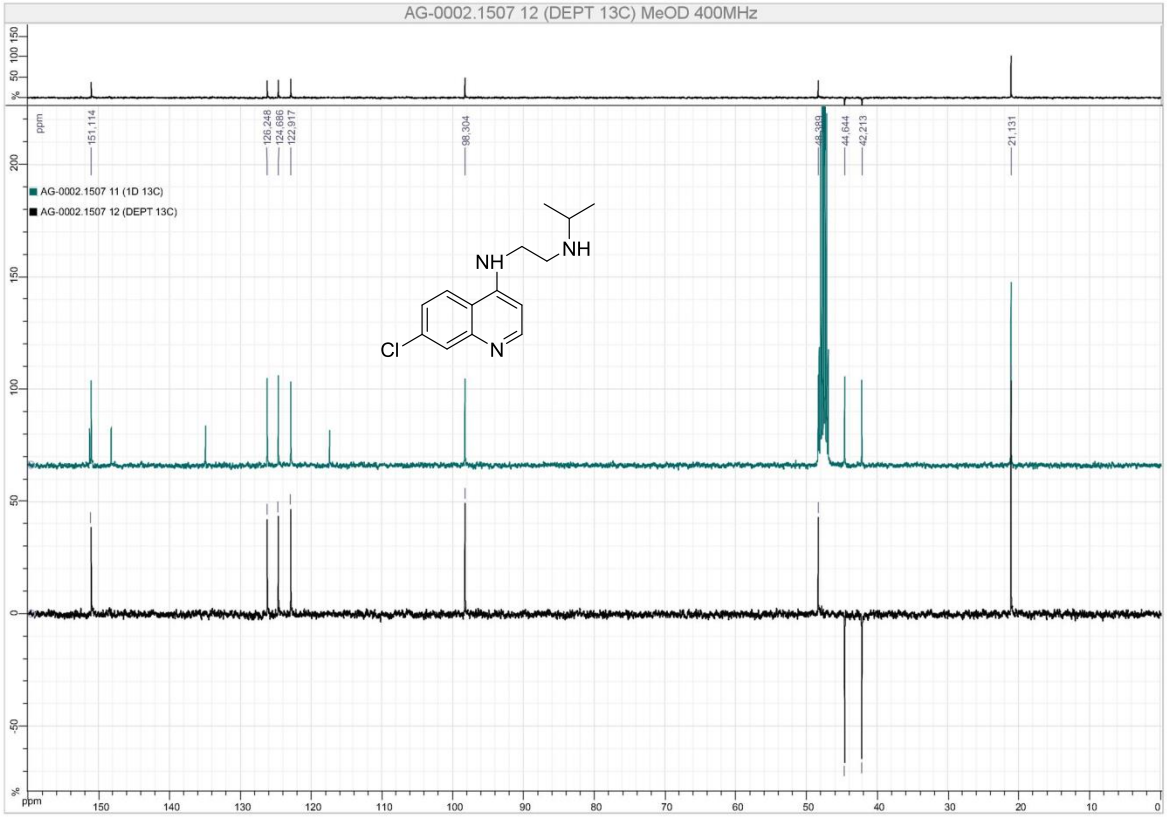
(2) ^{1}H NMR (400 MHz, MeOD)



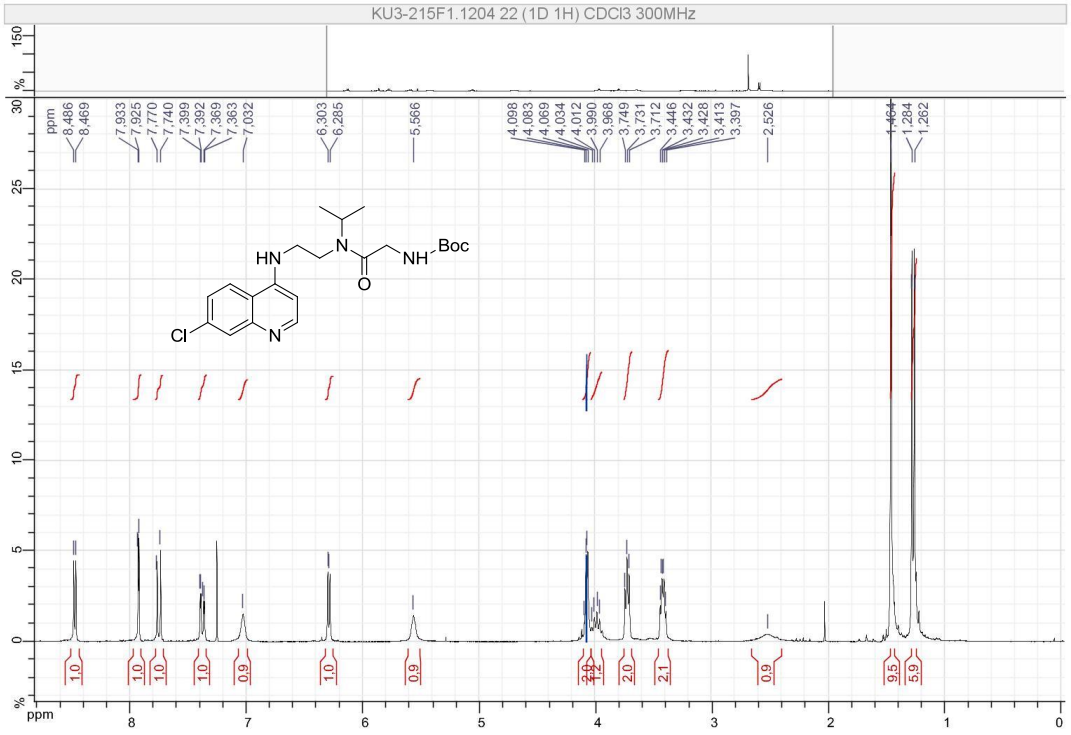
(2) ^{13}C NMR (100 MHz, MeOD)



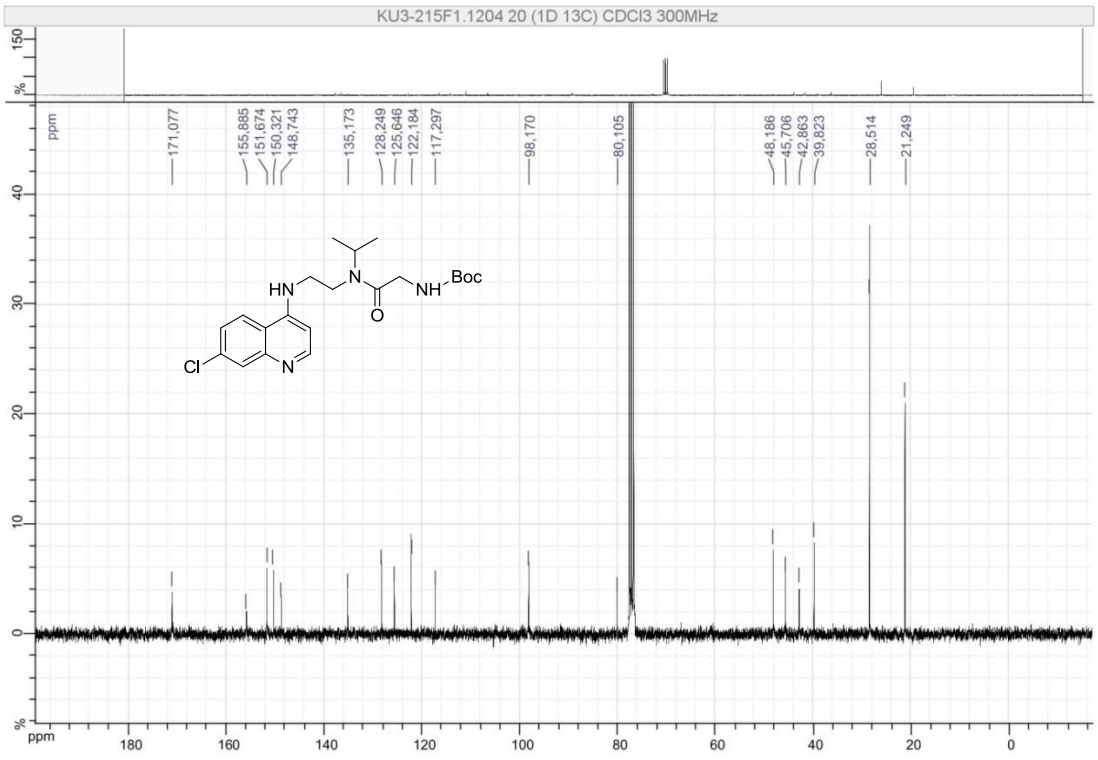
(2) ^{13}C DEPT NMR (100 MHz, MeOD)



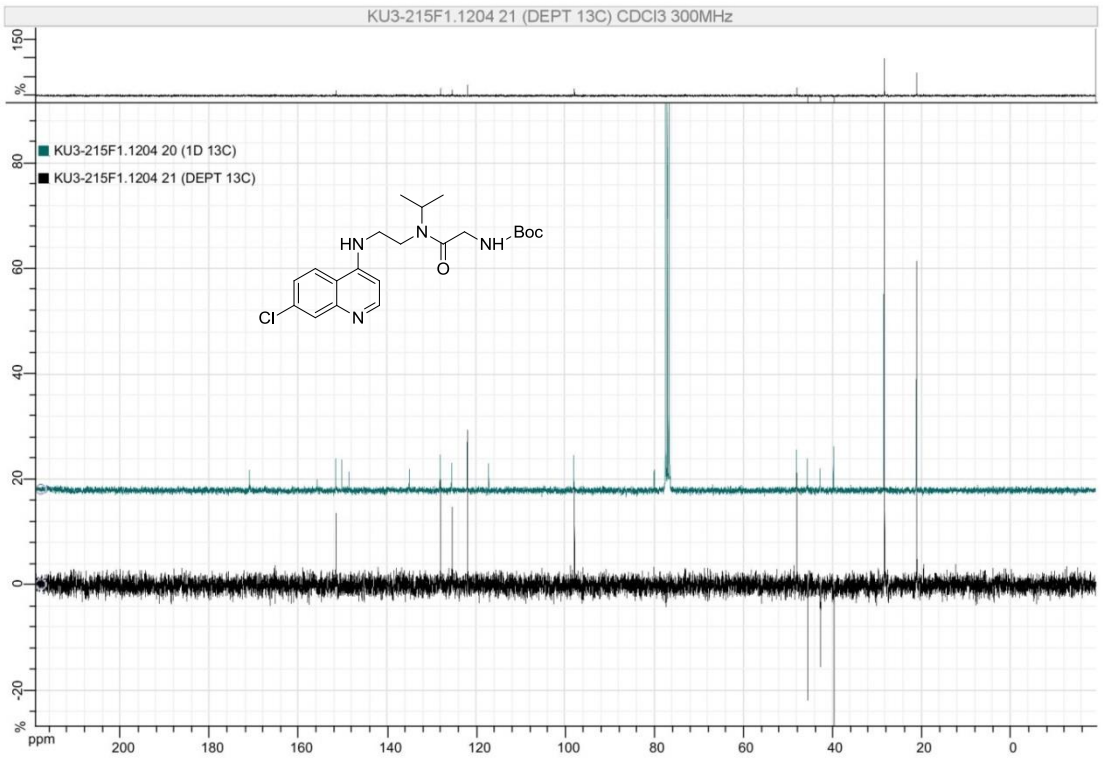
(3): ^{1}H NMR (300 MHz, $CDCl_3$)



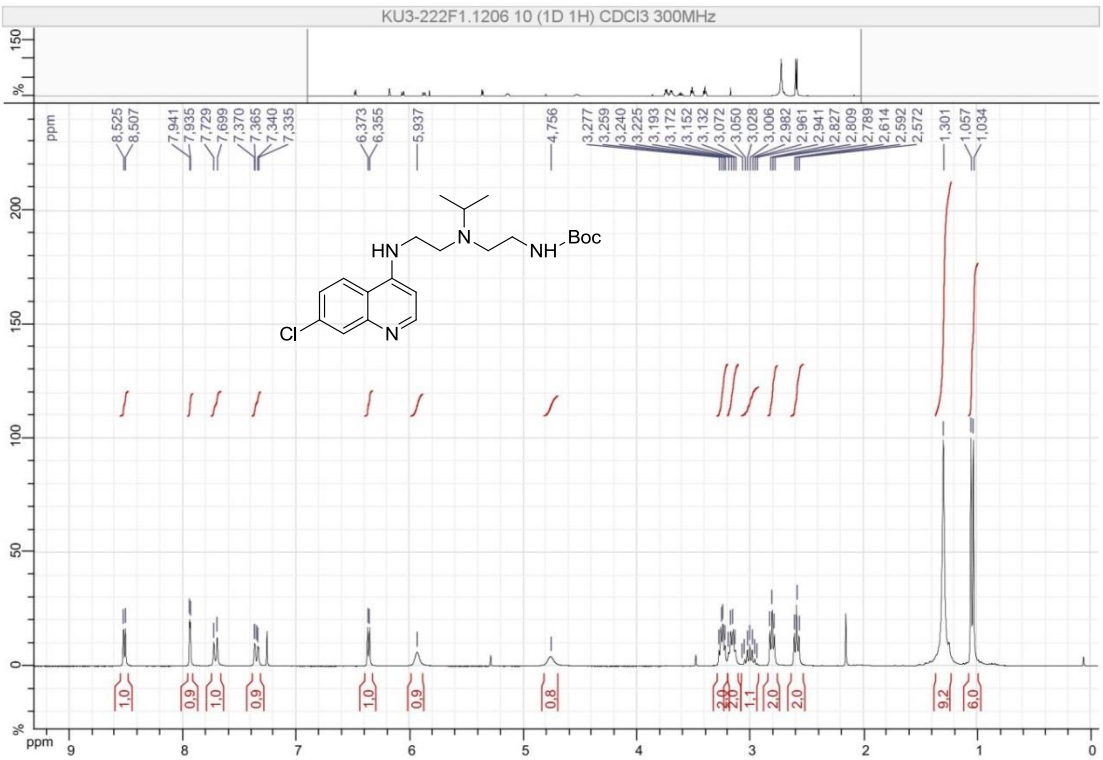
(3): ^{13}C NMR (75 MHz, $CDCl_3$)



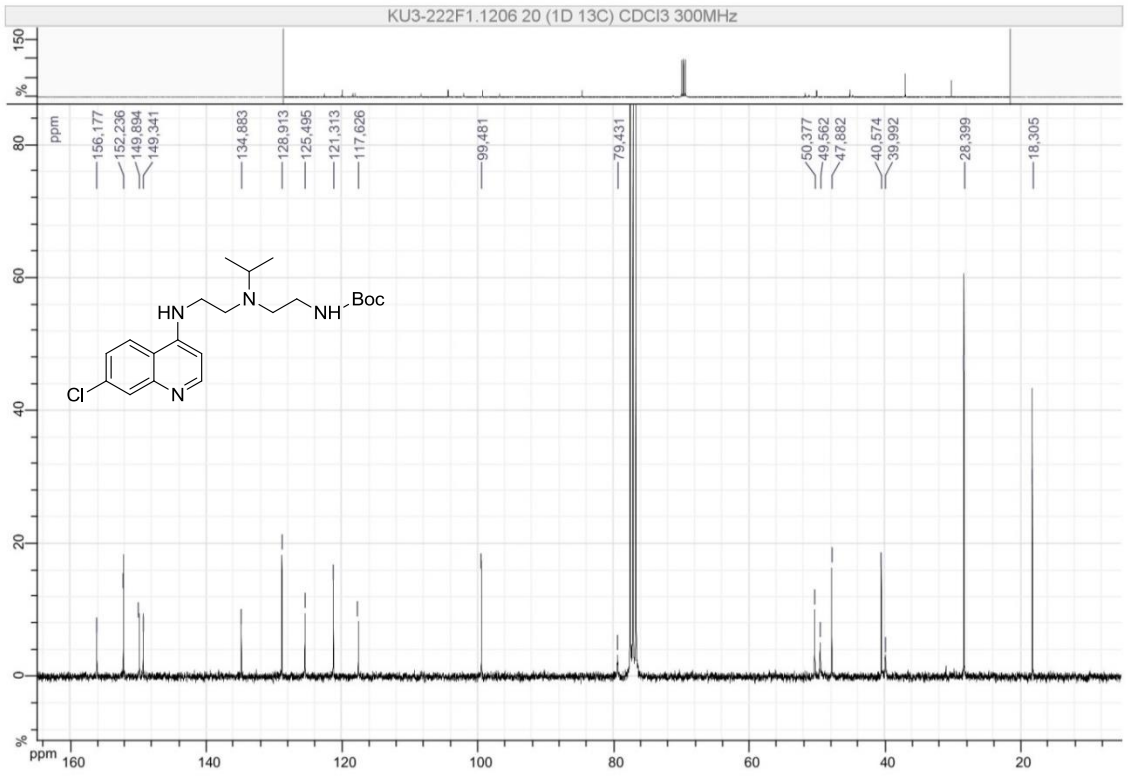
(3): ^{13}C Dept (75 MHz, $CDCl_3$)



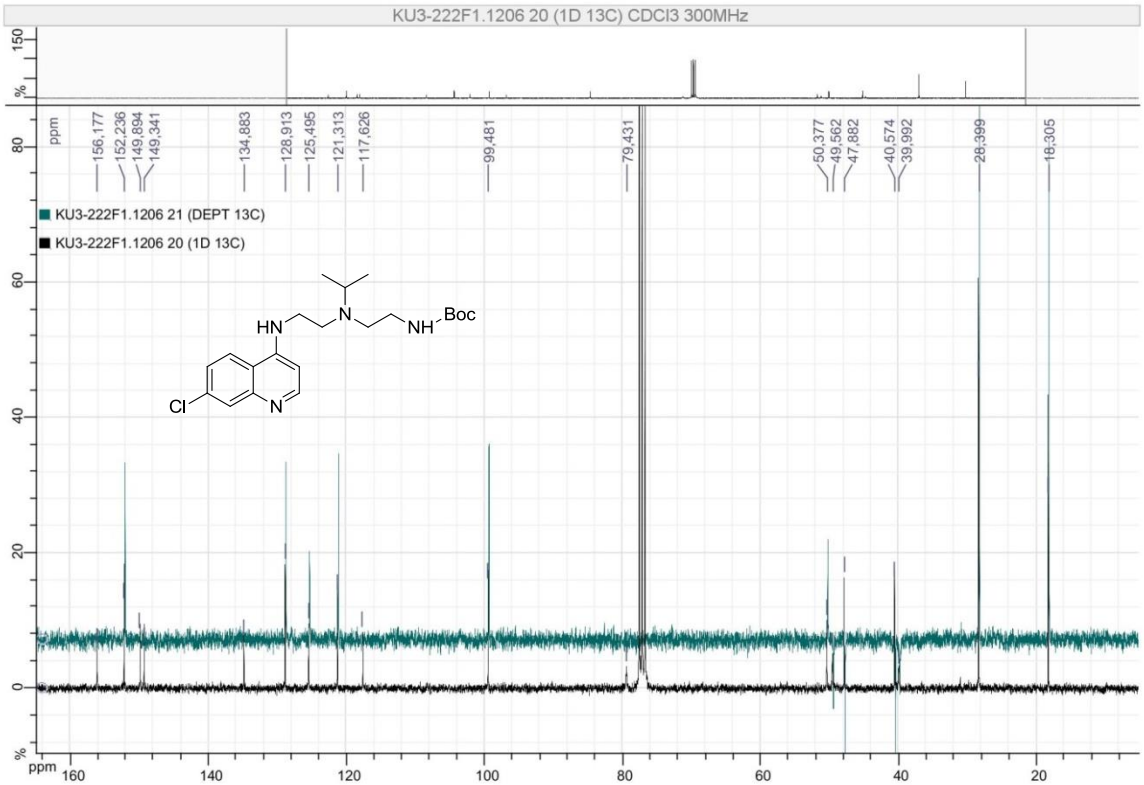
(4): ^{1}H NMR (300 MHz, $CDCl_3$)



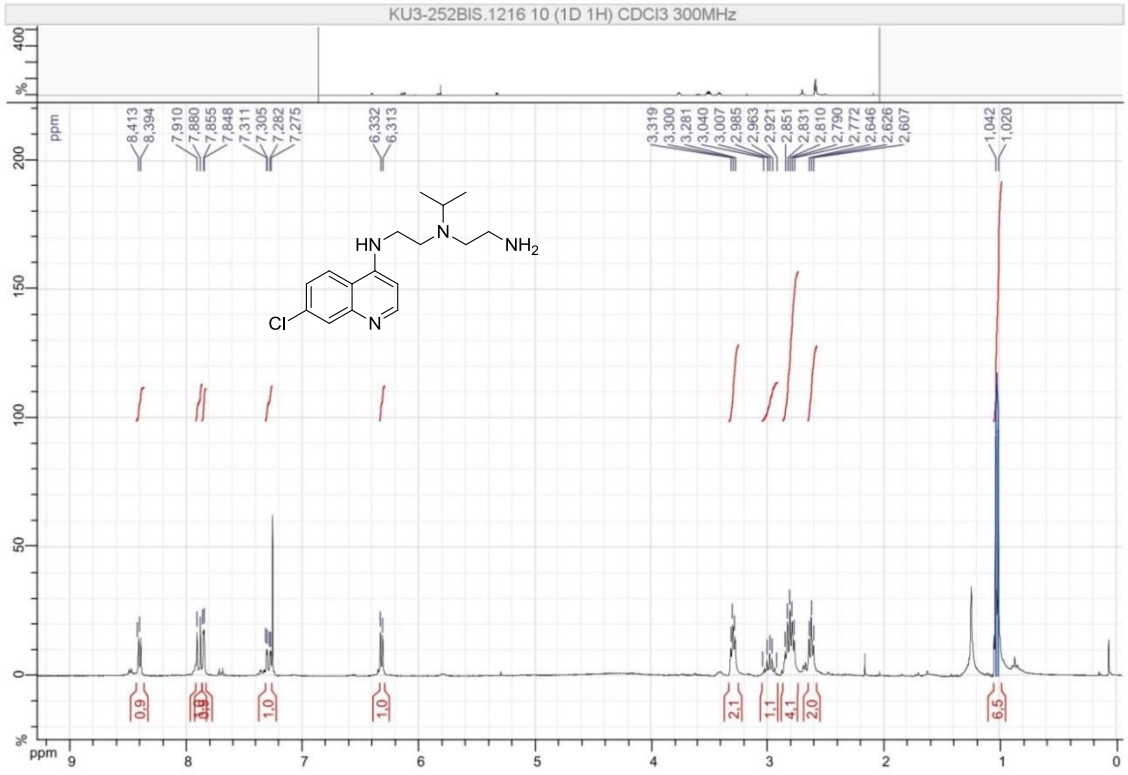
(4): ^{13}C NMR (75 MHz, $CDCl_3$)



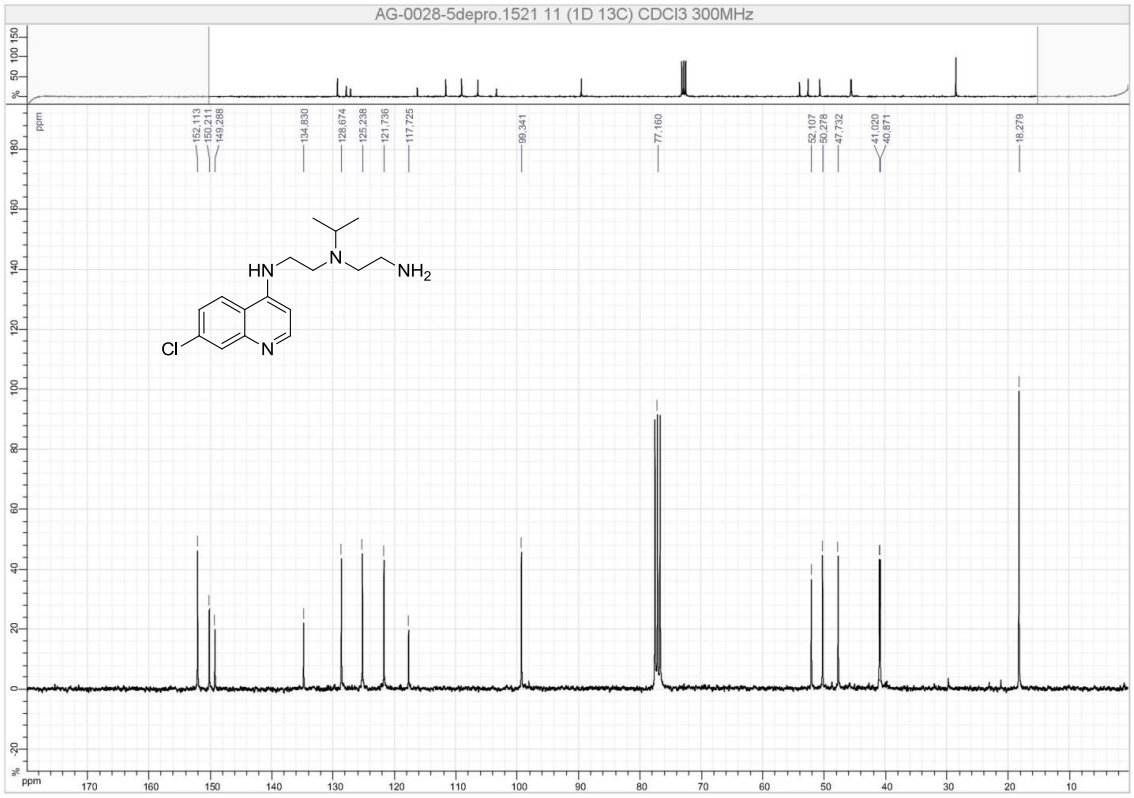
(4): ^{13}C Dept (75 MHz, $CDCl_3$)



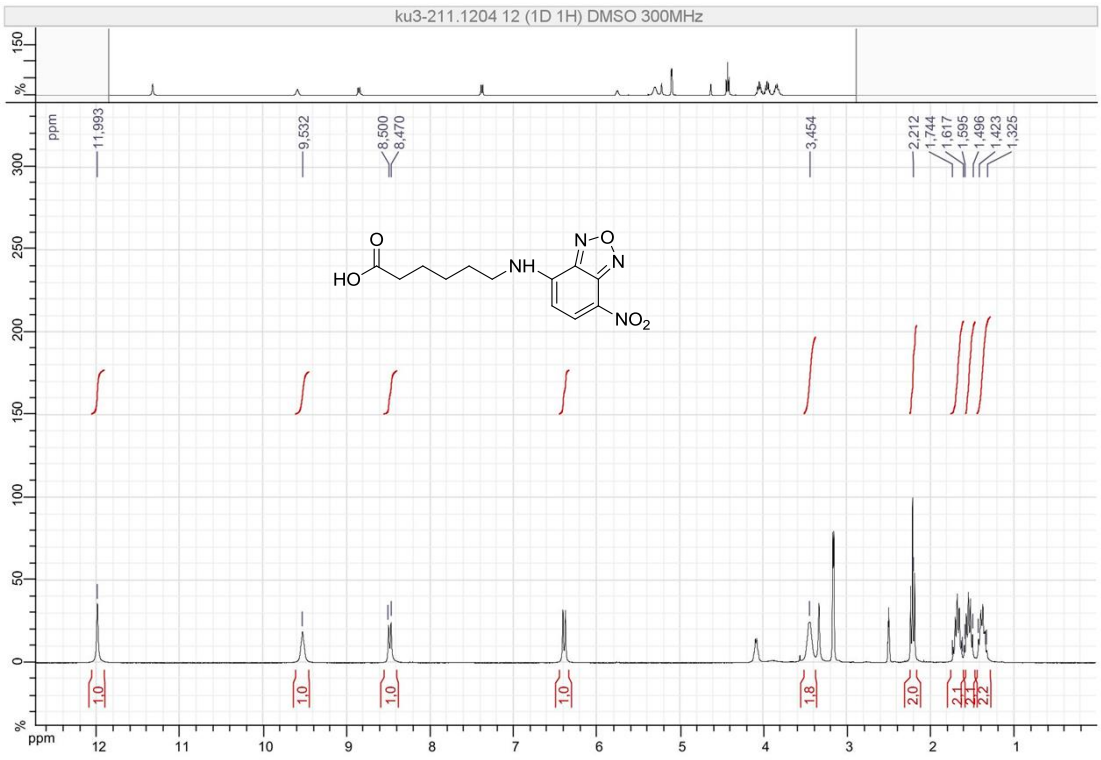
(5): ^{1}H NMR (300 MHz, $CDCl_3$)



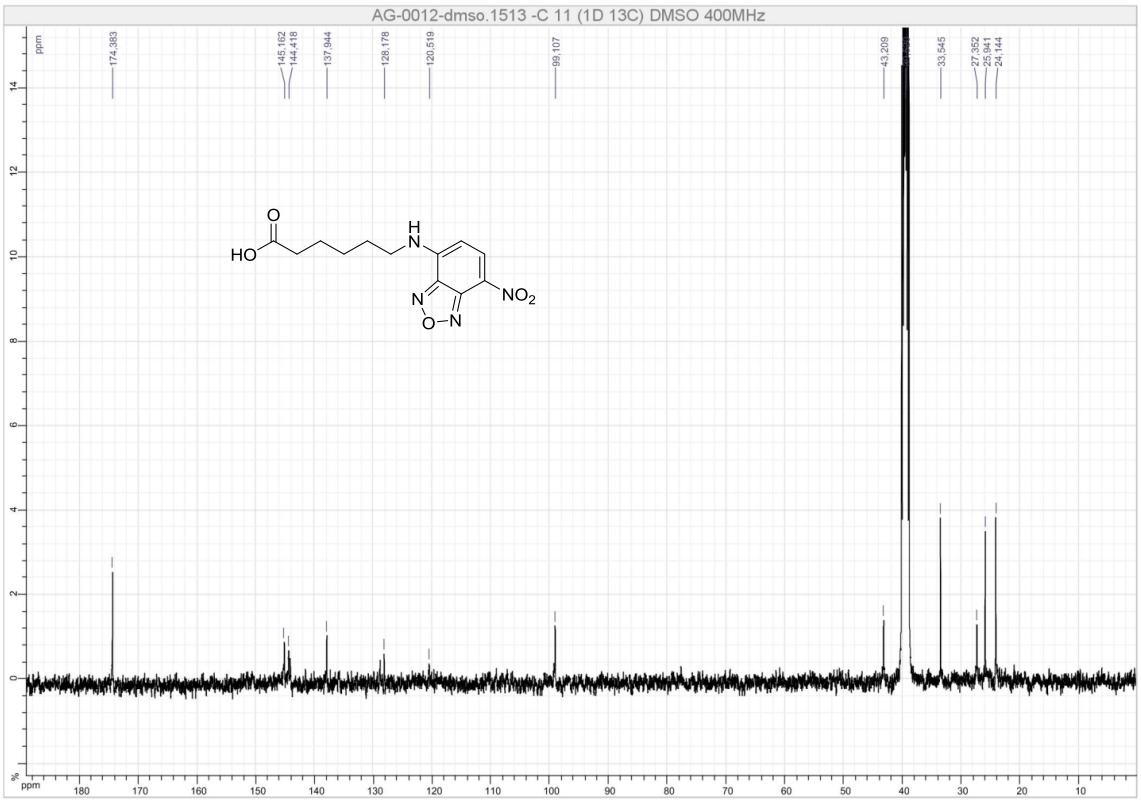
(5): ^{13}C NMR (75 MHz, $CDCl_3$)



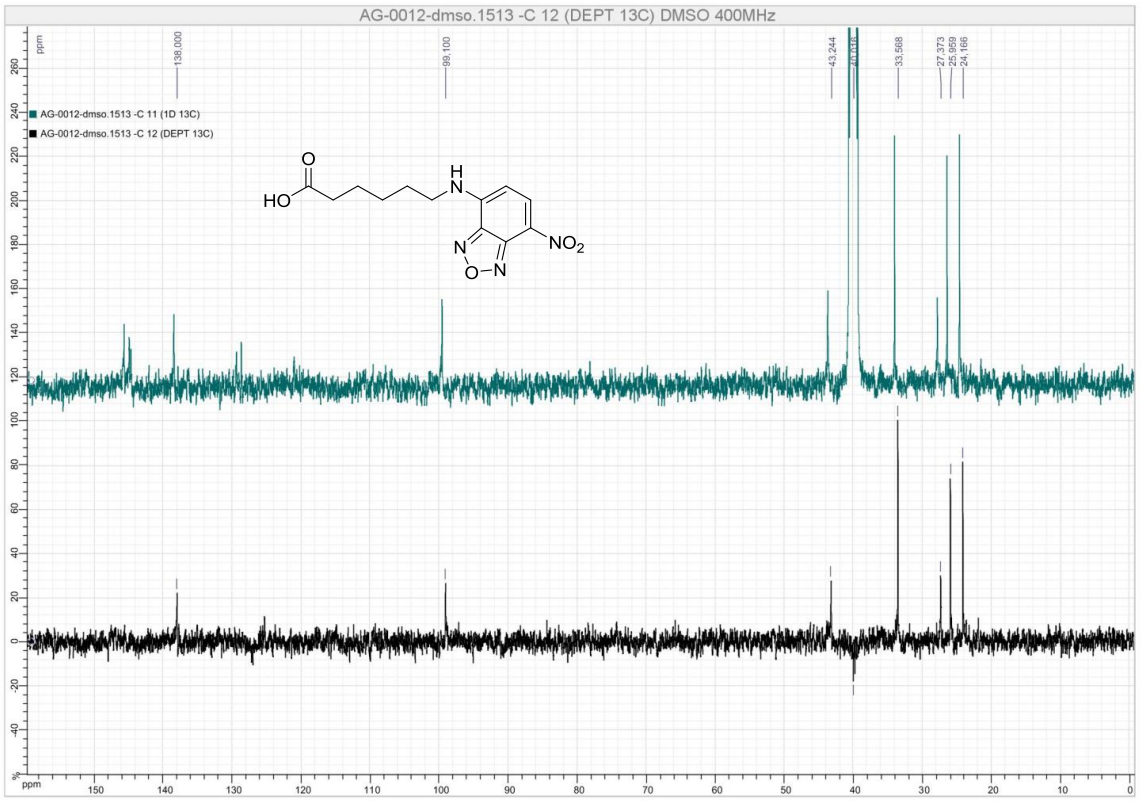
(7): ^{1}H NMR (300 MHz, $(CD_3)_2SO$)



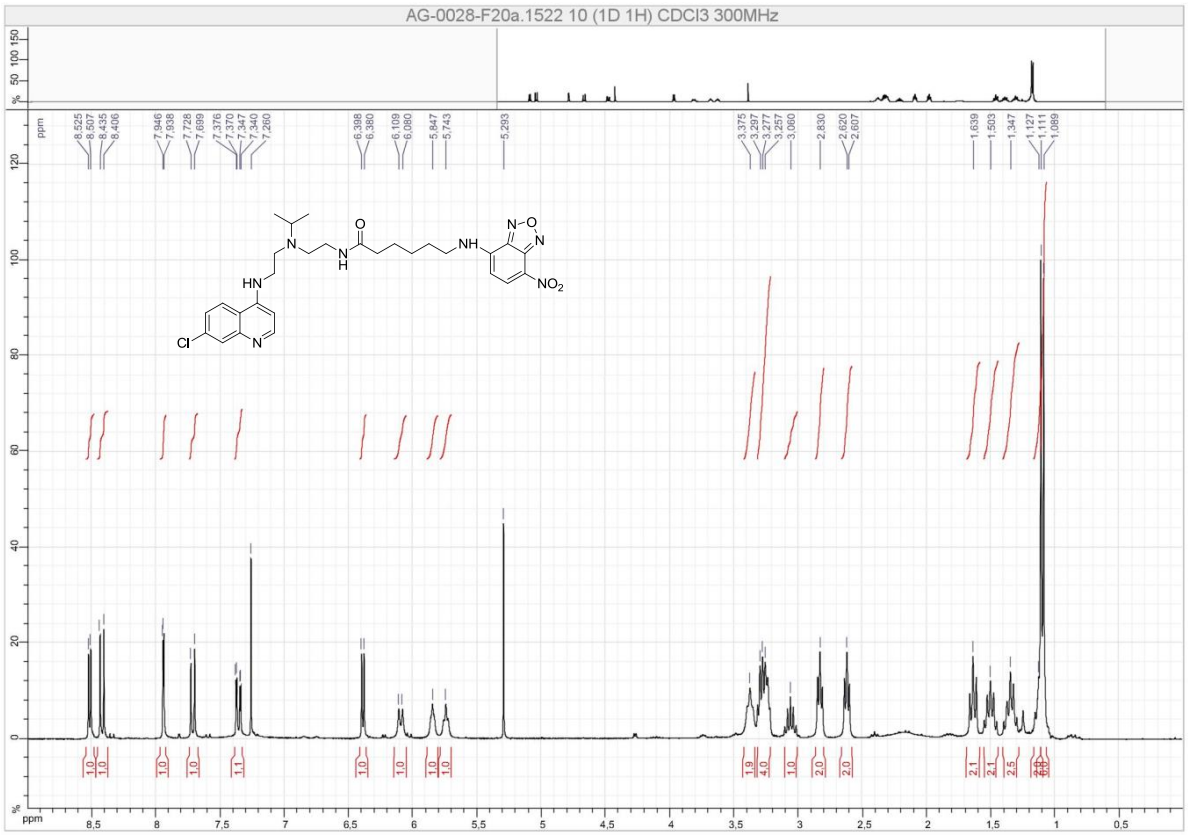
(7): ^{13}C NMR (100 MHz, MeOD)



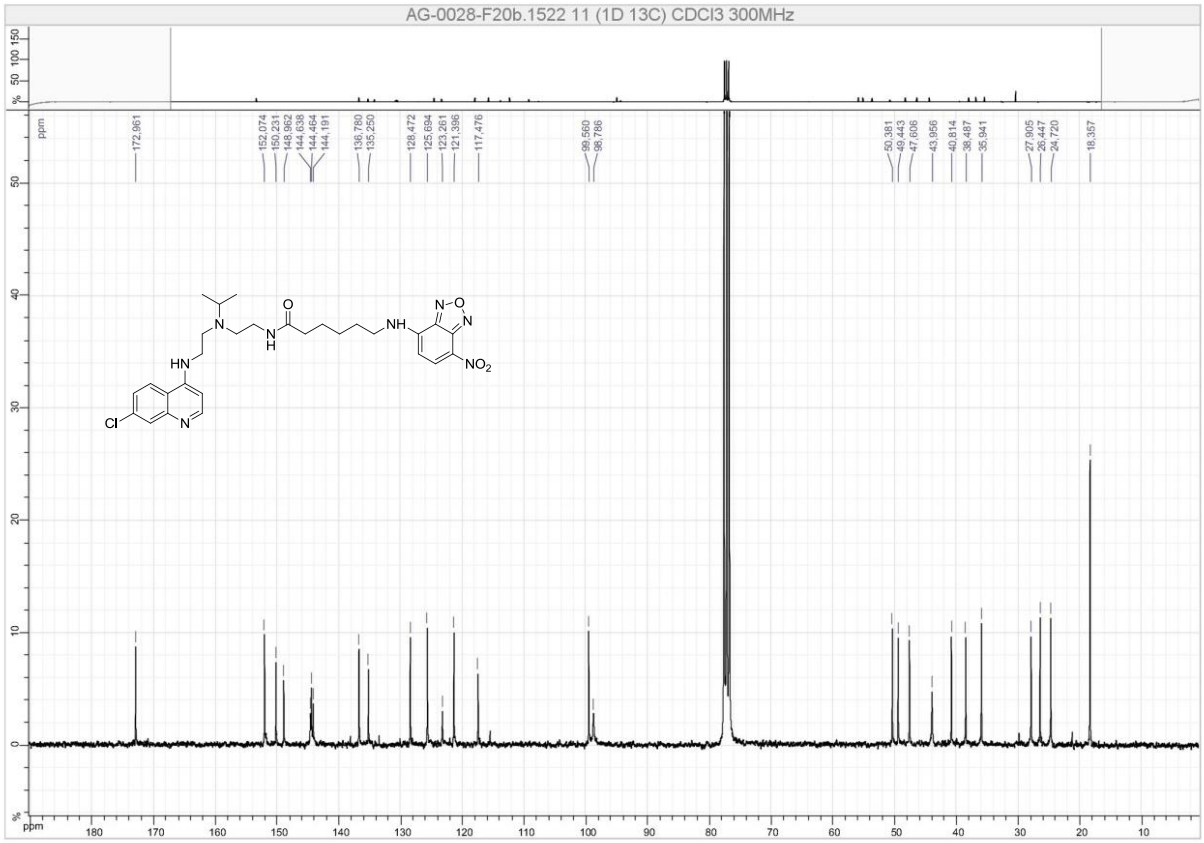
(7): ^{13}C DEPT NMR (100 MHz, MeOD)



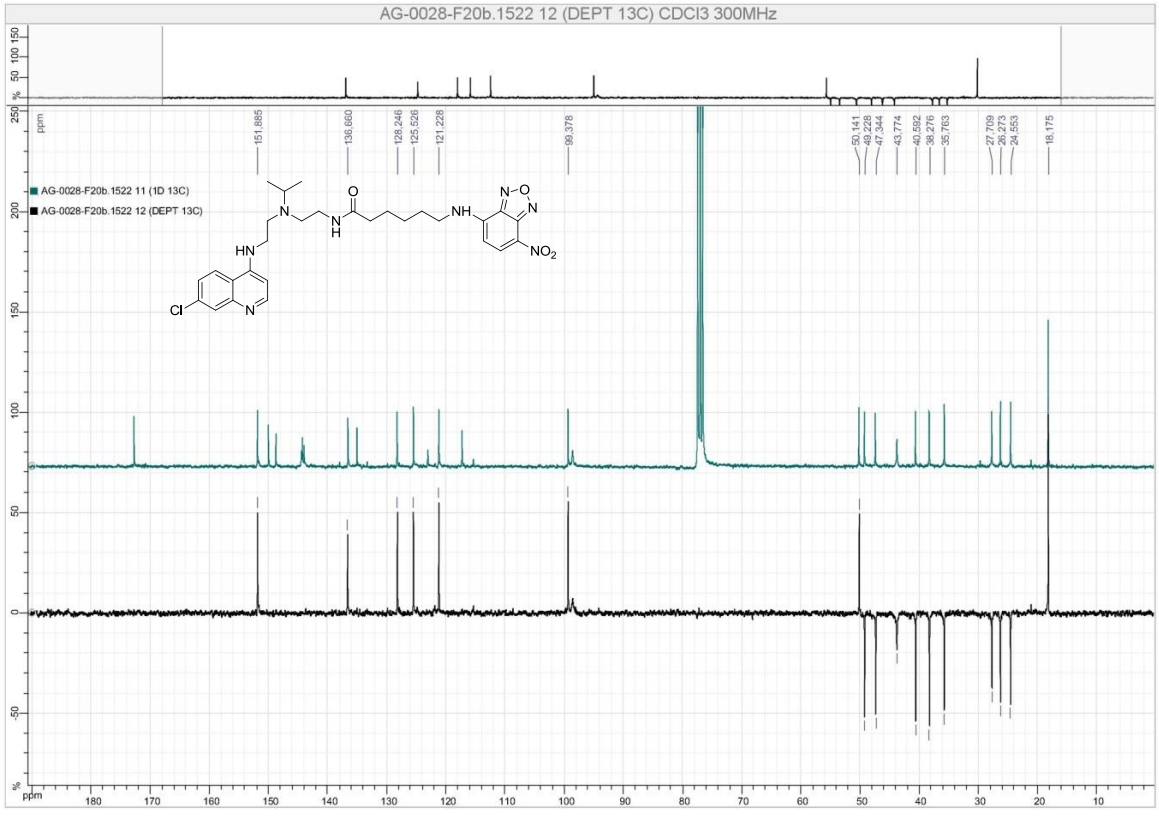
(6): ^{1}H NMR (300 MHz, $CDCl_3$)



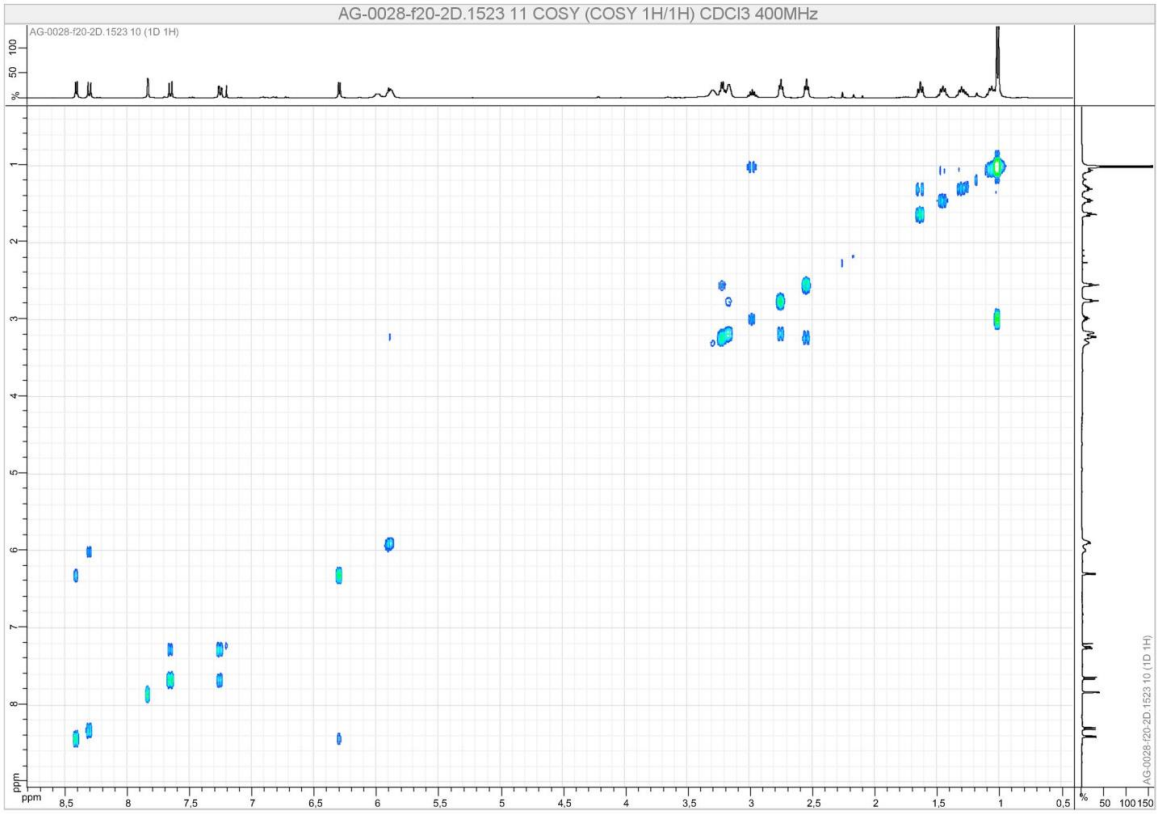
(6): ^{13}C NMR (75 MHz, $CDCl_3$)



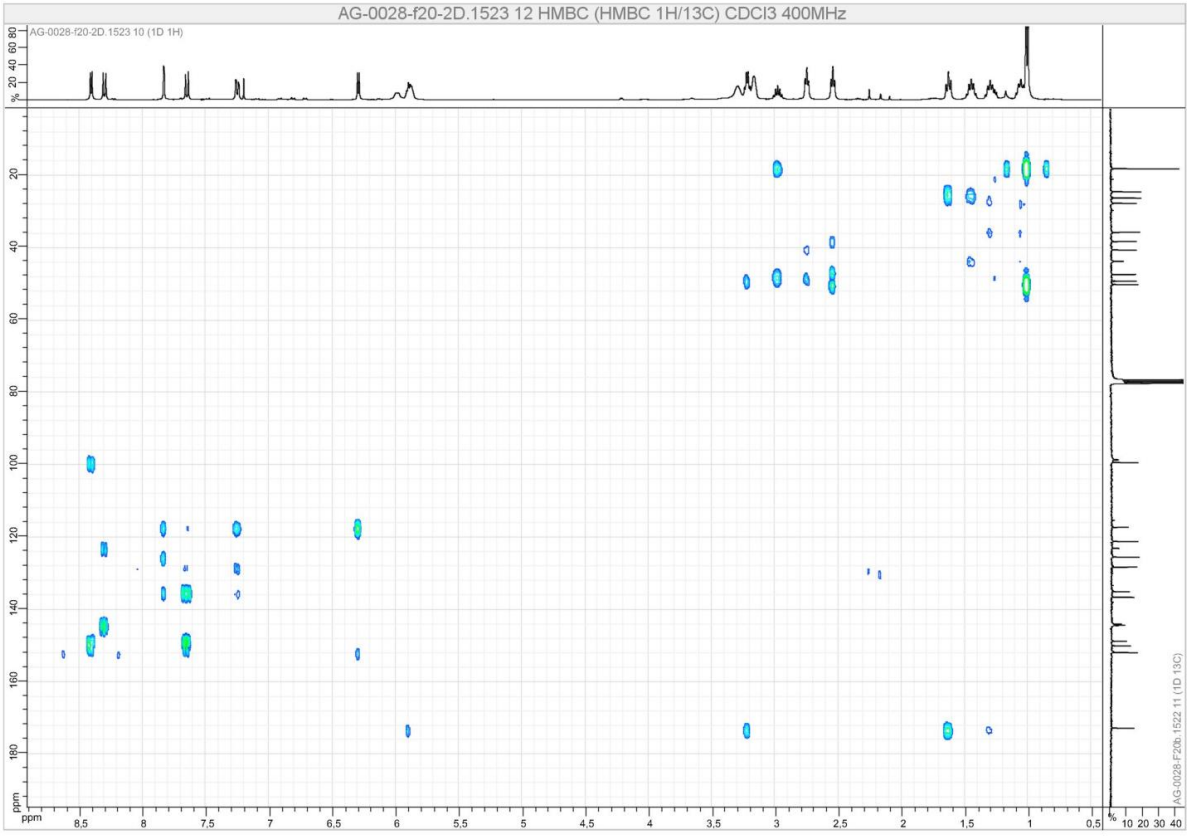
(6): ^{13}C Dept (75 MHz, $CDCl_3$)



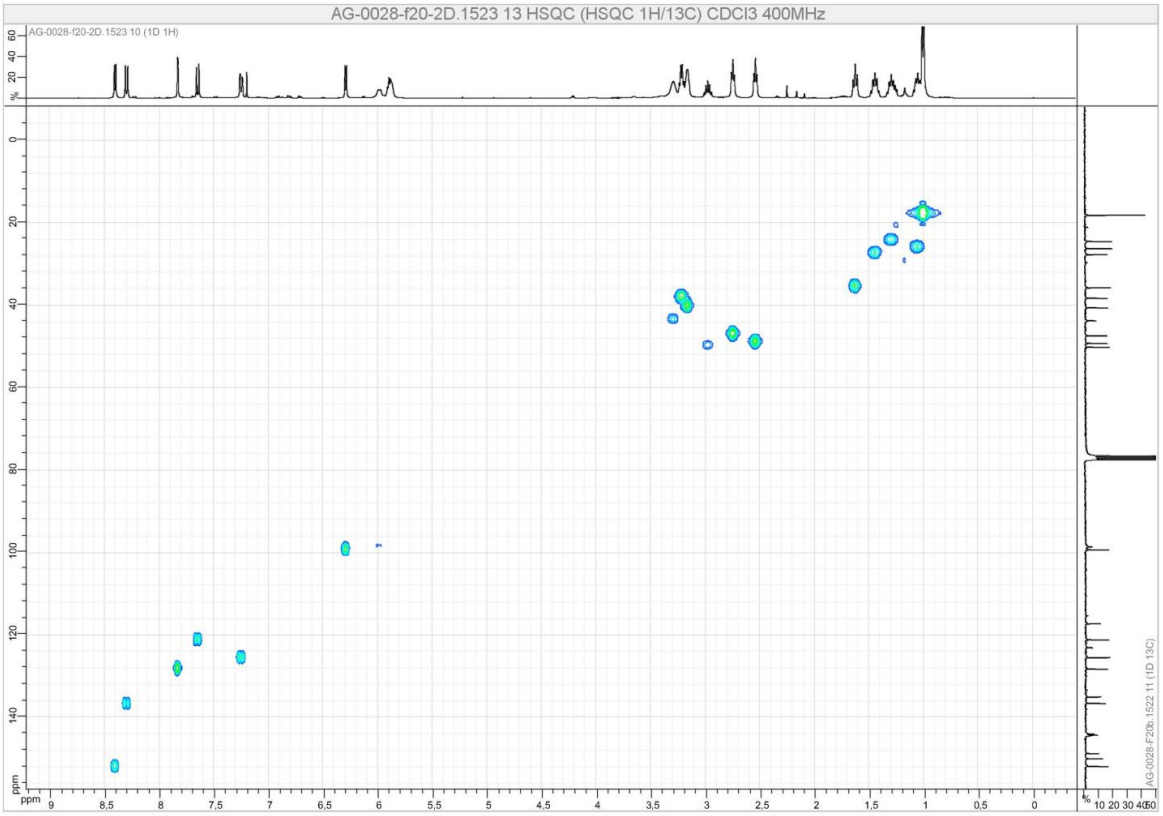
(6): COSY 2D NMR (400 MHz, $CDCl_3$)



(6): HMBC 2D NMR (75 MHz, $CDCl_3$)



(6): HSQC 2D NMR (75 MHz, $CDCl_3$)



IV. Physico-chemistry (materials and methods)

1- **Solvents and Material.** Chloroquine (Sigma, diphosphate salt) was purchased from commercial source and used without further purification. The CQ analogues (compound **2**, compound **4**, Fluo-CQ and **7**) were synthesized according to the procedures described above. All analyses were carried out with distilled water, spectroscopic grade dimethylsulfoxide DMSO, Bioreagent for molecular biology, >> 99.9%, Sigma), acetonitrile (E. Merck Uvasol, for spectroscopy) or with spectroscopic grade dichloromethane (Merck, 99.8% for spectroscopy). Distilled water was further purified by passing it through a mixed bed of ion-exchanger (Bioblock Scientific R3-83002, M3-83006) and activated carbon (Bioblock Scientific ORC-83005) and was de-oxygenated by CO_2- and O_2-free argon (Sigma Oxiclear cartridge) before use. The ionic strength was maintained at 0.1 M with sodium perchlorate ($NaClO_4.H_2O$, Merck, p.a.) whatever the solvent used (water or water/DMSO). Hematin ($Fe^{III}PPIX(OH)$) solution was prepared from hemin equine Type III ($Fe^{III}PPIXCl$, Sigma-Aldrich) and 50% aqueous ammonia (ESI-MS CID experiments) vigorously stirred at room temperature (RT) for 1 h. Stock solutions of the substrates for ESI-MS CID experiments for the different assays were freshly prepared in Eppendorf tubes just before the experiments. All solutions were protected from daylight to avoid any photochemical degradation. All the stock solutions were prepared by weighing solid products using a Mettler Toledo XA105 Dual Range (0.01/0.1 mg - 41/120 g) and the complete dissolution was achieved using an ultrasonic bath. The concentrations of the stock solutions of the compounds ($\approx 10^{-4}$ M) were calculated by quantitative dissolution of solid samples in the corresponding solutions. All the physico-chemical measurements were carried out at 25.0(2) °C.

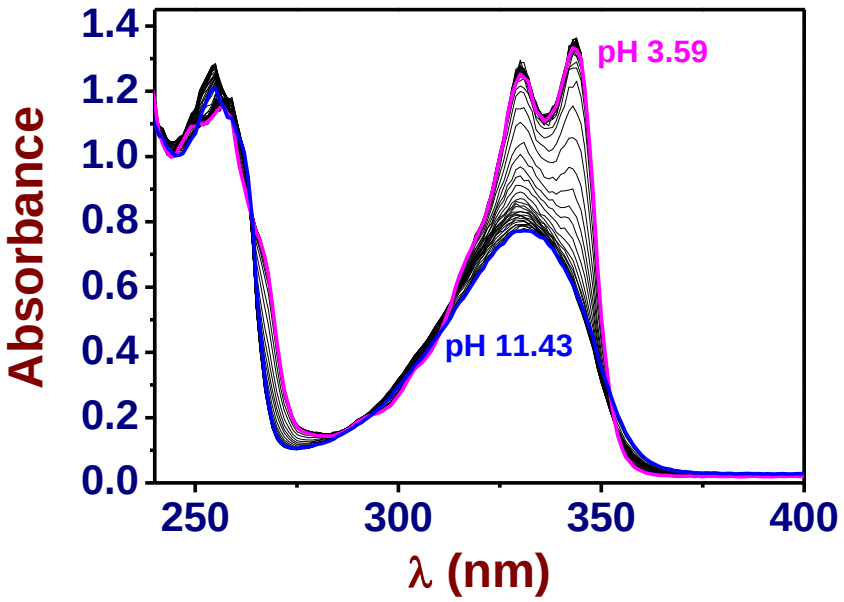
2- **Potentiometry.** The potentiometric measurements were performed using an automatic titrator system 794 Basic Titrino (Metrohm) with a combined glass electrode (Metrohm 6.0234.500, Long Life) filled with 0.1 M NaCl in water and connected to a microcomputer (Tiamo light 1.2 program for the acquisition of the potentiometric data). The combined glass electrode was calibrated as a hydrogen concentration probe by titrating known amounts of perchloric acid ($\sim 10^{-2}$ M from $HClO_4$, Prolabo, normapur, 70% min) with CO_2-free sodium hydroxide solution ($\sim 10^{-1}$ M from NaOH, BdH, AnalaR).[3] The $HClO_4$ and NaOH solutions were freshly prepared just before use and titrated with sodium tetraborate decahydrate ($B_4Na_2O_7.10H_2O$, Fluka, puriss, p.a.) and potassium hydrogen phthalate ($C_8H_5KO_3$, Fluka, puriss, p.a.), respectively, using methyl orange (RAL) and phenolphthalein (Prolabo, purum) as the indicators. The cell was thermostated at 25.0 ± 0.2 °C by the flow of a Lauda E200 thermostat. A stream of argon, pre-saturated with water vapor, was passed over the surface of the solution. The Glee program3 was applied for the glass electrode calibration (standard electrode potential E_0/mV and slope of the electrode/mV pH^{-1}) and to check carbonate levels of the NaOH solutions used (< 5%).

3- **Absorption Spectrophotometric Titrations versus pH.** Absorption spectrophotometric titrations as a function of pH of CQ and compound **2** were performed in water while those of Compound **4** and Fluo-CQ were performed in water/DMSO (1/1 v/v) for solubility reasons. Stock solutions of CQ (1.01 mM) and compound **2** (0.986 mM) were prepared by quantitative dissolution of the corresponding solid samples in deionised water and the ionic strength was adjusted to 0.1 M with $NaClO_4$ (Fluka, puriss). Stock solutions of compound **4** (1.01 mM) and Fluo-CQ (1.03 mM) were prepared by quantitative dissolution of the corresponding solid samples in pure DMSO. Prior to measurements, CQ and compound **2** were diluted with water containing 0.1 M of $NaClO_4$, while compound **4** (1.01 mM) and Fluo-CQ were diluted with a mixed solvent containing 50 % water with 0.2 M of $NaClO_4$ and 50% of DMSO (by volume). 40 mL of the solutions were introduced into a jacketed cell (METROHM) maintained at 25.0 ± 0.2 °C (Lauda E200). The free hydrogen ion concentration was measured with a combined glass electrode (METROHM 6.0234.500, Long Life) and an automatic titrator system 794 Basic Titrino (Metrohm). The Ag/AgCl reference glass electrode was filled with NaCl (0.1 M, Fluka, p.a.) and was calibrated as a hydrogen concentration probe as described above. The initial pH was adjusted to ~ 2 with $HClO_4$ (Prolabo, normapur, 70% min), and the

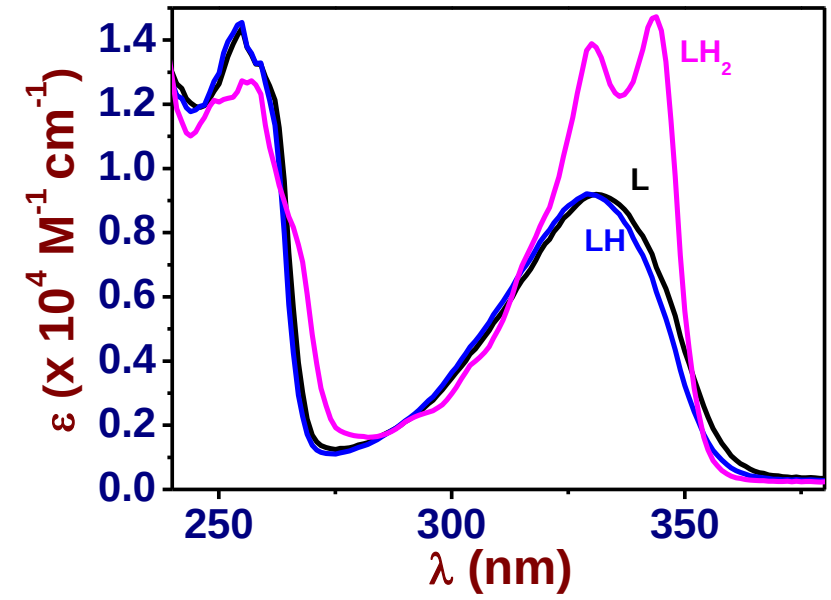
(3) Gans, P., and O'Sullivan, B. (2000) GLEE, a new computer program for glass electrode calibration *Talanta 51*, 33-37.

titrations of the free ligands were then carried out by addition of known volumes of NaOH solutions (BdH, AnalaR) with an Eppendorf microburette. Special care was taken to ensure that complete equilibration was attained. Absorption spectra versus pH were recorded using a Varian CARY 50 spectrophotometer fitted with Hellma optical fibers (Hellma, 041.002-UV) and an immersion probe made of quartz suprazil (Hellma, 661.500-QX). The temperature was maintained at 25.0(2) °C with the help of a Lauda E200 thermostat.

4- **Spectrofluorimetric Titrations versus pH.** Emission spectrophotometric titrations of the same set of ligands were thereafter carried out. The luminescence titrations were carried out on diluted solutions with an absorbance smaller than 0.1 at wavelengths $\geq \lambda_{exc}$ in order to avoid any errors due to the inner filter effect and to minimize re-absorption processes. The excitation wavelength corresponds to the smallest absorbance amplitudes measured along the absorption spectrophotometric titrations. 40 mL of solutions were introduced in a jacketed cell (METROHM) maintained at 25.0(2) °C (Lauda E200). The free hydrogen ion concentration was measured with a combined glass electrode (METROHM 6.0234.500, Long Life) and an automatic titrator system 716 DMS Titrino (Metrohm). The initial pH was adjusted to ~ 2 with $HClO_4$ (Prolabo, normapur, 70% min), and the titrations were then carried out by addition of known volumes of NaOH solutions (BdH, AnalaR) with an Eppendorf microburette. Special care was taken to ensure that complete equilibration was attained. The luminescence spectra were recorded on a Perkin-Elmer LS-50B maintained at 25.0(2) °C by the flow of a Haake FJ thermostat. The light source was a pulsed xenon flash lamp with a pulse width at half peak height < 10 μs and power equivalent to 20kW. The slit width was set at 15 nm for both the excitation and the emission.

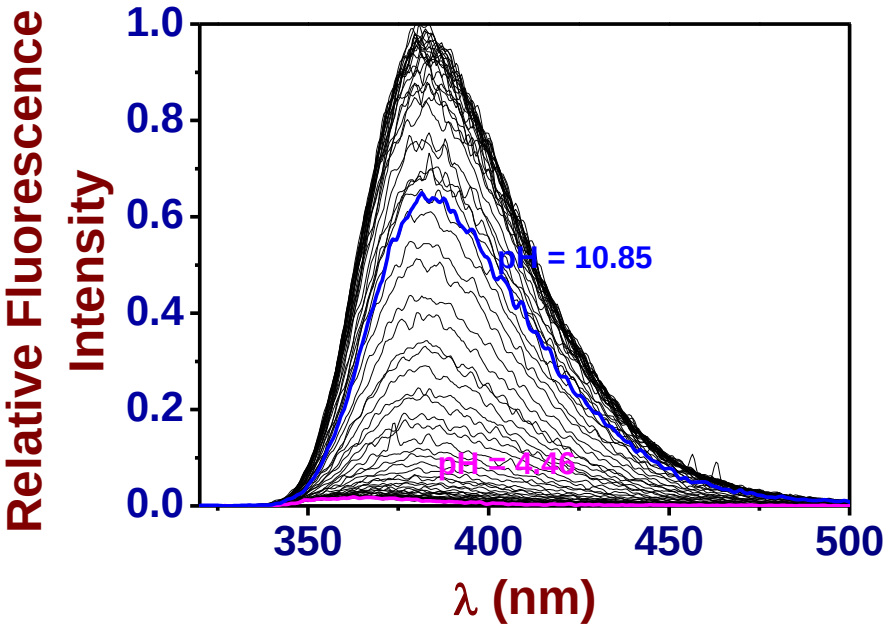


(A)

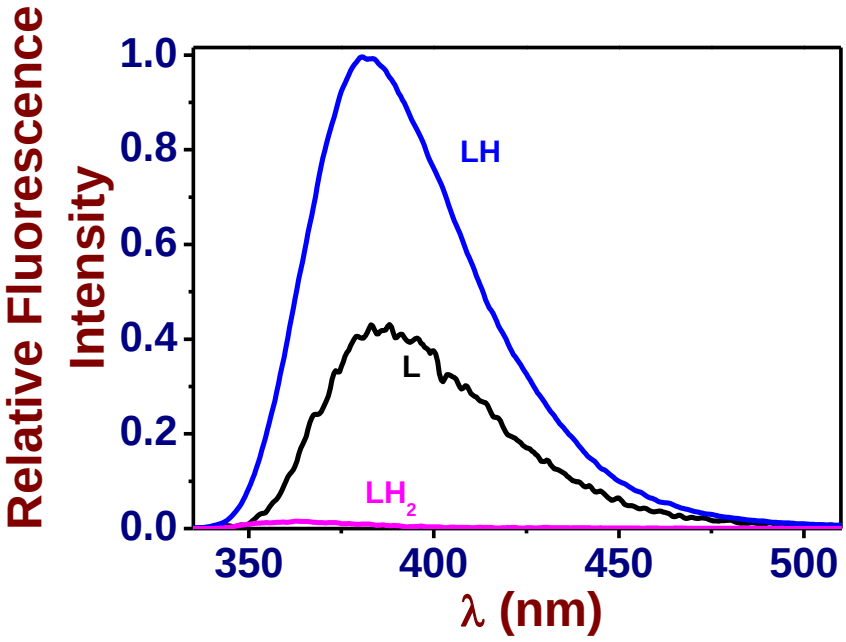


(B)

Figure S1. (A) Absorption spectrophotometric titration of chloroquine CQ (noted L) as a function of pH and (B) electronic spectra of the protonated species of CQ. Solvent: Water; I = 0.1 M $NaClO_4$; T = 25.0(2) °C; l = 1 cm; $[CQ]_{tot}$ = 9.18 x 10^{-5} M. The charges have been omitted for the sake of clarity.



(A)



(B)

Figure S2. (A) Spectrofluorimetric titration of chloroquine CQ (noted L) as a function of pH and (B) reconstituted relative fluorescence spectra of the protonated species of CQ. Solvent: Water; I = 0.1 M $NaClO_4$; T = 25.0(2) °C; l = 1 cm; $[CQ]_{tot}$ = 4.99 x 10^{-5} M. λ_{exc} = 264 nm; excitation and emission band widths = 2.5 nm; filter at 290 nm. The charges have been omitted for the sake of clarity.

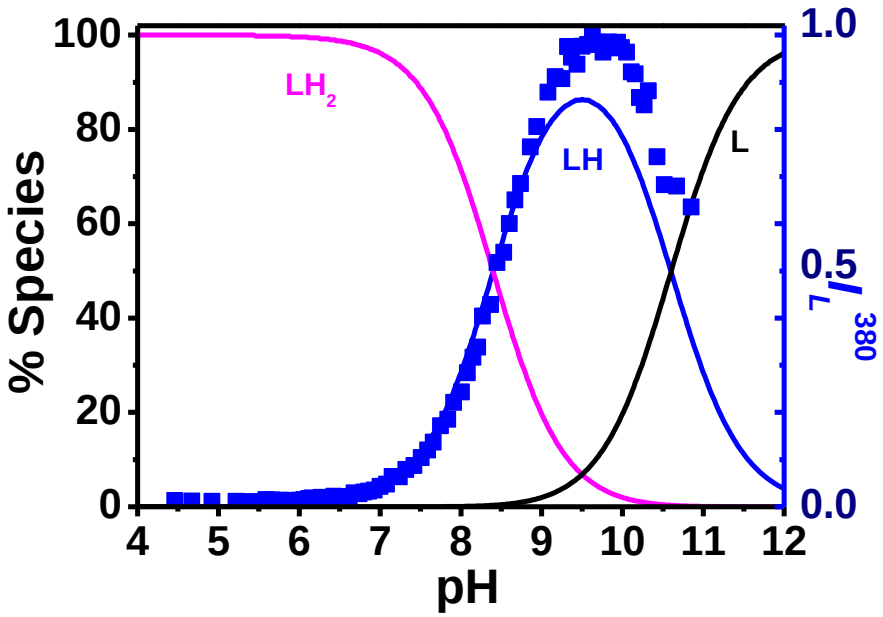
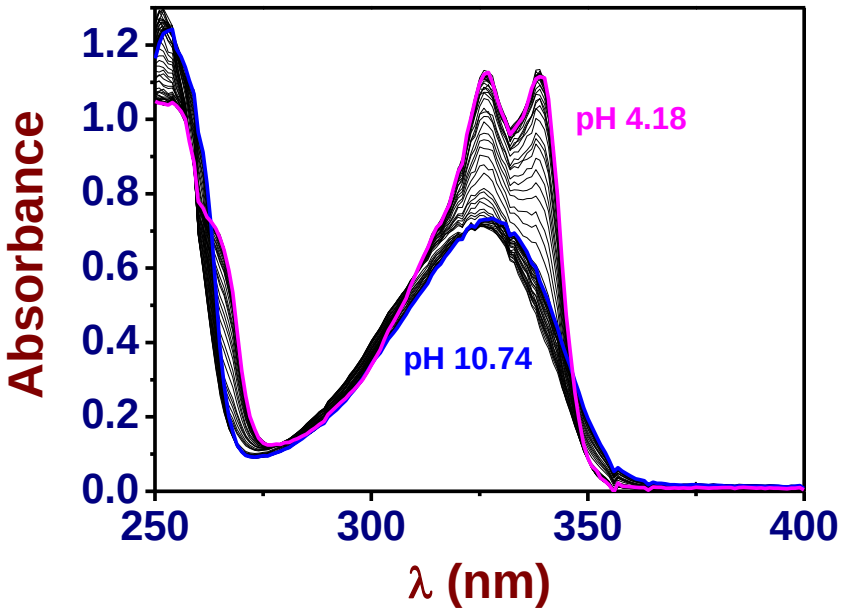
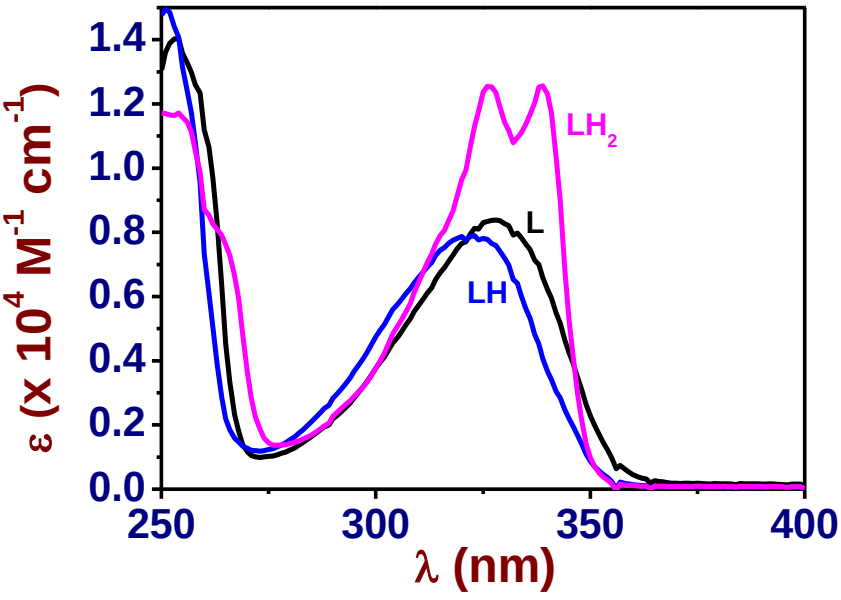


Figure S3. Distribution diagrams of the protonated species of chloroquine CQ (noted L) as a function of pH compared to the variation of the fluorescence intensity at 380 nm. Solvent: Water; I = 0.1 M $NaClO_4$; T = 25.0(2) °C; l = 1 cm; $[CQ]_{tot}$ = 4.99 x 10^{-5} M. λ_{exc} = 264 nm; excitation and emission band widths = 2.5 nm; filter at 290 nm. The charges have been omitted for the sake of clarity.

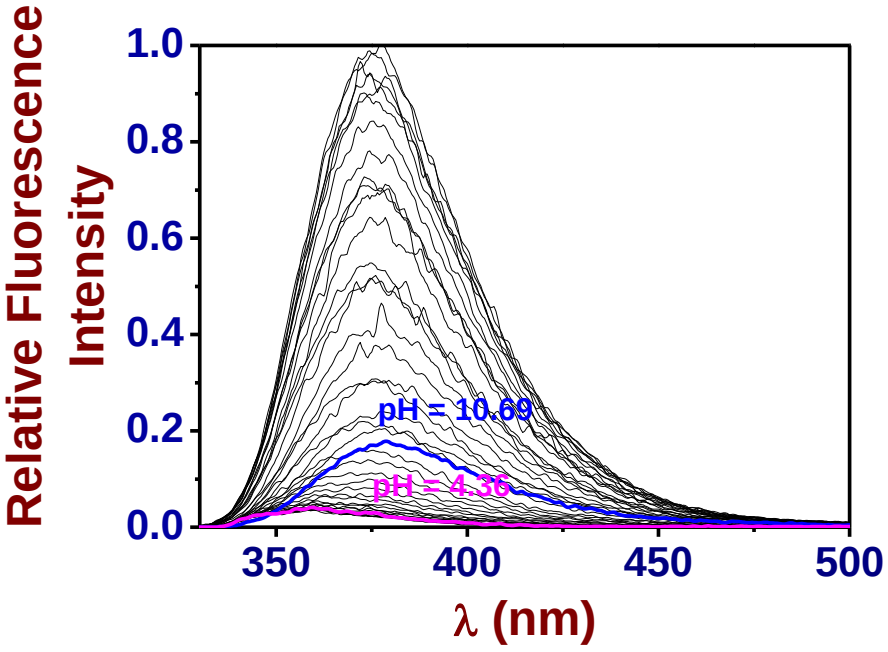


(A)

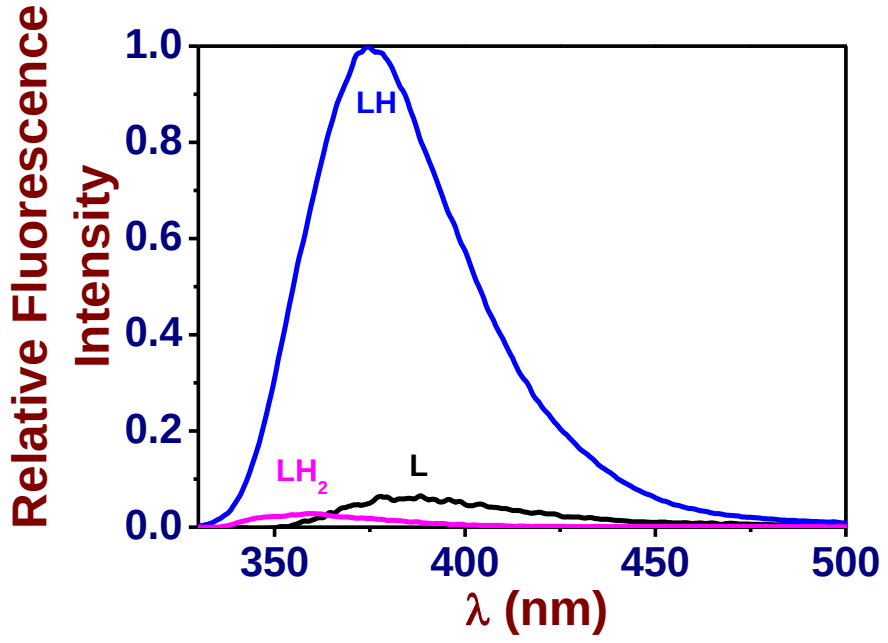


(B)

Figure S4. (A) Absorption spectrophotometric titration of compound **2** (noted L) as a function of pH and (B) electronic spectra of the protonated species of compound **2**. Solvent: Water; I = 0.1 M $NaClO_4$; T = 25.0(2) °C; l = 1 cm; $[\mathbf{2}]_{tot}$ = 8.96 x 10^{-5} M. The charges have been omitted for the sake of clarity.



(A)



(B)

Figure S5. (A) Spectrofluorimetric titration of compound **2** (noted L) as a function of pH and (B) reconstituted relative fluorescence spectra of the protonated species of compound **2**. Solvent: Water; I = 0.1 M $NaClO_4$; T = 25.0(2) °C; l = 1 cm; $[\mathbf{2}]_{tot}$ = 4.70 x 10^{-5} M. λ_{exc} = 260 nm; excitation and emission band widths = 4 nm; filter at 290 nm. The charges have been omitted for the sake of clarity.

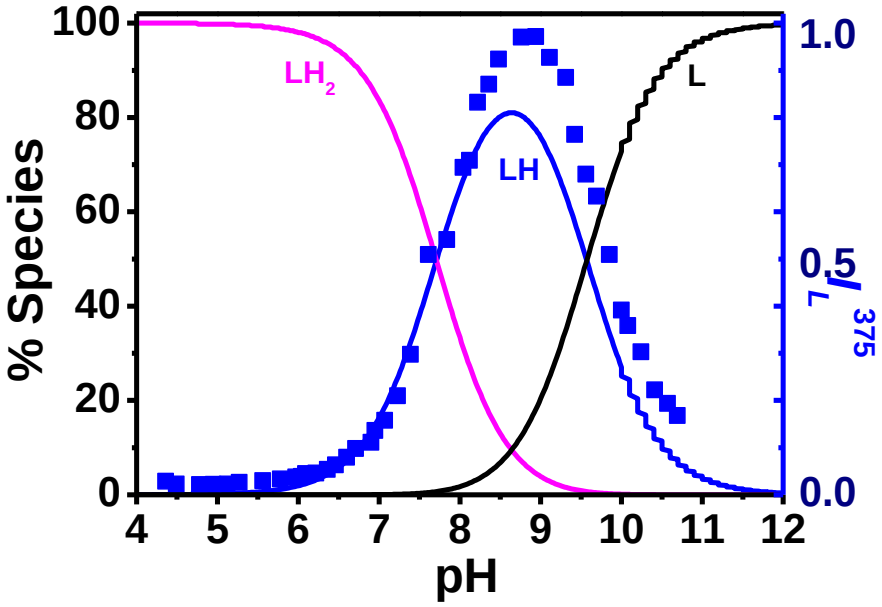
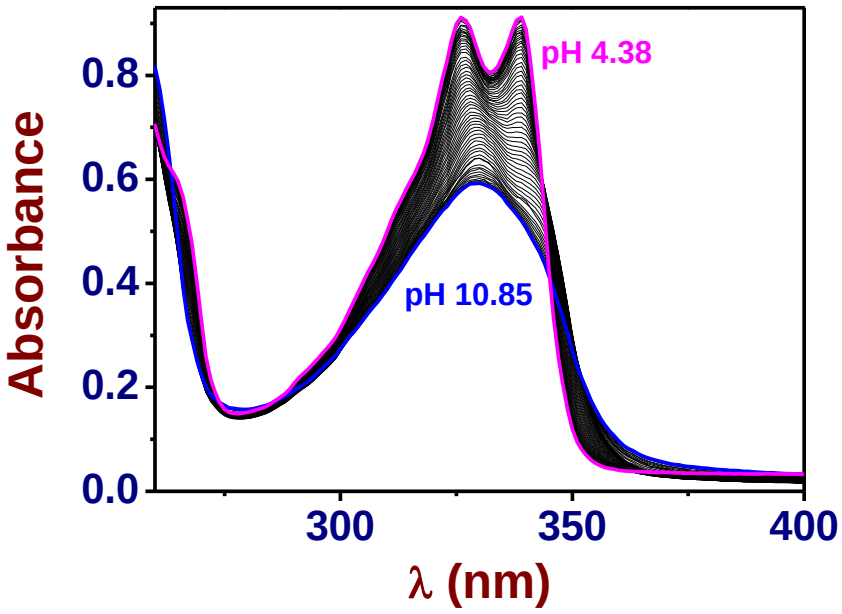
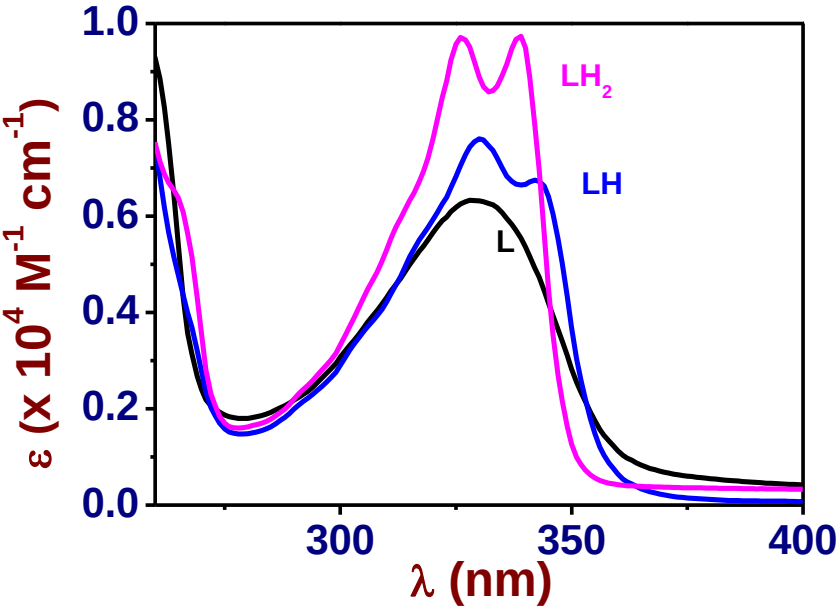


Figure S6. Distribution diagrams of the protonated species of compound **2** (noted L) as a function of pH compared to the variation of the fluorescence intensity at 375 nm. Solvent: Water; I = 0.1 M $NaClO_4$; T = 25.0(2) °C; l = 1 cm; $[\mathbf{2}]_{tot}$ = 4.70 x 10^{-5} M. λ_{exc} = 260 nm; excitation and emission band widths = 4 nm; filter at 290 nm. The charges have been omitted for the sake of clarity.

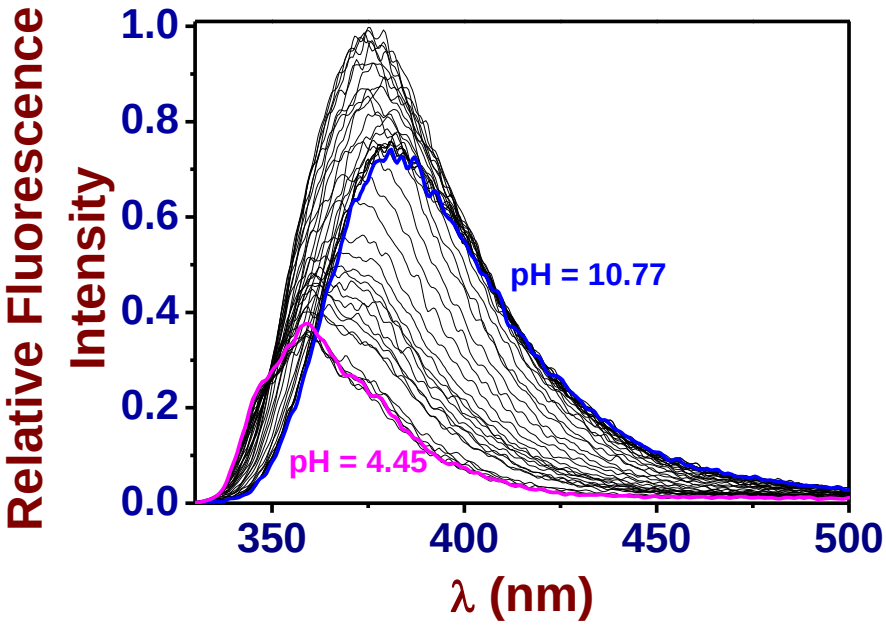


(A)

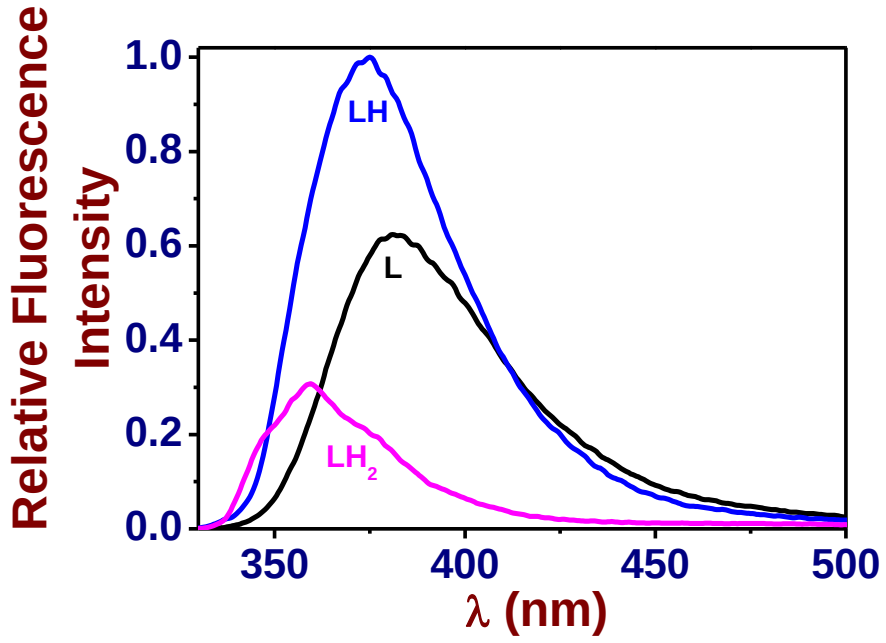


(B)

Figure S7. (A) Absorption spectrophotometric titration of compound **4** (noted L) as a function of pH and (B) electronic spectra of the protonated species of compound **4**. Solvent: Water:DMSO (1/1 *v/v*); I = 0.1 M $NaClO_4$; T = 25.0(2) °C; l = 1 cm; $[\mathbf{4}]_{tot}$ = 9.36 x 10^{-5} M. The charges have been omitted for the sake of clarity.



(A)



(B)

Figure S8. (A) Spectrofluorimetric titration of compound **4** (noted L) as a function of pH and (B) reconstituted relative fluorescence spectra of the protonated species of compound **4**. Solvent: Water:DMSO (1/1 *v/v*); I = 0.1 M $NaClO_4$; T = 25.0(2) °C; l = 1 cm; $[\mathbf{4}]_{tot}$ = 4.76 x 10^{-5} M. λ_{exc} = 262 nm; excitation and emission band widths = 5 nm; filter at 290 nm. The charges have been omitted for the sake of clarity.

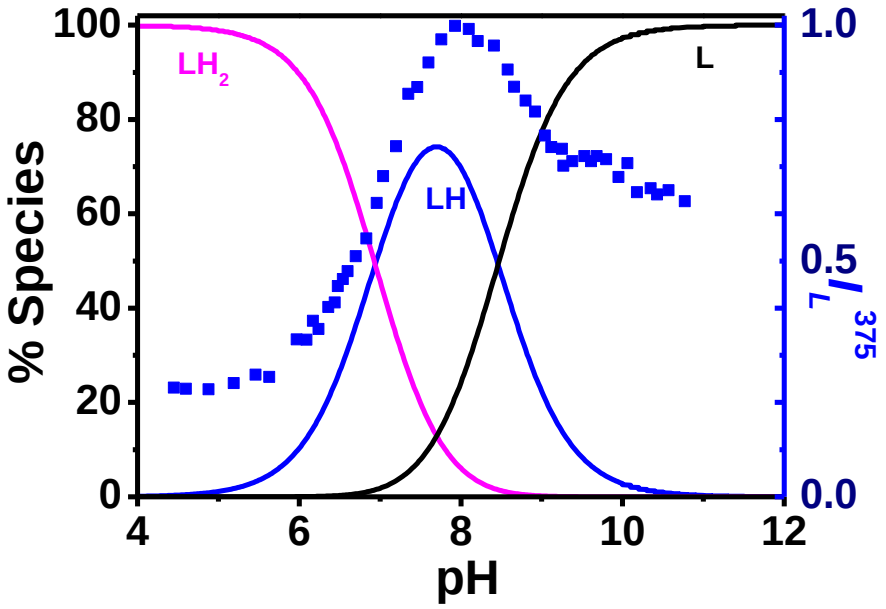
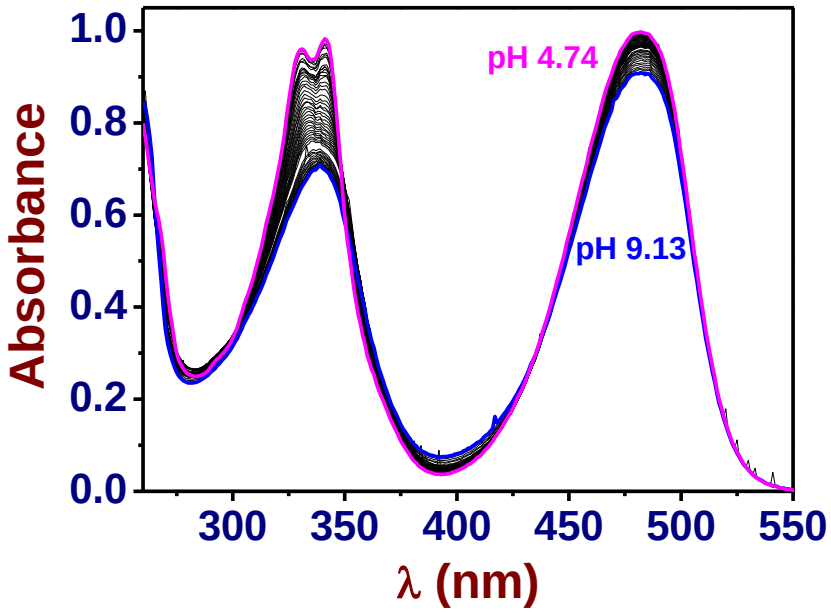
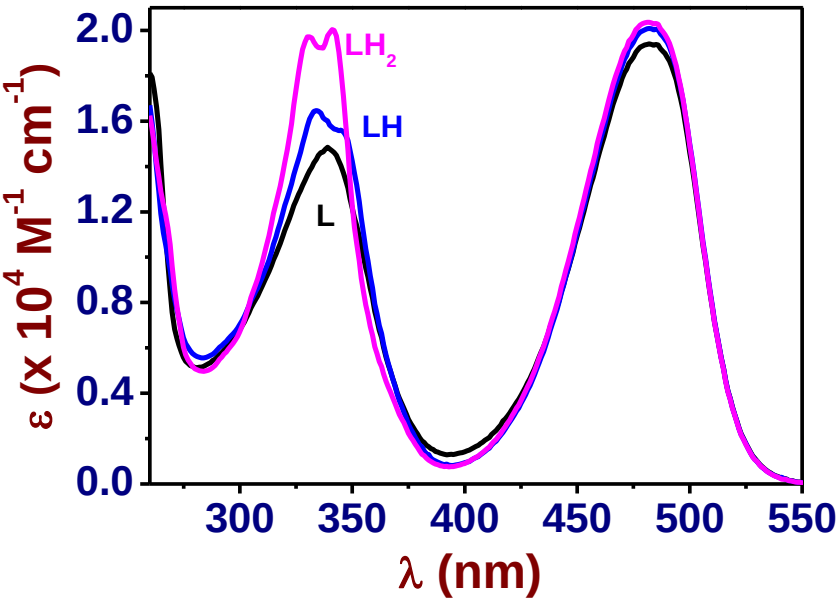


Figure S9. Distribution diagrams of the protonated species of compound **4** (noted L) as a function of pH compared to the variation of the fluorescence intensity at 375 nm. Solvent: Water:DMSO (1/1 *v/v*); I = 0.1 M $NaClO_4$; T = 25.0(2) °C; l = 1 cm; $[\mathbf{4}]_{tot}$ = 4.76 x 10^{-5} M. λ_{exc} = 262 nm; excitation and emission band widths = 5 nm; filter at 290 nm. The charges have been omitted for the sake of clarity.

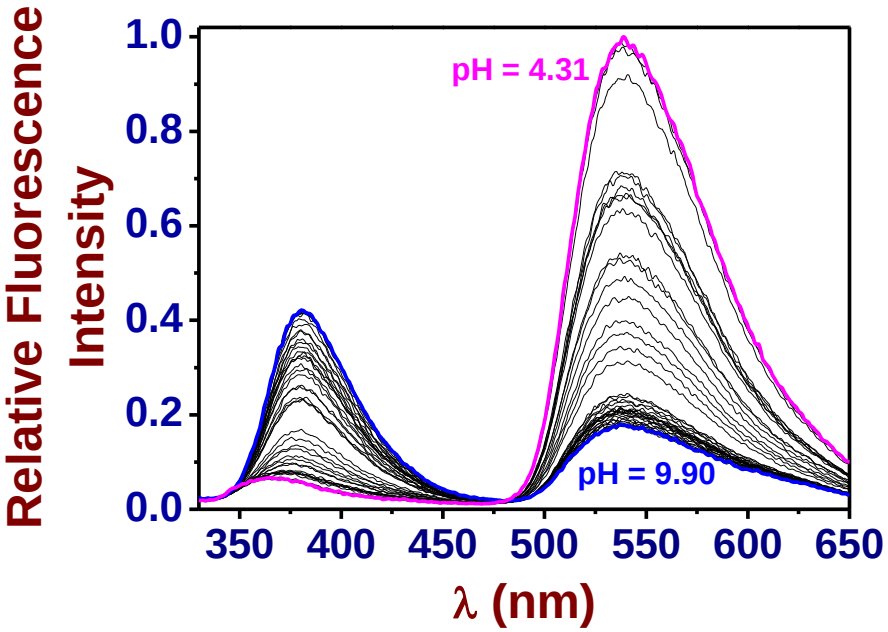


(A)



(B)

Figure S10. (A) Absorption spectrophotometric titration of Fluo-CQ **6** (noted L) as a function of pH and (B) electronic spectra of the protonated species of Fluo-CQ **6**. Solvent: Water:DMSO (1/1 *v/v*); I = 0.1 M $NaClO_4$; T = 25.0(2) °C; l = 1 cm; $[\mathbf{6}]_{tot}$ = 4.89 x 10^{-5} M. The charges have been omitted for the sake of clarity.



(A)

Figure S11. (A) Spectrofluorimetric titration of Fluo-CQ **6** (noted L) as a function of pH. The reconstituted relative fluorescence spectra of the protonated species of Fluo-CQ **6** was given in the manuscript. Solvent: Water:DMSO (1/1 *v/v*); I = 0.1 M $NaClO_4$; T = 25.0(2) °C; l = 1 cm; $[\mathbf{6}]_{tot}$ = 1.16 x 10^{-5} M. λ_{exc} = 265 nm; excitation and emission band widths = 5.5 nm; filter at 290 nm. The charges have been omitted for the sake of clarity.

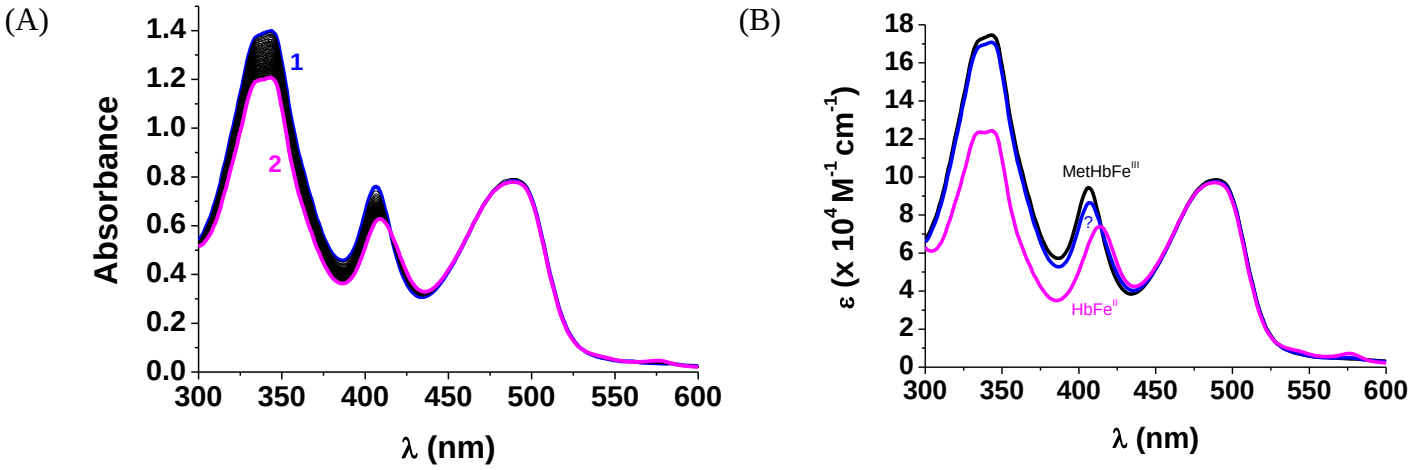
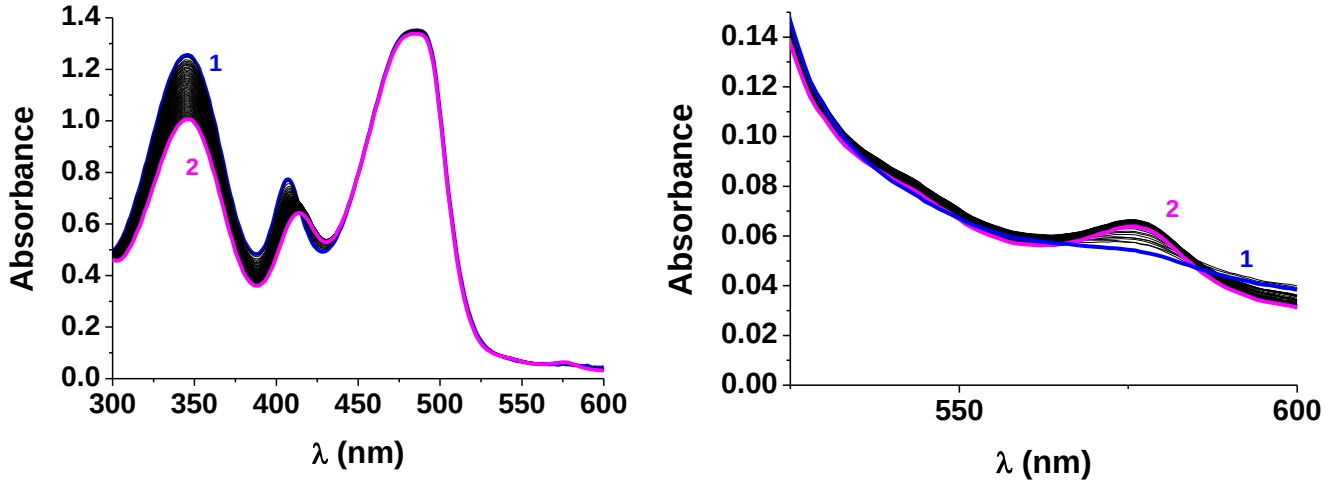


Figure S12. (A) UV-visible absorption spectra recorded as a function of time in the coupled metHb(Fe^{III}) reduction assay in the presence of the *Pf*GR/NADPH system and Fluo-CQ (**6**). (B) Electronic spectra of the reactants and the products of metHb(Fe^{III}) reduction. Solvent: water (47 mM phosphate buffer pH 6.9 + 1 mM EDTA + 200 mM KCl); *T* = 25.0°C; 120 µM NADPH + 1.72 nM *Pf*GR + 8 µM metHb + 20 µM GSSG + 40 µM Fluo-CQ (**6**); (1) t = 0; (2) t = 120 min.

(A)



(B)

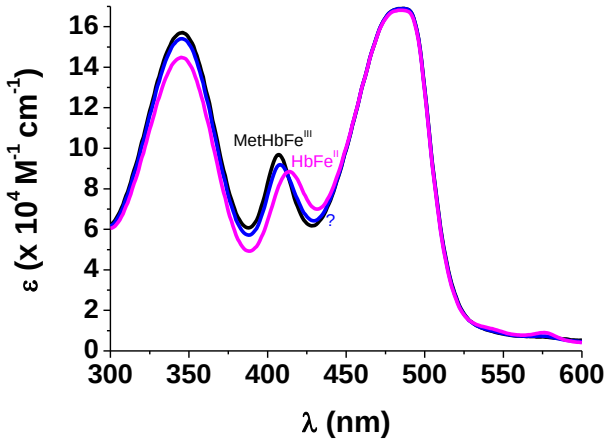
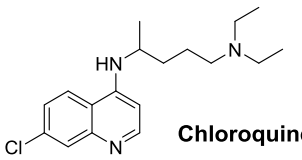
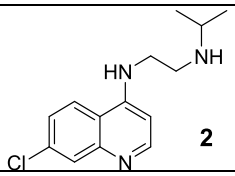
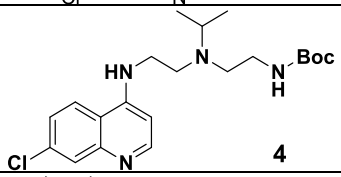
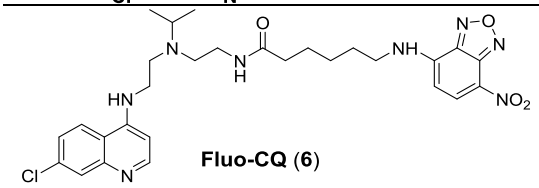


Figure S13. (A) UV-visible absorption spectra recorded as a function of time in the coupled metHb(Fe^{III}) reduction assay in the presence of the *Pf*GR/NADPH system and NBD-hexanoic acid (**7**). (B): Electronic spectra of the reactants and the products of metHb(Fe^{III}) reduction. Solvent: water (47 mM phosphate buffer pH 6.9 + 1 mM EDTA + 200 mM KCl); *T* = 25.0°C; 120 µM NADPH + 1.72 nM *Pf*GR + 8 µM metHb + 20 µM GSSG + 40 µM NBD-hexanoic acid (**7**); (1) t = 0; (2) t = 120 min.

Table S1. Base properties and *pKa* values of CQ, compound **2**, compound **4**, and Fluo-CQ **6** evaluated by coupled absorption spectrophotometric (or spectrofluorimetric) and potentiometric titrations.

Compound structure	LH_2^{2+} (pH=5) / LH_2^{2+} (pH=7.4) ratio	pK_a values[a]
 **Chloroquine (CQ)**	1.1	8.4 ± 0.2 10.6 ± 0.2
 **2**	1.49	7.7 ± 0.2 9.6 ± 0.2
 **4**	4.09	6.9 ± 0.3 8.5 ± 0.2
 **Fluo-CQ (6)**	14.4	6.4 ± 0.6 7.8 ± 0.5[b]

[a] Absorption titrations; I = 0.1 M $NaClO_4$, T = 25.0°C; Error = 1σ with σ = standard deviation.
[b] water/DMSO (1:1 v/v); I = 0.1 M $NaClO_4$.

Table S2. Characterization (ESI-MS) and association constants (K_D et DV_{50} CID-MS) of the heme-substrates species. These data are compared to the inhibition capacities of the corresponding substrates to prevent β-hematin (the synthetic hemozoin) formation.

Species	m/z_{exp} (m/z_{calc})	DV_{50} (V)	IC_{50} (Inhib. β-hematin)	K_D Heme/Substrate (μM) (p*K*a substrate)
$[Heme]^+$	616.35 (616.18)	360		
$[(Heme)(Heme\text{-}H)]^+$	1231.65 (1231.35)			
$[(Heme)_2(OH)]^+$	1249.60 (1249.36)			
$[(Heme)_2(HCO_2)]^+$	1277.6 (1277.36)			
$[(Heme)(\mathbf{CQ})]^+$	935.50 (935.36)	261	1.6[4]	0.85 (10.18/8.38)[5]
$[(Heme)(\mathbf{CQ})(H_2O)]^+$	953.86 (953.37)			
$[(Heme)(\mathbf{CQ})(HCO_2H)]^+$	981.60 (981.36)			
$[(Heme)(\mathbf{2})]^+$	879.50 (879.29)	236	-	0.41[a] (9.71/7.78[b])
$[(Heme)(\mathbf{2})(HCO_2H)]^+$	925.55 (925.30)			
$[(Heme)(\mathbf{4})]^+$	1022.65 (1022.39)	267	2.6[4]	
$[(Heme)(\mathbf{4})(HCO_2H)]^+$	1068.60 (1068.39)			
$[(Heme)(\mathbf{7})]^+$	910.45 (910.27)	144	-	
$[(Heme)(\mathbf{Fluo\text{-}CQ})]^+$	1198.70 (1198.42)	309	-	

Heme = $C_{34}H_{32}FeN_4O_4$; **CQ** = $C_{18}H_{26}ClN_3$; **2** = $C_{14}H_{18}ClN_3$; **4** = $C_{21}H_{31}ClN_4O_2$; **7** = $C_{12}H_{14}N_4O_5$; **Fluo-CQ** (**6**) = $C_{28}H_{35}ClN_8O_4$.

4 Friebolin W., Jannack B., Wenzel N., Furrer J., Oeser T., Sanchez C.P., Lanzer M., Yardley V., Becker K., Davioud-Charvet E. *J. Med. Chem.*, **2008**, *51*, 1260.

5 Warhurst D.C., Steele J.C.P., Adagu I.S., Craig J.C., Cullander C. *J. Antimicrob. Chemother.* **2003**, 52, 188.

Table S3. Averaged IC_{50} values (nM) for CQ and CQ1 analogues determined from growth inhibition assays with *Plasmodium falciparum* strain HB3 (CQ^S) and Dd2 (CQ^R).[a]

Compound	IC_{50} ± SEM (nM) HB3 (CQ^S) (n)[b]	IC_{50} ± SEM (nM) Dd2 (CQ^R) (n)[b]
CQ	15.6 ± 0.5 (3)	71.2 ± 3.0 (4)
2 (CQ1)	23.1 ± 1.3 (3)	27.5 ± 5.5 (4)
4 (CQ1-SPAC-Boc)	95.2 ± 10.2 (3)	77.4 ± 9.6 (4)
6 (**Fluo-CQ**) (= CQ1-SPAC-NBD)	22.7 ± 4.1 (3)	31.0 ± 9.3 (4)

[a] Test conditions: Dd2 or HB3 synchronous cultures, $SYBR_{green}$ assay, 72 h incubation. [b] Values are means of at least two independent determinations in triplicate. The IC_{50} value of the antimalarial drug chloroquine (CQ) is indicated as a reference. n: Number of measurements.