Supporting Information

Intrinsic Fluorescence Properties of Antimalarial Pyrido[1,2-a]benzimidazoles Facilitate Subcellular Accumulation and Mechanistic Studies in the Human Malaria Parasite *Plasmodium falciparum*

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1.0 Experimental

1.1 Reagents, solvents and equipment

All commercially available chemicals were purchased from either Sigma-Aldrich or Combi-Blocks Limited and were of analytical grade, thus used without further purification.
Compounds characterizations were done using ^{1}H NMR, ^{13}C NMR, and HPLC-MS.

^{1}H NMR spectra were recorded on a Varian Mercury (300 MHz), a Bruker Ultrashield-Plus (400 MHz) spectrometer or a Bruker (600 MHz). ^{13}C NMR spectra were recorded on the same instruments at 101 MHz or 151 MHz. NMR samples were dissolved in deuterated dimethyl sulfoxide (DMSO-d_6) or methanol (MeOD-d_4). Chemical shifts (δ) are reported in parts per million (ppm) to 2 decimal places. Coupling constants (*J*) are reported in Hertz (Hz) to one decimal place. Abbreviations used in assigning ^{1}H NMR signals are; d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), m (multiplet), q (quartet), s (singlet), t (triplet) or td (triplet of doublets).

Target compound's peak purities and mass spectrometry were determined on an Agilent HPLC system equipped with Agilent 1260® Infinity Binary Pump, Agilent 1260® Infinity Diode Array Detector, Agilent 1290® Infinity Column Compartment, Agilent 1260® Infinity Autosampler, Agilent 6120® Quadrupole LC/MS and Peak Scientific® Genius 1050 Nitrogen Generator. The column used was Kinetex Core C18, 2.6 μM, 3 x 50 mm, 100Å maintained at

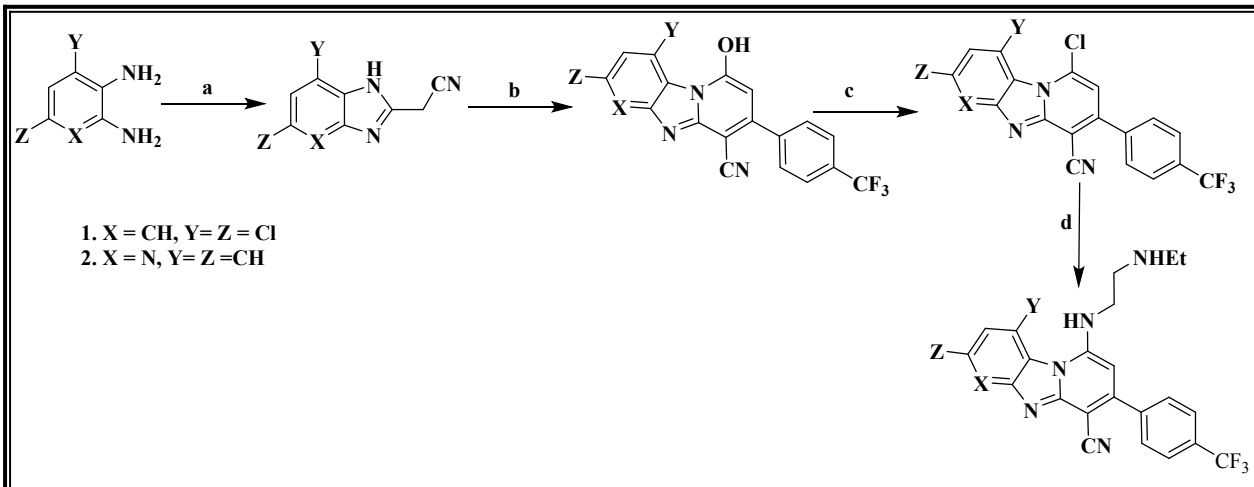
40 °C. The composition and gradient conditions of the mobile phase used at a flow rate of 0.7 mL/min is summarised in **Table 1**. The injection volume was 2 μL while the mass spectra were obtained in either positive or negative modes by Electron Spray ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI). The diode array detector was programmed to scan the eluents at an absorption wavelength range of 210-640 nm.

Table 1: HPLC Gradient Conditions

Time (min)	% A	% B	Composition	
			A	B
0.00	85.00	15.00	10 mM NH4OAc in buffer (0.4 % acetic acid)	10 mM NH4OAc (0.4 % acetic acid) in 90 % HPLC grade CH_3OH in H_2O
0.30	85.00	15.00		
1.20	0.00	100.00		
4.50	0.00	100.00		

Reactions were monitored by thin layer chromatography (TLC) using Fluka or Merck F254 aluminium-backed pre-coated silica gel plates and were visualized under ultraviolet light at 254 or 366 nm. Silica gel column chromatography was performed using Merck kieselgel 60: 70-230 mesh by gravity column chromatography or flash column chromatography on a Biotage Isolera™ system (Biotage AB, Uppsala, Sweden).

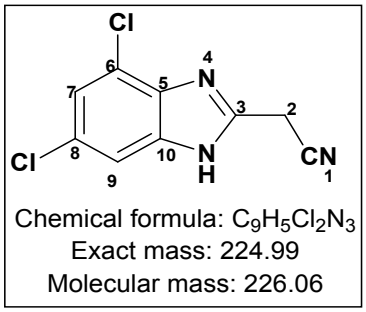
Scheme 1. Synthesis of Pyrido[1,2-a]benzimidazole intermediates and target compounds



Reagents and conditions. (a) Ethyl cyanoacetate. DMF, microwave, 110 ^oC, 15-45 min; (b)CF_3Ph, NH_4OA_C, 150 ^oC, 1 H; (c) $POCl_3$ 130 ^oC, 2h; (d) amine, Et3N, THF,80 ^oC, microwave, 20 min.

1.2 Synthesis and characterization

Procedure for the synthesis of intermediate a (scheme 1):A mixture of appropriate diamine (1.00 equiv.) and ethyl cyanoacetate (1.2 equiv.) was heated in a microwave reactor at 110 °C for 30-60 min. The reaction mixture was purified through column chromatography using MeOH: DCM as an eluent.



2-(4,6-dichloro-1H-benzo[d]imidazole-2-yl)acetonitrile (CKM01)

Obtained from 3,5-dichlorobenzene 1,2-diamine (1.00 g, 5.65 mmols) and ethyl 2-cyanoacetate (6.78 mmols) as brown solid (94%, 1.4307 g); R_f (DCM: MeOH: 9:1) 0.75; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.53 (d, J = 2.4 Hz, 1H, H^9), 6.50 (d, J = 2.4 Hz, 1H, H^7), 5.12 (s, 2H, H^2). ^{13}C NMR (101 MHz, DMSO) δ 138.00, 130.60, 120.67, 117.88, 115.91(2C), 112.20, 557.20 18.84. HPLC-MS (ESI): Purity = 99%, t_R = 2.57 min, m/z $[M+H]^+$ = 226.0

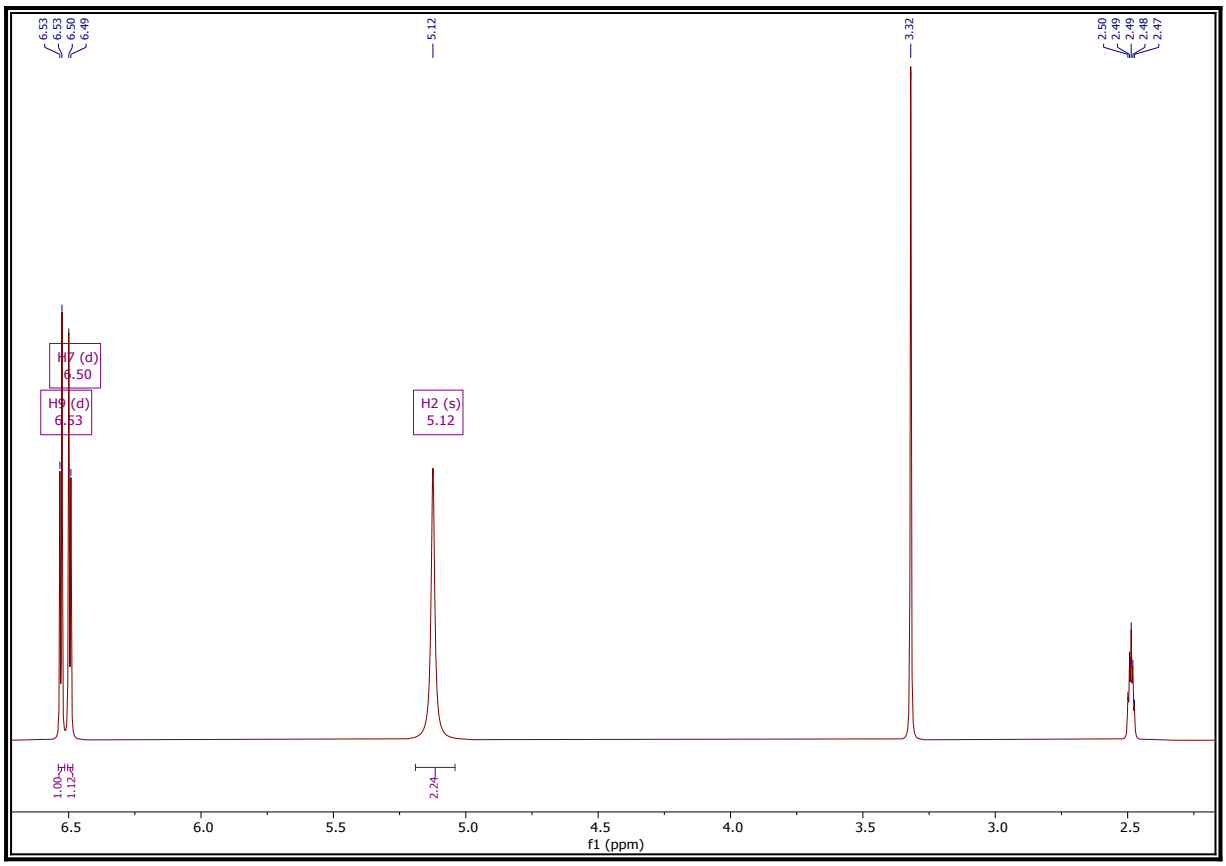
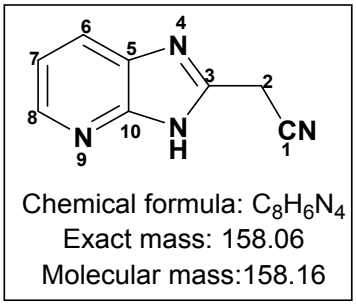


Figure 1: ^{1}H-nuclear magnetic resonance spectrum (NMR; 300 MHz, DMSO-*d6*) of the benzimidazole intermediate of compound **1** (**CKM03)**

2-(3H-imidazo[4,5-b]pyridin-2-yl)acetonitrile (CKM02)



Obtained from pyridine-2,3-diamine (1.00 g, 9.16 mmols) and ethyl 2-cyanoacetate (10.99 mmols) as brown solid (97%, 1.677 g); R_f (DCM: MeOH: 9:1) 0.3: ^{1}H NMR (600 MHz, DMSO-d6) δ 13.97 (br s, 1H, NH), 9.12 (m,1H, H^8), 8.78 (m, 1H, H^6),8.03 (dd, *J* = 8.0, 4.8 Hz, 1H, H^7), 5.23 (s, 2H, H^2). ^{13}C NMR (151 MHz, DMSO-D6) δ 148.33, 144.95 (2C), 124.71, 119.29 (2C), 118.98, 117.46, HPLC-MS (ESI): Purity = 99%, t_R = 0.207 min, m/z $[M+H]^+$ = 159.0

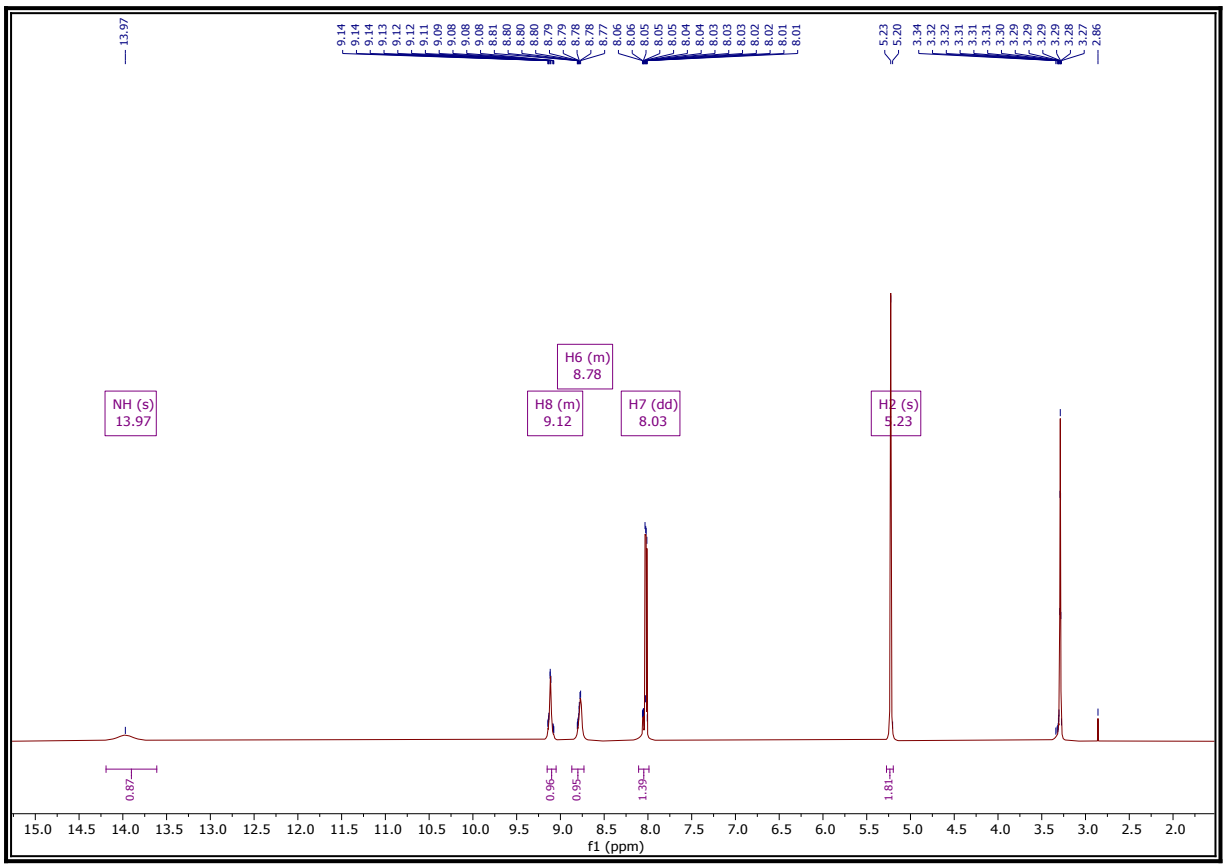
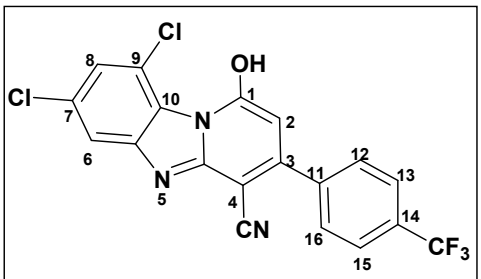


Figure 2: ^{1}H-nuclear magnetic resonance spectrum (NMR; 300 MHz, DMSO-*d6*) of the benzimidazole intermediate of compound **2** (**CKM02)**

1.2.1 Synthesis of hydroxylated intermediate b (scheme 1)

The appropriately substituted benzimidazole acetonitrile (1.0 equiv.), ethyl 3-oxo-3-(4-(trifluoromethyl)phenyl)propanoate or ethyl 4,4,4- trifluoro-3-oxobutanoate (1.2 equiv.) and ammonium acetate (2.0 equiv.) were heated at 150°C for 2 hours. Reaction mixture was cooled to room temperature and washed with cold acetonitrile. The mixture was filtered, and the product left to dry and used in subsequent step without further purification.

7,9-dichloro-1-hydroxy-3-(4-(trifluoromethyl)phenyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile (CKM03)



Obtained from **CKM01** (500 mg, 2.24 mmols) as a brown solid (78%, 733 mg); R_f (DCM: MeOH: 9:1) 0.3: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.50 (d, J = 2.0 Hz, 1H, H^6), 7.87 (d, J = 8.1 Hz, 2H, H^{13,15}), 7.82 (d, J = 8.1, Hz, 2H, H^{12,16}), 7.49 (d, J = 2.0 Hz, 1H, H^8), 5.60 (s, 1H, H^2). ^{13}C NMR (101 MHz, DMSO) δ 165.90, 164.93, 155.38, 144.01, 128.84 (4C), 126.86, 126.01, 125.67, 125.57, 125.53, 125.49, 125.46, 121.62, 107.94, 99.72, 74.51. HPLC-MS (ESI): Purity = 88%, t_R = 2.8 min, m/z $[M+H]^+$ = 422.0

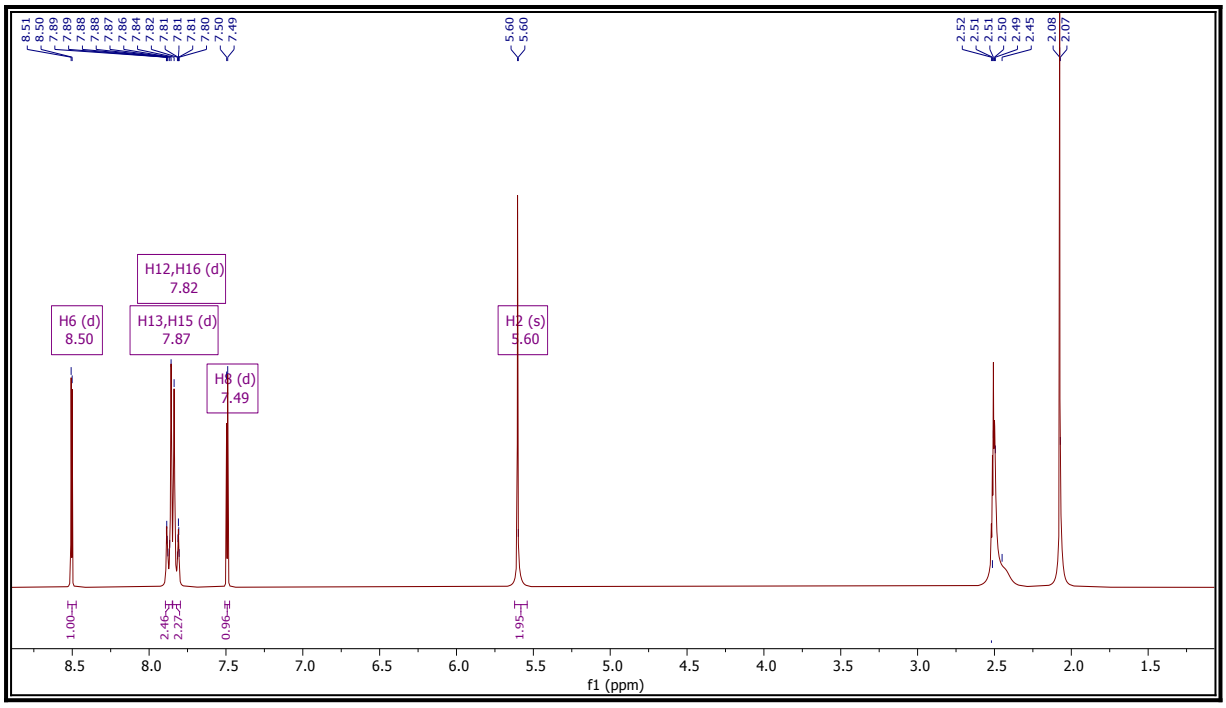
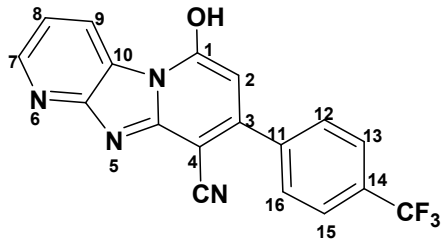


Figure 3: ^{1}H-nuclear magnetic resonance spectrum (NMR; 300 MHz, DMSO-*d6*) of the hydroxy intermediate of compound 1 **(CKM03)**

6-hydroxy-8-(4-(trifluoromethyl)phenyl)imidazo[1,2-a:4,5-b']dipyridine-9-carbonitrile (CKM04)



Chemical formula: $C_{18}H_9F_3N_4O$
Exact mass: 354.07
Molecular mass:354.29

Obtained from **CKM02** (1.00 g, 6.32 mmols) as a brown solid (80%, 1.8 g); R_f (DCM: MeOH: 9:1) 0.3: ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.90 (dd, J = 7.8, 1.4 Hz, 1H, H^7), 8.46 (dd, J = 5.7, 1.4 Hz, 1H, H^9), 7.89 (d, J = 8.3 Hz, 2H, $H^{13,15}$), 7.83 (d, J = 8.2 Hz, 2H, $H^{12,16}$), 7.39 (dd, J = 7.8, 5.7 Hz, 1H, H^8), 6.07 (s, 1H, H^2). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.34, 158.01, 153.92, 152.39, 151.41, 150.73, 142.43, 142.11, 141.64, 141.3, 129.62 (2C), 129.51, 125.93, 125.11, 118.10, 115.40 and 103.04. HPLC-MS (ESI): Purity = 84%, t_R = 2.6 min, m/z $[M+H]^+$ = 355.0

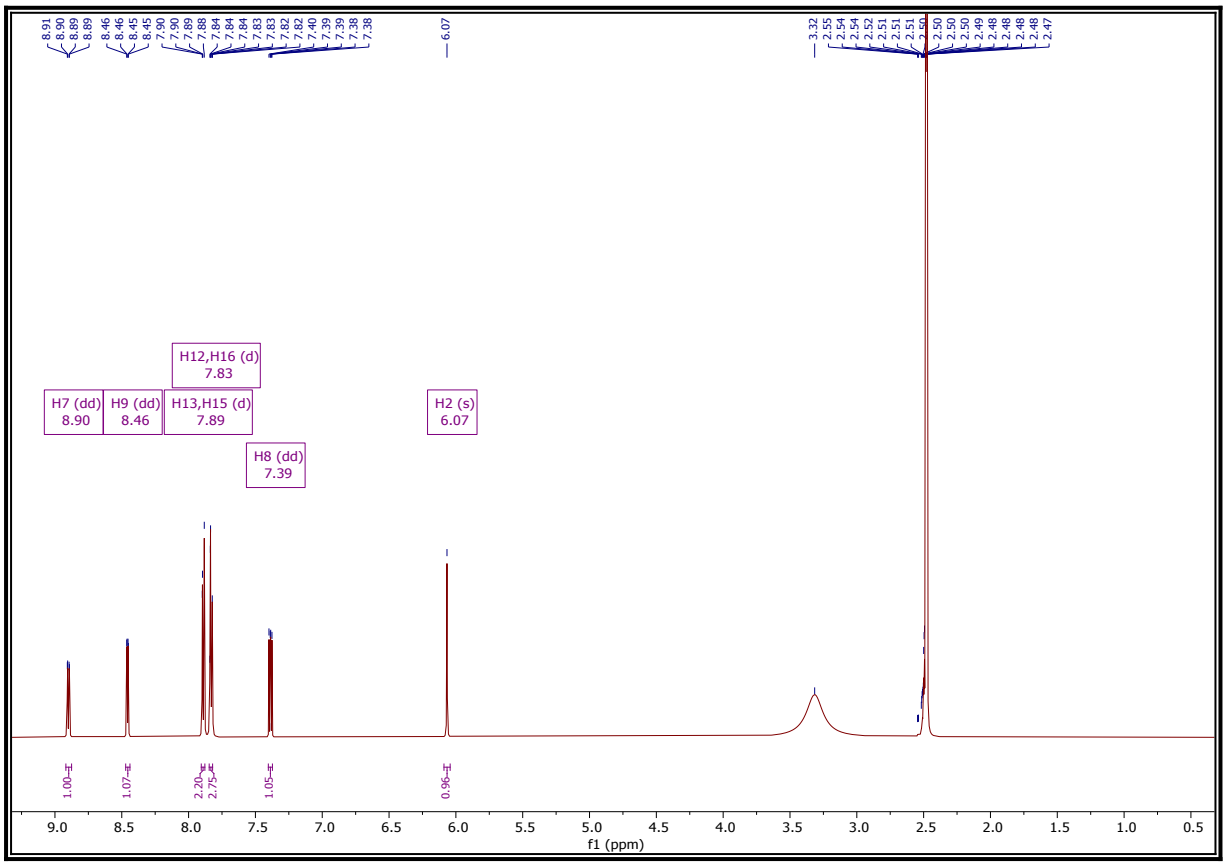
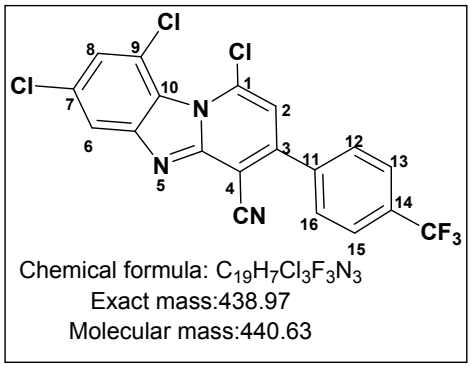


Figure 4: ^{1}H-nuclear magnetic resonance spectrum (NMR; 300 MHz, DMSO-*d6*) of the hydroxy intermediate of compound **2 (CKM04)**

1.2.3 Synthesis of Chloro intermediates c (scheme 1)

Phosphoryl oxychloride ($POCl_3$, 20 equiv.) was added to an appropriately substituted hydroxyl intermediate (1.00 equiv.). The mixture was heated in a sealed tube at 130 °C for 3 hours. Excess $POCl_3$ was removed under reduced pressure. On cooling to room temperature, ice cold water was added with consistent stirring for 15 minutes. The mixture was neutralized using sodium carbonate, the crude product filtered off, washed with water and dried. The product was purified using column chromatography.

1,7,9-trichloro-3-(4-(trifluoromethyl)phenyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile (CKM05)



Obtained from **CKM03** (500 mg, 1.18 mmols) as a brown solid (96%, 498 mg); R_f (DCM: MeOH: 9:1) 0.85: ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.68 (d, J = 2.8 Hz, 1H, H^{6}), 8.04 (d, J = 8.2 Hz, 2H, $H^{13,15}$), 8.00 (d, J = 8.3 Hz, 2H, $H^{12,16}$), 7.89 (d, J = 2.1 Hz, 1H, H^{8}), 7.63 (s, 1H, H^{2}). ^{13}C NMR (151 MHz, DMSO) δ 149.47, 148.93, 141.15, 139.16, 135.30, 130.87, 130.30 (2C), 127.07, 126.35, 126.32, 126.30, 126.27, 124.85, 115.10, 114.77, 114.17, 98.41. HPLC-MS (ESI): Purity = 99%, t_R = 2.6 min, m/z $[M+H]^+$ = 440.0

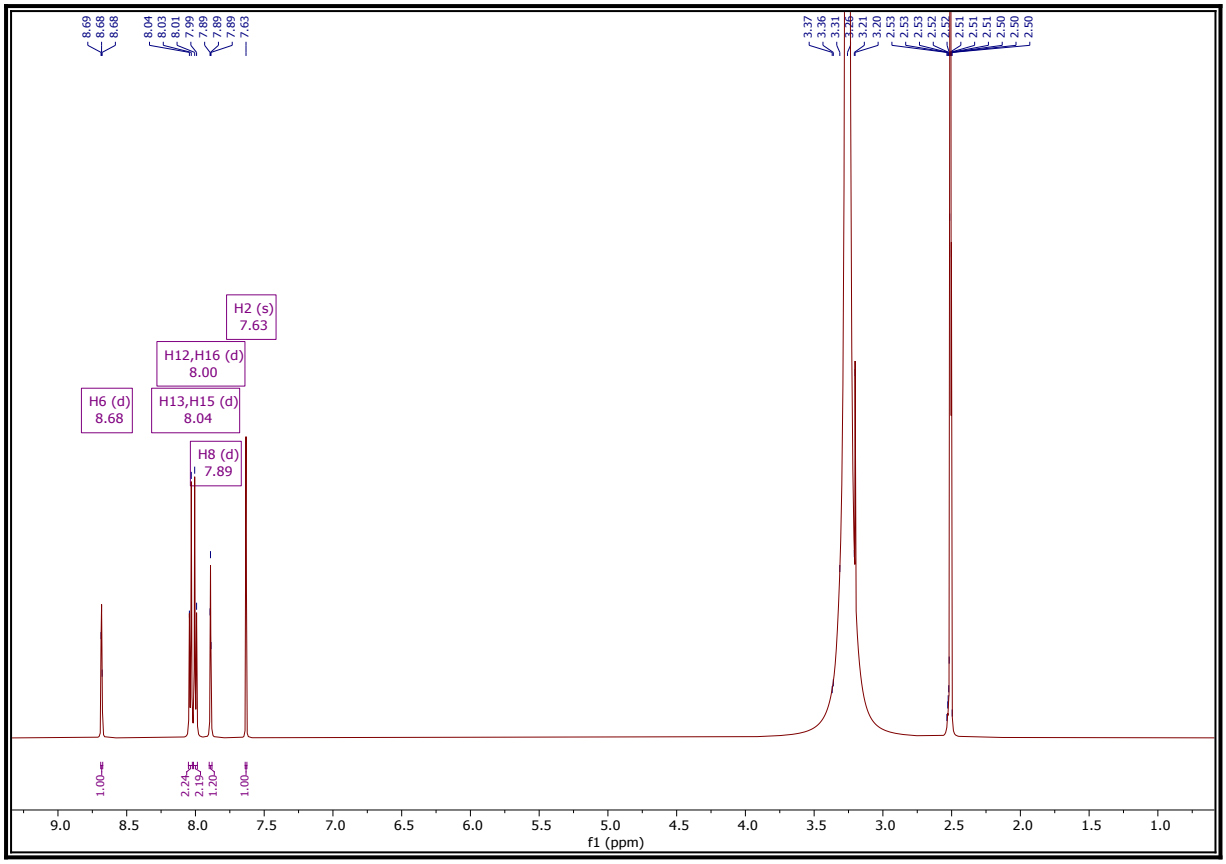
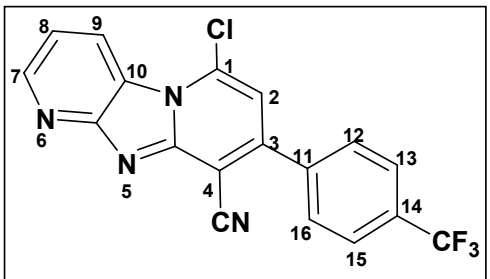


Figure 5: ^{1}H-nuclear magnetic resonance spectrum (NMR; 300 MHz, DMSO-*d6*) of the chloro intermediate of compound **1 (CKM05)**

6-chloro-8-(4-(trifluoromethyl)phenyl)imidazo[1,2-a:4,5-b']dipyridine-9-carbonitrile (CKM07)



Chemical formula: $C_{18}H_8ClF_3N_4$
Exact mass: 372.04
Molecular mass:372.74

Obtained from **CKM04** (500 mg, 1.41 mmols) as a yellow solid (98%, 515 mg); R_f (DCM: MeOH: 9:1) 0.75: ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.02 (dd, J = 8.5, 1.7 Hz, 1H, H^{7}), 8.85 (dd, J = 4.6, 1.6 Hz, 1H, H^{9}), 8.03 (d, J = 8.3 Hz, 2H, $H^{13,15}$), 7.99 (d, J = 8.2 Hz, 2H, $H^{12,16}$), 7.63 (s, 1H, H^{2}), 7.54 (dd, J = 8.4, 4.7 Hz, 1H, H^{8}). ^{13}C NMR (151 MHz, DMSO) δ 155.89, 149.95, 149.38, 149.14,

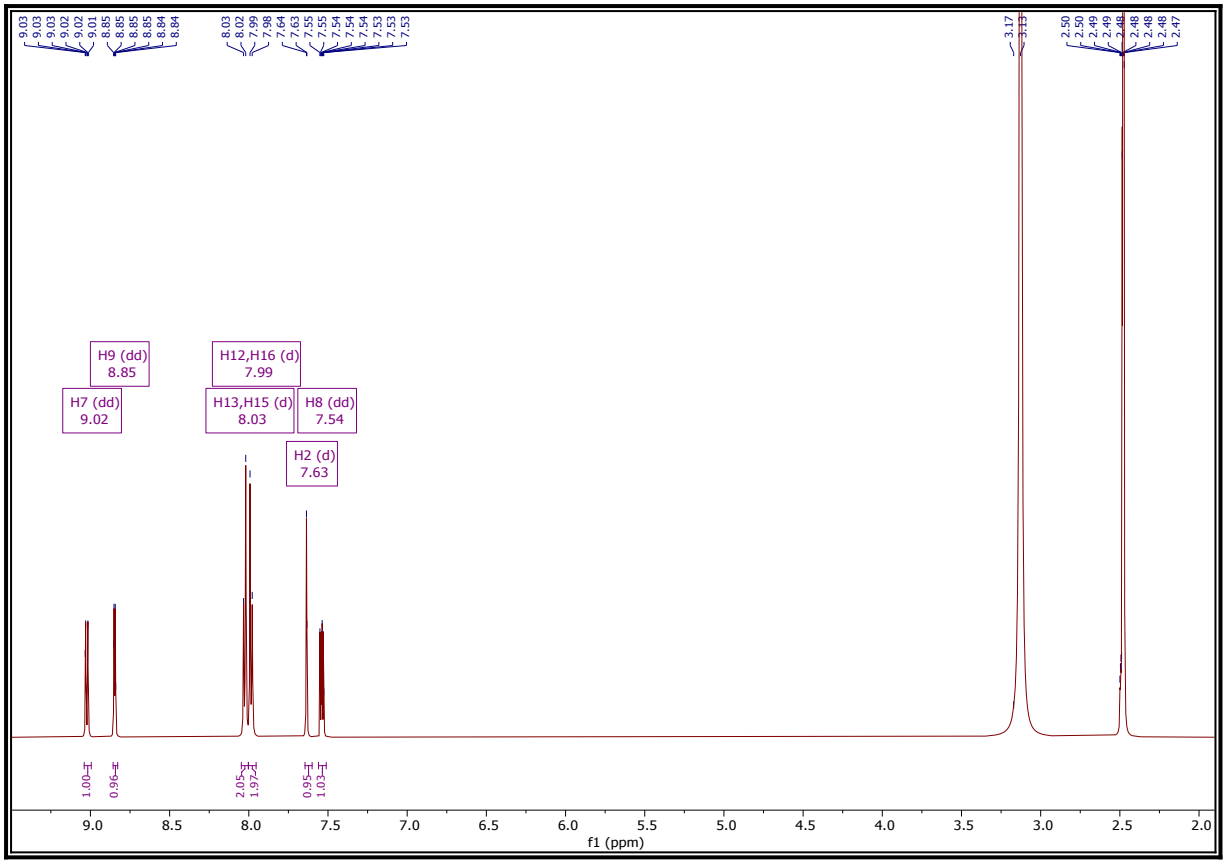
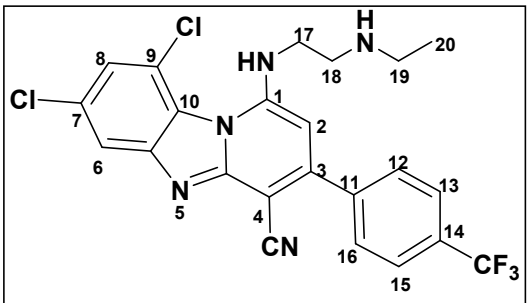


Figure 6: [1]H-nuclear magnetic resonance spectrum (NMR; 300 MHz, DMSO-*d6*) of the chloro-intermediate of compound **2** (**CKM07)**

1.2.4 Synthesis of Compounds 1 and 2.

To their chloro intermediates; **CKM05, 07** in triethylamine and tetrahydrofuran (THF), the appropriate amine was added, and the stirred mixture allowed to react in a microwave at 150W, 80-100 °C for 40-60 minutes. The cooled reaction mixture was transferred to a round bottom flask and concentrated. The crude reaction mixture was purified by column chromatography to obtain compounds **1** and **2.**

7,9-dichloro-1-((2-(ethylamino)ethyl)amino)-3-(4-(trifluoromethyl)phenyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile (1/CKM06)



Obtained from **CKM05** (100 mg, 0.23 mmols) as a yellow solid (43%, 53 mg); R_f (DCM: MeOH: 9:1) 0.6: ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.74 (d, J = 1.9 Hz, 1H, H^6), 7.87 (m, 4H, $H^{13,15}$, $H^{12,16}$), 7.50 (d, J = 1.9 Hz, 1H, H^8), 5.88 (s, 1H, H^2), 3.65 (t, J = 6.2 Hz, 2H, H^{17}), 3.24 (t, J = 6.2 Hz, 2H, H^{18}), 3.03 (q, J = 7.2 Hz, 2H, H^{19}), 1.25 (t, J = 7.2 Hz, 3H, H^{20}). ^{13}C NMR (151 MHz, DMSO) δ 152.97, 151.87, 149.12, 143.40, 141.75, 131.32, 129.68 (2C), 125.72, 125.70, 125.67, 125.65, 123.99, 123.74, 122.74, 120.84, 119.42, 115.26, 91.86, 47.62, 43.95, 42.96, 12.22. HPLC-MS (ESI): Purity = 99%, t_R = 2.8 min, m/z $[M+H]^+$ = 492.0

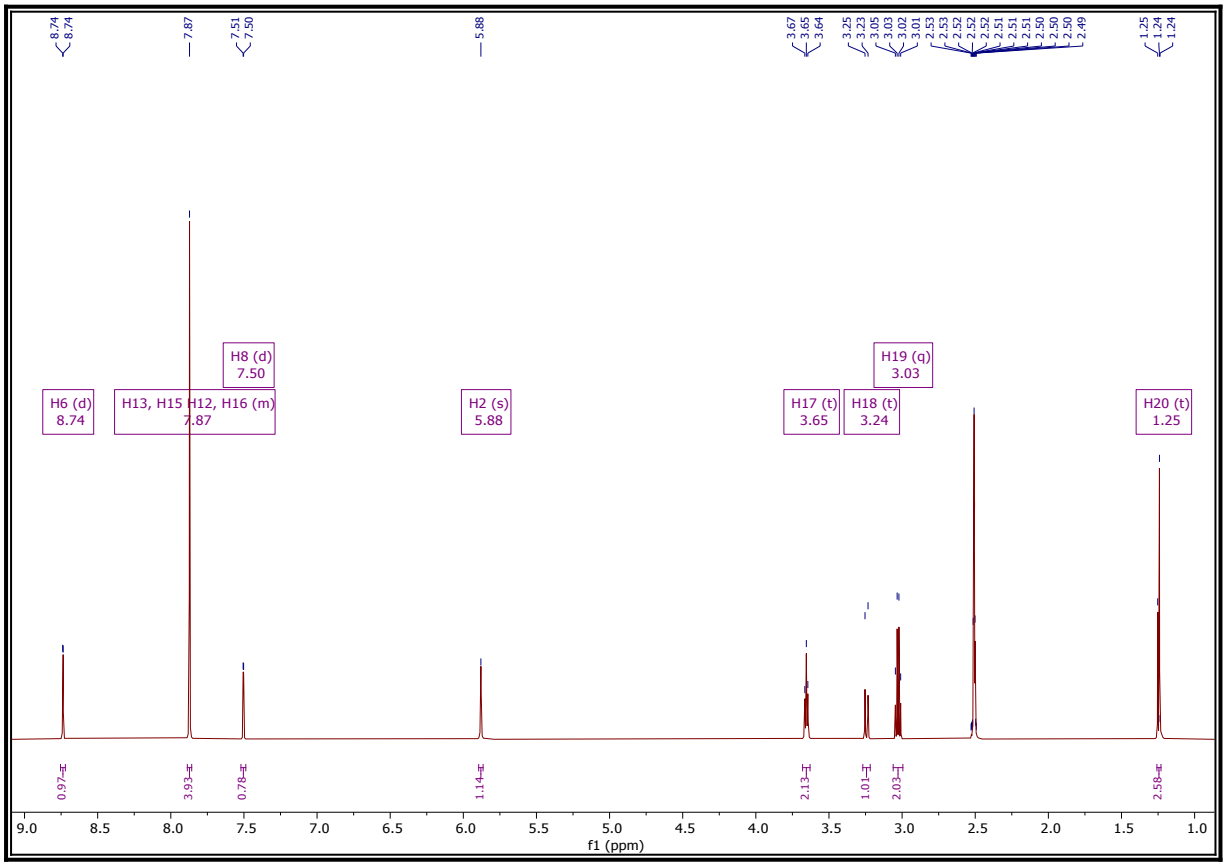
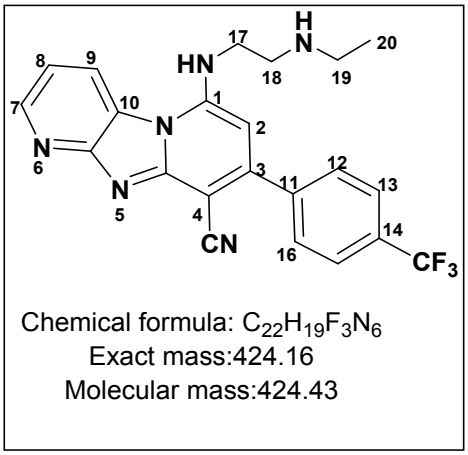


Figure 7: ^{1}H-nuclear magnetic resonance spectrum (NMR; 600 MHz, DMSO-*d6*) of compound **1**

6-((2-(ethylamino)ethyl)amino)-8-(4-(trifluoromethyl)phenyl)imidazo[1,2-a:4,5-b']dipyridine-9-carbonitrile (2/CKM08)



Obtained from **CKM07** (100 mg, 0.27 mmols) as a yellow solid (47%, 55 mg); R_f (DCM: MeOH: 9:1) 0.3: ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.43 (dd, J = 4.8, 1.3 Hz, 1H, H^7), 8.26 (dd, J = 8.2, 1.4 Hz, 1H, H^9), 7.99 (m, 4H, $H^{13,15}$, $H^{12,16}$), 7.64 (dd, J = 8.2, 4.8 Hz, 1H, H^8), 6.32 (s, 1H, H^2), 3.69 (t, J = 6.0 Hz, 2H, H^{17}), 2.98 (t, J = 6.0 Hz, 2H, H^{18}), 2.69 (q, J = 7.1 Hz, 2H, H^{19}), 1.09 (t, J = 7.1 Hz, 3H, H^{20}). ^{13}C NMR (151 MHz, DMSO) δ 152.44, 149.39, 149.09, 144.26, 142.04, 140.38, 137.92, 129.99 (2C), 126.55, 125.99 (2C), 122.22 (3C), 117.06, 90.08, 47.44, 43.49, 42.85, 15.51, 15.46. HPLC-MS (ESI): Purity = 99%, t_R = 2.8 min, m/z $[M+H]^+$ = 424.9.

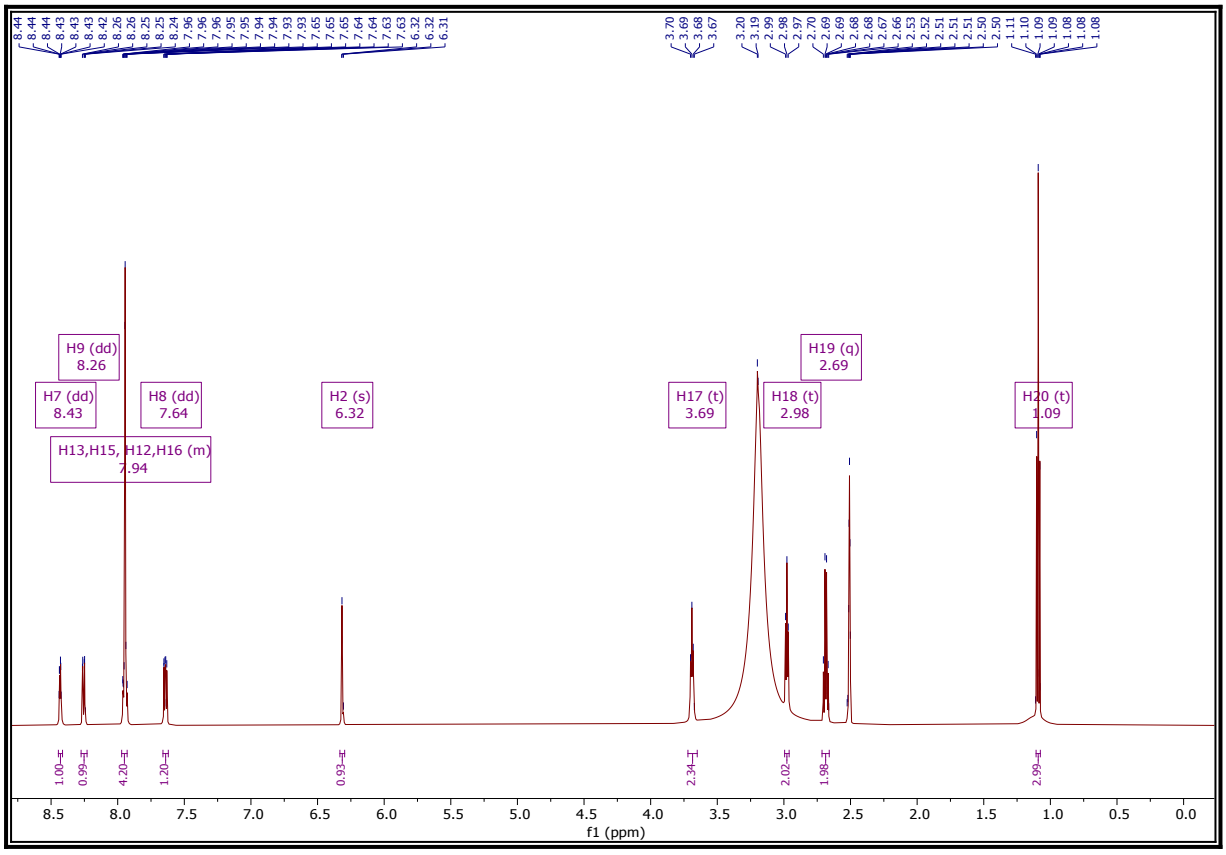


Figure 8: ^{1}H-nuclear magnetic resonance spectrum (NMR; 600 MHz, DMSO-*d6*) of compound **2**

1.3 Biological Testing

1.3.1 Antiplasmodial Assay

Modified [^{3}H]-hypoxanthine incorporation assay. Both compounds were screened against multidrug resistant (K1) and sensitive (NF54) strains of *P. falciparum* in vitro using the modified [^{3}H]-hypoxanthine incorporation assay[1]. *Plasmodium falciparum* was cultivated in a variation of the medium previously described[2,3] consisting of RPMI 1640 supplemented with 0.5% ALBUMAX® II, 25mM Hepes, 25mM $NaHCO_3$ (pH 7.3), 0.36mM hypoxanthine, and 100 microgram/ml neomycin. Human erythrocytes served as host cells. Cultures were maintained at 37 °C in an atmosphere of 3% O_2, 4% CO_2, and 93% N_2 in humidified modular chambers. Compounds were dissolved by sonication in DMSO (10mg/ml) and diluted in hypoxanthine-free culture medium. Infected erythrocytes (100 microliter per well with 2.5% hematocrit and 0.3% parasitaemia) were added to each drug titrated in 100 microliter duplicates over a 64-fold range. After 48 h incubation, 0.5 microCi of [^{3}H]hypoxanthine in 50 microliter media was added and plates were incubated for an additional 24 h. Parasites were harvested onto glass-fibre filters and radioactivity was counted using a Betaplate liquid scintillation counter (Wallac, Zurich). The results were recorded as counts per minute (cpm) per well at each drug concentration and expressed as a percentage of the untreated controls. Fifty percent inhibitory concentrations (IC_{50}) were estimated by linear interpolation[4].

1.3.2 Beta Haematin Inhibition Assay

Stock solutions of controls and test compounds were made to 20 mM in DMSO. A solution containing water/305.5 µM NP40/DMSO at a v/v ratio of 70%/20%/10%, respectively was added to every well in columns 1-11 while 140 µL of water and 40 µL of 305.5 µM NP40 were added to column 12 to mediate the formation of β-haematin. Twenty microliters of drug (20 mM) was added to column 12 and 100 µL of this solution serially diluted to column 2, with column 1 left as a blank (0 µM of compound). In case the compound was coloured (for instance AQ), a pre-reading of the plate was done by measuring absorbance at 405 nm on a SpectraMax plate reader. A 178.8 µL aliquot of hematin stock was suspended in 20 ml of a 1 M acetate buffer, pH 4.9 and 100 µL of this hematin suspension added into each well. Plates were then incubated for ±5 hrs at 37°C after which 32 µL of pyridine solution (20% water, 20% acetone, 10% 2M HEPES buffer (pH 7.4), 50% pyridine) was added followed by addition of 60 µL of acetone to all wells. Plates were again read at 405 nm and IC_{50}s plotted in GraphPad Prism.

1.3.3 Turbidimetric Solubility

Target compounds and controls were dissolved in DMSO to make 10 mM stock solutions. From this, a predilution plate was prepared in Nunc™ 96-well fluorescence plates in triplicate by serial dilution such that a concentration range of between 0.125 and 8 mM was achieved. Each predilution solution was used to make secondary dilutions in DMSO and PBS (0.01 M) in a second plate in triplicates such that each well contained 200 µL of solvent. After incubation at room temperature for 2 hours, absorbance was measured at 620 nm using a SpectraMax 340PC microplate reader (Molecular Devices, Sunnydale, CA). Hydrocortisone and reserpine were used as positive and negative controls, respectively. The assay measured solubility within the concentration range 0-200 µM.

2.0 *In vitro* fluorescent evaluation of 1 and 2

To obtain the fluorescent spectra of compounds **1** and **2**, a 2M stock solution of the compounds were prepared in Methanol, DMSO and DCM. Each stock solution was serial diluted to obtain a range of concentrations between ten to 1 micromolar. Fluorescence spectra was obtained for each concentration prepared using a Varian Cary Eclipse spectrofluorometer and fluorescent curves were plotted using excel. Absorption spectra of the compounds were also recorded on a Varian Cary 100 UV-Vis spectrophotometer at varying concentrations.

2.1 Fluorescence Emission profiles of 1

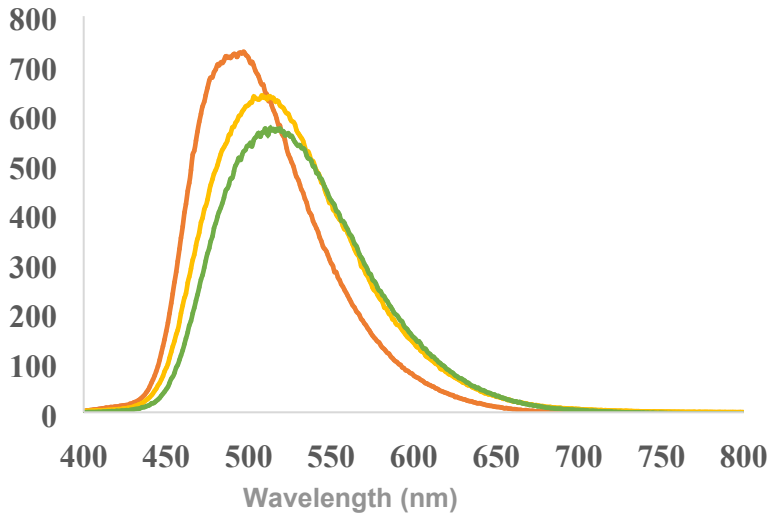


Figure 9: Emission spectra of **compound 1** in varying solvents; DCM, dichloromethane (red); DMSO, dimethyl sulfoxide (yellow), and Methanol (green) at 5 µM concentration.

2.2 Fluorescence Emission profiles of compound 2

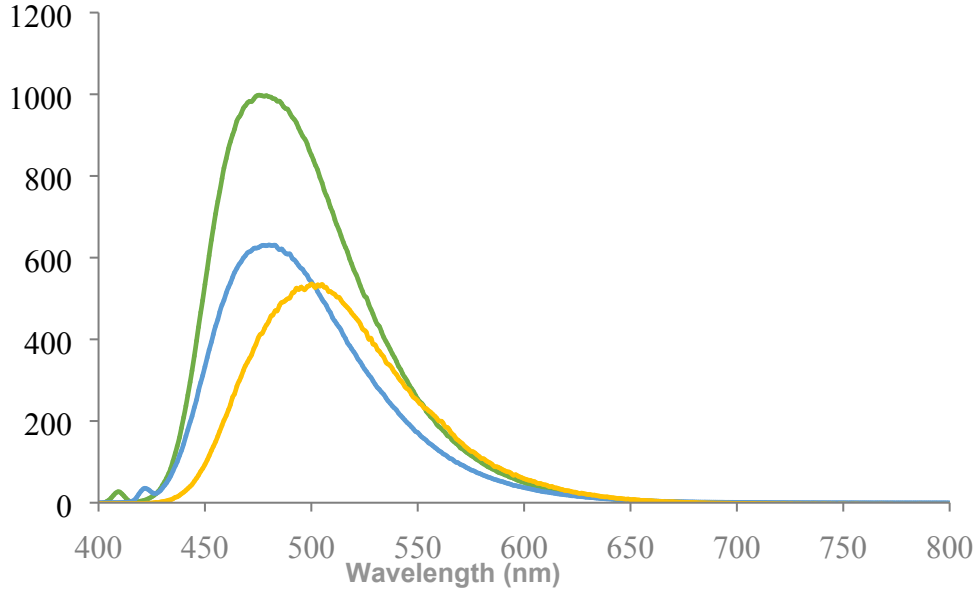


Figure 10: Emission spectra of **compound 2** in varying solvents; DCM, dichloromethane (green); DMSO, dimethyl sulfoxide (blue), and Methanol (yellow) at 5 µM concentration

3.0 Fluorescent Live-cell Imaging

3.1 Additional figures to support conclusions made

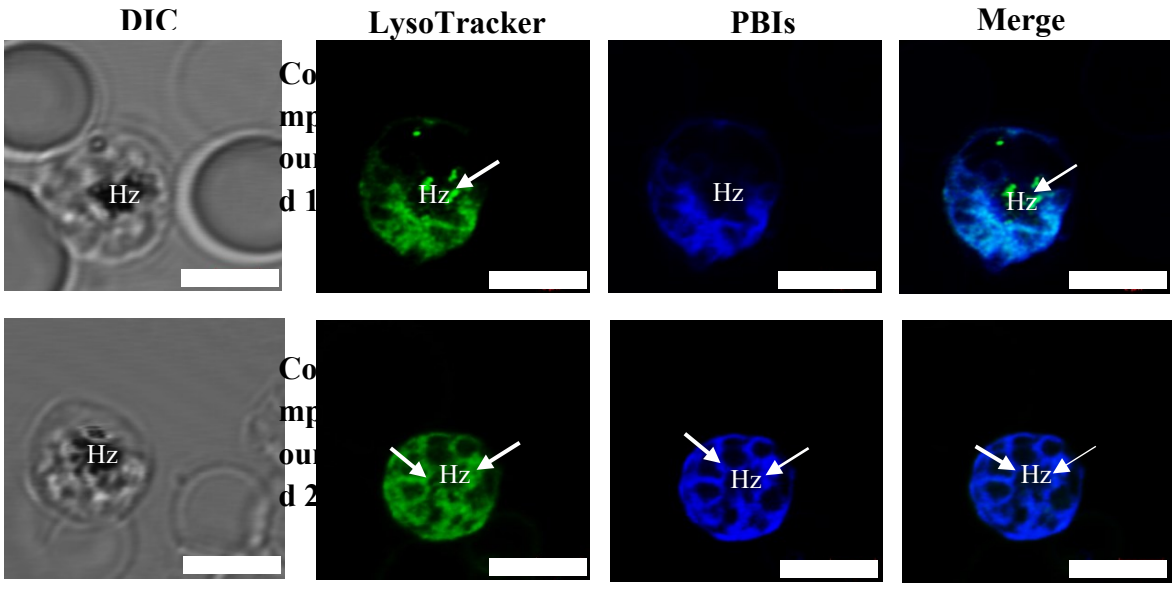


Figure 11: Live-cell confocal microscopy of P. falciparum-infected erythrocyte, treated with Compounds 1, 2 (blue) and LysoTracker Red (green). White arrows indicate areas of intense localization of the dye and regions of overlap shown in the merged image. Scale bars: 2 μm

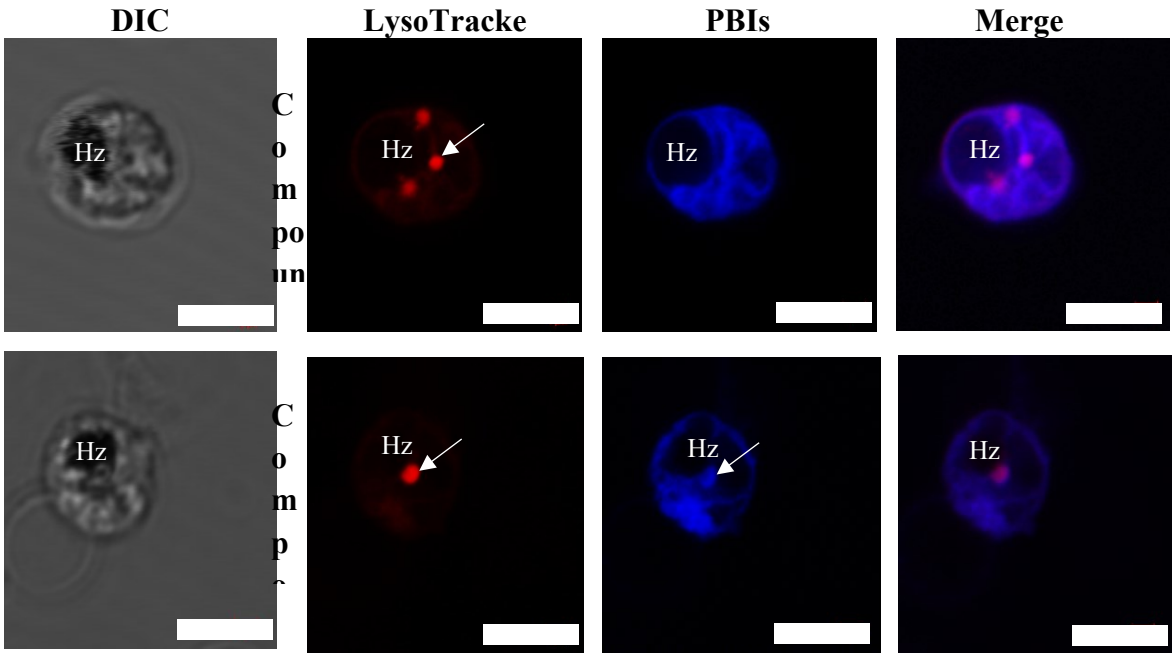


Figure 12: Live-cell confocal microscopy showing co-localization between Nile Red (red) and compounds 1 and 2 (blue) Scale bars: 2 μm.

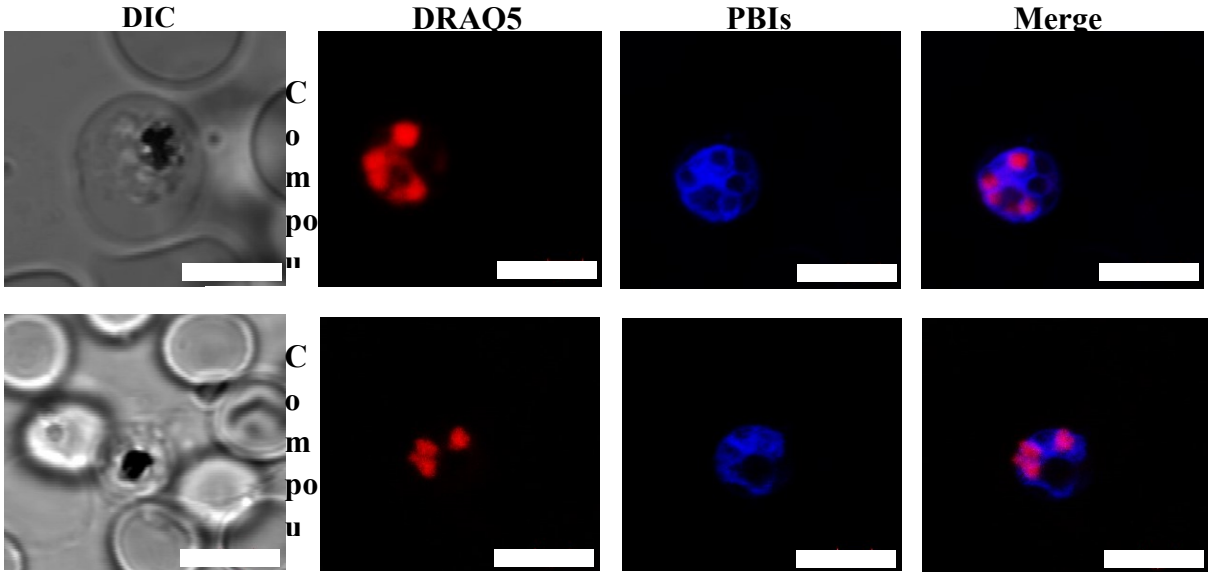


Figure 13: Live-cell confocal microscopy showing co-localization of compounds 1 and 2 (blue) with the nuclear marker DRAQ5 (red) Scale bars: 2 μm.

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