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Leveraging Synthetic Chlorins for Bio-imaging Applications

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Methods

General

Unless otherwise stated, all solvents and reagents were obtained from Sigma-Aldrich or Fisher Scientific and used without further purification. Acetonitrile (ACN) and water (H_2O) were of high-performance liquid chromatography (HPLC) grade and of liquid chromatography mass spectroscopy (LCMS) grade, respectively. Phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} was obtained from the Media Preparation Facility at Memorial Sloan Kettering Cancer Center and used when required. Reverse-phase (RP) HPLC purifications were performed on a Shimadzu HPLC system equipped with a DGU-20A degasser, SPD-M20A UV detector, LC-20AB pump system, and a CBM-20A communication BUS module using RP-HPLC columns (Atlantis T3 C18, 5 µm, 4.6 x 250 mm, P/N:186003748). For the HPLC analysis, an RP-HPLC column (Atlantis T3 C18, 5 µm, 4.6 x 250 mm, P/N:186003748) was used with a gradient of 5–95% B over 40 min, then 95% B for 20 min (A: 99.9% water/0.1% TFA, B: 99.9% ACN/0.1% TFA). Electrospray ionization mass spectroscopy (ESI-MS) spectra was recorded with a Waters Aquity UPLC (Milford, CA) with an electrospray ionization SQ detector. ^{64}Cu was produced at the Mallinckrodt Institute of Radiology (Washington University) on a 19 MeV-beam energy cyclotron (Advanced Cyclotron Systems Inc. British Columbia, Canada) via the $^{64}Ni(p,n)^{64}Cu$ nuclear reaction to yield ^{64}Cu with a specific activity of 298–1830 mCi/µg (978mCi/µg avg.). Luminescence and epifluorescence images were performed on an IVIS Spectrum (PerkinElmer). Confocal microscopy images were captured using a Leica SP6 inverted-stand confocal microscope equipped with a tunable white light laser with a 470–670 nm range. The microscope was also equipped with a 405 nm diode for detection of Hoechst 33342, an argon laser (with 476 nm, 488 nm, 496 nm and 514 nm laser lines) and a 610 nm laser for NIR imaging coupled with avalanche photo diode detectors (APDs), which were used for detection of chlorin.

^{1}H and ^{13}C solution NMR spectra were recorded using Bruker 500 MHz (spectrometer operating at 500 MHz for ^{1}H; 125 MHz, for ^{13}C). $CDCl_3$ was used as solvent and TMS as internal reference; the chemical shifts are expressed in (ppm) and the coupling constants (*J*) in Hertz (Hz). Trace solvent impurities (eg. water at approx. δ 1.6 ppm, dichloromethane (DCM) at approx. δ 5.2 ppm in ^{1}H NMR spectra) are detected. Some of the reactions were monitored by TLC with Analtech Uniplate silica gel G/UV 254 precoated plates

(0.2 mm). TLC plates were visualized by UV (254 nm), and by iodine vapour. Preparative thin-layer chromatography was carried out on 20x20 cm glass plates coated with silica gel (1 mm thick)

Synthesis of compound 2

To a solution of 100 mg of porphyrin-$TPPF_{20}$, (103 µmol) in 8 mL chlorobenzene at 120 ^{o}C, mixed-grinded ylide, comprised of 15.4 mg of glycine (205 µmol) and 6.1 mg of paraformaldehyde (203 µmol), were added to the reaction mixture, successively in aliquots every 6 h, for at least 24 h. Then, the reaction mixture was cooled down to room temperature and the solvent evaporated, the reaction mixture was separated with a preparatory TLC to remove unreacted $TPPF_{20}$, which can be recycled and to obtain a dark green pigment. The product, compound 2, was purified using RP-HPLC, showed a retention time at 30 min. Excess solvent was removed *in vacuo*, yielding compound 2 as a green powder (80 mg, 76% isolated yield). Final analysis of the RP-HPLC purification showed 95% purity. LC-MS (ESI) *m/z* calcd for $C_{93}H_{30}F_{40}N_{10}$ ($[M+H]^+$), 2046.10, found 2047.17.

Synthesis of compound 3

100 mg of compound 2 (49 µmol) in a 6 mL solution of 20% water and 80% DMSO at 120 ^{o}C, trifluoroacetic acid (75 µL, 1 µmol) was added and allow to react for at least 24 h. Then, the reaction mixture was cooled down to room temperature and the solvent evaporated. The product, compound 3, was purified using RP-HPLC, showed a retention time at 40 min. Excess solvent was removed *in vacuo*, yielding compound 3 as a green powder (50 mg, 50% isolated yield). Final analysis of the RP-HPLC purification showed 95% purity. LC-MS (ESI) *m/z* calcd for $C_{46}H_{15}F_{20}N_5$ ($[M+H]^+$), 1017.15, found 1018.17.

Synthesis of compound 4

100 mg of compound 3 (103 µmol) in 4 mL acetonitrile, 5-Iodo-1-pentyne (80 mg, 0.4 mmol) and K_2CO_3 (41 mg, 0.3 mmol) were added and allowed to reflux for 2 h. Then, water was added to the reaction mixture and the organic layer collected, solvents were evaporated. The product, compound 4, was purified using RP-HPLC, showed a retention time at 42 min. Excess solvent was removed *in vacuo*, yielding compound 4 as a green powder (70 µg, 70% isolated yield). Final analysis of the RP-HPLC purification showed 95% purity. ^{1}H NMR (500 MHz, CDCl3) δ 8.74 (d, *J*=5 Hz, 2H), 8.50 (s, 2H), 8.41 (d, *J*=10 Hz, 2H), 5.40-5.50 (m, 2H), 4.18 (t, *J*=10 Hz, 1H), 4.11 (t, *J*=10 Hz, 1H), 4.00-4.04 (m, 2H), 3.75-3.78 (dd, J_1=5, J_2=10 Hz, 2H), 2.53-2.60 (m, 2H), 2.34-2.45 (m, 1H), 2.13-2.18 (m, 1H), 1.85-1.90 (m, 2H), -1.85 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ; 159.80, 153.24, 147.36, 146.75, 145.41, 144.75, 143.41, 141.40, 139.23, 138.53, 137.19, 136.53, 135.36, 132.83, 128.23, 126.98, 124.35, 115.22, 106.86, 96.90, 53.47, 52.06, 51.73, 48.15, 32.12, 29.73. LC-MS (ESI) *m/z* calcd for $C_{51}H_{21}F_{20}N_5$ ($[M+H]^+$), 1083.73, found 1085.40.

Synthesis of compound 5

To a solution of 100 mg of compound 3, (98 µmol) in 4 mL of acetonitrile, 4-Bromobutyronitrile (30 µL, 44 mg, 0.3 mmol) and K_2CO_3 (41 mg, 0.3 mmol) were added and allowed to reflux for 2 h. Then, water was added to the reaction mixture and the organic layer collected, solvents were evaporated. An isolated green compound (80 mg) was dissolved in 3 mL tetrahydrofuran (THF) and $LiAlH_4$ (37 mg, 0.1 mmol) added to the reaction mixture. The reaction took place overnight; the organic compound was isolated in diethyl ether. The product, compound 5, was purified using RP-HPLC, showed a retention time at 40 min. Excess solvent was removed *in vacuo*, yielding compound 5 as a green powder (27 mg, 30% isolated yield). Final analysis of the RP-HPLC purification showed 95% purity. ^{1}H NMR (500 MHz, CDCl3) δ 8.65 (d, *J*=5 Hz, 2H), 8.41 (s, 2H), 8.33 (d, *J*=10 Hz, 2H), 5.36 (m, 2H), 5.19 (m, 2H), 3.57-3.61 (m, 2H), 3.50 (m, 2H), 2.14 (m, 4H), 2.10 (m, 2H), -1.90 (s, 2H); ^{13}C NMR (125 MHz, CDCl3) δ; 152.89, 147.28, 145.34, 144.31, 143.22, 141.33, 140.15, 138.53, 137.06, 136.50, 135.26, 128.36, 126.84, 124.30, 119.00, 115.23, 106.52, 96.95, 62.32, 52.39, 28.91, 32.12, 28.64, 27.99, 24.63, 14.21. LC-MS (ESI) *m/z* calcd for $C_{50}H_{24}F_{20}N_6$ ($[M+H]^+$), 1088.20, found 1089.50.

Synthesis of compound 6

To a 3 mL solution of 100 mg of compound 3, (98 µmol) in dichloromethane, succinic anhydride (98 mg, 98 µmol) and triethylamine (NEt_3, 40 µL, 0.3 µmol)) were added and allowed to react for at least 10 min. Then, water was added to the reaction mixture and the organic layer collected, solvents were evaporated. The product, compound 6, was purified using RP-HPLC, showed a retention time at 37 min. Excess solvent was removed *in vacuo*, yielding compound 6 as a green powder (75 mg, 68% isolated yield). Final analysis of the RP-HPLC purification showed 95% purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.77 (d, *J*=5 Hz, 2H), 8.52 (s, 2H), 8.44 (d, *J*=4 Hz, 2H), 5.51 (m, 2H), 4.21 (t, *J*=10.15 Hz, 2H), 3.82 (t, *J*=9.95 Hz, 2H), 3.81-3.80 (m, 2H), 2.61-2.60 (m, 2H), 2.40 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ; 152.77, 147.13, 145.57, 143.24, 141.29, 140.51, 138.76, 137.20, 136.42, 135.64, 139.23, 138.53, 137.19, 136.53, 135.64, 132.72, 128.45, 124.16, 115.241, 106.07, 96.53, 96.47, 51.57, 52.06, 29.58, 31,30, 22.57. LC-MS (ESI) *m/z* calcd for $C_{50}H_{19}F_{20}N_5O_3$ ($[M+H]^+$), 1117.11, found 1118.17.

Synthesis of compound 7

To a solution of 1 mg of compound 6, (0.89 µmol) dissolved in 3 mL of methanol, copper chloride (0.5 mg, 3.6 µmol) dissolved in 0.5 mL of water, was added and allowed to react for at least 1 h at room temperature. The product, compound 7, was purified using RP-HPLC, showed a retention time at 26 min. Excess solvent was removed *in vacuo*, yielding compound 7 as a light green powder (0.9 mg, 90% isolated yield). Final analysis of the RP-HPLC purification showed 95% purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.68 (d, *J*=5 Hz, 2H), 8.43 (s, 2H), 8.31 (d, *J*=4 Hz, 2H), 5.31 (m, 2H), 4.18 (t, *J*=10.15 Hz, 2H), 3.95 (t, *J*=9.95 Hz, 2H), 3.60-3.63 (m, 2H), 3.47-3.60 (m, 2H), 3.20 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ; 159.80, 153.24, 151.67, 147.36, 146.75, 145.40, 144.75, 143.41, 141.40, 140.66, 139.23, 138.53, 137.19, 136.53, 135.36, 132.84, 128.23, 124.35, 115.22, 106.86, 96.89, 96.47, 53.47, 52.06, 51.73, 48.15, 31,30, 29.73. LC-MS (ESI) *m/z* calcd for $C_{50}H_{16}F_{20}N_5CuO_3$ ($[M+H]^+$), 1180.15, found 1180.87.

Synthesis of compound 8

To a solution of 1 mg of compound 6, (0.89 μmol) dissolved in 3 mL of dimethylformamide (DMF), nickel chloride (0.46 mg, 3.6 μmol) dissolved in 0.5 mL of water, was added and allowed to reflux overnight. The product, compound 8, was purified using RP-HPLC, showed a retention time at 27 min. Excess solvent was removed *in vacuo*, yielding compound 8 as a light green-bluish powder (0.8 mg, 80% isolated yield). Final analysis of the RP-HPLC purification showed 95% purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.74 (d, *J*=5 Hz, 2H), 8.50 (s, 2H), 8.40 (d, *J*=4 Hz, 2H), 5.48 (m, 1H), 5.43 (m, 1H), 4.20 (m, 1H), 4.15 (m, 1H), 4.00 (m, 1H), 3.75 (m, 1H), 2.54-2.60 (m, 2H), 2.40 (m, 1H), 2.17 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ; 154.80, 153.68, 147.16, 146.75, 145.68, 144.15, 143.79, 141.77, 139.75, 138.03, 137.29, 137.00, 135.81, 132.83, 128.23, 126.18, 124.35, 113.92, 105.13, 96.80, 53.47, 50.74, 51.17, 49.71, 48.93, 32.12, 27.26. LC-MS (ESI) *m/z* calcd for $C_{50}H_{16}F_{20}N_5NiO_3$ ([M-2H]$^-$), 1172.00, found 1170.00.

Synthesis of compound 9

Radiolabeling of chlorin 6 (0.28 mM, 25 μg in 20 μL of DMSO) was performed using 3.8 μL of ^{64}Cu (103 MBq) in 300 μL ammonium acetate buffer for 30 min at 37 ºC. The [^{64}Cu]Cu-chlorin was trapped on a preconditioned C18 cartridge (ethanol/water), washed with H_2O to remove free copper-64, and then eluted with ethanol to obtain pure [^{64}Cu]Cu-chlorin. The specific activity of [^{64}Cu]Cu-chlorin throughout the study was measured to be between 2.52–4.51 MBq/μg.

iTLC quantification

The crude reaction mixture and purified ^{64}Cu-Chlorin solution were analyzed by iTLC (glass microfiber chromatography paper impregnated with silica gel, 100 10 mm) using 50 mM EDTA (pH 5.5) as the mobile phase. For the control, [^{64}Cu]$CuCl_2$ (approx. 0.074 MBq) was mixed with ammonium acetate (0.1 M, pH 6.0) and incubated for 30 min at 37 ºC. The same mobile phase was used for separation. The different iTLC plates were analyzed by iTLC and Laura 3 software (LabLogic, Sheffield, UK). [^{64}Cu]$CuCl_2$ R_f = 0.67–0.78; [^{64}Cu]Cu-chlorin R_f = 0.22–0.26.

Cell Line

For this study, small cell lung cancer were used and they were obtained from the American Type Culture Collection and grown under aseptic conditions in an incubator providing a humidified atmosphere of 5% CO_2 in air. H-82 small cell lung cancer cell lines were used between 29 and 31 passages after thawing to ensure complete revival. H-82 cells were cultured in RPMI media supplemented with 10% fetal bovine serum, 2 mM/L glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4,500 mg/L glucose, 1500 mg/L sodium bicarbonate and 100 U/mL penicillin and 100 µg/mL streptomycin.

Confocal Microscopy of chlorin

Staining experiments for fluorescence microscopy with chlorin were performed in small cell lung cancer, H-82. In brief, H-82 cancer cells were incubated with chlorin 6 (green, 67 nmol, 0.7 mM in 100 µL of PBS) for 2 h at 37^{o}C, the subsequent step was the conterstained with Hoechst (blue, 20 µM, 1 nmol in 50 µL of PBS) for one min as a control, fluorescence signal was observed after placing microscope slide under the microscope.

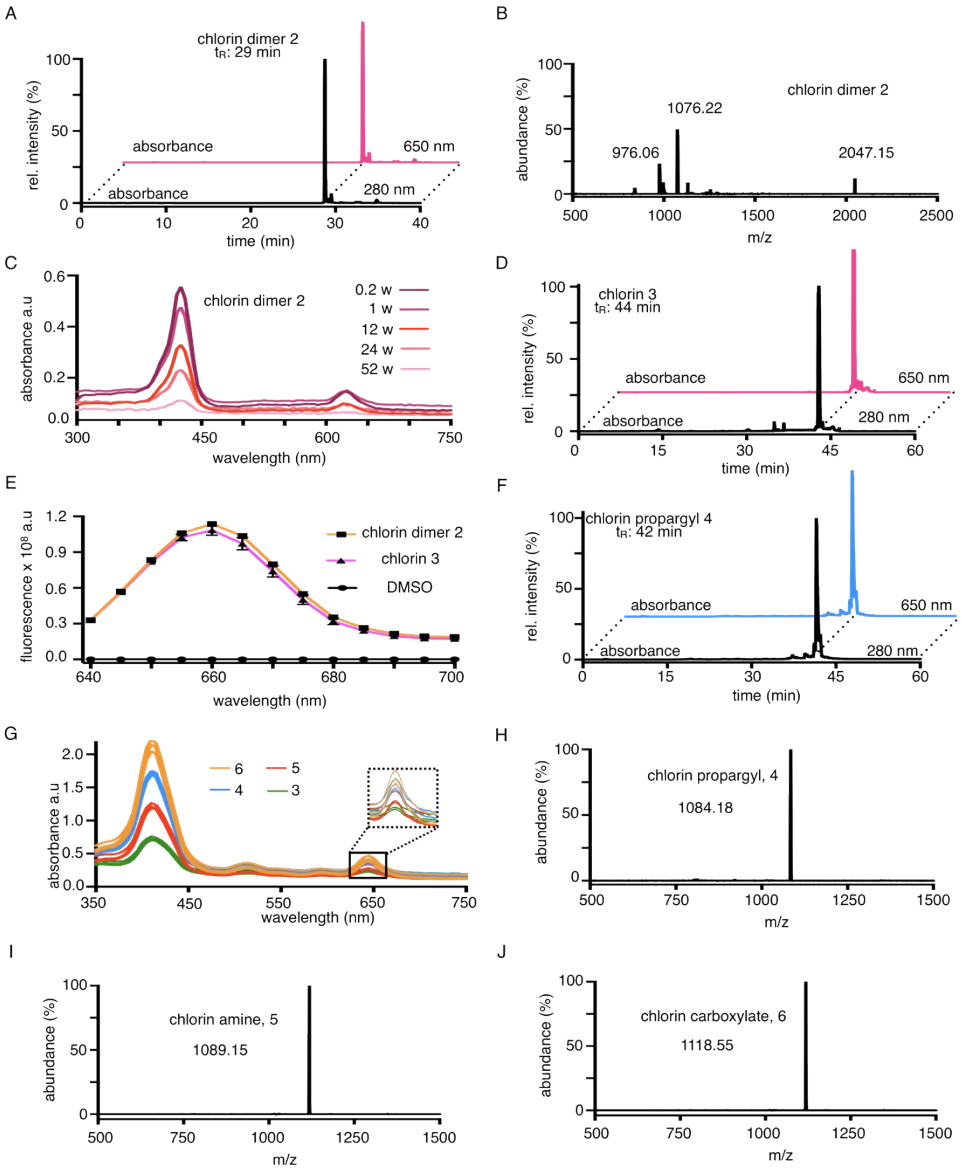

A
chlorin dimer 2
tR: 29 min
rel. intensity (%)
absorbance
650 nm
absorbance
280 nm
time (min)
B
abundance (%)
chlorin dimer 2
1076.22
976.06
2047.15
m/z
C
absorbance a.u
chlorin dimer 2
0.2 w
1 w
12 w
24 w
52 w
wavelength (nm)
D
chlorin 3
tR: 44 min
rel. intensity (%)
absorbance
650 nm
absorbance
280 nm
time (min)
E
fluorescence x 10^8 a.u
chlorin dimer 2
chlorin 3
DMSO
wavelength (nm)
F
chlorin propargyl 4
tR: 42 min
rel. intensity (%)
absorbance
650 nm
absorbance
280 nm
time (min)
G
absorbance a.u
6
5
4
3
wavelength (nm)
H
abundance (%)
chlorin propargyl, 4
1084.18
m/z
I
abundance (%)
chlorin amine, 5
1089.15
m/z
J
abundance (%)
chlorin carboxylate, 6
1118.55
m/z

Figure S1. Characterization of synthetic chlorins. A) HPLC profile of stable chlorin dimer 2 trace showing absorbances at 280 nm (black) and 650 nm (pink) with a retention time observed at 29 min. B) LC-MS trace profile of the reaction mixture that afforded the chlorin-dimer 2 showing an ion signal corresponding to the mass, 2047. C) Absorbance spectra of 0.25 μM chlorin dimer maintained in DMSO (1 mL) for a year observed from 350-750 nm, and where the classical 650 nm chlorin peak was observed at every withdraw for absorbance measurement. D) HPLC profile of stable chlorin 3 trace showing absorbances at 280 nm (black) and 650 nm (pink) with a retention time observed at 44 min. E) Florescence spectra of 0.1 μM chlorin dimer (orange), 0.1 μM chlorin pyrrolidine (pink) and 100 μL of DMSO (black) observed at a range of 635–700 nm, and an excitation wavelength of 610 nm. F) HPLC profile of chlorin 4 trace showing absorbances at 280 nm (black) and 650 nm (blue) with a retention time observed at 42 min. G) Absorbance spectra of 0.1 μM chlorin 3 (green), 0.1 μM chlorin 4 (blue), 0.1 μM chlorin 5 (red), and 0.1 μM chlorin 6 (orange) observed at a range of 350–750 nm. H), I) and J) LC-MS profile of stable chlorins 4, 5 and 6 traces showing ion peaks corresponding to the masses of 1084.18, 1089 and 1118, respectively.

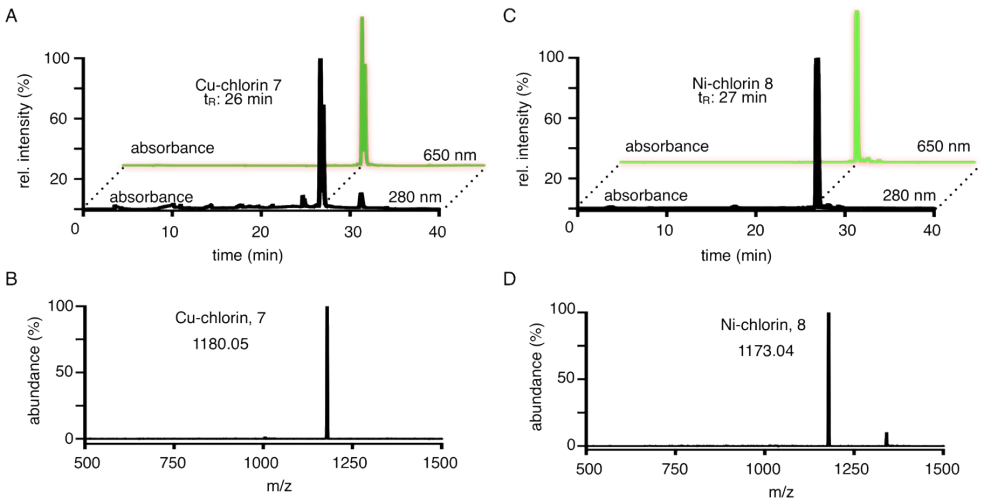


Figure S2. Characterization of chlorins 7 and 8. A) HPLC trace profile of chlorin 7 showing absorbances at 280 nm (black) and 650 nm (green) with a retention time observed at 26 min. B) LC-MS trace profile of chlorin 7, showing an ion signal corresponding to the mass, 1180.05. C) HPLC trace profile of chlorin 8 showing absorbances at 280 nm (black) and 650 nm (green) with a retention time observed at 27 min. D) LC-MS trace profile of chlorin 8, showing an ion signal corresponding to the mass, 1173.04.

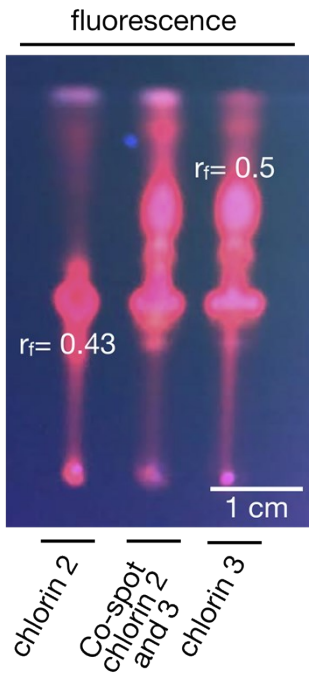


Figure S3. Fluorescence of chlorin 2 and chlorin 3. 2 µL of 0.1 µM of chlorin 2 and 2 µL of 0.1 µM of chlorin 3, same amounts for co-spot were loaded onto a thin layer chromatography plate (TLC). TLC experiment carried on in a 10 mL solvent phase, 15% methanol and 85% DCM. Experiment shows high polarity of chlorins, excitation at 410 nm.

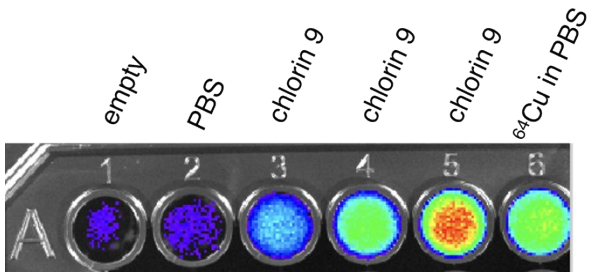
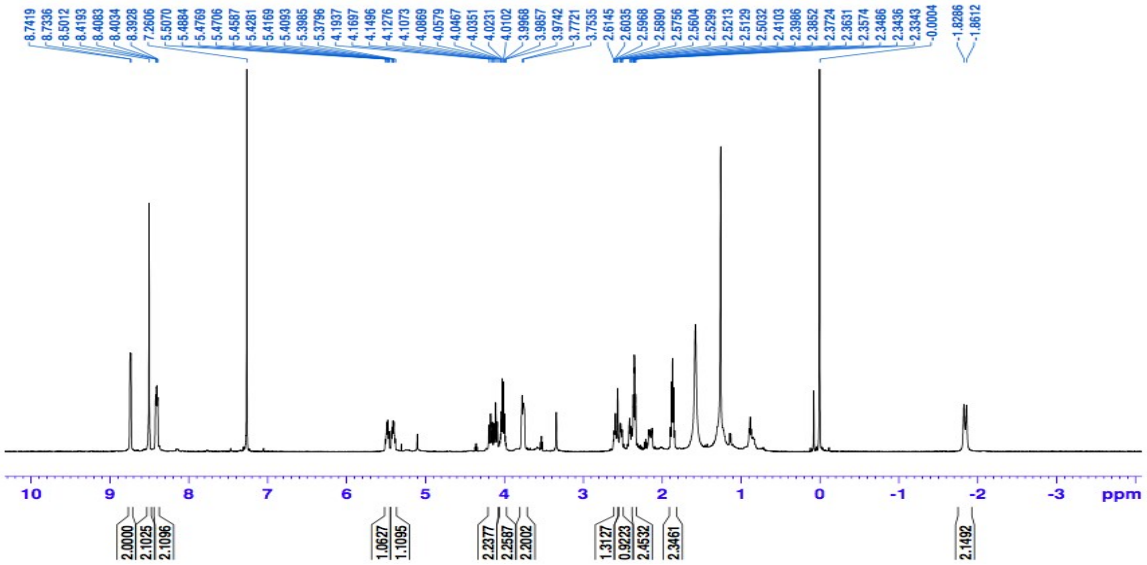


Figure S4. Cerenkov luminescence imaging of [^{64}Cu]Cu-chlorin 9, the chlorin 9 alone (2.52–4.51 MBq in 300 μL of PBS) in 3, 4 and 5 wells. Well 6 contains $^{64}CuCl_2$, (5.2 MBq in 100 μL), well 2 contains 100 μL of PBS and well 1 is empty.



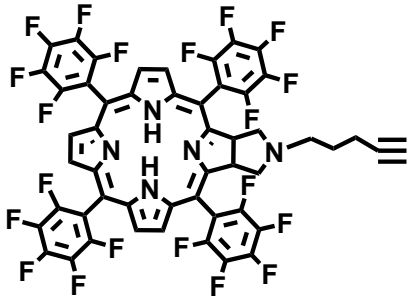


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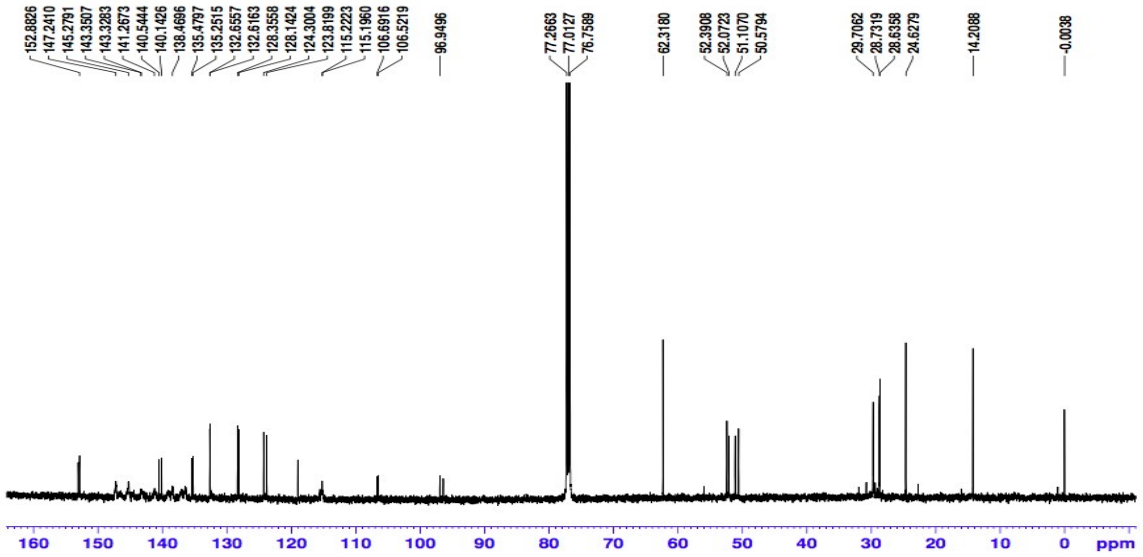
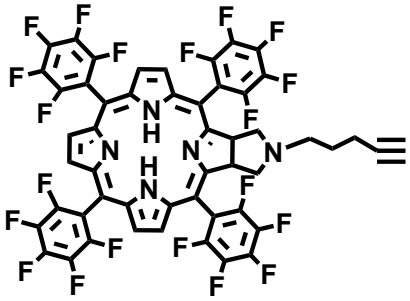
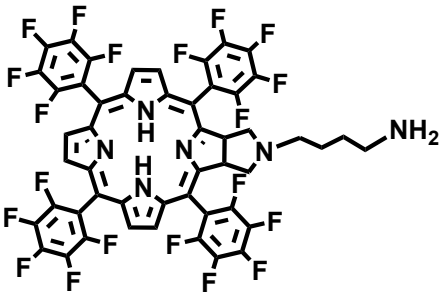
Figure S5. Proton NMR of Compound 4. Deuterated chloroform ($CDCl_3$, 7.26) was used as solvent and tetramethylsilane as internal reference (TMS, 0.00 ppm).



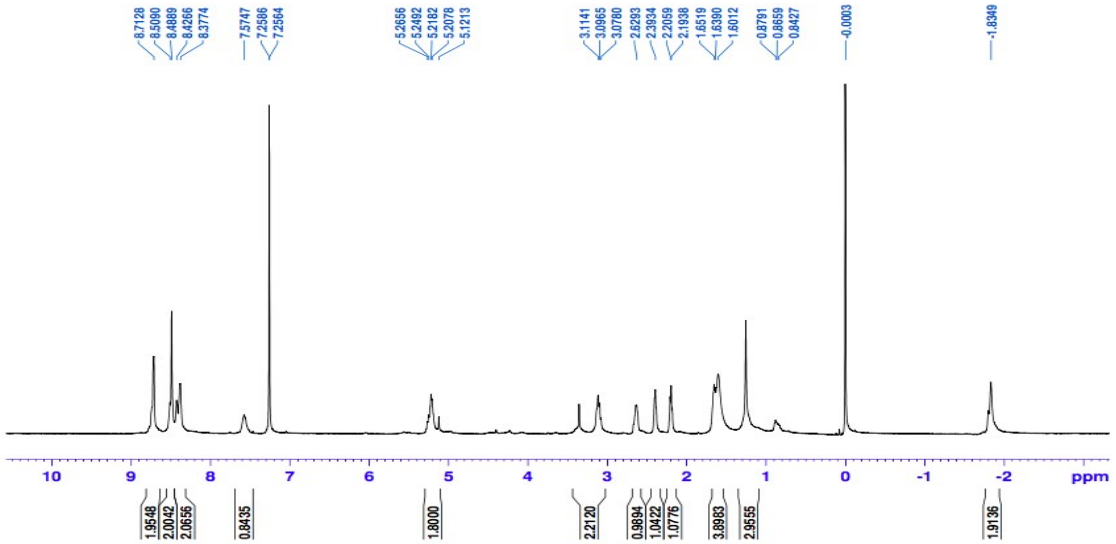


4

Figure S6. Carbon NMR of Compound 4 in $CDCl_3$, approx. 77.01 ppm.


F
N
H
NH2

5


8.7128
8.5090
8.4889
8.4266
8.3774
7.5747
7.2586
7.2564
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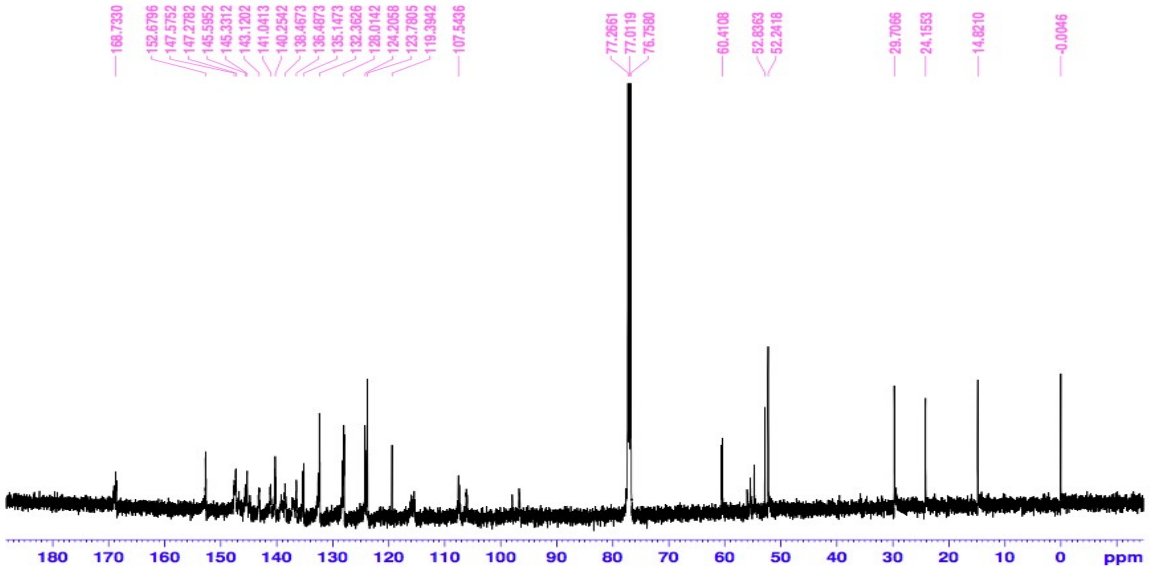
5.2078
5.1213
3.1141
3.0965
3.0780
2.6293
2.3934
2.2059
2.1938
1.6519
1.6390
1.6012
0.8791
0.8659
0.8427
-0.0003
-1.8349
10
9
8
7
6
5
4
3
2
1
0
-1
-2
ppm
1.9548
2.0042
2.0656
0.8435
1.8000
2.2120
0.9894
1.0422
1.0776
3.8983
2.9555
1.9136

Figure S7. Proton NMR of Compound 5. Deuterated chloroform ($CDCl_3$, 7.26) was used as solvent and tetramethylsilane as internal reference (TMS, 0.00 ppm).



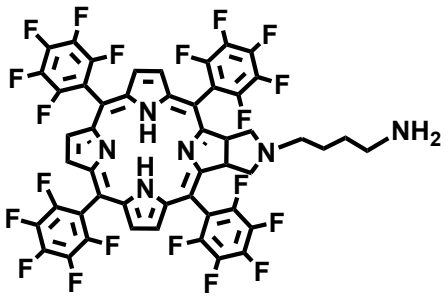


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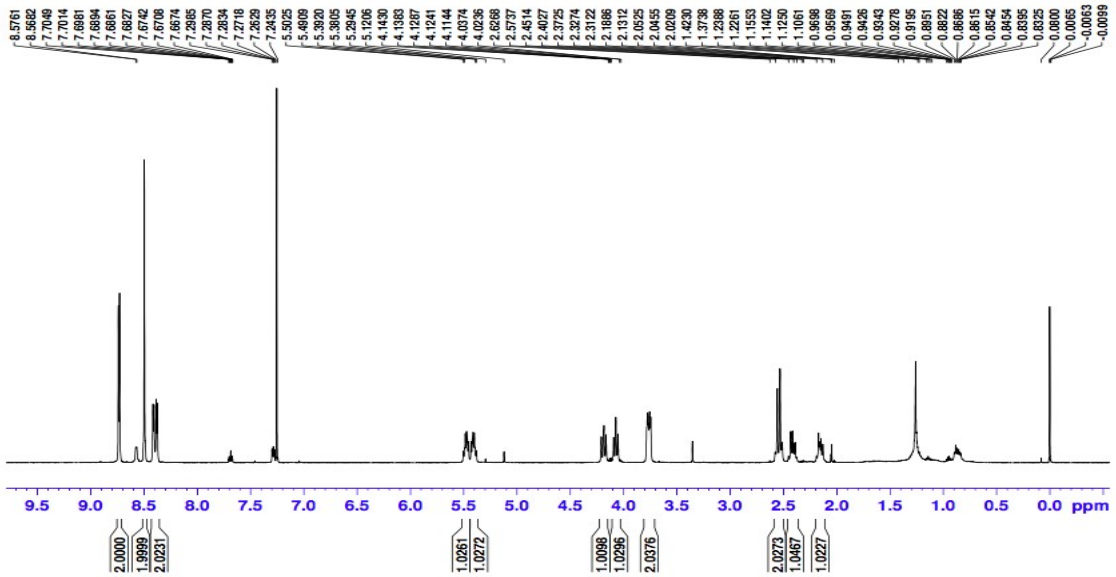
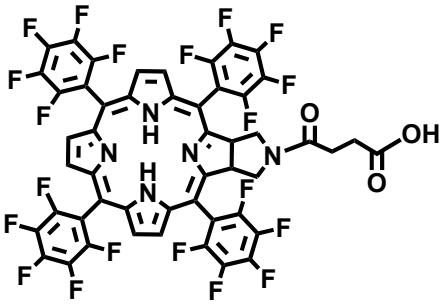
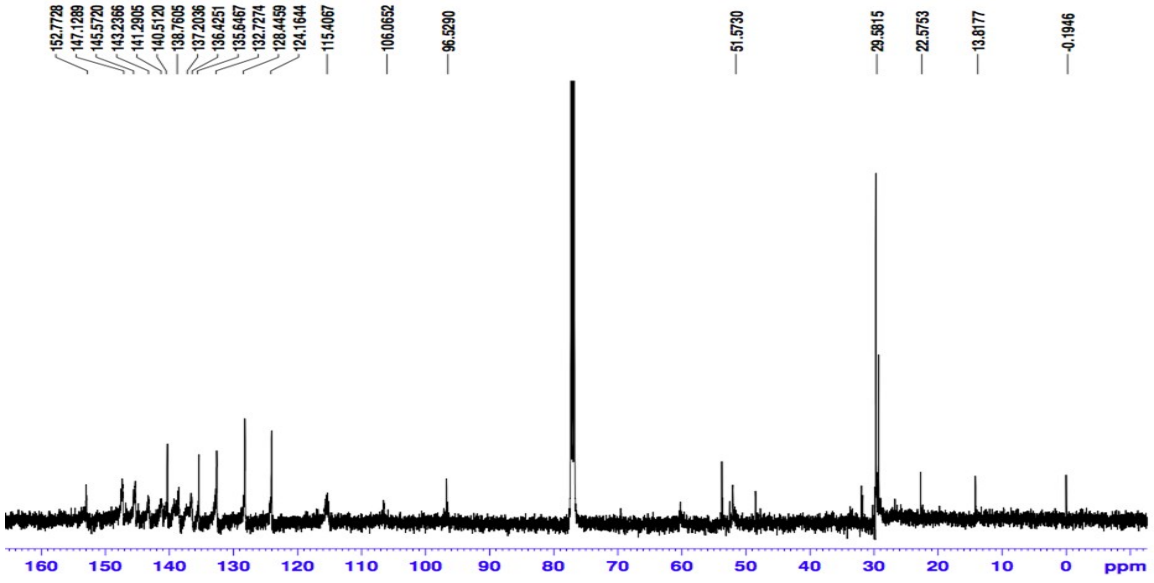
Figure S8. Carbon NMR of Compound 5 in $CDCl_3$, approx. 77.01 ppm.

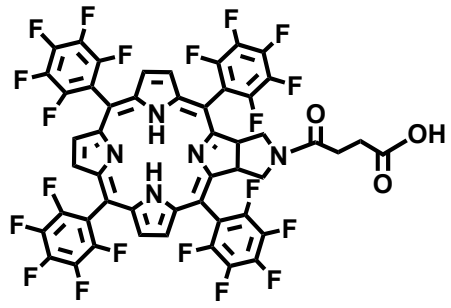




6

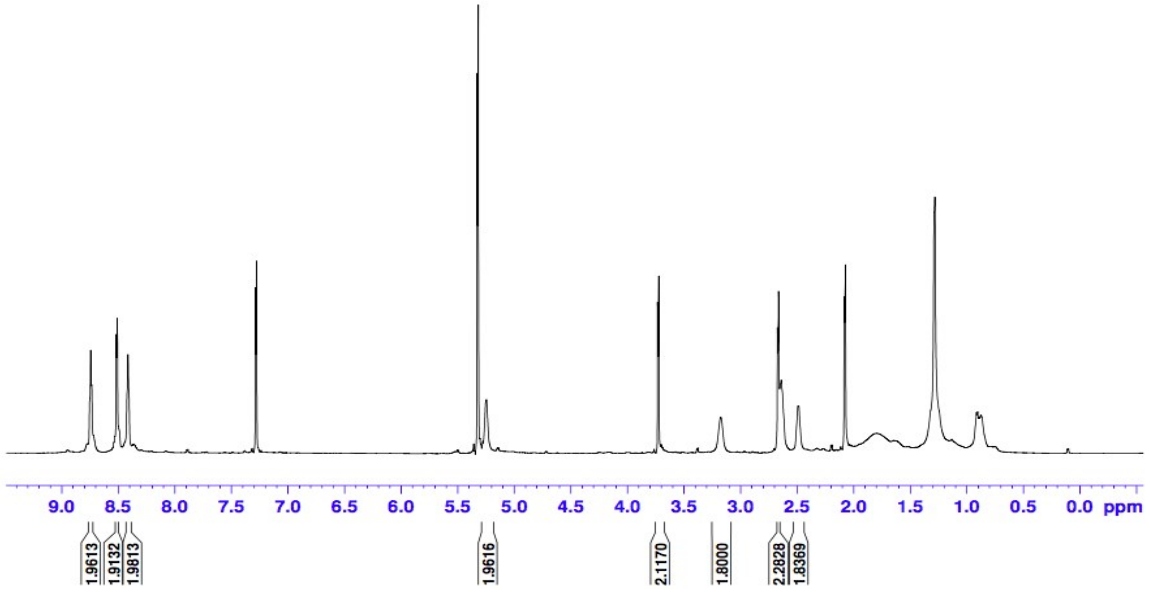
Figure S9. Proton NMR of Compound 6. Deuterated chloroform ($CDCl_3$, 7.26) was used as solvent and tetramethylsilane as internal reference (TMS, 0.00 ppm).

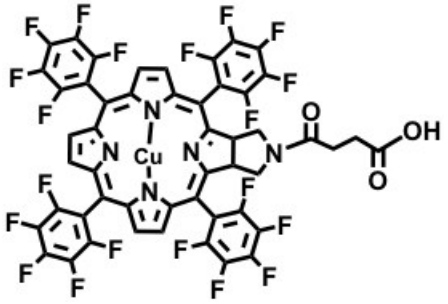




6

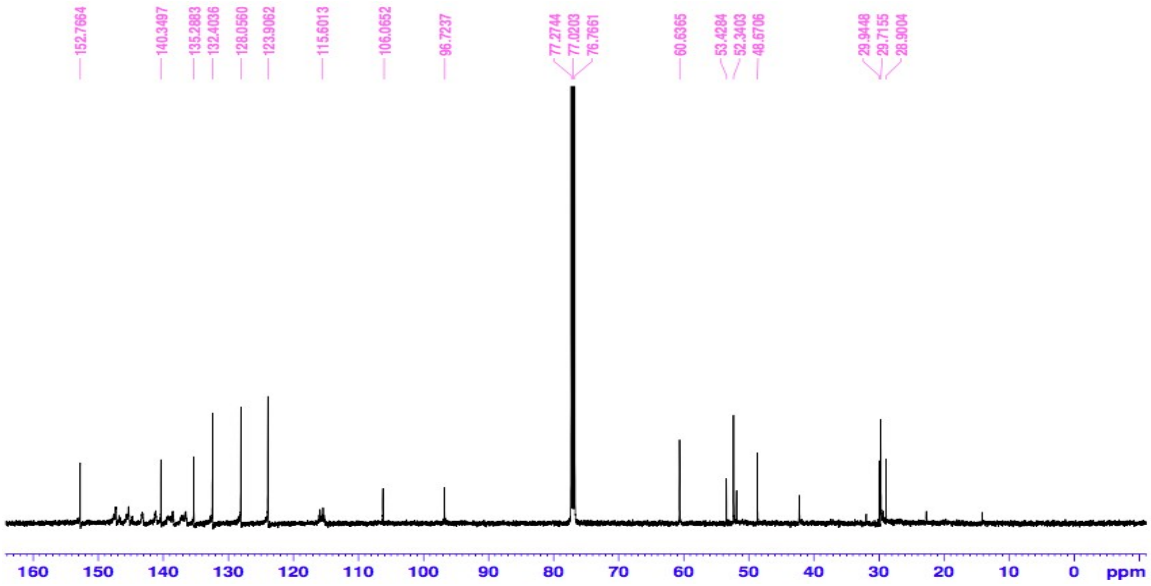
Figure S10. Carbon NMR of Compound 6 in $CDCl_3$, approx. 77.01 ppm.

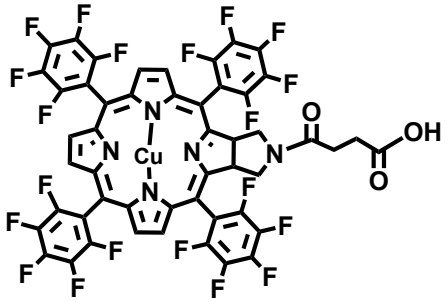




7

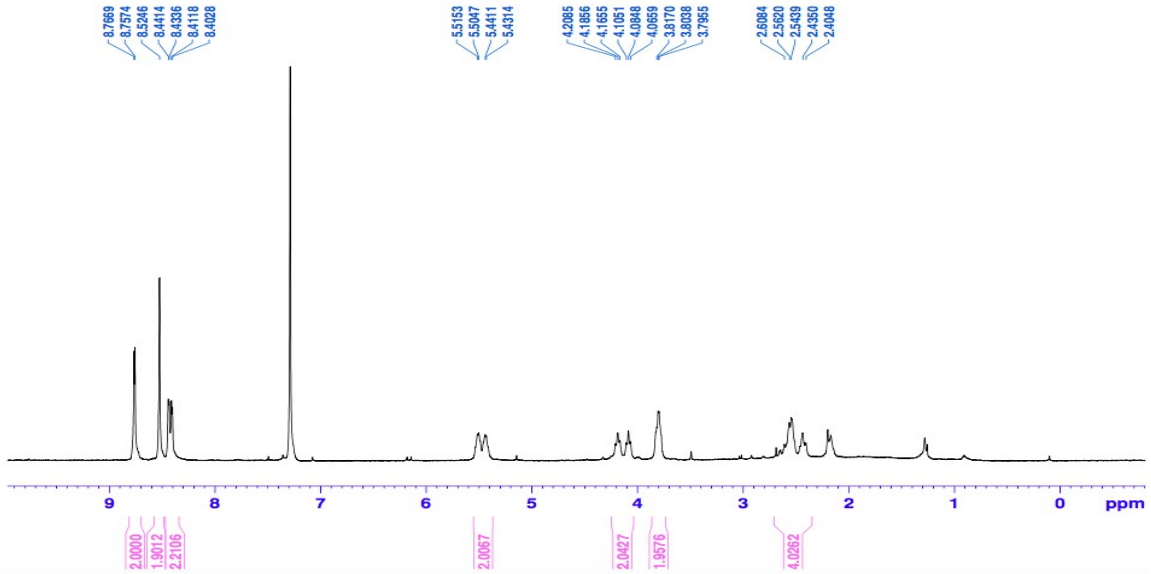
Figure S11. Proton NMR of Compound 7. Deuterated chloroform ($CDCl_3$, 7.26) was used as solvent and tetramethylsilane as internal reference (TMS, 0.00 ppm).

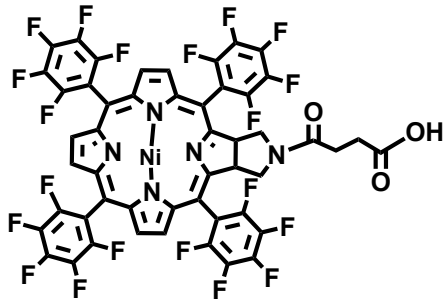




7

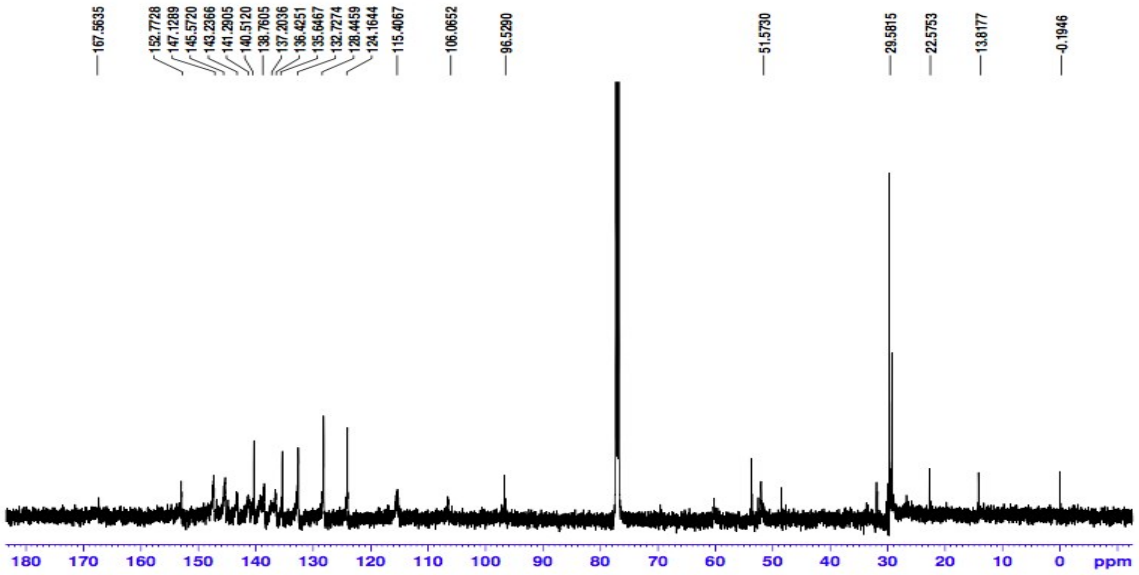
Figure S12. Carbon NMR of Compound 7 in $CDCl_3$, approx. 77.02 ppm.

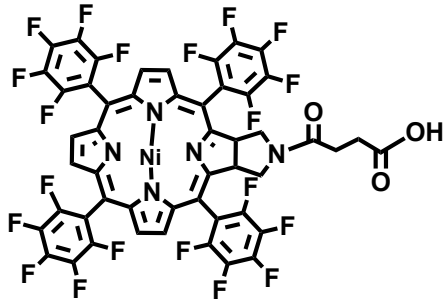




8

Figure S13. Proton NMR of Compound 8. Deuterated chloroform ($CDCl_3$, 7.26) was used as solvent and tetramethylsilane as internal reference (TMS, 0.00 ppm).





8

Figure S14. Carbon NMR of Compound 8 in $CDCl_3$, approx. 77.10 ppm.