Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.


# Supporting Information

## *RSC Medicinal Chemistry*

# 4-Amino-1,8-Naphthalimide-Ferrocene Conjugates as Potential Multi-targeted Anticancer and Fluorescent Cellular Imaging Agents

Alex D. Johnson[a] Joseph A. Buhagiar[b] and David C. Magri[a]

[a] Department of Chemistry, Faculty of Science, University of Malta, Msida, MSD 2080, Malta.
E-mail: david.magri@um.edu.mt.

[b] Department of Biology, Faculty of Science, University of Malta, Msida, MSD 2080, Malta.
E-mail: joseph.buhagiar@um.edu.mt

# Table of Contents

### 1.1 Chemicals

MTT Formazan (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan, thiazolyl blue tetrazolium bromide) was purchased from Sigma-Aldrich. LysoSensor™ Green DND-189 and RedoxSensor™ Red CC-1 were purchased from Thermo Fisher Scientific (local supplier E. J. Busuttil).

RPMI 1640 Medium was obtained as a powder from Gibco BRL, Life Technologies Ltd. UK. The medium was received in sealed aliquots used to prepare 1 L of medium containing 2 mM *L*-glutamine, 1000 mg/L *d*-glucose, 110 mg/L sodium pyruvate and 25 mM HEPES.

Penicillin-Streptomycin were obtained from Gibco BRL, Life Technologies Ltd., UK. The Penicillin-Streptomycin lyophilised preparation was rehydrated with sterile distilled water and stored at −20℃. The preparation provides 10 000 units/mL of Penicillin G (sodium salt) and 10,000 μg/mL of Streptomycin Sulphate. Gibco recommends use of 50-100 units /mL of sterilized medium or 1-2 mL of the rehydrated solution with every 200 mL of sterilized medium.

The Foetal Bovine Serum (FBS) was used as a serum-supplement for *in vitro* cell culture from Gibco BRL, Life Technologies Ltd., UK. It was heat treated, virus, and mycoplasma screened before use and stored at −20 °C and added to 200 mL medium aliquots. Other reagents included sodium hydrogen carbonate and sterile 1 M hydrochloric acid and 1 M sodium hydroxide. The cell cultures K562 and MCF-7 were obtained from the European Collection of Authenticated Cell Culture (ECACC).

### 1.2 Instrumentation

Sterile conditions for cell cultures were maintained by a Laminar Flow Hood (Microflow Advanced) with the provided High Efficiency Particulate Air (HEPA), and filtered sterile air circulate within an enclosed space. Instrumentation required for cell culture sterilisation, and preparation of medium included a Millipore™ Pressure Vessel for positive-pressure filtration system, Milli-Q$^{euTM}$ Deionized unit, pH meter with temperature probe, magnetic stirrer and plastic coated stirrer bars, and electronic balance. The following apparatus were used to sterilise the cell medium; Pipette filler Pump Operator (Automatic), filter units with filling bell, 0.22 $\mu$m Sterivex™ - GS (Sterile), and filter units, syringe adapted, 0.2 $\mu$m cellulose acetate membrane. Disposable syringes of 5 mL, 10 mL, and 20 mL along with 1 L measuring jar and beaker, 1 L Erlenmeyer flask, and 250 mL medium bottles (sterile).

An ELx – 808, Bio Tek microplate reader was used to shake and read the optical density at 650 nm of the 96-well plates after MTT assays. An Axiovert 40 CLF microscope, manufactured by Carl Zeiss MicroImaging, was used for viewing the cells during phase contrast cell counts. The ocular magnification is ×10, whilst the objective magnifications are in ×4, ×10, ×40 and ×60. The software for the cell imaging interface to the microscope called AxioVision Rel. Version 4.8.1 (11-2009). Rotofix 32A was required for the cytospin of suspension cells (K562) on glass slides for morphology fluorescent staining experiments.

Fluorescent imaging was captured using an EVOS FL Auto Cell Imaging system fluorescent microscope. The images were captured through one of three filters for blue (DAPI), green (FITC) or red (TRITC) and a merged image. LysoSensor$^{TM}$ Green DND-189, a naphthalimide-based pH sensor, and RedoxSensor$^{TM}$ Red CC-1, a rhodamine-based redox sensor, were used as controls to evaluate and compare possible staining within the cells.

### 1.3 Preparation of Medium for Cell Cultures

RPMI-1640 powdered medium was dissolved in 850 mL of deionized water along with 2 g of sodium hydrogen carbonate. The pH of the medium solution was adjusted to between 7.0–7.4 with 1 M HCl and 1 M NaOH. After the desired pH of the medium was obtained, the flask was topped up to 1 L deionized water. The medium was filter sterilised with a pressure vessel at 15 psi with a flow rate of 50 mL per minute. Penicillin-Streptomycin was added as an antibiotic to 200 mL aliquots of the medium, and stored at 4°C in $T_{25}$ flasks. The medium was treated with 22.5 mL FBS before being used with cell cultures.

The cell density was maintained at approximately between $10^5$ and $10^6$ viable cells/mL whilst ensuring the cell culture was assessed by both the medium colour and cell confluence using an inverted microscope. For K562, a suspension culture, the cells were sub-cultured by diluting a portion of the cell suspension into a new flask with fresh medium. This procedure was repeated every 2-3 days. For MCF-7, an adherent culture, the cells for sub-culturing required the use of 1 mL 2.5% trisodium citrate (prepared by adding 5 g of powdered trisodium citrate to 200 mL of buffered saline solution) and washing the $T_{25}$ flask after discarding the medium. 1 mL trypsin solution (prepared by diluting 2.5% of trypsin to 0.25% with phosphate-buffered saline (PBS)) was required to dislodge the MCF-7 from the bottom of the $T_{25}$ flasks, and incubated for 5 minutes. After the incubation period, the $T_{25}$ flask was tapped to dislodge the MCF-7 cells and sub-cultured

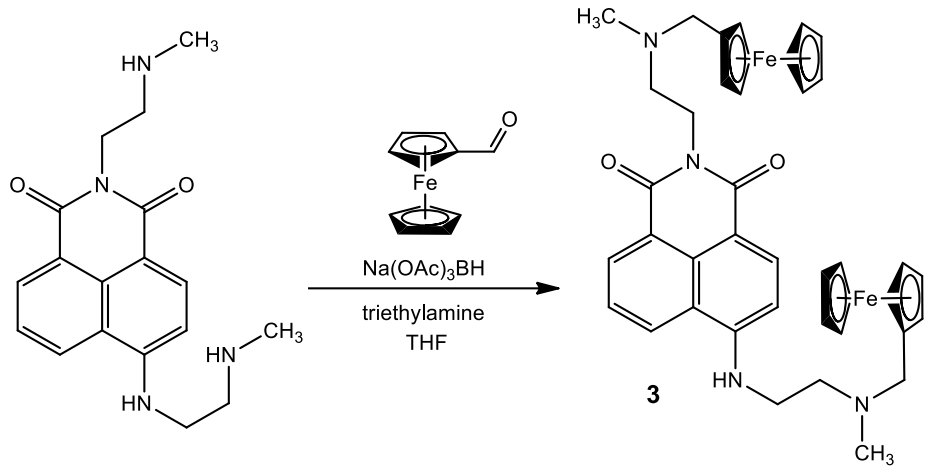
by diluting a portion of the cell suspension into a new flask with fresh medium. This procedure was repeated every 2-3 days.

The seeding of the 96-well plates was done in the log phase of the cell cycle in preparation for the MTT assays. Phase contrast microscopy was performed to measure the average viable cell count with a hemocytometer. The viable cell count for seeding 96-well plates was 8000 cells per well for K562, and 5000 cells per well for MCF-7. The calculation for viable cell count in 1000 µL is *'Viable cell count = mean viable cell count x $10^4$ x dilution factor = x'*.

**1.4 Cytotoxicity Studies**

A set of three trials were conducted for determining the $GI_{50}$ (the effectiveness of a substance in inhibiting a specific biological or biochemical function) of the ten compounds in both K562 and MCF-7 cells over 24, 48, and 72 hours at five concentrations between $10^{-4}$ M–$10^{-8}$ M with each concentration diluted by a factor of 10. K562 and MCF-7 were seeded in 96-well plates, and for each plate, four sensors where distributed to four wells for each dilution per probe. A total of nine 96-well plates were used for each cell line per trial. In addition, a separate 96-well plate was seeded with K562 and MCF-7 to establish a $T_0$ value as a control (the cell bioactivity on the day the cells were stained with probes) to provide a more accurate value for the $GI_{50.}$ After the plates were seeded, the cells were incubated (fixed at 37°C) for 24 hours to allow cell growth and time for the MCF-7 to adhere to base of the well. After the 24 hours, each well was treated with its respective probe and dilution. The plates were then incubated for another 24, 48, or 72 hour period. MTT was prepared by dissolving 1 g in 200 mL PBS solution. After each respective incubation period, MTT was added to the 96-wells, and incubated for a further 4 hours to allow the formation of formazan crystals. After a 4 hour period, the plates were centrifuged for 5 mins at 2500 rpm to fix the cells and formazan crystals to the bottom of the plates. The culture medium was removed by decantation, and to each well, 100 $\mu$L of DMSO with 25 $\mu$L of Sorenson's Glycine Buffer was added to dissolve the formazan crystals. The Sorenson's Glycine Buffer was prepared by dissolving 3.7 g of glycine and 2.9 g of NaCl in 500 mL sterile distilled water, and the pH adjusted to 10.5 with 1 M NaOH. A microplate reader was then used to determine the optical density of each well. Readings were plotted as dose-response curves and as 50% growth inhibition of the cell population ($GI_{50}$).

Synthesis of *N*-(2-(ferrocenylmethyl(methyl)amino)ethyl)-4-((2-(ferrocenylmethyl(methyl) amino)ethyl)amino)-naphthalimide (**3**)

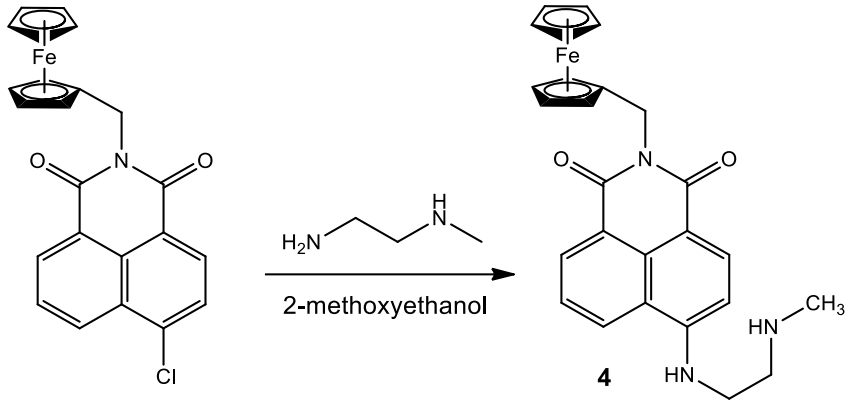



**Scheme S1** Synthesis of compound **3**.

4-*N*-methylethylenediamine-1,8-*N*-(*N*-methylethyldiamine) (500 mg, 1.5 mmol) was dissolved in 5 mL of dried THF with excess ferrocenecarboxaldehyde (690 mg, 3.2 mmol), sodium triacetoxyborohydride (1.3 g, 6 mmol) and triethylamine (430 $\mu$L, 4.2 mmol) over 4 Å molecular sieves under nitrogen atmosphere. The reaction was stirred for 48 hours in the dark at room temperature. The crude product was extracted with dichloromethane (3×20 mL), dried over $MgSO_4$ and filtered under vacuum through a glass sintered funnel. The crude brown/red oil was eluted through silica gel column using 9:1 (*v*/*v*) dichloromethane/methanol. The product was recrystallised from ethyl acetate (124 mg, 11% yield, dark red solid).

$R_f$ = 0.46 (9:1 (*v*/*v*) $CH_2Cl_2$/MeOH), m.p. = 122-126°C; $^1$H NMR (500 MHz, $CDCl_3$, ppm): $\delta$ 8.55 (d, 1H, *J* = 7.4 Hz, Ar-*H*), 7.67 (t, 1H, *J* = 7.9 Hz, Ar-*H*), 8.39 (d, 1H, *J* = 8.5 Hz, Ar-*H*), 8.48 (d, 1H, *J* = 8.1 Hz, Ar-*H*), 6.79 (d, 1H, *J* = 8.6 Hz, Ar-*H*), 3.61 (t, 2H, *J* = 6.3 Hz, -C*H*$_2$ spacer), 4.43 (t, 2H, *J* = 6.5 Hz, -C*H*$_2$ spacer), 2.91 (t, 2H, *J* = 6.1 Hz, -C*H*$_2$ spacer), 3.16 (t, 2H, *J* = 6.5 Hz, -C*H*$_2$ spacer), 2.44 (s, 3H, -C*H*$_3$), 2.64 (s, 3H, -C*H*$_3$), 3.72 (s, 2H, -C*H*$_2$ spacer), 4.06 (s, 2H, -C*H*$_2$ spacer), 4.28 (t, 2H, *J* = 1.8 Hz, Cp), 4.38 (t, 2H, *J* = 1.8 Hz, Cp), 4.16 (t, 2H, *J* = 1.7 Hz, Cp), 4.23 (t, 2H, *J* = 1.8 Hz, Cp), 4.13 (s, 5H, -C*H*$_3$), 4.18 (s, 5H, -C*H*$_3$); IR (KBr, cm$^{-1}$): 3093, 2941, 1687, 1653, 1582, 1384, 1367, 1297, 1218, 1100, 1040, 785; HRMS (ES-ToF): Calculated $C_{40}H_{42}Fe_2N_4O_2$ $[M+H]^+$ 723.2085, found 723.2096. A $^{13}$C NMR spectrum was not obtained due to limited solubility in $CDCl_3$ and DMSO-$d_6$.

Synthesis of *N*-ferrocenylmethyl-4-((2-(methylamino)ethyl)amino)-naphthalimide (**4)**




**Scheme S2** Synthesis of compound **4**.

*N*-ferrocenylmethyl-4-chloro-1,8-naphthalimide (200 mg, 0.5 mmol) was dissolved in 5 mL of 2-methoxyethanol with excess *N*-methylethylenediamine (160 $\mu$L, 1.9 mmol) and refluxed at 120°C for 5 hours. Upon completion, excess *N*-methylethylenediamine and solvent were removed by rotary evaporator. The crude product was extracted with dichloromethane (3×15 mL), dried over $MgSO_4$, filtered under vacuum through a glass sintered funnel and the solvent removed by rotary evaporator. The product **4** was recrystallised from ethyl acetate (85 mg, 39% yield, orange solid).

$R_f$ = 0.39 ($CH_2Cl_2$), m.p. = 201-204°C; $^1$H NMR (500 MHz, $CDCl_3$, ppm): $\delta$ 8.52 (d, 1H, *J* = 7.3 Hz, Ar-*H*), 7.55 (t, 1H, *J* = 7.9 Hz, Ar-*H*), 8.07 (d, 1H, *J* = 8.3 Hz, Ar-*H*), 8.39 (d, 1H, *J* = 8.4 Hz, Ar-*H*), 6.62 (d, 1H, *J* = 8.5 Hz, Ar-*H*), 5.09 (s, 2H, -C*H*$_2$ spacer), 4.51 (t, 2H, *J* = 1.8 Hz, Cp), 4.06 (t, 2H, *J* = 1.7 Hz, Cp), 4.19 (s, 5H, Cp), 6.15 (s, 1H, -N*H*), 3.38 (q, 2H, *J* = 5.2 Hz, -C*H*$_2$ spacer), 3.03 (t, 2H, *J* = 5.5 Hz, -C*H*$_2$ spacer), 1.6 (s, 1H, -N*H*), 2.5 (s, 3H, -C*H*$_3$); $^{13}$C NMR (126 MHz, $CDCl_3$, ppm) $\delta$ 164.5, 163.9, 149.6, 134.5, 132.1, 131.1, 129.7, 126.3, 124.6, 120.4, 110.2, 104.3, 83.8, 70.5, 67.9, 68.6, 49.5, 41.8, 38.9, 35.9 ; IR (KBr, cm$^{-1}$): 3092, 2947, 1692, 1656, 1583, 1388, 1369, 1248, 1150, 770; HRMS (ES-ToF): Calculated $C_{26}H_{25}FeN_3O_2$ $[M+H]^+$ 468.1374, found 468.1364.

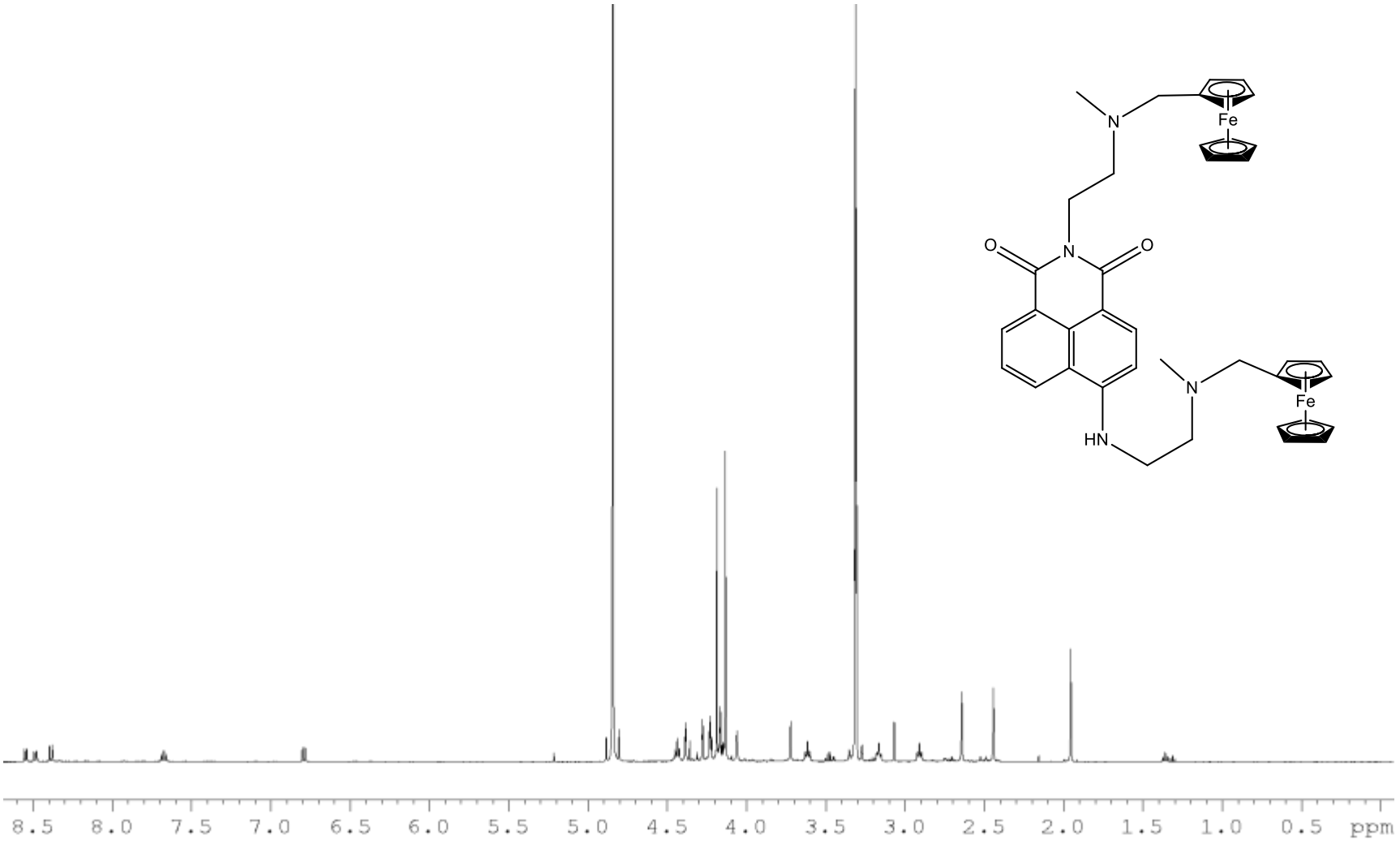



**Fig. S1** $^{1}$H NMR spectrum of compound **3** in $CDCl_3$ at 500 MHz.

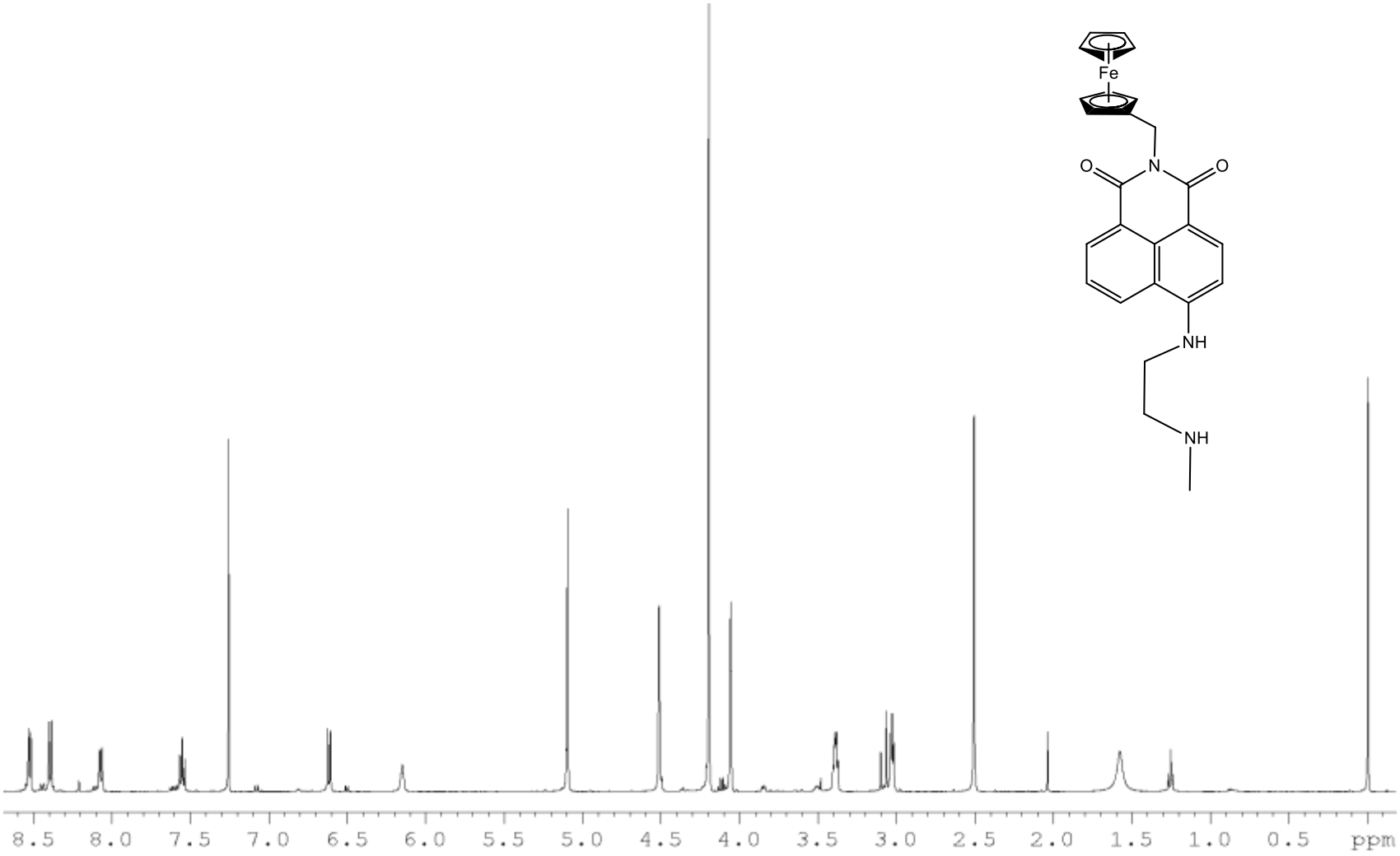



**Fig. S2** $^{1}$H NMR spectrum of compound **4** in $CDCl_3$ at 500 MHz.

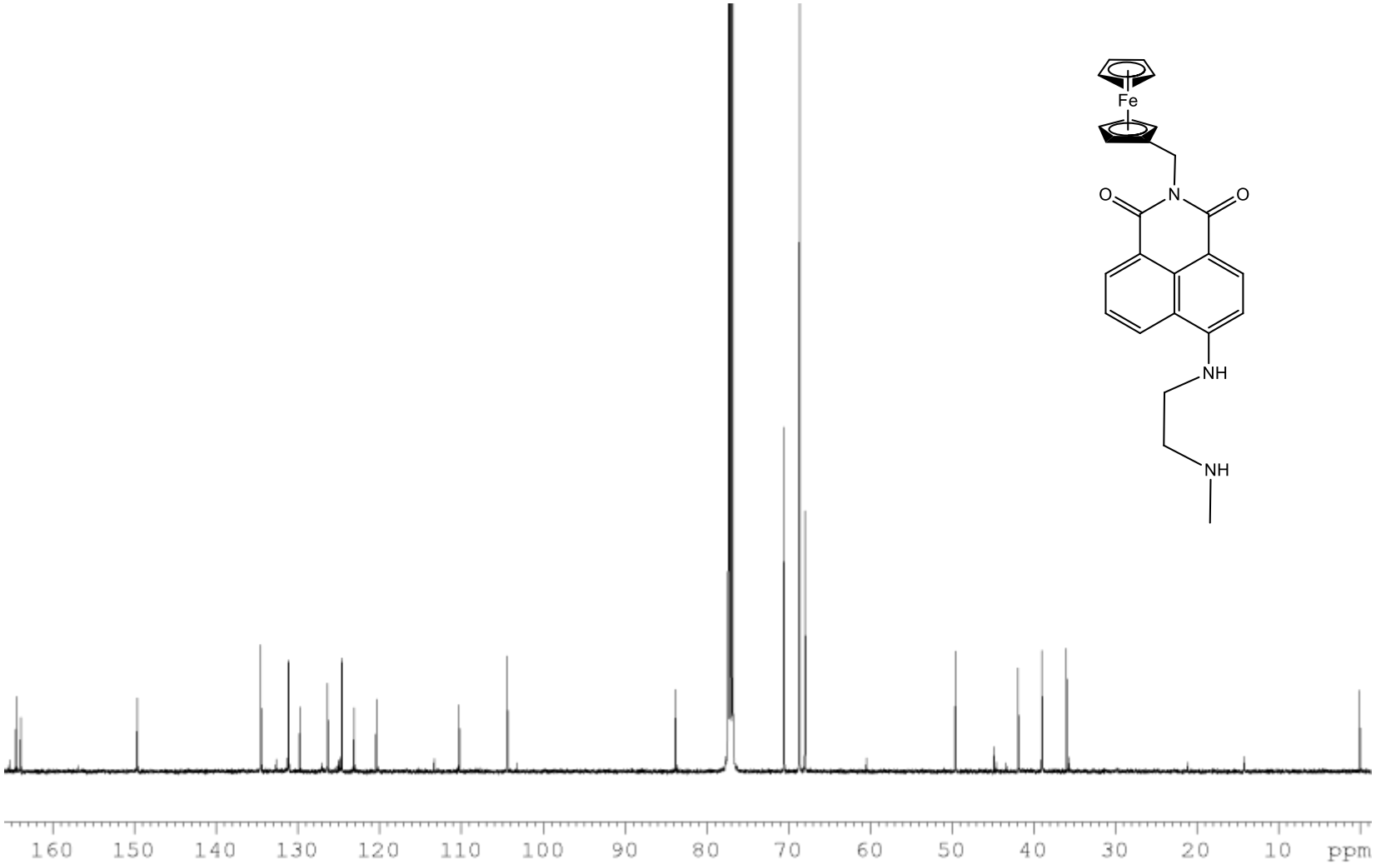



**Fig. S3** $^{13}C$ NMR spectrum of compound **4** in $CDCl_3$ at 126 MHz.

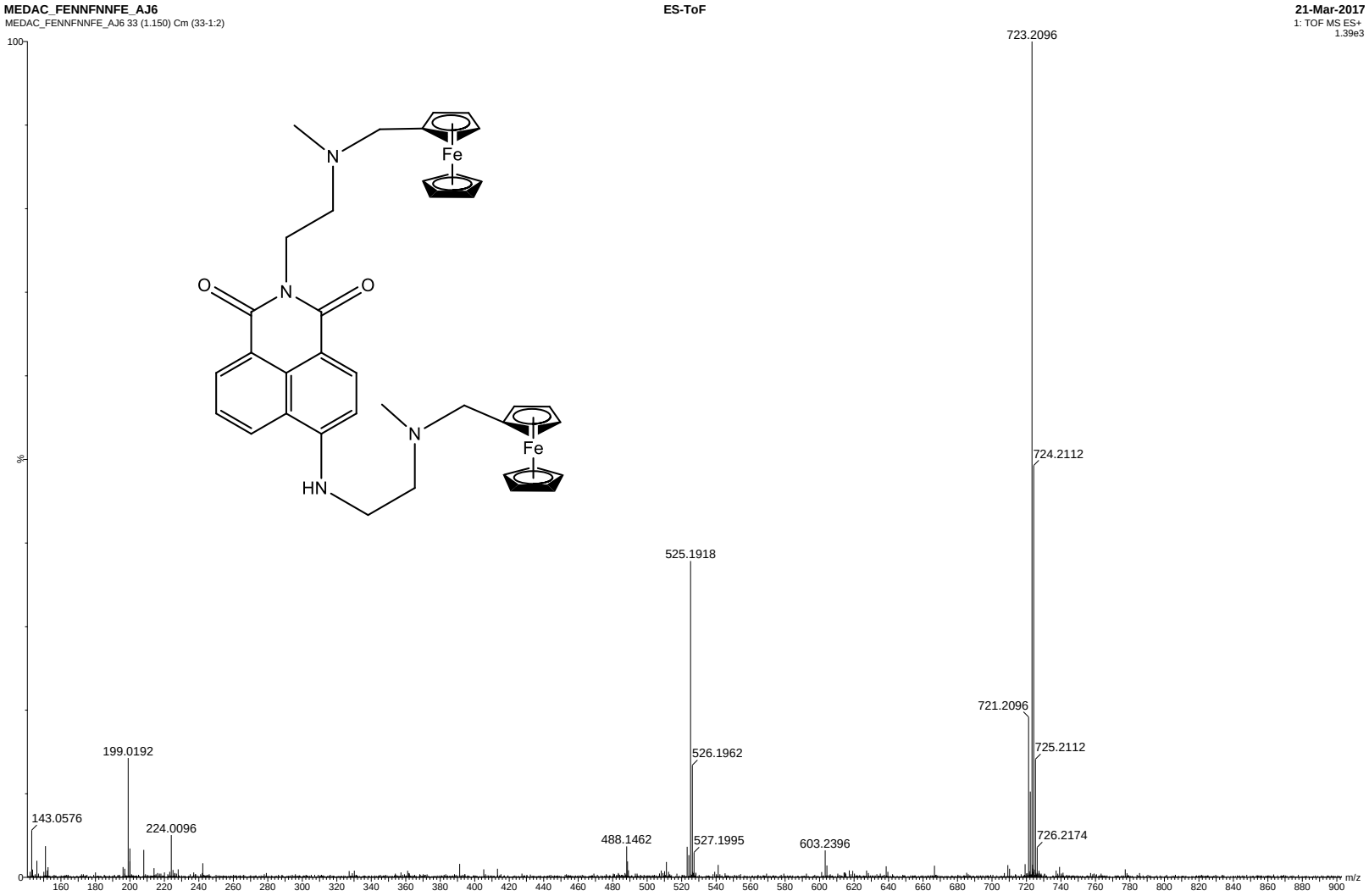



**Fig. S4** HRMS of compound **3**.

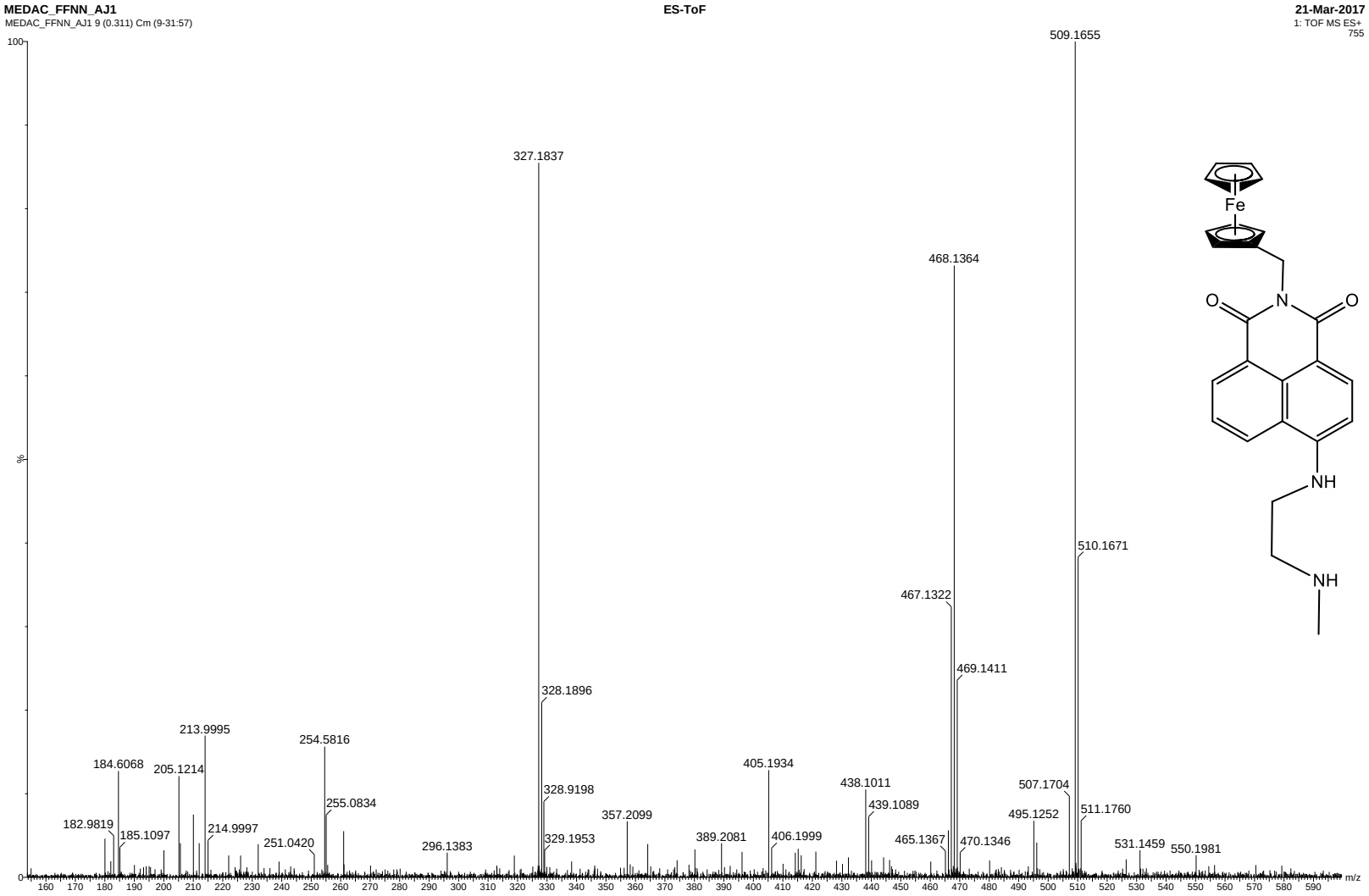



**Fig. S5** HRMS of compound **4**.