SI: Synthesis and Antimalarial Activity of Prodigiosenes

Marchal, Smithen, Uddin, Robertson, Jakeman, Mollard, Goodman, MacDougall, McFarland, McFadden and Thompson

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SUPPORTING INFORMATION

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1) *In vitro* antimalarial assays

The 3D7 strain of the human malaria parasite, *Plasmodium falciparum* was grown as previously described.[1]

The compounds were dissolved in DMSO at 1000x the maximum concentration and diluted with growth media to working concentrations. Parasite growth rates after 48 hours were assessed using a modified version of the SYBRgreen assay.[2-4] Calculation of IC_{50} values and statistical comparison between strains (Mann-Whitney test) were carried out using GraphPad Prism Software. . Three repeats were conducted for each compound.

Compounds	IC_{50}	95% confidence interval
CQ	14.9 nM	14.3-15.5 nM
Prodigiosin **1**	11 nM	7-16 nM
14	19 nM	10-38 nM
15	1.5 μM	0.9-2.4 μM
16	0.9 μM	0.8-1.1 μM
17	1.5 μM	1.1-2.1 μM
18	1.0 μM	0.8-1.2 μM
25	5.6 μM	4.4-7.0 μM
26	98 nM	84-114 nM
33	4.7 μM	2.9-7.7 μM
45	18 nM	15-21 nM
46	56 nM	47-68 nM
47	116 nM	95-142 nM
49	521 nM	369-738 nM
50	7.1 nM	6.5-7.7 nM
51	4.7 μM	3.5-6.2 μM
52	1.4 μM	1.0-2.0 μM

2) Isolation of prodigiosin from *Serratia marcescens*

Composition and preparation of media

MYM broth [maltose 4 g/L, yeast extract 4 g/L, malt extract 10 g/L]; MYM agar [maltose 4 g/L, yeast extract 4 g/L, malt extract 10 g/L, agar 15 g/L]; powdered peanut broth [2% powdered peanuts in distilled water]. Peanuts (unsalted and roasted) used in powdered peanut broth were purchased from the Atlantic Superstore, blended into a fine powder and sifted through a wire mesh to remove large particulates. 250 mL of each broth solution was prepared in 1 L glass Erlenmeyer flasks. 125 mL of MYM agar was prepared in 250 mL Erlenmeyer flasks and poured into standard petri dishes while molten. All media was adjusted to pH 7 with 5 M NaOH or 5 M HCl as required. All solutions were autoclaved at 120 °C for 20 minutes prior to use. (**Adapted method from:** "A Novel Medium for the Enhanced Cell Growth and Production of Prodigiosin from *Serratia marcescens* isolated from soil" *BMC Microbiol*., **2004**, *4*, 11).

Prodigiosin production and isolation

A 1 cm x 1 cm lawn of *Serratia marcescens* (Carolina Biological Supply Company) grown on MYM agar for 10 days at 25 °C was inoculated into 250 mL MYM liquid growth media and incubated at 28 °C, with shaking at 250 RPM for 16 hours (overnight). 2% (v/v) of the overnight growth was added to four 250 mL solutions of 2% (w/v) powdered peanut solution (5 mL seed growth per 250 mL of peanut solution). The growths were incubated at 28 °C, with shaking at 250 RPM for 48 hours. The pH of all solutions was maintained at 7 over the entire growth. After the 48 hour growth period the media was centrifuged at 4 °C, 3750 RPM for 2 hours. The supernatant was removed and extracted with 2 L (4 x 500 mL) of HPLC grade ethyl acetate. The combined organic layer was concentrated *in vacuo*. The remaining cell pellet was re-suspended in 300 mL of acetone and gently stirred at room temperature for 3 hours. To remove cellular debris, the acetone solution was centrifuged at 8500 RPM at 4 °C for 30 minutes. The supernatant was removed and combined with the ethyl acetate extract, which was then concentrated *in vacuo* resulting in a deep reddish-pink oil.

Prodigiosin purification

The crude prodigiosin-containing oil was purified using gravity-flow column chromatography over basic alumina (Brockmann III). Initial columns (2 x columns) were eluted with 0% then 0-10% ethyl acetate in hexanes (run over 2 hours), with collection of all orange-coloured fractions. Additional columns were eluted with 2-4% diethyl ether in hexanes (run over 5 hours) and 5-9% diethyl ether in hexanes (run over 3 hours) with only the major product-containing fractions (as determined visually) collected. The resulting purified prodigiosin (3 mg, orange film) was dried briefly in a vacuum oven (~40 °C, 1 hour).

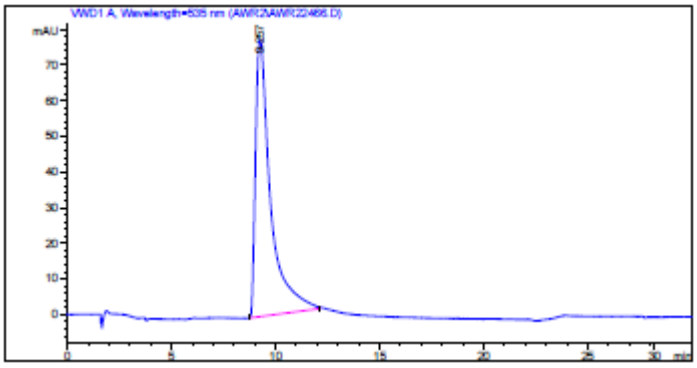
Analysis of isolated prodigiosin

Analysis of the isolated material using TLC revealed it to be comparable in terms of colour and Rf (~0.2; alumina plate, 40% ethyl acetate/hexanes) as an authentic sample (Figure 1). Further analysis using HPLC showed a single UV-active compound (monitoring λ 535 nm, Figure 2). ^{1}H NMR (CD_2Cl_2, 500 MHz) δ 6.77 (s, 1H, PyH), 6.74 (br s, 1H, PyH), 6.68 (dd, 1H, *J* = 3.5, 0.5 Hz PyH), 6.38 (s, 1H, PyH), 6.20 (t, 1H, *J* = 3.0 Hz, PyH), 6.05 (s. 1H, PyH), 3.93 (s, 3H, OCH_3), 2.27 (t, 2H, *J* = 7.5 Hz, $PyCH_2$), 1.91 (s, 3H, CH_3), 1.50-1.43 (m, 2H, CH_2), 1.32-1.27 (m, 4H, 2 x

CH_2), 0.87 (t, 3H, *J* = 7.0 Hz, CH_3) ppm: data comparable to that of an authentic sample. LRMS: 324.2 $(M+H)^+$; HRMS: 324.2058 Found, 324.2070 Calculated for $C_{20}H_{26}N_3O$.

Figure 1. TLC plate showing comparison of isolated material (left) with authentic sample (right) and a co-spot (centre)

Figure 2. HPLC trace of isolated prodigiosin



HPLC Conditions:
Reversed phase C18 analytical column
0-80% CH_3CN in pH 4 aq. buffer (see below) over 5 min then 80% CH_3CN/buffer for 25 min;
Flow rate 1.0 mL/min
UV detector monitoring λ 535 nm

pH 4 aq. buffer for HPLC (Millipore filtered):
3.87 g Tetrabutylammonium bromide (TBAB)
1.36 g Potassium phosphate monobasic (KH_2PO_4)
50 mL HPLC grade acetonitrile
Made up to 1 L final volume using Millipore water

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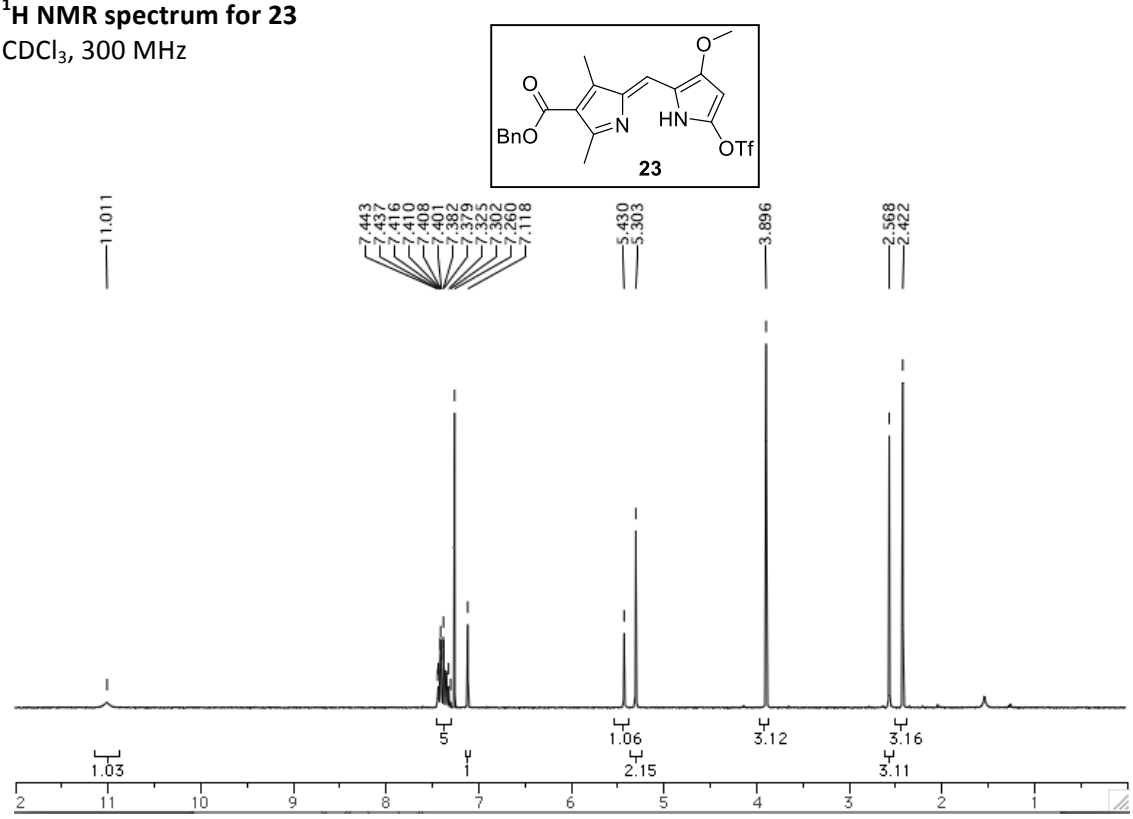
3) ^{1}H and ^{13}C NMR spectra

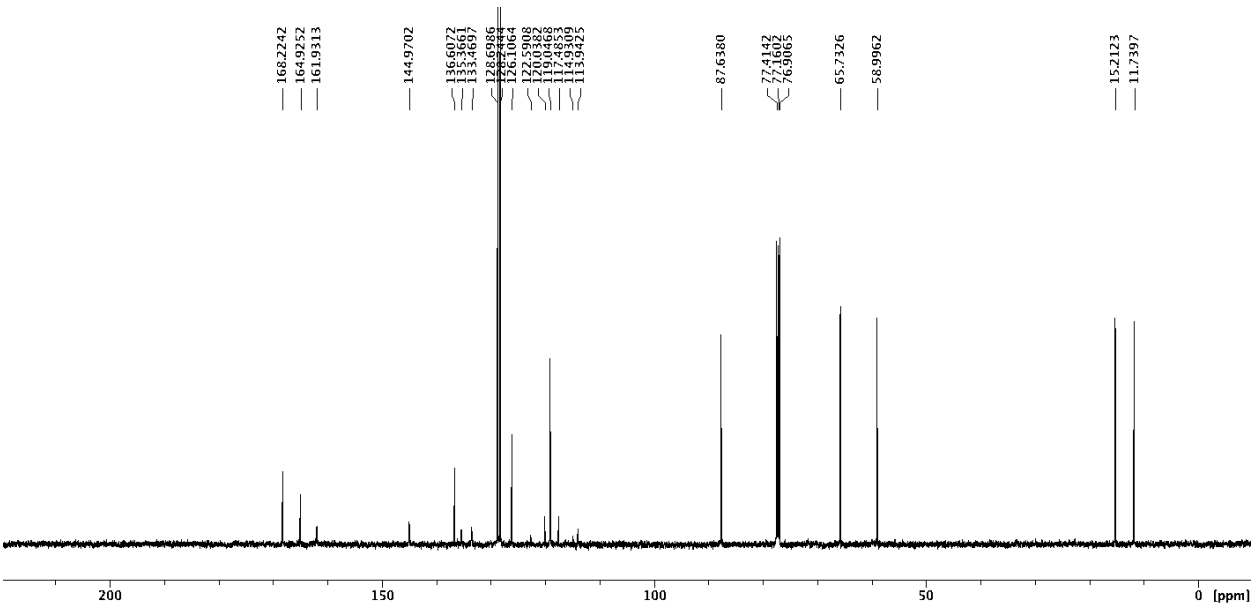
^{1}H NMR spectrum for 23

$CDCl_3$, 300 MHz



^{13}C NMR spectrum for 23

$CDCl_3$, 125 MHz



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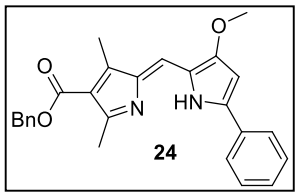
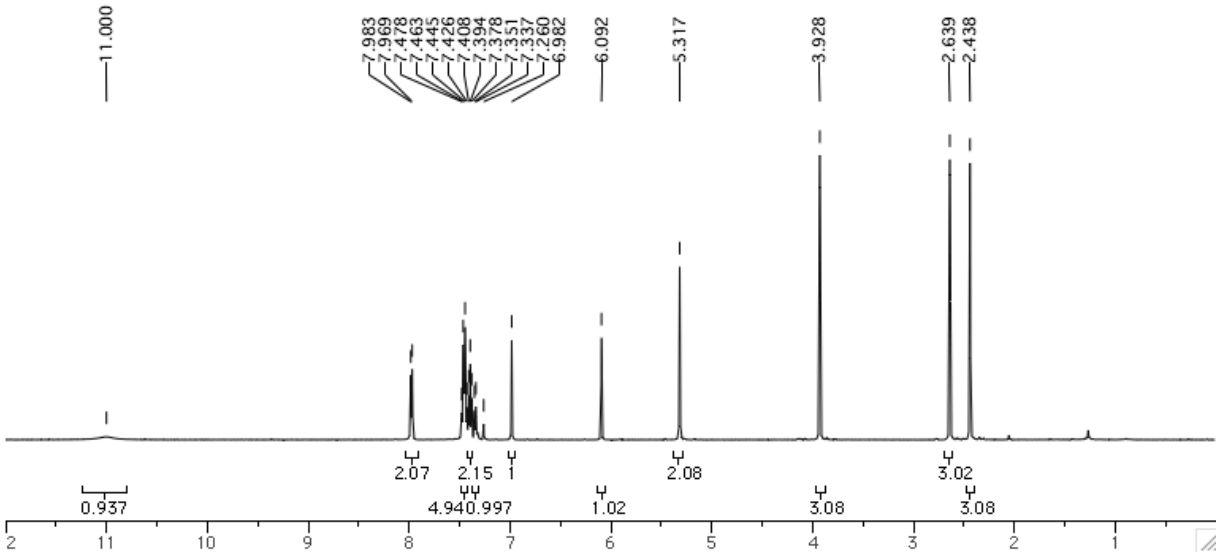
^{1}H NMR spectrum for 24

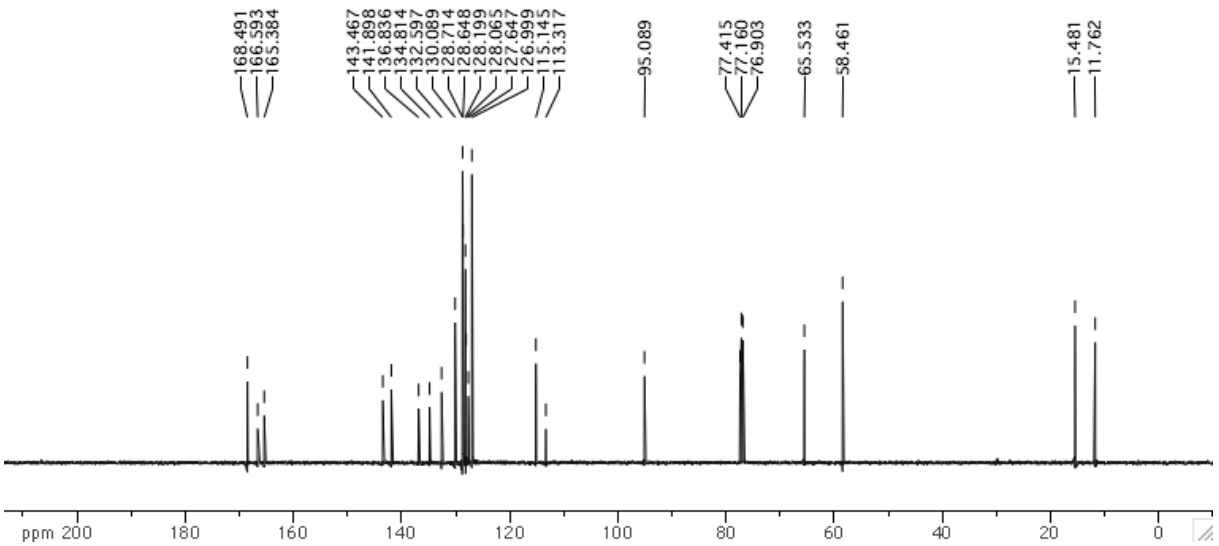
$CDCl_3$, 500 MHz





^{13}C NMR spectrum for 24

$CDCl_3$, 125 MHz



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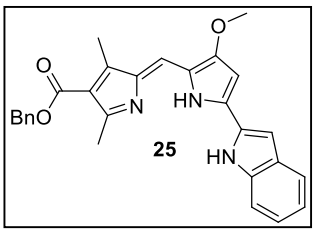
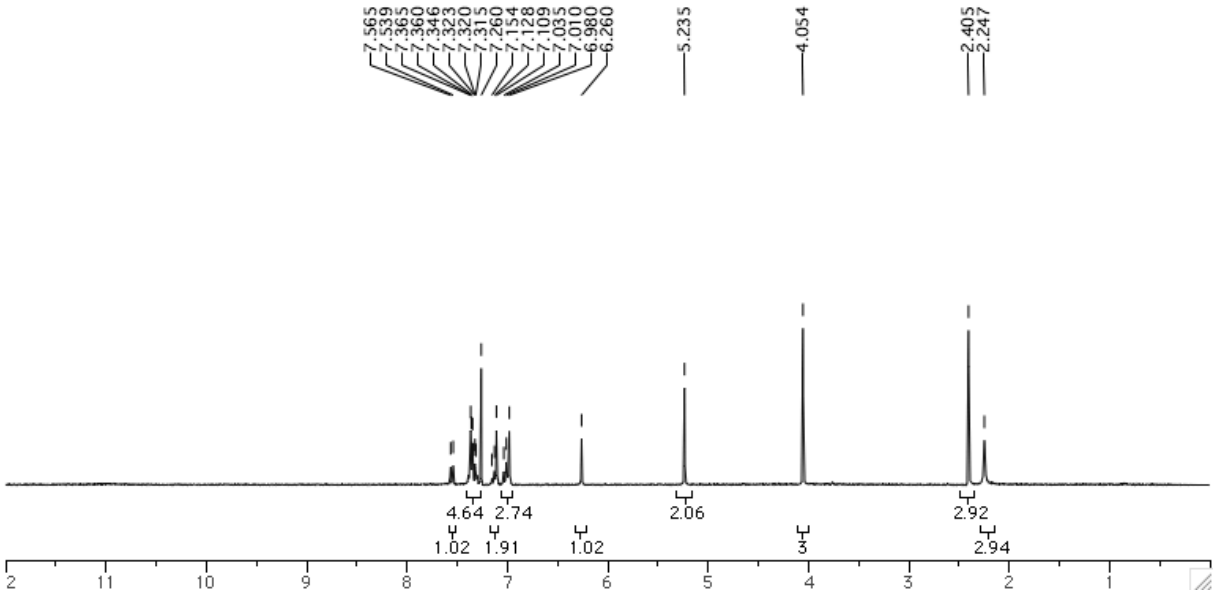
^{1}H NMR spectrum for 25

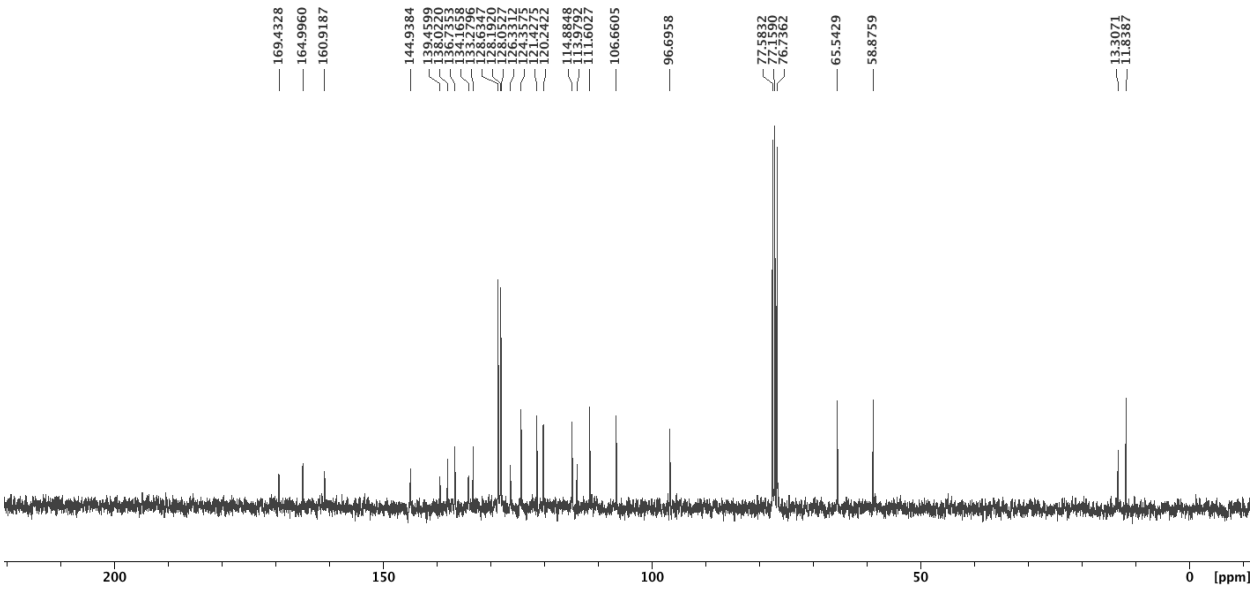
$CDCl_3$, 500 MHz





^{13}C NMR spectrum for 25

$CDCl_3$, 125 MHz



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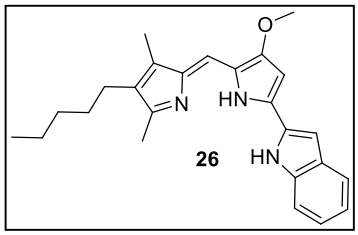
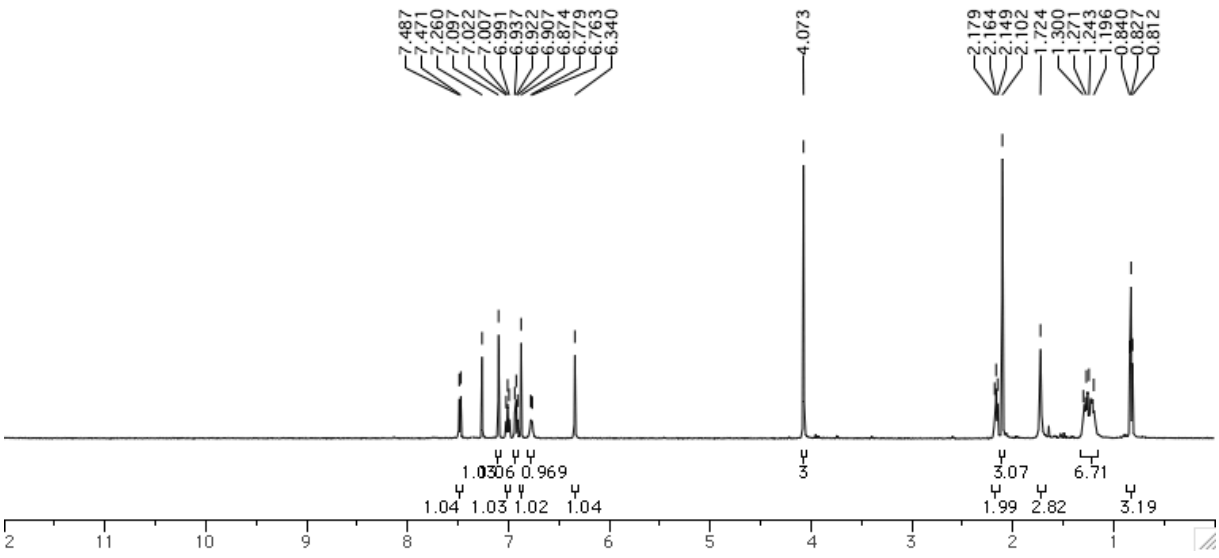
^{1}H NMR spectrum for 26

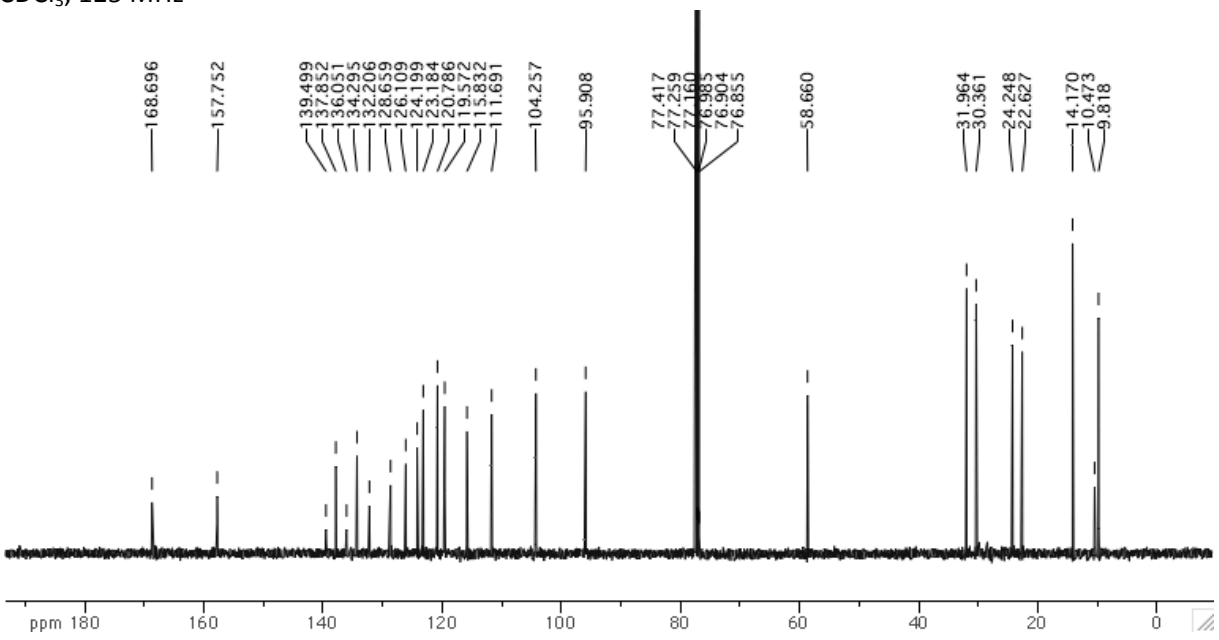
$CDCl_3$, 500 MHz





^{13}C NMR spectrum for 26

$CDCl_3$, 125 MHz



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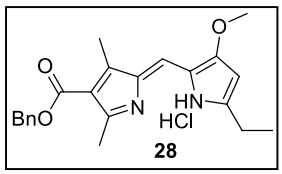
^{1}H NMR spectrum for 28

$CDCl_3$, 500 MHz



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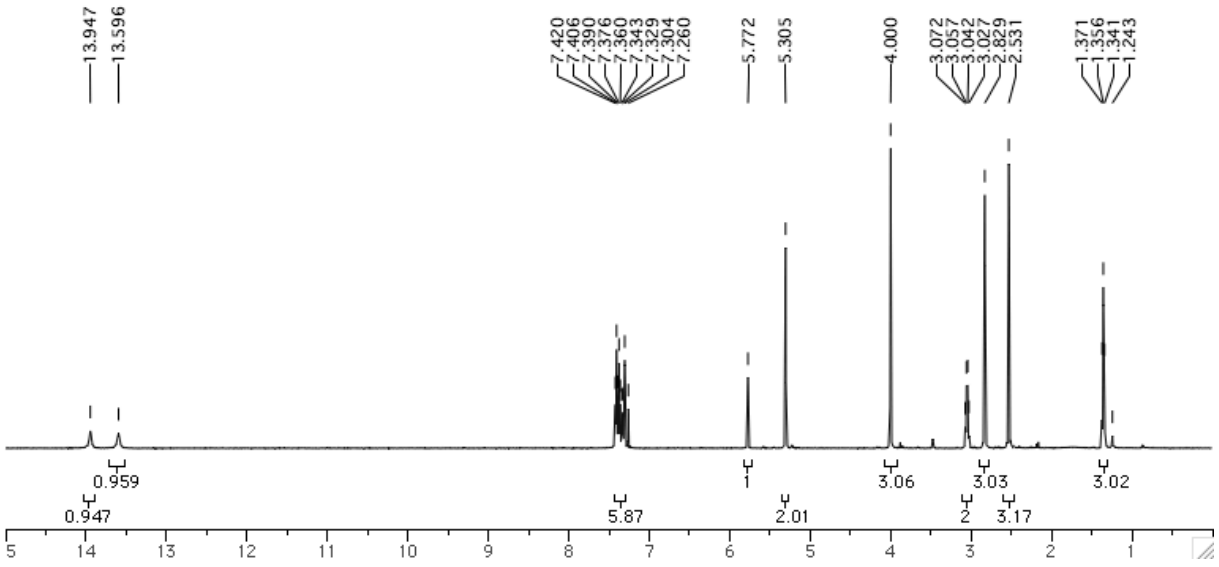
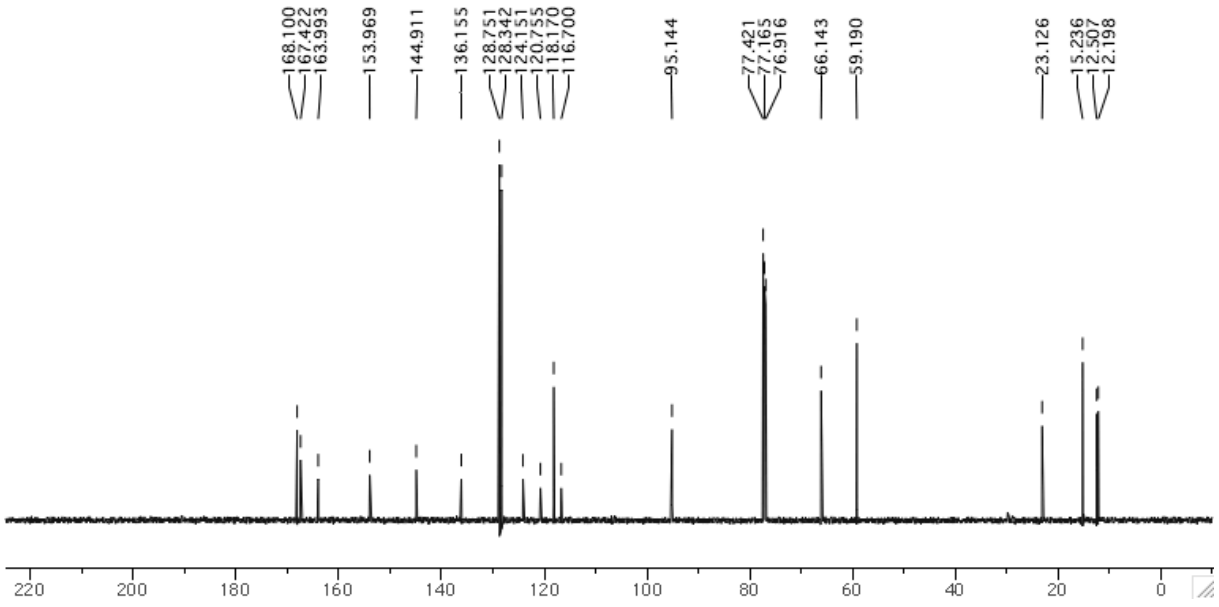


^{13}C NMR spectrum for 28

$CDCl_3$, 125 MHz



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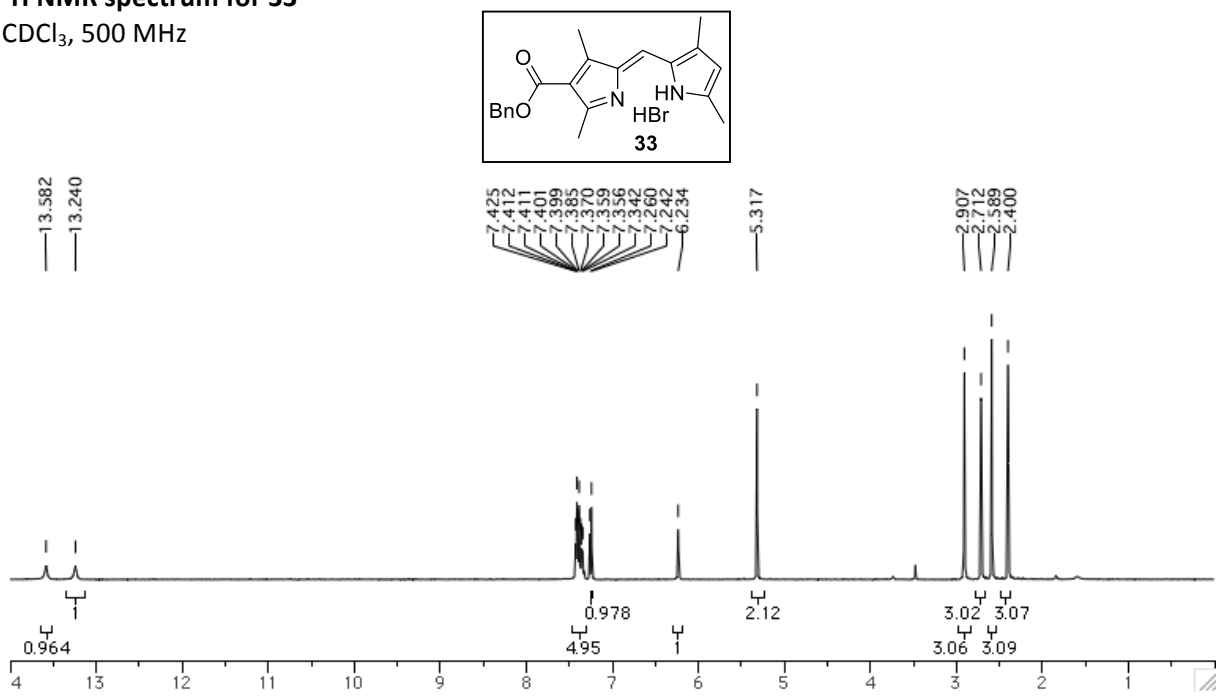
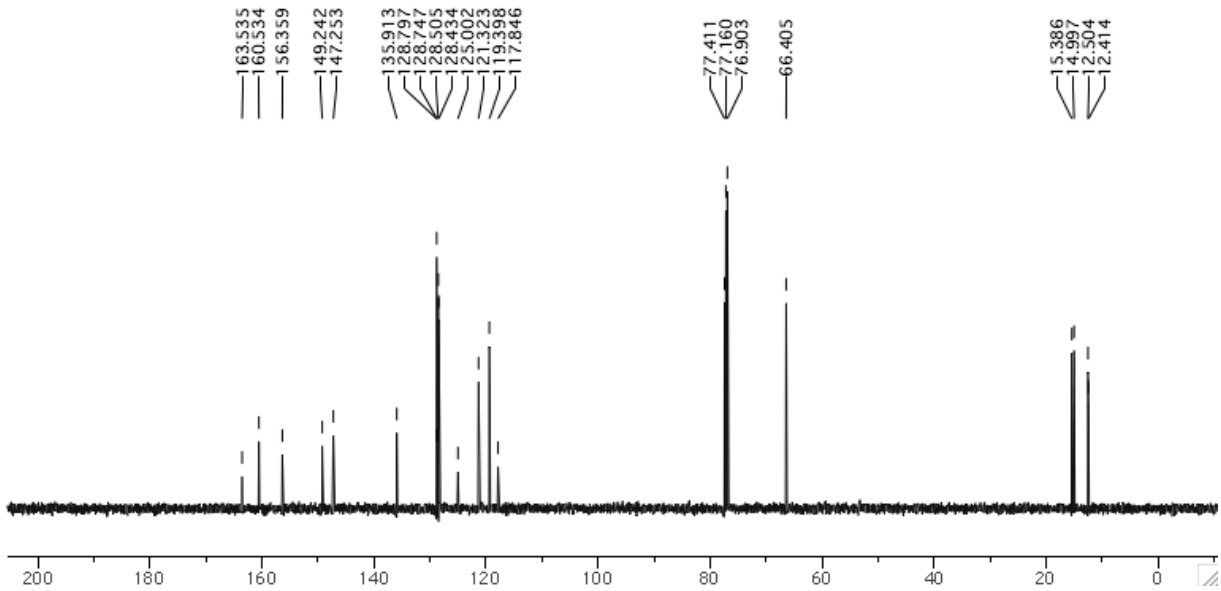
^{1}H NMR spectrum for 33

$CDCl_3$, 500 MHz



^{13}C NMR spectrum for 33

$CDCl_3$, 125 MHz



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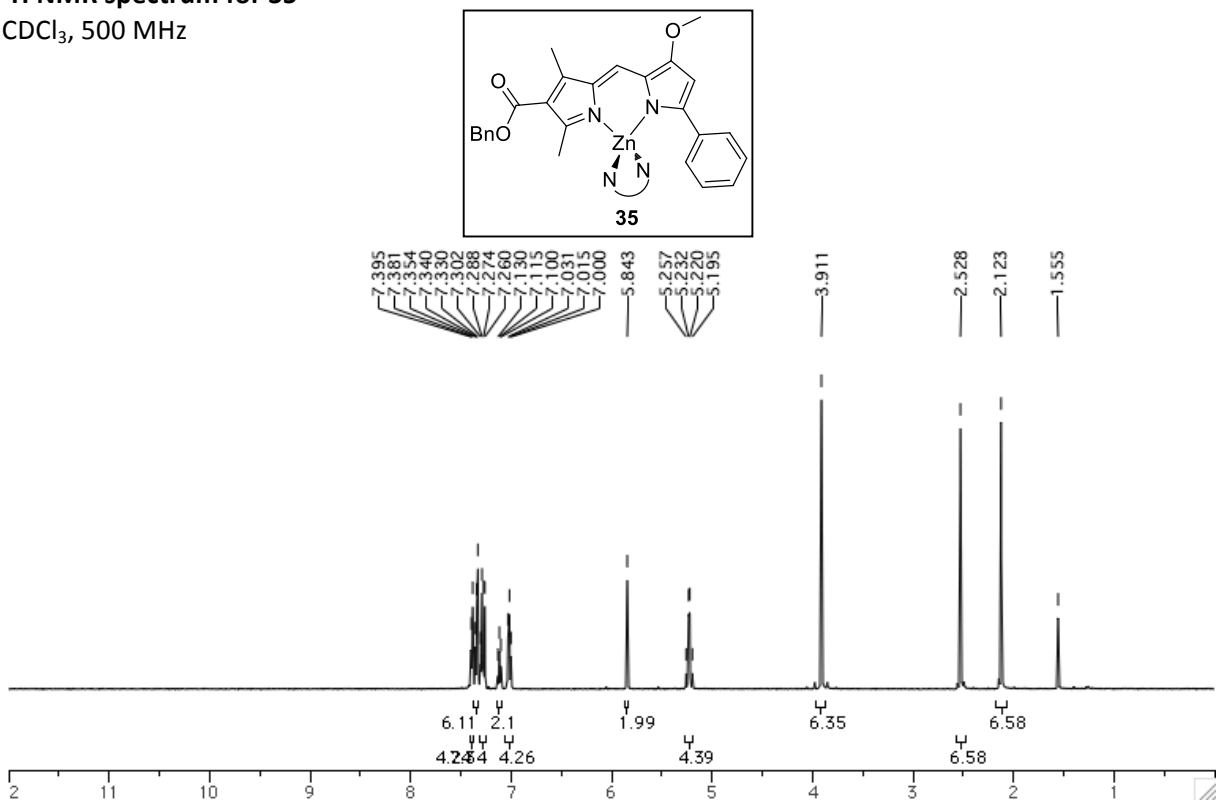
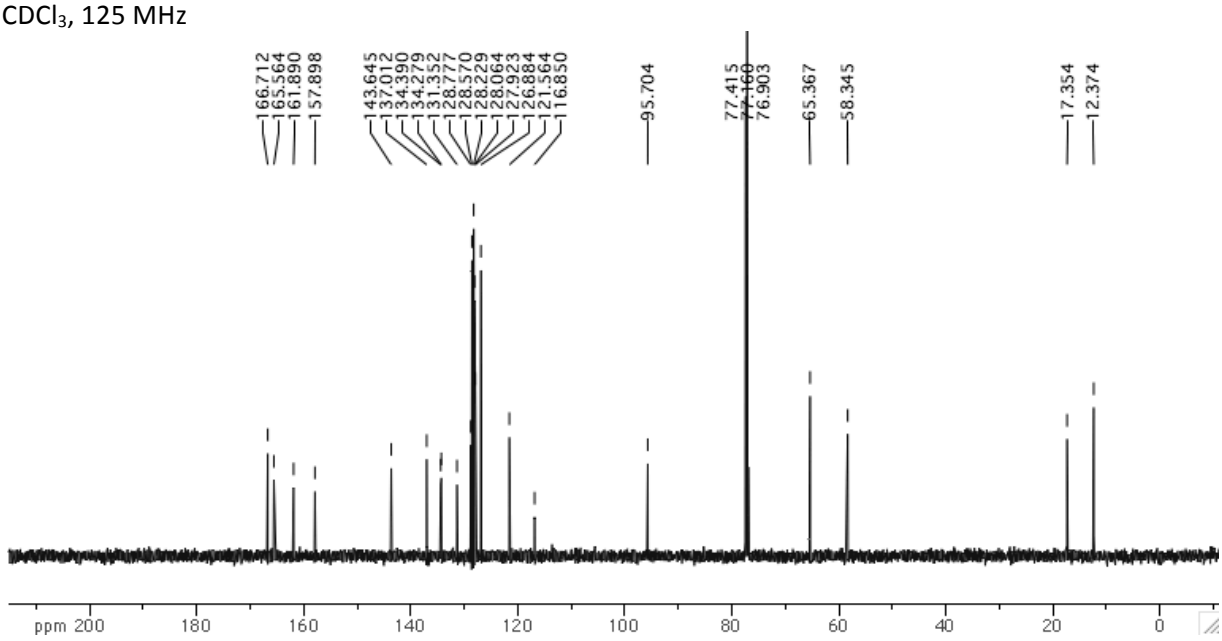
^{1}H NMR spectrum for 35

$CDCl_3$, 500 MHz



^{13}C NMR spectrum for 35

$CDCl_3$, 125 MHz



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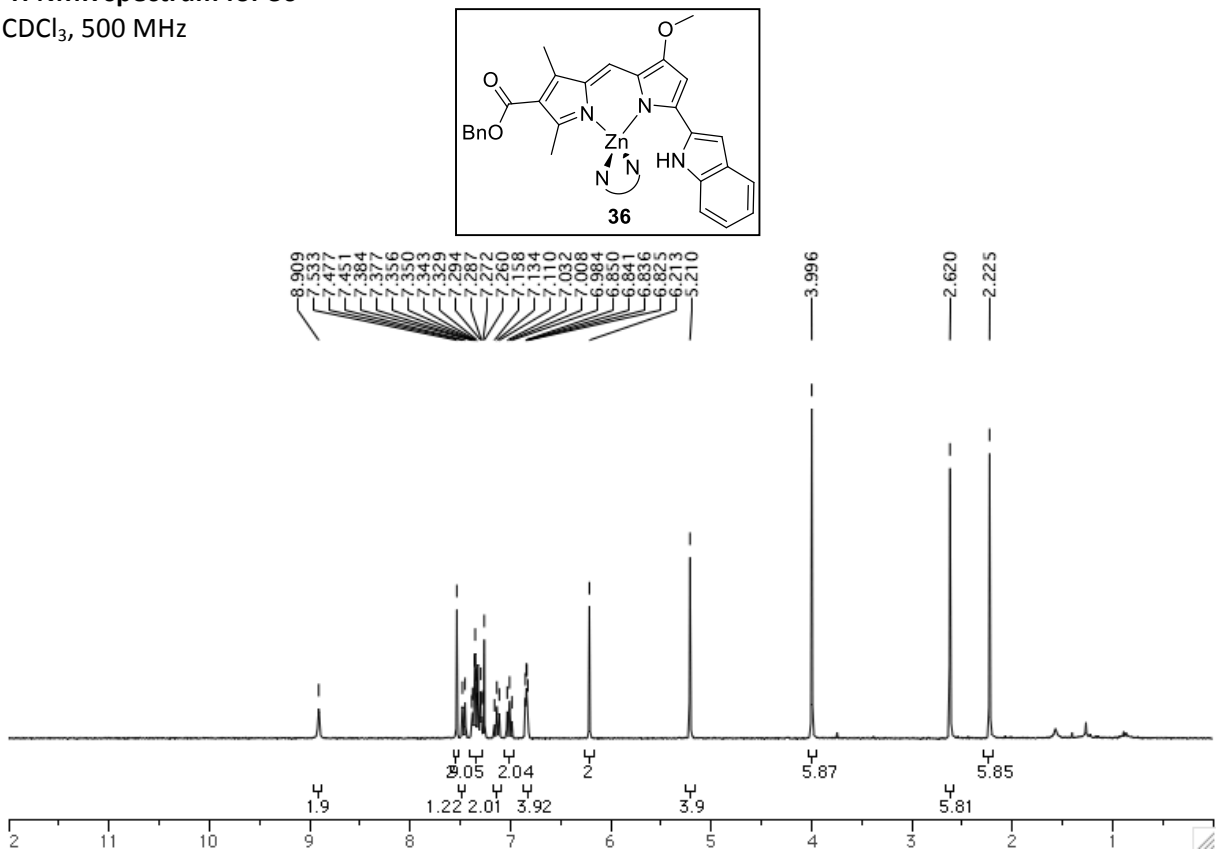
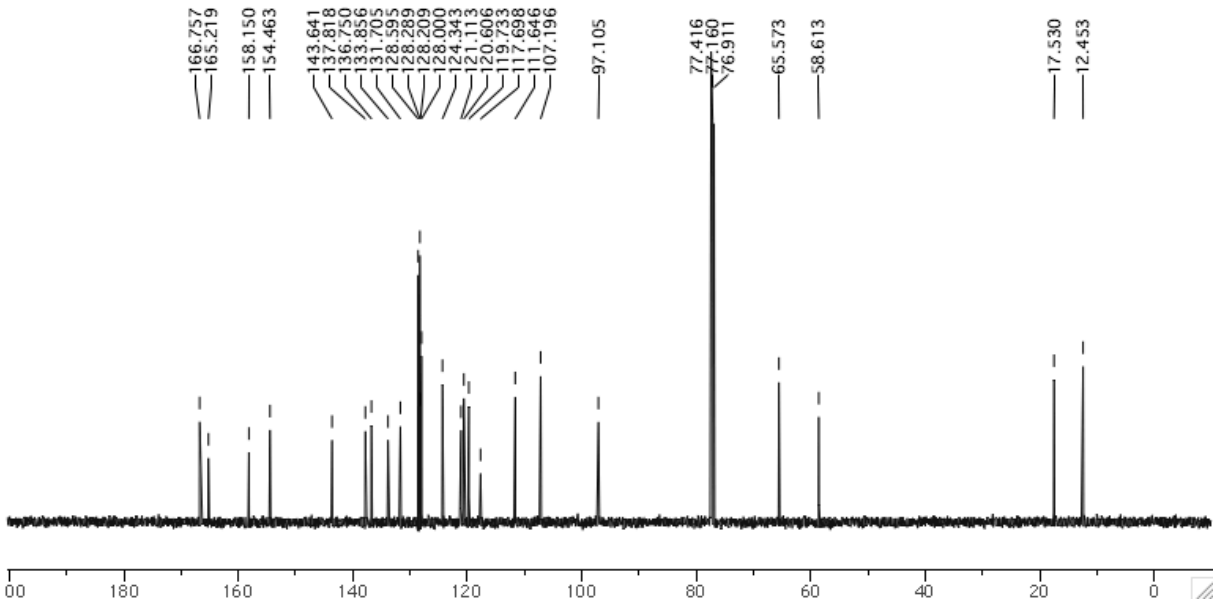
^{1}H NMR spectrum for 36

$CDCl_3$, 500 MHz



^{13}C NMR spectrum for 36

$CDCl_3$, 125 MHz



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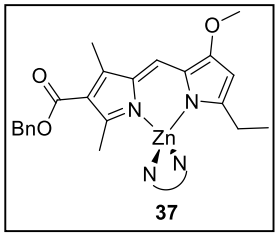
^{1}H NMR spectrum for 37

$CDCl_3$, 500 MHz



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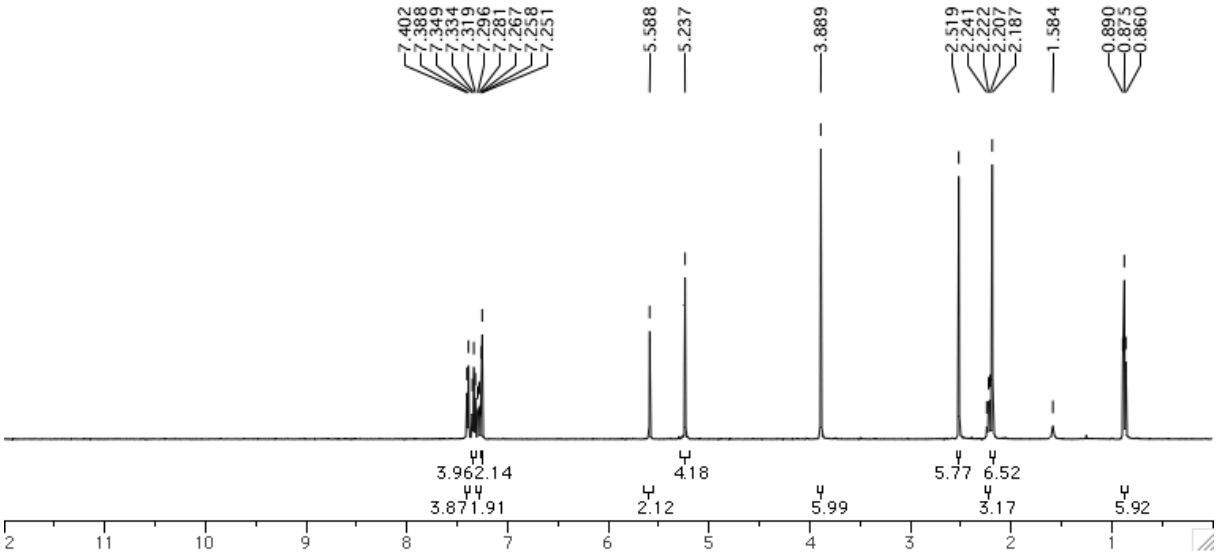
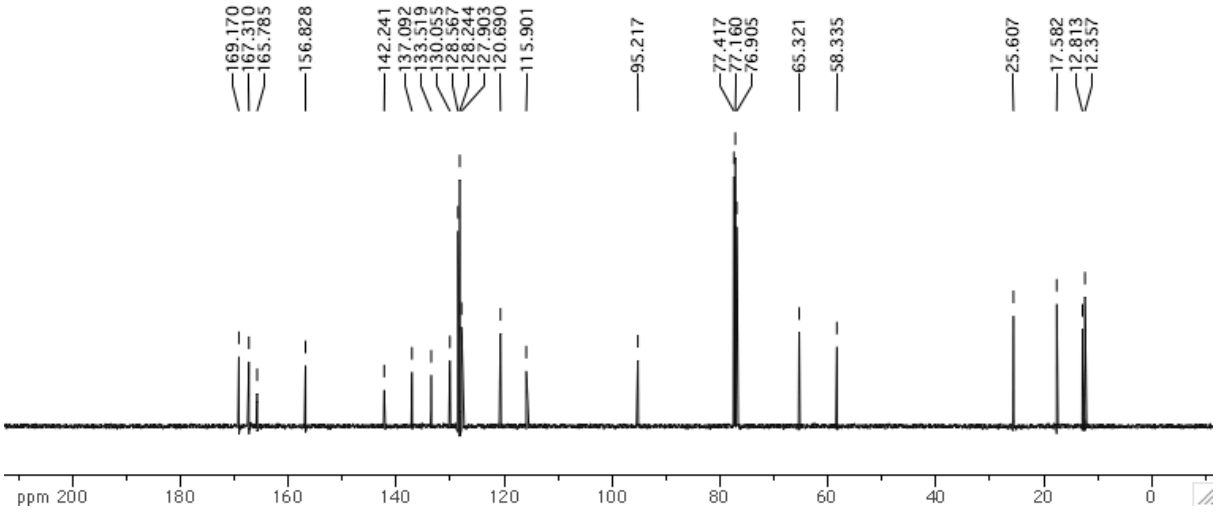


^{13}C NMR spectrum for 37

$CDCl_3$, 125 MHz



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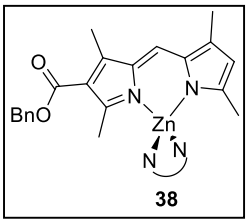
^{1}H NMR spectrum for 38

$CDCl_3$, 500 MHz



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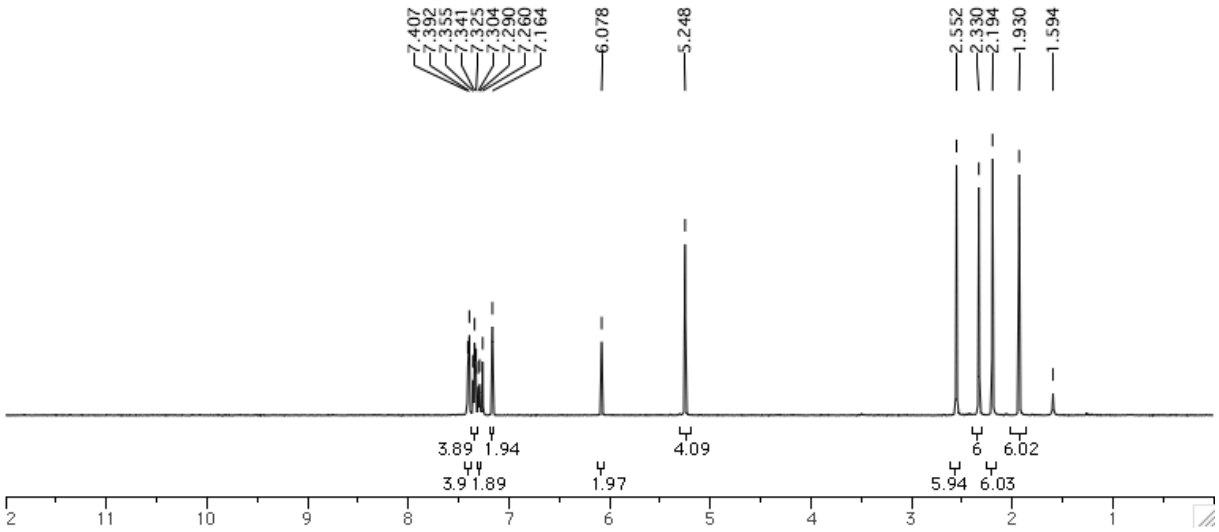
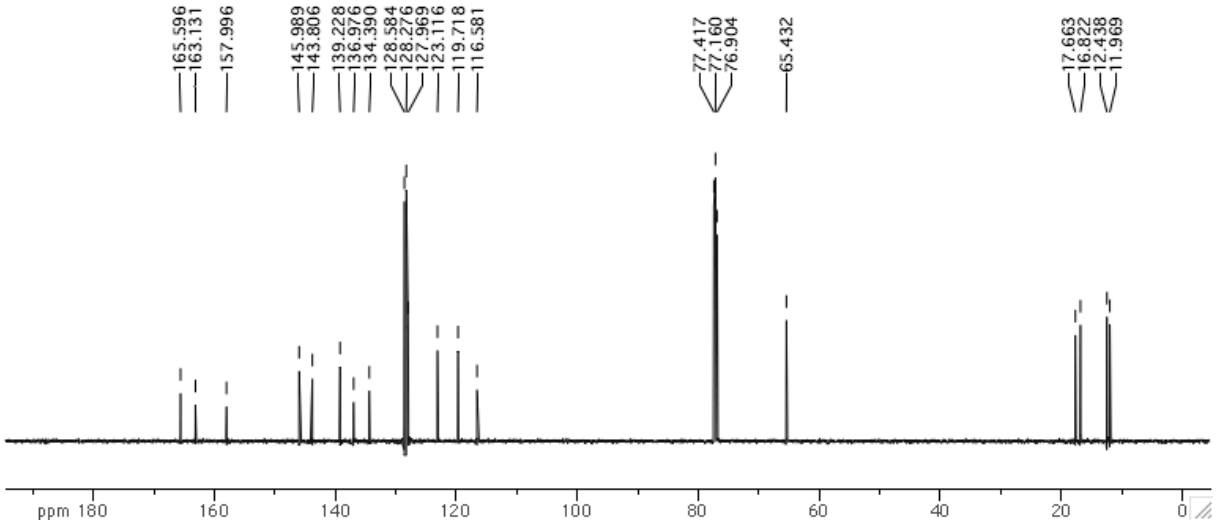


^{13}C NMR spectrum for 38

$CDCl_3$, 125 MHz



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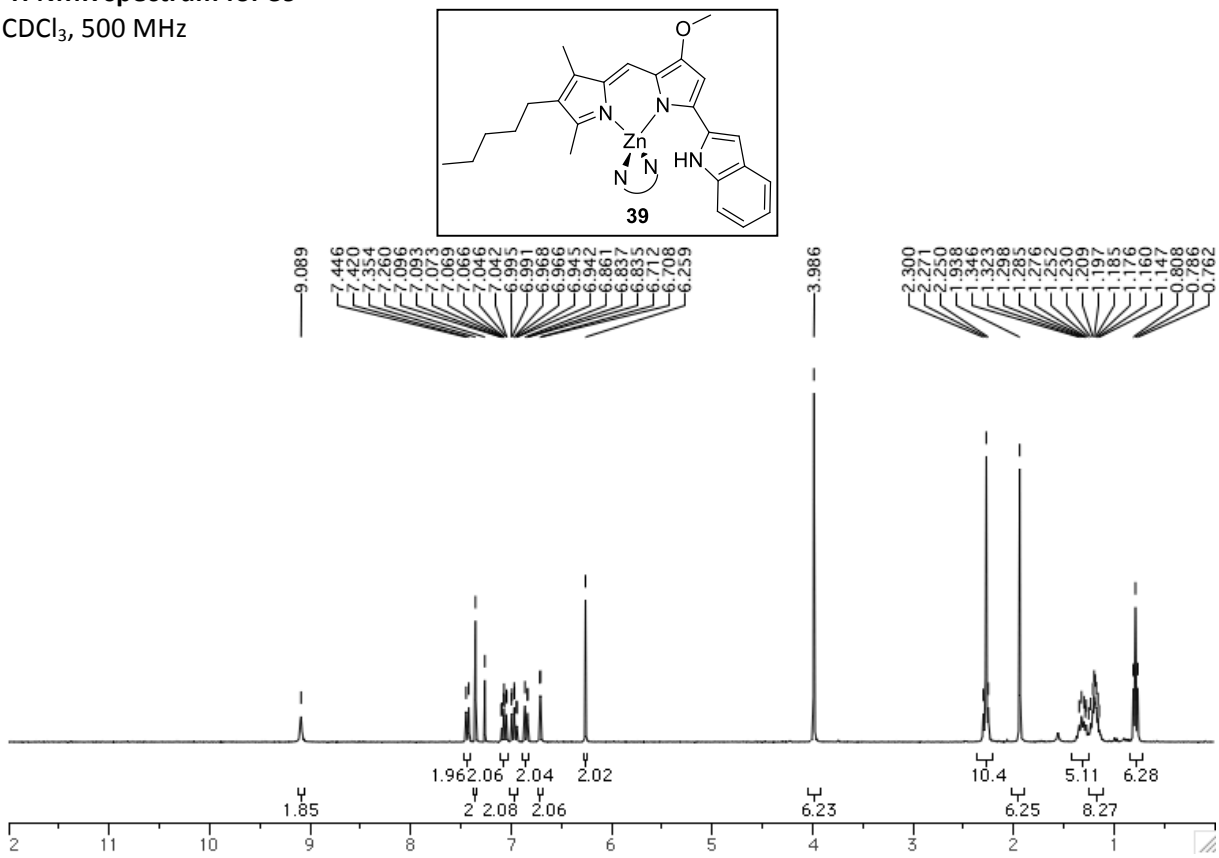
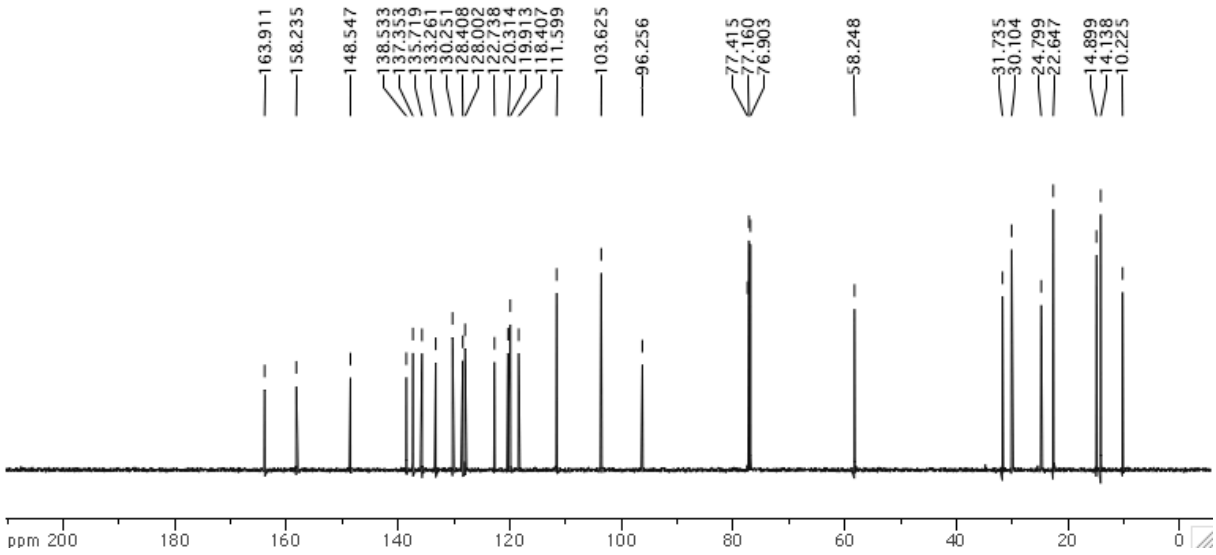
^{1}H NMR spectrum for 39

$CDCl_3$, 500 MHz



^{13}C NMR spectrum for 39

$CDCl_3$, 125 MHz



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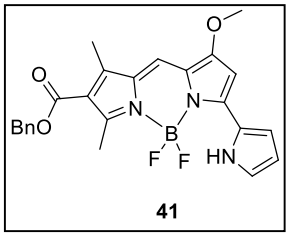
^{1}H NMR spectrum for 41

$CDCl_3$, 500 MHz



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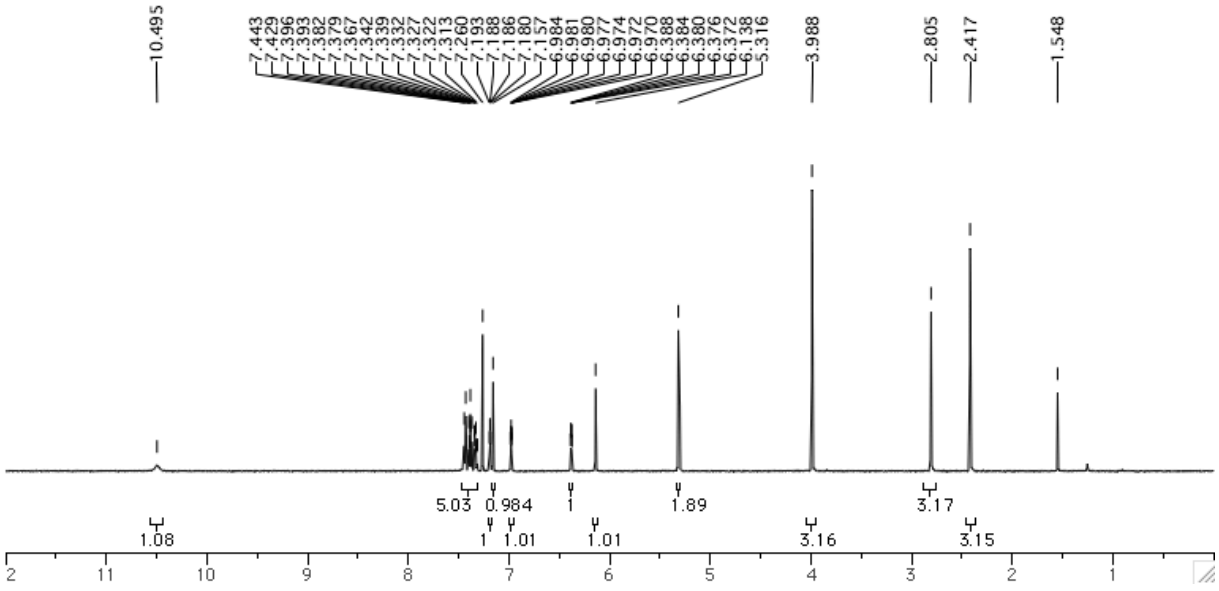
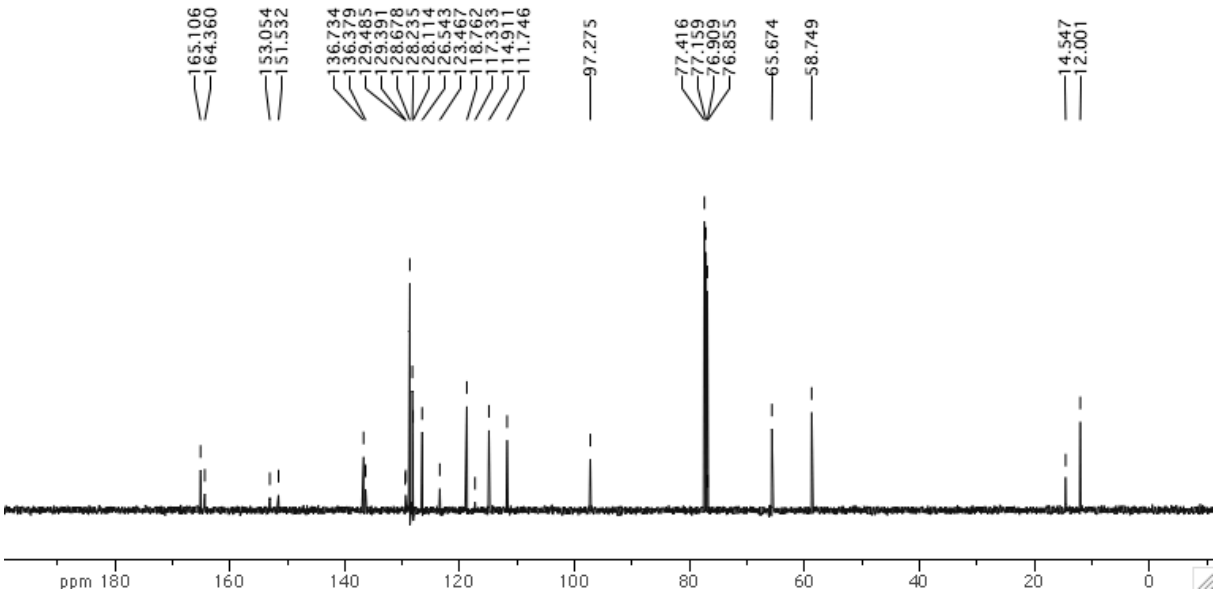


^{13}C NMR spectrum for 41

$CDCl_3$, 125 MHz



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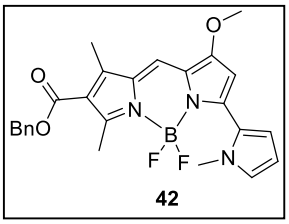
^{1}H NMR spectrum for 42

$CDCl_3$, 500 MHz



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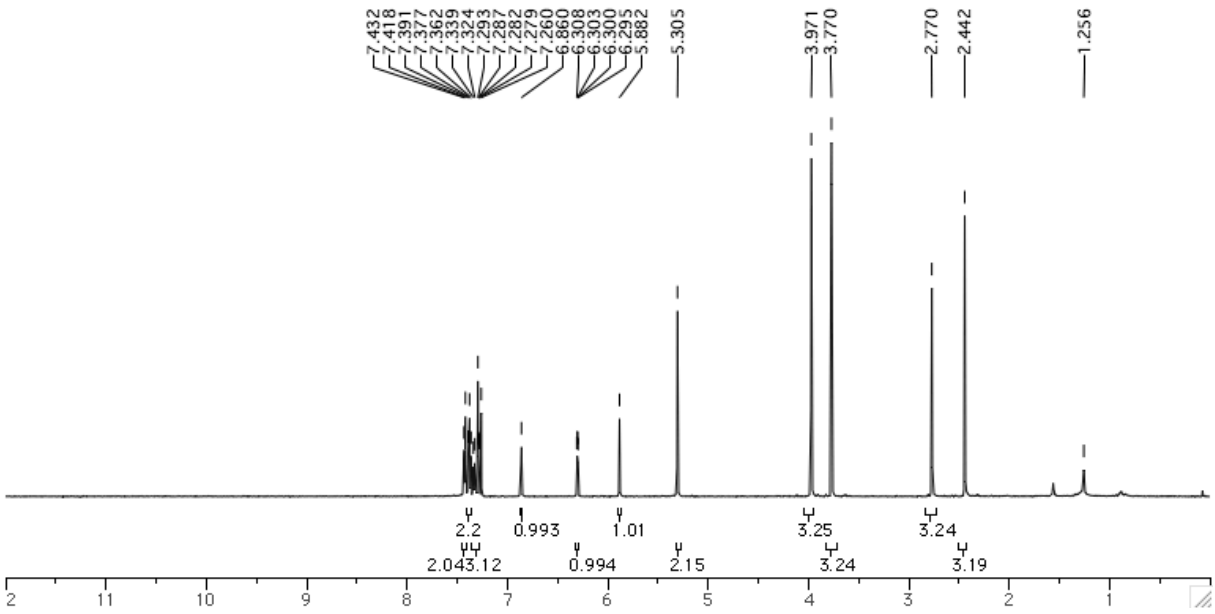
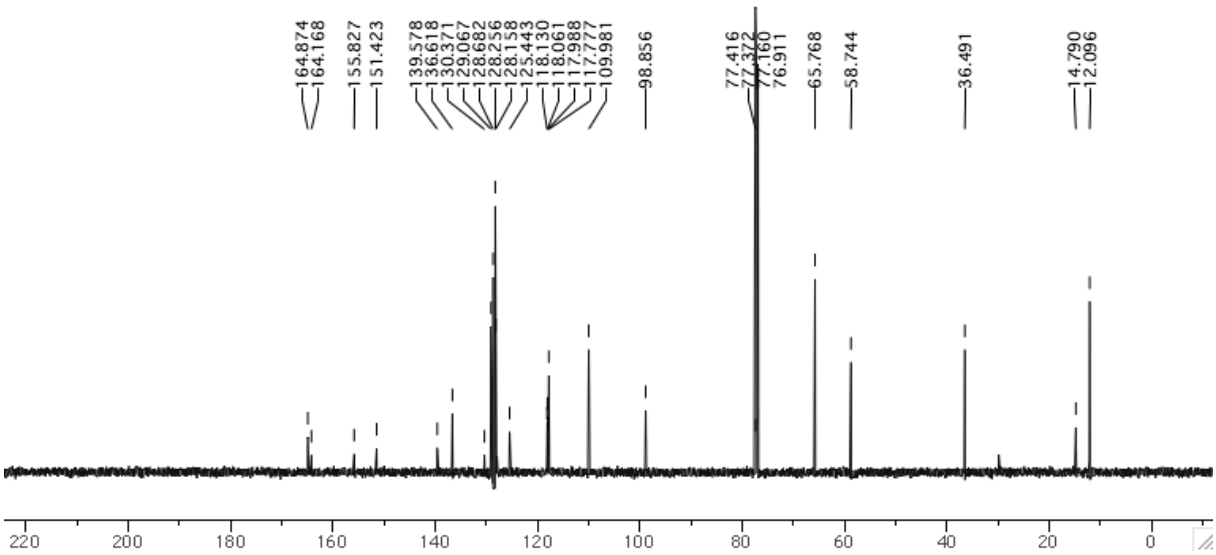


^{13}C NMR spectrum for 42

$CDCl_3$, 125 MHz



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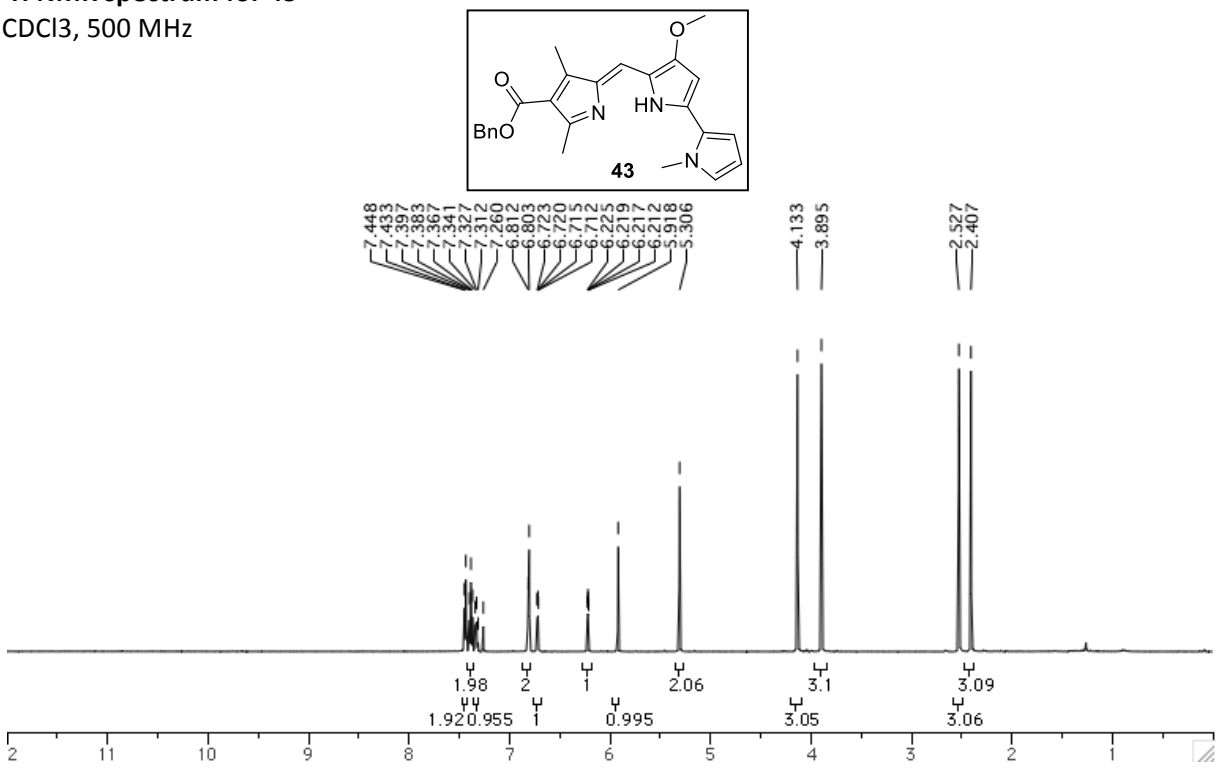
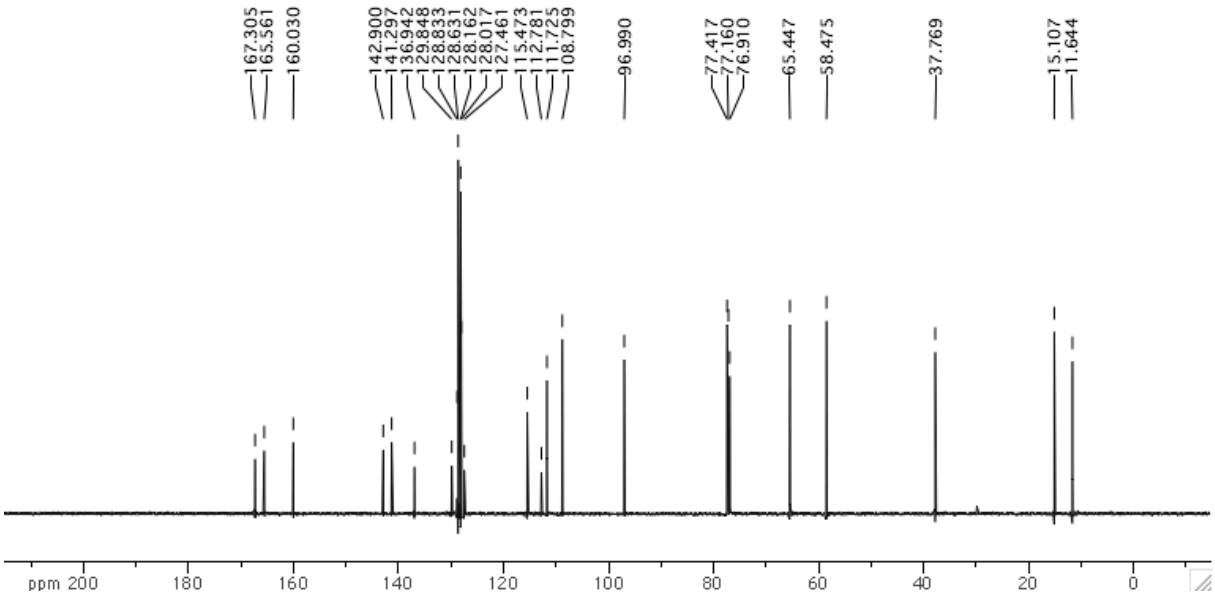
^{1}H NMR spectrum for 43

CDCl3, 500 MHz



^{13}C NMR spectrum for 43

$CDCl_3$, 125 MHz

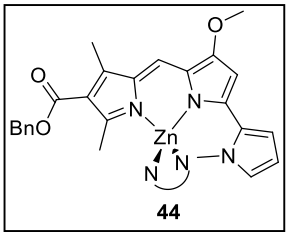


SI: Synthesis and Antimalarial Activity of Prodigiosenes

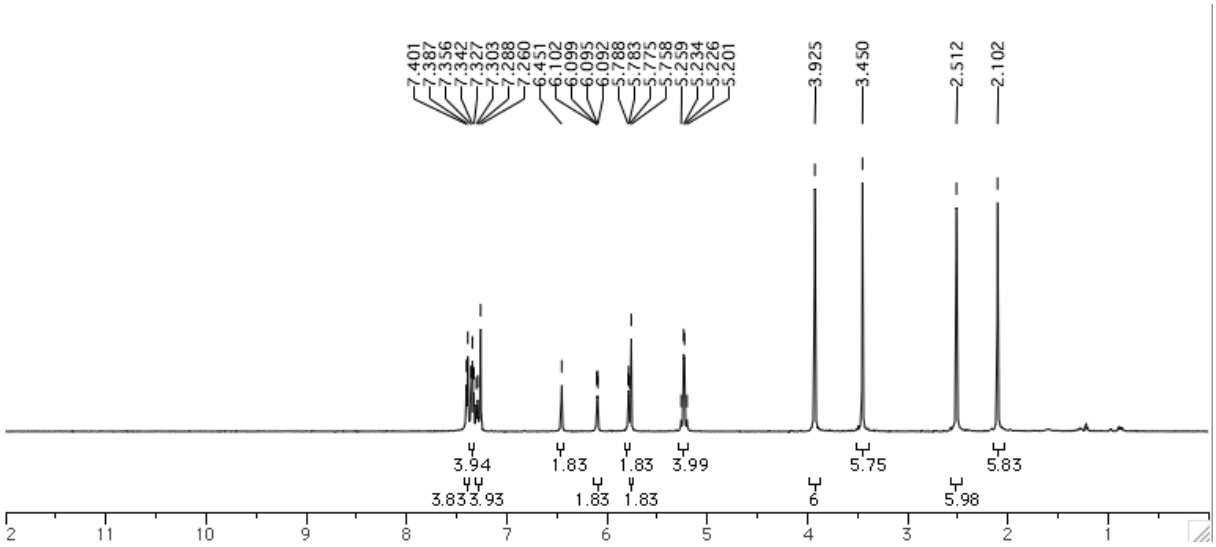
Marchal, Smithen, Uddin, Robertson, Jakeman, Mollard, Goodman, MacDougall, McFarland, McFadden and Thompson

^{1}H NMR spectrum for 44

$CDCl_3$, 500 MHz

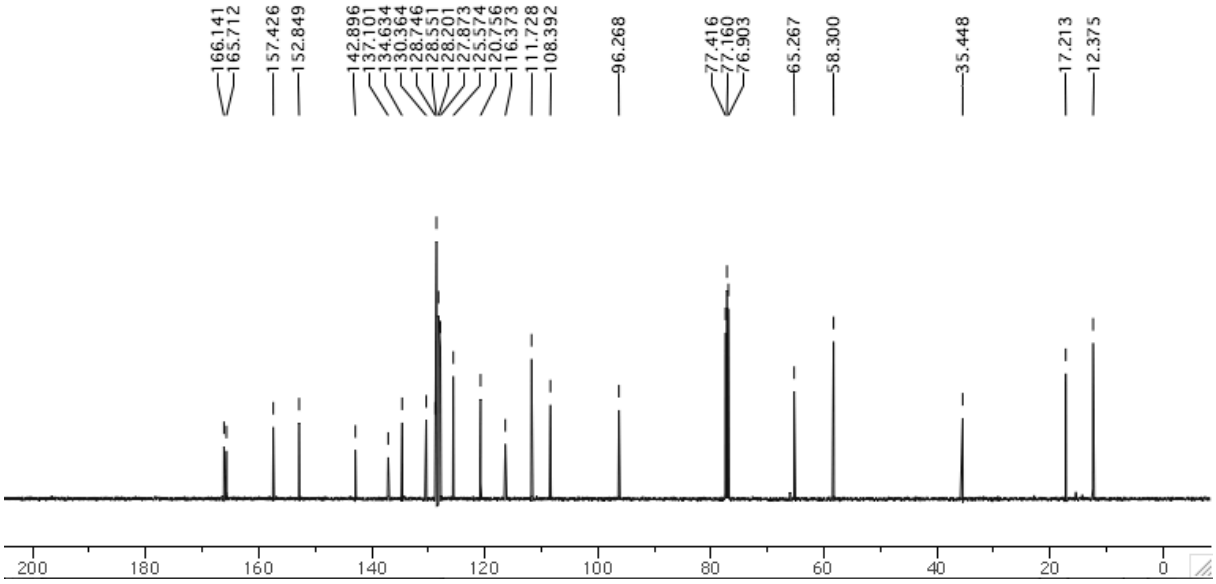


44



^{13}C NMR spectrum for 44

$CDCl_3$, 125 MHz

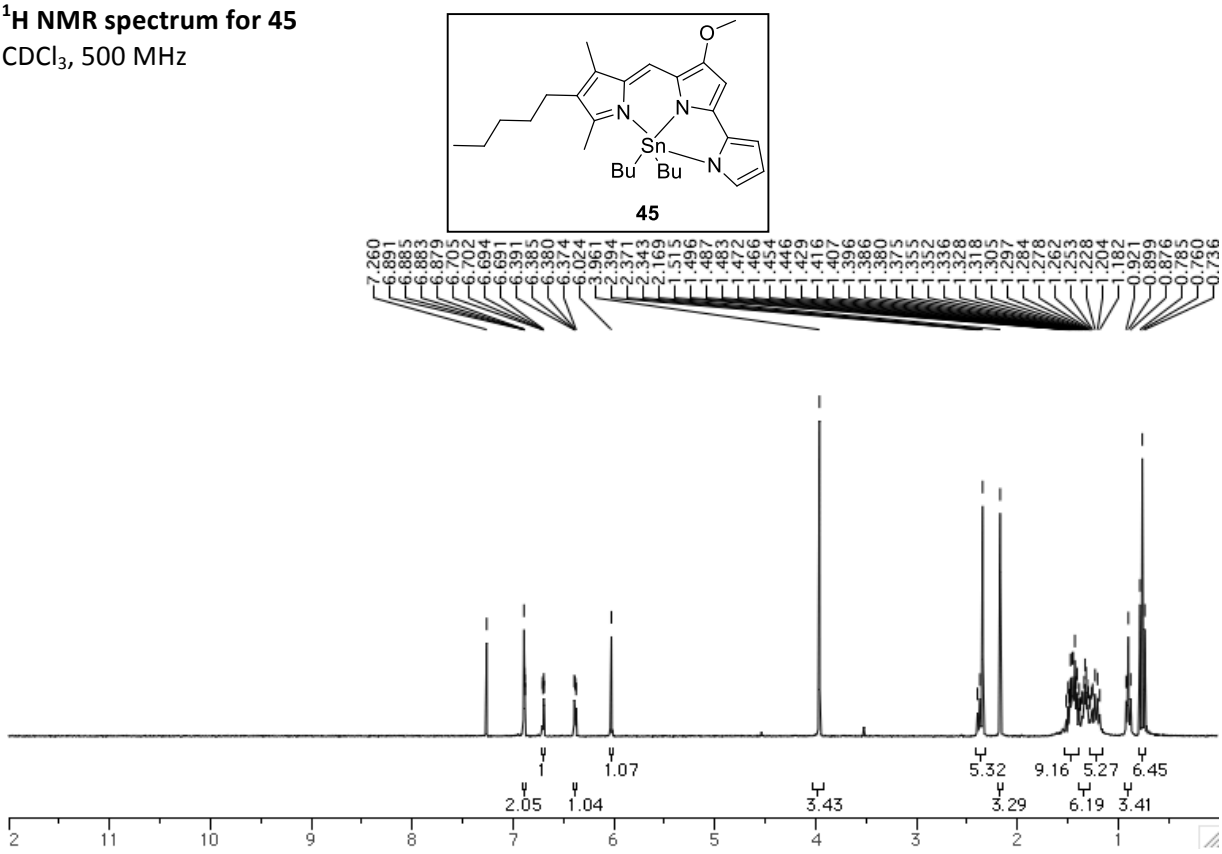


SI: Synthesis and Antimalarial Activity of Prodigiosenes

Marchal, Smithen, Uddin, Robertson, Jakeman, Mollard, Goodman, MacDougall, McFarland, McFadden and Thompson

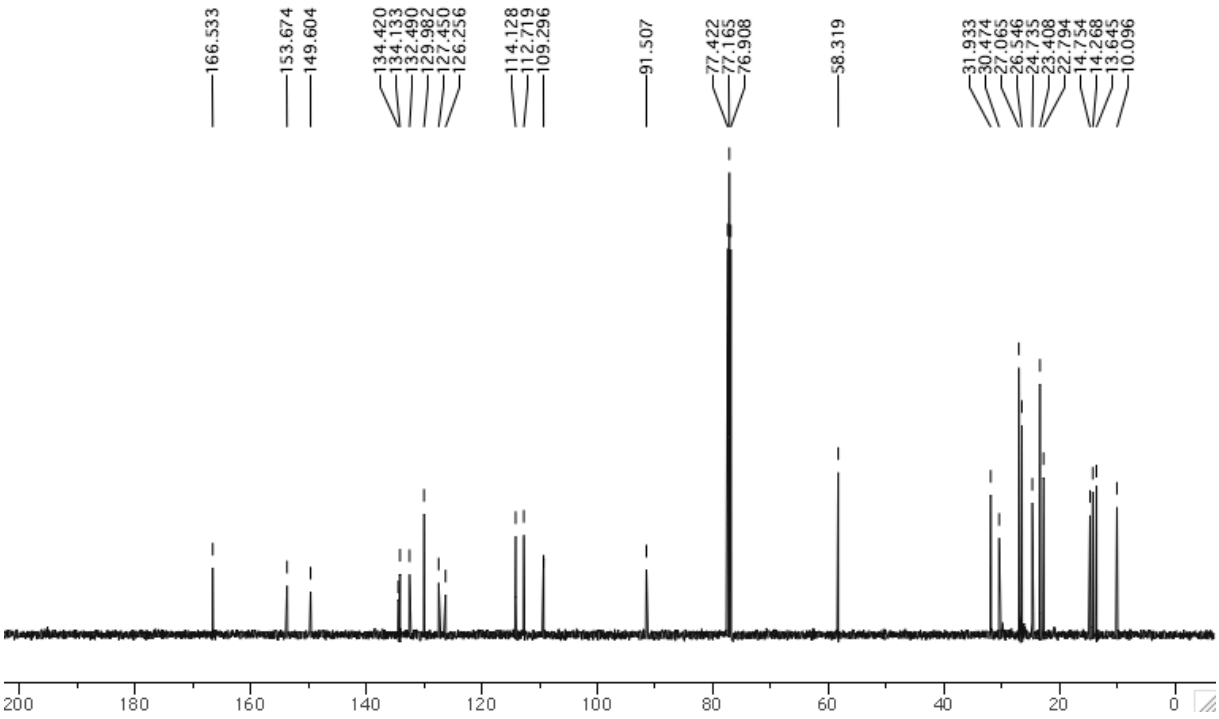
^{1}H NMR spectrum for 45

$CDCl_3$, 500 MHz



^{13}C NMR spectrum for 45

$CDCl_3$, 125 MHz

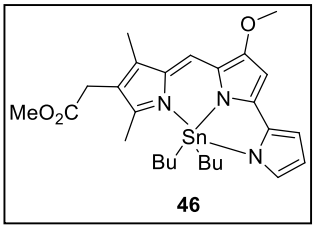


SI: Synthesis and Antimalarial Activity of Prodigiosenes

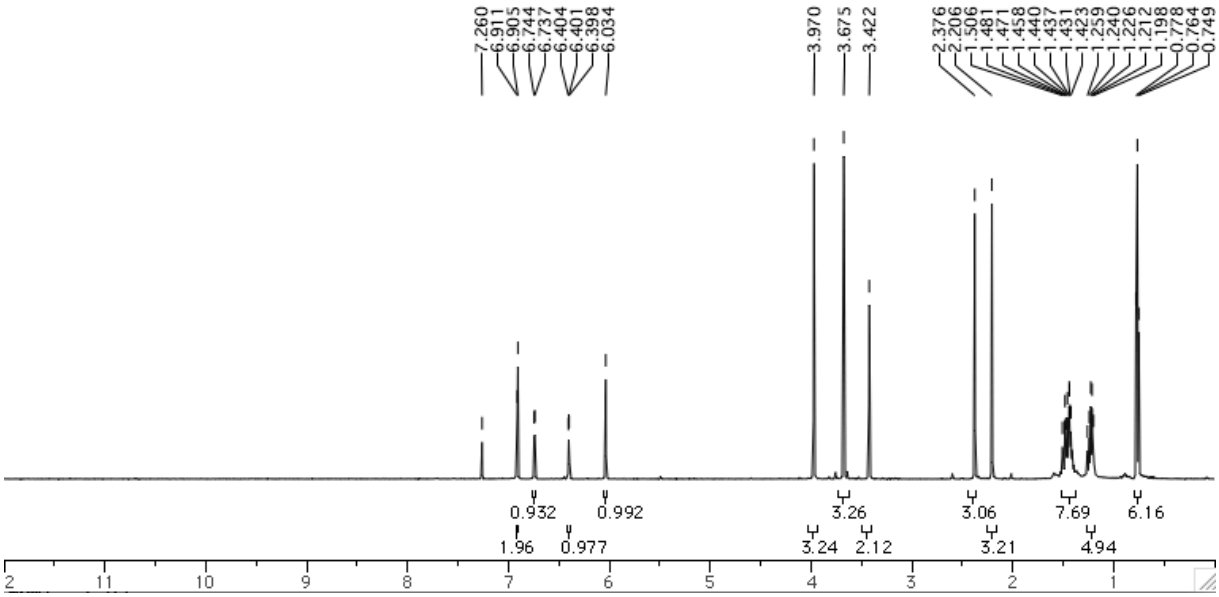
Marchal, Smithen, Uddin, Robertson, Jakeman, Mollard, Goodman, MacDougall, McFarland, McFadden and Thompson

^{1}H NMR spectrum for 46

$CDCl_3$, 500 MHz

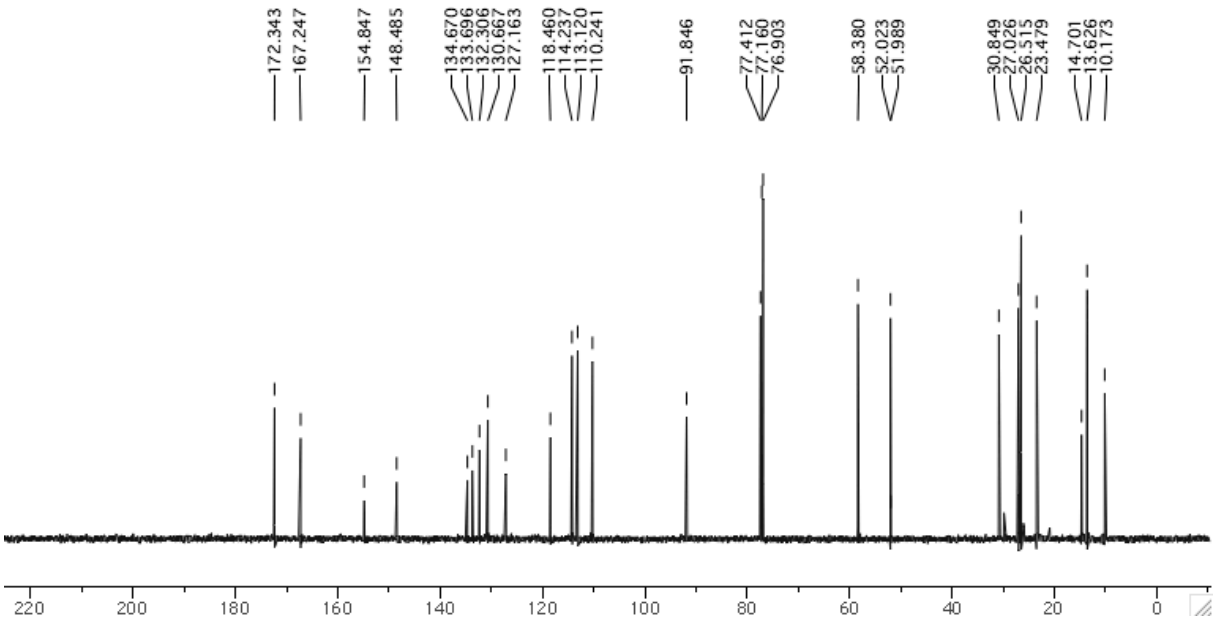


46



^{13}C NMR spectrum for 46

$CDCl_3$, 125 MHz

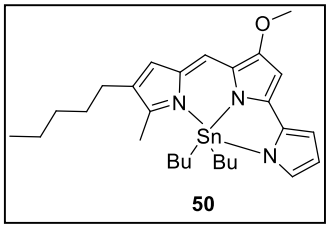


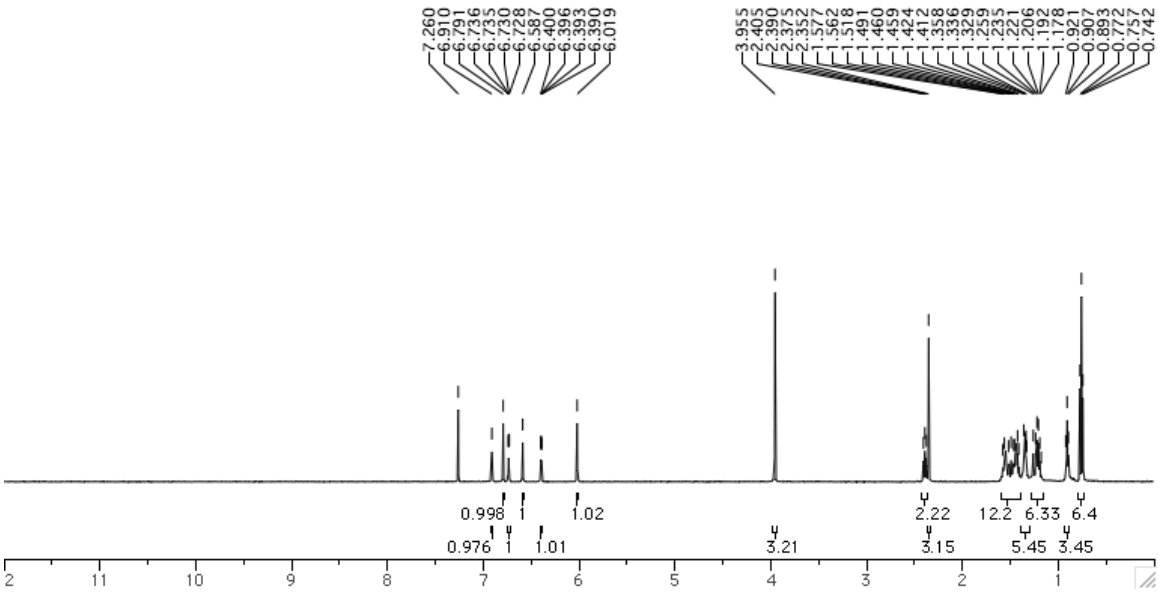
SI: Synthesis and Antimalarial Activity of Prodigiosenes

Marchal, Smithen, Uddin, Robertson, Jakeman, Mollard, Goodman, MacDougall, McFarland, McFadden and Thompson

^{1}H NMR spectrum for 50

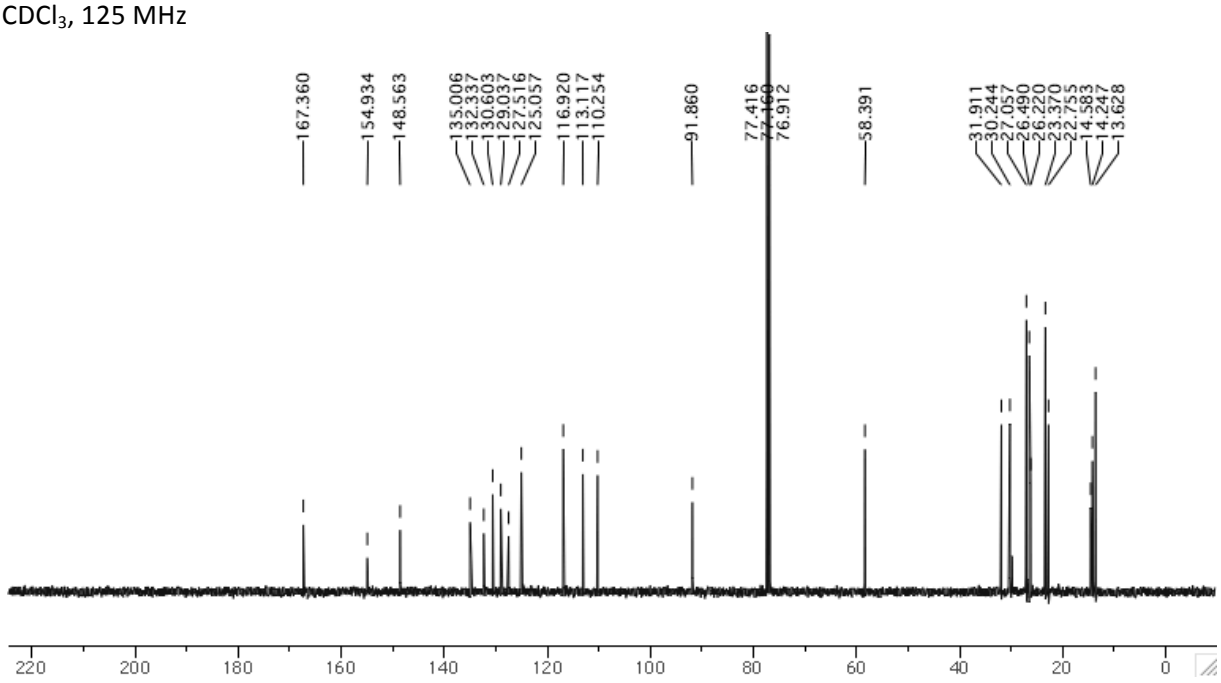
$CDCl_3$, 500 MHz





^{13}C NMR spectrum for 50

$CDCl_3$, 125 MHz

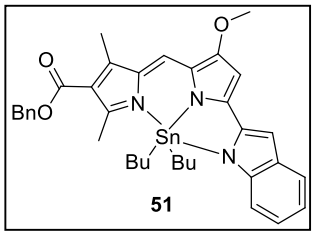


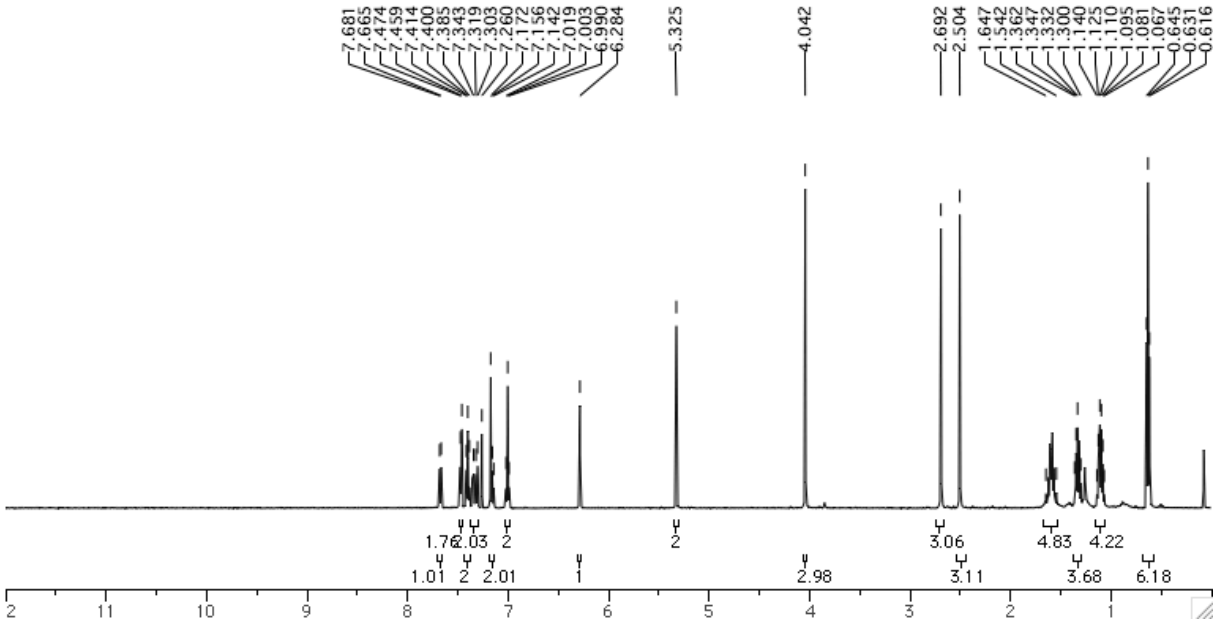
SI: Synthesis and Antimalarial Activity of Prodigiosenes

Marchal, Smithen, Uddin, Robertson, Jakeman, Mollard, Goodman, MacDougall, McFarland, McFadden and Thompson

^{1}H NMR spectrum for 51

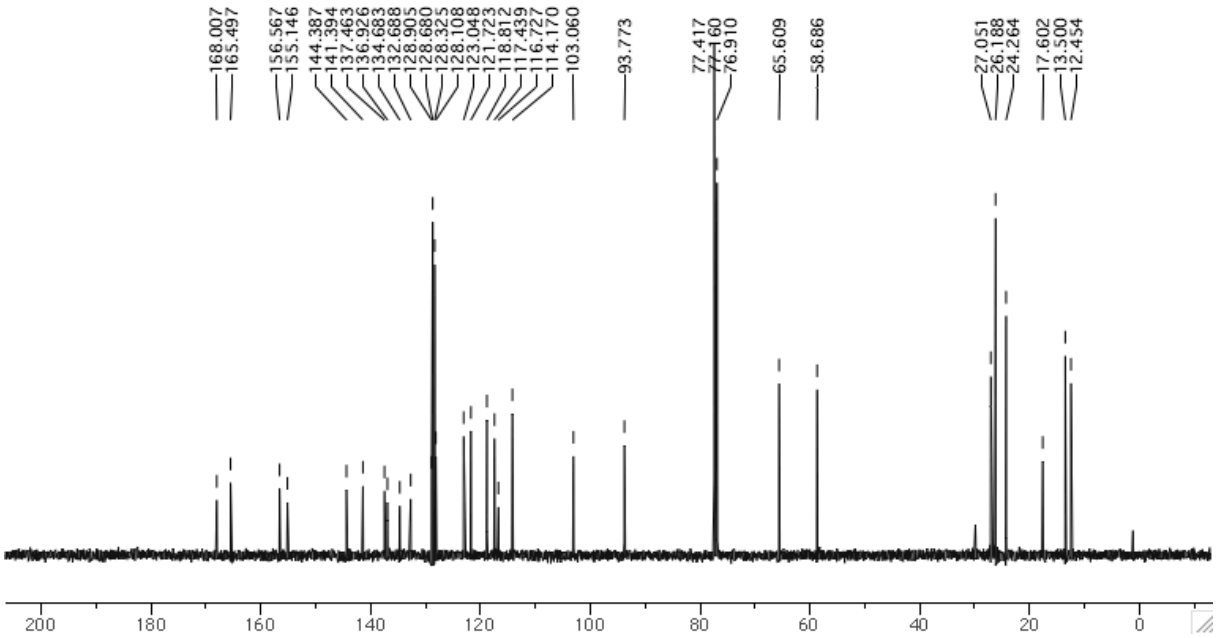
$CDCl_3$, 500 MHz





^{13}C NMR spectrum for 51

$CDCl_3$, 125 MHz

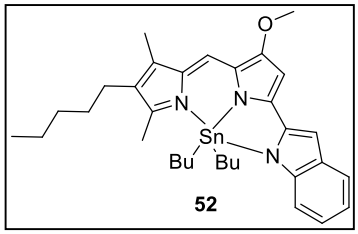


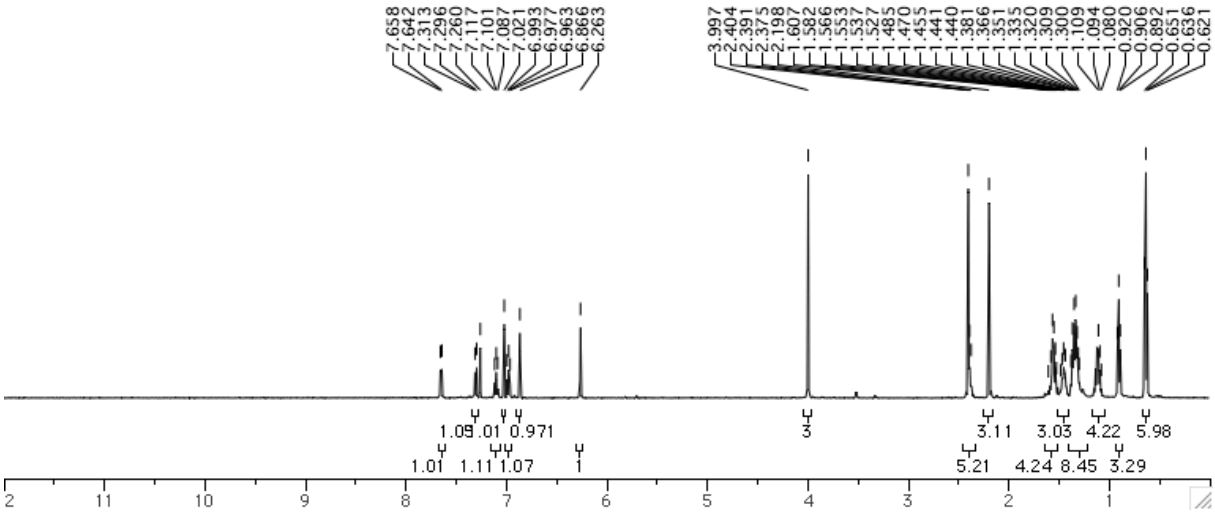
SI: Synthesis and Antimalarial Activity of Prodigiosenes

Marchal, Smithen, Uddin, Robertson, Jakeman, Mollard, Goodman, MacDougall, McFarland, McFadden and Thompson

^{1}H NMR spectrum for 52

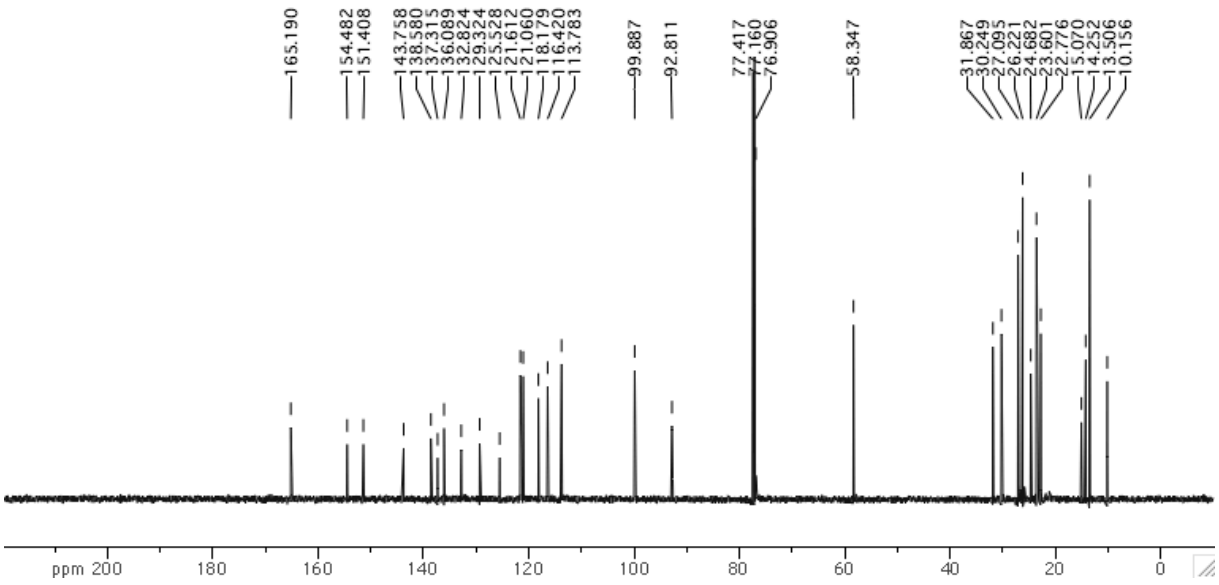
$CDCl_3$, 500 MHz



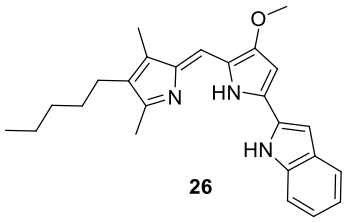


^{13}C NMR spectrum for 52

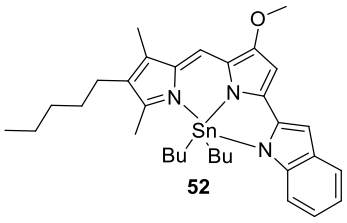
$CDCl_3$, 125 MHz



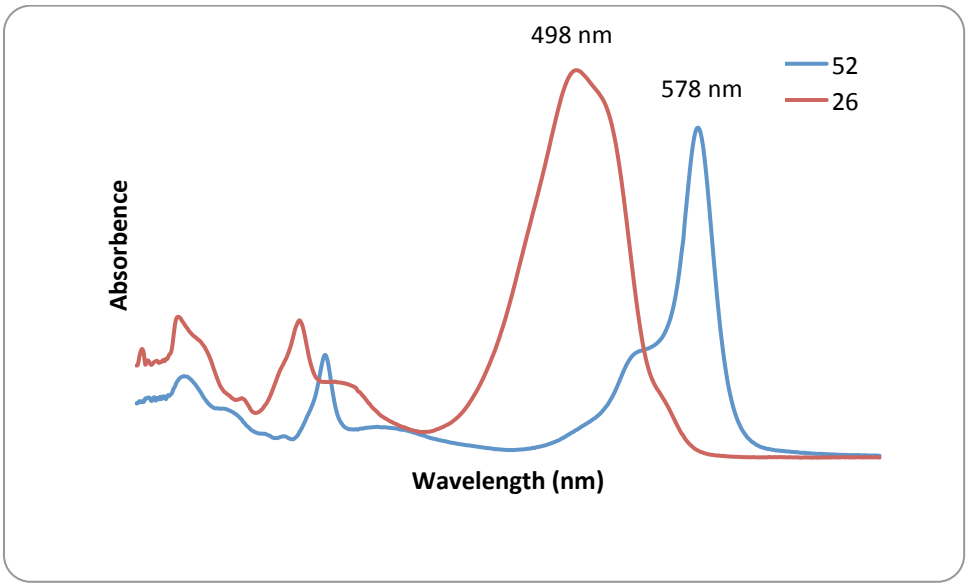
4) UV/Vis spectra for compounds 26 and 52



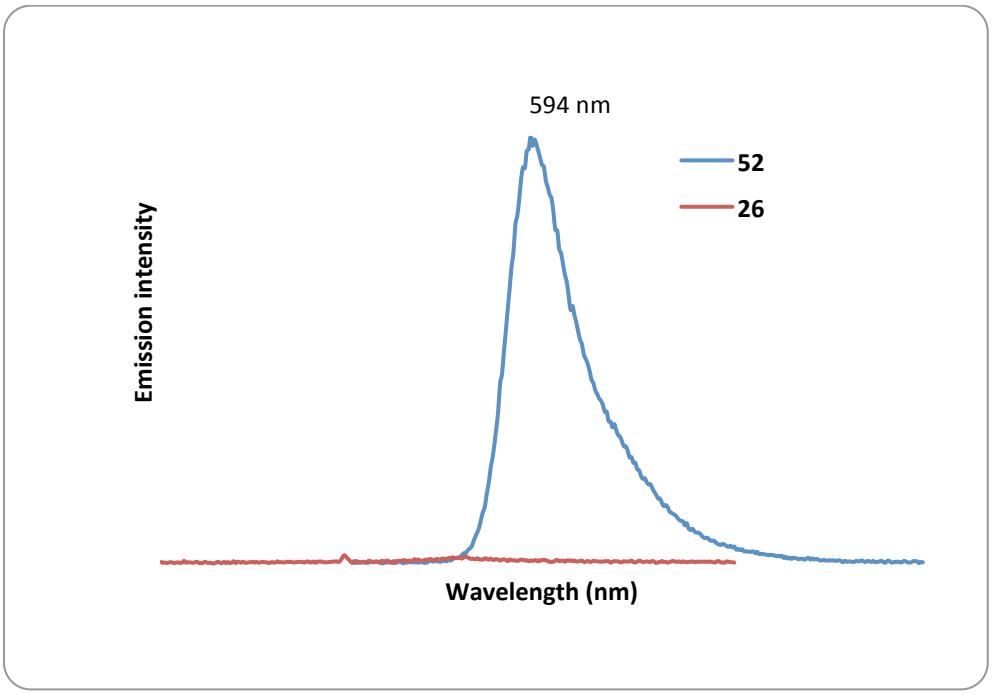
26



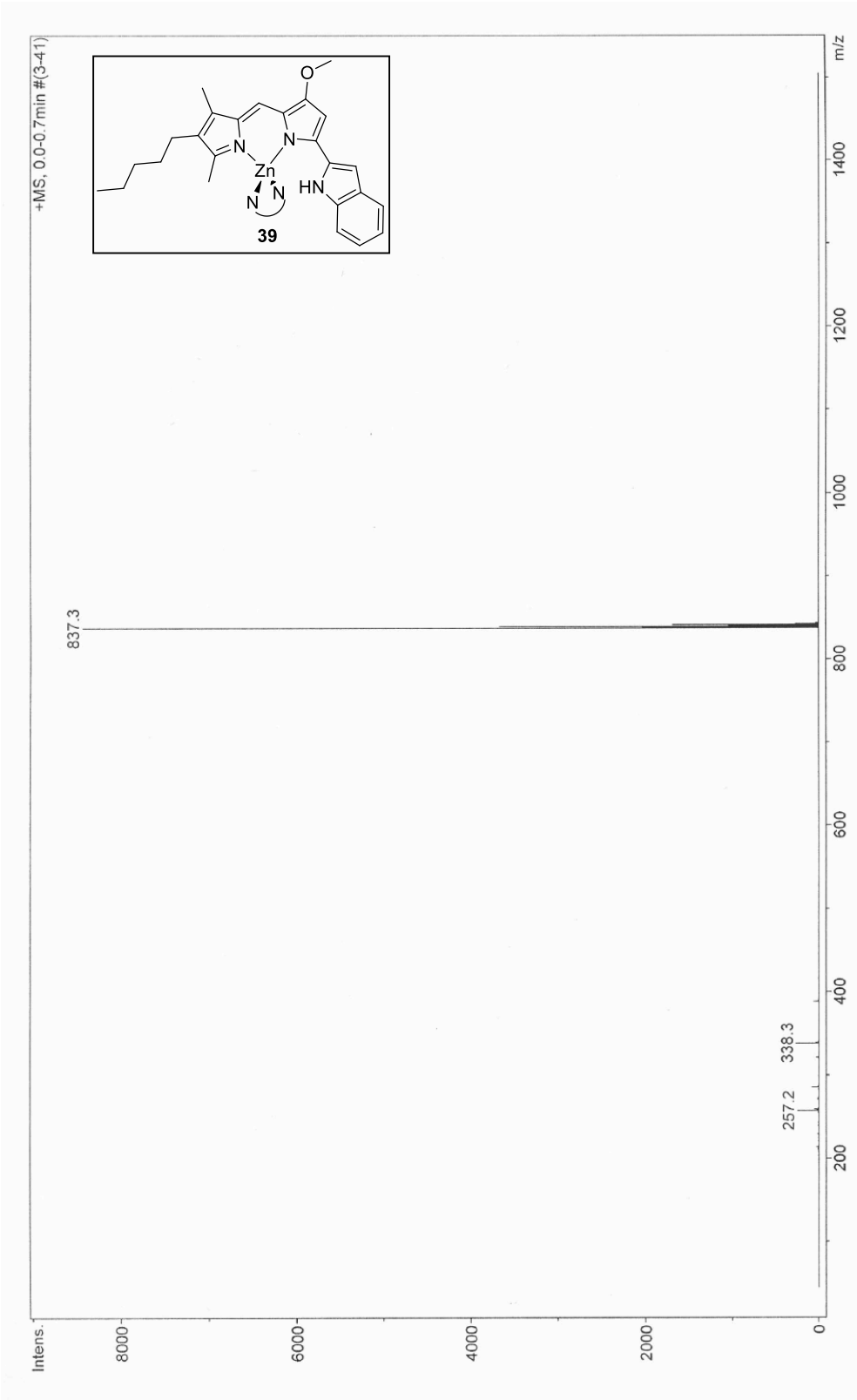
52

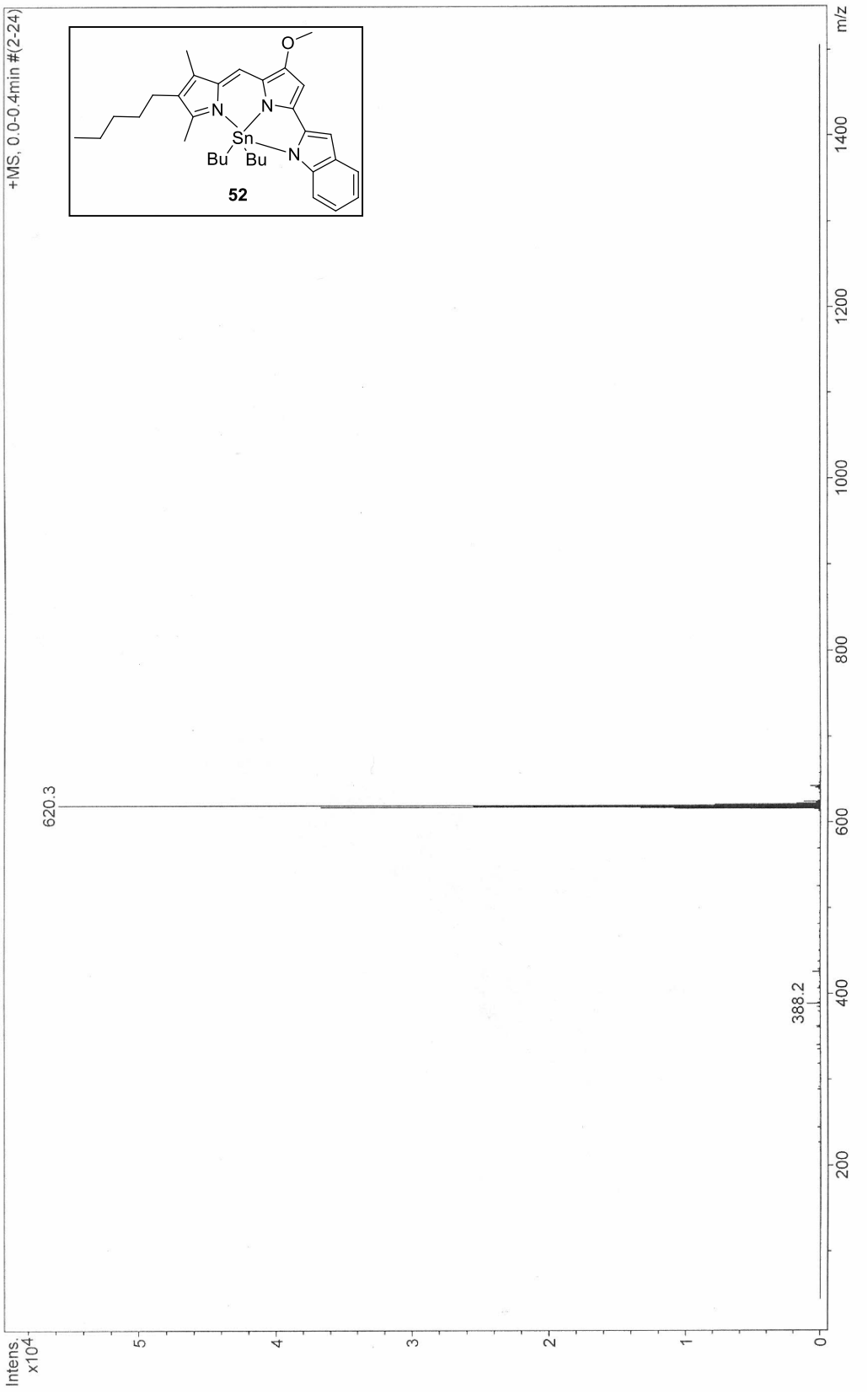


5) Fluorescence spectra for 26 and 52.



6) LRMS for compounds 39 and 52




+MS, 0.0-0.4min #(2-24)
O
N
N
Sn
Bu
Bu
N
52
620.3
388.2
m/z
1400
1200
1000
800
600
400
200
Intens.
x10^4
5
4
3
2
1
0

7) LogP values for 16 and 47

16 logP = 4.3,

48 logP = 6.7,

logP = partition coefficient between water and octanol at pH7, obtained using the online software vcclab: http://www.vcclab.org/).

8) DNA binding experiments using UV-VIS Spectroscopy

CT-DNA Titrations

Assessment of DNA binding using UV-VIS spectrometry was carried out for compounds **17**, **20** and **22** in 1.4 mL quartz cuvettes. Two blanks were prepared by adding in 293.6 µL of 109 mM MOPS, and 506.4 µL dI H_2O for a final concentration of 40 mM. The blanks were placed in the UV-VIS spectrophotometer and a baseline was collected. One of the blanks was removed from the sample compartment, and the appropriate amount of MOPS solution was pipetted out of the cuvette and the same volume of prodigiosene was added to achieve the desired concentration. The prodigiosenes were then titrated with CT DNA (12.1 mM base pairs) in 1 µL increments, with absorption spectra collected after each addition. Lyophilized CT-DNA (5 mg) was prepared for use by adding 1 mL of 40 mM MOPS and sonicated to shear the DNA into ~200 base pair fragments.

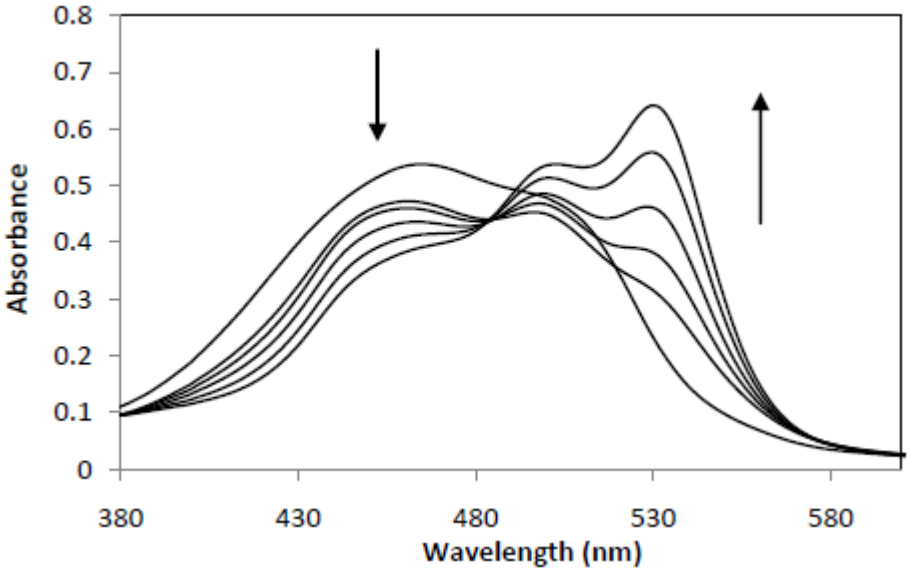


Figure S1: UV-VIS spectrum of CT-DNA (0-75 µM) titration of compound **17** (50 µM). Titration was carried out in 40 mM MOPS (pH 7.4). Arrows indicate changes in absorption.

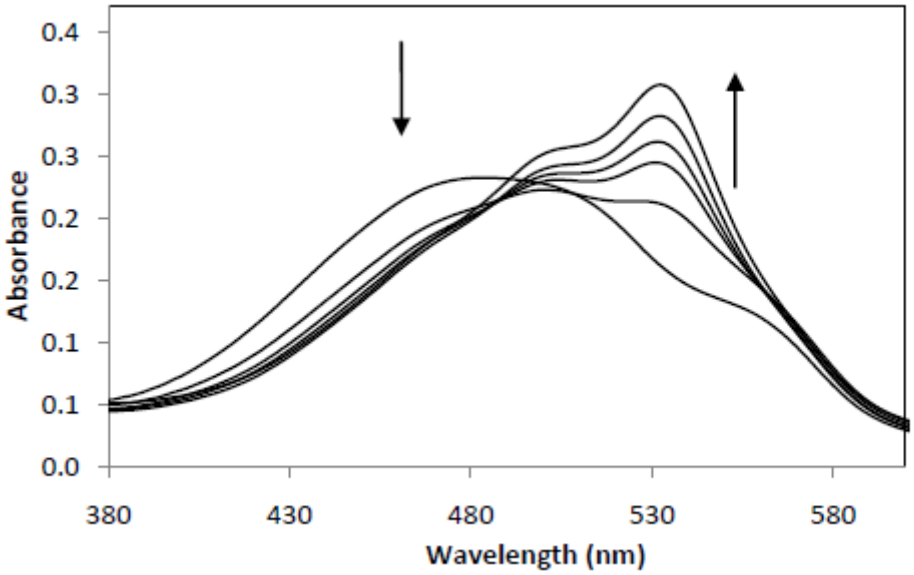


Figure S2: UV-VIS spectrum of CT-DNA (0-75 µM) titration of compound **20** (50 µM). Titration was carried out in 40 mM MOPS (pH 7.4). Arrows indicate changes in absorption.

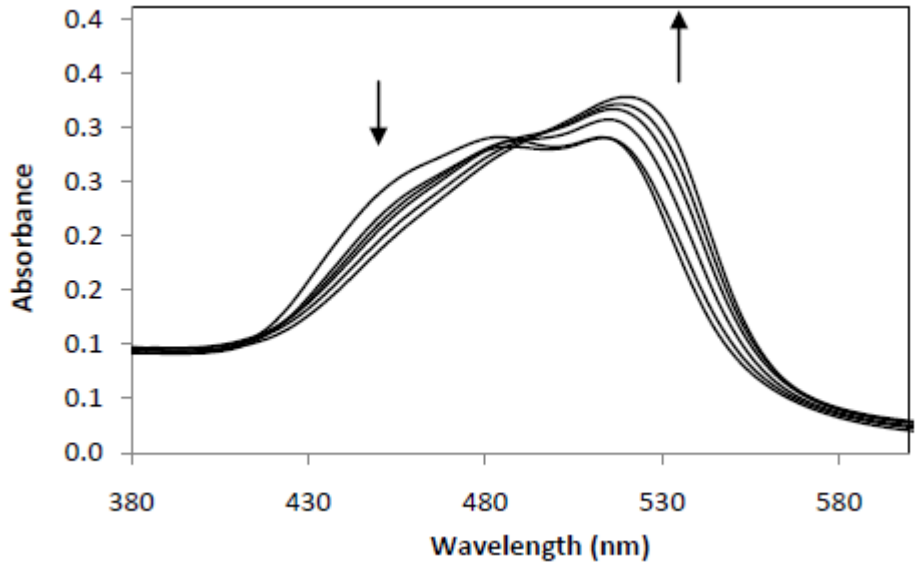


Figure S3: UV-VIS spectrum of CT-DNA (0-75 µM) titration of compound **22** (50 µM). Titration was carried out in 40 mM MOPS (pH 7.4). Arrows indicate changes in absorption.

The lack of distinct isosbestic points in the spectra indicates that multiple equilibria exist. Hypochromic and bathochromic shifts in the UV-VIS spectrum have been known to indicate strong DNA interactions, often via intercalation between base pairs or groove binding.[5] Such behaviour is exhibited for compounds **17**, **20** and **22** as indicated by the hypochromic shifts at around 460-500 nm after the addition of small amounts of CT-DNA.

Formation of the peak at approximately 530 nm suggests that protonation of the C-ring nitrogen may be facilitated by DNA binding. Protonated prodigiosin appears red in colour and absorbs around 530 nm, while the free base appears yellow in colour and absorbs

around 460 nm.[6] Because the minor-groove environment of DNA is more acidic than its surroundings, as much as two pH units lower than the bulk solvent,[7, 8] it is likely that binding or intercalation of the prodigiosene (pKa value of C-ring ester prodigiosene ≈ 6.5) takes place from the minor groove.

DNA Binding by Melt Curves

DNA binding studies were carried out for compounds **17**, **20** and **22** using a Jasco UV-Vis spectrometer equipped with an ETC-505T Peltier temperature controller. A stream of N_2 gas was introduced in the sample chamber to prevent condensation. Prodigiosenes (2 µL of 5mM **17**, **20** and **2**) were added to CT-DNA (3.97 µL of 12.1 mM base pairs) and topped up to 2 mL with 10 mM MOPS (pH 7.5). Absorption at 260 nm was recorded over a temperature range of 20-100 °C. The first-derivative of the curve was plotted in *Logger Pro 3.6.0*. and the melting temperature (T_m) was determined from the maximum of the first-derivative plot.

Table S1: DNA melting temperature studies.

Compound	T_m (°C)	ΔT_m (°C)
CT-DNA	59.6	
20	60.5	0.9
CT-DNA	55.6	
17	61.0	5.4
22	58.0	2.4

All of the prodigiosenes tested showed a shift in melting temperature indicating that all compounds are interacting with the DNA, most likely by intercalation or groove binding.

9) References

1. W. Trager and J. Jensen, *Science*, 1976, **193**, 673-675.
2. C. D. Goodman, V. Su and G. I. McFadden, *Mol. Biochem. Parasitol.*, 2007, **152**, 181-191.
3. T. Louie, C. D. Goodman, G. A. Holloway, G. I. McFadden, V. Mollard and K. G. Watson, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 4611-4613.
4. M. Smilkstein, N. Sriwilaijaroen, J. X. Kelly, P. Wilairat and M. Riscoe, *Antimicrob. Agents Ch.*, 2004, **48**, 1803-1806.
5. M. S. Melvin, D. C. Ferguson, N. Lindquist and R. A. Manderville, *J. Org. Chem.*, 1999, **64**, 6861-6869.
6. J. Q. H. La, A. A. Michaelides and R. A. Manderville, *J. Phys. Chem. B*, 2007, **111**, 11803-11811.
7. B. Nguyen, J. Stanek and W. D. Wilson, *Biophys. J.*, 2006, **90**, 1319-1328.
8. G. Lamm and G. R. Pack, *P. Natl. Acad. Sci.*, 1990, **87**, 9033-9036.