A change in metal cation switches selectivity of a phospholipid sensor from phosphatidic acid to phosphatidylserine

Supplementary Information

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Table of Contents

Supplementary Figures

Figure S1

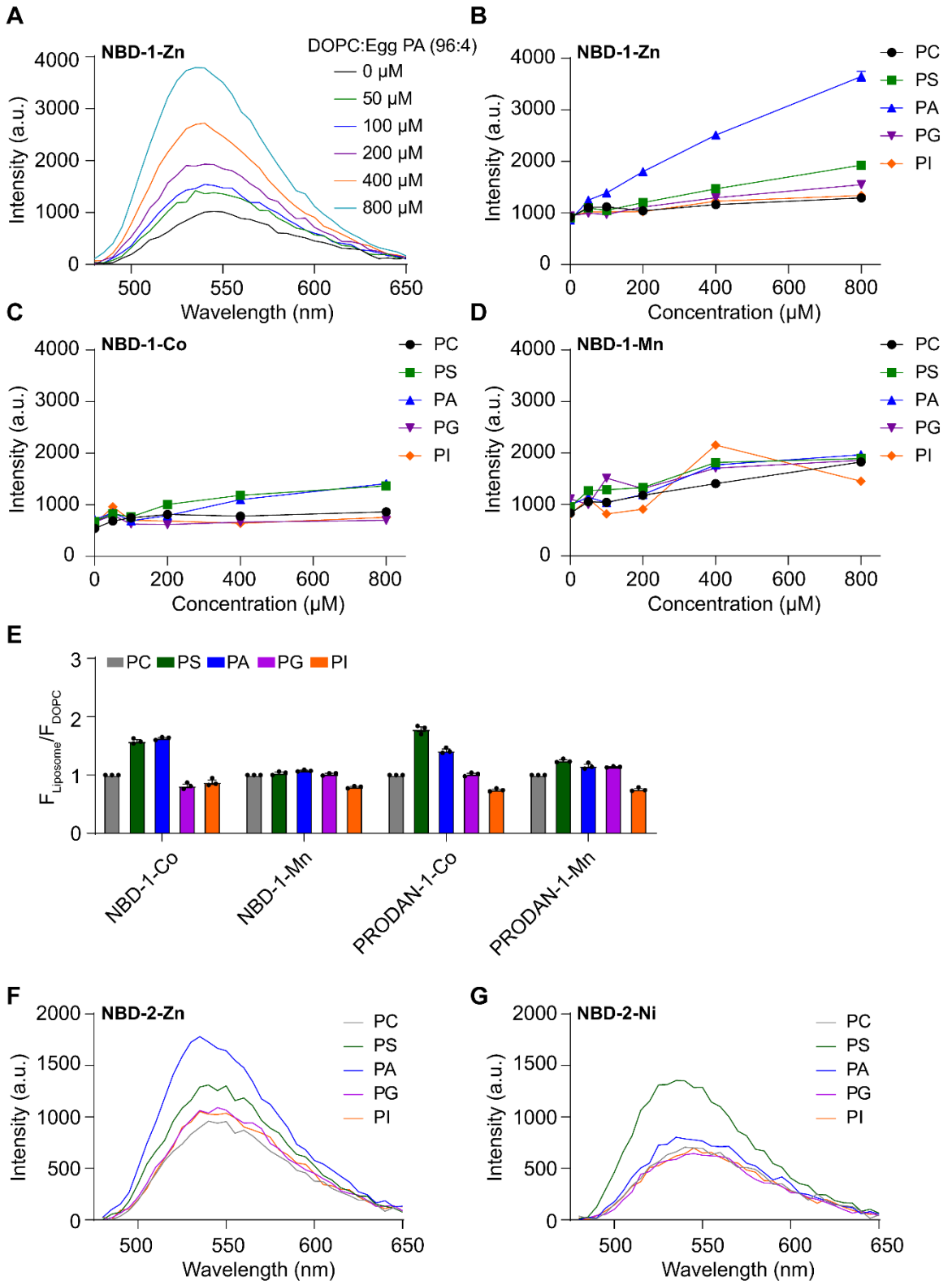


Figure S1—(A) Representative emission spectra of **NBD-1-Zn** (8 μM) in the presence of liposomes containing 96% PC and 4% PA with increasing liposome concentrations (0 μM, 50 μM, 100 μM, 200 μM, 400 μM, and 800 μM), 50 mM HEPES, 120 mM KOAc, pH 7.5, λ_{ex}= 460 nm; NBD fluorescence intensity measured at 530 nm for different membrane compositions (100% PC vs. 96% PC and 4% indicated phospholipids) as a function of total lipid concentration for: **(B) NBD-1-Zn**; **(C) NBD-1-Co**; **(D) NBD-1-Mn** [mean ± SEM, n=3 independent experiments for each condition]. Fluorescence intensity measured at 530 nm; **(E)** Comparison of fluorescence responses of metal complexes (Co(II) and Mn(II)) of sensors **NBD-1** and **PRODAN-1** to 800 μM of liposomes containing 4% of indicated phospholipids and 96% PC. Fluorescence intensity measured at 530 nm for NBD compounds and at 500 nm for PRODAN compounds. Data were normalised to the response obtained from 100% PC liposomes [mean ± SEM, n=3 independent experiments for each condition]; Representative emission spectra of: **(F) NBD-2-Zn** (8 μM) and **(G) NBD-2-Ni** (8 μM) in the presence of liposomes (800 μM) containing 100% PC or 96% PC and 4% of the indicated phospholipids (50 mM HEPES, 120 mM KOAc, pH 7.5).

Figure S2

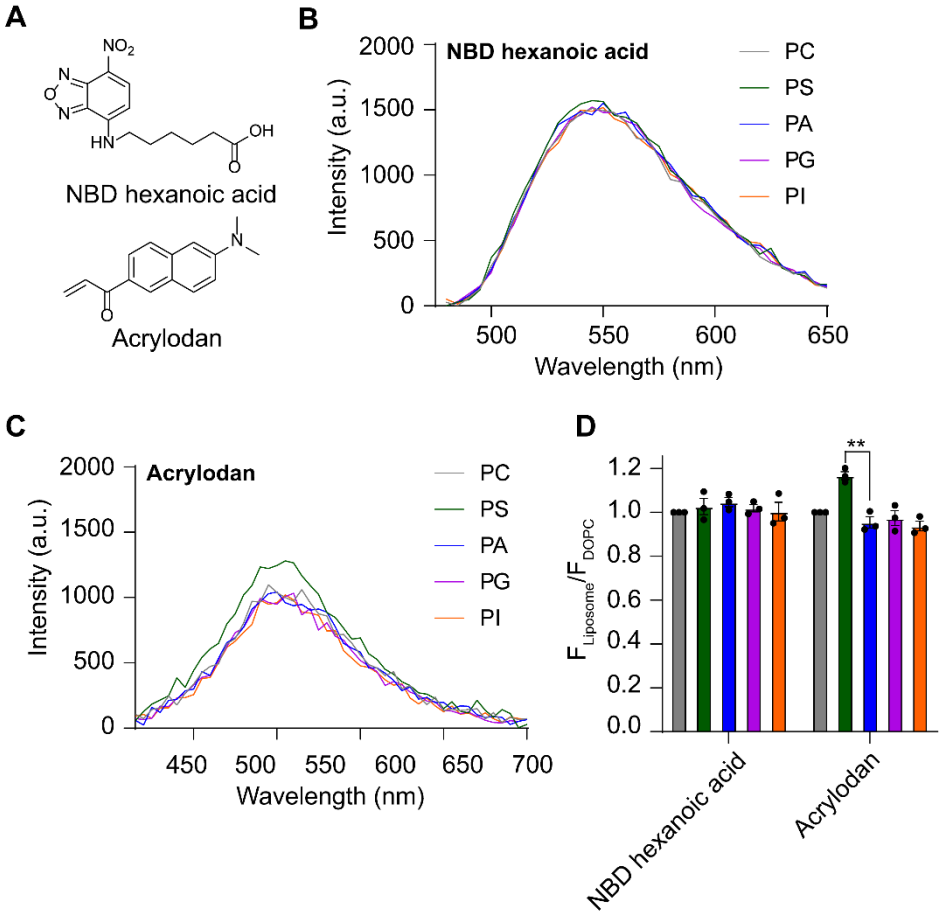


Figure S2—(A) Structures of NBD hexanoic acid and acrylodan. Representative emission spectra of: **(B)** NBD hexanoic acid (8 µM, λ_{ex} = 460 nm); and **(C)** acrylodan (8 µM, λ_{ex}= 392 nm) in the presence of liposomes (800 µM) containing 100% PC, or 96% PC and 4% indicated phospholipids (50 mM HEPES, 120 mM KOAc, pH 7.5); **(D)** Comparison of fluorescence responses of fluorophores to 800 µM of liposomes containing 4% of indicated phospholipids and 96% PC. Fluorescence intensity measured at 530 nm for NBD hexanoic acid and at 500 nm for acrylodan. Data were normalised to the response obtained from 100% PC liposomes [mean ± SEM, n=3 independent experiments for each condition; two-tailed unpaired Student's t-test, **P = 0.0024].

Figure S3

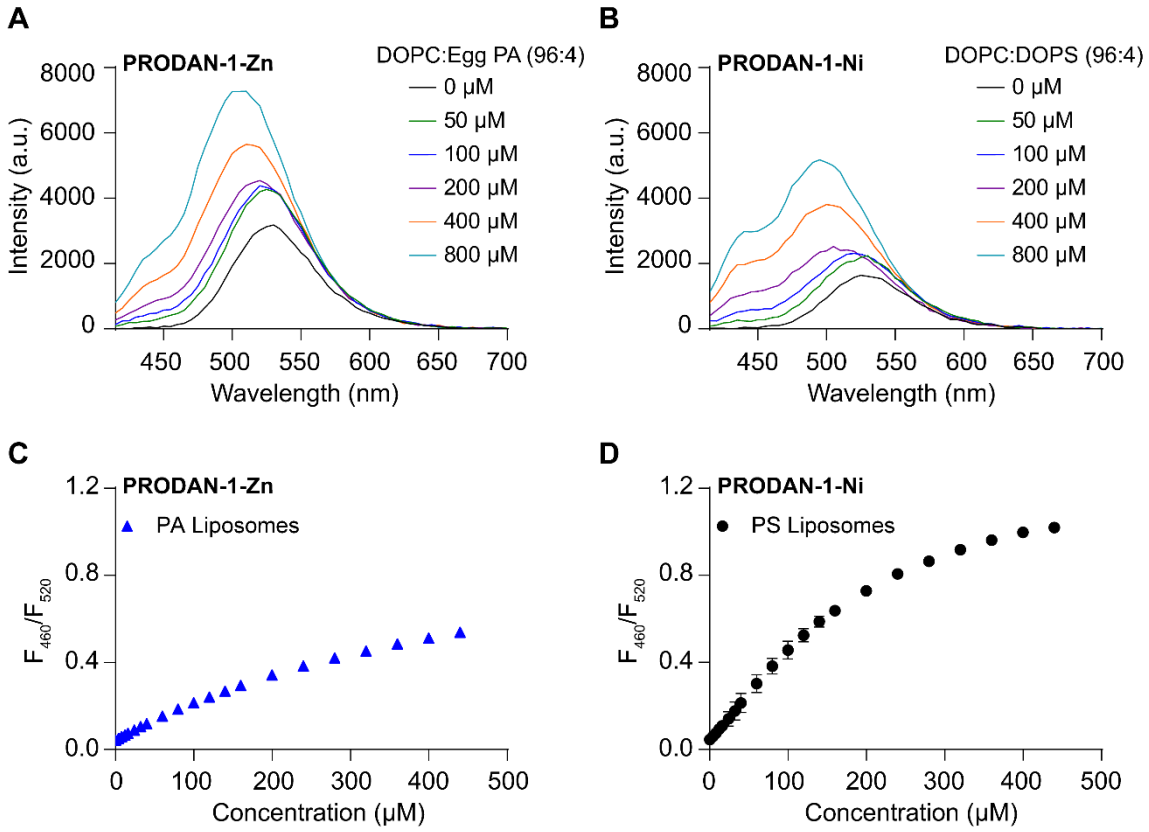


Figure S3—Representative emission spectra of: **(A) PRODAN-1-Zn** (8 µM) and **(B) PRODAN-1-Ni** (8 µM) in the presence of liposomes containing 96% PC and 4% PA with increasing liposome concentrations (0 µM, 50 µM, 100 µM, 200 µM, 400 µM, and 800 µM), 50 mM HEPES, 120 mM KOAc, pH 7.5, λ_{ex}= 392 nm. The plot of the ratio of emission wavelengths at 460 nm and 520 nm (F_{460}/F_{520}) versus liposome concentrations for **(C) PRODAN-1-Zn** and **(D) PRODAN-1-Ni** [mean ± SD, n=2 independent experiments for each condition.

Figure S4

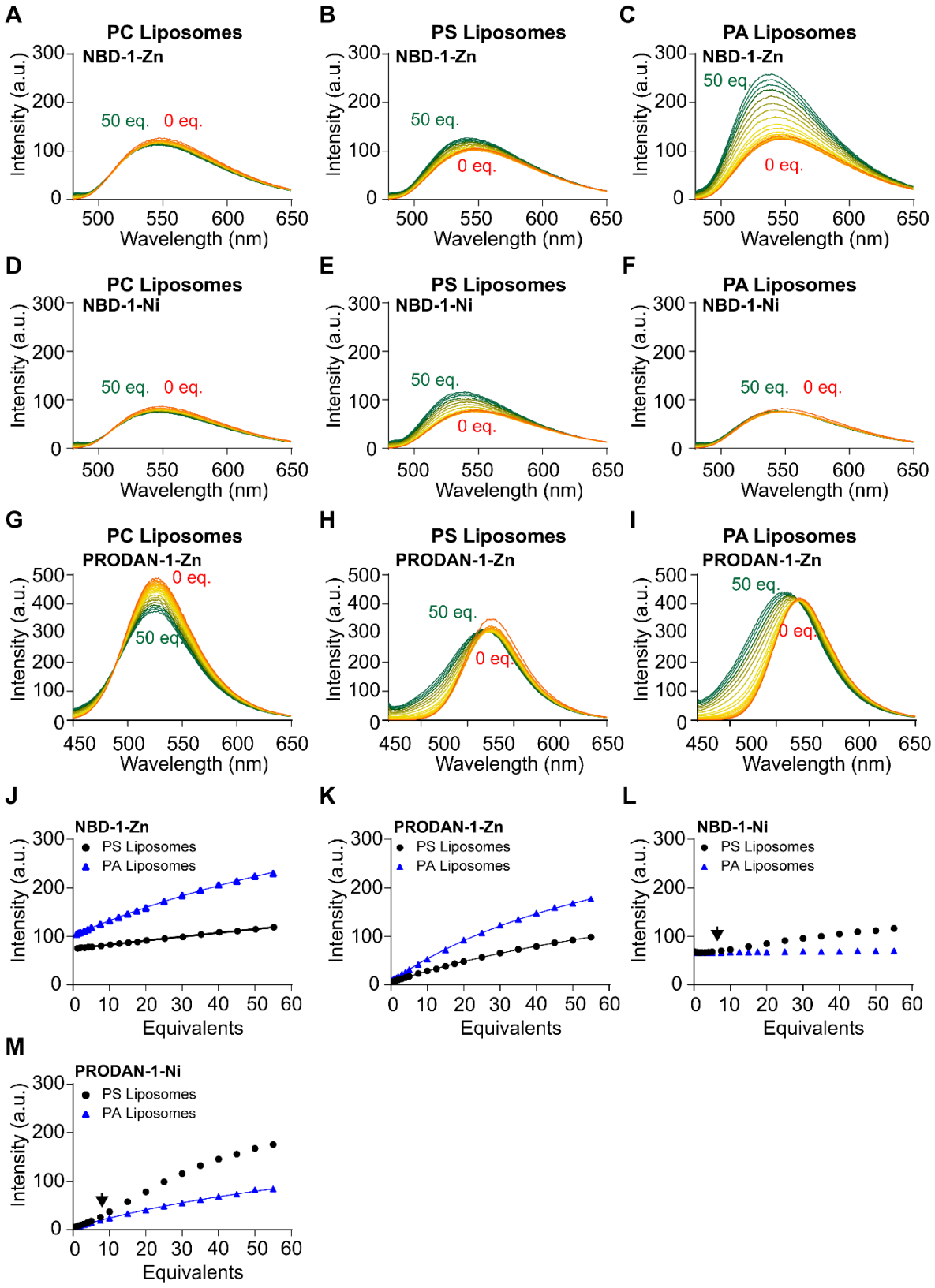

A
PC Liposomes
NBD-1-Zn
50 eq.
0 eq.
Intensity (a.u.)
Wavelength (nm)
B
PS Liposomes
NBD-1-Zn
50 eq.
0 eq.
C
PA Liposomes
NBD-1-Zn
50 eq.
0 eq.
D
PC Liposomes
NBD-1-Ni
50 eq.
0 eq.
E
PS Liposomes
NBD-1-Ni
50 eq.
0 eq.
F
PA Liposomes
NBD-1-Ni
50 eq.
0 eq.
G
PC Liposomes
PRODAN-1-Zn
0 eq.
50 eq.
H
PS Liposomes
PRODAN-1-Zn
50 eq.
0 eq.
I
PA Liposomes
PRODAN-1-Zn
50 eq.
0 eq.
J
NBD-1-Zn
PS Liposomes
PA Liposomes
Equivalents
K
PRODAN-1-Zn
PS Liposomes
PA Liposomes
Equivalents
L
NBD-1-Ni
PS Liposomes
PA Liposomes
Equivalents
M
PRODAN-1-Ni
PS Liposomes
PA Liposomes
Equivalents

Figure S4—A-C: Fluorescence titrations of **NBD-1-Zn** (in 50 mM HEPES, 120 mM KOAc buffer, pH 7.5) with increasing concentrations of liposomes containing: **(A)** (100) DOPC; **(B)** (4:96) DOPS:DOPC; and **(C)** (4:96) Egg PA:DOPC; λ_{ex} = 460 nm; **D-F** Fluorescence titrations of **NBD-1-Ni** (in 50 mM HEPES, 120 mM KOAc buffer, pH 7.5) with increasing concentrations of liposomes containing **(D)** (100) DOPC; **(E)** (4:96) DOPS:DOPC; and **(F)** (4:96) Egg PA:DOPC; λ_{ex} = 392 nm. **G-I:** Fluorescence titrations of **PRODAN-1-Zn** (in 50 mM HEPES, 120 mM KOAc buffer, pH 7.5) with increasing concentrations of liposomes containing **(G)** (100) DOPC; **(H)** (4:96) DOPS:DOPC; and **(I)** (4:96) Egg PA:DOPC; λex = 392 nm. **J-M:** Representative binding isotherms indicating change in fluorescence intensity of: **(J) NBD-1-Zn**; **(K) PRODAN-1-Zn**; **(L) NBD-1-Ni**; **(M) PRODAN-1-Ni** in response to increasing concentrations of PA- and PS-containing liposomes. Experimental data points are indicated by symbols, and the calculated binding model by lines (Bindfit). Arrows show points of inflection in data. NBD compounds were analysed at 530 nm, and PRODAN compounds at 450 nm.

Materials and Methods

Lipids

DOPC (850375), DOPS (840035), Egg PA (840101), Egg PG (841138), Soy PI (840044) were purchased from Avanti Polar Lipids.

Liposome preparation

Liposomes were prepared as previously described with slight modifications.[1] Briefly, lipids in chloroform were dried under a stream of N_2 gas, followed by further drying in the vacuum for 2 h. The dried lipid films were hydrated with HK buffer (50 mM HEPES, 120 mM potassium acetate, pH 7.5). Liposomes were then formed by five freeze-thaw cycles (liquid N_2 and 37°C water bath) followed by 11 times extrusion using Avanti Mini-Extruder with a pore size of 100 nm.

Liposome binding studies

The sensors (8 µM) were mixed with indicated liposomes in concentrations 0 µM, 50 µM, 100 µM, 200 µM, 400 µM, and 800 µM followed by NBD and PRODAN excitation at λ_{ex} = 460 nm and λ_{ex} = 392 nm, respectively. The fluorescent maxima at 530 nm (for NBD) and at 500 nm (for PRODAN) were plotted as a function of liposome concentration in Figure 2B and Figures S1B, S1C, and S1D.

Compound characterisation

Nuclear magnetic resonance (NMR) spectra were recorded at 300 K using either a 600 Bruker Avance (equipped with a high resolution cryogenic triple nucleus probehead), 500 Bruker Avance DPX 400 or a Bruker Avance 300 spectrometer. NMR spectra were calibrated to the residual proton solvent peak in $CDCl_3$ (δ 7.26 ppm), MeOD-d_4 (δ = 3.31 ppm) or DMSO-d_6 (δ = 2.50 ppm) at 300 K. ^{1}H NMR and ^{13}C NMR spectra were recorded at the indicated frequencies. Chemical shifts are expressed as parts per million (ppm) and are referenced to solvent residual signals. The data are reported as chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant *J* in Hz and relative integral. High resolution mass spectra were recorded on a Bruker Apex II Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometer with a 7.0 T magnet, fitted with an off-axis Analytic electrospray source with quadrupole mass analyser, and are reported as *m/z* (relative intensity).

Determination of binding affinities

To a 1 cm quartz glass cuvette was added a solution of the sensor (2.5 mL, 8 μM) and the fluorescence spectrum was recorded. Aliquots of a stock solution containing the sensor (8 μM) and the indicated liposomes (1840 μM lipid concentration) were then added to the cuvette. After each addition, the solution was mixed by stirring before the next fluorescence spectrum was taken. (For NBD, λ_{ex} = 460 nm, λ_{em} = 480−650 nm. For PRODAN, λ_{ex} = 392 nm, λ_{em} = 415-650 nm). Binding affinities were determined through analysis with the Bindfit web app.[2, 3] Data were fit by inputting the fluorescence intensities observed across a range of liposome concentrations, in the regions 470 – 500 nm (**PRODAN-1**) and 520 – 580 nm (**NBD-1**). The raw data and results of fitting may be accessed through the URLs in the table below.

Sensor	Target phospholipid	Replicate	URL
NBD-1-Zn	PA	1	http://app.supramolecular.org/bindfit/view/1d157e89-79a6-426d-9c1c-9deee1c85f3c
NBD-1-Zn	PA	2	http://app.supramolecular.org/bindfit/view/d8ef163a-f82b-4c18-81e0-2def32d2d862
NBD-1-Zn	PA	3	http://app.supramolecular.org/bindfit/view/cdce3ce1-dc08-4638-a453-72275d057c48
NBD-1-Zn	PS	1	http://app.supramolecular.org/bindfit/view/64c33a5b-c8f5-4db5-b391-94ff940f3636
NBD-1-Zn	PS	2	http://app.supramolecular.org/bindfit/view/e52667cb-f06f-49ff-bd77-3af35fcd47b7
NBD-1-Zn	PS	3	http://app.supramolecular.org/bindfit/view/bc03ddfb-3c69-44c2-adea-3040bd235e9e
NBD-1-Ni	PA	1	http://app.supramolecular.org/bindfit/view/3dcd97c0-70ce-4767-83b0-7777acb5ed2d
NBD-1-Ni	PS	1	http://app.supramolecular.org/bindfit/view/cffe51ea-9cea-4b17-9214-74cbaf664f61
NBD-1-Ni	PS	2	http://app.supramolecular.org/bindfit/view/452217f5-c49b-442d-9f29-28e2a8c5642f
NBD-1-Ni	PS	3	http://app.supramolecular.org/bindfit/view/1296d4c0-2fd1-4c14-a0e7-f6bfb08b31fe
PRODAN-1-Zn	PA	1	http://app.supramolecular.org/bindfit/view/42f7b102-fa28-4a0e-affb-6e8f021ad367
PRODAN-1-Zn	PA	2	http://app.supramolecular.org/bindfit/view/a3c02d7d-c47e-4350-a0cd-8ed0e8d6ef9c
PRODAN-1-Zn	PA	3	http://app.supramolecular.org/bindfit/view/4169f80c-da3d-4c9b-83b6-589e0ca2ae72
PRODAN-1-Zn	PS	1	http://app.supramolecular.org/bindfit/view/d212daf5-5e19-4548-9385-d6e4eaf0edc9
PRODAN-1-Zn	PS	2	http://app.supramolecular.org/bindfit/view/7048420f-81b3-4a9d-9cf8-77d9641837d5

PRODAN-1-Zn	PS	3	http://app.supramolecular.org/bindfit/view/fe1b5387-3275-4e09-889e-0356e7ded4b2
PRODAN-1-Ni	PA	1	http://app.supramolecular.org/bindfit/view/7341390d-691e-40db-a9f4-ddb69888132c
PRODAN-1-Ni	PA	2	http://app.supramolecular.org/bindfit/view/98343455-836c-4136-8b8f-930988c2b602
PRODAN-1-Ni	PA	3	http://app.supramolecular.org/bindfit/view/749eaa96-bd36-49cf-9ba3-5c4b223906db
PRODAN-1-Ni	PS	1	http://app.supramolecular.org/bindfit/view/80cf5bbe-790b-46a7-b2ce-633be567f854
PRODAN-1-Ni	PS	2	http://app.supramolecular.org/bindfit/view/bedba209-7668-4688-9bc5-28df3fed6d6c
PRODAN-1-Ni	PS	3	http://app.supramolecular.org/bindfit/view/0be8ba63-ca74-4897-b499-1e21d78b9323

Synthesis

All chemicals and solvents were of reagent grade (>95%) and used as received unless otherwise noted. NBD hexanoic acid was prepared as previously described.[4] Sensor **NBD-1** was synthesised by solid phase peptide synthesis (SPPS) from commercially available Fmoc-protected amino acids and NBD hexanoic acid on Rink amide resin. The DPA group was introduced on-resin by reductive amination with pyridine-2-carboxaldehyde onto the free amine of a Dab side chain, following deprotection of an allyloxycarbamate protecting group. The peptide backbone of sensor **NBD-2** was synthesised on Rink amide resin by SPPS, with on-resin introduction of the DPA group, and solution phase attachment of the NBD fluorophore with NBD-Cl. Sensor **PRODAN-1** was similarly synthesised on Rink amide resin by SPPS, with on-resin introduction of the DPA group, and solution-phase conjugation of the PRODAN fluorophore using acrylodan. The completed peptides were complexed with each divalent metal utilizing exactly one equivalent of metal salt. Complexation was confirmed by HRMS and by $^{1}H/^{13}C$ NMR (Zn only).

Loading of Rink amide resin

Rink amide resin (loading 0.463 mmol/g) in a fritted syringe was swollen in DMF for 15 minutes, then washed with DMF (x3), CH_2Cl_2 (x3), and DMF (x3). The resin was treated with 20% piperidine (v/v) in DMF (2x 5 mins) to remove the Fmoc protecting group, and the resin subsequently washed with DMF (x3), CH_2Cl_2 (x3), and DMF (x1). A solution of Fmoc-protected amino acid, preactivated for 5 mins with PyBOP (2.5 eq) and DIPEA (8 eq.) in DMF (0.05 M) was drawn into the syringe, and the resulting suspension agitated at r.t. for 1 h. The solution was ousted, and the resin washed with DMF (x3), CH_2Cl_2 (x3), and DMF (x3).

***N*-terminal Fmoc deprotection**

The resin was treated with 20% piperidine (v/v) in DMF (2x 5 mins) to remove the Fmoc protecting group, and the resin subsequently washed with DMF (x3), CH_2Cl_2 (x3), and DMF (x1). The free amine was immediately coupled with the next amino acid.

Estimation of resin loading

The ousted Fmoc deprotection solution was diluted with fresh 20% piperidine (v/v) in DMF, to produce two solutions of differing concentration, such that the concentration of the fulvene-piperidine adduct for each was in the range of 25 – 75 μM. A sample of each of these solutions was transferred to a clean 1 cm quartz glass cuvette, and the absorbance measured at 301 nm, using fresh 20% piperidine (v/v) in DMF solution as a reference. The concentration of fulvene adduct in each dilution was calculated using $\varepsilon = 7800\ M^{-1}\ cm^{-1}$, and the final resin loading calculated from the average calculated values of the original deprotection solution.

Amino acid coupling

A solution of Fmoc-protected amino acid, preactivated for 5 mins with PyBOP (2.5 eq) and DIPEA (8 eq.) in DMF (0.05 M) was drawn into the syringe, and the resulting suspension agitated at r.t. for 1 h. The solution was ousted, and the resin washed with DMF (x3), CH_2Cl_2 (x3), and DMF (x3).

Alloc deprotection

The resin was swollen in CH_2Cl_2 for 15 mins, then agitated with a solution of $Pd(PPh_3)_4$ (0.8 eq) and phenylsilane (25 eq) in CH_2Cl_2 (0.05 M) for 1 h at r.t.. The syringe was vented several times to avoid excess pressure build up. The resin was washed with CH_2Cl_2 (x3) and agitated with $Pd(PPh_3)_4$/phenylsilane solution for a further 1 h. The resin was washed with CH_2Cl_2 (x3), sodium diethyldithiocarbamate (20 mg/mL) with 1% NEt_3 (v/v) in DMF (x10), 1% NEt_3 (v/v) in DMF (x10), DMF (x3) and CH_2Cl_2 (x3).

Reductive amination

A suspension of $Na(OAc)_3BH$ (25 eq) and 2-pyridinecarboxaldehyde (20 eq) in 1% AcOH (v/v) in DMF (0.05 M) or in 1% AcOH (v/v) in 1:1 CH_2Cl_2:DMF was added to the resin *via* the back of the syringe, and the resulting suspension agitated overnight at r.t.. The resin was washed with methanol (x10), DMF (x3), and CH_2Cl_2 (x3).

Cleavage of peptides from resin

The resin was treated with a solution of trifluoroacetic acid/H_2O/triisopropylsilane (95:2.5:2.5 v/v/v) (0.05 M) for 1 h at r.t.. The solution was ousted into a weighed centrifuge tube. The resin was treated with the same solution twice more (15 mins, 5 mins) and the solution collected again. Chilled diethyl ether was added to the combined ousted deprotection solutions, and the resulting suspension centrifuged (4000 rpm, 5 mins). The supernatant solution was decanted off, and pellet dried under vacuum to afford the crude peptide as the trifluoroacetate salt.

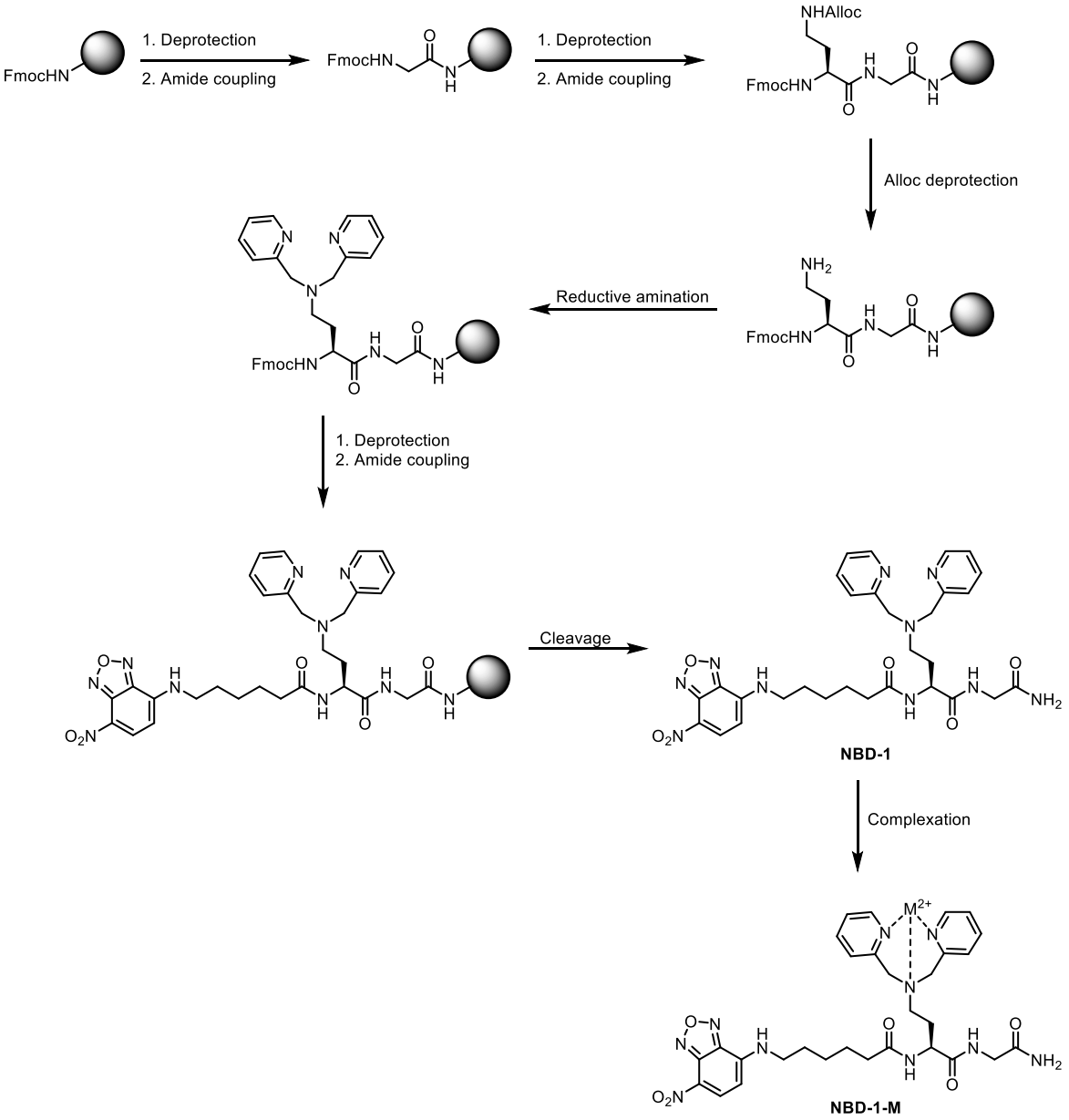
Purification

Peptides were purified by flash column chromatography on C8 silica, eluting with gradients of H_2O/MeCN (0.1% v/v of a 25% NH_4OH solution in H_2O was used as an additive). Fractions containing product were lyophilised, to afford the pure peptides as fluffy solids.

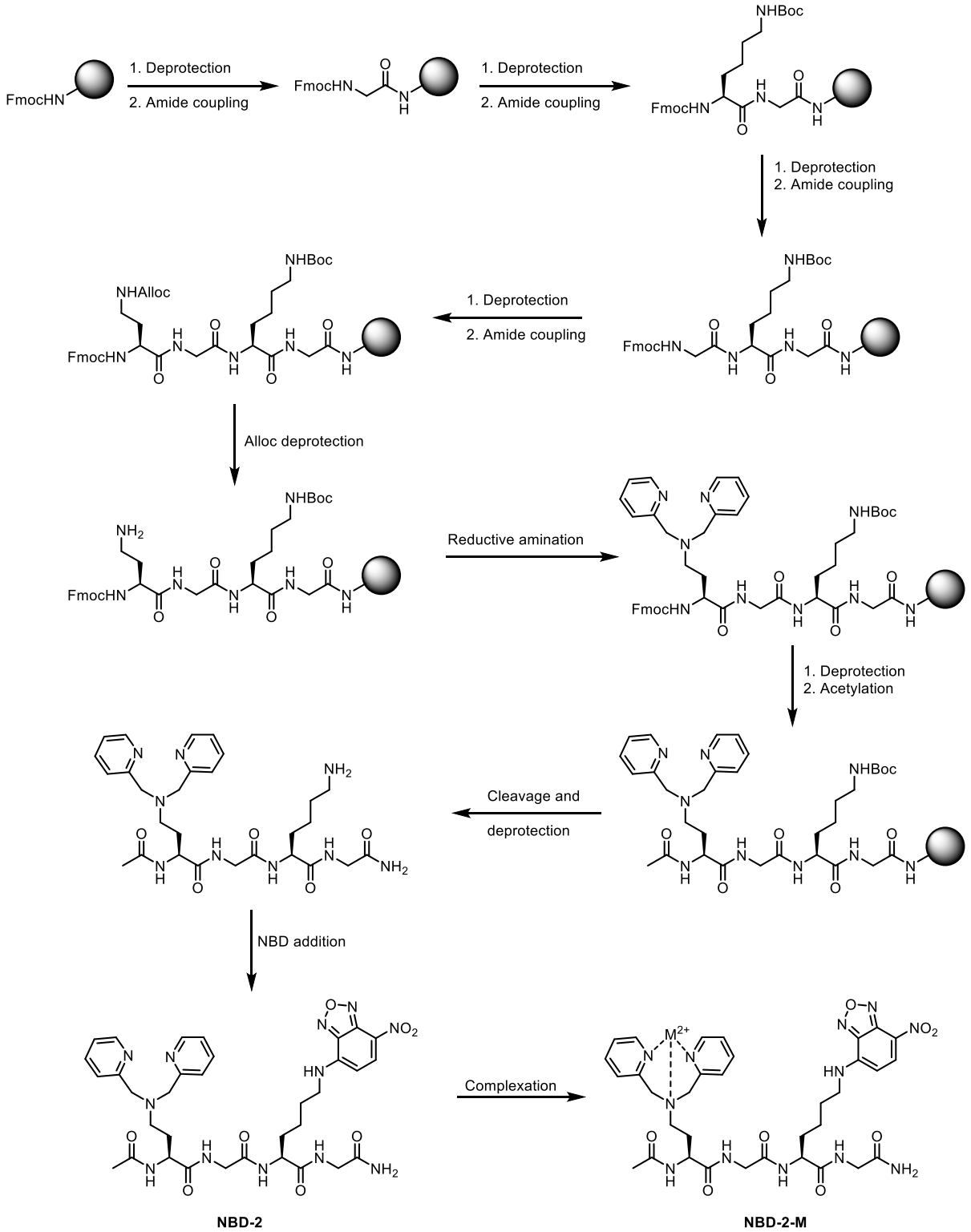
Complexation

An aqueous solution of $Zn(NO_3)_2.4H_2O$, $NiCl_2.6H_2O$, $MnCl_2.2H_2O$, or $CoCl_2.6H_2O$ (1 eq) was added to a solution of peptide in MeOH/H_2O (ratios and quantities were varied as necessary to dissolve the peptide). The solution was thoroughly mixed, and allowed to stand for 2 h at r.t.. The MeOH was removed under a flow of nitrogen, and the resulting residue dissolved in MeCN/H_2O, then lyophilised to afford the metal complex.

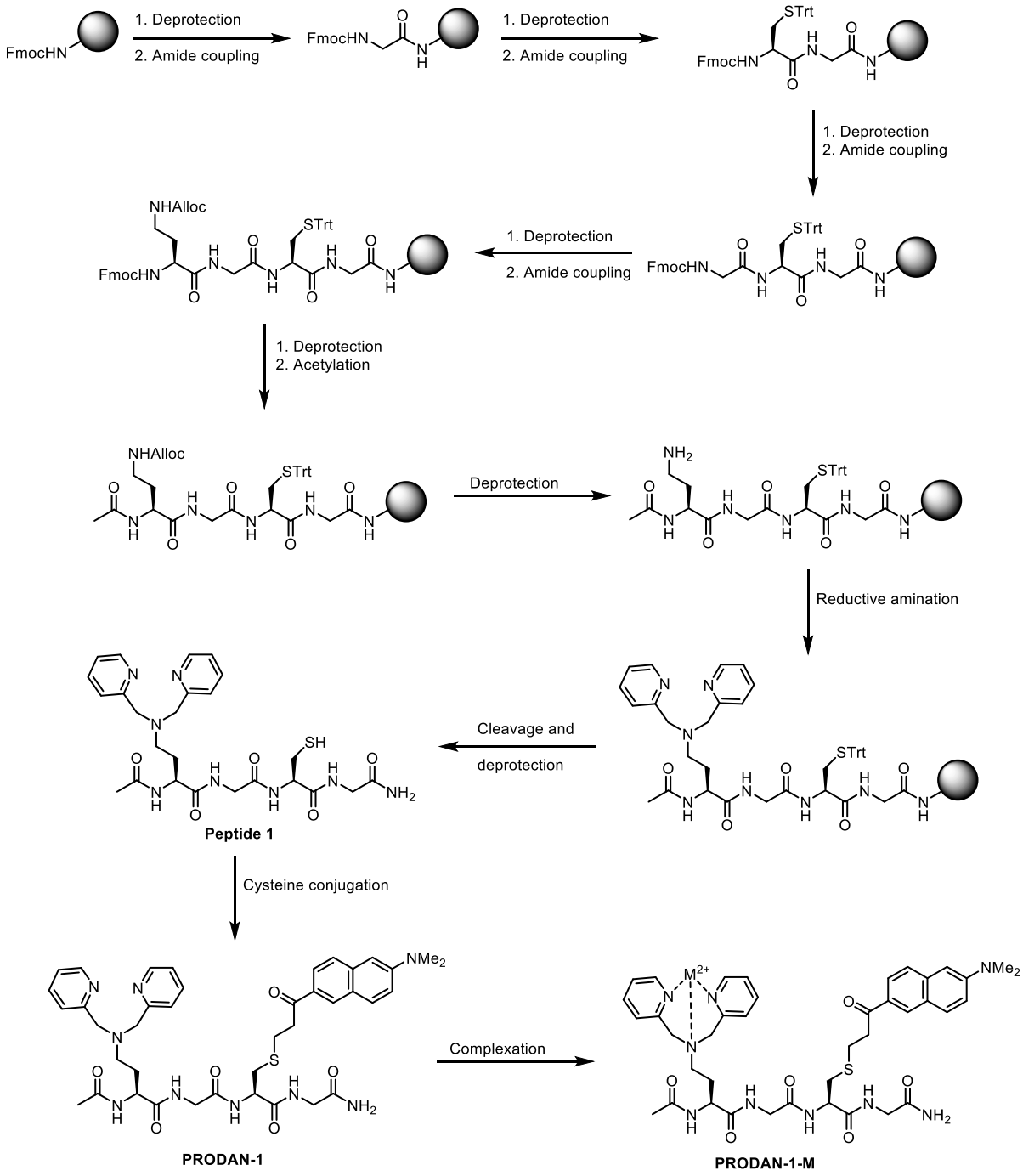
NBD-1-M Synthesis



NBD-2-M Synthesis



PRODAN-1-M Synthesis



NBD-1

Peptide 1 was synthesised on Rink amide resin (212 mg, 0.463 mol/g loading), following the procedure outlined above. Following purification and lyophilisation, the peptide was obtained as a fluffy orange solid (45 mg, 72 %). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.52 (broad s, 1H), 8.50 (d, *J* = 8.8 Hz, 1H), 8.47 – 8.43 (m, 2H), 8.08 – 8.02 (m, 2H), 7.75 – 7.69 (m, 2H), 7.45 (d, *J* = 7.7 Hz, 2H), 7.24 – 7.19 (m, 2H), 7.17 (s, 1H), 7.05 (s, 1H), 6.39 (d, *J* = 8.8 Hz, 1H), 4.31 – 4.22 (m, 1H), 3.83 – 3.50 (m, 6H), 3.49 – 3.39 (m, 2H), 2.64 – 2.50 (m, 2H), 2.12 – 2.04 (m, 2H), 2.02 – 1.91 (m, 1H), 1.79 – 1.60 (m, 3H), 1.56 – 1.43 (m, 2H) 1.38 – 1.27 (m, 2H); ^{13}C NMR (101 MHz, DMSO-*d6*) δ 172.5, 171.9, 170.8, 158.9*, 148.6, 145.1*, 137.9*, 136.4, 122.7, 122.0, 119.7*, 99.0*, 59.3, 51.2, 50.4, 43.2, 41.9, 34.9, 28.9, 27.3*, 26.0, 24.7; HRMS (ESI) Calc. for $C_{30}H_{37}N_{10}O_6$ $(M+H)^+$ 633.28921, found 633.28880; $[\alpha]_D^{22.7}$ = +9.4 (c = 0.25, MeOH).

*Broad peaks in ^{13}C NMR. Identified from HSQC and HMBC experiments.

NBD-1-Zn

A stock solution of $Zn(NO_3)_2.4H_2O$ in water (377 µL, 0.0353 M) was added to a solution of **NBD-1** (8.41 mg, 0.0133 mmol) in H_2O (4.0 mL) and MeOH (2.0 mL). The resulting solution was stirred at room temperature for 2 hours. Following removal of the organic solvent under a flow of nitrogen and subsequent lyophilisation, the peptide was obtained as a fluffy orange solid. ^{1}H NMR (400 MHz, MeOD) δ 8.75 – 8.69 (m, 2H), 8.50 (d, *J* = 8.8 Hz, 1H), 8.18 – 8.12 (m, 2H), 7.72 – 7.64 (m, 4H), 6.35 (d, *J* = 8.8 Hz, 1H), 4.37 (dd, *J* = 16.1 Hz, 7.1 Hz, 2H), 4.15 (dd, *J* = 16.1 Hz, 2.9 Hz, 2H), 4.11 – 4.06 (m, 1H), 3.83 – 3.72 (m, 2H), 3.60 – 3.45 (m, 2H), 2.84 – 2.74 (m, 2H), 2.25 – 2.16 (m, 2H), 2.13 – 2.02 (m, 1H), 1.99 – 1.87 (m, 1H), 1.80 – 1.70 (m, 2H), 1.65 – 1.55 (m, 2H), 1.46 – 1.36 (m, 2H); ^{13}C NMR (101 MHz, MeOD) δ 176.4, 174.0, 173.7, 156.6, 156.4, 149.4, 145.9*, 142.8, 142.7, 138.6, 126.4, 126.12, 126.08, 123.0*, 99.9*, 58.5, 57.9, 53.1, 52.3, 42.9, 36.4, 29.0*, 27.5, 26.8, 26.2; HRMS (ESI) Calc. for $C_{30}H_{36}N_{10}O_6Zn^{2+}$ $(M)^{2+}$ 348.10499, found 348.10497.

*Broad peaks in ^{13}C NMR. Identified from HSQC and HMBC experiments.

NBD-2

NBD-2 was synthesised on Rink amide resin (383 mg, 0.463 mol/g loading), following the procedure outlined above. The crude peptide pellet obtained from cleavage off the resin was dissolved in MeOH (2 mL), followed by addition of DIPEA (155 µL, 0.885 mmol) and 4-chloro-7-nitrobenzofurazan (46 mg, 0.230 mmol). The resulting solution was heated to 50 °C for 3 hours. Following completion of reaction, the solvent was removed *in vacuo*. The residue was partitioned between dichloromethane and dilute aqueous hydrochloric acid (pH 1). The aqueous phase was washed with dichloromethane (3x 30 mL), then lyophilised. Following purification and lyophilisation, the peptide was obtained as a fluffy orange solid (21 mg, 16 %). ^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.42 (broad s, 1H), 8.53 – 8.42 (m, 3H), 8.21 (t, *J* = 5.8 Hz, 1H), 8.16 – 8.08 (m, 2H), 7.93 – 7.86 (m, 1H), 7.77 – 7.67 (m, 2H), 7.49 – 7.42 (m, 2H), 7.26 –

7.19 (m, 2H), 7.16 (s, 1H), 7.05 (s, 1H), 6.43 – 6.35 (m, 1H), 4.31 – 4.17 (m, 2H), 3.85 – 3.54 (m, 11H), 2.61 – 2.52 (m, 2H), 1.99 – 1.92 (m, 2H), 1.77 (s, 3H), 1.76 – 1.53 (m, 5H), 1.45 – 1.30 (m, 2H); ^{13}C NMR (125 MHz, DMSO-*d6*) δ 172.3, 171.6, 170.8, 169.8, 169.0, 148.7, 145.1, 144.2, 138.0, 136.6, 122.8, 122.2, 120.5, 99.2, 59.2, 52.6, 51.3, 50.3, 43.2, 42.2, 41.8, 31.2, 28.7, 27.2, 22.6, 22.5; HRMS (ESI) Calc. for $C_{34}H_{43}N_{12}O_8$ $(M+H)^+$ 747.33300, found 747.33213.

NBD-2-Zn

A stock solution of $Zn(NO_3)_2.4H_2O$ in water (689 µL, 0.0302 M) was added to a solution of **NBD-2** (15.53 mg, 0.0208 mmol) in H_2O (5.0 mL) and MeOH (4.0 mL). The resulting solution was stirred at room temperature for 2 hours. Following removal of the organic solvent under a flow of nitrogen and subsequent lyophilisation, the peptide was obtained as a fluffy orange solid. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.53 (s, 1H), 8.71 – 8.39 (m, 3H), 8.30 – 7.39 (m, 6H), 7.76 – 7.39 (m, 4H), 7.19 (s, 1H), 7.05 (s, 1H), 6.49 – 6.30 (m, 1H), 4.35 – 4.08 (m, 4H), 4.04 – 3.88 (m, 2H), 3.84 – 3.51 (m, 6H), 2.83 – 2.58 (m, 2H), 2.18 – 2.02 (m, 1H), 1.95 – 1.49 (m, 5H), 1.77 (s, 3H), 1.47 – 1.24 (m, 2H); ^{13}C NMR (125 MHz, DMSO-*d6*) δ 171.7, 171.6, 170.8, 169.7, 168.8, 154.6, 145.2, 144.5, 144.2, 140.5, 138.0, 124.7, 124.5, 120.5, 99.2, 56.2, 52.7, 50.8, 43.2, 42.0, 41.8, 31.4, 27.2, 25.1, 22.7, 22.5; HRMS (ESI) Calc. for $C_{34}H_{42}N_{12}O_8Zn^{2+}$ $(M)^{2+}$ 405.12645, found 405.12668.

Peptide 1

Peptide 1 was synthesised on Rink amide resin (244 mg, 0.463 g/mol loading), following the procedure outlined above. Following purification and lyophilisation, the peptide was obtained as a fluffy, pale green solid (56 mg, 89 %). ^{1}H NMR (300 MHz, DMSO-*d6*) δ 8.50 – 8.44 (m, 2H), 8.28 (t, *J* = 5.7 Hz, 1H), 8.19 (t, *J* = 6.0 Hz, 1H), 8.16 – 8.12 (m, 1H), 7.94 (d, *J* = 7.7 Hz, 1H), 7.72 (dd, *J* = 7.6 Hz, *J* = 1.8 Hz, 2H), 7.47 (d, *J* = 7.6 Hz, 2H), 7.26 – 7.21 (m, 2H), 7.18 (s, 1H), 7.08 (s, 1H), 4.42 – 4.34 (m, 1H), 4.31 – 4.24 (m, 1H), 3.84 – 3.59 (m, 8H), 2.77 (broad s, 2H), 2.61 – 2.45 (m, 3H), 2.03 – 1.91 (m, 1H), 1.84 – 1.66 (m, 4H); ^{13}C NMR (101 MHz, DMSO-*d6*) δ 172.5, 170.6, 169.8, 169.7, 169.1, 159.1, 148.7, 136.4, 122.7, 122.0, 59.4, 55.2, 51.4, 50.2, 42.2, 42.0, 28.9, 26.0, 22.4; HRMS (ESI) Calc. for $C_{25}H_{33}N_8O_5S$ $(M-H)^-$ 557.23001, found 557.22977; $[\alpha]_D^{21.8}$ = +19.4 (c = 0.25, MeOH).

PRODAN-1

A solution of acrylodan (35 mg, 0.156 mmol) in MeCN (5 mL) was added to a solution of **Peptide 1** (29 mg, 0.0519 mmol) in 5 mM HEPES buffer (pH 7, 5 mL) at room temperature. The resulting solution was agitated for 4 hours, at which point the solvent was removed by lyophilisation. Following purification and lyophilisation, the peptide was obtained as a fluffy, pale green solid (22 mg, 54 %). ^{1}H NMR (400 MHz, MeOD) δ 8.46 – 8.36 (m, 3H), 7.88 – 7.81 (m, 2H), 7.75 – 7.68 (m, 2H), 7.63 (d, *J* = 8.8 Hz, 1H), 7.50 – 7.45 (m, 2H), 7.26 – 7.19 (m, 3H), 6.94 – 6.90 (m, 1H), 4.56 – 4.50 (m, 1H), 4.45 – 4.39 (m, 1H), 3.95 – 3.78 (m, 6H), 3.72 – 3.64

(m, 2H), 3.15 – 3.07 (m, 1H), 3.09 (s, 6H), 2.99 – 2.89 (m, 3H), 2.72 – 2.60 (m, 2H), 2.14 – 2.03 (m, 1H), 1.91 – 1.77 (m, 4H); ^{13}C NMR (101 MHz, MeOD) δ 200.5, 175.5, 174.1, 173.7, 172.1, 160.3, 152.2, 149.6, 139.4, 138.5, 131.9, 131.8, 131.5, 131.5, 127.4, 127.3, 126.6, 125.1, 123.70, 123.67, 117.6, 106.4, 61.1, 55.0, 54.5, 54.0, 52.4, 43.9, 43.6, 43.5, 40.5, 34.6, 30.2, 30.0, 27.9, 22.7; HRMS (ESI) Calc. for $C_{40}H_{50}N_9O_6S$ $(M+H)^+$ 784.35993, found 784.35927; $[\alpha]_D^{22.1}$ = -6.9 (c = 0.25, MeOH).

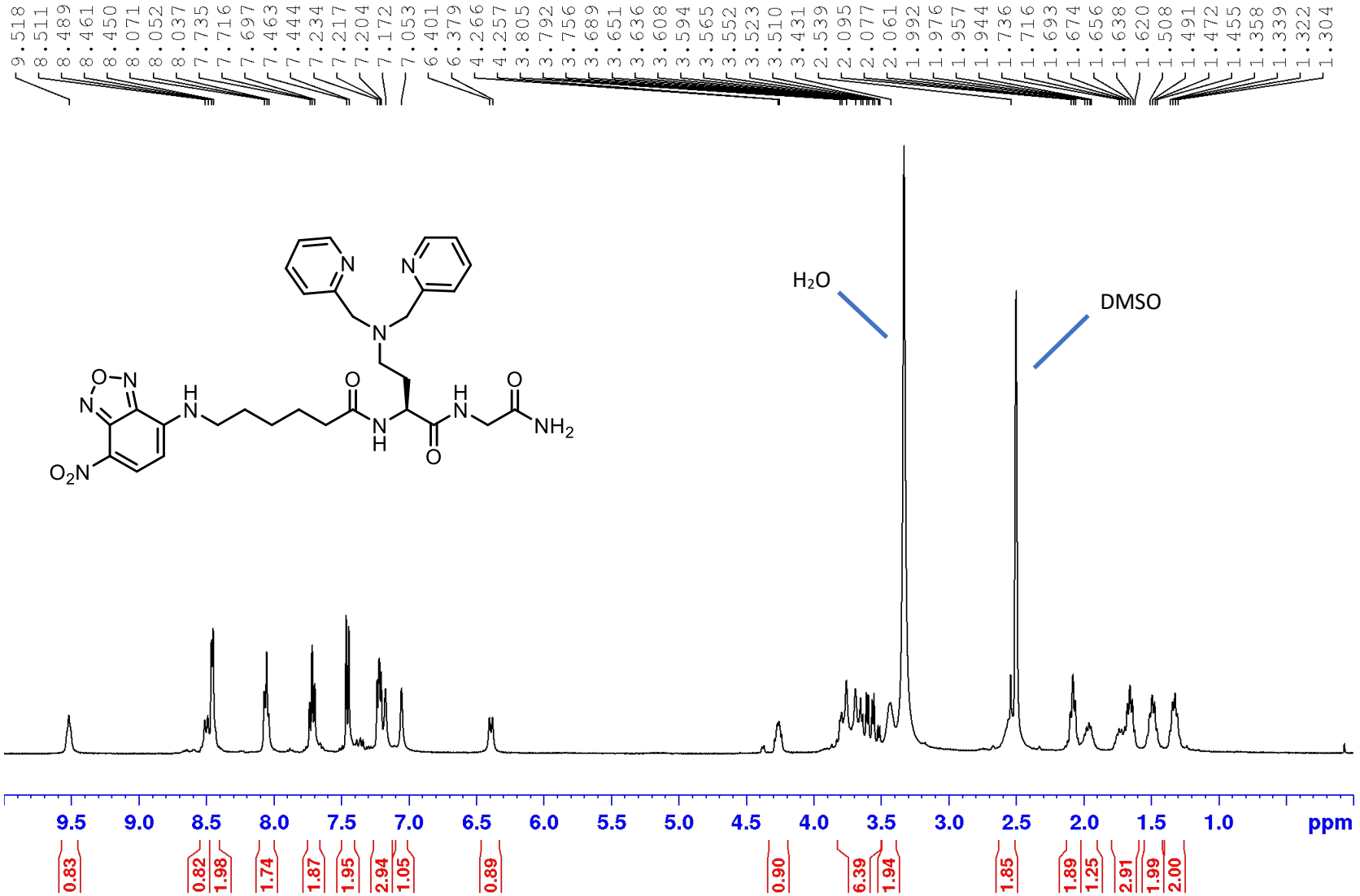
PRODAN-1-Zn

A stock solution of $Zn(NO_3)_2.4H_2O$ in water (614 µL, 0.0109 M) was added to a solution of **PRODAN-1** (5.23 mg, 0.00671 mmol) in H_2O (3.0 mL) and MeOH (3.0 mL). The resulting solution was stirred at room temperature for 2 hours. Following removal of the organic solvent under a flow of nitrogen and subsequent lyophilisation, the peptide was obtained as a fluffy pale green solid. ^{1}H NMR (400 MHz, MeOD) δ 8.73 – 8.64 (m, 2H), 8.42 (s, 1H), 8.18 – 8.09 (m, 2H), 7.86 (d, *J* = 8.8 Hz, 1H), 7.71 – 7.59 (m, 5H), 7.29 – 7.22 (m, 1H), 6.94 (s, 1H), 4.65 – 4.54 (m, 2H), 4.52 – 4.45 (m, 1H), 4.40 – 4.31 (m, 2H), 4.13 – 4.04 (m, 3H), 3.97 – 3.80 (m, 5H), 3.46 – 3.40 (m, 1H), 3.15 – 3.06 (m, 8H), 3.01 – 2.88 (m, 3H), 2.80 – 2.71 (m, 2H), 2.12 – 2.00 (m, 1H), 1.98 – 1.91 (m, 1H), 1.89 (s, 3H); ^{13}C NMR (101 MHz, MeOD)* δ 200.2, 174.3, 173.7, 173.1, 171.6, 156.5, 156.3, 152.2, 149.3, 149.2, 142.8, 142.7, 139.5, 131.9, 131.6, 127.4, 126.5, 126.4, 126.2, 126.15, 125.1, 117.6, 106.4, 58.6, 58.0, 54.8, 53.1, 52.1, 43.6, 43.3, 40.5, 39.4, 34.7, 27.9, 26.9, 22.5; HRMS (ESI) Calc. for $C_{40}H_{50}N_9O_6SZn^{2+}$ $(M)^{2+}$ 423.64035, found 423.64107; $[\alpha]_D^{22.5}$ = -3.4 (c = 0.25, MeOH).

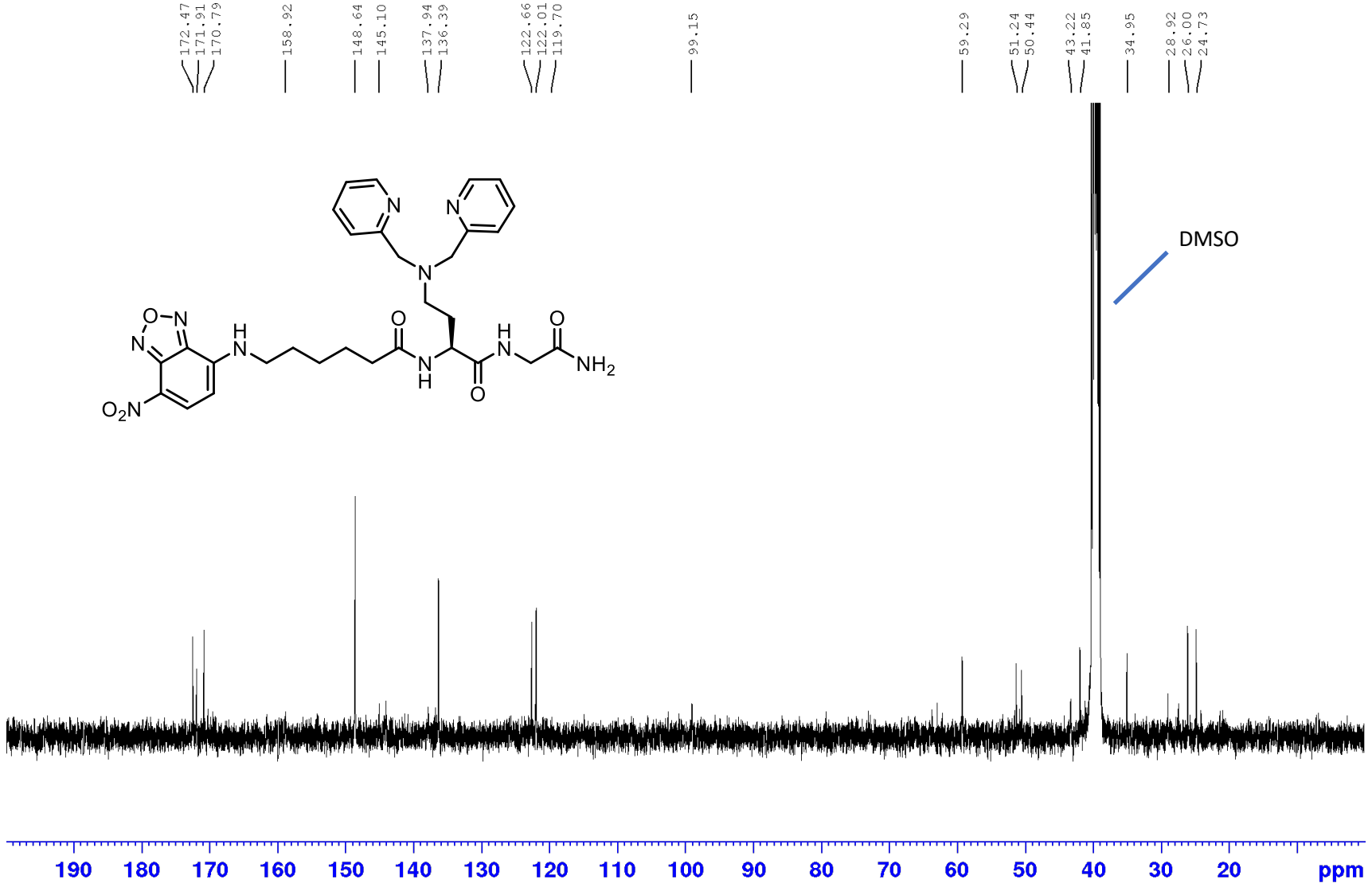
*Dipicolylamine groups appear as two separate sets of signals

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

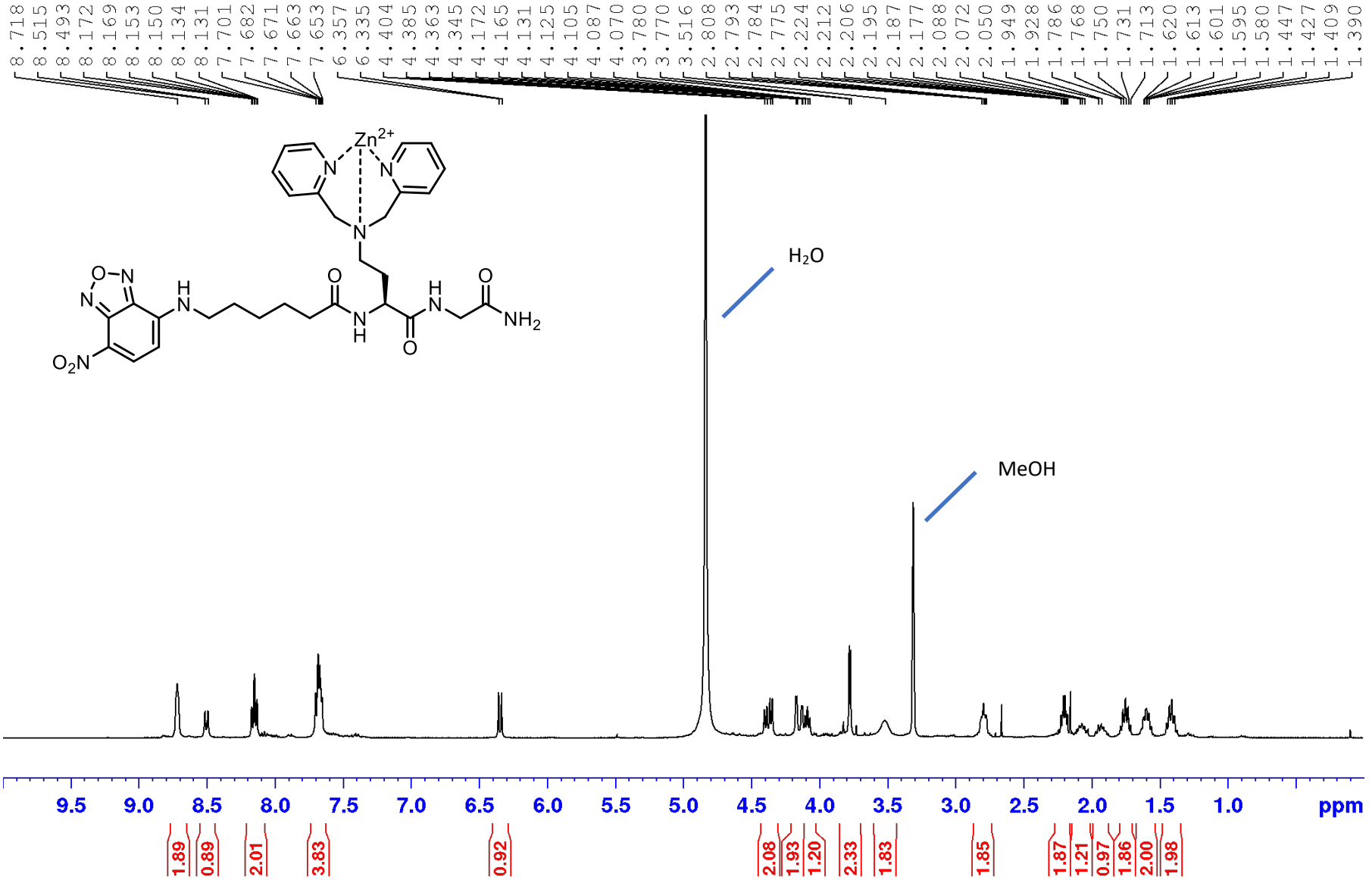
NBD-1—^{1}H NMR, 400 MHz, DMSO-*d6*



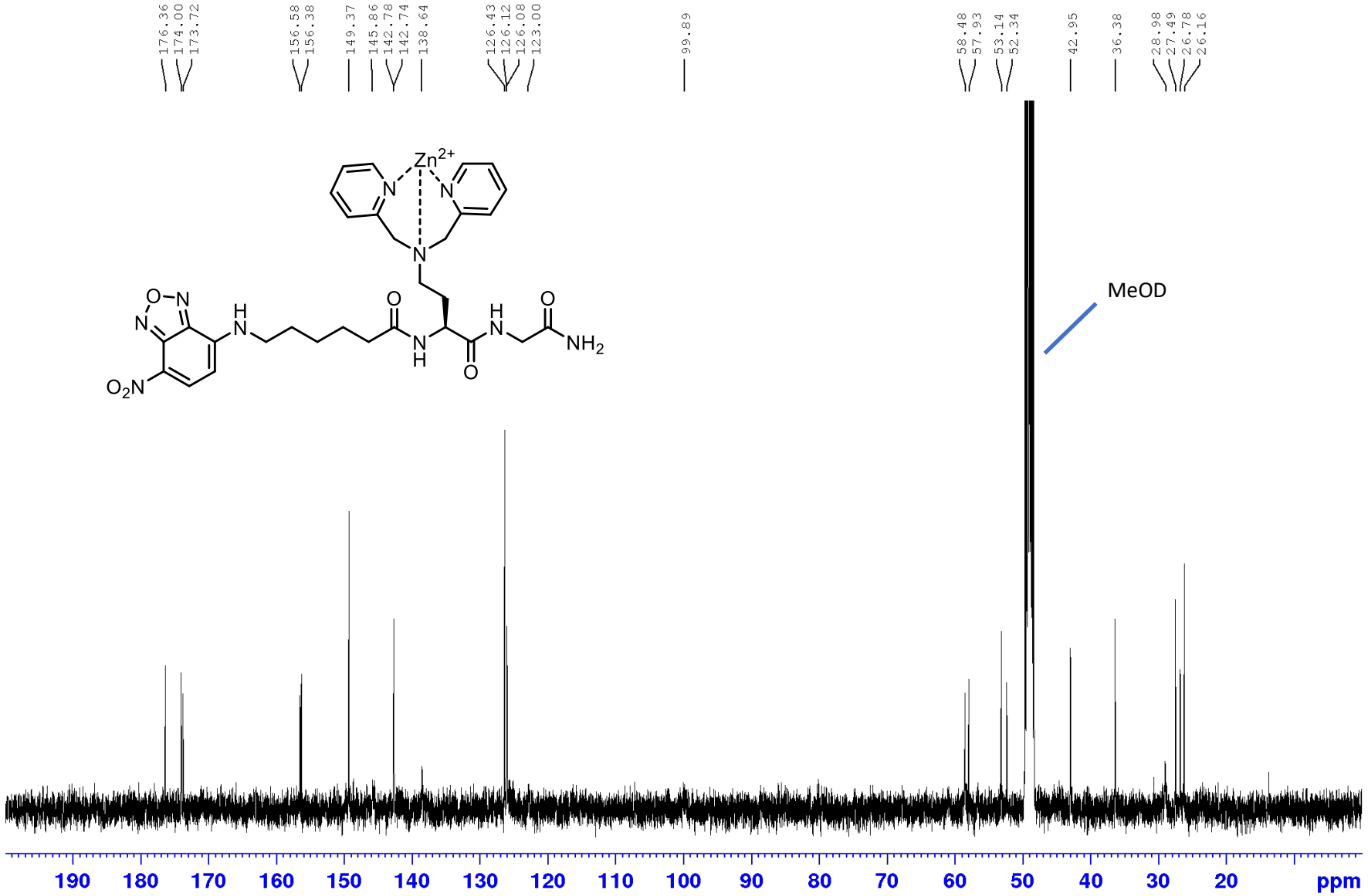
NBD-1—^{13}C NMR, 101 MHz, DMSO-*d6*



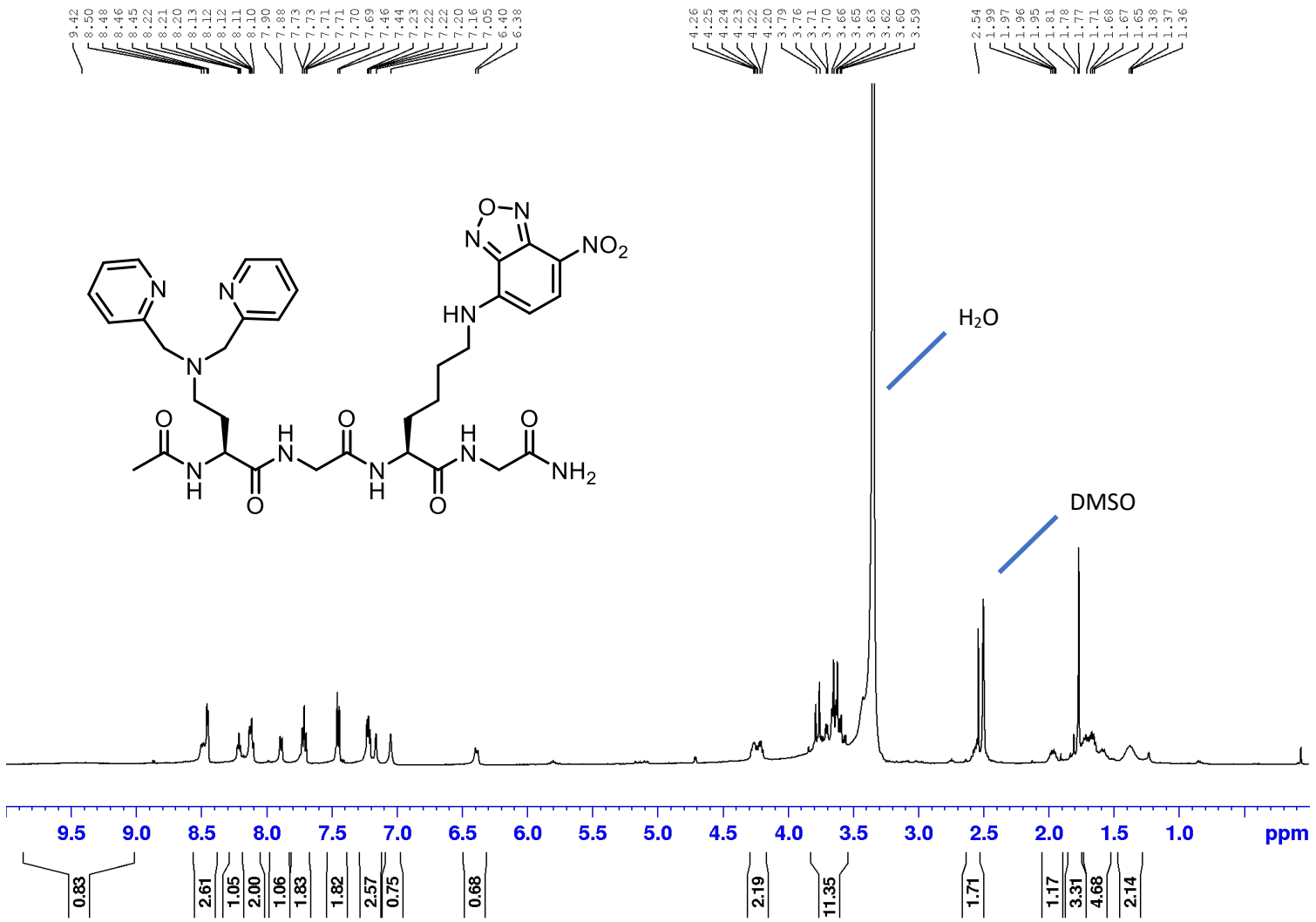
NBD-1-Zn—^{1}H NMR, 400 MHz, MeOD



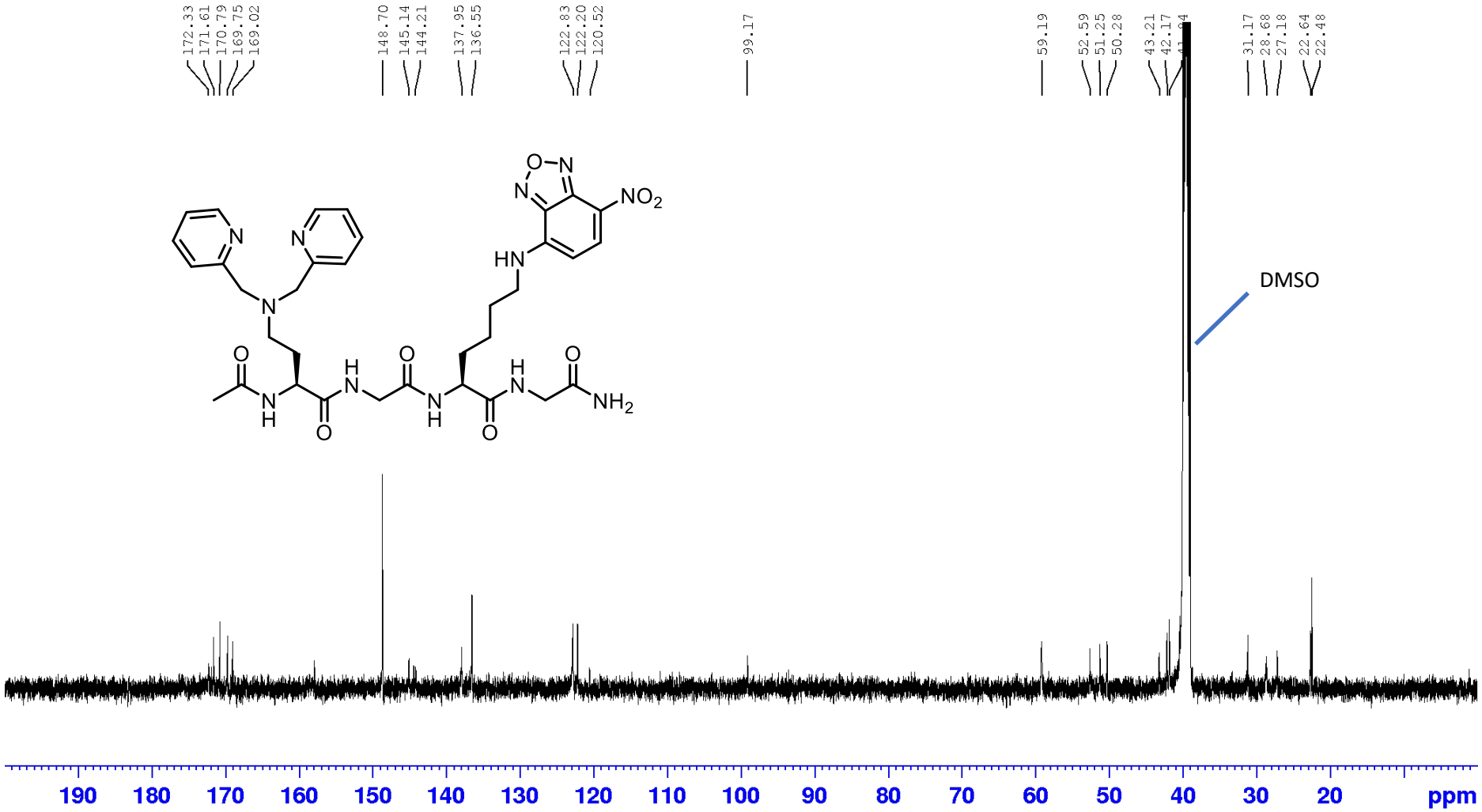
NBD-1-Zn—^{13}C NMR, 101 MHz, MeOD



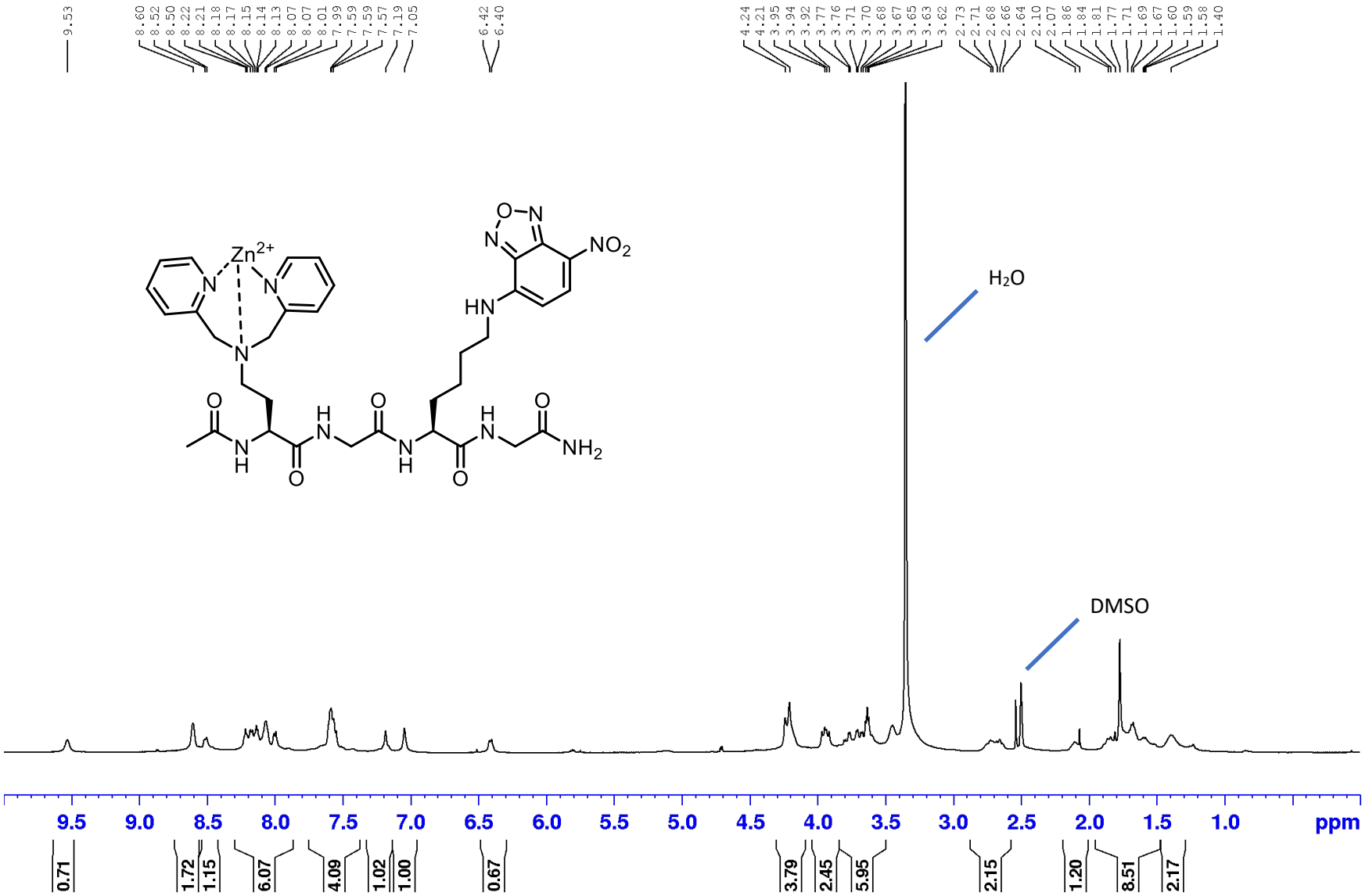
NBD-2—^{1}H NMR, 500 MHz, DMSO-*d6*



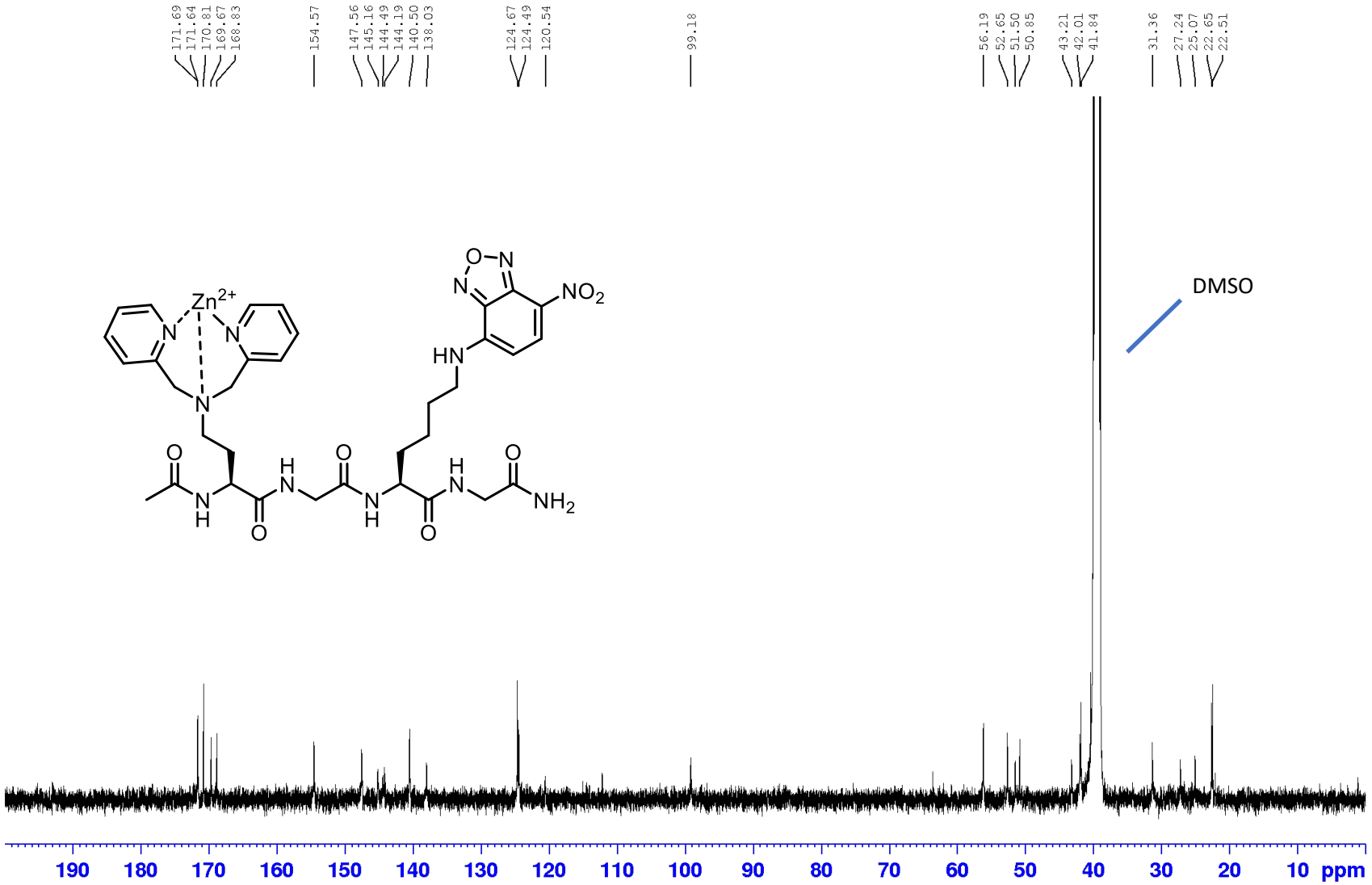
NBD-2—^{13}C NMR, 125 MHz, DMSO-*d6*



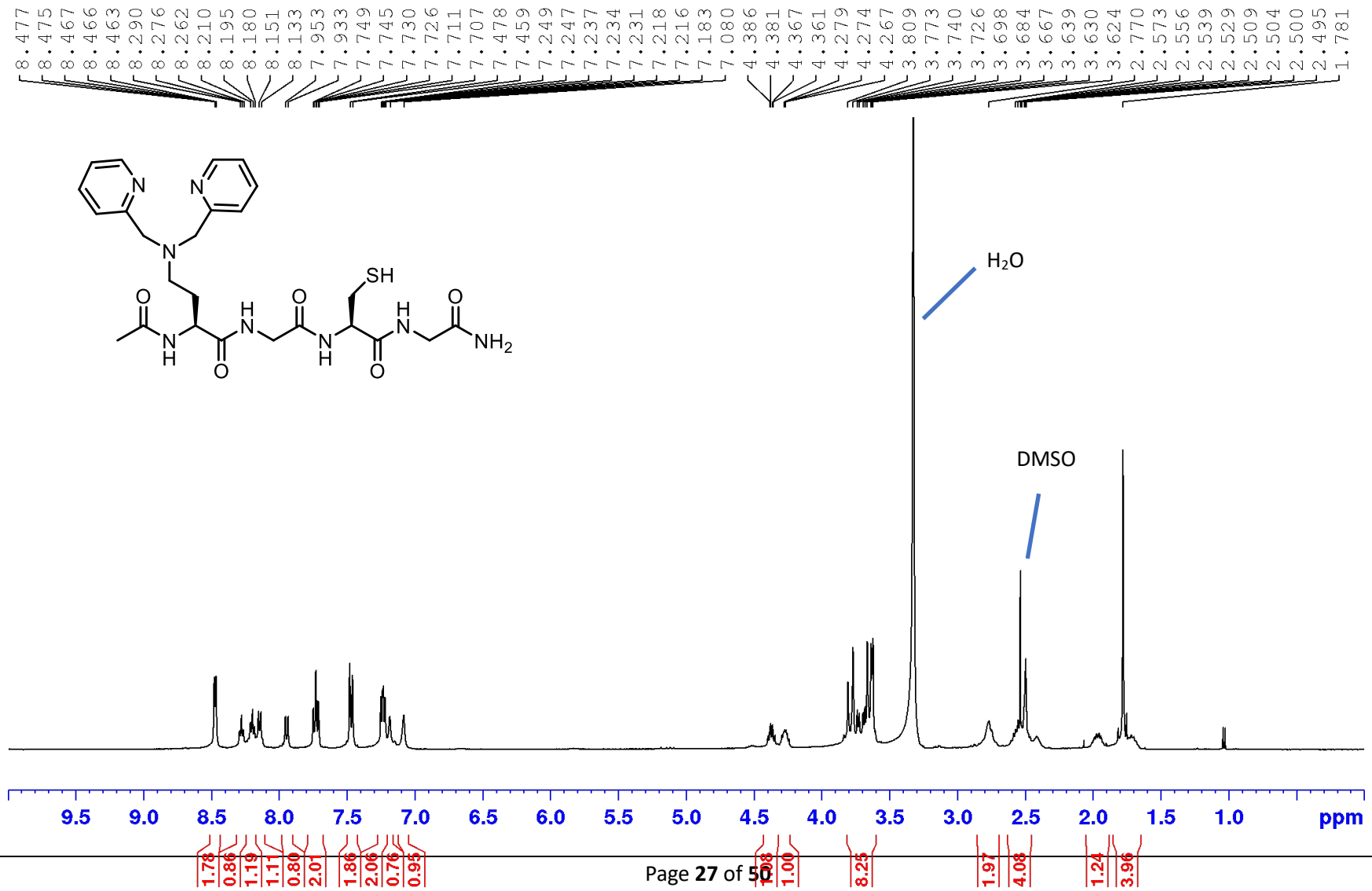
NBD-2-Zn—^{1}H NMR, 500 MHz, DMSO-*d6*



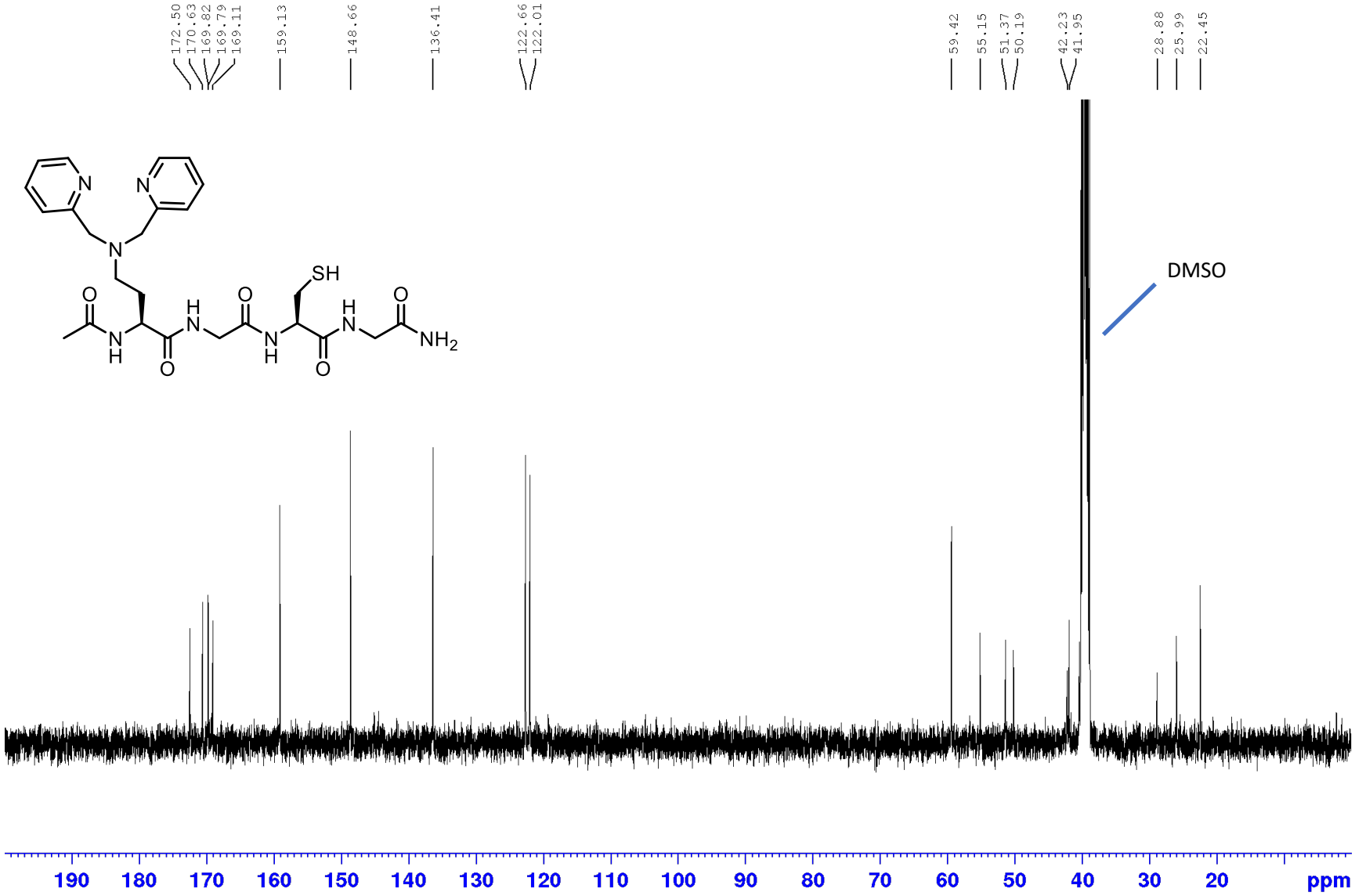
NBD-2-Zn—^{13}C NMR, 125 MHz, DMSO-*d6*



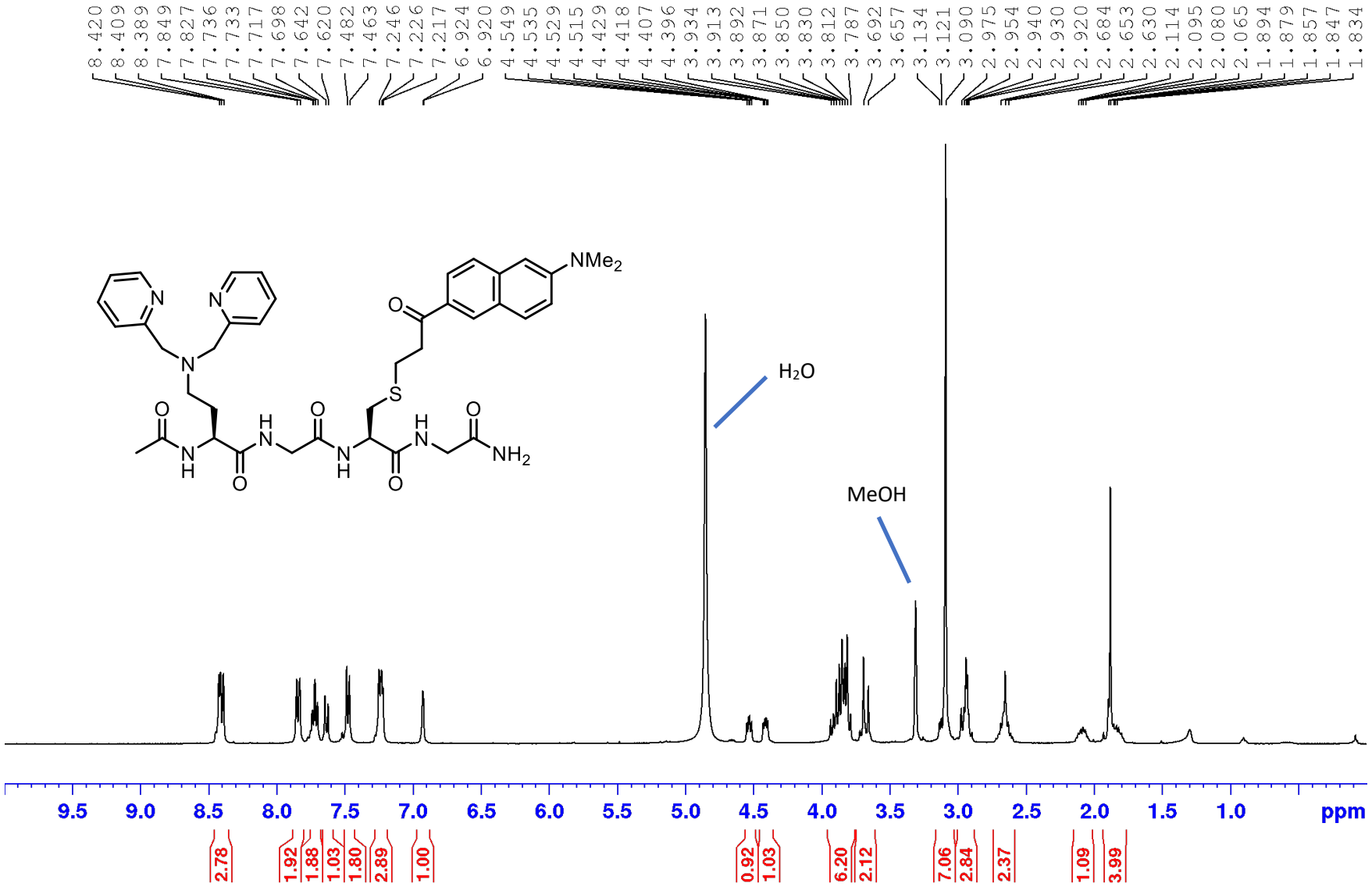
Peptide 1—^{1}H NMR , 400 MHz, DMSO-*d6*



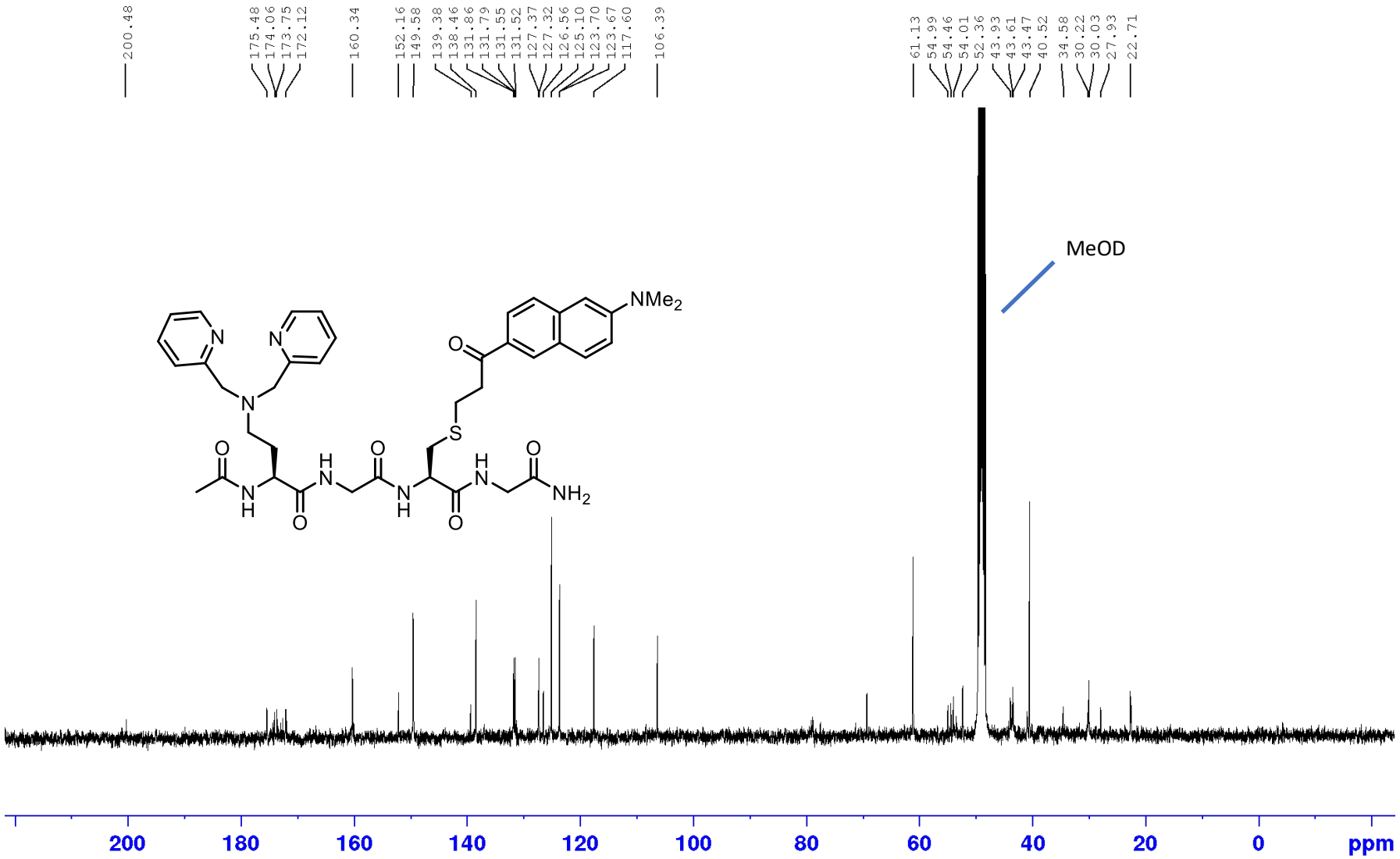
Peptide 1—^{13}C NMR , 101 MHz, DMSO-*d6*



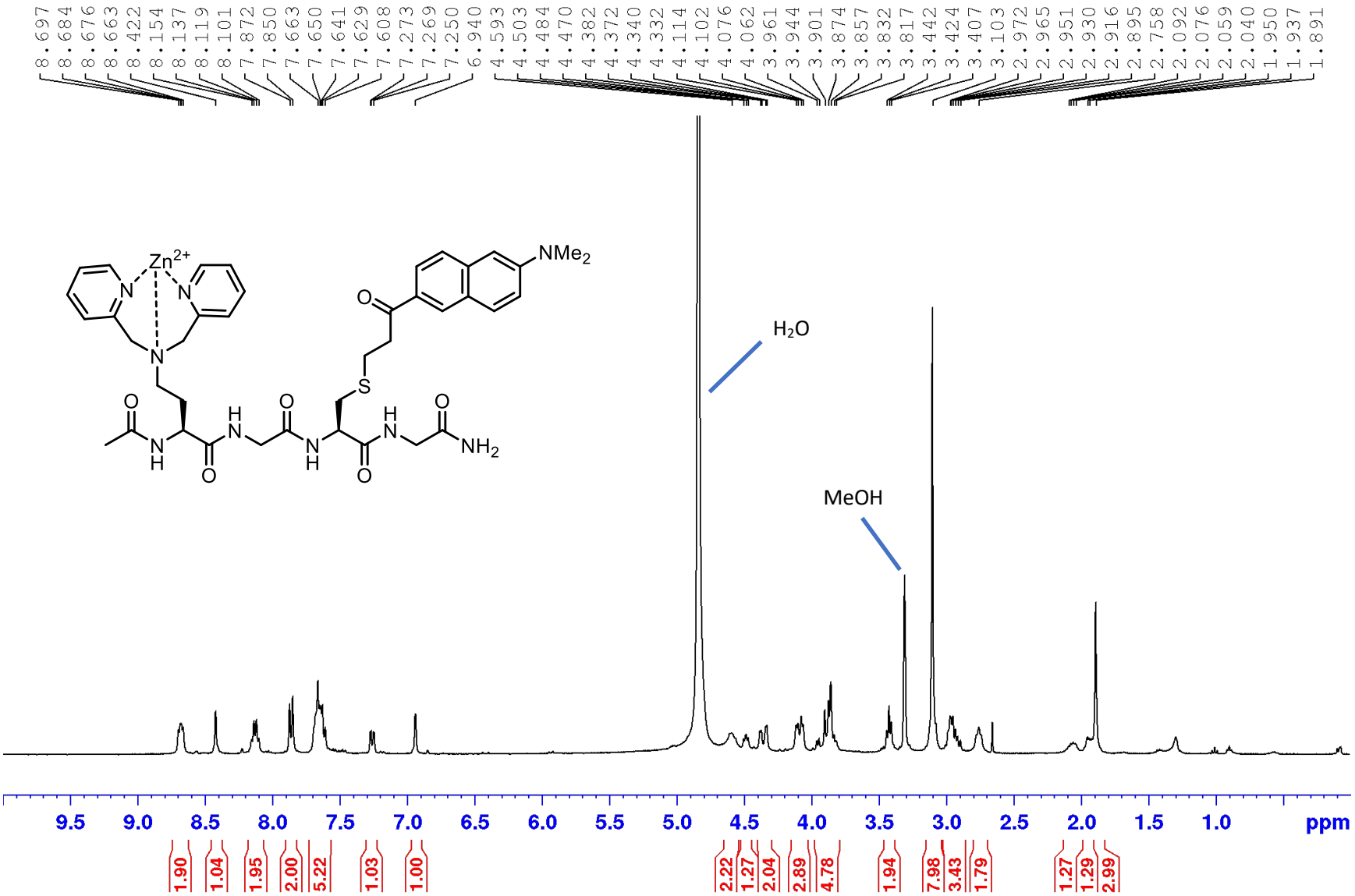
PRODAN-1—^{1}H NMR, 400 MHz, MeOD



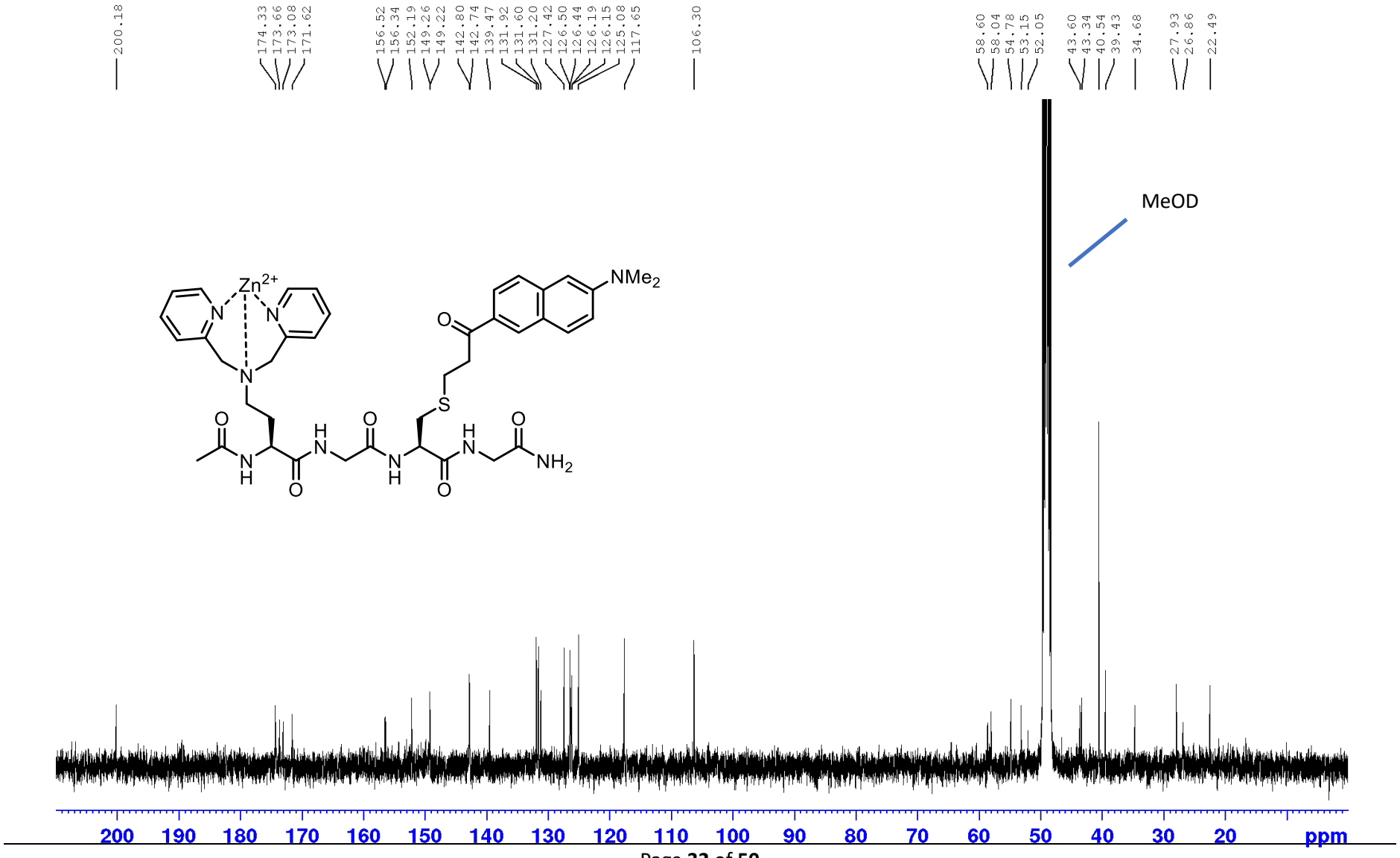
PRODAN-1—^{13}C NMR, 101 MHz, MeOD



PRODAN-1-Zn—^{1}H NMR, 400 MHz, MeOD



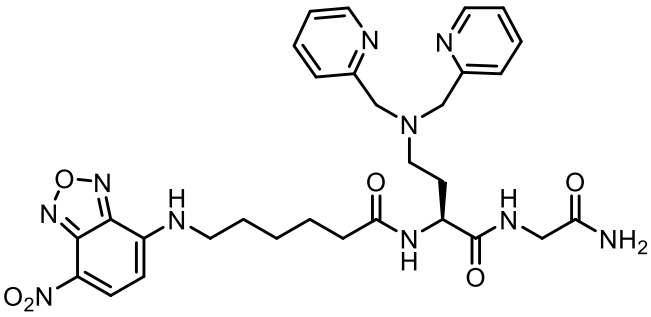
PRODAN-1-Zn—^{13}C NMR, 101 MHz, MeOD

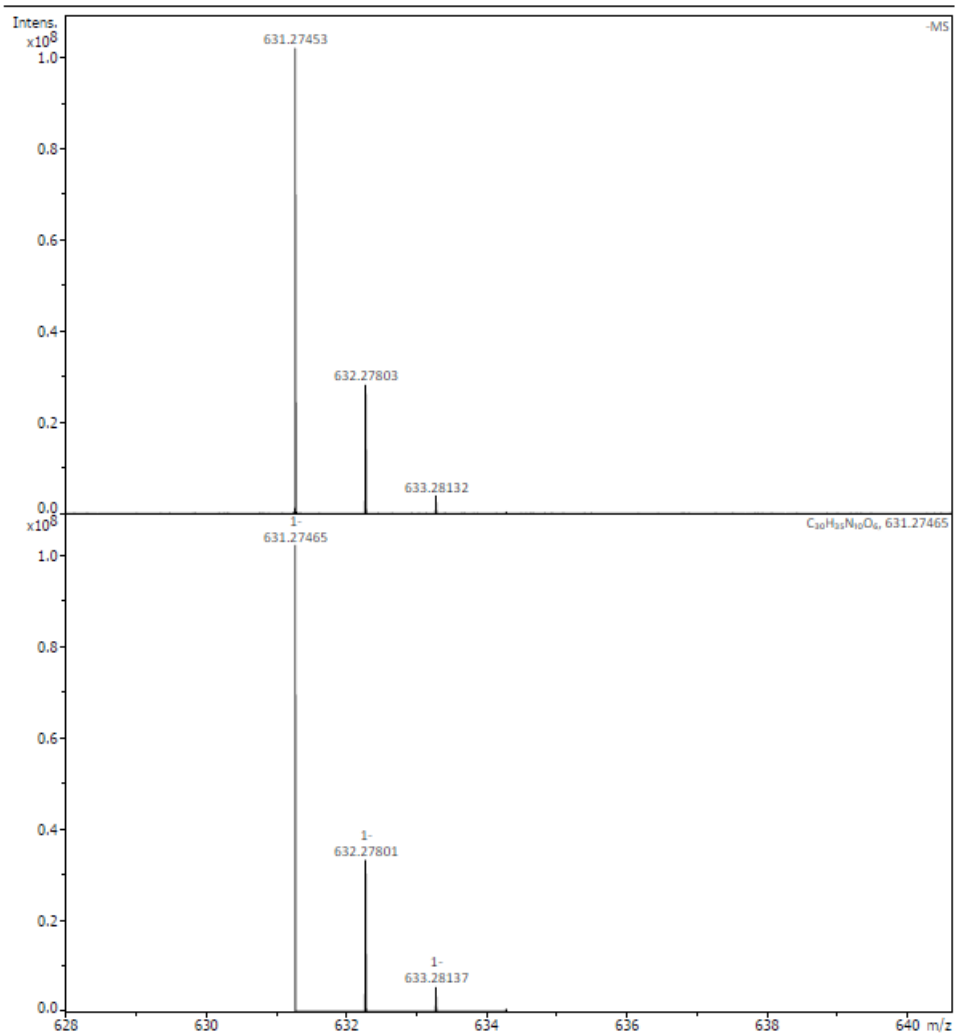


Mass Spectra

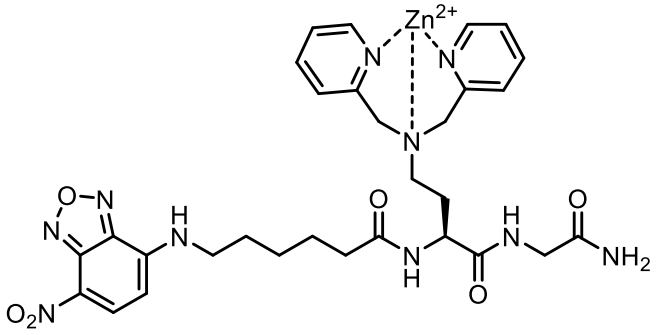
For all compounds, experimental data is shown above, and simulated spectra below.

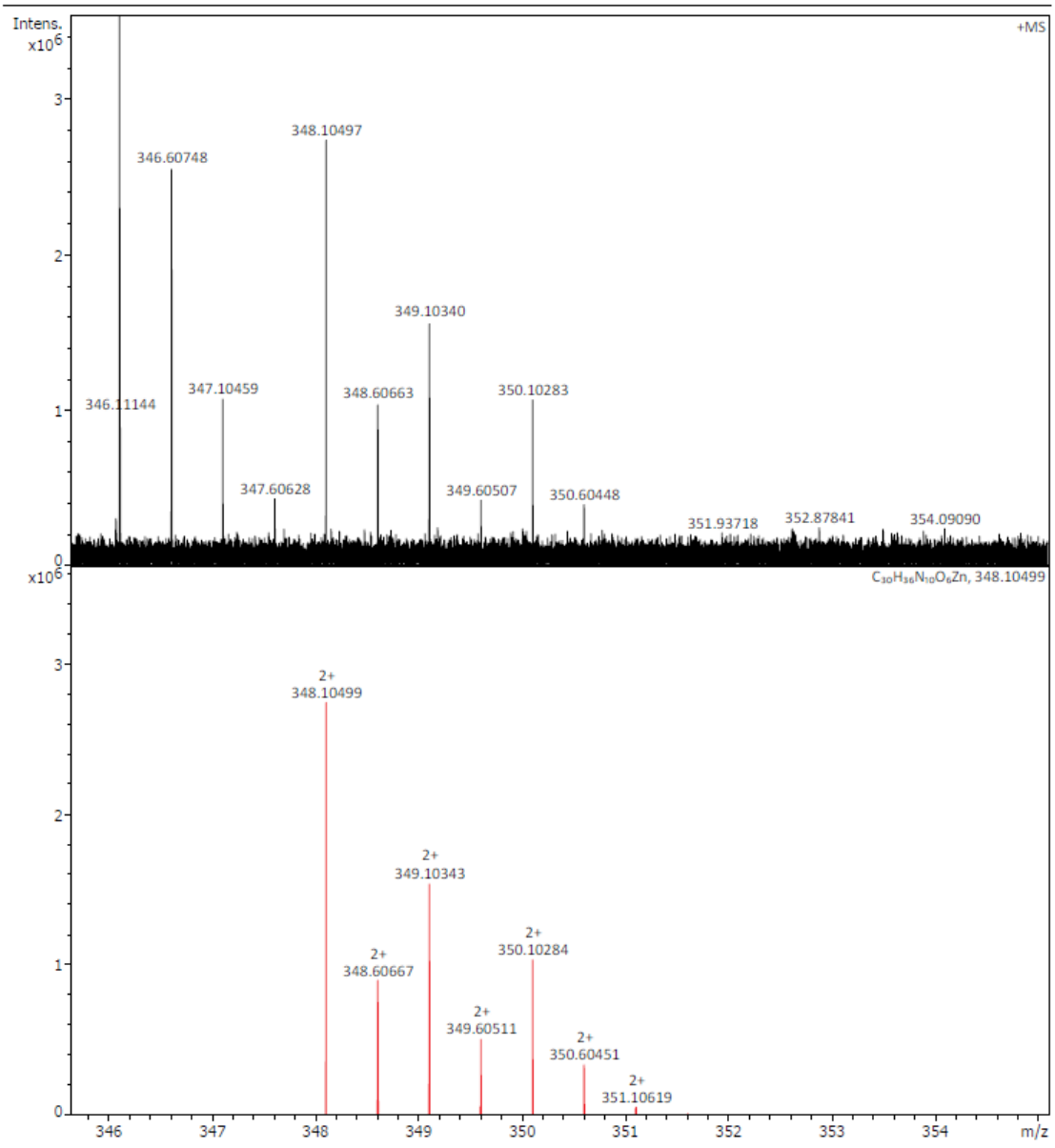
NBD-1: HRMS (ESI) Calc. for $C_{30}H_{35}N_{10}O_6$ $(M-H)^-$ 631.27465, found 631.27453;



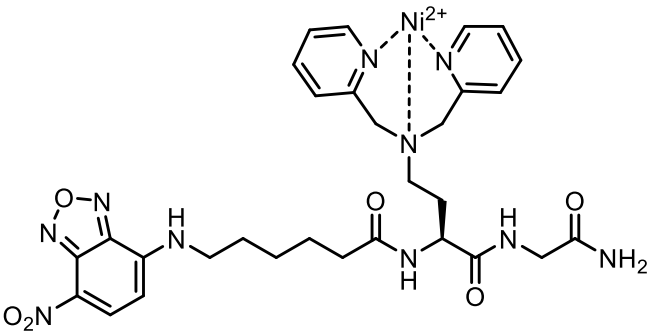


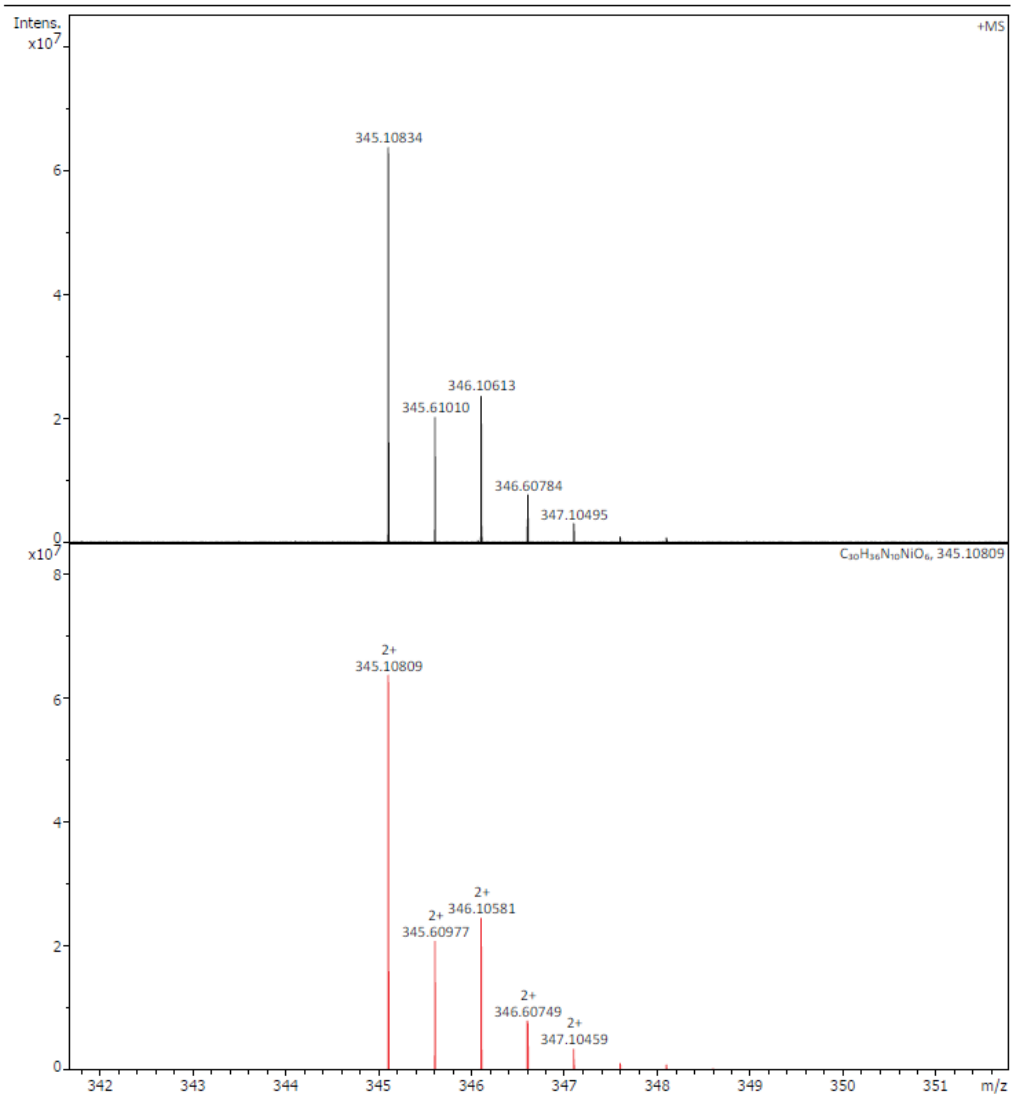
NBD-1-Zn: HRMS (ESI) Calc. for $C_{30}H_{36}N_{10}O_6Zn^{2+}$ $(M)^{2+}$ 348.10499, found 348.10497



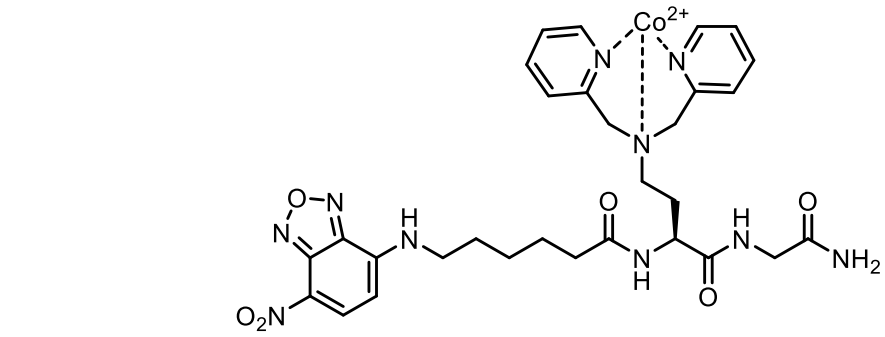


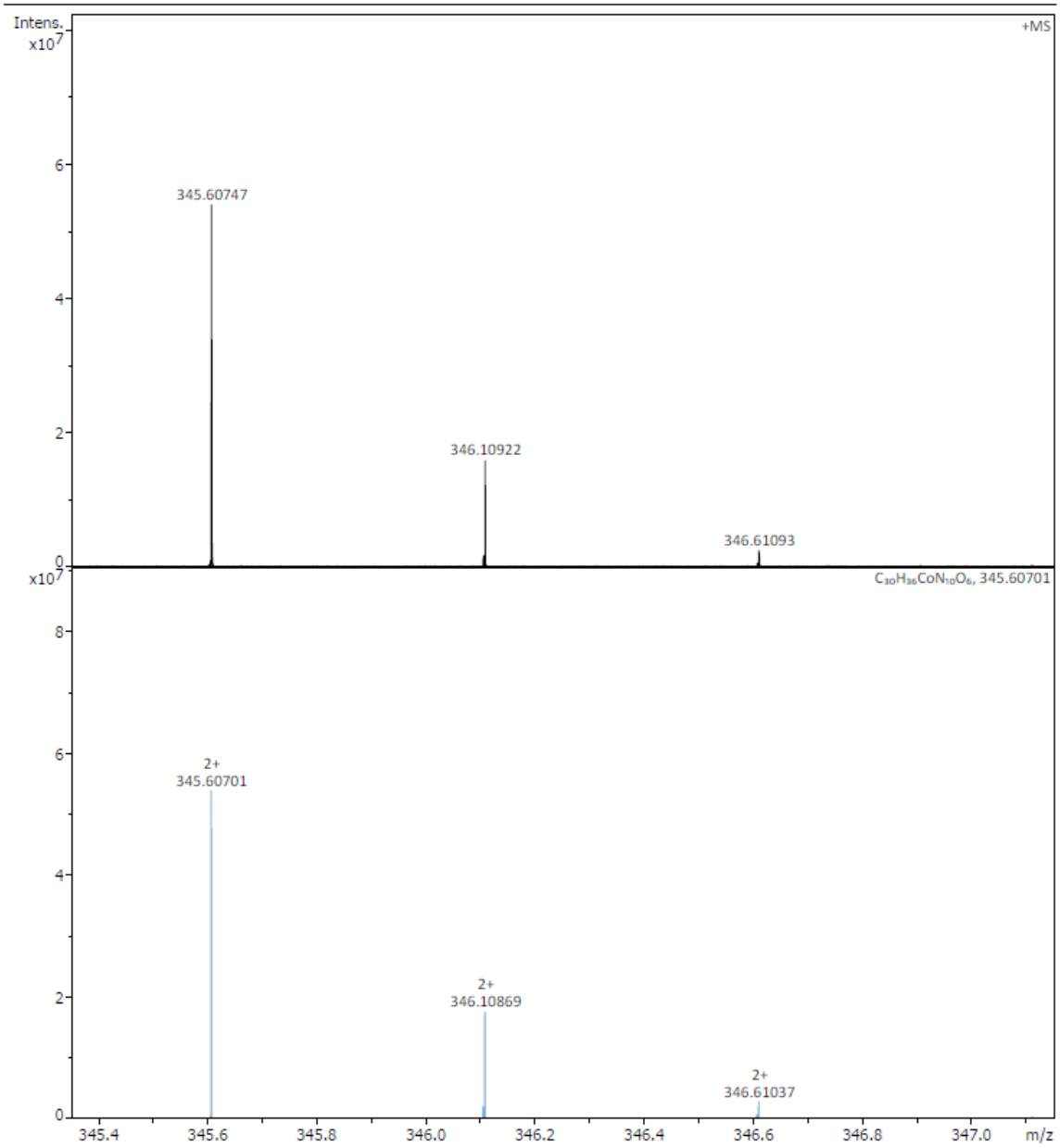
NBD-1-Ni: HRMS (ESI) Calc. for $C_{30}H_{36}N_{10}O_6Ni^{2+}$ $(M)^{2+}$ 345.10809, found 345.10834



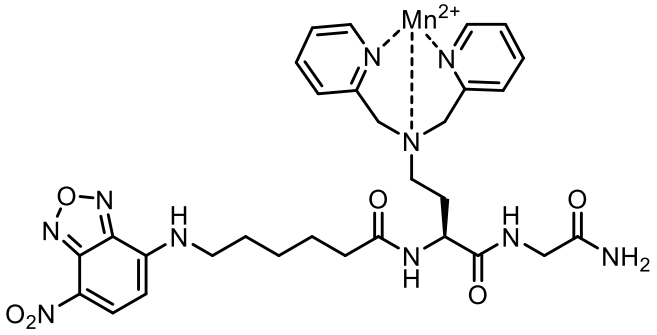


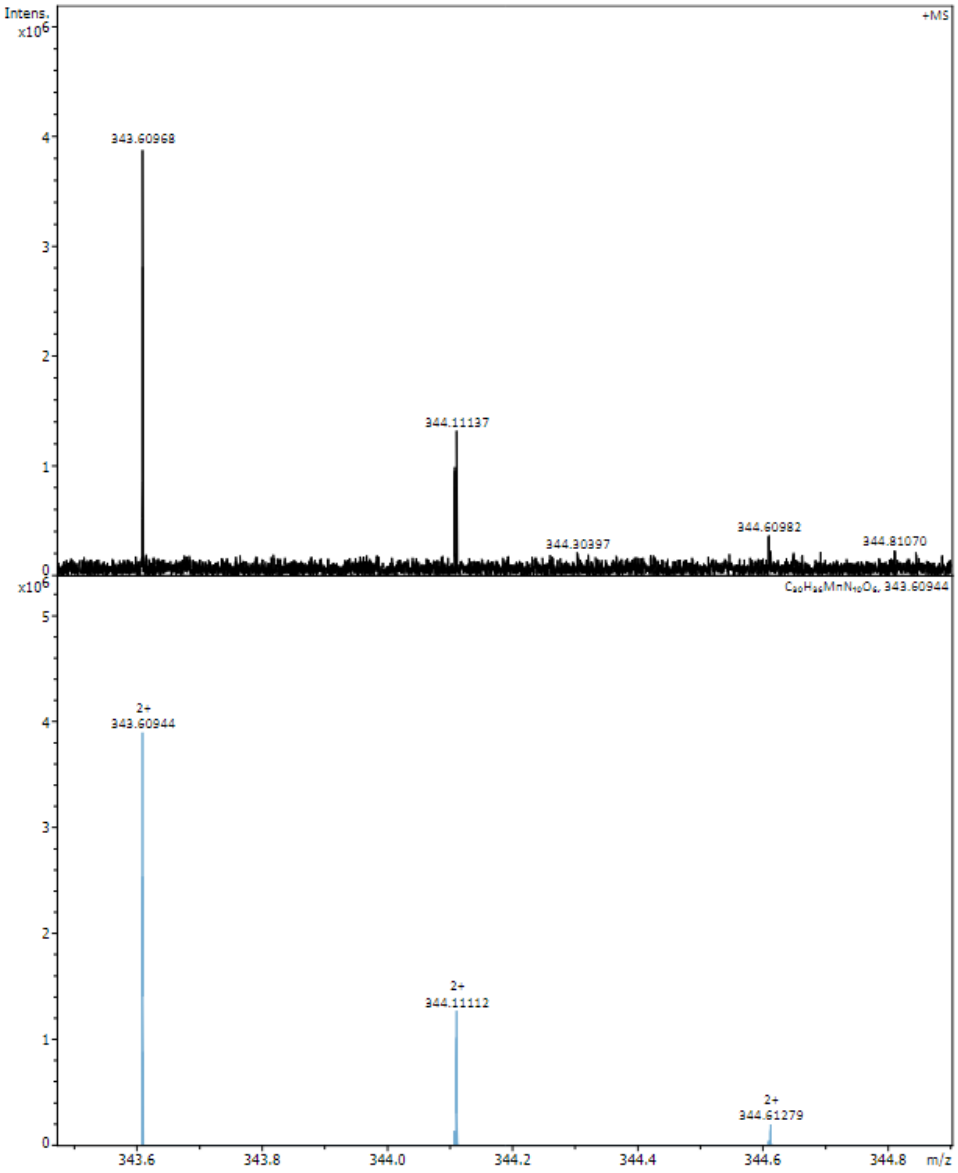
NBD-1-Co: HRMS (ESI) Calc. for $C_{30}H_{36}N_{10}O_6Co^{2+}$ $(M)^{2+}$ 345.60701, found 345.60747



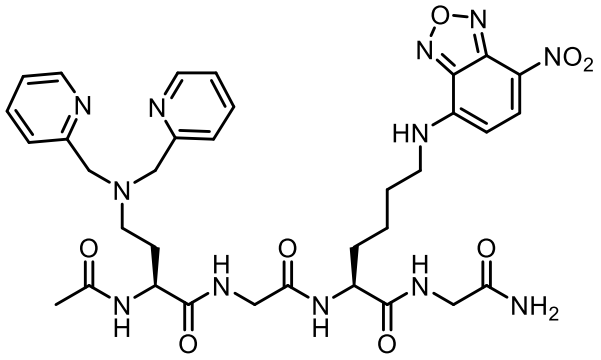


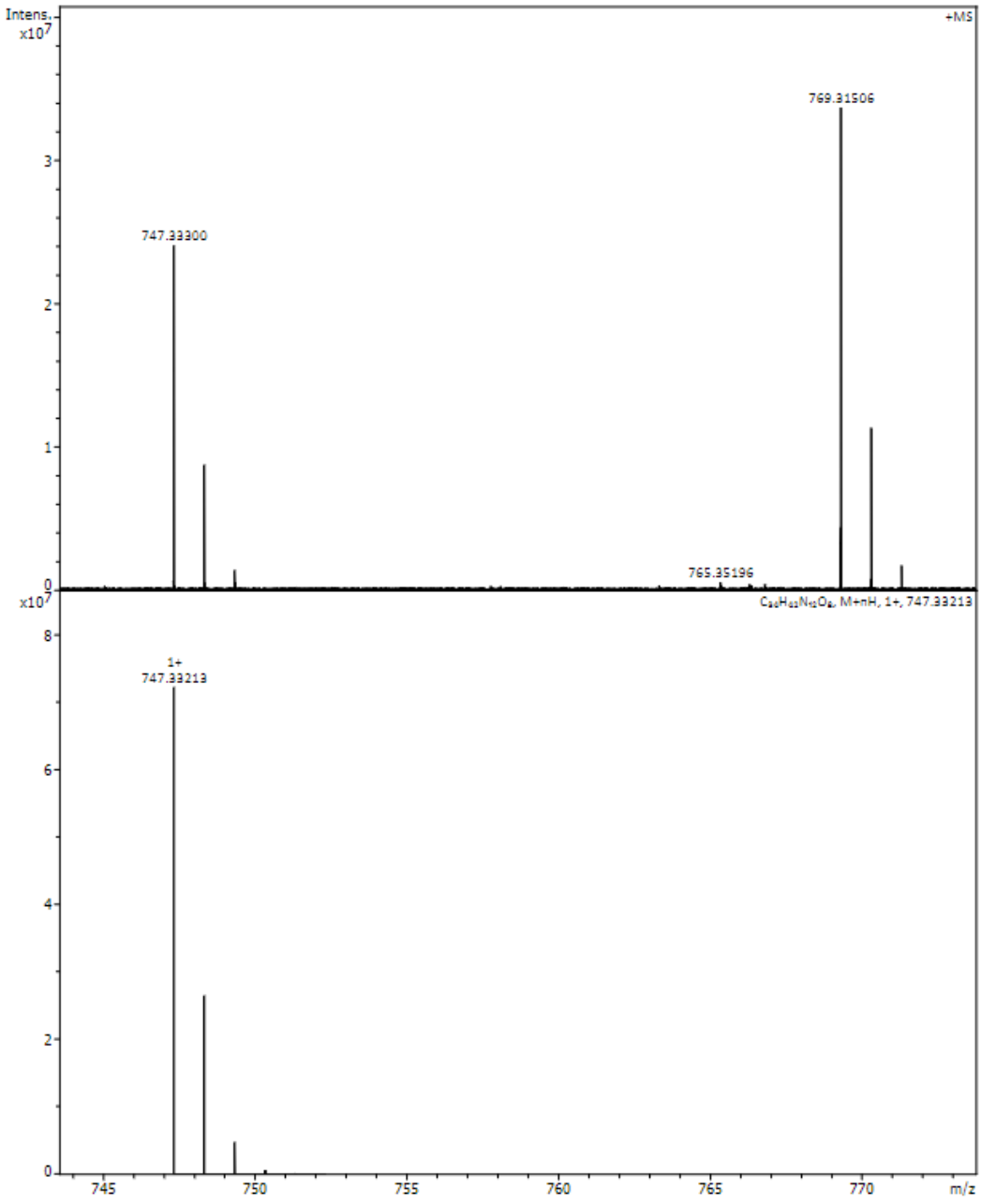
NBD-1-Mn: HRMS (ESI) Calc. for $C_{30}H_{36}N_{10}O_6Mn^{2+}$ $(M)^{2+}$ 343.60944, found 343.60968



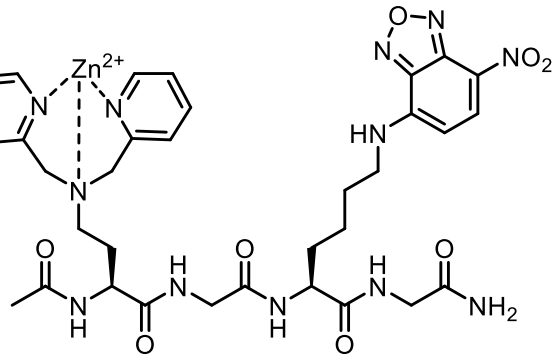


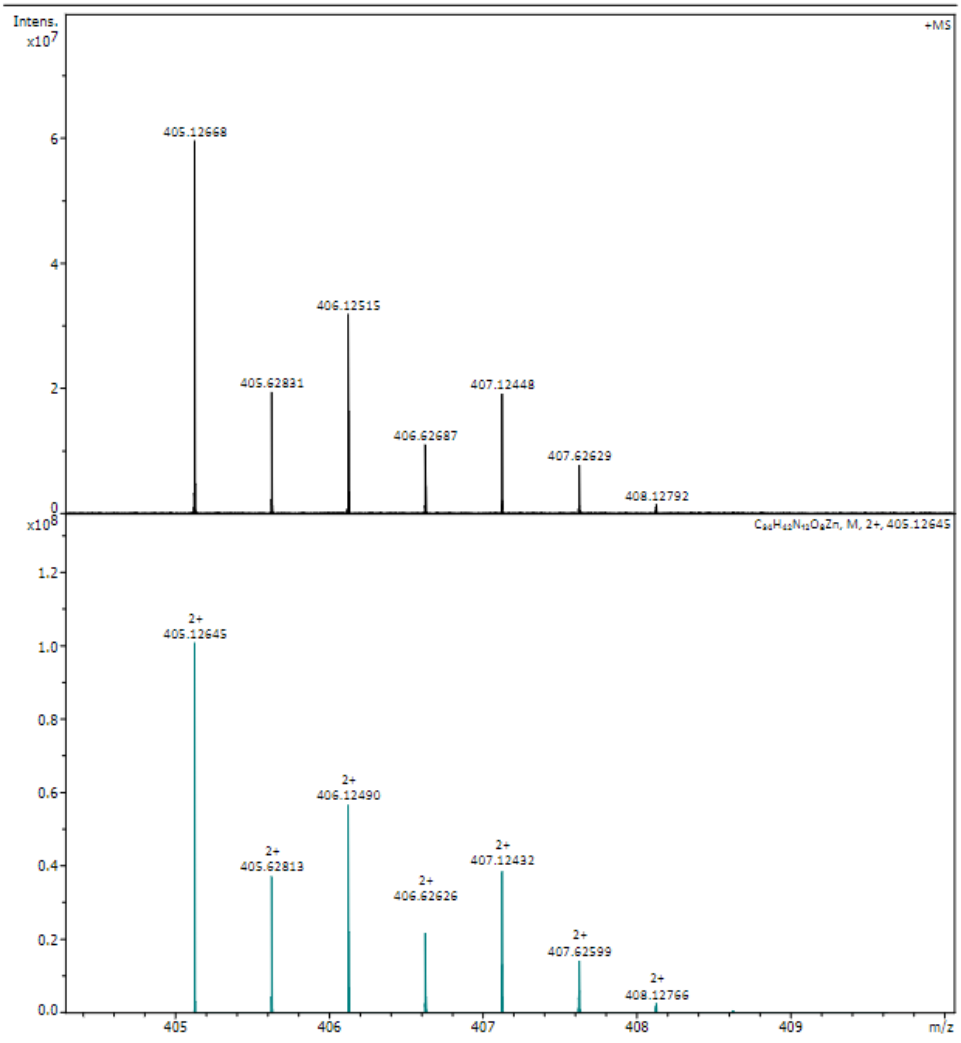
NBD-2: HRMS (ESI) Calc. for $C_{34}H_{43}N_{12}O_8$ $(M+H)^+$ 747.33300, found 747.33213



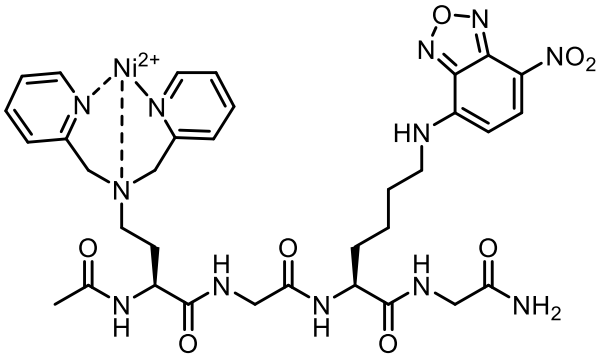


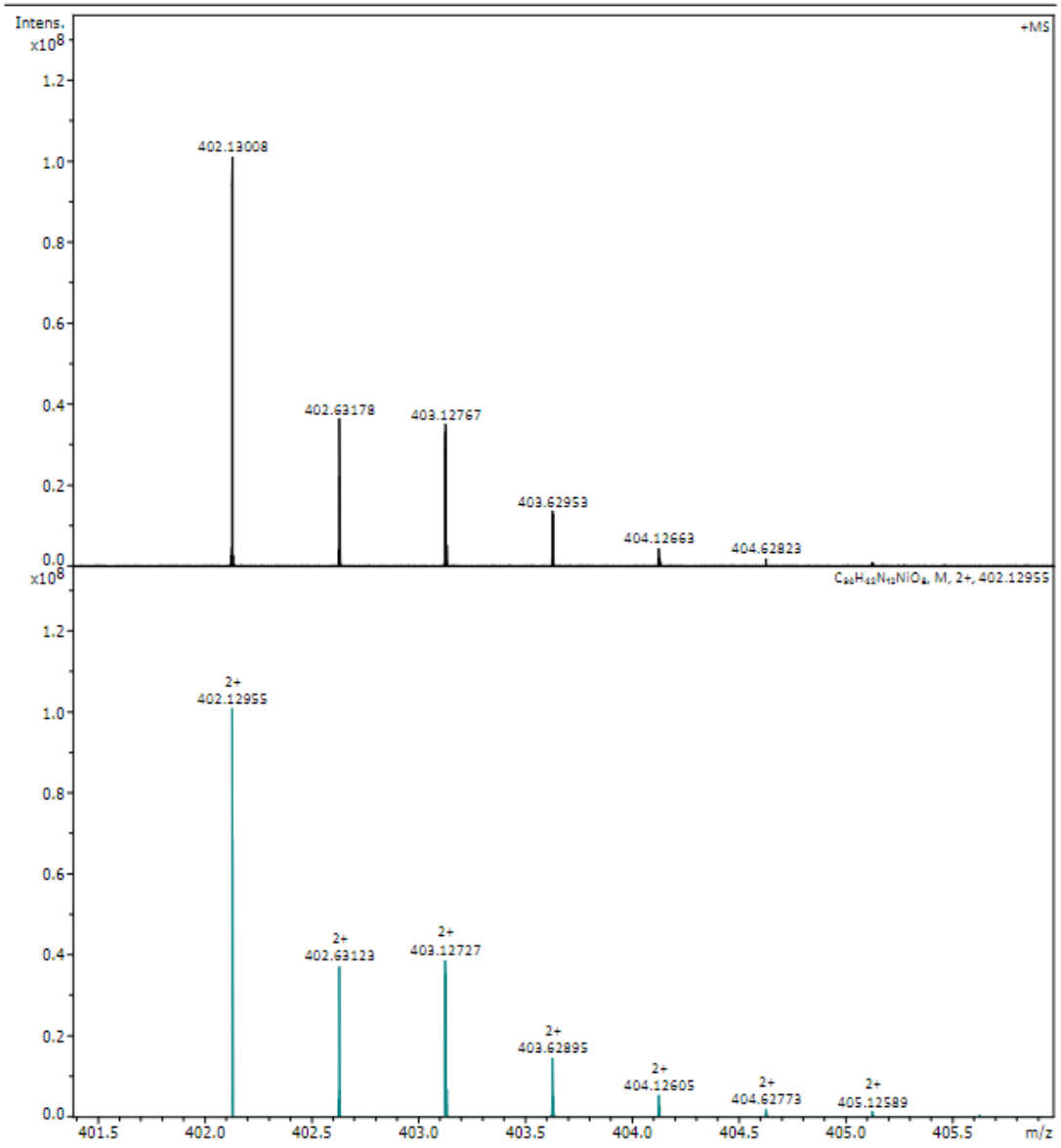
NBD-2-Zn: HRMS (ESI) Calc. for $C_{34}H_{42}N_{12}O_8Zn^{2+}$ $(M)^{2+}$ 405.12645, found 405.12668



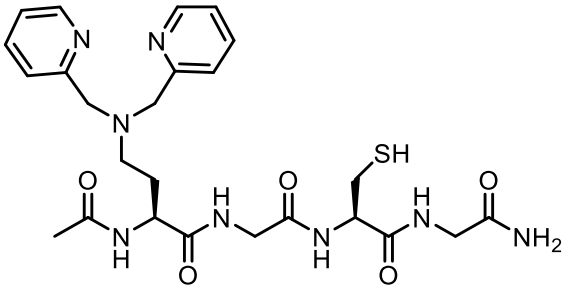


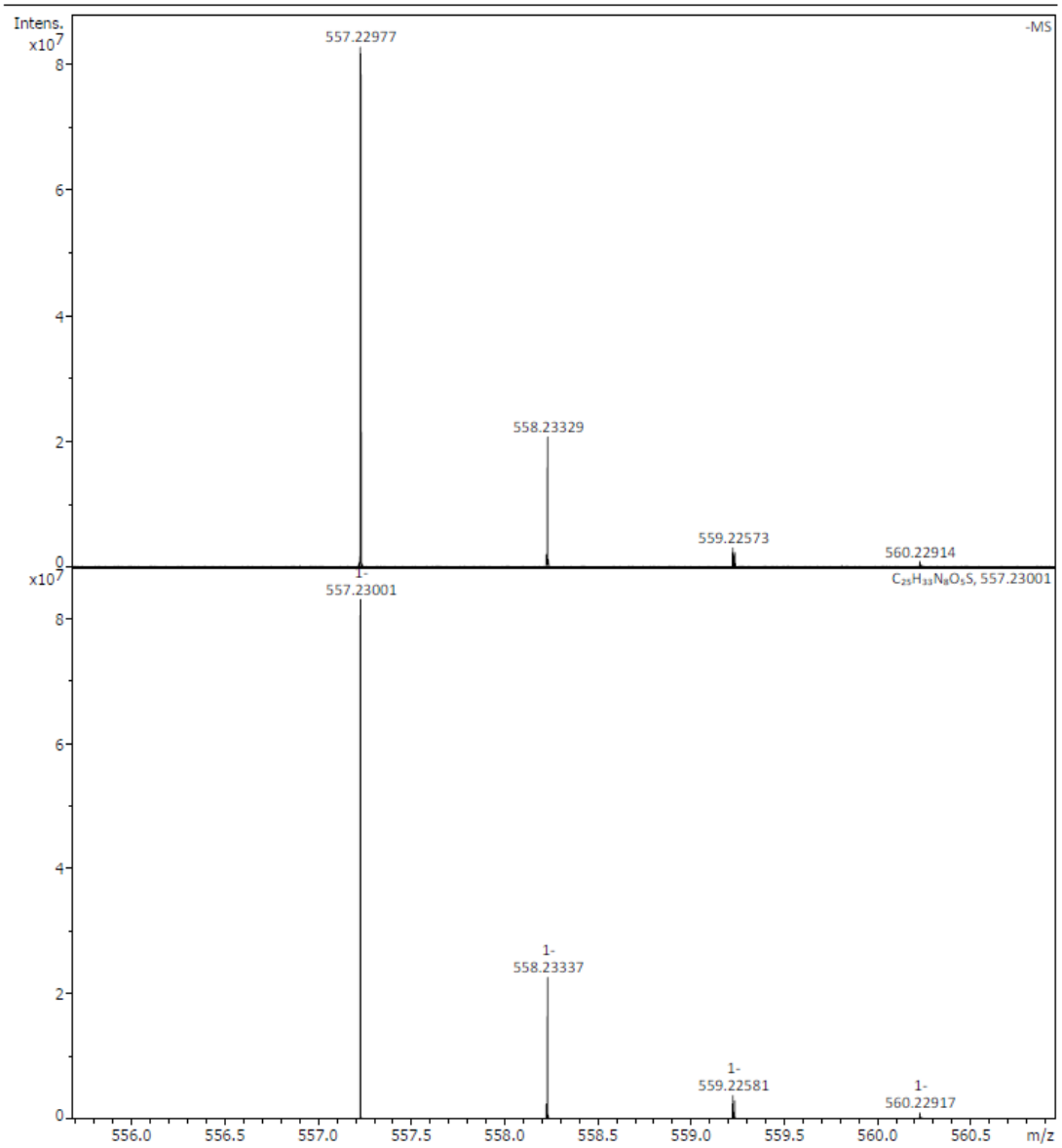
NBD-2-Ni: HRMS (ESI) Calc. for $C_{34}H_{42}N_{12}NiO_8$ $(M)^{2+}$ 402.12955, found 402.13008



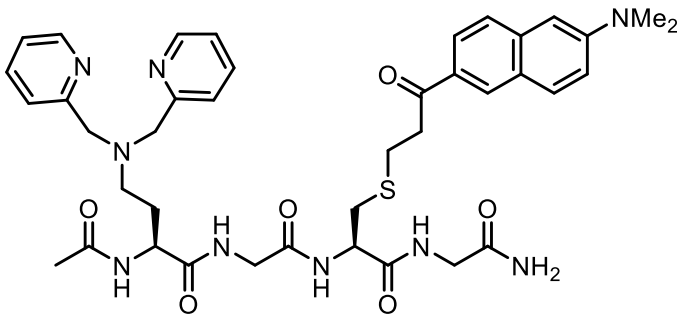


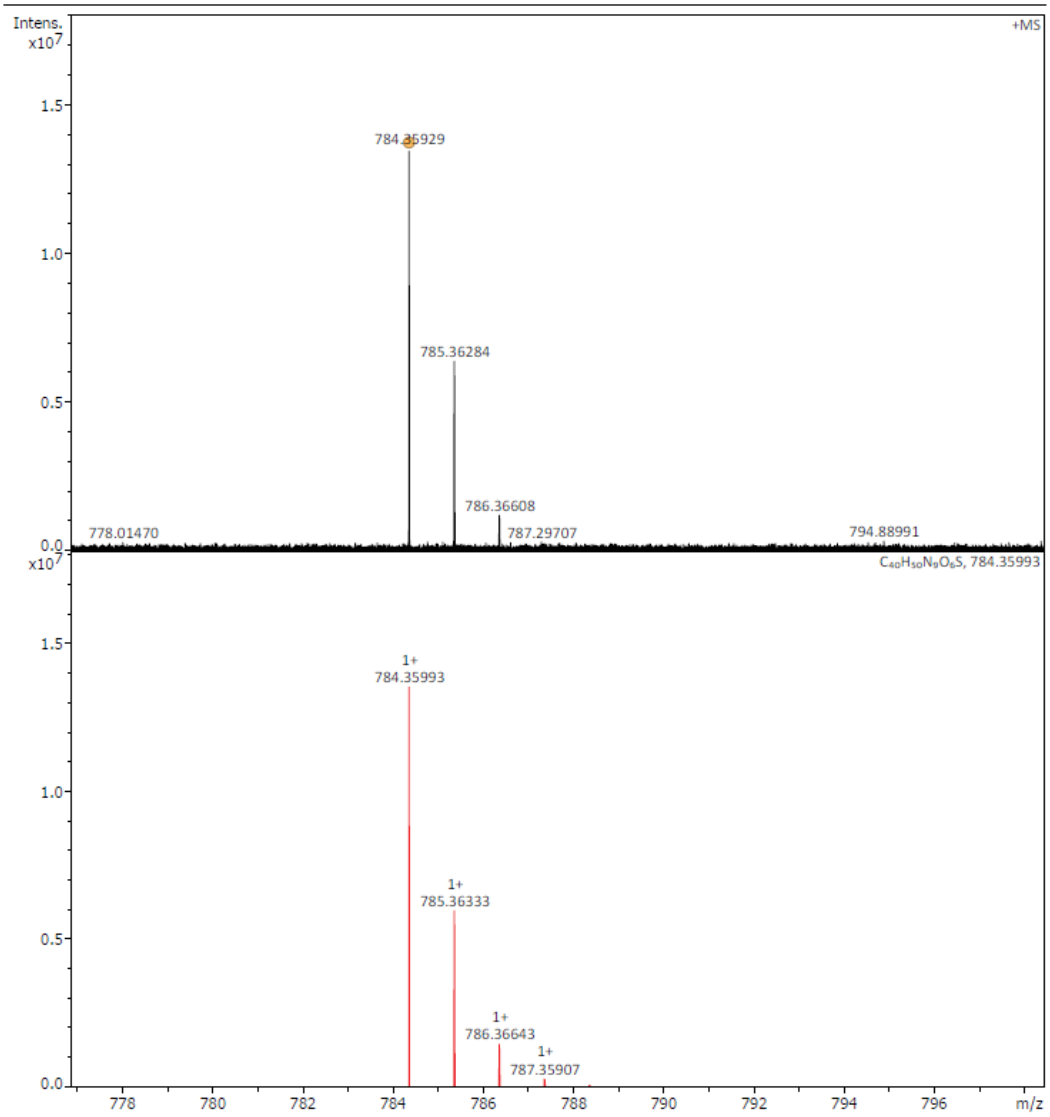
Peptide 1: HRMS (ESI) Calc. for $C_{25}H_{33}N_8O_5S$ $(M-H)^-$ 557.23001, found 557.22977



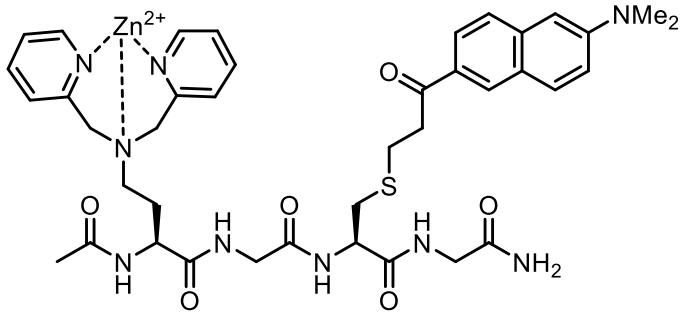


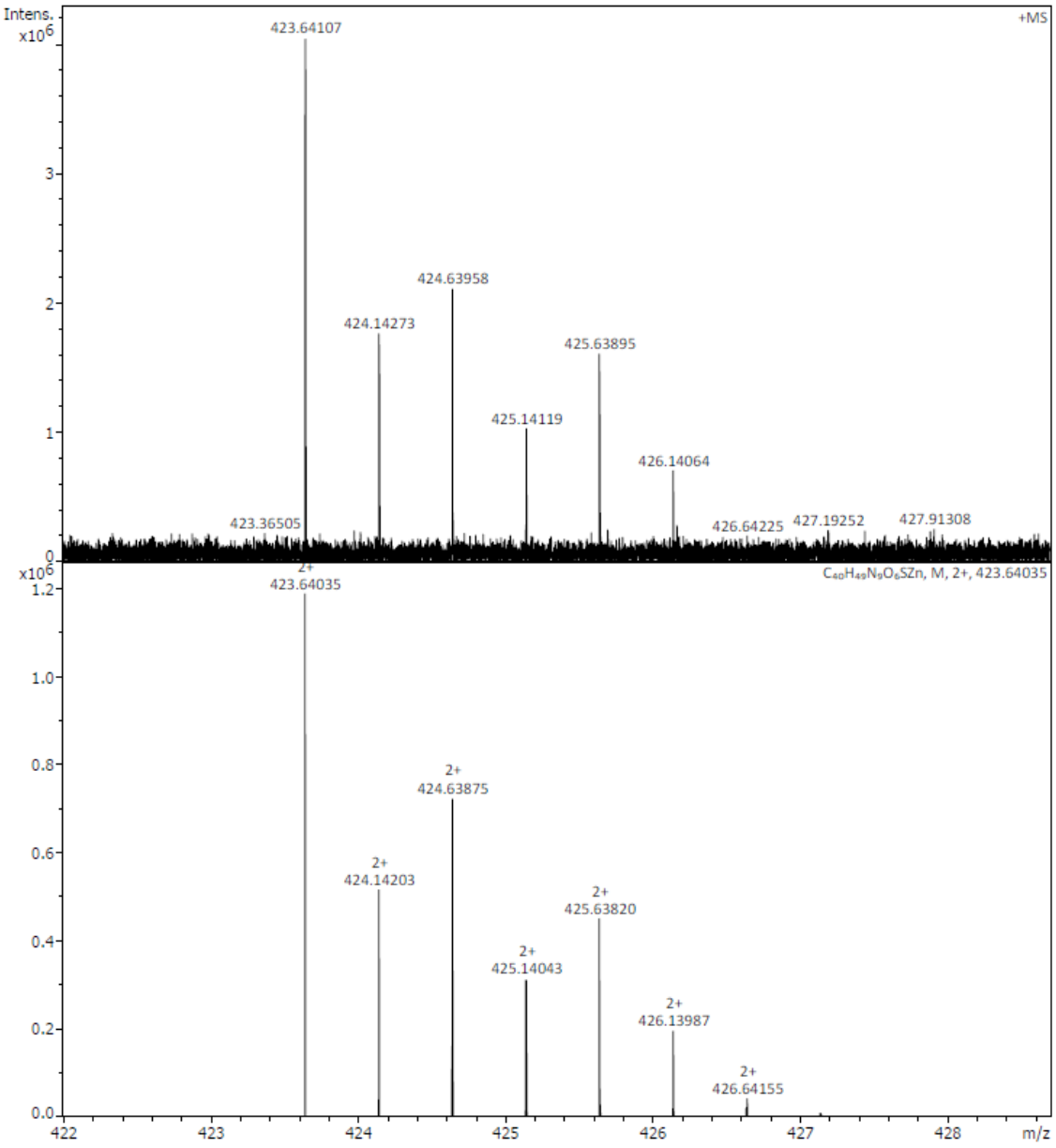
PRODAN-1: HRMS (ESI) Calc. for $C_{40}H_{50}N_9O_6S$ $(M+H)^+$ 784.35993, found 784.35927



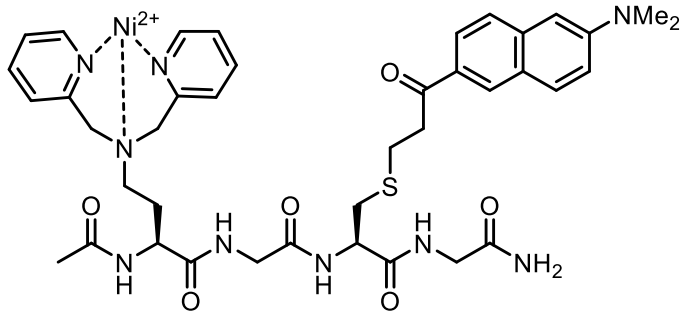


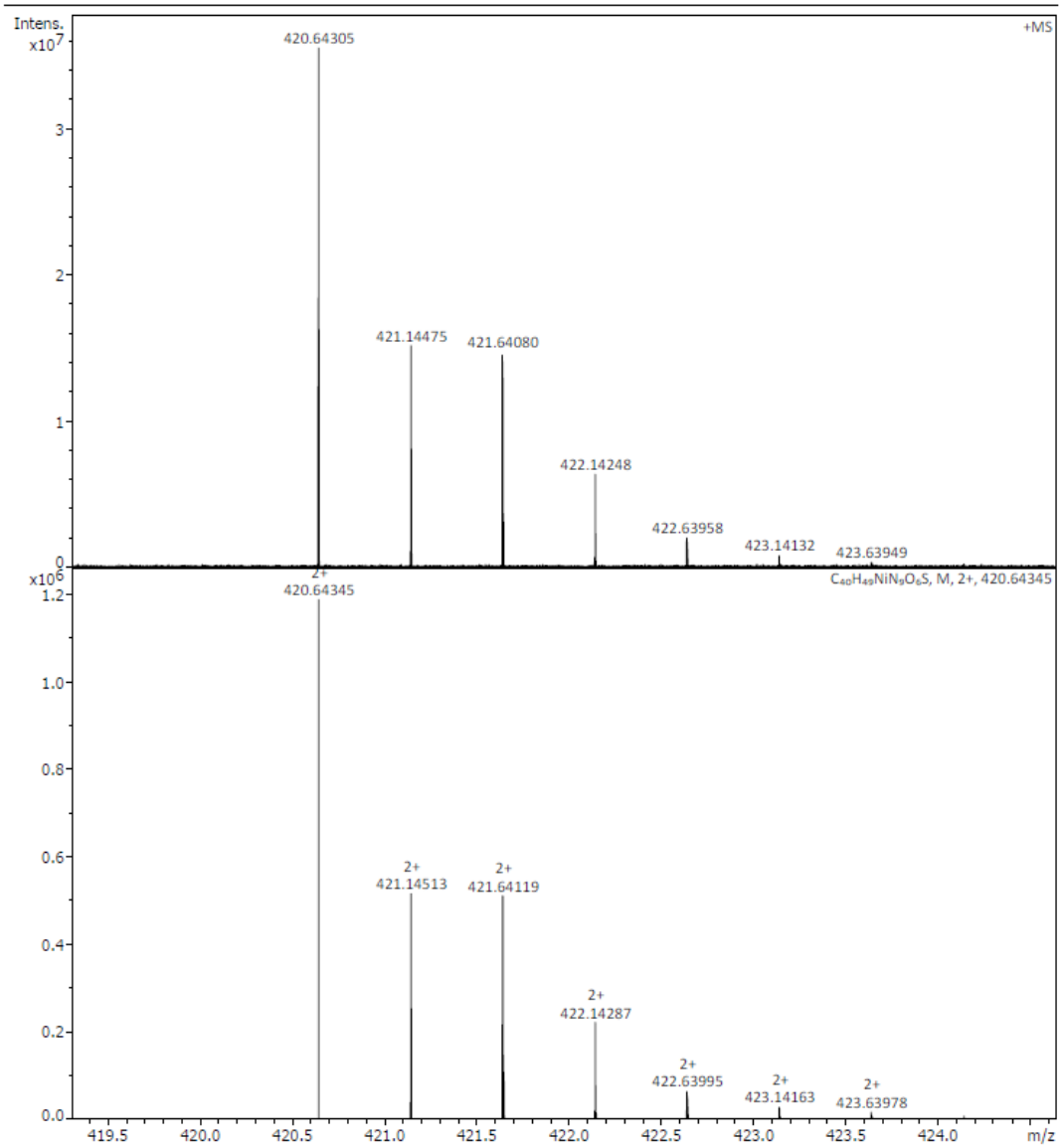
PRODAN-1-Zn: HRMS (ESI) Calc. for $C_{40}H_{50}N_9O_6SZn^{2+}$ $(M)^{2+}$ 423.64035, found 423.64107



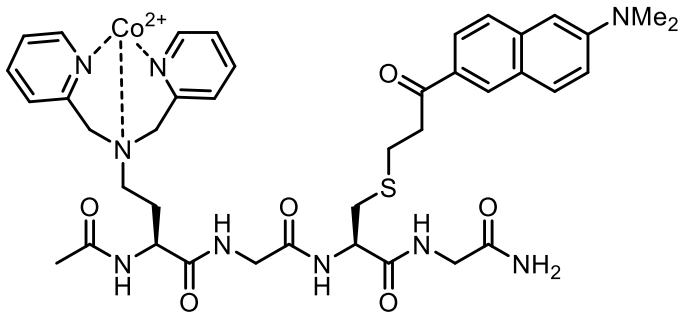


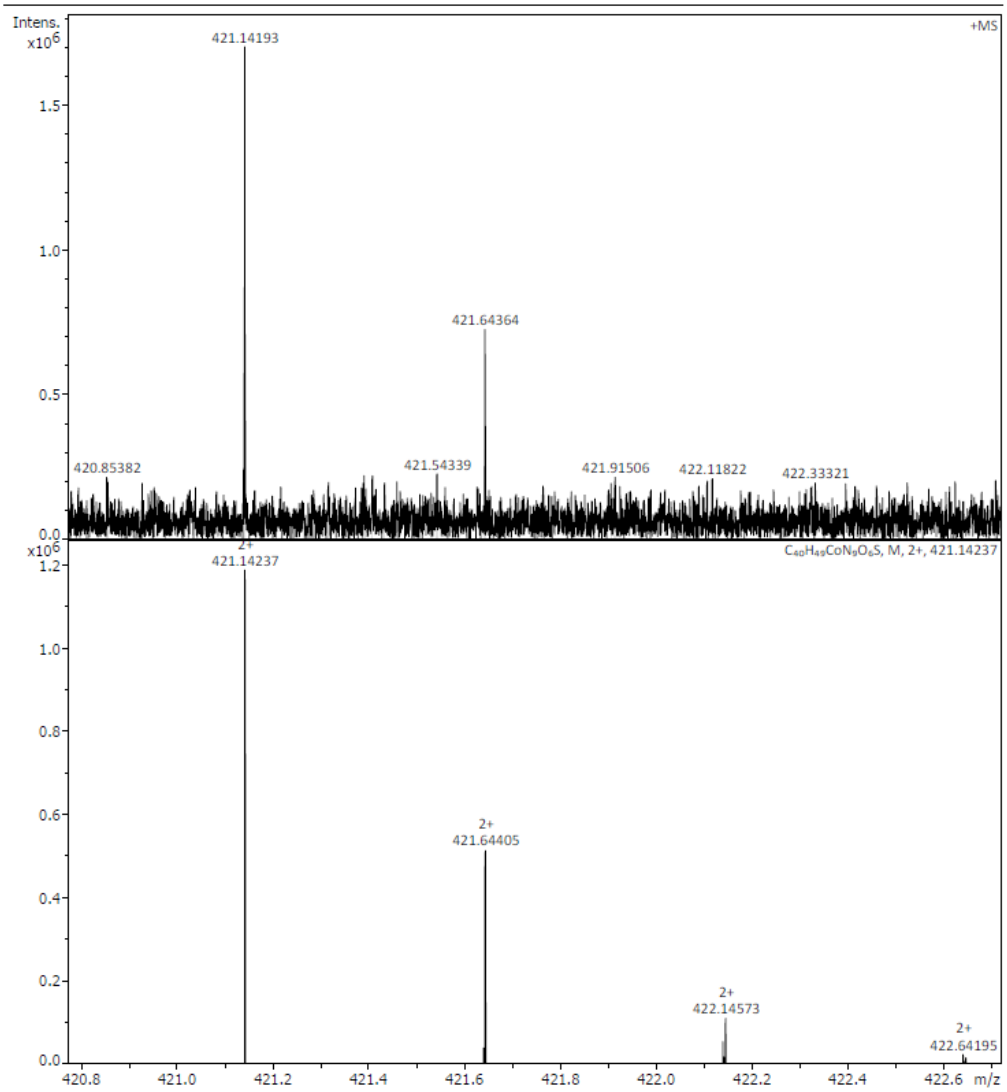
PRODAN-1-Ni: HRMS (ESI) Calc. for $C_{40}H_{50}N_9O_6SNi^{2+}$ $(M)^{2+}$ 420.64345, found 420.64305



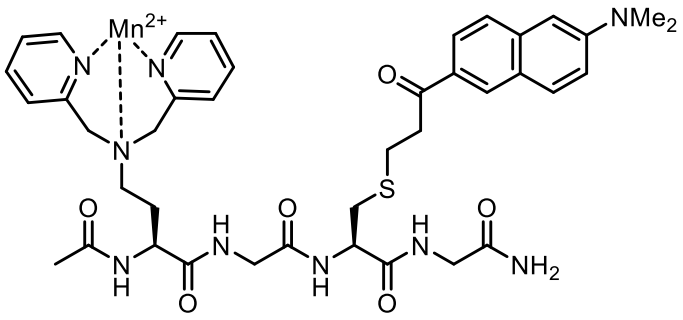


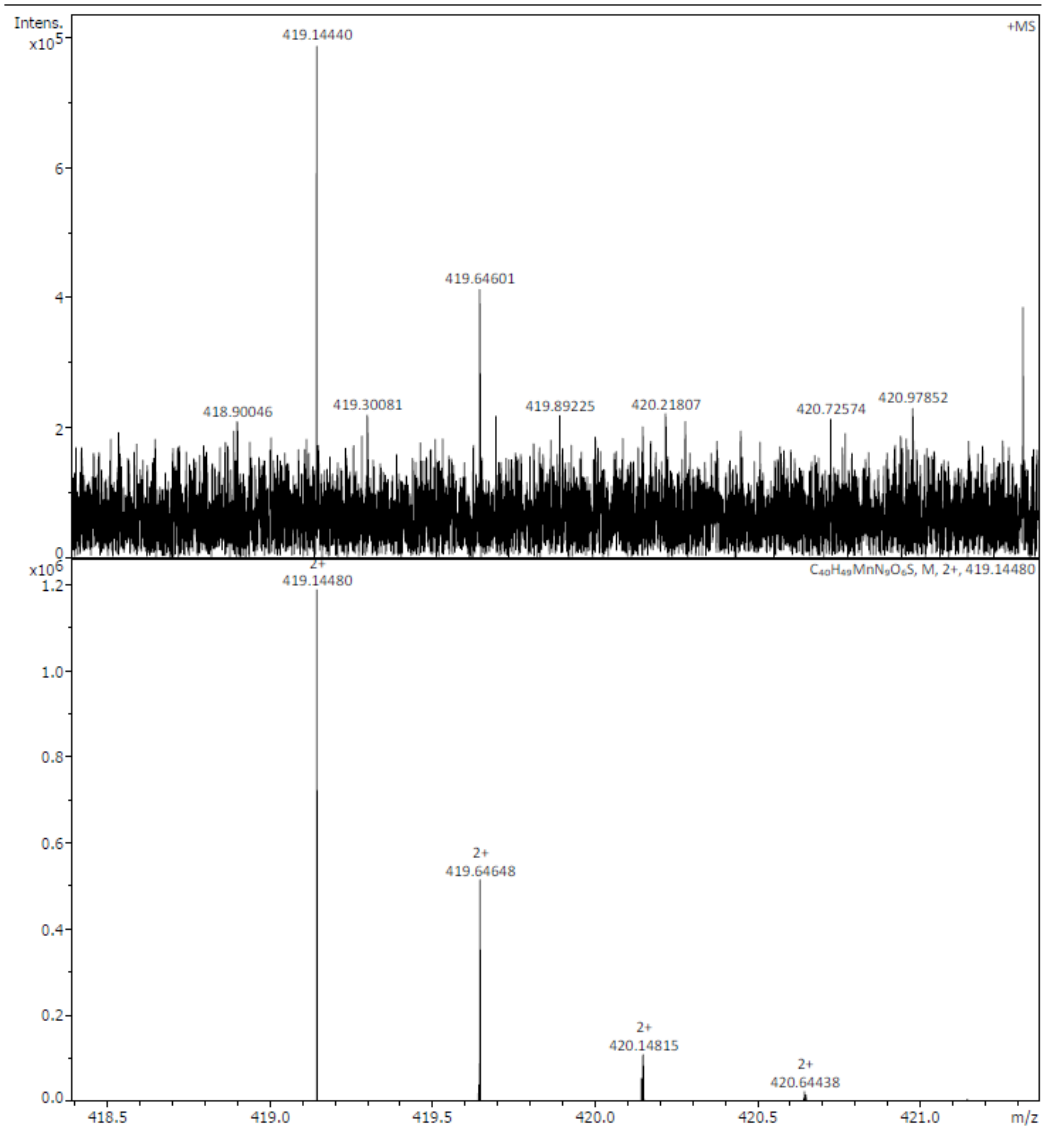
PRODAN-1-Co: HRMS (ESI) Calc. for $C_{40}H_{50}N_9O_6SCo^{2+}$ $(M)^{2+}$ 421.14237, found 421.14193





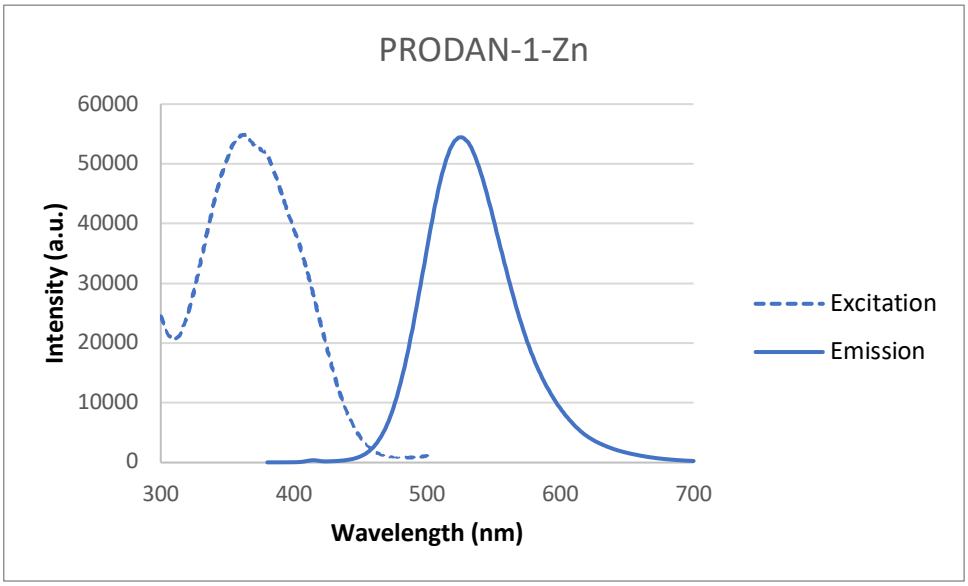
PRODAN-1-Mn: HRMS (ESI) Calc. for $C_{40}H_{50}N_9O_6SMn^{2+}$ $(M)^{2+}$ 419.14480, found 419.14440





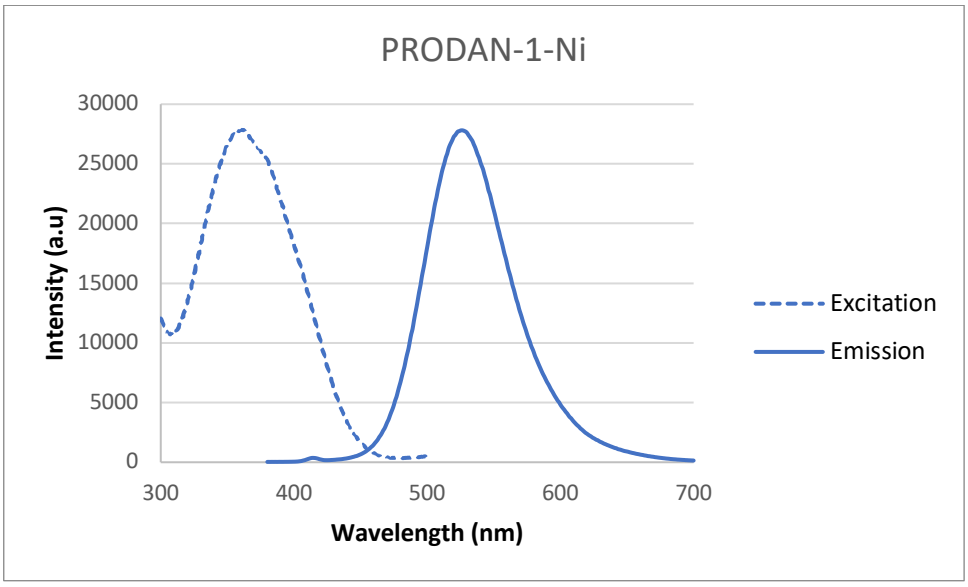
Excitation-Emission Spectra

PRODAN-1-Zn



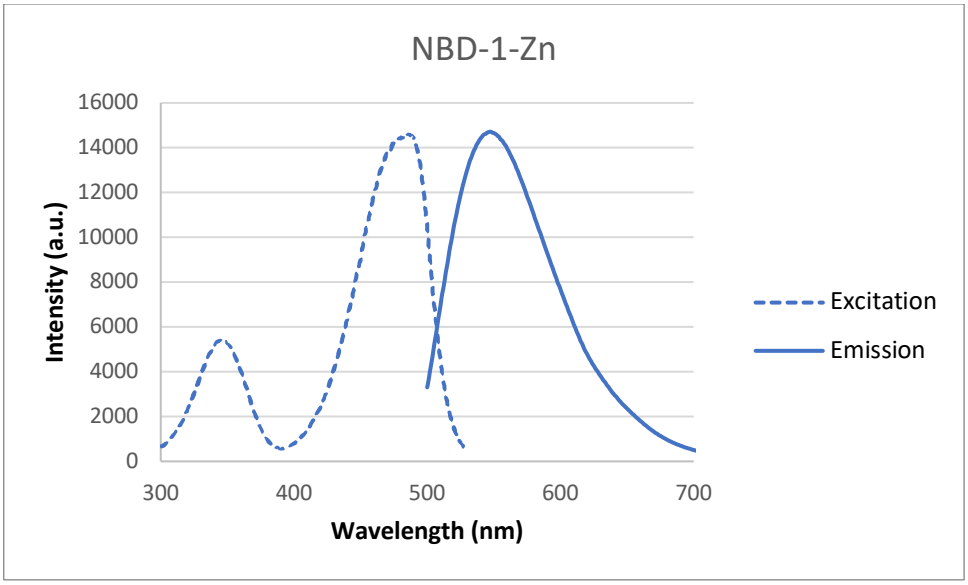
Excitation (λ_{em} = 528 nm) and emission (λ_{ex} = 362 nm) spectra of **PRODAN-1-Zn** (8 µM) in HEPES buffer (120 mM KOAc, 50 mM HEPES, pH 7.5).

PRODAN-1-Ni



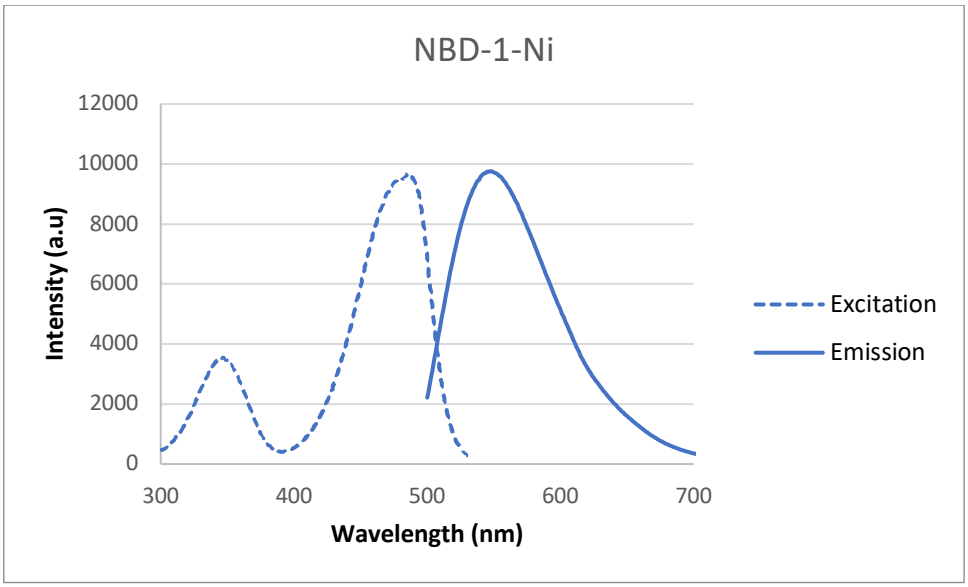
Excitation (λ_{em} = 528 nm) and emission (λ_{ex} = 362 nm) spectra of **PRODAN-1-Ni** (8 µM) in HEPES buffer (120 mM KOAc, 50 mM HEPES, pH 7.5).

NBD-1-Zn



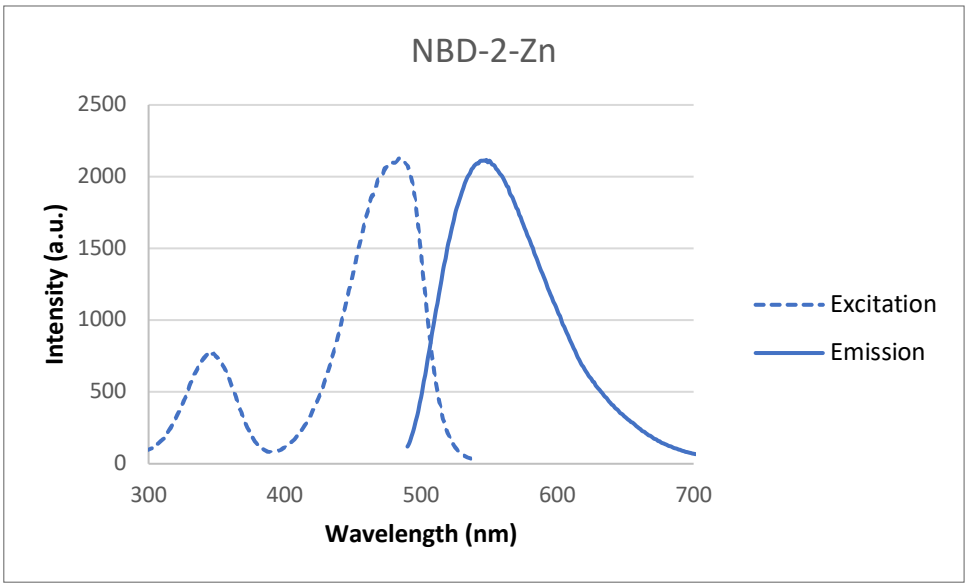
Excitation (λ_{em} = 548 nm) and emission (λ_{ex} = 484 nm) spectra of **NBD-1-Zn** (8 µM) in HEPES buffer (120 mM KOAc, 50 mM HEPES, pH 7.5).

NBD-1_Ni



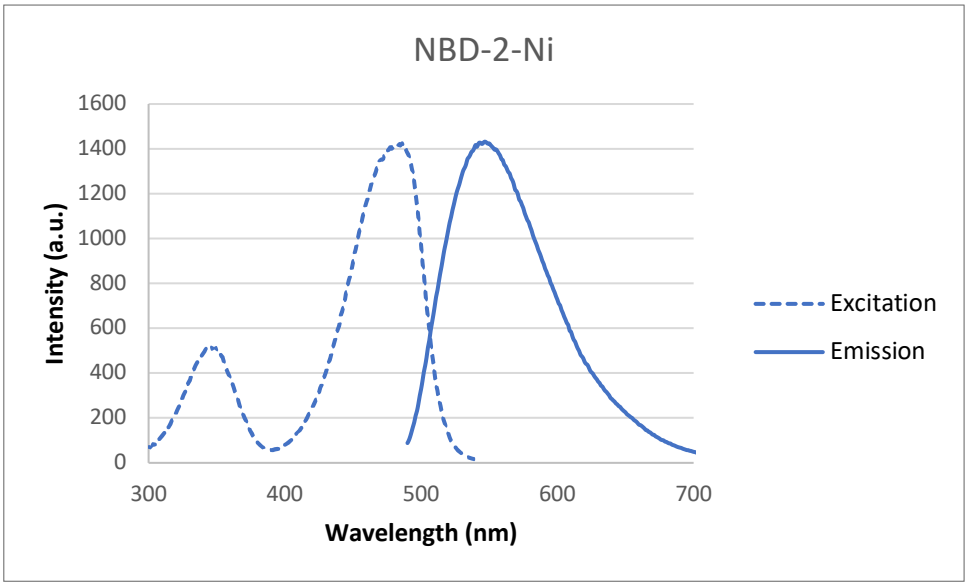
Excitation (λ_{em} = 548 nm) and emission (λ_{ex} = 484 nm) spectra of **NBD-1-Ni** (8 µM) in HEPES buffer (120 mM KOAc, 50 mM HEPES, pH 7.5).

NBD-2-Zn



Excitation (λ_{em} = 548 nm) and emission (λ_{ex} = 484 nm) spectra of **NBD-2-Zn** (8 µM) in HEPES buffer (120 mM KOAc, 50 mM HEPES, pH 7.5).

NBD-2-Ni



Excitation (λ_{em} = 548 nm) and emission (λ_{ex} = 484 nm) spectra of **NBD-2-Ni** (8 µM) in HEPES buffer (120 mM KOAc, 50 mM HEPES, pH 7.5).

References

1. B. Ercan, T. Naito, D. H. Z. Koh, D. Dharmawan and Y. Saheki, *The EMBO Journal*, 2021, **40**, e106524.
2. P. Thordarson, supramolecular.org, http://app.supramolecular.org/bindfit/, (accessed 24/01/24, 2024).
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4. K. M. DiVittorio, J. R. Johnson, E. Johansson, A. J. Reynolds, K. A. Jolliffe and B. D. Smith, *Org. Biomol. Chem.*, 2006, **4**, 1966-1976.