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In-gel detection of His-tagged proteins with 4-methoxy-1,8-naphthalimide based fluorescent probe

Marina A. Pavlova, Anna V. Vlaskina, Daria E. Tyupakova, Sergey V. Kutyakov, Yulia K. Agapova, Dmitryi E. Petrenko, Pavel A. Panchenko and Olga A. Fedorova

CONTENTS

General

Melting points were measured on Melt-temp melting point electrothermal apparatus and were uncorrected. LC-ESI-MS analyses were performed using acetonitrile solutions on a Shimadzu LCMS-2020 liquid chromatography mass spectrometer. Elemental analysis was conducted on a Carlo Erba 1108 elemental analyzer at the Laboratory of Microanalysis of the A. N. Nesmeyanov Institute of Organoelement Compounds of the Russian Academy of Sciences. ^{1}H and ^{13}C NMR spectra were recorded on an Avance 400 (Bruker) and Inova 400 (Agilent) spectrometers. The NMR chemical shifts (given as δ) were determined with an accuracy of 0.01 ppm relative to the signals corresponding to the residual solvents and recalculated to the internal standard (TMS); the spin-spin coupling constants (J) were measured with an accuracy of 0.1 Hz. The numbering of carbon atoms in the compounds used by us for the description of ^{1}H and ^{13}C NMR spectra is shown on Figure S1. The assignment of signals in the NMR spectra of compounds **2-4** was carried out using two-dimensional techniques ^{1}H COSY, HSQC and HMBC with pulsed field gradients. ^{1}H NMR spectra of **2** and **3** are presented on Fig. S2 and Fig. S3 respectively. Two-dimensional spectra, as well as ^{1}H and ^{13}C NMR spectra of the compound **4** are shown in the Figures S5-S9.

The absorption spectra were taken on a Cary 300 spectrophotometer (Agilent Technologies). The fluorescence measurements were performed using a Fluorolog3-221 spectrofluorimeter (Horiba Jobin Yvon) and Cary Eclipse spectrofluorimeter (Agilent

Technologies). Solvent used in spectroscopic studies was of HPLC grade. Spectral measurements were carried out in air-saturated solutions at ambient temperature. All measured fluorescence spectra were corrected for the nonuniformity of detector spectral sensitivity. For spectroscopic experiments **4** was dissolved in DMSO as millimolar stock solution and diluted by HEPES-buffer solution (pH = 7.4, 0.01M) to obtain micromolar solution.

Coumarin 481 in acetonitrile (φ^{fl} = 0.08)[S1] was used as the reference for the fluorescence quantum yield measurements. The fluorescence quantum yields were calculated by the Eq. (S1),[S2]

$$\varphi^{fl} = \varphi_R^{fl} \frac{S}{S_R} \cdot \frac{(1 - 10^{-A_R})n^2}{(1 - 10^{-A})n_R^2} \qquad \text{(S1)}$$

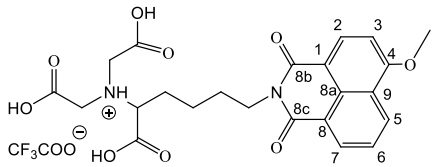
wherein φ^{fl} and φ_R^{fl} are the fluorescence quantum yields of the studied solution and the standard compound respectively; A and A_R are the absorptions of the studied solution and the standard respectively; S and S_R are the areas underneath the curves of the fluorescence spectra of the studied solution and the standard respectively; and n and n_R are the refraction indices of the solvents for the substance under study and the standard compound.

Complex formation of compound **4** with Ni^{2+} was studied by spectrofluorometric titration. The ratio of **4** to Ni^{2+} was varied by adding aliquots of a solution of nickel(II) perchlorate in water of known concentration to a solution of ligand **4** in the HEPES buffer (0.01M, pH = 7.4). The fluorescence spectrum of each solution was recorded, and the stability constants of the complexes were determined using the SPECFIT/32 program (Spectrum Software Associates, West Marlborough, MA).

To prepare the working solution of **4** for in-gel staining, a 20 μM stock solution of the dye in DMSO was diluted 100-fold (to a concentration of 0.2 μM) with 20 mM Na-phosphate buffer pH 7.6 and 5 equivalents of $NiSO_4$ were added. Electrophoresis was performed using the Laemmli method[S3]. Each of the test proteins was applied to the gel in duplicate: BSA 30 and 150 pmol, TEV 25 and 200 pmol, His-tagged bacterial endonuclease 70 and 140 pmol, bacterial endonuclease without His-tag 90 and 180 pmol. After incubation in a fixer solution (10% acetic acid, 40% ethanol, 50% water), the gel plate was washed twice - with distilled water and then with 20 mM Na-phosphate buffer pH 7.6. Staining was performed with a working solution of the dye for 60 minutes at room temperature. To wash off the background, the gel plate was washed twice with phosphate buffer (incubation for 10 minutes). Visualization was performed on a Gel Doc EZ Imager (Bio-Rad) in the Ethidium Bromide mode (excitation at 312 nm). HiGenoMB® (Himedia) was used as a molecular mass marker.

Synthesis

Sodium methoxide was prepared in advance by adding metallic sodium to methanol followed by evaporation of methanol. All other synthetic reagents were purchased from commercial sources. The reaction course and purity of the final products was followed by TLC on aluminium oxide (TLC Aluminium oxide 60 F_{254}, Merck). Column chromatography was conducted over aluminium oxide (Aluminium oxide, particle size 0.04-0.20 mm, ChromLab). Flash chromatography was performed at IsoleraPrime (Biotage).



4

Figure S1. A numbering of carbon atoms in compound **4**

Compound 2. 4-Nitro-1,8-naphthalic anhydride (0.166 g, 0.68 mmol) was reacted with 0.293 g (0.68 mmol) of N_α,N_α-bis(carboxymethyl)-L-lysine **1** in boiling ethanol for 14 h. Then the solvent was removed in vacuum to obtain heavy yellow oil. Product was isolated by flash column chromatography on Al_2O_3 (gradient elution from CH_2Cl_2 to CH_2Cl_2 : MeOH = 11:1 (v/v)) to give 0.142 g of product 1 as a yellow oil (32% yield). ^{1}H NMR (300 MHz, $CDCl_3$, 20 ºC): δ = 1.35-1.51 (m, 27H, 3×$(CH_3)_3$C), 1.50-1.83 (m, 6H, 3×CH_2), 3.25-3.39 (m, 1H, CH), 3.39-3.56 (m, 4H, 2×CH_2), 4.19 (t, 2H, J = 7.4, CH_2-NI), 7.95 – 8.03 (m, 1H, H(6)), 8.40 (d, 1H, J = 7.4, H(3)), 8.68 (d, 1H, J = 7.8, H(7)), 8.73 (d, 1H, J = 7.4, H(2)), 8.84 (d, J = 8.6, 1H, H(5)). ^{13}C NMR (100.60 MHz, $CDCl_3$, 18 ºC): 23.61 (CH_2), 27.83 (CH_2), 28.11 (9×CH_3), 30.47 (CH_2), 40.65 (CH_2-NI), 53.65 (2×CH_2N), 65.28 (CH), 80.54 (2×$\underline{C}(CH_3)_3$), 81.01 ($\underline{C}(CH_3)_3$), 123.12 (C(8)), 123.66 (C(4a)), 123.84 (C(3)), 127.11 (C(1)), 129.09 (C(8a)), 129.18 (C(5)), 129.70 (C(7)), 129.87 (C(6)), 132.35 (C(2)), 149.58 (C(4)), 159.73 (C(8c)), 159.92 (C(8b)), 170.62 (2×COOtBu), 172.33 (COOtBu). ESI-mass m/z: calculated: 655.31 [M], found: 656.40 ($[M+H]^+$).

Compound 3. MeONa (0.012 g, 0.22 mmol) was added to compound **2** (0.142 g, 0.22 mmol) in MeOH. The mixture was refluxed for 6.5 hours. Then the solvent was removed in vacuum. The resulting oil was dissolved in CH_2Cl_2, and the insoluble inorganic impurities were filtered off. After removing the solvent of filtrate in vacuum, the resulting oil was chromatographed on Al_2O_3 (gradient elution from CH_2Cl_2 to CH_2Cl_2 : MeOH = 99:1 (v/v)) to give 0.077 g of product **3** as yellow oil (55% yield). ^{1}H NMR (300 MHz, $CDCl_3$, 20 ºC): δ = 1.33 – 1.53 (m, 27H, 3×$(CH_3)_3$C), 1.65 – 1.83(m, 6H, 3×CH_2), 3.27-3.38 (m, 1H, CH), 3.40 – 3.58 (m, 4H, 2×CH_2), 4.11-4.21 (m, 5H, OMe, CH_2-NI), 7.05 (d, 1H, J = 8.2, H(3)), 7.67 – 7.75 (m, 1H, H(6)), 8.53 – 8.63 (m, 3H, H(2), H(7), H(5)).^{13}C NMR (100.60 MHz, $CDCl_3$, 18 ºC): δ = 27.96 (CH_2), 28.12

(9×CH_3), 30.54 (CH_2), 30.90 (CH_2), 40.05 (CH_2-NI), 53.69 (2×CH_2N), 56.16 (OCH3), 65.47 (CH), 80.52(2×$\underline{C}(CH_3)_3$), 81.08 ($\underline{C}(CH_3)_3$), 105.14 (C(3)), 115.21 (C(1)), 123.50 (C(4a), C(8)), 125.88 (C(6)), 128.52 (C(2)), 129.39 (C(8a)), 131.48 (C(7) or C(5)), 133.35 (C(7) or C(5)), 160.73 (C(4)), 163.89 (C(8c)), 164.42 (C(8b)), 170.69 (2×COOtBu)), 172.37 (C(COOtBu)). Elemental analysis, calculated (%) for $C_{35}H_{48}N_2O_9 \cdot 0.7CH_2Cl_2$: C, 61.23; H, 7.11; N, 4.00; found (%): C, 61.38; H, 7.19; N, 4.00. ESI-mass m/z: calculated: 640.34 [M], found: 641.30 ($[M+H]^+$).

Compound 4. Compound **3** (0.088 g, 0.14 mmol) was dissolved in a solvent mixture of water:methanol (15 ml, 1:1 v./v.). Then 1 ml of a concentrated trifluoroacetic acid was added. The reaction mixture was stirred for 22 hours at 80 °C. After removing the solvent in vacuum the resulting oil was recrystallized from methanol to give 46 mg of **4** (58% yield). ^{1}H NMR (300 MHz, DMSO-d_6, 18 ºC): δ = 1.50 – 1.75 (m, 6H, 3×CH_2), 3.36 – 3.79 (m, 5H, CH, 2×CH_2N)), 4.07 – 3.93 (m, 2H, CH_2-NI), 4.12 (s, 3H, OMe), 7.33 (d, 1H, J = 7.4, 1H, H(3)), 7.76 – 7.87 (m, 1H, H(6)), 8.42-8.57 (m, 3H, H(2), H(7), H(5)). ^{13}C NMR (100.60 MHz, DMSO-d_6, 18 ºC): δ = 27.30 (CH_2), 28.80 (CH_2), 29.12 (CH_2), 39.21 (CH_2-NI (with solvent signal)), 51.12 (2×CH_2N), 51.89 (CH), 56.62 (OCH_3), 106.27 (C(3)), 114.24 (C(1)), 121.90 (C(8)), 122.77 (C(4a)), 126.37 (C(6)), 128.25 (C(5)), 128.57 (C(8a)), 131.04 (C(7)), 133.29 (C(2)), 160.32 (C(4)), 162.91 (C(8b) or C(8c)), 163.49 (C(8b) or C(8c)), 172.92 (2×COOH), 173.29 (COOH). Elemental analysis, calculated (%) for $C_{25}H_{25}F_3N_2O_{11} \cdot 3H_2O$: C, 46.88; H, 4.88 N, 4.37; found (%): C, 46.79; H, 4.87; N, 4.35. ESI-mass m/z: calculated: 472.15 ($[M]^+$), found: 472.85 ($[M]^+$), 470.85 ($[M-H]^-$).

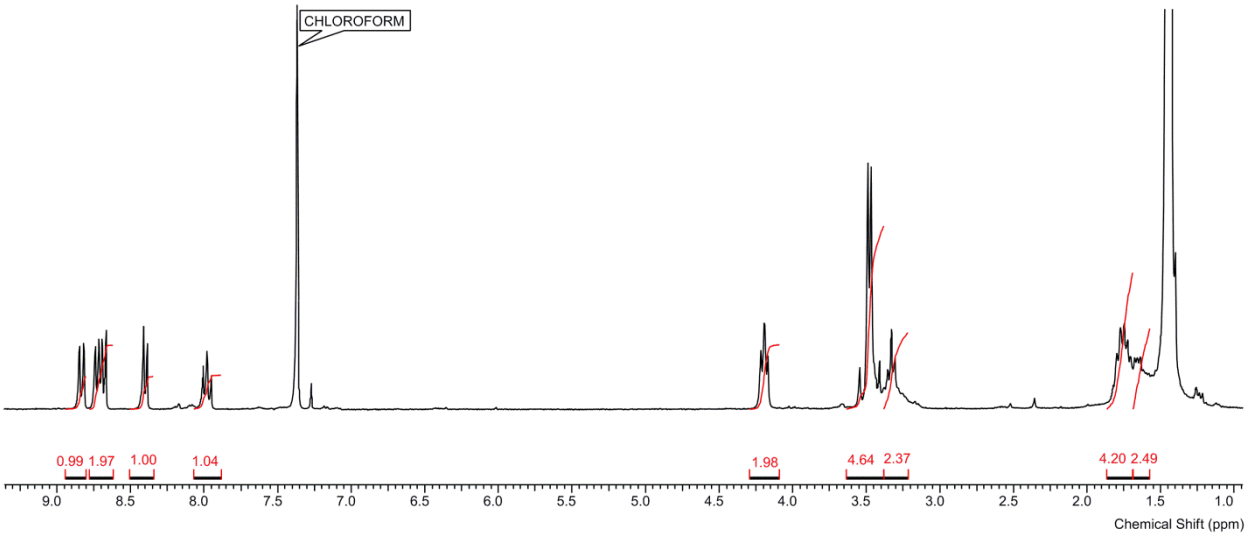


Figure S2. ^{1}H NMR spectrum of **2** in $CDCl_3$.

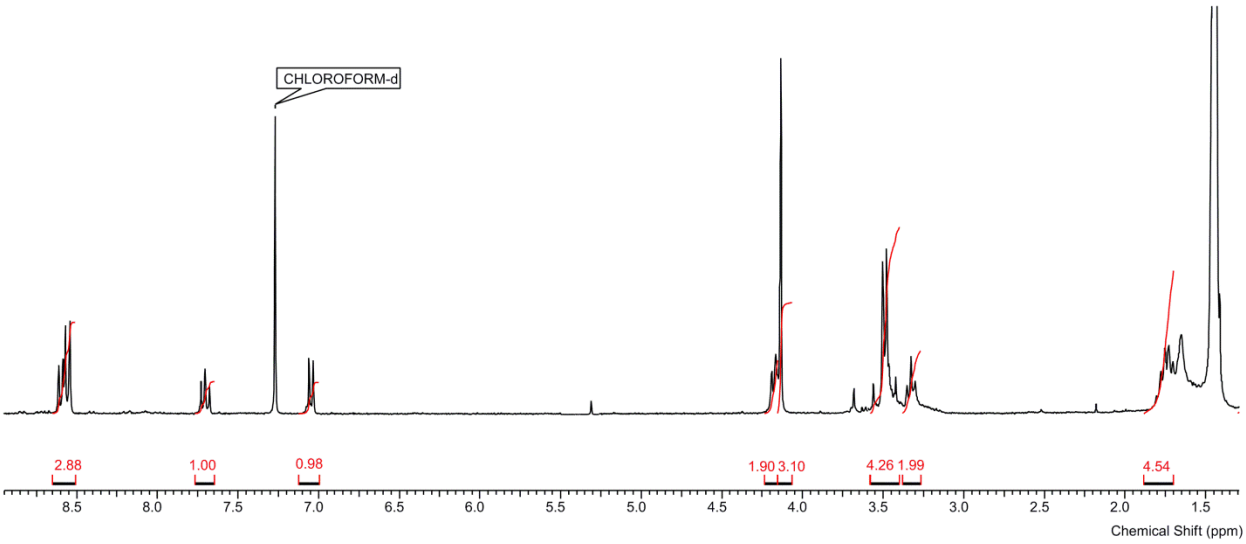


Figure S3. ^{1}H NMR spectrum of **3** in $CDCl_3$.

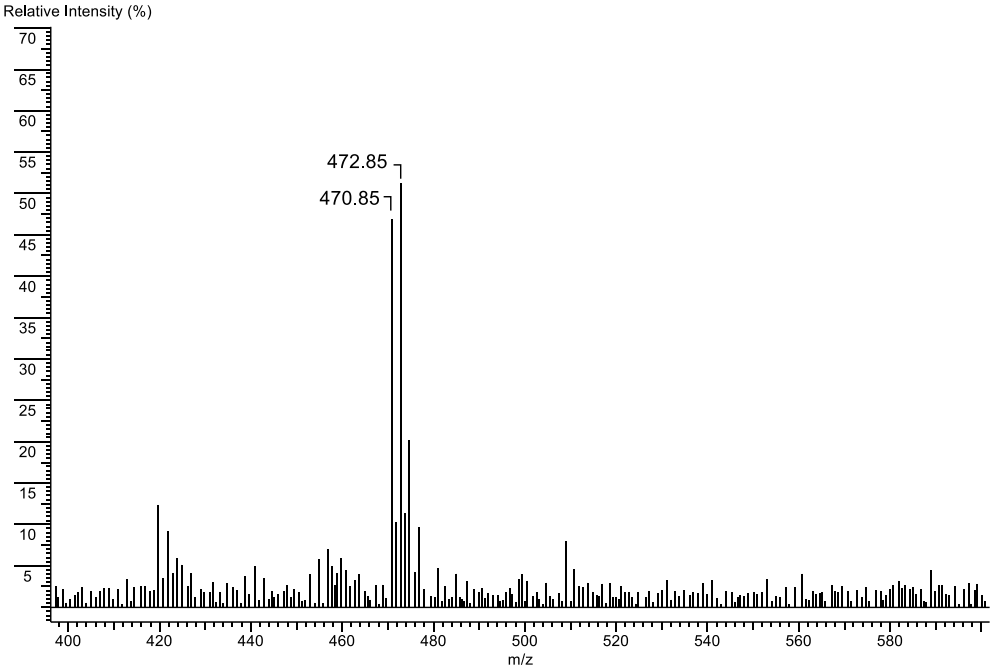


Figure S4. ESI-mass spectrum of **4** in MeCN.

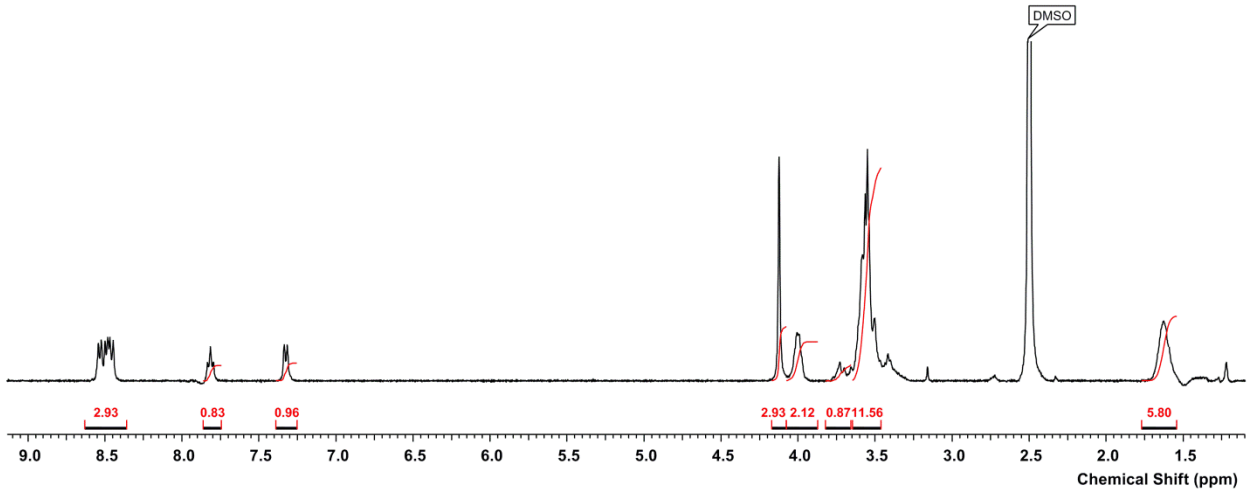


Figure S5. ^{1}H NMR spectra of **4** in DMSO-d_6.

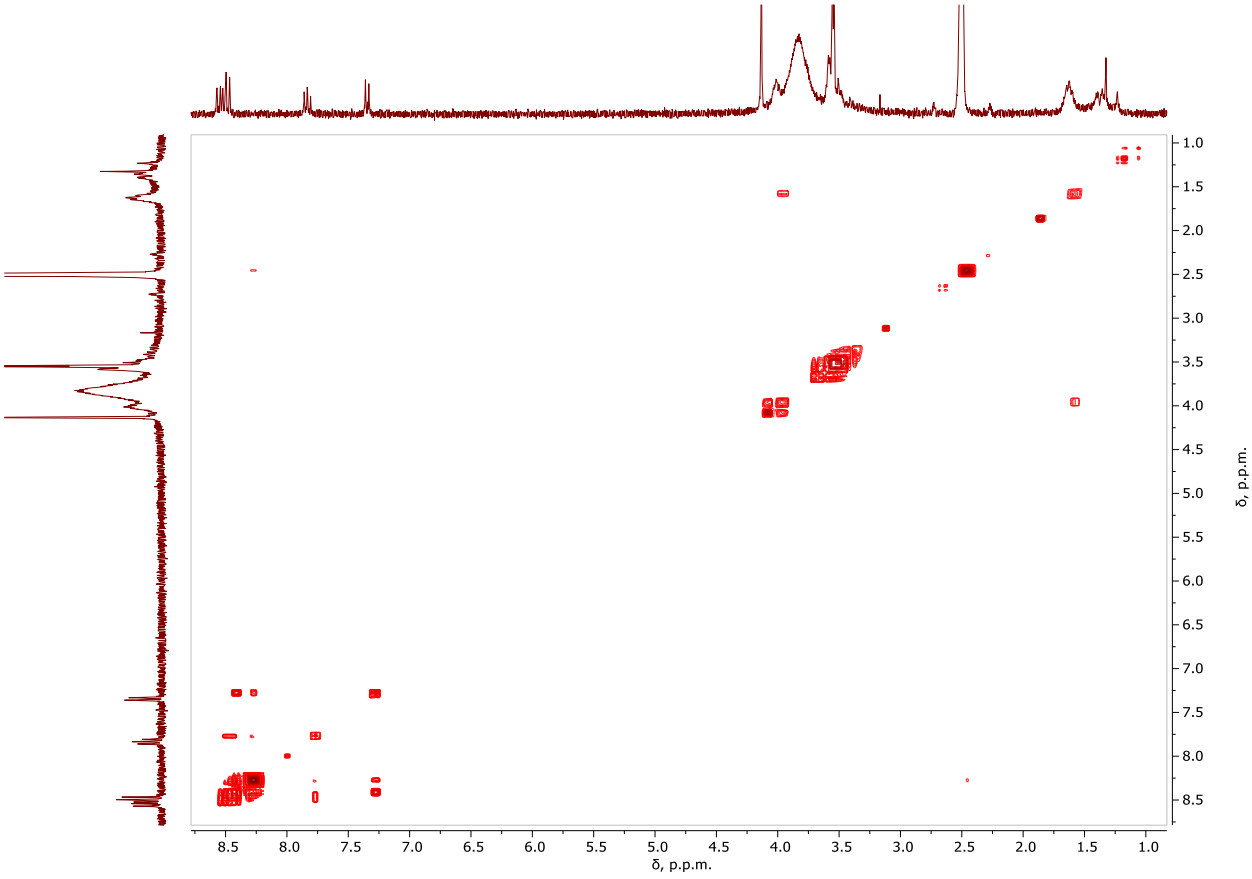


Figure S6. ^{1}H-^{1}H COSY spectra of **4** in DMSO-d_6

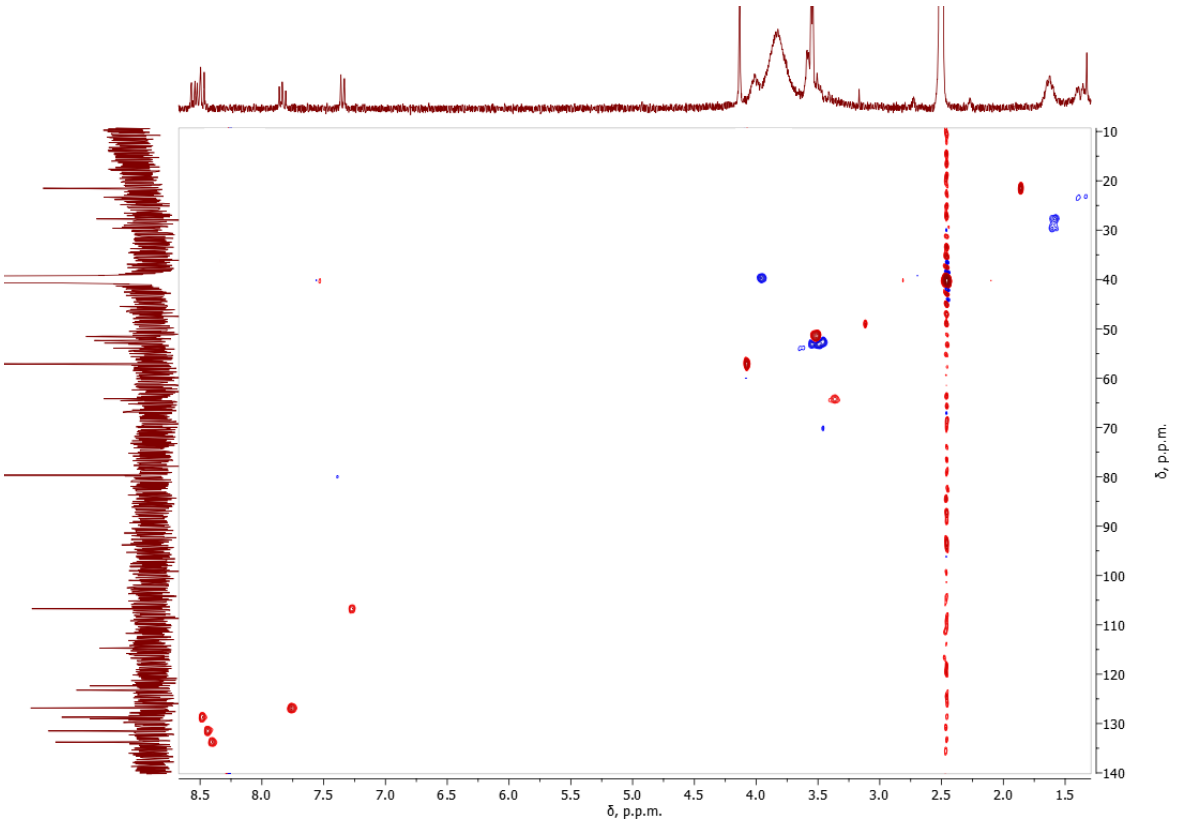


Figure S7. HSQC spectra of **4** in DMSO-d_6

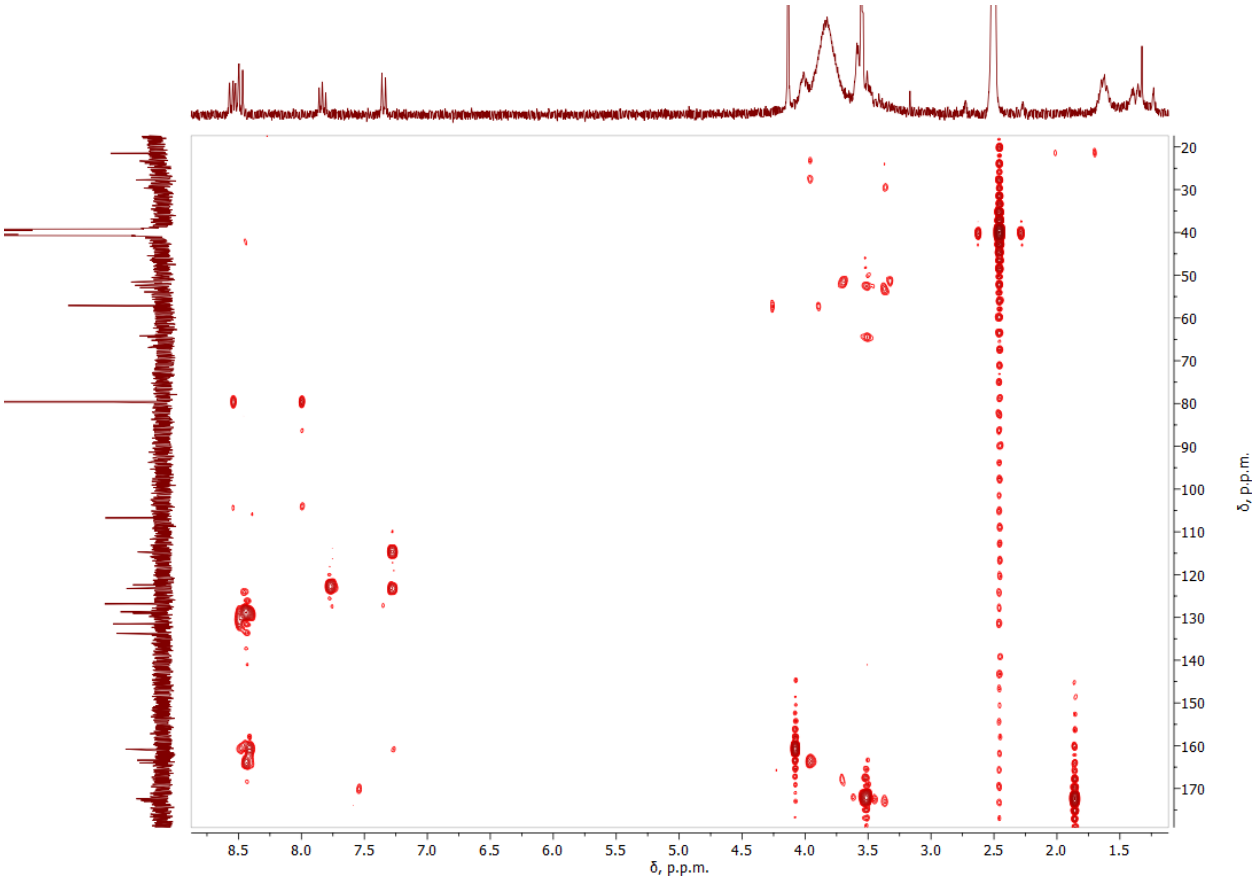


Figure S8. HMBC spectra of **4** in DMSO-d_6

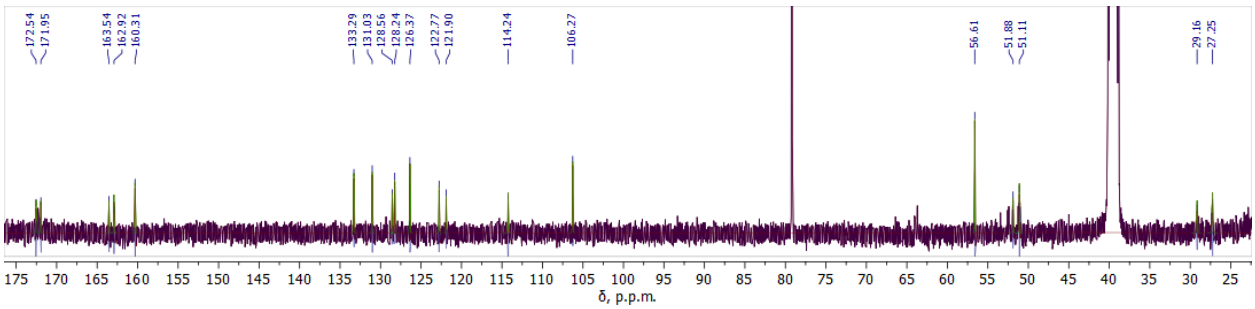


Figure S9. ^{13}C NMR spectra of **4** in DMSO-d_6

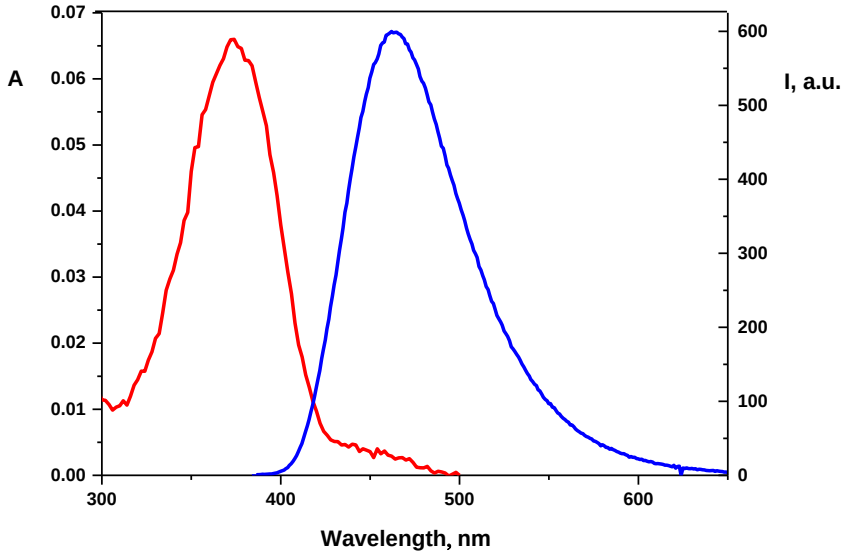


Figure S10. Absorption (red) and fluorescence (blue) spectra of **4** in HEPES buffer solution (0.01M, pH=7.4), excitation wavelength 375 nm, concentration $1 \cdot 10^{-5}$ M.

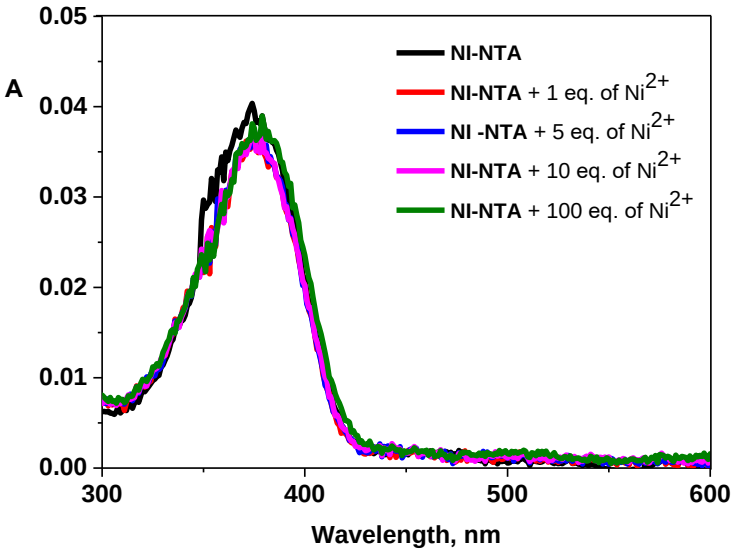


Figure S11. Changes in the absorption spectra of **4** with an increase in the concentration of Ni^{2+}, HEPES buffer solution (0.01M, pH = 7.4), concentration $1 \cdot 10^{-5}$ M, excitation wavelength 375 nm (b).

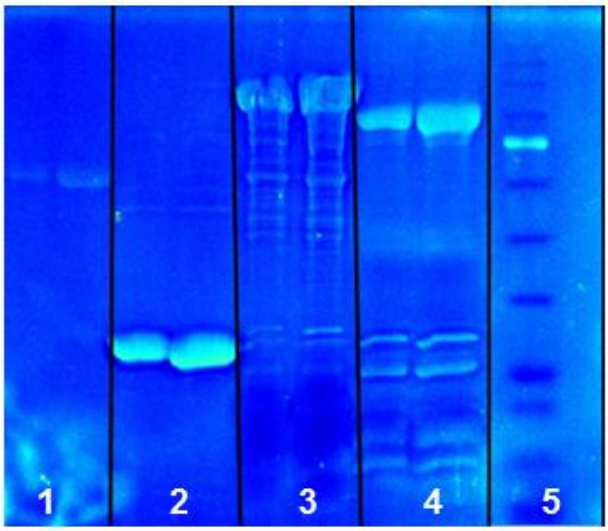


Figure S12. Detection of His-tagged reference proteins by InVision™ staining solution: 1) BSA (Bovine Serum Albumin); 2) TEV (Tobacco Etch Virus protease); 3) His-tagged bacterial endonuclease; 4) Bacterial endonuclease without His-tag; 5) Molecular mass marker.

References:

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S2. C. L. Renschler, L. A. Harrah, *Anal. Chem.*, 1983, **55**, 798; https://pubs.acs.org/doi/abs/10.1021/ac00255a050.
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