

Supplementary information:

Nitro-Pyrazinotriazapentalene scaffolds – nitroreductase quantification and *in vitro* fluorescence imaging of hypoxia

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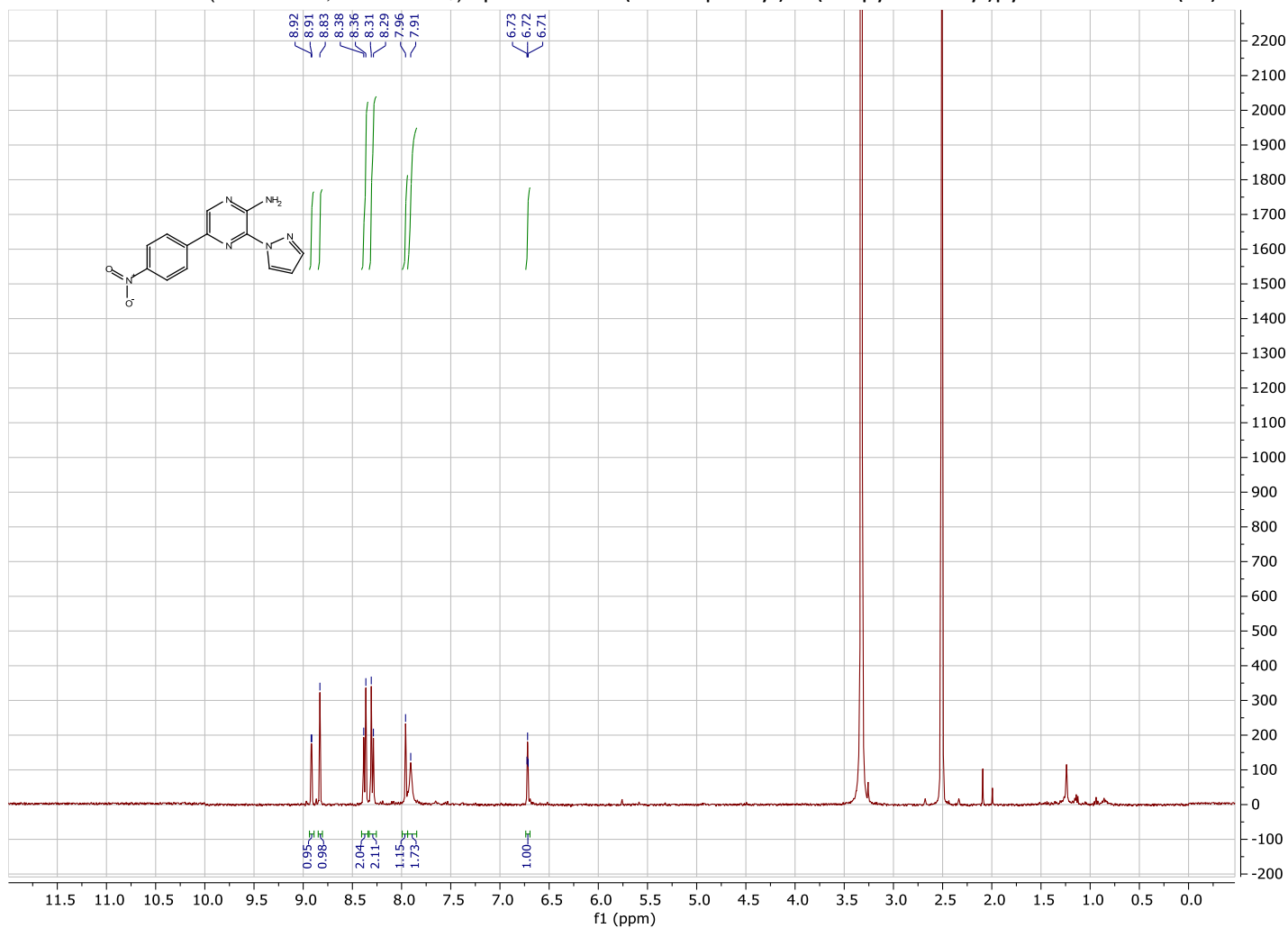
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Content

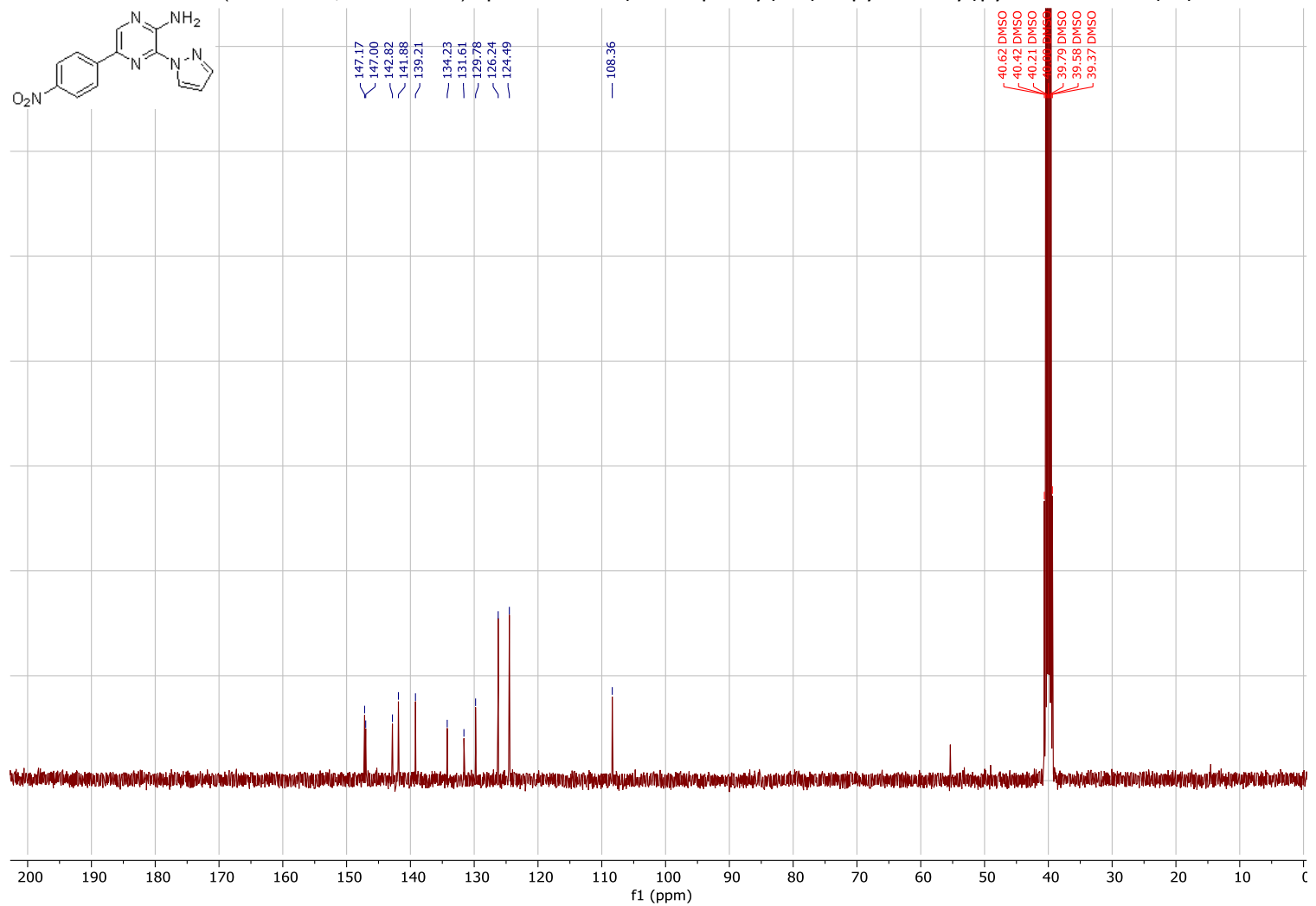
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1. Proton and carbon NMR spectra

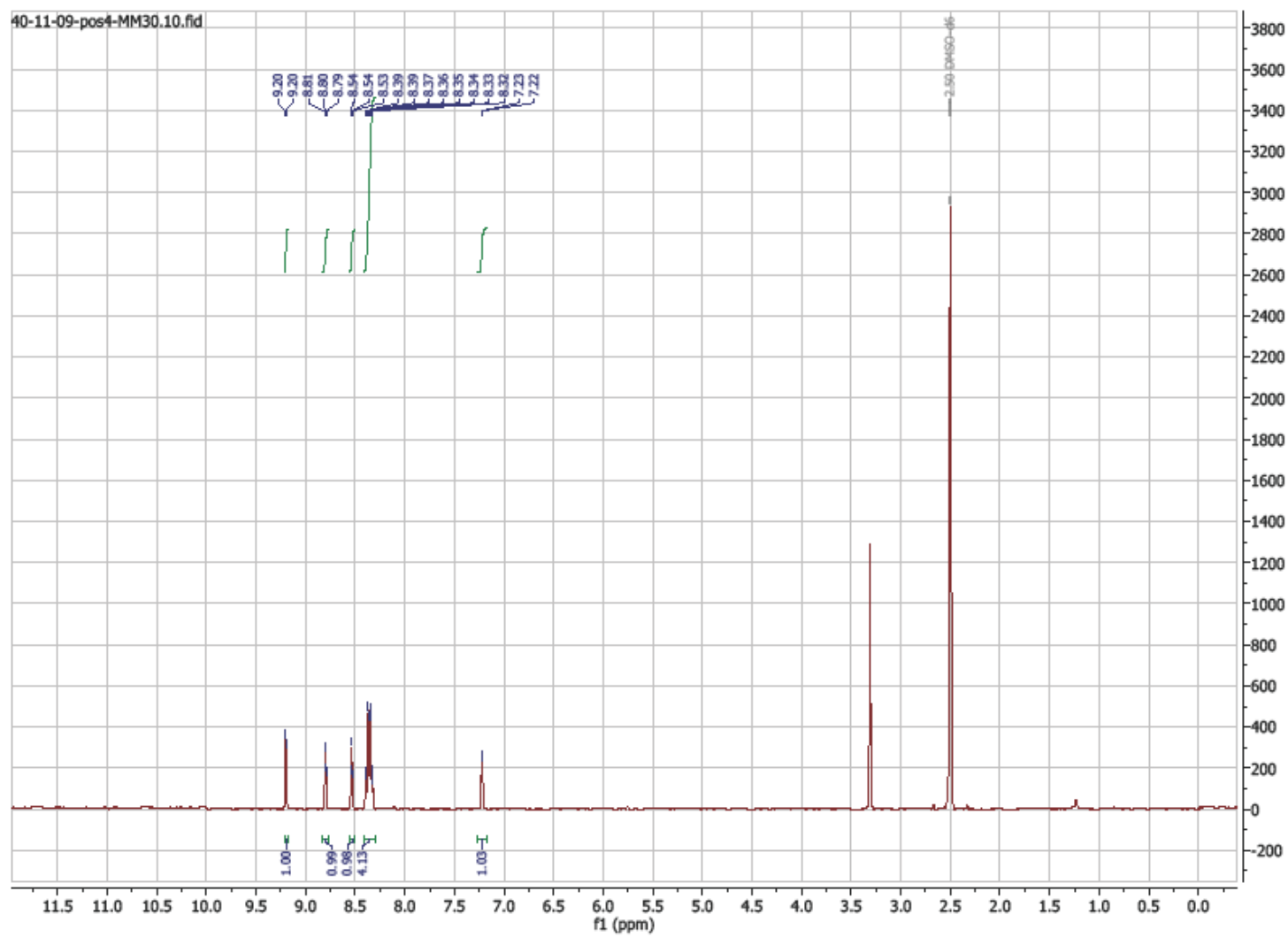
¹H NMR (400 MHz, DMSO-*d*₆) spectrum of 5-(4-nitrophenyl)-3-(1H-pyrazol-1-yl)pyrazin-2-amine (**1a**)



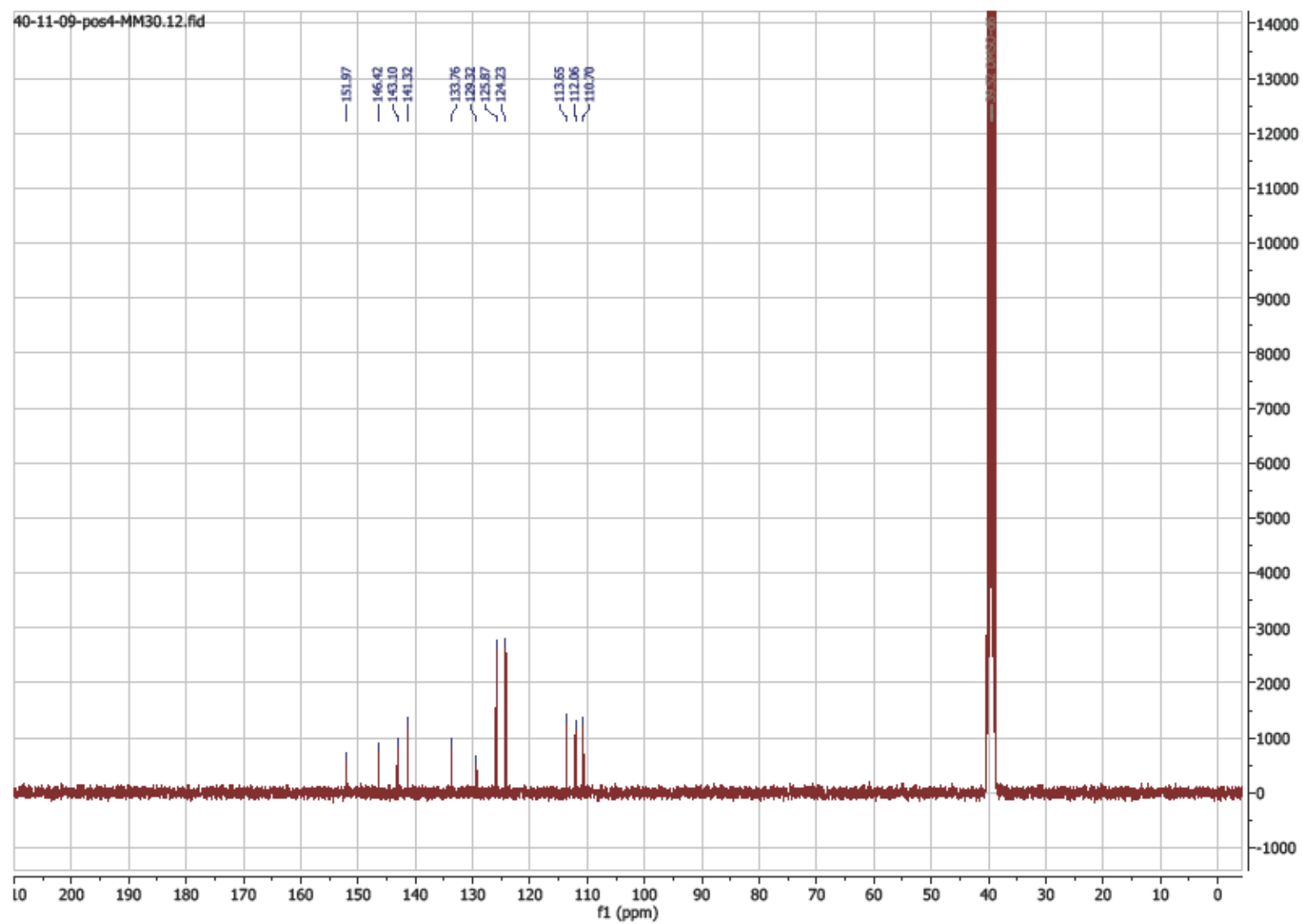
¹³C NMR (101 MHz, DMSO-*d*₆) spectrum of 5-(4-nitrophenyl)-3-(1*H*-pyrazol-1-yl)pyrazin-2-amine (**1a**)



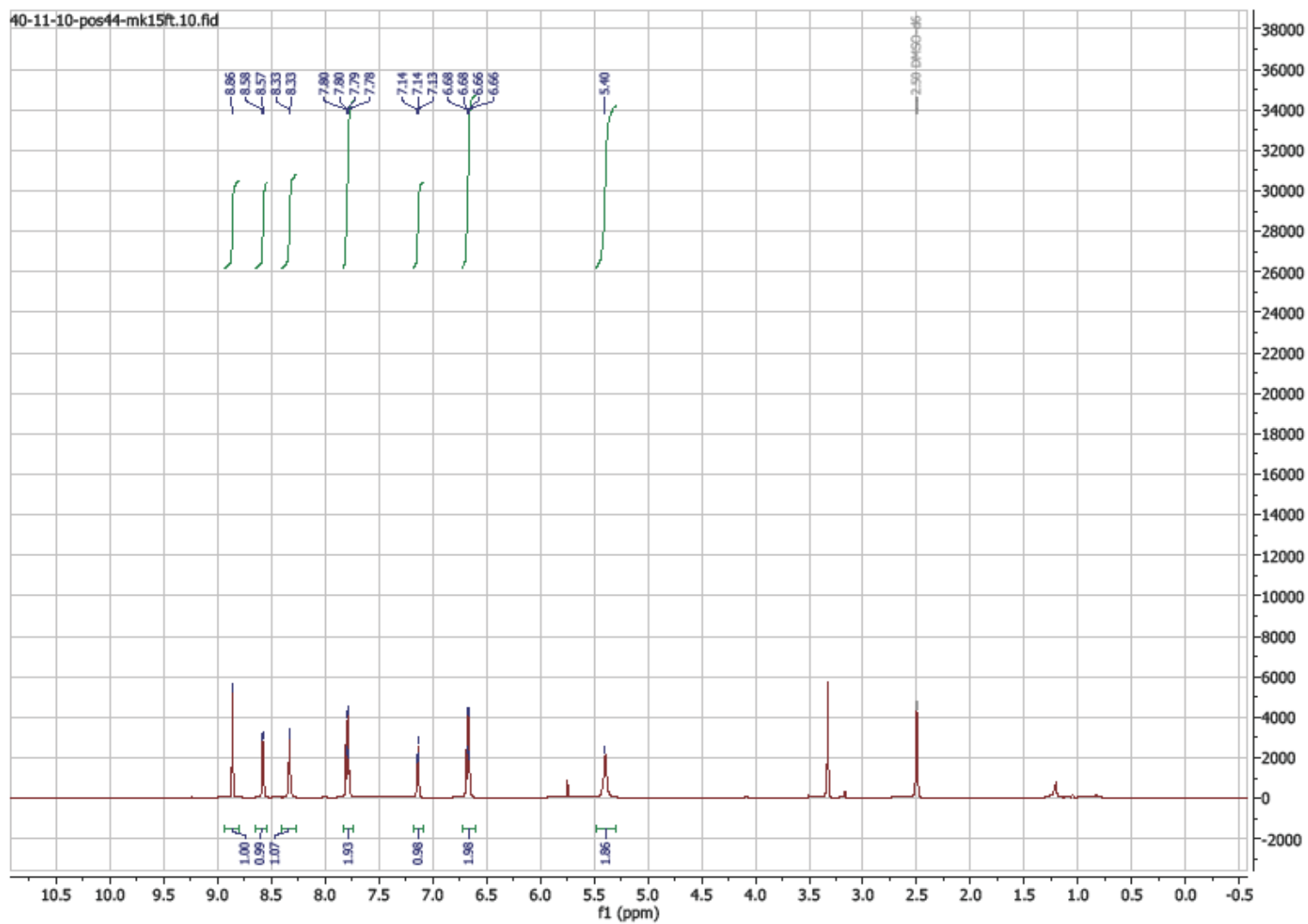
¹H NMR (400 MHz, DMSO-*d*₆) spectrum of 2-(4-nitrophenyl)pyrazolo[1',2':1,2][1,2,3]-triazolo[4,5-*b*]pyrazin-6-ium-5-ide (**1-NO₂**)



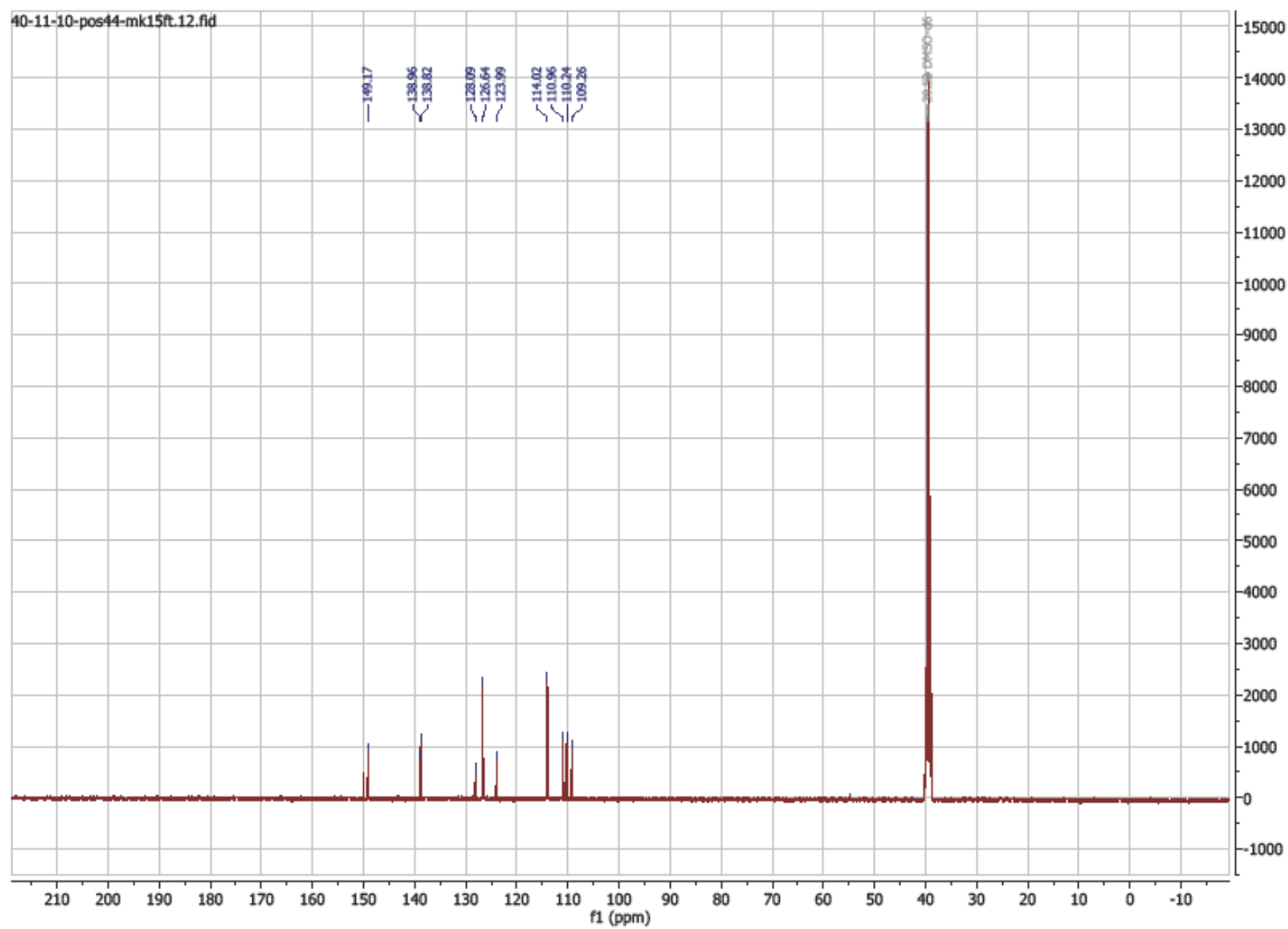
^{13}C NMR (101 MHz, CDCl_3) 2-(4-nitrophenyl)pyrazolo[1',2':1,2][1,2,3]triazolo[4,5-*b*]pyrazin-6-ium-5-ide (**1-NO₂**)



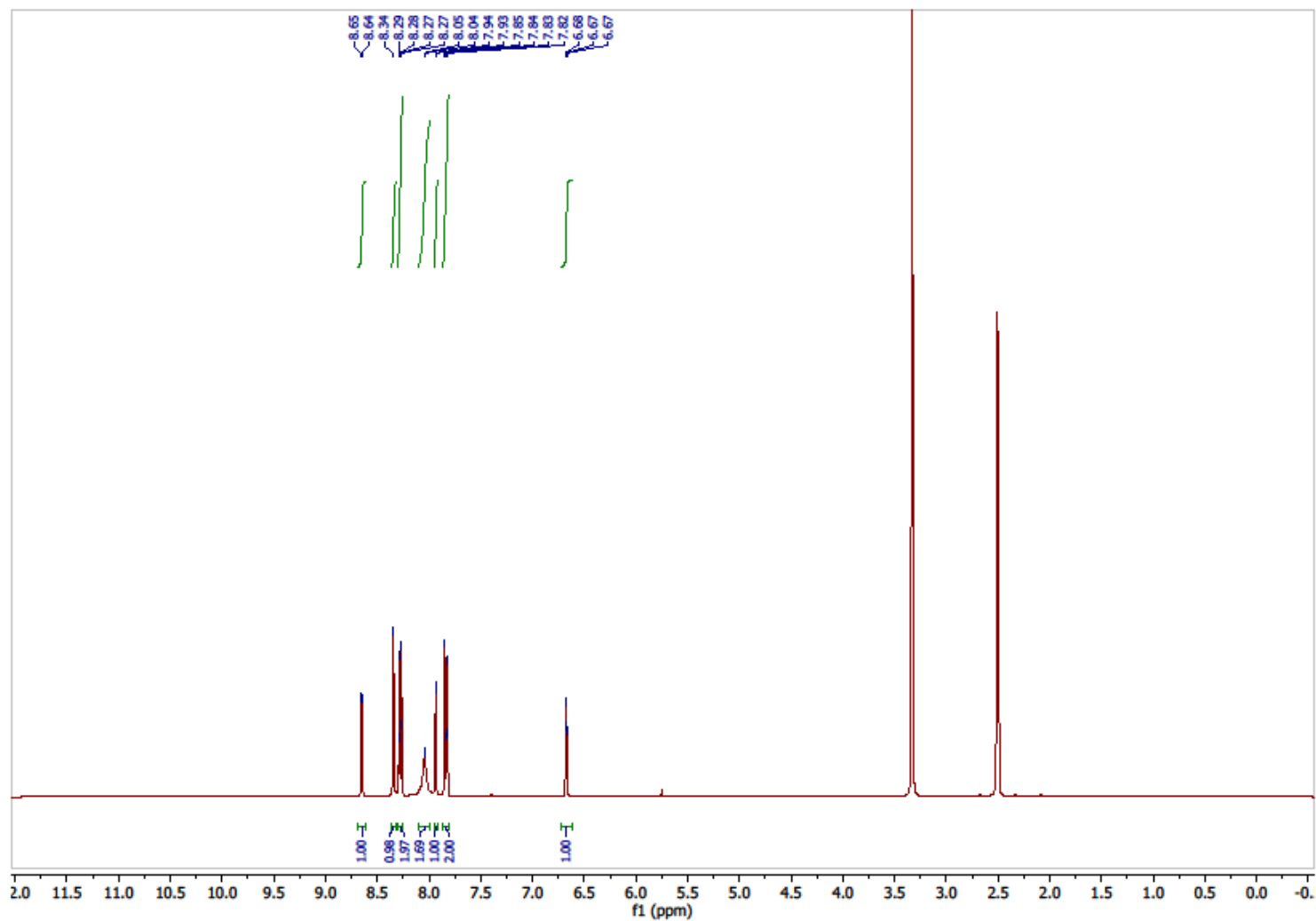
^1H NMR (400 MHz, $\text{DMSO-}d_6$) spectrum of 2-(4-aminophenyl)pyrazolo[1',2':1,2][1,2,3]-triazolo[4,5-*b*]pyrazin-6-ium-5-ide (**1-NH₂**)



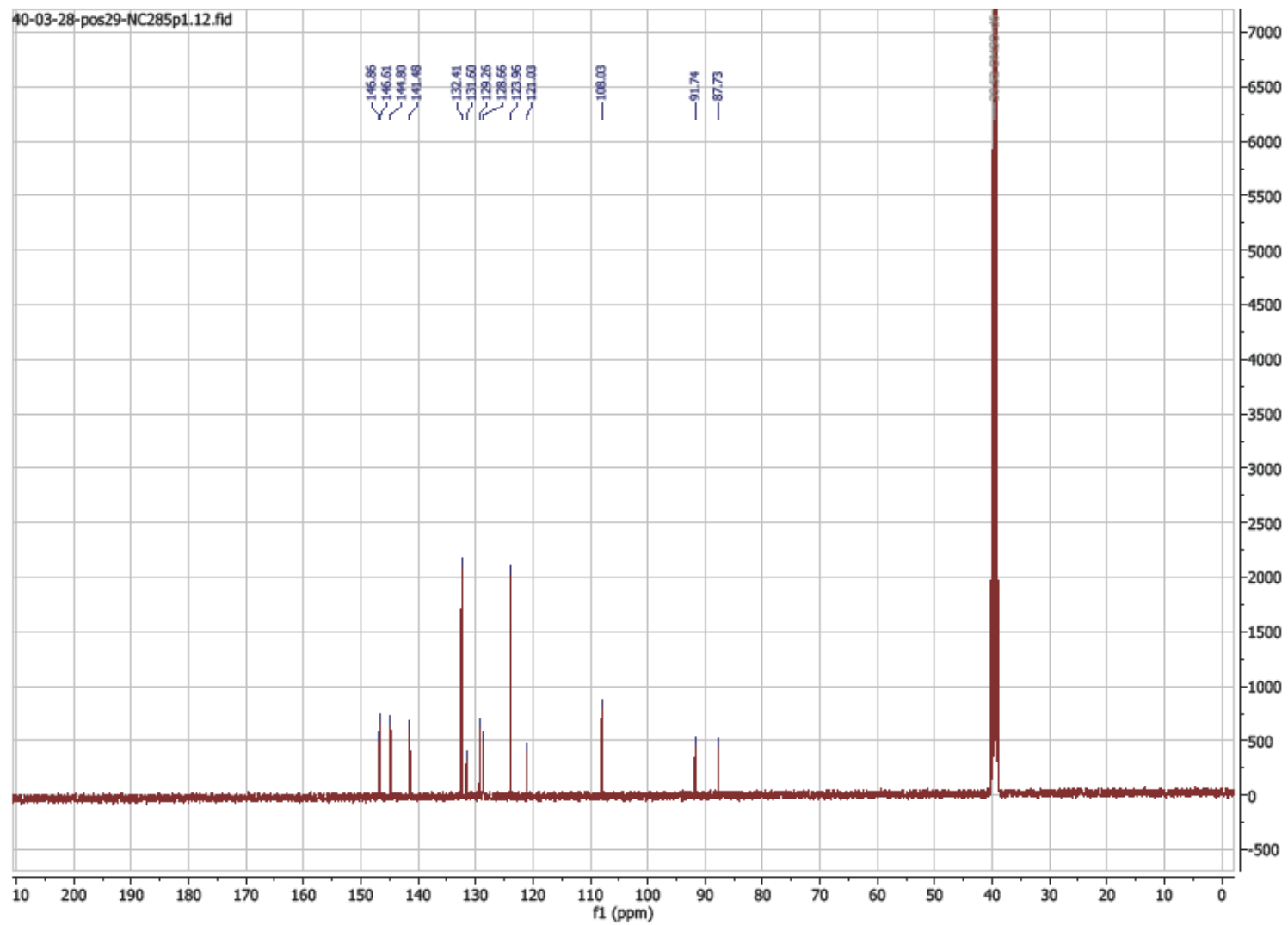
^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) 2-(4-aminophenyl)pyrazolo[1',2':1,2][1,2,3]-triazolo[4,5-*b*]pyrazin-6-ium-5-ide (**1-NH₂**)



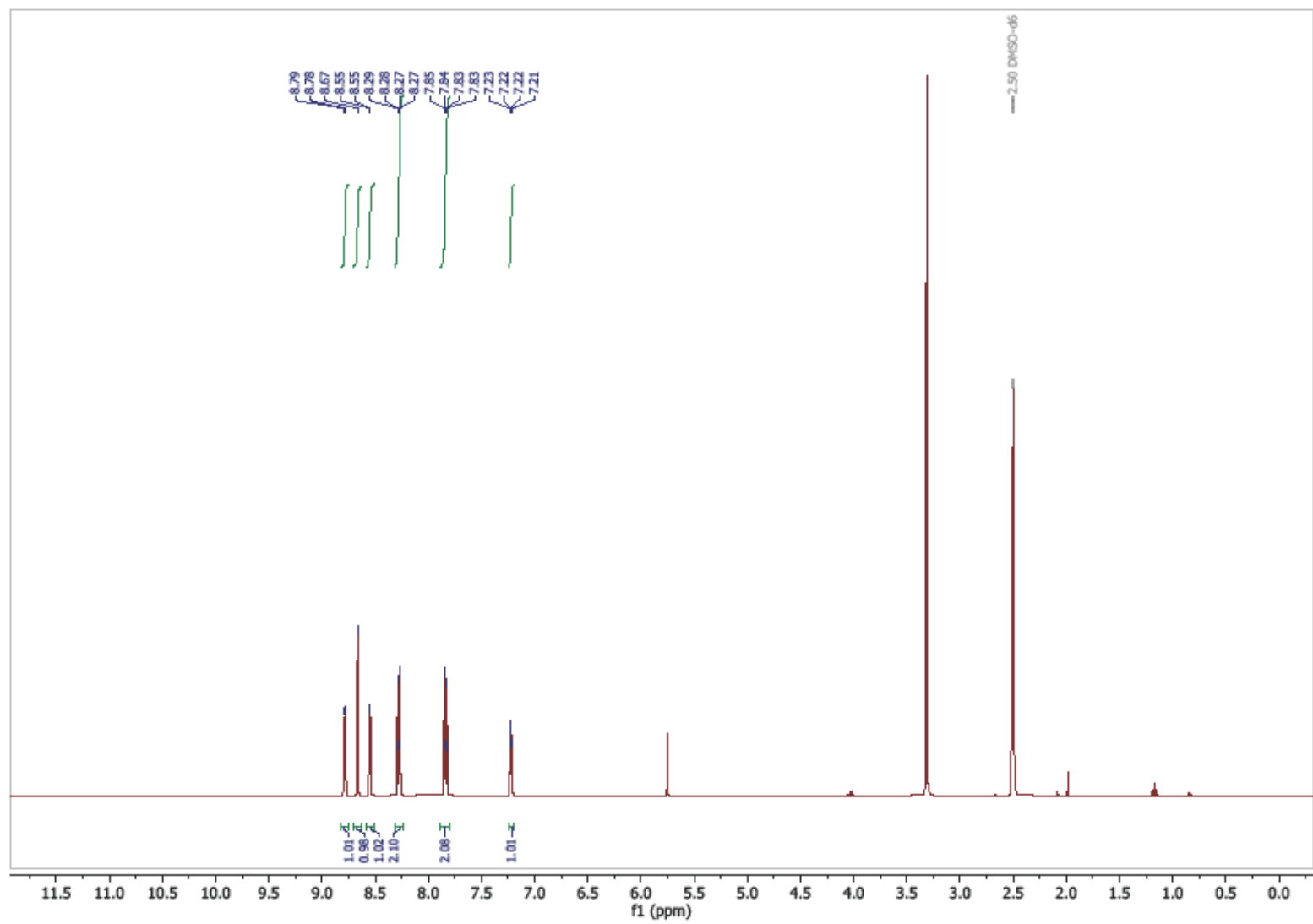
^1H NMR (400 MHz, $\text{DMSO-}d_6$) spectrum of 5-((4-nitrophenyl)ethynyl)-3-(*1H*-pyrazol-1-yl)pyrazin-2-amine (**2a**)



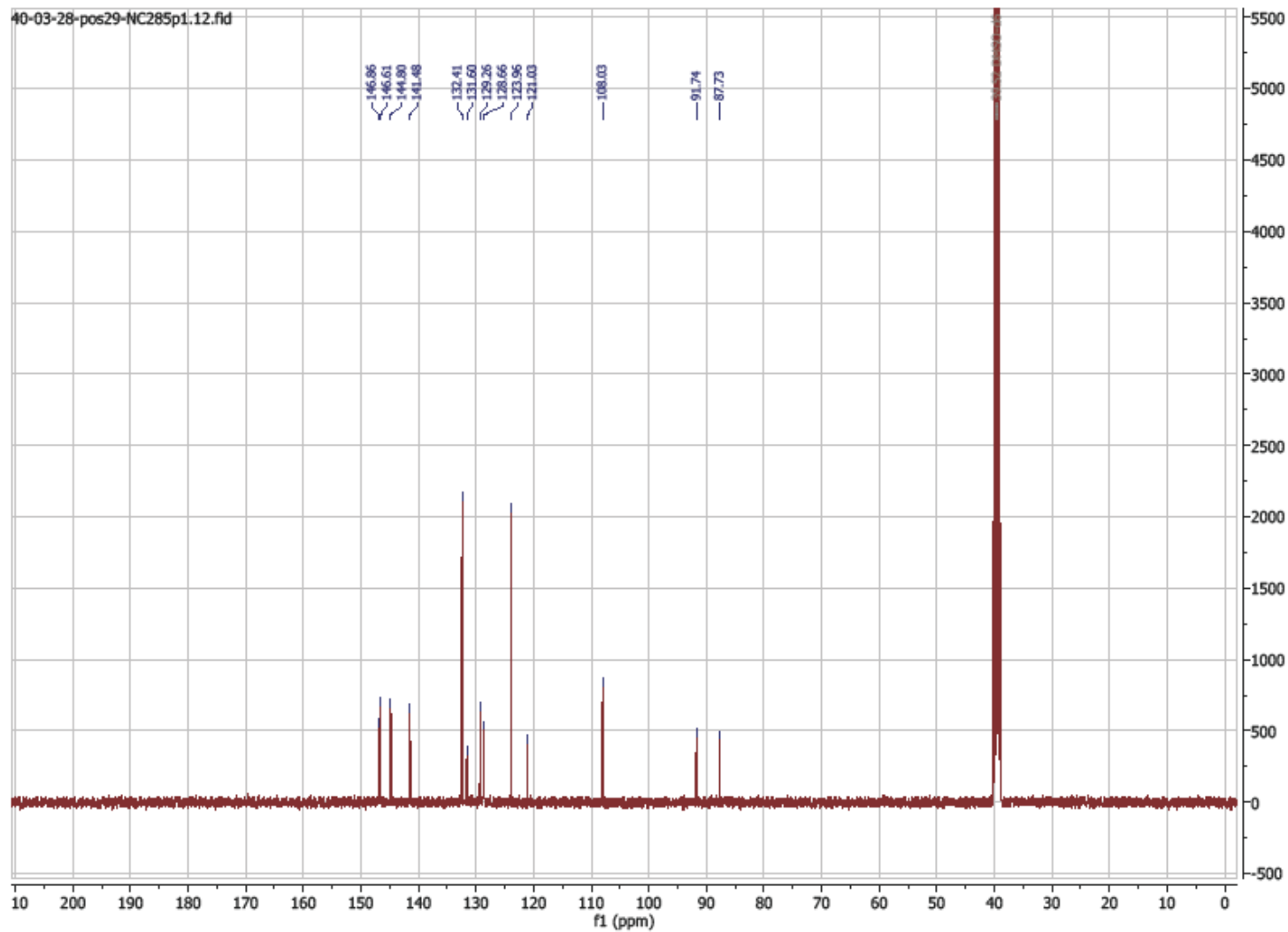
^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) spectrum of 5-((4-nitrophenyl)ethynyl)-3-(*1H*-pyrazol-1-yl)pyrazin-2-amine (**2a**)



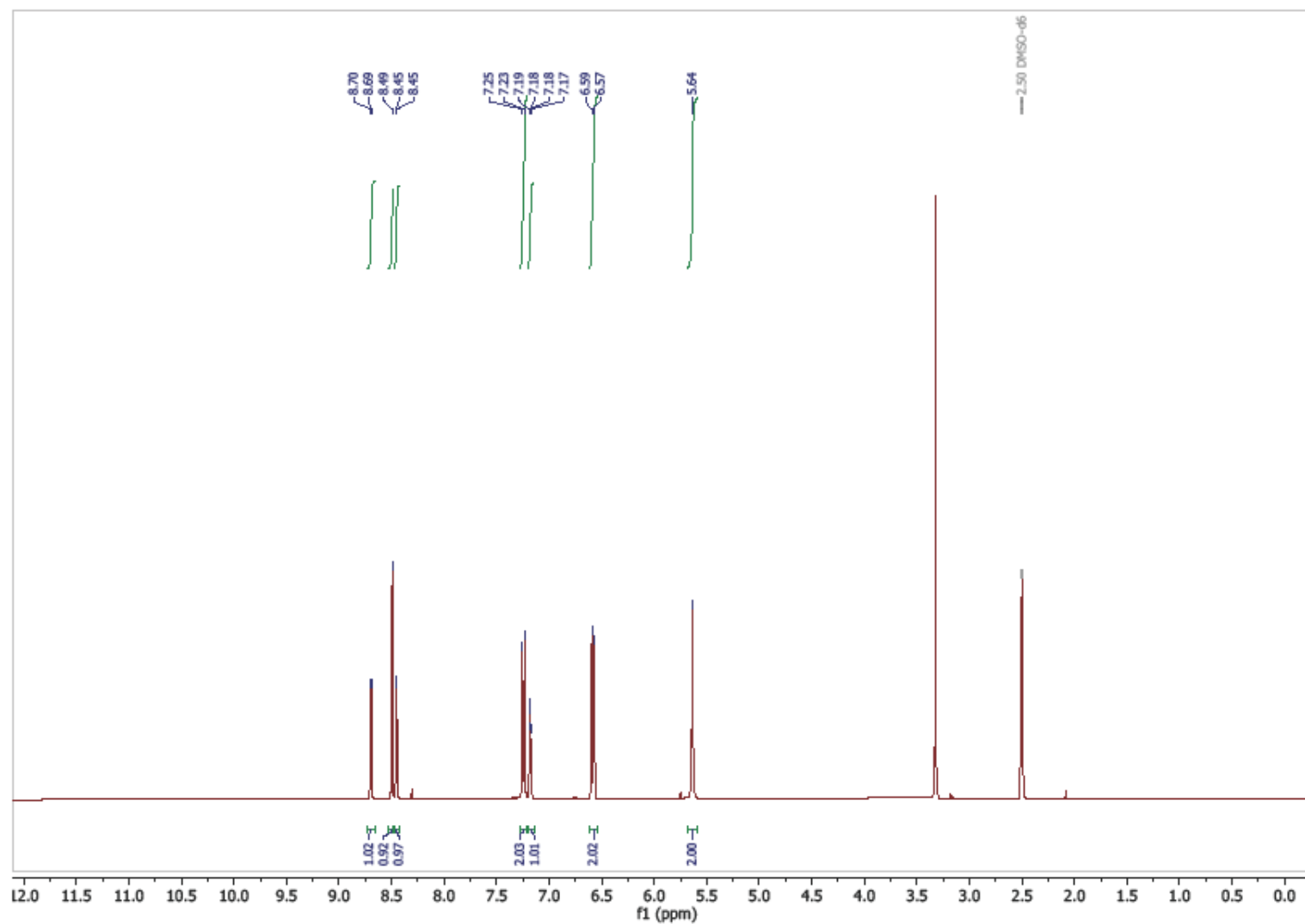
^1H NMR (400 MHz, $\text{DMSO-}d_6$) spectrum of 2-((4-nitrophenyl)ethynyl)pyrazolo[1',2':1,2]-[1,2,3]triazolo[4,5-b]pyrazin-6-ium-5-ide (**2-NO₂**)



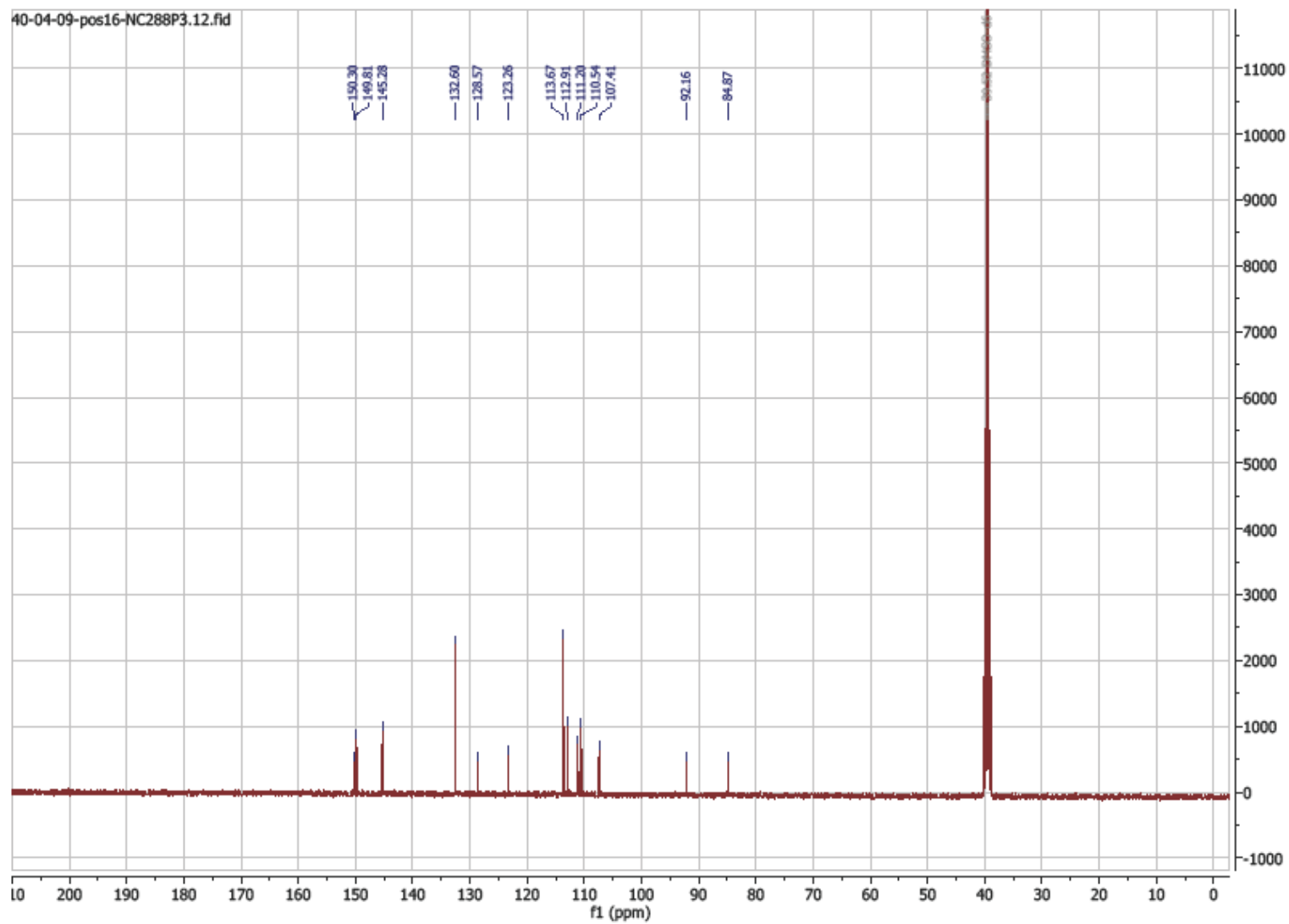
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2. Absorption and emission spectra

UV–Vis absorption spectra were recorded on a Perkin Elmer Lambda 35 spectrophotometer. Luminescence measurements were performed on a Perkin Elmer LS55 spectrofluorimeter. The spectra were recorded at the room temperature in DMSO or in aqueous solution containing small amount of DMSO (≤ 0.25 % v/v). Emission spectra were recorded between 470 and 800 nm upon excitation at the absorption maximum.

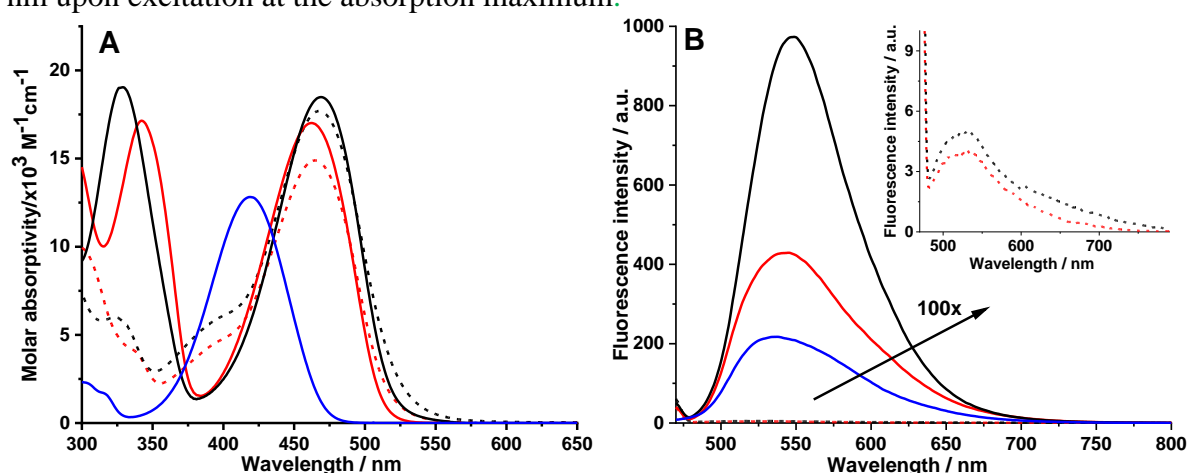


Figure A1. Absorption (A) and emission (B) spectra of **1-NO₂** (---), **1-NH₂** (—), **2-NO₂** (---), **2-NH₂** (—) and **PyrTAP** (—) in DMSO. Compounds were excited at their absorption maximum in visible region.

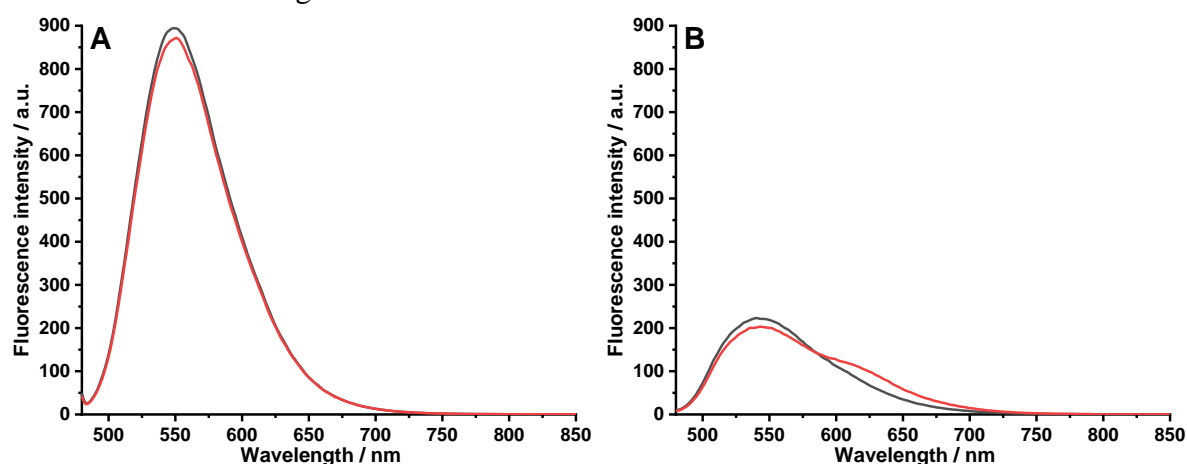


Figure A2. Fluorescence intensity ($\lambda_{\text{ex}} = 468$ nm) of (A) **1-NH₂** (1 μM) and (B) **2-NH₂** (1 μM) measured in DMSO in air-equilibrated (black) or deoxygenated conditions (red).

3. Determination of the quantum yield and lifetime of luminescence

For determination of the quantum yield of luminescence (ϕ), aqueous solutions of $[\text{Ru}(\text{bpy})_3]^{2+}$ with a small amount of DMSO were used as standards ($\phi = 0.028$ [1] for air-equilibrium conditions). The spectra were recorded for compounds at the concentration less than 0.05 absorbance unit at the excitation wavelength. Values were calculated according to the following equation[2]:

$$\phi = \phi_{\text{ref}} \cdot [A_{\text{ref}} / A] \cdot [I / I_{\text{ref}}] \cdot [n^2 / n_{\text{ref}}^2]$$

where I is the integrated intensity of luminescence, A is the optical density, n is the refractive index, ref apply to the values for reference. The average of three scans was subjected to smoothing. The mean value from minimum three independent experiments was calculated.

The luminescence lifetime measurements were carried out by a single photon counting technique using Fluorolog-3, Horiba Jobin Yvon at room temperature. The measurement was performed using 464 nm excitation wavelength (NanoLed diodes) while the average lifetime of luminescence was measured at emission maximum (546 nm and 542 for **1-NH₂** and **2-NH₂** respectively). Luminescence decays were collected with 1,000 counts in the peak. The instrument response functions were measured using a light scattering solution of Ludox (colloidal silica, Sigma-Aldrich). The DAS6 software (HORIBA Scientific) was used for deconvolution of the obtained decays and for calculation of the lifetime values.

4. Spectroscopic properties in aqueous solution

Table S1. Spectroscopic properties for the triazapentalene in aqueous solution.

	Absorption		Emission	
	λ_{max}^a [nm]	ϵ [M ⁻¹ cm ⁻¹]	λ_{max} [nm]	ϕ^b
1-NO₂	432	3700	-	-
1-NH₂	447	17800	564	0.0021
2-NO₂	446	4600	-	-
2-NH₂	449	20200	531	0.0009
PyTAP	408	12300	545	0.0045

^a Maximum of absorption band in visible region of spectrum. ^b ϕ is the relative fluorescence quantum yield determined using [Ru(bpy)₃]Cl₂ ($\phi = 0.028$ in H₂O) as a reference standard.

5. Photostability

The photostability of amine derivatives of triazapentalene was investigated following the measurement of their optical absorption and fluorescence intensity in TRIS-HCl buffer (pH 7.4, 50 mM). For this purpose, solutions of **1-NH₂** or **2-NH₂** (40 μ M) were prepared on a 96-well plate and irradiated with a lamp with an emission wavelength of 465 nm and power 7.35 mW/cm² for various periods of time. After the defined exposure time the reaction progress was monitored by Tecan Infinite 200 microplate reader. Additionally, absorbance and fluorescence spectra were performed before experiment and after 30 minutes of irradiation.

6. Reduction by NTR

6.1. Inhibition assay

To determine the ability of dicoumarol to inhibit the reduction of nitro compounds, different concentrations of dicoumarol (0-200 mM) were used. Typically 16 μ M **2-NO₂**, 160 μ M NADH and appropriate concentration of dicoumarol were mixed in 50 mM TRIS-HCl buffer pH 7.4 and incubated for 5 min at 37 °C. Then, 2.4 μ g/mL NTR from *Escherichia Coli* was added and the fluorescence intensity upon excitation at 447 nm were registered at 538 nm over 15 min.

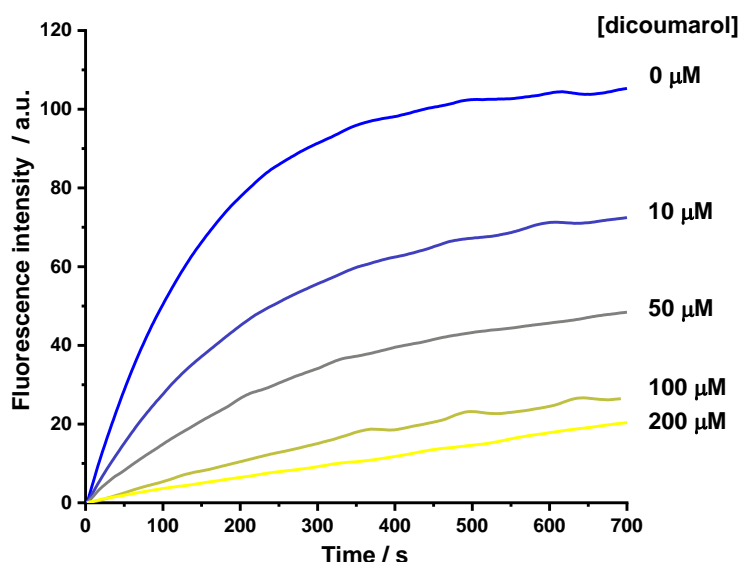


Figure A3. Time-dependent fluorescence emission intensity ($\lambda_{\text{ex}} = 447 \text{ nm}$, $\lambda_{\text{em}} = 538 \text{ nm}$) of **2-NO₂** (16 μM) for various concentrations of dicoumarol (0-200 μM) in the presence of 2.4 $\mu\text{g/mL}$ NTR and 160 μM NADH, TRIS-HCl buffer (pH 7.4, 50 mM), incubated at 37 $^{\circ}\text{C}$.

6.2. Analysis of products - spectrofluorimetry and UV-Vis spectroscopy

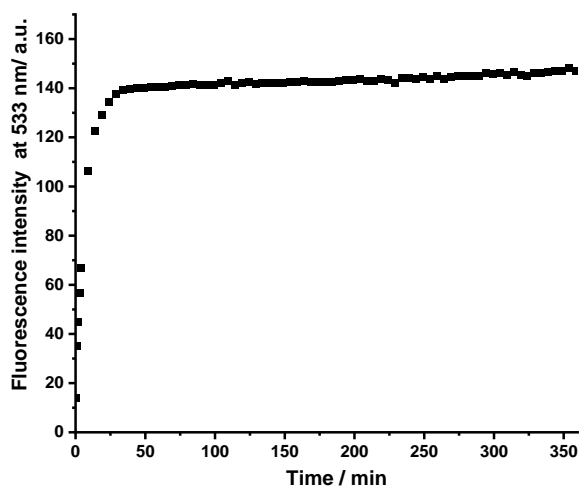


Figure A4. Time-dependent fluorescence intensity ($\lambda_{\text{ex}}=447 \text{ nm}$, $\lambda_{\text{em}}=533 \text{ nm}$) of **2-NO₂** (16 μM) in the presence of 2.4 $\mu\text{g/mL}$ NTR and 160 μM NADH. TRIS-HCl buffer (pH 7.4, 50 mM) at 37 $^{\circ}\text{C}$.

Time-dependent absorption spectra showed that the reduction proceeded in several steps. The first step of the reaction was fast (up to a few minutes under applied experimental conditions, (**Figures A5A and A6A**), and in the case of **2-NO₂** this step was *ca.* 5 times faster than for **1-NO₂**. This might be a significant issue in the further application of these probes. For both compounds, the initial step was followed by a slower step (completed within *ca.* 4 h), which corresponds to the gradual depletion of NADH. The subsequent step was quite fast (completed

within minutes), and it resulted in a pronounced increase in the absorption, registered in the visible part of spectra. The last stage of the process was particularly sluggish as it was not completed even after 20 hours.

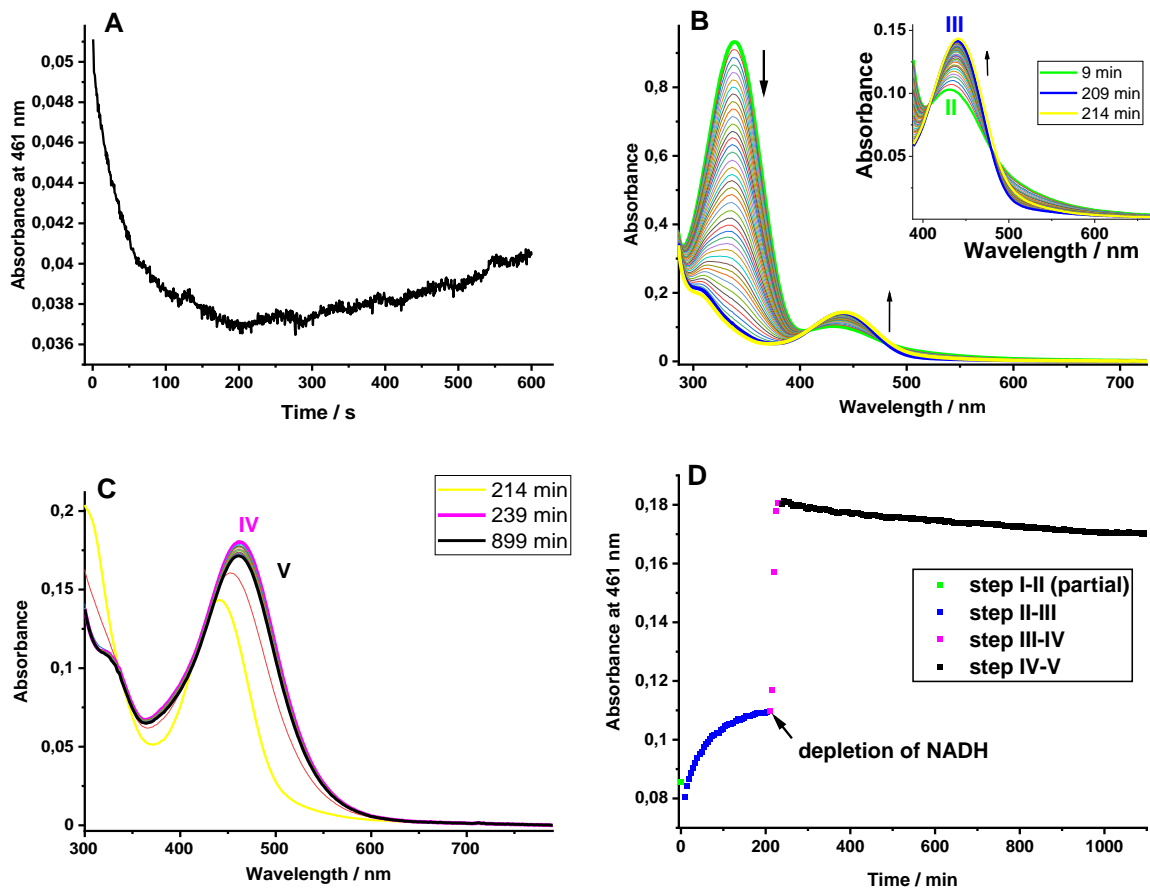
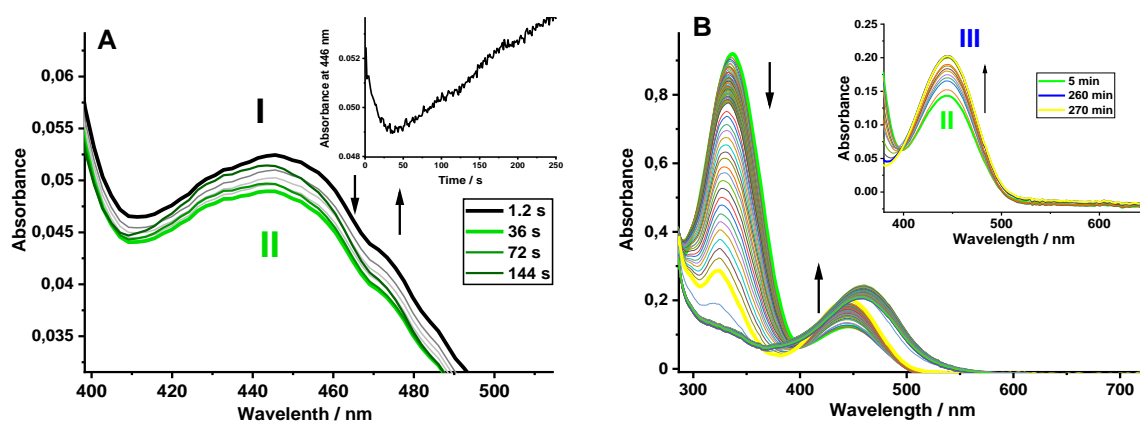


Figure A5. Time-dependent changes in absorption spectrum or absorbance at the selected wavelength for 1-NO₂ (16 μM) in the presence of 160 μM NADH and NTR (2.4 μg/mL) recorded at 37 °C, TRIS-HCl buffer (pH 7.4, 50 mM). A- measured on stopped flow, B-D – measured at classical spectrophotometer. A, D – time dependent changes at 461 nm and B, C - the corresponding changes in absorption spectrum.



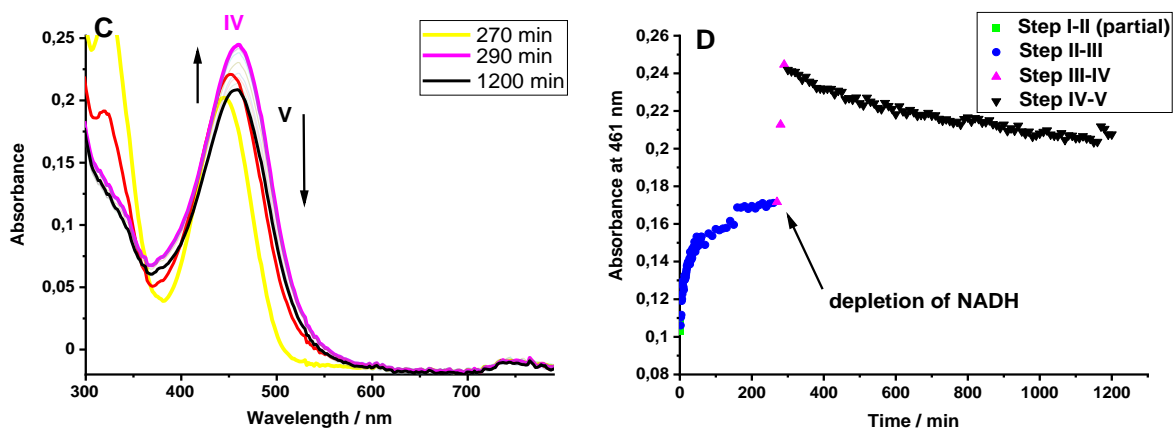


Figure A6. Time-dependent changes in absorption spectrum or absorbance at the selected wavelength for 2-NO₂ (16 μM) in the presence of 160 μM NADH and NTR (2.4 μg/mL) recorded at 37 °C, TRIS-HCl buffer (pH 7.4, 50 mM). A- measured on stopped flow, B-D – measured at classical spectrophotometer. A inset, D – time dependent changes at 461 nm and A, B, C -the corresponding changes in absorption spectrum.

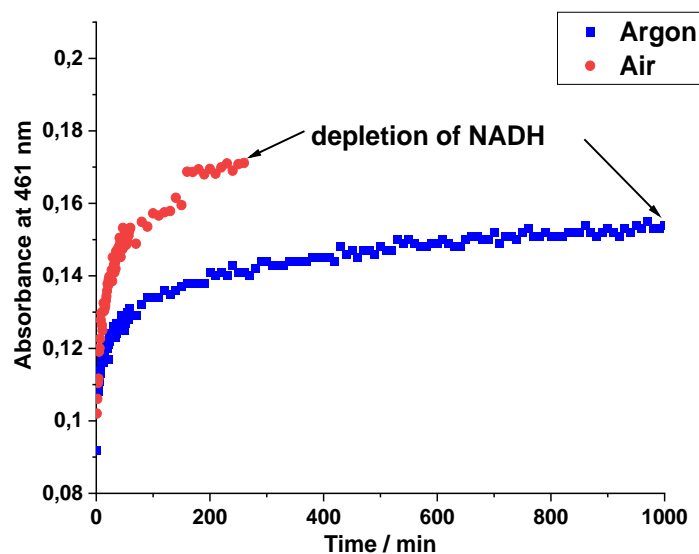


Figure A7. Time-dependent changes in absorbance at 461 nm for 2-NO₂ (16 μM) in the presence of 160 μM NADH and NTR (2.4 μg/mL) recorded at 37 °C, TRIS-HCl buffer (pH 7.4, 50 mM) measured under air-equilibrated (red circle) or argon (blue squares) conditions.

6.3. Analysis of products -HPLC

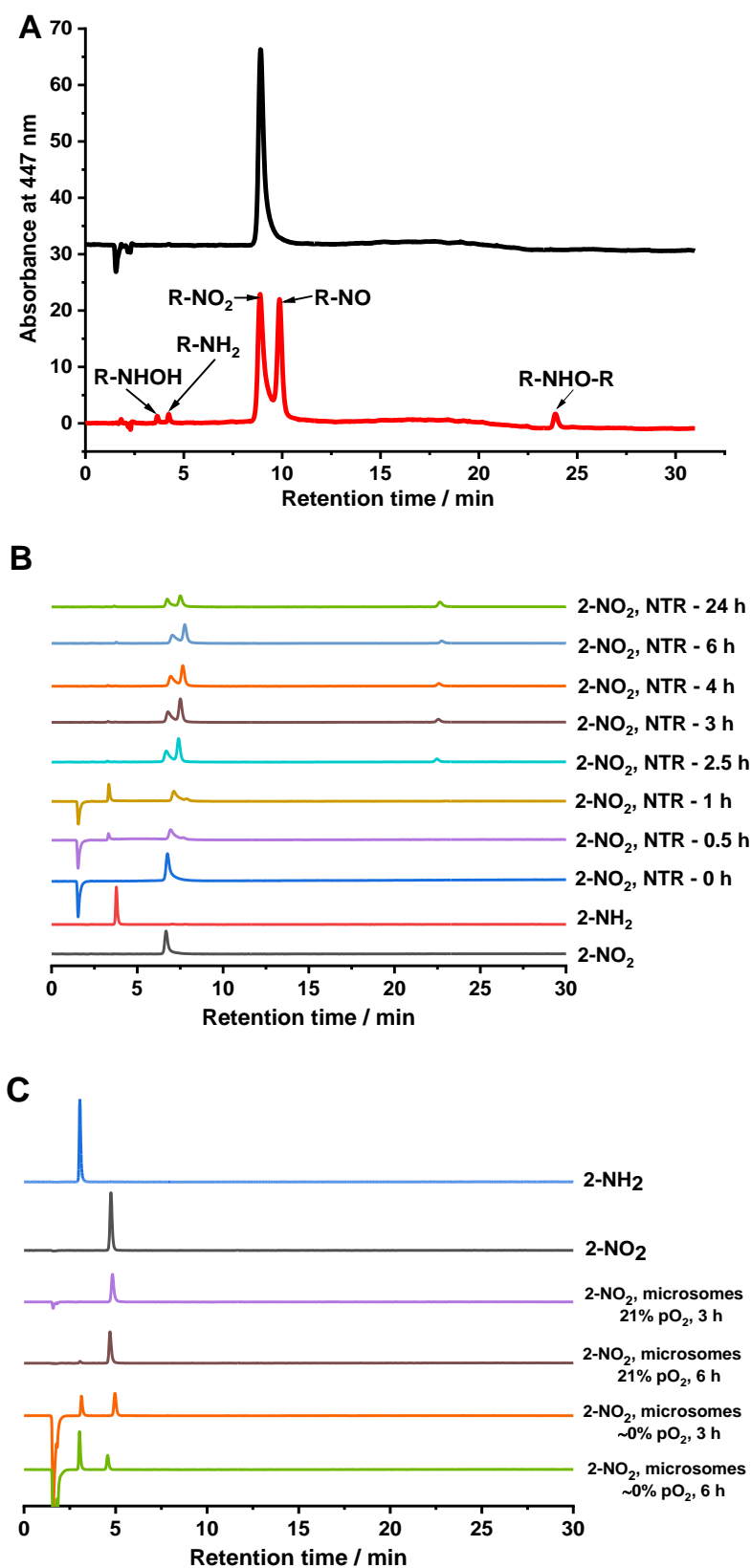


Figure A8. A) Comparison of chromatograms for reduction of the probe 2-NO₂ (16 μM) incubated only with NADH (160 μM) (black) or in the presence of NTR (2.4 μg/mL) for 5 h (red) and B) changes in product distribution with time under the same conditions. C)

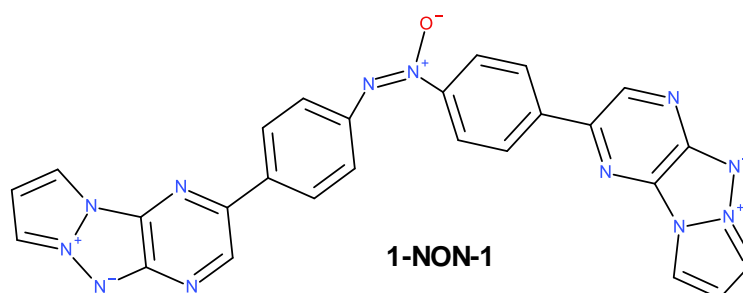
Comparison of chromatograms for reduction of the **2-NO₂** probe (16 μM) incubated with NADH (1 mM) and microsomes (1 mg/ml) carried out in normal (normoxia, 21% pO₂), or in the anaerobic environment (< 1 ppm of O₂, denoted as ~0% pO₂). Reaction conditions: 50 mM TRIS-HCl buffer pH 7.4, 37 °C Chromatograms were recorded using absorbance at 447 nm.

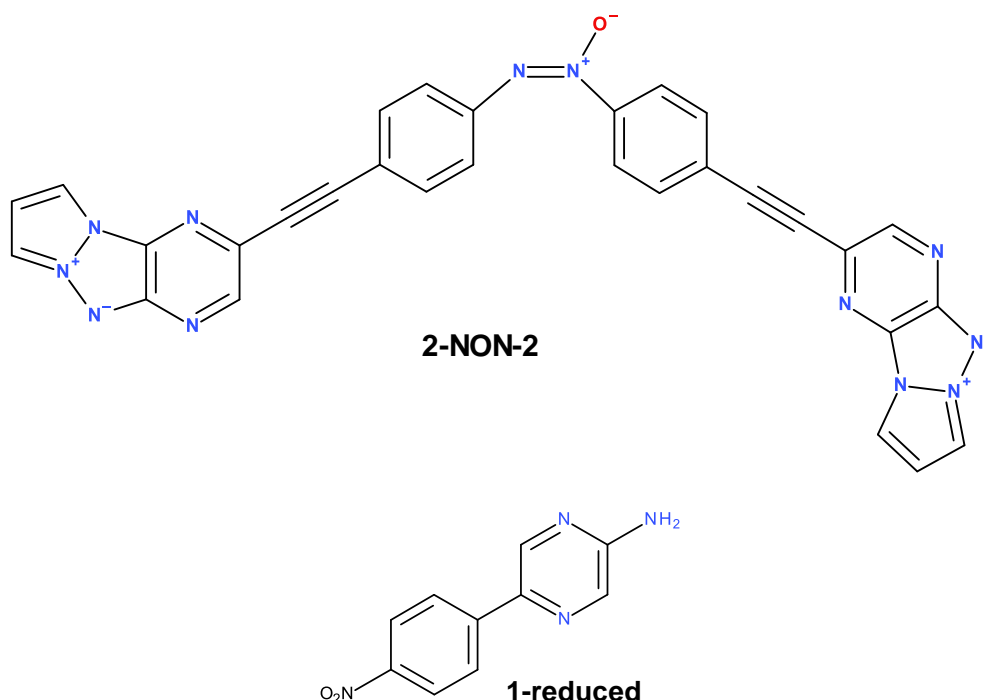
6.4. Analysis of products -LC-HRMS

Table A2. MS data for a mixture obtained after reduction of **1-NO₂** and **2-NO₂** probes with NADH in the presence of NTR.

Reaction	Chemical Formula	<i>m/z</i> theoretical	<i>m/z</i> found	Assigned product
1-NO₂ + NADH + NTR	C ₁₃ H ₈ N ₆ O ₂ +H	281.0781	281.0785	[1-NO ₂ +H] ⁺
	C ₁₃ H ₈ N ₆ O+H	265.0832	265.0832	[1-NO+H] ⁺
	C ₂₆ H ₁₆ N ₁₂ O+H	513.1643	513.1638	[1-NON-1+H] ⁺
	C ₂₆ H ₁₆ N ₁₂ O+2H	257.0858	257.0865	[1-NON-1+2H] ²⁺
	C ₁₃ H ₁₀ N ₆ +H	251.1040	251.1040	[1-NH ₂ +H] ⁺
	C ₁₃ H ₁₀ N ₆ +2H	126.0556	126.0559	[1-NH ₂ +H] ²⁺
	C ₁₃ H ₁₀ N ₆ O+H	267.0992	267.0989	*[1-NHOH+H] ⁺
C ₁₀ H ₈ N ₄ O ₂ +H	217.0468	217.0475	1-reduced	
2-NO₂ + NADH + NTR	C ₁₅ H ₈ N ₆ O ₂ +H	305.0781	305.0784	[2-NO ₂ +H] ⁺
	C ₁₅ H ₈ N ₆ O+H	289.0832	289.0835	[2-NO+H] ⁺
	C ₃₀ H ₁₆ N ₁₂ O+H	561.1643	561.1635	[2-NON-2+H] ⁺
	C ₃₀ H ₁₆ N ₁₂ O+2H	281.08581	281,0865	[2-NON-2+2H] ²⁺
	C ₁₅ H ₁₀ N ₆ +H	275.1040	275.1039	[2-NH ₂ +H] ⁺
	C ₁₅ H ₁₀ N ₆ +2H	138.0556	138.0557	[2-NH ₂ +2H] ⁺
	C ₁₅ H ₁₀ N ₆ O+H	291.0989	291.0988	*[2-NHOH+H] ⁺

*The hydroxylamine complex is not stable enough therefore within the course of analysis there is its conversion into nitroso derivatives as detected in mass spectra.





Scheme A1. The tentative structures of azoxy dimers denoted as **1-NON-1** and **2-NON-2** formed during the reduction of probe **1-NO₂** or **2-NO₂**, respectively, by NADH in the presence of nitroreductase under air-equilibrated conditions. The structures were suggested based on HRMS data as well as available literature data.[3, 4] **1-reduced** is a tentative structure of compound found upon reduction of triazapentalene moiety in **1-NO₂**.

6.5. NTR detection by 1-NO₂ and 2-NO₂

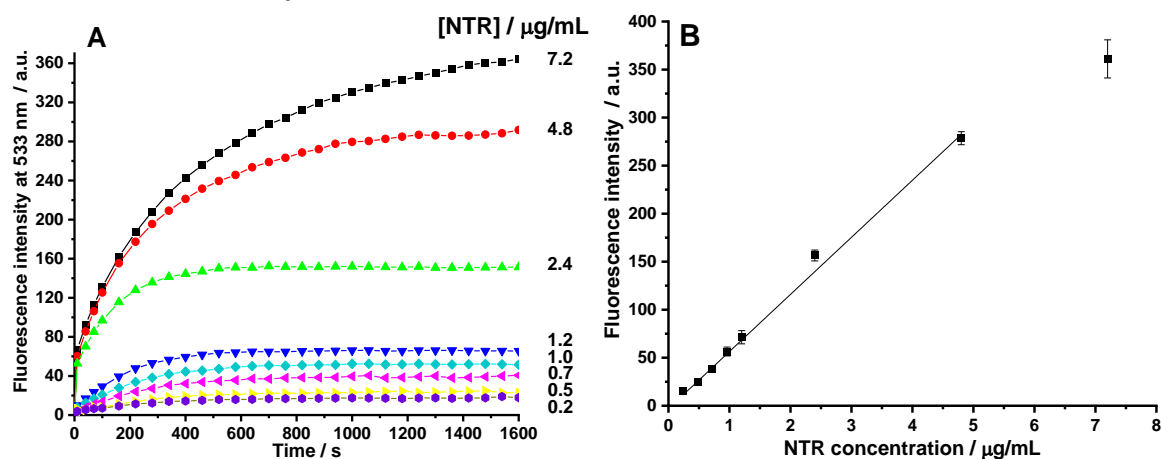


Figure A9. **A**) Time-dependent changes of fluorescence intensity ($\lambda_{\text{ex}} = 447 \text{ nm}$, $\lambda_{\text{em}} = 533 \text{ nm}$) of $16 \mu\text{M}$ **1-NO₂** in the presence of various concentrations of NTR ($0.2 - 7.2 \mu\text{g/mL}$) in the presence of $160 \mu\text{M}$ of NADH, TRIS-HCl buffer (pH 7.4, 50 mM), 37°C ; **B**) A correlation between fluorescence intensities and concentrations of NTR after 15 min reaction for **1-NO₂**.

7. Human serum albumin – interference with NTR detection

The effect of albumin on the fluorescence of the compound were conducted by measuring fluorescence spectra of compounds solution (5 μM) in the presence of different amounts of HSA (0-67 μM) in 50 mM TRIS-HCl buffer pH 7.4. The emission spectra were recorded between 480 and 800 nm upon excitation at 447 nm.

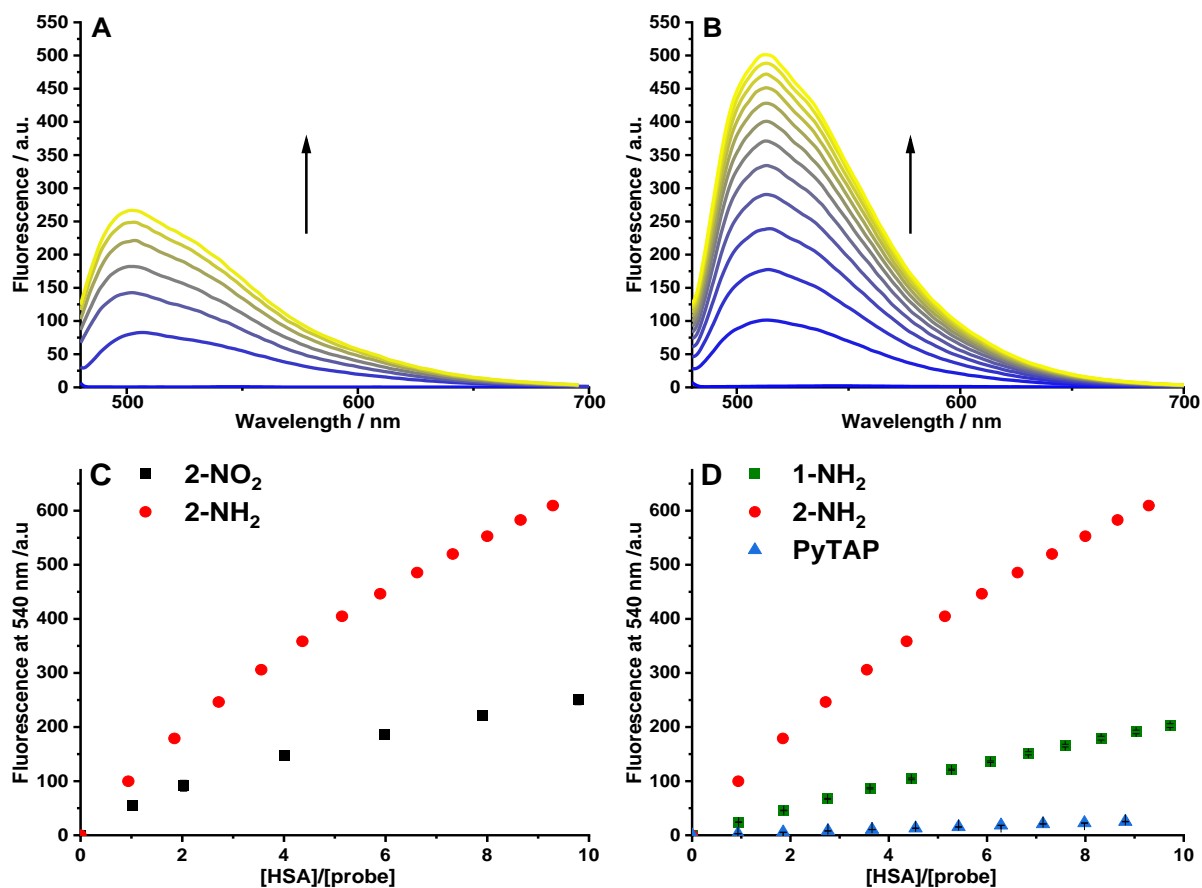


Figure A10. Fluorescence spectra of **2-NO₂** (A) and **2-NH₂** (B) in the presence of different amount of human serum albumin. (C-D) Fluorescence intensities at maximum of emission of compounds for various [HSA]/[probe] ratios. Experimental conditions: [1-NH₂] = [2-NO₂] = [2-NH₂] = [PyTAP] = 5 μM ; [HSA]= 0-52 μM ; [TRIS-HCl] = 0.05M, pH 7,4; 37°C; λ_{ext} = 447 nm.

Protein-binding experiments were carried out by measurement fluorescence spectra of protein solution (1 μM) in the presence of different amounts of compounds (0-7.5 μM) in TRIS-Cl buffer pH 7.4 at 37 °C. Before each measurement of emission spectra, the tested solutions were incubated for 5 min at 37 °C.

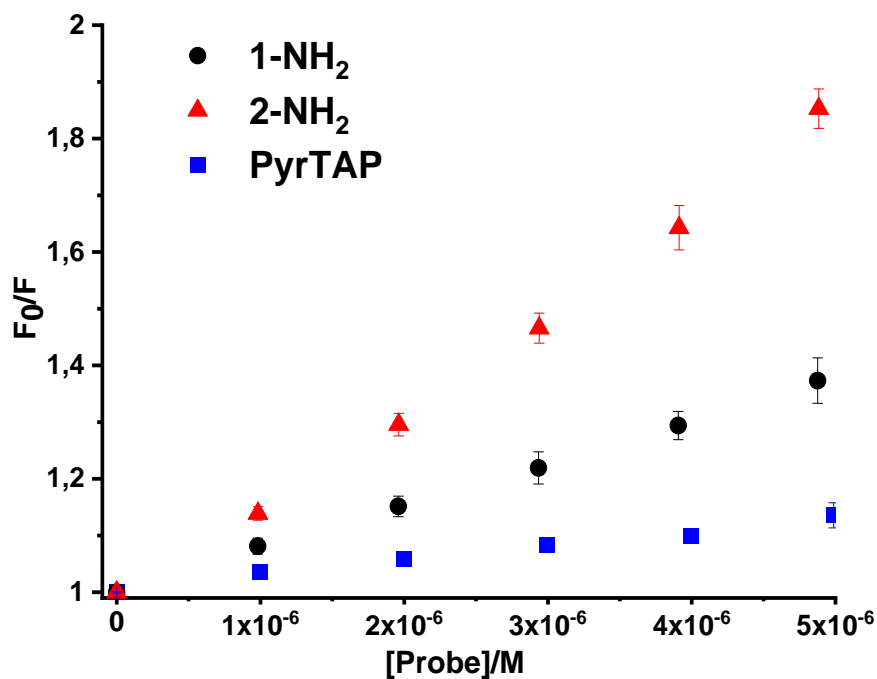


Figure A11. The Stern – Volmer plot showing the influence of the increasing concentration of the studied compound on the fluorescence intensity of HSA at $\lambda_{em} = 350$ nm. Experimental conditions: [HSA] = 1 μ M; [TRIS-HCl] = 0.05M, pH 7.4; 37°C; $\lambda_{ext} = 295$ nm.

Table A3. The association constants for formation HSA–ruthenium(II) polypyridyl complex adducts.

Compound	HSA association constant K_{sv} [10^5 M^{-1}]
1-NH ₂	0.758±0.006
2-NH ₂	1.874±0.022
PyrTAP	0.292±0.007

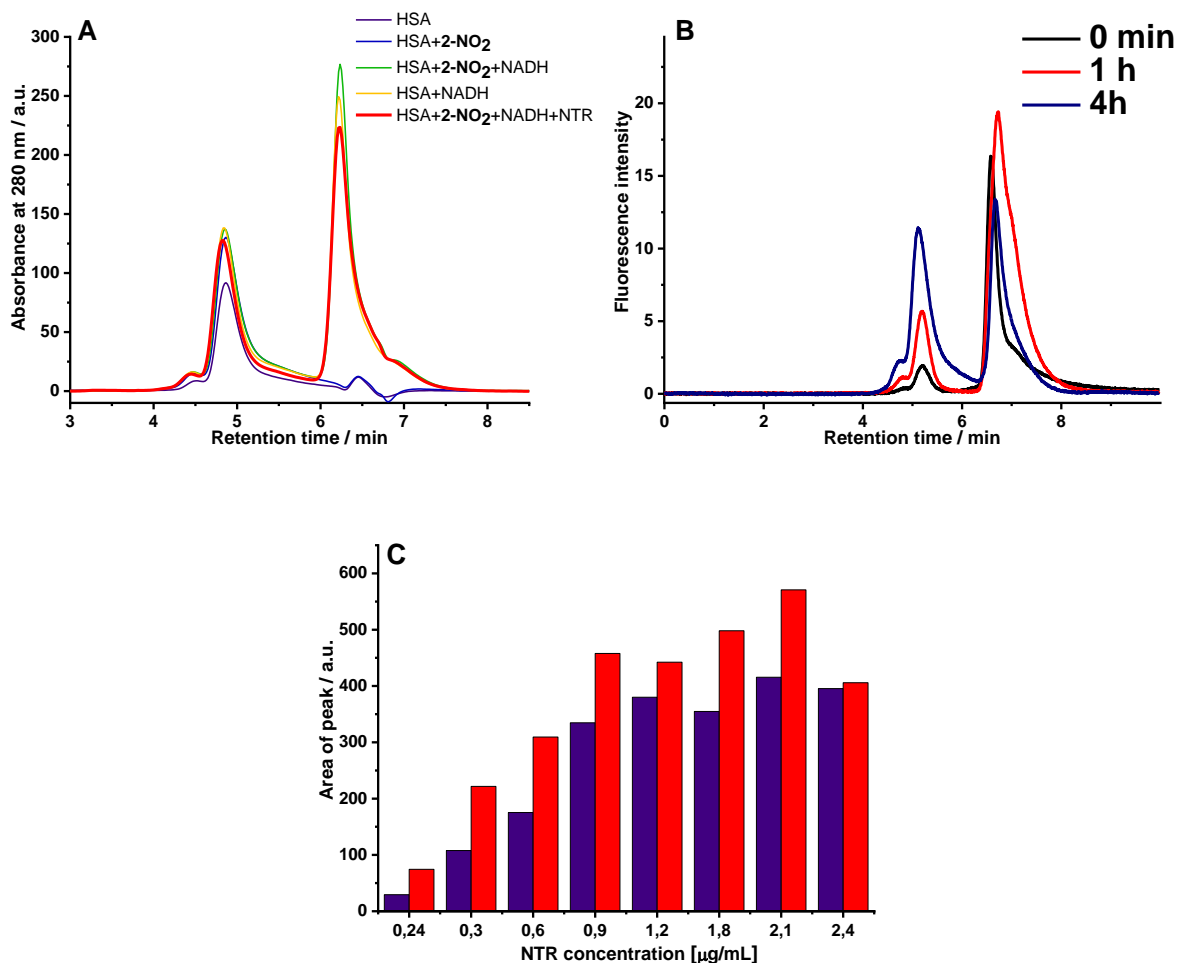


Figure A12. Size exclusion separation of 16 μ M human serum albumin (HSA) kept in the presence of **A)** 16 μ M 2-NO₂, 160 μ M NADH, 0.1 μ M NTR or their mixtures during 1h, Chromatograms were recorded using absorbance at 280 nm. **B)** a mixture of 16 μ M 1-NO₂, 160 μ M NADH, 0.1 μ M NTR during various time. **C)** Area peaks corresponding to the peaks obtained for the mixture of 16 μ M 2-NO₂, 160 μ M NADH, 16 μ M HSA and various concentration of NTR kept either 4 h (purple) or 24 h (red). Chromatograms were recorded using fluorescence detector with λ_{ext} 490 nm and λ_{em} 550 nm. Experimental conditions: TRIS-HCl buffer (pH 7.4; 50 mM), 37 °C

8. Cytotoxicity and photocytotoxicity

The evaluation of the cytotoxicity of the studied compound on A2058 cells, was conducted using resazurin assay. Cells were seeded on 96-well plate with density of 3×10^4 cells per cm^2 and cultured for 24 h in medium with FBS. Next, cells were incubated with various concentrations of compounds for 24 h. The tested compounds were dissolved in DMSO and then added to medium without FBS to achieve final concentrations. The final DMSO concentration was kept constant at 0.5 %. Upon completion of the incubation resazurin assay was carried out. For this purpose, cells after 24 h incubation with compounds were incubated in the resazurin sodium salt solution (50 μ M) for 3 h. The cell viability was quantified by monitoring emission at 605 nm using excitation light at 560 nm (Tecan Infinite 200 microplate reader). Experiments were performed in triplicate and repeated at least three times to get the

mean values \pm standard deviation. The viability was calculated with respect to the untreated cells control. The IC_{50} values were determined using the Hill equation (OriginPro2018)[5]

$$y = y_0 + \frac{(y_{100} - y_0)[c]^H}{[IC_{50}]^H + [c]^H}$$

For photocytotoxic experiments cells were preincubated with probes for 24 h and then were washed with PBS and irradiated with monochromatic blue light (465 nm 7.35 mW/cm^2) for different periods of time. After the irradiation cells were kept in fresh complete medium for 24 h, and then the viability assay was performed.

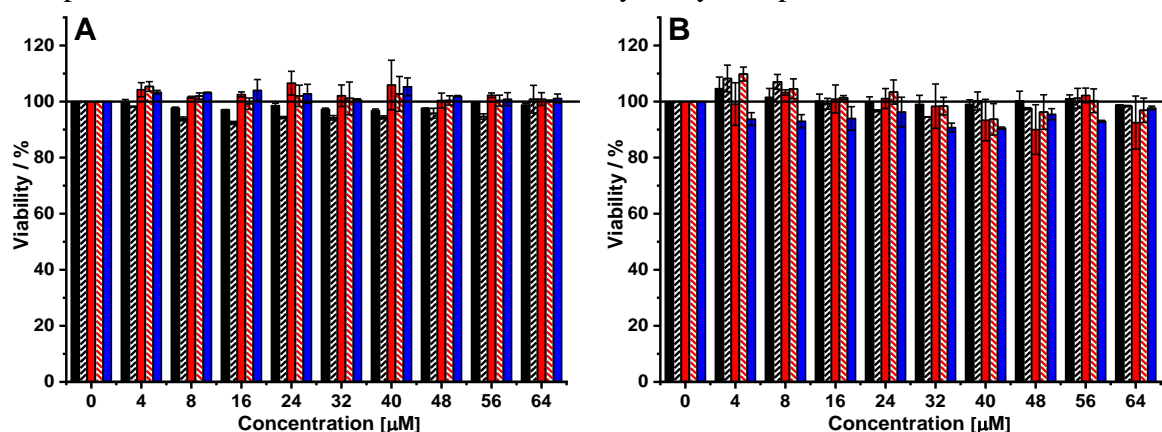
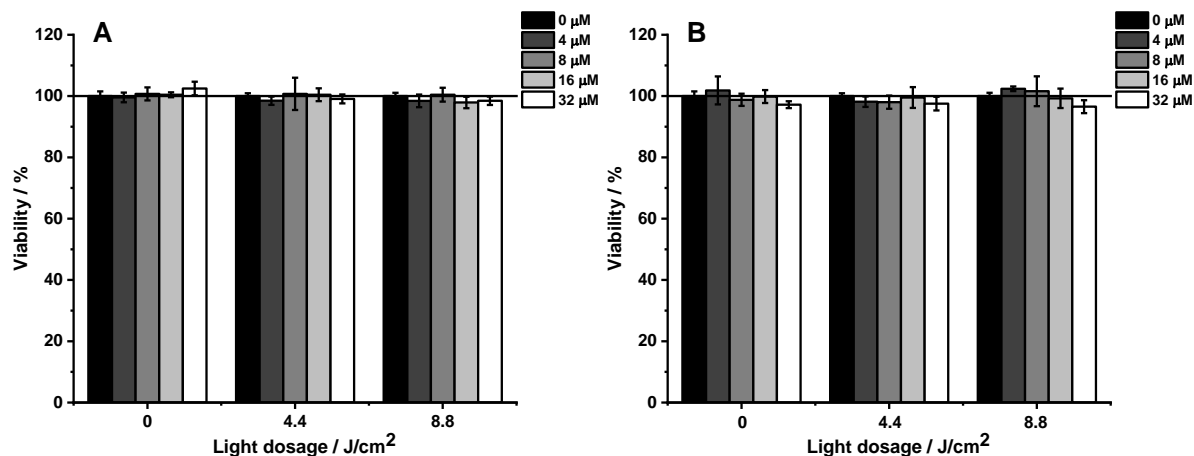


Figure A13. Viability of A2058 cell line incubated 24 h with the probe **1-NO₂** (black), **1-NH₂** (black dashed), **2-NO₂** (red) **2-NH₂** (red dashed) and **PyTAP** (blue) under normoxic (A) and hypoxic (B) conditions. Cell viability was assessed by resazurine assay.



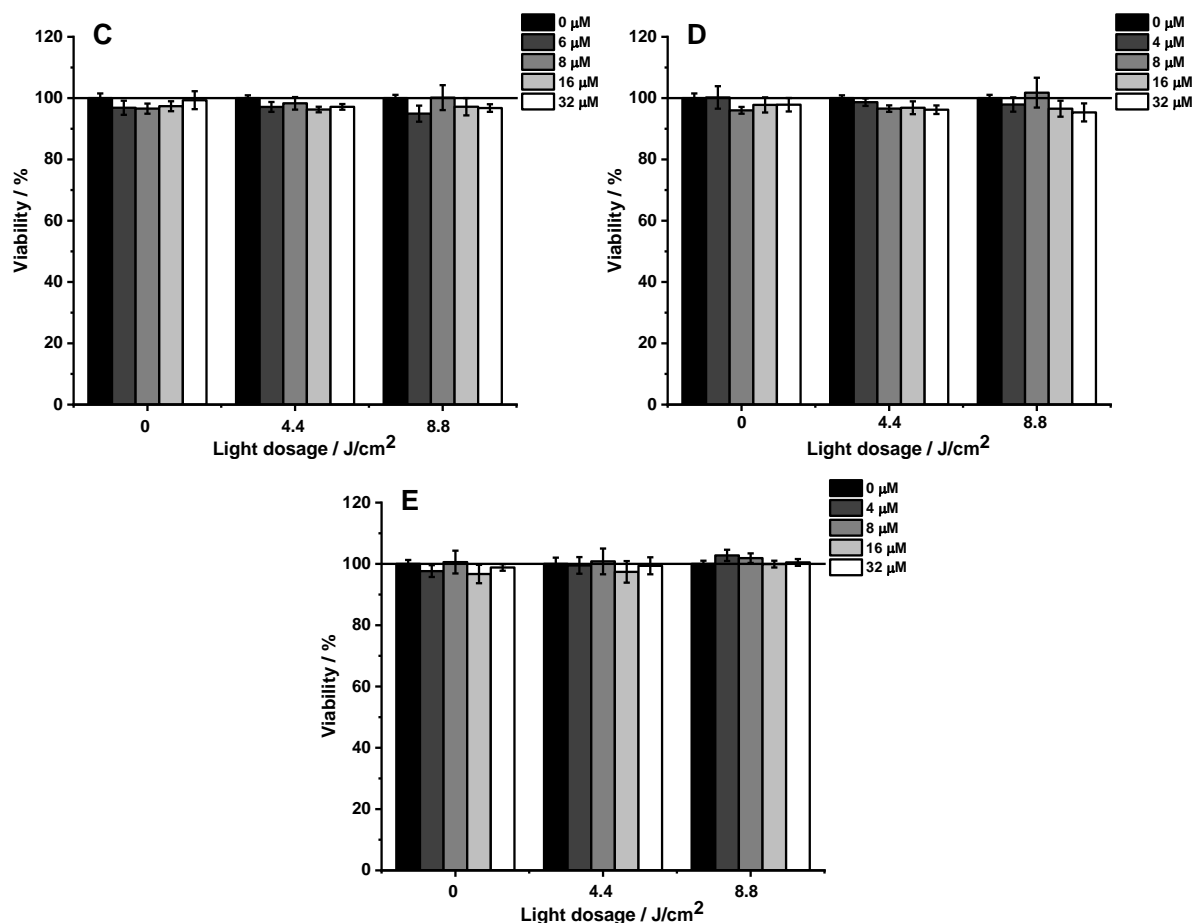


Figure A14. The photocytotoxic activity of **1-NO₂** (A) , **1-NH₂** (B), **2-NO₂** (C) **2-NH₂** (D) and **PyTAP** (E) against the cancer A2058 cell line measured after 24 h of incubation in medium without FBS in aerobic conditions in the dark and after irradiation. Cell viability was assessed by resazurin assay. Concentration of the studied compounds was in the range 0 – 32 μM . Lamp wavelength for irradiation: 465 nm; lamp power 7.35 mW/cm^2 .

9. Photostability

The photostability of amine derivatives of both probes i.e., **1-NH₂** and **2-NH₂** has been evaluated also under *in vitro* conditions. For this purpose, A2058 cells were seeded on a 96-wells culture plate with a density of 3×10^4 cell per cm^2 . A day later the cells were incubated with the tested compounds with a concentration of 40 μM for 24 h. After the incubation, cells were washed with PBS containing Mg^{2+} and Ca^{2+} . New PBS solution was added to the wells and the plates were irradiated for various periods of time using a lamp with an emission at 465 nm and power of 7.35 mW/cm^2 . After the defined exposure time the fluorescence intensity ($\lambda_{\text{ext}} = 447$ nm and $\lambda_{\text{em}} = 530$ nm) was monitored using Tecan Infinite 200 microplate reader.

10. Uptake

A2058 cells were seeded in a 24-well plate at a density of 3×10^4 cells per cm^2 . 24 hours after seeding, the **1-NO₂** or **2-NO₂** at two concentrations either 2 or 8 μM were added and incubated for the different period (10 min – 72h). Next, cells were washed with PBS, detached by trypsin treatment, and analyzed by BDLSR cytometer ($\lambda_{\text{ext}} = 488$ nm).

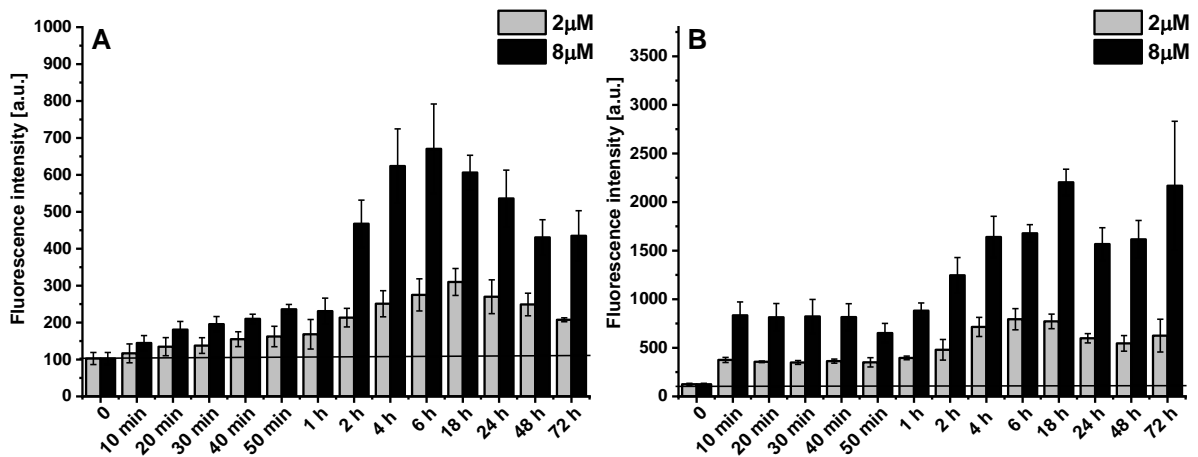


Figure. S15. Fluorescence intensity of A2058 cells incubated with 1-NO₂ (A) and 2-NO₂ (B) for different periods of time under normal oxygen conditions. 0 denotes the blank sample. ($\lambda_{ex} = 488 \text{ nm}$, $\lambda_{em} = 527 \pm 16 \text{ nm}$).

11. Detection of NTR in cell suspension

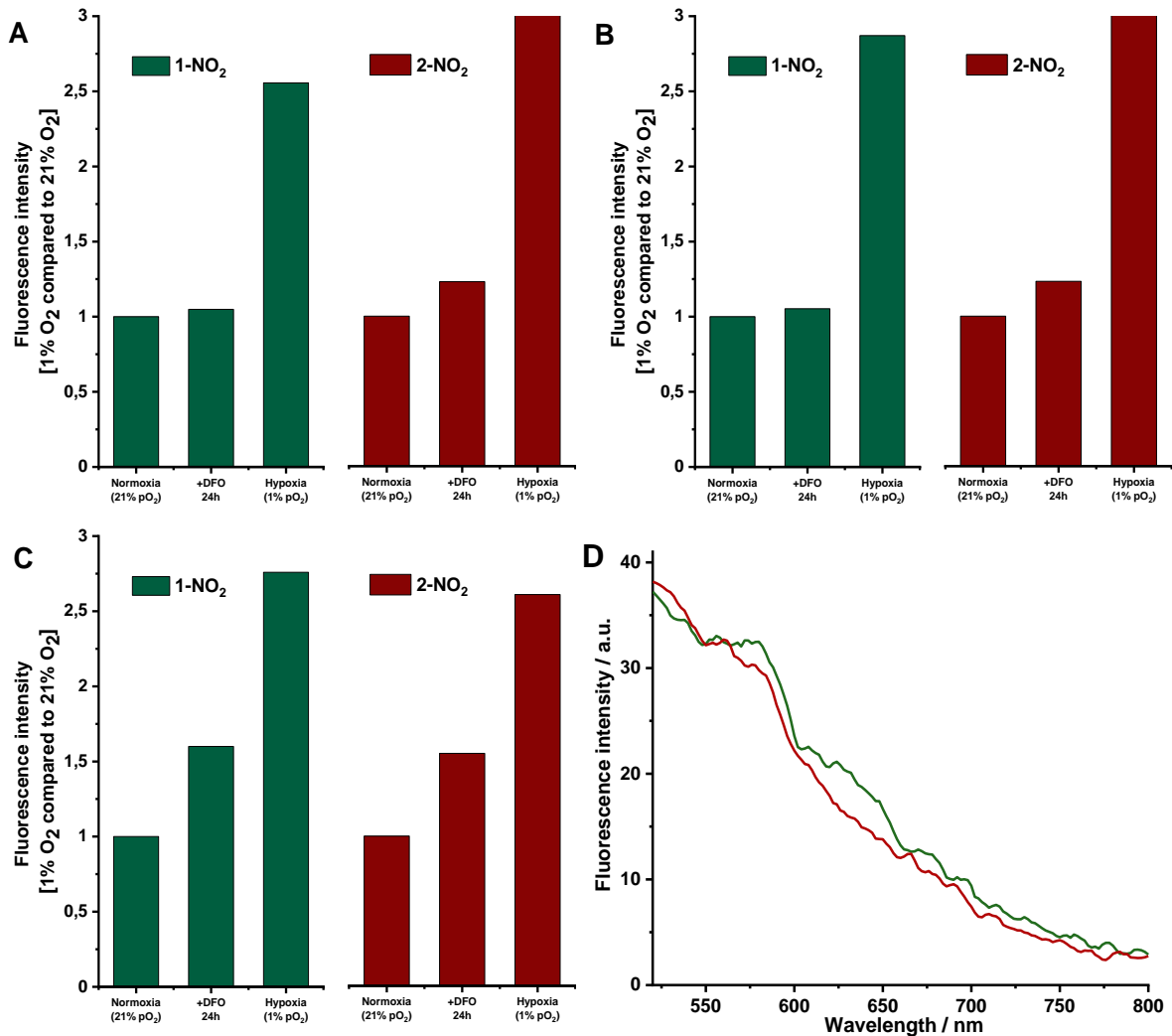


Figure. A16. Relative fluorescence intensity from 1-NO₂ and 2-NO₂ (2 μM) incubated 2h with A2058 cells under decreased oxygen concentration (pO₂ 1%) or with cells pre-incubated with

200 μM desferrioxamine (DFO) for 24 h under normoxia (pO_2 21%) compare to cells kept under normoxia. Measurement was performed using flow cytometry with different optical configurations (ex/em): **A** – FITC (488/527 \pm 16 nm), **B** – PE (488/586 \pm 21 nm), **C**- PerCP (488/700 \pm 27 nm). **D** – Fluorescence emission spectra of **1-NO₂** (green line) and **2-NO₂** (red line) after 2 h accumulation in A2058 under air-equilibrated conditions (pO_2 21%) after excitation at 488 nm.

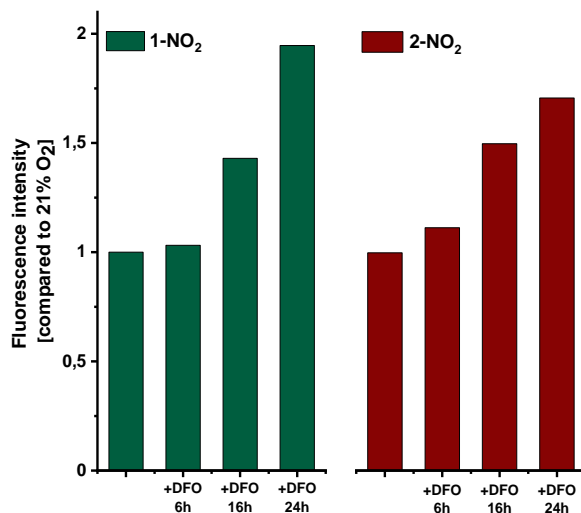


Figure A17. Relative fluorescence intensity from **1-NO₂** and **2-NO₂** (2 μM) incubated 2h with A2058 cells pre-incubated with 200 μM desferrioxamine (DFO) for 6, 16 or 24 h under normoxia (pO_2 21%) compare to cells kept under normoxia determined using flow cytometry.

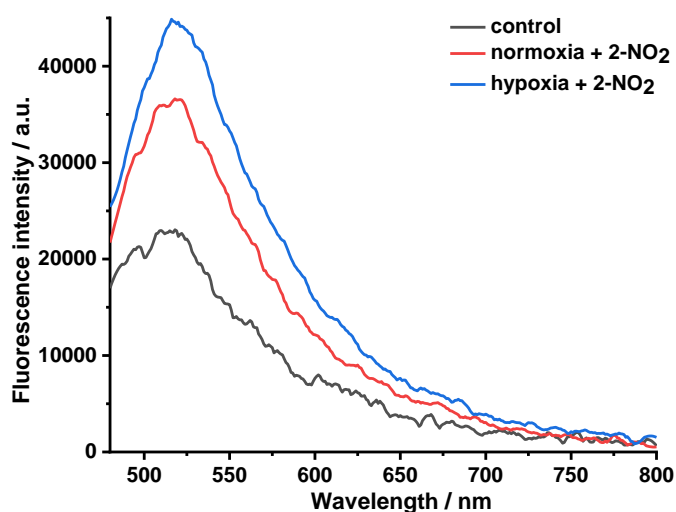
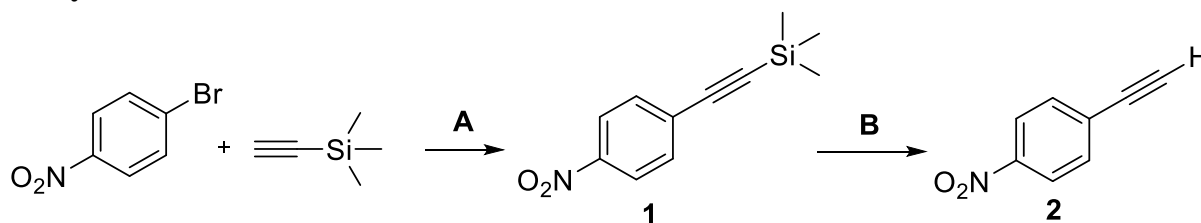


Figure. A18. Fluorescence emission spectra of A2058 cells (black line, control) and A2058 cells incubated 2h with **2-NO₂** under air-equilibrated conditions (red line, normoxia, pO_2 21%) or decreased oxygen concentration (blue line, hypoxia pO_2 1%) Measurements were performed using a plate reader with excitation at 446 nm.

12. Synthesis



Scheme A2. Preparation of 1-ethynyl-4-nitrobenzene (**2**). Reaction conditions: (A) Pd(PPh₃)₂Cl₂, CuI, Et₃N, THF, 60 °C, overnight (81% for **1**); (B) NaOH 10%, Et₂O/THF (1/1), RT, 15 min (77% for **2**).

trimethyl((4-nitrophenyl)ethynyl)silane (1)

Under argon atmosphere, 4-bromonitrobenzene (600 mg; 3.0 mmol), Pd(PPh₃)₂Cl₂ (42 mg; 0.1 mmol), CuI (23 mg; 0.1 mmol) were dissolved in THF. Et₃N (6 ml) and trimethylsilylacetylene were added and resulted mixture was stirred at 60°C during 16 h. After cooling the mixture to room temperature, filtration over Celite was carried out. The crude product was purified by column chromatography on silica gel with AcOEt/PE (97/3) as eluent to give **1** (533 mg, 81%) as brown solid. ¹H NMR (400 MHz, CDCl₃) δ: 8.18 (d, J = 8.9 Hz, 2H), 7.60 (d, J = 8.9 Hz, 2H), 0.28 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ: 147.27, 132.82, 130.11, 123.62, 102.82, 100.76, - 0.15. HRMS (ESI) for C₁₁H₁₄NO₂Si [M+H]⁺: found: 220.0787 *m/z*, calcd mass 220.0788. Mp: 88 °C.

1-ethynyl-4-nitrobenzene (2)

In a round-bottom flask, **1** (350 mg; 1.6 mmol) was dissolved in a mixture of diethyl ether (16 ml) and THF (16 ml) with addition of 10% aqueous NaOH (6.4 ml) under argon. Obtained solution was stirred during 15 minutes at room temperature and then it was neutralised with 2 M HCl to pH around 7. The extraction between AcOEt/water was performed and then organic phase was dried with MgSO₄, filtrated and concentrated. Column chromatography on silica gel with AcOEt/PE (4/96) as eluent afforded to isolate desired product **2** as an yellow solid (181 mg, 77 %). ¹H NMR (400 MHz, CDCl₃) δ: 8.18 (d, J = 8.9 Hz, 2H), 7.62 (d, J = 8.9 Hz, 2H), 3.36 (s, 1H). ¹³C NMR (101 MHz, CDCl₃) δ: 147.60, 133.06, 129.00, 123.65, 82.47, 81.70. HRMS for C₈H₅NO₂ [M+H]⁺: impossible to ionize this compound with ESI +/- and APCI +/- . Mp: 148 °C.

References

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- [2] J.R. Lakowicz, *Principles of fluorescence spectroscopy*, Springer, New York, 2006.
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- [4] H. Hosoya, L.C. Misal Castro, I. Sultan, Y. Nakajima, T. Ohmura, K. Sato, H. Tsurugi, M. Sugimoto, K. Mashima, *4,4'-Bipyridyl-Catalyzed Reduction of Nitroarenes by Bis(neopentylglycolato)diboron*, *Org. Lett.*, 21 (2019) 9812-9817.
- [5] W. J., D. Lochmann, A. Zimmer, *A practical note on the use of cytotoxicity assays*, *Int. J. Pharm.*, 288 (2005) 369-376.

Appendix. HPLC/HRMS data for reduction of both probes in the presence of NTR



Analysis Info

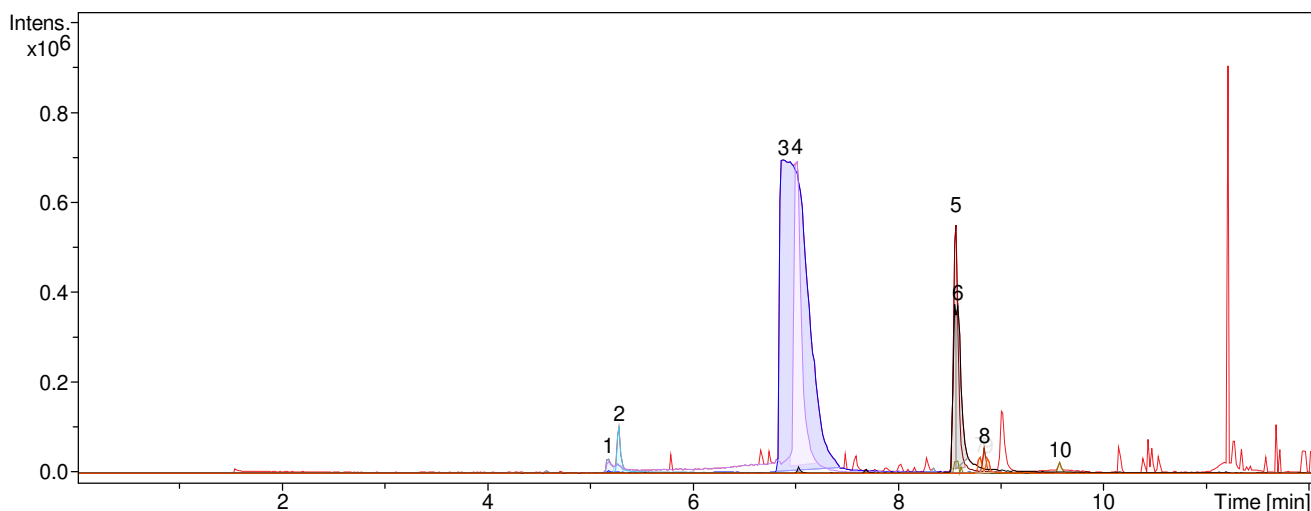
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Method

Acquisition Date <illegal time>

Laboratory
Instrument / Ser# MetaboliteDetect

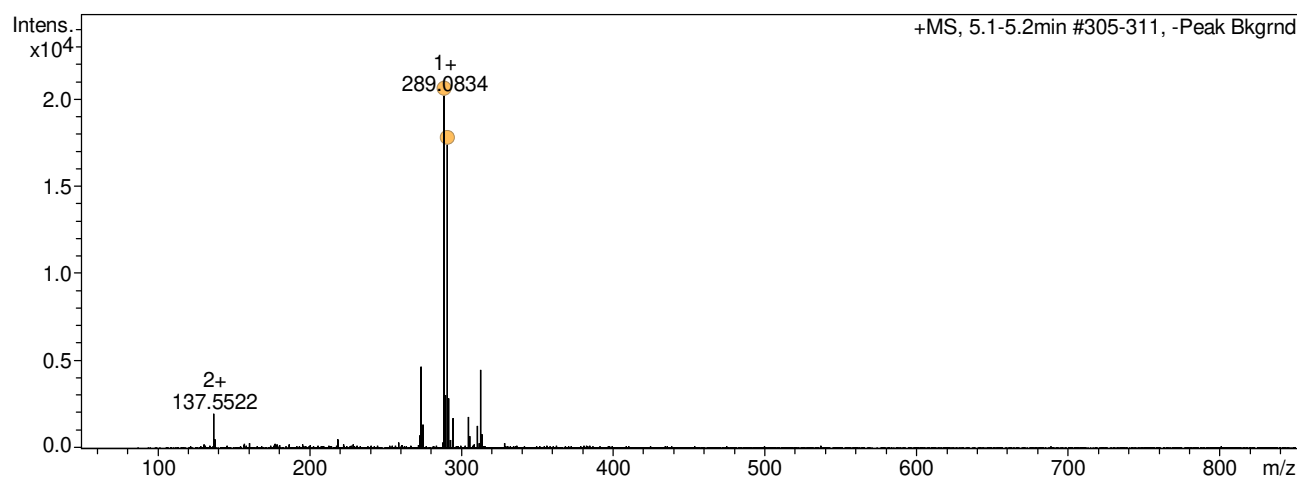
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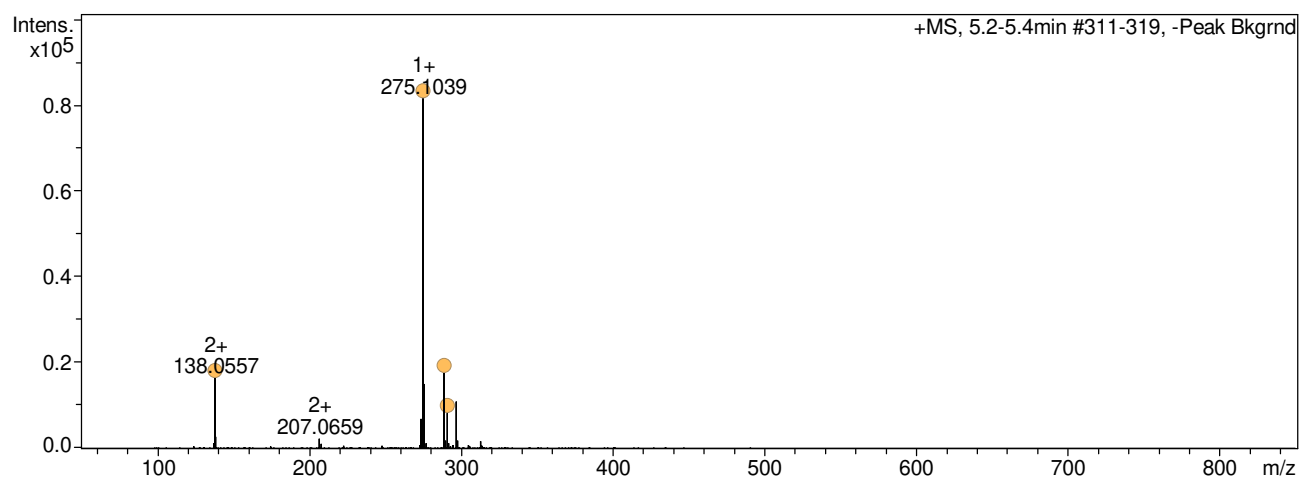
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2	5.3	273215	Manual	104901	239.8	EIC 275.1040±0.005 +All MS	275.1039	0.0
3	6.9	12818027	Manual	694084	680.3	EIC 305.0781±0.005 +All MS	305.0784	0.3
4	7.0	3469294	Manual	695423	669.5	EIC 289.0834±0.005 +All MS	289.0835	0.1
5	8.6	1723966	Manual	565759	1461.3	EIC 561.1634±0.005 +All MS	561.1635	0.0
6	8.6	1312653	Manual	372257	351.0	EIC 281.0858±0.005 +All MS	281.0865	0.0
7	8.8	88429	Manual	35940	101.9	EIC 288.0940±0.005 +All MS	288.0941	0.0
8	8.8	89811	Manual	55506	80.0	EIC 273.0890±0.005 +All MS	273.0891	0.0
9	8.9	75384	Manual	32853	89.2	EIC 288.0940±0.005 +All MS	288.0941	0.0
10	9.6	54937	Manual	23373	64.7	EIC 282.0941±0.005 +All MS	282.0942	0.0

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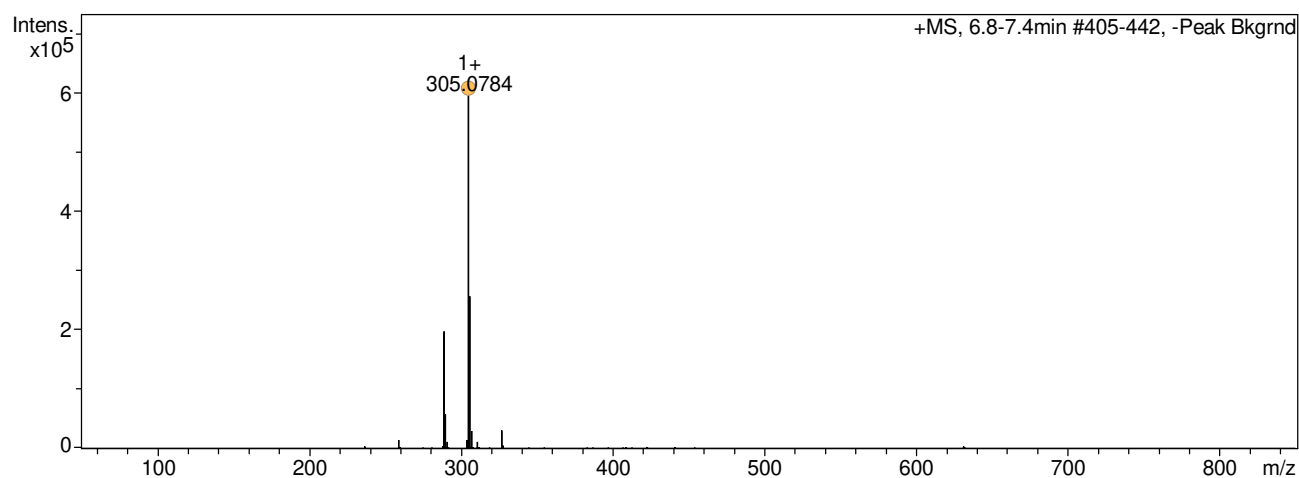
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291.0986	1	C ₁₅ H ₁₁ N ₆ O	291.0989	1.0	13.5	1	100.00	14.0	even		ok

Cmpd 2, 5.3 min



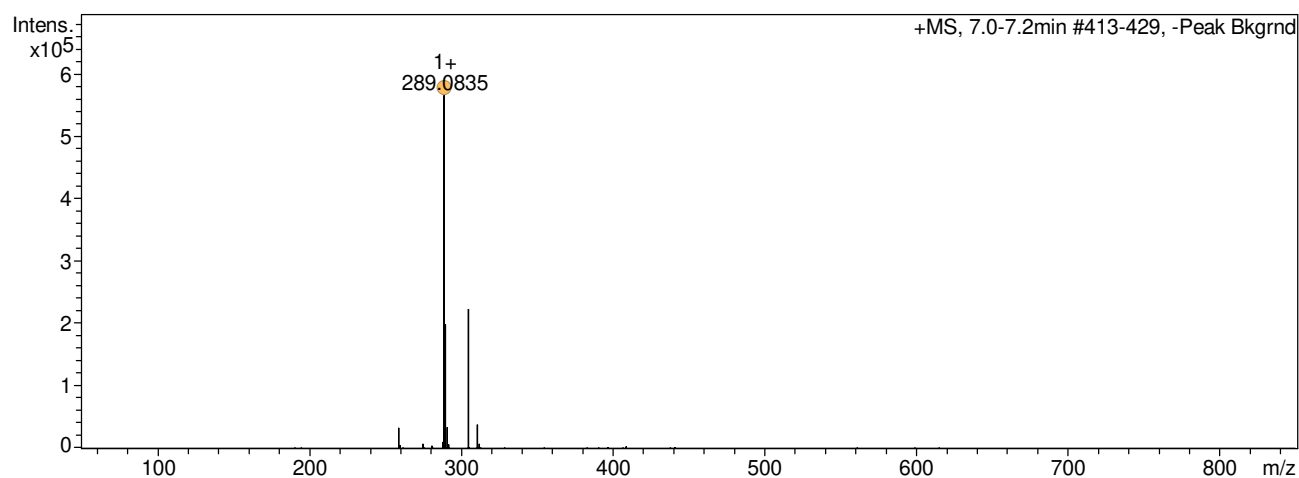
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275.1039	1	C ₁₅ H ₁₁ N ₆	275.1040	0.3	0.5	1	100.00	14.0	even		ok
289.0832	1	C ₁₅ H ₉ N ₆ O	289.0832	-0.0	49.3	1	100.00	15.0	even		ok
291.0988	1	C ₁₅ H ₁₁ N ₆ O	291.0989	0.4	27.7	1	100.00	14.0	even		ok

Cmpd 3, 6.9 min



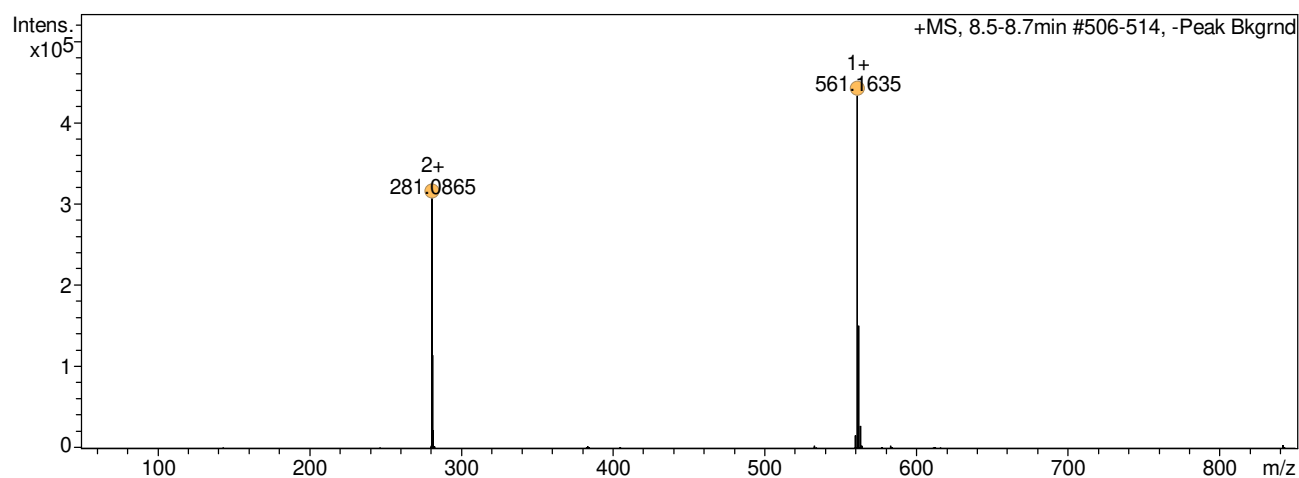
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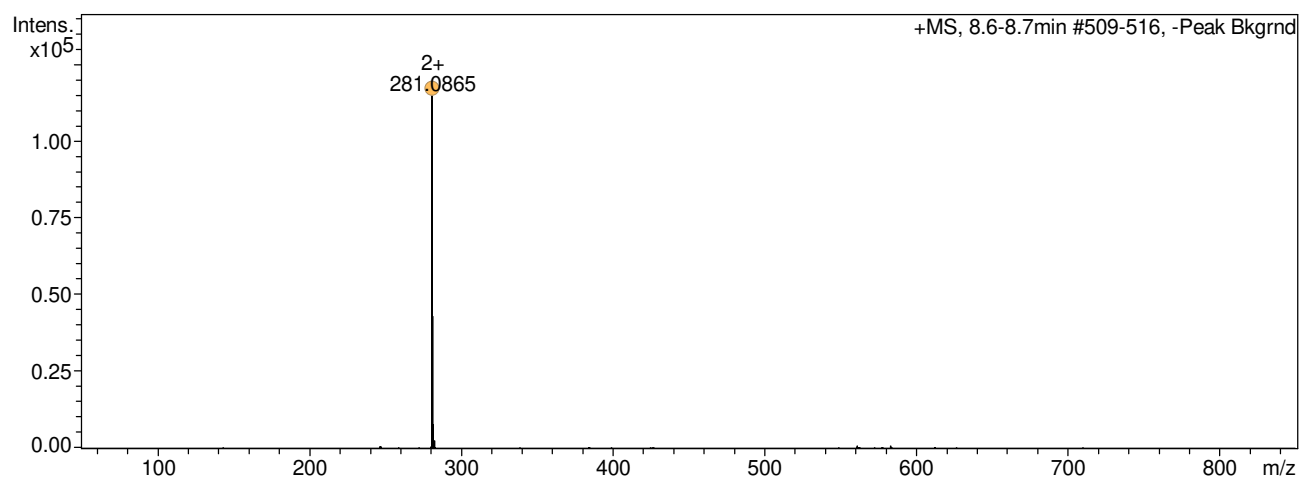
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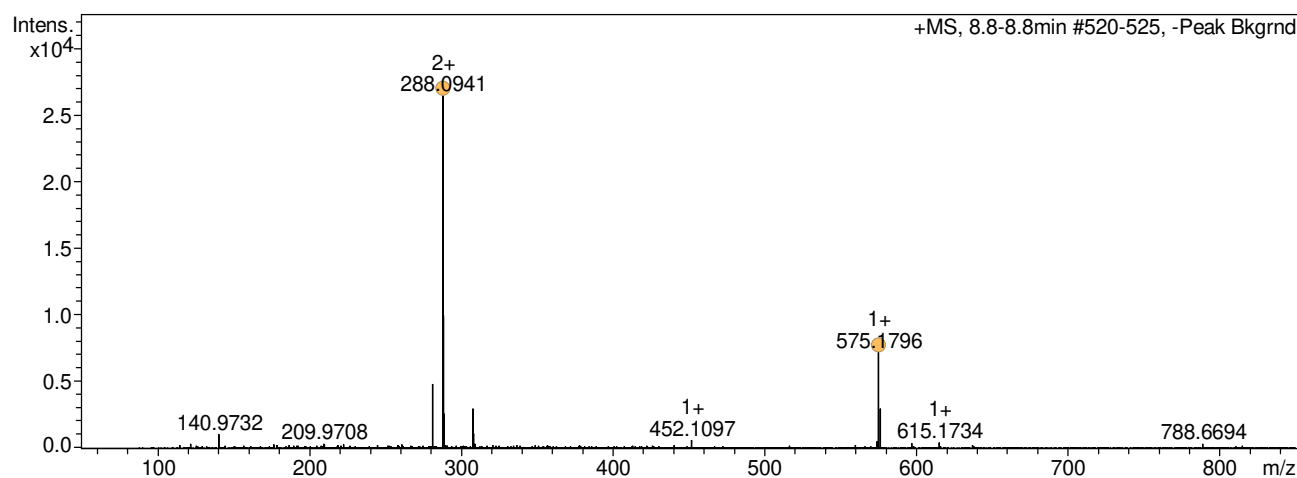
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281.0865	1	C30H18N12O	281.0858	-2.5	2.1	1	100.00	29.0	even		ok
561.1635	1	C30H17N12O	561.1643	1.4	11.5	2	74.62	29.0	even		ok

Cmpd 6, 8.6 min



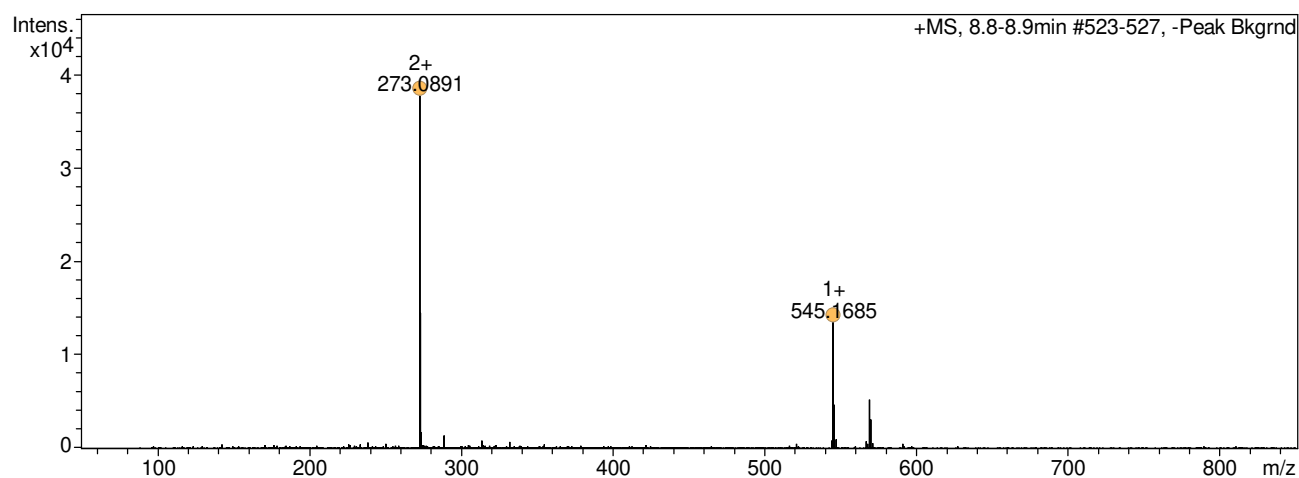
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Cmpd 7, 8.8 min



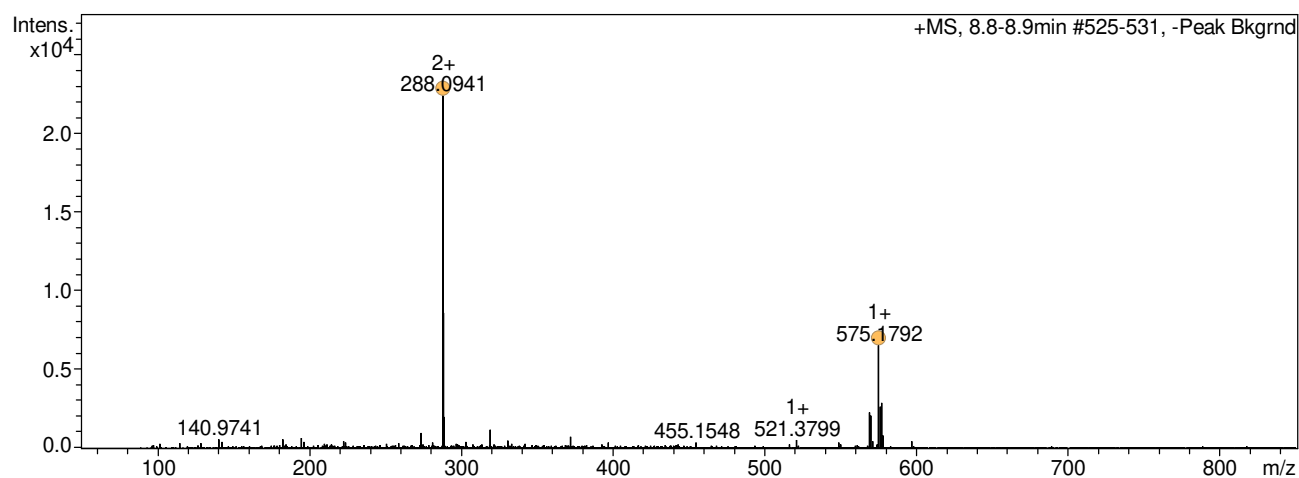
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288.0941	1	C ₃₁ H ₂₀ N ₁₂ O	288.0936	-1.6	12.7	1	100.00	29.0	even		ok
575.1796	1	C ₃₁ H ₁₉ N ₁₂ O	575.1799	0.5	40.8	1	100.00	29.0	even		ok

Cmpd 8, 8.8 min



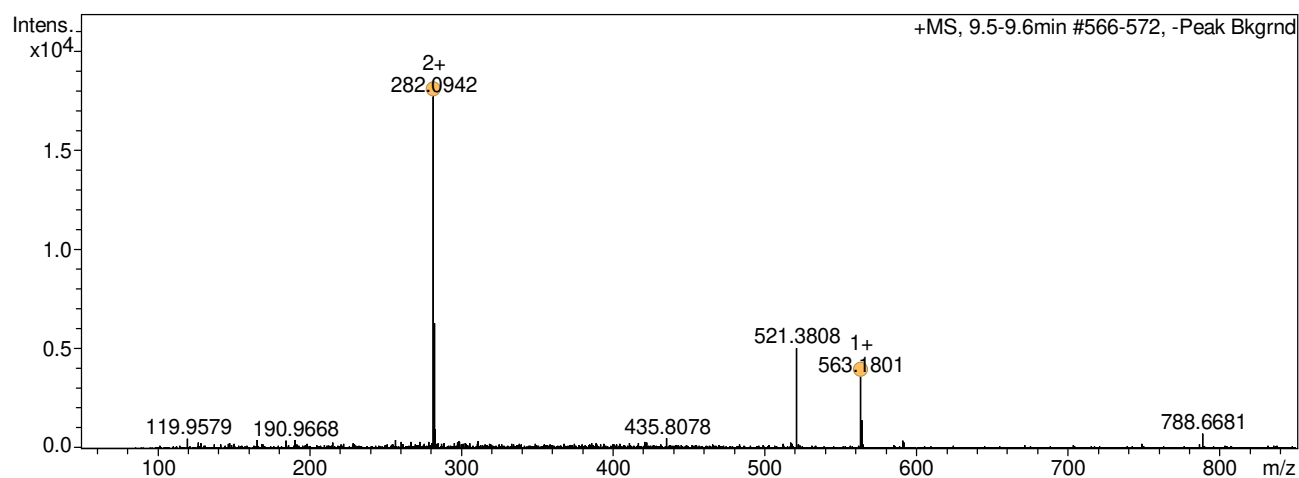
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273.0891	1	C30H18N12	273.0883	-2.9	12.3	1	96.63	29.0	even		ok
545.1685	1	C30H17N12	545.1694	1.6	12.1	1	100.00	29.0	even		ok

Cmpd 9, 8.9 min



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e ⁻	Conf	N-Rule
288.0941	1	C ₃₁ H ₂₀ N ₁₂ O	288.0936	-1.9	8.6	1	100.00	29.0	even		ok
575.1792	1	C ₃₁ H ₁₉ N ₁₂ O	575.1799	1.2	38.9	1	100.00	29.0	even		ok

Cmpd 10, 9.6 min



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e ⁻	Conf	N-Rule
282.0942	1	C30H20N12O	282.0936	-2.0	10.4	1	100.00	28.0	even		ok
563.1801	1	C30H19N12O	563.1799	-0.4	15.4	1	100.00	28.0	even		ok



Analysis Info

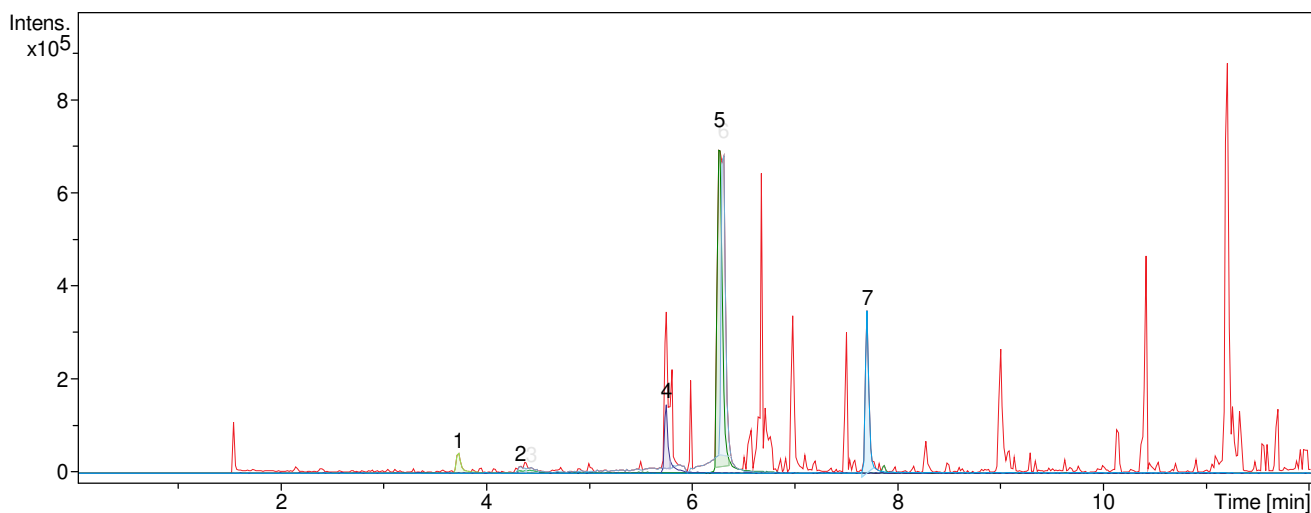
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Laboratory
Instrument / Ser# MetaboliteDetect

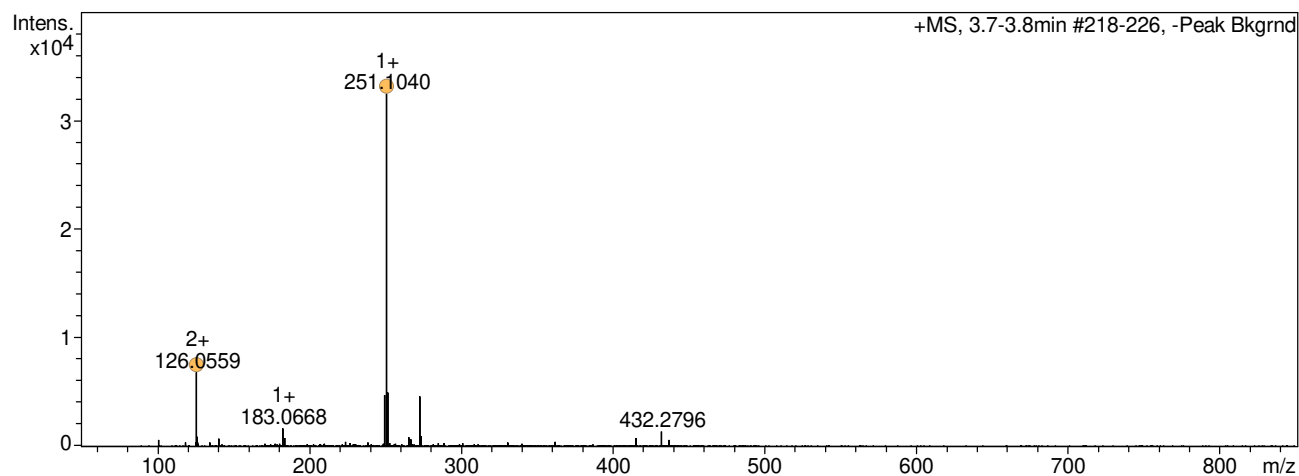
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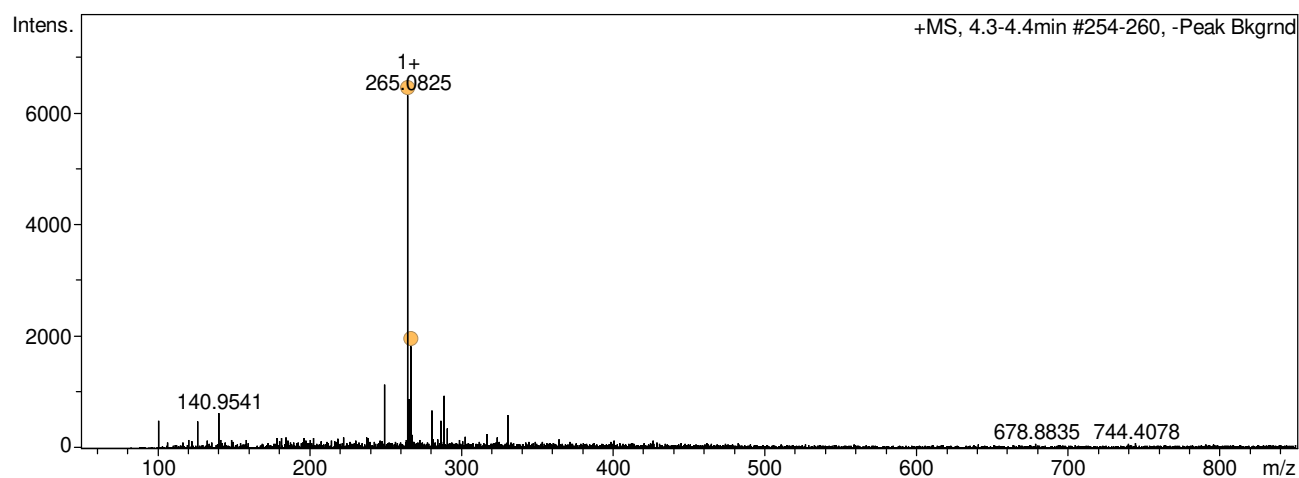
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2	4.3	54417	Manual	13933	12.9	EIC 265.0831±0.005 +All MS	265.0825	0.1
3	4.4	68955	Manual	11865	12.3	EIC 265.0831±0.005 +All MS	265.0823	0.1
4	5.8	313027	Manual	149450	365.8	EIC 217.0475±0.005 +All MS	163.0391	0.0
5	6.3	2434692	Manual	728519	1134.6	EIC 281.0781±0.005 +All MS	281.0785	0.1
6	6.3	1910893	Manual	705576	677.0	EIC 265.0831±0.005 +All MS	265.0834	0.0
7	7.7	876653	Manual	348927	719.0	EIC 257.0865±0.005 +All MS	257.0865	0.0

Cmpd 1, 3.7 min



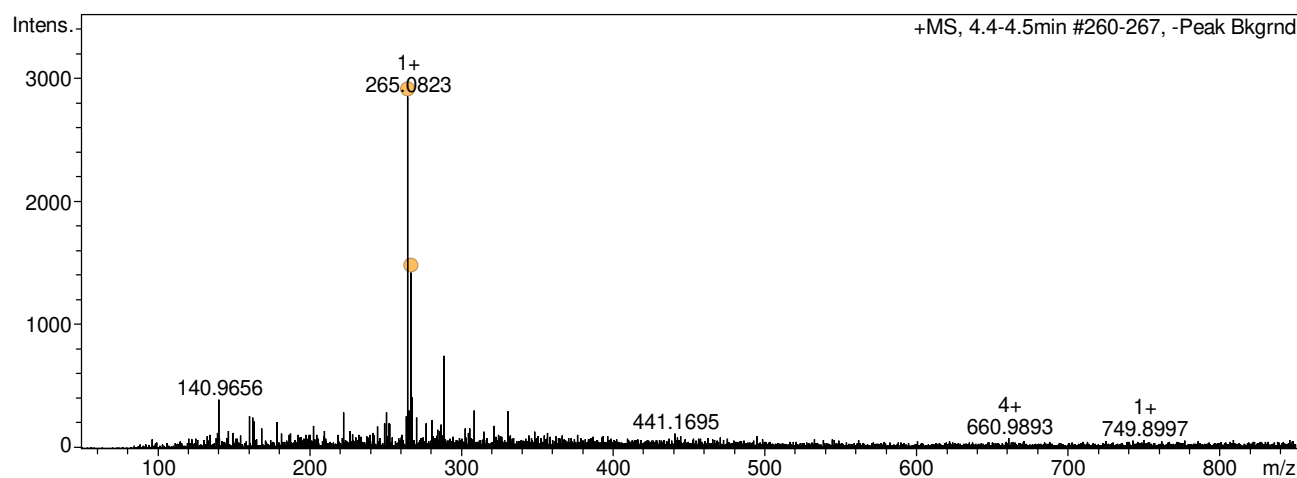
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251.1040	1	C ₁₃ H ₁₁ N ₆	251.1040	-0.2	6.5	1	100.00	12.0	even		ok

Cmpd 2, 4.3 min



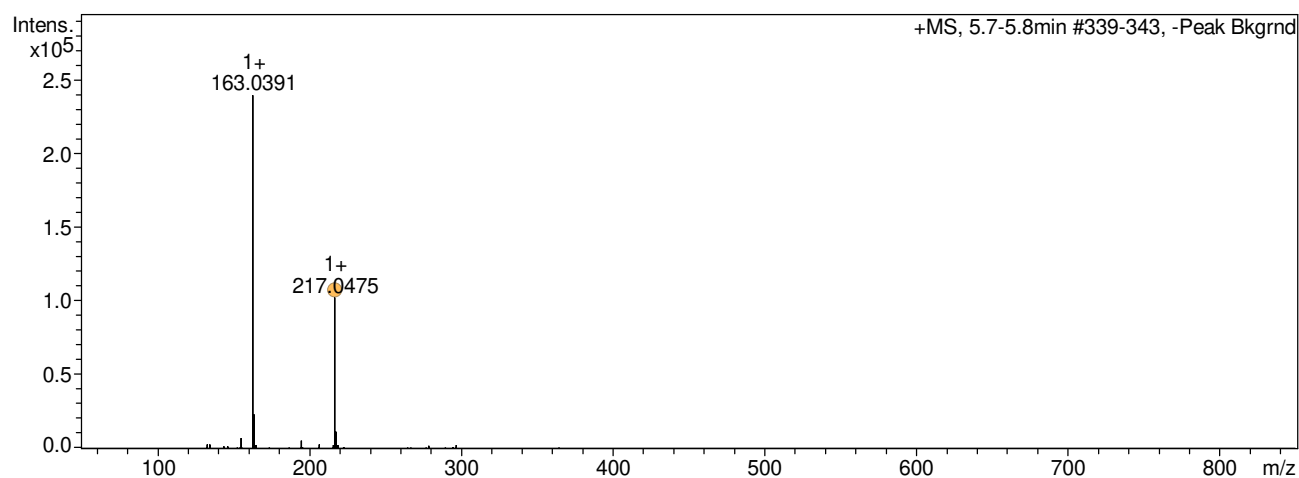
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267.0992	1	C13H11N6O	267.0989	-1.1	33.2	1	100.00	12.0	even		ok

Cmpd 3, 4.4 min



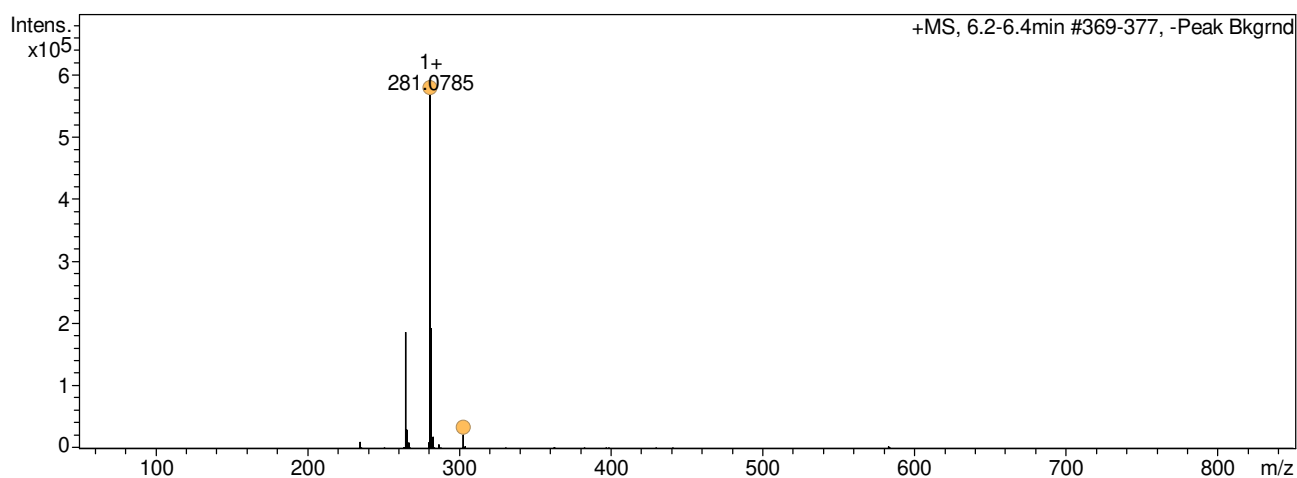
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267.0989	1	C13H11N6O	267.0989	-0.2	77.9	1	100.00	12.0	even		ok

Cmpd 4, 5.8 min



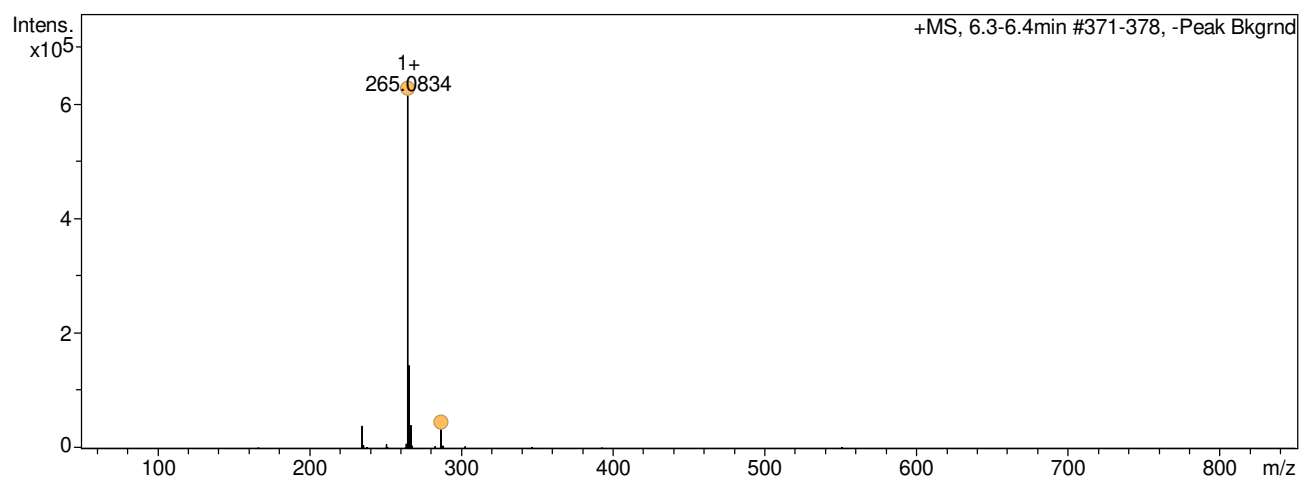
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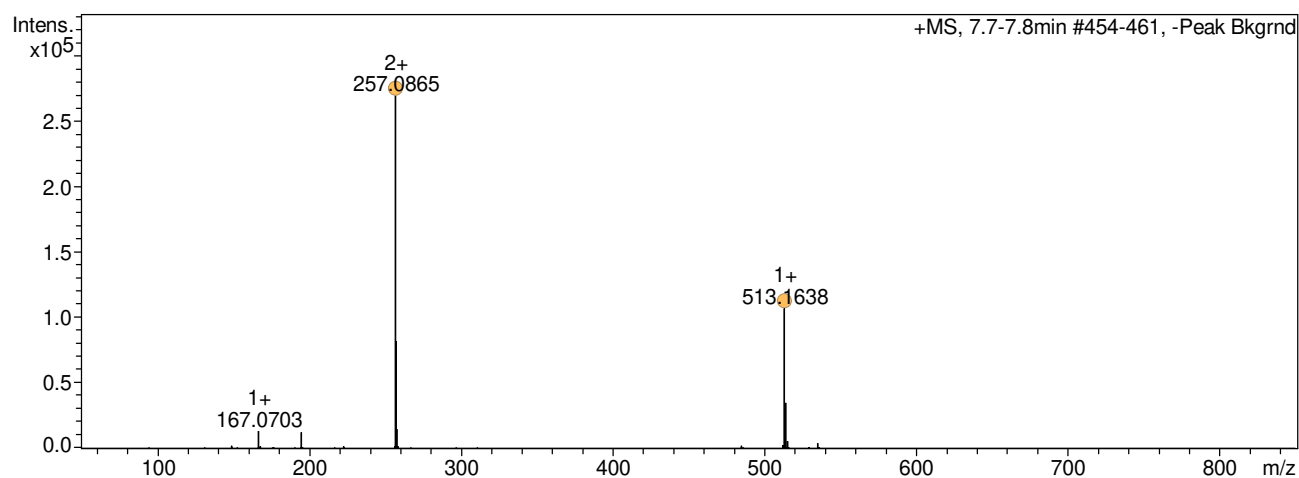
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303.0600	1	C ₁₃ H ₈ N ₆ NaO ₂	303.0601	0.3	9.5	1	100.00	13.0	even	ok

Cmpd 6, 6.3 min



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e ⁻	Conf	N-Rule
265.0834	1	C ₁₃ H ₉ N ₆ O	265.0832	-0.7	42.5	1	100.00	13.0	even		ok
287.0653	1	C ₁₃ H ₈ N ₆ NaO	287.0652	-0.5	4.9	1	100.00	13.0	even		ok

Cmpd 7, 7.7 min



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e ⁻	Conf	N-Rule
257.0865	1	C ₂₆ H ₁₈ N ₁₂ O	257.0858	-2.8	13.4	1	100.00	25.0	even		ok
513.1638	1	C ₂₆ H ₁₇ N ₁₂ O	513.1643	0.9	1.4	1	100.00	25.0	even		ok