Electronic Supplementary Information

A new genre of fluorescence recovery assay to evaluate polo-like kinase 1 ATP-competitive inhibitors

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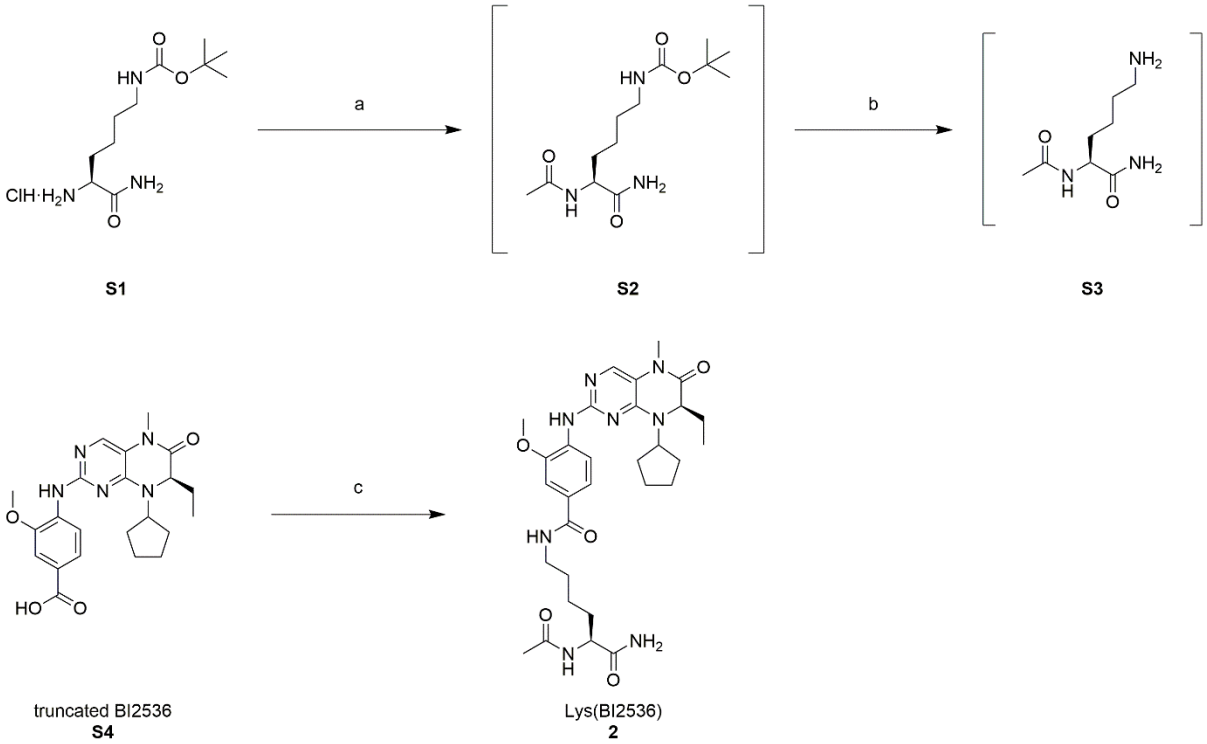
Experimental methods

General. All experiments involving moisture-sensitive compounds were conducted under anhydrous conditions (positive argon pressure) using standard syringe, cannula, and septa apparatus. Commercial reagents were purchased from Sigma, TCI America, Acros, Alfa Aesar, Chem-Impex, or Novabiochem. All solvents were purchased in anhydrous form (Aldrich) and used without further drying. HPLC-grade hexanes, EtOAc, dichloromethane (DCM), and MeOH were used for chromatography. Silica gel column chromatography employed a Telodyne CombiFlash Rf 200 instrument with either EtOAc/hexane or MeOH/DCM gradients. Nuclear Magnetic Resonance (NMR) spectra were recorded using a Varian Inova 400 MHz or 500 MHz spectrometer. Coupling constants are reported in Hertz, and peak shifts are reported in δ (ppm) relative to $CDCl_3$ (^1H 7.26 ppm, ^{13}C 77.16 ppm), MeOD (^1H 3.31 ppm, ^{13}C 49.00 ppm), or dimethyl sulfoxide (DMSO)-d_6 (^1H 2.50 ppm, ^{13}C 39.52 ppm). High resolution mass spectra (HRMS) were obtained by positive ion, electrospray ionization (ESI) analysis on a Thermo Scientific LTQ-XL Orbitrap mass spectrometer with high performance liquid chromatography (HPLC) sample introduction using a short narrow-bore C_{18} reversed-phase column with MeCN-H_2O gradients. Preparative HPLC purification was performed using a Waters 2545 binary pump (0.1% TFA in MeCN/0.1% trifluoroacetic acid (TFA) in H_2O gradient) with a Phenomenex Gemini-C_{18} (5 μm, 250 x 21 mm) preparative column with UV detection at 210 nm. Semi-preparative HPLC purification was performed using an Agilent 1200 series quaternary pump (0.1% TFA in MeCN/0.1% TFA in H_2O gradient) with a Phenomenex Kinetix-C_{18} (5 μm, 250 x 10 mm) semi-preparative column, 3 mL/min flow rate with UV detection at 210 nm. Analytical HPLC analyses of purified peptides were performed using an Agilent 1200 series quaternary pump (0.1% TFA in MeCN/0.1% TFA in H_2O gradient) with a Phenomenex Gemini-C_{18} (5 μm, 250 x 4 mm) analytical column, 1 mL/min flow rate with UV detection at 210 nm. UV spectra were measured using Shimadzu UV-2550 spectrophotometer and JASCO V-550. Fluorescence spectra were measured using JASCO FP-6600.

Synthesis of Lys(BI2536) (2).



Scheme S1. Synthesis of Lys(BI2536) (**2**). (a) Acetic anhydride, pyridine, THF/DMF = 2:1; (b) TFA, DCM; (c) **S3**, EDC·HCl, HOBt·H2O, DIPEA, DMF.

To a solution of H-Lys(Boc)-NH_2 (**S1**, 300 mg, 1.1 mmol) in tetrahydrofuran (THF, 11 mL) and *N*,*N*-dimethylformamide (DMF, 5.0 mL) was added acetic anhydride (0.50 mL, 5.3 mmol) and pyridine (0.43 mL, 5.3 mmol). The mixture was stirred at room temperature (3 h). The volatiles were evaporated. The crude material of Ac-Lys(Boc)-NH_2 (**S2**, 312 mg, quant.) was obtained as pale yellow powder. To the solution of **S2** (306 mg, 1.07 mmol) in DCM (5.0 mL) was added TFA (0.82 mL, 11 mmol) and stirred at room temperature (4 h). The volatiles were removed by nitrogen flow. The resulting crude material was added MeCN and H_2O then lyophilized. The crude material of Ac-Lys-NH_2·TFA (**S3**, 566.9 mg, 177%) was obtained as colorless oil containing H_2O determined by ^{1}H-NMR.

To a solution of truncated BI2536 (**S4**, 39 mg, 0.091 mmol)[1] in DMF (1.0 mL) was added 1-hydroxybenzotriazole (HOBt)·H_2O (12 mg, 0.091 mmol) followed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)·HCl (17 mg, 0.091 mmol) and the solution was stirred at room temperature (10 min). Separately, to a solution of Ac-Lys-NH_2·TFA (**S3**) in DMF (0.50 mL) was added *N*,*N*-diisopropylethylamine (DIPEA, 35 μL, 0.20 mmol) and the solution was added to the above and the combined reaction mixture was stirred at room temperature (6 h), then **S3** (1.1 eq.), DIPEA (2.2 eq.), EDC·HCl (1.0 eq.) and HOBt·H_2O (1.0 eq.) were added to the reaction mixture and stirred at room temperature (19 h). The mixture was partitioned between $CHCl_3$ and H_2O and the aqueous phase was extracted with $CHCl_3$ (three times) and the combined organic phase was washed with 1.0 M HCl in H_2O. The acidic aqueous solution was made alkaline by the addition of aqueous NaOH and this was then extracted with $CHCl_3$ (four times). The combined organic layer was washed with brine and dried (Na_2SO_4) and concentrated. The crude material was purified by CombiFlash silica gel

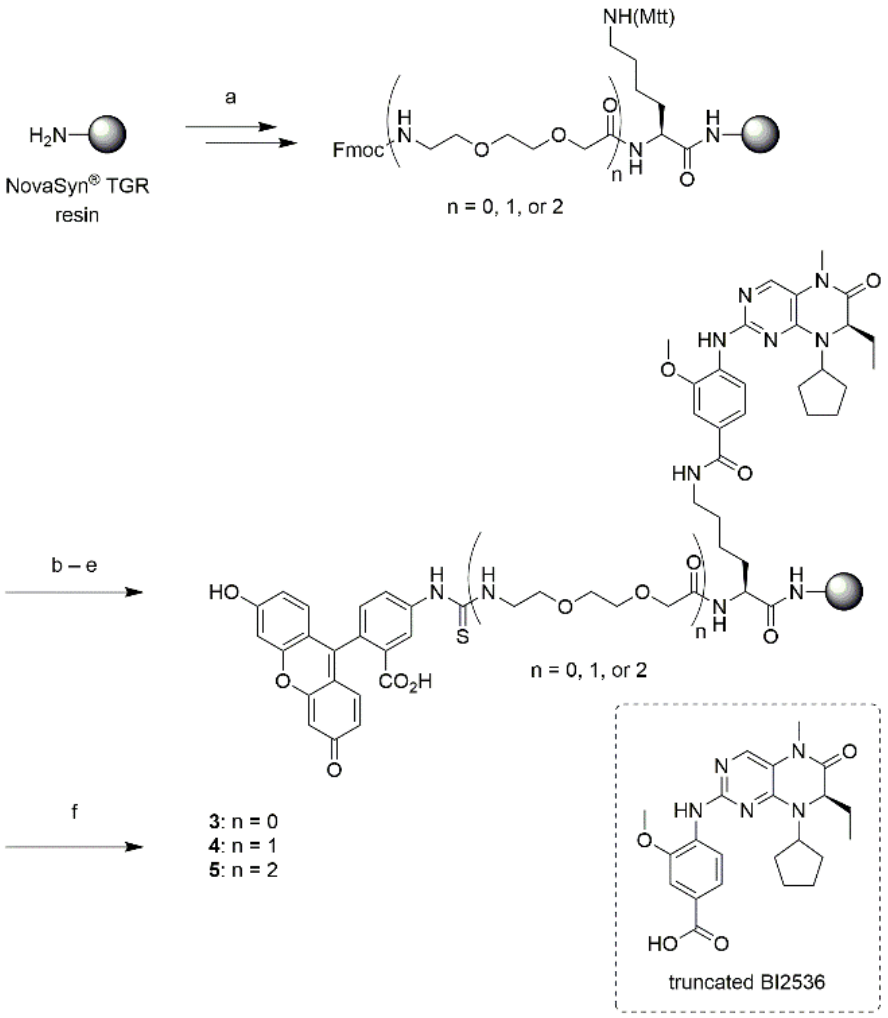
chromatography (MeOH/DCM gradient: 0-40% over 25 min) to afford Lys(BI2536) (**2**) as a white powder (14 mg, 26% yield).

^{1}H NMR (400 MHz, MeOD) δ 8.49 (d, *J* = 8.3 Hz, 1H), 7.78 (s, 1H), 7.49 – 7.46 (m, 2H), 4.55 – 4.47 (m, 1H), 4.33 – 4.27 (m, 2H), 4.00 (s, 3H), 3.40 (t, *J* = 7.0 Hz, 2H), 3.33 (s, 3H), 2.19 – 2.13 (m, 1H), 2.03 – 1.63 (m, 15H), 1.52 – 1.42 (m, 2H), 1.29 – 1.29 (m, 1H), 0.86 (t, *J* = 7.5 Hz, 3H).

^{13}C NMR (101 MHz, MeOD) δ 177.21, 173.36, 169.78, 165.55, 156.40, 153.76, 148.68, 139.30, 134.19, 127.79, 121.16, 117.67, 117.43, 110.03, 61.46, 60.34, 56.58, 54.49, 40.65, 32.83, 30.43, 30.26, 29.97, 28.59, 28.08, 24.36, 24.06, 22.48, 9.30.

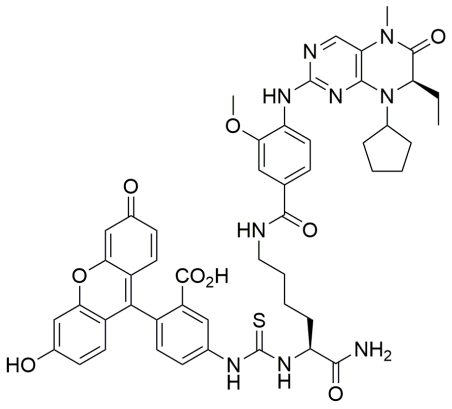
HRMS (ESI+) calculated for $C_{30}H_{43}N_8O_5$: 595.3351 $[M+H]^+$; found: 595.3342.

General solid-phase protocols for the synthesis of fluorescein isothiocyanate (FITC)-labeled probes 3 – 5. NovaSyn® TGR resin (Novabiochem, 0.25 mmol/g) was pre-swollen in *N*-methylpyrrolidone (NMP) for 20 min with shaking. The following loading procedure was used where applicable. Fmoc-protected amino acids (2.0 – 4.0 equivalents based on resin loading) were dissolved in NMP and pre-activated by the addition of 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 0.95 mole-equivalents relative to the amino acid) and DIPEA (2.0 mole-equivalents relative to the amino acid) with shaking (1 min). The resin was washed with NMP, and the HATU-activated amino acid solution was added to the washed resin. Coupling reactions were shaken at room temperature and allowed to proceed from 2 h to overnight, depending on the equivalents used and the steric bulk of each amino acid. Coupling reactions were routinely checked for completion using a Kaiser test. Once completed, the resin was filtered and washed with NMP, followed by Fmoc-deprotection using 20% piperidine in DMF for 10 min with shaking. Deprotection of the 4-methyltrityl (Mtt) group on the Lys ε-amine group was performed by treatment with DCM/TFA/triisopropylsilane (TIPS) = 95:1:4 (three times for 20 min each). The resin was subsequently coupled with truncated BI2536 (**S4**, 1.0 equivalents based on resin loading) using HATU (0.95 mole-equivalents relative to the amino acid) and DIPEA (2.0 mole-equivalents relative to the amino acid) at room temperature with shaking (from 3 h to overnight, twice). After completion of the coupling, the Fmoc group was removed using 20% piperidine in DMF with shaking (10 min). The resulting resin was treated with FITC (3.0 equivalents based on resin loading) in the presence of DIPEA (2.0 mole-equivalents relative to FITC) (from 3 h to overnight). Cleavage from the finished resin with global deprotection was achieved using a cocktail of TFA/TIPS/H_2O = 95:2.5:2.5 (4.0 mL, 2 h). The mixture was filtered and concentrated under a stream of N_2. The resulting crude material was dissolved in 0.1% TFA containing MeCN and H_2O and subjected to preparative reverse-phase HPLC purification. Further purification was conducted using semi-preparative reverse-phase HPLC when needed. HPLC eluents were A: 0.1% TFA in H_2O; B: 0.1% TFA in MeCN.



Scheme S2. Synthesis of probes **3** – **5**. (a) Fmoc-SPPS; (b) DCM/TFA/TIPS = 95:1:4; (c) truncated BI2536, HATU, DIPEA, NMP; (d) 20% piperidine in DMF; (e) FITC, DIPEA, NMP; (f) TFA/TIPS/H_2O = 95:2.5:2.5.

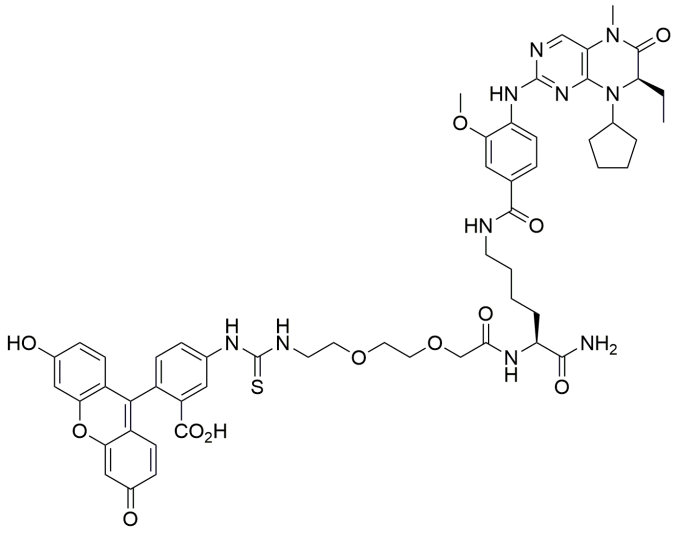
FITC-Lys(BI2536)-NH_2 (3).



3

This compound could not be obtained (as mentioned in the text).

FITC-miniPEG-Lys(BI2536)-NH_2 (4).



4

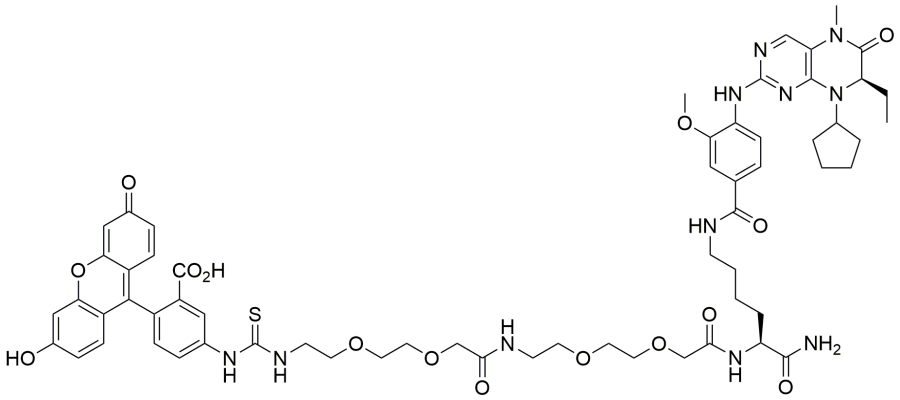
Compound **4** was obtained as a yellow powder (4.3 mg, 20% yield from 0.02 mmol resin) as shown in Scheme S2 according to the general solid-phase synthesis protocols described above.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.06 (bs, 2H), 9.37 (s, 1H), 8.46 (dd, J = 5.5 Hz and 5.5 Hz, 1H), 8.31 (s, 1H), 8.17 (m, 1H), 7.84 (d, J = 8.3 Hz, 1H), 7.75 – 7.74 (m, 2H), 7.61 – 7.50 (m, 4H), 7.17 (d, J = 8.3 Hz, 1H), 7.13 (s, 1H), 6.67 (d, J = 2.1 Hz, 2H), 6.61 – 6.54 (m, 4H), 4.47 – 4.45 (m, 1H), 4.32 – 4.27 (m, 1H), 4.16 (quin, J = 5.5 Hz, 1H), 3.94 (s, 2H), 3.90 (s, 3H), 3.70 – 3.60 (m, 8H), 3.51 (s, 2H), 3.27 – 3.21 (m, 5H), 1.92 – 1.31 (m, 18H), 0.75 (t, J = 7.4 Hz, 3H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 180.61, 173.37, 168.91, 168.51, 165.14, 162.49, 159.48, 158.81, 158.43, 158.08, 157.77, 152.22, 151.86, 149.43, 141.60, 141.38, 131.53, 129.46, 129.00, 128.35, 126.49, 124.00, 122.15, 119.49, 117.61, 116.33, 115.79, 114.68, 112.57, 110.21, 109.70, 102.21, 70.25, 69.77, 69.38, 68.50, 61.16, 60.92, 55.98, 51.56, 43.63, 32.17, 28.95, 28.10, 27.77, 27.65, 26.83, 22.89, 22.72, 8.08.

HRMS (ESI+) calculated for $C_{55}H_{63}N_{10}O_{12}S$: 1087.4342 $[M+H]^+$; found: 1087.4341.

FITC-(miniPEG)$_2$-Lys(BI2536)-NH$_2$ (5).



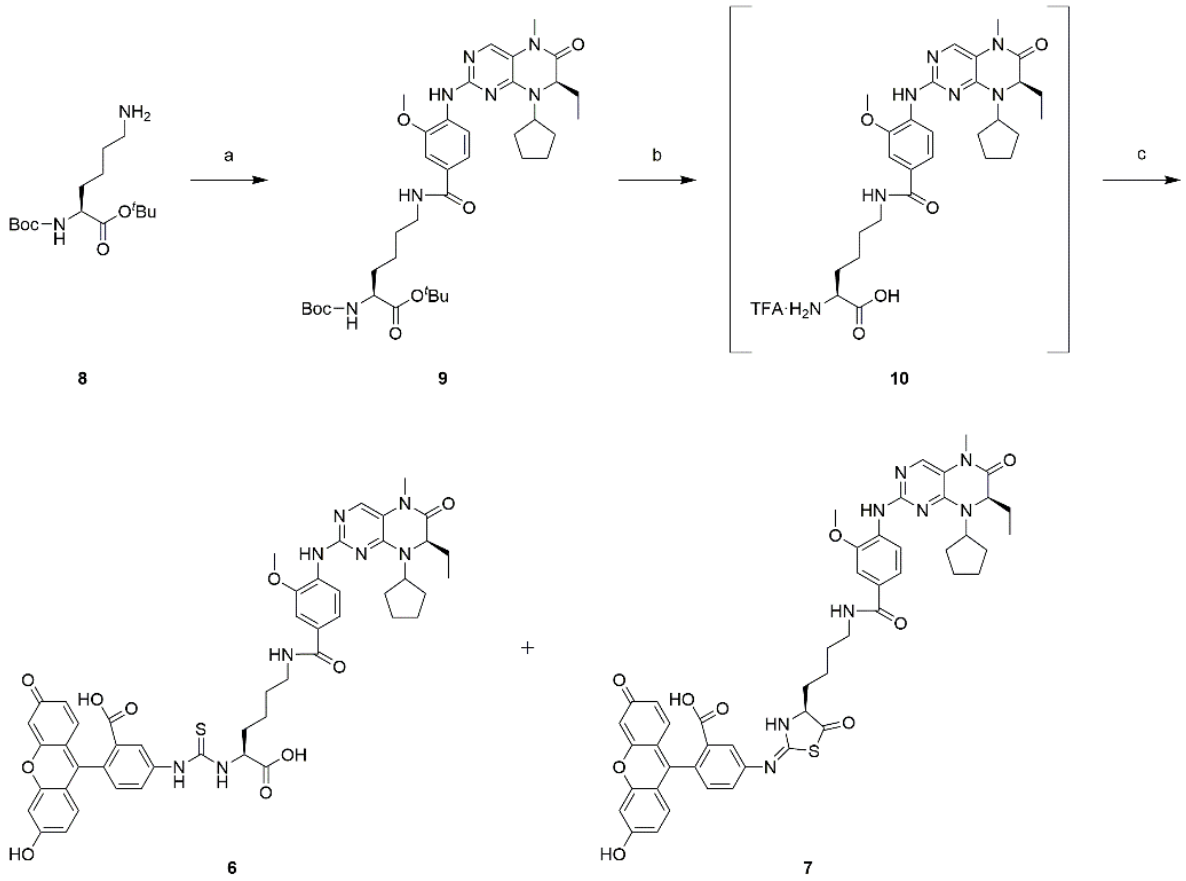
5

Compound **5** was obtained as a yellow powder (8.8 mg, 36% yield from 0.02 mmol resin) as shown in Scheme S2 according to general solid-phase synthesis protocols described above.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.10 (s, 1H), 9.48 (s, 1H), 8.48 (dd, J = 5.5 Hz, 1H), 8.29 (s, 1H), 8.17 (m, 1H), 7.81 (d, J = 8.3 Hz, 1H), 7.75 – 7.71 (m, 3H), 7.58 – 7.49 (m, 4H), 7.17 (d, J = 8.3 Hz, 1H), 7.09 (s, 1H), 6.67 (d, J = 2.2 Hz, 2H), 6.61 – 6.54 (m, 4H), 4.48 – 4.46 (m, 1H), 4.31 – 4.25 (m, 1H), 4.18 – 4.08 (m, 1H), 3.91 – 3.90 (m, 6H), 3.70 – 3.50 (m, 11H), 3.44 (t, J = 5.91 Hz, 2H), 3.30 – 3.21 (m, 8H), 1.93 – 1.68 (m, 8H), 1.63 – 1.23 (m, 10H), 0.74 (t, J = 7.4 Hz, 3H).

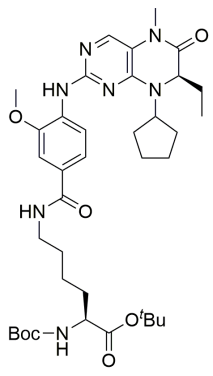
^{13}C NMR (101 MHz, DMSO-d_6) δ 180.60, 173.32, 169.31, 168.90, 168.53, 165.14, 162.49, 159.50, 158.56, 158.21, 157.86, 152.27, 151.88, 150.76, 149.16, 141.38, 131.81, 129.43, 129.02, 128.16, 126.51, 124.85, 124.05, 122.51, 119.49, 117.45, 116.36, 115.79, 114.53, 112.59, 110.29, 109.71, 102.24, 70.22, 70.20, 69.94, 69.79, 69.75, 69.42, 69.30, 68.95, 68.43, 61.29, 61.10, 55.99, 51.59, 43.66, 37.99, 32.17, 28.95, 28.13, 27.72, 27.62, 26.86, 22.91, 22.90, 22.74, 8.04.

HRMS (ESI+) calculated for $C_{61}H_{74}N_{11}O_{15}S$: 1232.5081 $[M+H]^+$; found: 1232.5088.



Scheme S3. Synthesis of carboxylic acid **6** by solution chemistries. (a) truncated BI2536, HATU, DIPEA, DMF; (b) TFA, TIPS, H_2O, DCM; (c) FITC, DIPEA, DMF.

Boc-Lys(BI2536)-O^{t}Bu (9).



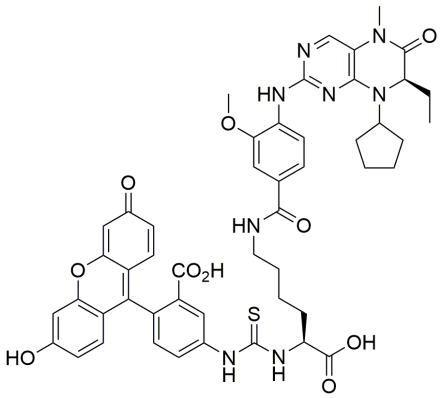
As shown in Scheme S3, to truncated BI2536 (**S4**, 50 mg, 0.10 mmol calculated as the 2HCl salt) and HATU (38 mg, 0.10 mmol) in DMF (300 μL) was added DIPEA (65 μL, 0.50 mmol) and the solution was shaken at room temperature (2 min). To the mixture was added Boc-L-Lys-O^{t}Bu (34 mg, 0.10 mmol) in DMF (200 μL) and the mixture was shaken at room temperature (3 h). The mixture was concentrated and purified by CombiFlash silica gel chromatography (EtOAc /hexane gradient: 0-30% over 2 min, 30-90% over 25 min, and then 90% over 5 min) to afford **9** as an off-white amorphous solid (73 mg, quantitative).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (d, *J* = 8.5 Hz, 1H), 7.67 (s, 1H), 7.63 (s, 1H), 7.43 (d, *J* = 1.6 Hz, 1H), 7.27 – 7.25 (m, 1H), 6.24 (t, *J* = 5.3 Hz, 1H), 5.07 (d, *J* = 8.0 Hz, 1H), 4.50 (quin, *J* = 7.94 Hz, 1H), 4.21 (dd, *J* = 7.9 Hz and 3.7 Hz, 1H), 4.17 – 4.16 (m, 1H), 3.96 (s, 3H), 3.45 – 3.44 (m, 2H), 3.32 (s, 3H), 2.17 – 2.09 (m, 1H), 2.02 – 1.96 (m, 1H), 1.88 – 1.60 (m, 12H), 1.49 – 1.42 (m, 20H), 0.87 (t, J = 7.5 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 172.06, 167.48, 163.88, 155.62, 155.17, 152.44, 147.34, 137.87, 133.20, 126.65, 119.12, 116.45, 116.16, 109.27, 82.01, 79.78, 60.04, 58.53, 56.06, 53.86, 39.96, 32.92, 29.86, 29.82, 29.39, 28.44, 28.31, 28.13, 27.30, 23.71, 23.29, 22.82, 9.32.

HRMS (ESI+) calculated for $C_{37}H_{56}N_7O_7$: 710.4236 $[M+H]^+$; found: 710.4234.

FITC-Lys(BI2536)-OH (6).



6

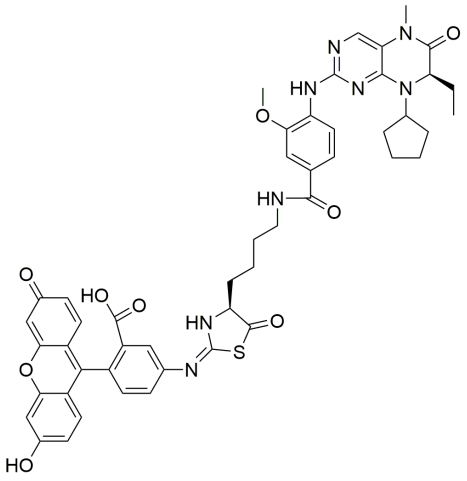
As shown in Scheme S3, to compound **9** (73 mg, 0.10 mmol, dissolved in 1.0 mL DCM) was added TIPS (105 µL, 0.51 mmol) and H_2O (9.3 µL, 0.51 mmol) followed by TFA (160 µL, 2.1 mmol). The reaction mixture was stirred at room temperature (3.5 h) and then concentrated under a N_2 stream. The resulting residue was dissolved in DMF (1.0 mL) and to this was added DIPEA (90 µL, 0.51 mmol) and FITC (60 mg, 0.15 mmol) and the mixture was stirred at room temperature (16 h). The crude mixture was purified by preparative HPLC (linear gradient, B in A: 20-55% over 30 min) and lyophilized to afford **6** as a yellow powder [12 mg, 13% yield (2 steps)).

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.20 (bs, 2H), 9.40 (s, 1H), 8.51 (dd, *J* = 5.5 Hz), 8.40 (s, 1H), 8.34 (d, *J* = 7.51 Hz, 1H), 7.81 (d, *J* = 8.23 Hz, 1H), 7.77 (dd, *J* = 8.37 Hz and 1.90 Hz, 1H), 7.73 (s, 1H), 7.58 (d, *J* = 1.60 Hz, 1H), 7.52 (dd, *J* = 8.3 Hz, 1.6 Hz, 1H), 7.18 (d, *J* = 8.3 Hz, 1H), 6.68 – 6.67 (m, 2H), 6.61 – 6.53 (m, 4H), 4.90 – 4.85 (m, 1H), 4.46 (dd, *J* = 6.5 Hz and 3.3 Hz, 1H), 4.13 (quin, *J* = 8.8 Hz, 1H), 3.89 (s, 3H), 3.33 – 3.27 (m, 2H), 3.21 (s, 3H), 1.93 – 1.75 (m, 9H), 1.58 – 1.43 (m, 9H), 0.74 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (101 MHz, DMSO- d_6) δ 180.75, 173.41, 168.53, 165.24, 162.53, 159.51, 158.55, 158.20, 152.25, 151.90, 150.72, 149.34, 147.17, 141.37, 131.70, 129.30, 129.03, 128.29, 126.52, 124.07, 122.41, 119.53, 117.68, 116.20, 115.81, 114.76, 112.60, 110.29, 109.72, 102.26, 61.27, 61.05, 56.43, 56.00, 31.12, 28.96, 28.13, 27.75, 27.65, 26.85, 22.92, 22.88, 8.07.

HRMS (ESI+) calculated for $C_{49}H_{51}N_8O_{10}S$: 943.3443 $[M+H]^+$; found: 943.3462.

FITC-Lys(BI2536) (cyclic form, 7).



7

The cyclized product **7** was also obtained as a side product during the synthesis of **6** (1.5 mg, 2% yield).

^{1}H NMR (500 MHz, DMSO- d_6) δ 10.69 (s, 1H), 10.12 (br, 2H), 9.14 (s, 1H), 8.46 – 8.44 (m, 1H), 7.92 (d, J = 1.7 Hz, 1H), 7.82 (d, J = 8.2 Hz), 7.69 (s, 1H), 7.65 (dd, J = 8.2 Hz and 1.8 Hz, 1H), 7.51 (d, J = 1.5 Hz, 1H), 7.46 (dd, J = 8.3 Hz and 1.6 Hz, 1H), 7.30 (d, J = 8.2 Hz, 1H), 6.63 (t, J = 2.2 Hz, 2H), 6.54 – 6.49 (m, 4H), 4.42 (t, J = 6.0 Hz, 1H), 4.38 – 4.36 (m, 1H), 4.10 (quin, J = 8.8 Hz, 1H), 3.83 (s, 3H), 3.27 – 3.22 (m, 1H), 3.12 (s, 3H), 1.85 – 1.70 (m, 8H), 1.54 – 1.38 (m, 9H), 0.68 (t, J = 7.4 Hz, 3H).

^{13}C NMR (126 MHz, DMSO- d_6) δ 181.68, 174.12, 167.87, 165.31, 162.56, 159.66, 158.26, 157.99, 157.73, 152.20, 151.80, 149.77, 145.64, 138.00, 136.06, 135.36, 134.84, 130.59, 128.87, 126.43, 124.86, 124.36, 121.72, 119.58, 117.73, 115.83, 115.30, 112.79, 110.17, 109.11, 102.37, 61.06, 60.76, 59.52, 56.01, 30.49, 28.95, 28.12, 27.89, 27.71, 26.85, 22.93, 21.59, 8.15.

HRMS (ESI+) calculated for $C_{49}H_{49}N_8O_9S$: 925.3338 $[M+H]^+$; found: 925.3359.

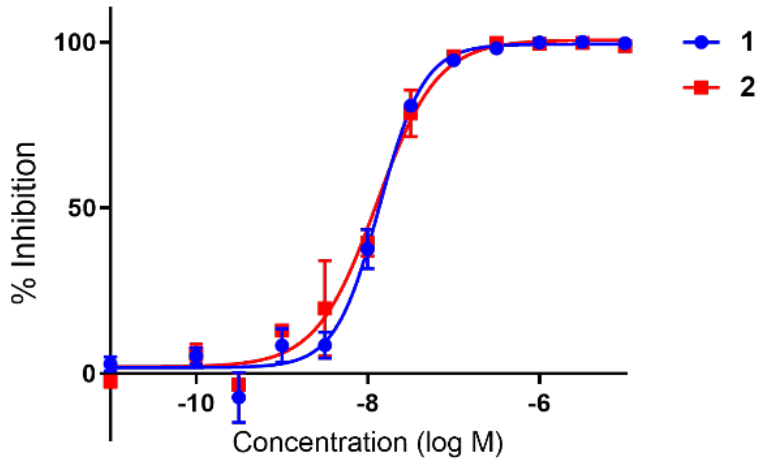
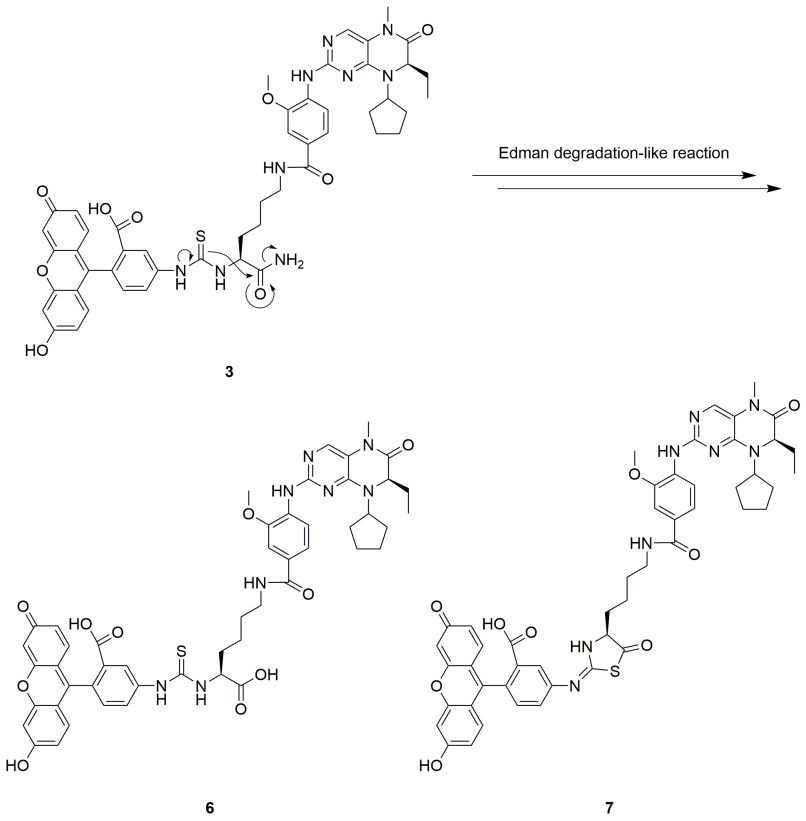


Figure S1. Results from kinase assays, which measured the inhibitory potencies of compounds **1** and **2** in a catalytic assay using full-length Plk1 (Z'-LYTE^{TM} kinase assay kit - Ser/Thr 16 Peptide, Invitrogen). The X axis represents inhibitor concentration (log M) and the Y axis represents % inhibition based on the % phosphorylation of the substrate. The calculation was conducted according to the manufacturer's instructions. Data points represent average ± SEM from three independent experiments and fit using non-linear regression in GraphPad Prism 7.



Scheme S4. Potential reaction mechanism to form hydrolyzed and cyclized byproducts (**6** and **7**, respectively).

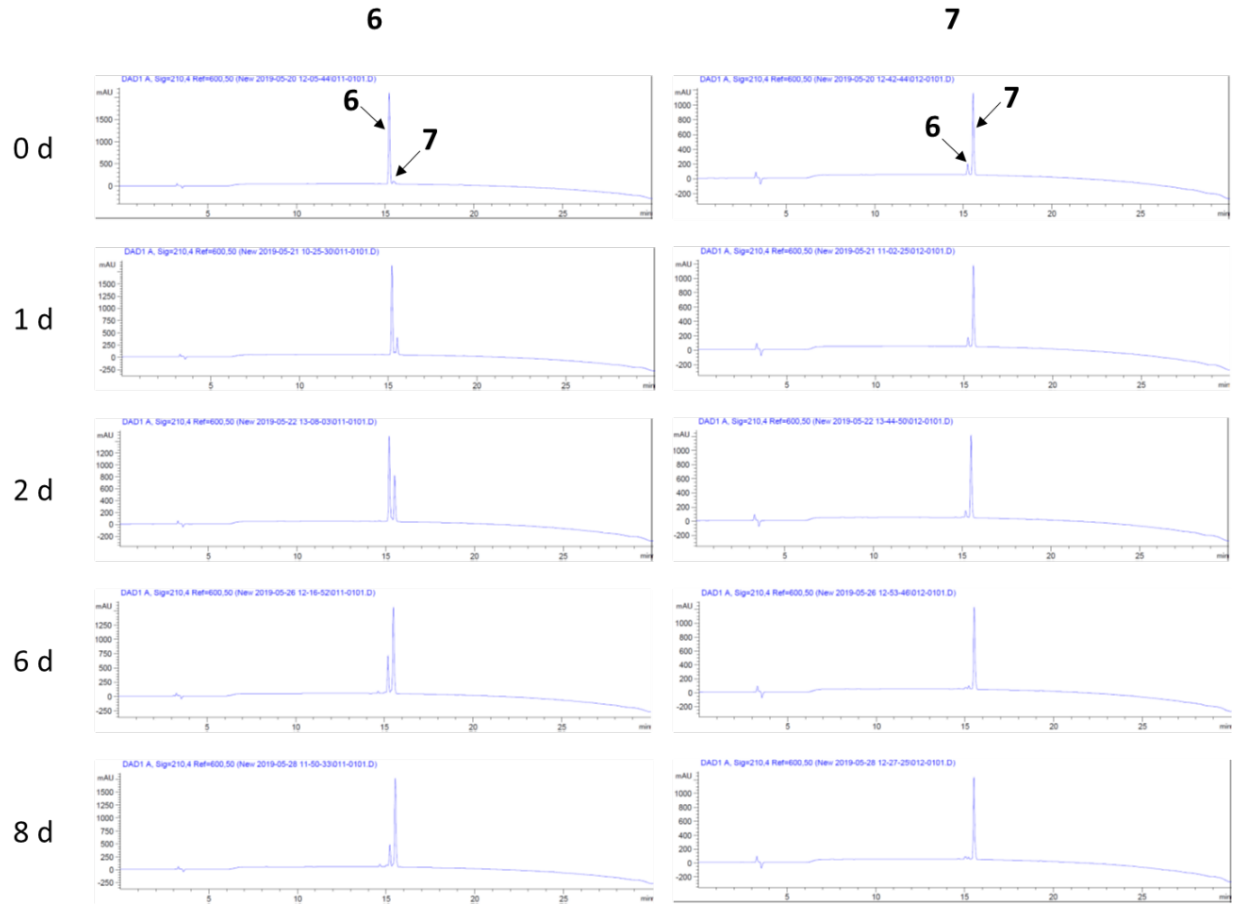


Figure S2. Stability of **6** in HPLC eluents. Analytical HPLC conditions: linear gradient elution (89.9/10/0.1 H_2O/acetonitrile/TFA to 99.9/0.1 acetonitrile/TFA over 30 min).

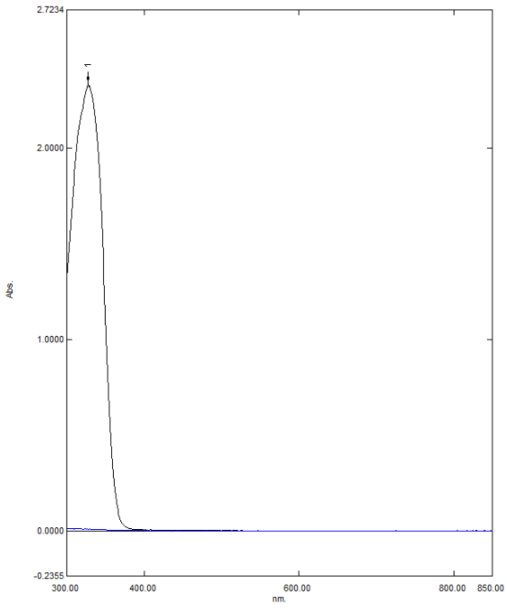


Figure S3. Absorbance of BI2536 (300 – 850 nm) measured by 100 µM in H_2O at room temperature. Black: BI2536, Blue: blank.

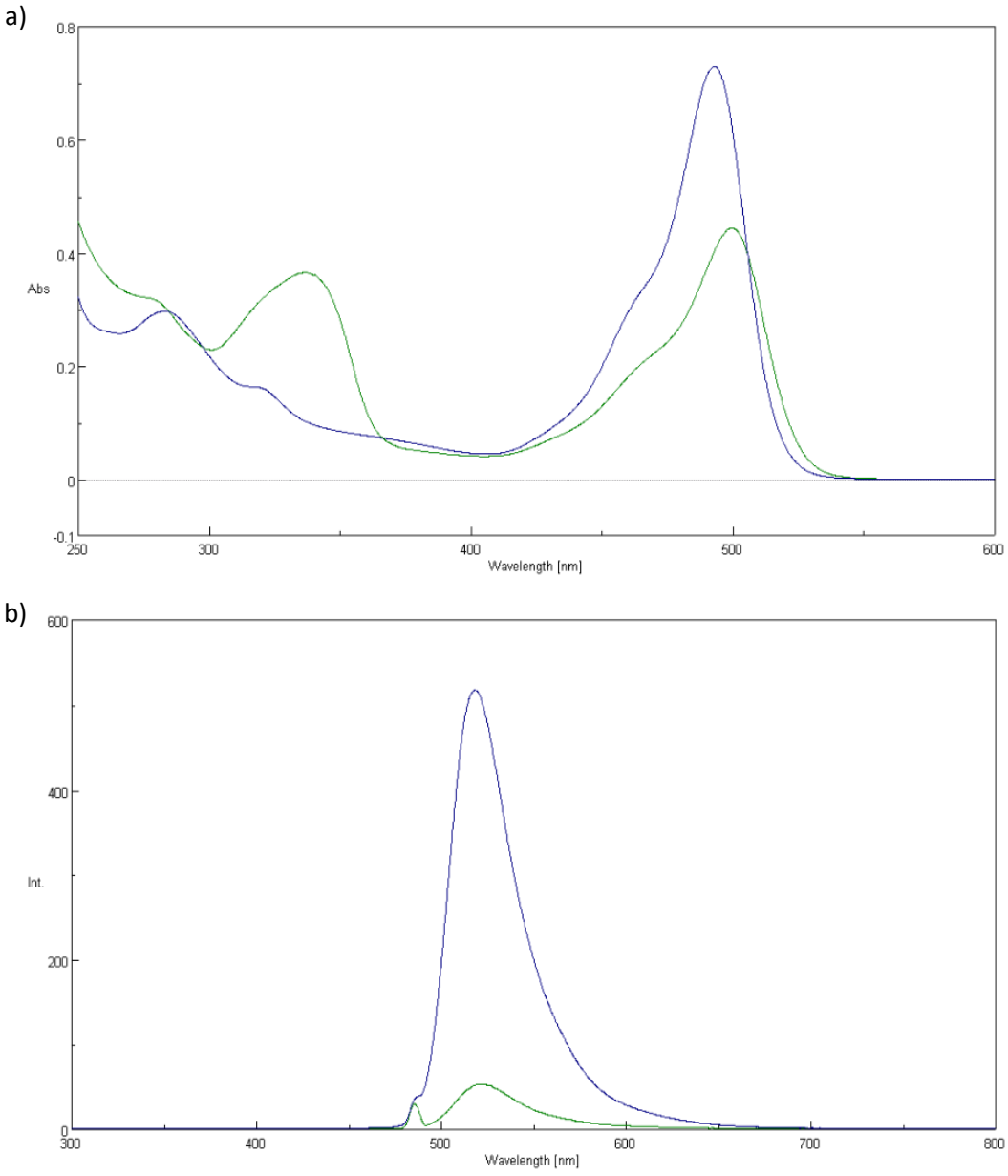


Figure S4. (a) UV spectra of FITC and **4** (250 – 600 nm) measured by 10 μM in HBST (HEPES buffered saline (HBS) containing 0.05% Tween-20, 1 mM DTT and 1 mM EDTA) at room temperature and (b) fluorescence spectra of FITC and **4** (300 – 800 nm) measured by 1 μM in HBS-T at room temperature. Blue: FITC, Green: **4**.

Expression and purification of full-length Plk1 for kinase assays and fluorescence recovery assays. As previously reported[2–4], a plasmid encoding myc-tagged full-length Plk1 was purchased from Addgene (Plasmid #41160). ~20 M HEK-293T cells (2 x 15 cm plates) were transfected with the plasmid using TurboFect reagent (Thermo Fisher Scientific) according to manufacturer's instructions. Following 48 h expression, cells were harvested, lysed in buffer [phosphate buffered saline (PBS, pH 7.4) containing 0.5% NP-40 and protease/phosphatase inhibitor (Pierce, Protease and Phosphatase Inhibitor Mini Tablets) cocktail] using freeze/thaw cycles (3x) and centrifuged at 12,500 x G for 10 min at 4 °C. The supernatant containing expressed protein was diluted into 8 mL of PBS (pH 7.4) containing protease/phosphatase inhibitor cocktail. This protein solution was added to a 1 mL bed of myc-agarose resin (Thermo Fisher Scientific) and allowed to bind for 2 h at 4 °C with gentle rotation. The lysate was removed by filtration and the resin was washed 4x with HBST (HEPES buffered saline (HBS) containing 0.05% Tween-20, 1 mM DTT and 1 mM EDTA) for 10 min with gentle rotation. The bound myc-tagged Plk1 protein was then eluted with a 1 mg/mL solution of myc peptide (EQKLISEEDL) in HBS + 1 mM DTT and 1 mM EDTA. The purified myc-tagged Plk1 was dialyzed 5x with HBS + 1 mM DTT and 1 mM EDTA using a 10 kDa MWCO filter (Sigma, fixed angle rotor at 7,500 x G, 4 °C, 10 min). The concentration of the final protein solution was determined by absorbance at 280 nm and purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie staining using NuPAGE™ 4 to 12%, Bis-Tris, 1.0 mm, Mini Protein Gel, 12-well NuPAGE™ Sample Reducing Agent (10X), NuPAGE™ MOPS SDS Running Buffer (20X), SeeBlue™ Plus2 Pre-stained Protein Standard (Invitrogen), and GelCode™ Blue Safe Protein Stain (Thermo Scientific).

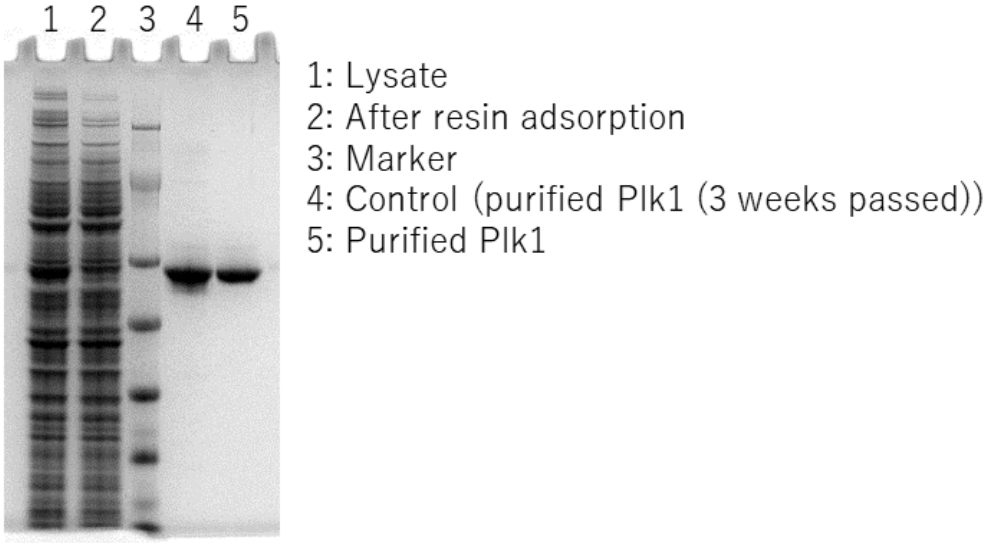


Figure S5. SDS-PAGE with Coomassie staining for purified Plk1.

Kinase enzymatic assays using purified full-length Plk1. The Z'-LYTE™ Kinase Assay Kit - Ser/Thr 16 Peptide (invitrogen) was used according to manufacturer's instructions using 50 nM Plk1, 20 μM ATP, 2 μM substrate (or 2 μM phosphorylated substrate as 100% control) in the presence of 1% DMSO and 1 mM dithiothreitol (DTT).

Fluorescence recovery assays using purified full-length Plk1. Purified Plk1 was diluted to a 2x working dilution in assay buffer (HEPES-buffered saline with 0.05% Tween-20, 1 mM DTT, and 1 mM EDTA) with the final protein concentration representing the approximate K_d values as determined for the probe **4**. Inhibitors were serially diluted to generate 4x working dilutions in assay buffer containing 4% DMSO. To each well of a 384-well plate was added 20 µL of 2x Plk1 solution (0% binding controls received 20 µL of assay buffer). A total of 10 µL of the 4x inhibitor solution (or DMSO blank) was added to corresponding wells and allowed to pre-incubate at room temperature for 30 min with shaking. Fluorescent probe **4** were diluted to 80 nM (4x) in assay buffer and then 10 µL was added to each well. The plate was allowed to equilibrate at room temperature for 30 min with shaking. The fluorescence intensity was read using a BioTek Synergy 2 plate reader with 485/20 excitation and 528/20 emission. The fluorescence intensity values were obtained in triplicate and normalized to 100% (no inhibitor) and 0% binding (no protein) controls. Normalized values were plotted versus concentration and analyzed using non-linear regression in GraphPad Prism 7 [log(inhibitor) vs response – variable slope (four parameter) model]. IC_{50} values represent average ± standard error of the mean (SEM).

For the validation of **4** and **5** as assay probes and determination of the K_d value of Plk1 (Figure 3), the probes were used with 20 nM as a final concentration and the Plk1 was serially diluted to generate 2x working dilutions in assay buffer. The incubation time for the assay was 30 min for equilibration. For the optimization of probe concentration (Figure 4a), the probes were used at 10, 20, 30, or 40 nM final concentration and the Plk1 was serially diluted to generate 2x working dilutions in assay buffer. The incubation time for the assay is 30 min for equilibration. For the optimization of incubation time (Figure 4b), the probes were used at 20 nM final concentration and the Plk1 was serially diluted to generate 2x working dilutions in assay buffer. The incubation times for assay for equilibration were 15, 30, or 60 min.

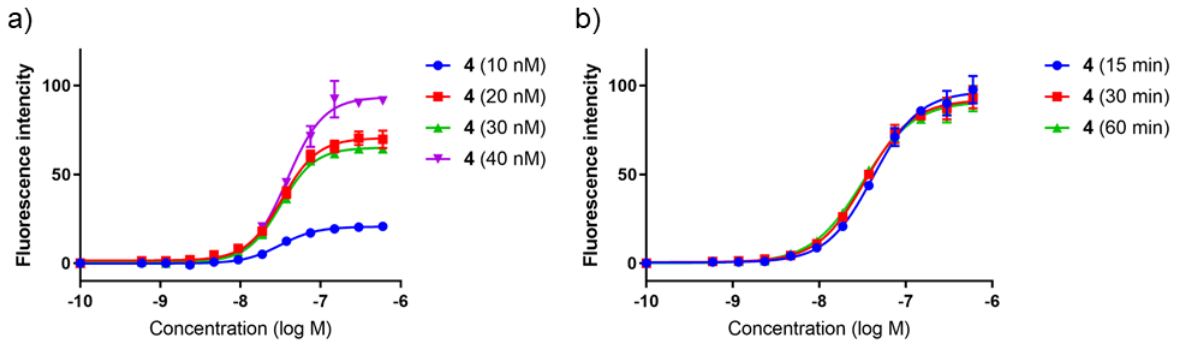
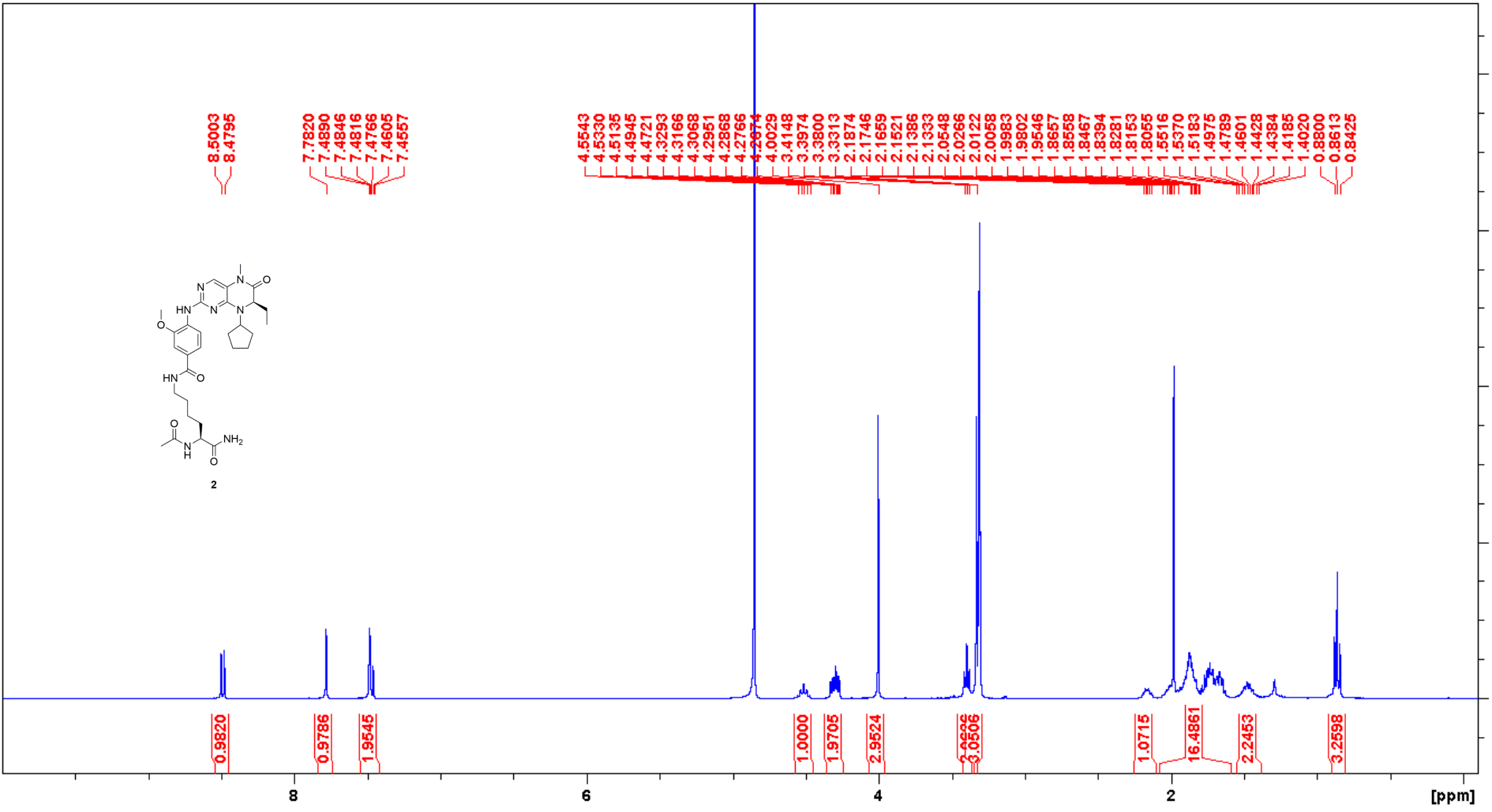


Figure S6. Results from fluorescence recovery assays to determine (a) optimum probe concentration and (b) optimum incubation time. The X axis represents Plk1 concentration (log M) and the Y axis represents fluorescence intensity (Ex: 485 nm, Em: 528 nm). Data points represent average ± SEM from three independent experiments and fit using non-linear regression in GraphPad Prism 7.

NMR Spectra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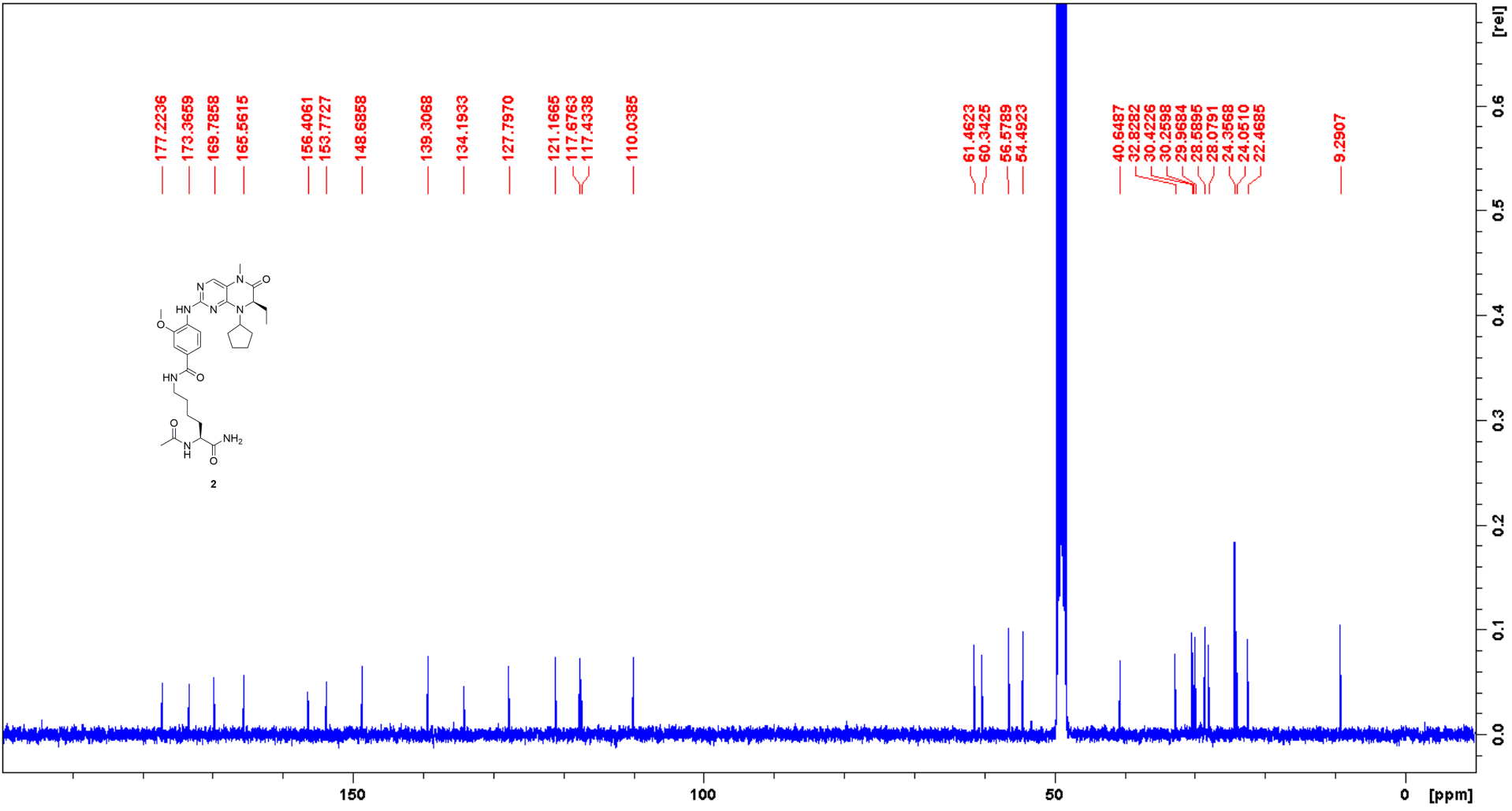
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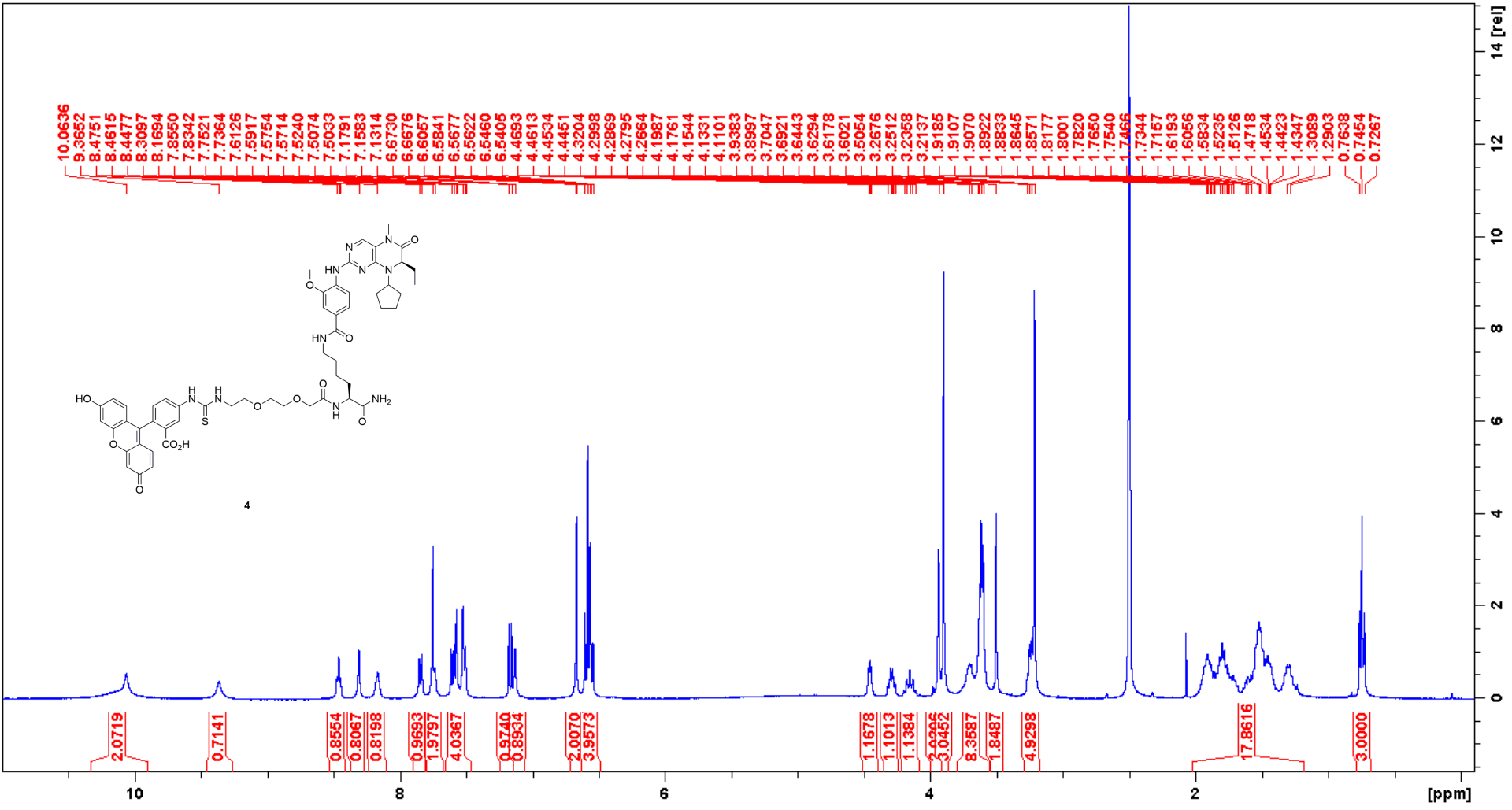
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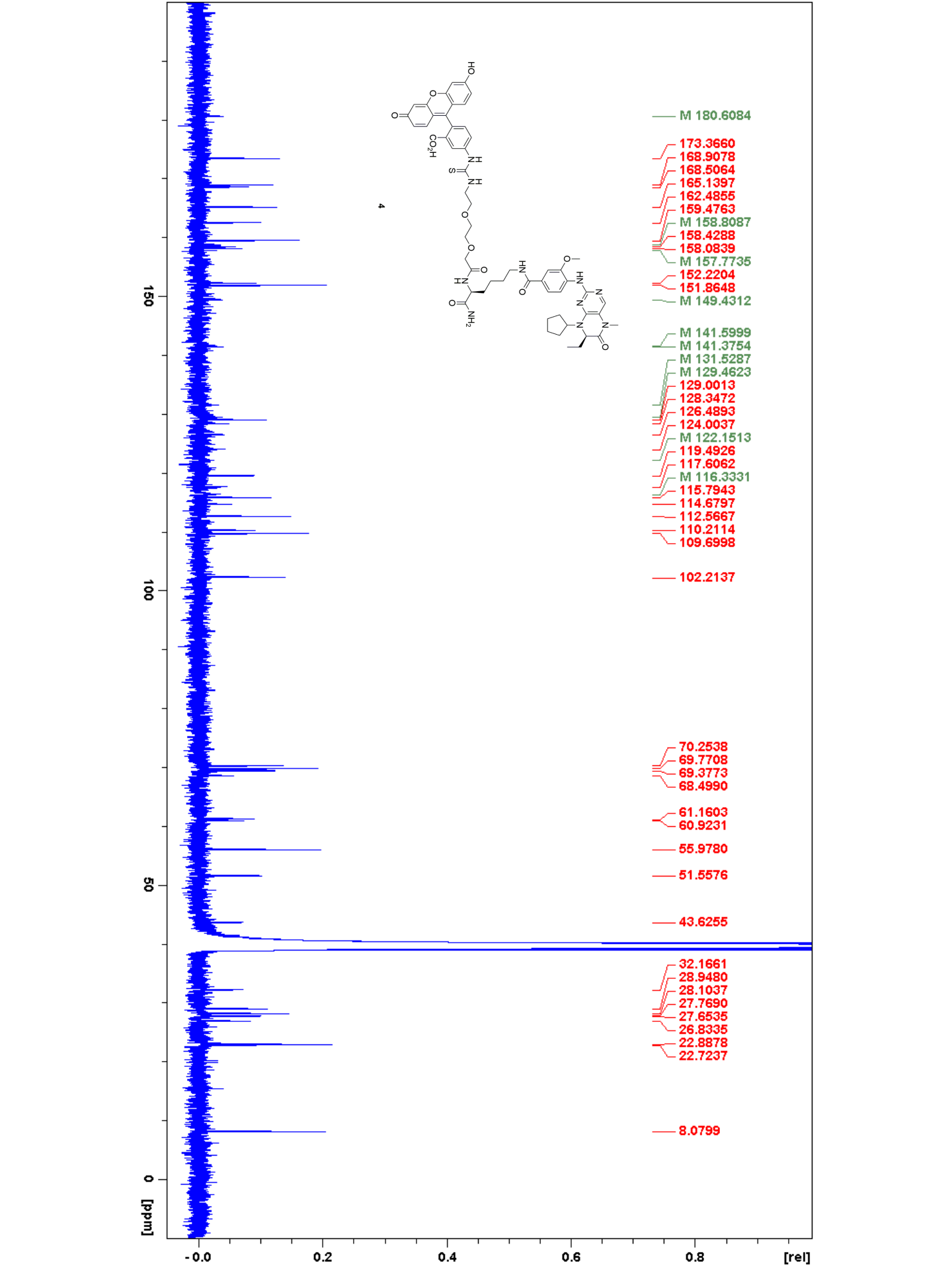
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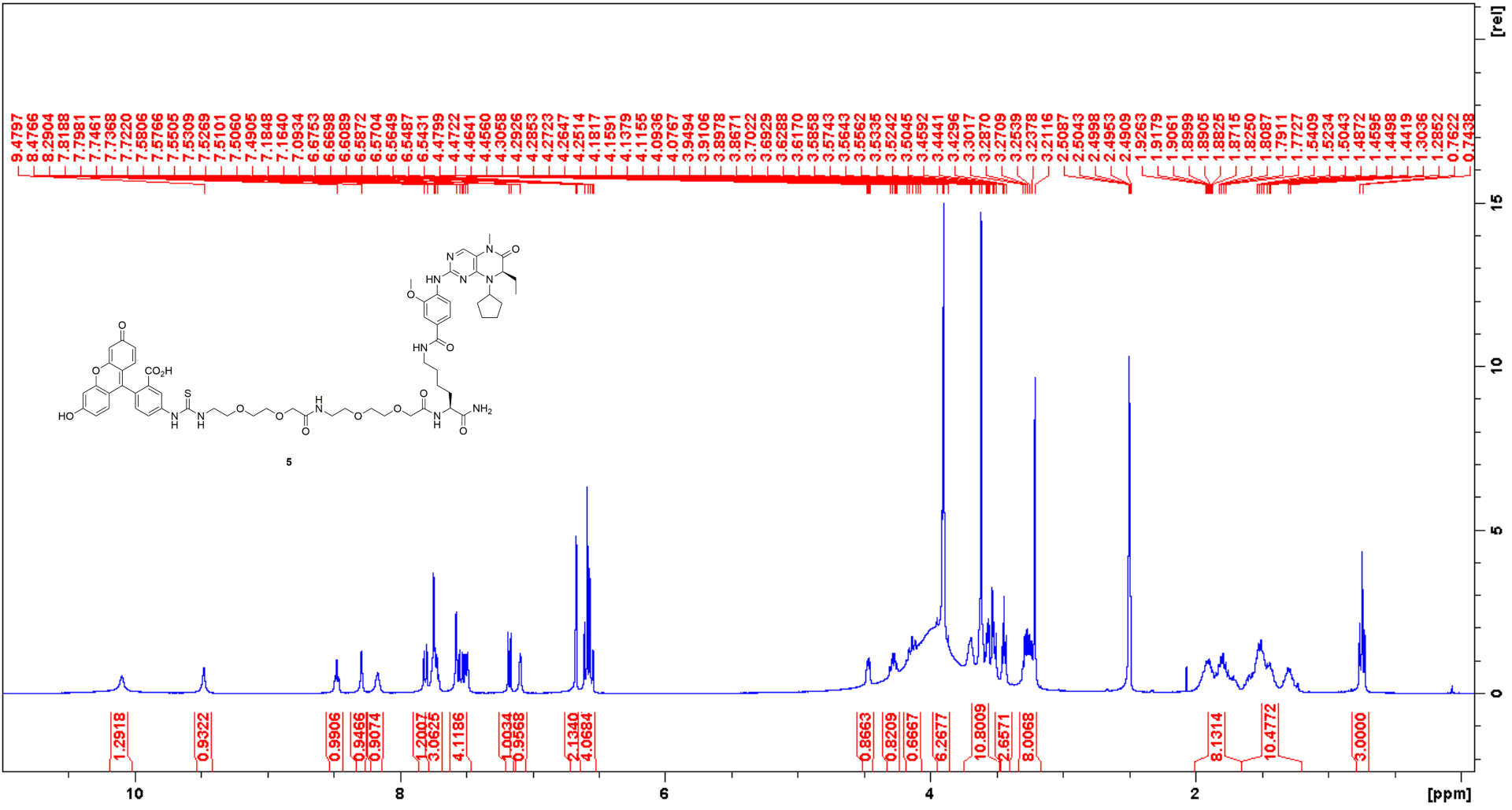
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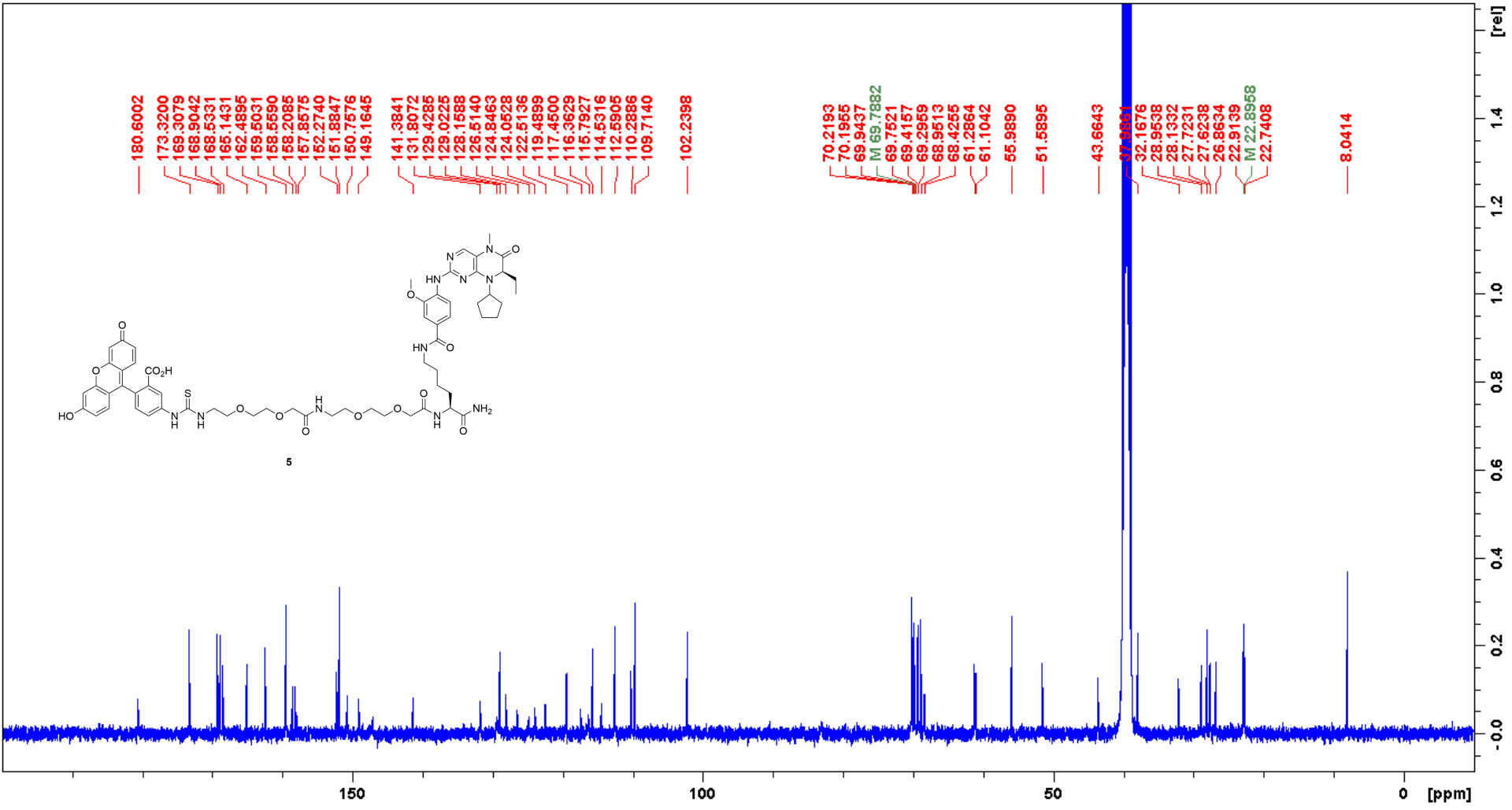
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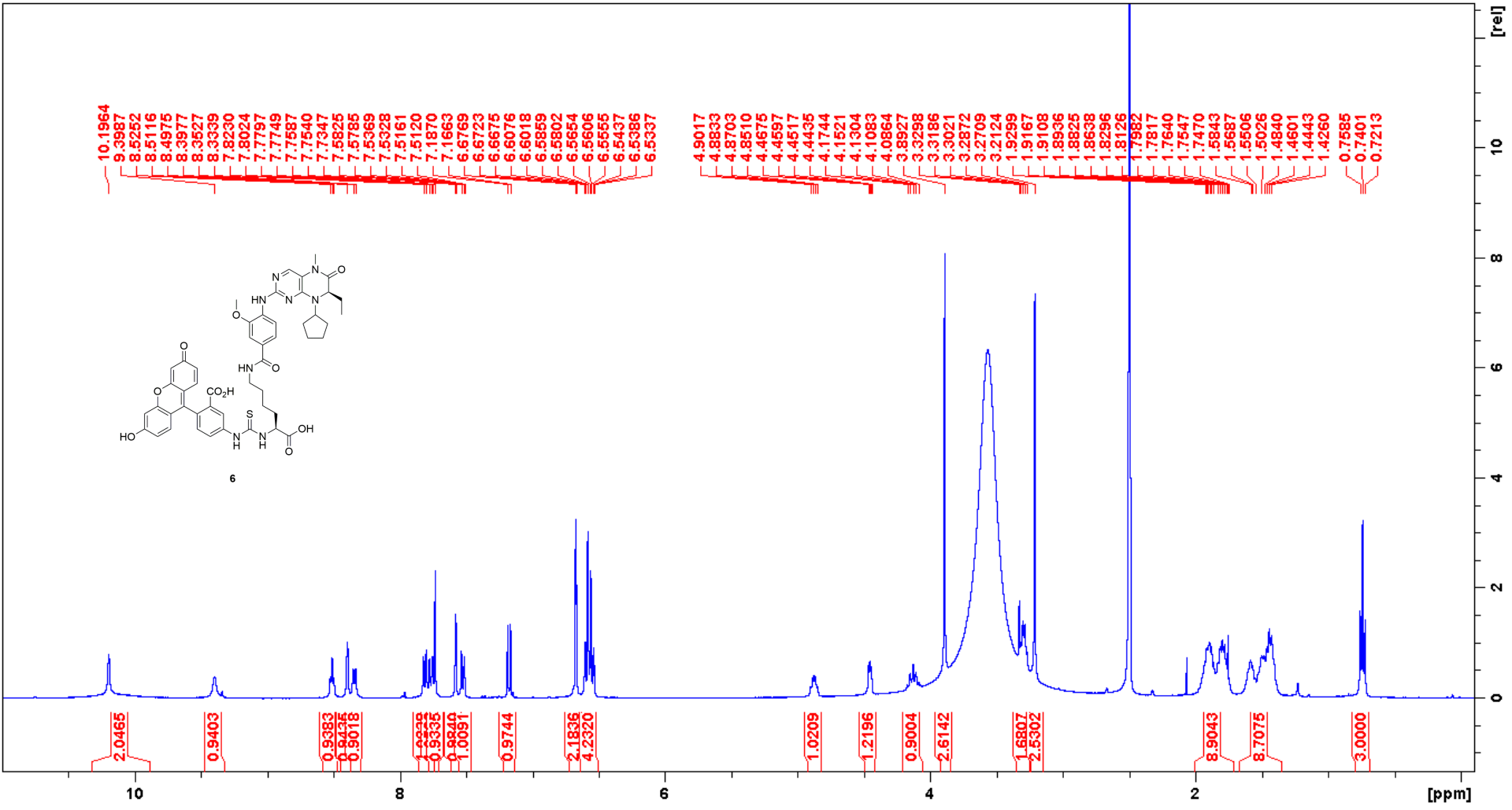
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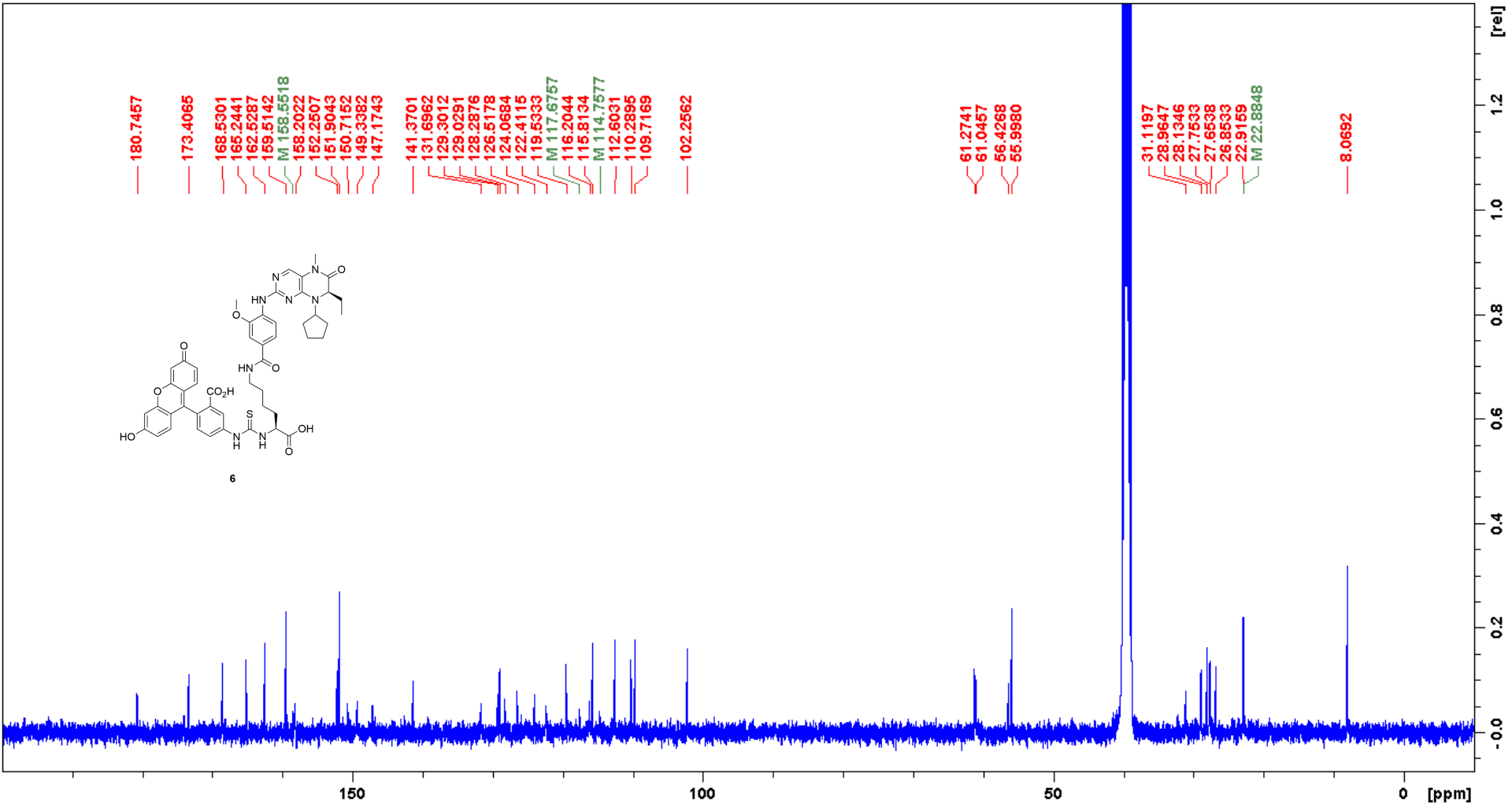
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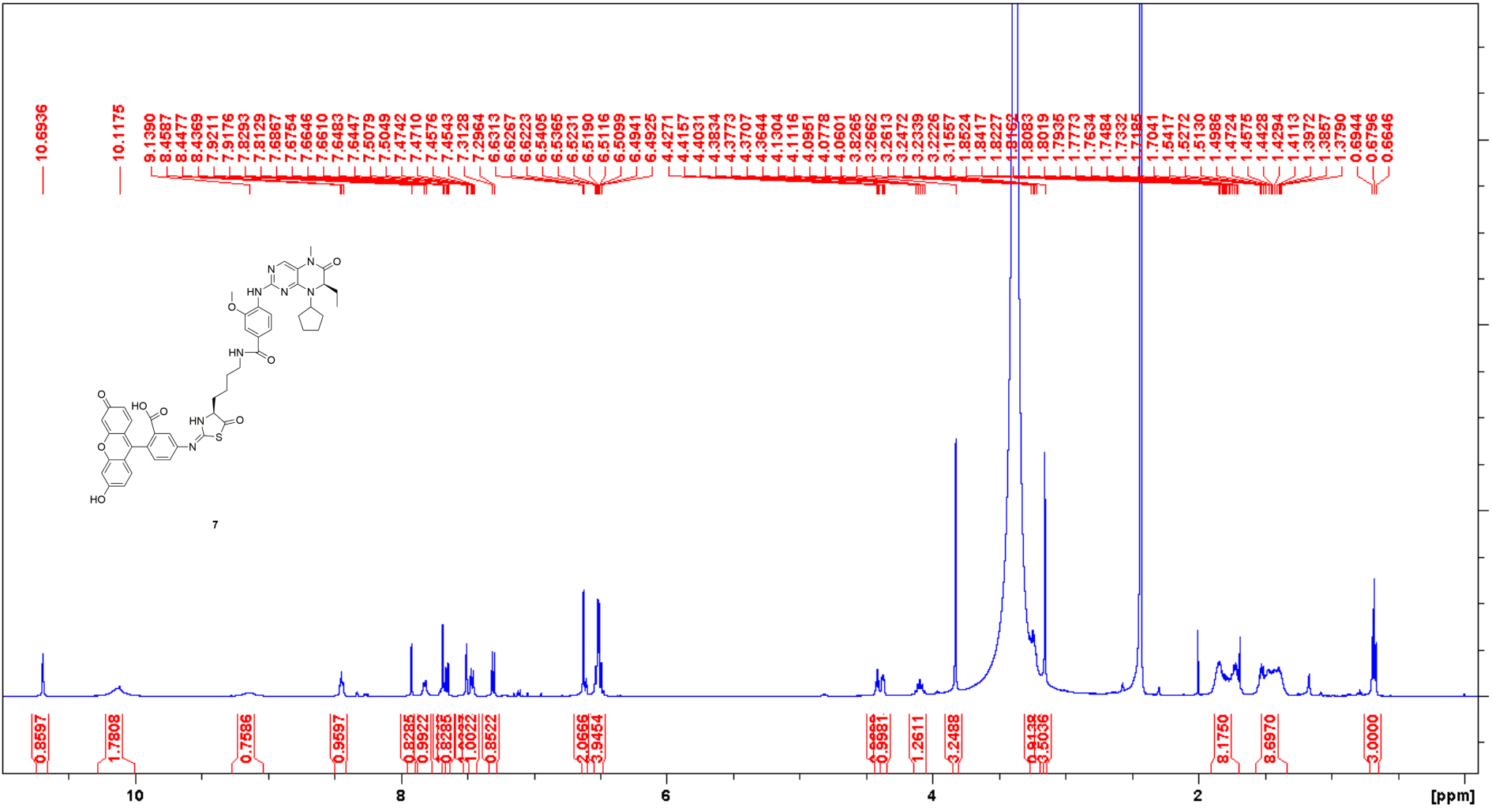
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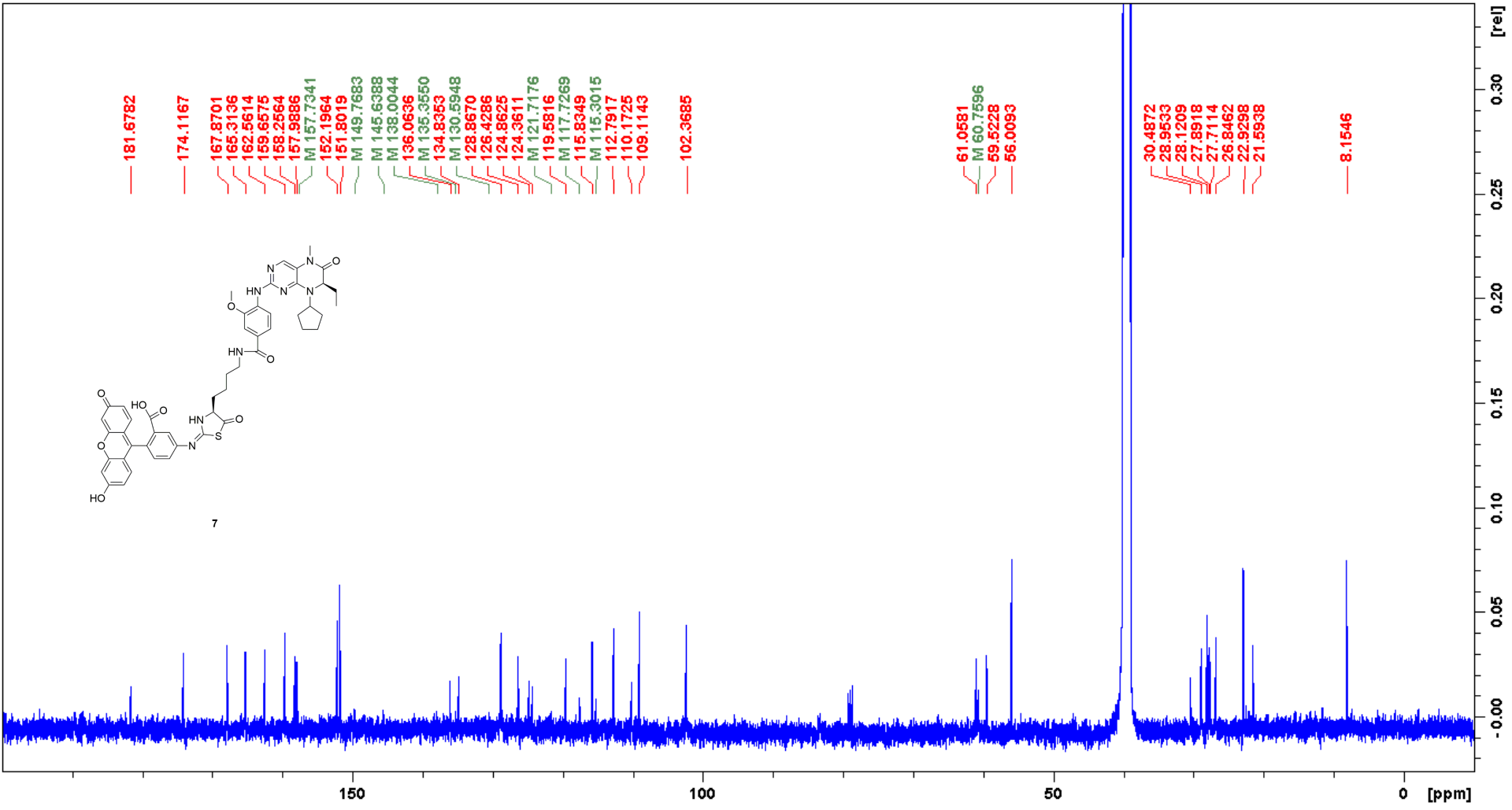
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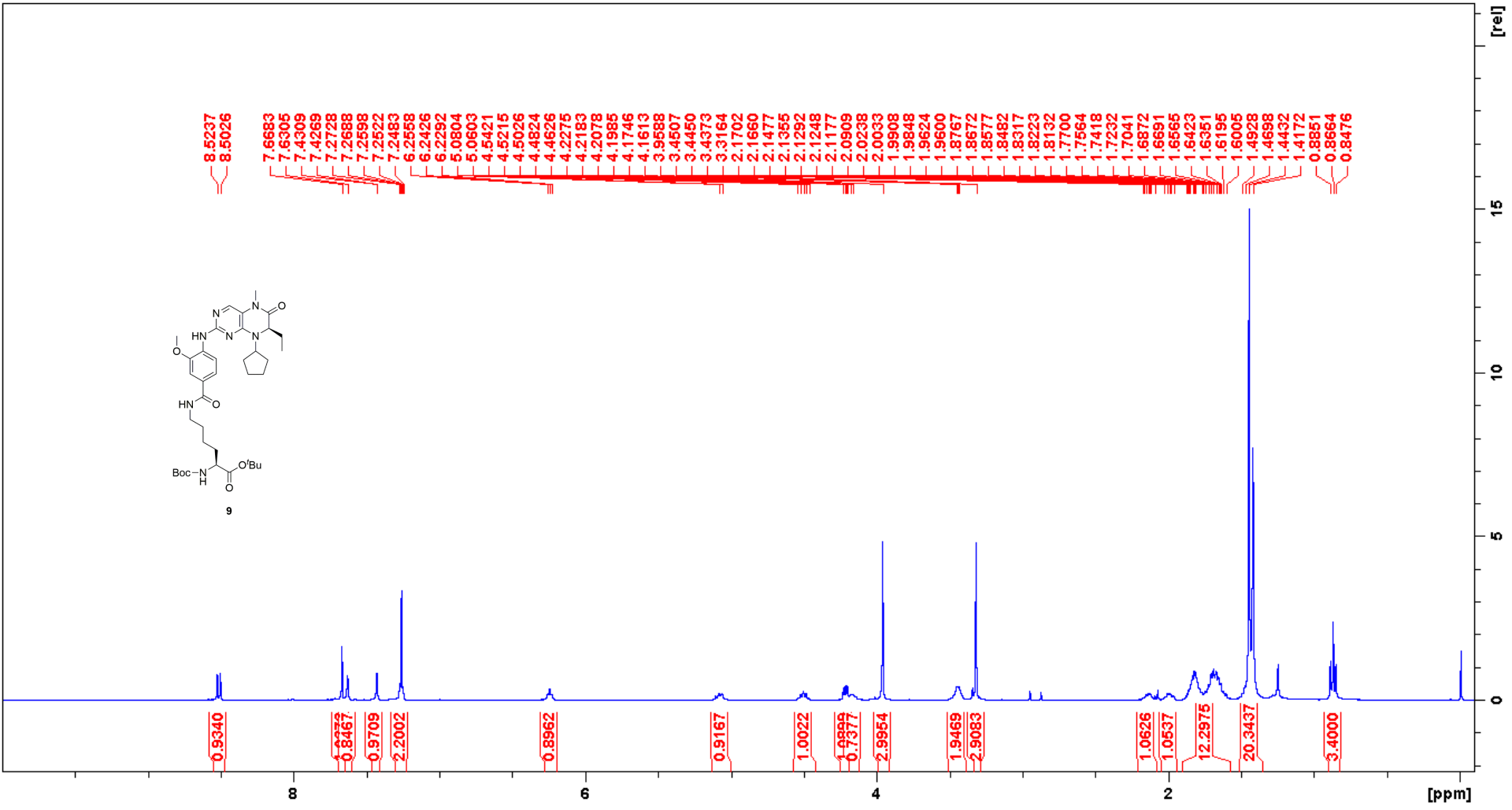
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H
O
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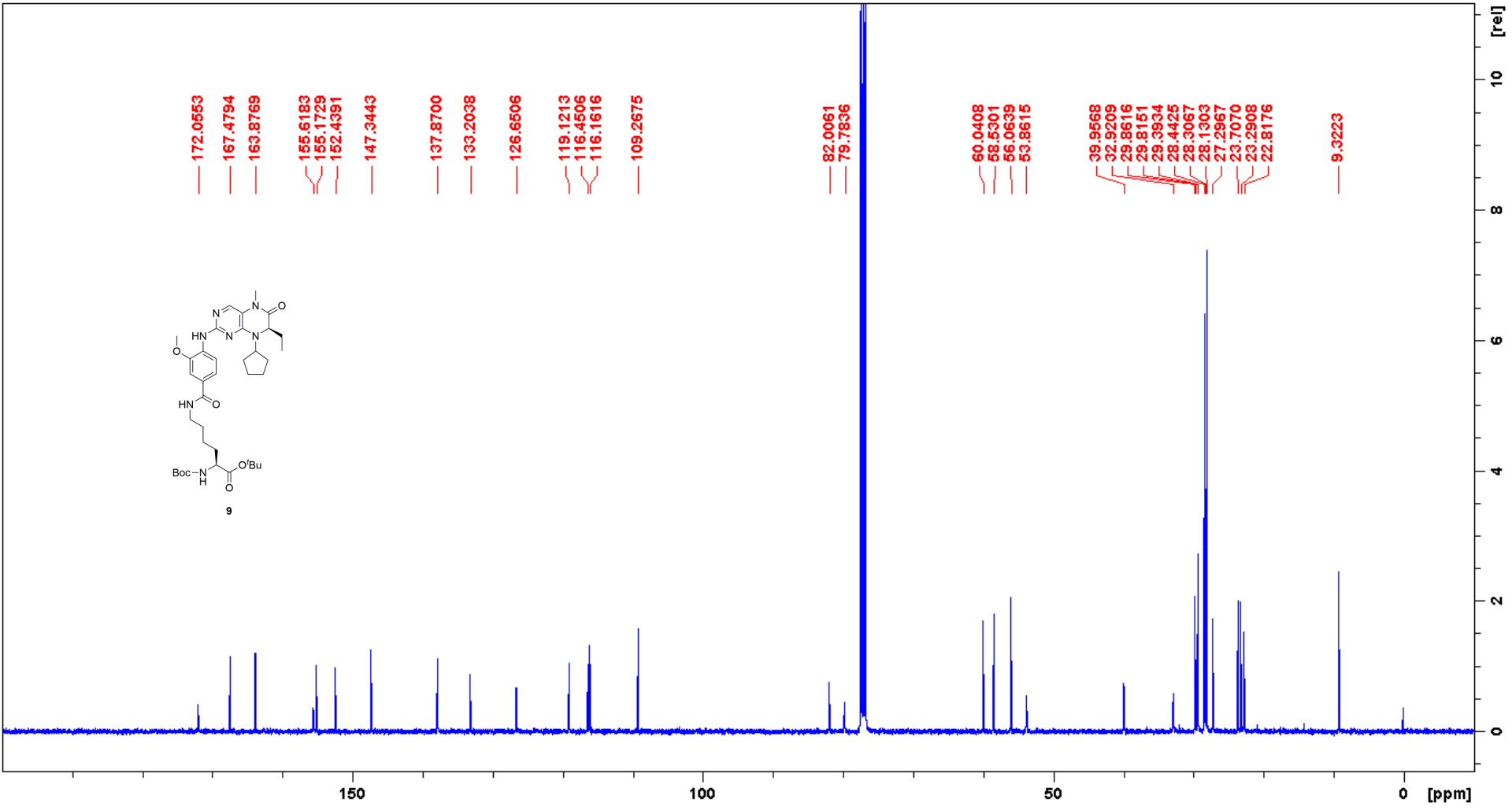
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9

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