Supporting Data

Toward Self-Adjuvanting Subunit Vaccines: Model Peptide and Protein Antigens Incorporating Covalently Bound Toll-Like Receptor-7 Agonistic Imidazoquinolines

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Experimental Methods

Chemistry. All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 ^{o}C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf 'Gold' high performance silica columns on CombiFlash Rf instruments unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel CCM pre-coated aluminum sheets. Purity for all final compounds was confirmed to be greater than 97% by LC-MS using a Zorbax Eclipse Plus 4.6 mm x 150 mm, 5 μm analytical reverse phase C_{18} column with H_2O-isopropanol or H_2O-CH_3CN gradients and an Agilent ESI-TOF mass spectrometer (mass accuracy of 3 ppm) operating in the positive ion acquisition mode.

Synthesis of Compound 2: 2-Butyl-1-(4-(isothiocyanatomethyl)benzyl)-1*H*-imidazo[4,5-c]quinolin-4-amine. To a solution of **1** (150 mg, 0.35 mmol) in anhydrous dichloromethane, were added carbon disulfide (266 mg, 3.5 mmol) and triethylamine (106 mg, 1.05 mmol). The reaction mixture was stirred for an hour and then was cooled to 0 ^{o}C. Di-*tert*-butyl dicarbonate (76 mg, 0.35 mmol) and a catalytic amount of DMAP were added to the reaction mixture. The reaction mixture was stirred for 18 hours and then the solvent was removed under vacuum. The residue was purified using column chromatography (7% MeOH/ dichloromethane) to obtain the compound **2** (105 mg, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 – 7.83 (m, 1H), 7.68 (dd, *J* = 8.3, 0.8 Hz, 1H), 7.51 – 7.45 (m, 1H), 7.32 (d, *J* = 8.2 Hz, 2H), 7.23 – 7.17 (m, 1H), 7.10 (d, *J* = 8.2 Hz, 2H), 6.52 (s, 2H), 5.78 (s, 2H), 4.71 (s, 2H), 2.94 – 2.86 (m, 2H), 1.82 (dt, *J* = 15.5, 7.6 Hz, 2H), 1.52 – 1.41 (m, 2H), 0.95 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.19, 150.27, 135.10, 134.65, 134.57, 128.20, 127.95, 126.10, 125.92, 124.08, 123.59, 119.94, 113.99, 48.74, 48.21, 29.71, 27.12, 22.47, 13.76. MS (ESI) calculated for $C_{23}H_{23}N_5S$, m/z 401.17, found 402.18 $(M + H)^+$.

Synthesis of Compound 3: Methyl 1-(4-((4-amino-2-butyl-1*H*-imidazo[4,5-c]quinolin-1-yl)methyl)phenyl)-6,9-dioxo-3-thioxo-2,4,7,10-tetraazadodecan-12-oate.
To a solution of **2** (15 mg, 0.037 mmol) in anhydrous MeOH, were added triethylamine (6 mg, 0.056 mmol) and methyl 2-(2-(2-aminoacetamido)acetamido)acetate hydrochloride (11mg, 0.044 mmol). The reaction was heated at 45 ^{o}C for 4 hours. The solvent was then removed under vacuum and the residue was purified using column chromatography (14 % MeOH/dichloromethane) to obtain the compound **3** (5 mg, 22%). ^{1}H NMR (400 MHz, DMSO) δ 8.36 – 8.14 (m, 3H), 7.79 (d, *J* = 7.7 Hz, 1H), 7.58 (d, *J* = 7.5 Hz, 2H), 7.33 (dd, *J* = 11.2, 4.1 Hz, 1H), 7.25 (d, *J* = 8.2 Hz, 2H), 7.05 (t, *J* = 7.1 Hz, 1H), 7.00 (d, *J* = 8.1 Hz, 2H), 6.59 (s, 2H), 5.85 (s, 2H), 4.61 (s, 2H), 4.11 (s, 2H), 3.83 (d, *J* = 5.9 Hz, 2H), 3.75 (d, *J* = 5.9 Hz, 2H), 3.62 (s, 3H), 2.96 – 2.86 (m, 2H), 1.72 (dt, *J* = 15.3, 7.6 Hz, 2H), 1.45 – 1.31 (m, 2H), 0.88 (t, *J* = 7.4 Hz, 3H). MS (ESI) calculated for $C_{30}H_{36}N_8O_4S$, m/z 604.26, found 605.27 $(M + H)^+$.

Synthesis of Compound 5: *N*-(4-((4-amino-2-butyl-1*H*-imidazo[4,5-c]quinolin-1-yl)methyl)benzyl)-3-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)propanamide. To a solution of **4** (30 mg, 0.18 mmol) in anhydrous DMF, were added triethylamine (50 mg, 0.49 mmol),

HBTU (68 mg, 0.18 mmol), a catalytic amount of DMAP and **1** (70 mg, 0.16 mmol). The reaction mixture was stirred for 14 hours and then the solvent was removed under vacuum. The residue was dissolved in ethyl acetate and washed with water. The ethyl acetate fraction was then dried using Na_2SO_4 and evaporated under vacuum to obtain the residue, which was purified using column chromatography (5% MeOH/dichloromethane) to obtain the compound **5** (65 mg, 80%). ^{1}H NMR (500 MHz, MeOD) δ 7.97 (dd, *J* = 8.4, 0.7 Hz, 1H), 7.74 – 7.71 (m, 1H), 7.64 (ddd, *J* = 8.4, 7.2, 1.2 Hz, 1H), 7.38 (ddd, *J* = 8.4, 7.2, 1.2 Hz, 1H), 7.26 (d, *J* = 8.3 Hz, 2H), 7.04 (d, *J* = 8.3 Hz, 2H), 6.73 (s, 2H), 5.93 (s, 2H), 4.27 (s, 2H), 3.75 (t, *J* = 7.0 Hz, 2H), 3.02 – 2.97 (m, 2H), 2.47 (t, *J* = 7.0 Hz, 2H), 1.85 (dt, *J* = 21.1, 7.6 Hz, 2H), 1.46 (dq, *J* = 14.8, 7.4 Hz, 2H), 0.94 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, MeOD) δ 172.88, 172.10, 159.09, 150.46, 140.15, 137.66, 135.44, 135.36, 135.21, 130.99, 129.71, 126.83, 126.50, 125.89, 123.02, 119.59, 114.25, 49.85, 43.65, 35.59, 35.40, 30.35, 27.78, 23.33, 14.12. MS (ESI) calculated for $C_{29}H_{30}N_6O_3$, m/z 510.24, found 511.25 $(M + H)^+$.

Synthesis of Compound 6b: (2*R*)-methyl 2-amino-5-((2*S*)-3-(1-(3-(4-((4-amino-2-butyl-1*H*-imidazo[4,5-c]quinolin-1-yl)methyl)benzylamino)-3-oxopropyl)-2,5-dioxopyrrolidin-3-ylthio)-1-(2-methoxy-2-oxoethylamino)-1-oxopropan-2-ylamino)-5-oxopentanoate. To a solution of **5** (15 mg, 0.03 mmol) in anhydrous MeOH and a few drops of anhydrous dichloromethane, were added triethylamine (8 mg, 0.08 mmol) and glutathione reduced dimethyl ester (20 mg, 0.06 mmol). [Glutathione-reduced dimethyl ester was obtained from glutathione-reduced by stirring in mixture of methanol and 1 ml of HCl/dioxane solution for 30 hours, followed by removal of the solvent under vacuum]. The reaction mixture was stirred for 30 minutes, followed by removal of solvent under vacuum. The residue was then purified using column chromatography (20% MeOH/dichloromethane) to obtain the compound **6b** (5 mg, 60%). ^{1}H NMR (500 MHz, MeOD) δ 7.75 (d, *J* = 8.3 Hz, 1H), 7.57 (d, *J* = 8.0 Hz, 1H), 7.36 (t, *J* = 7.3 Hz, 1H), 7.17 (d, *J* = 8.2 Hz, 2H), 7.06 (t, *J* = 7.7 Hz, 1H), 6.94 (d, *J* = 8.1 Hz, 2H), 5.79 (s, 2H), 4.19 (qd, *J* = 15.2, 6.7 Hz, 2H), 3.84 (s, 2H), 3.68 – 3.61 (m, 5H), 3.58 (s, 3H), 3.54 (dt, *J* = 10.7, 4.5 Hz, 1H), 3.14 – 2.93 (m, 3H), 2.93 – 2.82 (m, 2H), 2.39 (td, *J* = 6.9, 2.4 Hz, 2H), 2.36 – 2.27 (m, 3H), 2.05 – 1.91 (m, 1H), 1.90 – 1.78 (m, 1H), 1.75 – 1.66 (m, 2H), 1.40 – 1.30 (m, 2H), 1.21 (t, *J* = 7.3 Hz, 2H), 0.84 (t, *J* = 7.4 Hz, 3H). MS (ESI) calculated for $C_{41}H_{51}N_9O_9S$, m/z 845.35, found 868.33 $(M + Na^+)$.

Synthesis of Compound 7: To a solution of compound **1** (8 mg, 0.019mmol) in anhydrous DMF, were added 3-4 drops of acetic acid, maltoheptaose (20 mg, 0.018 mmol) and macroporous polystyrene-bound cyanoborohydride (15 mg, 0.033 mmol). The reaction mixture was heated at 50 ^{o}C for 24 hours. The solution was filtered to remove the solid resin and the filtrate was evaporated under vacuum to obtain the residue which was purified using C_{18} reverse-phase column chromatography (40% MeOH/H_2O) to obtain the compound **7** (12 mg, 45%). MS (ESI) calculated for $C_{64}H_{97}N_5O_{35}$, m/z 1495.60, found 1518.59 $(M + Na^+)$ and 759.83 $(M + H + Na)^{2+}$.

Immunoassays for Interferon (IFN)-α, IFN-γ, Interleukin (IL)-12, and IL-18. Fresh human peripheral blood mononuclear cells (PBMC) were isolated from human blood obtained by venipuncture with informed consent and as per institutional guidelines on Ficoll-Hypaque gradients as described elsewhere.[1] Aliquots of PBMCs (10^5 cells in 100 μL/well) were stimulated for 12 h with graded concentrations of test compounds. Supernatants were

isolated by centrifugation, diluted 1:20, and were assayed in triplicates using a high-sensitivity analyte-specific ELISA kits (PBL Interferon Source, Piscataway, NJ and R&D Systems, Inc., Minneapolis, MN).

NF-κB induction in human TLR7-expressing reporter gene assays: The induction of NF-κB was quantified using HEK-Blue-7 cells as previously described by us.[2;3] HEK293 cells were stably transfected with human TLR7, MD2, and secreted alkaline phosphatase (sAP), and were maintained in HEK-Blue™ Selection medium containing zeocin and normocin. Stable expression of secreted alkaline phosphatase (sAP) under control of NF-κB/AP-1 promoters is inducible by the TLR7 (or TLR8) agonists, and extracellular sAP in the supernatant is proportional to NF-κB induction. HEK-Blue cells were incubated at a density of ~10^5 cells/ml in a volume of 80 µl/well, in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluency was achieved, and subsequently graded concentrations of stimuli. sAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen (present in HEK-detection medium as supplied by the vendor) at 620 nm.

Protein Adduction and Mass Spectrometry Experiments: Bovine α-lactalbumin (Sigma-Aldrich Chemical Co., St. Louis, MO, and clinical grade human serum (Talecris Biotherapeutics, Research Triangle Park, NC) were incubated with **2** and **5**, respectively at a molar ratio of 1:5 (protein:imidazoquinoline) in aqueous carbonate buffer at pH 8.0 overnight. The adducted proteins were analyzed by reverse-phase LC-ESI-MS performed on a Shimadzu LC system (LC-10AD binary pumps, SCL-10A diode array detector) using a Zorbax 3.0 mm x 150 mm 3.5 µm stable-bond C_{18} reverse-phase column with a forty-minute binary gradient (CH_3CN/water, 0.1% HCOOH) from 5% to 95% of CH_3CN. ESI-MS data was acquired on an Agilent LC/MSD-TOF instrument with a mass accuracy of 20 ppm and a range of 100 - 3500 Daltons. Calibration drift was minimized on a scan-by-scan basis by using internal standards corresponding to 922.0001 and 2721.0201 marker ions infused concurrently through a second nebulizer in the ionization chamber. Deconvolution was performed using on-board Agilent MassHunter software.

Animal Experiments: All experiments were performed in accordance with animal care protocols approved by the University of Kansas IACUC Committee. Cohorts of 5 outbred CF-1 mice per group were immunized on Day 0 with vehicle (control 1), 50 µg/animal of bovine α-lactalbumin alone (control 2), or α-lactalbumin covalently coupled with 5 equivalents of **2**, or α-lactalbumin mixed with 5 equivalents of **1** (control 3). All antigen preparations were in sterile, physiological saline (vehicle). A volume of 0.2 ml was injected intramuscularly into the flank region. Animals were boosted once on Day 14, and bled by terminal cardiac puncture (under isoflurane anesthesia) on Day 21. Sera were obtained from clotted blood by centrifugation at 3000g X 10 min, and stored at -80°C until assayed.

Enzyme-linked immunosorbent assays (ELISA): A precision 2000 liquid handler (Bio-Tek, Winooski, VT) was used for all serial dilution and reagent addition steps, and a Bio-Tek ELx405 384-well plate washer was employed for plate washes; 100 mM phosphate-buffered saline (PBS) pH 7.4, containing 0.1% Tween-20 was used as wash buffer. Nunc-Immuno MaxiSorp (384-well) plates were coated with 30 mL of α-lactalbumin in 100 mM carbonate

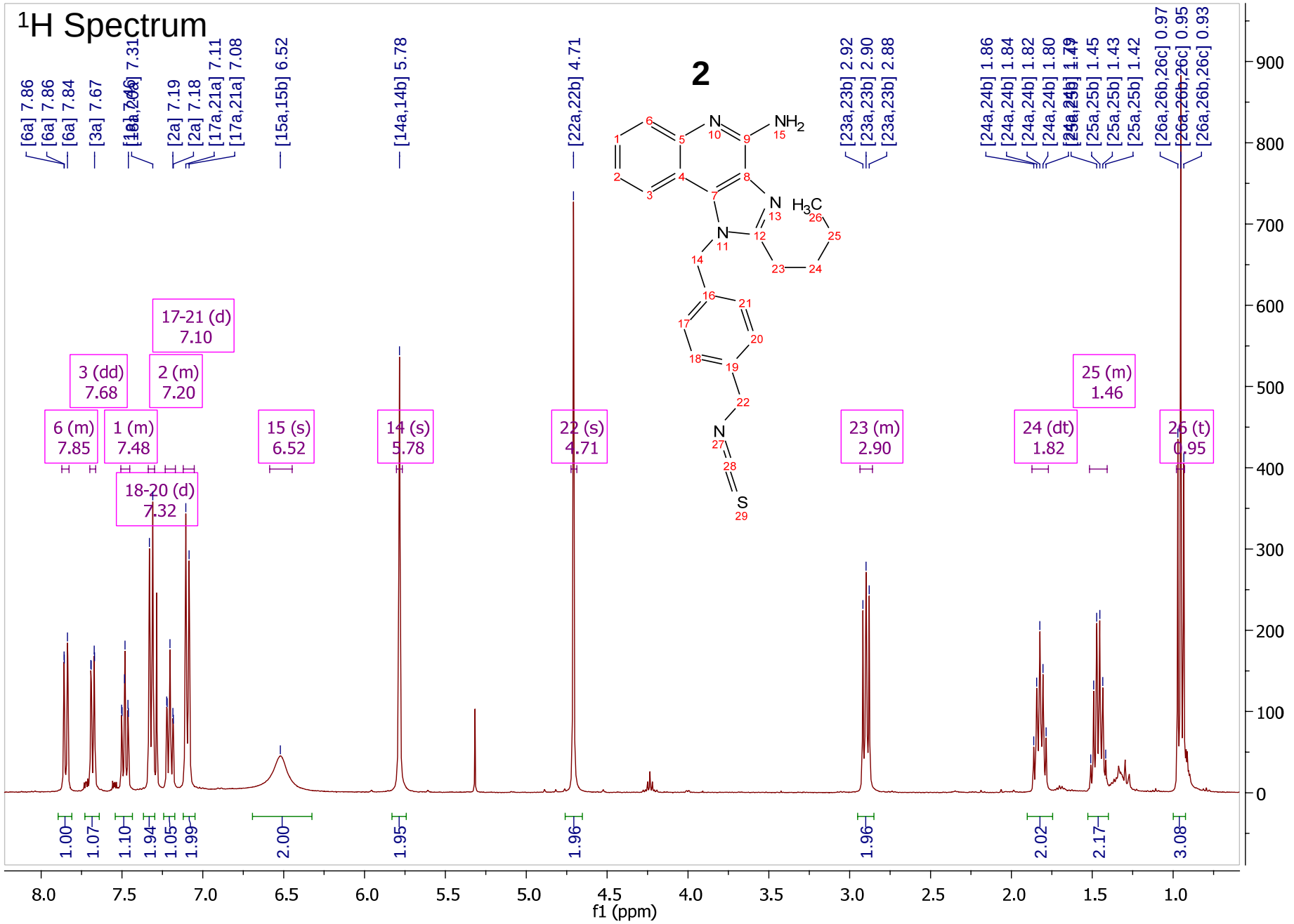
buffer, pH 9.0 overnight at 4^{o}C. After 3 washes, the plates were blocked with 3% bovine serum albumin (in PBS, pH 7.4) for 1 h at rt. Serum samples (in quadruplicate) were serially diluted in a separate 384-well plate using the liquid handler. After three additional washes of the assay plate, 30 μL of the serum dilutions were transferred using the liquid handler, and the plate incubated at 37^{o}C for 2 h. The assay plate was washed three times, and 30 μl of 1:10,000 diluted appropriate anti-mouse immunoglobulin (IgG [γ chain], IgM [μ chain], IgG1, IgG2a) conjugated with horseradish peroxidase was added to all wells. Following an incubation step at 37^{o}C for 1 h, and three washes, tetramethylbenzidine substrate was added at concentrations recommended by vendor (Sigma). The Chromogenic reaction was terminated at 30 min by the addition of 2M H_2SO_4. Plates were then read at 450 nm using a SpectraMax M4 device (Molecular Devices, Sunnyvale, CA). Data visualization and statistics (Student's T test for significance) were performed using Origin 7.0 (Northampton, MA).

Supporting Information References

(1) David, S. A.; Smith, M. S.; Lopez, G.; Mukherjee, S.; Buch, S.; Narayan, O. *AIDS Res.Human Retrovir.* **2001**, *17*, 59-68.

(2) Hood, J. D.; Warshakoon, H. J.; Kimbrell, M. R.; Shukla, N. M.; Malladi, S.; Wang, X.; David, S. A. *Hum.Vaccin.* **2010**, *6*, 1-14.

(3) Shukla, N. M.; Kimbrell, M. R.; Malladi, S. S.; David, S. A. *Bioorg.Med.Chem.Lett.* **2009**, *19*, 2211-2214.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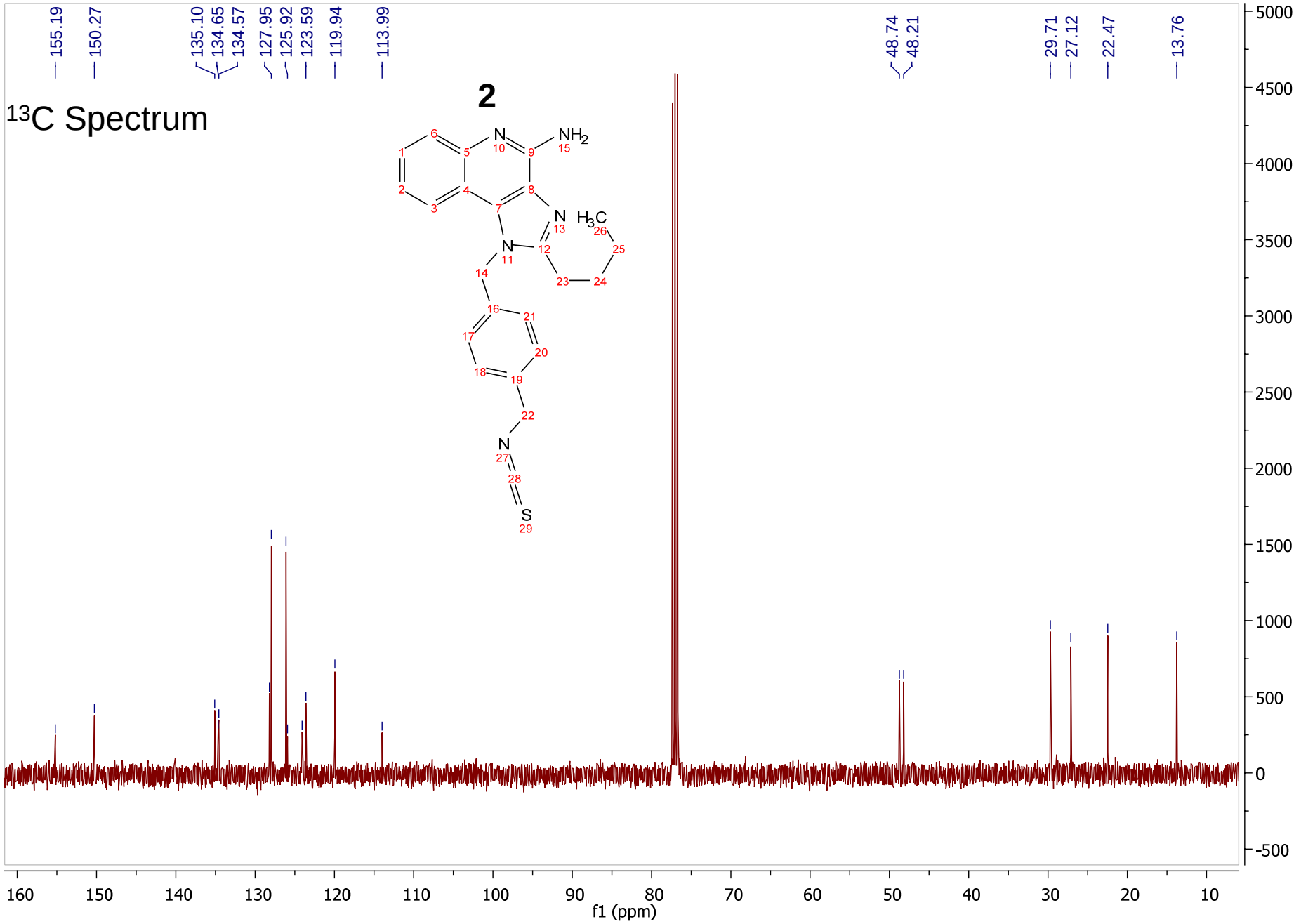
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22.47
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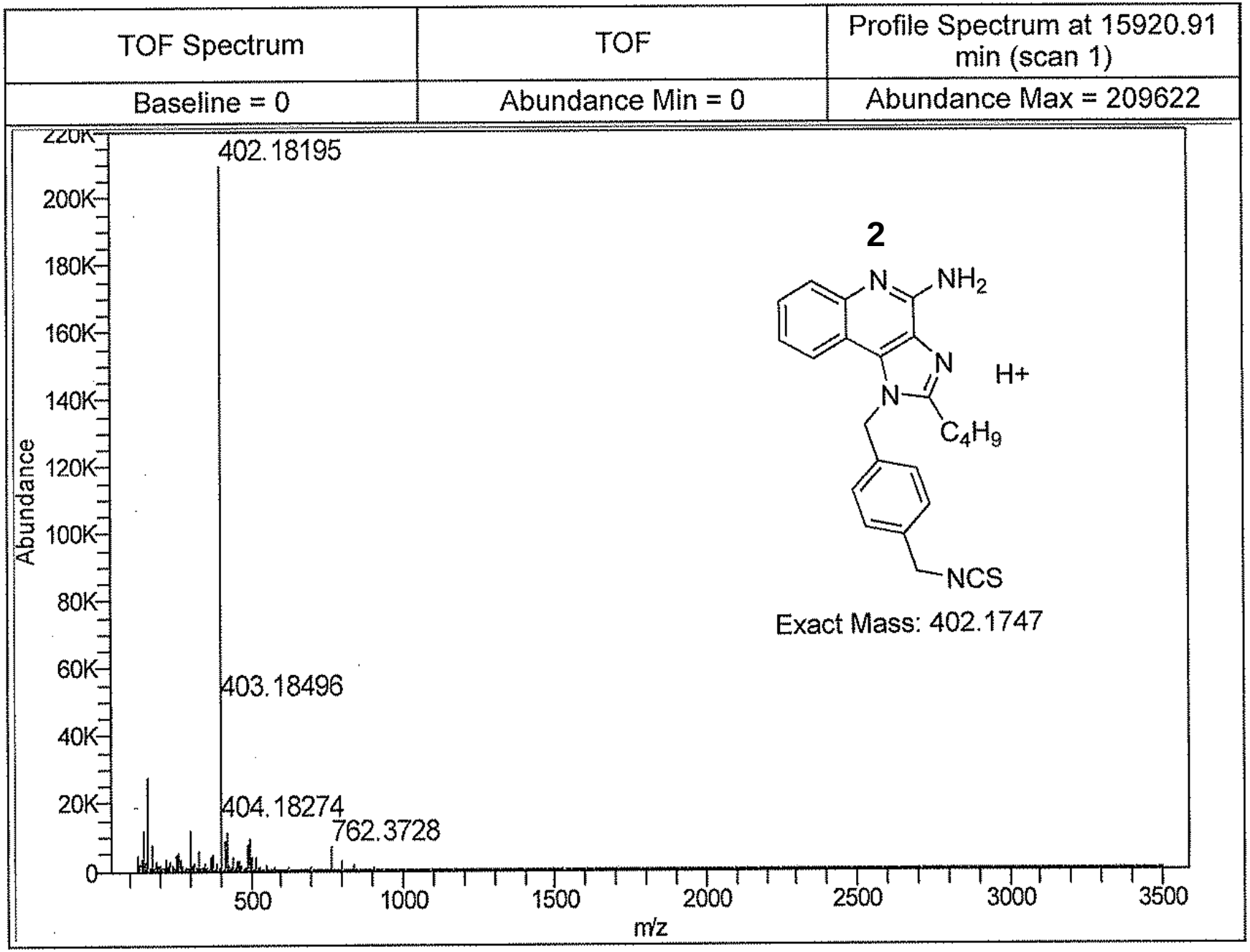
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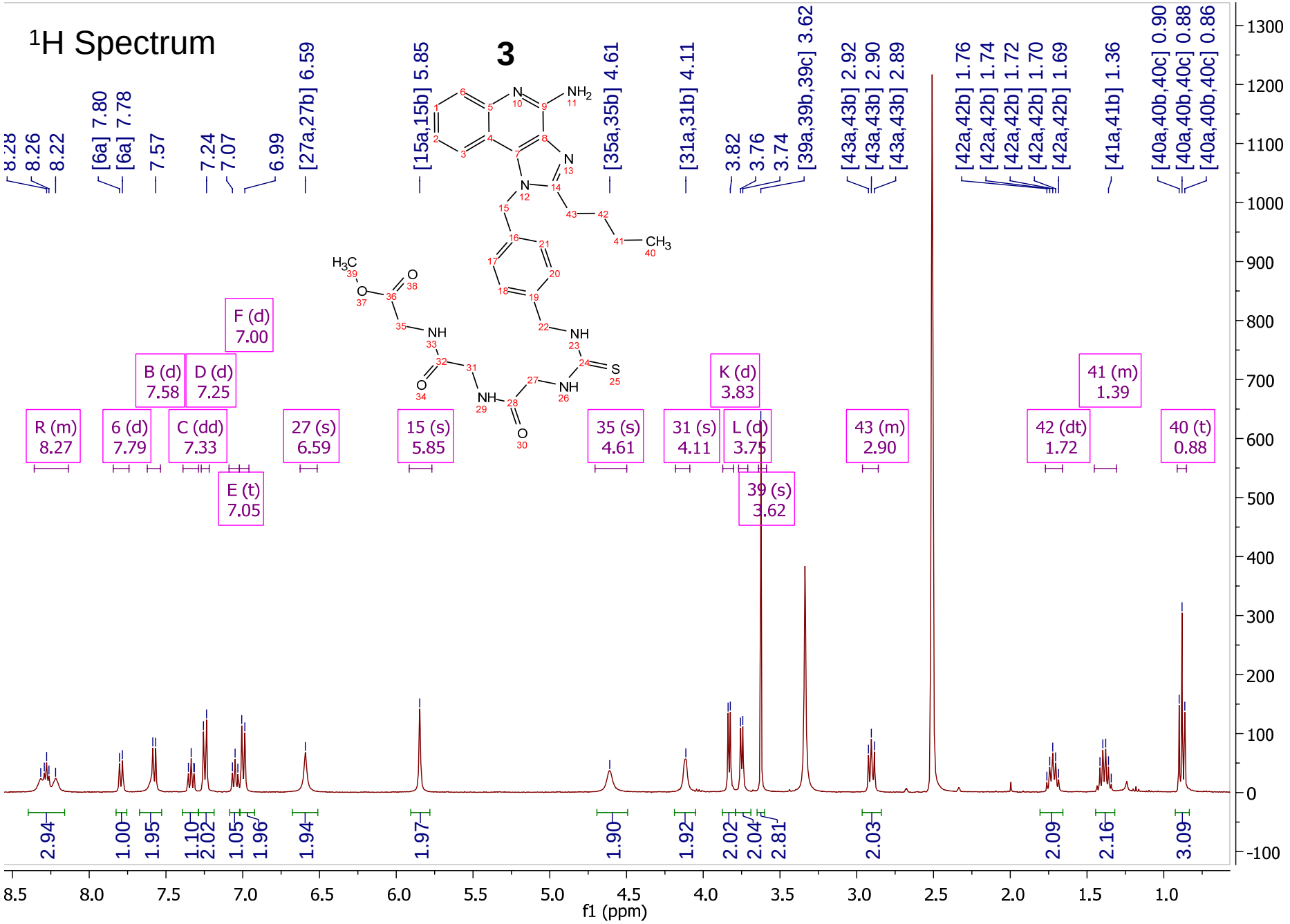
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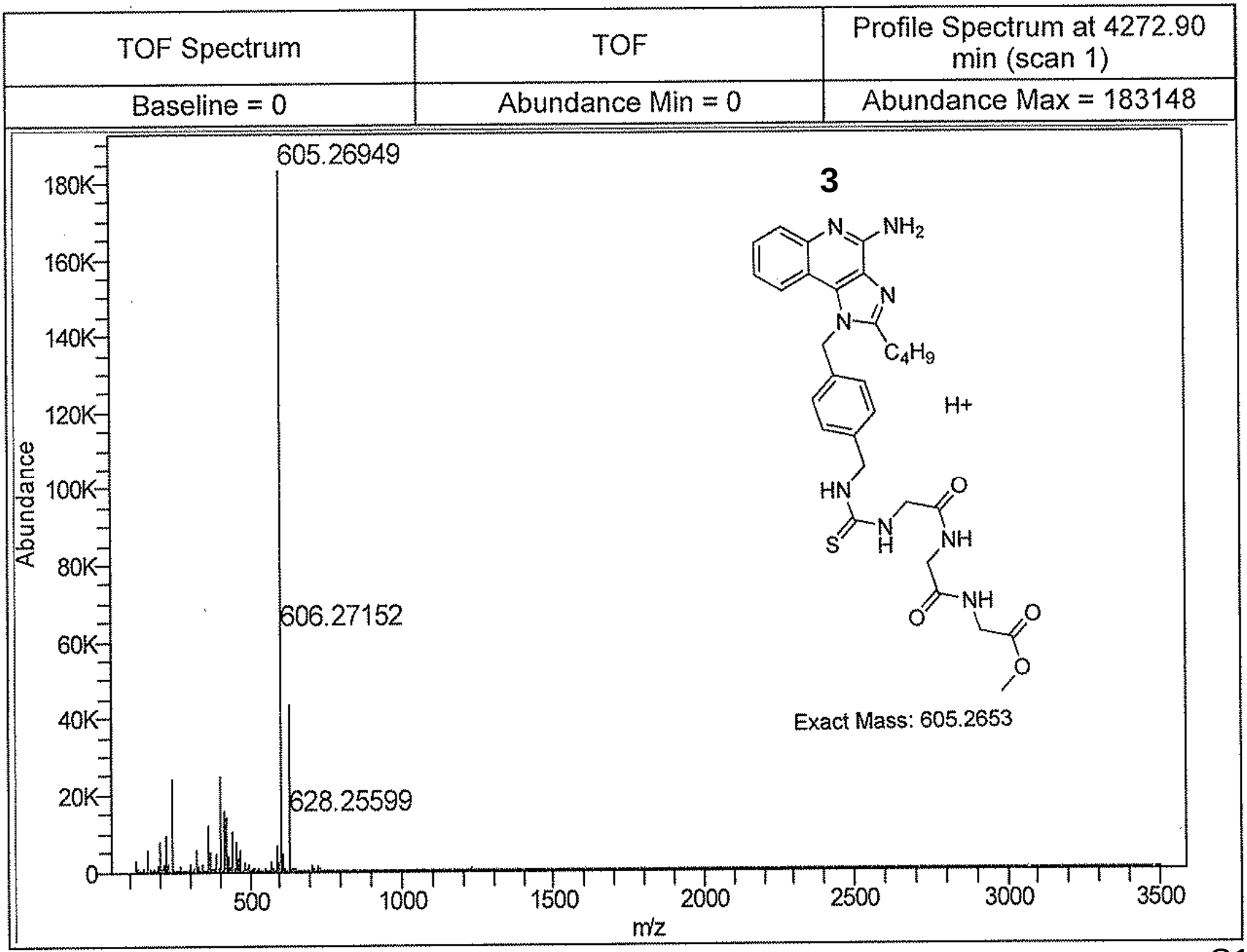
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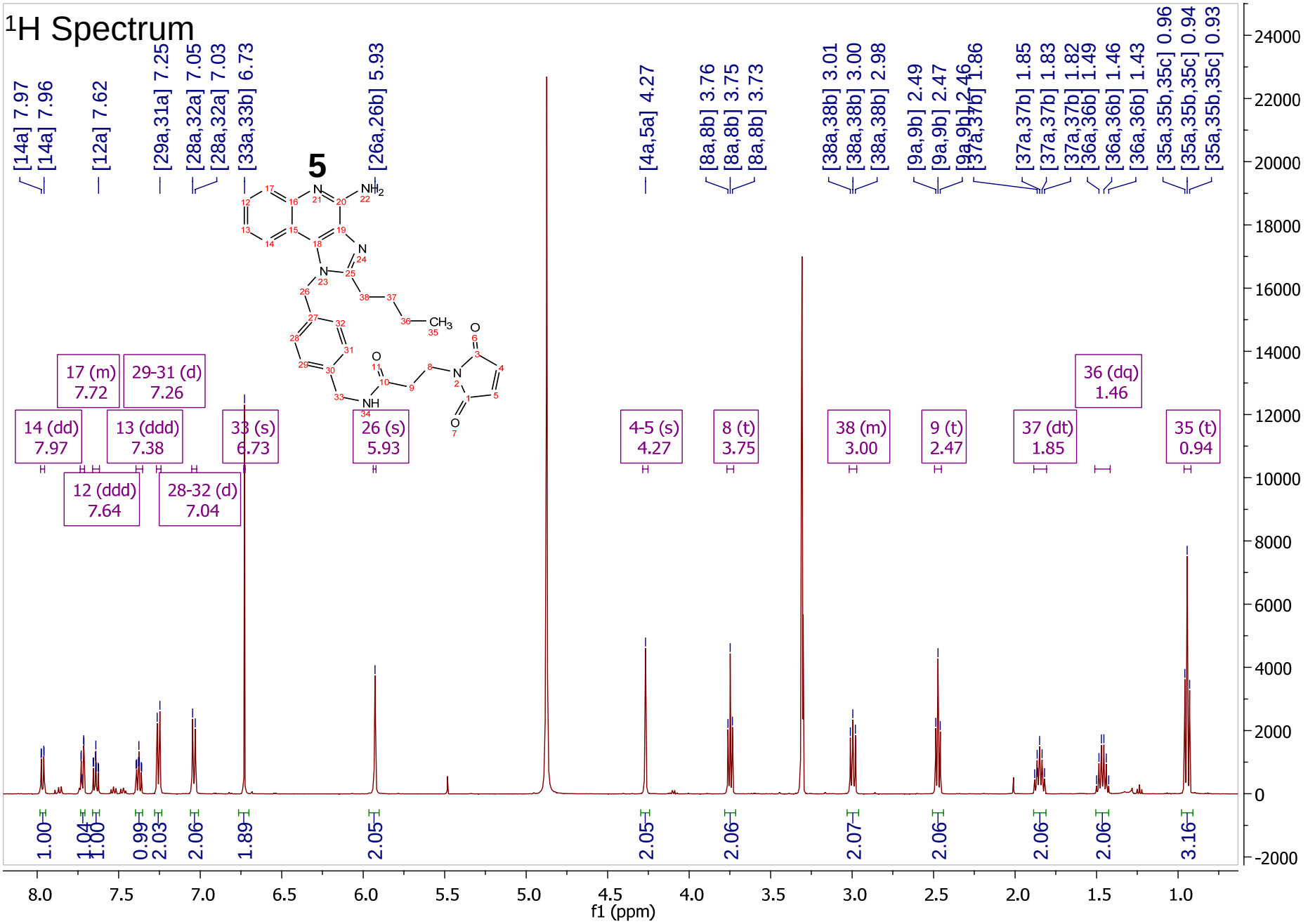
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14 (dd) 7.97
13 (ddd) 7.38
33 (s) 6.73
26 (s) 5.93
36 (dq) 1.46
12 (ddd) 7.64
28-32 (d) 7.04
4-5 (s) 4.27
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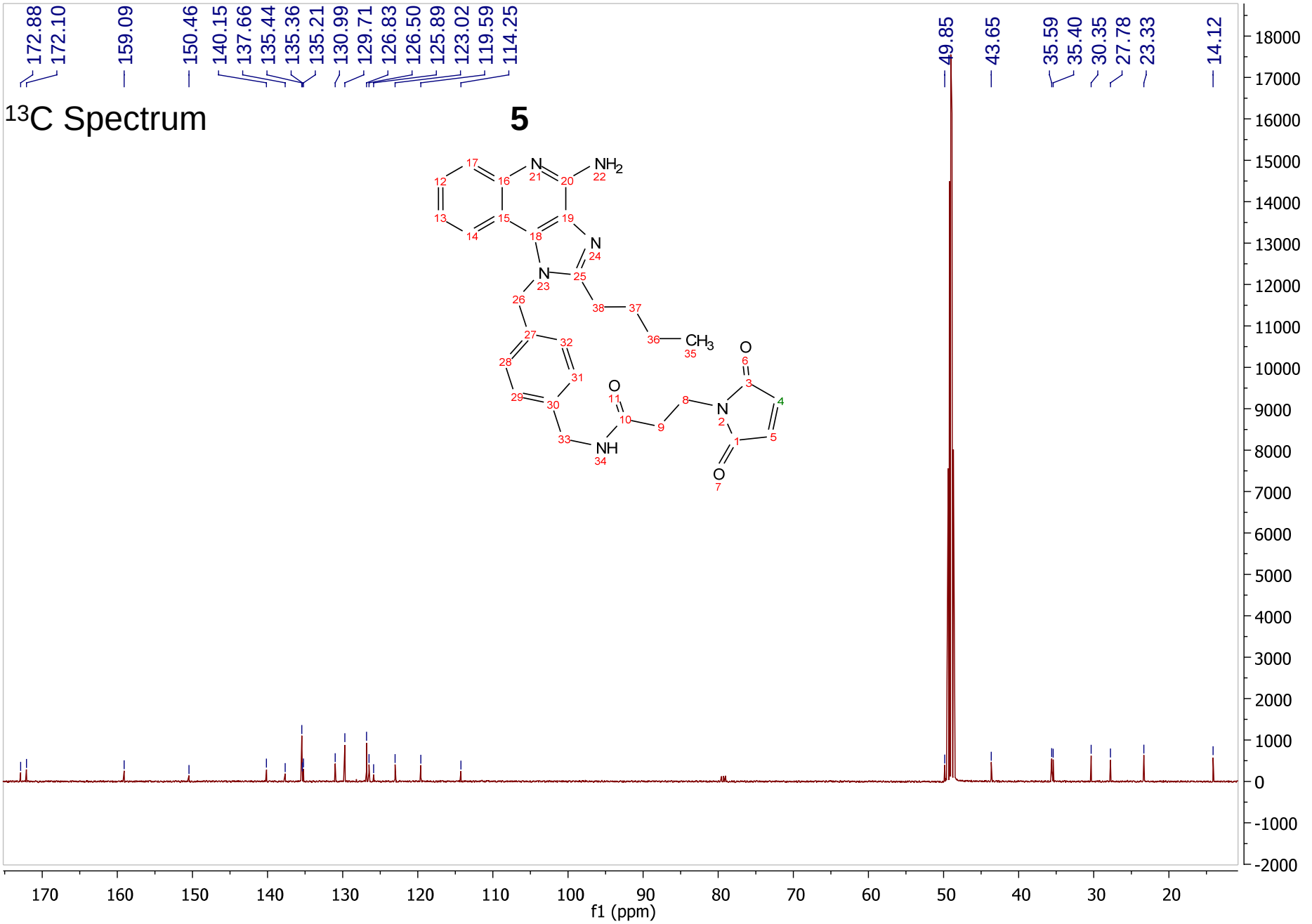
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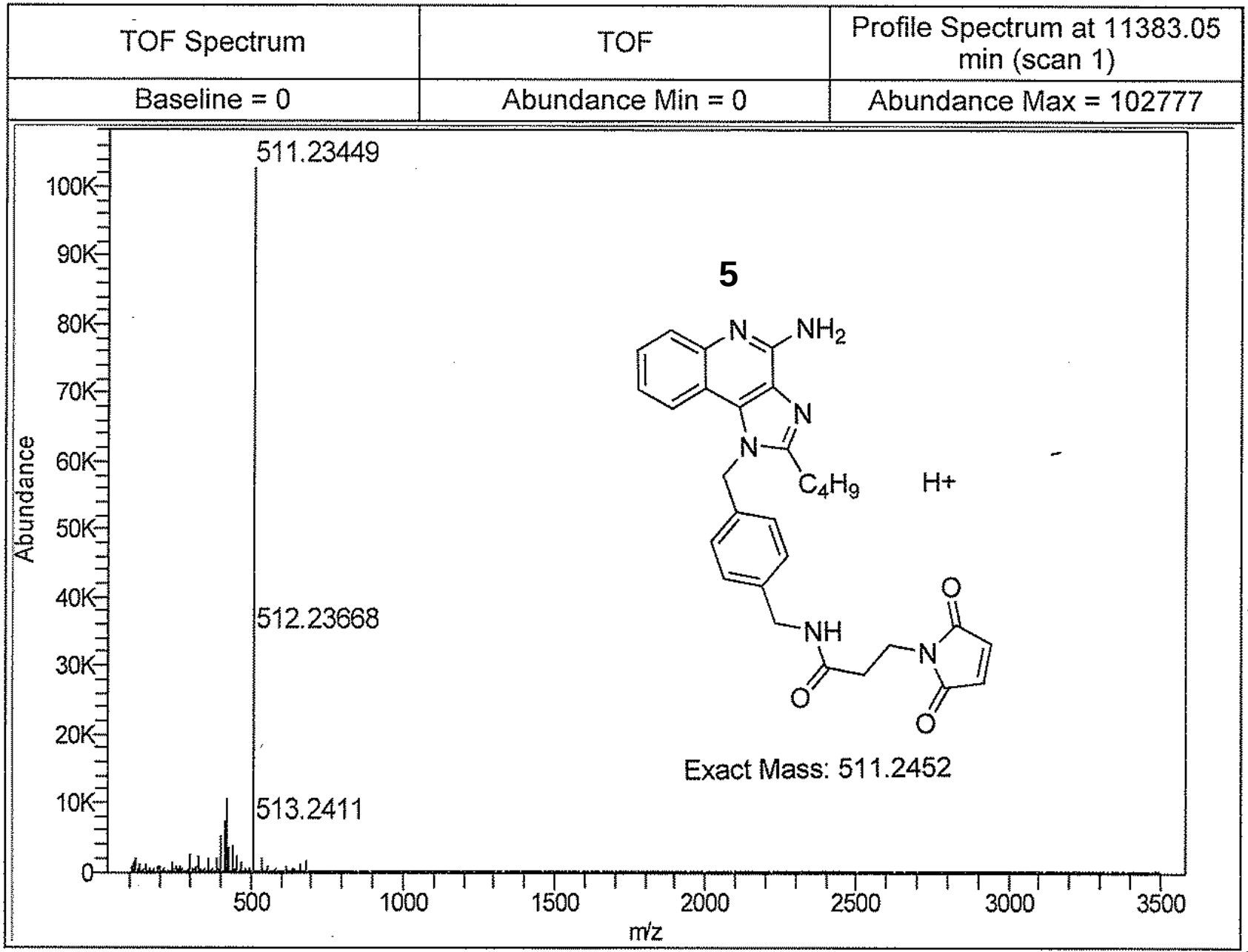
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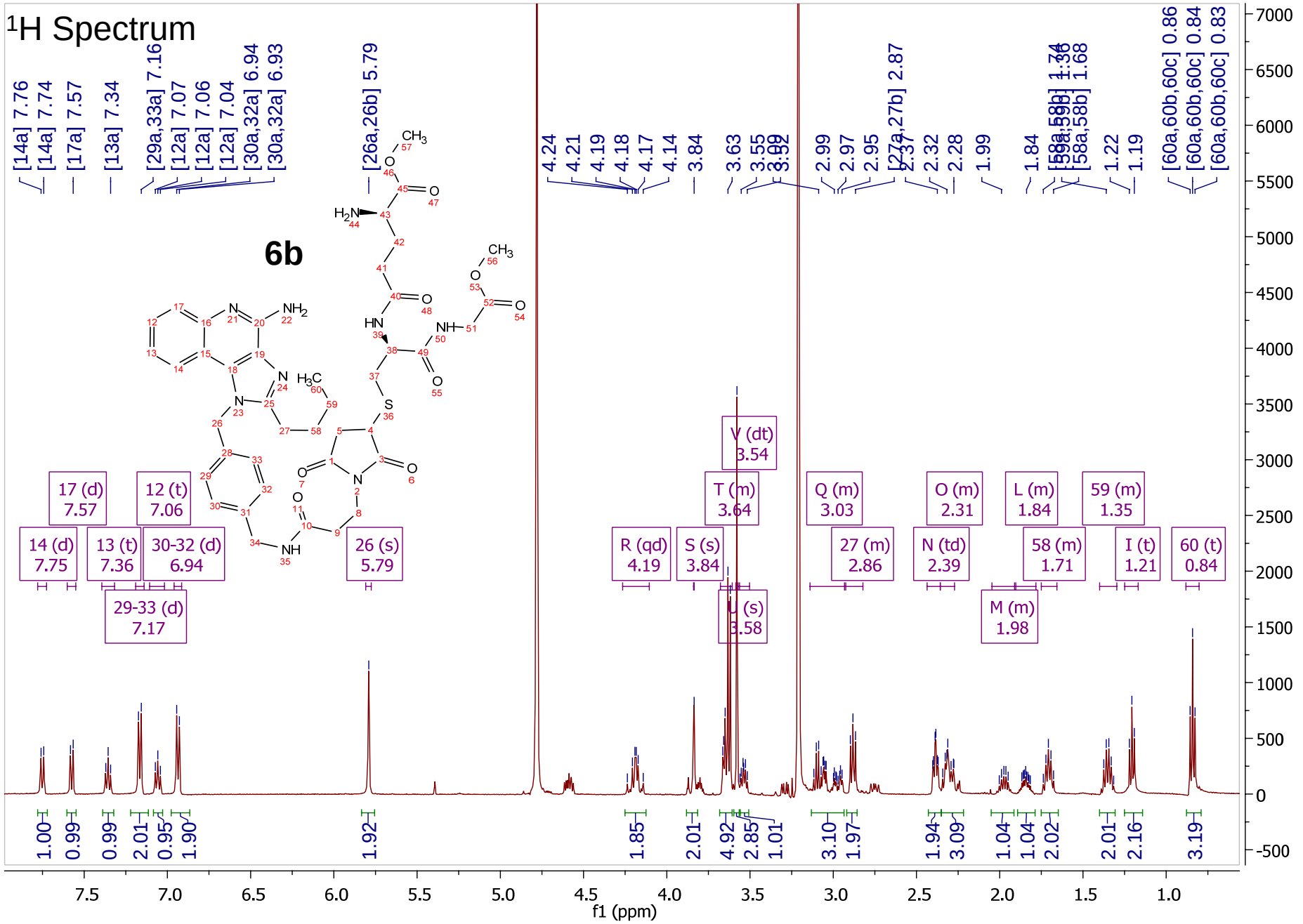
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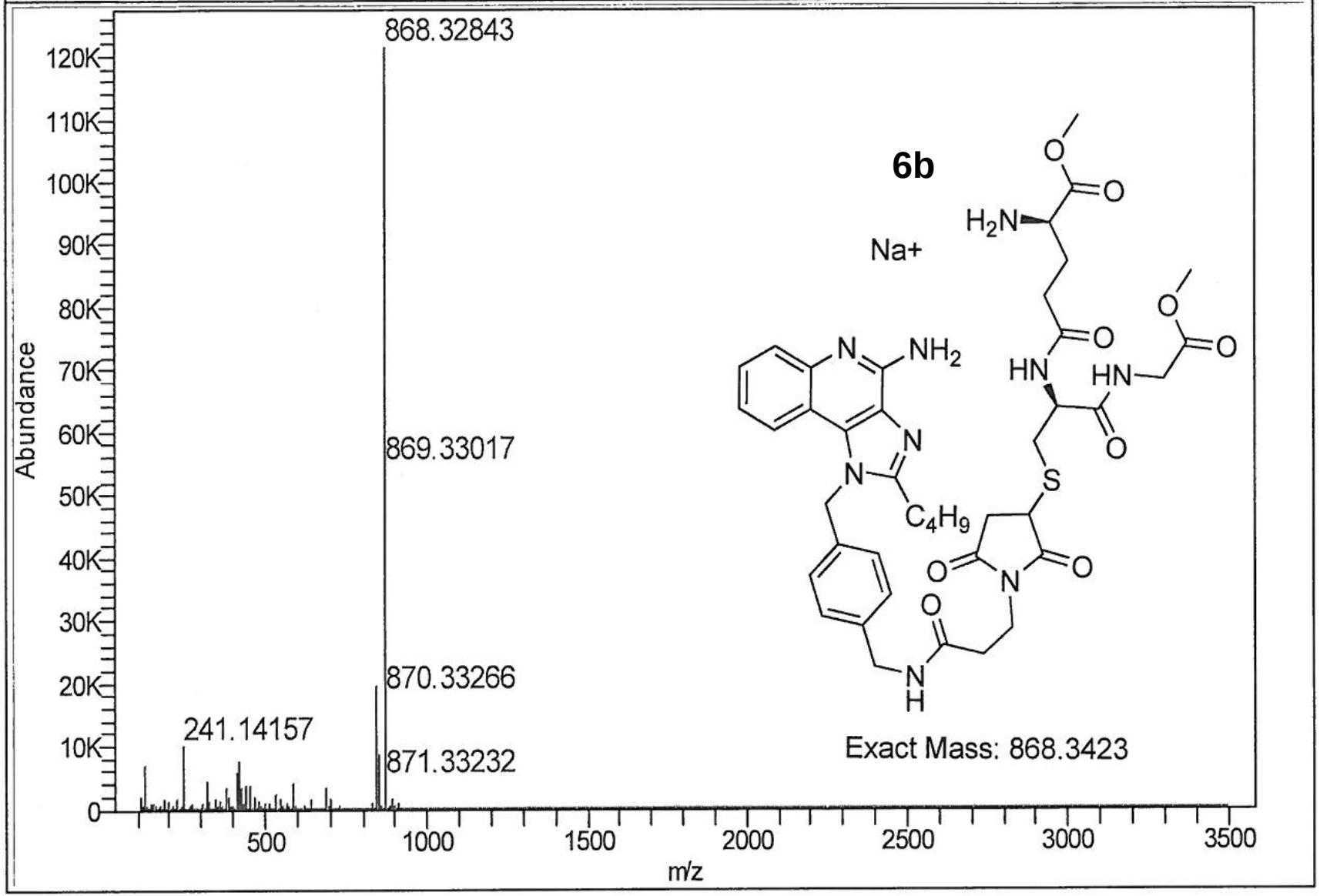
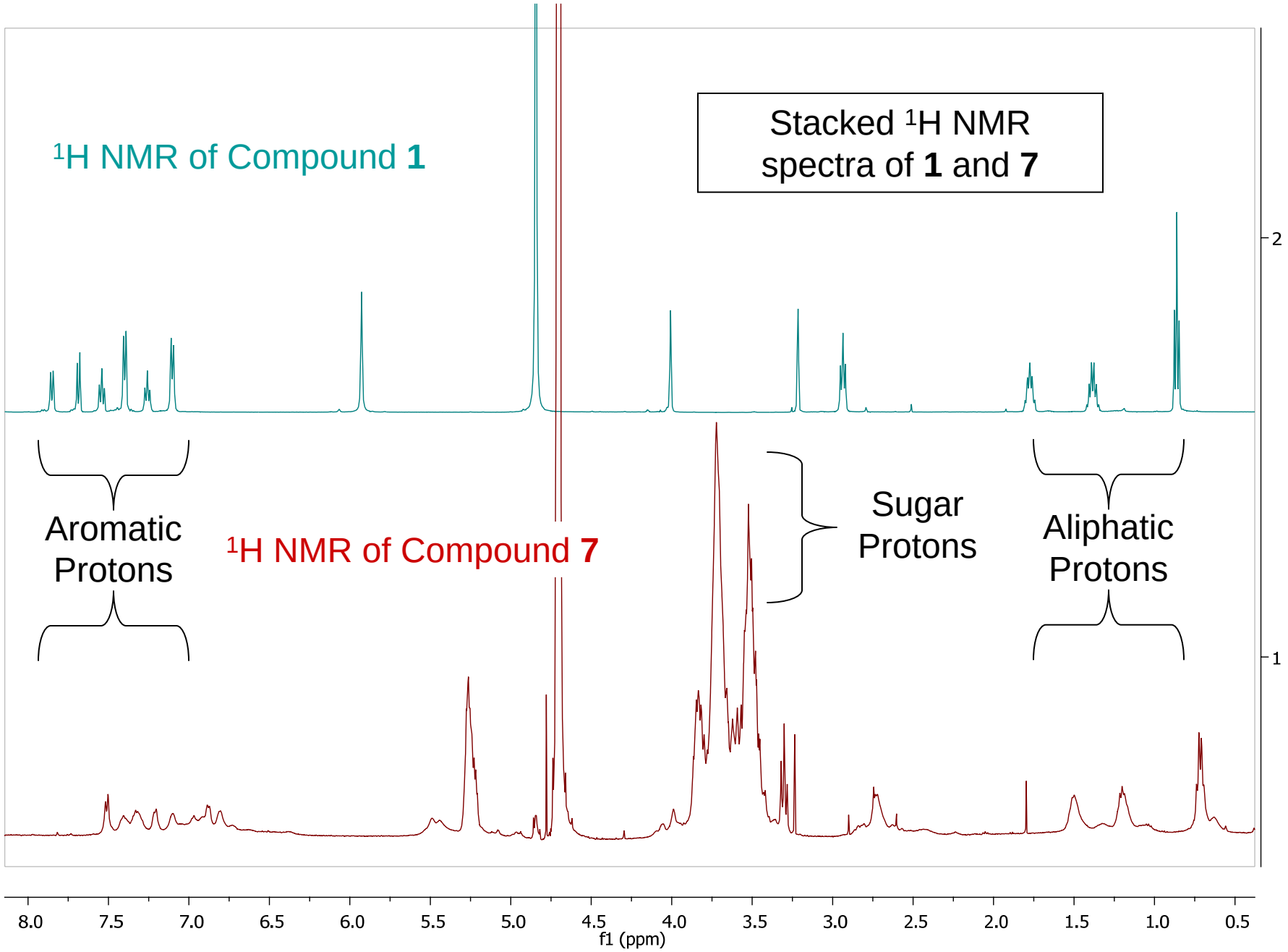
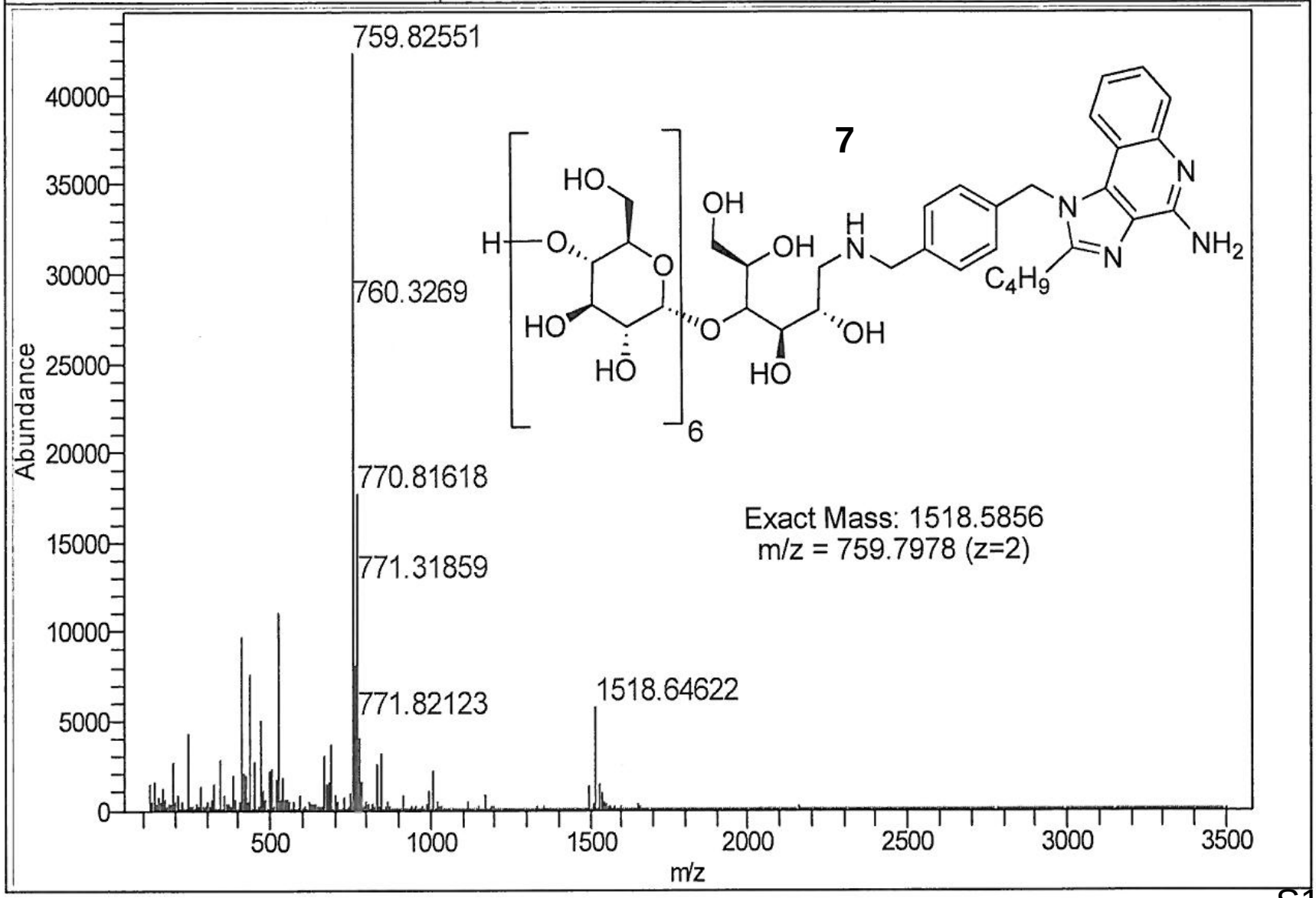
30-32 (d) 6.94
29-33 (d) 7.17
26 (s) 5.79
R (qd) 4.19
S (s) 3.84
V (dt) 3.54
T (m) 3.64
U (s) 3.58
Q (m) 3.03
27 (m) 2.86
O (m) 2.31
N (td) 2.39
L (m) 1.84
M (m) 1.98
58 (m) 1.71
59 (m) 1.35
I (t) 1.21
60 (t) 0.84
1.00
0.99
0.99
2.01
0.95
1.90
1.92
1.85
2.01
4.92
2.85
1.01
3.10
1.97
1.94
3.09
1.04
1.04
2.02
2.01
2.16
3.19
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f1 (ppm)
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500

TOF Spectrum	TOF	Profile Spectrum at 975.64 min (scan 1)
Baseline = 0	Abundance Min = 0	Abundance Max = 121269



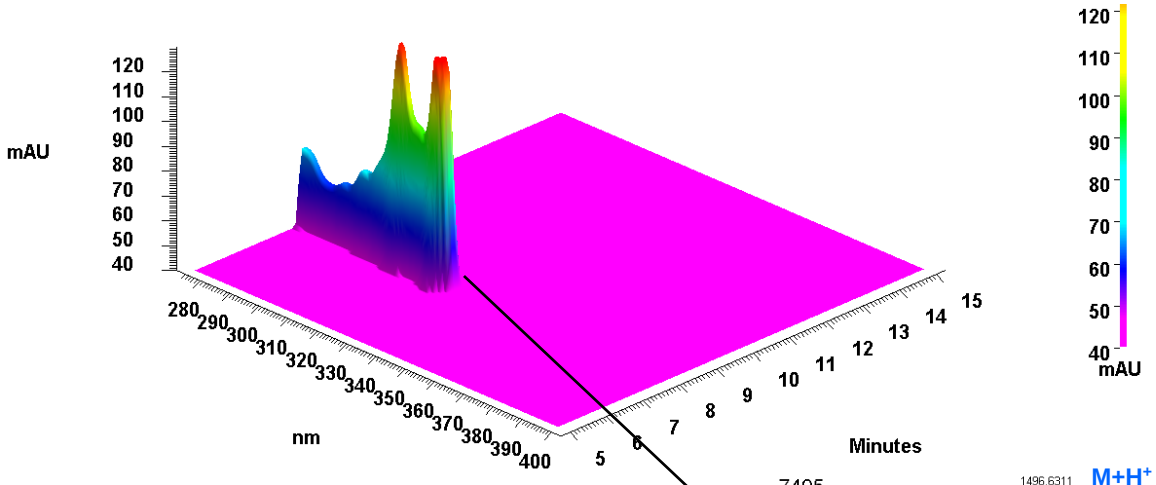

Stacked ^{1}H NMR spectra of **1** and **7**
^{1}H NMR of Compound **1**
^{1}H NMR of Compound **7**
Aromatic Protons
Sugar Protons
Aliphatic Protons
2
1
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)

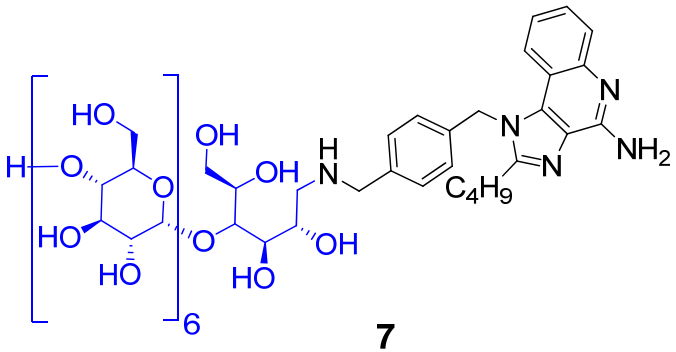
TOF Spectrum	TOF	Profile Spectrum at 941.83 min (scan 1)
Baseline = 0	Abundance Min = 0	Abundance Max = 42359



LC-MS Experiment:
Compound **7**

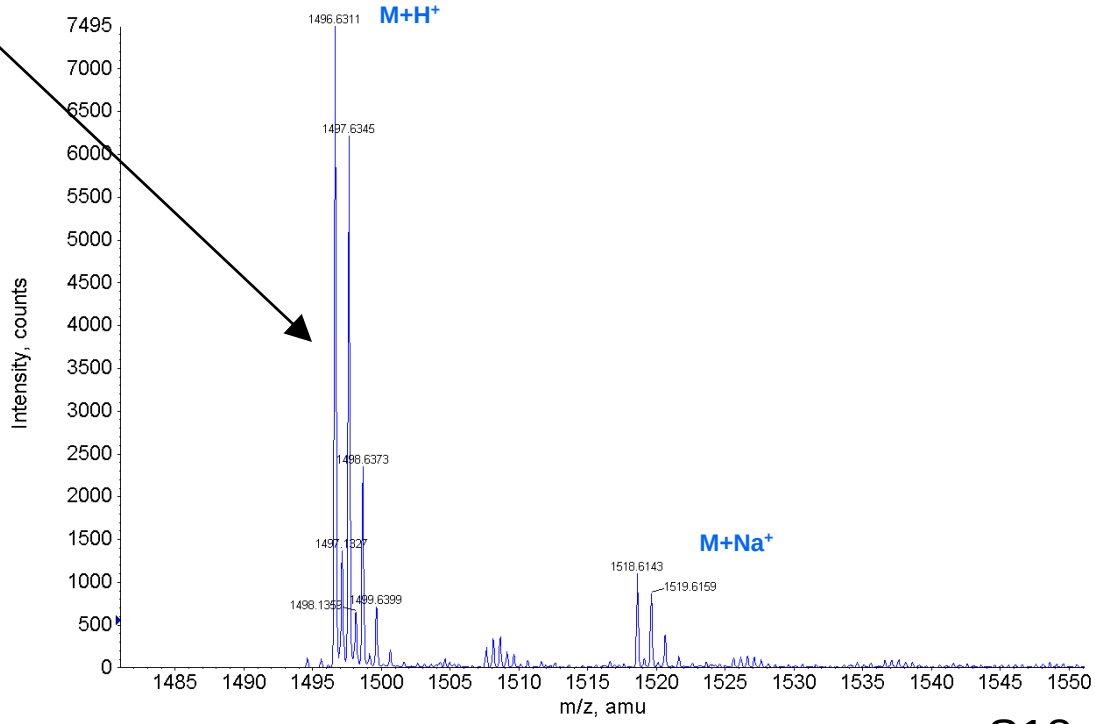
Photodiode array
detection

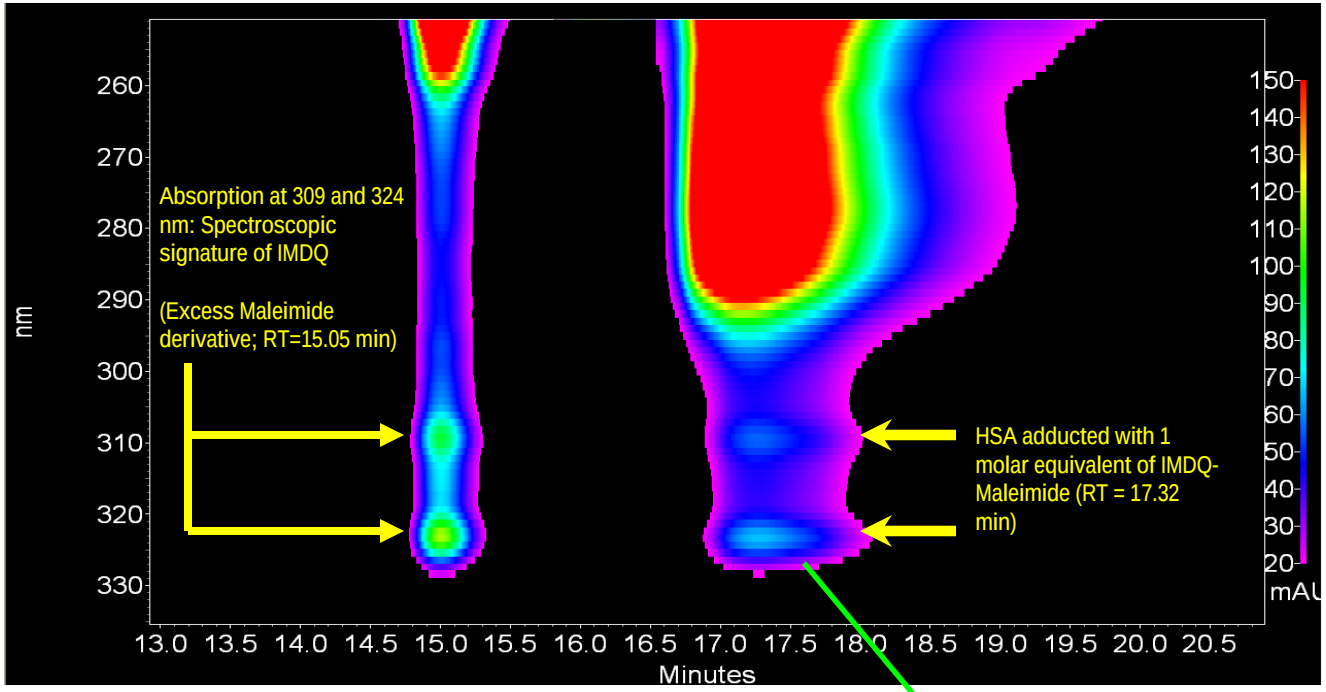




7

Exact Mass: 1495.5964





LC-MS Experiment: HSA+**5**

Photodiode array detection

LC-MS Experiment: HSA+**5**

Deconvoluted ESI-MS Data