Supporting Information

for

An antimycobacterial pleuromutilin analogue effective against dormant bacilli

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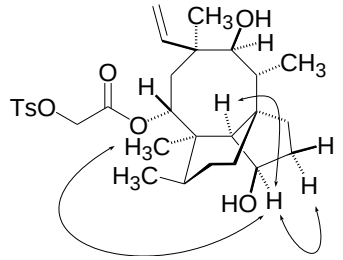
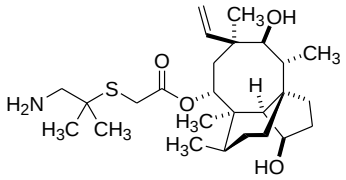
General

All chemicals were purchased from commercial sources and used without further purification unless otherwise noted. THF, CH_2Cl_2, and DMF were purified via Innovative Technology's Pure-Solve System. All reactions were performed under an Argon atmosphere. All stirring was performed with an internal magnetic stirrer. Reactions were monitored by TLC using 0.25 mm coated commercial silica gel plates (EMD, Silica Gel $60F_{254}$). TLC spots were visualized by UV light at 254 nm, or developed with ceric ammonium molybdate or anisaldehyde or copper sulfate or ninhydrin solutions by heating on a hot plate. Reactions were also monitored by using SHIMADZU LCMS-2020 with solvents: A: 0.1% formic acid in water, B: acetonitrile. Flash chromatography was performed with SiliCycle silica gel (Purasil 60 Å, 230-400 Mesh). Proton magnetic resonance (^{1}H-NMR) spectral data were recorded on 400, and 500 MHz instruments. Carbon magnetic resonance (^{13}C-NMR) spectral data were recorded on 100 and 125 MHz instruments. For all NMR spectra, chemical shifts (δH, δC) were quoted in parts per million (ppm), and *J* values were quoted in Hz. ^{1}H and ^{13}C NMR spectra were calibrated with residual undeuterated solvent ($CDCl_3$: δH = 7.26 ppm, δC = 77.16 ppm; CD_3CN: δH = 1.94 ppm, δC = 1.32ppm; CD_3OD: δH =3.31 ppm, δC =49.00 ppm; DMSO-d_6: δH = 2.50 ppm, δC = 39.52 ppm; D_2O: δH = 4.79 ppm) as an internal reference. The following abbreviations were used to designate the multiplicities: s = singlet, d = doublet, dd = double doublets, t = triplet, q = quartet, quin = quintet, hept = heptet, m = multiplet, br = broad. Infrared (IR) spectra were recorded on a Perkin-Elmer FT1600 spectrometer. HPLC analyses were performed with a Shimadzu LC-20AD HPLC system. All compounds were purified by reverse HPLC to be ≥95% purity.

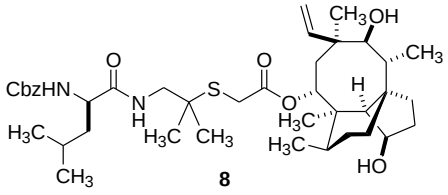
NOESY Map for **6** (A copy of NOESY data is attached):





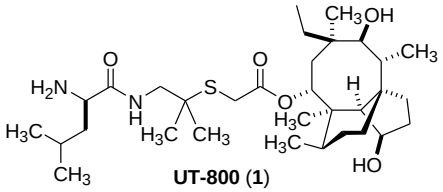
7

Data for **7**: TLC ($CHCl_3$/MeOH 90:10) R_f = 0.20; $[\alpha]^{21}_D$ -0.062 (*c* = 0.87, $CHCl_3$); IR (thin film) ν_{max} = 3421 (br), 2955, 2876, 1721, 1462, 1371, 1283, 1219, 1121, 1020, 1005, 932, 772 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 6.51 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.60 (d, *J* = 9.4 Hz, 1H), 5.32 (dd, *J* = 11.0, 1.7 Hz, 1H), 5.16 (dd, *J* = 17.4, 1.7 Hz, 1H), 4.55 (t, *J* = 5.5 Hz, 1H), 3.16 (d, *J* = 6.4 Hz, 1H), 3.13 (s, 2H), 2.61 (s, 2H), 2.26 – 2.18 (m, 1H), 2.15 (t, *J* = 6.8 Hz, 1H), 2.08 (dd, *J* = 15.8, 9.3 Hz, 1H), 2.00 – 1.91 (m, 1H), 1.85 (td, *J* = 13.7, 4.5 Hz, 1H), 1.74 – 1.58 (m, 4H), 1.51 (d, *J* = 5.2 Hz, 1H), 1.48 – 1.41 (m, 2H), 1.41 – 1.33 (m, 1H), 1.24 (s, 6H), 1.23 (s, 3H), 1.14 (s, 3H), 0.80 (d, *J* = 7.0 Hz, 3H), 0.71 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.38, 139.56, 116.66, 74.93, 71.25, 51.68, 51.01, 48.44, 46.00, 45.20, 44.86, 41.24, 36.62, 35.61, 34.31, 32.63, 31.76, 31.33, 27.66, 26.30, 26.25 (2C), 17.64, 17.21, 12.16; HRMS (ESI+) *m/z* calcd for $C_{26}H_{46}NO_4S$ [M + H] 468.3148, found: 468.3181 [1].



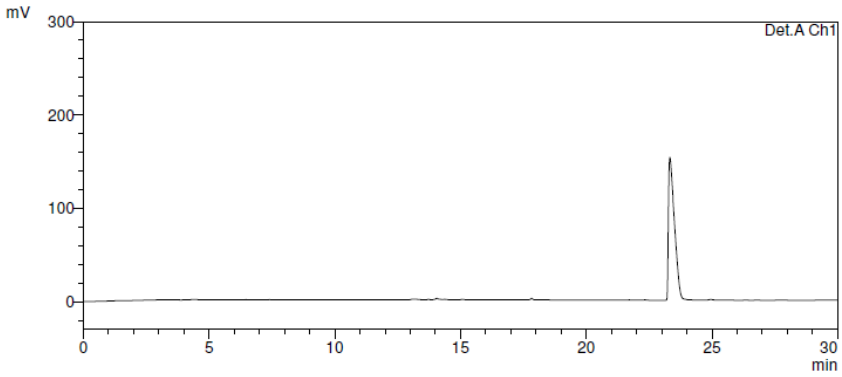
8

Data for **8**: TLC (hexanes/EtOAc 50:50) R_f = 0.5; $[\alpha]^{21}_D$ -0.168 (*c* = 0.95, $CHCl_3$); IR (thin film) ν_{max} = 3334 (br), 2956, 2871, 1722, 1665, 1525, 1468, 1415, 1388, 1370, 1282, 1121, 1062, 979, 932, 915, 774, 697 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 7.35 (d, *J* = 4.2 Hz, 5H), 7.14 (t, *J* = 6.3 Hz, 1H), 6.49 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.62 (d, *J* = 9.3 Hz, 1H), 5.35 (dd, *J* = 30.6, 9.6 Hz, 2H), 5.21 – 5.06 (m, 3H), 4.55 (t, *J* = 5.5 Hz, 1H), 4.27 (q, *J* = 8.0 Hz, 1H), 3.27 – 3.08 (m, 4H), 2.29 – 2.18 (m, 1H), 2.18 – 2.06 (m, 1H), 2.01 – 1.90 (m, 1H), 1.85 (td, *J* = 13.6, 4.7 Hz, 1H), 1.79 – 1.59 (m, 4H), 1.59 – 1.32 (m, 6H), 1.29 – 1.17 (m, 9H), 1.14 (s, 3H), 1.01 – 0.94 (m, 6H), 0.91 – 0.86 (m, 1H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.69 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.58, 170.31, 156.04, 139.23, 128.48 (2C), 127.99 (2C), 116.95, 74.85, 71.76, 66.93, 63.20, 60.43, 53.75, 50.84, 47.48, 47.03, 45.91, 45.22, 44.77, 42.18, 41.14, 36.41, 35.56, 34.25, 32.49, 31.70, 31.48, 27.62, 26.33, 26.16, 24.74, 22.95, 22.10, 21.07, 17.55, 17.22, 12.22; HRMS (ESI+) *m/z* calcd for $C_{40}H_{63}N_2O_7S$ [M + H] 715.4356, found: 715.4381.



UT-800 (1)

HPLC analysis of **1**.



Area % purity: 96.9%

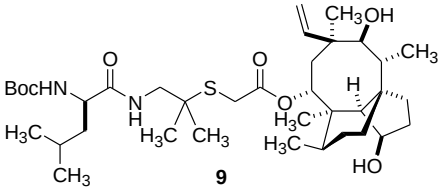
Conditions:

column: HYPERSIL GOLD™ 12 µm 175 Å 250 x 10 mm

solvents: a gradient elution of 25 : 75 to 55 : 45 MeOH : H_2O over 20 min then 55 : 45 MeOH : H_2O

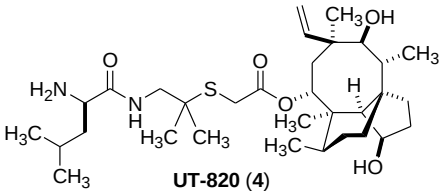
flow rate: 2.0 mL/min

UV: 220 nm



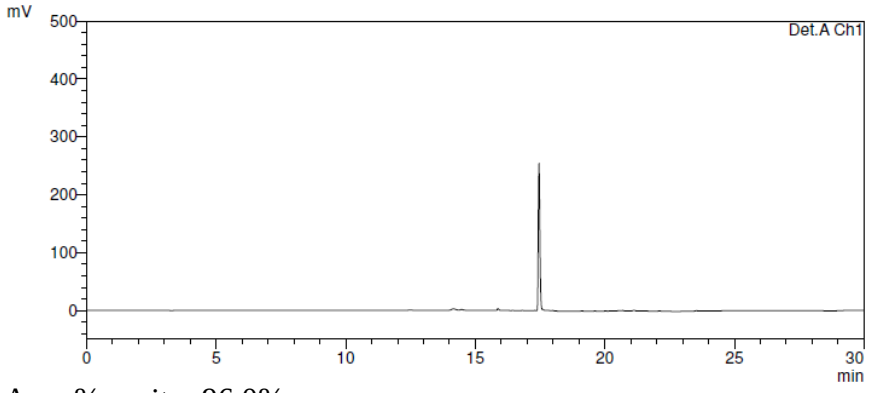
9

Data for **9**: TLC (hexanes/EtOAc 50:50) R_f = 0.40; $[\alpha]^{21}_D$ -0.087 (*c* = 3.10, $CHCl_3$); IR (thin film) ν_{max} = 3323 (br), 2956, 2934, 2872, 1704, 1666, 1526, 1455, 1390, 1367, 1286, 1249, 1166, 1120, 1020, 1005, 933, 755 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 7.11 – 7.02 (m, 1H), 6.49 (dd, *J* = 17.3, 11.0 Hz, 1H), 5.61 (d, *J* = 9.3 Hz, 1H), 5.31 (dd, *J* = 10.9, 1.7 Hz, 1H), 5.16 (dd, *J* = 17.4, 1.7 Hz, 1H), 4.98 (d, *J* = 8.1 Hz, 1H), 4.55 (t, *J* = 5.5 Hz, 1H), 4.19 – 4.10 (m, 1H), 3.22 (dd, *J* = 11.5, 6.1 Hz, 1H), 3.17 (s, 2H), 2.24 (ddd, *J* = 13.8, 6.8, 3.2 Hz, 1H), 2.17 – 2.04 (m, 2H), 1.94 (dddd, *J* = 16.3, 10.3, 7.4, 3.6 Hz, 1H), 1.89 – 1.80 (m, 1H), 1.76 – 1.57 (m, 4H), 1.54 – 1.48 (m, 1H), 1.44 (s, 12H), 1.27 – 1.21 (m, 12H), 1.14 (s, 3H), 0.96 (d, *J* = 6.0 Hz, 6H), 0.80 (d, *J* = 7.0 Hz, 3H), 0.69 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.96, 172.84, 170.06, 139.42, 116.78, 74.86, 71.67, 60.38, 50.89, 47.55, 47.06, 45.94, 44.81, 41.17, 36.44, 35.56, 34.24, 32.50, 31.72, 31.46, 28.34 (3C), 27.61, 26.21 (2C), 24.81, 22.95, 21.03, 17.54, 17.20, 14.17, 12.18; HRMS (ESI+) *m/z* calcd for $C_{37}H_{65}N_2O_7S$ [M + H] 681.4512, found: 681.4535.

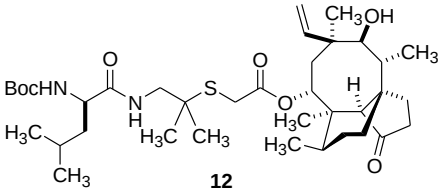


UT-820 (4)

HPLC analysis of **4**.

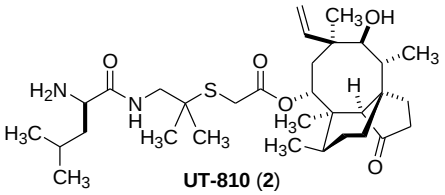


Area % purity: 96.0%
Conditions:
column: HYPERSIL GOLD™ 12 μm 175 Å 250 x 10 mm
solvents: a gradient elution of 70 : 30 to 100 : 0 MeOH : H_2O over 20 min
flow rate: 2.0 mL/min
UV: 220 nm



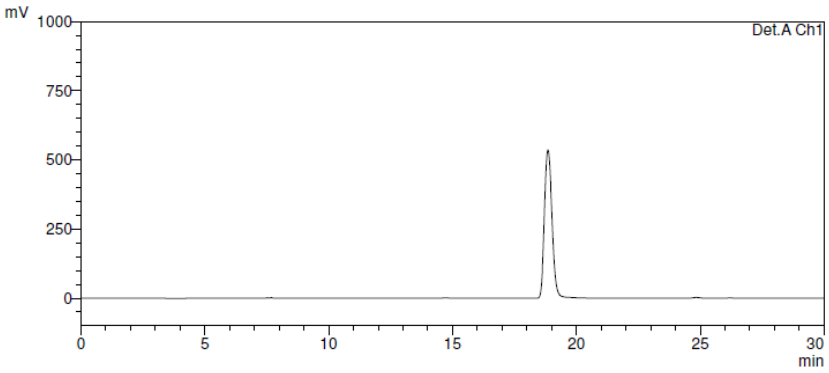
12

Data for **12**: TLC (hexanes/EtOAc 50:50) R_f = 0.40; $[\alpha]^{20}_D$ +0.497 (*c* = 2.32, $CHCl_3$); IR (thin film) ν_{max} = 3322 (br), 2957, 2931, 2868, 1722, 1662, 1525, 1454, 1388, 1366, 1279, 1247, 1164, 1116, 1018, 980, 917, 752 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 7.06 (t, *J* = 6.2 Hz, 1H), 6.45 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.75 (d, *J* = 8.4 Hz, 1H), 5.31 (dd, *J* = 11.0, 1.6 Hz, 1H), 5.19 (dd, *J* = 17.4, 1.6 Hz, 1H), 4.99 (d, *J* = 8.2 Hz, 1H), 4.19 – 4.11 (m, 1H), 3.39 – 3.32 (m, 1H), 3.21 (t, *J* = 6.2 Hz, 2H), 3.17 (d, *J* = 2.5 Hz, 2H), 2.36 – 2.28 (m, 1H), 2.28 – 2.15 (m, 2H), 2.13 – 2.05 (m, 2H), 1.80 – 1.73 (m, 1H), 1.72 – 1.59 (m, 3H), 1.59 – 1.46 (m, 2H), 1.44 (s, 3H), 1.43 (s, 9H), 1.40 – 1.33 (m, 1H), 1.29 (d, *J* = 16.1 Hz, 1H), 1.23 (s, 6H), 1.16 (s, 3H), 1.11 (dd, *J* = 13.9, 4.4 Hz, 1H), 0.95 (dd, *J* = 6.3, 1.7 Hz, 6H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.71 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.91, 172.89, 170.05, 138.89, 117.32, 74.59, 69.78, 58.14, 47.60, 47.08, 45.42, 44.81, 43.92, 41.77, 36.69, 36.00, 34.41, 31.44, 30.39, 28.33 (3C), 26.82, 26.33, 26.26, 26.17, 24.83, 24.81, 22.92, 16.81, 14.86, 11.50; HRMS (ESI+) *m/z* calcd for $C_{37}H_{63}N_2O_7S$ [M + H] 679.4356, found: 679.4328.

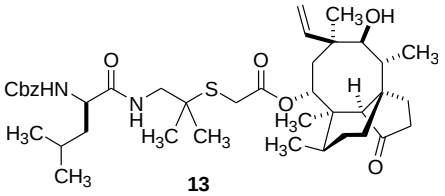


UT-810 (2)

HPLC analysis of **2**.

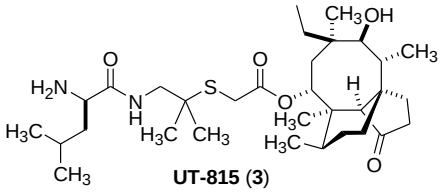


Area % purity: 98.0%
Conditions:
column: HYPERSIL GOLD™ 12 μm 175 Å 250 x 10 mm
solvents: a gradient elution of 70 : 30 to 100 : 0 MeOH : H_2O over 20 min,
flow rate: 2.0 mL/min
UV: 220 nm



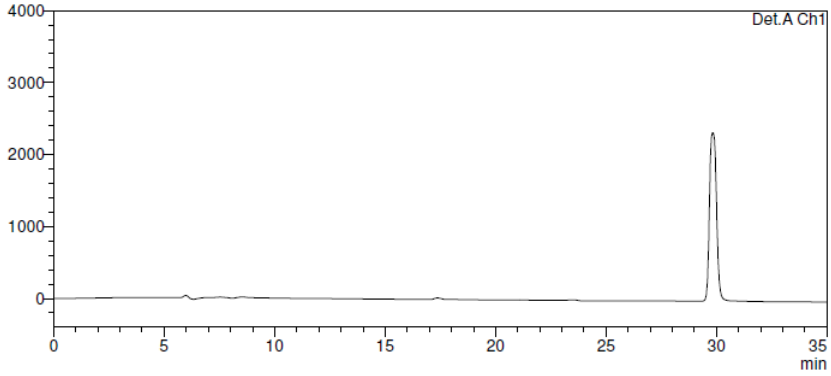
13

Data for **13**: TLC (hexanes/EtOAc 50:50) R_f = 0.50; $[\alpha]^{21}_D$ +0.070 (*c* = 0.93, $CHCl_3$); IR (thin film) ν_{max} = 3327 (br), 2957, 2933, 2869, 1721, 1670, 1534, 1454, 1386, 1369, 1324, 1284, 1212, 1142, 1117, 1055, 753, 698 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 7.37 – 7.32 (m, 5H), 7.18 (t, *J* = 6.2 Hz, 1H), 6.44 (dd, *J* = 17.4, 10.9 Hz, 1H), 5.76 (d, *J* = 8.4 Hz, 1H), 5.43 (d, *J* = 8.2 Hz, 1H), 5.32 (d, *J* = 11.4 Hz, 1H), 5.26 – 5.06 (m, 4H), 4.38 (q, *J* = 7.1 Hz, 1H), 4.27 (td, *J* = 8.6, 5.4 Hz, 1H), 3.36 (d, *J* = 6.6 Hz, 1H), 3.28 – 3.08 (m, 4H), 2.31 (dt, *J* = 10.8, 5.0 Hz, 1H), 2.27 – 2.18 (m, 1H), 2.14 – 2.06 (m, 2H), 1.80 – 1.59 (m, 4H), 1.59 – 1.51 (m, 1H), 1.51 – 1.47 (m, 1H), 1.45 (s, 3H), 1.32 – 1.19 (m, 6H), 1.16 (s, 3H), 1.00 – 0.93 (m, 6H), 0.88 (d, *J* = 7.2 Hz, 3H), 0.82 (t, *J* = 6.2 Hz, 2H), 0.71 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 217.14, 172.81, 170.39, 158.30, 156.11, 138.66, 128.50 (2C), 127.99 (2C), 117.55, 74.60, 69.87, 66.99, 63.27, 58.11, 47.08, 45.41, 44.78, 43.85, 42.06, 41.74, 36.64, 35.97, 34.43, 31.46, 30.36, 26.82, 26.33, 26.25, 26.14, 24.81, 24.74, 22.92, 22.08, 21.07, 16.85, 14.85, 14.00, 11.58; HRMS (ESI+) *m/z* calcd for $C_{40}H_{61}N_2O_7S$ [M + H] 713.4199, found: 713.4212.



UT-815 (**3**)

HPLC analysis of **3**.



Area % purity: 98.4%

Conditions:

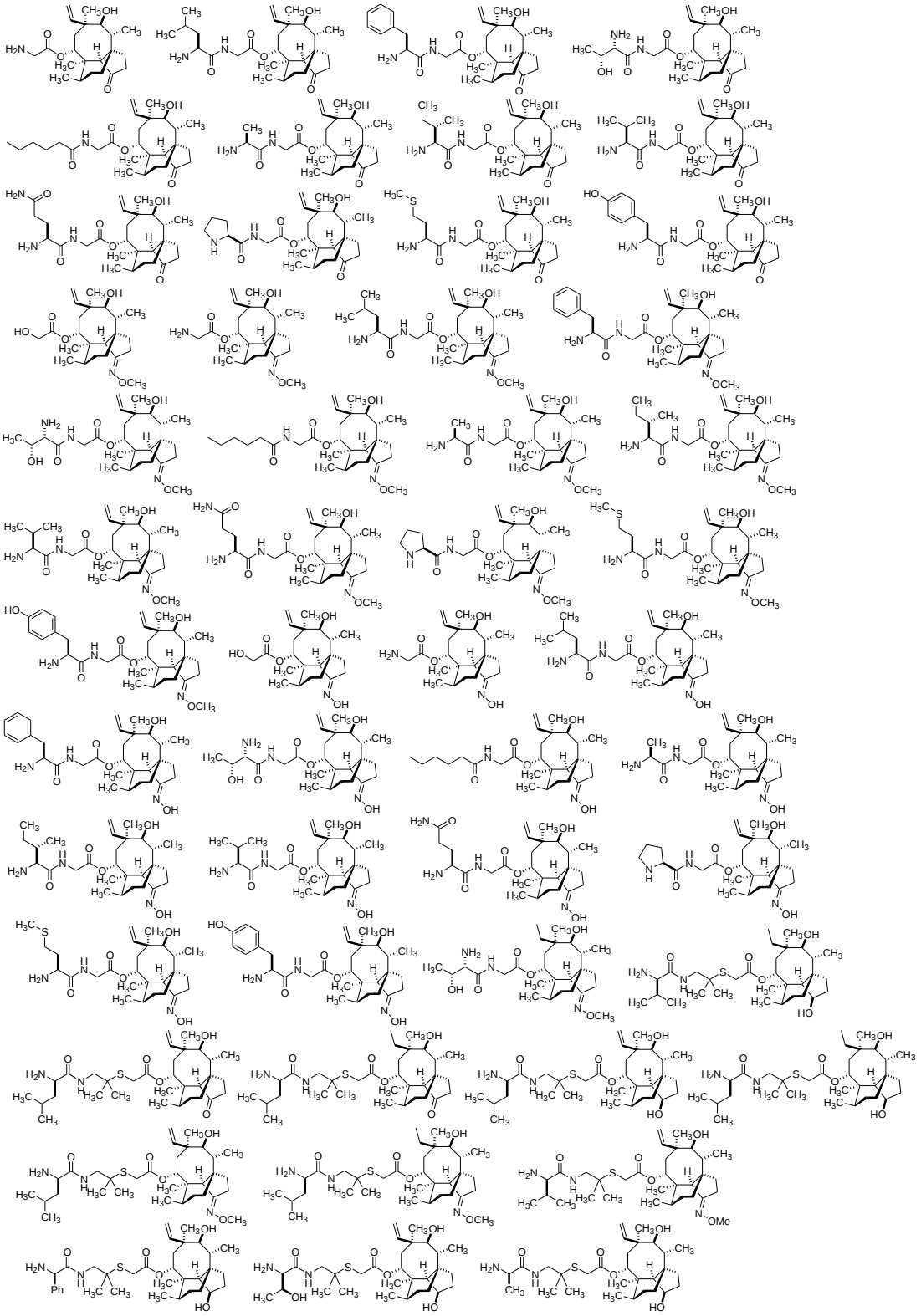
column: HYPERSIL GOLD™ 12 μm 175 Å 250 x 10 mm

solvents: a gradient elution of 25 : 75 to 55 : 45 MeOH : H_2O over 20 min then 55 : 45 MeOH : H_2O

flow rate: 2.0 mL/min

UV: 220 nm

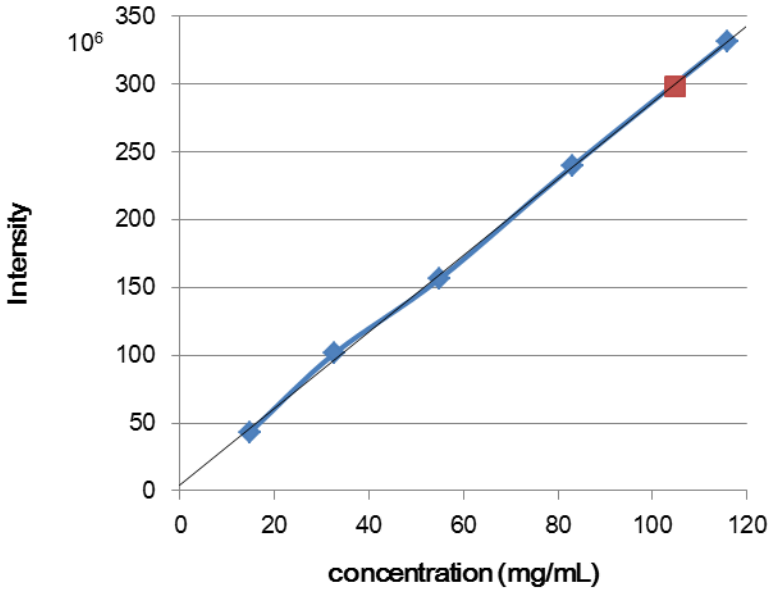
Compound library of pleuromutilin derivatives screened in this program



Determination of water-solubility of 1

A suspension of **1** (10.0 mg) in H_2O (50 μL) was stirred for 24h, and the precipitate was separated by centrifugation at 10,000 xg for 5 min. The upper solution (5 μL) was analyzed via C18 reverse-phase HPLC [column: HYPERSIL GOLD™ (175 Å, 12 μm, 250 x 10 mm), solvents: a gradient elution of 25:75 to 55:45 MeOH : H_2O over 20 min then 55:45 MeOH : H_2O, flow rate: 2.0 mL/min, UV: 220 nm, retention time: 25 min]. The area of the peak for **1** was quantified. The concentrations were determined via the HPLC intensity-concentration curves [2,3,4].

Concentration of **1**



Bacterial strains and growth of bacteria

Mycobacterium tuberculosis ($H_{37}Rv$) were obtained through BEI Resources, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). *Mycobacterium smegmatis* (ATCC 607), *Mycobacterium bovis* (BCG), *Staphylococcus aureus* (ATCC 25923, BAA 2094, BAA 44, and BAA 1683), *Enterococcus faecium* (ATCC 349), *Enterococcus faecalis* (ATCC 19433), *Streptococcus pneumoniae* (ATCC 6301), *Streptococcus salivarius* (ATCC 6301), *Bacillus subtilis* (ATCC 6051), *Clostridium difficile* (ATCC 700057), *Klebsielle pneumoniae* (ATCC 8047), *Pseudomonas aeruginosa* (ATCC 27853), *Acinetobacter baumannii* (ATCC 19606), and *Escherichia coli* (ATCC 10798) were obtained from American Type Culture Collection (ATCC).

Bacterial Culture Conditions

A single colony of each *Mycobacterium* strain (*M. tuberculosis* $H_{37}Rv$, *M. bovis* (BCG)

and *M. smegmatis* ATCC 607) was obtained on a Difco Middlebrook 7H10 nutrient agar enriched with 10% oleic acid, albumin, dextrose and catalase (OADC for *M.tuberculosis*), albumin, dextrose and catalase (ADC for *M. smegmatis* and *M. bovis*). Tryptic Soy agar was used for all other strains except *C. difficile*. A single colony of *C. difficile* was obtained on modified reinforced clostridial agar that was prepared anaerobically.

Seed cultures of each Mycobacterium strain were obtained in Middlebrook 7H9 broth enriched with OADC (for *M.tuberculosis*), ADC (for *M. smegmatis* and *M. bovis*). Tryptic Soy broth was used for all other strains except *C. difficile*. *C. difficile* was cultured in modified reinforced clostridial broth that was prepared anaerobically in an anerobic chamber under 10% H_2, 5% CO_2, and 85% N_2 conditions. Flasks containing each bacterial strain was incubated to mid-log phase in their respective culture media, in a shaking incubator at 37 °C with a shaking speed of 200 rpm and cultured to mid-log phase (Optical density - 0.5). The optical density was monitored at 600 nm using a microplate reader (Biotek Synergy XT).

Minimum Inhibitory Concentration Assays

Microplate Alamar Blue Assay (MABA). These were performed according to the published protocol [5]. Bacterial cultures at 0.5 optical density, was treated with serial dilutions of inhibitors in aerobic conditions and incubated at 37 °C for 15 days for *M.tuberculosis* (H37Rv) and *M. bovis* (BCG). Incubation time was 48h for *M. smegmatis* (ATCC 607). All other bacteria were incubated for 24 h. After incubation, 20 μL of resazurin (stock-0.02%) was added and incubated on a shaking incubator at 37 °C for 4 h for *M.tuberculosis* and *M. bovis*. For all other bacteria 20 μL was added from a 0.08% resazurin stock solution and left for 1 h. The lowest concentration at which the color of resazurin was completely retained as blue was read as the MIC_{100} (Pink = Growth, Blue = No growth). The absorbance measurements were also performed using a Biotek Synergy XT (Winooski, VT, USA), 96 well plate reader at 570 nm and 600 nm. Assay plates for *C. difficile* was incubated for 24 h in the anaerobic conditions and the optical density was measured at the end of incubation and wells with no visible growth was considered MIC.

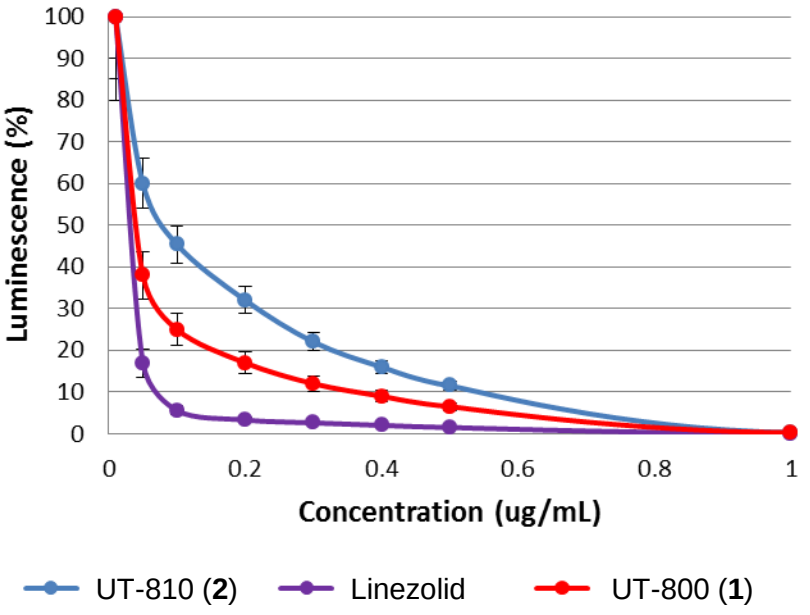
Luminescence-based Low-oxygen-recovery Assay (LORA). These assays were performed according to the reported procedures in the facility of Illinois TB Research Institute [6]. In brief, *M. tuberculosis* H37Rv cells were transformed by mixing at least 1 μg of the purified plasmid, pFCA-luxAB and incubating at room temperature for 30 min, followed by electroporation [7]. *M.tuberculosis* pFCA-luxAB strain cultured was diluted in Middlebrook 7H12 broth, and sonicated for 15s. The cultures were diluted to obtain an A570 of 0.03 to 0.05 and 3,000 to 7,000 RLUs per 100 μl. Twofold serial dilutions of antimicrobial agents were prepared in black 96-well microtiter plates (100 μl), and 100 μl of the cell suspension was added. The microplate was placed under anaerobic conditions (oxygen concentration, less than 0.16%) by using an Anoxomat model WS-8080 (MART Microbiology) and three cycles of evacuation and filling with a mixture of

10% H_2, 5% CO_2, and 85% N_2. Incubation was continued for 10 days, and transferred to an ambient gaseous condition (5% CO_2-enriched air) incubator for a 28h "recovery." 100 μl culture was transferred to white 96-well microtiter plates for determination of luminescence.

Entry	Compounds	MIC (μg/mL) against *Mtb*		IC_{50} (μg/mL) against Vero cells
		MABA[a]	LORA[b]	
1	UT-800	0.83	1.20	10.45
2	UT-810	1.56	1.98	13.96
3	UT-815	0.78	1.04	5.26
4	UT-522	3.06	3.46	25
5	UT-490	6.25	-	45.3
6	UT-820	5.81	2.76	5
7	UT-825	8.18	-	3.13
8	UT-910	12.5	-	5.26
9	Valnemulin	3.13-5.60	3.56	15.59
10	RIF	0.01-0.2	0.05	102.3
11	INH	0.57	50.24	>200
12	Tunicamycin	12.5	-	0.18

Table S 1 Anti-*Mtb* activity and cytotoxicity of pleuromutilin analogs
[a]The microplate alamar blue assay method was used; [b]Low oxygen recovery assay.

Transcription/Translation Coupled Assay. Transcription/translation coupled assays were performed using commercially available *E. coli* S30 circular DNA assay kit (L1020) purchased from Promega Corporation. The assay uses a control DNA template, pBESTluc™ Vector, containing the eukaryotic firefly luciferase gene positioned downstream from the tac promoter and a ribosome binding site. The protein product, luciferase was synthesized from 2 μg of pBESTluc™ DNA using 5 uL complete amino acid mixture, 15uL S30 extract (rNTPs, tRNAs, an ATP-regenerating system, IPTG and appropriate salts) and 20 uL premix provided with the assay kit, in a final reaction volume of 50 uL with nuclease free water. Inhibitor compounds were dissolved in DMSO. Assays were setup with a master mix consisting of S30 extract and water, amino acid mix, and the S30 Premix, followed by the addition of compound. Master mix was dispensed to microcentrifuge tubes and reactions were initiated by the addition of DNA template. Reaction mixture was vortexed gently, centrifuged for 5 sec and incubated at 37 ^oC for 60 min. Reactions were stopped by placing tubes in ice bath for 5 min. and diluted in two-fold dilution series using 50 uL of luciferase dilution reagent (Promega Corporation) containing luciferase assay substrate, and transferred to 96 well white plate. The luminescence readout was taken immediately on a luminescence reader according to the manufacturer's instructions.



(Promega Corporation)

Cytotoxicity Assays

Selected molecules were tested for cytotoxicity (IC_{50}) in Vero cells *via* a MTT colorimetric assay. Vero cell line was cultured in Complete eagle`s minimum essential growth medium (EMEM) containing L-glutamine, sodium pyruvate, minimum essential amino acids, penicillin-streptomycin and 10% fetal bovine serum. After 72h of exposure of molecules to this cell line at concentrations ranging from 0.78 to 200 μg/mL, the culture medium was changed to complete EMEM without phenol red before addition of yellow tetrazolium dye; MTT. Viability was assessed on the basis of cellular conversion of MTT into a purple formazan product. The absorbance of the colored formazan product was measured at 570 nm by BioTek Synergy HT Spectrophotometer.

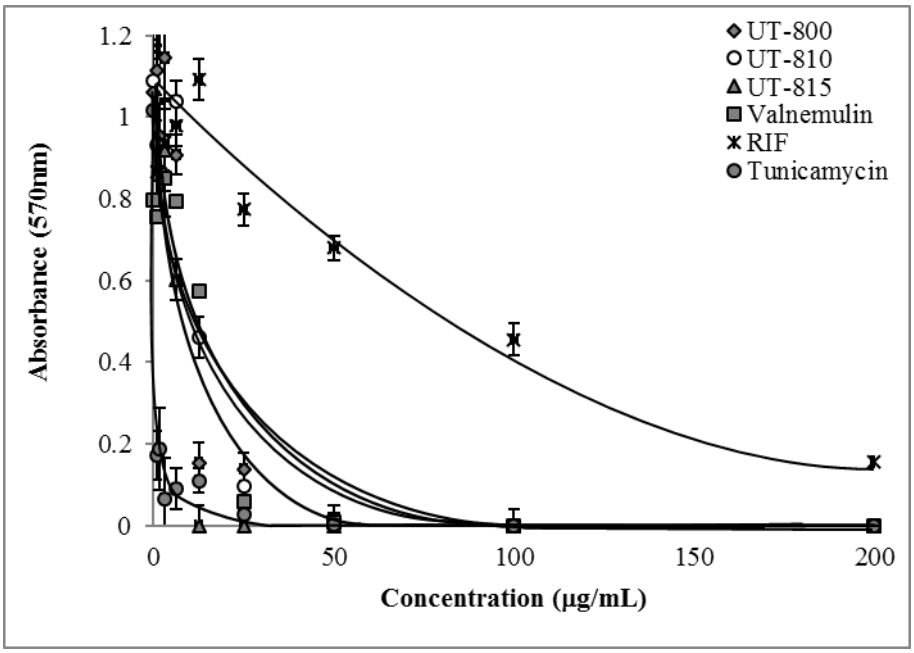


Figure S 1 Cytotoxicities (IC_{50}) of selected compounds in Vero monkey kidney cells.

Entry	Bacteria	MIC (μg/mL)[a]															
		UT-800 (**1**)		UT-810 (**2**)		UT-815 (**3**)		Valnemulin		Rifampicin		Isoniazid		Capreomycin		Linezolid	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	*M. tuberculosis* $H_{37}Rv$	-	0.83	-	1.46	-	0.77	3.13	6.25	0.20	0.01	0.04-0.10	0.57	3.6	3.13	0.60	1.61
2	*M. smegmatis* ATCC 607	6.25	25	6.25	>25	6.25	25	12.5	25	>25	>25	-	-	0.8	25	0.2	0.8
3	*S. aureus*	0.78	1.56	0.40	0.80	0.40	0.80	0.40	0.80	0.20	0.40	-	-	-	-	0.78	1.56
4	*S. aureus* ATCC 25923	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	-	-	>25	>25	>25	>25
5	*S. aureus* BAA 2094	>25	>25	>25	>25	>25	>25	>25	>25	>0.2	0.2	-	-	>25	>25	>25	>25
6	*S. aureus* BAA 1683	>25	>25	>25	>25	>25	>25	>25	>25	0.15	0.2	-	-	>25	>25	12.5	25
7	*S. aureus* BAA 44	>25	>25	>25	>25	>25	>25	>25	>25	6.25	25	-	-	>25	>25	>25	>25
8	*E. faecalis* ATCC 349	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	-	-	>25	>25	>25	>25
9	*E. faecalis* ATCC 19433	6.25	12.5	12.5	25	12.5	25	6.25	12.5	0.78	1.56	-	-	>25	>25	12.5	25
10	*C. difficile* ATCC 700057	-	1.56	-	1.56	-	1.56	-	1.56	-	0.78	-	-	-	-	-	3.13
11	*S. pneumoniae* ATCC 6301	25	>25	25	>25	25	>25	12.5	>25	25	>25	-	-	>25	>25	25	>25
12	*K. pneumoniae* ATCC 12019	12.5	25	25	>25	25	>25	12.5	25	1.56	3.13	-	-	25	>25	25	>25
13	*A. baumannii* ATCC 19606	1.56	3.13	12.5	25	9.38	12.5	4.69	6.25	2.35	3.13	-	-	>25	>25	>25	>25
14	*E. coli ATCC* 10798	12.5	>25	12.5	>25	12.5	>25	12.5	>25	3.13	25	-	-	>25	>25	25	>25
15	*S. salivarius* ATCC 6301	6.25	>25	6.25	>25	0.4	>25	0.4	>25	0.2	>25	-	-	>25	>25	3.13	>25
16	*B. subtilis* ATCC 6051	25	>25	12.5	25	12.5	25	12.5	25	0.3	0.4	-	-	>25	>25	3.13	6.25

Table S2. Spectrum of activity of UT-800, UT-810 and UT-815

[a]The microplate alamar blue assay method was used.

Killing Effect against Intracellular *M. tuberculosis*

J774A.1 cells were seeded at 2.5 × 105 cells/well in 24-well dishes or 1 × 105 cells/well in 8-well chamber slides and incubated overnight at 37 °C in DMEM. A transformant *M. tuberculosis* CDC1551 expressing tdTomato was grown in 7H9 Middlebrook medium supplemented with OADC. The *M. tuberculosis* cells were harvested at an optical density of 0.5, washed and re-suspended in saline. J774A.1 cells were maintained in cell culture medium and were infected by *M. tuberculosis* (106 bacteria in 0.2 mL of media): a multiplicity of infection (MOI) of ≈10 (bacteria/cell). The extracellular bacteria were removed by washing with PBS. The infected macrophages were treated with antibacterial agents at x2 and x4 MIC concentrations and the relative intensity of the fluorescence was measured [emission wavelength (581 nm)] via UV–vis spectroscopy in 24, 48, and 72 h for inhibition of intracellular bacterial growth. Surviving *M. tuberculosis* cells were confirmed by CFU method [8].

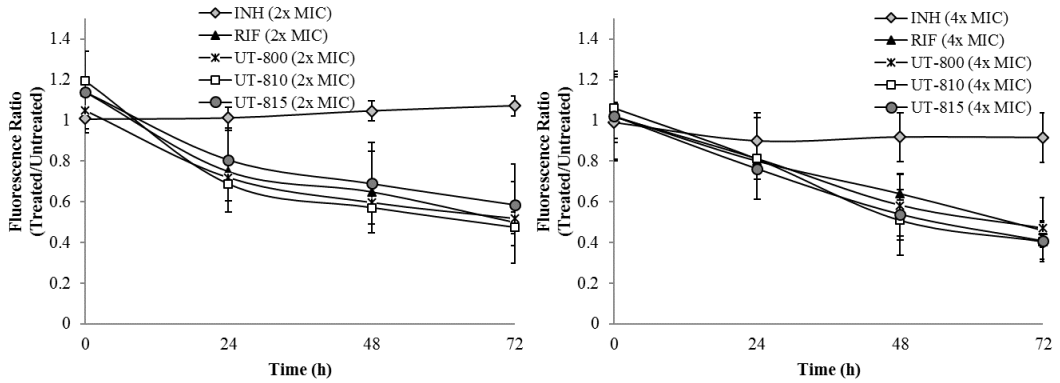


Figure S 2 Effect of UT-800, UT-810, UT-815 and representative TB drugs against intracellular Mtb CDC1551-tdTomato (a transformant Mtb CDC1551 containing tdTomato) in macrophages (J774A.1 cells)
A: Time-kill curve for intracellular Mtb at 2x MIC concentration; B: Time-kill curve for intracellular Mtb at 4x MIC concentration.

Kill-curve Graph: Determination of Colony Forming Units per Milliliter

M. tuberculosis H_{37}Rv cultures at mid-log phase (OD=0.5) were diluted to OD=0.25 and treated with inhibitor molecules at MIC, x2 MIC and x4 MIC. Each culture well was diluted 10, 100, 1000 and 10,000 fold every 24 h and 20 μ L from each dilution was plated on 7H10 agar plates supplemented with OADC enrichment. Plates were incubated for 15 days in a static incubator at 37 °C and colonies were counted.

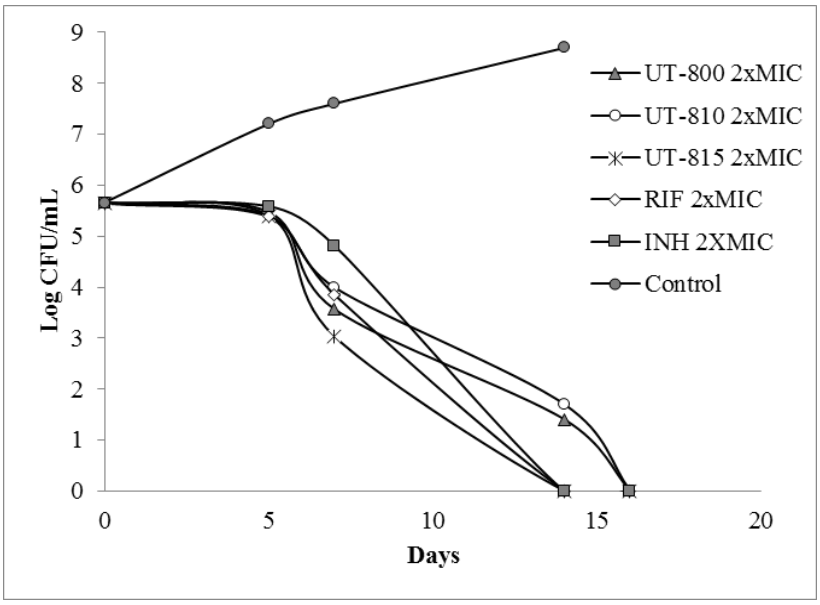


Figure S 3 Kill curve of selected compounds with activity against *Mycobacterium tuberculosis* $H_{37}Rv$

Microsomal Stability

Pooled Sprague-Dawley rat liver microsomes were purchased from Corning Life Sciences (Oneonta, NY, USA). Microsomes ((20 mg/mL) were thawed on ice and diluted using phosphate buffer (100 mM, pH: 7.4), resulting in a protein concentration of 1 mg/mL. Stock solutions (10 mg/L) of UT-800, UT-810, UT-815, valnemulin and verapamil (positive control) were prepared in DMSO (50%). A final concentration of 500 ng/mL was used for incubation with microsomes. NADPH (final concentration: 1 mM) was used as a co-factor. All the above solutions except NADPH were added to individual wells (12-well) in triplicate and were allowed to equilibrate for 5 min at 37°C. NADPH was then added. 50 µL aliquots in triplicate were drawn from the incubation mixture at 0, 5, 10, 20, 30, 45 and 60 min and immediately the reaction was quenched by addition of ice-cold methanol (4 volumes) [9]. The samples containing methanol was lyophilized to remove all volatiles. The residue was dissolved in 1N HCl aq. (10µL) and MeOH (40µL). The resulting solution (20µL) was injected to LC-MS. MS solvent 90:10 acetonitrile/0.05% formic acid in water. Flow rate: 0.5mL/min.

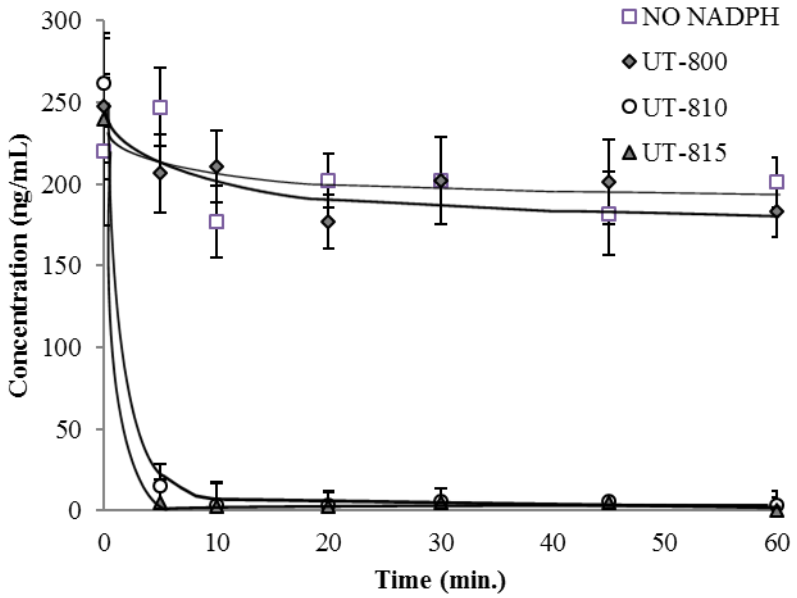


Figure S 4 ***In vitro*** **metabolic stability of pleuromutilin analogs (UT-800, UT-810, and UT-815)**
Verapamil was utilized as a control compound, and the half-life ($t_{1/2}$) of verapamil was determined to be 10 min

Prediction of Intestinal Permeability of UT-800 ***via*** **Caco-2 Permeability Assay**

The suitability of UT-800 for oral dosing was evaluated by using a bidirectional Caco-2 permeability assay by Cyprotex to predict *in vivo* intestinal permeability and to investigate drug efflux. This established *in vitro* model predicts the *in vivo* absorption of drugs across the gut wall by measuring the rate of transport across the Caco-2 cell line that resembles the intestinal epithelial cells [10]. UT-800 was added to the apical side of the membrane and the transport of the compound across the monolayer was monitored over a 2 h time period. The transport of UT-800 in the apical to basolateral direction (A-B) as well as the basolateral to apical direction (B-A) was assessed to determine an efflux ratio which provides an indicator as to whether UT-800 undergoes active efflux. The permeability coefficient (P_{app}) was determined to be 3.39 x 10^{-6} cm/s from A-B and 18.3 x 10^{-6} cm/s from B-A. Efflux ratio (P_{app}(B-A)/P_{app}(A-B)) was determined to be 5.44. Ranitidine was used as a low permeability control (P_{app} < 1) and warfarin was used as a high permeability control (P_{app} > 1). Talinolol, a known P-gp substrate, was screened as a control compound to confirm that the cells are expressing functional efflux proteins (P_{app}(B-A)/P_{app}(A-B) = 76.8). A P_{app} rate coefficient > 1 and efflux ratio apprx 5 indicates that UT-800 has permeability across the membrane with moderate levels of efflux (An efflux ratio greater than two indicates that drug efflux is occurring).

A

Compound	Test Concentration (μM)	Assay Duration (h)	Mean A → B P_{app} (10^{-6} cm/s)	Mean B → A P_{app} (10^{-6} cm/s)	Efflux Ratio
Ranitidine	10.0	2.00	0.237	1.14	5.00
Talinolol	10.0	2.00	0.0517	3.92	76.8
UT-800	10.0	2.00	3.39	18.3	5.44
Warfarin	10.0	2.00	35.2	28.0	0.794

B

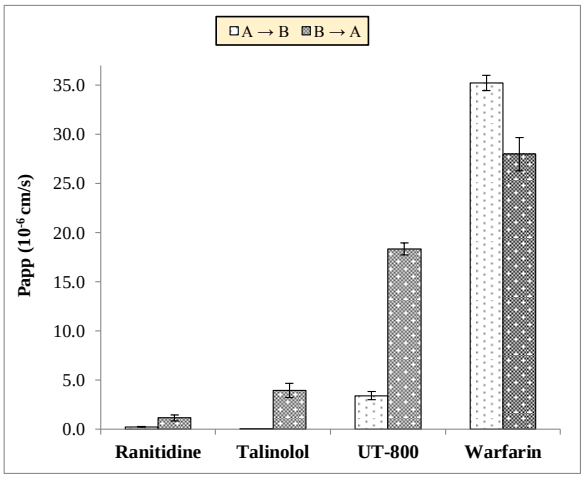


Figure S 5 ***In vitro*** **Caco-2 permeability assay to predict intestinal permeability of UT-800**

A: Experimental data for Caco-2 assay. B: UT-800 and control compounds were plotted against the permeability rate coefficient (P_{app} (10^{-6} cm/s).

Hemolysis Assay for UT-800, UT-810 and UT-815

Heparinized whole sheep blood was washed three times with saline (0.9% NaCl) and the pellet was suspended in PBS buffer (150mM NaCl, 5mM KCl, 10mM PBS, 2.5mM CaCl2, 10mM glucose, pH 7.4) [11]. 0.5 ml of 3% erythrocytes in PBS buffer, pH 7.4 was treated with UT-800, UT-810, UT-815, valnemulin, tunicamycin and aurantimycin A in concentrations ranging from 2-200 μg/mL). Triton X 100 was used as positive control in concentrations 0.01, 0.1 and 1%. As a negative control, 0.5% PEG:H_2O (2:1) (diluent for test compounds) was used in the same concentration as in samples (2.5 μL). The assay mixture was incubated at 37 ^{o}C for 2 h with gentle shaking. The assay mixture was centrifuged at 4700 rpm for 5 min and the released hemoglobin in the supernatant was determined at $OD_{540nm.}$ The percentage of hemolysis was calculated from the equation $(100 \times (A_{sample} - A_{negative\ control}) / (A_{positive\ control} - A_{negative\ control}))$.

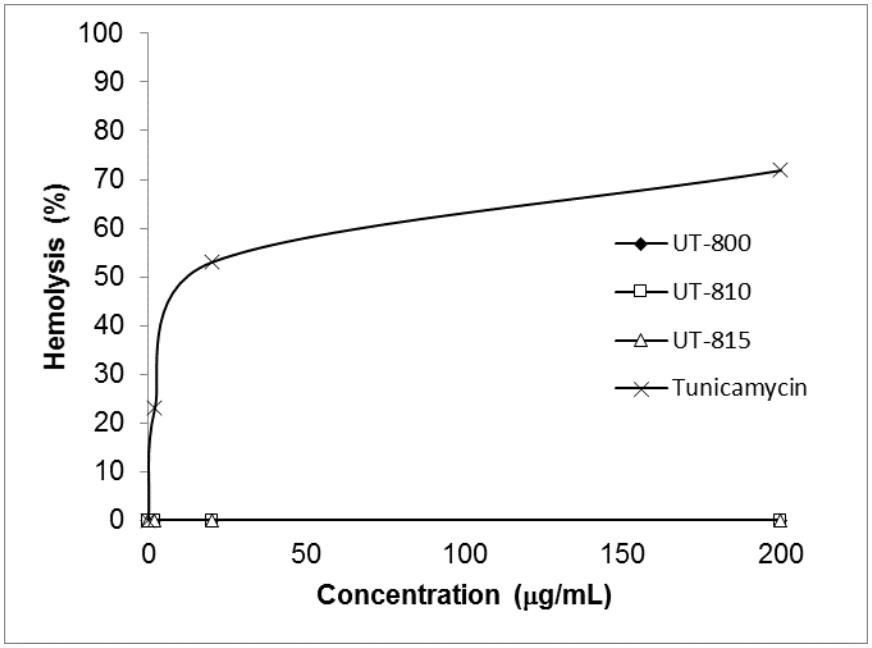


Figure S 6 Hemolysis assay using sheep blood

Triton X-100 was used as a positive control and displayed a 100 % hemolysis at 0.1 % v/v.

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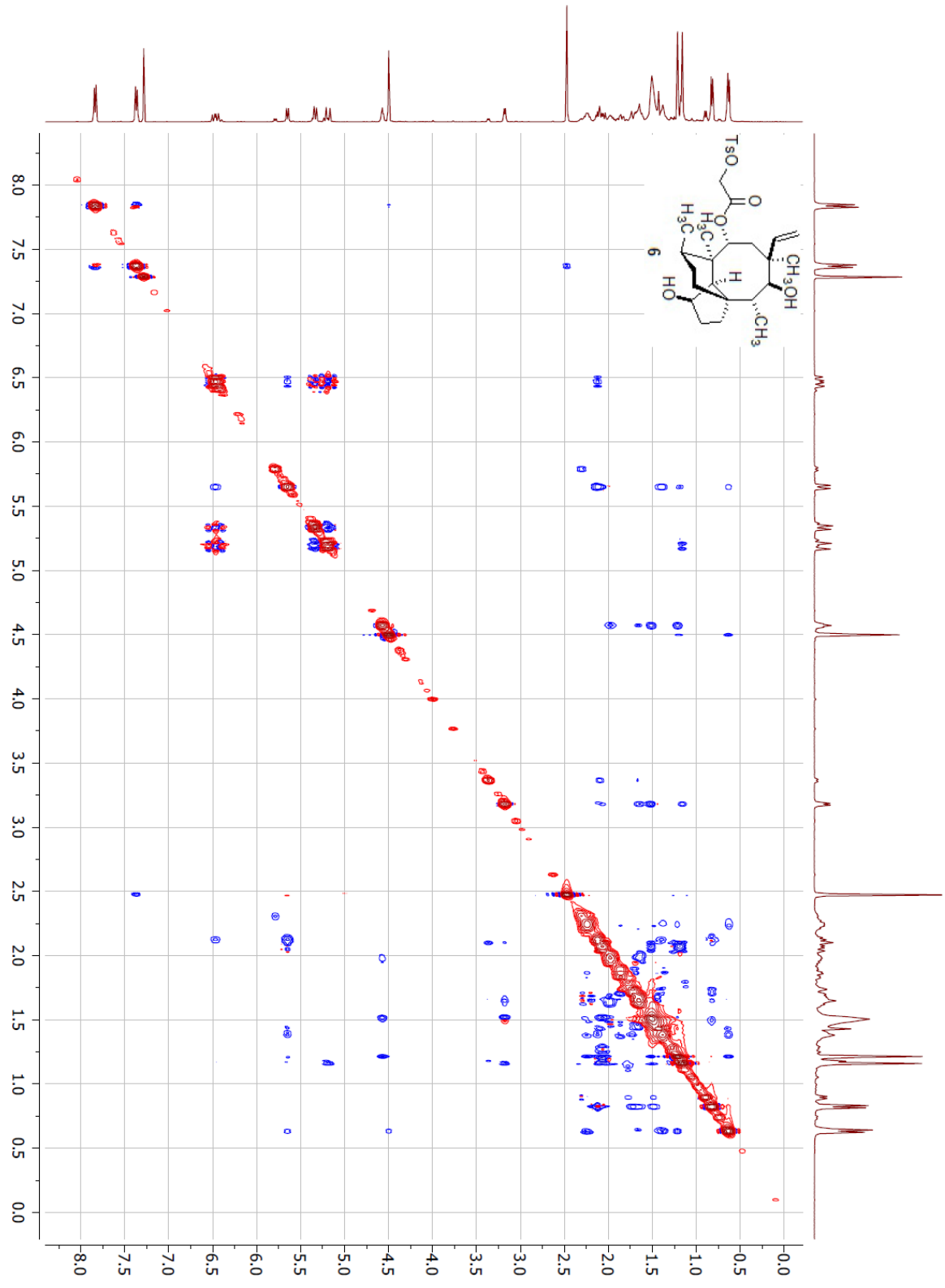
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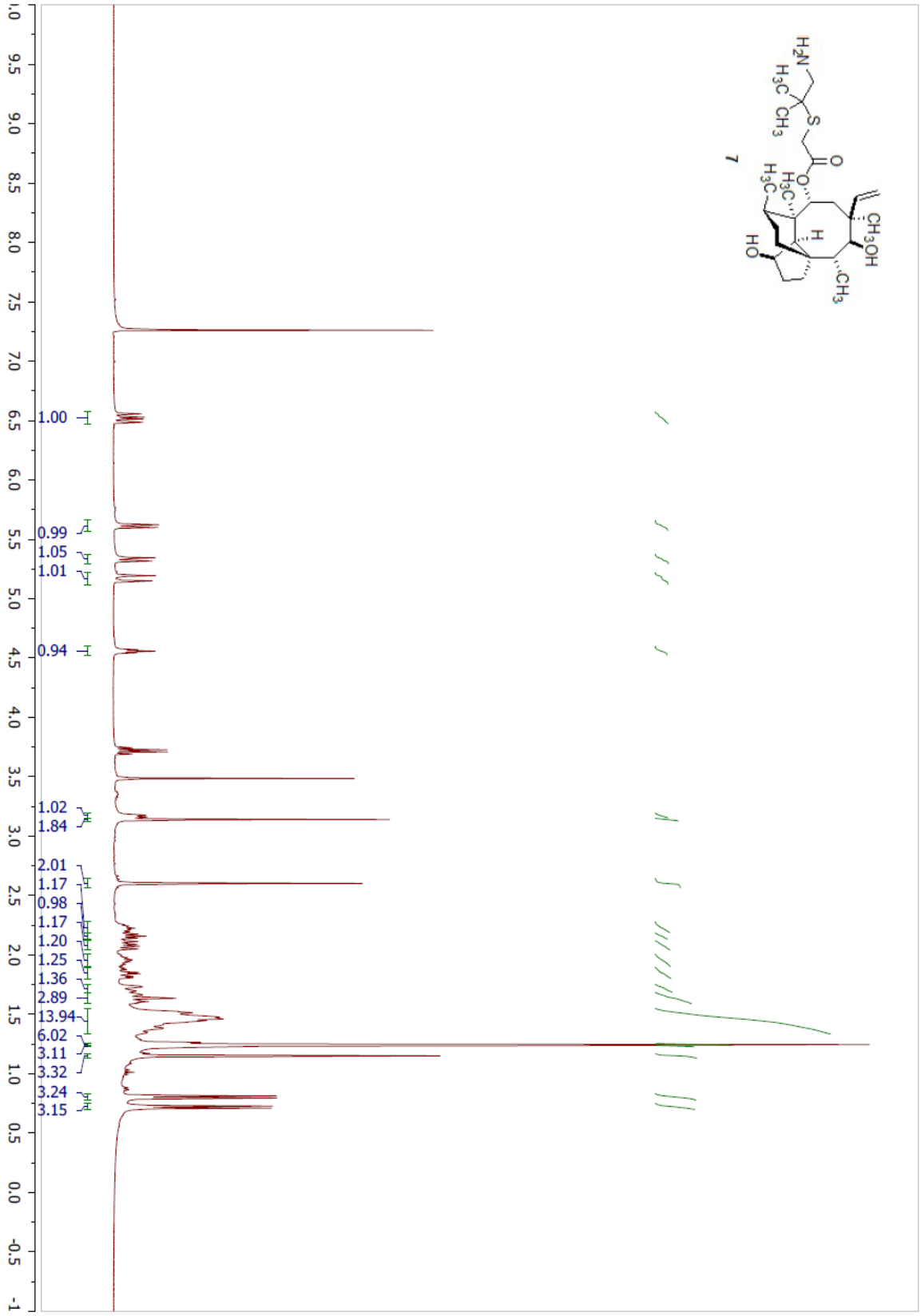
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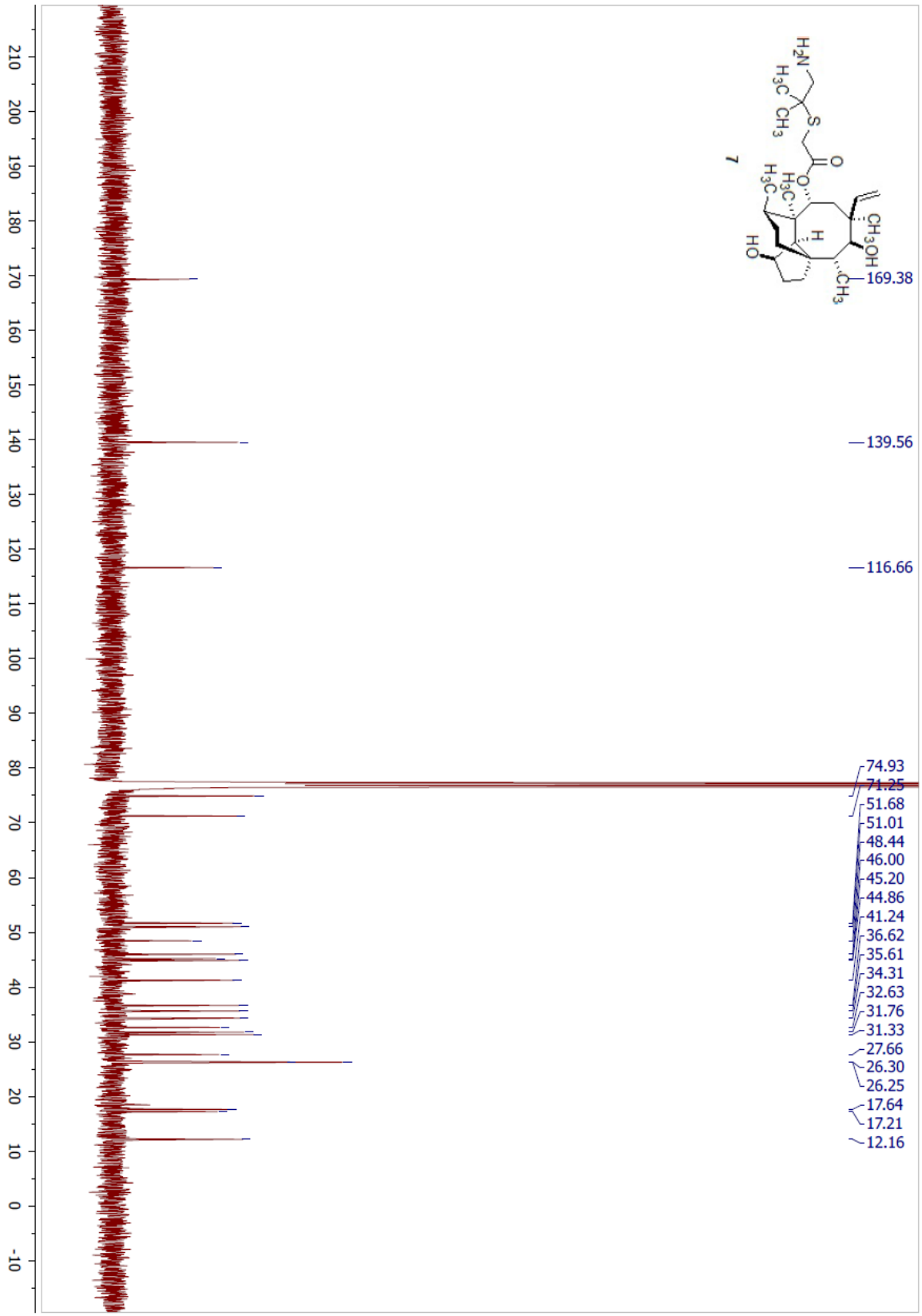
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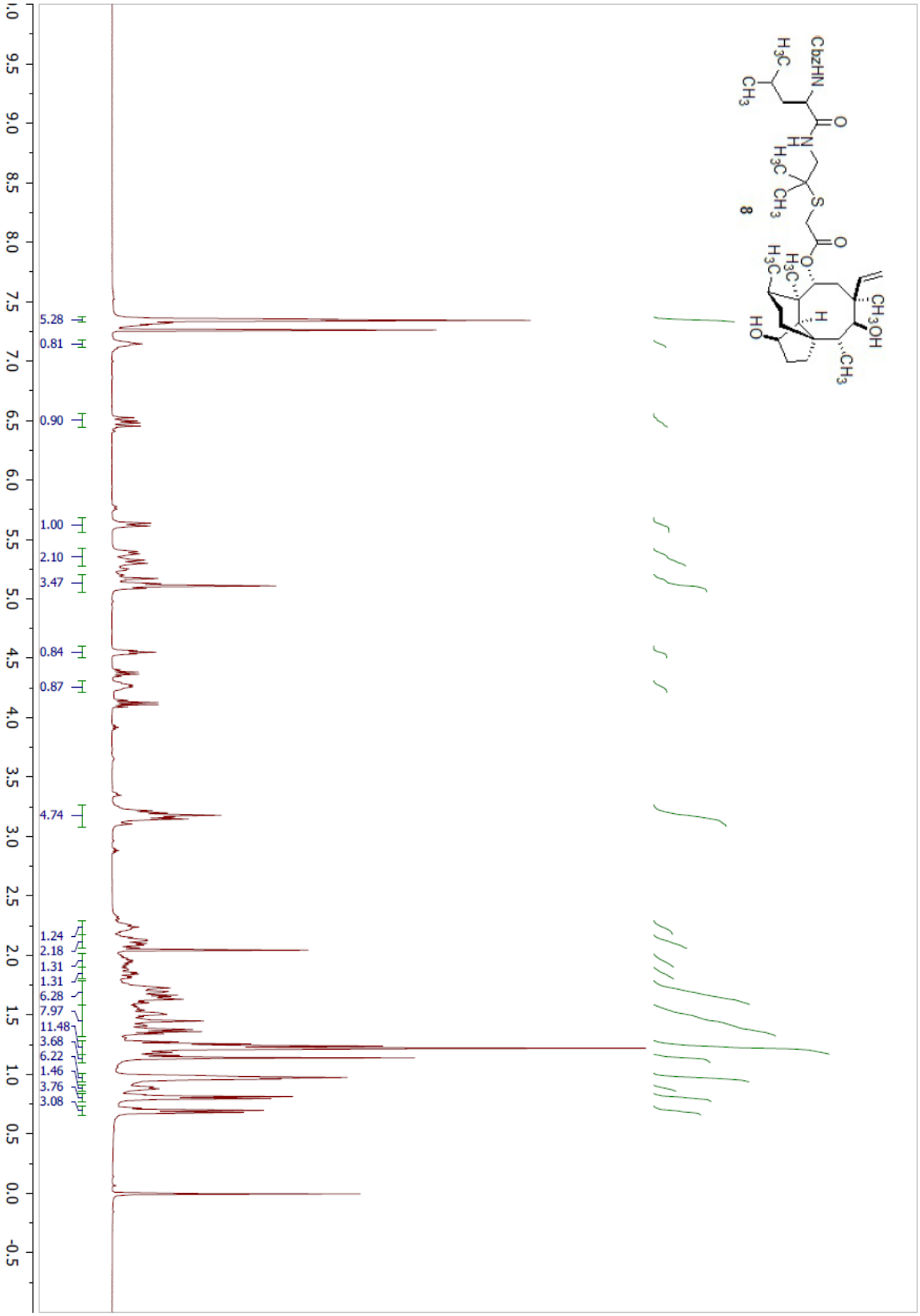
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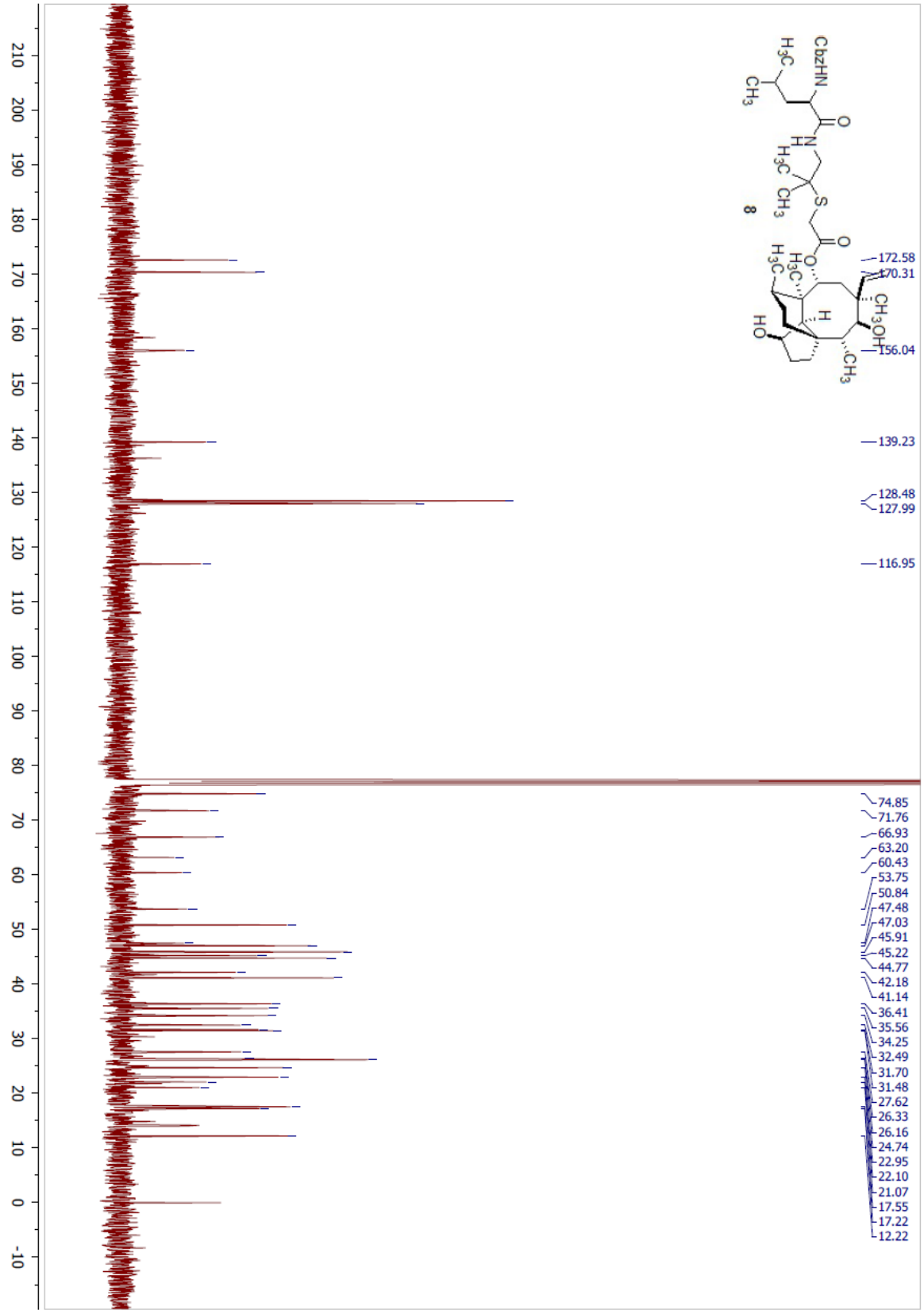
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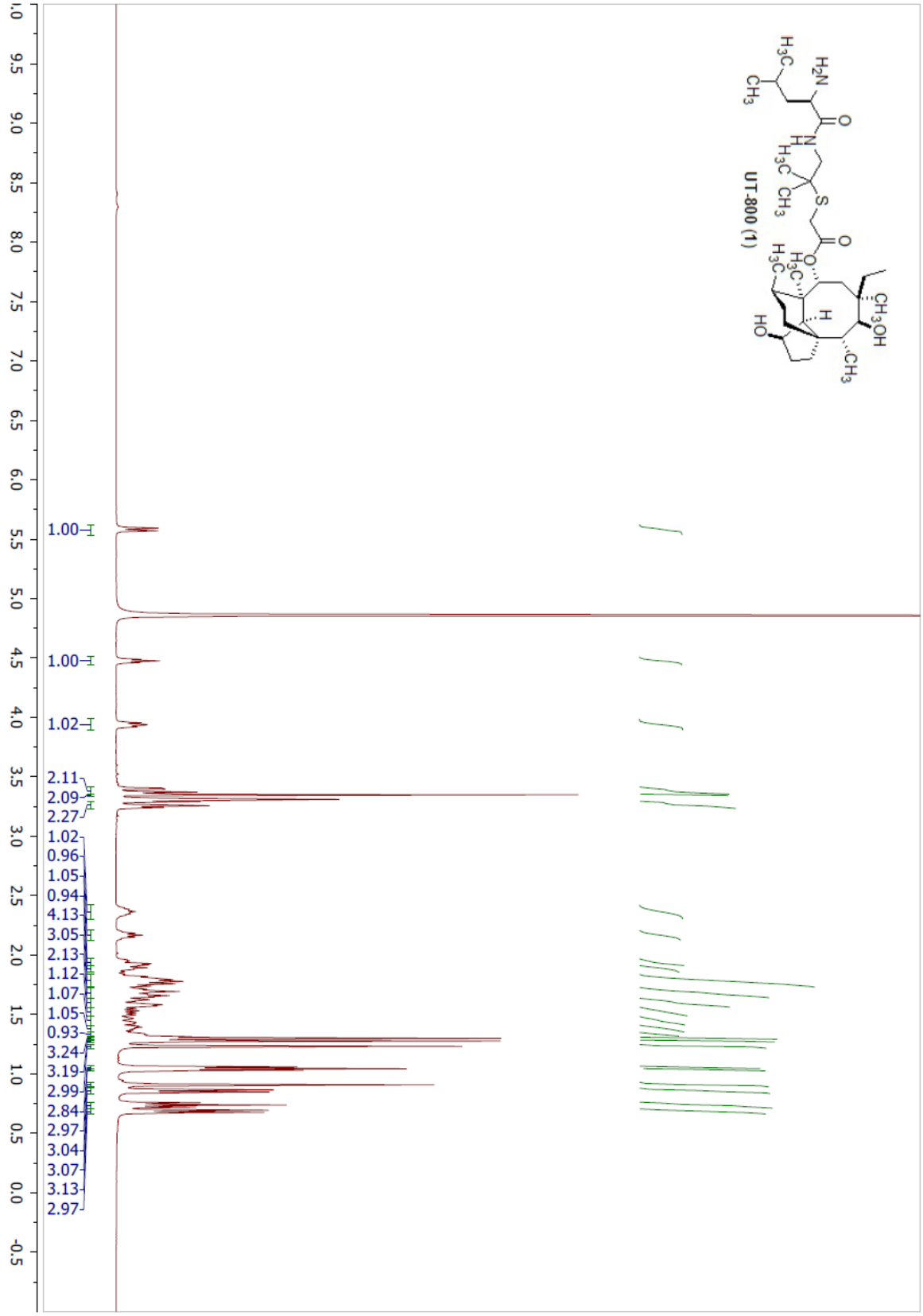
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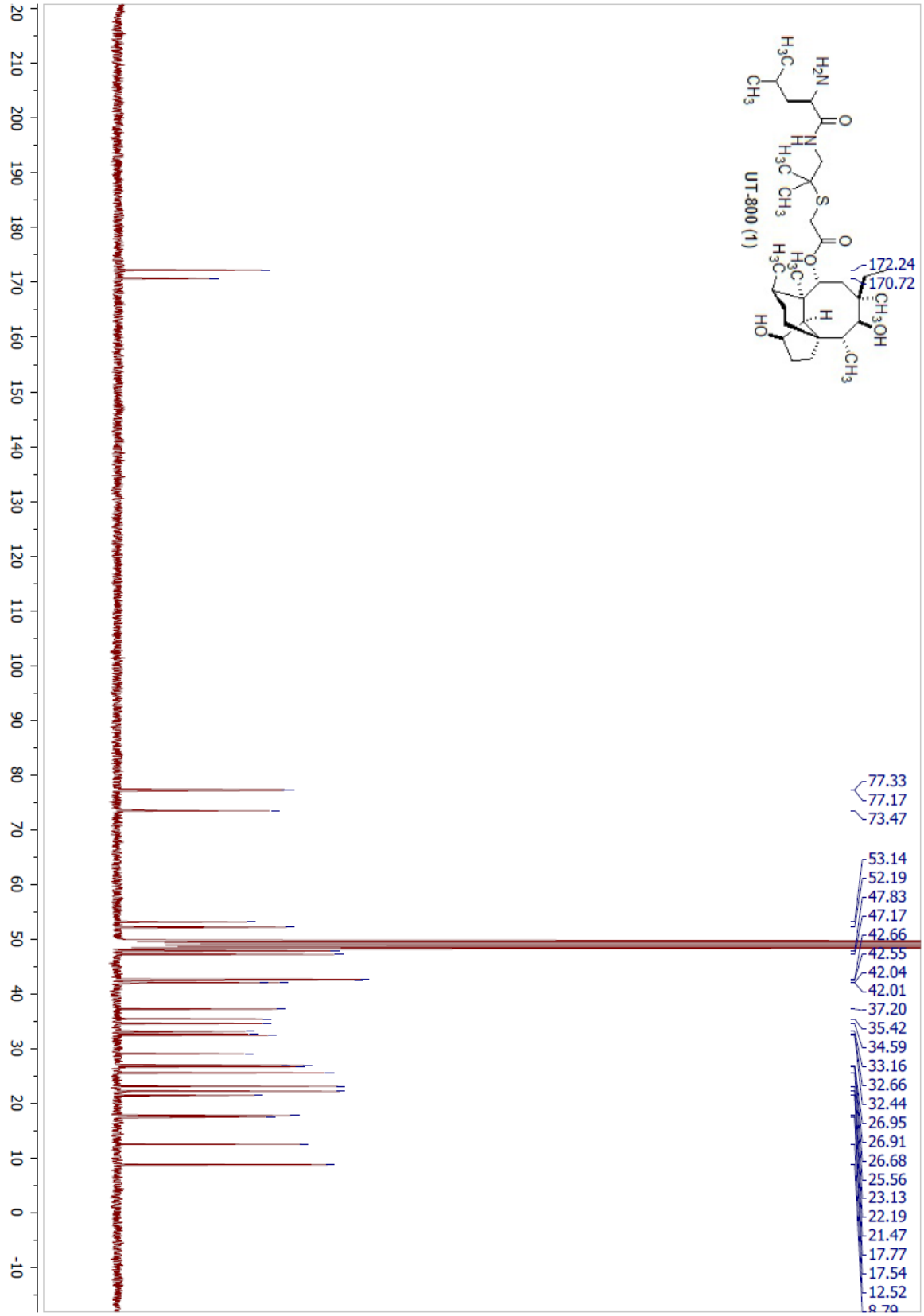
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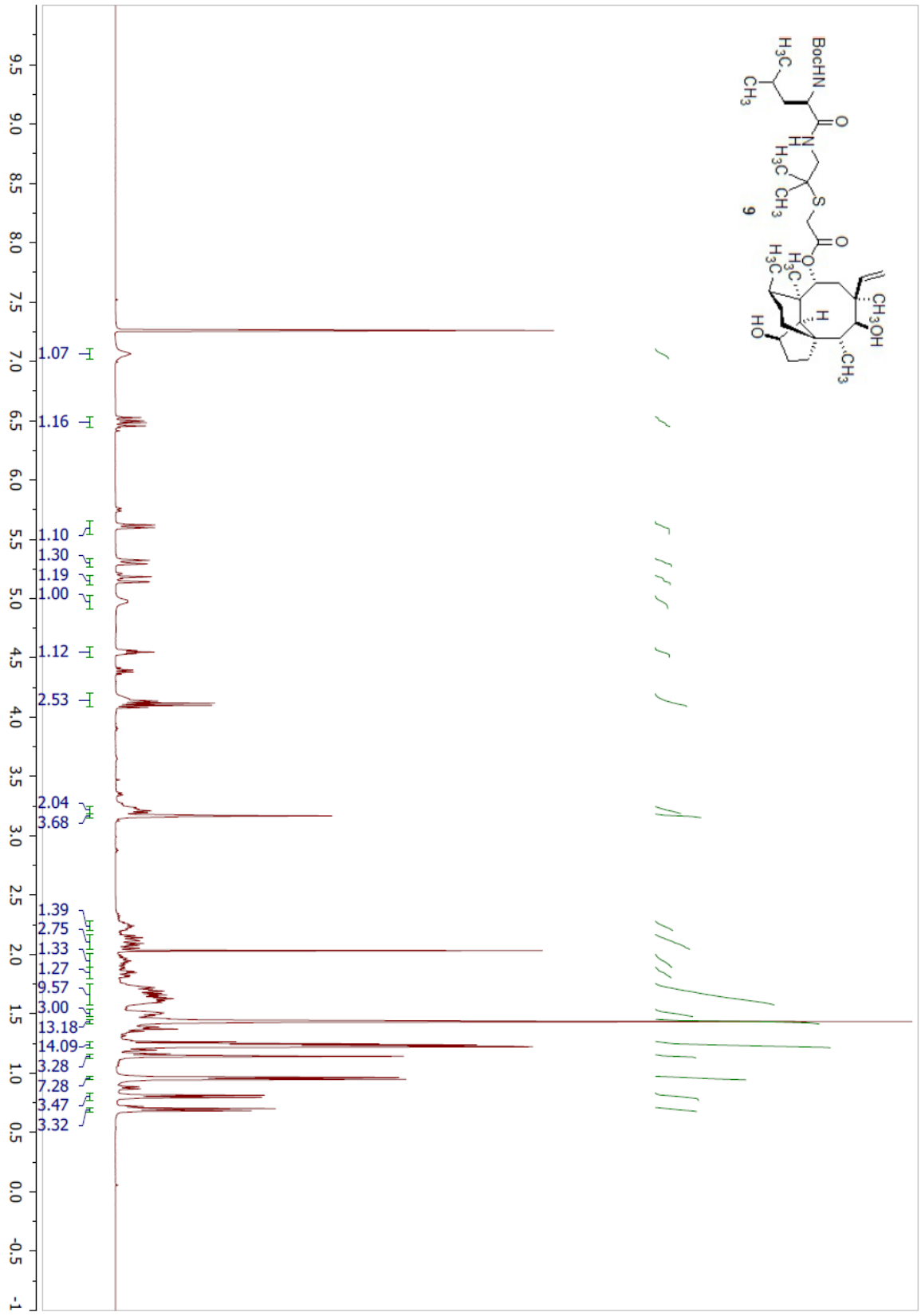
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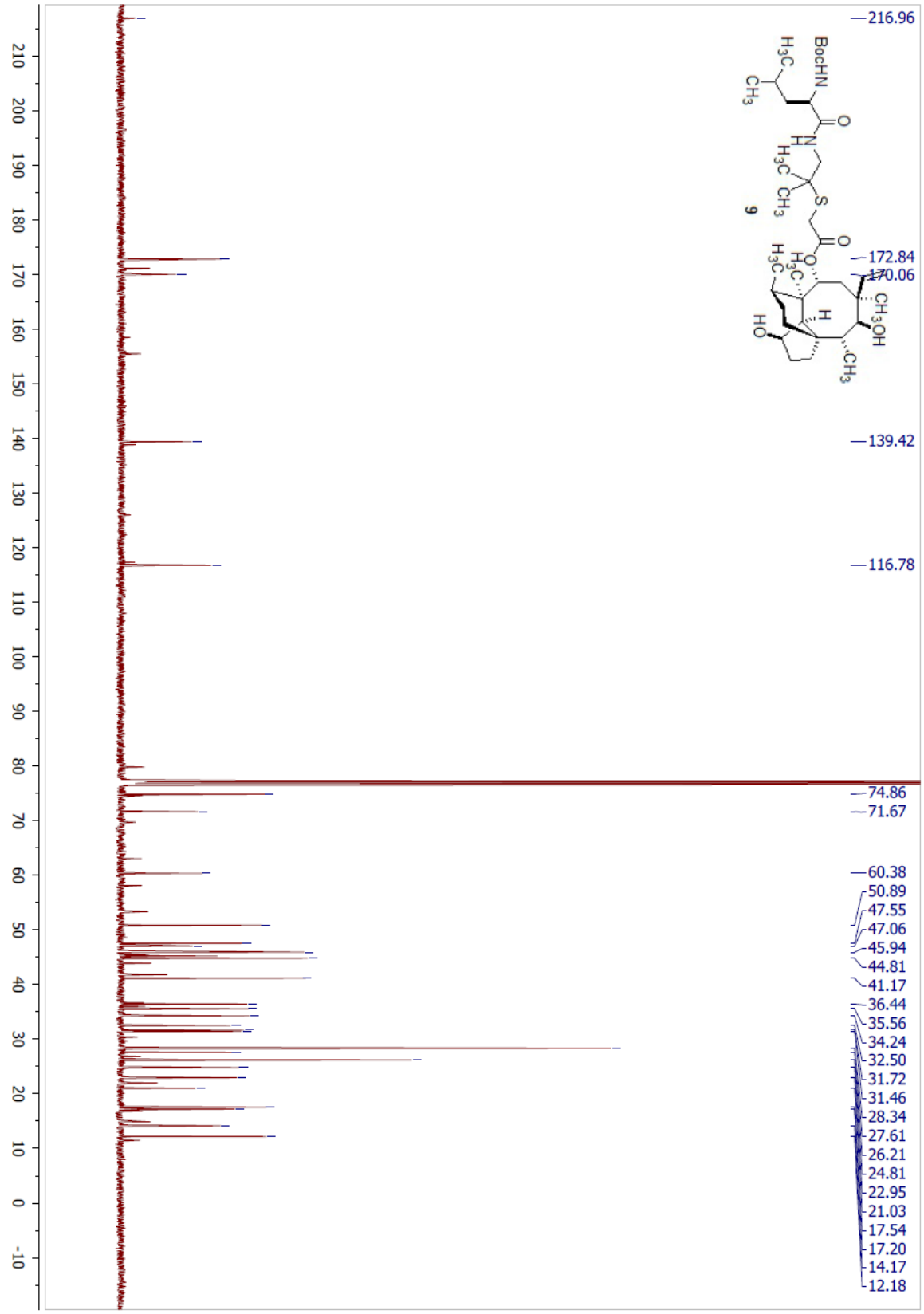
CH3OH
HO
CH3
9
1.07
1.16
1.10
1.30
1.19
1.00
1.12
2.53
2.04
3.68
1.39
2.75
1.33
1.27
9.57
3.00
13.18
14.09
3.28
7.28
3.47
3.32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172.84
170.06
139.42
116.78
74.86
71.67
60.38
50.89
47.55
47.06
45.94
44.81
41.17
36.44
35.56
34.24
32.50
31.72
31.46
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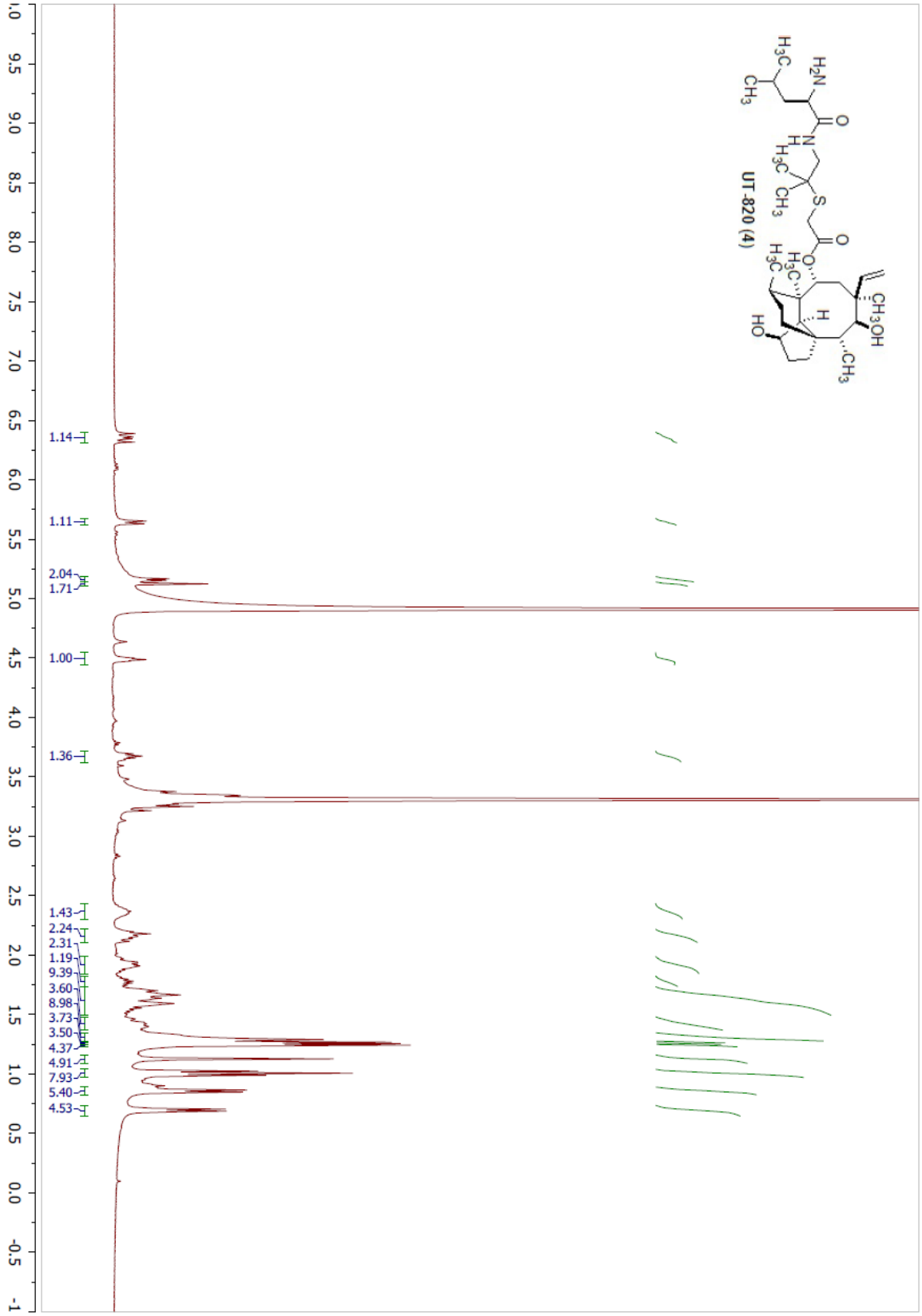
27.61
26.21
24.81
22.95
21.03
17.54
17.20
14.17
12.18
BocHN
9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1.14
1.11
2.04
1.71
1.00
1.36
1.43
2.24
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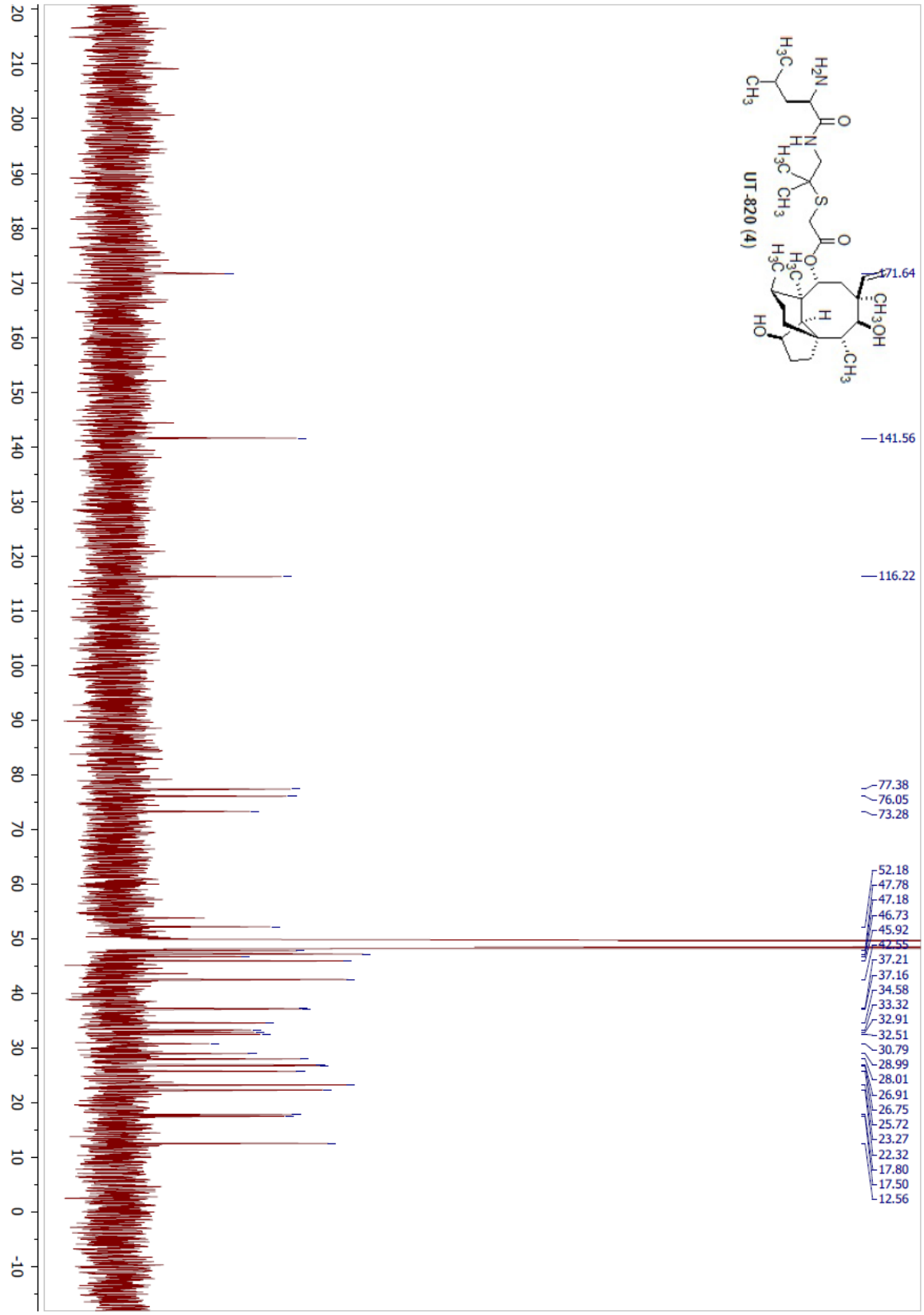
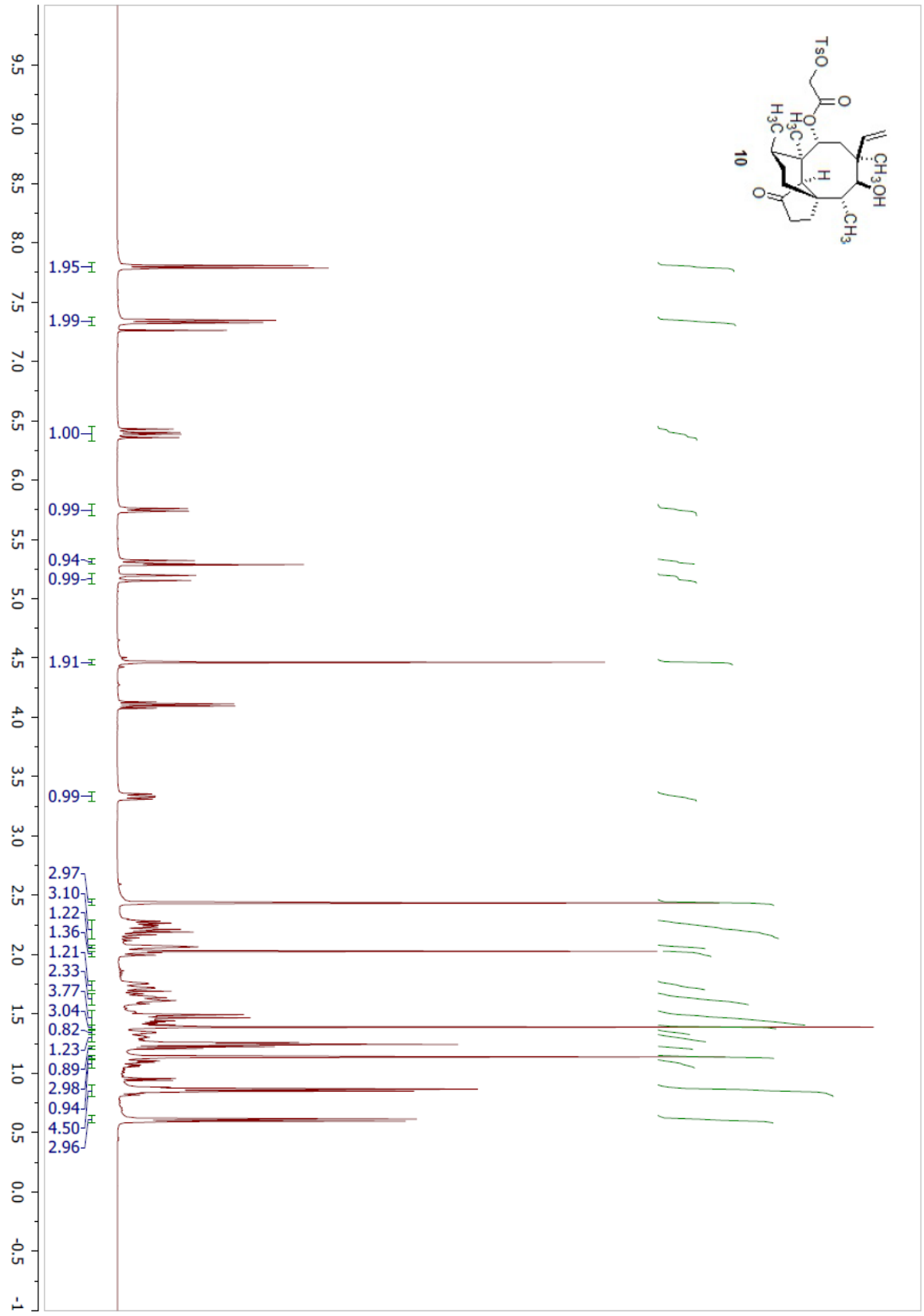
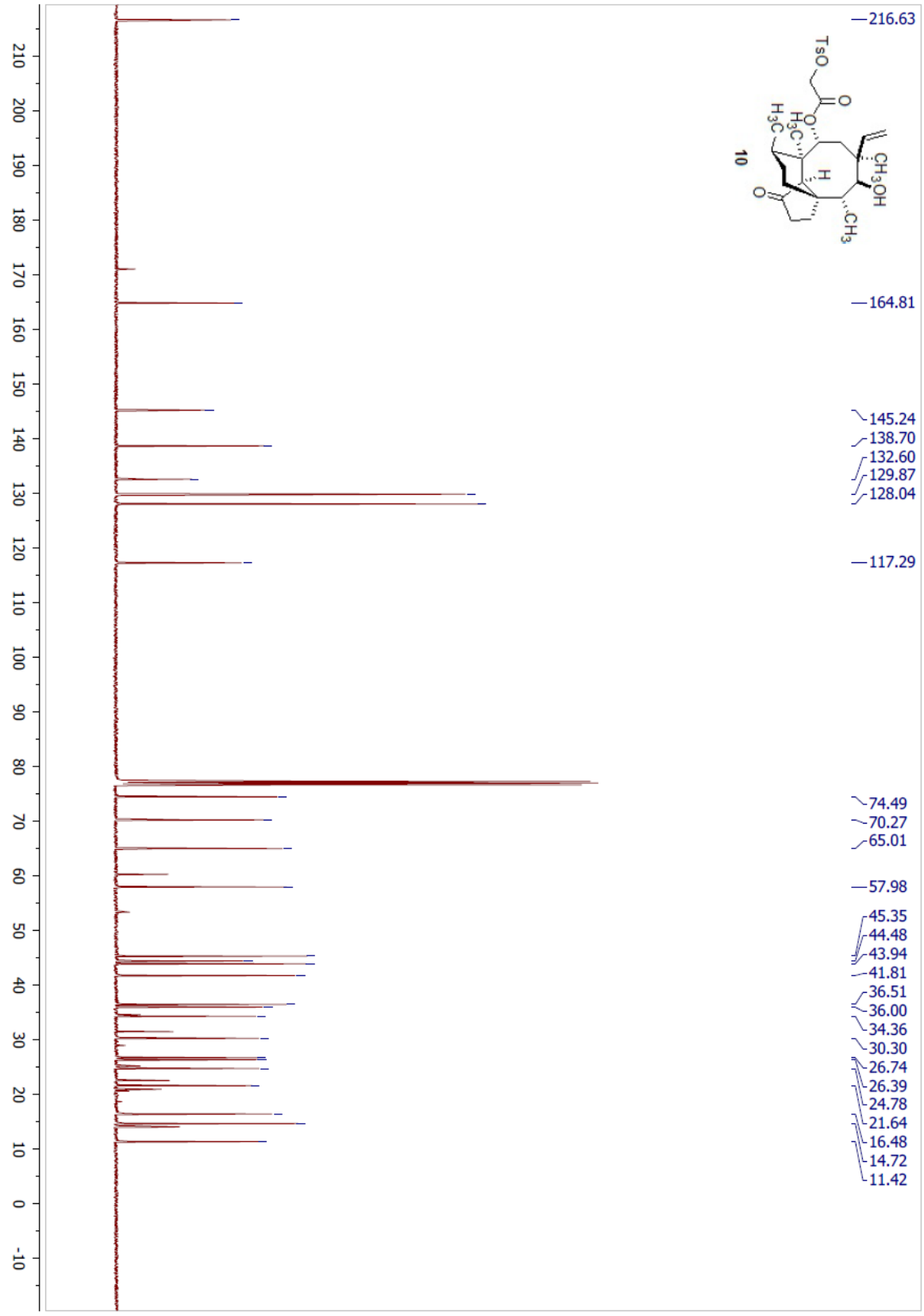
1.19
9.39
3.60
8.98
3.73
3.50
4.37
4.91
7.93
5.40
4.53


UT-820 (4)
171.64
141.56
116.22
77.38
76.05
73.28
52.18
47.78
47.18
46.73
45.92
42.55
37.21
37.16
34.58
33.32
32.91
32.51
30.79
28.99
28.01
26.91
26.75
25.72
23.27
22.32
17.80
17.50
12.56


TsO
O
O
H3C
H3C
H
CH3OH
CH3
O
10
1.95
1.99
1.00
0.99
0.94
0.99
1.91
0.99
2.97
3.10
1.22
1.36
1.21
2.33
3.77
3.04
0.82
1.23
0.89
2.98
0.94
4.50
2.96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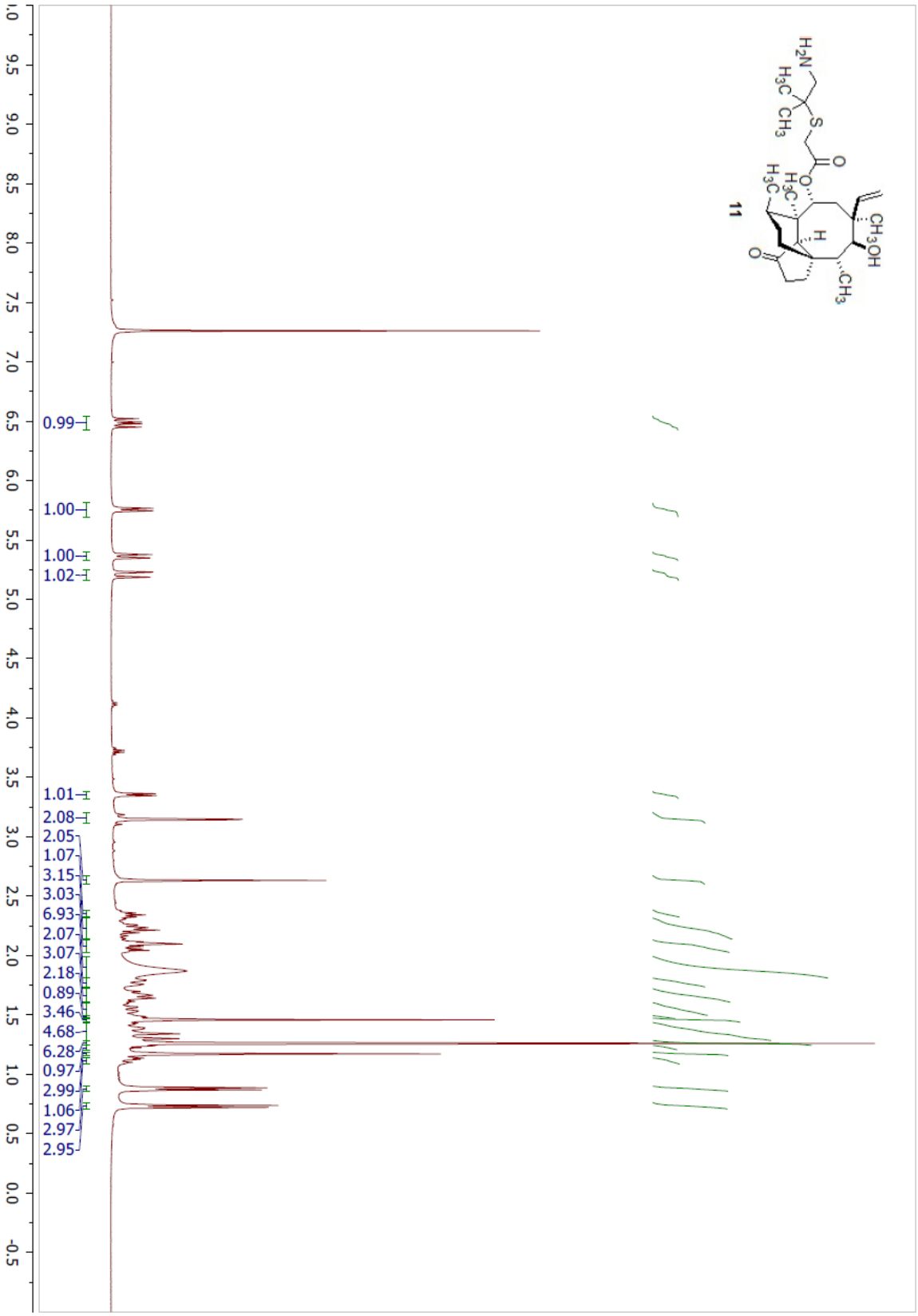
164.81
145.24
138.70
132.60
129.87
128.04
117.29
74.49
70.27
65.01
57.98
45.35
44.48
43.94
41.81
36.51
36.00
34.36
30.30
26.74
26.39
24.78
21.64
16.48
14.72
11.42
TsO
O
O
H3C
H3C
H
CH3OH
CH3
O
10


11
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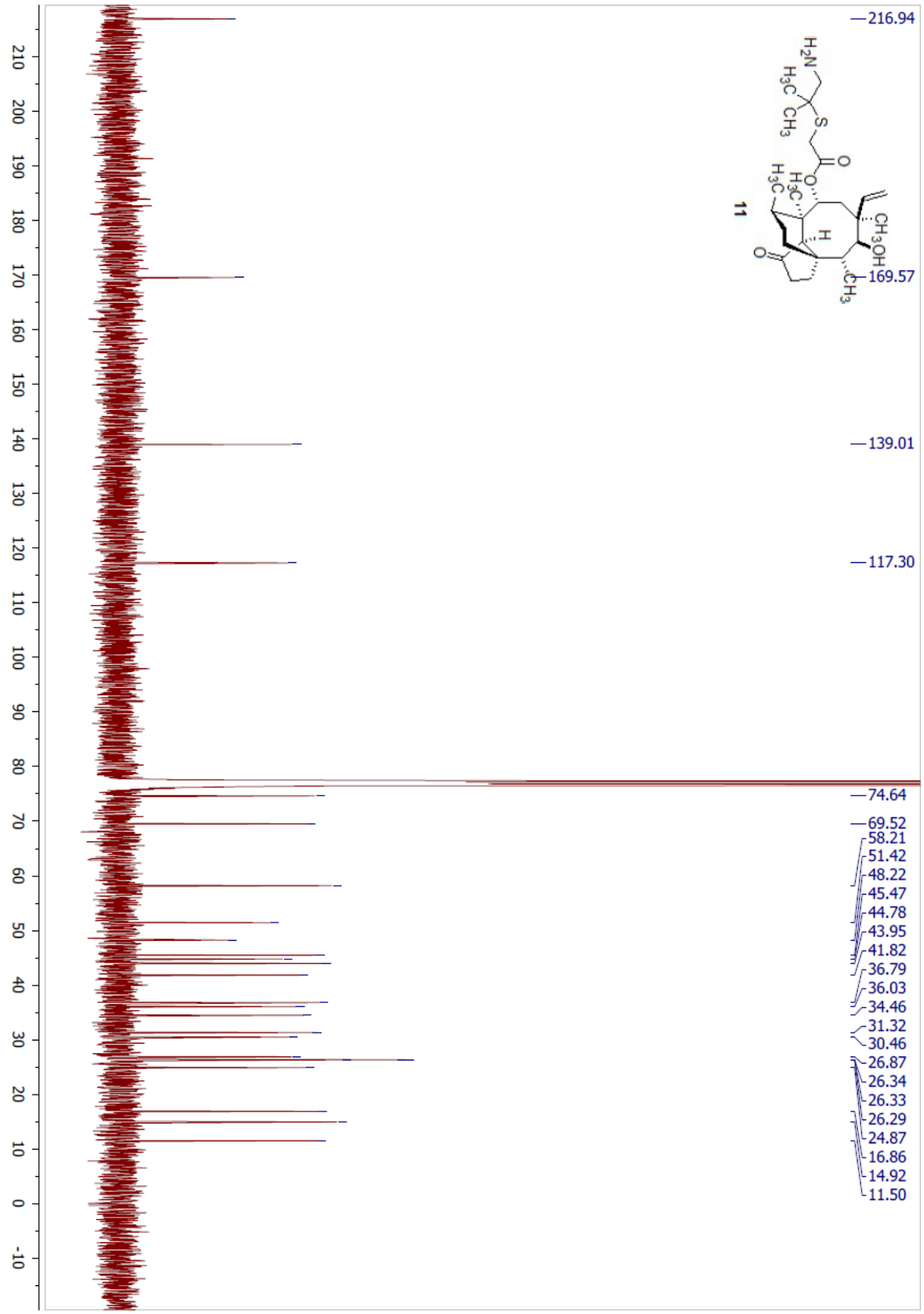
1.00
1.00
1.02
1.01
2.08
2.05
1.07
3.15
3.03
6.93
2.07
3.07
2.18
0.89
3.46
4.68
6.28
0.97
2.99
1.06
2.97
2.95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74.64
69.52
58.21
51.42
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44.78
43.95
41.82
36.79
36.03
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26.34
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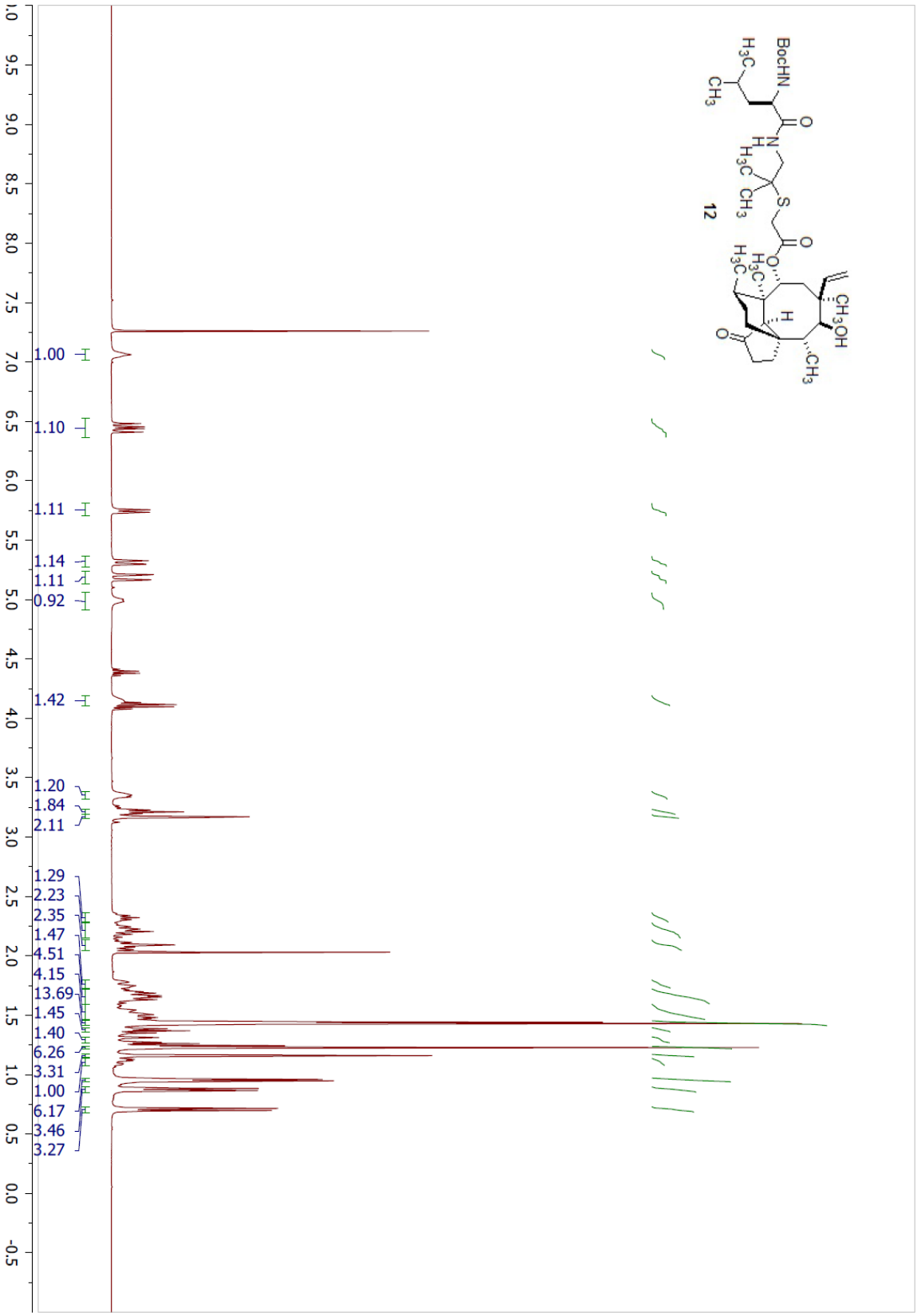
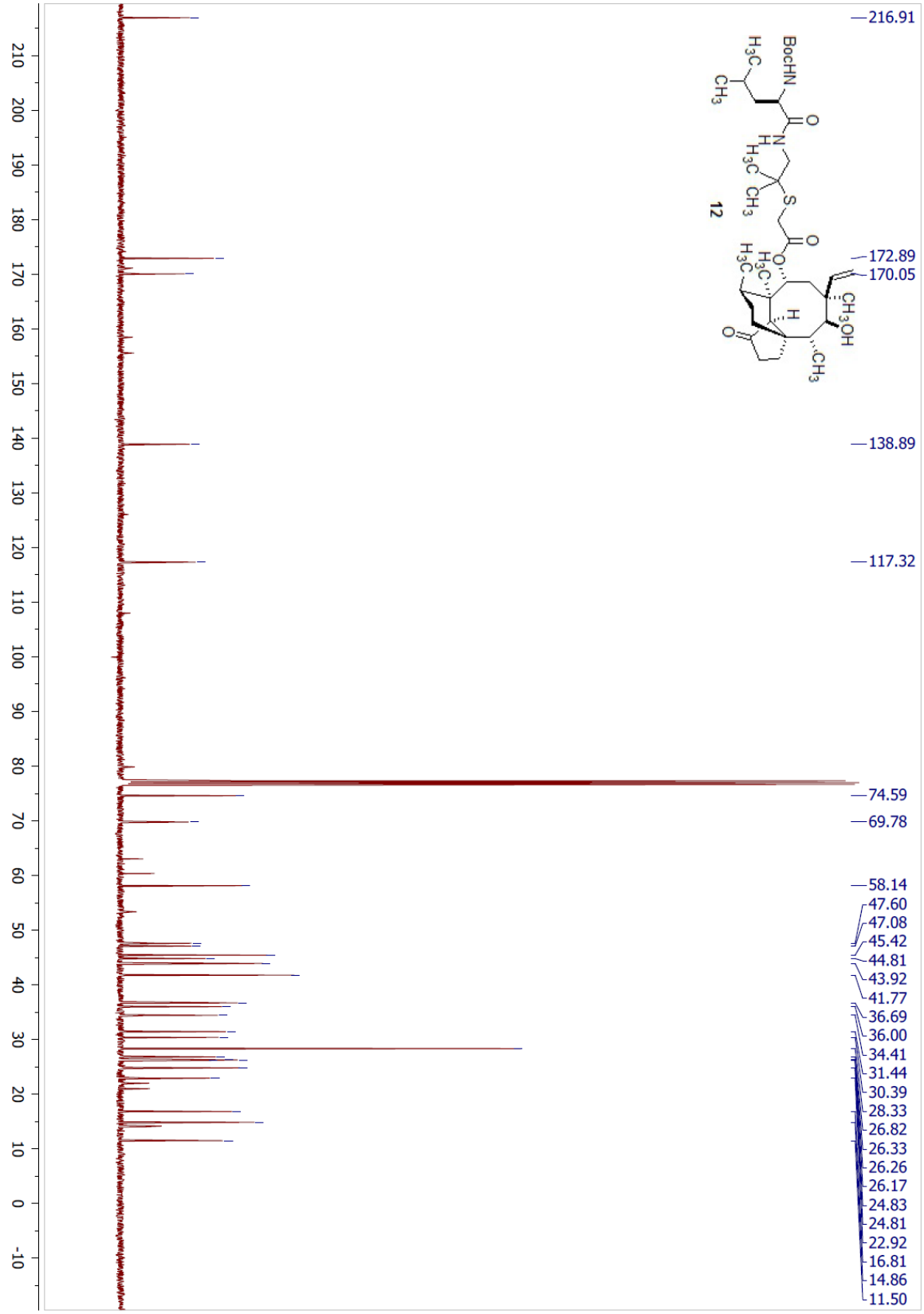
26.29
24.87
16.86
14.92
11.50
11


BocHN
H3C
CH3
O
N
H
H3C
CH3
S
O
O
H3C
H3C
O
H
CH3OH
CH3
O
12
1.00
1.10
1.11
1.14
1.11
0.92
1.42
1.20
1.84
2.11
1.29
2.23
2.35
1.47
4.51
4.15
13.69
1.45
1.40
6.26
3.31
1.00
6.17
3.46
3.27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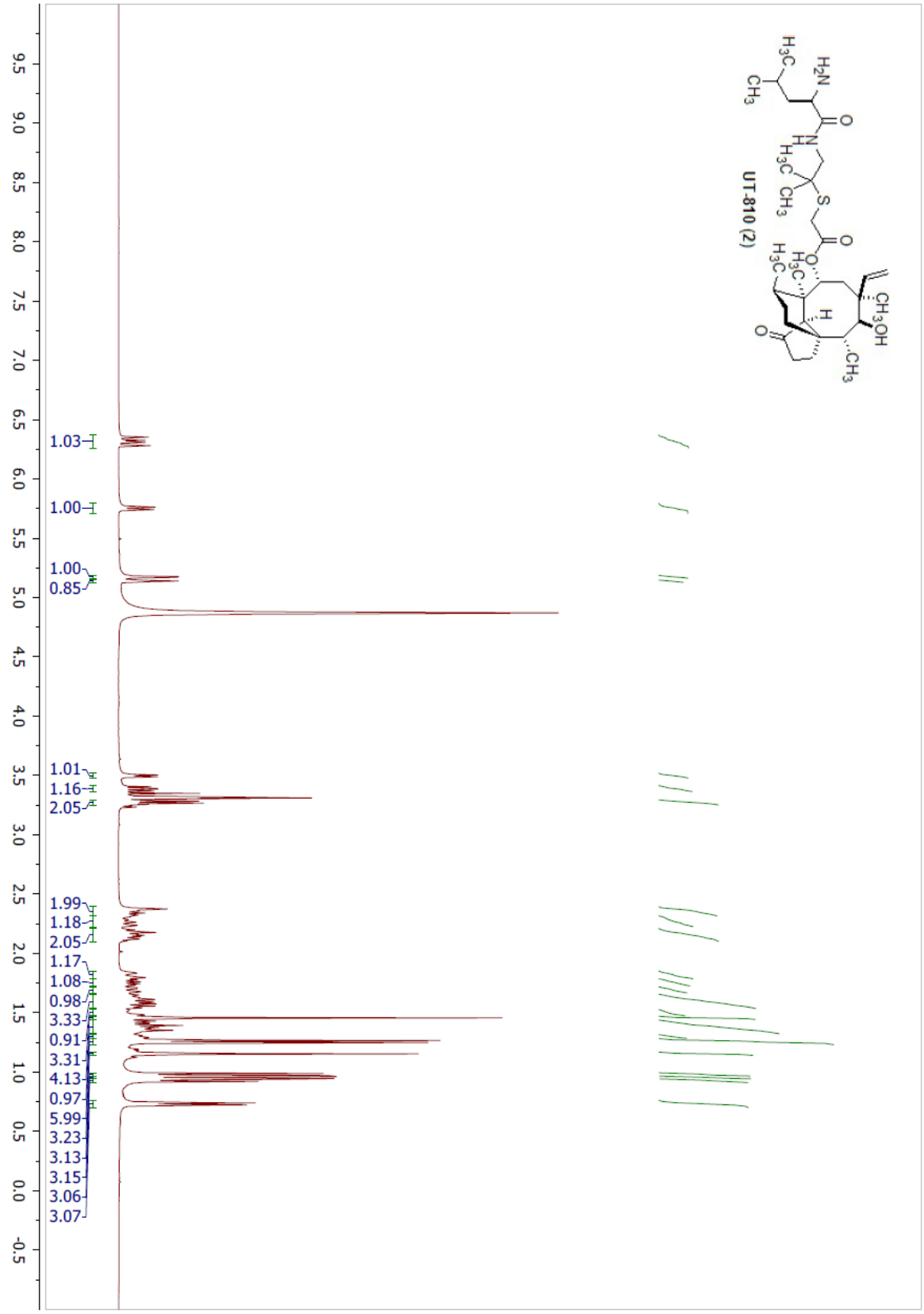
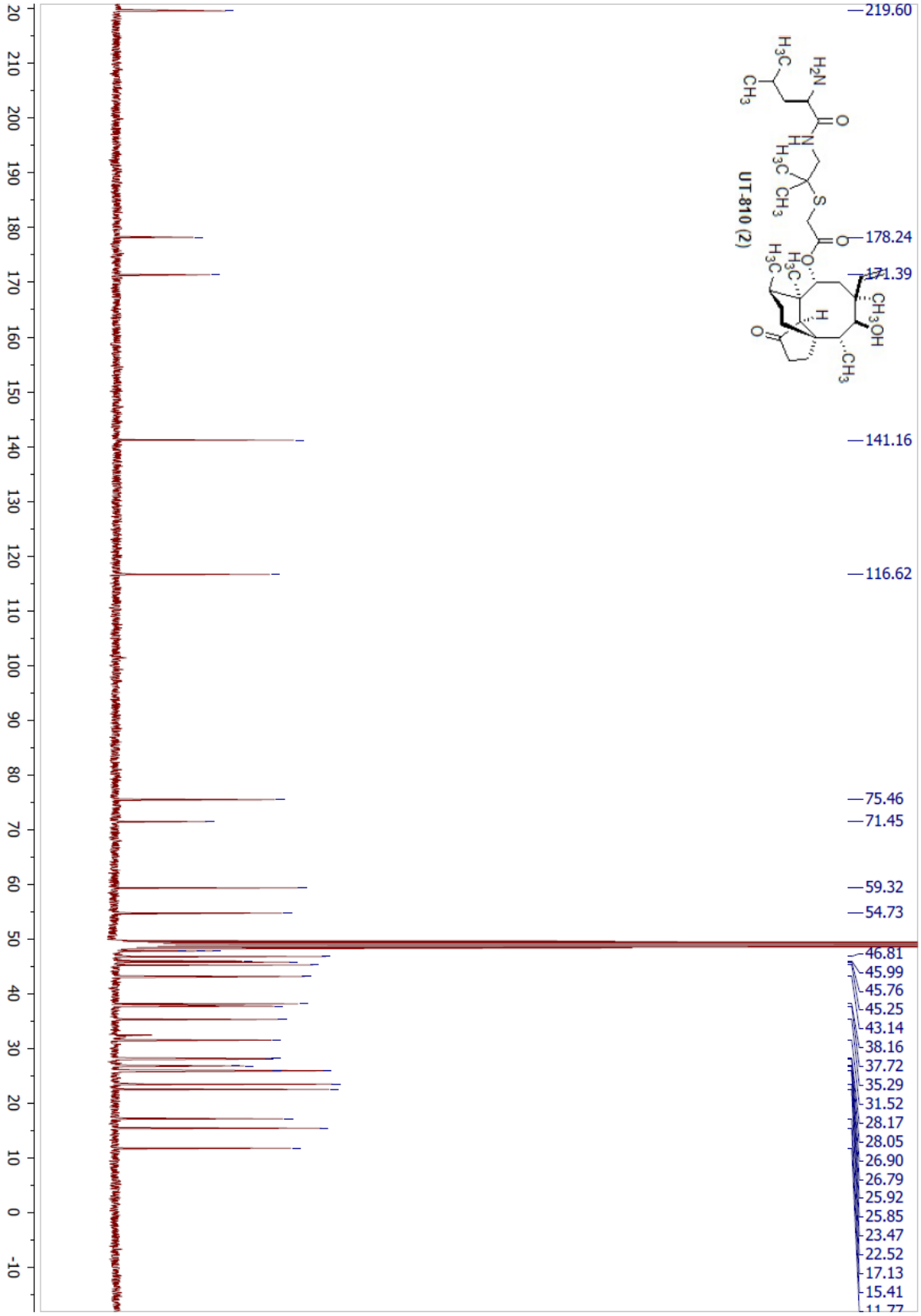
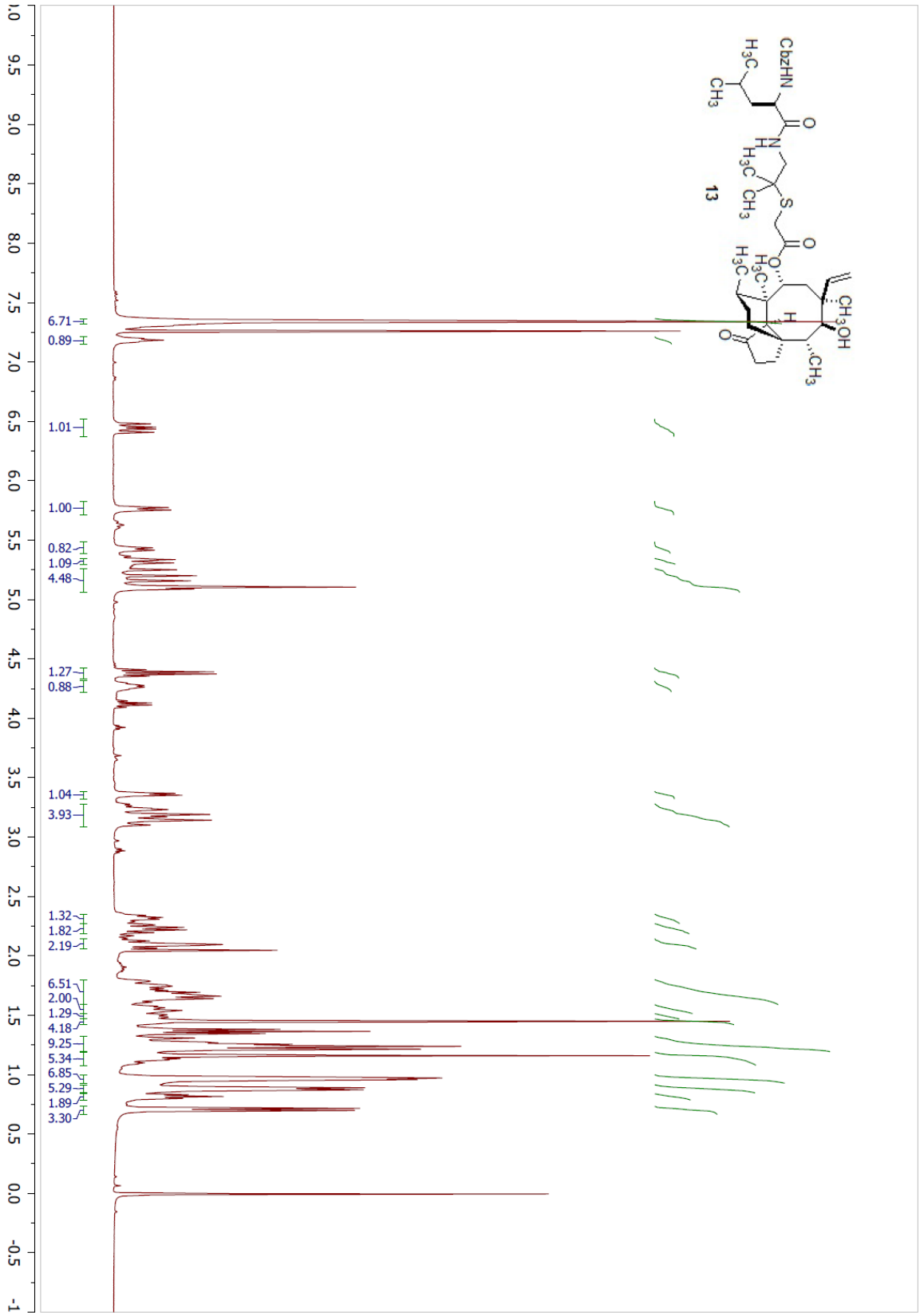
172.89
170.05
138.89
117.32
74.59
69.78
58.14
47.60
47.08
45.42
44.81
43.92
41.77
36.69
36.00
34.41
31.44
30.39
28.33
26.82
26.33
26.26
26.17
24.83
24.81
22.92
16.81
14.86
11.50
BocHN
12


UT-810 (2)
1.03
1.00
1.00
0.85
1.01
1.16
2.05
1.99
1.18
2.05
1.17
1.08
0.98
3.33
0.91
3.31
4.13
0.97
5.99
3.23
3.13
3.15
3.06
3.07


UT-810 (2)
219.60
178.24
171.39
141.16
116.62
75.46
71.45
59.32
54.73
46.81
45.99
45.76
45.25
43.14
38.16
37.72
35.29
31.52
28.17
28.05
26.90
26.79
25.92
25.85
23.47
22.52
17.13
15.41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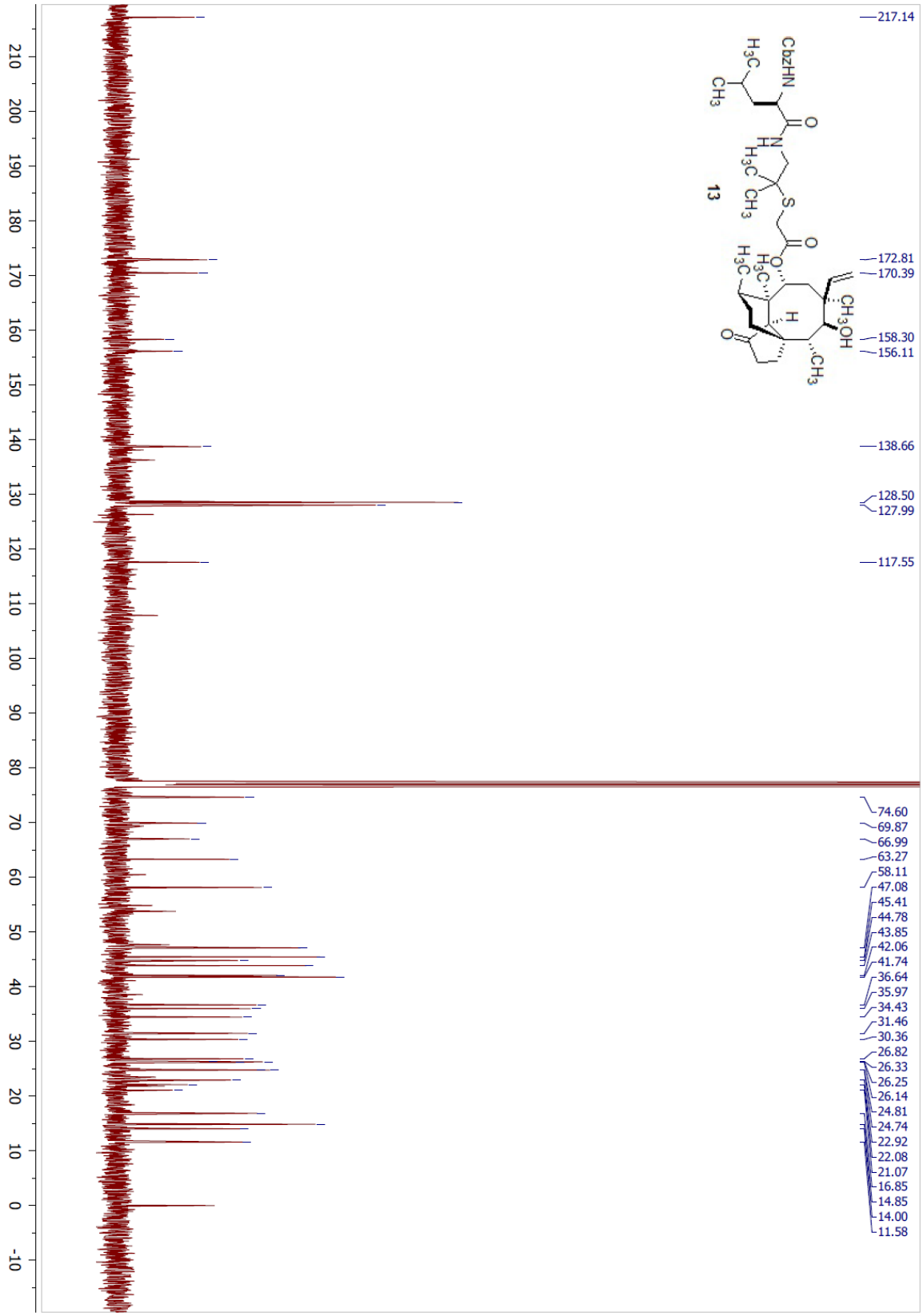
0.89
1.01
1.00
0.82
1.09
4.48
1.27
0.88
1.04
3.93
1.32
1.82
2.19
6.51
2.00
1.29
4.18
9.25
5.34
6.85
5.29
1.89
3.30
CbzHN
13
CH3OH


217.14
172.81
170.39
158.30
156.11
138.66
128.50
127.99
117.55
74.60
69.87
66.99
63.27
58.11
47.08
45.41
44.78
43.85
42.06
41.74
36.64
35.97
34.43
31.46
30.36
26.82
26.33
26.25
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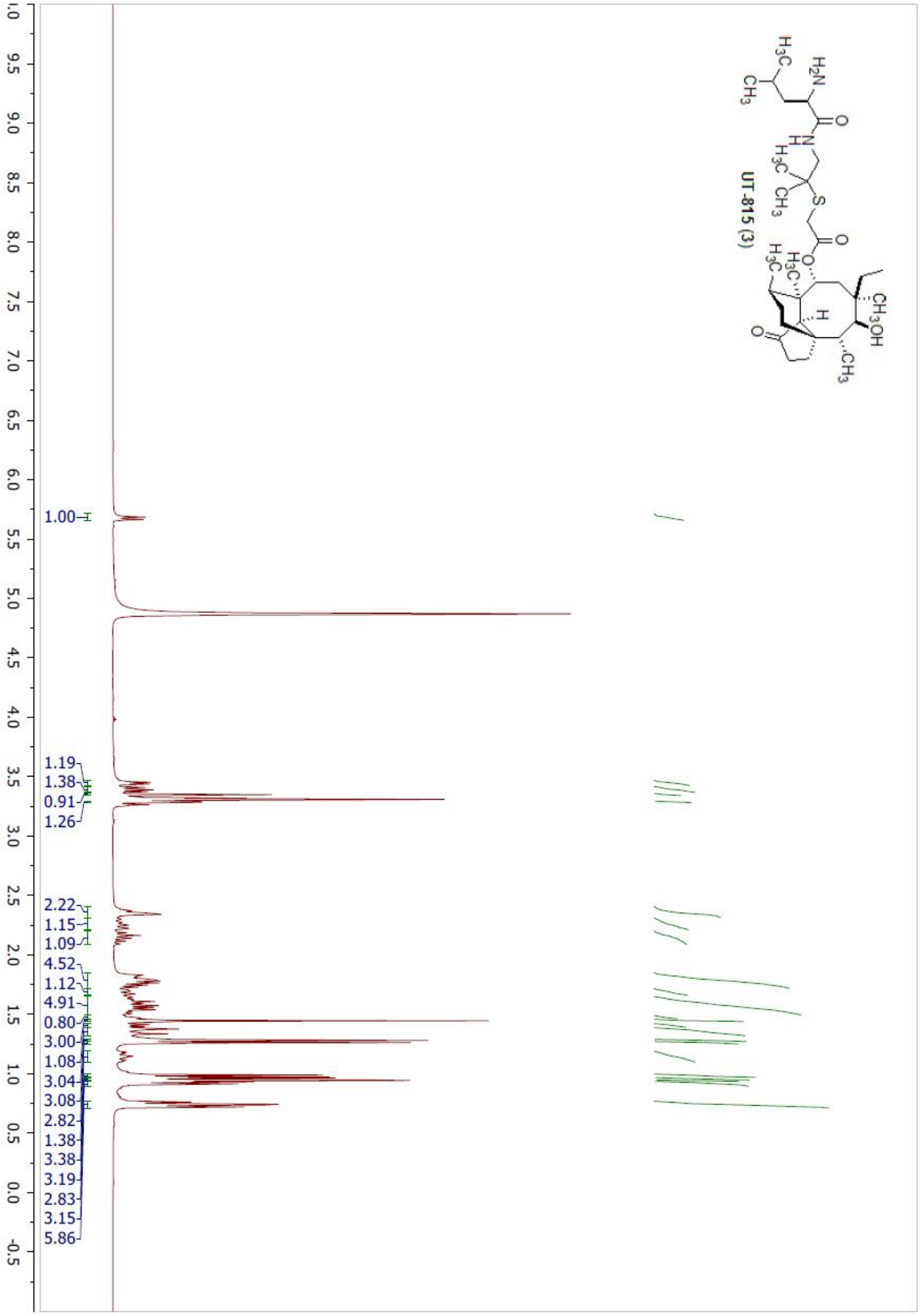
24.81
24.74
22.92
22.08
21.07
16.85
14.85
14.00
11.58
13


UT-815 (3)
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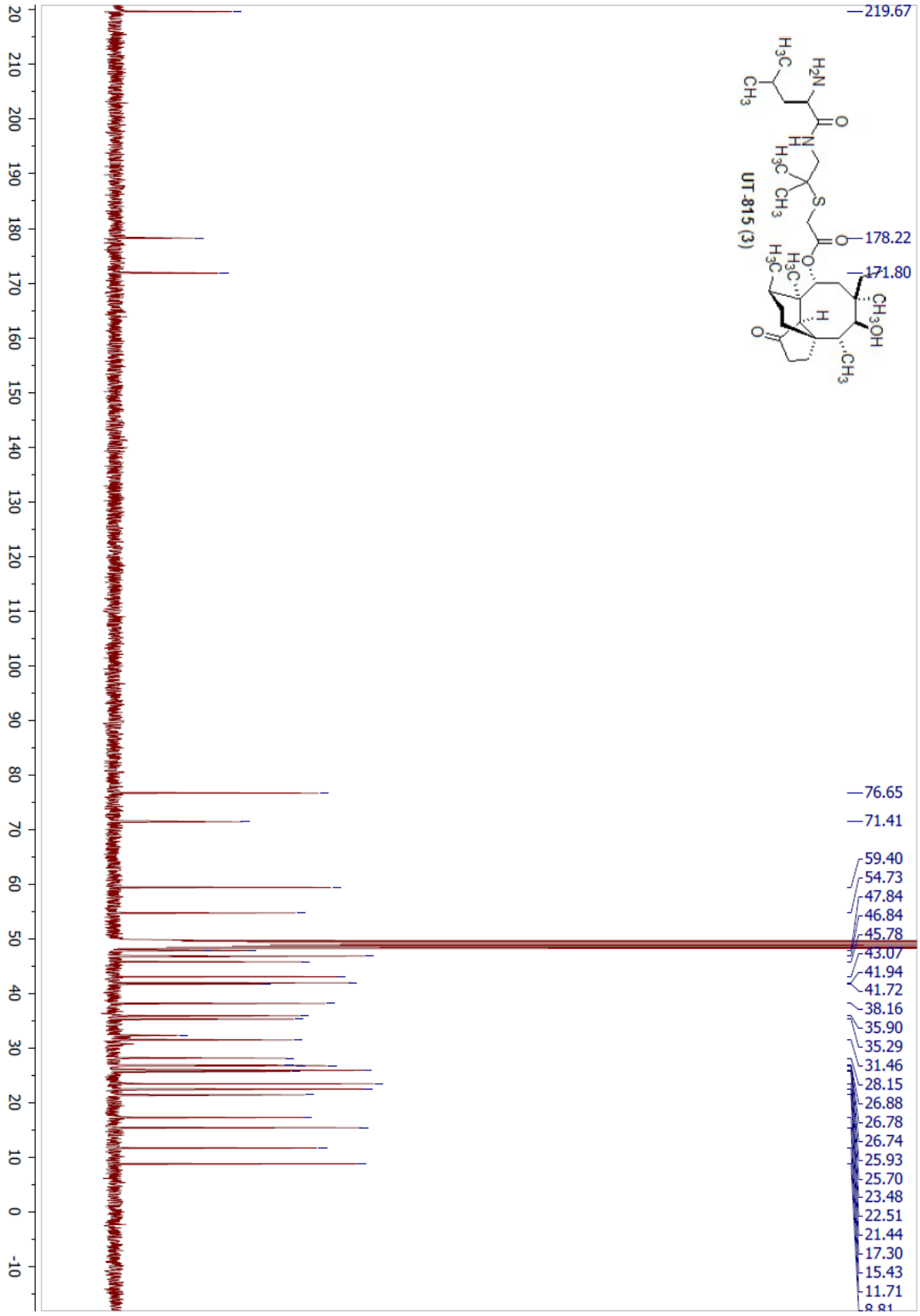
1.38
0.91
1.26
2.22
1.15
1.09
4.52
1.12
4.91
0.80
3.00
1.08
3.04
3.08
2.82
1.38
3.38
3.19
2.83
3.15
5.86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59.40
54.73
47.84
46.84
45.78
43.07
41.94
41.72
38.16
35.90
35.29
31.46
28.15
26.88
26.78
26.74
25.93
25.70
23.48
22.51
21.44
17.30
15.43
11.71
UT-815 (3)