Revised

Kinetic Studies and Bioactivity of Potential Manzamine Prodrugs

Supporting Information

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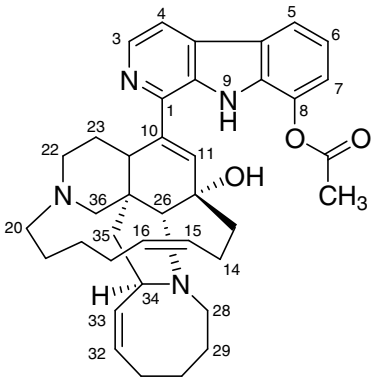
Experimental Section

General Experimental Procedures. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and CD_2Cl_2 on a Bruker DRX NMR spectrometer operating at 400 and 600 MHz for ^{1}H and 100 and 150 MHz for ^{13}C NMR, respectively. Chemical shift (δ) values are expressed in parts per million (ppm) and are referenced to the residual solvent signals of $CDCl_3$ and CD_2Cl_2 at δ_H/δ_C 7.26/77.0 and 5.32/54.0, respectively. IR spectra were obtained using an AATI Mattson, Genesis Series FTIR. Optical rotations were measured with a JASCO DIP-310 digital polarimeter. The HRMS spectra were measured using a Bioapex FTESI-MS with electrospray ionization. TLC analysis was completed using precoated silica gel G_{254}.

LC/MS Detection of analogs: Kinetic and detection measurements of 8-methoxy- and 8-acetoxymanzamine A were carried out using an LC/MS instrument. Analysis was conducted using a Bruker Daltonic GmbH LC/MS (Germany) equipped with Diode Array Detector (DAD) (AGILENT Techologies), Agilent 1100 binary pump and LC Autosampler (AGILENT). The electrospray ionization positive mass spectra were acquired on a microTOF series mass spectrometer. Conditions were optimized using flow injection of standard and sample solutions, were as follows: electrospray ionization capillary voltage, 4.5 kV; end plate offset voltage, -500 V; nebulizer pressure, 2 bar; dry gas flow, 6 L/min; dry gas temperature 180 °C; source detector voltage 1600 V; TOF detector voltage 2190 V. The electrospray ionization gas was nitrogen. All TOF measurements were performed at a high resolution setting and the TOF analyzer was scanned at 50-1000 *m/z* with a 1 sec integration time. A flow rate of 0.4 mL/min was used for the analytical column (C-8, Phenomenex, Luna, 5u) with dimensions of 4.6 × 150 mm. HPLC grade water and acetonitrile both with 0.1% formic acid were used as mobile phase for 20 min elution times.

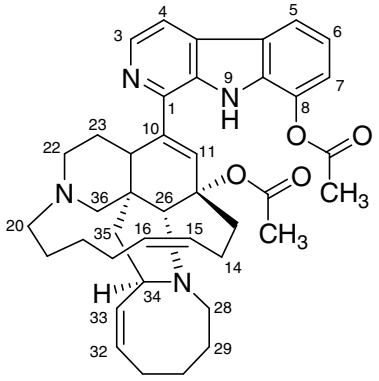
For stability studies we utilized extracted the ion chromatogram for 607 ± 0.5 (**3**) and 565 ± 0.5 (**2**) unit mass followed by manual integration to generate the relative peak area ratios %. The stability diagram was established by calculated 8-acetoxy peak area ratio *vs* dissolving time in various solvents .The experiments were performed three times for each sample to determine standard deviations.



8-Acetoxymanzamine A (3): To a solution of 8-hydroxymanzamine A as a free base (54.8 mg, 0.10 mmol) in anhydrous dichloromethane (10 mL) were added acetic anhydride (0.6 mL) and boron trifluoride-diethyl etherate (0.3 mL) at room temperature under nitrogen atmosphere. The mixture was stirred for 30 min, poured into cooled saturated NH_4OAc solution (20 mL), and extracted with dichloromethane (2×10 mL). The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure and the residue was subjected to silica gel flash column chromatography using hexanes/EtOAc (6:1) to obtain a monoacetylated product. Further purification was carried out with reverse-phase HPLC (C-8) using gradient MeCN/water to give 41 mg (68 %) of pure 8-acetate **3** as a white solid.

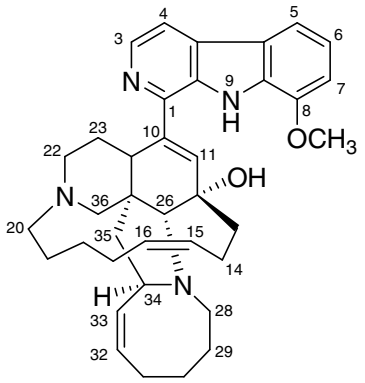
$[\alpha]^{25}_D$ – 12 (*c* = 0.1, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3198, 2929, 2850, 1762, 1653, 1559, 1506, 1419, 1372, 1218, 1197, 1071, 772 cm^{-1}; ^1H NMR (CD_2Cl_2, 400 MHz) δ 11.43 (1H, s, NH), 10.86 (1H, br s, NH^+), 8.35 (1H, d, *J* = 5.1 Hz, H-3), 8.01 (1H, dd, *J* = 6.1, 2.4 Hz, H-5), 7.87 (1H, d, *J* = 5.1 Hz, H-4), 7.26 (2H, m, H-6,7), 6.58 (1H, s, H-11), 6.31 (1H, m, H-32), 6.01 (1H, s, OH), 5.58 (2H, m, H-15,16), 5.47 (1H, t, *J* = 9.7 Hz, H-33), 4.85 (1H, m, H-34), 4.03 (1H, m, H-28), 3.71 (1H, d, *J* = 7.2 Hz, H-26), 3.26

(1H, m, H-28), 2.99 (3H, m), 2.69 (2H, m), 2.58 (1H, s, C*H*$_3$CO), 2.42 (4H, m), 2.27 (4H, m), 1.97 (7H, m), 1.68 (4H, m), 1.48 (3H, m), 1.24 (1H, m); ^{13}C NMR (CD_2Cl_2, 100 MHz) δ 170.1 (CO), 144.0, 142.2, 140.8, 138.0, 136.9, 135.9, 133.9, 133.5, 133.0, 129.6, 126.9, 124.2, 123.9, 120.9, 119.6, 118.5, 113.7, 77.7, 71.3, 70.4, 57.0, 53.3, 53.2, 49.3, 47.0, 44.6, 40.9, 39.0, 34.0, 28.3, 26.6, 26.3, 24.9, 24.6 (2C), 21.9 (*C*H$_3$CO), 20.7; HRESIMS *m/z* calcd for $C_{38}H_{47}N_4O_3$ $[M+H]^+$ 607.3642, found 607.3633.



8,12-Diacetoxymanzamine A (4): This reaction was performed as above for compound **3** except for continued stirring for an additional 12 hours at the same reaction conditions. After analogous work up and evaporation of solvent, the resulting residue was subjected to a reverse-phase HPLC (C-8) using gradient MeCN (2% DCM)/water to give 35 mg (54 %) of pure 8,12-diacetate **4** as a fluffy white solid. $[\alpha]^{25}_D$ + 35 (*c* = 0.2, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3317, 3009, 2927, 2850, 1769, 1713, 1635, 1567, 1422, 1261, 1197, 770 cm^{-1}; ^{1}H NMR (C_6D_6, 600 MHz) δ 10.43 (1H, s, NH), 8.53 (1H, d, *J* = 5.0 Hz, H-3), 7.70 (1H, d, *J* = 7.7 Hz, H-5), 7.51 (1H, d, *J* = 5.0 Hz, H-4), 7.31 (1H, d, *J* = 7.7 Hz, H-7), 7.01 (1H, t, *J* = 7.7 Hz, H-6), 6.26 (1H, s, H-11), 5.71 (1H, m, H-32), 5.64 (1H, m, H-15), 5.58 (1H, m, H-16), 5.12 (1H, t, *J* = 9.9 Hz, H-33), 3.96 (1H, t, *J* = 8.2 Hz, H-34), 3.44 (1H, s, H-26), 3.26 (1H, m, H-28), 3.00 (1H, m, H-23), 2.91 (1H, m, H-22), 2.61 (4H, m), 2.50 (1H, m), 2.41 (1H, dd, *J* = 8.1, 12.5 Hz), 2.29 (1H, m), 2.19 (1H, s, C*H*$_3$CO), 2.14 (2H, m), 2.03 (2H, m), 1.91 (2H, m), 1.78 (3H, s, C*H*$_3$CO), 1.75 (6H, m), 1.56 (3H, m), 1.28 (4H, m); ^{13}C NMR (C_6D_6, 150 MHz) δ 170.9 (CO), 168.0 (CO), 146.6, 138.9, 137.1, 134.7, 134.2, 133.7, 133.3, 132.5, 131.4, 129.0, 128.2, 127.6, 124.7, 120.3, 119.6, 119.1,

113.6, 84.3, 72.3, 68.7, 53.8, 53.1, 50.6, 49.5, 47.8, 44.7, 42.0, 40.5, 34.6, 31.8, 27.8, 27.2, 26.9, 25.6, 25.7, 22.8 (CH$_3$CO), 21.9, 21.1 (CH$_3$CO); HRESIMS *m/z* calcd for $C_{40}H_{49}N_4O_4$ $[M+H]^+$ 649.3753, found 649.3751.



8-Methoxymanzamine A (5): 8-OHMA hydrochloride salt (60.1 mg, 0.10 mmol) was dissolved in anhydrous dichloromethane (10 mL), followed by addition of a freshly *in situ* prepared diazomethane solution in dichloromethane and the mixture was stirred over night at room temperature under nitrogen atmosphere. Solvent was removed under nitrogen, yielding 59.7 mg (97%) of **5** as a pale yellow solid. $[\alpha]^{25}_D$ + 23 (c = 0.2, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3478, 3005, 2926, 2850, 1579, 1508, 1458, 1384, 1257, 1087, 1033, 757 cm^{-1}; ^{1}H NMR and ^{13}C NMR (see ref. 14); HRESIMS *m/z* calcd for $C_{37}H_{47}N_4O_2$ $[M+H]^+$ 579.3699, found 579.3703.

Assay for *in vitro* Antimalarial Activity and Cytotoxicity. A 200 µL suspension of red blood cells infected with D6 or W2 strain of *P. falciparum* (2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL Amikacin) was added to the wells of a 96-well plate containing 10 µL of serially diluted samples. The plate was flushed with a gas mixture of 90% N_2, 5% O_2, and 5% CO_2 and incubated at 37 ºC, for 72 h in a modular incubation chamber (Billups-Rothenberg, CA). Parasitic LDH activity was determined by using Malstat™ reagent (Flow Inc., Portland, OR) according to the procedure of Makler and Hinrichs.[i] 20 µL of the incubation

mixture was mixed with 100 µL of the Malstat™ reagent and incubated at room temperature for 30 min followed by addition of 20 µL of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) and further incubation in the dark for 1 h. The reaction was stopped by the addition of 100 µL of 5% acetic acid. The plate was read at 650 nm. Artemisinin and chloroquine were included as the drug controls. IC_{50} values were computed from the dose response curves. To determine the selectivity index of antimalarial activity of compounds their *in vitro* cytotoxicity to mammalian cells was also determined. The assay was performed as described earlier.[ii] Vero cells (monkey kidney fibroblasts) were seeded to the wells of 96-well plate at a density of 25,000 cells/well and incubated for 24 h. Samples at different concentrations were added and plates were again incubated for 48 h. The number of viable cells was determined by Neutral Red assay. IC_{50} values were obtained from dose response curves. Doxorubicin was used as a positive control.

Assay for Antimicrobial Activity. All organisms are obtained from the American Type Culture Collection (Manassas, VA) and include the fungi *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906 and the bacteria methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing is performed using a modified version of the CLSI (formerly NCCLS) methods.[iii-vi] *M. intracellulare* is tested using a modified method of Franzblau, et al.[vii] Samples are serially-diluted in 20% DMSO/saline and transferred in duplicate to 96 well flat bottom microplates. Microbial inocula are prepared by correcting the OD_{630} of microbe suspensions in incubation broth to afford final target inocula. Drug controls [Ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and Amphotericin B (ICN Biomedicals, Ohio) for fungi] are included in each assay. All organisms are read at either 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) or 544ex/590em, (*M. intracellulare*, *A. fumigatus*) using the

Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany) prior to and after incubation. Minimum fungicidal or bactericidal concentrations are determined by removing 5ul from each clear well, transferring to agar and incubating. The MFC/MBC is defined as the lowest test concentration that kills the organism (allows no growth on agar).

***In vitro* antileismanial assay.** Antileishmanial activity of the compounds was tested *in vitro* on a culture of *Leishmania donovani* promastigotes. In a 96 well microplate assay compounds with appropriate dilution were added to the leishmania promastigotes culture (2x 10^6 cell/mL). The plates were incubated at 26 C for 72 hours and growth of leishmania promastigotes was determined by Alamar blue assay.[viii] Pentamidine and Amphotericin B were used as the standard antileishmanial agents. IC_{50} value for each compound was computed from the growth inhibition curves.

***In vivo* antimalarial assay.** The *in vivo* antimalarial activity of the compounds was determined in mice infected with *Plasmodium berghei* (NK-65 strain) according to the Peter's 4-day suppressive test.[ix] Male mice (Swiss Webster strain) weighing 18-20 g were intraparitoneally inoculated with 2 $X10^7$ parasitized red blood cells obtained from a highly infected donor mouse. Mice were divided into different groups with 5 mice in each group. Test compounds were prepared in 0.1N HCl and administered orally to the mice about 2 h after the infection (Day 0). The test compounds were administered to the mice once a day for 3 consecutive days (Days 0-2). A control group was treated with equal volume of vehicle while another control group was treated with the standard antimalarial compound, β-arteether. The mice were closely observed after every dose for any apparent signs of toxicity. Blood smears were prepared on different days (till day 28 post infection) by clipping the tail end, stained with Giemsa and observed under microscope for determination of the parasitemia. Mice without parasitemia till day 28 post infection were considered as cured.

[i] Makler, M. T.; Hinrichs, D. J. *Am. J. Trop. Med. Hyg.* **1993**, *48*(2), 205-210.

[ii] Mustafa, J.; Khan, S. I.; Ma, G.; Walker, L. A.; Khan, I. A. *Lipids* **2004**, *39*(2), 167-172.

[iii] NCCLS, NCCLS Document M27-A2 **2002**, *22*(15), 1-51.

[iv] NCCLS, NCCLS Document M7-A7 **2006**, *26*(2), 1-64.

[v] NCCLS, NCCLS Document M38-A **2002**, *22*(16), 1-51.

[vi] NCCLS, NCCLS Document M24-A **2003**, *23*(18), 1-84.

[vii] Franzblau, S. G.; Witzig, R. S.; McLaughlin, J. C.; Torres, P.; Madico, G.; Hernandez, A.; Degnan, M. T.; Cook, M. B.; Quenzer, V. K.; Ferguson, R. M.; Gilman, R. H. *J. Clin. Microbiol.* **1998**, *36*(2), 362-366.

[viii] Mikus, J.; Steverding, D. *Parasitol. Int*. **2000**, *48*, 265-269.

[ix] Peters, W. *Ann. Trop. Med. Parasitol.* **1975**, *69*, 155-171.

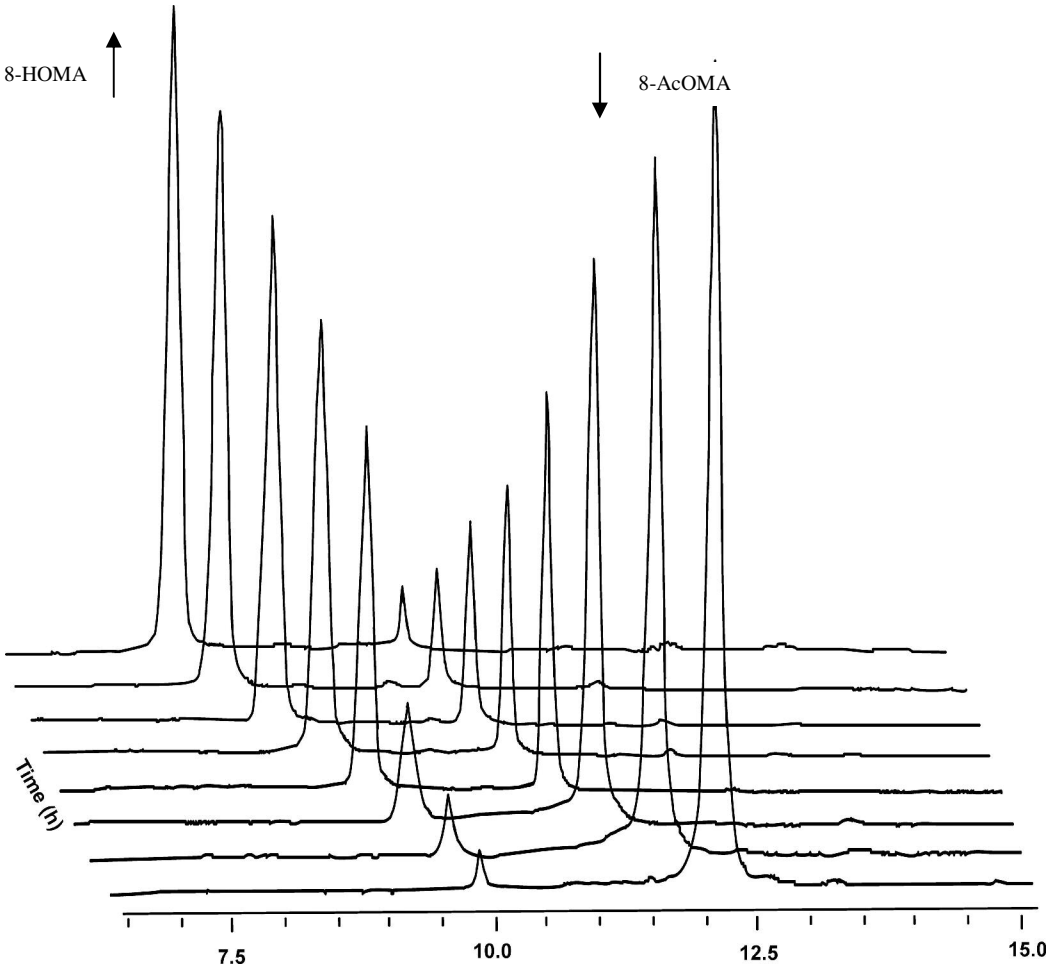


Figure 1. Time courses disappearance of **3** and appearance of **2** in methanol after 15 hours.

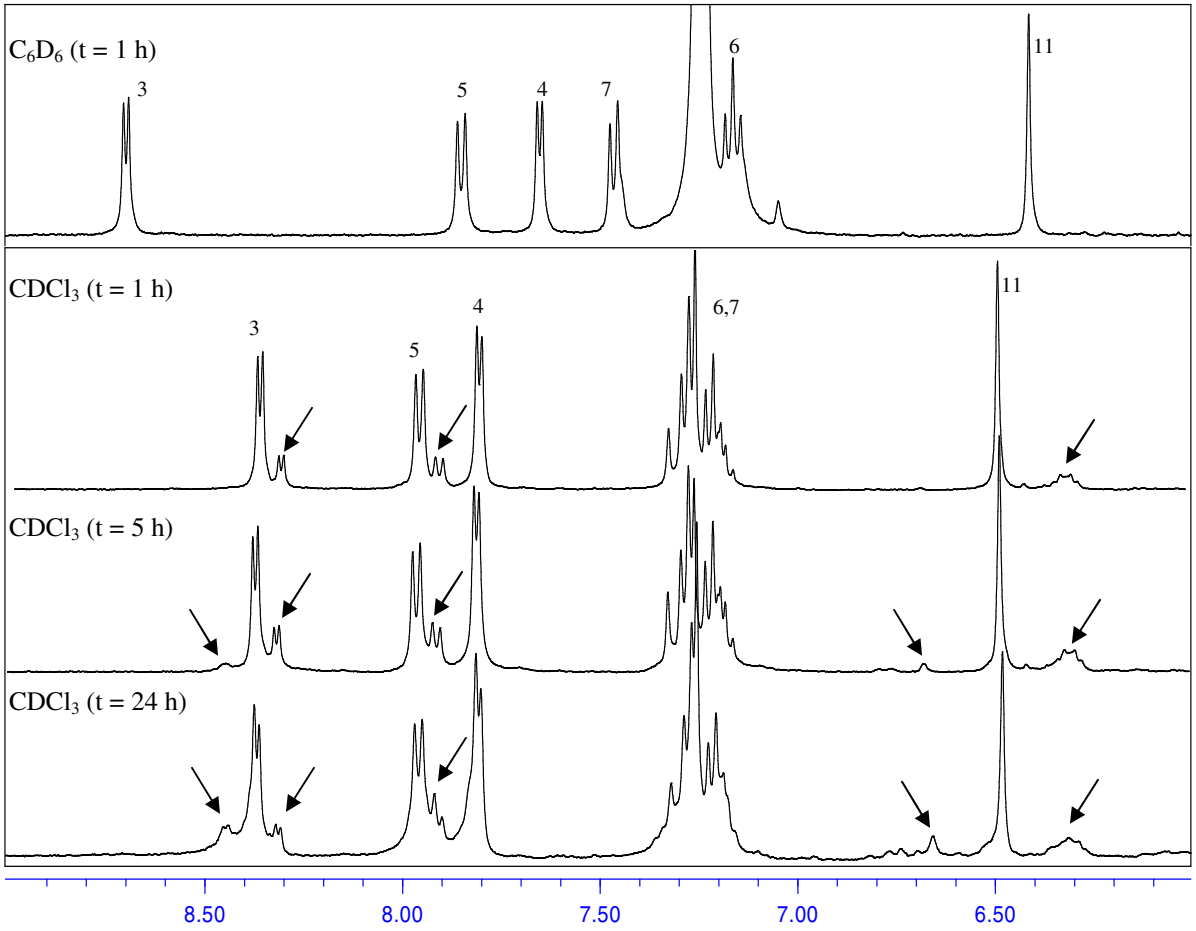
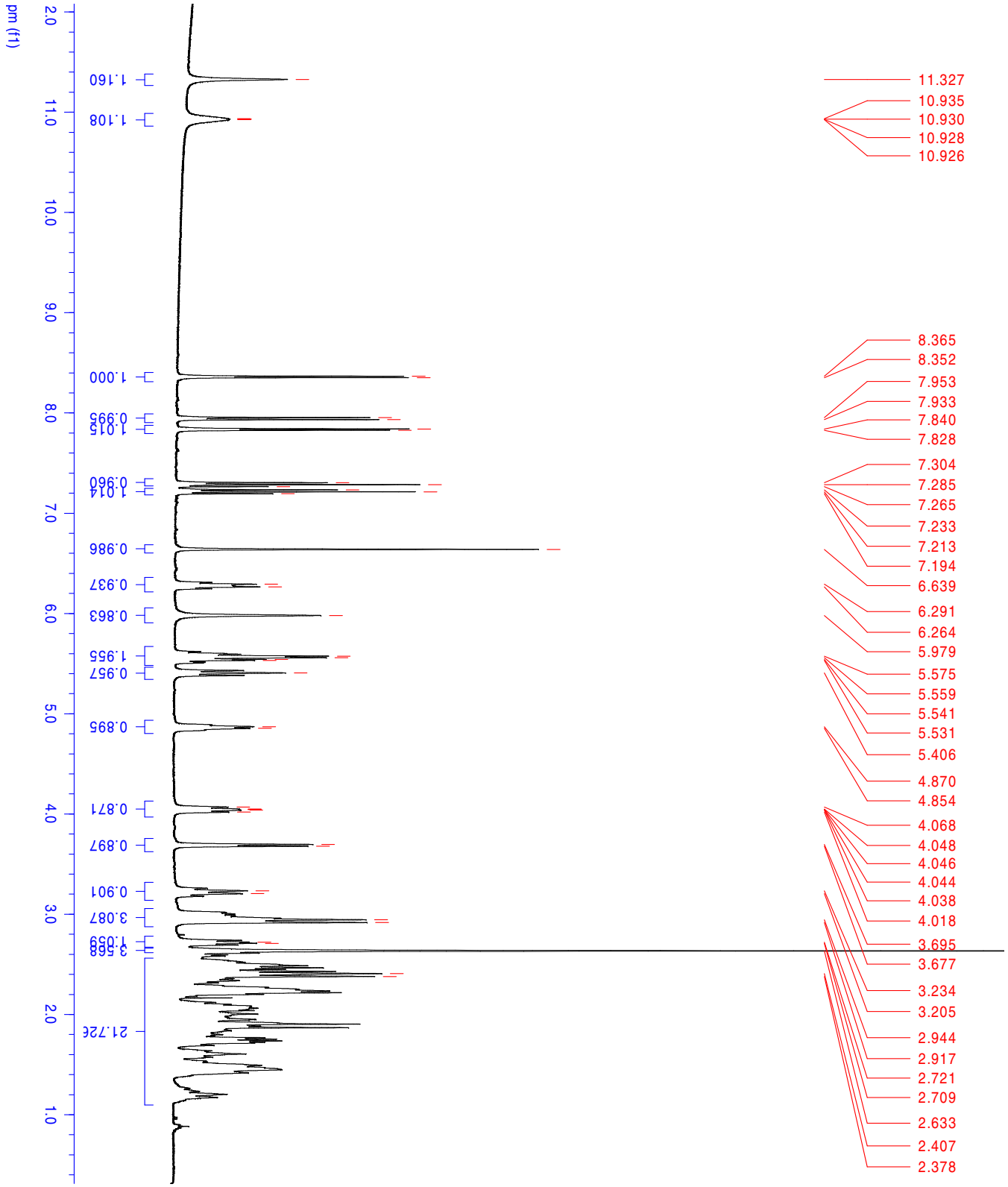


Figure 3. Solvent effect studies of compound **4** in deuterated chloroform and benzene. This product is stable in benzene (colorless solution) but decomposes in chloroform to a mixture of unknown species (brownish yellow) over 24 h period of time (→: impurities).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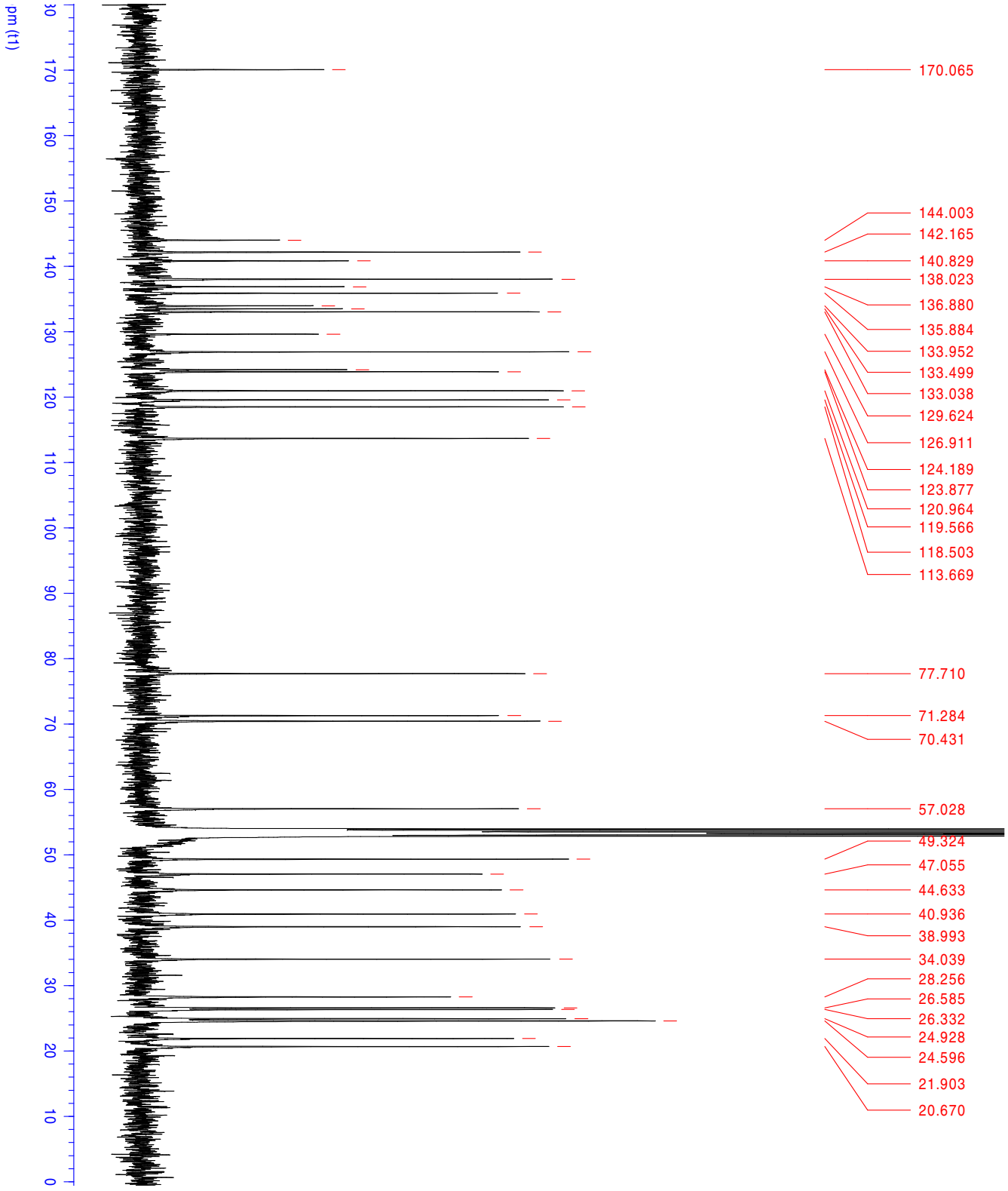
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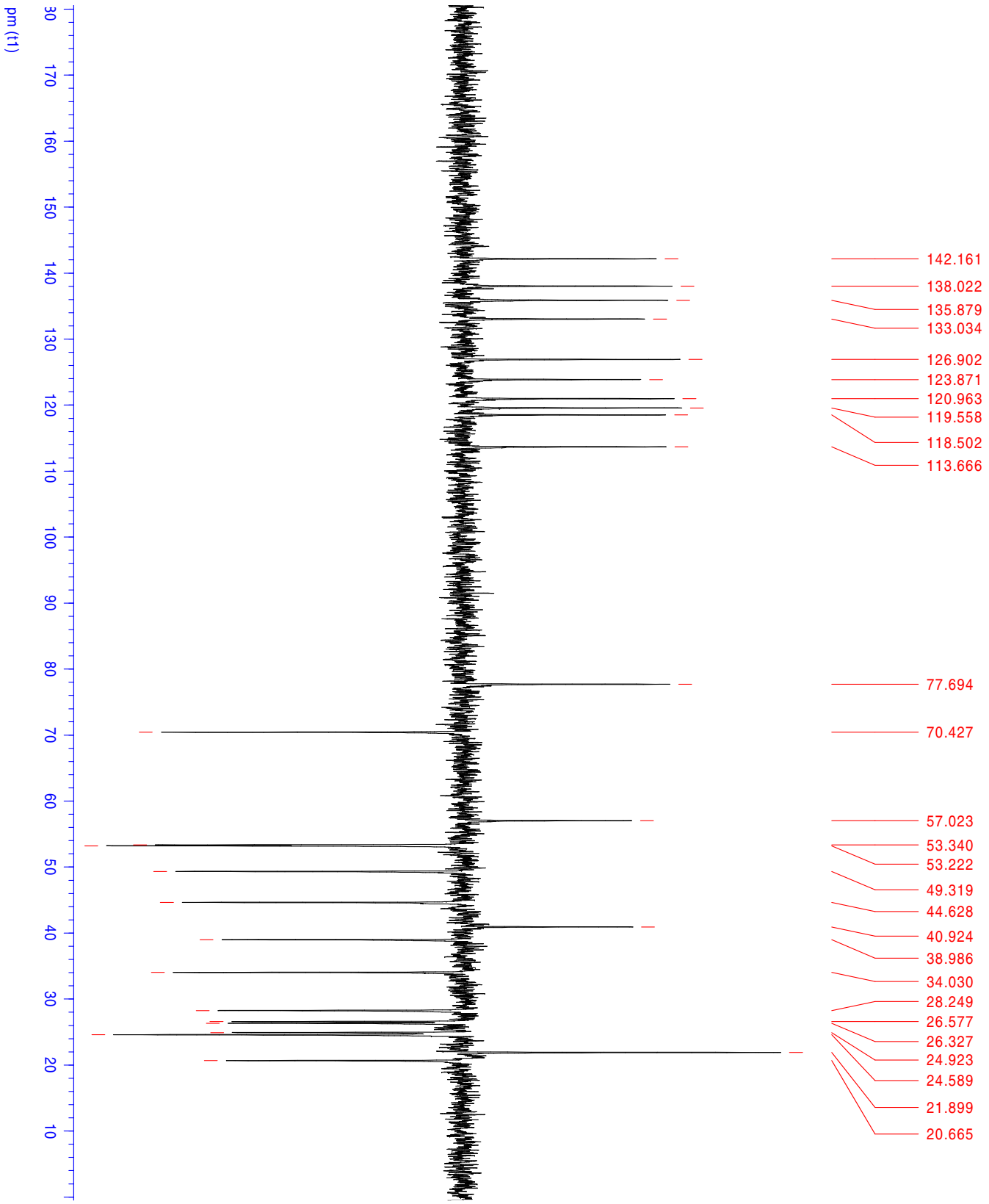
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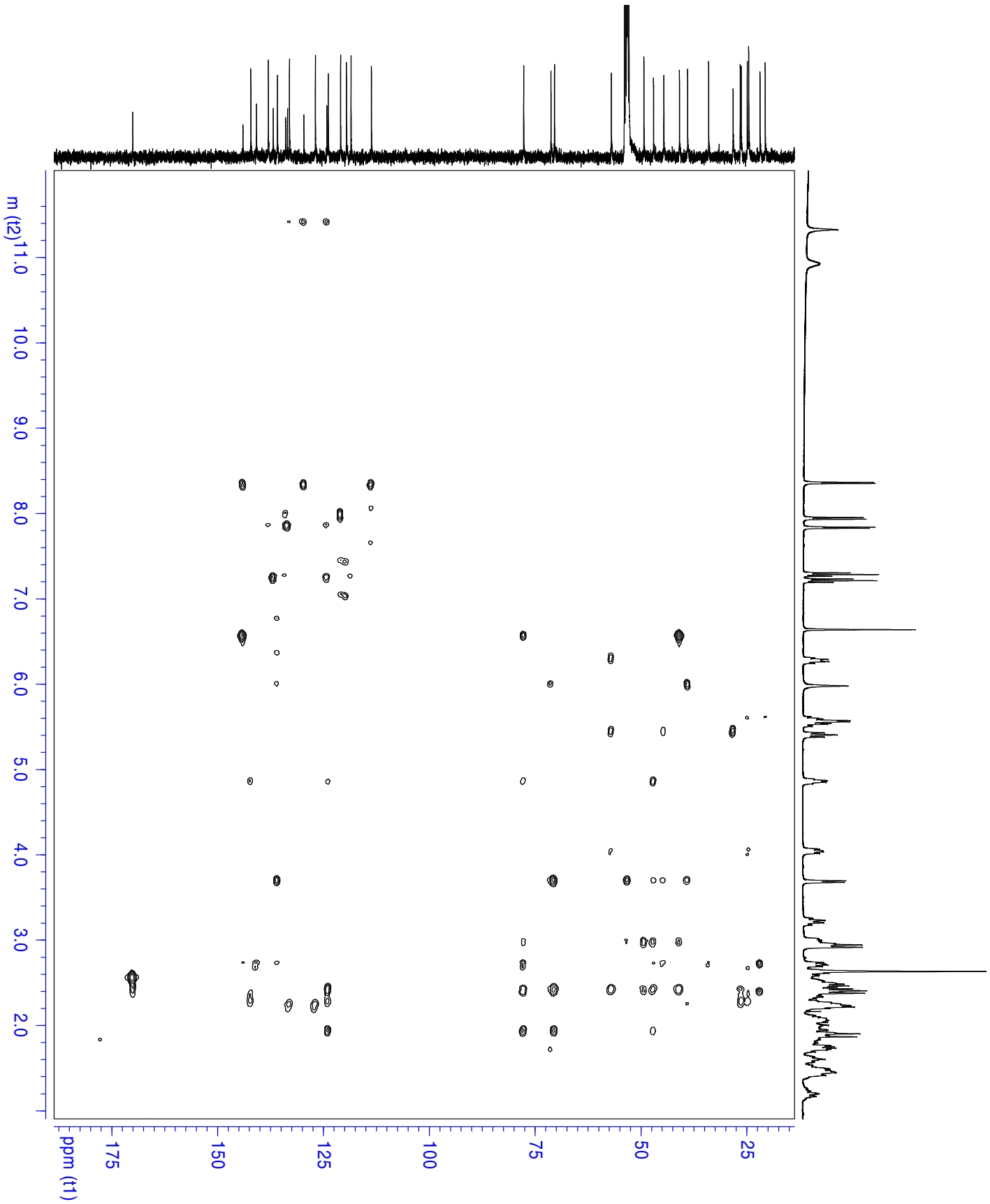
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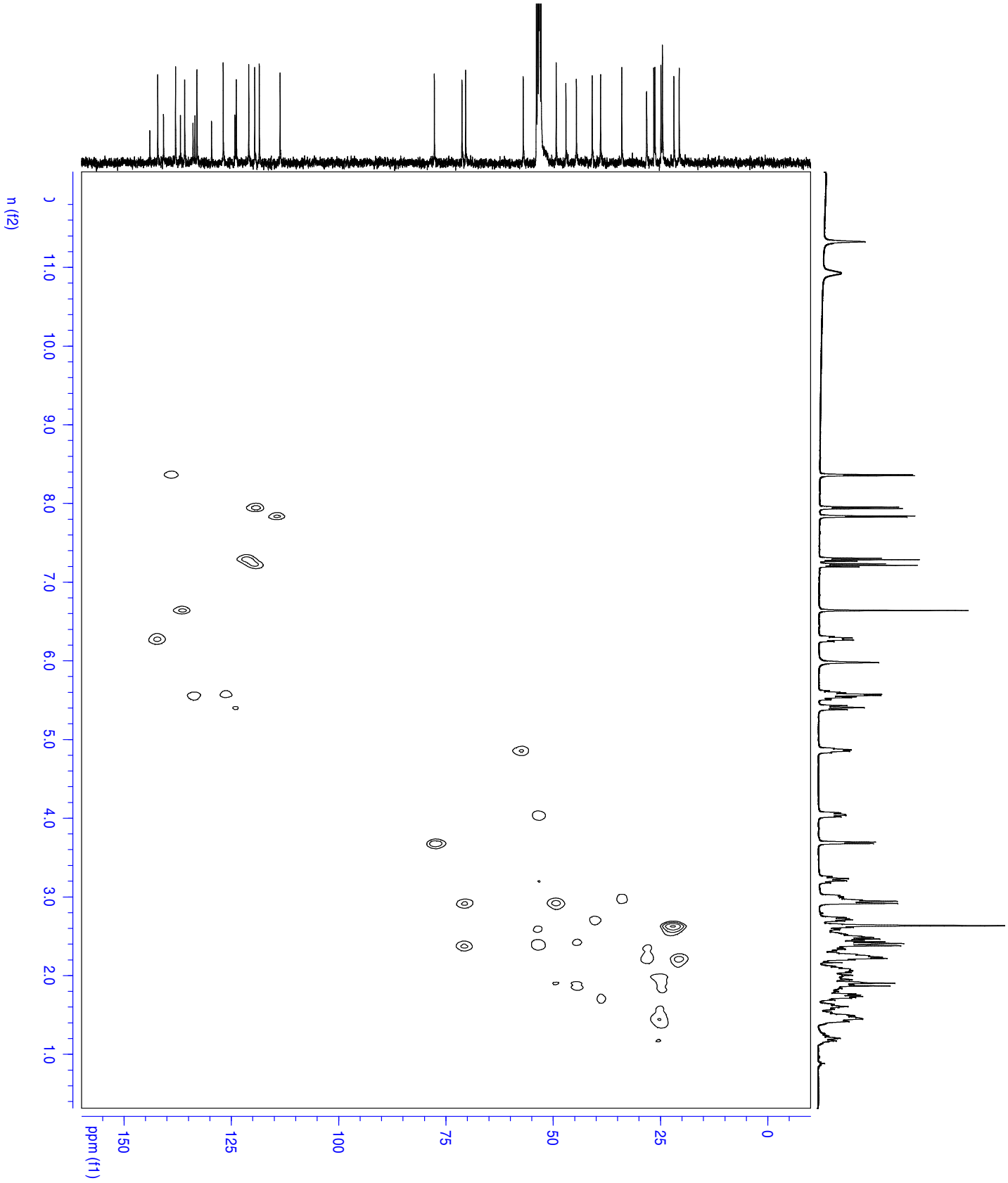
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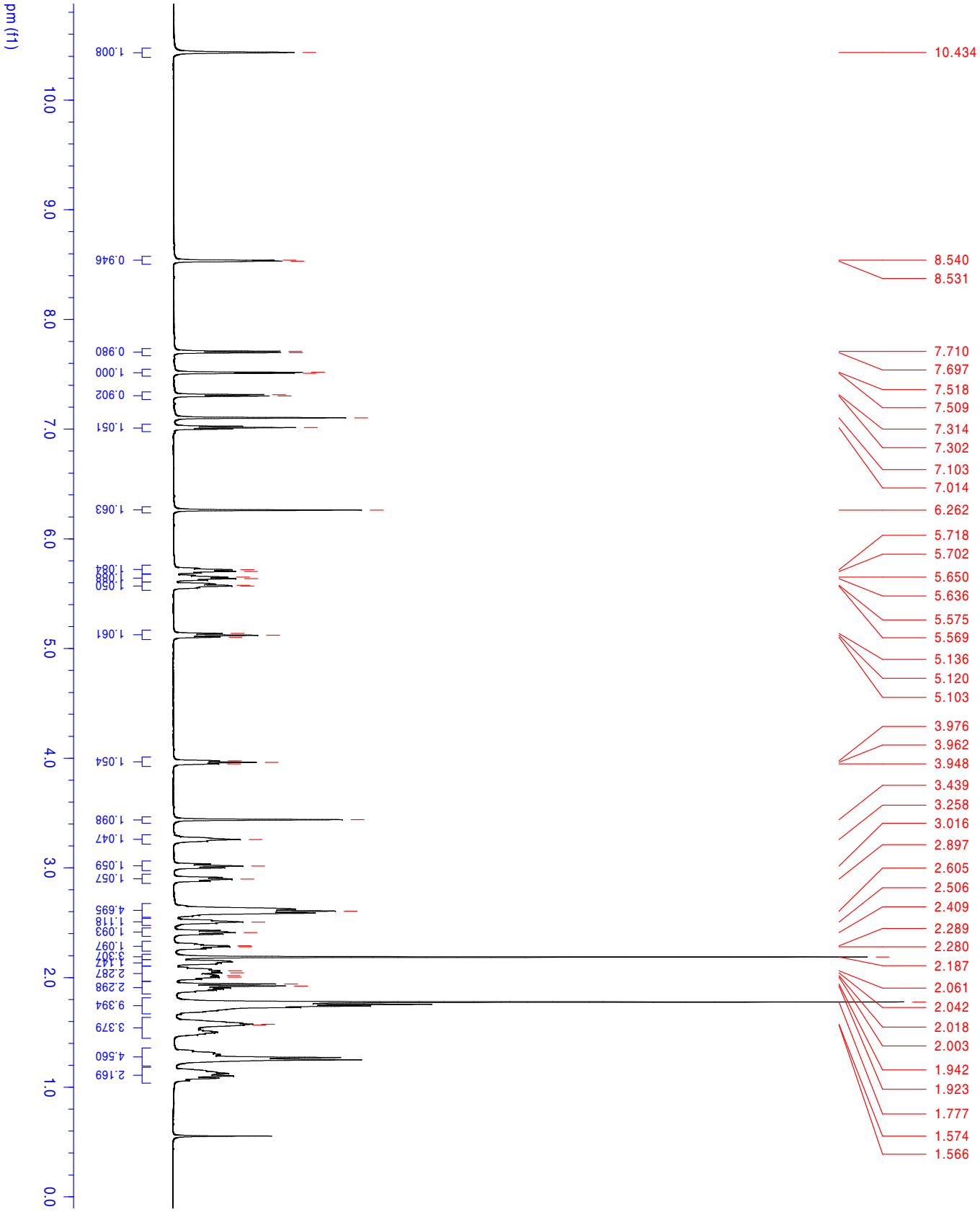
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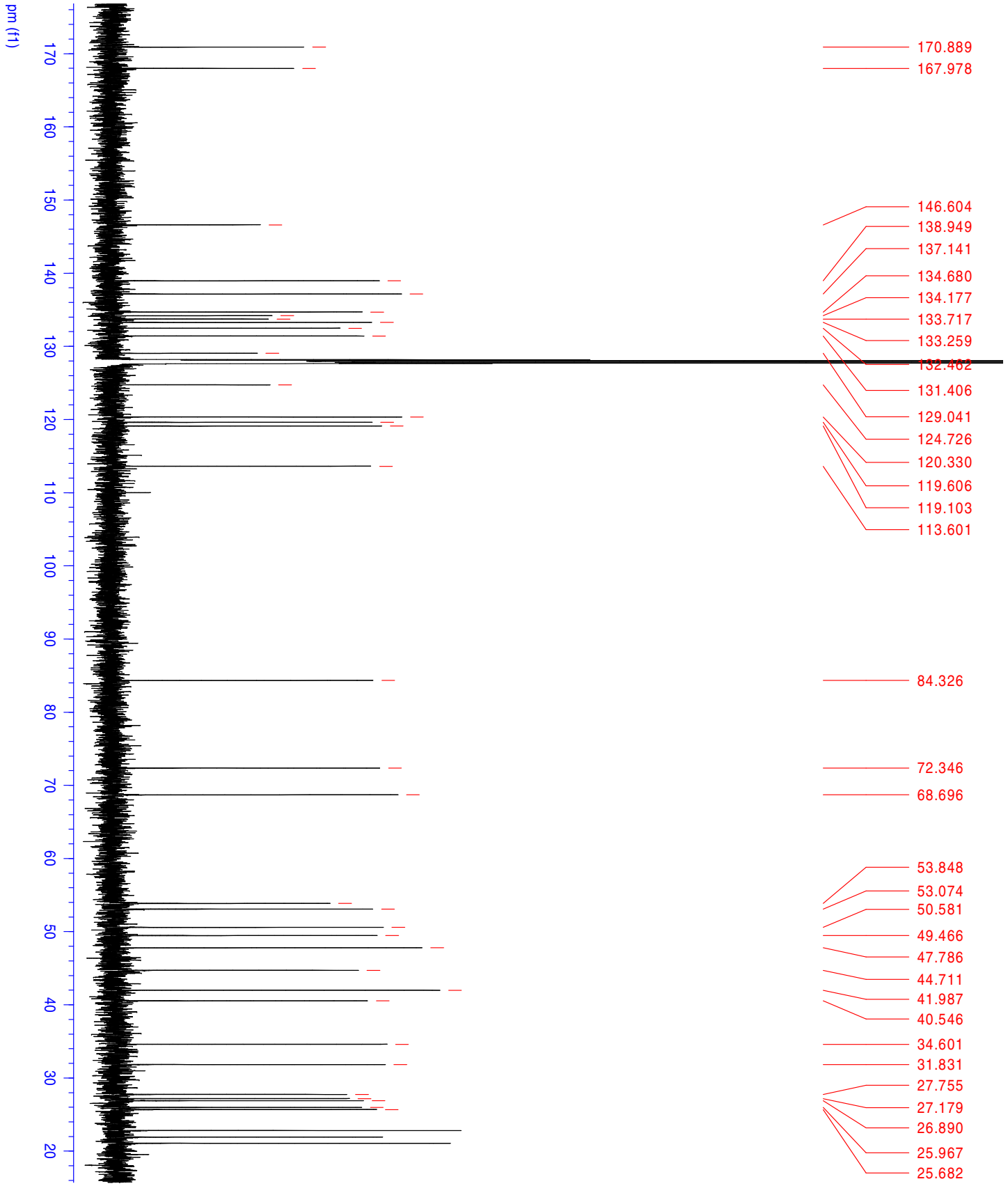
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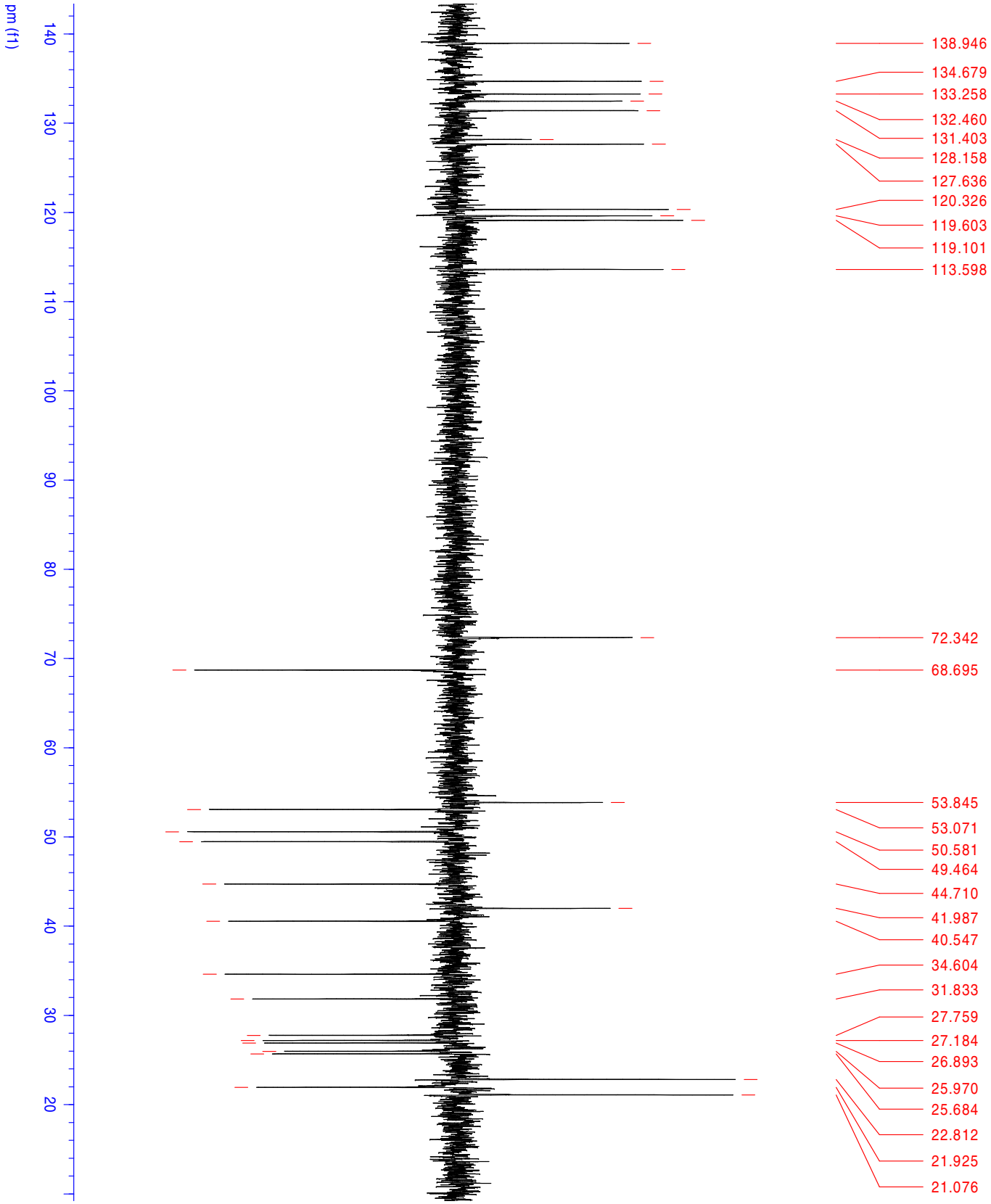
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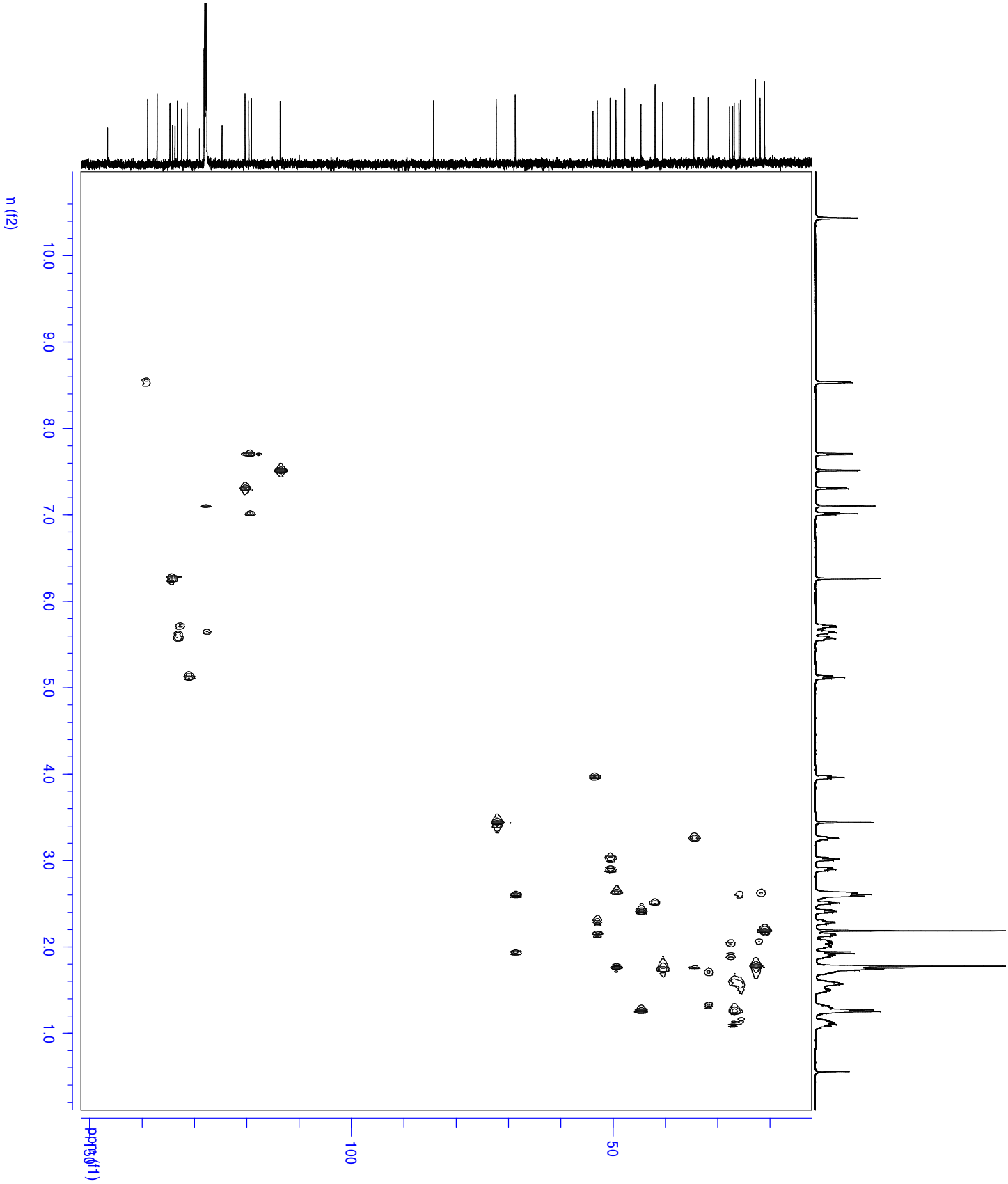
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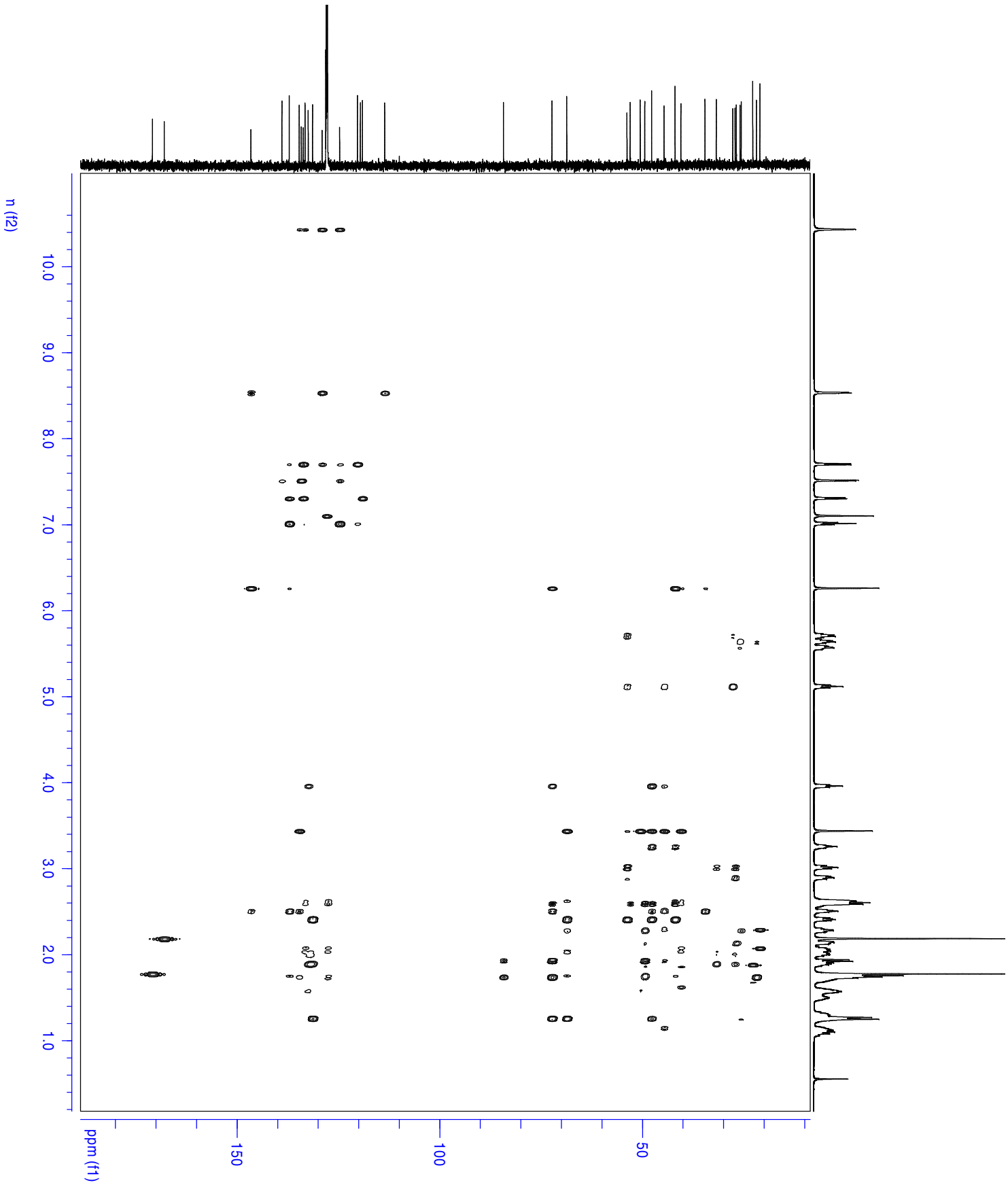
100
50


n (f2)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (f1)
150
100
50