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Towards more drug-like proteomimetics: Two-faced, synthetic α-helix mimetics based on a purine scaffold

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Supporting Information

Chemistry

General. Unless otherwise stated, all reaction were performed under an inert atmosphere (N_2). Reagents and solvents were ACS grade, and purchased from Sigma-Aldrich, Alfa Aesar, Oakwood and TCI America. Anhydrous solvents were used as provided from Sigma-Aldrich. Reactions were monitored by thin-layer chromatography (TLC), visualizing with a UV lamp and/or $KMnO_4$ stain. Flash column chromatography was performed with silica gel 60 Å (70-230 mesh, Merck). ^1H and ^{13}C NMR spectra were recorded on a Varian INOVA 400 MHz NMR spectrometer at 25 °C. Chemical shifts are reported in parts per million (ppm). Data for ^1H NMR are reported thus: chemical shift (δ ppm) (multiplicity, coupling constant (Hz), integration), where multiplicities are: s = singlet, d = doublet, t = triplet, m = multiplet. The residual solvent peak was used as an internal reference: $CDCl_3$ (δ_H 7.26; δ_C 77.21) and d_6-DMSO (δ_H 2.50; δ_C 39.51). Mass spectra were obtained on an Electrospray TOF (ESI-TOF) mass spectrometer (Bruker AmaZon X). All final molecules were deemed to be >95% pure by reversed-phased HPLC using a Waters 1525 analytical/preparative HPLC fitted with a C18 reversed-phase column (Atlantis T3: 4.6 mm x 150 mm) according to the following conditions with solvents (A) H_2O/0.1% TFA, (B) CH_3CN–H_2O, 9:1 with 0.1% TFA at 1 ml min^{-1}: (I) a gradient of 50% A to 100% B over 22 min; (II) an isocratic gradient of 100% B over 22 min. Data are presented as retention time (t_R (min)), purity (%), condition (I or II).

General Procedure A: Mitsunobu reaction at N9 position. *tert*-Butyl (6-chloro-9*H*-purin-2-yl)carbamate[1] (**2**; 1 eq) was dissolved in THF (0.07 M) at room temperature (RT). The requisite alcohol (1.1 eq) and PPh_3 (1.1 eq). Upon complete dissolution, DIAD (1.1 eq) was added dropwise. The reaction mixture was stirred for 1 h at RT, by which time TLC indicated the reaction was complete (Hex/EtOAc, 1:3). The reaction mixture was concentrated to dryness. Some $Ph_3P=O$ was triturated from the reaction mixture with Et_2O. The supernatant was dry-loaded onto silica gel, then purified by flash column chromatography, eluting with Hex/EtOAc, 1:3, to provide the N^9-alkylated product.

General Procedure B: Mitsunobu reaction at N2 position. The appropriate N^9-alkylated product (**3**; 1 eq) was dissolved in THF (0.07 M) at 35 °C, followed by the requisite alcohol (1.1 eq) and PPh_3 (1.1 eq). As soon as the reaction mixture became homogeneous, DIAD (2.5 eq) was added dropwise. The reaction mixture was allowed to stir at 35 °C overnight. The next day, TLC indicated the reaction was complete (Hex/EtOAc, 2:3). The reaction mixture was concentrated to dryness. Some $Ph_3P=O$ was triturated from the reaction mixture with Et_2O. The supernatant was dry-loaded onto silica gel, then purified by flash column chromatography, eluting with Hex/EtOAc, 2:3, to provide the N^2-alkylated product.

General Procedure C: Nucleophilic aromatic substitution at C6 position. The appropriate N^2,N^9-dialkylated purine (**4**; 1 eq) was dissolved in *i*-PrOH (0.1 M). Glycine (5 eq) and K_2CO_3 (5 eq) were added, then the reaction mixture was heated at reflux for 3 d, by which time TLC indicated the reaction was complete (CH_2Cl_2/MeOH/AcOH, 92:7:1). The solvent was removed *in vacuo*. The residue was partitioned between water and Et_2O. The ethereal layer was collected, then the aqueous layer was extracted once again with Et_2O. The aqueous layer was carefully acidifed to pH 5 with 0.1 M HCl, then diluted with sat. NaH_2PO_4. The product was extracted into EtOAc (x4). The pooled EtOAc extractions were washed with water, brine, dried (Na_2SO_4), filtered and concentrated to yield a crude residue that was purified by silica gel flash column chromatography (eluent: CH_2Cl_2/MeOH/AcOH, 92:7:1) to furnish the final molecules **5**.

tert-Butyl (6-chloro-9-isopropyl-9H-purin-2-yl)carbamate (**3a**). General procedure A on a 2 mmol scale with isopropanol. White solid (468 mg, 75%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.04 (s, 1H), 7.56 (s, 1H), 4.92-4.85 (q, *J* =6.8 Hz, 1H), 1.60 (d, *J* = 6.4 Hz, 6H), 1.53 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 152.4, 152.1, 151.0, 150.2, 141.9, 127.9, 81.5, 47.5, 28.2, 22.6; Calcd (M^+): 311.8, Found: 312.0 (M^+).

tert-Butyl (9-benzyl-6-chloro-9H-purin-2-yl)carbamate (**3b**). General procedure A on a 2 mmol scale with benzyl alcohol. White solid (575 mg, 80%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87 (s, 1H), 7.80 (s, 1H), 7.30-7.28 (m, 5H), 5.35 (s, 2H), 1.51 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 152.9, 152.6, 151.2, 150.3, 143.9, 134.6, 129.1, 128.7, 128.2, 127.6, 81.6, 47.7, 28.2; Calcd (M^+): 359.1, Found: 360.2 ($[M+H]^+$).

(2-((tert-Butoxycarbonyl)amino)-9-isobutyl-9H-purin-6-yl)glycine (**3c**). General procedure A on a 2 mmol scale with isobutanol. White solid (540 mg, 83%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.91 (s, 1H), 7.46 (s, 1H), 4.02 (d, *J* = 6.8 Hz, 2H), 2.30-2.23 (m, 1H), 1.53 (s, 9H), 0.94 (d, *J* = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.1, 152.3, 151.2, 150.2, 144.5, 127.8, 81.6, 51.4, 28.9, 28.2, 19.9; Calcd (M^+): 325.1, Found: 326.2 ($[M+H]^+$).

tert-Butyl (6-chloro-9-(naphthalen-1-ylmethyl)-9H-purin-2-yl)carbamate (**3d**). General procedure A on a 2 mmol scale with naphthalene-1-methanol. White solid (795 mg, 97%): ^{1}H NMR (400 MHz, d_6-DMSO): δ 10.38 (s, 1H), 8.56 (s, 1H), 8.41 (d, *J* = 7.6 Hz, 1H), 7.97 (d, *J* = 8 Hz, 1H), 7.91 (d, *J* = 8.4 Hz, 1H), 7.63-7.55 (m, 2H), 7.46 (t, *J* = 7.8 Hz, 1H), 7.33 (d, *J* = 7.2 Hz, 1H), 5.89 (s, 2H), 1.45 (s, 9H); ^{13}C NMR (100 MHz, d_6-DMSO): □ 153.4, 153.0, 151.3, 149.6, 146.6, 133.7, 132.1, 130.7, 129.1, 127.4, 127.1, 126.7, 126.5, 126.0, 123.8, 80.1, 79.6, 44.9, 28.4; Calcd (M^+): 409.1, Found: 410.2 ($[M+H]^+$).

tert-Butyl (6-chloro-9-isopropyl-9H-purin-2-yl)(isopropyl)carbamate (**4aa**). General procedure B on a 1 mmol scale with **3a** and isopropanol. Pale yellow gum, contaminated with DIAD-H_2 (350 mg, 99%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.12 (s, 1H), 4.88-4.82 (m, 1H), 4.63-4.57 (m, 1H), 1.64 (d, *J* = 7.2 Hz, 6H), 1.42 (s, 9H), 1.30 (d, *J* = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.9, 153.8, 151.9, 150.3, 142.9, 129.5, 80.8, 49.9, 48.1, 28.2, 22.4, 20.9; Calcd (M^+): 353.1, Found: 354.1 ($[M+H]^+$).

tert-Butyl (9-benzyl-6-chloro-9H-purin-2-yl)(sec-butyl)carbamate **(4ba).** General procedure B on a 1 mmol scale with **3b** and *sec*-butanol. Pale yellow gum, contaminated with DIAD-H_2 (362 mg, 87%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.04 (s, 1H), 7.33-7.26 (m, 5H), 5.38 (s, 2H), 4.39-4.34 (m, 1H), 1.83-1.76 (m, 1H) 1.59-1.52 (m, 1H), 1.42 (s, 9H), 1.27 (d, *J* = 7.2 Hz, 3H), 0.94 (t, *J* = 8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.8, 154.2, 152.4, 150.4, 144.7, 134.6, 129.2, 128.8, 127.9, 80.9, 56.0, 47.9, 28.3, 28.2, 21.9, 18.9, 11.4; Calcd (M^+): 415.2, Found: 416.2 ($[M+H]^+$).

tert-Butyl benzyl(6-chloro-9-isobutyl-9H-purin-2-yl)carbamate (**4cb**). General procedure B on a 1 mmol scale with **3c** and benzyl alcohol. Pale yellow gum (345 mg, 83%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.93 (s, 1H), 7.34 (d, *J* = 8.0 Hz, 2H), 7.24 (d, *J* = 7.2 Hz, 2H), 7.16 (t, *J* = 7.4 Hz 1H), 5.15 (s, 2H), 3.94 (d, *J* = 8 Hz, 2H) 2.20-2.14 (m, 1H), 1.45 (s, 9H), 0.89 (d, *J* = 6.4 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.1, 153.9, 152.3, 150.3, 144.9, 138.5, 128.2, 127.9, 127.5, 126.9, 81.9, 51.5 (2), 28.9, 28.1, 19.9; Calcd (M^+): 415.2, Found: 416.3 ($[M+H]^+$).

tert-Butyl benzyl(6-chloro-9-(naphthalen-1-ylmethyl)-9H-purin-2-yl)carbamate (**4db**). General procedure B on a 1 mmol scale with **3e** and benzyl alcohol. Pale yellow gum (356 mg, 87%): ^{1}H NMR (400 MHz, d_6-DMSO): δ 8.73 (s, 1H), 8.29-8.27 (m, 1H), 7.98-7.96 (m, 1H), 7.91 (d, J = 8.4 Hz, 1H), 7.57-7.53 (m, 2H), 7.43 (t, J = 7.8 Hz, 1H), 7.29 (d, J = 7.2 Hz, 1H), 7.20-7.17 (m, 5H), 5.93 (s, 2H), 5.01 (s, 2H), 1.32 (s, 9H); ^{13}C NMR (100 MHz, d_6-DMSO): δ 154.8, 153.8, 153.0, 149.1, 147.8, 138.6, 133.8, 131.7, 130.7, 129.2, 128.6, 128.0, 127.6, 127.4, 127.2, 126.9, 126.6, 125.9, 123.5, 81.6, 51.3, 45.2, 28.0; Calcd (M^+): 499.2, Found: 500.2 ($[M+H]^+$); t_R = 9.64 min (99.7%, II).

tert-Butyl (6-chloro-9-(naphthalen-1-ylmethyl)-9H-purin-2-yl)(isobutyl)carbamate (**4dc**)**.** General procedure B on a 1 mmol scale with **3d** and isobutanol. Pale yellow gum, contaminated with DIAD-H_2 after flash column chromatography. The material was used directly in the next step without further attempts of purification.

(2-((tert-Butoxycarbonyl)(isopropyl)amino)-9-isopropyl-9H-purin-6-yl)glycine (**5aa**). General procedure B on a 0.5 mmol scale with **4aa**. White solid (143 mg, 73%): ^{1}H NMR (400 MHz, d6-DMSO): δ 12.53 (s, 1H), 8.25 (s, 1H), 7.97 (s, 1H), 4.71-4.66 (m, 1H), 4.38-4.34 (m, 1H), 4.06 (d, J = 4.8 Hz, 2H), 1.53 (d, J = 6 Hz, 6H), 1.34 (s, 9H), 1.15 (d, J = 6.4 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.1, 154.8, 154.1, 153.6, 149.8, 139.9, 118.3, 79.3, 48.7, 47.1, 42.1, 28.4, 22.5, 21.1; mp = 178–182 ºC; Calcd (M^+): 392.2, Found: 393.1 ($[M+H]^+$); t_R = 12.4 min (100%, I).

(9-Benzyl-2-((tert-butoxycarbonyl)(sec-butyl)amino)-9H-purin-6-yl)glycine (**5ba**). General procedure B on a 0.5 mmol scale with **4ba**. White solid (150 mg, 66%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.52 (s, 1H), 8.26 (s, 1H), 8.01 (s, 1H), 7.29-7.24 (m, 5H), 5.31 (s, 2H), 4.04-3.99 (m, 3H), 1.59-1.52 (m, 1H), 1.40-1.33 (m, 1H), 1.27 (s, 9H), 1.08 (d, J = 6.4 Hz, 3H), 0.85 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.9, 154.8, 154.5, 154.3, 150.2, 141.8, 137.5, 129.0, 128.2, 128.0, 117.7, 79.3, 54.7, 46.7, 42.0, 28.3, 28.1, 19.3, 11.7; mp = 197–200 ºC; Calcd (M^-): 454.2, Found: 353.3 ($[M-Boc-H]^-$); t_R = 13.5 min (100%, I).

(2-(Benzyl(tert-butoxycarbonyl)amino)-9-isobutyl-9H-purin-6-yl)glycine (**5cb**). General procedure B on a 0.5 mmol scale with **4cb**. White solid (177 mg, 78%): ^{1}H NMR (400 MHz, d_6-DMSO): □ 12.55 (s, 1H), 8.09 (s, 1H), 8.00 (s, 1H), 7.32-7.18 (m, 5H), 4.92 (s, 2H), 4.07 (d, J = 5.6 Hz, 2H), 3.89 (d, J = 7.6 Hz, 2H), 2.19-2.15 (m, 1H), 1.38 (s, 9H), 0.83 (d, J = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, d_6-DMSO): δ 172.1, 155.2, 154.6, 150.4, 141.9 (2), 139.5, 128.4, 127.8, 127.0, 117.0, 80.3, 51.3, 50.5, 42.0, 28.7, 28.3, 20.1; mp = 168–171 ºC; Calcd (M^-): 454.2, Found: 353.3 ($[M-Boc-H]^-$); t_R = 12.3 min (98.8%, I).

(2-(Benzyl(tert-butoxycarbonyl)amino)-9-(naphthalen-1-ylmethyl)-9H-purin-6-yl)glycine (**5db**). General procedure B on a 0.5 mmol scale with **4db**. White solid (162 mg, 74%): ^{1}H NMR (400 MHz, d_6-DMSO): δ 12.58 (s, 1H), 8.31 (d, J = 7.2 Hz, 1H), 8.19 (s, 1H), 8.12 (s, 1H), 7.97 (d, J = 8 Hz, 1H), 7.90 (d, J = 8 Hz, 1H), 7.57-7.54 (m, 2H), 7.45 (t, J = 7.8 Hz, 1H), 7.29 (d, J = 7.2 Hz, 2H), 7.24-7.18 (m, 4H), 5.81 (s, 2H), 4.96 (s, 2H), 4.10 (d, J = 5.6 Hz, 2H), 1.29 (s, 9H); ^{13}C NMR (100 MHz, d_6-DMSO): δ 172.0, 155.5, 154.6, 154.5, 150.3, 141.6, 139.5, 133.8, 132.9, 130.8, 129.1, 128.9, 128.5, 127.7, 127.1, 127.0, 126.5, 126.3, 125.9, 123.6, 116.8, 80.4, 51.2, 44.2, 42.1, 28.2; mp = 194–197 ºC (dec.); Calcd (M^-): 538.23, Found: 437.3 ($[M-Boc-H]^-$); t_R = 16.3 min (100%, I).

(2-((tert-Butoxycarbonyl)(isobutyl)amino)-9-(naphthalen-1-ylmethyl)-9H-purin-6-yl)glycine (**5dc**). General procedure B on a 0.5 mmol scale with **4dc**. White solid (174 mg, 69%): ^{1}H NMR (400 MHz, d_6-DMSO): δ 12.51 (s, 1H), 8.32-8.30 (m, 1H), 8.18 (s, 1H), 8.06 (s, 1H), 7.98-7.95 (m, 1H), 7.90 (d, *J* = 7.6 Hz, 1H), 7.57-7.54 (m, 2H), 7.45 (t, *J* = 7.8 Hz, 1H), 7.26 (d, *J* = 6.8 Hz, 1H), 5.81 (s, 2H), 4.06 (d, *J* = 8 Hz, 2H), 3.53 (d, *J* = 6.8 Hz, 2H), 1.81-1.77 (m, 1H), 1.28 (s, 9H), 0.77 (d, *J* = 6 Hz, 6H); ^{13}C NMR (100 MHz, d_6-DMSO): δ 171.9, 155.9, 154.7, 151.2, 150.3, 141.6, 133.8, 132.9, 130.8, 129.1, 128.9, 127.1, 126.5, 126.4, 125.9, 123.6 (2), 79.7, 54.9, 44.2, 42.1, 28.2 (2), 20.3; mp = 170–173 ºC (dec.); Calcd (M^-): 504.3, Found: 403.5 ([M-Boc-H]$^-$); t_R = 15.7 min (96.9%, I).

(2-(Benzylamino)-9-(naphthalen-1-ylmethyl)-9H-purin-6-yl)glycine (**6db**). *(2-(Benzyl(tert-butoxycarbonyl)amino)-9-(naphthalen-1-ylmethyl)-9H-purin-6-yl)glycine* (**5db**; 30 mg, 0.056 mmol, 1 eq) was dissolved in anhydrous CH_2Cl_2 (1 mL), then TFA (1 mL) was added. The reaction mixture was stirred for 1 h at RT, by which time TLC confirmed the reaction was complete. All solvent was removed *in vacuo*, and the residual TFA was azeotroped with $CHCl_3$ (x3). The residue was suspended in ether, then collected by vacuum filtration to yield the title compound as an off-white solid (25 mg, 92%): ^{1}H NMR (400 MHz, d_6-DMSO): δ 8.25 (d, *J* = 8 Hz, 1H), 7.98 (d, *J* = 8.8 Hz, 2H), 7.90 (d, *J* = 7.6 Hz, 1H), 7.58–7.53 (m, 2H), 7.43 (t, *J* = 7.6 Hz, 1H), 7.32 (d, *J* = 6.8 Hz, 3H), 7.27-7.19 (m, 4H), 5.72 (s, 2H), 4.46-4.08 (m, 6H); ^{13}C NMR (100 MHz, d_6-DMSO): δ 171.2, 159.9, 159.6, 159.2, 156.7, 142.2, 140.2, 138.9, 133.8, 131.7, 130.7, 129.1, 128.6, 128.0, 127.2, 126.6, 126.0, 123.4, 117.7, 114.8, 45.5, 44.9, 42.5; t_R = 14.9 min (100%, I).

(2-Chloro-9H-purin-6-yl)glycine (**7**). 2,6-Dichloropurine (350 mg, 1.85 mmol, 1 eq) was dissolved in anhydrous DMSO (6 mL). Glycine *tert*-butyl ester.HCl (310 mg, 1.85 mmol, 1 eq) and DIPEA (967 μL, 5.55 mmol, 3 eq) were added to the reaction mixture, which was subsequently heated at 80 ºC for 16 h. TLC confirmed the reaction was complete. Upon cooling to RT, ice-cold water was added. The resulting beige precipitate was collected by vacuum filtration, washing with copious ice-cold water, then dried under vacuum at 50 ºC for 4 h to afford *tert*-butyl (2-chloro-9*H*-purin-6-yl)glycinate as a beige solid (509 mg, 97%): ^{1}H NMR (400 MHz, d_6-DMSO): δ ^{1}H NMR (400 MHz, d_6-DMSO, 60 ºC): δ 12.85 (s, 1H), 8.07 (s, 2H), 4.02 (s, 2H), 1.37 (s, 9H); ^{13}C NMR (100MHz, d_6-DMSO, 60 ºC): δ 168.5, 154.3, 152.2, 139.8, 117.2, 80.3, 42.6, 27.5. *tert*-Butyl (2-chloro-9H-purin-6-yl)glycinate (100 mg, 0.352 mmol) was suspended in anhydrous CH_2Cl_2 (3.5 mL), then TFA (3.5 mL) was added, which afforded complete dissolution of the *tert*-butyl ester. After stirring for 3 h at RT, TLC confirmed the reaction was complete. All solvent was removed *in vacuo*, and the residual TFA was azeotroped with $CHCl_3$ (x3). The residue was suspended in ether, then collected by vacuum filtration to yield the title compound as an off-white solid (76 mg, 95%): ^{1}H NMR (400 MHz, d_6-DMSO): δ ^{1}H NMR (400 MHz, d_6-DMSO, 60 ºC): δ 8.07–7.97 (m, 2H), 4.10 (s, 2H); ^{13}C NMR (100MHz, d_6-DMSO, 60 ºC): δ 171.5, 155.0, 153.2, 140.6, 118.0, 42.4. t_R = 1.87 min (100%, II).

Biology

Materials. All chemical reagents were ACS grade or higher unless otherwise indicated. All buffers were passed through Chelex-100 (Bio-Rad, Hercules, CA) to remove trace metals. The D_2O, D_6-DMSO, and $^{15}NH_4Cl$ were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Proteins. The pET30a expression vector (EMD Millipore, Billerica, MA) was used to express N-terminal His6-tagged Hdm2 N-terminal domain residues 1 to 155 ($Hdm2^{1-115}$) in HMS174 (DE3) cells (EMD Millipore). Briefly, the ^{15}N-labeled protein was purified (>98% final) from inclusion bodies by initial solubilizing in 6 M guanidium chloride in PBS, pH 7.4, with 0.5 mM TCEP. The denatured protein was refolded by rapid dilution into 6-fold volume of PBS, pH 7.4, with 0.5 mM TCEP. The folded protein was then captured on Q-sepharose resin and eluted with a linear gradient of 0 to 2 M NaCl in 10 mM Na_3PO_4, pH 7.0, and 1 mM DTT. Then $(NH_4)_2SO_4$ powder was added to reach a final concentration of 0.8 M by slow addition over 30 minutes and stirring for an additional 30 minutes. The sample was added to a butyl sepharose column preconditioned with 0.8 M $(NH_4)_2SO_4$, 10 mM Na_3PO_4, pH 7.0, and 1 mM DTT buffer. The sample was eluted with a linear gradient of decreasing $(NH_4)_2SO_4$ to a final buffer of 10 mM Na_3PO_4, pH 7.0, and 1 mM DTT. The protein was concentrated using a 10,000 MWCO centrifugal filter concentrator (EMD Millipore) and the concentrate stored frozen in the same buffer. The pLM302 expression vector was constructed to produce His6-MBP (maltose binding protein) tagged recombinant human Mcl-1 residues 172 to 327 ($Mcl\text{-}1^{172-327}$) in HMS174 (DE3) cells (EMD Millipore) using either LB or minimal media supplemented with $^{15}NH_4Cl$ to produce unlabeled or ^{15}N-labeled Mcl-1, respectively. The tagged protein was initially purified from the crude cell lysate by IMAC chromatography (GE Healthcare Life Sciences), and after dialysis to remove the imidazole the affinity tag was cleaved using PreScission Protease (GE Healthcare Life Sciences). A Sephacryl S-200 size exclusion column was used as a final purification step before the protein was concentrated with a 10,000 MWCO centifugal filter concentrator (EMD Millipore). The concentrations of the proteins were determined using the Bio-Rad Protein Assay (Bio-Rad Inc., Hercules, CA) using BSA of a known concentration as the standard (Pierce). The purity of the protein was confirmed using SDS-PAGE analysis and NMR HSQC experiments were done to confirm the protein was properly folded (data not shown).

Peptides. A 6-aminohexanoic acid linker was conjugated to the N-terminus of the Bak BH3 peptide (GQVGRQLAIIGDDINR), capped with fluorescein (on the amino group of the linker), and the peptide was amidated on the C-terminus to give FITC-Ahx-GQVGRQLAIIGDDINR-$CONH_2$, hereafter referred to as "$FITC\text{-}Bak^{71-89}$" (synthesized by Neo BioScience in >95% purity). The p53 peptides were derived from the N-terminal human p53, residues 15-29 (SQETFSDLWKLLPEN) with an amidated C-terminus with either an acetylated ($p53^{15–29}$) or TAMRA-labeled ($TAMRA\text{-}p53^{15–29}$) N-terminus. Each peptide was soluble and stored in H_2O at pH 7. The concentration of the stock solution of unlabeled peptides were determined by quantitative amino acid analysis (Biosynthesis Inc., Lewisville, TX), the concentration of FITC peptides was determined at pH 8.0 using the extinction coefficient for amide-linked FITC, $\varepsilon 494$ = 68,000 $cm^{-1}M^{-1}$, and the concentration of the TAMRA peptide was determined using the extinction coefficient for TAMRA, $\varepsilon 547$ = 65,000 $cm^{-1}M^{-1}$. All peptides were synthesized using solid-state peptide synthesis and their purity was determined to be >95% by high pressure liquid chromatography and mass spectrometry.

Fluorescence anisotropy experiments. Fluorescence anisotropy experiments were conducted using a PHERAstar FS (BMG Labtech) multimode microplate reader equipped with two PMTs for simultaneous measurements of the perpendicular and parallel fluorescence emission. In addition, the absolute anisotropy measurements were made on a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) equipped with automated polarizers.

The fluorescence anisotropy assays were performed in black polypropylene 384-well microplate (Costar) with a final volume of 20 μL. Initially the affinity (K_d) of the FITC-Bak$^{71-89}$ peptide was determined by titrating either Mcl-1$^{172-327}$ or Bcl-xL$^{2-212}$, into 10 nM FITC-Bak$^{71-89}$ peptide in 20 mM HEPES, pH 6.8, 50 mM NaCl, 3 mM DTT, 0.01% Triton X-100 and 5% DMSO at room temperature while monitoring the perpendicular and parallel fluorescence emission with a 485 nm excitation and 520 nm emission filters. The fluorescence anisotropy competition assay was performed using 100 nM Mcl-1$^{172-327}$ or 15 nM Bcl-xL$^{2-212}$ in the same buffer (10 nM FITC-Bak$^{71-89}$ peptide in 20 mM HEPES, pH 6.8, 50 mM NaCl, 3 mM DTT, 0.01% Triton X-100 and 5% DMSO) with varying concentrations of either unlabeled peptide or experimental compounds.

Similarly, the affinity of TAMRA-p53$^{15-29}$ was determined by the titration of Hdm2$^{1-115}$ into 10 nM TAMRA-p53$^{15-29}$ peptide in PBS with 0.01% Triton X-100 and 5% DMSO at room temperature with a 544 nm excitation and 590 nm emission filters. The fluorescence polarization assays (FPCA) were performed using 10 μM Hdm2$^{1-115}$ in the same buffer (PBS with 0.01% Triton X-100 and 5% DMSO) with varying concentrations of unlabeled p53$^{15-29}$ peptide or experimental compounds.

The initial binding affinities (K_d) were determined by fitting the binding data to the Dose Response function in the Origin software (OriginLab, Northampton, MA): $y = A_1 + (A_2 - A_1) / (1+10^{(LOGx0-x)p})$ such that dynamic range = abs (A_1-A_2) and the $K_d = 10^{LOGx0}$. The IC_{50} in the competition assays were determined by fitting the binding data to the One Site Competition function in the Origin software (OriginLab, Northampton, MA): $y = A_2 + (A_1 – A_2) / (1+10^{(x-logx_0)})$ such that dynamic range = abs (A_1-A_2) and the *IC50* = 10^(logx0). It has been shown that each of the proteins used here binds a single target peptide (1:1 stoichiometry) at the concentrations used in the competition assays.[2-4] Therefore, we are able to use an equation derived by Nikolovska-Coleska et al.[5] to calculate the K_d from the IC_{50} from the anisotropy competition assays. The affinity (K_d) of TAMRA-p53$^{15–29}$ for Hdm$^{21-115}$ was determined to be 6.51 ± 0.44 μM, and the affinities of FITC-Bak$^{71-89}$ for Mcl-1$^{172-327}$ and Bcl-xL$^{2-212}$ were determined to be 41.96 ± 2.78 nM and 6.67 ± 0.05 nM, respectively, in the assay conditions used.

The quality and suitability of the fluorescence anisotropy competition assays were evaluated using the Z-factor developed by Zhang et al.[6] The Z-factor = $1 - (3SD_b + 3SD_f) / (|\mu_b-\mu_f|)$ where μ_b and μ_f are the mean anisotropy (mA) values of the bound and free probe, respectively, and SD_b and SD_f are the standard deviations of those values for bound and free probe, respectively. The Z-factor can be any value ≤ 1, with a value of 1 being an ideal assay, ≥ 0.5 but < 1.0 being an excellent assay, and a value < 0.5 being unacceptable for our application.

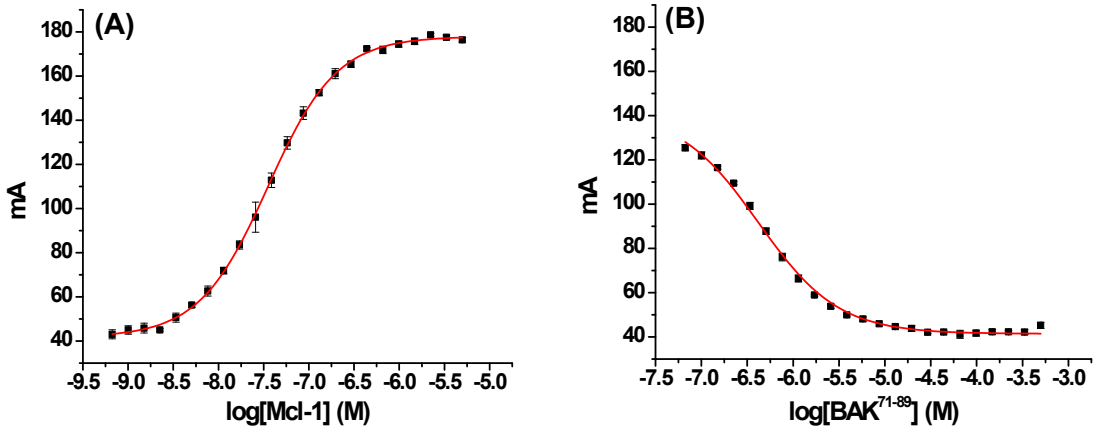

Figure S1. (A) Titration of Mcl-$1^{172-327}$ into 10 nM FITC-BAK^{71-89} gives a K_D of 41.96 ± 2.78 nM with the free FITC-BAK peptide having an absolute anisotropy value of 41.4 ± 1.4 mA (this is the same as Mcl-1 assay b/c same peptide, buffer and conditions) and the Mcl-1 bound peptide 178.0 ± 1.4 mA. (B) The FITC-BAK^{71-89} was competed off Mcl-$1^{172-327}$ with unlabeled BAK^{71-89} peptide with an IC_{50} of 418.19 ± 37.77 nM giving a calculated K_D of 101.84 ± 12.37 nM. For this competition assay 60 nM Mcl-$1^{172-327}$ was used and gives an excellent Z-factor of 0.79 with a dynamic range of 86.96 ± 0.32 mA.

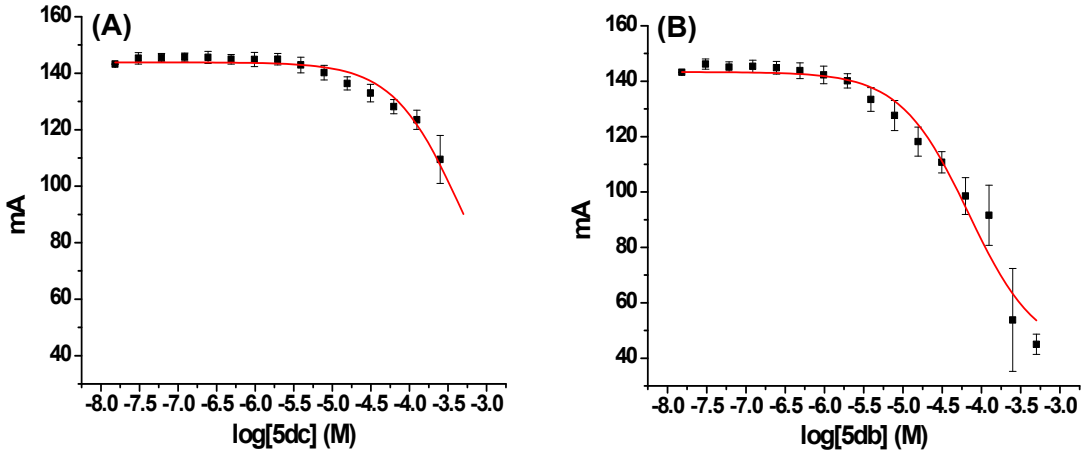

Figure S2. The FITC-BAK^{71-89} was competed off Mcl-$1^{172-327}$ with (A) **5dc** or (B) **5db** giving an estimated IC_{50} of 471.54 ± 123.12 μM and IC_{50} 72.17 ± 21.21 μM, respectively. For all the competition assays used to test compounds, 100 nM Mcl-$1^{172-327}$ was used giving an excellent Z-factor of 0.82 with a dynamic range of 101.8 ± 2.85 mA.

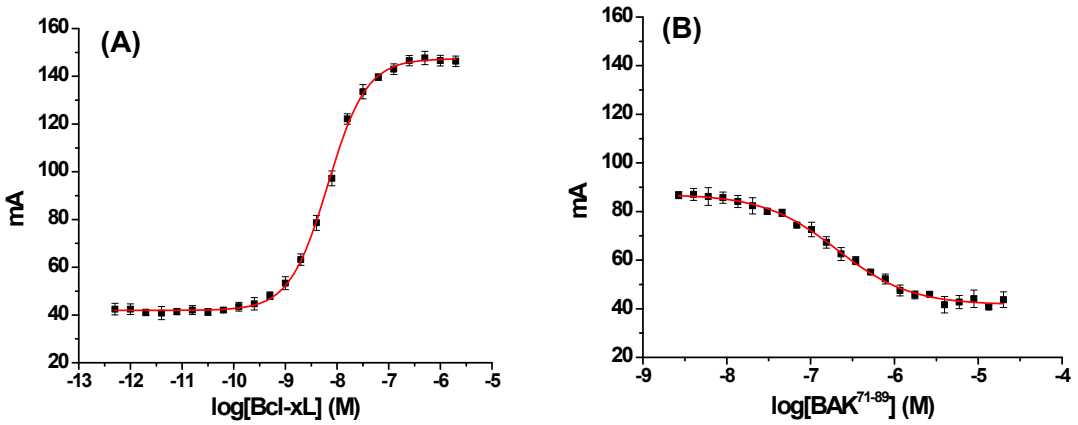

Figure S3. (A) Titration of Bcl-xL$^{2-212}$ into 10 nM FITC-BAK$^{71-89}$ gives a K_D of 6.67 ± 0.05 nM with the free FITC-BAK peptide having an absolute anisotropy value of 41.4 ± 1.4 mA (this is the same for MCL-1 assay because it is the same peptide, buffer and conditions) and the Bcl-xL$^{2-212}$ bound peptide 147.1 ± 1.4 mA. (B) The FITC-BAK$^{71-89}$ was competed off Bcl-xL$^{2-212}$ with unlabeled BAK$^{71-89}$ peptide with an IC_{50} of 206.28 ± 14.77 nM giving a calculated K_d of 57.17 ± 4.30 nM. For this competition assay 15 nM Bcl-xL$^{2-212}$ was used giving an excellent *Z*-factor of 0.91 with a dynamic range of 45.8 ± 0.6 mA.

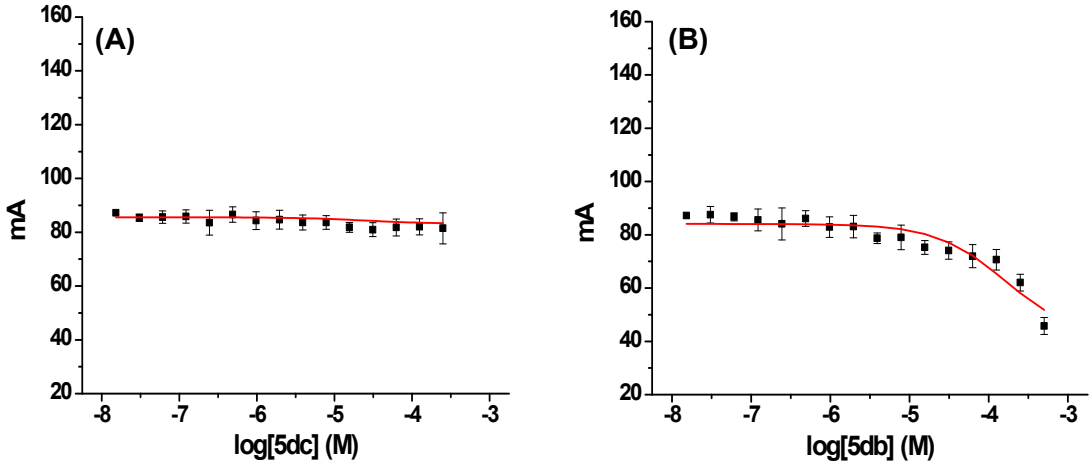

Figure S4. (A) **5dc** does not significantly compete with FITC-BAK$^{71-89}$ binding to Bcl-xL$^{2-212}$. (B) **5db** weakly competed with an estimated IC_{50} of 201.82 ± 51.53 μM. For all the competition assays used to test compounds, 15 nM Bcl-xL$^{2-212}$ was used giving an excellent Z-factor of 0.91 with a dynamic range of 45.8 ± 0.6 mA.

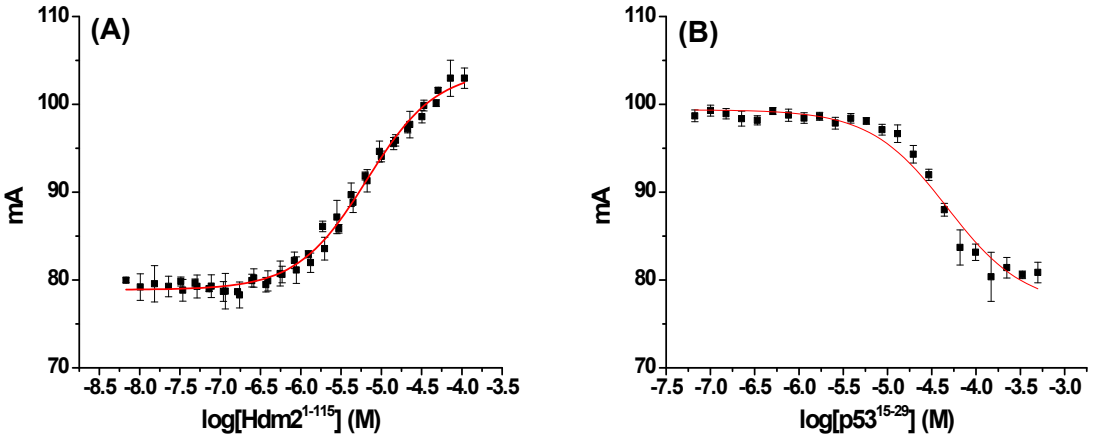

Figure S5. (A) Titration of Hdm2$^{1\text{-}115}$ into 10 nM TAMRA-p53$^{15\text{-}29}$ gives a K_d of 6.51 ± 0.44 μM with the free TAMRA-p53$^{15\text{-}29}$ peptide having an absolute anisotropy value of 80.1 ± 1.3 mA and the Hdm2$^{1\text{-}115}$ bound peptide 102.9 ± 1.5 mA. (B) The TAMRA-p53$^{15\text{-}29}$ was competed with unlabeled p53$^{15\text{-}29}$ with an IC_{50} of 49.36 ± 1.13 μM giving a calculated K_d of 16.64 ± 0.44 μM. Using 10 μM Hdm2$^{1\text{-}115}$ for the competition assay gives a good Z-factor of 0.58 and a dynamic range of 19.57 ± 0.64 mA. The same conditions were used to test all compounds but no competition was seen.

REFERENCES

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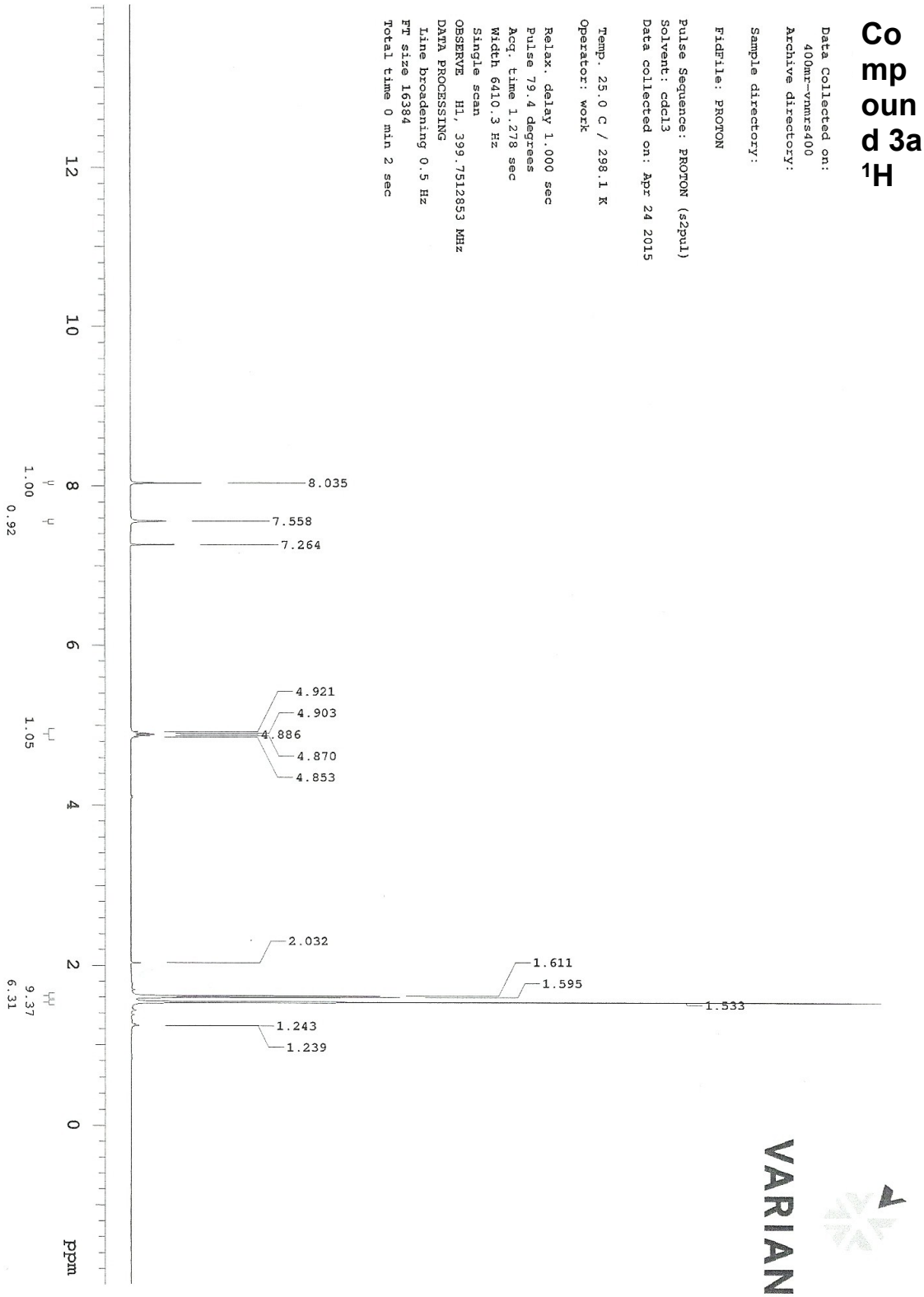
Compound 3a ^1H



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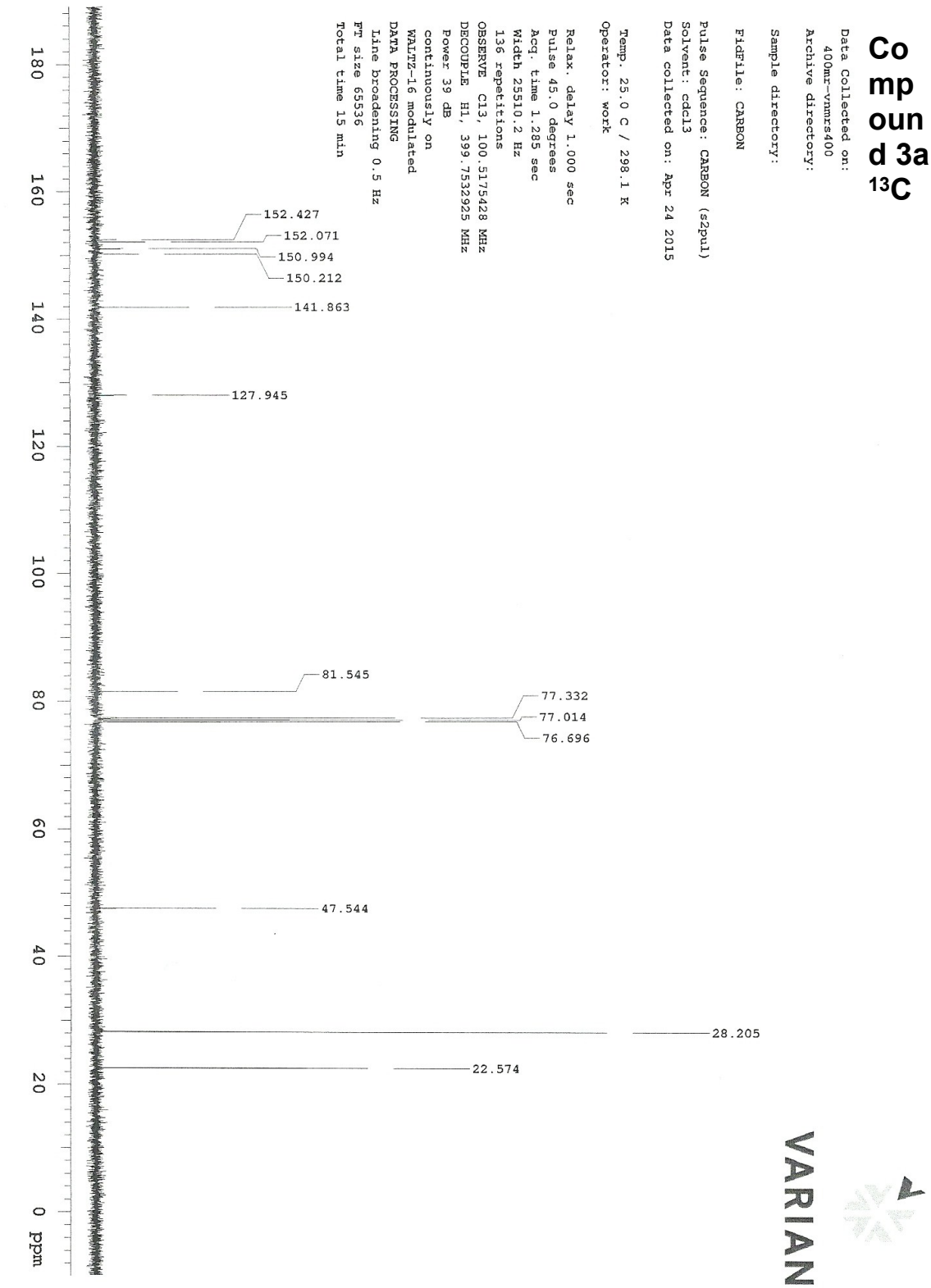

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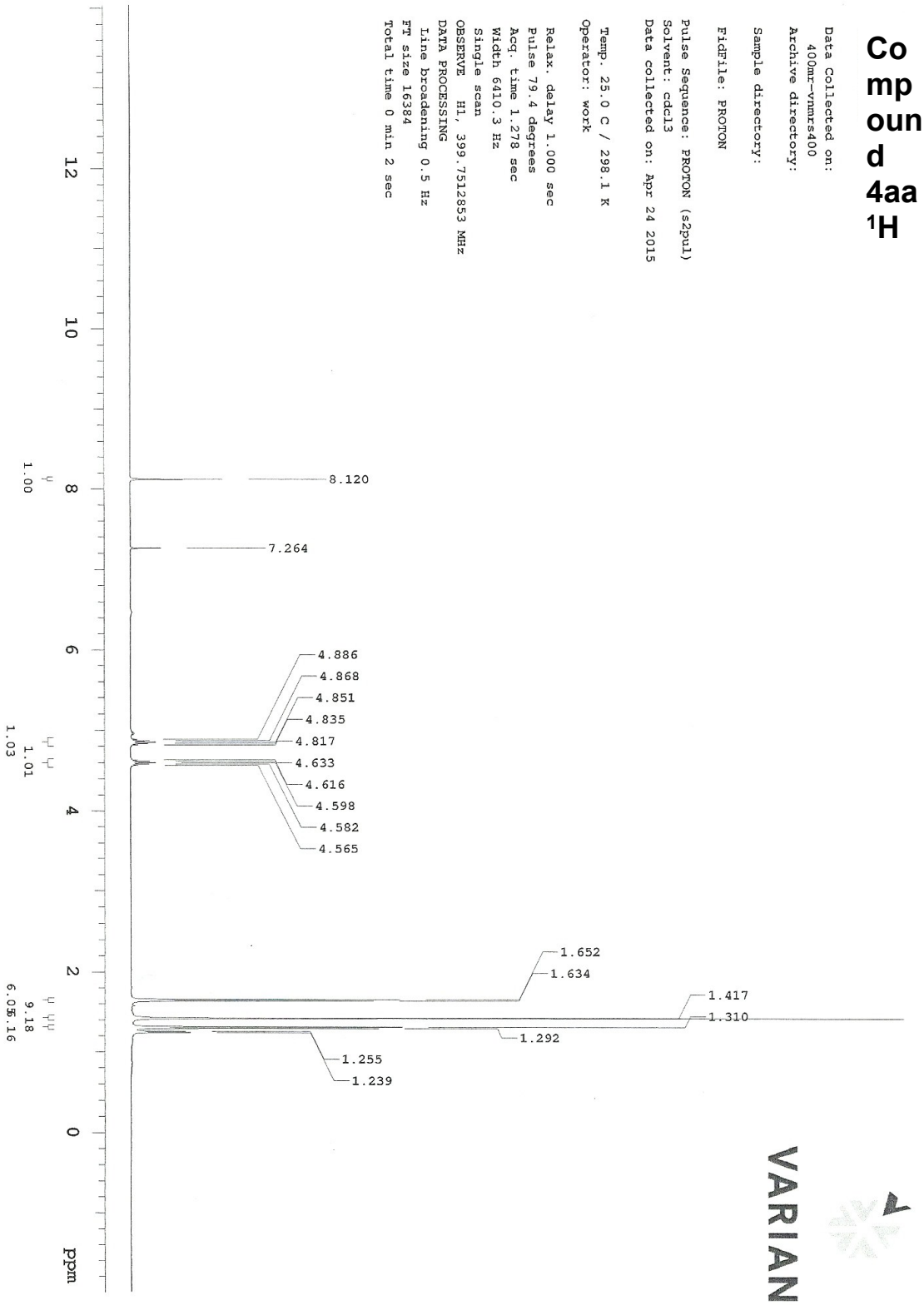

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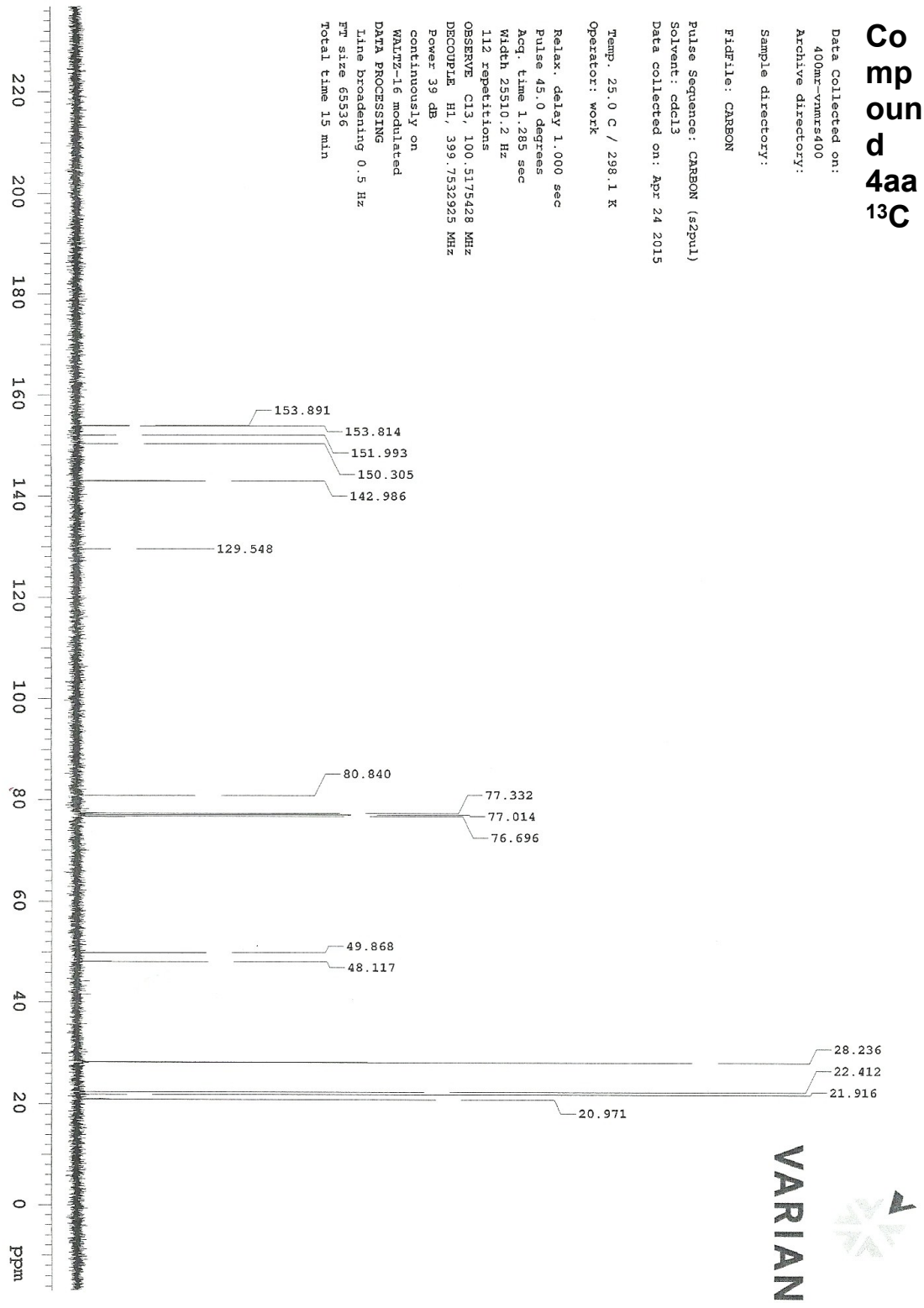

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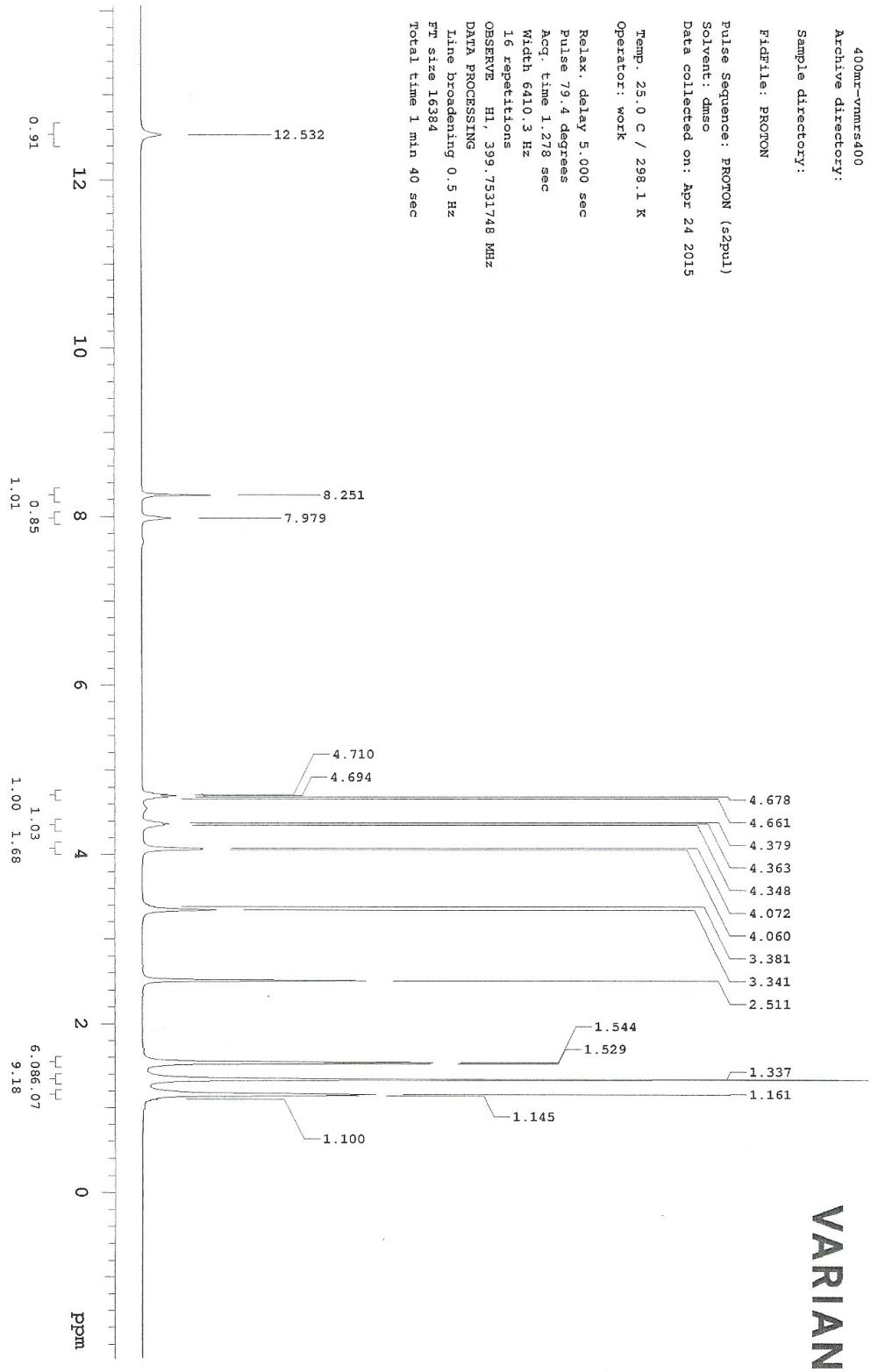

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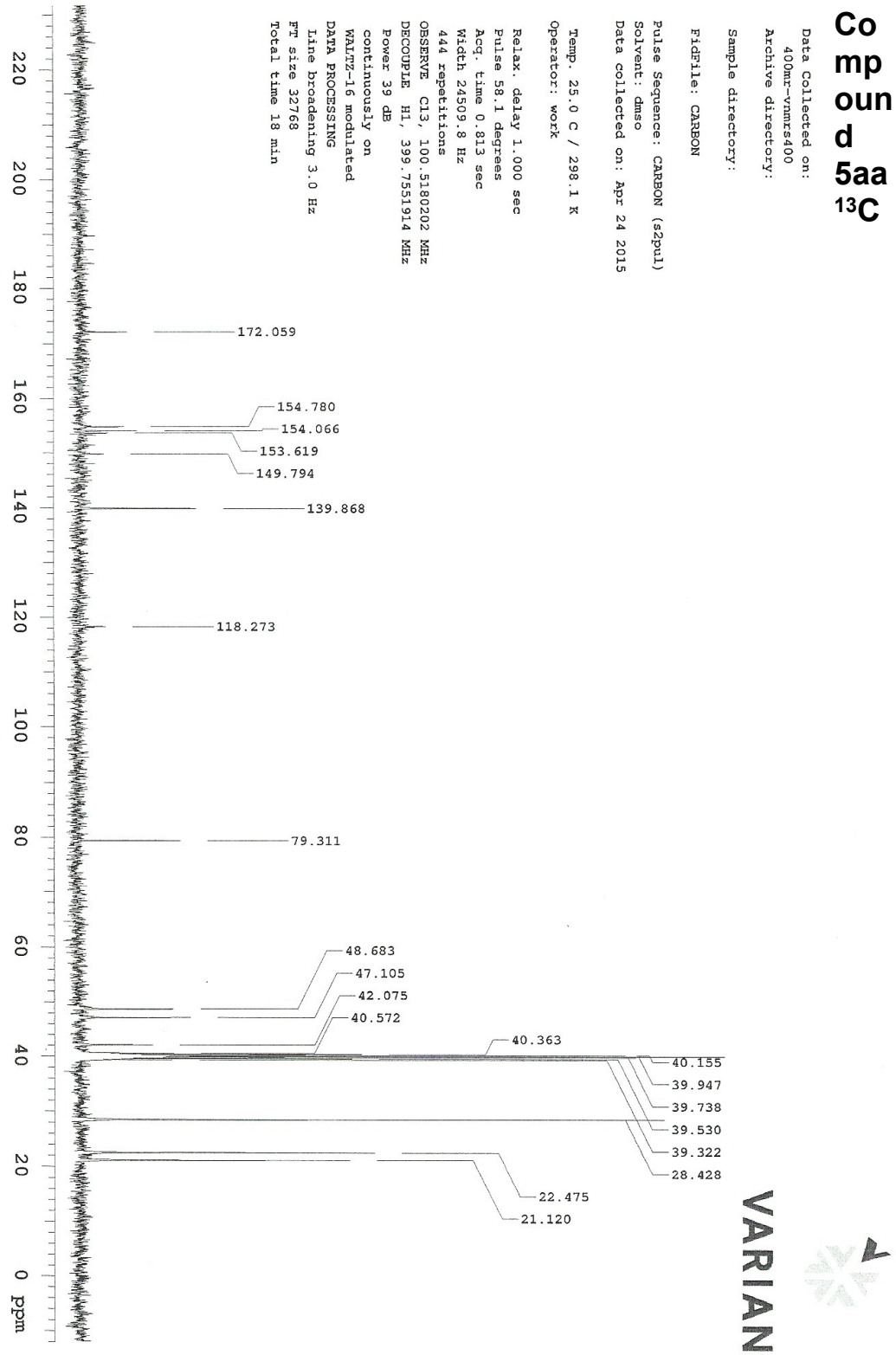

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Sample directory:

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Operator: work

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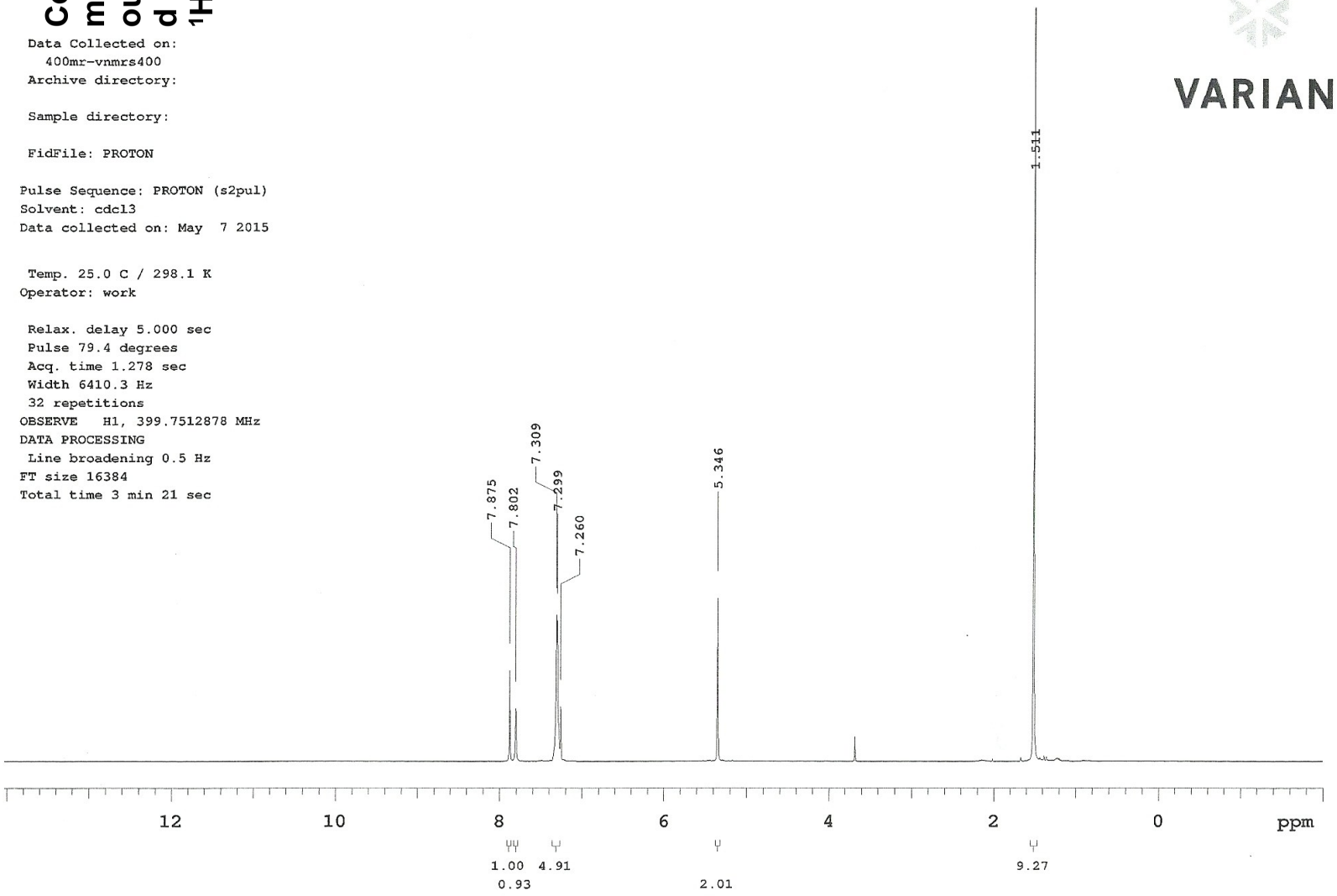

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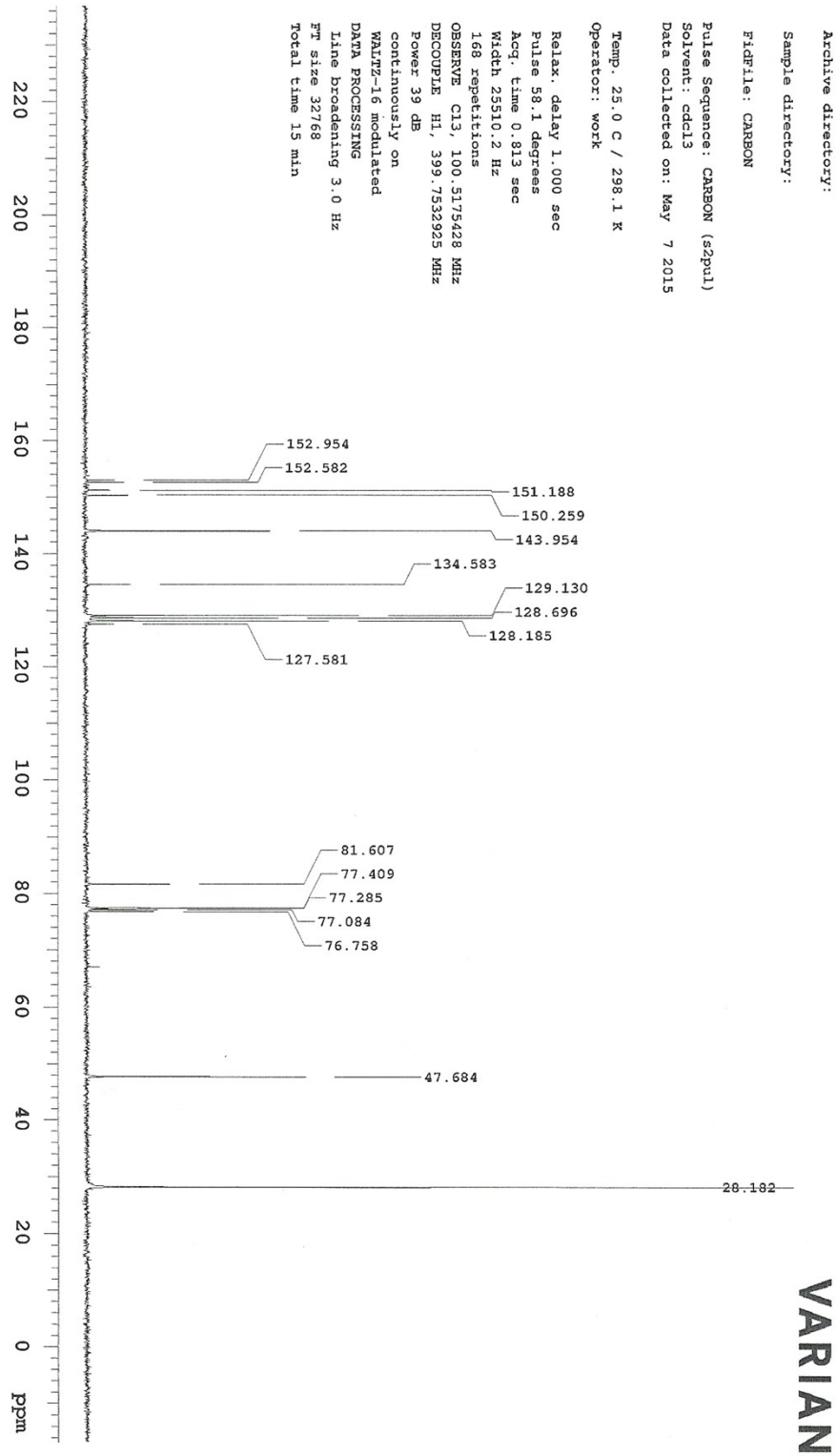

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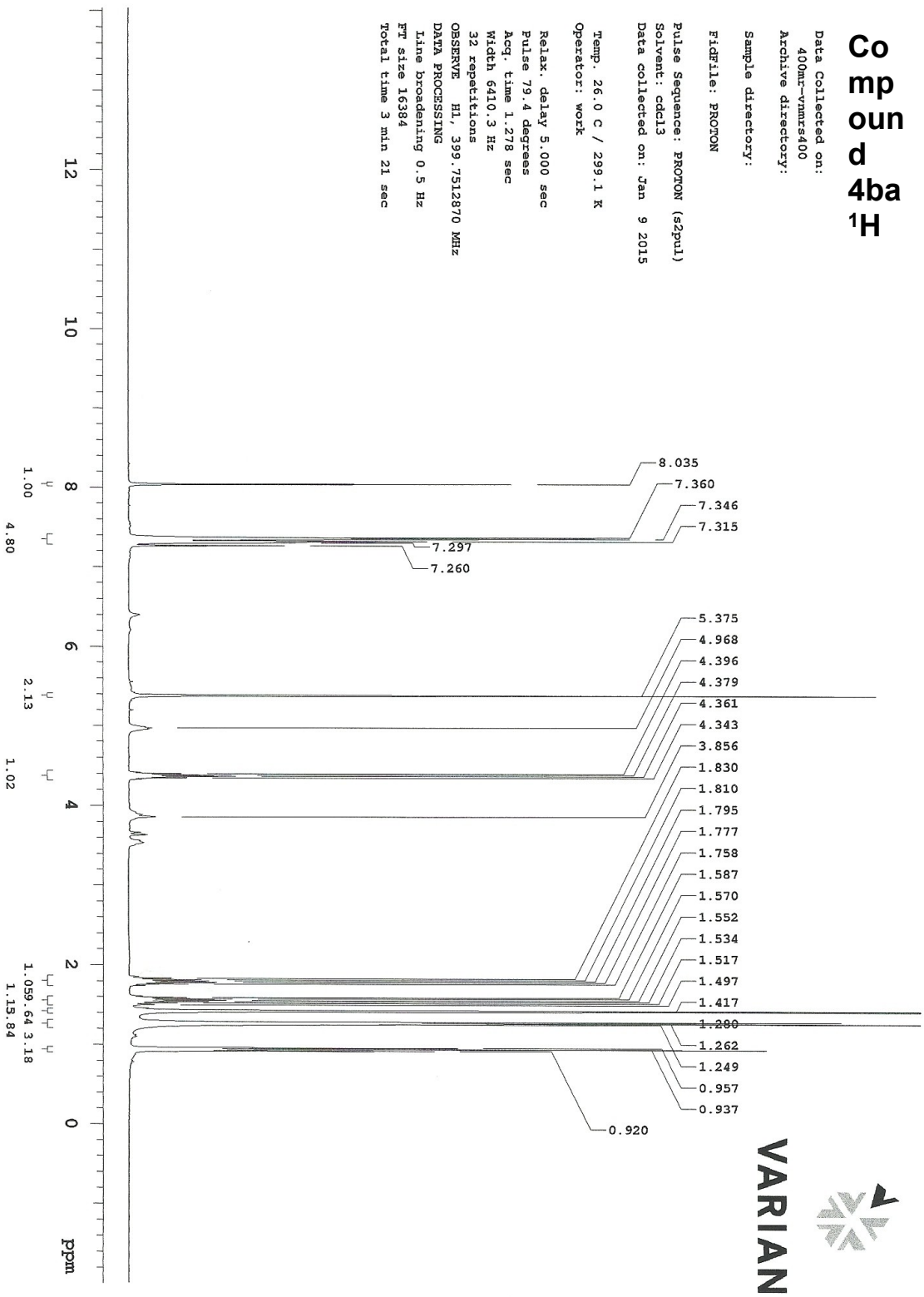

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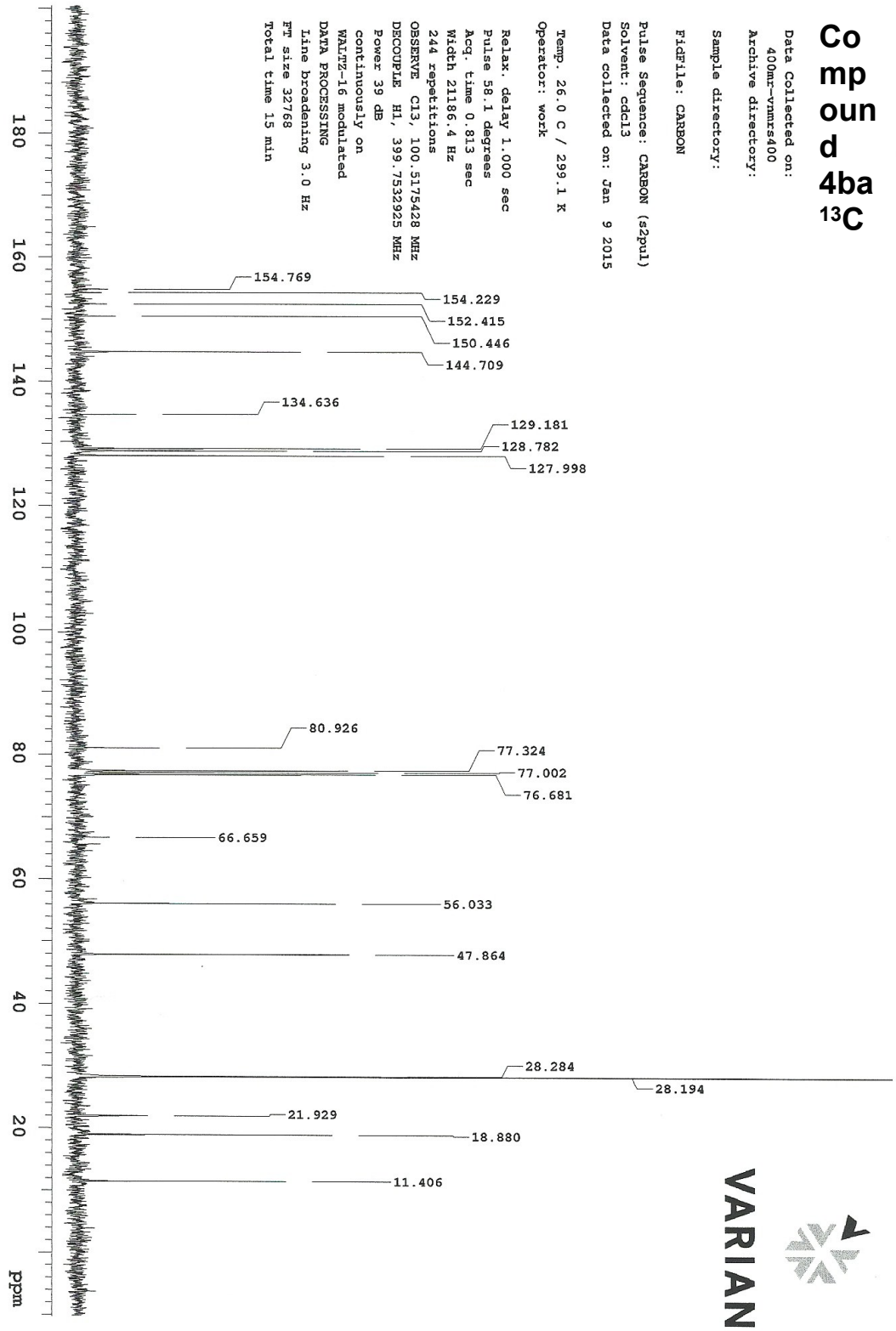

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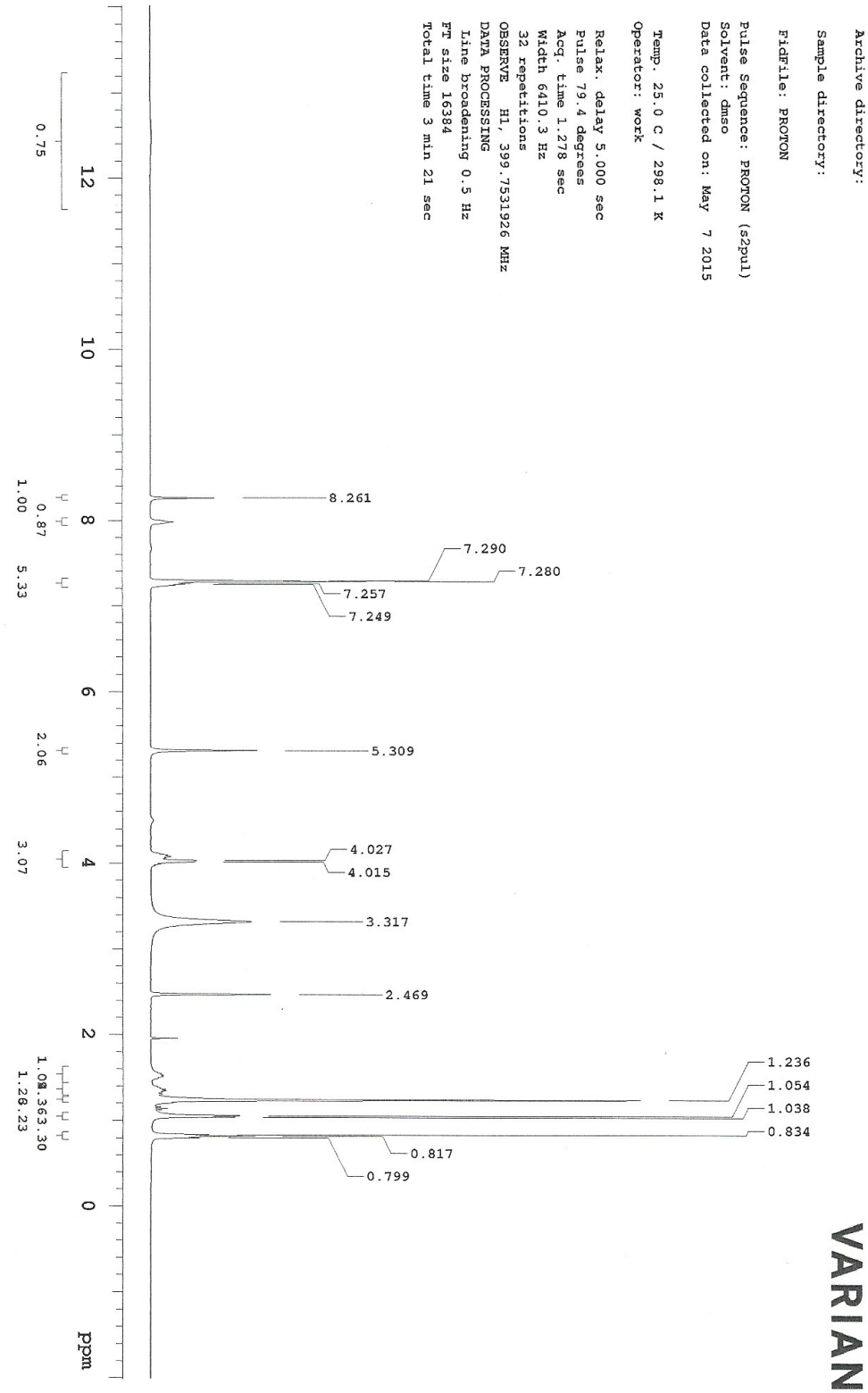

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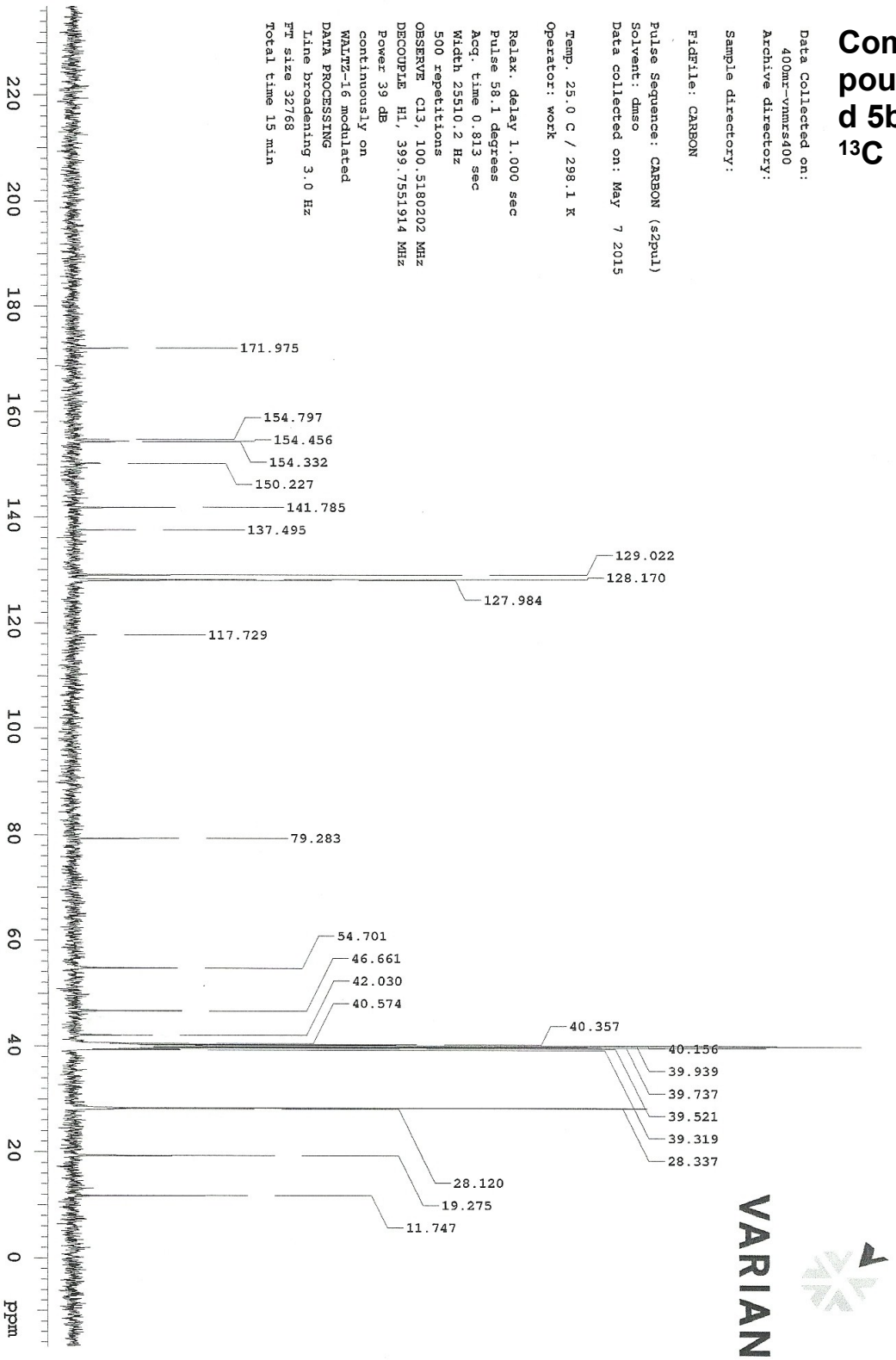

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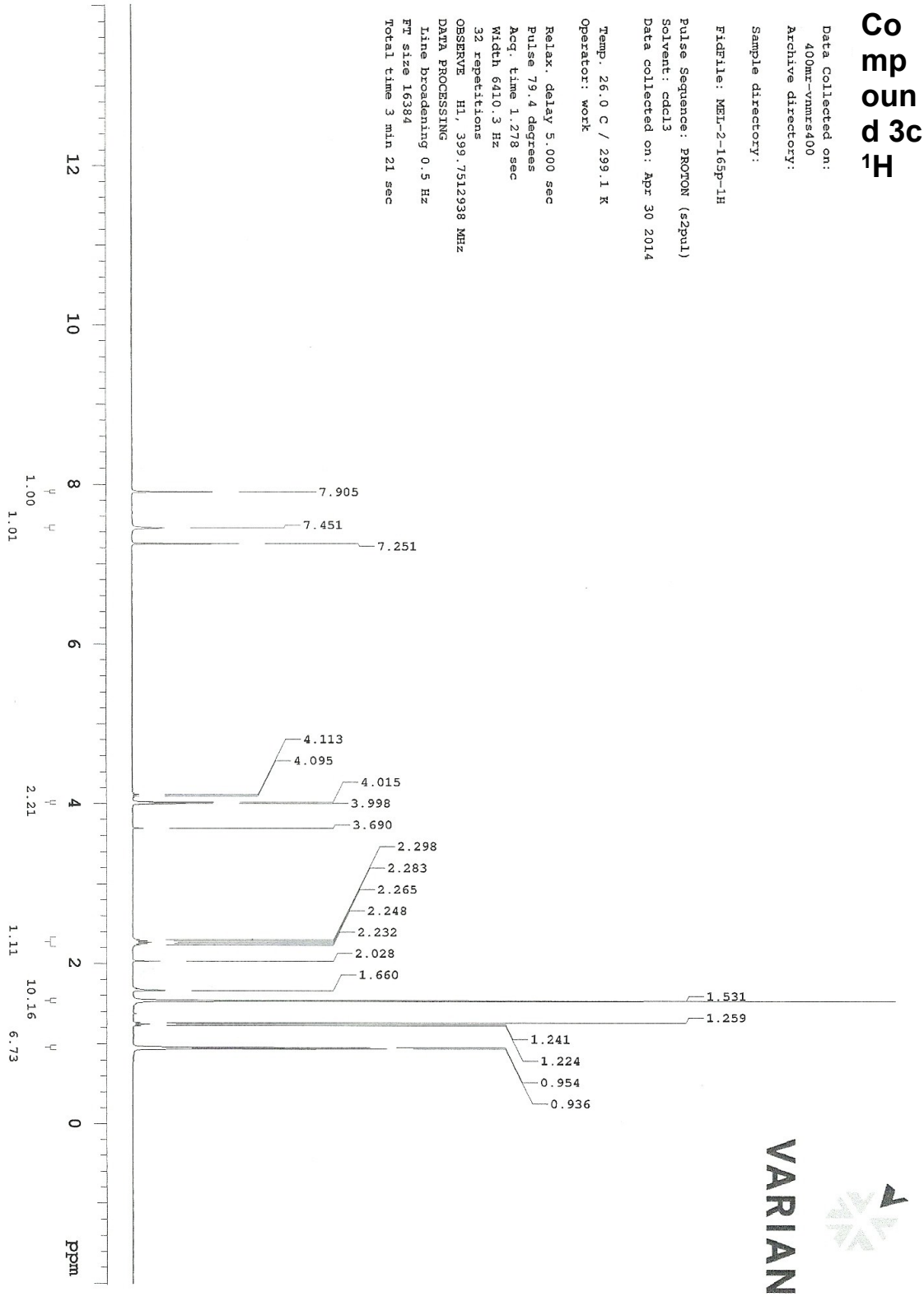

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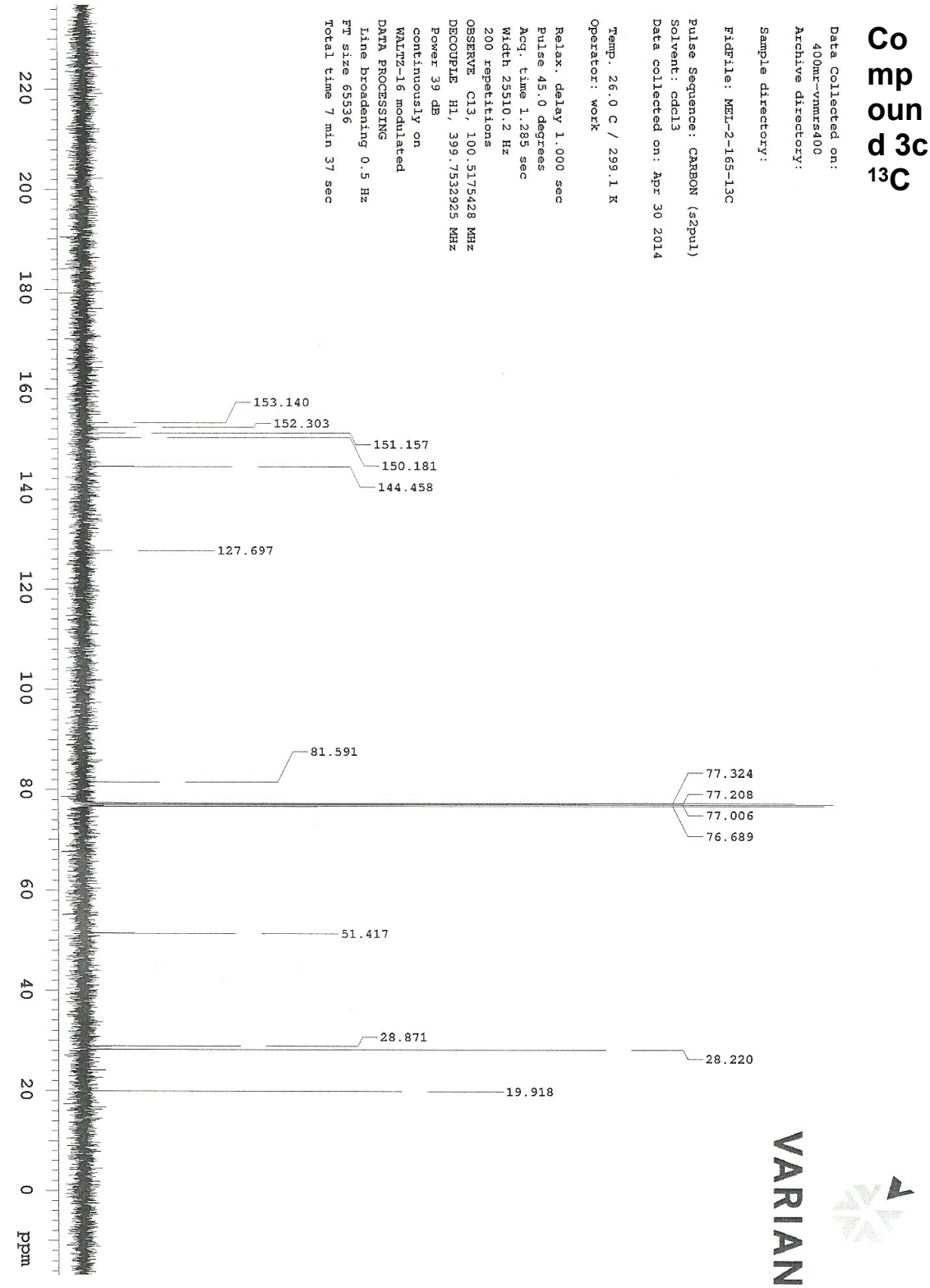

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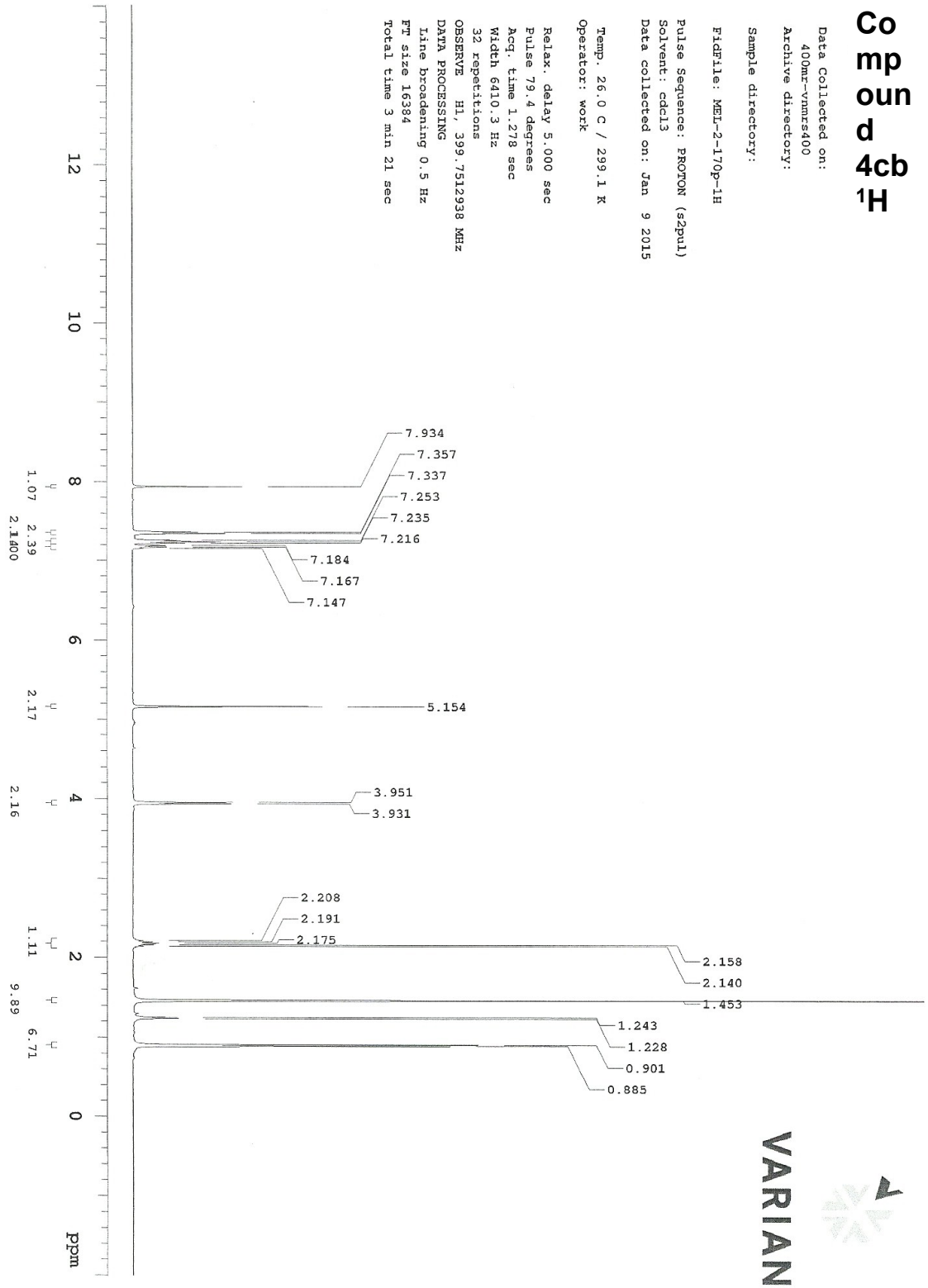

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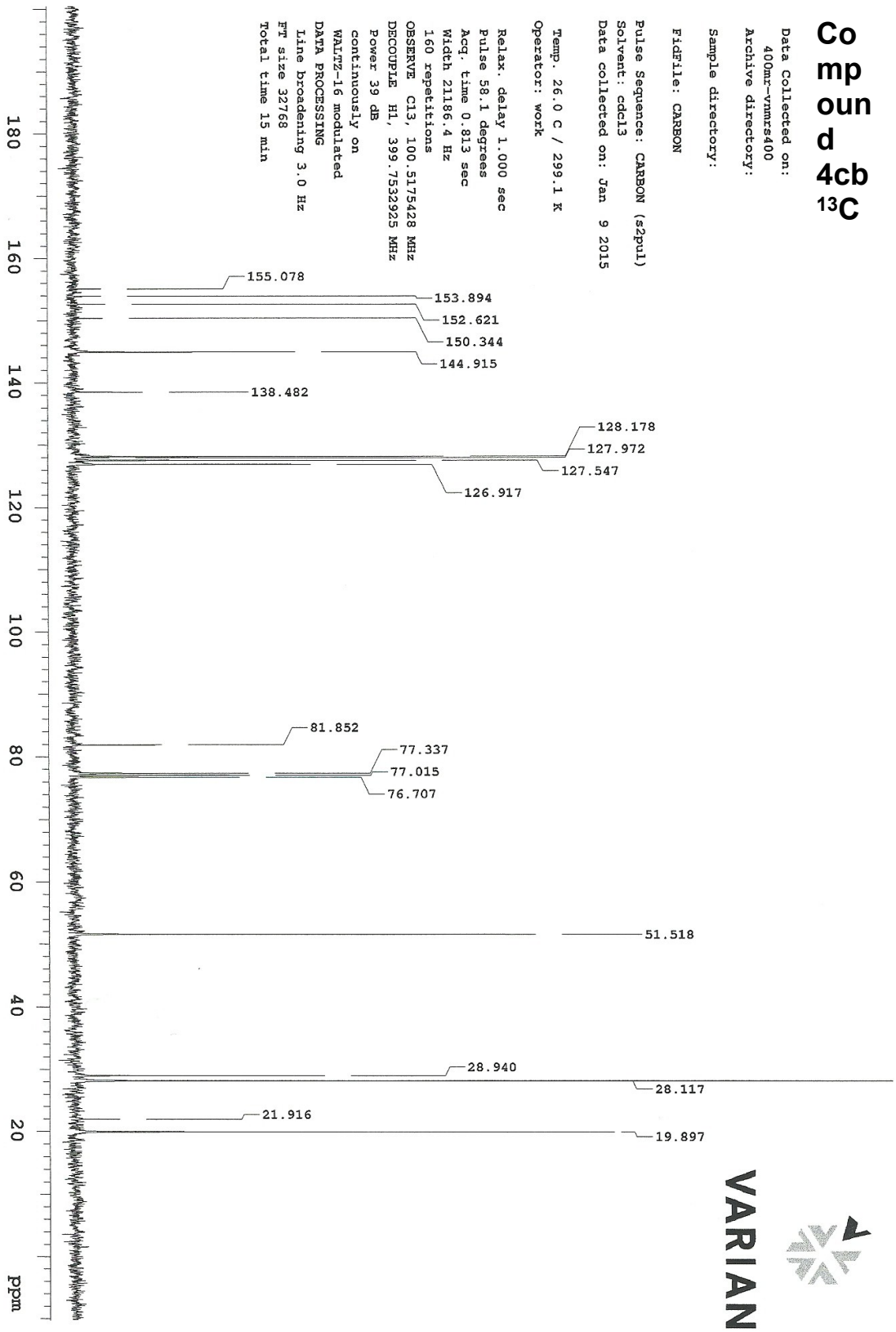

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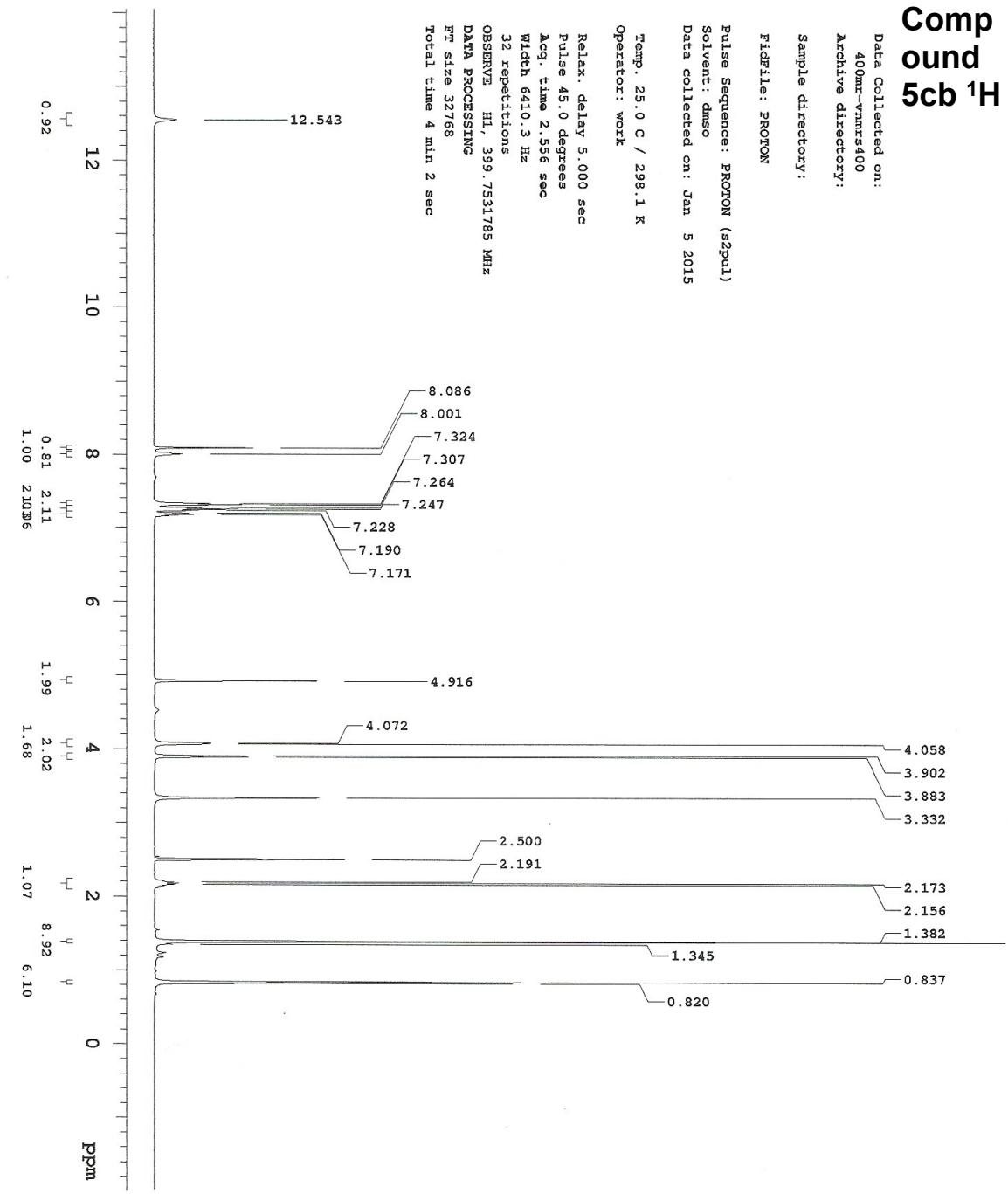

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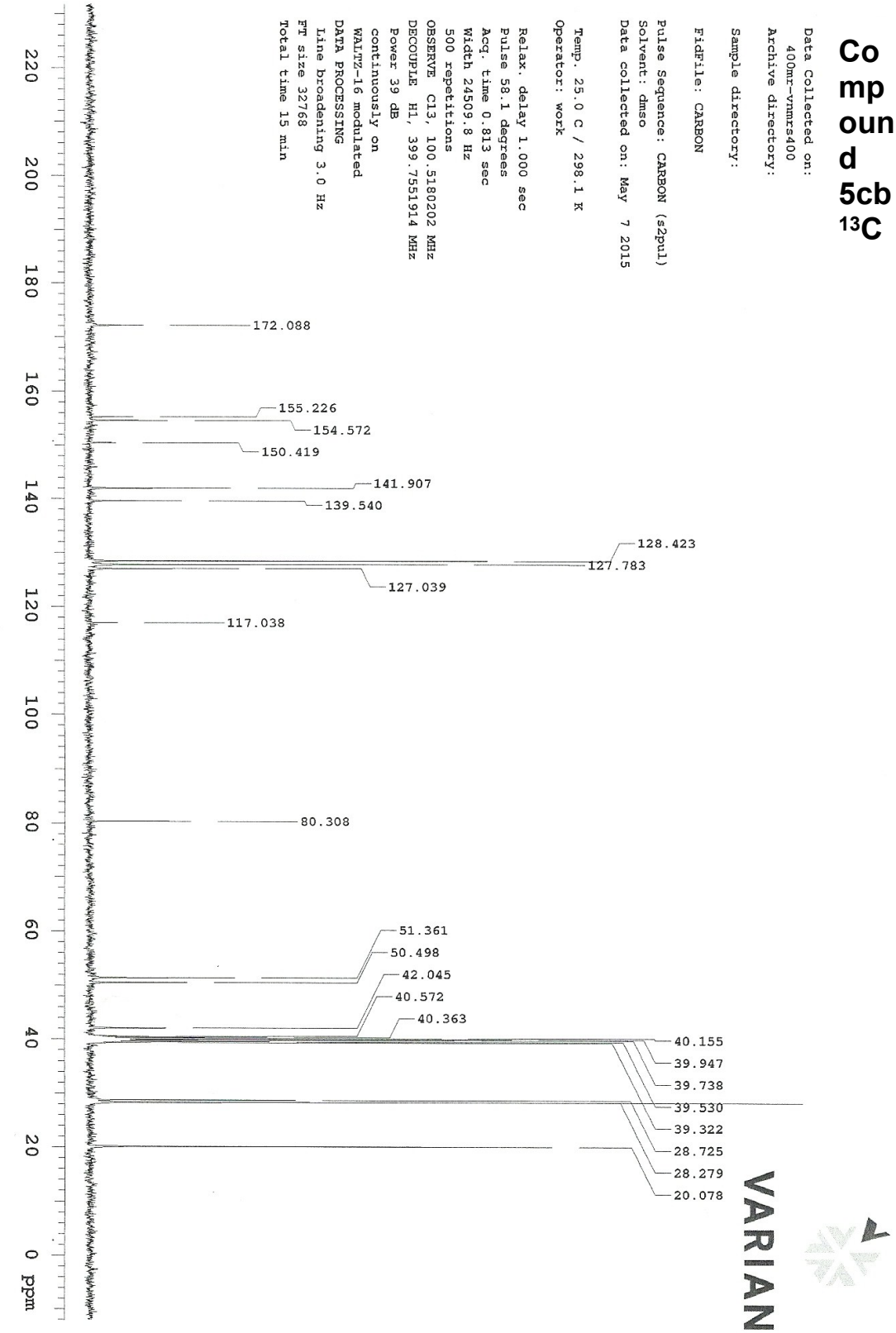

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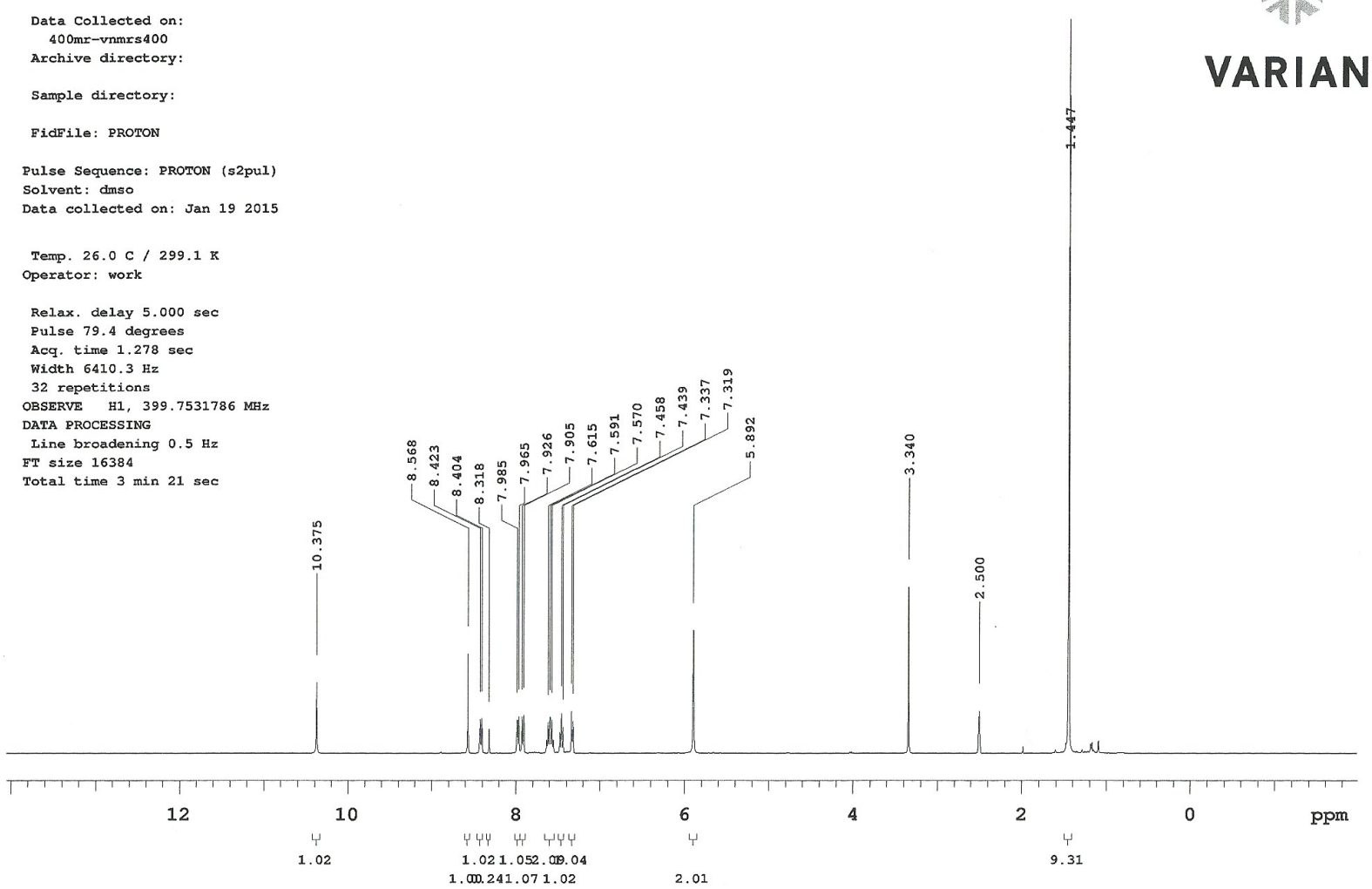

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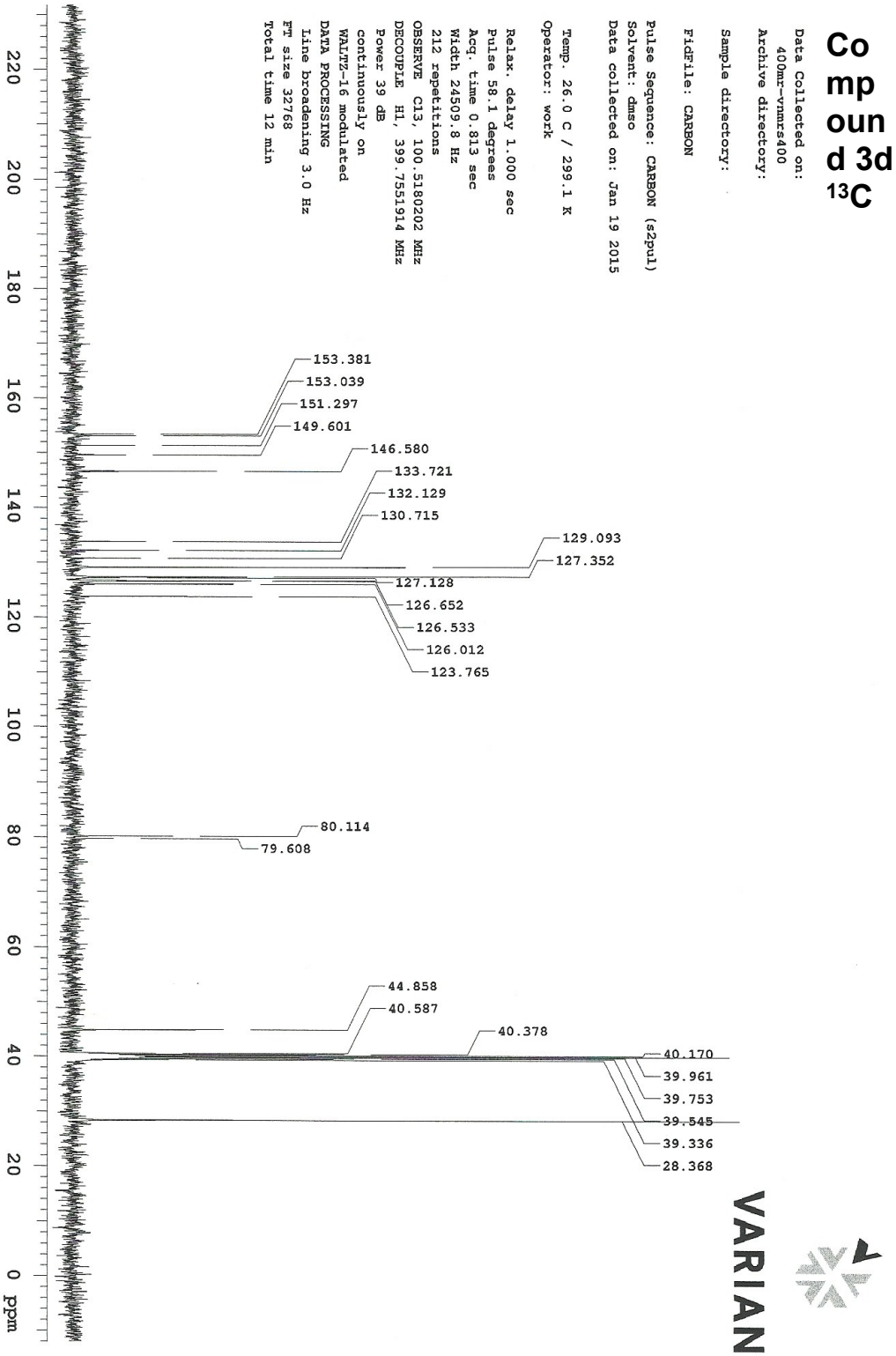

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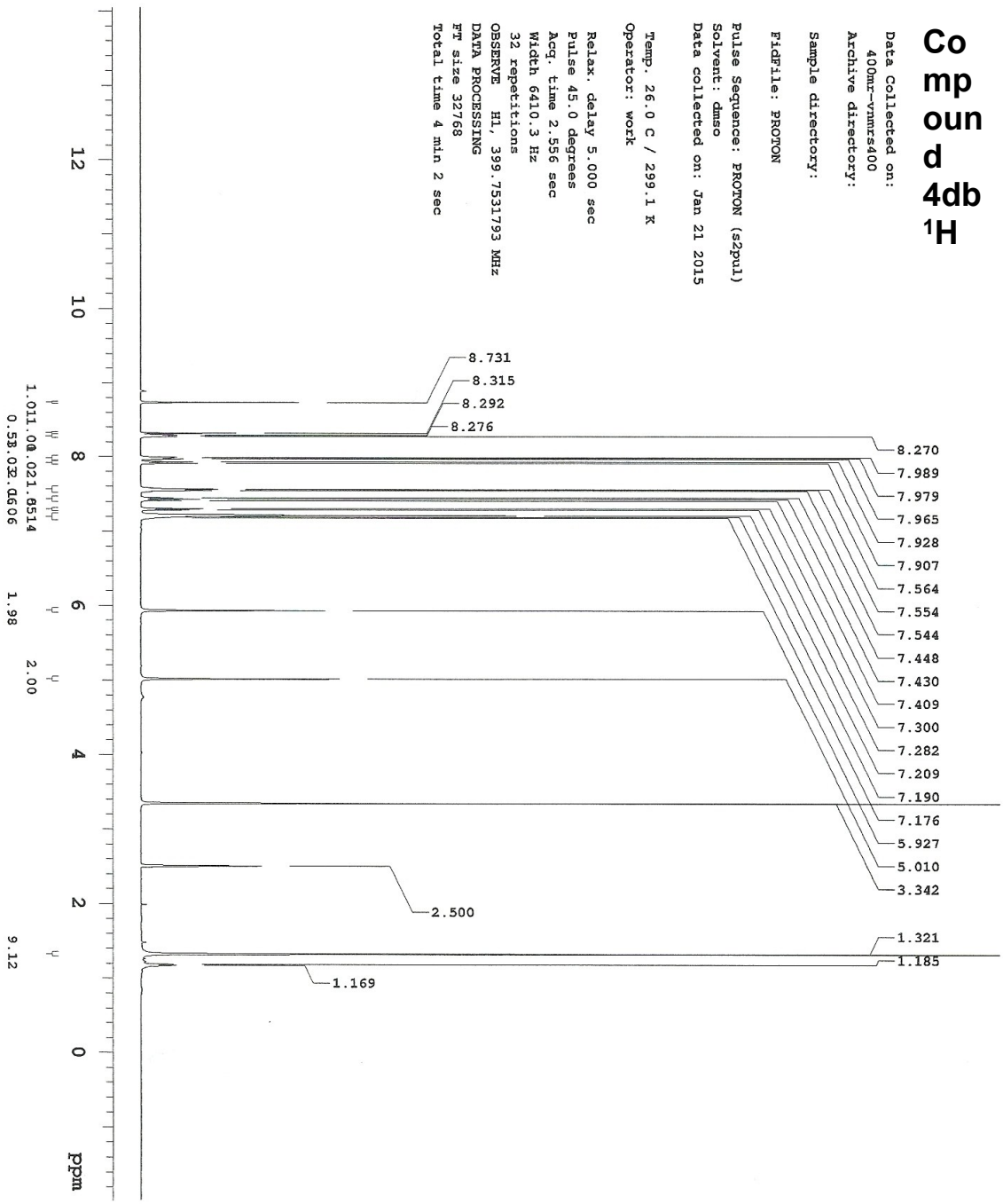

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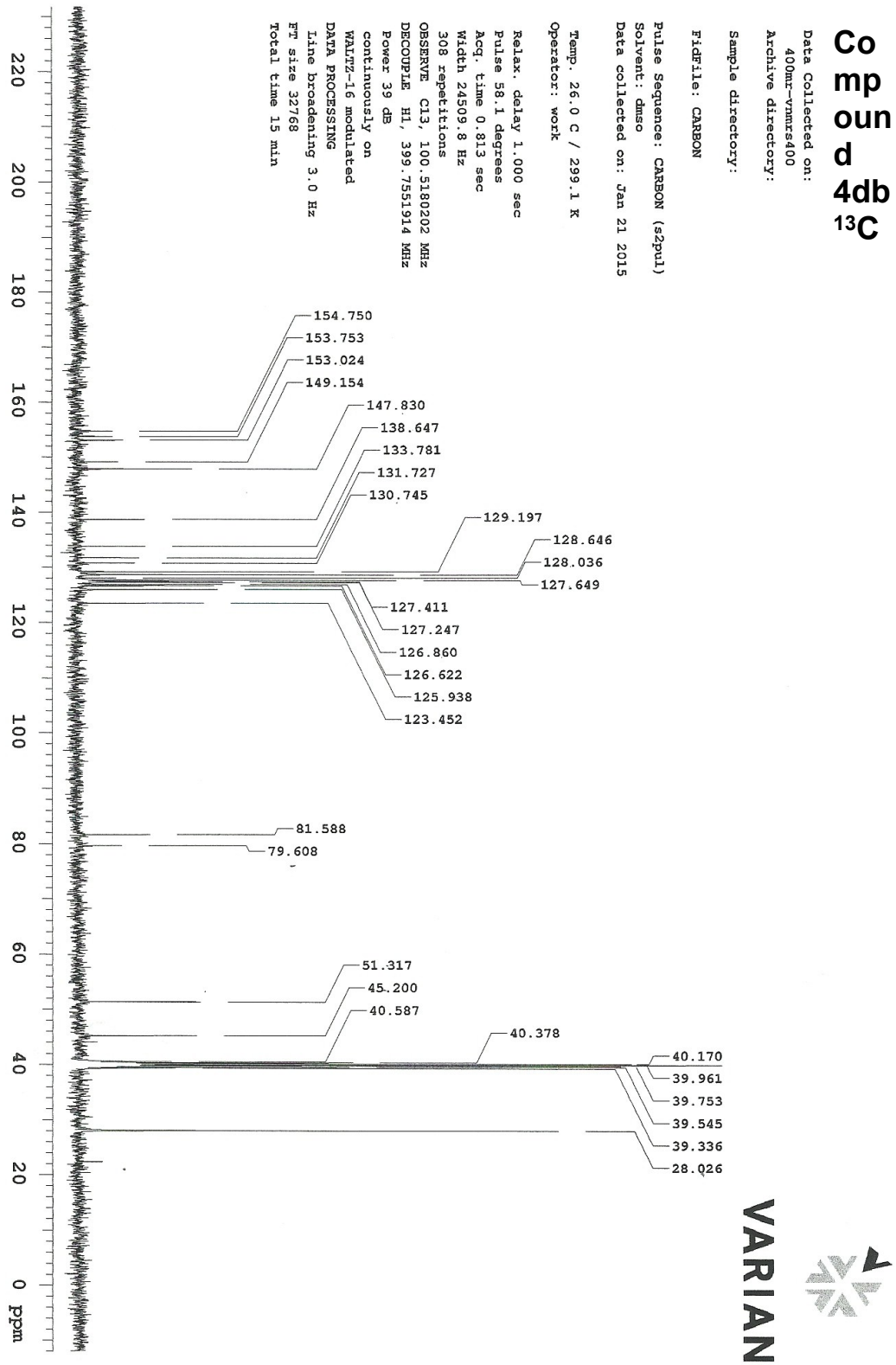

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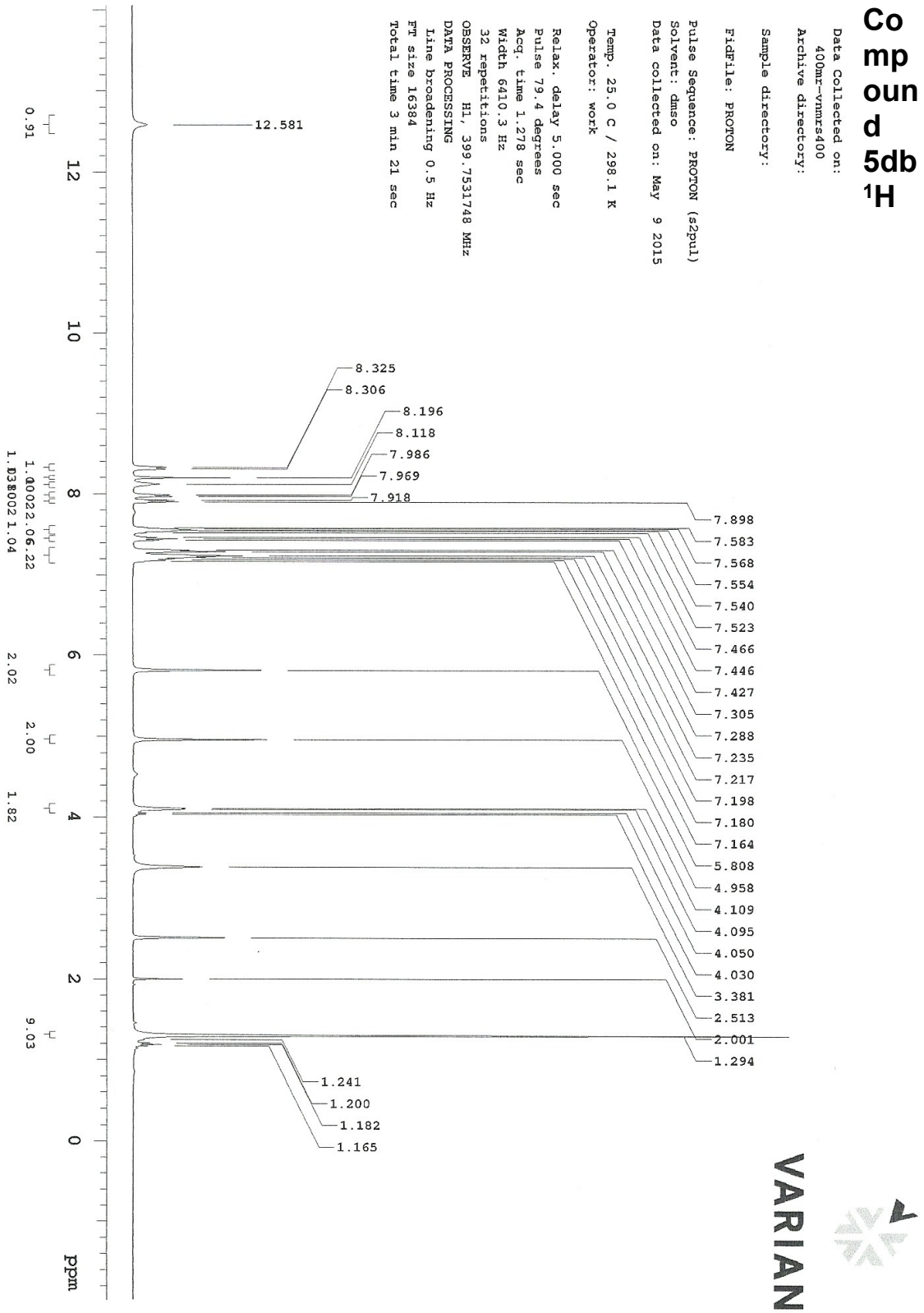

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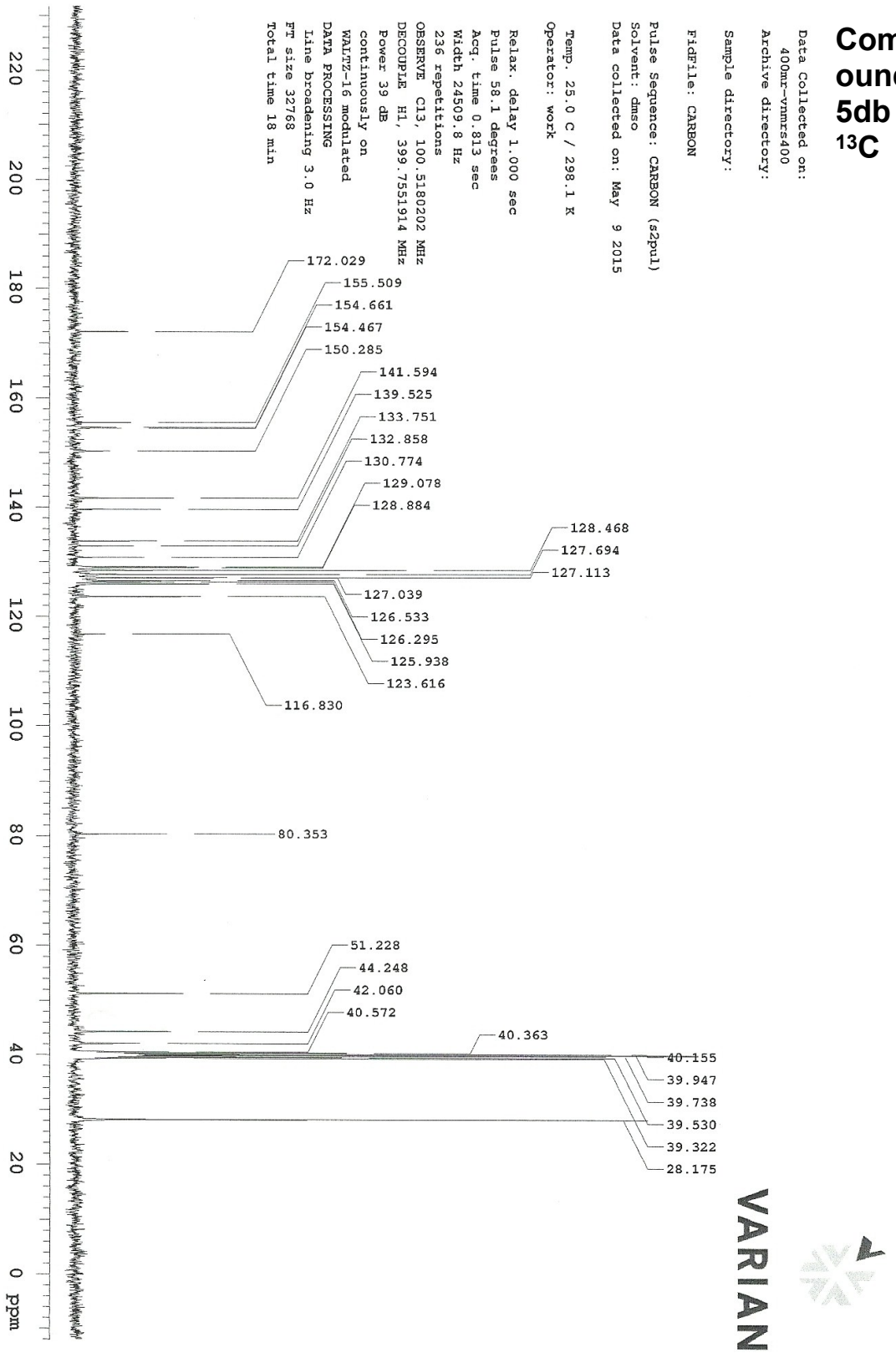

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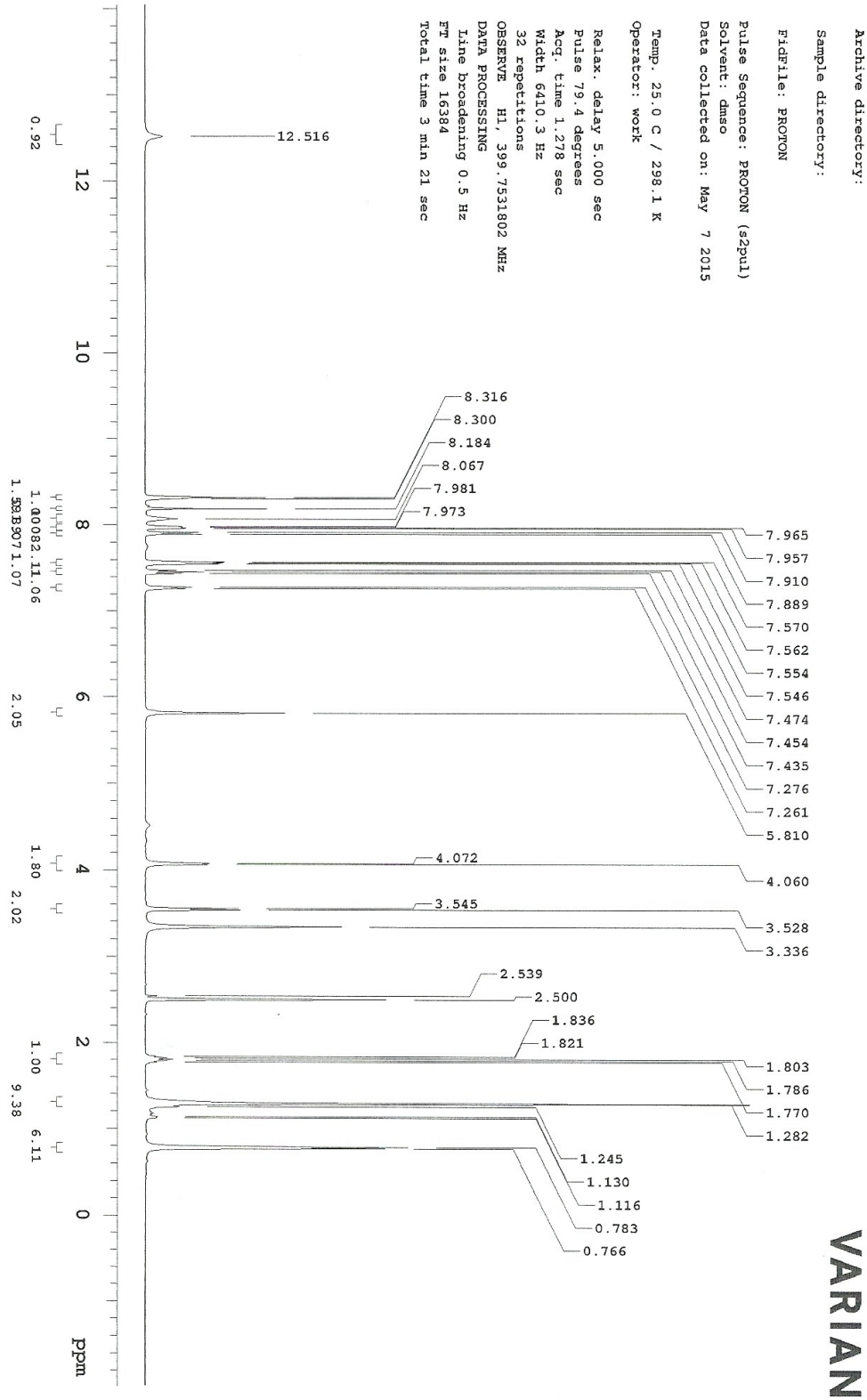

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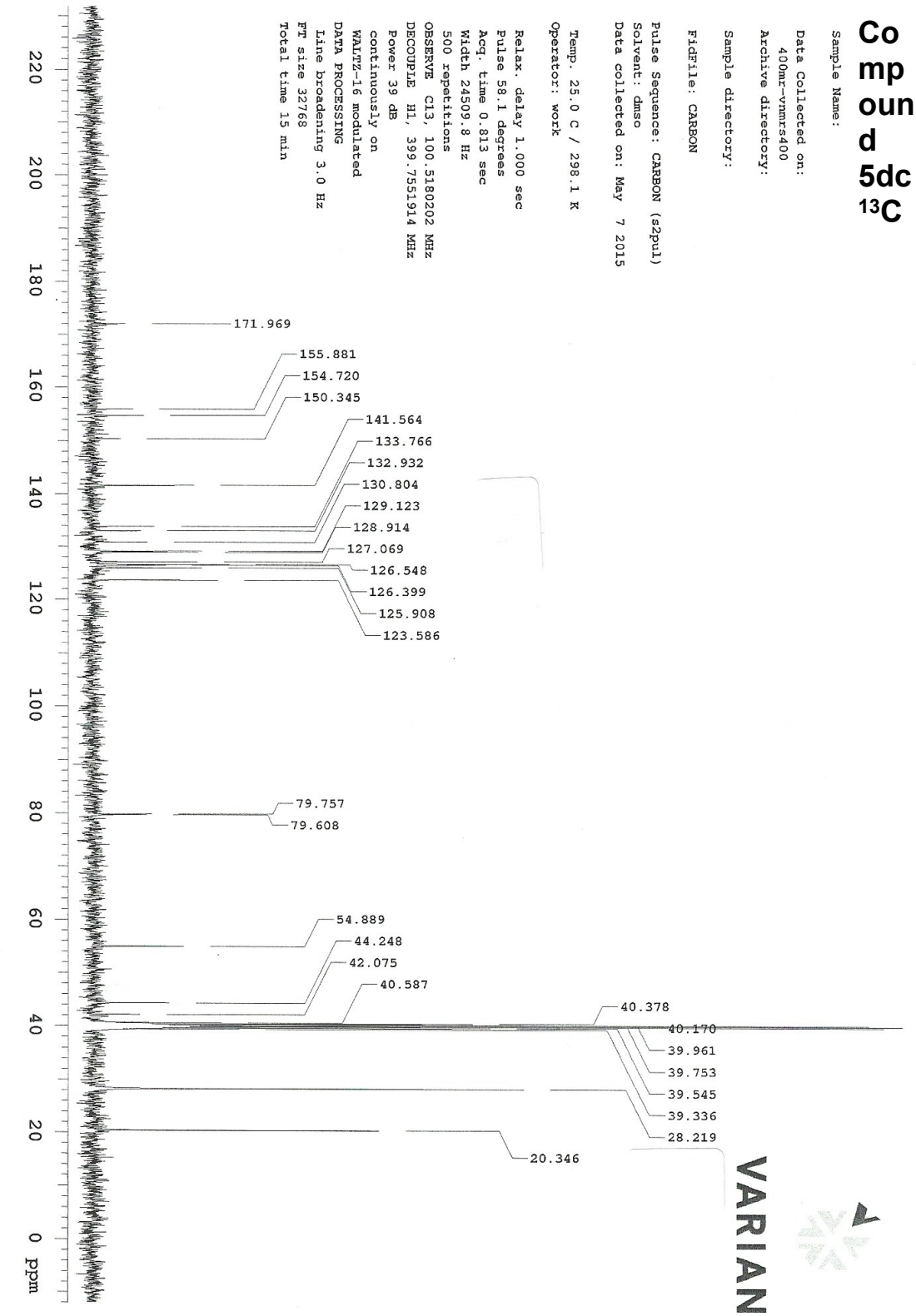

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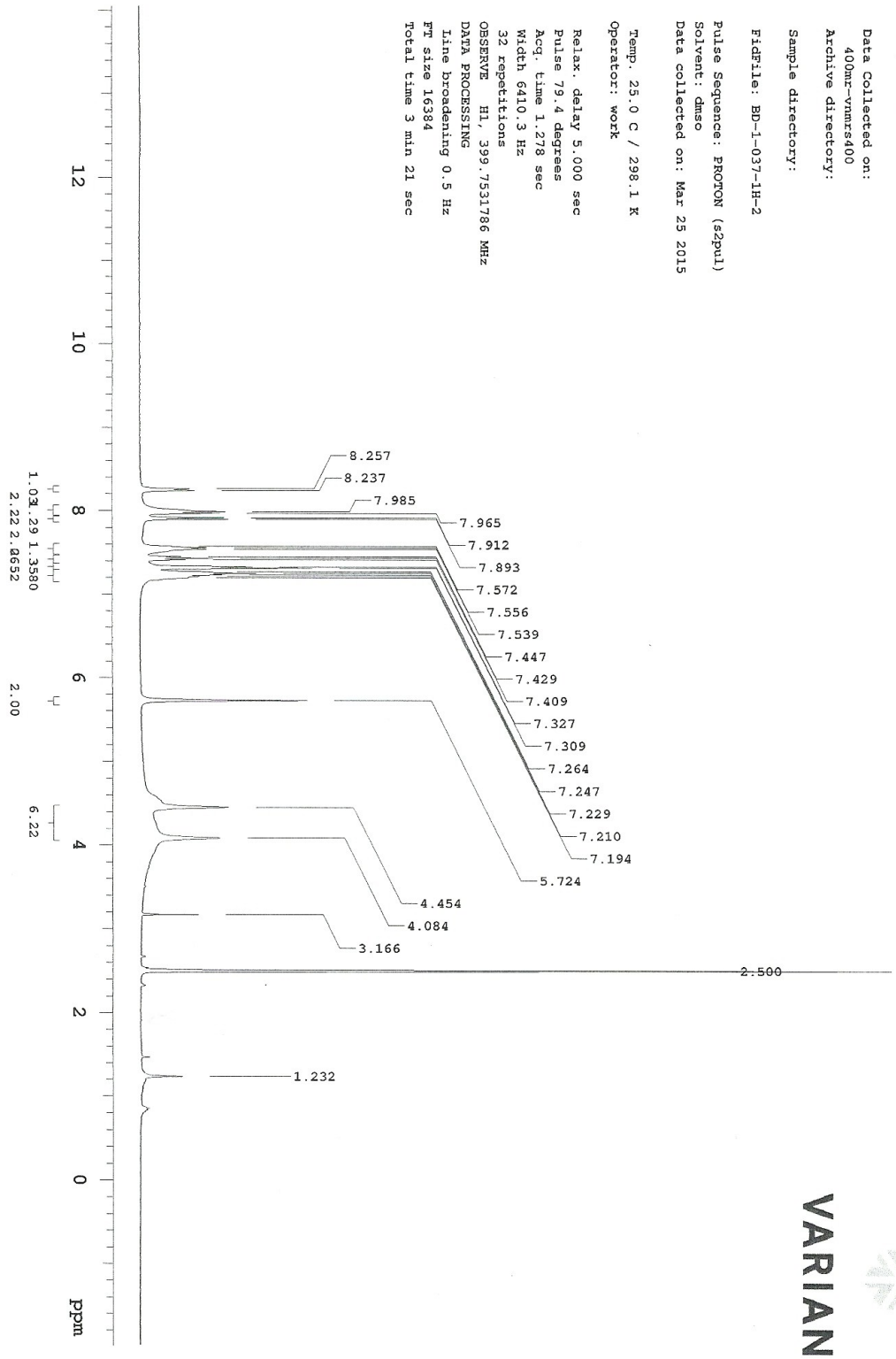

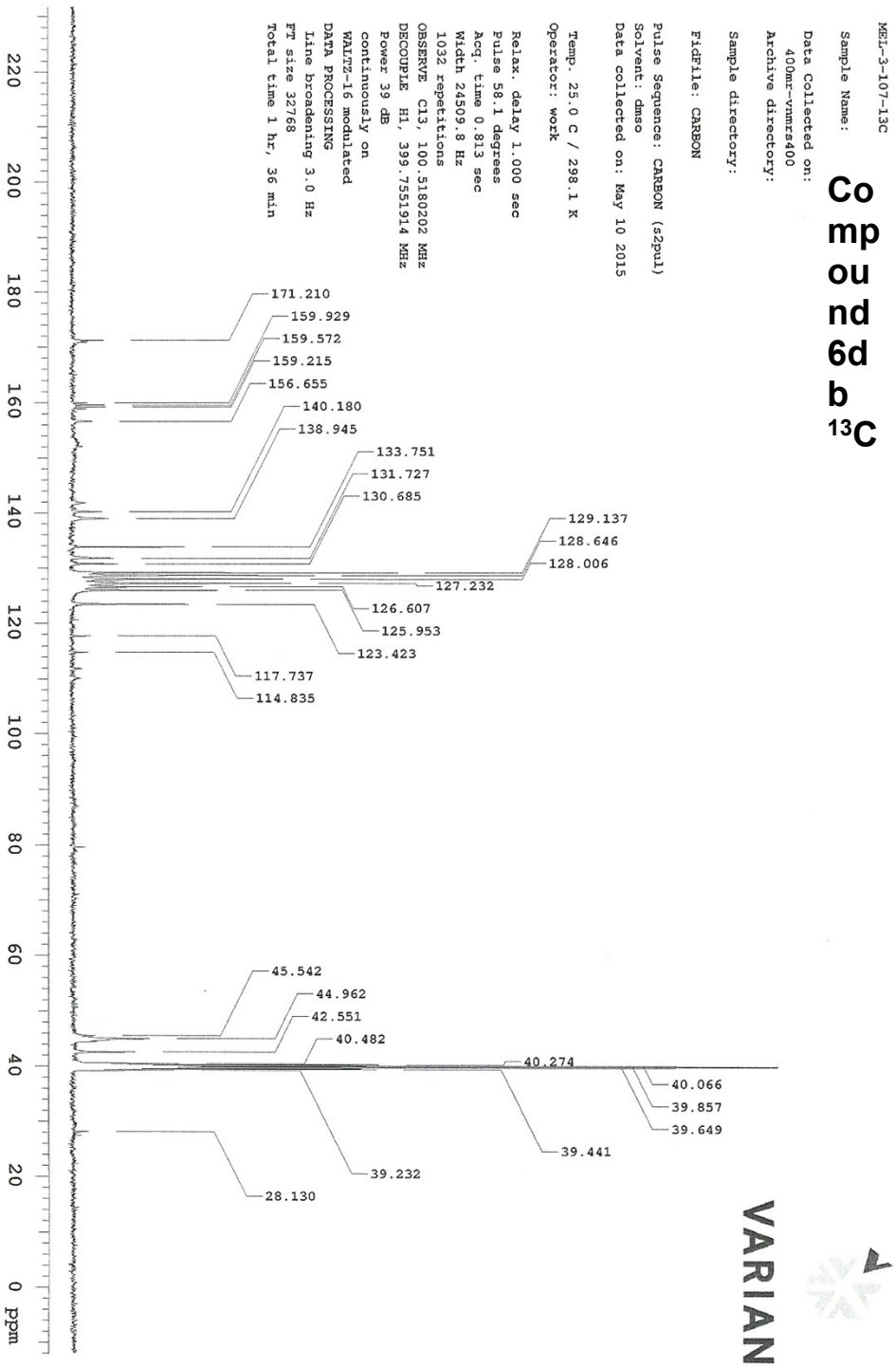
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Operator: work
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1032 repetitions
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DECOUPLE H1, 399.7551914 MHz
Power 39 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
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125.953
123.423
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114.835
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40.066
39.857
39.649
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120
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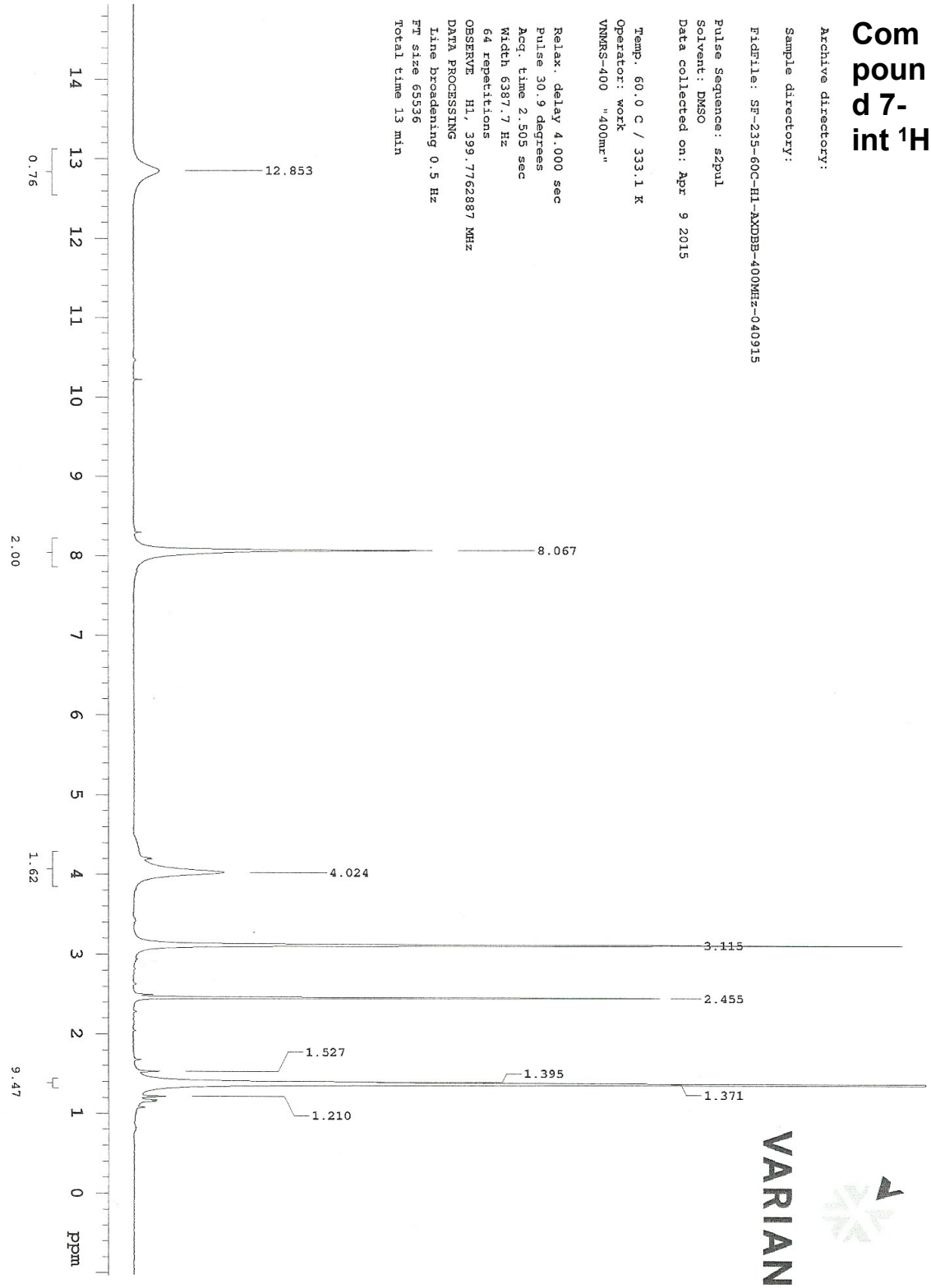
VARIAN

Compound 7-int ^{1}H



Compound 7-int ^{13}C

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Sample directory:

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Solvent: dmso
Data collected on: Apr 21 2015

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Operator: vnmrl

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Width 35714.3 Hz
16000 repetitions
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DECOUPLE H1, 599.6650334 MHz
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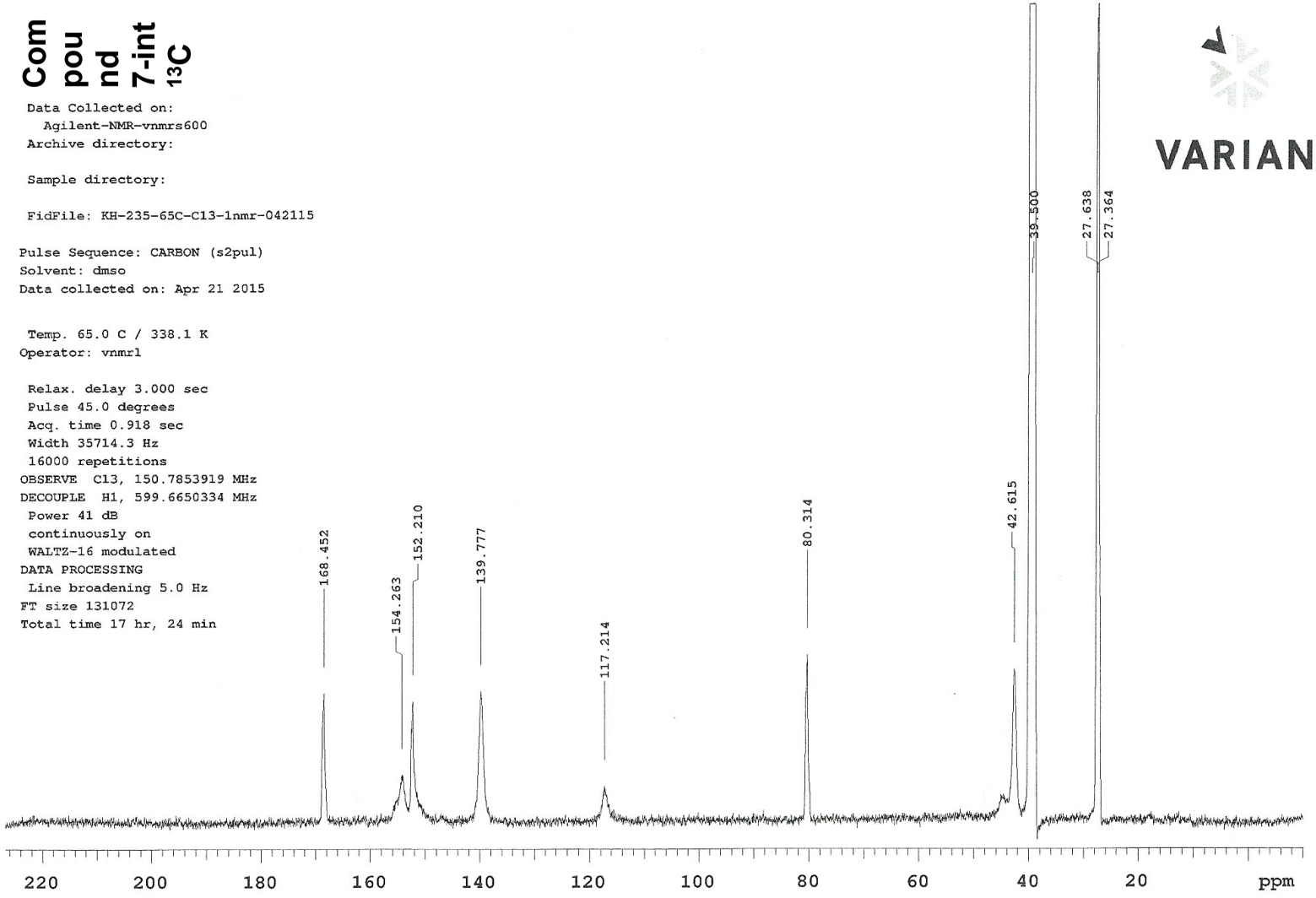
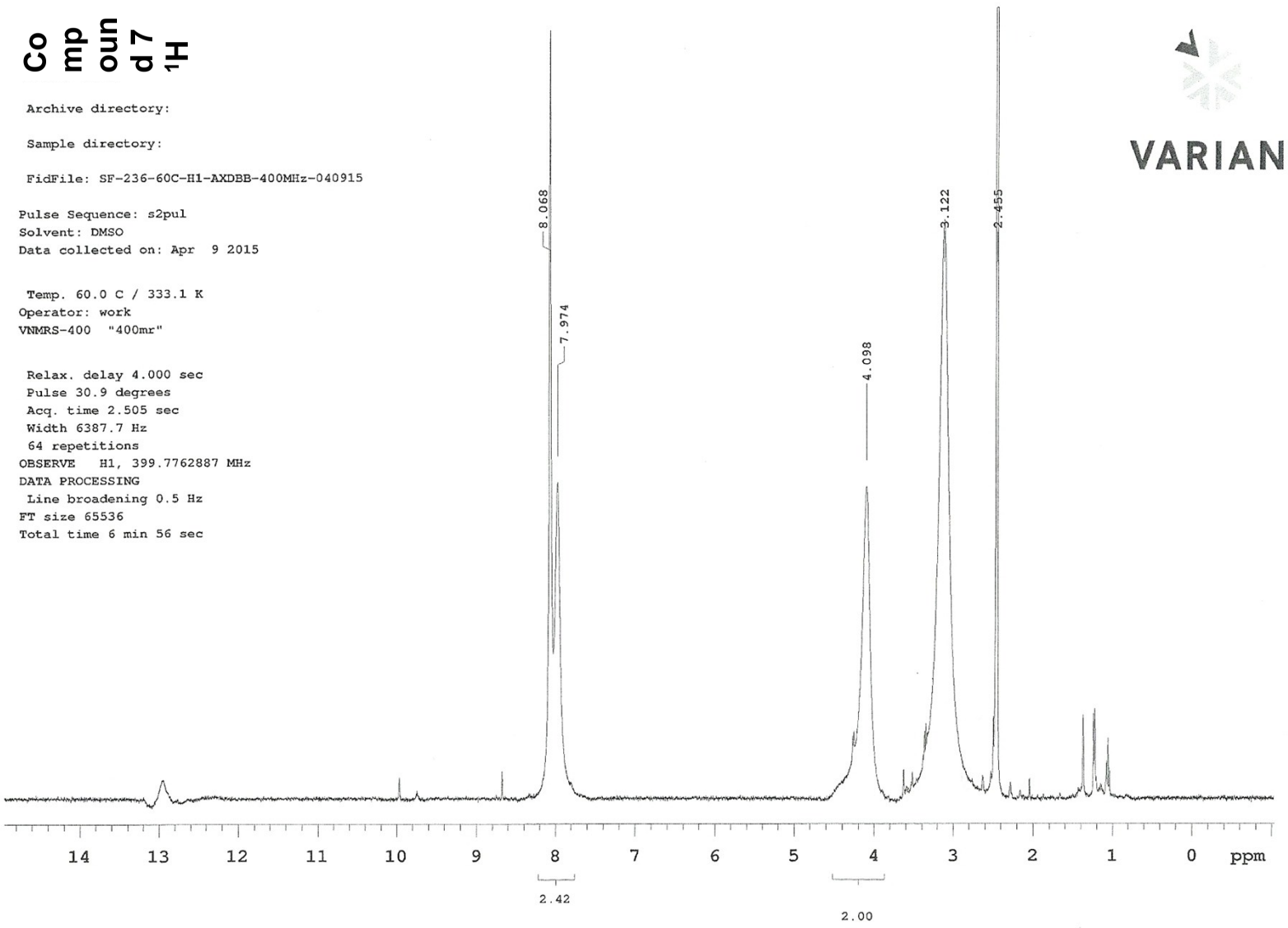
VARIAN



Compound 7 ^1H



Compound 7 ^{13}C