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for

Azido-BODIPY Acid Reveals Quantitative Staudinger-Bertozzi Ligation in Two-Step Activity Based Proteasome Profiling

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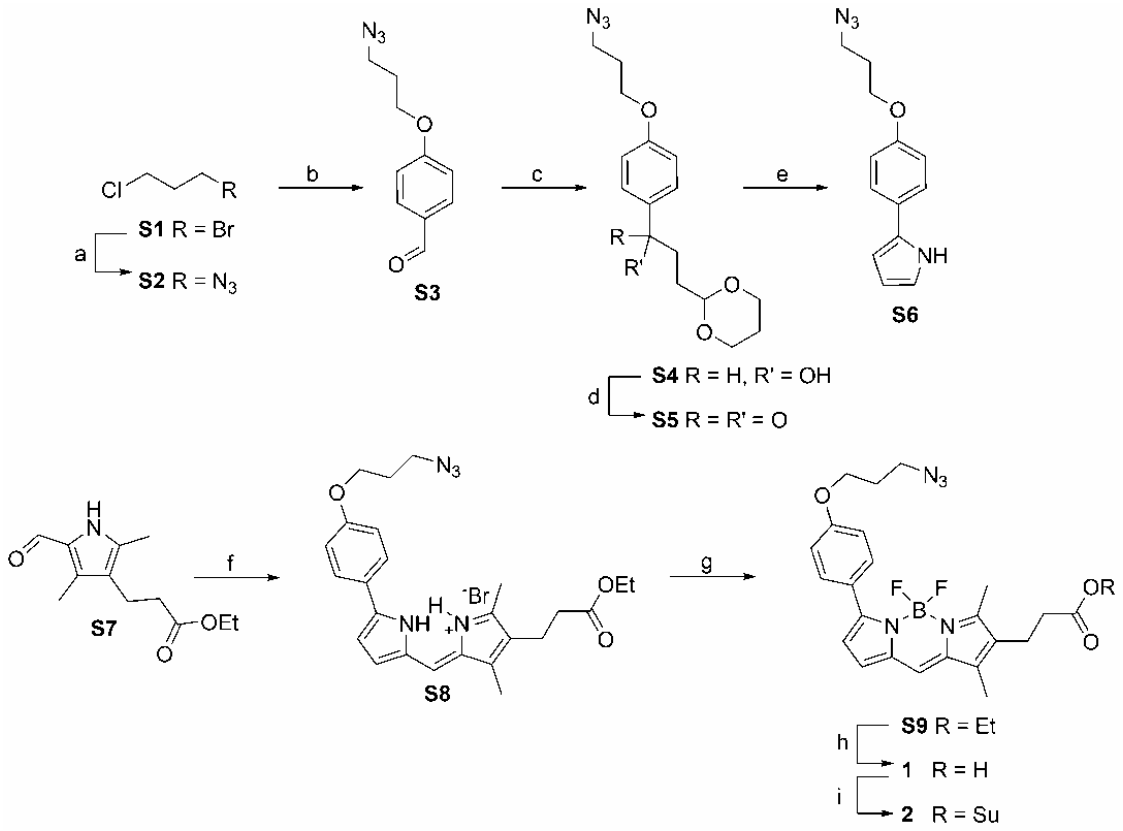
1. Complete Ref. [16]

M. Verdoes, B. I. Florea, V. Menendez-Benito, C. J. Maynard, M. D. Witte, W. A. van der Linden, A. M. C. H. van den Nieuwendijk, T. Hofmann, C. R. Berkers, F. W. van Leeuwen, T. A. Groothuis, M. A. Leeuwenburgh, H. Ovaa, J. J. Neefjes, D. V. Filippov, G. A. van der Marel, N. P. Dantuma, H. S. Overkleeft, *Chem. Biol.*, **2006**, *13*, 1217-1226.

2. Synthesis of Azido-BODIPY-OSu (2).

General

All reagents were commercial grade and were used as received unless indicated otherwise. Tol (Tol)(purum), ethyl acetate (EA) (puriss.), Diethyl ether (Et_2O) and light petroleum ether (PE) (puriss.) were obtained from Riedel-de Haën and distilled prior to use. Dichloroethane (DCE), dichloromethane (DCM), dimethyl formamide (DMF) and dioxane (Biosolve) were stored on 4Å molecular sieves. Methanol (MeOH) and *N*-methylpyrrolidone (NMP) were obtained from Biosolve. Tetrahydrofuran (THF) (Biosolve) was distilled from $LiAlH_4$ prior to use. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm), spraying with 20% H_2SO_4 in ethanol followed by charring at ~150°C, by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150°C or spraying with an aqueous solution of $KMnO_4$ (7%) and KOH (2%). Column chromatography was performed on Screening Divices (0.040 – 0.063 nm). LC/MS analysis was performed on a LCQ Adventage Max (Thermo Finnigan) equipped with an Gemini C18 column (Phenomenex). The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50), Bruker DPX-300 (300/75 MHz), Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory or a Bruker DMX-600 (600/150 MHz) with cryoprobe. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. UV spectra were recorded on a Perkin Elmer, Lambda 800 UV/VIS spectrometer.



Scheme S1. Reagents and conditions: a) NaN_3, DMSO, 12h, quant. b) 4-hydroxybenzaldehyde, K_2CO_3, DMF, 90°C, 48h, 86%. c) (1,3-dioxane-2-ylethyl)-magnesium bromide, THF, -10°C ? RT, 12h, 45%. d) MnO_2, DCM, 12h, 70%. e) NH_4OAc, Ac_2O, AcOH, reflux, 3h, 32%. f) **S6**, HBr (48% in H_2O), EtOH, 0°C, 2h. g) $BF_3{\cdot}Et_2O$, TEA, DCE, 90°C, 16h, 63% over 2 steps. h) 0.1 M NaOH/dioxane/MeOH 1/1/1, 15h, 35%. i) *N*-hydroxysuccinimide, EDC, DCM, 2h, 68%.

1-azido-3-chloro-propane (S2). 1-Bromo-3-chloro-propane (9.9 mL, 100 mmol) was dissolved in DMSO. NaN_3 (6.5g, 100 mmol, 1 equiv) was added and the solution was stirred for 12h before H_2O and pentane were added. The organic layer was separated, dried over $MgSO_4$ and concentrated to yield 1-azido-3-chloropropane (11.96g, quant.) as a colourless oil. ^{1}H NMR (200 MHz, $CDCl_3$): d 3.63 (t, *J* = 6.2 Hz, 2H), 3.50 (t, *J* = 6.6 Hz, 2H), 2.01 (m, 2H). ^{13}C NMR (50.1 MHz, $CDCl_3$): d 47.99, 41.35, 31.31.

4-(3-Azido-propoxy)-benzaldehyde (S3). 4-hydroxy-benzaldehyde (6.1g, 50 mmol) was dissolved in DMF (200 mL) before 1-azido-3-chloro-propane (**S2**, 11.96g, 100 mmol, 2 equiv) and potassium carbonate (13.82g, 100 mmol, 2 equiv) were added. The mixture was stirred 48h at 90°C before being concentrated. The residue was

taken up in DCM and washed with H_2O and brine, dried over $MgSO_4$ and concentrated. Silica column chromatography (0 ? 20% EtOAc in PetEt) yielded the title compound (8.85g, 43 mmol, 86%) as a colourless oil. ^{1}H NMR (200 MHz, $CDCl_3$): d 9.88 (s, 1H), 7.83 (d, *J* = 9.1 Hz, 2H), 7.00 (d, *J* = 8.8 Hz, 2H), 4.13 (t, *J* = 5.8 Hz, 2H), 3.53 (t, *J* = 6.2 Hz, 2H), 2.08 (m, 2H). ^{13}C NMR (50.1 MHz, $CDCl_3$): d 190.22, 163.29, 131.45, 129.66, 114.34, 64.64, 47.60, 28.29.

1-(4-(3-Azido-propoxy)-phenyl)-3-[1,3]dioxan-2-yl-propan-1-ol (S4). 4-(3-Azido-propoxy)-benzaldehyde (**S3**, 13.73 g, 67 mmol) was dissolved in freshly distilled THF (200 mL), put under an argon atmosphere and cooled to -10°C. (1,3-Dioxane-2-yleth-yl)-magnesium bromide (200 mL, 0.5 M in THF, 100 mmol, 1.5 equiv) was added dropwise over 1 h. The reaction mixture was allowed to warm to room temperature and was stirred 12 h before being quenched with sat. aq. NH_4Cl. and extracted with EtOAc. The organic layer was separated, dried over Na_2SO_4 and concentrated. Column chromatography (0% ? 50% EtOAc in PetEt) yielded **S4** (9.61g, 30 mmol, 45%) as a colourless oil. ^{1}H NMR (200 MHz, $CDCl_3$): d 7.24 (d, *J* = 8.8 Hz, 2H), 6.84 (d, *J* = 8.8 Hz, 2H), 4.63 – 4.50 (m, 2H), 4.11 – 3.99 (m, 4H), 3.73 (t, *J* = 11.5 Hz, 2H), 3.50 (t, *J* = 6.6 Hz, 2H), 2.92 (s, 1H), 2.13 – 1.25 (m, 8H). ^{13}C NMR (50.1 MHz, $CDCl_3$): d 157.24, 136.89, 126.55, 113.63, 101.47, 72.63, 66.14, 63.96, 47.62, 32.76, 30.88, 28.12, 25.08.

1-(4-(3-Azido-propoxy)-phenyl)-3-[1,3]dioxan-2-yl-propan-1-one (S5). 1-(4-(3-Azido-propoxy)-phenyl)-3-[1,3]dioxan-2-yl-propan-1-ol (**S4**, 0.8g, 2.48 mmol) was dissolved in DCM and MnO_2 (2.16g, 24.8 mmol, 10 equiv) was added. The reaction mixture was stirred for 12 h. before being filtered over HyFlo. The filtrate was concentrated in vacuo and purified by column chromatography (0% ? 25% EtOAc in PetEt) yielding **S5** (0.55g, 1.72 mmol, 70%) as a slight yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): d 7.96 (d, *J* = 9.1 Hz, 2H), 6.84 (d, *J* = 8.8 Hz, 2H), 4.66 (t, *J* = 4.9 Hz, 1H), 4.14 – 4.06 (m, 4H), 3.83 – 3.69 (m, 2H), 3.53 (t, *J* = 6.6 Hz, 2H), 3.06 (t, *J* = 7.3 Hz, 2H), 2.13 – 1.99 (m, 5H), 1.37 – 1.29 (m, 1H). ^{13}C NMR (50.1 MHz, $CDCl_3$): d 197.37, 162.05, 129.81, 113.68, 100.67, 66.31, 64.40, 47.63, 31.86, 29.16, 28.16, 25.40.

2-(4-(3-Azido-propoxy)-phenyl)-1H-pyrrole (S6). To a solution of 1-(4-(3-Azidopropoxy)-phenyl)-3-[1,3]dioxan-2-yl-propan-1-one (**S5**, 2.26g, 7.1 mmol) in AcOH (50 mL) were added NH_4OAc (6.55g, 85 mmol, 12 equiv) and Ac_2O (2.5 mL, 26.3 mmol, 3.7 equiv). The reaction mixture was refluxed for 3 h, poured into ice water, neutral-

ized with $NaHCO_3$ and extracted with DCM. The DCM layer was separated, dried over Na_2SO_4 and concentrated. Purification by column chromatography (0% ? 10% EtOAc in PetEt) gained an inseparable mixture of the title compound **S6** and 1,3-acetoxy-propane. The mixture was dissolved in MeOH and KO*t*Bu was added till pH 9. After stirring for 1 h. the reaction mixture was neutralized with AcOH, H_2O and DCM were added and the DCM layer was separated, dried over Na_2SO_4 and concentrated in vacuo to yield 2-(4-(3-Azido-propoxy)-phenyl)-1H-pyrrole (0.55g, 2.26 mmol, 32%) as a purplish foam. ^{1}H NMR (200 MHz, $CDCl_3$): d 8.39 (s, 1H), 7.39 (d, *J* = 8.4 Hz, 2H), 6.90 (d, *J* = 8.8 Hz, 2H), 6.82 (m, 1H), 6.41 (m, 1H), 6.28 (dd, J_1 = 2.6 Hz, J_2 = 5.5 Hz, 1H), 4.06 (t, *J* = 5.8 Hz, 2H), 3.52 (t, *J* = 6.6 Hz, 2H), 2.06 (m, 2H). ^{13}C NMR (50.1 MHz, $CDCl_3$): d 156.76, 131.49, 125.73, 124.67, 118.18, 114.54, 109.24, 104.32, 64.29, 47.83, 28.30.

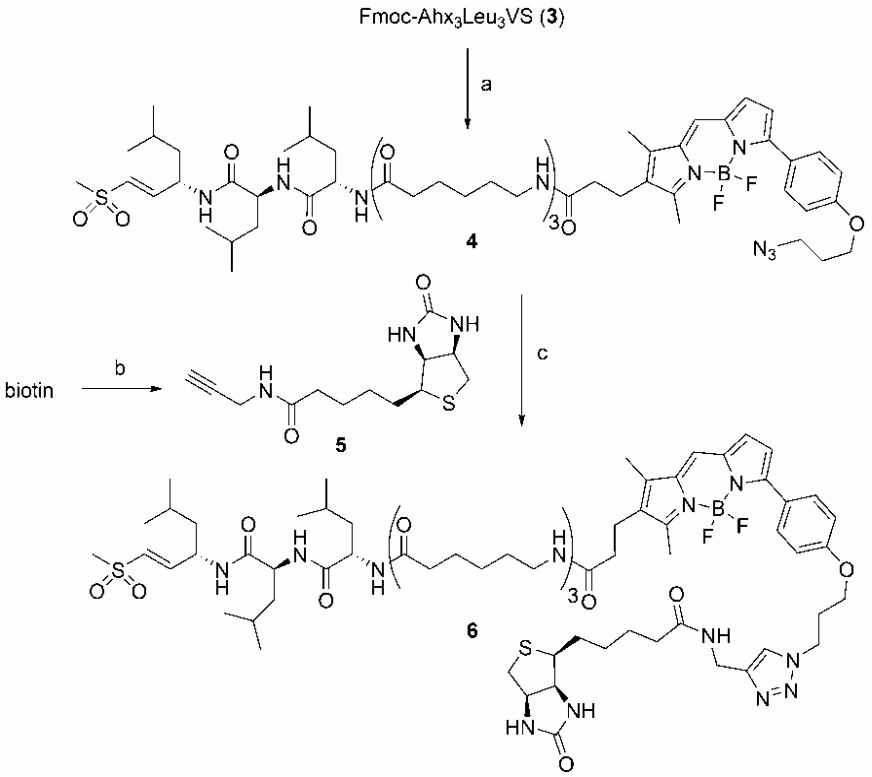
4,4-Difluoro-1,3-dimethyl-2-(2-(ethoxycarbonylethyl))-7-(4-(3-Azido-propoxy)-phenyl)-4-bora-3a,4a-diaza-s-indacene (S9). 2-(4-(3-Azido-propoxy)-phenyl)-1*H*-pyrrole **S6** (0.99 g, 4.1 mmol, 1 equiv) and carboxyaldehyde pyrrole **S7**[1] (0.92 g, 4.1 mmol, 1 equiv) were dissolved in EtOH (5 mL). The resulting mixture was cooled to 0°C, and hydrobromic acid, 48% solution in water (0.7 mL, 6.15 mmol, 1.5 equiv) was added. After 2h stirring, TLC analysis showed complete consumption of the starting materials. The reaction mixture was concentrated in vacuo, coevaporated with DCE (3×) and used without further purification. The crude dipyrrole **S8** was dissolved in DCE (50 mL) and put under an argon atmosphere. Triethylamine (1.7 mL, 12.3 mmol, 3 equiv) and $BF_3 \cdot Et_2O$ (5.4 mL, 20.5 mmol, 5 equiv) were added, and the reaction mixture was stirred for 16h, before being concentrated in vacuo and purified by column chromatography (0% ? 2.5% EtOAc in Tol.) yielding the title compound **S9** (1.28 g, 2.58 mmol, 63%). ^{1}H NMR (200 MHz, $CDCl_3$): d 7.87 (d, *J* = 8.8 Hz, 2H), 7.09 (s, 1H), 6.97 (d, *J* = 8.8 Hz, 2H), 6.96 (d, *J* = 4.4 Hz, 1H), 6.54 (d, *J* = 4.4 Hz, 1H), 4.12 (m, 4H), 3.54 (t, *J* = 6.6 Hz, 2H), 2.73 (t, *J* = 7.7 Hz, 2H), 2.54 (s, 3H), 2.44 (d, *J* = 7.7 Hz, 2H), 2.22 (s, 3H), 2.08 (dt, *J* = 6.3 Hz, 2H), 1.24 (d, *J* = 7.1 Hz, 2H). ^{13}C NMR (50.1 MHz, $CDCl_3$): d 172.07, 158.97, 158.51, 154.63, 139.50, 134.59, 133.74, 130.16, 129.52, 127.58, 125.07, 122.52, 117.61, 113.66, 64.02, 60.05, 47.62, 33.36, 28.09, 18.78, 13.53, 12.44, 8.71.

4,4-Difluoro-1,3-dimethyl-2-(2-carboxyethyl)-7-(4-(3-Azido-propoxy)-phenyl)-4-bora-3a,4a-diaza-s-indacene (1). Ethyl ester **S9** (89 mg, 0.18 mmol) was dissolved in dioxane (2 mL) and MeOH (2 mL). After addition of 0.1 M aqueous NaOH (0.2

mmol, 2 mL, 1.15 equiv) and stirring overnight, the purple suspension was diluted with EtOAc, extracted with 0.1 M HCl, dried and concentrated. Column chromatography (0% ? 1% EtOAc and 1% AcOH in Tol.) yielded acid **1** (30 mg, 64 µmol, 35%). ^{1}H NMR (200 MHz, $CDCl_3$): d 7.86 (d, *J* = 9.1 Hz, 2H), 7.09 (s, 1H), 6.96 (d, *J* = 8.8 Hz, 2H), 6.96 (m, 1H), 6.54 (d, *J* = 4.0 Hz, 1H), 4.10 (t, *J* = 6.0 Hz, 2H), 3.53 (t, *J* = 6.6 Hz, 2H), 2.74 (t, *J* = 7.7 Hz, 2H), 2.53 (s, 3H), 2.49 (t, *J* = 6.9 Hz, 2H), 2.22 (s, 3H), 2.07 (dt, *J* = 6.3 Hz, 2H). ^{13}C NMR (50.1 MHz, $CDCl_3$): d 175.32, 159.24, 155.18, 139.77, 134.80, 134.16, 130.49, 129.92, 127.80, 125.55, 122.79, 118.06, 114.03, 64.35, 48.01, 33.60, 28.48, 19.17, 12.80, 9.22.

4,4-Difluoro-1,3-dimethyl-2-(2-(succimidyloxycarbonylethyl))-7-(4-(3-Azidopropoxy)-phenyl)-4-bora-3a,4a-diaza-s-indacene (2). Azido-BODIPY-acid **1** (30 mg, 64 µmol) was coevaporated thrice with toluene, before being dissolved in DCM (1 mL). After the addition of *N*-hydroxysuccinimide (29 mg, 0.25 mmol. 4 equiv) and EDC (48 mg, 0.25 mmol, 4 equiv), the reaction mixture was stirred for 2h. Next, the reaction was diluted with EtOAc, washed with 0.5 M aq. HCl, dried over $MgSO_4$ and concentrated. Purification by column chromatography (0% ? 4% EtOAc in Tol.) furnished title compound **2** (24 mg, 43 µmol, 68%). ^{1}H NMR (200 MHz, $CDCl_3$): d 7.88 (d, *J* = 9.1 Hz, 2H), 7.12 (s, 1H), 6.97 (d, *J* = 8.8 Hz, 2H), 6.96 (m, 1H), 6.56 (d, *J* = 4.4 Hz, 1H), 4.11 (t, *J* = 5.8 Hz, 2H), 3.54 (t, *J* = 6.6 Hz, 2H), 2.79 (m, 8H), 2.56 (s, 3H), 2.14 (s, 3H), 2.08 (dt, *J* = 6.0 Hz, 2H). ^{13}C NMR (50.1 MHz, $CDCl_3$): d 168.98, 167.55, 159.45, 158.30, 155.91, 139.71, 135.10, 133.95, 130.65, 128.37, 128.22, 125.43, 123.12, 118.45, 114.09, 64.35, 48.07, 30.69, 28.60, 25.39, 18.96, 12.95, 9.47. HRMS: calcd for $C_{27}H_{27}BF_2N_6O_5H$ 565.21768, found 565.21783, for $C_{27}H_{27}BF-N_6O_5$ 545.21145, found 545.21130.

3. Synthesis of 4 and 6.



Scheme S2. Reagents and conditions: a) i) DBU, DMF, 5 min. ii) HOBt, 1 min. iii) **2**, DiPEA, 30 min., 86%. b) Propargylamine, HCTU, DiPEA, DMAP (cat.), DMF, 0°C, 3h. c) **5**, 10 mol% $CuSO_4$, 20 mol% sodium ascorbate, *t*BuOH/H_2O 1/1, RT, 15h, quant.

N_3-BODIPY-Ahx$_3$L$_3$VS (4). DBU (3.3 µl, 22 µmol, 1 equiv) was added to a solution of Fmoc-Ahx$_3$L$_3$VS (**3**)[2] (21.2 mg, 22 µmol) in DMF. After 5 min. of stirring, HOBt (13.4 mg, 0.1 mmol, 4.5 equiv) was added. To this mixture, **2** (12.4 mg, 22 µmol, 1 equiv) and DiPEA (22 µl, 0.13 mmol, 6 equiv) were added, and the mixture was stirred for 30 min. before being concentrated in vacuo. Purification by column chromatography (0.1% TEA in DCM ? 3% MeOH, 0.1% TEA in DCM) afforded N_3-Bodipy-Ahx$_3$L$_3$VS (**4**) (22.8 mg, 19 µmol, 86%). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD): d 7.86 (d, *J* = 8.85 Hz, 2H), 7.75-7.60 (m, 3H), 7.51-7.44 (m, 2H), 7.43-7.36 (m, 1H), 7.28-7.22 (m, 1H), 7.20 (s, 1H), 7.03-6.95 (m, 3H), 6.86-6.77 (m, 1H), 6.56 (m, 2H), 4.73-4.60 (m, 1H), 4.38-4.26 (m, 2H), 4.13 (t, *J* = 5.89 Hz, 2H), 3.55 (t, *J* = 6.62 Hz, 2H), 3.21-3.09 (m, 6H), 2.98 (s, 3H), 2.74 (t, *J* = 7.43 Hz, 2H), 2.54 (s, 3H), 2.31 (t, *J* = 7.34 Hz, 2H), 2.27-2.20 (m, 5H), 2.16 (t, *J* = 7.51 Hz, 2H), 2.13-2.05 (m, 4H), 1.73-1.18 (m, 27H), 1.03-0.84 (m, 18H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD): d 174.52, 174.45, 174.08, 173.20, 172.75, 172.72, 172.63, 159.31, 159.11, 154.85, 147.32, 139.97, 134.64,

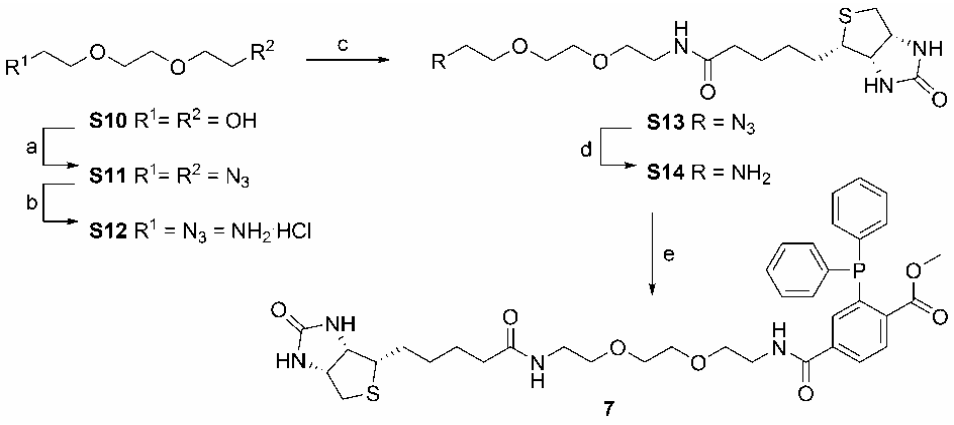
134.09, 130.27, 128.72, 127.62, 125.36, 122.67, 117.85, 113.82, 64.23, 51.89, 51.85, 47.58, 46.09, 42.06, 41.95, 40.06, 39.87, 39.85, 38.80, 38.69, 35.66, 35.53, 35.38, 35.33, 28.42, 28.31, 25.96, 25.91, 25.80, 24.96, 24.90, 24.80, 24.41, 24.38, 24.33, 22.33, 22.29, 22.26, 21.12, 21.08, 21.01, 19.91, 8.85, 8.10. HRMS: calcd for $C_{61}H_{94}BF_2N_{11}O_9SH$ 1206.70906, found 1206.71092, for $C_{61}H_{94}BF_2N_{11}O_9SNa$ 1228.69100, found 1228.69269, for $C_{61}H_{94}BF_2N_{11}O_9SK$ 1244.66494, found 1244.66770. l_{abs} = 541.94 nm, l_{em} = 570.00 nm, e = 62488 liter mol^{-1} cm^{-1}.

Biotin-propargylamide (5). Propargylamine (561 µl, 8.19 mmol, 1 equiv) was added dropwise to a cooled solution (0°C) of D-(+)-biotin (2.0 g, 8.19 mmol), HCTU (3.39 g, 8.19 mmol, 1 equiv), DiPEA (2.85 mL, 16.38 mmol, 2 equiv) and *N*,*N*-dimethyl-4-aminopyridin (cat.) in DMF (16 mL). The reaction mixture was allowed to reach RT over 3 h, after which TLC indicated the disappearance of the starting material. After reduction of the volume under reduced pressure (~1/2), excess EtOAc was added and the resulting suspension was stored overnight at -20°C. Filtration and rinsing with EtOAc and Et2O ultimately afforded the title compound as an off-white powder (2.03 g, 88%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): d 8.21 (t, *J* = 5.4 Hz, 1H), 6.42 (s, 1H), 6.35 (s, 1H), 4.30 (dd, J_1 = 5.0 Hz, J_2 = 7.5 Hz, 1H), 4.08 – 4.16 (m, 1H), 3.83 (dd, J_1 = 2.5 Hz, J_2 = 5.6 Hz, 2H), 3.08 – 3.14 (m, 1H), 3.07 (t, *J* = 2.5 Hz, 1H), 2.82 (dd, J_1 = 5.0, J_2 = 12.5 Hz, 1H), 2.57 (d, *J* = 12.3 Hz, 1H), 2.08 (t, *J* = 7.5 Hz, 2H), 1.20 – 1.67 (m, 7H). ^{13}C NMR (100 MHz, [D$_6$]DMSO): d 171.8, 162.7, 81.4, 72.8, 61.0, 59.2, 55.4, 34.9, 28.2, 28.0, 27.7, 25.1. HRMS: calcd for $C_{13}H_{19}N_3O_2S$ 282.12707, found 282.12714.

Biotin-BODIPY-Ahx$_3$L$_3$VS (6). N_3-BODIPY-Ahx$_3$L$_3$VS (**4**) (5.6 mg, 4.6 µmol) was dissolved in *t*BuOH (0.25 mL) before aqueous solutions of $CuSO_4$ (125 µL, 3.7 mM, 10 mol%) and sodium ascorbate (125 µL, 7.4 mM, 20 mol%) were added. The reaction mixture was stirred for 12 h, concentrated and purified by size-exclusion chromatography (Sephadex LH-20, eluent: MeOH) to give the title compound as a brown/red solid (6.9 mg, 4.6 µmol, quant.). ^{1}H NMR (600 MHz, $CDCl_3$/MeOD): d 7.88-7.85 (m, 1H), 7.82 (d, *J* = 8.74 Hz, 2H), 7.55 (s, 1H), 7.08 (d, *J* = 3.97 Hz, 1H), 6.95 (d, *J* = 8.77 Hz, 2H), 6.66 (dd, J_1 = 15.20 Hz, J_2 = 5.02 Hz, 1H), 6.62 (d, *J* = 3.97 Hz, 1H), 6.54 (d, *J* = 15.23 Hz, 1H), 4.57-4.48 (m, 3H), 4.34-4.27 (m, 3H), 4.27-4.19 (m, 2H), 4.13-4.08 (m, 1H), 4.01 (t, *J* = 5.89 Hz, 2H), 3.10-3.04 (m, 1H), 3.03-2.95 (m, 6H), 2.91 (s, 3H), 2.79 (dd, J_1 = 12.53 Hz, J_2 =5.00 Hz, 1H), 2.62 (t, *J* = 7.43 Hz, 2H), 2.58 (d, *J* = 12.54 Hz, 1H), 2.43 (s, 3H), 2.31-2.25 (m, 2H), 2.23-2.17 (m, 5H), 2.16-2.06 (m, 4H), 2.02 (t, *J* = 7.42 Hz, 2H), 1.99 (t, *J* = 7.47 Hz, 2H), 1.65-1.07 (m, 33H), 0.91-

0.74 (m, 18H). ^{13}C NMR (150 MHz, $CDCl_3$/MeOD): d 173.79, 173.23, 173.03, 172.32, 172.19, 160.34, 159.72, 154.49, 147.35, 146.45, 145.65, 141.19, 135.36, 134.72, 131.72, 131.37, 131.02, 130.99, 130.96, 130.93, 130.07, 129.52, 128.61, 125.91, 124.40, 124.36, 123.39, 123.33, 118.35, 114.77, 114.71, 65.09, 61.78, 60.00, 56.07, 52.08, 51.97, 47.12, 42.50, 42.46, 40.87, 40.79, 40.30, 35.97, 35.93, 35.72, 35.57, 34.65, 30.14, 29.42, 29.33, 28.81, 28.59, 26.68, 26.64, 25.79, 25.69, 25.66, 25.62, 24.89, 24.86, 24.73, 23.17, 23.14, 21.83, 21.76, 21.52, 13.44, 13.12, 9.38. HRMS: calcd for $C_{74}H_{113}BF_2N_{14}O_{11}S_2$ 1487.82885, found 1487.83093. l_{abs} = 551.94 nm, l_{em} = 574.05 nm, e = 59 325 L mol^{-1} cm^{-1}.

4. Synthesis of biotin-phosphane 7



Scheme S3. Reagents and conditions: a) i) Tosylchloride, TEA, DMAP (cat.), DCM, 16h. ii) NaN_3, TBAI (cat.), DMF, 80°C, 16h., 78% (2 steps). b) PPh_3, 5% HCl (aq.), Tol., 0°C, 16h., 79%. c) D-(+)-Biotin, BOP, DiPEA, DMF, 16h., 60%. d) i) PPh_3, 1h. ii) H_2O, DMF, 16h., 78%. e) 3-(diphenylphosphino)-4-(methoxycarbonyl)benzoic acid, EDC·HCl, DMF, 16h., 23%.

1,2-bis(2-azidoethoxy)ethane (S11). Triethyleneglycol (**S10**, 0.3 g, 2 mmol) was dissolved in DCM and put under an argon atmosphere, before tosylchloride (1.14 g, 6 mmol, 3 equiv), triethylamine (0.83 mL, 6 mmol, 3 equiv) and *N*,*N*-dimethyl-4-aminopyridin (12 mg, 0.1 mmol, 5 mol%) were added. After 16 h. the reaction mixture was washed with H_2O. The organic phase was separated, dried over $MgSO_4$ and concentrated in vacuo to result a yellowish oil which was dissolved in DMF. NaN_3 (0.26 g, 4 mmol, 2 equiv) and tetrabutylamoniomiodide (37 mg, 0.1 mmol, 5 mol%) were added and the reaction mixture was stirred at 80°C for 16h., before being washed with sat.

aq. $NaHCO_3$. The organic phase was separated, dried over $MgSO_4$ and concentrated to a yellow oil. Purification by column chromatography (Tol. ? 15% EtOAc in Tol.) afforded the bis-azide **S11** as a colourless oil (0.31 g, 1.56 mmol, 78%). ^{1}H NMR (200 MHz, $CDCl_3$): d 3.68 (m, 8H), t, 3.39 (*t*, *J* = 5.1 Hz, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): d 70.3, 69.8, 50.3.

***N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)biotinylamide (S13).** An aqueous 5% HCl solution (10 mL) was added to a cooled solution (0°C) of 1,2-bis(2-azidoethoxy)ethane (**S11**, 2.0 g, 10 mmol) in Tol. (10 mL), before triphenylphoshine (2.5 g, 9.5 mmol, 0.95 equiv) was added. The reaction mixture was allowed to warm up to RT and was stirred 16 h, after which the aqueous layer was separated and concentrated in vacuo to yield the crude 2-(2-(2-azidoethoxy)ethoxy)ethanamine HCl salt (1.67, 7.9 mmol, 79%). The HCl salt (0.83 g, 3.95 mmol) was coevaporated with Tol. (3x) and dissolved in DMF. D-(+)-Biotin (0.98 g, 4 mmol, 1.01 equiv), BOP (1.77 g, 4 mmol, 1.01 equiv) and DiPEA (1.99 mL, 12 mmol, 3.03 equiv) were added and the reaction mixture was stirred 16h. before the reaction volume was reduced under reduced pressure (~½). The crude title compound was crashed out with excess EtOAc. Recrystallization in MeOH/EtOAc resulted *N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)biotinylamide (**S13**) as an off-white powder (0.95 g, 2.37 mmol, 60%). ^{1}H NMR (200 MHz, MeOD): d 4.50 (ddd, J_1 = 7.8 Hz, J_2 = 4.8 Hz, J_3 = 0.8 Hz, 1H), 4.31 (dd, J_1 = 7.9 Hz, J_2 = 4.4 Hz, 1H), 3.72-3.60 (m, 6H), 3.56 (t, *J* = 5.6 Hz, 2H), 3.42-3.34 (m, 4H), 3.27-3.15 (m, 1H), 2.93 (dd, J_1 = 12.7 Hz, J_2 = 4.9 Hz, 1H), 2.70 (d, *J* = 12.8 Hz, 1H), 2.22 (t, *J* = 7.2 Hz, 1H), 1.82-1.34 (m, 6H). ^{13}C NMR (50 MHz, MeOD/[D_6]acetone): d 175.65, 71.36, 71.20, 70.96, 70.57, 63.11, 61.41, 56.88, 51.64, 41.09, 40.31, 36.70, 29.65, 29.40, 26.75.

***N*-(2-(2-(2-aminoethoxy)ethoxy)ethyl)biotinylamide (S14).** To a stirred solution of **S13** (0.95 g, 2.37 mmol) in DMF, triphenylphosphine (0.93 g, 3.56 mmol, 1.5 equiv) was added. After 1h. a drop of H_2O was added and the reaction mixture was stirred 16 h, before an excess EtOAc was added. The title compound was filtered and washed with EtOAc to result an off-white powder (0.7 g, 1.86 mmol, 78%). ^{1}H NMR (200 MHz, MeOD): d 4.50 (dd, J_1 = 7.6 Hz, J_2 = 4.8 Hz, 1H), 4.31 (dd, J_1 = 7.7 Hz, J_2 = 4.3 Hz, 1H), 3.77-3.12 (m, 12H), 3.03-2.79 (m, 2H), 2.71 (d, *J* = 12.7 Hz, 1H), 2.23 (t, *J* = 7.0 Hz, 2H), 1.85-1.33 (m, 6H). ^{13}C NMR (50 MHz, MeOD/[D_6]acetone): d 176.27, 165.81, 72.38, 71.29, 70.93, 70.35, 63.13, 61.34, 56.73, 41.39, 40.99, 40.66, 40.05, 36.60, 29.50, 29.20, 26.59.

Biotin-phosphane (7). *N*-(2-(2-(2-aminoethoxy)ethoxy)ethyl)biotinylamide (**S14**, 0.11 g, 0.3 mmol) was coevaporated with Tol. (3×), dissolved in DMF and put under argon atmosphere, before 3-(diphenylphosphino)-4-(methoxycarbonyl)benzoic acid[3] (0.17 g, 0.33 mmol, 1.1 equiv), EDC·HCl (85 mg, 0.45 mmol, 1.5 equiv) and *N*,*N*-dimethyl-4-aminopyridin (cat.) were added. After 16 h the reaction mixture was concentrated in vacuo. Purification by column chromatography (first acetone ? 3% H_2O in acetone, followed by DCM ? 6% MeOH in DCM) afforded biotin-phosphane (**7**; 51 mg, 70 µmol, 23%). ^{1}H NMR (200 MHz, [D_6]acetone, D_2O): d 7.99 (dd, J_1 = 8.1 Hz, J_2 = 3.5 Hz, 1H), 7.87 (dd, J_1 = 8.1 Hz, J_2 = 1.7 Hz, 1H), 7.47 (dd, J_1 = 3.9 Hz, J_2 = 1.7 Hz, 1H), 7.37-7.15 (m, 10H), 4.46 (dd, J_1 = 7.9 Hz, J_1 = 4.6 Hz, 1H), 4.26 (dd, J_1 = 7.9 Hz, J_2 = 4.4 Hz, 1H), 3.61 (s, 3H), 3.58-3.37 (m, 10H), 3.34-3.24 (m, 2H), 3.18-3.06 (m, 1H), 2.85 (dd, J_1 = 12.8 Hz, J_2 = 4.8 Hz, 1H), 2.64 (d, J = 12.7 Hz, 1H), 2.13 (t, J = 7.3 Hz, 2H), 1.79-1.14 (m, 6H). ^{13}C NMR (50 MHz, [D_6]acetone, D_2O): d 176.16, 168.39, 142.29, 141.74, 139.01, 138.83, 138.61, 135.51, 135.09, 134.81, 134.75, 132.03, 130.70, 130.34, 130.21, 128.42, 128.38, 71.32, 70.81, 70.66, 63.24, 61.56, 57.05, 53.39, 41.45, 41.15, 40.48, 37.01, 29.83, 29.55, 26.95. ^{31}P NMR (81.1 MHz, [D_6]acetone/D_2O): d -3.12. HRMS: calcd for $C_{37}H_{45}N_4O_7PSH$ 721.28193, found 721.28186.

5. Two-step labeling of fluorescently labeled proteasomes in living cells.

EL4 cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/mL penicillin and 10 µg/mL streptomycin in a 5% CO_2 humidified incubator at 37°C. Some $2 \cdot 10^6$ cells were seeded in 6 cm Petri dishes and allowed to grow O/N in 1 mL of medium. The cells were exposed to 0, 0.1, 1, 10 µM probe (1 µL 100 x solution in DMSO) for 2 h, before being washed with PBS (2x) and harvested. After flash freezing (N_2 (l)) the cells were lysed in 50 µl digitonin lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl, 1 mM DTT, 0.025% digitonin) for 5 min. on ice and centrifuged at 16 100 rcf. for 20 min at 4°C. The supernatant containing the cytosolic fraction was collected and the protein content was determined by Bradford assay. Some 25 µg protein was incubated with 100 µM biotin-phosphine **7** in 20 µL lysis buffer containing 5 mM DTT for 1 h at 37°C. The reaction was terminated by a chloroform / methanol precipitation of the proteins.[4] The pellet was solubilised by boiling for 5 min in 1x Laemli's sample buffer containing beta-mercapto ethanol. The pro-

teins were resolved by 12.5% SDS-PAGE. Labelled proteasome subunits were visualised by in-gel fluorescence scanning on a Typhoon variable mode imager (Amersham Biosciences) followed by western blotting. The blots were blocked with 1% BSA in TBS-Tween 20 (0.1 % Tween 20) for 30 min at RT, hybridised for 30 min with Streptavidin-HRP (1:10000) in blocking buffer, washed and visualised using an ECL+ kit (Amersham Biosciences).

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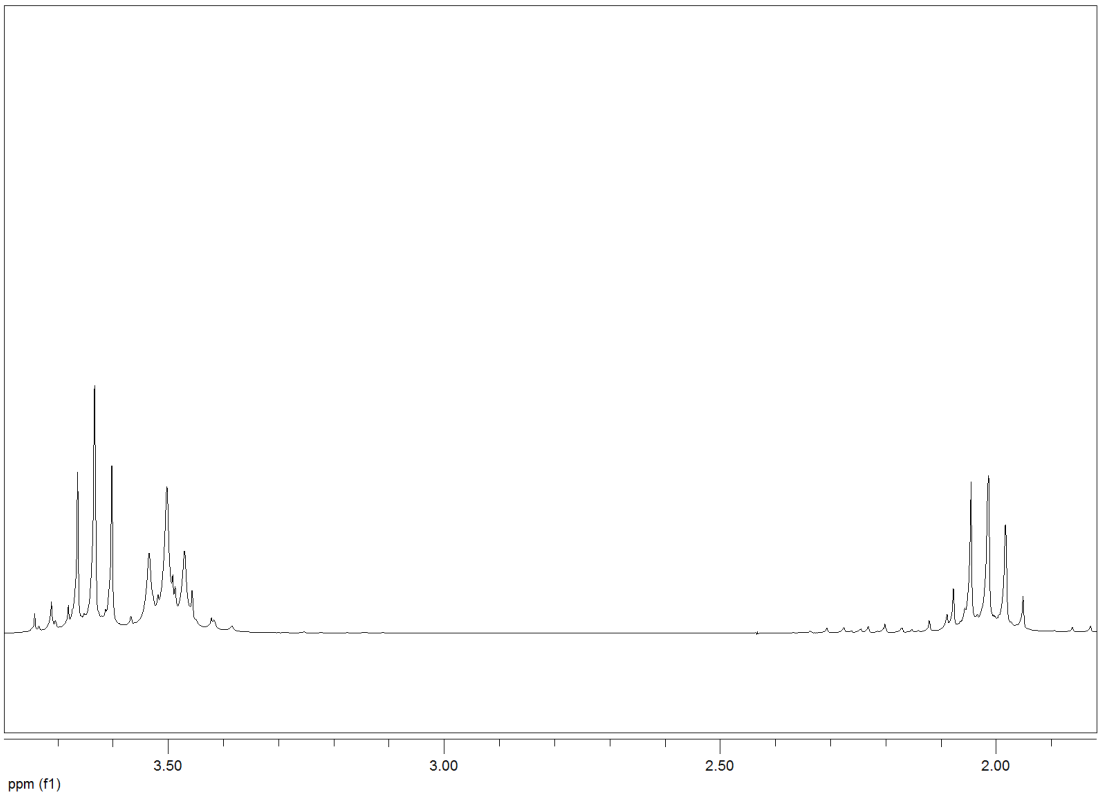
6. NMR spectra.

S2 ^{1}H NMR



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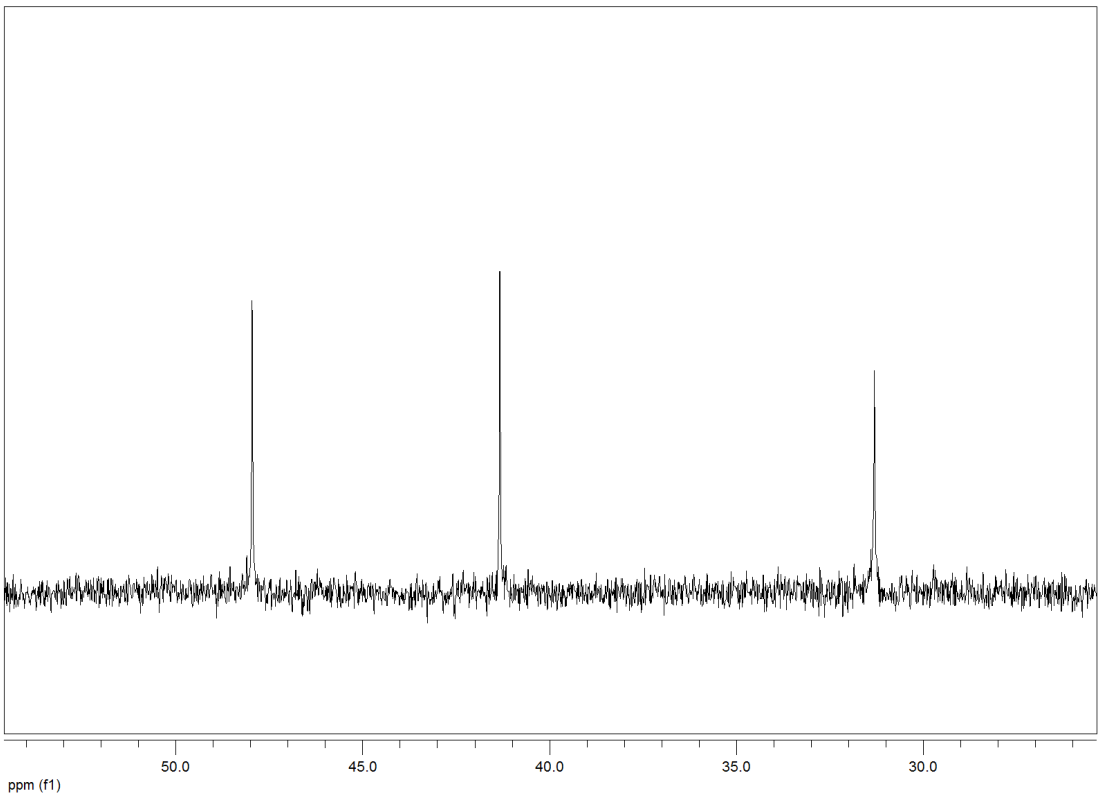

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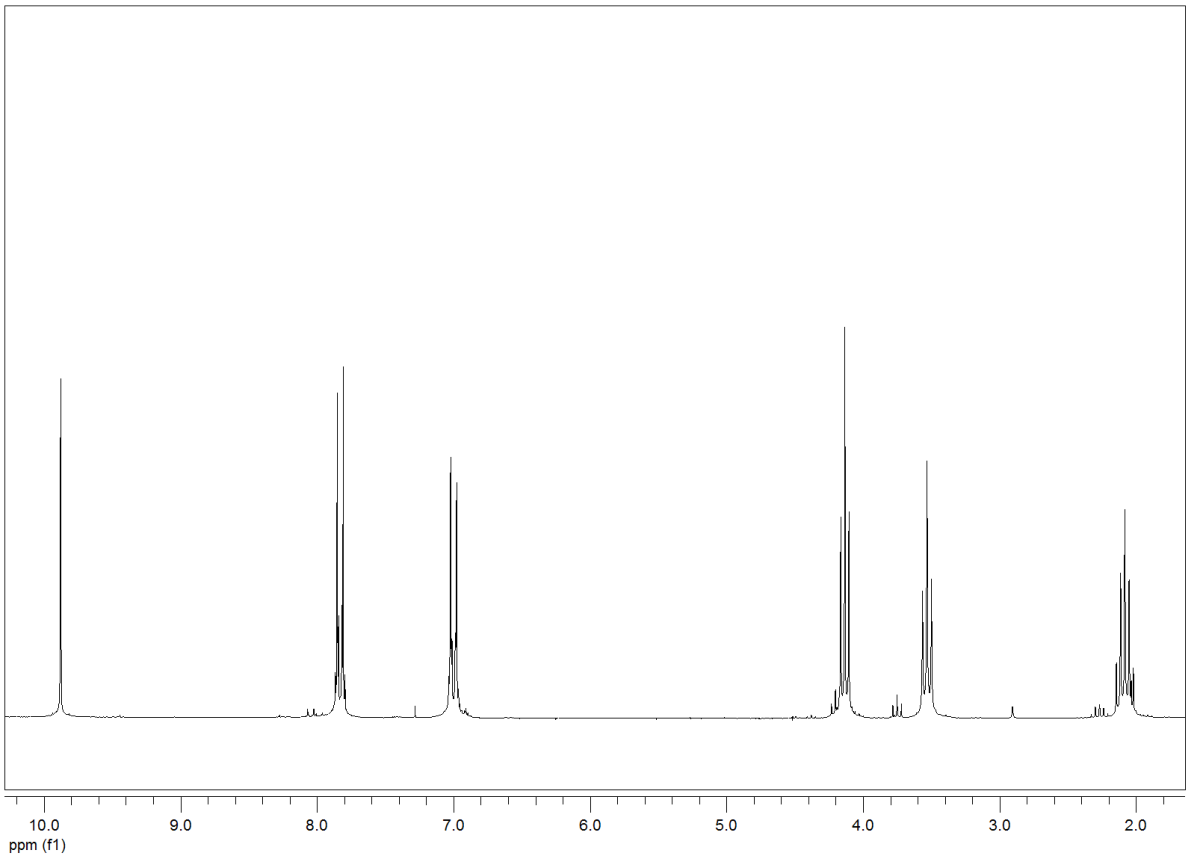

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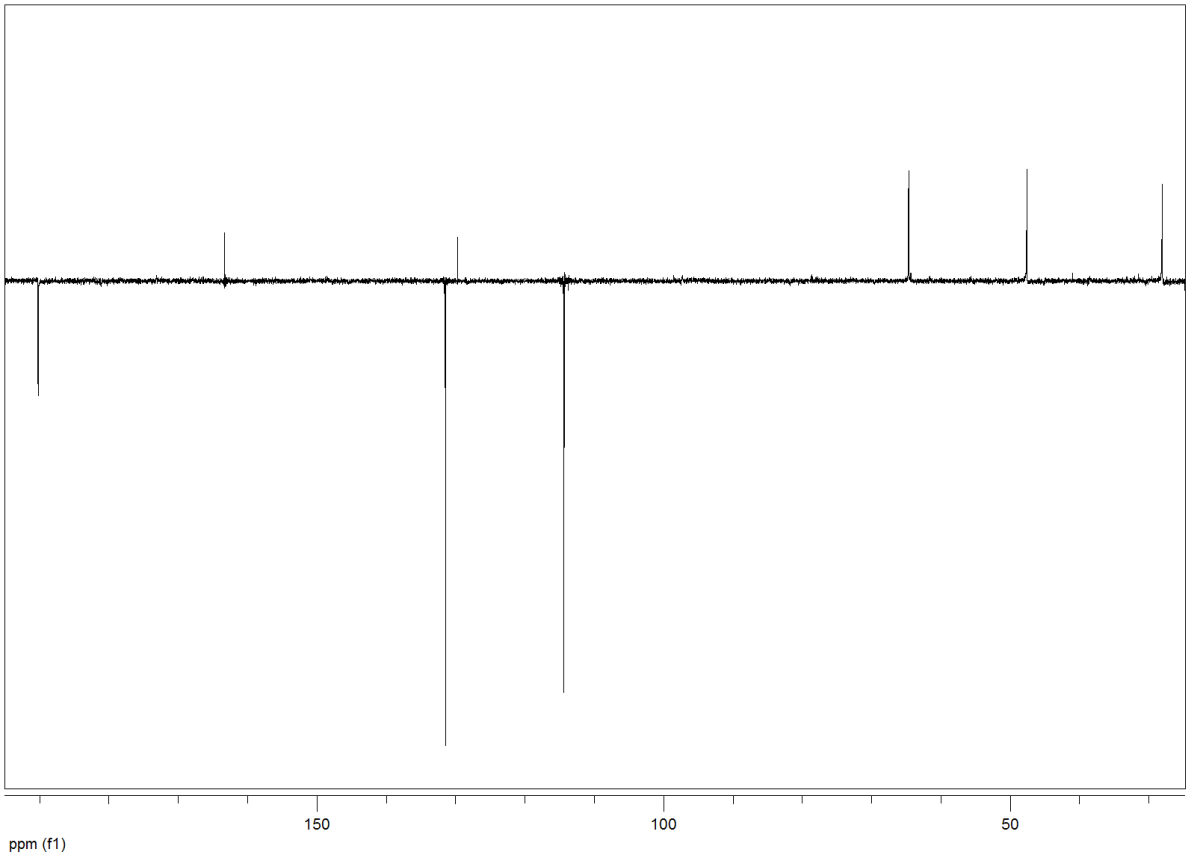

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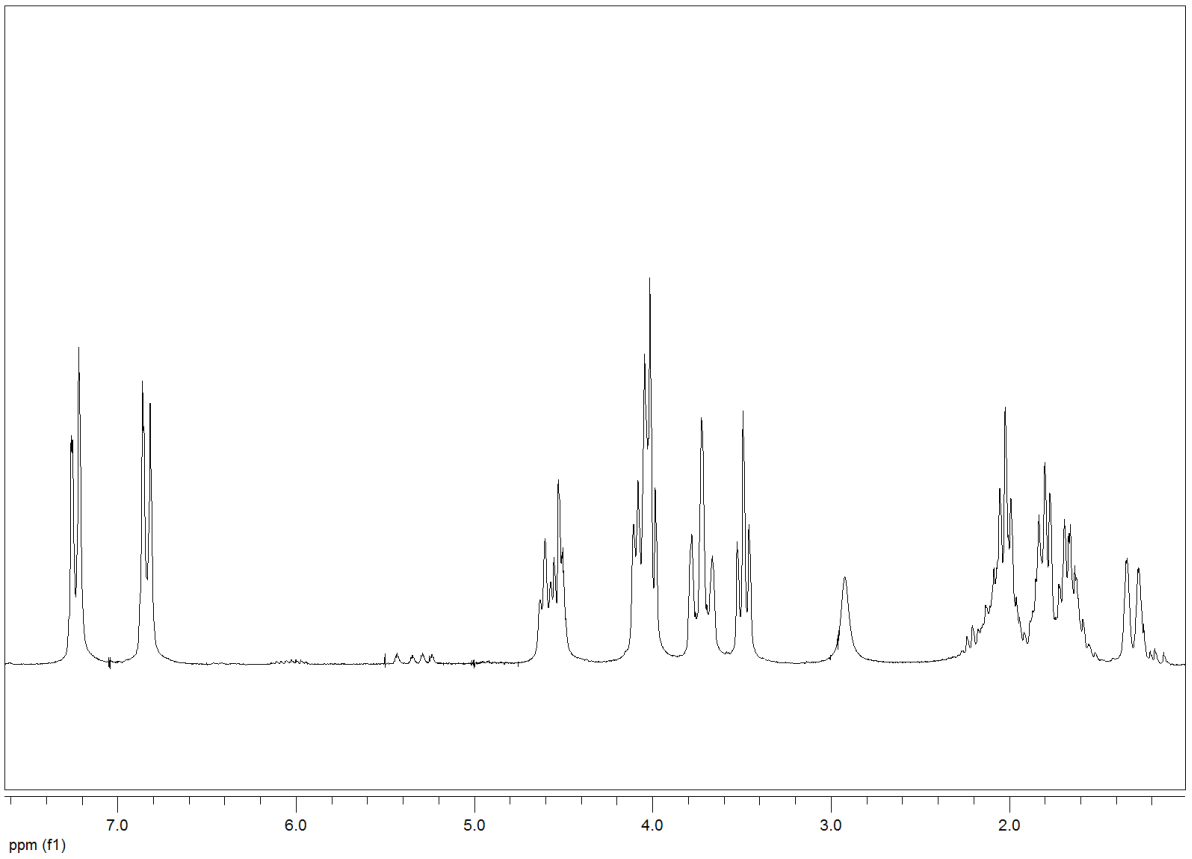

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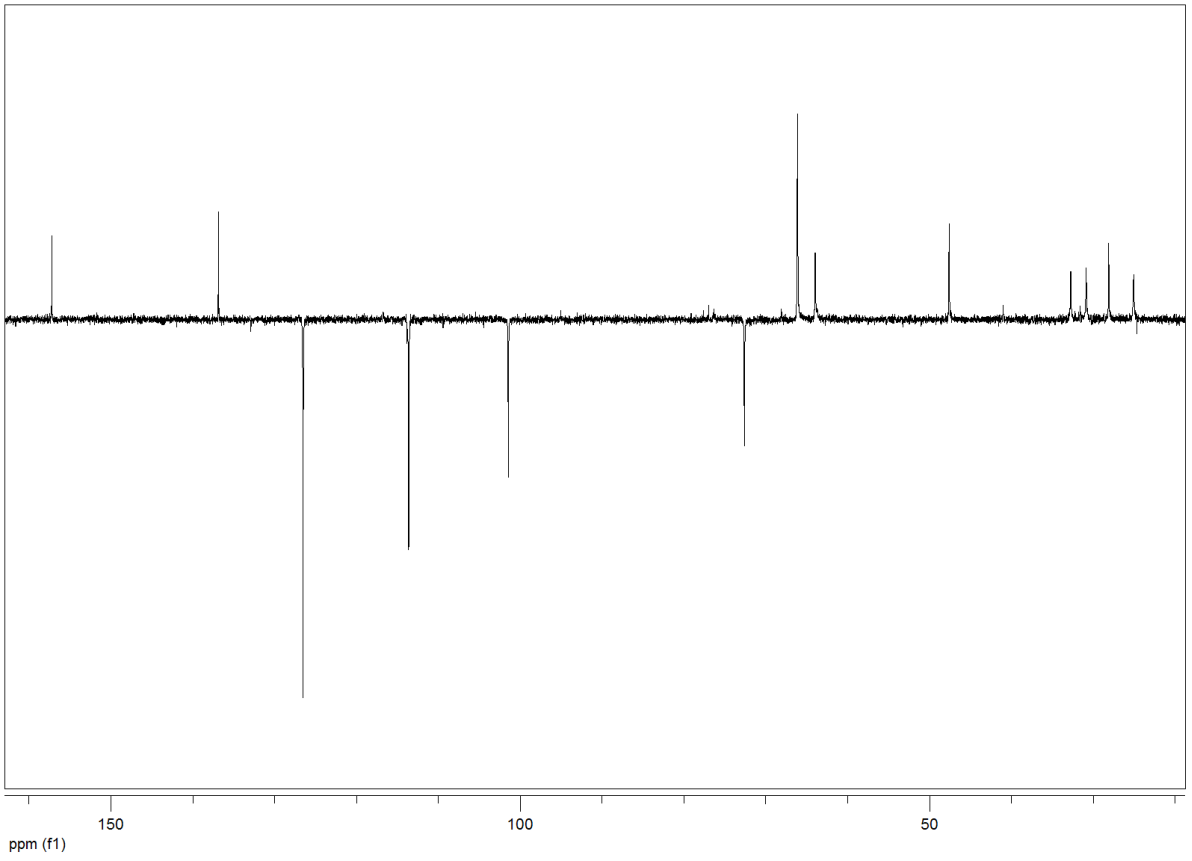

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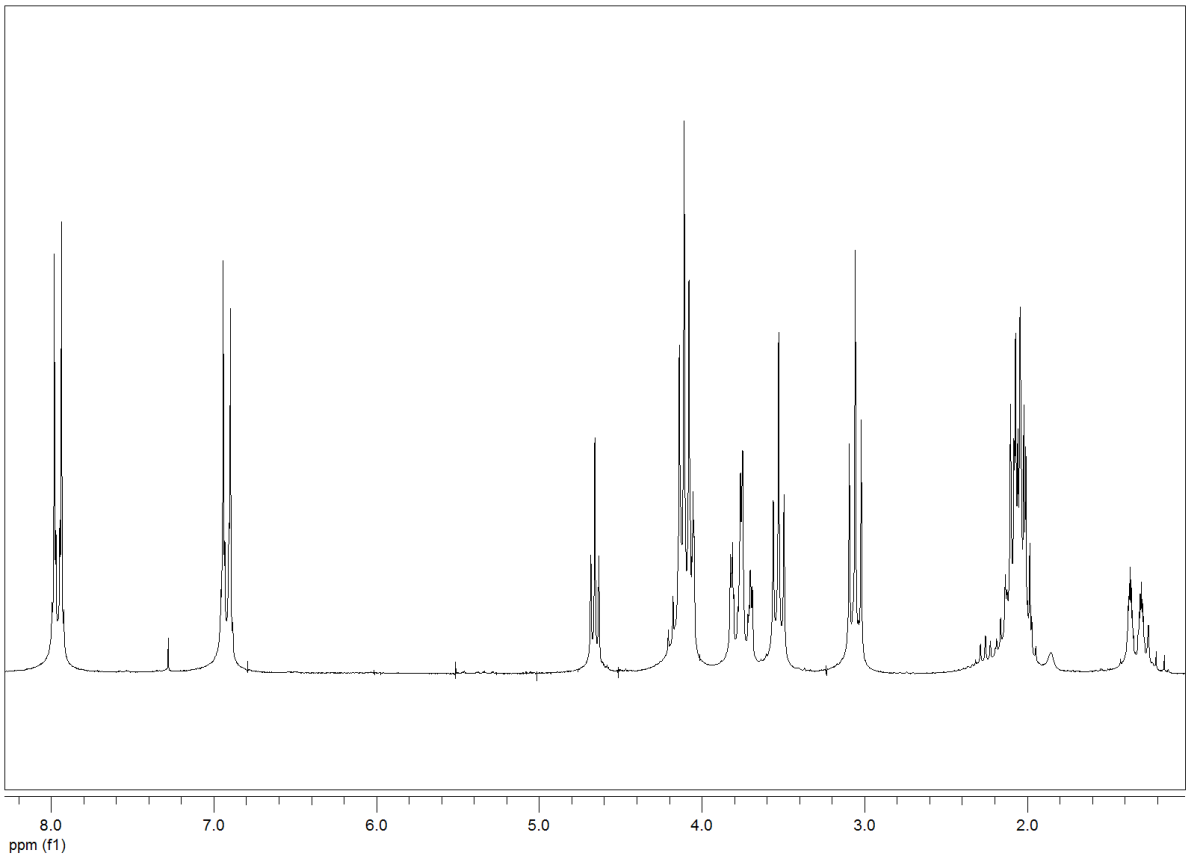

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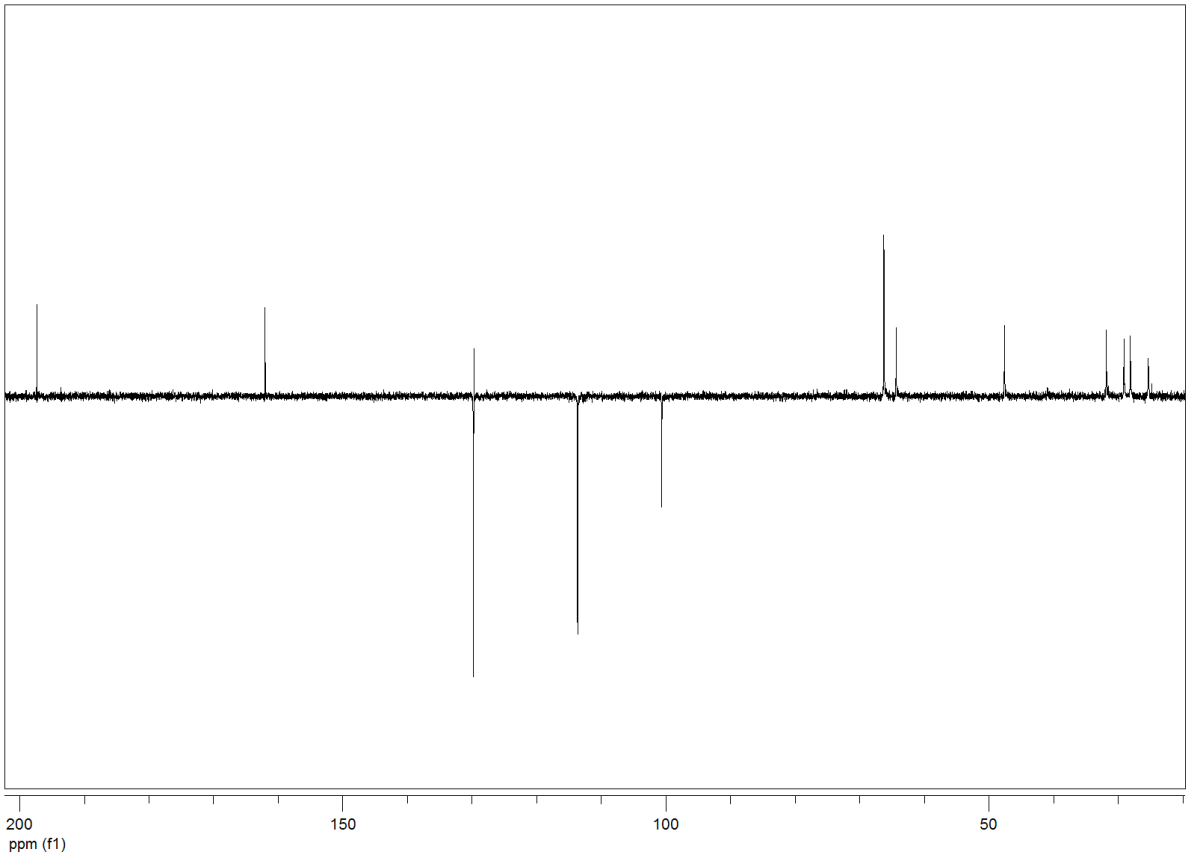

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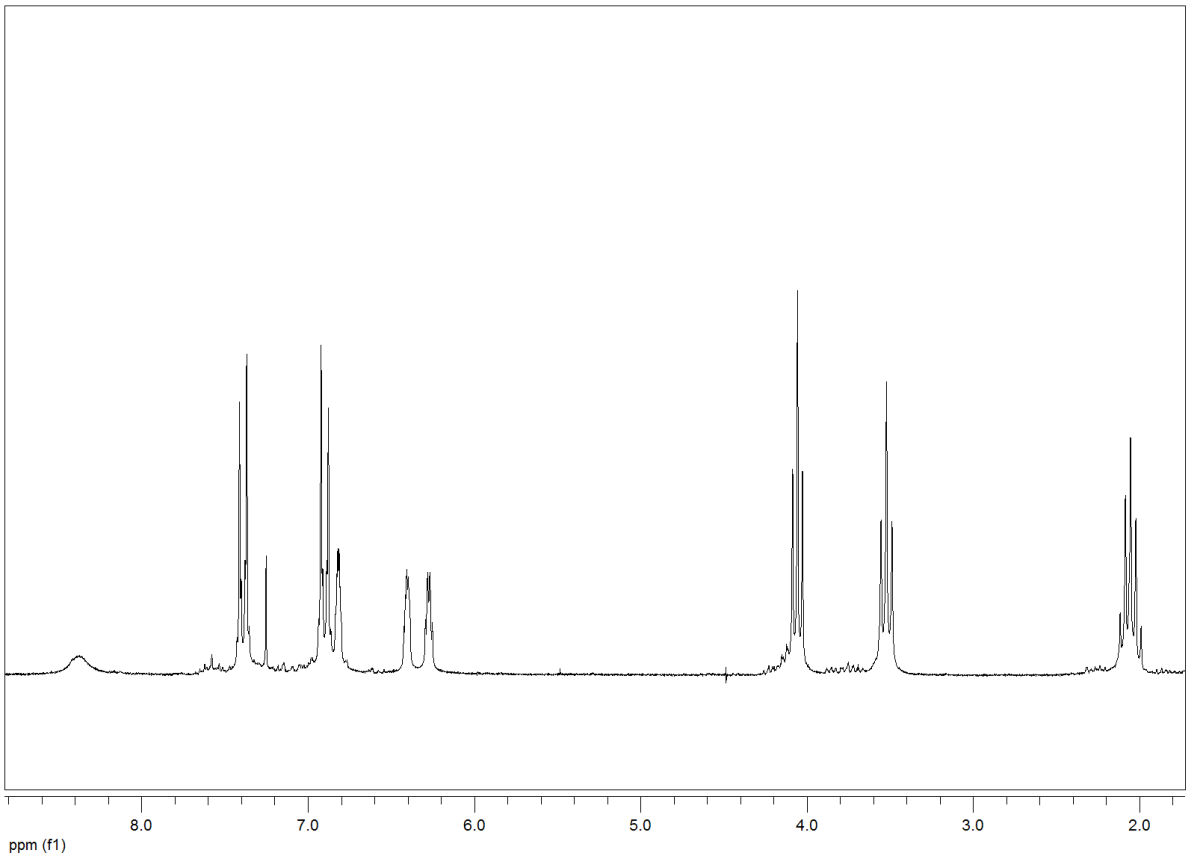

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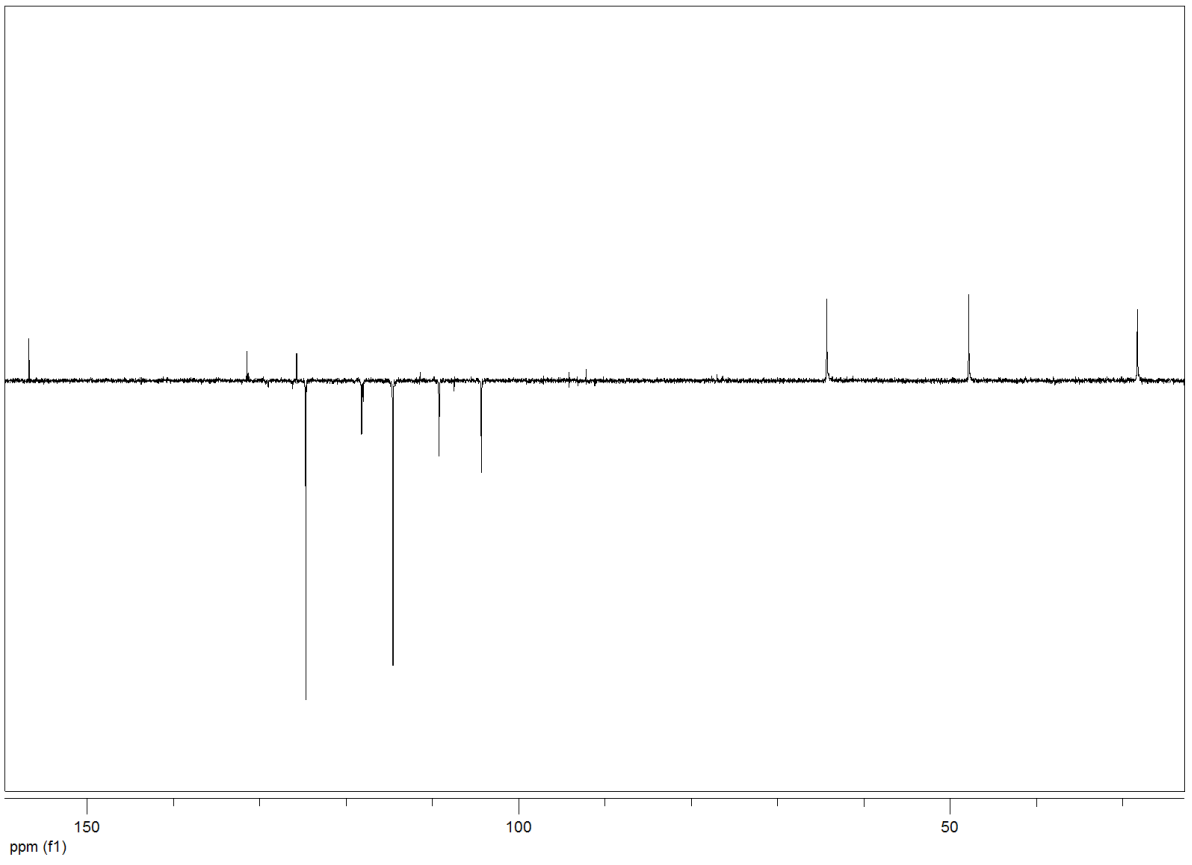

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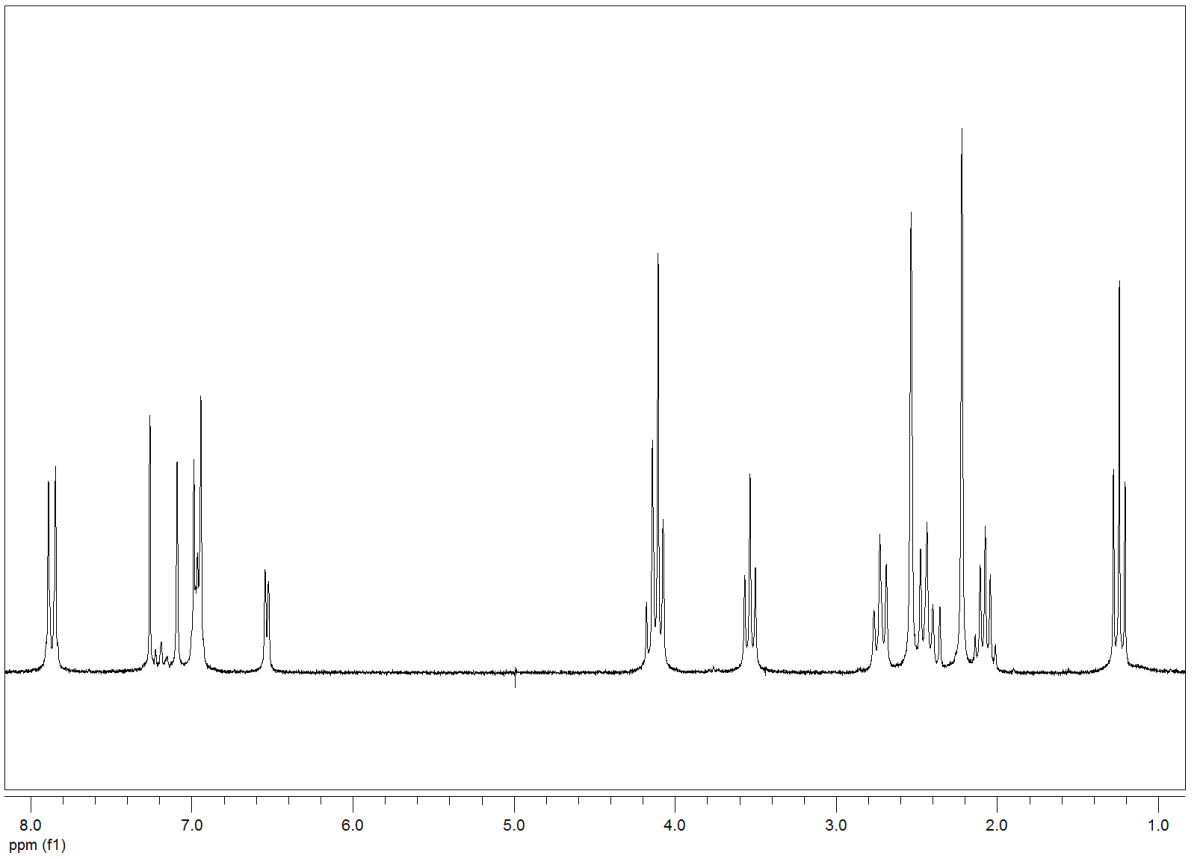

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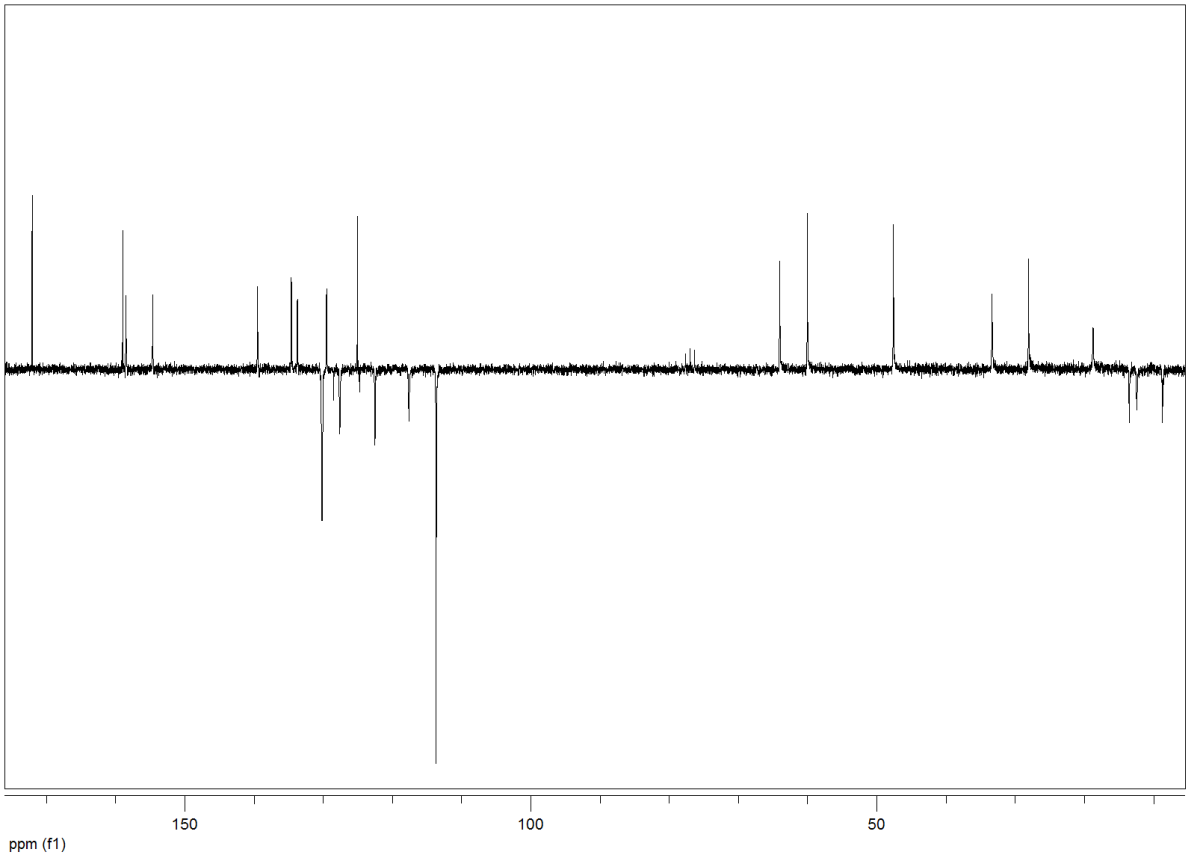

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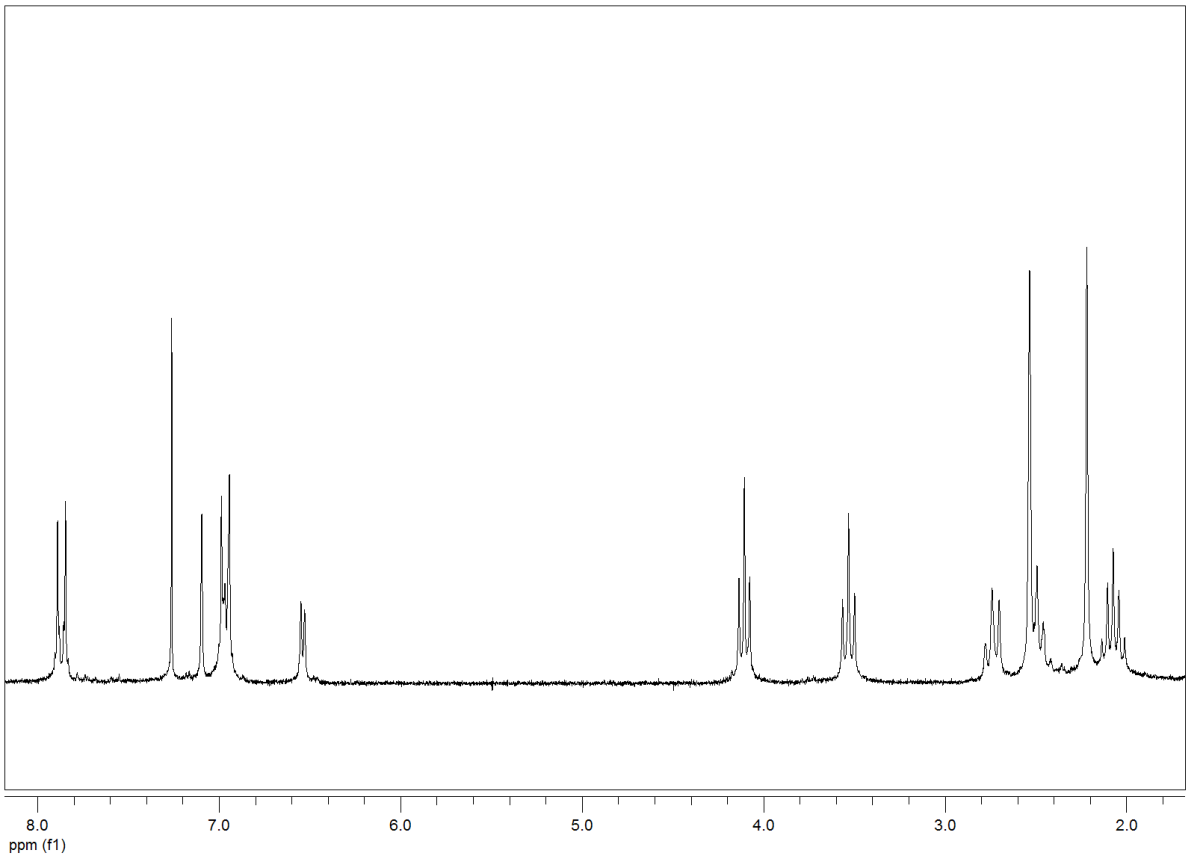

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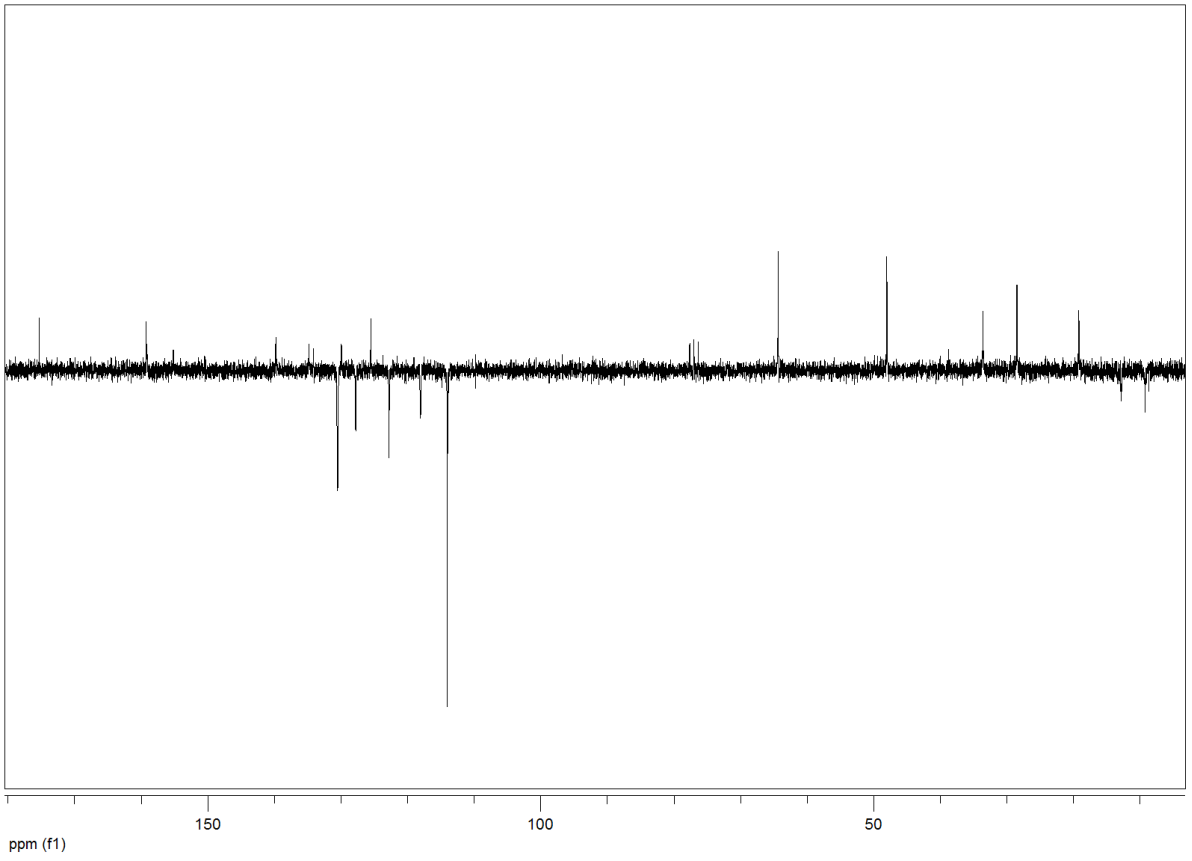

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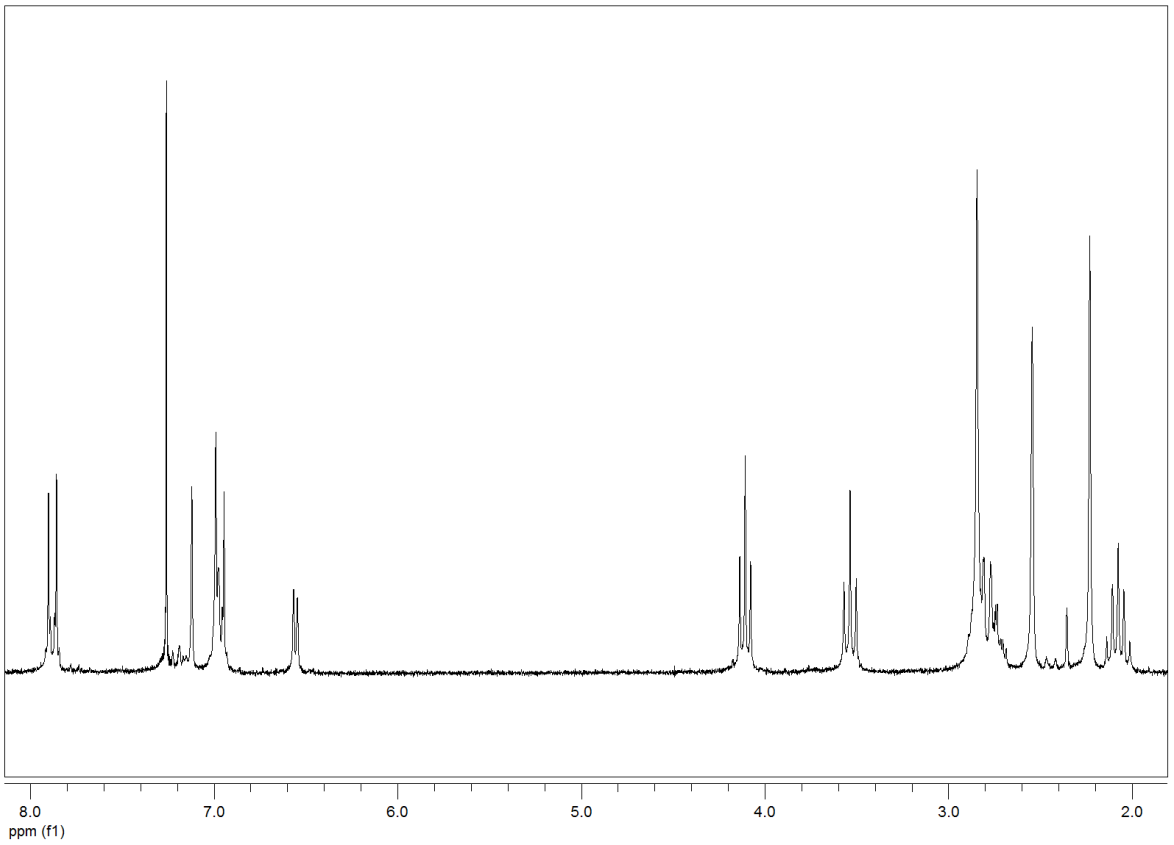

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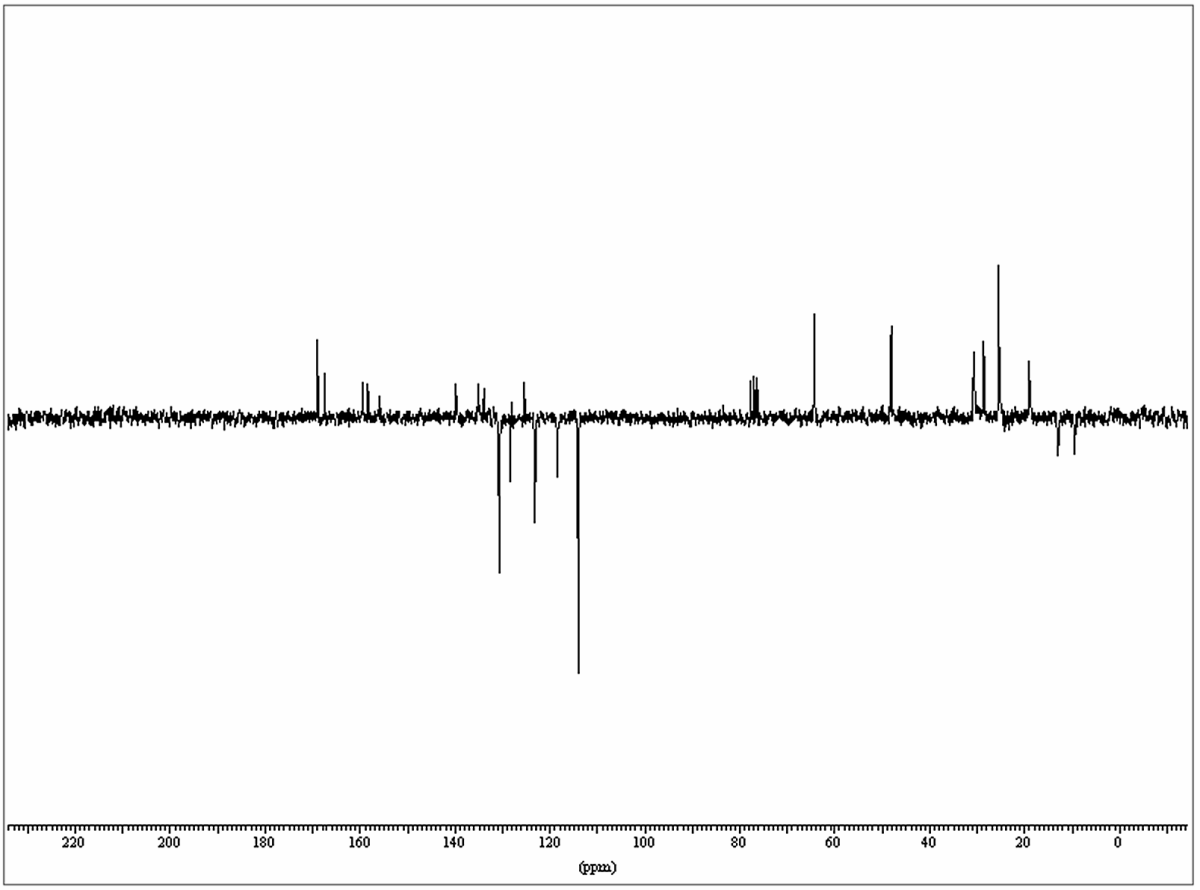

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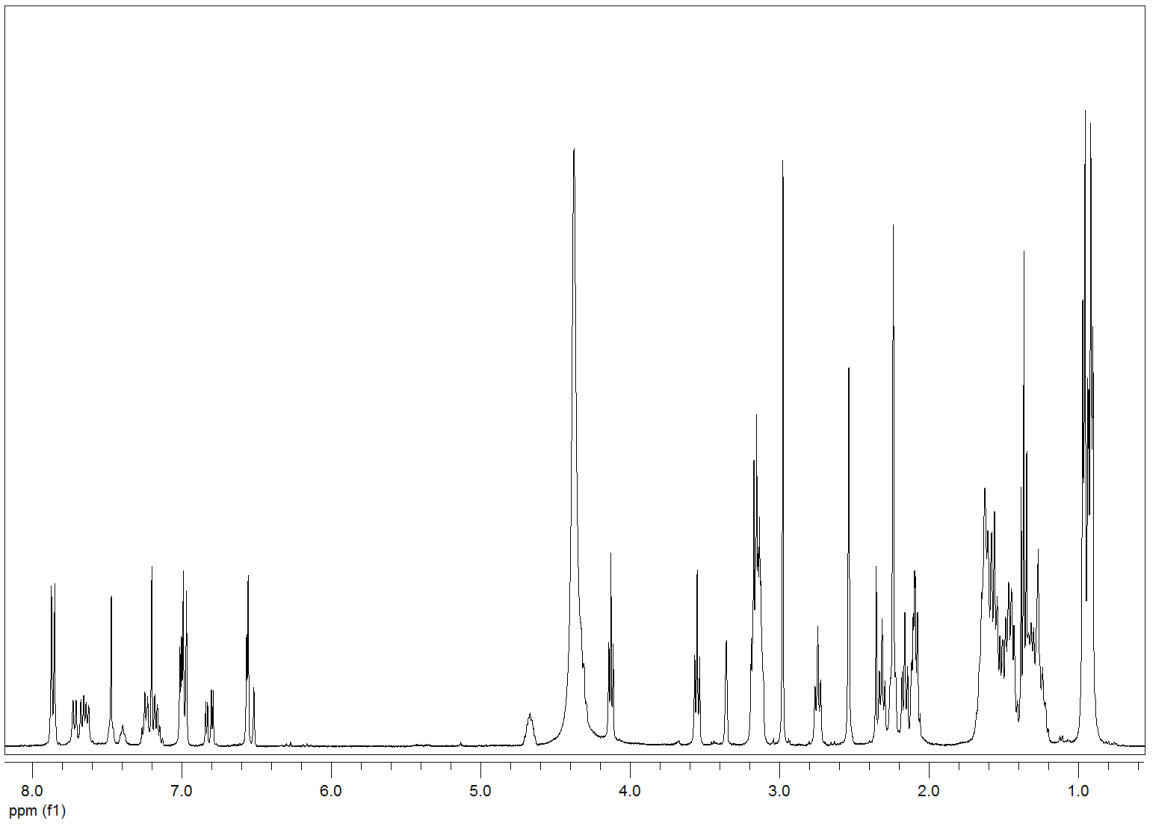

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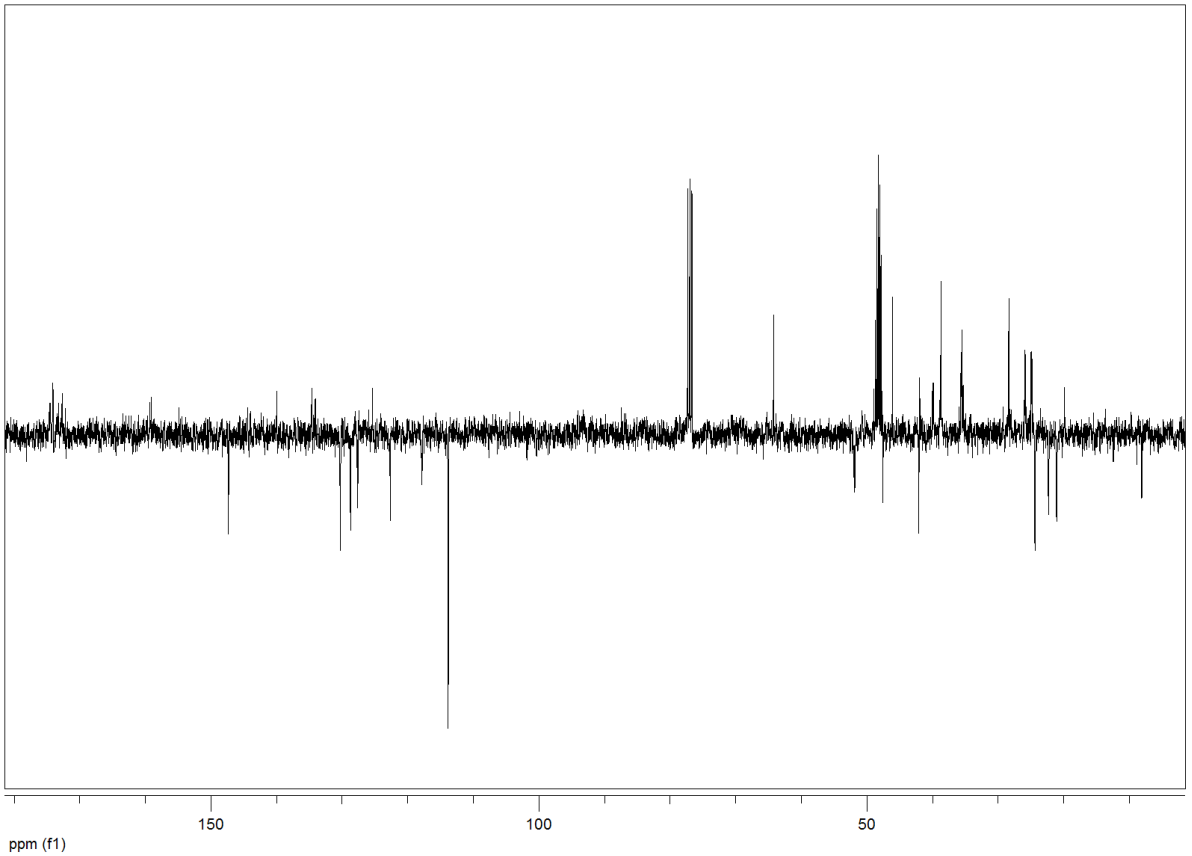

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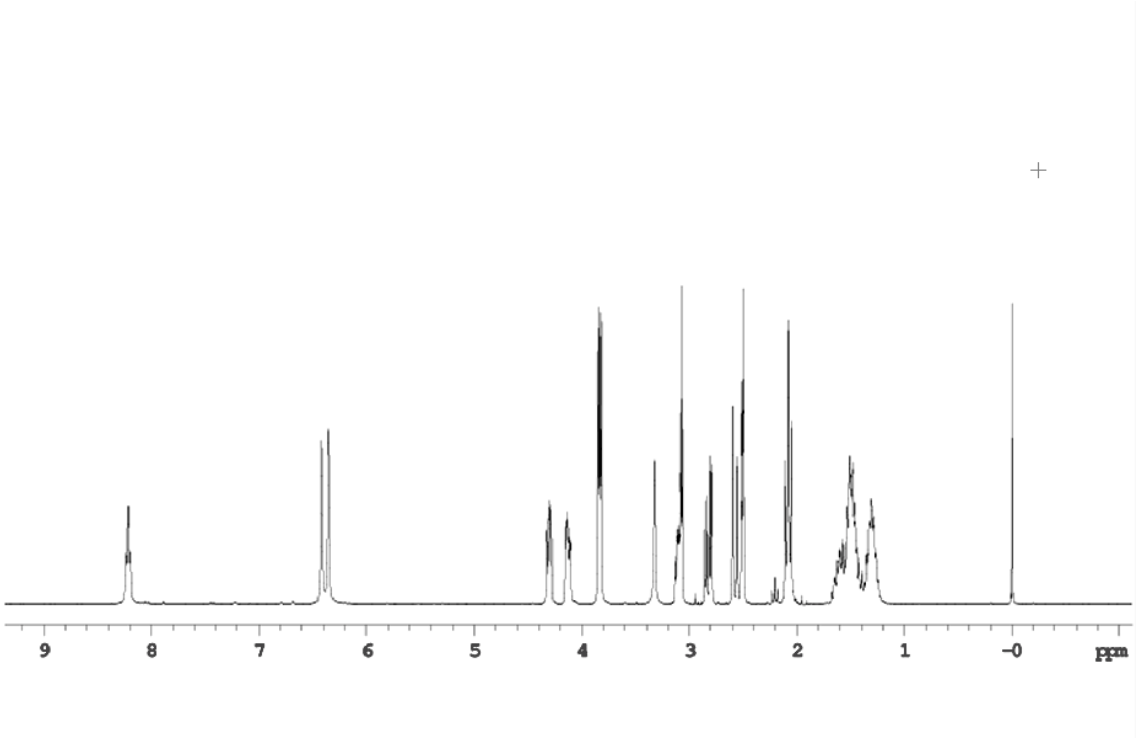

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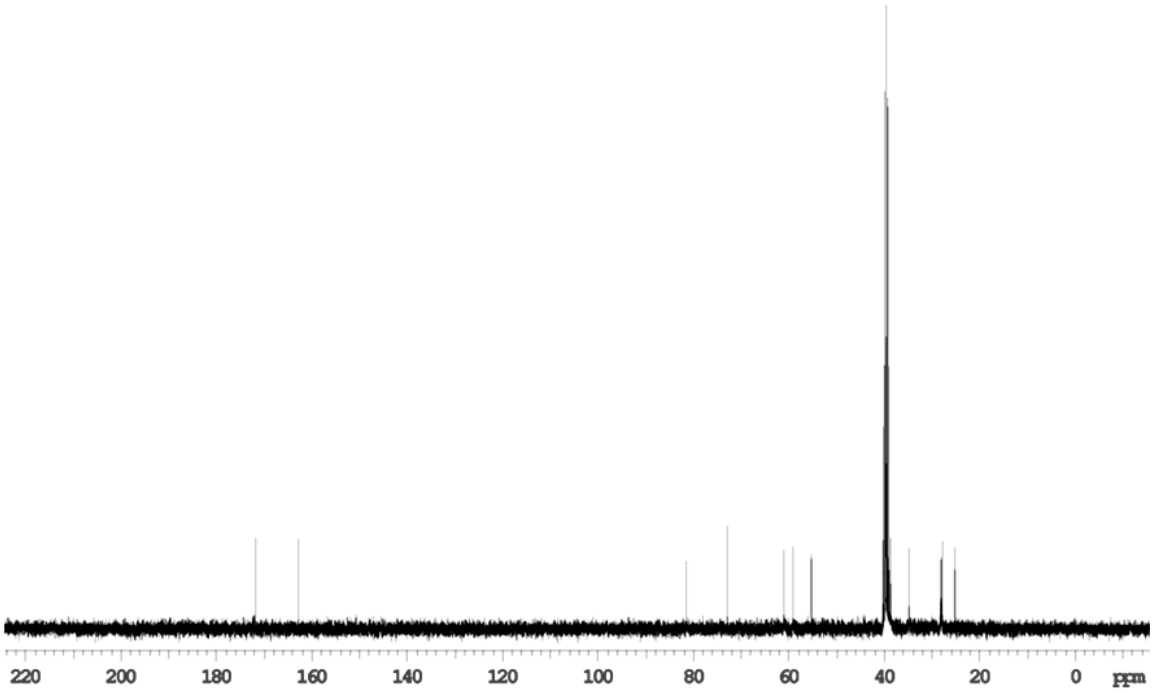

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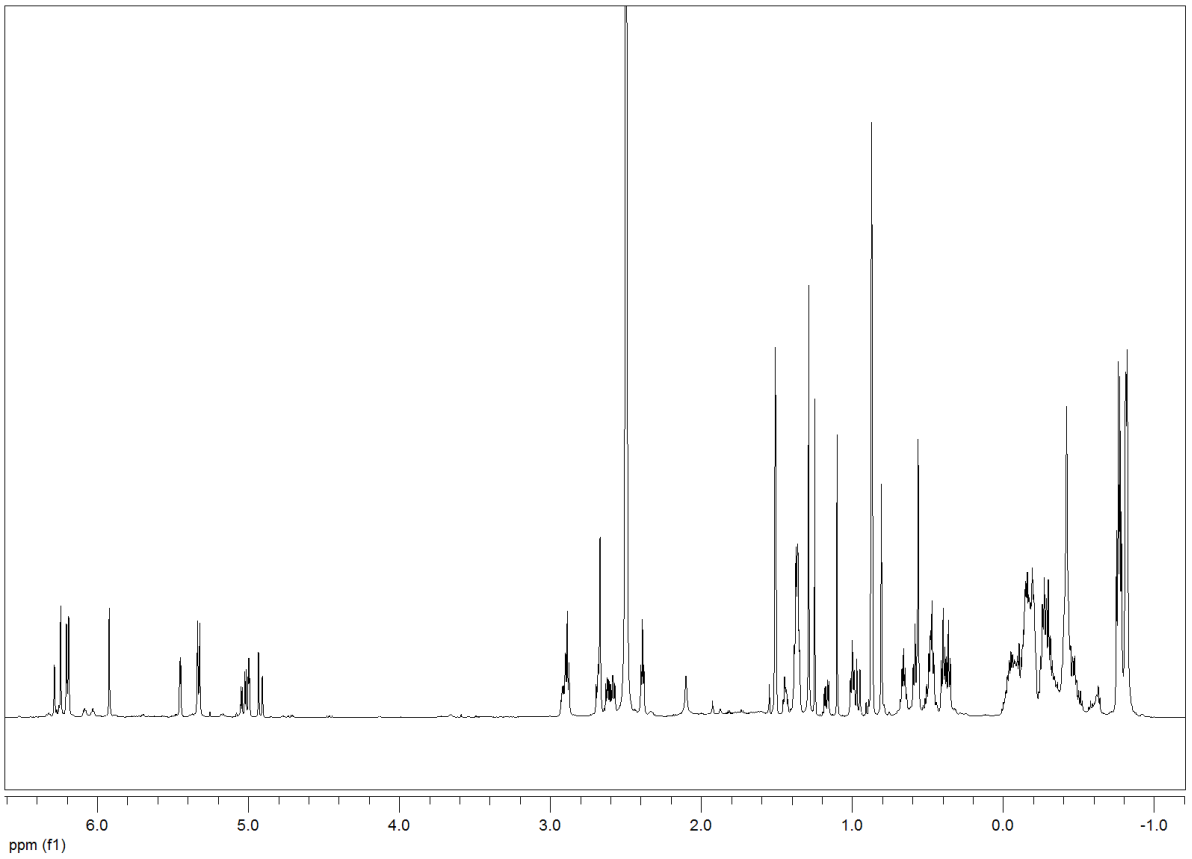

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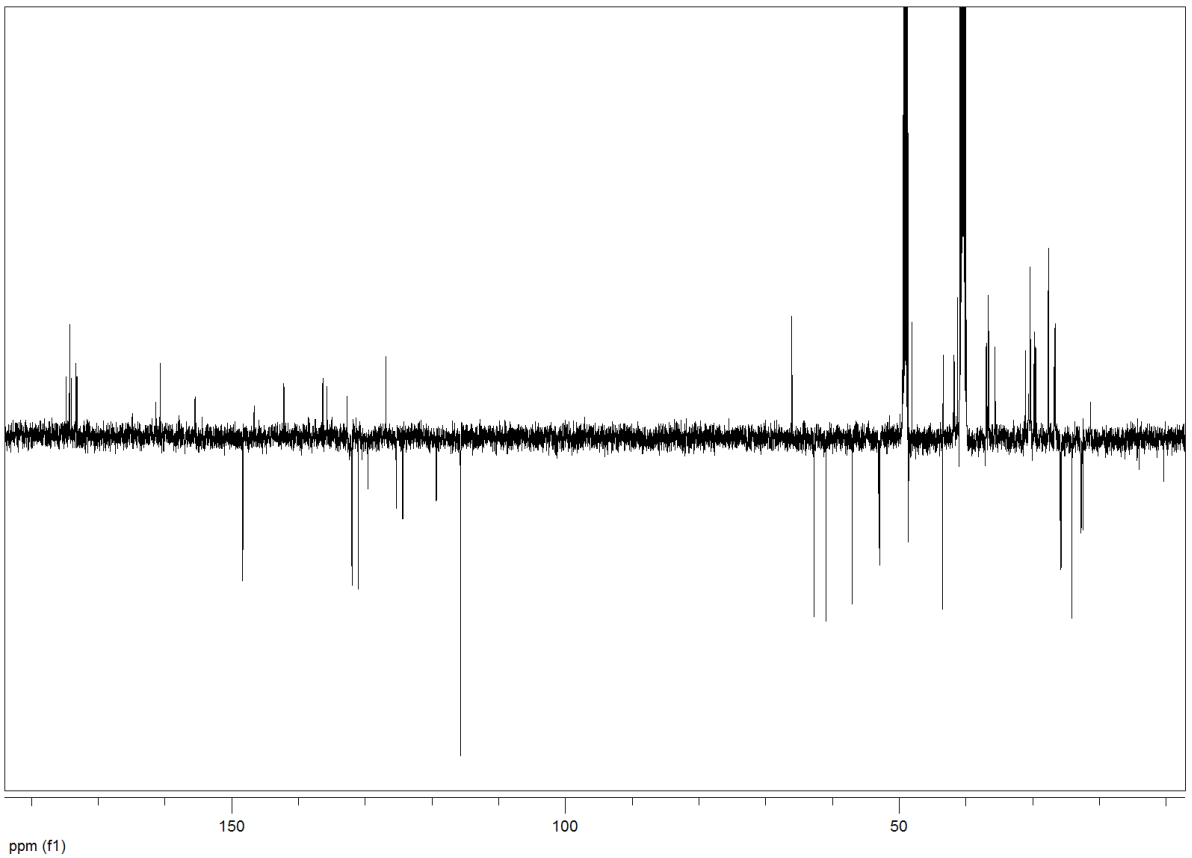

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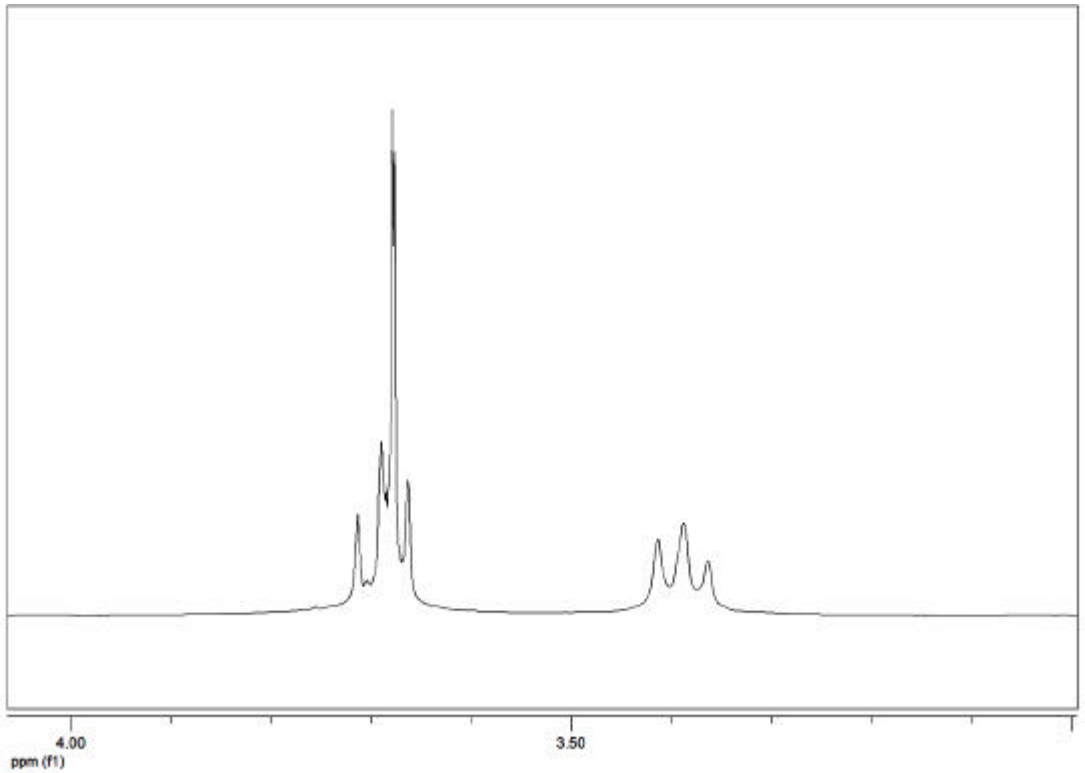

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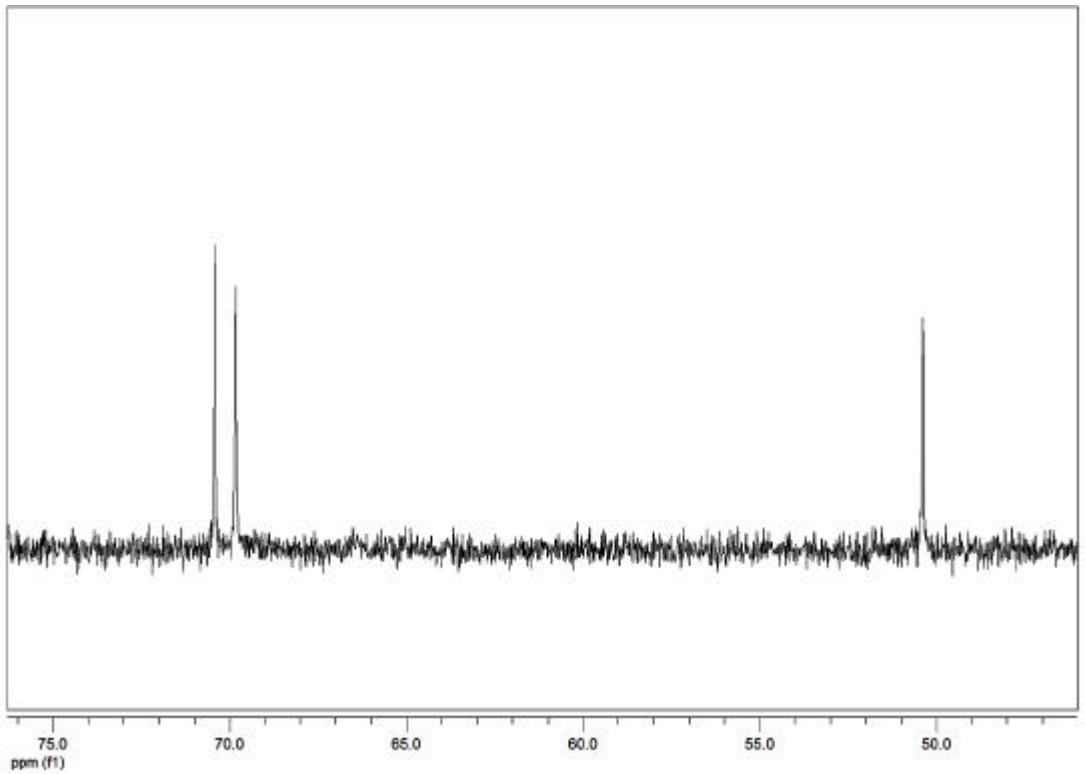

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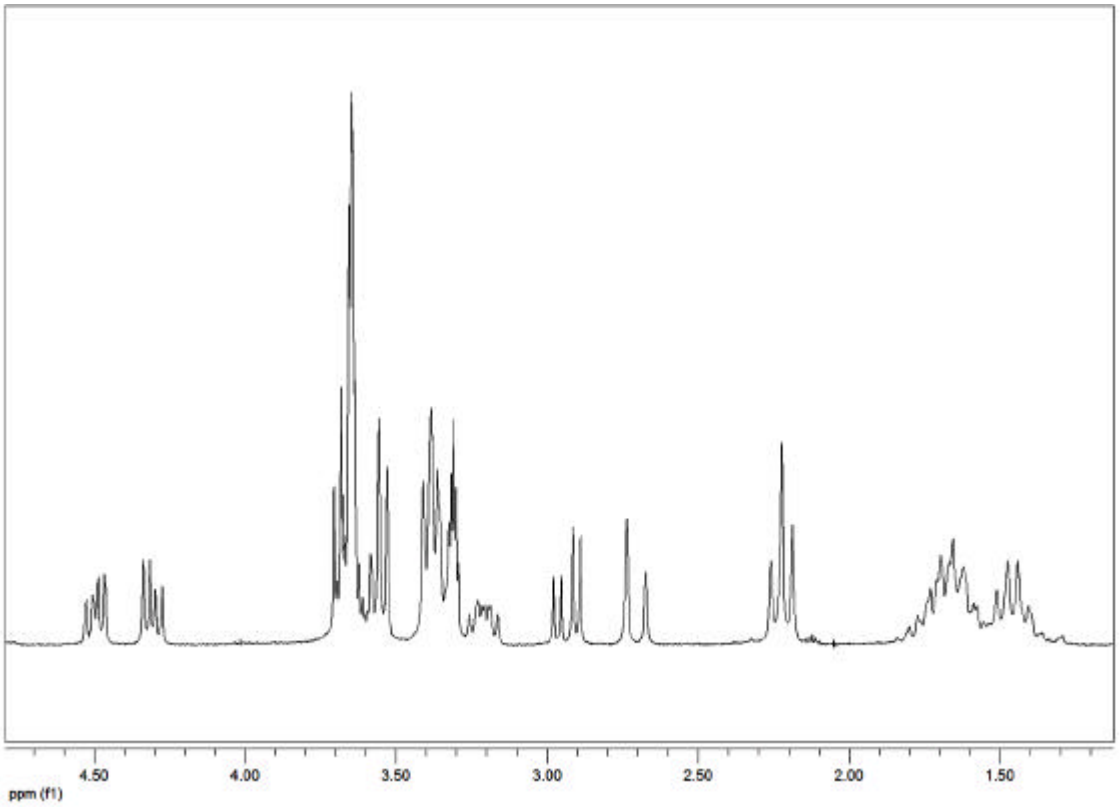

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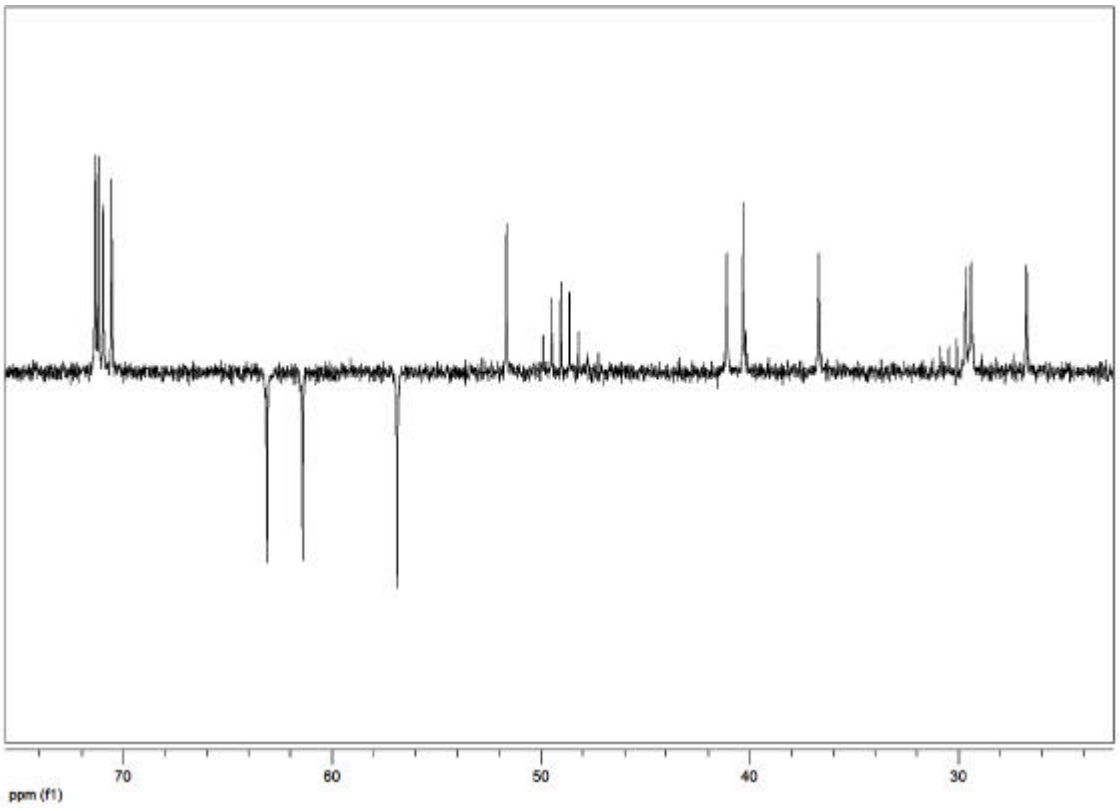

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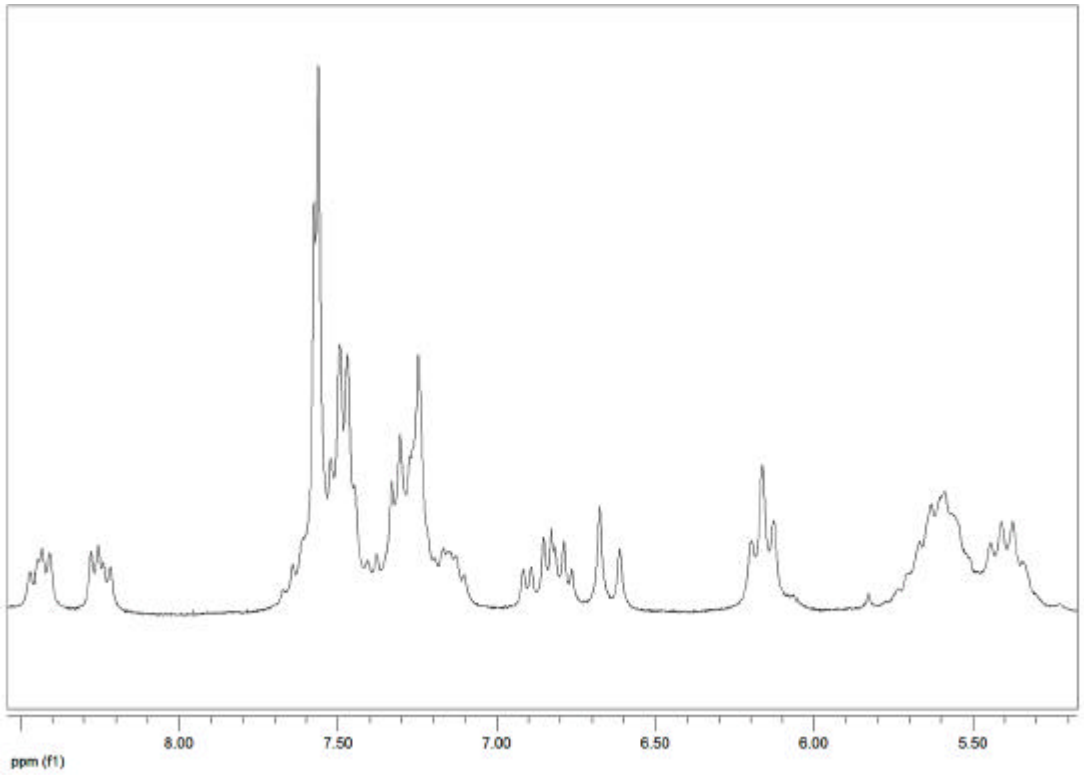

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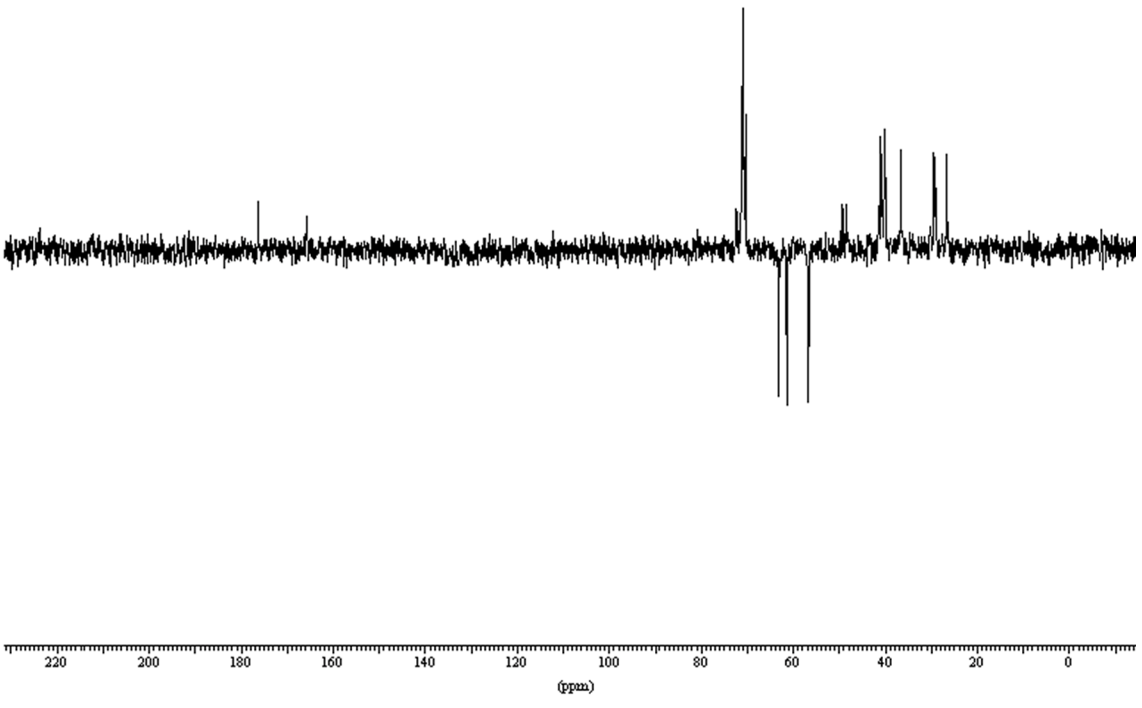

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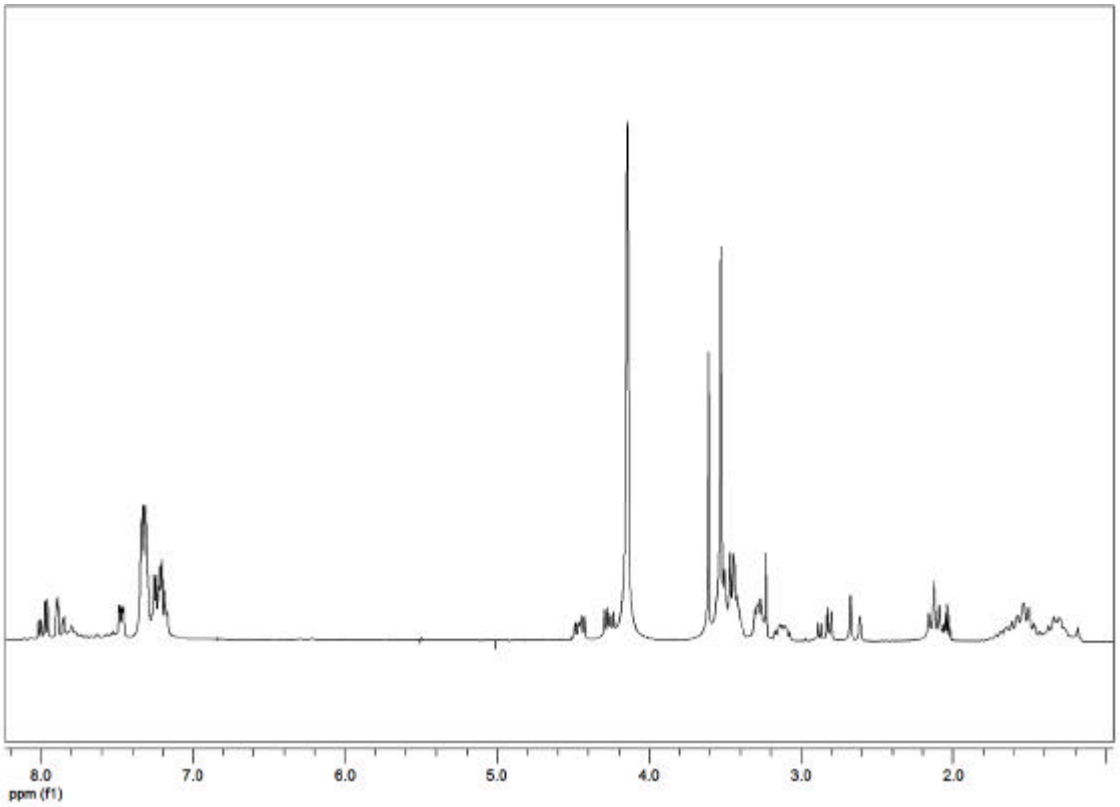

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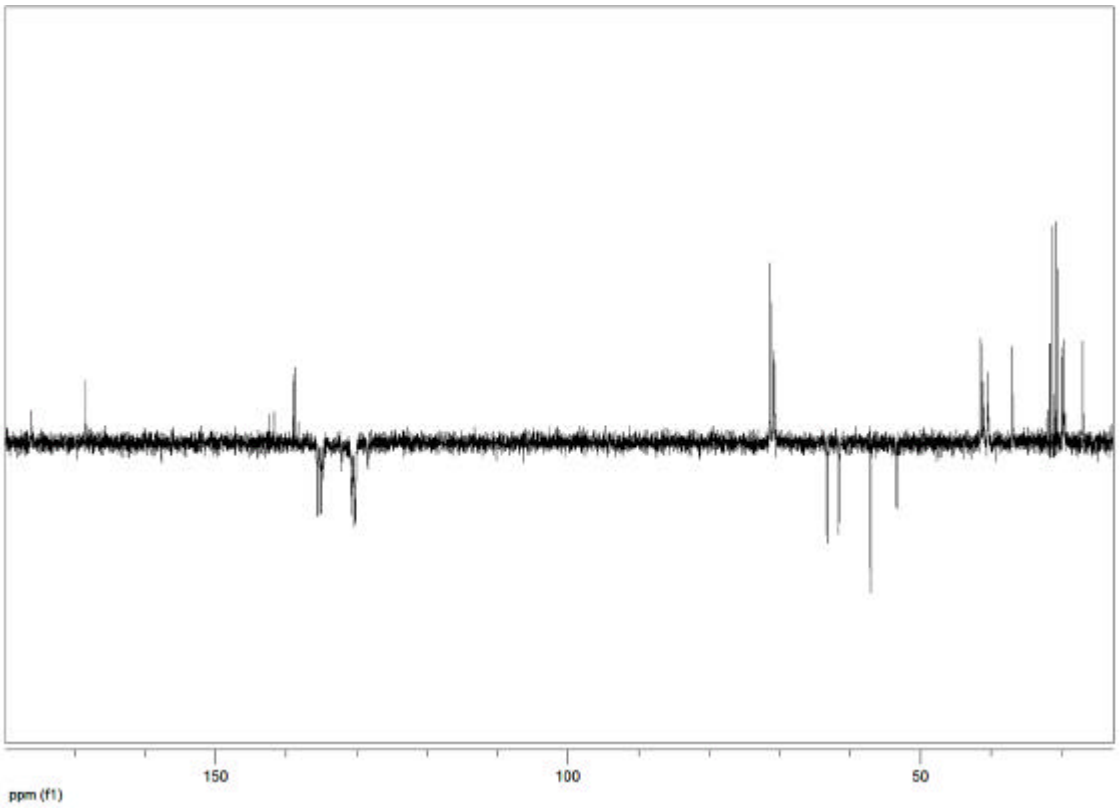

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