Supporting Materials for

Acylazetine as a dienophile in bioorthogonal inverse electron-demand Diels-Alder ligation

Sander B. Engelsma, Lianne I. Willems, Claudia E. van Paaschen, Sander I. van Kasteren, Gijsbert A. van der Marel, Herman S. Overkleeft*, Dmitri V. Filippov*

Leiden Institute of Chemistry, Gorlaeus Laboratories, Einsteinweg 55, 2333 CC Leiden

E-mail: h.s.overkleeft@lic.leidenuniv.nl, filippov@chem.leidenuniv.nl

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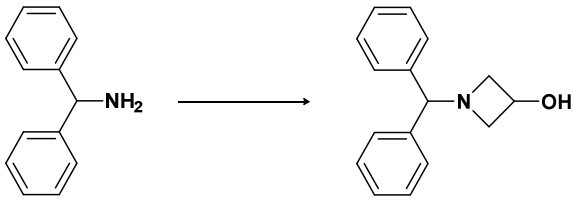
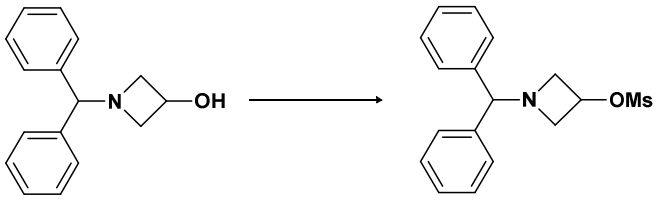
Synthetic Procedures

General: All solvents and reagents were obtained commercially and used as received. Reactions were executed at ambient temperatures unless stated otherwise. Reactions were monitored by TLC-analysis, spraying with varying stains; an aqueous solution of cerium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ 25 g/L), an aqueous solution of potassium permanganate (5 g $KMnO_4$, 25 g K_2CO_3 per L) or an ethanolic solution bromocresol (0.4 g in 1 L, addition of 0.1M $NaOH_{(aq)}$ until the solution turns blue). Column chromatography was performed on silica gel (40-63 µm). ^1H and ^{13}C-APT spectra were recorded on a Bruker AV-400 (400 MHz), Bruker DMX-600 (600 MHz) or Bruker BioSpin (850 MHz). All present ^{13}C-APT spectra are proton decoupled. The high resolution mass spectrometry were recorded by direct injection (2 µL of a 2µM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 ˚C) with resolution R = 600000 at m/z 400 (mass range m/z = 120-400).



1 **2**

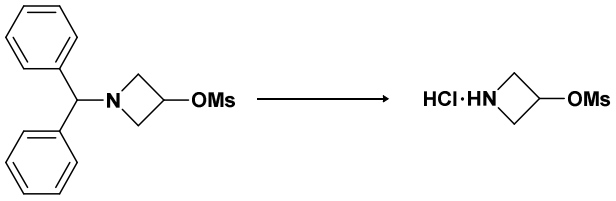
Epichlorohydrin (10.75 ml, 138 mmol, 1.1 eq) was added to a solution of benzhydrylamine (21.55 ml, 125 mmol) in isopropanol (125 mL) and the reaction mixture was stirred overnight at 30 °C. LC/MS analysis showed full conversion into the open-chain intermediate. The reaction mixture was concentrated *in vacuo* and redissolved in MeCN (200 mL). Sodium bicarbonate (11.44 g, 188 mmol, 1.5 eq) was added and the reaction mixture was refluxed for 30 h. The reaction mixture was filtered and reduced *in vacuo*, providing a pale-yellow solid. The solid cake was thoroughly pulverized, suspended in an Et_2O/Pentane/EtOAc mixture (9:9:2, 50 mL) and sonicated for 15 minutes. The white residue was collected by filtration and dried under reduced pressure, yielding 1-benzhydrylazetidin-3-ol as a white solid (24.85 g, 104 mmol, 83%), which was used in the next step without further purification. R_f = 0.49 (1:1 ; EtOAc:PE). ^1H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.12 (m, 10H), 4.42 (p, *J* = 5.8 Hz, 1H), 4.34 (s, 1H), 3.55 – 3.47 (m, 2H), 3.17 (s, 1H), 2.96 – 2.83 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.80, 128.42, 127.38, 127.14, 78.43, 63.30, 61.89.



2 **3**

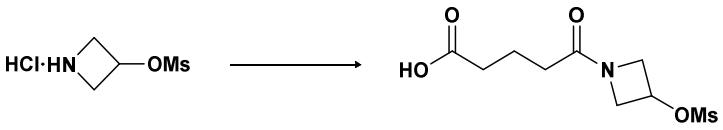
1-benzhydrylazetidin-3-ol (24.65 g, 103 mmol) and triethylamine (21.53 ml, 155 mmol, 1.5 eq) were dissolved in DCM (80 mL) under argon atmosphere and cooled to -40 °C. Methanesulfonyl chloride (9.63 ml, 124 mmol, 1.2 eq) in DCM (20 mL) was slowly added dropwise, while maintaining the temperature at -40 °C. The reaction mixture was stirred for 30 minutes, after which it was diluted with DCM (50 mL) and washed with water (2 x 100 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo* (if the concentrate did not solidify, the oil was diluted with EtOAc and re-concentrated). The residual yellow solid was pulverized and thoroughly rinsed with cold Et_2O. The solid was suspended in a mixture of acetone/water (1:1, 50 mL) and

sonicated for 15 minutes, filtered and vacuum dried, yielding the product as an off-white solid (26.3 g, 83 mmol, 80%). TLC R_f = 0.33 (1:3 ; EtOAc:PE). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.53 – 7.25 (m, 10H), 5.16 (p, *J*=5.8, 1H), 4.50 (s, 1H), 3.73 – 3.66 (m, 2H), 3.31 – 3.23 (m, 2H), 2.93 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.29, 128.46, 127.29, 127.19, 77.99, 67.86, 60.04, 37.91.



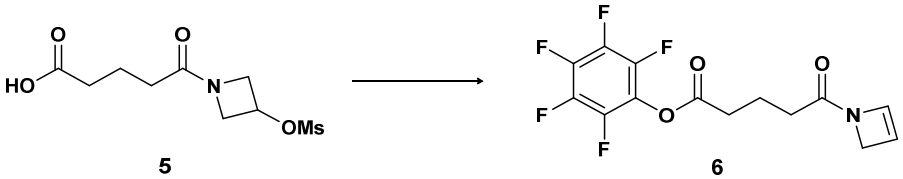
3 **4**

A solution of 1-benzhydrylazetidin-3-yl methanesulfonate (26.3 g, 83 mmol) in DCE (150 mL) was charged with 1-Chloroethyl chloroformate (9.96 ml, 91 mmol, 1.1 eq) and heated to reflux. After 1.5 hour, TLC (Rf = 0.38 - 2:3 ; EtOAc:PE) indicated conversion of the starting material in a lower running product. The solution was concentrated *in vacuo*, redissolved in MeOH (150 mL) and refluxed for an additional 2 h. The reaction mixture was concentrated *in vacuo*. The residual yellow cake was suspended in a mixture of Et_2O:EtOH (2:1, 50 mL) and sonicated for 15 minutes. The remaining white solid was isolated by filtration, washed with Et_2O and dried *in vacuo*, yielding azetidin-3-yl methanesulfonate hydrochloride (10.2 g, 54.4 mmol, 65.5 % yield) as a white solid. ^{1}H NMR (400 MHz, Methanol-d_4) δ 5.44 (ddd, *J* = 6.8, 4.8, 1.9 Hz, 1H), 4.52 (dt, *J* = 12.5, 4.4 Hz, 2H), 4.30 (dd, *J* = 12.6, 4.8 Hz, 2H), 3.23 (s, 3H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 70.08, 54.51, 37.92.



4 **5**

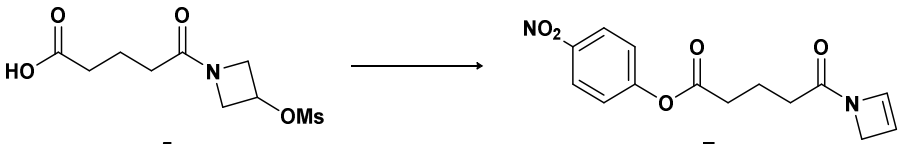
A suspension of azetidin-3-yl methanesulfonate hydrochloride (3.52 g, 18.8 mmol, 1.05 eq) and silver methanesulfonate (3.88 g, 19.1 mmol, 1.07 eq) in dry MeCN (25 mL) was prepared under argon atmosphere, and stirred vigorously at 40 °C for 15 minutes. Dry potassium carbonate (2.14, 35.7 mmol, 2 eq) and glutaric anhydride (2.04 g, 17.9 mmol, 1 eq) were added and the reaction was refluxed for 2 h. TLC indicated completion (10% EtOH in DCM), using bromocresol as a visualization agent. The reaction mixture was filtered over celite and carefully rinsed with water three times to remove product from the cake. The filtrate was diluted with 25% water in MeCN and acidified with Amberlite (IR120, H-form). After filtration, the solution was concentrated and purified by column chromatography (7% » 10% EtOH in DCM), yielding the title compound as a thick white solid (3.75 g, 14.1 mmol, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 5.28 (tt, *J*=6.7, 4.1, 1H), 4.52 (ddd, *J*=10.1, 6.6, 1.5, 1H), 4.41 (ddd, *J*=11.5, 6.6, 1.4, 1H), 4.36 – 4.29 (m, 1H), 4.20 – 4.14 (m, 1H), 3.12 (s, 3H), 2.45 (t, *J*=7.0, 2H), 2.23 (t, *J*=7.3, 2H), 1.97 (p, *J*=7.1, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.36, 172.75, 66.53, 57.47, 55.14, 38.37, 32.87, 30.29, 19.60. HRMS $[M+H]^+$ m/z calc. for $[C_{14}H_{10}F_5NO_3]$ = 266,06928, found 266,06919



5 **6**

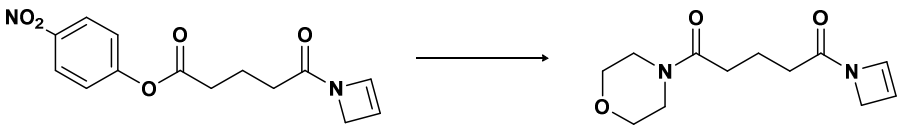
KO*t*Bu in THF (20.5 mL, 1.6 M, 32.9 mmol, 2.3 eq) was added to a solution of mesylazetidine pentanoic acid spacer **5** in DMF (150 mL), under argon atmosphere. The reaction mixture was stirred at 50 °C for 2 h. After

cooling to room temperature, EDC·HCl (8.23 g, 42.9 mmol, 3 eq.) and pentafluorophenol (4.58 mL, 42.9 mmol, 3 eq.) were added, and the reaction mixture was stirred for an additional 1.5 h. The mixture was poured into H_2O (150 mL) and extracted with DCM (150 mL). The organic layer was washed with brine (30 mL), dried over $MgSO_4$, filtrated and concentrated in *vacuo*. The crude product was purified by column chromatography (10% » 30% EtOAc in PE) to yield **6** as a colorless oil which solidified upon standing at -20°C (3 g, 9 mmol, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 6.93 – 6.69 (d, *J*=95.6, 1H), 5.75 (d, *J*=9.6, 1H), 4.52 (d, *J*=37.4, 2H), 2.90 – 2.76 (m, 2H), 2.42 (dt, *J*=36.0, 7.2, 2H), 2.15 (p, *J*=7.1, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.36, 172.75, 66.53, 57.47, 55.14, 38.37, 32.87, 30.29, 19.60. HRMS $[M+H]^+$ m/z calc. for $[C_{14}H_{10}F_5NO_3]$ = 336,06536, found 336,06512.



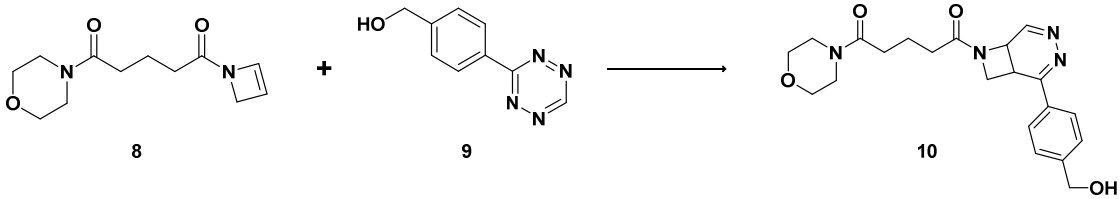
5 **7**

A solution of mesylazetidine pentanoic acid spacer **5** (0.58 g, 2.186 mmol) in DMF (25 mL) was prepared and put under argon atmosphere. KOtBu (3.01 ml, 4.81 mmol) was slowly added and the reaction mixture was warmed to 50 °C and stirred vigorously for 2 hours. The reaction was allowed to cool to room temperature, after which *p*-Nitrophenol (0.912 g, 6.56 mmol) was added. The reaction mixture was stirred until a clear yellow solution had formed. Then EDC·HCl (1.257 g, 6.56 mmol) was added and the reaction was stirred for additional 2 hours. The reaction mixture was poured into ether/EtOAc (2:1) and washed with water and brine. The organic layer was dried over $MgSO_4$, filtrated and concentrated in *vacuo*. The crude product was purified by column chromatography (30% » 80% EtOAc in PE) to yield **7** as a white crystalline substance (0.54 g, 1.86 mmol, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.27 (d, *J*=9.1, 2H), 7.29 (d, *J*=9.1, 2H), 6.80 (dd, *J*=100.2, 1.7, 1H), 5.73 (d, *J*=6.9, 1H), 4.51 (d, *J*=35.3, 2H), 2.74 (t, *J*=7.2, 2H), 2.46 (t, *J*=7.1, 1H), 2.37 (t, *J*=7.1, 1H), 2.13 (p, *J*=7.2, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 170.72, 155.28, 145.20, 137.40, 136.68, 125.13, 122.37, 113.71, 113.41, 58.53, 56.56, 33.23, 30.60, 29.56, 19.96, 19.78.

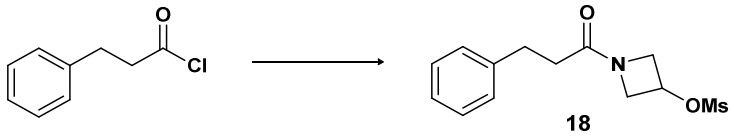


7 **8**

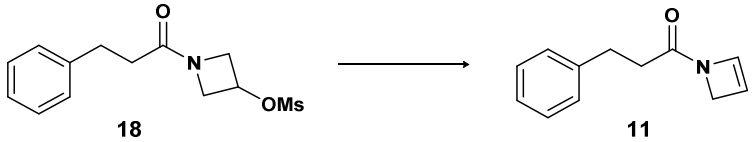
Morpholine (0.099 ml, 1.137 mmol) was added to a solution of 4-nitrophenyl 5-(azet-1(2H)-yl)-5-oxopentanoate **7** (0.11 g, 0.379 mmol) in DCM (1 mL). After 1 hour, the reaction mixture was directly purified by column chromatography (DCM » DCM:Acetone:EtOH 80:20:1 » 50:45:5) to give **8** (88 mg, 0.369 mmol, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.27 (d, *J*=9.1, 2H), 7.29 (d, *J*=9.1, 2H), 6.80 (dd, *J*=100.2, 1.7, 1H), 5.73 (d, *J*=6.9, 1H), 4.51 (d, *J*=35.3, 2H), 2.74 (t, *J*=7.2, 2H), 2.46 (t, *J*=7.1, 1H), 2.37 (t, *J*=7.1, 1H), 2.13 (p, *J*=7.2, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 170.72, 155.28, 145.20, 137.40, 136.68, 125.13, 122.37, 113.71, 113.41, 58.53, 56.56, 33.23, 30.60, 29.56, 19.96, 19.78. HRMS $[M+H]^+$ m/z calc. for $[C_{14}H_{14}N_2O_5]$ = 291,09755, found 291,09744. HRMS $[M+H]^+$ m/z calc. for $[C_{12}H_{18}N_2O_3]$ = 239,13902, found 239,13900.



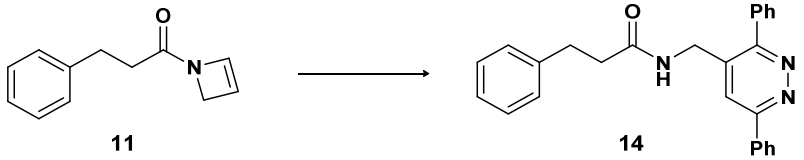
Tetrazine **9** was added to a solution of acylazetidine **8** in a H_2O:THF mixture (1:1, 1 mL). The reaction was stirred for 0.5 hour, diluted with water and extracted with EtOAc. The water layer was separated and reduced in volume and co-evaporated with dioxane. The residue was redissolved in DMSO (1 mL). Diethyl ether (10 mL) was added and the mixture was sonicated for 0.5 hour. The ether was decanted and the remaining off-yellow powder was dried in *vacuo* to give adduct **10**.



3-Phenylpropionyl chloride (2.228 ml, 15.00 mmol) was added to a cooled solution (-78 °C) of azetidin-3-yl methanesulfonate hydrochloride (2.81 g, 15 mmol) and TEA (4,60 ml, 33,0 mmol) in DCM (60 mL), under argon atmosphere. The reaction mixture was stirred for 30 minutes, before being quenched with water. The aqueous layer was extracted twice with DCM (30 mL). The organic layer was isolated and dried over $MgSO_4$, filtered and concentrated in *vacuo*. The residual oil was purified by flash column chromatography (70% » 100% EtOAc in pentane), yielding **18** (3.04 g, 10.73 mmol, 72%) as a white crystalline substance. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (dd, *J* = 8.1, 6.7 Hz, 2H), 7.24 – 7.16 (m, 3H), 5.12 (tt, *J* = 6.7, 4.1 Hz, 1H), 4.36 – 4.25 (m, 1H), 4.18 (ddd, *J* = 10.1, 6.6, 1.5 Hz, 1H), 4.11 – 3.97 (m, 2H), 3.03 (s, 3H), 2.93 (t, *J* = 7.6 Hz, 2H), 2.43 – 2.32 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 172.36, 140.81, 128.60, 128.41, 126.42, 66.61, 57.17, 54.89, 38.35, 33.77, 31.04.

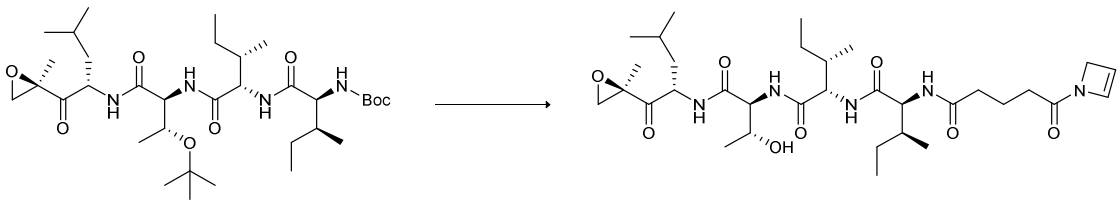


Potassium tert-butoxide (1,459 g, 13.00 mmol) was added to a solution of **18** (2.83 g, 10 mmol) in tBuOH (30 mL), under argon atmosphere. The reaction mixture was stirred for 4h at 50 °C. The reaction mixture was poured into a diluted ammonium chloride solution and extracted with DCM. The organic layers were dried over $MgSO_4$, filtered and concentrated in *vacuo*. Purification by silica gel column chromatography (40% » 50% EtOAc in pentane) yielded **11** (1.09 g, 5.84 mmol, 58%) as a colorless oil that solidified upon standing. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (dd, *J* = 7.1, 1.7 Hz, 2H), 7.62 (s, 1H), 7.53 (t, *J* = 6.0 Hz, 1H), 7.45 – 7.38 (m, 1H), 7.38 – 7.23 (m, 7H), 7.14 – 7.01 (m, 6H), 4.27 (d, *J* = 5.9 Hz, 2H), 2.88 (t, *J* = 7.6 Hz, 2H), 2.51 (t, *J* = 7.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.92, 159.03, 157.98, 140.49, 137.75, 135.72, 129.90, 129.07, 128.85, 128.72, 128.41, 128.27, 128.07, 126.92, 126.03, 122.63, 39.77, 37.48, 31.28.



3,6-diphenyl-1,2,4,5-tetrazine (62.6 mg, 0.267 mmol) was added to a solution of 1-(azet-1(2H)-yl)-3-phenylpropan-1-one (50 mg, 0.267 mmol) in H_2O:THF (1:4, 0.5 mL). The reaction mixture was stirred overnight. TLC (20% EtOAc in DCM) showed conversion into a single product. The solution was concentrated in *vacuo* and purified by column chromatography, yielding adduct **14** as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 7.1, 1.7 Hz, 2H), 7.62 (s, 1H), 7.53 (t, *J* = 6.0 Hz, 1H), 7.45 – 7.38 (m, 1H), 7.38 – 7.23 (m, 7H), 7.14 – 7.01 (m, 5H), 4.27 (d, *J* = 5.9 Hz, 2H), 2.88 (t, *J* = 7.6 Hz, 2H), 2.51 (t, *J* = 7.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.92,

159.03, 157.98, 140.49, 137.75, 135.72, 129.90, 129.07, 128.85, 128.72, 128.41, 128.27, 128.07, 126.92, 126.03, 122.63, 39.77, 37.48, 31.28.



15

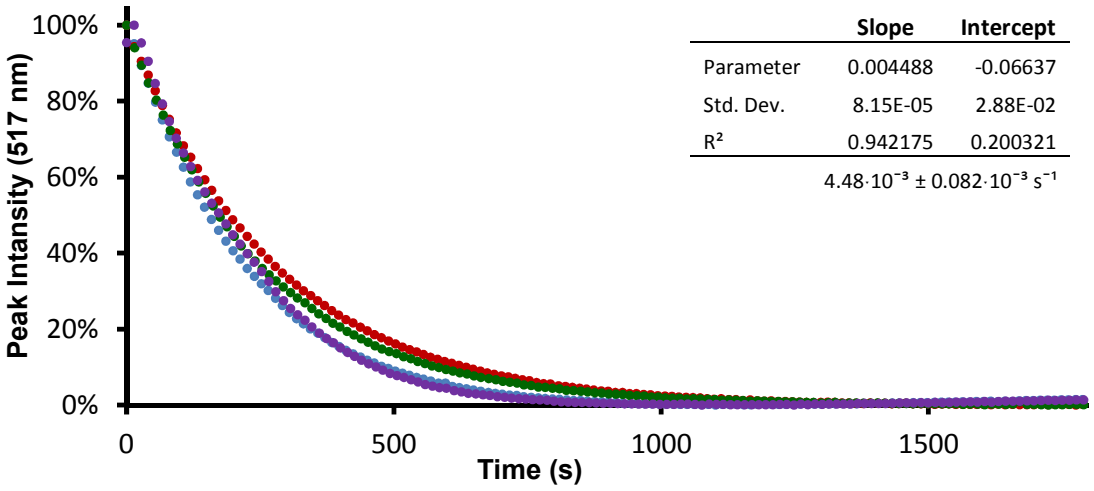
Epoxomicin-Thr(tBu)-Boc (0.05 g, 0.076 mmol) was dissolved in 0.5 ml TFA and stirred for 15 minutes. The reaction mixture was diluted with toluene and concentrated *in vacuo*. The residue was co-evaporated with toluene and redissolved in DMF (0.5 mL). DIPEA (0.053 ml, 0.305 mmol) was added, followed by perfluorophenyl 5-(azet-1(2H)-yl)-5-oxopentanoate (**6**) (0.026 g, 0.076 mmol). The reaction progress was followed by TLC-MS analysis. Upon completion, the reaction mixture was purified by HPLC to give **15** (15 mg, 0.023 mmol, 30%). ^{1}H NMR (600 MHz, DMSO-d6) δ = 7.96 – 7.74 (m, 4H), 6.95 (d, *J*=20.1, 1H), 5.81 (d, *J*=48.3, 1H), 4.84 – 4.71 (m, 1H), 4.54 (d, *J*=4.9, 1H), 4.42 – 4.35 (m, 1H), 4.31 (s, 1H), 4.27 – 4.15 (m, 3H), 3.87 (h, *J*=5.7, 1H), 3.18 (d, *J*=5.3, 1H), 3.00 (d, *J*=5.3, 1H), 2.27 – 2.08 (m, 3H), 1.81 – 1.58 (m, 3H), 1.39 (s, 3H), 1.36 – 1.24 (m, 2H), 1.12 – 1.02 (m, 2H), 0.99 (d, *J*=6.3, 3H), 0.89 (d, *J*=6.7, 3H), 0.85 – 0.73 (m, 15H). ^{13}C NMR (151 MHz, DMSO-d6) δ = 208.00, 171.63, 171.17, 170.85, 170.06, 138.49, 137.20, 113.98, 112.74, 66.43, 58.79, 58.36, 57.86, 56.86, 56.03, 51.55, 49.23, 43.30, 38.69, 36.44, 36.17, 34.69, 34.28, 32.55, 30.67, 29.53, 24.27, 23.16, 21.10, 21.05, 20.97, 19.70, 15.32, 15.19, 10.90, 10.84.

Kinetic Experiments

Determination of the pseudo-first order reaction rate constant

A 1.2 mM stock solution of tetrazine **9** in 12% DMSO:Water (v:v) and a 12 mM stock solution of acylazetine **8** in 12% DMSO:Water were prepared. 1 mL of the 1.2 mM tetrazine **9** stock solution was brought into a quarts cuvette (10 mm width, 2 mL volume), which was placed in the measurement chamber of a Cary 300 UV-Vis spectrophotometer (Agilant Technologies). Then, 1 mL of the 12 mM acylazetine **8** stock solution was added to the cuvette and the measurement was immediately started. Upon addition of the second solution, the concentration became 0.6 mM for **9** and 6 mM for **8**. The absorption decay was followed at 517 nm, measuring at 13 second intervals over 30 minutes. The experiments were conducted at uncontrolled room temperature (±20 ˚C).

The reaction rate was derived from four data sets using every measurement interval up to 600 seconds (absorption range from 100% to >10%). This gave 4 data points per value of time (x). This array was subjected to the LINEST function (Microsoft Excel 2013) to determine the slope and the standard deviation. The slope represents the pseudo first-order rate constant.

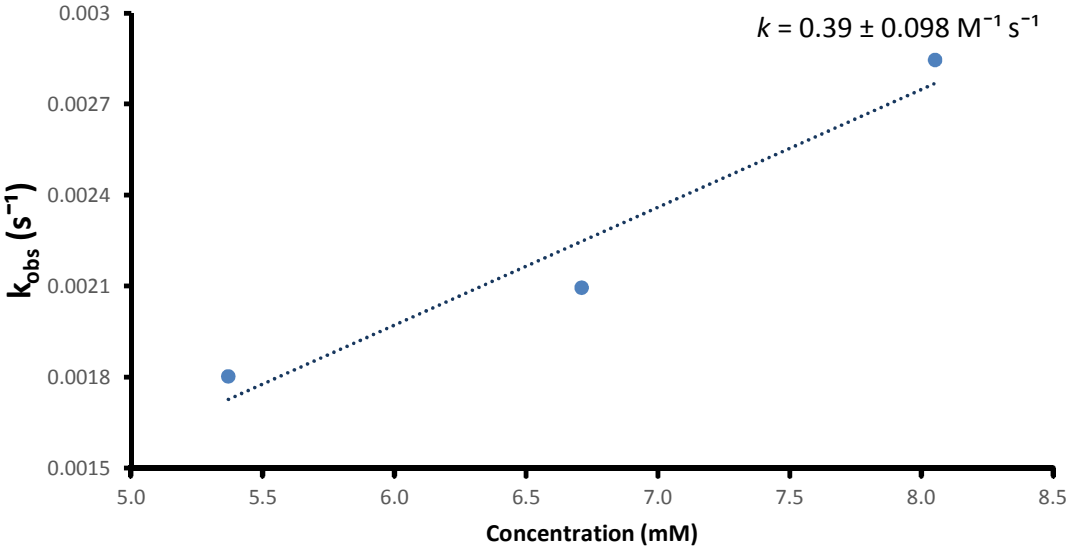


	Slope	Intercept
Parameter	0.004488	-0.06637
Std. Dev.	8.15E-05	2.88E-02
R^2	0.942175	0.200321

$4.48 \cdot 10^{-3} \pm 0.082 \cdot 10^{-3}\ s^{-1}$

Determination of the second order rate constant

The second order rate was derived from the observed pseudo-first order reaction rates at three different (excess) concentrations of **8**. These experiments were conducted at uncontrolled room temperature (±20 ˚C). The slope of this plot represents the second order rate constant.



Biological Essays

Preparation of cell extracts

Human Embryonic Kidney (HEK) cell extracts were prepared from cultured HEK-293T cells by harvesting, washing with PBS (2x) and cell lysis in digitonin lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.025% digitonin) for 30 min on ice followed by sonication on ice for 3x 10 s. After centrifugation of the cells at 16,100 g for 15 min at 4 °C, the supernatants were collected and the protein concentration was determined by Bradford assay.

Competition assay versus MV151

HEK cell lysates (20 µg total protein per experiment) in lysis buffer (9 µL) were exposed to the indicated concentrations of **15** (1 µL 10x solution in DMSO) for 1 hr at 37 °C, after which the lysates were incubated with 1 µM MV151 (1.1 µL 10 µM in DMSO) for 1 hr at 37 °C. The reaction mixtures were then boiled at 100 °C for 5 minutes with 4 µL 4x Laemmli's sample buffer containing 2-mercaptoethanol and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using a Typhoon Variable Mode Imager (Amersham Biosciences) with Cy3/TAMRA settings (excitation wavelength 532 nm, emission wavelength 580 nm). As a loading control gels were stained with Coomassie Brilliant Blue. As a protein standard the PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was used.

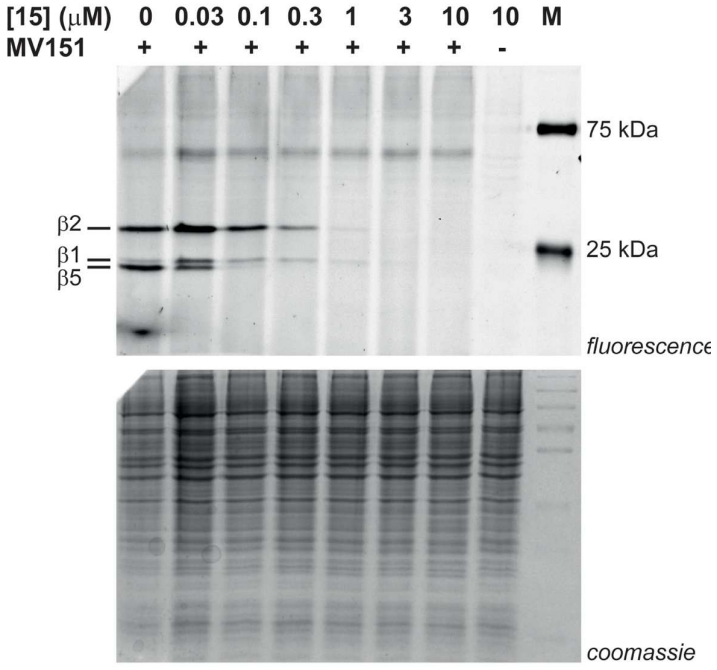


Figure S1. Competition experiment in HEK cell extracts. Cell extract were first exposed to 0 - 10 µM of azetidine-functionalized ABP **15** for 1 hr and then to fluorescent proteasome ABP MV151 for 1 hr. 12.5% SDS-PAGE with fluorescent readout followed by coomassie brilliant blue staining. Proteasome β-subunits are designated on the basis of reported labeling by MV151. 'M': protein marker.

Tetrazine ligation in cell extracts

HEK cell lysates (20 µg total protein per experiment) in lysis buffer (19 µL) were exposed to 5 µM of **12** or **11** (1 µL 100 µM in DMSO) for 1 hr at 37 °C. As a control, lysates were incubated with **11** in the presence of 100 µM epoxomicin (1 µL 2 mM in DMSO) or labeled with 1 µM MV151 (1 µL 20 µM in DMSO) for 1 hr at 37 °C. The cell extracts were then exposed to the indicated concentrations of **13** (1.1 µL 20x solution in DMSO) for the indicated time at 37 °C. After quenching by chloroform/methanol precipitation,[1] the proteins were taken up in 10 µL Laemmli's sample buffer containing 2-mercaptoethanol, boiled at 100 °C for 5 minutes and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using a Typhoon Variable Mode Imager (Amersham Biosciences) with Cy3/TAMRA settings (excitation wavelength 532 nm, emission wavelength 580 nm). As a loading control gels were stained with Coomassie Brilliant Blue. As a protein standard the PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was used.

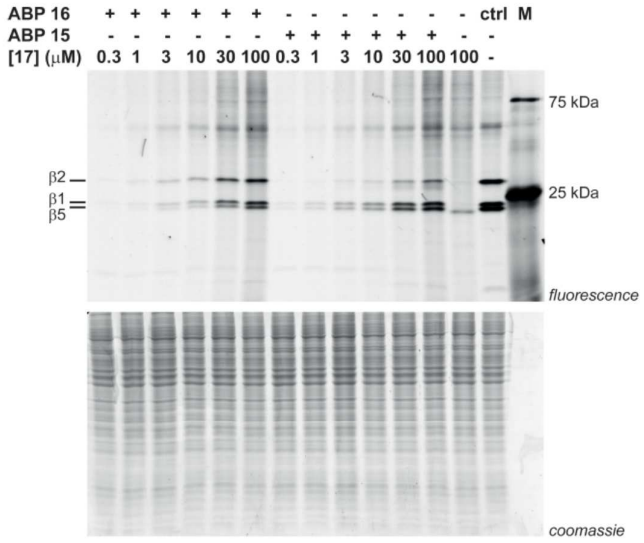


Figure S2. Full figure of Figure 4A.

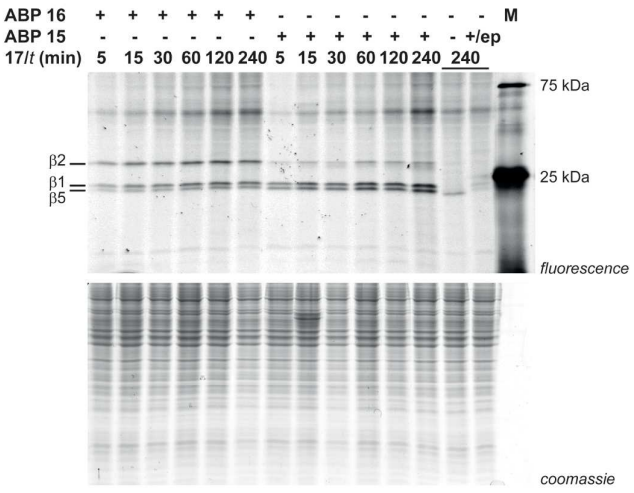


Figure S3. Full figure of Figure 4B.

Test of cross-reactivity in cell extracts

HEK cell lysates (20 µg total protein per experiment) in lysis buffer (19 µL) were exposed to 5 µM of **11** (1 µL 100 µM in DMSO) for 1 hr at 37 °C. The cell extracts were then exposed to 100 µM biotin-phosphine, 100 µM biotin-dibenzocyclooctyn, 50 µM azido-Bodipy, 50 µM Bodipy-alkyne or 50 µM tetrazine-Bodipy (each 1 µL 20x in DMSO) for 1 hr at 37 °C. After quenching by chloroform/methanol precipitation,[2] the proteins were taken up in 10 µL Laemmli's sample buffer containing 2-mercaptoethanol, boiled at 100 °C for 5 minutes and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using a Typhoon Variable Mode Imager (Amersham Biosciences) with Cy2/Blue Fam settings (excitation wavelength 488 nm, emission wavelength 520 nm). Next, the proteins were transferred onto a PVDF membrane for detection of biotinylated proteins. The membrane was blocked with 1% BSA in TBS-t(+) (0.1% Tween 20) for 1 hr at room temperature, hybridized with Streptavidin-HRP for 45 min at room temperature (1:10,000 in blocking buffer) (Molecular Probes, Life Technologies), washed with TBS-t(+) and TBS and then visualized using an ECL+ Western Blotting detection kit (Amersham Biosciences). As a loading control the membrane was stained with Coomassie Brilliant Blue.

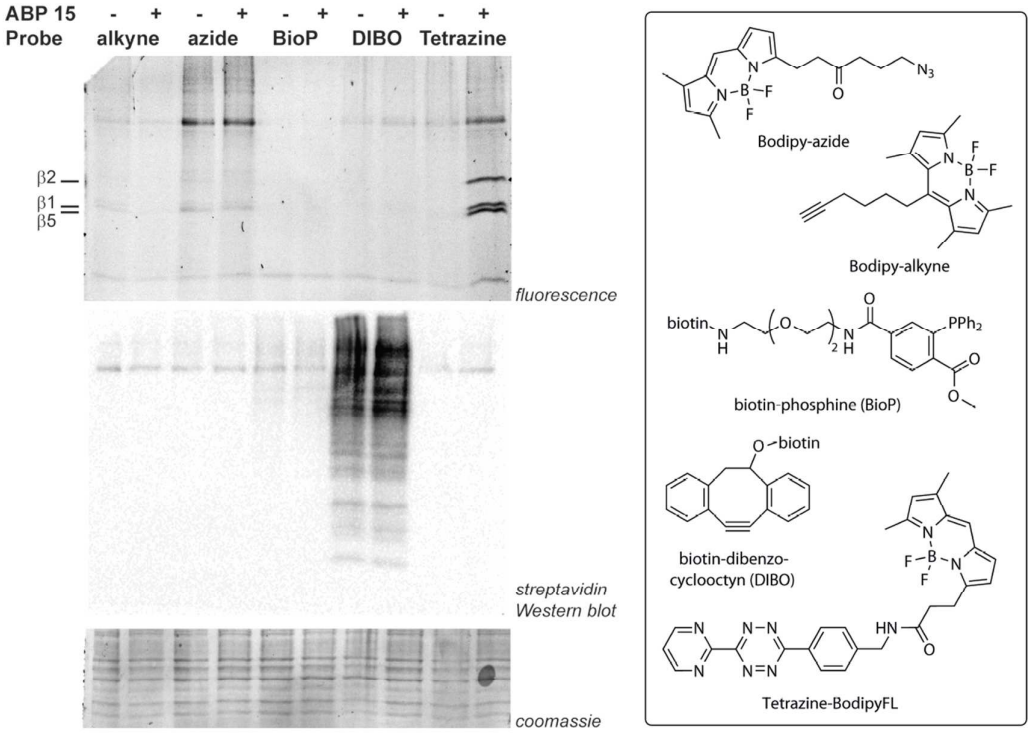
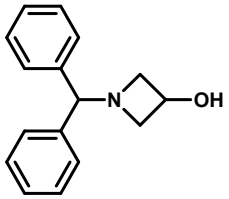


Figure S4. Test of cross-reactivity in HEK cell extracts. Cell extract were first exposed to 5 µM of azetidine-functionalized ABP **15** for 1 hr and then to Bodipy-azide (50 µM), Bodipy-alkyne (50 µM), tetrazine-BodipyFl (50 µM), biotin-phosphine ('BioP', 100 µM) or biotin-dibenzocyclooctyne ('DIBO', 100 µM) for 1 hr. 12.5% SDS-PAGE with fluorescent readout for detection of Bodipy-labeled proteins followed by streptavidin Western blotting to detect biotinylated proteins. Coomassie brilliant blue staining is shown as a loading control. 'M': protein marker.

Supplementary refeerences

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

(2) Wessel, D.; Flügge, U. I. *Anal. Biochem.* **1984**, *138*, 141.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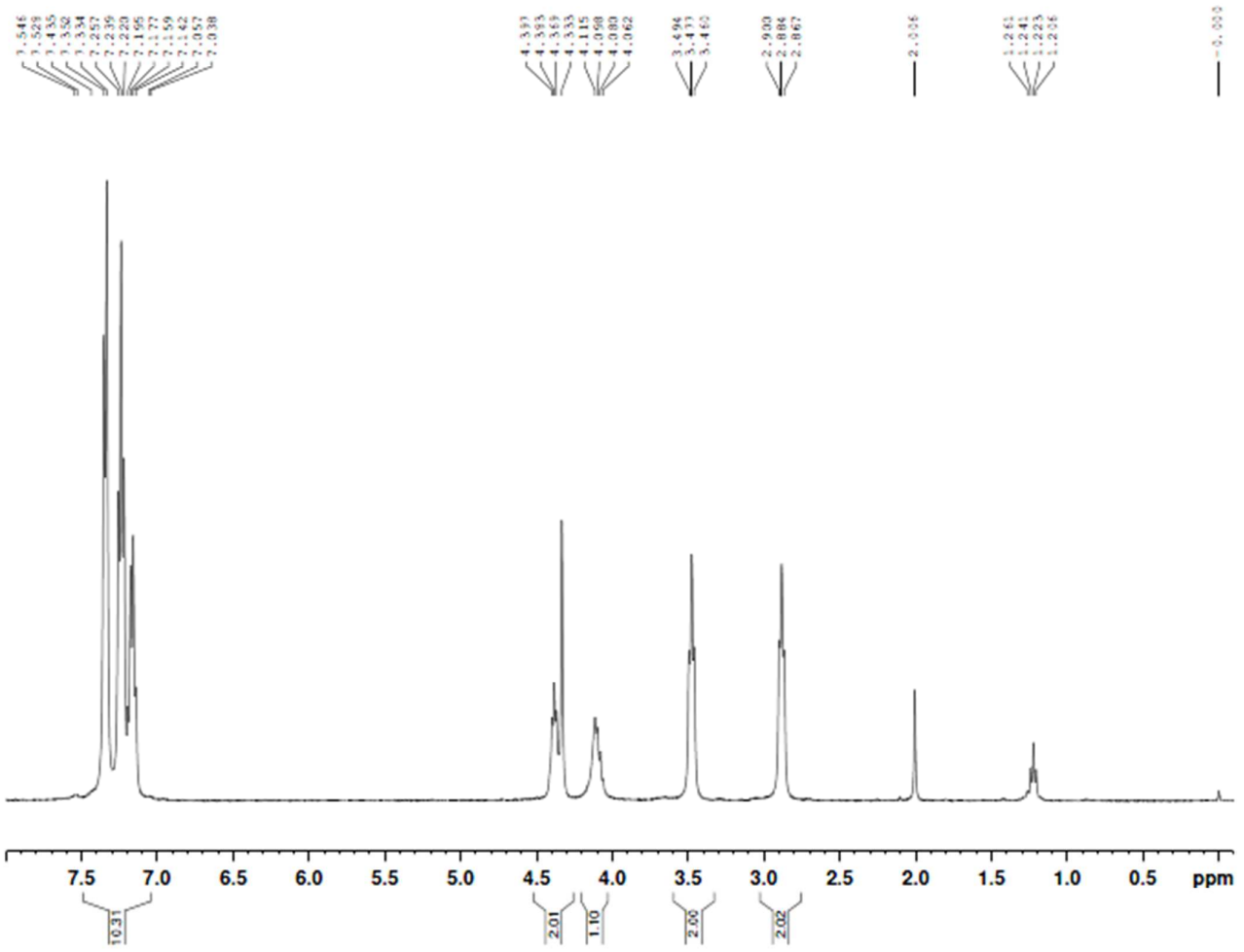
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1.206
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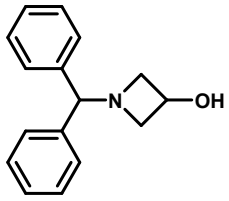
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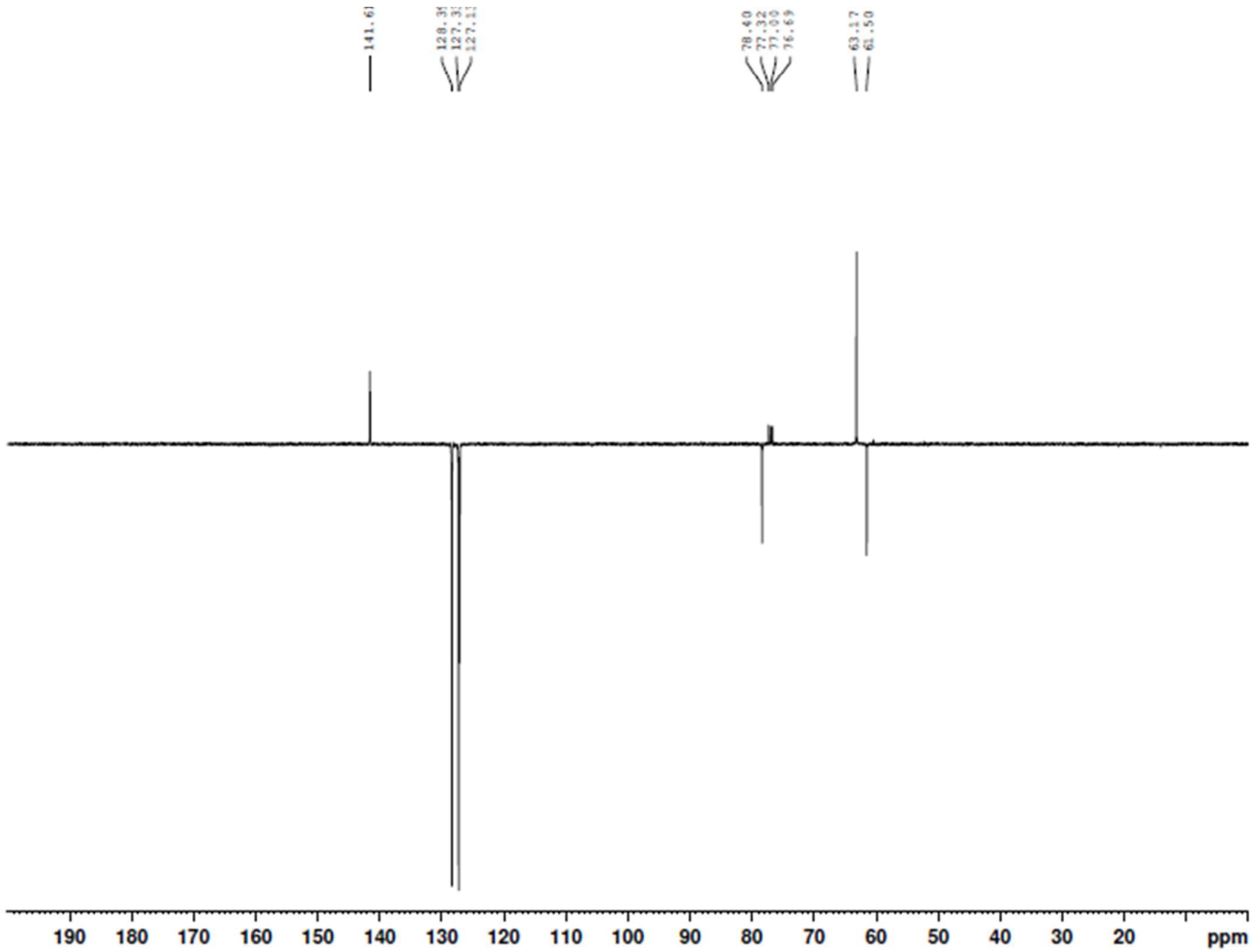
OH

2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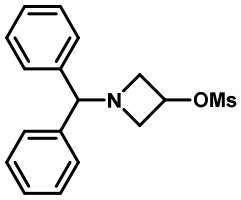
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OMs

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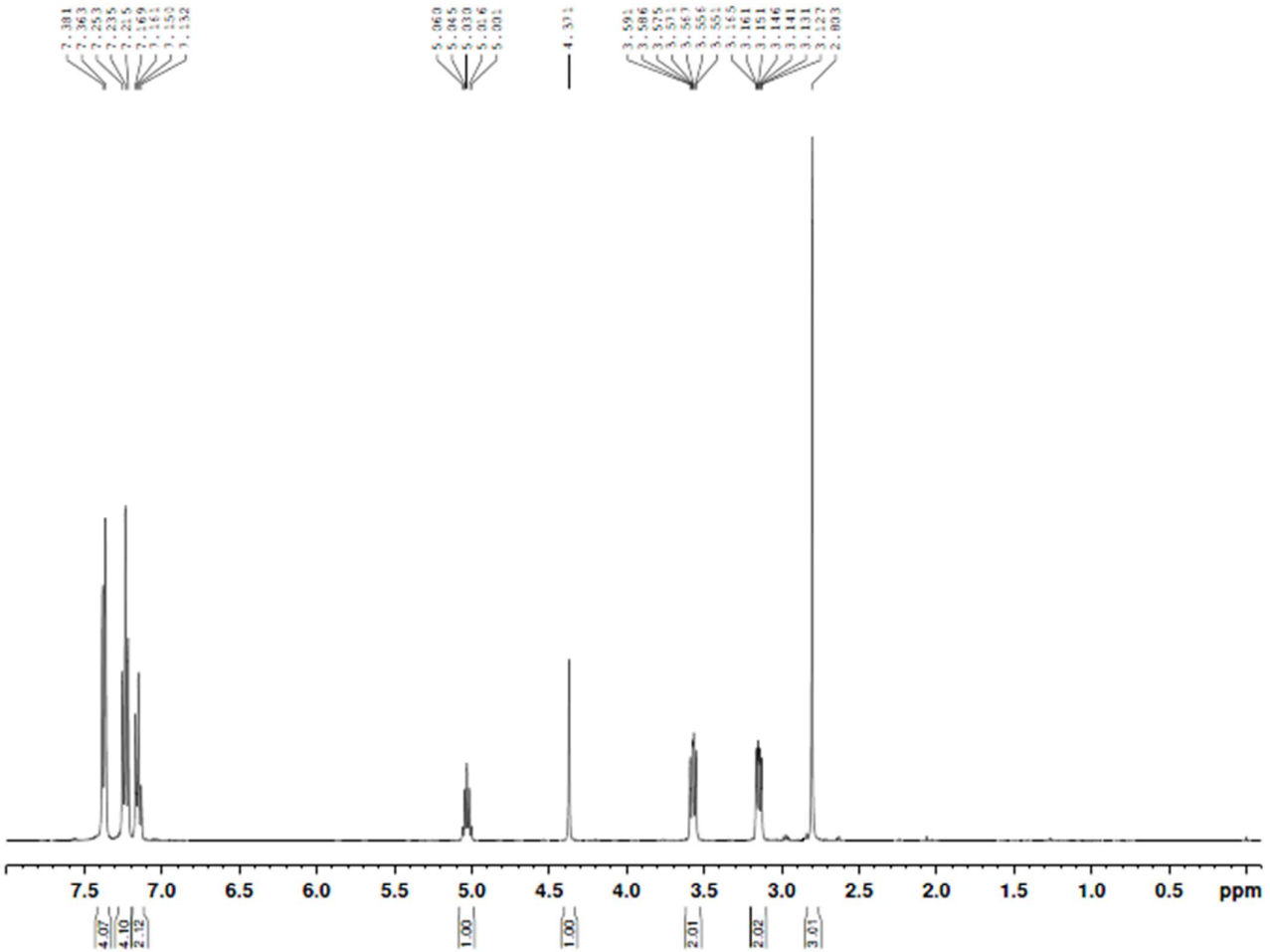
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3.151
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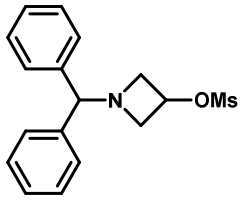
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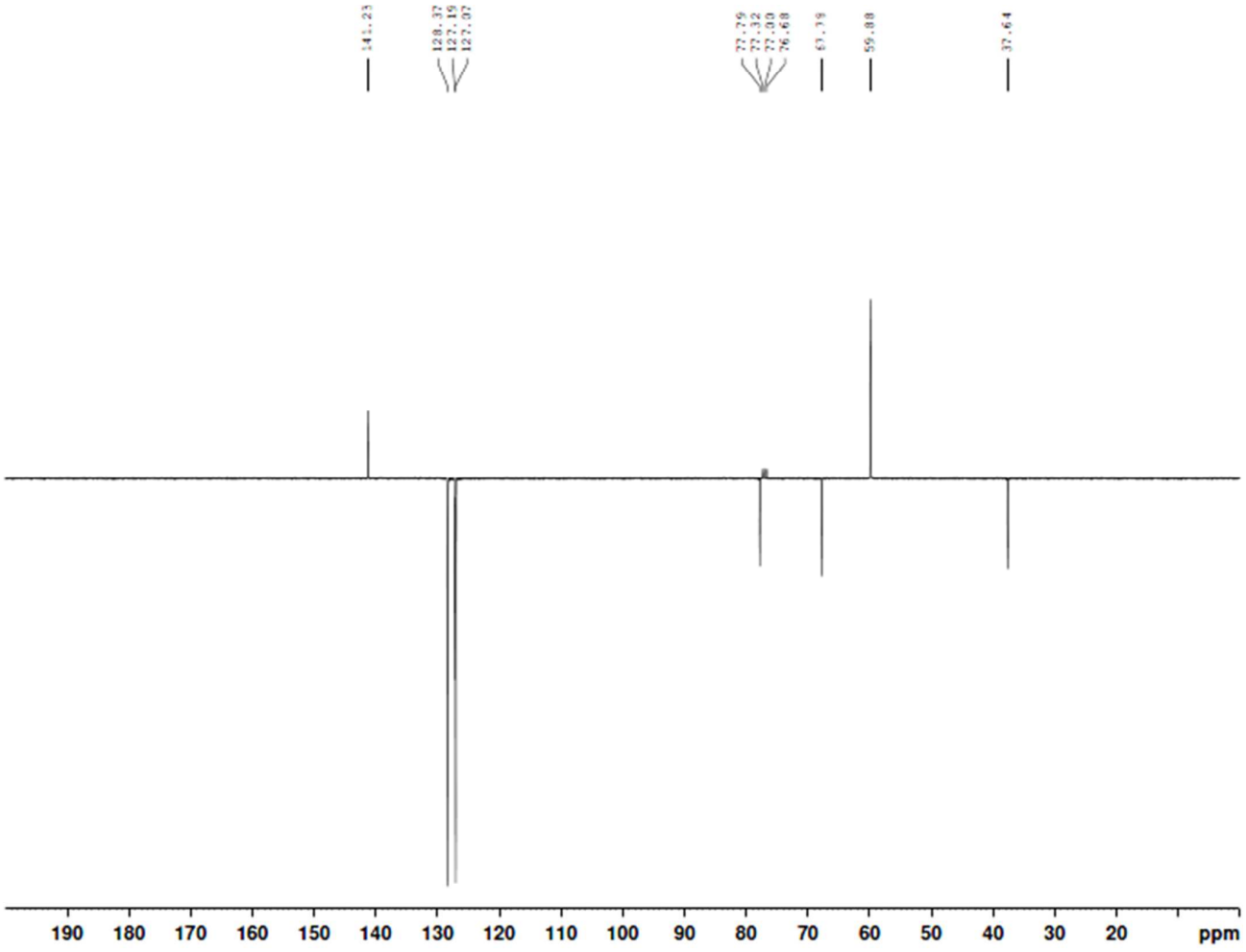
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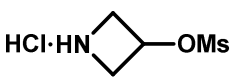
HCl·HN
OMs

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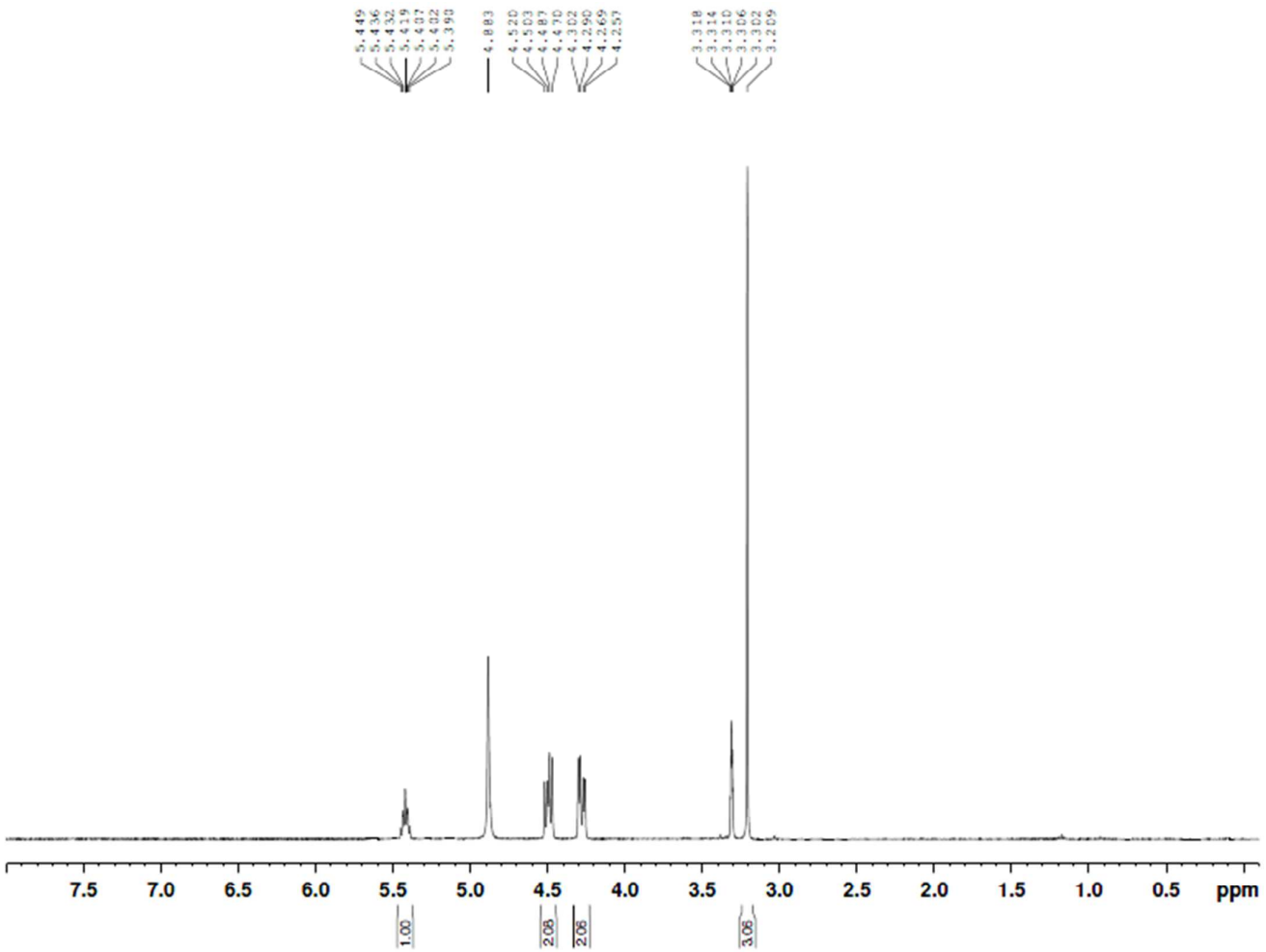
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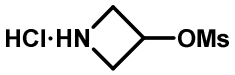
HCl·HN
OMs

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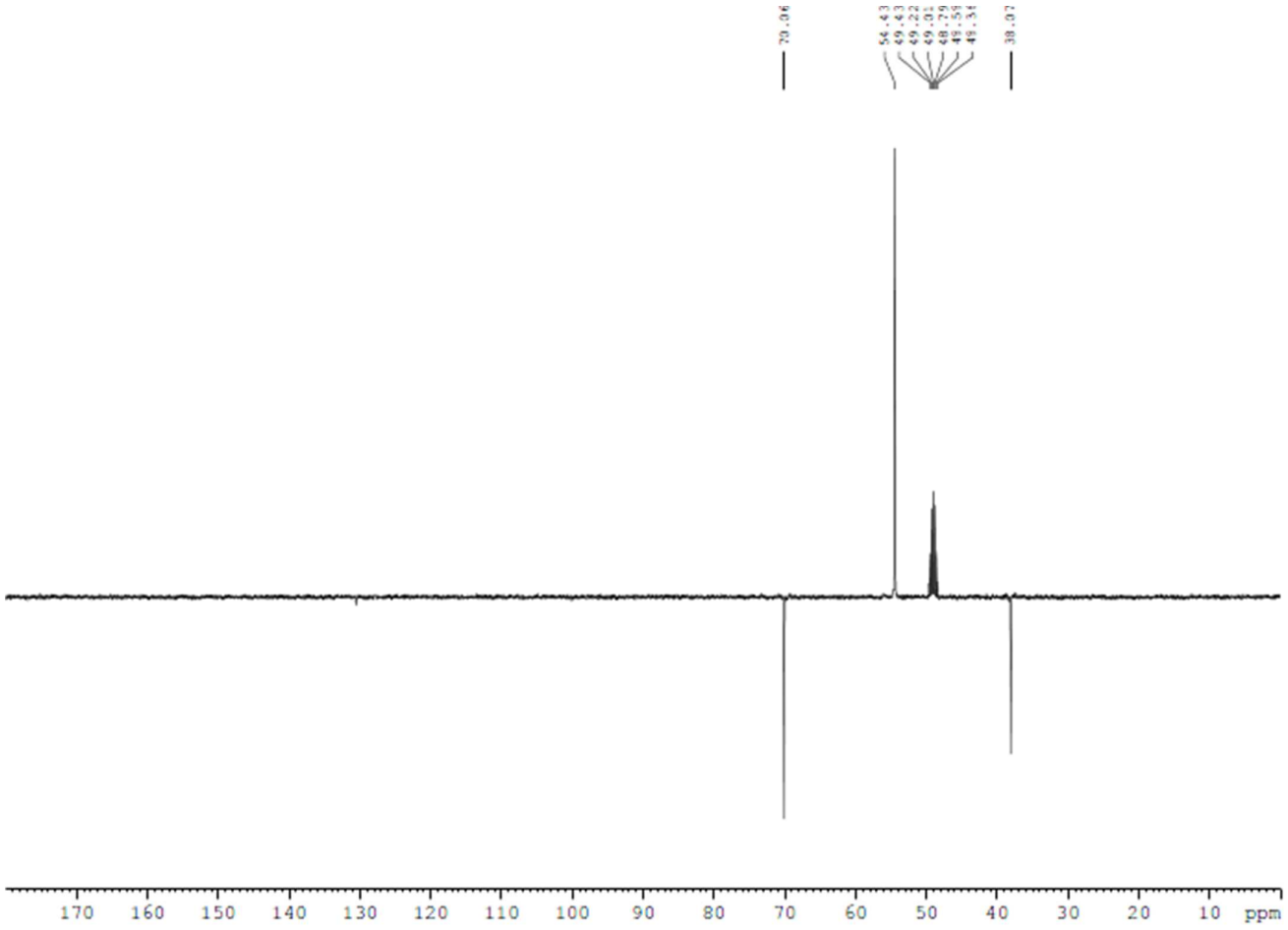
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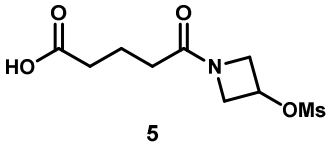
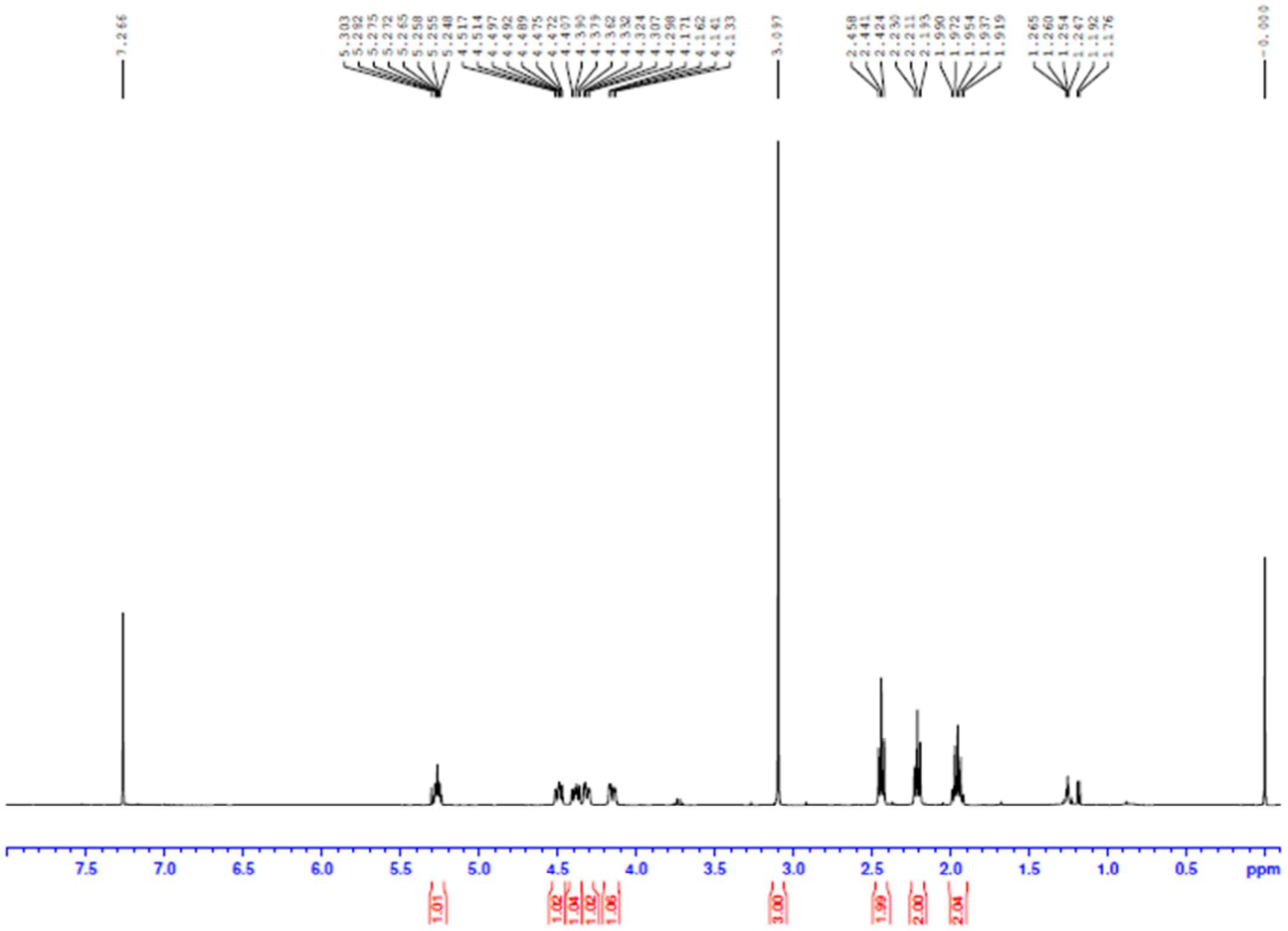
ppm


O
O
HO
N
OMs

5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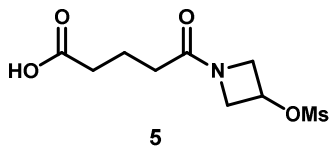
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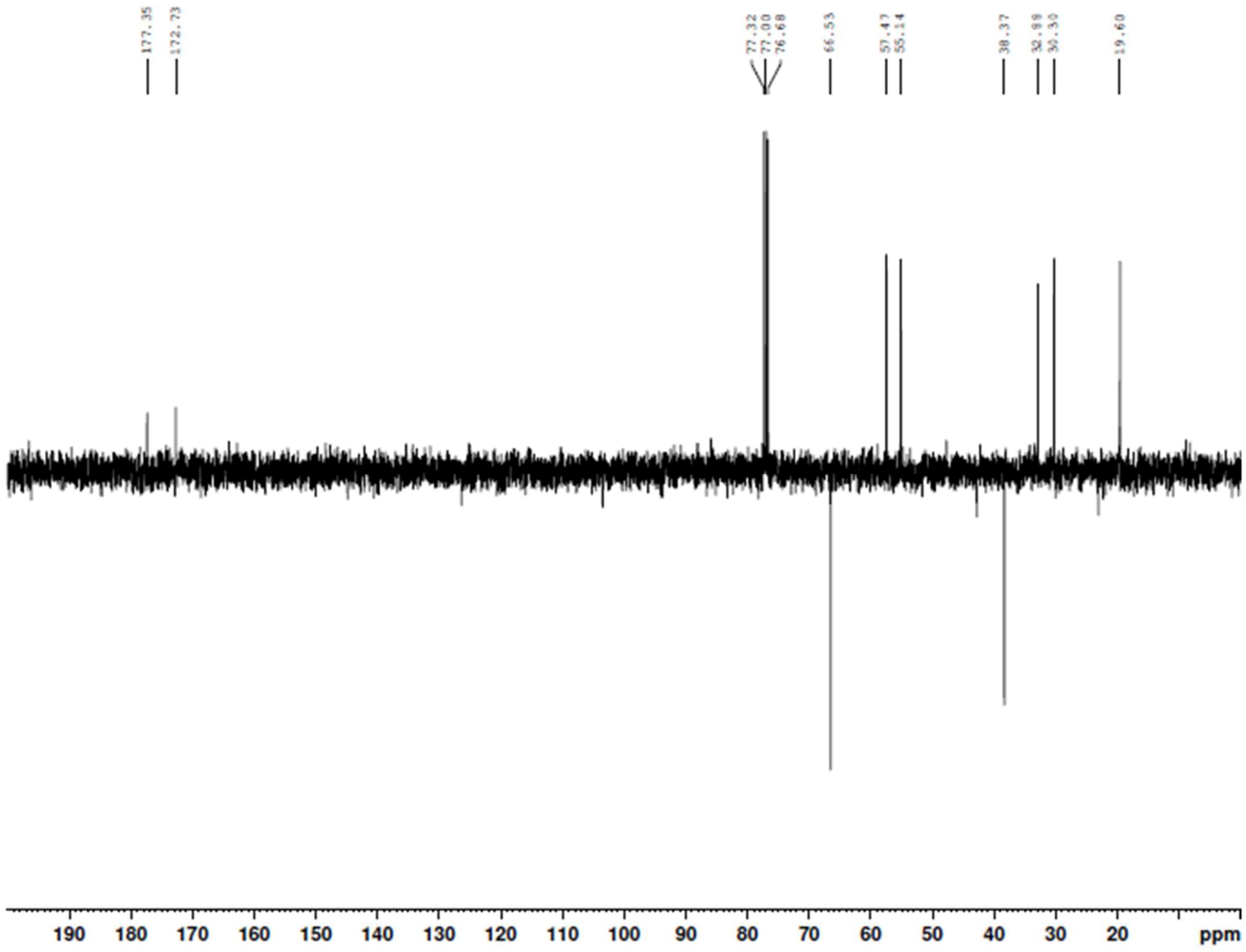
O
HO
N
OMs

5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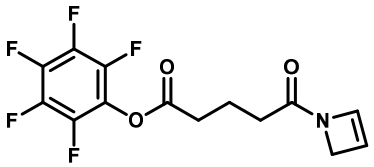

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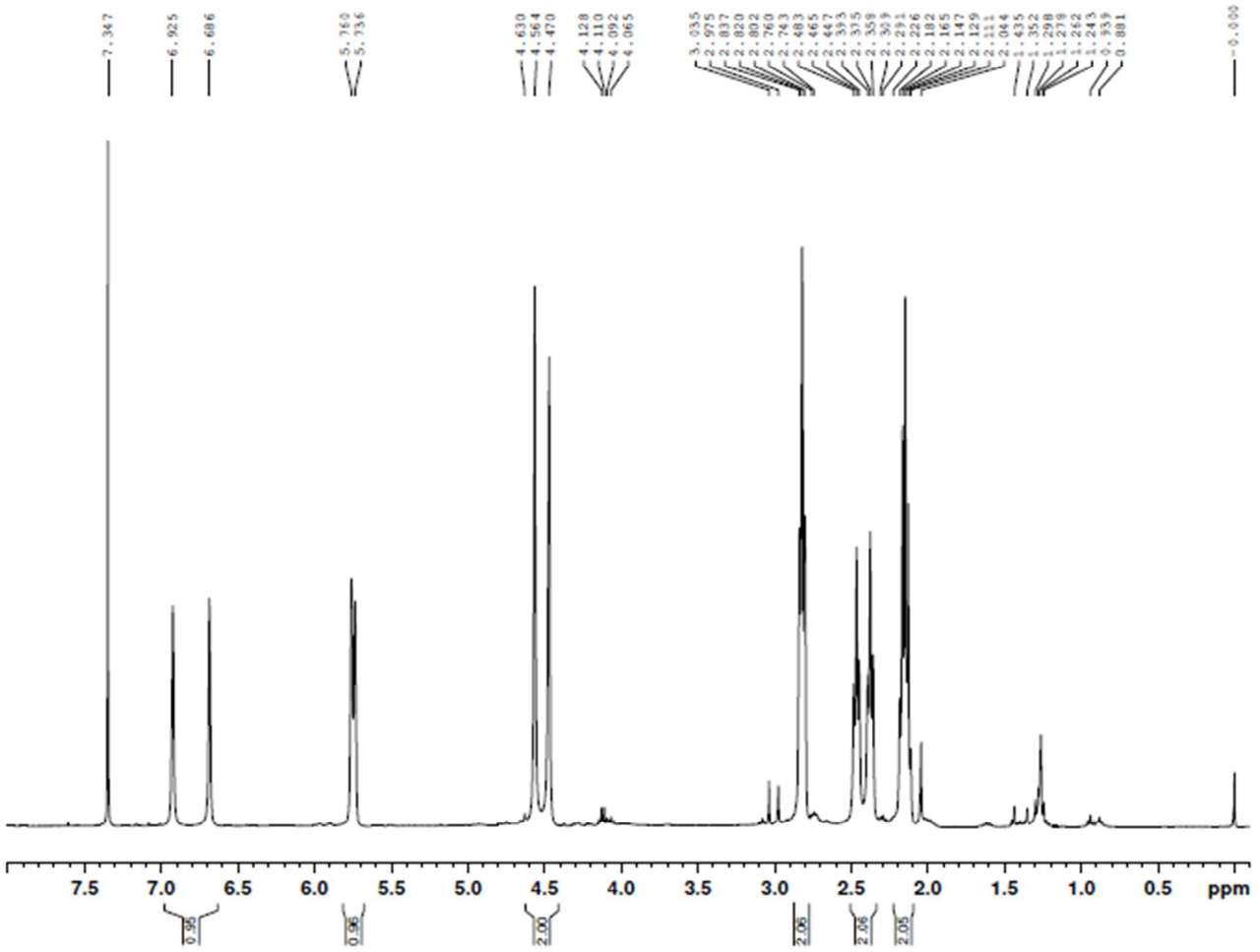
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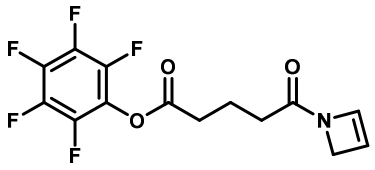
2.05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O
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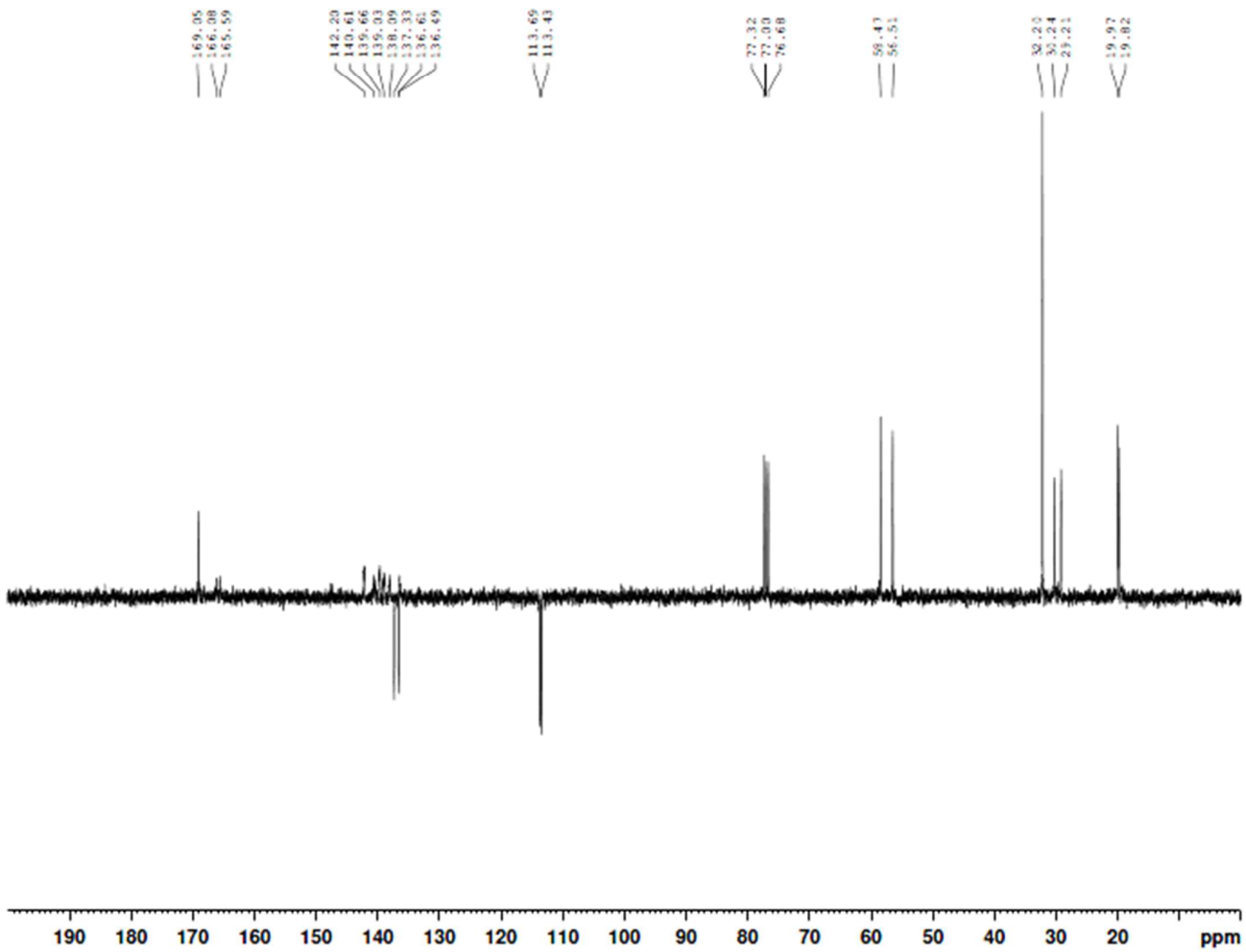
6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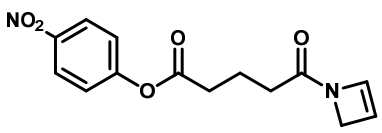
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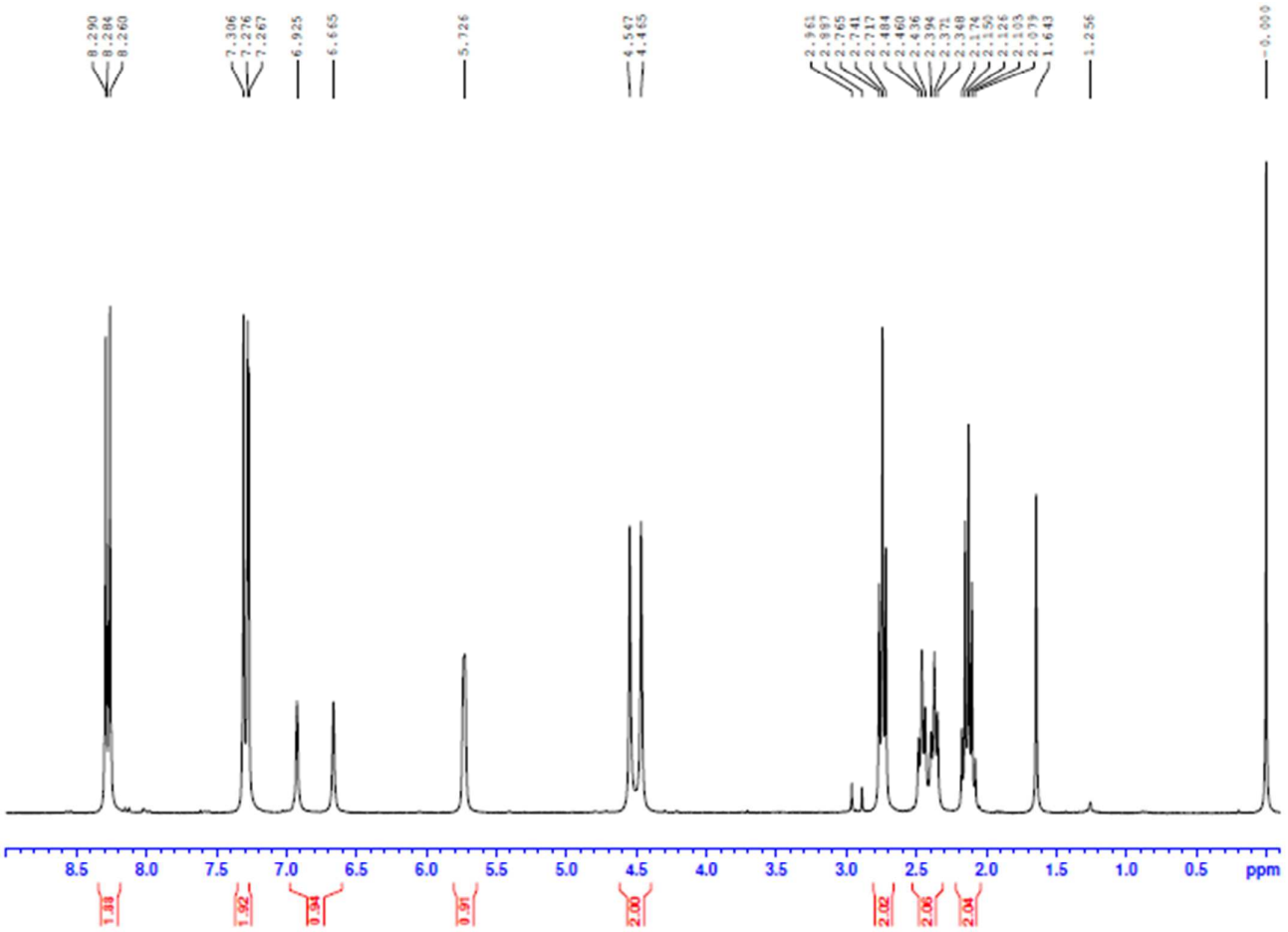
O
O
N

7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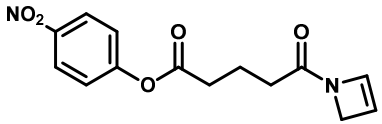
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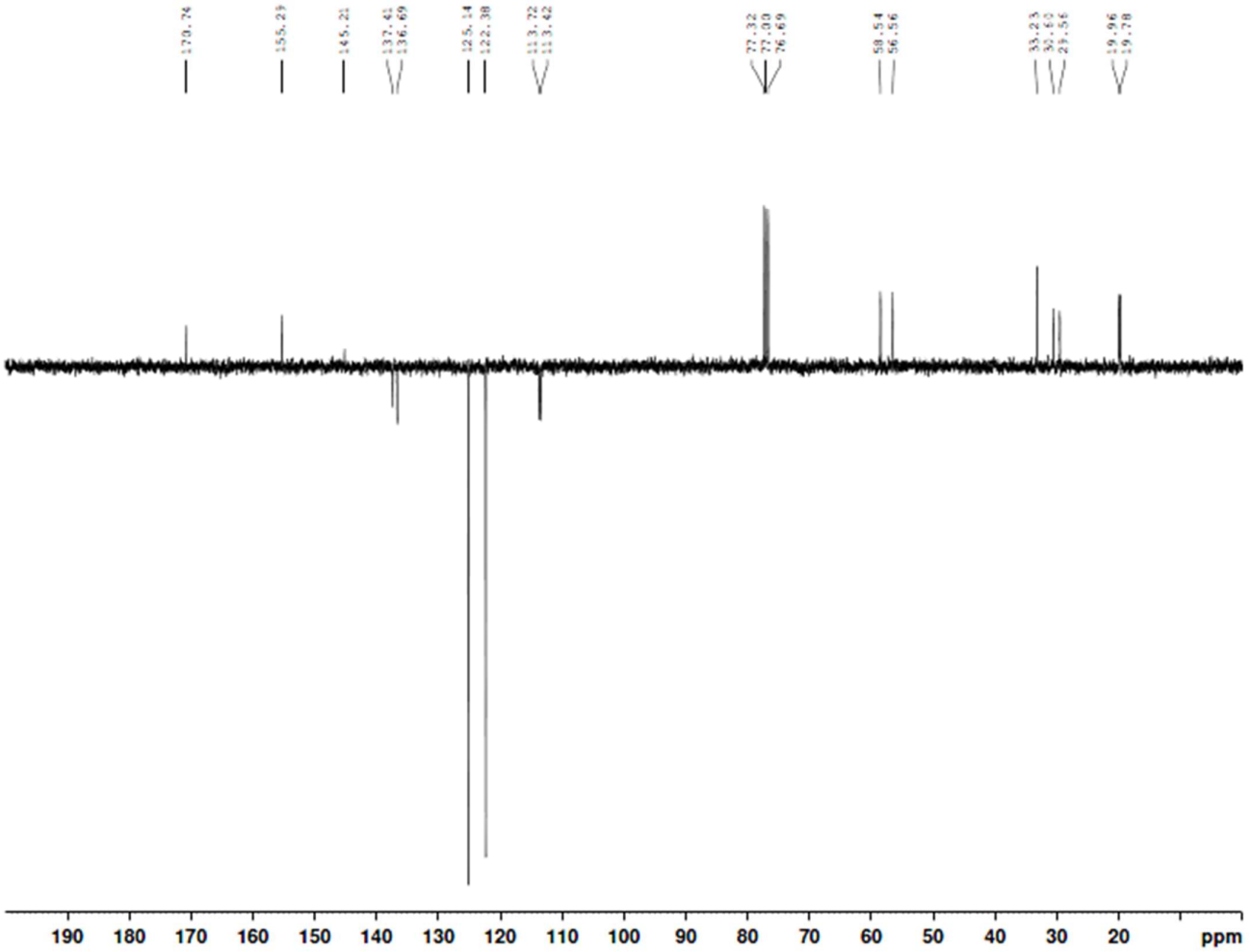
O
N

7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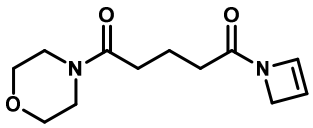
20
ppm



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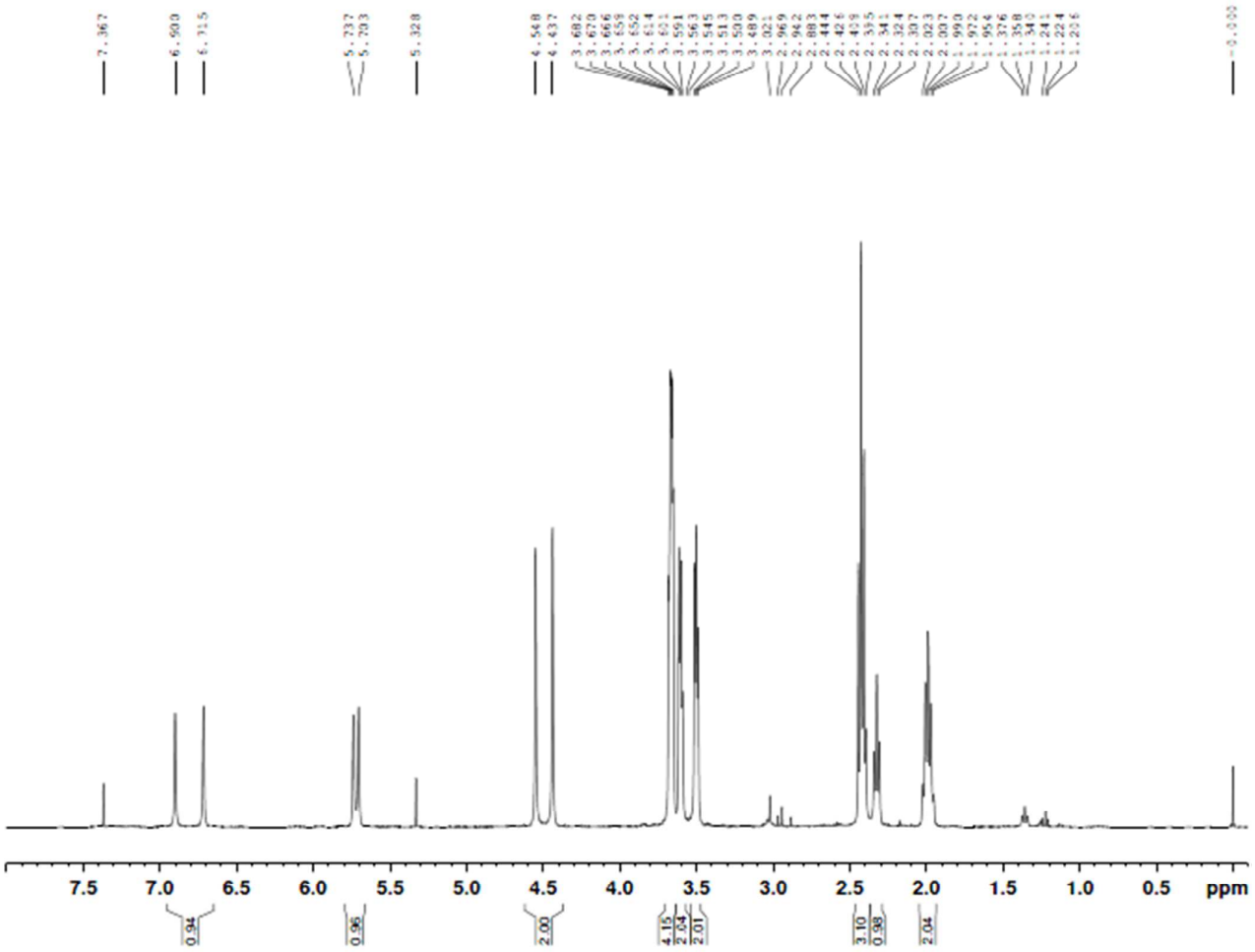
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1.241
1.224
1.206
-0.000
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
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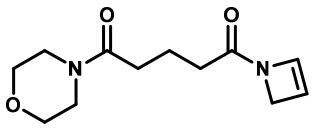
ppm
0.94
0.96
2.00
4.16
2.04
2.01
3.10
0.98
2.04



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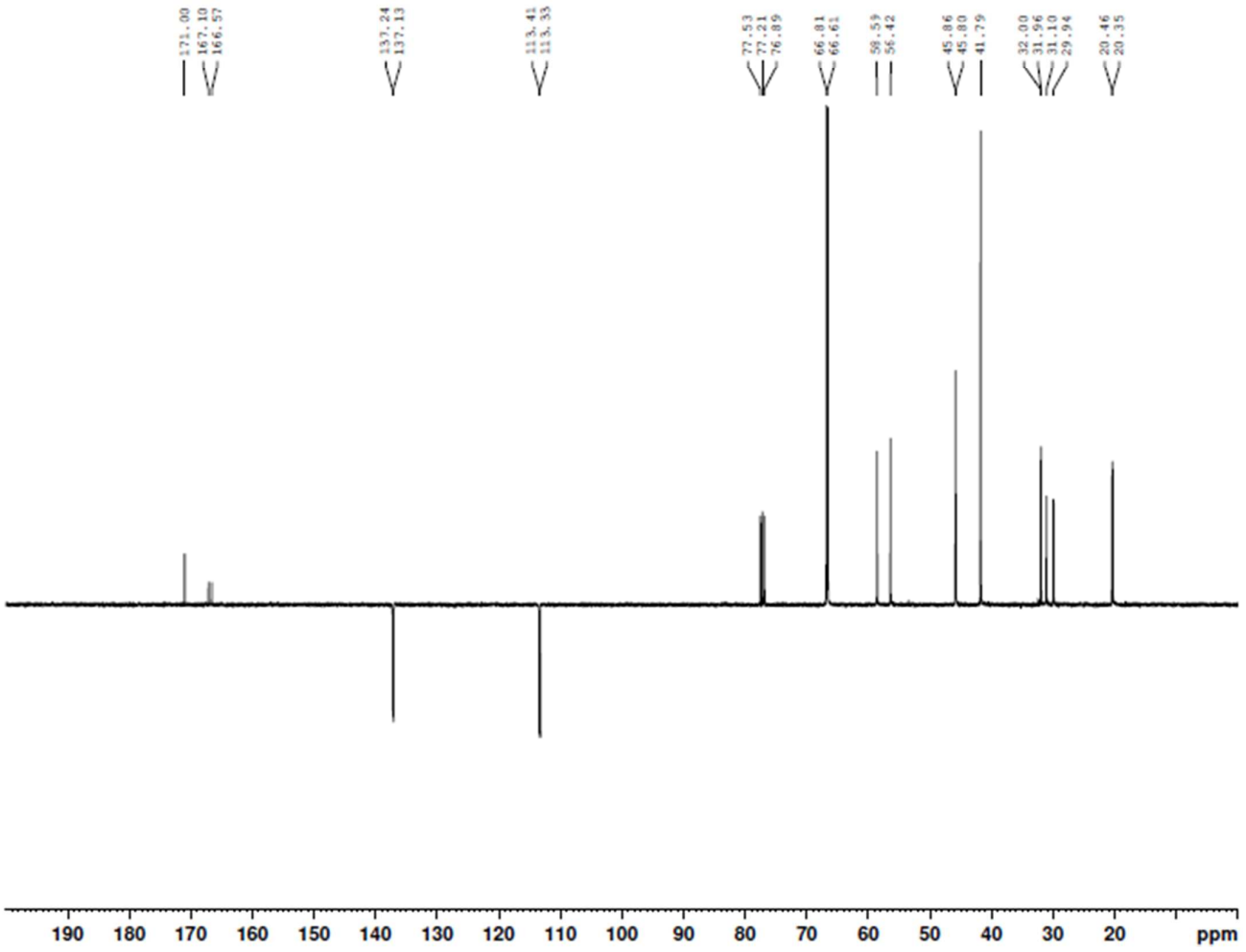
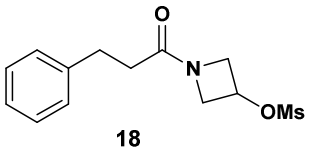
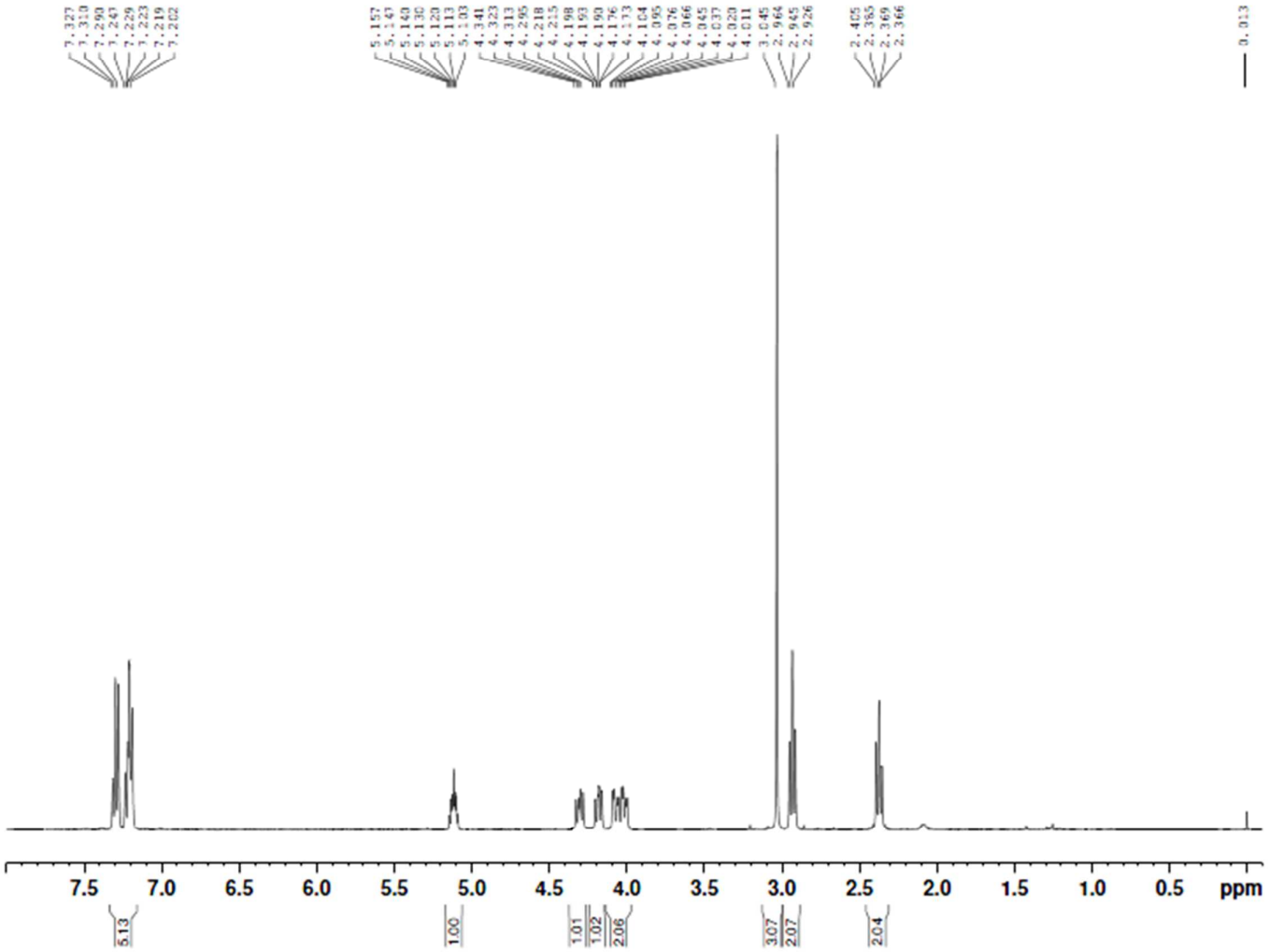
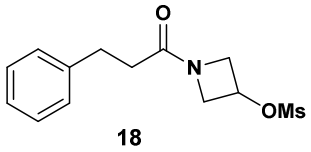

171.00
167.10
166.57
137.24
137.13
113.41
113.33
77.53
77.21
76.89
66.81
66.61
58.59
56.42
45.86
45.80
41.79
32.00
31.96
31.10
29.94
20.46
20.35
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm

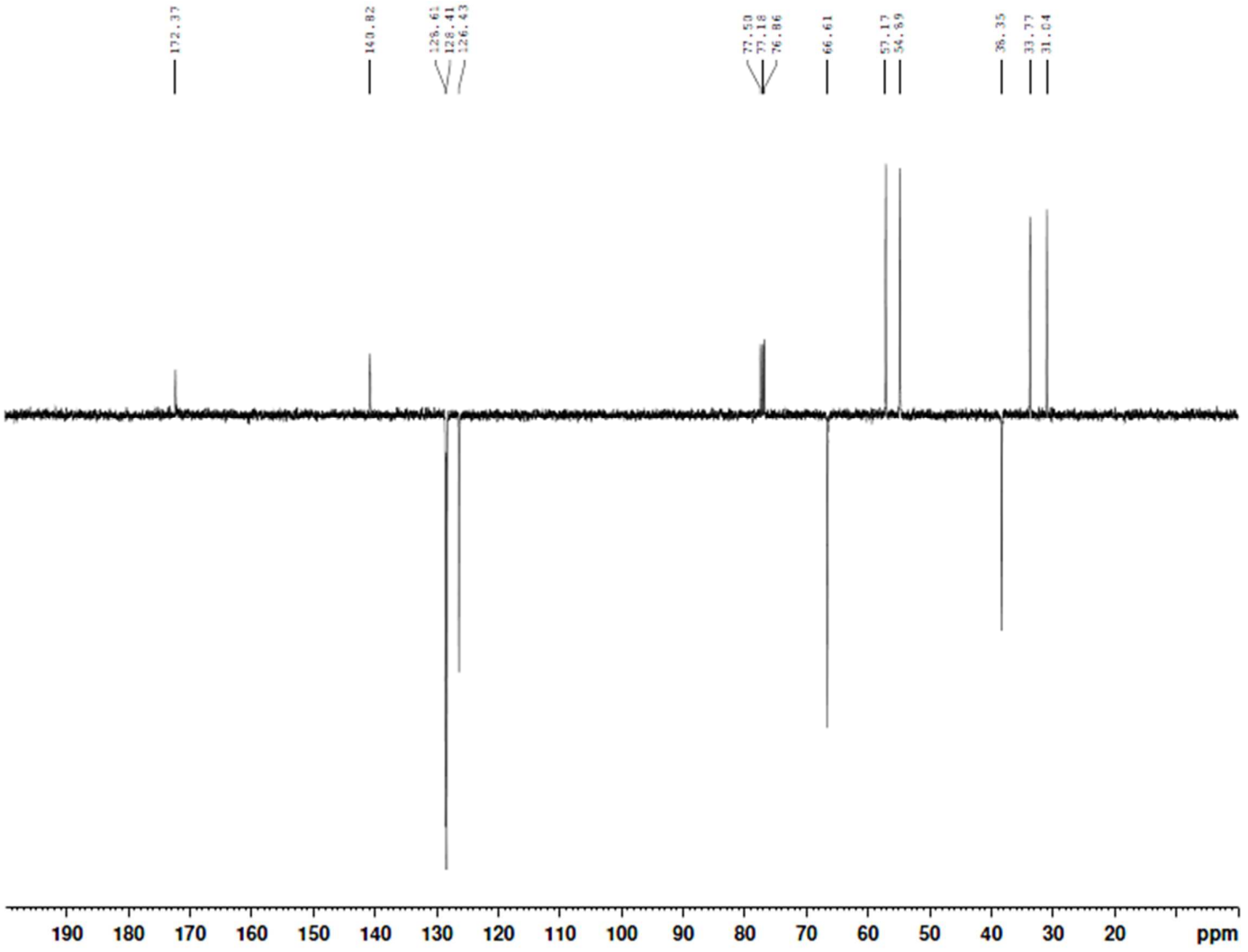

O
N
OMs

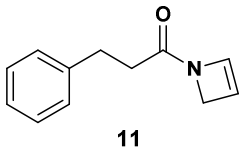
18


7.327
7.310
7.290
7.247
7.229
7.223
7.219
7.202
5.157
5.147
5.140
5.130
5.129
5.113
5.103
4.341
4.323
4.313
4.295
4.218
4.215
4.198
4.193
4.190
4.176
4.173
4.104
4.095
4.076
4.066
4.045
4.037
4.029
4.011
3.045
2.964
2.945
2.926
2.405
2.385
2.369
2.366
0.013
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
5.13
1.00
1.01
1.02
2.06
3.07
2.07
2.04

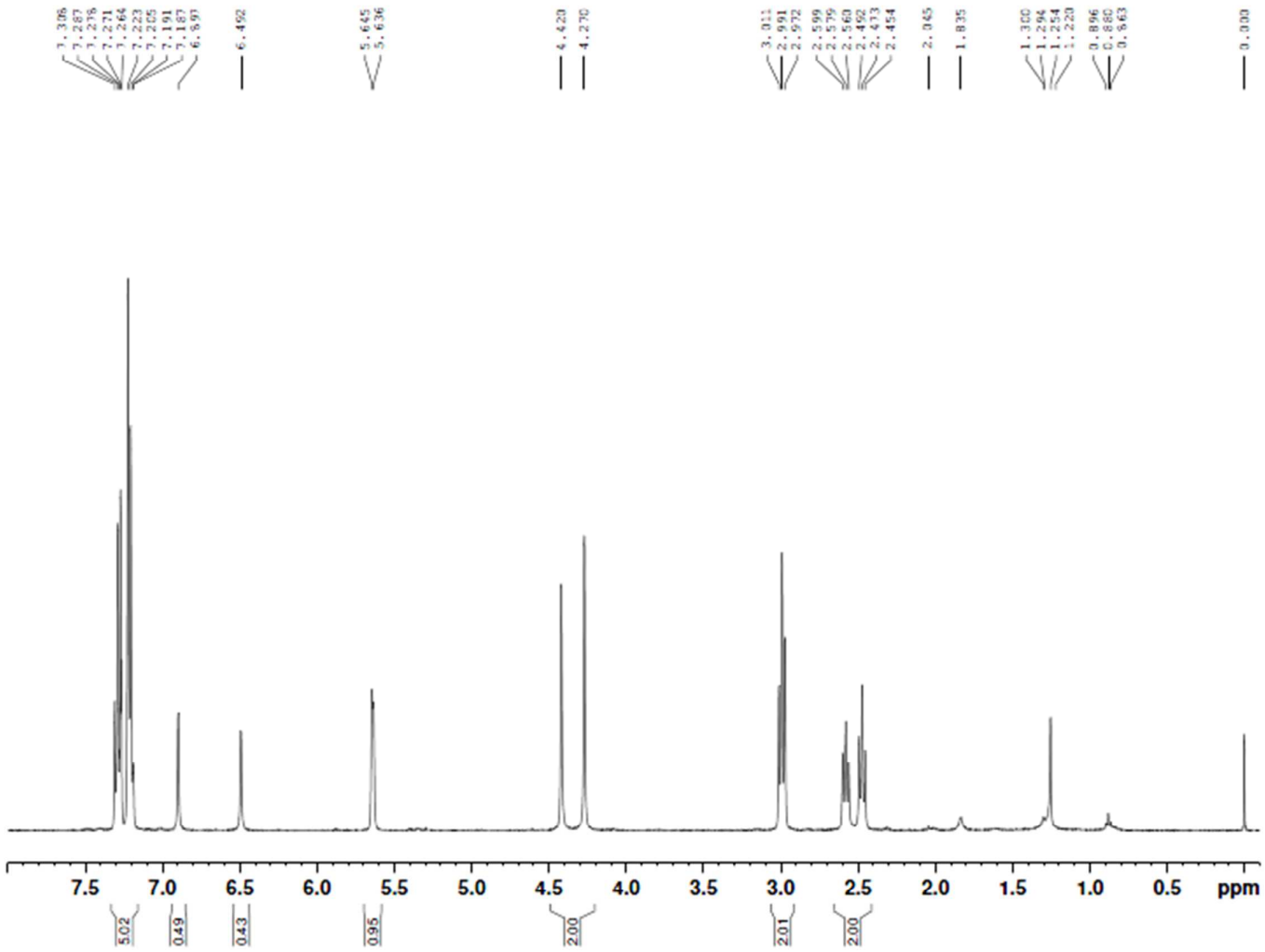

O
N
OMs

18


172.37
140.82
128.61
128.41
126.43
77.59
77.18
76.86
66.61
57.17
54.69
36.35
33.77
31.64
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm


O
N

11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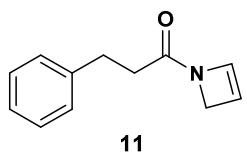
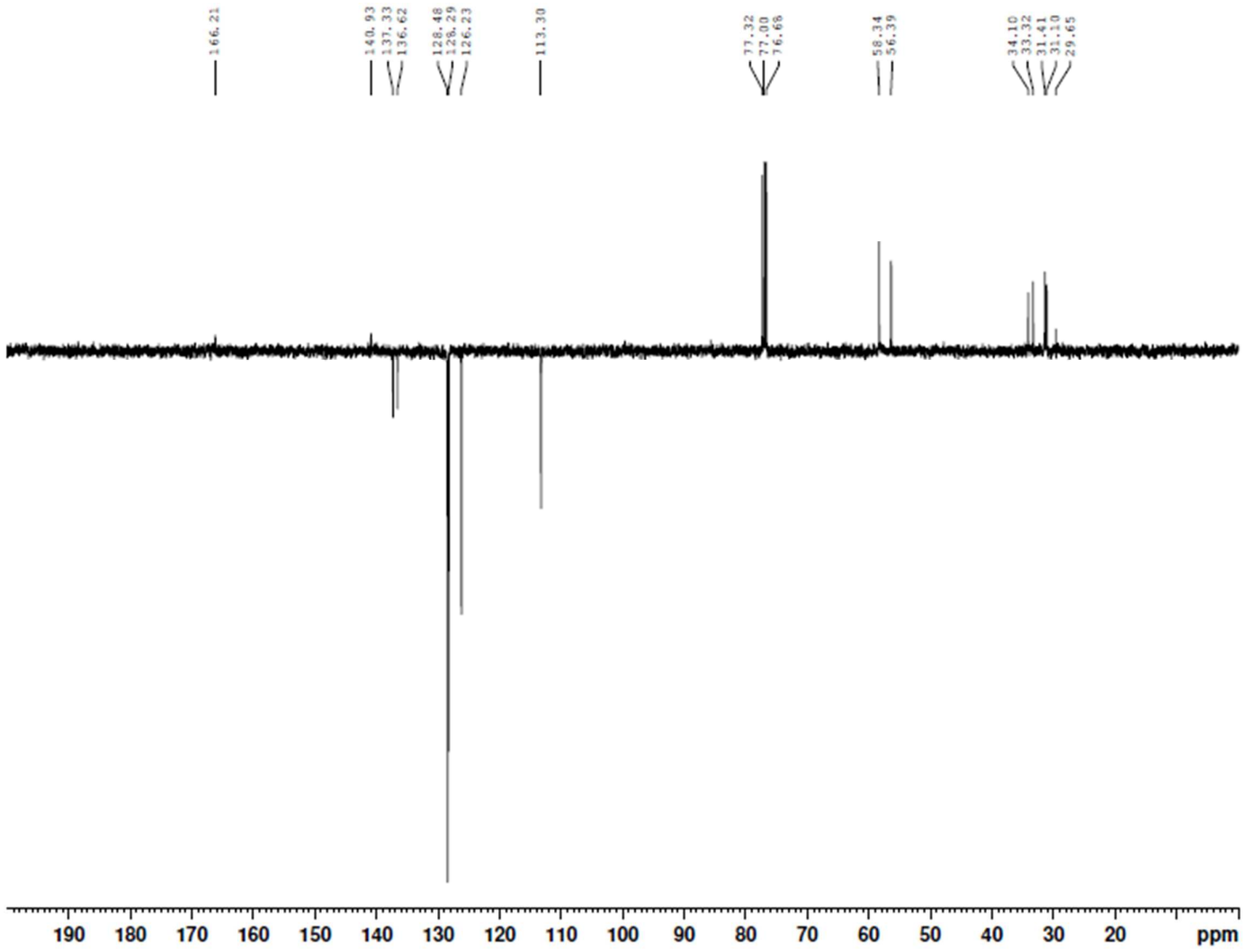
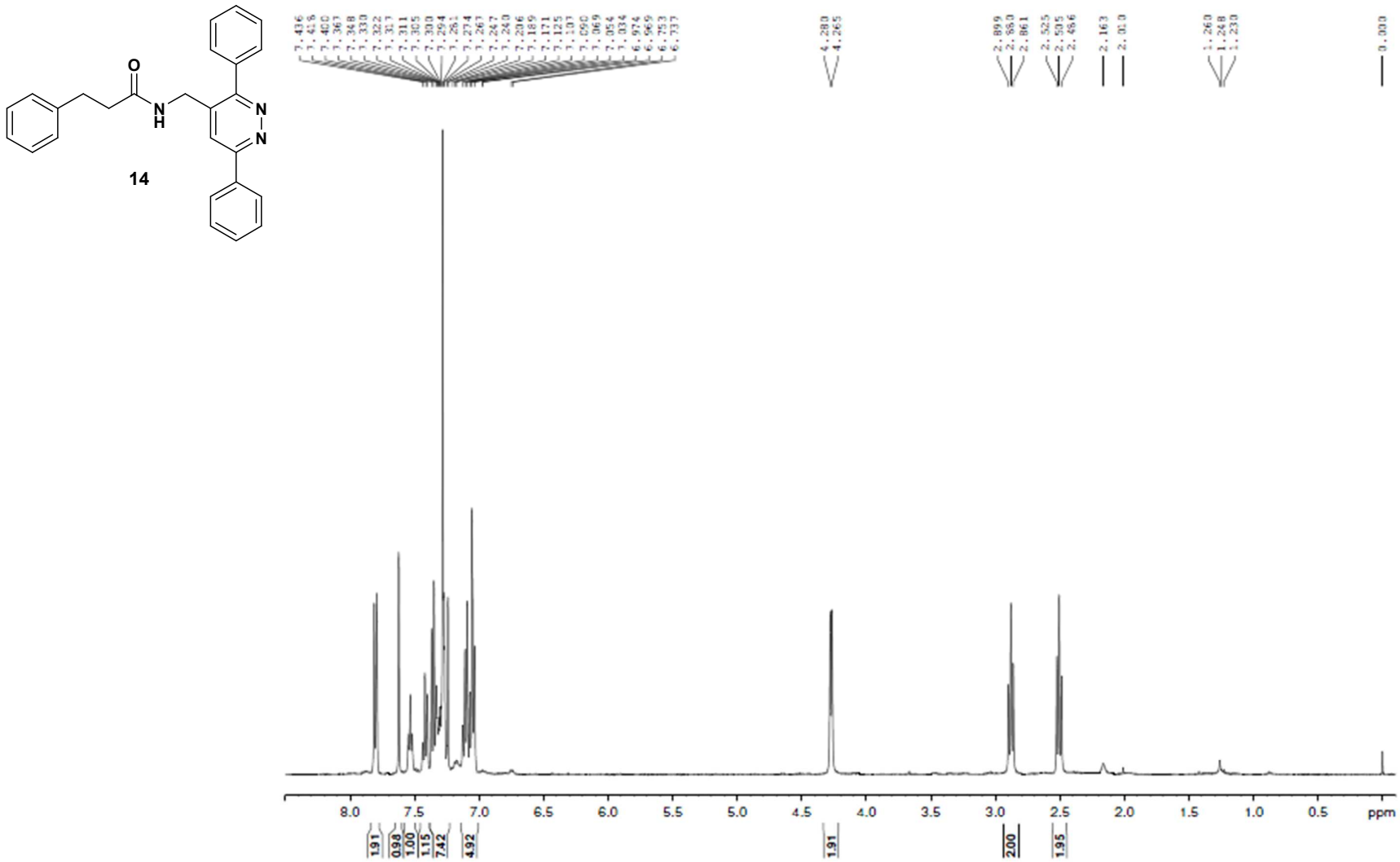
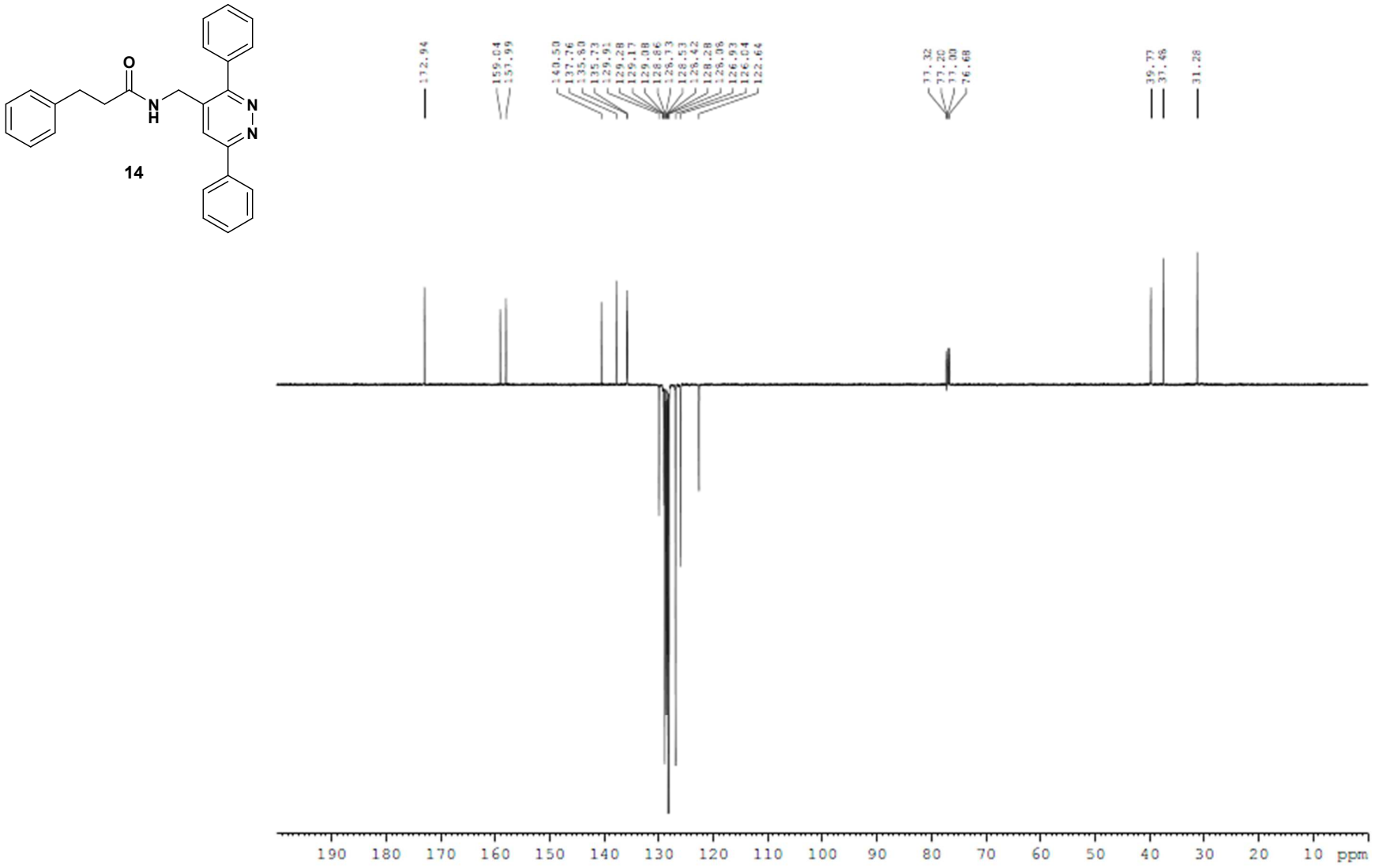
7.287
7.276
7.271
7.264
7.223
7.205
7.191
7.183
6.897
6.492
5.645
5.636
4.429
4.270
3.011
2.991
2.972
2.599
2.579
2.560
2.492
2.473
2.454
2.045
1.835
1.300
1.294
1.254
1.229
0.896
0.880
0.863
0.000
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
5.02
0.49
0.43
0.95
2.00
2.01
2.00

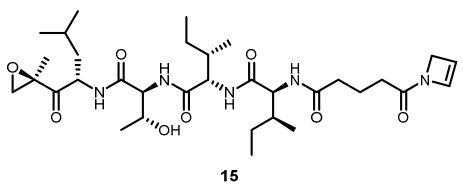

O
N

11

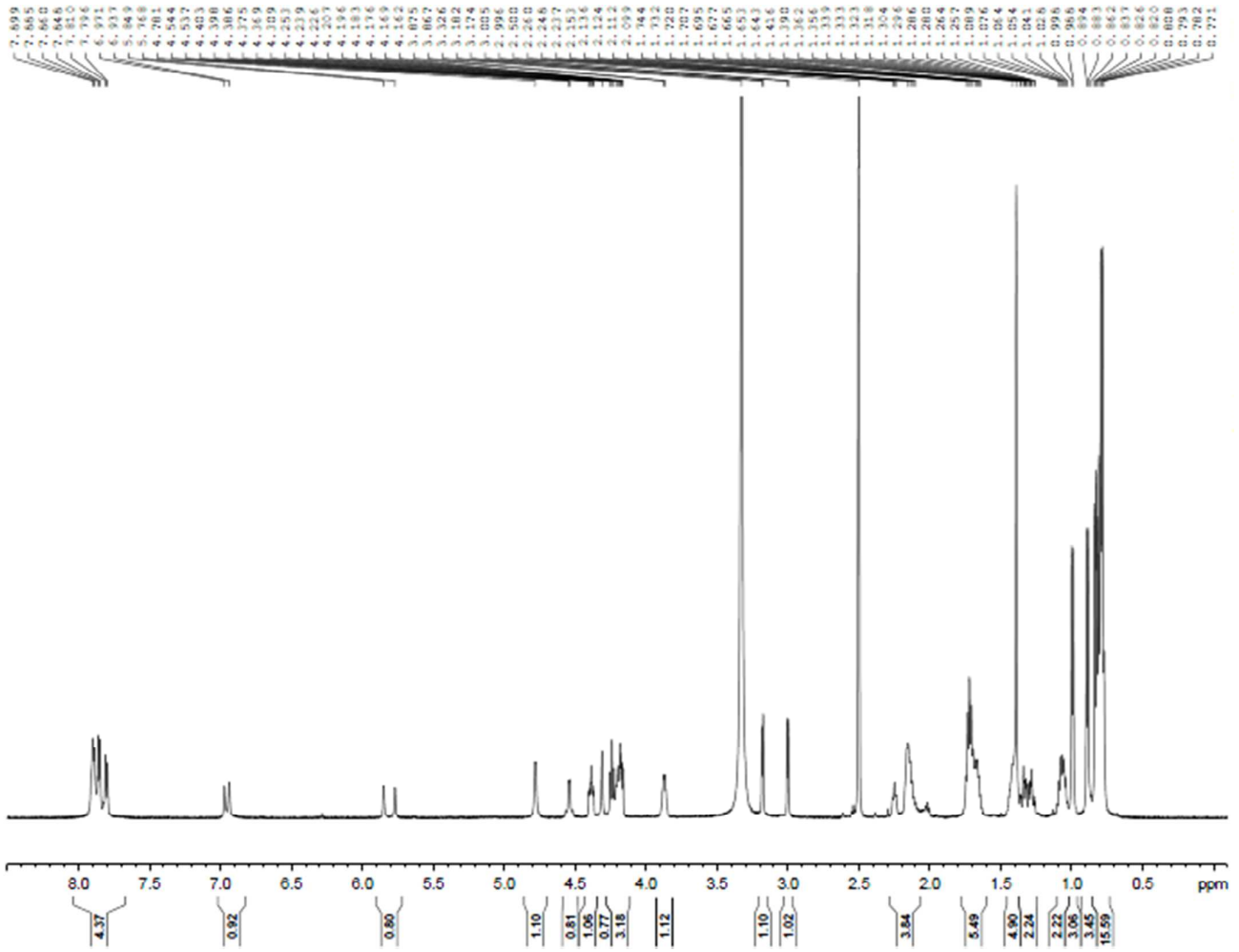

166.21
140.93
137.33
136.62
128.48
128.29
126.23
113.30
77.32
77.00
76.68
58.34
56.39
34.10
33.32
31.41
31.10
29.65
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm

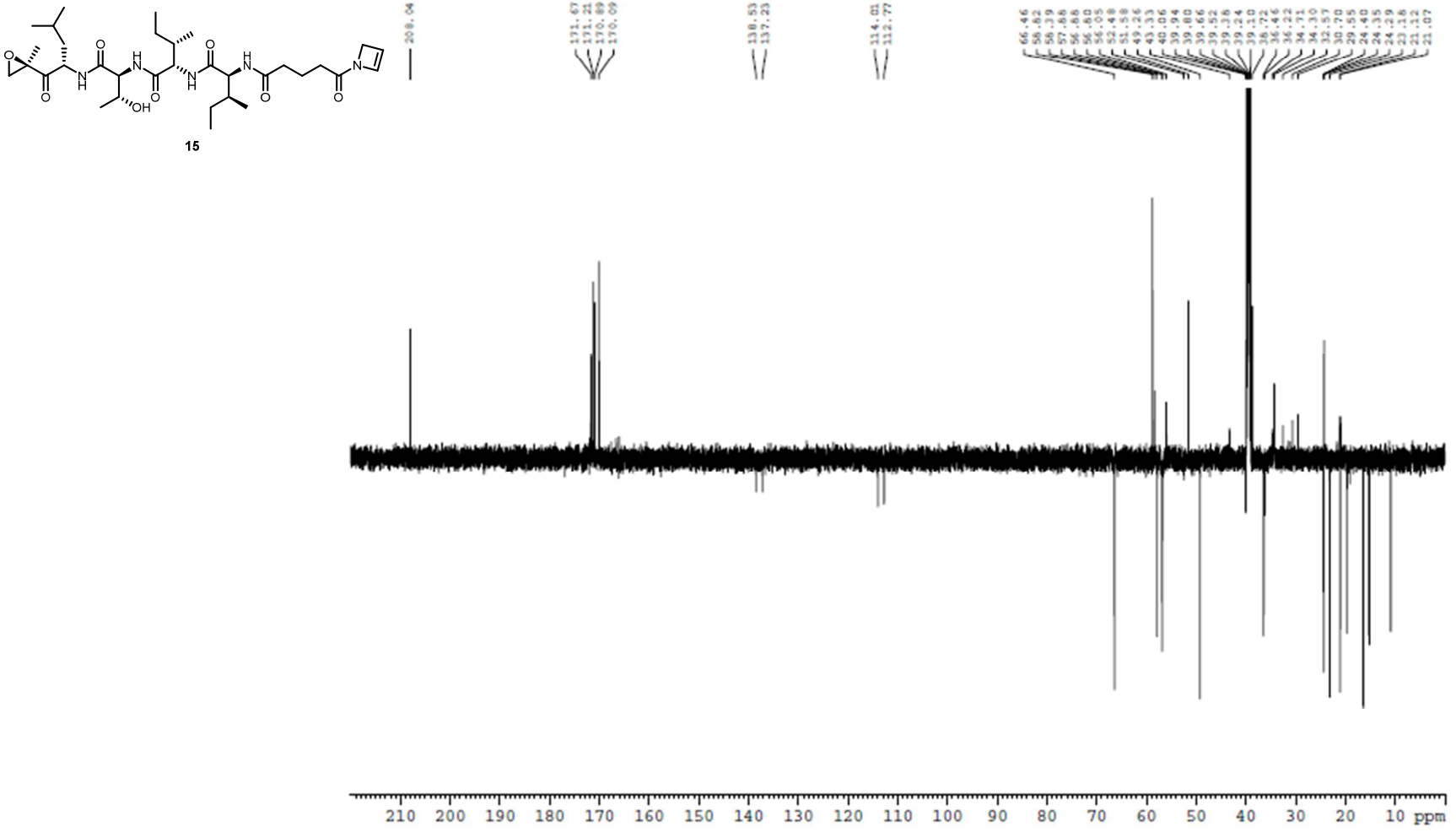

O
N
H
N
N
14
7.436
7.415
7.400
7.367
7.348
7.330
7.322
7.313
7.311
7.305
7.300
7.294
7.281
7.274
7.267
7.247
7.240
7.236
7.189
7.171
7.125
7.107
7.090
7.069
7.054
7.034
6.974
6.969
6.753
6.737
4.280
4.265
2.899
2.893
2.861
2.525
2.505
2.456
2.163
2.010
1.260
1.248
1.233
0.000
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
1.91
0.98
1.00
1.15
7.42
4.92
1.91
2.00
1.95

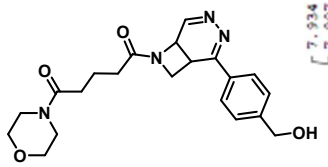

O
N
H
N
N
14
172.94
159.64
157.99
140.50
137.76
135.80
135.73
129.91
129.28
129.17
129.08
128.86
128.73
128.53
128.42
128.28
126.06
126.93
126.04
122.64
77.32
77.20
77.00
76.68
39.77
33.46
31.28
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm



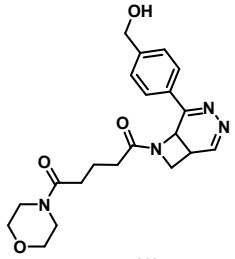
15


8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
4.37
0.92
0.80
1.10
0.81
1.06
0.77
3.18
1.12
1.10
1.02
3.84
5.49
4.90
2.24
2.22
3.06
3.45
15.59


15
209.04
171.67
171.21
170.89
170.09
138.53
137.23
114.01
112.77
66.46
58.62
58.39
57.66
57.46
56.80
56.05
52.48
51.58
49.26
43.33
40.06
39.94
39.80
39.66
39.52
39.36
39.24
39.10
38.72
38.46
36.22
34.71
34.30
32.57
30.70
29.55
24.40
24.35
24.29
23.16
21.12
21.07
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm



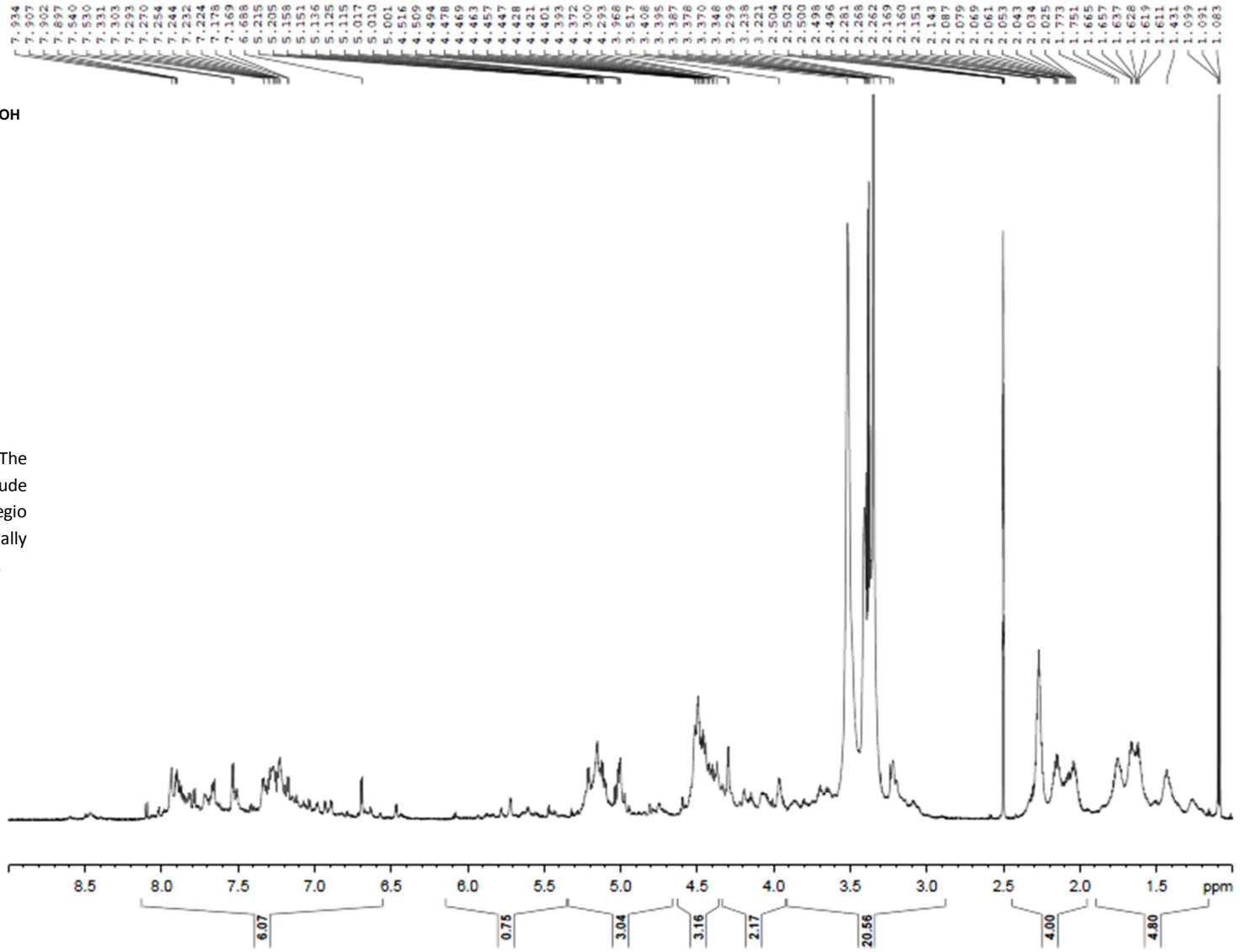
10a

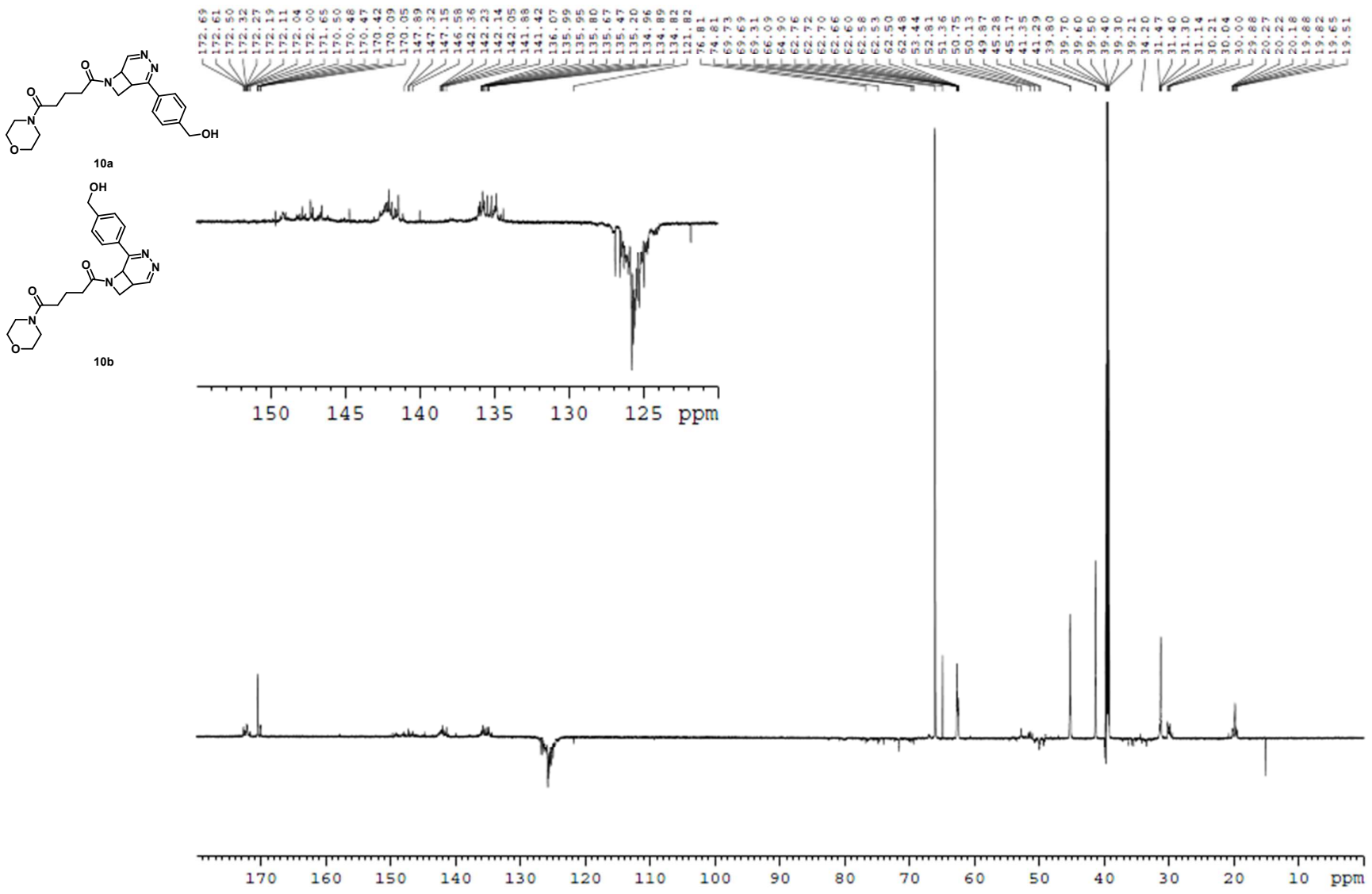


10b

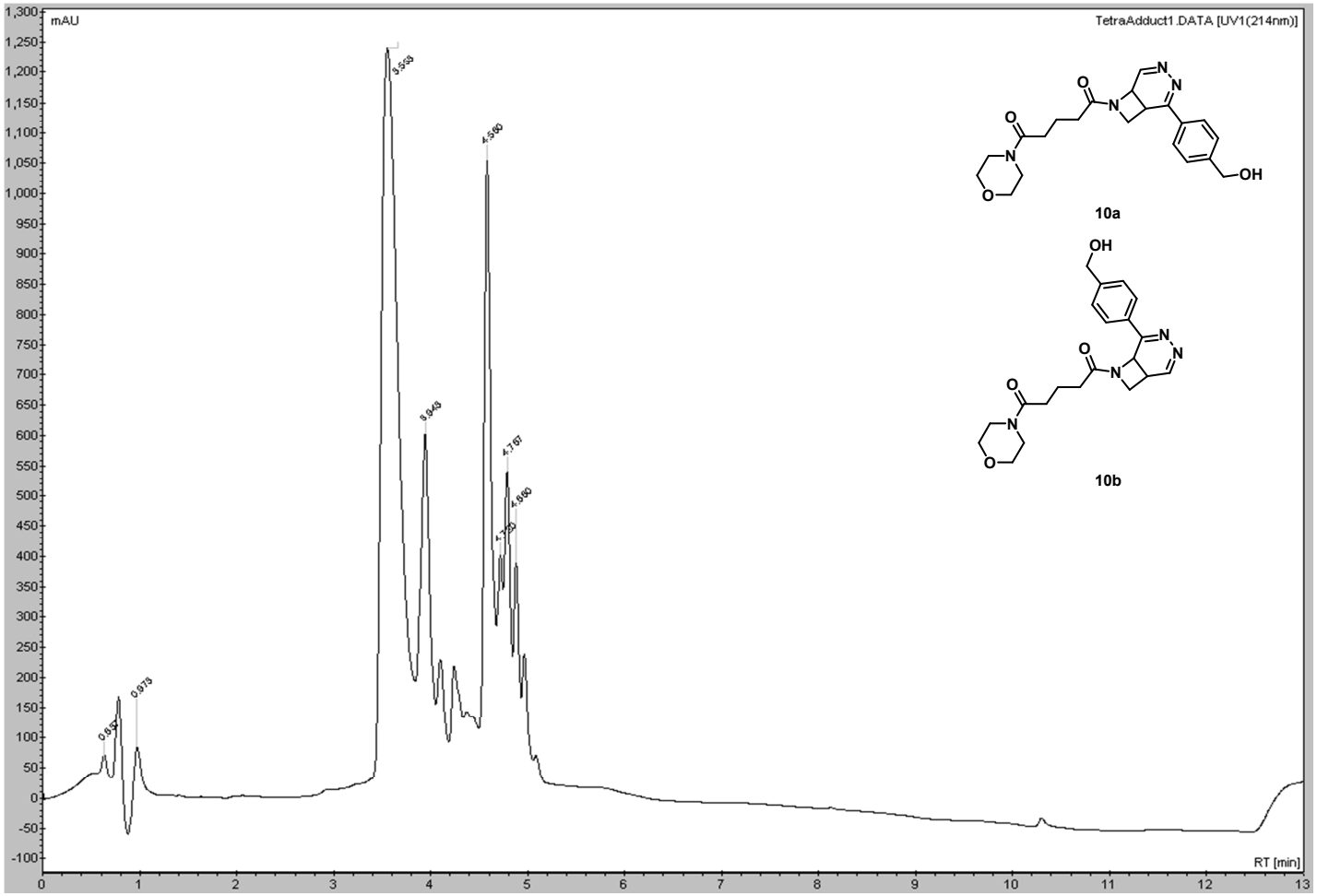
850 MHz NMR in DMSO:

Structures gives are putative. The spectra are taken from crude product. A mixture of regio isomers, rotamers and potentially tautomers are observed in NMR.

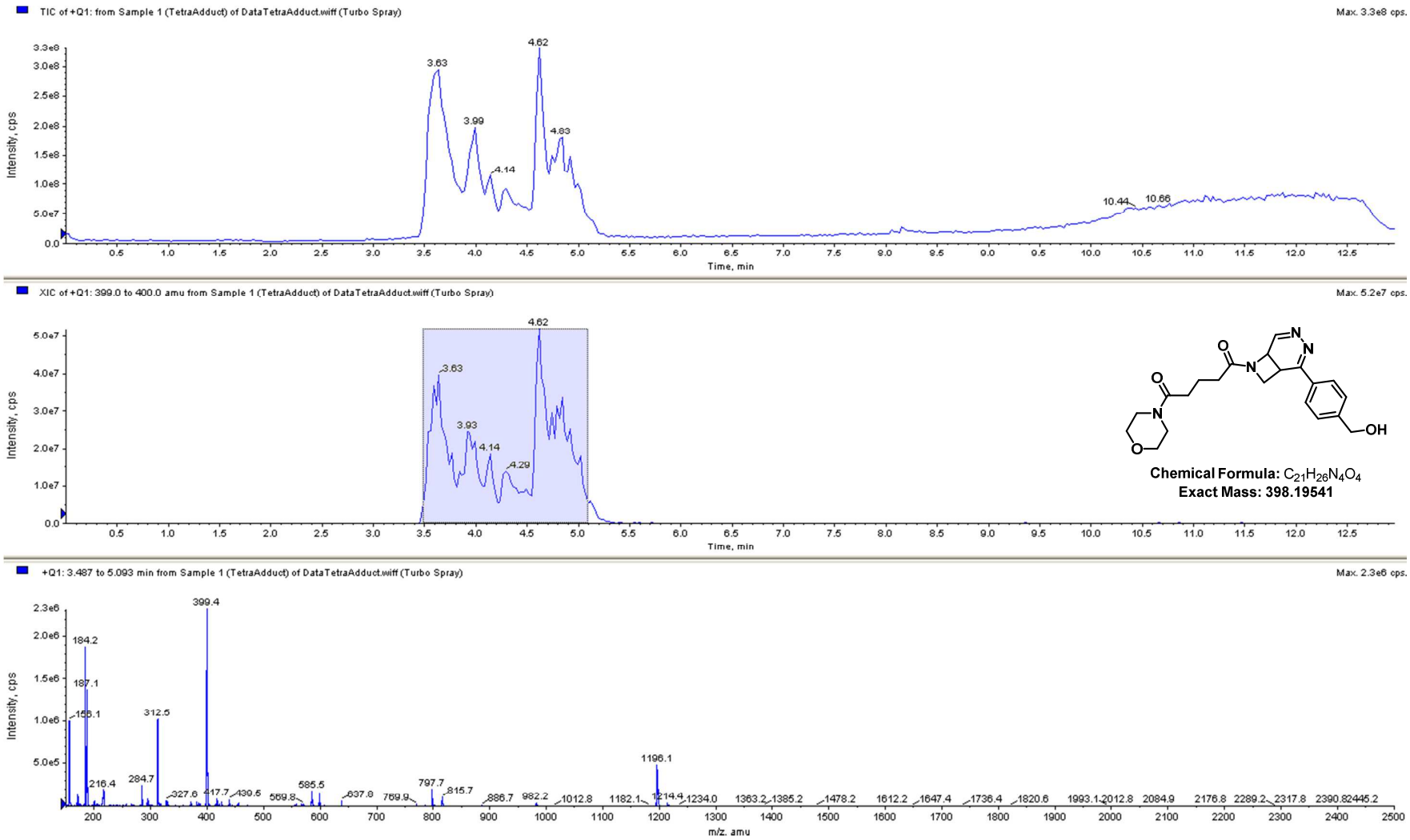



10a
10b
150 145 140 135 130 125 ppm
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

Mass Spectrometry Data



RP-HPLC: Gemini C_{18} column (3 µm particles, 110 Å pore size, 4.6 x 50 mm). A: H_2O, B: MeCN, C: 1% TFA; 8 minute gradient: 0% B to 50% B (10% C).


TIC of +Q1: from Sample 1 (TetraAdduct) of DataTetraAdduct.wiff (Turbo Spray)
Max. 3.3e8 cps.
Intensity, cps
Time, min
3.63
3.99
4.14
4.62
4.83
10.44
10.66
XIC of +Q1: 399.0 to 400.0 amu from Sample 1 (TetraAdduct) of DataTetraAdduct.wiff (Turbo Spray)
Max. 5.2e7 cps.
3.63
3.93
4.14
4.29
4.62
Chemical Formula: C21H26N4O4
Exact Mass: 398.19541
+Q1: 3.487 to 5.093 min from Sample 1 (TetraAdduct) of DataTetraAdduct.wiff (Turbo Spray)
Max. 2.3e6 cps.
m/z, amu
399.4
184.2
187.1
155.1
312.5
216.4
284.7
1196.1