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Supplementary Information

Chemoselective seleno-click amidation in kinetic target-guided synthesis

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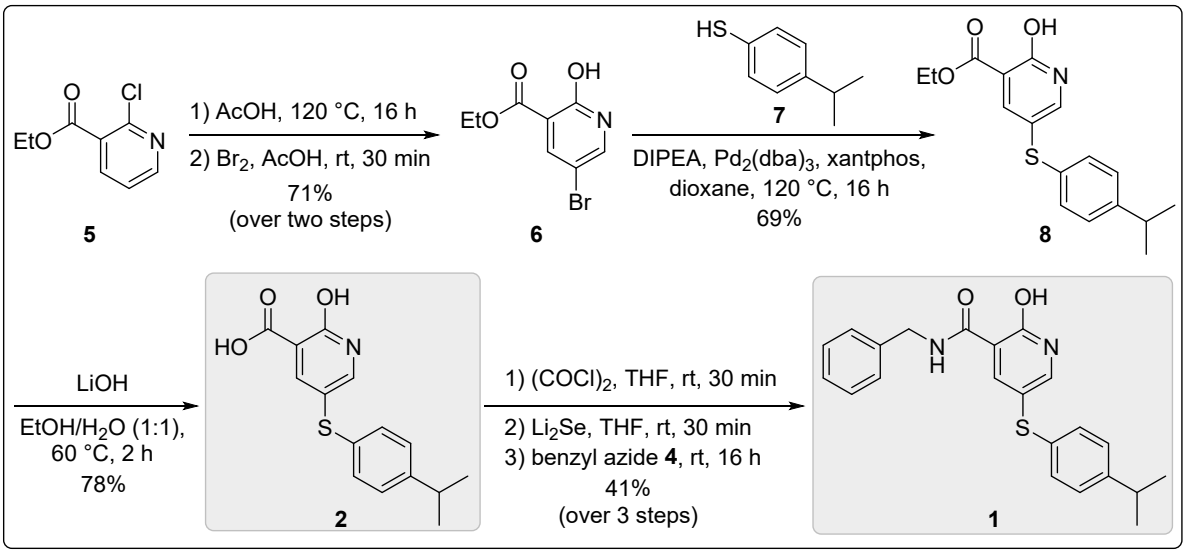
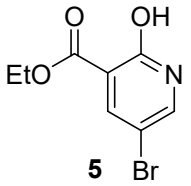
1. **General Information:** All reagents and solvents were obtained from Fisher Scientific, Sigma-Aldrich, or TCI America and used without further purification. Recombinant human Mcl-1 His protein (concentration 0.25 mg/mL, Catalog No. NBP25151025 – Novus Biologicals) was purchased from Fisher Scientific. Tetrahydrofuran (THF) was distilled from benzophenone and sodium metal under a positive-pressure argon atmosphere immediately before use. All other anhydrous solvents were purchased from VWR. Analytical thin layer chromatography (TLC) was performed on silica gel 60 F_{254} pre-coated plates (0.25 mm) from EMD Millipore Corp., and components were visualized by ultraviolet light (254 nm). Reported R_f values were determined by TLC. EMD silica gel 60 (particle size 40-63 μm) 230 – 400 mesh was used for column

chromatography. Preparatory HPLC purification was performed on an ACCQPrep HP150 equipped with a Phenomenex Luna C18(2) column (5 µm, 100 Å, 250 x 30 mm) (Part No: 00G-4252-U0-AX). ^{1}H-NMR spectra were recorded at ambient temperature on a 500 MHz Bruker NMR spectrometer in the indicated solvent. All ^{1}H NMR experiments are reported in δ units, parts per million (ppm) downfield of TMS and were measured relative to the signals for chloroform (7.26 ppm) and dimethylsulfoxide (2.50 ppm). Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, hep = heptet, m = multiplet), integration and coupling constant (Hz), whereas ^{13}C NMR analyses are reported in terms of chemical shift. NMR data was analyzed by using MestReNova Software ver. 12.0.3-21384. Low-resolution mass spectrometry (LRMS) was measured on an Agilent 1260 Infinity LC instrument coupled to an Agilent 6120 single quadrupole mass spectrometer with electrospray ionization. LC-MS/MS was measured on an Agilent 1290 UHPLC equipped with a thermostatted autosampler coupled to an Agilent 6460 QQQ-MS equipped with Agilent Jet Stream (AJS) electrospray ionization.

2. **Synthetic Procedures and Compound Characterization:**

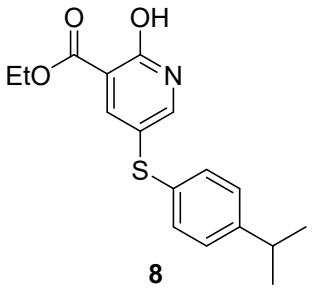


Scheme S1: Synthesis of the carboxylic acid fragment **2** and the nicotinamide derivative **1**.



5

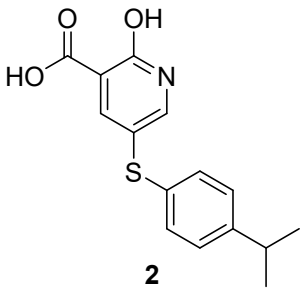
Ethyl 5-bromo-2-hydroxynicotinate (5): Performed according to the previously reported procedure.[1] Ethyl 2-chloronicotinate **5** (6.00 g, 31.7 mmol) in acetic acid (210 mL) was refluxed at 120 °C for 16 hours. The reaction mixture was cooled to room temperature, and bromine (7.7 mL, 151 mmol) was added dropwise over 30 minutes. The reaction mixture was stirred at room temperature for 3 hours. A solution of sodium pyrosulfite (20% in H_2O, 15.4 mL) was added to the reaction mixture and extracted with CH_2Cl_2 (3 × 80 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography eluting with CH_2Cl_2/MeOH 3:0.15 to yield **5** (5.53 g, 71%). R_f = 0.25 (CH_2Cl_2/MeOH 3:0.15). ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.39 (s, 1H), 8.40 (s, 1H), 8.29 (d, *J* = 2.4 Hz, 1H), 4.46 (q, *J* = 7.1 Hz, 2H), 1.44 (t, *J* = 7.1 Hz, 3H). LRMS-ESI (*m*/z): $[M + H]^+$ 245.7, 247.8.



8

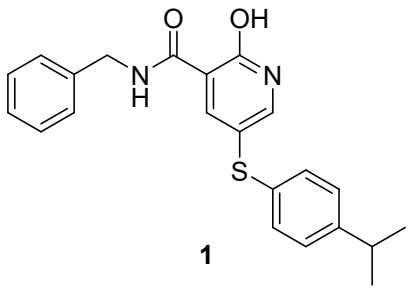
Ethyl 2-hydroxy-5-((4-isopropylphenyl)thio)nicotinate (8): To the degassed solution of ethyl 5-bromo-2-hydroxynicotinate **5** (2.00 g, 8.1 mmol) and DIPEA (2.8 mL, 16.3 mmol) in anhydrous 1,4-dioxane (27 mL) was added tris(dibenzylideneacetone)dipalladium(0) (186 mg, 203 µmol), xantphos (235 mg, 406 µmol), and 4-isopropylbenzenethiol **7** (2.6 mL, 17.1 mmol). The reaction mixture was repeatedly degassed (3 cycles) and stirred at 120 °C for 16 hours. Upon cooling to room temperature, water (150 mL) was added to the reaction mixture and extracted with EtOAc

(3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography eluting with CH_2Cl_2/MeOH 3:0.13 to yield **8** (1.78 g, 69%). R_f = 0.25 (CH_2Cl_2/MeOH 3:0.13). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.27 (d, *J* = 2.6 Hz, 1H), 8.11 (s, 1H), 7.22 – 7.12 (m, 4H), 4.38 (q, *J* = 7.1 Hz, 2H), 2.87 (hep, *J* = 6.9 Hz, 1H), 1.37 (t, *J* = 7.1 Hz, 3H), 1.23 (d, *J* = 6.9 Hz, 6H). LRMS-ESI (*m*/z): $[M + H]^+$ 318.1.



2

2-hydroxy-5-((4-isopropylphenyl)thio)nicotinic acid (2): To a solution of ethyl 2-hydroxy-5-((4-isopropylphenyl)thio)nicotinate **8** (1.78 g, 5.61 mmol) in the mixture of EtOH/H_2O (v/v 1:1, 54 mL) was added lithium hydroxide monohydrate (894 mg, 21.3 mmol), and the reaction mixture was stirred at 60 °C for 2 hours. The mixture was concentrated to remove the EtOH, and EtOAc (50 mL) was added to the aqueous layer. The organic layer was separated, and the aqueous layer was acidified to pH ~ 2 using 1 M aqueous HCl. The aqueous solution was extracted with EtOAc (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield **2** (1.27 g, 78%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.23 – 8.20 (m, 2H), 7.26 – 7.21 (m, 4H), 2.86 (hep, *J* = 13.8, 6.9 Hz, 1H), 1.17 (d, *J* = 6.9 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.1, 164.0, 152.0, 150.3, 149.7, 140.9, 131.4, 130.6, 128.1, 118.3, 34.0, 24.0. LRMS-ESI (*m*/z): $[M + H]^+$ 290.1.

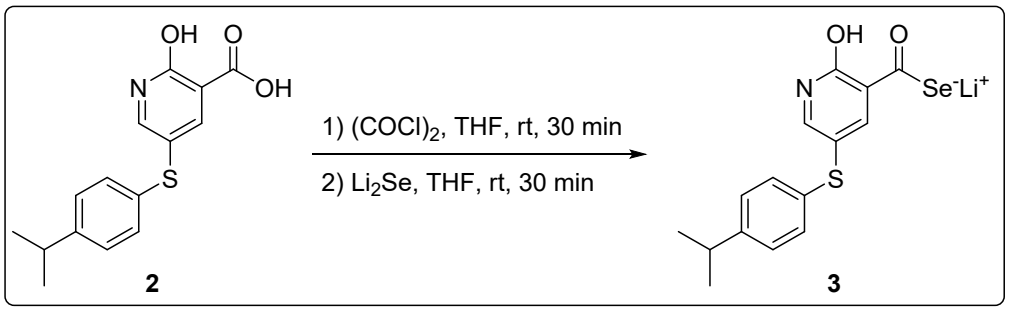


1

***N*-benzyl-2-hydroxy-5-((4-isopropylphenyl)thio)nicotinamide (1):** Performed according to the previously reported procedure.[2] To a solution of 2-hydroxy-5-((4-isopropylphenyl)thio)nicotinic acid **2** (50.0 mg, 173 µmol) in anhydrous THF (1.8 mL) was added oxalyl chloride (30.2 µL, 346 mmol), and the reaction mixture was stirred at room temperature for 30 minutes. The reaction mixture was concentrated and dissolved in anhydrous THF (1 mL). The resulting solution was added dropwise into the solution of lithium selenide (16 mg, 173 µmol) in anhydrous THF (0.8 mL). The reaction mixture was stirred at room temperature for 30 minutes, and then benzyl azide **4** (23.0 µL, 173 µmol) was added. The reaction mixture was stirred at room temperature for 16 hours. H_2O (50 mL) was added to the reaction mixture and extracted with EtOAc (3 × 20 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography eluting with CH_2Cl_2/MeOH 3:0.08 to yield impure **1**, which was then further purified by preparative HPLC to yield pure **1** (27.0 mg, 41%). R_f = 0.36 (CH_2Cl_2/MeOH 3:0.13). ^{1}H NMR data matches with that of data reported in the literature.[1] ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.77 (s, 1H), 8.69 (s, 1H), 7.68 (s, 1H), 7.33 (d, *J* = 6.9 Hz, 4H), 7.20 (d, *J* = 7.9 Hz, 2H), 7.14 (d, *J* = 7.6 Hz, 2H), 4.64 (d, *J* = 4.7 Hz, 2H), 2.85 (hep, *J* = 6.9 Hz, 1H), 1.21 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.1, 162.9, 150.8, 148.6, 141.2, 138.6, 132.3, 130.1, 128.8, 127.8, 127.7, 127.5, 122.2, 115.1, 43.7, 33.9, 24.0. LRMS-ESI (*m*/z): $[M + H]^+$ 379.0.

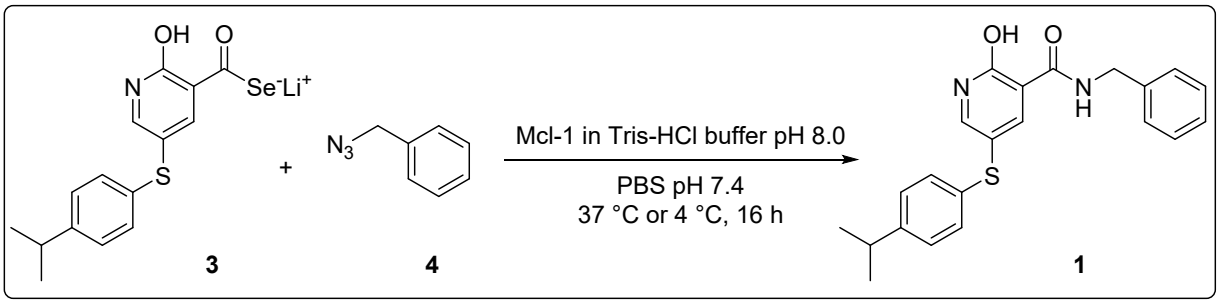
3. **KTGS Experimental Procedures:**

3.1 **Generation of selenocarboxylate fragment 3 prior to the KTGS:**



To a solution of 2-hydroxy-5-((4-isopropylphenyl)thio)nicotinic acid **2** (7.5 mg, 26.0 µmol) in anhydrous THF (250 µL) was added oxalyl chloride (4.6 µL, 51.8 µmol), and the reaction mixture was stirred at room temperature for 30 minutes. The reaction mixture was concentrated and dissolved in anhydrous THF (235 µL). The resulting solution was added dropwise into the solution of lithium selenide (2.4 mg, 25.9 µmol) in anhydrous THF (125 µL). The reaction mixture was stirred at room temperature for 30 minutes to give selenocarboxylate fragment **3**. The reaction mixture was diluted with the HPLC-grade MeOH to obtain a 2 mM stock solution of **3**, which was immediately used for KTGS experiments without further purification.

3.2 **General procedure for incubations of Mcl-1 with freshly prepared selenocarboxylate fragment 3 and benzyl azide fragment 4:**



In the 500 µL Eppendorf vials, selenocarboxylate fragment **3** (1 µL of a 2 mM solution in MeOH) and benzyl azide fragment **4** (1 µL of 2 mM solution in MeOH) were added to a solution of Mcl-1

(49 µL of a 6.72 µM of Mcl-1 in 20 mM Tris-HCl buffer pH 8.0 containing 0.2 M NaCl, 20% glycerol, 2 mM DTT) and 49 µL of PBS buffer pH 7.4 (1X). The Eppendorf vials were sealed with parafilm and incubated at 37 °C and 4 °C separately for 16 hours. The incubation samples (without work-up) were directly transferred to Agilent's autosampler vials and subjected to triple–quadrupole mass spectrometry analysis in the dynamic multiple reaction monitoring (dMRM) mode (LC-MS/MS-MRM, Phenomenex Kinetex PFP column (2.6 µm, 100 Å, 50 x 4.6 mm) (Part No: 00B-4477-E0) preceded by a Phenomenex SecurityGuard C18 Guard Cartridge (Part No: KJ0-4282), electrospray ionization and mass spectroscopic detection in the positive selected ion mode, tuned to the expected molecular mass of the product). The formation of the product, *N*-benzyl-2-hydroxy-5-((4-isopropylphenyl)thio)nicotinamide **1** was identified by the specific parent ion (*m/z* = 379 $[M+H]^+$) and its two distinct fragmentation product ions (*m/z* = 272 – acylium ion fragment and *m/z* = 91 – benzyl carbonium ion fragment). As a control, selenocarboxylate fragment **3** (1 µL of a 2 mM solution in MeOH) and benzyl azide fragment **4** (1 µL of 2 mM solution in MeOH) were incubated in 49 µL of 20 mM Tris-HCl buffer (pH 8.0 containing 0.2 M NaCl, 20% glycerol, 2 mM DTT) and 49 µL of PBS buffer pH 7.4 (1X) without Mcl-1 and subjected to LC/MS-MS-MRM analysis. Comparison of the LC-MS/MS chromatograms of these control incubations with the chromatograms of the Mcl-1 containing incubations allows one to determine whether the protein templates the corresponding seleno-click amidation reactions. Additionally, synthetically prepared *N*-benzyl-2-hydroxy-5-((4-isopropylphenyl)thio)nicotinamide **1** was subjected to LC-MS/MS analysis, and the retention time was compared with the retention time identified in the Mcl-1 containing incubation.

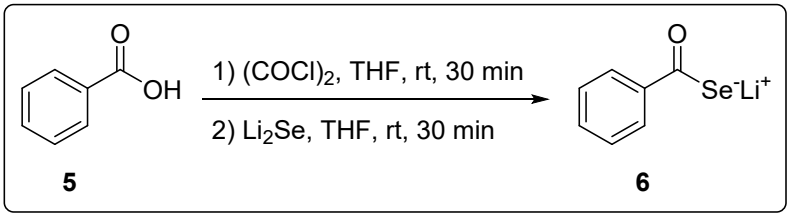
Table S1: Amount of product formed relative to standard reference compound (nicotinamide derivative **1**).

Temp. (°C)	**Protein**	**Area**	**Amount of product formed[a] (nM)**	**Amplification**

37	Mcl-1	5831	13.28	9.3
	No protein	628	1.43	
4	Mcl-1	4954	11.28	25.6
	No protein	193	0.44	

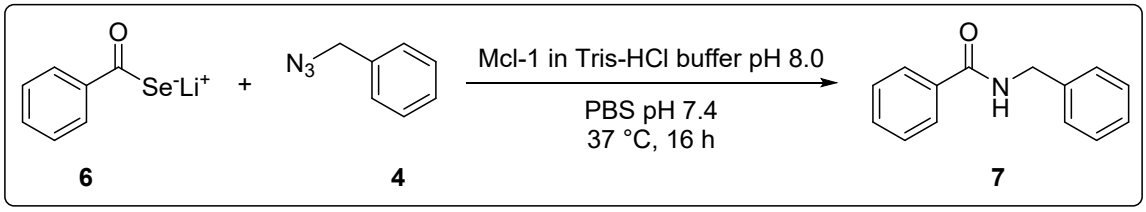
[a]The amount of product formed was calculated based on the area of the standard reference compound (chemically synthesized nicotinamide derivative **1** of a known concentration of 20 nM). The area of the standard reference compound is 8784.

3.3 **Generation of benzoic acid selenocarboxylate fragment 6 prior to the KTGS:**



To a solution of benzoic acid **5** (10.0 mg, 81.9 μmol) in anhydrous THF (820 μL) was added oxalyl chloride (14.0 μL, 163.8 μmol), and the reaction mixture was stirred at room temperature for 30 minutes. The reaction mixture was concentrated and dissolved in anhydrous THF (487 μL). The resulting solution was added dropwise into the solution of lithium selenide (7.6 mg, 81.9 μmol) in anhydrous THF (333 μL). The reaction mixture was stirred at room temperature for 30 minutes to give benzoic acid selenocarboxylate fragment **6**. The reaction mixture was diluted with the HPLC-grade MeOH to obtain a 2 mM stock solution of **6**, which was immediately used for KTGS experiments without further purification.

3.4 **General procedure for incubations of Mcl-1 with freshly prepared benzoic acid selenocarboxylate fragment 6 and benzyl azide fragment 4:**



In the 500 µL Eppendorf vials, benzoic acid selenocarboxylate fragment **6** (1 µL of a 2 mM solution in MeOH) and benzyl azide fragment **4** (1 µL of 2 mM solution in MeOH) were added to a solution of Mcl-1 (49 µL of a 6.72 µM of Mcl-1 in 20 mM Tris-HCl buffer pH 8.0 containing 0.2 M NaCl, 20% glycerol, 2 mM DTT) and 49 µL of PBS buffer pH 7.4 (1X). The Eppendorf vials were sealed with parafilm and incubated at 37 °C for 16 hours. The incubation samples (without work-up) were directly transferred to Agilent's autosampler vials and subjected to triple–quadrupole mass spectrometry analysis in the dynamic multiple reaction monitoring (dMRM) mode (LC-MS/MS-MRM, Phenomenex Kinetex PFP column (2.6 µm, 100 Å, 50 x 4.6 mm) (Part No: 00B-4477-E0) preceded by a Phenomenex SecurityGuard C18 Guard Cartridge (Part No: KJ0-4282), electrospray ionization and mass spectroscopic detection in the positive selected ion mode, tuned to the expected molecular mass of the product). The formation of the product, *N*-benzylbenzamide **7** was identified by the specific parent ion (*m/z* = 212 $[M+H]^+$) and its distinct fragmentation product ions (*m/z* = 91 – benzyl carbonium ion fragment). As a control, selenocarboxylate fragment **6** (1 µL of a 2 mM solution in MeOH) and benzyl azide fragment **4** (1 µL of 2 mM solution in MeOH) were incubated in 49 µL of 20 mM Tris-HCl buffer (pH 8.0 containing 0.2 M NaCl, 20% glycerol, 2 mM DTT) and 49 µL of PBS buffer pH 7.4 (1X) without Mcl-1 and subjected to LC/MS-MS-MRM analysis. Comparison of the LC-MS/MS chromatograms of these control incubations with the chromatograms of the Mcl-1 containing incubations allows one to determine whether the protein templates the corresponding seleno-click amidation reactions. Additionally, commercially

available *N*-benzylbenzamide **7** was subjected to LC-MS/MS analysis, and the retention time was compared with the retention time identified in the Mcl-1 containing incubation.

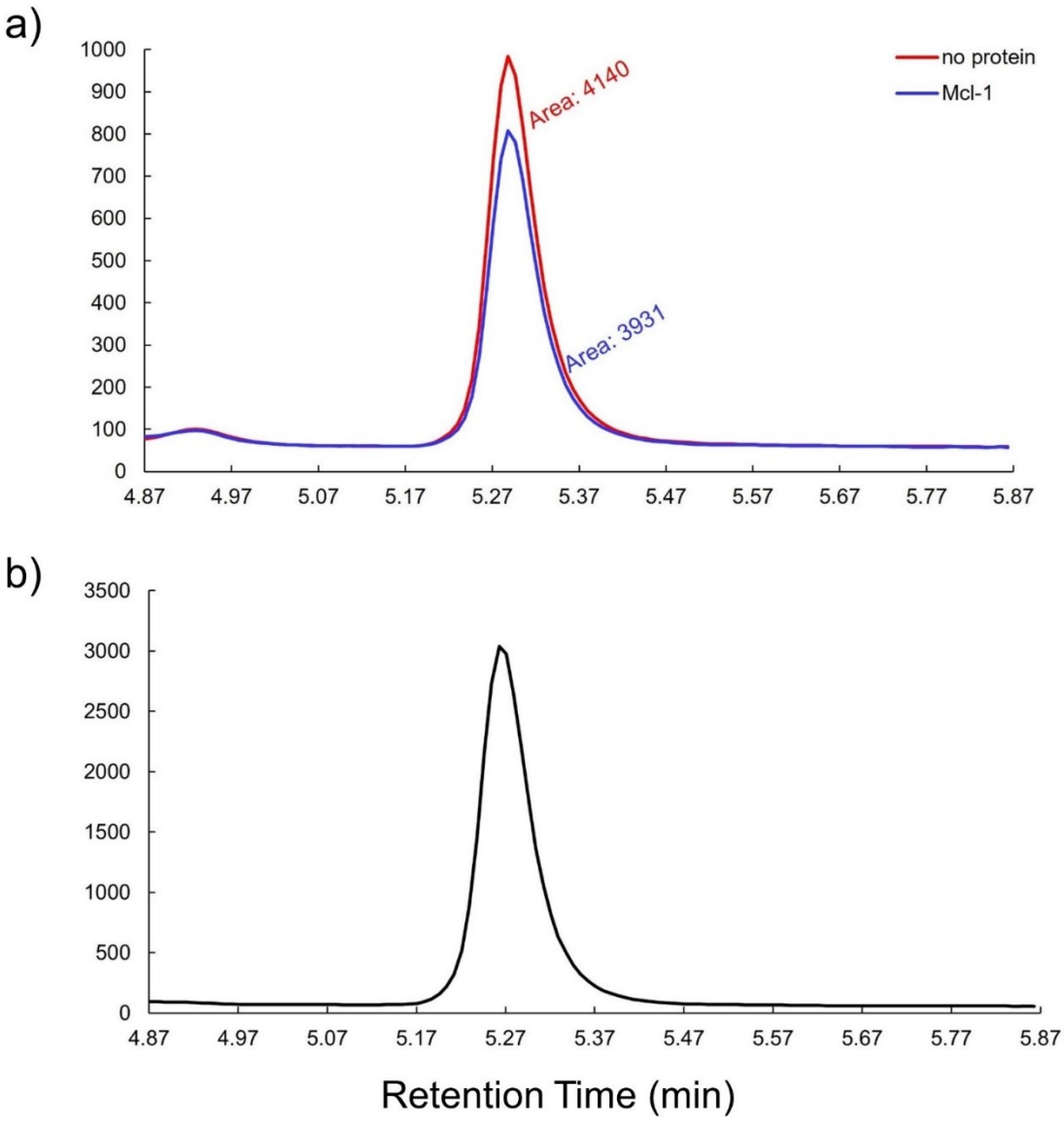


Figure S1: Identification and quantification of *N*-benzylbenzamide **7** by LC-MS/MS-dMRM analysis of KTGS incubations of benzoic acid selenocarboxylate fragment **6** with benzyl azide fragment **4** with and without Mcl-1. (a) LC-MS/MS trace of incubations at 37 °C. (b) LC-MS/MS trace of the commercially available *N*-benzylbenzamide **7** as a reference compound.

4. **Triple-Quadrupole Mass Spectrometry:**

4.1 **Fragmentation Studies of *N*-benzyl-2-hydroxy-5-((4-isopropylphenyl)thio)nicotinamide 1 and *N*-benzylbenzamide 7:** To detect small amounts of nicotinamide derivative **1** and *N*-benzylbenzamide **7** formation, it was essential to optimize its fragmentation parameters. We subjected an authentic sample of synthesized compounds **1** and **7** to multiple injections, varying the fragmentation voltages and collision energies to maximize the generation of the parent and product ions (Tables S1 and S2).

Table S2: Optimized and implemented detection parameters for nicotinamide derivative **1**.

Parent Ion	Fragment Voltage	Product Ion	Collision Energy	Retention Time (min)	Delta Retention Time (min)
379.15 $[M+H]^+$	140	91.1	28	6.15	1
		272	20		

Table S3: Optimized and implemented detection parameters for *N*-benzylbenzamide **7**.

Parent Ion	Fragment Voltage	Product Ion	Collision Energy	Retention Time (min)	Delta Retention Time (min)
212.10 $[M+H]^+$	85	91.1	24	5.37	1

4.2 **LC-MS/MS (triple quadrupole mass spectrometry) analysis in the dynamic multiple reaction monitoring (dMRM) mode:** The sample injection volume was 20 µL, wherein samples

were eluted with a gradient at a flow rate of 0.7 mL/min and a column compartment temperature of 37 °C. The chromatography was performed on a Phenomenex Kinetex PFP column (2.6 µm, 100 Å, 50 x 4.6 mm) (Part No: 00B-4477-E0), preceded by a Phenomenex SecurityGuard C18 Guard Cartridge (Part No: KJ0-4282). The first 2 minutes of eluted material was diverted to waste to minimize buffer contamination of the mass spectrometer.

Table S4: Elution gradient employed for analysis of KTGS incubations:

Time (min)	**% B***	**Flow rate** (mL min^{-1})
0	10	0.7
2	10	0.7
7	90	0.7
9	90	0.7
10	10	0.7

* eluent A: H_2O (0.1% TFA); eluent B: CH_3CN (0.1% TFA).

4.3 **Raw Data From Amplification Trials:**

Table S5. Raw data for injections, total ion current (TIC) analysis.

Sample	**Parent Ion (*m/z*)**	**Retention Time (min)**	**Area**
No protein, 37 °C	379.15	6.140	628
Mcl-1, 37 °C	379.15	6.132	5831

No protein, 4 °C	379.15	6.148	193
Mcl-1, 4 °C	379.15	6.157	4954

Table S6. Raw data for injections, MRM analysis of TIC (see Table S5).

Sample	Parent Ion (*m/z*)	Product Ion (*m/z*)	Retention Time (min)	Area
No protein, 37 °C	379.15	91	6.136	420
		272	6.137	181
Mcl-1, 37 °C	379.15	91	6.136	3714
		272	6.163	2085
No protein, 4 °C	379.15	91	6.144	116
		272	6.173	72
Mcl-1, 4 °C	379.15	91	6.144	1712
		272	6.165	3197

5. **References:**

1) Z. C. Zhang, C. W. Liu, X. Q. Li, T. Song, Z. Y. Wu, X. M. Liang, Y. Zhao, X. Y. Shen and H. B. Chen, *Eur. J. Med. Chem.*, 2013, **60**, 410-420.

2) L. Silva, A. R. Rosário, B. M. Machado and D. S. Lüdtke, *Tetrahedron*, 2021, **79**, 131834.

6. **Copies of ^{1}H, and ^{13}C NMR Spectra:**

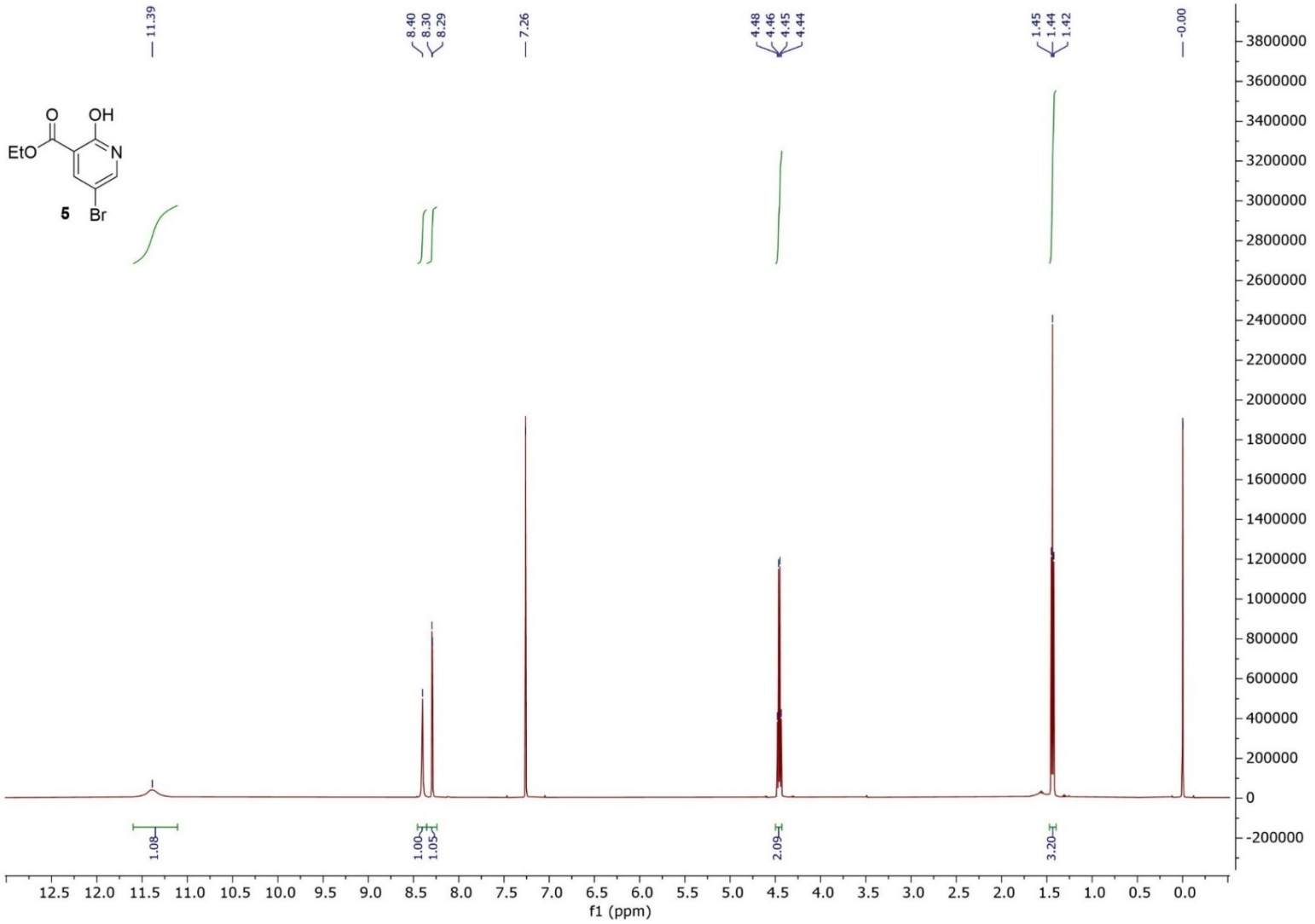


Figure S2: ^{1}H NMR of **5** in $CDCl_3$.

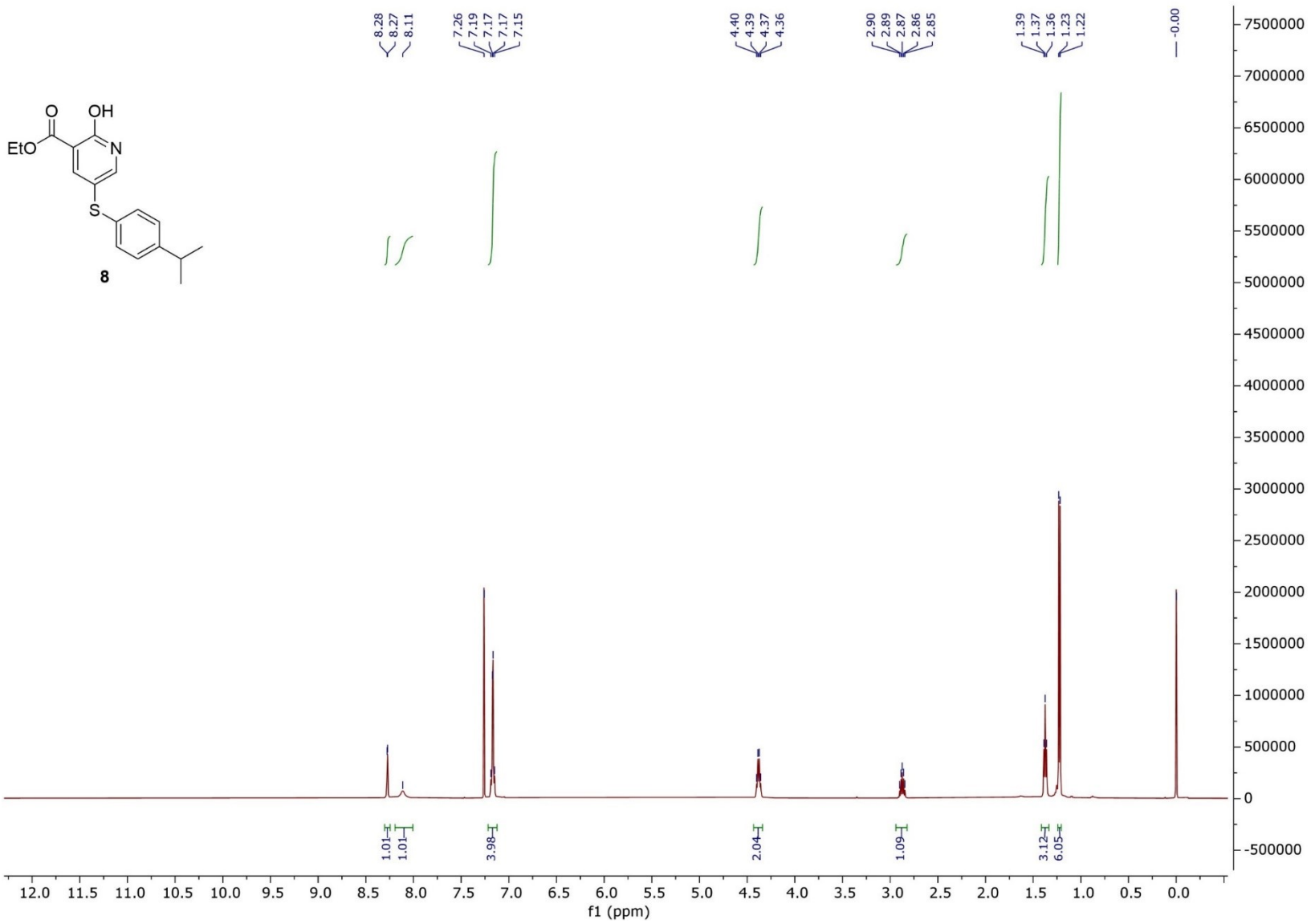


Figure S3: ^{1}H NMR of **8** in $CDCl_3$.

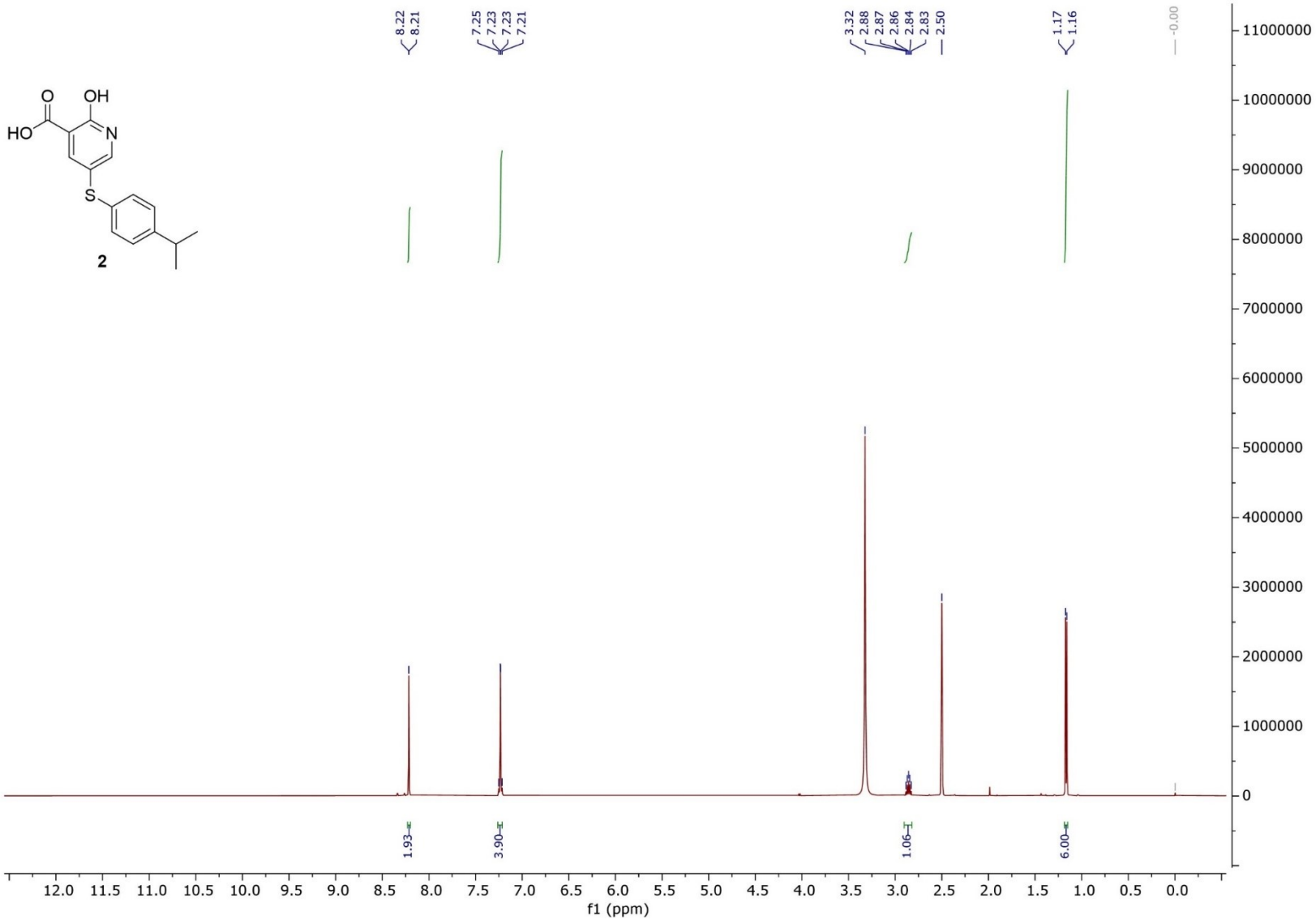


Figure S4: ^{1}H NMR of **2** in DMSO-d_6.

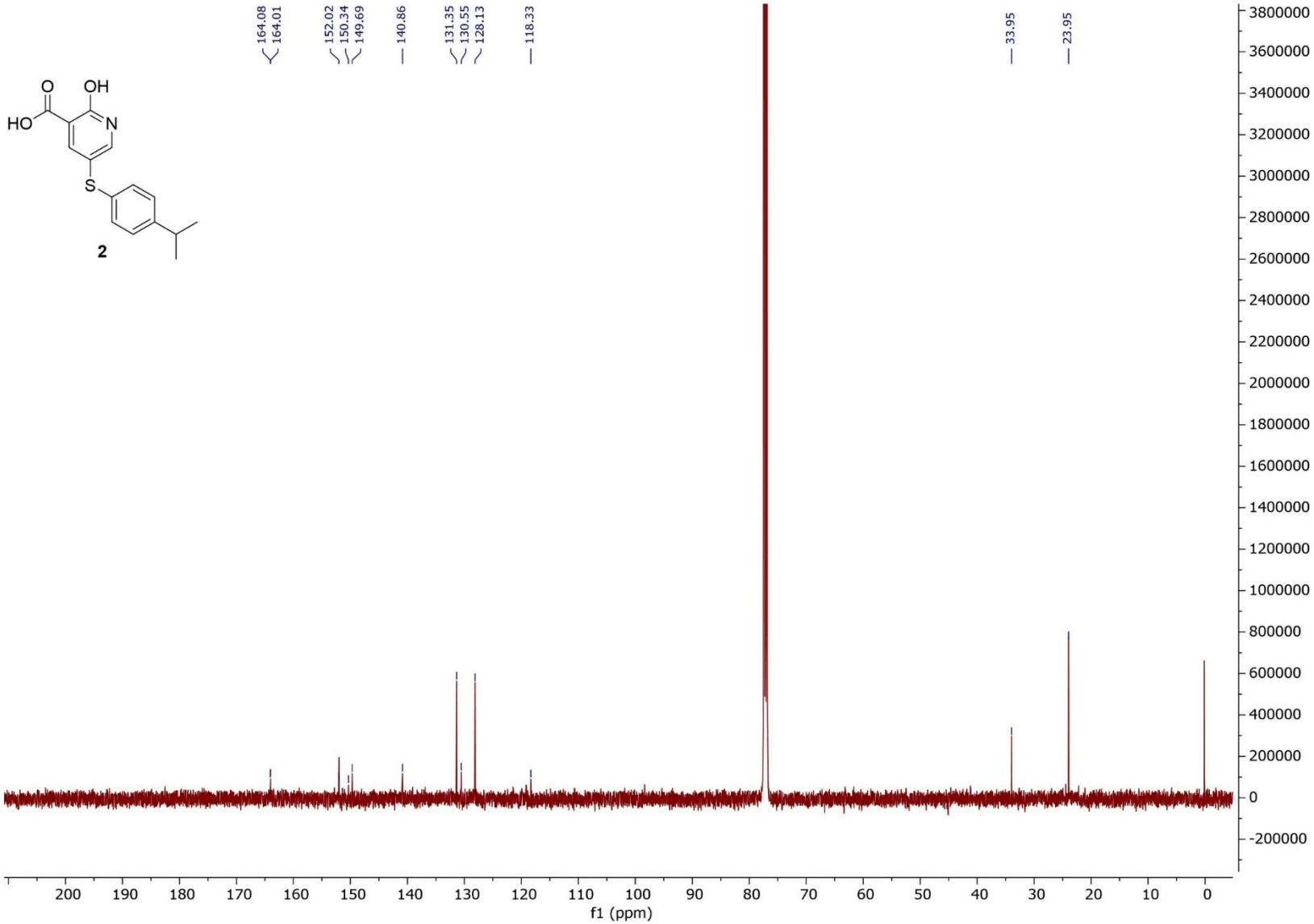


Figure S5: ^{13}C NMR of **2** in $CDCl_3$.

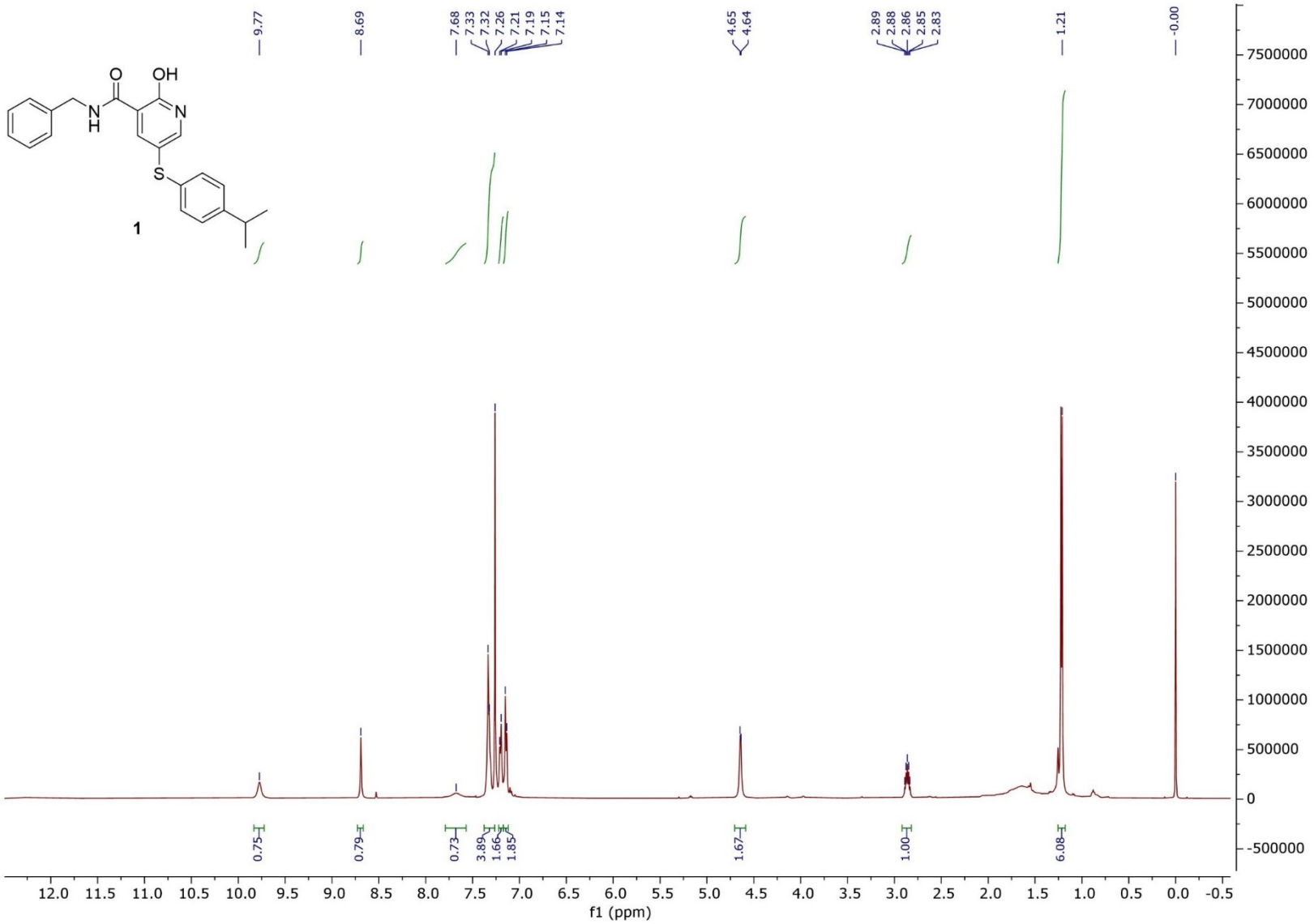


Figure S6: ^{1}H NMR of **1** in $CDCl_3$.

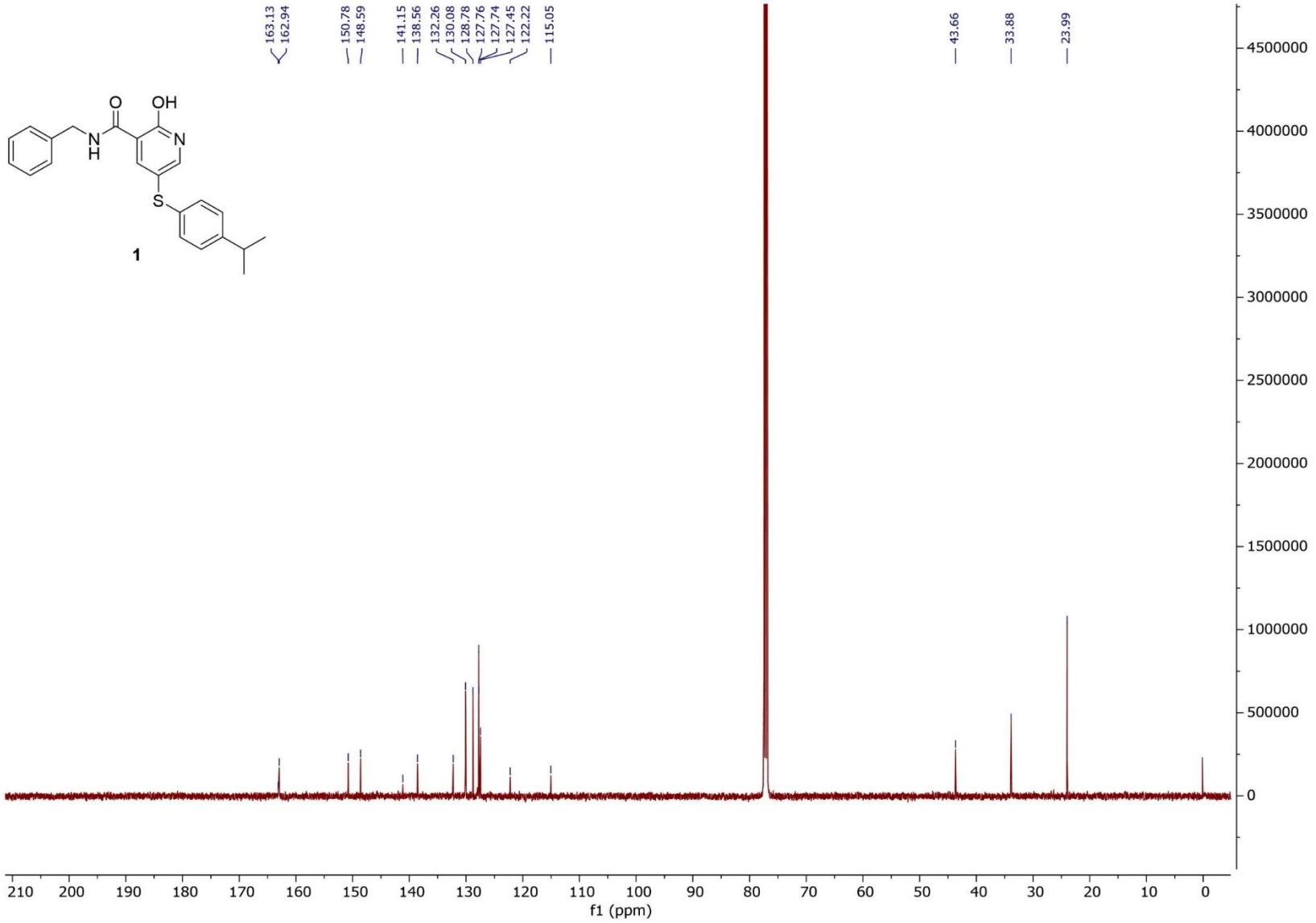


Figure S7: ^{13}C NMR of **1** in $CDCl_3$.