Supplementary Information for

Selenols: A new class of Carbonic Anhydrase inhibitors

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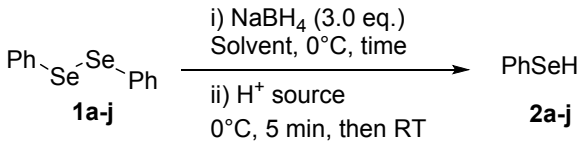
Experimental Procedures

General information

All reactions were carried out in an oven-dried glassware under inert atmosphere (N_2). Ethanol was dried using a solvent purification system (Pure-Solv™). All commercial materials were used as received without further purification. Diselenides **1b-l** were synthesized from the corresponding aryl bromides or iodides following literature reported procedures.[1] Flash column chromatography purifications were performed with Silica gel 60 (230-400 mesh). Thin layer chromatography was performed with TLC plates Silica gel 60 F_{254}. Mass spectra (MS) were determined by ESI. NMR spectra were recorded in $CDCl_3$ with Mercury 400, and Bruker 400 Ultrashield spectrometers operating at 400 MHz (for ^1H), 100 MHz (for ^{13}C), and 76 MHz (for ^{77}Se). NMR signals were referenced to nondeuterated residual solvent signals (7.26 ppm for ^1H, 77.0 ppm for ^{13}C). $(PhSe)_2$ was used as an external reference for ^{77}Se NMR (δ = 461 ppm). ^1H NMR data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, ap d = apparent doublet, m = multiplet, dd = doublet of doublet, bs = broad singlet, bd = broad doublet, ecc.), coupling constant (*J*) or line separation (ls), and assignment.

Optimization of the reaction conditions

Table S1 Optimization of the synthesis of aryl selenols



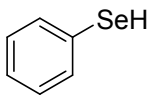
Entry	Solvent	Time (min)	H^+ source	Yield (%)
1	THF	30	HCl	15
2	THF	30	NH_4Cl	<5
3	THF	30	Citric acid	55
4	EtOH	10	HCl	10
5	EtOH	10	NH_4Cl	<5
6	EtOH	10	Citric acid	76
7	EtOH	10	Tartaric acid	<5
8	EtOH	10	Ascorbic acid	21

General Procedure for the preparation of aryl selenols 2 from diselenides 1

$NaBH_4$ (23 mg, 0.6 mmol, 3.0 eq.) was portionwise added to a stirred solution or suspension (depending on the nature of the diselenide) of diselenide 1 (0.2 mmol, 1.0 eq.) in EtOH (2 mL) at 0°C under inert atmosphere (N_2). After 15 min, solid citric acid (192 mg, 1.0 mmol, 5.0 eq.) was added and the reaction mixture was stirred at 0°C for 5 minutes. The mixture was then diluted with Et_2O (5 mL) and H_2O (3 mL) was added. The layers were separated and the organic layer was washed with saturated *aq*. NH_4Cl (2 mL) and with brine (2 mL), dried over Na_2SO_4, filtered and concentrated under vacuum to afford aryl selenols 2, pure enough to be used without further purification.

Product Characterisation

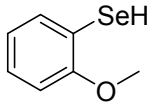
Synthesis of benzeneselenol (2a)



2a

Following the general procedure, diphenyl diselenide **1a** (125 mg, 0.40 mmol) gave benzeneselenol **2a** (95 mg, 76%).**^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.56 (1H, s, SeH); 7.22-7.25 (3H, m); 7.44-7.47 (2H, m). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 124.5; 126.5; 129.3; 132.8. **^{77}Se NMR** (76 MHz, $CDCl_3$) δ (ppm): 144.1. **HRMS** m/z calcd for C_6H_6Se 157.9635, found 157.9612.

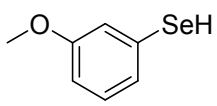
Synthesis of 2-methoxybenzeneselenol (2b)



2b

Following the general procedure, 1,2-bis(2-methoxyphenyl)diselane **1b** (75 mg, 0.20 mmol) gave 2-methoxybenzeneselenol **2b** (42.2 mg, 56%).**^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.53 (1H, s, SeH); 3.88 (3H, s); 6.80-6.85 (2H, m); 7.16-7.21 (1H, m); 7.39 (1H, dd, *J*=1.5, 7.5 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 55.8; 110.6; 115.7, 121.6, 127.4, 132.5, 156.4. **^{77}Se NMR** (76 MHz, $CDCl_3$) δ (ppm): 73.0. **HRMS** m/z calcd for C_7H_8OSe 187.9740, found 187.9764.

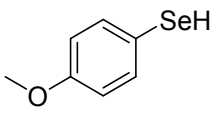
Synthesis of 3-methoxybenzeneselenol (2c)



2c

Following the general procedure, 1,2-bis(3-methoxyphenyl)diselane **1c** (75 mg, 0.20 mmol) gave 3-methoxybenzeneselenol **2c** (61.8 mg, 82%).**^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.58 (1H, s, SeH), 3.79 (3H, s, OCH_3), 6.76 (1H, ddd, *J*=0.8, 2.5, 8.3 Hz), 6.99-7.04 (2H, m), 7.13 (1H, ap t, *J*=8.3 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 55.2; 112.4; 118.1, 125.0, 125.4, 130.0, 159.9. **^{77}Se NMR** (76 MHz, $CDCl_3$) δ (ppm): 81.2. **HRMS** m/z calcd for C_7H_8NaOSe 210.9638, found 210.9653.

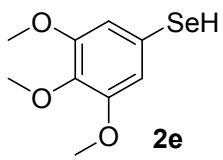
Synthesis of 4-methoxybenzeneselenol (2d)



2d

Following the general procedure, 1,2-bis(4-methoxyphenyl)diselane **1d** (75 mg, 0.20 mmol) gave 4-methoxybenzeneselenol **2d** (64.1 mg, 85%).**^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.45 (1H, s, SeH), 3.78 (3H, s, OCH_3), 6.79 (2H, ap d, ls=8.4 Hz), 7.41 (2H, ap d, ls=8.4 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 58.2; 112.9; 114.9, 135.3, 159.0. **^{77}Se NMR** (76 MHz, $CDCl_3$) δ (ppm): 119.9. **HRMS** m/z calcd for C_7H_8NaOSe 210.9638, found 210.9615.

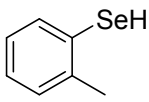
Synthesis of 3,4,5-trimethoxybenzeneselenol (2e)



2e

Following the general procedure, 1,2-bis(3,4,5-trimethoxyphenyl)diselane **1e** (75 mg, 0.15 mmol) gave 3,4,5-trimethoxybenzeneselenol **2e** (71.5 mg, 95%).**^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.62 (1H, s, SeH), 3.81 (3H, s, OCH_3), 3.84 (6H, s, OCH_3), 6.69 (2H, s). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 56.2; 60.9; 110.7; 117.7; 137. 3; 153.5. HRMS m/z calcd for $C_9H_{12}NaO_3Se$ 270.9849, found 270.9827.

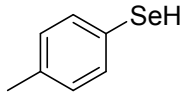
Synthesis of 2-methylbenzeneselenol (2f)



2f

Following the general procedure, 1,2-di-*o*-tolyldiselane **1f** (75 mg, 0.22 mmol) gave 2-methylbenzeneselenol **2f** (49.8 mg, 66%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.37 (1H, s, SeH), 2.36 (3H, s, CH_3), 7.02 (1H, ap t, *J*=7.4 Hz), 7.12-7.19 (2H, m), 7.46 (1H, ap d, *J*=7.7 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 23.0, 126.7, 126.9, 130.2, 133.2, 138.1. **^{77}Se NMR** (76 MHz, $CDCl_3$) δ (ppm): 63.4. **HRMS** m/z calcd for C_7H_8NaSe 194.9689, found 194.9707.

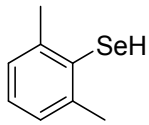
Synthesis of 4-methylbenzeneselenol (2g)



2g

Following the general procedure, 1,2-di-*p*-tolyldiselane 1g (75 mg, 0.22 mmol) gave 4-methylbenzeneselenol **2g** (45.3 mg, 60%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.47 (1H, s, SeH), 2.31 (3H, s, CH_3), 7.04 (2H, ap. D, ls=8.0 Hz), 7.36 (2H, ap.d, ls=8.0 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 21.0, 120.1, 130.1, 133.0, 136.1. **^{77}Se NMR** (76 MHz, $CDCl_3$) δ (ppm): 67.8. **HRMS** m/z calcd for C_7H_8NaSe 194.9689, found 194.9711.

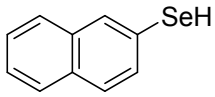
Synthesis of 2,6-dimethylbenzeneselenol (2h)



2h

Following the general procedure, 1,2-bis(2,6-dimethylphenyl)diselane **1h** (75 mg, 0.20 mmol) gave 2,6-dimethylbenzeneselenol **2h** (58.8 mg, 78%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.37 (1H, s, SeH); 2.39 (6H, s); 7.01-7.07 (3H, m). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 24.4; 125.8; 127.7; 138.1. **^{77}Se NMR** (76 MHz, $CDCl_3$) δ (ppm): 38.6. **HRMS** m/z calcd for $C_8H_{10}NaSe$ 208.9845, found 208.9863.

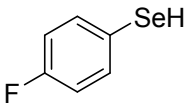
Synthesis of naphthalene-2-selenol (2i)



2i

Following the general procedure, 1,2-di(naphthalen-2-yl)diselane **1i** (75 mg, 0.18 mmol) gave naphthalene-2-selenol **2i** (63.3 mg, 84%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.69 (1H, s, SeH), 7.44-7.51 (3H, m), 7.68-7.74 (2H, m), 7.78-7.82 (1H, m), 7.94 (1H, s). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 122.0, 125.8, 126.6, 126.9, 127.8, 128.6, 130.6, 131.0, 131.8, 134.1. **HRMS** m/z calcd for $C_{10}H_8NaSe$ 230.9689, found 230.9702.

Synthesis of 4-fluorobenzeneselenol (2j)

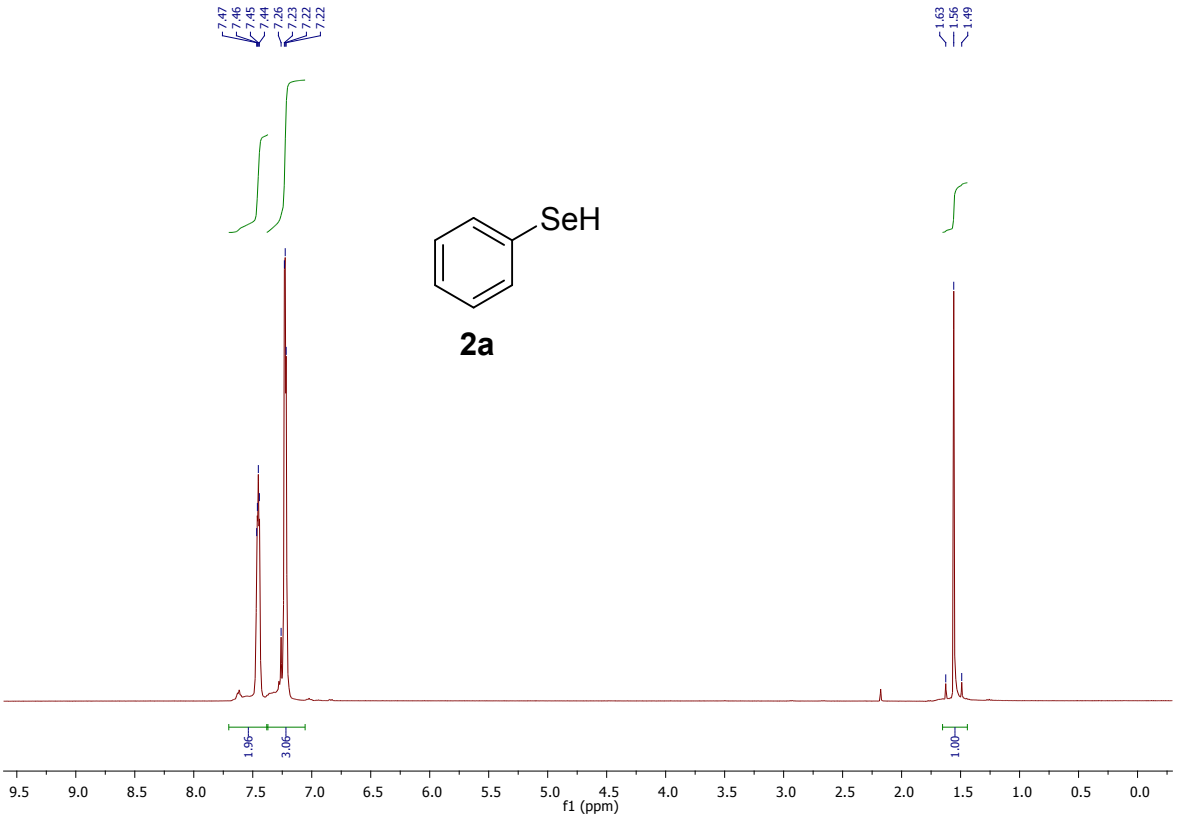


2j

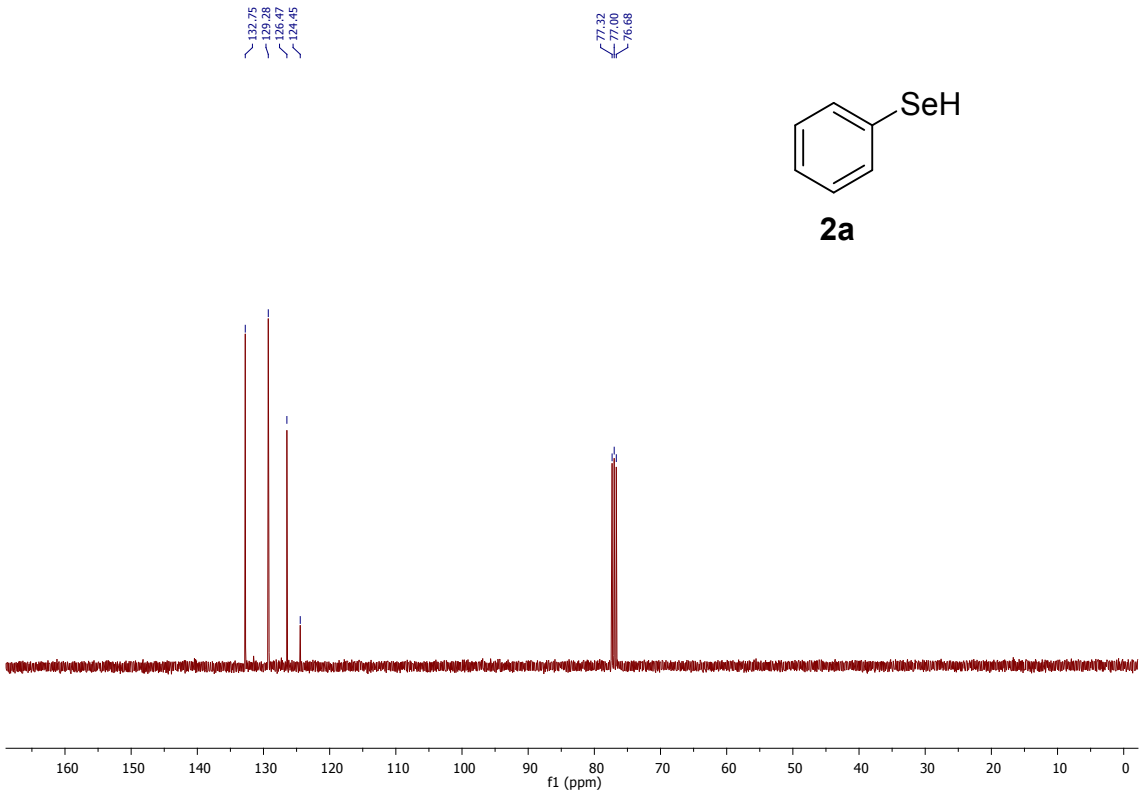
Following the general procedure, 1,2-bis(4-fluorophenyl)diselane **1l** (75 mg, 0.22 mmol) gave 4-fluorobenzeneselenol **2l** (48.3 mg, 64%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.55 (1H, s, SeH); 6.89-7.03 (2H, m), 7.40-7.47 (2H, m). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 116.4 (d, $^2J_{C\text{-}F}$=21.7 Hz), 118.0, 135.2 (d, $^3J_{C\text{-}F}$=7.8 Hz), 162.2 (d, $^1J_{C\text{-}F}$=246.4 Hz). **HRMS** m/z calcd for C_6H_5FNaSe 198.9438, found 198.9419.

NMR Spectra of synthesised compounds

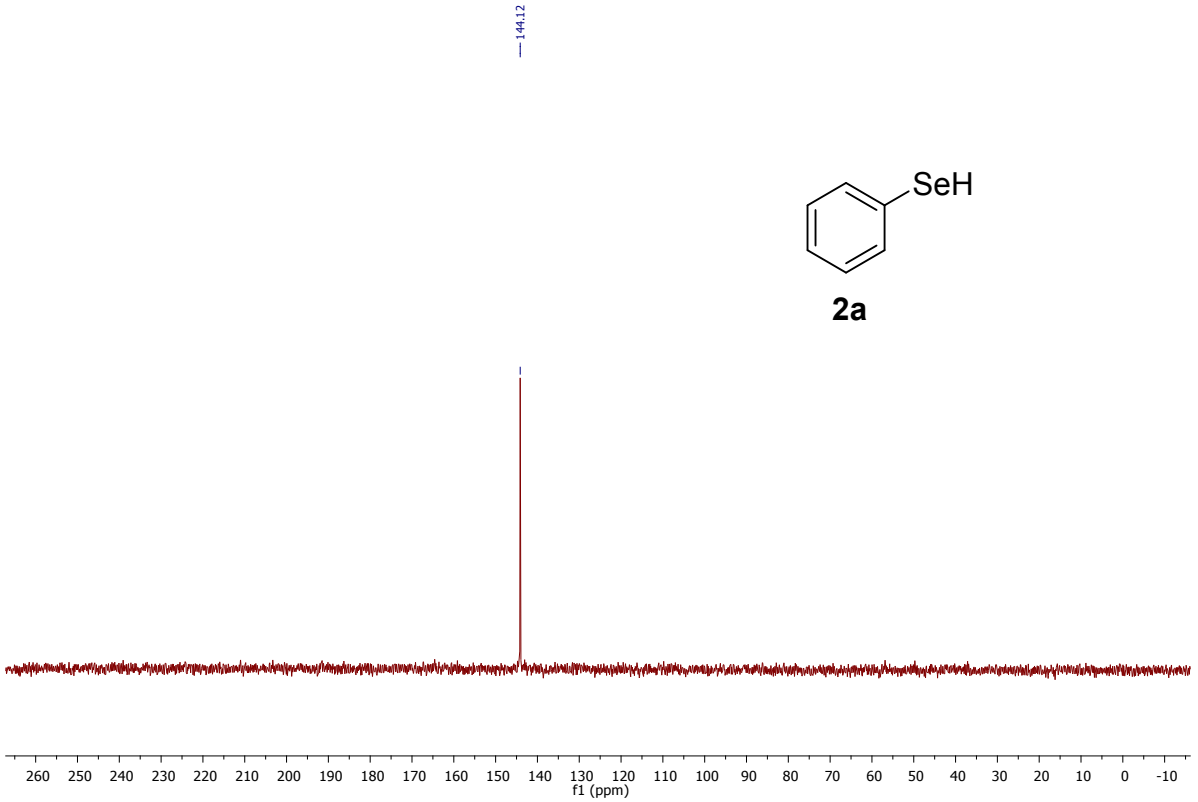
^{1}H NMR spectrum of compound **2a** ($CDCl_3$, 400 MHz)



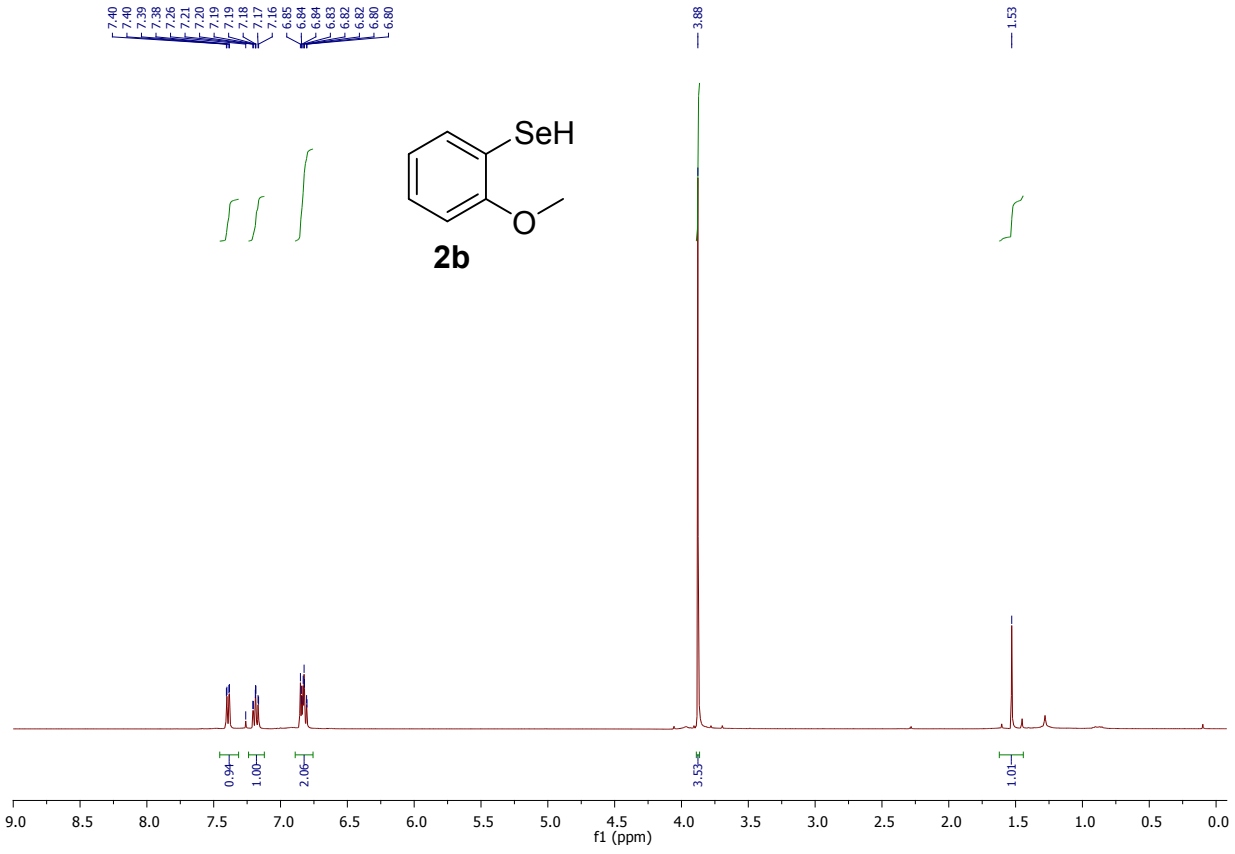
^{13}C NMR spectrum of compound **2a** ($CDCl_3$, 100 MHz)



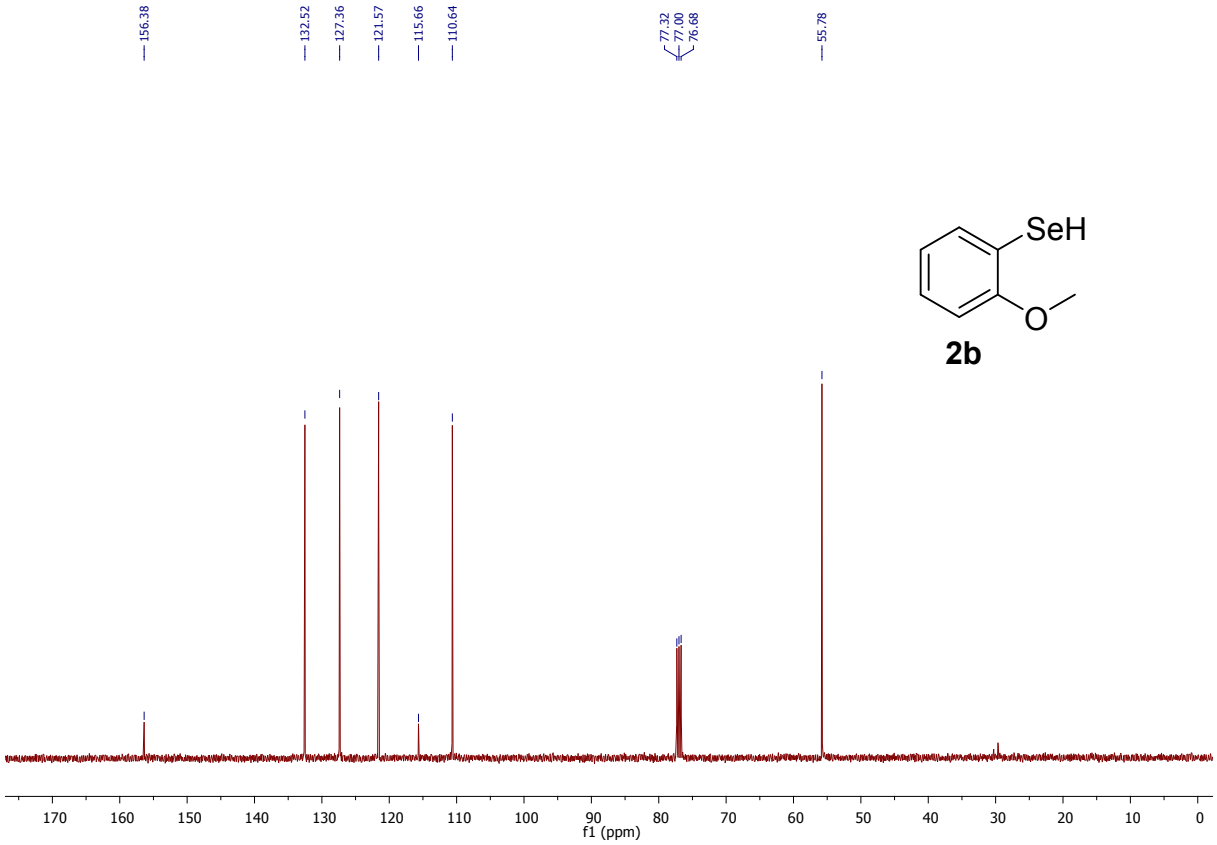
^{77}Se NMR spectrum of compound **2a** ($CDCl_3$, 76 MHz)



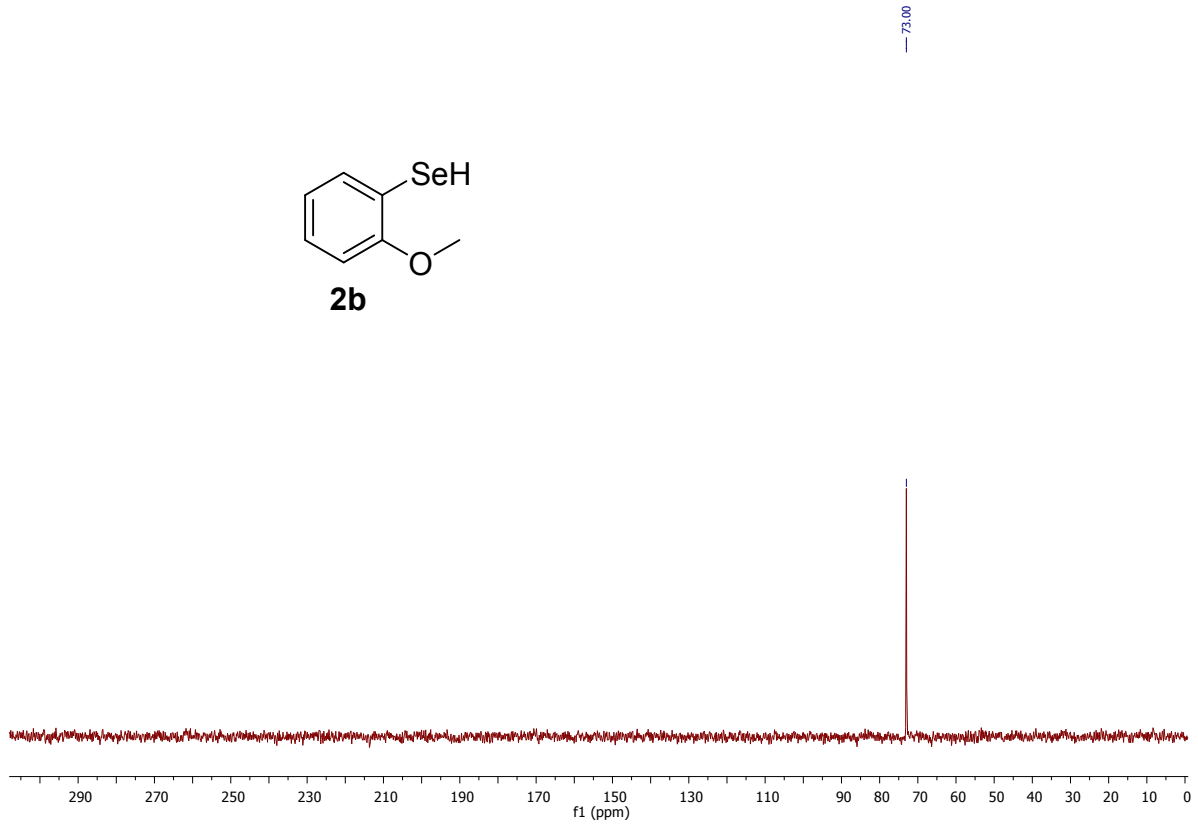
^{1}H NMR spectrum of compound **2b** ($CDCl_3$, 400 MHz)



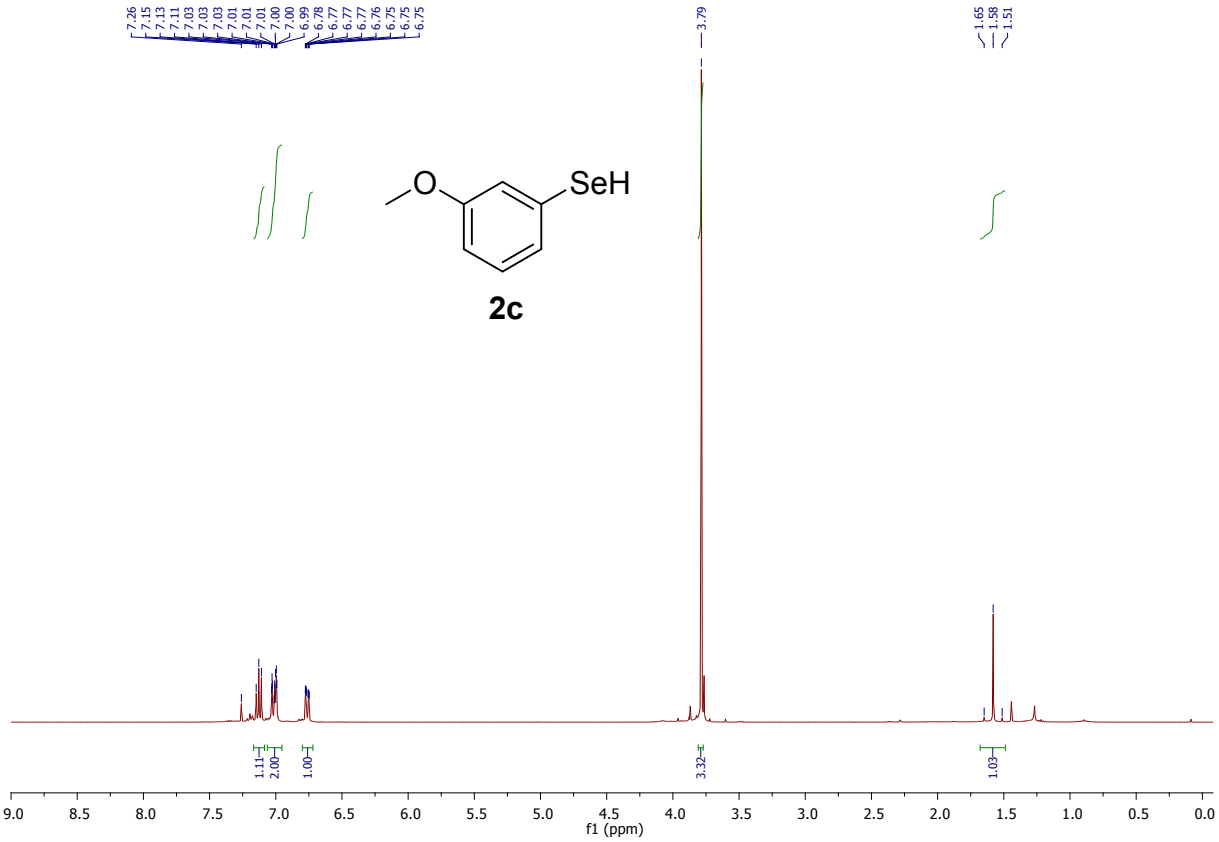
^{13}C NMR spectrum of compound **2b** ($CDCl_3$, 100 MHz)



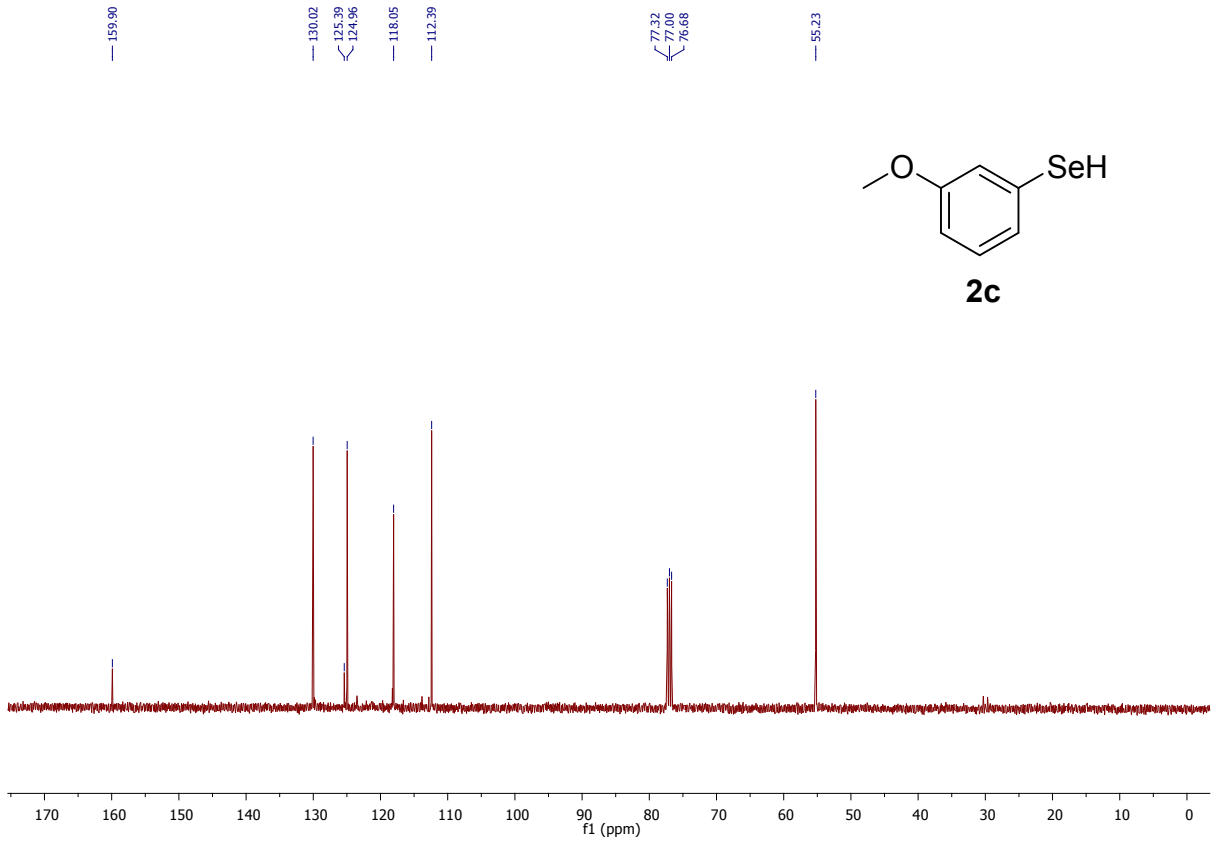
^{77}Se NMR spectrum of compound **2b** ($CDCl_3$, 76 MHz)



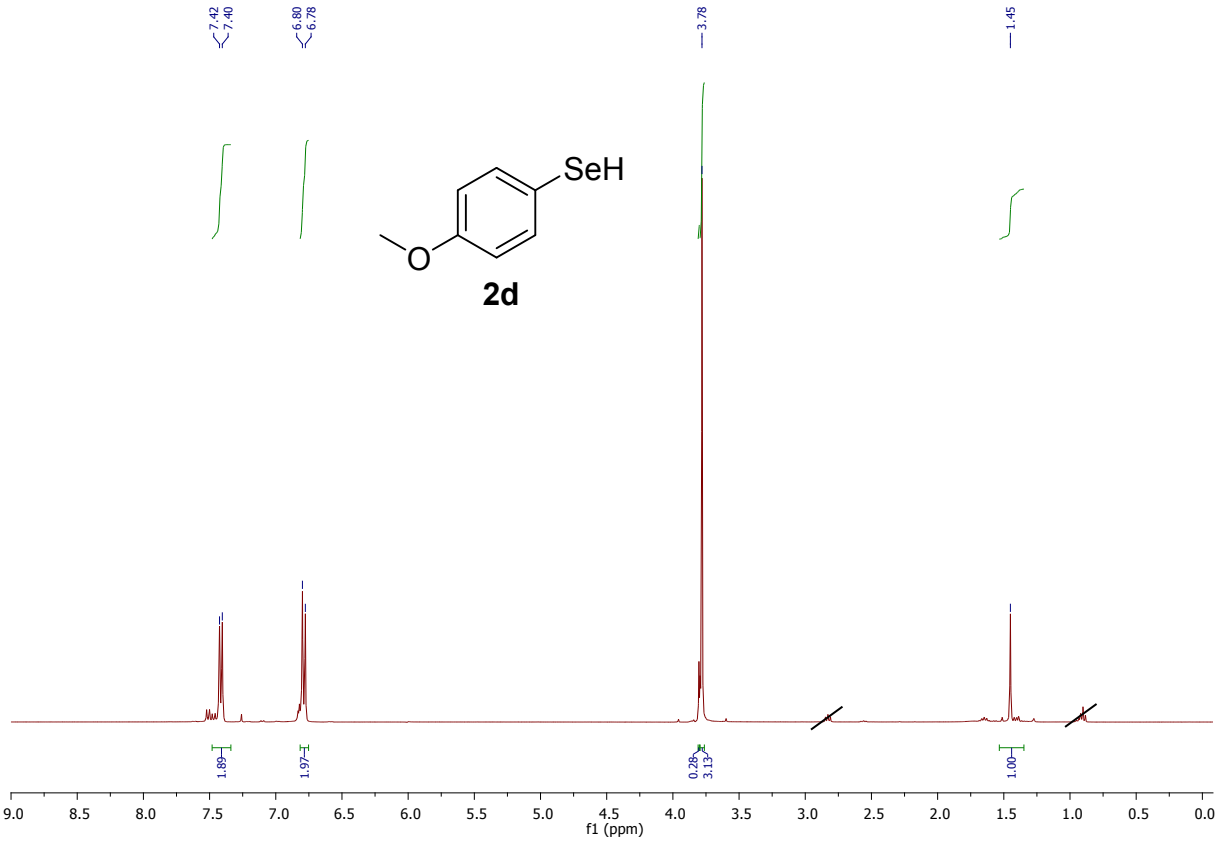
^{1}H NMR spectrum of compound **2c** ($CDCl_3$, 400 MHz)



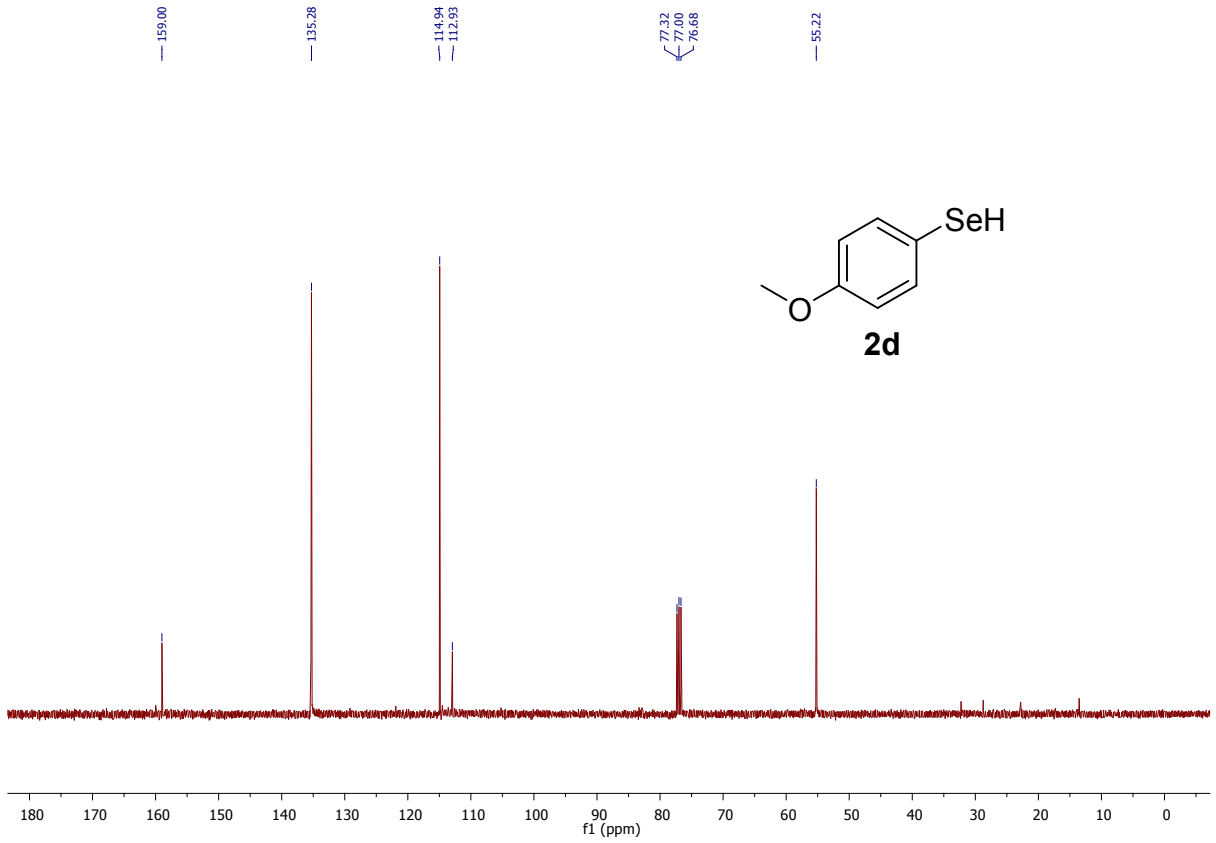
^{13}C NMR spectrum of compound **2c** ($CDCl_3$, 100 MHz)



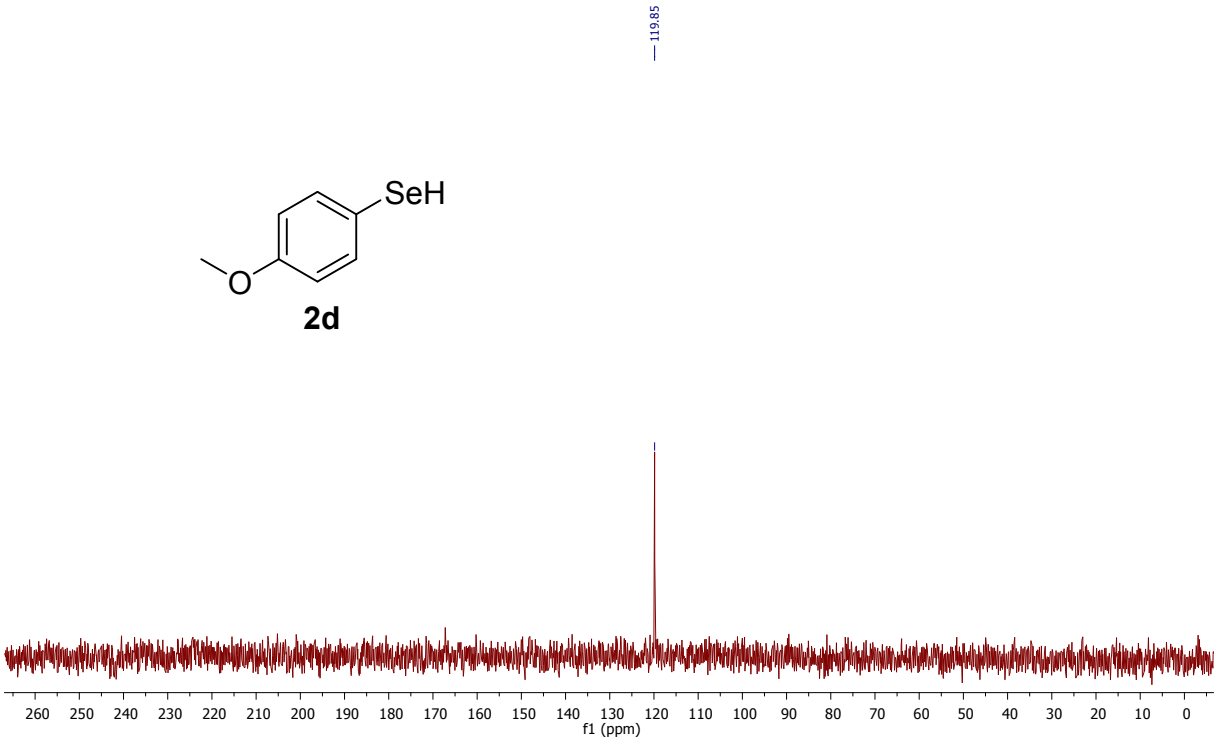
^{1}H NMR spectrum of compound **2d** ($CDCl_3$, 400 MHz)



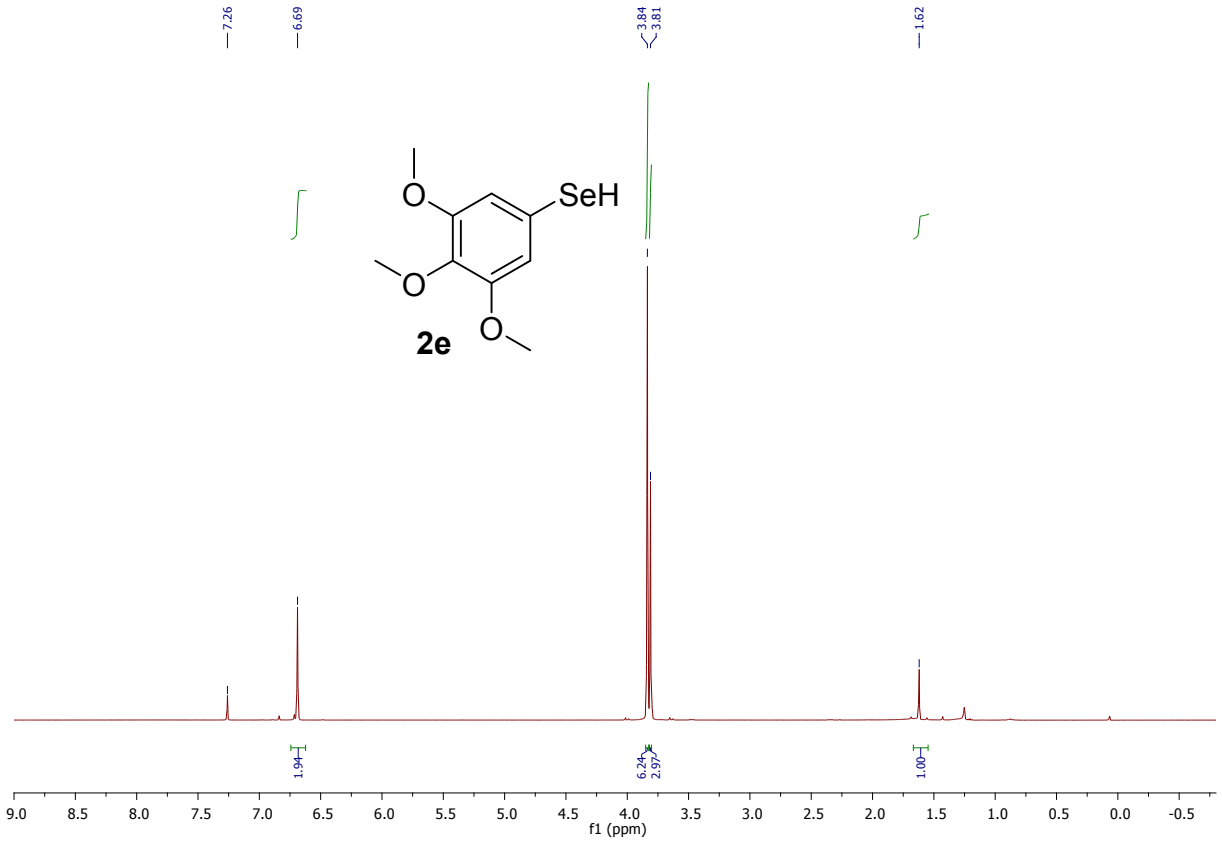
^{13}C NMR spectrum of compound **2d** ($CDCl_3$, 100 MHz)



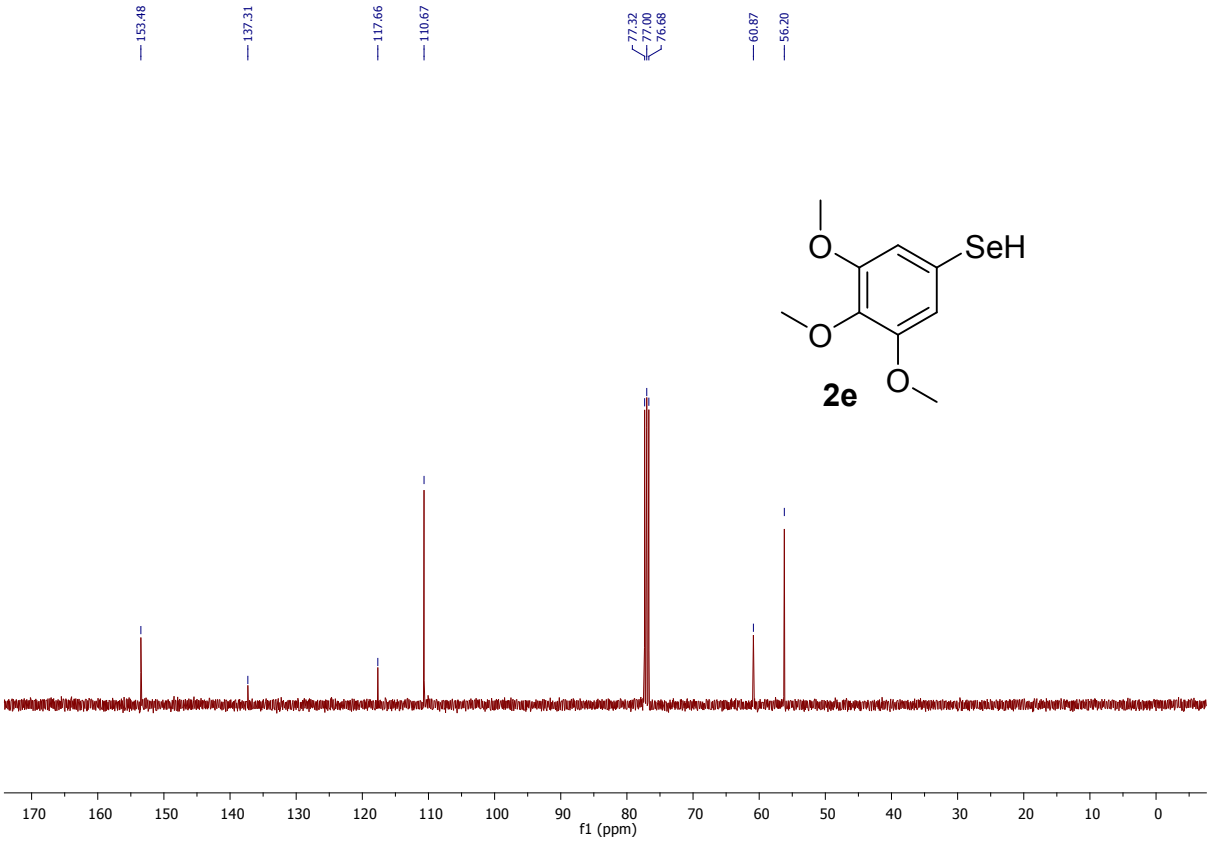
^{77}Se NMR spectrum of compound **2d** ($CDCl_3$, 76 MHz)



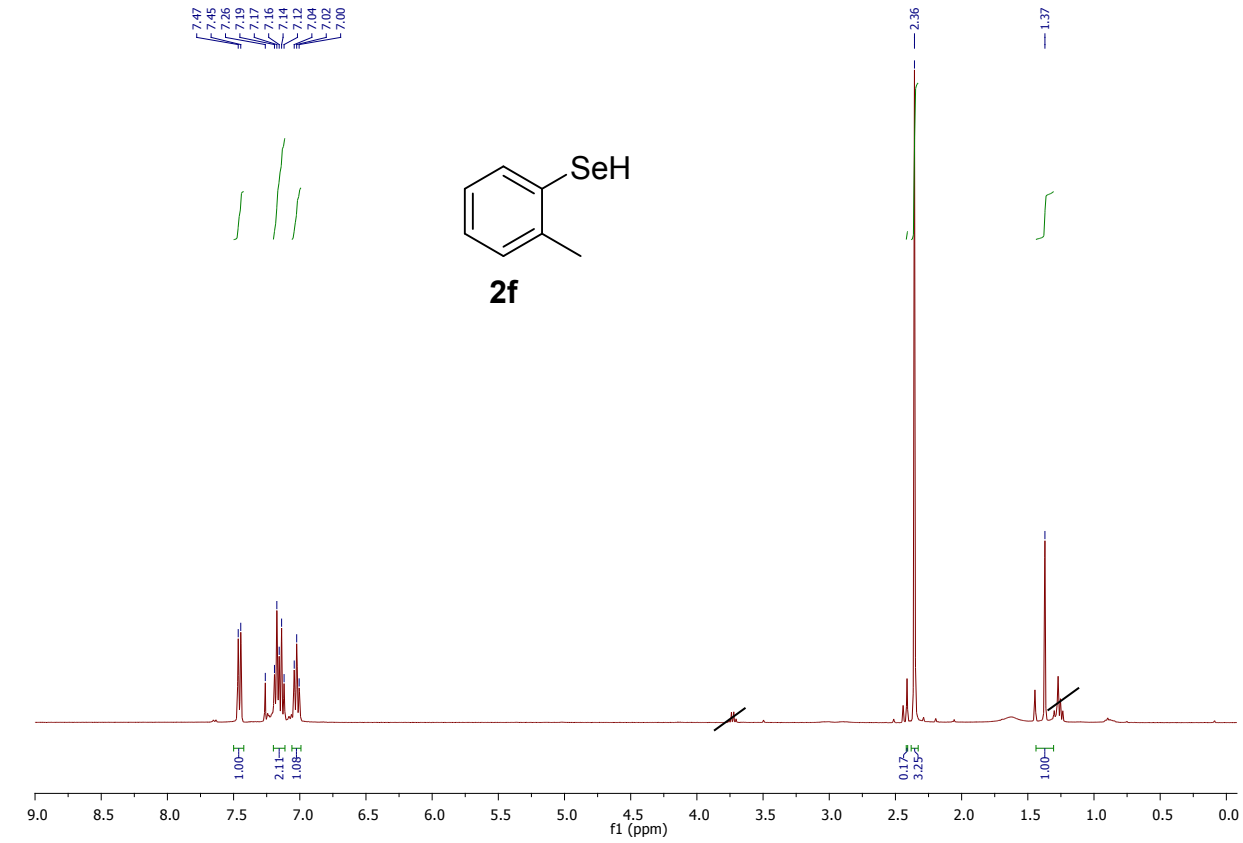
^{1}H NMR spectrum of compound **2e** ($CDCl_3$, 400 MHz)



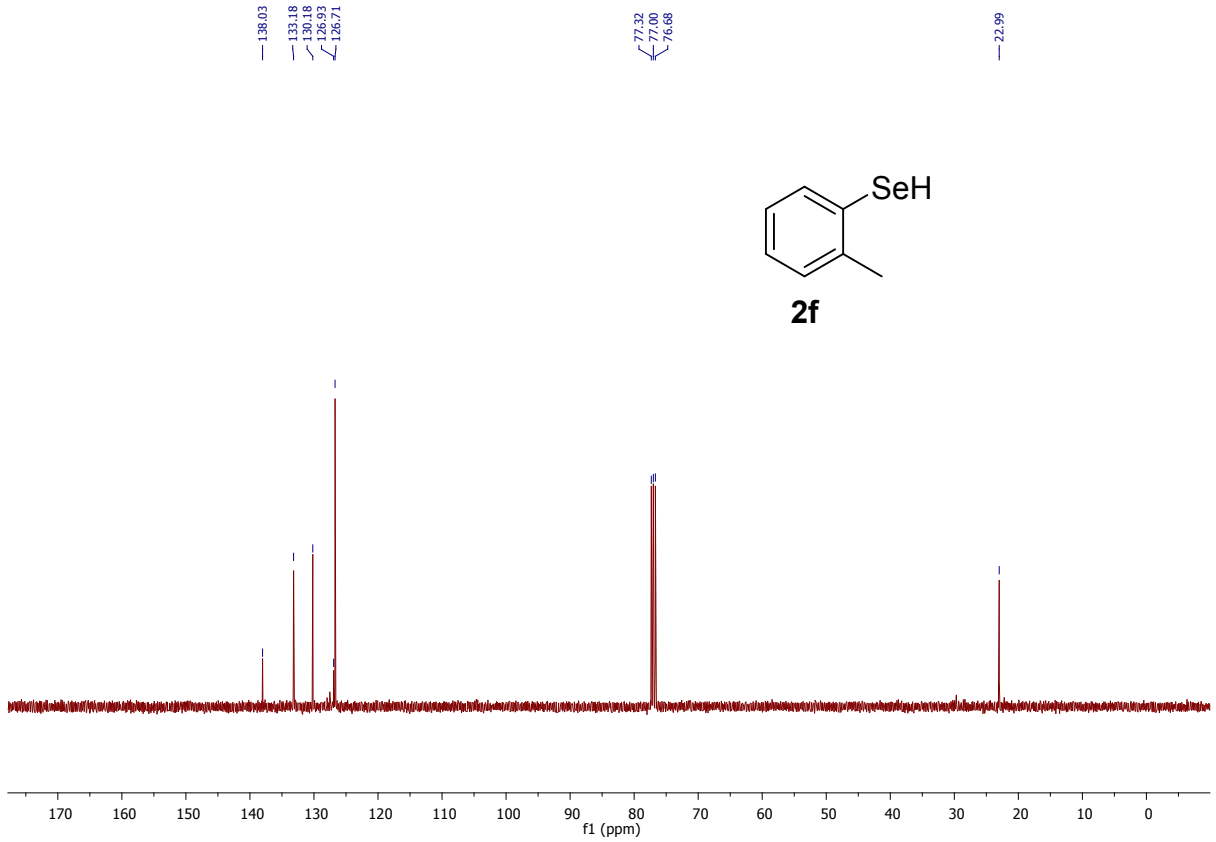
^{13}C NMR spectrum of compound **2e** ($CDCl_3$, 100 MHz)



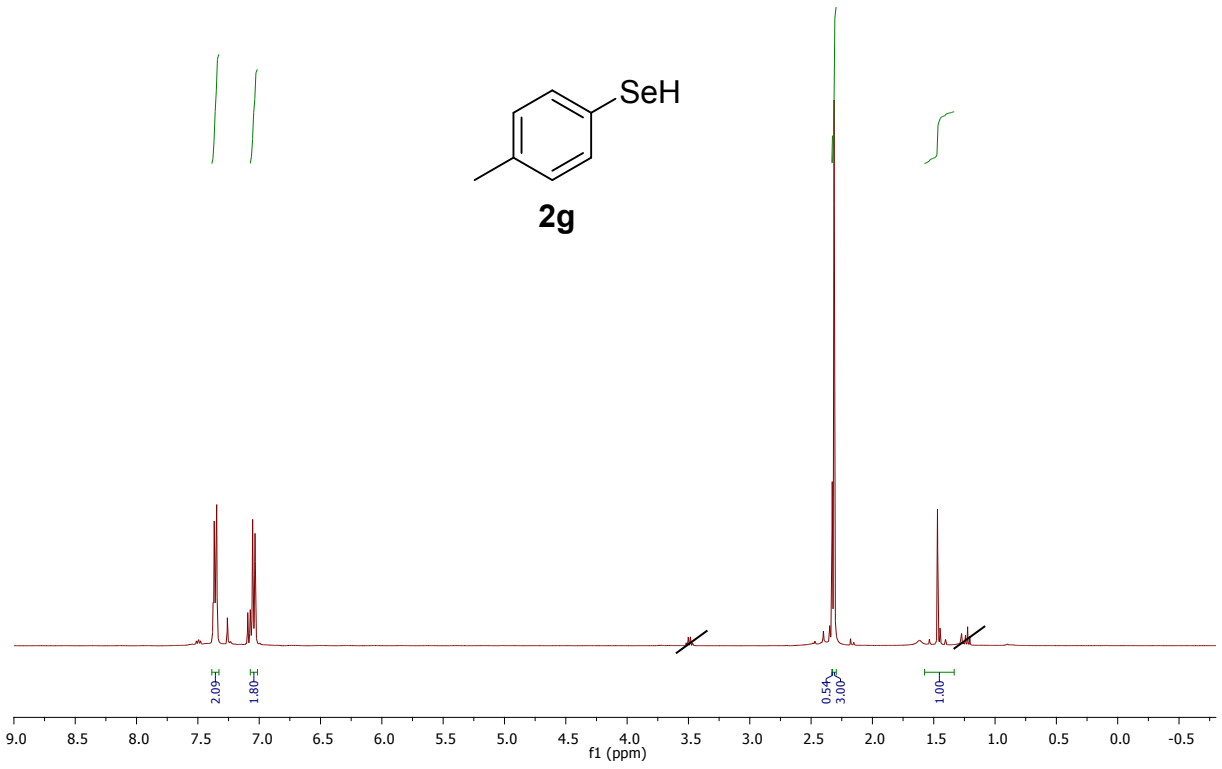
^{1}H NMR spectrum of compound **2f** ($CDCl_3$, 400 MHz)



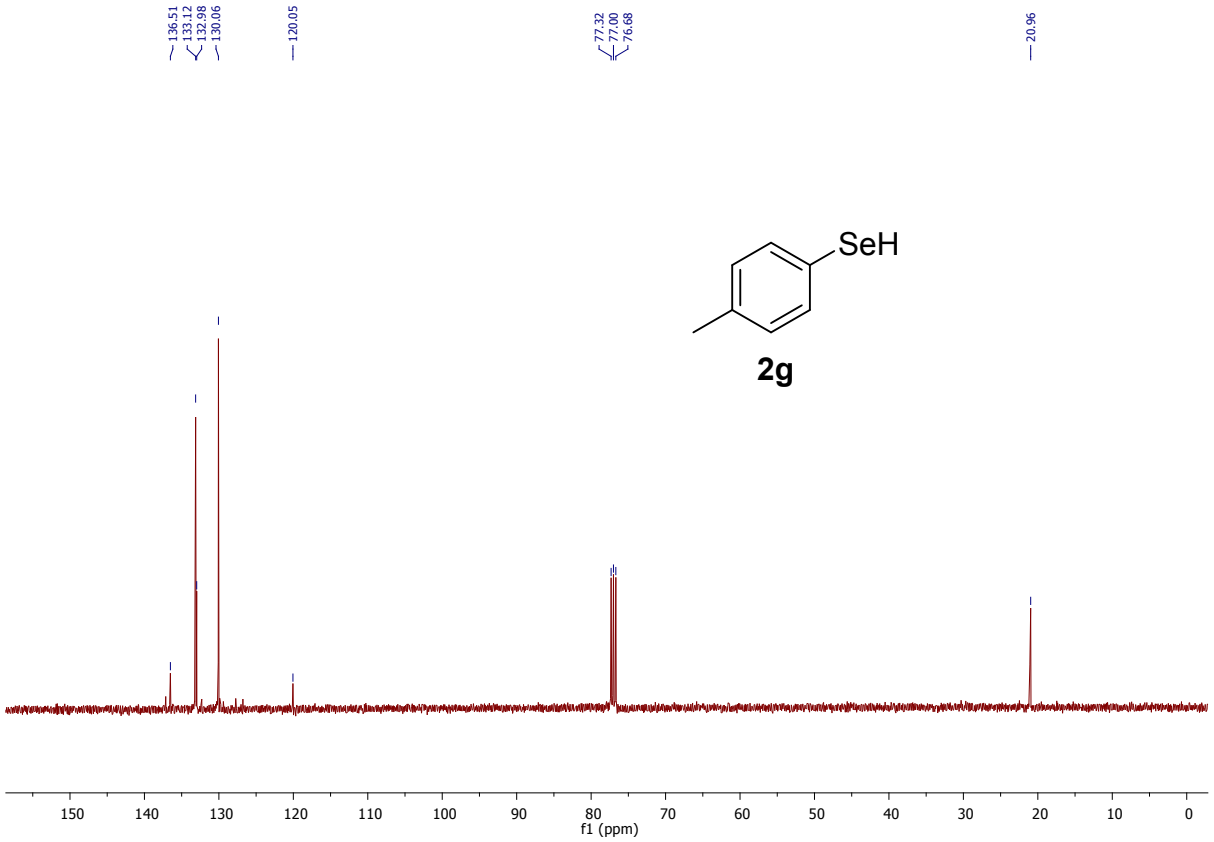
^{13}C NMR spectrum of compound **2f** ($CDCl_3$, 100 MHz)



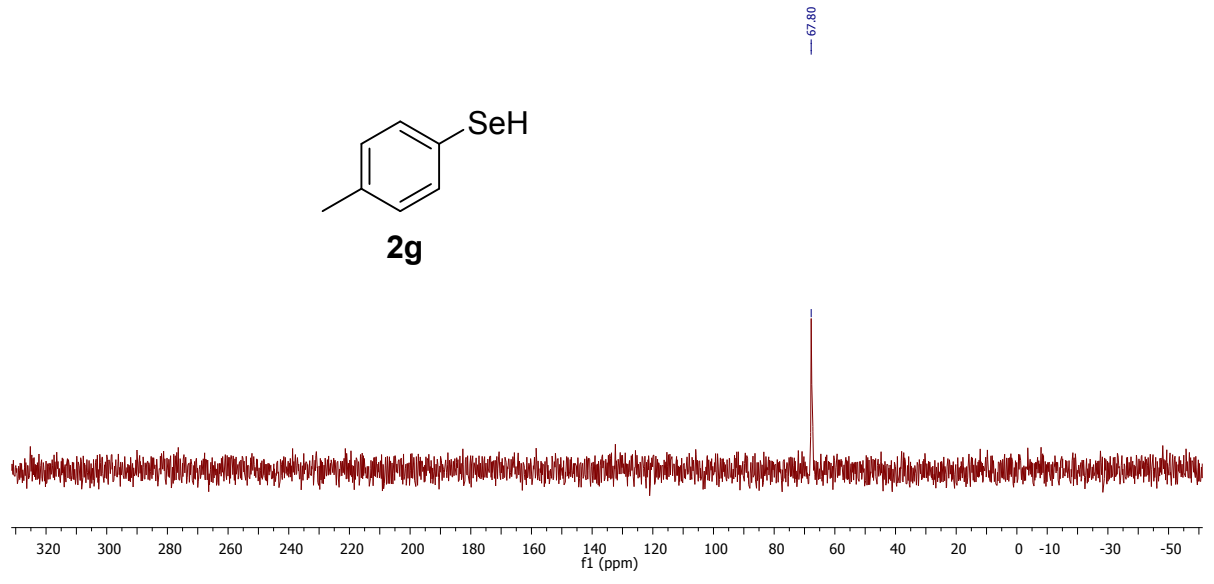
^{1}H NMR spectrum of compound **2g** ($CDCl_3$, 400 MHz)



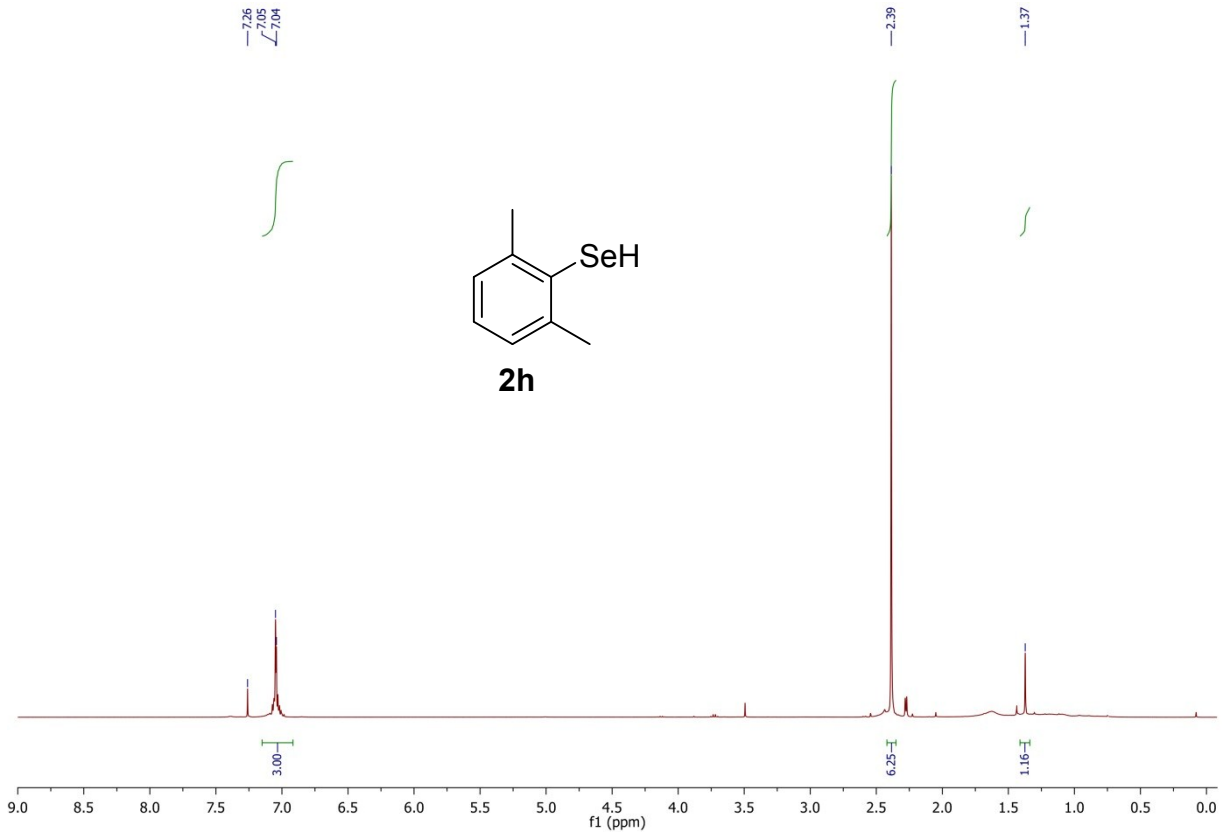
^{13}C NMR spectrum of compound **2g** ($CDCl_3$, 100 MHz)



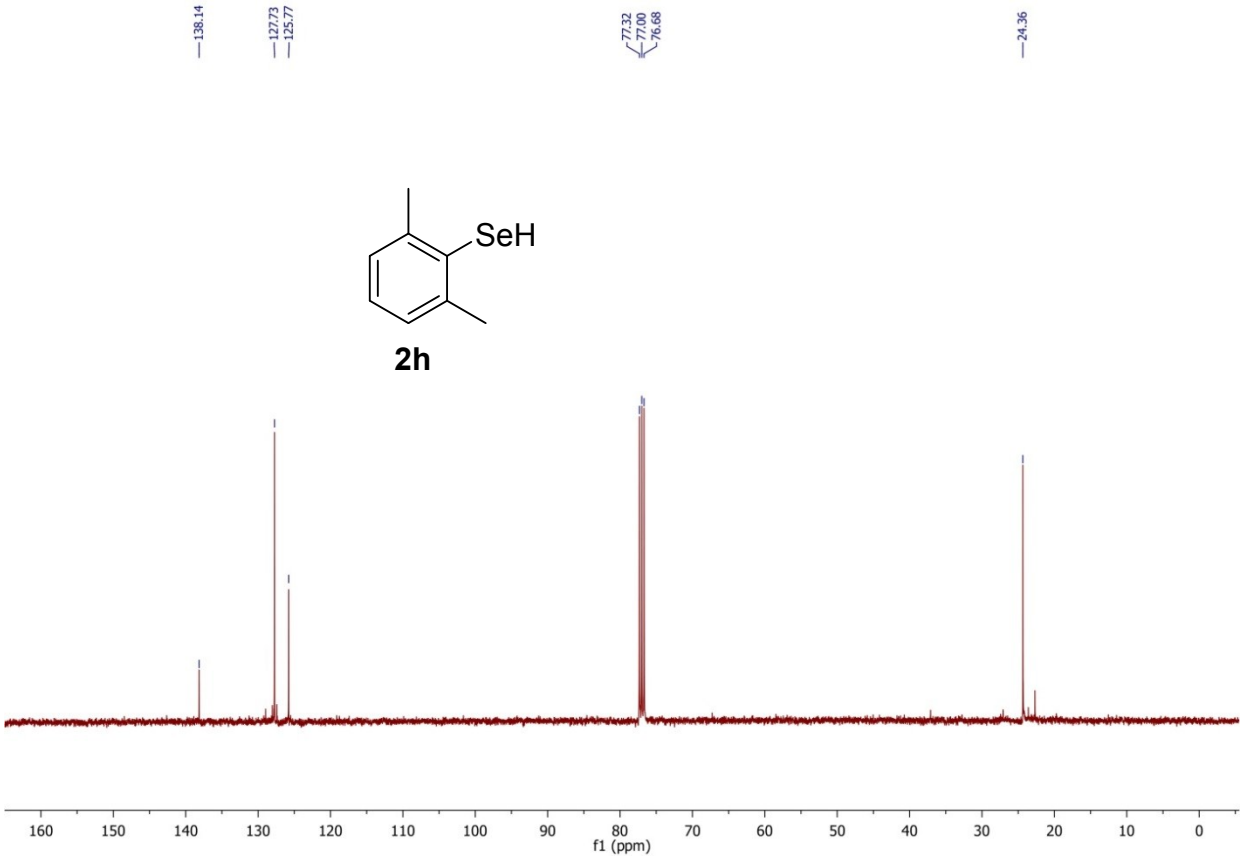
^{77}Se NMR spectrum of compound **2g** ($CDCl_3$, 76 MHz)



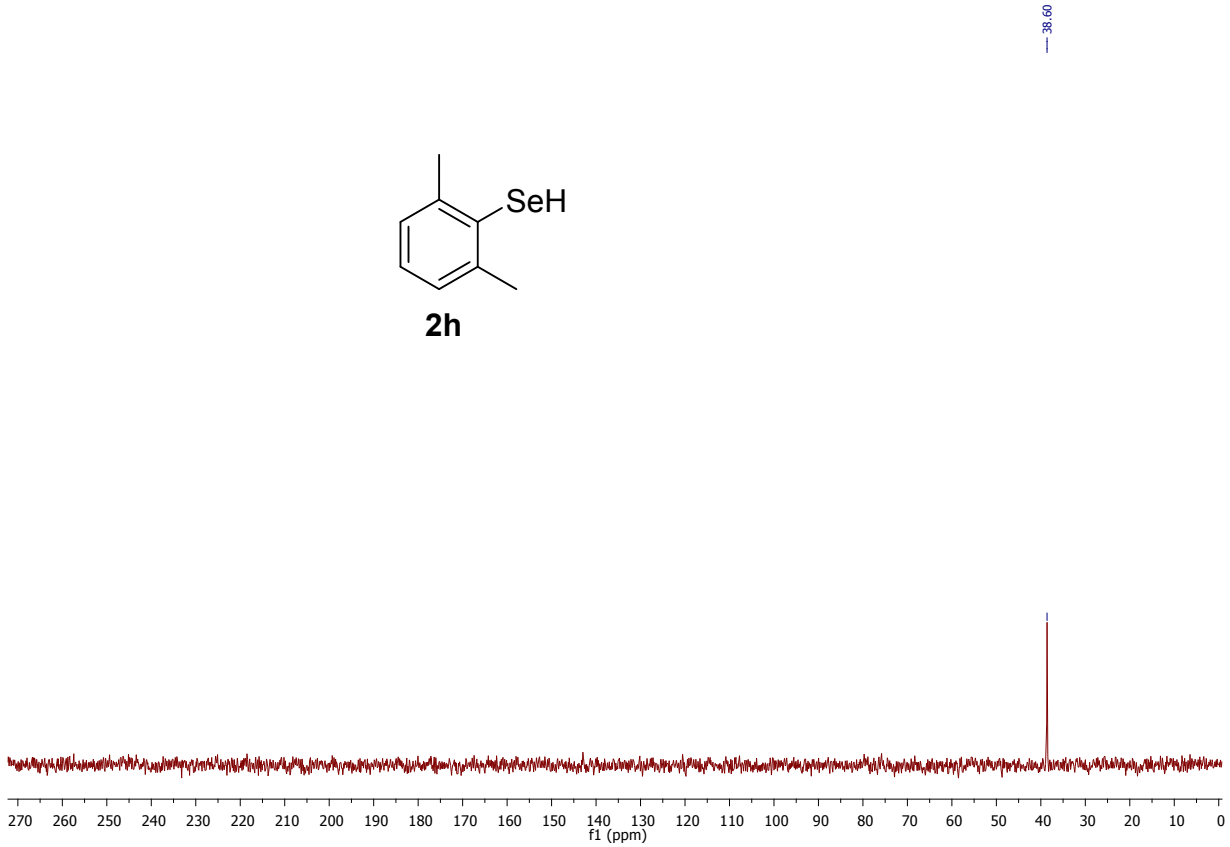
^{1}H NMR spectrum of compound **2h** ($CDCl_3$, 400 MHz)



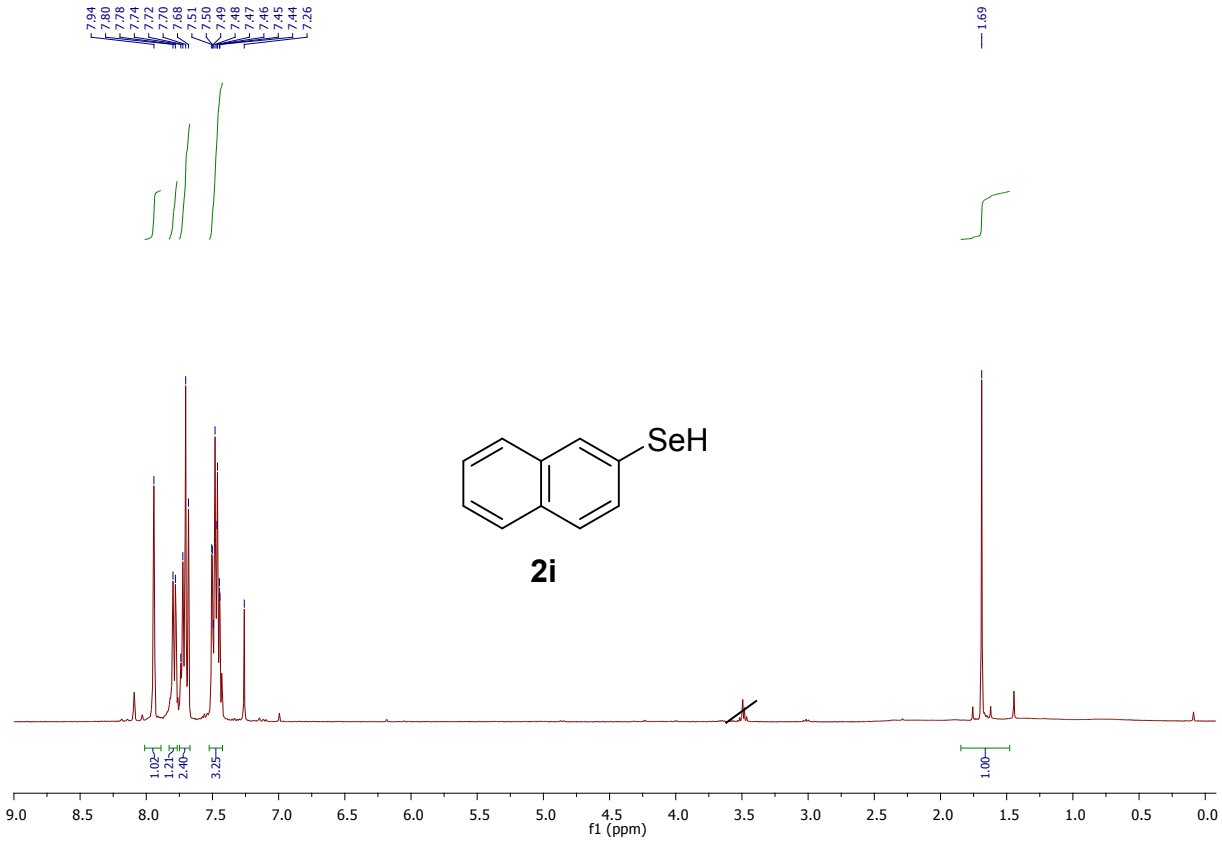
^{13}C NMR spectrum of compound **2h** ($CDCl_3$, 100 MHz)



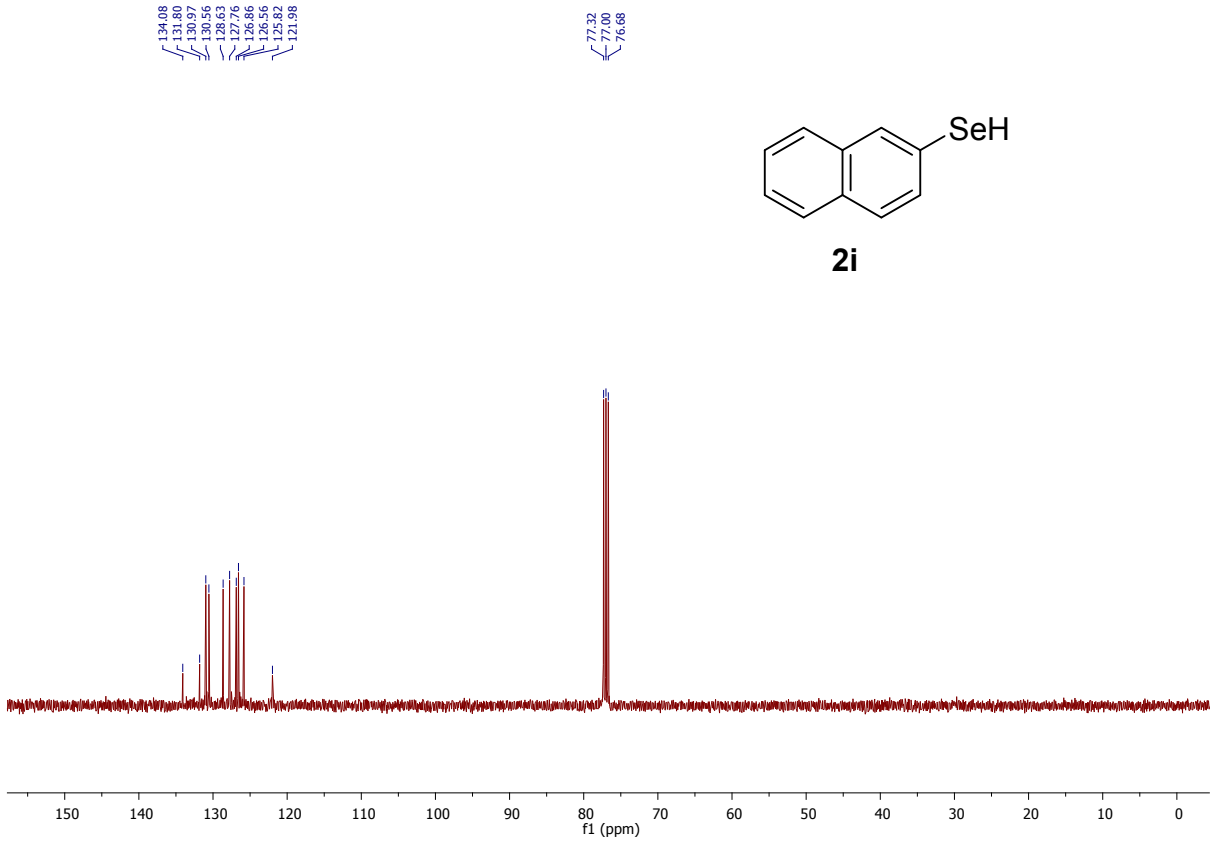
^{77}Se NMR spectrum of compound **2h** ($CDCl_3$, 76 MHz)



^{1}H NMR spectrum of compound **2i** ($CDCl_3$, 400 MHz)



^{13}C NMR spectrum of compound **2i** ($CDCl_3$, 400 MHz)



Carbonic anhydrase inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[2] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mMHepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation ($[IC_{50}=K_i(1+[S]/K_m)]$), as reported earlier,[3] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[3]

Data inhibition of diselenide 1a-j

Table S2 Inhibition data of human CA isoforms I, II, VII and IX with compounds **1a-j** and **AAZ** by a stopped flow CO_2 hydrase assay.[2]

	K_I (μM)*			
Cmp	**hCA I**	**hCAII**	**hCA VII**	**hCA IX**
1a	>100	>100	>100	>100
1b	>100	>100	>100	>100
1c	>100	>100	>100	>100
1d	>100	>100	>100	>100
1e	>100	>100	>100	>100
1f	>100	>100	>100	>100
1g	>100	>100	>100	>100
1h	>100	>100	>100	>100
1i	>100	>100	>100	>100
1j	>100	>100	>100	>100
AAZ	0.25	0.012	0.002	0.026

* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5-10 % of the reported values).

Crystallization and X-ray data collection

Crystals were obtained using the hanging drop vapor diffusion method using 24 well Linbro plate. 2 µl of 10 mg/ml solution of hCA I in Tris-HCl 20 mM pH 9.0 were mixed with 2 µl of a solution of 28-31% PEG4000, 0.2 M Sodium acetate, 0.1 M Tris pH 8.5-9.0 and were equilibrated against the same solution at 296 K. Crystals of the protein grew in fifteen days. Afterwards hCAI crystals were soaked in 5mM inhibitor solution for 3 days.

The crystals were flash-frozen at 100K using a solution obtained by adding 15% (v/v) glycerol to the mother liquor solution as cryoprotectant. Data on crystals of the complexes were collected using synchrotron radiation at the ID23-2 beamline at ESRF (Grenoble, France) with a wavelength of 0.873 Å and a PILATUS3 2M Dectris CCD detector.

2 µl of 0.8 mM solution of hCA II in Tris-HCl pH=8.0 were mixed with of a solution of 1.5, 1.6 and 1.7 M sodium citrate, 50 mM Tris pH 8.0 and were equilibrated against 500 µl of the same solution at 296 K. Crystals of the complexes grew in a few days. hCAII crystals were soaked in 5mM inhibitor solution for 2 days. The crystals were flash-frozen at 100K using a solution obtained by adding 25% (v/v) glycerol to the mother liquor solution as cryoprotectant. Data on crystals of the complexes were collected using synchrotron radiation at the ID-23-2 beamline at ESRF (Grenoble, France) with a wavelength of 0.873 Å and a PILATUS3 2M Dectris CCD detector. Data were integrated and scaled using the program XDS.[4]

Structure determination

The crystal structure of hCA I (PDB accession code: 1JV0) and hCA II (PDB accession code: 4FIK) without solvent molecules and other heteroatoms was used to obtain initial phases of the structures using Refmac5.[5] 5% of the unique reflections were selected randomly and excluded from the refinement data set for the purpose of Rfree calculations. The initial |Fo - Fc| difference electron density maps unambiguously showed the inhibitor molecules. Atomic models for inhibitors were calculated and energy minimized using the program JLigand 1.0.40.[6] Refinements proceeded using normal protocols of positional, isotropic atomic displacement parameters alternating with manual building of the models using COOT.[7] Solvent molecules were introduced automatically using the program ARP.[8] The quality of the final models were assessed with COOT and RAMPAGE.[9] Atomic coordinates were deposited in the Protein Data Bank (PDB accession code: 6HWZ and 6HX5). Graphical representations were generated with Chimera.[10]

Summary of Data Collection and Atomic Model Refinement Statistics.

Table S3 Summary of Data Collection and Atomic Model Refinement Statistics

	HCAII + 2a	HCAI + 2a
PDB ID	6HX5	6HWZ
Wavelength (Å)	0.873	0.873
Space Group	P21	P212121
Unit cell (a, b, c, α, β, γ) (Å,°)	42.31, 41.44, 72.11, 90.0, 104.3, 90.0	62.38, 71.52, 120.69, 90.0, 90.0, 90.0
Limiting resolution (Å)	41.00 -1.44 (1.58 – 1.44)	28.59 – 1.64 (1.73 - 1.64)
Unique reflections	43339 (9882)	66390 (9064)
Rsym (%)	9.9 (56.8)	15.0 (481.6)
Rmeas (%)	10.9 (65.0)	15.5 (499.3)
Redundancy	5.37 (4.17)	15.12 (13.87)
Completeness overall (%)	98.1 (92.9)	97.4 (83.6)
<I/σ(I)>	10.93 (2.85)	10.00 (0.40)
CC (1/2)	99.6 (84.3)	99.9 (28.4)
Refinement statistics		
Resolution range (Å)	41.0 – 1.44	28.59 – 1.64
Unique reflections, working/free	41176/2154	62499/3264
Rfactor (%)	18.82	22.45
Rfree(%)	20.98	26.91
r.m.s.d. bonds(Å)	0.018	0.0144
r.m.s.d. angles (°)	1.912	1.669
Ramachandran statistics (%)		
Most favored	96.9	96.5
additionally allowed	3.1	3.5
outlier regions	0.0	0.0
Average B factor (Å^2)		
All atoms	15.255	38.962
inhibitors	14.149	36.612
solvent	23.008	47.644

***In silico* studies**

2OSF[11] and 5FL4[12] crystal structures used for computational studies were prepared according to the Protein Preparation module in Maestro (residue numbers for hCA II according to 5LJT)[13] - Schrödinger suite, assigning bond orders, adding hydrogens, deleting water molecules, and optimizing H-bonding networks. Finally, energy minimization with a root mean square deviation (RMSD) value of 0.30 was applied using an Optimized Potentials for Liquid Simulation (OPLS-2005) force field.[14],[15] Grids for docking were centred in the centroid of the complexed ligand. Docking studies were carried out with the program Glide[14f] using the standard precision (SP) mode. 3D ligand structures were prepared by Maestro.[14a] QM geometry optimization and atomic electrostatic charges computation were performed with Jaguar[14g] fitting them to an electrostatic potential calculated at the B3LYP/cc/pvtz-f level of theory. ESP atomic charges were used in docking simulations. OPLS-2005 force field was modified according to Schrödinger to enable negatively charged selenate moieties for docking procedure in Glide. Indeed, the OPLS FF does not support parameters for the Lewis structure of selenates. The best docking pose for each compound was submitted to DFT (B3LYP/LACVP*$^{+}$) calculations with Jaguar within both built model systems. The QM optimized ligands were rescored in the complete hCA macromolecular environment with Glide.

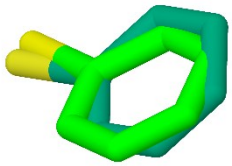


Figure S1. Superimposition of **2a** crystallographic (light green) and docked pose (dark green) – RMDS 0.54 Å.

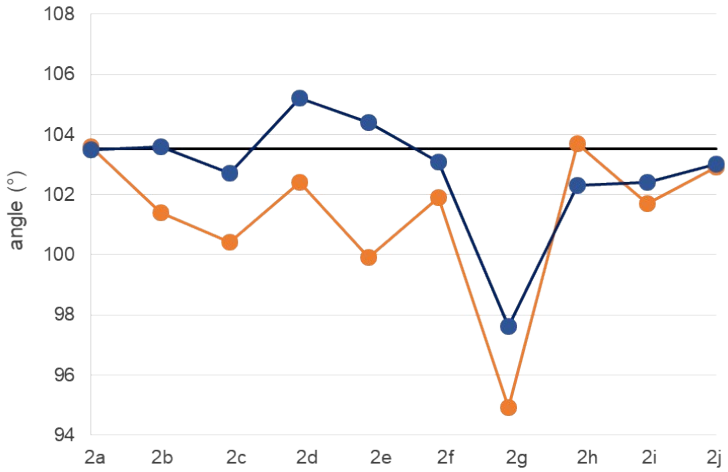
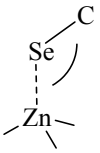


Figure S2 Deviation of the Zn-Se-C (°) angle from the CSD search reference value (103.53 °) for compounds 2a-j within hCA II (orange) and hCA IX (blue) binding site cavity.

Table S4: Se-Zn coordination properties (Se-Zn bond length and the Zn-Se-C angle) of derivatives **2a-j** obtained by docking/QM studies.

	hCA II (2OSF)[11]			hCA IX (5FL4)[12]		
Cmpd	Zn-Se (Å)	Se-C (Å)	Zn-Se-C (°)	Zn-Se (Å)	Se-C (Å)	Zn-Se-C (°)
2a	2,50	1,97	103,6	2.52	1.96	103.5
2b	2.48	1.96	101.7	2.52	1.96	102.4
2c	2.47	1.97	99.9	2.52	1.97	104.4
2d	2.50	1.97	100.4	2.51	1.96	102.7
2e	2.45	1.95	94.9	2.50	1.95	97.6
2f	2.48	1.96	101.9	2.51	1.97	103.1
2g	2,50	1,97	101,4	2.52	1.96	103.6
2h	2.49	1.98	102.9	2.49	1.98	103
2i	2.49	1.97	102.4	2.52	1.96	105.2
2j	2.46	1.96	103.7	2.52	1.96	102.3

Hydrolized Tioxolone (2OSF)	2.18 (Zn-S)	1.76 (S-C)	109.2 (Zn-S-C)
R-Se$^-$ (3WC5)[16]	2.39-2.43	1.98	99.4-101.7
		Se-C (Å)	Zn-Se-C (°)
Reference values from CSD search		1.93	103.53

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