Supporting Information

PIM kinase-responsive microsecond-lifetime photoluminescent probes based on selenium-containing heteroaromatic tricycle

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Content

1. Materials and methods

Solvents and chemicals were purchased from Rathburn and Sigma-Aldrich. They were used without further purification. Fmoc Rink-amide MBHA resin and Fmoc-protected amino acids for peptide synthesis were purchased from Iris Biotech. Fluorescent dyes PromoFluor-647 NHS ester and PromoFluor-555 ester were purchased from PromoKine.

^{1}H and ^{13}C NMR spectra were taken on spectrometers Bruker AC 200P (200 MHz) and Bruker Ascend (700 MHz). High-resolution mass spectra of all synthesized compounds were measured on Thermo Electron LTQ Orbitrap mass spectrometer. NanoDrop 2000c spectrometer (Thermo Scientific) was used for recording UV-Vis absorption spectra and quantification of the products. FluoroMax-4 (HORIBA Jobin Yvon) luminescence spectrometer and Cytation 5 (Biotek) plate reader were used for the measurement of the phosphorescence emission spectra. Peptide purification was performed with Schimadzu LC Solution (Prominence) HPLC system by using a manual injector and a diode array (SPD M20A) detector.

Full length protein kinases, PIM-1, PIM-2, PIM-3 [1,2] and PKAc [3] were produced as described previously.

Binding assays

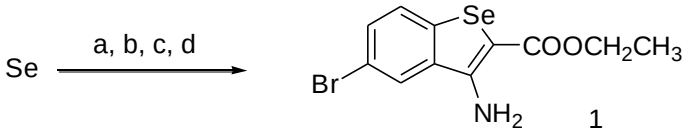
Biochemical assays were performed on black, low-volume, 384-well, nonbonding surface (NBS) microplates (code 3676, Corning) on a PHERAstar (BMG Labtech) plate reader using TRF or FP modules. The concentration of ARC-3157 and ARC-3160 was determined on the NanoDrop 2000c spectrometer (Thermo Scientific) using the molar absorption coefficient of 5700 $M^{-1}cm^{-1}$. The concentration of PromoFluor-555 and PromoFluor-647 labelled compounds were determined using the molar absorption coefficient of 150,000 $M^{-1}cm^{-1}$ and 250,000 $M^{-1}cm^{-1}$, respectively.

The concentration of the active form of the PK and K_D values of the probes were determined in binding assays using either (or both) FA[4] or TRF [5,6] read out. Briefly, the PK was two-fold serial diluted in the assay buffer (50 mM HEPES, 150 mM NaCl, 0.005% Tween 20, 5 mM DTT and 0.5 mg/ml BSA) in a row of wells of a microplate and the probe was added to the dilution series at a concentration of 1 nM, 2 nM or 10 nM, depending on the measurement. Thereafter the plate was incubated at 30 ^{o}C for 20 min and the signal was determined in FA and/or TRF mode, depending on the probe. The signal was plotted against concentration of the PK and the data were fit into equations described previously to calculate concentration of the PK or K_D value of the probe.[4] The probe ARC-3117[7] was used for the determination of

concentration of the active forms of PIM kinases in samples in the binding assay with fluorescence anisotropy read out.

2. Synthesis

Synthesis of 3-amino-5-bromobenzo[b]selenophene-2-carboxylic acid ethyl ester (1)

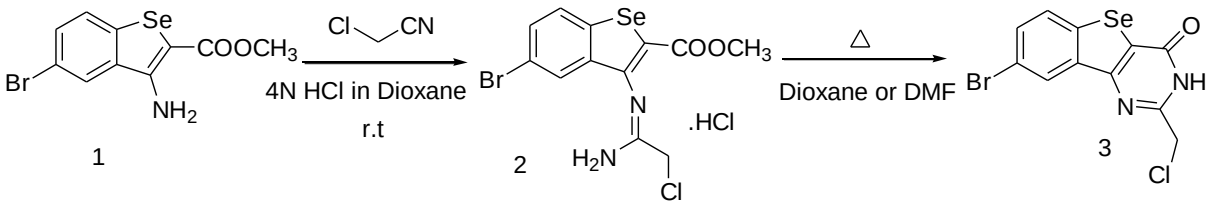


a) $NaBH_4/H_2O$, 0°C, b) 5-Bromo-2-fluorobenzonitrile, DMF, 0°C-20°C
c) $BrCH_2COOCH_2CH_3$, 0°C-20°C, d) 4M NaOH, 0-60°C

Selenium (195 mg, 2.47 mmol) and sodium borohydride (232 mg, 6.14 mmol) were suspended in water (0.9 mL) at 0 °C. A solution of 5-bromo-2-fluorobenzonitrile (156 mg, 0.78 mmol) in DMF (5 mL) was added at 0 °C to the formed suspension of sodium selenide and thereafter the mixture was stirred for 2 h at room temperature. Then ethyl bromoacetate (250 µL, 2.26 mmol) was added dropwise to the reaction mixture at 0 °C and the stirring was continued at room temperature. After 2 h a solution of 4 M sodium hydroxide (300 µL) was added dropwise and the mixture was stirred 3 h at 60 °C. The mixture was poured into cold water and solution was extracted with ethyl acetate. The organic layer was washed with water and dried over sodium sulfate. The solution was filtered and the solvent was evaporated. The residue was purified by column chromatography (chloroform/hexane, 3/2). The blue fluorescent spot with R_f = 0.25 was collected to obtain the product (32 mg, 12%) as a yellowish solid. UV_{max} at 288 nm and at 373 nm (DMF). ESI-HRMS m/z, calcd for $C_{11}H_{10}BrNO_2Se$ $[M+H]^+$ 347.9133, found 347.9127.

^{1}H NMR (200 MHz, $CDCl_3$) δ 1.38 (3H, t, *J* = 7.2 Hz), 4.33 (2H, q, *J* = 7.2 Hz), 5.96 (2H, br), 7.52 (1H, dd, *J* = 8.4 and 1.8 Hz), 7.66 (1H, d, *J* = 8.4 Hz), 7.75 (1H, d, *J* = 1.8 Hz).

^{13}C NMR (50 MHz, $CDCl_3$) δ 14.5, 60.7, 99.7, 118.5, 125.8, 128.0, 131.1, 136.2, 138.5, 149.1, 166.3.



Synthesis of 2-(chloromethyl)-seleno[2,3-d]pyrimidin-4(3H)-one (3)

A suspension of 3-amino-5-bromobenzo[b]selenophene-2-carboxylic acid ethyl ester (0.1 g, 0.3 mmol) in 4 N hydrochloric acid in dioxane (5 mL) was treated with 2-chloroacetonitrile

(37 µl, 0.6 mmol) at room temperature for 3 h and then the solvent was removed. The residual solid was refluxed with dry dioxane for 2 h, collected by filtration, washed with ethanol and dried to give the desired product in 65 % yield. ESI-HRMS m/z, calculated for $C_{11}H_6BrClN_2OSe$ $[M+H]^+$ 376.8590, found 376.8579.

^{1}H NMR (700 MHz, $DMSO_{6d}$) δ 4.66 (2H, s), 7.77 (1H, dd, J = 8.6 and 2.1 Hz), 8.24 (1H, d, J = 8.6 Hz), 8.31 (1H, d, J = 2.1 Hz), 13.17 (1H, br).

^{13}C NMR (234 MHz, $DMSO_{6d}$) δ 42.8, 119.2, 125.8, 127.2, 129.4, 131.7, 138.4, 139.9, 154.0, 155.5, 159.6.

Synthesis of peptide conjugates

Peptide fragments were prepared by using traditional Fmoc solid-phase peptide synthesis on Rink-amide MBHA resin. In general, protected amino acids (3 eq.) were dissolved in DMF and activated with HBTU/HOBt (2.8 eq. each) in the presence of N-methylmorpholine (9 eq.). After 3 minute coupling the solutions were added to the resin. After 1 h shaking the resin was washed 5-fold with DMF. The completeness of each coupling step was monitored with the Kaiser test. The N-terminal Fmoc group was removed with 20 % piperidine solution in DMF (20 min) and thereafter the resin was washed 5 times with DMF.

A solution of 2-(chloromethyl)-seleno[2,3-d]pyrimidin-4(3H)-one (3 eq.) in DMF and DIPEA (9 eq.) was added to N-terminal amino group of the corresponding peptide on the resin and the mixture was stirred slowly at 60 °C for 8 h.

Finally the resin was washed 5-fold with each solvent (DMF, isopropanol, DCE) and dried. The protection groups were removed and the conjugates cleaved from the resin by 2 h treatment with 90 % trifluoroacetic acid, 5 % triisopropylsilane, 5 % water. The conjugates were purified with C18 reversed phase HPLC and lyophilized.

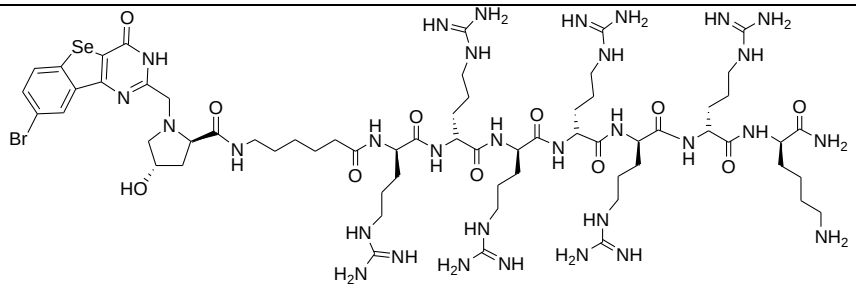
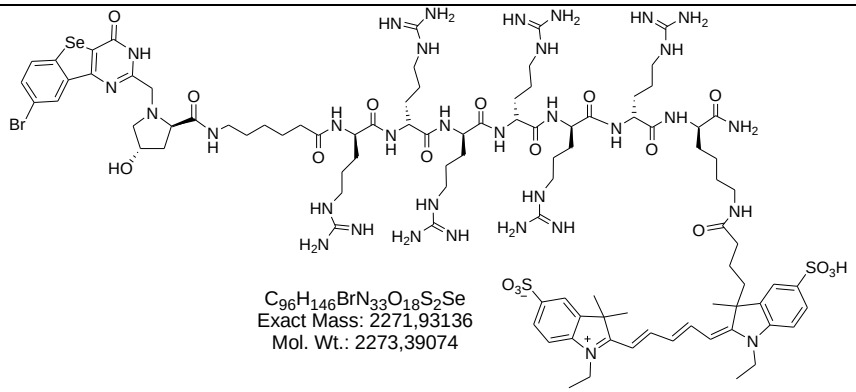
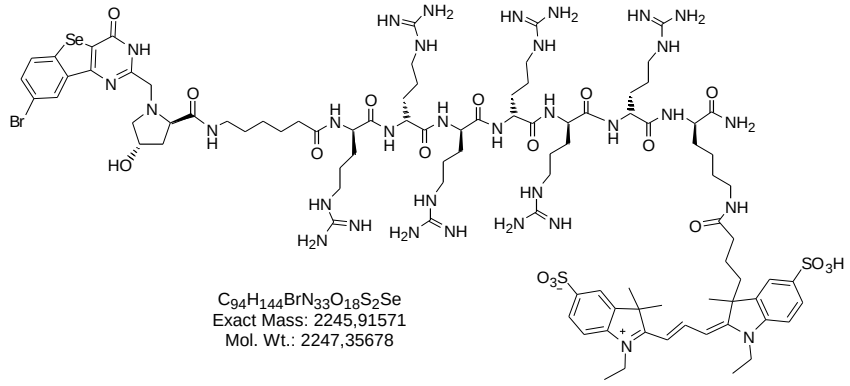
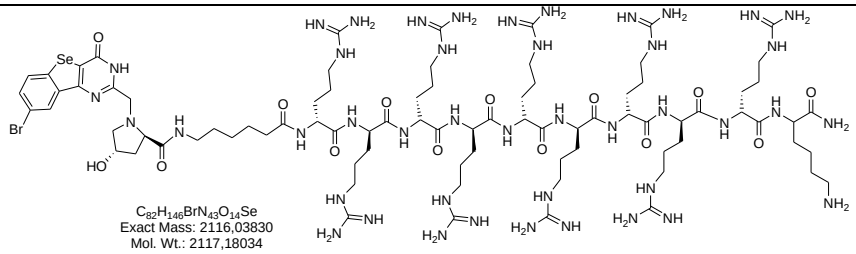
Labelling of peptide conjugates with the fluorescent dye PromoFlour-647

Lysine-containing peptide conjugate (ARC-3157, ARC-3160) and NHS ester of PromoFlour-647 or PromoFlour-555 fluorescence dye were dissolved in DMSO and Et_3N. After 3 h reaction the solvents were removed in the freeze dryer and the products were purified by HPLC with C18 reverse phase column to yield ARC-3158, ARC-3159 and ARC-3161.

4. Structures and HRMS data

Table S1. Structures and HRMS data of novel compounds

Structures and HRMS data of compounds reported in this study. Deconvoluted monoisotopic mass is presented

Compound	Structure	HRMS found
ARC-3157	 $C_{64}H_{110}BrN_{31}O_{11}Se$ Exact Mass: 1647,73496 Mol. Wt.: 1648,62330	1647.73973
ARC-3158	 $C_{96}H_{146}BrN_{33}O_{18}S_2Se$ Exact Mass: 2271,93136 Mol. Wt.: 2273,39074	2271.9312
ARC-3159	 $C_{94}H_{144}BrN_{33}O_{18}S_2Se$ Exact Mass: 2245,91571 Mol. Wt.: 2247,35678	2245.9143
ARC-3160	 $C_{82}H_{146}BrN_{43}O_{14}Se$ Exact Mass: 2116,03830 Mol. Wt.: 2117,18034	2116.04260

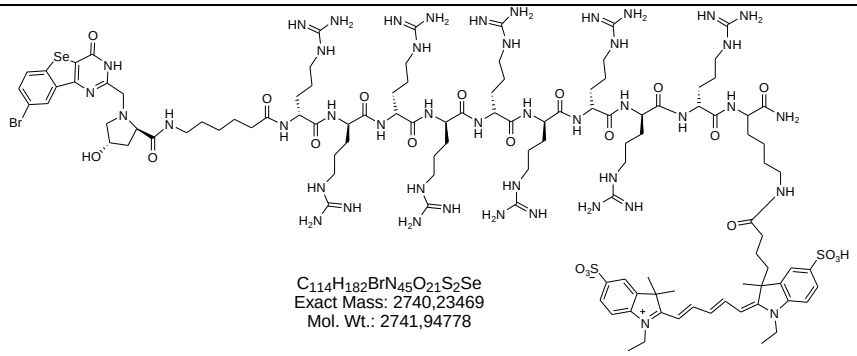
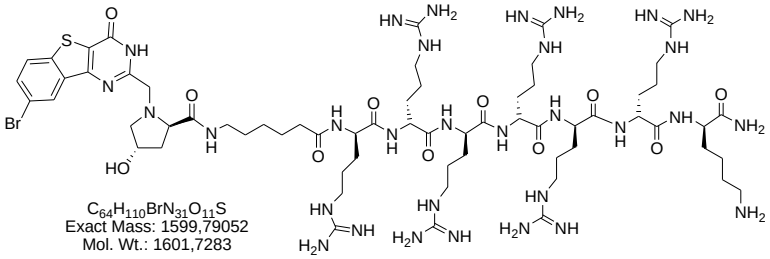
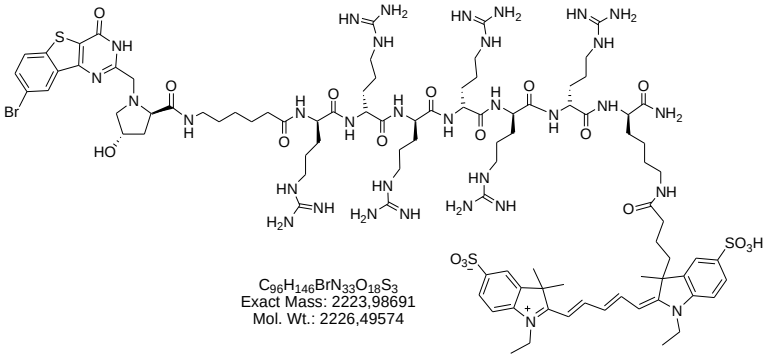
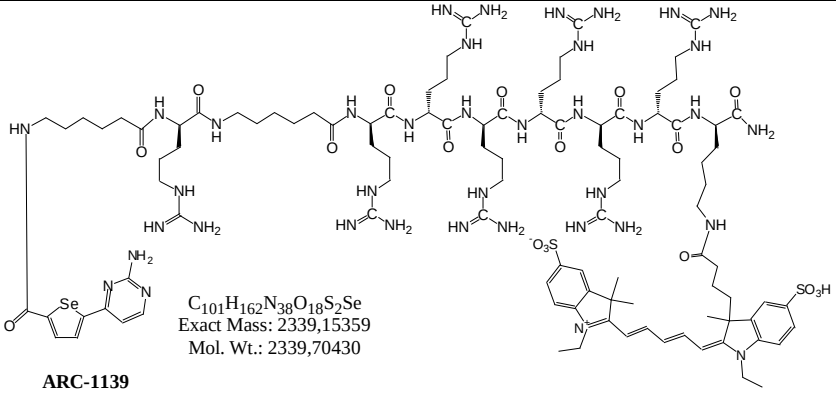
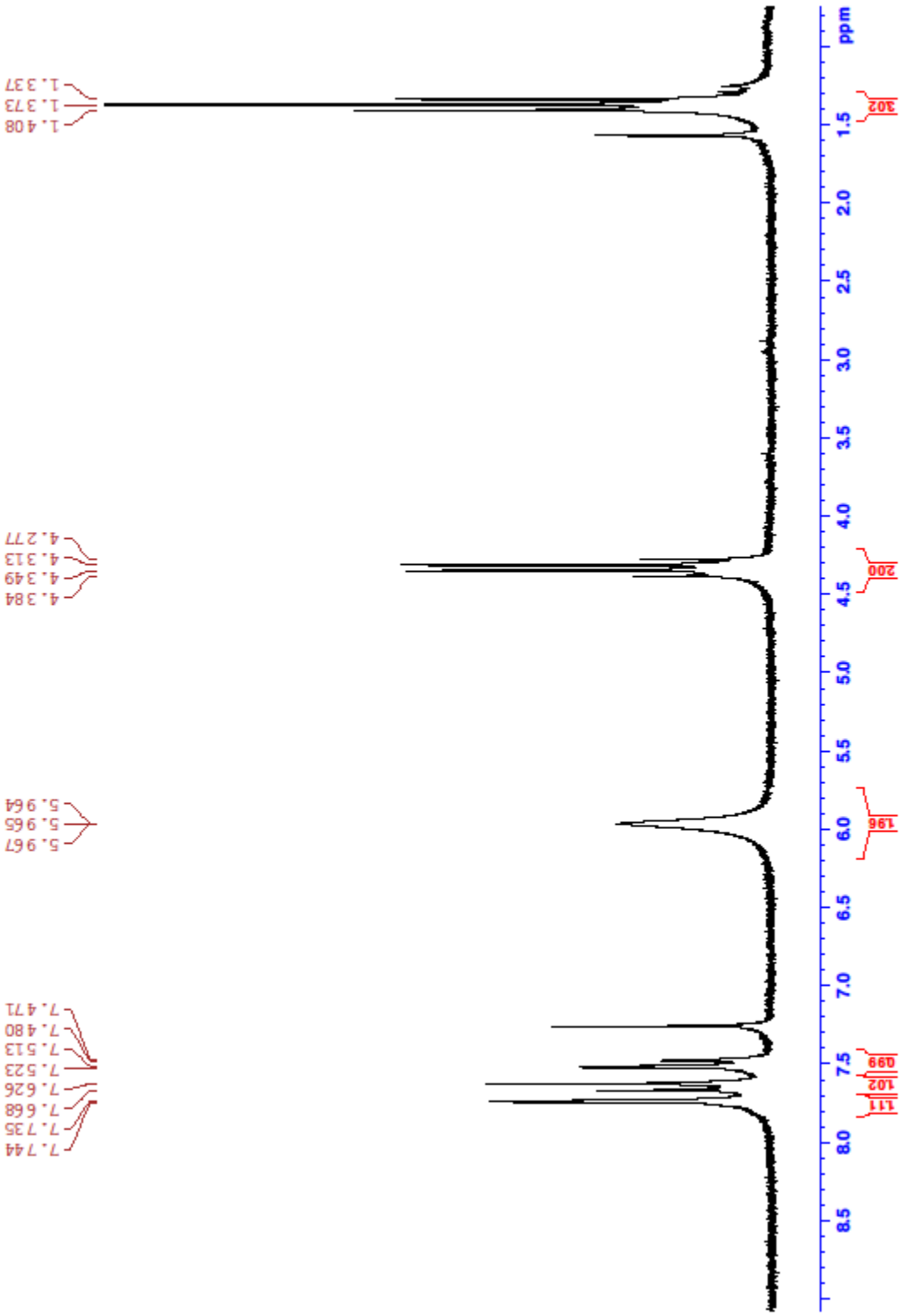
ARC-3161		2740.24042

Table S2. Structures of previously reported compounds

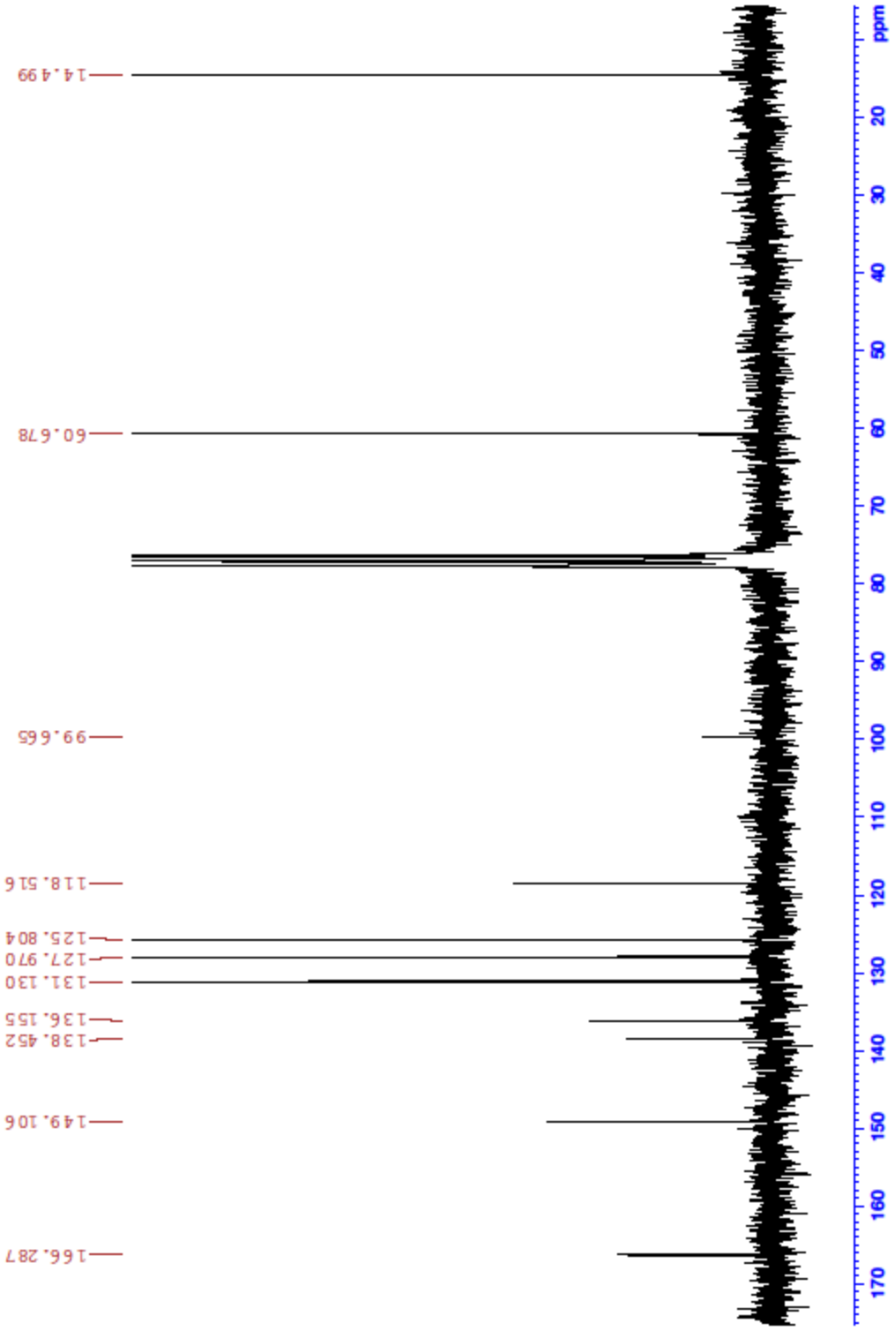
Compound	Structure	Reference
ARC-3104		(7)
ARC-3117		(7)
ARC-1139	 **ARC-1139**	(6)

4. NMR Spectra

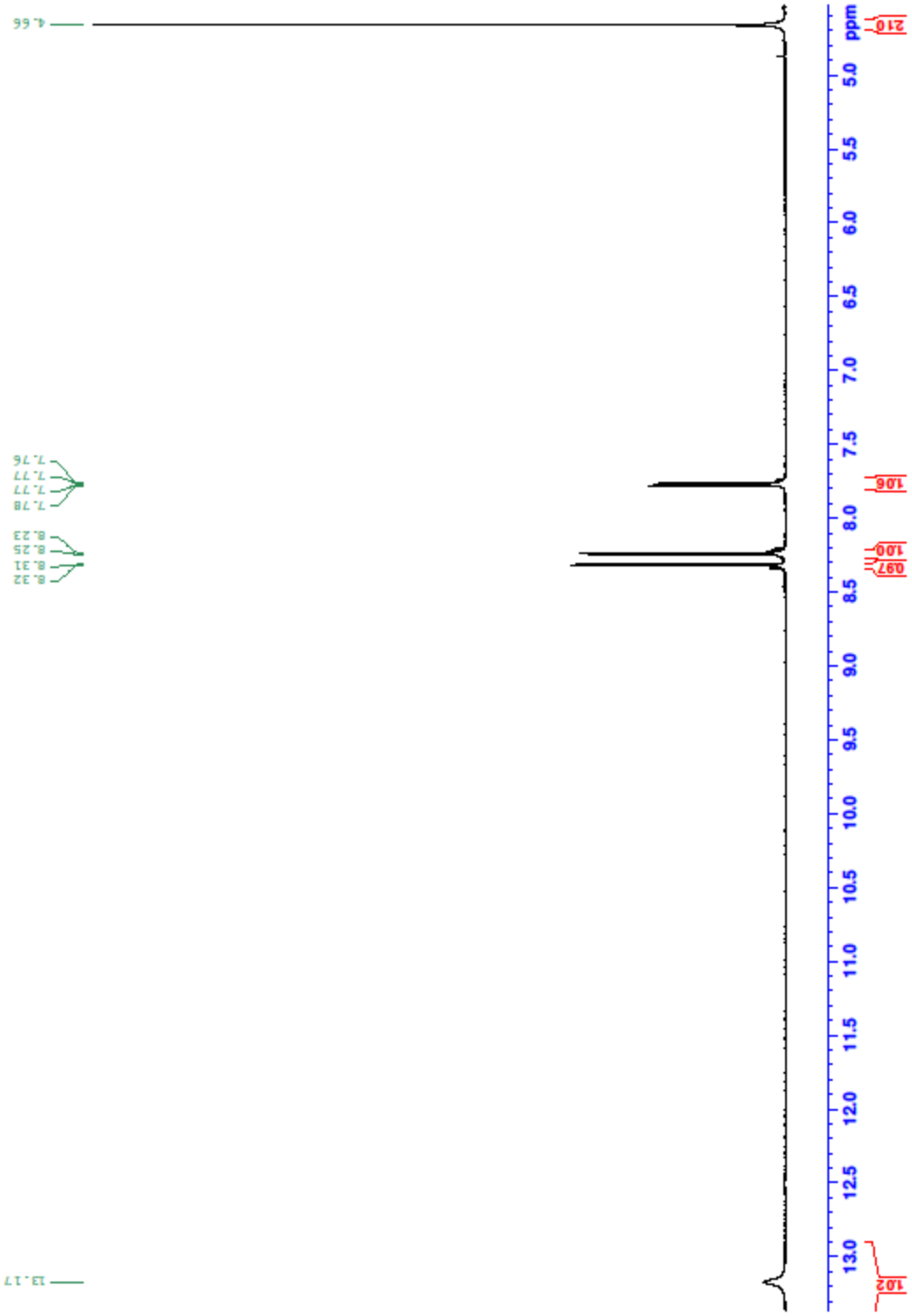
^{1}H NMR spectrum of 3-amino-5-bromobenzo[b]selenophene-2-carboxylic acid ethyl ester. (1)



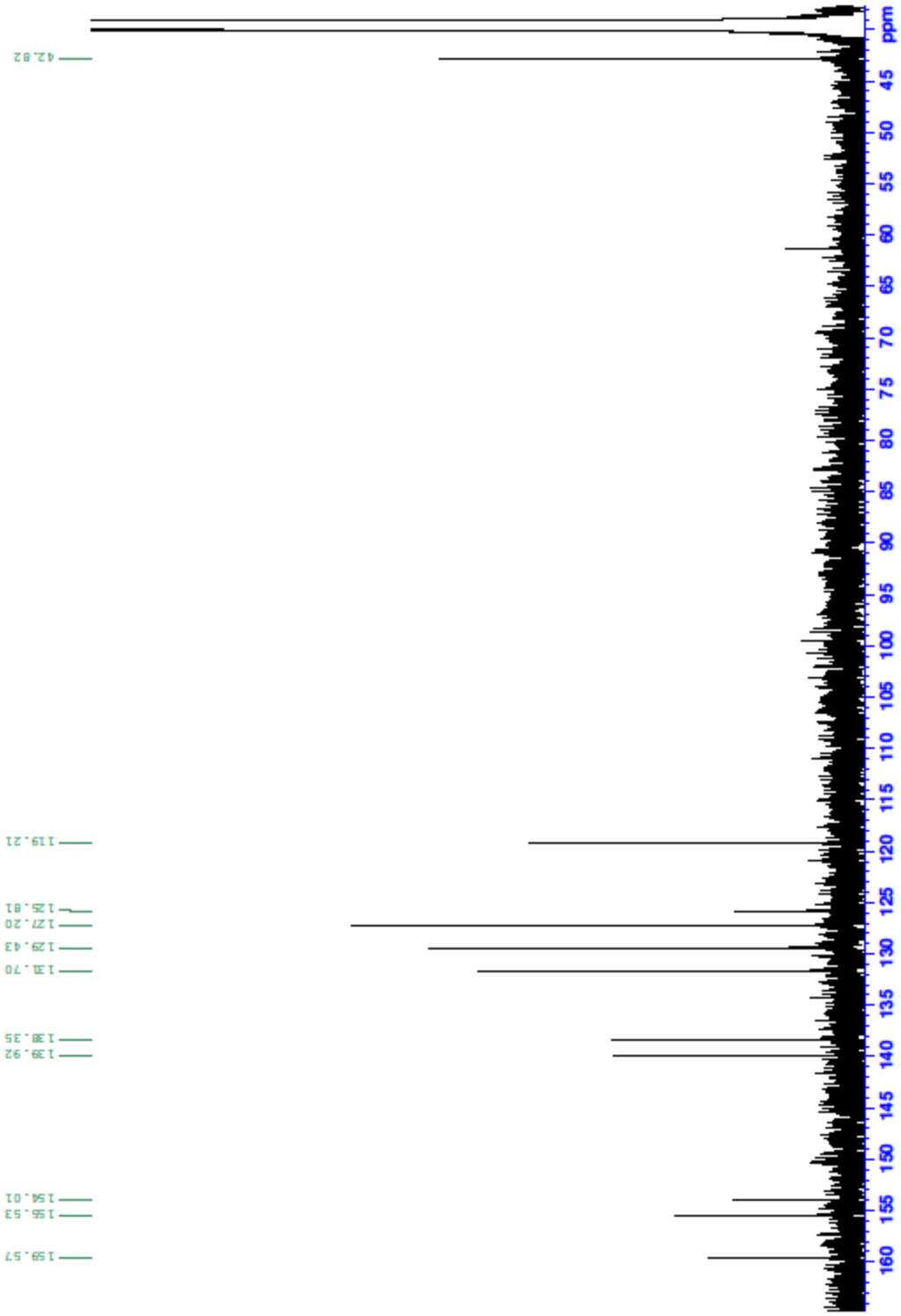
^{13}C spectrum of 3-amino-5-bromobenzo[b]selenophene-2-carboxylic acid ethyl ester. (1)



^{1}H spectrum of 2-(chloromethyl)-seleno[2,3-d]pyrimidin-4(3H)-one. (3)



^{13}C spectrum of 2-(chloromethyl)-seleno[2,3-d]pyrimidin-4(3H)-one. (3)



5. HPLC data of purified compounds

Purification of the compounds was performed with Schimadzu LC Solution (Prominence) HPLC system by using a manual injector and a diode array detector (SPM 20A). Separation was achieved with a Gemini C18 column (250X4.6, Phenomenex). Mobile phase was made up from solutions A (0.1 %TFA) and solution B (0.1%TFA in ACN). Elution flow rate was 1 ml/min. Linear gradient and elution were started at 3 min (injection time was also at 3 min) with % ACN as specified in the table.

Compound	**Molecular formula**	**Gradient speed Pump B ACN%**	**Retention time tR (min)**	**HPLC area purity%**
ARC-3157	$C_{64}H_{110}BrN_{31}O_{11}Se$	10%-40%/30 min	14.5	100
ARC-3158	$C_{96}H_{146}BrN_{33}O_{18}S_2Se$	10%-60%/30 min	16.4	96.5
ARC-3159	$C_{94}H_{144}BrN_{33}O_{11}Se$	10%-80%/30 min	12.9	95
ARC-3160	$C_{82}H_{146}BrN_{43}O_{14}Se$	10%-60%/30 min	12.9	92.4
ARC-3161	$C_{114}H_{182}BrN_{45}O_{21}S_2Se$	10%-80%/30 min	13.4	98.7

6. References

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(2) L. Harrington, S. Cheley, L. T. Alexander, S. Knapp and H. Bayley, Stochastic detection of PIM protein kinases reveals electrostatically enhanced association of a peptide substrate, *Proceedings of the National Academy of Sciences of the United States of America*, 2013, **110**, E4417-E4426.

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photoluminescent probes for protein kinases, *Chemical communications*, 2014, **50**, 4096-4098.

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(7) R. Ekambaram, E. Enkvist, A. Vaasa, M. Kasari, G. Raidaru, S. Knapp and A. Uri, Selective bisubstrate inhibitors with sub-nanomolar affinity for protein kinase PIM-1, *ChemMedChem*, 2013, **8**, 909-913.