SUPPORTING INFORMATION

Towards a RIOK2 chemical probe: Cellular potency improvement of a selective 2-(acylamino)pyridine series†

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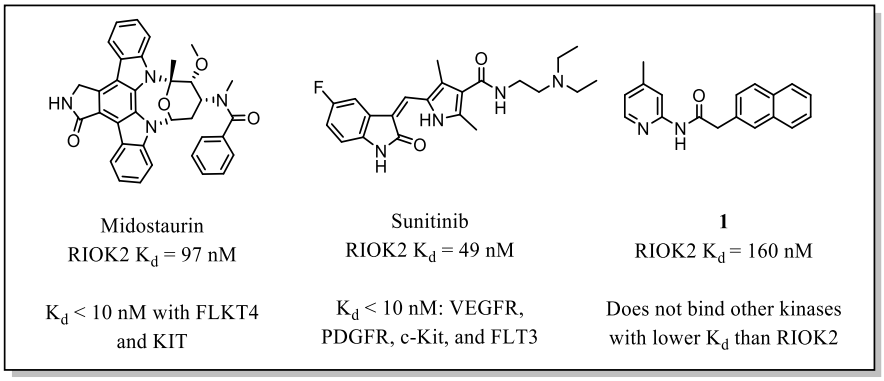
PREVIOUSLY REPORTED DATA

A)



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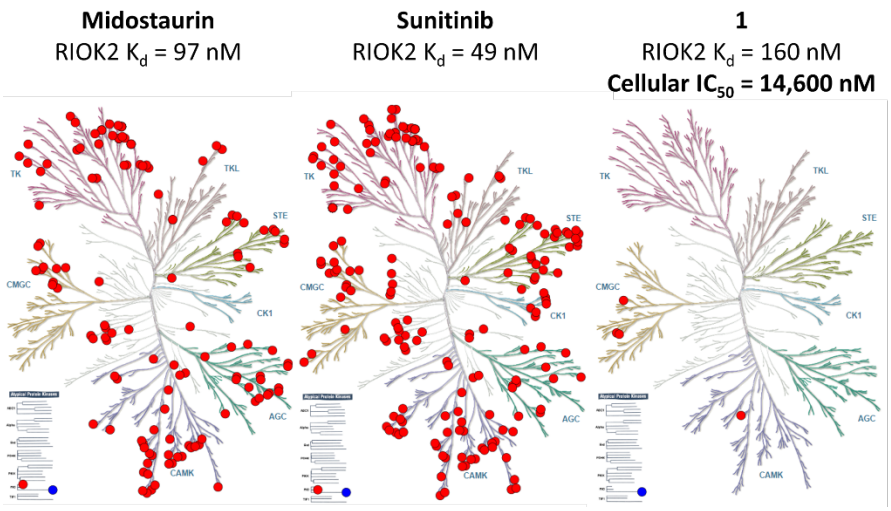
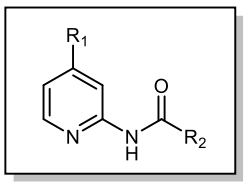


Supplementary Figure 1 Structures of previously published compounds known to bind RIOK2, including (**1**), with nanomolar biochemical affinity (Panel A). Kinome trees with profiles of reported RIOK2 inhibitors (Panel B).

Supplementary Table 1. K_d binding affinities (nM) of previously reported pyridine-amide compounds.



		R2 -				
R1	-H	5500	3200	1400	--	520
	-3-CH3	--	--	20000	--	20000
	-4-CH3	--	--	470	2500	160
	-2-CH3	--	--	13000	--	2900

BIOLOGICAL METHODS

Biological assays

***In vitro* kinase radiometric KinaseProfiler assays**

Eurofins kinase enzymatic radiometric assays were carried out at the K_m of ATP in dose-response (9-pt curve in duplicate) for each kinase for which it was offered. Details about the substrate used, protein constructs, controls, and assay protocol for each kinase assay can be found at https://www.eurofinsdiscoveryservices.com.

Cell culture:

HEK293 cells were cultured in DMEM supplemented with 10% FBS. Cells were incubated at 37˚C in 5% CO_2 and passaged every 72 hours with trypsin. Cells were not allowed to reach confluency.

NanoBRET measurements:

Constructs for NanoBRET measurements of RIOK2 were kindly provided by Promega. NanoBRET assays were executed as described previously.[1]

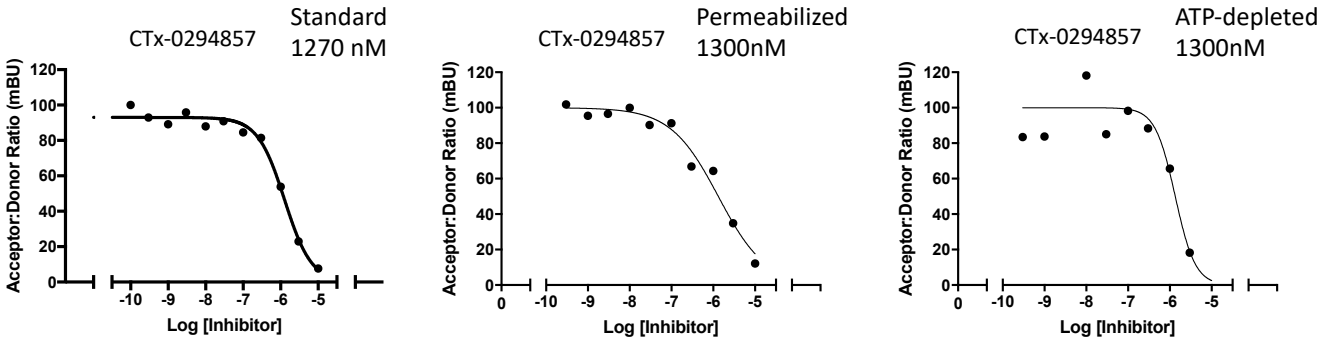
Example protocol for RIOK2: The *N*-terminal Nanoluciferase (NL)/ RIOK2 fusion (NL-RIOK2) was encoded in pFN32K expression vector, including a flexible Gly-Ser-Ser-Gly linker between NL and RIOK2 (Promega). For cellular NanoBRET target engagement experiments, a 10 µg/mL solution of DNA in Opti-MEM without serum was made containing 9 µg/mL of Carrier DNA (Promega) and 1 µg/mL of RIOK2-NL for a total volume of 1.05 mL. To this solution was then added 31.5 µL of FuGENE HD (Promega) to form a lipid:DNA complex. The solution was then mixed by inversion 8 times and incubated at room temperature for 20 min. The resulting transfection complex (1.082 mL) was then gently mixed with 21 mL of HEK-293 cells (ATCC) suspended at a density of 2 x 10^5 cells/mL in DMEM (Gibco) + 10% FBS (Corning). This solution was then dispensed (100 µL) into 96-well tissue culture treated plates (Corning #3917) followed by incubation (37 °C / 5 % CO_2) for 24 hours.

After 24 hours the media was removed and replaced with 85 µL of room temperature Opti-MEM without phenol red. NanoBRET Tracer K5 (Promega) was used at a final concentration of 1.0 µM as previously determined to be the optimal concentration in a titration experiment. A total of 5 µL per well (20x working stock of NanoBRET Tracer K5 [20 µM] in Tracer Dilution Buffer (Promega N291B)) was added to all wells, except the “no tracer” control wells. All test compounds were prepared initially as concentrated (30 mM) stock solutions in 100% DMSO (Sigma), and then diluted in Opti-MEM media (99%) to prepare 1% DMSO working stock solutions. A total of 10 µL per well of the 10-fold test compound stock solutions (final assay concentration of 0.1% DMSO) were added. For “no compound” and “no tracer” control wells, a total of 10 µL per well of Opti-MEM plus DMSO (9.9 µL Opti-MEM with 0.1 µL DMSO) was added for a final concentration of 0.1% DMSO. 96-well plates containing transfected cells, NanoBRET Tracer K5 [1 µM] and test compounds (100 µL total volume per well) were equilibrated (37 °C / 5 % CO_2) for 2 hours.

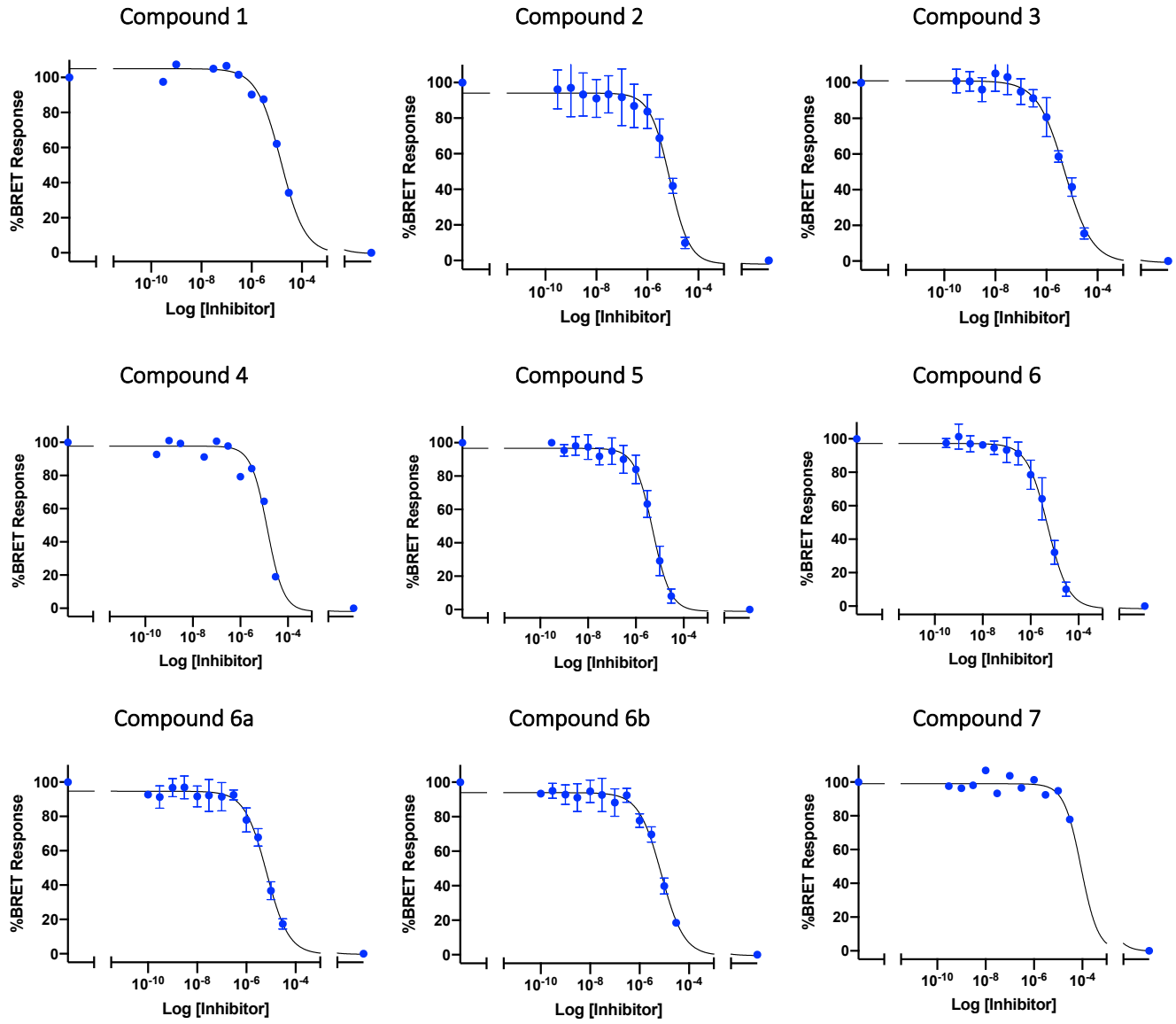
After 2 hours the plates were cooled to room temperature for 15 min. To measure NanoBRET signal, NanoBRET NanoGlo substrate (Promega) at a ratio of 1:166 to Opti-MEM media in combination with extracellular NanoLuc Inhibitor (Promega) diluted 1:500 (10 µL [30 mM stock] per 5 mL Opti-MEM plus substrate) were combined to create a 3X stock solution. A total of 50 µL of the 3X substrate/extracellular NL inhibitor were added to each well. The plates were read within 3 min on a GloMax Discover luminometer (Promega) equipped with 450 nm BP filter (donor) and 600 nm LP filter (acceptor), using 0.3 s integration time according to the “NanoBRET 618” protocol.

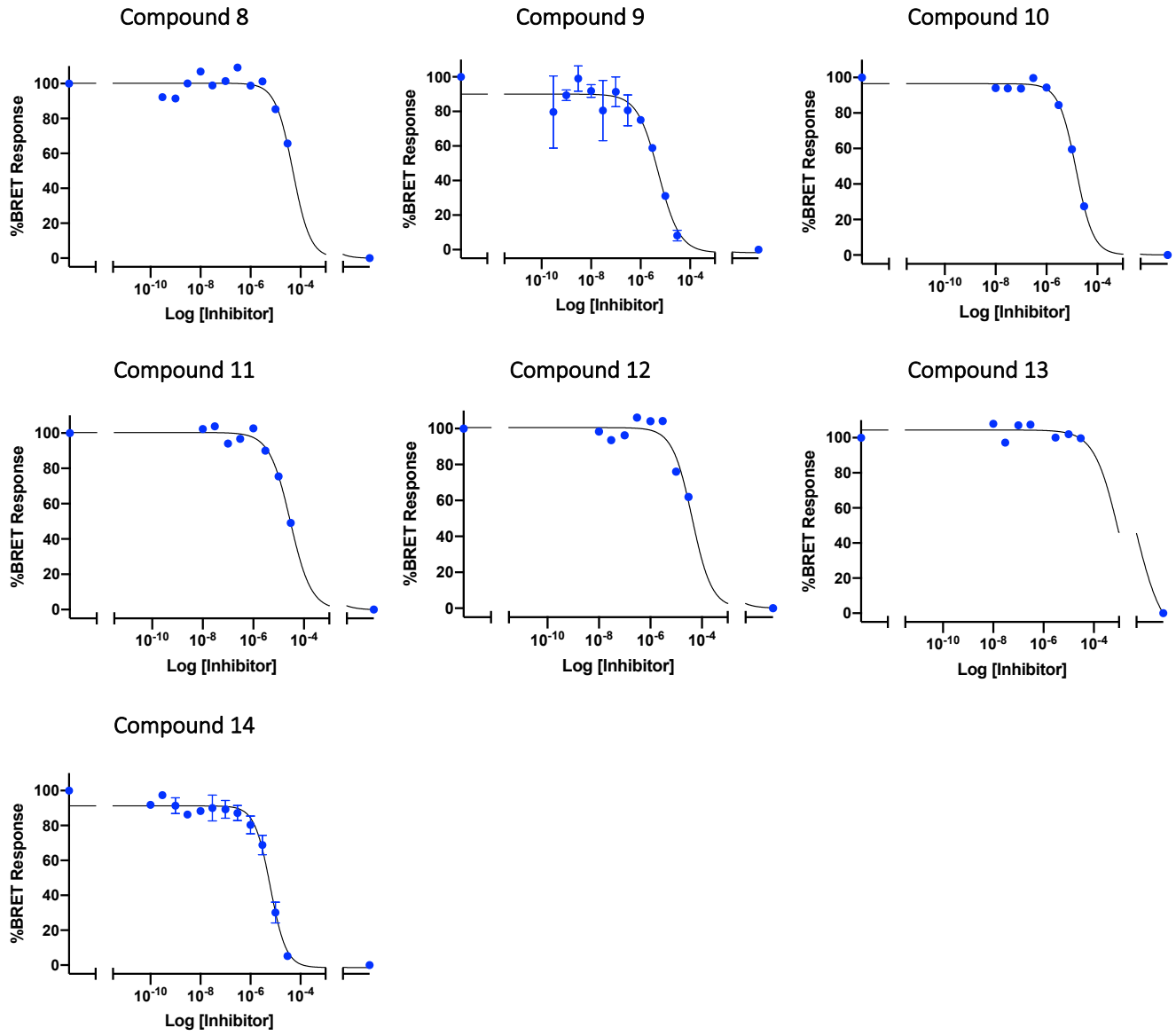
Test compounds were evaluated at eleven concentrations in competition with NanoBRET Tracer K5 in HEK-293 cells transiently expressing the NL-RIOK2 fusion protein. Raw milliBRET (mBRET) values were obtained by dividing the acceptor emission values (600 nm) by the donor emission values (450 nm), and then multiplying by 1000. Averaged control values were used to represent complete inhibition (no tracer control: Opti-MEM + DMSO only) and no inhibition (tracer only control: no compound, Opti-MEM + DMSO + Tracer K5 only) and were plotted alongside the raw mBRET values. The data with n=3 biological replicates was first normalized and then fit using Sigmoidal, 4PL binding curve in Prism Software (version 8, GraphPad, La Jolla, CA, USA). All error bars are based on n=3 and are +/- standard error (SE).

BIOLOGICAL RESULTS



Supplementary Figure 2. Representative curves from RIOK2 NanoBRET for non-specific inhibitor CTx-0294857 run in standard (non-permeabilized, adherent cells), permeabilized (non-adherent), or ATP depleted (non-permeabilized, non-adherent) formats.





Supplementary Figure 3. RIOK2 NanoBRET curves of synthesized compounds (**1**-**14**) in Table 4.

CHEMISTRY

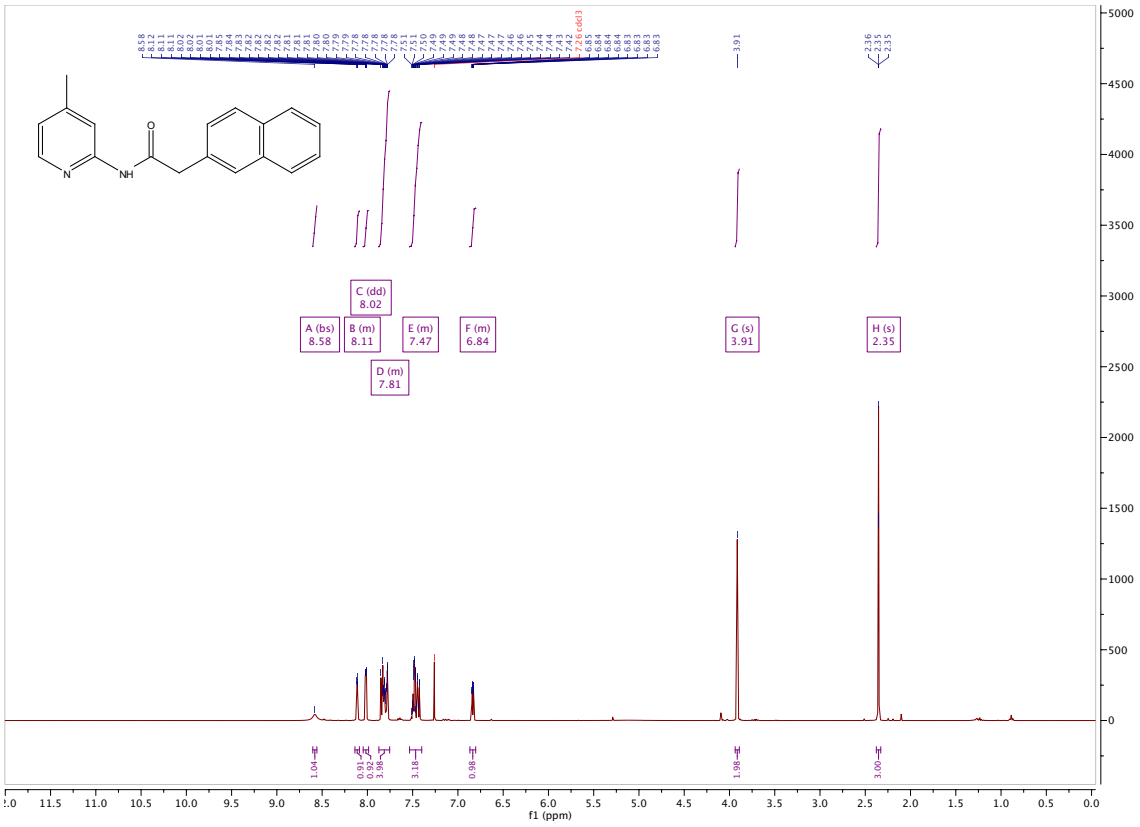
Mass spectrometry methods:

Samples were analyzed with a Q Exactive HF-X (ThermoFisher, Bremen, Germany) mass spectrometer. Samples were introduced via a heated electrospray source (HESI) at a flow rate of 20 µL/min. One hundred time domain transients were averaged in the mass spectrum. HESI source conditions were set as: nebulizer temperature 100 deg C, sheath gas (nitrogen) 15 arb, auxiliary gas (nitrogen) 5 arb, sweep gas (nitrogen) 0 arb, capillary temperature 250 degrees C, RF voltage 100 V. The mass range was set to 150-2000 m/z. All measurements were recorded at a resolution setting of 30,000. Solutions were analyzed at 0.1 mg/mL or less based on responsiveness to the ESI mechanism. Xcalibur (ThermoFisher, Breman, Germany) was used to analyze the data. Molecular formula assignments were determined with Molecular Formula Calculator (v 1.2.3). All observed species were singly charged, as verified by unit *m/z* separation between mass spectral peaks corresponding to the ^{12}C and $^{13}C^{12}C_{c-1}$ isotope for each elemental composition.

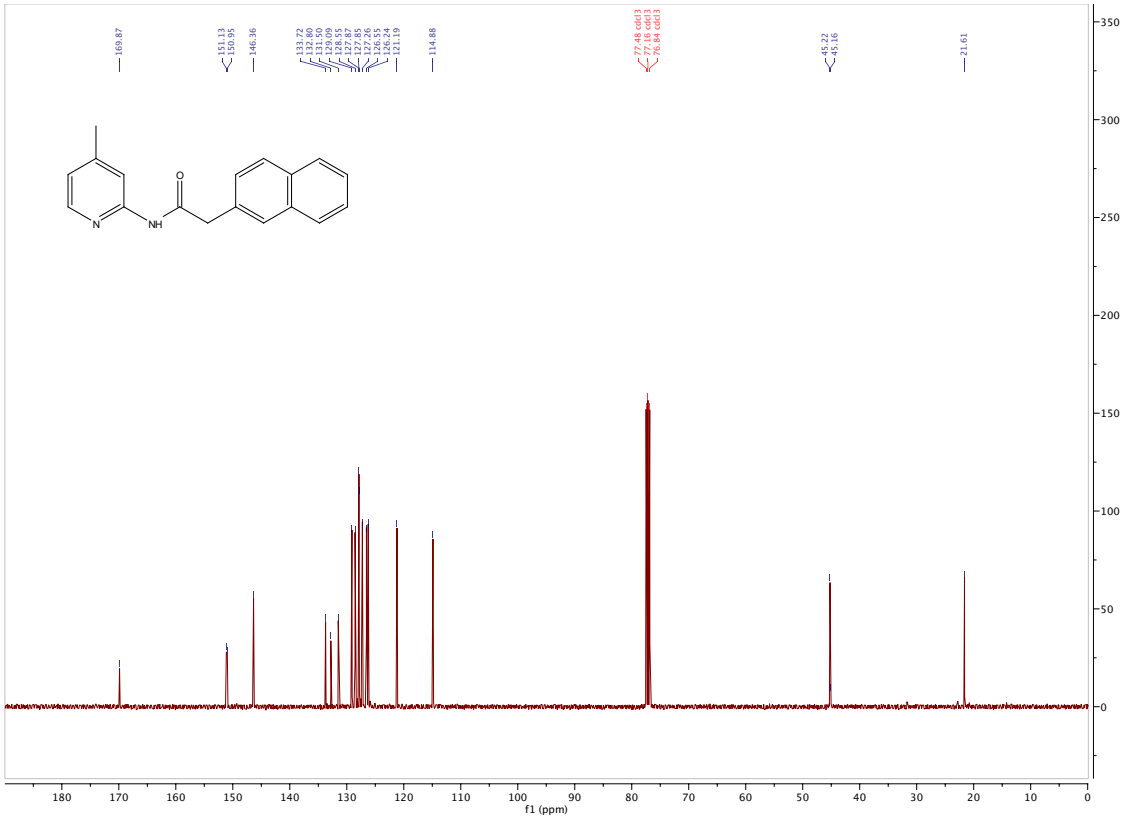
Preparation and characterization of *N*-(4-methylpyridin-2-yl)-2-(naphthalen-2-yl)acetamide (1):

4-methylpyridin-2-amine (150 mg, 1.39 mmol) and i-Pr_2NEt [DIPEA] (1.3 eq) were suspended in tetrahydrofuran [THF] (4 mL) and stirred at room temperature for 15 min prior to the addition of 2-(naphthalen-2-yl)acetyl chloride (341 mg, 1.66 mmol). The reaction solution was further stirred overnight at room temperature for 12 h. After this period, the reaction mixture was diluted with dichloromethane (DCM) and washed with water and saturated aqueous $NaHCO_3$ solution (2x). The combined organic layers were dried over anhydrous Na_2SO_4 and then, the solvent was evaporated via reduced pressure to afford a crude reaction mixture. Purification of the crude reaction mixture by column chromatography (silica gel, 0-25% EtOAc/n-hexanes) yielded a white solid (1, 77.1 mg, 20%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (bs, 1H), 8.14 – 8.09 (m, 1H), 8.02 (dd, *J* = 5.2, 0.8 Hz, 1H), 7.87 – 7.75 (m, 4H), 7.53 – 7.40 (m, 3H), 6.87 – 6.80 (m, 1H), 3.91 (s, 2H), 2.35 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.87, 151.13, 150.95, 146.36, 133.72, 132.80, 131.50, 129.09, 128.55, 127.87, 127.85, 127.26, 126.55, 126.24, 121.19, 114.88, 45.22, 45.16, 21.61. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{18}H_{17}N_2O$, 277.1341, found 277.1337. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (bs, 1H), 8.14 – 8.09 (m, 1H), 8.02 (dd, *J* = 5.2, 0.8 Hz, 1H), 7.87 – 7.75 (m, 4H), 7.53 – 7.40 (m, 3H), 6.87 – 6.80 (m, 1H), 3.91 (s, 2H), 2.35 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.87, 151.13, 150.95, 146.36, 133.72, 132.80, 131.50, 129.09, 128.55, 127.87, 127.85, 127.26, 126.55, 126.24, 121.19, 114.88, 45.22, 45.16, 21.61. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{18}H_{17}N_2O$, 277.1341, found 277.1337.

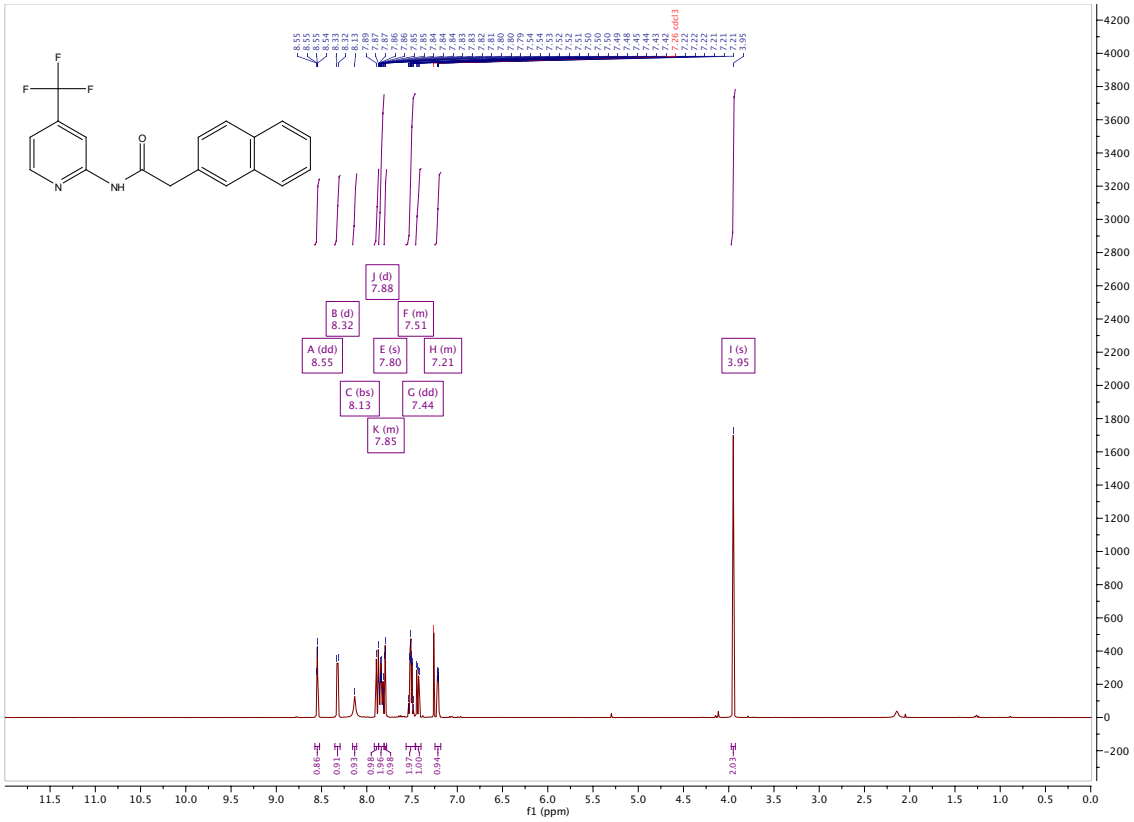
^{1}H NMR (400 MHz, $CDCl_3$) *N*-(4-methylpyridin-2-yl)-2-(naphthalen-2-yl)acetamide (1):



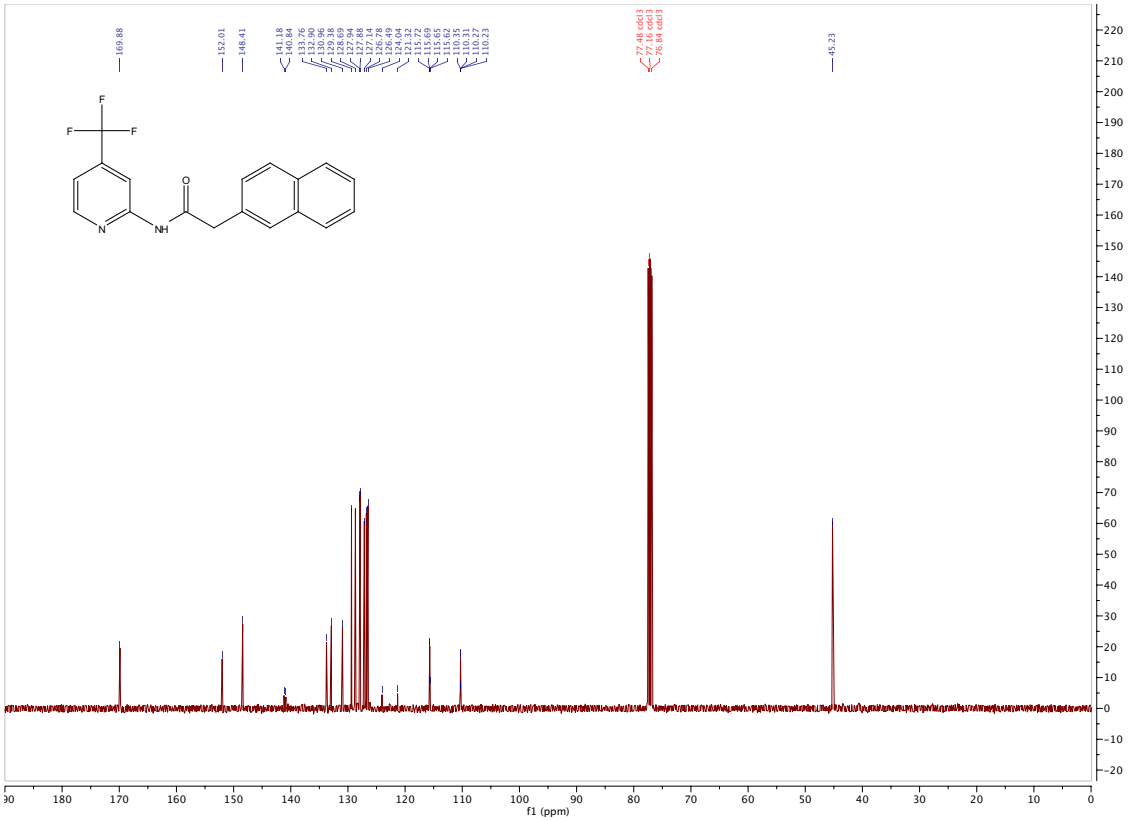
^{13}C NMR (100 MHz, $CDCl_3$) *N*-(4-methylpyridin-2-yl)-2-(naphthalen-2-yl)acetamide (1):



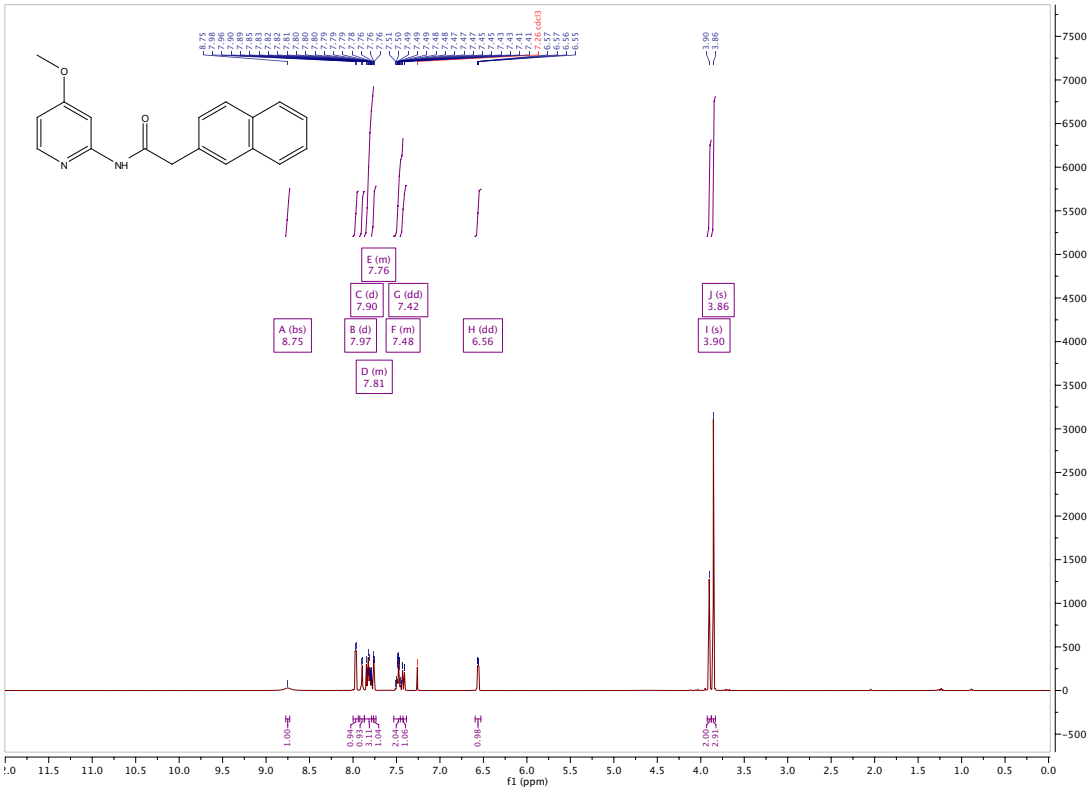
^{1}H NMR (400 MHz, $CDCl_3$) 2-(naphthalen-2-yl)-*N*-(4-(trifluoromethyl)pyridin-2-yl)acetamide (2):



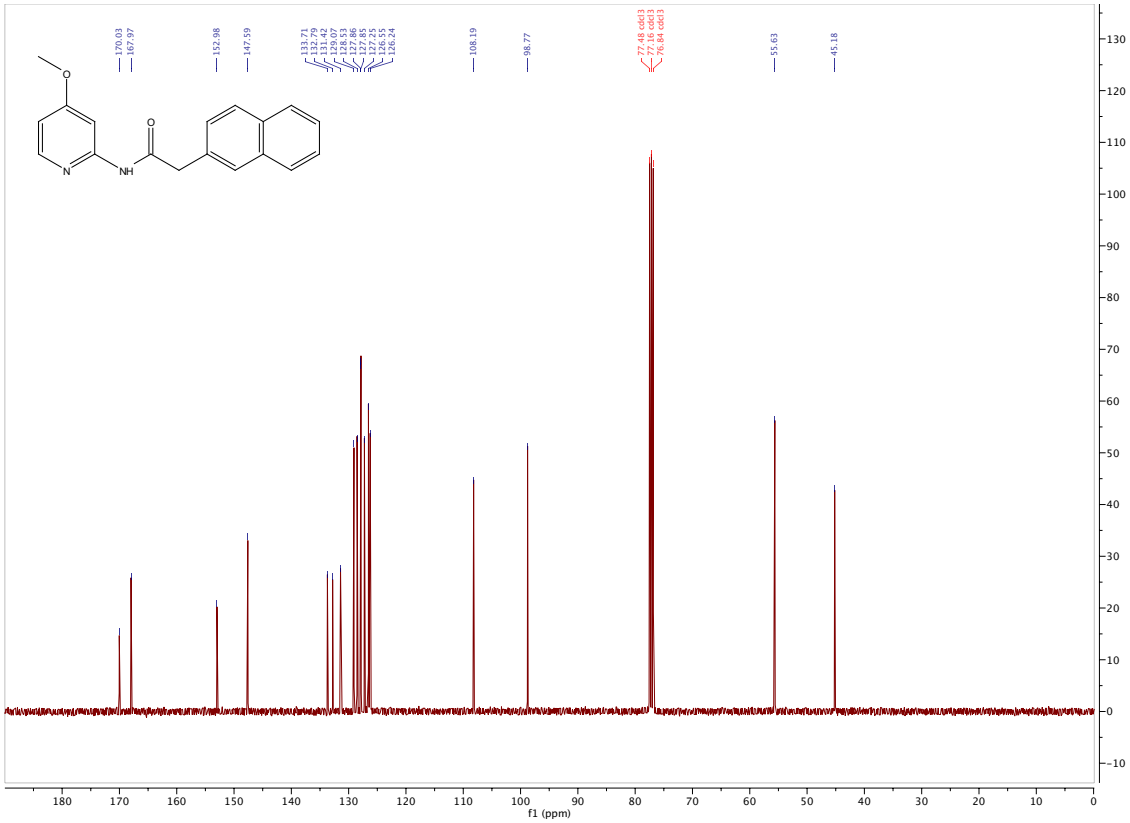
^{13}C NMR (100 MHz, $CDCl_3$) 2-(naphthalen-2-yl)-*N*-(4-(trifluoromethyl)pyridin-2-yl)acetamide (2):



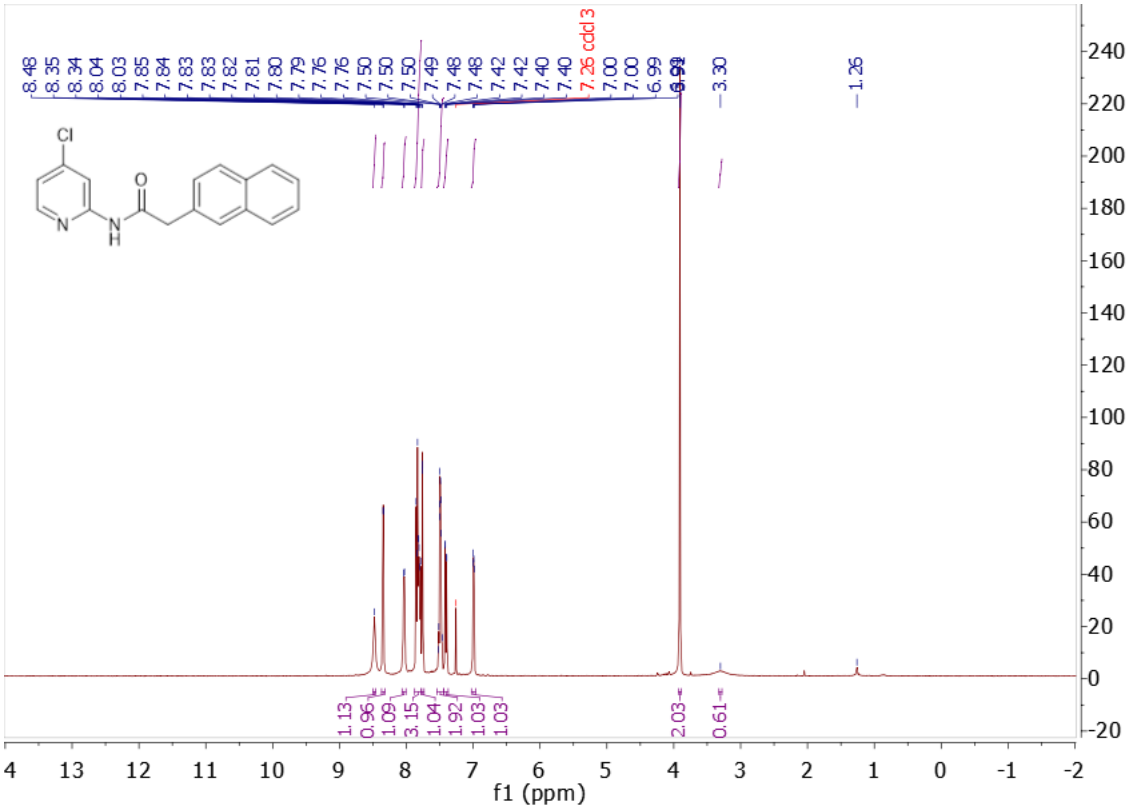
^{1}H NMR (400 MHz, $CDCl_3$) *N*-(4-methoxypyridin-2-yl)-2-(naphthalen-2-yl)acetamide (3):



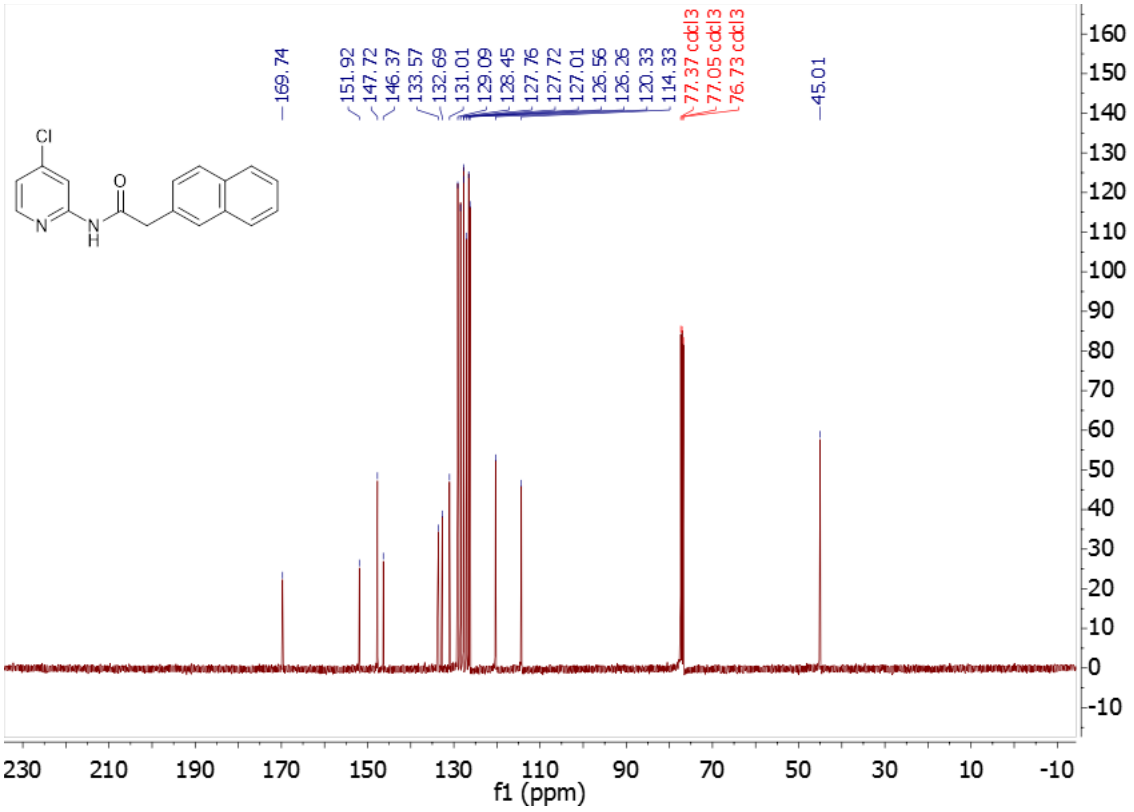
^{13}C NMR (100 MHz, $CDCl_3$) *N*-(4-methoxypyridin-2-yl)-2-(naphthalen-2-yl)acetamide (3):



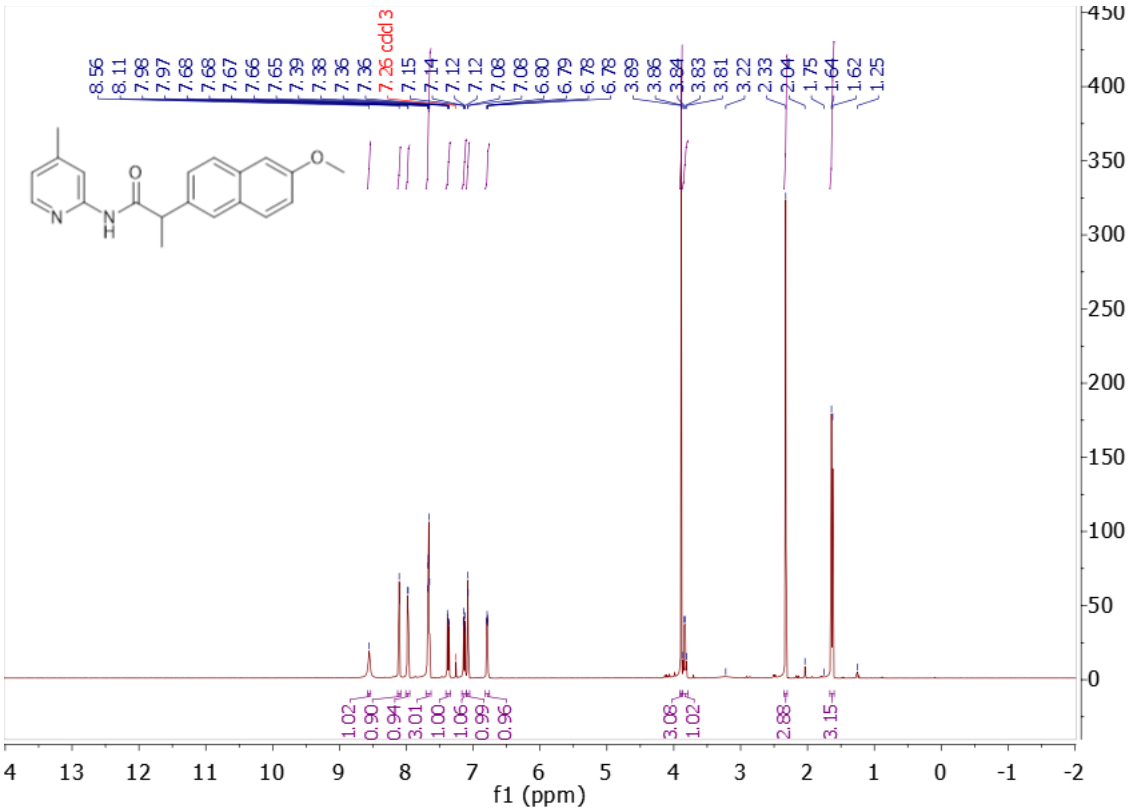
^{1}H NMR (400 MHz, $CDCl_3$) 2-Naphthaleneacetamide, N-(4-chloro-2-pyridinyl) (5):



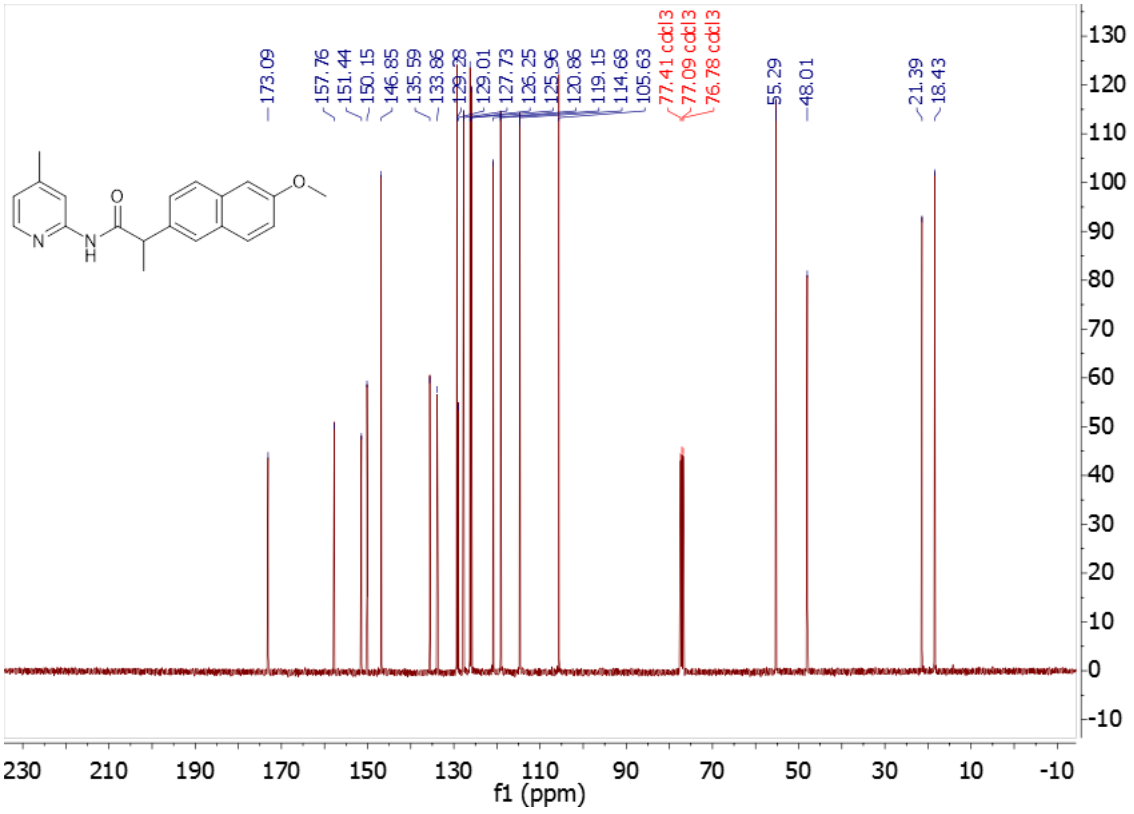
^{13}C NMR (101 MHz, $CDCl_3$) 2-Naphthaleneacetamide, N-(4-chloro-2-pyridinyl) (5):



^{1}H NMR (400 MHz, $CDCl_3$) 2-Naphthaleneacetamide, 6-methoxy-α-methyl-N-(4-methyl-2-pyridinyl): (6)



^{13}C NMR (101 MHz, $CDCl_3$) 2-Naphthaleneacetamide, 6-methoxy-α-methyl-N-(4-methyl-2-pyridinyl): (6)



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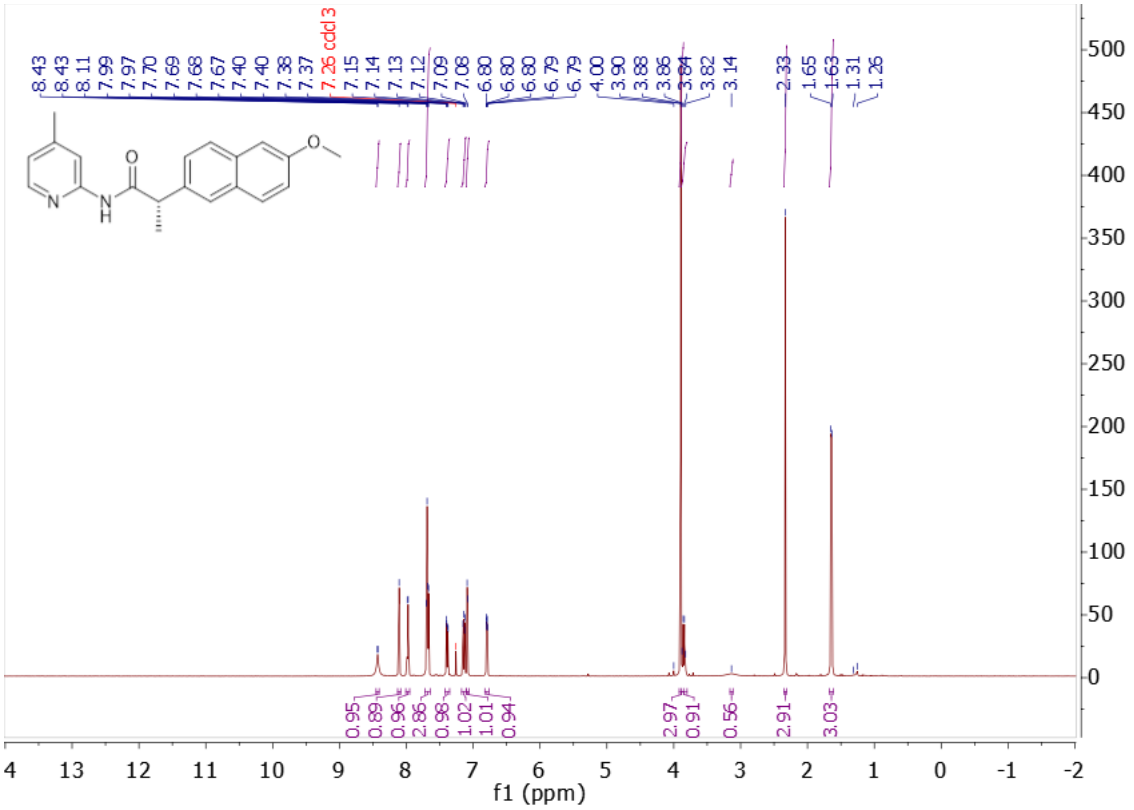
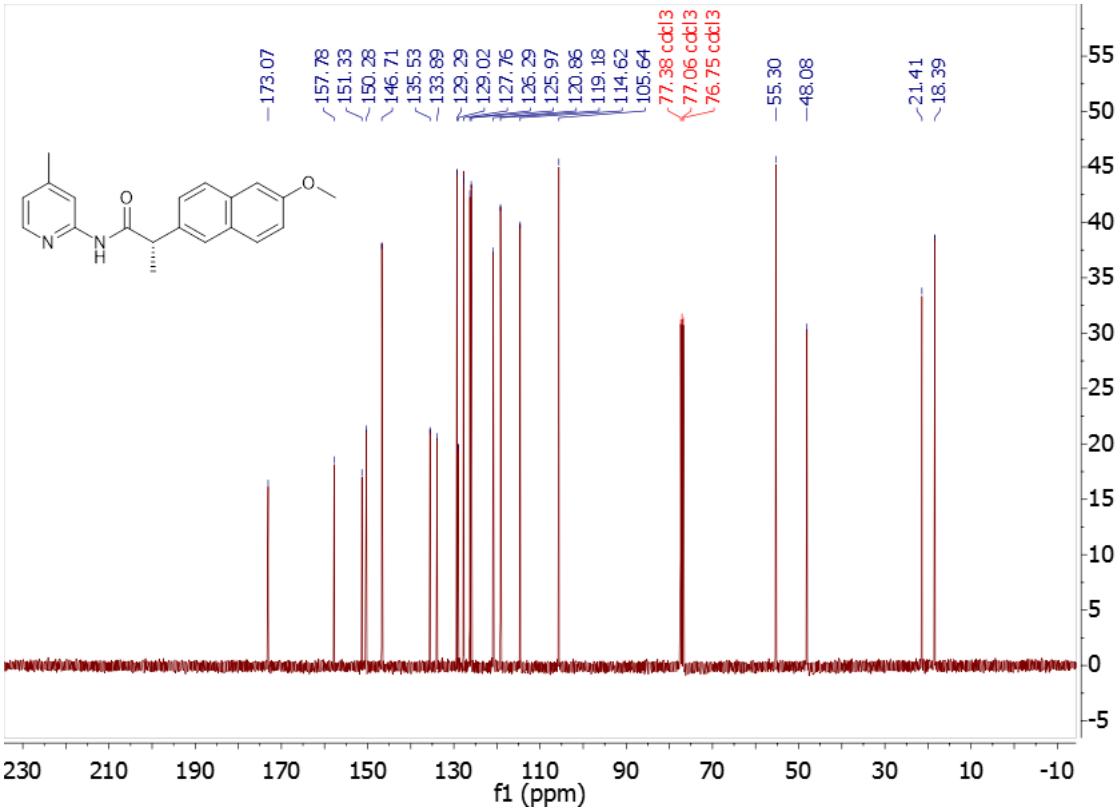
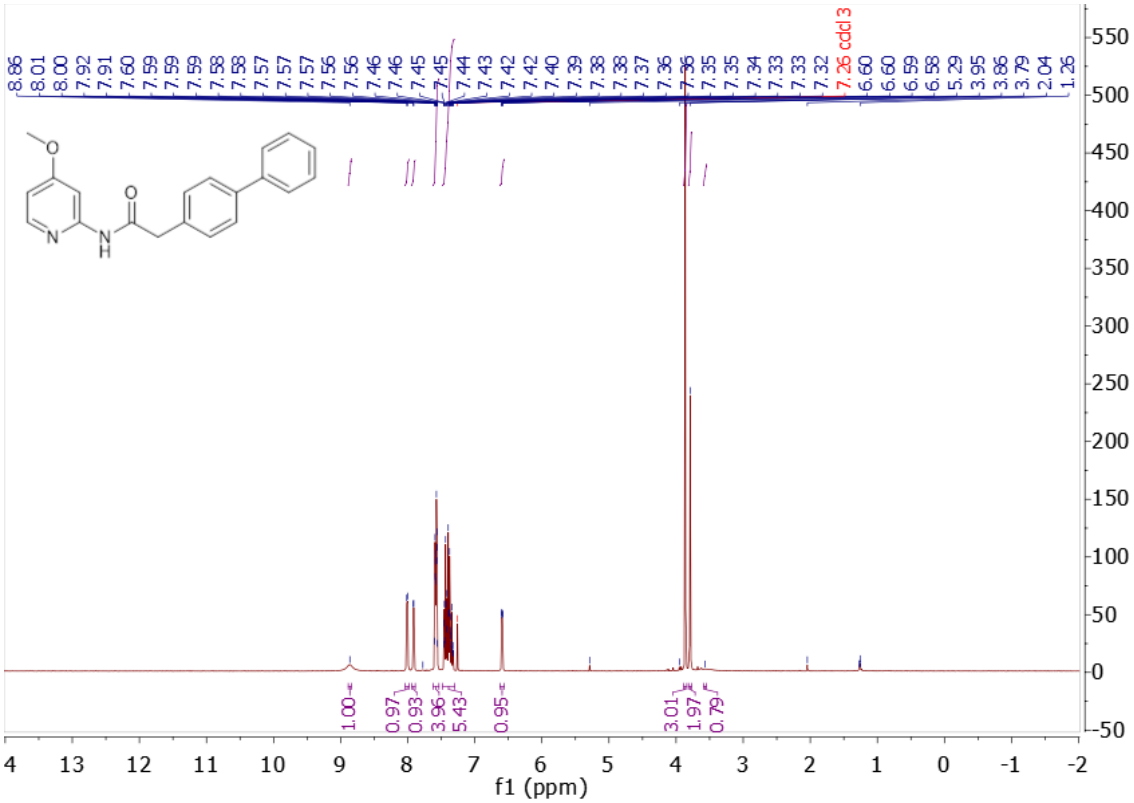


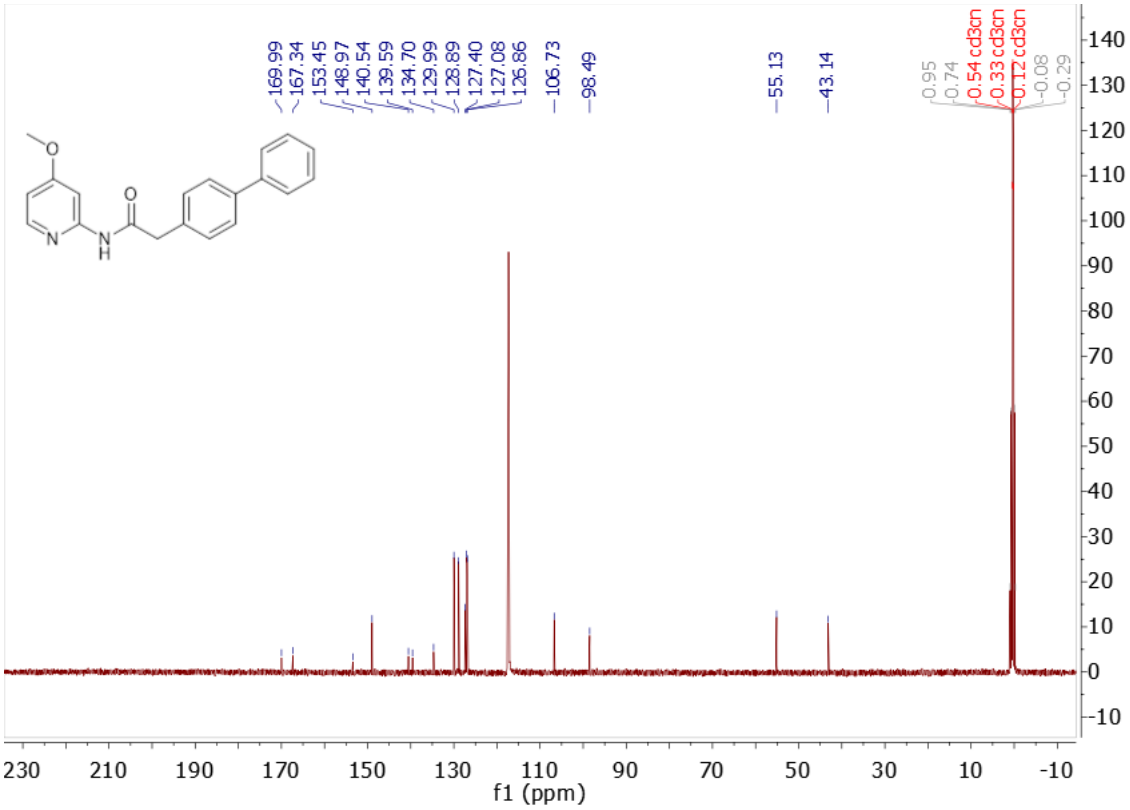
^{13}C NMR (101 MHz, $CDCl_3$) 2-Naphthaleneacetamide, 6-methoxy-α-methyl-N-(4-methyl-2-pyridinyl)-, (αS) (6a):



^{1}H NMR (400 MHz, $CDCL_3$) 2-([1,1'-biphenyl]-4-yl)-N-(4-methoxypyridin-2-yl)acetamide (14):



^{13}C NMR (101 MHz, CD_3CN) 2-([1,1'-biphenyl]-4-yl)-N-(4-methoxypyridin-2-yl)acetamide (14):



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1. Wells, C., Couñago, R. M., Limas, J. C., Almeida, T. L., Cook, J. G., Drewry, D. H., Elkins, J. M., Gileadi, O., Kapadia, N. R., Lorente-Macias, A., Pickett, J. E., Riemen, A., Ruela-de-Sousa, R. R., Willson, T. M., Zhang, C., Zuercher, W. J., Zutshi, R., and Axtman, A. D. (2019) SGC-AAK1-1: A Chemical Probe Targeting AAK1 and BMP2K, *ACS Med Chem Lett 11*, 340-345.