Supporting Information

Identification of a Novel Allosteric Binding Site on the Catalytic Domain of NF-κB Inducing Kinase (NIK)

Jared J. Anderson[a] and Daniel A. Harki[a,b,†]

[a]Department of Chemistry, University of Minnesota, 207 Pleasant St. SE, Minneapolis, MN 55455, United States.

[b]Department of Medicinal Chemistry, University of Minnesota, 2231 6th St SE, Minneapolis, MN 55455, United States.

***Contact Information:**

Daniel A. Harki, University of Minnesota, Department of Medicinal Chemistry, 2231 6th Street SE, Minneapolis, MN, 55455, United States

612-625-8687

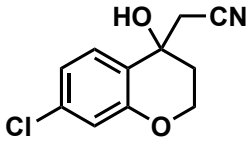
daharki@umn.edu

Table of Contents for Supporting Information

Chemistry – General: All air or moisture sensitive reactions were conducted in oven-dried glassware under inert argon atmosphere. Reactions were stirred with Teflon-coated stir bars. Solvents THF and CH_2Cl_2 were dried using an MBraun solvent purification system. Other solvents were purchased as ACS grade and were used as received. All reagents were purchased from commercial suppliers and were used without further purification. Water for reverse phase purification and SPR assays was purified using a MilliQ system (MilliporeSigma). Silica gel chromatography was performed using RediSep® Rf high performance silica gel columns (Teledyne-Isco) on a Combiflash NextGen 300+ instrument (Teledyne-Isco). C18 chromatography was performed using RediSep® high performance C18 columns (Teledyne-Isco) on a Combiflash NextGen 300+ instrument (Teledyne-Isco). ^{1}H and ^{13}C NMR spectra were collected on a Bruker Advance 500 MHz spectrometer at room temperature. Chemical shifts (δ) are reported in parts per million (ppm) and the residual solvent peak is used as a reference. Coupling constants (*J*) are reported in Hertz (Hz). High resolution mass spectrometry was performed on an Orbitrap HRMS LC-MS instrument at the Analytical Biochemistry Core Facility at the University of Minnesota Masonic Cancer Center. All assayed compounds were determined to be ≥ 95% purity at 215 nm and 254 nm by analytical reverse-phase HPLC analysis on an Agilent 1200-series instrument equipped with a diode array detector and a Luna C18 column (5 µm, 100 Å, 4.6 × 150 mm, Phenomenex). The analysis method (1.0 mL/min flow rate) involved isocratic H_2O (90% H_2O: 10% CH_3CN; 0-2 min) followed by a linear gradient to 85% CH_3CN (2-24 min) and finally a linear gradient to 95% CH_3CN (24-26 min). Safety statement: No unexpected or abnormally high safety hazards were encountered.

Chemistry – Experimental Procedures:

2-(7-chloro-4-hydroxychroman-4-yl)acetonitrile (2a)



To a solution of MeCN (486 mg, 11.8 mmol) in THF (15 mL) was added LDA (5.91 mL of 2.0 M solution in THF/heptane/ethylbenzene) at -78°C under Ar. The reaction was stirred at -78°C for 1 hour. Chromanone **1a** (1.08 g, 5.91 mmol) was dissolved in THF (5 mL) and was slowly added to the reaction at -78°C under Ar. The reaction was stirred at -78°C for 1 hour. The reaction was quenched with sat. aq. NH_4Cl solution (50 mL). The mixture was extracted with EtOAc (50 mL x

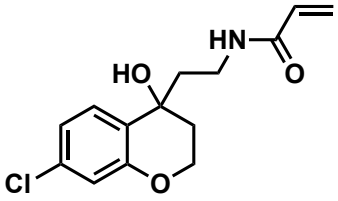
3), dried over anhydrous Na_2SO_4, and the filtrate was evaporated under reduced pressure. The crude material was purified by silica gel flash column chromatography on a gradient of EtOAc:Hexanes (1:4) to (4:1) to give **2a** as a white solid (589 mg, 45%).

^{1}H NMR (500 MHz, DMSO-*d*$_6$): δ 7.54 (d, *J* = 8.4 Hz, 1H), 6.98 (dd, *J* = 8.5, 2.2 Hz, 1H), 6.88 (d, *J* = 2.2 Hz, 1H), 5.95 (s, 1H), 4.30 – 4.22 (m, 2H), 3.13 (q, *J* = 16.7 Hz, 2H), 2.14 – 2.05 (m, 2H).

^{13}C NMR (500 MHz, DMSO-*d*$_6$): δ 154.7, 133.1, 128.9, 125.7, 120.3, 118.3, 116.2, 65.1, 63.0, 34.7, 30.3.

HRMS: $C_{11}H_{10}ClNO_2$ Calculated for $[M+H]^+$: 224.0473 Found: 224.0477.

***N*-(2-(7-chloro-4-hydroxychroman-4-yl)ethyl)acrylamide (2)**



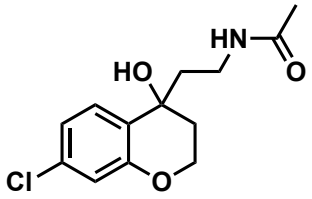
To a mixture of **2a** (723 mg, 3.23 mmol) in THF (5 mL) was added $LiAlH_4$ (307 mg, 8.08 mmol). The reaction mixture was stirred at room temperature for 30 minutes. The reaction was quenched with water (5 mL) and excess EtOAc. The reaction was filtered through a pad of celite and concentrated by rotary evaporation. The resulting residue was dissolved in DMF (2 mL) and added to a solution of acrylic acid (233 mg, 3.23 mmol), HATU (1.23 mg, 3.23 mmol), and DIPEA (2.09 g, 16.2 mmol) in DMF (10 mL). The reaction was stirred overnight at room temperature. The reaction mixture was concentrated by rotary evaporation. The crude material was purified by silica gel flash column chromatography on a gradient of EtOAc:Hexanes (1:9) to (4:1) followed by reverse phase purification using a gradient of 10% to 90% MeCN in H_2O to yield **2** as a white solid (448 mg, 49%).

^{1}H NMR (500 MHz, DMSO-*d*$_6$): δ 8.03 (t, *J* = 5.6 Hz, 1H), 7.42 (d, *J* = 8.5 Hz, 1H), 6.94 (dd, *J* = 8.5, 2.2 Hz, 1H), 6.82 (d, *J* = 2.1 Hz, 1H), 6.16 (dd, *J* = 17.2, 10.2 Hz, 1H), 6.04 (dd, *J* = 17.0, 2.2 Hz, 1H), 5.55 (dd, *J* = 10.2, 2.2 Hz, 1H), 5.32 (s, 1H), 4.25 – 4.17 (m, 2H), 3.21 – 3.13 (m, 2H), 2.07 – 1.88 (m, 4H).

^{13}C NMR (500 MHz, DMSO-*d*$_6$): δ 164.4, 154.6, 132.1, 131.8, 128.8, 128.1, 124.8, 120.0, 116.0, 65.8, 63.3, 40.4, 34.4, 34.0.

HRMS: $C_{14}H_{16}ClNO_3$ Calculated for $[M+H]^+$: 282.0891 Found: 282.0894.

***N*-(2-(7-chloro-4-hydroxychroman-4-yl)ethyl)acetamide (3)**



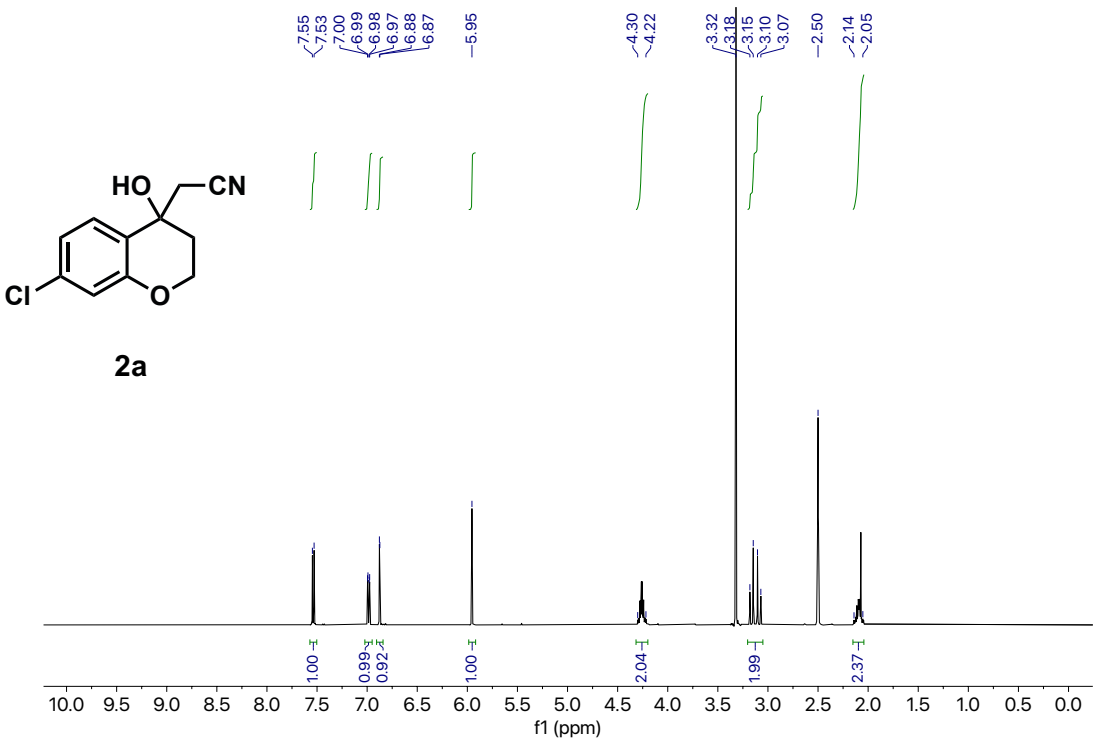
To a mixture of **2a** (704 mg, 3.15 mmol) in THF (5 mL) was added $LiAlH_4$ (299 mg, 7.87 mmol). The reaction mixture was stirred at room temperature for 30 minutes. The reaction was quenched with water (5 mL) and excess EtOAc. The reaction was filtered through a pad of celite and concentrated by rotary evaporation. The resulting residue was dissolved in THF (5 mL), Et_3N (319 mg, 3.15 mmol) was added, and the mixture was cooled to 0°C. Acetyl chloride (247 mg, 3.15 mmol) was slowly added and the reaction was stirred at room temperature for 30 minutes. The reaction was quenched with water (10 mL), extracted with EtOAc (50 mL), dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation. The crude material was purified by silica gel flash column chromatography on a gradient of EtOAc:Hexanes (1:9) to (4:1) followed by reverse phase purification using a gradient of 10% to 90% MeCN in H_2O to yield **3** as a white solid (492 mg, 58%).

^{1}H NMR (500 MHz, DMSO-*d*$_6$): δ 7.75 (t, *J* = 5.5 Hz, 1H), 7.40 (d, *J* = 8.5 Hz, 1H), 6.93 (dd, *J* = 8.3, 2.2 Hz, 1H), 6.82 (d, *J* = 2.3 Hz, 1H), 5.29 (s, 1H), 4.24 – 4.16 (m, 2H), 3.10 – 3.01 (m, 2H), 2.03 – 1.83 (m, 4H), 1.75 (s, 3H).

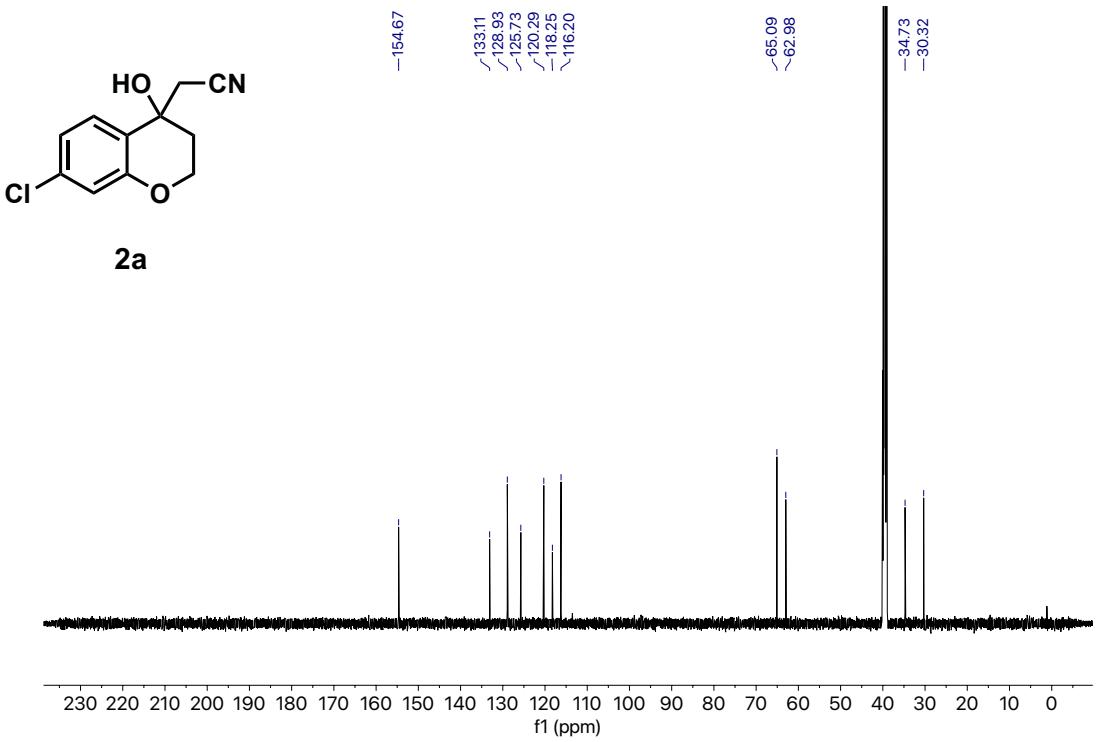
^{13}C NMR (500 MHz, DMSO-*d*$_6$): δ 168.9, 154.6, 132.1, 128.8, 128.2, 120.0, 116.0, 65.8, 63.3, 40.5, 34.4, 34.0, 22.6.

HRMS: $C_{13}H_{16}ClNO_3$ Calculated for $[M+H]^+$: 270.0891 Found: 270.0892.

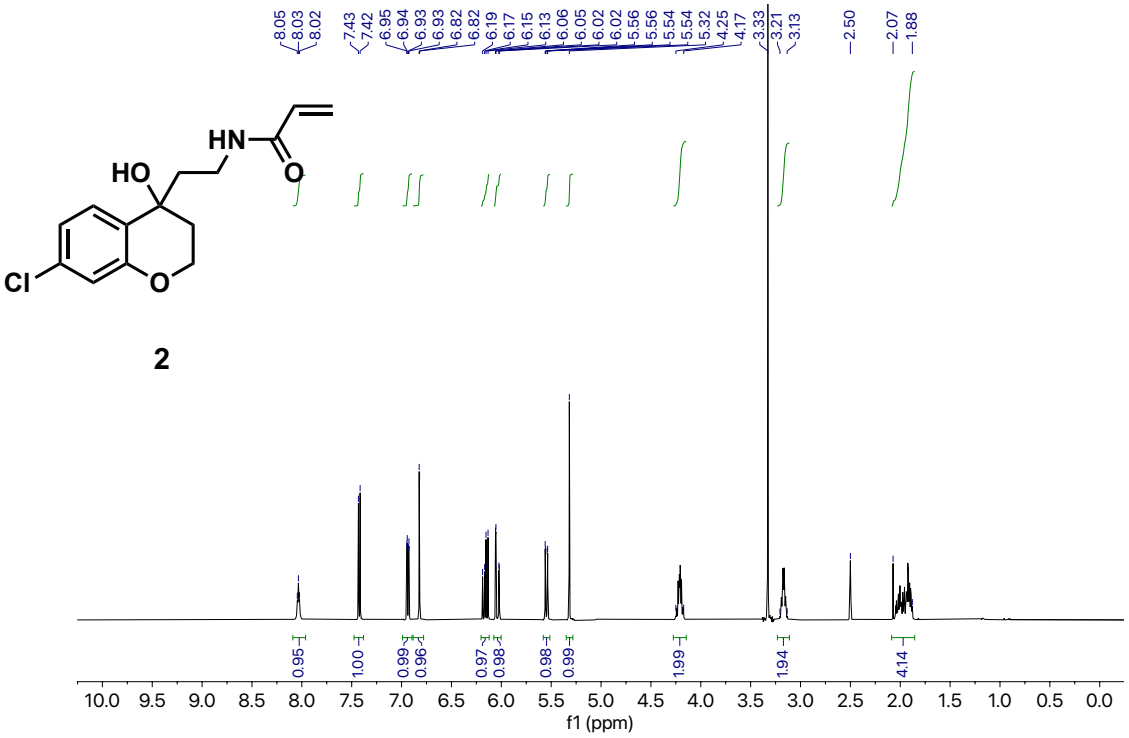
Chemistry – ^{1}H and ^{13}C NMR Spectra



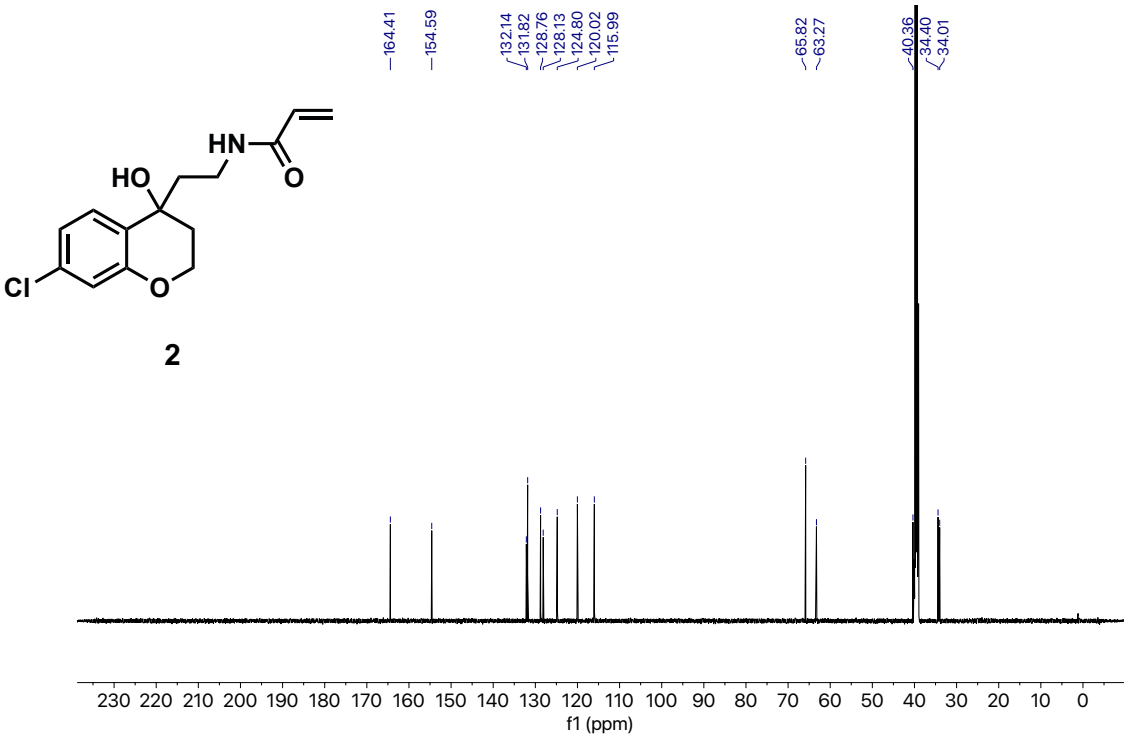
2a



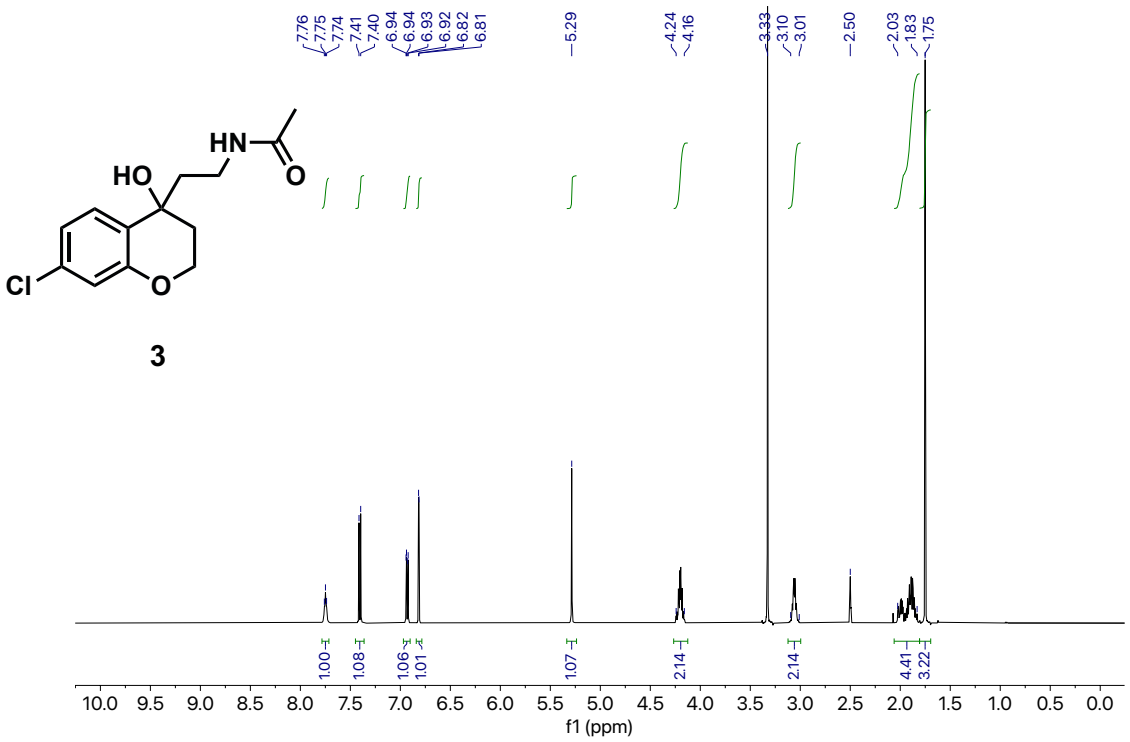
2a


8.05
8.03
8.02
7.43
7.42
6.95
6.94
6.93
6.82
6.82
6.19
6.17
6.15
6.13
6.06
6.05
6.02
6.02
5.56
5.54
5.54
5.32
4.25
4.17
3.33
3.21
3.13
2.50
2.07
1.88
HN
HO
O
Cl
O
0.95
1.00
0.99
0.96
0.97
0.98
0.98
0.99
1.99
1.94
4.14
10.0 9.5 9.0 8.5 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 0.0
f1 (ppm)

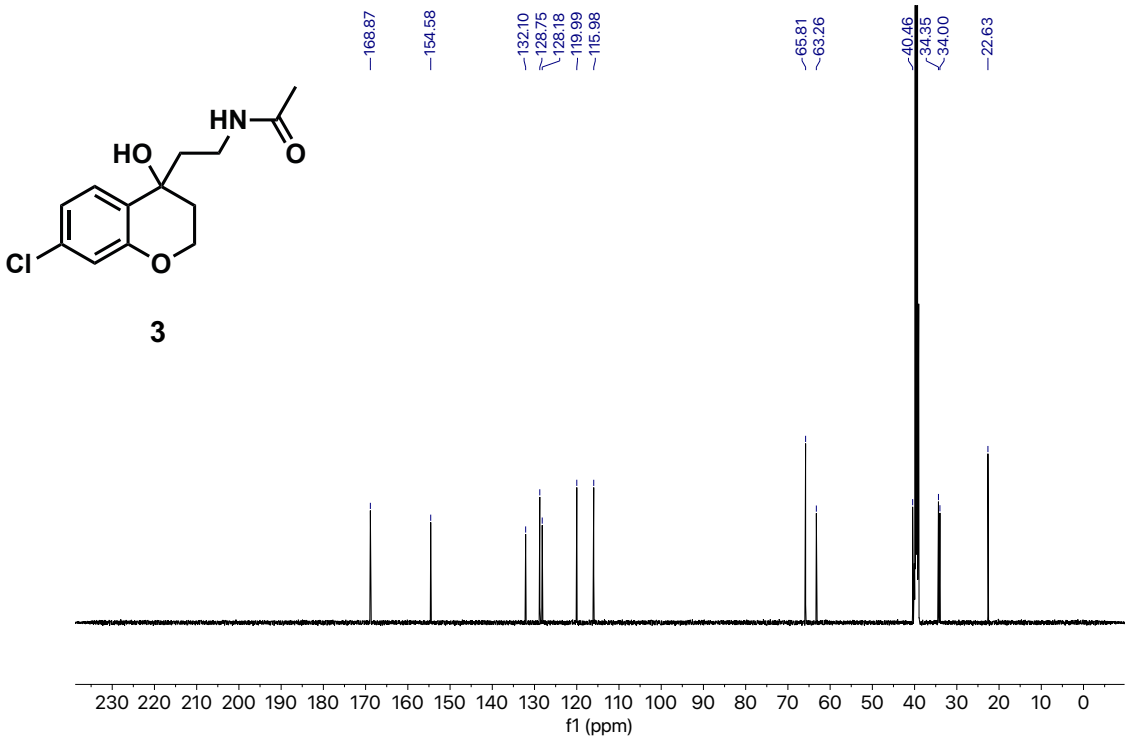
2


164.41
154.59
132.14
131.82
128.76
128.13
124.80
120.02
115.99
65.82
63.27
40.36
34.40
34.01
HN
HO
O
Cl
O
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

2


HN
O
HO
Cl
O
7.76
7.75
7.74
7.41
7.40
6.94
6.94
6.93
6.92
6.82
6.81
5.29
4.24
4.16
3.33
3.10
3.01
2.50
2.03
1.83
1.75
1.00
1.08
1.06
1.01
1.07
2.14
2.14
4.41
3.22
10.0 9.5 9.0 8.5 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 0.0
f1 (ppm)

3


HN
O
HO
Cl
O
168.87
154.58
132.10
128.75
128.18
119.99
115.98
65.81
63.26
40.46
34.35
34.00
22.63
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

3

Assay Procedures

NIK Protein for Assays

Human NIK protein was purchased from GenScript at ≥ 85% purity (residues 330-680, S549D mutation, with N-terminal 6xHis-tag and TEV cleavage site). The storage buffer for NIK protein consisted of 20 mM Tris (pH = 8.0), 300 mM NaCl, 10% glycerol, 0.25 mM TCEP. The 6xHis-tag was kept intact for all experiments. For SPR, NIK was buffer exchanged into 40 mM KH_2PO_4/K_2HPO_4 (pH = 8), 100 mM KCl, 10 mM $MgCl_2$, 0.01% Tween-20 using Amicon Ultra-0.5 centrifugal filter units (MilliporeSigma, 3 kDa filter). NIK concentration was measured on a NanoDrop 2000 spectrophotometer (Thermo Scientific). EZ-Link NHS-PEG4-Biotin (Thermo Scientific) was dissolved in H_2O and added to the NIK protein at a 1:1 ratio. The mixture was incubated overnight at 4°C. The mixture was buffer exchanged into 40 mM KH_2PO_4/K_2HPO_4 (pH = 8.0), 100 mM KCl, 10 mM $MgCl_2$, 0.01% Tween- 20 using Amicon Ultra-0.5 centrifugal filter units to remove the excess EZ-Link NHS-PEG4-Biotin reagent. The biotinylated NIK protein concentration was measured on a NanoDrop 2000 spectrophotometer and diluted into running buffer to be used for all SPR assays.

SPR

Surface plasmon resonance (SPR) was performed at the UMN Institute for Therapeutics Discovery and Development (ITDD) High-Throughput Screening Laboratory. A Series S NeutrAvidin-coated sensor chip (Cytiva, 29407997) was docked into a Biacore S200 instrument. The running buffer consisted of 10 mM HEPES (pH = 7.4), 150 mM NaCl, 10 mM $MgCl_2$, 3 mM DTT, 0.5 mg/mL γ-globulins, 0.005% (v/v) Tween20, 1% DMSO (when testing small molecules). Biotinylated human NIK protein at 100 µg/mL was injected over flow-cells 2 and 4 (flow-cells 1 and 3 were used for reference subtraction) with a contact time of 600 sec at a flowrate of 5 µL/min. Observed immobilization levels were 5000-8000 response units (RU). To block the remaining NeutrAvidin sites, 100 µM solution of biocytin was injected over all flow-cells with a contact time of 300 sec at a flowrate of 10 µL/min. For compound **2**, a single-cycle SPR method was used, and for compound **3**, a multi-cycle SPR method was used. Compound **2** was run at a top concentration of 50 µM, and compound **3** was run at a top concentration of 100 µM. 2-fold serial dilutions were made from the top concentration to obtain 7-dose response series. The activity of the chip surface was monitored by including reference compound staurosporine (pan-kinase inhibitor) first and last in each run. The assay was stable for one day at room temperature. K_d

values were determined using Biacore software with a steady-state fit. The mean ± SEM was calculated using Microsoft Excel (version 16.90).

LC-MS-MS Peptide Mapping

NIK was buffer exchanged into LC-MS-MS buffer consisting of 10 mM Tris (pH = 8), 50 mM NaCl, 1 mM TCEP using fast protein liquid chromatography (BioRad, NGC™ Chromatography System). The NIK protein concentration was measured on a NanoDrop 2000 spectrophotometer and diluted to 5 µM in LC-MS-MS buffer. For peptide mapping with **2**: 50 µL of 5 µM NIK was incubated with 100 µM of **2** for 30 minutes. For peptide mapping with heavy iodoacetamide (C_2D_4NOI): 50 µL of 5 µM NIK was incubated with 500 µM of heavy iodoacetamide (Cambridge Isotope Laboratories, DLM-7249) for 30 minutes. Excess compound was removed using Zeba™ Spin Desalting Columns, 7K MWCO (Thermo Scientific). The protein extract was frozen and dried on a Vacufuge Plus (Eppendorf). The dried protein samples were denatured and reduced with 20 µL of an aqueous solution consisting of 8 M urea and 20 mM DTT at 95°C for 10 minutes. The protein samples were then alkylated with 40 mM iodoacetamide (C_2H_4NOI) at room temperature for 30 minutes in the absence of light. The samples were diluted with 160 µL of 50 mM NH_4HCO_3 (pH = 8) and incubated with 0.5 µg of Pierce™ Trypsin Protease (Promega) overnight at 37°C. The protein sample digests were extracted using Pierce™ C18 tips, 100 µL (Thermo Scientific) following the manufacturer's protocol. The extracted peptides were frozen and dried on a Vacufuge Plus (Eppendorf). LC-MS-MS experiments were performed at the Analytical Biochemistry Core Facility at the University of Minnesota Masonic Cancer Center. The samples were dissolved in 10 µL of 2% CH_3CN, 0.1% formic acid in H_2O and 5 µL were loaded onto a home-packed analytical C18 reverse phase column with a 10 µm emission tip (75 µm x 200 mm [New Objective], with Luna C18 5 µm particles [Phenomenex]) via UltiMate™ 3000 liquid chromatography system (Thermo Scientific). Peptides were eluted with buffer A (0.1% formic acid in H_2O) and buffer B (0.1% formic acid in CH_3CN) with the following gradient profile: 0-5.5 min for loading, 2% B, flowrate 1.0 µL/min; 6-12 min, 2-10% B, 0.3 µL/min; 12-52 min, 10-25% B, 0.3 µL/min; 52-57 min, 25-40% B, 0.3 µL/min; 57-58 min, 40-85% B, 0.3 µL/min; 58-62 min 85% B, 0.3 µL/min; 62-63 min, 85-2% B, 0.3 µL/min; 63-65 min, 2% B, 1.0 µL/min. Mass spectrometry was obtained on an Orbitrap Fusion Tribrid Mass Spectrometer (ThermoFisher) in positive nanospray ionization mode. The mass spectrometer conditions were: spray voltage 2.2 kV, ion transfer tube temperature 300°C. MS survey scans were performed with a cycle time of 3 seconds. After each survey scan, the 10 to 20 most abundant precursor ions with $z > 1$ were selected for fragmentation using higher energy collisional dissociation (HCD). MS^1 resolution was

at 120,000 with a scan range of 300 – 1800. MS^2 resolution was fixed at 15,000. N = 2 biological replicates of the digestion and mass spectrometry analysis was performed with both **2** and heavy iodoacetamide. The resulting data was analyzed using Proteome Discoverer 3.0 (Thermo Scientific) for chromatogram processing and fragment spectra isolation. Peptides were analyzed by referencing the NIK protein sequences from a FASTA file created in-house, with cysteine carbamidomethylation, cysteine carbamidomethylation-d_2, and the compound **2** molecular weight set as dynamic modifications.