Supplementary Materials for

A Novel Chemical Attack on Notch-mediated transcription by targeting the NACK ATPase

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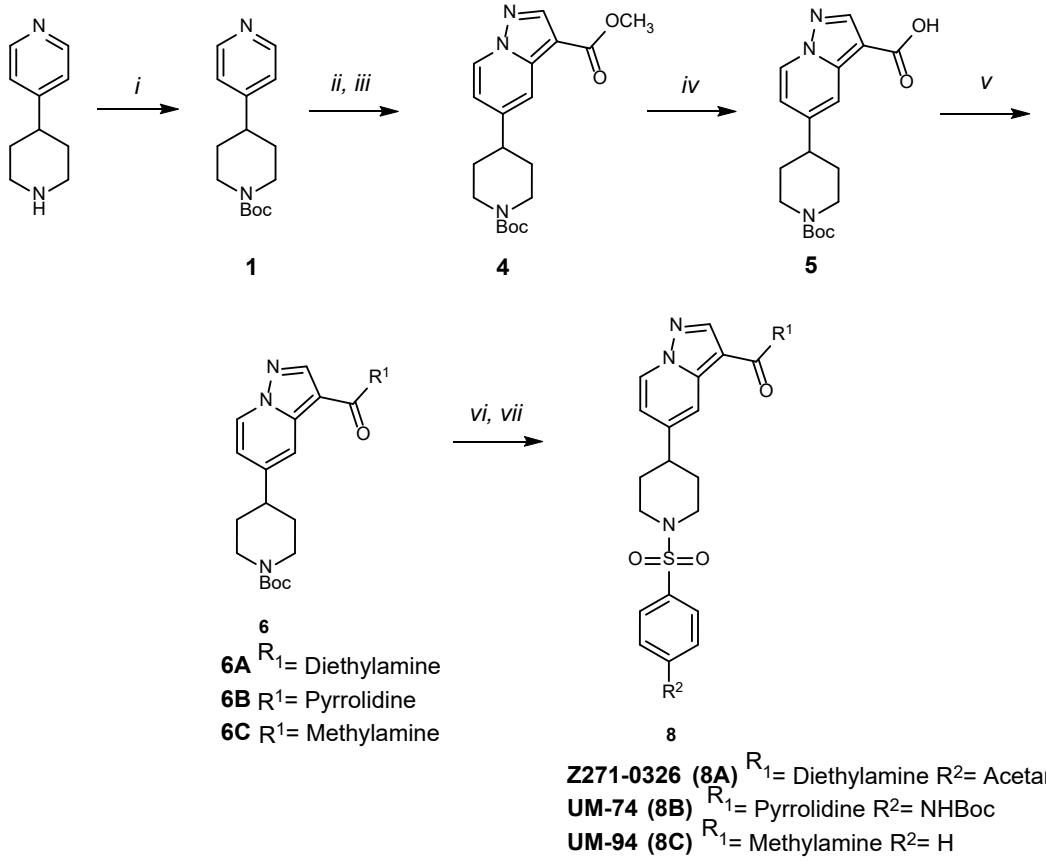
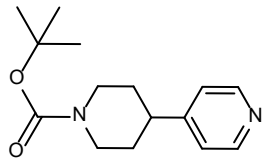
This PDF file includes:

Synthetic Methods



i. $BocO_2$, TEA, DMAP, DCM, 0 ºC to RT, 2 hrs (95% yield) ***ii***. MSH, DMF, RT, 16 hrs ***iii***. K_2CO_3, methylpropiolate, RT, 2 days (44% yield) ***iv***. LiOH, MeOH, H_2O, THF, 100 ºC, 8 hrs, microwave irradiation (81% yield) ***v***. HATU, DIPEA, DMF, RT, 1-16 hrs (Quantitative to 81% yield) ***vi***. TFA, DCM, 0 ºC to RT, 1 hr (Quantitative to 51% yield)
vii. Arylsulfonamide, TEA, DCM, 0 ºC to RT, 1 hr (78-58% yield)



Boc-4-pyridin-4-yl-piperidine (**1**):

Boc anhydride (1.61 g, 7.4 mmol) was added to a round-bottom flask and dissolved in DCM (10.3 mL). DMAP (7.33 mg, 0.06 mmol), 4-pyridin-4-yl-piperidine (1.00 g, 6.16 mmol), followed by TEA (1.05 mL) was added in that order and the reaction was allowed to stir at room temperature for 2 hours. At this point, all starting material had been converted to product as seen by TLC. Reaction was diluted with 20 ml EtOAc, washed with 10 mL water twice, and once again with 10 mL saturated brine solution, dried with sodium sulfate and concentrated *in vacuo*. Product was purified *via* flash chromatography using a 12 to100% EtOAc/Hexanes gradient. Yield was 95%, 1.53 g, white solid. Rf = 0.3 in 50/50 EtOAc/Hexanes.

^{1}H NMR (600 MHz, Chloroform-d) δ 8.59 – 8.43 (m, 2H), 7.13 – 6.85 (m, 2H), 4.19 (s, 2H), 2.74 (s, 2H), 2.58 (tt, J = 12.2, 3.6 Hz, 1H), 1.76 (d, J = 13.1 Hz, 2H), 1.54 (dtd, J = 16.8, 9.5, 7.0, 4.0 Hz, 2H), 1.41 (s, 9H).

^{13}CNMR:(151 MHz, Chloroform-d) δ 154.67, 154.21, 149.93, 122.13, 79.58, 41.91, 32.20, 28.39.

MS (ESI) m/z: 263.20 $(M+H)^{+}$.

N-Boc-O-(mesitylsulfonyl)hydroxylamine (2)

Prepared accordingly to J. Mendiola *et al* on a scale of 2.0 g of 2-Mesitylenesufonylchloride[33]. White waxy solid. Crude yield is quantitative, 2.88 g. Rf = 0.32 in 10% EtOAc in 90% Hexanes. NMR obtained matched reported literature values.

1HNMR: (400 MHz, Chloroform-d) δ 7.77 (1H, s), 6.98 (2H, s), 2.66 (6H, s), 2.31 (3H, s), 1.30 (9H, s).

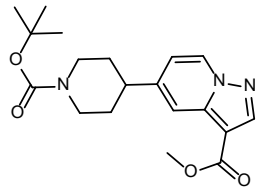
CNMR: 13C NMR (100 MHz, Chloroform-d) δ 154.5, 144.7, 142.2, 131.9, 128.7, 84.1, 27.9, 23.4, 21.4.

O-Mesitylenesulfonylhydroxylamine (MSH) (3)

Prepared accordingly to J. Mendiola *et al* on a scale of 2.88 g of N-Boc-O-(mesitylsulfonyl)hydroxylamine[33]. At the end of the reaction, MSH was filtered, washed with cold water, and stored in the freezer. No further purification was necessary. MSH is known to be unstable, and potentially explosive when fully dry. Compound stored wet (estimated 30% water), and used as is in the following step. Compound is a white solid, with a yield of 2.04 g. NMR obtained matched reported literature values.

^{1}HNMR: (400 MHz, Chloroform-d) δ 7.00 (s, 2H), 4.80 (s, 3H), 2.65 (s, 6H), 2.33 (s, 3H).

^{13}CNMR: (100 MHz, Chloroform-d): δ 21.1, 22.7, 128.9, 131.7, 141.0, 143.9



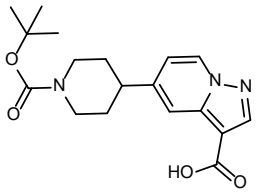
tert-butyl 4-[3-(methoxycarbonyl)pyrazolo[1,5-a]pyridin-5-yl]piperidine-1-carboxylate (4)

Boc-4-pyridin-4-yl-piperidine (1.56 g, 5.95 mmol) was dissolved in DMF (12 mL, 0.5M) and added to a round-bottom flask. MSH was added in two portions (1665 mg, 7.74 mmol) and the reaction stirred at RT for sixteen hours. After which, methyl prop-2-ynoate was added (792 µL, 8.9 mmol) followed by K_2CO_3 (1.23 g, 8.9 mmol). The reaction stirred for 2 days at RT, and was then diluted with 50 mL EtOAc and washed twice with 25 mL water, and twice with 25 mL saturated LiCl solution, and finally dried with brine, and Na_2SO_4. Organic extract was concentrated and purified *via* flash chromatography and eluted with a 6-50% gradient of EtOAc in hexanes. Fractions were concentrated *in-vacuo* and dried under high-vac overnight. Yellow solid, 957 mg, 44% yield. Rf = 0.30 in a 25% EtOAc in 75% hexanes.

^{1}H NMR (600 MHz, Chloroform-d) δ 8.43 (dd, J = 7.1, 0.9 Hz, 1H), 8.35 (s, 1H), 7.95 (dt, J = 1.9, 0.9 Hz, 1H), 6.81 (dd, J = 7.2, 2.0 Hz, 1H), 4.28 (s, 2H), 3.90 (s, 3H), 2.90 – 2.66 (m, 3H), 1.88 (d, J = 13.1 Hz, 2H), 1.66 (qd, J = 12.6, 4.4 Hz, 2H), 1.48 (s, 9H).

^{13}C NMR (151 MHz, Chloroform-d) δ 163.86, 154.61, 146.04, 145.05, 140.99, 128.96, 115.46, 113.69, 102.95, 79.62, 51.10, 42.25, 32.28, 28.38.

HRMS *m/z* $(M+H)^+$ Calcd for $C_{19}H_{26}N_3O_4$:360.1923, Found 360.1944



5-{1-[(tert-butoxy)carbonyl]piperidin-4-yl}pyrazolo[1,5-a]pyridine-3-carboxylic acid (5)

tert-butyl 4-[3-(methoxycarbonyl)pyrazolo[1,5-a]pyridin-5-yl]piperidine-1-carboxylate (932 mg, 2.59 mmol) was dissolved in a 2:1:2 solvent mixture of THF: MeOH: Water (5 mL, 3 mL, 5 mL) and added to a microwave vial, followed by LiOH was added (373 mg, 15.54 mmol) The reaction was irradiated at 100 °C for 8 hours. After which, all starting material had been converted, as seen by TLC. 20 ml EtOAc was added to the reaction, and then 1M HCl was added until solution was acidic. Aqueous layer was extracted with EtOAc x 3, and organic extracts were combined and washed with brine, followed by drying with Na2SO4 and then concentrated in vacuo. Crude product was purified via flash chromatography with an 18- 100% solvent gradient of EtOAc in hexanes. Yield was 723 mg, light yellow solid, 81% yield. Rf = 0.57 in 75% EtOAc in 25% hexanes

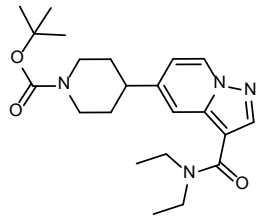
^{1}H NMR (600 MHz, Chloroform-d) δ 8.50 (dd, J = 7.1, 0.9 Hz, 1H), 8.45 (s, 1H), 8.02 (dt, J = 1.8, 0.9 Hz, 1H), 6.87 (dd, J = 7.2, 2.0 Hz, 1H), 5.30 (s, 1H), 4.31 (s, 2H), 2.89 – 2.76 (m, 3H), 1.91 (d, J = 13.0 Hz, 2H), 1.69 (qd, J = 12.6, 4.3 Hz, 2H), 1.50 (s, 9H).

^{13}C NMR (151 MHz, Chloroform-d) δ 168.62, 154.75, 146.84, 146.03, 141.53, 129.27, 115.84, 114.08, 102.49, 79.80, 60.43, 42.41, 32.39, 28.50.

MS (ESI) m/z: 344.20 $(M-H)^-$.

General procedure I

5-{1-[(tert-butoxy)carbonyl]piperidin-4-yl}pyrazolo[1,5-a]pyridine-3-carboxylic acid (1 equiv) was dissolved in DMF (0.25 M) and added to a scintillation vial. HATU (1.5 equiv), and DIPEA (6 equiv) were added to the vial in that order. The reaction was stirred at RT for ten minutes, and then corresponding amine was added (2 equiv). Reaction was stirred at RT for 2 hours, after which all starting material had been converted, as seen by TLC. 10 mL EtOAc was added to the reaction, and the organic extract was washed with water twice, followed by Saturated LiCl solution x 2, and lastly washed with saturated brine solution. Organic extract was dried with Na_2SO_4 and concentrated in vacuo. Crude product was purified via flash chromatography.



tert-butyl 4-[3-(diethylcarbamoyl)pyrazolo[1,5-a]pyridin-5-yl]piperidine-1-carboxylate (**6A**):

Following general procedure I, 5-{1-[(tert-butoxy)carbonyl]piperidin-4-yl}pyrazolo[1,5-a]pyridine-3-carboxylic acid (100 mg, 0.29 mmol) was dissolved in DMF (1.16 mL) and added to a scintillation vial. HATU (167.3 mg, 0.44 mmol), and DIPEA (304 μL, 1.74 mmol) were added to the vial in that order. The reaction was stirred at RT for ten minutes, and then diethylamine was added (60 μL, 0.58 mmol). Reaction was stirred at RT for 2 hours, after which

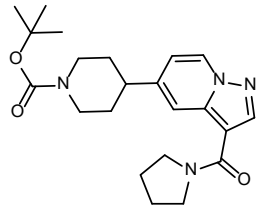
all starting material had been converted, as seen by TLC. After workup, crude product was purified via flash chromatography with an 18-100% solvent gradient of EtOAc/hexanes.

Yield was 115 mg, light yellow solid, quantitative yield. Rf = 0.58 in 75% EtOAc in 25% hexanes

^{1}H NMR: (400 MHz, Chloroform-d) δ 8.53 (d, J = 7.2 Hz, 1H), 8.14 (s, 1H), 7.81 (d, J = 1.9 Hz, 1H), 7.00 (dd, J = 7.2, 2.0 Hz, 1H), 4.24 (d, J = 13.2 Hz, 2H), 3.62 (q, J = 7.1 Hz, 4H), 2.96 – 2.82 (m, 3H), 1.92 (d, J = 12.9 Hz, 2H), 1.64 (qd, J = 12.4, 4.2 Hz, 2H), 1.48 (s, 9H), 1.30 (t, J = 7.1 Hz, 6H).

^{13}CNMR: (151 MHz, Methanol-d4) δ 166.39, 156.43, 146.58, 142.52, 142.16, 129.63, 116.39, 115.39, 106.87, 81.11, 49.42-48.47 (overlapping peak with CD_3OD) 43.14, 33.44, 28.71, 14.03.

HRMS m/z $(M+H)^+$ Calcd for $C_{22}H_{33}N_4O_3$:401.2553, Found 401.2545



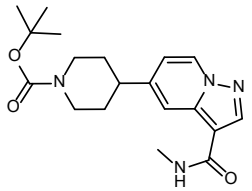
tert-butyl 4-[3-(pyrrolidine-1-carbonyl)pyrazolo[1,5-a]pyridin-5-yl]piperidine-1-carboxylate (**6B**):

Following general procedure I, 5-{1-[(tert-butoxy)carbonyl]piperidin-4-yl}pyrazolo[1,5-a]pyridine-3-carboxylic acid (100 mg, 0.29 mmol) was dissolved in DMF (1.16 mL) and added to a scintillation vial. HATU (167.3 mg, 0.44 mmol), and DIPEA (304 μL, 1.74 mmol) were added to the vial in that order. The reaction was stirred at RT for ten minutes, and then pyrrolidine was added (48 μL,0.58 mmol). Reaction was stirred at RT for 2 hours, after which all starting material had been converted, as seen by TLC. After workup, crude product was purified via flash chromatography with an 18-100% solvent gradient of EtOAc/hexanes.115 mg, yellow solid, quantitative yield. Rf = 0.38 in 75% EtOAc in 25% hexanes

^{1}H NMR (400 MHz, Chloroform-d) δ 8.56 (d, J = 7.2 Hz, 1H), 8.36 (s, 1H), 8.09 (s, 1H), 7.06 (dd, J = 7.2, 2.0 Hz, 1H), 4.27 (d, J = 13.3 Hz, 2H), 3.77 (m, J = 71.7 Hz, 4H), 3.02 – 2.86 (m, 2H), 2.12 – 1.99 (m, 4H), 1.96 (d, J = 13.2 Hz, 2H), 1.67 (qd, J = 12.6, 4.2 Hz, 2H), 1.51 (s, 9H).

^{13}C NMR (151 MHz, Methanol-d4) δ 163.75, 155.02, 145.59, 141.92, 141.41, 128.20, 115.57, 114.32, 105.95, 79.71, 41.76, 37.48, 32.08, 27.31, 26.12, 23.67.

HRMS m/z (M+H)+ Calcd for $C_{22}H_{31}N_4O_3$:399.2396, Found 399.2403



tert-butyl 4-[3-(methylcarbamoyl)pyrazolo[1,5-a]pyridin-5-yl]piperidine-1-carboxylate (6C)

Following general procedure I, 5-{1-[(tert-butoxy)carbonyl]piperidin-4-yl}pyrazolo[1,5-a]pyridine-3-carboxylic acid (100 mg, 0.29 mmol) was dissolved in DMF (1.16 mL) and added to a scintillation vial. HATU (167.3 mg, 0.44 mmol), and DIPEA (304 μL, 1.74 mmol) were added to the vial in that order. The reaction was stirred at RT for ten minutes, and then Methylamine in EtOH [8M] was added (73 μL,0.58 mmol). Reaction was stirred at RT for 2 hours, after which all starting material had been converted, as seen by TLC. After workup, crude product was purified via flash chromatography with an 18-100% solvent gradient of EtOAc/hexanes.115 mg, yellow solid, quantitative yield. Rf = 0.38 in 75% EtOAc in 25% hexanes. 87 mg, 81% yield, white solid. Rf = 0.45 in 75% EtOAc in 25% hexanes

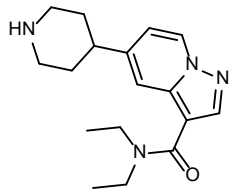
^{1}HNMR: (400 MHz, Methanol-d4) δ 8.51 (d, J = 7.2 Hz, 1H), 8.32 (s, 1H), 8.07 (s, 1H), 7.00 (dd, J = 7.2, 2.0 Hz, 1H), 4.25 (d, J = 13.2 Hz, 2H), 2.86 (m, 6H), 1.93 (d, J = 13.0 Hz, 2H), 1.65 (qd, J = 12.5 Hz, 4.2 Hz, 2H). 1.48 (s, 9H)

^{13}CNMR: (151 MHz, Methanol-d4) δ 166.43, 156.43, 147.10, 142.59, 141.74, 129.74, 116.42, 115.40, 107.24, 81.12, 49.42-48.47 (overlapping peak with CD_3OD), 43.18, 38.88, 33.46, 28.71, 26.25.

HRMS: m/z (M+H)+ Calcd for $C_{18}H_{27}N_4O_3$:359.2077, Found 399.2073

General procedure II

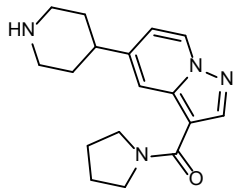
tert-butyl 4-[3-(diethylcarbamoyl)pyrazolo[1,5-a]pyridin-5-yl]piperidine-1-carboxylate (1 equiv) was dissolved in DCM (0.2M) and added to a round-bottom flask in an ice bath. Reaction was cooled to 0 °C, and TFA (0.4M) was added to the flask. Reaction was then warmed to RT and stirred for 1 hr. Reaction was concentrated, and 2 M NaOH was added dropwise until a white precipitate appeared and the solution was basic. The aqueous solution was extracted with EtOAc x 3. The organic extracts were combined and washed with brine, followed by drying with Na_2SO_4 and then concentrated in-vacuo. Crude product was clean (98% to 93% purity determined by LCMS) and utilized in the next step without further purification.



N,N-diethyl-5-(piperidin-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (**7A**)

Following general procedure II, tert-butyl 4-[3-(diethylcarbamoyl)pyrazolo[1,5-a]pyridin-5-yl]piperidine-1-carboxylate (100 mg, 0.25 mmol) was dissolved in DCM (1.25 mL) and added to a round-bottom flask in an ice bath. Reaction was cooled to 0 °C, and TFA (625 μL) was added to the flask. Reaction was then warmed to RT and stirred for 1 hr. Reaction was concentrated, and workup was performed as described. 87% yield, 66 mg, white solid.

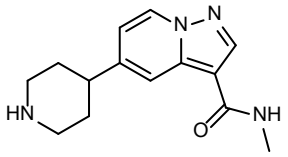
MS (ESI) m/z: 301.25 $(M+H)^+$



4-[3-(pyrrolidine-1-carbonyl)pyrazolo[1,5-a]pyridin-5-yl]piperidine (**7B**):

Following general procedure II, tert-butyl 4-[3-(pyrrolidine-1-carbonyl)pyrazolo[1,5-a]pyridin-5-yl]piperidine-1-carboxylate (100 mg, 0.23 mmol) was dissolved in DCM (1.15 mL) and added to a round-bottom flask in an ice bath. Reaction was cooled to 0 °C, and TFA (575 μL) was added to the flask. Reaction was then warmed to RT and stirred for 1 hr. Reaction was concentrated, and workup was performed as described. Yield was 35 mg, 51% yield. Compound is a light yellow solid.

MS (ESI) m/z: 299.24 $(M+H)^+$



N-methyl-5-(piperidin-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (**7C**):

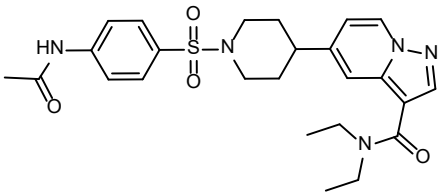
Following general procedure II, tert-butyl 4-[3-(methylcarbamoyl)pyrazolo[1,5-a]pyridin-5-yl]piperidine-1-carboxylate (50.2 mg, 0.14 mmol) was dissolved in DCM (700 μL) and added to

a round-bottom flask in an ice bath. Reaction was cooled to 0 °C, and TFA (350 μL) was added to the flask. Reaction was then warmed to RT and stirred for 1 hr. Reaction was concentrated, and workup was performed as described. Yield was 35 mg, quantitative yield. Compound is a white solid.

MS (ESI) m/z: 259.15 $(M+H)^+$

General procedure III

N,N-diethyl-5-(piperidin-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (1 equiv) was dissolved in DCM (0.3 M) and added to a round-bottom flask. The flask was cooled to 0 °C, and TEA (3.0M) was added, followed by corresponding arylsulfonyl chloride (1.1 equiv). The flask was then warmed to RT and stirred for 1 hr. The reaction was diluted with EtOAc and washed with water twice followed once with brine, dried with Na_2SO_4 and then concentrated *in-vacuo*. Crude product was purified via flash chromatography with an 18% to 100% solvent gradient of EtOAc/hexanes.



5-[1-(4-acetamidobenzenesulfonyl)piperidin-4-yl]-N,N-diethylpyrazolo[1,5-a]pyridine-3-carboxamide (**Z231-03265**, **8A**)

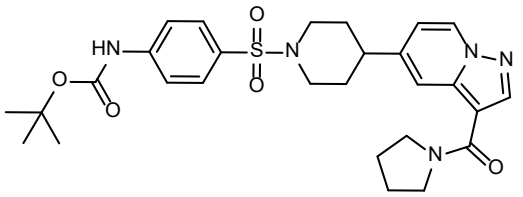
Following general procedure III, N,N-diethyl-5-(piperidin-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (82 mg, 0.27 mmol, 1 equiv) was dissolved in DCM (900 μL) and added to a

round-bottom flask. The flask was cooled to 0 °C, and TEA (90 μL) was added, followed by 4-acetamidobenzene-1-sulfonyl chloride (69 mg, 0.30 mmol). The flask was then warmed to RT and stirred for 1 hr. The reaction was worked up and crude product was purified via flash chromatography with an 18% to 100% solvent gradient of EtOAc in hexanes. 104 mg, white solid, 78% yield. Rf = 0.15 in a 75% EtOAc in 25% hexanes.

^{1}H NMR (400 MHz, Methanol-d_4) δ 8.51 (d, J = 7.2 Hz, 1H), 8.13 (s, 1H), 7.87 – 7.71 (m, 4H), 6.95 (dd, J = 7.3, 1.9 Hz, 1H), 3.91 (d, J = 11.7 Hz, 2H), 3.61 (q, J = 7.1 Hz, 4H), 2.63 (t, J = 12.0 Hz, 0H), 2.45 (t, J = 11.6 Hz, 2H), 2.17 (s, 2H), 1.96 (s, 1H), 1.82 (qd, J = 12.6, 4.0 Hz, 2H), 1.28 (t, J = 7.1 Hz, 6H).

^{13}CNMR: (151 MHz, Chloroform-d) δ 168.83, 164.35, 143.38, 142.37, 141.46, 140.95, 130.91, 128.76, 128.49, 119.52, 116.13, 113.12, 105.96, 46.34, 41.24, 29.62, 24.53, 13.81.

HRMS m/z $(M+H)^+$ Calcd for $C_{25}H_{32}N_5O_4S$: 498.2175, Found 498.2177



tert-butyl N-[4-({4-[3-(pyrrolidine-1-carbonyl)pyrazolo[1,5-a]pyridin-5-yl]piperidin-1-yl}sulfonyl)phenyl]carbamate (**UM-74**, **8B**)

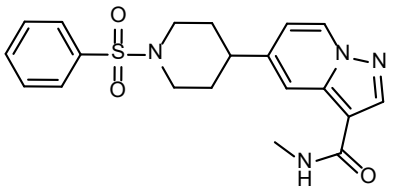
Following general procedure III, 4-[3-(pyrrolidine-1-carbonyl)pyrazolo[1,5-a]pyridin-5-yl]piperidine (82 mg, 0.27 mmol, 1 equiv) was dissolved in DCM (900 μL) and added to A

round-bottom flask. The flask was cooled to 0 °C, and TEA (90 μL) was added, followed by tert-butyl N-[4-(chlorosulfonyl)phenyl]carbamate (87.5 mg, 0.30 mmol). The flask was then warmed to RT and stirred for 1 hr. The reaction was worked up and crude product was purified via flash chromatography with an 18% to 100% solvent gradient of EtOAc in hexanes. 58 % yield, 87 mg, compound is a white solid. Rf = 0.33 in a 75% EtOAc in 25% hexanes.

^{1}H NMR (400 MHz, Methanol-d_4) δ 8.52 (d, J = 7.2 Hz, 1H), 8.32 (s, 1H), 8.02 (d, J = 1.9 Hz, 1H), 7.76 – 7.62 (m, 4H), 6.98 (dd, J = 7.2, 2.0 Hz, 1H), 3.90 (d, J = 11.7 Hz, 2H), 3.82 (s, 2H), 3.64 (s, 2H), 2.64 (t, J = 12.1 Hz, 1H), 2.49 – 2.38 (m, 2H), 1.98 (d, J = 13.3 Hz, 6H), 1.81 (qd, J = 12.5, 4.0 Hz, 3H), 1.54 (s, 9H).

^{13}CNMR:(151 MHz, Chloroform-d) δ 163.24, 152.27, 143.86, 142.88, 141.91, 141.79, 129.93, 128.98, 128.46, 118.28, 117.04, 113.42, 106.69, 81.42, 46.42, 41.46, 38.65, 31.56, 29.71, 28.26.

HRMS m/z $(M+H)^+$ Calcd for $C_{28}H_{36}N_5O_5S$:554.2431, Found 554.2430



5-[1-(benzenesulfonyl)piperidin-4-yl]-N-methylpyrazolo[1,5-a]pyridine-3-carboxamide (UM-94, 8C)

Following general procedure III, N-methyl-5-(piperidin-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (82 mg, 0.13 mmol,) was dissolved in DCM (900 μL) and added to a round-bottom flask. The flask was cooled to 0 °C, and TEA (90 μL) was added, followed by benzenesulfonyl chloride (87.5 mg, 0.30 mmol). The flask was then warmed to RT and stirred for 1 hr. The

reaction was worked up and crude product was purified via flash chromatography with an 18% to 100% solvent gradient of EtOAc in hexanes. Compound is a white solid. 36 mg, 63% yield. Rf = 0.23 in a 75% EtOAc in 25% hexanes.

^{1}H NMR (400 MHz, Chloroform-d) δ 8.40 (d, J = 7.1 Hz, 1H), 8.13 – 8.05 (m, 2H), 7.81 (d, J = 7.4 Hz, 2H), 7.67 – 7.51 (m, 3H), 6.81 – 6.64 (m, 1H), 5.84 (s, 1H), 4.04 – 3.89 (m, 2H), 3.01 (dd, J = 4.8, 2.0 Hz, 3H), 2.67 – 2.48 (m, 1H), 2.40 (td, J = 11.9, 3.0 Hz, 2H), 2.00 – 1.83 (m, 4H).

^{13}CNMR: (151 MHz, Chloroform-d) δ 163.93, 143.88, 140.55, 140.42, 136.09, 132.80, 129.05, 128.65, 127.60, 116.34, 112.97, 106.23, 46.37, 41.32, 31.66, 26.07.

HRMS m/z $(M+H)^+$ Calcd for $C_{20}H_{23}N_4O_3S$: 399.1485, Found 399.1484

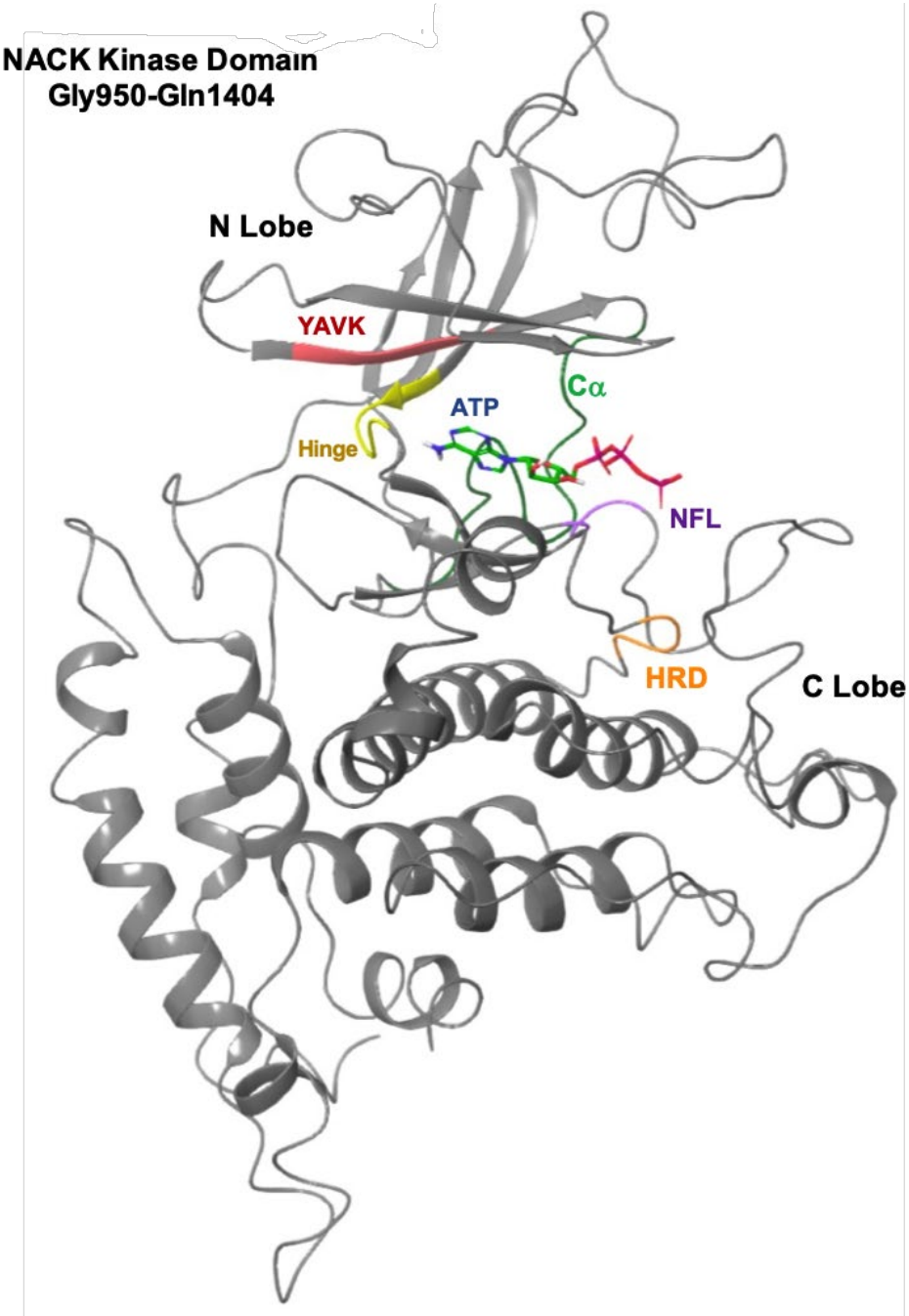


Figure S1.Homology model of NACK kinase domain with ATP docked.

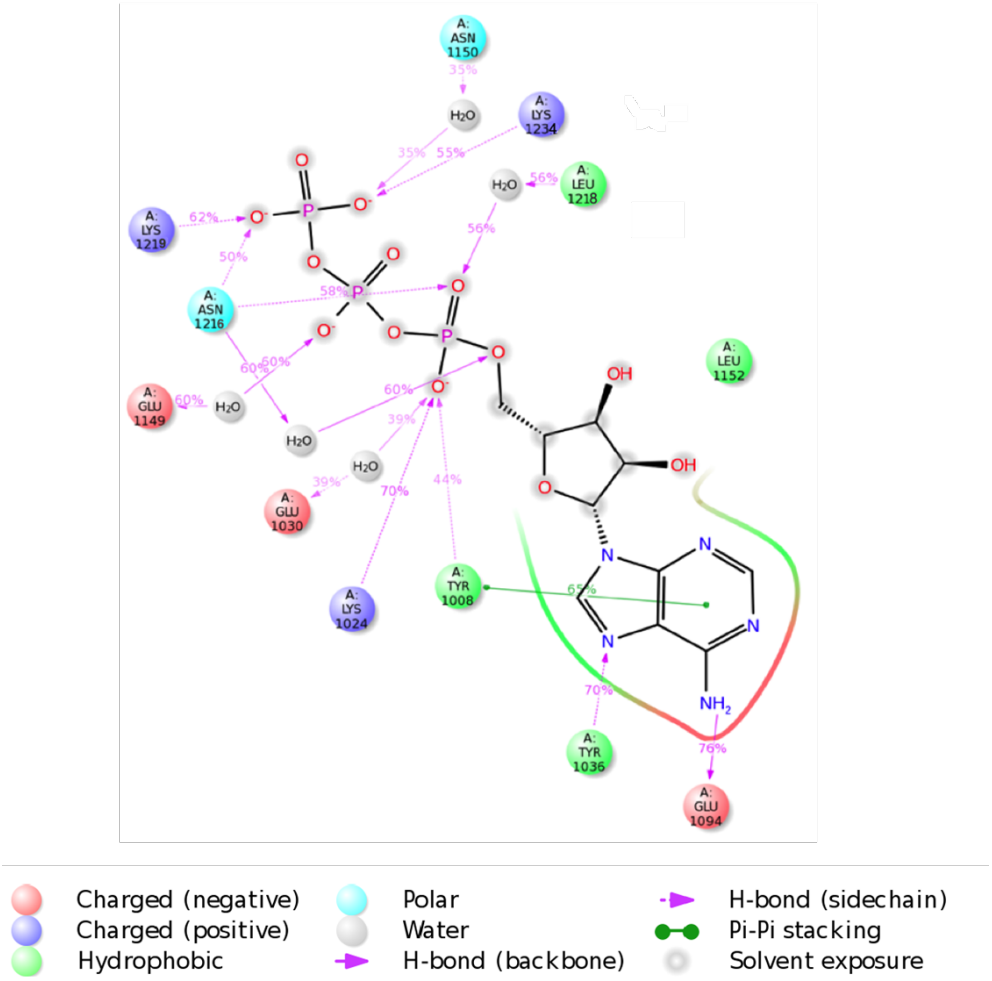


Figure S2. MD simulation of NACK coupled with ATP reveals the catalytic domain of NACK. Predicted key interactions between NACK and ATP throughout the 200 ns simulation. The predicted docking pose between ATP and NACK is stable throughout the simulation.

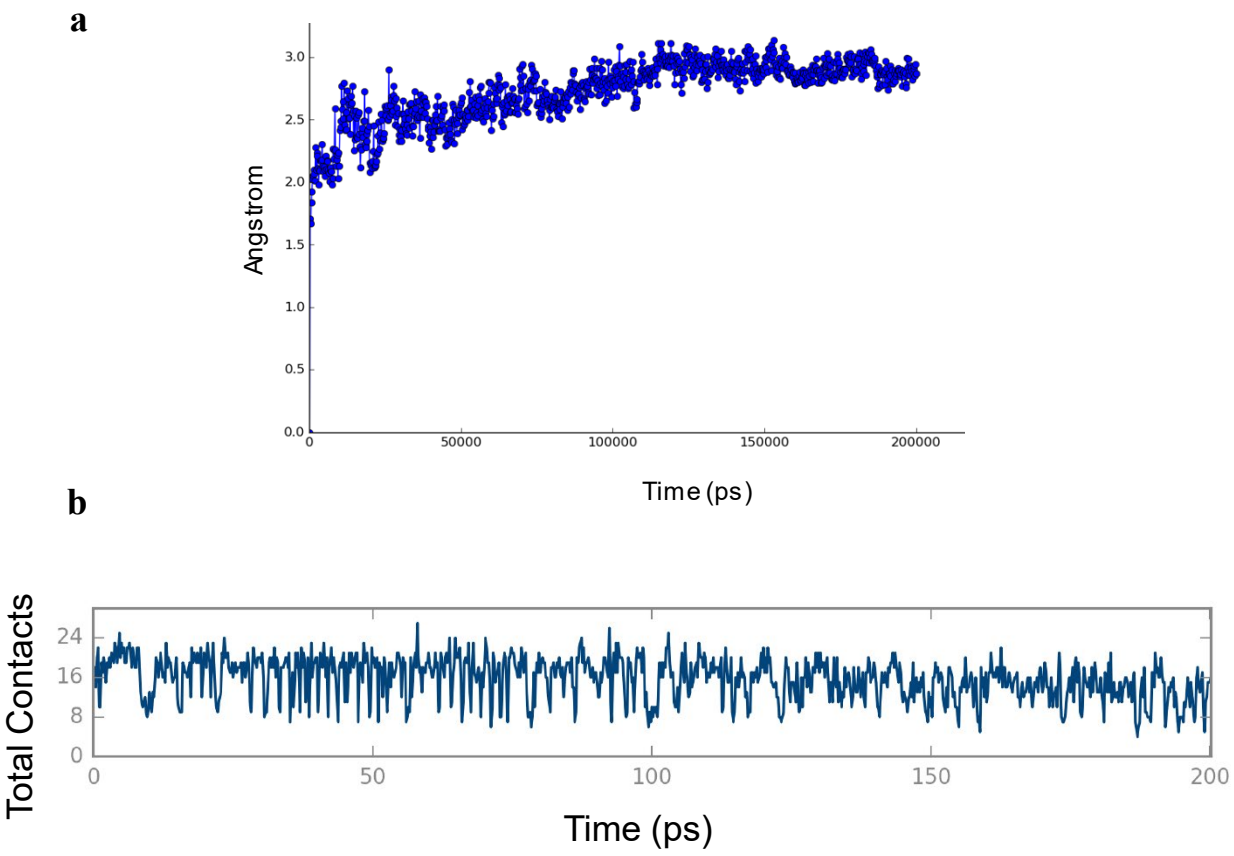


Figure. S3. MD simulation of NACK coupled with ATP is stable throughout a 200 ns MD simulation. a, The RMSD plot demonstrates that during the simulation the NACK-ATP binding pose remains stable throughout the entire 200 ns simulation. **b**, Total contacts of ATP with NACK remain stable throughout the 200 ns simulation.

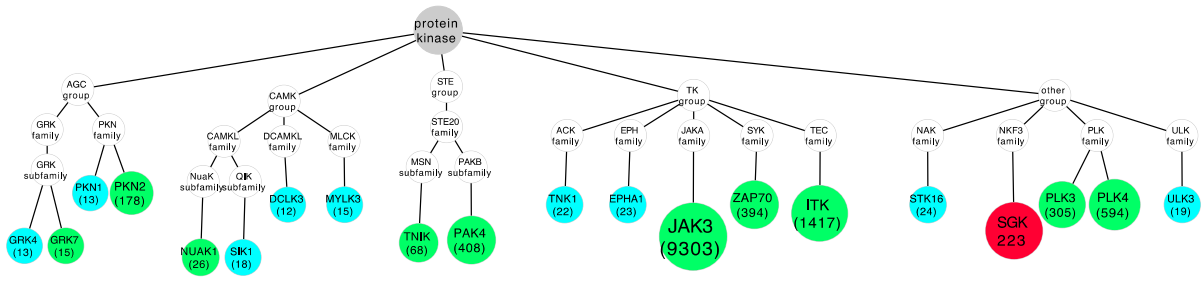
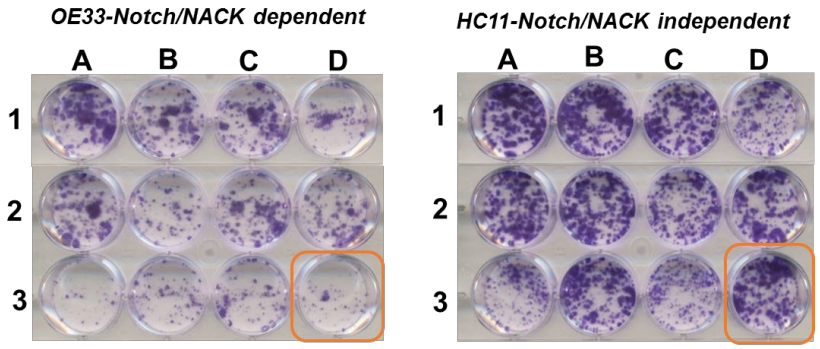


Figure S4. Machine learning kinase classifiers selected for identification of Z271-0326.

Classifiers were selected based on similarity of sequence homology and alignment of motifs.

a



Layout	A	B	C	D
1	DMSO	DAPT	Z269-0227	Z269-0327
2	Z269-0531	Z270-0137	Z270-0197	Z270-0248
3	Z271-0132	Z271-0191	Z271-0226	Z271-0326

b

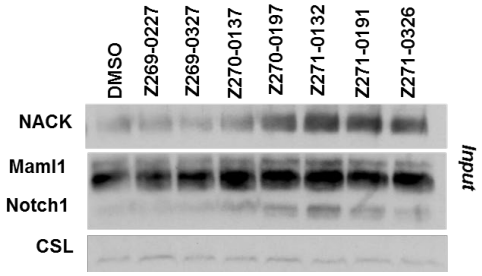
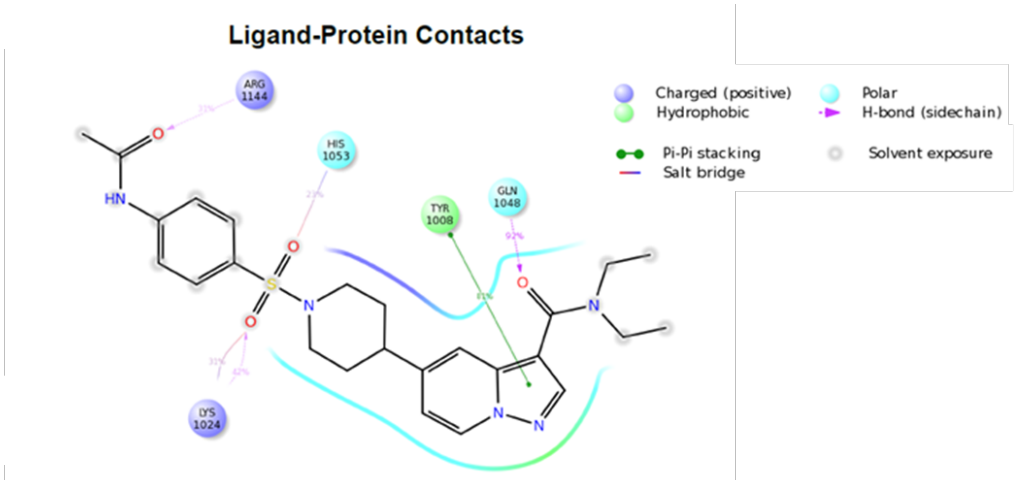
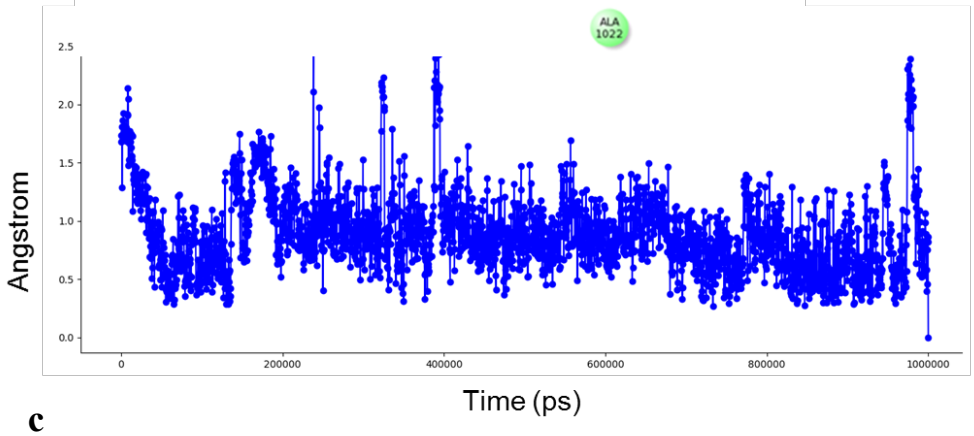


Figure S5. *In-vitro* and cell-based assay to screen compounds for NACK inhibitory activity. **a**, NACK Inhibitors selectively inhibit the viability of Notch/NACK dependent cell line. Cells were treated in colony formation assay with compounds every 48h for a total of 14 days. Then the colonies were stained. **b**, Input of Fig. 2C.

a


Ligand-Protein Contacts
Charged (positive)
Hydrophobic
Polar
H-bond (sidechain)
Pi-Pi stacking
Salt bridge
Solvent exposure
ARG 1144
HIS 1053
TYR 1008
GLN 1048
LYS 1024
HN
O
S
N

b


ALA 1022
Angstrom
Time (ps)
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1.0
1.5
2.0
2.5
0
200000
400000
600000
800000
1000000

c

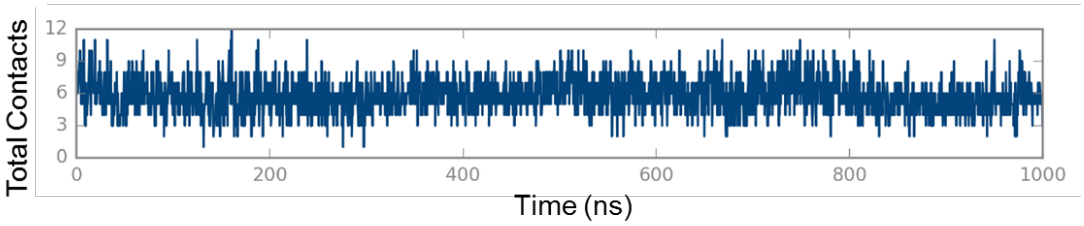

Total Contacts
Time (ns)
0
3
6
9
12
0
200
400
600
800
1000

Figure S6. MD simulation of NACK docked with Z271-0326 is stable throughout a 1 μs MD simulation. a, Predicted key interactions between NACK and Z271-0326 are demonstrated through a 1 μs MD simulation. The predicted docking pose of Z271-0326 and NACK is stable throughout the simulation. **b**, The ligand RMSD plot demonstrates that during the simulation the NACK-Z271-0326 docking pose stabilizes at 400 ns and remains in the docked pose throughout the entire simulation. **c**, Total contacts of Z271-0326 with NACK remain stable throughout the 1 μs simulation.

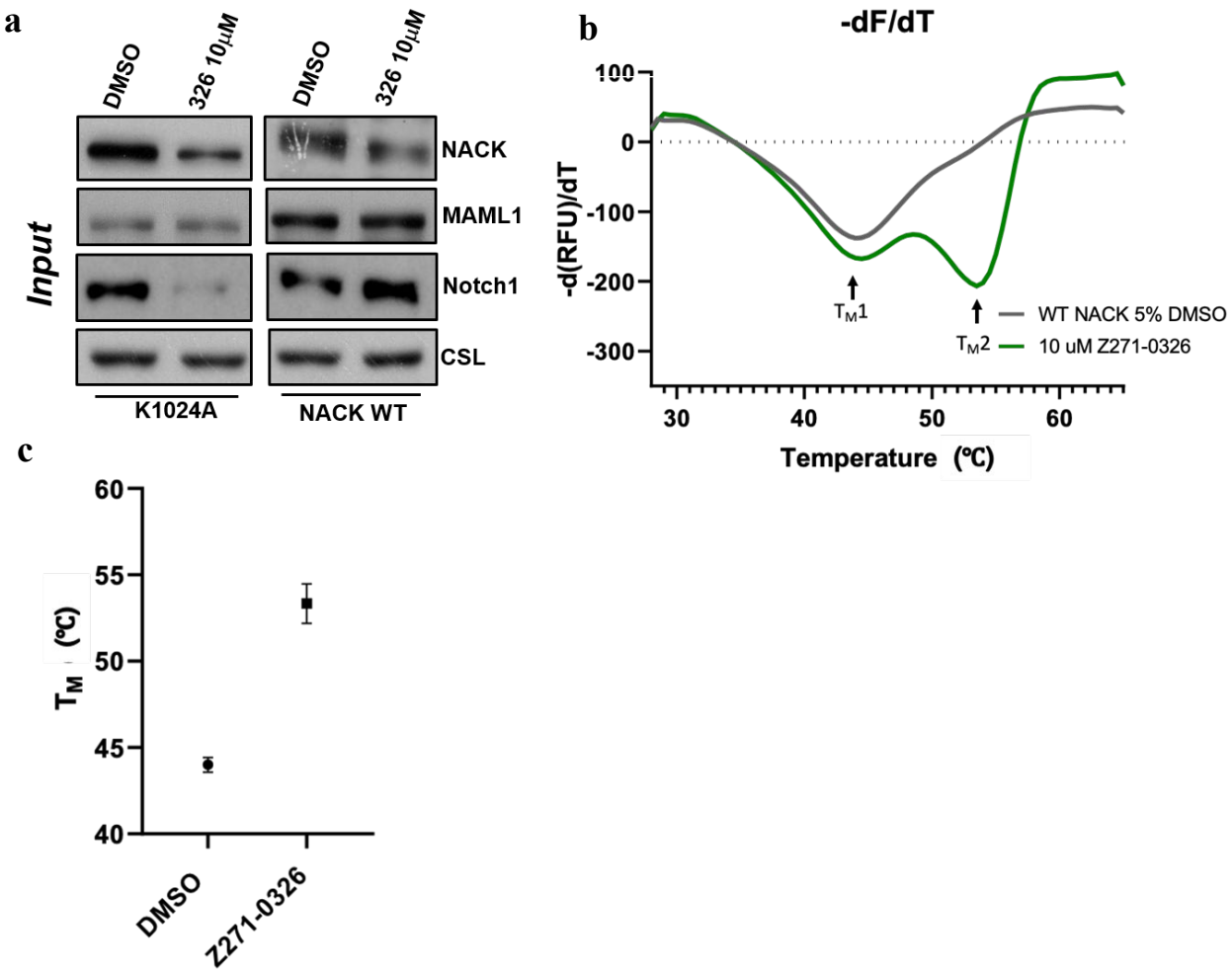


Figure S7. Thermal Shift analysis of T_M via first derivative of fluorescence.

a, Input of the Fig 3A. **b**, The first derivative plot for NACK treated with 10 μM Z271-0326, averaged for three separate experiments, each performed in triplicate, illustrates two melting temperatures (T_M). We believe these to be representative of bound and unbound states as the T_M1 coincides with the vehicle T_M of 44.4±0.13 °C. **c**, The T_M for protein treated with 10 μM Z271-0326 is 53.3±0.75 °C and demonstrates a NACK-Z271-0326 binding event.

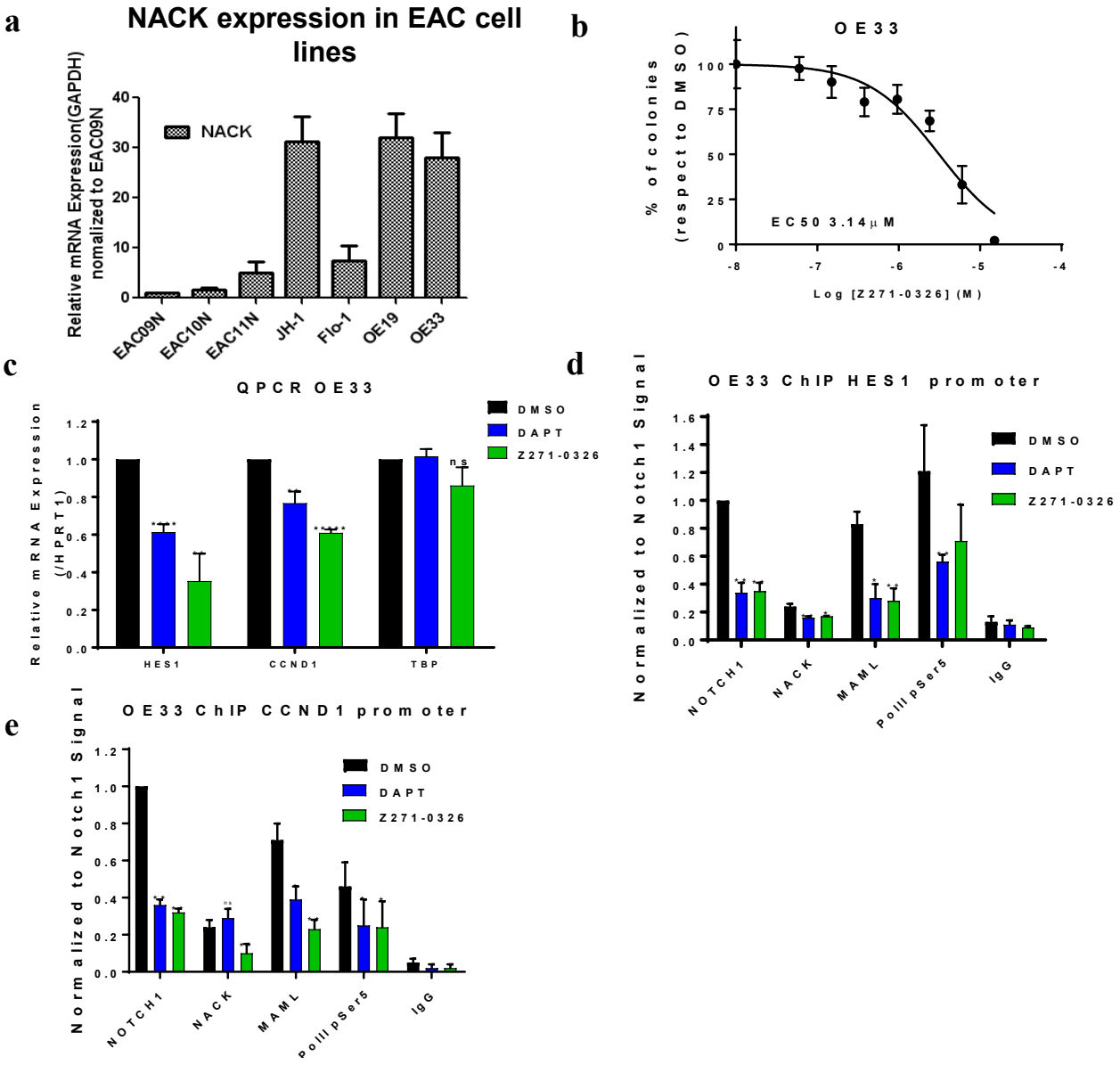

a
NACK expression in EAC cell lines
Relative mRNA Expression(GAPDH) nomalized to EAC09N
NACK
EAC09N
EAC10N
EAC11N
JH-1
Flo-1
OE19
OE33
b
OE33
% of colonies (respect to DMSO)
EC50 3.14μM
Log [Z271-0326] (M)
c
QPCR OE33
Relative mRNA Expression (/HPRT1)
DMSO
DAPT
Z271-0326
HES1
CCND1
TBP
ns
d
OE33 ChIP HES1 promoter
Normalized to Notch1 Signal
NOTCH1
NACK
MAML
PolII pSer5
IgG
e
OE33 ChIP CCND1 promoter
Normalized to Notch1 Signal

Figure S8. NACK is a novel therapeutic target in the Notch pathway. a, Expression of NACK in EAC cell lines OE33, O19, Flo-1, and JH-1 by qPCR. **b**, Colony formation assays were performed in OE33 EAC cell lines under treatment of different concentration of Z271-0326 every other day for ten days. The colony number was quantified and compared to the control (DMSO). **c**, mRNA levels of ES1, CCND1, or TBP gene expression of OE33 treated for 6h with DMSO, DAPT, or Z271-0326 were analyzed and normalized to HPRT1. **d** and **e**, ChIP assay on the HES1 and CCND1 promoter using the indicated antibodies (n=3) Error bars are representative of three independent experiments, and values indicate mean ± SD. p values ≤ 0.05 are considered statistically significant and indicated by an asterisk. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

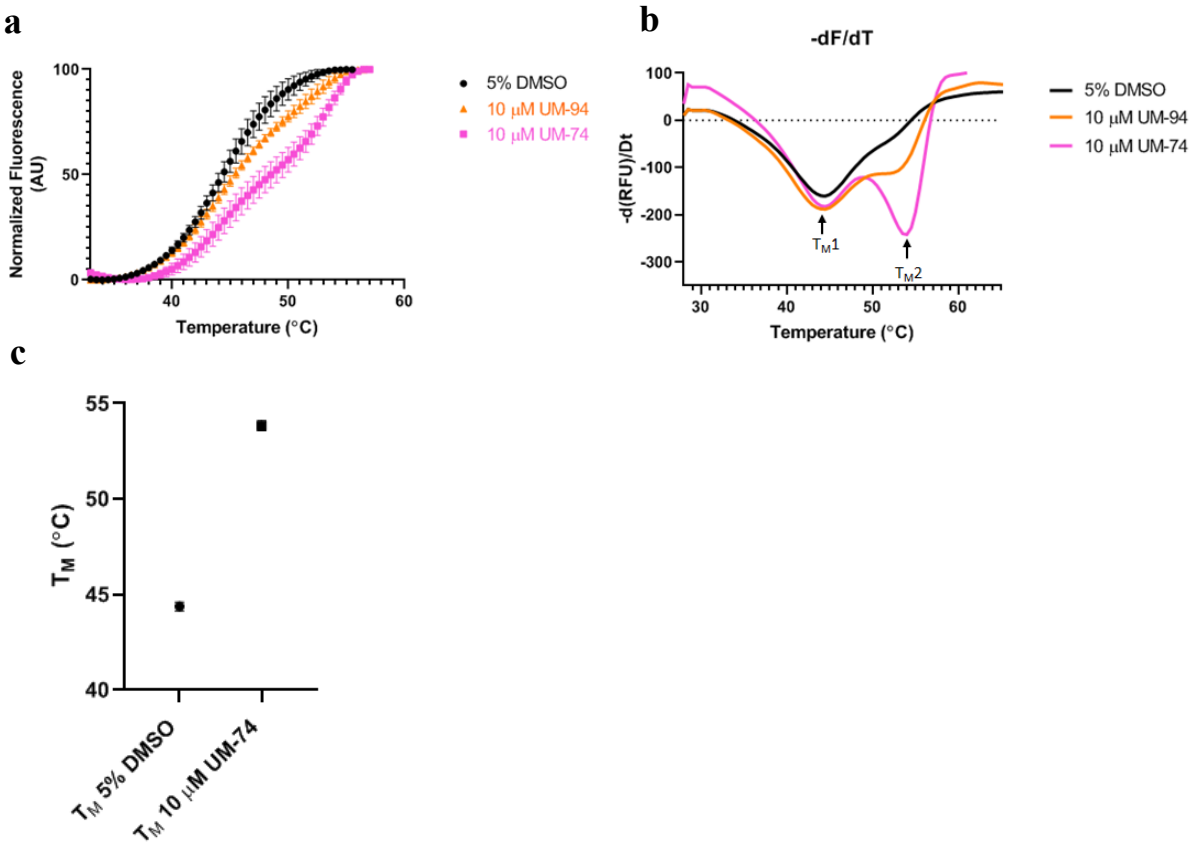


Figure S9. Thermal Shift analysis of NACK Treated with UM-74 and UM-94. a, When NACK is treated with 10 μM UM-74, a significant stabilization in T_M is demonstrated. However, NACK treatment with 10 μM UM-94 does not demonstrate a significant change in T_M. This experiment was performed twice with each experiment in triplicate. **b**, The first derivative plot for NACK treated with vehicle, 10 μM UM-94 and UM-74, averaged for two separate experiments, each performed in triplicate. NACK treated with UM-74 displays two significant $T_{M,}$ which we believe to correspond to bound and unbound states. **c**, The T_M for NACK treated with 10 μM UM-74 is 53.83 ±0.21 ºC and demonstrates a NACK-UM-74 binding event. Vehicle T_M is 44.38 ±0.13 ºC.

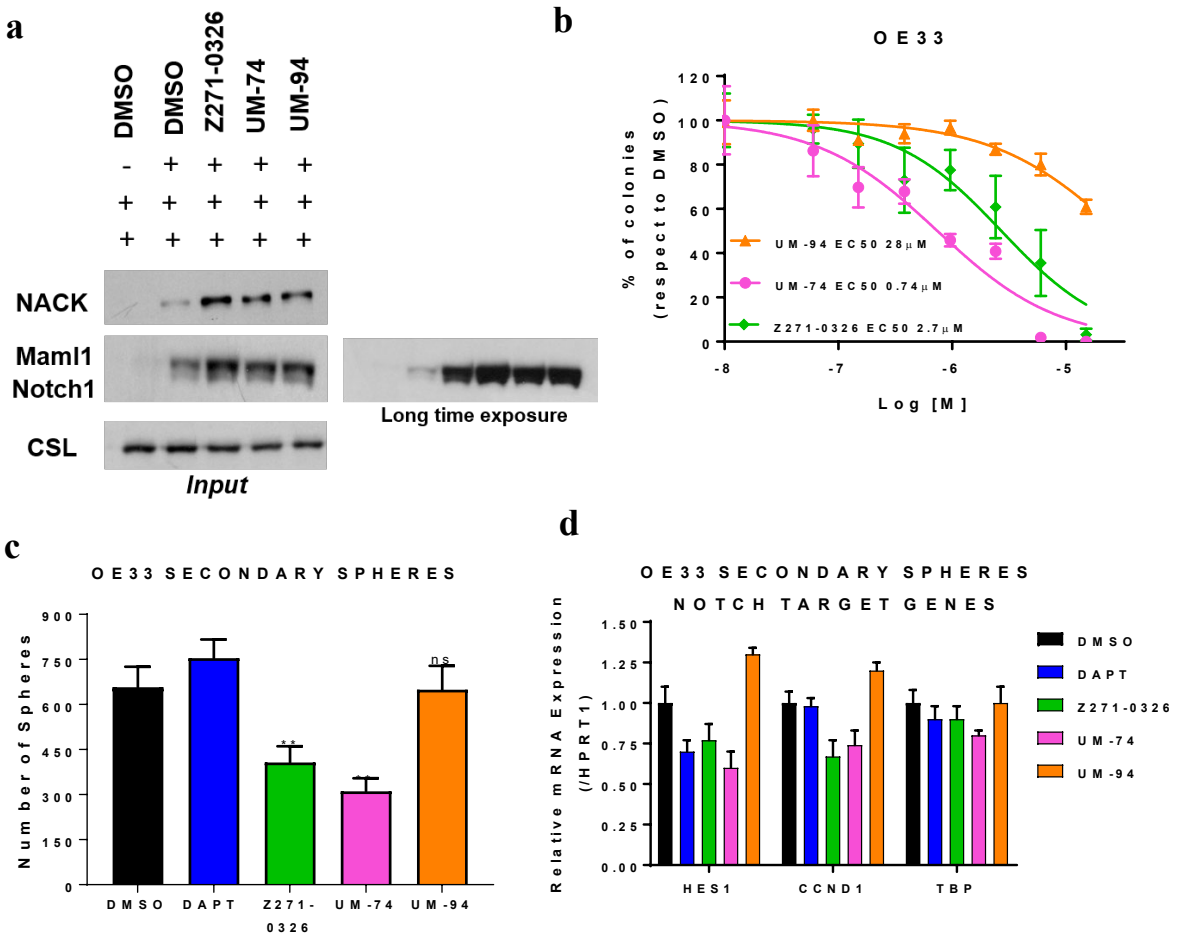


Figure S10. Validation of analogs UM-74 and UM-94 in cell-based-assays. a, Input of Fig 5A. **b**, Colony formation assays were performed in OE33 cell lines under treatment of different concentration of Z271-0326, UM-74 and UM-94 every other day for ten days. The colony number was quantified and compared to the control (DMSO). **c**, Tumor sphere formation assays were performed in OE19 treated every other day with DAPT (15 μM), Z271-0326, UM-74, and UM-94 (3 μM). **d**, mRNA levels of Notch targets were then determined in qPCR. Unpaired two-tailed t-test was used to determine significance. Error bars are representative of three independent experiments, and values indicate mean ± SD. p values ≤ 0.05 are considered

statistically significant and indicated by an asterisk. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns (no significant difference).

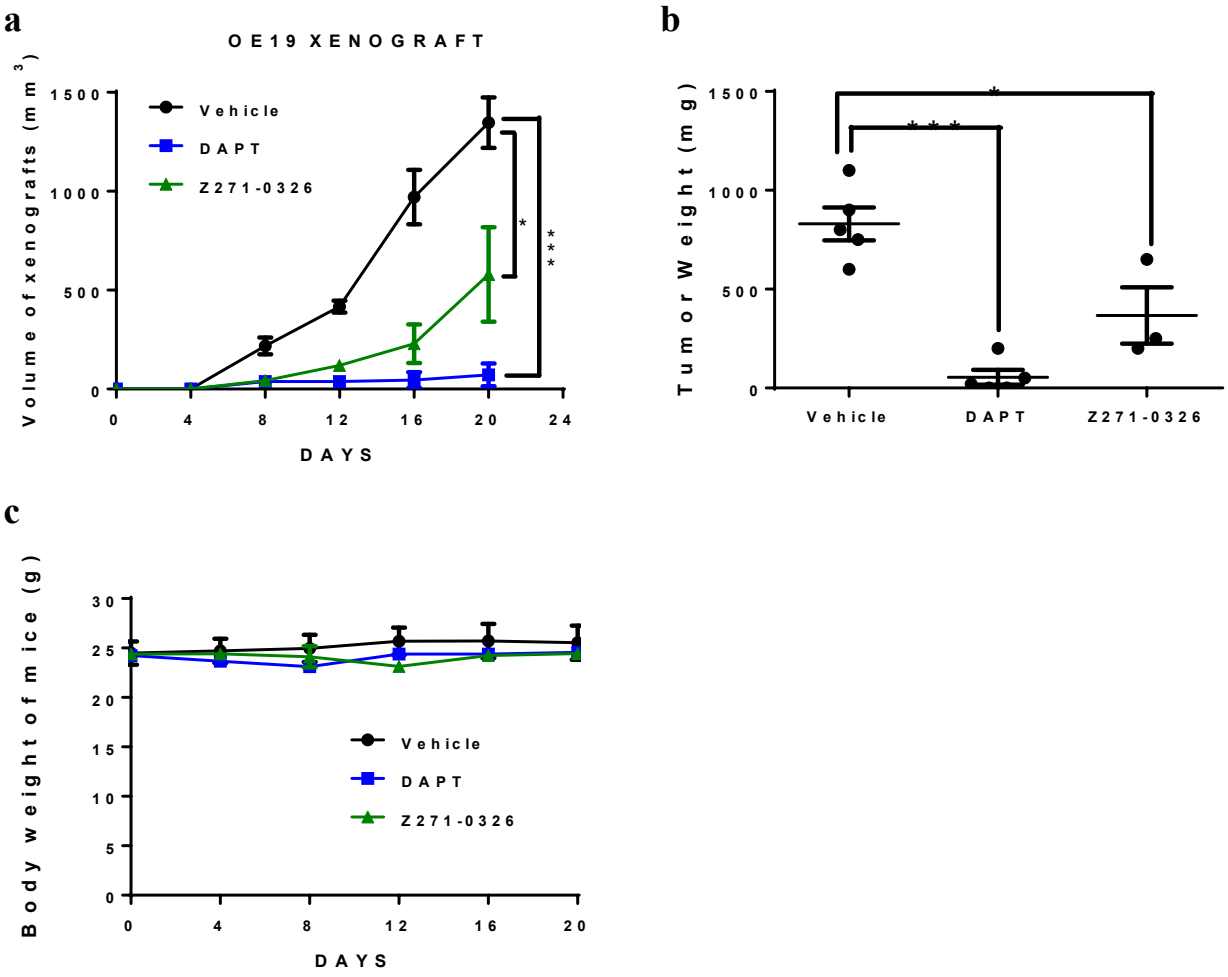


Figure S11. Inhibition of EAC tumor growth by Z271-0326. a, Z271-0326 treatment reduced tumor growth in the mice that bear OE19 xenograft (25mg/kg). Compounds were administered daily by intraperitoneal injection. n=5 per group. Statistical analysis was performed using two-way ANOVA, *P < 0.05. **b**, The corresponding tumor weight is illustrated. Unpaired two-tailed t-test was used to determine significance * P <0.05). **c**, Mice body weight along with the experiment n = 5 to 6 per group. t-test, *P <0.05.

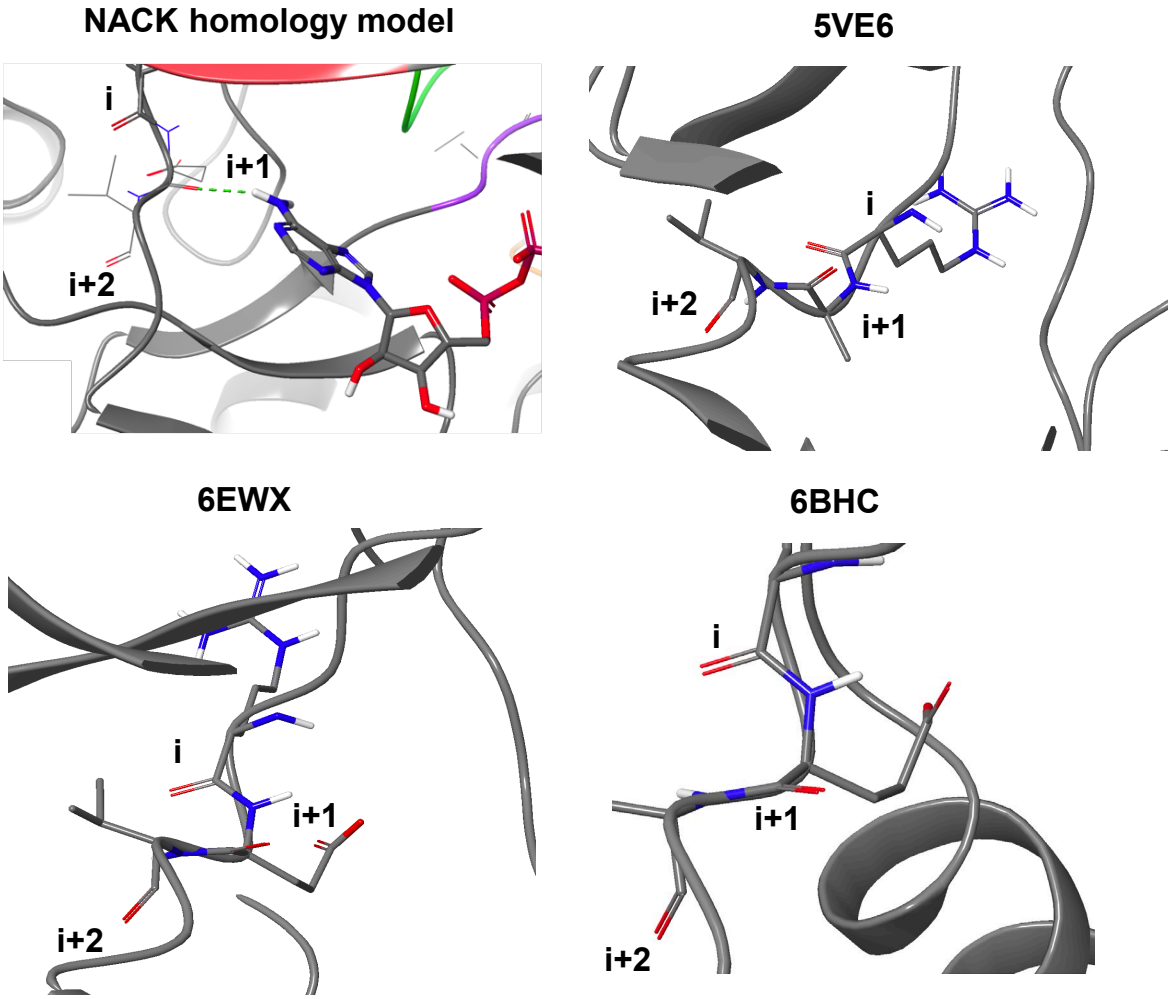
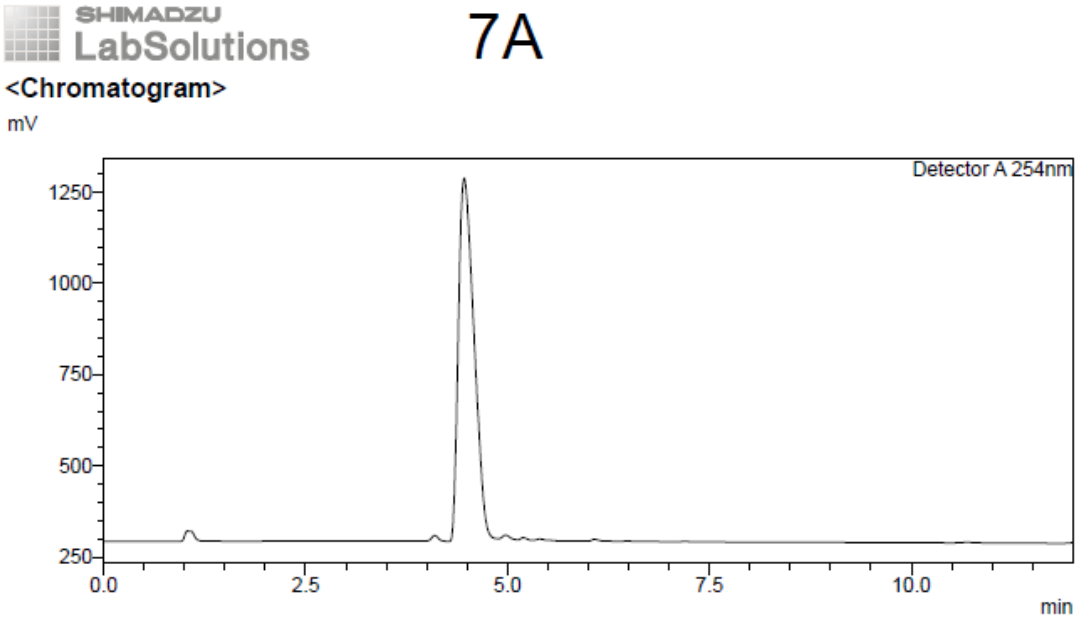


Figure S12. Analysis of the hinge region in NACK homology model, 5VE6, 6EWX, and 6BHC. Analysis of the NACK Homology model and apo crystal structures hinge region demonstrates expected dihedral angles at hinge when the gate- keeper (i) is assigned as R1093. ATP commonly binds to i+1 and 1+3 residues, and i+2 residue is normally a hydrophobic interaction. In both NACK apo structures deposited into the PDB (PDBID: 5VE6, 6EWX) and close homologue PEAK1 (PDBID: 6BHC) the dihedral angles of residues i+1 permits the adenine ring forming H-bonds, and the V1095 i+2 serves as the typical hydrophobic interaction if we consider R1093 the gate-keeper residue. However, this would lead to an unusual

assignment of a large hydrophilic residue serving as gate-keeper and an odd arrangement of the gate-keeper sidechain pointing away from the pocket.

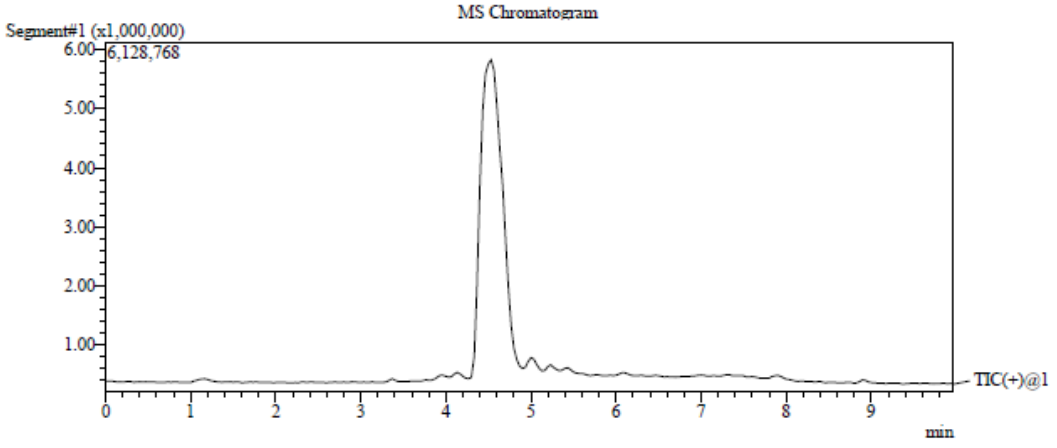
Selected Spectra



Peak Table

Detector A 254nm

Peak#	Ret. Time	Area	Height	Area%
1	1.035	223345	28592	1.635
2	4.094	87764	15598	0.643
3	4.459	13235230	990416	96.909
4	4.973	50551	9863	0.370
5	5.194	26232	5872	0.192
6	5.395	6479	1775	0.047
7	6.075	27762	5225	0.203
Total		13657363	1057341	100.000



MASS Peak Table TIC

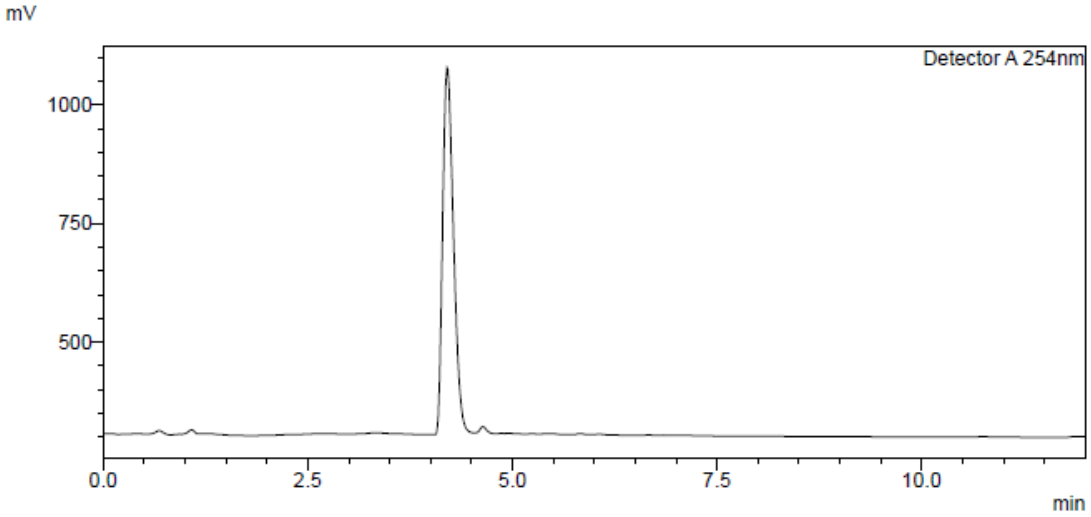
Peak#	Ret. Time	Area	Base Peak m/z
1	4.525	95860815	301.25
2	5.008	1829339	315.25
Total		97690154	

LCMS spectra for compound **7A**

SHIMADZU LabSolutions

7B

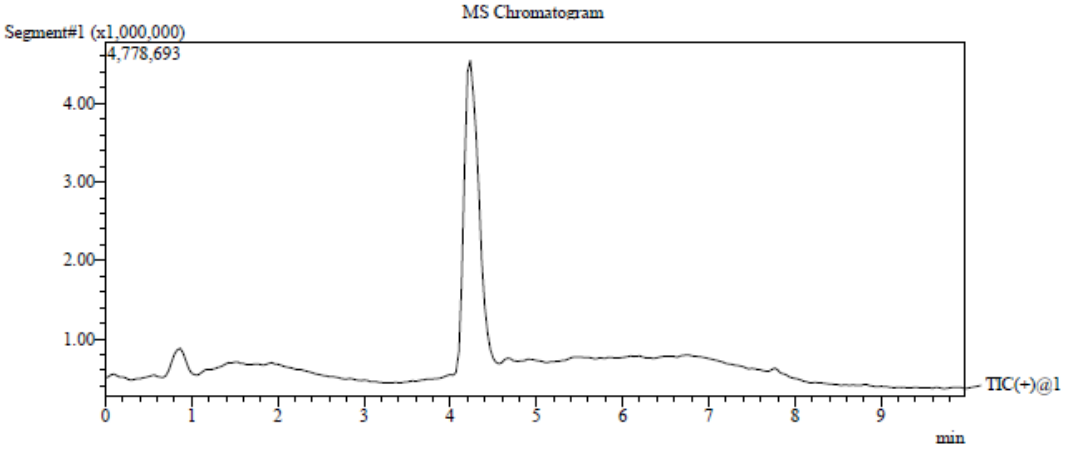
<Chromatogram>



Peak Table

Detector A 254nm

Peak#	Ret. Time	Area	Height	Area%
1	0.678	43594	7306	0.601
2	1.075	35710	8306	0.492
3	4.198	7103611	770765	97.898
4	4.634	73236	13509	1.009
Total		7256151	799886	100.000



MASS Peak Table TIC

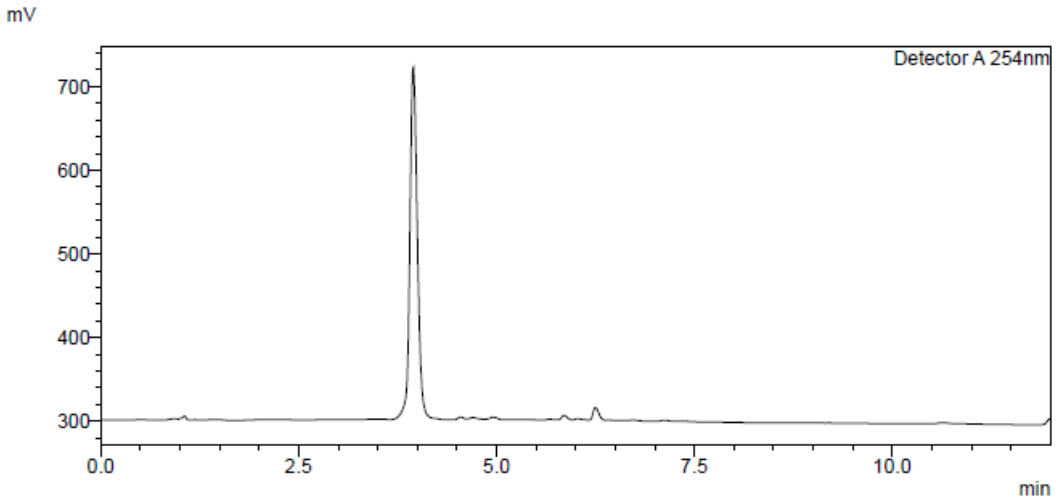
Peak#	Ret. Time	Area	Base Peak m/z
1	0.858	3686446	143.15
2	4.225	47115448	299.25
Total		50801894	

LCMS spectra for compound **7B**

SHIMADZU LabSolutions

7C

<Chromatogram>

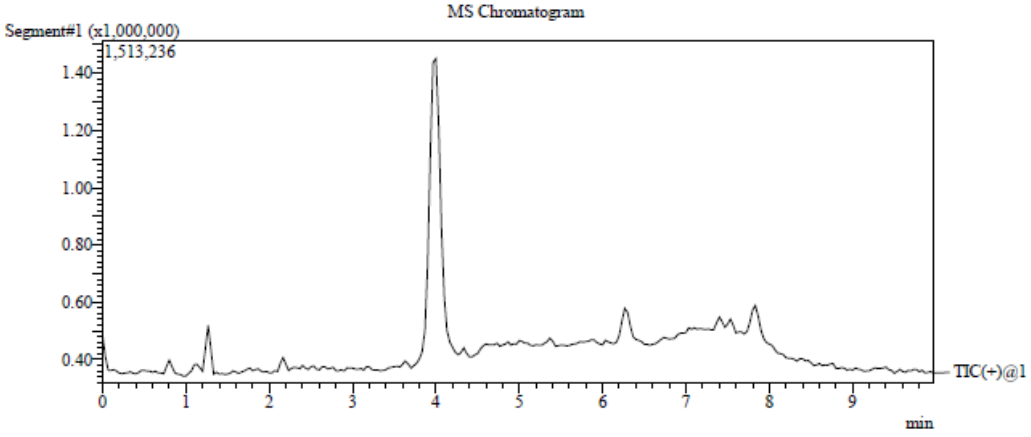


Peak Table

Detector A 254nm

Peak#	Ret. Time	Area	Height	Area%
1	0.922	4268	806	0.143
2	1.051	13449	4255	0.449
3	3.943	2807459	420490	93.748
4	4.543	16916	3181	0.565
5	4.697	19666	2526	0.657
6	4.954	22072	3171	0.737
7	5.847	27268	5519	0.911
8	6.016	10312	1756	0.344
9	6.246	73292	15216	2.447
Total		2994702	456920	100.000

MS Chromatogram



MASS Peak Table TIC

Peak#	Ret. Time	Area	Base Peak m/z
1	1.266	652932	739.45
2	3.985	9640645	259.15
Total		10293577	

LCMS spectra for compound **7C**

SHIMADZU LabSolutions

Analysis Report
Z271-0326

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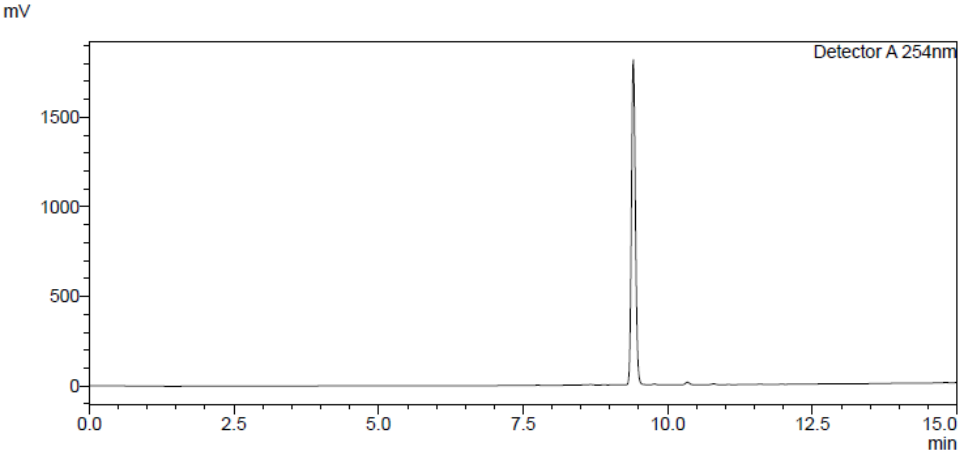

<Peak Table>

Detector A 254nm

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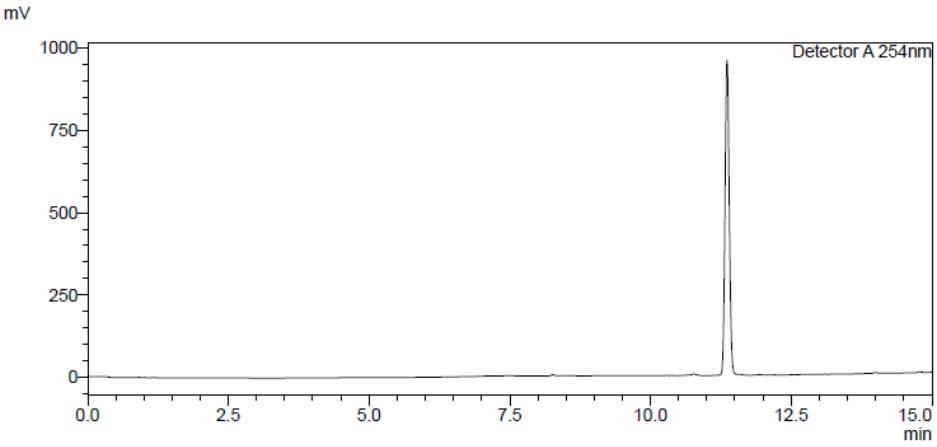
1	8.667	3537	0.218
2	9.406	1815989	98.648
3	9.770	3117	0.103
4	10.338	14255	0.823
5	10.793	4189	0.208
Total		1841088	100.000

UHPLC for **Z271-0326**

SHIMADZU LabSolutions

Analysis Report
UM-74

<Chromatogram>



<Peak Table>

Detector A 254nm

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2	8.509	168	0.038
3	9.452	836	0.072
4	10.288	113	0.018
5	10.767	4515	0.617
6	11.362	957516	98.676
7	11.939	1649	0.112
8	13.995	2861	0.298
Total		970450	100.000

UHPLC for **UM-74**

SHIMADZU LabSolutions

Analysis Report
UM-94

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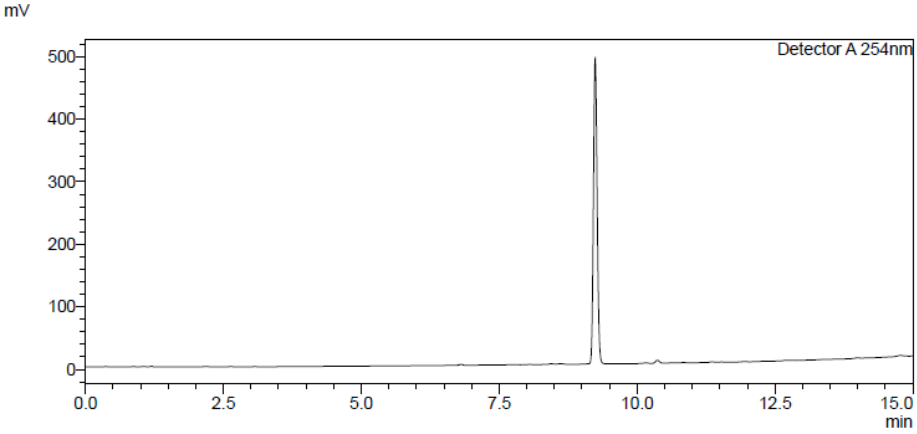

<Peak Table>

Detector A 254nm

Peak#	Ret. Time	Height	Area%
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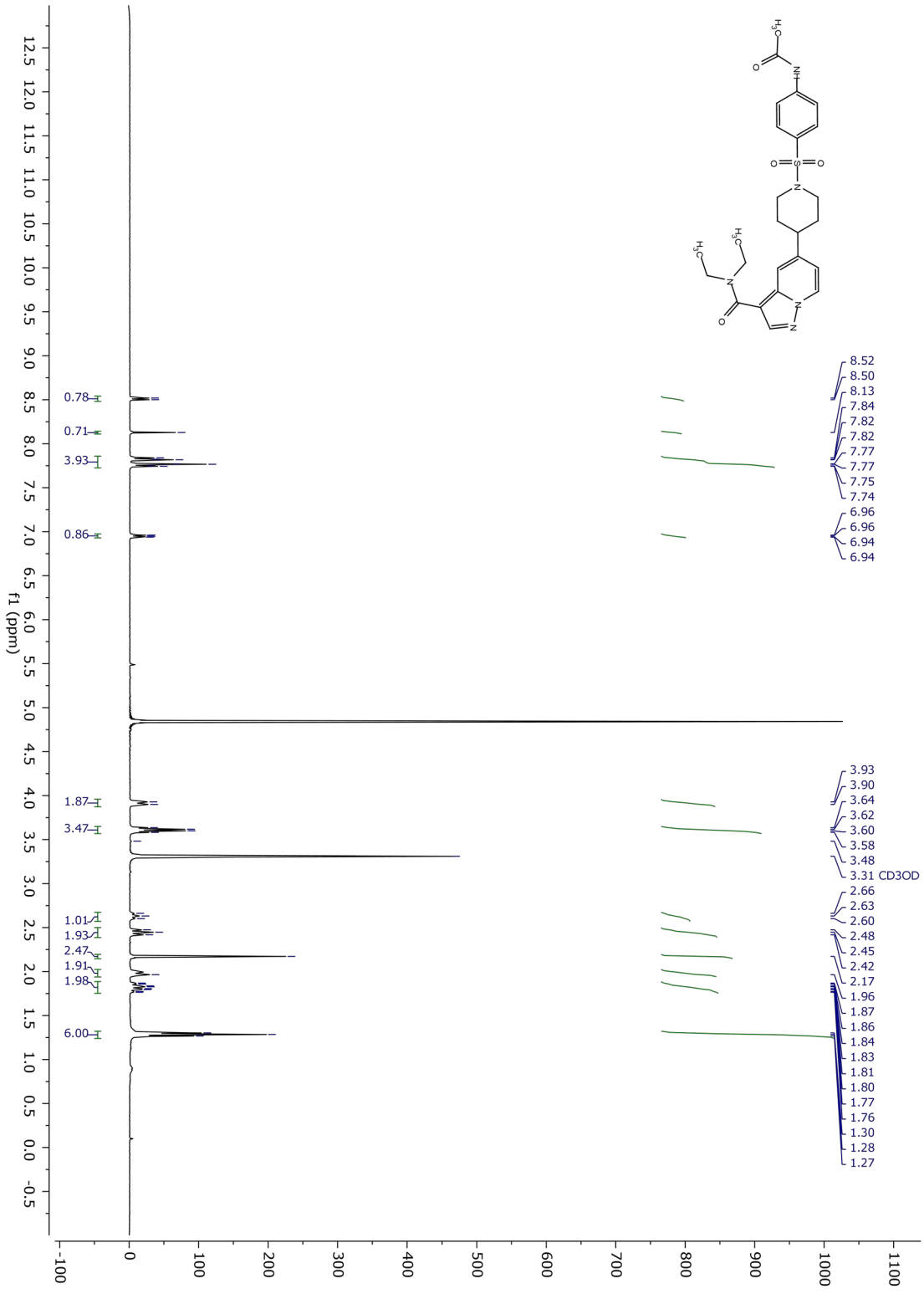
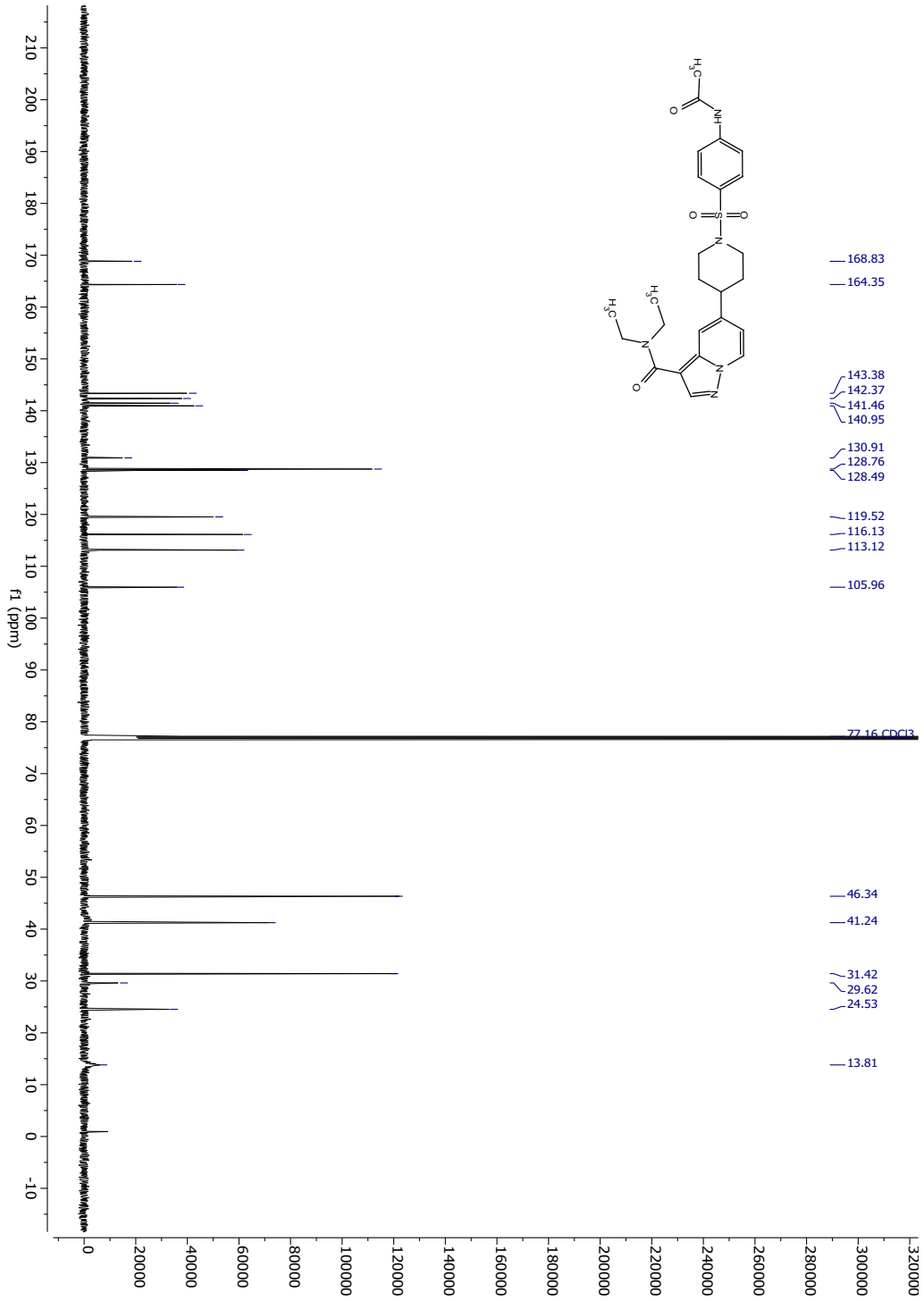
2	6.810	1481	0.220
3	8.439	1385	0.272
4	8.604	923	0.286
5	9.241	490156	97.087
6	10.158	835	0.183
7	10.368	4836	1.039
8	10.841	291	0.042
9	11.353	1118	0.235
10	14.775	1772	0.564
Total		503614	100.000

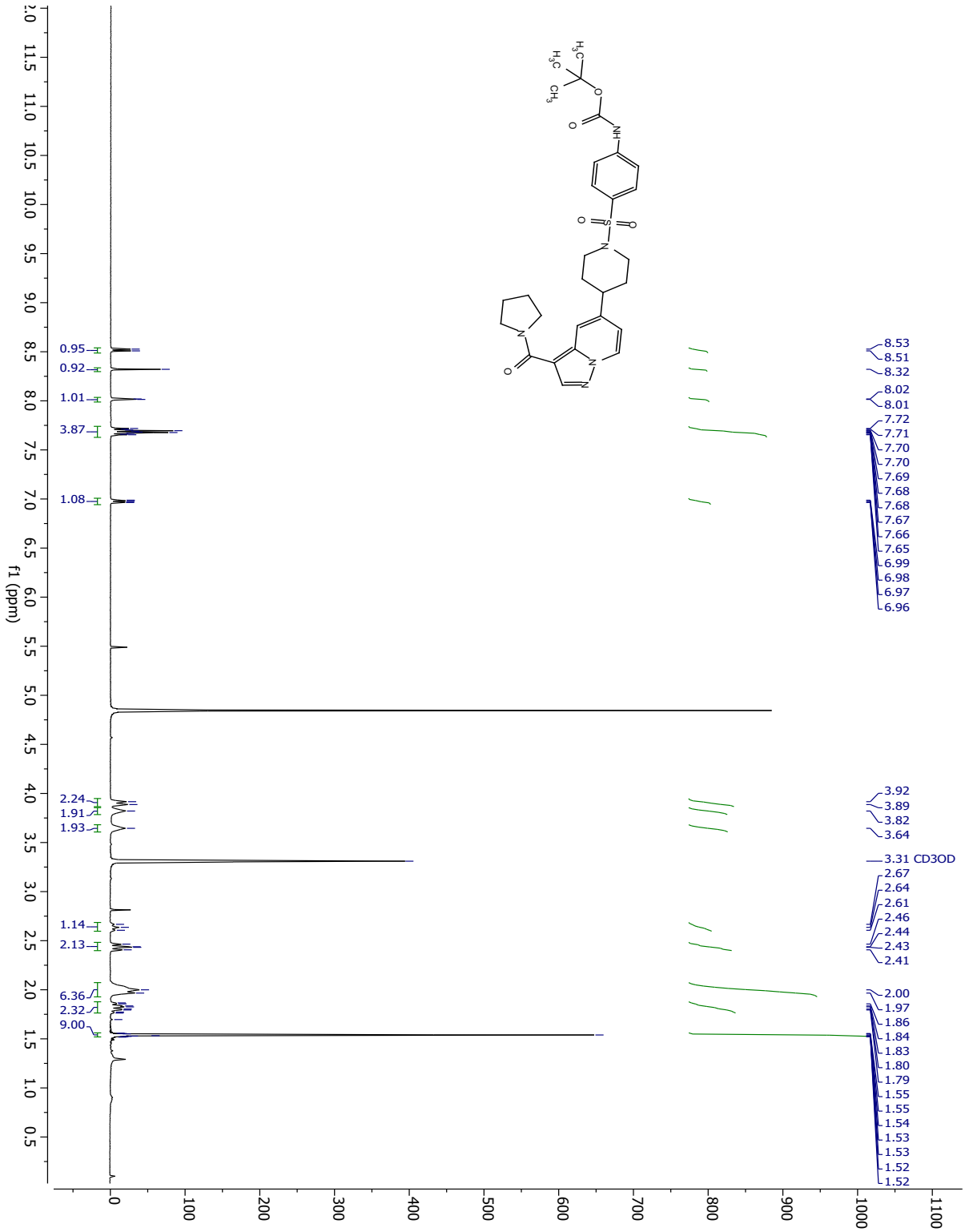
UHPLC for **UM-94**



^{1}HNMR **Z271-0326**



^{13}CNMR **Z271-0326**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1.93
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9.00
8.53
8.51
8.32
8.02
8.01
7.72
7.71
7.70
7.70
7.69
7.68
7.68
7.67
7.66
7.65
6.99
6.98
6.97
6.96
3.92
3.89
3.82
3.64
3.31 CD3OD
2.67
2.64
2.61
2.46
2.44
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2.41
2.00
1.97
1.86
1.84
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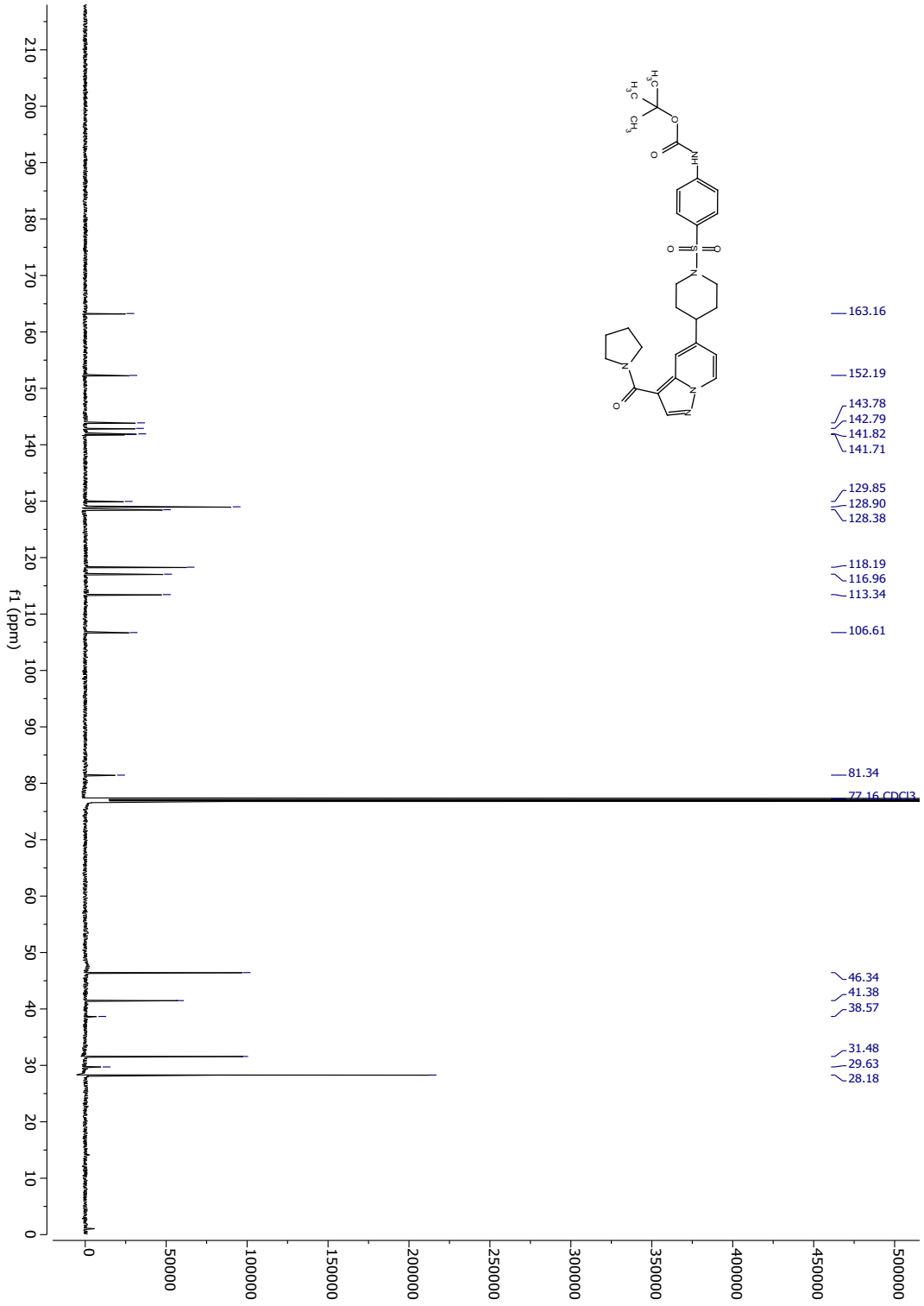
1.55
1.55
1.54
1.53
1.53
1.52
1.52

^{1}HNMR **UM-74**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143.78
142.79
141.82
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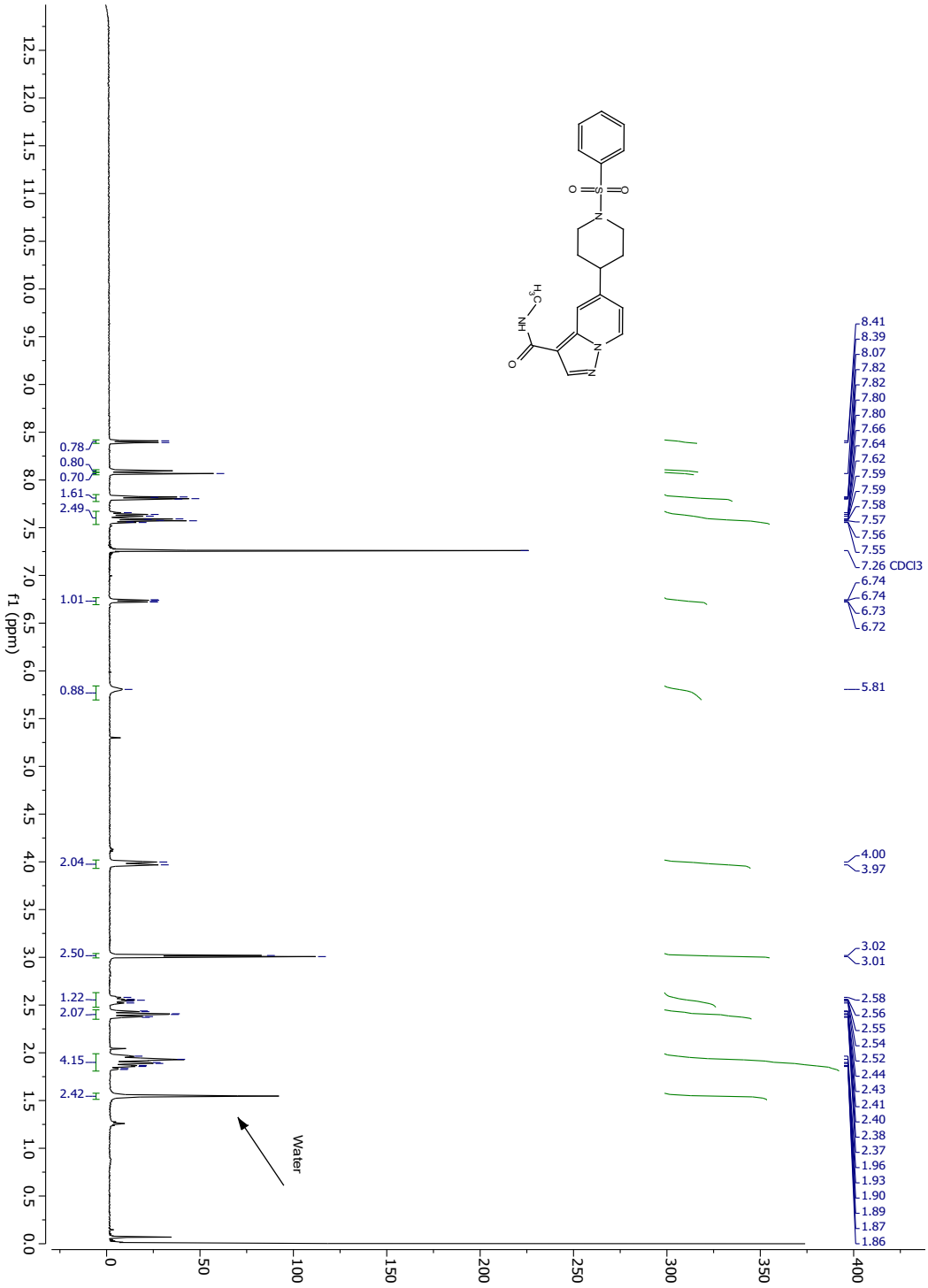
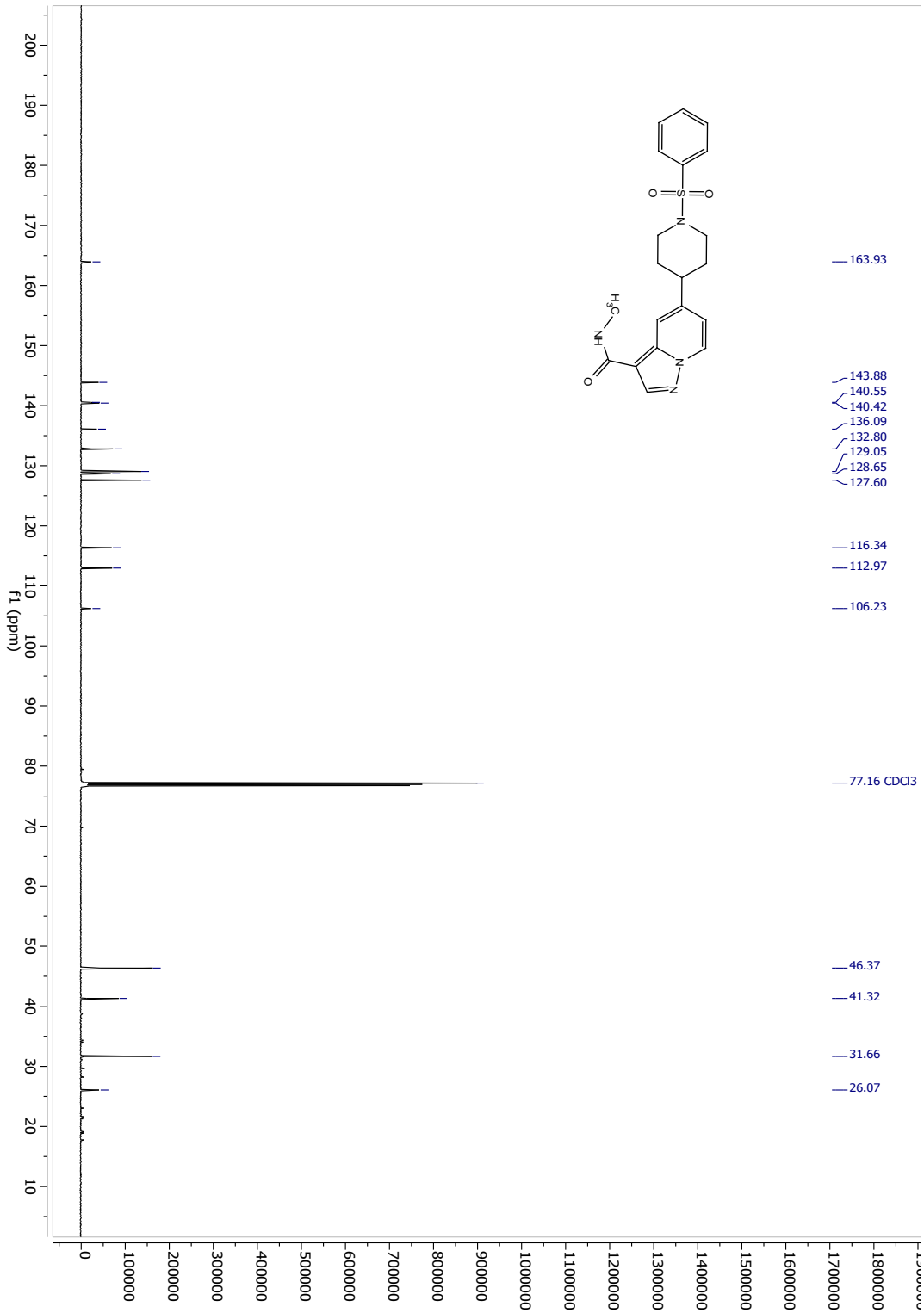
81.34
77.16 CDCl3
46.34
41.38
38.57
31.48
29.63
28.18

^{13}CNMR **UM-74**


f1 (ppm)
0.78
0.80
0.70
1.61
2.49
1.01
0.88
2.04
2.50
1.22
2.07
4.15
2.42
Water
8.41
8.39
8.07
7.82
7.82
7.80
7.80
7.66
7.64
7.62
7.59
7.59
7.58
7.57
7.56
7.55
7.26 CDCl3
6.74
6.74
6.73
6.72
5.81
4.00
3.97
3.02
3.01
2.58
2.56
2.55
2.54
2.52
2.44
2.43
2.41
2.40
2.38
2.37
1.96
1.93
1.90
1.89
1.87
1.86

[1]HNMR **UM-94**


163.93
143.88
140.55
140.42
136.09
132.80
129.05
128.65
127.60
116.34
112.97
106.23
77.16 CDCl3
46.37
41.32
31.66
26.07
f1 (ppm)

^{13}CNMR **UM-94**