Nicotinamide phosphoribosyltransferase positive allosteric modulators attenuate neuronal oxidative stress

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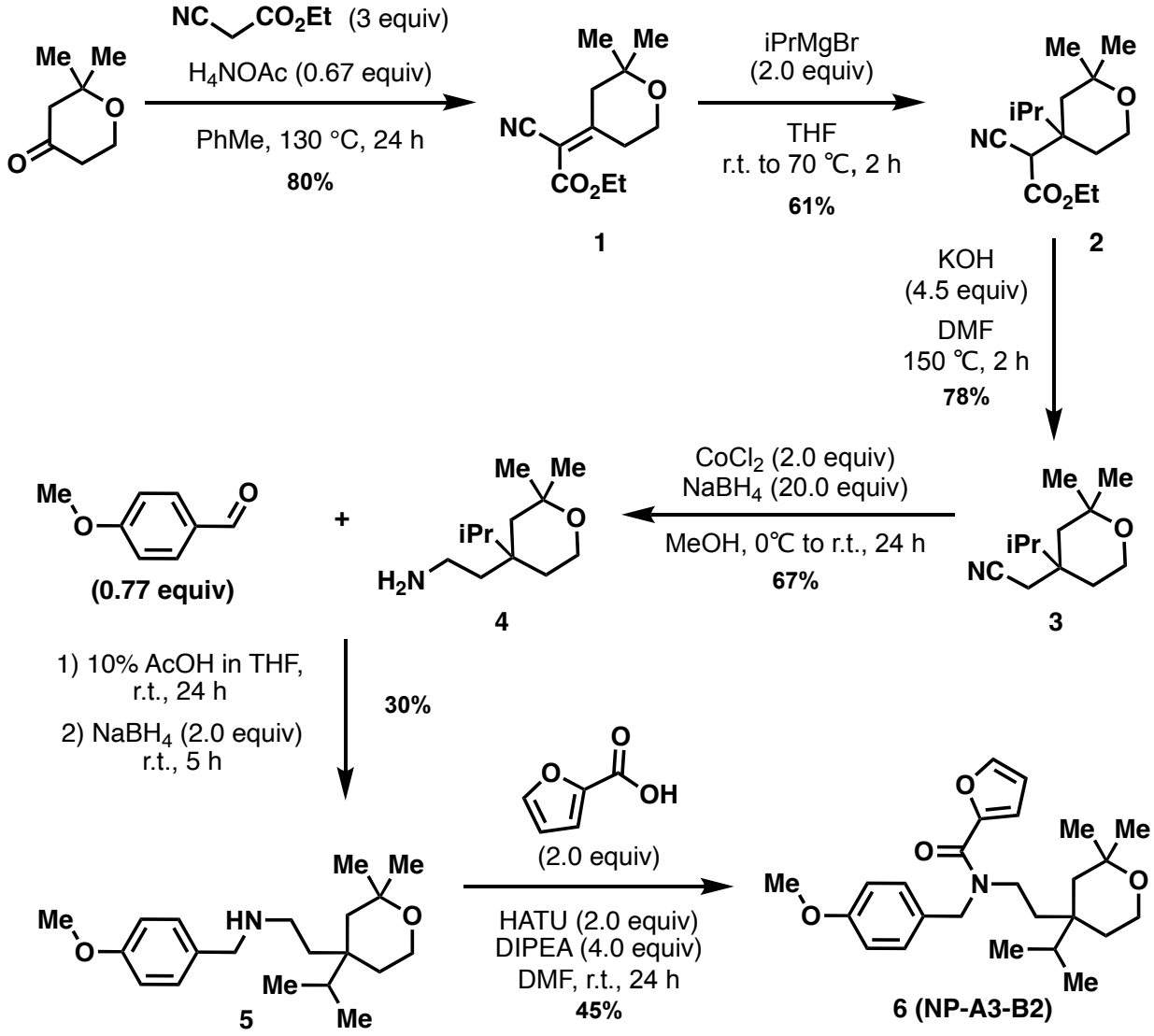
Safety statement. No unexpected or unusually high safety hazards were encountered.

I. Scheme I: Synthetic Procedures for NP-A3-B2 Resynthesis and Derivatization



Step 1. Ethyl-2-cyano-2-(2,2-dimethyl-tetrahydropyran-4-ylidene)acetate (1)[1]

A mixture 25.0 g of 2,2-dimethyltetrahydropyran-4-one (195 mmol, 1.00 equiv), 66.2 g of ethyl cyanoacetate (585 mmol, 3.00 equiv), and 10.1 g of ammonium acetate (131 mmol, 0.67 equiv) in 500 mL of anhydrous toluene (1.82 M) under argon was heated to 130 °C using a Dean–Stark trap. After 24 h, the mixture was cooled to room temperature and concentrated *in vacuo* to give a dark orange oil. The crude product was resuspended in 500 mL of EtOAc, and the resulting mixture was washed with 3 × 200 mL of brine. The organic phase was dried over $MgSO_4$, filtered, and the filtrate was concentrated *in vacuo*. Purification using silica column chromatography (10:90 EtOAc:hexanes) with MPLC afforded the product as a yellow oil (35 g, 80%). 1H NMR (400 MHz, CDCl3) δ 4.30 (ttd, J = 7.2, 5.0, 2.5 Hz, 8H), 3.93 (t, J = 5.7 Hz, 2H), 3.86 (t, J = 5.7 Hz, 2H), 3.47 (s, 4H), 3.14 (t, J = 5.7 Hz, 2H), 3.07 (s, 2H), 2.74 (t, J = 5.7 Hz, 2H), 2.68 (s, 2H), 1.40 – 1.32 (m, 12H), 1.29 (s, 6H), 1.26 (s, 6H). Molecular formula: $C_{12}H_{17}NO_3$. Expected ($[M+H]^+$): 224.1287 m/z. HRMS found: 224.1278 m/z.

Step 2. Ethyl-2-cyano-2-(4-isopropyl-2,2-dimethyl-tetrahydro-2H-pyran-4-yl)acetate (2)[1]

To a solution of 17.9 g of (Z)-ethyl-2-cyano-2-(2,2-dimethyl-tetrahydropyran-4-ylidene)acetate (80.2 mmol, 1.0 equiv) in 300 mL of anhydrous THF (0.27 M) at room temperature under argon was added 53.5 mL of isopropylmagnesium bromide solution (3.0 M in 2-Me-THF) (160.3 mmol, 2.0 equiv) dropwise at room temperature. The mixture was then heated to 70 °C for two hours and subsequently cooled by ice bath back to room temperature. The reaction was then quenched by slow addition of 50 mL of a saturated aqueous NH_4Cl solution. The mixture was further diluted with 100 mL of the saturated NH_4Cl solution and extracted with 3 x 50 mL EtOAc, dried over $MgSO_4$, filtered, and the filtrate was concentrated *in vacuo*. Purification using silica column chromatography (10:90 EtOAc:hexanes) with MPLC afforded the product as a dark yellow oil (13.0 g, 61%). 1H NMR (400 MHz, CDCl3) δ 4.36 – 4.22 (m, 4H), 4.00 (s, 1H), 3.94 (s, 1H), 3.83 – 3.75 (m, 3H), 3.70 (ddd, J = 12.5, 7.0, 4.4 Hz, 1H), 2.11 (p, J = 7.0 Hz, 1H), 1.99 – 1.93 (m, 1H), 1.91 (d, J = 1.9 Hz, 1H), 1.88 (dd, J = 4.4, 2.5 Hz, 1H), 1.86 – 1.72 (m, 4H), 1.68 (s, 1H), 1.64 (s, 1H), 1.40 – 1.26 (m, 20H), 1.06 (dd, J = 7.0, 1.5 Hz, 6H), 1.01 (dd, J = 6.9, 2.3 Hz, 6H). Molecular formula: $C_{15}H_{25}NO_3$. Expected ($[M+H]^+$): 268.1913 m/z. HRMS found: 268.1901 m/z.

Step 3. 2-(4-isopropyl-2,2-dimethyl-tetrahydro-2H-pyran-4-yl)acetonitrile (3)

A solution of 13.0 g of Ethyl-2-cyano-2-(4-isopropyl-2,2-dimethyl-tetrahydro-2H-pyran-4-yl)acetate (48.6 mmol, 1.0 equiv) and 12.3 g of potassium hydroxide (218 mmol, 4.5 equiv) in 200 mL of anhydrous DMF (1.33 M) under argon was heated to 150 °C for two hours, allowed to cool to room temperature, and quenched by careful addition of 50 mL of water. The mixture was further diluted with 500 mL of a saturated aqueous NH_4Cl solution and extracted with 5 x 50 mL EtOAc. The combined organic phase was washed with 5 x 200 mL brine, dried over $MgSO_4$, filtered, and the filtrate was concentrated *in vacuo*. Purification using silica column chromatography (10:90 EtOAc:hexanes) with MPLC afforded the product as a yellow oil (7.43 g, 78%). 1H NMR (400 MHz, CDCl3) δ 3.81 – 3.66 (m, 4H), 2.60 – 2.45 (m, 4H), 1.78 (p, J = 6.9 Hz, 2H), 1.72 (d, J = 1.8 Hz, 1H), 1.68 (d, J = 1.8 Hz, 1H), 1.66 – 1.60 (m, 1H), 1.53 (s, 1H), 1.50 (s, 1H), 1.43 (ddd, J = 3.9, 3.1, 1.8 Hz, 1H), 1.42 – 1.38 (m, 1H), 1.32 (s, 6H), 1.28 (d, J = 0.6 Hz, 7H), 0.99 (d, J = 6.9 Hz, 6H), 0.94 (d, J = 6.8 Hz, 6H). Molecular formula: $C_{12}H_{21}NO$. Expected ($[M+H]^+$): 196.1701 m/z. HRMS found: 196.1699 m/z.

Step 4. 2-(4-isopropyl-2,2-dimethyl-tetrahydro-2H-pyran-4-yl)ethanamine (4)[1]

To a 0 °C cooled solution of 7.43 g of 2-(4-isopropyl-2,2-dimethyl-tetrahydro-2H-pyran-4-yl)acetonitrile (38.0 mmol, 1.0 equiv) in 300 mL of anhydrous methanol (0.13 M) under argon was added 9.88 g of anhydrous cobalt(II) chloride (76.1 mmol, 2 equiv). To this mixture, 28.8 g of sodium borohydride (760.86 mmol, 20 equiv) was carefully added in small portions to accommodate considerable gaseous evolution from the mixture. Upon completion of sodium borohydride addition, the ice bath was removed, and the mixture allowed to warm to room temperature and stirred overnight. The reaction mixture was concentrated *in vacuo* the following day to give a black residue that was resuspended in 200 mL of an aqueous 1 M HCl solution and vigorously stirred until homogenous. This was then extracted with 3 x 100 mL EtOAc and the aqueous phase was alkalinized to pH 13 with 200 mL of an aqueous 2 M NaOH solution. The alkaline mixture was extracted with 3 x 100 mL EtOAc and the combined organic phase was washed with 50 mL of 2 M NaOH, dried over $MgSO_4$, filtered, and the filtrate was concentrated *in vacuo* to give a light a yellow oil (5.11 g, 67%) without the need for further purification. 1H NMR (501 MHz, CDCl3) δ 3.74 – 3.59 (m, 2H), 2.65 (dd, J = 10.3, 6.5 Hz, 2H), 2.02 – 1.85 (m, 1H), 1.80 (s, 2H), 1.65 – 1.54 (m, 3H), 1.46 (ddd, J = 13.9, 9.8, 4.3 Hz, 1H), 1.41 – 1.24 (m, 3H), 1.22 (s, 3H), 1.16 (s, 3H), 0.81 (td, J = 8.3, 3.8 Hz, 6H). Molecular formula: $C_{12}H_{26}NO$. Expected ($[M+H]^+$): 200.2014 m/z. HRMS found: 200.2011 m/z.

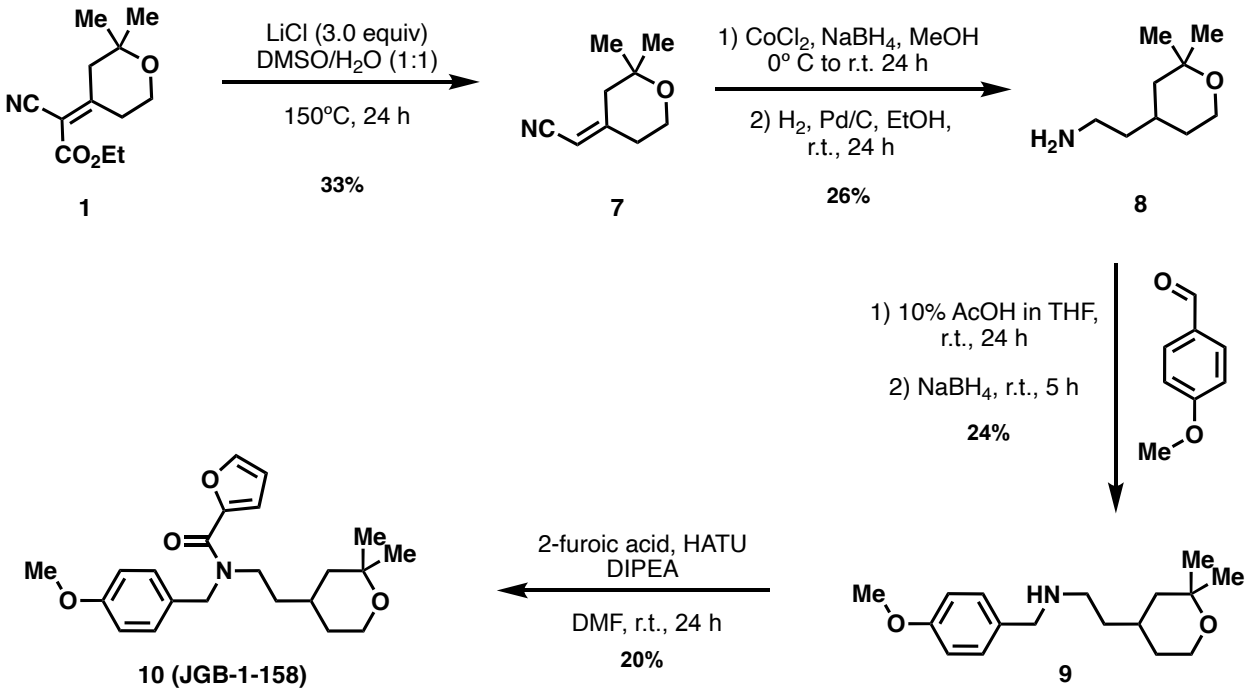
Step 5. [2-(4-Isopropyl-2,2-dimethyl-tetrahydro-pyran-4-yl)-ethyl]-(4-methoxy-benzyl)-amine (5)

To a solution of 2.22 mL of 4-methoxybenzaldehyde (18.3 mmol, 0.71 equiv) in 200 mL of 10% glacial AcOH in anhydrous THF (91.5 mM) at room temperature under argon was added 5.11 g of 2-(4-isopropyl-2,2-dimethyl-tetrahydro-2H-pyran-4-yl)ethanamine (25.6 mmol, 1 equiv) and the mixture was stirred overnight. After 24 h, 1.94 g of sodium borohydride (51.3 mmol, 2.0 equiv) was added and the mixture was stirred for 5 h. The mixture was then diluted with 100 mL of EtOAc and quenched with 100 mL of an aqueous 1 M NaOH solution. The resultant mixture was washed with 3 x 100 mL 1 M NaOH, dried over $MgSO_4$, filtered, and the filtrate was concentrated *in vacuo.* Purification using high-grade silica column chromatography (0.5:99.5 MeOH:DCM) with 0.75:99.25 triethylamine:DCM primed silica and MPLC afforded the product as light tan oil (2.42 g, 30%). 1H NMR (501 MHz, CDCl3) δ 7.34 (d, J = 8.5 Hz, 2H), 6.90 – 6.85 (m, 2H), 5.30 (s, 1H), 3.85 – 3.79 (m, 2H), 3.77 (s, 3H), 3.75 – 3.61 (m, 2H), 2.73 – 2.66 (m, 2H), 1.92 – 1.76 (m, 2H), 1.60 (h, J = 6.9 Hz, 1H), 1.50 (ddd, J = 14.0, 9.9, 4.3 Hz, 1H), 1.40 (d, J = 14.1 Hz, 1H), 1.32 – 1.23 (m, 3H), 1.22 (d, J = 3.9 Hz, 3H), 1.19 (s, 3H), 0.82 (dd, J = 6.9, 2.7 Hz, 6H). Molecular formula: $C_{20}H_{33}NO_2$. Expected ($[M+H]^+$): 320.2590 m/z. HRMS found: 320.2582 m/z.

Step 6. N-[(4-Methoxyphenyl)methyl]-N-[2-[tetrahydro-2,2-dimethyl-4-(1-methylethyl)-2H-pyran-4-yl]ethyl]-2-furancarboxamide (6, NP-A3-B2)

To a solution of 70.2 mg of 2-furoic acid (0.626 mmol, 2.0 equiv) in 2 mL of anhydrous DMF (0.31 M) at room temperature under argon was added 218 µL of diisopropylethylamine (1.25 mmol, 4.0 equiv). The mixture was stirred for 30 m, after which 238 mg of HATU (0.626 mmol, 2.0 equiv) was added and the mixture was stirred for another 30 m. At that time, 100 mg of [2-(4-Isopropyl-2,2-dimethyl-tetrahydro-pyran-4-yl)-ethyl]-(4-methoxy-benzyl)-amine (0.313 mmol, 1.0 equiv) was added and the mixture was stirred overnight. After 24 h, the reaction mixture was diluted with 30 mL of a saturated aqueous NH_4Cl solution and extracted with 5 x 5 mL EtOAc. The combined organic phase was washed with 5 x 50 mL brine, dried over $MgSO_4$, filtered, and the filtrate was concentrated *in vacuo*. Purification using silica column chromatography (20:80 EtOAc:hexanes) with MPLC afforded the product as a light grey oil (58.3 mg, 45%). 1H NMR (501 MHz, CDCl3) δ 7.43 (d, J = 2.0 Hz, 1H), 7.21 (d, J = 8.2 Hz, 2H), 7.06 – 7.02 (m, 1H), 6.91 – 6.85 (m, 2H), 6.46 (s, 1H), 4.79 (d, J = 15.4 Hz, 1H), 4.68 (d, J = 15.9 Hz, 1H), 3.80 (s, 3H), 3.65 (dt, J = 7.8, 3.7 Hz, 2H), 3.45 (s, 2H), 1.87 – 1.75 (m, 2H), 1.62 (p, J = 7.2 Hz, 1H), 1.56 – 1.47 (m, 2H), 1.41 (d, J = 14.4 Hz, 1H), 1.29 – 1.17 (m, 14H), 0.90 (d, J = 6.2 Hz, 1H), 0.85 (dd, J = 7.0, 3.6 Hz, 6H), 0.08 (s, 1H). Molecular formula: $C_{25}H_{35}NO_4$. Expected ($[M+H]^+$): 414.2644 m/z. HRMS found: 414.2637 m/z.

II. Scheme II: Synthetic Procedures for desisopropyl-NP-A3-B2



Step 1. 2-(2,2-dimethyltetrahydro-4*H*-pyran-4-ylidene)acetonitrile (7)

To a solution of 10.0 g of Ethyl-2-cyano-2-(2,2-dimethyl-tetrahydropyran-4-ylidene)acetate (44.79 mmol, 1.0 equiv), as prepared previously, in 100 mL (1:1) DMSO:H_2O (0.45 M) was added 5.70 g (134.37 mmol, 3.0 equiv) of LiCl. The mixture was heated to 150 °C for 24 h and allowed to cool to room temperature. It was then diluted with 100 mL of H_2O and extracted with 5 x 50 mL EtOAc. The combined organic phase was washed with 5 x 200 mL brine, dried over $MgSO_4$, filtered, and the filtrate was concentrated *in vacuo*. Purification using silica column chromatography (10:90 EtOAc:hexanes) with MPLC afforded the product as a yellow oil (2.21 g, 33%). 1H NMR (501 MHz, CDCl3) δ 5.82 (tq, J = 3.2, 1.6 Hz, 1H), 5.70 (p, J = 1.7 Hz, 1H), 4.16 (h, J = 2.3 Hz, 2H), 3.81 (t, J = 5.5 Hz, 2H), 3.77 (t, J = 5.7 Hz, 1H), 3.05 – 3.01 (m, 3H), 2.47 (s, 1H), 2.33 – 2.29 (m, 1H), 2.05 – 2.01 (m, 2H), 1.99 – 1.96 (m, 2H), 1.24 (d, J = 3.3 Hz, 7H), 1.22 (s, 5H). Molecular formula: $C_9H_{13}NO$. Expected ($[M+H]^+$): 152.1075 m/z. HRMS found: 152.1072 m/z.

Step 2. 2-(2,2-dimethyltetrahydro-2*H*-pyran-4-yl)ethan-1-amine (8)

1) To a 0 °C cooled solution of 2.21 g of 2-(2,2-dimethyltetrahydro-4*H*-pyran-4-ylidene)acetonitrile (14.62 mmol, 1.0 equiv) in 200 mL of anhydrous methanol (73.1 mM) under argon was added 15.18 g of anhydrous cobalt(II) chloride (116.92 mmol, 8 equiv). To this mixture, 44.23 g of sodium borohydride (1.17 mol, 80 equiv) was carefully added in small portions to accommodate considerable and rapid gaseous evolution from the mixture. Upon completion of sodium borohydride addition, the ice bath was removed, and the mixture allowed to warm to room temperature and stirred overnight. The reaction mixture was concentrated *in vacuo* the following day to give a black residue that was resuspended in 200 mL of an aqueous 1 M HCl solution and vigorously stirred until homogenous. This was then extracted with 3 x 100 mL EtOAc and the aqueous phase was alkalinized to pH 13 with 200 mL of an aqueous 2 M NaOH solution. The alkaline mixture was extracted with 3 x 100 mL EtOAc and the combined organic phase was washed with 50 mL of 2 M NaOH, dried over $MgSO_4$, filtered, and the filtrate was concentrated *in vacuo* to give a light a yellow oil (765 mg, 31%). ^{1}H NMR and LCMS

confirmed incomplete reduction of the double bond giving a mixture of the desired product and 2-(2,2-dimethyltetrahydro-4*H*-pyran-4-ylidene)ethan-1-amine.

2) The 765 mg mixture of 2-(2,2-dimethyltetrahydro-2*H*-pyran-4-yl)ethan-1-amine and 2-(2,2-dimethyltetrahydro-4*H*-pyran-4-ylidene)ethan-1-amine (~4.9 mmol, 1 equiv) was dissolved in 50 mL anhydrous ethanol along with 1.57 g Pd/C (10 wt. %, 1.48 mmol, 0.3 equiv) under H_2 and stirred vigorously. After 24 h the solution was filtered over cotton and silica to remove the catalyst and the filtrate was concentrated *in vacuo* to give a light a yellow oil (662 mg, 85 %). ^{1}H NMR and LCMS confirmed complete hydrogenation of the olefin was achieved. 1H NMR (501 MHz, CDCl3) δ 4.75 (s, 2H), 3.75 – 3.57 (m, 2H), 2.81 – 2.70 (m, 1H), 1.94 (s, 1H), 1.58 – 1.28 (m, 5H), 1.28 – 1.20 (m, 4H), 1.21 – 1.16 (m, 6H). Molecular formula: $C_9H_{19}NO$. Expected ($[M+H]^+$): 158.1545 m/z. HRMS found: 158.1550 m/z.

Step 3. 2-(2,2-dimethyltetrahydro-2*H*-pyran-4-yl)-*N*-(4-methoxybenzyl)ethan-1-amine (9)

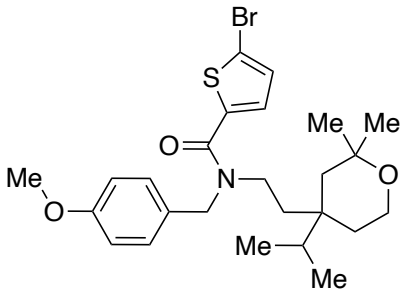
To a solution of 407 mg of 4-methoxybenzaldehyde (3 mmol, 0.71 equiv) in 25 mL of 10% glacial AcOH in anhydrous THF (0.12 M) at room temperature under argon was added 662 mg of 2-(4-isopropyl-2,2-dimethyl-tetrahydro-2H-pyran-4-yl)ethanamine (4.21 mmol, 1.0 equiv) by addition of 25 mL of a 0.17 M solution in 10% glacial AcOH in anhydrous THF and the mixture was stirred overnight. After 24 h, 319 mg of sodium borohydride (8.42 mmol, 2.0 equiv) was added and the mixture was stirred for 5 h. The mixture was then diluted with 50 mL of EtOAc and quenched with 100 mL of an aqueous 1 M NaOH solution. The resultant mixture was washed with 3 x 100 mL 1 M NaOH, dried over $MgSO_4$, filtered, and the filtrate was concentrated *in vacuo.* Purification using high-grade silica column chromatography (0.5:99.5 MeOH:DCM) with 0.75:99.25 triethylamine:DCM primed silica and MPLC afforded the product as light tan oil (277 mg, 24%). 1H NMR (400 MHz, CDCl3) δ 7.36 – 7.20 (m, 2H), 6.73 (t, J = 8.1 Hz, 2H), 3.84 – 3.69 (m, 1H), 3.65 (d, J = 3.6 Hz, 2H), 3.60 (d, J = 3.6 Hz, 1H), 3.57 – 3.40 (m, 3H), 2.76 – 2.59 (m, 3H), 1.49 – 1.13 (m, 8H), 1.04 – 0.98 (m, 6H). Molecular formula: $C_{17}H_{27}NO_2$. Expected ($[M+H]^+$): 278.2120 m/z. HRMS found: 278.2118 m/z.

Step 4. *N*-(2-(2,2-dimethyltetrahydro-2*H*-pyran-4-yl)ethyl)-*N*-(4-methoxybenzyl)furan-2-carboxamide (10)

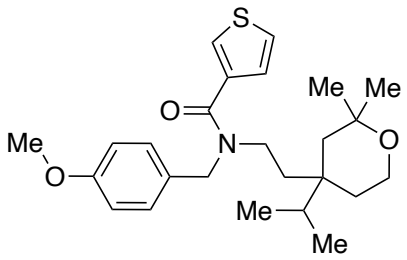
To a solution of 40.4 mg of 2-furoic acid (0.36 mmol, 2.0 equiv) in 1 mL of anhydrous DMF (0.36 M) at room temperature under argon was added 126 µL of diisopropylethylamine (0.72 mmol, 4.0 equiv). The mixture was stirred for 30 m, after which time 137 mg of HATU (0.36 mmol, 2.0 equiv) was added and the mixture was stirred for another 30 m. At that time, 50 mg of 2-(2,2-dimethyltetrahydro-2*H*-pyran-4-yl)-*N*-(4-methoxybenzyl)ethan-1-amine (0.18 mmol, 1.0 equiv) was added as a 0.18 M solution in 1 mL anhydrous DMF and the mixture was stirred overnight. After 24 h, the reaction mixture was diluted with 30 mL of a saturated aqueous NH_4Cl solution and extracted with 5 x 5 mL EtOAc. The combined organic phase was washed with 5 x 50 mL brine, dried over $MgSO_4$, filtered, and the filtrate was concentrated *in vacuo*. Purification using silica column chromatography (20:80 EtOAc:hexanes) with MPLC afforded the product as a light grey oil (16 mg, 20%). 1H NMR (501 MHz, DMSO) δ 7.88 – 7.65 (m, 0H), 7.18 (s, 2H), 6.89 (s, 2H), 6.59 (s, 1H), 4.60 (s, 1H), 3.74 – 3.66 (m, 2H), 3.56 – 3.36 (m, 2H), 3.32 – 3.24 (m, 1H), 1.54 (s, 1H), 1.40 (s, 3H), 1.22 (s, 1H), 1.04 (dd, J = 12.1, 5.4 Hz, 4H), 0.98 – 0.83 (m, 0H). Molecular formula: $C_{22}H_{29}NO_4$. Expected ($[M+H]^+$): 372.2175 m/z. HRMS found: 372.2173 m/z.

III. NP-A3-B2 Series Compound Synthesis and Characterization

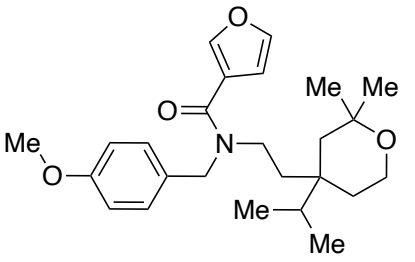
JGB-1-122 (5-bromo-N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxybenzyl)thiophene-2-carboxamide). Synthesized via HATU coupling 5-bromothiophene-2-carboxylic acid with intermediate **5** (same conditions as scheme **I** step 6). 1H NMR (501 MHz, DMSO) δ 7.73 (d, J = 5.1 Hz, 1H), 7.22 (d, J = 8.3 Hz, 2H), 7.11 (d, J = 5.1 Hz, 1H), 6.93 – 6.88 (m, 2H), 4.68 – 4.45 (m, 2H), 3.74 (s, 4H), 3.43 (s, 2H), 3.12 (s, 2H), 1.62 (s, 2H), 1.43 (s, 2H), 1.32 – 1.14 (m, 4H), 1.05 (s, 3H), 1.03 (s, 3H), 0.66 (s, 6H). Molecular formula: $C_{25}H_{34}BrNO_3S$. Expected ($[M+H]^+$): 508.1521 m/z. HRMS found: 508.1512 m/z.



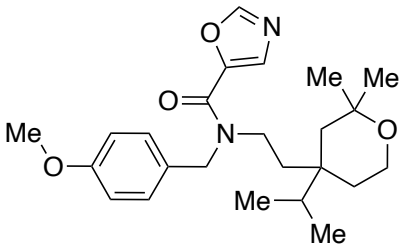
JGB-1-127 (N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxybenzyl)thiophene-3-carboxamide). Synthesized via HATU coupling thiophene-3-carboxylic acid with intermediate **5** (same conditions as scheme **I** step 6). 1H NMR (500 MHz, CDCl3) δ 7.49 (s, 1H), 7.24 (s, 4H), 6.88 (d, J = 8.6 Hz, 1H), 4.56 (s, 1H), 3.80 (s, 2H), 3.72 – 3.15 (m, 4H), 1.82 (s, 1H), 1.63 – 1.50 (m, 3H), 1.23 (s, 10H), 1.15 (s, 2H), 0.85 (th, J = 13.2, 6.8 Hz, 5H), 0.63 (s, 1H). Molecular formula: $C_{25}H_{35}NO_3S$. Expected ($[M+H]^+$): 430.2416 m/z. HRMS found: 430.2405 m/z.



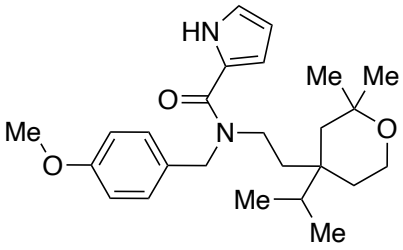
JGB-1-128 (N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxybenzyl)furan-3-carboxamide). Synthesized via HATU coupling furan-3-carboxylic acid with intermediate **5** (same conditions as scheme **I** step 6). 1H NMR (501 MHz, CDCl3) δ 7.67 (s, 1H), 7.38 (s, 1H), 7.16 (d, J = 8.1 Hz, 2H), 6.90 (dd, J = 9.1, 2.8 Hz, 2H), 6.58 (d, J = 1.7 Hz, 1H), 4.64 (q, J = 16.0 Hz, 2H), 3.81 (d, J = 2.8 Hz, 3H), 3.64 (q, J = 8.6 Hz, 2H), 3.38 (td, J = 12.0, 6.0 Hz, 2H), 1.78 (s, 2H), 1.59 (p, J = 7.0 Hz, 1H), 1.49 (dt, J = 14.2, 7.3 Hz, 1H), 1.40 (d, J = 14.1 Hz, 1H), 1.20 (s, 2H), 1.18 (s, 3H), 0.92 – 0.86 (m, 3H), 0.86 – 0.81 (m, 6H). Molecular formula: $C_{25}H_{35}NO_4$. Expected ($[M+H]^+$): 414.2644 m/z. HRMS found: 414.2633 m/z.



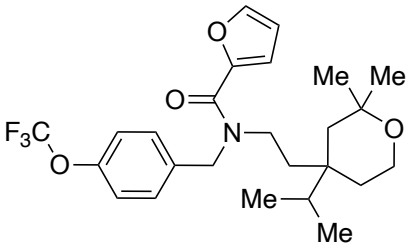
JGB-1-129 (N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxybenzyl)oxazole-5-carboxamide). Synthesized via HATU coupling oxazole-5-carboxylic acid with intermediate **5** (same conditions as scheme **I** step 6). 1H NMR (501 MHz, DMSO) δ 8.46 (s, 1H), 7.78 – 7.50 (m, 1H), 7.17 (s, 2H), 6.94 – 6.82 (m, 2H), 4.76 – 4.42 (m, 2H), 3.70 (d, J = 4.2 Hz, 3H), 3.40 – 3.17 (m, 2H), 1.63 (s, 2H), 1.47 (s, 2H), 1.34 – 0.94 (m, 10H), 0.79 – 0.62 (m, 6H). Molecular formula: $C_{24}H_{34}N_2O_4$. Expected ($[M+H]^+$): 415.2552 m/z. HRMS found: 437.2408 m/z. Calculated ($[M+Na]^+$): 437.2416 m/z.



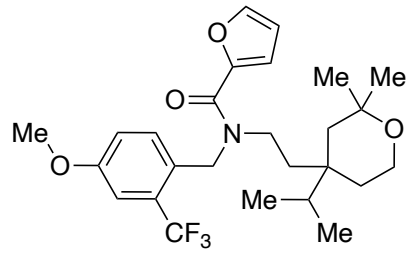
JGB-1-130 (N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxybenzyl)-1H-pyrrole-2-carboxamide). Synthesized via HATU coupling 1H-pyrrole-2-carboxylic acid with intermediate **5** (same conditions as scheme **I** step 6). 1H NMR (501 MHz, CDCl3) δ 9.51 (s, 1H), 7.20 (d, J = 8.3 Hz, 1H), 6.97 – 6.87 (m, 3H), 6.43 (s, 1H), 6.17 (s, 1H), 4.90 – 4.71 (m, 2H), 3.81 (s, 2H), 3.67 (q, J = 7.4 Hz, 2H), 3.50 – 3.39 (m, 2H), 3.35 (s, 1H), 2.23 (dd, J = 15.8, 8.2 Hz, 1H), 1.84 (dt, J = 17.0, 10.3 Hz, 2H), 1.55 (s, 4H), 1.22 (s, 3H), 1.19 (s, 3H), 0.88 – 0.86 (m, 6H). Molecular formula: $C_{25}H_{36}N_2O_3$. Expected ($[M+H]^+$): 413.2804 m/z. HRMS found: 413.2789 m/z.



JGB-1-131 (N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-(trifluoromethoxy)benzyl)furan-2-carboxamide).
Synthesized via reductive amination of 4-(trifluoromethoxy)benzaldehyde with intermediate **4** and subsequent HATU coupling with furoic acid (same conditions as scheme **I** steps 5 and 6). 1H NMR (501 MHz, CDCl3) δ 7.43 (s, 1H), 7.33 (d, J = 8.4 Hz, 2H), 7.19 (d, J = 8.2 Hz, 2H), 7.08 (s, 1H), 6.48 (s, 1H), 4.84 (d, J = 15.7 Hz, 1H), 4.73 (d, J = 15.8 Hz, 1H), 3.67 – 3.63 (m, 2H), 1.90 (s, 2H), 1.77 (dt, J = 13.4, 6.8 Hz, 2H), 1.63 (p, J = 6.8 Hz, 3H), 1.52 (dt, J = 14.1, 7.4 Hz, 4H), 1.44 (s, 1H), 1.41 (s, 1H), 1.20 (s, 3H), 1.19 (s, 3H), 0.84 (t, J = 6.4 Hz, 6H). 19F NMR (376 MHz, CDCl3) δ -59.14. Molecular formula: $C_{25}H_{32}F_3NO_4$. Expected ($[M+H]^+$): 468.2362 m/z. HRMS found: 468.2363 m/z.

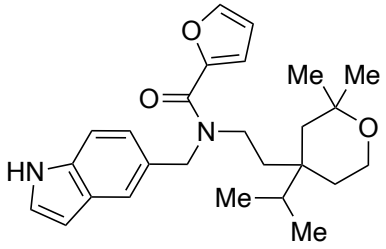


JGB-1-133 (N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxy-2-(trifluoromethyl)benzyl)furan-2-carboxamide).
Synthesized via reductive amination of 4-methoxy-2-(trifluoromethyl)benzaldehyde with intermediate **4** and subsequent HATU coupling with furoic acid (same conditions as scheme **I** steps 5 and 6). 1H NMR (501 MHz, CDCl3) δ 7.50 – 7.41 (m, 3H), 7.08 (d, J = 3.5 Hz, 1H), 6.99 (d, J = 8.4 Hz, 1H), 6.48 (dd, J = 3.5, 1.8 Hz, 1H), 4.81 (d, J = 15.5 Hz, 1H), 4.68 (d, J = 15.3 Hz, 1H), 3.90 (s, 3H), 3.65 (dq, J = 7.5, 3.1 Hz, 2H), 3.46 (s, 2H), 1.89 – 1.81 (m, 1H), 1.76 (td, J = 13.0, 5.6 Hz, 2H), 1.64 (p, J = 7.0 Hz, 2H), 1.52 (h, J = 7.2 Hz, 2H), 1.44 (d, J = 2.3 Hz, 1H), 1.20 (s, 3H), 1.19 (s, 3H), 0.85 (d, J = 4.8 Hz, 3H), 0.85 – 0.83 (m, 3H). 19F NMR (376 MHz, CDCl3) δ -62.95. Molecular formula: $C_{26}H_{34}F_3NO_4$. Expected ($[M+H]^+$): 482.2518 m/z. HRMS found: 482.2523 m/z.

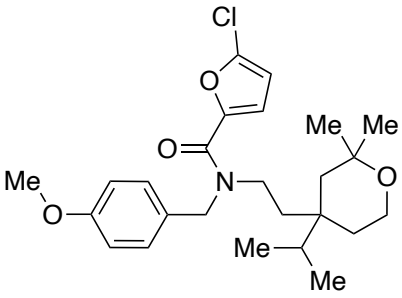


JGB-1-134 (N-((1H-indol-5-yl)methyl)-N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)furan-2-carboxamide).

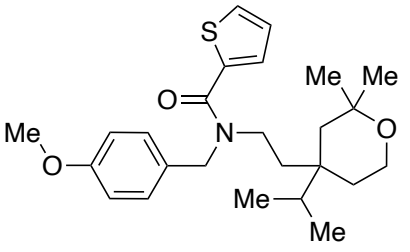
Synthesized via reductive amination of 1H-indole-5-carbaldehyde with intermediate **4** and subsequent HATU coupling with furoic acid (same conditions as scheme **I** steps 5 and 6). 1H NMR (500 MHz, CDCl3) δ 8.28 (s, 1H), 7.52 (s, 1H), 7.39 (d, J = 40.7 Hz, 2H), 7.23 – 6.96 (m, 3H), 6.53 – 6.39 (m, 2H), 4.98 (s, 0H), 4.85 (d, J = 17.2 Hz, 1H), 3.66 – 3.54 (m, 2H), 3.41 (dt, J = 11.9, 6.2 Hz, 1H), 1.87 – 1.61 (m, 4H), 1.55 (tt, J = 10.0, 5.6 Hz, 1H), 1.46 (p, J = 7.2 Hz, 1H), 1.36 (d, J = 14.4 Hz, 1H), 1.28 (d, J = 13.1 Hz, 1H), 1.23 (s, 3H), 1.16 (d, J = 15.2 Hz, 6H), 0.84 – 0.76 (m, 6H). Molecular formula: $C_{26}H_{34}N_2O_3$. Expected ($[M+H]^+$): 423.2648 m/z. HRMS found: 423.2635 m/z.



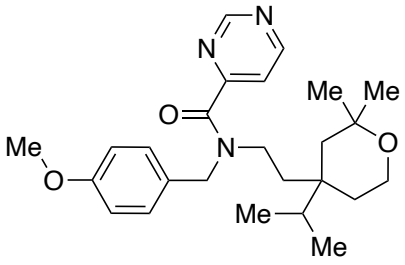
JGB-1-135 (5-chloro-N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxybenzyl)furan-2-carboxamide). Synthesized via HATU coupling 5-chlorofuran-2-carboxylic acid with intermediate **5** (same conditions as scheme **I** step 6). 1H NMR (501 MHz, CDCl3) δ 7.20 (d, J = 8.1 Hz, 2H), 7.06 – 7.01 (m, 1H), 6.90 – 6.86 (m, 2H), 6.26 (d, J = 3.5 Hz, 1H), 4.75 (d, J = 15.4 Hz, 1H), 4.65 (d, J = 15.7 Hz, 1H), 3.80 (s, 3H), 3.65 (dd, J = 7.0, 3.9 Hz, 2H), 3.46 (s, 2H), 1.87 – 1.71 (m, 3H), 1.53 (dt, J = 14.0, 6.9 Hz, 2H), 1.46 – 1.42 (m, 1H), 1.21 (s, 3H), 1.20 (s, 3H), 0.85 (dd, J = 6.9, 4.2 Hz, 6H). Molecular formula: $C_{25}H_{34}ClNO_4$. Expected ($[M+H]^+$): 448.2255 m/z. HRMS found: 448.2247 m/z.



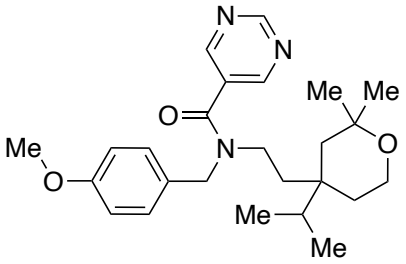
JGB-1-137 (N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxybenzyl)thiophene-2-carboxamide). Synthesized via HATU coupling thiophene-2-carboxylic acid with intermediate **5** (same conditions as scheme **I** step 6). 1H NMR (501 MHz, CDCl3) δ 7.41 (dd, J = 5.1, 1.4 Hz, 1H), 7.29 (d, J = 3.7 Hz, 1H), 7.20 (d, J = 8.1 Hz, 2H), 6.98 (t, J = 4.4 Hz, 1H), 6.90 (d, J = 8.1 Hz, 2H), 4.79 – 4.66 (m, 2H), 3.81 (t, J = 1.3 Hz, 2H), 3.64 – 3.60 (m, 2H), 3.42 (dh, J = 13.4, 7.0 Hz, 2H), 1.80 (ddd, J = 26.5, 12.2, 6.3 Hz, 2H), 1.58 (p, J = 6.9 Hz, 1H), 1.48 (dt, J = 14.1, 7.2 Hz, 1H), 1.36 (dd, J = 31.3, 14.2 Hz, 1H), 1.27 (s, 5H), 1.19 (d, J = 8.7 Hz, 6H), 0.82 (t, J = 7.1 Hz, 6H). Molecular formula: $C_{25}H_{35}NO_3S$. Expected ($[M+H]^+$): 430.2416 m/z. HRMS found: 430.2409 m/z.



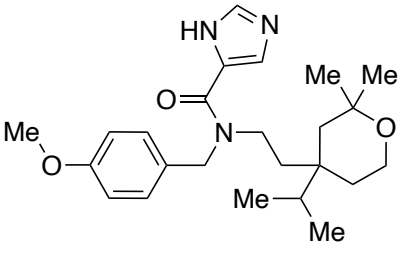
JGB-1-145 (N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxybenzyl)pyrimidine-4-carboxamide). Synthesized via HATU coupling pyrimidine-4-carboxylic acid with intermediate **5** (same conditions as scheme **I** step 6) 1H NMR (501 MHz, CDCl3) δ 9.22 (dd, J = 5.3, 3.3 Hz, 1H), 8.91 – 8.83 (m, 1H), 7.66 – 7.60 (m, 1H), 7.30 – 7.18 (m, 2H), 6.87 (ddd, J = 14.2, 8.8, 3.1 Hz, 2H), 4.84 – 4.48 (m, 2H), 3.82 – 3.77 (m, 2H), 3.64 (dt, J = 9.3, 3.0 Hz, 1H), 3.55 (dq, J = 9.4, 3.5 Hz, 1H), 3.44 – 3.38 (m, 1H), 3.36 – 3.26 (m, 1H), 1.82 (ddt, J = 17.2, 8.6, 3.1 Hz, 1H), 1.72 – 1.64 (m, 1H), 1.57 (ddd, J = 13.4, 8.8, 5.4 Hz, 1H), 1.49 (dt, J = 9.2, 4.2 Hz, 1H), 1.40 (ddd, J = 14.4, 9.0, 3.3 Hz, 2H), 1.29 (d, J = 3.1 Hz, 1H), 1.23 – 1.08 (m, 7H), 1.07 – 0.95 (m, 1H), 0.85 (ddd, J = 9.3, 6.4, 2.7 Hz, 4H), 0.65 (ddd, J = 15.8, 6.9, 3.1 Hz, 2H). Molecular formula: $C_{25}H_{35}N_3O_3$. Expected ($[M+H]^+$): 426.2757 m/z. HRMS found: 426.2742 m/z.



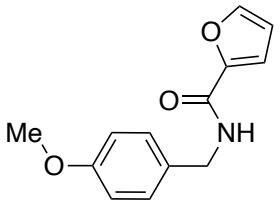
JGB-1-146 (N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxybenzyl)pyrimidine-5-carboxamide). Synthesized via HATU coupling pyrimidine-5-carboxylic acid with intermediate **5** (same conditions as scheme **I** step 6). 1H NMR (501 MHz, CDCl3) δ 9.30 – 9.19 (m, 1H), 8.82 (s, 2H), 7.06 (d, J = 8.1 Hz, 1H), 6.90 (d, J = 8.0 Hz, 2H), 4.71 (d, J = 9.5 Hz, 1H), 4.45 (s, 1H), 3.81 (s, 3H), 3.73 – 3.41 (m, 3H), 3.14 (s, 1H), 1.85 (t, J = 8.6 Hz, 1H), 1.75 – 1.32 (m, 6H), 1.22 – 0.91 (m, 6H), 0.90 – 0.82 (m, 4H), 0.65 – 0.54 (m, 2H).). Molecular formula: $C_{25}H_{35}N_3O_3$. Expected ($[M+H]^+$): 426.2757 m/z. HRMS found: 426.2737 m/z.



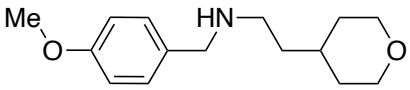
JGB-1-147 (N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxybenzyl)-1H-imidazole-5-carboxamide).
Synthesized via HATU coupling 1H-imidazole-5-carboxylic acid with intermediate **5** (same conditions as scheme **I** step 6). 1H NMR (501 MHz, CDCl3) δ 8.75 (s, 1H), 7.39 (s, 1H), 7.11 (d, J = 7.7 Hz, 2H), 6.93 (d, J = 7.9 Hz, 2H), 4.73 (q, J = 17.2 Hz, 2H), 3.80 (s, 3H), 3.71 (s, 2H), 3.64 – 3.34 (m, 3H), 2.12 (s, 1H), 1.93 – 1.69 (m, 3H), 1.70 – 1.53 (m, 3H), 1.46 (d, J = 13.9 Hz, 2H), 1.22 (s, 6H), 0.82 (d, J = 7.8 Hz, 6H). Molecular formula: $C_{24}H_{35}N_3O_3$. Expected ($[M+H]^+$): 414.2757 m/z. HRMS found: 414.2739 m/z.



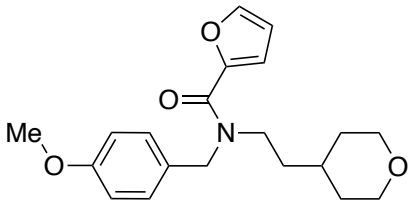
JGB-1-151 (N-(4-methoxybenzyl)furan-2-carboxamide). Synthesized via HATU coupling between (4-methoxyphenyl)methanamine and furoic acid (same conditions as scheme **I** step 6). 1H NMR (501 MHz, CDCl3) δ 7.40 (dd, J = 1.8, 0.9 Hz, 1H), 7.30 – 7.25 (m, 2H), 7.14 (dd, J = 3.5, 0.9 Hz, 1H), 6.91 – 6.85 (m, 2H), 6.58 (s, 1H), 6.49 (dd, J = 3.5, 1.7 Hz, 1H), 4.54 (d, J = 5.8 Hz, 2H), 3.80 (s, 3H). Molecular formula: $C_{13}H_{13}NO_3$. Expected ($[M+H]^+$): 232.1 m/z. LRMS found: 232.1 m/z.



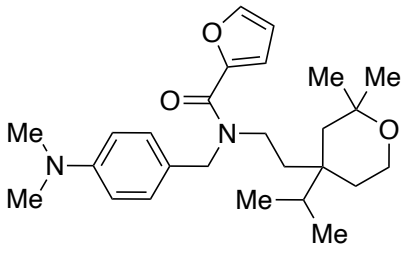
JGB-1-152 (N-(4-methoxybenzyl)-2-(tetrahydro-2H-pyran-4-yl)ethan-1-amine). Synthesized via reductive amination of 4-methoxybenzaldehyde with 2-(tetrahydro-2H-pyran-4-yl)ethan-1-amine (same conditions as scheme **I** step 5). 1H NMR (501 MHz, CDCl3) δ 7.23 – 7.18 (m, 2H), 6.87 – 6.81 (m, 2H), 3.93 – 3.88 (m, 2H), 3.77 (s, 3H), 3.70 (s, 2H), 3.33 (td, J = 11.8, 1.9 Hz, 2H), 2.62 (t, J = 7.4 Hz, 2H), 1.57 – 1.52 (m, 3H), 1.47 – 1.41 (m, 2H), 1.32 – 1.21 (m, 3H). Molecular formula: $C_{15}H_{23}NO_2$. Expected ($[M+H]^+$): 250.2 m/z. LRMS found: 250.2 m/z.



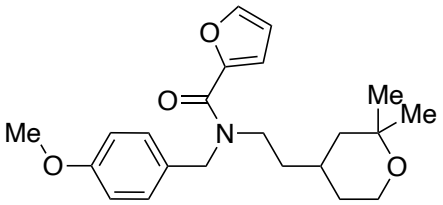
JGB-1-153 (N-(4-methoxybenzyl)-N-(2-(tetrahydro-2H-pyran-4-yl)ethyl)furan-2-carboxamide). Synthesized via reductive amination of 4-methoxybenzaldehyde with 2-(tetrahydro-2H-pyran-4-yl)ethan-1-amine and subsequent HATU coupling with furoic acid (same conditions as scheme **I** steps 5 and 6). 1H NMR (501 MHz, CDCl3) δ 7.44 (s, 1H), 7.20 (d, J = 7.8 Hz, 2H), 7.02 (s, 1H), 6.87 (d, J = 8.1 Hz, 2H), 6.46 (s, 1H), 4.71 (s, 2H), 3.94 – 3.87 (m, 2H), 3.79 (s, 3H), 3.47 (s, 2H), 3.32 (td, J = 11.7, 2.3 Hz, 2H), 2.79 (d, J = 2.1 Hz, 1H), 1.60 – 1.43 (m, 5H), 1.28 (dp, J = 16.5, 5.6 Hz, 3H). Molecular formula: $C_{20}H_{25}NO_4$. Expected ($[M+H]^+$): 344.2 m/z. LRMS found: 344.2 m/z.



JGB-1-155 (N-(4-(dimethylamino)benzyl)-N-(2-(4-isopropyl-2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)furan-2-carboxamide).
Synthesized via reductive amination of 4-(dimethylamino)benzaldehyde with intermediate **4** and subsequent HATU coupling with furoic acid (same conditions as scheme **I** steps 5 and 6). 1H NMR (501 MHz, CDCl3) δ 7.43 (s, 1H), 7.16 (d, J = 8.4 Hz, 2H), 7.02 (s, 1H), 6.76 (d, J = 8.3 Hz, 2H), 6.47 (d, J = 12.8 Hz, 1H), 4.82 – 4.57 (m, 2H), 3.64 (td, J = 11.0, 5.1 Hz, 2H), 3.45 (s, 2H), 2.95 (s, 6H), 1.82 (dt, J = 23.9, 7.2 Hz, 3H), 1.65 – 1.59 (m, 1H), 1.54 – 1.48 (m, 1H), 1.41 (d, J = 14.2 Hz, 1H), 1.29 – 1.18 (m, 13H), 0.86 (t, J = 7.6 Hz, 6H). Molecular formula: $C_{26}H_{38}N_2O_3$. Expected ($[M+H]^+$): 427.2961 m/z. HRMS found: 427.2949 m/z.



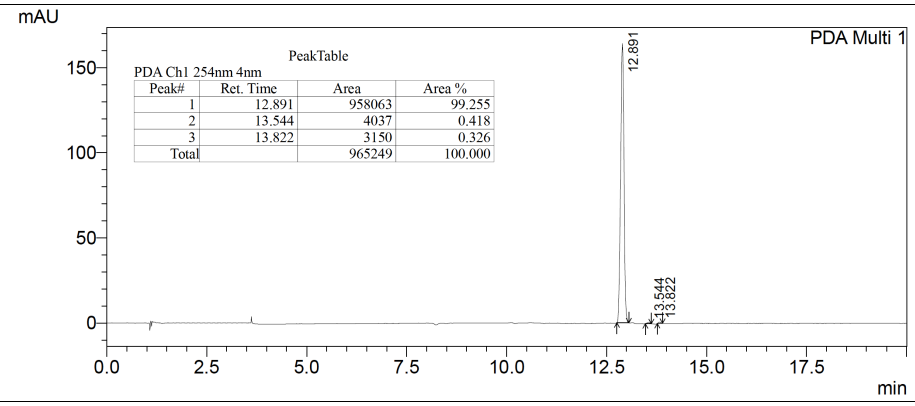
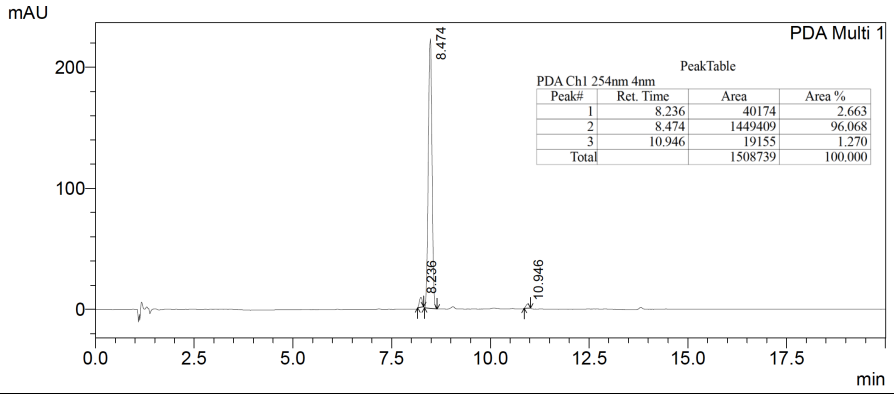
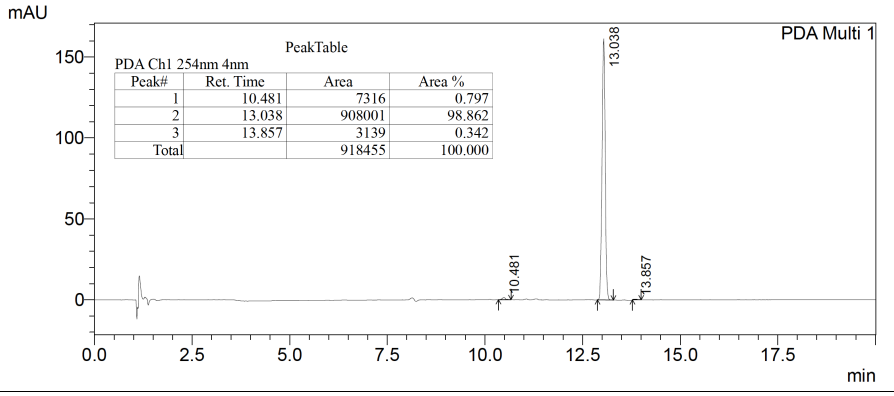
JGB-1-158 (N-(2-(2,2-dimethyltetrahydro-2H-pyran-4-yl)ethyl)-N-(4-methoxybenzyl)furan-2-carboxamide). Synthesized as described in scheme **II**. 1H NMR (501 MHz, DMSO) δ 7.85 – 7.74 (m, 1H), 7.19 (s, 2H), 7.02 (s, 1H), 6.90 (s, 2H), 6.60 (s, 1H), 4.62 (s, 2H), 3.75 – 3.68 (m, 3H), 3.56 – 3.36 (m, 3H), 3.34 – 3.27 (m, 2H), 1.41 (s, 5H), 1.23 (s, 1H), 1.06 (dd, J = 12.1, 5.4 Hz, 6H), 1.00 – 0.87 (m, 2H). Molecular formula: $C_{22}H_{29}NO_4$. Expected ($[M+H]^+$): 372.2175 m/z. HRMS found: 372.2173 m/z.

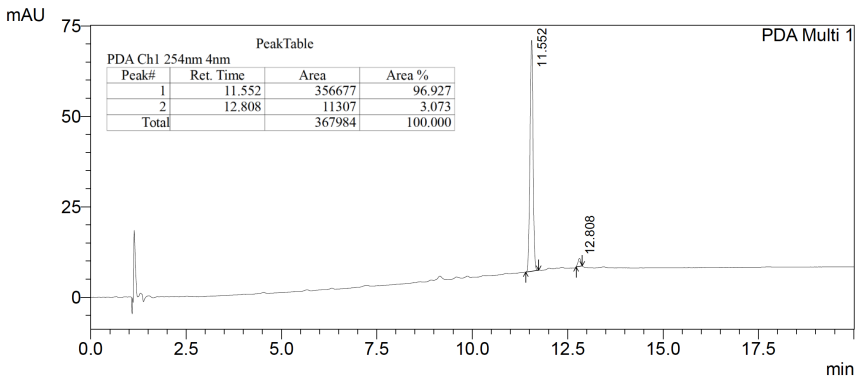
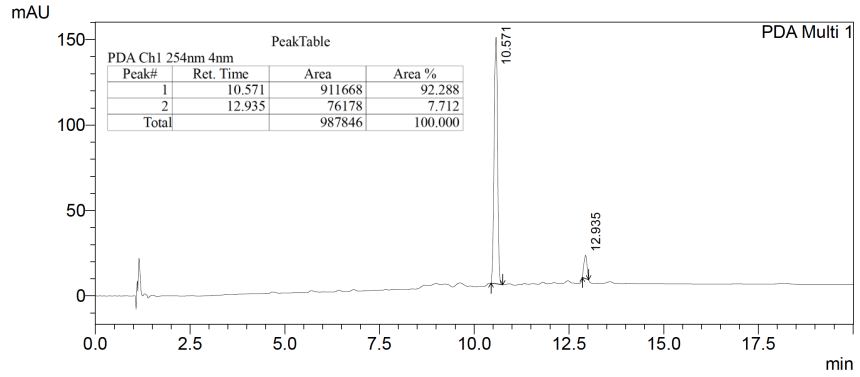
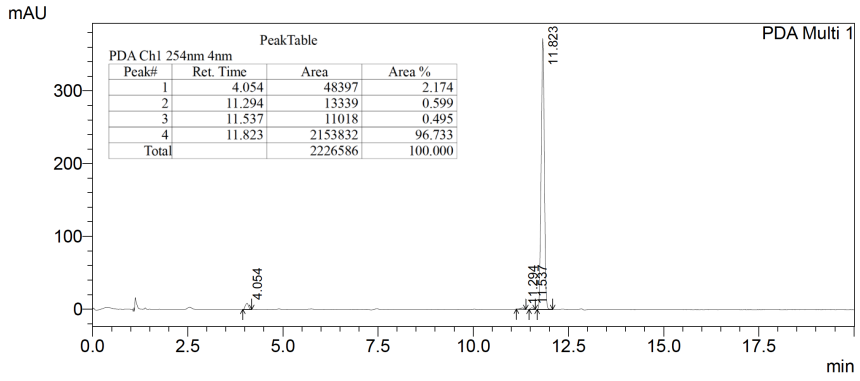
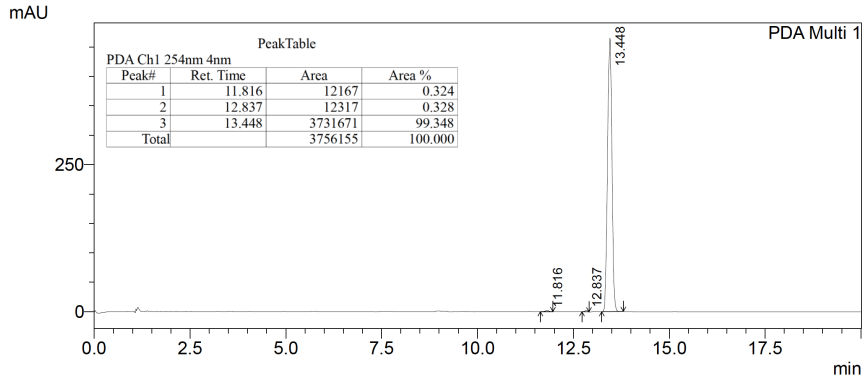


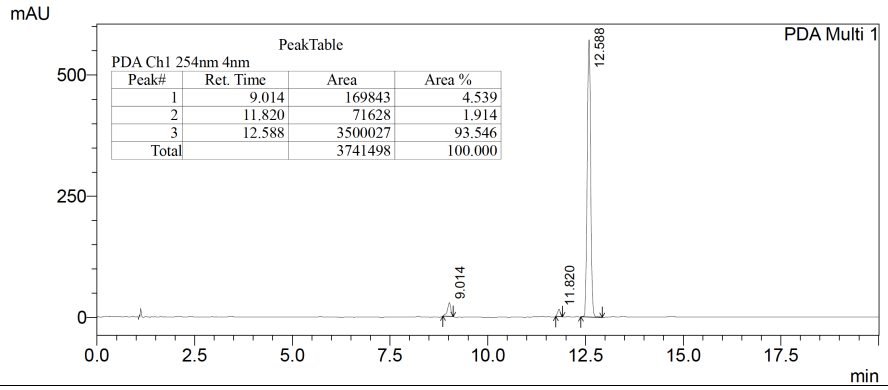
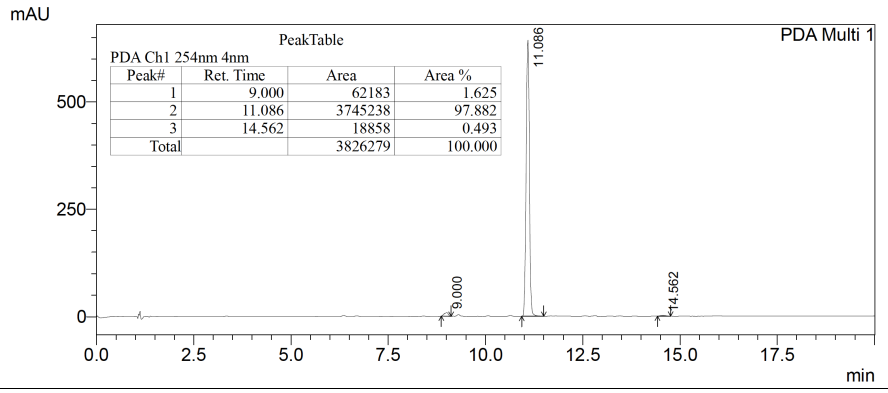
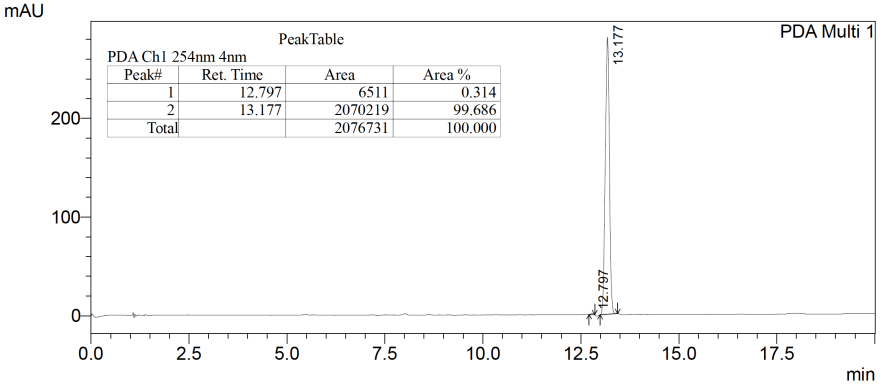
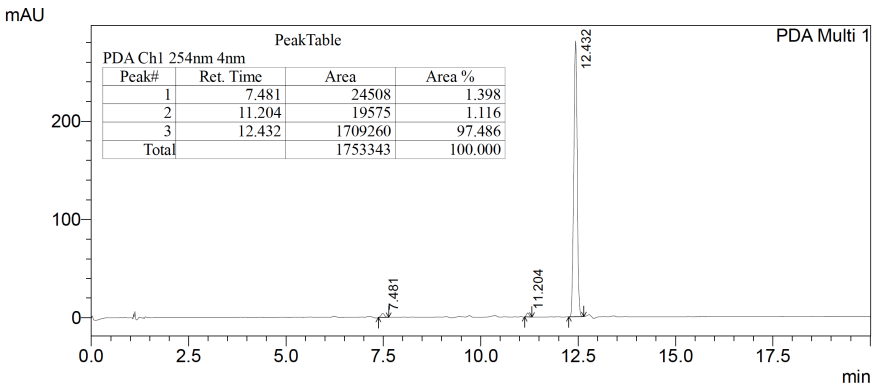
IV. Methods for Purity Determination

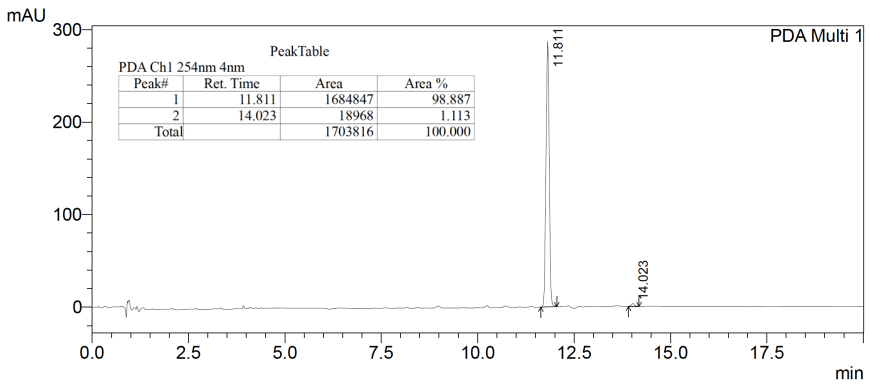
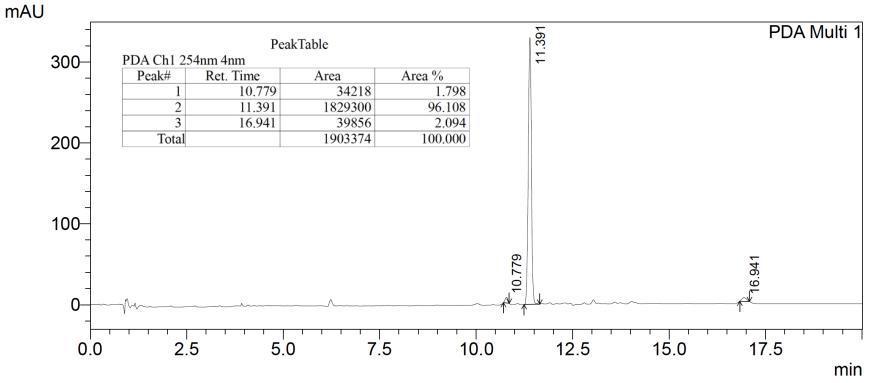
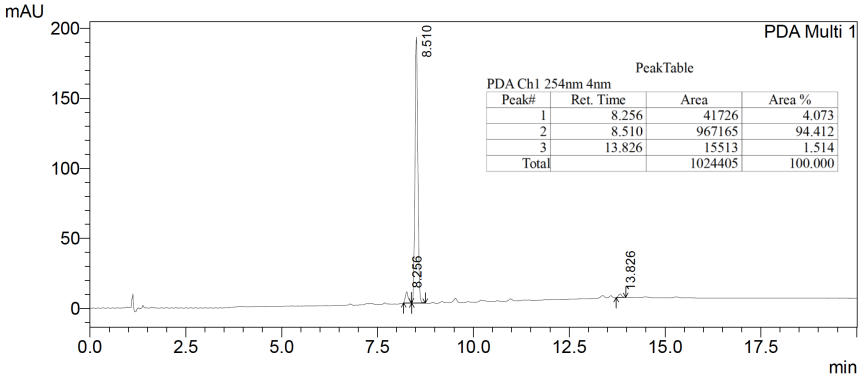
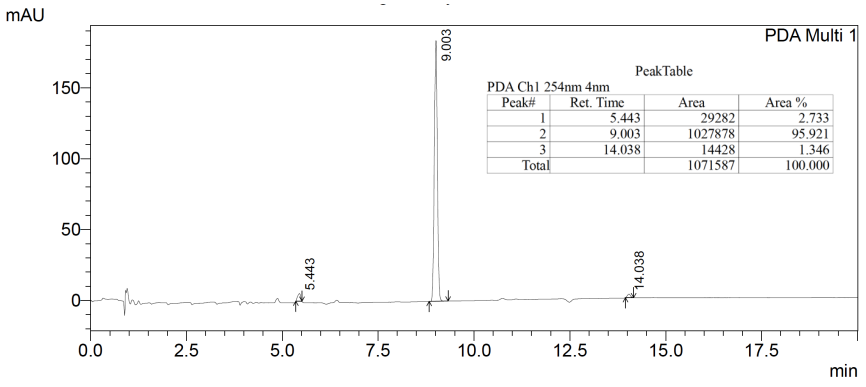
The purity of final compounds was determined by HPLC on a Shimadzu Nexera SIL-30AC Autosampler using an Agilent Eclipse plus C18 column (4.6 × 250 mm, 5 µm) with UV absorbance detection at 254 nm, eluting with a linear gradient from 2% aqueous MeCN to 95% MeCN over 15 min, holding at 95% MeCN for a further 5 min. Phase A 0.1% FA in water, phase B 0.1% FA in MeCN & Flow rate: 1.5 mL/min.

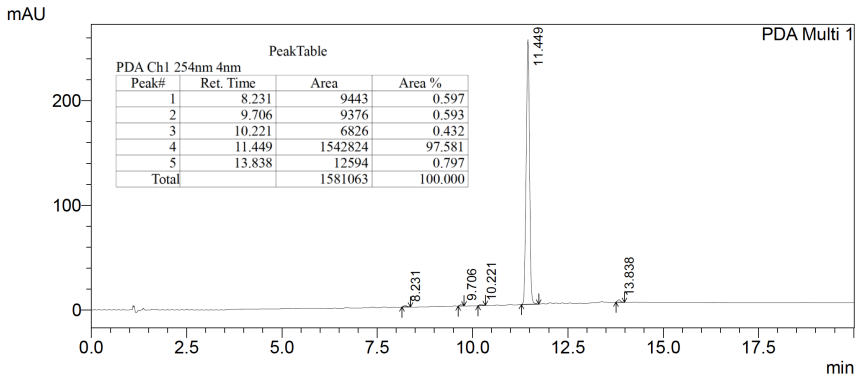
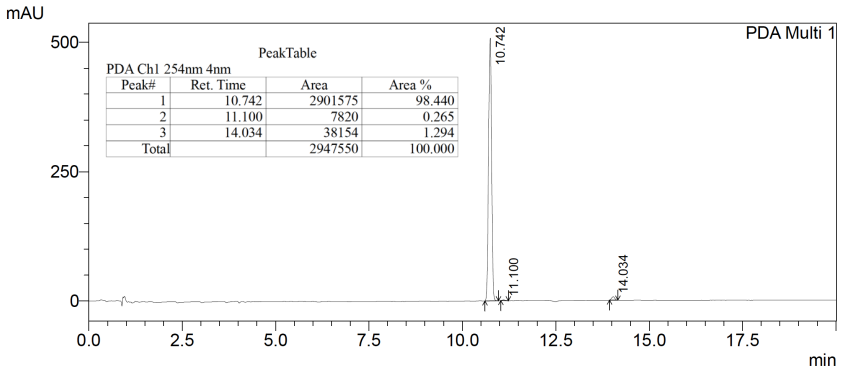
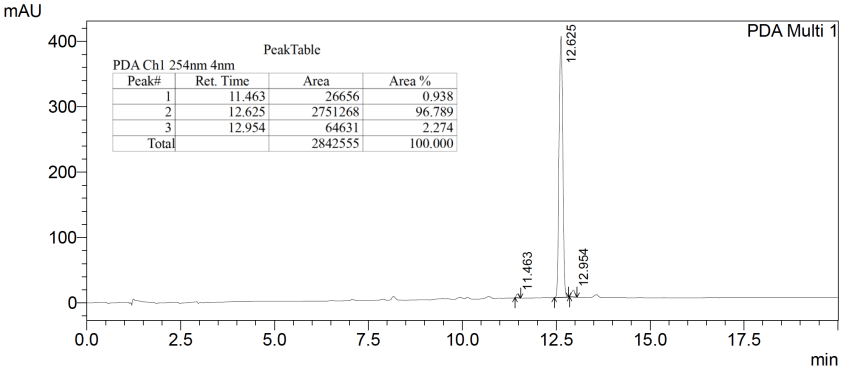
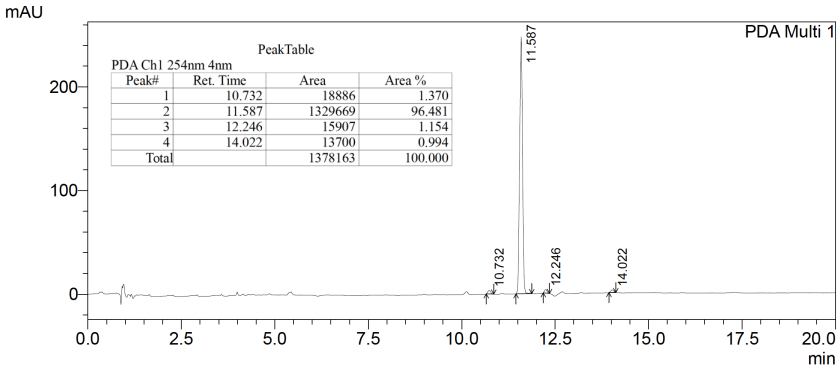
V. Table I: Purity Data for NP-A3-B2 Compounds Assayed

Compound ID	Purity (%)	HPLC Trace & Peak Table
NP-A3-B2	99	 PeakTable PDA Ch1 254nm 4nm Peak# | Ret. Time | Area | Area % 1 | 12.891 | 958063 | 99.255 2 | 13.544 | 4037 | 0.418 3 | 13.822 | 3150 | 0.326 Total | | 965249 | 100.000
JGB-1-122	96	 PeakTable PDA Ch1 254nm 4nm Peak# | Ret. Time | Area | Area % 1 | 8.236 | 40174 | 2.663 2 | 8.474 | 1449409 | 96.068 3 | 10.946 | 19155 | 1.270 Total | | 1508739 | 100.000
JGB-1-127	99	 PeakTable PDA Ch1 254nm 4nm Peak# | Ret. Time | Area | Area % 1 | 10.481 | 7316 | 0.797 2 | 13.038 | 908001 | 98.862 3 | 13.857 | 3139 | 0.342 Total | | 918455 | 100.000

Compound ID	Purity (%)	HPLC Trace & Peak Table
JGB-1-128	97	
JGB-1-129	92	
JGB-1-130	97	
JGB-1-131	99	

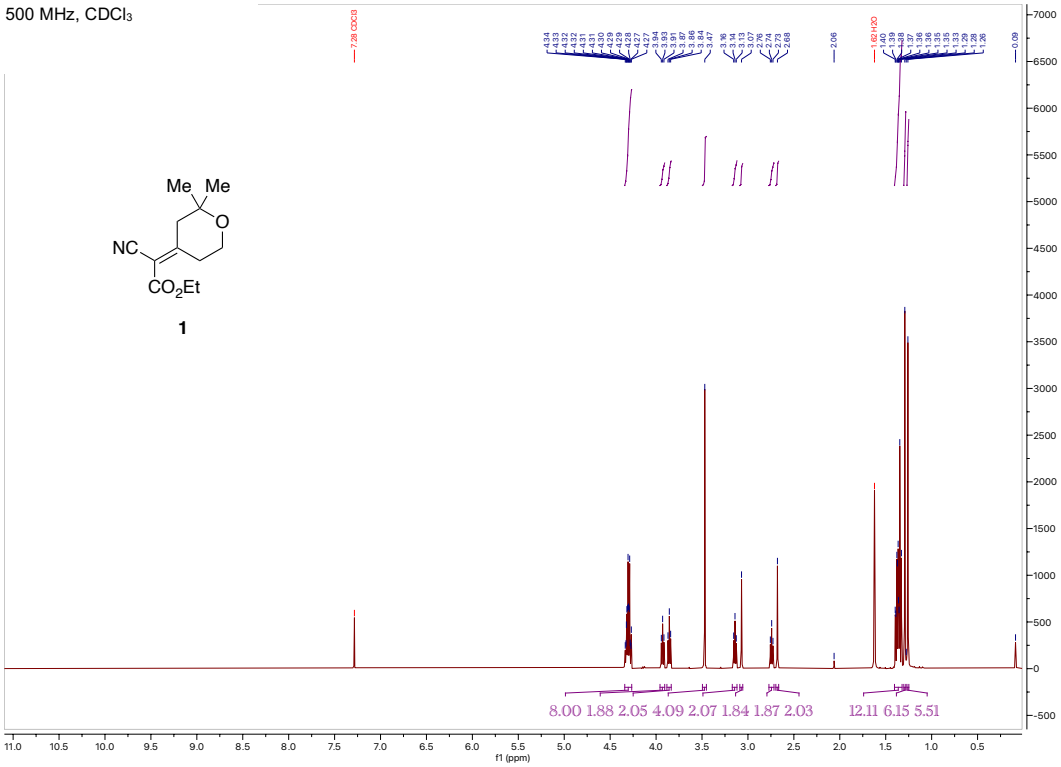
Compound ID	Purity (%)	HPLC Trace & Peak Table
JGB-1-133	94	
JGB-1-134	98	
JGB-1-135	99	
JGB-1-137	97	

Compound ID	Purity (%)	HPLC Trace & Peak Table
JGB-1-145	99	
JGB-1-146	96	
JGB-1-147	94	
JGB-1-151	96	

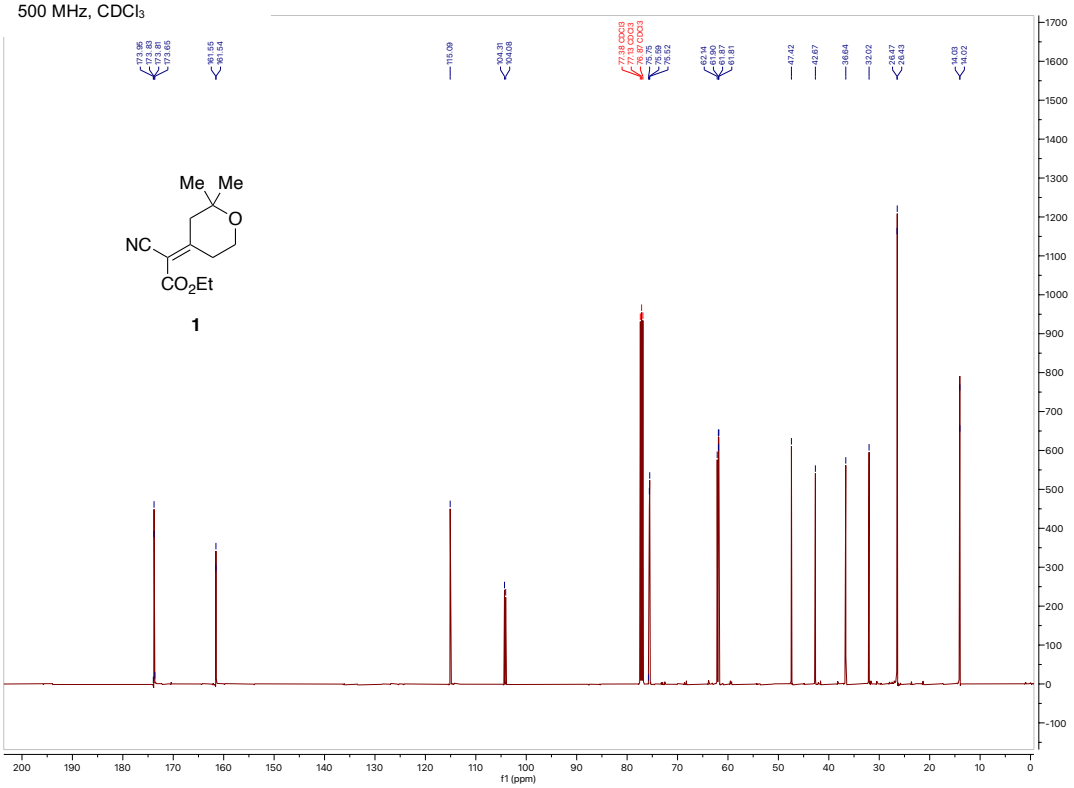
Compound ID	Purity (%)	HPLC Trace & Peak Table
JGB-1-152	98	
JGB-1-153	98	
JGB-1-155	97	
JGB-1-158	96	

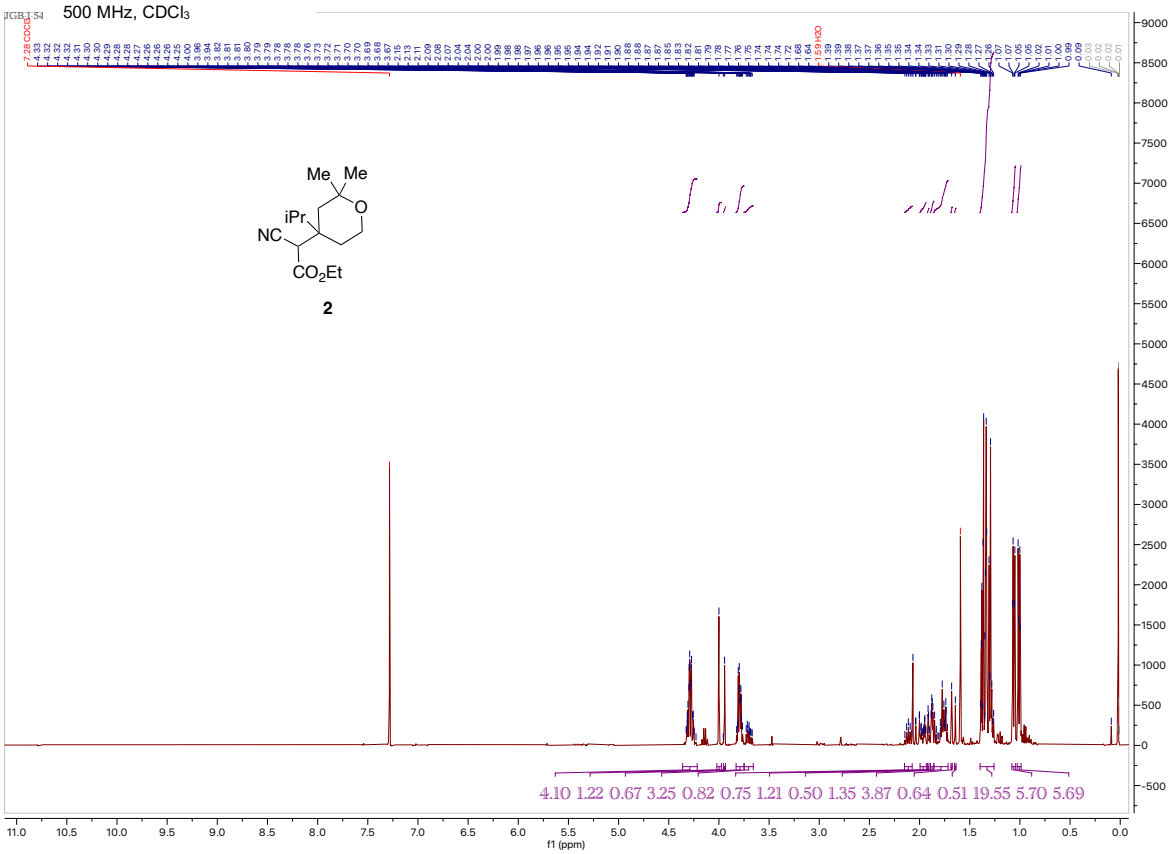
VI. ^{1}H and ^{13}C NMR Spectra for NP-A3-B2 Synthesis

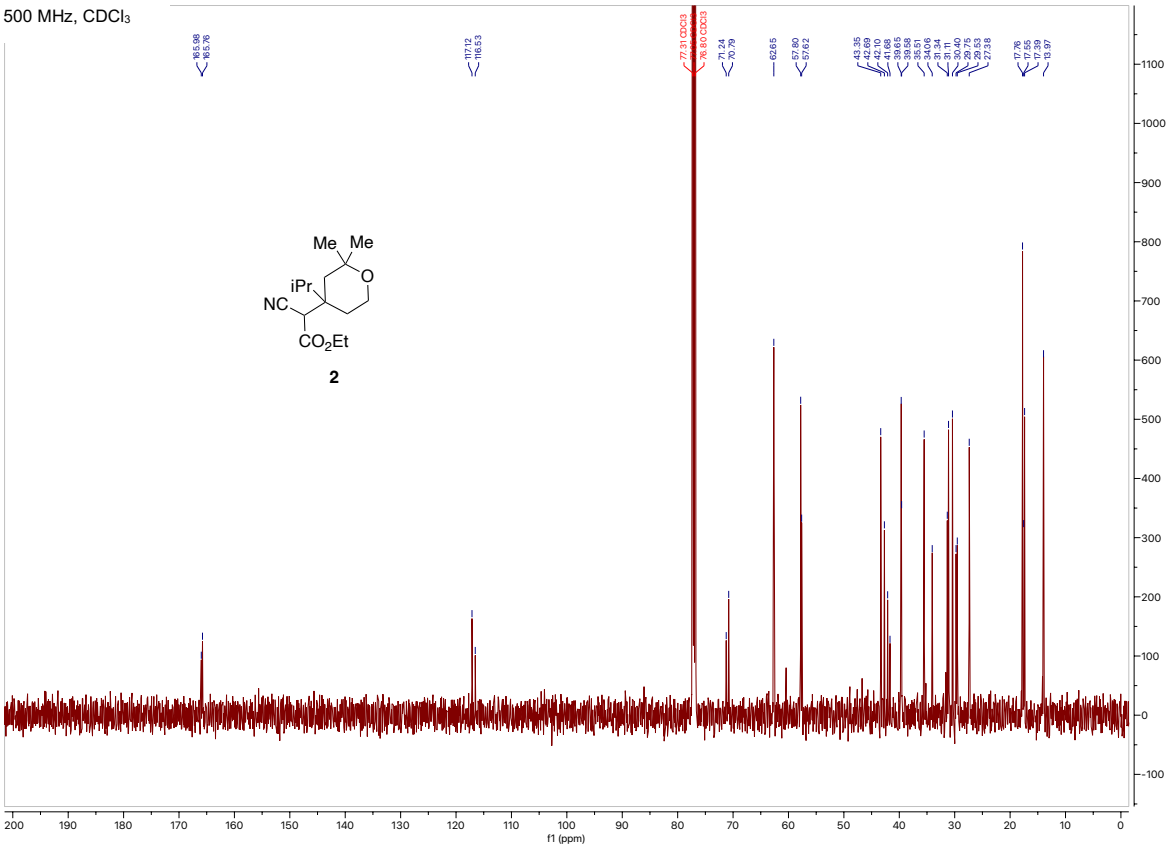
500 MHz, $CDCl_3$



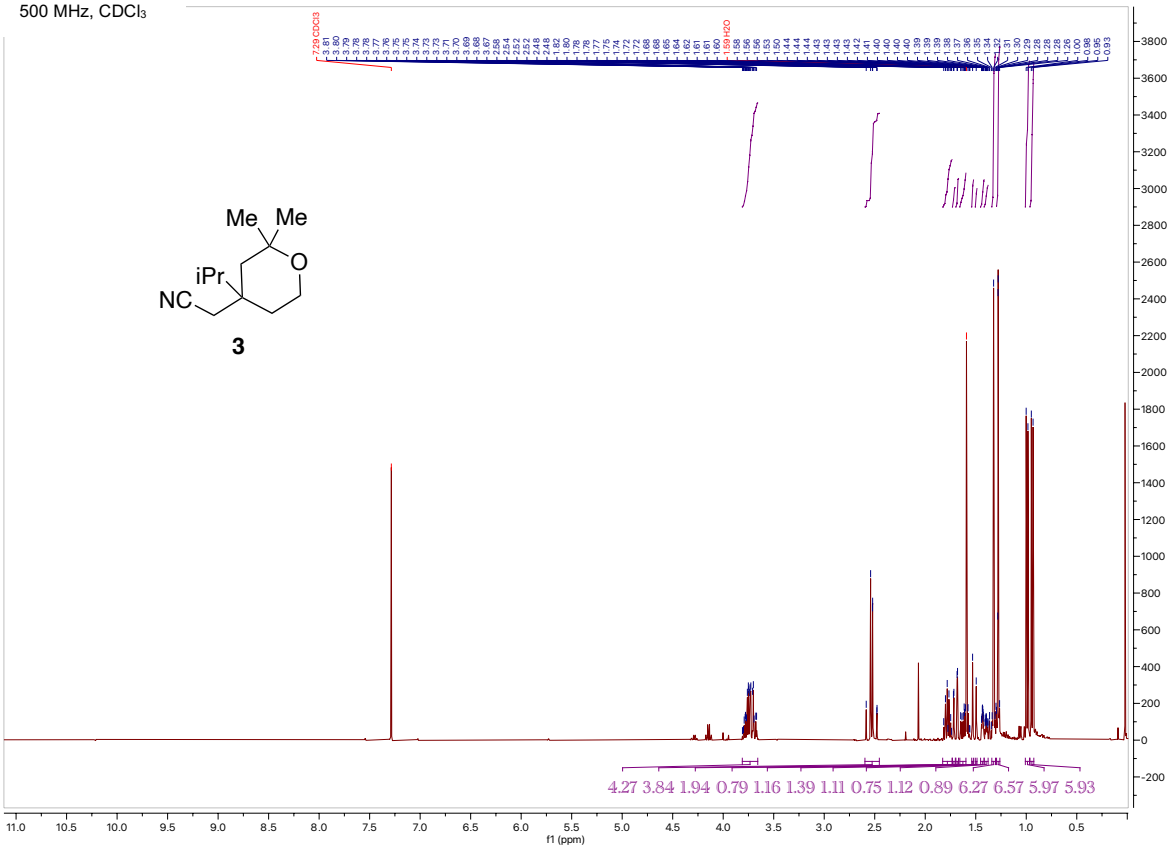
500 MHz, $CDCl_3$



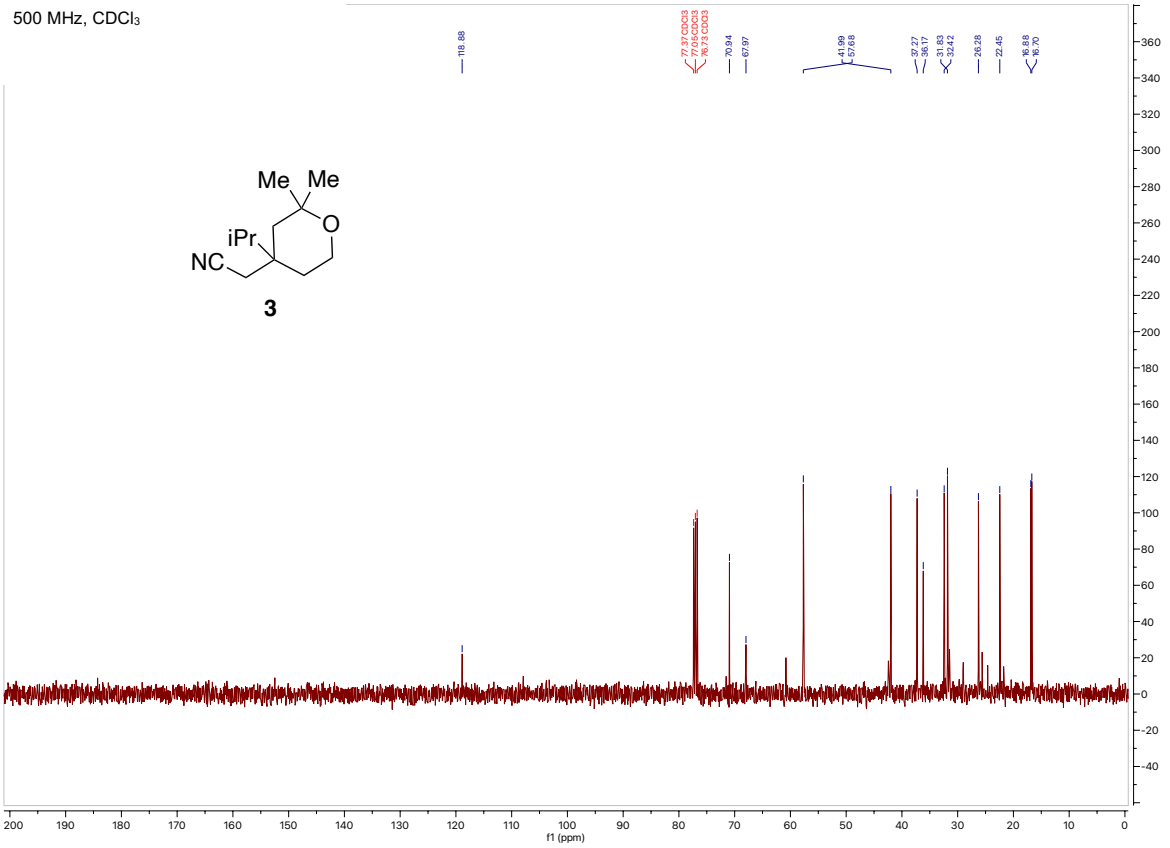

500 MHz, CDCl3
Me Me
O
iPr
NC
CO2Et
2
4.10 1.22 0.67 3.25 0.82 0.75 1.21 0.50 1.35 3.87 0.64 0.51 19.55 5.70 5.69
f1 (ppm)


500 MHz, CDCl3
Me Me
O
iPr
NC
CO2Et
2
f1 (ppm)

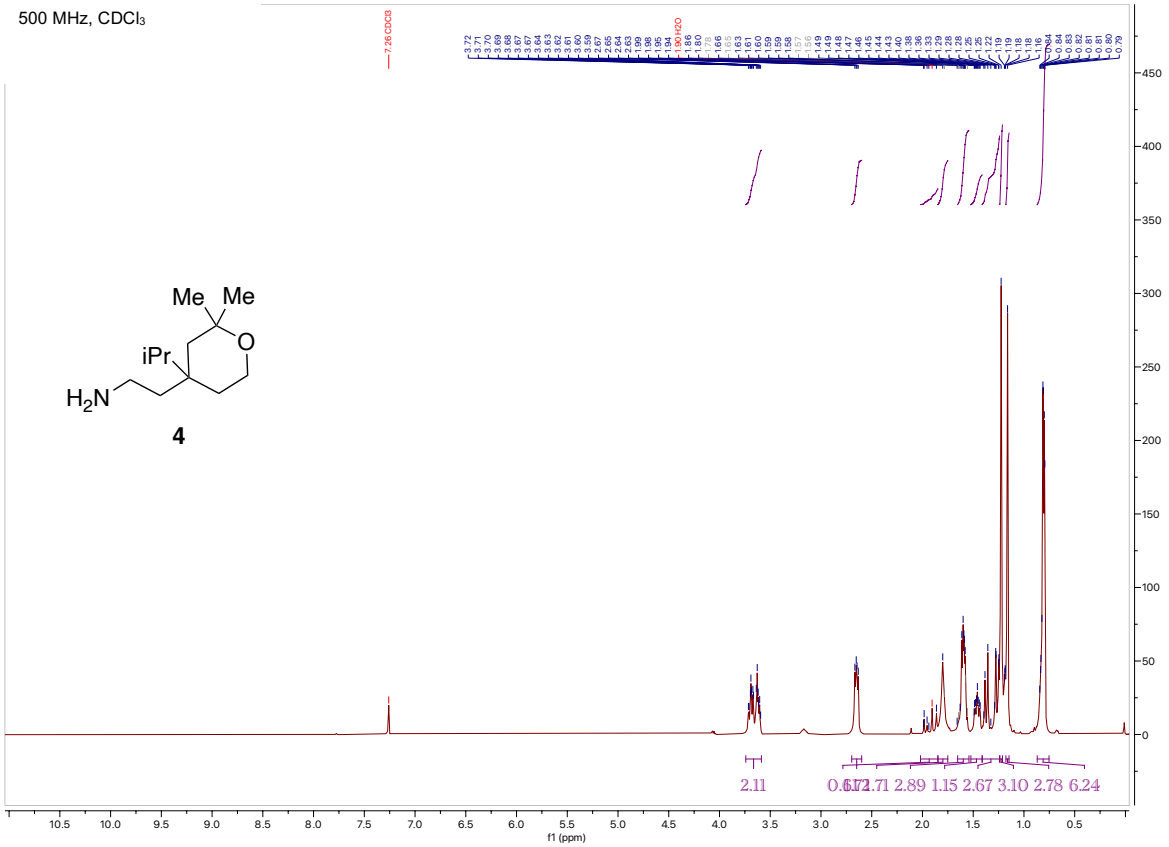
500 MHz, $CDCl_3$



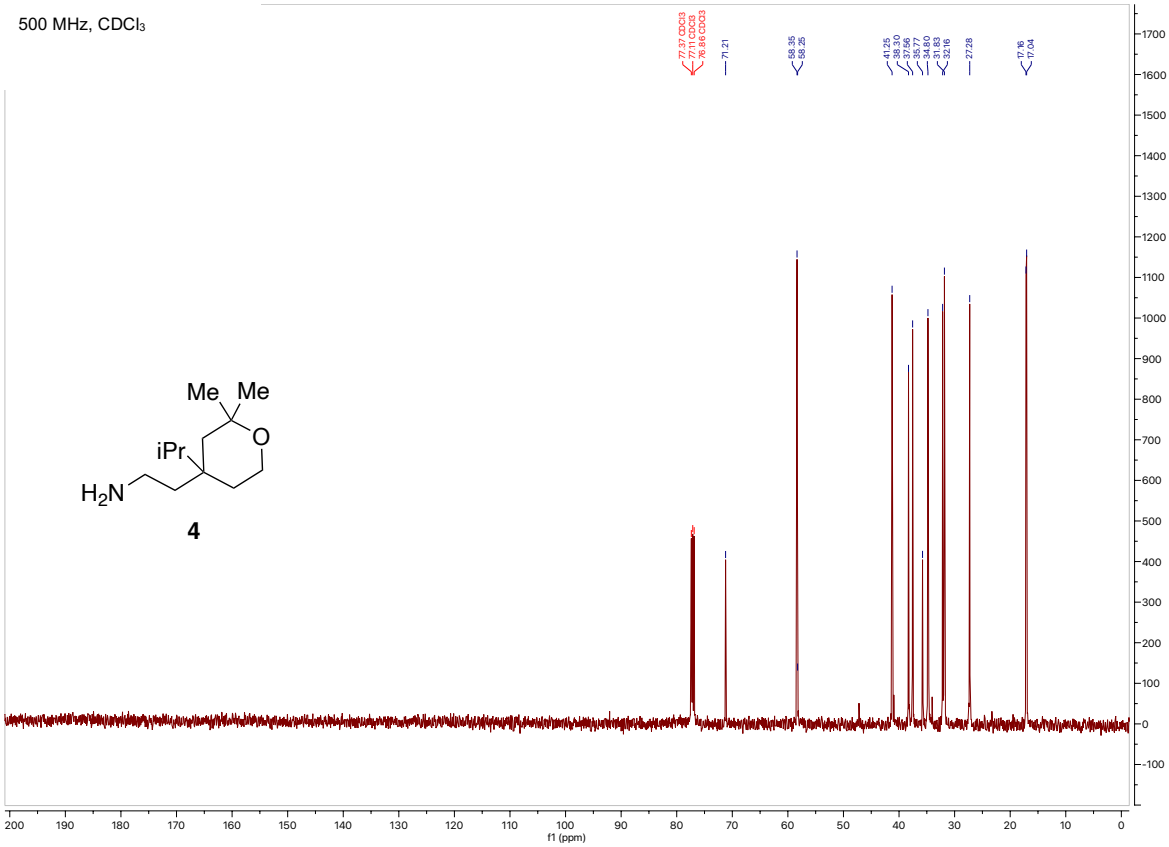
500 MHz, $CDCl_3$

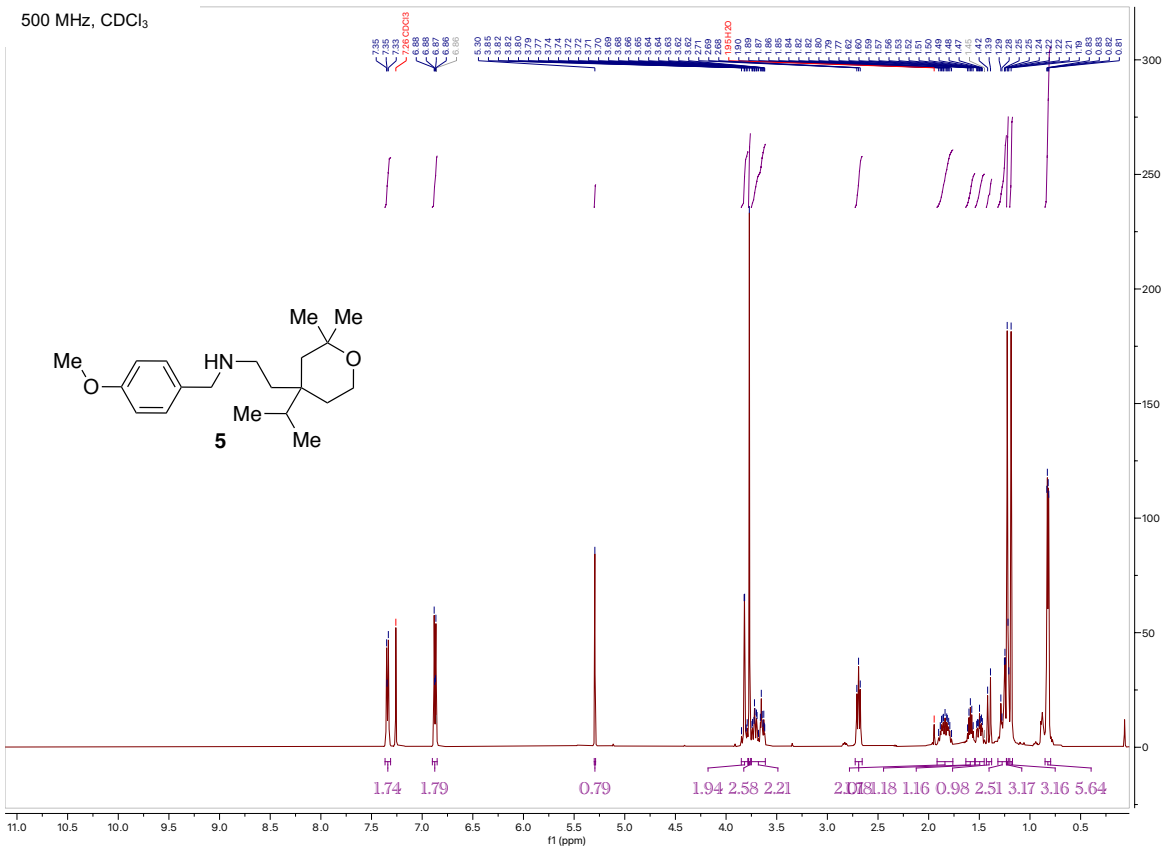


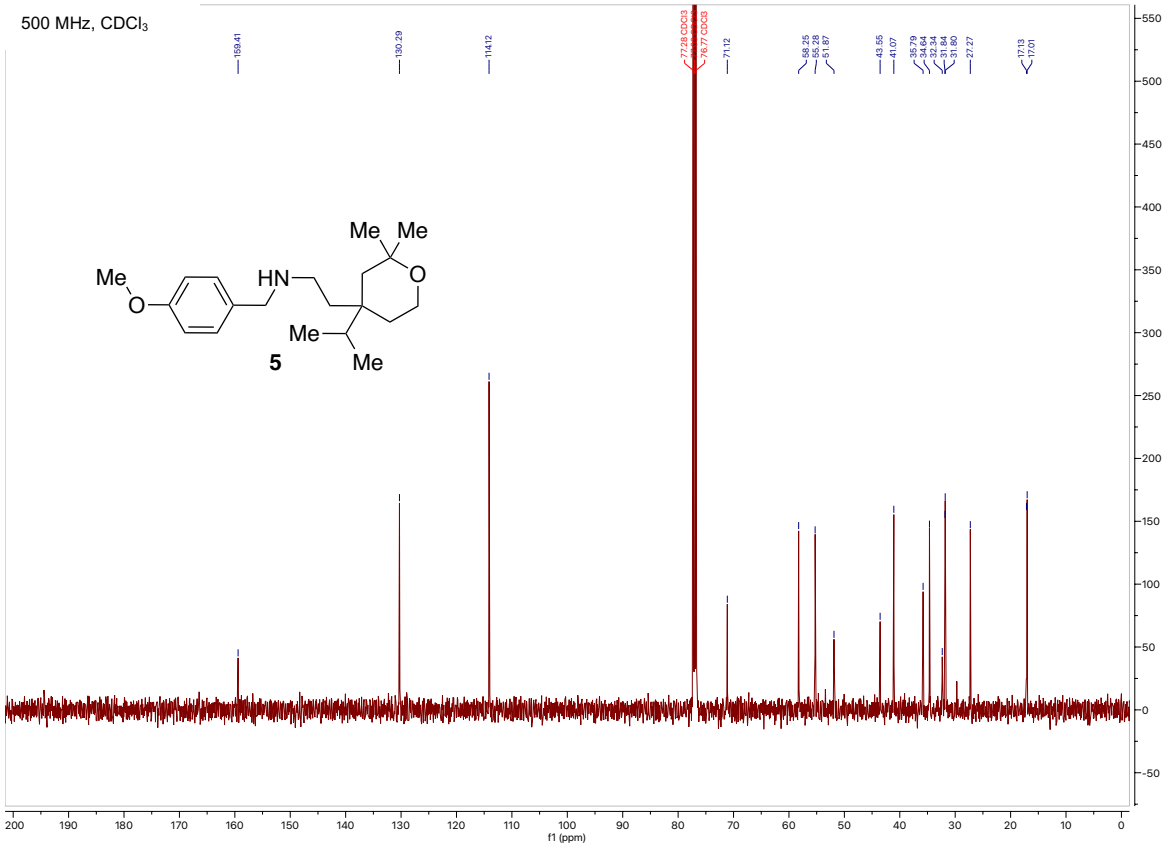
500 MHz, $CDCl_3$

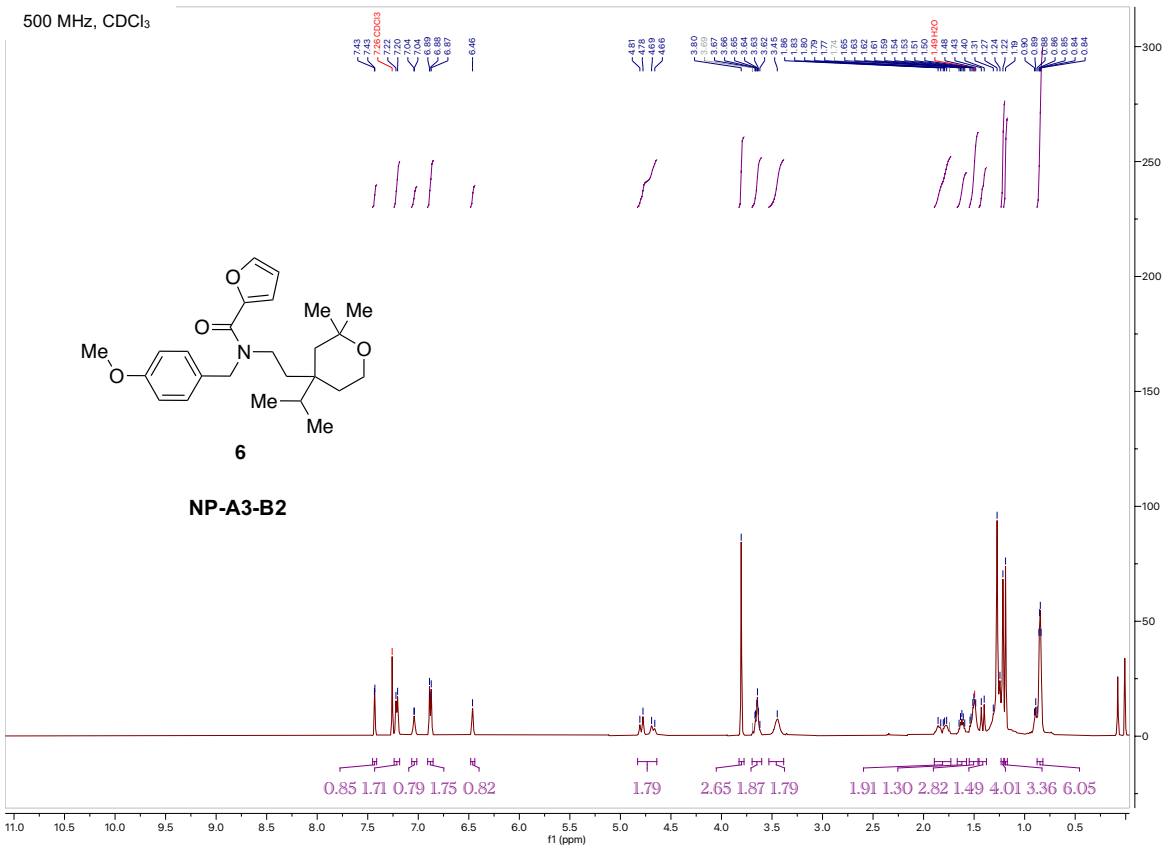


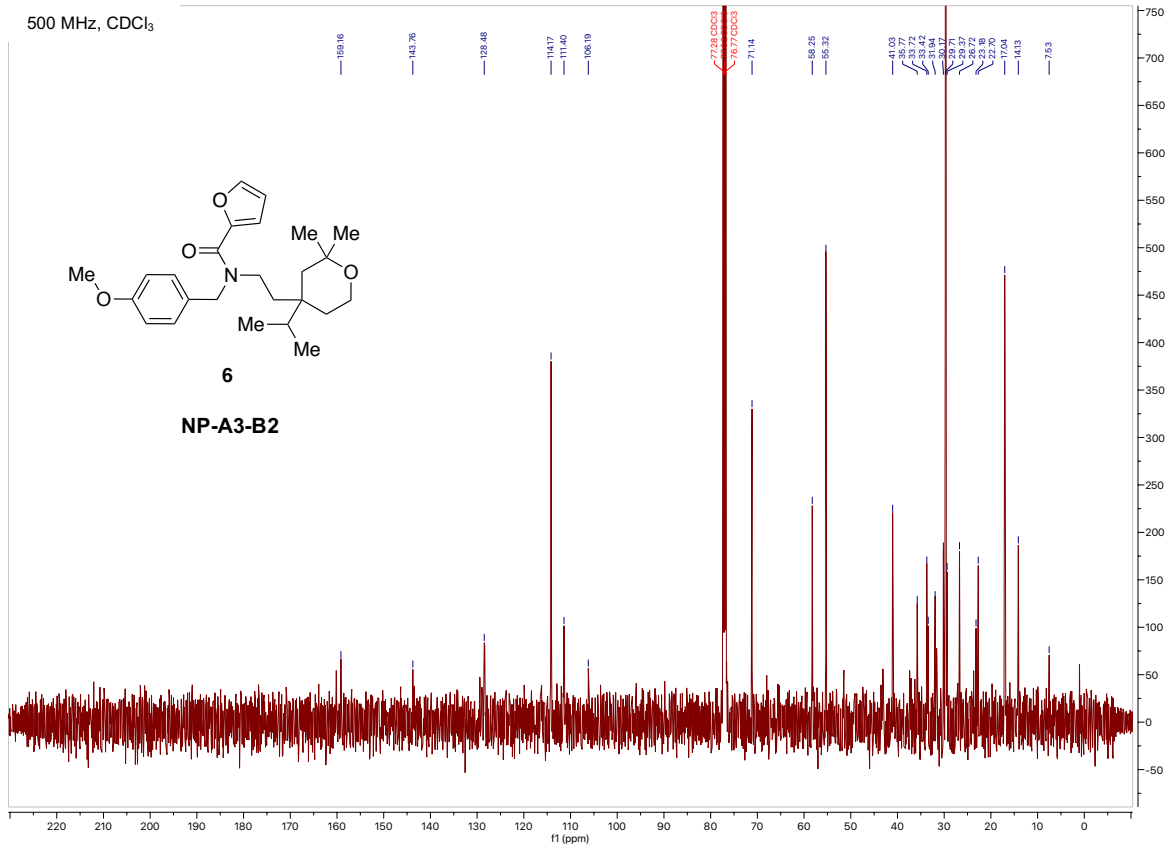
500 MHz, $CDCl_3$



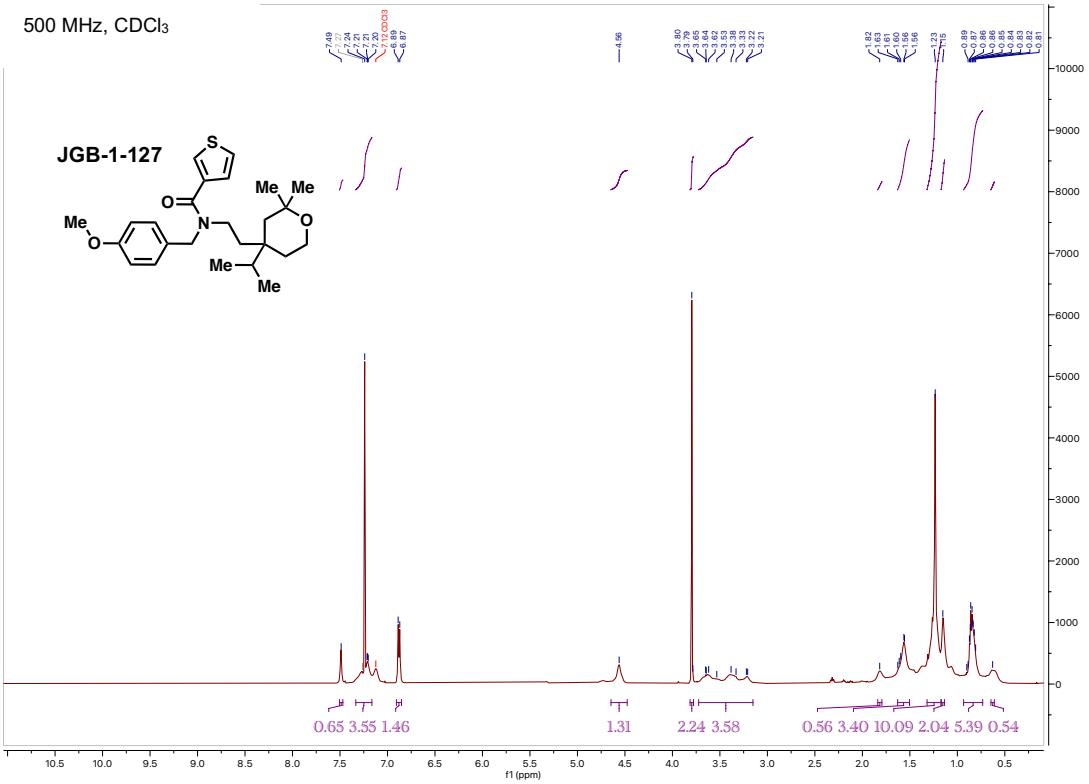

500 MHz, CDCl3
Me
Me
Me
O
HN
Me
Me
5
1.74
1.79
0.79
1.94
2.58
2.21
1.18
1.16
0.98
2.51
3.17
3.16
5.64
f1 (ppm)

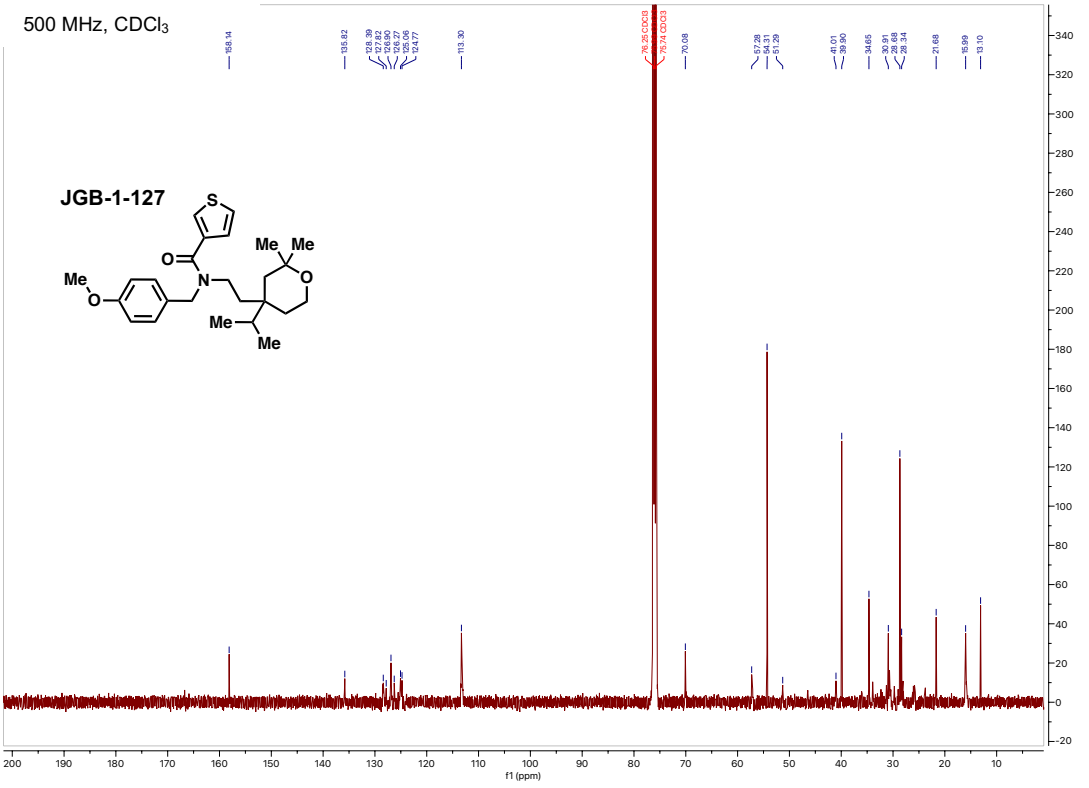

500 MHz, CDCl3
159.41
130.29
114.12
77.28 CDCl3
76.77 CDCl3
71.12
58.25
55.28
51.87
43.55
41.07
35.79
34.64
34.34
31.84
31.80
27.27
17.13
17.01
Me
Me
Me
O
HN
Me
Me
5
f1 (ppm)


500 MHz, CDCl3
Me
Me
Me
Me
Me
O
O
O
O
N
6
NP-A3-B2
0.85 1.71 0.79 1.75 0.82
1.79
2.65 1.87 1.79
1.91 1.30 2.82 1.49 4.01 3.36 6.05
f1 (ppm)

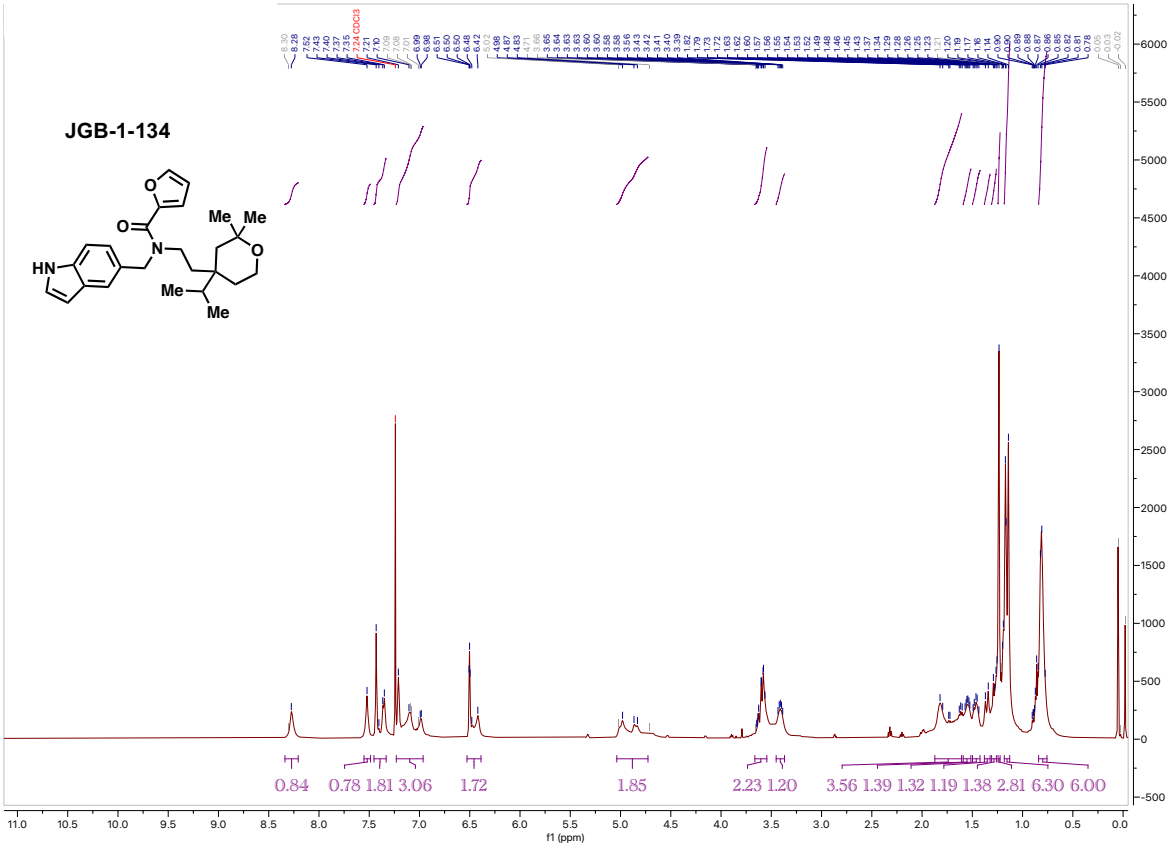

500 MHz, CDCl3
Me
Me
Me
Me
Me
O
O
O
O
N
6
NP-A3-B2
f1 (ppm)

VII. ^{1}H and ^{13}C NMR Spectra for Key Compounds

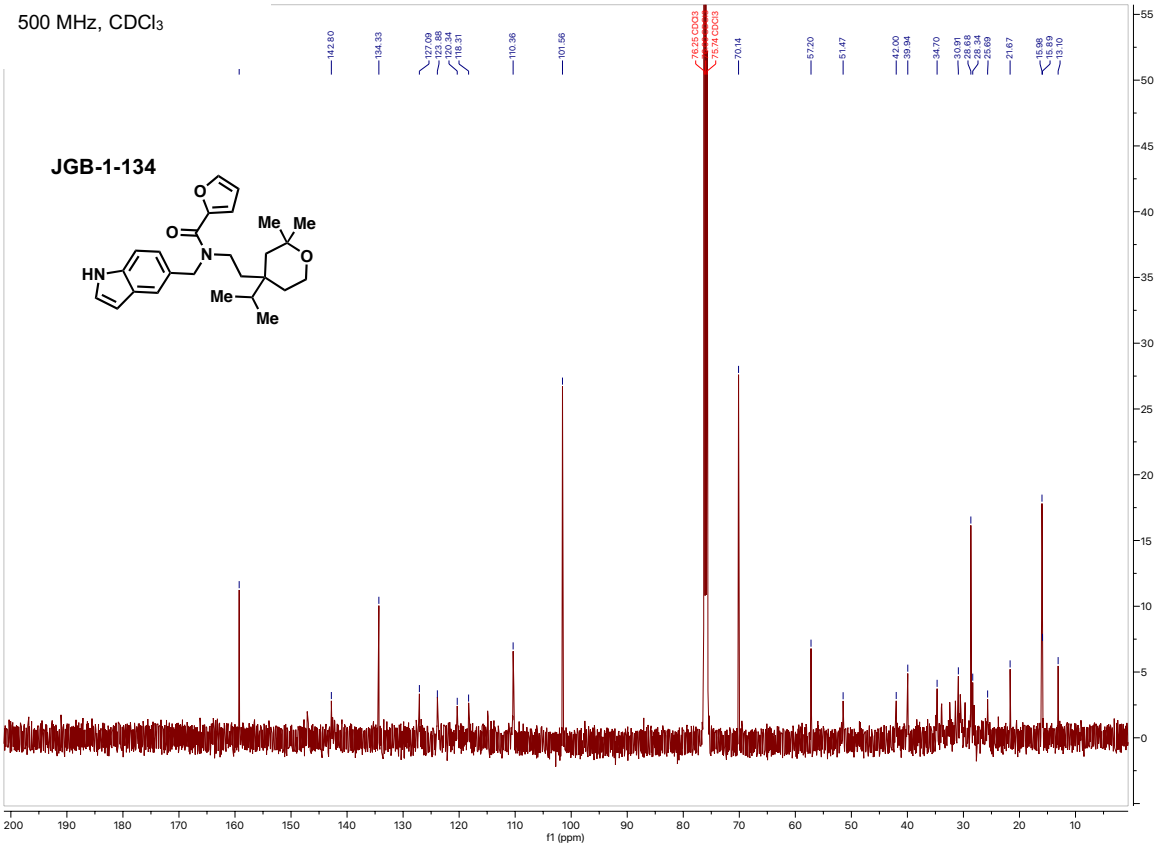


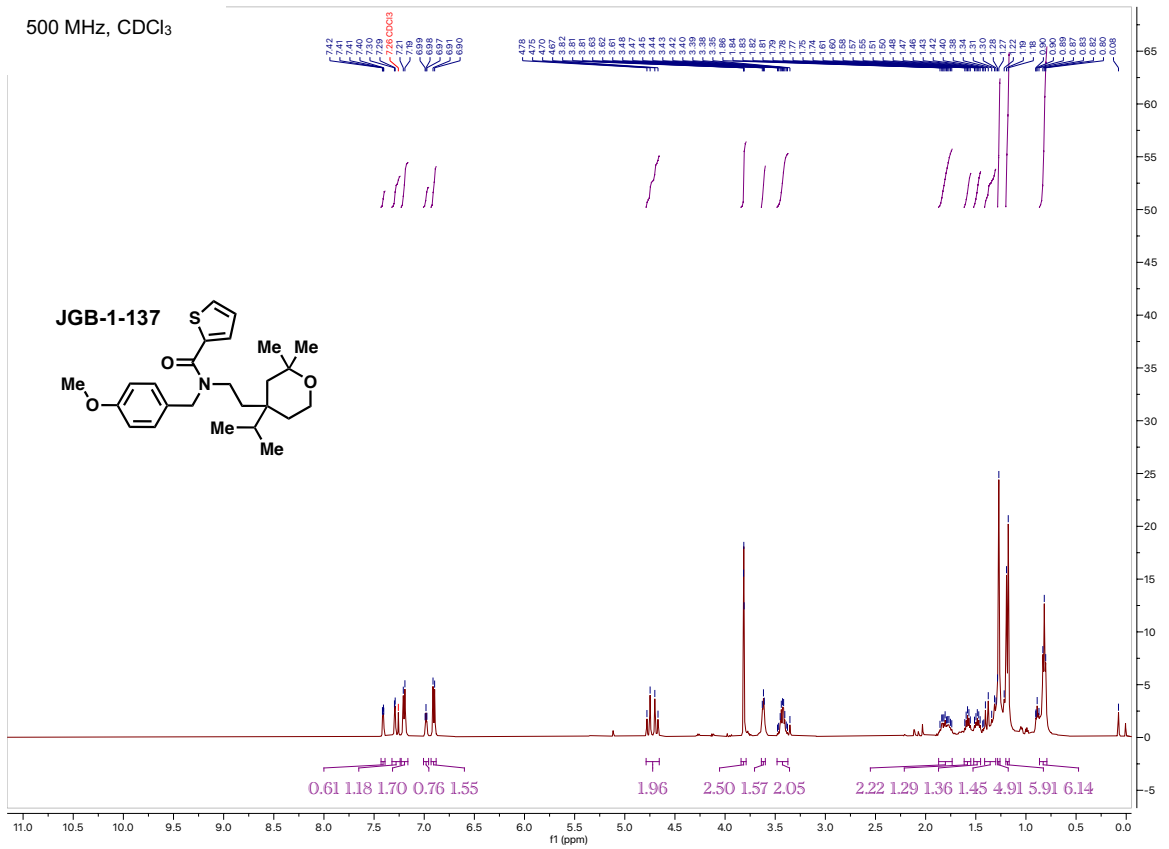


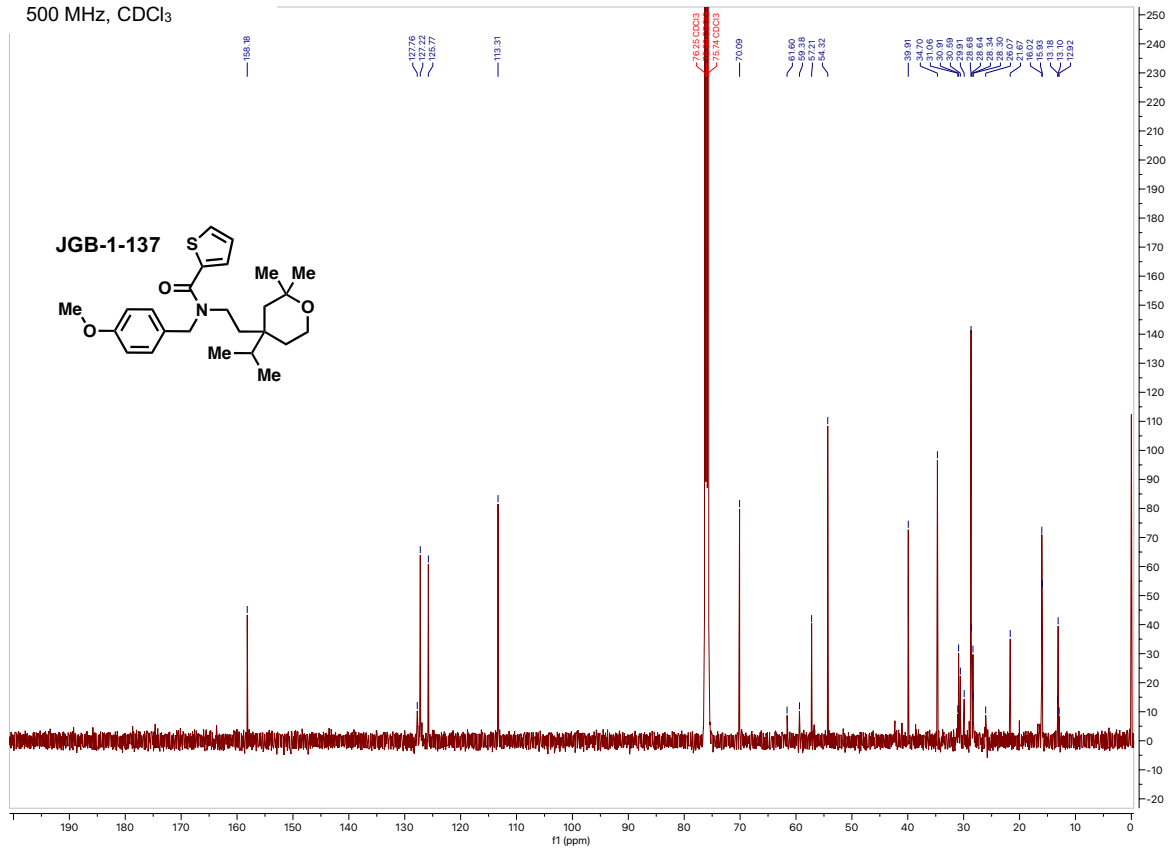
500 MHz, $CDCl_3$



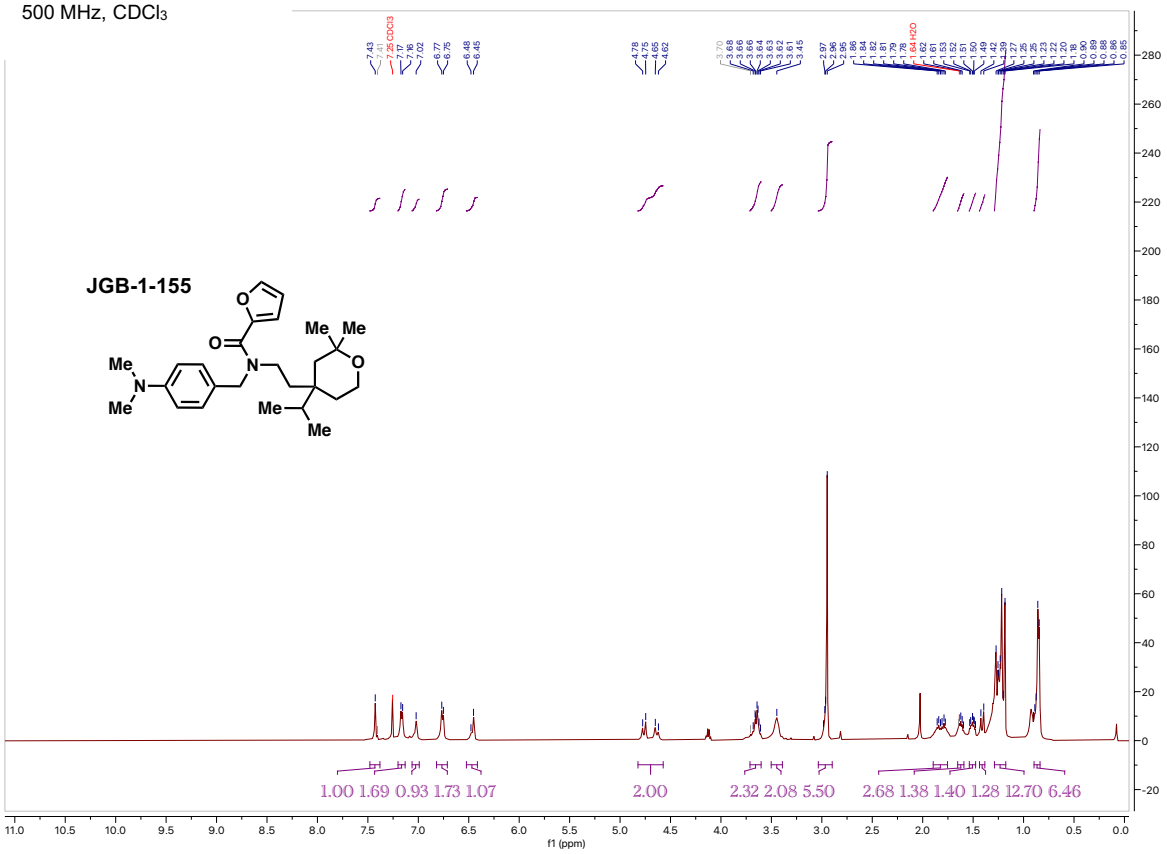
500 MHz, $CDCl_3$



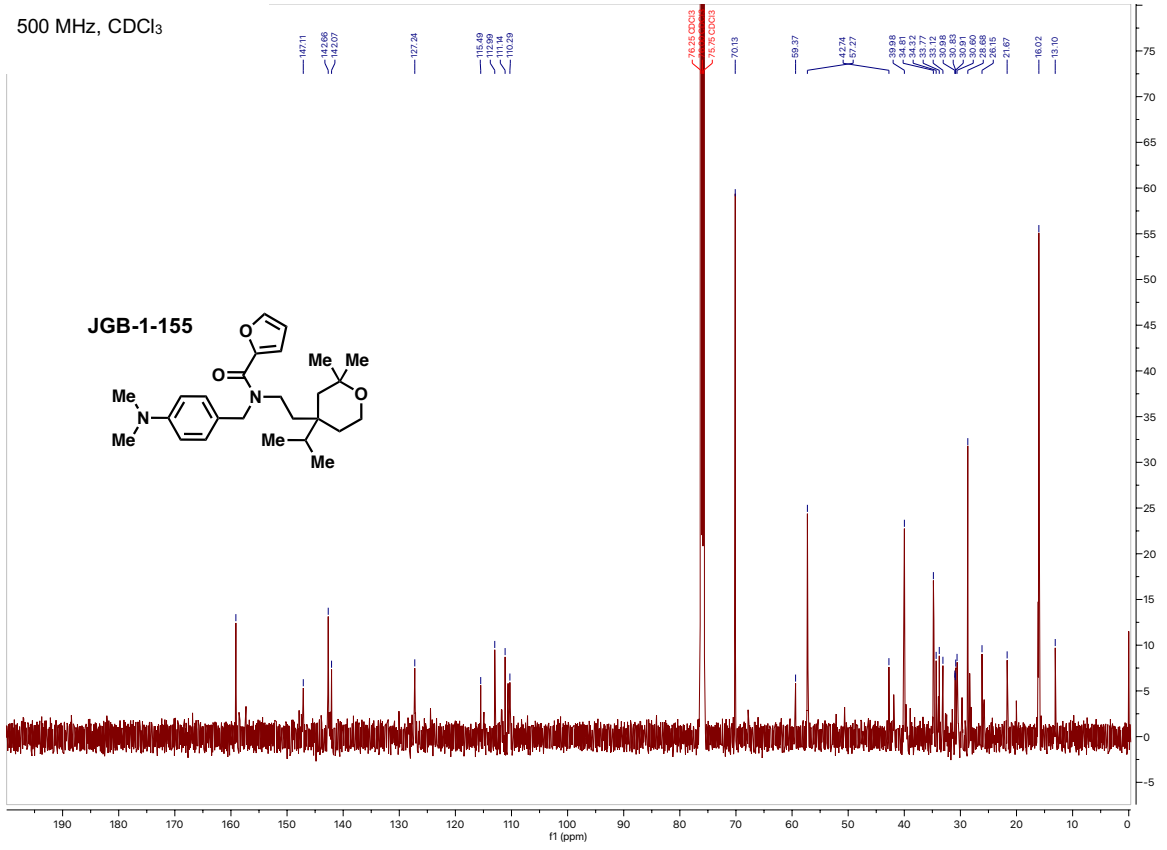

500 MHz, CDCl3
JGB-1-137
0.61 1.18 1.70 0.76 1.55
1.96
2.50 1.57 2.05
2.22 1.29 1.36 1.45 4.91 5.91 6.14
f1 (ppm)


500 MHz, CDCl3
JGB-1-137
f1 (ppm)

500 MHz, $CDCl_3$



500 MHz, $CDCl_3$



VIII. Experimental Methods for Crystallography and Bioassay

Crystallography

Compounds and chemical reagents. Synthetic reagents were purchased from Ambeed, Matrix Scientific, Enamine, ThermoFisher Scientific, and Sigma-Aldrich. All assay reagents were acquired from Sigma-Aldrich unless otherwise specified.

X-ray diffraction crystallography. Crystals of NAMPT complexed with NAM and N-PAM were grown by hanging drop vapor diffusion at 16°C. Prior to crystallization, 11 mg/mL NAMPT protein was incubated with 10 mM NAM and 1 mM compound for 30 min on ice. Crystals of the complex were grown by mixing 1- 2 µL of NAMPT complex with 2 µL of reservoir solution containing 0.1 M Tris-HCl, pH 8, 0.1 M KCl, and 24- 28% PEG 2000 MME *or* 0.1 M Tris-HCl, pH 8.5, 0.2 M NaCl, 20% glycerol and 13-18% PEG 3350. Crystals grew overnight from the PEG 2000 MME conditions and were used to streak seed drops of NAMPT complex equilibrating against the PEG 3350 conditions.

Data collection and structure refinement. The glycerol present in the crystallization solution was sufficient to cryo-protect crystals, which were flash-cooled in liquid nitrogen. Data were collected on a MAR300 detector at 0.979 Å at the Life Sciences Collaborative Access Team beamline 21-ID-F at the Advanced Photon Source, Argonne National Laboratory. Data indexing, integration, and scaling were performed using XDS, and phases were determined by molecular replacement using Molrep and a NAMPT-NAM co-crystal structure (PDB entry: 2E5D) as search model. Rigid body refinement followed by iterative rounds of restrained refinement and model building were performed with Phenix and Coot.

Table II: NAMPT:NP-A3:NAM co-crystal structure parameters

Data Collection	**NAMPT:NP-A3: NAM**
PDB ID	**8TM7**
Space Group	P1 2_1 1
Cell Dimensions	
a,b,c (Å)	60.56 106.86 83.22
α,β,γ (°)	90.00 96.71 90.00
Resolution (Å)	82.65 (1.79)*
Unique Observations	98,774 (4,745)
Completeness (%)	99.8 (97.3)
Redundancy	3.6 (2.1)
R_{merge}	0.087 (0.755)
I/σI	8.7 (1.2)
Source	LS-CAT ID-G
Collection date	11/2/19
Wavelength	0.9786
Refinement	
Resolution (Å)	1.79
R_{work} ; R_{free} (%)	17.8; 19.8
Number of atoms (protein/other/solvent)	7450/ 103/ 434
B-factors (Å^2) (protein/other/water)	19.4/30.2/24.2
Rmsd bond lengths (Å)	0.007
Rmsd bond angles (°)	1.34
Ramachandran	
Favored	97.30
Allowed	2.70
Forbidden	0.00
Rotamer outliers (%)	0.61
Molecules in ASU	2
Programs Used	
Processing	DIALS
Scaling	DIALS
Phasing	Molrep
Phasing Model	2E5D
Manual Build	Coot
Refinement	Refmac

* values in parentheses are for the highest resolution shell

Bioassay

NAMPT coupled enzyme activity assay. The NAMPT enzyme assay is based on conditions from Burgos and Schramm and adapted to include a cycling reaction to quantitate NAD^+ production colorimetrically[2]. The assay follows the NAMPT-catalyzed production of NMN from substrates NAM and PRPP by coupling NMN formation to the NMNAT reaction, which produces NAD^+ from NMN and ATP. The NAD^+ is then cycled by alcohol dehydrogenase (ADH) and diaphorase to continuously produce WST-1 formazan, which can be detected at 450 nm. Assays are performed at 25°C in clear 384-well plates, with a final assay volume of 30 µL, and contain the following: 50 mM HEPES, pH 7.5, 5 mM MgCl2, 50 mM NaCl, 0.01% Triton-X 100, 2.5 mM ATP, 40 µM PRPP, 30 µM NAM, 1.5 µL WST-1 (Roche Cell Proliferation Reagent), 1U/mL ADH, 0.083 U/mL diaphorase, 1.5% ethanol, 1% DMSO, 30 nM NAMPT, and 7.4 nM purified human NMNAT1. N-terminal His6-NMNAT1 and C-terminal His6-NAMPT were overexpressed and purified by metal chelate affinity chromatography. Following assay assembly, well signals were measured continuously at 450 nm on a Tecan Infinite M200 plate reader for 1h with intermittent shaking. Slopes of the linear portions of the reaction progress curves were recorded and corrected for background by subtracting the average slope of control wells containing NAMPT inhibitor FK866.

A counterassay to confirm NAMPT-specific activation was performed for all compounds using the enzyme assay described above, but lacking NAMPT, NAM and PRPP, and with the addition of 5 µM NMN (NMNAT substrate), and with a reduced NMNAT concentration (typically 1 nM). Biochemical assays were performed in two separate experiments and EC50 values were determined from non-linear regression analysis of the dose-response curve generated in GraphPad Prism 9.

Measurement of cellular NAD^+ levels in THP-1 cell cultures. Using FK-866 and NMN as negative and positive controls, respectively, we explored a number of cell lines to select a highly reproducible model system with good dynamic range. We optimized a commercial NAD-glo assay in the THP-1 human leukemic monocyte cell line in 96-well plates, measuring NAD^+ after incubation with test compounds for 24 hours. The human monocytic leukemia cell line (THP-1) was obtained from the American Type Culture Collection and were maintained in RPMI 1640 medium (ATCC) supplemented with 100 units of penicillin, 100 µg/ml streptomycin, and 10% heat inactive fetal bovine serum. All cells were grown at 37 °C, under 5% CO2 in a humidified incubator. Low passage THP-1 cells (37,500 cells/well) were seeded in 96-well plates and incubated at 37 °C and 5% CO2 for 1 1/2 h prior to a 24 h treatment. All compounds were dissolved in DMSO, and final DMSO concentrations never exceeded 1%. The NAD^+ levels in the cells are measured using the NAD^+/NADH-Glo™ assay (Promega). The assays were performed in three separate experiments for each concentration and the EC50 values were determined from non-linear regression analysis of the dose-response curve generated in GraphPad Prism 9.

Cell Culture and Drug Treatments. HT22 mouse hippocampal neuronal cells (a gift from the Salk Institute) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FBS and 1X Antibiotic-Antimycotic (ABAM). Cells were maintained in a humidified 5 % CO_2 atmosphere incubator at 37 °C, and were passaged when 60-80% confluent (1-2 days) by removing media, rinsing with phosphate buffered saline (PBS), and enzymatically detaching with trypsin solution. New culture dishes were seeded with 1/4 to 1/2 the cells from the previous dish.

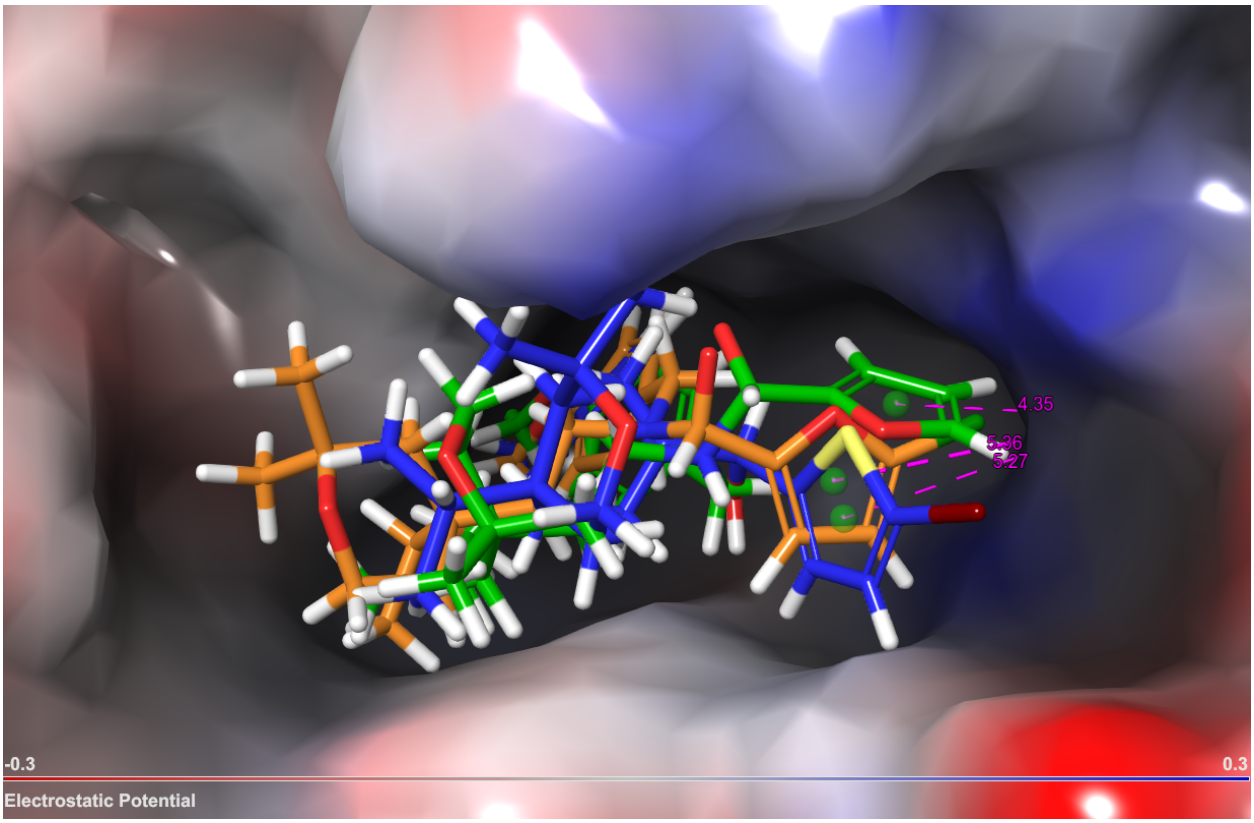
All assays were performed using cells with passage number 12-50. The inner 60 wells of black-walled (Corning® 3603/3604) 96-well plates were treated with poly D-lysine (30 µL/well) making sure to cover the entire well surface for 15-30 seconds and then washed thoroughly with sterile water (3X - 200 µL/well). TNFα stock solutions (10 µg/mL, PBS (0.1% BSA)) were made, aliquoted, and stored at -80 °C. Glutamate stock solutions were made fresh immediately before assay. All compound stock solutions (10 mM, DMSO) were made and stored at -20 °C.

Cells were plated at a seeding density of 3,000-5,000 cells/well and allowed to incubate overnight. The next day (18-24 hours later) cells were pretreated with N-PAMs, carefully ensuring equivalent vehicle (DMSO/PBS) composition in each well. After 18-24 hours, stressors (glutamate or TNFα) were treated. Live cell count, ROS level, and total cell count were then assessed after another 18-24 hours.

Chemicals and Reagents. Calcein-AM, Hoechst, and MitoSOX Red Superoxide Indicator dyes were purchased from ThermoFisher Scientific (Bedford, MA, USA). L- Glutamic Acid (99%, CAS 6106-04-3) was purchased from MP Biomedicals. Dulbecco's modified Eagle's medium (DMEM), trypsin, bovine serum albumin (BSA), and fetal bovine serum (FBS) were purchased from GIBCO Invitrogen (Carlsbad, CA, USA). Recombinant mouse TNFα was purchased from Shenandoah Biotechnology, Inc. (Warminster, PA, USA). SBI-797812 was purchased from MedChemExpress (Monmouth Junction, NJ, USA).

ROS Detection. ROS levels were quantified using MitoSOX Red Mitochondrial Superoxide Indicator (Invitrogen). At treatment endpoint, the cell culture media was removed and replaced with phosphate buffered saline (PBS) containing 1 μM calcein-AM, 5 μM MitoSOX, and 10 μg/mL Hoechst dyes. Cells were incubated for 30-45 minutes and then dye-containing PBS was dumped and replaced with 100 μL/well fresh PBS. The plate was immediately analyzed in the Celigo Imaging Cytometer using the Live-Dead-Total protocol, where MitoSOX was used in place of propidium iodide. The cell counts and fluorescence intensities for living (calcein), MitoSOX-stained, and total (Hoechst) were acquired. MitoSOX average mean fluorescence intensity ("dead" average mean intensity) was used to quantify mitochondrial ROS. All readouts used were transformed into fold-change over vehicle treatment for visualization. Experiments were performed in replicates of at least three separate experiments (different passage number and day), and each experiment contained at least three intraplate replicates for each treatment group.

Statistics. Data are represented as mean ± standard deviation or 95% confidence interval. Statistical significance (p-value < 0.05) was determined using one-way ANOVA or t-test analysis in GraphPad Prism.



Green - NP-A3-B2
Orange - JGB-1-135
Blue - JGB-1-122

Figure S1. Docking using Schrodinger Glide shows that JGB-1-122 and JGB-1-135 are displaced relative to NP-A3-B2 but still occupy the rear channel maintaining similar binding interactions to those identified for NP-A3-B2 in the co-crystal structure.

References

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