Supporting Information

A Novel Family of Small Molecules that Enhance the Intracellular Delivery and Pharmacological Effectiveness of Antisense and Splice Switching Oligonucleotides

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Supporting Table 1

In Vivo Toxicity Parameters

Cardiac blood samples were collected at the termination of the experiment described in Figure 6 (24 h after administration of the test compound). The UNC Animal Clinical Chemistry Core analyzed the samples for the following parameters. Blood urea nitrogen (BUN); alanine aminotransferase (ALT); aspartate aminotransferase (AST); hemoglobin (Hb); platelet count. None of the differences between experimental and control were significant at the 5% level using the paired t-Test.

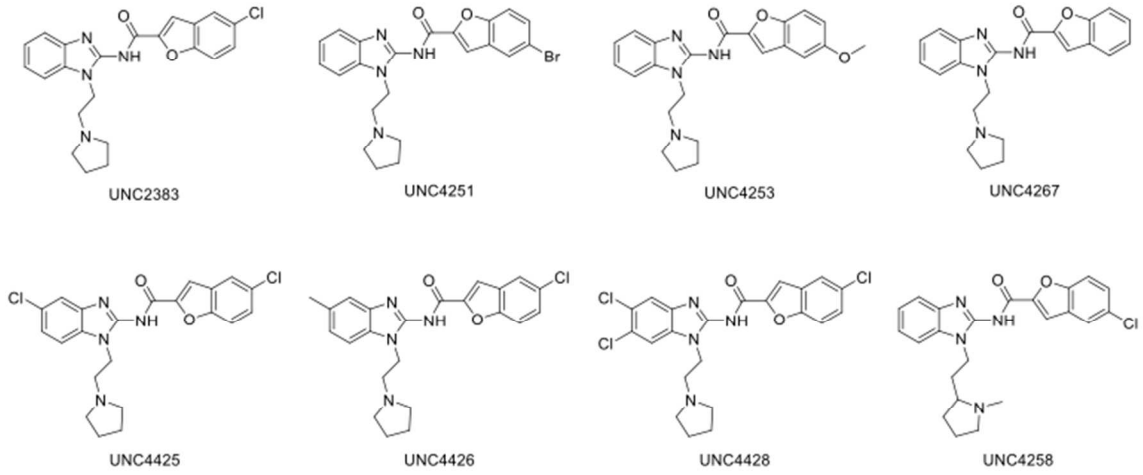
Mice	BUN	ALT	AST	Hb	Platelets
Control	22+/-3	53+/-8	84+/-18	13.2+/-0.7	838+/-15
SSO623 only	23 +/-2	51 +/- 8	65 +/-23	13.8 +/-0.8	824 +/-266
SSO623 + UNC2383	25 +/-11	116 +/-59	62 +/-5	10.0 +/-2.6	770 +/-587
UNC2383 only	20 +/-4	106 +/-32	76 +/-15	11.8 +/-0.4	711 +/-85

Values are means+/- SE. N=4. Units: BUN mg/dl, ALT U/L, AST U/L, Hb g/dl, Platelet $x10^3$ /ul

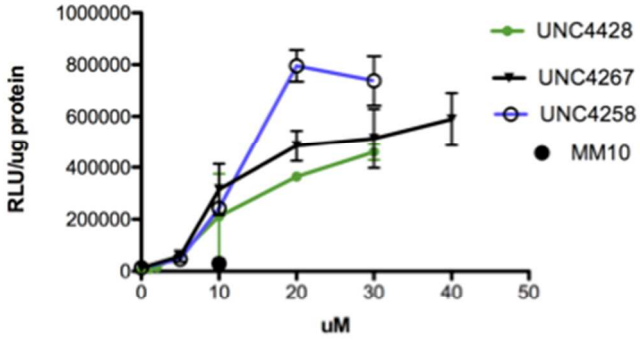
Supporting Table 2: Protein Content for Luciferase Assay in Figure 1b.

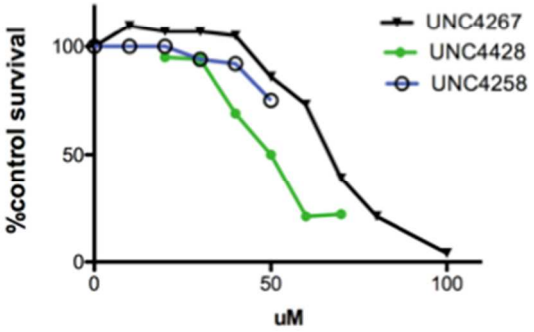
UNC2383 in uM	Protein Content in Luciferase Sample (ug) Mean Value (N=3)	S.E.
0	5.4	0.1
1	5.4	0.2
2	5.3	0.2
3	5.4	0.2
5	5.6	0.2
8	5.5	0.3
10	5.4	0.1
15	4.8	0.2

Supporting Fig 1. Structures of UNC2383 Analogs.



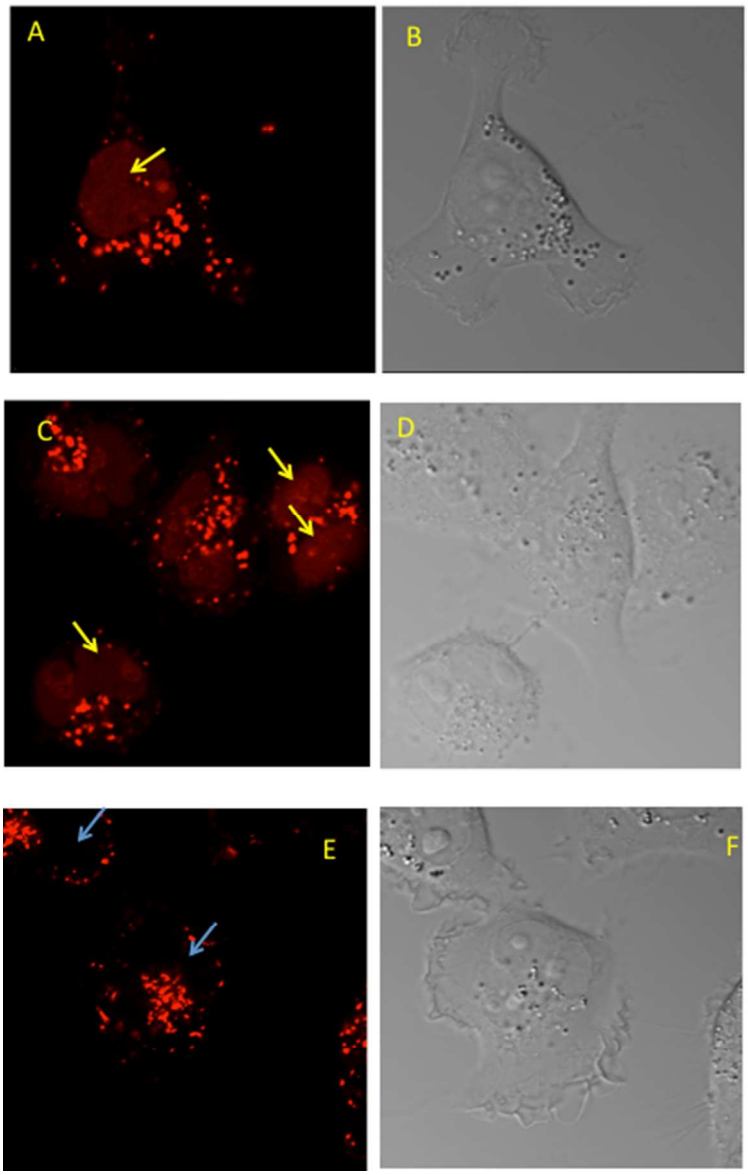
Supporting Fig 2. Effect and Cytotoxicity of Analogs. The effect of selected UNC2383 analogs on luciferase induction by SSO623 was measured as described in Methods and in the legend of Figure 1. Cytotoxicity was measured using the Alamar Blue method as in Fig 1 d. MM10 is the effect of 10 uM compound in the presence of a mismatched oligonucleotide. Means and SE. N=3.



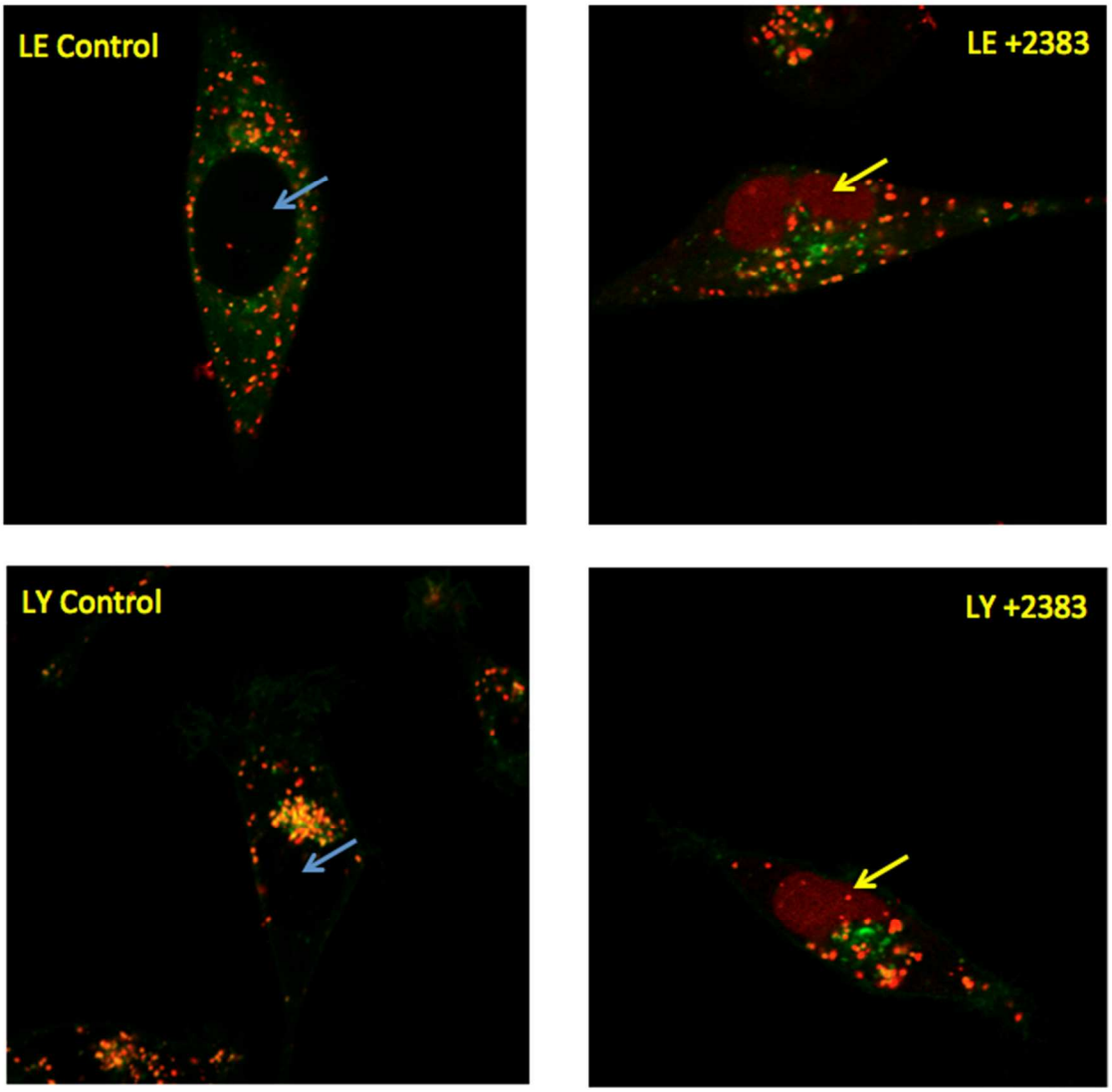


Supporting Fig 3. Confocal images of compound effects on nuclear distribution of a fluorescent oligonucleotide. Methods were similar to those used in Fig. 3 of the main text. Nuclei showing accumulation of fluorescent oligonucleotide (a TAMRA labeled SSO) are indicated with yellow arrows. Nuclei lacking fluorescence are indicated with blue arrows.

A,B UNC4267 30 uM; C, D UNC4258 30 uM; E,F controls



Supporting Fig 4. Confocal images of compound effects on overlap of a fluorescent oligonucleotide with GFP marker proteins for late endosomes (LE) or lysosomes (LY). Methods were similar to those used in Fig. 4 of the main text. Overlap of TAMRA-SSO (red) and GFP (green) is seen as yellow-orange. Control cells and cells treated with 5 uM UNC2383 are shown. Yellow arrows indicate nuclei showing TAMRA fluorescence; blue arrows indicate 'empty' nuclei. DIC images have been removed for clarity.



Additional Methods and Statistical Information:

PCR techniques. Total RNA was isolated using TriReagent (Molecular Research Center). Total RNA was normalized by measuring OD260 on a Nanodrop™ spectrophotometer and then converted into first-strand cDNA using an Enhanced Avian First Strand Synthesis Kit (Sigma). EGFP cDNA was amplified by PCR using forward (5'-CGTAAACGGCCACAAGTTCAGCG-3') and reverse (5'-GTGGTGCAGATGAACTTCAGGGTC-3') primers. The PCR products were separated on 1.5 % agarose gels and bands were visualized by staining with Gel Red™ and quantitated using a Biorad ChemiDoc XRS+ scanner.

Statistical Information. Confocal images were analyzed using the Fiji (Image J) software package. Overlap of red and green fluorescence was quantitated by calculation of the Manders Coefficient using the Jacop plug in. Quantitation of nuclear accumulation of TAMRA-labeled oligonucleotide was done by measuring grey scale intensity in the red channel in an area previously outlined using Hoechst stain. Evaluations of the significance of differences between experimental samples and controls in confocal and PCR measurements were done using the paired T-test. Calculation of the R^2 value for Figure 5b was done with Prism™ software.

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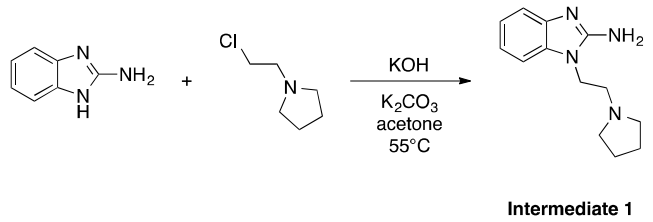
Supporting Chemical Information

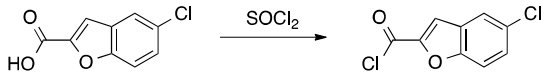
General Information

Analytical LCMS data for all compounds were acquired using an Agilent 6110 series system with the UV detector set to 220 and 254 nm. Samples were injected (<10 μL) onto an Agilent Eclipse Plus 4.6 × 50 mm, 1.8 um, C18 column at room temperature. A mobile phase of A (H_2O + 0.1% acetic acid) and B (MeOH + 0.1% acetic acid) was used with a linear gradient from 10% to 100% B in 5.0 min, followed by a flush at 100% B for another 2 minutes with a flow rate of 1.0 mL/min. Mass spectra data were acquired in positive ion mode using an Agilent 6110 single quadrupole mass spectrometer with an electrospray ionization source. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Mercury spectrometer at 400 MHz for proton (^{1}HNMR); chemical shifts are reported in ppm (δ). Analytical thin-layer chromatography (TLC) was performed with silica gel 60 F254, 0.25 mm pre-coated TLC plates, generally using a suitable MeOH in DCM solvent system. TLC plates were visualized using UV 254 nm, I_2 impregnated silica gel, potassium permanganate with charring, and phosphomolybdic acid with charring. Reverse phase or normal phase chromatography was used to purify reaction mixtures to obtain intermediate products using a Teledyne Isco CombiFlash Rf 200 chromatography unit equipped with the UV detector set to 220 nm and 254 nm. Suitable variations in the purification method (flow rate, solvent system) were made as needed to achieve ideal separation for each compound. All compounds that were evaluated in biochemical and biophysical assays had >95% purity as determined by ^{1}HNMR and LCMS.

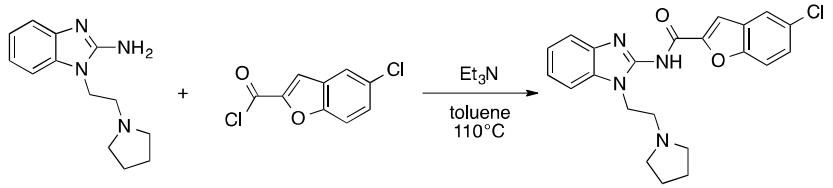
Synthesis of UNC2383



Intermediate 1



Intermediate 2



Intermediate 1 Intermediate 2 UNC2383

Synthesis of Intermediate 1

To a solution of 2-aminobenzimidazole (500 mg, 3.75 mmol, 1.0 eq.) and 1-(2-Chloroethyl)pyrrolidine hydrochloride (639 mg, 3.75 mmol, 1.0 eq.) in acetone (19 mL) was added powdered KOH (375 mg) and anhydrous K_2CO_3 (750 mg), and the reaction mixture was heated under reflux for 3 hours. Upon completion, solvent was removed under reduced pressure to obtain a pale yellow solid. The solid was dissolved in CH_2Cl_2 (30 mL) and washed with water (2×30 mL). The aqueous phase was extracted with CH_2Cl_2 (3×20 mL) and the organic layers were combined, washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to obtain a pale yellow crude material. The crude material was adsorbed onto silica gel, and purified by normal phase automated Teledyne Isco chromatography using a CH_2Cl_2/MeOH/NH_3 solvent system. Intermediate 1 was obtained as a white solid (569 mg, 66%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.15 – 7.09 (m, 2H), 6.89 (m, 2H), 4.07 (t, J = 6.8 Hz, 2H), 2.68 (t, J = 6.8 Hz, 2H), 2.52 - 2.49 (m, 4H), 1.70 – 1.61 (m, 4H).

LC-MS (λ = 254 nm): 99%, t_R = 0.6 min. MS (ESI+): 231 $[M+H]^+$

Synthesis of Intermediate 2

5-chlorobenzofuran-2-carboxylic acid (500 mg, 2.5 mmol) was added to thionyl chloride (5 mL) and heated under reflux for 2 hrs. Solvent was removed under reduced pressure to obtain a white solid, which then used in the next reaction without further purification (510 mg).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.76 (d, *J* = 0.9 Hz, 1H), 7.72 (dd, *J* = 2.0, 0.7 Hz, 1H), 7.56 – 7.48 (m, 2H).

Synthesis of UNC2383

To a solution of intermediate 1 (300 mg, 1.3 mmol, 1.0 eq.) and intermediate 2 (280 mg, 1.3 mmol, 1.0 eq.) in toluene (2 mL) was added triethylamine (0.7 mL, 5.2 mmol, 4.0 eq.), and the reaction mixture was heated under reflux for 3 hours. Upon completion, the reaction was quenched with sat. aqueous $NaHCO_3$ solution (10 mL). The aqueous phase was separated and extracted with CH_2Cl_2 (3 x 10 mL). All combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to obtain a pale yellow crude material. The crude material was adsorbed onto silica gel, and purified by normal phase automated Teledyne Isco chromatography using a CH_2Cl_2/MeOH/NH_3 solvent system. UNC2383 was obtained as a white solid (276 mg, 52%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.12 (brs, 1H), 7.63 (d, *J* = 2.0 Hz, 1H), 7.51 (d, *J* = 8.8 Hz, 1H), 7.47 (d, *J* = 1.0 Hz, 1H), 7.37 (dd, *J* = 6.4, 2.5 Hz, 1H), 7.33 – 7.24 (m, 4H), 4.45 – 4.31 (m, 2H), 2.98 – 2.86 (m, 2H), 2.71 – 2.64 (m, 4H), 1.88 – 1.73 (m, 4H).

LC-MS (λ = 254 nm): 99%, t_R = 5.2 min. MS (ESI+): 409 $[M+H]^+$

Compounds ***UNC4425***, ***UNC4426***, ***UNC4428***, ***UNC4253***, ***UNC4258***, ***UNC4267, and UNC4251*** *were prepared by a similar method as* ***UNC2383.***

Synthesis of UNC4425

Procedure for the synthesis of UNC2383 was followed with 5-chloro-2-aminobenzimidazole to obtain a pale yellow solid.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.14 (brs, 1H), 7.63 (d, *J* = 2.1 Hz, 1H), 7.53 – 7.49 (m, 1H), 7.48 (d, *J* = 0.9 Hz, 1H), 7.40 – 7.26 (m, 3H), 7.24 – 7.20 (m, 1H), 4.35 (q, *J* = 7.0, 6.6 Hz, 2H), 2.92 (t, *J* = 7.1 Hz, 2H), 2.68 (dd, *J* = 7.4, 4.4 Hz, 4H), 1.81 – 1.77 (m, 4H).

LC-MS (λ = 254 nm): 99%, t_R = 5.6 min. MS (ESI+): 444 $[M+H]^+$

Synthesis of UNC4426

Procedure for the synthesis of UNC2383 was followed with 5-methyl-2-aminobenzimidazole to obtain a pale yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.44 (brs, 1H), 7.85 (d, *J* = 2.2 Hz, 1H), 7.72 (d, *J* = 8.8 Hz, 1H), 7.62 (d, *J* = 0.9 Hz, 1H), 7.51 – 7.38 (m, 3H), 7.15 – 7.10 (m, 1H), 4.62 (t, *J* = 5.5 Hz, 2H), 3.96 – 3.86 (m, 2H), 3.69 – 3.62 (m, 2H), 3.26 – 3.16 (m, 2H), 2.42 (d, *J* = 10.8 Hz, 3H), 2.08 – 1.99 (m, 2H), 1.82 – 1.73 (m, 2H).

LC-MS (λ = 254 nm): 99%, t_R = 5.7 min. MS (ESI+): 424 $[M+H]^+$

Synthesis of UNC4428

Procedure for the synthesis of UNC2383 was followed with 5,6-dichloro-2-aminobenzimidazole to obtain a white solid.

^{1}H NMR (400 MHz, Methanol-d_4) δ 7.84 (s, 1H), 7.76 (d, *J* = 2.2 Hz, 1H), 7.71 (s, 1H), 7.63 (d, *J* = 0.9 Hz, 1H), 7.57 (d, *J* = 8.8 Hz, 1H), 7.44 (dd, *J* = 8.8, 2.2 Hz, 1H), 4.70 (t, *J* = 5.4 Hz, 2H), 3.73 (t, *J* = 5.4 Hz, 2H), 3.32 (m, 4H, overlapped with Methanol-d_4), 2.12 – 2.06 (m, 4H).

LC-MS (λ = 254 nm): 99%, t_R = 5.8 min. MS (ESI+): 478 $[M+H]^+$

Synthesis of UNC4253

Procedure for the synthesis of UNC2383 was followed with 5-methoxybenzofuran-2-carboxylic acid to obtain a pale yellow solid.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.20 (brs, 1H), 7.51 (d, *J* = 0.8 Hz, 2H), 7.41 – 7.35 (m, 1H), 7.34 – 7.29 (m, 1H), 7.30 – 7.26 (m, 1H), 7.26 – 7.23 (m, 1H), 7.10 (d, *J* = 2.6 Hz, 1H), 7.00 (dd, *J* = 8.9, 2.6 Hz, 1H), 4.49 – 4.36 (m, 2H), 3.87 (s, 3H), 2.95 (t, *J* = 7.4 Hz, 2H), 2.71 (t, *J* = 5.2 Hz, 4H), 1.89 – 1.76 (m, 4H).

LC-MS (λ = 254 nm): 99%, t_R = 4.5 min. MS (ESI+): 406 $[M+H]^+$

Synthesis of UNC4258

Procedure for the synthesis of UNC2383 was followed with 2-(2-chloroethyl)-1-methylpyrrolidine to obtain a pale yellow solid.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.11 (brs, 1H), 7.63 (d, *J* = 2.1 Hz, 1H), 7.56 – 7.46 (m, 1H), 7.46 (d, *J* = 1.0 Hz, 1H), 7.42 – 7.33 (m, 1H), 7.35 – 7.27 (m, 2H), 7.32 – 7.23 (m, 2H), 4.40 – 4.22 (m, 2H), 3.04-3.09 (m, 1H), 2.31 (s, 3H), 2.28 – 2.03 (m, 4H), 1.93 – 1.76 (m, 2H), 1.78 – 1.58 (m, 2H).

LC-MS (λ = 254 nm): 99%, t_R = 5.7 min. MS (ESI+): 424 $[M+H]^+$

Synthesis of UNC4267

Procedure for the synthesis of UNC2383 was followed with benzofuran-2-carboxylic acid to obtain a white solid.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.16 (brs, 1H), 7.62 (dd, *J* = 28.8, 8.1 Hz, 2H), 7.55 (s, 1H), 7.36 (td, *J* = 7.3, 1.8 Hz, 2H), 7.34 – 7.22 (m, 4H), 4.40 (t, *J* = 7.3 Hz, 2H), 2.94 (t, *J* = 7.3 Hz, 2H), 2.71-2.67 (m, 4H), 1.88 – 1.71 (m, 4H).

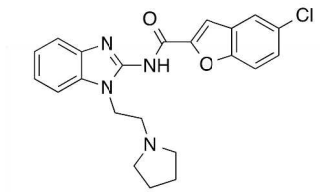
LC-MS (λ = 254 nm): 99%, t_R = 4.5 min. MS (ESI+): 375 $[M+H]^+$

Synthesis of UNC4251

Procedure for the synthesis of UNC2383 was followed with 5-bromobenzofuran-2-carboxylic acid to obtain a pale yellow solid.

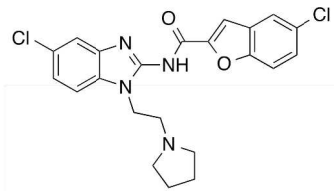
^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.12 (brs, 1H), 7.82 – 7.80 (m, 1H), 7.51 – 7.45 (m, 3H), 7.40 – 7.27 (m, 4H), 4.41 (t, *J* = 7.3 Hz, 2H), 2.95 (t, *J* = 7.3 Hz, 2H), 2.74 – 2.66 (m, 4H), 1.86 – 1.77 (m, 4H).

LC-MS (λ = 254 nm): 99%, t_R = 4.7 min. MS (ESI+): 453 + 455 $[M+H]^+$



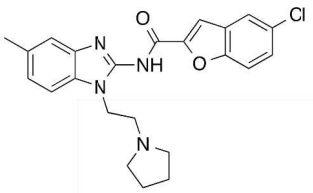
Chemical Formula: $C_{22}H_{21}ClN_4O_2$
Molecular Weight: 408.89

UNC2383



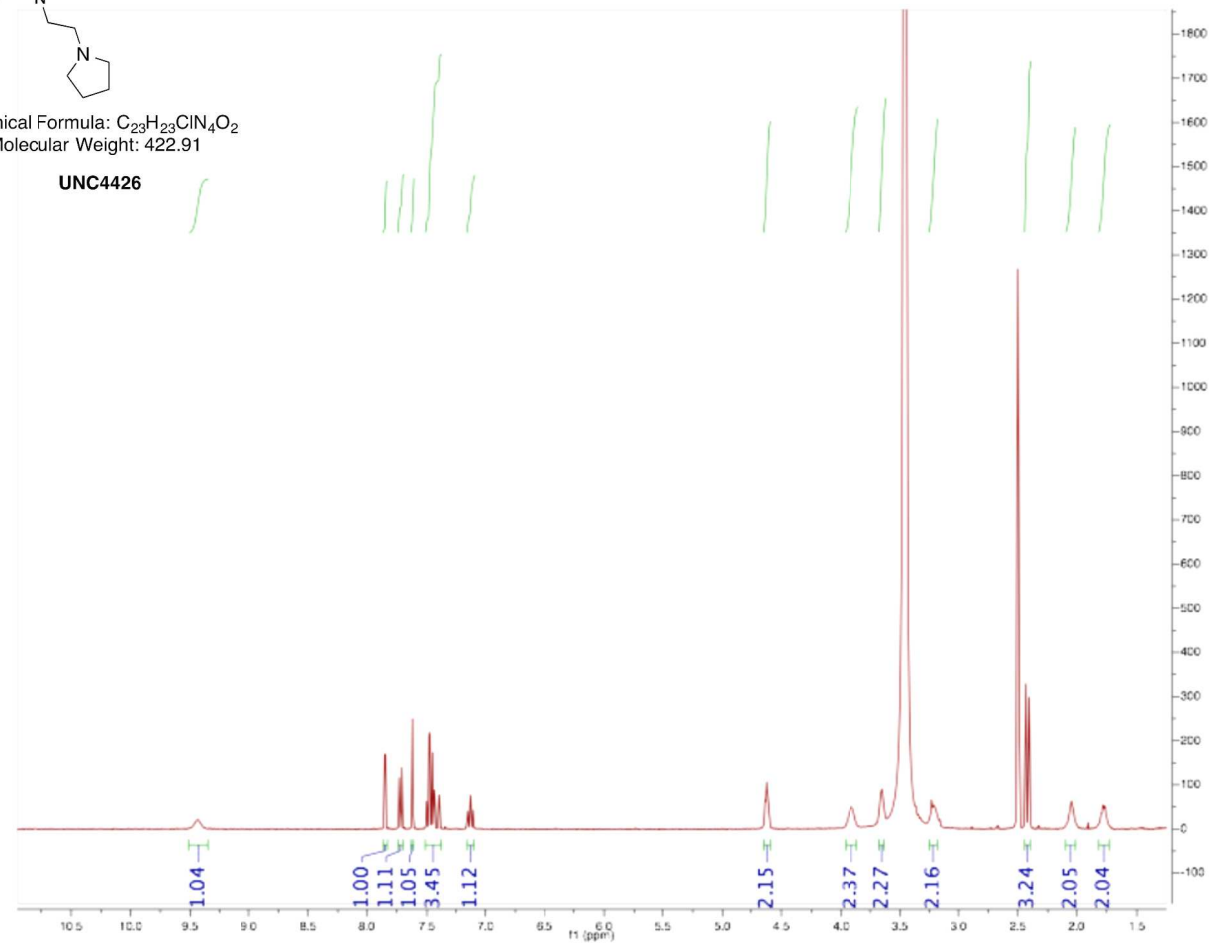
Chemical Formula: $C_{22}H_{20}Cl_2N_4O_2$
Molecular Weight: 443.33

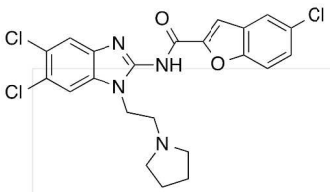
UNC4425



Chemical Formula: $C_{23}H_{23}ClN_4O_2$
Molecular Weight: 422.91

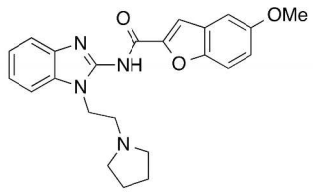
UNC4426





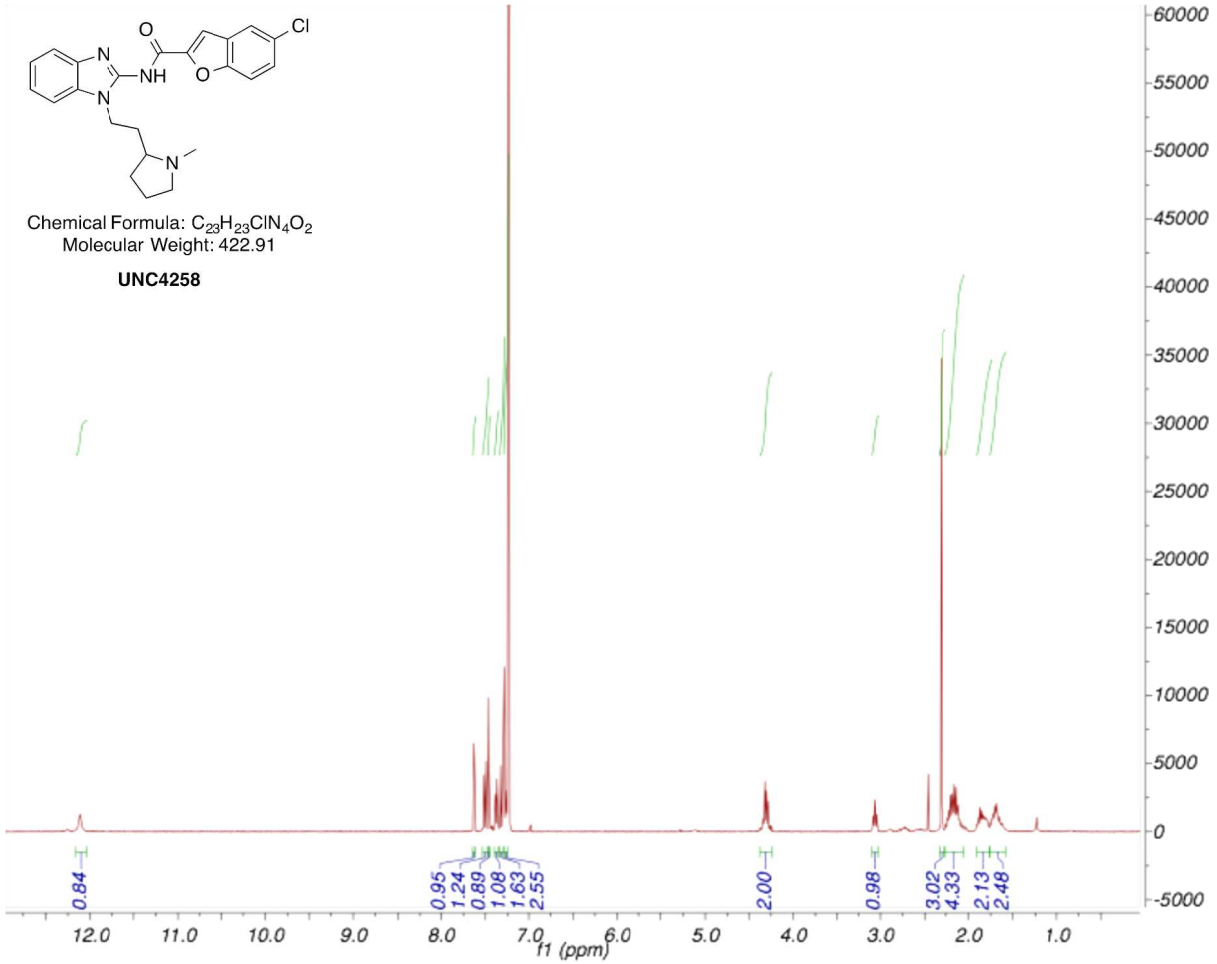
Chemical Formula: $C_{22}H_{19}Cl_3N_4O_2$
Molecular Weight: 477.77

UNC4428



Chemical Formula: $C_{23}H_{24}N_4O_3$
Molecular Weight: 404.47

UNC4253


Chemical Formula: $C_{23}H_{23}ClN_4O_2$
Molecular Weight: 422.91
UNC4258
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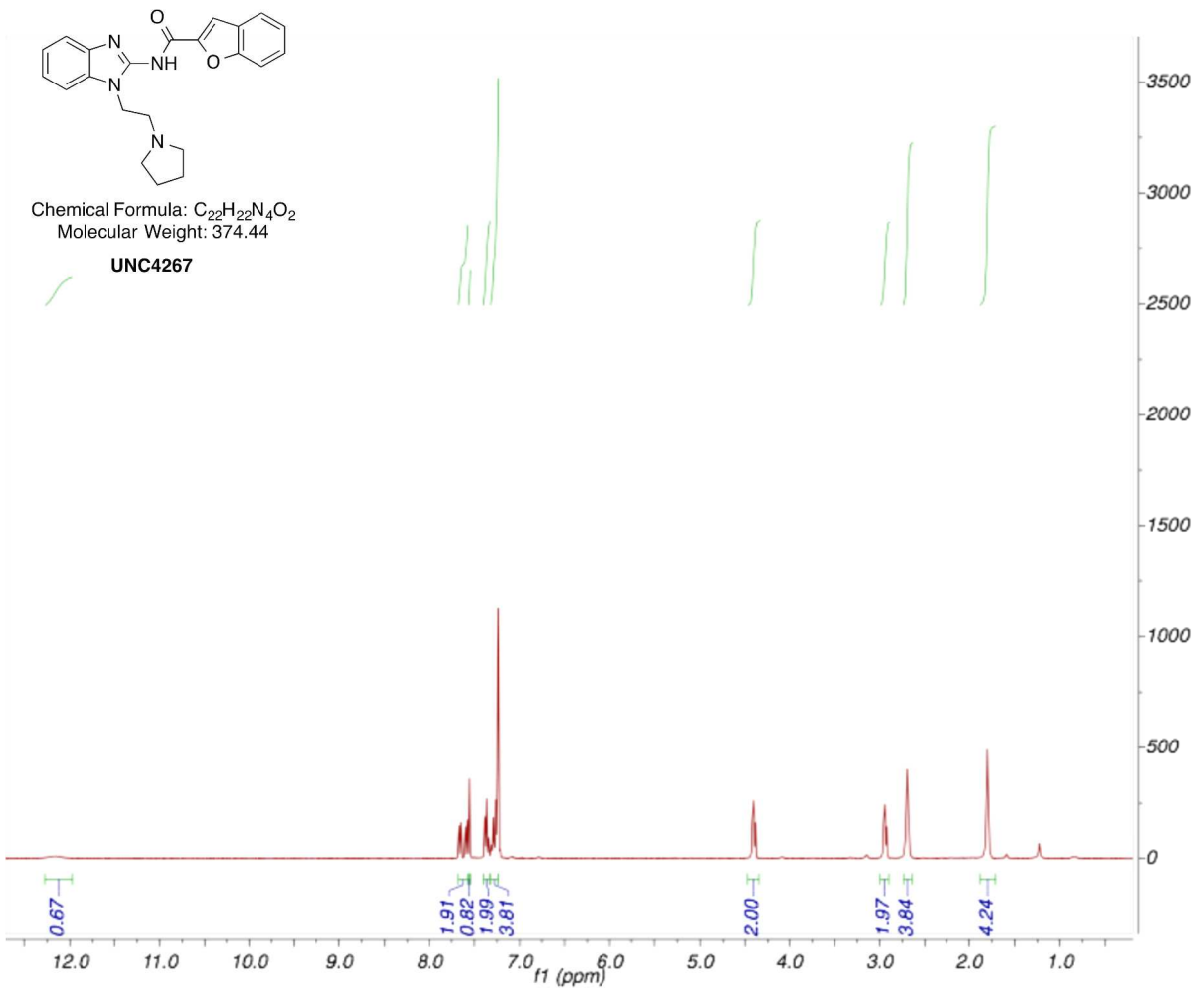
f1 (ppm)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UNC4267
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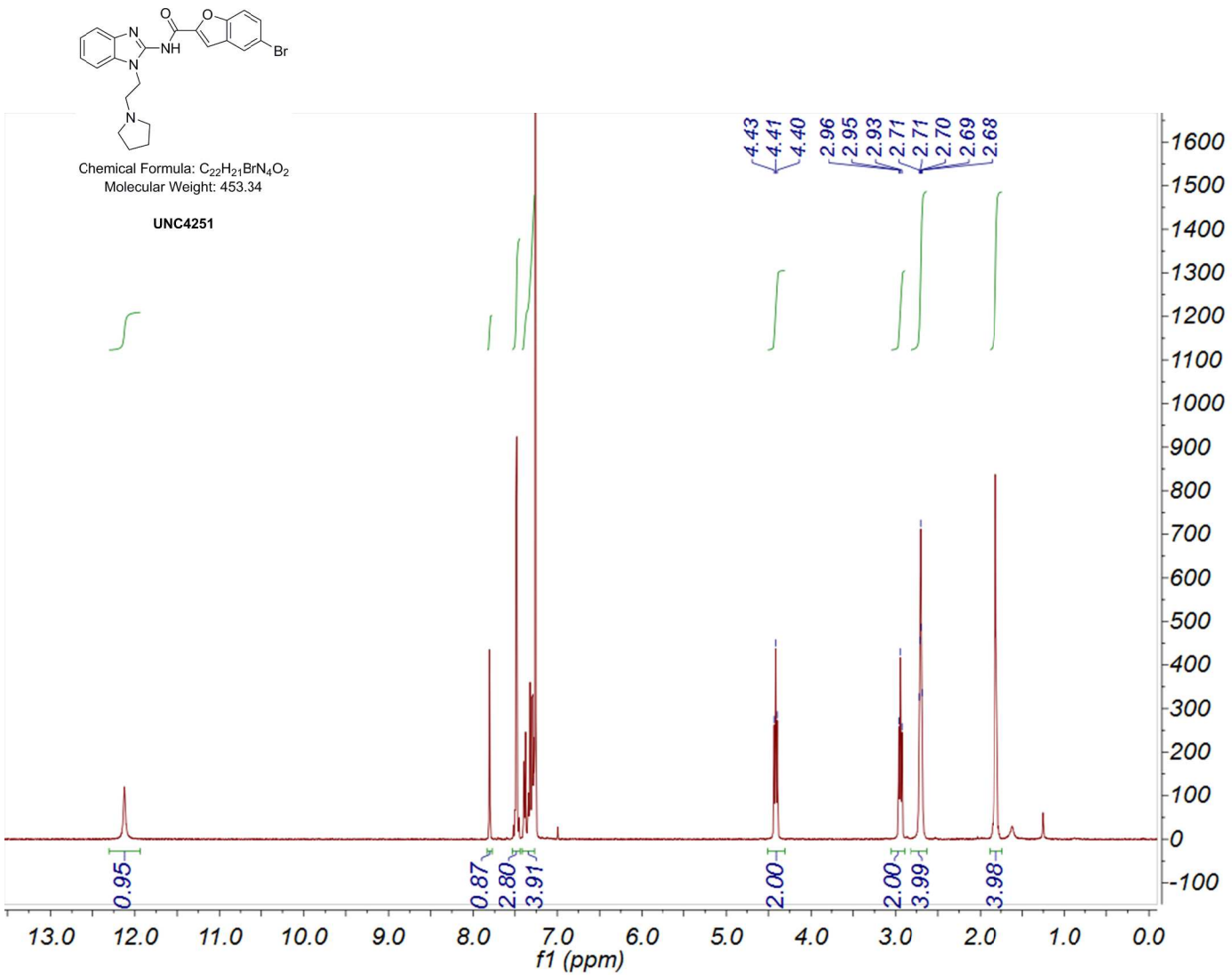
f1 (ppm)


Chemical Formula: $C_{22}H_{21}BrN_4O_2$
Molecular Weight: 453.34
UNC4251
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3.99
3.98
f1 (ppm)