Supporting Information for

Discovery of a Small-Molecule Modulator of Glycosaminoglycan Sulfation

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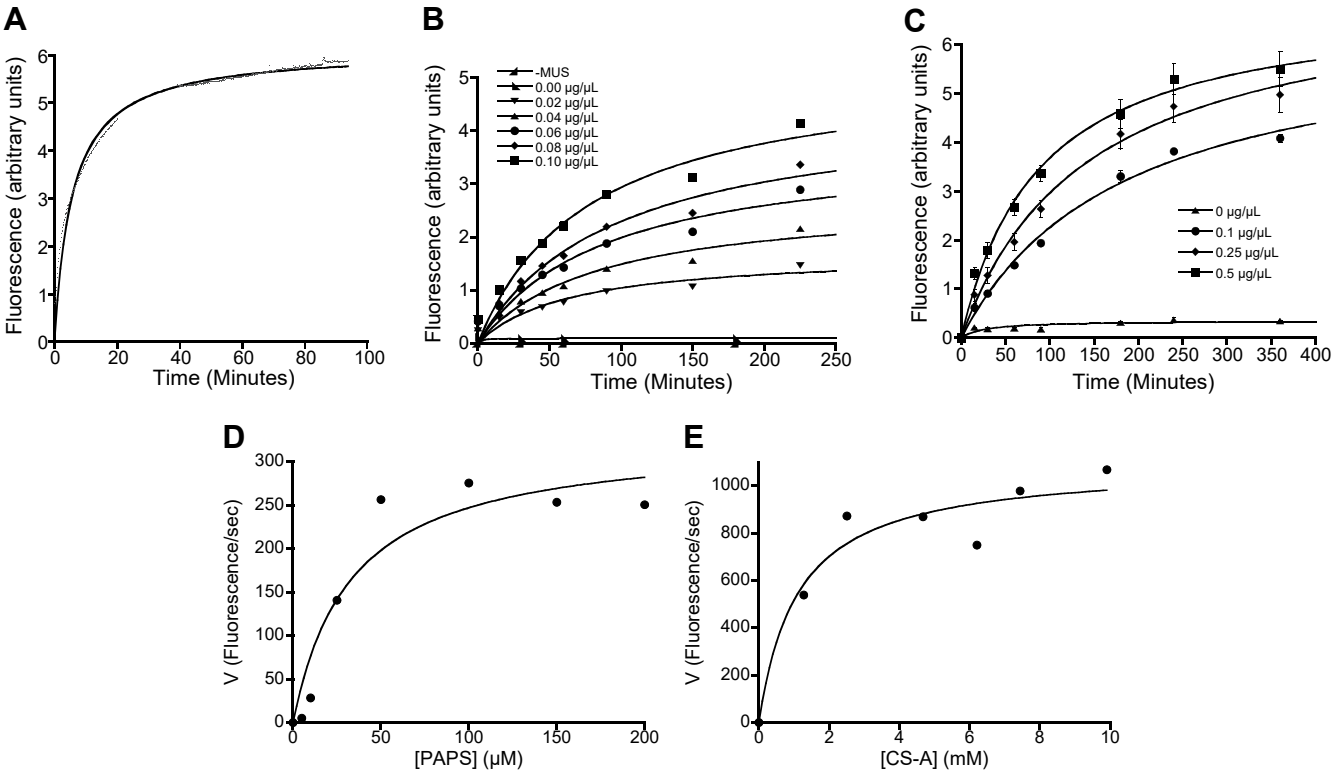


Figure S1. High-throughput assay optimization for the discovery of Chst15 small-molecule inhibitors. (A) A 60-min pre-incubation of Sult1c1 with all assay components except Chst15 was required to convert residual PAP to PAPS and minimize background fluorescence. Fluorimetry data fit to a Michaelis-Menten curve are shown. (B) Increasing the concentration of Sult1c1 led to a greater fluorescent output. A Sult1c1 concentration of 0.10 µg/µL was chosen to limit the final glycerol content in the assay. (C) Increasing the concentration of Chst15 also led to greater fluorescence. Larger amounts of Chst15 could be used to increase the assay signal-to-noise ratio (SNR) and Z-factor (Z'). SNR = 6.14 and Z' = 0.62 at 0.5 µg/µL Chst15 after 15 min in a 96-well plate format. n = 4 (mean ± s.d.) for each condition. The K_m of (D) PAPS and (E) CS-A were determined to be 33 µM and 1.1 mM respectively. Initial reaction rates for varying substrate concentrations were plotted and fit to a Michaelis-Menten curve.

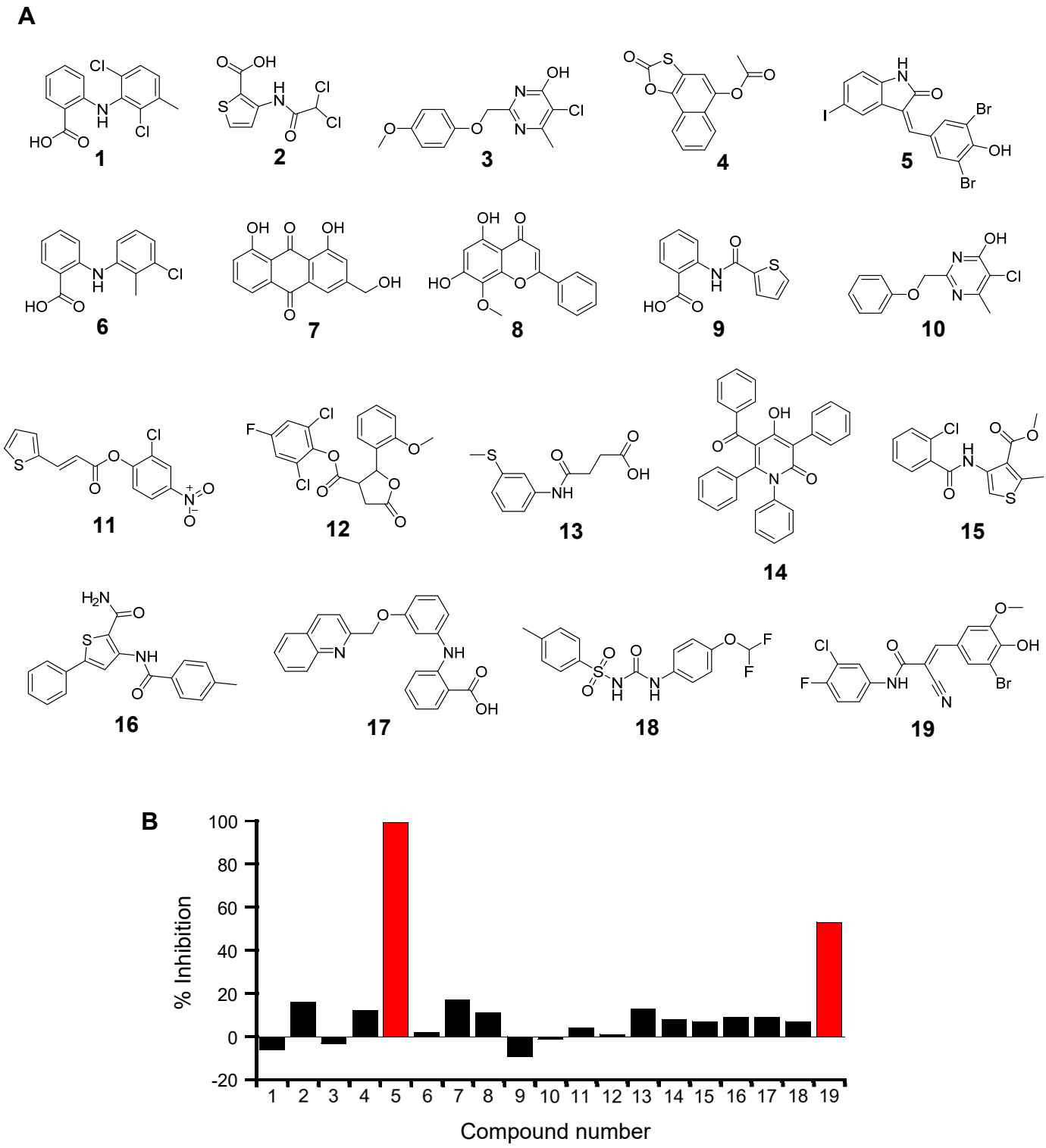


Figure S2. Validation of candidate compounds identified in the 70,000 compound primary screen. (A) Structures of the 19 hit compounds that were commercially available and obtained for further evaluation in secondary screening assays. (B) The compounds were tested directly against Chst15 in a radioisotope labeling assay at 100 μM to confirm the inhibitory activity of two compounds, **5** and **19**.

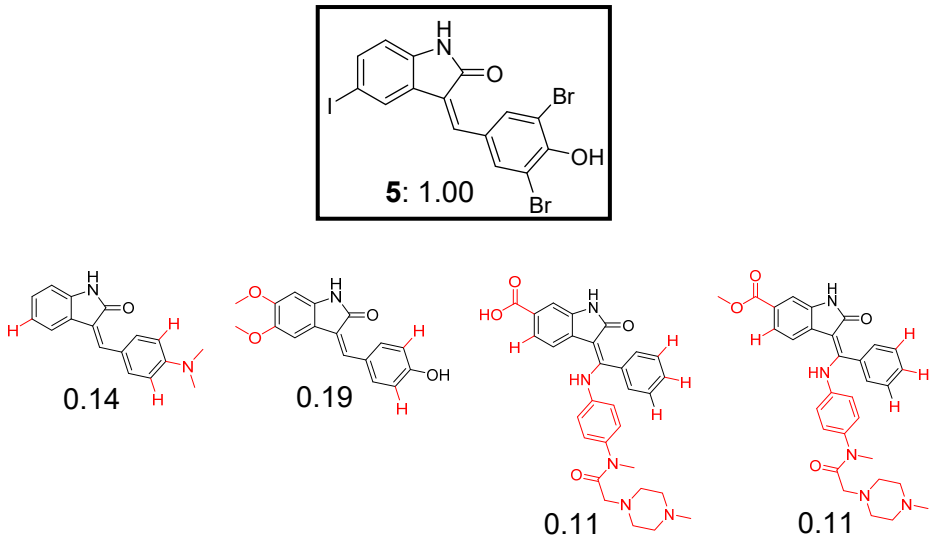


Figure S3. Comparison of chemical analogs to lead compound **5**. Relative inhibition values are given for each compound as compared to the original hit **5**. Compounds were assessed using the radiolabeling assay at 20 μM. Mean values of duplicate experiments were calculated.

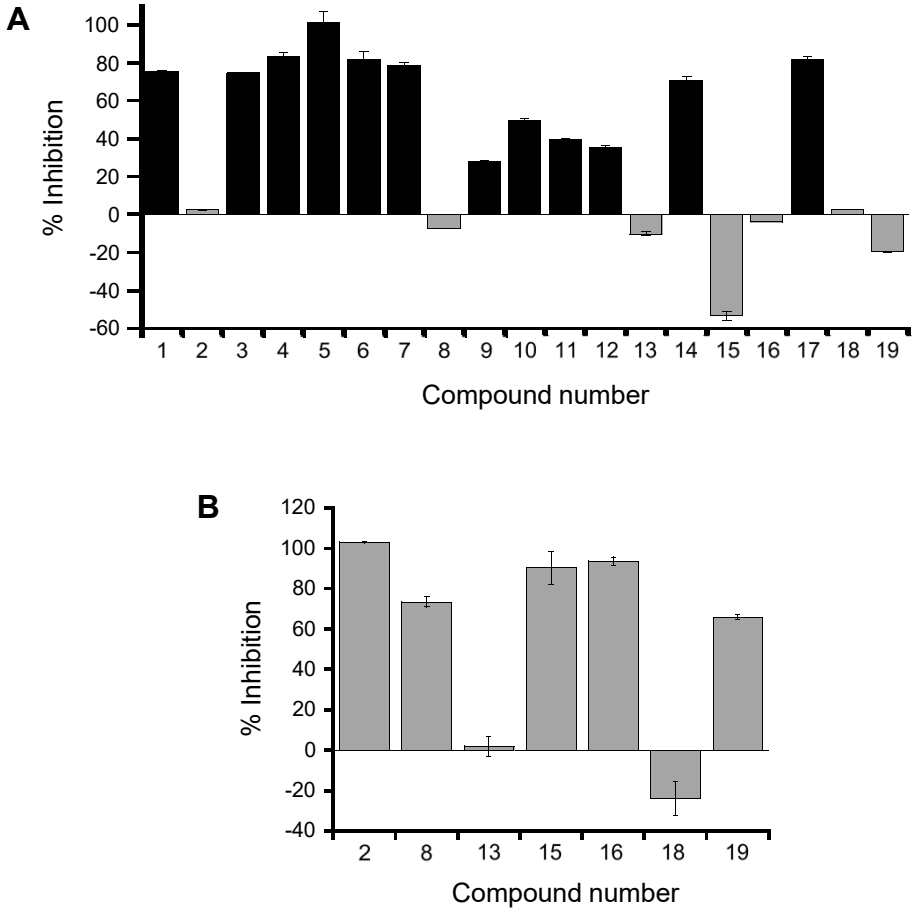


Figure S4. Analysis of candidate compounds using the counter-screen and fluorescence assays in a 96-well plate format resulted in six potential inhibitory compounds. (A) Re-testing of the compounds for Sult1c1 inhibition. Seven of the 19 compounds did not appreciably inhibit Sult1c1 at 12.5 μM. (B) The seven compounds that did not inhibit Sult1c1 were re-tested in the full fluorescence assay. Five of seven compounds inhibited Chst15 at 12.5 μM. Values shown are the mean ± S.E.M. for triplicate experiments.

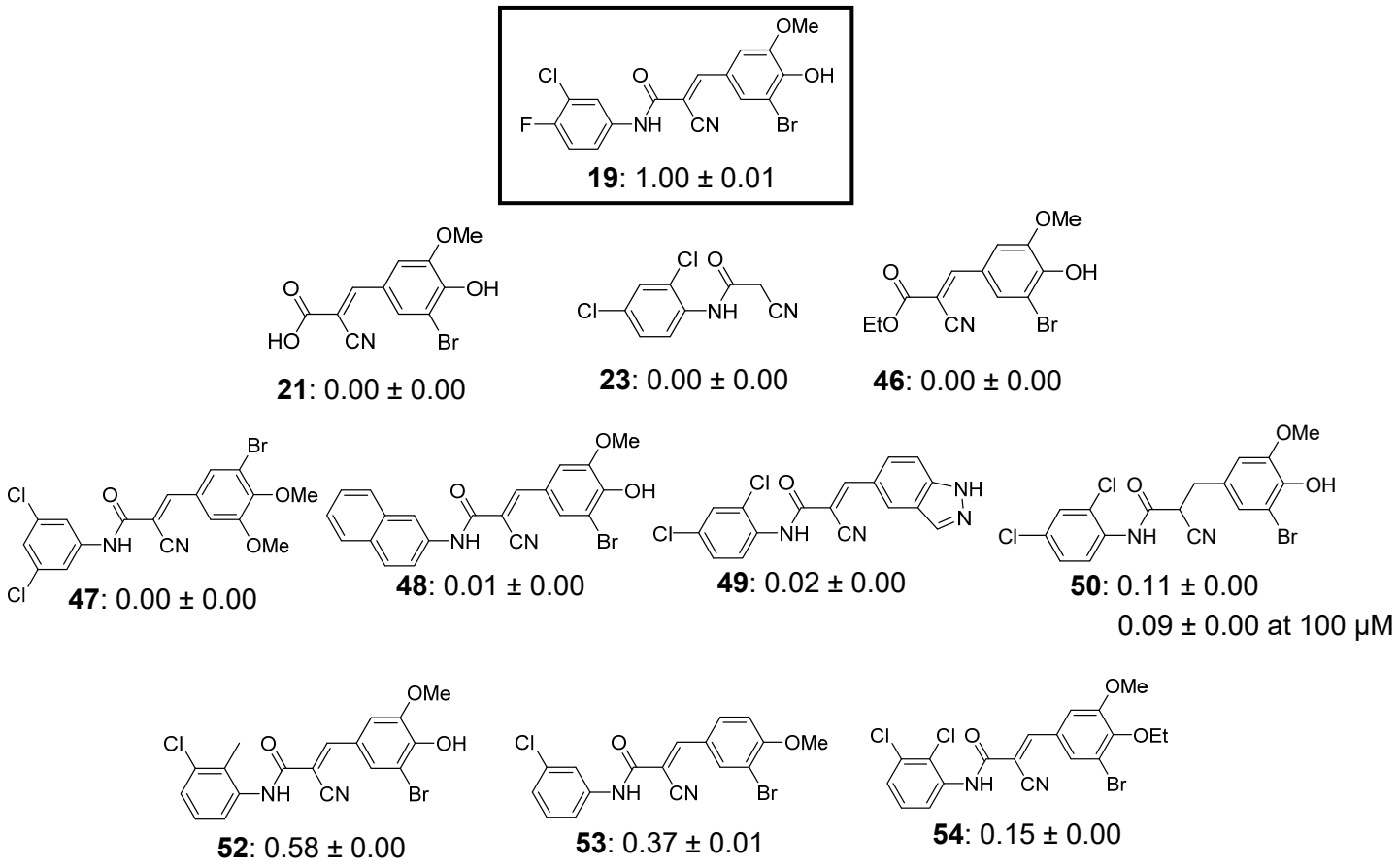


Figure S5. Comparison of additional chemical analogs to lead compound **19**. Relative inhibition values are given for each compound as compared to the original hit **19**. Compounds were assessed using the radiolabeling assay at 25 μM. Values represent the mean ± S.E.M. for triplicate experiments.

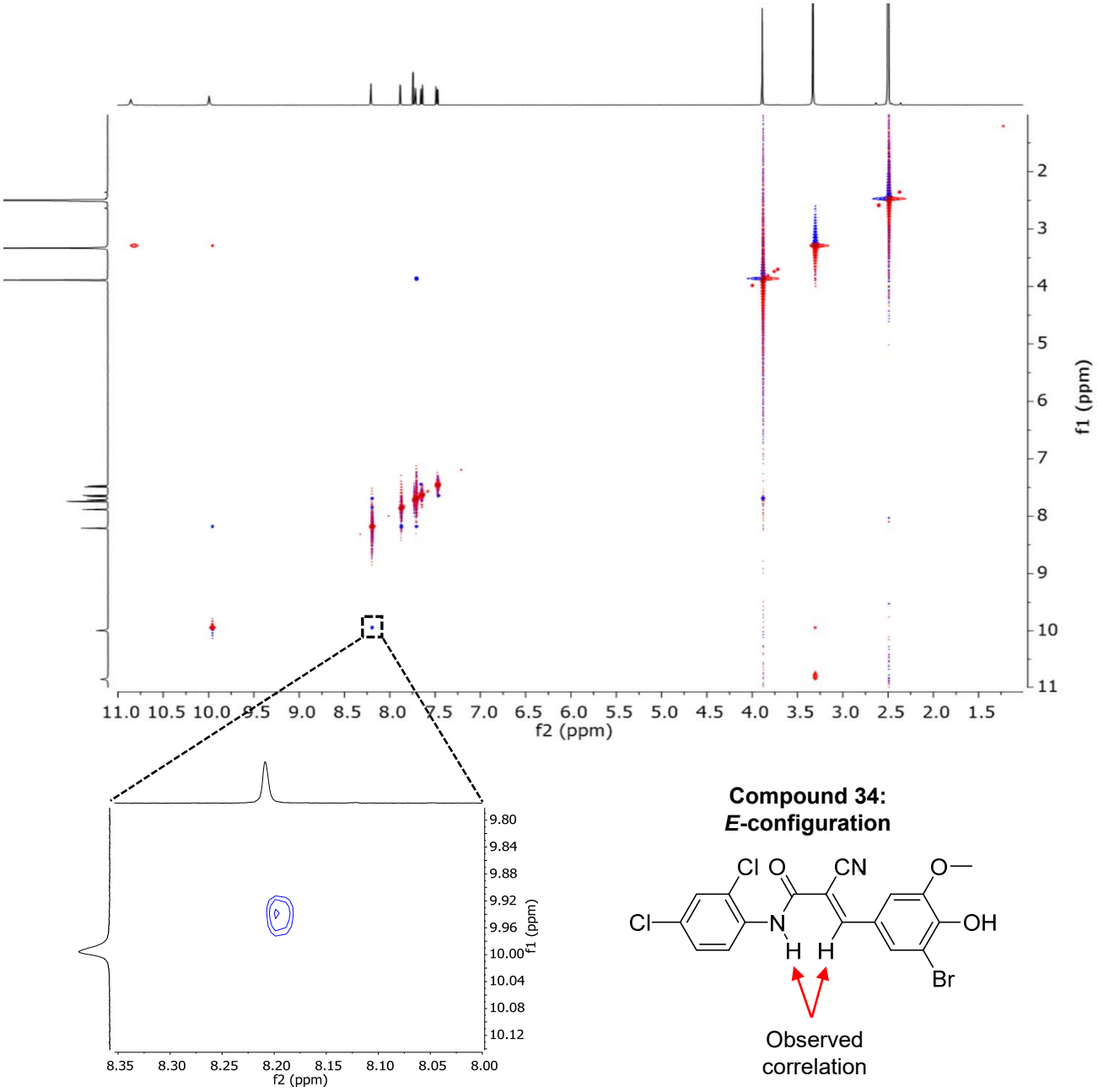


Figure S6. ROESY data of compound **34**. Correlation between the amide (δ 10.0 ppm) and alkene (δ 8.21 ppm) protons suggest the *E*-configuration of compound **34**. Cross peak should not be observed in the *Z*-configuration.

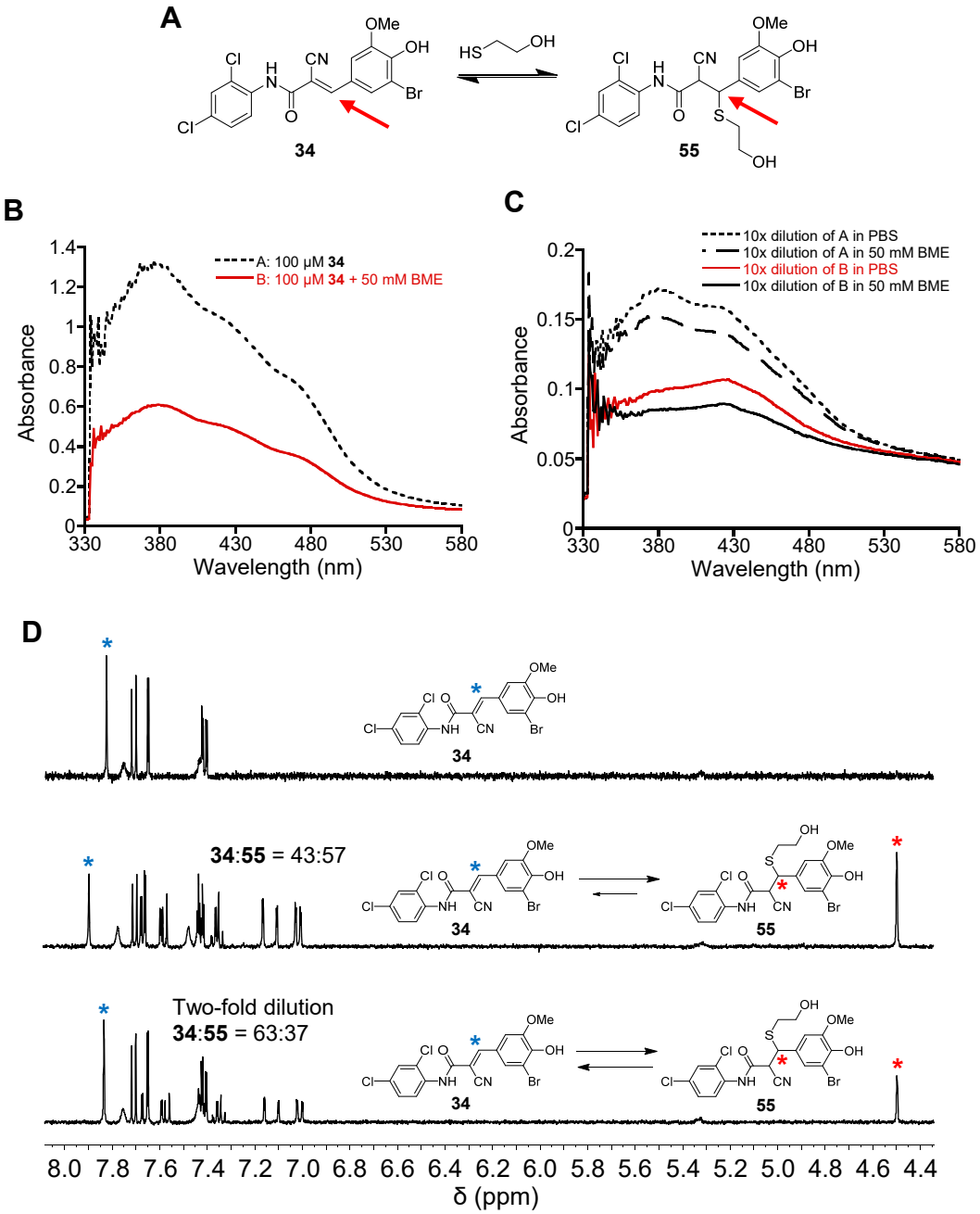


Figure S7. Compound **34** exhibits properties of a reversible covalent inhibitor when using β-mercaptoethanol (BME) as a surrogate nucleophile. (A) A conjugate-addition equilibrium exists when **34** is incubated with BME. (B) UV-Vis absorbance spectra suggest the reversible formation of a covalent thioether adduct. **34** was incubated with or without 50 mM BME in PBS. Adduct formation is suggested by the decrease in absorbance when incubated with BME (black to red). (C) Dilution of each reaction by 10-fold into PBS with or without 50 mM BME. Reversibility is evidenced by the reappearance of the cyanoacrylamide absorption peak after dilution of BME-containing samples into PBS (compare solid red and solid black curves). (D) ^{1}H NMR spectroscopy shows the reversible formation of thioether adduct **55**. **34** was incubated without (top) or with 100 mM BME in 3:1 DMSO-d_6:deuterated PBS (middle). The reaction containing βME was then diluted 2-fold into 3:1 DMSO-d_6:deuterated PBS (bottom). Peak integration of the olefinic proton (blue asterisk) and aliphatic β-proton (red asterisk) was used to determine the indicated ratios of cyanoacrylamide to thioether adduct.

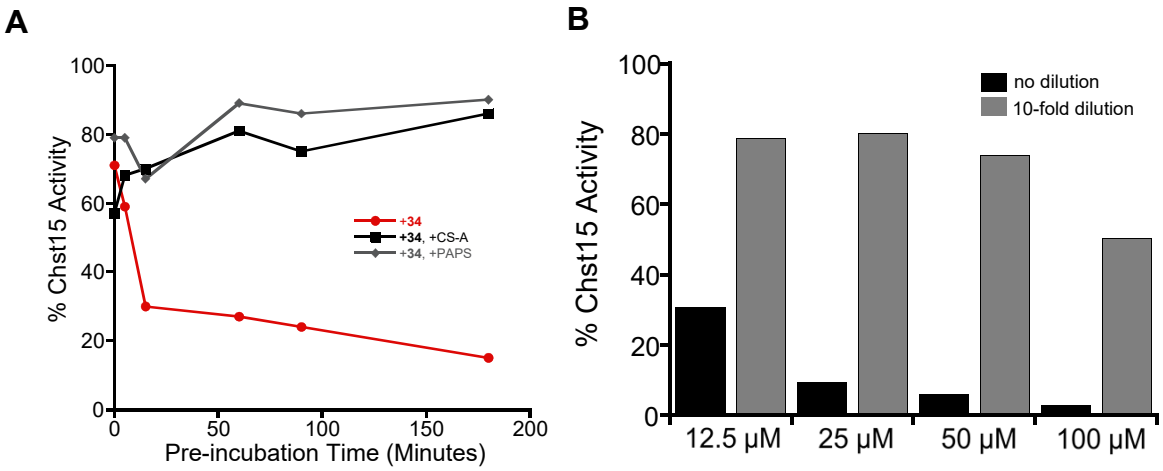


Figure S8. The inhibitory activity of **34** depends on the pre-incubation time and shows reversibility. (A) Chst15 was pre-incubated with **34** (final inhibitor concentration = 25 μM) for varying lengths of time prior to initiation of the [^{35}S]-labeling assay. Longer pre-incubation times resulted in greater inhibition by **34** (red). Compound **34** is out-competed by the substrates CS-A (1 mM) and PAPS (30 μM) when pre-incubated at the same time. (B) In a rapid dilution assay, Chst15 is pre-incubated with the indicated concentrations of **34**. After incubation, the mixture is diluted ten-fold with substrates to initiate the [^{35}S]-labeling assay (gray bars, $[Chst15]_{final}$ = 0.067 mg/mL). Compared to a reaction in which Chst15 is pre-incubated at the indicated concentrations and initiated without ten-fold dilution (black bars, $[Chst15]_{final}$ = 0.067 mg/mL), there was a recovery in Chst15 activity upon dilution. This recovery strongly suggests rapid reversibility of the E·I complex and a dependence of **34** on the final equilibrium concentration.

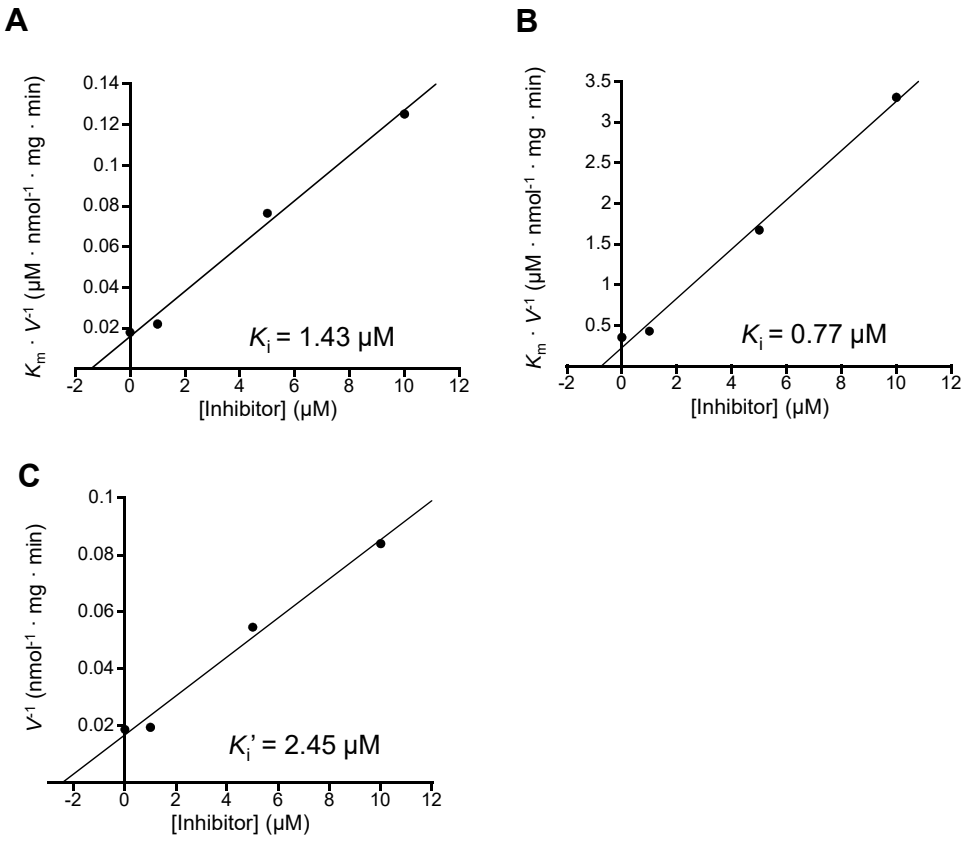


Figure S9. K_i and K_i' determination. Parameters were determined by measuring the initial reaction velocities at varying substrate concentrations in the presence of **34** at concentrations of 0, 1, 5, and 10 μM. K_i was derived from a secondary plot of K_m/V versus [**34**] for (A) CS-A and (B) PAPS. (C) K_i' was derived from a secondary plot of $1/V$ versus [**34**] for CS-A.

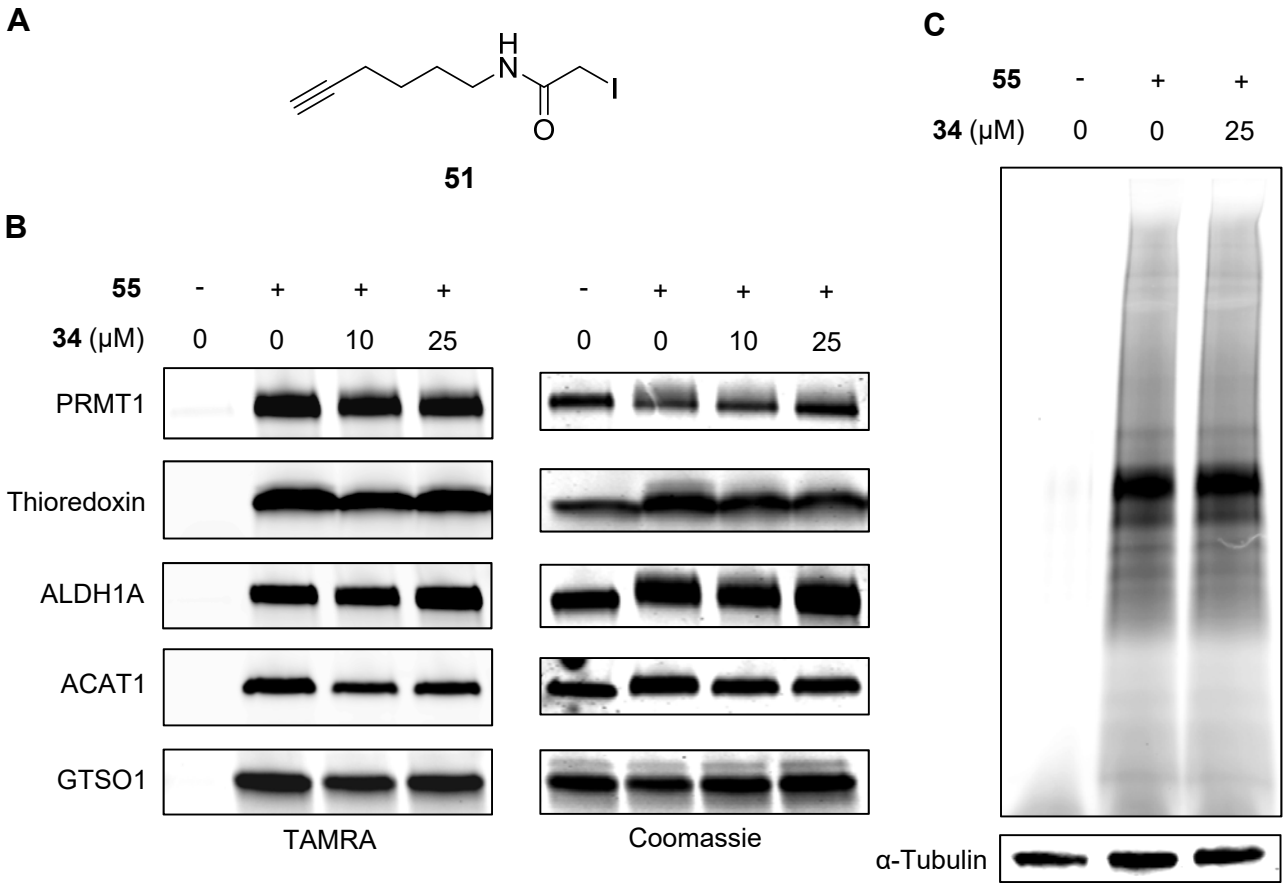


Figure S10. Compound **34** does not react nonspecifically with proteins containing hyper-reactive cysteine residues. (A) Structure of iodoacetamide probe (**51**) used to label reactive cysteine residues. (B) Purified proteins known to contain hyper-reactive cysteine residues were incubated with iodoacetamide probe **51** (25 μM) and compound **34** (0, 10, 25 μM). Reactions were then subjected to copper-catalyzed azide alkyne cycloaddition (CuAAC) with azido-TAMRA and visualized by in-gel fluorescence. (C) Neu7 astrocytes were treated with compound **34** (0, 25 μM) for 24 h prior to treatment with iodoacetamide probe **51** (25 μM). The soluble fraction of lysed cells was subjected to CuAAC with azido-TAMRA and visualized by in-gel fluorescence. Tubulin levels (assessed by immunoblotting) or protein levels (assessed by Coomassie staining) are used to show approximately equal protein loading in each lane.

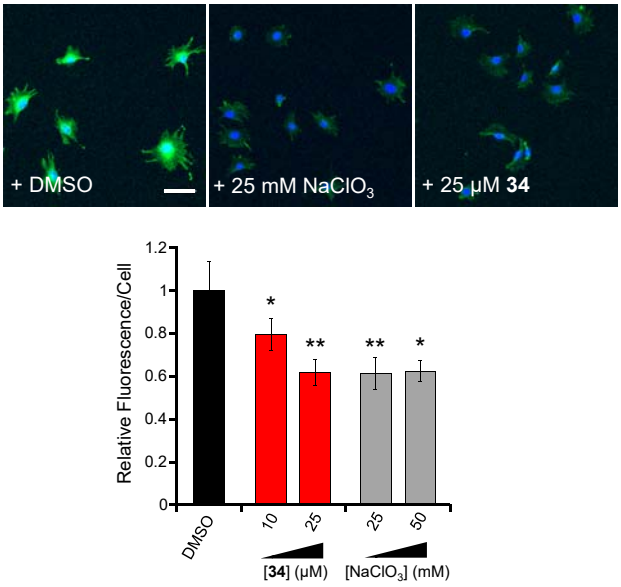


Figure S11. Treatment of NIH3T3 cells with **34** leads to a significant, dose-dependent decrease in CS-E expression. NIH3T3 fibroblasts were grown in the presence of DMSO or the indicated inhibitors for 40 h and co-stained with a CS-E-specific antibody (green) and DAPI (blue). Quantification of the average fluorescence from anti-CS-E immunostaining under each condition. Values represent mean ± S.E.M. from triplicate experiments. *n* = 20-100 cells per experiment. **P* < 0.05, ***P* < 0.01. (Scale bar: 100 μm).

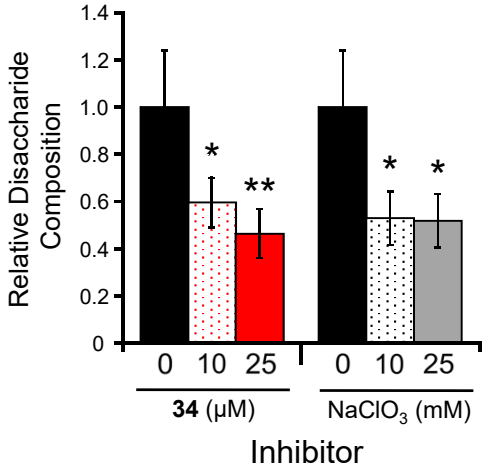


Figure S12. Relative CS-C disaccharide composition of CSPGs secreted from treated Neu7 astrocytes. CS disaccharide composition was analyzed by reverse-phase ion-pairing high performance liquid chromatography (RPIP-HPLC). CS-D was not detected. Values were normalized with respect to the disaccharide content on CSPGs from untreated astrocytes and are reported as the mean ± S.E.M. for triplicate experiments. *$P < 0.05$, **$P < 0.01$.

2. Supporting Tables

Table S1. Inhibition Kinetic Parameters for Chst15[a]

Varied Substrate	34 (μM)	k_{cat} (sec^{-1})	$K_{m,app}$ (μM)	K_i (μM)	K_i'(μM)
PAPS	0	0.126	29.49	0.77	-
	1	0.125	35.86		
	5	0.114	124.57		
	10	0.121	263.12		
Varied Substrate	**34 (μM)**	**k_{cat} (sec^{-1})**	**$K_{m,app}$ (mM)**	**K_i (mM)**	**K_i'(mM)**
CS-A	0	0.081	0.96	1.43	2.45
	1	0.078	1.12		
	5	0.028	1.40		
	10	0.018	1.49		

[a]Kinetic parameters were determined under pseudo first-order conditions by measuring the initial reaction velocities at varying PAPS or CS-A concentrations in the presence of **34** (0, 1, 5, and 10 μM). k_{cat} and K_m values were determined from Lineweaver-Burk plots. K_i and K_i' values were derived from secondary plots of K_m/V versus [**34**] or $1/V$ versus [**34**], respectively.

Table S2. Percent Disaccharide Composition of CSPGs from Neu7 Astrocytes Treated with the Indicated Inhibitors[a]

Disaccharide	No Treatment	10 μM 34	25 μM 34	10 mM $NaClO_3$	25 mM $NaClO_3$
uCS	38.8 ± 4.2	48.7 ± 7.4	63.2 ± 6.6	49.4 ± 6.1	54.2 ± 6.1
CS-A	37.1 ± 3.1	33.8 ± 6.4	27.2 ± 6.3	33.3 ± 4.9	31.4 ± 4.5
CS-C	13.0 ± 3.1	7.9 ± 1.4	7.7 ± 0.2	6.9 ± 1.5	6.7 ± 2.2
CS-D	n.d.	n.d.	n.d.	n.d.	n.d.
CS-E	11.2 ± 0.7	9.7 ± 0.6	3.6 ± 1.2	10.4 ± 1.0	7.7 ± 1.2

[a]Percent disaccharide composition from secreted CSPGs were determined by RPIP-HPLC after AMAC conjugation (428 nm) from three independent experiments. n.d. = not detected.

Table S3. *In Vitro* Microsomal Stability of **19** and **34**

	CL_{int} (μL/min/mg)	
Microsome Species	**19**	**34**
Human	<6.9	<6.9
Mouse	<6.9	<6.9
Rat	<6.9	<6.9

Table S4. Pharmacokinetic Parameters of **34** in Rats[a]

Route	Dose (mg/kg)	$AUC_{0-\infty}$ (h·nM)	CL (mL/min/kg)	V_{ss} (L/kg)	C_{max} (nM)	T_{max} (h)	$T_{1/2}$ (h)
IV	3.0	5486	21	0.97	11847	0.03	1.6

[a]IV = intravenous injection, AUC = area under the curve, CL = plasma clearance, V_{ss} = volume of distribution, C_{max} = maximum plasma concentration, T_{max} = time of maximum plasma concentration, $T_{1/2}$ = half life

3. Experimental Methods

3-1 Materials and Methods

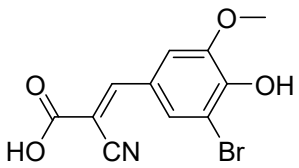
All reactions were carried out under an argon atmosphere unless otherwise specified. Reagents were purchased from Sigma-Aldrich and used without purification unless otherwise specified. ^{1}H NMR spectra were recorded with a Varian Inova 500 (500 MHz) and are reported relative to residual solvent peaks. ^{13}C NMR spectra were recorded with Varian Inova 500 (125 MHz) or Bruker AVANCE AV400 (100 MHz) and are reported relative to residual solvent peaks. ROESY NMR spectra were recorded with a Varian Inova 600 (600 MHz) spectrometer. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm) (multiplicity, coupling constant (Hz), integration, assignment). Multiplicities are reported as follows: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. Data for ^{13}C NMR are reported in terms of chemical shifts (δ ppm). High-resolution mass spectra (HRMS) were acquired using an Agilent 6200 Series TOF with and Agilent G1978A Multimode source using mixed electrospray ionization/atmospheric pressure chemical ionization (MM: ESI-APCI). High-performance liquid chromatography (HPLC) experiments were carried out using an Agilent 1100 Series HPLC.

Commercially available lead compounds were obtained from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), or Enamine LLC (Monmouth Junction, NJ).

Carbazole assays were performed as previously reported.[1]

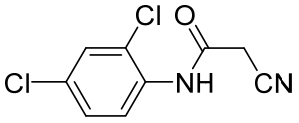
3-2. Synthetic Procedures

(*E*)-3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-2-cyanoacrylic acid (21)



Ethyl 3-(3-bromo-4-hydroxy-5-methoxyphenyl)-2-cyanoacyrlate (**46**, 0.10 g, 0.31 mmol) was dissolved into 4 mL of THF, and 2 N NaOH (10 mL) was added. The mixture was stirred at room temperature for 15 h. After 15 h, the reaction was neutralized with concentrated HCl. The reaction mixture was then extracted with EtOAc (3 x 10 mL), and the organic layer was washed with brine (10 mL), dried over $MgSO_4$, and concentrated *in vacuo*. The resulting crude solid was redissolved into 10:1 DCM/EtOAc and purified by silica flash chromatography using 10:1 DCM/EtOAc and 0.1% AcOH. A yellow solid was obtained after concentration *in vacuo* (0.071 g, 77%). R_f 0.3 (9:1 DCM/MeOH). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.21 (s, 1 H), 7.93 (s, 2 H), 7.77 (s, 1 H), 3.88 (s, 3H), 3.12 (s, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 165.43, 163.86, 151.12, 148.37, 128.51, 123.15, 117.65, 116.11, 112.45, 109.96, 56.16. HRMS: $[M-H]^-$ calculated: 295.9564, found: 295.9578.

2-Cyano-*N*-(2,4-dichlorophenyl)acetamide (23)



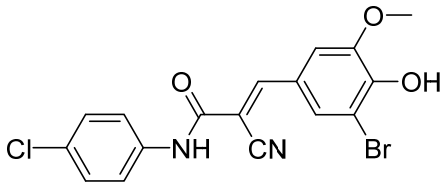
Cyanoacetic acid (0.26 g, 3.1 mmol) was dissolved in 10 mL of THF at room temperature. A solution of 2,4-dichloroaniline (0.50 g, 3.1 mmol) in 5 mL of THF was added dropwise at room temperature. After 5 min, a solution of *N*,*N*'-diisopropylcarbodiimide (DIC, 0.48 mL, 3.1 mmol) in 5 mL of THF was added dropwise. The reaction was refluxed under argon for 1 h. The mixture was allowed to return to room temperature, filtered, and the filtrate was concentrated *in vacuo*. The crude solid was redissolved into EtOAc (20 mL), and the organic layer was extracted with 6 N HCl (20 mL x 3) and saturated $NaHCO_3$ (20 mL x 3) successively. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The solid was recrystallized in DCM to afford a white solid (0.32 g,

45%). R_f 0.2 (8:2 Hex/EtOAc). ^{1}H NMR (500 MHz, CD_3OD) δ 8.25 (d, *J* = 8.9 Hz, 1 H), 8.21 (br, 1 H), 7.44 (d, *J* = 2.4 Hz, 1 H), 7.30 (dd, *J* = 2.4, 8.9 Hz, 1 H), 3.62 (s, 2 H); ^{13}C NMR (125 MHz, d_6-DMSO $_3$) δ 159.23, 132.46, 131.18, 129.47, 128.51, 124.68, 123.00, 114.20, 27.47. HRMS: $[M-H]^-$ calculated: 226.9784, found: 226.9792.

General Procedure for Amide Coupling:

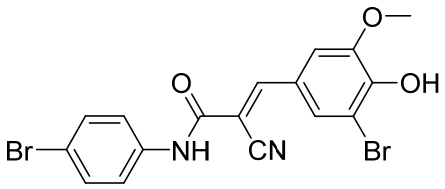
(*E*)-3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-2-cyanoacrylic acid (**21**, 0.016 g, 0.053 mmol) and an aniline-derivative (0.53 mmol, 10 eq) were dissolved into 0.5 mL of THF. DIC (8.2 µL, 1 eq) was added slowly, and the solution was stirred at room temperature for 2.5 h. Afterwards, the reaction mixture was diluted with 10 mL of DCM, and the organic layer was extracted sequentially with 6 N HCl (3 x 10 mL), saturated $NaHCO_3$ (3 x 10 mL), and brine (1 x 10 mL). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The resulting crude solid was redissolved into DCM with 0.1% AcOH and purified by silica flash chromatography using DCM and 0.1% AcOH (R_f 0.8 (100:0.01 DCM/AcOH)). A colored solid was obtained after concentration *in vacuo.*

(*E*)-3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-*N*-(4-chlorophenyl)-2-cyanoacrylamide (26)



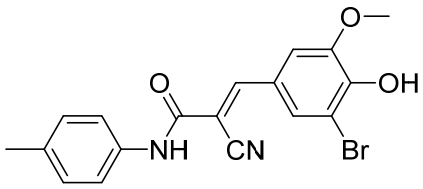
4-Chloroaniline (0.29 g, 1.7 mmol, 10 eq) was used in the same previous amide coupling reaction conditions (5.0 mg, 23%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.82 (br, 1 H), 10.41 (s, 1 H), 8.15 (s, 1 H), 7.85 (d, *J* = 1.9 Hz, 1 H), 7.70 (m, 3 H), 7.43 (m, 2 H), 3.89 (s, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.83, 149.95, 148.31, 148.23, 137.31, 128.68, 128.15, 127.94, 123.82, 122.11, 116.67, 112.30, 109.63, 103.76, 56.30. HRMS: $[M-H]^-$ calculated: 404.9647, found: 404.9661.

(*E*)-3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-*N*-(4-bromophenyl)-2-cyanoacrylamide (27)



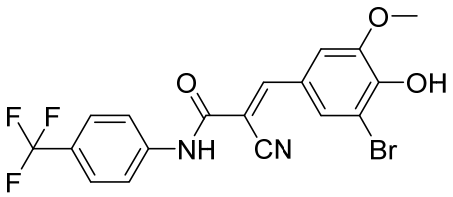
4-Bromoaniline (0.29 g, 1.7 mmol, 10 eq) was used in the same previous amide coupling reaction conditions (0.020 g, 26%). ^{1}H NMR (500 MHz, CD_3CN) δ 8.58 (s, 1 H), 8.10 (s, 1 H), 7.78 (d, *J* = 2.0 Hz, 1 H), 7.70 (d, *J* = 2.0 Hz, 1 H), 7.59 (m, 2 H), 7.52 (m, 2 H), 3.93 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.86, 149.70, 149.43, 148.38, 137.69, 131.41, 128.42, 122.93, 122.38, 116.71, 115.86, 112.20, 109.88, 102.62, 56.17. HRMS: $[M-H]^-$ calculated: 448.9142, found: 448.9157.

(*E*)-3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-2-cyano-*N*-(p-tolyl)acrylamide (28)



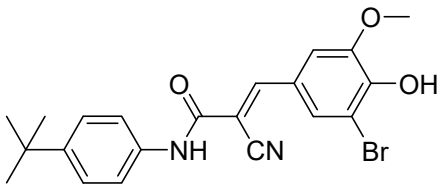
p-Toluidine (0.18 g, 1.7 mmol, 10 eq) was used in the same previous amide coupling reaction conditions (0.023 g, 36%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.03 (s, 1 H), 7.73 (s, 2 H), 7.49 (m, 2 H), 7.17 (m, 2 H), 3.91 (s, 3 H), 2.33 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 161.01, 152.51, 149.13, 148.86, 135.92, 132.91, 129.31, 128.92, 120.70, 120.46, 117.53, 111.83, 110.74, 99.88, 55.96, 20.37. HRMS: $[M-H]^-$ calculated: 385.0193, found: 385.0208.

(*E*)-3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-2-cyano-*N*-(4-(trifluoromethyl)phenyl)acrylamide (29)



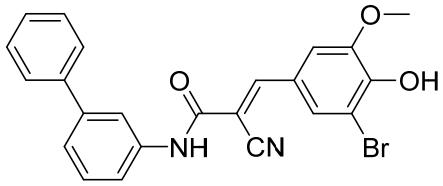
4-(Trifluoromethyl)aniline (0.21 mL, 1.7 mmol, 10 eq) was used in the same previous amide coupling reaction conditions (0.033 g, 45%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.61 (s, 1 H), 8.19 (s, 1 H), 7.90 (d, *J* = 8.5 Hz, 2 H), 7.86 (d, *J* = 1.9 Hz, 1 H), 7.74 (d, *J* = 8.7 Hz, 2 H), 7.72 (d, *J* = 2.0 Hz, 2 H), 3.90 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.76, 161.14, 150.12, 148.58, 148.25, 141.90, 128.24, 125.89, 125.86, 123.55, 120.35, 116.46, 112.31, 109.63, 103.43, 56.24, 20.89. HRMS: $[M-H]^-$ calculated: 438.9911, found: 438.9950.

(*E*)-3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-*N*-(4-(*tert*-butyl)phenyl)-2-cyanoacrylamide (30)



4-*tert*-Butylaniline (0.27 mL, 1.68 mmol, 10 eq) was used in the same previous amide coupling reaction conditions (0.040 g, 56%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.14 (s, 1 H), 8.10 (s, 1 H), 7.81 (d, *J* = 1.9 Hz, 1 H), 7.69 (d, *J* = 2.0 Hz, 1 H), 7.54 (m, 2 H), 7.33 (m, 2 H), 3.86 (s, 3 H), 1.23 (s, 9 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 149.36, 148.19, 148.08, 146.60, 135.60, 127.90, 125.19, 123.86, 120.31, 116.62, 112.28, 109.53, 104.10, 56.24, 33.98, 31.06. HRMS: $[M-H]^-$ calculated: 427.0663, found: 427.0688.

(*E*)-*N*-([1,1'-Biphenyl]-3-yl)-3-(3-bromo-4-hydroxy-5-methoxyphenyl)-2-cyanoacrylamide (33)

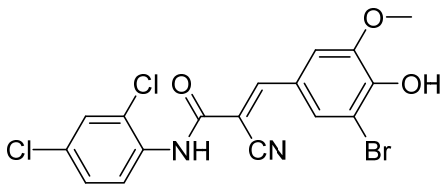


3-Aminobiphenyl (0.14 g, 0.84 mmol, 10 eq) was used in the same previous amide coupling reaction conditions (0.017 g, 44%). ^{1}H NMR (500 MHz, DMSO-d_6) 10.37 (s, 1 H), 8.19 (s, 1 H), 7.98 (t, *J* = 1.8 Hz, 1 H), 7.87 (d, *J* = 2.0 Hz, 1 H), 7.73 (d, *J* = 2.1 Hz, 1 H), 7.70 (dt, *J* = 1.9, 7.5 Hz, 1 H), 7.65 (m, 2 H), 7.50 (m, 2 H), 7.46 (m, 2 H), 7.40 (m, 1 H), 3.90 (s, 3 H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.27, 150.28, 148.75, 148.69, 141.17, 140.36, 139.36, 129.85, 129.49, 128.60, 128.15, 127.09, 124.30, 123.07, 119.99, 119.32, 117.21, 112.71, 110.10, 104.35, 56.75. HRMS: $[M-H]^-$ calculated: 447.0350, found: 447.0253.

General Procedure for Aldol Reaction:

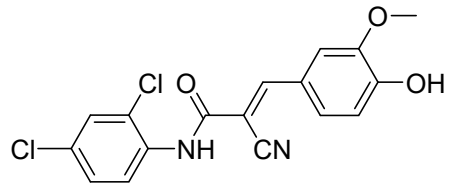
2-Cyano-*N*-(2,4-dichlorophenyl)acetamide (**23**, 0.24 g, 1.1 mmol) and benzaldehyde-derivative (1.0 mmol) was added to 5 mL of toluene. While stirring this mixture, piperidine (0.016 mL, 0.16 eq) and glacial acetic acid (0.074 mL, 1.3 eq) were added. The solution was stirred and heated to reflux for 3 h. After 3 h and cooling to room temperature, the precipitate was filtered, collected, and rinsed with ice-cold methanol to yield a colored solid.

(*E*)-3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-2-cyano-*N*-(2,4-dichlorophenyl)acrylamide (34)



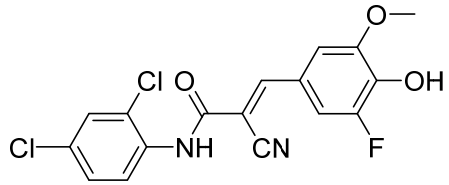
5-Bromovanillin (0.23 g, 1.0 mmol) was used in the same previous aldol reaction conditions (0.41 g, 93%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.88 (s, 1 H), 10.01 (s, 1 H), 8.22 (s, 1 H), 7.90 (d, *J* = 2.0 Hz, 1 H), 7.76 (d, *J* = 2.4 Hz, 1 H), 7.73 (d, *J* = 2.0 Hz, 1 H), 7.66 (d, *J* = 8.6 Hz, 1 H), 7.49 (dd, *J* = 2.4, 8.6 Hz, 1 H), 3.89 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.59, 150.95, 148.63, 148.24, 133.64, 131.02, 129.84, 129.17, 128.57, 128.34, 127.85, 123.70, 116.75, 112.61, 109.71, 102.32, 56.33. HRMS: $[M-H]^-$ calculated: 438.9257, found: 438.9258.

(*E*)-2-Cyano-*N*-(2,4-dichlorophenyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (37)



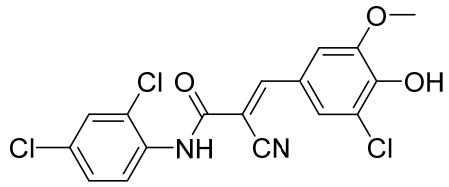
Vanillin (0.032 g, 0.21 mmol) was used in the same previous aldol reaction conditions (0.067 g, 89%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.40 (s, 1 H, ArO*H*), 9.92 (s, 1 H), 8.23 (s, 1 H), 7.73 (m, 2 H), 7.67 (d, *J* = 8.6 Hz, 1 H), 7.57 (d, *J* = 2.1, 8.4 Hz, 1 H) 7.48 (dd, J = 2.4, 8.7 Hz, 1 H), 6.98 (d, *J* = 8.3 Hz, 1 H), 3.86 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.78, 152.06, 152.00, 147.76, 133.64, 130.67, 129.45, 128.96, 128.17, 127.66, 126.29, 123.00, 117.00, 115.99, 113.67, 100.45, 55.58. HRMS: $[M-H]^-$ calculated: 361.0152, found: 361.0172.

(*E*)-2-Cyano-*N*-(2,4-dichlorophenyl)-3-(3-fluoro-4-hydroxy-5-methoxyphenyl)acrylamide (38)



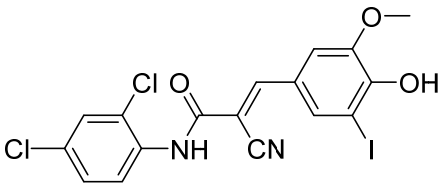
3-Fluoro-4-hydroxy-5-methoxybenzaldehyde (AstaTech Inc., 0.035 g, 0.21 mmol) was used in the same previous aldol reaction conditions (0.068 g, 86%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.94 (s, 1 H), 8.20 (s, 1 H), 7.74 (d, *J* = 2.4 Hz, 1 H), 7.68 (d, *J* = 8.7 Hz, 1 H), 7.58 (m, 2 H) 7.48 (dd, *J* = 2.4, 8.7 Hz, 1 H), 3.86 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 161.16, 152.56, 151.64, 150.66, 150.24, 150.19, 134.18, 131.20, 129.89, 129.53, 128.60, 128.25, 117.49, 112.29, 112.13, 111.19, 56.76. HRMS: $[M-H]^-$ calculated: 379.0058, found: 379.0074.

(*E*)-3-(3-Chloro-4-hydroxy-5-methoxyphenyl)-2-cyano-*N*-(2,4-dicholorophenyl)acrylamide (39)



3-Chloro-4-hydroxy-5-methoxybenzaldehyde (0.078 g, 0.42 mmol) was used in the same previous aldol reaction conditions (0.15 g, 91%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.83 (s, 1 H), 9.95 (s, 1 H), 8.20 (s, 1 H), 7.75 (d, *J* = 2.2 Hz, 2 H), 7.68 (d, *J* = 8.6 Hz, 2 H), 7.49 (dd, *J* = 2.4, 8.6 Hz, 1 H), 3.88 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 161.00, 151.47, 149.09, 148.22, 134.08, 131.40, 130.18, 129.57, 128.91, 128.26, 125.82, 123.29, 120.86, 117.18, 112.67, 102.73, 56.77. HRMS: $[M-H]^-$ calculated: 394.9762, found: 394.9780.

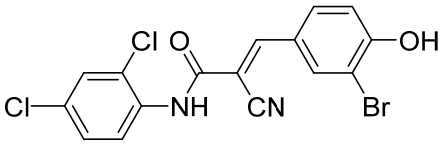
(*E*)-2-Cyano-*N*-(2,4-dichlorophenyl)-3-(4-hydroxy-3-iodo-5-methoxyphenyl)acrylamide (40)



5-Iodovanillin (0.058g, 0.21 mmol) was used in the same previous aldol reaction conditions (0.072 g, 71%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.87 (s, 1 H), 9.90 (br, 1 H), 8.16 (s, 1 H), 8.04 (s, 1 H), 7.74 (d, *J* = 2.4 Hz, 1 H),

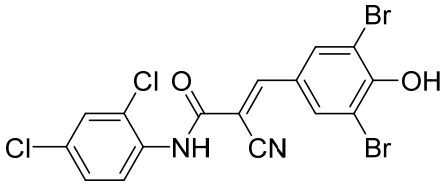
7.72 (s, 1 H), 7.68 (d, J = 8.7 Hz, 1 H), 7.48 (dd, J = 2.4, 8.6 Hz, 1 H), 3.86 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.54, 151.21, 150.53, 146.81, 134.42, 133.56, 130.78, 129.52, 129.00, 127.69, 124.60, 116.67, 113.06, 101.77, 84.94, 56.11. HRMS: $[M-H]^-$ calculated: 486.9119, found: 486.9132.

(*E*)-3-(3-Bromo-4-hydroxyphenyl)-2-cyano-*N*-(2,4-dichlorophenyl)acrylamide (41)



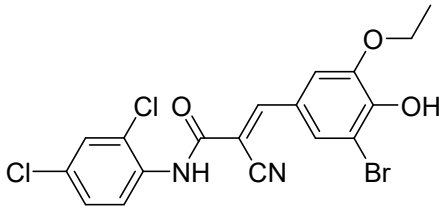
3-Bromo-4-hydroxybenzaldehyde (0.042 g, 0.21 mmol) was used in the same previous aldol reaction conditions (0.044 g, 52%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.96 (s, 1 H), 8.26 (d, J = 2.2 Hz, 1 H), 8.19 (s, 1 H), 7.93 (dd, J = 2.3, 8.7 Hz, 1 H), 7.74 (d, J = 2.4 Hz, 1 H), 7.66 (d, J = 8.7 Hz, 1 H) 7.48 (dd, J = 2.4, 8.6 Hz, 1 H), 7.10 (d, J = 8.6 Hz, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 161.11, 159.51, 159.48, 150.85, 136.11, 134.06, 132.45, 131.32, 130.14, 129.54, 128.90, 128.23, 124.24, 117.40, 117.13, 110.86. HRMS: $[M-H]^-$ calculated: 408.9152, found: 408.9166.

(*E*)-2-Cyano-3-(3,5-dibromo-4-hydroxyphenyl)-*N*-(2,4-dichlorophenyl)acrylamide (42)



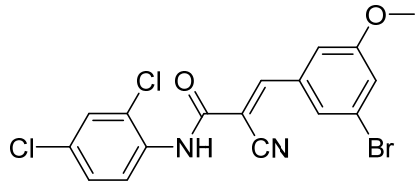
3,5-Dibromo-4-hydroxybenzaldehyde (0.058 g, 0.21 mmol) was used in the same previous aldol reaction conditions (0.084 g, 82%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.03 (s, 1 H), 8.27 (s, 2 H), 8.20 (s, 1 H), 7.75 (d, J = 2.4 Hz, 1 H), 7.67 (d, J = 8.7 Hz, 1 H), 7.49 (dd, J = 2.4, 8.6 Hz, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 159.98, 154.88, 148.72, 134.22, 133.24, 130.69, 129.32, 128.83, 128.05, 127.52, 125.08, 115.92, 111.84, 103.44. HRMS: $[M-H]^-$ calculated: 486.8257, found: 486.8264.

(*E*)-3-(3-Bromo-5-ethoxy-4-hydroxyphenyl)-2-cyano-*N*-(2,4-dichlorophenyl)acrylamide (43)



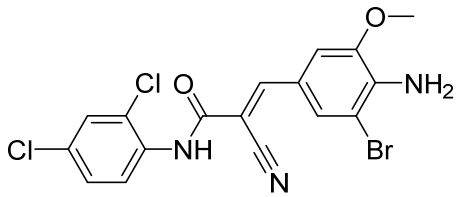
3-Bromo-5-ethoxy-4-hydroxybenzaldehyde (Alfa Aesar, 0.051 g, 0.21 mmol) was used in the same previous aldol reaction conditions (0.081 g, 85%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.62 (br, 1 H), 10.00 (s, 1 H), 8.21 (s, 1 H), 7.87 (d, *J* = 1.7 Hz, 1 H), 7.75 (d, *J* = 2.3 Hz, 1 H), 7.72 (d, *J* = 1.7 Hz, 1 H), 7.65 (d, *J* = 8.7 Hz, 1 H), 7.49 (dd, *J* = 2.3, 8.6 Hz, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.93, 151.34, 149.26, 147.81, 134.00, 131.32, 129.86, 129.49, 128.80, 128.56, 128.21, 124.25, 117.08, 113.84, 110.16, 102.89, 65.31, 14.79. HRMS: $[M-H]^-$ calculated: 452.9414, found: 452.9429.

(*E*)-3-(3-Bromo-5-methoxyphenyl)-2-cyano-*N*-(2,4-dichlorophenyl)acrylamide (44)



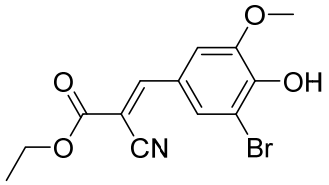
3-Bromo-5-methoxybenzaldehyde (AstaTech Inc., 0.10 g, 0.47 mmol) was used in the same previous aldol reaction conditions (0.14 g, 70%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.23 (s, 1 H), 8.30 (s, 1 H), 7.76 (d, *J* = 1.6 Hz, 1 H), 7.75 (d, *J* = 2.4 Hz, 1 H), 7.63 (d, *J* = 8.6 Hz, 1 H), 7.58 (t, *J* = 1.7 Hz, 1 H), 7.49 (dd, *J* = 2.4, 8.6 Hz, 1 H), 7.43 (t, *J* = 1.6 Hz, 1 H), 3.84 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.76, 160.45, 150.82, 134.99, 133.80, 131.72, 130.45, 129.66, 129.22, 128.32, 125.07, 123.25, 121.03, 116.15, 115.42, 108.11, 56.39. HRMS: $[M-H]^-$ calculated: 422.9308, found: 422.9204.

(*E*)-3-(4-Amino-3-bromo-5-methoxyphenyl)-2-cyano-*N*-(2,4-dichlorophenyl)acrylamide (45)



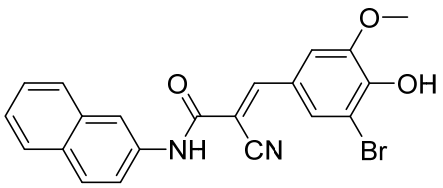
4-Amino-3-bromo-5-methoxybenzaldehyde (**58**, 0.048 g, 0.21 mmol) was used in the same previous aldol reaction conditions (0.081 g, 88%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.77 (s, 1 H), 8.13 (s, 1 H), 7.84 (d, *J* = 1.7 Hz, 1 H), 7.73 (d, *J* = 2.4 Hz, 1 H), 7.68 (d, *J* = 8.6 Hz, 1 H), 7.63 (d, *J* = 1.5 Hz, 1 H), 7.47 (dd, *J* = 2.4, 8.7 Hz, 1 H), 6.25 (s, 1 H), 3.87 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 161.45, 151.62, 146.42, 141.74, 134.25, 131.09, 130.16, 129.83, 129.47, 128.55, 128.19, 120.31, 117.93, 110.97, 105.80, 98.83, 56.49. HRMS: $[M-H]^-$ calculated: 437.9417, found: 437.9300.

(*E*)-Ethyl 3-(3-bromo-4-hydroxy-5-methoxyphenyl)-2-cyanoacyrlate (46)



Ethyl cyanoacetate (1.1 mL, 11 mmol) and 5-bromovanillin (2.3 g, 11 mmol) were dissolved into 10 mL of toluene at room temperature. Piperidine (0.16 mL, 1.6 mmol) and glacial acetic acid (0.74 mL, 13 mmol) were then added to the previous mixture. The mixture was refluxed for 3 h and allowed to cool to room temperature. The precipitate was filtered, collected, and rinsed with ice-cold methanol to afford a yellow solid (3.0 g, 91%). R_f 0.2 (1:1 Hex/EtOAc). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.16 (s, 1 H), 7.79 (s, 2 H), 4.35 (q, *J* = 7.1 Hz, 2 H), 3.94 (s, 3 H), 1.37 (t, *J* = 7.2 Hz, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.62, 154.03, 149.62, 148.64, 129.49, 123.90, 116.61, 113.56, 110.15, 99.54, 62.57, 56.78, 14.47. HRMS: $[M-H]^-$ calculated: 323.9877, found: 323.9883.

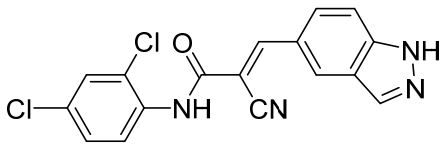
(*E*)-3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-2-cyano-*N*-(naphthalen-2-yl)acrylamide (48)



2-Naphthylamine (0.12 g, 0.84 mmol, 10 eq) was used in the same previous amide coupling reaction conditions (6.0 mg, 17%). ^{1}H NMR (500 MHz, DMSO-d_6) 10.81 (br, 1 H), 10.49 (s, 1 H), 8.30 (d, *J* = 2.0 Hz, 1 H), 8.20 (s,

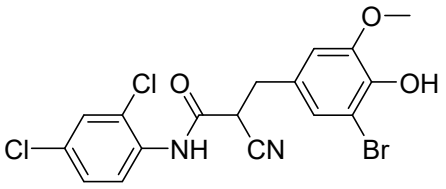
1 H), 7.91 (d, *J* = 9.0 Hz, 1 H), 7.87 (m, 3 H), 7.73 (m, 2 H), 7.47 (m, 2H), 3.90 (s, 3 H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.02, 149.75, 148.42, 148.28, 135.99, 133.20, 130.22, 128.38, 128.21, 127.53, 127.51, 126.56, 125.15, 123.80, 120.81, 117.03, 116.82, 112.25, 109.70, 103.90, 56.31. HRMS: $[M-H]^-$ calculated: 421.0193, found: 421.0121.

(*E*)-2-Cyano-*N*-(2,4-dichlorophenyl)-3-(1*H*-indazol-5-yl)acrylamide (49)



Indazole-5-carboxaldehyde (0.016 g, 0.11 mmol) was used in the same previous aldol reaction conditions (0.024 g, 62%). ^{1}H NMR (500 MHz, DMSO-d_6) 13.53 (s, 1 H), 10.07 (s, 1 H), 8.51 (s, 1 H), 8.45 (s, 1 H), 8.32 (s, 1 H), 8.09 (dd, *J* = 1.7, 8.9 Hz, 1 H), 7.75 (m, 2 H), 7.68 (d, *J* = 8.7 Hz, 1 H), 7.49 (dd, *J* = 2.4, 8.6 Hz, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 161.26, 153.41, 136.12, 134.11, 131.44, 130.32, 129.59, 129.09, 128.28, 127.32, 126.90, 124.72, 123.63, 117.32, 111.74, 109.99, 102.96. HRMS: $[M-H]^-$ calculated: 355.0159, found: 355.0169.

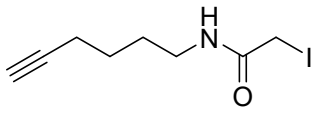
3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-2-cyano-*N*-(2,4-dichlorophenyl)propanamide (50)



Palladium on carbon (5.0 mg, 10% wt % loading) was added to a round bottom flask and purged with argon. (*E*)-3-(3-bromo-4-hydroxy-5-methoxyphenyl)-2-cyano-*N*-(2,4-dichlorophenyl)acrylamide (**34**, 0.050 g, 0.11 mmol) was dissolved into a minimal amount of dry acetone and added to the flask. The reaction was stirred under an atmosphere of H_2 (1.0 atm) for 18 h at room temperature. The mixture was then filtered through Celite and purified by preparative TLC using 4:6 EtOAc/hexanes (R_f 0.2) (5.0 mg, 10%). ^{1}H NMR (500 MHz, CD_3CN) δ 8.34 (s, 1 H), 7.87 (d, *J* = 8.8 Hz, 1 H), 7.53 (d, 1 H), 7.35 (dd, *J* = 2.4, 8.8 Hz, 1 H), 7.05 (d, J = 1.8 Hz, 1 H), 6.91 (d, *J* = 1.8 Hz, 1 H), 4.10 (t, J = 7.5 Hz, 1 H), 3.82 (s, 1 H), 3.20 (m, 2 H); ^{13}C NMR (100 MHz, CD_3CN) δ

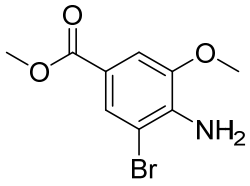
164.89, 148.86, 144.00, 133.81, 131.63, 130.11, 129.57, 128.66, 127.74, 126.52, 125.94, 112.78, 108.77, 57.09, 42.07, 36.41. HRMS: $[M-H]^-$ calculated: 440.9414, found: 440.9434.

N-(hex-5-yn-1-yl)-2-iodoacetamide (51)



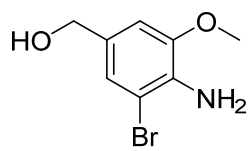
This compound was prepared as previously reported.[2] ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.12 (br, 1 H), 3.70 (s, 2 H), 3.30 (m, 2 H), 2.24 (td, *J* = 2.7, 6.7 Hz, 2 H), 1.97 (t, *J* = 2.7 Hz, 1 H), 1.62 (m, 6 H).

Methyl 4-amino-3-bromo-5-methoxybenzoate (56)



This compound was prepared as previously reported.[3] *N*-bromosuccinimide (0.49 g, 2.8 mmol) was added to an ice-cold solution of methyl 4-amino-3-methoxybenzoate (AstaTech Inc., 0.50 g, 2.8 mmol) in 3 mL of $CHCl_3$. After stirring at 4 °C for 4 h, the white precipitate was filtered. The filtrate was extracted with water (3 x 10 mL), dried over $MgSO_4$, and concentrated *in vacuo* to yield a white solid (0.71 g, 99%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.81 (d, *J* = 1.7 Hz, 1 H), 7.38 (d, *J* = 1.6 Hz, 1 H), 4.65 (s, 2 H), 3.91 (s, 3 H), 3.87 (s, 3 H); HRMS: $[M+H]^-$ calculated: 259.9917, found: 259.9916.

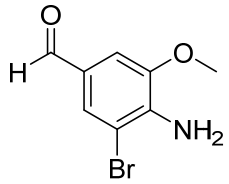
(4-Amino-3-bromo-5-methoxyphenyl)methanol (57)



$LiAlH_4$ (0.052 g, 1.4 mmol, 2 eq) was added to a round bottom flask under argon and placed into an ice bath. Dry diethyl ether (1.5 mL) was slowly added to the flask with stirring. A solution of the methyl 4-amino-3-bromo-5-

methoxybenzoate (**55**, 0.18 g, 0.69 mmol) in diethyl ether (1.5 mL) was added dropwise to the flask over 15 min. The ice bath was then removed and the reaction was allowed to stir at room temperature. After 2 h, the reaction was quenched by the slow addition of EtOAc (1.5 mL). The reaction mixture was then poured into water (9 mL) and extracted with three portions of EtOAc (15 mL). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. The resulting crude solid was redissolved into 4:6 EtOAc/Hex and purified by silica flash chromatography using 6:4 EtOAc/Hex to give a clear, yellow oil (0.13 g, 84%). R_f 0.6 (6:4 EtOAc/Hex). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.93 (d, J = 1.8 Hz, 1 H), 6.67 (d, J = 1.7 Hz, 1 H), 4.45 (s, 2 H), 4.14 (s, 2 H), 3.79 (s, 3 H), 2.92 (br, 1 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 147.62, 133.93, 131.56, 122.90, 108.40, 108.31, 64.73, 55.94. HRMS: $[M-H]^-$ calculated: 229.9822, found: 229.9821.

4-Amino-3-bromo-5-methoxybenzaldehyde (58)



4-Amino-3-bromo-5-methoxyphenyl)methanol (**56**, 0.13 g, 0.58 mmol) was dissolved into dry DMF (3.4 mL). MnO_2 (0.28 g, 3.2 mmol, 5.5 eq) was added, and the reaction mixture was stirred for 50 h at room temperature. The mixture was filtered through Celite, rinsed with EtOAc (10 mL), and extracted with water (3 x 50 mL). The resulting organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to give a tan solid (0.12 g, 88%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.64 (s, 1 H), 7.50 (d, J = 1.5 Hz, 1 H), 7.21 (s, J = 1.4 Hz, 1 H), 4.88 (s, 2 H), 3.90 (s, 3 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.34, 146.94, 141.20, 130.25, 127.24, 107.13, 106.31, 56.14. HRMS: $[M+H]^-$ calculated: 229.9811, found: 229.9812.

HPLC Purification. Crude compound was dissolved into the minimum amount of MeOH. Purification was achieved by using an Eclipse XDB-C18 column (9.4 x 250 mm, Agilent) at a flow rate of 5 mL/min using the following method: Linear gradient of 70:30 MeCN:H_2O to 88:12 MeCN:H_2O for 8 min, followed by 100%

MeCN for 2 min. Elution of the compound was monitored by absorbance at 254 nm. Collected fractions were pooled, flash frozen, and lyophilized.

3-3. Biological/Biochemical Procedures

Expression and Purification of hChst15. The luminal catalytic domain of human Chst15 (Ser^{99}-Thr^{561}) was cloned into pcDNA3.3 containing a C-terminal hexahistidine tag. The completed construct was transiently transfected into FreeStyle 293-F cells (ThermoFisher) using polyethylenimine and secreted into the media. After centrifugation of the collected media, the supernatant was passed through a pre-equilibrated HisTrap HP column (GE Healthcare) at 4 °C. The column was then washed with five column volumes of a buffer containing 25 mM Tris pH 7.5, 500 mM NaCl, 10 mM imidazole and eluted with twenty-five column volumes of a buffer containing 25 mM Tris pH 7.5, 500 mM NaCl, 500 mM imidazole. The protein was then concentrated using a 50-kDa MWCO spin filter, buffer exchanged into 100 mM Tris pH 7.6, and stored at -80 °C. Human Chst15 was used for high-throughput screening, and murine Chst15 was used for subsequent validation and characterization of the hits in biochemical and cell-based assays. Human and murine Chst15 share 93% sequence identity and completely identical sequences in known parts of the active site. Importantly, no appreciable differences between human and murine inhibition by the compounds were observed.

Expression and Purification of mChst15. The catalytic domain of mouse Chst15 (Ser^{151}-Thr^{561}) was cloned into the pMAL-c2X vector (New England BioLabs) using the BamHI and EcoRI sites to generate a maltose-binding protein (MBP)-Chst15 fusion protein. Expression of Chst15 was achieved in Origami B(DE3) cells (Novagen) harboring the pGro7 (TaKaRa) and pMAL-c2X-Chst15 constructs. The cells were grown in LB medium (1L) at 37 °C until they reached an OD_{600} of 0.8, after which they were moved to 22 °C. After 30 min, isopropyl β-*D*-1-thiogalactopyranoside (IPTG) and L-(+)-arabinose were added to the cells at final concentrations of 0.2 mM and 1 mg/mL, respectively, and the cells were then incubated for an additional 18 h at 22 °C. The pelleted cells were lysed in ice-cold buffer containing 20 mM Tris pH 7.5, 500 mM NaCl, 1 mM EDTA, and 1x Complete™ protease inhibitors (Roche) by sonication. After centrifugation, the clarified lysate was added to pre-equilibrated amylose resin beads (New England Biolabs) and incubated at 4 °C for 1 h with end-over-end rotation. The beads were washed 4 times with a buffer containing 20 mM Tris pH 7.5, 500 mM NaCl, and 1 mM EDTA and eluted with a

buffer containing 20 mM Tris pH 7.5, 500 mM NaCl, 1 mM EDTA, and 10 mM D-(+)-maltose. After SDS-PAGE analysis, the purified protein fractions were concentrated with a 50 kDa molecular weight cut-off (MWCO) spin filter (Millipore), buffer exchanged into 20 mM Tris pH 7.5, 500 mM NaCl, and 1 mM EDTA, and 50% glycerol, and stored at -20 °C.

Expression and Purification of Sult2b1a and Sult2b1b. BL21(DE3) cells (Novagen) harboring the pGEX-6P-3-Sult2b1a or pGEX-6P-3-Sult2b1b[4] constructs were kindly provided by Dr. Lars Pedersen (NIEHS/NIH). Cells were grown in LB medium (1L) at 37 °C until they reached an OD_{600} of 0.8, after which they were moved to 16 °C. After 30 min, IPTG (0.2 mM final concentration) was added to the cells, and the cells were incubated for an additional 18 h at 16 °C. The pelleted cells were lysed in ice-cold PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.3, 140 mM NaCl, 2.7 mM KCl, and 1x Complete™ protease inhibitors) by sonication. After centrifugation, the clarified lysate was added to pre-equilibrated glutathione Sepharose 4B resin beads (GE Healthcare) and incubated at 4 °C for 1 h with end-over-end rotation. The beads were then washed 4 times with a buffer containing ice-cold PBS and eluted with a buffer containing 50 mM Tris pH 8.0 and 10 mM reduced glutathione. After SDS-PAGE analysis, the purified protein fractions were concentrated using a 30 kDa MWCO spin filter, buffer exchanged into PBS and 50% glycerol, and stored at -20 °C.

Expression and Purification of Sult1c1, Chst11, and Ust. The catalytic domains of human Chst11 (Ser^{72}-Glu^{352}) and human Ust (Asn^{84}-Arg^{406}) were cloned into the pET32b vector (Novagen) using NcoI/EcoRI and NcoI/HindIII sites, respectively, to generate the thioredoxin-fusion proteins. Rabbit Sult1c1 was constructed as previously reported.[5] Expression of Chst11 and Ust was achieved in Origami B (DE3) cells (Novagen). Expression of Sult1c1 was achieved in BL21(DE3)RIL cells (Stratagene). The cells were grown in LB medium (1L) at 37 °C until they reached an OD_{600} of 0.8, after which they were moved to 22 °C. After 30 min, IPTG (0.2 mM final concentration) was added to the cells, and the cells were incubated for an additional 18 h at 22 °C. The pelleted cells were lysed in ice-cold buffer containing 25 mM Tris pH 7.5, 500 mM NaCl, 10 mM imidazole and 1x EDTA-free Complete™ protease inhibitors by sonication. After centrifugation, the clarified lysate was added

to pre-equilibrated Ni-NTA resin beads (Qiagen) and incubated at 4 °C for 1 hour with end-over-end rotation. The beads were then washed 3 times with a buffer containing 25 mM Tris pH 7.5, 500 mM NaCl, 10 mM imidazole, washed 1 time with 25 mM Tris pH 7.5, 500 mM NaCl, 50 mM imidazole, and eluted with a buffer containing 25 mM Tris pH 7.5, 500 mM NaCl, 250 mM imidazole. After SDS-PAGE analysis, the purified protein fractions were concentrated using a 30,000 Da MWCO spin filter, buffer exchanged into 25 mM Tris pH 7.5, 500 mM NaCl and 50% glycerol, and stored at -20 °C.

Enzymatic Synthesis and Purification of PAPS and ^{35}S-PAPS. Adenosine 5'triphosphate (ATP, 11.8 mM), $MgCl_2$ (11.8 mM), phosphoenolpyruvic acid (PEP, 24 mM), and $NaSO_4$ (118 mM) were dissolved into 137 µL of 50 mM Tris pH 8.0. For ^{35}S-PAPS, 25 µL of ^{35}S-$NaSO_4$ (10 mCi/mL stock, American Radiolabeled Chemicals Inc.) was added to the reaction. For cold PAPS, 25 µL of water was added instead. After, 1 µL each of pyruvate kinase (700 units/mL, Sigma-Aldrich), inorganic pyrophosphatase (250 units/mL, Sigma-Aldrich), ATP sulfurylase (2 units/mL, Sigma-Aldrich), and APS kinase[6] (5 mg/mL, generously donated from Dr. Andrew Fisher (UC-Davis)) were added to the reaction mixture. The reaction was allowed to incubate at 37 °C for 9 h, after which an additional 1 µL of the same four enzymes was added, and the reaction was allowed to proceed an additional 12 h at 37 °C. Purification of PAPS was achieved using a DEAE-cellulose (Whatman) column (2.5 mL resin) pre-equilibrated with water and eluted using a gradient of 0-500 mM NaCl. Fractions were lyophilized, reconstituted in 170 µL of 1 mM Tris pH 8.0, and analyzed with HPLC using a Zorbax-NH_2 column (4.6 mm x 150 mm, Agilent). The following method was applied: 100% water for 10 min, followed by a linear gradient of 0-100% 1 M KH_2PO_4 for 30 min, followed by 100% 1 M KH_2PO_4 for 15 min at a flow rate of 1 mL/min with UV (254 nM) detection. Fractions containing PAPS were stored at -80 °C.

1536-Well Plate High-Throughput Screening Protocol. For the full assay, 2 µL of human Chst15 (0.46 mg/mL) was added to each well in black 1536-well plates pre-plated with a small-molecule library (12.5 µM final concentration) containing 70,000 publicly-disclosed molecules. The mixture was incubated for 5 min with shaking and then 2 µL of a mixture containing 100 mM Tris pH 7.6, 1 mM β-mercaptoethanol, 5 mM 4-

methylumbelliferyl sulfate, 1 mM CS-A, 30 μM PAPS, and 0.1 mg/mL Sult1c1 was added to the plate. The plate was read as the 0 minute time point on a PheraStar Plate Reader with an excitation wavelength of 350 nm and emission wavelength of 460 nm. After incubating at 37 °C for 30 min, the plate was read again at the same wavelengths. For the counter-screen, Chst15 was omitted. Compounds were considered hits with >10% inhibition of Chst15 and <20% inhibition of Sult1c1.

Chst15 Radioisotope Labeling Assay. Chst15 activity was determined by incubating mouse Chst15 (30 μg) with 150 μg of CS-A (Sigma-Aldrich) and 30 μM of ^{35}S-PAPS in 150 μL of reaction buffer (100 mM Tris pH 7.6, 5 mM β-mercaptoethanol) for 30 min at 37 °C. For assaying the potency of inhibitors, 1.875 μL of inhibitor stock solutions in DMSO (0.25-16 mM) were pre-incubated with Chst15 alone for 1 h at room temperature prior to the addition of the substrates in reaction buffer. The samples were then subjected to DEAE-Sepharose chromatography (200 μL slurry per reaction) to purify the ^{35}S-labeled product. Samples were washed 3 times with 1 mL of a buffer containing 50 mM NaOAc pH 5.5, 150 mM NaCl, 6 M urea, 1 mM EDTA, and 0.01% Triton X-100, washed 2 times with 1 mL of a buffer containing 50 mM NaOAc pH 5.5, 250 mM NaCl, and 0.01% Triton X-100, and eluted with 1 mL of a buffer containing 50 mM NaOAc pH 5.5, 1 M NaCl, and 0.01% Triton X-100. The elution was mixed with 7 mL of Ecoscint XR scintillation fluid (National Diagnostics), and activity was quantified with a liquid scintillation counter (Beckman LS6500). IC_{50} values were determined by varying the inhibitor concentration and fit to a Michaelis-Menten model using Kaleidagraph software (version 4.1.2).

96-Well Plate Enzyme-Coupled, Fluorescent Assay. For the enzyme-coupled assay, the substrates PAPS and CS-A were added to a solution containing 100 mM Tris pH 7.6, 1 mM β-mercaptoethanol, and 5 mM 4-methylumbelliferyl sulfate at concentrations of 30 μM and 1 mM, respectively, to a total volume of 74 μL. 5 μL of Sult1c1 (2 mg/mL) was added, and the mixture was incubated at 37 °C for 1 h. Mouse Chst15 (20 μL, 1 mg/mL), pre-incubated with 1 μL of DMSO or inhibitor stock (0.3-20 mM) for 30 min, was then added to the mixture to initiate the reaction. The 96-well plate was read using either a Victor3 Plate Reader (Perkin Elmer) or Flexstation 3 (Molecular Devices) at excitation and emission wavelengths of 360 nm and 449 nm, respectively.

For the counter-screen, the substrates PAP (Sigma-Aldrich) and CS-A were added to a solution containing 100 mM Tris pH 7.6, 1 mM β-mercaptoethanol, and 5 mM 4-methylumbelliferyl sulfate at final concentrations of 30 μM and 1 mM, respectively, to a total volume of 75 μL. The reaction was initiated by adding a mixture of 5 μL of Sult1c1, 19 μL of 100 mM Tris pH 7.6, and 1 μL of DMSO or inhibitor stock (1.25 mM, 12.5 μM final concentration) that had been pre-incubated at room temperature for 30 min.

For enzyme and substrate specificity determinations, Chst15 was replaced with Chst11, Ust, Hs3st1, Sult1e1, Sult2b1a, or Sult2b1b. Likewise, CS-A was replaced with unsulfated chondroitin (Seikagaku), CS-C (Sigma-Aldrich), heparan sulfate (Neoparin), β-estradiol (Sigma-Aldrich), 5-pregnen-3β-ol-20-one (Sigma-Aldrich), or cholesterol (Sigma-Aldrich) and used with their respective enzymes. IC_{50} values were determined by varying the inhibitor concentration and fit to a Michaelis-Menten model using Kaleidagraph software.

For kinetic analysis of Chst15, reactions were performed in triplicate with varying substrate PAPS (10 μM to 200 μM, fixed 10 mM CS-A) or CS-A (0.5 mM to 10 mM, fixed 200 μM PAPS) concentrations. Product formation was monitored for 10 min, and kinetic parameters were determined from Lineweaver-Burk, K_m/V versus [inhibitor], and 1/V versus [inhibitor] plots.

Enzyme-Coupled, Fluorescence Assay Substrate Optimization with Fluorimetry. The substrates PAPS and CS-A were added to a solution containing 100 mM Tris pH 7.6, 1 mM β-mercaptoethanol, and 5 mM 4-methylumbelliferyl sulfate at concentrations of 30 μM and 1 mM, respectively, to a total volume of 75 μL. 5 μL of Sult1c1 (2 mg/mL) was added, and the mixture was incubated at 37 °C for 1 h. 20 μL of mouse Chst15 (1 mg/mL) was then added to the mixture to initiate the reaction. The reaction mixture was added to a sub-micro fluorometer cell (Starna Cells), and product formation was monitored for 10 min at excitation and emission wavelengths of 360 nm and 449 nm, respectively, using a steady-state fluorimeter (Jobin Yvon Spex Fluorolog-3-11). The K_m values of the substrates were determined by varying substrate PAPS (10 μM to 200 μM, fixed 10 mM CS-A) or CS-A (0.5 mM to 10 mM, fixed 200 μM PAPS) concentrations, and nonlinear regression analysis of initial velocity versus substrate concentration using Kaleidagraph software.

UV-Vis Spectroscopy Assay for Reversible Covalent Inhibition. Compound **34** in 1% DMSO/PBS (100 μM) was incubated with or without BME (50 mM) for 24 h at room temperature. After incubation, UV-visible absorption spectra were recorded by performing a wavelength scan using a UVIKON XS spectrophotometer. Each reaction was then diluted 10-fold into PBS or PBS with BME (50 mM), and absorption spectra were recorded again.

^{1}H NMR Spectroscopy Assay for Reversible Covalent Inhibition. To a solution of compound **34** (1 mg, 2.26 μmol) in 0.75 mL DMSO-d_6 was added deuterated PBS (0.25 mL) with or without 100 mM BME. The reaction mixture was incubated for 10 min at room temperature and then analyzed by ^{1}H NMR. The reaction containing BME was then diluted 2-fold by addition of a 3:1 *v/v* DMSO-d_6:deuterated PBS solution (1 mL) and analyzed again after 10 min at room temperature. Analysis of the peak integrations of the olefinic proton (δ = 7.83 ppm) and aliphatic β-proton (δ = 4.49 ppm) provided the cyanoacrylamide:thioether adduct ratios at equilibrium.

Time-dependent Inhibition Assay. To determine the time-dependent activity of **34**, mouse Chst15 (0.67 mg/mL) was pre-incubated with DMSO or compound **34** (250 μM) for varying lengths of time (0-180 min) at room temperature in a final volume of 15 μL. After pre-incubation, reactions were initiated by the addition 135 μL of a solution containing 1.1 mM CS-A (by carbazole assay) and 33.3 μM ^{35}S-PAPS, 100 mM Tris pH 7.6, and 5.6 mM BME. To examine the effects of the substrates in the pre-incubation step, CS-A or PAPS were excluded from the initiation step and included in the pre-incubation step at concentrations of 10 mM and 300 μM, respectively. After reaction initiation, the samples were incubated for 30 min at 37 °C. The samples were then purified and analyzed as in the radioisotope labeling assay.

Rapid Dilution Assay. For ten-fold diluted reactions, mouse Chst15 (0.67 mg/mL) was pre-incubated with DMSO or at varying concentrations of compound **34** (12.5-100 μM) for 1 h at room temperature at a final volume of 15 μL. After pre-incubation, reactions were initiated by the addition 135 μL of a solution containing 1.1 mM CS-A (by carbazole assay) and 33.3 μM ^{35}S-PAPS, 100 mM Tris pH 7.6, and 5.6 mM BME. For undiluted

reactions, Chst15 (0.069 mg/mL) in 100 mM Tris pH 7.6 was pre-incubated with DMSO or at varying concentrations of compound **34** (12.5-100 μM) for 1 h at room temperature at a final volume of 145 μL. Reactions were then initiated by the addition of 5 μL of a solution containing 30 mM CS-A (by carbazole assay) and 900 μM ^{35}S-PAPS, 100 mM Tris pH 7.6, and 150 mM BME. After reaction initiation, all samples were incubated for 30 min at 37 °C. The samples were then purified and analyzed as in the radioisotope labeling assay.

Cell Culture. NIH3T3 fibroblasts and Neu7 astrocytes were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Gibco). Cells were passaged and dissociated with 0.25% trypsin (Life Technologies). Cells were incubated in a 5% CO_2 humidified chamber at 37 °C. NIH3T3 fibroblasts were obtained from American Type Culture Collection (ATCC), and Neu7 astrocytes[7] were a generous gift from Dr. Herbert Geller (NIH).

Hyper-Reactive Cysteine Protein and Proteome Profiling. Experiments were performed as previously reported with slight modifications.[2] For the labeling of purified proteins, 1 μg of glutathione S-transferase omega-1 (GTSO1, Abcam), acetyl-CoA acetyltransferase-1 (ACAT1, Abcam), protein arginine methyltransferase 1 (PRMT1, Cayman Chemical), thioredoxin (R&D Systems), or aldehyde dehydrogenase 1A (ALDH1A, R&D Systems) were incubated with 25 μM of the iodoacetamide probe and compound **34** (0, 10, 25 μM) in 100 μL of 100 mM Tris pH 7.6 for 2 h at room temperature. CuAAC was then performed with the addition of 25 μM azido-TAMRA (Click Chemistry Tools), 3 mM THPTA (Click Chemistry Tools), 1 mM $CuSO_4$, and 2 mM sodium ascorbate. Proteins were then extracted with methanol/chloroform, resuspended in 1% SDS, analyzed by SDS-PAGE, and visualized by both in-gel fluorescence and Coomassie staining.

For cell labeling studies, Neu7 astrocytes were seeded at 8,000 cells/cm^2 and grown in media premixed with DMSO or 25 μM of compound **34** diluted from a 100x DMSO stock solution. After 24 h, cells were treated with or without 25 μM of the iodoacetamide probe for 1 h at 37 °C. Cells were removed with a cell scraper in ice-cold PBS and pelleted by centrifugation at 4 °C. The pellet was washed twice with ice cold PBS, lysed by sonication, and centrifuged at 15,000 x g to collect soluble proteins. CuAAC was then performed with the

addition of 25 μM azido-TAMRA, 3 mM THPTA, 1 mM $CuSO_4$, and 2 mM sodium ascorbate. Proteins were extracted with methanol/chloroform, resuspended in 1% SDS, analyzed by SDS-PAGE, and visualized by both in-gel fluorescence and Western blotting for α-tubulin (Cell Signaling).

CS Expression by Immunofluorescence. NIH3T3 fibroblasts or Neu7 astrocytes were seeded at 4,000 cells/cm^2 and grown in media premixed with inhibitors diluted from a 100x DMSO stock solution. After 48 h, the media was aspirated, and cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. For chondroitinase ABC (ChABC) background determination, cells were treated with ChABC (Seikagaku) at a final concentration of 2 mU/μL in 33 mM Tris, 33 mM NaOAc pH 8.0 for 20 min at 37 °C and 5% CO_2 prior to fixing. Cells were rinsed twice with PBS and blocked with a solution containing 1% BSA and 0.1% Triton X-100 in PBS for 30 min at room temperature. A monoclonal mouse anti-CS-E antibody[8] (1:250 dilution) in a buffer containing 1% BSA and 0.1% Triton X-100 in PBS was then applied overnight at 4 °C. Cells were then washed twice with PBS and incubated with an anti-mouse AF488 antibody (Life Technologies, 1:1000 dilution) in a buffer containing 1% BSA and 0.1% Triton X-100 in PBS for 1 h at room temperature. Cells were washed twice with PBS, imaged under a Zeiss 700 confocal microscope, and quantified by the NIH software ImageJ.

CSPG Purification. Media from Neu7 astrocytes was collected 48 h after treatment. The media was centrifuged at 4,000 x g for 10 min to remove cells and debris. Urea (2 M, final) was added to the supernatant and incubated at 4 °C for 1 h. For each 10 mL of media, 2 mL of pre-equilibrated DEAE Sepharose beads were added, and the mixture was incubated at 4 °C. After 1 h, the mixture was added to a column and allowed to drain. The beads were washed with 10 mL of a buffer containing 50 mM Tris pH 7.5, 2 mM EDTA, 2 M urea, and 0.25 M NaCl. The CSPGs were eluted with 3 mL of a buffer containing 50 mM Tris pH 7.5, 2 mM EDTA, 2 M urea, and 2 M NaCl. The eluate was then dialyzed overnight at 4 °C into PBS using dialysis tubing with a 10-12 kDa MWCO (Spectrum Labs). The samples were then concentrated using 50 kDa MWCO spin filters, and uronic acid concentrations of the samples were determined using the carbazole assay.

Disaccharide Analysis. The analysis was performed with slight modifications to a previously reported protocol.[9] CSPGs purified from Neu7 astrocytes were dialyzed into a buffer containing 100 mM Tris pH 7.5 using a spin filter with a 3,000 MWCO. 150 µL of a buffer containing 100 mM Tris pH 7.5, 0.5% SDS, and 10 mM $CaCl_2$ was added to the CSPGs (50 µL). Pronase (Roche) was added to a final concentration of 2 mg/mL, and the reaction was incubated at 37 °C for 18 h. The samples were boiled for 10 min and centrifuged at 12,000 x g for 10 min at room temperature. The supernatant was then added to a DEAE Sepharose (500 µL) column, washed 3 times with 1 mL of a buffer containing 50 mM Tris pH 7.5, 2 mM EDTA, 2 M urea, and 0.25 M NaCl, and eluted with 1.5 mL of a buffer containing 2 mM EDTA, 2 M urea, and 2 M NaCl. The eluate was buffer exchanged into a buffer containing 33 mM Tris and 33 mM NaOAc pH 8.0 using a spin filter with a 3,000 MWCO. The CSPGs (50 µL) were diluted to 200 µL with the same buffer. 50 mU of chondroitinase ABC (Seikagaku) was added to each CSPG sample and allowed to incubate at 37 °C for 18 h. The reaction was put into a 3,000 MWCO spin filter, centrifuged at 12,000 x g for 10 min, and the eluate was collected. This procedure was repeated twice more by adding 200 µL of water to the retentate. The eluates were pooled, analyzed using the carbazole assay, and lyophilized. For 2-aminoacridone (AMAC) conjugation, 5 µL of a solution containing 0.1 M AMAC in 3:18 glacial acetic acid:DMSO was added to 1 µg of CS disaccharide and incubated for 15 min at room temperature. 5 uL of 1 M $NaBH_3CN$ was then added, and the reaction was incubated for 4 h at 45 °C. The reaction was quenched with 1:1 DMSO:water and analyzed by HPLC with a Poroshell 120 EC-C18 column (4.6 x 50 mm) with the following method at a flow rate of 1 mL/min and detection at 428 nm: linear gradient of 98% 60 mM NH_4OAc and 2% MeCN to 70% 60 mM NH_4OAc and 30% MeCN for 50 min, followed by 15 min of 98% 60 mM NH_4OAc and 2% MeCN. *P*-values were determined using one-way ANOVA with Tukey's HSD post hoc analyses, and the results from three experiments were shown.

Neurite Outgrowth Assays. For the neurite outgrowth studies, 96-well poly-lysine Cellware plates (BD BioCoat^{TM}) were coated with laminin (10 µg/mL, Life Technologies) in Neurobasal medium (Life Technologies) for 2 h at 37 °C and 5% CO_2. Dorsal root ganglia neurons (DRGs) were dissected from P5 Sprague Dawley rats, incubated in 0.25% trypsin with EDTA for 15 min at 37 °C, followed by collagenase (Worthington; 4 mg/mL) for

15 min at 37 °C, triturated to dissociate to single cell suspensions, filtered using a 40 μm cell strainer (Fisher) to remove nondissociated cells, and seeded at approximately 60,000 cells/cm^2 in Neurobasal medium supplemented with B27 and GlutaMAX^{TM} (Life Technologies). After 48 h, the media was aspirated, and cells were fixed with 4% PFA for 15 min at room temperature. A monoclonal rabbit anti-β3 tubulin antibody (1:1000 dilution, Cell Signaling (D71G9)) in a buffer containing 1% BSA and 0.1% Triton X-100 in PBS was then applied overnight at 4 °C. The cells were then washed twice with PBS and incubated with an anti-rabbit AF568 antibody ((Life Technologies, 1:1000 dilution) in a buffer containing 1% BSA and 0.1% Triton X-100 in PBS for 1 h at room temperature. The cells were washed twice with PBS, imaged under a Zeiss 700 confocal microscope, and quantified using MetaMorph neurite outgrowth software (Molecular Devices).

For inhibition studies using CSPGs derived from Neu7 astrocytes, glass coverslips were coated with poly-lysine in borate buffer pH 8.5 (0.5 mg/mL) overnight at 37 °C and 5% CO_2, followed by laminin (10 μg/mL) in Neurobasal medium for 2 h at 37 °C and 5% CO_2, followed by CSPGs purified from Neu7 astrocytes (1 μg/mL based on uronic acid content determined by carbazole assay) in PBS for 2 h at 37 °C and 5% CO_2. DRGs were dissected, plated, imaged, and quantified as above. *P*-values were determined using one-way ANOVA with Tukey's HSD post hoc analyses (n = 20-80 neurons per experiment), and the results from three experiments were shown.

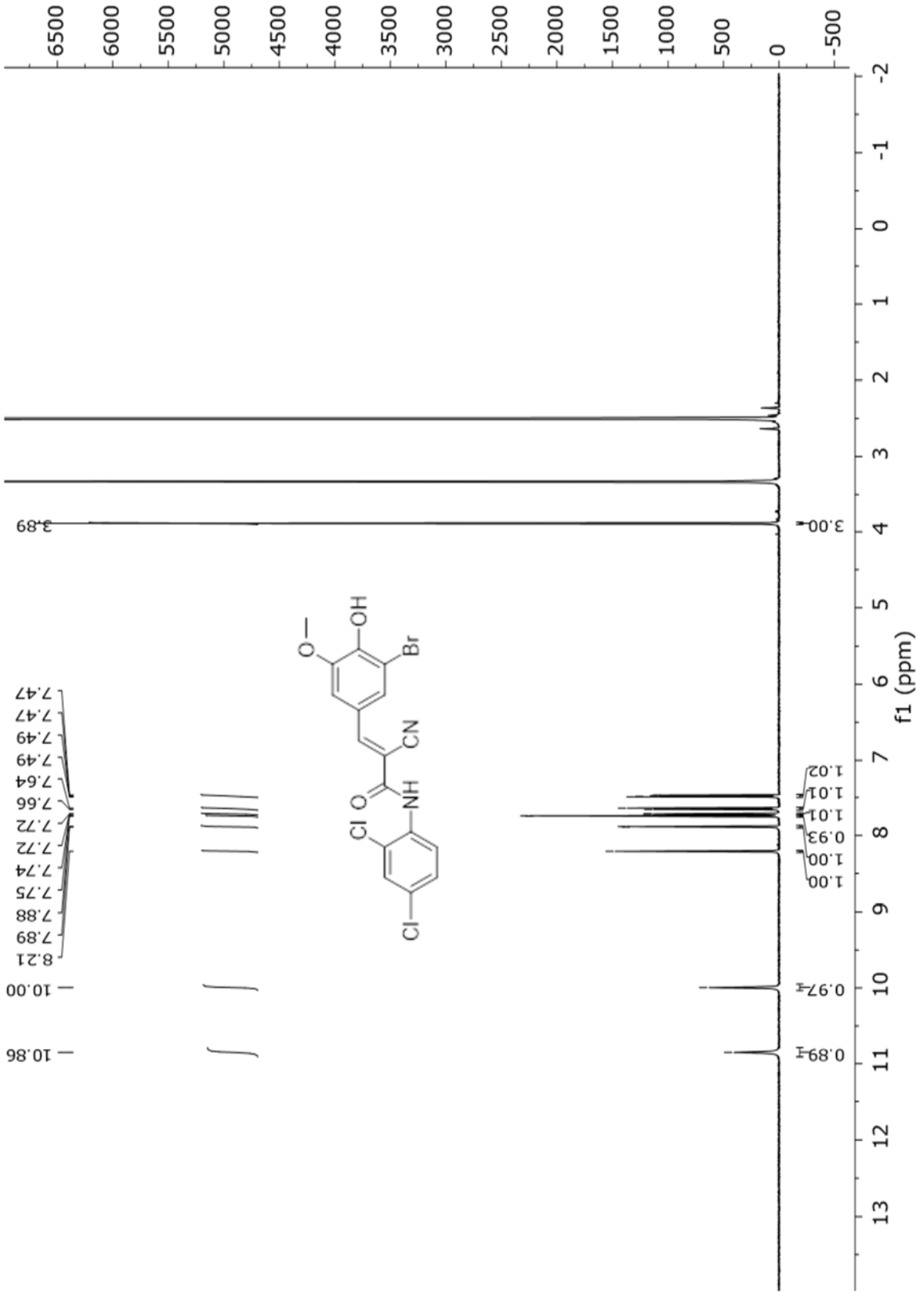
Boundary Assay. CSPGs purified from Neu7 astrocytes (5 μg/mL based on uronic acid content determined by carbazole assay) were mixed with Texas Red (0.5 mg/mL; Life Technologies) in PBS, spotted on poly-lysine and laminin (10 μg/mL) coated coverslips, and incubated for 2 h at 37 °C and 5% CO_2. DRGs were dissected from P5 Sprague Dawley rats, incubated in 0.125% trypsin with EDTA for 15 min at 37 °C, followed by collagenase (4 mg/mL) for 15 min at 37 °C, triturated to dissociate to single cell suspensions, filtered using a 40-μm cell strainer to remove nondissociated cells and seeded at approximately 60,000 cells/cm^2. Cells were cultured in Neurobasal medium supplemented with B27 and GlutaMAX^{TM}. After 48 h, the media was aspirated, and cells were fixed with 4% PFA for 15 min at room temperature. A monoclonal rabbit anti-β3 tubulin antibody (1:1000 dilution, Cell Signaling (D71G9)) in a buffer containing 1% BSA and 0.1% Triton X-100 in PBS was then applied overnight at

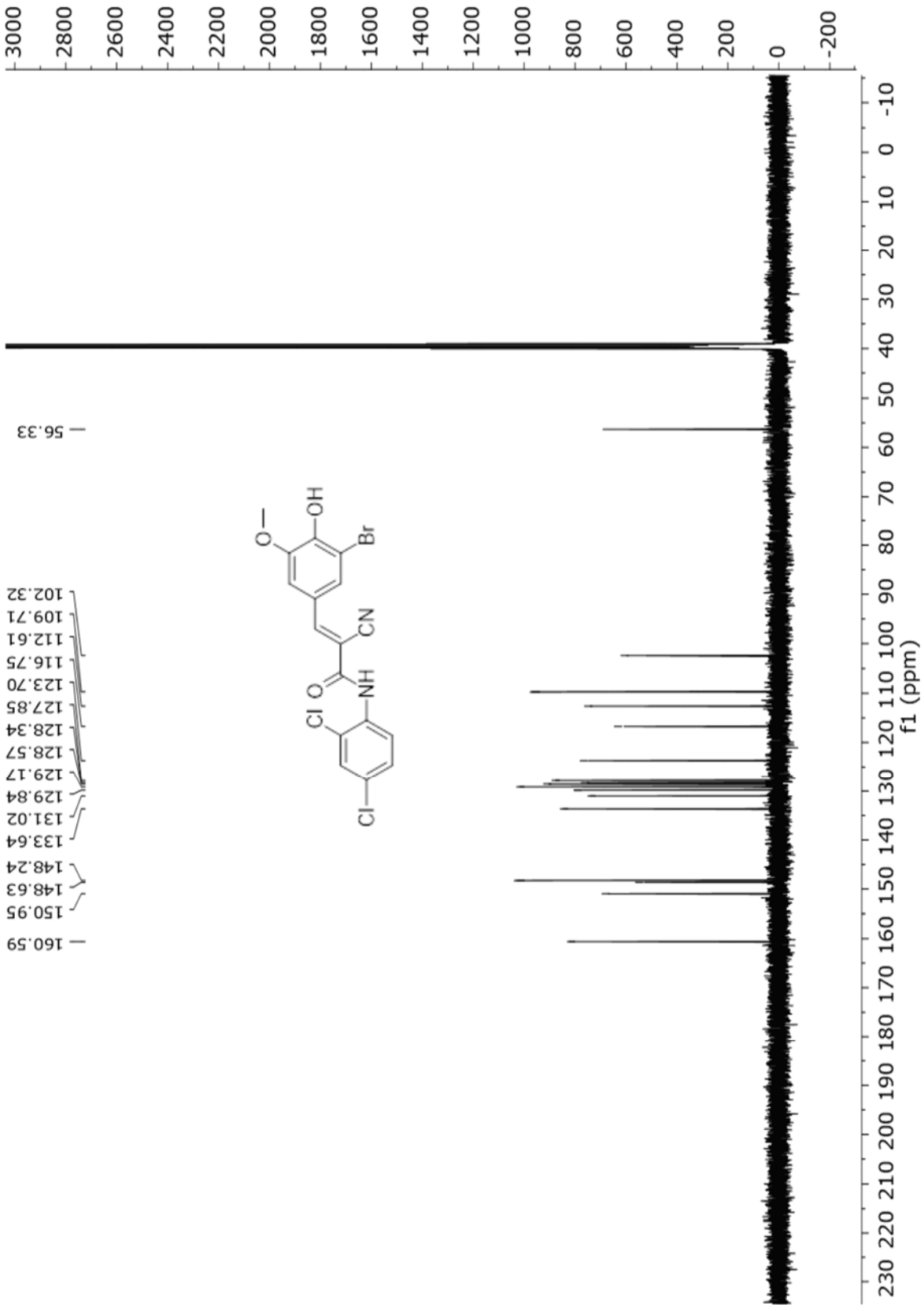
4 °C. The cells were then washed twice with PBS and incubated with an anti-rabbit AF488 antibody ((Life Technologies, 1:1000 dilution) in a buffer containing 1% BSA and 0.1% Triton X-100 in PBS for 1 h at room temperature. Neurites growing toward and within 10 μm of the boundary were evaluated using a Zeiss 700 confocal microscope. The percentage of neurites that crossed the boundary over the total neurites was quantified. *P*-values were determined using one-way ANOVA with Tukey's HSD post hoc analyses (n = 20-40 neurons per experiment), and the results from five experiments were shown.

Microsomal Stability Assay. Performed as previously reported.[10]

In vivo Pharmacokinetics. Performed as previously reported.[11]

3-4. Representative NMR Spectra




56.33
160.59
150.95
148.63
148.24
133.64
131.02
129.84
129.17
128.57
128.34
127.85
123.70
116.75
112.61
109.71
102.32
O
OH
Br
CN
NH
O
Cl
Cl
f1 (ppm)

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