Supporting information

Design and synthesis of benzenesulfonamide derivatives as potent anti-influenza hemagglutinin inhibitors

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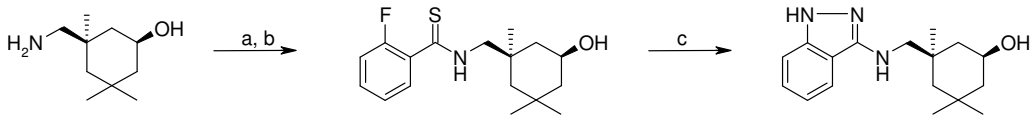
Experimental details:

All starting materials were obtained commercially and were used without further purification. All reported yields are of isolated products and are not optimized. All the final compounds were purified by preparative HPLC on reversed phase column using X Bridge^{TM} Perp C_{18} (OBD^{TM} 30 × 100 mm) column or SunFire^{TM} Perp C_{18} (5 m, OBD^{TM} 30 × 100 mm) column. LC/MS spectra were obtained using a MicroMass Plateform LC (WatersTM alliance 2795-ZQ2000). And NMR Spectra were obtained using Bruker Avance 400MHz. All the target compounds (**7**–**28**, **31**–**33** and **38**) have purities of > 95% based upon LC/MS, and ^{1}H-NMR.

Synthesis of analog 7 [a]



5 **6** **7**

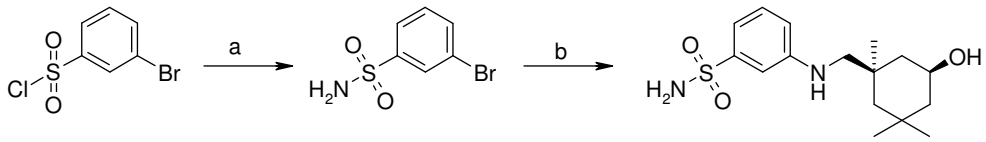
[a] Reagents and conditions: (a): 2-fluoro-benzoic acid, HATU, NEt_3, DCM, r.t, 3 h, 98%; (b): Lawesson's reagent, toluene, refluxing, 30%; (c): $NH_2NH_2.H_2O$, DMSO, 150 ˚C, 62%.

To a solution of 2-fluoro-benzoic acid (1.40 g, 10 mmol) and amine **5** (1.71 g, 10 mmol) in 20 mL of DCM was added HATU (3.8 g, 10 mmol) and NEt_3 (1.4 mL, 10 mmol). The reaction mixture was stirred at r.t. for 3 h before partitioned between water and DCM. The organic phase was dried and concentrated. The residue was purified with silica-gel column chromatography to afford 2.89 g of amide. It was treated with Lawesson's reagent (4.0 g) in toluene (20 mL) under refluxing overnight. The solvent was removed and the residue was purified with preparative HPLC to give 0.91 g of thioamide **6**.

To a solution of **6** (40 mg, 0.13 mmol) in DMSO (2 mL) was added hydrazine (0.5 mL). The reaction mixture was stirred at 150 ˚C for 2 h. The mixture was purified with preparative HPLC to give **7** (23

mg) as powder. ^{1}H NMR (d_4-MeOD, 400MHz), 7.74 (d, 1H, J = 8.0 Hz), 7.30-7.25 (m, 2H), 6.97 (t, 1H, J = 7.6 Hz), 3.96-3.93 (m, 1H), 3.18 (s, 2H), 1.80-1.72 (m, 2H), 1.34-1.24 (m, 3H), 1.21 (s, 3H), 1.10 (s, 3H), 1.09-1.04 (m, 1H), 0.99 (s, 3H). LCMS $(M+H)^+$ 288.2 (98% purity). HR-MS($C_{17}H_{25}N_3O$): calc'd $(M+H)^+$ 288.2076, exp $(M+H)^+$ 288.2082.

Synthesis of sulfonamide 28 [a]

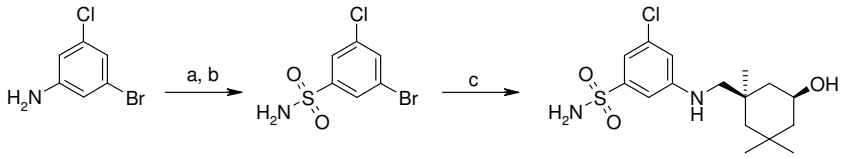


28

[a] Reagents and conditions: (a): NH_3, DCM; (b): **5**, CuI, K_3PO_4, L-proline, microwave, DMSO, 80 ˚C, 56%.

To a solution of 3-bromo-benzenesulfonyl chloride (1.28 g, 0.5 mmol) in 20 mL of DCM was added 1 mL of aqueous ammonia (25%) at 0 ˚C, and the mixture was brought to r.t. and stirred for 2 h. The reaction mixture was washed with water, and dried under vacuum to give 1.1 g of 3-bromo-benzenesulfonamide. A mixture of 3-bromo-benzenesulfonamide (137 mg, 0.58 mmol), amine **5** (150 mg, 0.88 mmol), K_3PO_4 (250 mg, 1.16 mmol), CuI (11 mg, 0.058 mmol), and L-proline (13 mg, 0.116 mmol) in 3 mL of DMSO was heated in microwave at 80 ˚C for 30 min. The reaction mixture was sent to RP-HPLC separation and 106 mg of benzenefulfonamide **28** was obtained as white powder. ^{1}H NMR (d_6-DMSO, 400MHz), 7.19 (t, 1H, J = 8.0 Hz), 7.13 (s, 2H), 7.09 (t, 1H, J = 1.8 Hz), 6.92 (m, 1H), 6.81 (dd, 1H, J_1 = 8.0 Hz, J_2 = 1.8 Hz), 6.00 (br, 1H), 3.76 (m, 1H), 2.79 (s, 2H), 1.62 (m, 2H), 1.24 (d, 1H, J = 14 Hz), 1.12 (d, 1H, J = 14 Hz), 1.05 (s, 3H), 1.01 (s, 3H), 0.97-0.91 (m, 2H), 0.85 (s, 3H). ^{13}C NMR (d_6-DMSO, 100MHz), 150.4, 145.1, 129.6, 115.1, 112.3, 108.8, 63.8, 57.8, 49.2, 47.5, 45.0, 37.3, 35.7, 32.4, 28.5, 24.4. LCMS $(M+H)^+$ 327.2 (99% purity). HR-MS($C_{16}H_{26}N_2O_3S$): calc'd $(M+H)^+$ 327.1737, exp $(M+H)^+$ 327.1735.

Synthesis of substituted benzenesulfonamide 32 [a]



29 **30** **32**

[a] Reagents and conditions: (a): 1. $NaNO_2$, HCl; 2. CuCl, AcOH, SO_2; (b): NH_3, DCM, 46%; (c): **5**, K_3PO_4, L-proline, DMSO, microwave, 80 ˚C, 51%.

The synthesis of **32** can be applied to many other analogs including **31** and **33**. As an example, **32** was prepared by the following procedures. To a stirring mixture of 3-bromo-5-chloro-phenylamine **29** (2 g,

9.6 mmol) in 10 mL of HCl, was added a solution of $NaNO_2$ (0.8 g, 11.6 mmol) in 10 mL of water with the reaction temperature kept below 5 ˚C. The diazonium salt solution was added into an acetic acid solution of CuCl (0.28 g, 2.88 mmol) that was saturated with SO_2 gas. The mixture was stirred at r.t. for 2 h before partitioned between EtOAc and water. The organic phase was washed with water and aqueous $NaHCO_3$ solution, dried over Na_2SO_4, and concentrated. The crude product was dissolved in 50 mL of DCM and then NH_3 was bubbled into this solution at -78 ˚C for 10 min. The reaction mixture was brought to r.t. and stirred for 2 h. It was washed with water, and dried under vacuum to give 1.2 g of **30** (46% yield for two steps). The copper catalyzed coupling reaction between amine **5** and **30** gave designed compound **32** as white solid. ^{1}H NMR (d_4-MeOD, 400MHz), 7.03 (t, 1H, J = 2.0 Hz), 6.96 (t, 1H, J = 1.6 Hz), 6.77 (t, 1H, J = 2.0 Hz), 3.94-3.85 (m, 1H), 2.85 (s, 2H), 1.73-1.65 (m, 2H), 1.27 (d, 1H, J = 13.6 Hz), 1.18 (d, 1H, J = 13.6 Hz), 1.08 (s, 3H), 1.06-0.95 (m, 5H), 0.94 (s, 3H). LCMS $(M+H)^+$ 361.0 (99% purity). HR-MS($C_{16}H_{25}ClN_2O_3S$): calc'd $(M+H)^+$ 361.1352, exp $(M+H)^+$ 361.1364.

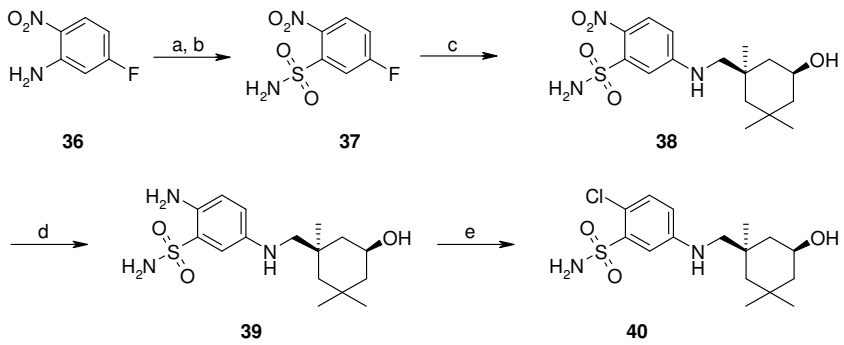
Analog **31**: ^{1}H NMR (d_4-MeOD, 400MHz), 6.95 (t, 1H, J = 1.6 Hz), 6.69 (dt, 1H, J_1 = 8.4 Hz, J_2 = 0.8 Hz), 6.51 (dt, 1H, J_1 = 12.0 Hz, J_2 = 0.8 Hz), 3.94-3.85 (m, 1H), 2.86 (s, 2H), 1.83-1.67 (m, 2H), 1.28 (d, 1H, J = 13.6 Hz), 1.17 (d, 1H, J = 13.6 Hz), 1.08 (s, 3H), 1.06-0.96 (m, 5H), 0.94 (s, 3H). LCMS(ESI) $(M+H)^+$ 345.1 (98% purity). HR-MS($C_{16}H_{25}FN_2O_3S$): calc'd $(M+H)^+$ 345.1648, exp $(M+H)^+$ 345.1659.

Analog **33**: ^{1}H NMR (d_4-MeOD, 400MHz), 7.32 (s, 1H), 7.25 (s, 1H), 7.06 (s, 1H), 3.97-3.89 (m, 1H), 2.93 (s, 2H), 1.76-1.68 (m, 2H), 1.29 (d, 1H, J = 13.2 Hz), 1.20 (d, 1H, J = 13.2 Hz), 1.13-1.00 (m, 8H), 0.96 (s, 3H). LCMS $(M+H)^+$ 394.9 (98% purity). HR-MS($C_{17}H_{25}F_3N_2O_3S$): calc'd $(M+H)^+$ 395.1616, exp $(M+H)^+$ 395.1628.

Analog **34**: ^{1}H NMR (d_4-MeOD, 400MHz), 8.11 (d, 1H, J = 2.4 Hz), 7.4 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.4 Hz), 7.59 (d, 1H, J = 8.4 Hz), 4.02-3.93 (m, 1H), 3.25 (d, 2H, J = 5.6 Hz), 2.71 (s, 3H), 1.85-1.72 (m, 2H), 1.48 (d, 1H, J = 13.6 Hz), 1.31 (s, 3H), 1.29-1.12 (m, 3H), 1.10 (s, 3H), 1.02 (s, 3H). LCMS $(M+H)^+$ 341.1 (99% purity). HR-MS($C_{17}H_{28}N_2O_3S$): calc'd $(M+H)^+$ 341.1899, exp $(M+H)^+$ 341.1916.

Analog **35**: ^{1}H NMR (d_4-MeOD, 400MHz), 7.17 (s, 1H), 6.99 (d, 1H, J = 8.8 Hz), 6.87 (d, 1H, J = 8.8 Hz), 3.94-3.89 (m, 4H), 2.84 (s, 2H), 1.75-7.71 (m, 2H), 1.29-0.98 (m, 13H). LCMS $(M+H)^+$ 357.2 (98% purity). HR-MS($C_{17}H_{28}N_2O_4S$): calc'd $(M+H)^+$ 357.1848, exp $(M+H)^+$ 357.1860.

Synthesis of sulfonamide 38 [a]



[a] Reagents and conditions: (a): 1. $NaNO_2$, HCl; 2. CuCl, AcOH, SO_2; (b): NH_3, DCM, 65% for two steps; (c): **5**, K_2CO_3, DMSO, 50 ˚C, 43%; (d): $SnCl_2{\cdot}H_2O$, EtOH, reflux, 99%; (e): $CuCl_2{\cdot}2H_2O$, *t*-BuONO, DMF, 45 ˚C, 22%.

To a mixture of 2-nitro-5-fluoroaniline **36** (60.0 g , 0.384 mol) in 400 mL of con. HCl was added an aqueous solution of $NaNO_2$ (14.6 g, 0.422 mmol) at -5 ˚C. The reaction mixture was stirred at -5 ˚C for 30 min before added dropwise into a solution of $CuCl_2{\cdot}2H_2O$ (17.2 g, 0.101 mol) and $NaHSO_3$ (160.0 g, 1.536 mol) in 240 mL of con. HCl. After the mixture was stirred for 2 h at r.t., the suspension solid was collected and dissolved in 600 mL of THF. To this solution was added 100 mL of aqueous ammonia (25%, 0.364 mol) at 0 ˚C. The mixture was stirred at r.t. for 30 min before 600 mL of EtOAc and 600 mL of water were added. The organic phase was washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The crude powder product was washed with a mixture of petroleum ether and EtOAc to afford 55.4 g of **37**.

To a solution of **37** (65.6 g, 0.298 mol) in 250 mL DMSO was added amine **5** (51.0 g, 0.298 mol) and K_2CO_3 (82.4 g, 0.596 mol). The reaction mixture was stirred at 50 ˚C overnight before poured into 1 L of water, and the resulted aqueous solution was extracted with EtOAc twice. The organic phase was washed with saturated brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude product was purified by flash chromatography to give 47.2 g of **38** as yellowish solid [MS(ESI): 372.1 $(M+H)^+$].

To a solution of **38** (47.2 g, 0.127 mol) in 500 mL of EtOH was added $SnCl_2{\cdot}2H_2O$ (143 g, 0.635 mol), and the mixture was stirred under refluxing for 30 min. After removal of solvent, the residue was diluted with EtOAc and poured into 500 mL of ice water. The mixture was neutralized to pH 8 by addition of $NaHCO_3$ and the formed thick slurry was filtered through Celite. The organic phase was separated, dried over anhydrous Na_2SO_4, and concentrated to give 43 g of **39** as yellow solid.

To a suspension of $CuCl_2{\cdot}2H_2O$ (4.98 g, 29.2 mmol) in 25 mL of DMF was added 5.0 g of **39** (14.6 mmol) at 0 ˚C under N_2 atmosphere. 1.76 g of *tert*-BuONO (15.4 mmol) was added into the flask and the reaction mixture was stirred at 45 ˚C for 1.5 hrs. The reaction mixture was poured into water and EtOAc. After filtration through Celite, the organic phase was separated, dried over anhydrous Na_2SO_4, and concentrated. Column and HPLC purification gave 1.16 g of **40** as solid. ^1H NMR (d_6-DMSO, 400MHz), 7.34 (s, 2H), 7.31 (d, 1H, J = 3.2 Hz), 7.22 (d, 1H, J = 8.8 Hz), 6.77 (dd, 1H, J_1 = 8.8 Hz, J_1

= 3.0 Hz), 6.19 (t, 1H, *J* = 6.0 Hz), 4.37 (d, 1H, *J* = 4.8 Hz), 3.75 (m, 1H), 2.77 (d, 2H, *J* = 6.0 Hz), 1.61 (m, 2H), 1.22 (d, 1H, *J* = 13.6 Hz), 1.07 (d, 1H, *J* = 13.6 Hz), 1.03 (s, 3H), 1.00 (s, 3H), 0.98-0.93 (m, 2H), 0.90 (s, 3H). ^{13}C NMR (*d*$_6$-DMSO, 100MHz), 149.1, 141.3, 132.0, 115.2, 114.6, 112.8, 63.8, 57.7, 49.2, 44.9, 47.4, 37.4, 35.7, 32.4, 28.5, 24.4. LCMS $(M+H)^+$ 361.1 (99% purity). HR-MS($C_{16}H_{25}ClN_2O_3S$): calc'd $(M+H)^+$ 361.1337, exp $(M+H)^+$ 361.1347.

Biological assays and results:

Cells and viruses. Madin-Darby canine kidney cell (MDCK) was purchased from American type culture collection (ATCC) and was maintained in minimal essential medium (MEM) containing 10% fetal bovine serum and antibiotics. Influenza A/Weiss/43 (H1N1), A/PR/8/34 (H1N1), and A/Hongkong/8/68 (H3N2) were purchased from ATCC and propagated in 10-day-old embryonated chicken eggs at 37 ˚C. Virus was harvested 48h after inoculation as pooled allantoic fluid. After a brief centrifugation (3,000 rpm at r.t. for 20 min) and virus titer measurement by a hemagglutination test, virus was aliquoted and stored at a -80 ˚C freezer.

Viral cytopathic effect (CPE) assay: To measure anti-influenza activity of compounds, MDCK cells were seeded into 96-well plates at a density of 5,000 cells per well. Next day, compounds were serially half-log diluted with Gibco SFM containing trypsin. Compounds and 50 pfu of virus were added into corresponding wells to make m.o.i at 0.01 and a final trypsin concentration of 2.5 µg/ml. The testing plates also contained medium control, cell control, virus control, and compound toxicity control. After a 3-day treatment, cell viability was measured with a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. Briefly, 20 µl of MTT diluted in culture medium was added into each wells and incubated at 37 ˚C for 4 hrs. Reduced MTT (formazan) was extracted with acidic isopropanol and absorbance at wavelengths of 570 nm and 630 nm (OD_{570} and OD_{630}) was read on a microtiter plate reader. After subtraction of background OD values, dose response curves of half-log concentration vs. percent protection were generated, on which half maximal effective concentration (EC_{50}) and half maximal toxic concentration (CC_{50}) were calculated.

Red blood cell (RBC) hemolysis assay and results: In this assay, fresh chicken RBCs were collected and incubated with influenza A/Weiss/43 viruses. After the binding of HA to host receptors on the surface of chicken RBC, a brief acidification (pH ~5) triggered an irreversible conformational change of HA protein, and resulted in pore formations on the RBC membrane and subsequent release of hemoglobin from RBCs to the supernatant. The following table shows inhibitory activity (IC_{50} value) of selected compounds to the influenza A/Weiss/43 virus mediated hemolysis of chicken RBCs. The reported IC_{50} data are half maximal inhibition concentration to prevent hemolysis of chicken RBCs,

values are mean of two duplicate experiments.

Table SI-1. Inhibitory activities of selected compounds to the influenza A/Weiss/43 virus mediated hemolysis of chicken RBCs.

	Compound ID																	
	1	**9**	**11**	**15**	**16**	**17**	**18**	**20**	**23**	**25**	**26**	**28**	**31**	**32**	**33**	**34**	**35**	**40**
IC_{50} (μM) [a]	0.25	2.22	> 31.6	1.11	1.50	0.19	1.06	0.04	0.06	> 31.6	> 31.6	0.27	0.58	0.23	2.91	1.13	4.16	0.25

[a] Half maximal inhibition concentration to prevent hemolysis of chicken RBCs, values are mean of two duplicate experiments.

Hemagglutinin (HA) trypsin sensitivity assay: HA protein was isolated from viral preparations after cleavage with bromelain. In a trypsin sensitivity assay, 6 μg of purified HA was incubated with compounds at 31 ˚C for 15 minutes. Then the pH of the solution was adjusted to ~5.0 to trigger HA conformational changes. After neutralization with Tris buffer, the solution was treated with 4 μg of trypsin at 37 ˚C for 30 minutes. The extent of trypsin digestion of HA was revealed on a 10% SDS-PAGE gel that was stained with Coomassie blue G-250.

Liver microsomal stability test: The stability of compounds in the presence of human or mouse liver microsomes was determined by incubation of compound (1 μM), sodium phosphate buffer (100 mM, pH 7.4), microsomal protein (0.5 mg/ml), and NADPH (1 mM) together. Hepatic clearance (CL_h) is calculated using the well-stirred model: $CL_h=Q_h*f_u*CL_{int}/(Q_h+f_u* CL_{int})$, where Q_h is hepatic blood flow (human liver blood flow is about 21 mL/min/kg and mouse liver blood flow is about 91.3 mL/min/kg), f_u is the free fraction of compound in blood (assuming f_u is 1).

Single dose PK study: To evaluate PK parameters of compounds in male CD-1 mice, **1**, **28**, **32** and **40** were administered to mice at an intravenous dose of 2.0 mg/kg and an oral dose of 10.0 mg/kg, respectively. The plasma samples were collected after dosing, and the drug concentrations in plasma were determined by LC-MS/MS.

Table SI-2. SDPK study of **1**, **28**, **32** and **40**.

Compd. id	Cl (mL/min/kg) [a]	$T_{1/2}$	Vd_{ss} (L/kg)	AUC(0-t) (ng*h/mL) [b]	F (%)
1 [c]		< 5 min			
28	82	3.1 h	6.3	1485	75
32	42	3.5 h	4.6	2195	55

40	53	2.6 h	6.9	2202	70

[a] Single dose i.v. administration of 2 mg/kg to CD-1 mice.
[b] Single dose p.o. administration of 10 mg/kg to CD-1 mice.
[c] PK parameters were not determined due to fast clearance of **1** and limited number of data points available during the distribution and elimination phases.