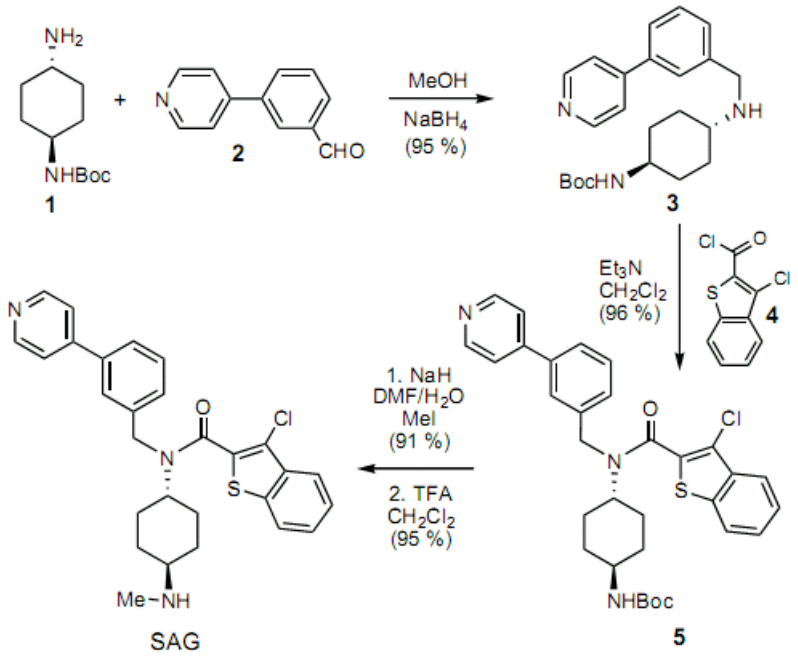

Fig S1. Concise synthesis of SAG. **1** and **2** were mixed in methanol at room temperature for half an hour without adding any molecular sieves; the resultant imine was then reduced by $NaBH_4$ to give product **3** through a direct reductive amination. Then **3** was treated with acyl chloride **4** in CH_2Cl_2 with triethyl amine as the base to afford desired product **5**. The methylated product was eventually obtained by treatment of substrate **5** with NaH in the presence of catalytic amount of water in DMF at 0 ºC for 1 h, followed by reaction with methyl iodide at room temperature for 5h. Thus, our final target **SAG** was generated by treatment of the methylated product with TFA in CH_2Cl_2.

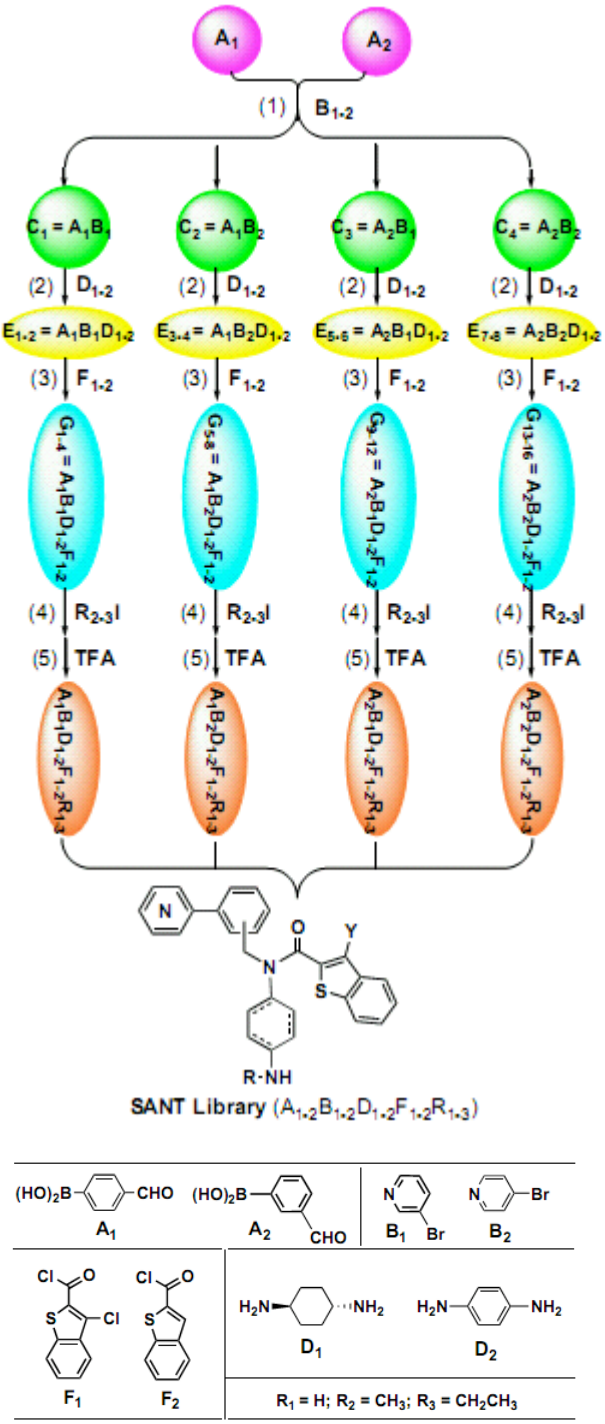

Fig S2. Parallel synthesis of initial SANT library. The scaffold of SAG could be constructed by combining together five distinct modules ($A_{1\text{-}2}$, $B_{1\text{-}2}$, $D_{1\text{-}2}$, $F_{1\text{-}2}$, $R_{1\text{-}3}$). Following the synthesis of SAG, the design elements for SANT library included replacing the cyclohexane-1,4-diamine with benzene-1,4-diamine, changing the substitution pattern of the biaryl fragment, varying the isosteric substituents on the benzo[*b*]thiophene ring, and replacing the methyl group by other alkyl groups. (if R = H, step (4) could be omitted)

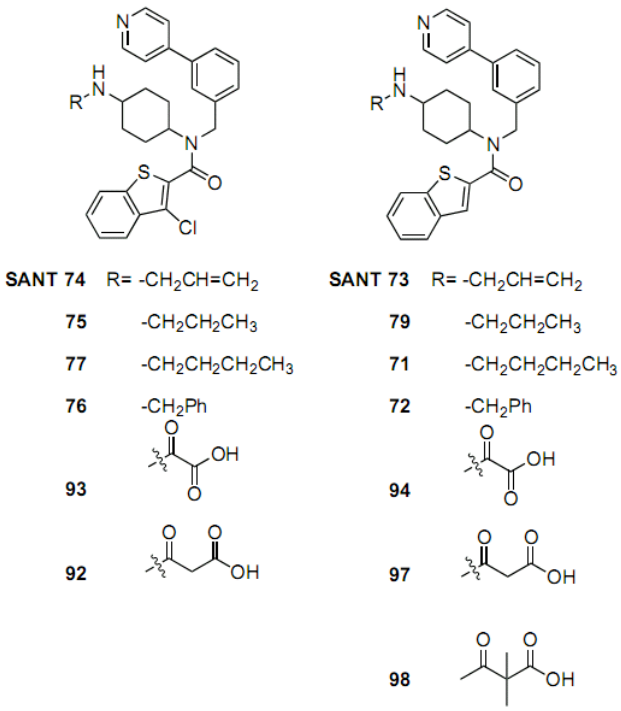

Fig S3. Stuctures of the 13 compounds in the focused library.

Table S1. The lowest effective concentrations of compounds in the focused library on Flk:GFP embryos(3 independent assay)

Compound	Lowest effective concentration (μM)	Compound	Lowest effective concentration (μM)
SANT 19	80		
SANT 74	5	SANT 73	>100
SANT 75	5	SANT 79	40
SANT 77	30	SANT 71	30
SANT 76	>100	SANT 72	>100
SANT 93	80	SANT 94	>100
SANT 92	40	SANT 97	>100
		SANT 98	40

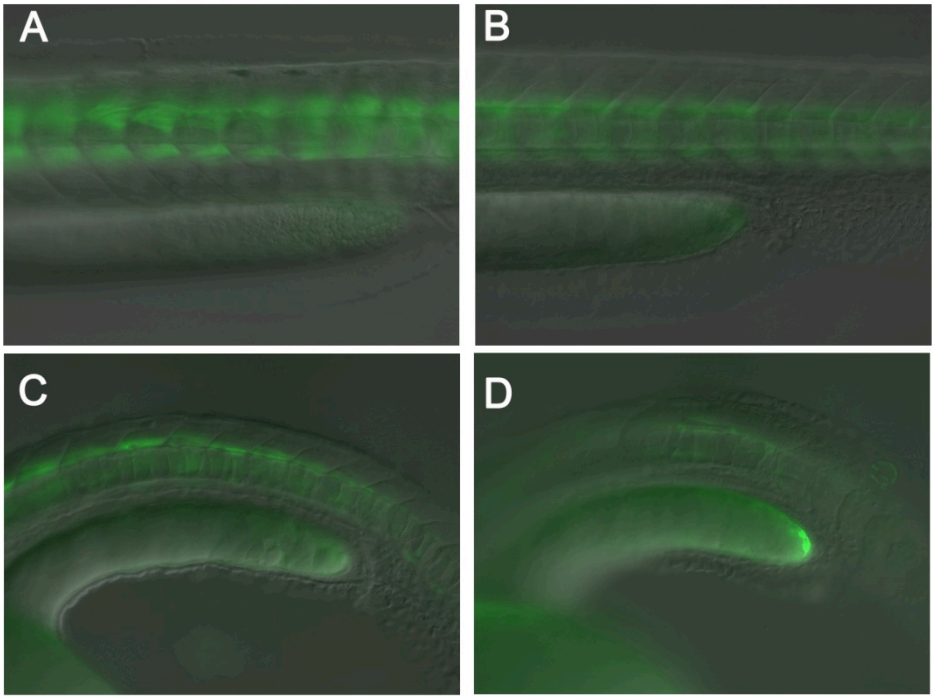

Fig S4 Ablation of Gli:GFP by different concentration of SANT75.

A. Gli:GFP expression treated with DMSO in 2dpf embryos. B C and D. Ablation of Gli:GFP by 5, 10, 15μM SANT75 in 2dpf embryos .

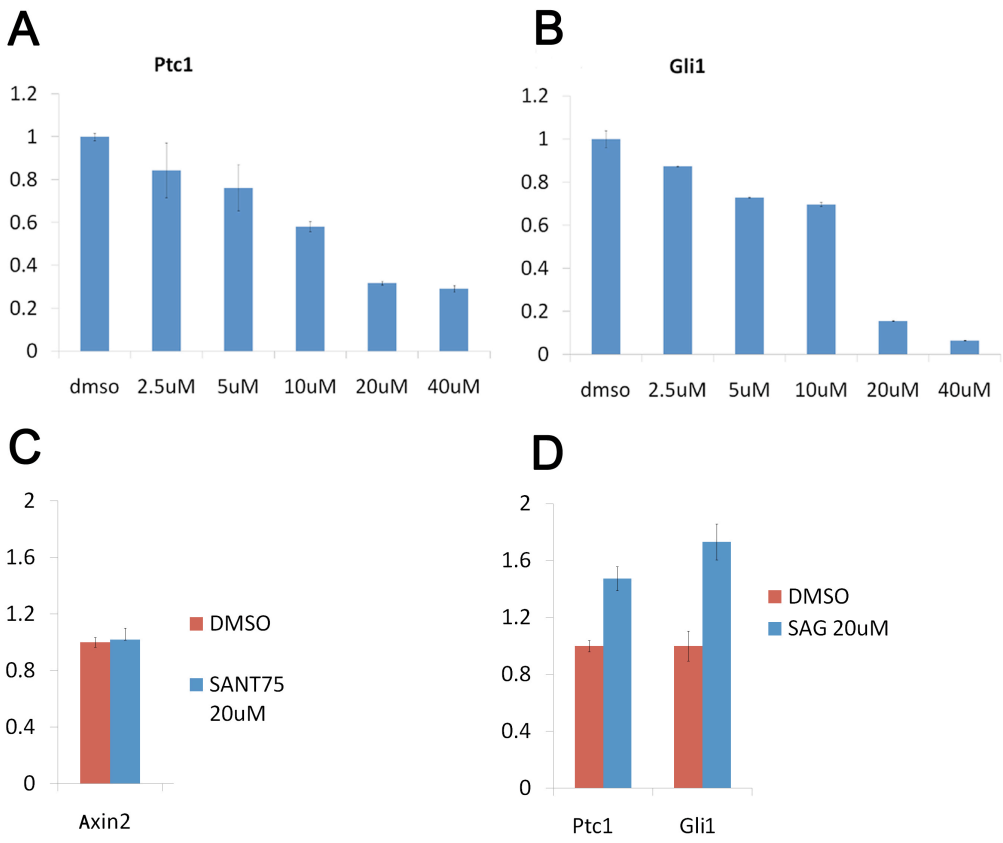

Fig S5. The expression level of Gli1 and Ptc1 of embryos treated with SANT74 and SAG.

A and B Reverse transcription followed by real-time PCR shows the dose-dependent down-regulation of Hh signal pathway target genes mRNA levels after treatment with different concentration of SANT74. A and B show Ptc1/beta-actin and Gli1/beta-actin mRNA ratio, respectively. C shows no changes of Axin2 expression between embryos treated with 20 μM SANT74 and DMSO. D shows higher level of Ptc1 and Gli1 expression between embryos treated with 20 μM SAG and DMSO. The error bars indicate the standard deviation determined from three independent measurements.

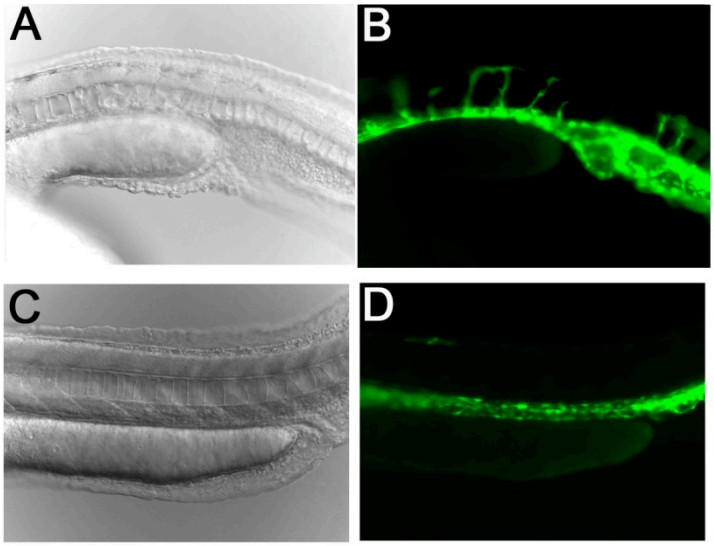

Fig S6 A and B. embryos treated with 40μM SANT74 at 1 hpf but washed out at 17 hpf, A shows the U-shaped somite of treated embryos. B shows the sprouting of ISV of treated embryos. C and D. Embryos treated with 40μM SANT74 at 17 hpf and washed away at 24hpf C shows the normal somite of treated embryos at 36hpf. D shows no ISVs sprouting of treated embryos at 36hpf.

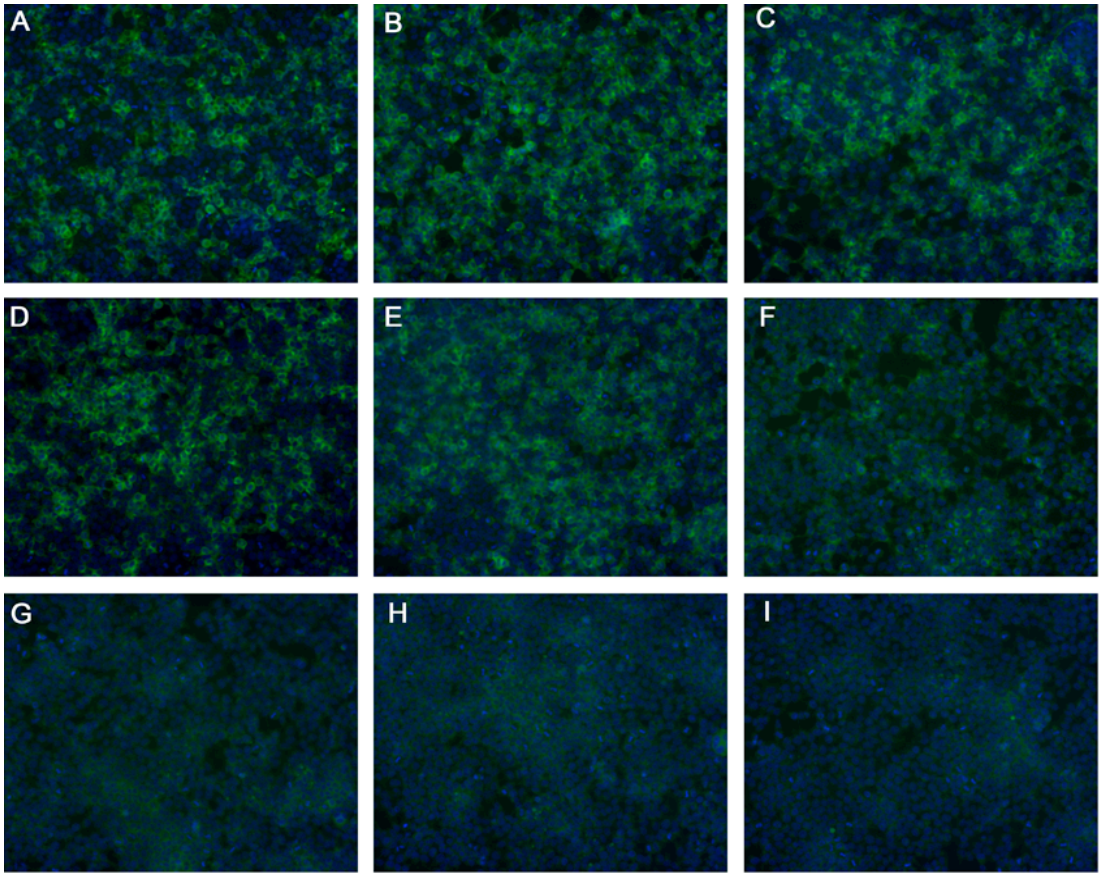

Fig S7 SANT75 competitively blocks the binding of BODIPY-cyclopamine to Smo.
A. Smo-overexpressing HEK 293T cells were incubated with 5 nM BODIPY-cyclopamine (green) and DMSO and counterstained with Hoescht 33342 (blue). B to I. Smo-overexpressing HEK 293T cells were incubated with 5 nM BODIPY-cyclopamine (green) and the indicated with different concentrations of SANT75 (2.5nM 5nM 12.5nM 25nM 50nM 125nM 250nM and 500nM are shown here).

Synthetic Methods:

Part I: Synthetic Methods for SAG

1. Preparation of (4-Amino-cyclohexyl)-carbamic Acid tert-Butyl Ester (1 in Fig S1). A solution of Boc2O (1.0 g, 4.6 mmol) in methanol (25 mL) was slowly added to trans-1, 4-diaminocyclohexane (1.0 g, 8.8 mmol) in methanol (100 mL), and the reaction mixture was stirred at room temperature for 1 h. After filtration, the filtrate was concentrated under vacuum to ~5 mL, and then cooled to -20 °C. The crystallized product was collected. The filtrate was resubmitted to the same reaction condition. After the second cycle, the desired product 1 was obtained in 56% yield (1.05 g). IR (cm-1): 3365, 2933, 1686, 1520. 1H NMR (300 MHz, CDCl3): δ 4.90-5.02 (br, 1H), 3.30-3.42 (br, 1H), 2.58-2.66 (m, 1H), 1.92-2.00 (br, 2H), 1.85-1.97 (m, 4H), 1.43 (s, 9H), 1.10-1.25 (m, 4H). 13C NMR (75 MHz, CDCl3): δ 154.0, 77.9, 48.7, 48.0, 34.2, 34.1, 31.0, 30.9, 27.3. MS (EI) calcd for C11H22N2O2 (M+) 214; found 214.

2. Preparation of 3-Pyridin-4-yl-benzaldehyde (2 in Fig S1). To a solution of 4-bromopyridine hydrochloride (533.4 mg, 2.7 mmol) in water (4.0 mL) and toluene (4.8 mL) was added slowly a solution of Na2CO3 (714 mg, 6.7 mmol) in water (7.0 mL) at room temperature. The solution was then mixed with 3-formylbenzeneboronic acid (431 mg, 2.9 mmol), Pd(PPh3)4 (100 mg, 0.086 mmol). The reaction mixture was stirred at 85 °C for 24 h, and then cooled to room temperature. The reaction was worked up by extraction of the mixture with CH2Cl2 (4 × 5 mL). The combined organic phase was dried over anhydrous Na2SO4. The solvent was removed under vacuum, and the residue was purified by a flash chromatography (silica gel, petroleum ether/EtOAc = 1/4) to give product 2 (420 mg) in 85% yield. 1HNMR (300 MHz, CDCl3): δ 10.1 (s, 1H), 8.72-8.74 (m, 2H), 8.17 (s, 1H), 7.90-7.99 (m, 2H), 7.69 (t, J = 11.4 Hz, 1 H), 7.57 (d, J = 6.6 Hz, 2H). 13C NMR (75 MHz, CDCl3): δ 191.5, 150.3, 146.5, 138.8, 136.8, 132.5, 130.1, 129.6, 127.5, 121.3. HRMS (ESI) calcd for C12H9NO (M+H+) 184.07569; found 184.07538.

3. Preparation of [4-(3-Pyridine-4-yl-benzylamino)-cyclo-hexyl]-carbamic Acid tert-Butyl Ester (3 in Fig S1). To a solution of 3-pyridinyl benzaldehyde (2) (205 mg, 1.1 mmol) in methanol (20 mL) was added N-Boc-1,4-diaminocyclohexane (1) (300 mg, 1.4 mmol), and the mixture was stirred at room temperature for 30 min. To this solution was added NaBH4 (0.5 g, 13.2 mmol) in portions at 0 °C, and the reaction mixture was stirred at room temperature overnight. The reaction was worked up by addition of saturated aqueous Na2CO3 (2 mL), and the mixture was then extracted with chloroform (3 × 6 mL). The combined organic layers were dried over anhydrous MgSO4. The solvent was removed under vacuum, and the residue was purified by a flash chromatography on silica gel (CH2Cl2/MeOH = 10/1) to give 3 (398 mg) in 95% yield. 1H NMR (300 MHz, CDCl3): δ 8.66 (d, J = 8.1 Hz, 2H), 7.39-7.61 (m, 6H), 4.30-4.50 (br, 1H), 3.88(s, 2H), 3.30-3.50 (br, 1H), 2.40-2.60 (m, 1H), 1.90-2.10 (m, 4H), 1.70-1.90(br, 1H), 1.44 (s, 9H), 1.06- 1.40(m, 4H). 13C NMR (75 MHz, CDCl3): δ 155.2, 150.0, 148.3, 141.5, 138.1, 129.1 128.7, 126.6, 125.5, 121.6, 79.0, 55.6, 51.0, 49.4, 31.9, 28.3. HRMS (ESI) calcd for C23H31N3O2 (M + H+) 382.24890; found 382.24896.

4. Preparation of {4-[(3-Chloro-benzo[b]thiophene-2-carbonyl)-(3-pyridin-4-yl-benzyl)-amino]-cyclohexyl}-Carbamic Acid tert-Butyl Ester (5 in Fig S1). To a solution of 3 (410mg, 1.1 mmol) and Et3N (280 μL, 2.0 mmol) in CH2Cl2 (10mL) was added

3-chlorobenzo[b]thiophene-2- carbonyl chloride 4 (278 mg, 1.2 mmol), and the reaction mixture was stirred at room temperature for 0.5 h. The solvent was removed, and the residue was purified by a flash chromatography on silica gel (acetone/PE = 5:1) to give the desired product 19 (587 mg) in 96% yield. 1H NMR (400 MHz, CDCl3): δ 8.65 (br, 2H), 7.20-8.20 (m, 10H), 3.70-5.00 (m, 4H), 3.20-3.40 (br, 1H), 1.75-2.20 (m, 4H), 1.42-1.75 (br, 2H), 1.38 (s, 9H), 0.90-1.30 (br, 2H). 13C NMR (100 MHz, CDCl3): 163.7, 155.1, 150.2, 148.1, 147.7, 139.2, 138.4, 137.2, 135.6, 130.0, 129.3, 127.6, 126.5, 126.2, 125.5, 122.7, 122.5, 121.6, 119.0, 79.2, 58.7, 48.5, 45.1, 32.1, 30.5, 29.7, 29.3, 28.3. HRMS (EI) calcd for C32H34ClN3O3S (M+) 575.2009; found 575.2018.

5. Preparation of 3-Chloro-benzo[b]thiophene-2-carboxylic acid (4-Methylamino-cyclohexyl)-(3-pyridin-4-yl-benzyl) amide (SAG). To a solution of compound 5 (61 mg, 0.1 mmol) in DMF (6.0 mL) was added water (2 μL), followed by addition of NaH (~60 mg, 60% suspension in mineral oil), and the reaction mixture was stirred at 0 °C for 1 h. To this solution was added MeI (15 uL), and the resultant mixture was stirred at room temperature for 5 h. The reaction was worked up by addition of saturated solution of NaHCO3 (10 mL) extracted with Et2O (3 × 20 mL). The combined organic layers were dried over Na2SO4. The solvent was removed under vacuum, and the residue was purified by semipreparative HPLC (94 × 250 XDB C18 column (100% CH3CN, 3 mL/min)) to give the methylated product 6 (57 mg) in 91% yield. 1H NMR (300 MHz, DMSO, T = 333 K): δ 8.63 (d, J = 9.1 Hz, 2H), 8.04-8.22 (m, 1H), 7.84-7.89 (m, 1H), 7.46-7.69 (m, 8H), 4.80 (s, 2H), 3.80-4.18 (br, 1H), 3.60-3.66 (br, 1H), 2.57 (s, 3H), 1.79-1.90 (m, 4H), 1.33-1.55 (m, 2H), 1.33 (s, 9H), 1.26-1.32 (m, 2H). 13C NMR (75 MHz, CDCl3, 323 K): δ 163.6, 155.9, 150.2, 147.8, 139.2, 138.4, 137.2, 135.6, 130.0, 29.3, 127.5, 126.5, 125.4, 122.7, 122.5, 121.6, 118.7, 79.4, 58.7, 52.6, 45.0, 30.8, 29.4, 28.6, 28.3.

To a solution of the methylated product 6 in CH2Cl2 (1.0 mL) was added trifluoroacetic acid (1.0 mL) at 0 °C, and the reaction mixture was stirred at room temperature for 1 h. The reaction was worked up by addition of a saturated solution of Na2CO3 (2 mL) and then extracted with CH2Cl2 (3 × 5 mL), and the combined organic layers were finally dried over anhydrous Na2SO4. The solvent was removed under vacuum, and the residue was purified by a flash chromatography on silica gel (CH2Cl2/acetone/TEA = 40/10/1) to give SAG (45 mg) in 95% yield. 1H NMR (300 MHz, DMSO): δ 8.64 (d, J = 6.0 Hz, 2H), 8.07 (d, J = 7.5 Hz, 1H), 7.85-7.88 (m, 1H), 7.47-7.70 (m, 8H), 4.78 (s, 2H), 3.70-3.90 (br, 1H), 2.20 (s, 3H), 1.85-1.89 (m, 2H), 1.60-1.80 (m, 4H), 0.8-1.0 (m, 2H). 13C NMR (75 MHz, CDCl3): 163.7, 150.3, 148.0, 139.3, 138.5, 137.3, 135.7, 129.3, 127.6, 126.5, 125.8, 125.6, 125.5, 122.7, 122.5, 121.6, 119.0, 59.0, 57.4, 45.8, 3.4, 31.8, 29.8. HRMS (EI) calcd for C28H28ClN3OS (M+) 489.1642; found 489.1651.

Part II: General Synthetic Methods for SANT Library

1. General Procedure for Preparation of E1~E8 (in Fig S2). To a solution of pyridinyl benzaldehyde (C1~C4) (1.0 mmol) in methanol (15 mL) was added boc-protected D1~D2 (1.1 mmol), and the mixture was stirred at room temperature for 30 min. To this solution was added NaBH4 (100 mg, 2.5 mmol) in portion at 0 °C, and the mixture was stirred at room temperature for 1 h. The reaction was worked up by addition of a saturated aqueous Na2CO3 (2 mL); the mixture was then extracted with chloroform (3 × 6 mL), and the combined

organic layer was dried over anhydrous Na2SO4. The solvent was removed under vacuum, and the residue was purified by a flash chromatography on silica gel (CH2Cl2/MeOH = 10/1) to give compound E1 to E8.

2. General Procedure for Preparation of G1~G16. To a solution of substrate E1~E8 (1.0 mmol) and Et3N (280 μL, 2.0 mmol) in CH2Cl2 (10 mL) were added the corresponding acyl chlorides F1~F2 (1.1 mmol, prepared by reaction of the individual acid with SOCl2), and the mixture was stirred at room temperature for 0.5 h. The reaction was worked up by removal of solvent, and the residue was purified by a flash chromatography on silica gel (acetone/PE = 5/1) to give the products G1~G16.

3. General Procedure for Preparation of SANT Library. To a solution of compound G1~G16 (0.1 mmol) in DMF (6.0 mL) was added water (2 μL), followed by addition of NaH (~60 mg, 60% suspension in mineral oil), and the mixture was stirred at 0 °C for 1 h. To this solution was added organohalides at 0 °C, and the mixture was stirred at room temperature for 5 h. The reaction was worked up by addition of a saturated solution of NaHCO3 (10 mL); the mixture was first extracted with Et2O(3 × 20 mL), and the combined extract was then dried over anhydrous Na2SO4. The solvent was removed under vacuum, and the residue was purified by semipreparative HPLC (96 × 250 XDB C18 column (100% CH3CN, 3 mL/min)) to give the alkylated product, which was dissolved in CH2Cl2 (1.0 mL). Trifluoroacetic acid (1.0 mL) was added at 0 °C, and the mixture was stirred at room temperature for 4 h. The reaction was worked up by addition of a saturated solution of Na2CO3 (2 mL); the mixture was extracted with CH2Cl2 (3 × 5 mL), and the combined organic layers were dried over anhydrous Na2SO4. The solvent was removed under vacuum, and the residue was purified by a flash chromatography on silica gel (CH2Cl2/acetone/TEA = 40/10/1) to give compounds of SANT library.