Rapid Discovery of Potent Sulfotransferase Inhibitors by Diversity-Oriented Reaction in Microplates Followed by in situ Screening

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Rapid diversity-oriented microplate library synthesis and in situ screening with a high-throughput fluorescence-based assay were used to develop potent inhibitors of β-arylsulfotransferase IV (β-AST-IV). This strategy leads to facile inhibitor synthesis and study as it allows protecting-group manipulation and product isolation from other library components to be avoided. Through repeated library formation, three aspects of inhibitor makeup, the identities of the two binding groups and the length of the linker between them, were independently optimized. Several potent inhibitors were obtained, one of which was determined to have an inhibition constant K_i of 5 nM. This compound is the most potent β-AST-IV inhibitor developed to date, with a K_i value more than five orders of magnitude lower than the Michaelis constant K_m for the substrate whose binding it inhibits.

Introduction

Sulfotransferases (STs) catalyze sulfuryl group transfer from the donor adenosine 3′-phosphate-5′-phosphosulfate (PAPS) to a range of acceptor substrates.[1] This functional group transfer is involved in a variety of biological processes, including molecular recognition, detoxification, hormone regulation, drug processing, and modulation of receptor binding.[2–8] Various STs have been implicated in the development of disease states. For instance, estrogen ST helps regulate estrogen concentration, which is related to breast cancer,[9,10] heparan 3-O-ST-3 sulfonates heparan sulfate glycosaminoglycan and thereby allows herpes simplex virus-1 cell entry,[11] and tyrosylprotein STs (TPSTs) sulfonate CCR-5[12,13] and the N terminus of P-selectin glycoprotein-1, processes that lead to cell entry by HIV and to diseases involving chronic inflammation,[14] respectively. As a result, these enzymes have emerged as promising therapeutic targets and the search for potent inhibitors is of interest.

We used the enzyme β-arylsulfotransferase IV (β-AST-IV) to study the requirements for ST inhibition because this enzyme is a tractable cytosolic ST and the recombinant protein is available in large amounts.[15] β-AST-IV represents an effective model for studying ST inhibition in lieu of the membrane-associated STs involved in pathophysiological events, which are of limited availability. The effectiveness of such a model is to be expected as a result of the observed structural homology of STs and is particularly anticipated in the case of TPSTs, which also sulfonate aryl substrates. To facilitate screening, we developed a simple and sensitive high-throughput assay for sulfotransferase inhibitor screening that involves the reverse process of the enzymatic reaction of adenosine 3′,5′-diphosphate (PAP, Scheme 1) with 4-methylumbelliferone sulfate (4MUS).[16] This assay was used to screen a library of 35 000 purine and pyrimidine analogues, which resulted in the discovery of compound **4a** (inhibition constant, $K_i = 96$ nM, Table 1), an inhibitor that binds to the aryl site of the enzyme.[17] Structural improvement of **4a** yielded **4b** ($K_i = 70$ nM). Inhibitors of STs such as estrogen ST,[18–21] carbohydrate STs,[2] and tyrosylprotein ST[22] have already been reported.

Results and Discussion

In the study reported herein, rapid diversity-oriented amide-bond-forming reactions and in situ screening[23,24] were employed in the optimization of β-AST-IV inhibitors. This strategy, that is, synthesis and in situ screening in microplates without isolation or protecting-group manipulation, has proven very effective in the rapid identification of potent inhibitors. To develop more potent inhibitors, the linker length and the identities of the two binding groups (adenine and napthalene in the case of **4**) were independently optimized through repetitive library formation and analysis.

The study commenced with an 80-compound library consisting of adenine coupled to various acids by linkers containing even numbers of carbon atoms between 2 and 12, as illustrated by structures **5a–f** (Scheme 2A). The synthesis of the original core structure for the library began with 6-chloropurine (**6**). Mono-Boc-protected diamino linkers were attached to **6** by treatment with **7a–f** to form compounds **8a–f**. The protecting groups were then removed by acid cleavage to give **9a–f**, which were isolated as trifluoroacetate salts. Amide bond formation was performed in microplates and resulted in compounds **5a–f**. The crude reaction product mixtures were ana-

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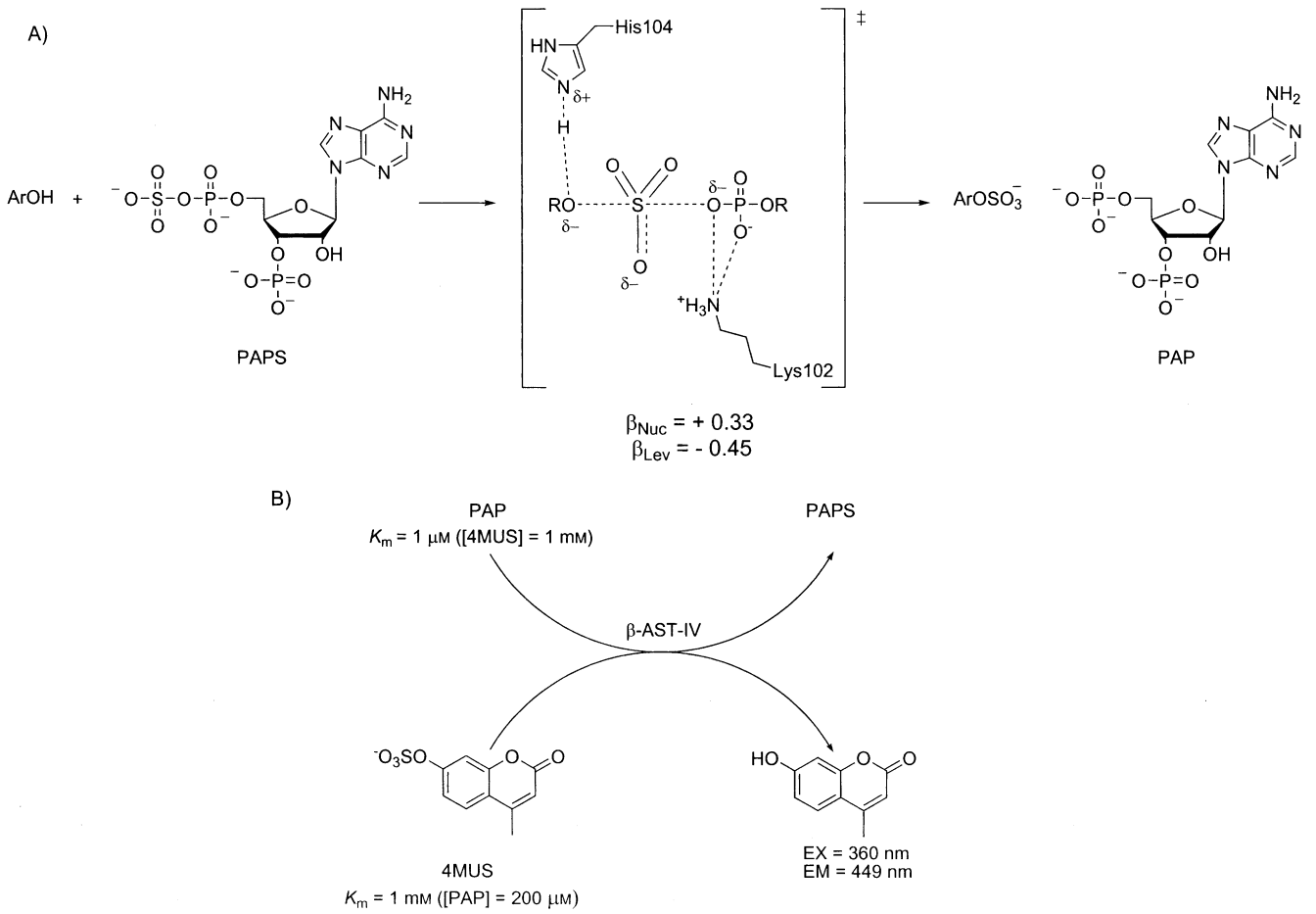
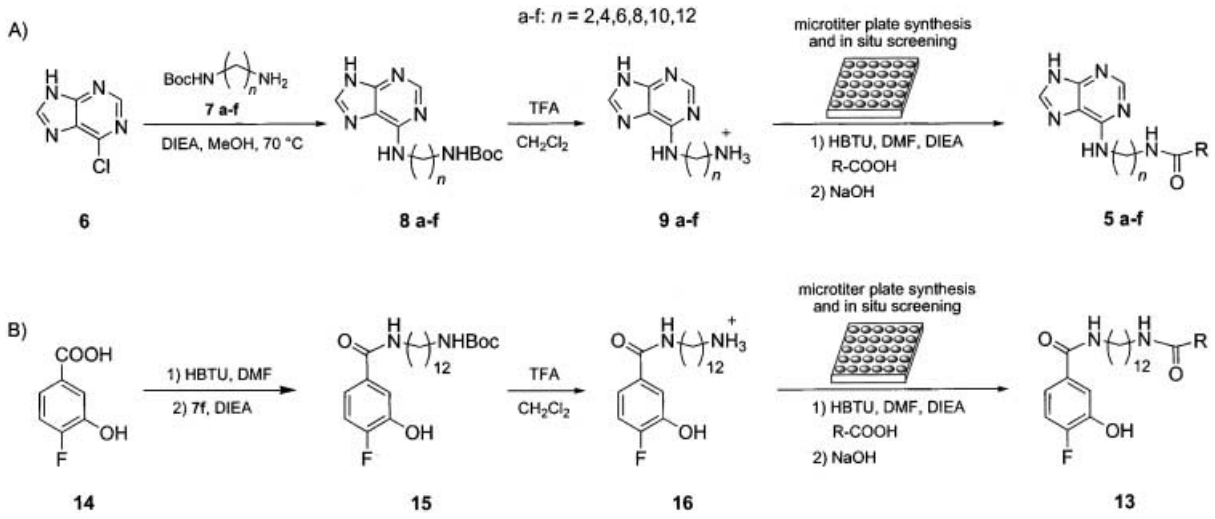
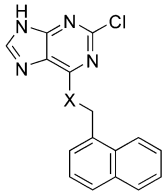
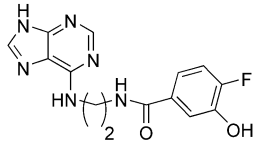
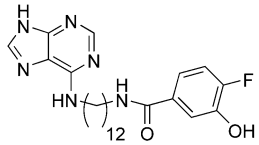
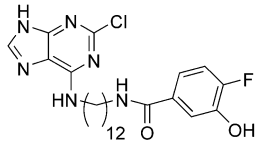
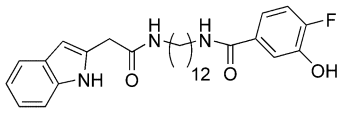
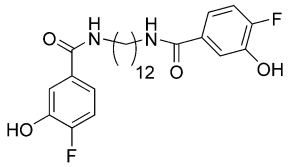
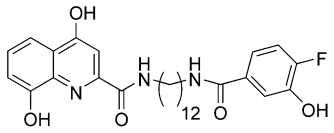
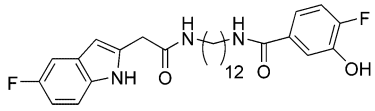


Scheme 1. *A) Arylsulfotransferase-catalyzed reaction showing proposed transition state structure. B) A reverse reaction designed for high throughput screening using 4MUS as substrate. The product was detected by fluorescence emission at 449 nm.*



Scheme 2. *Synthetic route for the A) adenine-based combinatorial library and B) Follow-up library replacing the purine group. Boc, tert-butoxycarbonyl; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; HBTU, O-benzotriazol-1-yl)-N,N,N′, N′-tetramethyluronium hexafluorophosphate; DMF, dimethylformamide.*

Table 1. *Inhibitor K_i values.*

Compound	Structure	K_i [nm]
4a: X = NH **4b**: X = 0		96 70
10		311
11		30
12		28
17		12
3		9
2		6
1		5

lyzed by ESI-MS to ensure the presence of the desired product. The solutions were then diluted and screened directly for β-AST-IV inhibition.

In preliminary studies, compounds of type **5a**–**f** with linker lengths of 2, 10, and 12 carbon atoms (**5a**, **e**, **f**) were found to provide the best inhibition. We focused on compounds containing a 10-carbon linker (**5e**) and created an expanded library by using acids **A1**–**A38** (Table 2). An initial 100 μM screen revealed that the compounds produced by coupling benzoic acids containing a hydroxy group (**A31**, **A35**) to adenine exhibited by far the best inhibition (Figure 1A). A 10 μM follow-up screen was undertaken with hydroxybenzoic acids **A39**–**A60**. Evaluation of the coupling reaction products, initially by ESI-MS and then by product isolation, revealed that the hydroxy-containing acids yielded minor amounts of products formed by oligomerization at the oxygen atom. However, treatment of the crude reaction products with base led to ester hydrolysis to form the desired products. Reaction mixtures that had been treated with base were generally about 40% more effective at inhibiting the enzyme than those not treated in this way, which indicates that the monomeric species are better inhibitors than the oligomers. In this follow-up screen, acids containing a hydroxy group in the 3-position relative to the carboxy group were consistently found to be the best inhibitors (Figure 1B). Maximal inhibition was detected for the coupling product of 4-fluoro-3-hydroxybenzoic acid (**A50**), although treatment of the enzyme with this acid by itself yielded no inhibition.

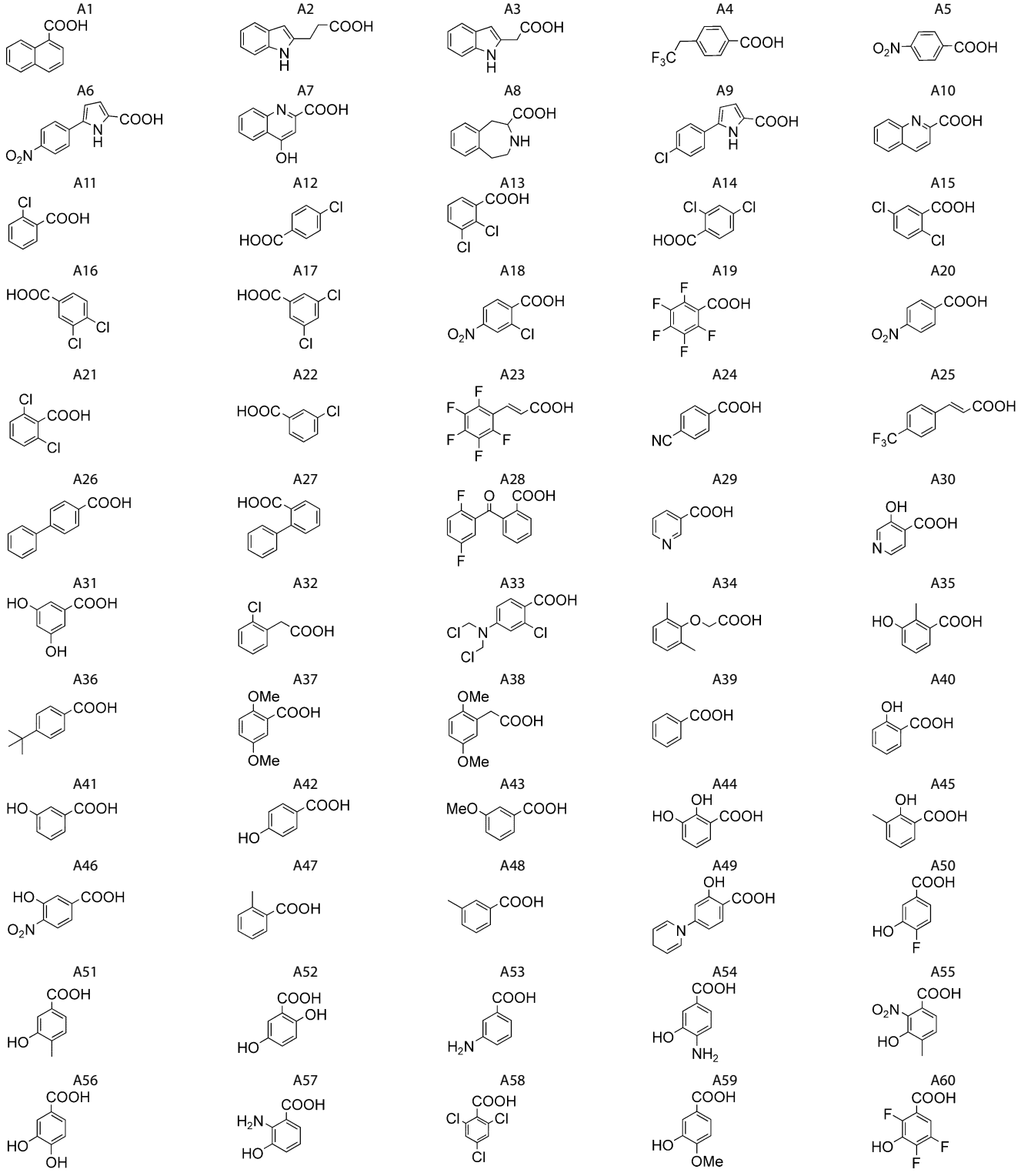
We next re-evaluated the effect of linker length on inhibition by screening the compounds produced by coupling amines **9a**–**f** with 4-fluoro-3-hydroxybenzoic acid. The results obtained by screening 1 μM inhibitor samples are summarized in Figure 1C. A linker length of two carbon atoms (**10**) leads to good inhibition, while use of a four-carbon linker leads to a significantly lower inhibitory activity. The results then follow a trend of gradually increasing inhibition with increasing linker length up to the longest linker tested (12 carbon atoms, **11**), which had the maximal effect on enzyme activity.

After this screen, the two best inhibitors, **10** and **11**, were re-synthesized and isolated for further study. Upon analysis, both inhibitors showed competitive inhibition of the enzyme in the presence of 4MUS (Figure 2A) and mixed-type inhibition in the presence of PAP (Figure 2B). The data shown in the figure were fitted to the competitive inhibitor equation by using the Pro Fit program (Quantum Soft), and K_i values (Table 1) of 311 and 30 nM were determined for **10** and **11**, respectively. This result validates the crude screening data, which also suggested that **11** is a stronger inhibitor than **10**. Mechanistic studies involving β-AST-IV have indicated a rapid equilibrium random bi bi kinetic mechanism for the enzyme.[25] A bisubstrate inhibitor would be expected to have a competitive inhibition pattern in the presence of each substrate. The fact that the two inhibitors show the same inhibition patterns as each other with both PAP and 4MUS indicates that the inhibitors have similar binding modes, despite the 10-carbon difference in linker length.

These data contradict the hypothesis that the longer inhibitor, **11**, reaches out to inhibit enzyme sites beyond the aryl site. Instead, it is possible that the 12-carbon chain of **11** allows the molecule to fold in such a way that both the aryl and adenine groups reach into the aryl binding site. The increased binding affinity could arise from the flexibility of this inhibitor, which allows the binding groups the freedom to reach a conformation that results in strong interaction with the residues within the active site. This theory is supported by the assay data showing that the activity of the inhibitor increases with linker length and thus with linker flexibility for linkers of between 4 and 12 carbon atoms. Inhibitors with longer chain lengths could also benefit from increased hydrophobicity.

We next sought to improve upon the structure of the 30 nM β-AST-IV inhibitor developed through library screening by

Table 2. *Acids used for formation of the original combinatorial library.*



using rational modification. We knew that the presence of the 2-chloro group in **4** caused increased inhibitory activity. We therefore synthesized and evaluated **12**, which consists of **11** and an additional 2-chloro group. The synthesis of **12** is analogous to that of **11**, except 2,6-dichloropurine was used as the starting material for **12**. Compound **12** was found to have a K_i value of 18 nM.

We also sought to improve inhibition of the enzyme through replacement of the adenine group in the inhibitor. To do this, a "reverse library" was synthesized that contained mol-

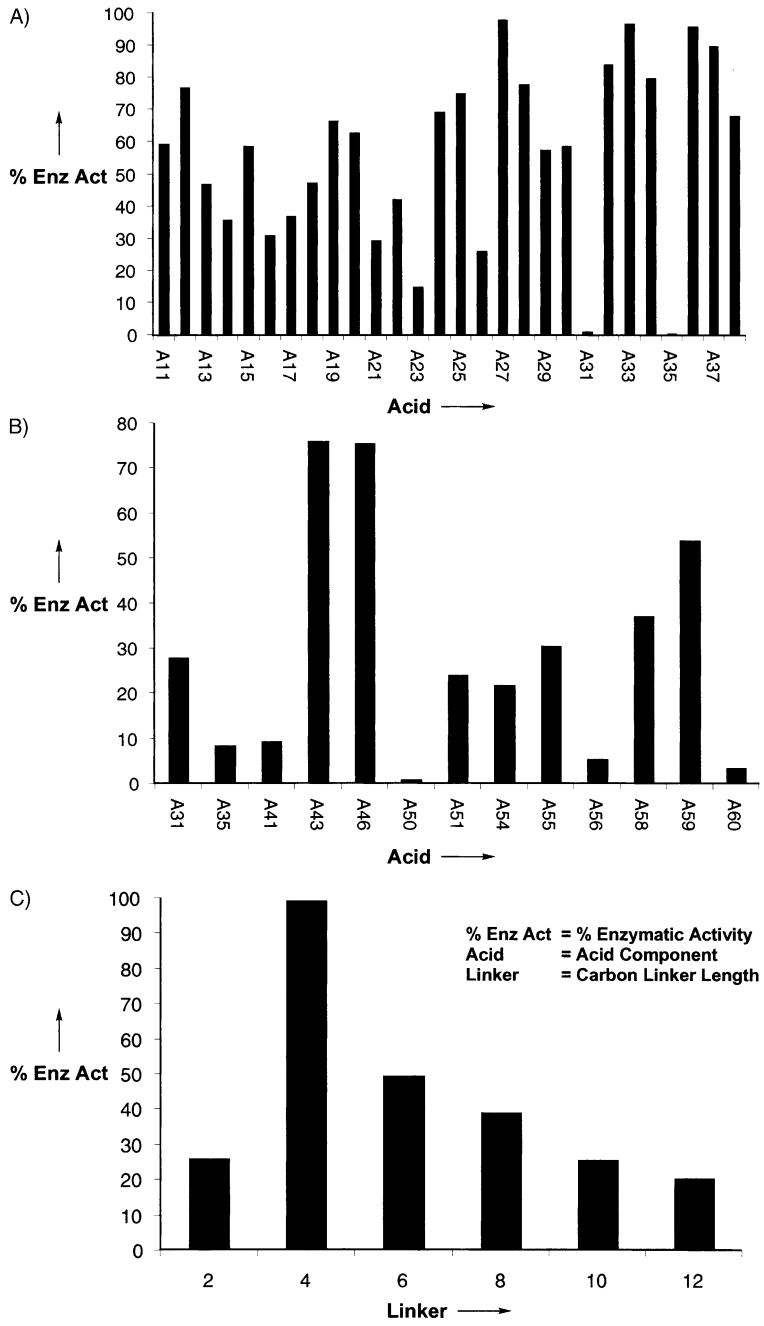


Figure 1. *β-AST-IV inhibitor screens. A) Screen of initial library at 100 μM inhibitor. B) Screen of follow-up library at 10 μM inhibitor. C) Linker-length study with optimal aryl group coupling products, screened at 1 μM.*

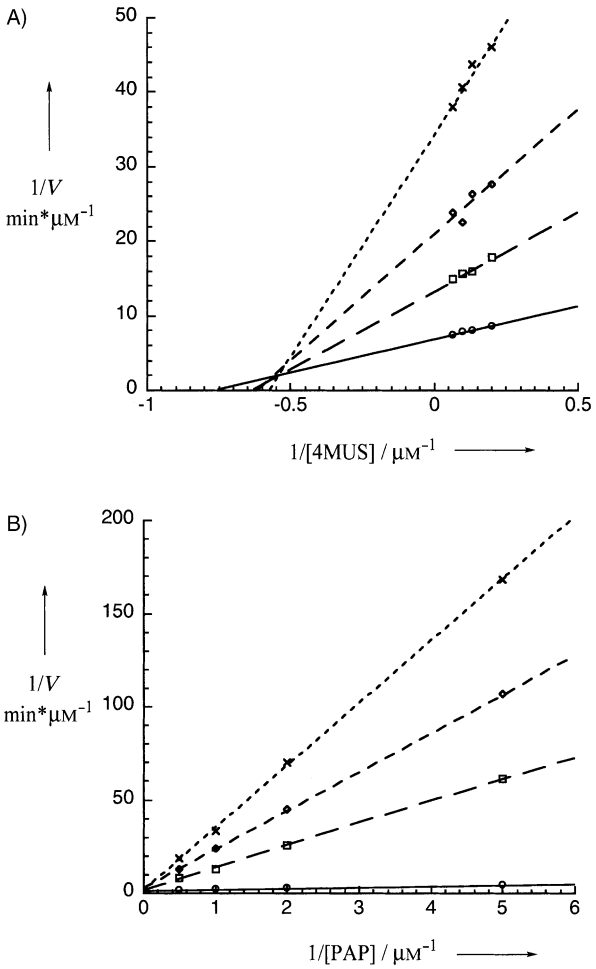


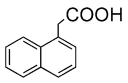
Figure 2. *Analysis of β-AST-IV inhibition by **11**. A) Reciprical rate vs. reciprical 4MUS concentration with 0, 0.5, 0.75, and 1 μM **11**. B) Reciprical rate vs. reciprical PAP concentration with 0, 150, 200, and 250 nM **11**.*

ecules of type **13** (Scheme 2B), in which the 4-fluoro-3-hydroxybenzoic acid group is coupled to an acid by a 12-carbon linker. We focused this library on heteroatom-containing biaryl acids because of the success of adenine. A series of screens were performed with acids **A61**–**A95** (Table 3). Coupling of 4-fluoro-3-hydroxybenzoic acid to indole 3-acetic acid (**A68**) yielded a product (**17**, $K_i = 12$ nM) that showed promise. The corresponding products of indole-3-propionic acid (**A75**) and indole-3-butyric acid (**A81**) gave lower inhibition, which suggests that the optimal linker length is that found in **17**.

A follow-up screen (Figure 3) was carried out with compounds produced from indole-containing and related acids **A96**–**A106**. Coupling to 5-fluoroindole-2-carboxylic acid (**A105**), 4,8-dihydroxyquinoline (**A103**), and 4-fluoro-3-hydroxybenxoic acid (**A106**) yielded compounds **1**, **2**, and **3**, with K_i values of 5, 6, and 9 nM, respectively. Compound **1** represents the most potent inhibitor of β-AST-IV developed to date, with a K_i value over five orders of magnitude lower than the Michaelis constant, K_m, of the substrate whose binding it inhibits.

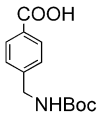
Overall, three factors involved in inhibitory activity, linker length and the identities of the two binding groups, were optimized rapidly by using repetitive library formation and in situ screening on microplates. We focused on one factor at a time and used multiple rounds of studies to improve the inhibitor structure. Once each factor had been optimized, we moved on to improve the next part of the inhibitor. In doing so, we developed not only a very potent inhibitor, but an understanding of the requirements for inhibition, since all the best inhibitors contained two heteroatom or halogen-functionalized aryl moi-

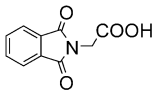
Table 3. *Heteroatom-containing biaryl acids used to replace the original adenine group in reverse library formation.*

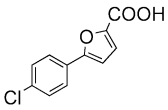
A61 

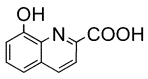
A62 

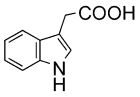
A63 

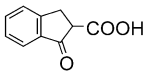
A64 

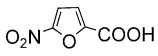
A65 

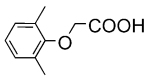
A66 

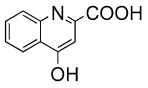
A67 

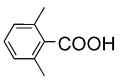
A68 

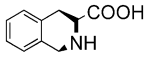
A69 

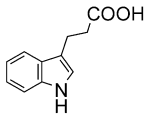
A70 

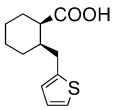
A71 

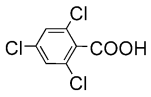
A72 

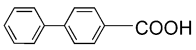
A73 

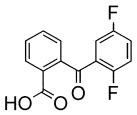
A74 

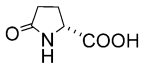
A75 

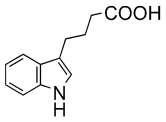
A76 

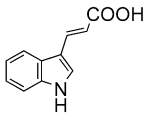
A77 

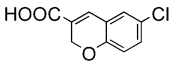
A78 

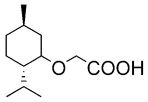
A79 

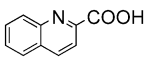
A80 

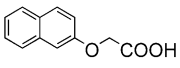
A81 

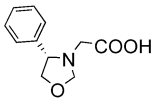
A82 

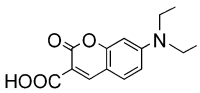
A83 

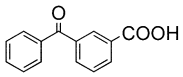
A84 

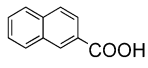
A85 

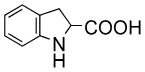
A86 

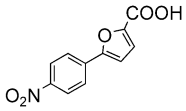
A87 

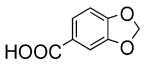
A88 

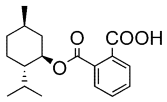
A89 

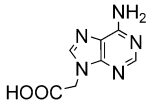
A90 

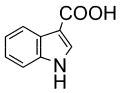
A91 

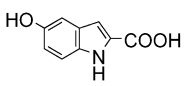
A92 

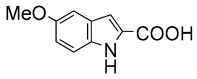
A93 

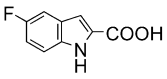
A94 

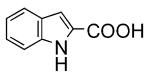
A95 

A96 

A97 

A98 

A99 

A100 

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eties attached to one another by 12-carbon linkers. This approach effectively provides a blueprint for the inhibition of β-AST-IV and a strategy for the inhibition of other sulfotransferases such as the tyrosylprotein STs with aromatic sulfonate substrates. We are currently evaluating the usefulness of this approach for the inhibition of other STs. We are also developing novel methods for the inhibition of β-AST-IV.

Experimental Section

General: Compounds were generally purchased from Aldrich. The solvents MeOH and DMF were obtained as anhydrous products from Aldrich. THF was distilled over sodium and benzophenone ketyl, and dichloromethane was distilled over calcium hydride. NMR spectra were obtained on Varian Mercury 300 and Inova 400 instruments. Mass spectrometry results were obtained by using an IonSpec Ultima instrument for MALDI-FTMS measurements, and a Perkin Elmer API 100 Sciex single quadrupole instrument for ESI-

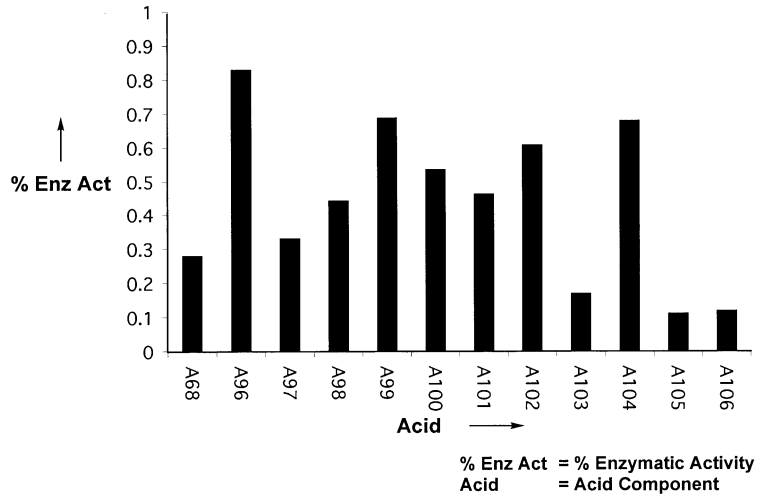

Figure 3. *Final screen for reverse library in which the adenine group is replaced.*

MS experiments. Products were generally placed under a high vacuum overnight prior to characterization. Enzymatic reaction rates were determined by measurement of the production of 4-methylumbelliferone with a Packard Fusion 96-well plate reader and a Hitachi F-2000 fluorescence spectrophotometer. Some studies were also performed by using *p*-nitrophenolsulfate as a substrate and taking measurements with a Beckman DU-650 absorbance spectrophotometer.

***N*-Boc-1,12-diaminododecane (7 f)**: 1,12-Diaminododecane (3.86 g, 19.24 mmol) was added to a 250 mL flame-dried round-bottomed flask and dissolved in distilled dichloromethane (25 mL). Boc anhydride (0.7 g, 3.21 mmol) was dissolved separately in dichloromethane (75 mL) in a 100 mL flame-dried round-bottomed flask. The Boc anhydride solution was then placed in a 100 mL addition funnel and added dropwise to the diamine solution. The resulting mixture was stirred overnight. The crude reaction product was then extracted with water (3×100 mL), dried with magnesium sulfate, and filtered, and the solvent was removed by rotary evaporation. The resulting white solid was purified by column chromatography (SiO_2, 6% MeOH/dichloromethane) then dissolved in 3% MeOH/dichloromethane and filtered to remove any SiO_2 and leave pure **7 f** (685 mg, 71%). ^{1}H NMR: (CD_3OD, 300 MHz): $\delta = 3.02$ (t, 2H, $J = 6.9$ Hz), 2.60 (t, 2H, $J = 6.9$ Hz), 1.44 (s, 13H), 1.31 ppm (s, 16H). ^{13}C NMR: (CD_3OD, 300 MHz): $\delta = 157.24$, 78.59, 41.55, 40.35, 32.81, 29.99, 29.72, 29.70, 29.64, 29.45, 27.84, 27.06, 26.89 ppm. MALDI-FTMS: $[M+Na]^+$ calcd: 323.2669; found: 323.2669.

6-(2-*N*-Boc-aminoethylamino)purine (8 a): 6-Chloropurine (**6**, 181 mg, 1.2 mmol), mono-*N*-Boc-ethylenediamine (370 μL, 2.3 mmol), and diisopropylethylamine (409 μL, 2.3 mmol) were dissolved in distilled methanol (15 mL) in a flame-dried 100 mL round-bottomed flask. The contents of the flask were heated to 70°C under reflux for 48 h and the solvent was then removed by rotary evaporation. The product was purified by column chromatography (SiO_2, 6% MeOH/dichloromethane) then dissolved in 3% MeOH/dichloromethane, filtered to remove any SiO_2, and isolated as a white solid (170 mg, 52%). ^{1}H NMR: (CD_3OD, 300 MHz): $\delta =$ 8.21 (s, 1H), 8.04 (s, 1H), 3.72 (t, 2H, $J = 6.9$ Hz), 3.34 (t, 2H, $J =$ 6.9 Hz), 1.38 ppm (s, 9H). MALDI-FTMS: $[M+H]^+$ calcd: 279.1564; found: 279.1567.

6-(12-*N*-Boc-aminododecylamino)purine (8 f): 6-Chloropurine (**6**, 170 mg, 1.1 mmol), mono-*N*-Boc-1,12-diaminododecane (**7 f**, 331 mg, 1.1 mmol), and diisopropylethylamine (191 μL, 2.2 mmol) were dissolved in distilled methanol (12 mL) in a flame-dried 100 mL round-bottomed flask. The contents of the flask were heated to 70°C under reflux for 48 h and the solvent was then removed by rotary evaporation. The product was purified by column chromatography (SiO_2, 5% MeOH/dichloromethane), dissolved in 3% MeOH/dichloromethane, and filtered to remove any SiO_2. **8 f** was isolated as a white solid (461 mg, 51%). ^{1}H NMR: (CD_3OD, 300 MHz): $\delta = 8.42$ (s, 1H), 7.97 (s, 1H), 3.68 (m, 2H), 3.08 (m, 2H), 1.71 (m, 2H), 1.44 (s, 13H), 1.24 ppm (s, 14H). MALDI-FTMS: $[M+H]^+$ calcd: 419.3129; found: 419.3128.

6-((2-ammonium trifluroacetate)ethylamino)purine (9 a): Compound **8 a** (170 mg, 611 μmol) was dissolved in dichloromethane (2 mL) and trifluoroacetic acid (2 mL) and stirred at RT for three hours. The solvent was then removed and the resulting product was coevaporated twice with methanol and benzene, then once with dichloromethane. The resulting trifluoroacetate salt was obtained as an orangish solid (175 mg, 99%). ^{1}H NMR: (CD_3OD, 300 MHz): $\delta = 8.55$ (s, 1H), 8.42 (s, 1H), 3.36 (m, 2H), 1.37 ppm (m, 2H). MALDI-FTMS: $[M+H-TFA\ salt]^+$ calcd: 179.104; found: 179.1037.

6-((12-ammonium trifluroacetate)dodecylamino)purine (9 f): Compound **8 f** (235 mg, 56 μmol) was dissolved in dichloromethane (2 mL) and trifluoroacetic acid (2 mL) and stirred at RT for three hours. The solvent was then removed and the resulting product was coevaporated twice with methanol and benzene, then once with dichloromethane. The resulting trifluoroacetate salt was obtained as a white solid (240 mg, 99%). ^{1}H NMR: (CD_3OD, 300 MHz): $\delta = 8.48$ (s, 1H), 8.32 (s, 1H), 2.90 (m, 2H), 1.65 (m, 4H), 1.27 ppm (s, 18H). MALDI-FTMS: $[M+H-TFA\ salt]^+$ calcd: 319.2605; found: 319.2603.

6-(2-(*N*-4-fluoro-3-hydroxybenzoylamino)ethylamino)purine (10): 4-Fluoro-3-hydroxybenzoic acid (**14**; 24 mg, 154 μmol) was dissolved in DMF (1 mL) in a conical vial. Compound **9 a** (30 mg, 103 μmol) was dissolved separately in DMF (2 mL) then diisopropylethylamine (27 μL, 205 μmol) was added. HBTU (59 mg, 154 μmol) was added to the solution of acid **14**. The amine **9 a** solution was added to this mixture after 2 minutes. The reaction mixture was stirred overnight then 1 M NaOH (2.05 mL, 20 equiv) was added. The solution was stirred overnight again and the solvent was then removed by rotary evaporation. The product was purified by column chromatography (SiO_2, 7% MeOH/dichloromethane/0.1% acetic acid), dissolved in 3% MeOH/dichloromethane, and filtered to remove any SiO_2. Compound **10** was obtained as a white solid (18 mg, 55%). ^{1}H NMR: (*d*-6 dimethylsulfoxide (DMSO), 300 MHz): $\delta = 8.59$ (s, 1H), 8.17 (s, 1H), 7.46 (dd, 1H, $J = 7.0$, 1.8 Hz), 7.20 (m, 1H), 7.16 (dt, 1H, $J = 9.9$, 2.7 Hz), 3.48 (m, 2H), 3.23 ppm (m, 2H). MALDI-FTMS: $[M+H]^+$ calcd: 317.1157; found: 317.1159.

6-(12-(*N*-4-fluoro-3-hydroxybenzoylamino)dodecylamino)purine (11): 4-Fluoro-3-hydroxybenzoic acid (**14**; 11 mg, 69 μmol) was dissolved in DMF (1 mL) in a conical vial. Compound **9 f** (20 mg, 46 μmol) was dissolved separately in DMF (2 mL) then diisopropylethylamine (16 μL, 92 μmol) was added. HBTU (26 mg, 69 μmol) was added to the solution of acid **14**, and the amine **9 f** solution was added after 2 minutes. The reaction mixture was stirred overnight then 1 M NaOH (925 μL, 20 equiv) was added. The solution was stirred overnight again and the solvent removed by rotary evaporation. The product was purified by column chromatography

(SiO_2, 5% MeOH/dichloromethane/0.1% acetic acid), dissolved in 3% MeOH/dichloromethane, and filtered to remove any SiO_2. Compound **11** was obtained as a white solid (21 mg, 49%). ^{1}H NMR: ([D_6]DMSO, 300 MHz): $\delta = 8.19$ (s, 1H), 8.03 (s, 1H), 7.36 (dd, 1H, $J = 8.7$, 2.1 Hz), 7.22 (m, 1H), 7.08 (dt, 1H, $J = 10.8$, 2.4 Hz), 3.53 (m, 2H), 3.30 ppm (m, 2H). MALDI-FTMS: $[M+H]^+$ calcd: 457.2722; found: 457.2731.

2-Chloro-6-(12-(*N*-4-fluoro-3-hydroxybenzoylamino)dodecylamino)purine (12): 4-Fluoro-3-hydroxybenzoic acid (**14**; 51 mg, 328 μmol) was dissolved in DMF (2 mL) in a conical vial. 2-Chloro-6-((12-ammonium trifluroacetate)dodecylamino)purine (92 mg, 197 μmol) was dissolved separately in DMF (2 mL) then diisopropylethylamine (114 μL, 655 μmol) was added. HBTU (124 mg, 328 μmol) was added to the solution of acid **14**, and the amine solution was added after 2 minutes. The reaction mixture was stirred overnight then 1 M NaOH (2.18 mL, 10 equiv) was added. The solution was stirred overnight again and the solvent removed by rotary evaporation. The product was purified by column chromatography (SiO_2, 4% MeOH/dichloromethane/0.1% acetic acid). Compound **12** was obtained as a white solid (9 mg, 9%). ^{1}H NMR: (CD_3OD, 300 MHz): $\delta = 7.91$ (s, 1H), 7.27 (dd, 1H, $J = 8.4$ Hz, 1.5 Hz), 7.13 (m, 1H), 6.98 (m, 1H), 3.53 (m, 2H), 3.27 (m, 2H), 1.58 (m, 4H), 1.19 ppm (s, 16H). MALDI-FTMS: $[M+H]^+$ calcd: 491.2332; found: 491.2332.

12-*N*-Boc-1-*N*-(4-fluoro-3-hydroxybenzoyl)diaminododecane (15): 4-fluoro-3-hydroxybenzoic acid (**14**; 139 mg, 889 μmol) was dissolved in DMF (4 mL). *N*-Boc-1,12-diaminododecane (**7 f**, 178 mg, 592 μmol) was dissolved in DMF (4 mL) and DIEA (207 μL, 1.18 mmol) was added. HBTU (337 mg, 889 μmol) was added to the acid solution and after 2 minutes, the amine solution was added. The reaction mixture was stirred at RT overnight then 1 M NaOH (5.92 mL, 5.92 mmol) was added. The solution was stirred overnight again, the solvent was removed by rotary evaporation, and the product was purified by column chromatography (SiO_2, 40% EtOAc/hexanes). Compound **15** was obtained as a white solid (260 mg, 60%). ^{1}H NMR (CD_3OD, 300 MHz): $\delta = 7.39$ (dd, $J = 6.3$ Hz, 1.8 Hz, 1H), 7.25 (m, 1H), 7.11 (dt, $J = 7.2$ Hz, 1.8 Hz, 1H), 6.55 (br s, 1H), 3.32 (t, $J = 7.2$ Hz, 2H), 3.00 (t, $J = 6.6$ Hz, 2H), 1.59 (m, 4H), 1.42 (s, 9H), 1.29 ppm (m, 16H). MALDI-FTMS: $[M+Na]^+$ calcd: 461.2786; found: 461.2789.

12-*N*-(4-fluoro-3-hydroxybenzoylamino)dodecylammonium trifluoroacetate (16): Compound **15** (157 mg, 358 μmol) was dissolved in dichloromethane (2 mL) and trifluoroacetic acid (2 mL) and the reaction mixture was stirred for 3 h. The solvent was removed and the crude product was dissolved twice in 50% benzene/methanol and once in dichloromethane. The solvent was again removed and compound **16** was obtained as a white solid (141 mg, 87%). ^{1}H NMR: (CD_3OD, 300 MHz): $\delta = 7.39$ (dd, $J = 8.4$, 2.1 Hz, 1H), 7.24 (m, 1H), 7.09 (dt, $J = 9.75$, 2.1 Hz, 1H), 3.33 (t, $J = 6.6$ Hz, 2H), 2.89 (t, $J = 7.8$ Hz, 2H), 1.61 (m, 4H), 1.31 ppm (s, 16H). MALDI-FTMS: $[M+H]^+$ calcd: 339.2442; found: 339.2448.

1-*N*-(4-fluoro-3-hydroxybenzoyl)-1-*N*-(indole-3-acetyl)diaminododecane (17): 3-indoleacetic acid (**A68**; 5.8 mg, 33 μmol) was dissolved in DMF (500 μL) and HBTU (12.6 mg, 33 μmol) was added. After 2 minutes, amine **16** (10 mg, 22 μmol) and DIEA (8 μL, 44 μmol) were added. The reaction mixture was stirred overnight and the solvent removed by rotary evaporation. The product was purified by column chromatography (SiO_2, 4% MeOH/dichloromethane) and compound **17** was obtained as a white solid (6.7 mg, 61%). ^{1}H NMR: (CD_3OD, 300 MHz): $\delta = 7.96$ (br s, 1H), 7.63 (br s, 1H), 7.51 (d, $J = 7.8$ Hz, 1H), 7.38 (dd, $J = 8.4$, 2.1 Hz, 1H), 7.33 (d, $J = 8.1$ Hz, 1H), 7.23 (m, 1H), 7.16 (s, 1H), 7.09 (m, 2H), 7.02 (dt, $J = 7.8$, 0.9 Hz, 1H), 3.64 (s, 2H), 3.17 (m, 4H), 1.61 (m, 4H), 1.23 ppm (m, 16H). MALDI-FTMS: $[M+H]^+$ calcd: 496.2970; found: 496.2951.

1-*N*-(4-fluoro-3-hydroxybenzoyl)-1-*N*-(5-fluoroindole-3-acetyl)diaminododecane (1): 5-Fluoroindole-3-acetic acid (**A105**; 16 mg, 83 μmol) was dissolved in DMF (1 mL) and HBTU (31 mg, 83 μmol) was added. After 2 minutes, amine **16** (25 mg, 55 μmol) and DIEA (19 μL, 110 μmol) were added and the reaction mixture was stirred for 6 h. 1 M NaOH (1.1 mL, 1.1 mmol) was added and the solution was left overnight. The solvent was then removed and the product purified by column chromatography (SiO_2, 4% MeOH/dichloromethane), which yielded compound **1** as a white solid (23 mg, 82%). ^{1}H NMR: (CD_3OD, 300 MHz): $\delta = 7.96$ (br s, 1H), 7.78 (br s, 1H), 7.39 (d, $J = 8.4$ Hz), 7.22 (m, 4H), 7.09 (dt, $J = 10.2$, 1.8 Hz, 1H), 6.85, t, $J = 9.3$ Hz, 1H), 3.59 (s, 2H), 3.31 (t, $J = 6.6$ Hz, 2H), 3.27 (t, $J = 7.8$ Hz, 2H), 1.61 (m, 2H), 1.23 ppm (m, 18H). MALDI-FTMS: $[M+H]^+$ calcd: 514.2876; found: 514.2866.

1-*N*-(4-fluoro-3-hydroxybenzoyl)-1-*N*-(4,8-dihydroxyquinoline-2-carbonyl)-diaminododecane (2): The same procedure as that described for **1** was applied, except 4,8-dihydroxyquinoline-2-carboxylate (**A103**; 17 mg, 83 μmol) was used. The product was purified by column chromatography (SiO_2, 5–6% MeOH/dichloromethane) and obtained as a white solid (29 mg, 68%). ^{1}H NMR: (CD_3OD, 300 MHz): $\delta = 7.97$ (s, 1H), 7.66 (d, $J = 8.7$ Hz, 1H), 7.38 (dd, $J = 8.1$, 1.5 Hz, 1H), 7.32 (t, $J = 8.1$ Hz, 1H), 7.24 (m, 1H), 7.11 (m, 2H), 3.33 (t, $J = 6.9$ Hz, 2H), 3.20 (t, $J = 7.8$ Hz, 2H), 1.56 (m, 4H), 1.20 ppm (s, 16H). MALDI-FTMS: $[M+H]^+$ calcd: 526.2712; found: 526.2715.

1,12-*N*,*N*-bis(4-fluoro-3-hydroxybenzoyl)-diaminododecane (3): The same procedure as that used for **1** was applied, except 4-fluoro-3-hydroxybenzoic acid (**14**; 13 mg, 83 μmol) was used. The product was purified by column chromatography (SiO_2, 3% MeOH/dichloromethane) and obtained as a white oily solid (4.4 mg, 17%). ^{1}H NMR: (CD_3OD, 300 MHz): $\delta = 7.38$ (dd, $J = 8.1$, 1.8 Hz, 2H), 7.24 (m, 2H), 7.09 (dt, $J = 9.6$, 2.4 Hz, 2H), 3.31 (t, $J = 6.8$ Hz, 4H), 1.60 (m, 4H), 1.32 ppm (m, 16H). MALDI-FTMS: $[M+H]^+$ calcd: 477.2559; found: 477.2562.

Library formation: Library synthesis reactions were run in 1 mL Eppindorf tubes. The acids (1–2 mg, around 5 μmol) were placed in the tubes. Two stock solutions were made up, one containing HBTU (63 mg, 166 μmol) in DMF (100 μL) and the other containing the desired amine (20–40 mg, about 100 μmol) and diisopropylethylamine (about 300 μmol) in DMF (100 μL). The two stock solutions and DMF were added to each of the eppindorf tubes containing the acids in the amounts necessary to produce reaction mixtures containing 2 equiv acid and HBTU, 1 equiv amine, 3 equiv DIEA, and a total volume of 100μL. The mixtures were shaken overnight then 1 M NaOH (20 equiv) was added and the solutions were again shaken overnight. Reaction mixtures were analyzed by ESI-MS to verify the presence of the desired product. The crude mixtures were then diluted and screened.

Enzymatic assay: A 2X stock solution of 1 M tris(hydroxymethyl)-aminomethane buffer (2 mL, 200 mM, pH 7.6), 250 mM β-mercaptoethanol (400 μL, 10 mM), enzyme (10 μL), and water (7.59 mL) was formulated. The stock solution also contained PAP in experiments in which the PAP concentration had to remain constant. Inhibitor, PAP, and 4MUS solutions were diluted (10×) to the desired final concentration. The inhibitors were dissolved in DMSO. For plate reader measurements, a final volume of 200 μL was used, while 500-μL solutions were studied on the Hitachi spectrophotometer. For the plate reader measurements, enzyme stock solution (100 μL), inhibitor and PAP solutions (20 μL each), and water

(40 μL) were combined, mixed, and left to stand for 10 minutes. The reaction was initiated by addition of 4MUS (20 μL) solution and reaction rates were recorded for 5 minutes. Experiments with the Hitachi spectrophotometer were similar: stock solution (250 μL), inhibitor, PAP, and 4MUS (50 μL each), and water (100 μL) were used. Inhibitor concentrations were chosen such that the enzyme-catalyzed reaction rates were linearly related to these concentrations. Inhibitor concentrations between 100 nM and 1 μM were employed for K_i measurements. The resulting data were analyzed to determine K_i values by using the Pro Fit software (Quantum Soft). Multiple K_i values were determined and the results were averaged to yield the reported values.

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