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Boronic acids as inhibitors of steroid sulfatase

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Abstract—Steroid sulfatase (STS) catalyzes the hydrolysis of steroidal sulfates such as estrone sulfate (ES1) to the corresponding steroids and inorganic sulfate. STS is considered to be a potential target for the development of therapeutics for the treatment of steroid-dependent cancers. Two steroidal and two coumarin- and chromenone-based boronic acids were synthesized and examined as inhibitors of purified STS. The boronic acid analog of estrone sulfate bearing a boronic acid moiety at the 3-position in place of the sulfate group was a good competitive STS inhibitor with a K_i of 2.8 μM at pH 7.0 and 6.8 μM at pH 8.8. The inhibition was reversible and kinetic properties corresponding to the mechanism for slow-binding inhibitors were not observed. An estradiol derivative bearing a boronic acid group at the 3-position and a benzyl group at the 17-position was a potent reversible, non-competitive STS inhibitor with a K_i of 250 nM. However, its 3-OH analog, a known STS inhibitor, exhibited an almost identical affinity for STS and also bound in a non-competitive manner. It is suggested that these compounds prefer to bind in a hydrophobic tunnel close to the entrance to the active site. The coumarin and chromenone boronic acids were modest inhibitors of STS with IC_{50}s of 86 and 171 μM, respectively. Surprisingly, replacing the boronic acid group of the chromenone derivative with an OH group yielded a good reversible, mixed type inhibitor with a K_i of 4.6 μM. Overall, these results suggest that the boronic acid moiety must be attached to a platform very closely resembling a natural substrate in order for it to impart a beneficial effect on binding affinity compared to its phenolic analog.

1. Introduction

Steroid sulfatase (STS), also known as aryl sulfatase C (ARSC) or estrone sulfatase, catalyzes the hydrolytic desulfation of steroidal sulfates, such as dehydroepiandrosterone sulfate (DHEAS) or estrone sulfate (E1S), to the corresponding steroids and inorganic sulfate.[1] These sulfated species are considered to be the storage form of estrone (E1) and dehydroepiandrosterone (DHEA) in breast tumor and other cells.[1] Approximately one-third of breast tumors in post-menopausal women require stimulation by estrogens for optimal growth.[1] Consequently, considerable interest has arisen in designing inhibitors of STS anticipating that such inhibitors could be developed into therapeutics for the treatment of estrogen-dependent breast cancer and other estrogen-dependent diseases.[2]

Many inhibitors of STS have been reported.[2,3] The vast majority are aryl sulfamates ($Ar–SO_2NH_2$) which act as irreversible suicide inhibitors.[2] The S–O bond of the sulfamate is hydrolyzed by the enzyme resulting in the release of the phenolic portion of the inhibitor and sulfamic acid which then irreversibly inhibits ES by a yet unknown mechanism. EMATE (**1**),[4] 667-COUMATE (**2**)[5], and chromenone **3**[6] are examples of this class of highly potent inhibitors. 667-COUMATE has recently successfully completed phase I clinical trials for the treatment of breast cancer.[7]

In comparison to irreversible sulfamate inhibitors, far fewer reversible STS inhibitors have been developed.[2] Perhaps the most noteworthy are those developed by Poirier and coworkers who reported that certain 17α-benzyl-substituted estradiol derivatives, such as **4** and **5**, are reversible STS inhibitors. Some of these compounds, which were tested using homogenates of JEG-3 cells, are the most potent reversible inhibitors reported to date with IC_{50}s in the low-nM region.[8,9] The modality of inhibition was not reported. Several non-steroidal competitive and non-competitive reversible STS inhibitors have been obtained from high throughput screening of compound libraries.[10–12] Although some of these compounds exhibited good potency in vitro, their potency in cell-based assays was much lower.

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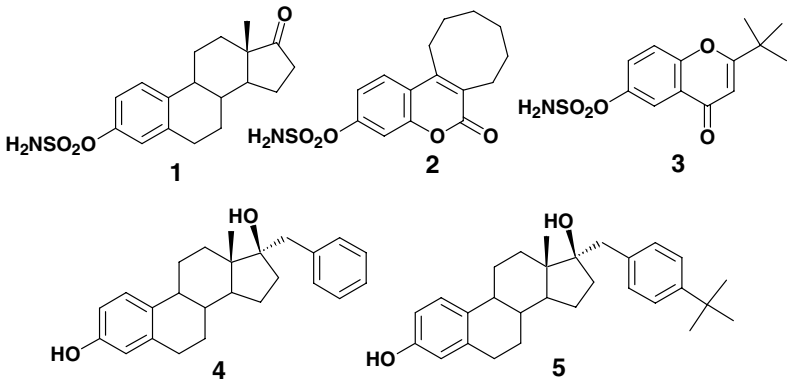
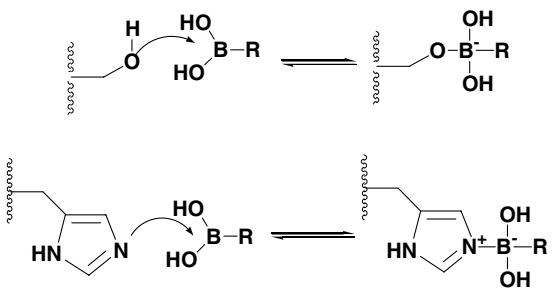
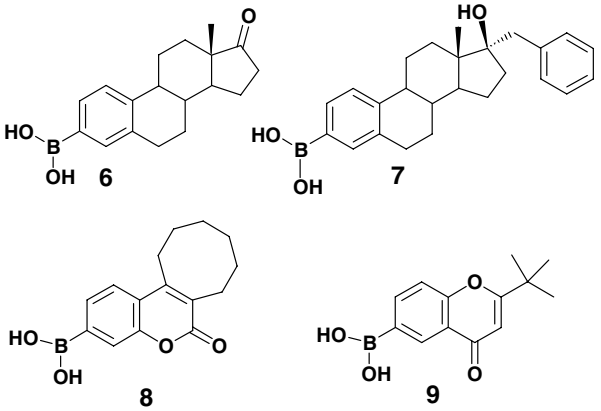


Many reversible STS inhibitor inhibitors have been obtained by replacing the sulfate group of estrone or estradiol with O-, N-, or S-linked sulfate surrogates.[2] However, for the most part, these have not proven to be highly effective inhibitors with the vast majority exhibiting K_is or IC_{50}s in mid-to low μM range with the better ones being around 10 μM. However, the vast majority of these were never examined with pure enzyme and the modality of inhibition was not determined.[13]

Boronic acids have been used as inhibitors and probes of enzymes and proteins, such as serine proteases, for many years and recently, a highly potent and selective protease inhibitor in the form of a peptidyl boronic acid has recently been approved by the FDA for treatment of relapsed and refractory multiple myeloma.[16] These inhibitors, some of which have K_is in the subnanomolar range, function by forming reversible covalent adducts with active site residues, such as the crucial serine residue or an active site histidine residue as shown in Scheme 1.[16] Unlike serine proteases, ARSs do not have an active site serine residue when in their catalytically active form. Instead, all ARSs have an active site formylglycine hydrate which is a result of a post-translational enzymatic modification of a cysteine or serine residue. Addition of water to the aldehyde yields the stable formylglycine hydrate.[17,18] The structures of STS[19] and several other sulfatases[20] have been elucidated by X-ray crystallography. There is a very high degree of sequence and structural homology at the active site such that their active sites are almost superimposable hence it is believed that all ARSs function by a similar mechanism. Several mechanisms have been proposed for aryl sulfatases.[21] On the basis of crystal structures of aryl sulfatase A (ASA) as well as on kinetic studies on the wild type and specific mutants, a mechanism has been proposed by von Figura and coworkers that is now the most widely accepted mechanism for ARSs (Scheme 2).[22,23] One of the hydroxyls of the formylglycine hydrate attacks the sulfur atom of the substrate resulting in cleavage of the S–O bond, release of the hydroxyl or phenolic portion of the substrate, and formation of a sulfated hydrate. The sulfate group is then eliminated from the hydrate to give inorganic sulfate and formyl glycine which is then rehydrated (Scheme 2). Several other active site residues, including two conserved histidines, are believed to function as general acids and bases during the reaction. In light of this proposed mechanism, we reasoned that boronic acids might act as potent inhibitors of STS by forming reversible covalent adducts with the active site hydrate and/or histidine residues in a manner similar to serine proteases. Here, we report the results of our studies on the inhibition of STS with steroidal and non-steroidal boronic acids **6**–**9**.



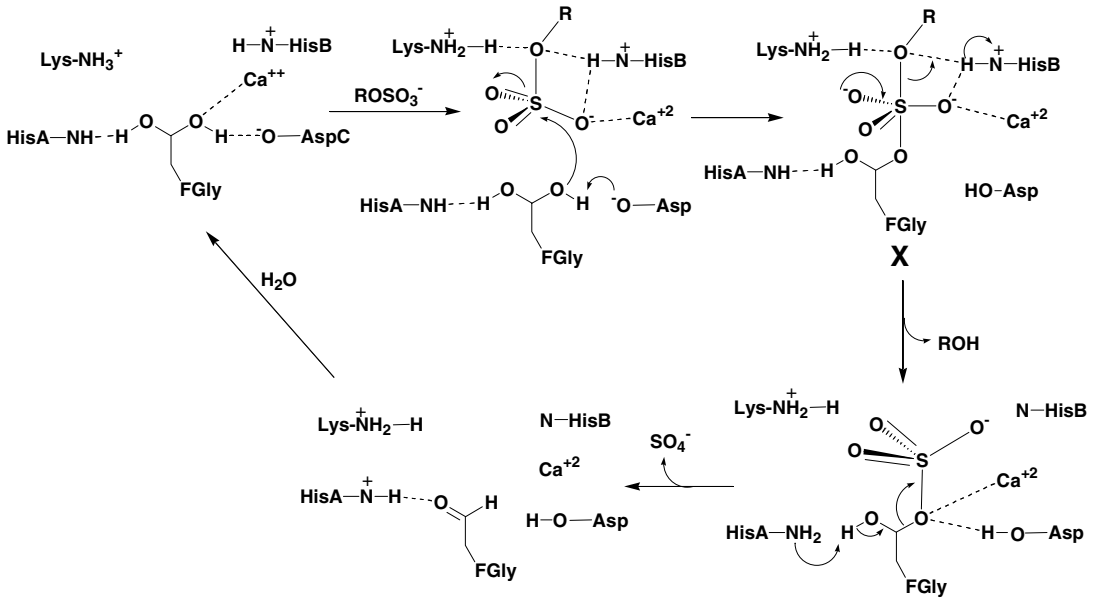
Scheme 1. Mechanism of inhibition of serine proteases by boronic acids.



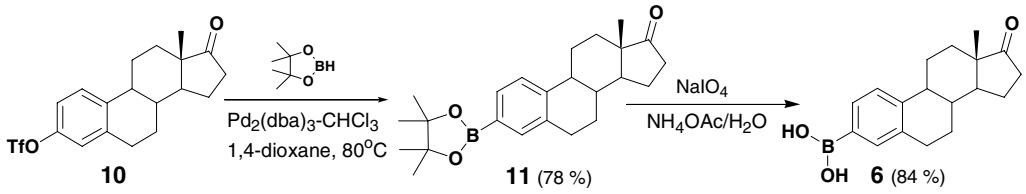
2. Results and discussion

2.1. Syntheses

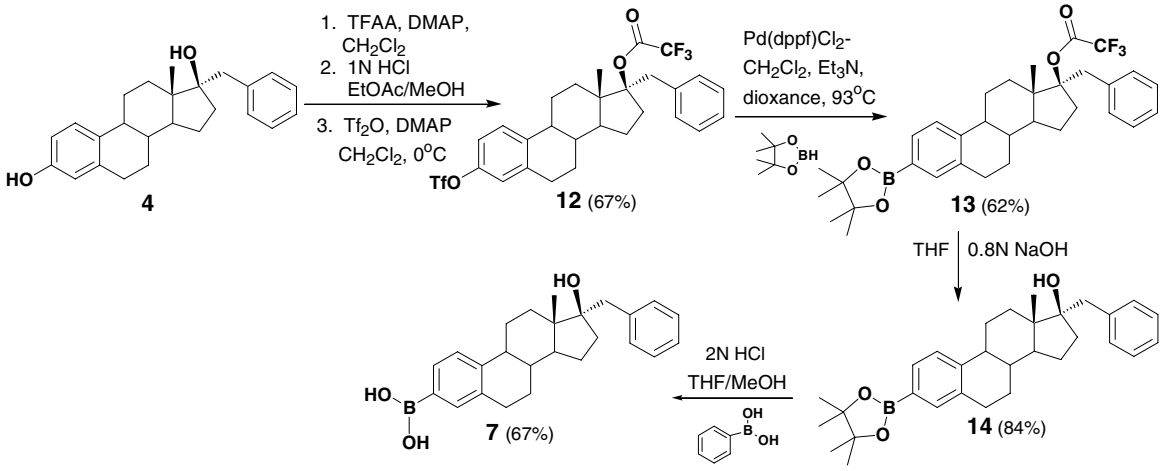
Two steroidal boronic acids were prepared. Steroid derivative **6** in which the 3-OH of estrone was replaced



Scheme 2. Proposed mechanism for aryl sulfatases.



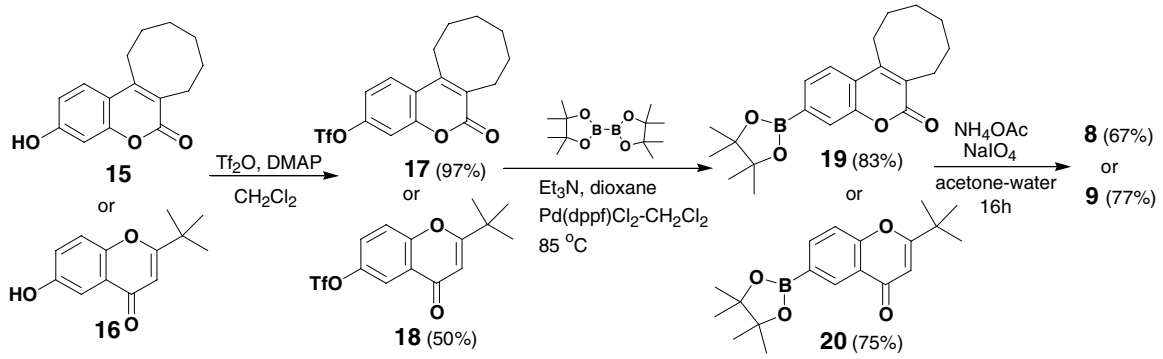
Scheme 3. Synthesis of boronic acid **6**.



Scheme 4. Synthesis of compound **7**.

with $B(OH)_2$ was readily synthesized by first reacting triflate **10**[24] with pinacolborane in the presence of a palladium catalyst to give boronate ester **11** in 78% yield. Reaction of the ester with $NaIO_4$ in ammonium acetate–water[25] for 10 days gave compound **6** in 84% yield (Scheme 3).

Since it has been shown that compound **4** is a very good inhibitor of STS,[8,9] we prepared the boronic acid analog of **4**, compound **7**, as outlined in Scheme 4. Compound **4**[9] was reacted with trifluoroacetic anhydride which gave the trifluoroacetate diester of **4** at 3- and 17-positions. Selective hydrolysis of the ester at the 3-position with aq 1 N HCl in EtOAc–MeOH followed by reaction with triflic anhydride gave compound **12** in 67% yield (three steps). Introduction of the pinacol borate ester as described for compound **11** gave compound **13** in 62% yield.[26] Hydrolysis of the ester at the 17-position with aq NaOH in THF gave compound **14** in 84% yield. Hydrolysis of the pinacol boronate ester with 2 N aq



Scheme 5. Synthesis of compounds **8** and **9**.

HCl in the presence of phenylboronic acid in THF–MeOH[27] gave the desired boronic acid **7** in 67% yield.

Since the coumarin and chromenone platforms have proven to be effective for obtaining highly potent sulfamate inhibitors of STS, we also prepared coumarin boronic acid **8** and chromenone boronic acid **9** as outlined in Scheme 5. Coumarin **15**[28] and chromenone **16**[29] were converted to triflates **17** and **18** in 97% and 50% yield, respectively. Attempts to convert these triflates to the corresponding boronate esters **19** and **20** using the conditions described for **11** and **14** proved unsuccessful. However, when bis(pinacolato)diboron was used in place of pinacolborane the coumarin and chromenone derivative **19** and **20** were readily prepared in 83% and 75% yield, respectively. Removal of the pinacol group as described above for **6** gave the desired boronate esters **8** and **9** in 67% and 77% yield, respectively.

2.2. Inhibition studies

Inhibition studies were carried out using purified STS in Tris buffer containing 5% DMSO with 4-methylumbelliferyl-6-*O*-sulfate (MUS) as substrate. K_is or IC_{50}s determined at pH 7.0 for boronic acids **6**–**9** as well as for estrone, steroid derivative **4**, and chromenone **16** are given in Table 1.

Table 1. K_is or IC_{50}s for compounds **4**, **6**–**9**, **16**, and estrone

Inhibitor	pH	K_i or IC_{50} (μM)	αK_i (μM)[e]
6	7.0	2.8 ± 0.4[a]	NA[c]
6	7.5	2.1 ± 0.3[a]	NA[c]
6	8.0	3.8 ± 0.5[a]	NA[c]
6	8.5	7.0 ± 0.6[a]	NA[c]
6	8.8	6.8 ± 0.8[a]	NA[c]
7	7.0	0.25 ± 0.02[a]	0.30 ± 0.02
8	7.0	171 ± 9[b]	ND[d]
9	7.0	86 ± 4[b]	ND[d]
Estrone	7.0	63 ± 2[a]	111 ± 14
4	7.0	0.25 ± 0.02[a]	0.42 ± 0.01
16	7.0	4.6 ± 0.8[a]	40 ± 4

[a] K_i.
[b] IC_{50}.
[c] NA, not applicable.
[d] ND, not determined.
[e] Dissociation constant from ES complex.

Compound **6** is a competitive STS inhibitor with a K_i of 2.8 μM at pH 7.0. Comparing the potency of **6** with those of many other estrone derivatives bearing sulfate surrogates that have appeared in the literature is difficult since very few have been examined with pure enzyme and the modality of inhibition was rarely determined. Nevertheless, a comparison of **6** with other estrone derivatives bearing sulfate surrogates whose K_is or IC_{50}s have been determined using pure enzyme or placental microsomes[2] reveals that compound **6** is one of the most potent inhibitors of this class. Moreover, compound **6** is a purely competitive inhibitor which is in contrast to many other estrone derivatives bearing sulfate surrogates which often exhibit noncompetitive or mixed inhibition.[30–33] Estrone phosphate is one of the better estrone-based STS competitive inhibitors bearing a sulfate surrogate.[14,15] Under our assay conditions, we determined estrone phosphate to have an IC_{50} of 5 μM at pH 7.0. Thus, compound **6** is as potent as estrone phosphate at pH 7.0.

As mentioned earlier, boronic acids are competitive inhibitors of serine proteases and function by forming covalent adducts with active site residues such as serine and histidine.[16] Simple aryl boronic acids are generally modest serine protease inhibitors with K_is ranging from the millimolar to low micromolar depending on the enzyme and the substituents on the boronic acid. However, peptide boronic acids that have the primary specificity requirement for the protease are much more potent inhibitors with K_is sometimes extending into the subnanomolar range and exhibit an affinity for the protease that is many orders of magnitude greater than the corresponding substrate. Moreover, these inhibitors show kinetic properties corresponding to the mechanism for slow-binding inhibitors.[34] Compound **6** can be considered the boronic acid analog of estrone sulfate, a natural substrate of STS. The K_m for estrone sulfate in 0.1 M Tris–HCl, 0.1% Triton X-100, at pH 7.5 is 95 μM.[35] Dibbelt et al. have shown, using [^{35}S]-labeled DHEAS as substrate, that estrone sulfate is an inhibitor of STS exhibiting mixed inhibition with a K_i of about 1 μM in 0.1 M Tris–acetate at pH 7.0.[32] Thus, it appears that compound **6** has an affinity for STS that is about equal to estrone sulfate. Estrone itself is mainly a non-competitive inhibitor with a K_i of 63 μM and αK_i of 110 μM. Thus, replacing the 3-OH of estrone with a boronic acid

moiety resulted in about 23-fold increase in potency and changes the modality of inhibition from noncompetitive to competitive. STS assays performed in the presence of compound **6** exhibited a linear increase in product with time during the entire time course of the assays (10 min). Preincubation of **6** with STS for up to 60 min did not result in an increase in potency. These results suggest that compound **6** is not a slow-binding inhibitor. Moreover, the inhibition was found to be reversible as demonstrated by experiments in which the activity was recovered by dilution into a solution containing a large excess of substrate.

In some instances, the inhibition of serine proteases with boronic acids is very pH-dependent. Bender has reported that the inhibition of subtilisin with benzene boronic acid increases significantly between pH 6.0 and 8.0, and then rapidly decreases at pH > 8.0.[36] The increase in inhibition from pH 6.0 to 8.0 was attributed to the increase in the concentration of a reactive residue in the active site such as histidine or serine while the decrease was attributed to a decrease in the concentration of the reactive trigonal form of the boronic acid inhibitor. We have observed a modest *decrease* in inhibitor potency as the pH increases from pH 7.5 to 8.5 then levels off as the pH increases to 8.8. The inhibition remains competitive throughout this pH range and even at pH 8.8 compound **6** is still a good inhibitor with a K_i of 6.8 μM. It is unlikely that it is the tetrahedral hydrated form of **6** that binds since the concentration of this species should increase with increasing pH and one would expect an increase in inhibition as the pH increases. Moreover, this decrease in inhibition is not due to a decrease in the trigonal form of the inhibitor since its pK_a should be similar to that of 4-methylphenyl boronic acid which is 9.3.[37] The slight increase in K_i with pH found with compound **6** is in contrast to the large increase in K_i with pH exhibited by estrone phosphate whose K_i increases over 50-fold between pH 7.0 and 8.5 (52 μM).[15] This large increase is a result of the enzyme's preference to bind the monoanionic form of estrone phosphate.

Our results do not allow us to determine whether a reversible covalent adduct is formed between **6** and STS. Although it is a good inhibitor, it does not exhibit an affinity for STS that is orders of magnitude greater than that of estrone sulfate. If it does form a reversible adduct this adduct is not as stable as that observed with the peptide boronic acid inhibitors of serine proteases. Boronic acid inhibitors of serine proteases are considered to be transition state analog inhibitors in that they mimic the tetrahedral transition state formed during the reaction. The reaction of STS with ES does not proceed by a tetrahedral transition state. The transition state for the cleavage of the S–O bond of the substrate probably resembles a trigonal bipyramidal intermediate **X** as shown in Scheme 2. This may explain why compound **6** is not as potent an inhibitor of STS as the peptide boronic acids are of serine proteases. Nevertheless, compound **6** is still one of the best estrone-based STS inhibitors bearing a sulfate surrogate ever reported.

Since boronic acid inhibitor **6** was considerably more potent than estrone, compound **7** was prepared in the anticipation that substitution of the 3-OH moiety in **4** with a boronic acid would also result in a significant increase in inhibitory potency. Compound **7** is a 10-fold more potent inhibitor than compound **6** with a K_i of 252 nM. However, compounds **7** and **4** exhibited almost identical K_is and both were mainly non-competitive inhibitors of STS with both inhibitors exhibiting similar affinities for both the free and substrate bound forms of the enzyme. These results suggest that both **7** and **4** preferably bind in a region outside the active site. It has been suggested that the high affinity of estrone derivatives bearing benzyl groups at the 17-position for STS is a result of the benzyl group extending into a hydrophobic channel between the two antiparallel helices that are believed to insert into the membrane of the endoplasmic reticulum.[2a] Since our results with compounds **4** and **7** suggest that these compounds bind in an area outside the active site, it is possible that they bind in the channel between the helices and block the entrance to the active which is a small tunnel at the top of the two helices.[19]

IC_{50}s were determined for coumarin and chromenone boronic acids **8** and **9** at pH 7.0. Both of these compounds were relatively poor STS inhibitors with the coumarin having an IC_{50} of 171 μM and the chromenone having an IC_{50} of 86 μM. We also determined the IC_{50} of chromenone **16** to see if the substitution of the phenolic group in **16** with a boronic acid group had any effect on inhibitor potency as found with compound **6**.[38] Surprisingly, compound **16** exhibited an IC_{50} of 6 μM which is 14 times less than its boronic acid analog **9**. A more detailed kinetic analysis revealed that it exhibits mixed inhibition with a K_i of 4.6 μM and an αK_i of 40 μM and the inhibition is reversible. Thus, this compound is much more potent than its boronic acid analog and almost as potent as steroid boronic acid **6**. Like the compounds reported by Poirier and coworkers (such as **4** and **5**), chromenone **16** does not require a sulfate mimic to exhibit good potency. Horvath and coworkers have demonstrated the potential of the chromenone platform for obtaining reversible STS inhibitors.[39] These workers examined a series of 6-substituted 2-(1-adamantyl)-4-(thio)chromenones as STS inhibitors.[39] Those bearing non-ionizable or very weakly acidic moieties at the 6-position were modest reversible inhibitors of STS with K_is generally greater than 50 μM. The 6-COOH derivatives were the most potent of the series having K_is of about 500 nM. The 6-OH derivative was not examined. Although chromenone **16** is 20-fold less potent than compound **4** it is a smaller, non-steroidal compound and could be used as a lead to the development of yet more potent reversible STS inhibitors.

In summary, we have prepared several novel steroidal and non-steroidal boronic acids and examined them as inhibitors of STS. The potency of these compounds and their mode of inhibition varied dramatically. It appears that the boronic acid moiety must be attached to a platform very closely resembling a natural substrate in order for it to impart any beneficial effect on binding affinity. Thus, estrone boronic acid **6** is a good compet-

itive inhibitor of STS even at basic pH. The fact that this compound exhibits a good affinity for STS even at basic pHs may be significant in terms of obtaining the crystal structure of an inhibitor-STS complex. The structure of an STS-inhibitor complex has not yet been reported. Such a structure would be very useful for rational inhibitor design. However, one of the potential difficulties in obtaining the structure of an STS-inhibitor complex is that the enzyme is crystallized at pH 8.5 where its affinity for ligands is often far from optimal.[30] However, since **6** binds to STS with a good affinity at basic pHs, this compound may enable one to obtain the structure of an STS inhibitor complex and such studies are in progress.[40] Compound **4**, an estradiol derivative which, in addition to the boronic acid group at the 3-position also bears a benzyl group at the 17-position, was a potent non-competitive STS inhibitor. However, the fact that compound **7** and its phenolic precursor **4** exhibit a similar affinity and are non-competitive inhibitors suggests that these compounds bind in a region outside the active site, possibly in the hydrophobic channel between the two membrane-spanning helices. The coumarin and chromenone boronic acids **8** and **9** were not good inhibitors. However, the chromenone precursor to **8**, compound **16**, is a remarkably good STS inhibitor and may be useful as a lead to the development of yet more potent reversible STS inhibitors.

3. Experimental

3.1. General methods

All starting materials and reagents were obtained from Aldrich Chemical Company. THF and dioxane were distilled from sodium-benzophenone. CH_2Cl_2 was distilled from calcium hydride under nitrogen. Silica-gel chromatography was performed using silica gel 60 Å (230–400 mesh) obtained from Silicycle (Laval, Quebec, Canada). ^1H, ^{13}C, ^{11}B, and ^{19}F NMR spectra were recorded on a Bruker Avance 300 spectrometer. NMR spectra are reported in parts per million (ppm) relative to internal standards or solvent peaks. For NMR spectra run in $CDCl_3$, chemical shifts (δ) for ^1H NMR spectra are reported relative to internal Me_4Si (δ 0.0 ppm), chemical shifts for ^{13}C spectra are relative to the residual solvent peak (δ 77.0 ppm, central peak), ^{11}B NMR are relative to external triethylborate (δ 0.0 ppm), and ^{19}F NMR relative to an external fluoroform (δ 0.0 ppm). For NMR spectra run in DMSO-d_6, chemical shifts for ^1H NMR spectra are reported relative to the residual solvent peak (δ 2.49 ppm, central peak), chemical shifts for ^{13}C spectra are relative to the residual solvent peak (δ 39.5 ppm, central peak), and ^{11}B NMR are relative to external triethylborate (δ 0.0 ppm). For NMR spectra run in CD_3OD, chemical shifts for ^1H NMR spectra are reported relative to the residual solvent peak (δ 3.31, central peak), chemical shifts for ^{13}C spectra are relative to the residual solvent peak (δ 49.00, central peak), and ^{11}B NMR are relative to external triethylborate (δ 0.0 ppm). Low-resolution (LRMS) and high-resolution (HRMS) electron impact (EI) mass spectra were obtained on a JEOL HX110 double focusing mass spectrometer. Chemical ionization (CI) mass spectra were run in the positive mode using ammonia as ionizing agent. Positive ion electrospray (ESI) mass spectra were obtained with a Waters/Micromass QTOF Ultima Global mass spectrometer. Infrared spectra were obtained on a Perkin-Elmer Spectrum RX Fourier transform spectrophotometer. Melting points were determined on a Fisher–Johns melting point apparatus and are uncorrected. Steroid sulfatase from human placenta was purified as previously described.[41]

3.2. Syntheses

3.2.1. 3-Pinacolatoboroestra-1,3,5-(10)-triene-17-one (11). To a mixture of estrone triflate **10**[24] (4.02 g, 10 mmol) and Pd(dppf)Cl_2–CH_2Cl_2 (320 mg, 0.39 mmol, 3.9 mol %) in dioxane (50 ml) under argon were added Et_3N (8.4 ml, 60 mmol) and 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (4.2 ml, 28.9 mmol). The reaction mixture was heated at 100 °C for 16 h. The reaction was cooled to room temperature and concentrated. Purification of the residue by flash chromatography (elution with ethyl acetate/hexane 1:3) gave compound **11** (2.969 g, 78%) as a white solid: mp 193 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 7.58 (d, J = 8.0 Hz, 1H), 7.55 (s, 1H), 7.29 (d, J = 7.8 Hz, 1H), 2.95–2.88 (m, 2H), 2.65–2.40 (m, 2H), 2.40–2.25 (m, 1H), 2.20–1.90 (m, 4H), 1.70–1.35 (m, 6H), 1.32 (s, 12 H), 0.89 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 220.6, 143.1, 135.8, 135.6, 132.2, 124.7, 83.6, 50.6, 47.9, 44.7, 38.0, 35.8, 31.6, 29.1, 26.5, 25.6, 24.8, 21.6, 13.8; LRMS (EI) m/z (%) 381 (M+1, 25), 380 (M^+, 100), 379 (24), 323 (13), 294 (30), 281 (24); HRMS (EI) calcd for $C_{24}H_{33}BO_3$ 380.2523; found 380.2531.

3.2.2. Estra-1,3,5-(10)-triene-17-one-3-boronic acid (6). To a mixture of estrone boronate **11** (1.14 g, 3 mmol) in acetone (450 ml) was added a solution of ammonium acetate (924 mg, 12 mmol) and sodium periodate (2.568 g, 12 mmol) in H_2O (360 ml). The reaction mixture was stirred for 10 days at room temperature. After removal of acetone by rotary evaporation, the remaining aqueous solution was extracted with ethyl acetate. The combined extracts were dried over sodium sulfate and concentrated. Purification of the residue by flash chromatography (elution with acetone/hexane 1:3) gave boronic acid **6** (839 mg, 84%) as a white solid: mp 181–182 °C; ^1H NMR (300 MHz, DMSO-d_6) δ 7.77 (s, 2H, $B(OH)_2$), 7.48 (d, J = 7.5 Hz, 1H), 7.45 (s, 1H), 7.18 (d, J = 7.5 Hz, 1H), 2.80 (br s, 2H), 2.40–1.85 (m, 6H), 1.74 (br s, 1H), 1.65–1.20 (m, 6H), 0.78 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 220.0, 141.9, 135.4, 135.3, 131.2, 124.6, 50.2, 47.7, 44.5, 38.1, 35.8, 31.8, 29.3, 26.5, 25.7, 21.6, 13.9; LRMS (ESI) m/z (%) 299 (M+1, 100), 298 (M^+, 25), 281 (27), 231 (82); HRMS (ESI) calcd for $C_{18}H_{24}BO_3$ 298.1855; found 298.1862.

3.2.3. 3-*O*-[(Trifluoromethylsulfonyl)oxy]-17α-benzyl-17β-trifluoroacetyloxyestra-1,3,5-(10)-triene (12). To a solution of estradiol **4**[9] (2.888 g, 8 mmol) and 4-(dimethylamino)pyridine (2.44 g, 20 mmol) in methylene chloride (120 ml) at 0 °C was added trifluoroacetic anhydride (2.7 ml) over 30 min. The mixture was stirred

for 7 h at room temperature before quenching with water. The layers were separated and the aqueous phase was extracted with methylene chloride and the combined organics were dried over Na_2SO_4. After removal of solvent, the residue was dissolved in ethyl acetate (150 ml) and methanol (30 ml). 40 ml of 1 N HCl was added and the resulting mixture was stirred 1 h at room temperature. Reaction mixture was diluted with water and extracted with ethyl acetate. The combined extracts were dried over Na_2SO_4 and concentrated. Flash chromatography (elution with ethyl acetate/hexane 1:8 to 1:6) afforded crude 17-trifluoroacetoxy estrone as a white foam. This material was dissolved in methylene chloride (120 ml) and *N*,*N*-dimethylaminopyridine (1.22 g, 10 mmol) was added. The resulting mixture was cooled to 0 °C and triflic anhydride (1.5 ml, 8.9 mmol) was added over 10 min. After stirring for 1 h at 0 °C, the reaction mixture was quenched with ice-cold water and extracted with methylene chloride. The combined extracts were washed with water, brine, dried over Na_2SO_4, and concentrated. Purification of the residue by flash chromatography (elution with ethyl acetate/hexane 1:8) gave compound **12** (3.13 g, 67%) as a white solid: mp 112–113 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35–7.24 (m, 4 H), 7.11 (d, J = 7.1 Hz, 2H), 7.02 (d, J = 8.8 Hz, 1H), 6.97 (s, 1H), 3.90 (d, J = 14.6 Hz, 1H), 2.96–2.85 (m, 2H), 2.77 (d, J = 14.6 Hz, 1H), 2.40–2.20 (m, 4H), 2.00–1.70 (m, 4H), 1.70–1.35 (m, 5H), 0.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.8 (q, J = 38 Hz, CF_3C═O), 147.6, 140.3, 139.2, 135.8, 130.4, 128.4, 127.2, 126.9, 121.2, 118.8 (q, J = 333 Hz, CF_3), 118.2, 114.6 (q, J = 285 Hz, CF_3), 99.9, 50.5, 48.2, 43.6, 38.9, 37.8, 33.0, 32.7, 29.4, 26.9, 26.1, 23.0, 14.2; ^{19}F NMR (282 MHz, $CDCl_3$) δ −72.7, −75.1; LRMS (CI) *m*/*z* (%) 608 (M+18, 25), 494 (40), 477 (87), 476 (100), 461 (29), 385 (70), 329 (41); HRMS (ESI) calcd for $C_{28}H_{28}F_6O_5S+NH_4^+$ 608.1905; found 608.1927.

3.2.4. 3-*O*-Pinacolatoboro-17α-benzyl-17β-trifluoroacetyloxyestra-1,3,5-(10)-triene (13). To a mixture of triflate **12** (1.60 g, 2.71 mmol) and Pd(dppf)Cl_2–CH_2Cl_2 (110 mg, 0.135 mol, 5 mmol %) in dioxane (15 ml) under argon was added Et_3N (3.4 ml, 24 mmol) and 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (1.7 ml, 11.7 mmol). The reaction mixture were heated at 92–96 °C for 3 h, cooled to room temperature, diluted with water, and extracted with ethyl acetate. The combined extracts were dried over Na_2SO_4 and concentrated. Purification of the residue by flash chromatography (elution with ethyl acetate/hexane, 1:10) gave compound **13** (952 mg, 62%) as a white solid: mp 143–144 °C ; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.59 (d, J = 7.8 Hz, 1H), 7.54 (s, 1H), 7.36–7.27 (m, 4H), 7.11 (d, J = 6.5 Hz, 2H), 3.87 (d, J = 14.7 Hz, 1H), 2.92–2.88 (m, 2H), 2.78 (d, J = 14.7 Hz, 1H), 2.55–2.20 (m, 4H), 2.00–1.75 (m, 4H), 1.70–1.40 (m, 5H), 1.32 (s, 12H), 0.90 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.7 (q, J = 41 Hz, C═O), 143.2, 136.0, 135.7, 135.7, 132.3, 130.5, 128.4, 126.9, 126.2 (br, *C*–B), 124.8, 114.8 (q, J = 286 Hz, CF_3), 100.1, 83.6, 50.7, 48.3, 44.2, 39.2, 38.0, 33.2, 32.8, 29.3, 27.4, 26.1, 24.9, 23.0, 14.3; ^{19}F NMR (282 MHz, $CDCl_3$) δ −75.1; ^{11}B NMR (96 MHz, $CDCl_3$) δ 33.2; LRMS (EI) *m*/*z* (%) 568 (M^+, 8), 478 (9), 454 (100), 439 (28), 363 (62), 267 (32); HRMS (EI) calcd for $C_{33}H_{40}BF_3O_4$ 568.2972; found 568.2972.

3.2.5. 3-*O*-Pinacolatoboro-17α-benzyl-17β-hydroxyestra-1,3,5-(10)-triene (14). To a solution of **13** (480 mg, 0.845 mmol) in THF (240 ml) at room temperature was added 0.8 N NaOH (24 ml) slowly over 10 min. After stirring for 10 min, water (60 mL) was added and the mixture was extracted with Et_2O. The combined extracts were washed with water, brine, dried over Na_2SO_4, and concentrated. Purification of the residue by flash chromatography (elution with ethyl acetate/hexane, 1:6 to 1:5) gave **14** (335 mg, 84%) as a white solid: mp 211–213 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.63 (d, J = 7.7 Hz, 1H), 7.59 (s, 1H), 7.37–7.24 (m, 6H), 3.00–2.90 (m, 3H), 2.95 (d, J = 12.8 Hz, 1H), 2.71–2.66 (m, 1H), 2.30–2.20 (m, 1H), 2.05–1.91 (m, 2H), 1.80–1.50 (m, 7H), 1.50–1.25 (m, 2H, overlapping), 1.36 (s, 12H, overlapping), 0.97 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 143.8, 138.5, 136.0, 135.7, 132.2, 131.1, 128.4, 126.3, 126.0 (br, *C*–B), 124.8, 83.6, 83.0, 49.8, 46.9, 44.7, 42.5, 39.4, 33.7, 31.5, 29.4, 27.6, 26.1, 25.0, 23.4, 14.6; ^{11}B NMR (96 MHz, $CDCl_3$) δ 33.7; LRMS (EI) *m*/*z* (%) 472 (M^+, 8), 454 (7), 381 (96), 380 (100), 363 (79), 323 (18), 237 (22); HRMS (EI) calcd for $C_{31}H_{41}BO_3$ 472.3149; found 472.3151.

3.2.6. 17α-Benzyl-17β-hydroxyestra-1,3,5-(10)-triene-3-boronic acid (7). To a solution of **14** (47 mg, 0.1 mmol) and phenylboronic acid (13 mg, 0.105 mg) in THF (8 ml)/MeOH (3 ml) was added 2 N HCl (2 ml). The resulting mixture was stirred overnight and then quenched with water. The mixture was extracted with ethyl acetate and the combined extracts were washed with water, brine, dried over Na_2SO_4, and concentrated. Purification of the residue by flash chromatography (elution with acetone/hexane 1:4 to 1:2) gave **7** (26 mg, 67%) as a white solid: mp 216–218 °C; ^{1}H NMR (300 MHz, DMSO-d_6 with one drop of D_2O) δ 7.46 (d, J = 7.4 Hz), 7.41 (s, 1H), 7.20–7.05 (m, 6H), 3.10 (s, 2H, B(O*H*)$_2$), 2.80–2.75 (m, 3H), 2.54 (d, J = 13.2 Hz, 1H), 2.35–2.25 (m, 1H), 2.25–2.15 (m, 1H), 1.85–1.75 (m, 1H), 1.70–1.20 (m, 10H), 0.78 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6 with one drop of D_2O) δ 142.6, 139.9, 135.5, 135.3, 131.8, 131.6, 131.0 (br, *C*-B), 127.8, 126.0, 124.7, 82.6, 49.5, 47.2, 44.4, 42.4, 39.7, 32.2, 31.3, 29.5, 27.6, 26.2, 23.3; LRMS (ESI, +LiOAc) 787 (2M+Li, 100), 397 (M+Li, 47); HRMS (ESI) calcd for $C_{25}H_{31}LiBO_3$ 396.2563; found 396.2549.

3.2.7. 3-[(trifluoromethylsulfonyl)oxy]-6-oxo-8,9,10,11-tetrahydro-7*H*-cyclohepta[*c*][*1*]-benzopyran (17). To a solution of coumarin **15**[27] (3.00 g, 13.0 mmol) and 4-(dimethylamino)pyridine (400 mg, 3.28 mmol) in methylene chloride (100 ml) at 0 °C were added 2,6-lutidine (3.05 ml, 26 mmol) then triflic anhydride (2.65 ml, 15.6 mmol) over 10 min. The reaction mixture was stirred for 1 h at 0 °C then quenched with ice and 0.5 N HCl (30 ml). The layers were separated and the aqueous phase was extracted with methylene chloride. The combined organics were washed with 0.5 N HCl, 5% NaH-

CO_3, dried over Na_2SO_4, and then concentrated. Purification of the residue by flash chromatography (elution with ethyl acetate/hexane 1:4) gave triflate **17** (4.562 g, 97%) as a white solid: mp 86–87 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 (d, J = 8.9 Hz, 1H), 7.21 (d, J = 2.0 Hz, 1H), 7.18 (dd, J = 8.9 Hz, 2.0 Hz, 1H), 2.95–2.87 (m, 4H), 1.94–1.85 (m, 2H), 1.70–1.55 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.9, 152.9, 152.4, 149.8, 129.8, 125.8, 119.9, 118.6 (q, J = 315 Hz, CF_3), 117.0, 110.1, 31.7, 28.1, 26.8, 25.3, 24.7; ^{19}F NMR (282 MHz, $CDCl_3$) δ −72.3; LRMS (EI) m/z (%) 362 (M^+, 60), 347 (7), 230 (15), 229 (100), 201 (18); HRMS (EI) calcd for $C_{15}H_{13}F_3O_5S$ 362.0436; found 362.0438.

3.2.8. 3-Pinacolatoboro-6-oxo-8,9,10,11-tetrahydro-7*H*-cylohepta-[*c*][*1*]benzopyran (19). To a mixture of coumarin triflate **17** (1.81 g, 5 mmol), bis(pinacolato)diboron (1.435 g, 5.65 mmol), Pd(dppf)Cl_2–CH_2Cl_2 (123 mg, 0.15 mmol, 3 mol%), and dry KOAc (735 mg, 7.5 mmol) under argon was added dioxane (30 ml). The resulting mixture was heated at 85–90 °C overnight. After cooling to room temperature, the reaction mixture was loaded directly onto a silica column. Purification by flash chromatography (elution with 100% hexane, then ethyl acetate/hexane 1:4) afforded boronate **19** (1.41 g, 83%) as a white solid: mp 119–120 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.63 (s, 1H), 7.59 (d, J = 8.6 Hz, 1H), 7.55 (d, J = 8.6 Hz, 1H), 2.90–2.82 (m, 4H), 1.83 (quint, J = 5.5 Hz, 2H), 1.62–1.49 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.8, 153.1, 151.9, 129.8, 129.7, 123.0, 122.8, 121.8, 84.2, 31.9, 27.8, 26.8, 25.5, 24.9, 24.8; ^{11}B NMR (96 MHz, $CDCl_3$) δ 30.4; LRMS (EI) m/z (%) 340 (M^+, 100), 339 (30), 325 (19), 312 (17), 254 (10), 241 (18); HRMS (EI) calcd for $C_{20}H_{25}BO_4$ 340.1846; found 340.1838.

3.2.9. 6-Oxo-8,9,10,11-tetrahydro-7*H*-cylohepta-[*c*][*1*]benzopyran-3-boronic acid (8). To a mixture of coumarin boronate **19** (342 mg, 1 mmol) in acetone (150 ml) was added a solution of ammonium acetate (308 mg, 4 mmol) and sodium periodate (856 mg, 4 mmol) in H_2O (120 ml). The reaction mixture was stirred overnight at room temperature. After removal of acetone by rotary evaporation, the residue was extracted with ethyl acetate and then concentrated. Purification of the residue by flash chromatography (elution with ethyl acetate/hexane 1:1 to 100% ethyl acetate) gave coumarin boronic acid **8** (172 mg, 67%) as a white solid: mp > 260 °C; ^{1}H NMR (300 MHz, DMSO-d_6 with one drop of D_2O) δ 8.32 (s, 2H, B(OH)$_2$), 7.82 (d, J = 7.7 Hz, 1 H), 7.70–7.60 (m, 2H), 3.00 (m, 2H), 2.78 (m, 2H), 1.80 (m, 2H), 1.56 (m, 2H), 1.47 (m, 4H); ^{13}C NMR (75 MHz, DMSO-d_6 with one drop of D_2O) δ 161.4, 153.9, 151.8, 130.0, 128.8, 123.9, 121.9, 120.9, 31.7, 27.5, 26.6, 25.5, 25.0; ^{11}B NMR (96 MHz, DMSO-d_6 with one drop of D_2O) δ 30.3; LRMS (ESI) m/z (%) 259 (M+1, 100), 258 (M^+, 29); HRMS (ESI) calcd for $C_{14}H_{15}BO_4$ 258.1178; found 258.1177.

3.2.10. 2-*tert*-Butyl-6-[(trifluoromethylsulfonyl)oxy]-4*H*-1-benzopyran-4-one (18). To a solution of chromenone **16**[28] (2.16 g, 10.0 mmol) and 4-(dimethylamino)pyridine (305 mg, 2.5 mmol) in methylene chloride (80 ml) at 0 °C were added 2,6-lutidine (2.33 ml, 20 mmol) then triflic anhydride (2.02 ml, 12 mmol) over 10 min. After addition, the reaction mixture was stirred for 1.5 h at 0 °C then quenched with ice and 0.5 N HCl (30 ml). The layers were separated and the aqueous phase was extracted with methylene chloride. The combined organics were washed with 0.5 N HCl, 5% $NaHCO_3$, dried over Na_2SO_4, and then concentrated. Purification of the residue by flash chromatography (elution with ethyl acetate/hexane 1:2 to 1:1) gave chromenone triflate **18** (1.76 g, 50%) as a white solid and recovered starting material (350 mg, 16%): mp 88–89 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.98 (s, 1H), 7.52 (t, J = 9.3 Hz, 2H), 6.24 (s, 1H), 1.29 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.2, 176.8, 155.5, 145.8, 126.7, 124.5, 120.5, 118.6 (q, J = 315 Hz, CF_3), 117.9, 106.5, 36.5, 27.7; ^{19}F NMR (282 MHz, $CDCl_3$) δ −72.5; MS (EI) m/z (%) 350 (M^+, 65), 217 (100); HRMS (EI) calcd for $C_{14}H_{13}F_3O_5S$ 350.0436; found 350.0442.

3.2.11. 2-*tert*-Butyl-6-pinacolatoboro-4*H*-1-benzopyran-4-one (20). To a mixture of chromenone triflate **18** (1.044 g, 3 mmol), bis(pinacolato)diboron (861 g, 3.39 mmol), Pd(dppf)Cl_2–CH_2Cl_2 (74 mg, 0.09 mmol, 3 mol%) and dry potassium acetate (441 mg, 4.5 mmol) under argon was added dioxane (18 ml). The resulting mixture was heated at 85–90 °C for 16 h. After cooling to room temperature, the reaction mixture was loaded onto a silica column. Purification by flash chromatography (elution with 100% hexane, then ethyl acetate/hexane 1:4) afforded chromenone boronate **20** (725 mg, 75%) as a light yellow solid: mp 135–136 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.61 (s, 1H), 7.98 (d, J = 8.4 Hz, 1H), 7.36 (d, J = 8.4 Hz, 1H), 6.22 (s, 1H), 1.29 (s, 21H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.7, 175.8, 158.3, 139.1, 133.2, 122.6, 117.0, 107.0, 84.0, 36.4, 27.8, 24.8; ^{11}B NMR (96 MHz, $CDCl_3$) δ 30.4; MS (EI) m/z (%) 328 (M, 100), 327 (95), 313 (75), 285(48), 271 (39), 229 (75); HRMS (EI) calcd for $C_{19}H_{25}BO_4$ 328.1846; found 328.1852.

3.2.12. 2-*tert*-Butyl-4*H*-1-benzopyran-4-one-6-boronic acid (9). To a mixture of chromenone boronate **20** (328 mg, 1 mmol) in acetone (150 ml) was added a solution of ammonium acetate (308 mg, 4 mmol) and sodium periodate (856 mg, 4 mmol) in H_2O (120 ml). The reaction mixture was stirred overnight at room temperature. After removal of acetone by rotary evaporation, the residue was extracted with ethyl acetate and then concentrated. Purification of the residue by flash chromatography (elution with ethyl acetate/hexane 1:1 to 100% ethyl acetate) gave chromenone boronic acid **9** (189 mg, 77%) as a white solid: mp 176–178 °C; ^{1}H NMR (300 MHz, DMSO-d_6 with one drop of D_2O) δ 8.47 (s, 1 H), 8.28 (s, 2 H, partially exchanged with D_2O, B(OH)$_2$), 8.09 (d, J = 8.2 Hz, 1H), 7.53 (d, J = 8.3 Hz, 1H), 6.07 (s, 1H), 1.27 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6 with one drop of D_2O) δ 178.0, 176.0, 157.6, 140.0, 131.8, 122.3, 117.4, 106.7, 36.5, 27.8; ^{11}B NMR (96 MHz, DMSO-d_6 with one drop of D_2O) δ 28.6; LRMS (ESI) m/z (%) 248 (M + 1, 12),

247 (M^+, 100); HRMS (ESI) calcd for $C_{13}H_{15}BO_4$ 246.1178; found 246.1185.

3.3. Inhibition studies

3.3.1. Determination of K_i and αK_i values for compounds 4, 6, 7, and 16. An appropriate volume of a MUS stock solution in 0.1 M Tris–HCl of desired pH was added to the wells of a 96-well microtiter plate containing 0.1 M Tris–HCl buffer of the same pH such that the total volume was 80 μl. To the wells was added 10 μl of a stock solution of inhibitor in 50% DMSO. For a control, 10 μl of 50% DMSO was added instead. The assay was initiated by the addition of 10 μl STS (115 nM stock solution in 20 mM Tris–HCl, pH 7.4, 0.1% Triton X-100). To detect non-enzymatic hydrolysis of the substrate 10 μl of enzyme storage buffer was added instead. The final volume of the assay was 100 μl. The final concentration of buffer was 92 mM TrisHCl, 0.01% Triton X-100, and 5% DMSO. The final enzyme concentration was 11.5 nM. For studies at pH 7.0, 7.5, and 8.0, the final concentration of MUS was 83.3–500 μM, or approximately 0.5–3 times the K_m value at pH 7 and 7.5 (145 μM at pH 7.0; 170 μM at pH 7.4) and 0.25–1.5 times the K_m value at pH 8.0 (338 μM). For studies at pH 8.5 and 8.8, the final concentration of MUS ranged between 100 and 2500 μM or approximately 0.1–3 times the K_m value (830 μM, pH 8.5; 980 μM, pH 8.8). The final concentration of inhibitor was 0.5–4 times K_i. The reactions were followed by detection of fluorescent product, 4-methylumbelliferone (excitation 360 nm, emission, 460 nm), over 10 min using a Spectramax GeminiXS plate reader (Molecular Devices, CA) at 30 °C. Each reaction was performed in triplicate. Controls were performed in an identical manner but did not contain STS. Initial rates (v) were determined by taking the slopes of plots of the change in relative fluorescence units with time. These data were plotted as Lineweaver–Burk graphs and K_i values were calculated from replots of the slopes or intercepts of the Lineweaver–Burk graphs according to the equations for mixed and competitive inhibition.

3.3.2. Determination of IC_{50}s for compounds 8, 9, and 16. Ten microliters of inhibitor stock solutions in 50% DMSO were added to the wells of a 96-well microtiter plate containing 70 μl of 0.1 M Tris, pH 7.0. Ten microliters of a 2.0 mM MUS stock solution in 0.1 M Tris–HCl, pH 7.0, was added. The assay was initiated by adding 10 μL STS (115 nM stock solution in 20 mM Tris–HCl, pH 7.4, 0.1% Triton X-100). The concentration of inhibitor ranged from 7 to 250 μM. The final concentration of MUS was 200 μM. The reaction was followed as described above. Eleven concentrations of inhibitor bracketing the IC_{50} value were used for each compound. The initial rates of enzyme activity in relative fluorescence units per second (RFU/s) were used to determine the IC_{50}. The ratio of the initial rate in the presence of inhibitor (V_i) to that in the absence of inhibitor (V_o) was calculated and plotted as a semi-log curve in Grafit, from which the IC_{50} value was calculated based on the following equation: $V_i = V_o/[1 + ([I]/IC_{50})^S] + B$, where: V_i is the initial rate of reaction at an inhibitor concentration of [I]; V_o is the velocity in the absence of inhibitor; B is background and s is the slope factor.

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