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Substituted thiophene-anthranilamides as potent inhibitors of human factor Xa☆

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Abstract—A series of thiophene-containing non-amidine factor Xa inhibitors is described. Simple methyl-substituted thiophene analogs were relatively weak inhibitors. However, introduction of hydrophilic substituents at C-4 or C-5 of the thiophene afforded inhibitors with low nanomolar potency. Optimization of the thiophene substituent at C-4 afforded subnanomolar inhibitors with improved in vitro anticoagulant activity. Incorporating basic amine substituents on the thiophene increased hydrophilicity and improved anticoagulant activity. The pharmacokinetic profile of one inhibitor was evaluated in dogs, and the X-ray crystal structure of this compound bound to factor Xa provides insight into the observed SAR for binding to factor Xa.

1. Introduction

Anticoagulant therapy is the primary strategy for treatment and prevention of thromboembolic disease. Warfarin (Coumadin), a vitamin K antagonist, is the only oral anticoagulant in clinical use. Coumadin is highly efficacious, but suffers from a delayed onset of action, and the need for careful clinical monitoring. Alternate anticoagulants such as the low molecular weight heparins and the direct thrombin inhibitor hirudin must be administered parenterally. The unmet clinical need for a safe and orally active anticoagulant has resulted in a widespread drug discovery effort.

Factor Xa (fXa) is a trypsin-like serine protease located at the convergence point of the intrinsic and extrinsic blood coagulation pathways.[2] As part of the prothrombinase complex, factor Xa catalyzes the conversion of prothrombin to thrombin (fIIa). Thrombin catalyzes the formation of fibrin, which crosslinks to form the matrix of blood clots. Thrombin also stimulates platelet aggregation and is essential for platelet plug formation after vascular injury. Because inhibition of fXa prevents thrombin formation but does not affect pre-existing thrombin, fXa inhibitors are predicted to cause less impairment of hemostasis than direct thrombin inhibitors, leading to a wider therapeutic window.[3] Both proteinaceous and small molecule inhibitors of fXa have been shown to be effective and safe in preclinical animal thrombosis models.[4]

Our high capacity screening efforts led to the identification of the benzothiophene-substituted anthranilamide **1** as a novel, potent ($K_{i,app}$ = 10 nM), non-amidine inhibitor of human fXa.[5] We have previously described our early optimization efforts on this template, including the discovery that substitution of chlorine for fluorine afforded a 30-fold increase in potency (**2**, $K_{i,app}$ = 0.33 nM).[6] While compound **2** maintained high selectivity versus thrombin and trypsin ($K_{i,app}$ > 5 μM), it had no measurable anticoagulant activity, as measured by the concentration of compound required to cause a doubling of the prothrombin time in vitro in human plasma (2× PT). This lack of anticoagulant activity

Keywords: Factor Xa inhibitor; Anticoagulant; Non-amidine; Thrombosis.
☆ See Ref. 1.
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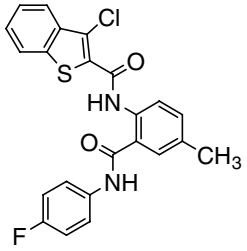
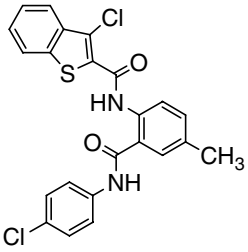
was attributed to the poor physiochemical properties of **2**, including high lipophilicity which likely results in high plasma protein binding. Related results have been reported for a similar series of lipophilic fXa inhibitors[7] and for a series of lipophilic thrombin inhibitors.[8] In these reports, potency of compounds in PT (fXa inhibitors) or in vitro activated partial thromboplastin time (APTT, thrombin inhibitors) assays did not correlate solely with activity against fXa or thrombin, but was additionally a function of lipophilicity. In order to improve solubility and decrease lipophilicity, we focused on the introduction of basic and/or hydrophilic substituents. In this paper, we will report our investigation of substitution on the benzothiophene ring, and the subsequent design and synthesis of a series of potent fXa inhibitors bearing a C-4 or C-5 substituted thiophene in place of the benzothiophene.[9] The latter compounds were found to have improved anticoagulant activity in vitro.



1

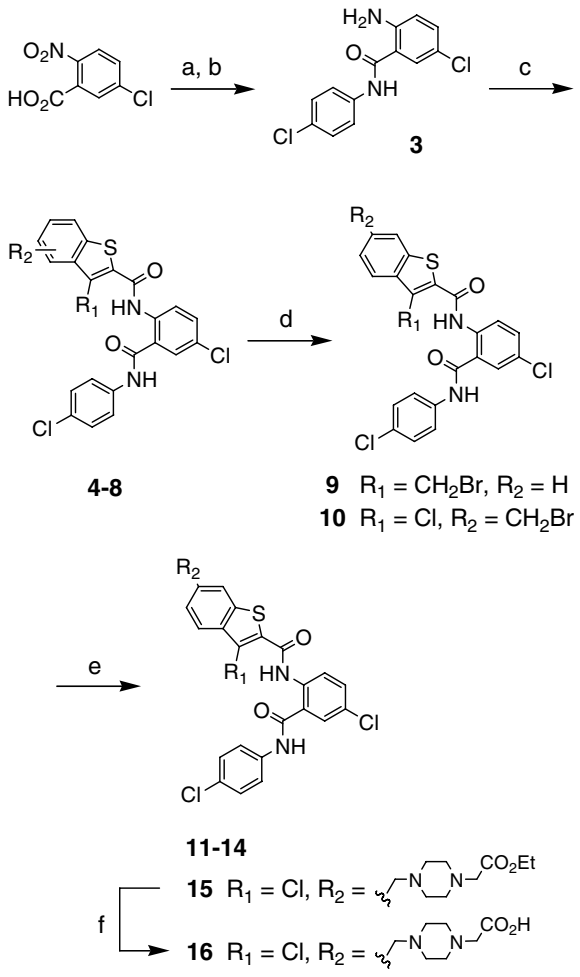


2

2. Chemistry

The synthesis of all inhibitors described in this report proceeded through the common anthranilamide intermediate **3**. Synthesis of **3** and its conversion to benzothiophene-containing compounds is outlined in Scheme 1. 5-Chloro-2-nitrobenzoic acid was converted to the acid chloride and coupled with 4-chloroaniline. The nitro group was then reduced with tin chloride to give **3**. Reaction of the aniline with a benzothiophene acid chloride provided compounds **4**–**8**. Methyl-substituted compounds **5** and **8** (Table 1) were brominated with *N*-bromosuccinimide (NBS) to give intermediates **9** and **10**. Displacement of the bromide with amines afforded inhibitors **11**–**15** (Table 2), and hydrolysis of the ester group of **15** afforded compound **16**.

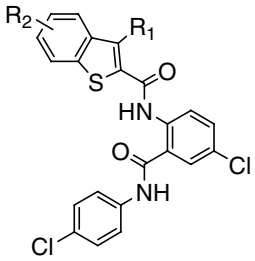
Methylthiophene analogs **20** and **24** were prepared in a similar manner, as outlined in Scheme 2. The 3-amino group of methylthiophenes **17** and **21** was first converted to chloride by a Sandmeyer reaction.[10] Ester hydrolysis to provide acids **19** and **23**, followed by conversion to the acid chloride and reaction with aniline **3**, afforded **20** and **24**. The 5-methyl compound **24** was cleanly converted to the bromomethyl intermediate **25** with NBS, and displacement with amine nucleophiles afforded inhibitors **32**, **34**, and **36** (Table 3). Similar attempts to halogenate the 4-methyl compound **20** were unsuccessful, providing mixtures of the starting material, mono- and dihalogenated compounds.



Scheme 1. Reagents and conditions: (a) $(COCl)_2$, DMF (cat.), CH_2Cl_2, 16 h, concentrate; then NEt_3, 4-chloroaniline, CH_2Cl_2; (b) $SnCl_2{\cdot}2H_2O$, EtOAc, 70 °C; (c) substituted benzothiophene-2-carbonyl chloride, pyridine; (d) *N*-bromosuccinimide, benzoyl peroxide, C_6H_6, irradiation; (e) amine, CH_2Cl_2; (f) $LiOH{\cdot}H_2O$, THF–MeOH–H_2O.

The 4-chloromethylthiophene intermediate **30** was used for preparation of most of the inhibitors discussed in this report. Since compound **30** could not be obtained by direct halogenation of **20**, an alternate synthetic route was used, as outlined in Scheme 3. The 4-methylthiophene compound **18** was chlorinated with sulfuryl chloride. When carried to complete consumption of **18**, a mixture of the mono- and dichloro compounds was obtained which was difficult to separate. The best result was obtained by carrying the reaction to only ca. 60% completion. This gave minimal quantities of the dichloro product, and allowed chromatographic isolation of **26** and recycling of starting material. Direct hydrolysis of the ester of **26** under all attempted conditions gave a mixture of the desired acid and compound **28**, resulting from hydrolysis of the chloromethyl substituent. Attempts to push the reaction completely to compound **28** afforded poor yields. To circumvent this, the chloride was first displaced using sodium acetate to give intermediate **27**,

Table 1. Addition of methyl groups on benzothiophene



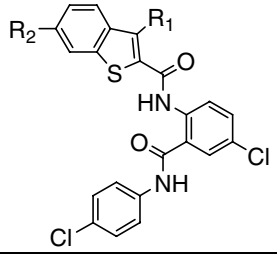
Compound	R_1	R_2	fXa $K_{i,app}$[a,b] (nM)
4	Cl	H	0.60
5	CH_3	H	0.82
6	Cl	4-CH_3	>5000
7	Cl	5/7-CH_3[c]	54
8	Cl	6-CH_3	0.95

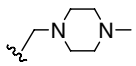
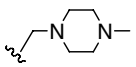
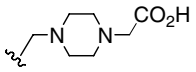
[a] $K_{i,app}$ is the apparent K_i value, defined as $IC_{50}/2$ when the IC_{50} value is determined at a substrate concentration equal to the K_m. $K_{i,app}$ values are averaged from multiple determinations ($n \geqslant 2$), and the standard deviations are <30% of the mean.

[b] All compounds had $K_{i,app}$ values for human thrombin and bovine trypsin of >5000 nM.

[c] Inseparable mixture of 5-methyl and 7-methyl compound, ca. 3:2 ratio by ^1H NMR, assayed as a mixture.

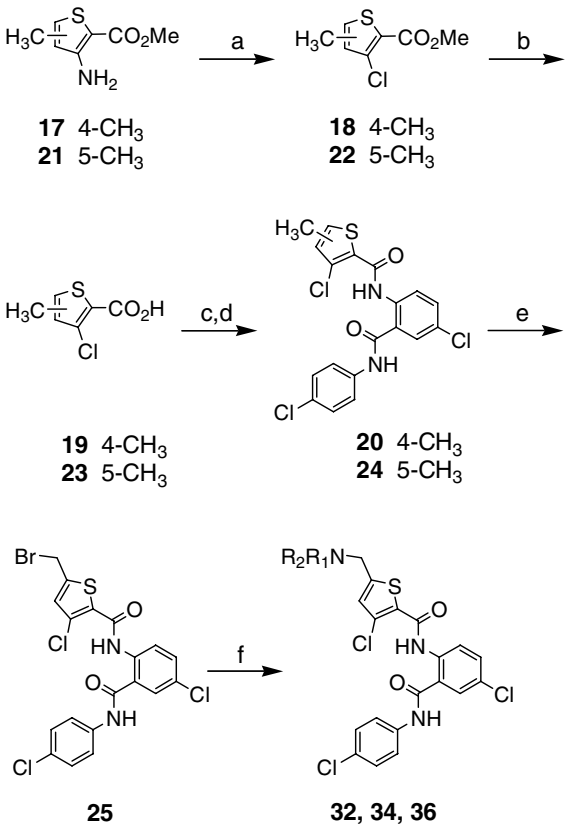
Table 2. Effect of amine-substituted benzothiophenes on enzyme inhibition



Compound	R_1	R_2	$K_{i,app}$[a,b] (nM)	
			fXa	fIIa
5	CH_3	H	0.82	>5000
8	Cl	CH_3	0.95	>5000
11	CH_2NMe_2	H	9.0	1200
12	Cl	CH_2NMe_2	14	>5000
13		H	20	1000
14	Cl		21	>5000
16	Cl		9.5	>5000

[a] $K_{i,app}$ is the apparent K_i value, defined as $IC_{50}/2$ when the IC_{50} value is determined at a substrate concentration equal to the K_m. $K_{i,app}$ values are averaged from multiple determinations ($n \geqslant 2$), and the standard deviations are <30% of the mean.

[b] All compounds had $K_{i,app}$ values for bovine trypsin of >5000 nM.



Scheme 2. Reagents and conditions: (a) $NaNO_2$, HCl (aq), 0 °C; then CuCl, HCl, 0 °C; (b) 1 N NaOH, EtOH, reflux; (c) $(COCl)_2$, DMF (cat.), CH_2Cl_2; (d) **3**, pyridine; (e) *N*-bromosuccinimide, benzoyl peroxide, C_6H_6, irradiation; (f) amine, CH_2Cl_2.

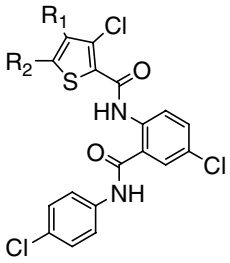
which underwent clean hydrolysis to the hydroxy acid **28**.[11] Reaction of **28** with thionyl chloride effected both acid chloride formation and conversion of the alcohol to the chloride to give **29**. Reaction of **29** with aniline **3** afforded chloromethyl intermediate **30**. Finally, the halogen was displaced by a variety of nucleophiles to afford the 4-substituted inhibitors (Tables 3–5). When necessary, a subsequent deprotection, oxidation or reductive amination step was carried out to afford the product of interest.

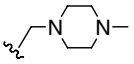
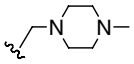
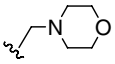
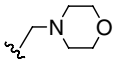
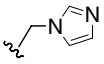
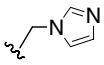
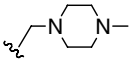
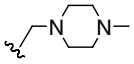
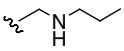
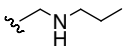
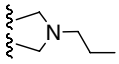
Scheme 4 illustrates the preparation of compounds substituted at both positions 4 and 5 of the thiophene. Thiophenes **18**, **22**, and **45** reacted with chloromethyl methyl ether and $TiCl_4$ to give chloromethyl intermediates **37**, **41**, and **46**.[12] Compounds **37** and **41** were reacted with 1-methylpiperazine, followed by hydrolysis, acid chloride formation, and reaction with aniline **3**, to give inhibitors **40** and **44**. The bis(chloromethyl) compound **46** was coupled to aniline **3** via the acid chloride to give intermediate **47**. Reaction with *n*-propylamine afforded a mixture of the disubstituted and cyclized compounds **48** and **49**.

3. Results and discussion

We initially looked for positions on the benzothiophene amenable to substitution by introducing a methyl group

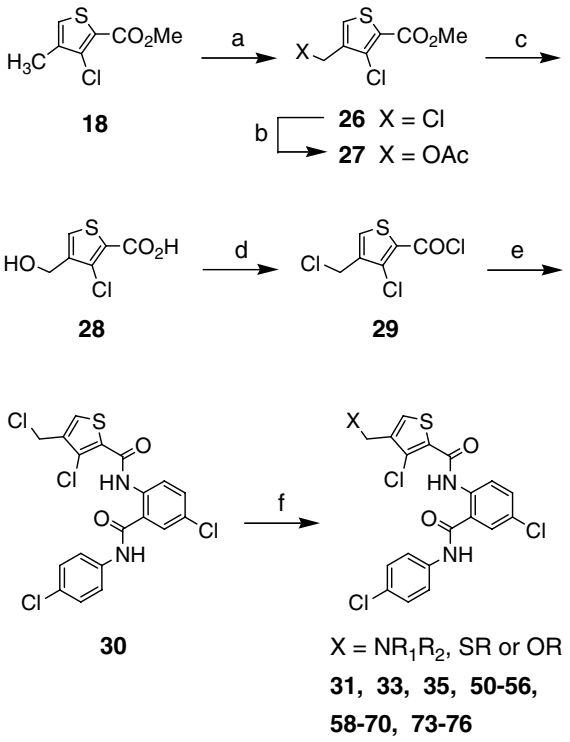
Table 3. Effect of thiophene substitution on enzyme inhibition and anticoagulant activity



Compound	R_1	R_2	$K_{i,app}$[a,b] (nM)		2× PT[c] (μM)	$c\log D$ (7.4)[d]
			fXa	fIIa		
20	CH_3	H	18	>5000	ND[e]	6.6
24	H	CH_3	24	>5000	ND	6.6
31		H	1.0	910	12	4.6
32	H		5.6	1000	13	4.8
33		H	6.2	2300	59	5.3
34	H		34	>5000	>500	5.4
35		H	1.9	1000	18	5.4
36	H		7.2	510	24	5.4
40		CH_3	12	4400	25	5.1
44	CH_3		10	1500	58	5.2
48			7.5	1500	19	3.0
49	R_1, R_2 = 		2.2	3200	110	6.0

[a] $K_{i,app}$ is the apparent K_i value, defined as $IC_{50}/2$ when the IC_{50} value is determined at a substrate concentration equal to the K_m. $K_{i,app}$ values are averaged from multiple determinations ($n \geqslant 2$), and the standard deviations are <30% of the mean.
[b] All compounds had $K_{i,app}$ values for bovine trypsin of >5000 nM.
[c] Concentration which gives a 2-fold extension of human prothrombin time in vitro.
[d] See Ref. 14.
[e] ND, not determined.

at each position. Because we planned to use the added methyl group as a handle for further substitution via a radical halogenation reaction, the methyl substituent at C-5 on the central anthranilamide ring needed to be replaced with a non-reactive group. Our previous research had shown that chlorine could be substituted for methyl at this position with no change in fXa potency (**4**).[6] Table 1 shows the results of methyl substitution at positions 3–7 of the benzothiophene. Replacement of the 3-chloro substituent (**5**) or addition of methyl at C-6 (**8**) maintained subnanomolar potency against fXa, while substitution at C-4 (**6**) caused complete loss of



Scheme 3. Reagents and conditions: (a) SO_2Cl_2, benzoyl peroxide, CCl_4, reflux; chromatography; (b) NaOAc, HOAc, reflux; (c) NaOH, 1,4-dioxane-H_2O; (d) $SOCl_2$, reflux; (e) **3**, pyridine, CH_2Cl_2; (f) nucleophile, DMF.

activity. The C-5 and C-7 methyl compounds (**7**) were obtained as an inseparable mixture in ca. 3:2 ratio by ^{1}H NMR. The rather weak inhibitory activity of the mixture indicated that neither position was promising for further substitution.

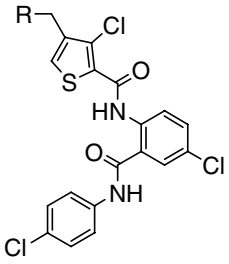
Compounds **5** and **8** were brominated and reacted with amines. The results in Table 2 demonstrate that substitution at either position afforded ca. 10- to 20-fold loss in inhibitory potency. This effect appeared to be relatively insensitive to steric influences, as the piperazine-substituted analogs (**13**, **14**, **16**) had similar potency to the dimethylamino compounds (**11**, **12**). This result suggests that these compounds bind to fXa with the benzothiophene in the S4 binding site, rather than the more tightly defined S1 pocket.[13] The C-3 substituted compounds also had $K_{i,app}$ values of ca. 1 μM for thrombin; this was the first observation of thrombin inhibition for compounds of this template. Although the compounds in Table 2 were moderately potent fXa inhibitors and were more hydrophilic than the unsubstituted benzothiophenes, they showed no significant improvement in in vitro anticoagulant activity, failing to cause two-fold prolongation of PT at concentrations up to 50 μM.

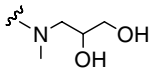
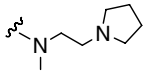
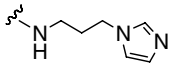
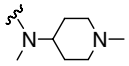
Our early work had shown that replacement of the chlorobenzothiophene with 3-chlorothiophene caused a ca. 50-fold loss in fXa potency.[6] We hypothesized that a substituent at C-4 or C-5 on thiophene could occupy the same region in the active site as the benzene ring of the benzothiophene. Proper choice of a thiophene substituent might maintain fXa potency, while increasing hydrophilicity, thus leading to improved anticoagulant activity. The C-4 and C-5 methylthiophenes were prepared as intermediates. Not surprisingly, these compounds were significantly less potent than the benzothiophene (Table 3, compounds **20** and **24** vs Table 1, compound **4**). However, attaching an *N*-methylpiperazine to the methyl group at C-4 (**31**) improved potency nearly 20-fold, affording an inhibitor with similar potency to benzothiophene **4**. More importantly, this was the first compound in this series to show measurable in vitro anticoagulant activity, prolonging PT 2-fold at a concentration of 12 μM. Substitution with *N*-methylpiperazine on the C-5 methyl also improved potency against fXa (**32** vs **24**, ca. 4-fold increase), although to a lesser extent than for C-4 substitution. This proved to be a general trend, with greater potency resulting from substitution at C-4 versus C-5 for a variety of amine substituents (compounds **31**–**36**). We also looked at the effect of introducing substituents at both C-4 and C-5. With a piperazine at C-4, addition of methyl at C-5 caused a ca. 10-fold loss in potency (**40** vs **31**). This may be due to steric interaction of the added C-5 substituent with Phe174 (Fig. 1), forcing the piperazine to adopt a less favorable position for binding. When the piperazine was at C-5, adding a methyl group at C-4 had essentially no effect on activity (**44** vs **32**). Introducing amine substituents at both C-4 and C-5 also did not improve potency (**48**), while incorporation of a basic ring across C-4 and C-5 (**49**) gave improved binding affinity but poor anticoagulant activity.

The PT results are consistent with the hypothesis that the physiochemical properties of this series of inhibitors have a large effect on their anticoagulant activity. Increasing hydrophilicity, as determined by the calculated log *D* at pH 7.4 (*c*log *D*), by changing to amine-substituted thiophenes afforded compounds that were active as anticoagulants, in contrast to benzothiophene analogs having equal or greater fXa inhibitory activity. For example, compounds **32**, **36**, **40**, and **44** have 2× PT values of 13–60 μM, compared to compounds **2**, **4**, **5**, and **8** that have calculated log *D* values >7.5, and fail to cause 2-fold prolongation of PT in vitro at concentrations up to 500 μM.[14] Further, compound pairs having the same lipophilicity also showed the same effect on PT despite differences in fXa potency (**31** and **32**, **35** and **36**). The exception to this is the morpholine-substituted pair (**33** and **34**). In this case, compound **34** may not be a potent enough fXa inhibitor to cause PT prolongation. Finally, compound **49** had similar fXa potency to compounds **31** and **35** but was a much weaker anticoagulant, consistent with its predicted greater lipophilicity.

Based on these results, we further explored SAR around the 4-substituent on the thiophene (Table 4). Within this series, inhibitory activity against fXa increased with increasing basicity of the nitrogen substituent.[14] Substitution with *N*-ethylpiperazine (**50**, cpKa = 7.8) was similar to *N*-methylpiperazine (**31**, cpKa = 7.7). However, neutral heterocyclic amine substituents (**33**, cpKa = 7.1;

Table 4. SAR of amine substituents at thiophene C-4



Compound	R	$K_{i,app}$[a,b] (nM)		2× PT[c] (μM)	*c*log *D* (7.4)[d]
		fXa	fIIa		
31	4-Methylpiperazin-1-yl	1.0	910	12	4.6
33	Morpholin-4-yl	6.2	2300	59	5.3
50	4-Ethylpiperazin-1-yl	1.6	>5000	12	5.1
51	4-Acetylpiperazin-1-yl	6.0	4400	ND[e]	6.6
52	Thiomorpholin-4-yl	4.8	>5000	400	6.2
53	$N(Me)CH_2CO_2Et$	5.2	2300	160	6.6
54	$N(Me)CH_2CH_2OH$	0.80	830	7.0	4.4
55		1.4	990	11	4.2
56	$–NHCH_2CH_2OCH_2CH_2OH$	3.4	3200	20	3.7
57	$–N(Me)CH_2CH_2OCH_2CH_2OH$	1.5	1100	8.8	3.7
58	$–N(Me)CH_2CH_2NMe_2$	0.45	160	4.6	5.1
59	$–N(Me)CH_2CH_2NEt_2$	0.52	120	3.9	6.0
60	$–N(Me)(CH_2)_3NMe_2$	0.60	190	2.9	4.0
61		0.30	160	5.0	5.3
62		4.6	1800	24	3.4
63		0.38	310	3.2	4.1
35	1*H*-Imidazol-1-yl	1.9	1000	18	5.4
64	1*H*-Pyrazol-1-yl	2.1	550	49	6.0
65	1*H*-1,2,4-Triazol-1-yl	1.6	2400	18	4.8
66	1*H*-1,2,3-Triazol-1-yl	1.4	2700	15	5.3
67	2*H*-1,2,3-Triazol-2-yl	3.5	3300	83	5.3
68	1*H*-Tetrazol-1-yl	1.7	>5000	29	5.1
69	2*H*-Tetrazol-2-yl	2.0	>5000	63	4.6

[a] $K_{i,app}$ is the apparent K_i value, defined as $IC_{50}/2$ when the IC_{50} value is determined at a substrate concentration equal to the K_m. $K_{i,app}$ values are averaged from multiple determinations ($n \geqslant 2$), and the standard deviations are <30% of the mean.

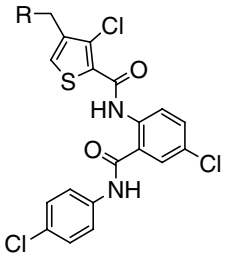
[b] All compounds had $K_{i,app}$ values for bovine trypsin of >5000 nM.

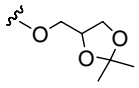
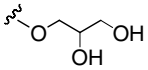
[c] Concentration which gives a 2-fold extension of human prothrombin time in vitro.

[d] See Ref. 14.

[e] ND, not determined.

Table 5. SAR of sulfur and oxygen substituents on enzyme inhibition and anticoagulant activity



Compound	R	$K_{i,app}$[a,b] (nM)		2× PT[c] (μM)	$c\log D$ (7.4)[d]
		fXa	fIIa		
70	–SMe	0.80	>5000	>200	6.5
71	–S(O)Me	0.40	970	5.6	4.3
72	$-SO_2Me$	2.1	>5000	14	4.6
73	$-SCH_2CH_2NMe_2$ $pK_a = 8.9$	2.7	560	19	5.2
74	$-OCH_2CH_2OMe$	12	>5000	110	5.9
75	$-OCH_2CH_2OCH_2CH_2OMe$	10	3600	95	6.0
76		20	>5000	ND[e]	6.2
77		5.7	2400	30	4.6

[a] $K_{i,app}$ is the apparent K_i value, defined as $IC_{50}/2$ when the IC_{50} value is determined at a substrate concentration equal to the K_m. $K_{i,app}$ values are averaged from multiple determinations ($n \geqslant 2$), and the standard deviations are <30% of the mean.

[b] All compounds had $K_{i,app}$ values for bovine trypsin of >5000 nM.

[c] Concentration which gives a 2-fold extension of human prothrombin time in vitro.

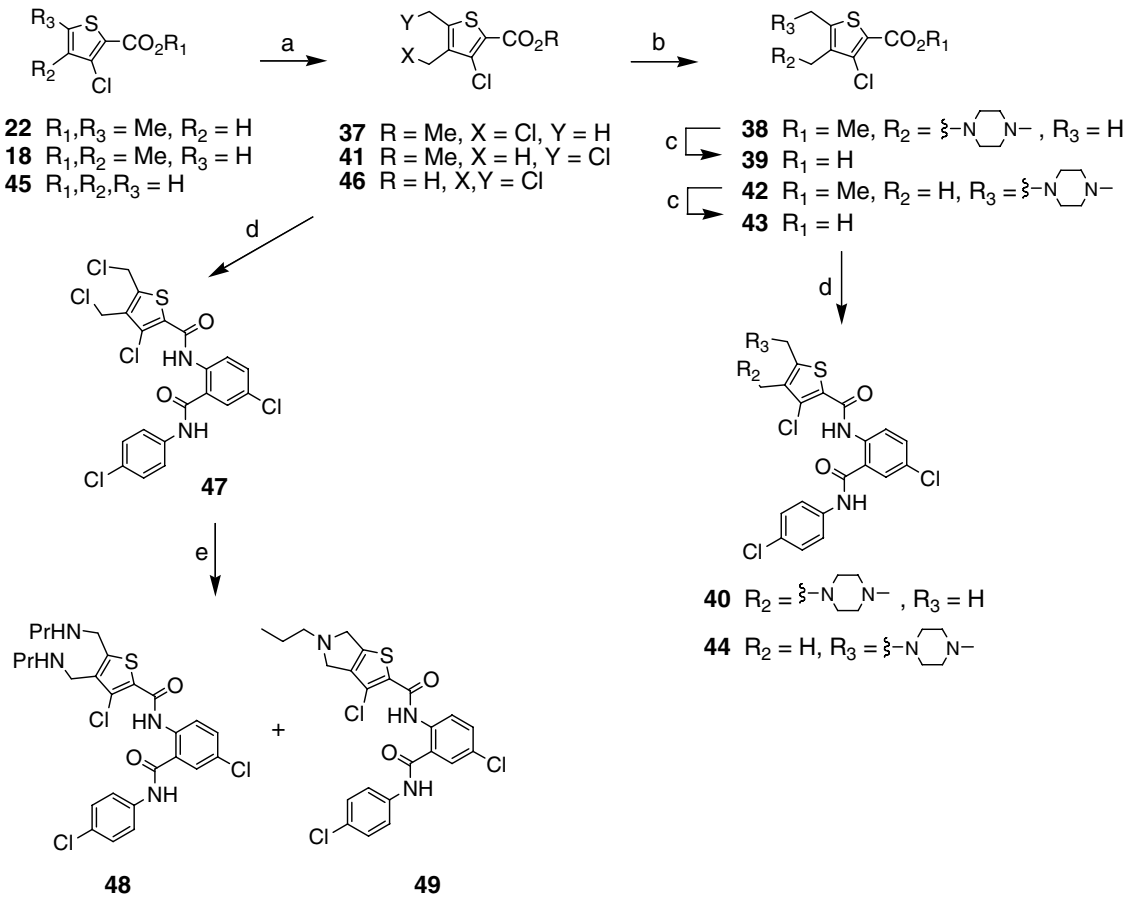
[d] See Ref. 14.

[e] ND, not determined.

51, cpKa = 5.6; and **52**, cpKa = 7.1) gave decreased fXa potency. Aliphatic mono- and diamines were also investigated. We selected monoamines bearing at least one additional polar substituent in order to increase hydrophilicity. Again, compounds with a more basic tertiary nitrogen (**54**, **55**, **57**, cpKa = 8.0–8.1) were more potent inhibitors than a compound without a basic nitrogen (**53**, cpKa = 6.6). Increasing the number of hydroxy or ether substituents had little effect on either fXa or PT potency in this series. Introduction of a basic substituent in addition to the linking nitrogen gave slight improvements in both fXa and PT potency relative to the monoamine substituents (**58**–**61**, **63**). For these compounds, the terminal basic group may be positioned to interact with the acidic side chain of Glu 97 in the S4 pocket. The fXa potency for this series was relatively unaffected by chain length (**58** vs **60**), substituent size (**58**, **59**, **61**) or configuration (**63**), indicating significant flexibility in binding to the S4 site. The exception was the imidazole-substituted compound (**62**), which was an order of magnitude less potent than the aliphatic diamines. The lack of a methyl group on the linking nitrogen atom is not expected to significantly impact the potency of this compound (cf. **56** vs **57**). Calculations indicate that the benzylic nitrogen of this group is preferentially protonated, and the imidazole is neutral. This may result in loss of interaction with Glu 97, resulting in a similar potency for **62** as for the monoamine compounds **53**–**57**. The diamine-substituted compounds also showed increased affinity for thrombin relative to the monoamines, but maintained at least 200-fold selectivity for fXa. Since the $c\log D$ values for the diamine compounds fall in the same range as those of the monoamines, the improvement in anticoagulant activity for the diamines is attributed to their increased affinity for fXa.

A series of five-membered nitrogen-containing aromatic heterocycles was also investigated (**35**, **64**–**69**). All of these compounds had essentially the same potency against fXa, suggesting that none of the ring atoms are positioned properly to act as hydrogen bond acceptors. The affinity of these compounds for fXa may result from stacking interactions with the aromatic residues in the S4 binding pocket.

Finally, we investigated substituents on the thiophene in which the heteroatom linked to the methylene group was sulfur or oxygen rather than nitrogen (Table 5). Despite their shorter length, the methylthio- (**70**) and methyl sulfoxide (**71**)-substituted inhibitors had similar fXa potency to the aliphatic mono- and diamines, indicating that it is not necessary to fill the S4 pocket to retain good binding affinity in this inhibitor series. The more hydrophilic sulfoxide (**71**) and sulfone (**72**) analogs also had improved anticoagulant activity relative to the sulfide (2× PT = 5.6 and 14 μM vs >200 μM). In general, however, the oxygen- or sulfur-substituted inhibitors were less potent than the amino compounds. In cases where substituents can be directly compared (**73** vs **58**, **77** vs **55**), the amines were superior both as fXa inhibitors and as anticoagulants.



Scheme 4. Reagents and conditions: (a) $TiCl_4$, CH_3OCH_2Cl; (b) NEt_3, 1-methylpiperazine, CH_2Cl_2; (c) 2 N HCl, 90 °C; (d) $SOCl_2$, reflux, or $(COCl)_2$, CH_2Cl_2; then **3**, pyridine; (e) *n*-propylamine, CH_3CN, 50 °C, sealed vessel.

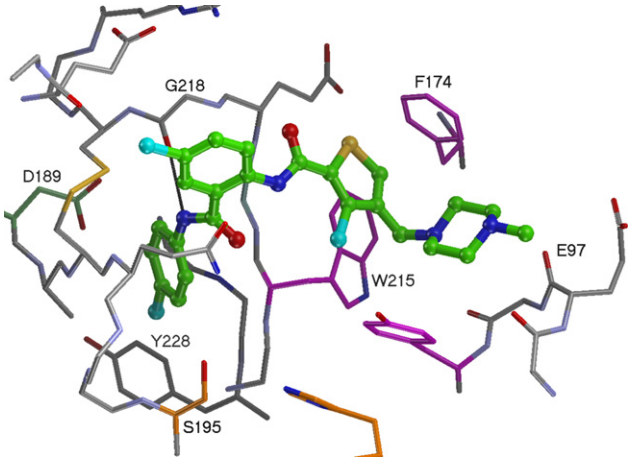


Figure 1. A view of compound **31** bound to fXa. The figure is derived from the coordinates of pdb entry 1MQ5.[16] Carbon atoms of specific residues have been colored. Compound **31** is colored in green and its chlorine atoms are shown in cyan. Residues that form the S4 pocket, Tyr99, Phe174, and Trp215 are magenta. Asp189 at the bottom of the S1 pocket is light green. Gly218 is light blue. Two residues from the catalytic triad, Ser195 and His57, are shown in orange. The black line denotes the single H-bond between the inhibitor and the protein.

The pharmacokinetic profile of compound **31** was evaluated in dogs to obtain a preliminary assessment of this template. The compound was dosed in conscious dogs intravenously at 1 mg/kg and by oral gavage at 10 mg/kg. Plasma samples were taken and inhibitor concentrations determined ex vivo using an established chromogenic assay measuring factor Xa inhibition.[15] After iv dosing, compound **31** had a half-life of 2 h, clearance of 0.3 L/h/kg, and a volume of distribution at steady state of 0.7 L/kg. The peak plasma level after oral dosing was 6.3 μM, and the oral bioavailability was estimated to be 41%.

The crystal structure of compound **31** bound to factor Xa is shown in Figure 1.[16] The inhibitor binds in an L-shaped conformation with the chlorobenzene in the S1 pocket and the thiophene extending into the S4 binding site. The chlorine atom makes contact with Tyr228 in the S1 pocket; this chloro binding mode has been reported for other factor Xa inhibitors,[17] as well as for inhibitors of thrombin,[8b] trypsin,[18] and urokinase.[19] The binding of **31** to fXa arises primarily from hydrophobic interactions and steric fit. Only one well-defined hydrogen bond to the protein is present between the amide NH attached to the chlorobenzene ring and the carbonyl of glycine 218.

The crystal structure provides an explanation for the relative affinity of some of the compounds in the thiophene series. Table 3 indicates that substituents attached to thiophene C-5 are less potent than the same group

attached to C-4 (compounds **31**–**36**). Our modeling studies of the bound conformation of **32** (C-5) indicate the thiophene ring would rotate by 180° compared to **31**. Once the thiophene is rotated, the *N*-methylpiperazine group can fold into the S4 pocket the same way as **31**. The thiophene rotation breaks the intramolecular hydrogen bond between the chlorine on the thiophene ring and the amide nitrogen (3.2 Å in **31**).[20] The rotation also places the chlorine in close proximity to the acidic side chain of Glu217 (~4.5Å). These alterations in the binding contribute to the 5-fold loss in potency for the C-5 piperazine group.

It has been more difficult to explain electrostatic interactions in the S4 pocket. The published structures of the majority of potent (<1 nM) fXa inhibitors have basic groups in the S4 pocket and the inhibitor has at least one atom in close contact with the indole ring of Trp215 (structures 1FJS, 1EZQ, 1KSN, 1MQ6).[16,21] The SAR shown in Tables 4 and 5 is mostly consistent with this result. Our own work has shown that an additional basic group on the S4 substituent improves the potency of these compounds (**58**–**61**, **63**). These groups may interact with the side chain of Glu97 in a fashion similar to fXa/DX-9065a (pdb code 1FAX[21a]). However, ionic interaction with Glu97 is notably absent in the other published structures of human fXa and the structures do not show any firm correlations between potency and electrostatics.

4. Conclusion

In summary, we have discovered thiophene-substituted anthranilamides as a series of potent non-amidine fXa inhibitors. Initial inhibitors in this series had subnanomolar fXa inhibitory potency, but were inactive as anticoagulants. Replacement of the benzothiophene with a thiophene having a basic substituent at C-4 afforded improved anticoagulant activity. Optimization of the thiophene substituent by addition of aliphatic diamines afforded a series of subnanomolar fXa inhibitors with low micromolar anticoagulant activity. Inhibitors in this series demonstrate that anticoagulant activity is linked to both fXa affinity and lipophilicity. This suggests that the anticoagulant activity of a compound, and therefore its antithrombotic potential, can be improved by introducing changes which are neutral with respect to binding affinity but result in a better physiochemical profile. During our investigation of this series, it was discovered that certain modifications to the chlorobenzene and anthranilic acid rings of this template afforded compounds with improved fXa and PT potency, and good pharmacokinetic properties. This work will be detailed in future publications.

5. Experimental

5.1. General

All starting materials not described below were purchased from commercial sources or synthesized following the noted literature procedure. All reagents and solvents were used as received from commercial sources without additional purification. Elemental analyses were performed by Robertson Microlit Laboratories; Madison, NJ, and results were within ±0.4% of the calculated values. NMR spectra were recorded with a Varian XL-300 spectrometer and were consistent with the assigned structures. HPLC was performed with a Rainin SD-1 Dynamax system and a C-18 reverse phase Dynamax 60A column using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA). Human fXa and human fIIa were from Enzyme Research Lab., South Bend, IN, and bovine trypsin was from Boehringer–Mannheim Corp., Indianapolis, IN. All peptide *p*-nitroanilide substrates were purchased from Kabi Pharmacia Hepar, Inc., Franklin, OH. Tris–HCl, NaCl, and $CaCl_2$ were from J. T. Baker Inc., Jackson, TN, and polyethylene glycol 6000 was from BDH Laboratory Supplies, Poole, England.

5.2. 2-Amino-5-chloro-*N*-(4-chlorophenyl)benzamide (3)

To a suspension of 5-chloro-2-nitrobenzoic acid (21 g, 100 mmol) in CH_2Cl_2 (200 mL) at 0 °C were added several drops of DMF, followed by oxalyl chloride (13 mL, 150 mmol). The reaction mixture was warmed to room temperature. After 16 h, the mixture was concentrated. The crude acid chloride was dissolved in CH_2Cl_2 (200 mL) and cooled to 0 °C. Triethylamine (16 mL, 115 mmol) was added, followed by 4-chloroaniline (14 g, 110 mmol). The mixture was stirred for 20 min at 0 °C, then warmed to room temperature. After 5 h, the mixture was concentrated. The residual solid was dissolved in EtOAc (400 mL) and washed with H_2O (200 mL), aqueous 1 N HCl (2× 200 mL), aqueous 1 N $NaHCO_3$ (200 mL), and brine (200 mL), and dried over $MgSO_4$. Concentration and drying afforded 5-chloro-*N*-(4-chlorophenyl)-2-nitrobenzamide as a pale yellow solid (30 g, 93%). ^{1}H NMR ($CDCl_3$) δ 8.1 (d, 1H), 7.7 (br s, 1H), 7.6 (m, 2H), 7.5 (d, 2H), 7.3 (d, 2H).

N-(4-Chlorophenyl)-5-chloro-2-nitrobenzamide (13.2 g, 42.4 mmol) and $SnCl_2{\cdot}2H_2O$ (48 g, 213 mmol) were combined in EtOAc (90 mL) and the mixture was heated at 70 °C under a nitrogen atmosphere. After 15 min, the mixture was cooled to room temperature, then poured onto H_2O (750 mL) and EtOAc (750 mL). The aqueous layer was adjusted to pH 8 by addition of aqueous 1 N NaOH and a saturated aqueous $NaHCO_3$ solution, and the layers were separated. The aqueous layer was further extracted with EtOAc (500 mL). The combined organic extracts were washed with H_2O (1 L), then brine (500 mL), dried over $MgSO_4$, filtered, and concentrated to afford **3** as an off-white solid (11.6 g, 97%). ^{1}H NMR ($CDCl_3$) δ 7.7 (br s, 1H), 7.2–7.5 (m, 6H), 6.7 (d, 1H), 5.5 (br s, 2H).

5.3. 3-Chloro-4-methyl-2-thiophenecarboxylic acid methyl ester (18)

To a suspension of 3-amino-4-methyl-2-thiophenecarboxylic acid methyl ester, **17**, (2.0 g, 12 mmol) in aqueous 6 N HCl (5 mL) at 0 °C was added a solution of $NaNO_2$

(0.80 g, 12 mmol) in H_2O (2 mL). The resulting solution was stirred for 1 h, then added slowly to a 0 °C solution of CuCl (1.2 g, 12 mmol) in 12 N HCl (5 mL) (Note: vigorous foaming). After complete addition, the mixture was warmed to room temperature, poured onto H_2O (250 mL), and extracted with EtOAc (2× 100 mL). The combined extracts were washed with H_2O and brine, dried over Na_2SO_4, and concentrated to afford **18** as a yellow solid (1.9 g, 84%), used without purification. ^{1}H NMR ($CDCl_3$) δ 7.2 (s, 1H), 3.9 (s, 3H), 2.2 (s, 3H).

5.4. 3-Chloro-5-methyl-2-thiophenecarboxylic acid methyl ester (22)

Prepared from 3-amino-5-methyl-2-thiophenecarboxylic acid methyl ester (**21**)[22] as described for compound **18**. Purification of the crude product by flash chromatography on silica gel gave **22** as a white solid (0.87 g, 51%). ^{1}H NMR ($CDCl_3$) δ 6.7 (s, 1H), 3.9 (s, 3H), 2.5 (s, 3H).

5.5. 3-Chloro-4-methyl-2-thiophenecarboxylic acid (19)

To a solution of ester **18** (1.8 g, 9.3 mmol) in EtOH (21 mL) was added aqueous 1 N NaOH (21 mL, 21 mmol). The solution was refluxed for 0.5 h, then cooled and concentrated. The residue was dissolved in H_2O (25 mL) and acidified by slow addition of aqueous 1 N HCl (25 mL) with stirring. The precipitate was collected by filtration and dried to afford **19** as a white solid (1.4 g, 88%). ^{1}H NMR ($CDCl_3$) δ 7.3 (s, 1H), 2.2 (s, 3H).

5.6. 3-Chloro-5-methyl-2-thiophenecarboxylic acid (23)

Prepared from ester **22** as described for compound **19**, white solid (0.34 g, 90%). ^{1}H NMR ($CDCl_3$) δ 6.8 (s, 1H), 2.5 (s, 3H).

5.7. Coupling of benzothiophenes or thiophenes with anthranilamide 3. General method for preparation of compounds 4–8, 20, and 24

To a solution of the appropriate benzothiophene or thiophene acid chloride (1.0–2.0 equiv) in pyridine (0.1 M) at 0 °C was added **3**, and the mixture warmed to room temperature. After 1–18 h, the mixture was concentrated, and the crude solid washed with H_2O, dried, and purified by trituration or crystallization. If necessary, the acid chloride was prepared from the corresponding carboxylic acid by reaction with oxalyl chloride in CH_2Cl_2 as described in the preparation of compound **3**. In general, the crude acid chlorides were used without purification.

5.8. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-benzo[*b*]thiophene-2-carboxamide (4)

Prepared from 3-chlorobenzo[*b*]thiophene-2-carbonyl chloride (1.2 equiv) and **3** using CH_2Cl_2 as solvent, with addition of pyridine (2.0 equiv). Trituration with CH_3CN gave **4** as a white solid (0.12 g, 64%). ^{1}H NMR (DMSO-d_6/TFA) δ 11.4 (s, 1H), 10.8 (s, 1H), 8.4 (d, 1H), 7.4–8.1 (m, 10H). Anal. Calcd for $C_{22}H_{13}Cl_3N_2O_2S{\cdot}0.1CH_2Cl_2$: C, 54.81; H, 2.75; N, 5.78. Found: C, 54.86; H, 2.64; N, 5.42.

5.9. *N*-[4-Chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-3-methylbenzo[*b*]thiophene-2-carboxamide (5)

Prepared from 3-methylbenzo[*b*]thiophene-2-carbonyl chloride[23] (1.5 equiv) and **3**. Crystallization from CH_3CN gave **5** as colorless needles (0.14 g, 29%). ^{1}H NMR (DMSO-d_6) δ 11.2 (s, 1H), 10.7 (s, 1H), 8.4 (d, 1H), 7.4–8.0 (m, 10H), 2.8 (s, 3H). Anal. Calcd for $C_{23}H_{16}Cl_2N_2O_2S$: C, 60.67; H, 3.54; N, 6.15. Found: C, 60.61; H, 3.40; N, 6.02.

5.10. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-methylbenzo[*b*]thiophene-2-carboxamide (6)

Prepared from 3-chloro-4-methylbenzo[*b*]thiophene-2-carbonyl chloride[24] (2.0 equiv) and **3**. Crystallization from CH_3CN gave **6** as colorless needles (0.082 g, 47%). ^{1}H NMR (DMSO-d_6) δ 11.4 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.3–7.9 (m, 9H), 2.8 (s, 3H). Anal. Calcd for $C_{23}H_{15}Cl_3N_2O_2S$: C, 56.40; H, 3.09; N, 5.72. Found: C, 56.20; H, 2.95; N, 5.61.

5.11. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)aminocarbonyl]phenyl]-5-methylbenzo[*b*]thiophene-2-carboxamide and 3-chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino] carbonyl]phenyl]-7-methylbenzo[*b*]thiophene-2-carboxamide (7)

Synthesis of 3-chloro-5-methylbenzo[*b*]thiophene-2-carbonyl chloride according to published methods[24] afforded an inseparable mixture of the 5- and 7-methyl compounds in ca. 3:2 ratio based on ^{1}H NMR integration of the methyl signals. Reaction with **3** and crystallization from CH_3CN gave a white solid (0.11 g, 61%, inseparable mixture of 5- and 7-methyl compounds). ^{1}H NMR (DMSO-d_6) δ 11.4 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.4-8.0 (m, 9H), 2.5 (s, masked by solvent).

5.12. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino] carbonyl]phenyl]-6-methylbenzo[*b*]thiophene-2-carboxamide (8)

Prepared from 3-chloro-6-methylbenzo[*b*]thiophene-2-carbonyl chloride[24] (1.3 equiv) and **3**. Crystallization from CH_3CN gave **8** as a white solid (0.038 g, 22%). ^{1}H NMR (DMSO-d_6) δ 11.3 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.4–7.9 (m, 9H), 2.5 (s, 3H). Anal. Calcd for $C_{23}H_{15}Cl_3N_2O_2S$: C, 56.40; H, 3.09; N, 5.72. Found: C, 56.51; H, 2.75; N, 5.65.

5.13. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino] carbonyl]phenyl]-4-methyl-2-thiophenecarboxamide (20)

Prepared from **19** (1.0 equiv) and **3**. Trituration with CH_3CN gave **20** as a light brown solid (0.61 g, 78%). ^{1}H NMR (DMSO-d_6) δ 11.0 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (d, 1H), 7.4–7.7 (m, 6H), 2.1 (s, 3H). Anal. Calcd for $C_{19}H_{13}Cl_3N_2O_2S$: C, 51.90; H, 2.98; N, 6.37. Found: C, 52.14; H, 2.71; N, 6.64.

5.14. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-5-methyl-2-thiophenecarboxamide (24)

Prepared from **23** (1.0 equiv) and **3**. Trituration with CH_3CN gave **24** as a light brown solid (0.49 g, 75%).

^{1}H NMR (DMSO-d_6) δ 11.0 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.4-7.9 (m, 6H), 7.0 (d, 1H), 2.5 (d, 3H). Anal. Calcd for $C_{19}H_{13}Cl_3N_2O_2S$: C, 51.90; H, 2.98; N, 6.37. Found: C, 51.76; H, 2.80; N, 6.14.

5.15. 5-(Bromomethyl)-3-chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-2-thiophenecarboxamide (25)

To a suspension of **24** (2.6 g, 6.0 mmol) in benzene (250 mL) were added NBS (1.2 g, 6.6 mmol) and benzoyl peroxide (0.15 g, 0.6 mmol). The mixture was refluxed while irradiating with a 250 W lamp. After 28 h, the reaction mixture was concentrated and the resulting solid triturated with benzene. Purification by flash chromatography on silica gel afforded **25** as a white solid (2.3 g, 75%). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.7 (s, 1H), 8.3 (d, 1H), 7.9 (s, 1H), 7.7 (d, 2H), 7.6 (d, 1H), 7.4 (d, 2H), 7.3 (s, 1H), 4.9 (s, 2H).

5.16. 3-(Bromomethyl)-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]benzo[*b*]thiophene-2-carboxamide (9)

Prepared from **5** as described for compound **25**, white solid (0.087 g, 74%). ^{1}H NMR (DMSO-d_6) δ 11.4 (s, 1H), 10.8 (s, 1H), 8.4 (d, 1H), 7.4–8.2 (m, 10H), 5.3 (s, 2H).

5.17. 6-(Bromomethyl)-3-chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]benzo[*b*]thiophene-2-carboxamide (10)

Prepared from **8** as described for compound **25**, white solid (0.23 g, 39%). ^{1}H NMR (DMSO-d_6) δ 11.4 (s, 1H), 10.8 (s, 1H), 7.4–8.4 (m, 10H), 4.9 (s, 2H).

5.18. *N*-[4-Chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-3-[(dimethylamino)methyl]benzo[*b*]thiophene-2-carboxamide (11)

To a suspension of **9** (0.065 g, 0.12 mmol) in CH_2Cl_2 (1.5 mL) in a pressure vessel was added dimethylamine hydrochloride (0.035 g, 0.43 mmol), followed by Bio-Rad AG1-X8 anion exchange resin (0.55 g, 0.7 mmol equivalents, OH^- form). The vessel was sealed and the mixture stirred at room temperature for 3.5 h. The vessel was opened and the reaction mixture diluted with CH_2Cl_2 (25 mL) and CH_3CN (25 mL), filtered, and concentrated. Purification by flash chromatography on silica gel, followed by crystallization from CH_3CN, afforded **11** as a white solid (0.030 g, 50%). ^{1}H NMR (DMSO-d_6/TFA) δ 13.2 (s, 1H), 10.7 (s, 1H), 7.3-8.1 (m, 11H), 3.9 (s, 2H), 2.1 (s, 6H). Anal. Calcd for $C_{25}H_{21}Cl_2N_3O_2S$: C, 60.24; H, 4.25; N, 8.43. Found: C, 60.19; H, 4.23; N, 8.38.

5.19. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-6-[(dimethylamino)methyl]benzo[*b*]thiophene-2-carboxamide (12)

Prepared from **10** as described for compound **11**, white powder (0.047 g, 50%). ^{1}H NMR (DMSO-d_6/TFA) δ 11.4 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 8.2 (s, 1H), 7.4–8.0 (m, 8H), 4.0 (br s, 2H), 2.5 (s, 6H, masked by solvent). Anal. Calcd for $C_{25}H_{20}Cl_3N_3O_2S$: C, 53.34; H, 3.58; N, 7.46. Found: C, 53.01; H, 3.55; N, 7.26.

5.20. General method for preparation of amine-substituted compounds 13, 14, 32, and 34

To a suspension of the appropriate bromomethyl precursor in CH_2Cl_2 (0.1 M) was added an amine (5 equiv). The mixture was stirred at room temperature for 18 h, then diluted with CH_2Cl_2, washed with saturated aqueous $NaHCO_3$, and the aqueous layer back-extracted with CH_2Cl_2. The combined organics were dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography on silica gel. Alternatively, the product was purified by HPLC using a Dynamax column and a gradient of CH_3CN in H_2O with 0.1% TFA. The resulting fractions were combined and the solvent was removed by lyophilization to afford the product as a trifluoroacetic acid salt.

5.21. *N*-[4-Chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-3-[(4-methyl-1-piperazinyl)methyl]benzo[*b*]thiophene-2-carboxamide (13)

Prepared from **9** and 1-methylpiperazine. Purified by flash chromatography, pale yellow solid (0.043 g, 83%). ^{1}H NMR (DMSO-d_6/TFA) δ 11.8 (s, 1H), 10.8 (s, 1H), 8.4 (d, 1H), 8.2 (m, 2H), 8.0 (d, 1H), 7.4–7.7 (m, 7H), 4.9 (s, 2H), 3.5 (br s, 8H), 2.8 (s, 3H). Anal. Calcd for $C_{28}H_{26}Cl_2N_4O_2S{\cdot}0.3C_4H_8O_2$: C, 60.47; H, 4.94; N, 9.66. Found: C, 60.80; H, 5.01; N, 9.31.

5.22. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-6-[(4-methyl-1-piperazinyl)methyl]benzo[*b*]thiophene-2-carboxamide (14)

Prepared from **10** and 1-methylpiperazine. Purified by flash chromatography, white solid (0.054 g, 52%). ^{1}H NMR (DMSO-d_6/TFA) δ 11.4 (s, 1H), 10.8 (s, 1H), 8.4 (d, 1H), 8.3 (s, 1H), 8.0 (d, 1H), 7.9 (d, 1H), 7.4–7.7 (m, 6H), 4.6 (s, 2H), 3.5 (br s, 8H), 2.9 (s, 3H). Anal. Calcd for $C_{28}H_{25}Cl_3N_4O_2S{\cdot}0.6CH_2Cl_2$: C, 53.77; H, 4.13; N, 8.77. Found: C, 53.66; H, 3.92; N, 8.69.

5.23. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-5-[(4-methyl-1-piperazinyl)methyl]-2-thiophenecarboxamide trifluoroacetic acid salt (32)

Prepared from **25** and 1-methylpiperazine. Purified by HPLC, white solid (0.089 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.7 (s, 1H), 8.3 (d, 1H), 7.7 (d, 2H), 7.6 (dd, 1H), 7.3 (s, 2H), 7.2 (dd, 1H), 4.5 (s, 2H), 3.6–3.2 (br m, 8H), 2.8 (s, 3H). Anal. Calcd for $C_{24}H_{23}Cl_3N_4O_2S{\cdot}2.8C_2HF_3O_2{\cdot}H_2O$: C, 40.62; H, 3.20; N, 6.40. Found: C, 40.92; H, 3.00; N, 6.58.

5.24. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-5-(4-morpholinylmethyl)-2-thiophenecarboxamide (34)

Prepared from **25** and morpholine. Purified by flash chromatography, pale yellow solid (0.40 g, 79%). ^{1}H NMR ($CDCl_3$) δ 11.0 (s, 1H), 9.2 (s, 1H), 8.3 (d, 1H), 7.8 (d, 2H), 7.5 (d, 1H), 7.4 (d, 2H), 7.2 (dd, 1H), 6.9

(s, 1H), 3.75 (br m, 4H), 3.68 (s, 2H), 2.55 (br m, 4H). Anal. Calcd for $C_{23}H_{20}Cl_3N_3O_3S{\cdot}0.2H_2O$: C, 52.28; H, 3.89; N, 7.95. Found: C, 52.07; H, 3.85; N, 7.93.

5.25. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-5-(1*H*-imidazol-1-ylmethyl)-2-thiophenecarboxamide (36)

Prepared from **25** and imidazole sodium salt. Purified by flash chromatography, light brown solid (0.15 g, 31%). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.7 (s, 1H), 9.2 (s, 1H), 8.3 (d, 1H), 7.9 (d, 1H), 7.8 (s, 1H), 7.3–7.2 (m, 4H), 7.4 (s, 1H), 7.3 (s, 2H), 5.6 (s, 2H). Anal. Calcd for $C_{22}H_{15}Cl_3N_4O_2S{\cdot}0.5H_2O$: C, 51.33; H, 3.13; N, 10.88. Found: C, 51.23; H, 3.09; N, 10.80.

5.26. 4-[[3-Chloro-2-[[[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]amino]carbonyl]benzo[*b*]thien-6-yl]methyl]-1-piperazineacetic acid trifluoroacetic acid salt (16)

Treatment of **10** with 1-piperazineacetic acid ethyl ester (5 equiv) as described for the preparation of **13** afforded ester **15** (85 mg, 49%). ^{1}H NMR (DMSO-d_6) δ 11.4 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.4–8.0 (m, 9H), 4.1 (m, 2H), 3.6 (s, 2H), 3.2 (s, 2H), 3.1 (m, 1H), 2.7 (m, 1H), 2.4 (br m, 6H), 1.2 (t, 3H).

Compound **15** (84 mg, 0.13 mmol) was dissolved in a mixture of 3:1:1 (v/v/v) THF–MeOH–H_2O (5 mL) and treated with $LiOH{\cdot}H_2O$ (20 mg, 0.48 mmol) at room temperature for 1 h. The mixture was poured into H_2O (20 mL), acidified with aqueous 1 N HCl, and the resulting solid collected by filtration. Purification by HPLC and lyophilization afforded **16** as a white solid, (0.027 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.4 (s, 1H), 10.8 (s, 1H), 8.4 (d, 1H), 8.3 (br s, 1H), 8.1 (d, 1H), 8.0 (d, 1H), 7.4–7.7 (m, 6H), 4.6 (s, 2H), 4.2 (s, 2H), 3.5 (br s, 8H). Anal. Calcd for $C_{29}H_{25}Cl_3N_4O_4S{\cdot}2.5C_2HF_3O_2$: C, 44.53; H, 3.02; N, 6.11. Found: C, 44.87; H, 3.11; N, 6.29.

5.27. 3-Chloro-4-chloromethyl-2-thiophenecarboxylic acid methyl ester (26)

To 1.5 L of dry CCl_4 were added **18** (100 g, 0.53 mol), SO_2Cl_2 (65 mL, 0.81 mol), and benzoyl peroxide (2.5 g, 10 mmol). The reaction mixture was refluxed for 17 h (HPLC analysis showed a product to starting material ratio of 1.4:1). The reaction mixture was cooled to room temperature and concentrated. The resulting oil was purified by flash chromatography on silica gel to give **26** as a pale yellow oil which crystallized to fine needles upon standing (63 g, 54%), mp 55–56 °C. ^{1}H NMR ($CDCl_3$) δ 7.6 (s, 1H), 4.6 (s, 2H), 3.9 (s, 3H). Also recovered was unreacted **18** (36 g, 36%).

5.28. 4-Acetoxymethyl-3-chloro-2-thiophenecarboxylic acid methyl ester (27)

To **26** (48 g, 0.21 mol) in glacial HOAc (500 mL) was added NaOAc (35 g, 0.42 mol). The reaction mixture was refluxed for 24 h, cooled, and concentrated. The mixture was made basic by addition of saturated aqueous $NaHCO_3$, and the resulting solution was extracted with EtOAc (4× 150 mL). The combined extracts were dried over Na_2SO_4 and concentrated to give **27** as a light brown oil (47 g, 90%). ^{1}H NMR ($CDCl_3$) δ 7.6 (s, 1H), 5.1 (s, 2H), 3.9 (s, 3H), 2.1 (s, 3H).

5.29. 3-Chloro-4-hydroxymethyl-2-thiophenecarboxylic acid (28)

To 350 mL of 1,4-dioxane was added **27** (83 g, 0.33 mol) followed by a solution of NaOH (26.5 g, 0.66 mol) in H_2O (200 mL). After 1 h, the dioxane was removed in vacuo and the aqueous phase washed with EtOAc (2× 100 mL). The aqueous layer was adjusted to pH 2 with 12 N HCl and extracted with *n*-butanol (4× 200 mL). The combined organics were concentrated and dried to afford **28** as a light brown solid (63 g, 90%). ^{1}H NMR (DMSO-d_6) δ 7.7 (s, 1H), 4.4 (s, 2H).

5.30. 3-Chloro-4-chloromethyl-2-thiophenecarbonyl chloride (29)

Compound **28** (83 g, 0.43 mmol) was added to $SOCl_2$ (200 mL) and the mixture refluxed for 6 h. After cooling, the mixture was concentrated to dryness, followed by concentration from 1,2-dichloroethane to complete removal of the $SOCl_2$. The crude product was dissolved in CH_2Cl_2 (250 mL) and filtered to remove solids. The filtrate was concentrated to give **29** as a light brown waxy solid (100 g, 89%). ^{1}H NMR ($CDCl_3$) δ 7.8 (s, 1H), 4.6 (s, 2H).

5.31. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-(chloromethyl)-2-thiophenecarboxamide (30)

To a solution of **29** (3.1 g, 13.5 mmol) in CH_2Cl_2 (40 mL) at 0 °C was added **3** (3.8 g, 13.5 mmol), followed after 5 min by pyridine (1.6 mL, 16 mmol). The mixture was warmed to room temperature. After 17 h, the mixture was concentrated, and the resulting solid was triturated with H_2O and a small amount of CH_3CN, and dried to afford **30** as a light brown solid (5.1 g, 80%). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 8.2 (s, 1H), 7.9 (s, 1H), 7.7 (d, 2H), 7.6 (dd, 1H), 7.4 (d, 2H), 4.8 (s, 2H).

5.32. 3-Chloro-4-(chloromethyl)-5-methyl-2-thiophenecarboxylic acid methyl ester (37)

To a solution of **22** (1.4 g, 7.5 mmol) in chloromethyl methyl ether (1.4 mL, 18 mmol) at 0 °C under a nitrogen atmosphere was added $TiCl_4$ (0.84 mL, 7.7 mmol) dropwise over 10 min. The mixture solidified. The cooling bath was removed, an additional 0.6 mL (7.9 mmol) of chloromethyl methyl ether added, and the resulting slurry stirred at room temperature. After 2 h, the mixture was poured onto CH_2Cl_2 (30 mL), washed with H_2O and brine, dried over $MgSO_4$, and concentrated. Purification by flash chromatography on silica gel afforded **37** as a white solid (1.6 g, 90%). ^{1}H NMR ($CDCl_3$) δ 4.6 (s, 2H), 3.9 (s, 3H), 2.6 (s, 3H).

5.33. 3-Chloro-5-(chloromethyl)-4-methyl-2-thiophenecarboxylic acid methyl ester (41)

Prepared from **18** as described for compound **37**. Purification by flash chromatography on silica gel afforded **41** as a white solid (1.2 g, 95%). ^{1}H NMR ($CDCl_3$) δ 4.7 (s, 2H), 3.9 (s, 3H), 2.2 (s, 3H).

5.34. 3-Chloro-4,5-bis(chloromethyl)-2-thiophenecarboxylic acid (46)

Prepared from 3-chloro-2-thiophenecarboxylic acid (**45**) as described for compound **37**, using 11 equiv of chloromethyl methyl ether and 3 equiv of $TiCl_4$. The crude product was purified by HPLC using a Dynamax column and a gradient of CH_3CN in H_2O with 0.1% TFA. The resulting fractions were combined, partially concentrated, and the product extracted into EtOAc and concentrated to afford **46** as a white solid (0.83 g, 26%). ^{1}H NMR ($CDCl_3$) δ 4.8 (s, 2H), 4.6 (s, 2H).

5.35. 3-Chloro-5-methyl-4-[(4-methyl-1-piperazinyl)methyl]-2-thiophenecarboxylic acid methyl ester (38)

To a solution of **37** (0.75 g, 3.1 mmol) in CH_2Cl_2 (15 mL) at 0 °C was added triethylamine (0.44 mL, 3.2 mmol), followed by 1-methylpiperazine (0.42 mL, 3.8 mmol). The mixture was warmed to room temperature and stirred for 24 h. The mixture was concentrated and the residue partitioned between EtOAc and H_2O. The aqueous phase was further extracted with EtOAc, and the combined organics washed with brine, dried over $MgSO_4$, and concentrated. Purification by flash chromatography on silica gel afforded **38** as a yellow solid (0.75 g, 79%). ^{1}H NMR ($CDCl_3$) δ 3.9 (s, 3H), 3.4 (s, 2H), 2.5 (s, 3H), 2.4 (br m, 8H), 2.2 (s, 3H).

5.36. 3-Chloro-4-methyl-5-[(4-methyl-1-piperazinyl)methyl]-2-thiophenecarboxylic acid methyl ester (42)

Prepared from **41** as described for compound **38**. Purification by flash chromatography on silica gel afforded **42** as a yellow solid (1.1 g, 75%). ^{1}H NMR ($CDCl_3$) δ 3.9 (s, 3H), 3.6 (s, 2H), 2.5 (br m, 8H), 2.4 (s, 3H), 2.2 (s, 3H).

5.37. 3-Chloro-5-methyl-4-[(4-methyl-1-piperazinyl)methyl]-2-thiophenecarboxylic acid dihydrochloride (39)

A solution of **38** (0.74 g, 2.5 mmol) in aqueous 2 N HCl (15 mL) was heated at 90 °C for 24 h, then cooled and concentrated to afford **39** as a white solid (0.89 g, quant.). ^{1}H NMR (DMSO-d_6/TFA) δ 4.4 (s, 2H), 3.6 (br m, 8H), 2.9 (s, 3H), 2.7 (s, 3H).

5.38. 3-Chloro-4-methyl-5-[(4-methyl-1-piperazinyl)methyl]-2-thiophenecarboxylic acid dihydrochloride (43)

Prepared from **42** as described for compound **39**, white solid (1.2 g, quant.). ^{1}H NMR (DMSO-d_6/TFA) δ 4.4 (s, 2H), 3.4 (br m, 8H), 2.8 (s, 3H), 2.2 (s, 3H).

5.39. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-5-methyl-4-[(4-methyl-1-piperazinyl) methyl]-2-thiophenecarboxamide trifluoroacetic acid salt (40)

A suspension of **39**, dihydrochloride salt (0.40 g, 1.1 mmol) in $SOCl_2$ (10 mL), was heated at reflux. After 30 h, the mixture was cooled to room temperature and concentrated. The resulting solid was dissolved in pyridine (4 mL) and **3** (0.28 g, 1.0 mmol) was added. After stirring for 18 h at room temperature, the mixture was poured into aqueous CH_3CN to form a homogeneous solution. The CH_3CN was evaporated and the resulting solid collected by filtration. The crude product was purified by HPLC using a Dynamax column and a gradient of CH_3CN in H_2O with 0.1% TFA. The resulting fractions were combined and the solvent was removed by lyophilization to afford **40** as a white solid (0.22 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (d, 1H), 7.4–7.7 (m, 5H), 4.3 (s, 2H), 3.5 (br m, 8H), 2.9 (s, 3H), 2.6 (s, 3H). Anal. Calcd for $C_{25}H_{25}Cl_3N_4O_2S{\cdot}2.4C_2HF_3O_2$. H_2O: C, 42.43; H, 3.51; N, 6.64. Found: C, 42.52; H, 3.33; N, 6.87.

5.40. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-methyl-5-[(4-methyl-1-piperazinyl)methyl]-2-thiophenecarboxamide trifluoroacetic acid salt (44)

Prepared from **43**, dihydrochloride salt as described for compound **40**. Purification by HPLC and lyophilization gave **44** as a white solid (0.022 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (d, 1H), 7.4–7.7 (m, 5H), 4.4 (s, 2H), 3.4 (br m, 8H), 2.8 (s, 3H), 2.2 (s, 3H). Anal. Calcd for $C_{25}H_{25}Cl_3N_4O_2S{\cdot}1.7C_2HF_3O_2$: C, 45.37; H, 3.57; N, 7.40. Found: C, 45.40; H, 3.70; N, 7.15.

5.41. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4,5-bis(chloromethyl)-2-thiophenecarboxamide (47)

Compound **46** was converted to the acid chloride with oxalyl chloride in CH_2Cl_2. Reaction of the crude acid chloride with **3** as described for compound **30** gave **47** as a yellow solid (0.69 g, 86%). ^{1}H NMR (DMSO-d_6) δ 11.2 (s, 1H), 10.7 (s, 1H), 8.3 (d, 1H), 7.4–7.9 (m, 6H), 5.1 (s, 2H), 4.8 (s, 2H).

5.42. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4,5-bis[(propylamino)methyl]-2-thiophenecarboxamide trifluoroacetic acid salt (48) and 3-chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-5,6-dihydro-5-propyl-4*H*-thieno[2,3-*c*]pyrrole-2-carboxamide trifluoroacetic acid salt (49)

To a suspension of **47** (0.075 g, 0.14 mmol) in CH_3CN (3 mL) in a pressure vessel was added 1-aminopropane (0.025 mL, 0.30 mmol). The vessel was sealed and the suspension heated at 50 °C for 10 days, with additional 0.025 mL portions of 1-aminopropane being added after 3 and 9 days. The mixture was cooled to room temperature and concentrated. The resulting solid was purified by HPLC on a Vydac column with a gradient of CH_3CN in H_2O with 0.1% TFA. The resulting fractions were

combined and the solvent was removed by lyophilization to afford the trifluoroacetic acid salts of **48** and **49**. The more polar compound **48** eluted first.

Compound **48**: white solid (0.018 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.3 (s, 1H), 10.8 (s, 1H), 8.9 (br s, 2H), 8.7 (br s, 2H), 8.4 (d, 1H), 7.4–8.0 (m, 6H), 4.6 (s, 2H), 4.3 (s, 2H), 3.0 (m, 4H), 1.6 (m, 4H), 0.9 (m, 6H). Anal. Calcd for $C_{26}H_{29}Cl_3N_4O_2S{\cdot}2.5C_2HF_3O_2$: C, 43.65; H, 3.72; N, 6.57. Found: C, 43.40; H, 3.53; N, 6.55.

Compound **49**: white solid (0.030 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (d, 1H), 7.4–7.7 (m, 5H), 4.6 (br m, 4H), 3.4 (m, 2H), 1.6 (m, 2H), 1.0 (t, 3H). Anal. Calcd for $C_{23}H_{20}Cl_3N_3O_2S{\cdot}1.5C_2HF_3O_2$: C, 45.93; H, 3.19; N, 6.18. Found: C, 45.98; H, 3.06; N, 6.35.

5.43. General method for preparation of amine-substituted compounds 31, 33, 50–55, 58–63

To a solution of **30** in dry DMF (0.1 M) at 0 °C was added an amine (5 equiv), and the mixture stirred for 0.5 h at 0 °C, then warmed to room temperature. After 4–18 h, the reaction mixture was poured into H_2O and the resulting solid collected by filtration. The crude product was purified by flash chromatography on silica gel. Alternatively, the product was purified by HPLC using a Dynamax column and a gradient of CH_3CN in H_2O with 0.1% TFA. The resulting fractions were combined and the solvent was removed by lyophilization to afford the product as a trifluoroacetic acid salt.

5.44. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[(4-methyl-1-piperazinyl)methyl]-2-thiophenecarboxamide (31)

Purified by flash chromatography, light brown solid (4.5 g, 76%). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (s, 1H), 7.8 (s, 1H), 7.7 (d, 2H), 7.6 (d, 1H), 7.4 (d, 2H), 3.4 (s, 2H), 2.4 (m, 4H), 2.3 (m, 4H), 2.1 (s, 3H). Anal. Calcd for $C_{24}H_{23}Cl_3N_4O_2S{\cdot}0.5H_2O$: C, 52.71; H, 4.42; N, 10.24. Found: C, 52.97; H, 4.34; N, 10.23.

5.45. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-(4-morpholinylmethyl)-2-thiophenecarboxamide (33)

Purified by crystallization from 1-butanol, light brown solid (0.26 g, 13%). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (s, 1H), 7.8 (s, 1H), 7.7 (d, 2H), 7.6 (d, 2H), 7.4 (d, 3H), 3.6 (s, 2H), 3.3 (br m, 4H), 2.4 (br m, 4H). Anal. Calcd for $C_{23}H_{20}Cl_3N_3O_3S{\cdot}1.5H_2O$: C, 50.06; H, 4.20; N, 7.61. Found: C, 50.23; H, 3.95; N, 7.22.

5.46. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[(4-ethyl-1-piperazinyl)methyl]-2-thiophenecarboxamide trifluoroacetic acid salt (50)

Purified by HPLC, white solid (0.082 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (d, 2H), 7.7 (m, 3H), 7.4 (d, 2H), 3.6 (s, 2H), 3.4 (br m, 3H), 3.2 (m, 2H), 3.0 (m, 3H), 2.4 (m, 2H), 1.1 (t, 2H). Anal. Calcd for $C_{25}H_{25}Cl_3N_4O_2S{\cdot}1.2C_2HF_3O_2$: C, 47.78; H, 3.83; N, 8.13. Found: C, 48.07; H, 3.73; N, 8.25.

5.47. 4-[(4-Acetyl-1-piperazinyl)methyl]-3-chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-2-thiophenecarboxamide trifluoroacetic acid salt (51)

Purified by HPLC, white solid. ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.8 (s, 1H), 8.4 (d, 1H), 8.2 (s, 1H), 8.0 (s, 1H), 7.7 (m, 3H), 7.4 (d, 2H), 4.3 (br m, 2H), 2.8–4.0 (br m, 8H), 2.0 (s, 3H). Anal. Calcd for $C_{25}H_{23}Cl_3N_4O_3S{\cdot}1.3C_2HF_3O_2$: C, 46.42; H, 3.43; N, 7.85. Found: C, 46.51; H, 3.22; N, 7.74.

5.48. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-(4-thiomorpholinylmethyl)-2-thiophenecarboxamide (52)

Purified by flash chromatography, white solid (0.22 g, 30%). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (s, 1H), 7.8 (s, 1H), 7.6 (m, 3H), 7.4 (d, 2H), 3.5 (s, 2H), 2.6 (m, 8H). Anal. Calcd for $C_{23}H_{20}Cl_3N_3O_2S_2{\cdot}0.5H_2O$: C, 50.23; H, 3.84; N, 7.64. Found: C, 50.37; H, 3.69; N, 7.53.

5.49. [[[4-Chloro-5-[[[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]amino]carbonyl]-3- thienyl]methyl]methylamino]acetic acid ethyl ester (53)

Purified by flash chromatography, pale yellow solid (0.30 g, 98%). ^{1}H NMR ($CDCl_3$) δ 11.1 (s, 1H), 8.6 (s, 1H), 8.3 (d, 1H), 7.7 (d, 2H), 7.6 (s, 1H), 7.5 (d, 1H), 7.4 (d, 2H), 7.3 (dd, 1H), 4.2 (q, 2H), 3.7 (s, 2H), 3.3 (s, 2H), 2.5 (s, 3H), 1.3 (t, 3H). Anal. Calcd for $C_{24}H_{22}Cl_3N_3O_4S$: C, 51.86; H, 4.17; N, 7.56. Found: C, 51.87; H, 4.00; N, 7.43.

5.50. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[(2-hydroxyethyl)methylamino]methyl]-2-thiophenecarboxamide (54)

Purified by flash chromatography, off-white solid (0.23 g, 82%). ^{1}H NMR ($CDCl_3$) δ 11.1 (s, 1H), 8.7 (s, 1H), 8.3 (d, 1H), 7.7 (d, 2H), 7.5 (s, 2H), 7.4 (d, 2H), 7.3 (dd, 1H), 3.7 (t, 2H), 3.6 (s, 2H), 2.7 (t, 2H), 2.3 (s, 3H). Anal. Calcd for $C_{22}H_{20}Cl_3N_3O_3S$: C, 51.42; H, 4.12; N, 8.18. Found: C, 51.11; H, 3.84; N, 8.07.

5.51. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[(2,3-dihydroxypropyl)methylamino]methyl]-2-thiophenecarboxamide trifluoroacetic acid salt (55)

Purified by HPLC, white solid (0.98 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 8.0 (d, 2H), 7.8 (d, 2H), 7.7 (d, 1H), 7.4 (d, 2H), 4.4 (s, 2H), 3.6 (m, 1H), 3.5 (m, 1H), 3.2–3.4 (br m, 3H), 2.5 (s, 3H). Anal. Calcd for $C_{23}H_{22}Cl_3N_3O_4S{\cdot}C_2HF_3O_2$: C, 45.78; H, 3.38; N, 6.41. Found: C, 45.66; H, 3.52; N, 6.31.

5.52. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[[2-(2-hydroxyethoxy)ethyl]amino]methyl]-2-thiophenecarboxamide trifluoroacetic acid salt (56)

Purified by HPLC, white solid (0.33 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.8 (s, 1H), 9.0 (br s, 2H), 8.3 (d, 1H), 8.2 (s, 1H), 8.0 (s, 1H), 7.8 (d, 2H), 7.7 (d, 1H), 7.4 (d, 2H), 4.2(s, 2H), 3.7 (m, 2H), 3.5 (m, 4H), 3.2 (br s, 2H). Anal. Calcd for $C_{23}H_{22}Cl_3N_3O_4S{\cdot}1.1C_2HF_3O_2{\cdot}0.5H_2O$: C, 44.69; H, 3.59; N, 6.20. Found: C, 44.77; H, 3.40; N, 6.07.

5.53. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[[2-(dimethylamino)ethyl]methylamino]methyl]-2-thiophenecarboxamide (58)

Purified by flash chromatography, off-white solid (0.26 g, 57%). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (d, 1H), 7.8 (s, 1H), 7.7 (d, 2H), 7.6 (dd, 1H), 7.4 (d, 2H), 3.5 (s, 2H), 2.5 (d, 2H), 2.3 (d, 2H), 2.2 (s, 3H), 2.1 (s, 6H). Anal. Calcd for $C_{24}H_{25}Cl_3N_4O_2S{\cdot}0.3CH_2Cl_2$: C, 51.62; H, 4.56; N, 9.90. Found: C, 51.60; H, 4.44; N, 9.68.

5.54. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[[2-(diethylamino)ethyl]methylamino]methyl]-2-thiophenecarboxamide (59)

Purified by flash chromatography, white solid (0.16 g, 67%). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.8 (s, 1H), 8.3 (m, 2H), 8.0 (s, 1H), 7.7 (m, 3H), 7.4 (d, 2H), 4.4 (s, 2H), 3.5 (m, 3H), 3.2 (q, 4H), 2.8 (s, 3H), 1.2 (t, 6H). Anal. Calcd for $C_{26}H_{29}Cl_3N_4O_2S$: C, 54.98; H, 5.15; N, 9.86. Found: C, 54.66; H, 5.01; N, 9.62.

5.55. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[[3-(dimethylamino)propyl]methylamino]methyl]-2-thiophenecarboxamide trifluoroacetic acid salt (60)

Purified by HPLC, white solid (0.32 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 8.2 (s, 1H), 7.9 (d, 1H), 7.7 (d, 2H), 7.6 (dd, 1H), 7.4 (d, 2H), 4.4 (m, 2H), 3.1 (m, 4H), 2.8 (s, 9H), 2.1 (m, 2H). Anal. Calcd for $C_{25}H_{27}Cl_3N_4O_2S{\cdot}3.0C_2HF_3O_2$: C, 41.56; H, 3.37; N, 6.25. Found: C, 41.79; H, 3.34; N, 6.38.

5.56. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[[3-(1*H*-imidazol-1-yl)propyl]amino]methyl]-2-thiophenecarboxamide (62)

Purified by flash chromatography, pale yellow solid (0.65 g, 73%). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.8 (s, 1H), 9.1 (s, 1H), 8.4 (d, 1H), 8.2 (s, 1H), 7.9 (d, 1H), 7.8–7.6 (m, 5H), 7.4 (d, 2H), 4.3 (t, 2H), 4.2 (br s, 2H), 3.0 (br s, 2H), 2.2 (m, 3H). Anal. Calcd for $C_{25}H_{22}Cl_3N_5O_2S$: C, 53.34; H, 3.94; N, 12.44. Found: C, 53.33; H, 3.84; N, 12.16.

5.57. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[methyl(1-methyl-4-piperidinyl)amino]methyl]-2-thiophenecarboxamide trifluoroacetic acid salt (63)

Purified by HPLC, white solid (0.19 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.8 (s, 1H), 8.4 (d, 2H), 8.0 (s, 1H), 7.7 (m, 3H), 7.4 (d, 2H), 3.6 (m, 2H), 3.0 (m, 2H), 2.8 (s, 3H), 1.8–2.4 (br m, 4H). Anal. Calcd for $C_{26}H_{27}Cl_3N_4O_2S{\cdot}2.0C_2HF_3O_2$: C, 45.38; H, 3.68; N, 7.06. Found: C, 44.98; H, 3.59; N, 7.01.

5.58. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[[2-(2-hydroxyethoxy)ethyl]methylamino]methyl]-2-thiophenecarboxamide trifluoroacetic acid salt (57)

To a solution of **56** (0.25 g, 0.46 mmol) in CH_3CN (5 mL) was added an aqueous 37% solution of formaldehyde (0.19 mL, 2.3 mmol), followed by $NaCNBH_3$ (0.045 g, 0.69 mmol), and the mixture stirred at room temperature. After 2 h, the mixture was concentrated and the product was purified by HPLC using a Dynamax column and a gradient of CH_3CN in H_2O with 0.1% TFA. The resulting fractions were combined and the solvent was removed by lyophilization to afford **57** as a white solid (0.12 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.8 (s, 1H), 9.6 (br s, 1H), 8.4 (d, 1H), 8.3 (s, 1H), 7.9 (s, 1H), 7.7 (d, 2H), 7.6 (d, 1H), 7.4 (d, 2H), 4.5 (d, 1H), 4.3 (d, 1H), 3.8 (m, 2H), 3.5 (m, 4H), 3.4 (br s, 1H), 2.8 (s, 3H). Anal. Calcd for $C_{24}H_{24}Cl_3N_3O_4S{\cdot}0.8C_2HF_3O_2{\cdot}0.9H_2O$: C, 46.28; H, 4.04; N, 6.33. Found: C, 45.87; H, 3.66; N, 6.71.

5.59. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[methyl[2-(1-pyrrolidinyl)ethyl]amino]methyl]-2-thiophenecarboxamide (61)

Compound **30** was treated with 1-pyrrolidineethanamine as described for compound **31**. Following purification by flash chromatography on silica gel, the product was treated with formaldehyde and $NaCNBH_3$ as described for compound **57**. Purification by flash chromatography afforded **61** as a white solid (0.48 g, 21% over 2 steps). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.8 (s, 1H), 10.0 (br s, 1H), 8.4 (d, 1H), 8.3 (s, 1H), 8.0 (s, 1H), 7.8 (d, 2H), 7.7 (d, 1H), 7.4 (d, 2H), 4.4 (s, 2H), 3.6 (m, 4H), 3.3 (br s, 2H), 2.8 (s, 3H), 2.0 (br s, 4H). Anal. Calcd for $C_{26}H_{27}Cl_3N_4O_2S{\cdot}0.5H_2O$: C, 54.31; H, 4.91; N, 9.74. Found: C, 54.41; H, 4.72; N, 9.56.

5.60. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-(1*H*-imidazol-1-ylmethyl)-2-thiophenecarboxamide trifluoroacetic acid salt (35)

To a solution of **30** (0.30 g, 0.64 mmol) in dry DMF (10 mL) was added sodium imidazole (0.17 g, 1.9 mmol) and the reaction mixture stirred at room temperature. After 16 h, the mixture was poured into H_2O and extracted with EtOAc. The combined organics were washed with H_2O, aqueous 1 N HCl, and brine, dried over $MgSO_4$, and concentrated. The crude product was purified by HPLC using a Dynamax column and a gradient of CH_3CN in H_2O with 0.1% TFA. The resulting fractions were combined and the solvent was removed by lyophilization to afford **35** as an off-white solid containing residual imidazole (0.080 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.1 (s, 1H), 10.8 (s, 1H), 9.2 (s,

1H), 8.3 (d, 1H), 8.1 (s, 1H), 7.9 (d, 1H), 7.7 (m, 5H), 7.4 (d, 2H), 5.4 (s, 2H). Anal. Calcd for $C_{22}H_{15}Cl_3N_4O_2S{\cdot}2.2C_2HF_3O_2{\cdot}0.3C_3H_4N_2$: C, 42.20; H, 2.38; N, 8.29. Found: C, 42.10; H, 2.47; N, 8.31.

5.61. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[(methylthio)methyl]-2-thiophenecarboxamide (70)

Prepared from **30** and sodium thiomethoxide as described for compound **35**. The crude product was purified by flash chromatography, pale yellow solid (0.36 g, 68%). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (d, 1H), 7.8 (s, 1H), 7.7 (d, 2H), 7.6 (dd, 1H), 7.4 (d, 2H), 3.7 (s, 2H), 2.0 (s, 3H). Anal. Calcd for $C_{20}H_{15}Cl_3N_2O_2S_2$: C, 49.44; H, 3.11; N, 5.77. Found: C, 49.70; H, 3.12; N, 5.78.

5.62. General method for the preparation of heteroaryl-substituted compounds 64–69

To a solution of the appropriate *N*-heterocycle in dry DMF (0.5–1 M) was added NaH (60% dispersion in mineral oil, 1.0 equiv) and the mixture stirred at room temperature. After 10 min, a solution of **30** (0.2 equiv) was added in sufficient dry DMF to bring the concentration of **30** to 0.1 M, and stirring continued. After 18 h, the mixture was poured onto H_2O and extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$ and concentrated, and the crude product purified by flash chromatography on silica gel.

5.63. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-(1*H*-pyrazol-1-ylmethyl)-2-thiophenecarboxamide (64)

0.72 g, 84%. ^{1}H NMR (DMSO-d_6/TFA) δ 11.1 (s, 1H), 10.7 (s, 1H), 7.3-8.4 (m, 10H), 6.3 (s, 1H), 5.3 (s, 2H). Anal. Calcd for $C_{22}H_{15}Cl_3N_4O_2S$: C, 52.24; H, 2.99; N, 11.08. Found: C, 52.23; H, 2.99; N, 10.87.

5.64. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-(1*H*-1,2,4-triazol-1-ylmethyl)-2-thiophenecarboxamide (65)

White solid (0.99 g, 77%). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.7 (s, 1H), 9.3 (s, 1H), 8.5 (s, 1H), 8.3 (d, 1H), 8.0 (s, 1H), 7.9 (d, 1H), 7.7 (d, 2H), 7.6 (dd, 1H), 7.4 (d, 2H), 5.5 (s, 2H). Anal. Calcd for $C_{21}H_{14}Cl_3N_5O_2S$: C, 49.77; H, 2.78; N, 13.82. Found: C, 49.81; H, 2.61; N, 13.49.

5.65. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-(1*H*-1,2,3-triazol-1-ylmethyl)-2-thiophenecarboxamide (66) and 3-chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-(2*H*-1,2,3-triazol-2-ylmethyl)-2-thiophenecarboxamide (67)

Reaction of **30** with 1*H*-1,2,3-triazole afforded a mixture of compounds **66** and **67**. The two products were separated by flash chromatography on silica gel, with compound **66** eluting first as the less polar isomer. The two products were assigned based on comparison of the ^{1}H NMR spectra with calculated spectra[25] and with known triazole compounds.[26]

Compound **66**: white solid (0.49 g, 57%). ^{1}H NMR (DMSO-d_6/TFA) δ 11.1 (s, 1H), 10.7 (s, 1H), 7.3–8.4 (m, 9H), 5.3 (s, 2H). Anal. Calcd for $C_{21}H_{14}Cl_3N_5O_2S$: C, 49.77; H, 2.78; N, 13.82. Found: C, 49.58; H, 2.94; N, 13.62.

Compound **67**: white solid containing residual triazole (0.072 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.9 (s, 1H), 7.3-8.5 (m, 10H), 5.6 (br s, 2H). Anal. Calcd for $C_{21}H_{14}Cl_3N_5O_2S{\cdot}0.35C_2H_3N_3$: C, 49.08; H, 2.86; N, 15.96. Found: C, 49.01; H, 2.46; N, 16.16.

5.66. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-(1*H*-tetrazol-1-ylmethyl)-2-thiophenecarboxamide (68) and 3-chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-(2*H*-tetrazol-2-ylmethyl)-2-thiophenecarboxamide (69)

Reaction of **30** with 1*H*-tetrazole afforded a mixture of compounds **68** and **69**. The two products were separated by flash chromatography on silica gel, with compound **69** eluting first as the less polar isomer. The two products were assigned based on comparison of the ^{1}H NMR spectra with calculated spectra[25] and with known tetrazole compounds.[26,27]

Compound **68**: white solid (0.29 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.7 (s, 1H), 9.4 (s, 1H), 8.3 (d, 1H), 8.0 (s, 1H), 7.85 (d, 1H), 7.7 (d, 2H), 7.5 (dd, 1H), 7.3 (d, 2H), 5.7 (s, 2H). Anal. Calcd for $C_{20}H_{13}Cl_3N_6O_2S$: C, 47.31; H, 2.58; N, 16.55. Found: C, 47.40; H, 2.50; N, 16.22.

Compound **69**: white solid (0.65 g). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.7 (s, 1H), 9.4 (s, 1H), 8.3 (d, 1H), 8.1 (s, 1H), 7.9 (d, 1H), 7.7 (d, 2H), 7.6 (dd, 1H), 7.4 (d, 2H), 5.9 (s, 2H). Anal. Calcd for $C_{20}H_{13}Cl_3N_6O_2S{\cdot}0.2C_4H_8O_2$: C, 47.55; H, 2.80; N, 16.00. Found: C, 47.40; H, 2.50; N, 15.86.

5.67. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[[2-(dimethylamino)ethyl]thio]methyl]-2-thiophenecarboxamide (73)

To a solution of **30** (0.70 g, 1.5 mmol) in dry DMF (5 mL) was added 2-(dimethylamino)ethanethiol (2.1 g, 15 mmol), followed by K_2CO_3 (1.0 g, 7.2 mmol), and the reaction mixture stirred at room temperature. After 24 h, the mixture was poured into H_2O (100 mL) and the resulting solid collected by filtration, washed with H_2O, and dried. Purification by flash chromatography on silica gel afforded **73** as an off-white solid (0.28 g, 35%). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (d, 1H), 7.8 (s, 1H), 7.7 (d, 2H), 7.6 (dd, 1H), 7.4 (d, 2H), 3.7 (s, 2H), 2.5 (t, 2H), 2.4 (t, 2H), 2.1 (s, 6H). Anal. Calcd for $C_{23}H_{22}Cl_3N_3O_2S_2$: C, 50.88; H, 4.08; N, 7.74. Found: C, 50.59; H, 4.27; N, 7.49.

5.68. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[2-(2-methoxyethoxy)ethoxy]methyl]-2-thiophenecarboxamide (75)

To 2-(2-methoxyethoxy)ethanol (20 mL) at 0 °C was added NaH (0.45 g, 11 mmol). The solution was warmed to room temperature and stirred for 16 h. Compound **30** (1.0 g, 2.3 mmol) was added and the mixture stirred at room temperature for 3 h, then heated at 65 °C for 4 h. The mixture was then poured onto H_2O, and the resulting solid was collected by filtration, washed with H_2O and 50% ether/hexanes, and dried to afford **75** as a pale yellow solid (0.65 g, 52%). ^{1}H NMR (DMSO-d_6/TFA) δ 11.1 (s, 1H), 10.7 (s, 1H), 8.3 (d, 1H), 7.9 (d, 2H), 7.7 (d, 2H), 7.6 (d, 1H), 7.4 (s, 1H), 7.4 (s, 1H), 4.4 (s, 2H), 3.5 (m, 6H), 3.4 (m, 2H), 3.2 (s, 3H). Anal. Calcd for $C_{24}H_{23}Cl_3N_2O_5S{\cdot}0.3H_2O$: C, 51.17; H, 4.22; N, 4.97. Found: C, 50.90; H, 3.85; N, 4.78.

5.69. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[(2-methoxyethoxy)methyl]-2-thiophenecarboxamide (74)

Prepared in 2-methoxyethanol as described for compound **75**; pale yellow solid (0.79 g, 67%). ^{1}H NMR (DMSO-d_6/TFA) δ 11.2 (s, 1H), 10.7 (s, 1H), 8.3 (d, 1H), 7.9 (d, 2H), 7.7 (d, 2H), 7.6 (d, 1H), 7.4 (s, 1H), 7.4 (s, 1H), 4.4 (s, 2H), 3.5 (m, 4H), 3.2 (s, 3H). Anal. Calcd for $C_{22}H_{19}Cl_3N_2O_4S$: C, 51.43; H, 3.73; N, 5.45. Found: C, 51.10; H, 3.51; N, 5.27.

5.70. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[[(2,2-dimethyl-1,3-dioxolan-4-yl)methoxy]methyl]-2-thiophenecarboxamide (76)

Prepared in 2,2-dimethyl-1,3-dioxolane-4-methanol as described for compound **75**, white solid (0.58 g, 48%). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 8.0 (d, 2H), 7.7 (d, 2H), 7.6 (d, 1H), 7.4 (d, 2H), 4.5 (s, 2H), 4.2 (t, 1H), 4.0 (t, 1H), 3.6 (m, 1H), 3.4 (d, 2H), 1.2 (d, 6H). Anal. Calcd for $C_{25}H_{23}Cl_3N_2O_5S$: C, 52.69; H, 4.06; N, 4.91. Found: C, 52.33; H, 3.94; N, 4.82.

5.71. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[(methylsulfinyl)methyl]-2-thiophenecarboxamide (71)

To a suspension of **70** (0.40 g, 0.83 mmol) in MeOH (30 mL) at 0 °C was added a solution of OXONE® (1.0 g, 1.7 mmol) in H_2O (10 mL). The mixture was stirred at 0 °C for 30 min, then warmed to RT and stirred for 1.5 h. The mixture was poured into H_2O (50 mL) and extracted with CH_2Cl_2 (2× 50 mL). The combined organics were washed with H_2O and brine, dried over $MgSO_4$ and concentrated. The crude product was purified by HPLC using a Dynamax column and a gradient of CH_3CN in H_2O with 0.1% TFA. The resulting fractions were combined and the solvent was removed by lyophilization to afford **71** as a white solid (0.018 g). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 7.9 (s, 1H), 7.9 (d, 1H), 7.4–7.7 (m, 5H), 4.0 (dd, 2H), 2.5 (s, 3H). Anal. Calcd for $C_{20}H_{15}Cl_3N_2O_3S_2{\cdot}0.8H_2O$: C, 46.53; H, 3.24; N, 5.43. Found: C, 46.63; H, 3.07; N, 5.36.

5.72. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[(methylsulfonyl)methyl]-2-thiophenecarboxamide (72)

Reaction of **70** with OXONE® (3.0 equiv) at room temperature for 18 h, followed by purification by HPLC, afforded **72** as a white solid (0.049 g). ^{1}H NMR (DMSO-d_6) δ 11.1 (s, 1H), 10.8 (s, 1H), 8.3 (d, 1H), 8.1 (s, 1H), 7.9 (s, 1H), 7.7 (d, 2H), 7.6 (dd, 1H), 7.4 (d, 2H), 4.6 (s, 2H), 3.0 (s, 3H). Anal. Calcd for $C_{20}H_{15}Cl_3N_2O_4S_2{\cdot}H_2O$: C, 44.83; H, 3.20; N, 5.23. Found: C, 45.03; H, 2.82; N, 5.19.

5.73. 3-Chloro-*N*-[4-chloro-2-[[(4-chlorophenyl)amino]carbonyl]phenyl]-4-[(2,3-dihydroxypropoxy)methyl]-2-thiophenecarboxamide (77)

Compound **76** (0.10 g, 0.17 mmol) was stirred in a mixture of aqueous 1 N HCl (1.0 mL) and THF (1.0 mL) at room temperature. After 16 h, the mixture was poured onto H_2O and extracted with EtOAc. The organic layer was dried over Na_2SO_4 and concentrated. The product was purified by HPLC using a Dynamax column and a gradient of CH_3CN in H_2O with 0.1% TFA. The resulting fractions were combined and the solvent was removed by lyophilization to afford **77** as a white solid (0.070 g). ^{1}H NMR (DMSO-d_6) δ 11.0 (s, 1H), 10.7 (s, 1H), 8.3 (d, 1H), 7.9 (d, 2H), 7.7 (d, 2H), 7.6 (d, 1H), 7.4 (d, 2H), 4.4 (s, 2H), 3.5 (m, 1H), 3.4 (m, 1H), 3.3 (m, 2H). Anal. Calcd for $C_{22}H_{19}Cl_3N_2O_5S$: C, 49.87; H, 3.61; N, 5.29. Found: C, 49.91; H, 3.69; N, 5.28.

5.74. Enzyme assay procedures[28]

The activities of human fXa, human thrombin, and bovine trypsin were determined kinetically as the initial rate of cleavage of a peptide *p*-nitroanilide by the enzyme. The assay was performed at room temperature in flat-bottomed microtiter plates in a final volume of 200 μL. The reaction mixture consisted of 50 mM Tris–HCl, 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% polyethylene glycol 6000, pH 7.5, with enzyme and substrate at the following concentrations: (1) fXa assay: 0.04–1 nM fXa and 164 μM S-2222; (2) thrombin assay: 16 nM thrombin and 300 μM S-2302; (3) trypsin assay: 16 nM bovine trypsin and 127 μM S-2266. Standard techniques with at least four substrate dilutions were used to determine the K_m for a given enzyme and substrate. The substrate concentration listed is equal to the K_m. Controls without the test inhibitors or with a reference compound were also run in each assay plate. Enzyme was incubated with test compounds for 10 min; the reaction was then started by the addition of the substrate. Reaction rates were determined by measuring the rate of the absorbance change at 405 nm in a ThermoMax microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA).

5.75. Data analysis methods

IC_{50} values for inhibitors were determined from the dose–response curve by fitting the data to the Hill equation with an automated analysis method using a computer spreadsheet. $K_{i,app}$ values were calculated as the $IC_{50}/2$, with the IC_{50} value determined at a substrate concentration equal to K_m ($K_{i,app} = IC_{50}/(1 + [S]/K_m) = IC_{50}/2$). For inhibitors with $K_{i,app}$ values less than 3 nM, IC_{50} values were determined by fitting data to a modification of the Morrison equation to correct for the proportion of inhibitor bound to the enzyme relative to the free inhibitor.[29] $K_{i,app}$ values are means of multiple determinations ($n \geqslant 2$). Standard deviations are <30 % of the mean.

5.76. Prothrombin time (PT) assay

For in vitro coagulation studies, pooled, lyophilized human plasma (SARP, Helena Laboratories, Beaumont, TX) was used following reconstitution. Thromboplastin HS (Sigma, St. Louis, MO) was used for PT assays. For construction of concentration–response curves, plasma was serially diluted at half-log increments ranging from 0.01 to 100 μg/mL. Clot time for all coagulation assays was measured by taking the average of two measurements using an Electra 1400 C automated coagulometer (Medical Laboratory Automation, Inc., Pleasantville, NY).

5.77. Pharmacokinetic study in dogs

Fasted (18 h) male beagle dogs were instrumented with indwelling jugular catheters on the day of the experiment for the purpose of withdrawing blood samples. At the end of the experiment, the catheters were removed and the dogs were returned to the kennel. Dogs were allowed water ad lib during the experiment and were housed in individual cages. Drugs were administered orally by gavage via a feeding tube temporarily inserted into the stomach, or intravenously, by a bolus (ca. 30 s) injection via the cephalic vein. For oral dosing, the compound was dissolved in a 40% w/w solution of hydroxypropyl-β-cyclodextrin in water acidified to pH 5 with 1 N HCl. Final compound concentration was 10 mg/mL, and dosing volume was 1 mL/kg. For intravenous dosing, the compound was dissolved in a 10% v/v solution of DMSO in unbuffered saline, acidified to pH 5 with 0.1 N HCl. Final compound concentration was 1 mg/mL, and dosing volume was 1 mL/kg.

Blood samples were drawn at the following time points: pre-dose, 1 min (iv dose only), 5, 15, 30, and 45 min, and hourly from 1 h out to 6 h. For each time point, 2.7 mL of blood was collected from the jugular vein catheter in a 3 cc syringe previously washed with 5% sodium citrate solution in phosphate-buffered saline (without calcium or magnesium) and placed in a test tube containing 0.3 mL of the same 5% citrate solution. Prior to obtaining the blood sample, blood was withdrawn into the indwelling catheter to remove any saline from the catheter and dead space. After obtaining the blood sample, the catheter was flushed with approx. 2 mL of saline. The blood samples were kept on ice until centrifuged at 1000*g* for 10 min at 4 °C to obtain plasma. The plasma samples were kept frozen (−20 °C) until assayed for determination of drug levels.

Anti-fXa activity of the test compound in plasma was determined ex vivo using a chromogenic assay.[15] The assay was performed at room temperature in flat-bottomed microtiter plates in a final volume of 200 μL. The assay buffer consisted of 50 mM Tris–HCl, 150 mM NaCl, and 0.1% polyethylene glycol 6000, pH 7.5. Solutions of human fXa (6 nM in assay buffer) and substrate S-2222 (948 μM in assay buffer) were prepared. A standard curve was prepared with five dilutions of test compound in pooled dog plasma. Plasma samples were thawed and diluted 1:100 with assay buffer. To each well of a 96-well microtiter plate were added 20 μL of diluted plasma sample, 30 μL of buffer, and 50 μL of substrate solution. Two wells of each plate received pooled dog plasma instead of diluted plasma sample (negative control), and four wells received a diluted solution of a standard fXa inhibitor of known activity (positive control). The plate was shaken for 30 s, then 100 μL of fXa solution added. Reaction rates were determined by measuring the rate of the absorbance change at 405 nm at 10 s intervals for 2 min. Plasma samples and standards were tested in duplicate. The rate of reaction in mOD/min was calculated based on the kinetic readings. The rate was converted to compound concentration (μM) based on the standard curve using log–logit curve fit analysis.

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