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Design and Synthesis of 4,5-Disubstituted-thiophene-2-amidines as Potent Urokinase Inhibitors

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Abstract—A study of the S1 binding of lead 5-methylthiothiophene amidine **3,** an inhibitor of urokinase-type plasminogen activator, was undertaken by the introduction of a variety of substituents at the thiophene 5-position. The 5-alkyl substituted and unsubstituted thiophenes were prepared using organolithium chemistry. Heteroatom substituents were introduced at the 5-position using a novel displacement reaction of 5-methylsulfonylthiophenes and the corresponding oxygen or sulfur anions. Small alkyl group substitution at the 5-position provided inhibitors equipotent with **3** but possessing improved solubility.

Urokinase-type plasminogen activator (uPA) is a serine protease that is involved in many cellular processes including angiogenesis, tissue remodeling, tumor invasion, and cancer metastasis.[1,2] The ability of tumor cells to invade and metastasize can be downregulated by uPA inhibitors, making this an attractive target for drug discovery.[3,4] Several novel inhibitors of this enzyme have been reported, all having a common guanidine or amidine functionality. Included are: cyclohexylthio-phene-2-carboxamidines,[5] naphthylamidines,[6] isoquino-linyl-guanidines,[7] and benzo[b]thiophene-2-carboxamidines,[8,9] for example **1** (Eisai, B428, $K_i = 0.32$ μM). The inhibitor **1** has been used in recent X-ray crystal and NMR structure studies of uPA.[10,11] The structural studies show the effects of varying templates and the trajectory into the proximal pocket.[12] This trypsin-like serine protease prefers a positively charged P1 that makes a salt bridge with Asp189 in the S1 pocket.[13] Our primary goal was to optimize S1 binding of any emerging leads prior to optimizing binding in the proximal pocket.

By screening amidine libraries, 5-methylthiothiophene-2-carboxamidine **2** was identified as an inhibitor with a K_i of 6 μM. A molecular modeling comparison of template **2** to inhibitor **1** indicated that substitution off the 4-position of the thiophene should lead to increased potency.[6] This ultimately led to the new lead inhibitor **3** with a K_i of 101 nM.[14] In addition to its modest potency, **3** possessed marginal solubility. It was decided to further explore groups that would be tolerated at the thiophene 5-position in the S1 pocket and develop an SAR (Fig. 1).

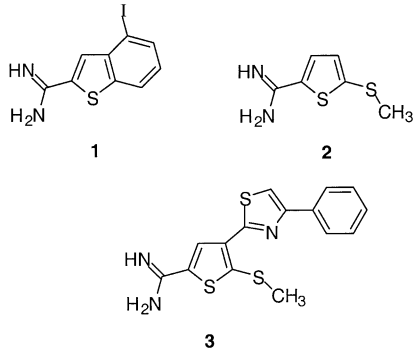

Figure 1. Lead thiophene amidine compounds.

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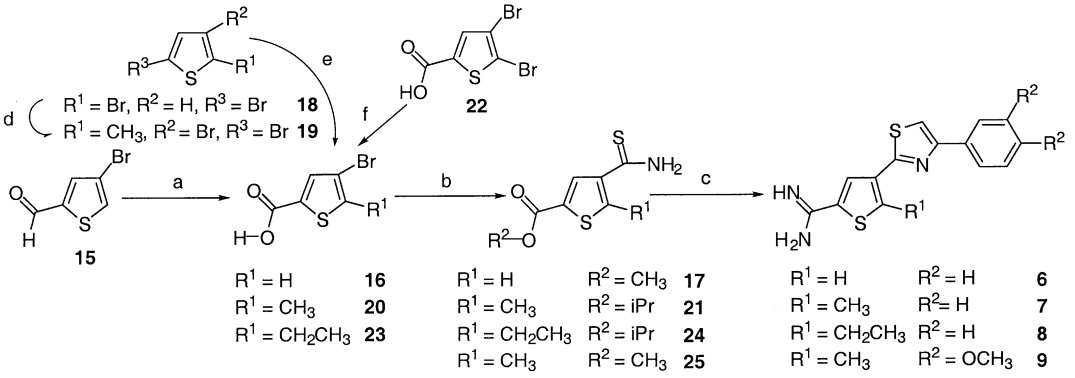
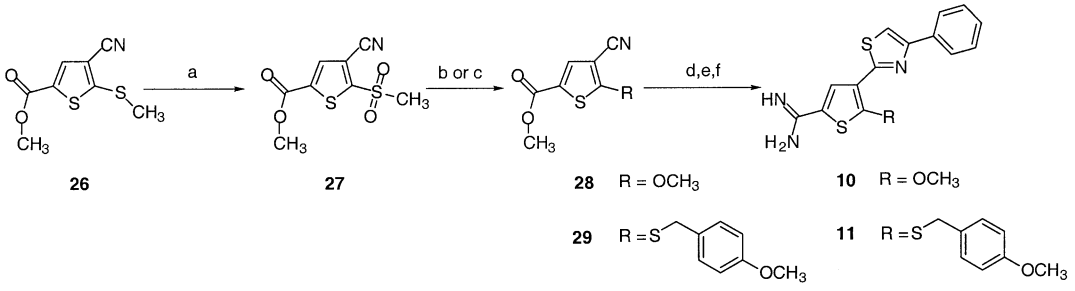


Scheme 1. (a) $NaClO_2$, 20% NaH_2PO_4, *t*-BuOH, 2-methyl-2-butene, 0 °C to rt (93%); (b) (i) $SOCl_2$, CH_3OH, −20 °C to reflux (95%) for **16**; or (i) CH_2Cl_2, oxalyl chloride, then *i*-PrOH, Na_2CO_3 (32%) for **20**; or 2 M $TMSCHN_2$ in hexanes (100%) for **20**; or (i) CH_2Cl_2, oxalyl chloride, then *i*-PrOH, CH_2Cl_2, pyridine (48%) for **23**; (ii) CuCN, DMF, reflux (58% for $R^1=H$, 63% for $R^1=CH_3$, 53% for $R^1=CH_2CH_3$, 68% for $R^1=CH_3$, $R^2=CH_3$; (iii) H_2S, TEA, MeOH (82% for **17**, 56% for **21**, 74% for **24**, 48% (mixture with 60% starting nitrile) for **25**); (c) (i) α-bromoacetophenone, acetone, reflux (90% for **17**, 66% for **21**, 49% for **24**, 53% for **25**); (ii) $Al(CH_3)_3$, NH_4Cl, toluene, reflux (45% for **6**, 100% for **7**, 67% for **8**, 9% for **9**); (d) (i) LDA, −78 °C, (ii) CH_3I (59%); (e) (i) *n*-butyllithium, −78 °C; (ii) CO_2; (g) (iii) 6 N HCl (aq) (94%); (f) (i) *n*-BuLi (2 equiv); (ii) CH_3CH_2I (85%).



Scheme 2. (a) *m*-CPBA, CH_2Cl_2, rt, 2 h (95%); (b) $NaOCH_3$, CH_3OH, reflux, 15 min (73%); (c) *p*-methoxybenzyl thiol, TEA, CH_3OH, reflux, 15 min (95%); (d) H_2S, TEA, MeOH (59% for $R=OCH_3$; 48% for $R=p$-CH_3O-benzyl); (e) 2-bromobenzophenone, acetone, reflux (10% for $R=OCH_3$; 49% for $R=p$-CH_3O-benzyl); (f) $Al(CH_3)_3$, NH_4Cl, toluene, reflux (69% for **10**; 91% for **11**).

Modeling suggested that since the orientation of the thiophene in S1, and therefore the resulting trajectory off the thiophene 4-position, would be expected to change for different 5-position substituents, this optimization had to be undertaken first. The introduction of 5-alkyl substituents would require their incorporation early in the synthesis. For 5-heteroatom substitutions, the strategy was to develop chemistry to carry out single-step modifications on the intact inhibitor.[15]

The inhibitors (Table 1) were synthesized in a straightforward manner and the synthesis of 4-aryl-5-methylthiothiophene compounds **3**, **4**, and **5** were described previously.[16]

The 5-unsubstituted thiophene **6** was synthesized (Scheme 1) starting with 4-bromothiophene-2-aldehyde **15**. Oxidation to the acid via the method of Kraus[17] gave acid **16**. Esterification[18] and treatment with copper(I) cyanide gave the nitrile,[19] which was then converted to thioamide **17** with hydrogen sulfide.[20] Hantsch condensation of **17** with 2-bromo-acetophenone[21] and treatment of the resulting ester with trimethylaluminum and ammonium chloride[22] gave amidine **6**.

The 5-methylthiophene **7** was prepared (Scheme 1) starting with 2,5-dibromothiophene **18**. Treatment with one equivalent of LDA resulted in a stable 5-lithio-2,4-dibromothiophene intermediate via a 'base-catalyzed halogen dance' mechanism which was trapped by iodomethane and gave 2,4-dibromo-5-methylthiophene **19**.[23] Selective lithiation of **19**, and treatment of the intermediate with excess CO_2 gas gave the acid **20**.[24] Esterification with oxalyl chloride and isopropanol, then treatment with copper cyanide resulted in the nitrile, which was converted to thioamide **21** as before. The preparation of the more methanol-insoluble isopropyl esters was necessary since the methyl esters did not precipitate during the reaction, leading to an inseparable mixture of unreacted nitrile and thioamide. Precipitation of the thioamide product from solution under these conditions was necessary to push this reaction to completion. Hantzch condensation and amidinylation gave **7**.

The 5-ethylthiophene **8** was synthesized (Scheme 1) starting with commercially available 4,5-dibromothiophene-2-carboxylic acid[25] **22**. Selective lithiation with *n*-butyl lithium and trapping with iodoethane gave **23**. Subsequent transformations were carried out as above to give **8**.

The 3,4-dimethoxyphenyl-5-methylthiophene **9** was prepared (Scheme 1) in a manner similar to **7**, but using

3′,4′-dimethoxy-α-bromoacetophenone as the condensation partner during the Hantzch synthesis.[16]

For the introduction of heteroatoms in the 5-position, a different approach was taken. We reasoned that a sufficiently electron-deficient thiophene might allow direct displacement of a sulfone moiety which would be easily derived from a readily available sulfide. Thus, synthesis (Scheme 2) of 5-methoxythiophene **10** started with nitriloester **26**.[26] Oxidation with *m*-chloroperbenzoic acid gave the sulfone **27**.[18] In practice, the sulfone was readily displaced by refluxing with sodium methoxide in methanol to give 5-methoxythiophene nitrile **28**.[27] The 5-methoxy derivative **10** was then prepared as described above.

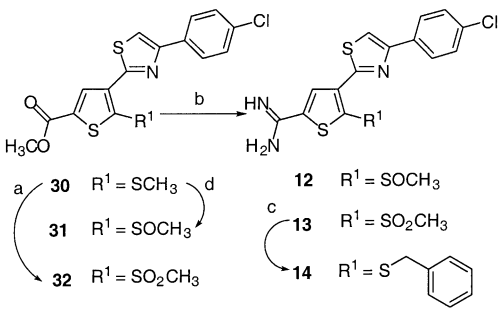
The synthesis (Scheme 2) of 5-(4-methoxybenzylmercapto)thiophene **11** was also carried out by the displacement of sulfone **27** with the triethylammonium salt of 4-methoxybenzylmercaptan in refluxing methanol and gave **29**.[27] Following the subsequent transformations for **7** gave **11**.

The synthesis (Scheme 3) of the 5-sulfoxide **12** started with commercially available[26] 5-methylthio ester **30**. Selective mono-oxidation to sulfoxide **31** was carried out with 30% hydrogen peroxide and 1,1,1,3,3,3-hexafluoro-2-propanol in dichloromethane.[28] This ester **31** was amidinylated as above to give **12**.[22]

The 5-methylsulfonylthiophene **13** (Scheme 3) was synthesized by peroxidation of 5-methylthiothiophene ester **30** with mCPBA, which gave 5-methylsulfonylthiophene ester **31**,[18] then amidinylated to give **13**.[22]

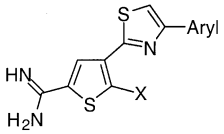
The 5-benzylmercaptothiophene **14** (Scheme 3) was synthesized by the same sulfone displacement used to make **28**, but by carrying out this transformation on preformed amidine **13**. Thus, **13** was treated with the triethylammonium salt of benzyl mercaptan in refluxing methanol to yield **14**.[27] Interestingly, subjecting sulfonyl ester **32** to the same conditions resulted in unchanged starting material. The absence of the electron-withdrawing nitrile group in **32** was compensated for by the amidine group in **13**. Further exploitation of this useful transformation is in progress.

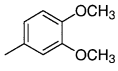
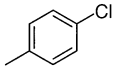
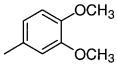
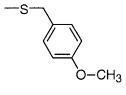
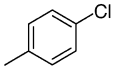
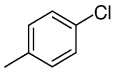
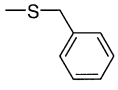
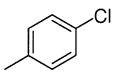
All compounds were assayed against human kidney cell urokinase,[29] and the SAR results are shown in Table 1. Estimated solubilities of the most potent inhibitors were measured.[30] During the course of this project, three different substitutions were employed for the aryl component. Whereas the *p*-chlorophenyl derivative **5** (K_i 102 nM) is essentially equipotent with the unsubstituted parent phenyl derivative **3**, the 3,4-dimethoxyphenyl derivative **4** is almost twice as potent, presumably as a result of filling the proximal pocket to a greater degree. Modeling suggested that one method to improve potency would be to optimize the interaction of the amidine with the protein. Replacement of the methylthio group with smaller groups was investigated to see if the thiophene ring would slide deeper into the S1 pocket bringing the amidine closer to Asp189 to form a better salt bridge. However, unsubstituted thio-



Scheme 3. (a) *m*-CPBA, CH_2Cl_2, 1.5 h, rt (77%); (b) $Al(CH_3)_3$, NH_4Cl, toluene, reflux (31% for **12**, 53% for **13**); (c) benzyl mercaptan, TEA, CH_3OH, reflux, 15 min (85%); (d) 30% H_2O_2, 1,1,1,3,3,3-hexafluoro-2-propanol, CH_2Cl_2, 45 h, rt (90%).

Table 1. SAR of the 5-substituted thiophene



Compd	X	Aryl	K_i (μM)
3	—SCH_3		0.101
4	—SCH_3		0.058
5	—SCH_3		0.102
6	—H		5.49
7	—CH_3		0.103
8	—CH_2CH_3		0.138
9	—CH_3		0.169
10	—OCH_3		0.531
11			1.52
12			1.42
13			2.5
14			2.5

phene **6** lost significant potency. Likewise, **10** with the smaller 5-methoxy group should allow formation of a better salt bridge relative to parent 5-methylthio-substituted **3** but, in fact, **10** was 5-fold less potent than **3**. Modeling studies show that a perpendicular geometry for the 5-position substituent fits better into the S1 pocket. The S–Me with a lower barrier to rotation out of the plane of the ring can achieve the perpendicular geometry more easily than the O–Me. Replacement of the heteroatom in the 5-substitutent of **3** with a carbon atom resulted in 5-ethyl-substituted thiophene **8**. Interestingly, this compound was nearly equipotent with **3**, but showed a 9-fold increase in water solubility (2.7 mM aqueous solubility for **8** vs 0.3 mM for **3**). Compound **7**, with the smaller 5-methyl group was also equipotent with **3** and showed a significant 30-fold increase in solubility (9.0 mM aqueous solubility). It is interesting to note that the 5-methyl version containing 3,4-dimethoxyphenyl **9** was now 1.5 times less potent than the corresponding 5-methylthio analogue **4**. This may indicate that the phenyl rings in **7** and **9** may be going off at different vectors due to subtle positioning differences of the thiophene ring within the S1 pocket. Mono- or dioxidation of the methylthio in the 5-methylthiothiophene **5** was explored to attenuate the pK_a of the amidine and to potentially pickup additional hydrogen-bonding interactions with nearby Ser195 at the edge of the S1 pocket. However, **12** and **13** were less potent inhibitors relative to **5**, probably because these groups are sterically too large. Substitution of the 5-methylthio group with larger arylmethylthio groups in **11** and **14** also resulted in less potent inhibitors due to their steric size.

To conclude, although the modifications explored in this study did not result in the expected improvements in potency, equipotent analogues **7** and **8** were found that demonstrated markedly (up to 30-fold) improved solubility. The ease with which useful substitutions can be made at the 5-position of electron-deficient 5-methylsulfonylthiophenes was also shown. Further modifications to other regions of these compounds are being pursued and will be disclosed in due course.

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29. Human kidney cell urokinase (Sigma U5004) was purchased from Sigma Chemical Company (St. Louis, MO, USA). All assays were based on the ability of the test compound to inhibit the enzyme catalyzed hydrolysis of a peptide *p*-nitroanilide substrate. In a typical K_i determination, into each well of a 96-well plate is pipetted 280 μL of *N*-CBz-Val-Gly-Arg-*p*-nitroanilide solution, 10 μL of the test compound solution, and the plate allowed to thermally equilibrate at 37 °C in a Molecular Devices plate reader for >15 min. Reactions were initiated by the addition of a 10 μL aliquot of enzyme and the absorbance increase at 405 nm is recorded for 15 min. Data corresponding to less than 10% of the total substrate hydrolysis were used in the calculations. The ratio of rate of change in absorbance as a function of time for a sample containing no test compound is divided by the velocity of a sample containing test compound, and is plotted as a function of test compound concentration. The data are fit into a linear regression, and the value of the slope of the line calculated. The inverse of the slope is the experimentally determined K_i value.

30. Concentrated solutions (10 mM) of the inhibitors were prepared, shaken and incubated at 37 °C for 24 h, then filtered through a 0.22 μ pore size, nylon membrane, polypropylene syringe filter (Phenomenex, Torrance, CA). Analysis of the solutions by HPLC was carried out using a SPER HTS, 60 Å, 5 μ, 50×4.6 mm column (Princeton Chromatography, Princeton, NJ, USA), running a gradient of 5–100% acetonitrile in 0.1% TFA (aq) over 7.5 min, with UV detection at 215 nm. The rank order of solubility was determined according to the area under the curve and accounting for any dilution factor.