Novel Thieno[2,3-*d*][1,3]oxazin-4-ones as Inhibitors of Human Leukocyte Elastase

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A series of thieno[2,3-*d*][1,3]oxazin-4-ones was synthesized and evaluated in vitro for inhibitory activity toward human leukocyte elastase. New synthetic routes to 2-alkoxy-, 2-alkylthio-, and 2-*sec*-amino-substituted derivatives are reported. This study demonstrates the versatility of 2-aminothiophenes prepared by Gewald reaction as a synthetic entry to serine protease-inhibiting, fused 1,3-oxazin-4-ones. Introduction of ethoxy, *n*-propoxy, and ethylthio groups at C-2 delivered the most potent inhibitors of this series with K_i values lower than 11 nM. Kinetic studies and product analyses revealed the formation of acyl-enzymes as a result of the attack of the active site serine at the carbon C-4 and subsequent deacylation. This mode of action is similar to the inhibition of serine proteases by 4*H*-3,1-benzoxazin-4-ones. Replacement of the benzene ring in benzoxazinones by a (substituted) thiophene led to improved hydrolytic stability and retained inhibitory potency.

Introduction

Human leukocyte elastase (HLE) is a serine protease contained in the azurophilic granules of human neutrophils. By inflammatory stimuli, HLE is released into the extracellular environment. Extracellular HLE is capable of proteolytic degradation of structural components of connective tissues. When ineffectively regulated by endogenous inhibitors, the resulting unrestrained proteolytic activity of HLE is associated with several pathological states. HLE has been shown to contribute to the pathogenesis of destructive lung diseases, such as pulmonary emphysema, cystic fibrosis, and adult respiratory distress syndrome, and inflammatory disorders, such as rheumatoid arthritis and periodontitis.[1] In recent years, much attention has focused on the inhibition of HLE by low-molecular-weight inhibitors that might serve as therapeutic agents. Many types of peptidic and nonpeptidic inhibitors, employing both reversible and nonreversible mechanisms of action, have been reported.[2,3]

The primary specificity pocket (S_1) of HLE is relatively small and lined with hydrophobic residues; thus HLE preferentially cleaves proteins or peptidic substrates with small aliphatic residues at P_1, such as alanine, leucine, or valine.[2−4] The latter amino acid has been frequently incorporated at the P_1 position of potent peptidic inhibitors of HLE. The S_1-P_1 interaction was established by X-ray crystallographic analyses of the complexes of such inhibitors with HLE or porcine pancreatic elastase (PPE), a closely related enzyme.[5] In the crystal structure of the complex between a pyridinone trifluoromethyl ketone and PPE, the isopropyl group of the valine-derived inhibitor was also located in the S_1 pocket.[6]

Many types of synthetic HLE inhibitors have been reported in the literature,[2] including transition-state analogue inhibitors, mechanism-based inhibitors, and acyl-enzyme inhibitors (alternate substrate inhibitors), all of which bind covalently to HLE. Mechanism-based inhibitors possess an electrophilic carbonyl that is attacked by Ser-195, forming an intermediate acyl-enzyme which unmasks a previously concealed functional group that can further react covalently at the active site. This class includes isocoumarins,[4,7] sulfonyloxy succinimides and phthalimides,[8−10] and cephem sulfones and monocyclic β-lactams,[11−14] as well as benzisothiazolones.[15,16]

Proteases that are only acylated will recover activity spontaneously upon deacylation. However, for such acyl-enzyme inhibitors, strong inhibition can be achieved by increasing the rate of acylation or decreasing the rate of deacylation, or both. 3,1-Benzoxazin-4-ones represent a class of heterocyclic acyl-enzyme inhibitors which has been reported to inhibit various serine proteases, such as chymotrypsin,[17−20] cathepsin G,[21] C1r serine protease of the complement system,[22] HSV-1 protease,[23] human cytomegalovirus protease,[24] PPE,[25] and HLE.[26−30] In an extended study, Krantz et al.[28] have demonstrated the design of highly potent HLE inhibitors by introducing favorable substituents in both rings of the benzoxazinone skeleton. Small alkyl groups linked via heteroatoms to C-2 enhanced acylation and limited deacylation rates. Electron withdrawal by the 2-substituents led to an accelerated acylation, but also to a reduced stability as measured by alkaline hydrolysis rates. However, both stability and inhibitory potency could be further improved by substitution at the fused benzene ring (e.g., alkyl groups at position 5).

In the present study, we describe the synthesis and HLE inhibition of a series of thieno[2,3-*d*][1,3]oxazin-4-ones. The strategy to replace the benzene ring in benzoxazinones by thiophene is based on the consideration that the enhanced electron density at the thiophene carbon atoms might result in an improved intrinsic stability of an isosteric thieno[1,3]oxazin-4-one system. Among the three possible bicyclic systems, the thieno-

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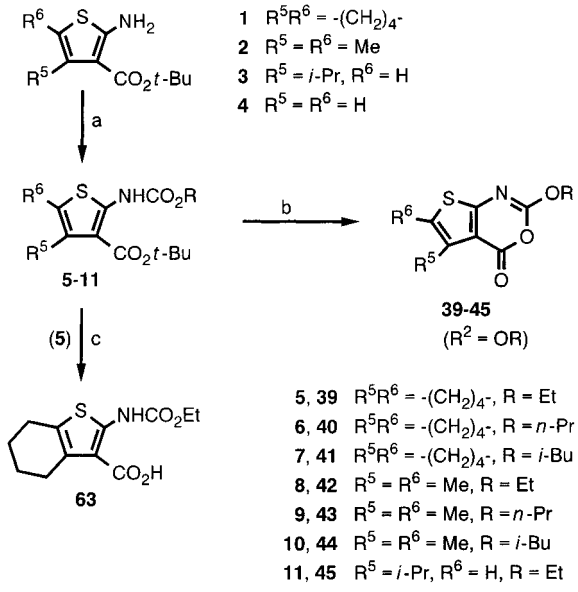
Scheme 1[a]



[a] Reagents: (a) $ClCO_2R$, EtOH, room temperature; (b) TFA, trifluoroacetic anhydride, 0−25 °C; (c) TFA, 0−25 °C.

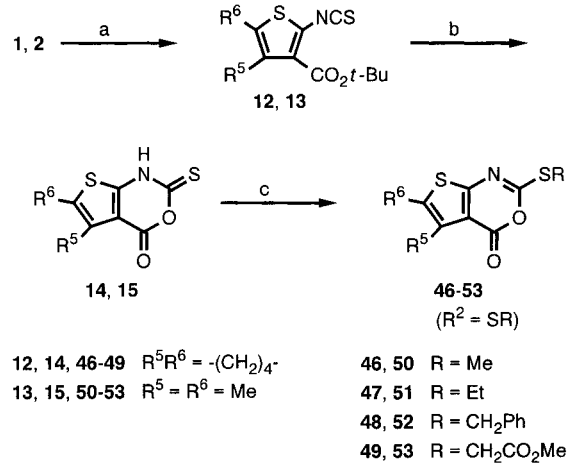
[2,3-*d*][1,3]oxazin-4-one system was chosen because of the expected synthetic entry through the Gewald thiophene synthesis. As a conclusion from the results of HLE inhibition by 3,1-benzoxazin-4-ones, compounds with alkoxy, alkylthio, and amino residues at position 2 have been prepared.

Results and Discussion

Synthesis. Alkyl 2-aminothiophenecarboxylates are available by Gewald synthesis.[31,32] Usually, ethyl 2-aminothiophenecarboxylates are prepared by reacting ethyl cyanoacetate with sulfur and a carbonyl compound in the presence of an organic base. We have performed the reaction with *tert*-butyl cyanoacetate in place of ethyl cyanoacetate to obtain *tert*-butyl thiophenecarboxylates **1**−**4** (Scheme 1), which were found to provide ready access to 2-alkoxy- and 2-(alkylthio)thieno[2,3-*d*]-[1,3]oxazin-4-ones. Compounds **1** and **2**[33] were obtained from the appropriate ketones in the one-pot version of the Gewald reaction. To afford **3**, the Knoevenagel adduct of 3-methyl-2-butanone and *tert*-butyl cyanoacetate was prepared and subsequently treated with sulfur under Gewald conditions. Compound **4** was obtained by reacting the mercaptoacetaldehyde dimer (2,5-dihydroxy-1,4-dithiane) with *tert*-butyl cyanoacetate.

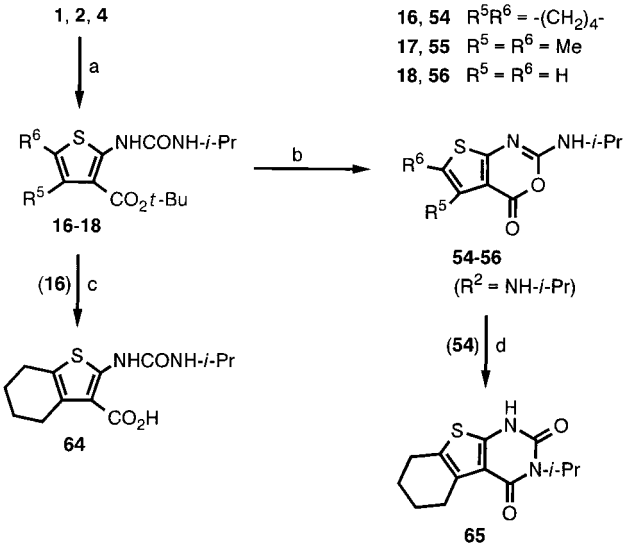
The synthetic route to the new class of 2-alkoxythieno-[2,3-*d*][1,3]oxazin-4-ones is shown in Scheme 1. Urethane derivatives **5**−**11** were easily obtained from aminothiophenes and the appropriate alkyl chloroformates. Deprotection of **5** furnished **63**, which was needed as a reference compound for enzymatic studies. Treatment of **5**−**11** with a mixture of trifluoroacetic acid and trifluoroacetic anhydride resulted in deesterification and cyclocondensation, to obtain the desired final products **39**−**45** in 32−97% yield (Table 1). Besides 5,6-tetramethylene- and 5,6-dimethyl-substituted derivatives, the 5-isopropyl derivative **45** was prepared by this route. Its substitution pattern was chosen in light of the highly potent benzisothiazolones developed by Hlasta et al.; an isopropyl group in the peri position to the carbonyl of the scissile amide bond led to rapid inactivation due to a proposed hydrophobic interaction with the S_1 pocket of HLE.[15]

Scheme 2[a]



[a] Reagents: (a) $CSCl_2$, $CaCO_3$, CH_2Cl_2, H_2O, 0 °C; (b) TFA, CH_2Cl_2, 0 °C; (c) MeI or RBr, Na_2CO_3, acetone, room temperature.

Scheme 3[a]

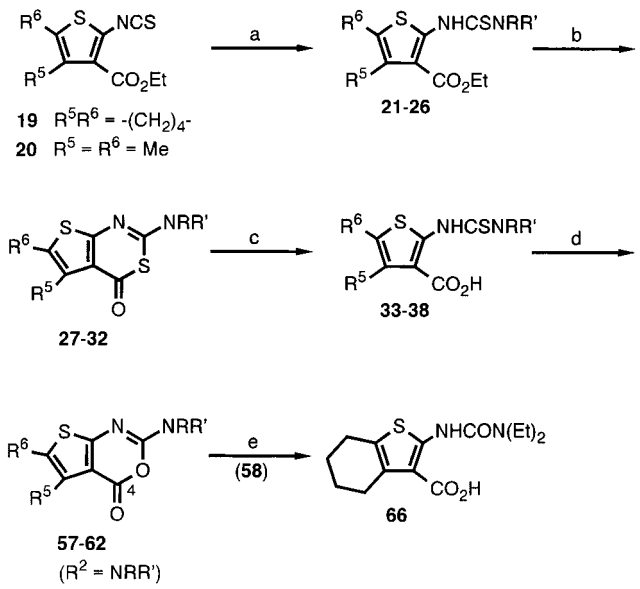


[a] Reagents: (a) isopropyl isocyanate, pyridine, 50 °C; (b) TFA, trifluoroacetic anhydride, 0−25 °C; (c) TFA, 0−25 °C; (d) NaOEt, EtOH, reflux.

The route to the new class of 2-(alkylthio)thieno[2,3-*d*][1,3]oxazin-4-ones is outlined in Scheme 2. Aminothiophenes **1** and **2** were converted to isothiocyanatothiophenes **12** and **13** by the action of thiophosgene. Deprotection of **12** and **13** resulted directly to ring closure of the intermediate isothiocyanatothiophenecarboxylic acids. Thus, 2-thioxooxazinones **14** and **15** were obtained. These key intermediates could be alkylated conveniently with appropriate alkyl halides to furnish the final 2-alkylthio derivatives **46**−**53**, mostly in high yields (67−97%, except **49**: 36%). Attempts to cleave the oxazinone ring in **50** under mild alkaline conditions failed; a product mixture was observed, besides unreacted starting material.

2-Isopropylamino-substituted thienooxazinones (Scheme 3) were prepared according to the procedure of Hallenbach et al.[34] Aminothiophenes **1**, **2**, and **4** were reacted with isopropyl isocyanate in pyridine, and the resulting ureas **16**−**18** were subsequently cyclized

Scheme 4[a]



21, 27, 33, 57 R^5R^6 = -$(CH_2)_4$-, RR' = -$(CH_2)_2O(CH_2)_2$-
22, 28, 34, 58 R^5R^6 = -$(CH_2)_4$-, R = R' = Et
23, 29, 35, 59 R^5R^6 = -$(CH_2)_4$-, R = Me, R' = cyclohexyl
24, 30, 36, 60 R^5 = R^6 = Me, RR' = -$(CH_2)_2O(CH_2)_2$-
25, 31, 37, 61 R^5 = R^6 = Me, R = R' = Et
26, 32, 38, 62 R^5 = R^6 = R = Me, R' = cyclohexyl

[a] Reagents: (a) HNRR′, CH_2Cl_2, room temperature; (b) concd H_2SO_4, room temperature; (c) NaOH, dioxane−H_2O, reflux; (d) HgO, CH_2Cl_2, room temperature; (e) NaOH, acetone−H_2O, reflux.

to afford **54**−**56** in 65−98% yield. Two reference compounds were prepared, **64** by cleavage of the *tert*-butyl ester of **16** and **65** by sodium ethoxide-promoted Dimroth rearrangement of **54**.

The synthesis of 2-*sec*-amino-substituted thienooxazinones is outlined in Scheme 4. In contrast to the synthetic routes to afford the products **39**−**56** via *tert*-butyl precursors (Schemes 1−3), 2-*sec*-amino derivatives **57**−**62** were prepared by a route using common ethyl 2-aminothiophenecarboxylates as starting materials. Conversion to the isothiocyanates **19** and **20**, followed by treatment with secondary amines gave the thiourea derivatives **21**−**26**. Ring closure to **27**−**32** was then performed upon the action of concentrated sulfuric acid, according to a previously reported procedure.[35] The formal replacement of the thiazine sulfur atom by oxygen was accomplished by a new two-step procedure. The thiazinone ring was first cleaved hydrolytically upon the action of sodium hydroxide to afford the carboxylic acids **33**−**38**. Subsequent treatment with yellow mercury(II) oxide in dichloromethane at room temperature furnished the final products **57**−**62** directly in an unexpectedly smooth reaction. The yields of the 2-*sec*-amino derivatives **57**−**62** were 79−94%. The reference compound **66** was prepared by alkaline ring cleavage of **58**.

Alkaline Hydrolysis. The criteria used to evaluate the present set of thienooxazinones were (i) their stability in alkaline buffer to determine their relative chemical stability and (ii) their HLE inhibitory potency. Aqueous alkaline hydrolysis of the thienooxazinones **39**−**62** was assumed to proceed by hydroxide attack at C-4 (e.g., in Scheme 4), as has been reported for 2-alkoxy- and 2-aminobenzoxazinones,[20,28] and can therefore be used as a model for enzyme acylation, as well as to estimate the stability of the compounds. The rate constants k_{OH^-} were obtained either spectrophotometrically or by means of HPLC control to determine the disappearance of the thienooxazinones. The compounds exhibited considerable hydrolytic stability, unexceptionally with values of log $k_{OH^-} < 1$ (Table 1). Amino-substituted derivatives **54**−**62** possessed extraordinarily low susceptibility to alkaline hydrolysis, clearly reflecting the dependence of k_{OH^-} on electron withdrawal of the 2-substituent.

Having in mind that the 5,6-dialkyl substitution in most of the present thienooxazinones may additionally account for their stability toward alkaline hydrolysis, the data demonstrate the advantageous effect of the fused thiophene ring. The rate constants of the present thienooxazinones are lower by around 1 order of magnitude relative to the values of 5-methyl-3,1-benzoxazin-4-ones bearing identical 2-substituents (OEt, SMe, SEt, NH-*i*-Pr).[28] Comparison to 5-unsubstituted 3,1-benzoxazin-4-ones (R^2 = OEt, O-*n*-Pr, O-*i*-Bu, SMe, SEt, NH-*i*-Pr, morpholino)[20,21,28] revealed 1.5−2.5 lower log k_{OH^-} values in the case of the thienooxazinone analogues.

Inhibition Kinetics. HLE inhibition by thienooxazinones **39**−**62** was assayed in the presence of MeOSuc-Ala-Ala-Pro-Val-pNA or Suc-Ala-Ala-Pro-Val-AMC as chromogenic or fluorogenic substrates, respectively. Progress curves were characterized by an initial exponential phase, followed by a linear steady-state turnover of the substrate, and could be analyzed by slow-binding kinetics.[36] Progress curves were fitted to eq 1,

$$[P] = v_s t + (v_i - v_s)[1 - \exp(-k_{obs}t)]/k_{obs} + \text{offset} \quad (1)$$

where v_s and v_i are the steady-state velocity and the initial velocity and k_{obs} is the first-order rate constant for the approach to the steady state. According to the mechanism of inhibition (Scheme 5), the observed rate constant, k_{obs}, is given by eq 2:[26]

$$k_{obs} = k_1[I]/(K_d' + [I]) + k_{off} \quad (2)$$

The dissociation constant of an enzyme−inhibitor complex, K_d, the second-order rate constant, k_{on}, the first-order rate constant, k_{off}, and the steady-state inhibition constant, K_i, are related by eqs 3−8:

$$K_d = K_d'/(1 + [S]/K_m) \quad (3)$$

$$k_{on} = k_1/K_d \quad (4)$$

$$k_{on} = k_{on}'(1 + [S]/K_m) \quad (5)$$

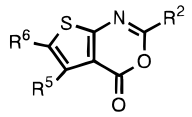
$$k_{off} = k_{-1} + k_2 \quad (6)$$

$$K_i = K_i'/(1 + [S]/K_m) \quad (7)$$

$$k_{off} = K_i k_{on} \quad (8)$$

Typically, investigations on acyl-enzyme inhibitors of serine proteases were performed under experimental conditions with [I] much lower than K_d', which do not allow for the detection of the presence of a pre-

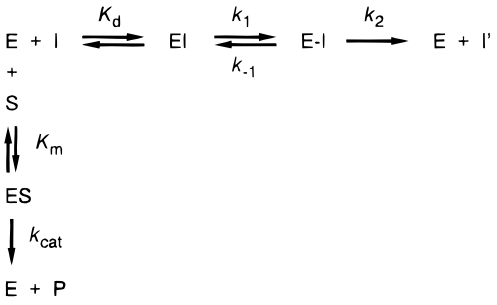
Table 1. 4*H*-Thieno[2,3-*d*][1,3]oxazin-4-ones Prepared and Kinetic Data of the Alkaline Hydrolysis and HLE Inhibition



compd	R^2	R^5	R^6	k_{OH^-} (M^{-1} s^{-1})	log k_{OH^-}	k_{on} (10^3 M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_i (nM)	pK_i
39	OEt	$-(CH_2)_4-$		2.77	0.44	93.6	1.27	1.36	8.87
40	O-*n*-Pr	$-(CH_2)_4-$		2.75	0.44	99.3	1.42	1.43	8.84
41	O-*i*-Bu	$-(CH_2)_4-$		1.83	0.26	1.01	0.515	51	7.29
42	OEt	Me	Me	2.82	0.45	12.2	1.3	10.7	7.97
43	O-*n*-Pr	Me	Me	2.48	0.39	15.6	0.628	4.02	8.40
44	O-*i*-Bu	Me	Me	2.55	0.41	0.219	0.292	133	6.87
45	OEt	*i*-Pr	H	2.98	0.47	76.4	4.29	5.61	8.25
46	SMe	$-(CH_2)_4-$		1.63	0.21	272	15.6	5.74	8.24
47	SEt	$-(CH_2)_4-$		0.59	−0.22	nd[a]		5.7	8.24
48	SCH_2Ph	$-(CH_2)_4-$		0.355	−0.45	0.239	0.692	290	6.54
49	SCH_2CO_2Me	$-(CH_2)_4-$		0.92	−0.04	1.63	0.292	17.9	7.75
50	SMe	Me	Me	1.65	0.22	16.2	5.67	35	7.46
51	SEt	Me	Me	1.27	0.10	1310	35.8	2.73	8.56
52	SCH_2Ph	Me	Me	0.69	−0.16	3.54	3.58	101	7.00
53	SCH_2CO_2Me	Me	Me	1.03	0.01	0.685	1.63	238	6.62
54	NH-*i*-Pr	$-(CH_2)_4-$		0.323	−0.49	1.39	2.01	145	6.84
55	NH-*i*-Pr	Me	Me	0.073	−1.14	1.13	0.85	75.5	7.12
56	NH-*i*-Pr	H	H	0.332	−0.48	3.2	1.34	41.8	7.38
57	4-morpholinyl	$-(CH_2)_4-$		0.083	−1.08	<0.8		nd	
58	$N(Et)_2$	$-(CH_2)_4-$		0.025	−1.59	3.75	0.612	16.3	7.79
59	N(Me)cyclohexyl	$-(CH_2)_4-$		nd		<0.6		nd	
60	4-morpholinyl	Me	Me	0.085	−1.07	<0.6		nd	
61	$N(Et)_2$	Me	Me	0.023	−1.63	<0.6		nd	
62	N(Me)cyclohexyl	Me	Me	0.077	−1.11	<0.8		nd	

[a] nd, not determined.

Scheme 5. Kinetic Model for Acyl-Enzyme Inhibition of Serine Proteases in the Presence of Substrate



association complex, EI. Then, eq 2 takes on the form (9):

$$k_{obs} = k_{on}'[I] + k_{off} \quad (9)$$

In the present study on HLE inhibition by compounds **39**−**62**, with the exception of compound **56**, a preassociation complex, EI, did not accumulate in the inhibitor concentration range used. This was indicated by similar initial velocities, v_i, that equaled the velocity in the absence of inhibitor, v_0, as well as by a linear dependence of k_{obs} on [I]. The values obtained for k_{obs} were plotted versus [I], and a linear regression gave the apparent rate constant, k_{on}', as the slope (eq 9). The values for k_{on} were calculated from eq 5. The apparent inhibition constant, K_i', was obtained using the steady-state velocities, v_s, together with v_0, and fitting them to the equation of a competitive inhibition (eq 10):

$$v_s = v_0/[([I]/K_i') + 1] \quad (10)$$

K_i and k_{off} values were calculated from eqs 7 and 8, respectively. As an example, the analysis of the inhibition kinetics of HLE by the thienooxazinone **42** is illustrated in Figures 1−3. The kinetic data of the whole set of thienooxazinones are outlined in Table 1. It was concluded from their kinetic behavior as well as from product analysis experiments (see below) that the title compounds act as acyl-enzyme inhibitors of HLE. In this case, the rate constants, k_{on} and k_{off}, reflect the acylation and deacylation steps, respectively, and not the simple association−dissociation equilibrium of a competitive inhibition. Acyl-enzymes, E-I, are formed, and a slow consumption of the inhibitors occurs to release the modified inhibitors, I′, that are a priori inactive. However, since a steady-state condition is reached, and as long as the inhibitor concentration is

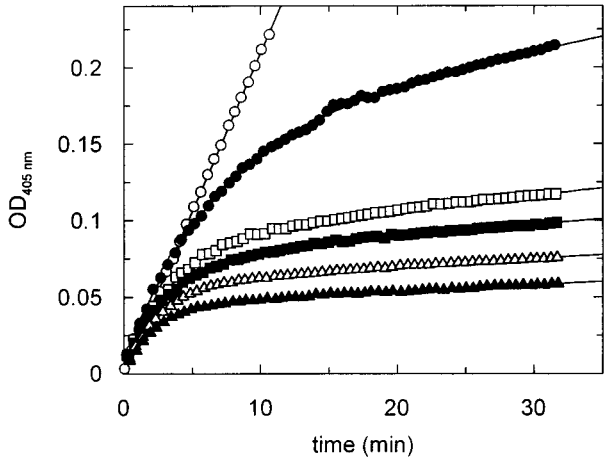


Figure 1. Slow-binding inhibition of HLE by compound **42** in 50 mM sodium phosphate, 500 mM NaCl, pH 7.8. Substrate: MeOSuc-Ala-Ala-Pro-Val-pNA. Data were fitted to eq 1 to obtain the best-fit parameters for v_i, v_s, k_{obs}, and offset: ○, [I] = 0; ●, [I] = 0.5 μM; □, [I] = 1 μM; ■, [I] = 1.5 μM; △, [I] = 2 μM; ▲, [I] = 2.5 μM.

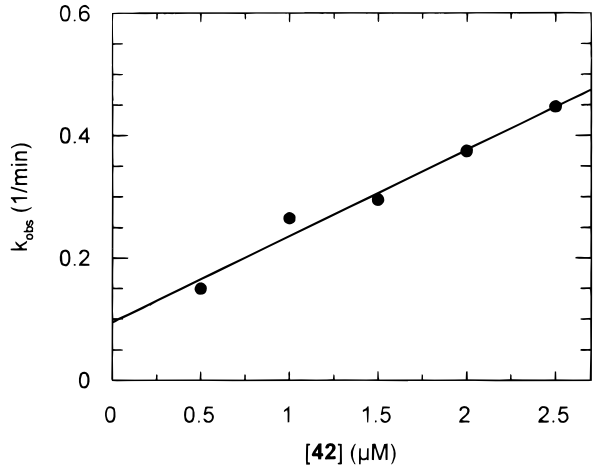


Figure 2. Plot of k_{obs} versus [I] for the inhibition of HLE by compound **42**. The values for k_{obs} were obtained from fits to the data shown in Figure 1. The solid line was drawn using the best-fit parameters to eq 9, and the slope corresponds to a value for $k_{on}' = 2350\ M^{-1}\ s^{-1}$.

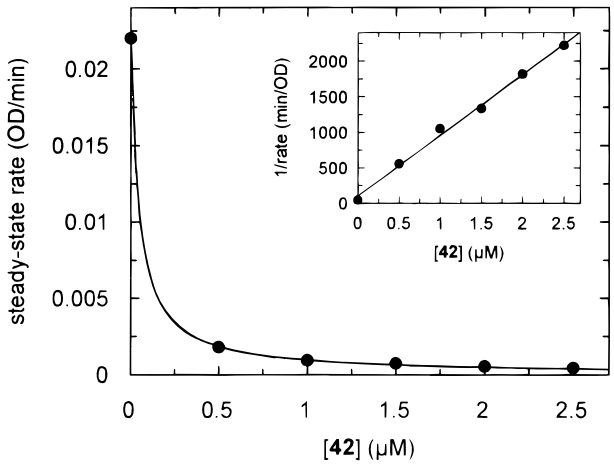


Figure 3. Plot of the steady-state rates versus [I] for the inhibition of HLE by compound **42**. The data were obtained from fits of the curves shown in Figure 1. The solid line was drawn using the best-fit parameters from a fit according to eq 10, which gave $K_i' = 46.0 \pm 1.5$ nM. The insert is a Dixon plot to show the linearity.

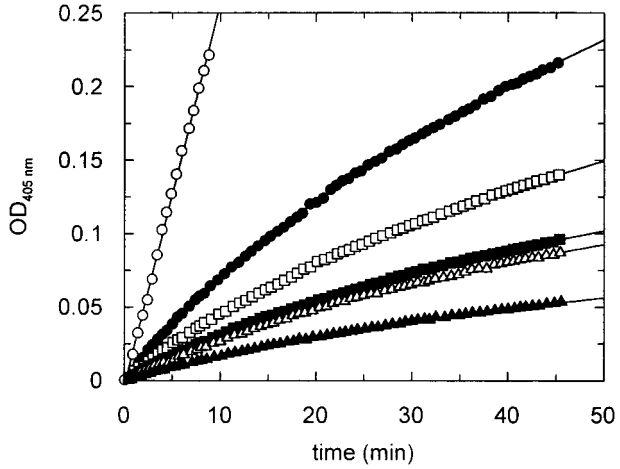


Figure 4. HLE inhibition by compound **56** in 50 mM sodium phosphate, 500 mM NaCl, pH 7.8. Substrate: MeOSuc-Ala-Ala-Pro-Val-pNA. Data were fitted to eq 1 to obtain the best-fit parameters for v_i, v_s, k_{obs}, and offset: ○, [I] = 0; ●, [I] = 1 μM; □, [I] = 2 μM; ■, [I] = 3 μM; △, [I] = 4 μM; ▲, [I] = 5 μM.

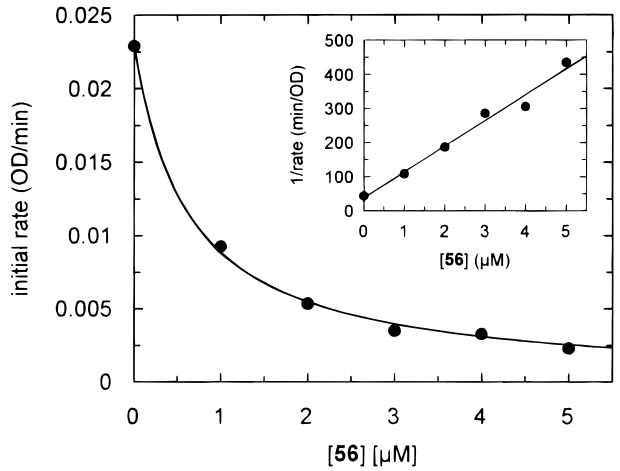


Figure 5. Plot of the initial rates versus [I] for the inhibition of HLE by compound **56**. The values for v_i were obtained as average of duplicate assays from fits to eq 1. The solid line was drawn using the best-fit parameters to eq 11, which gave $K_d' = 628 \pm 23$ nM. The insert is a Dixon plot to show the linearity.

not depleted due its turnover by HLE, K_i values will reflect the dissociation of the enzyme-bound inhibitor complexes[26] and are used to express the potency of acyl-enzyme inhibitors of serine proteases.[27,28]

HLE inhibition by the isopropylamino derivative **56** is illustrated in Figures 4−6. This compound, but not the 5,6-substituted analogues **54** and **55**, showed a different kinetic behavior. Two facts indicated that a preassociation complex, EI, did accumulate under the experimental conditions used: (i) the initial velocities, v_i, decreased with increased inhibitor concentrations, and (ii) the k_{obs} values were identical. The initial velocities, v_i, together with the value for the reaction without inhibitor, v_0, were fitted to eq 11,

$$v_i = v_0/[([I]/K_d') + 1] \quad (11)$$

and $K_d = 0.182$ μM for **56** was calculated from eq 3. The final inhibition constant, K_i, was obtained from eqs 10 and 7. Under conditions with K_d' much lower than [I], eq 2 is simplified to eq 12:

$$k_{obs} = k_1 + k_{off} \quad (12)$$

An expression to calculate k_{on} (eq 13) is given by combining eqs 4, 12, and 8:

$$k_{on} = k_{obs}/(K_d + K_i) \quad (13)$$

Finally, k_{off} was calculated from eq 8. Since **56** is the only 5,6-unsubstituted compound of the present series of thienooxazinones, the small size of the inhibitor probably accounts for the increased affinity to accumulate the preassociation complex. Compared to the 5,6-tetramethylene- and dimethyl-substituted isopropylamino derivatives (**54** and **55**), compound **56** exhibited similar acylation and deacylation rates.

Product Analysis. The action of thienooxazinones as acyl-enzyme inhibitors of HLE was established by product analysis experiments (Scheme 6). Selected inhibitors were incubated with HLE at pH 7.8, and the

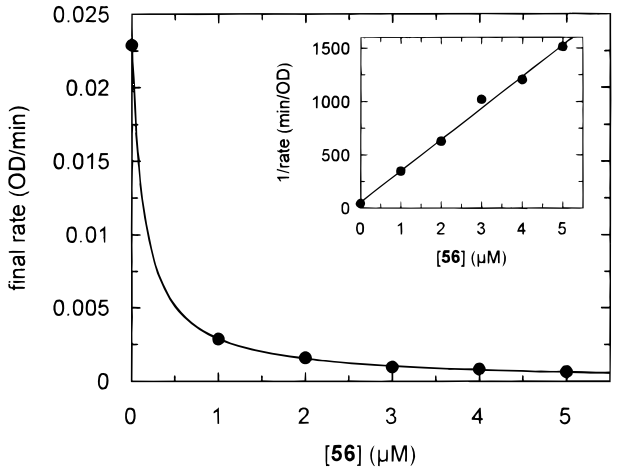
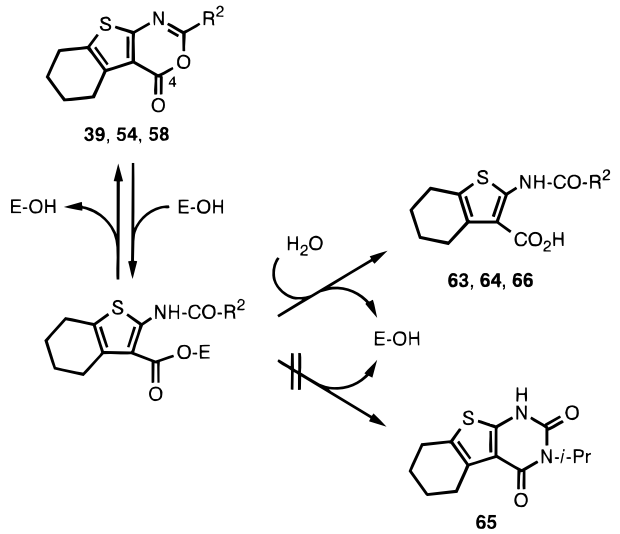


Figure 6. Plot of the steady-state rates versus [I] for the inhibition of HLE by compound **56**. The data were obtained as average of duplicate assays from fits to eq 1. The solid line was drawn using the best-fit parameters from a fit according to eq 10, which gave $K_i' = 145 \pm 2.1$ nM.

Scheme 6. Reactions of Compounds **39**, **54**, and **58** with HLE



mixtures were analyzed with HPLC. Reaction of HLE with the 2-ethoxythienooxazinone **39** and the 2-diethylamino derivative **58** afforded exclusively the thiophenecarboxylic acids **63** and **66**, respectively. These results are consistent with the proposed mechanism of inhibition, namely, the attack of the active site serine at the C-4 atom of the inhibitor and ring cleavage to the corresponding acyl-enzyme that deacylates hydrolytically. In the case of the isopropylamino derivatives, the possibility of an intramolecular deacylation has to be taken into consideration. The isopropylamino derivative **54**, however, gave the ureidothiophenecarboxylic acid **64** as the only reaction product, and the pyrimidinedione **65** was not formed. The branched group in **54** (NH-*i*-Pr) obviously blocks pyrimidine cyclization of the acyl-enzyme as has been reported for the reaction of the analogous (isopropylamino)benzoxazinone with HLE,[27] cathepsin G, and chymotrypsin.[21] Incubation of HLE with the alkylthio derivatives **46**−**48** (each $R^5R^6 = -(CH_2)_4-$) led to the formation of three distinct reaction products (retention times 15.5, 18, and 25 min, respectively), accompanied by one identical side product (13 min). This is interpreted as a result of an analogous C-4 attack and the release of distinct thiophenecarboxylic acids with thiolurethane substituents. The hydrolysis of thiolurethanes (−NH−CO−SR) is reported to be faster by 3−4 orders of magnitude compared to that of urethanes (−NH−CO−OR).[37,38] Likely, the formation of the side product is a result of a subsequent decomposition of the thiolurethanes. Accordingly, reaction of HLE with the alkylthio derivatives **50** and **51** (each $R^5 = R^6 =$ Me) afforded two distinct products (retention times 12 and 15 min, respectively) together with an identical side product (10 min). In summary, these results confirm the expected mode of action of the thienooxazinones as acyl-enzyme inhibitors of HLE.

Effects of R^2, R^5, and R^6 on Inhibitory Potency. Within the 2-alkoxy series (**39**−**45**), ethoxy and *n*-propoxy substitution led to fast acylation of HLE (Table 1), whereas an extension to isobutoxy decreased the k_{on} values by 2 orders of magnitude (**39** and **40** versus **41**; **42** and **43** versus **44**). This indicates that in the initial enzyme−inhibitor complex, the 2-substituent is accommodated at the primary specificity site S_1. Similar results were reported within a series of 3-alkoxy-7-amino-4-chloroisocoumarins; the decrease in the inhibition rates as the size of the 3-alkoxy group is increased was consistent with the preference of elastases for small hydrophobic residue in the S_1 pocket.[4,39] The tetramethylene derivatives **39**−**41** exhibited stronger activity than the dimethyl derivatives **42**−**44**. Compounds **39** and **40** are the most potent inhibitors of the whole series with K_i values lower than 2 nM. Inhibition by the 5-isopropyl derivative **45** also showed fast acylation but was adversely affected by a rather fast deacylation. 5-Alkyl substitution in 3,1-benzoxazin-4-ones was found to decelerate the deacylation step in HLE inhibition drastically. This was interpreted as a result of conformational restrictions and steric hindrance of the water attack at the ester carbonyl.[28] One might suspect that the replacement of a 5-methyl group or the tetramethylene chain by a 5-isopropyl substituent (**45**) could result in a similar effect. This was, however, not the case.

Within the 2-alkylthio series (**46**−**53**), large substituents (SCH_2Ph, SCH_2CO_2Me) diminished the acylation rate compared to methylthio and ethylthio residues. As was already concluded in the 2-alkoxy series, the 2-substituent might interact with the S_1 subsite of HLE. When comparing the two methylthio derivatives, the tetramethylene compound **46** has a more than 10-fold higher k_{on} value than the 5,6-dimethyl derivative **50**. Three inhibitors of the alkylthio series (**46**, **47**, and **51**) were found to exhibit extraordinarily high acylation rates. In the case of **47**, we were unable to determine k_{on}, not even by means of fluorescence measurement, but a value $> 1 \times 10^6$ M^{-1} s^{-1} is assumed. The deacylation in the case of **46** and **51** is accelerated, and an estimation for **47** gave $k_{off} > 50 \times 10^{-4}$ s^{-1}. These data indicate that the specific recognition of the 2-substituent might improve geometries for both the acylation and deacylation steps. However, compounds **46**, **47**, and **51** are potent inhibitors with K_i values lower than 10 nM.

Amino-substituted thienooxazinones (**54**−**62**) generally showed a decreased acylation rate, an effect that can be easily explained from their enhanced intrinsic

stability due to the electron-donating 2-substituents. Among the 2-*sec*-amino derivatives, only **58** exhibited a remarkable capacity to acylate HLE. Since the acyl-enzyme formed in the reaction of **58** with HLE is highly stable ($k_{off} < 1 \times 10^{-4}$ s^{-1}), a rather strong inhibition was achieved.

Effect of the Thiophene Introduction. The present thienooxazinones exhibit K_i values in the range obtained for the HLE inhibition by analogous 3,1-benzoxazin-4-ones.[28] The potency of the most active (5-alkyl-substituted) benzoxazinones is not achieved by the present inhibitors. Introduction of the (mostly substituted) thiophene ring in place of a (unsubstituted) benzene unit decreased both acylation and deacylation rates. The reduced susceptibility to attack by an oxygen nucleophile, as was demonstrated in alkaline hydrolysis, may also account for the diminished acylation rates. As in the case of benzoxazinones, attack of the active site serine at the carbonyl C-4 of the thienooxazinones leads to the formation of corresponding acyl-enzymes. The acyl-enzyme ester bond as a part of a heteroaromatic β-enamino ester[40] is assumed to be less reactive toward nucleophiles. As a consequence, the deacylation rate should be reduced leading to the desired enhanced stability of the thiophene-derived acyl-enzymes. Our data support this hypothesis.

There have been few attempts to investigate the serine protease inhibition by heterocyclic fused 1,3-oxazin-4-ones reported so far. 2-Methyl-4*H*-pyrido[2,3-*d*][1,3]oxazin-4-one,[28] being less active toward HLE than the corresponding 2-methylbenzoxazinone, showed an increased k_{OH^-} value. Since pyridine introduction should have the opposite effect on electron density than the introduction of thiophene, the latter effect can be explained thereby. The interaction of HLE with 7-aza-isatoic anhydride was reported to form an acyl-enzyme that deacylates rapidly.[9] Previously, a series of pyrrolo-[2,3-*d*][1,3]oxazine-2,4-diones, further analogues of isatoic anhydride, was described to inhibit HLE and chymotrypsin in the micromolar range.[41] More recently, derivatives of the thieno[3,2-*d*][1,3]oxazin-4-one ring system (with −CH(Me)NHCbZ substitution at position 2) were reported as inhibitors of herpes proteases.[42] The most effective inhibitor toward HSV-1 protease showed a strongly enhanced aqueous stability relative to the corresponding benzoxazinone. Thus, as can be concluded from this result and our data, both thieno[3,2-*d*][1,3]oxazin-4-one and thieno[2,3-*d*][1,3]oxazin-4-one systems provide increased stability of the oxazinone ring as a result of increased electron density.

Conclusions

A series of thieno[2,3-*d*][1,3]oxazin-4-ones was prepared including potent inhibitors of HLE that possess high stability toward alkaline hydrolysis.

The most obvious trend in the present set of thienooxazinones is the strong dependence of the acylation rate on the size of the 2-substituent. Bulky residues decreased k_{on}; this effect is particularly impressive when comparing the *n*-propoxy to isobutoxy compounds. Optimal size of R^2 in 5,6-dimethyl- and tetramethylene-substituted thienooxazinones may be reached with ethoxy, propoxy, and ethylthio substituents. These results indicate that the 2-substituent interacts with the S_1 subsite of the enzyme.

A comparison of 5,6-tetramethylene to 5,6-dimethyl substitution revealed that in most cases, but not generally, tetramethylene substitution was advantageous to accelerate enzyme acylation and improve inhibition. However, it should be noted that the Gewald synthesis provides access to a variety of substituted aminothiophenes. Different thiophene precursors may be successfully used in the synthetic routes presented herein. Such an approach will allow for the controlled introduction of further 5- and 6-substituents.

2-*sec*-Amino-substituted thienooxazinones possessed extraordinarily high stability toward alkaline hydrolysis. For example, the morpholino derivatives **57** and **60** exhibited more than 300-fold lower k_{OH^-} values compared to 2-morpholino-4*H*-3,1-benzoxazin-4-one.[20,28] However, among the six *sec*-amino derivatives, only **58** showed HLE inhibitory activity. This compound is an interesting candidate for further investigation, combining good activity and high stability. The preparation of analogous 2-diethylamino derivatives with new 5,6-substituents to optimize HLE-inhibiting activity is in progress in our laboratories.

Experimental Section

General Methods and Materials. Melting points were determined on a Boetius apparatus and are not corrected. Thin-layer chromatography was performed on Merck aluminum sheets, silica gel 60 F_{254}. ^{13}C NMR spectra (75 MHz) and ^1H NMR spectra (300 MHz) were recorded on a Varian Gemini 300 spectrometer. ^{13}C NMR signals were assigned on the basis of $^{13}C,^1H$ coupled spectra and $^{13}C/^1H$ correlation experiments. IR spectra were measured with a Perkin-Elmer 16 PC FTIR spectrometer. UV spectra were recorded on a Shimadzu UV−vis spectrophotometer (UV-160A). Mass spectra (70 eV) were obtained using a Varian MAT CH6 spectrometer. Spectrophotometric assays were done on a Varian Cary 3 Bio spectrophotometer with a six-cell holder. Fluorimetric assays were performed on a Perkin-Elmer LS50B instrument. Analytical HPLC was performed on a ThermoSeparationProducts liquid chromatograph with PC1000 software. A 5-μm Hypersil ODS 200 × 4.6-mm column was used at a flow rate of 0.5 mL/min. Mobile phase A was 50 mM triethylammonium acetate buffer, pH 7.0, and mobile phase B was the same containing 85% acetonitrile. A gradient of 20−100% B in 30 min was utilized. Elastase was prepared from human leukocytes and purified by affinity chromatography using an immobilized synthetic inhibitor.[43] The substrates MeOSuc-Ala-Ala-Pro-Val-pNA and Suc-Ala-Ala-Pro-Val-AMC were from Bachem, Bubendorf, Switzerland.

***tert*-Butyl 2-Amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (1).** A mixture of sulfur (2.24 g, 70 mmol), cyclohexanone (6.9 g, 70 mmol), *tert*-butyl cyanoacetate (9.9 g, 70 mmol), and EtOH (20 mL) was treated dropwise with morpholine (6.1 g, 70 mmol) at 45 °C. After being stirred for 5 h at 45 °C, the mixture was diluted with 0.4 M acetic acid (300 mL) and extracted with ether (4 × 100 mL). The organic layer was washed with H_2O and dried (Na_2SO_4). Removal of the solvent in vacuo yielded **1** (15.8 g, 89%) as an oil. Recrystallization from MeOH gave yellowish crystals: mp 43−44 °C; ^1H NMR ($CDCl_3$) δ 1.55 (s, 9H, CH_3), 1.65−1.85 (m, 4H, CH_2), 2.45−2.73 (m, 4H, CH_2), 5.88 (br s, 2H, NH_2). Anal. ($C_{13}H_{19}NO_2S$) C, H, N, S.

***tert*-Butyl 2-Amino-4,5-dimethylthiophene-3-carboxylate (2).** Compound **2** was prepared by the foregoing procedure, but using 2-butanone (5.0 g, 70 mmol) in place of cyclohexanone. The mixture was stirred for 7 h at 45 °C and for an additional 2 days at room temperature. The crude product was recrystallized from MeOH to yield **2** (5.6 g, 35%): mp 84−86 °C (lit.[33] mp 66−67 °C); ^1H NMR ($CDCl_3$) δ 1.56 (s, 9H, CH_3), 2.15 (s, 6H, 4- and 5-CH_3), 5.84 (s, 2H, NH_2). Anal. ($C_{11}H_{17}NO_2S$) H, N, S; C: calcd, 58.12; found, 57.59.

***tert*-Butyl 2-Amino-4-isopropylthiophene-3-carboxylate (3).** Triethylamine (7.1 g, 70 mmol) was added dropwise over a period of 15 min at 45 °C to a mixture of sulfur (2.24 g, 70 mmol), (*E*/*Z*)-2-cyano-3,4-dimethyl-2-propenoic acid *tert*-butyl ester,[44] and DMF (28 mL). The mixture was stirred at 45 °C for an additional 75 min, diluted with 0.2 M acetic acid (350 mL), extracted with ether (4 × 70 mL), washed with H_2O, and dried (Na_2SO_4). Evaporation of the solvent and recrystallization from hexane yielded **3** (8.96 g, 53%): mp 77−78 °C; ^{1}H NMR ($CDCl_3$) δ 1.19 (d, J = 6.7 Hz, 6H, CH_3), 1.58 (s, 9H, CH_3), 3.40 (sept, J = 6.7 Hz, 1H, CH), 5.88 (s, 1H, H-5), 6.02 (s, 2H, NH_2). Anal. ($C_{12}H_{19}NO_2S$) C, H, N, S.

***tert*-Butyl 2-Aminothiophene-3-carboxylate (4).** By the procedure outlined above, but using 2,5-dihydroxy-1,4-dithiane (5.33 g, 35 mmol) and *tert*-butyl cyanoacetate (9.9 g, 70 mmol) in place of sulfur and (*E*/*Z*)-2-cyano-3,4-dimethyl-2-propenoic acid *tert*-butyl ester, compound **4** (8.1 g, 58%) was prepared: mp 53−54 °C (hexane); ^{1}H NMR ($CDCl_3$) δ 1.56 (s, 9H, CH_3), 5.85 (s, 2H, NH_2), 6.16 (d, J = 5.8 Hz, 1H, H-5), 6.93 (d, J = 5.8 Hz, 1H, H-4). Anal. ($C_9H_{13}NO_2S$) C, H, N, S.

***tert*-Butyl 2-[(Ethoxycarbonyl)amino]-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (5): General Procedure for Urethanes 5−11.** Compound **1** (2.53 g, 10 mmol), dissolved in EtOH (25 mL), was treated dropwise with ethyl chloroformate (10.9 g, 100 mmol). The mixture was stirred at room temperature for 30 min and cooled overnight. The precipitate was collected by filtration to yield **5** (2.62 g, 81%): mp 146−147 °C; IR (KBr, cm^{-1}) 1718, 1658 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.32 (t, J = 7.1 Hz, 3H, CH_3), 1.57 (s, 9H, CH_3), 1.70−1.90 (m, 4H, CH_2), 2.55−2.80 (m, 4H, CH_2), 4.26 (q, J = 7.1 Hz, 2H, OCH_2), 10.53 (s, 1H, NH). Anal. ($C_{16}H_{23}NO_4S$) C, H, N; S: calcd, 9.89; found, 10.42.

***tert*-Butyl 2-[(Propoxycarbonyl)amino]-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (6).** This compound was prepared from **1** and propyl chloroformate (12.3 g, 100 mmol) in 95% yield: mp 138−139 °C; ^{1}H NMR ($CDCl_3$) δ 0.98 (t, J = 7.4 Hz, 3H, CH_3), 1.57 (s, 9H, CH_3), 1.65−1.83 (m, 6H, CH_2), 2.60−2.78 (m, 4H, CH_2), 4.17 (t, J = 6.7 Hz, 2H, OCH_2), 10.52 (s, 1H, NH). Anal. ($C_{17}H_{25}NO_4S$) C, H, N, S.

***tert*-Butyl 2-[(Isobutoxycarbonyl)amino]-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (7).** Compound **1** was reacted with isobutyl chloroformate (13.7 g, 100 mmol): yield 79%; mp 119−120 °C; ^{1}H NMR ($CDCl_3$) δ 0.97 (d, J = 6.7 Hz, 6H, CH_3), 1.57 (s, 9H, CH_3), 1.70−1.83 (m, 4H, CH_2), 1.92−2.06 (m, 1H, CH), 2.58−2.77 (m, 4H, CH_2), 3.99 (d, J = 6.7 Hz, 2H, OCH_2), 10.51 (s, 1H, NH). Anal. ($C_{18}H_{27}NO_4S$) C, H, N, S.

***tert*-Butyl 2-[(Ethoxycarbonyl)amino]-4,5-dimethylthiophene-3-carboxylate (8).** The reaction was performed using compound **2** (2.27 g, 10 mmol) and ethyl chloroformate to give **8** in 51% yield: mp 104−105 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ 1.33 (t, J = 7.1 Hz, 3H, CH_3), 1.58 (s, 9H, CH_3), 2.20 and 2.24 (each s, 3H, 4- and 5-CH_3), 4.26 (q, J = 7.1 Hz, 2H, OCH_2), 10.53 (s, 1H, NH). Anal. ($C_{14}H_{21}NO_4S$) C, H, N, S.

***tert*-Butyl 2-[(Propoxycarbonyl)amino]-4,5-dimethylthiophene-3-carboxylate (9).** Compound **2** was reacted with propyl chloroformate. The solution was concentrated to 15 mL and cooled to obtain **9** in 87% yield: mp 75−76 °C; ^{1}H NMR ($CDCl_3$) δ 0.98 (t, J = 7.4 Hz, 3H, CH_3), 1.58 (s, 9H, CH_3), 2.20 and 2.24 (each s, 3H, 4- and 5-CH_3), 1.65−1.70 (m, 2H, CH_2), 4.16 (t, J = 6.6 Hz, 2H, OCH_2), 10.51 (s, 1H, NH). Anal. ($C_{15}H_{23}NO_4S$) C, H, N, S.

***tert*-Butyl 2-[(Isobutoxycarbonyl)amino]-4,5-dimethylthiophene-3-carboxylate (10).** Compound **10** was prepared from **2** and isobutyl chloroformate in 43% yield: mp 94−95 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 0.97 (d, J = 6.8 Hz, 6H, CH_3), 1.59 (s, 9H, CH_3), 1.91−2.07 (m, 1H, CH), 2.20 and 2.24 (each s, 3H, 4- and 5-CH_3), 3.99 (d, J = 6.7 Hz, 2H, OCH_2), 10.50 (s, 1H, NH). Anal. ($C_{16}H_{25}NO_4S$) C, H, N, S.

***tert*-Butyl 2-[(Ethoxycarbonyl)amino]-4-isopropylthiophene-3-carboxylate (11).** The reaction was performed using compound **3** (2.41 g, 10 mmol) and ethyl chloroformate to give **11** in 56% yield: mp 55−56 °C; ^{1}H NMR ($CDCl_3$) δ 1.21 (d, J = 6.7 Hz, 6H, CH_3), 1.33 (t, J = 7.1 Hz, 3H, CH_3), 1.60 (s, 9H, CH_3), 3.45 (sept, J = 6.7 Hz, 1H, CH), 4.28 (q, J = 7.1 Hz, 2H, OCH_2), 6.37 (s, 1H, H-5), 10.70 (s, 1H, NH). Anal. ($C_{15}H_{23}NO_4S$) H, N, S; C: calcd, 57.49; found, 56.63.

***tert*-Butyl2-Isothiocyanato-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carboxylate (12).** A mixture prepared from thiophosgene (4.6 g, 40 mmol), $CaCO_3$ (4 g, 40 mmol), CH_2Cl_2 (20 mL), and H_2O (40 mL) was stirred at 0 °C. A solution of compound **1** (10.1 g, 40 mmol) in CH_2Cl_2 (70 mL) was added dropwise over a period of 40 min. The mixture was stirred for an additional 3 h at 0 °C. The organic layer was washed with water and dried (Na_2SO_4). After removal of the solvent, the residue was triturated with silica gel (2 g) and boiling hexane (2 × 125 mL). The combined filtrates were concentrated and cooled to obtain **12** (5.3 g, 45%) as yellow crystals: mp 79−80 °C; IR (KBr, cm^{-1}) 2136 (NCS), 1698 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.59 (s, 9H, CH_3), 1.66−1.83 (m, 4H, CH_2), 2.60−2.79 (m, 4H, CH_2). Anal. ($C_{14}H_{17}NO_2S_2$) C, H, N; S: calcd, 21.71; found, 20.84.

***tert*-Butyl 2-Isothiocyanato-4,5-dimethylthiophene-3-carboxylate (13).** By the foregoing procedure, compound **13** was prepared from **2** (9.1 g, 40 mmol) in 51% yield: mp 139−140 °C; IR (KBr, cm^{-1}) 2158 (NCS), 1698 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.61 (s, 9H, CH_3), 2.22 and 2.28 (each s, 3H, 4- and 5-CH_3). Anal. ($C_{12}H_{15}NO_2S_2$) C, H, N, S.

1,2,5,6,7,8-Hexahydro-2-thioxo-4*H*-[1]benzothieno[2,3-*d*][1,3]oxazin-4-one (14). Compound **12** (1.77 g, 6 mmol) was dissolved in CH_2Cl_2 (4 mL). Trifluoroacetic acid (2 mL) was added dropwise over 3 min at 0 °C under argon atmosphere. Stirring was continued for 2 min, and the precipitate was collected by filtration to yield **14** (400 mg, 28%): mp 159−161 °C (lit.[45] mp 152−154 °C); IR (KBr, cm^{-1}) 1725 (br, C=O); ^{1}H NMR (DMSO-d_6) δ 1.65−1.85 (m, 4H, CH_2), 2.60−2.76 (m, 4H, CH_2); MS (EI) *m*/*z* (rel intensity) 239 (M^+, 100). Anal. ($C_{10}H_9NO_2S_2$) C, H, N; S: calcd, 26.79; found, 27.29.

1,2-Dihydro-5,6-dimethyl-2-thioxo-4*H*-thieno[2,3-*d*][1,3]-oxazin-4-one (15). By the foregoing procedure, but using **13** (1.62 g, 6 mmol) in CH_2Cl_2 (24 mL), compound **15** was prepared in 56% yield: mp 225−230 °C (conversion 175−182 °C); IR (KBr, cm^{-1}) 1732 (br, C=O); ^{1}H NMR (DMSO-d_6) δ 2.22 and 2.29 (each s, 3H, 5- and 6-CH_3); MS (EI) *m*/*z* (rel intensity) 213 (M^+, 100). Anal. ($C_8H_7NO_2S_2$) C, H, N; S: calcd, 30.07; found, 30.88.

***tert*-Butyl 2-(3-Isopropylureido)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (16).** A mixture of **1** (5.1 g, 20 mmol), isopropyl isocyanate (3.9 g, 46 mmol), and pyridine (40 mL) was stirred at 50 °C for 19 h. It was poured onto a mixture of 3 M HCl (230 mL) and ice (46 g). The precipitate was collected by filtration, dried, and recrystallized from cyclohexane to yield **16** (4.9 g, 72%): mp 188−191 °C (lit.[34] mp 182 °C); ^{1}H NMR ($CDCl_3$) δ 1.21 (d, J = 6.5 Hz, 6H, CH_3), 1.55 (s, 9H, CH_3), 1.70−1.83 (m, 4H, CH_2), 2.55−2.75 (m, 4H, CH_2), 3.91−4.06 (m, 1H, CH), 4.66 (d, J = 7.7 Hz, 1H, NH), 10.61 (s, 1H, NH). Anal. ($C_{17}H_{26}N_2O_3S$) C, H, N; S: calcd, 9.47; found, 8.89.

***tert*-Butyl 2-(3-Isopropylureido)-4,5-dimethylthiophene-3-carboxylate (17).** According to the procedure outlined above, **2** (4.55 g, 20 mol) was used. The crude product was recrystallized twice from cyclohexane: yield 31%; mp 181−185 °C (lit.[34] mp 186 °C); ^{1}H NMR ($CDCl_3$) δ 1.21 (d, J = 6.5 Hz, 6H, CH_3), 1.58 (s, 9H, CH_3), 2.19 and 2.22 (each s, 3H, 4- and 5-CH_3), 3.95−4.10 (m, 1H, CH), 4.55 (d, J = 7.7 Hz, 1H, NH), 10.64 (s, 1H, NH). Anal. ($C_{15}H_{24}N_2O_3S$) C, H, N, S.

***tert*-Butyl 2-(3-Isopropylureido)thiophene-3-carboxylate (18).** The method was the same as for **16**, but using compound **4** (3.99 g, 20 mmol), and provided **18** in 88% yield: mp 207−208 °C (cyclohexane/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.22 (d, J = 6.5 Hz, 6H, CH_3), 1.56 (s, 9H, CH_3), 3.92−4.08 (m, 1H, CH), 4.76 (d, J = 7.4 Hz, 1H, NH), 6.55 (d, J = 5.8 Hz, 1H, H-5), 7.06 (d, J = 5.8 Hz, 1H, H-4), 10.30 (s, 1H, NH). Anal. ($C_{13}H_{20}N_2O_3S$) C, H, N, S.

Ethyl 2-(3-Cyclohexyl-3-methylthioureido)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (23): General Procedure for Thioureas 21−26. *N*-Methylcyclohexylamine

(2.55 g, 22.5 mmol) was added dropwise to a solution of compound **19**[46] (4 g, 15 mmol) in CH_2Cl_2 (15 mL). The mixture was stirred at room temperature for 5 h and acidified with 1 M HCl/EtOH, and the precipitate was collected by filtration to yield **23** (5.5 g, 96%): mp 156−157 °C (EtOH); IR (KBr, cm^{-1}) 1648 (C═O); ^{1}H NMR ($CDCl_3$) δ 1.05−1.90 (m, 14H, CH_2), 1.38 (t, J = 7.1 Hz, 3H, CH_3), 2.59−2.80 (m, 4H, CH_2), 3.18 (s, 3H, NCH_3), 4.32 (q, J = 7.1 Hz, 2H, OCH_2), 4.91−5.08 (m, 1H, CH), 12.25 (s, 1H, NH). Anal. ($C_{19}H_{28}N_2O_2S_2$) C, H, N, S.

Ethyl 2-(3,3-Diethylthioureido)-4,5-dimethylthiophene-3-carboxylate (25). This compound was prepared from isothiocyanate **20**[46] (3.6 g, 15 mmol) and diethylamine (1.65 g, 22.5 mmol) in 89% yield: mp 96−97 °C (EtOH/H_2O); ^{1}H NMR ($CDCl_3$) δ 1.32 (t, J = 7.1 Hz, 6H, CH_3), 1.39 (t, J = 7.1 Hz, 3H, CH_3), 2.24 (s, 6H, 4- and 5-CH_3), 3.81 (q, J = 7.1 Hz, 4H, NCH_2), 4.34 (q, J = 7.1 Hz, 2H, OCH_2), 12.31 (s, 1H, NH). Anal. ($C_{14}H_{22}N_2O_2S_2$) C, H, N, S.

Ethyl 2-(3-Cyclohexyl-3-methylthioureido)-4,5-dimethylthiophene-3-carboxylate (26). Compound **20** was reacted with *N*-methylcyclohexylamine. The crude product was recrystallized from EtOH to give **26** in 73% yield: mp 154−155 °C; ^{1}H NMR ($CDCl_3$) δ 1.05−1.89 (m, 10H, CH_2), 1.39 (t, J = 7.1 Hz, 3H, CH_3), 2.23 (s, 6H, 4- and 5-CH_3), 3.17 (s, 3H, NCH_3), 4.34 (q, J = 7.1 Hz, 2H, OCH_2), 4.91−5.07 (m, 1H, CH), 12.28 (s, 1H, NH). Anal. ($C_{17}H_{26}N_2O_2S_2$) C, H, N; S: calcd, 18.09; found, 18.79.

2-(*N*-Cyclohexyl-*N*-methylamino)-5,6,7,8-tetrahydro-4*H*-[1]benzothieno[2,3-*d*][1,3]thiazin-4-one (29): General Procedure for Thieno[2,3-*d*][1,3]thiazin-4-ones 27−32. A mixture of compound **23** (5.7 g, 15 mmol) and concentrated H_2SO_4 (30 mL) was kept at room temperature for 3 days and poured into ice−water (800 mL). The precipitate was collected by filtration, washed with H_2O, dried, and recrystallized from ethyl acetate/EtOH to yield **29** (2.6 g, 52%): mp 154−156 °C; IR (KBr, cm^{-1}) 1652 (C═O); ^{1}H NMR ($CDCl_3$) δ 1.04−1.90 (m, 14H, CH_2), 2.61−2.90 (m, 4H, CH_2), 3.03 (s, 3H, NCH_3), 4.12−4.28 (m, 1H, CH). Anal. ($C_{17}H_{22}N_2OS_2$) C, H, N, S.

2-(Diethylamino)-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]-thiazin-4-one (31). Compound **31** was prepared from **25** in 90% yield: mp 136−136.5 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.24 (t, J = 7.1 Hz, 6H, CH_3), 2.26 and 2.35 (each s, 3H, 5- and 6-CH_3), 3.57 (q, J = 7.1 Hz, 4H, NCH_2). Anal. ($C_{12}H_{16}N_2OS_2$) C, H, N, S.

2-(*N*-Cyclohexyl-*N*-methylamino)-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]thiazin-4-one (32). Compound **32** was prepared from **26** in 68% yield: mp 154−155 °C (ethyl acetate/EtOH); ^{1}H NMR ($CDCl_3$) δ 1.05−1.90 (m, 10H, CH_2), 2.29 and 2.35 (each s, 3H, 5- and 6-CH_3), 3.03 (s, 3H, NCH_3), 4.10−4.25 (m, 1H, CH). Anal. ($C_{15}H_{20}N_2OS_2$) H, N, S; C: calcd, 58.41; found, 57.98

2-(3,3-Diethylthioureido)-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carboxylic Acid (34): General Procedure for Thioureas 33−38. A mixture of compound **28**[35] (2.94 g, 10 mmol), 3 M NaOH (40 mL), and dioxane (80 mL) was refluxed for 80 min. After cooling, 1 M HCl (200 mL) was added, and the precipitate was collected by filtration to yield **34** (2.71 g, 87%): mp 144−144.5 °C; IR (KBr, cm^{-1}) 1630 (C═O); ^{1}H NMR ($CDCl_3$) δ 1.33 (t, J = 7.1 Hz, 6H, CH_3), 1.72−1.85 (m, 4H, CH_2), 2.60−2.82 (m, 4H, CH_2), 3.81 (q, J = 7.1 Hz, 4H, NCH_2), 12.11 (s, 1H, NH). Anal. ($C_{14}H_{20}N_2O_2S_2$) C, H, N; S: calcd, 20.52; found, 19.88.

2-(3-Cyclohexyl-3-methylthioureido)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic Acid (35). Compound **35** was prepared from **29**. The mixture was refluxed for 160 min: yield 66%; mp 158−160 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.04−1.99 (m, 14H, CH_2), 2.60−2.83 (m, 4H, CH_2), 3.23 (s, 3H, NCH_3), 4.63−4.79 (m, 1H, CH), 12.05 (s, 1H, NH). Anal. ($C_{17}H_{24}N_2O_2S_2$) C, H, N, S.

2-[(Morpholinothiocarbonyl)amino]-4,5-dimethylthiophene-3-carboxylic Acid (36). Compound **36** was prepared from **30**[35] in 63% yield: mp 172−173 °C (ethyl acetate/EtOH); ^{1}H NMR (DMSO-d_6) δ 2.20 (s, 6H, 4- and 5-CH_3), 3.68−3.72 (m, 4H, NCH_2), 3.84−3.88 (m, 4H, OCH_2), 12.60 (s, 1H, NH). Anal. ($C_{12}H_{16}N_2O_3S_2$) C, H, N, S.

2-(3,3-Diethylthioureido)-4,5-dimethylthiophene-3-carboxylic Acid (37). Compound **37** was prepared from **31** in 84% yield: mp 144−145 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.33 (t, J = 7.1 Hz, 6H, CH_3), 2.26 (s, 6H, 4- and 5-CH_3), 3.81 (q, J = 7.1 Hz, 4H, NCH_2), 12.15 (s, 1H, NH). Anal. ($C_{12}H_{18}N_2O_2S_2$) C, H, N; S: calcd, 22.39; found, 22.94.

2-(3-Cyclohexyl-3-methylthioureido)-4,5-dimethylthiophene-3-carboxylic Acid (38). Compound **38** was prepared from **32** in 80% yield: mp 142−144 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.05−1.86 (m, 10H, CH_2), 2.26 and 2.27 (each s, 3H, 4- and 5-CH_3), 3.23 (s, 3H, NCH_3), 12.07 (s 1H, NH). Anal. ($C_{15}H_{22}N_2O_2S_2$) C, H, N, S.

2-Ethoxy-5,6,7,8-tetrahydro-4*H*-[1]benzothieno[2,3-*d*]-[1,3]oxazin-4-one (39): General Procedure for 2-Alkoxythieno[2,3-*d*][1,3]oxazin-4-ones 39−45. A mixture of trifluoroacetic acid (5 mL) and trifluoroacetic anhydride (1.47 g, 7 mmol) was stirred at 0 °C under argon atmosphere. Compound **5** (1.63 g, 5 mmol) was added in portions over 30 min. Stirring was continued at room temperature for 90 min. The mixture was poured into chilled saturated aqueous $NaHCO_3$ (75 mL). The precipitate was collected by filtration to yield **39** (1.1 g, 88%): mp 119−120 °C (hexane); IR (KBr, cm^{-1}) 1775 (br, C═O); UV (EtOH) λ_{max} (nm) (log ϵ) 234 (4.26), 316 (3.77); ^{1}H NMR ($CDCl_3$) δ 1.43 (t, J = 7.1 Hz, 3H, CH_3), 1.77−1.92 (m, 4H, CH_2), 2.66−2.89 (m, 4H, CH_2), 4.47 (q, J = 7.1 Hz, 2H, OCH_2); ^{13}C NMR ($CDCl_3$) δ 14.05 (CH_3), 22.06 and 23.05 (C-6 and C-7), 24.84 and 25.04 (C-5 and C-8), 66.33 (OCH_2), 112.36 (C-4a), 130.72 and 131.41 (C-4b and C-8a), 154.44 (C-9a), 156.71 (C-2), 165.35 (C-4). Anal. ($C_{12}H_{13}NO_3S$) C, H, N, S.

2-Propoxy-5,6,7,8-tetrahydro-4*H*-[1]benzothieno[2,3-*d*]-[1,3]oxazin-4-one (40). Compound **40** was prepared from **6** and recrystallized from hexane: yield 32%; mp 70−71 °C; ^{1}H NMR ($CDCl_3$) δ 1.03 (t, J = 7.4 Hz, 3H, CH_3), 1.76−1.91 (m, 6H, CH_2), 2.68−2.89 (m, 4H, CH_2), 4.37 (t, J = 6.7 Hz, 2H, OCH_2). Anal. ($C_{13}H_{15}NO_3S$) C, H, N, S.

2-Isobutoxy-5,6,7,8-tetrahydro-4*H*-[1]benzothieno[2,3-*d*][1,3]oxazin-4-one (41). Compound **41** was prepared from **7** and recrystallized from hexane: yield 42%; mp 64−66 °C; ^{1}H NMR ($CDCl_3$) δ 1.02 (d, J = 6.9 Hz, 6H, CH_3), 1.78−1.91 (m, 4H, CH_2), 2.05−2.18 (m, 1H, CH), 2.67−2.89 (m, 4H, CH_2), 4.19 (d, J = 6.6 Hz, 2H, OCH_2). Anal. ($C_{14}H_{17}NO_3S$) C, H, N, S.

2-Ethoxy-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (42). Compound **42** was prepared from **8** in 93% yield: mp 96−97 °C (hexane); ^{1}H NMR ($CDCl_3$) δ 1.43 (t, J = 7.1 Hz, 3H, CH_3), 2.33 and 2.35 (each s, 3H, 5- and 6-CH_3), 4.46 (q, J = 7.1 Hz, 2H, OCH_2); ^{13}C NMR ($CDCl_3$) δ 12.73 and 12.79 (5- and 6-CH_3), 14.05 (CH_3), 66.30 (OCH_2), 113.36 (C-4a), 127.42 and 129.25 (C-5 and C-6), 154.73 (C-7a), 156.65 (C-2), 164.48 (C-4). Anal. ($C_{10}H_{11}NO_3S$) C, H, N; S: calcd, 14.23; found, 14.83.

2-Propoxy-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (43). Compound **43** was prepared from **9** in 92% yield: mp 34.5−35.5 °C (hexane); ^{1}H NMR ($CDCl_3$) δ 1.03 (t, J = 7.4 Hz, 3H, CH_3), 1.76−1.89 (m, 2H, CH_2), 2.34 and 2.36 (each s, 3H, 5- and 6-CH_3), 4.36 (t, J = 6.7 Hz, 2H, OCH_2). Anal. ($C_{11}H_{13}NO_3S$) C, H, N, S.

2-Isobutoxy-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (44). Compound **44** was prepared from **10** and recrystallized from hexane: yield 38%; mp 61−62 °C; ^{1}H NMR ($CDCl_3$) δ 1.02 (d, J = 6.8 Hz, 6H, CH_3), 2.04−2.19 (m, 1H, CH), 2.33 and 2.35 (each s, 3H, 5- and 6-CH_3), 4.17 (d, J = 6.6 Hz, 2H, OCH_2). Anal. ($C_{12}H_{15}NO_3S$) C, H, N, S.

2-Ethoxy-5-isopropyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (45). Compound **45** was prepared from **11** in 97% yield: mp 71−73 °C (hexane); ^{1}H NMR ($CDCl_3$) δ 1.28 (d, J = 6.8 Hz, 6H, CH_3), 1.45 (t, J = 7.1 Hz, 3H, CH_3), 3.48 (sept, J = 6.8 Hz, 1H, CH), 4.50 (q, J = 7.1 Hz, 2H, OCH_2), 6.65 (s, 1H, H-6). Anal. ($C_{11}H_{13}NO_3S$) C, H, N, S.

2-(Methylthio)-5,6,7,8-tetrahydro-4*H*-[1]benzothieno-[2,3-*d*][1,3]oxazin-4-one (46): General Procedure for

2-(Alkylthio)thieno[2,3-*d*][1,3]oxazin-4-ones 46−53. Methyl iodide (568 mg, 4 mmol) was added to a mixture of compound **14** (479 mg, 2 mmol), Na_2CO_3 (318 mg, 3 mmol), and acetone (20 mL). The mixture was stirred at room temperature for 6 h and poured into ice−water (60 mL). The precipitate was collected by filtration and washed with H_2O to yield **46** (430 mg, 85%): mp 157−159 °C (ether/hexane); IR (KBr, cm^{-1}) 1774 (C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 237 (4.19), 333 (4.05); ^{1}H NMR ($CDCl_3$) δ 1.78−1.92 (m, 4H, CH_2), 2.57 (s, 3H, SCH_3), 2.70−2.90 (m, 4H, CH_2); ^{13}C NMR ($CDCl_3$) δ 14.17 (SCH_3), 22.02 and 22.93 (C-6 and C-7), 24.92 and 25.14 (C-5 and C-8), 114.06 (C-4a), 131.77 and 132.47 (C-4b and C-8a), 154.73 (C-9a), 162.52 (C-4), 165.92 (C-2); MS (EI) *m*/*z* (rel intensity) 253 (M^+, 46), 206 (100). Anal. ($C_{11}H_{11}NO_2S_2$) C, H, N, S.

2-(Ethylthio)-5,6,7,8-tetrahydro-4*H*-[1]benzothieno[2,3-*d*][1,3]oxazin-4-one (47). Compound **47** was prepared from **14** and ethyl bromide (436 mg, 4 mmol) in 76% yield: mp 128−129 °C (ether/hexane); ^{1}H NMR ($CDCl_3$) δ 1.43 (t, J = 7.4 Hz, 3H, CH_3), 1.78−1.93 (m, 4H, CH_2), 2.70−2.90 (m, 4H, CH_2), 3.15 (q, J = 7.4 Hz, 2H, SCH_2); MS (EI) *m*/*z* (rel intensity) 267 (M^+, 21), 206 (100). Anal. ($C_{12}H_{13}NO_2S_2$) C, H, N, S.

2-(Benzylthio)-5,6,7,8-tetrahydro-4*H*-[1]benzothieno-[2,3-*d*][1,3]oxazin-4-one (48). Compound **14** was reacted with benzyl bromide (684 mg, 4 mmol) to give **48** in 94% yield: mp 115−116 °C (ether/hexane); ^{1}H NMR ($CDCl_3$) δ 1.78−1.94 (m, 4H, CH_2), 2.70−2.90 (m, 4H, CH_2), 4.38 (s, 2H, SCH_2), 7.26−7.44 (m, 5 ArH); MS (EI) *m*/*z* (rel intensity) 329 (M^+, 10), 206 (100). Anal. ($C_{17}H_{15}NO_2S_2$) C, H, N; S: calcd, 19.46; found, 18.75.

2-[[(Methoxycarbonyl)methyl]thio]-5,6,7,8-tetrahydro-4*H*-[1]benzothieno[2,3-*d*][1,3]oxazin-4-one (49). Compound **49** was prepared from **14** and methyl bromoacetate (612 mg, 4 mmol) and recrystallized from ether/hexane: yield 36%; mp 147−149 °C; ^{1}H NMR ($CDCl_3$) δ 1.78−1.92 (m, 4H, CH_2), 2.70−2.90 (m, 4H, CH_2), 3.78 (s, 3H, CH_3), 3.94 (s, 2H, SCH_2); ^{13}C NMR ($CDCl_3$) δ 21.97 and 22.88 (C-6 and C-7), 24.92 and 25.08 (C-5 and C-8), 33.40 (S-CH_2), 53.04 (CH_3), 114.27 (C-4a), 131.86 and 133.04 (C-4b and C-8a), 154.24 (C-9a), 161.86 (C-4), 163.59 (C-2), 168.25 (CO_2CH_3); MS (EI) *m*/*z* (rel intensity) 311 (M^+, 23), 206 (100). Anal. ($C_{13}H_{13}NO_4S_2$) C, H, N, S.

2-(Methylthio)-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (50). Compound **50** was prepared from **15** (427 mg, 2 mmol) and methyl iodide in 97% yield: mp 120−122 °C (ether/hexane); ^{1}H NMR ($CDCl_3$) δ 2.37 (s, 6H, 5- and 6-CH_3), 2.57 (s, 3H, SCH_3); ^{13}C NMR ($CDCl_3$) δ 12.85 and 12.91 (5-CH_3 and 6-CH_3), 14.15 (SCH_3), 115.03 (C-4a), 129.21 and 129.66 (C-5 and C-6), 154.99 (C-7a), 161.63 (C-4), 165.84 (C-2); MS (EI) *m*/*z* (rel intensity) 227 (22, M^+), 180 (100). Anal. ($C_9H_9NO_2S_2$) H, N, S; C: calcd, 47.56; found, 48.01.

2-(Ethylthio)-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (51). Compound **15** was reacted with ethyl bromide to give **51** in 85% yield: mp 65−66 °C (ether/hexane); ^{1}H NMR ($CDCl_3$) δ 1.42 (t, J = 7.4 Hz, 3H, CH_3), 2.35 (s, 6H, 5- and 6-CH_3), 3.14 (q, J = 7.4 Hz, 2H, SCH_2); MS (EI) *m*/*z* (rel intensity) 241 (M^+, 49), 180 (100). Anal. ($C_{10}H_{11}NO_2S_2$) C, H, N, S.

2-(Benzylthio)-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (52). Compound **52** was prepared from **15** and benzyl bromide in 97% yield: mp 77.5−78.5 °C (ether/hexane); ^{1}H NMR ($CDCl_3$) δ 2.37 (s, 6H, 5- and 6-CH_3), 4.38 (s, 2H, SCH_2), 7.26−7.44 (m, 5 ArH); MS (EI) *m*/*z* (rel intensity) 303 (M^+, 13), 180 (100). Anal. ($C_{15}H_{13}NO_2S_2$) H, N, S; C: calcd, 59.36; found, 58.64.

2-[[(Methoxycarbonyl)methyl]thio]-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (53). Compound **53** was prepared from **15** and methyl bromoacetate and recrystallized from ether/hexane: yield 67%; mp 84−84.5 °C; ^{1}H NMR ($CDCl_3$) δ 2.36 (s, 6H, 5- and 6-CH_3), 3.79 (s, 3H, OCH_3), 3.94 (s, 2H, SCH_2); ^{13}C NMR ($CDCl_3$) δ 12.81 and 12.94 (5- and 6-CH_3), 33.37 (SCH_2), 53.04 (OCH_3), 115.27 (C-4a), 129.78 (C-5 and C-6), 154.51 (C-7a), 161.00 (C-4), 163.52 (C-2), 168.25 (CO_2-CH_3); MS (EI) *m*/*z* (rel intensity) 285 (M^+, 9), 180 (100). Anal. ($C_{11}H_{11}NO_4S_2$) C, H, N; S: calcd, 22.47; found, 21.80.

2-(Isopropylamino)-5,6,7,8-tetrahydro-4*H*-[1]benzothieno[2,3-*d*][1,3]oxazin-4-one (54). A mixture of trifluoroacetic acid (5 mL) and trifluoroacetic anhydride (1.47 g, 7 mmol) was stirred at 0 °C under argon atmosphere. Compound **16** (1.69 g, 5 mmol) was added in portions over 30 min. Stirring was continued at 0 °C for 30 min and at room temperature overnight. The mixture was poured into chilled saturated aqueous $NaHCO_3$ (75 mL). The precipitate was collected by filtration to yield **54** (1.29 g, 98%): mp 208−210 °C (ethyl acetate/hexane) (lit.[34] mp 203 °C); IR (KBr, cm^{-1}) 1732 (C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 265 (3.87), 337 (4.01); ^{1}H NMR ($CDCl_3$) δ 1.27 (d, J = 6.5 Hz, 6H, CH_3), 1.75−1.92 (m, 4H, CH_2), 2.64−2.84 (m, 4H, CH_2), 4.03−4.15 (m, 1H, CH), 4.92 (d, J = 7.1 Hz, 1H, NH). Anal. ($C_{13}H_{16}N_2O_2S$) H, N, S; C: calcd, 59.07; found, 58.46.

2-(Isopropylamino)-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]-oxazin-4-one (55). By the foregoing procedure, but using compound **17** (1.56 g, 5 mmol), **55** was prepared in 92% yield: mp 200−202 °C (ethyl acetate/hexane) (lit.[34] mp 200 °C dec); ^{1}H NMR ($CDCl_3$) δ 1.26 (d, J = 6.4 Hz, 6H, CH_3), 2.28 and 2.32 (each s, 3H, 5- and 6-CH_3), 4.00−4.16 (m, 1H, CH), 4.98 (d, J = 6.7 Hz, 1H, NH). Anal. ($C_{11}H_{14}N_2O_2S$) C, H, N, S.

2-(Isopropylamino)-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (56). This compound was synthesized from **18** (1.42 g, 5 mmol) according to the procedure followed in the preparation of **54**. Recrystallization from hexane provided **56** in 65% yield: mp 164−167 °C; ^{1}H NMR ($CDCl_3$) δ 1.29 (d, J = 6.5 Hz, 6H, CH_3), 4.05−4.19 (m, 1H, CH), 4.97 (br s, 1H, NH), 6.79 (d, J = 5.8 Hz, 1H, H-6), 7.21 (d, J = 5.8 Hz, 1H, H-5); MS (EI) *m*/*z* (rel intensity) 210 (M^+, 83), 152 (100). Anal. ($C_9H_{10}N_2O_2S\cdot0.5H_2O$) C, H, N, S.

2-Morpholino-5,6,7,8-tetrahydro-4*H*-[1]benzothieno-[2,3-*d*][1,3]oxazin-4-one (57): General Procedure for 2-*sec*-Aminothieno[2,3-*d*][1,3]oxazin-4-ones 57−62. A mixture of compound **33**[35] (816 mg, 2.5 mmol), yellow HgO (866 mg, 4 mmol), and CH_2Cl_2 (50 mL) was stirred at room temperature for 48 h. Silica gel (1.8 g) was added, and the mixture was stirred for 2 min. The inorganic material was filtered from the solution and washed with CH_2Cl_2 (25 mL). The solvent was removed in vacuo to yield **57** (592 mg, 81%): mp 205−207 °C (ethyl acetate/hexane); IR (KBr, cm^{-1}) 1768 (br, C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 266 (3.92), 340 (4.07); ^{1}H NMR ($CDCl_3$) δ 1.76−1.90 (m, 4H, CH_2), 2.62−2.84 (m, 4H, CH_2), 3.69−3.79 (m, 8H, NCH_2 and OCH_2). Anal. ($C_{14}H_{16}N_2O_3S$) C, H, N; S: calcd, 10.97; found, 11.53.

2-(Diethylamino)-5,6,7,8-tetrahydro-4*H*-[1]benzothieno-[2,3-*d*][1,3]oxazin-4-one (58). Compound **58** was prepared from **34** in 94% yield: mp 95−95.5 °C (ethyl acetate/hexane); ^{1}H NMR ($CDCl_3$) δ 1.23 (t, J = 7.1 Hz, 6H, CH_3), 1.76−1.89 (m, 4H, CH_2), 2.59−2.83 (m, 4H, CH_2), 3.54 (q, J = 7.1 Hz, 4H, NCH_2). Anal. ($C_{14}H_{18}N_2O_2S$) C, N, S; H: calcd, 6.52; found, 7.12.

2-(*N*-Cyclohexyl-*N*-methylamino)-5,6,7,8-tetrahydro-4*H*-[1]benzothieno[2,3-*d*][1,3]oxazin-4-one (59). Compound **59** was synthesized from **35** in 92% yield: mp 182−183 °C (ethyl acetate/hexane); ^{1}H NMR ($CDCl_3$) δ 1.05−1.90 (m, 14H, CH_2), 2.60−2.84 (m, 4H, CH_2), 2.99 (s, 3H, NCH_3), 4.22−4.29 (m, 1H, CH). Anal. ($C_{17}H_{22}N_2O_2S$) C, H, N, S.

2-Morpholino-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (60). Compound **60** was prepared from **36** in 81% yield: mp 194−195 °C (ethyl acetate/hexane); ^{1}H NMR ($CDCl_3$) δ 2.29 and 2.32 (each s, 3H, 5- and 6-CH_3), 3.68−3.81 (m, 8H, NCH_2 and OCH_2). Anal. ($C_{12}H_{14}N_2O_3S$) C, H, N, S.

2-(Diethylamino)-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]-oxazin-4-one (61). This compound was prepared from **37** in 93% yield: mp 95.5−96 °C (ethyl acetate/hexane); ^{1}H NMR ($CDCl_3$) δ 1.24 (t, J = 7.1 Hz, 6H, CH_3), 2.27 and 2.31 (each s, 3H, 5- and 6-CH_3), 3.54 (q, J = 7.1 Hz, 4H, NCH_2). Anal. ($C_{12}H_{16}N_2O_2S$) C, H, N, S.

2-(*N*-Cyclohexyl-*N*-methylamino)-5,6-dimethyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (62). Compound **62** was prepared from **38** in 79% yield: mp 161−162 °C (ethyl acetate/

hexane); ^{1}H NMR ($CDCl_3$) δ 1.04−1.90 (m, 10H, CH_2), 2.27 and 2.31 (each s, 3H, 5- and 6-CH_3), 2.99 (s, 3H, NCH_3), 4.22−4.36 (m, 1H, CH). Anal. ($C_{15}H_{20}N_2O_2S$) C, H, N, S.

2-[(Ethoxycarbonyl)amino]-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carboxylic Acid (63). Compound **5** (1.63 g, 5 mmol) was added in portions over 20 min to trifluoroacetic acid (5 mL) at 0 °C. After being stirred for an additional 90 min at room temperature, the mixture was poured into ice−water (75 mL). The precipitate was collected by filtration and washed with H_2O to yield **63** (1.3 g, 97%): mp 210−212 °C; IR (KBr, cm^{-1}) 1728, 1636 (C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 226 (4.31), 306 (3.94); ^{1}H NMR (DMSO-d_6) δ 1.26 (t, J = 7.1 Hz, 3H, CH_3), 1.63−1.76 (m, 4H, CH_2), 2.50−2.71 (m, 4H, CH_2), 4.20 (q, J = 7.1 Hz, 2H, OCH_2), 10.57 (s, 1H, NH). Anal. ($C_{12}H_{15}NO_4S$) C, H, N, S.

2-(3-Isopropylureido)-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carboxylic Acid (64). This compound was prepared from **16** (1.69 g, 5 mmol) using the procedure outlined above. The crude product was dissolved in 1 M NaOH, and the filtrate was acidified with 3 M HCl. The precipitate was collected by filtration to yield **64** (900 mg, 64%): mp 182−184 °C; IR (KBr, cm^{-1}) 1644 (br, C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 228 (4.27), 313 (3.94); ^{1}H NMR (DMSO-d_6) δ 1.09 (d, J = 6.4 Hz, 6H, CH_3), 1.60−1.78 (m, 4H, CH_2), 2.45−2.67 (m, 4H, CH_2), 3.64−3.81 (m, 1H, CH), 7.69 (d, J = 7.1 Hz, 1H, NH), 10.26 (s, 1H, NH). Anal. ($C_{13}H_{18}N_2O_3S$) C, H, N, S.

3-Isopropyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidine-2,4(1*H*,3*H*)-dione (65). Compound **54** (100 mg, 0.38 mmol) was refluxed in 1 M ethanolic sodium ethoxide (5 mL) for 60 min. The mixture was poured into 1 M HCl (10 mL), and the precipitate was collected by filtration to yield **65** (80 mg, 80%): mp 233−235 °C; IR (KBr, cm^{-1}) 1710, 1646 (C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 233 (4.43), 297 (3.60); ^{1}H NMR ($CDCl_3$) δ 1.52 (d, J = 6.9 Hz, 6H, CH_3), 1.70−1.90 (m, 4H, CH_2), 2.60−2.96 (m, 4H, CH_2), 5.28 (sept, J = 6.9 Hz, 1H, CH), 11.14 (s, 1H, NH). Anal. ($C_{13}H_{16}N_2O_2S$) C, H, N, S.

2-(3,3-Diethylureido)-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carboxylic Acid (66). A mixture prepared from compound **58** (100 mg, 0.36 mmol), 1.5 M NaOH (10 mL), and acetone (5 mL) was refluxed for 5 min. After being cooled, the mixture was filtrated into 3 M HCl (10 mL), and the precipitate was collected by filtration to yield **66** (90 mg, 77%): mp 168−169 °C; IR (KBr, cm^{-1}) 1664, 1628 (C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 228 (4.40), 314 (4.03); ^{1}H NMR ($CDCl_3$) δ 1.25 (t, J = 7.1 Hz, 6H, CH_3), 1.70−1.86 (m, 4H, CH_2), 2.55−2.75 (m, 4H, CH_2), 3.43 (q, J = 7.1 Hz, 4H, NCH_2), 10.91 (s, 1H, NH). Anal. ($C_{14}H_{20}N_2O_3S\cdot1.5H_2O$) C, N, S; H: calcd, 7.17; found, 6.60.

Determination of the Kinetic Parameters of the Alkaline Hydrolysis. Alkaline hydrolysis was followed spectrophotometrically at a fixed wavelength (330−355 nm) by monitoring the disappearance of the thienooxazinones in 50 mM CAPS, pH 10.5 or 11.5, at 25 °C. Stock solutions of the compounds were prepared in DMSO; the final inhibitor concentration was 10−25 μM, and the final DMSO concentration was 5%. Curves were analyzed as first-order reactions. The hydrolysis of compounds **46**−**48**, **50**−**52**, **57**, **58**, and **60**−**62** at pH 10.5 was analyzed by HPLC. The reverse-phase HPLC equipment and conditions described under General Methods and Materials were utilized. Inhibitor solutions were prepared as outlined above. The reaction was followed over 24 h at 25 °C. Aliquots were injected onto the HPLC every 3 h, and the absorbance was measured at 240 nm. The disappearance of the compounds was monitored by measuring peak areas. Values were fitted to a first-order equation.

HLE Inhibition Assay. HLE inhibition by compounds **39**−**62** was assayed by the progress curve method at 25 °C. Assay buffer was 50 mM sodium phosphate, 500 mM NaCl, pH 7.8. A stock solution of MeOSuc-Ala-Ala-Pro-Val-pNA (20 mM in DMSO) was diluted with assay buffer. An enzyme stock solution (50 μg/mL in 100 mM sodium acetate buffer, pH 5.5) was freshly diluted with assay buffer. At least five different inhibitor concentrations were used, with the exception of compounds **57** and **59**−**62**, which were analyzed at a single concentration (10 μM) due to limits of solubility. Progress curves were fitted, and data were analyzed as described above.

In the spectrophotometric assay, inhibitors, dissolved in DMSO (5 μL), were added into a cuvette containing 845 μL of assay buffer and 100 μL of MeOSuc-Ala-Ala-Pro-Val-pNA (final concentration 200 μM). After thermal equilibration, the reaction was initiated by addition of 50 μL of HLE solution. Progress curves were monitored at 405 nm over 30−75 min.

HLE inhibition by compounds **46**, **47**, and **51** was assayed fluorimetrically, using Suc-Ala-Ala-Pro-Val-AMC (final concentration 125 μM) as the substrate. Reactions were initiated by addition of HLE, and the progress curves were monitored (λ_{ex} 340 nm, λ_{em} 460 nm) over 4−5 min.

Product Analysis. The reaction of HLE with compounds **39**, **46**−**48**, **50**, **51**, **54**, and **58** was analyzed by HPLC using conditions described under General Methods and Materials. Inhibitor solution (15 μL) (2 mM stock in DMSO) was added to 255 μL of assay buffer. The reactions were initiated by the addition of 30 μL of HLE solution (1 μg/mL). These mixtures were incubated at 25 °C for 16 h, during which time 25-μL aliquots were injected onto the HPLC every 4 h. The absorbance was monitored at 240 nm. As a control, the chromatograms of the intact compounds were recorded in the absence of HLE. For comparison, authentic samples of **63**−**66** were analyzed.

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