2-(Diethylamino)thieno[1,3]oxazin-4-ones as Stable Inhibitors of Human Leukocyte Elastase

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A series of 2-(diethylamino)thieno[1,3]oxazin-4-ones was synthesized and evaluated in vitro for inhibitory activity toward human leukocyte elastase (HLE). The Gewald thiophene synthesis was utilized to obtain several ethyl 2-aminothiophene-3-carboxylates. These precursors were subjected to a five-step route to obtain thieno[2,3-*d*][1,3]oxazin-4-ones bearing various substituents at positions 5 and 6. Both thieno[2,3-*d*] and thieno[3,2-*d*] fused oxazin-4-ones possess extraordinary chemical stability, which was expressed as rate constants of the alkaline hydrolysis. The kinetic parameters of the HLE inhibition were determined. The most potent compound, 2-(diethylamino)-4*H*-[1]benzothieno[2,3-*d*][1,3]oxazin-4-one, exhibited a K_i value of 5.8 nM. 2-(Diethylamino)thieno[1,3]oxazin-4-ones act as acyl-enzyme inhibitors of HLE, similar to the inhibition of serine proteases by 4*H*-3,1-benzoxazin-4-ones. The isosteric benzene−thiophene replacement accounts for an enhanced stability of the acyl-enzyme intermediates.

Introduction

Human leukocyte elastase (HLE, EC 3.4.21.37), a potent serine proteinase, is capable of degradating a variety of structural proteins, including elastin, collagens, laminin, fibronectin, and cartilage proteoglycans, as well as immunoglobulins and complement components, and can indirectly favor the breakdown of matrix proteins by proteolytic activation of matrix metalloproteinases.[1] HLE is stored in the azurophilic granules of polymorphonuclear leukocytes and is released in response to inflammatory stimuli. Under normal circumstances, the proteolytic activity of HLE is effectively controlled by its natural inhibitors such as α_1-proteinase inhibitor, α_2-macroglobulin, human mucus proteinase inhibitor, and elafin. An imbalance between free HLE and its endogenous inhibitors may result in several pathological states. HLE is thought to play a role in various disorders including pulmonary emphysema, cystic fibrosis, chronic bronchitis, adult respiratory distress syndrome, rheumatoid arthritis, and periodontitis.[1,2]

Several strategies have been pursued for the development of HLE inhibition[3] and various types of inhibitors have been reported during the last years, e.g., anionic oligomers and polymers,[4] hydrazinopeptides,[5] or anthraquinones.[6] Most of the potent, low molecular weight inhibitors form covalent adducts with the active-site serine. Examples include electrophilic ketone-based inhibitors and acylating agents. Electrophilic ketones, such as peptidyltrifluoromethyl or pentafluoroethyl ketones,[7] are assumed to act as transition-state analogue inhibitors. Acylating agents react with the active-site serine in a substratelike manner by eliminating a leaving group to form a stable acyl-enzyme.

The inactivation by mechanism-based inhibitors (suicide substrates) is assumed to result from the unmasking of a latent reactive function in the acyl-enzyme intermediate. This function reacts with an available nucleophilic amino acid in the active site to irreversibly inactivate the enzyme. A variety of heterocyclic structures have been described as suicide substrates of HLE. They are usually activated enzymatically by an esterolytic reaction, as for halo enol and ynenol lactones[8] and isocoumarins,[9] or by an amidolytic reaction, as for cephem sulfones,[10] monocyclic β-lactams,[11] sulfonyloxy phthalimides,[12] and succinimides,[13] 1,2,5-thiadiazolidin-3-ones,[14] and benzisothiazolones.[15] With other types of mechanism-based inhibitors, the acylation reaction leads to stable acyl-enzymes, but a modification reaction does not occur. The resulting acyl-enzymes, which have the potential for further conversion to products of the enzymatic turnover, may be trapped in potential energy wells.[16] For such acyl-enzyme inhibitors (alternate substrate 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 of several mammalian serine proteases such as HLE,[17−23] porcine pancreatic elastase,[24] cathepsin G,[25] proteinase 3,[22] thrombin,[26] C1r serine protease of the complement system,[27−29] and chymotrypsin.[30−33] Inhibition of the viral serine proteases HSV-1 protease[34] and human cytomegalovirus protease[35] by benzoxazinones has also been reported.

Efforts in the design of heterocyclic HLE inhibitors have focused on developing compounds that would react with the enzyme after binding in the active site but would be sufficiently stable to hydrolysis by nonspecific nucleophiles. Krantz et al.[19] have examined the effect of electron-donating groups on hydrolytic stability in a series of benzoxazinone HLE inhibitors. Another strategy to improve the chemical stability is the replacement

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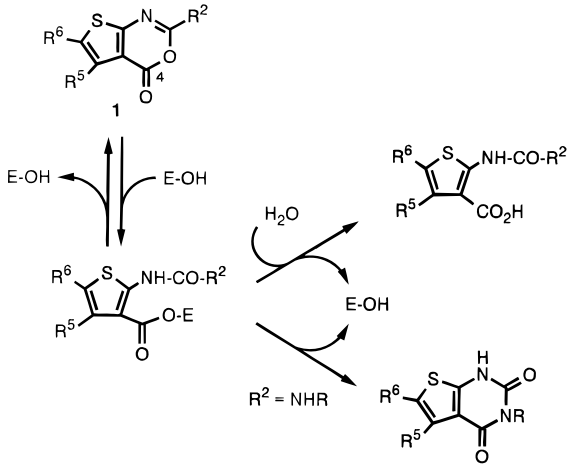
Scheme 1. Reaction of Thieno[2,3-*d*][1,3]oxazin-4-ones with HLE: Formation of Acyl-Enzymes and Possible Ways of Deacylation



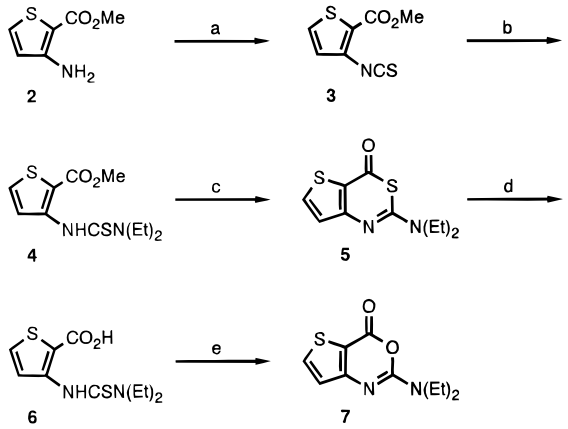
of the benzene ring in benzoxazinones by an isosteric thiophene unit. Recently, the inhibitory efficacy of thieno[2,3-*d*][1,3]oxazin-4-ones **1** (Scheme 1) toward HLE has been demonstrated.[36] These heterocycles act as acyl-enzyme inhibitors, similar to benzoxazinones: Nucleophilic attack of the active-site serine (E-OH) at the C-4 atom of the inhibitor and ring cleavage forms the corresponding acyl-enzymes that deacylate hydrolytically. As in the class of benzoxazinones,[19,28] substitution at position 2 is a sensitive parameter that affects chemical stability of thieno[2,3-*d*][1,3]oxazinones.[36] Introduction of an amino substituent at position 2 was advantageous to improve stability in both classes. With a NHR group at position 2, an intramolecular deacylation may occur (Scheme 1, R^2 = NHR). Thus, deacylation of benzoxazinone-derived acyl-enzymes was adversely accelerated by an intramolecular quinazoline cyclization.[18,25,32,33] Therefore, fused [1,3]-oxazin-4-ones bearing a disubstituted amino group at position 2[37] represent an attractive structure for the ongoing efforts in the design of inhibitors for HLE and other serine proteases.

On the basis of these considerations and previous results,[36] we have prepared a series of thieno[1,3]-oxazinones with a 2-diethylamino group as a fixed structural feature and various substituents at positions 5 and 6. The synthesis and kinetic data of the alkaline hydrolysis and in vitro inhibition of HLE are reported herein.

Results and Discussion

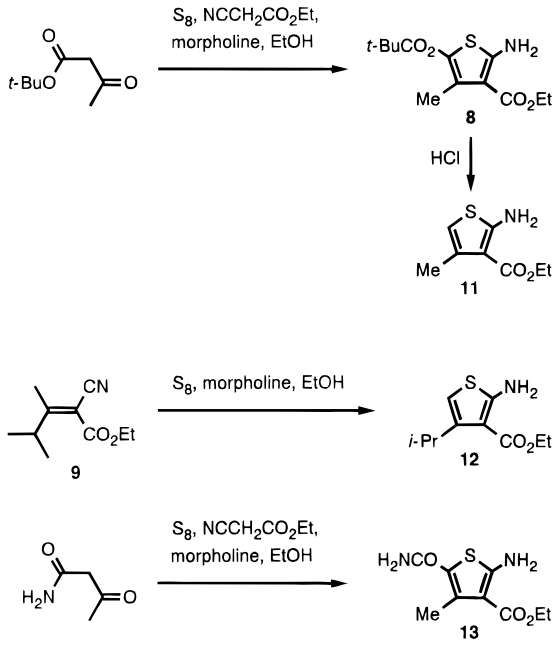
Synthesis. The preparation of 2-diethylaminothieno-[3,2-*d*][1,3]oxazin-4-one **7** (Scheme 2) was achieved following a route that has been established for the synthesis of thieno[2,3-*d*]oxazinone derivatives.[36] Conversion of the aminothiophene **2** to the isothiocyanate **3**, followed by treatment with diethylamine, gave the thiourea derivative **4**. Ring closure to the thiazinone **5** was then performed upon the action of concentrated sulfuric acid, according to a previously reported procedure.[38] To replace the thiazine sulfur atom in **5** by oxygen, the thiazinone ring was first cleaved hydrolytically upon the action of sodium hydroxide to afford the

Scheme 2[a]



[a] Reagents: (a) $CSCl_2$, $CaCO_3$, CH_2Cl_2, H_2O, 0 °C; (b) diethylamine, CH_2Cl_2, room temperature; (c) concd H_2SO_4, room temperature; (d) NaOH, dioxane−H_2O, reflux; (e) HgO, CH_2Cl_2, room temperature.

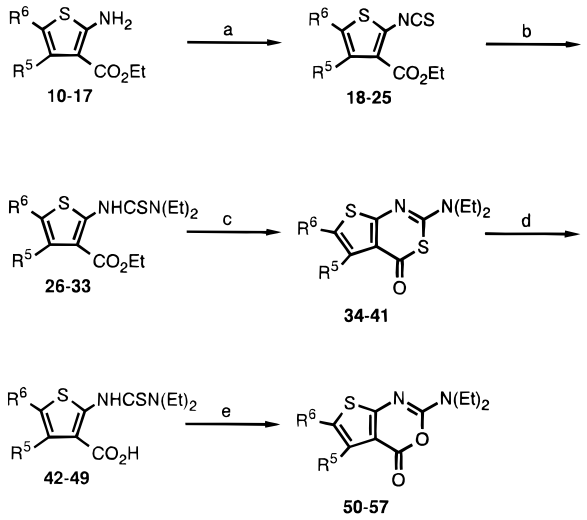
Scheme 3



carboxylic acid **6**. Subsequent treatment with yellow mercury(II) oxide furnished the final product **7** in 86% yield.

The Gewald thiophene synthesis[39,40] allows for a facile synthetic entry to the class of thieno[2,3-*d*][1,3]oxazin-4-ones. Ethyl 2-aminothiophene-3-carboxylates **10**−**17** (Scheme 4) were used as starting materials to prepare the final thieno[2,3-*d*][1,3]oxazin-4-ones **50**−**57**. The requisite aminothiophenes **14**−**16** were synthesized from the appropriate ketone, sulfur, and ethyl cyanoacetate in the presence of an organic base by an one-pot thiolation−heterocyclization reaction.[39] The 4,5-unsubstituted aminothiophene **10** was obtained by reacting the mercaptoacetaldehyde dimer (2,5-dihydroxy-1,4-dithiane) with ethyl cyanoacetate. To prepare the benzothiophene **17**,[41] the corresponding tetrahydrobenzo derivative was first acetylated to protect the amino group and then dehydrogenated with sulfur in diethyl phthalate, followed by alkaline deprotection. The synthesis of three further precursors is shown in Scheme 3. Compound **11** was synthesized by a facile

Scheme 4[a]



10, **18**, **26**, **34**, **42**, **50** $R^5 = R^6 = H$
11, **19**, **27**, **35**, **43**, **51** R^5 = Me, $R^6 = H$
12, **20**, **28**, **36**, **44**, **52** R^5 = *i*-Pr, $R^6 = H$
13, **21**, **29**, **37**, **45**, **53** R^5 = Me, $R^6 = CONH_2$
14, **22**, **30**, **38**, **46**, **54** $R^5R^6 = -(CH_2)_3-$
15, **23**, **31**, **39**, **47**, **55** $R^5R^6 = -(CH_2)_5-$
16, **24**, **32**, **40**, **48**, **56** $R^5R^6 = -CH(Me)CH_2CH_2CH_2-$
17, **25**, **33**, **41**, **49**, **57** $R^5R^6 = -CH=CH-CH=CH-$

[a] Reagents: (a) $CSCl_2$, $CaCO_3$, CH_2Cl_2, H_2O, 0 °C, or room temperature; (b) diethylamine, CH_2Cl_2, room temperature; (c) concd H_2SO_4, room temperature, or polyphosphoric acid, 170 °C; (d) NaOH, dioxane−H_2O, reflux; (e) HgO, CH_2Cl_2, room temperature, or reflux.

two-step route: The thiophene diester **8** was prepared from *tert*-butyl acetoacetate, sulfur, and ethyl cyanoacetate. Treatment of **8** with hydrochloric acid resulted in selective deesterification and decarboxylation, to obtain the desired methylthiophene **11**. To afford **12**, the Knoevenagel adduct of 3-methyl-2-butanone and ethyl cyanoacetate was prepared and subsequently treated with sulfur. To prepare the 5-carbamoyl thiophene **13**, acetoacetamide was subjected to the conditions of the Gewald reaction.

The synthesis of a series of thieno[2,3-*d*] fused [1,3]-oxazinon-4-ones **50**−**57** is outlined in Scheme 4. The route corresponds to the preparation of the thieno[3,2-*d*] fused [1,3]-oxazin-4-one **7** described above. Cyclocondensation of ethyl 2-thioureidothiophene-3-carboxylates **26**−**33** was performed either with concentrated sulfuric acid or upon treatment with polyphosphoric acid to furnish **34**−**41**. Ring cleavage of **34**−**41** was carried out in refluxing NaOH−dioxane to produce the thiophene-3-carboxylic acids **42**−**49**. A prolonged reaction time, compared to the standard methodology,[36] did improve the yields in certain cases but was less satisfactory to prepare **49** due to the formation of byproducts. Finally, compounds **42**−**49** were converted to thieno[2,3-*d*][1,3]-oxazin-4-ones **50**−**57** upon treatment with yellow mercury(II) oxide in dichloromethane. The ring closure is similar to known heterocyclizations in which carbodiimides react with nucleophiles.[42] Since the formation of an intermediate carbodiimide is not possible in the present transformation, the thiourea carbon, activated by the action of HgO, is believed to be attacked by the carboxylate oxygen. With the exception of **53**, the HgO-promoted cyclization was performed at room temperature to furnish the products in 69−83% yield, but more vigorous conditions were needed to obtain **53** in only 31% yield. This might be attributed to the electron-withdrawing effect of the carbamoyl group to reduce nucleophilicity of the thiourea sulfur, which is assumed to interact with mercury oxide.

Alkaline Hydrolysis. Stability of the compounds was determined spectrophotometrically in aqueous buffer, pH 11.25 at 25 °C. Alkaline hydrolysis rate constants of thieno[1,3]oxazin-4-ones **7** and **50**−**58** were determined at 335 nm by the disappearance of the long-wavelength ultraviolet chromophore (330−346 nm) that followed the first-order exponential decay for all compounds. The second-order rate constants, k_{OH^-}, were calculated and are outlined in Table 1. In addition, the alkaline hydrolysis of the reference 3,1-benzoxazin-4-one **59**, bearing the same 2-diethylamino substituent, was also determined. The k_{OH^-} value was in accordance with reported data for comparable amino-substituted benzoxazinones.[19] As described for compound **58**,[36] aqueous alkaline hydrolysis of the thieno[1,3]oxazinones **7** and **50**−**57** was assumed to proceed by hydroxide attack at C-4 to produce the corresponding ureidothiophenecarboxylic acids. An analogous hydrolytic ring opening for 3,1-benzoxazin-4-ones is well established.[19,33,37,43] Both the alkaline hydrolysis of fused 1,3-oxazin-4-ones and the process by which they inhibit HLE involve attack of a nucleophilic oxygen upon the lactone carbonyl carbon. Therefore, recognition of structural features promoting enzyme inhibition over chemical reactivity is crucial in the design of such mechanism-based inhibitors. The amino-substituted thieno[1,3]-oxazin-4-ones **7** and **50**−**58** possessed extraordinary low susceptibility to alkaline hydrolysis, with values $k_{OH^-} \leq 0.4\ M^{-1}\ s^{-1}$ (Table 1). A comparison of the benzoxazinone **59** with the two 5,6-unsubstituted thieno-[1,3]oxazinones **7** and **50** revealed the favorable effect of the benzene−thiophene replacement; the second-order rate constants of both thieno compounds were 50-fold lower. Except for **53**, 5,6-substituted thieno[2,3-*d*][1,3]oxazin-4-ones **51**−**58** were found to be even more stable.

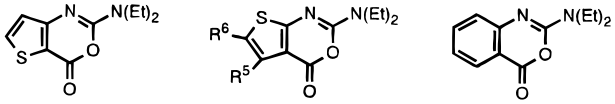
HLE Inhibiting Activity. HLE inhibition by thieno-[1,3]oxazin-4-ones **7** and **50**−**58** and the 3,1-benzoxazin-4-one **59** was assayed in the presence of Suc-Ala-Ala-Pro-Val-pNA as a chromogenic substrate. Progress curves were characterized by an initial exponential phase, followed by a linear steady-state turnover of the substrate, and could be analyzed by slow-binding kinetics.[44] Progress curves were fitted to

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

where v_s and v_i are the steady-state and the initial velocity and k_{obs} is the first-order rate constant for the approach to the steady state. The second-order rate constant, k_{on}, the first-order rate constant, k_{off}, and the steady-state inhibition constant, K_i, were obtained as described.[17,36]

In the present study on HLE inhibition by compounds **7** and **50**−**59**, except for compounds **51** and **53**, a preassociation complex did not accumulate at the inhibitor concentration range used. This was indicated by

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



7 50-58 59

compd	R^5	R^6	k_{OH^-} (M^{-1} s^{-1})	log k_{OH^-}	k_{on} (M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_i (nM)	pK_i
7			0.11	−0.95	330	0.43	130	6.88
50	H	H	0.12	−0.91	530	1.4	260	6.59
51	Me	H	0.073	−1.14	140	0.64	450[a]	6.35
52	*i*-Pr	H	0.054	−1.27	10000	1.3	13	7.88
53	Me	$CONH_2$	0.40	−0.40	4000	3.6	91[b]	7.04
54	$-(CH_2)_3-$		0.094	−1.03	3600	0.45	13	7.90
55	$-(CH_2)_5-$		0.038	−1.42	990	0.43	44	7.36
56	$-CH(Me)CH_2CH_2CH_2-$		0.039	−1.41	63	0.13	210	6.69
57	$-CH=CH-CH=CH-$		0.075	−1.13	9500	0.55	5.8	8.24
58	$-(CH_2)_4-$		0.032	−1.49	2500	0.32	13	7.90[c]
59			5.9	0.77	2600	7.9	310	6.52[d]

[a] K_d = 5.5 μM. [b] K_d = 620 nM. [c] Reference 36: pK_i = 7.79, obtained with MeOSuc-Ala-Ala-Pro-Val-pNA as substrate. [d] Reference 19: pK_i = 6.43, obtained with MeOSuc-Ala-Ala-Pro-Val-pNA as substrate.

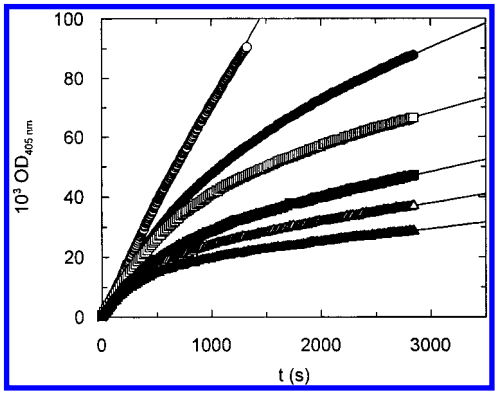


Figure 1. Slow-binding inhibition of HLE by compound **52** in 50 mM sodium phosphate and 500 mM NaCl, pH 7.8. Substrate was Suc-Ala-Ala-Pro-Val-pNA. Data were fitted to eq 1 to obtain the best-fit parameters for v_i, v_s, k_{obs}, and offset. (○) [I] = 0; (●) [I] = 100 nM; (□) [I] = 200 nM; (■) [I] = 300 nM; (△) [I] = 400 nM; (▲) [I] = 500 nM.

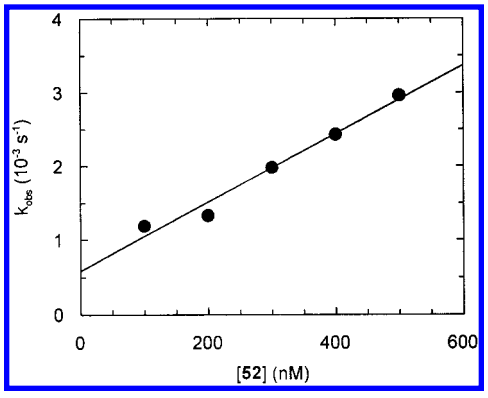


Figure 2. Plot of k_{obs} versus [I] for the inhibition of HLE by compound **52**. The values for k_{obs} were obtained from fits to the data shown in Figure 1. The slope corresponds to a value for $k_{on}/(1 + [S]/K_m)$ = 4640 ± 430 M^{-1} s^{-1}.

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]. As an example, the analysis of the HLE inhibition kinetics by the thieno[2,3-*d*][1,3]-oxazinone **52** is illustrated in Figures 1−3.

HLE inhibition by compounds **51** and **53** was characterized by initial velocities, v_i, that decreased with

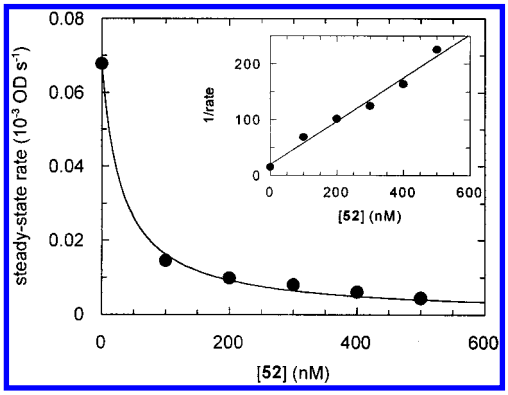


Figure 3. Plot of the steady-state rates versus [I] for the inhibition of HLE by compound **52**. The data were obtained from fits of the curves shown in Figure 1. The solid line was drawn by using the best-fit parameters from a fit according to an equation of a competitive inhibition, which gave K_i (1 + $[S]/K_m$) = 31.5 ± 2.6 nM. The inset is a Dixon plot to show the linearity.

increased inhibitor concentrations, whereas the k_{obs} values were identical. This indicated the accumulation of a preassociation complex under the experimental conditions used. The dissociation constants of a preassociation complex, K_d, and the final inhibition constants, K_i, as well as k_{on} and k_{off} values, were obtained as described.[36]

The kinetic data of the HLE inhibition by compounds **7** and **50**−**59** are outlined in Table 1. It was concluded from their kinetic behavior as well as from product analysis experiments[36] that the compounds act as acyl-enzyme inhibitors of HLE. In this case, the rate constants, k_{on} and k_{off}, reflect the acylation and deacylation step, respectively, and not the simple association−dissociation equilibrium of a competitive inhibition. Inhibition constants, K_i, are equal to the deacylation rate constant, k_{off}, divided by the acylation rate constant, k_{on}. Since a steady-state condition is reached, and as long as the inhibitor is not depleted due to its turnover by HLE, K_i values will reflect the dissociation of all enzyme-bound inhibitor complexes that accumulate in the steady state[17] and are used to express the potency of acyl-enzyme inhibitors of serine proteases.[18−20,22,25,36]

The present thieno[1,3]oxazinones **7** and **50**−**58** exhibited K_i values in the nanomolar range (Table 1). Considering their extraordinary stability in alkaline hydrolysis, 2-(diethylamino)thieno[1,3]oxazin-4-ones constitute an interesting class of HLE inhibitors. Studies on a series of 2-alkoxy-, 2-alkylthio-, and 2-aminothieno-[2,3-*d*][1,3]oxazin-4-ones[36] have demonstrated the mode of action as acyl-enzyme inhibitors of HLE (Scheme 1), similar to benzoxazinones. Likely, the inhibitors of the present series act in the same manner, by acylating the active-site serine to form an acyl-enzyme intermediate that deacylates slowly. The replacement of the benzene ring in 2-(diethylamino)-3,1-benzoxazinone by an unsubstituted thiophene (**59** versus **7** and **50**) resulted in around a 10-fold deceleration of both the acylation and deacylation step. These results support a general trend[36] in which the kinetic parameters of the HLE inhibition were affected by the isosteric benzene−thiophene replacement. The reduced susceptibility to be attacked by an oxygen nucleophile, as it was demonstrated in alkaline hydrolysis, is assumed to account for the diminished acylation rates. On the other hand, in the thiophene-derived acyl-enzymes, the ester bond, as a part of a heteroaromatic β-enamino ester,[45] is expectedly less reactive toward nucleophiles, leading to a reduced deacylation rate and an enhanced stability of the acyl-enzyme intermediates.

Substitutions at position 5 and 6 of the parent thieno-[2,3-*d*][1,3]oxazin-4-one **50** provided inhibitors with lower K_i values. Remarkably, replacement of the 5-methyl group by isopropyl (**51** versus **52**) strongly increased the acylation rate. A comparable effect has been reported in a series of highly potent benzisothiazolones:[15a,b] An isopropyl group in the *peri* position to the carbonyl of the scissile lactam bond led to rapid inactivation and a hydrophobic interaction with the S_1 pocket of HLE was proposed. The tetramethylene derivative **58**[36] was already part of a previous series of thieno compounds investigated as HLE inhibitors. The potency of **58** was retained by removing one methylene unit from the cycloaliphatic chain (**58** versus **54**), and was slightly diminished by extending to the pentamethylene bridge (**58** versus **55**). Introduction of a 5-methyl group was disadvantageous and resulted in a 40-fold decelaration of the acylation step [**58** versus (*R*,*S*)-**56**]. This might be attributed to a restricted conformation of the methyl group relative to the thieno-[2,3-*d*][1,3]oxazinone skeleton. When the C(7)−C(8) ethylene unit was removed to allow for a free rotation of the isopropyl group around the C−CH bond, a 150-fold increased k_{on} value was observed (**56** versus **52**). Annellation of a benzene ring in **57** provided the most potent inhibitor of the present series. Formally, in this compound, a thiophene unit is placed between the two rings of the benzoxazinone **59**. Introduction of a carbamoyl moiety at the 6-position in **53** resulted in an increased affinity of **53** to HLE as detected by an accumulation of a preassociation complex (K_d = 620 nM). However, although influenced by K_d,[36] the k_{on} value of **53** was in the range obtained for the potent compounds of the present series. Both acylation and deacylation rates might also be increased due to the electron-withdrawing effect of the carbamoyl substituent.

The thieno[1,3]thiazin-4-ones **5** (Scheme 2) and **34**−**41** (Scheme 4) were also evaluated as inhibitors of HLE. Due to limits of solubility, assays were performed only at a single inhibitor concentration (2 μM). For none of the compounds was enzyme inhibition detected, probably as a result of the further decreased reactivity of the sulfur analogues. As described,[33] replacement of the ring oxygen of 3,1-benzoxazin-4-ones by sulfur affected both the intrinsic stability and the parameters of the chymotrypsin inhibition. Resonance stabilization was assumed to account for the enhanced stability of the resulting 3,1-benzothiazin-4-ones. However, benzothiazinones were found to acylate chymotrypsin, but with much lower rate constants. Toward complement C1r protease, the oxygen−sulfur exchange in benzenesulfonamide-derived 3,1-benzoxazin-4-ones led to inactive compounds.[29] HLE inhibition with micromolar K_i values was reported for some 2-amino-substituted indolo[1,3]thiazin-4-ones.[46]

Conclusions

A series of thieno[1,3]oxazin-4-ones was prepared and evaluated in vitro as inhibitors of human leukocyte elastase. The present study demonstrates the versatility of the Gewald thiophene synthesis to provide ethyl 2-aminothiophene-3-carboxylates as precursors to thieno-[2,3-*d*][1,3]oxazin-4-ones with various substituents at positions 5 and 6. On the basis of the inhibitory efficacy of 3,1-benzoxazin-4-ones toward serine proteases, the title compounds were designed as a result of an isosteric benzene−thiophene replacement. Both thieno[2,3-*d*] and thieno[3,2-*d*] fused oxazin-4-ones exhibit remarkable chemical stability of the oxazinone ring as a result of the enhanced electron density at the thiophene carbons. A 2-diethylamino group provided further improvement of the stability, compared to 2-alkoxy- or 2-(alkylthio)-thieno[2,3-*d*][1,3]oxazin-4-ones.[36] From the data of the alkaline hydrolysis at 25 °C, pH 11.25, half-lives at pH 8 can be calculated, being in the range of 2−8 months, for the most potent inhibitors **52**, **54**, **57**, and **58**. The design of stable compounds is a special challenge in the development of covalently reacting inhibitors for serine proteases. It has been addressed by many authors[7a,10a,11a,15c,47] that hydrolytic stability is a prerequisite for stability in biological fluids.

In summary, 2-(diethylamino)thieno[1,3]oxazin-4-ones represent an attractive new class of acyl-enzyme inhibitors for human leukocyte elastase. Their acylation rates were moderate, but the acyl-enzyme intermediates were stable with half-lives of 1−6 h at 25 °C, pH 7.8, for the most potent compounds **52**, **54**, **57**, and **58**. Consequently, the consumption of a given inhibitor concentration by the enzyme will occur slowly. This appears as a favorable feature for acyl-enzyme inhibitors.

The strategy of the benzene−thiophene replacement was also followed to develop thiophene-derived inhibitors for viral serine proteases. Thieno[1,3]oxazin-4-ones with -CH(Me)NHCOR substitution at position 2 were found to strongly inhibit HSV-1, HSV-2, VZV, and CMV herpes proteases.[48] Thus, the thieno[1,3]oxazin-4-one systems are suitable scaffolds that might find further applicability for the design of nonpeptidic inhibitors of other serine proteases.

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}. Preparative column chromatography was performed on silica gel 60 (Merck) 70−230 mesh, with an ethyl acetate/hexane mixture (1:4). ^{13}C NMR spectra (75 MHz) and ^{1}H NMR spectra (300 MHz) were recorded on a Varian Gemini 300. ^{13}C NMR signals were assigned on the basis of $^{13}C/^{1}H$ 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 on a Varian MAT CH6 spectrometer. Spectrophotometric assays were done on a Perkin-Elmer Lambda 16 UV/VIS spectrophotometer. Elastase was prepared from human leukocytes and purified by affinity chromatography using an immobilized synthetic inhibitor.[49] The substrate Suc-Ala-Ala-Pro-Val-pNA was from Bachem, Heidelberg, Germany.

Methyl 3-aminothiophene-2-carboxylate (**2**) was purchased from Aldrich, Steinheim, Germany. 2-(Diethylamino)-5,6,7,8-tetrahydro-4*H*-[1]benzothieno[2,3-*d*][1,3]oxazin-4-one (**58**) was prepared as reported.[36] 2-(Diethylamino)-4*H*-3,1-benzoxazin-4-one (**59**)[19] was prepared as reported.[37]

Ethyl 2-Amino-4-methyl-5-(*tert*-butoxycarbonyl)thiophene-3-carboxylate (8). A mixture of *tert*-butyl acetoacetate (15.8 g, 100 mmol), ethyl cyanoacetate (11.3 g, 100 mmol), sulfur (3.5 g, 110 mmol), and EtOH (25 mL) was stirred at 45 °C. Morpholine (10 g, 115 mmol) was added dropwise over 15 min. The mixture was stirred at 60 °C for 5 h and filtered. The filtrate was diluted with water (50 mL) and cooled. The precipitate was collected by filtration, washed with 30% EtOH, and dried to obtain **8** (21.2 g, 74%): mp 116−117 °C (cyclohexane/hexane); IR (KBr, cm^{-1}) 1670, 1588 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.37 (t, J = 7.1 Hz, 3H), 1.53 (s, 9H), 2.67 (s, 3H), 4.31 (q, J = 7.1 Hz, 2H), 6.47 (s, 2H). Anal. ($C_{13}H_{19}NO_4S$) C, H, N, S.

Ethyl 2-Aminothiophene-3-carboxylate (10). Triethylamine (3.6 g, 50 mmol) was added dropwise over 10 min to a mixture of 2,5-dihydroxy-1,4-dithiane (7.6 g, 50 mmol), ethyl cyanoacetate (11.3 g, 100 mmol), and DMF (40 mL). The mixture was stirred at 45 °C for 30 min, diluted with 0.4 M acetic acid (200 mL), extracted with ether (4 × 40 mL), washed with water (2 × 40 mL), and dried (Na_2SO_4). After removal of the solvent, the residue was cooled and washed with cold hexane to obtain **10** (13.6 g, 80%): mp 47−48 °C (ethyl acetate/hexane) (lit.[50] mp 46−48 °C); ^{1}H NMR ($CDCl_3$) δ 1.33 (t, J = 7.1 Hz, 3H), 4.27 (q, J = 7.1 Hz, 2H), 6.17 (d, J = 5.8 Hz, 1H), 6.70 (d, J = 5.8 Hz, 1H), 5.86 (br s, 2H); ^{13}C NMR ($CDCl_3$) δ 14.53, 59.77, 106.91, 107.17, 125.93, 162.74, 165.50.

Ethyl 2-Amino-4-methylthiophene-3-carboxylate (11). A mixture of compound **8** (14.3 g, 50 mmol) and EtOH (100 mL) was treated with 1 M HCl (100 mL). It was refluxed for 1 h, cooled, and neutralized with 1 M NaOH. The mixture was cooled for 48 h and the precipitate was collected by filtration to obtain **11** (7.9 g, 85%): mp 76−78 °C (hexane); IR (KBr, cm^{-1}) 1646 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.36 (t, J = 7.1 Hz, 3H), 2.29 (s, 3H), 4.30 (q, J = 7.1 Hz, 2H), 5.84 (s, 1H), 6.02 (s, 2H). Anal. ($C_8H_{11}NO_2S$) H, N, S; C: calcd, 51.87; found, 51.45.

Ethyl 2-Amino-4-isopropylthiophene-3-carboxylate (12). A mixture of (*E*,*Z*)-2-cyano-3,4-dimethyl-2-pentenoic acid ethyl ester **9**,[51] (18.1 g, 100 mmol), sulfur (3.2 g, 100 mmol), and EtOH (25 mL) was stirred at 45 °C. Morpholine (8.7 g, 100 mmol) was added dropwise over 15 min. The mixture was stirred at 45 °C for 4 h, cooled, poured into 0.4 M acetic acid (400 mL), extracted with ether (4 × 60 mL), washed with water (2 × 60 mL), dried (Na_2SO_4), and evaporated under reduced pressure. The residue was purified by column chromatography to obtain **12** (7.9 g, 37%): mp 54−55 °C (cyclohexane); IR (KBr, cm^{-1}) 1650 (br, C=O); ^{1}H NMR ($CDCl_3$) δ 1.19 (d, J = 6.8 Hz, 6H), 1.36 (t, J = 7.1 Hz, 3H), 3.42 (septet, J = 6.8 Hz, 1H), 4.31 (q, J = 7.1 Hz, 2H), 5.89 (s, 1H), 6.06 (br s, 2H). Anal. ($C_{10}H_{15}NO_2S$) C, H, N, S.

Ethyl 2-Amino-5-carbamoyl-4-methylthiophene-3-carboxylate (13). A mixture of sulfur (2.24 g, 70 mmol), acetoacetamide (7.08 g, 70 mmol), ethyl cyanoacetate (7.9 g, 70 mmol), and EtOH (20 mL) was treated dropwise with morpholine (6.1 g, 70 mmol) at 45 °C over 15 min. After being stirred for 5 h at 45 °C, the precipitate was collected by filtration. The filtrate was diluted with H_2O to obtain an additional amount of product. The combined product was washed with 50% EtOH, dried, and recrystallized from ethyl acetate to obtain **13** (11 g, 69%): mp 181−182 °C; IR (KBr, cm^{-1}) 1652, 1638 (C=O); ^{1}H NMR (DMSO-d_6) δ 1.27 (t, J = 7.1 Hz, 3H), 2.47 (s, 3H), 4.20 (q, J = 7.1 Hz, 2H), 7.06 (s, 2H), 7.63 (s, 2H). Anal. ($C_9H_{12}N_2O_3S$) C, H, N; S, calcd, 14.05; found, 14.68.

(*R*,*S*)-Ethyl 2-Amino-4-methyl-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (16). A mixture of sulfur (6.4 g, 200 mmol), (*R*,*S*)-2-methylcyclohexanone (22.4 g, 200 mmol), ethyl cyanoacetate (22.6 g, 200 mmol), and EtOH (57 mL) was treated dropwise with morpholine (17.4 g, 200 mmol) at 45 °C over 15 min. The mixture was stirred for 5 h at 45 °C and 24 h at room temperature. Unreacted sulfur was removed by filtration, and the filtrate was evaporated under reduced pressure. Compound **16** (20.5 g, 43%) was obtained by column chromatography: mp 68−70 °C (MeOH) (lit.[52] mp 73 °C); IR (KBr, cm^{-1}) 1643 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.16 (d, J = 6.8 Hz, 3H), 1.35 (t, J = 7.1 Hz, 3H), 1.58−1.92 (m, 4H), 2.46−2.52 (m, 2H), 3.20−3.30 (m, 1H), 4.18−4.38 (m, 2H), 5.99 (s, 2H).

Ethyl 2-(Isothiocyanato)thiophene-3-carboxylate (18): General Procedure for Isothiocyanates 18−25. 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 **10** (6.84 g, 40 mmol) in CH_2Cl_2 (70 mL) was added dropwise over a period of 40 min. The mixture was stirred for 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 to 50 mL and cooled. The precipitate was collected by filtration to obtain **18** (2.5 g, 29%): mp 40−42 °C (hexane) (lit.[53] mp 43−44 °C); IR (KBr, cm^{-1}) 2143 (NCS), 1699 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.40 (t, J = 7.1 Hz, 3H), 4.37 (q, J = 7.1 Hz, 2H), 6.92 (d, J = 5.9 Hz, 1H), 7.29 (d, J = 5.5 Hz, 1H).

Ethyl 2-(Isothiocyanato)-4-methylthiophene-3-carboxylate (19). Yield 22%; mp 50.5−51 °C; IR (KBr, cm^{-1}) 2126 (NCS), 1694 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.42 (t, J = 7.1 Hz, 3H), 2.38 (s, 3H), 4.38 (q, J = 7.1 Hz, 2H), 6.59 (s, 1H). Anal. ($C_9H_9NO_2S_2$) C, H, N, S.

Ethyl 2-(Isothiocyanato)-4-isopropylthiophene-3-carboxylate (20). Compound **20** was prepared from **12**. The combined filtrates were evaporated under reduced pressure. The residue was purified by column chromatography to give **20** as an oil (1.53 g, 15%): IR (KBr, cm^{-1}) 2113 (NCS), 1716 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.21 (d, J = 6.8 Hz, 6H), 1.43 (t, J = 7.1 Hz, 3H), 3.50 (septet, J = 6.8 Hz, 1H), 4.34 (q, J = 7.1 Hz, 2H), 6.66 (s, 1H).

Ethyl 5-Carbamoyl-2-(isothiocyanato)-4-methylthiophene-3-carboxylate (21). Compound **13** was reacted with thiophosgene. The reaction mixture was stirred for 3 h at 0 °C and for an additional 48 h at room temperature. The precipitate was collected by filtration and thoroughly washed with H_2O to obtain **21** in 55% yield: mp 171−173 °C (CH_3CN); IR (KBr, cm^{-1}) 2122 (NCS), 1700, 1668 (C=O); ^{1}H NMR (DMSO-d_6) δ 1.34 (t, J = 7.1 Hz, 3H), 2.49 (s, 3H), 4.31 (q, J = 7.1 Hz, 2H), 7.72 (s, 2H). Anal. ($C_{10}H_{10}N_2O_3S_2$) C, H, N, S.

Ethyl 5,6-Dihydro-2-(isothiocyanato)-4*H*-cyclopenta[4,5]thiophene-3-carboxylate (22). Compound **22** was prepared from **14**[39] in 15% yield: mp 55−56 °C; IR (KBr, cm^{-1}) 2102 (NCS), 1710 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.39 (t, J = 7.1 Hz, 3H), 2.32−2.44 (m, 2H), 2.81−2.96 (m, 4H) 4.34 (q, J = 7.1 Hz, 2H). Anal. ($C_{11}H_{11}NO_2S_2$) C, H, N; S: calcd, 25.31; found, 24.24.

Ethyl 2-(Isothiocyanato)-5,6,7,8-tetrahydro-4*H*-cyclohepta[4,5]thiophene-3-carboxylate (23). Compound **23** was prepared from **15**.[39] The combined filtrates were evaporated under reduced pressure. The residue was purified by column chromatography to give **23** in 35% yield: mp 49−50 °C (hexane); IR (KBr, cm^{-1}) 2132 (NCS), 1708 (C=O); ^1H NMR ($CDCl_3$) δ 1.42 (t, J = 7.1 Hz, 3H), 1.56−1.67 (m, 4H), 1.81−1.91 (m, 2H), 2.69−2.74 (m, 2H), 2.93−2.98 (m, 2H), 4.36 (q, J = 7.1 Hz, 2H). Anal. ($C_{13}H_{15}NO_2S_2$) C, H, N; S, calcd, 22.79; found, 21.95.

(*R*,*S*)-Ethyl 2-(Isothiocyanato)-4-methyl-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (24). Compound **24** was prepared from **16** and purified by column chromatography to obtain an oil in 37% yield; IR (KBr, cm^{-1}) 2121 (NCS), 1716 (C=O); ^1H NMR ($CDCl_3$) δ 1.15 (d, J = 6.9 Hz, 3H), 1.41 (t, J = 7.1 Hz, 3H), 1.62−1.92 (m, 4H), 2.55−2.72 (m, 2H), 3.32−3.42 (m, 1H), 4.32−4.40 (m, 2H).

Ethyl 2-(Isothiocyanato)benzo[*b*]thiophene-3-carboxylate (25). Compound **17**[41] dissolved in CH_2Cl_2 (100 mL) was reacted with thiophosgene to obtain **25** in 45% yield. An analytical sample was purified by column chromatography: mp 70−71 °C; IR (KBr, cm^{-1}) 2130 (NCS), 1706 (C=O); ^1H NMR ($CDCl_3$) δ 1.49 (t, J = 7.1 Hz, 3H), 4.49 (q, J = 7.1 Hz, 2H), 7.41−7.49 (m, 2H), 7.66−7.70 and 8.44−8.49 (each m, 1H). Anal. ($C_{12}H_9NO_2S_2$) C, H, N, S.

Methyl 3-(3,3-Diethylthioureido)thiophene-2-carboxylate (4): General Procedure for Thioureas 4 and 26−33. Diethylamine (2.55 g, 22.5 mmol) was added dropwise to a solution of compound **3**[54] (2.36 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 **4** (4.0 g, 98%): mp 81−82 °C (EtOH/H_2O); IR (KBr, cm^{-1}) 1678 (C=O); ^1H NMR ($CDCl_3$) δ 1.36 (t, J = 7.1 Hz, 6H), 3.84 (q, J = 7.1 Hz, 4H), 3.88 (s, 3H), 7.43 (d, J = 5.6 Hz, 1H), 8.84 (d, J = 5.6 Hz, 1H), 10.55 (s, 1H). Anal. ($C_{11}H_{16}N_2O_2S_2$) C, H, N, S.

Ethyl 2-(3,3-Diethylthioureido)thiophene-3-carboxylate (26). Yield 70%; mp 80.5−82.5 °C (hexane); IR (KBr, cm^{-1}) 1667 (C=O); ^1H NMR ($CDCl_3$) δ 1.34 (t, J = 7.1 Hz, 6H), 1.36 (t, J = 7.1 Hz, 3H), 3.83 (q, J = 7.1 Hz, 4H), 4.34 (q, J = 7.1 Hz, 2H), 6.59 (d, J = 5.9 Hz, 1H), 7.22 (d, J = 5.9 Hz, 1H), 11.95 (s, 1H). Anal. ($C_{12}H_{18}N_2O_2S_2$) C, H, N, S.

Ethyl 2-(3,3-Diethylthioureido)-4-methylthiophene-3-carboxylate (27). Yield 90%; mp 114−116 °C; IR (KBr, cm^{-1}) 1657 (C=O); ^1H NMR ($CDCl_3$) δ 1.33 (t, J = 7.1 Hz, 6H), 1.40 (t, J = 7.1 Hz, 3H), 2.36 (s, 3H), 3.82 (q, J = 7.1 Hz, 4H), 4.36 (q, J = 7.1 Hz, 2H), 6.26 (s, 1H), 12.32 (s, 1H). Anal. ($C_{13}H_{20}N_2O_2S_2$) C, H, N, S.

Ethyl 2-(3,3-Diethylthioureido)-4-isopropylthiophene-3-carboxylate (28). Yield 94%; mp 67.5−69.5 °C; IR (KBr, cm^{-1}) 1653 (C=O); ^1H NMR ($CDCl_3$) δ 1.21 (d, J = 6.8 Hz, 6H), 1.33 (t, J = 7.1 Hz, 6H), 1.40 (t, J = 7.1 Hz, 3H), 3.49 (septet, J = 6.8 Hz, 1H), 3.82 (q, J = 7.1 Hz, 4H), 4.38 (q, J = 7.1 Hz, 2H), 6.32 (s, 1H), 12.43 (s, 1H). Anal. ($C_{15}H_{24}N_2O_2S_2$) C, H, N, S.

Ethyl 5-Carbamoyl-2-(3,3-diethylthioureido)-4-methylthiophene-3-carboxylate (29). Yield 80%; mp 206−207 °C (EtOH); IR (KBr, cm^{-1}) 1660 (br, C=O); ^1H NMR ($CDCl_3$) δ 1.33 (t, J = 7.1 Hz, 6H), 1.41 (t, J = 7.1 Hz, 3H), 2.70 (s, 3H), 3.77−3.85 (m, 4H), 4.38 (q, J = 7.1 Hz, 2H), 5.66 (s, 2H), 12.55 (s, 1H); MS (EI) m/z (rel intensity) 343 (53, M^+), 116 (100). Anal. ($C_{14}H_{21}N_3O_3S_2$) H, N, S; C, calcd, 48.96; found, 48.35.

Ethyl 2-(3,3-Diethylthioureido)-5,6-dihydro-4*H*-cyclopenta[4,5]thiophene-3-carboxylate (30). Yield 92%; mp 93−94 °C; IR (KBr, cm^{-1}) 1662 (C=O); ^1H NMR ($CDCl_3$) δ 1.33 (t, J = 7.1 Hz, 6H), 1.36 (t, J = 7.1 Hz, 3H), 2.28−2.41 (m, 2H), 2.78−2.93 (m, 4H), 3.82 (q, J = 7.1 Hz, 4H), 4.31 (q, J = 7.1 Hz, 2H), 11.92 (s, 1H). Anal. ($C_{15}H_{22}N_2O_2S_2$) H, N, S; C: calcd, 55.19; found, 54.31.

Ethyl 2-(3,3-Diethylthioureido)-5,6,7,8-tetrahydro-4*H*-cyclohepta[4,5]thiophene-3-carboxylate (31). Yield 86%; mp 93−95 °C (EtOH); IR (KBr, cm^{-1}) 1650 (C=O); ^1H NMR ($CDCl_3$) δ 1.31 (t, J = 7.1 Hz, 6H), 1.36 (t, J = 7.1 Hz, 3H), 1.57−1.70 (m, 4H), 1.78−1.88 (m, 2H), 2.68−2.73 (m, 2H), 2.99−3.05 (m, 2H), 3.80 (q, J = 7.1 Hz, 4H), 4.34 (q, J = 7.1 Hz, 2H), 12.21 (s, 1H). Anal. ($C_{17}H_{26}N_2O_2S_2$) C, H, N, S.

(*R*,*S*)-Ethyl 2-(3,3-Diethylthioureido)-4-methyl-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (32). Yield 81%; mp 72−73 °C; IR (KBr, cm^{-1}) 1643 (C=O); ^1H NMR ($CDCl_3$) δ 1.16 (d, J = 6.6 Hz, 3H), 1.31 (t, J = 7.1 Hz, 6H), 1.39 (t, J = 7.1 Hz, 3H), 1.62−1.92 (m, 4H), 2.50−2.72 (m, 2H), 3.28−3.39 (m, 1H), 3.81 (q, J = 7.1 Hz, 4H), 4.25−4.44 (m, 2H), 12.38 (s, 1H). Anal. ($C_{17}H_{26}N_2O_2S_2$) C, H, N, S.

Ethyl 2-(3,3-Diethylthioureido)benzo[*b*]thiophene-3-carboxylate (33). Yield 96%; mp 137−138 °C (EtOH); IR (KBr, cm^{-1}) 1654 (C=O); ^1H NMR ($CDCl_3$) δ 1.37 (t, J = 7.1 Hz, 6H), 1.52 (t, J = 7.1 Hz, 3H), 3.80−3.95 (m, 4H), 4.50 (q, J = 7.1 Hz, 2H), 7.24−7.31 and 7.36−7.42 (each m, 1H), 7.70−7.75 and 8.23−8.28 (each m, 1H), 12.80 (s, 1H). Anal. ($C_{16}H_{20}N_2O_2S_2$) C, H, N; S, calcd, 19.06; found, 18.45.

2-(Diethylamino)-4*H*-thieno[3,2-*d*][1,3]thiazin-4-one (5): General Procedure for Thieno[1,3]thiazin-4-ones 5, 37, 39, and 40. A mixture of compound **4** (4.1 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, and dried to give **5** (3.2 g, 89%): mp 76−78 °C; IR (KBr, cm^{-1}) 1648 (C=O); ^1H NMR ($CDCl_3$) δ 1.26 (t, J = 7.1 Hz, 6H), 3.62 (q, J = 7.1 Hz, 4H), 7.07 (d, J = 5.3 Hz, 1H), 7.72 (d, J = 5.3 Hz, 1H). Anal. ($C_{10}H_{12}N_2OS_2$) C, H, N, S.

6-Carbamoyl-2-(diethylamino)-5-methyl-4*H*-thieno[2,3-*d*][1,3]thiazin-4-one (37). Yield 89%; mp 193−194 °C (EtOH); IR (KBr, cm^{-1}) 1660, 1654 (C=O); ^1H NMR ($CDCl_3$) δ 1.27 (t, J = 7.1 Hz, 6H), 2.78 (s, 3H), 3.55−3.65 (m, 4H), 5.65 (br s, 2H). Anal. ($C_{12}H_{15}N_3O_2S_2$) C, H, N, S.

2-(Diethylamino)-6,7,8,9-tetrahydro-4*H*,5*H*-cyclohepta-[4,5]thieno[2,3-*d*][1,3]thiazin-4-one (39). Yield 99%; mp 128−129 °C (EtOH); IR (KBr, cm^{-1}) 1652 (C=O); ^1H NMR ($CDCl_3$) δ 1.23 (t, J = 7.1 Hz, 6H), 1.60−1.73 (m, 4H), 1.82−1.91 (m, 2H), 2.70−2.74 (m, 2H), 3.17−3.21 (m, 2H), 3.56 (q, J = 7.1 Hz, 4H). Anal. ($C_{15}H_{20}N_2OS_2$) C, H, N; S, calcd, 20.79; found, 20.12.

(*R*,*S*)-2-(Diethylamino)-5-methyl-5,6,7,8-tetrahydro-4*H*-[1]benzothieno[2,3-*d*][1,3]thiazin-4-one (40). Yield 90%; mp 97.5−98 °C (EtOH); IR (KBr, cm^{-1}) 1636 (C=O); ^1H NMR ($CDCl_3$) δ 1.22 (d, J = 6.8 Hz, 3H), 1.24 (t, J = 7.1 Hz, 6H), 1.62−1.72 (m, 4H), 2.54−2.70 (m, 2H), 3.26−3.38 (m, 1H), 3.53−3.62 (m, 4H). Anal. ($C_{15}H_{20}N_2OS_2$) C, H, N, S.

2-(Diethylamino)-4*H*-thieno[2,3-*d*][1,3]thiazin-4-one (34): General Procedure for Thieno[2,3-*d*][1,3]thiazin-4-ones 34, 35, 36, 38, and 41. A mixture of compound **26** (2.86 g, 10 mmol) and polyphosphoric acid (100 g) was stirred at 170 °C for 20 min. It was then poured into a mixture of ice (200 g) and EtOH (200 mL) and stirred to obtain a solid precipitate, which was filtered off. The precipitate was thoroughly washed with H_2O and dried to give **34** (2.1 g, 87%): mp 78−82 °C; IR (KBr, cm^{-1}) 1653 (C=O); ^1H NMR ($CDCl_3$) δ 1.27 (t, J = 7.1 Hz, 6H), 3.61 (q, J = 7.1 Hz, 4H), 6.77 (d, J = 6.0 Hz, 1H), 7.25 (d, J = 6.0 Hz, 1H). Anal. ($C_{10}H_{12}N_2OS_2$) C, H, N, S.

2-(Diethylamino)-5-methyl-4*H*-thieno[2,3-*d*][1,3]thiazin-4-one (35). Yield 79%; mp 94−95 °C; IR (KBr, cm^{-1}) 1659 (C=O); ^1H NMR ($CDCl_3$) δ 1.25 (t, J = 7.1 Hz, 6H), 2.45 (s, 3H), 3.59 (q, J = 7.1 Hz, 4H), 6.34 (s, 1H). Anal. ($C_{11}H_{14}N_2OS_2 \cdot 0.5H_2O$) C, H, N, S.

2-(Diethylamino)-5-isopropyl-4*H*-thieno[2,3-*d*][1,3]-thiazin-4-one (36). Yield 86%; mp 71−73 °C (EtOH); IR (KBr, cm^{-1}) 1659 (C=O); ^1H NMR ($CDCl_3$) δ 1.23 (d, J = 6.8 Hz, 6H), 1.25 (t, J = 7.2 Hz, 6H), 3.50−3.65 (m, 5H), 6.44 (s, 1H). Anal. ($C_{13}H_{18}N_2OS_2$) C, H, N, S.

2-(Diethylamino)-6,7-dihydro-4*H*,5*H*-cyclopenta[4,5]-thieno[2,3-*d*][1,3]thiazin-4-one (38). Compound **38** was prepared from **30**. The crude product was recrystallized from EtOH with charcoal to give **38** in 33% yield: mp 147−149 °C; IR (KBr, cm^{-1}) 1662 (C=O); ^1H NMR ($CDCl_3$) δ 1.25 (t, J = 7.1 Hz, 6H), 2.33−2.44 (m, 2H), 2.80−3.05 (m, 4H), 3.58 (q, J = 7.1 Hz, 4H). Anal. ($C_{13}H_{16}N_2OS_2$) H, N, S; C: calcd, 55.69; found, 55.10.

2-(Diethylamino)-4*H*-[1]benzothieno[2,3-*d*][1,3]thiazin-4-one (41). Yield 86%; mp 123−125 °C (EtOH); IR (KBr, cm^{-1}) 1654 (C=O); ^1H NMR ($CDCl_3$) δ 1.27 (t, $J = 7.1$ Hz, 6H), 3.54−3.68 (m, 4H), 7.28−7.35 and 7.39−7.45 (each m, 1H), 7.65−7.69 and 8.43−8.46 (each m, 1H); ^{13}C NMR ($CDCl_3$) δ 12.74, 44.42, 107.73, 121.35, 123.82, 124.78, 125.90, 131.41, 134.32, 163.37, 173.58, 174.15. Anal. ($C_{14}H_{14}N_2OS_2$) H, N, S; C: calcd, 57.90; found, 57.37.

3-(3,3-Diethylthioureido)thiophene-2-carboxylic Acid (6): General Procedure for Thiophenecarboxylic Acids 6 and 42−49. A mixture of compound **5** (2.4 g, 10 mmol), 3 M NaOH (40 mL), and dioxane (80 mL) was stirred and refluxed for 80 min. After cooling, it was poured onto ice−water (133 mL). Insoluble material was removed by filtration, and 3 M HCl (67 mL) was added at 0 °C to the stirred filtrate. The precipitate was collected by filtration to yield **6** (2.2 g, 85%): mp 119−128 °C; IR (KBr, cm^{-1}) 1640 (C=O); ^1H NMR ($CDCl_3$) δ 1.36 (t, $J = 7.1$ Hz, 6H), 3.84 (q, $J = 7.1$ Hz, 4H), 7.56 (d, $J = 5.6$ Hz, 1H), 8.90 (d, $J = 5.6$ Hz, 1H), 10.42 (s, 1H). Anal. ($C_{10}H_{14}N_2O_2S_2$) C, H, N; S: calcd, 24.82; found, 24.31.

2-(3,3-Diethylthioureido)thiophene-3-carboxylic Acid (42). Yield 87%; mp 142−147 °C (EtOH/hexane); IR (KBr, cm^{-1}) 1637 (C=O); ^1H NMR ($CDCl_3$) δ 1.36 (t, $J = 7.1$ Hz, 6H), 3.83 (q, $J = 7.1$ Hz, 4H), 6.63 (d, $J = 5.9$ Hz, 1H), 7.27 (d, $J = 5.9$ Hz, 1H), 11.74 (s, 1H). Anal. ($C_{10}H_{14}N_2O_2S_2$) C, H, N, S.

2-(3,3-Diethylthioureido)-4-methylthiophene-3-carboxylic Acid (43). Yield 88%; mp 146−148 °C; IR (KBr, cm^{-1}) 1632 (C=O); ^1H NMR ($CDCl_3$) δ 1.34 (t, $J = 7.1$ Hz, 6H), 2.39 (s, 3H), 3.82 (q, $J = 7.1$ Hz, 4H), 6.29 (s, 1H), 12.13 (s, 1H). Anal. ($C_{11}H_{16}N_2O_2S_2$) C, H, N, S.

2-(3,3-Diethylthioureido)-4-isopropylthiophene-3-carboxylic Acid (44). Compound **36** was refluxed in NaOH/dioxane for 120 min. The crude product was partitioned between ethyl acetate (100 mL) and 0.25 M NaOH (200 mL). The aqueous layer was cooled and acidified, and the precipitate was filtered off to give **44** in 30% yield: mp 142−145 °C; IR (KBr, cm^{-1}) 1631 (C=O); ^1H NMR ($CDCl_3$) δ 1.22−1.37 (m, 12H), 3.40−3.60 (m, 1H), 3.70−3.90 (m, 4H), 6.36 (s, 1H), 12.29 (s, 1H); MS (EI) *m/z* (rel intensity) 300 (38, M^+), 117 (100). Anal. ($C_{13}H_{20}N_2O_2S_2 \cdot H_2O$) C, H, N, S.

5-Carbamoyl-2-(3,3-diethylthioureido)-4-methylthiophene-3-carboxylic Acid (45). A mixture of compound **37** (2.97 g, 10 mmol), 3 M NaOH (80 mL), and dioxane (160 mL) was refluxed and then acidified with 3 M HCl (134 mL) to yield **45** (3.1 g, 93%): mp 197−200 °C (EtOH); IR (KBr, cm^{-1}) 1647 (C=O); ^1H NMR (DMSO-d_6) δ 1.22 (t, $J = 7.0$ Hz, 6H), 2.53 (s, 3H), 3.70−3.80 (m, 4H), 7.35 (s, 2H), 12.73 (s, 1H, NH); MS (EI) *m/z* (rel intensity) 315 (5, M^+), 58 (100). Anal. ($C_{12}H_{17}N_3O_3S_2 \cdot H_2O$) C, H, N, S; C: calcd, 48.96; found, 48.35.

2-(3,3-Diethylthioureido)-5,6-dihydro-4*H*-cyclopenta[4,5]thiophene-3-carboxylic Acid (46). Yield 90%; mp >149 °C (dec, EtOH); IR (KBr, cm^{-1}) 1637 (C=O); ^1H NMR ($CDCl_3$) δ 1.34 (t, $J = 7.1$ Hz, 6H), 2.32−2.43 (m, 2H), 2.80−2.98 (m, 4H), 3.81 (q, $J = 7.1$ Hz, 4H), 11.76 (s, 1H). Anal. ($C_{13}H_{18}N_2O_2S_2$) C, H, N, S.

2-(3,3-Diethylthioureido)-5,6,7,8-tetrahydro-4*H*-cyclohepta[4,5]thiophene-3-carboxylic Acid (47). Compound **39** was refluxed in NaOH/dioxane for 170 min to give **47** in 60% yield: mp 141−145 °C (EtOH); IR (KBr, cm^{-1}) 1628 (C=O); ^1H NMR ($CDCl_3$) δ 1.32 (t, $J = 7.1$ Hz, 6H), 1.57−1.70 (m, 4H), 1.80−1.90 (m, 2H), 2.68−2.77 (m, 2H), 3.04−3.10 (m, 2H), 3.80 (q, $J = 7.1$ Hz, 4H), 12.09 (s, 1H). Anal. ($C_{15}H_{22}N_2O_2S_2$) H, N, S; C: calcd, 55.19; found, 54.60.

(*R*,*S*)-2-(3,3-Diethylthioureido)-4-methyl-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic Acid (48). Compound **40** was refluxed in NaOH/dioxane for 4 h to give **48** in 63% yield: mp 142−145 °C (EtOH); IR (KBr, cm^{-1}) 1620 (C=O); ^1H NMR ($CDCl_3$) δ 1.20 (d, $J = 6.7$ Hz, 3H), 1.33 (t, $J = 7.1$ Hz, 6H), 1.63−1.92 (m, 4H), 2.52−2.74 (m, 2H), 3.30−3.38 (m, 1H), 3.81 (q, $J = 7.1$ Hz, 4H), 12.24 (s, 1H). Anal. ($C_{15}H_{22}N_2O_2S_2$) C, H, N, S.

2-(3,3-Diethylthioureido)benzo[*b*]thiophene-3-carboxylic Acid (49). Compound **41** was treated with NaOH/dioxane. The crude product was partitioned between ethyl acetate (100 mL) and 0.25 M NaOH (200 mL). The aqueous layer was cooled and acidified, and the precipitate was filtered off to give **49** in 15% yield: mp 142−144 °C; IR (KBr, cm^{-1}) 1630 (C=O); ^1H NMR (DMSO-d_6) δ 1.26 (t, $J = 6.8$ Hz, 6H), 3.70−3.90 (m, 4H), 7.22−7.29 and 7.30−7.46 (each m, 1H), 7.82−7.90 and 8.21−8.30 (each m, 1H), 12.13 (s, 1H). Anal. ($C_{14}H_{16}N_2O_2S_2$) C, H, N, S.

2-(Diethylamino)-4*H*-thieno[3,2-*d*][1,3]oxazin-4-one (7): General Procedure for Thieno[1,3]oxazin-4-ones 7 and 50−57. A mixture of compound **6** (646 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 **7** (480 mg, 86%): mp 36−37 °C (hexane); IR (KBr, cm^{-1}) 1759 (br, C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 239 (4.42), 340 (4.03); ^1H NMR ($CDCl_3$) δ 1.23 (t, $J = 7.1$ Hz, 6H, CH_3), 3.54 (q, $J = 7.1$ Hz, 4H, CH_2), 6.93 (d, $J = 5.2$ Hz, 1H, H-7), 7.72 (d, $J = 5.2$ Hz, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 13.26 (CH_3), 42.60 (CH_2), 106.39 (C-4a), 123.62 (C-7), 137.28 (C-6), 155.79 and 157.19 (C-2 and C-7a), 161.96 (C-4); MS (EI) *m/z* (rel intensity) 224 (68, M^+), 152 (100). Anal. ($C_{10}H_{12}N_2O_2S$) C, H, N, S.

2-(Diethylamino)-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (50). Yield 73%; mp 77−79 °C (hexane); IR (KBr, cm^{-1}) 1744 (C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 232 (4.31), 266 (3.85), 330 (3.94); ^1H NMR ($CDCl_3$) δ 1.25 (t, $J = 7.0$ Hz, 6H, CH_3), 3.56 (q, $J = 7.0$ Hz, 4H, CH_2), 6.72 (d, $J = 5.9$ Hz, 1H, H-6), 7.18 (d, $J = 5.9$ Hz, 1H, H-5); ^{13}C NMR ($CDCl_3$) δ 13.21, 13.26 (CH_3), 42.58 (CH_2), 109.34 (C-4a), 116.49 (C-6), 122.22 (C-5), 155.65 and 155.81 (C-2 and C-7a), 171.19 (C-4); MS (EI) *m/z* (rel intensity) 224 (M^+, 82), 152 (100). Anal. ($C_{10}H_{12}N_2O_2S$) C, H, N, S.

2-(Diethylamino)-5-methyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (51). Yield 77%; mp 102−103 °C (hexane); IR (KBr, cm^{-1}) 1752 (C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 238 (4.34), 268 (3.88), 332 (4.03); ^1H NMR ($CDCl_3$) δ 1.24 (t, $J = 7.1$ Hz, 6H, CH_3CH_2), 2.41 (s, 3H, 5-CH_3), 3.55 (q, $J = 7.1$ Hz, 4H, CH_2), 6.30 (s, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 13.29, 13.23 (CH_3CH_2), 16.17 (5-CH_3), 42.46 (CH_2), 108.64 (C-4a), 110.99 (C-6), 134.41 (C-5), 155.69 and 155.86 (C-2 and C-7a), 171.48 (C-4); MS (EI) *m/z* (rel intensity) 238 (M^+, 74), 166 (100). Anal. ($C_{11}H_{14}N_2O_2S$) C, H, N; S, calcd, 13.46; found, 14.09.

2-(Diethylamino)-5-isopropyl-4*H*-thieno[2,3-*d*][1,3]-oxazin-4-one (52). Yield 73%; mp 38−40 °C (hexane); IR (KBr, cm^{-1}) 1743 (C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 239 (4.36), 268 (3.85), 333 (4.06); ^1H NMR ($CDCl_3$) δ 1.25 (t, $J = 7.1$ Hz, 6H, CH_3CH_2), 1.26 (d, $J = 6.7$ Hz, 6H, CH_3CH), 3.42 (septet, $J = 6.7$ Hz, 1H, CH), 3.55 (q, $J = 7.1$ Hz, 4H, CH_2), 6.36 (s, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 13.32 (CH_3CH_2), 22.64 (CH_3CH), 28.88 (CH), 42.38 (CH_2), 107.73 (C-4b), 108.31 (C-6), 146.29 (C-5), 155.14 and 155.74 (C-2 and C-7a), 171.99 (C-4); MS (EI) *m/z* (rel intensity) 266 (M^+, 100), 194 (41). Anal. ($C_{13}H_{18}N_2O_2S$) C, H, N, S.

6-Carbamoyl-2-(diethylamino)-5-methyl-4*H*-thieno[2,3-*d*][1,3]oxazin-4-one (53). A mixture of compound **45** (834 mg, 2.5 mmol), yellow HgO (1.08 g, 5 mmol), CH_2Cl_2 (100 mL), and DMF (5 mL) was stirred and refluxed for 30 h. The mixture was stirred for an additional 60 h at room temperature. The crude product was recrystallized from ethyl acetate to give **53** (220 mg, 31%). An analytical sample was obtained after a second recrystallization from acetone: mp 185−190 °C; IR (KBr, cm^{-1}) 1750, 1650 (C=O); UV (EtOH) λ_{max} (nm) (log ϵ) 242 (4.26), 331 (4.31); ^1H NMR ($CDCl_3$) δ 1.26 (t, $J = 7.1$ Hz, 6H, CH_3CH_2), 2.75 (s, 3H, 5-CH_3), 3.45−3.60 (m, 4H, CH_2), 5.79 (br s, 2H, NH_2); ^{13}C NMR ($CDCl_3$) δ 12.75, 13.63 (CH_3-CH_2), 14.90 (5-CH_3), 42.20, 43.06 (CH_2), 109.97 (C-4a), 121.15 (C-6), 139.33 (C-5), 155.20 and 155.41 (C-2 and C-7a), 164.48 (C=O), 171.51 (C-4); MS (EI) *m/z* (rel intensity) 281 (M^+, 100), 209 (85). Anal. ($C_{12}H_{15}N_3O_3S$) C, H, N, S.

2-(Diethylamino)-6,7-dihydro-4*H*,5*H*-cyclopenta[4,5]-thieno[2,3-*d*][1,3]oxazin-4-one (54). Yield 72%; mp 92−93

°C (hexane); IR (KBr, cm^{-1}) 1753 (C═O); UV (EtOH) λ_{max} (nm) (log ϵ) 242 (4.22), 269 (3.92), 346 (4.02); ^{1}H NMR ($CDCl_3$) δ 1.24 (t, J = 7.1 Hz, 6H, CH_3), 2.36−2.46 (m, 2H, $CH_2CH_2CH_2$), 2.80−2.96 (m, 4H, $CH_2CH_2CH_2$), 3.55 (q, J = 7.1 Hz, 4H, NCH_2); ^{13}C NMR ($CDCl_3$) δ 13.33 (CH_3), 27.67, 28.91, and 29.42 (C-5, C-6, and C-7), 42.44 (NCH_2), 105.32 (C-4a), 131.12 (C-7a), 139.60 (C-4b), 155.43, 155.53 (C-2, C-8a), 174.91 (C-4); MS (EI) *m*/*z* (rel intensity) 264 (M^+, 100), 192 (83). Anal. ($C_{13}H_{16}N_2O_2S$) C, H, N, S.

2-(Diethylamino)-6,7,8,9-tetrahydro-4*H*,5*H*-cyclohepta-[4,5]thieno[2,3-*d*][1,3]oxazin-4-one (55). Yield 78%; mp 103−104 °C (hexane); IR (KBr, cm^{-1}) 1761 (C═O); UV (EtOH) λ_{max} (nm) (log ϵ) 241 (4.35), 272 (4.01), 341 (4.05); ^{1}H NMR ($CDCl_3$) δ 1.23 (t, J = 7.1 Hz, 6H, CH_3), 1.62−1.72 (m, 4H, CH_2), 1.82−1.93 (m, 2H, CH_2), 2.68−2.74 (m, 2H, CH_2), 3.06−3.11 (m, 2H, CH_2), 3.53 (q, J = 7.1 Hz, 4H, NCH_2); ^{13}C NMR ($CDCl_3$) δ 13.34 (CH_3), 27.25, 27.91, 28.03, 29.48, and 32.38 (C-5, C-6, C-7, C-8, and C-9), 42.32 (NCH_2), 109.25 (C-4a), 130.04 (C-9a), 135.91 (C-4b), 155.43 and 155.71 (C-2 and C-10a), 167.84 (C-4); MS (EI) *m*/*z* (rel intensity) 292 (M^+, 100), 220 (45). Anal. ($C_{15}H_{20}N_2O_2S$) C, H, N, S.

(*R*,*S*)-2-(Diethylamino)-5-methyl-5,6,7,8-tetrahydro-4*H*-[1]benzothieno[2,3-*d*][1,3]oxazin-4-one (56). Yield 83%; mp 78−79 °C (hexane); IR (KBr, cm^{-1}) 1742 (C═O); UV (EtOH) λ_{max} (nm) (log ϵ) 240 (4.35), 267 (3.95), 340 (4.07); ^{1}H NMR ($CDCl_3$) δ 1.23 (t, J = 7.1 Hz, 6H, CH_3CH_2), 1.26 (d, J = 6.9 Hz, 3H, 5-CH_3), 1.65−1.74 (m, 4H, CH_2), 2.56−2.66 (m, 2H, CH_2), 3.17−3.25 (m, 1H, CH), 3.53 (q, J = 7.1 Hz, 4H, CH_2-CH_3); ^{13}C NMR ($CDCl_3$) δ 13.32 (CH_3CH_2), 21.19 (5-CH_3), 18.67, 24.93, and 29.54 (C-6, C-7, and C-8), 28.92 (C-5), 42.35 (NCH_2), 107.58 (C-4a), 125.96 (C-8a), 135.86 (C-4b), 154.76 and 155.71 (C-2 and C-9a), 170.17 (C-4); MS (EI) *m*/*z* (rel intensity) 292 (M^+, 100), 220 (32). Anal. ($C_{15}H_{20}N_2O_2S$) C, H, N; S: calcd, 10.96; found, 11.50.

2-(Diethylamino)-4*H*-[1]benzothieno[2,3-*d*][1,3]oxazin-4-one (57). Yield 69%; mp 114−116 °C (hexane); IR (KBr, cm^{-1}) 1748 (C═O); UV (EtOH) λ_{max} (nm) (log ϵ) 234 (4.38), 257 (4.63), 344 (4.25); ^{1}H NMR ($CDCl_3$) δ 1.30 (t, J = 7.1 Hz, 6H, CH_3), 3.52−3.69 (m, 4H, CH_2), 7.27−7.33 and 7.40−7.55 (each m, 1H, H-6 and H-7), 7.65−7.70 and 8.16−8.21 (each m, 1H, H-5 and H-8); ^{13}C NMR ($CDCl_3$) δ 12.83, 13.62 (CH_3), 42.37, 43.02 (CH_2), 102.59 (C-4a), 122.07 and 122.44 (C-5 and C-8), 124.54 and 125.90 (C-6 and C-7), 132.83 and 134.27 (C-4b and C-8a), 154.61 and 156.87 (C-2 and C-9a), 173.29 (C-4); MS (EI) *m*/*z* (rel intensity) 274 (M^+, 96), 202 (100). Anal. ($C_{14}H_{14}N_2O_2S$) C, H, N, S.

Determination of the Kinetic Parameters of the Alkaline Hydrolysis. Alkaline hydrolysis was followed spectrophotometrically at 335 nm in 50 mM CAPS, pH 11.25, at 25 °C by monitoring the disappearance of the thieno[1,3]-oxazin-4-ones **7** and **50**−**58** and the 3,1-benzoxazin-4-one **59** for at least 1.5 half-lives. Stock solutions of the compounds were prepared in DMSO; the final inhibitor concentration was 15−20 μM and the final DMSO concentration was 5%. Curves were analyzed as first-order reactions.

HLE Inhibition Assay. HLE inhibition by compounds **5**, **7**, **34-41**, and **50**−**59** was assayed spectrophotometrically by the progress curve method at 25 °C. Assay buffer was 50 mM sodium phosphate and 500 mM NaCl, pH 7.8. A stock solution of Suc-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. Thieno[1,3]thiazin-4-ones **5** and **34**−**41** were analyzed at a single concentration (2 μM). For thieno[1,3]-oxazin-4-ones **7** and **50**−**58** and 3,1-benzoxazin-4-one **59**, at least five different inhibitor concentrations were used. Progress curves were fitted, and data were analyzed as described.[36] Inhibitors, dissolved in DMSO (10 μL), were added into a cuvette containing 890 μL of assay buffer and 50 μL of Suc-Ala-Ala-Pro-Val-pNA (final concentration 100 μM = 1.3 K_m). After thermal equilibration, the reaction was initiated by addition of 50 μL of HLE solution. Progress curves were monitored at 405 nm over 33−66 min.

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