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# Synthesis and *in-vitro* Evaluation of Novel Low Molecular Weight Thiocarbamates as Inhibitors of Human Leukocyte Elastase

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**A series of novel low molecular weight thiocarbamate esters (*1e–6e*) were synthesized and evaluated as inhibitors of human leukocyte elastase (HLE). The thiocarbamate esters studied consist of a substituted primary or secondary aliphatic or aromatic amine and a 1-phenyl-*1H*-tetrazole-5-thiol (Table I). The HLE catalyzed hydrolysis of *N*-methoxysuccinyl- L-Ala-L-Ala-L-Pro-L-Val-*p*-nitroanilide substrate was utilized as the measure of inhibition. *N-n*-butyl, 1-phenyl-*1H*-tetrazole-5-thiocarbamate (*1e*) exhibited the highest inhibitory activity ($k_{obs}/[I] = 2.1 \times 10^5\ M^{-1} \cdot min^{-1}$) and *N*-allyl, 1-phenyl-*1H*-tetrazole-5-thiocarbamate (*2e*) ($K_{obs}/[I] = 6.1 \times 10^4\ M^{-1} \cdot min^{-1}$) exhibited the second highest inhibitory activity of all the thiocarbamates. The aromatic *N*-phenyl, 1-phenyl-*1H*-tetrazole-5-thiocarbamate (*4e*) showed the lowest inhibitory activity ($K_{obs}/[I] = 1.9 \times 10^2\ M^{-1} \cdot min^{-1}$) among the *N*-monosubstituted derivatives, similar to that of *N*-ethyl-*N-n*-butyl, 1-phenyl-*1H*-tetrazole-5-thiocarbamate (*5e*) ($K_{obs}/[I] = 1.8 \times 10^2\ M^{-1} \cdot min^{-1}$). The *N*-isopropyl, 1-phenyl-*1H*-tetrazole-5-thiocarbamate (*3e*) ($K_{obs}/[I] = 3.3 \times 10^3\ M^{-1} \cdot min^{-1}$) was about 10 fold more active than (*4e*) and *N, N*-diisopropyl, 1-phenyl-*1H*-tetrazole- 5-thiocarbamate (*6e*) showed no inhibitory activity against HLE. In the present work less than 3% of HLE specific activity was regained after 24 hours incubation with each of the tested *N*-monosubstituted thiocarbamates (*1e–4e*). The time-dependent inhibition of HLE by the thiocarbamate compounds (*1e–5e*) seems to involve the interaction and possible chemical modification of one enzyme residue. Straight chain nonpolar aliphatic substituents on the nitrogen of the thiocarbamate functionality may be essential for high inhibitory activity. As the degree of substitution (branching) on the nitrogen of the thiocarbamate functionality increases the inhibitory activity of the compounds decreases. The time-dependent inhibition of HLE and the slow deacylation rates by the *N*-monosubstituted thiocarbamates are consistent with irreversible inhibition.**

*Keywords*: Human leukocyte elastase, Thiocarbamates, Irreversible inhibition, HLE

## INTRODUCTION

Human leukocyte elastase (HLE; EC 3.4.21.37) is an active component of the inflammatory response in the phagocyte system of polymorphonuclear leukocytes.[1,2] HLE elastinolysis and

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general proteolysis, as an initial step of tissue repair, activates polymorphonuclear leukocyte migration through connective tissue in the interstitium.[1] Elastase digests immune complexes, bacteria cell walls, and proteins intracellularly after phagocytosis.[1,2] Extracellularly elastase is capable of degrading a variety of structural proteins including elastin, collagen and articular cartilage.[1,2] The natural inhibitors $\alpha_1$-proteinase ($\alpha_1$-PI), $\alpha_2$-macroglobulin and secretory leukocyte proteinase inhibitor (SLPI) on mucosal surfaces regulate the immune response by maintaining physiological levels of HLE and other lysosomal enzymes.[3–6] In a pathological situation the balance between HLE and its natural inhibitors could be disrupted towards high levels of HLE with uncontrolled proteolysis and destruction of connective tissue.[7] Subsequent increased vascular permeability, plasma leakage and accumulation of fluid lead to potentially life threatening inflammatory edema. Edema may contribute to additional tissue damage by distorting tissue structures and impeding the delivery of oxygen.[7] A synthetic inhibitor of HLE could arrest or prevent inflammatory edema in several pathological situations: neurosurgery, cosmetic, reconstructive, transplantation, trauma and emergency room surgery, burns and other wounds, rheumatoid arthritis, cystic fibrosis, multiple sclerosis, acute asthma and respiratory distress syndrome.[7–10] The rational design of potent HLE inhibitors requires an understanding of both the specificity and the catalytic mechanism at the active site of the enzyme. The substrate or inhibitor, natural or synthetic, binds to the specificity site of the enzyme to give an enzyme-substrate or an enzyme-inhibitor physical complex.[11] The higher the affinity and the tighter the binding with the specificity site the higher the probability of forming a covalent bond in the active site of the enzyme, producing inactive acylated enzyme.[12] Elastin is the natural substrate of HLE and a component of connective tissue present in virtually every organ of the body.[13]

The polypeptide chain of elastin consists of alternating elastic and inelastic areas (beta helix). The elastic areas are composed of largely hydrophobic (non-polar) aminoacids such as alanine, proline and valine. The inelastic areas are composed of polyalanine sequences with interspersed lysine residues. Properly designed, low molecular weight organic molecules,[14–19] with hydrophobic side chains similar to those of natural substrates may interact at the primary specificity subsite of the active site and could inhibit selectively individual serine proteases. A prospective inhibitor of HLE should meet the following general requirements based on the structure of the substrate elastin: (1) Simulate the natural substrate elastin in incorporating aliphatic non-polar substituents with similar hydrophobic character. (2) Incorporate a reactive moiety towards the aminoacid residues of the active site so that it can form an acylated enzyme complex. (3) Exhibit fast acylation and slow deacylation rates at the active site of the enzyme.

Peptidyl carbamates with a secondary amine substituent have been reported by our group[20–23] as being specific elastase inhibitors. The present work involves the design and synthesis of low molecular weight monosubstituted (**1e–4e**) and disubstituted (**5e–6e**) thiocarbamate esters consisting of an electrophilic thiocarbamate functionality and a primary or secondary aliphatic or aromatic amine substituent (Table I).

## MATERIALS AND METHODS

### Instrumentation

All common chemicals and solvents utilized were reagent grade. Infrared (IR) spectra were determined in KBr discs, as Nujol mulls or neat liquid films on a Perkin-Elmer 567 ratio recording infrared spectrophotometer. NMR spectra were acquired on Varian Model

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EM-360, EM-390, or VXR-300 spectrometers. Tetramethylsilane was used as internal standard in deuturated chloroform ($CDCl_3$). UV spectrophotometric analyses were performed using a Cary 2200 spectrophotometer, Varian Associates, connected to a MGW Lauda RM3 circulating water bath. Melting points were determined on a Fischer-Johns melting point apparatus and reported uncorrected. Elemental analyses were conducted by the Atlantic Microlab, Inc., Atlanta, GA and the results were within ±0.4% of their calculated values. The pH determinations were performed using a Corning Digital 135 pH/ion meter. Incubations were carried out in a Dubnoff metabolic shaking incubator, GSA Corporation.

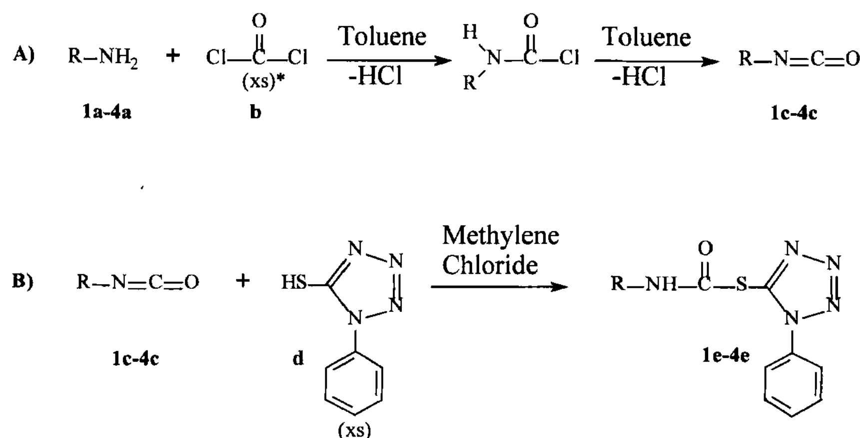
## Synthesis

### *n-Butylisocyanate (1c)*

A solution of *n*-butylamine (**1a**) (3.7 g, 0.05 moles) in 50 mL of dry toluene was added dropwise in 20% phosgene (**b**) solution (33.7 mL, 0.07 moles) in toluene, over a period of 1 h at a temperature below −15 °C (dry ice-isopropanol bath) (Scheme 1). The reaction mixture was stirred overnight at ambient laboratory conditions. The excess of phosgene was removed by a stream of dry nitrogen and finally trapped and destroyed in 6 N KOH. The precipitated *n*-butylamine hydrochloride was filtered and washed with chloroform. The combined filtrate and washings were then distilled *in vacuo* and characterized by IR. The distillate with the largest characteristic peak at 2360 $cm^{-1}$ was further purified with an additional distillation to yield 3.22 g (0.03 moles) (65% yield) **1c** as a clear liquid. IR (neat) 2360, 1770 $cm^{-1}$.

### *Isopropylisocyanate (3c)*

Compound **3c** was prepared in a manner analogous to that described above for the synthesis of **1c**. A solution of isopropylamine (**3a**) (11.5 mL, 0.14 moles) in about 50 mL of dry toluene was reacted with 20% phosgene (**b**) solution in toluene (91.7 mL, 0.18 moles) (Scheme 1). Treatment in an identical manner as above afforded 8.9 g (0.11 moles) (75% yield)




SCHEME 1 Synthesis of *N*-monosubstituted thiocarbamates.

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**3c** as a clear liquid with a characteristic IR peak at $2260\,cm^{-1}$.

### *Ethyl, n-butyl carbamoyl chloride (5c)*

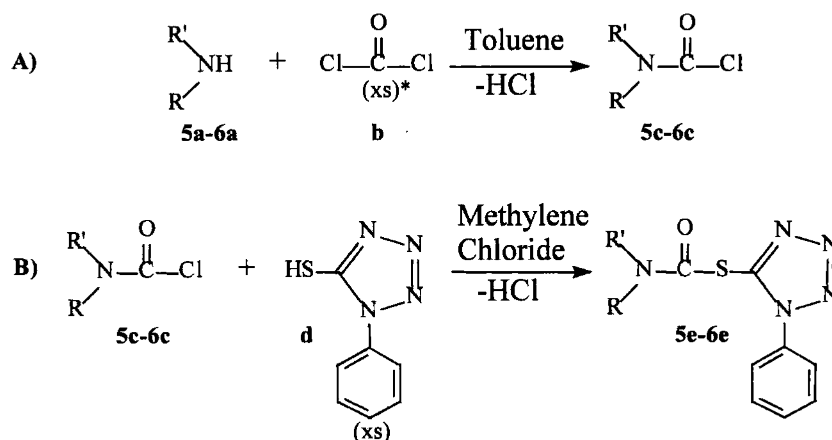
Compound **5c** was prepared in a manner analogous for **1c** and **3c**. A solution of ethyl, *n*-butylamine (**5a**) 4.5 mL (3.33 g, 0.03 moles) in about 50 mL of dry toluene was reacted with 20% phosgene (**b**) solution in toluene (18 mL, 0.04 moles) (Scheme 2). The isolated clear distillate was 2.9 g (0.018 moles) (60% yield) of **5c** which was used without further purification in subsequent steps. IR (neat) 2950, $1738\,cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$ 0.96 (t, 3H, $CH_3$), 1.22 (t, 3H, $CH_3$), 1.34 (m, 2H, $CH_2$), 1.61 (m, 2H, $CH_2$), 3.39 (m, 4H, $CH_2CH_2$). $^{13}$C-NMR ($CDCl_3$) $\delta$ 12.7, 13.7, 19.9, 30, 45.5, 50, 148.8.

### *N-n-Butyl, 1-phenyl-1H-tetrazole-5-thiocarbamate (1e)*

Compound **1c** (1.63 mL, 0.014 moles), synthesized or purchased commercially, was added dropwise over a period of 1 h into a solution of 3.1 g (0.017 moles) of 1-phenyl-*1H*-tetrazole-5-thiol (**d**) in 120 mL methylene chloride (Scheme 1). The reaction was stirred overnight at ambient laboratory conditions. The reaction mixture was then concentrated in a rotary evaporator to give a yellow oily residue which then was triturated and reconcentrated with hexane to give a crude white solid. The crude white solid residue was recrystallized from 2-propanol to give a white crystalline solid 3.1 g (0.012 moles) (80% yield) **1e**, mp 61°–62 °C. $^1$H-NMR ($CDCl_3$) $\delta$ 1.0 (t, 3H, $CH_3$), 1.48 (sext., 2H, $CH_2$), 1.69 (p, 2H, $CH_2$), 3.57 (q, 2H, $CH_2$), 7.6–7.8 (m, 5H, $C_6H_5$), 9.70 (t, 1H, NH). $^{13}$C-NMR ($CDCl_3$) $\delta$ 12.8, 20.0, 31, 40.8, 124.6, 129.8, 130.5, 134, 146, 162.3. Elemental analysis ($C_{12}H_{15}N_5OS$) C, H, N, S.

### *N-Allyl, 1-phenyl-1H-tetrazole-5-thiocarbamate (2e)*

Allylisocyanate (**2c**) (1.23 mL, 0.013 moles) was reacted with **d** (2.85 g, 0.016 moles) in a manner analogous to that for **1e** to afford 2.72 g (0.01 moles) (80% yield) of **2e**, a white crystalline solid, recrystallized from isopropyl ether, mp 92 °–93 °C. IR: 3020, $1765\,cm^{-1}$; $^1$H-NMR ($CDCl_3$)




SCHEME 2 Synthesis of *N*, *N*-disubstituted thiocarbamates.

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δ 4.2 (t, 2H, $CH_2$), 5.3 (d, 1H, CH), 5.4 (d, 1H, $CH_2$), 5.9 (m, 1H, $CH_2$), 7.5–7.9 (m, 5H, $C_6H_5$), 9.9 (m, 1H, NH). Elemental analysis ($C_{11}H_{11}N_5OS$) C, H, N.

### *N-Isopropyl, 1-phenyl-1H-tetrazole-5-thiocarbamate (3e)*

This compound was prepared by reacting **3c** (1.5 mL, 0.015 moles), with **d** (3.2 g, 0.018 moles) in a similar manner to that for **1e** and **2e** to afford 2.96 g (0.011 moles) (75% yield) of **3e**, a white crystalline solid which was recrystallized from isopropyl alcohol and water, mp 89°–90 °C. $^1$H-NMR ($CDCl_3$) δ 1.5 (d, 6H, $2CH_3$), 3.48 (m, 1H, CH), 7.6–7.8 (m, 5H, $C_6H_5$). Elemental analysis ($C_{11}H_{13}N_5OS$) C, H, N, S.

### *N-Phenyl, 1-phenyl-1H-tetrazole-5-thiocarbamate (4e)*

This compound was prepared by reacting phenylisocyanate (**4c**) (1.5 mL, 0.014 moles), with **d** (2.78 g, 0.016 moles) in a similar manner to that for **1e**, **2e**, and **3e** to afford 2.71 g (0.009 moles) (65% yield) of **4e**, a white crystalline solid, recrystallized from acetone and water, mp 120°–121 °C. $^1$H-NMR ($CDCl_3$) δ 7.4–8.0 (m, 10H, $2C_6H_5$). Elemental analysis ($C_{14}H_{11}N_5OS$) C, H, N, S.

### *N-Ethyl-N-n-butyl, 1-phenyl-1H-tetrazole-5-thiocarbamate (5e)*

Compound **5c** (5.4 g, 0.03 moles) was added dropwise over a period of 30 min to a solution of **d**, 7.13 g (0.04 moles) in 240 mL methylene chloride (Scheme 2). The reaction was stirred overnight at ambient laboratory conditions. The reaction mixture was then concentrated *in vacuo* to obtain a brown, oily residue. The residue was purified on Alumina gel (Alumina Woelm B-Super I) column chromatography (2 × 60 cm) using methanol in methylene chloride mixtures to obtain 4.2 g (0.014 moles, 42% yield) of **5e** as a light brown oil. $^1$H-NMR ($CDCl_3$) δ 0.8–1.6 (m, 10H, $2CH_3CH_2$), 3.3 (m, 4H, $CH_2CH_2$), 7.56 (m, 5H, $C_6H_5$). $^{13}$C-NMR ($CDCl_3$) δ 12.7, 13.7, 19.8, 30.5, 43.5, 48, 125.1, 128.3, 129.3, 130.5, 134, 147, 159. Elemental analysis ($C_{14}H_{19}N_5OS$) C, H, N, S.

### *N, N-Diisopropyl, 1-phenyl-1H-tetrazole-5-thiocarbamate (6e)*

Compound **6e** was prepared by reacting diisopropyl carbamoyl chloride (**6c**) (2.14 g, 0.013 moles), with **d** (2.46 g, 0.014 moles) in a similar manner to that for **5e** to afford 1.86 g (0.006 moles) (47% yield) **6e** (Scheme 2). The product was obtained as a white solid recrystallized from a mixture of isopropyl alcohol and water or a mixture of dimethyl formamide and water, mp 105°–106 °C. $^1$H-NMR ($CDCl_3$) δ 1.2 (d, 12H, $4CH_3$), 3.4 (m, 1H, CH), 3.85 (m, 1H, CH), 7.45–7.6 (m, 5H, $C_6H_5$). Elemental analysis ($C_{14}H_{19}N_5OS$) C, H, N, S.

## Enzyme Inhibition Kinetics – Materials and Methods

Human leukocyte elastase (HLE) from human sputum was obtained from the Elastin Company (St. Louis, MO). HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, dimethyl sulfoxide (DMSO) and sodium acetate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Substrate, *N*-methoxysuccinyl-L-Ala-L-Ala-L-Pro-L- Val-*p*-nitroanilide, was purchased from Sigma Chemical Co. (St. Louis, MO).

### *Enzyme Inactivation*

The method followed was a modification of that described originally by Nakajima *et al.*[24] (1979) and used by many others.[20–28] In a typical assay, 2.9 mL HEPES buffer (0.1 M, pH 7.50, containing 10% DMSO, and 0.05 M NaCl), 5 μL HLE enzyme solution ($3.7 \times 10^{-5}$ M) in sodium acetate buffer (0.05 M, pH 5.50) and 50 μL thiocarbamate compound (**1e–6e**) (Table I) solution in dimethyl sulfoxide (DMSO) were pipetted into a 3 mL

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sample quartz cuvette cell. The reference cuvette contained 2.9 mL of the same HEPES buffer, 5 µL acetate buffer (0.05 M, pH 5.50) and 50 µL thiocarbamate compound solution in DMSO. The two cuvette cells were shaken for 10 s to mix and placed in the jacketed cuvette holders of the spectrophotometer to thermally equilibrate and incubate at 25 °C for various time intervals (3, 4, 5, 7, 10, 15, 20, 30, 45, 60, 90 and 120 min). At the end of each incubation interval and after balancing the instrument at 410 nm, the enzyme was assayed by pipetting 50 µL substrate solution in DMSO ($2.78 \times 10^{-2}$ M) into both the reference and sample cuvettes, with the addition to the sample cuvette marking the end of the incubation interval. The cuvettes were shaken for 10 s to mix, placed in the spectrophotometer, and the absorbance was recorded at 410 nm after 2 min. Subsequently, a control experiment was conducted in the same manner as above and for the same incubation intervals by replacing the 50 µL of the thiocarbamate compound solution with 50 µL solvent DMSO in both cuvettes. The recorded absorbance at each incubation interval for each compound (2 min after substrate addition) was the absolute remaining activity of HLE ($A$). The recorded absorbance at the same incubation intervals for control (2 min after substrate addition) was the total activity of HLE ($A_0$). % Relative Remaining Activity = $(A/A_0) \times 100$.

The percent relative remaining activity of the HLE was plotted against the time of incubation for each thiocarbamate compound (Figure 1).

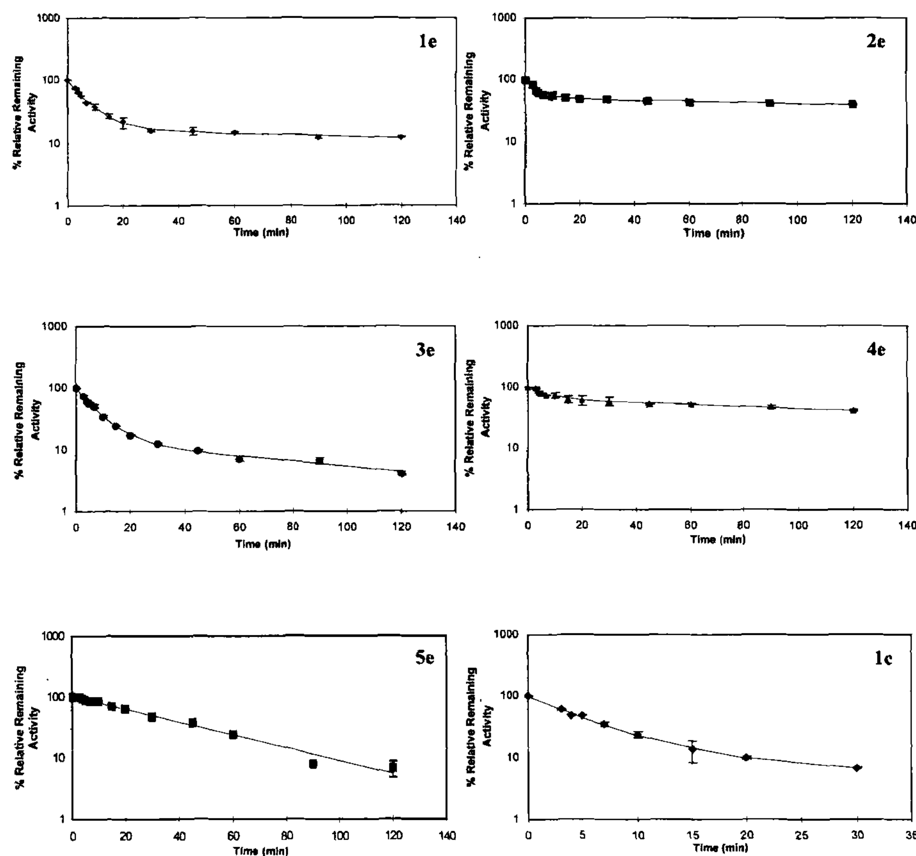


FIGURE 1 Semilogarithmic plots of percentage relative remaining activity of HLE *vs* time after incubation with the thiocarbamate compounds (**1e–5e**), and *n*-butylisocyanate (**1c**).

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In addition compounds **d**, and (**1a–5a**) were tested at a compound to enzyme ratio of 100:1 while compound (**1c**) was tested at an enzyme to compound ratio of 1000:1 in exactly the same manner. Inhibitory activity was assumed present when the rate of substrate hydrolysis was reduced by 10% from that of the control. The inhibition enzymatic assay was conducted three times at each incubation interval for each thiocarbamate compound and the control. The final DMSO concentration never exceeded 12% v/v.

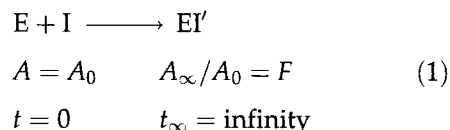
### *Enzyme Reactivation*

The recovery of HLE activity was monitored with time upon standing of the incubation solution in excess of (**1c**) and (**1e–4e**) compounds without removing the excess.[25,26,28] This became possible because the half-lives of spontaneous hydrolysis for these compounds were much shorter than the reactivation rate for inhibited HLE. The experiment was conducted in a similar manner to that for enzyme inactivation using the same concentrations of HLE enzyme and substrate and about 10- to 100-fold greater concentrations of (**1c**) and (**1e–4e**). After complete enzyme inactivation ($<10\%$ remaining activity) (observed in usually less than 5 min) and at different time intervals (up to 24 h) substrate solution was pipetted into both the reference and sample cuvettes and the increase in absorbance was recorded at 410 nm at 2 min after substrate addition. A control experiment was also conducted for the same time intervals by replacing the thiocarbamate compound solution with DMSO solvent in the enzyme incubation mixture.

## RESULTS AND DISCUSSION

The present work involves the synthesis and evaluation of novel compounds as HLE inhibitors. The compounds studied are low molecular weight thiocarbamate esters consisting of a substituted primary or secondary aliphatic or aromatic amine and a 1-phenyl-*1H*-tetrazole-5-thiol (Table I). The substituents on the compounds were selected to test the requirement of HLE for an aliphatic non polar residue in the substrate or inhibitor. *n*-Butyl, isopropyl, allyl, phenyl, ethyl-*n*-butyl, and diisopropyl amine substituents were selected to determine the proper alignment for optimum interaction with the primary subsite of the active site of the enzyme.[11,29–31] The 1-phenyl-*1H*-tetrazole-5-thiol (**d**) (Schemes 1, 2) was selected as a good leaving group due to its thione–thiol tautomerization and its partial aromatic character.[32,33] Generally, incorporating a good leaving group on the reactive carbamate moiety of the inhibitor may result in fast carbamoylation and irreversible inhibition of the enzyme.[16,34–35]

Fitting of the inhibition data, percentage relative remaining activity *vs* time, in an exponential model suggested time-dependent loss of HLE catalytic activity for compounds (**1e–5e**) while (**6e**) showed no inhibitory activity against HLE (Figure 1, Table I). According to the model the free enzyme (E) reacts with excess of inhibitor (I) in a pseudo-first order rate process, where $k_{obs}$ is the pseudo-first order rate constant, to produce the modified partially active enzyme (EI′) which maintains residual or fractional activity (F):



$$\begin{array}{ll} E + I \longrightarrow EI' & \\ A = A_0 \quad A_\infty/A_0 = F & (1) \\ t = 0 \quad t_\infty = \text{infinity} & \end{array}$$

At the beginning of incubation, ($t = 0$), the absolute remaining activity ($A$) will equal the total enzyme activity ($A_0$). As time progresses approaching infinity, ($t_\infty = \text{infinity}$), the relative remaining activity ($A_\infty/A_0$) approaches the fractional activity ($F$). The proposed model can be best described by Equation (2):

$$A/A_0 = (1 - F)e^{-k_{obs}t} + F \qquad (2)$$

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TABLE I Novel thiocarbamates and their inhibition rate constants against HLE

| | R | R′ | [I] M | $k_{obs}$ min$^{-1}$ | $k_{obs}/[I]$ M$^{-1}\cdot$min$^{-1}$ |
|---|---|---|---|---|---|
| **1e** | $CH_3$–$CH_2$–$CH_2$–$CH_2$— | H— | $6.88\times10^{-7}$ | 0.146 | $2.1\times10^{5}$ |
| **2e** | $CH_2$=CH–$CH_2$— | H— | $4.13\times10^{-6}$ | 0.250 | $6.1\times10^{4}$ |
| **3e** | ($CH_3$)$_2$CH— | H— | $4.10\times10^{-5}$ | 0.136 | $3.3\times10^{3}$ |
| **4e** | $C_6H_5$— | H— | $8.25\times10^{-4}$ | 0.161 | $1.9\times10^{2}$ |
| **5e** | $CH_3$–$CH_2$— | $CH_3$–$CH_2$–$CH_2$–$CH_2$— | $1.38\times10^{-4}$ | 0.024 | $1.8\times10^{2}$ |
| **6e** | ($CH_3$)$_2$CH— | ($CH_3$)$_2$CH— | $2.48\times10^{-2}$ | N.I.* | N.I.* |

*N.I. no inhibitory activity.

In such a case a semilog plot of the percentage relative remaining activity *vs* time of incubation would describe a curve approaching the terminal percentage fractional activity (Figure 1). Fitting the inhibition data in a biexponential model allows determination of the percentage fractional activity as the intercept of the second exponential term and estimation of $k_{obs}$ as the slope of the first exponential term. Accurate determination of $k_{obs}$ is achieved from the slope of the linear semilog plot of the rearranged Equation (2) shown in Equation (3):

$$\ln((A/A_0 - F)/(1 - F)) = -k_{obs}t \qquad (3)$$

The above enzymatic model developed by Ray and Koshland[36] and later expanded by Kitz and Wilson[37] was based on the assumption that the enzyme is a reagent with one or more reactive amino acid residues. These could be modified by a sufficient excess of inhibitor for pseudo-first order conditions to apply and react sufficiently slowly to allow accurate time courses to be determined. The pseudo-first order rate constant $k_{obs}$ divided by the concentration of the inhibitor ([I]) can be used as a measure of the inhibitory activity of the compounds (Equation 4) (Table I):

$$K_{sec} = k_{obs}/[I] \qquad (4)$$

It follows that the larger the number of $k_{obs}/[I]$ the greater the inhibiting capacity of the compound. The *N*-monosubstituted thiocarbamates (**1e–4e**) exhibited time-dependent inhibition against HLE according to the previously described model (Figure 1). The *N,N*-disubstituted thiocarbamate (**5e**) exhibited time-dependent inhibition with a pseudo-first order rate profile

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with no fractional activity (*F*) observed after 2 hours of incubation (Figure 1). Compound (**6e**) did not exhibit inhibitory activity (Figure 1). According to the data of Table I *N*-*n*-butyl, 1-phenyl-*1H*-tetrazole-5-thiocarbamate (**1e**) exhibited the highest inhibitory activity ($k_{obs}/[I] = 2.1 \times 10^5 M^{-1} \cdot min^{-1}$) and the *N*-allyl, 1-phenyl-*1H*-tetrazole-5-thiocarbamate (**2e**) exhibited the second highest inhibitory activity ($k_{obs}/[I] = 6.1 \times 10^4 M^{-1} \cdot min^{-1}$) of all thiocarbamates. The aromatic *N*-phenyl, 1-phenyl-*1H*-tetrazole-5-thiocarbamate (**4e**) showed lowest inhibitory activity ($k_{obs}/[I] = 1.9 \times 10^2 M^{-1} \cdot min^{-1}$) among the *N*-monosubstituted derivatives, similar to that of *N*-ethyl-*N*-*n*-butyl, 1-phenyl-*1H*-tetrazole-5-thiocarbamate (**5e**) ($k_{obs}/[I] = 1.8 \times 10^2 M^{-1} \cdot min^{-1}$). The *N*-isopropyl, 1-phenyl-*1H*-tetrazole-5-thiocarbamate (**3e**) ($k_{obs}/[I] = 3.3 \times 10^3 M^{-1} \cdot min^{-1}$) was about 10-fold more active than (**4e**) and *N*, *N*-diisopropyl, 1-phenyl-*1H*-tetrazole-5-thiocarbamate (**6e**) showed no inhibitory activity against HLE. The recovery of HLE activity was monitored in an excess of the *N*-monosubstituted thiocarbamate inhibitors. This became possible because the half-life ($t_{1/2}$) for spontaneous hydrolysis of the *N*-monosubstituted thiocarbamates under the enzymatic assay conditions were much shorter than the half-life for the reactivation of inhibited HLE.[26] In the present work less than 3% of HLE specific activity was regained after 24 hours incubation with each of the tested *N*-monosubstituted thiocarbamates (**1e–4e**). In addition, none of the corresponding amines *n*-butylamine (**1a**), allylamine (**2a**), isopropylamine (**3a**), aniline (**4a**), ethyl-*n*-butylamine (**5a**) and 1-phenyl-*1H*-tetrazole-5-thiol (**d**) inhibited HLE after incubation with the enzyme at a compound to enzyme ratio of 100:1. Using a final concentration upto 13% methyl sulfoxide as aqueous inhibitor diluent no influence on the rate of enzymatic hydrolysis of substrate was observed.

The starting reagent for the preparation of **1e**, *n*-butylisocyanate (**1c**), exhibited time-dependent inhibition against HLE similar to the inhibition profile for the primary thiocarbamate (Figure 1). The inhibition rate constant of (**1c**) ($k_{obs}/[I] = 2.8 \times 10^5 M^{-1} \cdot min^{-1}$), determined in the same manner as for the thiocarbamates, was found to be of the same order of magnitude as the inhibition rate constant of (**1e**). An average of 3.3% HLE activity was regained after 24 hours incubation with excess of *n*-butylisocyanate.

Enzymes in general, lower selectively the activation energy of enzymatic reactions. This can be accomplished only by the formation of an enzyme-reactant complex during the course of the enzymatic reaction. Initially the lock and key theory was suggested to explain the high degree of specificity in the enzyme–substrate interaction. However, there were several phenomena that the hypothesis could not explain. The induced-fit theory[29,30,38–41] suggested that the active site of the enzyme is complementary in structure to the structure of the transition state of the substrate. On binding, the enzyme is strained or distorted to achieve the transition state. The work by Stein *et al.*[30,42,43] strongly suggested that the induced fit theory is confirmed with HLE. The charge relay system of HLE is uncoupled in the ground state and full functioning during the transition state and only for transition states that fulfill exact structural requirements.[41–44] The inactivation of HLE by the novel thiocarbamate compounds (**1e–5e**), seems to involve the interaction and possible chemical modification of one enzyme residue. Compound (**6e**) did not show any inhibitory activity. Straight chain nonpolar aliphatic substituents on the nitrogen of the thiocarbamate functionality may be essential for high inhibitory activity. Several investigators have confirmed in a variety of elastase inhibitors that nonpolar aliphatic side chains of three to four carbons give optimal fit in the primary specificity subsite of the HLE active site which leads to covalent bond formation. In the past alkyl isocyanates have been found to inhibit serine proteases with some degree of specificity. Although elastase[31] is not inhibited by *n*-octylisocyanate it is inhibited by

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stoichiometric amounts of *n*-butylisocyanate. This degree of specificity strongly suggests a reaction with the active site of elastase, which apparently depends on specific recognition of the *n*-butyl alkyl chain. This observation has been attributed to the shallow binding pocket of the active site of elastase due to steric interference of valine and threonine side chains.[11,29–31] The observation that *N,N*-disubstituted thiocarbamates, (**5e**) and (**6e**), displayed the least or no inhibitory activity along with the low activity of (**3e**) indicates that as the degree of substitution (branching) on the nitrogen of the side chain of the thiocarbamate functionality increases the inhibitory activity of the compounds decreases. Thus human leukocyte elastase appears to react with thiocarbamates with short, straight, hydrophobic alkyl chains giving optimal inactivation. Deacylation enzyme kinetics were performed in an excess of the more potent *N*-monosubstituted thiocarbamates (**1e–4e**) inhibitors to distinguish between irreversible and reversible inhibition of HLE.[39] The slow deacylation rates and the time-dependent inhibition of HLE by the *N*-monosubstituted thiocarbamates are consistent with irreversible inhibition and possible covalent bond formation at the active site of the enzyme. Highly specific synthetic peptidyl carbamate inhibitors[16,20–23,25,34] allow the formation of a very tight Michaelis-Menten complex such as a tetrahedral intermediate at the active site of the enzyme leading to a covalent bond between enzyme and inhibitor at the Ser195 catalytic site. The catalytic site is composed of three hydrogen bonded amino acid residues, His57, Asp102 and Ser195 that form the so-called charge – relay system or catalytic triad.[25,45–47] The current thiocarbamates possibly carbamoylate the catalytic site at the Ser195 similarly to synthetic peptidyl carbamate specific elastase inhibitors. The rate of carbamoylation is affected by the good leaving group properties of 1-phenyl-1H-tetrazole-5-thiol due to its thione–thiol tautomerization and its partial aromatic character and the amine substituents which allow proper alignment for optimum interaction with the primary specificity subsite of the enzyme.

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