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SYNTHESIS AND MECHANISM OF ACTION OF NOVEL THIOCARBAMATE INHIBITORS OF HUMAN LEUKOCYTE ELASTASE

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Several peptidyl thiocarbamate inhibitors of human leukocyte elastase were synthesized in the molecular weight range of 700–800. Two different sequences with lysine at the $\mathbf{P}_3$ and ornithine at the $\mathbf{P}_4$ positions were synthesized. Most of the inhibitors with large molecular weights showed high inhibitory capacity with Ki values as low as 10^{-8} M. Compounds immobilized on poly,α,β-[*N*-(2-hydroxyethyl)-D,L-aspartamide] (PHEA) polymers with an average molecular weight of 36,000 showed higher inhibitory capacity than their free forms.

Keywords: Human leukocyte elastase (HLE); Peptidyl thiocarbamate inhibitors; Polymers

INTRODUCTION

Human leukocyte elastase (HLE) is a serine protease that has been involved in the abnormal degradation of connective tissue proteins associated with diseases such as rheumatoid arthritis,[1] adult respiratory distress syndrome,[2] and pulmonary emphysema.[3] Our laboratory has been involved in the design and synthesis of HLE inhibitors of carbamate types for the past several years.[4–9]

The objective of this study was to develop highly potent and specific active-site directed inhibitors of HLE. The inhibitors were designed as peptidyl thiocarbamates (PC), RR_1NCOSR', where NRR_1 was a polypeptide.

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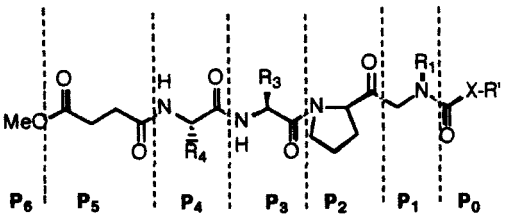


FIGURE 1

Peptidyl carbamate esters of the type shown in Figure 1 were synthesized earlier.

Introduction of an isopropyl group at $\mathbf{P}_1$ and *p*-nitro phenyl group at position $\mathbf{P}_0$ led to potent inhibitors of HLE.[4] Introduction of a desmosine like amino acid (lysine or ornithine) into a peptidyl carbamate at the $\mathbf{P}_3$ or $\mathbf{P}_4$ position generated more potent HLE inhibitors.[6] Furthermore, the introduction of derivatized lysine or ornithine made these compounds more specific to HLE and not PPE (porcine pancreatic elastase). All these studies were carried out with *p*-nitrophenol as the leaving group at the $\mathbf{P}_0$ position.[4-7]

The leaving group, *p*-nitrophenol, is a relatively toxic group and was therefore replaced by phenyl thiotetrazole. This group is less toxic than *p*-nitrophenol[10] and at the same time it is a better leaving group. As a consequence, the resulting compounds (thiocarbamates) were expected to be more susceptible towards enzymatic attack than their corresponding carbamate derivatives.

Based on the observations mentioned above, a new generation of peptidyl thiocarbamates (PTC inhibitors) were designed which had derivatized lysine or ornithine at the $\mathbf{P}_3$ or $\mathbf{P}_4$ positions, respectively. In this series of compounds an isovaline residue was introduced at the $\mathbf{P}_1$ position and *p*-nitrophenol at the $\mathbf{P}_0$ position was replaced by phenyl thiotetrazole.

EXPERIMENTAL

Organic reagents used in the syntheses of these compounds were purchased from Aldrich or Fluka. The amino acids were bought from Sigma. Silica gel used for CC was obtained from Fisher Scientific and TLC plates were purchased from Whatman, Inc.

Melting points were determined on a Fisher-Johns Uni-Melt apparatus. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross,

GA. Infra Red (IR) spectra were recorded on a Perkin-Elmer 1430 model. NMR spectra were recorded on a Varian Model EM-360 60 MHz, EM-390 90 MHz, and VXR-300 300 MHz spectrometer. Deuterated chloroform was used as solvent for the NMR spectra. Ultraviolet (UV) spectra were recorded using a Cary model 2200 UV–VIS spectrophotometer.

t-Boc-*N*-δ-CBZ-ornithine (1b)

This compound was prepared according to the methods of Itoh *et al.*[13] and was found to be spectrally and chromatographically identical with an authentic sample.

Preparation of *t*-Boc-alanine-*N*-hydroxy succinimide ester (2)

In a 250 mL round-bottomed flask, DCC (2.06 g, 10.0 mmol) was added to a solution of *t*-Boc-alanine (**1**) (1.89 g, 10.0 mmol), *N*-hydroxy succinimide (1.15 mL, 10.0 mmol) in 100 mL of THF at 5°C. The resulting reaction mixture was stirred for 10 min and then stored in the refrigerator overnight. The insoluble material was then filtered off. The filtrate was evaporated under vacuum and the residue washed with diethyl ether. The resulting solid was recrystallized from THF and ether to obtain a white solid **2** (2.17 g, 75%); m.p. 163–164°C; IR (KBr) 1830, 1800, 1780, 1725 cm^{-1}.

t-Boc-*N*-δ-CBZ-ornithine-*N*-hydroxy succinimide ester (2b)

This compound was synthesized following the same procedure used for the synthesis of **2**. The yield was quantitative; m.p. 126–127°C.

Preparation of *t*-Boc-alanyl-*N*-ε-CBZ-lysine (3)

To a solution of **2** (1.5 g, 5.2 mmol) and *N*-ε-CBZ-lysine (1.4 g, 5.1 mmol) in 15 mL of tetrahydrofuran at 5°C, triethylamine (0.70 mL, 5.1 mmol), was added and the suspension was stirred overnight at room temperature. The insoluble material was then filtered off and the filtrate was evaporated under reduced pressure. The residue obtained was dissolved in 60 mL of 5% $NaHCO_3$, and extracted with ethyl acetate (2 × 20 mL). The organic layer was evaporated and the crude yellowish oil was crystallized by triturating with diethyl ether and hexane to produce **3** (1.72 g, 77%), m.p. 65–70°C. ^1H NMR (300 MHz) δ 1.25 (d, $J = 6$ Hz, 3H), 1.35 (s, 9H), 1.5–1.9 (m, 6H), 3.15 (m, 2H), 4.25 (m, 1H), 4.55 (m, 1H), 4.8 (s, 1H), 5.2 (s, 1H), 5.6 (s, 1H), 5.1 (s, 2H), 7.3 (s, 5H), 9.4 (s, 1H). IR (Nujol) 3500, 750, 1690, 1680 cm^{-1}.

t-Boc-*N*-δ-CBZ-ornithinyl-alanine (3b)

This compound was synthesized in a similar manner to that for compound **3** except that compound **2b** and alanine were used. Yield: 71%, m.p. 58°C. ^{1}H NMR (300 MHz) δ 1.2 (d, $J = 6$ Hz, 3H), 1.35 (s, 9H), 1.5–1.7 (m, 4H), 1.8 (m, 2H), 4.15 (s, 1H), 4.5 (s, 1H), 4.85 (s, 1H), 5.1 (s, 2H), 5.3 (s, 1H), 5.6 (s, 1H), 7.3 (s, 1H) 9.6 (s, 1H). IR (Nujol) 3500, 1760, 1690, 1680 cm^{-1}.

S-1-Phenyl-5-tetrazolyl-*N*-(L-brolylmethyl)-*N*-isopropylthiocarbamate hydrochloride (4)

This compound was prepared according to the method of Digenis *et al.*[4] and was found to be spectrally and chromatographically identical with the authentic sample.

Synthesis of *S*-1-phenyl-5-tetrazolyl-*N*-(*t*-Boc-alanyl-*N*-ε-CBZ-lysyl-prolylmethyl)-*N*-isopropylthiocarbamate (5)

Compound **3** (0.132 g, 0.292 mmol), NMM (70 μL, 0.584 mmol), and 15 mL of THF were mixed and stirred at −10°C to −20°C. Isobutyl chloroformate (43 μL, 0.292 mmol) was then added followed by **4** (0.12 g, 0.292 mmol and the mixture stirred for 3 h at room temperature. *N*-methylmorpholinium hydrochloride was then filtered off and the filtrate evaporated *in vacuo*. The residue obtained was purified by column chromatography using silica gel and methanol–methylene chloride system. The white residue obtained was triturated with ethyl acetate and hexane and then crystallized to obtain the desired product **5** (0.16 g, 68%), m.p. 76–78°C. ^{1}H NMR (90 MHz) δ 1.15–1.65 (m, 24H), 2.1 (m, 4H), 2.3–3.6 (m, 4H), 3.9–4.9 (m, 6H), 5.1 (s, 2H), 5.5–7.0 (m, 3H), 7.25–7.56 (m, 10H). IR ($CHCl_3$) 1780, 1740, 1690, 1640, 1600, 1500, 1450 cm^{-1}. Found: C, 57.52; H, 6.45; N, 15.26; S, 3.68. Calcd. for $C_{39}H_{53}N_9O_8S$: C, 57.98; H, 6.62; N, 15.60; S, 3.96%.

Synthesis of *S*-1-phenyl-5-tetrazolyl-*N*-(*t*-Boc-*N*-δ-CBZ-ornithyl-alanyl-prolylmethyl)-*N*-isopropylthiocarbamate (5b)

This compound was synthesized following the literature procedure for the synthesis of compound **5** except that compounds **3b** and **4** were used instead of compounds **3** and **4**. m.p. 82–85°C. ^{1}H NMR (90 MHz) δ 1.1–1.45 (m, 18H), 1.6 (m, 4H), 2.0 (m, 4H), 2.9–3.2 (m, 4H), 3.9–4.8 (m, 6H), 5.1 (s, 2H), 5.4–6.9 (m, 3H), 7.25–7.55 (m, 10H). IR ($CHCl_3$) 1780, 1740, 1690,

1650, 1600, 1500, 1450, 1360 cm^{-1}. Found: C, 57.51; H, 6.39; N, 15.67; S, 3.96. Calcd. for $C_{38}H_{51}N_9O_8S$: C, 57.49; H, 6.47; N, 15.88; S, 4.03%.

Synthesis of *S*-1-phenyl-5-tetrazolyl-*N*-(alanyl-*N*-ε-CBZ-lysyl-prolylmethyl)-*N*-isopropylthiocarbamate hydrochloride (6)

Compound **5** (0.55 g, 0.65 mmol) was dissolved in 20 mL of ethyl acetate. Dried HCl gas was bubbled through the solution at −5°C for 2 min. The solution was then stirred at the same temperature for 15 min and was then evaporated *in vacuo*. The residue obtained was triturated with ether to generate the desired product **6** as a white solid (0.4 g, 85%), m.p. 113–115°C. ^{1}H NMR (90 MHz) δ 1.1–1.55 (m, 15H), 2.2 (m, 4H), 2.3–3.6 (m, 4H), 3.7–5.0 (m, 8H), 5.1 (s, 2H), 5.3–6.45 (m, 2H), 7.35–7.56 (m, 10H). IR (Nujol) 3300, 1740, 1690, 1635, 1590 cm^{-1}.

Synthesis of *S*-1-phenyl-5-tetrazolyl-*N*-(*N*-δ-CBZ-ornithyl-alanyl-prolylmethyl)-*N*-isopropylthiocarbamate hydrochloride (6b)

This compound was prepared from compound **5b** following the same procedure as that used for synthesis of compound **6** from compound **5**, m.p. 126–129°C. ^{1}H NMR (90 MHz) δ 1.12–1.55 (m, 9H), 1.8 (m, 4H), 2.2 (m, 4H), 2.8–3.4 (m, 4H), 3.8–4.7 (m, 8H), 5.1 (s, 2H), 5.2–6.8 (m, 2H), 7.35–7.55 (m, 10H). IR (Nujol) 3340, 1740, 1690, 1645, 1600, 1360 cm^{-1}.

Synthesis of *S*-1-phenyl-5-tetrazolyl-*N*-(succinyl-alanyl-*N*-ε-CBZ-lysyl-prolylmethyl)-*N*-isopropylthiocarbamate (7)

Triethylamine (70 μL, 0.53 mmol) was added to a stirred solution of succinyl anhydride (0.09 g, 0.9 mmol) and **6** (0.34 g, 0.45 mmol) in 10 mL of acetonitrile. The resulting reaction mixture was stirred for 6 h at room temperature and evaporated *in vacuo*. The residue was purified by column chromatography (silica gel and chloroform–methanol system). A crystalline product **7** was obtained after treating the purified residue with ether and hexane (0.31 g, 85%), m.p. 85–90°C. ^{1}H NMR (300 MHz) δ 1.1–1.8 (m, 15H), 1.85–2.2 (m, 4H), 2.3–2.6 (m, 4H), 3.1–3.3 (m, 2H), 3.6 (m, 2H), 3.7–4.0 (m, 2H), 4.1–4.7 (m, 4H), 5.1 (d, J = 9 Hz, 2H), 6.35 (m, 1H), 6.45 (m, 1H), 7.0 (m, 1H), 7.35–7.56 (m, 10H), 9.8 (s, 1H). IR ($CHCl_3$) 3450, 1770, 1740, 1690, 1680, 1600, 1500, 1450, 1360 cm^{-1}. Found: C, 56.24; H, 6.40; N, 14.03; S, 3.43. Calcd. for $C_{38}H_{49}N_9O_9S \cdot Et_2O \cdot H_2O$: C, 56.05; H, 6.82; N, 14.01; S, 3.55%.

Synthesis of *S*-1-phenyl-5-tetrazolyl-*N*-(succinyl-*N*-δ-CBZ-ornithyl-alanyl-prolylmethyl)-*N*-isopropylthiocarbamate (7b)

This compound was synthesized in a similar to that for compound **7** except that compound **6b** was the precursor. Yield: 79%, m.p. 95°C. ^{1}H NMR (90 MHz) δ 1.1–1.4 (m, 9H), 1.5–1.7 (m, 4H), 1.8–2.2 (m, 4H), 2.55 (d, $J = 4.5$ Hz, 4H), 3.2 (m, 2H), 3.55 (m, 2H), 3.75 (m, 2H), 3.9–4.2 (m, 3H), 4.5 (m, 1H), 5.07 (m, 2H), 4.7 (m, 1H), 5.5 (m, 1H), 6.9 (m, 1H), 7.3–7.56 (m, 10H), 9.8 (s, 1H). IR ($CHCl_3$) 3450, 1775, 1740, 1690, 1675, 1640, 1600, 1500, 1450, 1360 cm^{-1}. Found: C, 56.50; H, 6.63; N, 13.98; S, 3.42. Calcd. for $C_{37}H_{47}N_9O_9S \cdot ET_2O \cdot 1/4H_2O$: C, 56.45; H, 6.65; N, 14.45; S, 3.67%.

*N*α-Methoxysuccinylalanyl-L-*N*ε-carbobenzoxy lysine (8)

This compound was prepared according to the methods of Digenis *et al.*[6] and was found to be spectrally and chromatographically identical with the authentic sample.

*N*α-Methoxysuccinyl-*N*δ-carbobenzoxy ornithyl alanine (9)

This compound was prepared according to the methods of Digenis *et al.*[6] and was found to be spectrally and chromatographically identical with the authentic sample.

Synthesis of *S*-1-phenyl-5-tetrazolyl-*N*-(methoxy-succinyl-alanyl-*N*-ε-CBZ-lysyl-prolylmethyl)-*N*-isopropylthiocarbamate (10)

Compound **8** (295 mg, 0.63 mmol) was dissolved in 15 mL of THF at −15°C and NMM (180 μL, 1.3 mmol) and IBCF (82 μL, 0.63 mmol) added. Compound **4** (250 mg, 0.61 mmol) was then added and the resulting mixture was stirred at room temperature for 2 h and then filtered. The filtrate was evaporated under reduced pressure to give a yellow oily residue. This residue was purified on silica gel column using chloroform–methanol and triturated with hexane to obtain the desired compound as a crystalline solid **10** (0.2 g, 38%), m.p. 54°C. ^{1}H NMR (300 MHz) δ 1.12 (d, $J = 6$ Hz, 3H), 1.18 (d, $J = 6$ Hz, 3H), 1.31 (d, $J = 6$ Hz, 3H), 1.45–1.8 (m, 6H), 1.84–2.16 (m, 4H), 2.51 (d, $J = 6$ Hz, 4H), 3.19 (d, $J = 6$ Hz, 4H), 3.66 (s, 3H), 3.5–4.0 (m, 2H), 4.12 (m, 1H), 4.44–4.54 (m, 2H), 4.64–4.76 (m, 1H), 5.07 (m, 2H), 5.42 (s, 1H), 6.39 (d, $J = 9$ Hz, 1H), 6.95 (d, $J = 9$ Hz, 1H), 7.34–7.56 (m, 10H). IR ($CHCl_3$) 1770, 1740, 1690, 1680, 1650, 1640, 1600, 1500, 1450, 1360 cm^{-1}. Found: C, 57.65; H, 6.45; N, 14.26; S, 3.57. MS (−FAB) *m/e* 821 (M+);

(+FAB) *m/e* 822(MH^+). Calcd. for $C_{39}H_{51}N_9O_9S \cdot Et_2O$: C, 57.59; H, 6.82; N, 14.07; S, 3.57%. MS *m/e* 821.

Synthesis of *S*-1-phenyl-5-tetrazolyl-*N*-(methoxy-succinyl-*N*-δ-CBZ-ornithyl-alanyl-prolylmethyl)-*N*-isopropylthiocarbamate (10b)

Compound **10b** was synthesized in a manner similar to compound **10** except that compounds **9** and **4** were used instead of compounds **8** and **4**. Yield 37%, m.p. 75°C. ^{1}H NMR (90 MHz) δ 1.45–1.8 (m, 9H), 1.6 (m, 4H), 2.0 (m, 4H), 2.55 (m, 4H), 2.9 (m, 2H), 3.15 (m, 2H), 3.6 (s, 3H), 3.9–4.7 (m, 6H), 5.07 (m, 2H), 5.5 (m, 2H), 6.3–7.1 (m, 1H), 7.3–7.56 (m, 10H). IR ($CHCl_3$) 1770, 1740, 1690, 1680, 1640, 1600, 1500, 1450, 1360 cm^{-1}. Found: C, 56.59; H, 6.16; N, 15.53; S, 3.92. Calcd. for $C_{38}H_{49}N_9O_9S$: C, 56.50; H, 6.11; N, 15.60; S, 3.96%.

Synthesis of compounds 11 and 11b

Compounds **11** and **11b** were synthesized by coupling compounds **7** and **7b** with a linear hydrophilic polymer PHEA (poly,α,β,-[*N*-(2-hydroxyethyl)-D,L-aspartamidel]) respectively. These compounds were prepared according to the methods of Rypacek *et al.*[8]

Enzymatic Studies: Materials

HLE and its substrate (methoxysuccinyl-L-alanyl-L-alanyl-L-propyl-L-valine *p*-nitroanilide) and Hepes buffer were purchased from Sigma Chemical Co., St Louis, MO. HLE from sputum was obtained from Elastin Product Company, Pacific, MO. pH values were determined using a Corning Digital 135 pH/ion meter. Centrifugations were carried out on a IEC clinical centrifuge. Incubations were conducted using a Dubnoff metabolic shaking incubator. HPLC was conducted using Waters C-18 columns on a Water's HPLC system.

Stability Studies and HPLC Assays

The HPLC assay was developed to check the stability of these compounds in buffers (at different pH values) and rat plasma. A stock solution of compounds **7** and **10** was prepared in propylene glycol (5 mg/mL) and aliquots of 100 μL were taken and diluted with 5 mL of specific buffer and the mixture incubated at 37°C. Aliquots of 100 μL were withdrawn and neutralized, internal standard (phenylbutazone) added and analyzed on C18 RP HPLC

using acetonitrile and sodium acetate buffer. In the case of the stability study with plasma, 10 μL aliquots from the stock solution were mixed with 1 mL of plasma. After incubation, these samples were neutralized and extracted with methylene chloride. The samples were analyzed under the standard conditions described above.

Enzyme Assays

All enzyme assays were performed spectrophotometrically at 25°C using a Varian 2200 Cary spectrophotometer. The activity of HLE was assayed using methoxysuccinyl-L-alanyl-L-alanyl-L-propyl-L-valine *p*-nitroanilide and monitoring the absorbance at $\lambda = 410$ nm.[14]

Initial Screening for Inhibitory Activity

In a typical experiment, the inhibitor (0.05 mL, 1 μM to 1 mM in DMSO or Hepes buffer) and the substrate (0.05 mL, 28 mM in DMSO) were added to 0.1 M Hepes buffer (2.9 mL, pH 7.5) in a quartz cuvette and thermally equilibrated in the spectrophotometer for 2 min. The absorbance was balanced at the desired wavelength, after which the enzyme (0.05 mL, 3.7 μM in 0.05 M acetate buffer, pH 5.5) was added to the sample cuvette. The mixture was shaken for 20 s, and the increase in absorbance was monitored for 10 min. A comparison of the velocities produced by monitoring the absorbance in the presence and absence of an inhibitor determined if the compound was an inhibitor.

Determination of Km Value for HLE Substrate

In a standard experiment, Hepes buffer (2.9 mL), substrate (0.05 mL, 28 mM in DMSO), and 50 μL of DMSO were added to cuvettes. The absorbance was balanced at 410 nm wavelength, after which the enzyme (0.05 mL, 3.7 μM in 0.05 M acetate buffer, pH 5.5) was added to the sample cuvette. The mixture was shaken for 20 s, and the increase in absorbance was recorded at 1, 2, and 3 min after the addition of the enzyme. Inhibitory potency Ki was recorded at various substrate concentrations. A Lineweaver–Burke plot was used to determine the Km value. Various concentrations of inhibitors could be used to generate Dixon plots.

Determination of Incubation Time

This experiment was conducted to determine the minimum incubation time required for measuring the Ki and IC_{50} values. In a typical experiment,

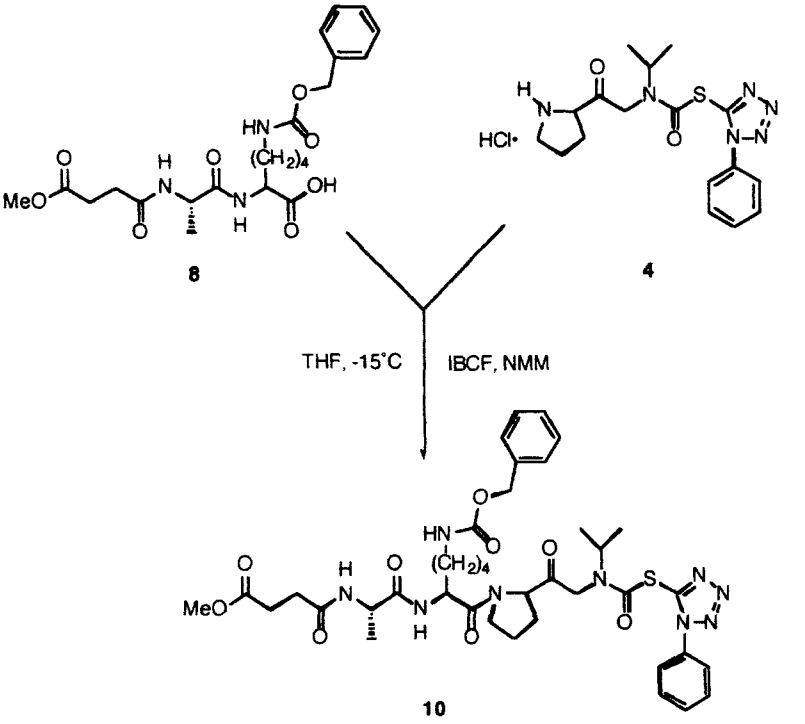
0.01 mL (1 μM–1 mM) of the inhibitor was added to acetate buffer (5 mL) in a thermally equilibrated quartz cuvettes. Enzyme (0.05 mL, 3.7 μM) was added to the sample cuvette and incubated for various time intervals (2.5–90 min) at 25°C. At the end of the incubation period, aliquots of 560 μL were transferred to the substrate (2.55 mL). The absorbance was balanced at 410 nm, and the substrate (0.05 mL) was added. The mixture was shaken for 20 s, and the increase in absorbance was recorded for 3 min. Percentage remaining activity of the enzyme versus incubation time was then plotted for each inhibitor concentration. An adequate incubation period was chosen as the shortest incubation time necessary for the lowest percentage remaining activity.

Determination of the Ki Value

In a typical experiment, 0.5 mL of acetate buffer and 0.01 mL of inhibitor solution (1 μM–1 mM) were added to quartz cuvettes at 25°C and thermally equilibrated for the incubation time determined from the above experiment after the addition of the enzyme solution (0.05 mL, 3.7 μM). The absorbance was balanced at 410 nm, the substrate (0.05 mL) was added to the sample cuvette and the mixture shaken for 20 s. The increase in absorbance was recorded at 1 and 2 min. The value obtained from a control experiment (without inhibitor) was considered as 100% activity of the enzyme. The inhibition curve was generated as a plot of percentage remaining HLE activity versus inhibitor concentration. This curve was then converted into a Easson–Stedman plot[12] from which the Ki value was obtained from the slope.

RESULTS AND DISCUSSION

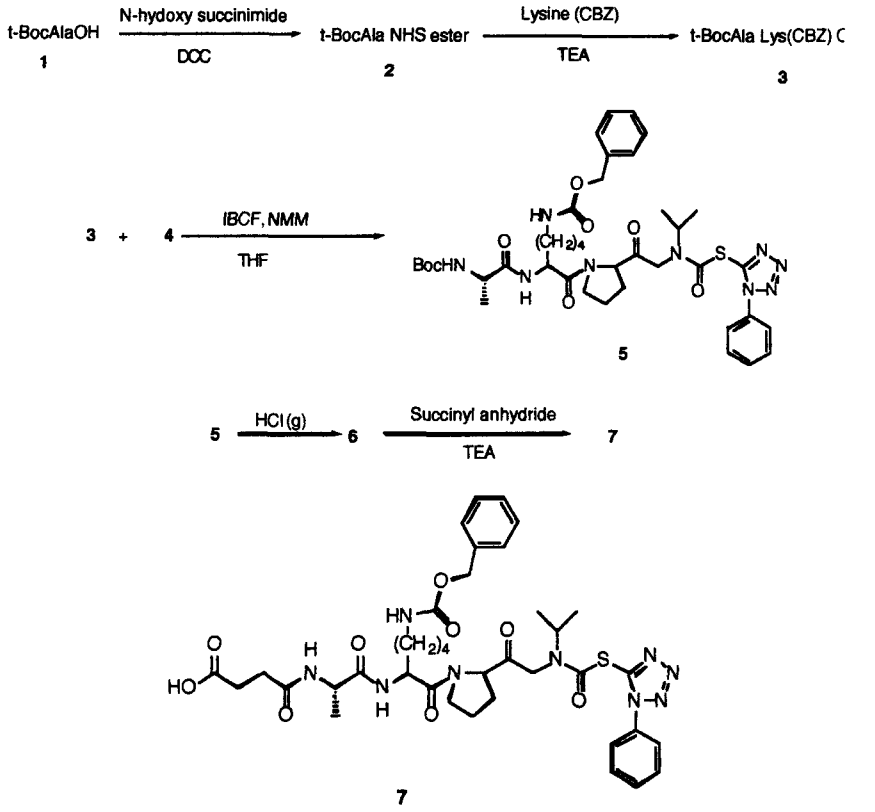
Synthesis of compound **8** (*N*α-methoxysuccinyl alanyl-L-*N*ε-carbobenzoxy lysine),[6] compound **4** (*S*-1-phenyl-5-tetrazolyl *N*-(L-prolylmethyl)-*N*-isopropylthiocarbamate hydrochloride),[4] and compound **9** (*N*α-methoxysuccinyl-L-*N*δ-carbobenzoxy ornithyl alanine)[6] have been reported earlier. Compound **10** (*S*-1-phenyl-5-tetrazolyl-*N*-(methoxysuccinyl alanyl-L-*N*ε-carbobenzoxy lysyl prolylmethyl)-*N*-isopropyl thiocarbamate) was synthesized by the mixed anhydride method from compounds **8** and **4** as shown in Scheme 1.



SCHEME 1 Synthesis of compound **10**.

Based on the studies from *p*-nitrophenol derivatives,[7] it was anticipated that compounds **6** and **7** would be the metabolites of compound **10** and so they were synthesized as shown in Scheme 2. Boc Ala (**1**) was reacted with *N*-hydroxy succinimide (NHS) to obtain its NHS ester (**2**). This compound was reacted with lysine (with the side chain protected as the CBZ group) to obtain compound **3**. Compound **3** was reacted with compound **4** via the mixed anhydride method to obtain compound **5**. Using HCl(g), the N-terminal Boc group of compound **5** was deprotected to give compound **6** which was then reacted with succinyl anhydride in the presence of triethylamine to produce compound **7**.

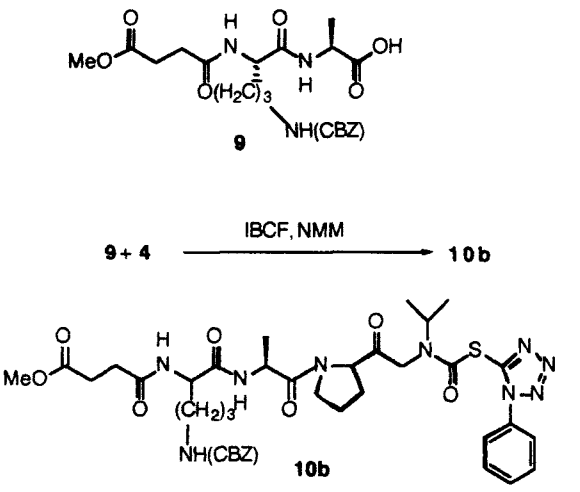
Thus, we were able to synthesize compounds **6**, **7**, and **10** which had phenyl thiotetrazole at the $\mathbf{P_0}$ position and protected lysine at the $\mathbf{P_3}$ and alanine at the $\mathbf{P_4}$ position. Another series of compounds were prepared where alanine was at the $\mathbf{P_3}$ position and protected ornithine was at the $\mathbf{P_4}$ position (Scheme 3). The synthesis of compound (**10b**) was similar to that for compound **10**.



SCHEME 2 Synthesis of compound 7.

Based on earlier results[7] some expected metabolites of compound **10b**, such as **6b** and **7b**, were also synthesized (see Scheme 4). The starting material for the synthesis of compounds **6b** and **7b** was *t*-Boc-*N*-α-ornithine-*N*-δ-CBZ (**1b**). Compound **1b** was reacted with DCC and *N*-hydroxy succinimide to form the activated ester (**2b**) which was subsequently reacted with alanine to produce compound **3b**. Compound **3b** was then reacted with compound **4** via the mixed anhydride method to give compound **5b**. The Boc group at the N-terminus of compound **5b** was removed to give compound **6b** which was then reacted with succinyl anhydride in the presence of triethylamine to give compound **7b** (Scheme 4).

Compounds **11** and **11b** were synthesized by coupling **7** and **7b** with a linear hydrophilic polymer poly,α,β-[*N*-(2-hydroxyethyl)-D, L-aspartamide] (PHEA) through an ethylene diamine spacer, respectively.[8,9]



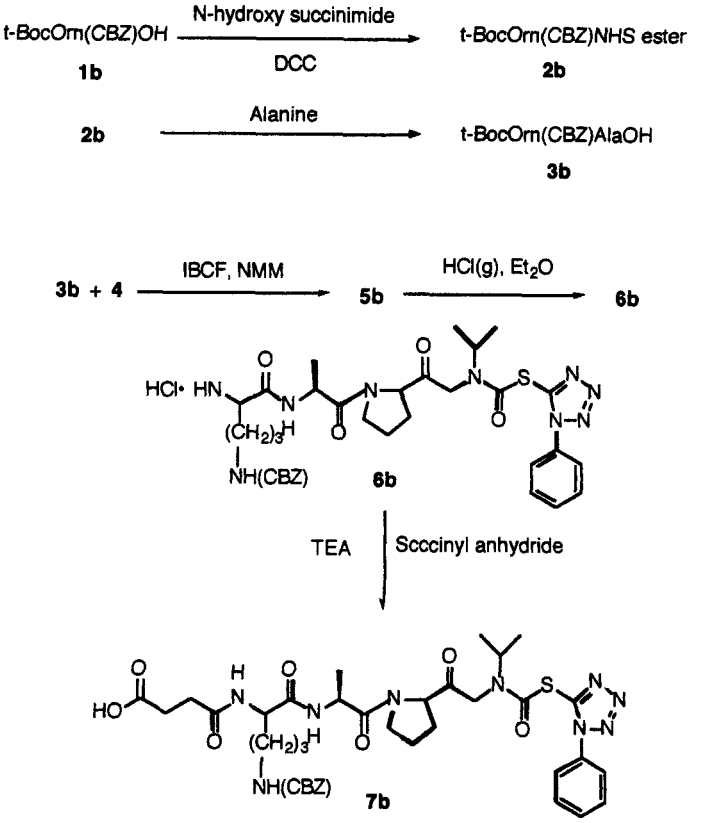
SCHEME 3 Synthesis of compound **10b**.

Carbamates are known to decompose particularly in alkaline media[11] and therefore stability studies for these thiocarbamates were carried out in buffers (at different pH values) and plasma at 37°C.

As seen from the data shown in Table I, compound **10** was very stable in acidic media. At neutral pH (7.4) its half-life was 24 min. The degradation product, which happens to be compound **7**, was more stable than its parent compound as expected at all the pH values examined.

The inhibitors shown in Table II were found to be slow binding inhibitors of HLE and therefore steady-state kinetics could not be applied for the determination of the enzymatic parameters. This observation was consistent with the earlier reported results for carbamate inhibitors.[8] In order to determine Ki values, a preincubation method was chosen.[12] The data obtained with these studies were used to determine the Ki value using Bieth's method.[12]

All the novel inhibitors synthesized in this work exhibited inhibitory activity as shown in Table II. The thiocarbamate derivatives were more potent than their *p*-nitrophenol counterparts suggesting that the phenyl thiotetrazole group had a better influence on the inhibitory activity than the *p*-nitrophenol group. The polymer bound compounds were more potent than their free forms (the polymer itself was ineffective). Impressively, the covalent linkage between the flexible linear polymer ($M_r = 36{,}000$) and the



SCHEME 4 Synthesis of compound **7b**.

TABLE I Stability studies on compounds **10** and **7** in buffers and plasma at 37°C

Compound	$t_{1/2}$ *(in buffer)*				$t_{1/2}$ *(in plasma)*
	pH 2.0[a]	*pH* 5.5[b]	*pH* 7.4[c]	*pH* 9.0[c]	
10	7 days	16 days	5.5 h	24 min	7 min
7	21 days	—	15 h	1 h	12.5 min

[a] 0.05 M KCl/HCl buffer; [b] McIlvaine buffer; [c] Clark and Lubs buffer.

peptidyl thiocarbamate inhibitors of HLE did not compromise their *in vitro* inhibitory capacity. Similar results were obtained with the PHEA bound HLE inhibitors of the carbamate type.[8] These macromolecules represent a novel class of HLE inhibitors.

TABLE II Inhibitory activity of synthesized compounds against HLE

Compound	M_r	*Avg.* IC_{50} (M)*	*Avg.* Ki (M)**
5	807.9	5.30×10^{-8}	6.70×10^{-8}
6	744.7	2.25×10^{-8}	2.55×10^{-8}
5b	793.8	6.45×10^{-8}	9.70×10^{-8}
6b	730.7	9.32×10^{-8}	6.24×10^{-7}
7	807.9	5.83×10^{-8}	9.11×10^{-8}
7b	793.8	9.28×10^{-8}	3.23×10^{-7}
10	821.9	3.25×10^{-8}	4.91×10^{-8}
10b	807.9	3.93×10^{-8}	7.09×10^{-8}
11	36,000	1.32×10^{-8}	4.40×10^{-9}
11b	36,000	2.14×10^{-8}	8.72×10^{-9}

* IC_{50} was estimated using the inhibition curve. ** Ki value was calculated from an Easson–Stedman plot.

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