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PEPTIDYL CARBAMATES AS HUMAN LEUKOCYTE ELASTASE INHIBITORS: DESIGN AND SYNTHESIS OF DESMOSINE-LIKE TETRAPEPTIDYL CARBAMATE INHIBITORS[†*]

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The rational design and synthesis of a series of peptidyl carbamates incorporating a derivatized ornithyl or lysyl residue at the P_3 or P_4 subsite is described. The derivatized residues were chosen as mimics of desmonsine cross-links ubiquitously found in mature elastin. The alteration of specific residues of the peptidyl carbamate, in addition to the realization of a stereospecific synthesis, required utilization of two convergent synthetic approaches. When tested for inhibitory activity against the serine dependent enzymes, human leukocyte elastase, porcine pancreatic elastase, trypsin, chymotrypsin, as well as acetyl cholinesterase, the compounds were found to be specific inhibitors of the elastases. Thus the series was found to exhibit inhibitor dissociation constants as low as 0.2×10^{-6}M and 3.0×10^{-6}M for human leukocyte and porcine pancreatic elastase, respectively. Michaelis-Menten kinetics demonstrated active site inhibition. Placement of N∂-Bz-L-Orn at P_4 with *p*-nitrophenol at P_1'(23a) resulted in the most active human leukocyte elastase inhibitor within this series (K_i 0.19×10^{-6}M). The most active inhibitor against porcine pancreatic elastase of this series resulted from placement of N_ε-Cbz-L-Lys at P_3 with *p*-nitrophenol at P_1'(24a) (K_i 3.61×10^{-6}M).

KEY WORDS: Human leukocyte elastse, inhibitor, peptidyl carbamate, porcine pancreatic elastase, desmosine

INTRODUCTION

Human leukocyte elastase (HLE) is a serine dependent enzyme found primarily in the granulocytic fractions of the human leukocyte (HL), to lesser extents in monocytes[1], and in the lung and joints[2]. The subject of HLE and the rational design of synthetic

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inhibitors has witnessed intense investigation as of late because of mounting evidence suggesting a role of HLE in tissue destruction associated with pulmonary emphysema, arthritis, and inflammation[3,4]. Elevated enzymatic activity or depressed antiprotease (i.e. α_1-antitrypsin) levels and/or ill-functioning antiproteases may result in a protease/antiprotease imbalance affording detrimental consequences to the host. Therapeutic approaches aimed at regulation of the activity of HLE offer potential of therapeutic intervention of the previously mentioned pathological situations, and consequently amelioration of symptoms[4,5].

Small molecular weight synthetic HLE inhibitors, being of the peptidyl carbamate (PC) type, have been designed and previously described by these laboratories[6,7,8]. The present report will describe further modifications of these PCs which led to greater HLE specificity and improved inhibitory activity.

Substrate requirements of HLE, namely S_1 preferring valine (P_1) and S_2 requiring proline (P_2), have been described by Powers[9]. The incorporation of a carbamate as the electrophilic site of enzyme attack required the placement of an isostere of valine at the P_1 position. Utilization of this strategy allowed Tsuji *et al.* to develop the selective inhibitor **1** which possessed K_i values of 11 and 42 $\times$ 10^{-6}M for HLE and porcine pancreatic elastase (PPE), respectively[6,7] (Figure 1).

In further continuation of our efforts, the present studies were directed at modifications of **1**, whereby the P_3 and P_4 residues were altered for optimal recognition by the S_3 and S_4 sites of HLE. Mature elastin, a natural substrate of HLE, possesses a desmosine ring (1,3,4,5-alkyl substituted pyridinium ring) containing alkyl branches of varying lengths, originating from four lysine residues[10]. The N_ε-amino group and the original chain length of lysine are present in one of four branches. Therefore the effect of chain length on the affinity of HLE was studied by incorporating derivatives of lysine and ornithine, utilizing the carbobenzoxy (CBZ) or benzoyl (BZ) functionalities as the aromatic moiety. The general structure of the desmosine tetrapeptidyl carbamates is illustrated in Figure 2. Futhermore, the effect of stereochemistry at the P_2 site was studied by using both the L- and D-isomers of proline at this site. The obtained PCs were evaluated *in vitro* against PPE and HLE, and trypsin, chymotrypsin, and acetyl cholinesterase for specificity.

MATERIALS AND METHODS

Chemistry

Melting points were determined on a Fisher-Johns Mel Temp apparatus and are reported uncorrected. UV measurements were recorded using a Varian 2200 Spectrophotometer. Infrared (ir) spectra were procured from a Perkin Elmer Model 1430 ratio recording spectrophotometer. Nuclear magnetic resonance spectra were recorded on either a Varian VXR 300 or XL 200 spectrometer. Microanalyses were performed by Atlantic Microlab and were within $\pm$ 0.4% of theoretical. HLE was purchased from the Elastin Company, St. Louis, MO. α-Chymotrypsin, trypsin, and acetyl cholinesterase were purchased from Sigma Chemicals, St. Louis, MO. The substrate t-boc alanine-p-nitrophenyl ester, methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-

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L-valine-*p*-nitro-anilide, N-benzoyl-L-tyrosine ethyl ester, N-benzoyl-L-arginine ethyl ester, and acetylcholine chloride were procured from Sigma Chemical Co., St. Louis, MO, and were stored desiccated at –10°C.

t-Butyl methoxysuccinyl alanine (**3**)

To an ice cooled solution of methylsuccinimide succinate[7] **2** (3.6 g, 16.0 mmol) and L-ala-t-butyl ester (2.3 g, 16.0 mmol) in THF (50 mL), a solution of TEA (2.0 g, 16.0 mmol) in THF (1.0 mL) was added dropwise. After stirring at 5°C for 3.5 h, all solids were filtered and washed with EtOAc. Concentration *in vacuo* afforded an oil which was purified by column chromatography (silica gel-G, 40 g); CH_2Cl_2 (50 mL) then 2% $MeOH/CH_2Cl_2$. Evaporation and crystallization from EtOAc/petroleum ether afforded 3(2.9 g, 71% yield) as a colorless powder (mp 91–92°C). ^{1}H-NMR ($CDCl_3$) ∂ 1.36(3H, d, J = 8Hz); 1.46 (9H, s); 2.44 (2H, app. t, J = 8Hz); 3.66 (3H, s); 4.20–4.60 (1H,m); 6.85 (1H, m, rotamers of amide-NH). IR ($CHCl_3$) 3400, 1815, 1785, 1740 cm^{-1}.

Methoxysuccinylalanine (**4**)

Using the method of Anderson and Callahan[11] the t-butyl ester **3** was hydrolyzed to afford the free acid **4** in 98% yield after chromatography (silica gel, 4% $MeOH/CH_2Cl_2$) as a transparent oil. ^{1}H-NMR ($CDCl_3$) ∂ 1.36 (3H, d, J = 8Hz); 2.56 (4H, app.t, J = 8Hz); 3.66 (3H, s); 4.20–4.60 (1H, m); 6.56 (1H, m, rotamers of amide-NH); 9.50 (1H, s); IR ($CHCl_3$) 3280, 1735, 1690, 1630, 1540 cm^{-1}.

N_α-Methoxysuccinyl alanine-N_ε-carbobenzoxylysine phenacyl ester (**6**)

Methoxysuccinyl alanine **4** (0.37 g, 1.8 mmol) was added to a solution of N-hydroxy succinimide (0.2g, 1.8 mmol) in THF (3 mL) cooled to 5°C. A solution dicyclohexylcarbodiimide (0.4g, 1.8 mmol) in THF (2 mL) was added dropwise with stirring. Stirring of the mixture was allowed to continue overnight at 5°C, after which the urea by-product was removed by vacuum filtration. The filtrate was cooled to 5°C followed by the addition of N_ε-carbobenzoxylysine phenacyl ester.HCl (0.7 g, 1.7 mmol) and triethyl amine (0.17 g, 17 mmol) in THF (0.5 mL). Upon completion of the reaction, as determined by tlc (10% $MeOH/CHCl_3$), the mixture was filtered and the solids washed with EtOAc (4 mL) and the filtrate was concentrated *in vacuo*. Chromatography (silica gel g (15 g), 2% $MeOH/CH_2Cl_2$) afforded a hygroscopic solid. Crystallization from $EtOAc/Et_2O$) gave 0.8 g (8.4%) of a colorless powder; mp 114–116°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.36 (3H, d, J = 8Hz); 1.50–2.20 (6H, m); 2.44 (2H, app. t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.23 (2H, m); 3.66 (3H, s); 4.30–4.90 (2H, m); 5.10 (2H, s); 5.46 (2H, s); 5.80 (1H, m, rotamers of amide-NH): 6.56 (1H, m, rotamers of amide-NH); 7.10 (1H, m, rotamers of amide-NH): 7.40 (5H, s); 7.63 (3H, app.dd, J = 8Hz, J = 2Hz); 8.06 (2H, app.dd, J = 8Hz, J = 2Hz). IR ($CHCl_3$) 3330, 1755, 1730, 1645, 1600, 1540 cm^{-1}.

N_α-Methoxysuccinyl alanyl-N_ε-benzoyl-L-lysine (**5**)

In an analogous procedure to that described for the production of **6**, the reaction of N_ε-benzoyl-L-lysine with methoxysuccinyl-L-alanine N-hydroxysuccinate in DMF afforded **5** in moderate yield (47%) as a transparent oil after chromatography. ^{1}H-NMR($CDCl_3$) ∂ 1.36 (3H, d, J = 8HZ); 1.50–1.86 (6H, m); 2.56 (4H, app.t, J = 8Hz); 3.23 (2H, m); 3.66 (3H, s); 4.00–4.70 (2H, m); 5.80 (1H, m, rotamers of amide-NH): 7.23 (1H, m, rotamers of amide-NH); 7.34 (2H, app.dd, J = 8Hz, J = 2Hz); 7.43 (1H, app.dd, J = 8Hz, J = 2Hz). 7.86 (2H, app.dd, J = 8Hz,

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J = 2Hz); 8.10 (1H, m, rotamers of amide-NH); 9.56 (1H, s). IR ($CHCl_3$) 3330, 1730, 1645, 1600, 1540 cm^{-1}.

N_α-Methoxysuccinyl alanyl-L-N_ε-carbobenzoxy lysine (**7**)

To a stirred solution of phenacyl ester **6** (0.6 g, 1.1 mmol) in glacial HOAc (10 mL), was added zinc metal (2 g) portion wise over 1 h. After an additional hour at room temperature, the reaction was diluted with MeOH/$CHCl_3$ (20% v/v, 50 mL), filtered, and the solid rinsed with the above solvent combination (50 mL). Concentration of the filtrate *in vacuo* produced an oil which was purified by chromatography (silica gel g, 5% MeOH/CH_2Cl_2). The oily product was crystallized by trituration with EtOAc/hexane to afford **7** (9.37 g) in 74% yield as a colorless powder; mp 116–118°C. ^{1}H-NMR($CDCl_3$) ∂ 1.36 (3H, d, J = 8Hz); 1.40–2.20 (6H, m); 2.56 (4H, app.t, J = 8Hz); 3.23 (2H, m); 3.66 (3H, s); 4.30–4.90 (2H, m); 5.10 (2H, s); 5.60 (1H, m, rotamers of amide-NH): 6.80–7.20 (2H, m, rotamers of amide-NH); 7.34 (5H, s); 9.60 (1H, s). IR ($CHCl_3$) 3330, 1730, 1710, 1590, 1540 cm^{-1}.

N_α-Methoxysuccinyl-L-alanyl-N_∂-carbobenzoxy ornithine phenacyl ester (**8**)

In an identical reaction to that presented for **6**, the title compound was prepared from N^∂-carbobenzoxy-L-ornithine in 46% yield (0.46 g) as a colorless powder which was recrystallized from EtOAc/Et_2O; mp 112–113°C; ^{1}H-NMR ($CDCl_3$) ∂ 1.36 (3H, d, J = 8Hz); 1.40–2.20 (4H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m); 3.66 (3H, s); 4.36-5.00 (2H, m); 5.10 (2H, s); 5.46 (2H, s); 5.80 (1H, m, rotamers of amide-NH): 6.56 (1H, m, rotamers of amide-NH); 7.10 (1H, m, rotamers of amide-NH); 7.34 (5H, s); 7.63 (3H, app.dd, J = 8Hz), J = 2Hz); 8.06 (2H, app.dd, J = 8Hz, J = 2Hz). IR ($CHCl_3$) 3330, 1755, 1730, 1645, 1600, 1540 cm^{-1}.

N_α-Methoxysuccinyl-L-alanyl-N_∂-carbobenzoxy-L-ornithine (**9**)

In an analogous manner as that described for **7**, the title compound was prepared from **8** as a transparent oil in 68% yield. ^{1}H-NMR ($CDCl_3$) ∂ 1.36 (3H, d, J = 8Hz); 1.40–1.90 (4H, app.t, J = 8Hz); 3.23 (2H, m); 3.66 (3H, s); 4.40–4.80 (2H, m); 5.10 (2H, s); 6.80–7.10 (2H, m, rotamers of amide-NH); 7.34 (5H, s); 10.10 (1H, s). IR ($CHCl_3$) 3330, 1730, 1710, 1590, 1545 cm^{-1}.

N_α-Methoxysuccinyl-N_∂-carbobenzoxy-L-ornithine (**10**)

N_∂-Carbobenzoxy-L-ornithine (1.0 g, 3.8 mmol) was added with stirring to a mixture of **2** (0.86 g, 3.8 mmol) and TEA (0.38 g, 3.8 mmol) in DMF (4.0 mL) at room temperature. After 24 h, filtration was followed by azeotropic removal of the DMF with toluene *in vacuo*. The residue was dissolved in EtOAc (15 mL) and washed with HCl (0.01 m, 15 ml), H_2O (30 mL), brine (30 mL), dried over $MgSO_4$ and concentrated *in vacuo*. Chromatography (30 g silica gel g, 4% MeOH/$CHCl_3$) afforded **10**, 1.4 g (96.7%), as an oil; ^{1}H-NMR ($CDCl_3$) ∂ 1.50–2.00 (4H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.23 (2H, m); 3.66 (3H, s); 4.30–4.80 (1H, m); 5.10 (2H, s); 7.20 (1H, m, rotamers of amide-NH): 7.40 (5H, s); 8.13 (1H, m, rotamers of amide-NH); 9.46 (1H, s). IR ($CHCl_3$) 3320, 1720, 1700, 1660, 1540 cm^{-1}.

N_α-Methoxysuccinyl-N_ε-carbobenzoxy-L-lysine (**13**)

Using an anlagous procedure as that for the production of **10**, **13** was prepared from N_ε-carbobenzoxy-L-lysine in a 90% yield as a colorless crystalline powder; (from $CHCl_3$/hexane)

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mp 85–86°. ^{1}H-NMR ($CDCl_3$) ∂ 1.50-2.00 (6H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.23 (2H, m); 3.66 (3H, s); 4.30-4.80 (1H, m); 5.10 (2H, s); 7.20 (1H, m, rotamers of amide-NH): 7.40 (5H, s); 8.13 (1H, m, rotamers of amide-NH); 9.46 (1H, s). IR ($CHCl_3$) 3320, 1720, 1660, 1535 cm^{-1}.

N_α-Methoxysuccinyl-N_ε-carbobenzoxylysyl-L-alanine t-butyl ester (**14**)

To an ice cooled solution of **13** (1.5 g, 3.8 mmol) in THF (10 mL) was added N-hydroxysuccinimide (0.4 g, 3.8 mmol) and DCC (0.86 g, 3.8 mmol). The reaction was stirred at 0°C overnight, followed by filtration of the urea. To an ice cooled solution of the above activated ester was added, portionwise, L-ala-t-butyl ester (0.7 g, 3.8 mmol), followed by TEA/(0.38 g, 3.8 mmol) in THF (0.5 mL). After 1 h the solution was filtered, and the filtrate concentrated in *in vacuo*. Purification, by chromatography (silica gel-g, 20 g, 3% MeOH/CH_2Cl_2), afforded **14** as an oil. Trituration with EtOAc/hexane gave 1.67 g (82% yield) of a colorless crystalline powder, mp 155–157°C ^{1}H-NMR ($CDCl_3$) ∂ 1.36 (3H, dd, J = 8Hz); 1.46 (9H, s); 1.50–2.20 (6H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.23 (2H, m); 3.66 (3H, s); 4.00–4.52 (2H, m); 5.10 (2H, s); 6.80–7.20 (3H, m, rotamers of amide-NH); 7.40 (5H, s). IR ($CHCl_3$) 3330, 1735, 1725, 1590, 1540 cm^{-1}.

N_α-Methoxysuccinyl-N_ε-carbobenzoxy-L-ornithinyl-L-alanine t-butyl ester (**11**)

Compound **11** was synthesized from **10** in 87% yield using analogous procedure to that described for **14**; mp 133–135°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.36 (3H, dd, J = 8Hz); 1.46 (9H, s); 3.66 (3H, s); 4.00-4.52 (2H, m); 5.10 (2H, s); 6.80-7.20 (3H, m, rotamers of amide-NH); 7.40 (5H, s). IR ($CHCl_3$) 3330, 1735, 1725, 1590, 1540 cm^{-1}.

N_α-Methoxysuccinyl-N_ε-carbobenzoxy-L-lysyl-L-alanine (**15**)

Formic acid (98%) (0.75 mL) was added in one portion to an ice cooled solution of **14** (0.54 g, 1 mmol) in EtOAc (5.0 mL). Hydrogen chloride was bubbled through the solution in two 30 s intervals. After warming to room temperature, the reaction was allowed to stir until the reaction was complete (tlc, 10% MeOH/$CHCl_3$). Azeotropic removal of excess formic acid using n-heptane afforded 0.4 g (88% yield) of a crystalline powder (from EtOAc/heptane); mp 192–194°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.36 (3H, d, J = 8Hz); 1.50–2.20 (6H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.23 (2H, m); 3.66 (3H, s); 4.00–4.52 (2H, m); 5.10 (2H, s); 6.56 (1H, m, rotamers of amide-NH); 6.80–7.20 (2H, m, rotamers of amide-NH); 7.34 (5H, s); 9.36 (1H, s). IR ($CHCl_3$) 3330, 1730, 1645, 1590, 1540 cm^{-1}.

N_α-Methoxysuccinyl-N_∂-carbobenzoxy-L-ornithyl-L-alanine (**12**)

Compound **12** was synthesized from **11** in 48% yield in a similar manner to that described for the production of **15**. Recrystallization (EtOAc/hexane) afforded an amorphous solid which melted at 147–148°C; ^{1}H-NMR ($CDCl_3$) ∂ 1.36 (3H, d, J = 8Hz); 1.50–2.20 (4H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.23 (2H, m); 3.66 (3H, s); 4.00–4.52 (2H, m); 5.10 (2H, s); 6.56 (1H, m, rotamers of amide-NH); 6.80–7.20 (2H, m, rotamers of amide-NH); 7.34 (5H, s); 9.36 (1H, s). IR ($CHCl_3$) 3330, 1730, 1645, 1590, 1540 cm^{-1}.

N_α-Methoxysuccinyl-N_ε-benzoyl-L-lysine (**16**)

Prepared as above for **10** to afford **16** as a colorless oil (88%. ^{1}H-NMR ($CDCl_3$) ∂ 1.50–1.90 (6H, m); 2.70 (2H, app.t, J = 8Hz); 2.90 (2H, app.t, J = 8Hz); 3.33 (2H, m); 3.66 (3H, s); 4.10–

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4.80 (1H, m); 6.90 (2H, m, rotamers of amide-NH); 7.34 (2H, app.dd, J = 8Hz, J = 2Hz); 7.43 (1H, app.dd, J = 8Hz, J = 2Hz); 7.86 (2H, dd, J = 8Hz, J = 2Hz); 8.90 (1H, s). IR ($CHCl_3$) 3520, 3360, 1840, 1790, 1735 1650, 710, 640 cm^{-1}.

*N_α-Methoxysuccinyl-N_ε-benzoyl-L-lysyl-L-alanine-t-butyl ester (**17**)*

Ester **17** was prepared from **16** (47%), as described above for **14** as a white amorphous powder, mp 156–158°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.36 (3H, d, J = 8Hz); 1.46 (9H, s); 1.50–2.20 (6H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.23 (2H, m); 3.66 (3H, s); 4.10–4.70 (2H, m); 6.60–7.10 (2H, m, rotamers of amide-NH); 7.34 (1H, app.dd, J = 8Hz, J = 2Hz); 7.42 (1H, app.dd, J = 8Hz, J = 2Hz); 8.10 (1H, m, rotamers of amide-NH). IR ($CHCl_3$) 3330, 1730, 1630, 1540, 1460, 1380 cm^{-1}.

*N_α-Methoxysuccinyl-N_ε-benzoyl-L-lysine-L-alanine (**18**)*

Hydrolysis of ester **17** using the method described above for **15** afforded **18** (96%) as an amorphous colorless powder (EtOAc/hexane). ^{1}H-NMR ($CDCl_3$) ∂ 1.36 (3H, d, J = 8Hz); 1.50–2.20 (6H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.23 (2H, m); 3.66 (3H, s); 4.10–4.70 (2H, m); 6.60–7.10 (2H, m, rotamers of amide-NH); 7.34 (2H, app.dd, J = 8Hz, J = 2Hz); 7.43 (1H, app.dd, J = 8Hz, J = 2Hz); 7.83 (2H, app.dd, J = 8Hz, J = 2Hz); 8.10 (1H, m, rotamers of amide-NH); 9.60 (1H, s). IR ($CHCl_3$) 3330, 1730, 1630, 1590, 1540, 1460 1380 cm^{-1}.

*p-Nitrophenyl-N-(L-prolylmethyl)-N-isopropyl carbamate hydrochloride (**19**)*

Hydrochloride **19** was synthesized using the method of Digenis *et al*[7]. The hygroscopic tan powder was found to exhibit identical spectral properties as an authentic standard.

*p-Nitrophenyl-N-[Methoxysuccinyl-(N_ε-carbobenzoxy)-L-lysyl-L-alanyl-D-prolylmethyl]-N-isopropyl carbamate (**20b**)*

Isobutyl chloroformate (0.13 g, 0.94 mmol) in CH_3CN (3 mL) was added dropwise to a cooled (–15°C) solution of acid **15** (0.39 g, 0.85 mmol) and an equimolar amount of N-methylmorpholine in THF (5 mL). After 10 min, **19** (0.4 g, 1.0 mmol) and NMM (0.1 g, 1.0 mmol) in CH_3CN (2 mL) were added and the mixture allowed to warm to 5°C and stirring allowed to continue (3h). At the end of this period, the reaction was filtered and the filtrate evaporated *in vacuo* to afford an oil. Methylene chloride (25 mL) was added and the resulting solution was washed with H_2O (25 mL), 10% citric acid (25 mL), brine (25 mL) H_2O (25 mL), dried ($MgSO_4$), decanted, and concentrated *in vacuo*. Chromatography (silica gel g (10 g), CH_2Cl_2–8% MeOH/CH_2Cl_2) afforded an amorphous powder comprised of **20a–b**. The product was dissolved in a minimal amount of $CHCl_3$ applied to preparative tlc plates for separation of the diastereomers (100 mg/plate). The plates were eluted three times with 4% MeOH/EtOAc and the two bands were scraped and extracted using 20% MeOH/$CHCl_3$ (3 × 25 mL). Combination of the lower bands afforded **20b** (the LLD isomer) 0.23 g (35%) as a colorless amorphous powder, mp 46–47°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.13 (3H, app.d, J = 7Hz, rotamer of carbamate); 1.20 (3H, app.d, J = 7Hz, rotamer of carbamate); 1.36 (3H, J = 8Hz; 1.40-2.22 (10H, m); 2.44 (2H, app.t, J = 8Hz), 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m); 3.33 (2H, app.t, J = 8Hz); 3.66 (3H, s); 4.30 (2H, center of a set of dd, J = 20 Hz, rotamers of CH_2 geminal system); 4.53 (1H, app.t, J = 8Hz); 4.63 (1H, app.t, J = 8Hz); 4.80 (2H, m); 5.10 (2H, s); 5.42 (1H, m); 6.70–7.10 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H,

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app.d, J = 10 Hz); 7.34 (5H, app.s); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.25 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3310, 1730, 1650, 1520, 735, 700 cm^{-1}. Anal. Found: C, 58.19; H, 6.50; N, 10.64. Requires $C_{38}H_{50}N_6O_{12}$: C, 58.30; H, 6.44; N, 10.74%.

p-Nitrophenyl-N-[Methoxysuccinyl-(N_ε-carbobenzoxy)-L-lysyl-L-alanyl-L-prolymethyl]-N-isopropyl carbamate (**20a**)

Prepared and purified as above for **20a–b**, isolated from the upper tlc bands, as a colorless powder (50%) mp 50–57°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.06–1.28 (6H, m, rotamer of carbamate); 1.32 (3H, J = 8Hz); 1.38–2.32 (10 H, m); 2.46 (2H, app.t, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.20 (2H, m); 3.36 (2H, m); 3.66 (3H, s); 4.20, 4.32 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of the CH_2 geminal system); 4.53 (1H, app.t, J = 8Hz); 4.63 (1H, app.t, J = 8Hz); 4.80 (2H, m); 5.10 (2H, s); 5.42 (1H, m); 6.36–7.08 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3310, 1730, 1650, 1520, 735, 700 cm^{-1}. Anal. Found: C, 58.47; H, 6.48; N, 10.67. Requires $C_{38}H_{50}N_6O_{12}$: C, 58.30; H, 6.44; N, 10.74%.

p-Nitrophenyl-N-[Methoxysuccinyl-(N_ε-benzoyl)-L-lysyl-L-alanyl-L-prolylmethyl]-N-isopropylcarbamate (**21b**)

Prepared and described above for **20b** from **18** and **19** to afford **21b** from the lower bands resulting from three successive elutions of the plates with 15% iPrOH/$CHCl_3$. Extraction afforded **21b** (40%) of a colorless powder, mp 63–64°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.13 (3H, app.d, J = 7Hz, rotamer of carbamate); 1.20 (3H, app.d, J = 7Hz, rotamer of carbamate); 1.36 (3H, d, J = 8Hz); 1.40–2.22 (10H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m); 3.33 (2H, app.t, J = 8Hz); 3.66 (3H, s); 4.30 (2H, center of sets of dd, J = 20 Hz, rotamers of the CH_2 = geminal system); 4.53 (1H, app.t, J = 8Hz); 4.63 (1H, app.t, J = 8Hz); 4.80 (2H, m); 5.42 (1H, m); 6.70–7.10 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.34 (2H, app.dd, J = 8Hz, J = 2Hz); 7.43 (1H, app.dd, J = 8Hz, J = 2Hz); 7.83 (2H, app.dd, J = 8Hz, J = 2Hz); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.25 ($1H_t$; app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3440, 1730, 1645, 1525, 1440, 1345, 1215, 750, 665 cm^{-1}. Anal. Found: C, 58.59; H, 6.76; N, 10.24. Requires $C_{37}H_{48}N_6O_{11}$: C, 58.90; H, 6.37; N, 11.14%.

p-Nitrophenyl-N-[Methoxysuccinyl-(N_ε-benzoyl)-L-lysyl-L-alanyl-L-prolylmethyl]-N-isopropylcarbamate (**21a**)

Prepared and purified as above for **21b**; however, isolation of the upper bands afforded **21a** (41%) as a colorless amorphous powder, mp 45–46°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.06–1.28 (6H, m, rotamer of carbamate); 1.32 (3H, d, J = 8Hz); 1.38–2.32 (10 H, m); 2.46 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.20 (2H, m); 3.53–3.88 (2H, m); 3.66 (3H, s); 4.20, 4.32 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.53 (1H, app.t, J = 8Hz); 4.63 (1H, app.t, J = 8Hz); 4.80 (2H, m); 5.42 (1H, m); 6.70–7.10 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.34 (2H, app.dd, J = 8Hz, J = 2Hz); 7.43 (1H, app.dd, J = 8Hz, J = 2Hz); 7.83 (2H, app.dd, J = Hz, J = 2Hz); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.25 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3400, 1730, 1645, 1525, 1440, 1345, 1215, 750, 665 cm^{-1}. Anal. Found: C, 59.18; H, 6.76; N, 10.22. Requires $C_{37}H_{48}N_6O_{11}$: C, 58.90; H, 6.37; N, 11.14%.

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*p-Nitrophenyl-N-[Methoxysuccinyl-(N∂-carbobenzoxy)-L-ornithyl-L-alanyl-D-prolylmethyl]-N-isopropylcarbamate (**22b**)*

Prepare from **12** and **19** as above for **20b**. Preparative thin layer chromatography (3 × 10% iPrOH/$CHCl_3$ afforded **22b** (15%) from the lower bands as a colorless amorphous powder, mp 50-51°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.13 (3H, app.d, J = 8Hz, rotamer of carbamate); 1.20 (3H, app.d, J = 7Hz, rotamer of carbamate); 1.36 (3H, d, J = 8Hz; 1.40-2.22 (8H, m); 2.44 (2H, app.t, J = 8Hz), 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m); 3.33 (2H, app.t, J = 8Hz); 3.66 (3H, s); 4.30 (2H, center of a set of dd, J = 20 Hz, rotamers of CH_2 geminal system); 4.53 (1H, app.t, J = 8Hz); 4.63 (1H, app.t, J = 8Hz); 4.80 (2H, m); 5.10 (2H, s); 5.42 (1H, m); 6.70-7.10 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz); 7.34 (5H, app.s); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.25 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3310, 1730, 1650, 1520, 735, 700 cm^{-1}. Anal. Found: C, 57.80; H, 6.36; N, 10.82. Requires $C_{37}H_{48}N_6O_{12}$: C, 57.80; H, 6.25; N, 10.94%.

*p-Nitrophenyl-N-[Methoxysuccinyl-(N∂-carbobenzoxy)-L-ornithyl-L-alanyl-L-prolylmethyl]-N-isopropyl carbamate (**22a**)*

Prepared as above for **22b** to afford **22a** (48%) from the upper bands of the eluted plates as a colorless powder, mp 55–56°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.06–1.28 (6H, m, rotamer of carbamate); 1.32 (3H, d, J = 8Hz); 1.38-2.32 (8H, m); 2.46 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.20 (2H, m); 3.36 (2H, m); 3.66 (3H, s); 4.20, 4.32 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of the CH_2 geminal system); 4.53 (1H, app.t, J = 8Hz); 4.63 (1H, app.t, J = 8Hz); 4.80 (2H, m); 5.10 (2H, s); 5.42 (1H, m); 6.37-7.08 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz); 7.35 (5H, app.s); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.25 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3310, 1730, 1650, 1520, 735, 700 cm^{-1}. Anal. Found: C, 57.81; H, 6.36; N, 10.68. Requires $C_{37}H_{48}N_6O_{12}$: C, 57.80; H, 6.25; N, 10.94%.

*p-Nitrophenyl-N-[Methoxysuccinyl-(N∂-benzoyl-L-ornithyl-L-alanyl-D-prolylmethyl]-N-isopropylcarbamate (**23b**)*

Hydrogen bromide in HOAc (30%) (0.3 mL) was added to a solution of the diastereomers **22a–b** (0.16 g, 0.2 mmol) in glacial HOAc (0.3 mL). The resulting solution was stirred at room temperature for 90 min after which dry Et_2O (10 mL) was added. Decantation of the turbid mixture followed by the addition and decantation with Et_2O (3 × 20 mL) afforded a tan hygroscopic solid which was dried *in vacuo* over P_2O_5. The above powder was dissolved in CH_3CN (2 mL) and set aside for the next reaction. Carbonyl diimidazole (0.09 g, 0.6 mmol) was added to benzoic acid (0.07 g, 0.6 mmol) in CH_3CN (1 mL). After stirring at room temperature for 10 min, the solution of the above amine salt in CH_3CN and N-methyl morpholine (0.08 g, 0.8 mmol) was added dropwise and stirred overnight. Concentration *in vacuo* afforded an oil which was taken up in CH_2Cl_2 (20 mL). The solution was washed with H_2O (20 mL) 10% citric acid (20 mL), brine (20 mL), H_2O (20 mL) dried ($MgSO_4$), decanted, and evaporated *in vacuo*. Preparative tlc (3 × 15% iPrOH/$CHCl_3$) afforded **23b** from the lower band (0.04 g, 25%), mp 65–66°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.13 (3H, app.d, J = 7Hz, rotamer of carbamate); 1.20 (3H, app. d, J = 7Hz, rotamer of carbamate); 1.36 (3H, d, J = 8Hz); 1.40–2.22 (8H, m); 2.44 (2H, app.t, J = 8Hz), 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m); 3.33 (2H, app.t, 8Hz); 3.66 (3H, s); 4.30 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.53 (1H, app.t, J = 8Hz); 4.63 (1H, app.t, J = 8Hz); 4.80 (2H, m); 5.42 (1H, m); 6.70–7.10 (2H,

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m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz); 7.83 (2H, app.dd, J = 8Hz, 2Hz); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.25 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3340, 1730, 1645, 1525, 1440, 1345, 1215, 750, 665 cm^{-1}. Anal. Found: C, 58.53; H, 6.37; N, 11.31. Requires $C_{36}H_{46}N_6O_{11}$: C, 58.50; H, 6.20; N, 11.38%.

*p-Nitrophenyl-N-[Methoxysuccinyl-(N_∂-benzoyl-L-ornithyl-L-alanyl-L-prolylmethyl]-N-isopropylcarbamate (**23a**)*

The antipode of **23b** was prepared under identical conditions to that presented above to afford **23a** (0.06g, 43%) as an amorphous colorless powder, mp 55–56°C, from the upper tlc band. ^{1}H-NMR ($CDCl_3$) ∂ 1.06-1.28 (6H, m, rotamer of carbamate); 1.32 (3H, d, J = 8Hz); 1.36–2.32 (8H, m); 2.46 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.20 (2H, m); 3.53-3.88 (2H, m); 3.66 (3H, s); 4.20, 4.32 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.53 (1H, app.t, J = 8Hz); 4.63 (1H, app.t, J = 8Hz); 4.80 (2H, m); 5.42 (1H, m); 6.70-7.10 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz); 7.34 (2H, app.dd, J = 8Hz, 2Hz); 7.43 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.83 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.25 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3340, 1730, 1645, 1525, 1440, 1345, 1215, 750, 665 cm^{-1}. Anal. Found: C, 58.53; H, 6.37; N, 11.31. Requires $C_{36}H_{46}N_6O_{11}$: C, 58.54; H, 6.36; N, 11.18%.

*p-Nitrophenyl-N-[Methoxysuccinyl-L-alanyl-(N_ε-carbobenzoxy-L-lysyl-D-prolylmethyl]-N-isopropylcarbamate (**24b**)*

Compound **24b**, synthesized from **7** and **19** following a procedure which was directly analogous to that described for **20b**, was isolated from the lower tlc band (3 × 4% MeOH/EtOAc) as an amorphous powder (36% yield), mp 44–45°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.13 (3H, app.d, J = 7Hz, rotamer of carbamate); 1.20 (2H, app.d, J = 7Hz, rotamer of carbamate); 1.36 (3H, d, J = 8Hz); 1.40–2.22 (10H, m); 2.44 (2H, app.t, J = 8Hz), 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m); 3.56–3.84 (2H, m); 3.66 (3H, s); 4.23 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.34–4.58 (4H, m); 4.60-4.64 (1H, m); 4.80 (2H, m); 5.10 (2H, s) 6.34–7.16 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz); 7.22 (1H, app.dd, J = 10 Hz); 7.34 (5H, app.s); 8.22 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.245 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3305, 1720, 1645, 1520, 735, 700 cm^{-1}. Anal. Found: C, 58.49; H, 6.47; N, 10.63. Requires $C_{38}H_{50}N_6O_{12}$: C, 58.30; H, 6.44; N, 10.74%.

*p-Nitrophenyl N-[Methoxysuccinyl-L-alanyl-(N_ε-carbobenzoxy-L-lysyl-D-prolylmethyl]-N-isopropylcarbamate (**24a**)*

Compound **24a**, was prepared as above (56% yield) to afford a colorless amorphous solid after preparative tlc (upper band), mp 56–57°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.06–1.28 (6H, m, rotamer of carbamate); 1.32 (3H, d, J = 8Hz); 1.38–2.32 (10H, m); 2.46 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.20 (2H, m); 3.52-3.88 (2H, m); 3.66 (3H, s); 4.20 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.50 (2H, app.t, J = 8Hz); 4.52–4.80 (2H, m); 5.10 (2H, s); 5.42 (1H, m); 6.36-7.08 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz); 7.22 (1H, app.dd, J = 10 Hz); 7.35 (5H, app.s); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.25 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3305, 1720, 1640, 1520, 735, 700 cm^{-1}. Anal. Found: C, 58.12; H, 6.55; N, 10.64. Requires $C_{38}H_{50}N_6O_{12}$: C, 58.30; H, 6.44; N, 10.74%.

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*p-Nitrophenyl N-[Methoxysuccinyl-L-alanyl-(N_ε-benzoyl-L-lysyl-D-prolylmethyl]-N-isopropylcarbamate (**25b**)*

Compound **25b**, synthesized from **5** and **19** following a procedure which was directly analogous to that described for **20b**, was isolated from the upper tlc band (3 × 15% iPrOH/$CHCl_3$) as an amorphous powder (41% yield), mp 45–46°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.13 (3H, app.d, J = 7Hz, rotamer of carbamate); 1.20 (2H, app.d, J = 7Hz, rotamer of carbamate); 1.36 (3H, d, J = 8Hz); 1.40–2.22 (10H, m); 2.44 (2H, app.t, J = 8Hz), 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m); 3.56–3.84 (2H, m); 3.66 (3H, s); 4.23 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.34–4.58 (4H, m); 4.60–4.64 (1H, m); 6.34–7.16 (2H, m, rotamers of amide-NH); 7.22 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.26 (1H, app.d, J = 10 Hz); 7.34 (2H, app.dd, J = 8Hz, J = 2Hz); 7.43 (1H, app.d, J = 8Hz); 8.22 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.24 (1H, app.d, J = 8Hz, J = 2Hz, rotamer of carbamate). IR ($CHCl_3$) 3340, 1735, 1645, 1520, 1435, 1345, 1260, 735, 700 cm^{-1}. Anal. Found: C, 59.58; H, 6.47; N, 11.30. Requires $C_{37}H_{48}N_6O_{11}$: C, 58.90; H, 6.37; N, 11.14%.

*p-Nitrophenyl N-[Methoxysuccinyl-L-alanyl-(N_ε-benzoyl)-L-lysyl-L-prolylmethyl]-N-isopropylcarbamate (**25a**)*

Compound **25a**, was prepared as above (45% yield) to afford a colorless amorphous solid after preparative tlc (upper band), mp 61–62°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.06–1.28 (6H, m, rotamer of carbamate); 1.32 (3H, J = 8Hz); 1.38–2.32 (10H, m); 2.46 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.20 (2H, m); 3.52–3.88 (2H, m); 3.66 (3H, s); 4.20, 4.32 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of the CH_2 geminal system); 4.50 (2H, app.t, J = 8Hz); 4.50–4.80 (2H, m); 5.42 (1H, m); 6.36–7.08 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz); 7.34 (2H, app.d, J = 8Hz); 7.43 (1H, app.d, J = 8Hz, J = 2Hz); 7.83 (2H, app.d, J = 8Hz, J = 2Hz); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3340, 1730, 1645, 1520, 1435, 1260, 735, 700 cm^{-1}. Anal. Found: C, 59.58; H, 6.47; N, 11.30. Requires $C_{37}H_{48}N_6O_{11}$: C, 58.83; H, 6.44; N, 11.36%.

*p-Nitrophenyl N-[Methoxysuccinyl-L-alanyl-($N\delta$-carbobenzoxy)-L-ornithyl-D-prolylmethyl]-N-isopropylcarbamate (**26b**)*

Prepared as above for **20b** from **9** and **19** to afford **26b** as an amorphous powder (15% yield) after preparative tlc (upper band) (3 × 4%) MeOH/EtOAc); mp 65-66°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.13 (3H, app.d, J = 7Hz, rotamer of carbamate); 1.20 (2H, app.d, J = 7Hz, rotamer of carbamate); 1.36 (3H, d, J = 8Hz); 1.40–2.22 (8H, m); 2.44 (2H, app.t, J = 8Hz), 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m); 3.56–3.84 (2H, m); 3.66 (3H, s); 4.23 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.34–4.58 (4H, m); 4.60–4.64 (1H, m); 4.80 (2H, m); 5.10 (2H, s) 6.34-7.16 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz); 7.22 (1H, app.dd, J = 10 Hz); 7.34 (5H, app.s); 8.22 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.24 (1H, app.d, J = 10 Hz), rotamer of carbamate). IR ($CHCl_3$) 3305, 1720, 1645, 1520, 735, 700 cm^{-1}. Anal. Found: C, 58.12; H, 6.55; N, 10.64. Requires $C_{37}H_{48}N_6O_{12}$: C, 57.80; H, 6.25; N, 10.94%.

*p-Nitrophenyl N-[Methoxysuccinyl-L-alanyl-($N\delta$-carbobenzoxy)-L-ornithyl-L-prolylmethyl]-N-isopropylcarbamate (**26a**)*

Prepared as above for **26b** to afford **26a** as an amorphous powder (16% yield) after preparative tlc (lower band); mp 55–56°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.06-1.28 (6H, m, rotamer of carbamate);

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1.32 (3H, d, J = 8Hz); 1.38-2.32 (8H, m); 2.46 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.20 (2H, m); 3.52-3.88 (2H, m); 3.66 (3H, s); 4.20 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of the CH_2 geminal system); 4.50 (2H, app.t, J = 8Hz); 4.52–4.80 (2H, m); 5.10 (2H, s); 5.42 (1H, m); 6.36–7.08 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz); 7.22 (1H, app.dd, J = 10 Hz); 7.35 (5H, app.s); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.25 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3305, 1720, 1645, 1520, 735, 700 cm^{-1}. Anal. Found: C, 57.68; H, 6.39; N, 10.89. Requires $C_{37}H_{48}N_6O_{12}$: C, 57.80; H, 6.25; N, 10.94%.

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p-Nitrophenyl N-[Methoxysuccinyl-L-alanyl-(Nδ-benzoyl)-L-ornithyl-D-prolylmethyl]-N-isopropylcarbamate (**27b**)

Compound **27b**, was prepared as described above for **23b** from mixture of diastereomers **26a–b** and was isolated as an amorphous solid (26% yield) after preparative tlc (upper band) (15% iPrOH/$CHCl_3$); mp 54–55°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.13 (3H, app.d, J = 7Hz, rotamer of carbamate); 1.20 (2H, app.d, J = 7Hz, rotamer of carbamate); 1.36 (3H, d, J = 8Hz); 1.40–2.22 (8H, m); 2.44 (2H, app.t, J = 8Hz), 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m); 3.56-3.84 (2H, m); 3.66 (3H, s); 4.23 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.34–4.58 (4H, m); 4.60-4.64 (1H, m); 6.34–7.16 (2H, m, rotamers of amide-NH); 7.22 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.26 (1H, app.d, J = 10 Hz); 7.34 (2H, app.dd, J = 8Hz, J = 2Hz); 7.43 (1H, app.d, J = 8Hz); 8.22 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.24 (1H, app.d, J = 8Hz, J = 2Hz, rotamer of carbamate). IR ($CHCl_3$) 3340, 1735, 1645, 1520, 1435, 1345, 1260, 735, 700 cm^{-1}. Anal. Found: C, 58.43; H, 6.29; N, 11.24. Requires $C_{36}H_{46}N_6O_{11}$: C, 58.50; H, 6.20; N, 11.38%.

p-Nitrophenyl N-[Methoxysuccinyl-L-alanyl-(Nδ-benzoyl)-L-ornithyl-L-prolylmethyl]-N-isopropylcarbamate (**27a**)

Compound **27a**, was prepared as described above for **23b** from mixture of diastereomers **26a–b** and was isolated as an amorphous solid (32% yield) after preparative tlc (upper band) (15% iPrOH/$CHCl_3$); mp 56–58°C. ^{1}H-NMR ($CDCl_3$) ∂ 1.06-1.28 (6H, m, rotamer of carbamate); 1.32 (3H, d, J = 8Hz); 1.38–2.32 (8H, m); 2.46 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.20 (2H, m); 3.52–3.88 (2H, m); 3.66 (3H, s); 4.20, 4.32 (2H, center of a set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.50 (2H, app.t, J = 8Hz); 4.50–4.80 (2H, m); 5.42 (1H, m); 6.36–7.08 (2H, m, rotamers of amide-NH); 7.24 (1H, app.d, J = 10 Hz, rotamer of carbamate); 7.28 (1H, app.d, J = 10 Hz); 7.34 (2H, app.d, J = 8Hz); 7.43 (1H, app.d, J = 8Hz, J = 2Hz); 7.83 (2H, app.d, J = 8Hz, J = 2Hz); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate); 8.20 (1H, app.d, J = 10 Hz, rotamer of carbamate). IR ($CHCl_3$) 3340, 1730, 1645, 1520, 1435, 1260, 735, 700 cm^{-1}. Anal. Found: C, 58.38; H, 6.36; N, 11.38. Requires $C_{36}H_{46}N_6O_{11}$: C, 58.50; H, 6.20; N, 11.38%.

STEREOSPECIFIC APPROACH TO INHIBITORS **24A–B**

N_α-Methoxysuccinylalanyl-N_ε-carbobenzoxy-L-lysyl-D-proline phenacyl ester (**28b**)

A solution of N,N'-dicyclohexylcarbodiimide (DCC) (0.17 g, 0.8 mmol) in THF (2 mL) was added dropwise to an ice cooled solution of 7 (0.4 g, .8 mmol) and N-hydroxysuccinimide (0.1 g, 0.8 mmol) in THF (4 mL). The resultant mixture was stirred overnight at 5°C. On the next day, all solids were filtered, and was the filtrate used without further treatment in the next step.

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To a stirred mixture of the cooled (5°C) solution from above and D-proline phenacyl ester hydrochloride (0.22 g, 0.8 mmol) was added triethylamine (0.08 g, 0.8 mmol) in THF (0.5 mL). The mixture was allowed to stir (5 h), at this temperature, after which the reaction was filtered, and the precipitate was washed with EtOAc (5 mL). The filtrates were combined and concentrated *in vacuo*, and the oil thus obtained was purified over silica gel (20 g) (3% $MeOH/CH_2$). Concentration of the desired fractions afforded an oil which was induced to crystallize, via trituration with EtOAc/hexane, as a colorless crystalline powder (0.5 g, 96% yield); mp 114–116°C. ^1H NMR ($CDCl_3$) ∂ 1.36 (3H, J = 8Hz); 1.40–2.22 (10H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m); 3.56 (2H, m); 3.66 (3H, s); 4.50 (2H, app.t, J = 8Hz); 4.53–4.80 (1H, m); 5.10 (2H,s); 5.43 (2H, s); 5.76 (1H, m, rotamers of amide-NH); 6.70–7.30 (2H, m, rotamers of amide-NH); 7.36 (5H, s); 7.60 (3H, app dd, J = 8Hz, J = 2Hz); 8.06 (2H, app dd., J = 8Hz, J = 2Hz). IR ($CHCl_3$) 3280, 1725, 1680, 1630, 725, 635 cm^{-1}.

N_α-Methoxysuccinylalanyl-$N_\varepsilon$carbobenzoxy-L-lysyl-L-proline phenacyl ester (**28a**)

The title compound was prepared in 74% yield by an analogous procedure to that described above for the preparation of **28b**, with the exception of the use of L-pro-phenacyl ester hydrochloride. Compound **28a** was shown to elute at a lower R_f value when compared to its antipode **28b** (4% $EtOAc/CH_2Cl_2$, and in 5% $MeOH/CH_2Cl_2$); mp 116–118°C. ^{1}NMR ($CDCl_3$) 21.36 (3H, J = 8Hz); 1.40–2.22 (l0H, m); 2.44 (2H, app. t, J = 8Hz): 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m); 3.56 (2H, m); 3.66 (3H, s); 4.34–4.58 (3H, m); 4.60-4.64 (1H, m); 5.10 (2H,s); 5.43 (2H, s); 6.70–7.30 (2H, m, rotamers of amide-NH); 7.36 (5H, s); 7.60 (3H, app dd, J = 8Hz, J = 2Hz); 8.06 (2H, app dd., J = 8Hz, J = 2Hz). IR ($CHCl_3$) 3280, 1725, 1680, 1630, 725, 635 cm^{-1}.

N_α-Methoxysuccinylalanyl-$N_\varepsilon$carbobenzoxy-L-lysyl-D-proline (**29b**)

The title compound was prepared as a colorless powder in 57% yield by an analogous procedure to that described above for the preparation of **7**; mp 119–120°C. ^1H NMR ($CDCl_3$) ∂ 1.36 (3H, J = 8Hz); 1.40–2.22 (10H, m); 2.44 (2H, app. t, J-8Hz); 2.64 (2H, app.t, J = 8Hz); 3.18 (2H, m), 3.56 (2H, m); 3.66 (3H, s); 4.50 (2H, app. t, J = 8Hz); 4.83 (1H, m); 5.10 (2H, s); 5.60 (1H, m, rotamers of amide-NH); 6.70–7.26 (2H, m, rotamers of amide-NH); 7.36 (5H, s); 10.20 (1H, s). IR ($CHCl_3$) 3400, 1740, 1680, 1630 cm^{-1}.

N_α–Methoxysuccinylalanyl-N_ε-carbobenzoxy-L-lysyl-L-proline (**29a**)

The title compound was prepared as a colorless powder in 98% yield by an analogous procedure to that described above for the preparation of **7**; Compound **29a** was shown to elute at a lower R_f value when compared to its antipode **29b** (4% $MeOH/CH_2Cl_2$) mp 119–120°C. ^1H NMR ($CDCl_3$) ∂ 1.36 (3H, J = 8Hz); 1.40–2.22 (10H, m); 2.44 (2H app. t, J = 8Hz); 2.46 (2H app.t, J = 8Hz); 3.18 (2H, m); 3.56 (2H, m); 3.66 (3H, s); 4.34–4.5 (3H, m); 4.60–4.64 (lH, m, rotamers of amide-NH); 5.10 (2H, s); 6.70–7.26 (2H, m, rotamers of amide-NH); 7.36 (5H, s), 10.21 (1H, s).

N_α-Methoxysuccinylalanyl-N_ε-carbobenzoxy-L-lysyl-D-prolyl chloromethyl ketone (**30b**)

Isobutyl chloroformate (0.11 g, 0.8 mmol) in THF (2 mL) was added to a cooled solution of **29b** (0.45 g, 0.8 mmol) and N-methyl morpholine (0.81 g, 0.8 mmol) in THF (4.5 mL), and the mixture was stirred for 10 min at –15°C. A chilled solution of previously prepared diazomethane

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(~0.13 g, 3.2 mmol) in Et_2O (20 mL) was added and the mixture was stirred at −10°C for 10 min and then at 5°C for an additional 90 min. Dilution of the reaction with EtOAc (40 mL) was followed by washing with saturated $NaHCO_3$ (2 × 30 mL), water (30 mL) and brine (30 mL), and finally drying over $MgSO_4$. Concentration *in vacuo* afforded a pale yellow oil which was used without further treatment.

Anhydrous hydrogen chloride (HCl) was briefly (30 s) introduced through a solution of the diazomethylene (0.8 mmol) intermediate in EtOAc (10 mL) at 5°C. The resulting solution was stirred an additional 10 min at this temperature, and nitrogen gas was introduced for 5 min to remove excess HCl. Concentration *in vacuo* afforded a pale yellow oil (0.25 g, 54% yield) after purification by column chromatography (silica gel, 5 g, 3% $MeOH/CH_2Cl_2$); mp 46–48°C (from $CHCl_3$) petroleum ether). ^1H NMR($CDCl_3$) ∂ 1.36 (3H, d, J = 8Hz); 1.40–2.22 (10H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.20 (2H, m); 3.56 (2H, m); 3.6 (3H, s); 4.2, 4.32 (2H, center of set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.50 (2H, app. t, J = 8Hz); 4.8 (1H, m); 5.10 (2H, s); 5.60 (1H, m, rotamers of amide-NH); 6.70-7.26 (2H, m, rotamers of amide-NH); 7.6 (5H, s). IR ($CHCl_3$) 3300, 1725, 1635, 1625, 1535, cm^{-1}.

*N_α-Methoxysuccinylalanyl-N_ε-carbobenzoxy-L-lysyl-L-prolyl chloromethyl ketone (**30a**)*

Prepared in similar fashion as above for **30b**, to afford **30a** (52% yield) as a crystalline powder after chromatography, mp 58–60°C. ^1H NMR($CDCl_3$) ∂ 1.32 (3H, d, J = 8Hz); 1.38–2.32 (10H, m); 2.44 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.20 (2H, m); 3.52–3.88 (3H, m); 3.66 (3H, s); 4.20, 4.32 (2H, center of set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.50 (2H, app. t, J = 8Hz); 4.50–4.80 (2H, m); 5.10 (2H, s); 5.42 (1H, m, rotamers of amide-NH); 6.36–7.08 (2H, m, rotamers of amide-NH); 7.35 (5H, s).

*N_α-[Methoxysuccinylalanyl-N_ε-carbobenzoxy-L-lysyl-D-prolylmethyl]-isopropylamine(**31b**)*

Isopropyl amine (1.4 g, 24.2 mmol) was added to a cooled solution (0°C) of **30b** (1.45 g, 2.4 mmol) in THF (5 mL). The reaction mixture was stirred at 0°C for 12 h, filtered, and the filtrate concentrated *in vacuo* to afford a crude yellow oil. Chromatography, (silica gel, 25 g, 7% $MeOH/CH_2Cl_2$) afforded 0.31 g (21% yield) of **31b** as a pale yellow oil. ^1H NMR($CDCl_3$) ∂ 1.06–1.28 (6H, m, rotamers of isopropyl amine); 1.32 (3H, d, J = 8Hz); 1.38–2.32 (10H, m); 2.46 (2H, app.t, J = 8Hz); 2.64 (2H, app.t, J = 8Hz); 3.20 (2H, m); 3.52–3.88 (3H, m); 3.66 (3H, s); 4.20, 4.32 (2H, center of set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.50 (2H, app. t, J = 8Hz); 4.50–4.80 (2H, m); 5.10 (2H, s); 5.42 (1H, m, rotamers of amide-NH); 6.37–7.08 (2H, m, rotamers of amide-NH); 7.35 (5H, s).

*N_α-[Methoxysuccinylalanyl-N_ε-carbobenzoxy-L-lysyl-D-prolylmethyl]-isopropylamine(**31a**)*

Prepared in similar fashion as above for **31b**, to afford **31a** (27% yield) as an oil after chromatography. ^1H NMR ($CDCl_3$) ∂ 1.06–1.28 (6H, m, rotamers of isopropyl amine); 1.32 (3H, d, J-8 Hz); 1.38–2.32 (10H, m); 2.46 (2H, app. t, J = B Hz); 2.64 (2H, app. t, J = 8 Hz); 3.20 (2H, m); 3.52–3.88 (3H m); 3.66 (3H, s); 4.32 (2H, center of set of dd, overlapping with another set, J = 20 Hz, rotamers of CH_2 geminal system); 4.34–4.58 (3H, m); 4.60–4.64 (lH, m); 5.10 (2H, s); 5.42 (1H, m, rotamers of amide-NH); 6.36–7.0 (2H, m, rotamers of amide-NH); 7.35 (5H s).

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p-Nitrophenyl-N_α-[Methoxysuccinylalanyl-$N_\varepsilon$carbobenzoxy-L-lysyl-D-prolylmethyl]-isopropyl carbamate (**24b**)

An ice cooled solution of **31b** (0.3 g, O.5 mmol), N-methylmorpholine (0.07 g, 0.7 mmol), and 4-nitrophenyl chloroformate (0.15 g, 0.75 mmol) in THF (3 mL) was stirred at 0°C for 2 h. The reaction was quenched by dilution with CH_2Cl_2 (15 mL) and washing with water (15 mL), 10% citric acid (15 mL), brine (15 mL) and water (15 mL). The organic layer was next dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford a pale yellow oil. Column chromatography (silica gel, 5 g, CH_2Cl_2 (10 mL); 2% MeOH/CH_2Cl_2 (20 mL); 4% MeOH/CH_2Cl_2 afforded **24b** as a colorless amorphous solid. The product was further purified by preparative tlc (4% MeOH/EtOAc), and was found to elute homogenously. Scraping of the desired band and extraction afforded **24b** (89.9 mg, 23%) as an amorphous solid; mp 44–45°C. ^1H NMR ($CDCl_3$) identical to **24b** from above. Anal. Found: C, 58.52; H, 6.43; N,10.66. Requires $C_{38}H_{50}N_6O_{12}$: C, 58.30; H, 6.44; N, 10.74%.

p-Nitrophenyl-N_α-[Methoxysuccinylalanyl-N_ε- carbobenzoxy-L-lysyl-D-prolylmethyl]-isopropyl carbamate (**24a**)

Prepared in similar fashion as above for **24b**, to afford **24a** (27% yield) as an amorphous solid after chromatography; mp 56–57°C. ^1H NMR ($CDCl_3$) identical to **24a** from above. Anal. Found: C, 58.49; H, 6.47; N,10.63. Requires $C_{38}H_{50}O_{12}$: C, 58.37; H, 6.40; N, 10.81%.

Enzymatic Studies.

PPE, bovine trypsin, chymotrypsin, acetyl cholinesterase and their respective substrates, as well as the substrate for HLE were purchased from the Sigma Chemical Company (St. Louis, MO). Human sputum elastase (HSE)* was purchased from the Elastin Products Company, Inc., Pacific. MO.

Enzyme Assays

All enzyme assays were performed spectrophotometrically at 25°C on a 2200 Varian spectrophotometer. The activity of PPE was measured using t-Boc-L-alanine p-nitrophenyl ester (Boc-Ala-ONP) as the substrate and monitoring the absorbance at 398 nm (*p*-nitrophenol). The activity of HLE was measured using methoxy succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide (MeO-Suc-Ala-Ala-Pro-Val-NA) as the substrate, and following the absorbance at 410 nm (*p*-nitroaniline). Active inhibitors were tested against other serine-dependent proteolytic enzymes such as trypsin and chymotrypsin, using their respective substrates, N-benzoyl-L-arginine ethyl ester, and N-benzoyl-L-tyrosine ethyl ester by monitoring the absorbance at 253 and 256 nm, respectively.

Screening for Inhibitory Activity.

In a typical experiment the inhibitor and the substrate, both in DMSO, were added to buffer in a quartz cuvette, and the system was thermally equilibrated in the spectrophotometer for 2 min. The absorbance was balanced at the desired wavelength. The enzyme was added to sample cuvette, the mixture shaken, and the increase in the absorbance was monitored over a pre-determined time interval.

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HL Elastase.

The substrate, MeO-Suc-Ala-Ala-Pro-Val-NA (10 mM, 0.033 mL) and inhibitor (5.8×10^{-4}M, 0.033 mL were added to two quartz cuvette cells containing the following buffer (1.9 mL): 0.1 M Hepes buffer (pH 7.4) containing 0.05 M NaCl and 10% DMSO, and the system was thermally equilibrated in the spectrophotometer for 2 min. The enzyme (2.1×10^{-5}M, 0.033 mL) dissolved in 0.05 M sodium acetate buffer (pH 5.5), was added to the sample cuvette and mixed for 15 s. The absorbance was monitored at 410 nm and 25°C for 5 min. Inhibitory activity was assumed when the rate of substrate hydrolysis was reduced by 10%. Control experiments were conducted in-which the inhibitor solution was replaced by a solution of DMSO.

PP Elastase.

Two quartz cuvettes containing inhibitor in DMSO (10 mM, 0.1 mL) and substrate in MeOH (5.7×10^{-4}M, 0.1 mL) were diluted with 0.05 M phosphate buffer (pH 6.5) (2.7 mL), mixed for 20 s, and thermally equilibrated in the spectrophotometer for 2 min. A solution of the enzyme (1.0×10^{-5}M, 0.1 mL) was added to the sample cuvette, and the increase in absorbance at 398 nm and 25°C was monitored for 5 min. Inhibitory activity was assumed when the rate of substrate hydrolysis was reduced by 10%. Control experiments were conducted in-which the inhibitor solution was replaced by a solution of DMSO.

Trypsin.

The substrate (5.8×10^{-4}M), Bz-Arg-OEt, was dissolved in 0.01 M Tris-HCl (8.3×10^{-6}M, buffer (pH 8.0) which was also used as the buffer medium. The enzyme was dissolved in 2 M HCl. In a typical assay, buffer (0.2 mL), inhibitor (0.1 mL) and trypsin (0.1 mL) were mixed (20 s) and incubated in the sample cuvette for 2 min. The increase in absorbance at 253 nm at 25°C was monitored for 1 min after the addition of substrate (2.6 mL). Inhibitory activity was assumed when the rate of substrate hydrolysis was reduced by 10%.

Chymotrypsin.

The substrate (1.0×10^{-3}M), Bz-Tyr-OEt, was dissolved in 50% aqueous CH_3OH and the enzyme (3.66×10^{-5}M) was dissolved in 0.001 M HCl. The buffer medium consisted of 0.1 M Tris-HCl containing 0.1 M $CaCl_2$ (pH 7.8). Buffer (1.2 mL), DMSO, or inhibitor solution (9.2×10^{-3}M, 0.1 mL), and substrate solution (1.5 mL) in quartz cuvettes were thermally equilibrated at 30°C. Absorbance was monitored at 256 nm and 30°C for two min after addition of the enzyme (0.1 mL) or water (0.1 mL). Inhibitory activity was assumed when the rate of substrate hydrolysis was reduced by 10%.

Acetyl Cholinesterase.

In a typical experiment, inhibitor solution (1.8×10^{-3}M, 0.1 mL) was added to a quartz sample cuvette and DMSO (0.1 mL) to the reference cuvette containing

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0.1 M $NaHCO_3$ (pH 7.8) buffer (2.5 mL), substrate (acetyl choline hydrochloride, 4.1×10^{-3}M, 0.1 mL) and indicator, m-nitrophenol (5.4×10^{-3}M in buffer, 0.1 mL), and the cuvettes were mixed (20 s) and equilibrated. The increase in absorbance at 420 nm and 25°C was monitored for 30 s. Inhibitory activity was assumed when the rate of substrate hydrolysis was reduced by 10%.

Kinetic Studies

In a typical experiment, the inhibitor in DMSO and the substrate were added to the medium buffer in a quartz cuvette and thermally equilibrated in the spectrophotometer for 2 min and the absorbance was balanced at the desired wavelength. The enzyme was added to the sample cuvette, the mixture shaken for 20 s, and the increase in absorbance monitored for 2 min. K_i values were obtained from Dixon plots and by plotting the slopes from Lineweaver-Burk curves versus inhibitor concentration. In a different experiment (preincubation), the inhibitor and elastase were incubated in buffer in a quartz cuvette at 25°C. At specified time intervals, the substrate was added and the absorbance monitored for 2 min. A control experiment represented 100% enzyme activity was conducted in which DMSO was used in place of inhibitor. Pseudo-first-order rate constants (k_{obs}) for the inhibition were determined from the slope of a plot of percent activity remaining versus incubation time. K_i values were obtained from the intercept of a double reciprocal plot of k_{obs} inhibitor concentration.

PPE

For a typical experiment, 0.1 mL of inhibitor ($0.6–4.6 \times 10^{-4}$M) in DMSO and 0.1 mL of Boc-Ala-ONp ($3–18 \times 10^{-3}$M) in MeOH were added to 0.05 M phosphate buffer (2.7 mL; pH 6.5) in two quartz cuvette cells and the mixture thermally equilibrated for 2 min in the spectrophotometer. After balancing the instrument at 398 nm, PPE (0.1 mL; 1×10^{-5}M) was added to one cell, the mixture shaken for 20 s, and the increase in absorbance was monitored for 5 min. K_i values were determined by plotting the slopes from Lineweaver-Burk vs inhibitor concentration plots.

Preincubation

A reservoir of enzyme and inhibitor comprised of PPE (1.0×10^{-5}M) in buffer (0.8 mL), inhibitor ($0.6–4.6 \times 10^{-4}$M) in 50% DMSO (0.8 mL) and buffer (0.4 mL) were incubated at 37°C. Remaining activity was assessed by sampling (0.2 mL) at predetermined time intervals and adding to a cuvette containing buffer (2.7 mL) and substrate (1.0×10^{-2}M) (0.1 mL) pre-equilibrated in the spectrophotometer at 25°C and mixed for 20 s. The reference cell contained buffer (2.9 mL) and substrate (0.1 mL). The increase in absorbance (398 nm) was measured for 2 min. Pseudo-first-order rate constants (k_{obs}) for the inhibition were determined from the slope of a plot of percent activity remaining versus incubation time. K_i values were obtained from the intercept of a double reciprocal plot of k_{obs} vs inhibitor concentration. In control experiments, DMSO did not effect the rate of hydrolysis of substrate.

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HLE

For a typical experiment, 0.033 mL substrate (0.8-9.3 × 10^{-3}M) and 0.033 mL inhibitor (2.3–18.4 × 10^{-5}M) in DMSO were added to two quartz cells containing 0.1 M HEPES buffer containing 0.05 M NaCl and 10% DMSO (1.9 mL) (pH 7.5). After equilibrating at 25°C for 2 min, the spectrophotometer was balance at 410 nm, and 0.033 mL of the enzyme solution (2.1 × 10^{-5}M) was added with mixing as above for 20 s. Absorbance increases were monitored for 5 min. K_i values were determined by plotting the slopes from Lineweaver-Burk vs inhibitor concentration plots.

Preincubation

In a typical assay HLE (4.6 × 10^{-6}M) (0.033 mL), and inhibitor (1.2-58 × 10^{-6}M) (0.033 mL) were added to a sample cuvette containing buffer (1.9 mL). The cuvettes were equilibrated at 25°C and the absorbance was balanced at 410 nm. At predetermined time intervals, substrate (8.5 × 10^{-3}M) (0.033 mL) was added to the sample cuvette which was then mixed for 15 s. The increase in absorbance was measured for 2 min. Pseudo-first-order rate constants (k_{obs}) for the inhibition were determined from the slope of a plot of percent activity remaining versus incubation time. K_i values were obtained from the intercept of a double reciprocal plot of k_{obs} vs inhibitor concentration. In control experiments, DMSO did not effect the rate of hydrolysis of substrate.

Determination of K_i Values for 50:50 Diastereomeric Mixture of **22a** *and* **22b**

The K_i value was determined as described above when a solution of equal amounts of **22a** and **22b** was used.

Dialysis Experiment

Reagents used for this experiment were as described above for the determination of K_i values for HLE. Briefly, HLE (10 μ mole/0.25 mL 0.05 M NaOAc (pH 5.5) and 0.75 mL 0.1 M HEPES buffer containing 0.05 M NaCl and 10% DMSO (pH 7.5)), was inhibited by **24a** (250 μ mole) by incubation at 25°C for 30 min (99.9% inhibition as determined spectrophotometrically). Samples of this solution (1 mL) were placed in pre-soaked dialysis tubing (MW cutoff 10,000), and then placed directly into the buffer described above (300 mL) at 5°C. The dialysis medium was constantly stirred at this temperature and replaced with fresh buffer every 8 h for 3 days. At predetermined time intervals, the contents of the dialysis bag was removed, placed in a sealed vial and incubated at 25°C for 60 min. Enzyme activity was then determined as above. The results from these dialysis experiment appear in Table 4.

RESULTS

Chemistry

A highly convergent synthetic approach was utilized to produce the tetrapeptidyl carbamates **20–27**. This strategy relied on the independent construction of the P_6–P_3

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fragments (compounds, **5**, **7**, **9**, **12**, **15** and **18**) and the subsequent coupling with the P_2–P_1, moiety. The synthesis of the P_2–P_1, carbamate portion was achieved using the method of Digenis *et al.*. The P_6–P_3 segments of the molecules were synthesized as seen in Scheme 2. Methyl succinimide succinate[7] was allowed to react with either the t-butyl or phenacyl ester of amino acid corresponding to the appropriate P_5 residue to afford the carboxyl protected analogue of P_6–P_4. Hydrolysis of the ester functionality, using either anhydrous HCl for the t-butyl or Zn/HOAc for the phenacyl esters[12], afforded the P_6–P_4 residues (compounds **4**, **10**, **13**, and **16**) in useful yields. Subsequent N-hydroxysuccinimide activation, using DCC, followed by treatment with the esterified P_3 group resulted in the formation of the carboxyl protected heterogenous dipeptides (compounds **5**, **7**, **9**, **12**, **15** and **18**) in moderate to good yields.

Scheme 1 illustrates the method used for the formation of the peptide linkage between the methoxysuccinyl-dipeptides (P_6–P_3) and the P_2–P_1, carbamate moiety[6,7]. This was accomplished utilizing the *in situ* formation of the mixed carbonic anhydride between the carboxyl terminus on P_6–P_3 and isobutyl chloroformate in THF. Acetonitrile was necessary as a co-solvent to insure complete dissolution of the N-methylmorpholine and **19**. Chromatography over silica gel-G was utilized to afford the PCs **20–22** and **24–26** in moderate yields.

The desmosine-like PCs **23** and **27**, were synthesized in a somewhat different manner from compounds **22** and **26** respectively. This was achieved by selective cleavage

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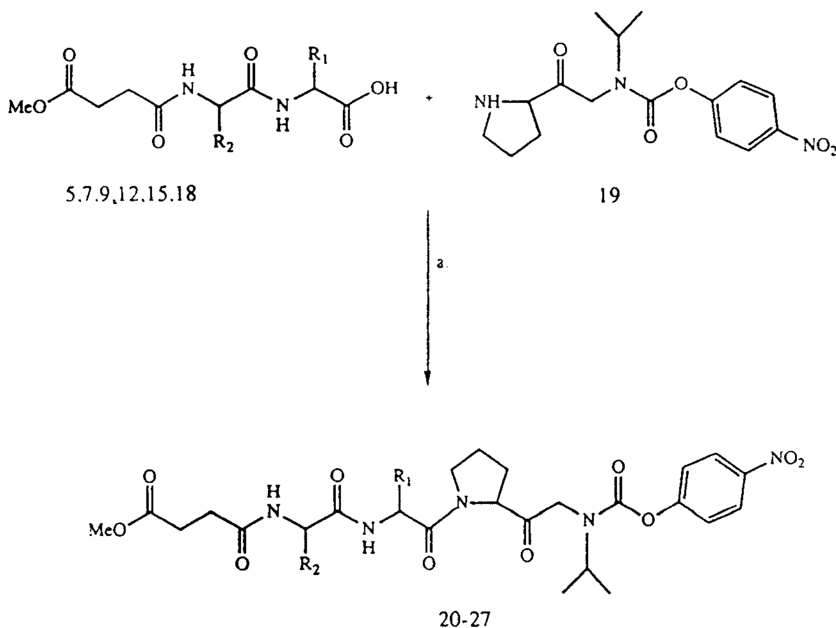

FIGURE 1 Scheme 1. General synthesis of desmonsine-like peptidyl carbamates via coupling of P_6P_3 fragments with P_2-P_1, moeity.

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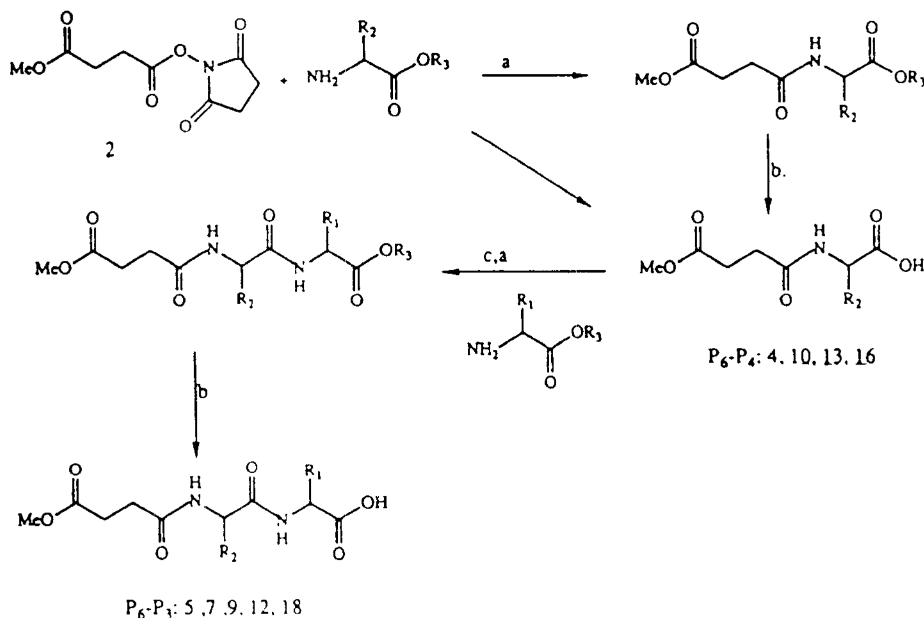

FIGURE 2 Scheme 2. Synthesis of P_6–P_3 fragment of desmosine-like inhibitors.

of the CBZ moiety on the side chain using 30% HBr in glacial acetic acid. The hygroscopic HBr salts were next reacted with benzoyl imidazolide, generated *in situ* from carbonyl diimidazole and benzoic acid, producing the desired products **23** and **27** in low to moderate yields after preparative thin layer chromatography (tlc).

After column chromatography, each of the final products **20–27** was subjected to further purification using preparative tlc. During these final purifications, it was noted that the one band isolated by column chromatography corresponded to two spots of slightly differing R_f values. Isolation of these afforded two amorphous solids, **20a–b** – **27a–b** which were analytically characterized; additionally their inhibitory activity against the enzymes PPE and HLE was assessed. The two compounds, designated as **a** and **b** of each arabic numeral **20–27**, possessed different melting points and ^{1}H-NMR spectra; however, gave similar IR and UV spectra, in addition to identical elemental analyses.

The similarities in spectral properties and elemental analyses led to speculation of the presence of optical isomers. Amino acids are known to retain their chirality under standard conditions utilized in peptide synthesis; however, conversion of the prolyl carboxylate moiety into a ketone (see compound **19**) renders the α-prolyl protons more susceptible to abstraction, thus enhancing racemization under relatively mild acidic or basic conditions. Since HCl gas was used twice in the overall synthesis, chloromethylation of the diazomethyl ketone of t-boc-proline[7], and cleavage of

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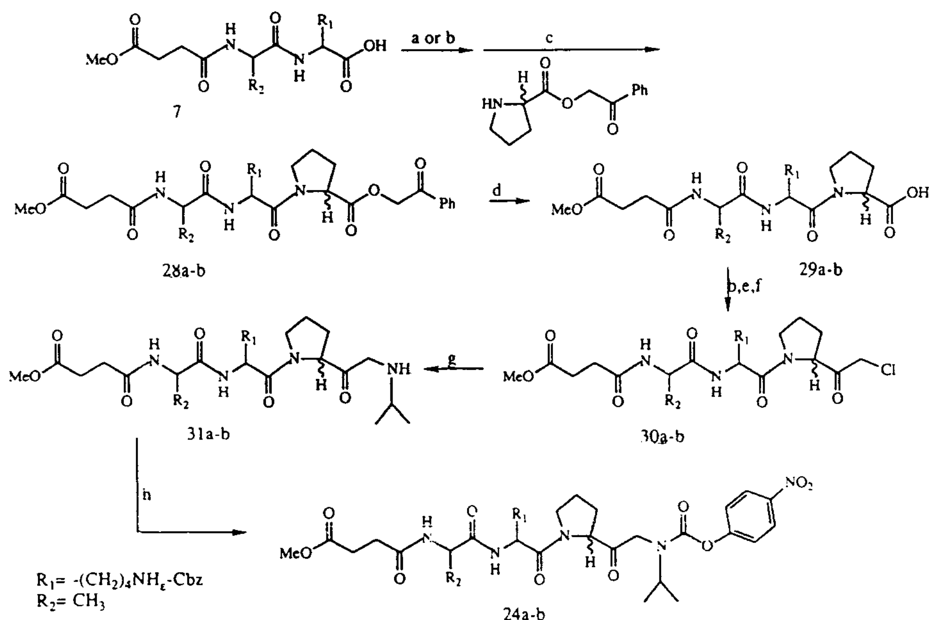

FIGURE 3 Scheme 3. Stereospecific approach to **24a–b**

the t-boc protecting group) it was thought that racemization may have occurred at either or both of these steps. Indeed in an independent experiment, racemization at the α-position of the prolyl ketone was seen to proceed in acidic (HCl) media; however, it was not detected in the presence of a weak non-nucleophilic base (N-methyl morpholine)[13].

Unambiguous assignment of the correct R_f value with the corresponding isomer (LLD $= n$**b** or LLL $= n$**a**; where n represents arabic numerals **20–27**), necessitated that a stereospecific synthesis of compounds and **20a–b** and **24a–b** be achieved. It was interesting that for **24a** (LLL isomer), with the desmosine residue placed at P_3, the R_f value corresponded to that of the slower moving (or lower) constituent. However, **20a** (LLL isomer), having the derivatized alkyl amino functionality at P_4, exhibited inverse chromatographic behavior relative to the above instance, namely the LLL isomer **20a** was now the faster moving (or higher) component. The reason for this dichotomy remains unclear, however, this relative substituent position/chromatographic behavior correlation was used to assign the stereochemistry at the prolyl residue of the obtained derivatives **20a–b** – **27a–b**. Summarizing this trend, products having the desmosine-like function at P_3 were labelled as follows: LLL = lower R_f = **24a–27a**; LLD = upper R_f = **24b–27b**. Using an analogous approach, those derivatized at P_4 were labelled; LLL = upper R_f = **20a–23a**; LLD = lower R_f = **20b–23b**.

Scheme 3 illustrates the methods used for the stereospecific approach to compounds **24a–b**. Construction of the P_6–P_3 fragment of the inhibitors was achieved

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using the phenacyl esters of the respective amino acids, as reported above. The phenacyl ester[12] of either L- or D-proline was then coupled with the P_6–P_3 residue **7**, using either the isobutyl chloroformate mixed anhydride or N-hydroxy succinimide activation strategies. Cleavage of the phenacyl ester[12] (**28a–b**) with Zn/HOAc afforded the free acids **29a–b**, after chromatography, in moderate to excellent yields. Treating the above acids with isobutyl chloroformate and subsequent reaction with diazomethane[14] gave the intermediate diazomethylketones. Conversion of the diazo-functionality into the respective chloromethyl ketones **30a–b** in 50-60% yields was affected by bubbling HCl gas briefly (30 s) into a chilled (5°C) solution of the unstable diazomethylketones. Aminolysis of the chloromethylketones **30a–b** with isopropyl amine gave the unstable aminomethylketones **31a–b** in low but useable yields (20%). The final products (**24a–b**) were prepared by acylation of **31a–b** with 4-nitrophenyl chloroformate in again low but useable yields (20%) after preparative thin layer chromatography. Using an analogous approach, **20a–b** was prepared from P_6–P_3 residue **15** in similar yields (data not shown).

Enzymatic Studies

The inhibitory activities of the peptidyl cabamates listed in Tables 1–2 were evaluated against porcine pancreatic elastase (PPE) and human leukocyte elastase (HLE). Enzymatic assays were conducted spectrophotometrically utilizing the synthetic substrates Boc-Ala-ONP[15] for PPE and MeO-Suc-Ala-Ala-Pro-Val-NA[16,17] for HLE, by following the rate of production of *p*-nitrophenolate anion and *p*-nitroaniline at 398 and 410 nm, respectively.

Initial screening of these novel compounds revealed that only four peptidyl carbamates (**24–27**) inhibited PPE and all eight peptidyl carbamates (**20–27**) exhibited inhibitory activity against HLE. The specificity of these inhibitors towards elastase was illustrated by the lack of inhibitory activity towards the other serine-dependent enzymes such as trypsin and chymotrypsin, as well as acetyl cholinesterase. Specificity within the elastases, ie. exclusive inhibitory activity towards HLE, was achieved by placement of the bulky desmosine-like residue at the P_4 position.

Kinetic studies were conducted for the assessment of the mode and potency of inhibition of active compounds. Inhibition was found to be rapid and irreversible. The dissociation constants of the enzyme-inhibitor complex (K_i) were determined from Dixon plots[18] utilizing four inhibitor concentrations which ranged from 1.9–15.2 $\times 10^{-6}$M for PPE and 3.8-30.4 $\times 10^{-7}$M, as well as four substrate concentrations which ranged from 10–60 $\times 10^{-6}$M for PPE and 1.4–15.4 $\times 10^{-4}$M for HLE. The K_i values were in agreement with those determined from Kitz and Wilson[19] double reciprocal plots of the psuedo-first order rate constant of inhibition (k_{obs}) versus inhibitor concentrations[19]. The k_{obs} values were determined from a separate set of experiments in which the enzyme and the inhibitor were preincubated for different time intervals prior to the addition of substrate.

The mode of inhibition as determined from Dixon[18] and Lineweaver-Burk[20] plots appeared to be of the competitive type (only K_m, the affinity of the enzyme for substrate was affected). During the screening and kinetic analysis of these inhibitors

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TABLE 1
Inhibition of HLE and PPE by Desmosine-like Peptidyl Carbamates: Variations at P_4.

						$K_i(\mu M)^c$	
No.	P_4[a]	R_1[a]	P_3	R_2	isomer[b]	HLE	PPE
20a	N_ε-Cbz-L-Lys	$-(CH_2)_4NHCbz$	L-Ala	$-CH_3$	L	0.47	NI[d]
20b	N_ε-Cbz-L-Lys	$-(CH_2)_4NHCbz$	L-Ala	$-CH_3$	D	7.63	nd[e]
21a	N_ε-Bz-L-Lys	$-(CH_2)_4NHBz$	L-Ala	$-CH_3$	L	0.38	NI
21b	N_ε-Bz-L-Lys	$-(CH_2)_4NHBz$	L-Ala	$-CH_3$	D	3.13	nd
22a	N_δ-Cbz-L-Orn	$-(CH_2)_3NHCbz$	L-Ala	$-CH_3$	L	0.36	NI
22b	N_δ-Cbz-L-Orn	$-(CH_2)_3NHCbz$	L-Ala	$-CH_3$	D	7.95	nd
23a	N_δ-Bz-L-Orn	$-(CH_2)_3NHBz$	L-Ala	$-CH_3$	L	0.19	NI
23b	N_δ-Bz-L-Orn	$-(CH_2)_3NHBz$	L-Ala	$-CH_3$	D	17.70	nd
1[f]	L-Ala	$-CH_3$	L-Ala	$-CH_3$		11.0	42.0

[a] Cbz = carbobenzoxy; Bz = Benzoyl; Lys = Lysine; Orn = Ornithine; Ala = Alanine. [b] configuration at α-prolyl carbon. [c] K_i values determined from Lineweaver Burk slope replots; slopes were obtained from lines possessing linear regression analysis correlations of $r^2 > 0.99$. [d] no inhibition at [I]/[E] = 260. [e] not determined. [f] ref 7.

TABLE 2
Inhibition of HLE and PPE by Desmosine-like Peptidyl Carbamates: Variations at P_3.

						$K_i(\mu M)^c$	
No.	P_4[a]	R_1[a]	P_3	R_2	isomer[b]	HLE	PPE
24a	L-Ala	$-CH_3$	N_ε-Cbz-L-Lys	$-(CH_2)_4NHCbz$	L	0.22	3.61
24b	L-Ala	$-CH_3$	N_ε-Cbz-L-Lys	$-(CH_2)_4NHCbz$	D	11.33	nd[d]
25a	L-Ala	$-CH_3$	N_ε-Bz-L-Lys	$-(CH_2)_4NHBz$	L	0.31	3.85
25b	L-Ala	$-CH_3$	N_ε-Bz-L-Lys	$-(CH_2)_4NHBz$	D	38.5	nd
26a	L-Ala	$-CH_3$	N_δ-Cbz-L-Orn	$-(CH_2)_3NHCbz$	L	1.05	11.28
26b	L-Ala	$-CH_3$	N_δ-Cbz-L-Orn	$-(CH_2)_3NHCbz$	D	19.25	nd
27a	L-Ala	$-CH_3$	N_δ-Bz-L-Orn	$-(CH_2)_3NHBz$	L	2.14	25.0
27b	L-Ala	$-CH_3$	N_δ-Bz-L-Orn	$-(CH_2)_3NHBz$	D	100.0	nd
1[e]	L-Ala	$-CH_3$	L-Ala	$-CH_3$		11.0	42.0

[a] Cbz = carbobenzoxy; Bz = Benzoyl; Lys = Lysine; Orn = Ornithine; Ala = Alanine. [b] configuration at α-prolyl carbon. [c] K_i values determined from Lineweaver Burk slope replots; slopes were obtained from lines possessing linear regression analysis correlations of $r^2 > 0.99$. [d] no inhibition at [I]/[E] = 260. [e] not determined. [f] ref 7.

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TABLE 3
Inhibition of HLE and PPE by Desmosine-like Peptidyl Carbamates: Variations in Configuration at P_2.

No.	P_4[a]	R_1[a]	P_3	R_2	isomer[b]	$K_i(\mu M)$[c] HLE
22a	Nδ-Cbz-L-Orn	$-(CH_2)_3NHCbz$	L-Ala	$-CH_3$	L-L-L	0.36
22b	Nδ-Cbz-L-Orn	$-(CH_2)_3NHCbz$	L-Ala	$-CH_3$	L-L-D	7.95
22ab	Nδ-Cbz-L-Orn	$-(CH_2)_3NHCbz$	L-Ala	$-CH_3$	L-L-DL	0.42

[a])Cbz = carbobenzoxy; Orn = Ornithine; Ala = Alanine. [b])configuration at α-prolyl carbon. [c])K_i values determined from Lineweaver Burk slope replots; slopes were obtained from lines possessing linear regression analysis correlations of $r^2 > 0.99$.

no absorbance was observed at 398 nm (indicating no release of *p*-nitrophenol), even with incubation times approaching 2 h. However, even though no *p*-nitrophenol was produced from the inhibitors, HLE was completely inhibited, indicating that no carbamoylation of the active site serine took place during inactivation.

The reversibility of inhibition of HLE by **24a** was examined by conducting dialysis experiments: the enzyme was incubated with 25-fold excess of inhibitor for 30 min and the reaction mixture was dialyzed. After removal of excess inhibitor, return of activity was monitored as a function of time (Table 4). Dialysis up to 120 h resulted in only 25% restoration of the enzymatic activity. A control experiment illustrated that the activity of the native enzyme was not affected under these dialysis conditions.

TABLE 4
Enzyme Activity Recovered by Dialysis as a Function of Time

Time (h)	% Enzyme Activity Remaining
0.5	0.094
24	0.24
48	3.13
72	7.30
84	16.1
96	18.2
108	28.6*
120	25.0

Data collected from three experiments.* Standard deviation of ±15%, all other samples were ±10%

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DISCUSSION

Many peptidyl-type elastase inhibitors have appeared in the literature. These include di- and tripeptides[21–23], peptide chloromethyl ketones[24–26], peptide aldehydes[27–29] and peptide carbazate esters[30–33] to name a few. More recently peptidyl carbamates were reported[6–8] by our group as being specific elastase inhibitors, the mechanism of inhibition involving the carbamoylation of the active site serine residue of elastase.

The effect of structural variations in the P_3 or P_4 position of peptidyl carbamates were studied by comparing the K_i values for these tetrapeptides on the inhibition of PPE and HLE activity. The potency of one of the compounds was also assessed when the chirality at the P_2 position of the peptidyl carbamates was altered to the unnatural D-isomer.

The design of a peptidyl carbamate containing a desmosine-like amino acid derivative was intended to mimic the natural substrate elastin. The primary result has been improved specificity and inhibitory capacity towards elastase. Thus the desmosine-like peptidyl carbamates have no effect on the substrate hydrolysis capabilities of other serine-dependent enzymes tested ie., trypsin and chymotrpsin as well as acetylcholinesterase.

Insertion of a derivatized amino acid into the P_4 position afforded inhibitors which selectively inhibited HLE without affecting PPE. Thus selectivity among the elastases was also seen. All eight compounds **(20a–b–23a–b)** containing the desmosine-like functionality at the P_4 position were active against HLE (Table 1). The LLL diastereomer in each case was more active than its counterpart possessing the unnatural D-isomer. For example, the K_i values ranged from $0.19–0.47 \times 10^{-6}$M for the LLL diastereomer and from $3.0–18.0 \times 10^{-6}$M for the LLD diastereomer (HLE). Analysis of the results indicated that the benzoyl (Bz) group was preferred over carbobenzyloxy (Cbz) (compare for example **21a** and **23a** with **20a** and **22a**, respectively). An additional structural preference for the shorter alkyl chain of L-ornithine verses the L-lysine residue was observed by inspection of **21a**, **23a** and **20a**, **22a**. Thus combination of the N-benzoyl substituent on the L-ornithine residue in the P_4 position afforded the best HLE inhibitor of this series (**23a**). Among this series of potent and selective HLE inhibitors, the rank order of activity was **23a** > **22a** ≥ **21a** > **20a** > **21b** > **20b** ≥ **22b** > **23b**.

Incorporation of either of the bulky residues into the P_3 position of the peptidyl carbamates, produced inhibitors of PPE and HLE. The inhibitory capacity of the inhibitors in this series was always greater for the HLE than for PPE (Table 2). Although not determined for PPE, K_i values obtained for the LLL isomers were smaller ($0.22–2.14 \times 10^{-6}$M) than the corresponding LLD diastereomer ($11.3–100 \times 10^{-6}$M) when tested against HLE. When the bulky residue was positioned at P_3, both enzymes showed a preference of lysyl over ornithyl which was inverse to substitution at P_4 (compare **24a–b** and **25a–b** with **26a–b** and **27a–b**). Analysis of the results indicated that the carbobenzoxyl (Cbz) group was preferred over benzoyl (Bz) (compare for example **24a** and **26a** with **25a** and **27a**, respectively), which again was in contrast to results from analogues substituted at P_4. The rank order of activity for HLE inhibition for

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P_3 derivitized inhibitors was **24a** > **25a** > **26a** > **27a** > **24b** > **26b** > **25b** > **27b**, and for PPE inhibition it was **24a** > **25a** > **26a** > **27a**.

The observed affinity pattern for derivatized lysine residues in the P_3 and P_4 positions of these inhibitors was in contrast contrary to that reported for elastase substrates[34]. Inspection of Table 2 indicated that HLE inhibitory activity by peptidyl carbamates substituted at P_3 was more dependent on the methylene chain length of the amino acid residue than it was on the choice of terminal amino substituent. For example comparing **24a** and **25a**, which differ only in the terminal amino substituent resulted in only an approximate 1.4 times more potent inhibitor. However, a near order of magnitude difference in potency arose with the addition of one methylene group (**24a** vs **26a**). Substitution at P_4 afforded inhibitors of HLE which were all within the same order of magnitude regardless of chain length or amino substituent. Taken together, the P_3 position of these peptidyl carbamate inhibitors appears to be more sensitive to structural variants, and that the P_4 position seems to be more tolerant. Future analogues, therefore should focus on the functionalization of the P_4 position.

Enzymatic Studies

Steady-state kinetic studies were conducted to determine the dissociation constant of the enzyme-inhibitor complex (K_i). The mode of inhibition as shown from Dixon[18] and Lineweaver-Burke[29] plots was of the competitive type (only K_m affected). Thus the K_i values were obtained from slope and intercept replots. Additional kinetic parameters were evaluated under presteady state conditions because the inhibition seemed to be irreversible during the assay time. These pre-steady state kinetic studies were conducted whereby the enzyme and the inhibitor were preincubated prior to the addition of the substrate.

Initially, the inhibition could be represented by:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_3} E\text{–}I \quad (1)$$

where EI is the noncovalently bound enzyme-inhibitor complex and E-I is the final product with the inhibitor covalently bound to the enzyme. The kinetic data were analyzed according to Kitz and Wilson[19]. The equilibrium constant for inactivation ($K_i = k_2/k_1$ was obtained from a reciprocal plot of the apparent pseudo-first-order rate constant (k_{obs}) versus inhibitor concentration [I] according to the equation:

$$1/K_{obs} = K_i/k_3/[I] + 1/k_3 \quad (2)$$

The values of K_i appear in Tables 1–2.

Time dependent inactivation was obtained by incubation of inhibitor and HLE at 25°C. At specified time intervals, the incubate was assayed for enzyme activity by the addition of substrate, and its hydrolysis was followed spectrophotometically (410 nm). Peptidyl carbamate **24a** (at a concentration of 240 nM) exhibited 50% inhibition of HLE (76 nM) in less than 5 min of preincubation ($k_{obs} = 0.128\ min^{-1}$). The second order rate constant ($k_{obs}/[I]$) for **24a** was 8888 $M^{-1}s^{-1}$. For comparison, the suicide inhibitor,[16] MeO-Suc-Ala-Ala-ProCH$_2$Cl, possessed a $k_{obs}/[I]$ value of 1560 $M^{-1}s^{-1}$.

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Using three inhibitor concentrations (240–960 nM), a reciprocal plot of $-k_{obs}$ vs [I] indicated the possible occurence of reversible complex formation between the enzyme (E) and **24a** (I) prior to irreversible inhibition[19]. Because acylation was not observed during inhibition (no production of *p*-nitrophenol) equation (1) was modified to (3) as a proposed explanation for the observed pattern of inhibition.

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_3} E\text{-}I^* \quad (3)$$

EI now represents the reversibly bound enzyme-inhibitor complex and E-I* is an irreversibly bound enzyme inhibitor complex. Note that E-I* is not the same as E-I from equation (1) and no covalent bond exists. $K_I(k_1/k_2)$ represents the affinity constant obtained from the double reciprocal plot of $-1/k_{obs}$ vs 1/[I]. The affinity constant (K_I) obtained from these plots was slightly greater than the dissociation (K_i) constant ($K_I = 0.73 \times 10^{-6}$ vs $K_i = 0.22 \times 10^{-6}$M) determined by steady-state analysis. Conversion of EI, the reversibly inhibited enzyme, into E-I*, the irreversibly inhibited enzyme, apparently proceeded very rapidly with a determined rate value (k_3) of 0.5 min^{-1}. The overall rate constant for irreversible inhibition was $6.15 \times 10^5 M^{-1} min^{-1}$.

The same experiment conducted with **27a** (concentration of 190 nM) demonstrated a k_{obs} of 0.058 min^{-1} and a $k_{obs}/[I]$ value of 5114 $M^{-1}s^{-1}$. The reciprocal plot of three concentrations (190–950 nM) afforded the following parameters: $K_I = 2.4 \times 10^{-6}$M (vs $K_i = 2.1^4 \times 10^{-6}$M); $k_3 = 0.80$ min^{-1}; and an overall rate of inhibition of $3.28 \times 10^5 M^{-1} min^{-1}$. These parameters are summarized in Table 5.

Presteady state kinetic experiments suggest a rapid equilibrium established between free inhibitor and enzyme. The formation of the irreversible complex occurs at a slower rate rendering the enzyme irreversible inactivated. The observed presteady state affinity constants (K_I) were greater than the steady state dissociation values (K_i) and has been observed previously[19]. Further work on the mechanism of irreversible inactivation of HLE by desmosine-like peptidyl carbamate inhibitors is forthcoming[13].

In conclusion, novel and specific elastase inhibitors are reported that are carbamate esters (R_1R_2NCOOR) where R_1 is a tetrapeptide containing an amino acid (valine) isostere P_1. Incorporation of a desomsine-like residue at either P_3 or P_4 provides highly specific HLE inhibitors with increased albeit irreversible potency than analogues reported to date[6-8]. As a result of this work, more potent inhibitors ($K_i \sim 10^{-9}$M) of HLE have been recently synthesized in these laboratories[34]. These P_4 and P_3 substituted analogues have been shown to prevent corneal ulceration and vascularization after prolonged soft contact lens wear in the rabbit[35].

TABLE 5
Kinetic parameters for inhibitors **24a** and **27a**

No.	$K_i(10^{-6}M)$	$K_I(10^{-6}M)$	$k_{obs}(min^{-1})$	$k_{obs}/[I](M^{-1}s^{-1})$	$k_3(min^{-1})$	Rate $(10^5M^{-1}s^{-1})$
24a	0.22	0.73	0.128	8888	0.50	6.85
27a	2.14	2.4	0.058	5114	0.80	3.28

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