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PEPTIDYL CARBAMATES AS NOVEL ELASTASE INHIBITORS: STRUCTURE-ACTIVITY RELATIONSHIP STUDIES

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Twenty-six novel peptidyl carbamates and thiocarbamates were synthesized and evaluated as elastase inhibitors. Eighteen compounds inhibited porcine pancreatic elastase, whereas only eleven of the newly synthesized compounds inhibited human leukocyte elastase. Neither of the other serine dependent proteases, trypsin or chymotrypsin, were affected by any of the active inhibitors. Structure-activity relationship studies indicated that inhibition was dependent on P_1 and P'_1 substitution as well as on the presence of the carbamate functionality. Placement of an isostere of valine at P_1 and a 1-(phenyl mercaptotetrazole at P'_1 resulted in the most active human leukocyte elastase inhibitor within this series of compounds ($K_i - 3.0 \times 10^{-7}$ M).

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

INTRODUCTION

Synthetic human leukocyte elastase (HLE) inhibitors have recently attracted considerable interest due to the involvement of this enzyme in the tissue destruction associated with such disease states as arthritis, bronchitis, and emphysema.[1-4] Such inhibitors have the advantage of being relatively easily prepared, purified and studied. Furthermore, their structures are amenable to alterations in order to induce more desirable properties. Additionally, being relatively small molecules, they may be targeted to sites that are ordinarily inaccessible to macromolecules.

Our group has been involved in the rational design and study of peptidyl carbamates representing a novel class of synthetic elastase inhibitors.[5-7] These molecules incorporate a peptide portion which affords specificity for the enzyme, in addition

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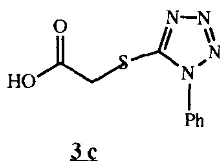
to an electrophilic carbamate functionality (ROCONR′R″) which may carbamoylate the serine residue at the active site of the enzyme.

In continuation of our efforts, this report describes the design and synthesis of a new series of peptidyl carbamates, and the effect of variations in P_1 and P'_1 on their inhibitory activity against elastase.

MATERIALS AND METHODS

Chemistry

Melting points were determined on a Fisher-Johns Uni-Melt apparatus and are uncorrected. Microanalyses were performed by Atlantic Microlab, Inc., Atlanta, Georgia. Analytical results obtained for all compounds were within ±0.4% of the theoretical values unless otherwise stated. ^{1}H-NMR spectra were recorded on a Varian EM-360 (60 MHz) or EM-390 (90 MHz) spectrometer, using tetramethylsilane (TMS) as an internal standard. IR spectra were determined as KBr discs, Nujol mulls or liquid films on a Perkin Elmer 567 spectrophotometer. Reactions were routinely followed by thin layer chromatography (TLC) using Whatman K6F silica gel plates; spots were detected by UV irradiation at 254 nm, iodine, or HBr-Ninhydrin spraying. Column chromatography was carried out using Silica Gel 60 from E. Merck, Darmstadt, Germany. Methyl succinyl-L-alanyl-L-alanine (*6*), and N-(N′-*t*-Boc-L-prolyl)methyl-N-isopropylamine (*2*) were prepared as previously described.[7] 1,2-Dihydro-1-propyl-5H-tetrazole-5-thione was synthesized according to the procedure of Lieber *et al.*[34]



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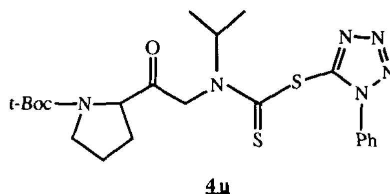

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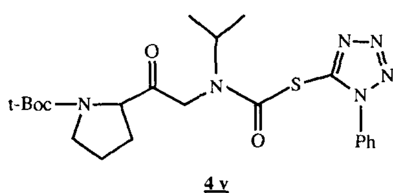

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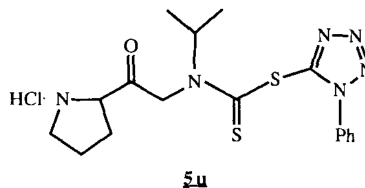

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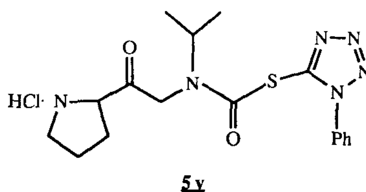

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Table 1 N(N′-*t*-Boc-L-prolyl)methyl-N-alkylamines *2*: Spectral data[a]

t-BOC-N-(pyrrolidine)-C(=O)-CH₂-NHR₁

No.	R_1	Yield[b]	IR cm^{-1c}	^{1}H-NMR (δ, $CDCl_3$)
2a	CH_3	30	3340, 1695	1.40 (s, 9H), 1.7–2.1 (m, 4H), 2.4 (s, 3H), 3.3–3.7 (m, 4H), 4.10–4.50 (m, 1H)
2b	CH_2CH_3	54	3300, 1710, 1690, 1380	0.9 (t, 3H, J = 7 Hz), 1.43 (s, 9H), 1.62–2.31 (m, 6H), 2.67 (t, 2H, J = 7 Hz), 3.33–3.70 (m, 4H), 4.33 (m, 1H)
2c	$CH(CH_3)_2$	80	(ref. 7)	(ref. 7)
2d	$cycC_3H_5$	55	3260, 1720, 1680	0.83 (m, 4H), 1.46 (s, 9H), 2.01–2.3 (m, 6H), 3.36–3.92 (m, 6H), 4.42–4.54 (m, 1H)
2e	$CH_2CH=CH_2$	56	3290, 1690	0.8 (t, 3H), 1.42 (s, 9H), 1.90 (m, 4H), 3.30–3.70 (m, 6H), 4.20 (m, 1H)
2f	$CH_2C\equiv CH$	51	3290, 1690	1.42 (s, 9H), 1.70–2.70 (m, 4H), 2.40 (s, 1H), 3.30–3.70 (m, 6H), 4.20 (m, 1H)
2g	$(CH_2)_3CH_3$	80	3300, 1690	1.01–1.24 (m, 3H), 1.57 (s, 9H), 1.22–1.80 (m, 4H), 1.81–2.24 (m, 4H), 2.66–2.80 (m, 2H), 3.10–3.90 (m, 5H), 4.33 (m, 1H)
2h	$CH_2CH(CH_3)_2$	70	3340, 1700	0.93 (d, 3H, J = 6 Hz), 1.46 (s, 9H), 1.36–1.67 (m, 1H), 2.01–2.20 (m, 4H), 2.43–2.55 (m, 2H), 3.31–4.30 (m, 4H), 4.42 (m, 1H)
2i	cyc-C_6H_{11}	44	3320, 1690	0.9–2.51 (m, 15H), 1.47 (m, 9H), 3.31 (m, 4H), 4.33 (m, 1H)
2j	CH_2Ph	64	3310, 1700, 1605, 1585	1.43 (s, 9H), 1.96–2.10 (m, 4H), 3.30–4.03 (m, 6H), 4.33 (m, 1H), 7.36 (s, 5H)

[a]Compounds were isolated as oils. [b]Yields from the chloromethylketone *1*. [c]Film.

*N-(N′-*t*-Boc-L-prolyl)methyl-N-methylamine (2a)*

Sodium iodide (635 mg, 4.23 mmol) and 40% aqueous methyl amine (6.9 mL, 80.6 mmol) were added to an ice-cooled solution of chloromethyl ketone (*1*) (2.0 g, 4.03 mmol), in EtOH (10 mL). The mixture was shaken in a steel bomb at 65 °C for 12 h, after which solvent was removed *in vacuo*. To the residue, was added saturated $NaHCO_3$ (5 ml) and saturated NaCl (5 mL) solutions, and the resulting mixture was extracted into ether. The volatiles were evaporated and the residue was purified by column chromatography, eluting with $CHCl_3$: MeOH (50:1) to afford 290 mg (30%) of *2a*. Spectral data are listed in Table 1.

*N-(N′-*t*-Boc-L-prolyl)methyl-N-alkylamines (2b–2j)*

General procedure. To an ice cooled solution of chloromethyl ketone *1* (12.1 mmol) in ether (40 mL), was added an excess of the appropriate amine (60 mmol). The solution was allowed to warm to room temperature and stirring was continued overnight. The amine hydrochloride salt was filtered and washed with ether. Concentration of the filtrate *in vacuo* and purification by column chromatography (silica gel 45 g, 2% MeOH-$CHCl_3$) afforded compounds *2b–2j* as colorless oils. The products (*2b–2j*) thus obtained were identified by their IR and NMR spectra. Yields and spectral data are listed in Table 1.

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p-Nitrophenyl-N-(N'-Boc-L-prolyl)methyl-N-alkyl carbamate (4a–4h)
General procedure. A mixture of the alkylaminomethyl ketones *2* (3.76 mmol) in THF (15 mL) and triethylamine (TEA) (0.62 mL, 4.7 mmol) was cooled to 5 °C. A solution of *p*-nitrophenyl chloroformate (0.75 g, 3.76 mmol) in THF (5 mL) was added dropwise over a period of 15 min. The reaction mixture was stirred at 5 °C for 5 h and diluted with CH_2Cl_2 (14 mL). The organic layer was washed with 10% citric acid (3 mL), then water (5 mL), and dried over $MgSO_4$. Filtration and concentration *in vacuo* was followed by isolation of the desired materials by silical gel column chromatography (CH_2Cl_2). The oily carbamates (*4a–4h*) thus obtained were identified by their IR and NMR spectra. Yields and spectral data appear in Table 2.

S-1-Methyl-5-tetrazolyl chlorothioformate 3a
Triethylamine (0.62 mL, 4.64 mmol) in THF (2 mL) was added dropwise to a solution of 1-methyl-5-mercaptotetrazole (450 mg, 3.87 mmol) in THF (5 mL) and 12.5% w/w phosgene in benzene (4.7 mL, 6 mmol) at 0–5 °C. The reaction mixture was stirred at 0–5 °C for 30 min and at room temperature for 2 h. Filtration of the mixture and

Table 2 *p*-Nitrophenyl-N-(N'-*t*-Boc-prolyl)methyl-N-alkyl carbamates *4*: Spectral data

No.	R_1	Yield[a]	IR cm^{-1}[b]	^{1}H-NMR (δ, $CDCl_3$)
4a	CH_3	52	1740–1670, 1615 1590, 1520, 1497	1.49 (s, 9H), 1.8–2.2 (m, 4H), 3.09–3.21 (brs, 3H), 3.40–3.80 (m, 2H), 4.30–4.60 (m, 3H), 7.00–7.20 (m, 2H), 8.20–8.50 (m, 2H)
4b	$CH_2CH_2CH_3$	52	1740, 1700, 1670, 1610	0.93 (t, 3H, J = 7 Hz), 1.46 (s, 9H), 1.55–2.50 (m, 6H), 3.56–3.70 (m, 4H), 4.32–4.62 (m, 3H), 7.56 (d, 2H, J = 8 Hz), 8.40 (d, 2H, J = 8 Hz)
4c	$CH(CH_3)_2$	69	(ref. 7)	(ref. 7)
4d	$CH_2C{\equiv}CH$	98	1730, 1680, 1610, 1590	1.43 (s, 9H), 2.02–2.33 (m, 4H), 2.43 (s, 1H), 3.64–4.82 (m, 7H), 7.43 (d, 2H, J = 8 Hz), 8.33 (d, 2H, J = 8 Hz)
4e	$cycC_3H_5$	45	1740, 1720, 1700, 1610, 1590	0.86–0.95 (m, 4H), 1.53 (s, 9H), 1.90 (m, 4H), 1.74–2.56 (m, 4H), 3.00–3.70 (m, 1H), 3.32–3.56 (m, 2H), 4.42–4.72 (m, 3H), 7.43 (d, 2H, J = 8 Hz), 8.33 (d, 2H, J = 8 Hz)
4f	$CH_2CH(CH_3)_2$	93	1730, 1615, 1595	0.96 (d, 6H, J = 6 Hz), 1.43 (s, 9H), 1.33–1.60 (m, 1H), 2.00–2.31 (m, 4H), 3.12–3.82 (m, 4H), 4.13–4.16 (m, 3H), 7.31 (d, 2H, J = 8 Hz), 8.33 (d, 2H, J = 8 Hz)
4g	cyc-C_6H_{11}	80	1725, 1690, 1612, 1590	0.95–2.30 (m, 15H), 1.47 (s, 9H), 3.33–4.06 (m, 3H), 7.13 (d, 2H, J = 8 Hz), 8.33 (d, 2H, J = 8 Hz)
4h	CH_2Ph	56	1730, 1690, 1615, 1595	1.43 (s, 9H), 1.70–2.30 (m, 4H), 3.54–3.57 (m, 2H), 4.02–5.00 (m, 5H), 7.31 (d, 2H, J = 8 Hz), 8.33 (d, 2H, J = 8 Hz)

[a]Yields from compoundd *2*. [b]Film.

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concentration of the filtrate *in vacuo* gave the title compound *3a* as an amorphous powder (0.62 g, 3.4 mmole, 90%); IR (film) 1770 cm^{-1}; NMR ($CDCl_3$) δ 4.23 (s).

S-1-Methyl-5-tetrazolyl N-(N'-5-Boc-L-prolyl)methyl-N-alkylthiocarbamates (4i–4m) General procedure. To an ice-cooled solution of the appropriate alkylaminomethyl ketone *2* (7.2 mmol) and TEA (1.24 mL, 9.36 mmol) in THF (15 mL) was added a suspension of 1-methyl-5-tetrazoyl chlorothioformate *3a* (1.67, 9.36 mmol) in THF (10 mL) over a period of 10 min. the reaction mixture was stirred for 1 h at 5 °C, diluted with cold water (12 mL) and extracted with $CHCl_3$ (30 mL). The organic layer

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Table 3 N-(N'-*t*-Boc-prolyl)methyl-N-alkyl thiocarbamates *4*: Spectral data

t-BOC–N(pyrrolidine)–C(=O)–CH₂–N(R₁)–C(=O)–XR₂

No.	R_1	XR_2[b]	Yield[c]	IR cm^{-1}[d]	¹H-NMR (δ, $CDCl_3$)
4i	$CH(CH_3)_2$	SMT	74	(ref. 7)	(ref. 7)
4j	$CH_2CH_2CH_3$	SMT	24	1730, 1680	1.05 (t, 3H, J = 7 Hz), 1.46 (s, 9H), 1.63–2.32 (m, 6H), 3.23–3.72 (m, 4H), 4.17 (s, 3H), 4.30–4.60 (m, 3H)
4k	$cycC_3H_5$	SMT	30	1730, 1680	1.01–1.23 (m, 4H), 1.45 (s, 9H), 1.74–2.35 (m, 4H), 2.98 (m, 1H), 3.53–3.86 (m, 2H), 4.06 (s, 3H), 4.34 (m, 3H)
4l	$CH_2CH{=}CH_2$	SMT	40	1735, 1685	1.47 (s, 9H), 2.02–2.30 (m, 4H), 3.5 (m, 2H), 4.13 (s, 3H), 4.40–4.62 (m, 5H), 5.10–5.50 (m, 2H), 5.60–5.90 (m, 1H)
4m	$(CH_2)_3CH_3$	SMT	35	1735, 1670	0.96 (t, 3H, J = 6 Hz), 1.46 (s, 9H), 1.20–1.85 (m, 4H), 2.03–2.42 (m, 4H), 3.24–3.71 (m, 4H), 4.07 (d, 3H), 4.20–4.65 (m, 3H)
4n	$CH(CH_3)_2$	SPT	74	(ref. 7)	(ref. 7)
4o	$CH_2CH{=}CH_2$	SPT	81	1740, 1685, 1590	1.43 (s, 9H), 1.95–2.32 (m, 4H), 3.36–4.53 (m, 7H), 5.02–5.54 (s, 2H), 5.70–6.30 (m, 1H), 7.55 (s, 5H)
4p	$CH_2C{\equiv}CH$	SPT	92	3290, 1720, 1680	1.43 (s, 9H), 1.80–2.60 (m, 5H), 3.33–3.85 (m, 2H), 4.02–4.75 (s, 5H), 7.80 (s, 5H)
4q	$CH(CH_3)_2$	SPrT	30	(ref. 7)	(ref. 7)
4r	$CH(CH_3)_2$	SBzT	55	1735, 1680	1.27 (d, 6H, J = 7 Hz), 1.56 (s, 9H), 1.82–2.22 (m, 4H), 3.50 (m, 2H), 4.05–4.72 (m, 4H), 7.23–7.66 (m, 2H), 7.84–8.24 (m, 2H)
4s	$CH(CH_3)_2$	SPyr	40	1735, 1690, 1615	1.23 (d, 6H, J = 7 Hz), 1.53 (s, 9H), 1.90–2.36 (m, 4H), 3.30–3.81 (m, 2H), 4.19–5.06 (m, 4H), 7.33–7.65 (m, 1H), 7.82–8.05 (m, 2H), 8.72 (d, 1H, J = 8 Hz)
4t	$CH(CH_3)_2$	TTC	50	1690, 1615	0.93–1.54 (m, 6H), 1.46 (s, 9H), 1.84–2.21 (m, 4H), 3.13–3.91 (m, 4H), 3.94–5.01 (m, 6H)

[a]Compounds *2* isolated as oils. [b]SMT = N-methyl-2-thiotetrazolyl; SPT = N-phenyl-2-thiotetrazolyl; SPrT = N-propyl-2-thiotetrazolyl; SBzT = S-2-thiazolyl; S-2-pyridyl; TTC = 2-thioxo-3-thiazolidinyl. [c]Yields from *1*. [d]Film.

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was separated, dried over $MgSO_4$, filtered and evaporated. Purification of the residue by column chromatography (silica gel, $CHCl_3$) afforded the corresponding N-alkylthiocarbamates *4i–4m* as an oil. The oils were induced to crystallize from ether or EtOAc-hexane. Compounds *4i–4m* thus obtained were identified by IR, NMR and elemental analyses, and these results appear in Table 3.

S-1-Phenyl-5-tetrazolyl chlorothioformate (3b)

1-Phenyl-1H-tetrazol-5-thiol (2.44 g, 13.7 mmol) was added to a solution of TEA (2.0 mL, 15.1 mmol) in Et_2O (15 mL) and CH_3CN (8 mL) at room temperature. The resulting turbid solution was added dropwise over 10 min to 20% w/w phosgene in toluene (13.5 mL, 2 mmol) at $-10\,^{\circ}C$. The mixture was stirred at $5\,^{\circ}C$ for 40 min. The precipitate was filtered and washed with THF (10 mL). Concentration of the filtrate gave compound *3b* (3.3 g, 11.36 mmol, 64%) as an oil which solidified on standing; IR (film) 1775, 1720, 1640 cm^{-1} ^1H-NMR ($CDCl_3$) δ 7.41–7.83 (m). Compound *3b* was used in the next step without further purification.

S-(1-phenyl-5-tetrazoyl) N-(N′-Boc-L-prolyl)methyl N-alkylthiocarbamates (4n–4p)

General procedure. To a solution of the appropriate alkylaminomethyl ketone *2* (3.0 mmol) and TEA (0.48 mL, 3.6 mmol) in THF (14 mL) at $-10\,^{\circ}C$ was added, in dropwise manner, a solution of chloroformate *3b* (1.3 g, 5.4 mmol) in CH_3CN (12 mL) over 12 min. The reaction mixture was stirred at $5\,^{\circ}C$ for 30 min and at room temperature for an additional 2 h. After filtration of the solids, the filtrate was diluted with CH_2Cl_2 (14 mL). The organic layer was washed with water (5 mL), dried over $MgSO_4$, filtered, and concentrated. The oily residue was purified by silica gel column chromatography ($CHCl_3$) to give the alkylthiocarbamate (*4n–4p*) as a colorless oil. Spectral data and yields are listed in Table 3.

S-1-Phenyl-5-tetrazolylthioacetic acid (3c)

To an ice-cooled suspension of 1-phenyl-1H-tetrazol-5-thiol (2.0 g, 11.2 mmol) and TEA (1.80 mL, 13.5 mmol) in THF (15 mL), was added, dropwise, a solution of chloroacetic acid (1.06 g, 11.2 mmol) in THF (10 mL). The reaction mixture was stirred at $5\,^{\circ}C$ for 2 h, was allowed to warm to room temperature, and stirring was continued overnight. The precipitate was collected and dissolved in water (10 mL). Acidification of the aqueous solution with concentrated HCl afforded a precipitate which was extracted with EtOAc. The organic layer was dried ($MgSO_4$), filtered, and the solvent was concentrated *in vacuo* until precipitation ensued. Filtration and washing with a small amount of EtOAc gave the title compound *3c* (2.1 g, 8.9 mmol, 80%); IR (Nujol) 3000, 2500, 1715, 1585 cm^{-1}; ^1H-NMR ($CDCl_3$) δ 4.33 (s, 2H), 7.66 (s, 5H), 9.59 (1H, brs), which was used in subsequent reactions without further purification.

N-(N′-Boc-prolyl)methyl-N-isopropyl(1-phenyl-5-tetrazolyl) thioacetamide (4v)

To an ice-cooled solution of thioacetic acid *3c* (2.0 g, 8.46 mmol) and N-methylmorpholine (NMM) (1.10 mL, 10.0 mmol) in THF (7 mL) was added a solution of isobutylchloroformate (1.15 mL, 8.40 mmol) in THF (2 mL) with stirring over 15 min. A solution of N-(*t*-Boc-L-prolyl)methyl-N-isopropylamine *2c* (2.28 g, 8.46 mmol), and N-methylmorpholine (1.10 mL, 10.0 mmol) in THF (5 mL) was then added, and the reaction mixture was stirred at $5\,^{\circ}C$ for 2 h and at room temprature for an

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additional 3.5 h. After filtration of the precipitated salts, the filtrate was diluted with CH_2Cl_2 (20 ml) and washed with water (12 mL). The organic layer was dried over $MgSO_4$, filtered, and the solvent evaporated to dryness. Thioacetamide *4v* was isolated by silica gel column chromatography ($CHCl_3$) as a colorless oil (1.7 g, 3.4 mmol, 41.1%); IR (Film) 1735, 1685, 1635, 1590 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.23 (d, 6H), 1.43 (s, 9H), 2.01–2.33 (m, 4H), 3.53 (m, 2H), 4.02–4.51 (m, 4H), 4.66 (s, 2H), 7.66 (s, 5H).

1-Phenyl-5-tetrazolyl chlorodithioformate (3d)
To an ice-cooled solution of thiophosgene (1.29 g, 11.2) in THF (40 mL) was added a solution of 1-phenyl-1H-tetrazole-5-thiol (2.0 g, 11.2 mmol) and TEA (1.78 mL, 13.4 mmol) in THF (30 mL). The reaction mixture was stirred at 5 °C for 30 min and for an additional 1.5 h at room temperature. Filtration and concentration of the filtrate *in vacuo* gave compound *3d* as a solid (0.912, 3.1 mmole, 27.6%); IR (Nujol) 1725, 1585 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 7.44–7.83 (m). The crude powder was used in the next reaction without purification.

1-Phenyl-5-tetrazolyl N-(N′-5-Boc-L-prolyl)methyl-N-isopropyldithiocarbamate (4u)
To an ice-cooled solution of isopropylaminomethylketone *2c* (1.30 g, 4.81 mmol) and TEA (0.75 mL, 5.7 mmol) in THF (14 mL) was added dropwise a solution of the dithioformate *3d* (1.40 g, 4.77 mmol) in THF (14 mL). The solution was stirred at 5 °C for 2 h and at room temperature for 2 h. The precipitate was filtered and washed with THF (14 mL). Concentration of the filtrate and isolation of the desired compound by chromatography (silica gel, $CHCl_3$) gave isopropyldithiocarbamate *4u* as an oil (1.30 g, 2.66 mmol, 51.8%); IR (Film) 1740, 1690, 1595, 1395 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.00–1.63 (m, 6H), 1.50 (s, 9H), 1.81–2.30 (m, 4H), 3.30–4.80 (m, 6H), 7.63 (s, 5H).

S-(1-Propyl-5-tetrazolyl) chlorothioformate (3e)
A solution of 5-mercapto-1-propyltetrazole (2.5 g, 17.4 mmol) and TEA (1.33 mL, 10.1 mmol) in CH_3CN (10 mL) was added dropwise to a solution of phosgene (2.58 g, 2.60 mmol) in toluene (13.5 mL) at 5 °C over a period of 50 min. The reaction mixture was stirred at 5 °C for an additional 50 min. The precipitate was filtered, washed with THF (14 mL), and the filtrate was evaporated to give thioformate *3e* as an oil (3.1 g, 15.0 mmol, 86.2%); IR (Film) 1775, 1720 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.00 (t, 3H, J = 7Hz), 2.10 (m, 2H), 4.50 (m, 2H). The crude product *3e* was used in the next reaction without purification.

S-(2-Pyridyl) chlorothioformate (3f)
A solution of 2-mercaptopyridine (1.50 g, 13.5 mmol) and TEA (2.4 mL, 18.0 mmol) in THF (24 mL) was added dropwise to a solution of phosgene (1.53 g, 15.0 mmol) in toluene (8 mL) at 5 °C over 5 min. The reaction mixture was stirred at 5 °C for 5 min and at ambient temperature for 5 min. The resulting turbid mixture was then filtered and the filtrate evaporated to dryness. Trituration of the residue with Et_2O and evaporation to dryness was done twice to remove excess phosgene. Chlorothioformate *3f* (1.5 g, 8.6 mmol, 63.9%) was obtained as an oil; IR (Neat) 1765 cm^{-1}, compound *3f* was used in the next reaction without further purification.

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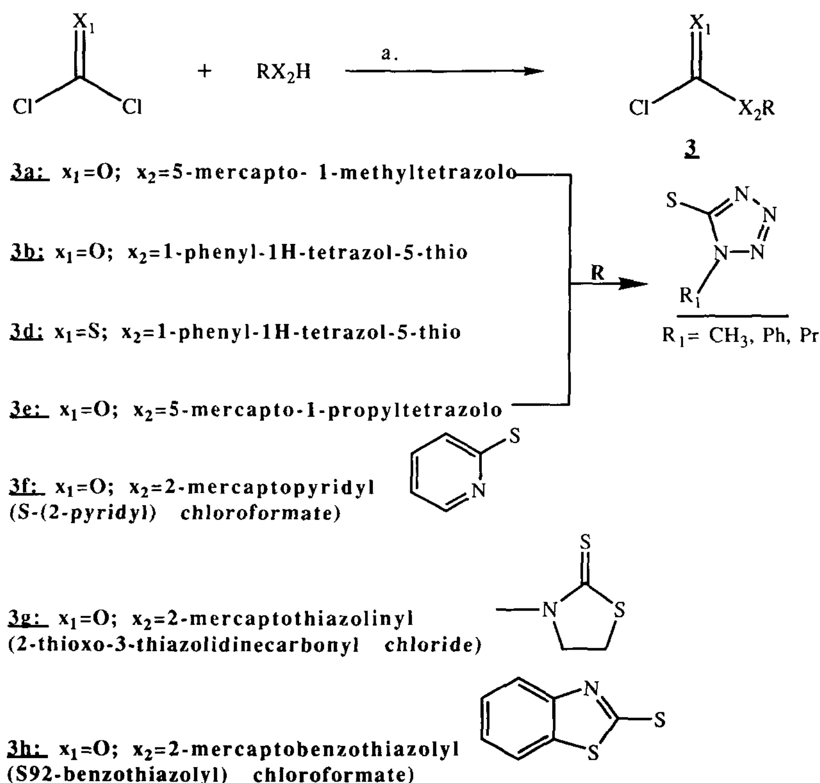
2-Thioxo-3-thiazolidinecarbonyl chloride (3g)

A solution of 2-mercaptothiazoline (2.0 g, 16.8 mmol) and TEA (2.8 mL, 21.0 mmol) in THF (30 mL) was added dropwise to a solution of phosgene (2.37 g, 23.9 mmol) in toluene (12 mL) at 5 °C over 7 min. The mixture was stirred at 5 °C for 5 min and the filtrate evaporated to produce a solid. Et_2O (10 mL) was added to the residue and the solvent removed *in vacuo*. This operation was performed twice to ensure removal of phosgene. 2-Thioxo-3-thiazolidine-carbonyl chloride *3g* (2.0 g, 11.0 mmol, 65.5% yield) was obtained as a crude solid; IR (Nujol) 1780 cm^{-1}, and was used in the reaction without purification.

S-(2-Benzothiazolyl)chlorothioformate (3h)

A solution of 2-mercaptobenzothiazole (0.669 g, 4.0 mmol) TEA (0.60 mL, 4.4 mmol) in THF (5 mL) was added to a solution of phosgene 20% w/w (0.59 g, 6.0 mmol) in toluene (3.1 mL) at 5 °C over a period of 15 min. The reaction mixture was diluted with CH_3CN (3 mL) and stirring continued at 5 °C for an additional 30 min. The precipitate was filtered and washed with THF. The filtrate and washings were evaporated to give crude chlorothioformate *3h*, (0.9 g, 3.9 mmol, 97%); IR (Nujol) 1775 cm^{-1}, which was used in the next step without further purification.



Scheme I Synthesis of chloroformate and chlorothioformate derivatives *3*

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S-(N-(N'-Boc-L-prolyl)methyl-N-isopropylthiocarbamates (4g–4t)

General procedure. To an ice-cooled solution of isopropylaminomethyl ketone *2* (2.39 g, 8.8 mmol) and TEA (1.91 g, 14.4 mmol) in CH_3CN (10 mL) was added a solution of the appropriate chlorothioformate (*3e–3h*) (12.0 mmol) in CH_3CN (8 mL) or THF (12 mL). The mixture was stirred at 5 °C for 1 h and at room temperature for 2 h. The reaction mixture was evaporated affording a residue which was dissolved in $CHCl_3$. The organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and the solvent evaporated. The residue was purified by column chromatography (silica gel, 10% $EtOAc/CHCl_3$) affording the desired product as a colorless oil. The thiocarbamates obtained in this manner were identified by IR and ^{1}H-NMR spectral analyses. Yields and spectral data appear in Table 3.

p.*Nitrophenyl-N-(L-prolylmethyl)-N-alkylcarbamate hydrochlorides (5a–5h)*

General procedure. Hydrogen chloride gas was bubbled through a solution of the appropriate *p*-nitrophenylcarbamate (*4a–4h*) in Et_2O (30 mL) at 25 °C for 3 min. The solution was allowed to stand at room temperature for 1.5 h after which the solvent

Table 4 *p*-Nitrophenyl-N-(L-prolyl)methyl-N-alkyl carbamate hydrochlorides *5*: Spectral data

No.	R_1	Yield[a]	Mp (°C)	IR cm^{-1}[b]	^{1}H-NMR (δ, DMSO-d_6)
5a	CH_3	74	177–181 (dec)	3530, 3440, 1735 1612, 1592, 1515	1.7–2.3 (m, 4H), 2.90–3.70 (m, 5H), 4.40–4.80 (m, 3H), 7.30–7.70 (m, 2H), 8.20–8.50 (m, 2H)
5b	$CH_2CH_2CH_3$	80	155–160	3400, 1720, 1610, 1590, 1520	0.99 (t, 3H, J = 9 Hz), 1.18–2.27 (m, 6H), 3.02–3.75 (m, 4H), 4.36–4.80 (m, 3H), 7.46 (d, 2H, J = 9 Hz), 8.37 (d, 2H, J = 9 Hz)
5c	$CH(CH_3)_2$	78	165–174	(ref. 7)	(ref. 7)
5d	$CH_2C{\equiv}CH$	30	141–152	3350, 1740, 1720, 1620, 1590, 1515	2.06 (m, 4H), 2.46 (bs, 1H), 3.30–3.72 (m, 2H), 4.05–4.82 (m, 5H), 7.40 (d, 2H, J = 8 Hz), 8.30 (d, 2H, J = 8 Hz)
5e	cycC_3H_5	63	150–153	1720, 1610, 1590, 1515	0.81 (m, 4H), 1.75–2.33 (m, 4H), 2.90 (m, 1H), 3.23 (m, 2H), 4.46 (m, 3H), 4.66 (m, 3H), 7.46 (d, 2H, J = 9 Hz), 8.33 (d, 2H, J = 8 Hz)
5f	$CH_2CH(CH_3)_2$	47	135–149	3500, 1740, 1710 1615, 1595	0.95 (d, 6H, J = 6 Hz), 1.37–2.21 (m, 7H), 3.04–3.61 (m, 4H), 4.17–4.82 (m, 3H), 7.43 (d, 2H, J = 9 Hz), 8.36 (d, 2H, J = 9 Hz)
5g	cycC_6H_{11}	77	146–158	1720, 1612	1.17–2.61 (m, 14H), 3.39–4.94 (m, 6H), 7.47 (dd, 2H, J = 2, J = 8 Hz), 8.43 (d, 2H, J = 2, J = 8 Hz)
5h	CH_2Ph			1730, 1670	2.05 (m, 4H), 3.30 (brs. 2H), 4.21–5.04 (m, 5H), 7.33–7.42 (m, 7H, J = 8 Hz), 8.33 (d, 2H, J = (Hz)

[a]Yields from compounds *4*. [b]Nujol mull.

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was evaporated *in vacuo*. Excess acid was removed by adding EtOAc followed by removal of volatiles. The solid carbamate were filtered and washed with ether. The alkyl carbamate hydrochlorides *5a–5h* were identified by their IR and ^{1}H-NMR spectra. Yields, melting points, and spectral data are listed in Table 4.

1-Methyl-5-tetrazolyl-(N-L-prolylmethyl)N-alkylthiocarbamate hydrochlorides (5i–5m)
General procedure. Hydrogen chloride gas was bubbled through a solution of the appropriate N-methyltetrazolothiocarbamate (*4i–4m*) in EtOAc/THF (30 mL) for 3 min. The solution was allowed to stand at room temperature for 5 min. Removal of the solvent *in vacuo* followed by trituration with Et_2O gave the desired thiocarbamate hydrochloride as an amorphous powder. Compounds (*5i–5m*) were identified from their IR and ^{1}H-NMR spectra. Yields, melting points and spectral information are listed in Table 5.

1-Phenyl-5-tetrazolyl-N-(L-propylmethyl)-N-alkylthiocarbamate hydrochlorides (5n–5p)
General procedure. Hydrogen chloride gas was bubbled through a solution of the appropriate alkyl thiocarbamate (*4n–4p*) in Et_2O (30 mL) at ambient temperature. The solution was stirred for 15 min and evaporated to dryness. The resulting residue was triturated with Et_2O to afford the alkyl thiocarbamate hydrochloride (*5n–5p*) as a hygroscopic powder. Yields and analytical results appear in Table 5.

N-(L-prolylmethyl)-N-isopropylthiocarbamate hydrochloride (*5q–5s, 5u–5v*)
General procedure. Hydrogen chloride gas was bubbled through a solution of the appropriate thiocarbamate (*4q–4s, 4u–4v*) in EtOAc (7 mL) at 5 °C for 5 min. The solution was allowed to stand at room temperature for 10 min and then concentrated to dryness. EtOAc (15 mL) was added to the oily residue and the solvent evaporated. This operation was repeated three times, after which the residue was triturated with Et_2O to give a powder which was filtered and washed with Et_2O. The resulting thiocarbamate hydrochlorides were identified by their IR and ^{1}H-NMR spectra (Table 5). Yields, and melting also appear in Table 5.

p-*Nitrophenyl-N-[(methoxysuccinyl)-L-alanyl-L-alanyl-L-prolyl methyl]-N-isopropyl carbamate (7c)*[7]
To a stirred mixture of *6* (213 mg, 0.778 mmol) and N-methylmorpholine (86 μL, 0.778 mmol) in THF (2.5 mL) cooled to −15 °C to −30 °C, was added dropwise, a solution of isobutyl chloroformate (101 μL, 0.778 mmol) in THF (1 mL) and the mixture stirred for 30 min. A solution of *5c* (244 mg, 0.779 mmol), N-methylmorpholine (86 μL, 0.778 mmol), and bis(trimethylsilyl)-acetamide (1 mL) in THF (3 mL) was then added dropwise to the stirred mixture which had been previously cooled to −50 °C. The reaction mixture was allowed to warm gradually, stirred overnight at room temperature, diluted with $CHCl_3$ (20 mL), washed successively with 10% citric acid and 4% $NaHCO_3$ solutions, and evaporated. The resulting oil was purified by column chromatography ($CHCl_3$/MeOH, 50:1) to give colorless crystals of *7c* (83%).[7]

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Table 5 N-(L-prolyl)methyl-N-alkyl thiocarbamates *5*: Spectral Data

No.	R_1	XR_2[a]	Yield[b]	Mp (°C)	IR cm^{-1}[c]	^{1}H-NMR (δ, DMSO-d_6)
5i	$CH(CH_3)_2$	SMT	52	145–153	(ref. 7(	(ref. 7)
5j	$CH_2CH_2CH_3$	SMT	84	107–112	3350, 1740, 1680	1.00–1.20 (m, 3H), 1.20–2.00 (m, 6H), 3.00 (m, 2H), 4.07 (s, 3H), 4.20–5.00 (m, 5H)
5k	$cycC_3H_5$	SMT	80	144–148	1725, 1670, 1260	1.01–1.20 (m, 4H), 1.50 (m, 4H), 3.20 (m, 3H), 4.00 (m, 1H), 4.07 (s, 3H), 4.50–4.80 (m, 3H)
5l	$CH_2CH=CH_2$	SMT	94	112–121	1730, 1670	1.90 (m, 4H), 3.20–3.50 (m, 2H), 4.07 (s, 3H), 3.90–4.90 (m, 5H), 5.00–5.60 (m, 2H), 5.70–6-30 (m, 1H)
5m	$(CH_2)_3CH_3$	SMT	92	120-129	3350, 1735, 1665, 1560	0.96 (m, 3H), 1.20–2.3 (m, 8H), 3.00–3.60 (m, 4H), 4.07 (s, 3H), 4.40–5.0 (m, 3H)
5n	$CH(CH_3)_2$	SPT	46	—	(ref. 7)	(ref. 7)
5o	$CH_2CH=CH_2$	SPT	68	—	1730, 1690, 1675	1.72–2.67 (m, 5H), 3.33–4.71 (m, 7H), 7.66 (s, 5H)
5p	$CH_2C\equiv CH$	SPT	44	—	1730, 1690, 1680	1.88–2.58 (m, 4H), 3.37–4.66 (m, 7H), 5.10–6.45 (m, 3H), 7.66 (s, 5H)
5q	$CH(CH_3)_2$	SPrT	40	112–115	1745, 1665, 1545	0.94–1.61 (m, 9H), 1.73–2.32 (m, 6H), 3.21–4.06 (m, 2H), 4.07–5.02 (m, 6H)
5r	$CH(CH_3)_2$	SBzT	82	150–256	1745, 1655, 1545	1.23 (d, 6H, J = 7 Hz), 1.76–2.25 pm, 4H), 3.04–3.60 (m, 2H), 4.00–5.03 (m, 4H), 7.43–7.55 (m, 2H), 7.90–8.33 (m, 2H)
5s	$CH(CH_3)_2$	SPyr	76	163–171	1740, 1645, 1605, 1545	1.15–1.55 (m, 6H), 1.86–2.23 (m, 4H), 3.14–3.51 (m, 2H), 4.08–5.07 (m, 4H), 7.55–8.24 (m, 3H), 8.73 (m, 1H)

[a]SMT = N-methyl-2-thiotetrazolyl; SPT = N-phenyl-2-thiotetrazolyl; SPrT = N-propyl-2-thiotetrazolyl; SBzT = S-2-thiazolyl; S-2-pyridyl; TTC = 2-thioxo-3-thiazolidinyl. [b]Yields from *4*. [c]Nujol mull.

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p-*Nitrophenyl N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl methyl N-methylcarbamate (7a)*

Compound *7a* was prepared from carbamate hydrochloride *5a* in an analogous procedure to that described for *7c*. The carbamate was recrystallized from EtOAc-hexane (0.14 g, 0.25 mmol, 18.3%) mp 166–168 °C; IR ($CHCl_3$) 3420, 1755, 1645, 1580, 1545 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.4 (br d, 6H, J = 7 Hz), 1.57–2.34 (m, 4H), 2.63 (br s, 4H), 3.1–3.2 (brs, 3H) 3.25–4.05 (m, 2H), 3.70 (s, 3H), 4.23–5.03 (m, 5JH), 7.02 (br d, 1H, J = 6.5 Hz), 7.13–7.65 (m, 3H), 8.32 (br d, 2H, J = 9 Hz). Anal. Found: C, 53.30; H, 5.88; N, 12.47. Requires $C_{25}H_{33}N_5O_{10}$: C, 53.27; H, 5.90; N, 12.43%.

p-*Nitrophenyl N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl methyl N-propylcarbamate (7b)*

Compound *7b* was prepared from carbamate hydrochloride *5b* in an analogous procedure to that described for *7c*. The carbamate was recrystallized from EtOAc-hexane (0.16 g, 0.27 mmol, 20.1%) mp 158–161 °C; IR ($CHCl_3$) 3320, 1740, 1650, 1580, 1550 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.96–1.10 (m, 3H), 1.36 (br d, 6H, J = 6.5 Hz), 1.57–2.35 (m, 6H), 2.63 (br s, 4H), 3.25–4.05 (m, 4H), 3.70 (s, 3H), 4.26–5.08 (m, 5H), 7.03 (br d, 1H, J = 6.5 Hz), 7.14–7.66 (m, 3H) 8.30 (br d, 2H, J = 9 Hz). Anal. Found: C, 54.78; H, 6.31; N, 11.88. Requires $C_{27}H_{37}N_5O_{10}$: C, 54.80; H, 6.13; N, 11.89%.

p-*Nitrophenyl N-(methoxysuccinyl-L-alanyl-L-alanyl prolylmethyl)-N-propynylcarbamate (7d)*

Compound *7d* was prepared from carbamate hydrochloride *5d* in an analogous procedure to that described for *7c*. The oily product *7d* was crystalized from EtOAc-hexane (0.21 g, 0.35 mmol, 37.6%) mp 160–163 °C; IR ($CHCl_3$) 3340, 1740, 1690, 1655, 1635, 1595, 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.33 (d, 6H, J = 7 Hz), 2.03–2.23 (m, 4H), 2.40 (br s, 1H), 2.60–2.94 (m, 4H), 3.51–4.06 (m, 2H), 3.70 (s, 3H), 4.14–5.08 (m, 7H), 6.40–6.62 (m, 1H), 7.10 (d, 1H, J = 7 Hz), 7.33–7.54 (m, 2H), 8.30 (d, 2H, J = 9 Hz). Anal. Found: C, 55.33; H, 5.73; N, 11.70. Requires $C_{27}H_{33}N_5O_{10}$: C, 55.19, H, 5.62; N, 11.92%.

p-*Nitrophenyl N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl methyl)-N-cyclopropylcarbamate (7e)*

Compound *7e* was prepared from carbamate hydrochloride *5e* in an analogous procedure to that described for *7c*. The oily product *7e* crystallized from EtOAc-hexane (0.3 g, 0.51 mmol, 58.4%) mp 154–158 °C; IR ($CHCl_3$) 3340, 1740, 1690, 1655, 1640, 1590, 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.83–1.16 (m, 4H), 1.40 (d, 6H, J = 7 Hz), 1.85–2.32 (m, 4H), 2.60–2.80 (m, 4H), 3.05–3.23 (m, 1H), 3.50–3.66 (m, 2H), 3.70 (s, 3H), 4.22–5.05 (m, 5H), 6.38 (br d, 1H, J = 8 Hz), 7.03 (br t, 1H, J = 8 Hz), 7.36 (d, 2H, J = 9 Hz). Anal. Found: C, 54.80; H, 5.99; N, 11.77. Requires $C_{27}H_{35}N_5O_{10}$: C, 55.0; H, 5:98; N, 11.88%.

p-*Nitrophenyl N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl methyl)-N-(2-methyl)-propyl carbamate (7f)*

Compound *7f* was prepared from carbamate hydrochloride *5f* in an analogous

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procedure to that described for *7c*. The oily product was crystallized from EtOAc-hexane (0.5 g, 0.82 mmol, 57%) mp 177–179 °C; IR (Nujol) 3350, 1735, 1695, 1660, 1635, 1595 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.96 (d, 6H, J = 7 Hz), 1.35 (d, 6H, J = 7 Hz), 1.51 – 2.02 (m, 1H), 2.03–2.25 (m, 4H), 2.50–2.77 (m, 4H), 3.30–3.55 (m, 3H), 3.70 (s, 3H), 3.80–4.03 (m, 2H), 4.11–5.02 (m, 5H), 6.50 (br d, 1H), J = 7 Hz), 7.00–7.15 (m, 1H), 7.30 (d, 2H, J = 9 Hz), 8.30 (d, 2H, J = 9 Hz). Anal. Found: C, 55.29; H, 6.55; N, 11.46. Requires $C_{28}H_{39}N_5O_{10}$: C, 55.53; H, 6.44; N, 11.57%.

p-*Nitrophenyl N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-cyclohexylcarbamate (7g)*

Compound *7g* was prepared from carbamate hydrochloride *5g* in an analogous procedure to that described for *7c*. The oily product was crystallized from EtOAc-hexane (0.15 g, 0.238 mmol, 54.3%) mp 150–154 °C; IR (Nujol) 3345, 1735, 1690, 1655, 1595 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.26–1.64 (m, 14H), 1.80 (d, 6H, J = 7.5 Hz), 2.66–2.85 (m, 4H), 3.73 (s, 3H), 3.55–5.03 (m, 8H), 5.42 (m, 1H), 7.07–7.28 (m, 1H), 7.43 (d, 2H, J = 9 Hz), 8.33 (d, 2H, J = 9 Hz). Anal. Found: C, 56.87; H, 6.54; N, 11.0. Requires $C_{30}H_{41}N_5O_{10}$: C, 57.05; H, 6.49; N, 11.09%.

p-*Nitrophenyl N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-phenylmethylcarbamate (7h)*

Compound *7h* was prepared from carbamate hydrochloride *5h* in an analogous procedure to that described for *7c*. The oily product was crystallized from EtOAc-hexane (0.21 g, 0.32 mmol, 39.5%) mp 141–143 °C; IR (Nujol) 3340, 1730, 1690, 1660, 1630, 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.27–1.83 (m, 6H), 2.06–2.35 (m, 4H), 3.60 (s, 3H), 3.70–3.84 (m, 2H), 4.20–5.02 (m, 7H), 6.46 (br d, 1H, J = 7 Hz) 7.10 (br d, 1H, J = 7 Hz), 7.31–7.63 (m, 7H), 8.33 (d, 2H, J = 9 Hz). Anal. Found: C, 57.97; H, 5.85; N, 10.89. Requires $C_{31}H_{37}N_5O_{10}$: C, 58.21; H, 5.79; N, 10.95%.

1-Methyl-5-tetrazolyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-propylthiocarbamate (7i)

Compound *7i* was reported previously by Digenis *et al.*[7]

1-Methyl-5-tetrazolyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropylthiocarbamate (7j)

Compound *7j* was prepared from carbamate hydrochloride *5j* in an analogous procedure to that described for *7c*. The oily product *7j* crystallized from EtOAc-hexane (0.08 g, 0.14 mmol, 22.1%) mp 138–140 °C; IR (Nujol) 3300, 1735, 1650, 1170 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.00 (br t, 3H, J = 7 Hz), 1.40 (d, 6H, J = 7 Hz) 1.24–1.86 (m, 2H), 2.07–2.31 (m, 4H), 2.66–2.84 (m, 4H), 3.50–3.75 (m, 2H), 3.80 (s, 3H), 4.10 (s, 3H), 4.22–5.05 (m, 5H), 6.96–7.67 (m, 1H), 7.43–7.60 (m, 1H). Anal. Found: C, 48.37; H, 6.36; N, 19.64. Requires $C_{23}H_{36}N_8O_7$: C, 48.58; H, 6.38; N, 19.71%.

1-Methyl-5-tetrazolyl N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl) N-cyclopropylthiocarbamate (7k)

Compound *7k* was prepared from carbamate hydrochloride *5k* in an analogous procedure to that described for *7c*. The oily product *7k* crystallized from EtOAc-hexane (0.5 g, 0.88 mmol, 68.4%) mp 107–109 °C; IR ($CHCl_3$) 3400–33—, 1730,

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1710, 1690, 1510 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.06–1.22 (m, 4H), 1.36 (d, 6H, J = 6 Hz), 1.80–2.43 (m, 4H), 2.63–2.83 (m, 4H), 3.04 (m, 1H), 3.56–3.72 (m, 2H), 3.73 (s, 3H), 4.08 (s, 3H), 4.33–4.93 (m, 5H). Anal. Found: C, 48.56; H, 6.07; N, 19.57. Requires $C_{23}H_{34}N_8O_7S$: C, 48.76; H, 6.05; N, 19.77%.

1-Methyl-5-tetrazolyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-allythiocarbamate (7l)

Compound *7l* was prepared from carbamate hydrochloride *5l* in an analogous procedure to that described for *7c*. The oily product *7l* crystallized from EtOAc-hexane (0.15 g, 0.26 mmol, 24.1%) mp 143–145 °C; IR (Nujol) 3340, 1735, 1660, 1530, 1450 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.40 (d, 5H, J = 7 Hz), 2.10–2.44 (m, 4H), 2.66–2.96 (m, 4H), 3.73 (s, 3H), 3.62–3.85 (m, 2H), 4.10 (s, 3H), 4.13–5.05 (m, 7H), 5.16–5.68 (m, 2H), 5.80 (br d, 1H, J = 7 Hz), 5.60–6.32 (m, 1H), 7.20 (m, 1H). Anal. Found: C, 48.51; H, 6.08; N, 19.68. Requires $C_{23}H_{34}N_8O_7S$: C, 48.75; H, 6.05; N, 19.78%.

1-Methyl-5-tetrazolyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-butylthiocarbamate (7m)

Compound *7m* was prepared from carbamate hydrochloride *5m* in an analogous procedure to that described for *7c*. The oily product *7m* was crystallized from EtOAc-hexane (0.14 g, 0.24 mmol, 29%) mp 140–143 °C; IR (Nujol) 3340, 1730, 1690, 1655, 1630, 1535 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.00–1.23 (m, 3H), 1.22–1.86 (m, 4H), 1.37 (d, 6H, J = 7 Hz), 2.07–2.31 (m, 4H), 2.63–2.86 (m, 4H), 3.33–3.92 (m, 4H), 3.83 (s, 3H), 4.10 (s, 3H), 4.27–5.04 (m, 5H), 6.50 (br d, 1H, J = 7 Hz), 7.10 (br d, 1H, J = 7 Hz). Anal. Found: C, 49.52; H, 5.58; N, 19.08. Requires $C_{24}H_{38}N_8O_7S$: C, 49.47; H, 5.57; N, 19.23%.

1-Phenyl-5-tetrazolyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropylthiocarbamate (7n)

Compound *7n* was reported previously by Digenis *et al.*[7]

1-Phenyl-5-tetrazolyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-allylthiocarbamate (7o)

Compound *7o* was prepared from carbamate hydrochloride *7o* in an analogous procedure to that described for *7c*. The oily product *7o* was crystallized from EtOAc-hexane (0.14 g, 0.22 mmol, 18.5%) mp 101–104 °C; IR (Nujol) 3350, 1750, 1740, 1710, 1680, 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.30 (s, 6H), 2.03–2.25 (m, 4H), 2.32–2.70 (m, 5H), 3.70 (s, 3H), 4.03–5.04 (m, 7H), 6.86 (m, 1H), 7.61 (s, 5H). Anal. Found: C, 53.58; H, 5.80; N, 17.73. Requires $C_{28}H_{36}N_8O_7S$: C, 53.50; H, 5.73; N, 17.83%.

1-Phenyl-5-tetrazolyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-propynylthiocarbamate (7i)

Compound *7p* was prepared from carbamate hydrochloride *5p* in an analogous procedure to that described for *7c*. The oily product *7p* crystallized from $CHCl_3$-hexane (0.18 g, 0.28 mmol, 20.0%), mp 115–119 °C; IR (Nujol) 3345, 1735, 1725, 1680, 1670, 1650, 1640, 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.26 (d, 6H, J = 7 Hz), 2.03–2.26 (m, 4H),

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2.30–2.70 (m, 5H), 3.70 (s, 3H), 3.70-3.85 (m, 2H), 4.00–5.00 (m, 7H), 7.03 (d, 1H, J = 7 Hz), 7.60 (s, 5H), 7.66 (m, 1H). Anal. Found: C, 53.75; H, 5.47; N, 17.84. Requires $C_{28}H_{34}N_8O_7S$: C, 53.66; H, 5.47; N, 17.88%.

S-(2-Benzothiazolyl)-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropylthiocarbamate (7r)

Compound *7r* was prepared from carbamate hydrochloride *5r* in an analogous procedure to that described for *7c*. The oily product *7r* was crystallized from EtOAc-hexane (0.35 g, 0.56 mmol, 60.4%); mp 167–171 °C; IR (Nujol) 3350, 1730, 1690, 1655, 1530 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.06–1.64 (m, 12H), 2.13–2.37 (m, 4H), 2.63-2.80 (m, 4H) 3.53–4.03 (m, 2H), 3.70 (s, 3H), 4.05–5.14 (m, 6H), 7.03 (br d, 1H, J = 7 Hz), 7.30–8.20 (m, 5H). Anal. Found: C, 54.15; H, 6.03; N, 11.22. Requires $C_{28}H_{37}N_5O_7S$: C, 54.27; H, 6.02; N, 11.30%.

S-(2-Pyridyl N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropylthiocarbamate (7s)

Compound *7s* was prepared from carbamate hydrochloride *5s* in an analogous procedure to that described for *7c*. The oily product *7s* was crystallized from $CHCl_3$-hexane (0.16 g, 0.28 mmol, 19.5%) mp 98–102 °C. IR ($CHCl_3$) 3300, 1730, 1650, 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.10–1.62 (m, 12H), 2.03–2.23 (m, 4H), 2.60–2.80 (m, 4H), 3.52–4.06 (m, 2H), 3.66 (s, 3H), 4.11–5.20 (m, 6H), 6.76 (d, 1H, J = 8 Hz), 7.15–7.53 (m, 2H), 7.66–7.85 (m, 2H), 8.60 (br d, 1H, J = 8 Hz). Anal. Found: C, 55.55; H, 6.67; N, 12.69. Requires $C_{26}H_{37}N_5O_7S$: C, 55.41; H, 6.57; N, 12.43%.

1-Pentafluorophenyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropylcarbamate (7t)

Compound *7t* was reported previously by Digenis *et al.*[7]

1-Phenyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropylcarbamate (7u)

Compound *7u* was reported previously by Digenis *et al.*[7]

1-(2,2,3,3,4,4,4-Heptafluorobutyl)-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropylcarbamate (7v)

Compound *7v* was reported previously by Digenis *et al.*[7]

1-S-Benzyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropylcarbamate (7w)

Compound *7w* was reported previously by Digenis *et al.*[7]

N-(Methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropyl-2-thioxo-3-thiazolidinecarboxamide (7x)

Compound *7x* was prepared from urea hydrochloride *5t* in an analogous procedure to that described for *7c*. The oily product *7x* was crystallized from $CHCl_3$-hexane (0.35 g, 0.6 mmol, 44.3%) mp 140–145 °C; IR ($CHCl_3$) 3300, 1730. 1680, 1640, 1520,

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1435 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.92–1.63 (m, 12H), 2.10–2.37 (m, 4H), 2.63–2.85 (m, 4H), 3.08–5.06 (m, 12H), 3.73 (s, 3H), 6.60 (br d, 1H, J = 7 Hz), 7.16–7.33 (m, 1H). Anal. Found: C, 50.13; H. 6.30; N, 12.34. Requires $C_{22}H_{37}N_5O_7S$: C, 50.43; H, 6.48; N, 12.26%.

1-Phenyl-5-tetrazolyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropyldithiocarbamate (7y)
Compound *7y* was prepared from carbamate hydrochloride *5u* in an analogous procedure to that described for *7c*, and was obtained as a very hygroscopic powder. IR (Nujol) 3340, 1738, 1680, 1650 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.96–1.68 (m, 12H), 2.09–2.35 (m, 4H), 2.53 (m, 4H), 3.55–3.80 (m, 2H), 3.63 (s, 3H), 4.03–5.05 (m, 6H), 7.06-7.28 (m, 1H), 7.33–7.80 (m, 5H). The extreme hygroscopic nature of this compound impeded determination of melting point and elemental analysis.

1-Phenyl-5-tetrazolyl-N-(methoxysuccinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropyl thioacetamide (7z)
Compound *7z* was prepared from amide hydrochloride *5v* in an analogous procedure to that described for *7c*. The oily product *7z* was recrystallized from EtOAc-hexane (0.4 g, 0.62 mmol, 42.5%) mp 188–190 °C; IR (Nujol) 3340, 1735, 1690, 1655, 1530 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 0.90–1.33 (m, 12H), 1.95–2.20 (m, 4H), 2.50–2.73 (m, 4H), 3.60 (s, 3H), 3.66–3.84 (m, 2H), 3.90–4.66 (m, 6H), 4.66 (2H), 7.76 (s, 5H), 8.10 (d, 2H, J = 7 Hz). Anal. Found: C, 54.28; H, 6.29; N, 17.51. Requires $C_{29}H_{40}N_8O_7S$: C, 54.04; H, 6.21; N, 17.34%.

Enzymatic Studies

PPE, bovine trypsin, chymotrypsin 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 using a CARY 219 or 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 347.5 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.

* Human sputum elastase has been reported to possess similar enzymatic, inhibitory, and physical chemical properties as elastase from human leukocytes (HLE).[35]

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Screening for inhibitory activity

In a typical experiment, the inhibitor (0.1 mL, 1 mM in DMSO) and the substrate (0.1 mL, 10 mM) were added to 2.7 mL of 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 (0.1 mL, 3.85 μM) was added to sample cuvette, the mixture was shaken for 20 s, and the increase in the absorbance was monitored over a 30 min interval.

PP elastase

The substrate, Boc-Ala-ONp, and the enzyme, were dissolved in CH_3OH and 0.05 M phosphate buffer (pH 6.5) respectively. The same system was also used as the buffer medium. The absorbance was monitored at 398 nm and 25°.

Trypsin

The substrate, Bz-Arg-OEt, was dissolved in 0.01 M Tris-HCl buffer (pH 8.0), which was also used as the buffer medium. The enzyme was dissolved in 2 M HCl and the absorbance monitored at 253 nm and 25 °C.

Chymotrypsin

The substrate, Bz-Tyr-OEt, was dissolved in 50% aqueous CH_3OH and the enzyme 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). Absorbance was monitored at 256 nm and 30 °C.

HL Elastase

The substrate, MeO-Suc-Ala-Ala-Pro-Val-NA, was dissolved in DMSO. The enzyme was dissolved in 0.05 M sodium acetate buffer, pH 5.5. The medium buffer consisted of 0.1 M. Hepes buffer (pH 7.4) containing 0.5 M NaCl and 10% DMSO. The absorbance was monitored at 410 nm and 25 °C.

Kinetic studies

In a typical experiment, 0.1 mL of the inhibitor, (19.3–2.4 μM) in DMSO and 0.1 mL of the substrate (700–160 μM) were added to the medium buffer (2.7 mL) in a quartz cuvette and thermally equilibrated in the spectrophotometer for 2 min and the absorbance was balanced at the desired wavelength. The enzyme (0.1 mL of 0.3 mg/mL buffer) 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, 0.1 mL of the inhibitor (19.3–2.4 μM) and 0.1 mL of elastase (0.3 mg/mL) were incubated in buffer (2.7 mL) in a quartz cuvette at 25 °C. At specified time intervals, 0.1 mL of the substrate (9.0 mM) 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 percentage activity remaining versus incubation time. K_i values were obtained from the intercept of a double reciprocal plot of k_{obs} vs inhibitor concentration.

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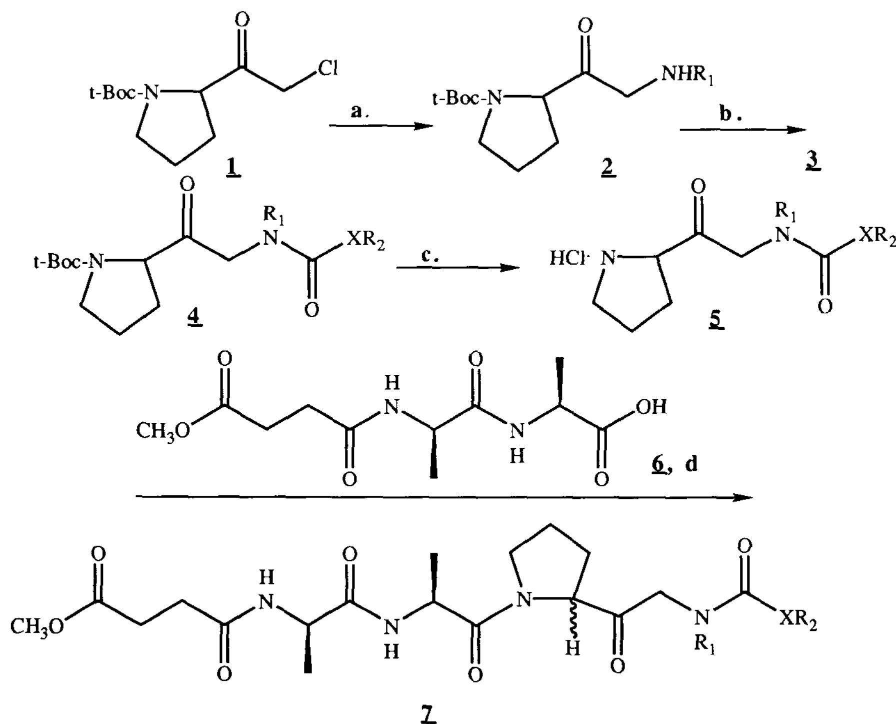
RESULTS

Chemistry

The peptidyl carbamate esters *7* were synthesized according to the general procedure described earlier[7] (Scheme II). Chloroformate derivatives *3* were prepared by the reaction of phosgene ($COCl_2$) with the corresponding alcohol or thiol in THF (or Et_2O or CH_3CN) containing Et_3N (Scheme II). The crude chloroformates were each reacted with the appropriate aminomethyl ketone *2* (Table 1), yielding the corresponding carbamates/thiocarbamates *4* (Tables 2 and 3). Removal of the *t*-BOC group of *4* was accomplished by acidolysis with anhydrous HCl gas in various solvents (Tables 4 and 5). The resulting propyl carbamates (or thiocarbamates) *5* were coupled with the appropriate protected dipeptides *6*, via the mixed anhydride method,[8] affording the target products *7*. In general, the *p*-nitrophenyl carbamates were produced in higher overall yields than the more reactive thiocarbamates.

Enzymatic Studies

The inhibitory activities of the peptidyl carbamates listed in Tables 6–9 were evaluated against porcine pancreatic elastase (PPE) and human leukocyte elastase (HLE).



Scheme II Preparation of peptidyl carbamates *7* (XR_2 = thiaazole, thiotetrazole, or *p*-nitrophenol). a) RNH_2, Et_2O, 4 °C – > 25 °C, or CH_3NH_2/H_2O, KI, 65 °C, bomb. b) *3*, TEA, THF or CH_3CN, $^-10$ or 5 °C. c) HCl (g), Et_2O or EtOAc/THF or EtOAc 5 °C or 25 °C. d) NMM, THF, *6*, isobutyl chloroformate, $^-15$ – $^-30$ °C. Then *5*, NMM, bistrimethylsilylacetamide, $^-50$ °C, THF.

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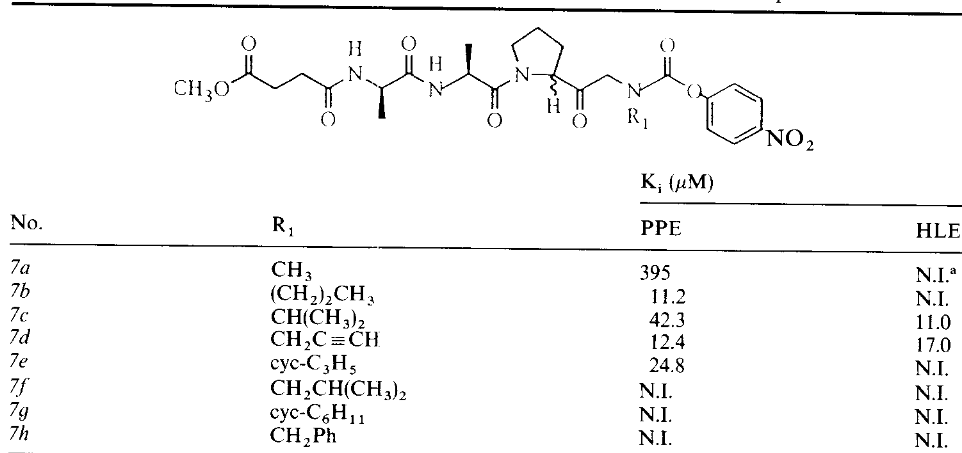
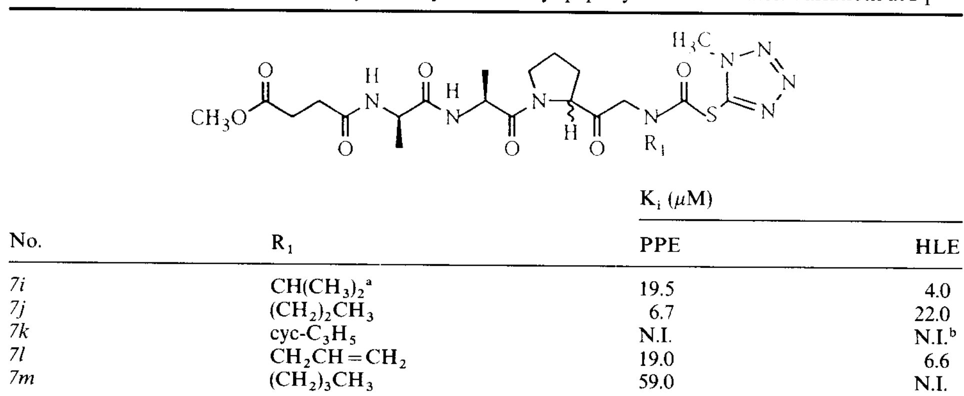
Table 6 Inhibition of PPE and HLE by *p*-nitrophenylcarbamates. Variations at P_1



No.	R_1	K_i (μM) PPE	HLE
7a	CH_3	395	N.I.[a]
7b	$(CH_2)_2CH_3$	11.2	N.I.
7c	$CH(CH_3)_2$	42.3	11.0
7d	$CH_2C{\equiv}CH$	12.4	17.0
7e	cyc-C_3H_5	24.8	N.I.
7f	$CH_2CH(CH_3)_2$	N.I.	N.I.
7g	cyc-C_6H_{11}	N.I.	N.I.
7h	CH_2Ph	N.I.	N.I.

[a]N.I. no inhibition at [I]/[E] = 260.

Table 7 Inhibition of PPE and HLE by 1-methyl-5-tetrazolyl-peptidyl thiocarbamates. Variations at P_1



No.	R_1	K_i (μM) PPE	HLE
7i	$CH(CH_3)_2$[a]	19.5	4.0
7j	$(CH_2)_2CH_3$	6.7	22.0
7k	cyc-C_3H_5	N.I.	N.I.[b]
7l	$CH_2CH{=}CH_2$	19.0	6.6
7m	$(CH_2)_3CH_3$	59.0	N.I.

[a]From Digenis *et al.*[7] [b]N.I., no inhibition at [I]/[E] = 260.

Enzymatic assays were conducted spectrophotometrically utilizing the synthetic substrates Boc-Ala-ONP[9] for PPE and MeO-Suc-Ala-Ala-Pro-Val-NA[10-11] for HLE, by following the rate of production of *p*-nitrophenolate anion and *p*-nitroaniline at 398 and 410 nm, respectively.

Initial screening of twenty-seven newly synthesized compounds revealed that eighteen peptidyl carbamates inhibited PPE and eleven elicited 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.

Kinetic studies were conducted for the assessment of the mode and potency of inhibition of active compounds. The dissociation constants of the enzyme-inhibitor

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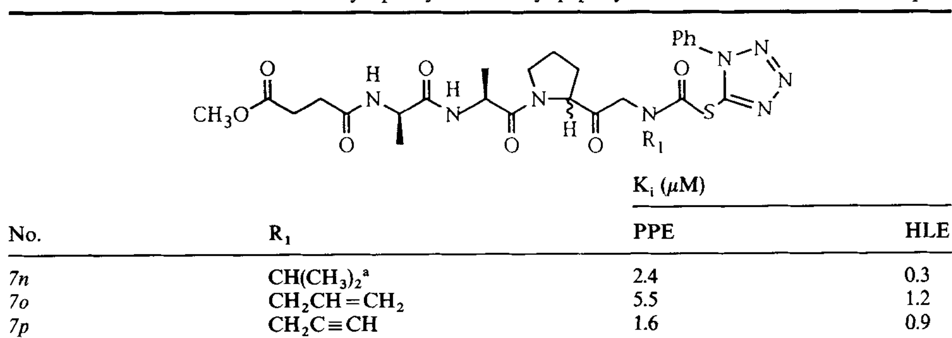
Table 8 Inhibition of PPE and HLE by 1-phenyl-5-tetrazolyl-peptidyl thiocarbamates. Variations at P_1



No.	R_1	K_i (μM) PPE	HLE
7n	$CH(CH_3)_2$[a]	2.4	0.3
7o	$CH_2CH=CH_2$	5.5	1.2
7p	$CH_2C\equiv CH$	1.6	0.9

[a]From Digenis *et al.*[7]

complex (K_i) was determined from Dixon plots[12] utilizing four inhibitor, and three substrate concentrations which ranged from 395.0–0.75 μM for PPE and 22.0–0.3 μM for HLE. The K_i values were in agreement with those determined from Kitz and Wilson[13] double reciprocal plots of the pseudo-first order rate constant of inhibition (k_{obs}) versus inhibitor concentration.[13] 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[12] and Lineweaver-Burk[14] plots appears to be of the mixed type [both K_m and V_{max} were affected]. None of the peptidyl carbamate inhibitors produced a burst of nucleofuge when incubated with either enzyme.

DISCUSSION

Several peptidyl-type elastase inhibitors have been reported. These include, among others, di- and tripeptides,[15-17] peptide chloromethyl ketones,[18-20] peptide aldehydes[21-23] and peptide carbazate esters.[24-28] More recently peptidyl carbamates were reported[5-7] by our group as being specific elastase inhibitors, the mechanism of inhibition involving the carbamoylation of the active site serine residue of elastase (Scheme III).

The resulting carbamoylated enzyme is expected to be more resistant towards deacylation relative to the acylated enzyme obtained from the proteolytic reaction of a serine protease and a peptide substrate. This incurred stability may be a result of the decreased electrophilicity of the carbonyl group due to the potential for electron release (resonance effect) by the adjacent nitrogen atom, which is not native to the acylated enzyme resulting from reaction with a peptide substrate.

Additionally, the sp^2 character of the carbamoyl nitrogen atom may force the N-CO-O moiety into a planar configuration. This twisting of the carbonyl group may invoke conformational stability of the carbamoylated enzyme relative to the optimal spacial requirements necessary for the hydrolytic deacylation reaction (decarbamoylation).

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Table 9 Inhibition of PPE and HLE by peptidylcarbamates incorporating a valine isostere at P_1. Variations at P_1

No.	XR_2	K_i (μM) PPE	HLE
7q		2.0	0.8
7r		0.75	4.5
7s		N.I.[a]	N.I.
7t[b]		35.0	0.4
7u[b]		31.5	N.I.
7v[b]	$OCH_2(CF_2)CF_3$	82.0	N.I.
7w[b]	SCH_2Ph	N.I.	N.I.
7x		N.I.	N.I.
7y		78.0	N.I.
7z		N.I.	N.I.
7aa[b]		N.I.	N.I.

[a]N.I., no inhibition at [I]/[E] = 260. [b]From Digenis *et al.*[7]

Structure-Activity Relationship Studies

Novel peptidyl carbamates (Tables 6–9) were designed based on earlier efforts illustrating that tetrapeptide analogs incorporating α-amino acid isosteres at P_1 were good inhibitors of PPE.[7] In the present report, the inhibitory activity of this class of compounds against HLE was examined. Additionally, novel peptidyl carbamates

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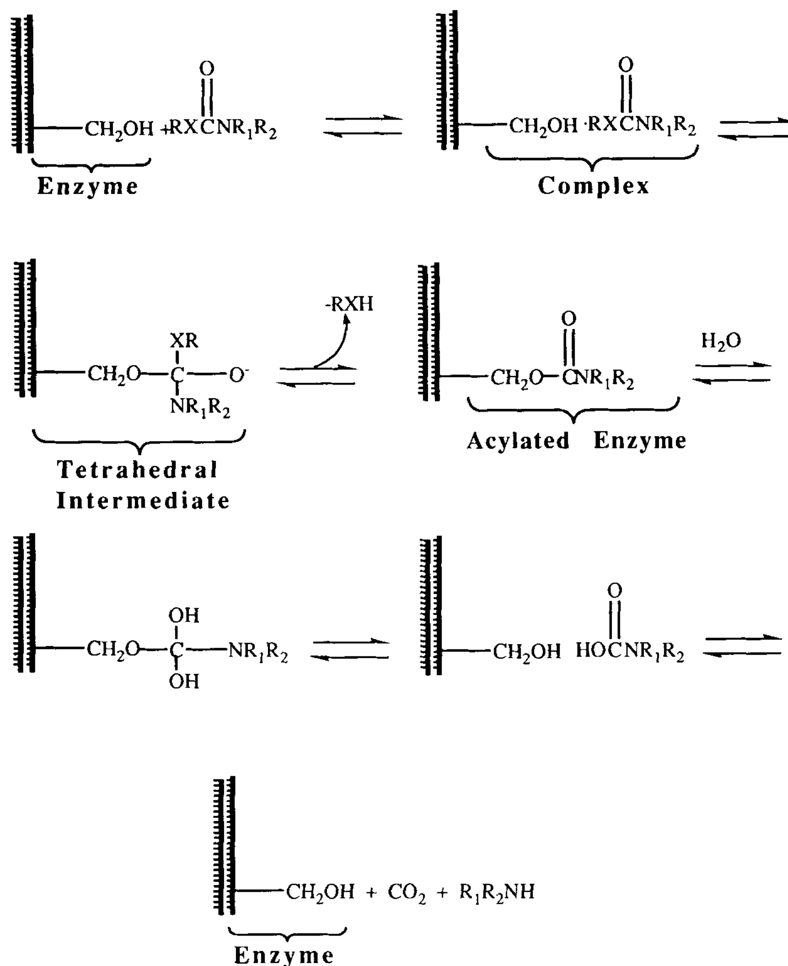

Scheme III Proposed mechanism of HLE inhibition by peptidyl carbamates 7
a) TEA, THF or Et_2O or CH_3CN, 0–5 °C.

and thiocarbamates were synthesized, and the effect of structural variations at P_1 and P'_1 on the elastase inhibitory capacity (EIC) was investigated.

As described earlier,[7] the nature of the substituent at P'_1 is an important determinant of the inhibitory activity of this class of compounds. Thus, according to the mechanism depicted in Scheme IIII, a good leaving group at P'_1 (i.e., lower pK_a of the alcohol) should result in faster enzyme acylation. The same trend had been observed in the reaction of azapeptides with HLE.[28] However, the present data indicate that steric requirements may be equally if not more important in determining the enzyme inhibitory capacity of these compounds. Thus, when P_1 was a valine isostere and P'_1 was varied (Tables 6, 7, 8, and 9), the order of inhibitory activity of peptidyl carbamates against PPE was *7r* > *7q* > *7n* > *7i* > *7u* > *7t* > *7c* > *7v*, where *7v* was the least potent inhibitor (highest K_i value). A different order (*7n* > *7t* > *7q* > *7i* > *7r* > *7c*)

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was observed for HLE inhibition where compounds *7u* and *7v* lacked inhibitory activity against the enzyme. If the determinant factor was purely electronic, the same order of activity against both enzymes would be expected. However, these data implicate the importance of the fit of the P'_1 residue in the S'_1 subsite of the enzyme, illustrating that an incongruency exists between HLE and PPE in the steric requirements at S'_1.

These results also suggest that the nature of P'_1 affects the specificity at P_1. Compound *7b* (Table 6), where P'_1 incorporated a *n*-propyl substituent, inhibited PPE ($K_i = 11.2\ \mu M$) without affecting HLE; however, when P'_1 was changed from a *p*-nitrophenyl to a 5-methylmercaptotetrazoyl group (*7i*, Table 7), the compound was a good inhibitor of HLE ($K_i = 22.0\ \mu M$). Similarly, a cyclopropyl substitution at P_1 resulted in a compound which lacked inhibitory activity against either enzyme when P'_1 was 5-methylmercaptotetrazoyl (*7k*, Table 7), yet provided a moderate inhibitor of PPE ($K_i = 24.8\ \mu M$) when P'_1 was *p*-nitrophenyl (*7e*, Table 6).

The effects of structural variations at P_1 on the EIC of the peptidyl carbamates are illustrated in Tables 6–8. Marked changes in the EIC of these compounds could be effected by altering the P_1 residue. Thus, neither enzyme was inhibited when P_1 incorporated a cyclohexyl substituent (*7g*) or with P_1 being an isostere of leucine (*7f*), tert-leucine (data not shown), or phenylalanine (*7h*). These data are in accordance with our finding that the PPE catalyzed hydrolysis rate of simple carbamate and carbonate esters is markedly depressed with branched, relative to straight chained substituents.[29] However, the optimal chain length at P_1 for the hydrolysis of peptidyl carbamates by PPE was three carbons as compared to four carbons for simple carbonate[29] and carbamate[29,30] esters. Thus compounds *7b* and *7j*, having an isostere of norvaline at P_1, afforded the best inhibitors of PPE ($K_i = 11.2$ and $19.5\ \mu M$ respectively), whereas compound *7m* containing a norleucine isostere at P_1, was only a moderate PPE inhibitor ($K_i = 59.0\ \mu M$). In contrast, a valine isostere at P_1 resulted in the best HLE inhibitors. This finding is in agreement with substrate specificity studies showing that HLE possesses a high affinity for valine at P_1.[18,19] Surprisingly, compound *7a* where P_1 was an isostere of alanine, showed poor inhibition of PPE ($K_i = 395\ \mu M$) and no inhibition of HLE, whereas previous reports had shown that peptidyl chloromethyl ketones,[19] and peptide nitroanilides[30] having alanine at P_1 exhibited good specificity for PPE. Unsaturation at P_1 did not appear to have a significant effect on the inhibitory activity of these compounds. the best HLE inhibitor, *7n* ($K_i = 0.3\ \mu M$). (Table 8) incorporated an isostere of valine at P_1 and a 5-phenylmercaptotetrazoyl group at P'_1.

Compound *7y* (Table 9), a dithiocarbamate, was a weak inhibitor of PPE ($K_i = 78.0\ \mu M$). This lower activity could be attributed to: (a) the larger size of the sulfur atom, which may sterically affect specificity, or (b) the lower electrophilicity of the C=S moiety compared to C=O group, rendering the nucleophilic attack by the active-site serine residue less facile.

Thiocarbamate *7s* (Table 9), having a thiopyridine moiety at P'_1, did not inhibit either enzyme. It is believed that the basicity of the pyridyl nitrogen atom may in part be responsible for this observed lack of activity.

Absence of activity in compound *7z* (Table 9), the amide analogue of *7n*, may be attributed to structural modifications (steric factors) introduced by the additional methylene group, possibly affecting the "fit" in the S'_1-S'_2-S'_3 subsites of the enzyme. However, an important factor may be the absence of the carbamate functionality found in *7z*, amides being more resistant towards hydrolysis than either carbamate

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or thiocarbamate esters.[30] The lack of conjugation of the electrophilic carbonyl center with the heteroaromatic ring (leaving group) as a result of insertion of the methylene group may, (1) serve to decrease the electrophilicity, and (2) decrease the nucleofugacity of the leaving group as carbon-carbon bonds are less susceptable to cleavage (as compared to carbon-sulfur bonds). Furthermore, if enzymatically hydrolyzed, amides would lead to acylated enzymes which are more easily deacylated than carbamoylated enzymes, thus regenerating the active enzyme. In this respect, compounds *7x* and *7aa* (Table 9) are both ureas that are expected to be relatively stable toward hydrolysis. The lack of inhibitory activity of these two compounds therefore was not surprising.

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[12] and Lineweaver-Burke[14] plots was of the mixed type (both K_m and V_{max} are affected). Thus the K_i values were obtained from slope and intercept replots. Since the inhibition seemed to be irreversible during the assay time, pre-steady state kinetic studies were carried out whereby the enzyme and the inhibitor were preincubated prior to the addition of the substrate. The inhibition could be represented by equation (1)

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \overset{k_3}{\rightarrow} E - I \quad (1)$$

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.[12] 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 equation (2),

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

The values of K_i (from Dixon Plots) and K_i (from Kitz and Wilson plots) were in agreement, and ranged from 395.0 μM − 0.75 μM for PPE and 22.0 μM − 0.3 μM for HLE.

The proposed mechanism of elastase inhibition by the peptidyl carbamates (Scheme III) involves an acylation (carbamoylation) step leading to a burst of the leaving group (alcohol or thiol). Thus, elastase was incubated with the inhibitors and the rate of enzyme acylation was monitored spectrophotometrically by following the appearance of the leaving group. Although the *p*-nitrophenyl ester showed a continual release of the leaving group, no burst effect was observed for any of the tested compounds. This observation led to speculation that rapid elastase inhibition observed for this class of compounds was not attributable to active site acylation, but may be due to the formation of a tight binding enzyme-inhibitor complex, or a stable tetrahedral intermediate.[7] Another possibility is a rapid formation of a Michaelis-Menten type complex, with subsequent slow acylation of the enzyme. (This is substantiated by the data obtained from the *p*-nitrophenyl esters). Slow acylation may arise from the lack of hydrogen bonding between the enzyme and the P_1 residue of the inhibitor, which in turn may result in non-productive interactions. Indeed, it is believed that during the binding of substrates and inhibitors with serine proteases,

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the NH of the peptide bond at the P_1 position undergoes hydrogen bonding with the back-bone peptidyl carbonyl oxygen of the active site serine residue.[28] Furthermore when the NH group of the P_1 residue of azapeptides was substituted with a methyl group, no acylation of either of the elastases occurred.[28] In peptidyl carbamates, the NH of the P_1 residue replaced with a $-CH_2$, which cannot hydrogen-bond to the enzyme.

The active inhibitors were shown to be specific for elastase as demonstrated by their lack of inhibitory activity towards the other serine-dependent enzymes trypsin, and chymotrypsin.

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 isostere at P_1. The incorporation of amino acid isosteres into biologically active peptides is expected to incur a higher degree of stability towards specific peptidases, thereby increasing their duration of *in-vivo* activity. Similar observations have been reported for tripeptides containing aza-amino acids and D-amino acids.[32] This stability, coupled with with proven clinical usefulness of carbamate esters,[33] make peptidyl carbamates promising reagents for *in-vivo* studies. As a result of the present study, more potent inhibitors ($K_i \sim 10^{-9}$ M) of HLE have been recently synthesized in these laboratories.[36] One of these derivatives, which incorporated a lysine residue at the P_3 or P_4 residue of its peptidyl carbamate moiety, has been shown to prevent corneal ulceration and vascularization after prolonged soft contact lens wear in the rabbit.[37] Furthermore, the covalent bonding of *7c* (Table 6) to a hydrophilic polymer[38] has resulted in a product which was shown to be effective in preventing HLE induced emphysema and secretory cell metaplasia in the hamster.

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References

1. Janoff, A. (1985) *Anm. Rev. Respir. Dis.*, **132**, 417–433.
2. Ekerot, L. and Ohlsson, K. (1984) *Adv. Exp. Med. Biol.*, **167**, 335–344.
3. C. Mittmann, Ed., (1972) "Pulmonary Emphysema and Proteolysis," pp. 1–537. New York: Academic Press.
4. Hance, A.J. and Crystal, R.G. (1975) *Am. Rev. Respir. Dis.*, **112**, 656-711.
5. Tsuji, K., Agha, B.J., Shinogi, M. and Digenis, G.A. (1984) *Biochem. Biophys. Res. Commun.*, **12**, 571–576.
6. Digenis, G.A., Agha, B.J., Kato, M. and Abdul.Raheem, A. (1985) *The Pharmacologist*, **27**, 202–205.
7. Digenis, G.A., Agha, B.J., Tsuji, K., Kato, M. and Shinogi, M. (1986) *J. Med. Chem.*, **29**, 1468–1476.
8. Penke, B., Czombos, J., Balaspiri, L., Peters, J. and Kovacs, K. (1970) *Hlev. Chim. Acta*, **53**, 1057–1068.
9. Visser, L. and Blout, E.R. (1972) *Biochim. Biophys. Acta*, **26**, 257–266.
10. Powers, J.C., Gupton, B.F., Harley, A.D., Nishino, N. and Whitely, R.J. (1977) *Biochim. Biophys. Acta*, **485**, 156–166.
11. Nakajima, K., Powers, J.C., Ashe, B.M. and Zimmerman, M. (1979) *J. Biol. Chem.*, **253**, 4027–4032.
12. Dixon, M. (1953) *Biochem. J.*, **55**, 1970–1975.
13. Kitz, R. and Wilson, I.B. (1962) *J. Biol. Chem.*, **237**, 3245–3249.
14. Lehninger, A.L. (1975) *Biochemistry*, 2nd ed., p. 197. New York: Worth Publishers, Inc.
15. Dzioloszynski, L. and Hofmann, T. (1973) *Biochim. Biophys. Acta*, **302**, 406–411.
16. Hassall, C.H., Johnson, W.H. and Roberts, N.A. (1979) *Bioorgan. Chem.*, **8**, 299–309.
17. Fric, P., Kasafirek, E. and Slaby, G. (1983) *L Experientia*, **39**, 374–378.
18. Powers, J.C. and Tuhy, P.M. (1972) *J. Am. Chem. Soc.*, **94**, 6344–6351.

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19. Powers, J.C. and Tuhy, P.M. (1973) *Biochemitry*, **12**, 4767–4774.
20. Thompson, R.C. and Blout, E.R. (1973) *Biochemistry*, **12**, 51–56.
21. Thompson, R.C. (1973) *Biochemistry*, **12**, 47–51.
22. Umezawa, H., Aoyagi, T., Okura, A., Morishima, H., Takeuchi, T. and Okami, Y. (1973) *J. Antibiot.*, **26**, 787–793.
23. Feinstein, G., Malemud, C.J. and Janoff, A. (1976) *Biochim. Biophys. Acta*, **429**, 925–934.
24. Powers, J.C. and Carroll, D.L. (1975) *Biochem. Biophys. Res. Commun.*, **67**, 639–644.
25. Powers, J.C., Carroll, D.L. and Tuhy, P.M. (1975) *Ann. N.Y. Acad. Sci.*, **256**, 420–427.
26. Dorn, C.P., Zimmerman, M., Yang, S.S., Yurewicz, E.C., Ashe, B.M., Frankshun, R. and Jones, H. (977) *J. Med. Chem.*, **20**, 1464–1468.
27. Gupton, B.F., Carroll, D.L., Tuhy, P.M., Kam, C.M. and Powers, J.C. (1984) *J. Biol. Chem.*, **259**, 4279–4287.
28. Powers, J.C., Boone, R., Carroll, D.L., Gukpton, G.F., Kam, C.-M., Nishino, N., Sakamoto, M. and Tuhy, P.M. (1984) *J. Biol. Chem.*, **259**, 4288–4293.
29. Nassar, M. (1984) Ph.D. Thesis, University of Kentucky, Lexington.
30. Scofield, R.E., Werner, R.P. and Wold, F. (1977) *Biochemistry*, **16**, 2492–2496.
31. Harper, J., Cook, R. and Powers, J.C. (1984) *Biochemistry*, **23**, 2995–3002.
32. Dutta, A.S. and Giles, M.B. (1976) *J. Chem. Soc. Perkin I*, 244–247.
33. Gilman, A.G., Goodman, L.S. and Gilman, A. (1980) *The Pharmacology Basis of Therapeutics*, p. 101. McMillan Publishing Co.
34. Lieber, E. and Ramchandran, I. (1959) *J. Can. J. Chem.*, **37**, 101–104.
35. Twumasi, D.Y. and Liener, I.E. (1977) *J. Biol. Chem.*, **252**, 1917–1924.
36. Digenis, G.A. and Agha, B.J. (1991) U.S. Patent 5,008,245.
37. Cejkova, J., Lojda, Z., Vacik, J., Digenis, G.A. and Dropcova, S. (1992) *Histochemistry*, **97**, 69–78.
38. Banks, W.R., Rypacek, G. and Digenis, G.A. (1991) *J. Labelled Comp. Radiopharm.*, **29**(4), 381–391.
39. Stone, P.J., Lucey, E.C., Noskova, D., Digenis, G.A. and Snider, G.L. (1992) *Am. Rev. Respir. Dis.*, **148**, 457–461.

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