138.9 ppm (sym m, 2 F, F-3,5). Anal. ($C_{12}H_{14}F_4N_2O_2$) C, H, F, N.

Enzyme Preparation and Assay Procedures. The reagents and conditions for the assays for inhibitory activity against desmolase and aromatase were exactly those described in ref 10. Binding spectra were determined as follows. Difference spectra were obtained at room temperature with a Pye Unicam SP8-150 spectrophotometer in the range 360–520 nm. The human placental microsomes were diluted with 0.1 M potassium phosphate buffer (pH 7.4) to give a final protein concentration of 2 mg/mL. The ligands were dissolved in dimethyl sulfoxide and equal volumes of solvent were added to the reference and sample cuvettes. Dimethyl sulfoxide had no effect on the binding spectra with addition up to 10% of the final suspension volume. Spectra were recorded at 1 nm/s with a slit width of 2 nm and a full-scale absorbance of 0.02. The K_s values were determined from a Scatchard plot (absorbance difference vs. absorbance difference/concentration: slope K_s) and are the average of three determinations (±10%).

Acknowledgment. This investigation was supported by grants from the Medical Research Council and the Cancer Research Campaign (to the Institute of Cancer Research) and from S.E.R.C. (to P.S.). We thank M. H. Baker for skilled technical assistance.

Registry No. 4, 92788-14-2; 4·picrate, 92788-15-3; 5, 92788-10-8; 6, 13121-99-8; 7, 92788-11-9; 8, 72434-23-2; 8·picrate, 92788-13-1; 9, 92788-12-0; 10, 92788-16-4; 11, 92788-18-6; 12, 700-16-3; 13, 92788-23-3; 14, 1619-58-5; 15, 92788-19-7; 16, 92788-20-0; 17, 92788-21-1; 18, 96-33-3; 19, 92788-22-2; EtI, 75-03-6; CH_2=CHCN, 107-13-1; 3-pyridineacetonitrile, 6443-85-2; desmolase, 9044-50-2; aromatase, 9039-48-9; 4-(4-pyridyl)-4-carboxamidohexanoic acid hydrazide, 92788-17-5.

Amino Acid Derived Latent Isocyanates: Irreversible Inactivation of Porcine Pancreatic Elastase and Human Leukocyte Elastase

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Several amino acid derived azolides (I) have been synthesized and investigated for their inhibitory activity toward human leukocyte elastase and porcine pancreatic elastase. The inhibitory activity was found to be dependent on the nature of the precursor amino acid ester. Thus, compounds derived from L-valine methyl ester **3**, L-norvaline methyl ester **5**, DL-norleucine methyl ester **9**, and L-methionine methyl ester **10** were found to inhibit irreversibly both enzymes. Compound **10** was found to be a specific and selective inhibitor of human leukocyte elastase. In contrast to these, inhibitors derived from glycine methyl ester **1**, D-valine methyl ester **4**, and D-norvaline methyl ester **6** were found to be inactive. The results of the present study show that latent isocyanates derived from appropriate amino acids can serve as selective inhibitors of serine proteases and are of potential pharmacological value.

Pulmonary emphysema is a disease characterized by alterations in physiological lung function related to the loss of elastic recoil.[1,2] The proteinase–proteinase inhibitor imbalance hypothesis has been proposed as a model for the development of emphysema. It suggests that either an increase in destructive proteolytic activity due to human leukocyte elastase (HLE) and cathepsin G or a decrease in protective serum proteinase inhibitor (α-1-PI) can lead to emphysema.[3,4] The progress of the disease involves the destruction of the lung connective tissues. A similar model may be involved in other related connective tissue diseases, such as arthritis.[5] Thus, the development of biospecific inhibitors of HLE and cathepsin G devoid of cytotoxic effects remains of extreme pharmacological importance.

In designing inhibitors of HLE cognizance must be taken of the fact that potential pharmacological agents must possess the ability to inhibit selectivity HLE and none of the other closely related proteinases. Although HLE, cathepsin G, porcine pancreatic elastase (PPE), and α-chymotrypsin have many features in common, such as a similar catalytic apparatus, an extended binding site, and a preference for hydrophobic substrates (or inhibitors), they differ from each other in their substrate specificities.[6,7] These differences in substrate preference arise from variations in the size of the binding cleft at the catalytic sites.

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Table I. Amino Acid Derived Azolides I

compd	R	mp °C (% yield)	anal.	formula
1	H	80–81 (70)	C, H, N	$C_8H_{11}N_3O_3$
2	L-CH_3	*a* (67)	C, H, N	$C_9H_{13}N_3O_3$
3	L-$(CH_3)_2CH$	52–53 (90)	C, H, N	$C_{10}H_{15}N_3O_3$
4	D-$(CH_3)_2CH$	*a* (88)	C, H, N	$C_{10}H_{15}N_3O_3$
5	L-$CH_3CH_2CH_2$	86–88 (96)	C, H, N	$C_{10}H_{15}N_3O_3$
6	D-$CH_3CH_2CH_2$	*a* (98)	C, H, N	$C_{10}H_{15}N_3O_3$
7	L-$(CH_3)_2CHCH_2$	80–81 (99)	C, H, N	$C_{11}H_{17}N_3O_3$
8	L-CH_3CH_2-$CH(CH_3)$	*a* (82)	C, H, N	$C_{11}H_{17}N_3O_3$
9	DL-CH_3CH_2-CH_2CH_2	*a* (99)	C, H, N	$C_{11}H_{17}N_3O_3$
10	L-$CH_3SCH_2CH_2$	*a* (96)	C, H, N, S	$C_{10}H_{15}N_3O_3S$

[a] Oil.

The results of several studies[8] related to the specificities of HLE, PPE, and cathepsin G reveal that PPE can acommodate small hydrophobic side chains at its primary specificity site S_1.[9] The S_2–S_4 subsites of PPE are similar to those of HLE while the S_1 subsite of HLE is larger, enabling the accommodation of larger side chains at S_1. The N-terminal of HLE is homologous to that of PPE. Cathepsin G is similar to α-chymotrypsin (α-CT) in its substrate specificity and shows a preference for Phe and Met as the P_1 residue.

The elegant work of Powers and co-workers as well as that of others[8] on the mapping of the active sites of these enzymes has paved the way toward the development of irreversible[10-24] and reversible[25-29] inhibitors that can discriminate between individual members of this class of enzymes. These studies have demonstrated cogently that

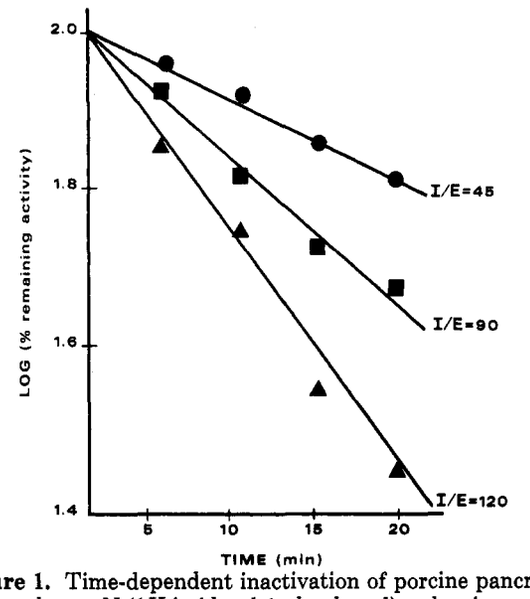

Figure 1. Time-dependent inactivation of porcine pancreatic elastase by DL-*N*-(1*H*-imidazol-1-ylcarbonyl)norleucine methyl ester (**9**). The enzyme preparation was incubated at 25 °C in 50 mM phosphate buffer solution, pH 6.5, and acetonitrile. At specified time intervals, aliquots were withdrawn and assayed for remaining enzyme activity with use of (*tert*-butoxycarbonyl)-L-alanyl-*p*-nitrophenyl ester.

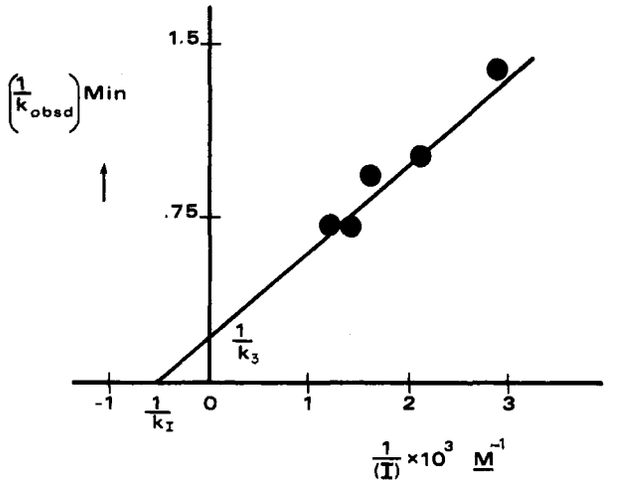

Figure 2. Inhibition of porcine pancreatic elastase by DL-*N*-(1*H*-imidazol-1-ylcarbonyl)norleucine methyl ester (**9**). Double-reciprocal plot of the effect of inhibitor concentration on the observed rate constant of inactivation.

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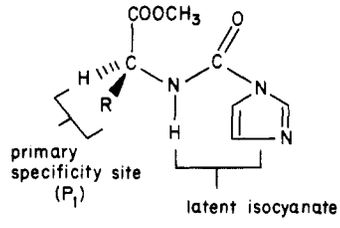
Table II. K_i and k_3 Values for the Inhibition of Porcine Pancreatic Elastase and Human Leukocyte Elastase by Azolides I

	porcine pancreatic elastase			human leukocyte elastase		
compd	K_i, mM	k_3, min^{-1}	k_3/K_i, M^{-1} s^{-1}	K_i, mM	k_3, min^{-1}	k_3/K_i, M^{-1} s^{-1}
1	inactive					
2						
3	1.11	0.183	2.8	1.80	20.8	193
4	inactive					
5	0.84	0.322	6.3	0.32	9.60	500
6	inactive					
7	1.17	0.040	0.5			
8	1.22	0.070	1.0			
9	1.94	0.255	2.2	1.00	10.0	167

properly designed small organic molecules can be used to inhibit selectively individual serine proteases, even though these molecules cannot participate in secondary subsite interactions.

Wold and Brown[15] have previously shown that aliphatic isocyanates can be used to inhibit selectively the serine proteases. However, the high toxicity of isocyanates has prompted us to investigate the inhibitory activity of compounds that function as latent isocyanates. 1-(Alkylcarbamoyl)imidazoles and -benzimidazoles, sulfated hydroxamic acids, and others are known to function as latent isocyanates.[30] In this paper we report on the use of azolides (I) derived from appropriate amino acid esters in the irreversible inhibition of PPE and HLE and demonstrate that masked isocyanates can be used as inhibitors of the serine proteinases.

Chemistry. Compounds represented by structure I were prepared by using published procedures.[31] Compounds II were prepared by reacting equimolar amounts of the appropriate isocyanate with imidazole, benzimidazole, or other heterocyclic bases. The 1-(dialkyl-



I

carbamoyl)imidazoles were synthesized by reacting the appropriate amine with carbonyldiimidazole in dry THF[32] (Table V).

Table III. Inhibition of Porcine Pancreatic Elastase and Human Leukocyte Elastase by Amino Acid Derived Isocyanates and Compound **10**

	% inhibition[a]	
compd	PPE	HLE
10	0[b]	100[c]
L-$CH_3SCH_2CH_2CH(COOCH_3)N{=}C{=}O$ (11)	27	100
L-$CH_3CH_2CH_2CH(COOCH_3)N{=}C{=}O$ (12)	98	100

[a] Incubation time: 20 min. [b] Fifty microliters of porcine pancreatic elastase, 4.50×10^{-6} M in 2.00 mL of phosphate buffer, pH 6.5, and 50 μL of inhibitor, 2.50×10^{-3} M in acetonitrile, were incubated at 25 °C. Enzyme was assayed by adding 50 μL of (*tert*-butoxycarbonyl)-L-alanyl-*p*-nitroanilide, 2.22×10^{-2} M. Ten microliters of human leukocyte elastase, 1.53×10^{-4} M in Tris-HCl buffer, pH 7.5, and 10 μL of inhibitor. Enzyme was assayed by adding 1.00 mL of succinyltrialanyl-*p*-nitroanilide, 2.3×10^{-3} M in Tris buffer.

Results and Discussion

Figure 1 is a typical plot showing the time-dependent inactivation of PPE by compound **9** (Table II). Double-reciprocal plotting of the observed rate constants for inactivation (Figure 2) yielded K_i, the dissociation constant for the enzyme–inhibitor complex and k_3, the limiting rate constant for the irreversible loss of enzyme activity. In comparing the relative inhibitory activity of each inhibitor, the k_3/K_i ratio was computed for each inhibitor and the results are listed in Table II. The irreversibility of the inactivation was demonstrated by dialyzing PPE against 0.05 M phosphate buffer, pH 6.5, for 24 h. No detectable recovery of enzymatic activity was observed.

It is clearly seen from Table II that *N*-(1*H*-imidazol-1-ylcarbonyl)glycine methyl ester (**1**) failed to inactivate either PPE or HLE. This is to be expected since the structure of the inhibitor is such so that binding of the inhibitor to the active site of either enzyme is precluded. Surprisingly, compound **2** showed marginal inhibitory activity toward PPE despite the known preference of PPE for an alanine side chain as a P_1 residue. *N*-(1*H*-Imidazol-1-ylcarbonyl)valine methyl ester (**3**) was found to be an effective inhibitor of HLE (k_3/K_2 = 193 M^{-1} s^{-1}) and a rather poor inhibitor of PPE. As anticipated, the corresponding D-isomer **4** of compound **3** had no effect on either PPE or HLE, reflecting unproductive binding and/or wrong stereochemical alignment of the inhibitor reactive site and the catalytic residues. In accord with previous observations related to the preference of HLE for a three- or four-carbon straight alkyl chain at P_1, *N*-(1*H*-imidazol-1-ylcarbonyl)norvaline methyl ester (**5**) and *N*-(1*H*-imidazol-1-ylcarbonyl)norleucine methyl ester (**9**) were found to be effective inhibitors of HLE. Thus, compounds **3**, **5**, **9** show an avidity for HLE but not PPE. The rate of inactivation of PPE and HLE by L-norvaline methyl ester isocyanate was too rapid to measure. We were unable

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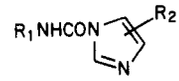
Table IV. Inhibition of Porcine Pancreatic Elastase by Substituted 1-(Alkylcarbamoyl)imidazoles



compd	R_1	R_2	mp, °C (% yield)	porcine pancreatic elastase[a] K_i, mM	porcine pancreatic elastase[a] $k_3 \times 10^3$ sec^{-1}
13	$CH_3(CH_2)_3$	H[b]		1.4	11
14	$CH_3(CH_2)_3$	2-methyl	(77)[a]	2.41	5
15	$CH_3(CH_2)_3$	4-methyl	(73)[a]	7.23	1
16	$CH_3(CH_2)_3$	2-ethyl	(79)[a]	0.47	2
17	$CH_3(CH_2)_3$	2-isopropyl	(80)[c]		
18	$CH_3(CH_2)_3$	2-*n*-butyl	(67)[d]		
19	$CH_3(CH_2)_3$	2-*n*-heptyl	35–36 (74)	2.41	2

[a] 50 mM phosphate buffer, pH 6.5, 25 °C, Boc-L-Ala-*p*-nitrophenyl ester, 2.50×10^{-3} M in acetonitrile, porcine pancreatic elastase, 9.0×10^{-6} M. The inhibitor to enzyme ratio varied between 20 and 150. [b] K_i was 9.7 mM with HLE. 2 M Tris-HCl buffer, pH 8.0, 25 °C, succinyltri-L-Ala-Ala-Ala-*p*-nitroanilide as substrate, 1.6×10^{-7} M human leukocyte elastase. Inhibitor was dissolved in *N*-methylpyrrolidinone. [c] Unstable in buffer, pH 8.0. [d] Inactive.

to make measurments at high pH values because of the instability of the inhibitors.

The results with *N*-(1*H*-imidazol-1-ylcarbonyl)-methionine methyl ester (**10**) proved to be significant and interesting. Human α_1-proteinase inhibitor (α_1-PI) is a serum protein which functions as a specific inhibitor of the serine proteinases.[33] The interaction of a serine proteinase with α_1-PI results in the formation of a highly stable 1:1 complex involving the active site serine of the proteinase and a methionine–serine peptide bond in the α_1-PI. The oxidation of a critical methionine residue at the active site of α_1-PI to the corresponding sulfoxide inactivates the proteinase inhibitor, creating a proteinase–α_1-PI imbalance.[34,35] Reduction of the methionine sulfoxide residue by methionine sulfoxide peptide reductase restores the biological function of the inhibitor.[36] The importance and role of the methionine residue in α_1-PI has prompted us to examine the inhibitory activity of *N*-(1*H*-imidazol-1-ylcarbonyl)methionine methyl ester (**10**) toward PPE and HLE. This compound has been found to show remarkable specificty and high potency toward HLE. PPE is unaffected by the inhibitor under the same conditions (Table III). It is not intuitively apparent why such a high specificity is shown by compound **10**, although it is likely that the side chain of methionine is highly complementary to the S_1 subsite of HLE. Steric effects probably play a large role as far as the lack of inhibitory activity toward PPE is concerned. This finding is of pharmacological significance since the isosteric replacement of a CH_2 group by sulfur gives rise to a highly biospecific inhibitor. The precursor isocyanate of compound **10** inhibited PPE weakly. However, incubation of **12** with HLE resulted in total loss of enzyme activity (Table III).

In contrast to the amino acid derived inhibitors, simpler molecules such as 1-(butylcarbamoyl)imidazole **13** (Table IV) were found to be poor inhibitors of both PPE and HLE. The inhibition of PPE by simple 1-(alkylcarbamoyl)heterocycles has established the following.

(a) Both substituted and unsubstituted 1-(alkylcarbamoyl)imidazoles (Table IV) are capable of inhibiting PPE (Table IV). The presence of hydrophobic substituents in the imidazole ring did not result in a significant enhancement in the inhibitory activity of these compounds. The 2-ethyl-substituted compound **16** had a K_i of 4.7×10^{-4} M. The K_i values of the rest of the compounds were in the millimolar range. Interestingly, compound **18** (Table IV) was found to be inactive. This can probably be ascribed to possible binding of the *n*-butyl side chain in the imidazole ring which would juxtapose the reactive groups incorrectly relative to the catalytic residues at the active site.

(b) In contrast to these compounds, the 1-(alkylcarbamoyl)pyrazoles and 1,2,4-triazoles (Table V) were devoid of any inhibitory activity. These compounds are not known to function as latent isocyanates.[30] Similarly, the 1-(alkylcarbamoyl)benzimidazoles (Table V) failed to inactivate PPE despite the known propensity of these compounds to dissociate readily into the corresponding isocyanates. The bulky benzimidazole ring apparently imposes severe steric constraints in the vicinity of the S'_1 subsite which affects adversely the binding of the inhibitor to the active site of the enzyme.

(c) The 1-(alkylcarbamoyl)heterocycles such as **20–22** (Table V), for example, having alkyl chains consisting of more than four carbon atoms or a bulky aromatic ring were found to be inactive. The introduction of bifunctionality (Table V, compounds **23** and **24**) also failed to yield active compounds. The size of the alkyl chain is of paramount importance and reflects the size of the binding cleft at the active site of PPE.

(d) 1-(Dialkylcarbamoyl)imidazoles (Table V), which lack the ability to function as latent isocyanates, failed to inhibit PPE under comparable conditions.

The inhibition of HLE by the amino acid derived azolides (I) can be depicted as taking place either by direct acylation of the active site serine or by reaction of the active site serine with an enzyme-generated isocyanate. Although further work is needed in order to delineate fully the mechanism of the inactivation process, this study demonstrates that masked isocyanates can be useful probes and selective inhibitors of the serine proteinases. Compounds **3**, **5**, and **9** are currently undergoing in vivo evaluation to ascertain their effectiveness in alleviating elastase-induced emphysema in hamsters. These compounds also offer the means of circumventing host toxicity and are therefore of potential pharmacological value.

Experimental Section

Porcine pancreatic elastase was purchased from Worthington Biochemicals Co. Human leukocyte elastase was purified from isolated neutrophils which were obtained from freshly drawn human peripheral blood by published procedures.[37] *tert*-(Butoxycarbonyl)alanyl-*p*-nitriphenol and succinyltrialanyl-*p*-nitroaniline were purchased from Sigma Co. The infrared spectra of the synthesized compounds were recorded on either a Beckman Acculab or a Perkin-Elmer 1330 infrared spectrophotometer and the ^1H NMR spectra were recorded on a Hitachi Perkin-Elmer R-24B spectrometer using tetramethylsilane as an internal

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Table V. 1-(Alkylcarbamoyl)heterocycles and 1-(Dialkylcarbamoyl)imidazoles, R_1R_2N-CO-L (II)

compd	R_1	R_2	L	mp, °C (recrystn solvent)	% yield
20	$CH_3(CH_2)_5$	H	imidazole	39-41 (hex/CH_2Cl_2)	73
21	$CH_3(CH_2)_7$	H	imidazole	oil	77
22	$CH_3(CH_2)_{11}$	H	imidazole	62-64 (hex/ethyl ether)	70
23	$Br(CH_2)_{10}$	H	imidazole	82-84	79
24	I-$(CH_2)_5$	H	imidazole	47	82
25	phenyl	H	imidazole	112-114 (hex/CH_2Cl_2)	84
26	$CH_3(CH_2)_2$	H	benzimidazole	60-62 (hex/CH_2Cl_2)	70
27	$CH_3(CH_2)_3$	H	benzimidazole	40-42 (hex/CH_2Cl_2)	77
28	$CH_3(CH_2)_5$	H	benzimidazole	40-42 (hex/CH_2Cl_2)	79
29	$CH_3(CH_2)_7$	H	benzimidazole	39-40 (hex/CH_2Cl_2)	70
30	cyclohexyl	H	benzimidazole	112-115 (hex/CH_2Cl_2)	67
31	$CH_3(CH_2)_2$	H	1,2,4-triazole	67-68	48
32	$(CH_3)_2CH$	H	1,2,4-triazole	58-61	59
33	$CH_3(CH_2)_3$	H	1,2,4-triazole	68-70	44
34	$CH_3(CH_2)_2$	H	pyrazole	46-48	63
35	$(CH_3)_2CH$	H	pyrazole	64-65	59
36	$CH_3(CH_2)_3$	H	pyrazole	31-33	64
37	CH_3CH_2	CH_3CH_2	imidazole	45-47	94
38	$CH_3(CH_2)_2$	$CH_3(CH_2)_2$	imidazole	oil	77
39	$CH_3(CH_2)_3$	$CH_3(CH_2)_3$	imidazole	oil	65
40	$(CH_2)_4$		imidazole	56-59	76
41	$(CH_2CH_2OCH_2CH_2)$		imidazole	91-92	81
42	$(CH_2)_5$		imidazole	61-64	82

standard. Melting points were recorded on a Mel-Temp apparatus and are uncorrected. A Gilford 2600 UV/visible spectrophotometer equipped with a Hewlett-Packard XY plotter was used in the enzyme assays and inhibition studies.

Synthesis. Representative syntheses for all of the compounds are described below.

L-*N*-(Imidazol-1-ylcarbonyl)norvaline Methyl Ester (5). A 1.36-g (0.02 mol) sample of imidazole was mixed with 3.14 g (0.02 mol) of L-norvaline methyl ester isocyanate[31] in 20 mL of anhydrous ethyl ether under a nitrogen atmosphere. The reaction mixture was refluxed with stirring for 2 h. Removal of the solvent in vacuo left a colorless oil which solidified upon refrigeration. The isolated solid was homogeneous by TLC (silica gel:ethyl acetate/chloroform, 1:1), melted at 86–88 °C, and was obtained in 96% yield (4.3 g). Anal. ($C_{10}H_{15}N_3O_3$) C, H, N.

The rest of the compounds listed in Table I were prepared in a similar manner.

1-(Dodecylcarbamoyl)imidazole (22). A solution of imidazole (0.68 g, 10 mmol) in 15 mL of dry ethyl was treated with *n*-dodecyl isocyanate (2.11 g, 10 mmol) under a nitrogen atmosphere. After the solution was refluxed for 3 h, the solvent was evaporated off in vacuo leaving a white solid which was recrystallized from hexane/ethyl ether (2.20 g, 70% yield), mp 62–64 °C.

1-(Diethylcarbamoyl)imidazole (37). Carbonyldiimidazole (1.62 g, 10 mmol) in 10 mL of dry THF was added to a solution of diethylamine (0.73 g, 10 mmol) in 10 mL of THF at room temperature under nitrogen. Stirring was continued for 2 h. Evaporation of the solvent in vacuo left a residue which was taken up in methylene chloride and washed with water (2 × 35 mL). After the organic layer was dried over anhydrous sodium sulfate, the solvent was removed on a rotary evaporator, leaving a solid which was purified by recrystallization from ethyl ether, mp 45–47 °C (1.26 g, 75% yield).

Enzyme Assays. Porcine pancreatic elastase was assayed spectrophotometrically at 347.5 nm with (*tert*-butoxycarbonyl)-alanyl-*p*-nitrophenol in 0.05 M phosphate buffer, pH 6.5, 25 °C, or by using succinyl-L-trialanyl-*p*-nitroanilide in 0.1 M Tris buffer, pH 8.0, at 410 nm.[5] Human leukocyte elastase was assayed spectrophotometrically at 410 nm with succinyl-L-trialanyl-*p*-nitroanilide in 0.1 M Tris buffer, pH 8.0.[5]

Inactivation Studies. The appropriate amount of inhibitor in acetonitrile was mixed with porcine pancreatic elastase in phosphate buffer solution and placed in a constant-temperature bath. Aliquots were withdrawn at different time intervals and transferred to a cuvette containing 100 μL of substrate, 140 μL of acetonitrile, and 2 mL of phosphate buffer, pH 6.5. After incubating for 30 s, the absorbance change was monitored for 2 min at 347.5 nm. The inhibitor to enzyme ratios varied between 50 and 200 and the incubation intervals were shortened or lengthened depending on the potency of the inhibitor. Control reactions for 100% PPE activity were run in the presence of acetonitrile.

The reaction of an irreversible inhibitor with PPE and HLE may be illustrated by the scheme shown below

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

where EI is the noncovalently bound enzyme–inactivator complex and E–I is the final product with the inhibitor covalently bound to the enzyme. The kinetics data were analyzed according to Kitz and Wilson.[38] The apparent pseudo-first-order inactivation rate constants were determined from the slopes of the semilogarithmic plots of enzymatic activity remaining vs. time using eq 1, where

$$\ln (E_t/E_0) = k_{obsd}t \quad (1)$$

E_t/E_0 is the amount of active enzyme remaining after time t. The kinetic constants in Table II were determined by linear regression analysis and represent the average of two rate determinations of each inhibitor concentration. Correlation coefficients were greater than 0.980. Replotting of the data using equation 2 yielded values for the parameters K_i, the dissociation constants for the enzyme–inhibitor complex, and k_3, the limiting rate constant for the irreversible loss of enzyme activity. The inhibition of HLE

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

was also studied as a pseudo-first-order reaction to 100% completion of inhibition at pH 8.0, 25 °C, 0.1 M Tris buffer. The synthetic substrate succinyl L-trialanyl-*p*-nitroanilide was used to follow continuously the proportion of enzyme remaining active and the resulting pseudo-first-order rate constants determined according to the method of Tsou.[39]

Dialysis Studies. One milliliter of phosphate buffer (pH 6.5), 230 μL of the elastase stock solution, and 308 μL of the stock inihibitor solution were mixed together in a test tube. A control test tube was prepared in a similar manner except 308 μL of acetonitrile was used in place of the inhibitor. The two test tubes were incubated for 1 h in a 25 °C water bath. After the incubation period, 50 μL of the inhibitor solution was delivered into a cuvette containing 2 mL of phosphate buffer (pH 6.5), 110 L of substrate, and 140 μL of acetonitrile. The cuvette was shaken and incubated for 30 s, and the absorbance change was monitored for 2 min at 347.5 nm. The same process was repeated for the control solution.

Once the enzyme assay was completed, the two solutions were dialyzed against a 0.05 M sodium phosphate buffer, pH 6.5, for 24 h. Upon completion of the dialysis period, 50-μL aliquots of each solution were removed, and an enzyme assay was run exactly

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like the assay at the 1-h incubation period.

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Registry No. 1, 93644-93-0; 2, 93644-94-1; 3, 93605-47-1; 4, 93605-48-2; 5, 93605-49-3; 6, 93605-50-6; 7, 93605-51-7; 8, 93605-52-8; 9, 93605-53-9; 10, 93605-54-0; 11, 93778-88-2; 12, 93712-83-5; 13, 63678-26-2; 14, 93605-55-1; 15, 93605-56-2; 16, 93605-57-3; 17, 93605-58-4; 18, 93605-59-5; 19, 93605-60-8; 20, 93605-61-9; 21, 93605-62-0; 22, 6475-71-4; 23, 93644-95-2; 24, 93605-63-1; 25, 33876-94-7; 26, 93605-64-2; 27, 57964-56-4; 28, 93605-65-3; 29, 93605-66-4; 30, 58425-49-3; 31, 93605-67-5; 32, 93605-68-6; 33, 93605-69-7; 34, 93605-70-0; 35, 93605-71-1; 36, 93605-72-2; 37, 61985-24-8; 38, 66772-82-5; 39, 66772-80-3; 40, 93605-73-3; 41, 93605-74-4; 42, 72741-21-0; imidazole, 288-32-4; dodecyl isocyanate, 4202-38-4; carbonyldiimidazole, 530-62-1; elastase, 9004-06-2.

Chemistry and Antitumor Evaluation of Selected Classical 2,4-Diaminoquinazoline Analogues of Folic Acid

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A series of six 2,4-diaminoquinazoline analogues of folic acid which bear close structural resemblance to methotrexate, **1a**, were synthesized by unequivocal routes. Three of these have not been described previously, while complete structural characterization of the remaining compounds is presented for the first time. Each of the compounds was a potent inhibitor of dihydrofolate reductase (DHFR) from rat liver or L1210 leukemia cells having I_{50} values in a range similar to that of **1a**. However, a wide divergence in inhibitory activity toward the growth of human gastrointestinal adenocarcinoma or L1210 leukemia cells in vitro was observed. Compounds having a normal folate configuration at positions 9 and 10 were more inhibitory than their isomeric reversed-bridge counterparts. The *N*-formyl modifications were the least active of the compounds studied. Unsubstituted or *N*-methyl modifications competed effectively with tritiated **1a** for uptake into L1210 leukemia cells, while *N*-formyl modifications did not. Against an L1210 cell line resistant to **1a** by virtue of altered transport and overproduction of DHFR, partial but not complete cross-resistance was observed for certain analogues. Of the three compounds selected for in vivo evaluation against L1210 leukemia in mice, two had a similar level of antitumor activity to that of **1a**. The compound 5,8-dideazamethopterin, **2b**, however, was slightly more active than **1a** but at substantially reduced dose levels.

The folic acid antagonists methotrexate, **1a**, and aminopterin, **1b**, were among the earliest cancer chemotherapeutic agents to be developed on a rational basis.[1] Compound **1a** remains a mainstay in the treatment of certain human neoplastic diseases, but the drug has numerous shortcomings. With the exception of a few high growth fraction cancers such as acute lymphocytic leukemia, choriocarcinoma, and Hodgkin's disease, most human tumors are only marginally responsive or completely refractory.[2] The use of high doses of **1a** followed by rescue with calcium leucovorin has expanded somewhat the clinical utility of this drug.[3]

Mechanisms of tumor resistance to **1a** include (i) overproduction of the target enzyme DHFR,[4] which has been linked to the amplification of DNA sequences containing the enzyme structural gene;[5] (ii) alterations in the membrane transport proteins for folates giving rise to reduced uptake of **1a**;[6] (iii) production of a structurally altered DHFR having dramatically decreased binding affinity for **1a**.[7-9] In addition, recent studies suggest that differences in the degree of intracellular poly-γ-glutamate formation may be a determinant of tumor cell responsiveness.[10] Longer chain polyglutamates of natural folates and **1a** appear to be selectively retained by most mammalian cell lines.[11-13] Differential synthesis of polyglutamates of **1a**

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