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Proteinase Inhibitors. 1. Inhibitors of Elastase

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A series of peptides and depsipeptides containing 2-methylcarbazic acid (H-Mec-OH), the 2-aza analogue of alanine, was prepared and tested as inhibitors of pancreatic and human granulocyte elastases. A requirement for a minimum chain length as well as specific amino acid sequence was observed which correlates well with both substrate and inhibitor studies by others in this field. The most active inhibitors have the structure Ac-Ala-Ala-Pro-Mec-Lac-R. When Lac-R is an ester, only the pancreatic enzyme is inhibited. When Lac-R is an amide or hydrazide, then both enzymes are inhibited. The inhibitory activity is reversible; the inhibitors are not hydrolyzed by the enzyme and the inhibition is noncompetitive with synthetic substrates of similar structure, suggesting that binding at the sites adjacent to the carboxyl group of the amino acid analogue, 2-methylcarbazic acid, is important for this inhibition. The data further demonstrate the differences between pancreatic and granulocyte elastases.

Our interest in the synthesis of elastase inhibitors arises from the probable involvement of this enzyme in tissue destruction associated with arthritis,[1] inflammation,[1] emphysema,[1] and pancreatitis.[2] Effective inhibition of elastase would, therefore, appear to be a worthwhile goal in the amelioration of these disease states.

Several types of elastase inhibitors have been reported in the literature. These include certain di- and tripeptides,[3] peptide chloromethyl ketones,[4-7] and peptide aldehydes,[8] including elastatinal.[9] More recently, Powers has described a new type of elastase inhibitor, namely, peptide carbazate *p*-nitrophenyl esters such as Cbz-Ala-Ala-Pro-Mec-*p*-NP (**2**).[10] In these compounds, the alanine moiety at position P_1[11] of the substrate–inhibitor has been replaced by its nitrogen isostere 2-methylcarbazic acid (Mec). Such compounds acylate the hydroxy group of the active site serine.[12] The resulting carbazoyl enzyme deacylates slowly because of the influence of the nitrogen atom adjacent to the bond which is cleaved.

Because the reactivity of the *p*-nitrophenyl ester group makes its in vivo usefulness doubtful, we prepared a series of peptide carbazates incorporating at the P_1 position the alanine isostere 2-methylcarbazic acid but lacking a terminal activated ester bond such as Ac-Ala-Ala-Pro-Mec-OEt (**3**). The compounds were designed primarily as inhibitors of pancreatic elastase since this enzyme has been well studied and the structural features of its substrates and inhibitors are well known. Human granulocyte elastase was previously believed to be similar to the pancreatic enzyme, but differences between the two with respect to both substrate specificity and sensitivity to inhibitors are now becoming apparent[12-15] and, indeed, were observed in our studies.

Our first objective was to determine whether or not a peptide carbazate terminated by a simple ester instead of the activated *p*-nitrophenyl group was still capable of acylating the enzyme and, if not, could such a compound function as a competitive inhibitor. Compounds **3–10** served to answer this question and also to verify the structural requirements of the peptide backbone as reported by Thompson and Blout[6,8,16] for porcine pancreatic elastase.

Another objective was to make use of the $S_{1'}$ and $S_{2'}$ subsites on the enzyme ($P_{1'}$ and $P_{2'}$ on the inhibitor) to increase the strength of binding between enzyme and inhibitor. The terminology S and P is that of Schechter and Berger.[11]

$\cdots S_2\text{–}S_1\text{–}S_{1'}\text{–}S_{2'}\cdots$ refers to subsites on both sides of the catalytic site of the enzyme. Each subsite may be composed of several amino acid residues arranged in a particular geometric pattern which allows for interaction with an amino acid residue of the substrate–inhibitor. The notation P on the substrate (or inhibitor) denotes the amino acids which bind to these enzyme subsites such that $P_1\text{–}P_{1'}$ represents the bond which is cleaved. Moreover, the utilization of these additional binding sites might confer increased selectivity for inhibiting elastase but not other serine proteases. At the time we initiated our efforts, no study had been reported which characterized the nature of the amino acids preferred at the $P_{1'}$ and $P_{2'}$ subsites of an elastase substrate or inhibitor. During the course of our study, however, Atlas published the finding that lysine and phenylalanine are favored at the $P_{1'}$ and $P_{2'}$ sites, respectively,[17] of synthetic substrates for the porcine pancreatic enzyme.

Our exploration of the $P_{1'}$ subsite was guided by the observation of Thompson[16] that the tetraalanine amide, Ac-Ala-Ala-Ala-Ala-NH_2, could bind to pancreatic elastase in the S_{54321} or $S_{43211'}$ mode, giving rise to ammonia or alaninamide, respectively. This suggested to us the synthesis of a carbazate ester resembling alanine at the $P_{1'}$ site. Accordingly, we prepared compounds **11–13** which contain the alanine isostere lactic acid (Lac) at the $P_{1'}$ site. Compounds **14–19** interchange alanine and its oxygen and nitrogen isosteres, lactic acid, and 2-methylcarbazic acid at the P_1 and $P_{1'}$ sites. Compounds **20–23** verify both length and amino acid requirements of the peptide backbone in the carbazate lactate series. Compounds **24–36** were prepared to evaluate the effect of various substituents on the lactate moiety.

Compounds **2–36** were evaluated for their ability to inhibit the cleavage of the synthetic substrate Ac-Ala-Ala-Pro-Ala-*p*-nitroanilide (**1**) by the enzyme under the conditions specified in the Experimental Section. Results are shown in Table I. K_i values for the most active compounds are shown in Table II.

The majority of the compounds listed in Table I were prepared according to one of the following routes shown

Table I. Peptide Carbazates as Inhibitors of Elastase

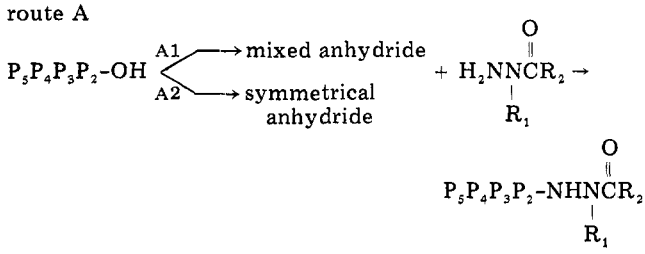
Compd no.	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	ID_{50} vs. elastase, μg/mL: Porcine pancreatic	ID_{50} vs. elastase, μg/mL: Human leucocyte
1	Ac	Ala	Ala	Pro	Ala	*p*-NA			Substrate	Substrate
2	Cbz	Ala	Ala	Pro	Mec	*p*-NP			1	
3	Ac	Ala	Ala	Pro	Mec	OEt			20	Inactive
4	Ac	Pro	Ala	Pro	Mec	OEt			60	Inactive
5	Boc	Ala	Ala	Pro	Mec	OEt			90	
6	Cbz	Ala	Ala	Pro	Mec	OEt			30	
7	Ac	Ala	Ala	Pro	Car	OEt			Inactive	
8	Ac	Pro	Ala	Pro	Car	OEt			Inactive	Inactive
9	Boc	Ala	Ala	Pro	Car	OEt			Inactive	
10			Ac	Ala	Mec	OEt			Inactive	Inactive
11	Ac	Ala	Ala	Pro	Mec	Lac	OEt		1	Inactive
12	Ac	Pro	Ala	Pro	Mec	Lac	OEt		6	Inactive
13	Ac	Pro	Ala	Pro	Mec	DL-Lac	OEt		4.5	Inactive
14	Ac	Ala	Ala	Pro	Mec	Ala	OEt		100	
15	Ac	Pro	Ala	Pro	Mec	Ala	OEt		Inactive	Inactive
16	Ac	Ala	Ala	Pro	Ala	Lac	OEt		167	Inactive
17	Ac	Pro	Ala	Pro	Ala	Lac	OEt		Inactive	Inactive
18	Ac	Ala	Ala	Pro	Lac	Lac	OEt		33	Inactive
19	Ac	Pro	Ala	Pro	Lac	Lac	OEt		167	Inactive
20	Ac	Ala	Ala	Leu	Mec	Lac	OEt		9	Inactive
21	Ac	Pro	Ala	Leu	Mec	Lac	OEt		15	Inactive
22				Ac	Mec	Lac	OEt		Inactive	Inactive
23			Ac	Ala	Mec	Lac	OEt		Inactive	Inactive
24	Ac	Ala	Ala	Pro	Mec	Lac	OCH_2Ph		0.5	Inactive
25	Ac	Ala	Ala	Pro	Mec	3-Ph-Lac	OEt		2	Inactive
26	Ac	Ala	Ala	Pro	Mec	Lac	Lac	OEt	1.5	Inactive
27	Ac	Pro	Ala	Pro	Mec	Lac	Lac	OEt	1.5	Inactive
28	Ac	Ala	Ala	Pro	Mec	Lac	Phe	OEt	2	Inactive
29	Ac	Ala	Ala	Pro	Mec	Lac	OH		110	Inactive
30	Ac	Ala	Ala	Pro	Mec	Lac	NH_2		0.4	8
31	Ac	Pro	Ala	Pro	Mec	DL-Lac	NH_2		0.3	9
32	Ac	Ala	Ala	Pro	Mec	Lac	$NHCH_3$		0.4	10
33	Ac	Ala	Ala	Pro	Mec	Lac	NHPh		0.4	10
34	Ac	Ala	Ala	Pro	Mec	Lac	$NHCH_2Ph$		0.3	10
35	Ac	Ala	Ala	Pro	Mec	Lac	$NHNH_2$		0.25	5
36	Ac	Ala	Ala	Pro	Mec	Lac	$N(CH_3)NH_2$		2	50

Table II. K_i of Inhibition of Elastases by Peptide Carbazate Derivatives

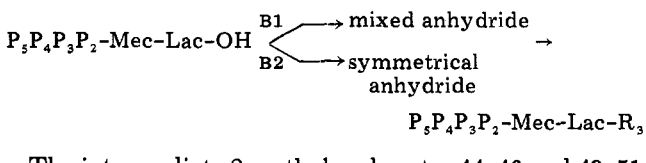
Compd no.	$K_i \times 10^7$, pancreatic elastase	$K_i \times 10^5$, granulocyte elastase
30	5.0	3.9
31	4.5	3.9
32	6.5	3.6
33	4.5	3.9
34	8.5	2.2
35	6.5	3.8

below. Other syntheses are described in the Experimental Section.

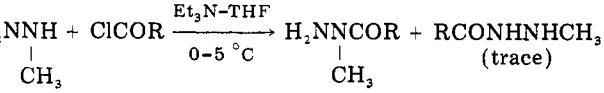
route A



route B



The intermediate 2-methyl carbazates **44–46** and **49–51** were prepared by direct acylation of an excess of 2-methylhydrazine with the appropriate chloroformate derivative in the cold.



Under these conditions acylation occurred almost exclusively on the methylated nitrogen. Column chromatography afforded the desired product free from isomeric material. Structure proof was based on the chemical shift of the NCH_3 peak which was observed at ~3.2 ppm. The chemical shift of the NCH_3 peak of the isomeric compound would be expected at ~2.5 ppm.[18] In addition, further acylation of **44–46** and **49–51** did not result in any significant change in the NCH_3 peak which was then observed at 3.04–3.20 ppm.

Structure–Activity Relationships. Results. Pancreatic Elastase. Replacement of the *p*-nitrophenyl ester group by a simple ethyl ester gives compounds **3–6** which are weak inhibitors of pancreatic elastase. In this series there appears to be a slight advantage with alanine in place of proline at P_4. Increasing bulk at P_5 decreases inhibitory activity. Replacement of 2-methylcarbazic acid (Mec) by carbazic acid (Car), the nitrogen isostere of glycine (compounds **7–9**), results in decreased or no activity. This is in accord with previous studies[19] which show that some side chain binding is required at P_1. Compound **10** verifies the need for an extended chain of at least three or four amino acids.[20,21]

The introduction of lactic acid ethyl ester into the P_1' site (**11–13**) results in a dramatic increase in inhibitory activity. If this lactate is replaced by the amino acid

Table III. Synthesis and Properties of Peptide Carbazates and Key Intermediates

Compd no.	Method	% yield	Mp, °C	Formula	Analyses
3	A1	55	167-169	$C_{17}H_{29}N_5O_6$	C, H, N
4	A1	47	105-110	$C_{19}H_{31}N_5O_6 \cdot 0.5H_2O$	C, H, N
5	A1	10		$C_{20}H_{35}N_5O_7 \cdot H_2O$	C, H; N[a]
6		44	152-155	$C_{23}H_{33}N_5O_7$	C, H, N
7	A1	50	177-179	$C_{16}H_{27}N_5O_6$	C, H, N
8	A1	46		$C_{18}H_{29}N_5O_6 \cdot H_2O$	C, H, N
9	A1	31		$C_{19}H_{33}N_5O_7$	C, H, N
10		84	124-126	$C_9H_{17}N_3O_4 \cdot 0.25H_2O$	C, H, N
11	A1	70	95-100	$C_{20}H_{33}N_5O_8$	C, H, N
12	A1	35	80-85	$C_{22}H_{35}N_5O_8 \cdot 0.5H_2O$	C, H, N
13	A1	75	81-87	$C_{22}H_{35}N_5O_8 \cdot 0.5H_2O$	C, H, N
14	A1	71	165-172	$C_{20}H_{34}N_6O_7$	C, H, N
15	A1	70	161-165	$C_{22}H_{36}N_6O_7$	C, H, N
16	A1	35	72-76	$C_{21}H_{34}N_4O_8 \cdot 0.25H_2O$	C, H, N
17	A1	50	69-74	$C_{22}H_{36}N_4O_8 \cdot 0.25H_2O$	C, H, N
18	A2	25		$C_{21}H_{33}N_3O_9$	C, H, N
19	A2	65		$C_{23}H_{35}N_3O_9$	C, H, N
20	A1	48	220-225	$C_{21}H_{37}N_5O_8$	C, H, N
21	A1	32	207-212	$C_{23}H_{39}N_5O_8$	C, H, N
22		85		$C_9H_{16}N_2O_5$	C, H, N
23		76	93-98	$C_{12}H_{21}N_3O_6 \cdot 0.25H_2O$	C, H, N
24	A1	67	95-105	$C_{25}H_{35}N_5O_8$	C, H, N
25	A1	50		$C_{26}H_{37}N_5O_8$	C, H, N
26	A1	50		$C_{23}H_{37}N_5O_{10}$	C, H, N
27	A1	48		$C_{25}H_{39}N_5O_{10}$	C, H, N
28	B1	50		$C_{29}H_{42}N_6O_9$	C, H, N
29		95		$C_{18}H_{29}N_5O_8 \cdot 0.5H_2O$	C, H, N
30	A1	55		$C_{18}H_{30}N_6O_7 \cdot H_2O$	C, H, N
31		64	105-111	$C_{20}H_{32}N_6O_7 \cdot 0.5H_2O$	C, H, N
32	B1	52		$C_{19}H_{32}N_6O_7 \cdot 2H_2O$	C, H; N[b]
33	B1	70		$C_{24}H_{34}N_6O_7$	C, H, N
34	B2	48		$C_{25}H_{36}N_6O_7$	C, H, N
35	B2	75		$C_{18}H_{31}N_7O_7 \cdot H_2O$	C, N; H[c]
36	B1	42		$C_{19}H_{33}N_7O_7$	C, H; N[d]
38		44		$C_{13}H_{21}N_3O_5$	
39		46		$C_{16}H_{27}N_3O_6 \cdot 0.75H_2O$	C, H, N
40		29	185-186	$C_{14}H_{25}N_3O_5$	C, H, N
41		32	205-207	$C_{16}H_{27}N_3O_5$	C, H, N
44		41		$C_7H_{14}N_2O_4$	C, H, N
45		31		$C_7H_{14}N_2O_4$	
46		45	77-79.5	$C_7H_{15}N_3O_3$	C, H, N
47		78		$C_8H_{15}NO_4$	
49		42		$C_{12}H_{16}N_2O_4$	C, H, N
50		32		$C_{13}H_{18}N_2O_4$	C, H, N
51		52		$C_{10}H_{18}N_2O_6$	C, H, N

[a] N: calcd, 14.72; found, 14.28. [b] N: calcd, 17.06; found, 16.30. [c] H: calcd, 6.99; found, 6.46. [d] N: calcd, 20.79; found, 19.95.

alanine (14 and 15) activity decreases. Likewise, replacement of Mec at P_1 with alanine while maintaining lactate at $P_{1'}$ (16 and 17) results in a loss of inhibitory activity. Replacement of Mec at P_1 with isosteric lactate (18 and 19) also gives less active inhibitors. Substituting leucine for proline at P_2 (20 and 21) results in only a slight decrease in activity as expected from the work of Powers and Tuhy.[22] Decreasing the length of the peptide backbone (22 and 23) results in a loss of activity as expected. Replacing ethyl lactate at $P_{1'}$ with the corresponding benzyl ester 24 results in an active compound. Some measure of bulk tolerance exists at $P_{1'}$ as shown by the fact that 3-phenyllactic acid (25), the oxygen isostere of phenylalanine, is tolerated at this site. Elongation of the molecule to extend binding into the $P_{3'}$ site gives active compounds 26–28. Compound 28 was prepared with phenylalanine at the $P_{2'}$ position based on the preference of pancreatic elastase for this amino acid as shown by Atlas.[17] Removal of the terminal ester group to give a free carboxylic acid 29 results in a decrease in activity.

The most potent inhibitors of pancreatic elastase were those compounds in which the lactic acid at $P_{1'}$ was terminated as an amide or simple hydrazide (30–35). Compound 36, which is a methyl analogue of the potent hydrazide 35, was less active (see Table III).

Human Granulocyte Elastase. Most of the compounds (3–29) prepared by us failed to inhibit granulocyte elastase. Only when the inhibitor was a carbazate lactic acid, terminated as an amide (30–34) or a simple hydrazide (35) rather than an ester, was there reasonably good inhibition of the granulocyte enzyme.

Mechanism of Enzyme Inhibition. The carbazate esters described herein do not appear to function as acylating agents. Incubation of either the carbazate lactate 11 or the carbazate lactamide 31 (1 mg) with pancreatic elastase (50 μg) in 0.05 M phosphate buffer (pH 7.5) for up to 24 h at room temperature had no effect on the structural integrity of the inhibitor as monitored by TLC. In contrast, compounds 16 and 17 which contain the natural amino acid alanine at P_1 are completely hydrolyzed within 5 min when incubated with pancreatic elastase under similar conditions. There is no recovery of enzyme activity upon prolonged incubation of enzyme and inhibitor (31). In addition, removal of excess inhibitor by rapid passage of the mixture of enzyme and inhibitor over Sephadex G-25 results in a fully active enzyme. This

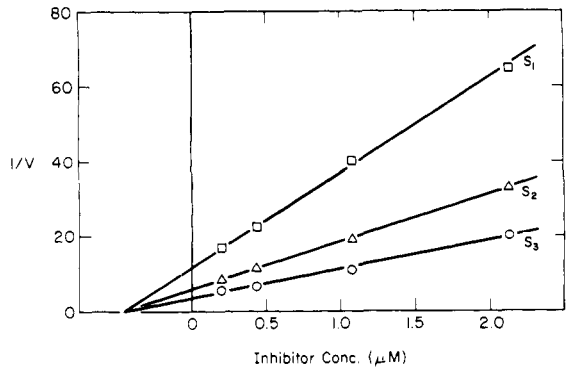


Figure 1. Dixon plot for inhibition of hog pancreatic elastase by Ac-Pro-Ala-Pro-Mec-DL-Lac-NH_2. The substrate is Ac-Ala-Ala-Pro-Ala-*p*-nitroanilide in 0.05 M TES buffer, pH 7.5, containing 10% Me_2SO: S_1 = 0.1 mM, S_2 = 0.2 mM, S_3 = 0.4 mM. V = micromoles of substrate cleaved per milliliter per minute. The enzyme concentration is 0.08 mM.

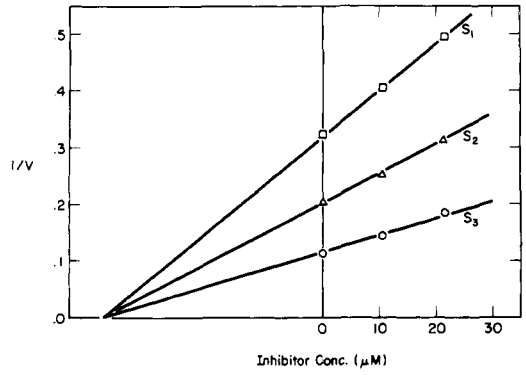


Figure 2. Dixon plot for inhibition of human granulocyte elastase by Ac-Pro-Ala-Pro-Mec-DL-Lac-NH_2. The substrate is as described for Figure 1. The enzyme concentration is 0.4 mM.

observation is in contrast with that of Powers who reported a gradual recovery of enzyme activity when pancreatic elastase was inhibited by the acylating carbazate *p*-nitrophenyl ester Cbz-Ala-Ala-Pro-Mec-*p*-NP[12] due to hydrolysis of the carbazoyl enzyme at pH 7.5. Reactivation was slower at lower pH values. Studies carried out on Ac-Pro-Ala-Pro-Mec-Lac-NH_2 (**31**) indicated noncompetitive inhibition of the enzyme as evidenced by Dixon plots shown in Figures 1 and 2. A possible explanation of this can be offered from the studies of Green and Work.[23] If the K_i of a tightly bound inhibitor is much smaller than the K_m of the substrate used in the assay, then the substrate will not substantially perturb the enzyme–inhibitor equilibrium and the inhibition will appear to be noncompetitive. For porcine elastase the K_m for Ac-Ala-Ala-Pro-Ala-*p*-NA (9.5×10^{-4})[13] is greater than the K_i values ($4.5–8.5 \times 10^{-7}$) of our inhibitors as shown in Table II. In the case of granulocyte elastase the K_m value (1.5×10^{-3})[13] is also greater than the K_i values ($2.2–3.9 \times 10^{-5}$) reported in Table II. We attribute these differences in K_m (substrate) and K_i (inhibitor) to the increased binding of the inhibitor at the $S_{1'}$ and $S_{2'}$ subsites of the enzyme. In addition, the marked differences between pancreatic enzyme and granulocyte enzyme with respect to the requirement of the latter for an amide bond at the P_1–$P_{2'}$ junction further points out a greater complexity of closely related serine proteases than might have been expected.

Experimental Section

General. Melting points were determined on a Thomas-Hoover Uni-Melt apparatus. Lack of melting point data indicates an indefinite melting point or noncrystallinity. Intermediates and final products were examined by infrared, ^1H NMR, mass spectroscopy, and elemental analysis and gave results consistent with the expected structures.

Organic Synthesis. Ac-Pro-Ala-Pro-OH (**37**) was prepared according to the procedure of Thompson and Blout.[19] Similarly prepared were Ac-Ala-Ala-Pro-OH (**38**), Boc-Ala-Ala-Pro-OH (**39**), Ac-Ala-Ala-Leu-OH (**40**) (mp 185–186 °C), and Ac-Pro-Ala-Leu-OH (**41**) (mp 205–207 °C). H-Car-OEt (**42**) was purchased from Aldrich Chemical Co., Inc. H-Mec-OEt (**43**) was prepared according to the methods of Pedersen.[24]

H-Mec-Lac-OEt (44). To a stirred solution of phosgene in benzene (12.5%, 105 mL, 120 mmol) at 0–5 °C was added over a period of 1 h a solution of ethyl 1-(+)-lactate (11.8 g, 100 mmol) in dry THF (100 mL) containing triethylamine (10.6 g, 105 mmol). The reaction mixture was stirred at 0–5 °C for 30 min and then at room temperature for 1 h and filtered and the filtrate concentrated in vacuo to yield ClCO-Lac-OEt (13 g, 67%) which was used without further purification.

To a stirred solution of methylhydrazine (5.8 g, 126 mmol) and triethylamine (10.6 g, 105 mmol) in dry THF (60 mL) at 0–5 °C was added over a period of 1 h a solution of ClCO-Lac-OEt (13 g, 67 mmol) in dry THF (50 mL). The mixture was stirred for 1 h at 0–5 °C and then overnight at room temperature and filtered and the filtrate concentrated in vacuo. Chromatography of the residue (13 g) on silica gel (270 g) and elution with 1% MeOH in $CHCl_3$ gave **44** as an oil (8.0 g, 62%).

In a similar manner were prepared H-Mec-DL-Lac-OEt (**45**), H-Mec-Ala-OEt (**46**) (mp 70–77 °C), H-Mec-Lac-OCH_2Ph (**49**), H-Mec(3-Ph)-Lac-OEt (**50**), and H-Mec-Lac-Lac-OEt (**51**).

H-Ala-Lac-OEt (47). To a solution of Boc-Ala-OH (5.68 g, 30 mmol) in dry THF (50 mL) at −20 °C was added *N*-methylmorpholine (3.40 mL, 30 mmol) followed by isobutyl chloroformate (4.50 mL, 30 mmol). After 30 min there was added to the above mixture a solution of ethyl 1-(+)-lactate (6.0 mL, 60 mmol) and *N*-methylmorpholine (3.40 mL, 30 mmol) in dry THF (20 mL). The mixture was stirred at −20 °C for 1 h and at room temperature overnight and then concentrated in vacuo. The residue was dissolved in $CHCl_3$ (250 mL) and washed with saturated $NaHCO_3$ solution (100 mL). The organic layer was concentrated in vacuo to give crude Boc-Ala-Lac-OEt (7.5 g) which was deblocked without further purification.

A solution of Boc-Ala-Lac-OEt (3.5 g, 12 mmol) in EtOAc (150 mL) was saturated with HCl gas at 0 to −10 °C. After 3 h at room temperature the reaction mixture was stripped in vacuo and the residue triturated with Et_2O to give H-Ala-Lac-OEt as its HCl salt (2.5 g, 91%).

H-Lac-Lac-OEt (48) was prepared by the self-alcoholysis of ethyl lactate according to the procedure of Rehberg and Dixon.[25]

Method A1. Ac-Ala-Ala-Pro-Mec-OEt (3). To a stirred solution of Ac-Ala-Ala-Pro-OH (560 mg, 2.0 mmol) and *N*-methylmorpholine (230 μL, 2.0 mmol) in dry THF (20 mL) at −20 °C was added isobutyl chloroformate (290 μL, 2.0 mmol). After 10 min a solution of ethyl 2-methylcarbazate (240 mg, 2.0 mmol) in THF (2 mL) was added. The mixture was stirred at −20 °C for 1 h and then at room temperature overnight after which it was filtered and the filtrate evaporated to dryness. The residue (800 mg) was chromatographed on silica gel (50 g). Elution with 10% MeOH in $CHCl_3$ gave Ac-Ala-Ala-Pro-Mec-OEt (450 mg, 55%).

Method A2. Ac-Pro-Ala-Pro-Lac-Lac-OEt (19). To a solution of Ac-Pro-Ala-Pro-OH (651 mg, 2.0 mmol) in CH_3CN (20 mL) and CH_2Cl_2 (80 mL) was added DCC (200 mg, 1.0 mmol). The mixture was stirred at room temperature for 1 h and filtered to remove dicyclohexylurea and the filtrate evaporated to give crude $(Ac\text{-}Pro\text{-}Ala\text{-}Pro)_2O$. To this anhydride was added ethyl lactyllactate (500 mg, 2.6 mmol) and pyridine (5 mL). The mixture was heated on the steam bath for 1 h and concentrated to dryness and the residue chromatographed on silica gel (100 g). Elution with 3% MeOH in $CHCl_3$ gave Ac-Pro-Ala-Pro-Lac-Lac-OEt (325 mg, 65%).

Method B1. Ac-Ala-Ala-Pro-Mec-Lac-Phe-OEt (28). To a solution of Ac-Ala-Ala-Pro-Mec-Lac-OH (665 mg, 1.5 mmol) and *N*-methylmorpholine (360 μL, 3.1 mmol) in CH_2Cl_2 (6 mL) and THF (200 mL) cooled to −20 °C was added isobutyl chloroformate (230 μL, 1.5 mmol). After 10 min a cold suspension of phenylalanine ethyl ester hydrochloride (350 mg, 1.5 mmol)

and N-methylmorpholine (180 μL, 1.5 mmol) in THF (80 mL) was added. The mixture was stirred overnight at room temperature and then filtered and the filtrate evaporated to dryness. The residue was chromatographed on 40 g of silica gel. Elution with 10% MeOH in $CHCl_3$ gave Ac-Ala-Ala-Pro-Mec-Lac-Phe-OEt (450 mg, 50%).

Method B2. Ac-Ala-Ala-Pro-Mec-Lac-NH-CH_2Ph (34). To a suspension of Ac-Ala-Ala-Pro-Mec-Lac-OH (220 mg, 0.5 mmol) in CH_3CN (5 mL) was added DCC (70 mg, 0.35 mmol) in 20 mL of CH_2Cl_2 and the mixture then stirred for 1 h at room temperature. To this anhydride-containing mixture was added benzylamine (54 mg, 0.5 mmol). The reaction mixture was stirred overnight at room temperature and then evaporated to dryness. Preparative TLC on silica gel gave Ac-Ala-Ala-Pro-Mec-Lac-$NHCH_2$Ph (130 mg, 48%).

Cbz-Ala-Ala-Pro-Mec-OEt (6). A. Boc-Ala-Pro-Mec-OEt. To a mixture of Boc-Ala-Pro-OH[26] (4.58 g, 16 mmol) and N-methylmorpholine (1.61 g, 16 mmol) in dry THF (80 mL) which was cooled to −20 to −30 °C was added isobutyl chloroformate (2.18 g, 16 mmol), followed after 10 min by the addition of H-Mec-OEt (**43**) (1.89 g, 16 mmol) in $CHCl_3$ (60 mL). After stirring overnight at room temperature, the reaction mixture was concentrated in vacuo, EtOAc (100 mL) added, and the insoluble material removed by filtration. Concentration of the filtrate in vacuo gave crude Boc-Ala-Pro-Mec-OEt (5.80 g, 100%).

B. TFA·Ala-Pro-Mec-OEt. A solution of the above material in CH_2Cl_2 (15 mL) was cooled to 0 °C and TFA (15 mL) added. The reaction mixture was stirred for 1 h at 0 °C and then 1 h at room temperature. Concentration in vacuo gave crude TFA·Ala-Pro-Mec-OEt (7.56 g).

C. Cbz-Ala-Ala-Pro-Mec-OEt (6). To a mixture of Cbz-Ala-OH (1.12 g, 5 mmol) and N-methylmorpholine (0.51 g, 5 mmol) in dry THF (100 mL) which was cooled to −20 to −30 °C was added isobutyl chloroformate (0.68 g, 5 mmol), followed after a period of 10 min by a solution of TFA·Ala-Pro-Mec-OEt (2.0 g, 5 mmol) and N-methylmorpholine (0.51 g, 5 mmol) in $CHCl_3$ (60 mL). After stirring overnight at room temperature the reaction mixture was worked up as in part A. Chromatography of the residue (3.9 g) on silica gel (200 g) using EtOAc as the eluent gave Cbz-Ala-Ala-Pro-Mec-OEt (**6**) (1.1 g, 44%).

Ac-Ala-Mec-OEt (10). To a stirred solution of Ac-Ala-OH (490 mg, 3.3 mmol) in THF (20 mL) at −20 °C was added N-methylmorpholine (0.38 mL, 3.3 mmol). After 5 min, isobutyl chloroformate (0.47 mL, 3.3 mmol) was added and the mixture stirred for 10 min after which time ethyl 2-methylcarbazate (H-Mec-OEt) (460 mg, 3.3 mmol) was added. The reaction mixture was stirred at room temperature for 3 h and filtered, and the filtrate was concentrated in vacuo. Trituration with Et_2O followed by recrystallization from $CHCl_3$–Et_2O gave Ac-Ala-Mec-OEt (**10**) (640 mg, 84%).

Ac-Mec-Lac-OEt (22). To H-Mec-Lac-OEt (1.9 g, 10 mmol) in THF (25 mL) was added Ac_2O (1.3 g, 10 mmol). The reaction mixture was stirred for 1 h at room temperature and concentrated in vacuo and the residue chromatographed on silica gel (100 g). Elution with EtOAc–hexane (50–85%) gave Ac-Mec-Lac-OEt (**22**) (1.98 g, 85%).

Ac-Ala-Mec-Lac-OEt (23). To Ac-Ala-OH (393 mg, 3.0 mmol) in THF (120 mL) which was cooled to −20 °C was added N-methylmorpholine (0.35 mL, 3.0 mmol) followed by isobutyl chloroformate (0.45 mL, 3.0 mmol). The mixture was stirred cold for 10 min and H-Mec-Lac-OEt (695 mg, 3.0 mmol) was added. After stirring at room temperature for 3 h the mixture was filtered and concentrated in vacuo. Chromatography of the residue (1.3 g) on silica gel (75 g) using MeOH in $CHCl_3$ (1–7.5%) gave Ac-Ala-Mec-Lac-OEt (**23**) (700 mg, 76%).

Ac-Ala-Ala-Pro-Mec-Lac-OH (29). A solution of Ac-Ala-Ala-Pro-Mec-Lac-OCH_2Ph (**24**) (1.26 g, 2.4 mmol) in EtOH (60 mL) was hydrogenated over 10% Pd/C (0.13 g) at 40 psi for 30 min. The mixture was filtered through a pad of Celite and the filtrate concentrated to dryness. Trituration of the residue with Et_2O–hexane (1:1) gave Ac-Ala-Ala-Pro-Mec-Lac-OH (**29**) (1.0 g, 95%).

Ac-Pro-Ala-Pro-Mec-DL-Lac-NH_2 (31). Ac-Pro-Ala-Pro-Mec-DL-Lac-OEt (**13**) (995 mg, 2.0 mmol) was treated with liquid NH_3 (20 mL) in a glass lined bomb at room temperature for 20 h. After evaporation of the solvent, the residue was chromatographed on silica gel (70 g). Elution with MeOH in $CHCl_3$ (12–18%) gave Ac-Pro-Ala-Pro-Mec-DL-Lac-NH_2 (**31**) (600 mg, 64%).

Enzyme Assays. Hog pancreatic elastase (chromatographically purified) was purchased from Miles Laboratories; human granulocyte (PMN) elastase was prepared essentially according to the procedure of Taylor and Crawford.[27] The substrate, Ac-Ala-Ala-Pro-Ala-p-nitroanilide, was prepared in this laboratory.[13] Elastase activity was determined at 25 °C using 2×10^{-4} M substrate dissolved in pH 7.5 potassium phosphate buffer containing 10% Me_2SO. The liberation of p-nitroaniline was followed using a Gilford spectrophotometer by measuring the change in absorption at 410 nm. Inhibitors were dissolved in Me_2SO. Inhibition is reported (Table I) as the concentration of inhibitor giving 50% inhibition under the specified conditions. Inhibition constants (K_i) were determined only for the most active compounds in the series using Dixon plots.[28]

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