Synthetic Macromolecular Inhibitors of Human Leukocyte Elastase. 1. Synthesis of Peptidyl Carbamates Bound to Water-Soluble Polymers: Poly-α,β-[*N*-(2-hydroxyethyl)-D,L-aspartamide] and Poly-α-[N^5-(2-hydroxyethyl)-L-glutamine]⊥

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The design and synthesis of macromolecular peptidyl carbamate inhibitors of human leukocyte elastase (HLE), based on coupling of a low-molecular-weight peptidyl carbamate, succinylalanylalanylprolylmethyl isopropyl carbamate, with a linear hydrophilic polymer, poly-α,β-[*N*-(2-hydroxyethyl)-D,L-aspartamide] or poly-α-[N^5-(2-hydroxyethyl)-L-glutamine], is described. The covalent linkage between a flexible linear polymer and a peptidyl carbamate inhibitor of HLE did not compromise *in vitro* inhibitory capacity. The macromolecular peptidyl carbamates reported here represent a novel class of elastase inhibitors with a K_i ranging from 35.5 to 2.0 nM.

Introduction

Human leukocyte elastase (HLE) is a serine protease that has been implicated in the abnormal degradation of connective tissue proteins associated with diseases such as rheumatoid arthritis, adult respiratory distress syndrome, and pulmonary emphysema.[1] The generally accepted explanation of the observed pathogenesis is a proteinase–antiproteinase imbalance. Normally, the level of HLE in human tissues is regulated by the naturally occurring inhibitors α_1-proteinase inhibitor (α_1PI) and α-macroglobulin (α_2M) present in plasma[2] as well as secretory leukocyte protease inhibitor (SLPI) on mucosal surfaces.[3] It is believed that administration of an effective HLE inhibitor might be beneficial in order to restore proteinase–inhibitor balance. Considering the properties of HLE and its natural inhibitors, it is clear that different inhibitors should be developed for short- and long-term therapy. Different approaches have been pursued by the research groups investigating HLE inhibition. Some laboratories focused on the simple supplement of α_1PI produced by recombinant method.[4] This will be certainly an appropriate therapy for the acute states. Others, including us, paid attention to the design and development of low-molecular-weight synthetic inhibitors. Several types of reversible or irreversible synthetic elastase inhibitors have been reported. Among these are peptidyl aldehydes,[5] peptide ketones,[6] peptidyl boronic acids,[7] sulfonate salts,[8] latent isocyanates,[9] and chloroisocoumarins,[10] yenol lactones,[11] benzoxazinones,[12] 2-pyrones,[13] hydantoins,[14] and cephalosporin sulfones.[15,16] The advantage of irreversible HLE inhibitors is that protease cannot be reactivated once inhibition has occurred. In contrast, reversible HLE inhibitors must always be present in sufficient concentration since protease regains its activity.

Our laboratory has been involved in the design and synthesis of HLE inhibitors of carbamate type for the past several years.[17–20] These are low-molecular-weight peptidyl carbamate (PC) inhibitors designed as site-directed inhibitors. In spite of a high inhibitory potency *in vitro*, the therapeutic potential of these low-molecular-weight inhibitors has been offset by short biological half-lives. Rapid elimination from the target compartment or inhibitor decomposition are largely responsible for the short *in vivo* half-lives. Macromolecular forms of peptidyl carbamate inhibitors (PPC) of HLE were proposed for prolongation of their *in vivo* residence times. The rational strategy in the design of synthetic macromolecular inhibitors of HLE was based on consideration of the role of the inhibitor in the pathophysiology of disease. HLE is liberated from neutrophils, migrates into the area of inflammation, and acts to degrade elastin in the extracellular matrix. Hence the inhibitor, the beneficial effects of which arise from curbing enzyme activity and preventing it from spreading, does not need to undergo cellular penetration. The inhibitor therefore can be macromolecular in nature and would be more effective with a long residence time in the interstitial compartment. It follows from our previous structure–function relationship studies on peptidyl carbamate inhibitors that the tetrapeptide sequence adjacent to the N-side of the carbamate bond is crucial for HLE specificity and therefore must remain intact.[19] However, it may be possible to attach any uncharged group to the succinic acid residue of PC with no impediment to inhibitory activity or specificity toward the enzyme. A hydrophilic linear polymer, bound at this position via a suitable spacer, should provide enough flexibility for the interaction of the inhibitory structure

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⊥ The following abbreviations have been used within the text: α_1PI = α-1-antitrypsin; DMSO = dimethyl sulfoxide; EDC = *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide; EEDQ = 2-ethoxy-1-(ethoxycarbonyl)-1,2-dihydroquinoline; GPC = gel permeation chromatography; Hepes = *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HLE = human leukocyte elastase; M_n = number average molecular weight; M_w = weight average molecular weight; α_2M = α-macroglobulin; NMM = *N*-methylmorpholine; PC = peptidyl carbamate; PPC = polymer-bound peptidyl carbamate; pd = polydispersity number; PBLG = poly-γ-(O^5-benzyl L-glutamate); PHEA = poly-α,β-[*N*-(2-hydroxyethyl)-D,L-aspartamide]; PHEG = poly-α-[N^5-(2-hydroxyethyl)-L-glutamine]; BOC-ON = 2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile; BOC = *tert*-butoxycarbonyl; SLPI = secretory leukocyte protease inhibitor; TEA = triethylamine; TFA = trifluoroacetic acid; TNBS = 2,4,6-trinitrobenzenesulfonic acid.

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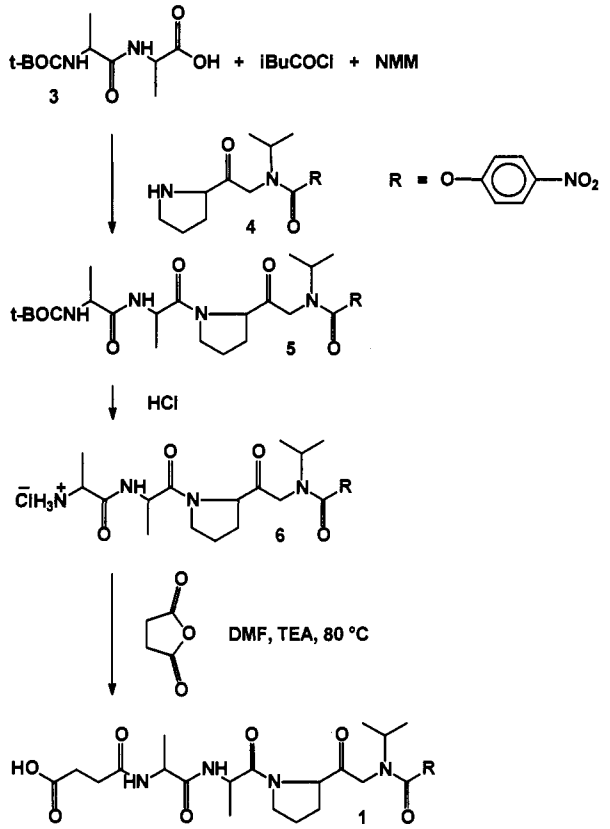
Scheme 1



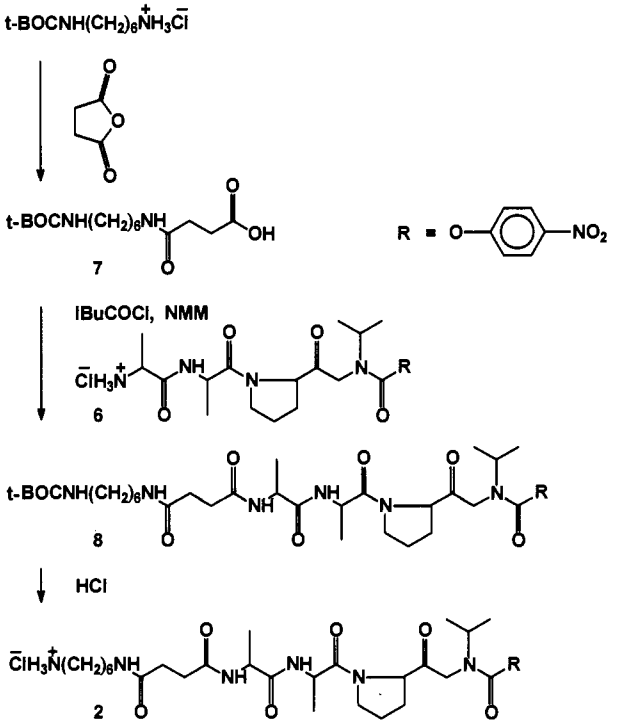
with the active site of the enzyme. Indeed, when coupled with a hydrophilic polymer, the selected PC has been recently shown to protect the hamster lung from HNE-induced emphysema ($t_{1/2}$ 421 min) after intratracheal administration.[21] In contrast, the unbound PC exhibited rapidly decreasing functional activity in the lavageable compartment of the lungs ($t_{1/2}$ 4 min).[21] On the basis of these results, we decided to conduct a mechanistic study using doubly labeled [^{3}H/^{14}C] polymer-bound PC. This experiment indicated that this HLE inhibitor elicits its anti HLE activity without prior release of PC moiety from polymer.[20] This pivotal finding then led to the synthesis of several analogs and to the evaluation of their *in vitro* inhibitory activity against HLE presented in this report.

Results

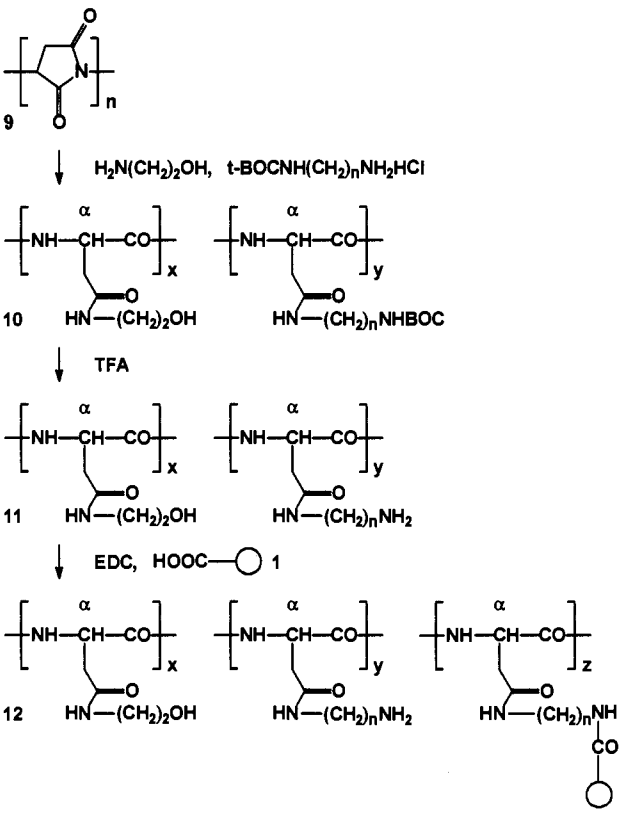
Chemistry. The peptidyl carbamates utilized in this study were of two types: (a) a hemisuccinate **1** (Scheme 1), exhibiting a free carboxyl as a binding group, and (b) its derivative **2** (Scheme 2), providing a primary amino functional group on a hexamethylene chain. Schemes 1 and 2 depict the synthetic routes followed for the preparation of these compounds. Coupling of BOC-Ala-Ala **3** with the carbamate hydrochloride **4** afforded the pivotal BOC-protected derivative **5**. Acidolysis using anhydrous HCl afforded the isolable intermediate **6**, which was subsequently converted either to hemisuccinate **1** (PC-COOH), upon reaction with succinic anhydride (Scheme 1), or to the corresponding BOC-protected intermediate **8**, upon reaction with **7** (Scheme 2). Deprotection of **8** in anhydrous acidic media as above yielded the (6-aminohexyl)succinamido derivative **2** (PC-NH_2, Scheme 2).

Peptidyl carbamate **1** was bound to PHEA(n)NH_2 (**11**) (Scheme 3, n = 2, 6), and peptidyl carbamate **2** was bound to PHEG-N_3 (**15**) (Scheme 4). PHEA(n)NH_2 (**11**) was

Scheme 2



Scheme 3



synthesized from polysuccinimide (PSI) **9** by the sequence of reactions appearing in Scheme 3. Reaction of **9** with mono-BOC-1,2-diaminoethyl benzoate or (mono-BOC-1,6-diaminohexane hydrochloride) and aminoethanol gave BOC-protected PHEA(n)NH (**10**). Deprotection of aminoalkyl groups under acidic conditions afforded PHEA-(n)NH_2 (**11**).

In the synthesis of macromolecular peptidyl carbamate **12**, PHEA(n)NH_2 (**11**) was reacted with peptidyl carbam-

Scheme 4

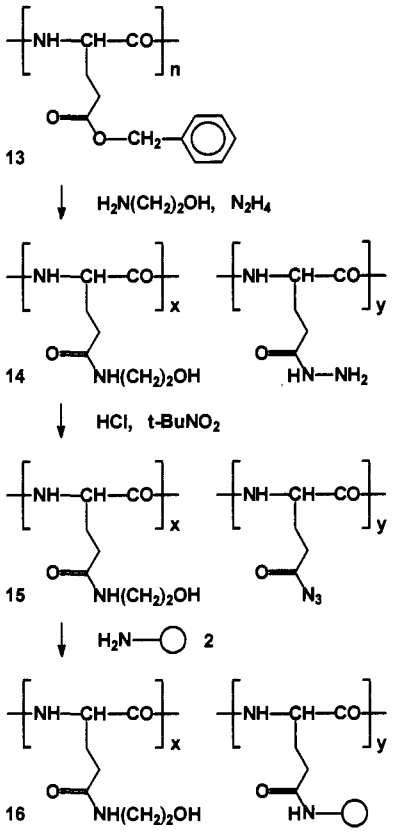


Table 1. Molecular Characteristics of Carrier Polymers and Polymer-Bound Inhibitors

compd[a]	structure[a]	composition[b] (mol %) x	y	z	$\bar{M}_w$[c] (kDa)	$\bar{M}_n$[d] (kDa)
11 (2)	PHEA(2)NH_2	91.2	8.8	0.0	31.6	21.0
11 (2)	PHEA(2)NH_2	94.8	5.2	0.0	30.4	22.0
11 (6)	PHEA(6)NH_2	89.5	10.5	0.0	70.5	52.0
14	PHEG-NH_2	84.1	15.9	0.0	62.0	34.0
12 (2)	PHEA(2)PC	91.2	4.2	4.6	38.0	24.0
12 (2)	PHEA(2)PC	94.4	3.6	1.6	34.0	22.5
12 (6)	PHEA(6)PC	89.5	3.2	7.3	74.5	52.0
16	PHEG-PC			7.0	64.0	36.0

[a] Numbers in parentheses denote the number of methylene groups in the spacer (n). [b] Composition of the polymer according to Schemes 3 and 4, x = number of OH side chains calculated ($x + y = 1$) in starting polymer, y = number of NH_2 side chains determined by coupling with TNBS (see the Experimental Section), z = number of PC side chains determined spectrophotometrically using molar absorptivity of 1 (see the Experimental Section). [c] $\bar{M}_w$ = weight average molecular weight. [d] $\bar{M}_n$ = number average molecular weight.

ate 1 utilizing *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide (EDC) activation of the carboxylate terminus of peptidyl carbamate 1. The excess carbodiimide, as well as direct reaction with the polymer, without preincubating carbodiimide with PC-COOH (1), usually provided a partially cross-linked polymer. An optimum preincubation period of EDC with PC-COOH (1) was determined to be between 40 and 60 min prior to the addition of polymer 11. Yields of the reaction (percent fraction of bound 1), when starting from equal mole amounts of carboxylic groups of 1 and amino groups of the polymer 11, were typically 70%. Addition of water to the reaction mixture (40% v/v) affected neither the rate nor the yield of the coupling reaction. The data in Table 1 illustrate the content of amino groups (y) in the polymer as determined by coupling with 2,4,6-trinitrobenzenesulfonic acid (2,4,6-TNBS),[22] before and after reaction with 1. Good agreement was found between the amount of amino groups not involved in the reaction (2,4,6-TNBS) and the content of 1 (z) determined spectrophotometrically using the molar absorptivity of 1.

PHEG-NH_2 (14) was synthesized from poly-γ-(O^5-benzyl L-glutamate) (PBLG) (13) by reaction with hydrazine and aminoethanol (Scheme 4). The resulting polymer 14 was comprised of randomly distributed glutamic acid hydrazide and (hydroxyethyl)-L-glutamine units. Oxidation of the hydrazide units under acidic conditions afforded the desired azido moieties of PHEG-N_3 (15). Reaction of 15 with peptidyl carbamate 2 yielded macromolecular peptidyl carbamate 16 (Scheme 4). A detailed investigation of the reaction conditions indicated that virtually 100% of hydrazide groups were converted into azido functions, and the maximum yield of the coupling of 2 with polymer 15 was attained within 1 h. Under these conditions, the amount of *p*-nitrophenol cleaved during a 1-h reaction at pH 8 was below the detection limit (1%). Extended reaction times were not beneficial and did not increase the yield of the coupling; rather, they invoked an enhanced cleavage of the *p*-nitrophenyl leaving group from 2. The reaction of azido groups of 15 with 2 afforded better results in nonaqueous systems. In contrast, the addition of water (10% v/v) decreased the yield of the coupling reaction between 2 and 15 by 50%. Data on the composition and mean molecular parameters of the parent polymers and the polymer-bound peptidyl carbamates are presented in Table 1. Analysis of the reaction mixture composition was achieved using gel permeation chromatography (GPC), which also allowed the assessment of the time course of binding of PCs to the polymer carriers and to set up optimum reaction conditions.

Enzymatic Studies. The inhibitory activity of the above polymer peptidyl carbamates 12 and 16 was tested against HLE, and kinetic studies were carried out to determine the mode and potency (K_i values) of the inhibitors. With the use of three different inhibitor concentrations (1–127 μM) and four substrate concentrations, Lineweaver–Burk[23] and Dixon[24] plots were constructed. Nonlinearity of Dixon plot (low-molecular-weight inhibitors as well as their macromolecular derivatives) indicated that the steady-state method could not be used for the determination of the inhibition constants (Figure 1). Time-dependent loss of enzymatic activity, when the enzyme was preincubated with the inhibitor (Figure 3), also suggested the use of the alternate method.[25] K_i values, shown in Table 2, were therefore determined from Easson–Stedman plot[25] (Figure 5) using the data obtained from the inhibition curves (Figure 4). Native polymers 11 and 14 were inactive and did not inhibit HLE under experimental conditions, whereas polymer-bound peptidyl carbamates (Table 2) exhibited good inhibitory activity against the enzyme as is indicated by their K_i values (35.5–2.0 nM).

Discussion

Two synthetic methods were described for spacer construction. The first approach involved incorporation of the spacer (ethylenediamine or hexamethylenediamine) into the polymer (Scheme 3). The second approach incorporated the spacer into the peptidyl carbamate prior to attachment onto the polymer (Scheme 4). Both approaches have been shown to produce water-soluble

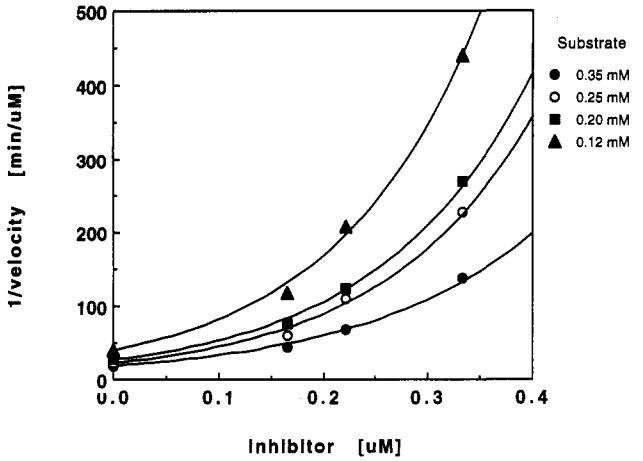


Figure 1. Dixon plot for the inhibition of HLE by PPC 16 (load 4.5%). Enzyme assay performed in triplicate at 27 °C in 0.1 M Hepes buffer pH 7.5, containing 0.05 M NaCl and 10% DMSO. Enzyme (61.5 nM), substrate (0.12, 0.20, 0.25 and 0.35 mM), inhibitor (0.16, 0.22 and 0.33 μM).

Table 2. Inhibition of HLE by Polymer-Bound Peptidyl Carbamates

compd[a]	structure[a]	loading[b] (mol %)	$\bar{M}_w$[c] (kDa)	K_i (nM)
12 (2)	PHEA(2)PC	4.6	38.0	2.0
12 (2)	PHEA(2)PC	1.6	34.0	8.5
12 (6)	PHEA(6)PC	7.3	74.5	4.2
16	PHEG-PC	7.0	62.0	3.2
16	PHEG-PC	4.5	62.0	27.3
16	PHEG-PC	1.9	62.0	35.5
1	PC-COOH			68.0
α_1PI			52.0	1.0
11	PHEA(2)NH_2	-	31.6	NI[d]
11	PHEA(6)NH_2	-	70.5	NI
14	PHEG-NH_2	-	64.0	NI

[a] Numbers in parentheses denote the number of methylene groups in the spacer. [b] Mole % of PC unit in the polymer-bound inhibitor. [c] $\bar{M}_w$ = weight average molecular weight. [d] NI = no inhibition.

macromolecular derivatives exhibiting a sufficient degree of loading (1–7%) with peptidyl carbamate inhibitor moieties. However, the first method was found to be more efficient (higher yields and better chemically defined products) since possible side reaction were likely to occur with the low-molecular-weight component, and the resulting side products could be easily separated from the polymer. None of the coupling reactions was accompanied by any change in the molecular weight distribution of the starting polymer (values of M_w, weight average molecular weight; M_n, number average molecular weight; and pd, polydispersity number, pd = M_w/M_n). Such a change would be reflected either by appearance of new peaks with increased retention times or by elution curve broadening. The former indicates degradation processes during the reaction or isolation of polymer-bound PC, and the latter suggests cross-linking. Increases of the average molecular weights (M_w) of starting polymers 11 or 14 (Table 1) after the binding of 1 or 2 (12 and 16), determined by GPC, corresponded well to the addition of 1 (2).

In order to study the inhibitory activity of this class of macromolecular inhibitors toward HLE, macromolecular peptidyl carbamates (Table 2) were prepared using the above described synthetic approaches and screened against HLE. The first analyses used to evaluate the mechanism of inhibition were steady-state methods. The velocities that occurred during the first 2 min were assumed to represent "initial velocities", and from these values a

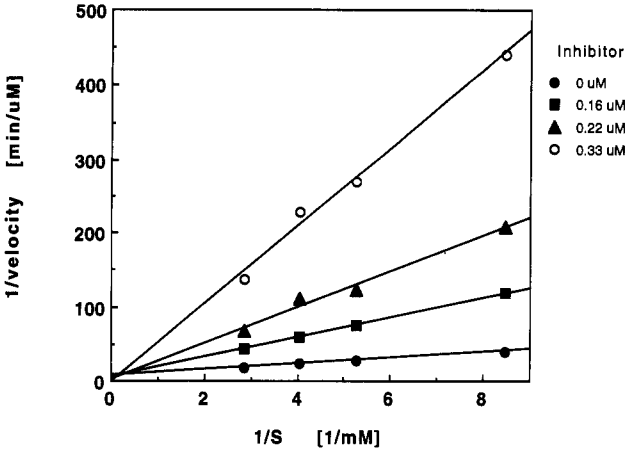


Figure 2. Lineweaver–Burk plot for the inhibition of HLE by PPC 16 (load 4.5%). Enzyme assay performed in triplicate at 27 °C in 0.1 M Hepes buffer pH 7.5, containing 0.05 M NaCl and 10% DMSO. Enzyme (61.5 nM), substrate (0.12, 0.20, 0.25 and 0.35 mM), inhibitor (0.16, 0.22 and 0.33 μM).

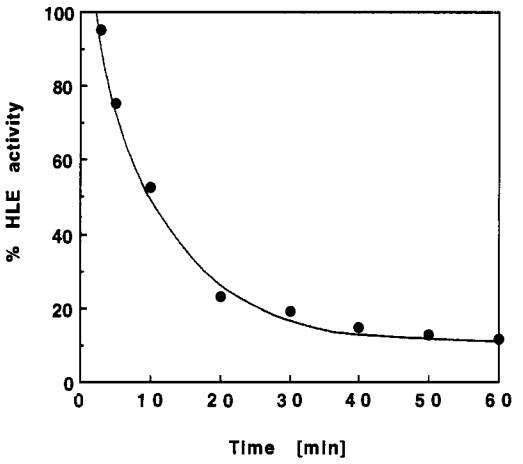


Figure 3. Time-dependent inhibition of HLE by PPC 16 (load 4.5%). Percent HLE activity is the residual enzyme activity, i.e., the ratio between the enzyme activity in the presence and absence (100%) of the inhibitor. Each data point represents % HLE activity at different incubation time intervals obtained with inhibitor concentration of 0.6 μM. Enzyme assay performed in triplicate at 27 °C in 0.1 M Hepes buffer pH 7.5, containing 0.05 M NaCl and 10% DMSO. Enzyme (61.5 nM), substrate (0.49 mM), inhibitor (0.6 μM).

Lineweaver–Burk[23] plot was constructed (Figure 2). This classical plot might readily be considered to fit a simple competitive inhibition, and an attempt might be made to estimate the value of K_i. However, it was obvious from further examination of the time-dependent loss of enzymatic activity (Figure 3) that the enzymatic activity gradually decreased with the prolonged incubation time, and therefore the data points used for the Lineweaver–Burk plot of Figure 2 did not represent steady-state velocities. Further construction of a Dixon[24] plot showed a nonlinear replot of the reciprocal velocity versus the inhibitor concentration as seen in Figure 1. The data in Figure 1 clearly demonstrated the inadequacy of the classic Lineweaver–Burk methods based on steady-state assumptions for the determination of either the inhibition mechanism or the K_i values of our PC or polymer-bound PC. The same pattern was observed for the low-molecular-weight PC as well as its macromolecular derivatives. When the classic plots are inadequate for the determination of K_i values, other methods such as the Easson–Stedman[25]

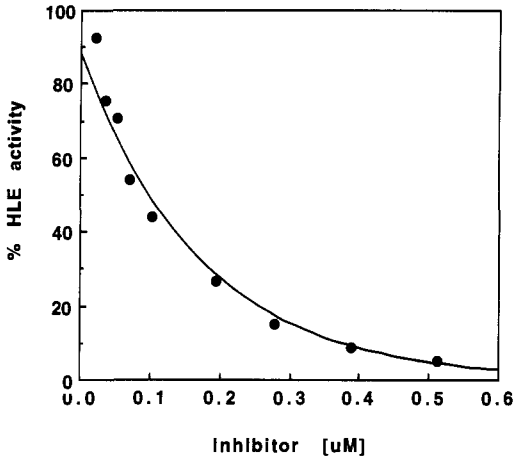


Figure 4. Concentration-dependent inhibition of HLE by PPC 16 (load 4.5%). Percent HLE activity is the residual enzyme activity, i.e., the ratio between the enzyme activity in the presence and absence (100%) of the inhibitor. Enzyme assay performed in triplicate at 27 °C in 0.1 M Hepes buffer pH 7.5, containing 0.05 M NaCl and 10% DMSO. Enzyme (61.5 nM), substrate (0.49 mM), inhibitor (0.02–0.6 μM).

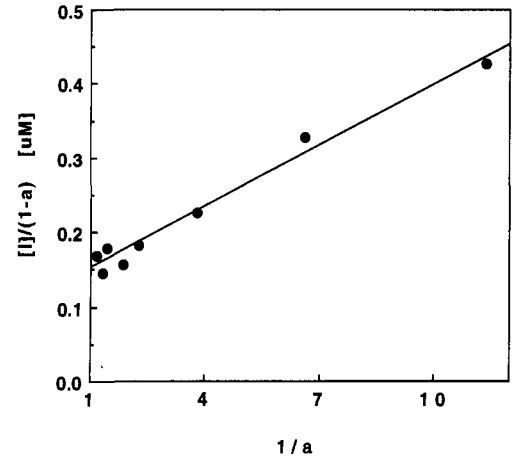


Figure 5. Determination of K_i. Easson–Stedman plot for the inhibition of HLE by PPC 16 (load 4.5%). Representation of the data plotted in Figure 4 in accordance with the equation $[I]/(1-a) = K_i/a + [E°]$, where I is inhibitor concentration and a is the fractional activity, i.e., the ratio between the enzyme activity in the presence and absence ($a = 1$) of the inhibitor. Enzyme assay performed in triplicate at 27 °C in 0.1 M Hepes buffer pH 7.5, containing 0.05 M NaCl and 10% DMSO.

plot may be applied. This method requires a sufficient incubation period. The time-dependent loss of the enzymatic activity experiment provided the incubation time at which the residual enzyme activity showed no further change with time. This inhibition time needed for the minimum enzymatic activity was used to obtain the inhibition curve, percent remaining enzyme activity versus the inhibitor concentration (Figure 4). This inhibition curve was then converted into the Easson–Stedman plot (Figure 5), and K_i values were obtained from the slope of this plot.

As seen from K_i values in Table 2, all polymer-bound inhibitors exhibited very high potency compared to the low-molecular-weight peptidyl carbamate. In contrast, both native polymers PHEA(n)NH_2 (11) and PHEG-N_3 (5) did not inhibit HLE. More work is underway to determine the mechanism of HLE inhibition and evaluate the effect of loading and molecular size of the polymer on the inhibitory activity. Although, at present, the mechanism of HLE inactivation is not clear, the enzymatic work presented here tends to indicate that these peptidyl carbamate inhibitors act as tight-binding inhibitors.

Experimental Section

Melting points were determined on a Fisher-Johns Mel Temp apparatus and are reported uncorrected. Infrared spectra were procured from a Perkin-Elmer Model 1430 ratio recording spectrophotometer. Nuclear magnetic resonance spectra were recorded using either a Varian EM 360 or VXR-300 spectrophotometer with tetramethylsilane as an internal standard. UV spectra were recorded either with a Cary Model 2200 or with a Varian Superscan 3 spectrophotometer. Microanalyses were performed by Atlantic Microlab and were within ±0.4% of theoretical values.

All amino acids and protected amino acids were procured from Sigma Chemical Co., St. Louis, MO. 2-Ethoxy-1-(ethoxycarbonyl)-1,2-dihydroquinoline (EEDQ, 97%), 2[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile (BOC-ON), and succinic anhydride (gold label) were purchased from Aldrich Chemical Co., Milwaukee, WI, and used as received. *N*-Ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), BOC-1,6-diaminohexane hydrochloride, 1,2-diaminoethane, and 1-amino-2-ethanol were purchased from Fluka Chemical Co., Hauphauge, NJ. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were predistilled from LAH and finally distilled from sodium/benzophenone ketyl prior to using. *N,N'*-Dimethylformamide (DMF) was predistilled from KOH and finally distilled *in vacuo*.

Mono-BOC-1,2-diaminoethyl Benzoate. This compound was prepared by reaction of BOC-ON with the monobenzoate salt of 1,2-diaminoethane in 1:1 water/dioxane. Mono-BOC-1,2-diaminoethane was extracted from the aqueous solution (pH 12) into ethyl acetate and crystallized as the benzoate salt: mp 122–123 °C; ^1H NMR (D_2O) δ 2.9 (2 H, t, J = 6 Hz, $CH_2NH_3^+$), 3.18 (2 H, t, J = 6 Hz, $NHCH_2$), 7.21–7.41 (5 H, m, ArH), 7.62–7.72 (5 H, m, ArH), 1.24 (9 H, s, $(CH_3)_3C$); IR (KBr) 3220, 3040–2800, 1720, 1625, 1590, 1515, 1465, 1340, 730 cm^{-1}. Anal. ($C_{14}H_{22}N_2O_4$) C, H, N.

***N*-BOC-L-alanyl-L-alanine (3).** Compound **3** was prepared according to the method previously described by Kato et al.[19] and was found to be spectrally and chromatographically identical with an authentic sample.

***p*-Nitrophenyl *N*-[L-Prolylmethyl]-*N*-isopropylcarbamate Hydrochloride (4).** Compound **4** was prepared according to the method of Kato et al.[19] and was found to be spectrally and chromatographically identical with an authentic sample.

***p*-Nitrophenyl *N*-[*N*-BOC-L-alanyl-L-alanyl-L-prolylmethyl]-*N*-isopropylcarbamate (5).** Compound **5** was prepared according to the method of Banks et al.[26] and was found to be spectrally and chromatographically identical with an authentic sample.

***p*-Nitrophenyl *N*-[L-Alanyl-L-alanyl-L-prolylmethyl]-*N*-isopropylcarbamate Hydrochloride (6).** Compound **6** was also prepared according to the method of Banks et al.,[26] and the resulting pale yellow oil was used in subsequent reactions without further purification.

***p*-Nitrophenyl *N*-[Succinyl-L-alanyl-L-alanyl-L-prolylmethyl]-*N*-isopropylcarbamate (PC-COOH, 1).** Compound **1** was prepared according to the modified method of Banks et al.[26] To a solution of **6** (1.52 g, 2.96 mmol) in DMF (20.0 mL) was added TEA (362 mg, 3.6 mmol). Succinic anhydride (358 mg, 3.6 mmol) was added in one portion and the mixture heated to 80 °C for 1.5 h. The reaction was allowed to cool to room temperature, and Et_2O (60 mL) was added to induce precipitation of TEA·HCl. Filtration and concentration of the filtrate *in vacuo* afforded a pale yellow solid. Trituration with 2% HCl, filtration, and recrystallization (THF/Et_2O) afforded **1** (1.6 g, 93.7%) as a colorless amorphous powder which was dried *in vacuo* over P_2O_5: mp 185–187 °C; ^1H NMR (DMSO-d_6) δ 1.0–1.6 (16 H, m), 1.7–2.4 (4 H, m), 3.6–3.8 (3 H, m), 4.0–4.7 (5 H, m), 7.2 (2 H, appd, J = 9 Hz), 8.2 (2 H, appd, J = 9 Hz); IR (KBr) 3400, 1770, 1750, 1680, 1650, 1560, 1224 cm^{-1}. Anal. ($C_{26}H_{35}N_5O_{10}\cdot{}^1/_2H_2O$) C, H, N.

1-(*N*-t-BOC-amino)-6-hemisuccinamidohexane (7). To a solution of succinic anhydride (40.0 mg, 0.395 mmol) in CH_3CN

(2.0 mL) was added a solution of *N*-BOC-1,6-diaminohexane hydrochloride (100.0 mg, 0.395 mmol) and TEA (44.0 mg, 0.395 mmol) in CH_3CN (2.0 mL). After 30 min, **7** precipiated from the reaction mixture. Filtration and drying *in vacuo* afforded **7** (92 mg, 74%): mp 132–134 °C; ^{1}H NMR (DMSO-d_6) δ 1.0–1.7 (17 H, 2 s), 2.2–2.6 (5 H, m), 2.8–3.1 (4 H, m), 6.5 (1 H, br s), 7.6 (1 H, br s). Anal. ($C_{15}H_{28}N_2O_5$) C, H, N.

***p*-Nitrophenyl *N*-[[[6-(*N*-BOC-amino)hexyl]succinamido]-L-alanyl-L-alanyl-L-prolylmethyl]-*N*-isopropylcarbamate (8).** To a solution of **7** (677 mg, 2.10 mmol) and NMM (258 μL, 2.30 mmol) in THF (20.0 mL) cooled to −20 °C was added isobutyl chloroformate (303 μL, 2.30 mmol) in THF (2.0 mL). The turbid mixture was stirred for 15 min at this temperature, after which the reaction mixture was cooled to −40 °C and a solution of **6** (1.0 g, 1.9 mmol) and NMM (258 μL, 2.30 mmol) in THF (70 mL) was added. After the mixture was slowly warmed to room temperature over 5 h, $CHCl_3$ (40 mL) was added, and the solution was washed with 10% citric acid (2 × 20 mL) and 4% $NaHCO_3$ (10 mL). The organic layer was dried ($MgSO_4$), decanted, and concentrated *in vacuo* to afford an oil. Compound **8** was isolated by chromatography (silica gel, 40 g; R_f = 0.42, 10% MeOH/$CHCl_3$) (gradient, 100 mL of each: 20% hexane/$CHCl_3$–10% hexane/$CHCl_3$–2% methanol/$CHCl_3$–5% methanol/$CHCl_3$) as a colorless glassy solid (1.12 g, 76.5%): mp 107–110 °C; ^{1}H NMR ($CDCl_3$) δ 1.17 (3 H, app d), 1.21 (3 H, app d), 1.33 (11 H, m), 1.44 (13 H, br s), 2.01–2.09 (2 H, m), 3.10 (2 H, d), 3.22 (2 H, app d), 3.63 (1 H, app d), 3.77 (1 H, app d), 4.19 (1 H, app d), 4.29 (1 H, app d), 4.49 (2 H, t), 4.60–4.72 (3 H, m), 6.31 (1 H, br s, NH), 6.84 (1 H, m, NH), 7.21–7.34 (2 H, dd, J = 9 Hz), 8.21–8.27 (2 H, dd, J = 9 Hz); IR (KBr) 1745, 1670, 1610, 1535, 1510, 1345, 1190, 1150 cm^{-1}. Anal. ($C_{37}H_{57}N_7O_{11}$) C, H, N.

***p*-Nitrophenyl *N*-[[(6-Aminohexyl)succinamido]-L-alanyl-L-alanyl-L-prolylmethyl]-*N*-isopropylcarbamate Hydrochloride (PC-NH$_2$, 2).** A solution of **8** (0.040 mmol) in ethyl acetate (4.0 mL) was combined with 0.44 M HCl in diethyl ether, and the mixture (4.5 mL) was stirred for 1–2 h at room temperature. BOC acidolysis progress was monitored by TLC (silica gel; Silufol, Kavalier, 10% methanol/chloroform). Typically, 2 h was sufficient to affect complete cleavage of the BOC moiety. The reaction mixture was concentrated *in vacuo*, and residual HCl was removed by repeated evaporation with ethyl acetate. The dry residue of **2** was dissolved in DMSO and used immediately for binding to polymer **15**.

An analytical sample was prepared and isolated using the following procedure. The protected amine **8** (100 mg, 0.13 mmol) was dissolved in ethyl acetate (2.0 mL) and cooled to 5 °C. The stirring mixture was treated with formic acid (125 μL) and anhydrous HCl gas. Reaction progress was monitored using silica gel TLC (10% MeOH/$CHCl_3$) by observing the disappearance of **8** (R_f 0.42) and the concomitant formation of **9** (R_f 0.05). Volatiles were removed *in vacuo*. Excess formic acid was removed with repeated azeotropic evaporations with *n*-heptane to afford a glassy hygroscopic solid after crystallization from anhydrous EtOH (74.5 mg, 81%): mp 144–146 °C; ^{1}H NMR ($CDCl_3$) (salt) δ 0.91–1.30 (7 H, m), 1.41–1.42 (10 H, br s), 1.77 (9 H, br s), 2.01–2.32 (3 H, m), 2.52–3.41 (7 H, m), 3.62 (1 H, app q), 3.83 (1 H, app q), 4.09–4.81 (5 H, m), 7.21–7.27 (2 H, d, J = 9 Hz), 8.13–8.19 (2 H, d, J = 9 Hz). Anal. ($C_{32}H_{46}N_7O_9Cl\cdot{}^1/_2H_2O$) C, H, N.

Polymers. Poly-D,L-succinimide (PSI, 9, Scheme 3). Polymer **9** was prepared and fractionated by fractional precipitation in a DMF/water system according to previously developed procedures.[27] The viscosity average molecular weight (M_v) of the PSI fractions were determined by viscometry.[27]

Poly-γ-(*O*^{5}-benzyl L-glutamate) (PBLG, 13, Scheme 4). Polymer **13** was prepared by polymerization of *O*^{5}-benzyl-L-glutamate *N*-carboxyanhydride in dioxane according to the method of Blout and Karlson.[28]

PHEA(*n*)NH-BOC (10, Scheme 3, n = 2, 6). Compound **10** was prepared by reaction of mono-BOC-1,2-diaminoethyl benzoate (n = 2) or mono-BOC-1,6-diaminohexane hydrochloride (n = 6)[26] and 2-aminoethanol with PSI (**9**). In a typical example, 10 g of the chosen fraction of **9** (M_v) was dissolved in DMF (50 mL), and mono-BOC-1,2-diaminoethyl benzoate (2.80 g, 0.01 mol) and TEA (0.80 mL, 0.01 mol) were added. The reaction was carried out at room temperature for 4 days, after which 2-aminoethanol (11.0 mL, 0.18 mol) was added and the reaction continued an additional 24 h. The mixture was neutralized with acetic acid and dialyzed against double-distilled water (Visking Dialysis Tubing, Serva Feinbiochemica), and the polymer was isolated by lyophilization to yield 9.20 g of PHEA(2)-NH-BOC (**10**). Polymer **10** (8.50 g) was next treated with trifluoroacetic acid (30 mL) (Scheme 3) and subsequently purified as described previously.[26] The deprotected polymer was isolated from the retentate by freeze drying to yield 6.5 g of PHEA(2)-NH_2 (**11**).

PHEA(6)-NH$_2$ (11). Polymer **11** was prepared from **9** using essentially the same procedure as described for PHEA(2)-NH_2, with an exception that mono-t-BOC-1,6-diaminohexane hydrochloride was utilized in place of mono-BOC-1,2-diaminoethyl benzoate to afford 6.9 g of PHEA(6)-NH_2 (**11**).

Copolymer Poly-α-[*N*^{5}-(2-hydroxyethyl)-L-glutamine-*co*-L-glutamic Acid Hydrazide] (PHEG-NH$_2$, 14). Polymer **14** was prepared by a polymer modification reaction of anhydrous hydrazine with poly-γ-(*O*^{5}-benzyl L-glutamate) **13** in the presence of 2-aminoethanol (Scheme 4). The mole ratio of hydrazine to 2-aminoethanol was 0.005, and there was a 98-fold excess of both hydrazine and 2-aminoethanol over the benzyl ester groups of **13** in the reaction mixture. The reaction was carried out at 60 °C for 48 h, and the polymer product **14** was purified by dialysis and GPC similarly to polymer **11**.

Polymer-Bound Inhibitors. Procedure A (Scheme 3). This coupling procedure previously described by Banks et al.[26] for [^{3}H/^{14}C]PHEA(6)-PC was modified in the following way. In a typical example, peptidyl carbamate hemisuccinate (PC-COOH, **1**) (0.586 g, 1.0 mmol) and *N*-ethyl-*N*′-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDC) (0.23 g, 1.2 mmol) were dissolved in cold DMF (4.0 mL) and allowed to stir for 50 min in an ice bath. An ice-cold solution of polymer **11** containing 1.2 mmol of NH_2 groups (i.e., 2.20 g of **11**, n = 2) and TEA (0.167 mL, 1.2 mmol) in DMF (12 mL) was added, and the reaction mixture was stirred at 0–4 °C for up to 24 h. The polymer product was purified by dialysis and by GPC chromatography as described previously.[26] The resulting polymer inhibitor was isolated from the retentate by freeze drying to yield 1.35 g of PHEA(2)-PC, **12**.

Polymer-Bound Inhibitors. Procedure B (Scheme 4). In a typical example, polymer **14** (1.03 g) was dissolved in DMSO such that the concentration of hydrazide groups in the final solution was 1 M. A solution of anhydrous HCl in methanol (2.4 M) was added in a ratio of 10 mol of HCl per mol of hydrazide groups. The reaction mixture was stirred at −10 °C, and *tert*-butyl nitrite (8 mol per 1 mol of hydrazide groups) was added. The reaction mixture was stirred at −10 °C for 15 min. At this time virtually no remaining hydrazide groups were detected in an aliquot using spectrophotometry after reaction with 2,4,6-TNBS.[22] The reaction mixture containing PHEG-N_3 (**15**) was neutralized with TEA, and PC-NH_2 (**2**) in DMSO was added. Polymer product **16** was purified by dialysis against water with the pH of the dialysis maintained at pH 4 by acetic acid. The polymer-bound peptidyl carbamate **16** was obtained by lyophillization to yield: 1.19 g of PHEG-PC (**16**).

Analyses. The mole fractions of aminoalkyl side chains in polymers **11** and **12** and of hydrazide groups in polymer **14** were determined spectrophotometrically after the reaction with 2,4,6-trinitrobenzenesulfonic acid, modifying the method according to Brown.[22] The mole fractions of polymer structural units covalently modified with PC in the side chains were determined from the absorption spectrum of the polymer-bound PC (polymers **12** and **16**). It should be mentioned that it was assumed that the absorption coefficient of PC, i.e., 9700 mol^{-1} L cm^{-1} at λ = 276 nm, remained unchanged after immobilization to the polymer.

The molecular weight averages (M_w and M_n) and molecular-weight distributions of all polymer samples were determined by GPC according to the method of Banks et al.[26]

Enzymatic Studies. Materials. HLE and its substrate (methoxysuccinyl-L-alanyl-L-alanyl-L-propyl-L-valine *p*-nitroanilide) and Hepes buffer were purchased from Sigma Chemical Co., St. Louis, MO.

Enzyme Assays. All enzyme assays were performed spectrophotometrically at 25 °C using a Varian 2200 Cary spectrophotometer. The activity of HLE was assayed with use of MeO-Suc-Ala-Ala-Pro-Val-NA and monitoring the absorbance at λ = 410 nm.[29]

Screening for Inhibitory Activity. In a typical experiment, the inhibitor (0.05 mL, 2 μM to 2 mM in DMSO or Hepes buffer) and the substrate (0.05 mL, 27 mM in DMSO) were added to 0.1 M Hepes buffer (2.9 mL, pH 7.5) in a quartz cuvette and thermally equilibrated in the spectrophotometer for 2 min. The absorbance was balanced at the desired wavelength, after which the enzyme (0.005 mL, 37 μM in 0.05 M acetate buffer, pH 5.5) was added to the sample cuvette. The mixture was shaken for 20 s, and the increase in absorbance was monitored for 30 min.

Kinetic Studies. Steady-State Study. In a typical experiment, 0.05 mL of inhibitor (1–125 μM in DMSO or Hepes buffer) and 0.05 mL of the substrate (7–27 mM in DMSO) were added to 0.1 M Hepes buffer (2.9 mL, pH 7.5) in a quartz cuvette, thermally equilibrated in the spectrophotometer for 2 min, and the absorbance was balanced at 410 nm. The enzyme (0.005 mL, 37 μM in acetate buffer, pH 5.5) was added to the sample cuvette. The mixture was shaken for 20 s, and the increase in the absorbance was monitored for 5 min.

Kinetic Studies. Bieth Method.[25] Substrate, buffer, and enzyme concentrations were used as reported above in the screening experiment. Inhibitor concentrations ranging from 1 to 80 μM were used.

Determination of Incubation Time. In a typical experiment, 0.05 mL of the inhibitor and 0.005 mL of the enzyme were added to Hepes buffer (2.9 mL) in a thermally equilibrated quartz cuvette and incubated for various time intervals (3–60 min). At the end of the incubation period, the absorbance was balanced at 410 nm, and the substrate (0.05 mL) was added. The mixture was shaken for 20 s, and the increase in absorbance was recorded for 3 min. Percent remaining activity of the enzyme versus incubation time was then plotted for each inhibitor concentration. An adequate incubation period was chosen as the shortest incubation time necessary for the lowest percent remaining activity.

Determination of the Inhibition Curve. In a typical experiment, 0.05 mL of the inhibitor and 0.005 mL of the enzyme were added to Hepes buffer (2.9 mL) in a quartz cuvette and thermally equilibrated for the incubation time determined from above experiment. The absorbance was balanced at 410 nm, the substrate (0.05 mL) was added, and the sample cuvette was shaken for 20 s. The increase in absorbance was recorded for 3 min. The value obtained from a control experiment (without inhibitor) was considered as 100% activity of the enzyme. The inhibition curve was generated as a plot of percent remaining HLE activity versus inhibitor concentration. This curve was then converted into a Easson–Stedman plot[25] from which the K_i value was obtained from the slope.

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