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Human Neutrophil Elastase Phosphonic Inhibitors with Improved Potency of Action

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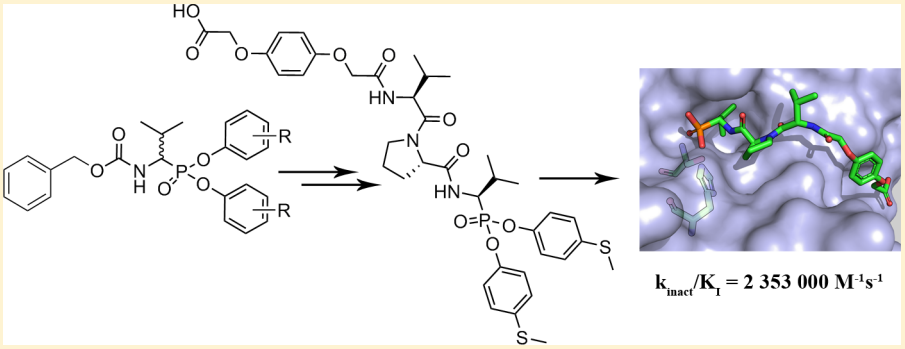
Supporting Information



ABSTRACT: Herein, we present the synthesis and the measurement of the inhibitory activity of novel peptidyl derivatives of α-aminoalkylphosphonate diaryl esters as human neutrophil elastase inhibitors. Their selectivity against other serine proteases, including porcine pancreatic elastase, chymotrypsin, and trypsin, was also demonstrated. We also describe the preparation of single peptide diastereomers. The most active and selective compound developed possessed a k_{inact}/K_I of 2353000 M^{-1} s^{-1}, which is the most potent irreversible peptidyl inhibitor of human neutrophil elastase reported to date. The peptidyl inhibitors were demonstrated to be stable in PBS buffer and human plasma, as were their complexes with HNE.

INTRODUCTION

Human neutrophil elastase (HNE, EC 3.4.21.37; also referred to as human leukocyte elastase, HLE) belongs to the chymotrypsin-like serine protease superfamily and is one of the most destructive enzymes present in the human body. It can easily degrade almost every component of the extracellular matrix, including elastin, collagen types I–IV, proteoglycans, fibronectin, and laminin.[1−5] Neutrophil elastase is stored in the azurophilic granules of polymorphonuclear leukocytes and is secreted along with reactive oxygen species, cationic peptides, and eicosanoids after an inflammatory stimulation.[1] One of the major physiological functions of HNE is host defense against invading microbial pathogens.[1] It was also found that elastase can lead to activation of metalloproteinases and up-regulation of inflammation.[6−8] Under normal physiological conditions the proteolytic activity of elastase is controlled by natural endogenous serine protease inhibitors (serpins) such as α_1-proteinase inhibitor (α_1-PI), secretory leukocyte peptidase inhibitor (SLPI), and α_2-macroglobulin (α_2-MG).[9−11] The imbalance between active elastase and its specific endogenous inhibitors is in part the result of the so-called "oxidative burst" of neutrophils which destroys serpins.[12] The disruption of this delicate balance between active elastase and its inhibitors may lead to the development of pulmonary inflammatory disorders such as chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), pulmonary emphysema, adult respiratory syndrome, and chronic bronchitis.[13,14] Importantly, the number of deaths attributed to COPD has increased rapidly in recent years, and it is now considered one of the major worldwide causes of mortality.[15−17] In addition to its essential role in the development of pulmonary inflammatory disorders, HNE has also been demonstrated to contribute to the metastasis and progression of some cancers via its degradation of extracellular matrix components.[18] Clearly, lack of an effective therapy to control the activity of HNE may complicate the treatment of chronic diseases. Thus, there is a need to develop novel biologically active molecules that target the proteolytic function of HNE to allow for more effective treatments for chronic pulmonary inflammation and metastatic cancer.

Various synthetic HNE inhibitors such as chloromethyl ketones, trifluoromethyl ketones, sivelestat sodium hydrate (ONO-5046, Elaspol), *N*-benzoylpyrazoles, benzoxazinones, and thiadiazolidines have been developed over the past few decades.[19−24] Most of these elastase inhibitors were designed to include a β-lactam scaffold in their structure, and some were determined to be orally available and stable in aqueous solutions.[25−29] Unfortunately, none of the β-lactams specific

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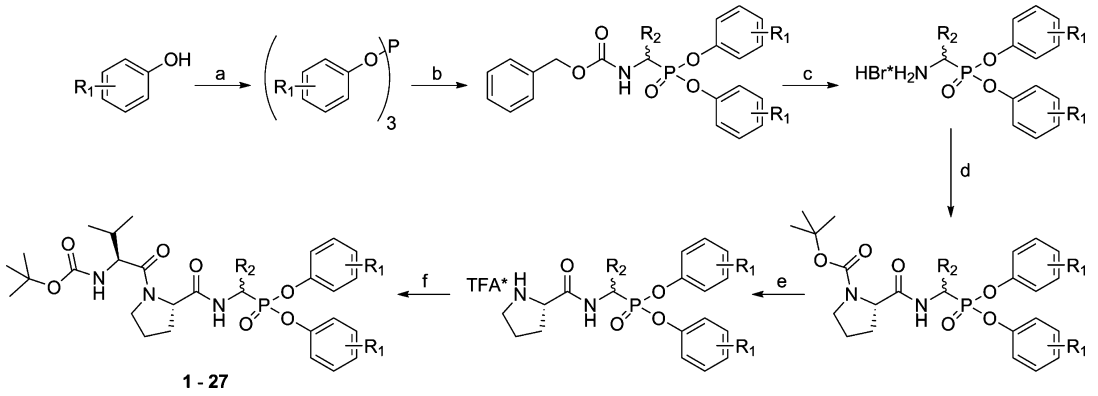
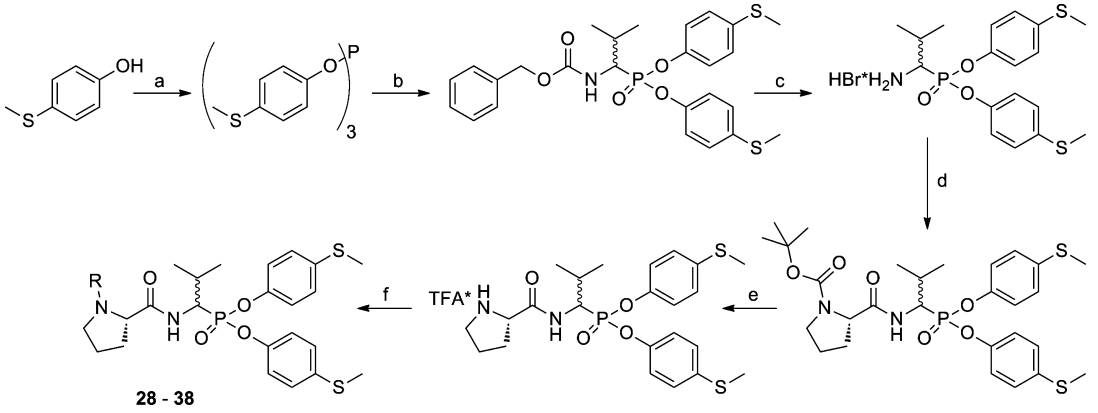
Scheme 1. Synthesis of Phosphonic Peptides with General Formula Boc-Val-Pro-Aaa^{P}(OAr)$_2$[a]



[a]Reagents and conditions: (a) PCl_3, MeCN, reflux, 6 h; (b) benzyl carbamate, R_2CHO, AcOH, 80−90 °C, 2 h; (c) 33% HBr/AcOH, 22 °C, 2 h; (d) *N*-Boc-L-Pro-OH, HBTU, DIPEA, MeCN, 12 h, 22 °C; (e) 50% TFA/DCM (v/v), 2 h, 22 °C; (f) *N*-Boc-L-Val-OH, HBTU, DIPEA, MeCN, 12 h, 22 °C.

Scheme 2. Synthesis of Cbz- or Boc-Protected Phosphonic Peptides with General Structure Cbz/Boc-Aaa-Pro-Val^{P}(OC_6H_4-*S*-CH_3)$_2$[a]



[a]Reagents and conditions: (a) PCl_3, MeCN, reflux, 6 h; (b) benzyl carbamate, $(CH_3)_2CHCHO$, AcOH, 80−90 °C, 2 h (60%); (c) 33% HBr/AcOH, 2 h, 22 °C (95%); (d) *N*-Boc-L-Pro-OH, HBTU, DIPEA, MeCN, 12 h, 22 °C (88%); (e) 50% TFA/DCM (v/v), 2 h, 22 °C; (f) *N*-Cbz/Boc-Aaa-OH, HBTU, DIPEA, MeCN, 12 h, 22 °C.

for HNE have successfully passed clinical trials for use in human patients.

Aromatic esters of 1-aminoalkylphosphonates (1-AAP) and their peptidyl derivatives are well-known inhibitors of serine proteases.[30] They irreversibly bind to the active site serine with high specificity and selectivity of action. In this respect, they differ significantly from previously reported elastase inhibitors. They also lack the ability to inactivate cysteine, threonine, and metalloproteinases. Importantly, the reactivity of 1-AAP peptidyl derivatives can be easily adjusted by modification of their amino acid sequences, allowing for the development of compounds that target one specific protease but are inactive against structurally and/or functionally similar serine proteases. In addition, through the introduction of different substituents into phenyl ester rings, the electrophilic properties of the phosphorus atom can be modulated.[31,32] In this fashion, peptidyl derivatives of phosphonic amino acid analogues have been developed that display high potency of action toward serine proteases including trypsin, thrombin, uPA, DPPIV, granzymes, or kallikreins as well as bacterial proteases containing noncanonical catalytic triad such as subtilisin.[32−36]

Although phosphonic inhibitors of HNE have been reported for at least 30 years, an in-depth structure−activity relationship analysis of the P1−P4 positions of the inhibitor and analysis of the structural requirements for the phosphonate diaryl ester have yet to be performed. Moreover, the inhibitory activities of single phosphonic peptide diastereoisomers toward HNE have yet to be reported. The most potent peptidyl phosphonic inhibitor of neutrophil elastase reported thus far is Boc-Val-Pro-Val^{P}(OPh)$_2$, which displayed a $k_{obs}/[I]$ of 27000 M^{-1} s^{-1}. Our previous studies on simple Cbz-protected phosphonic HNE inhibitors showed that the preferred amino acid analogues in the P1 position are the phosphonic analogues of Abu and Val.[37]

In this report, we describe the development of peptidyl phosphonic-type HNE inhibitors designed on a Val-Pro-Aaa^{P}(OAr)$_2$ scaffold that display improved potency of action. Additionally, since the starting phosphonic analogues of amino acids were obtained as racemic mixtures, we were able to isolate single diastereoisomers that displayed high inhibition activity. As a result, several new highly potent human neutrophil elastase inhibitors that displayed k_{inact}/K_I of 10^6 M^{-1} s^{-1} were developed. Moreover, since 1-aminoalkylphosphonate diaryl esters and their peptidyl derivatives have poor solubility in aqueous media that could limit their practical application, we also generated water-soluble derivatives.

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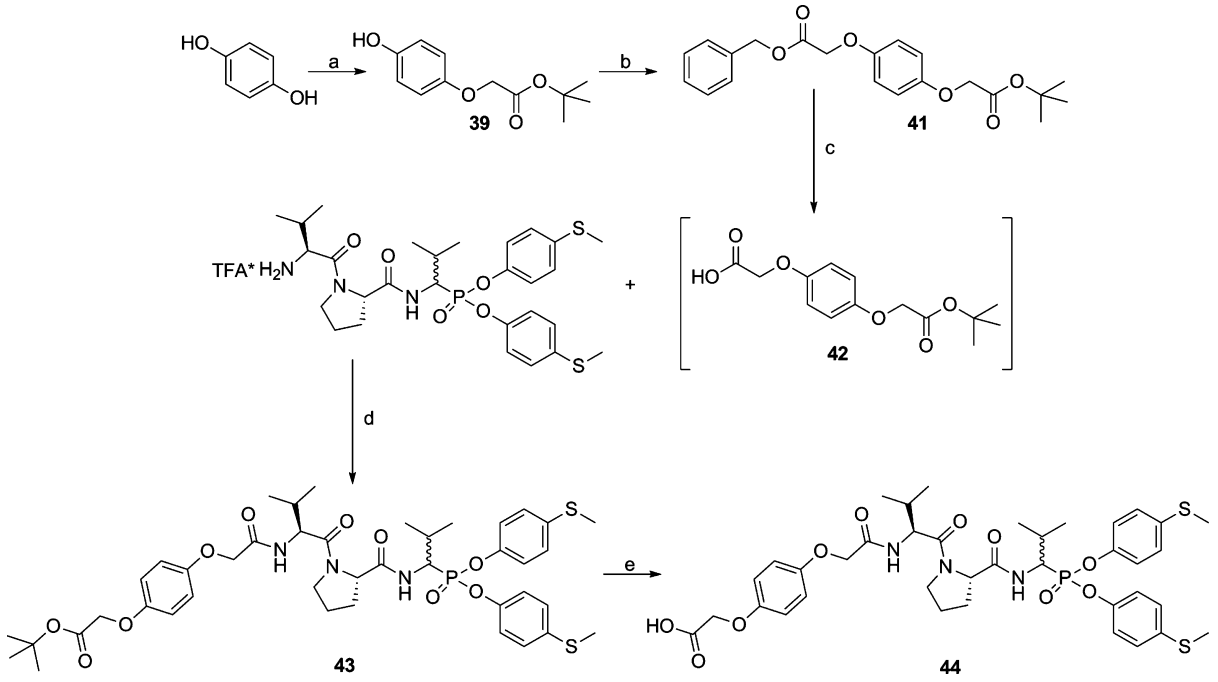
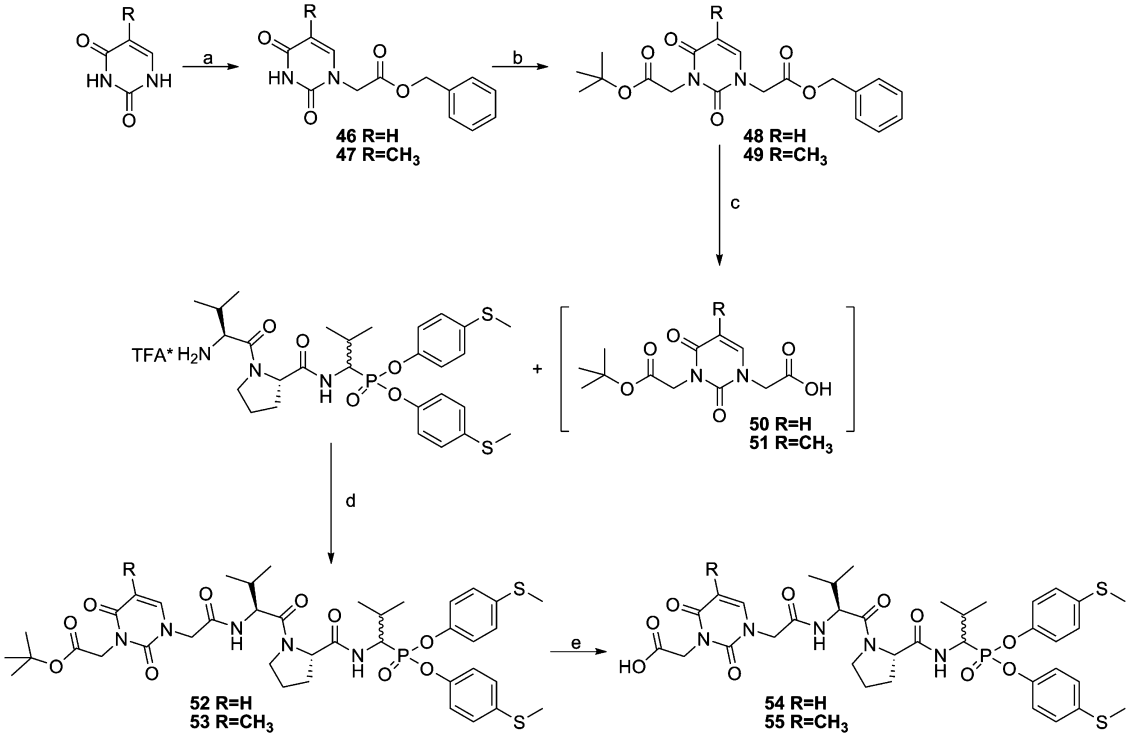
Scheme 3. Synthesis of Peptide 1-Aminoalkylphosphonates Containing a Hydroquinone Moiety[a]



[a]Reagents and conditions: (a) *tert*-butyl bromoacetate, NaOH, dioxane/H_2O, N_2, 22 °C, 1 h (35%); (b) Na_2CO_3, **40**, DMF, 22 °C, 24 h (73%); (c) Pd/C, H_2, AcOEt, 22 °C (92%); (d) HBTU, DIPEA, MeCN, 12 h, 22 °C (40%); (e) TFA, CH_2Cl_2, 2 h, 22 °C (86%).

Scheme 4. Synthesis of Peptide 1-Aminoalkylphosphonates Containing a Thymine or Uracil Moiety[a]



[a]Reagents and conditions: (a) Na_2CO_3, **40**, DMF, 24 h, 22 °C (42%); (b) *tert*-butyl bromoacetate, NaH, 2 h, 0−22 °C (58%); (c) Pd/C, H_2, AcOEt, 22 °C (88%); (d) HBTU, DIPEA, MeCN, 12 h, 22 °C; (e) TFA, CH_2Cl_2, 2 h, 22 °C.

CHEMISTRY

As shown in Scheme 1, the synthesis of peptidyl derivatives of 1-aminoalkylphosphonate diaryl esters (**1**−**27**) began with the preparation of simple Cbz-protected phosphonic analogues of amino acids as previously described.[37] Briefly, the starting triaryl phosphite was prepared from phosphorus trichloride and appropriate phenol in refluxing acetonitrile. The obtained crude triaryl phosphite was used in the next step without purification. The Cbz-protected phosphonates were obtained as racemic

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mixtures in an α-amidoalkylation reaction of triaryl phosphite with benzyl carbamate and an aldehyde and were crystallized from cold methanol. After removal of the Cbz protective group using a 33% solution of hydrobromic acid in acetic acid, the resulting N-deprotected phosphonates were crystallized from methanol/diethyl ether and were coupled with *N*-Boc-L-Pro-OH in acetonitrile using HBTU in the presence of DIPEA as a coupling reagent.[36] Deprotection of the Boc group was performed in 50% trifluoroacetic acid in dichloromethane, and coupling with *N*-Boc-L-Val-OH was achieved using the HBTU/DIPEA method as described above, generating derivatives **1**–**27**.

In order to obtain derivatives **28**–**38** with different amino acid residues in the P3 position or in the fixed P2 and P1 positions, a similar approach was applied (Scheme 2). In short, after deprotection of the Cbz-group in Cbz-Val^{P}(O-C_6H_4-*S*-CH_3)$_2$, the resulting phosphonate was coupled first with *N*-Boc-L-Pro-OH, the Boc group was removed, and the resulting dipeptide was conjugated to different Boc- or Cbz-protected amino acids including glycine, alanine, aminoisobutyric acid, valine, leucine, isoleucine, methionine, or serine.

The synthesis of Boc-Val-Pro-Val^{P}(O-C_6H_4-*S*-CH_3)$_2$ derivatives (**44**, **45**, **54**, **55**) with improved solubility in aqueous media started with the removal of the Boc-protective group in **24**, and the resulting tripeptide was then coupled using HBTU/DIPEA with 4-methoxyphenylacetic acid, hydroquinone (**42**), thymine (**50**), or uracil (**51**) derivatives (Scheme 3) which were prepared as described previously.[20,38] Briefly, the synthesis of the hydroquinone derivative (**44**) began with the condensation of the *tert*-butyl bromoacetate with hydroquinone leading to a *tert*-butyl (4-hydroxyphenoxy)acetate (**39**) which then reacted with benzyl bromoacetate (**40**) to generate **41**. The final product (**42**) was obtained by the removal of the benzyl group using hydrogenolysis in the presence of 10% Pd/C.

Derivatives of thymine (**54**) and uracil (**55**) were obtained in a similar fashion (Scheme 4). First, thymine or uracil was reacted with *tert*-butyl bromoacetate and then with benzyl bromoacetate (**40**). The benzyl group was further removed by hydrogenation in the presence of 10% palladium on activated carbon, affording the desired derivative **50** or **51** that were then used to synthesize phosphonic peptides.

To obtain target compounds as single diastereoisomers (**58**–**71**), the diastereomeric mixture of peptidyl 1-aminoalkylphosphonate derivative **24** was purified by column chromatography on silica gel. The separated fractions containing single diastereoisomers were then concentrated, and the presence of the desired compound was confirmed by ^{31}P NMR spectroscopy and HPLC. Simple Boc deprotection of compound **56** or **57** resulted in the generation of starting material for the synthesis of the final peptides using HBTU as the coupling reagent.

RESULTS AND DISCUSSION

All of the target compounds were tested for their inhibition of HNE activity as well as for selectivity of reaction versus related serine proteases such as porcine pancreatic elastase (PPE), chymotrypsin, and trypsin. For screening purposes the enzymes were first incubated with the tested compound at 25 μM for 15 min before addition of substrate. For compounds that displayed less than 5% enzyme inhibition we assigned an inhibition value of zero (NI). For the inhibitors that showed an activity between 5% and 40% we presumed the k_{inact}/K_I to be <50 M^{-1} s^{-1}. For compounds that displayed HNE inhibition at a minimum of 40% after 15 min of incubation with enzyme, we calculated k_{inact}/K_I values using a linear model of inhibition.[39,40] Control curves in the absence of inhibitor were linear. The standard deviation for the presented values was calculated using the mean of three independent experiments and does not exceed 10%. A typical progress curve (for compound **44**) at different inhibitor concentrations is presented in Figure 1.

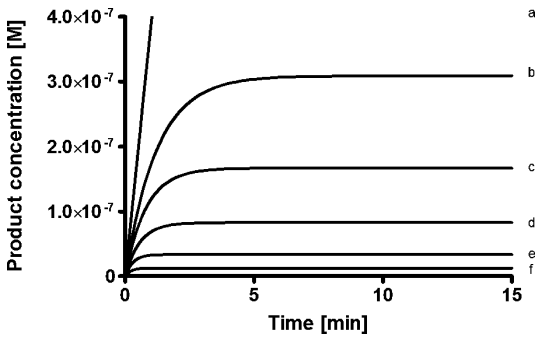

Figure 1. Progress curves for the inhibition of HNE (0.0025 U/mL) by **44**: (a) control; (b) 9 nM; (c) 13 nM; (d) 20 nM; (e) 30 nM; (f) 44 nM.

Our obtained biological results are summarized in Tables 1, 2, and 4. As expected, introduction of the peptide chain into the inhibitor structure resulted in greatly increased potency of action when compared to simple Cbz-protected 1-aminoalkylphosphonates diaryl esters.[37] The reference compound used in this study (Boc-Val-Pro-Val^{P}(OPh)$_2$, **21**) displayed a k_{inact}/K_I of 46100 M^{-1} s^{-1}. Peptides containing the phosphonic analogue of alanine at the P1 position demonstrated poor inhibition (Table 1). Among derivatives containing the phosphonic leucine analogue in the structure, the highest activity against HNE was observed for compound **14** (k_{inact}/K_I = 122500 M^{-1} s^{-1}), but it was more than 1.3 times as effective toward chymotrypsin (k_{inact}/K_I = 165900 M^{-1} s^{-1}) and was also able to inhibit trypsin (k_{inact}/K_I = 2800 M^{-1} s^{-1}). This lack of selectivity was observed for all peptides containing the phosphonic leucine analogue, and all displayed more reactivity toward chymotrypsin than elastase (**11**, k_{inact}/K_I = 325 M^{-1} s^{-1} and k_{inact}/K_I = 1900 M^{-1} s^{-1}; **13**, k_{inact}/K_I = 4100 M^{-1} s^{-1} and k_{inact}/K_I = 21900 M^{-1} s^{-1}; **15**, k_{inact}/K_I = 3400 M^{-1} s^{-1} and k_{inact}/K_I = 18100 M^{-1} s^{-1}; respectively). We observed a slight increase in the selectivity of the peptidyl derivatives of Nle and Nva. For example, compound **19** was approximately 2 times more active against HNE (k_{inact}/K_I = 8100 M^{-1} s^{-1}) than chymotrypsin (k_{inact}/K_I = 4250 M^{-1} s^{-1}). Although compound **16** was almost 20 times more active against HNE (k_{inact}/K_I = 56200 M^{-1} s^{-1}) than chymotrypsin (k_{inact}/K_I = 2800 M^{-1} s^{-1}), it also inhibited porcine pancreatic elastase with k_{inact}/K_I of 54800 M^{-1} s^{-1}. Among the series of phosphonic aminobutyric acid derivatives the highest potency of action was observed for compound **8** (Boc-Val-Pro-Abu^{P}(OC_6H_4-4-*S*-CH_3)$_2$), which inhibited neutrophil elastase with a k_{inact}/K_I of 165100 M^{-1} s^{-1} and displayed little to no activity against chymotrypsin or trypsin. Among all of the obtained peptides with the sequence Boc-Val-Pro-Aaa^{P}(OAr)$_2$ (where Aaa^{P} is the phosphonic analogue of amino acid; Ar is the aromatic ester ring) compound **24** (Boc-Val-Pro-Val^{P}(OC_6H_4-4-*S*-CH_3)$_2$) displayed the highest potency (k_{inact}/K_I of 217300 M^{-1} s^{-1}) and was the most active HNE inhibitor in this series (Table 1). An

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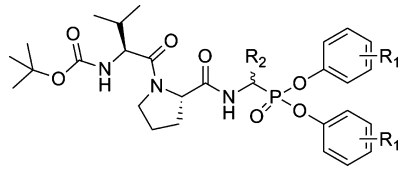
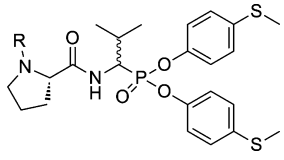
Table 1. Inhibitory Activity of Phosphonic Peptides with the General Formula Boc-Val-Pro-Aaa^{P}(OAr)$_2$[a]



			k_{inact}/K_I (M^{-1} s^{-1})			
compd	R_1	R_2	HNE	PPE	chymotrypsin	trypsin
1	4-ethyl	methyl (Ala)	870 (±5)	1100 (±70)	<50	NI
2	4-*tert*-butyl	methyl (Ala)	<50	<50	<50	NI
3	4-isopropyl	methyl (Ala)	<50	300 (±30)	<50	NI
4	4-*S*-methyl	methyl (Ala)	3500 (±300)	11450 (±260)	<50	<50
5	H	ethyl (Abu)	7100 (±60)	12680 (±240)	<50	<50
6	4-ethyl	ethyl (Abu)	1380 (±5)	4210 (±200)	<50	<50
7	4-isopropyl	ethyl (Abu)	<50	580 (±20)	<50	<50
8	4-*S*-methyl	ethyl (Abu)	165100 (±5300)	60100 (±1100)	365 (±70)	460 (±5)
9	3,4-dimethyl	ethyl (Abu)	460 (±15)	1250 (±45)	<50	<50
10	4-*O*-methyl	ethyl (Abu)	11000 (±460)	9900 (±240)	<50	<50
11	H	2-methylopropyl (Leu)	325 (±5)	1300 (±130)	1900 (±20)	<50
12	4-*O*-methyl	2-methylopropyl (Leu)	270 (±5)	960 (±20)	1700 (±30)	<50
13	4-*S*-methyl	2-methylopropyl (Leu)	4100 (±60)	11200 (±50)	21900 (±660)	280 (±5)
14	3-carbomethoxy	2-methylopropyl (Leu)	122500 (±1800)	76900 (±7200)	165900 (±3700)	2800 (±40)
15	4-chloro	2-methylopropyl (Leu)	3400 (±100)	12800 (±550)	18100 (±600)	<50
16	4-*S*-methyl	*n*-propyl (Nva)	56200 (±2000)	54800 (±900)	2800 (±10)	420 (±5)
17	H	*n*-propyl (Nva)	2600 (±100)	10900 (±150)	140 (±10)	<50
18	4-*tert*-butyl	*n*-propyl (Nva)	<50	<50	NI	NI
19	4-*S*-methyl	*n*-butyl (Nle)	8100 (±800)	9700 (±300)	4250 (±150)	<50
20	H	*n*-butyl (Nle)	650 (±25)	1700 (±10)	400 (±20)	<50
21	H	1-methylethyl (Val)	46100 (±1000)	22800 (±400)	<50	<50
22	4-*tert*-butyl	1-methylethyl (Val)	3450 (±100)	700 (±50)	NI	NI
23	4-ethyl	1-methylethyl (Val)	6800 (±200)	6000 (±70)	NI	NI
24	4-*S*-methyl	1-methylethyl (Val)	217300 (±2500)	14000 (±1400)	900 (±60)	<50
25	4-chloro	1-methylethyl (Val)	92100 (±2500)	5000 (±230)	250 (±5)	<50
26	4-isopropyl	1-methylethyl (Val)	4200 (±170)	2000 (±130)	<50	<50
27	4-*O*-methyl	1-methylethyl (Val)	42700 (±1000)	19500 (±1100)	<50	<50

[a]NI: less than 5% of inhibition was observed after 15 min of incubation of enzyme with the inhibitor (25 μM) at 37 °C.

Table 2. Influence of N-Terminal Residue Structure on the Inhibitory Activity of Phosphonic Peptides with the General Formula Boc/Cbz-Aaa-Pro-Val^{P}(OC_6H_4-*S*-CH_3)$_2$[a]



		k_{inact}/K_i (M^{-1} s^{-1})			
compd	R	HNE	PPE	chymotrypsin	trypsin
28	Boc-Gly	35000 (±550)	5100 (±190)	<50	<50
29	Cbz-Gly	39000 (±150)	1200 (±20)	200 (±20)	<50
30	Boc-D-Ala	39400 (±700)	900 (±30)	<50	<50
31	Cbz-L-Ala	143500 (±2600)	12000 (±900)	1500 (±60)	<50
32	Cbz-Aib	1300 (±130)	<50	<50	<50
33	Cbz-L-(^{t}Bu)Ser	162200 (±10600)	4300 (±110)	500 (±10)	<50
34	Cbz-L-Val	231100 (±13500)	6200 (±120)	4400 (±440)	<50
35	Boc-L-Leu	127600 (±5200)	7000 (±100)	150 (±60)	<50
36	Cbz-L-Leu	86200 (±1700)	1000 (±50)	800 (±70)	NI
37	Cbz-L-Ile	145100 (±3700)	1800 (±70)	1600 (±160)	<50
38	Cbz-L-Met	223200 (±1700)	6300 (±150)	3700 (±20)	<50

[a]NI: less than 5% of inhibition was observed after incubation of enzyme with the inhibitor (25 μM) at 37 °C.

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introduction of the *S*-methyl substituent into the ester ring structure at the para position resulted in a 4.7× greater increase in potency of action against HNE (compared to the unsubstituted compound **21**, k_{inact}/K_I = 46100 M^{-1} s^{-1}) and a 7.6× increase in selectivity versus porcine pancreas elastase (PPE) (compound **21** was 2× more active against HNE vs PPE; compound **24** was 15× more active against HNE vs PPE). Replacing the *S*-methyl group with a chlorine atom (**25**) or an *O*-methyl group (**27**) decreased the potency of action (k_{inact}/K_I of 92100 and 42700 M^{-1} s^{-1}, respectively), though these compounds displayed a similar selectivity for HNE vs PPE and chymotrypsin. On the basis of our initial results, derivative **24** was selected for further development of potent and selective HNE inhibitors.

Next, we evaluated the influence of the P3 residue as well as the N-terminal protective group on the inhibitory profile of phosphonic peptides (Table 2). Replacing the Boc protective group (**24**) with a Cbz group increased the overall potency of action (**34**, k_{inact}/K_I = 231100 M^{-1} s^{-1}) but also increased reactivity with chymotrypsin (**24**, k_{inact}/K_I = 900 M^{-1} s^{-1}; **34**, k_{inact}/K_I = 4400 M^{-1} s^{-1}). With the exception of one compound (**38**), exchanging the Val residue at the P3 position with any of the selected amino acids (Gly, Ala, Ile, Leu, ^{t}Bu-Ser) resulted in decreased inhibitory properties. Surprisingly, compound **38** (Cbz-Met-Pro-Val^P(OC_6H_4-*S*-CH_3)$_2$) displayed activity and selectivity profiles similar to compound **24**.

A serious disadvantage of phosphonates, which limits their practical application as potential therapeutics, is their low solubility in aqueous media. To address this, we introduced N-terminal groups, which were shown to increase compound solubility, into the inhibitor structure.[20] H-Val-Pro-Val^P(OC_6H_4-*S*-CH_3)$_2$ was used as the starting material and was further coupled either with 4-methoxybenzoic acid or with a derivative of hydroquinone, uracil, or thymine. In addition to increased solubility (Table 3) all of the obtained compounds in this group displayed increased activity against HNE (Table 4) and relatively low activity against chymotrypsin and trypsin.

Table 3. Solubility of HNE Phosphonic Inhibitors in Buffer and Human Plasma

	solubility (mM)	
compd	PBS, pH 7.4	human plasma
24	0.36	0.36
44	6.23	6.23
45	0.17	0.17
54	6.22	6.22
55	6.11	6.11

The most selective compound identified within this group was a 4-methoxybenzoic acid derivative (**45**) that displayed a k_{inact}/K_I of 304000 M^{-1} s^{-1} against HNE and 4700 M^{-1} s^{-1} against PPE and was almost completely inactive against chymotrypsin and trypsin. The highest inhibition potency against HNE was observed for derivatives of thymine (**55**, k_{inact}/K_I = 965000 M^{-1} s^{-1}) and hydroquinone (**44**, k_{inact}/K_I = 970000 M^{-1} s^{-1}). Although compound **55** was among the most active HNE inhibitors in this series, it was only 8.2 times more specific toward HNE than PPE and 42 times more specific to HNE than to chymotrypsin. The lowest selectivity was observed for compound **54** (k_{inact}/K_I of 460000 and 28300 M^{-1} s^{-1} for HNE and PPE, respectively). Derivative **44** displayed the highest inhibitory potency against HNE in this series, as its observed reactivity against HNE was 50.8 times greater than its reactivity to PPE (k_{inact}/K_I = 19100 M^{-1} s^{-1}) and it was over 245 times less active against chymotrypsin (k_{inact}/K_I = 4000 M^{-1} s^{-1}).

Previously, an attempt to generate and biologically evaluate the reactivity of diastereomerically pure phosphonic peptides against serine proteases (DPP IV) was reported by Boduszek et al.[34] Subsequently, the Haemers group presented their investigations of diastereomerically pure phosphonic dipeptides as DPP IV inhibitors.[41,42] In 2000, Walker et al. reported an advanced biochemical evaluation of enantiomerically pure Cbz-protected phosphonic analogues of Phe and Val as inhibitors of cathepsin G, chymotrypsin, and human neutrophil elastase.[43] These results clearly showed that (*R*)-epimers are more potent inhibitors of target serine proteases than their corresponding (*S*)-epimers. In order to generate target inhibitors with a high potency of action against HNE in an optically pure form, we separated compound **24** into single diastereoisomers, which, after Boc-group removal, were used for the synthesis of derivatives **58**−**71**. This approach was found to be very convenient, since the desired products could be obtained in great quantities. Silica gel column chromatography method was found to be very useful for separating of **24** into single diastereoisomers even on a multigram scale. A typical HPLC analysis of the final synthesized compounds including ^{31}P NMR data, obtained as racemic mixture or separated single diastereisomers, is shown in Figure 2.

The results obtained from our enzyme inhibition studies of compounds **56**−**59**, **61**, **63**, **65**, **67**, **69**, and **71** (Table 4) clearly show that the *R*-diastereoisomer is capable of protease inactivation, while diastereoisomer *S* is almost completely inactive against the target enzyme. The most potent and selective inhibitor of HNE found in this study was **61** which displayed a k_{inact}/K_I of 2353000 M^{-1} s^{-1} and was 60 times less active against PPE (k_{inact}/K_I = 39200 M^{-1} s^{-1}) and 365 times less active against chymotrypsin (k_{inact}/K_I = 6450 M^{-1} s^{-1}). Although **69** displayed high potency against HNE (k_{inact}/K_I = 1760000 M^{-1} s^{-1}) it was only 7 times less active against PPE (k_{inact}/K_I = 242900 M^{-1} s^{-1}) and 39 times less active against chymotrypsin (k_{inact}/K_I = 44700 M^{-1} s^{-1}). Similar to its diastereomeric mixture (**54**), the uracil derivative (**65**) displayed the lowest selectivity of action, since it was only 3.5 times more active against HNE than PPE and 27 times more active against HNE than chymotrypsin.

To examine the stability of the irreversible complex formed between HNE and compound **61**, we first incubated the protease with an excess of inhibitor. After complete inactivation the excess of inhibitor was removed by dialysis and the activity of protease was monitored at different time intervals (Figure 3). In parallel, a control HNE sample incubated only with DMSO instead of **61** was prepared and dialyzed in the same way. These results demonstrated that after 50 days in complex with **61**, HNE recovered around 15% of its original activity whereas the activity of the control HNE sample after this time remained at approximately 90% of its original value. The recovery of enzymatic activity at different time points is expressed as the v_{max} [units/s] (Figure 3).

The rate of hydrolysis of compounds **24**, **44**, **45**, **54**, and **55** was evaluated either in PBS buffer (pH 7.4) or in 80% human plasma at 37 °C.[29] The stability of the inhibitors was monitored by HPLC at different time points (Table 5). The evaluation of compound stability in PBS buffer showed that the half-life of all tested compounds was longer than 48 h. Of note, at 48 h, only

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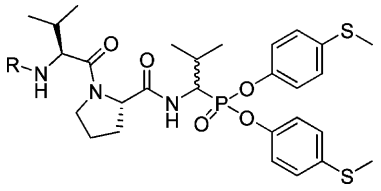
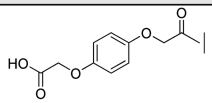
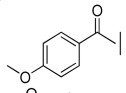
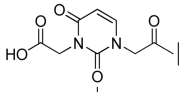
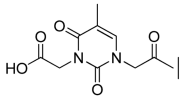
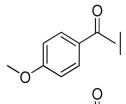
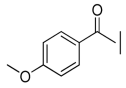
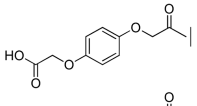
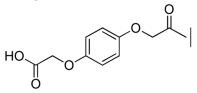
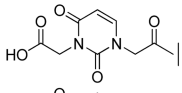
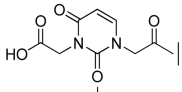
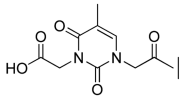
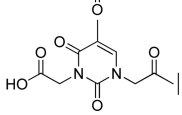
Table 4. Inhibitory Properties of Phosphonic Peptides as Single Diastereisomers[a]



Compound	R	Diastereoisomer	k_{inact}/K_I [$M^{-1}s^{-1}$]			
			HNE	PPE	Chymotrypsin	Trypsin
44		*R,S*	970000 (±83000)	19100 (±1300)	4000 (±100)	<50
45		*R,S*	304000 (±13600)	4700 (±100)	<50	<50
54		*R,S*	460000 (±23000)	139800 (±2900)	28300 (±1000)	130 (±5)
55		*R,S*	965000 (±55500)	118000 (±300)	22900 (±50)	140 (±5)
56		*R*	431400 (±14200)	22300 (±1800)	850 (±25)	<50
57		*S*	2650 (±25)	<50	<50	NI
58		*R*	539700 (±3000)	6700 (±120)	100 (±10)	<50
59		*S*	4800 (±10)	<50	<50	NI
61		*R*	2353000 (±89000)	39200 (±4200)	6450 (±300)	<50
63		*S*	1550 (±35)	85 (±5)	<50	<50
65		*R*	1079000 (±20000)	309400 (±540)	40200 (±1400)	235 (±10)
67		*S*	2000 (±100)	200 (±10)	<50	NI
69		*R*	1760000 (±75000)	242900 (±2200)	44700 (±1400)	280 (±5)
71		*S*	4100 (±400)	300 (±5)	<50	NI

[a]NI: less than 5% of inhibition was observed after 15 min of incubation of enzyme with the inhibitor (25 μM) at 37 °C.

16% hydrolysis of compound **44** was measured, and even after 6 days, over 50% of **44** remained detectable. In addition, compound **44** also displayed the highest stability in 80% human plasma, with a half-life of 8 h. Compound **24** was also stable in human plasma, with a $t_{1/2}$ of 6.2 h. Derivatives of 4-methoxybenzoic acid, uracil, and thymine were less stable in 80% human plasma, with measured half-lives of 3.8, 4.55, and 3.55 h, respectively. The main products of hydrolysis were their corresponding monoaryl esters. These results indicate that designing (4-*S*-methyl)phenyl as the ester group represents an optimal balance between compound stability and inhibitory potency which is a consequence of the increased electrophilicity of the phosphorus atom. When stability in human plasma is taken into consideration, along with selectivity and high potency of action against HNE, the synthesized peptidyl 1-aminoalkylphosphonate diaryl esters represent a superior class of inhibitors with potential for practical applications.

In order to evaluate the inhibitory properties of the most active HNE inhibitors and gain insight into the binding mode of compounds **61**, **65**, **69**, we performed a molecular docking

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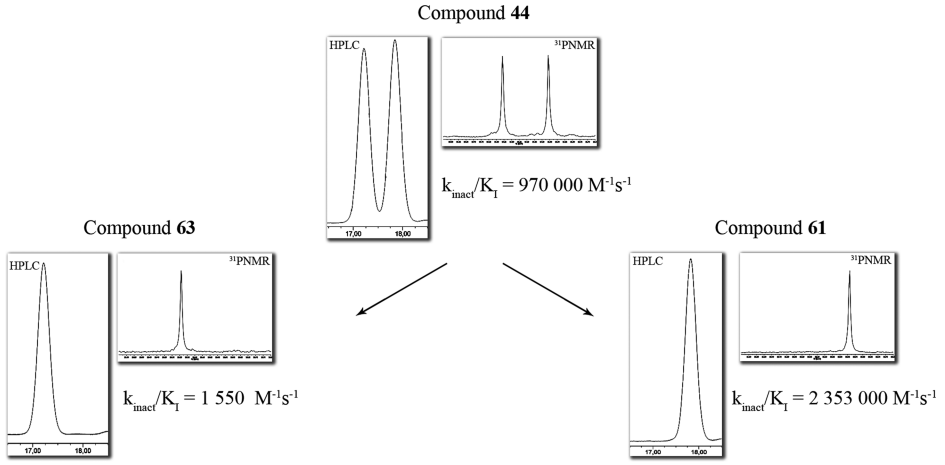

Figure 2. HPLC and ^{31}P NMR confirmation of separation of diastereisomers with corresponding k_{inact}/K_I values.

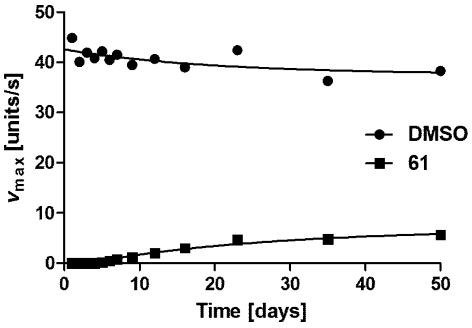

Figure 3. Recovery of human neutrophil elastase activity from HNE complexed with **61**.

Table 5. Stability of Compounds 24, 44, 45, 54, and 55 in Aqueous Solution and 80% Human Plasma

	$t_{1/2}$ (h)	
compd	PBS, pH 7.4	80% human plasma
24	28	6.20
44	150	8.00
45	26	3.80
54	100	4.55
55	100	3.95

analysis of inhibitors bound to the active site of HNE using an AutoDock Vina and structural template 1PPG.pdb.[44,45] Since the crystal structures of phosphonic inhibitor in complex with serine proteases showed that the inhibitor is devoid of ester phenyl rings (aged complex), we docked inhibitors **61**, **65**, **69** as phosphonic acids (Figure 4).[46] The overall positioning pattern observed for all analyzed inhibitors is essentially identical. The oxygen atom of the phosphonic group is hydrogen-bonded with the oxyanion hole formed by the main chain amide groups of Gly193 and Ser195. The phosphorus−oxygen distance ranges from 2.7 to 2.9 Å. Inhibitor side chains at the P1−P3 positions occupy the corresponding enzyme binding pockets (S1−S3). In contrast to the chloromethyl template inhibitor, compounds **61**, **65**, **69** do not seem to fully occupy the S4 binding pocket and they tend to display different conformational binding to the cavity formed by Arg177, Arg217, Cys168, and Leu166. The highly inhibitory properties of the hydroquinone derivative **61** could be the result of its extended length which results in the formation of additional interactions with HNE, especially between the hydrophobic forces of the aromatic ring and the hydrogen bonds with Arg177 and Arg217. Studies focused on solving the HNE crystal structure in complex with obtained phosphonic inhibitors are ongoing.

CONCLUSIONS

We propose that theoretically most reversible inhibitors of elastase are unable to correct the elastase−α_1-PI imbalance in vivo. Although the equilibrium of the inhibition reaction by reversible inhibitor is shifted substantially to the formation of an EI complex, a small amount of free active enzyme remains. Under normal conditions free HNE would be bound by α_1-PI, but because of an insufficient amount of reactive serpin, the activity of HNE is not completely blocked. Thus, reversible elastase inhibitors do not display therapeutic efficiency in vivo. In contrast, phosphonates form covalent and extremely stable EI complexes that release free enzyme only after an extended period of time.

The HNE inhibitors described here represent the most potent peptidyl irreversible inactivators of this enzyme reported to date. Our initial findings regarding the structural requirements for phosphonic peptides (**1**−**38**) led to the development of highly potent derivatives of hydroquinone, uracil, and thymine. The most active compounds were further obtained as single diastereisomers with increased inhibitory properties. Overall, compound **61** displayed the best balance of high inhibitory activity against HNE and selectivity for HNE over porcine pancreatic elastase, chymotrypsin, and trypsin. Furthermore, the analysis of the elastase−inhibitor **61** complex revealed that even after 50 days HNE recovered only 15% of its initial activity. Additionally, our obtained inhibitors displayed high stability in either PBS or human plasma. Therefore, it may be possible to apply our generated derivatives in vivo to overcome HNE−α_1-PI imbalance under physiological conditions.

EXPERIMENTAL SECTION

Enzymatic Studies. *Mathematical Model.*[39,40] The inhibitory activity of the synthesized compounds was determined by the progress curve method. The nonlinear progress curves observed with time-dependent inhibitors of HNE were fit to eq 1 to obtain the first-order rate constant (k_{obs}):

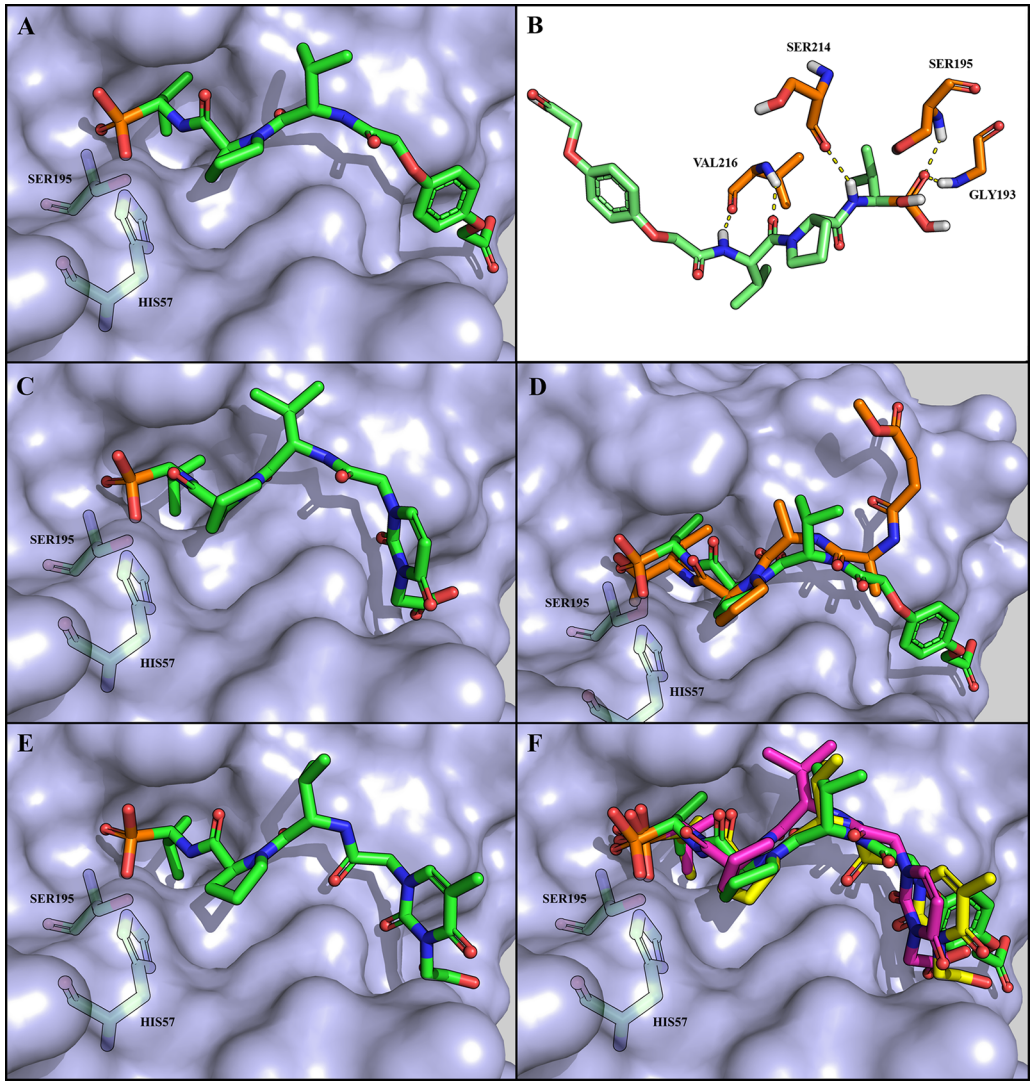

Figure 4. Schematic representation of HNE inhibitors bound to the protease active site (PDB accession code 1PPG): (A) proposed binding mode for compound **61**; (B) representation of the HNE residues surrounding the docked **61**, showing the potential hydrogen bonds (dashed yellow lines); (C) proposed binding mode for compound **65**; (D) representation of compound **61** and MeO-Suc-AAPV-CMK superimposed in the binding site of HNE; (E) proposed binding mode for compound **69**; (F) HNE binding site superimposition of **61** (green), **65** (purple), and **69** (yellow).

$$[\mathrm{P}] = v_s t + \frac{v_i - v_s}{k_{obs}}[1 - e^{(-k_{obs}t)}] \quad (1)$$

where [P] is the product concentration at time t, v_i is the initial velocity, and v_s is the steady state velocity.

Second-order rate constants (k_{inact}/K_I) were estimated by calculating $k_{obs}/[I]$ and then correcting for the substrate concentration ([S]) and Michaelis–Menten constant (K_M) according to eq 2:

$$\frac{k_{obs}}{[I]} = \frac{k_{inact}}{K_I\left(1 + \frac{[S]}{K_M}\right)} \quad (2)$$

Control curves in the absence of inhibitor were linear. The standard deviation for the presented values was calculated using the mean of three independent experiments and does not exceed 10%.

General. The rates of inhibition of human neutrophil elastase (HNE, 2.5 mU/mL, Biocentrum, Kraków, Poland), porcine pancreatic elastase (PPE, 12.5 nM, Serva, Polgen, Łódź, Poland), chymotrypsin (0.5 nM, Sigma-Aldrich, Poznań, Poland), and trypsin (12.5 nM, AppliChem, Polgen, Łódź, Poland) were measured in 0.1 M HEPES, 0.5 M NaCl, 0.03% Triton X-100 (pH 7.5) at 37 °C. All enzymes were assayed using fluorogenic substrates (Calbiochem, Merck, Warszawa, Poland): MeO-Suc-Ala-Ala-Pro-Val-AFC (40 μM, HNE; 40 μM, PPE), Cbz-Arg-AFC (50 μM, trypsin), and Suc-Ala-Ala-Pro-Phe-AMC (20 μM, chymotrypsin). All compounds were screened for inhibitory activity against HNE, porcine pancreatic elastase (PPE), chymotrypsin, and trypsin at 25 μM. First, the enzyme was incubated for 15 min with each of the tested compounds and measurements were taken for 15 min. When less than 5% inhibition was observed for any of the molecules, the level of inhibition was assigned as zero. For the inhibitors that showed an activity between 5% and 40%, we presumed $k_{inact}/K_I < 50\ M^{-1}\ s^{-1}$. We calculated k_{inact}/K_I values for compounds that displayed HNE inhibition at a minimum of 40% after 10 min of incubation with the enzyme. The inhibitory activity of the synthesized compounds was determined by the progress curve method. Control curves in the absence of the inhibitor were linear. The standard deviation for presented values is calculated from the mean of three independent experiments and does not exceed 10%. All measurements were performed using Spectra Max Gemini XPS spectrofluorometer (Molecular Devices, U.S.).

HNE–Inhibitor Complex Stability. Human neutrophil elastase (1.91 μM) was incubated with **61** (200 μM, final DMSO concentration in the reaction mixture was 1%) for 15 min at 37 °C in 0.1 M HEPES, 0.5 M NaCl, 0.03% Triton X-100, pH 7.5.After incubation the enzyme showed no proteolytic activity as determined using a fluorogenic substrate (MeOSuc-AAPV-AFC, 40 μM).

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Completely inactivated elastase was further dialyzed (D-Tube Dializer Mini, MWCO 6−8 kDa, Novagen) against the reaction buffer (0.1 M HEPES, 0.5 M NaCl, 0.03% Triton X-100, pH 7.5) at 4 °C with 5 times change of buffer (dilution factor of 10^{15}). The control sample was prepared in parallel using the same approach with the exception that the enzyme was incubated in the reaction buffer with DMSO instead of **61** before dialysis. The activity of both elastase solutions incubated at 37 °C was monitored using a fluorogenic substrate at different time points within 50 days. The recovery of enzymatic activity is expressed as v_{max} [unit/s] at different time points.

Hydrolysis in PBS Buffer. The susceptibility of compounds to hydrolysis in buffer solution was examined by HPLC. Stock solutions of the tested compounds (50 mM) prepared in DMSO were added to 100 mM phosphate buffered saline (PBS, pH 7.4) to a final concentration of 3 mg/mL (**44**, **54**, **55**) or 0.1 mg/mL (**24**, **45**) and then incubated at 37 °C. At different time points the mixtures were analyzed by HPLC using a C8 column.

Hydrolysis in Human Plasma. The stability of compounds in human plasma was examined as described before with minor changes.[29] Freshly isolated human plasma pooled from healthy donors was used to study the kinetics of hydrolysis of specific compounds. A stock DMSO solution of each tested compound (50 mM) was added to 80% human plasma (diluted with PBS, pH 7.4) to a final concentration of 3 mg/mL (**44**, **54**, **55**) or 0.1 mg/mL (**24**, **45**) and incubated at 37 °C. At different time points, 100 μL aliquots of compound solution were added to 200 μL of DMSO and precipitated proteins were centrifuged. The resulting supernatant was subjected to HPLC analysis (200 μL) to determine compound stability.

Molecular Docking. Molecular docking studies of compounds **61**, **65**, **69** bound to the active site of HNE with MeO-Suc-Ala-Ala-Pro-Val chloromethyl ketone (MeO-Suc-Ala-Ala-Pro-Val-CMK) inhibitor removed from the structure (PDB accession code 1PPG) were performed with the AutoDock Vina.[44,45] Each ligand was initially energy minimized. Pictures ware prepared using Pymol.[47]

Chemistry. *General.* All chemical reagents and solvents were obtained from commercial sources and, unless otherwise noted, were used without further purification. Silica gel chromatography was performed using glass columns packed with silica gel 60 (70−230 mesh). Analytical thin-layer chromatography was performed on Macherey-Nagel silica gel Polygram SIL G plates, and compounds were visualized with UV light at 254 nm. All final compounds were purified to >95% purity at 220 and 254 nm as determined by HPLC. HPLC analyses and purifications were carried out on a Varian ProStar 210 with a dual λ absorbance detector system using SunFire Prep C8 column (10 mm × 250 mm, 5 μm) at 40 °C with a 4 mL/min flow rate using a gradient 5−95% [0.05% TFA in acetonitrile] in [0.05% TFA in water] over 40 min. The nuclear magnetic resonance spectra (^{1}H and ^{31}P) were recorded on either a Bruker Avance DRX-300 (300.13 MHz for ^{1}H NMR, 121.50 MHz for ^{31}P NMR) or Bruker Avance 600 MHz (600.58 MHz for ^{1}H NMR, 243.10 MHz for ^{31}P NMR) spectrometer. Chemical shifts are reported in parts per million (ppm) relative to a tetramethylsilane internal standard. Mass spectra were recorded using a Biflex III MALDI TOF mass spectrometer (Bruker, Germany). The cyano-4-hydroxycinnamic acid (CCA) was used as a matrix. High resolution mass spectra (HRMS) were acquired either on a Waters Acquity Ultra Performance LC, LCT Premier XE or Bruker micrOTOF-Q II mass spectrometer.

Solubility Assay. Compounds **24**, **44**, **45**, **54**, and **55** were dissolved in PBS buffer or freshly isolated human plasma from healthy donor (pooled) in increasing concentrations ranging from 15 to 0.016 mg/mL. The turbidimetry in reference to the buffer or plasma solution of the solutions was measured at 620 nm using a microplate reader (Molecular Devices, SpectraMax Plus 384).

Triaryl Phosphites (General Procedure).[37] Phosphorus trichloride (10 mmol) was added to a solution of substituted phenol (30 mmol) in acetonitrile (50 mL) at room temperature, and the mixture was refluxed for 6 h. The volatile components were removed under reduced pressure, and the resulting crude phosphite was used directly in the next step without purification.

Cbz-Protected 1-Aminoalkylphosphonate Diaryl Esters (General Procedure).[37] Triaryl phosphite (10 mmol) was dissolved in glacial acetic acid (25 mL), and an appropriate aldehyde (11 mmol) and benzyl carbamate (11 mmol, 1.66 g) were subsequently added. The mixture was then heated at 80−90 °C for 2 h, and the solvent was evaporated. The resulting oil was dissolved in methanol (100 mL) and left at −20 °C for crystallization. The product was filtered, washed with cold methanol, and dried at room temperature. If necessary, the product was recrystallized from chloroform/methanol.

Deprotection of Cbz Group (General Procedure). The Cbz-protected 1-aminoalkylphosphonate diaryl ester (10 mmol) was dissolved in 33% HBr/AcOH solution (4 mL). The reaction was performed at room temperature for 2 h. The volatile components of the mixture were removed under reduced pressure. The resulting oil was dissolved in methanol (1 mL), and diethyl ether (100 mL) was added and was left for crystallization at 4 °C. The hydrobromide salt of 1-aminoalkylphosphonate diaryl ester was filtered and washed extensively with cold diethyl ether. If necessary, the product was recrystallized using a methanol/diethyl ether system.

Phosphonic Dipeptides (General Procedure). The hydrobromide salt of 1-aminoalkylphosphonate diaryl ester (10 mmol) was suspended in acetonitrile (25 mL), and *N*,*N*′-diisopropylethylamine (25 mmol, 1.75 mL) was added. After complete dissolution of the substrate, *N*-Boc-L-Pro-OH (10 mmol, 2.15 g) was added followed by the addition of HBTU (12 mmol, 4.55 g). The reaction was performed at room temperature for 12 h and its progress monitored by thin-layer chromatography. When the reaction was completed, the solvent was evaporated under reduced pressure and the resulting oil was dissolved in ethyl acetate (100 mL). The white precipitate was filtered off, and the solution was washed with 5% $NaHCO_3$ (aq) (50 mL), 5% $KHSO_4$ (aq) (50 mL), and brine (50 mL). After drying over anhydrous sodium sulfate, the solution was filtered and concentrated in a vacuum. The final compounds were purified using silica gel column chromatography.

Deprotection of the ^{t}Bu/Boc Group (General Procedure). The *N*-Boc-protected phosphonic di- or tripeptides (10 mmol) were dissolved in dichloromethane (5 mL), and an equal volume of trifluoroacetic acid was added dropwise. The reaction was performed at room temperature for 2 h, and the progress of the reaction was monitored by TLC. When the reaction was completed, the volatile components of the mixture were removed under reduced pressure. In order to remove the traces of trifluoroacetic acid, the resulting oil was dissolved in toluene and evaporated a total of three times. The obtained phosphonic dipeptides with a free N-terminal amino group were used directly in the next step without further purification.

Phosphonic Tripeptides (General Procedure). The trifluoroacetate salt of phosphonic dipeptide derivative (10 mmol) was dissolved in acetonitrile followed by the addition of *N*,*N*′-diisopropylethylamine (25 mmol, 1.75 mL). Next, the N-protected amino acid (10 mmol) and HBTU (12 mmol, 4.55 g) were added. The reaction was performed at room temperature for 12 h and progress monitored by TLC. After completion of the reaction the solvent was evaporated and the resulting oil was dissolved in ethyl acetate (100 mL). The solution was then washed with 5% $NaHCO_3$ (aq) (50 mL), 5% $KHSO_4$ (aq) (50 mL), and brine (50 mL), dried over Na_2SO_4, filtered, and concentrated resulting in a crude phosphonic tripeptide which was purified by column chromatography on silica gel.

Diastereoisomers Separation. Single diastereoisomers of 1-aminoalkyphosphonate aromatic ester peptidyl derivatives, the starting material for further synthesis, were prepared using compound **24** (Boc-Val-Pro-Val^P(OC_6H_4-4-*S*-CH_3)$_2$). Diastereoisomer separation was performed using column chromatography on silica gel. Collected fractions containing single diastereoisomer were concentrated, and the optical purity was confirmed by ^{31}P NMR spectroscopy and HPLC analysis. Compounds **58**−**71** were synthesized as described above with the exception that a single diastereoisomer (**56** or **57**) was used as the starting substrate.

*tert-Butyl (S)-1-((S)-2-((R,S)-1-(Bis(4-(methylthio)phenoxy)-phosphoryl)-2-methylpropylcarbamoyl)pyrrolidin-1-yl)-3-methyl-1-oxobutan-2-ylcarbamate (**24**).* **24** was prepared using general

methods described above and purified by column chromatography on silica gel using 20% ethyl acetate in chloroform as the eluent to yield a colorless oil which formed a white solid foam under reduced pressure (73%). R_f = 0.15 (separate diastereoisomer), 0.07 (separate diastereoisomer) ($CHCl_3$/AcOEt, 4:1, v/v). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.03−7.23 (m, 8H), 5.30 (dd, J = 61.7, 9.3 Hz, 1H), 4.49−4.78 (m, 2H), 4.20−4.34 (m, 1H), 3.43−3.80 (m, 2H), 2.44 (d, J = 2.7 Hz, 6H), 2.04−2.41 (m, 2H), 1.74−2.03 (m, 4H), 1.42 (d, J = 2.0 Hz, 9H), 0.81−1.14 (m, 12H). ^{31}P NMR (121 MHz, $CDCl_3$) δ 18.15 (s, 52%), 18.48 (s, 48%). HRMS: calcd for $(C_{33}H_{48}N_3O_7PS_2)H^+$, 694.2750; found, 694.2738.

*Benzyl Bromoacetate (**40**).* To the solution of benzyl alcohol (4 g, 37 mmol) in DMF (100 mL) were subsequently added 2-bromoacetic acid (10.26 g, 74 mmol), DCC (15.26 g, 74 mmol), and 4-dimethylaminopyridine (1.35 g, 11.1 mmol), and the reaction was performed at room temperature for 24 h. The reaction mixture was diluted with distilled water (1 L) and extracted four times with ethyl acetate (500 mL). The organic fraction was dried over $MgSO_4$, filtered, and concentrated in vacuum, and the product was purified by chromatography on silica gel, eluting with ethyl acetate in hexane (20:1, v/v) to provide **40** (5.7 g, 67%) as a pale yellow oil. R_f = 0.40 (Hex/AcOEt, 20:1, v/v). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32−7.46 (m, 5H), 5.21 (s, 2H), 3.88 (s, 2H).

*Benzyl 2-(2,4-Dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetate (**46**).* Uracil (1 g, 9 mmol), **40** (2.5 g, 11 mmol), and anhydrous potassium carbonate (2.5 g, 18.1 mmol) were suspended in anhydrous DMF. The reaction was performed at room temperature for 24 h. The reaction mixture was diluted with distilled water (100 mL) and extracted three times with ethyl acetate (30 mL). Combined organic fractions were washed with brine (50 mL), dried over $MgSO_4$, filtered, and the solvent was removed in vacuum. Next, the resulting oil was dissolved in diethyl ether (100 mL) and left for crystallization, leading to the generation of **46** as a white solid (0.98 g, 42%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.43 (s, 1H), 7.65 (d, J = 7.9 Hz, 1H), 7.34−7.40 (m, 5H), 5.63 (dd, J = 7.9, 2.1 Hz, 1H), 5.20 (s, 2H), 4.59 (s, 2H). HRMS: calcd for $(C_{13}H_{12}N_2O_4)H^+$, 261.0870; found, 261.0853

*Benzyl 2-(3-(2-tert-Butoxy-2-oxoethyl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetate (**48**).* A suspension of **46** (0.98 g, 3.8 mmol) in anhydrous DMF (10 mL) was cooled in an ice bath, and 60% sodium hydride solution (0.18 g, 4.5 mmol) was slowly added. After 15 min *tert*-butyl bromoacetate (0.68 mL, 4.6 mmol) was added and the reaction mixture was allowed to warm to room temperature. After 2 h saturated ammonium chloride solution (100 mL) was added and the mixture was extracted three times with ethyl acetate (50 mL). Combined organic fractions were washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated in vacuum. Purification by column chromatography on silica gel using chloroform/ethyl acetate (4:1, v/v) as the eluent afforded **48** as a colorless oil (0.82 g, 58%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.76 (d, J = 7.9 Hz, 1H), 7.28−7.41 (m, 6H), 5.82 (d, J = 7.9 Hz, 1H), 5.18 (s, 2H), 4.67 (s, 2H), 4.41 (s, 2H), 1.38 (s, 9H).

*2-(3-(2-tert-Butoxy-2-oxoethyl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetic Acid (**50**).* 10% palladium on carbon (100 mg) was added to a solution of **48** (0.82 g, 2.2 mmol) in ethyl acetate (20 mL), and a stream of hydrogen was passed through. The progress of the reaction was monitored by thin-layer chromatography. After the reaction was completed, the reaction mixture was passed through a pad of Celite and concentrated in vacuum to yield pure **50** as a colorless oil which formed a white solid foam under reduced pressure (0.55 g, 88%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.18 (s, 1H), 7.72 (d, J = 7.9 Hz, 1H), 5.79 (d, J = 7.9 Hz, 1H), 4.48 (s, 2H), 4.40 (s, 2H), 1.38 (s, 9H). HRMS: calcd for $C_{12}H_{16}N_2O_6)Na^+$, 307.0901; found, 307.0894.

*tert-Butyl (S)-1-((S)-2-((R)-1-(Bis(4-(methylthio)phenoxy)-phosphoryl)-2-methylpropylcarbamoyl)pyrrolidin-1-yl)-3-methyl-1-oxobutan-2-ylcarbamate (**56**).* **56** was obtained as a colorless oil which formed a white solid foam under reduced pressure (17%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.57 (d, J = 10.3 Hz, 1H), 7.00−7.20 (m, 8H), 5.58 (d, J = 9.3 Hz, 1H), 4.54−4.76 (m, 2H), 4.22−4.31 (m, 1H), 3.69−3.81 (m, 1H), 3.56−3.67 (m, 1H), 2.41 (s, 3H), 2.40 (s, 3H), 2.21−2.37 (m, 1H), 2.02−2.21 (m, 2H), 1.76−1.99 (m, 3H), 1.40 (s, 9H), 0.97−1.05 (m, 6H), 0.94 (d, J = 6.7 Hz, 3H), 0.88 (d, J = 6.7 Hz, 3H). ^{31}P NMR (121 MHz, $CDCl_3$) δ 18.25 (s). HRMS: calcd for $(C_{33}H_{48}N_3O_7PS_2)Na^+$, 716.2569; found, 716.2537.

*tert-Butyl 2-(4-(2-((S)-1-((S)-2-((R)-1-(Bis(4-(methylthio)phenoxy)-phosphoryl)-2-methylpropylcarbamoyl)pyrrolidin-1-yl)-3-methyl-1-oxobutan-2-ylamino)-2-oxoethoxy)phenoxy)acetate (**60**).* The TFA salt of **56** obtained after *N*-Boc deprotection under conditions described above (0.34 g, 0.48 mmol) was dissolved in acetonitrile (5 mL), and *N*,*N*′-diisopropylethylamine (236 μL, 1.44 mmol) was added. Next, **42** (163 mg, 0.58 mmol) was added into the mixture which was followed by the addition of HBTU (218 mg, 0.58 mmol). The postreaction workup was done as described for **58**, and purification by column chromatography on silica gel using 2% methanol in chloroform as the eluent yielded **60** as a colorless oil which formed a white solid foam under reduced pressure (185 mg, 45%). R_f = 0.14 ($CHCl_3$/MeOH, 50:1, v/v). ^{1}H NMR (601 MHz, DMSO-d_6) δ 8.49 (d, J = 9.9 Hz, 1H), 8.05 (d, J = 8.7 Hz, 1H), 7.21−7.35 (m, 4H), 7.14 (d, J = 8.6 Hz, 2H), 7.09 (d, J = 8.6 Hz, 2H), 6.82−6.90 (m, 4H), 4.60 (s, 2H), 4.50−4.57 (m, 2H), 4.48 (s, 2H), 4.39−4.45 (m, 1H), 3.68−3.77 (m, 1H), 3.55−3.61 (m, 1H), 2.47 (s, 3H), 2.46 (s, 3H), 2.24−2.33 (m, 1H), 1.95−2.07 (m, 2H), 1.84−1.95 (m, 1H), 1.74−1.84 (m, 1H), 1.55−1.62 (m, 1H), 1.41 (s, 9H), 1.09 (d, J = 6.8 Hz, 3H), 1.03 (d, J = 6.8 Hz, 3H), 0.92 (d, J = 6.7 Hz, 3H), 0.84 (d, J = 6.7 Hz, 3H). ^{31}P NMR (243 MHz, DMSO-d_6) δ 18.94 (s). HRMS: calcd for $(C_{42}H_{56}N_3O_{10}PS_2)Na^+$, 880.3043; found, 880.3027.

*2-(4-(2-((S)-1-((S)-2-((R)-1-(Bis(4-(methylthio)phenoxy)-phosphoryl)-2-methylpropylcarbamoyl)pyrrolidin-1-yl)-3-methyl-1-oxobutan-2-ylamino)-2-oxoethoxy)phenoxy)acetic Acid (**61**).* The *tert*-butyl protective group of **60** was removed by the general method described above. The product was further dissolved in 50% acetonitrile in water solution and lyophilized to afford **61** as a white solid (152 mg, 88%). ^{1}H NMR (601 MHz, DMSO-d_6) δ 8.51 (d, J = 10.0 Hz, 1H), 8.03 (d, J = 8.6 Hz, 1H), 7.34−7.23 (m, 4H), 7.14 (d, J = 8.5 Hz, 2H), 7.09 (d, J = 8.6 Hz, 2H), 6.93−6.81 (m, 4H), 4.60 (s, 2H), 4.57−4.51 (m, 2H), 4.50 (s, 2H), 4.46−4.41 (m, 1H), 3.75−3.69 (m, 1H), 3.62−3.55 (m, 1H), 2.47 (s, 3H), 2.46 (s, 3H), 2.31−2.23 (m, 1H), 2.05−1.95 (m, 2H), 1.94−1.86 (m, 1H), 1.85−1.77 (m, 1H), 1.61−1.55 (m, 1H), 1.09 (d, J = 6.8 Hz, 3H), 1.03 (d, J = 6.8 Hz, 3H), 0.92 (d, J = 6.7 Hz, 3H), 0.84 (d, J = 6.7 Hz, 3H). ^{31}P NMR (243 MHz, DMSO-d_6) δ 18.94 (s). HRMS: calcd for $(C_{38}H_{48}N_3O_{10}PS_2)Na^+$, 824.2416; found, 824.2455.

*tert-Butyl 2-(3-(2-((S)-1-((S)-2-((R)-1-(Bis(4-(methylthio)phenoxy)-phosphoryl)-2-methylpropylcarbamoyl)pyrrolidin-1-yl)-3-methyl-1-oxobutan-2-ylamino)-2-oxoethyl)-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)acetate (**64**).* The TFA salt of **56** obtained after *N*-Boc deprotection under conditions described above (0.1 g, 0.14 mmol) was dissolved in acetonitrile (5 mL), and *N*,*N*′-diisopropylethylamine (69 μL 0.42 mmol) was added. Next, **50** (0.15 mmol, 44 mg) was added into the mixture which was followed by the addition of HBTU (64 mg, 0.17 mmol). The postreaction workup was performed as described for **58**, and purification by column chromatography on silica gel using 3% methanol in chloroform as the eluent yielded **64** as a colorless oil which formed a white solid foam under reduced pressure (36 mg, 29%). R_f = 0.06 ($CHCl_3$/MeOH, 30:1, v/v). ^{1}H NMR (601 MHz, DMSO-d_6) δ 12.92 (s, 1H), 8.44−8.56 (m, 2H), 7.66 (d, J = 7.8 Hz, 1H), 7.27−7.32 (m, 4H), 7.12 (d, J = 8.7 Hz, 2H), 7.10 (d, J = 8.6 Hz, 2H), 5.77 (d, J = 7.8 Hz, 1H), 4.45−4.60 (m, 4H), 4.38−4.45 (m, 2H), 4.32−4.38 (m, 1H), 3.66−3.72 (m, 1H), 3.54−3.59 (m, 1H), 2.47 (s, 3H), 2.46 (s, 3H), 2.21−2.30 (m, 1H), 1.91−2.02 (m, 2H), 1.87−1.91 (m, 1H), 1.75−1.87 (m, 1H), 1.54−1.62 (m, 1H), 1.40 (s, 9H), 1.09 (d, J = 6.8 Hz, 3H), 1.03 (d, J = 6.7 Hz, 3H), 0.93 (d, J = 6.8 Hz, 3H), 0.89 (d, J = 6.7 Hz, 3H). ^{31}P NMR (243 MHz, DMSO-d_6) δ 18.94 (s). HRMS: calcd for $(C_{40}H_{54}N_3O_{10}PS_2)Na^+$, 882.2947; found, 882.2968.

*2-(3-(2-((S)-1-((S)-2-((R)-1-(Bis(4-(methylthio)phenoxy)-phosphoryl)-2-methylpropylcarbamoyl)pyrrolidin-1-yl)-3-methyl-1-oxobutan-2-ylamino)-2-oxoethyl)-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)acetic Acid (**65**).* The *tert*-butyl protective group of **64** was removed by the general method described above. The product was further dissolved in 50% acetonitrile in water solution and lyophilized

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to afford **65** as a white solid (30 mg, 83%). ^{1}H NMR (601 MHz, DMSO-d_6) δ 12.92 (s, 1H), 8.45−8.54 (m, 2H), 7.67 (d, J = 7.9 Hz, 1H), 7.27−7.32 (m, 4H), 7.14 (d, J = 8.6 Hz, 2H), 7.10 (d, J = 8.4 Hz, 2H), 5.76 (d, J = 7.9 Hz, 1H), 4.47−4.58 (m, 4H), 4.40−4.47 (m, 2H), 4.34−4.40 (m, 1H), 3.66−3.71 (m, 1H), 3.54−3.60 (m, 1H), 2.47 (s, 3H), 2.46 (s, 3H), 2.23−2.31 (m, 1H), 1.93−2.02 (m, 2H), 1.85−1.93 (m, 1H), 1.77−1.85 (m, 1H), 1.54−1.60 (m, 1H), 1.09 (d, J = 6.8 Hz, 3H), 1.03 (d, J = 6.7 Hz, 3H), 0.93 (d, J = 6.8 Hz, 3H), 0.89 (d, J = 6.7 Hz, 3H). ^{31}P NMR (243 MHz, DMSO-d_6) δ 18.94 (s). HRMS: calcd for $(C_{36}H_{46}N_5O_{10}PS_2)H^+$, 804.25020; found, 804.24623.

*tert-Butyl 2-(3-(2-((S)-1-((S)-2-((R)-1-(Bis(4-(methylthio)phenoxy)-phosphoryl)-2-methylpropylcarbamoyl)pyrrolidin-1-yl)-3-methyl-1-oxobutan-2-ylamino)-2-oxoethyl)-5-methyl-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)acetate (**68**).* The TFA salt of **56** obtained after *N*-Boc deprotection under conditions described above (0.1 g, 0.14 mmol) was dissolved in acetonitrile (5 mL), and *N*,*N*′-diisopropylethylamine (69 μL 0.42 mmol) was added. Next, **51** (0.15 mmol, 46 mg) was added into the mixture followed by the addition of HBTU (64 mg, 0.17 mmol). The postreaction workup was performed as described for **58**, and purification by column chromatography on silica gel using 3% methanol in chloroform as the eluent yielded **68** as a colorless oil which formed a white solid foam under reduced pressure (51 mg, 41%). R_f = 0.12 ($CHCl_3$/MeOH, 30:1, v/v). ^{1}H NMR (601 MHz, DMSO-d_6) δ 12.78 (s, 1H), 8.33−8.44 (m, 2H), 7.47 (d, J = 1.3 Hz, 1H), 7.19−7.24 (m, 4H), 7.05 (d, J = 8.6 Hz, 2H), 6.99 (d, J = 8.3 Hz, 2H), 4.39−4.50 (m, 2H), 4.32−4.39 (m, 4H), 4.25−4.31 (m, 1H), 3.55−3.63 (m, 1H), 3.45−3.55 (m, 1H), 2.38 (s, 3H), 2.37 (s, 3H), 2.16−2.25 (m, 1H), 1.83−1.96 (m, 2H), 1.77−1.83 (m, 1H), 1.75 (s, 3H), 1.67−1.74 (m, 1H), 1.44−1.53 (m, 1H), 1.40 (s, 9H), 1.00 (d, J = 6.9 Hz, 3H), 0.94 (d, J = 6.9 Hz, 3H), 0.84 (d, J = 6.8 Hz, 3H), 0.80 (d, J = 6.7 Hz, 3H). ^{31}P NMR (243 MHz, DMSO-d_6) δ 18.94 (s). HRMS: calcd for $(C_{41}H_{56}N_5O_{10}PS_2)Na^+$, 896.3104; found, 896.3075.

*2-(3-(2-((S)-1-((S)-2-((R)-1-(Bis(4-(methylthio)phenoxy)-phosphoryl)-2-methylpropylcarbamoyl)pyrrolidin-1-yl)-3-methyl-1-oxobutan-2-ylamino)-2-oxoethyl)-5-methyl-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)acetic Acid (**69**).* The *tert*-butyl protective group of **68** was removed by the general method described above. The product was further dissolved in 50% acetonitrile solution in water and lyophilized to afford **69** as a white solid (42 mg, 83%). ^{1}H NMR (601 MHz, DMSO-d_6) δ 12.78 (s, 1H), 8.36−8.44 (m, 2H), 7.48 (d, J = 1.1 Hz, 1H), 7.18−7.24 (m, 4H), 7.05 (d, J = 8.9 Hz, 2H), 7.01 (d, J = 8.3 Hz, 2H), 4.38−4.48 (m, 2H), 4.34−4.38 (m, 4H), 4.25−4.30 (m, 1H), 3.56−3.63 (m, 1H), 3.45−3.52 (m, 1H), 2.38 (s, 3H), 2.37 (s, 3H), 2.14−2.23 (m, 1H), 1.84−1.94 (m, 2H), 1.78−1.84 (m, 1H), 1.74 (s, 3H), 1.69−1.73 (m, 1H), 1.45−1.51 (m, 1H), 1.00 (d, J = 6.8 Hz, 3H), 0.94 (d, J = 6.7 Hz, 3H), 0.84 (d, J = 6.7 Hz, 3H), 0.80 (d, J = 6.7 Hz, 3H). ^{31}P NMR (243 MHz, DMSO-d_6) δ 18.94 (s). HRMS: calcd for $(C_{37}H_{48}N_5O_{10}PS_2)H^+$, 818.26585; found, 818.26387.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis and analytical data of all intermediates and final compounds not described in the Experimental Section. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

1-AAP, 1-aminoalkylphosphonate; Aaa, amino acid residue; Aib, aminoisobutyric acid; Abu, aminobutyric acid; AFC, 7-amino-4-trifluoromethylcoumarin; AMC, 7-amino-4-methylcoumarin; ARDS, acute respiratory distress syndrome; CMK, chloromethyl ketone; COPD, chronic obstructive pulmonary disease; HNE, human neutrophil elastase; DIPEA, *N*,*N*′-diisopropylethylamine; α_1-PI, α1 proteinase inhibitor; SLPI, secretory leucocyte peptidase inhibitor; α_2-MG, α2 macroglobulin; PPE, porcine pancreatic elastase; Suc, succinyl group

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