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Development of a highly water-soluble peptide-based human neutrophil elastase inhibitor; AE-3763 for treatment of acute organ injury

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ABSTRACT

A series of peptide-based transition-state human neutrophil elastase (HNE) inhibitors with N-terminal acidic moieties were synthesized and their inhibitory activity against HNE was evaluated both in vitro and in vivo. Our results show that compounds containing cyclic amide bridged acidic moieties at the N-terminal have not only improved water solubility but also high in vivo potency. Among these compounds, **AE-3763** showed remarkable efficacy in hamster models of elastase-induced lung hemorrhage and lipopolysaccharide (LPS)-induced lung injury as well as in a mouse model of LPS/galactosamine-induced acute multiple organ dysfunctions. The water solubility of **AE-3763** (>1000 mg/ml in H_2O) was also far superior to that of any of the other compounds synthesized. Thus, it is believed that **AE-3763** would be useful for treatment of HNE-associated respiratory disorders, such as acute respiratory distress syndrome (ARDS), acute lung injury (ALI), and acute exacerbation of chronic obstructive pulmonary disease (COPD).

1. Introduction

Human neutrophil elastase (HNE, EC 3.4.21.37), a neutral serine protease with broad substrate specificity, is a major constituent in the azurophilic granules of human neutrophils. It is one of the many proteolytic enzymes released to combat invading foreign bodies during inflammation.[1,2] HNE has potent catalytic activity to hydrolyze a variety of extracellular matrix proteins, including elastin, which plays a major role in lung elasticity and proteolytic resistance. Under physiological conditions, organs are protected from HNE elastolytic activity by endogenous inhibitors, such as α_1-protease inhibitor, α_2-macrogloblin, and secretory leukocyte protease inhibitor. However, in the course of a pathological condition, such as emphysema, cystic fibrosis, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), or acute lung injury (ALI), the balance between HNE and its endogenous inhibitors is displaced in favor of the enzyme.[3] Such unbalance leads to massive infiltration of neutrophils into the lungs and other organs and subsequently tissue injury.

Although numerous HNE inhibitors, including peptidic and non-peptidic compounds, have been reported with some studied at clinical level, sivelestat[4] is so far the only marketed agent (Japan and Korea only) for the treatment of ALI associated with systemic inflammatory response syndrome (SIRS). Generally, treatment of acute destructive diseases requires the use of parenteral drugs as patients are usually in a critical condition. Therefore, high solubility and good stability in aqueous solution are essential physicochemical properties required for any potential HNE inhibitor. We have previously reported peptide-based carboxylic acid-containing transition-state inhibitors of HNE and have shown that the presence of both a benzene ring and an amide bond at the N-terminal position are preferred for good HNE inhibitory activity.[5] On the basis of these findings, exchange of the lipophilic benzene ring with a hydrophilic ring was further studied with the aim of improving compounds water solubility. In this study, we first replaced the aromatic group at the N-terminal of the parent compound **14a**[5] with various heterocyclic aliphatic rings having an acidic residue with hydrophilic five- or six-membered cyclic amides as water-soluble group (WG). Next, we examined the effects of substitution of the P1′ carbonyl-activating group (CAG) at the C-terminal and evaluated various combination of WG and CAG to optimize compounds efficacy/solubility balance. Herein, we describe the SAR of the synthesized compounds and the development of a highly water-soluble HNE inhibitor **14v** (**AE-3763**) from the lead compound **14a** (Fig. 1).

2. Chemistry

Synthesis of the key intermediates **5** and **8** is illustrated in Schemes 1 and 2, respectively. The cyanohydrin **1**[6] was converted to the tripeptides **5** in five steps as shown in Scheme 1. In brief, the

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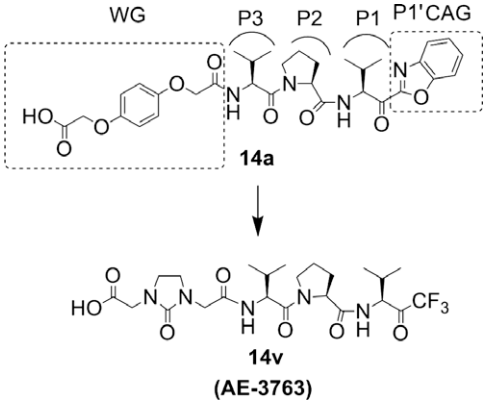
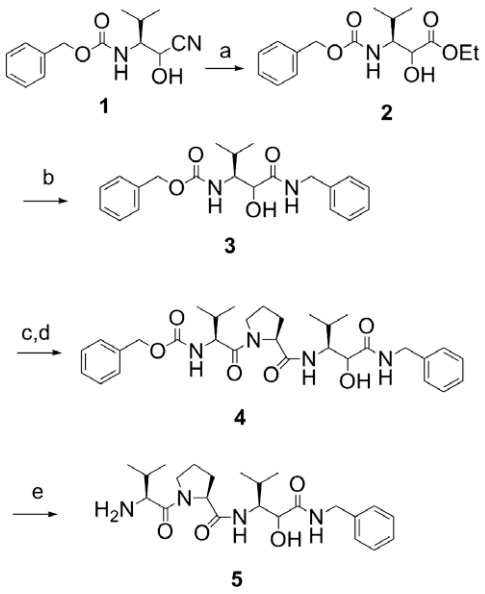
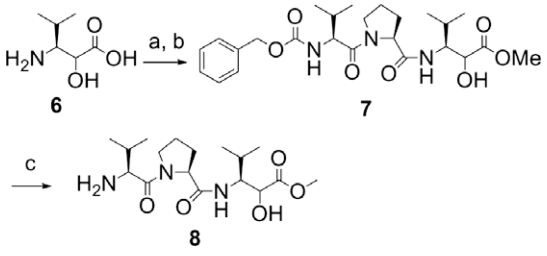


Figure 1. Lead compound (**14a**) to **14v** (**AE-3763**).



Scheme 1. Reagents: (a) HCl, EtOH; (b) $PhCH_2NH_2$, $LiAlH_4$; (c) H_2, $Pd(OH)_2$-C; (d) Z-Val-Pro-OH, EDC; (e) H_2, $Pd(OH)_2$-C.



Scheme 2. Reagents: (a) MeOH, HCl; (b) Z-Val-Pro-OH, EDC; (c) H2, $Pd(OH)_2$-C.

cyanohydrin **1** was directly transformed into the ethyl ester **2** by treatment with dry ethanolic hydrogen chloride followed by in situ hydrolysis of the intermediate imidate hydrochloride. Aminolysis[7] of the ethyl ester **2** gave the amide **3**, which was hydrogenated and then condensed with Z-Val-Pro-OH to give the Z-tripeptides **4**. Deprotection of the Z-group in **4** in a usual manner afforded the tripeptides **5**.

The α-hydroxyl-β-amino acid **6**[8] was converted to the tripeptides **8** in three steps as shown in Scheme 2. First, the diastereomeric mixture of α-hydroxyl-β-amino acid **6** was esterified with HCl/MeOH and then condensed with Z-Val-Pro-OH to afford the Z-tripeptides **7**. Deprotection of the Z-group in **7** in a usual manner afforded the tripeptides **8**. Similar tripeptides, that is, **9a** (2*S*,3*S*-isomer),[9] **9b** (*RS*-mixture at 2- and 3-position)[10] and **10**,[6,11] were also synthesized by the reported methods (Fig. 2).

Scheme 3 shows the final three steps in the synthesis of the target compounds **14a**–**w**. The synthetic methods and physical data for the half esters **11b**–**o** have already been described previously.[9] After coupling of the tripeptides (**5**, **8**, **9a**, **9b**, and **10**) and the half esters **11a**–**o**, **11u**[12] using EDC, oxidation of the secondary hydroxyl group with Dess–Martin periodinane reagent (DMP) yielded the ketones **13a**–**w**. Finally, TFA cleavage of the *tert*-butyl ester provided the target compounds **14a**–**w**.

3. Results and discussion

Firstly, several N-terminal acidic compounds appended with a benzoxazole ketone moiety as P1′ CAG were synthesized, and the in vivo efficacy of each compound was evaluated by iv administration in a lung hemorrhage hamster model produced by treatment with HNE. As shown in Table 1, replacement of the benzene ring of the starting compound **14a** with a six-membered heterocyclic amide (compound **14b**) decreased the in vitro inhibitory activity for HNE, however, iv bolus administration of the same compound resulted in an increase in the in vivo efficacy. Furthermore, water solubility of compound **14b** (>100 mg/ml at pH 7) was superior to that of compound **14a**.

Based on these results, various compounds containing a cyclic amide moiety (**14c–o**) were synthesized, and both their in vitro inhibitory activity for HNE and in vivo efficacy in the hamster model were evaluated. Most compounds showed in vitro inhibitory activity and in vivo efficacy similar to those of compound **14b**. For example, the position of carbonyl group in the cyclic amide moiety was tolerable (**14b** vs **14c**, **14h** vs **14i**, **14j** vs **14k**, **14m** vs **14n**). Decrease of ring size in the heterocyclic amides from 6 to 5 (**14e** vs **14i**) or deletion of the carbonyl group (**14h** and **14i** vs **14l**) did not affect the in vitro inhibitory activity. These results suggest that the cyclic amide portion does not bind to human neutrophil elastase tightly, although similar physicochemical properties may contribute to favorable in vivo efficacy.

As drug infusion is considered to be the most favorable route of administration for patients with acute respiratory disorders, we examined the in vivo efficacy of compounds that showed over 60% inhibition at 10 mg/kg by iv bolus administration (**14b**, **14d**, **14h–l**, and **14l–o**) following iv infusion for over 70 min in the lung hemorrhage hamster model produced by treatment with HNE. As expected, some compounds showed better efficacy by iv infusion than by iv bolus administration, especially compounds **14b** and **14m**, which exhibited prominent efficacy at a low dose (over 50% inhibition at 1 mg/kg/h).

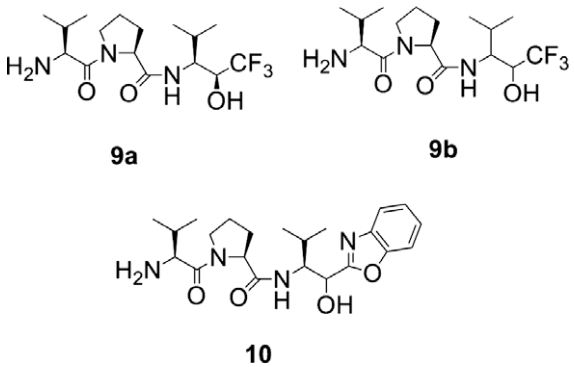
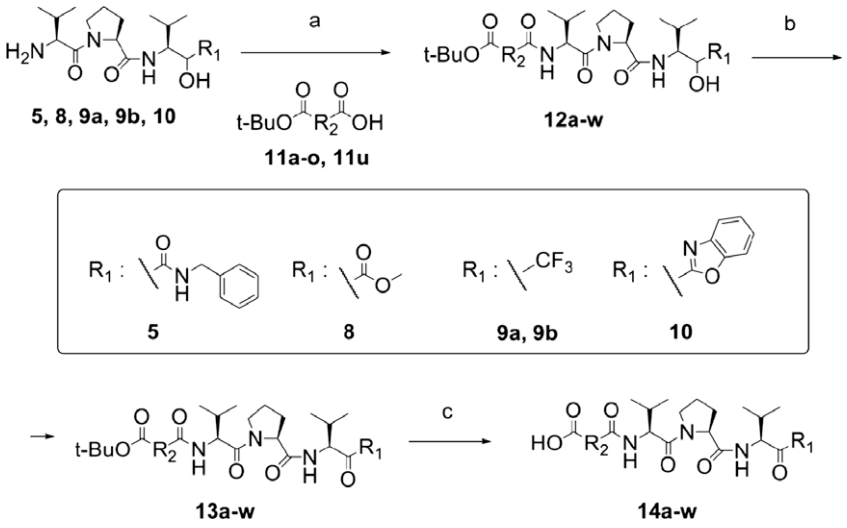


Figure 2. Tripeptides **9a**, **9b**, and **10**.



Scheme 3. Reagents: (a) EDC; (b) DMP, *t*-BuOH; (c) TFA.

Next, the effects of substitution of P1′ CAG, which had been reported[10,13] as an important group in the development of various serine protease inhibitors, were examined (Table 2). Compounds in vivo efficacy was determined following iv infusion to the earlier mentioned hamster models. The in vitro inhibitory activity of the diketoester derivatives **14r** and **14s** for HNE was superior to that of other compounds, although their in vivo efficacy was not much different from that of other compounds. This discrepancy is probably due to the fact the diketoester moiety is metabolically less stable. On the other hand, deletion of the acetic acid moiety in **14t** led to a loss of inhibitory activity against HNE (compound **14u**, Table 2). This indicates that the N-terminal carboxylic acid is indispensable not only for increasing water solubility but also for HNE inhibitory activity. In addition, conversion of the stereochemistry at the P1 position in **14v** (**AE-3763**) gave complete loss of in vitro inhibitory activity (compound **14w**); presumably because the conformational requirement of the isopropyl group at the P1 position in **AE-3763** for binding to HNE is crucial to the inhibitory activity. Preliminary study on stability has indicated that a compound having a trifluoromethylketone group was more stable than that having α-ketobenzoxazole group. In the case of **AE-3763**, which has a trifluoromethylketone at the C-terminal, generation of the epimer was less than 1% after 24 h incubation in phosphate buffer solution (pH 5.5) at 25 °C. In addition, it has also been reported that placement of a large lipophilic group in the P1′ position increases the in vitro inhibitory activity, although small P1′ substituents are preferable to large lipophilic groups in order to maintain good aqueous solubility.[14] In fact, the water solubility of **AE-3763** (>1000 mg/ml in H_2O) was far superior to that of other compounds. Thus, **AE-3763**, which is characterized by high solubility and stability in water, was chosen as candidate for further therapeutic evaluation in the treatment of other destructive acute diseases associated with excess HNE.

Edema and leukocytes infiltration into the lung, induced by LPS administration in hamsters, were significantly inhibited by infusion of **AE-3763** (Fig. 3). The lowest doses of **AE-3763** that produced statistically significant inhibition were 3 mg/kg/h (lung edema), and 1.5 mg/kg/h (leukocyte infiltration). These inhibitory effects were also observed when **AE-3763** administration was started after the establishment of the disease. Furthermore, **AE-3763** significantly improved survival rate by 24 h in a mouse model of fatal shock associated with multiple organ dysfunction induced by LPS/D-galactosamine administration (Fig. 4). In the lung hemorrhage hamster model produced by treatment with HNE, **AE-3763** dose-dependently prevented hemorrhage when given intravenously by infusion (ED_{50}: 0.42 mg/kg/h) or by bolus injection (1.2 mg/kg). With regard to the toxicity of **AE-3763** in mice, the results of a preliminary study have shown no overt toxic effect even at the high dose of 300 mg/kg, iv. These results suggest that **AE-3763** would be of therapeutic value in the treatment of excess HNE-associated destructive acute diseases, such as ARDS, septic shock, and acute organ injury.

4. Conclusion

A series of peptide-based transition-state inhibitors of HNE with a carboxylic acid group at the N-terminal were synthesized and their pharmacological activity and solubility in water were evaluated. Marked in vitro and in vivo HNE inhibitory activities as well as high water solubility were observed in several compounds possessing a carboxylic acid group with an aliphatic cyclic amide moiety at the N-terminal (e.g., compound **14b**). Optimization of the cyclic amide part at the N-terminal and then the P1′ CAG at the C-terminal led to the discovery of **AE-3763**, which exhibited potent in vitro inhibitory activity against HNE as well as extremely high solubility and stability in water. Pharmacological and toxicological evaluation of in vivo **AE-3763** revealed that this compound would be therapeutically useful in the treatment of destructive acute diseases associated with excess HNE.

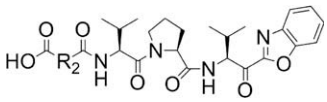
5. Experimental

5.1. Chemistry

5.1.1. General methods

Unless otherwise noted, reagents were obtained from commercial suppliers and used without further purification. Melting points were determined on a cover glass with an electrothermal melting point apparatus and are given as uncorrected values. ^{1}H NMR spectra were recorded on a JNMLA300 (JEOL, 300 MHz) and chemical shifts are expressed in ppm downfield from internal TMS. Mass spectra were recorded with electron spray ionization (ESI) or atmospheric pressure chemical ionization (APCI) and liquid secondary ion (LSI) on LTQ Orbitrap Discovery (Thermo Fisher Scientific) or M-1000 (Hitachi). Optical rotation was measured with P-1020 (JASCO). Silica gel column chromatography was performed on a Silica Gel 60 (70–230 mesh, Merck). Reactions requiring anhydrous

Table 1
Effects of modification in the N-terminal WG (R_2) on HNE inhibitory activity



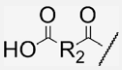
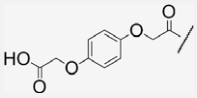
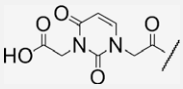
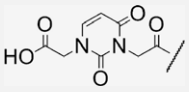
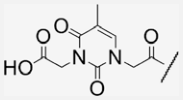
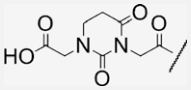
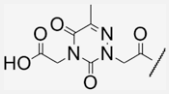
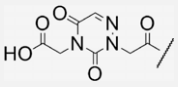
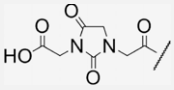
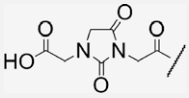
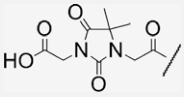
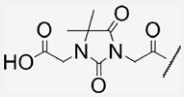
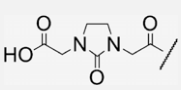
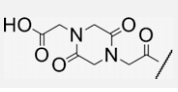
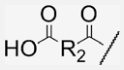
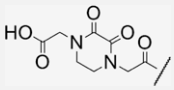
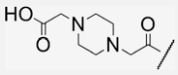
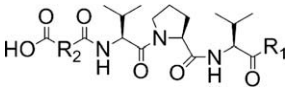
Compd[a]		IC_{50}[b] (nM)	% Inhibition after iv administration[c]					
			Bolus (mg/kg)			Infusion (mg/kg/h)		
			30	10	3	10	3	1
14a		7.6	56	38				
14b		24	97	66	24	93	62	50
14c		27	94	47	34			
14d		14	92	70	39	56	38	33
14e		33		34	38			
14f		27		48				
14g		43		33	14			
14h		42	91	67	32	78	44	−7
14i		35	81	72	37	53	57	39
14j		47		42	40			
14k		23		59	20			
14l		17		68	22	90	31	25
14m		62		67	20	90	73	55

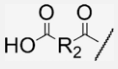
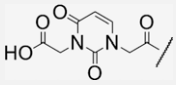
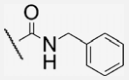
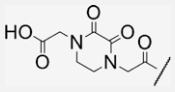
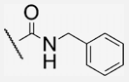
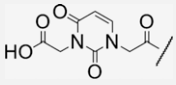
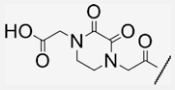
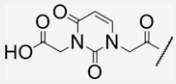
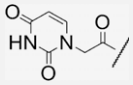
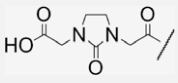
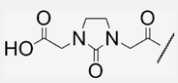
Table 1 (*continued*)

Compd[a]		IC_{50}[b] (nM)	% Inhibition after iv administration[c]					
			Bolus (mg/kg)			Infusion (mg/kg/h)		
			30	10	3	10	3	1
14n		30		88	57	95	76	41
14o		62		70	44	87	48	34

[a] All compounds had more than 90% of the diastereomers in the *S*-configuration at the P1 position.
[b] Inhibition of HNE-catalyzed hydrolysis of the synthetic substrate Suc-Ala-Pro-Ala-MCA.[2]
[c] Inhibition of HNE-induced lung hemorrhage in hamsters. See the Section 5 for further details.[4]

Table 2
Effects of modification in the N-terminal WG (R_2) and the C-terminal CAG (R_1) on HNE inhibitory activity



Compd[a]		R_1	IC_{50}[b] (nM)	% Inhibition after iv administration[c]		
				Infusion (mg/kg/h)		
				10	3	1
14p			21	90	78	32
14q			20	89	47	31
14r			6.6	94	43	32
14s			6.6	94	48	
14t			28	79	7.6	−1.2
14u			1000			
14v (AE-3763)			29	97	84	45
14w		*R*-Configuration of **14v** at P1 position	>1000			

[a] All compounds except for **14t**, **14u**, and **14w** had more than 90% of diastereomers in the *S*-configuration at the P1 position.
[b,c]See footnotes in Table 1.

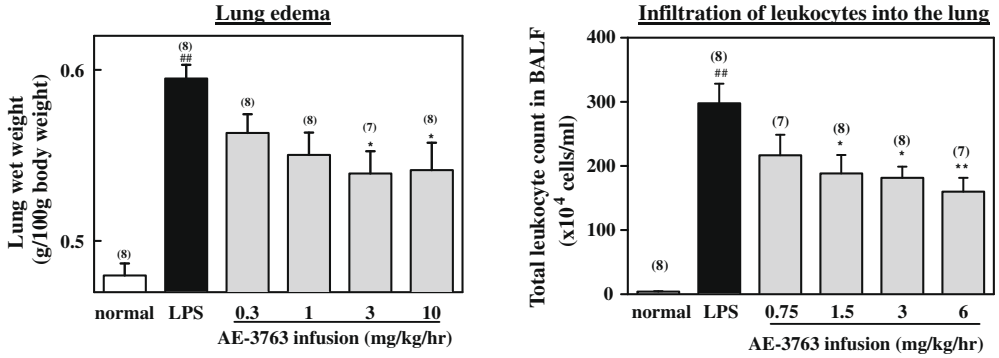


Figure 3. Effects of **AE-3763** on LPS-induced lung injury in hamsters. The results are shown as means ± SEM (n = 7–8) $^{\#\#}p$ <0.01, significantly different from the control group (edema: Student's t-test; total cell count: Welch test) $^{*}p$ <0.05, $^{**}p$ <0.01, significantly different from LPS group (Dunnett's multiple comparison test). See Section 5 for further details.

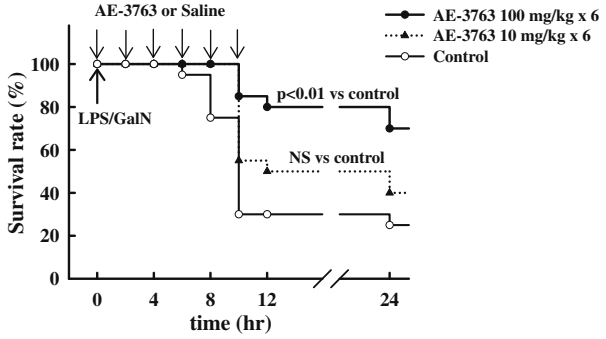


Figure 4. Effects of **AE-3763** on LPS/galactosamine-induced shock in mice Log-Rank test was used to test statistical differences in survival rate between the control group and each **AE-3763**-treated group. See Section 5 for further details.

conditions were performed under argon atmosphere. Solutions were evaporated under reduced pressure on a rotary evaporator. Synthetic yields of all compounds were not optimized. The following abbreviations are used: NaCl, sodium chloride; HCl, hydrochloric acid; $MgSO_4$, anhydrous magnesium sulfate; $NaHCO_3$, sodium hydrogen carbonate; K_2CO_3, potassium carbonate; THF, tetrahydrofuran; DMF, *N,N*-dimethylformamide; CH_2Cl_2, methylene chloride; $CHCl_3$, chloroform; DMSO, dimethyl sulfoxide; MeOH, methanol; EtOH, ethanol; AcOEt, ethyl acetate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; $CDCl_3$, chloroform-*d*; TFA, trifluoroacetic acid; Et_3N, triethylamine.

5.1.2. (3*S*)-3-Benzyloxycarbonylamino-2-hydroxy-4-methylbutyric acid ethyl ester (2)

To (3*S*)-3-benzyloxycarbonylamino-2-hydroxy-4-methylpentanenitrile **1** (62.0 g) was added 30% HCl in EtOH (500 ml) at 0 °C, and the mixture was stirred at room temperature for 15 h. The reaction mixture was then concentrated, and to the resulting residue was added THF (250 ml) and water (250 ml), and the mixture was stirred at room temperature for 30 min. The reaction mixture was concentrated, poured into water and extracted with AcOEt. The combined organic layer was washed with saturated $NaHCO_3$, brine, dried over $MgSO_4$, and evaporated in vacuo. The residue was purified by silica gel column chromatography [solvent; *n*-hexane–AcOEt (7:3)] to give the desired **2** (40.0 g, 55%) as colorless oil: MS (LSI, positive) *m*/*z* 310 [(M+H)$^+$]; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.92–1.05 (6H, m), 1.21–1.30 (3H, m), 1.84–1.93 (1H, m), 3.01–3.10 (1H, m), 3.76–3.90 (1H, m), 4.14–4.36 (3H, m), 4.96–5.16 (3H, m), 7.26–7.38 (5H, m).

5.1.3. (1*S*)-3-Benzylamino-1-benzyloxycarbonylamino-1-isopropyl-2-hydroxy-3-oxopropane (3)

A suspension containing lithium aluminum hydride[7] (1.2 g) and THF (200 ml) was stirred under reflux for 1.5 h. The reaction solution was then cooled to room temperature, and thereto was added dropwise benzylamine (17.3 g). To this mixture was added dropwise THF (150 ml) containing compound **2** (10.0 g), obtained in the above step, and the new mixture was stirred at room temperature for 12 h. Water was carefully added to the reaction solution, and the mixture was extracted with AcOEt. The extract was dried over $MgSO_4$, and the solvent was removed by evaporation under reduced pressure. The residue was purified by silica gel column chromatography [solvent; *n*-hexane–AcOEt (1:1)] to give the desired **3** (9.0 g, 75%) as colorless oil: MS (LSI, positive) *m*/*z* 371 [(M+H)$^+$]; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.97 (3H, d), 1.04 (3H, d), 2.28 (1H, m), 3.45 (1H, br t), 4.29–4.49 (4H, m), 5.02 (2H, dd), 5.13 (1H, d), 5.43 (1H, d), 7.07 (1H, m), 7.21–7.38 (10H, m).

5.1.4. *N*-Benzyloxycarbonyl-L-valyl-*N*-[(1*S*)-3-benzylamino-2-hydroxy-1-isopropyl-3-oxopropyl]-L-prolinamide (4)

To EtOH (200 ml) containing compound **3** (9.0 g), obtained in the above step, was added a catalytic amount of 20% palladium hydroxide, and the mixture was subjected to hydrogenolysis at room temperature. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure. To the remaining oil was added CH_2Cl_2 (200 ml) and then *N*-(benzyloxycarbonyl)-L-valyl-L-proline (10.2 g) and EDC (5.6 g). The mixture was stirred at room temperature for 15 h and concentrated under reduced pressure at room temperature. To the residue was added AcOEt, and the mixture was washed successively with 1.0 N HCl, saturated $NaHCO_3$, and brine, and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure, and the residue was purified by silica gel column chromatography [solvent; $CHCl_3$–MeOH (100:1)] to give the desired **4** (4.6 g, 33%) as colorless oil: MS (LSI, positive) *m*/*z* 567 [(M+H)$^+$]; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.79–1.06 (12H, m), 1.77–2.35 (5H, m), 3.56 (1H, m), 3.73 (1H, m), 4.23–4.45 (4H, m), 5.06 (1H, d), 5.11 (1H, d), 5.48 (1H, br t), 7.17–7.42 (11H, m).

5.1.5. L-Valyl-*N*-[(1*S*)-(3-benzylamino-1-isopropyl-2-hydroxy-3-oxo-propyl)-L-prolinamide (5)

To a solution of compound **4** (4.6 g), obtained in the above step, in EtOH (100 ml) was added a catalytic amount of 20% palladium hydroxide on carbon, and the mixture was stirred at room temperature under hydrogen current for 2 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired **5** (3.5 g, quant.) as colorless oil. MS (LSI, positive) *m*/*z* 433 [(M+H)$^+$]. This product was used as starting compound in the next step without further purification.

5.1.6. *N*-Benzyloxycarbonyl-L-valyl-*N*-[(1*S*)-3-methoxy-2-hydroxy-1-isopropyl-3-oxopropyl]-L-prolinamide (7)

To (3*S*)-3-amino-2-hydroxy-4-methylpentanoic acid hydrochloride salt **6** (1.0 g) was added 10% HCl in MeOH (30 ml) at 0 °C, and the mixture was stirred at room temperature for 16.5 h. The reaction mixture was then concentrated under reduced pressure. To the remaining oil was added pyridine (20 ml) and then *N*-(benzyloxycarbonyl)-L-valyl-L-proline (1.9 g) and EDC (1.6 g). The mixture was stirred at room temperature for 15 h and concentrated under reduced pressure at room temperature. To the residue was added AcOEt, and the mixture was washed successively with 10% HCl, saturated $NaHCO_3$, and brine, and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure, and the residue was purified by silica gel column chromatography [solvent; $CHCl_3$–MeOH (50:1→20:1)] to give the desired **7** (0.64 g, 24%) as foam: MS (LSI, positive) *m*/*z* 492 [(M+H)$^+$]; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.89–1.03 (12H, m), 1.81–2.35 (6H, m), 3.38–4.57 (10H, m), 5.02–5.13 (2H, m), 5.61 (0.5H, d), 5.74 (0.5H, d), 6.82 (0.5H, d), 6.99 (0.5H, d), 7.34 (5H, m).

5.1.7. L-Valyl-*N*-[(1*S*)-(3-benzylamino-1-isopropyl-2-hydroxy-3-oxo-propyl)-L-prolinamide (8)

To a solution of compound **7** (0.3 g), obtained in the above step, in AcOEt (30 ml) was added a catalytic amount of 20% palladium hydroxide on carbon, and the mixture was stirred at room temperature under hydrogen current for 4 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired **8** (0.19 g, 90%) as colorless oil: MS (LSI, positive) *m*/*z* 358 [(M+H)$^+$]. This product was used as starting compound in the next step without further purification.

5.1.8. Typical procedure for the synthesis of half esters: [4-(2-*tert*-butoxy-2-oxoethoxy)phenoxy]acetic acid (11a)

5.1.8.1. Benzyl *tert*-butyl 2,2′-[benzene-1,4-diylbis(oxy)]diacetate. To anhydrous DMF (200 ml) containing *tert*-butyl(4-hydroxyphenoxy)acetate[15] (26.0 g) were added benzyl bromoacetate (32.0 g) and K_2CO_3 (32.0 g) at room temperature, and the mixture was stirred at room temperature for 15 h. The reaction solution was then poured into water, and the mixture was extracted with AcOEt. The extract was washed with brine and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure, and the residue was purified by silica gel column chromatography [solvent; *n*-hexane–AcOEt (8:1)] to give the desired compound (40.4 g, 94%) as colorless oil: MS (LSI, positive) *m*/*z* 373 [(M+H)$^+$].

5.1.8.2. [4-(2-*tert*-Butoxy-2-oxoethoxy)phenoxy]acetic acid (11a). To AcOEt (150 ml) containing benzyl *tert*-butyl 2,2′-[benzene-1,4-diylbis(oxy)]diacetate (40.4 g) obtained in the above step was added 20% palladium hydroxide on carbon (4.04 g), and the mixture was stirred at room temperature under hydrogen atmosphere for 1 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired **11a** (30.0 g, 98%) as colorless powder: Mp 95–97 °C; MS (LSI, positive) *m*/*z* 283 [(M+H)$^+$]; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 1.42 (9H, s), 4.56 (2H, S), 4.59 (2H, s), 6.83 (4H, s), 12.93 (1H, s).

5.1.9. Typical procedure for the synthesis of half esters: [3-(2-*tert*-butoxy-2-oxoethyl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl]-acetic acid (11b)

5.1.9.1. Benzyl (2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetate. To anhydrous DMF (10 ml) containing 2,4-dioxopyrimidine (1.0 g) were added benzyl bromoacetate (2.5 g) and K_2CO_3 (2.5 g), and the mixture was stirred at room temperature for 15 h. The reaction solution was poured into water, and the mixture was extracted with AcOEt. The extract was washed with brine and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure, and the precipitated crystals were washed with diethyl ether to give the desired compound (1.4 g, 60%) as colorless crystals: Mp 192–194 °C ; MS (LSI, positive) *m*/*z* 261 [(M+H)$^+$]; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 4.59 (2H, s), 5.20 (2H, s), 5.62 (1H, d), 7.37 (5H, m), 7.65 (1H, d), 11.4 (1H, s).

5.1.9.2. Benzyl *tert*-butyl 2,2′-(2,4-dioxopyrimidine-1,3(2H,4H)-diyl)diacetate. To anhydrous DMF (10 ml) containing benzyl (2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetate (1.0 g) obtained in the above step was gradually added sodium hydride (purity; 60%, 0.18 g) under ice-cooling, and the mixture was stirred under ice-cooling for 15 min. To this mixture was added *tert*-butyl bromoacetate (0.9 g), and the mixture was stirred at room temperature for 1 h. Saturated aqueous ammonia chloride solution was added to the reaction mixture, and the mixture was extracted with AcOEt. The extract was washed with brine dried over $MgSO_4$, and the solvent was removed by evaporation under reduced pressure. The residue was purified by silica gel column chromatography [solvent; *n*-hexane–AcOEt (2:1→1:1)] to give the desired compound (1.2 g, 84%) as colorless oil: MS (LSI, positive) *m*/*z* 375 [(M+H)$^+$]; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.46 (9H, s), 4.51 (2H, s), 4.58 (2H, s), 5.21 (2H, s), 5.81 (1H, d), 7.10 (1H, d), 7.36 (5H, m).

5.1.9.3. [3-(2-*tert*-Butoxy-2-oxoethyl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl]acetic acid (11b). To AcOEt (20 ml) containing benzyl *tert*-butyl 2,2′-(2,4-dioxopyrimidine-1,3(2H,4H)-diyl)diacetate (1.2 g) obtained in the above step was added 20% palladium hydroxide on carbon (50 mg), and the mixture was stirred at room temperature under hydrogen atmosphere for 1 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired **11b** (0.7 g, 77%) as colorless powder: MS (LSI, positive) *m*/*z* 285 [(M+H)$^+$]; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.46 (9H, s), 4.51 (2H, s), 4.59 (2H, s), 5.86 (1H, d), 7.17 (1H, d).

5.1.10. Typical procedure for the synthesis of half esters: [3-(2-*tert*-butoxy-2-oxoethyl)-2-oxoimidazolidin-1-yl]acetic acid (11l)

5.1.10.1. Di-*tert*-Butyl 2,2′-(2-oxoimidazolidine-1,3-diyl)diacetate. To anhydrous DMF (200 ml) containing 2-oxo-imidazolidine (10.0 g) and *tert*-butyl bromoacetate (50.7 g) was added lithium *tert*-butoxide (20.5 g) under ice-cooling, and the mixture was stirred for 1 h at room temperature. The reaction mixture was poured into ice, and the precipitated solids were washed with water and air-dried overnight. Crude solid was crystallized from AcOEt to give the desired compound (26.0 g, 69%) as white crystals: Mp 100–102 °C; MS (LSI, positive) *m*/*z* 315 [(M+H)$^+$]; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.46 (18H, s), 3.54 (4H, s), 3.89 (4H, s).

5.1.10.2. [3-(2-*tert*-Butoxy-2-oxoethyl)-2-oxoimidazolidin-1-yl]acetic acid (11l). To a mixture of EtOH and water in equal volume (20 ml) containing di-*tert*-butyl 2,2′-(2-oxoimidazolidine-1,3-diyl)diacetate (1.14 g) obtained in the above step was added potassium hydroxide (0.27 g), and the mixture was stirred at 70 °C for 5 h. EtOH was removed by evaporation under reduced pressure, and to the residue was added aqueous saturated $NaHCO_3$. The aqueous layer was washed with AcOEt, acidified with 10% HCl, and extracted with AcOEt. The extract was dried over $MgSO_4$, and the solvent was removed by evaporation under reduced pressure. The precipitated crystals were recrystallized from AcOEt to give the desired **11l** (0.30 g, 32%) as colorless crystals: Mp 112–113 °C; MS (LSI, positive) *m*/*z* 259 [(M+H)$^+$]; ^{1}H NMR (300 MHz,

$CDCl_3$, δ): 1.46 (9H, s), 3.53 (4H, s), 3.89 (2H, s), 4.01 (2H, s), 7.11 (1H, br s).

5.1.11. Typical procedure for the synthesis of AE-3763 (14v)

5.1.11.1. *N*-{[3-(2-*tert*-Butoxy-2-oxoethyl)-2-oxoimidazolidin-1-yl]acetyl}-L-valyl-*N*-[(2S,3S)-1,1,1-trifluoro-2-hydroxy-4-methylpentan-3-yl]-L-prolinamide (12v). To CH_2Cl_2 (1000 ml) containing the half ester **11l** (46.4 g) obtained in the above step and L-valyl-*N*-[(2*S*,3*S*)-1,1,1-trifluoro-2-hydroxy-4-methylpentan-3-yl]-L-prolinamide hydrochloride salt **9a** (72.0 g) were added EDC (41.4 g) and Et_3N (38.5 g), and the mixture was stirred at room temperature for 15 h and concentrated under reduced pressure at room temperature. To the residue was added AcOEt, and the mixture was washed successively with 1.0 N HCl, saturated $NaHCO_3$, and brine, and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure, and the residue was purified by silica gel column chromatography [solvent; $CHCl_3$–MeOH (50:1→30:1)] to give the desired **12v** (79.5 g, 73%) as colorless powder: MS (APCI, positive) *m*/*z* 608 [(M+H)$^+$]; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.77–0.89 (12H, m), 1.40 (9H, s), 1.66–2.11 (6H, m), 3.34 (4H, m), 3.53 (1H, m), 3.67–3.80 (6H, m), 3.99 (1H, ddd), 4.27–4.35 (2H, m), 6.37 (1H, d), 7.69 (1H, d), 8.07 (1H, d).

5.1.11.2. *N*-{[3-(2-*tert*-Butoxy-2-oxoethyl)-2-oxoimidazolidin-1-yl]acetyl}-L-valyl-*N*-[(3S)-1,1,1-trifluoro-4-methyl-2-oxopentan-3-yl]-L-prolinamide (13v). To CH_2Cl_2 (1000 ml) containing compound **12v** (79.5 g), obtained in the above step, was added Dess–Martin reagent (112.3 g), and the mixture was stirred at room temperature for 4 h and concentrated under reduced pressure. To the residue was added AcOEt, and the mixture was washed successively with saturated aqueous sodium thiosulfate solution, saturated $NaHCO_3$, and brine, and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure, and the residue was purified by silica gel column chromatography [solvent; $CHCl_3$–MeOH (100:3)] to give the desired **13v** (67.2 g, 85%) as colorless foam: MS (APCI, positive) *m*/*z* 606 [(M+H)$^+$]; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.83–1.02 (12H, m), 1.47 (9H, s), 1.89–2.32 (6H, m), 3.48–4.06 (10H, m), 4.58 (1H, dd), 4.64 (1H, dd), 4.84 (1H, dd), 7.35 (1H, d), 7.72 (1H, d).

5.1.11.3. *N*-{[3-(Carboxymethyl)-2-oxoimidazolidin-1-yl]acetyl}-L-valyl-*N*-[(3S)-1,1,1-trifluoro-4-methyl-2-oxopentan-3-yl]-L-prolinamide (AE-3763, 14v). To CH_2Cl_2 (200 ml) containing compound **13v** (34.9 g), obtained in the above step, was added TFA (200 ml) at room temperature, and the mixture was stirred at room temperature for 1 h and concentrated under reduced pressure. To the residue was added diisopropyl ether, and the precipitated crystals were collected by filtration, and recrystallized from AcOEt to give **AE-3763** (**14v**) (14.0 g, 44%) as colorless crystals. According to high performance liquid chromatography, the purity of the product was 98.71% and the percentage of isomer was 0.78%: Mp 177–178 °C; $[\alpha]_D^{20}$: −63.0 (*c* 1.0, $CHCl_3$); HRMS (ESI, negative) *m*/*z* calcd for $C_{23}H_{33}O_7N_5F_3$ [(M−H)$^-$] 548.2327, found 548.2329; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.85–1.02 (12H, m), 1.90–2.31 (6H, m), 3.51 (4H, m), 3.68 (1H, m), 3.81–4.13 (5H, m), 4.58 (2H, m), 4.92 (1H, dd), 7.45 (2H, m).

The same synthetic routes were used to synthesize compounds **14a**–**x** (Tables 1 and 2).

5.1.11.4. *N*-{[4-(Carboxymethoxy)phenoxy]acetyl}-L-valyl-*N*-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14a). Compound **14a** (3.2 g, 40.6%) was prepared from **10** and **11a** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{32}H_{37}O_9N_4$ [(M−H)$^-$] 621.2555, found 621.2565; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.90–1.13 (12H, m), 1.99–2.26 (5H, m), 2.50 (1H, m), 3.71 (1H, m), 3.94 (1H, m), 4.45–4.68 (6H, m), 5.61 (1H, dd), 6.77–6.87 (4H, m), 7.25 (1H, d), 7.47 (1H, ddd), 7.56 (1H, ddd), 7.66 (2H, d), 7.92 (1H, d).

5.1.11.5. *N*-{[3-(Carboxymethyl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl]acetyl}-L-valyl-*N*-[(2S)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14b). Compound **14b** (12.0 g, 61.2%) was prepared from **10** and **11b** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{30}H_{35}O_9N_6$ [(M−H)$^-$] 623.2460, found 623.2458; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.85–1.05 (12H, m), 1.70–2.05 (5H, m), 2.38 (1H, m), 3.55 (1H, m), 3.64 (1H, m), 4.32 (1H, dd), 4.40–4.55 (5H, m), 5.28 (1H, dd), 5.75 (1H, dd), 7.55 (1H, dd), 7.64 (2H, m), 7.90 (1H, d), 8.01 (1H, d), 8.45 (2H, m), 12.9 (1H, s).

5.1.11.6. *N*-{[3-(Carboxymethyl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl]acetyl}-L-valyl-*N*-[(2S)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14c). Compound **14c** (0.92 g, 41.6%) was prepared from **10** and **11c** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{30}H_{35}O_9N_6$ [(M−H)$^-$] 623.2460, found 623.2462; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.82–1.01 (12H, m), 1.71–2.05 (5H, m), 2.38 (1H, m), 3.53 (1H, m), 3.62 (1H, m), 4.29 (1H, dd), 4.41–4.52 (5H, m), 5.27 (1H, dd), 5.75 (1H, d), 7.55 (1H, ddd), 7.65 (1H, ddd), 7.72 (1H, d), 7.90 (1H, d), 8.02 (1H, d), 8.35 (1H, d), 8.45 (1H, d), 13.1 (1H, br s).

5.1.11.7. *N*-{[3-(Carboxymethyl)-5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl]acetyl}-L-valyl-*N*-[(2S)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14d). Compound **14d** (2.0 g, 68.1%) was prepared from **10** and **11d** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{31}H_{37}O_9N_6$ [(M−H)$^-$] 637.2617, found 637.2628; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.86–1.01 (12H, m), 1.68–2.04 (5H, m), 1.81 (3H, s), 2.38 (1H, m), 3.36–3.68 (6H, m), 4.31 (1H, dd), 4.50 (1H, dd), 5.28 (1H, dd), 7.53–7.68 (3H, m), 7.90 (1H, d), 8.02 (1H, d), 8.45 (2H, m), 12.9 (1H, br s).

5.1.11.8. *N*-{[3-(Carboxymethyl)-2,6-dioxotetrahydropyrimidin-1(2H)-yl]acetyl}-L-valyl-*N*-[(2S)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14e). Compound **14e** (1.7 g, 52.1%) was prepared from **10** and **11e** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{30}H_{37}O_9N_6$ [(M−H)$^-$] 625.2617, found 625.2624; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.82–1.00 (12H, m), 1.70–2.04 (5H, m), 2.38 (1H, m), 2.70 (2H, t), 3.46 (2H, t), 3.50 (1H, m), 3.63 (1H, m), 4.07 (2H, s), 4.25 (2H, s), 4.29 (1H, m), 4.51 (1H, dd), 5.27 (1H, dd), 7.55 (1H, dd), 7.65 (1H, dd), 7.90 (1H, d), 8.02 (1H, d), 8.19 (1H, d), 8.44 (1H, d), 12.8 (1H, s).

5.1.11.9. *N*-{[4-(Carboxymethyl)-6-methyl-3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3H)-yl]acetyl}-L-valyl-*N*-[(2S)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14f). Compound **14f** (1.25 g, 45.7%) was prepared from **10** and **11f** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{30}H_{36}O_9N_7$ [(M−H)$^-$] 638.2569, found 623.2586; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.85–1.01 (12H, m), 1.71–2.06 (5H, m), 2.15 (3H, s), 2.36–2.42 (1H, m), 3.48–3.66 (2H, m), 4.31 (1H, dd), 4.47 (2H, s), 4.52 (1H, dd), 4.60 (2H, s), 5.28 (1H, dd), 7.53–7.68 (2H, m), 7.96 (2H, dd), 8.46 (2H, m), 13.1 (1H, br s).

5.1.11.10. *N*-{[4-(Carboxymethyl)-3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3H)-yl]acetyl}-L-valyl-*N*-[(2S)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14g). Compound **14g** (0.50 g, 44.5%) was prepared from **10** and **11g** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{29}H_{34}O_9N_7$ [(M−H)$^-$] 624.2413, found 624.2413; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.83–1.00 (12H, m), 1.71–2.09 (5H, m), 2.39 (1H, m), 3.34–3.56 (14H, m), 3.69 (1H, m), 4.33 (1H, dd), 4.50 (1H, m), 5.29 (1H, m), 7.55 (1H, dd), 7.65 (1H, dd), 7.90 (1H, d), 8.02 (1H, d), 8.26 (1H, m), 8.43 (2H, m).

5.1.11.11. *N*-{[3-(Carboxymethyl)-2,4-dioxoimidazolidin-1-yl]acetyl}-L-valyl-*N*-[(2*S*)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14h). Compound **14h** (1.80 g, 65.8%) was prepared from **10** and **11h** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{29}H_{35}O_9N_6$ [(M−H)$^-$] 611.2460, found 611.2466; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.83–1.01 (12H, m), 1.72–2.09 (5H, m), 2.36–2.42 (1H, m), 3.51–3.74 (2H, m), 4.05–4.14 (6H, m), 4.34 (1H, t), 4.52 (1H, dd), 5.29 (1H, dd), 7.53–7.69 (2H, m), 7.96 (2H, dd), 8.37 (1H, d), 8.44 (1H, d), 13.0 (1H, br s).

5.1.11.12. *N*-{[3-(Carboxymethyl)-2,5-dioxoimidazolidin-1-yl]acetyl}-L-valyl-*N*-[(2*S*)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14i). Compound **14i** (1.24 g, 47.2%) was prepared from **10** and **11i** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{29}H_{35}O_9N_6$ [(M−H)$^-$] 611.2460, found 611.2475; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.84–1.01 (12H, m), 1.72–2.04 (5H, m), 2.35–2.40 (1H, m), 3.49–3.69 (2H, m), 4.04–4.12 (6H, m), 4.31 (1H, t), 4.52 (1H, dd), 5.26–5.30 (1H, m), 7.53–7.68 (2H, m), 7.96 (2H, dd), 8.42 (2H, m), 13.0 (1H, br s).

5.1.11.13. *N*-{[3-(Carboxymethyl)-5,5-dimethyl-2,4-dioxoimidazolidin-1-yl]acetyl}-L-valyl-*N*-[(2*S*)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14j). Compound **14j** (1.27 g, 31.0%) was prepared from **10** and **11j** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{31}H_{39}O_9N_6$ [(M−H)$^-$] 639.2773, found 639.2789; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.83–1.01 (12H, m), 1.29 (6H, s), 1.72–2.02 (4H, m), 2.33–2.44 (1H, m), 3.50–3.72 (2H, m), 3.99 (2H, s), 4.09 (2H, s), 4.35 (1H, dd), 4.50 (1H, dd), 5.27 (1H, dd), 7.53–7.68 (2H, m), 7.96 (2H, dd), 8.22 (1H, d), 8.44 (1H, d), 13.0 (1H, br s).

5.1.11.14. *N*-{[3-(Carboxymethyl)-4,4-dimethyl-2,5-dioxoimidazolidin-1-yl]acetyl}-L-valyl-*N*-[(2*S*)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14k). Compound **14k** (1.04 g, 32.1%) was prepared from **10** and **11k** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{31}H_{39}O_9N_6$ [(M−H)$^-$] 639.2773, found 639.2781; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.84–1.01 (12H, m), 1.31 (6H, s), 1.71–2.07 (5H, m), 2.34–2.42 (1H, m), 3.49–3.65 (2H, m), 4.04–4.06 (4H, m), 4.35 (1H, dd), 4.52 (1H, dd), 5.28 (1H, dd), 7.53–7.68 (2H, m), 7.96 (2H, dd), 8.36 (1H, d), 8.45 (1H, d), 12.8 (1H, br s).

5.1.11.15. *N*-{[3-(Carboxymethyl)-2-oxoimidazolidin-1-yl]acetyl}-L-valyl-*N*-[(2*S*)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14l). Compound **14l** (0.3 g, 41.3%) was prepared from **10** and **11l** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{29}H_{37}O_8N_6$ [(M−H)$^-$] 597.2667, found 597.2668; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.82–1.13 (12H, m), 1.88–2.28 (5H, m), 2.50 (1H, m), 3.25–3.74 (5H, m), 3.82–4.12 (5H, m), 4.54–4.71 (2H, m), 5.65 (1H, m), 7.35–7.59 (4H, m), 7.66 (1H, d), 7.91 (1H, d).

5.1.11.16. *N*-{[4-(Carboxymethyl)-2,5-dioxopiperazin-1-yl]acetyl}-L-valyl-*N*-[(2*S*)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14m). Compound **14m** (1.93 g, 70.0%) was prepared from **10** and **11m** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{30}H_{37}O_9N_6$ [(M−H)$^-$] 625.2617, found 625.2613; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.86–1.11 (12H, m), 1.91–2.25 (5H, m), 2.51 (1H, m), 3.19 (1H, br s), 3.69 (1H, m), 3.83 (1H, m), 4.00–4.31 (8H, m), 4.55–4.69 (2H, m), 5.65 (1H, dd), 7.36–7.59 (3H, m), 7.66 (2H, d), 7.91 (1H, d).

5.1.11.17. *N*-{[4-(Carboxymethyl)-2,3-dioxopiperazin-1-yl]acetyl}-L-valyl-*N*-[(2*S*)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14n). Compound **14n** (0.7 g, 30.0%) was prepared from **10** and **11n** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{30}H_{37}O_9N_6$ [(M−H)$^-$] 625.2617, found 625.2619; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.82–1.13 (12H, m), 1.90–2.73 (6H, m), 3.56–3.90 (6H, m), 4.13–4.36 (4H, m), 4.54–4.69 (2H, m), 5.64 (1H, dd), 7.39 (1H, br d), 7.47 (1H, t), 7.55 (1H, t), 7.66 (1H, d), 7.74 (1H, br d), 7.91 (1H, d).

5.1.11.18. *N*-{[4-(Carboxymethyl)piperazin-1-yl]acetyl}-L-valyl-*N*-[(2*S*)-1-(1,3-benzoxazol-2-yl)-3-methyl-1-oxobutan-2-yl]-L-prolinamide (14o). Compound **14o** (0.82 g, 20.6%) was prepared from **10** and **11o** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{30}H_{41}O_7N_6$ [(M−H)$^-$] 597.3037, found 597.3038; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.84–1.00 (12H, m), 1.71–2.06 (5H, m), 2.39 (1H, m), 3.29–4.03 (14H, m), 4.37 (1H, dd), 4.51 (1H, m), 5.28 (1H, dd), 7.56 (1H, dd), 7.66 (1H, dd), 7.91 (1H, d), 8.02 (1H, d), 8.47 (1H, m), 8.69 (1H, m).

5.1.11.19. *N*-{[3-(Carboxymethyl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl]acetyl}-L-valyl-*N*-[(3*S*)-1-(benzylamino)-4-methyl-1,2-dioxopentan-3-yl]-L-prolinamide (14p). Compound **14p** (3.0 g, 58.0%) was prepared from **5** and **11b** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{31}H_{39}O_9N_6$ [(M−H)$^-$] 639.2773, found 639.2775; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.75–1.06 (12H, m), 1.88–2.50 (6H, m), 3.65 (1H, m), 3.78 (1H, m), 4.35–4.69 (7H, m), 4.94 (1H, d), 5.82 (1H, d), 7.15 (1H, d), 7.18–7.40 (5H, m), 8.07 (1H, br s).

5.1.11.20. *N*-{[4-(Carboxymethyl)-2,3-dioxopiperazin-1-yl]acetyl}-L-valyl-*N*-[(3*S*)-1-(benzylamino)-4-methyl-1,2-dioxopentan-3-yl]-L-prolinamide (14q). Compound **14q** (0.65 g, 41%) was prepared from **5** and **11n** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{31}H_{41}O_9N_6$ [(M−H)$^-$] 641.2930, found 641.2947; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.73–1.00 (12H, m), 1.67–2.30 (6H, m), 4.00–4.20 (5H, m), 4.23–4.42 (4H, m), 4.49 (1H, m), 4.95 and 5.01 (1H, each dd), 8.16 (1H, d), 8.32 (1H, d), 9.24 (1H, t).

5.1.11.21. *N*-{[3-(Carboxymethyl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl]acetyl}-L-valyl-*N*-[(3*S*)-1-methoxy-4-methyl-1,2-dioxopentan-3-yl]-L-prolinamide (14r). Compound **14r** (43 mg, 16.3%) was prepared from **8** and **11b** as white powder: MS (APCI, positive) *m*/*z* 566 [(M+H)$^+$]; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.81–0.92 (12H, m), 1.69–2.27 (7H, m), 3.77 (3H, s), 4.31–4.61 (7H, m), 5.74 (1H, d), 7.66 (1H, d), 8.38–8.48 (2H, m).

5.1.11.22. *N*-{[4-(Carboxymethyl)-2,3-dioxopiperazin-1-yl]acetyl}-L-valyl-*N*-[(3*S*)-1-methoxy-4-methyl-1,2-dioxopentan-3-yl]-L-prolinamide (14s). Compound **14s** (30 mg, 5.2%) was prepared from **8** and **11n** as white powder: MS (APCI, positive) *m*/*z* 568 [(M+H)$^+$]; ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.81–1.38(12H, m), 1.86–3.00 (7H, m), 3.53–3.95 (9H, m), 4.03–4.35 (4H, m), 4.47–4.69 (2H, m), 5.02 (1H, m).

5.1.11.23. *N*-{[3-(Carboxymethyl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl]acetyl}-L-valyl-*N*-[(3R,*S*)1,1,1-trifluoro-4-methyl-2-oxopentan-3-yl]-L-prolinamide (14t). Compound **14t** (1.25 g, 62.3%) was prepared from **9b** and **11b** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{24}H_{31}O_8N_5F_3$ [(M−H)$^-$] 574.2119, found 574.2130; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.79–0.95 (12H, m), 1.69–2.23 (8H, m), 3.53–3.72 (2H, m), 4.24–4.64 (6H, m), 5.72–8.63 (5.5H, m), 12.9 (1H, br s).

5.1.11.24. *N*-[(2,4-Dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetyl]-L-valyl-*N*-[(3R,S)-1,1,1-trifluoro-4-methyl-2-oxopentan-3-yl]-L-prolinamide (14u). Compound **14u** (0.18 g, 8.4%) was prepared from **9b** and **11u** as white powder: HRMS (ESI, negative) *m*/*z* calcd for $C_{22}H_{29}O_6N_5F_3$ [(M−H)$^-$] 516.2064, found 516.2072; ^{1}H NMR (300 MHz, DMSO-d_6, δ): 0.79–0.94 (12H, m), 1.69–2.25

(6H, m), 3.56–3.69 (2H, m), 4.01–4.64 (5H, m), 5.54 (1H, d), 6.70–8.62 (2.5H, m), 7.54 (1H, d), 11.2 (1H, s).

5.1.11.25. *N*-{[3-(Carboxymethyl)-2-oxoimidazolidin-1-yl]acetyl}-L-valyl-*N*-[(3R)-1,1,1-trifluoro-4-methyl-2-oxopentan-3-yl]-L-prolinamide (14w). Compound **14w** (0.25 g, 14.4%) was prepared from **9b** and **11l** as white powder. This compound was purified by MCI GEL CHP20P (Mitsubishi Chemical Co.) column chromatography [solvent; MeCN–H_2O (0:100→30:70)] to isolate from diastereomeric mixture: HRMS (ESI, negative) *m*/*z* calcd for $C_{23}H_{33}O_7N_5F_3$ [$(M-H)^-$] 548.2332, found 548.2328; ^1H NMR (300 MHz, $CDCl_3$, δ): 0.86–1.06 (12H, m), 1.88–2.40 (6H, m), 3.47–4.02 (10H, m), 4.57 (1H, dd), 4.68 (1H, dd), 4.88 (1H, dd), 7.33 (1H, d), 7.71 (1H, d).

5.2. Biological study

5.2.1. Compounds inhibitory activity for HNE

HNE (0.2 unit/ml) was preincubated with each test-compound in the assay solution (100 nM HEPES, pH 7.5 containing 1 M NaCl and 0.001% Brij-35) at 37 °C for 4 min. The reaction was started by addition of a substrate, Suc(OMe)-Ala-Ala-Pro-Val-pNA (0.5 mM), for HNE. Hydrolysis of the substrate to pNA was continuously measured spectrophotometrically by monitoring absorbance at 404 nm. IC_{50} value was estimated using non-linear regression of data to a logistic function.

5.2.2. Compounds effects in HNE-induced lung hemorrhage model

5.2.2.1. (a) Intravenous bolus injection. Each test-compound was administrated into the jugular vein 5 min before intratracheal instillation of HNE (29 μg). One hour after HNE instillation, the bronchoalveolar lavage fluid (BALF) was collected, centrifuged, and absorbance of the supernatant at 414 nm was measured. Hemorrhage in BALF was expressed as absorbance at 414 nm.

5.2.2.2. (b) Continuous intravenous infusion. Each test-compound was infused (for over 70 min) into the femoral vein of the animal model starting 10 min before intratracheal instillation of HNE (29 μg) and finishing at the end of the experimental period. One hour after HNE instillation, BALF was collected, centrifuged, and absorbance of the supernatant at 414 nm was measured. Hemorrhage in BALF was expressed as absorbance at 414 nm.

5.2.3. AE-3763 effects in LPS-induced lung injury model

5.2.3.1. (a) Lung edema. LPS (50 μg/kg) was administrated intratracheally to the animal model. **AE-3763** infusion into the jugular vein was started immediately after LPS treatment and continued up to 6 h after LPS treatment. The wet weight of the whole lung was measured 6 h after LPS treatment and corrected for each individual animal based on body weight.

5.2.3.2. (b) Leukocyte infiltration. LPS (50 μg/kg) was administrated intratracheally to the animal model. **AE-3763** infusion into the jugular vein was started immediately after LPS treatment and maintained up to 6 h after LPS treatment. BALF was then collected, and total leukocytes in BALF were counted.

5.2.4. AE-3763 effects on D-galactosamine-induced shock

D-Galactosamine shock was induced in C3H/HeN mice (6–7 w, 22–25 g) by administration of D-galactosamine (GalN) and LPS (20 mg/mouse and 0.3 μg/mouse, respectively) into the tail vein. Immediately after LPS/GalN administration, **AE-3763** (10 or 100 mg/kg) was administrated intraperitoneally six times at 2 h interval. Control animals received the vehicle (PBS) instead of **AE-3763**. Animal's survival rate was observed up to 24 h after shock induction.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.09.020.

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