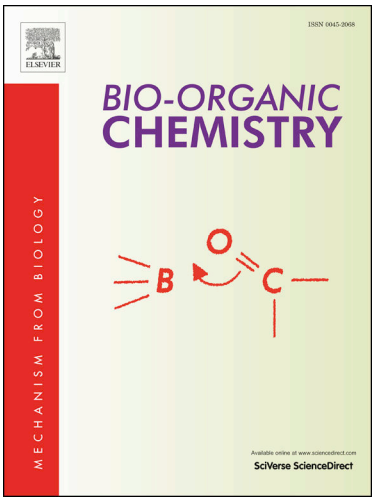
Journal Pre-proofs



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Structure-activity relationship studies of dipeptide-based hepsin inhibitors with Arg bioisosteres

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Abstract

Hepsin is a type II transmembrane serine protease (TTSP) associated with cell proliferation and overexpressed in several types of cancer including prostate cancer (PCa). Because of its significant role in cancer progression and metastasis, hepsin is an attractive protein as a potential therapeutic and diagnostic biomarker for PCa. Based on the reported Leu-Arg dipeptide-based hepsin inhibitors, we performed structural modification and determined in vitro hepsin- and matriptase-inhibitory activities. Comprehensive structure-activity relationship studies identified that the p-guanidinophenylalanine-based dipeptide analog 22a exhibited a strong hepsin-inhibitory activity (K_i = 50.5 nM) and 22-fold hepsin selectivity over matriptase. Compound 22a could be a prototype molecule for structural optimization of dipeptide-based hepsin inhibitors.

Keywords: Prostate cancer, Hepsin, Dipeptide, Bioisostere, Structure-activity relationship

Abbreviations: ACN, acetonitrile; Boc, tert-butoxycarbonyl; BPH, benign prostate hyperplasia; Cbz, benzyloxycarbonyl; DIPEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ESI, electrospray ionization; HOBt, 1-hydroxybenzotriazole; HRMS, high resolution mass spectrometry; NMR, nuclear magnetic resonance; PCa, prostate cancer; PMB, para-methoxybenzyl; PTFE, polytetrafluoroethylene; RP-HPLC, reversed-phase high performance liquid chromatography; SAR, structure-activity relationship; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TMEDA, N,N,N',N',-tetramethylethylenediamine; TTSP, type II transmembrane serine protease.

1. Introduction

Hepatocyte Growth Factor (HGF) is a pleiotropic factor secreted by tumor-associated fibroblasts and plays an important role in cancer metastasis [1]. It is activated through the cleavage of inactive pro-HGF mediated by trypsin-like serine proteases such as hepsin, hepatocyte growth factor activator (HGFA), and matriptase [2-9]. Dysregulated HGF-mediated activity in the HGF / Met signaling pathway leads to oncogenesis, cancer cell proliferation, invasion, and resistance to cancer therapy [10]. HGF-activating proteases, including hepsin, matriptase, and HGFA, are upregulated in cancer cells [11-13]. HGF is inhibited by endogenous HGF activator inhibitors (HAI-1 and HAI-2) [9, 14-16]. The poor prognosis of patients with advanced cancer is closely associated with an increased HGF and reduced HAIs levels [17-22]. Thus, the inhibition of the HGF activation pathway has been considered as a potential therapeutic strategy of cancer intervention.

Hepsin, a type II transmembrane serine protease (TTSP) [23], is composed of 417 amino acids with a C-terminal serine protease domain localized on the surface [24]. Beyond the pro-HGF activation, hepsin also contributes to the activation of matriptase, another pro-HGF activator [25]. Furthermore, hepsin is associated with cell motility and basement membrane components disruption, promoting cancer cell metastasis [26]. Hepsin is predominantly overexpressed in several cancer cells including prostate, breast, ovarian cancer [27, 28]. Especially, mRNA expression of hepsin was significantly upregulated in 90% of the prostate cancer (PCa) specimens and the expression levels were 10-fold higher than those in normal prostate or benign prostate hyperplasia (BPH) [12, 28-32]. The PCa-related hepsin overexpression continues from the early to the later stages [33]. Although localized PCa can be treated effectively with chemotherapy, surgery, and radiation, the treatment is extremely difficult and the mortality rate increases drastically once it metastasizes to other organs including lymph nodes or bones [34]. Therefore, hepsin is an attractive potential biomarker and

prognostic factor of PCa metastasis [31, 33] due to its structural characteristics and significant role in metastasis [35-38].

Several low-molecular-weight (M. W.) hepsin inhibitors have been reported including benzamidine-, indolecarboximidamide-, and peptide-derived analogs [39-47]. Moreover, some comprehensive reviews on small molecular hepsin inhibitors were published recently [48, 49]. Previously, we reported the Leu-Arg dipeptide-based hepsin inhibitors (1–2, Fig. 1), which exhibited strong binding affinity to hepsin with K_i values of nM concentration [41], demonstrating that the dipeptide is the minimal structural requirement for hepsin-inhibitory activity. Janetka and co-workers also confirmed this trend in a recent report [47]. In addition, a NIR fluorescent dye was conjugated successfully with peptide-based hepsin inhibitors, which were accumulated in the hepsin-overexpressing cells in vitro and in vivo [50, 51].

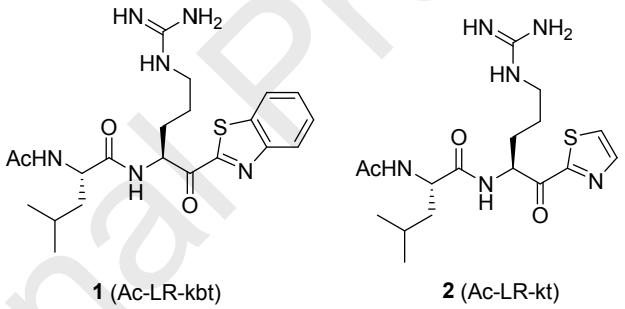


Fig. 1. Previously reported Leu-Arg dipeptide-based hepsin inhibitors

Although peptide-based agents have many attractive aspects, they continue to have some drawbacks as drug candidates, especially in systemic delivery [52]. Low specificity to the target site due to the high conformational flexibility of peptide-based molecule is another challenge to overcome [53, 54]. In addition, hydrophilic properties of peptide-based molecules result in poor permeability through biological membranes and are responsible for rapid clearance from the circulation by the liver and kidney [53, 54]. For these reasons, additional structural modifications have been required for peptide-based compounds.

Herein, we have performed the structure-activity relationship (SAR) studies of compound 1 aiming at identifying a new bioisostere for Arg in the P1 position and the optimal amino acid in the P2 position. The demonstrations in this study would support the further development of hepsin-targeted candidate compounds for the diagnosis or treatment of metastatic PCa.

2. Results and discussion

2.1. Design strategy

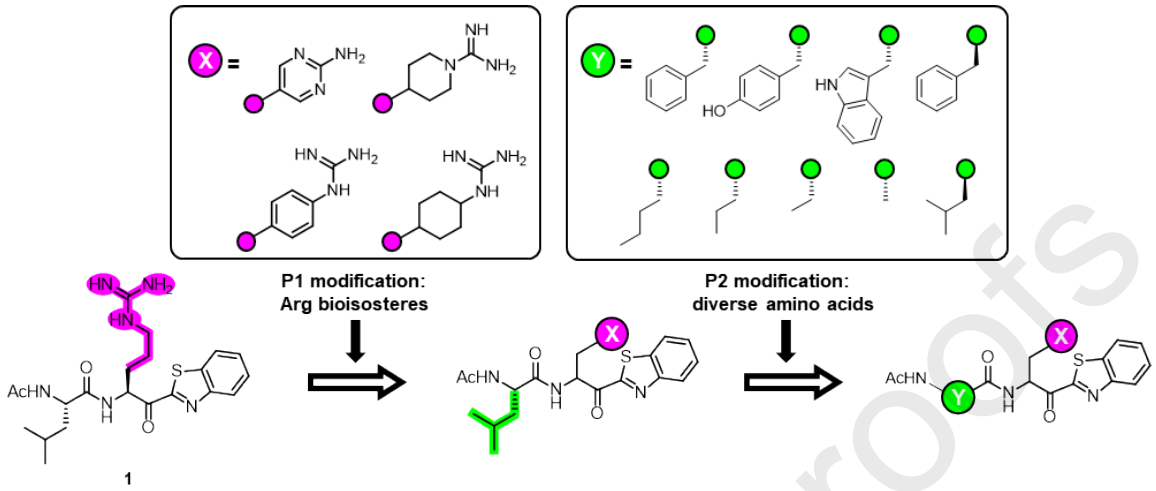
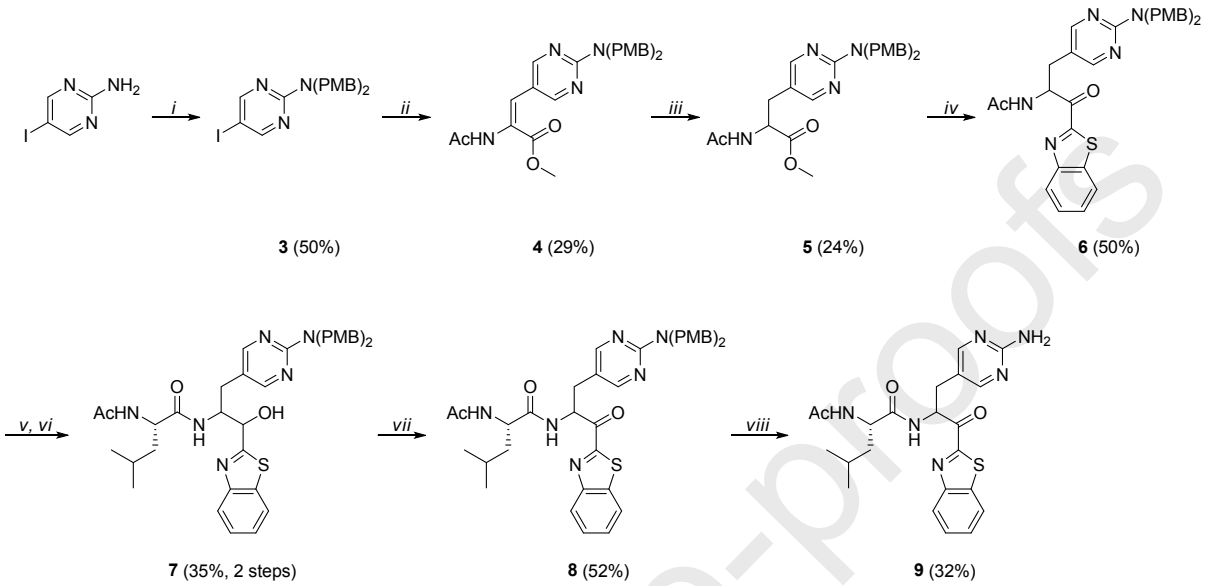


Fig. 2. Design strategy for the structural modification of Ac-LR-kbt (**1**)

The starting point of our research for structural modification was based on the previously reported dipeptide derivative, Ac-LR-kbt (1). Based on the chemical structure of 1, new hepsin inhibitors were designed to replace Arg with a bioisostere that enhances rotational rigidity and increases the lipophilicity of the parent molecules. The guanidine group of compound 1 was located at the S1 sub-pocket and formed a salt bridge with Asp189, contributing to the potent hepsin-inhibitory activity [41]. The guanidine group of Arg interacted with Asp189, acting as an anchor group for binding to the hepsin active site, while the other part of the compound could restrict the catalytic triad composed of His57, Asp102, and Ser195. Although the Arg of the dipeptide derivative at the P1 position plays a critical role for binding to hepsin, it remains as a natural-type amino acid with high rotational flexibility at the side chain. Therefore, we introduced a cyclic ring structure at the side chain of Arg to confer the rotational rigidity at the P1 region (Fig. 2). As shown in Fig. 2, the cyclic ring moieties such as aminopyrimidine, piperidine-1-carboximidamide, cyclohexylguanidine and phenylguanidine were introduced as potential bioisosteres of the Arg in compound 1.

2.2. Chemistry and in vitro biological evaluation

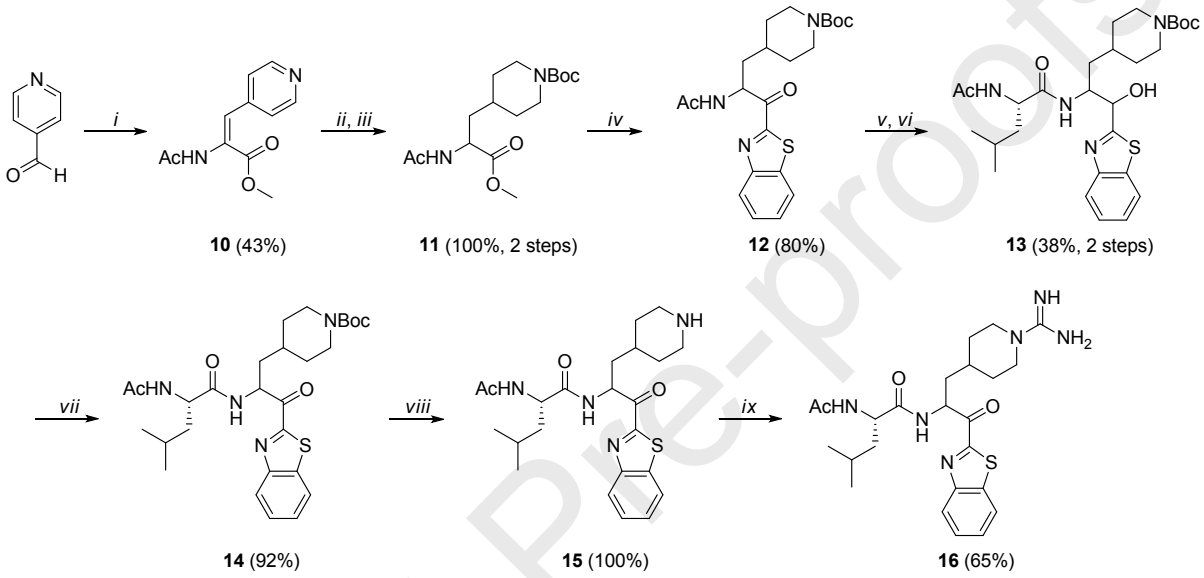


Scheme 1. Synthesis of aminopyrimidine-based compound 9[a]

[a] Reagents and conditions: (i) NaH (60% in oil), 4-methoxybenzyl chloride, THF, 40 ºC, 15 h; (ii) methyl-2-acetamidoacrylate, $NaHCO_3$, Bu_4NCl, $(o\text{-tol})_3P$, $PdCl_2$, DMF, 110 ºC, overnight; (iii) 10% Pd/C, H_2 (gas), MeOH/1,4-dioxane (5/2, v/v), rt, 24 h; (iv) benzothiazole, TMEDA, n-BuLi, THF, −78 ºC, 1.5 h; (v) Cp_2ZrHCl, THF, rt, 20 min; (vi) N-acetyl-L-leucine, HOBt, EDCI·HCl, THF, rt, 12 h; (vii) Dess-Martin periodinane, CH_2Cl_2, rt, 3 h; (viii) TFA/water (97.5/2.5, v/v), 50 ºC, 13 h.

Compounds substituted with the Arg bioisostere were prepared as described in Schemes 1–3. Commercial 2-amino-5-iodopyrimidine was used as a starting material for the synthesis of compound 9 (Scheme 1). Compound 4 was prepared from the starting material by applying para-methoxybenzyl (PMB) protection and subsequent Heck-vinylation [55]. The double bond of 4 was reduced by catalytic hydrogenation using 10% Pd/C and H_2 gas to afford compound 5. The reaction of 5 with benzothiazolyl lithium solution afforded compound 6

yielding 50% as reported previously [41]. Deacetylation of compound 6 using Schwartz's reagent (Cp_2ZrHCl), followed by amide coupling using EDCI·HCl and HOBt, provided compound 7 in 35% yield (2 steps). Compound 8 was obtained through oxidizing the alcohol group of 7 by a Dess-Martin periodinane treatment. The PMB groups of 8 were removed using trifluoroacetic acid (TFA) to yield the final compound 9.

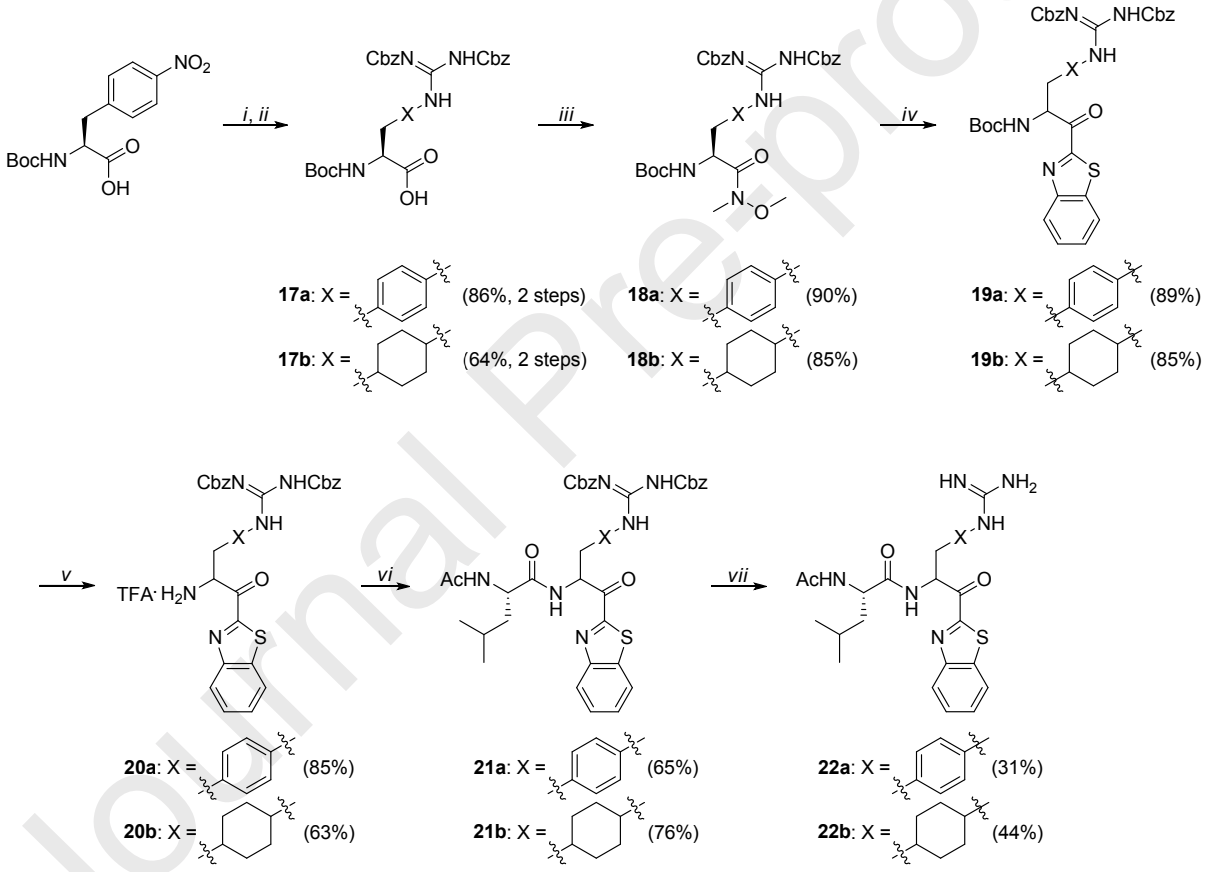


Scheme 2. Synthesis of piperidine-1-carboximidamide-based compound 16[a]

[a] Reagents and conditions: (i) N-acetylglycine, sodium acetate, acetic anhydride, MeOH, 100 ºC, 1 min; (ii) PtO_2(IV), AcOH, H_2 (gas), rt, 24 h; (iii) di-tert-butyl dicarbonate, 4-dimethylaminopyridine, THF, rt, 2 h; (iv) benzothiazole, TMEDA, n-BuLi, THF, −78 ºC, 1.5 h; (v) Cp_2ZrHCl, THF, rt, 20 min; (vi) N-acetyl-L-leucine, HOBt, EDCI·HCl, THF, rt, 12 h; (vii) Dess-Martin periodinane, CH_2Cl_2, rt, 3 h; (viii) TFA/CH_2Cl_2 (1/2, v/v), rt, 1 h; (ix) 1H-pyrazole-1-carboxamidine, DIPEA, DMF, rt, 12 h.

By applying a similar synthetic strategy to the aminopyrimidine 9 described in Scheme 1, a variety of compounds with Arg bioisostere moiety at the P1 position were prepared. The synthesis of piperidine-1-carboximidamide analog is described in Scheme 2. Briefly, the dehydropyridylalanine 10 was obtained by reacting 4-pyridylaldehyde with N-acetylglycine

[56]. Conversion of the pyridine into piperidine using Adam's catalyst (PtO_2) with acetic acid and the subsequent tert-butoxycarbonyl (Boc) protection provided the piperidinylalanine 11 in quantitative yield. The reaction of 11 with lithium-benzothiazole afforded compound 12 in 80% yield. Deacetylation of 12, followed by the amide coupling, provided compound 13 in 38% yield (2 steps). Oxidation of 13 and the subsequent Boc deprotection afforded 15 in 92% yield (2 steps). The reaction of 15 with 1H-pyrazole-1-carboxamidine provided the final piperidine-1-carboximidamide compound 16 in 65% yield [57].



Scheme 3. Synthesis of p-phenylguanidine-based compound 22a and cyclohexylguanidine-based compound 22b[a]

[a] Reagents and conditions: (i) H_2 (gas), 10% Pd/C (for 17a) or PtO_2(IV) (for 17b), AcOH (for 17b), MeOH, rt, 3 h (for 17a) or 24 h (for 17b); (ii) N,N'-bis(carbobenzoxy)-1H-pyrazole-1-carboxamidine, Et_3N, CH_2Cl_2, rt, 6 h; (iii) N,O-dimethylhydroxylamine HCl, HOBt, EDCI·HCl, DIPEA, THF, rt, 13 h; (iv) benzothiazole, TMEDA, n-BuLi, THF, −78 ºC, 1.5 h; (v) TFA,

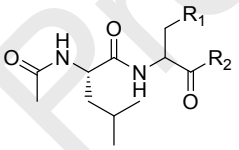
triethylsilane, water, CH_2Cl_2, rt, 2 h; (vi) N-acetyl-L-leucine, HATU, DIPEA, rt, CH_2Cl_2, 5 h; (vii) TFA, thioanisole, rt, 12 h.

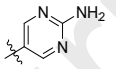
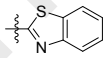
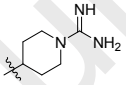
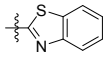
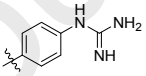
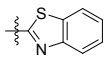
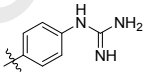
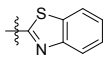
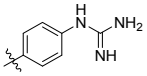
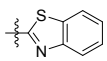
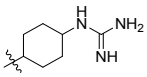
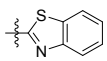
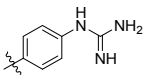
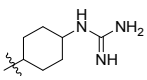
The synthesis of phenylguanidine- and cyclohexylguanidine-based analogs were achieved via a 7-step synthetic process (Scheme 3). The catalytic hydrogenation reaction using Adam's catalyst and 50% acetic acid in methanol reduced not only the nitro group but also the benzene ring of N-(tert-butoxycarbonyl)-4-nitro-L-phenylalanine [58], while the hydrogenation using 10% Pd/C in methanol only reduced nitro group to the amine group. The respective reduced compounds were reacted with N,N'-bis(carbobenzoxy)-1H-pyrazole-1-carboxamidine to give compounds 17a and 17b, respectively. During the guanidine formation, the mono-N-Cbz-protected 1H-pyrazole-1-carboxamidine gave a detrimental effect on the yield of the next Weinreb amide synthesis step as compared with the bis-Cbz-protected 1H-pyrazole-1-carboxamidine. Introduction of the Weinreb amide and the subsequent addition of lithium-benzothiazole provided compounds 19a and 19b, respectively. The Boc-protecting groups of 19a–19b were removed with 25% TFA in CH_2Cl_2. Recrystallization of the crude compounds 20a and 20b were successfully achieved using diethyl ether. Conjugation of 20a–20b with N-acetyl-L-leucine was achieved using HATU as a coupling reagent. The Cbz groups of 21a and 21b were removed using TFA and thioanisole to afford the final compounds 22a and 22b, respectively [59]. The final compounds were purified by reversed-phase (RP)-HPLC and analyzed by NMR and ESI-MS. Some compounds showed two diastereomeric peaks at HPLC spectra due to the epimerization of the α-carbon in the P1 Arg portion during the addition reaction with lithium–benzothiazole solution. The hepsin-inhibitory activities of the synthesized compounds were determined by applying the reported fluorescence-based enzymatic assay [40, 41]. To investigate the selectivity of synthesized compounds for hepsin over matriptase, the compounds were also evaluated matriptase-inhibitory activities as

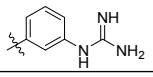
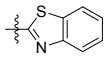
previously reported [41]. We monitored substrate proteolysis inhibition kinetically for two hours. Hepsin and matriptase enzymatic activities of the synthesized compounds are summarized in Table 1.

The first set of compounds (9, 16, 22a, 22b) included Leu-Arg analogs substituted with the bioisosteres of the guanidine group (Table 1). Among them, compound 22a with the phenylguanidine exhibited the strongest hepsin-inhibitory activities with a K_i value of 50.5 nM. In addition, 22a showed higher hepsin selectivity over matriptase as compared to compound 1 (22-fold vs, 14-fold). However, the potencies of compounds 9 and 16 were too weak to act as an Arg bioisostere. Compound 22b with the cyclohexylguanidine displayed moderate hepsin inhibition (K_i = 505 nM).

Table 1. In vitro hepsin- and matriptase-inhibitory activities of the synthesized compounds



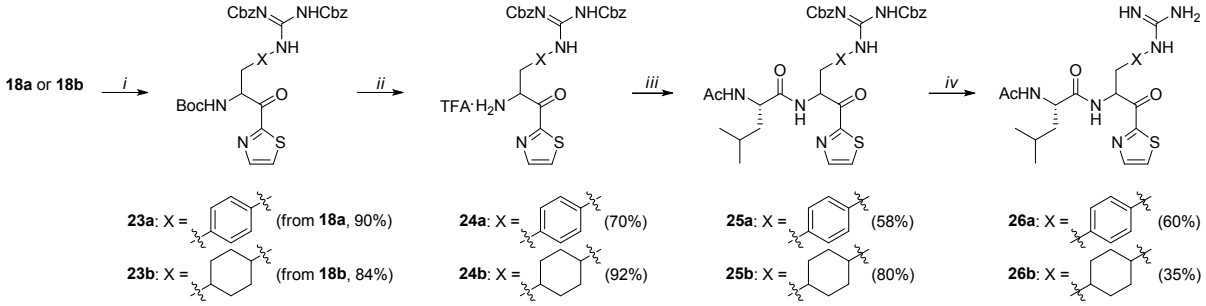
Cmpd	R_1	R_2	Hepsin K_i (nM)	Matriptase K_i (nM)	Selectivity Index	cLogP[a]
	1 (Ac-LR-kbt)		11.7 ± 4.9	169 ± 38.9	14	1.06
9			>10000	>10000		1.68
16			>10000	>10000		2.06
22a[b,c]			50.5 ± 12.2	1120 ± 57.6	22	2.52
22a-1[b,c]			104 ± 30.4	2190 ± 115	21	2.52
22a-2[b,c]			35.2 ± 6.2	853 ± 98.3	24	2.52
22b			505 ± 39.2	>10000	>20	2.55
26a			527 ± 92.2	>10000	>19	0.92
26b			2110 ± 1200	>10000	>4.7	0.96

32			>10000	>10000	2.52

[a]calculated using ChemDraw Ultra v12.0.2.1076.
[b]HPLC retention time: 22a = 3.79 and 4.36 min; 22a-1 = 3.91 min; 22b-1 = 4.47 min.
[c]analytical HPLC was performed with Phenomenex Gemini-NX C18 column (150×4.6 mm, 3 μm, 110 Å), isocratic elution of 35% of solvent B (A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile), flow rate of 1.0 mL/min, monitored by UV detector at 220 nm.

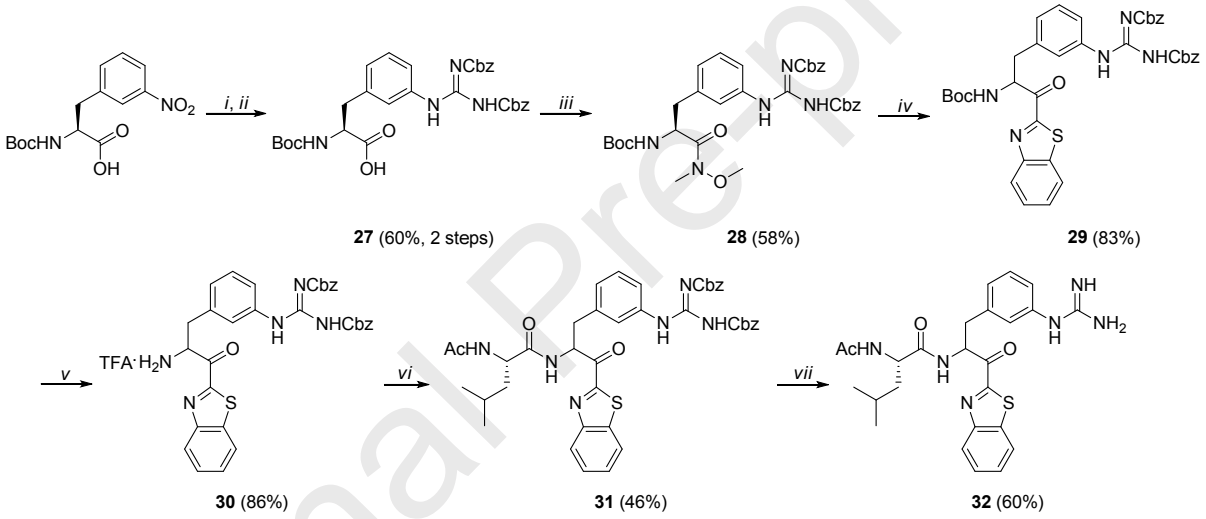
To clarify the effect of the absolute configuration at the P1 position on hepsin inhibition, each epimer of the mixture compound 22a was separated and purified (See Supplementary data). A pure epimer 22a-1 eluted at 3.91 min in the HPLC experiment and displayed hepsin-inhibitory activity with a K_i value of 104 nM (Table 1). The other epimer 22a-2, which eluted at 4.47 min, exhibited a little stronger hepsin-inhibitory activity (K_i = 35.2 nM) than 22a-1. We previously reported that the α-carbon epimerization of Arg at the P1 position makes a little difference in hepsin inhibition [41]. Because the difference in hepsin inhibition and hepsin/matriptase selectivity was little between the two epimers (Table 1), hepsin-inhibitory activity was determined using an epimeric mixture of inhibitors modified at the P1 position.

In our previous study, Ac-LR-kt (ketothiazole) (2) was also a potent hepsin inhibitor with possessing hepsin-inhibitory activities comparable to 1 [41]. Thus, we assumed that dipeptide derivatives substituted C-terminus with a thiazole ring might exhibit hepsin-inhibitory activities. Compounds 26a and 26b were synthesized to investigate the effect of the changes from benzothiazole to thaizole on hepsin binding affinity. In addition, compound 32 with m-phenylguanidinylalanine, was synthesized to determine the effect of the orientation of the guanidine group in 3-D space on hepsin affinity. As shown in Schemes 4 and 5, compounds 26a, 26b, and 32 were prepared by applying a synthetic strategy similar to 22a.



Scheme 4. Synthesis of thiazole-substituted compounds 26a and 26b[a]

[a] Reagents and conditions: (i) Thiazole, TMEDA, n-BuLi, THF, −78 ºC, 1.5 h; (ii) TFA, triethylsilane, water, CH_2Cl_2, rt, 2 h; (iii) N-acetyl-L-leucine, HATU, DIPEA, rt, CH_2Cl_2, 5 h; (iv) TFA, thioanisole, rt, 12 h.



Scheme 5. Synthesis of m-phenylguanidine-based compound 32[a]

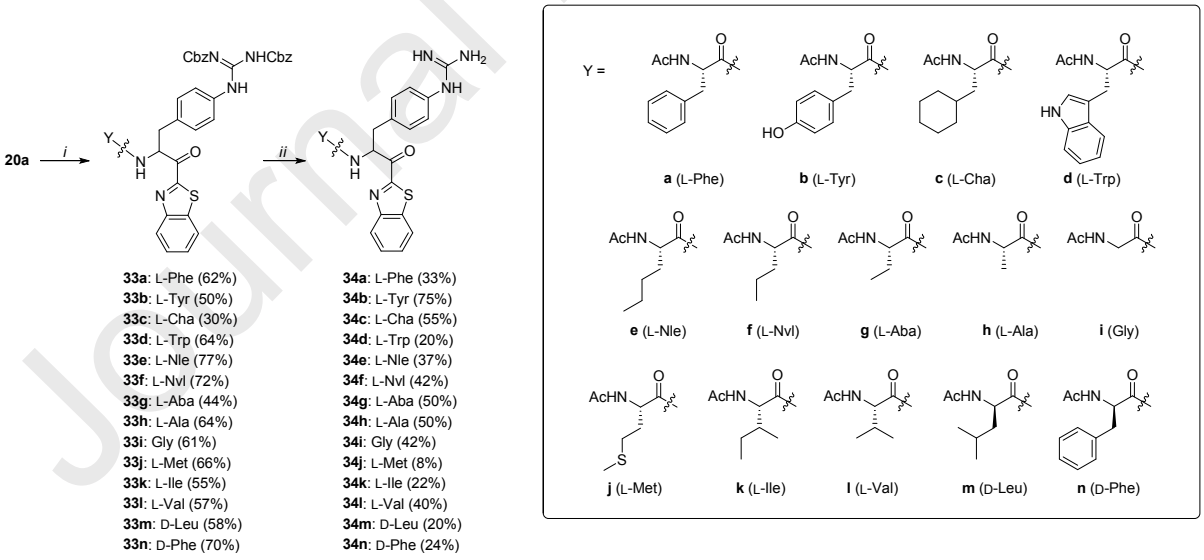
[a] Reagents and conditions: (i) H_2 (gas), 10% Pd/C, MeOH, rt, 3 h; (ii) N,N'-bis(carbobenzoxy)-1H-pyrazole-1-carboxamidine, Et_3N, CH_2Cl_2, rt, 6 h; (iii) N,O-dimethylhydroxylamine HCl, HOBt, EDCI·HCl, DIPEA, THF, rt, 13 h; (iv) benzothiazole, TMEDA, n-BuLi, THF, −78 ºC, 1.5 h; (v) TFA, triethylsilane, water, CH_2Cl_2, rt, 2 h; (vi) N-acetyl-L-leucine, HATU, DIPEA, rt, CH_2Cl_2, 5 h; (vii) TFA, thioanisole, rt, 12 h.

As summarized in Table 1, the thiazole-bearing compounds 26a (K_i = 527 nM) and 26b (K_i = 2110 nM) exhibited lower hepsin binding affinity than the corresponding

benzothiazole compounds 22a and 22b, indicating the importance for the benzothiazole moiety at the C-terminus region. Moreover, the m-phenylguanidine 32 (K_i >10 μM) completely lost hepsin-inhibitory activity, indicating that the positional location of the guanidine group in 3-D space is critical for binding to hepsin.

With the p-phenylguanidinylalanine moiety fixed in the P1 position, diverse natural and non-natural amino acids were introduced in the P2 position. As shown in Scheme 6, the compounds 34a–34n were synthesized by applying a synthetic procedure similar to 22a. N-acetylated non-natural amino acids were prepared as described in the Supplementary data. During the synthetic process of compound 34j, the sulfide group of 33j acted as a soft base and underwent a nucleophilic attack on the electron-deficient benzylic carbon of the Cbz group [59]. According to the in vitro enzymatic studies of compounds 34a–34d with a bulky side chain at the P2 position, 34b with a tyrosine residue displayed the most potent hepsin-inhibitory activity (K_i = 129 nM, shown in Table 2), consistent with the claim that Tyr is permissive at the P2 position [60]. Compounds bearing both a bulky ring moiety and hydrogen-bonding donor capability at the P2 residue (34b, 34d) exhibited stronger hepsin-binding affinity than 34a and 34c (K_i = 149 nM for 34d vs. 357 nM for 34a; 539 nM for 34c). Next, the compounds having residues with diverse lengths of carbon chain at the P2 region were synthesized. Compounds 34e (K_i = 302 nM) with norleucine and 34f (K_i = 333 nM) with norvaline showed weaker hepsin-inhibitory activity than 34d. In addition, hepsin inhibition decreased as the length of linear alkyl chain residue at P2 position became shorter (K_i = 453 nM for 34g; 1720 nM for 34h; K_i = 2450 nM for 34i). Interestingly, hepsin selectivity over the matriptase of these compounds (34h, 34i) was maintained as also reported by the Janetka and co-workers [47]. Changing the ε-carbon of 34e to the sulfur atom (34j) made little influence on hepsin-binding

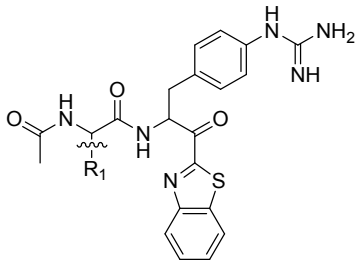
affinity (K_i = 296 nM for 34j). As shown in Table 2, compounds bearing bifurcated carbon chain residues with an additional methyl group at the β-position (34k, 34l) showed higher hepsin-inhibitory activity (K_i = 162 nM for 34l; 287 nM for 34k) than those with similar lengths of linear carbon chains (34f, 34g). Compound 22a, which has a carbon chain bifurcated at the γ-position, showed higher hepsin-inhibitory activity than 34k and 34l, suggesting that the bulky lipophilic part of the P2 residue might extend over a certain range for efficient interaction at hepsin active site. This trend was also observed in the linear carbon length change mentioned above. Compound 34m (K_i = 151 nM), with an (R)-configuration of the P2 residue of 22a, displayed lower hepsin-binding affinity and selectivity over matriptase than 22a. We also observed this trend between the phenylguanidine-conjugated compounds 34a (K_i = 357 nM) and 34n (K_i = 551 nM), demonstrating that amino acids with an (S)-configuration at the P2 position were preferred over those with an (R)-configuration.

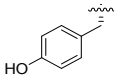
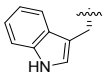


Scheme 6. Synthesis of 4-phenylguanidine-based dipeptide analogs[a]

[a] Reagents and conditions: (i) N-acetylated appropriate amino acids, HATU, DIPEA, CH_2Cl_2, rt, 5 h; (ii) TFA, thioanisole, rt, 12 h.

Table 2. In vitro hepsin- and matriptase-inhibitory activities of the 4-phenylguanidinylalanine-based dipeptide analogs



Cmpd	R_1	Hepsin K_i (nM)	Matriptase K_i (nM)	Selectivity Index	cLogP[a]
1 (Ac-LR-kbt)		11.7 ± 4.9	169 ± 38.9	14	1.06
34a		357 ± 101	488 ± 88.3	1.4	2.48
34b		129 ± 21.1	398 ± 49.4	3.1	1.81
34c		539 ± 66.5	3020 ± 975	5.6	3.71
34d		149 ± 9.20	211 ± 19.6	1.4	2.47
34e		302 ± 15.6	396 ± 11.8	1.3	2.65
34f		333 ± 41.7	421 ± 8.0	1.3	2.12
34g		453 ± 38.0	312 ± 48.9	0.7	1.59
34h		1720 ± 355	69.0 ± 1.5	0.04	1.06
34i	-H	2450 ± 501	46.2 ± 2.2	0.02	0.75
34j		296 ± 26.5	306 ± 15.5	1.0	1.21
34k		287 ± 48.9	2346 ± 656	8.2	2.52
34l		162 ± 10.0	465 ± 109	2.9	1.99
34m		151 ± 11.8	2780 ± 138	18	2.52
34n		551 ± 37.5	529 ± 92.7	1.0	2.48

[a] calculated using ChemDraw Ultra v12.0.2.1076

2.3. In silico docking studies

To elucidate the binding modes of the prepared compounds, we conducted in silico virtual docking studies with the hepsin X-ray crystal structure (PDB ID: 1O5E [61]) using the Surflex-Dock GeomX module in SYBYL-X software (v2.1.1, Tripos Inc., NJ, USA). In the Ligand preparation process, we fixed the configuration of P1 position of the prepared compounds to be an (S)-configuration. The docking results revealed that compound 22a formed hydrogen bonds with Asp189 and Gly219 as well as with Ser195, Gly193, and His57 within a range of 3.0 Å (Fig. 3A). The benzothiazole ring of 22a made van der Waals interaction with Pro60, Leu41, and the disulfide bridge between Cys42 and Cys58, which supported the benzothiazole preference to the thiazole ring in dipeptide-derived hepsin inhibitors. The Leu residue of the P2 position was projected to the aromatic amino acid residues surrounded by Trp215 and His57. The side chain of Leu interrupted a catalytic triad which consists of Asp102, His57, and Ser195. When the hydrophobic moieties bulkier than Leu were introduced at the side chain of the P2 residue, they made steric repulsion with Asn99 and Asp102, leading to a dramatic change of the binding pose (See Supplementary data). The branched methyl group at the β-carbon in Val or Ile affected the conformation of the parent molecules in the hepsin active site. However, the non-branched linear side chain in the P2 region made a minimal effect on the surface of the catalytic triad. In the case of 22b, the guanidine group presented strong interaction with Asp189 and Gly219 at the S1 site (Fig. 3B). However, it was located away from Ser195 out of the 3.0-Å range. The Leu residue of P2 position of 22b was projected to Leu41 to generate lipophilic interaction, thus the surface of catalytic triad and S2 sub-pocket were relatively more exposed than 22a. In the case of compound 32, in which the guanidine group is substituted at meta-position, 32 did not fully interrupt the catalytic triad and S2 sub-pocket due to the positional change in the projection of the guanidine group (Fig. 3C). Compound 32 made several hydrogen bonds within a 3.0-Å range; however, to a lesser extent than 22b. Furthermore, two

residues, Tyr146 and Gln192, formed strong repulsive contacts with the benzothiazole ring of 32, resulting in a decrease of hepsin-binding affinity. The orientation of the benzothiazole moieties of 22b and 32 was different from that of 22a because it was located far away from the hydrophobic residues (Leu 41 and Cys42-Cys58 disulfide bridge) due to the distortion of the ligand dipeptide backbone.

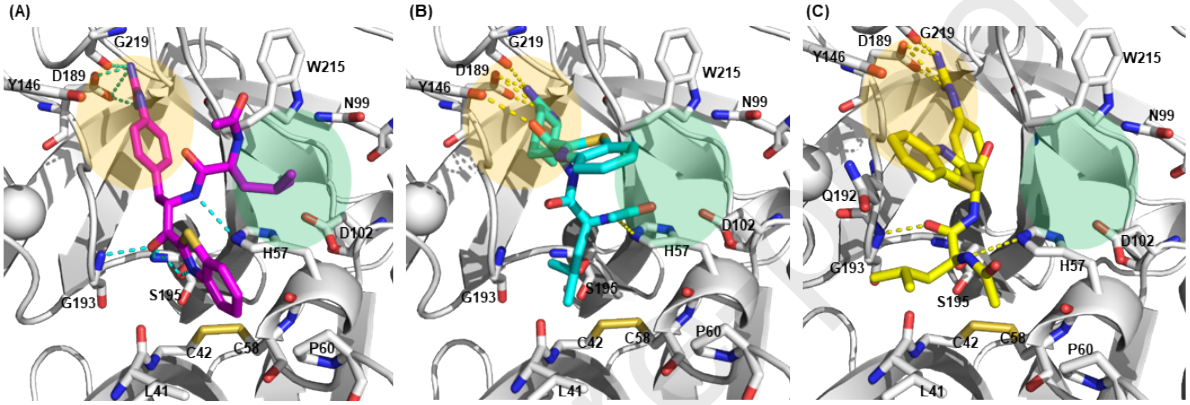


Fig. 3. Docked poses of compounds 22a (A), 22b (B), and 32 (C) in the active site of hepsin (PDB ID: 1O5E). S1 and S2 sub-pockets are marked in yellow and green, respectively.

In the case of molecular docking simulation with matriptase (PDB ID: 3NCL [62]), both 22a and 1 bind to matriptase in a similar binding mode (Fig. 4). The guanidine group of 1 presented hydrogen bonds with the Asp202 and Gly232 of matriptase, and also made an ionic interaction with Asp202 in a range of 2.00 Å. However, the hydrogen-bonding distance of 22a was much longer than that of 1. In particular, 22a lacked hydrogen-bonding interaction with Asp202 within the range of 2.50 Å from the matriptase active site. These results explained the weak matriptase-inhibitory activity of 22a as compared to that of 1, indicating that the restricted flexibility of the P1 residue enhances the selectivity of hepsin over matriptase.

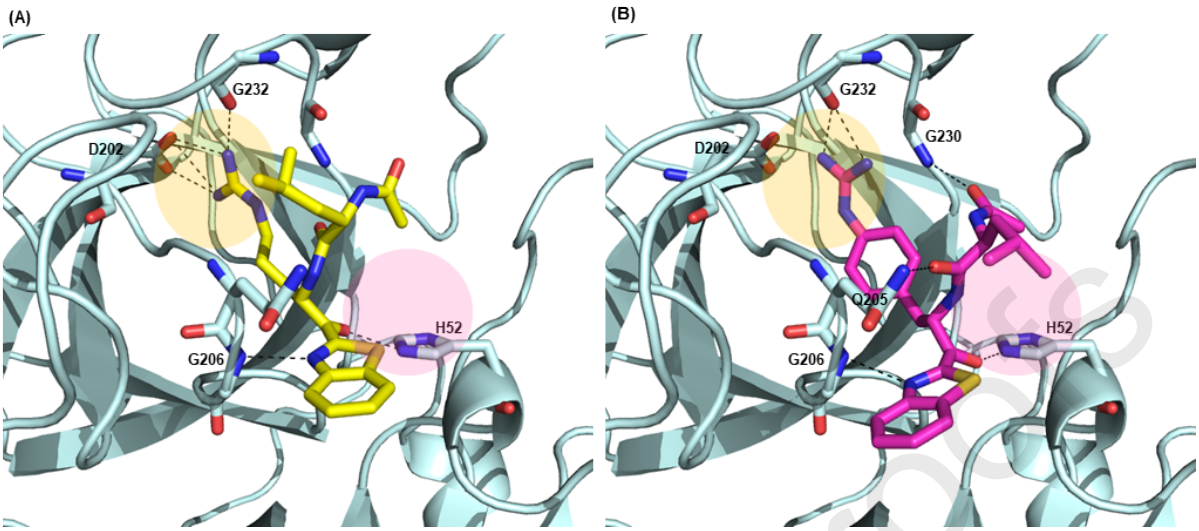


Fig. 4. Docked poses of compounds 1 (A) and 22a (B) in the active site of matriptase (PDB ID: 3NCL). S1 and S2 sub-pockets are marked in yellow and pink, respectively.

The results indicated that the flexibility of peptidomimetic compounds in the P1 region is required for inhibitory activities of hepsin and matriptase. Although compound 22a displayed slightly lower hepsin-inhibitory activity than 1, it possesses several merits for further structural optimization. Compound 22a showed higher hepsin selectivity over matriptase (22-fold) than 1 (14-fold). In vitro and in silico studies indicated that flexibility in P1 residue appears to be somewhat important for matriptase than hepsin. In addition to the reduced number of rotational bonds, 22a was more lipophilic property than 1 (cLogP = 2.52 for 22a; 1.06 for 1). The prolonged RP-HPLC retention time of 22a compared to 1 also supported the enhanced lipophilicity of 22a (See Supplementary data). The parallel artificial membrane permeability assay (PAMPA) also showed an increase in permeability of 22a compared to compound 1 (0.06 nm/s for 22a and 0.04 nm/s for 1, See Supplementary data). However, the absolute cell permeation values of both compounds were very low due to the hydrophilic nature of 1 and 22a. The 4-phenylguanidine group of 22a is less basic (pKa = 10.01) than the guanidine of 1 (pKa = 11.69) [63-65], which may be favorable for membrane permeability. Therefore,

compound 22a has a potential as a prototype molecule for further structural optimization of more potent dipeptide-based hepsin inhibitors with better lipophilicity and permeability properties.

3. Conclusions

We designed and synthesized hepsin-targeted inhibitors derived from Leu-Arg dipeptides and evaluated their in vitro hepsin-inhibitory activities. We introduced the Arg bioisosteres at the P1 residue in order to restrict the rotational flexibility of the dipeptide-based hepsin inhibitors. Comprehensive SAR studies demonstrated that the p-phenylguanidine moiety acts as a promising Arg bioisostere at the P1 residue. For the variation of the P2 residue, several natural and non-natural amino acids were introduced in order to investigate the effect of the side chain and the absolute configuration of the α-carbon. The Leu with (S)-configuration was the most potent residue for the P2 residue. Compound 22a with the p-phenylguanidine at the P1 region and L-Leu at the P2 region exhibited the most potent hepsin binding affinity (K_i = 50.5 nM) and a 22-fold higher hepsin selectivity compared to matriptase. In addition, compound 22a showed a slight increase in membrane permeability compared to 1. Compound 22a can be used as a prototype molecule for structural modification of dipeptide-based hepsin inhibitors for metastatic PCa treatment or diagnosis.

4. Experimental section

4.1. General

All the chemicals and solvents used in the reaction were purchased from Sigma-Aldrich, TCI, or Alfa Aesar and were used without further purification. Reactions were monitored by TLC on 0.25 mm Merck precoated silica gel plates (60 F_{254}). Reaction progress was monitored by TLC analysis using a UV lamp and/or $KMnO_4$ staining for detection purposes. Column chromatography was performed on silica gel (230−400 mesh, Merck, Darmstadt, Germany). ^{1}H and ^{13}C NMR spectra were recorded at room temperature (298 K) in $CDCl_3$ (7.26 ppm/77.16 ppm), CD_3OD (3.31 ppm/49.00 ppm) or CD_3CN/D_2O (2.53 and 4.79 ppm/1.32 and 118.26 ppm) on either Bruker BioSpin Avance 300 MHz NMR or Bruker Ultrashield 600 MHz Plus spectrometer and referenced to an internal solvent. NMR solvents including $CDCl_3$, CD_3OD, CD_3CN and D_2O were used as received from the Eurisotop company. Chemical shifts are reported in parts per million (ppm). Coupling constants (J) are given in Hertz. Splitting patterns are indicated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, double of doublet; dt, double of triplet; br, broad for ^{1}H NMR data. High resolution mass spectra (HRMS) were recorded on an Agilent 6530 Accurate mass Q-TOF LC/MS spectrometer. Low resolution mass spectra (LRMS) analyses were obtained from an API 150EX ESI-MS spectrometer. Reversed-phase high-performance liquid chromatography (RP-HPLC) purification using semi-preparative column (Phenomenex Gemini-NX C18, 110 Å, 150 mm × 10 mm, 5 μm) was performed on Agilent 1260 Infinity (Agilent). The purity of all final compounds was measured by analytical RP-HPLC on an Agilent 1260 Infinity (Agilent) with a C18 column (Phenomenex, 150 mm × 4.6 mm, 3 μm, 110 Å) using water (containing 0.1% TFA) and acetonitrile (ACN; containing 0.1% TFA) as mobile phase. All compounds were monitored at UV detector: 220 nm. The purities of the tested compounds were >95%.

4.2. Synthesis

Typical procedure A for benzothiazole or thiazole addition: To benzothiazole or thiazole (10.2 eq) and N,N,N',N'-trimethylethylenediamine (TMEDA; 10.0 eq) in THF at –78 ℃ was added n-BuLi (1.6M in hexane, 10.0 eq) dropwise over 10 min. The lithium-benzothiazole or -thiazole solution was stirred at –78 ℃ for 35 min. The appropriate Weinreb amide or methyl ester compound (1.0 eq) was dissolved in THF, then added via syringe to the lithium-benzothiazole or -thiazole solution at –78 ℃ dropwise over 15 min. The reaction mixture was stirred at –78 ℃ for 1.5 h. The reaction mixture was quenched by pouring into a saturated aqueous ammonium chloride solution and shaking vigorously. The aqueous layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (eluting with hexane/EtOAc) on silica gel.

Typical procedure B for Weinreb amide formation: To a solution of appropriate carboxylic acid (1.0 eq) in dry THF (tetrahydrofuran) was added N,O-dimethylhydroxylamine hydrochloride (2.0 eq), hydroxybenzotriazole (HOBt; 1.4 eq), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI·HCl; 1.4 eq). N,N-diisopropylethylamine (DIPEA; 6.0 eq) was added to the reaction mixture under argon atmosphere. The reaction mixture was stirred at room temperature for 13 h. The reaction mixture was partitioned and diluted with EtOAc and water. The organic layer was partitioned between EtOAc and brine. The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (eluting with hexane/EtOAc) on silica gel.

Typical procedure C for amide coupling: To a CH_2Cl_2 solution of appropriate N-acetylated amino acid (2.0 eq) and HATU (3.0 eq) was added DIPEA (4.5 eq) under argon

atmosphere. The reaction mixture was stirred at room temperature for 30 min, then was added CH_2Cl_2 suspension of Boc-deprotected Arg analog TFA salt (1.0 eq) with DIPEA (1.5 eq). The reaction mixture was stirred at room temperature for 5 h. A saturated aqueous sodium bicarbonate solution was added to the mixture to quench the reaction. The organic layer was diluted by EtOAc and extracted by water and brine, then combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (eluting with hexane/EtOAc) on silica gel.

Typical procedure D for Cbz deprotection: Thioanisole (100 eq) was added to Cbz di-protected compound (1.0 eq). TFA (560 eq) was added dropwise to reaction mixture at 0 ℃. The reaction mixture was stirred at room temperature for 12 h then evaporated in vacuo. The residue was added diethyl ether, then the precipitated crude product was collected by centrifugation, followed by carefully decanting out the ether solvent. The crude product was purified by RP-HPLC (eluting with 0.1% TFA in water/ACN).

4.2.1. 5-Iodo-N,N-bis(4-methoxybenzyl)pyrimidin-2-amine (3)

This compound was prepared by previously reported method and the analytical data were same as reference [55].

4.2.2. (E)-methyl 2-acetamido-3-(2-(bis(4-methoxybenzyl)amino)pyrimidin-5-yl)acrylate (4)

This compound was prepared by previously reported method and the analytical data were same as reference [55].

4.2.3. Methyl 2-acetamido-3-(2-(bis(4-methoxybenzyl)amino)pyrimidin-5-yl)propanoate (5)

To a solution of compound 4 (168 mg, 0.4 mmol, 1.0 eq) in 7.0 mL methanol/1,4-dioxane (5:2, v/v) was added 10% Pd/C (4.3 mg, 0.04 mmol, 0.1 eq). The reaction mixture was stirred at room temperature under 1 atm of hydrogen gas for 24 h. The reaction mixture was filtered through celite then evaporated under reduced pressure. The residue was purified by flash column chromatography (eluting with hexane/EtOAc, 2:1 to 1:2) on silica gel to afford the product (40 mg, 0.1 mmol) in 24% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 8.09 (s, 2H), 7.15 (d, J = 8.4 Hz, 4H), 6.84 (d, J = 8.4 Hz, 4H), 6.07 (d, J = 6.9 Hz, 1H), 4.84 (d, J = 6.2 Hz, 1H), 4.79 (s, 4H), 3.79 (s, 9H), 3.00 (q, J = 5.1 Hz, 1H), 2.98 (q, J = 5.1 Hz, 1H), 2.05 (s, 3H). HRMS (ESI): $[M + H]^+$ calculated for $C_{26}H_{31}N_4O_5^+$, 479.2289; found, 479.2307.

4.2.4. N-(1-(benzo[d]thiazol-2-yl)-3-(2-(bis(4-methoxybenzyl)amino)pyrimidin-5-yl)-1-oxopropan-2-yl)acetamide (6)

This compound was prepared in 50% yield by typical procedure A with compound 5 and benzothiazole. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 8.22 (d, J = 7.5 Hz, 1H), 8.05 (s, 2H), 7.98 (d, J = 6.9 Hz, 1H), 7.63–7.56 (m, 2H), 7.12 (d, J = 8.4 Hz, 4H), 6.82 (d, J = 8.4 Hz, 4H), 6.52 (d, J = 7.5 Hz, 1H), 6.13 (q, J = 5.4 and 7.5 Hz, 1H), 4.71 (s, 4H), 3.78 (s, 6H), 3.32 (q, J = 5.1Hz, 1H), 3.30 (q, J = 5.1Hz, 1H), 2.08 (s, 3H). ^{13}C NMR (75 MHz, CDCl3): δ ppm 192.37, 169.80, 163.29, 161.67, 158.74, 158.43, 153.53, 137.28, 130.19, 128.91, 128.33, 127.40, 125.94, 122.47, 116.60, 113.97, 113.88, 56.41, 55.27, 48.12, 32.69, 23.29. HRMS (ESI): $[M – H]^–$ calculated for $C_{32}H_{30}N_5O_4S^-$, 580.2019; found, 580.2050.

4.2.5. (2S)-2-acetamido-N-(1-(benzo[d]thiazol-2-yl)-3-(2-(bis(4-methoxybenzyl)amino)pyramidin-5-yl)-1-hydroxypropan-2-yl)-4-methylpentanamide (7)

To a solution of compound 6 (105 mg, 0.2 mmol, 1.0 eq) in THF (7.0 mL) was added Schwartz's reagent (Cp_2ZrHCl, 139 mg, 0.5 mmol, 3.0 eq), then the reaction mixture was vigorously stirred at room temperature for 20 min. The reaction was quenched by 20 μL of water and the mixture was filtered through short silica gel pad eluting with CH_2Cl_2/methanol (10:1, v/v). The filterate was evaporated in vacuo, then dissolved in THF (3.0 mL). N-acetyl-L-leucine (40 mg, 0.2 mmol, 1.3 eq) and HOBt (39 mg, 0.3 mmol, 1.6 eq) were added to the aforementioned THF solution under argon atmosphere, then cooled to 0 ℃. To a reaction mixture was added suspension of EDCI·HCl (56 mg, 0.3 mmol, 1.6 eq) in THF (4.5 mL) then stirred at room temperature for 12 h. A saturated aqueous sodium bicarbonate solution was added to the mixture to quench the reaction. The organic layer was diluted by EtOAc and extracted by water and brine, then combined organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography (eluting with CH_2Cl_2/methanol, 100:1 to 20:1) on silica gel to afford the product (44 mg, 0.1 mmol) in overall 35% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 8.33–8.18 (m, 2H), 8.00–7.92 (m, 2H), 7.54–7.47 (m, 1H), 7.45–7.39 (m, 1H), 7.16–7.08 (m, 4H), 6.87–6.81 (m, 4H), 5.01 (d, J = 6.7 Hz, 1H), 4.77–4.61 (m, 4H), 4.60–4.55 (m, 1H), 4.32 (dd, J = 5.8 and 9.8 Hz, 0.2H), 4.28 (dd, J = 6.1 and 8.6 Hz, 0.8H), 3.78 (d, J = 2.8 Hz, 6H), 2.97 (dd, J = 3.8 and 14.4 Hz, 1H), 2.80–2.73 (m, 1H), 1.36–1.29 (m, 1H), 1.27–1.19 (m, 2H), 0.80 (dd, J = 4.1 and 6.5 Hz, 1H), 0.80–0.58 (m, 6H). HRMS (ESI): $[M + H]^+$ calculated for $C_{38}H_{45}N_6O_5S^+$, 697.3167; found, 697.3148.

4.2.6. (2S)-2-acetamido-N-(1-(benzo[d]thiazol-2-yl)-3-(2-(bis(4-methoxybenzyl)amino)pyrimidin-5-yl)-1-oxopropan-2-yl)-4-methylpentanamide (8)

To a solution of compound 7 (52 mg, 0.1 mmol, 1.0 eq) in CH_2Cl_2 (2.0 mL) was added Dess-Martin periodinane (0.3M in CH_2Cl_2, 0.5 mL, 0.2 mmol, 2.0 eq), then was stirred at room

temperature for 3 h. The reaction mixture was poured to silica gel directly then was purified by flash column chromatography (eluting with hexane/EtOAc, 5:1 to 1:1). The product can be afforded (27 mg, 0.04 mmol) in 52% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 8.23–8.12 (m, 3H), 8.01–7.94 (m, 1H), 7.60–7.54 (m, 2H), 7.12 (d, J = 8.1 Hz, 4H), 6.82 (d, J = 8.1 Hz, 4H), 6.12 (d, J = 6.1 Hz, 0.5H), 5.99 (d, J = 5.1 Hz, 1H), 5.92 (d, J = 7.7 Hz, 0.5H), 4.81–4.57 (m, 5H), 3.78 (s, 6H), 3.33–3.28 (m, 1H), 3.09–3.02 (m, 1H), 1.95 (d, J = 16.8 Hz, 3H), 1.63–1.50 (m, 3H), 0.91 (d, J = 3.9 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 191.94, 191.81, 172.13, 171.73, 170.49, 170.40, 163.47, 161.65, 161.56, 158.70, 158.45, 153.48, 137.27, 130.34, 130.30, 128.94, 128.86, 128.25, 128.19, 127.34, 127.28, 125.85, 122.43, 116.79, 113.86, 56.47, 55.27, 51.64, 48.22, 48.08, 41.04, 40.54, 32.86, 32.58, 24.85, 24.75, 23.12, 22.97, 22.80, 22.24. HRMS (ESI): $[M + H]^+$ calculated for $C_{38}H_{43}N_6O_5S^+$, 695.3010; found, 695.2980.

4.2.7. (2S)-2-acetamido-N-(3-(2-aminopyrimidin-5-yl)-1-(benzo[d]thiazol-2-yl)-1-oxopropan2-yl)-4-methylpentanamide (9)

To a solution of compound 8 (27 mg, 0.04 mmol) in CH_2Cl_2 (2.0 mL) was added TFA/water mixture (97.5:2.5, v/v) 2.0 mL then stirred at 50 ℃ for 18 h. The reaction mixture was concentrated under reduced pressure and diluted with a mixture of ACN and water (1:1, v/v). The solution was filtered through a 0.45 μm PTFE filter and the filter was washed with a mixture of ACN and water (1:1, v/v). The filtrate was purified by RP-HPLC using 0.1% TFA in water (A)/ACN (B) to give the product (6.0 mg, 0.01 mmol) in 32% yield as ivory power. The mobile phase on semi-preparative RP-HPLC was consisted of 30% B at 2 mL/min flow rate. R_t = 7.05 and 8.37 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.45 (s, 2H), 8.20 (dd, J = 8.2 and 11.9 Hz), 8.13 (t, J = 8.3 Hz), 7.68–7.58 (m, 2H), 5.85 (q, J = 4.3 Hz, 0.5H), 5.58 (q, J = 4.8 Hz, 0.5H), 4.30–4.23 (m, 1H), 3.39–3.34 (m, 1H), 3.08–3.00 (m, 1H), 1.94 (d, J = 10.6Hz,

3H), 1.59–1.25 (m, 3H), 0.84–0.76 (m, 6H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 192.42, 192.21, 175.13, 174.99, 173.45, 173.39, 165.34, 159.00, 158.81, 154.71, 154.62, 138.50, 138.31, 137.04, 136.87, 129.47, 129.31, 128.59, 128.49, 126.54, 126.43, 124.30, 123.83, 123.78, 123.17, 121.02, 120.76, 57.27, 56.59, 53.45, 53.31, 41.52, 41.47, 41.36, 31.61, 31.14, 25.73, 23.15, 23.01, 22.34, 22.30, 22.08, 21.94, 21.89. HRMS (ESI): $[M + Na]^+$ calculated for $C_{22}H_{26}N_6NaO_3S^+$, 477.1679; found, 477.1669.

4.2.8. (E)-methyl 2-acetamido-3-(pyridin-4-yl)acrylate (10)

N-acetylglycine (1000mg, 8.5 mmol, 1.0 eq), 4-pyridinecarboxaldehyde (0.8 mL, 8.5 mmol, 1.0 eq), sodium acetate (701 mg, 8.5 mmol, 1.0 eq), and acetic anhydride (1.5 mL, 25.6 mmol, 3.0 eq) were added to dried round bottom flask. The reaction mixture was plunged quickly at 90–100 ℃ for exactly 1 min. The mixture became deep purple color, then the reaction mixture was cooled at room temperature. The reaction mixture was washed several times by hexane then suspended in methanol (20 mL). The reaction mixture was heat up to 100 ℃ until desolated completely. The color of mixture turned to deep orange. The solvent was evaporated under reduced pressure and The residue was diluted by EtOAc and partitioned between EtOAc and water. The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (eluting with CH_2Cl_2/methanol, 80:1 to 25:1) on silica gel to afford the product (816 mg, 3.7 mmol) in 43% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 8.52 (d, J = 4.2 Hz, 2H), 8.18 (s, 1H), 7.28 (d, J = 13.2 Hz, 2H), 7.19 (s, 1H), 3.86 (s, 3H), 2.09 (s, 3H). LRMS (ESI): $[M + Na]^+$ calculated for $C_{11}H_{12}N_2NaO_3^+$, 243.1; found, 243.1.

4.2.9. tert-Butyl 4-(2-acetamido-3-methoxy-3-oxopropyl)piperidine-1-carboxylate (11)

Compound 10 (240 mg, 1.1 mmol, 1.0 eq) was dissolved in acetic acid (10 mL), then was added PtO_2(IV) (15 mg, 0.1 mmol, 0.06 eq). The mixture was stirred at room temperature for 24 h under hydrogen gas (1 atm) atmosphere using balloon. The reaction mixture was filtered through celite then evaporated in vacuo. The residue was washed by toluene several times to remove acetic acid completely. The residue dissolved in THF was added DMAP (27 mg, 0.2 mmol, 0.2 eq) and di-tert-butyldicarbonate (240 mg, 1.1 mmol, 1.0 eq) then stirring at room temperature for 2 h. The reaction mixture was diluted by EtOAc and partitioned between EtOAc and water. The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (eluting with CH_2Cl_2/methanol, 50:1 to 30:1) on silica gel to afford the product in quantitative yield (358 mg, 1.1 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 6.76 (d, J = 8.4 Hz, 1H), 4.54–4.46 (m, 1H), 3.92 (d, J = 10.5 Hz, 2H), 3.58 (s, 3H), 2.52 (t, J = 11.4Hz, 2H), 1.88 (s, 3H), 1.65–1.40 (m, 5H), 1.30 (s, 9H), 1.09–0.89 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 173.23, 170.21, 154.64, 79.22, 52.41, 49.70, 38.92, 32.48, 32.14, 31.25, 28.31, 22.78. HRMS (ESI): $[M - H]^-$ calculated for $C_{16}H_{27}N_2O_5^-$, 327.1920; found, 327.1928.

4.2.10. tert-Butyl 4-(2-acetamido-3-(benzo[d]thiazol-2-yl)-3-oxopropyl)piperidine-1-carboxylate (12)

This compound was afforded by typical procedure A with compound 11 and benzothiazole in 80% yield. ^{1}H NMR (300 MHz, CDCl3): δ ppm 8.16 (dd, J = 1.4 and 7.5 Hz, 1H), 7.95 (dd, J = 1.4 and 7.3 Hz, 1H), 7.62–7.48 (m, 2H), 6.53 (d, J = 8.6 Hz, 1H), 5.94 (dt, J = 3.5 and 7.8 Hz, 1H), 4.19–3.93 (br, 2H), 2.68 (q, J = 8.5 and 11.2 Hz, 2H), 2.06 (s, 3H), 2.02–1.90 (m, 2H), 1.70–1.51 (m, 3H), 1.42 (s, 9H), 1.31–1.10 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$):

δ ppm 193.86, 170.05, 163.61, 154.80, 153.48, 137.19, 128.09, 127.19, 125.83, 122.35, 79.29, 53.15, 39.88, 33.00, 32.61, 31.24, 28.46, 23.22. HRMS (ESI): $[M - H]^-$ calculated for $C_{22}H_{28}N_3O_4S^-$, 430.1801; found, 430.1827.

4.2.11. tert-Butyl 4-(2-((S)-2-acetamido-4-methylpentanamido)-3-(benzo[d]thiazol-2-yl)-3-hydroxypropyl)piperidine-1-carboxylate (13)

This compound was afforded in 38% yield, following the same procedure described for synthesis of compound 7 with 12 instead of 6. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 7.94–7.82 (m, 2H), 7.51-7.30 (m, 2H), 7.08 (d, J = 8.1 Hz, 0.2H), 6.73 (d, J = 7.9 Hz, 0.5H), 6.35 (d, J = 7.7 Hz, 0.3H), 5.78 (d, J = 5.7 Hz, 0.6H), 5.44 (d, J = 5.6 Hz, 0.4H), 5.26–5.12 (m, 2H), 4.73–4.35 (m, 2H), 3.99 (brs, 2H), 2.57 (dd, J = 12.7 and 14.6 Hz, 2H), 1.95 (s, 1H), 1.91 (s, 2H), 1.80–1.45 (m, 6H), 1.40 (s, 9H), 1.31–1.15 (m, 1H), 1.13-1.00 (m, 1H), 0.93 (dd, J = 4.4 and 6.1 Hz, 3H), 0.85–0.81 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 173.41, 173.09, 171.06, 170.58, 154.77, 154.71, 153.25, 153.03, 134.80, 134.69, 126.12, 126.04, 125.12, 124.96, 122.92, 121.80, 121.76, 79.28, 74.15, 53.04, 52.27, 52.14, 41.15, 40.88, 35.93, 34.69, 32.81, 32.65, 28.43, 24.91, 24.76, 23.10, 22.97, 22.73, 22.61, 22.44, 22.27. HRMS (ESI): $[M + Na]^+$ calculated for $C_{28}H_{42}N_4NaO_5S^+$, 569.2768; found, 569.2786.

4.2.12. tert-Butyl 4-(2-((S)-2-acetamido-4-methylpentanamido)-3-(benzo[d]thiazol-2-yl)-3-oxopropyl)piperidine-1-carboxylate (14)

This compound was afforded in 92% yield, following the same procedure described for synthesis of compound 8 with 13 instead of 7. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 8.20–8.10 (m, 1H), 7.99–7.89 (m, 1H), 7.60–7.48 (m, 2H), 7.44 (d, J = 8.4 Hz, 0.5H), 7.10 (d, J =

8.0 Hz, 0.5H), 6.46 (dd, J = 8.5 and 11.2 Hz, 1H), 5.91–5.74 (m, 1H), 4.70–4.49 (m, 1H), 2.67 (t, J = 12.3 Hz, 2H), 2.02–1.86 (m, 2H), 1.97 (d, J = 0.7 Hz, 3H), 1.75–1.45 (m, 7H), 1.43 (d, J = 3.5 Hz, 9H), 1.32–1.03 (m, 3H), 0.93 (t, J = 6.0 Hz, 3H), 0.87 (d, J = 6.0 Hz, 3H). HRMS (ESI): $[M + Na]^+$ calculated for $C_{28}H_{40}N_4NaO_5S^+$, 567.2612; found, 567.2627.

4.2.13. (2S)-2-acetamido-N-(1-(benzo[d]thiazol-2-yl)-1-oxo-3-(piperidin-4-yl)propan-2-yl)-4-methylpentan-amide (15)

To a solution of compound 14 (112 mg, 0.2 mmol) in CH_2Cl_2 (2.0 mL) was dropwisely added TFA (1.0 mL). The reaction mixture was stirred at room temperature for 1 h, then was evaporated in vacuo. The residue was purified by flash column chromatography (eluting with CH_2Cl_2/methanol, 80:1 to 10:1) on silica gel to afford the product in quantitative yield (93 mg, 0.2 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 8.17 (d, J = 7.7 Hz 1H), 8.13–8.06 (m, 1H), 7.67–7.53 (m, 2H), 5.81–5.60 (m, 1H), 4.37 (q, J = 8.6 Hz, 1H), 3.47–3.29 (m, 2H), 3.06–2.86 (m, 2H), 2.07–1.87 (m, 3H), 1.96 (s, 3H), 1.88–1.69 (m, 2H), 1.68–1.41 (m, 5H), 0.95–0.85 (m, 6H). HRMS (ESI): $[M + H]^+$ calculated for $C_{23}H_{33}N_4O_3S^+$, 445.2268; found, 445.2287.

4.2.14. (2S)-2-acetamido-N-(1-(benzo[d]thiazol-2-yl)-3-(1-carbamimidoylpiperidin-4-yl)-1-oxopropan-2-yl)-4-methylpen-tanamide (16)

To a solution of compound 15 (58 mg, 0.1 mmol) and 1-amidinopyrazole hydrochloride (134 mg, 0.9 mmol, 7.0 eq) in N,N-dimethylformamide (DMF, 2.0 mL) was added DIPEA (0.15 mL, 0.9 mmol, 7.0 eq) under argon atmosphere. The reaction mixture was stirred at room temperature for 12 h then concentrated under reduced pressure. The residue was added diethyl ether, then the precipitated crude product was collected by centrifugation,

followed by carefully decanting out the ether solvent. The methanol solution of precipitated crude was filtered through a 0.45 μm PTFE filter and the filter was washed with methanol. The filtrate was purified by RP-HPLC using 0.1% TFA in water (A)/ACN (B) to give compound 16 (41 mg, 0.1 mmol) in 65% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 35% B at 2 mL/min flow rate. R_t = 7.66 and 8.85 min. ^{1}H NMR (300 MHz, CD_3OD): δ ppm 8.23–8.15 (m, 1H), 8.12 (dd, J = 1.3 and 6.4 Hz, 1H), 7.69–7.56 (m, 2H), 5.85–5.66 (m, 1H), 4.41 (dd, J = 6.6 and 8.2 Hz, 1H), 3.89 (t, J = 14.5 Hz, 2H), 3.18–2.98 (m, 2H), 2.16–1.90 (m, 2.5H), 1.98 (s, 3H), 1.90–1.71 (m, 2.5H), 1.71–1.59 (m, 1H), 1.58–1.48 (m, 2H), 1.46–1.23 (m, 2H), 0.94 (d, J = 6.4 Hz, 3H), 0.90 (dd, J = 1.8 and 6.2 Hz, 3H). ^{13}C NMR (75 MHz, CD_3OD): δ ppm 192.76, 192.51, 173.89, 173.71, 172.05, 171.98, 164.22, 164.15, 156.22, 153.39, 153.35, 136.97, 127.92, 127.09, 125.36, 125.04, 122.77, 122.37, 52.92, 52.09, 45.60, 45.55, 40.37, 36.95, 36.73, 32.27, 32.16, 31.61, 30.16, 24.55, 24.41, 21.86, 21.05, 20.96, 20.68, 20.62. HRMS (ESI): $[M + H]^+$ calculated for $C_{24}H_{35}N_6O_3S^+$, 487.2486; found, 487.2510.

4.2.15.

(2S)-3-{4-[({[(benzyloxy)carbonyl]amino}({[(benzyloxy)carbonyl]imino})methyl)amino]phenyl}-2-{[(tert-butoxy)carbonyl]amino}propanoic acid (17a)

To a solution of N-(tert-butoxycarbonyl)-4-nitro-L-phenylalanine (133 mg, 0.4 mmol) in dry methanol (4.0 mL) was added 10% Pd/C (5 mg, 0.04 mmol, 0.1 eq) and stirred under 50 psi of hydrogen gas for 3 h using Parr Hydrogenation Apparatus. The mixture was filtered through celite and concentrated under reduced pressure. The reaction mixture was added N,N'-bis(barbobenzoxy)-1H-pyrazole -1-carboxamidine (195 mg, 0.5 mmol, 1.2 eq) and CH_2Cl_2 (3.0 mL). To the reaction mixture was added triethylamine (0.09 mL, 0.7 mmol, 1.5 eq) under argon

atmosphere then stirred 6 h at room temperature. The mixture was partitioned between water and CH_2Cl_2 then neutralized by 1N HCl. The combined organic layer was collected, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (eluting with CH_2Cl_2/methanol, 100:1 to 11:1, v/v) on silica gel to afford the product (218 mg, 0.4 mmol) in 86% yield. ^{1}H NMR (300 MHz, CD_3OD): δ ppm 7.38 (d, J = 7.7 Hz, 2H), 7.35–7.20 (m, 10H), 7.14 (d, J = 8.3 Hz, 2H), 5.12 (s, 4H), 4.30–4.12 (m, 1H), 3.15–3.02 (m, 1H), 2.93–2.76 (m, 1H), 1.32 (s, 9H). ^{13}C NMR (75 MHz, CD_3OD): δ ppm 175.77, 156.30, 153.65, 135.08, 134.70, 129.50, 128.18, 127.95, 122.56, 78.98, 67.70, 55.66, 37.03, 27.35. HRMS: $[M + H]^+$ calculated for $C_{31}H_{35}N_4O_8^+$, 591.2449; found, 591.2473.

4.2.16.

(2S)-3-{4-[({[(benzyloxy)carbonyl]amino}({[(benzyloxy)carbonyl]imino})methyl)amino]cyclohexyl}-2-{[(tert-butoxy)carbonyl]amino}propanoic acid (17b)

To a solution of N-(tert-butoxycarbonyl)-4-nitro-L-phenylalanine (161 mg, 0.5 mmol, 1.0 eq) in methanol/acetic acid (3.0 mL, 1:1, v/v) was added PtO_2(IV) (12 mg, 0.05 mmol, 0.1 eq). The reaction mixture was stirred under 50 psi of hydrogen gas for 24 h using Parr Hydrogenation Apparatus. The mixture was filtered through celite and concentrated under reduced pressure. The reaction mixture was added N,N'-bis(barbobenzoxy)-1H-pyrazole-1-carboxamidine (216 mg, 0.6 mmol, 1.1 eq) and DMF (3.0 mL). To the reaction mixture was added triethylamine (0.1 mL, 0.8 mmol, 1.5 eq) under argon atmosphere then stirred 6 h at room temperature. The mixture was evaporated in vacuo and partitioned between water and EtOAc. The combined organic layer was collected, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (eluting with CH_2Cl_2/methanol, 100:1 to 11:1, v/v) on silica gel to afford the

product as a colorless solid (198 mg, 0.3 mmol) in 64% yield. The analytical data were same as reference [58].

4.2.17. Benzyl N-({[(benzyloxy)carbonyl]amino}({4-[(2S)-2-{[(tert-butoxy)carbonyl]amino}-2-[methoxy(methyl)carbamoyl]ethyl]phenyl}amino)methylidene)carbamate (18a)

This compound was afforded by typical procedure B with compound 17a in 90% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 11.92 (s, 1H), 10.24 (s, 1H), 7.52 (d, J = 8.4 Hz, 2H), 7.46–7.28 (m, 10H), 7.16 (d, J = 8.3 Hz, 2H), 5.24 (s, 2H), 5.20 (brs, 1H), 5.16 (s, 2H), 4.94 (d, J = 6.5 Hz, 1H), 3.69 (s, 3H), 3.23 (s, 3H), 3.04 (dd, J = 5.9 and 13.6 Hz, 1H), 2.87 (dd, J = 7.1 and 13.5 Hz, 1H), 1.42 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 172.16, 163.83, 155.21, 153.94, 153.55, 136.59, 135.02, 134.48, 133.72, 129.91, 128.76, 128.56, 128.43, 128.06, 127.98, 122.49, 79.64, 68.48, 67.40, 61.60, 51.51, 38.14, 32.09, 28.35. HRMS: $[M + H]^+$ calculated for $C_{33}H_{40}N_5O_8^+$, 634.2871; found, 634.2894.

4.2.18. Benzyl N-({[(benzyloxy)carbonyl]amino}({4-[(2S)-2-{[(tert-butoxy)carbonyl]amino}-2-[methoxy(methyl)carbamoyl]ethyl]cyclohexyl}amino)methylidene)carbamate (18b)

This compound was afforded by typical procedure B with compound 17b in 89% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 11.78 (s, 1H), 8.58 (d, J = 7.9 Hz, 0.5H), 8.19 (d, J = 8.1 Hz, 0.5H), 7.43-7.23 (m, 10H), 5.16 (d, J = 6.2 Hz, 2H), 5.13-5.04 (m, 3H), 4.73 (brs, 1H), 4.27 (m, 0.5H), 3.95 (m, 0.5H), 3.77 (d, J = 5.6 Hz, 3H), 3.19 (d, J = 6.0 Hz, 3H), 2.02 (m, 2H), 1.89-1.50 (m, 5H), 1.43 (s, 9H), 1.39-0.95 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 163.90,

163.87, 155.66, 155.21, 155.07, 154.01, 153.87, 136.93, 136.92, 134.69, 134.63, 128.75, 128.68, 128.54, 128.40, 128.38, 128.18, 128.09, 128.06, 127.86, 79.59, 68.04, 67.09, 61.57, 49.64, 48.44, 46.61, 40.08, 39.03, 33.12, 32.59, 32.42, 32.32, 32.18, 30.42, 29.69, 29.38, 29.10, 28.73, 28.36, 26.91. HRMS (ESI): $[M+Na]^+$ calculated for $C_{33}H_{45}N_5NaO_8^+$, 662.3160; found, 662.3174.

4.2.19. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-{[(tert-butoxy)carbonyl]amino}-3-oxopropyl]phenyl}amino) ({[(benzyloxy)carbonyl]amino})methylidene]carbamate (19a)

This compound was afforded by typical procedure A with compound 18a and benzothiazole in 89% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 11.89 (s, 1H), 10.22 (s, 1H), 8.23 (d, J = 8.0 Hz, 1H), 7.99 (d, J = 7.8 Hz, 1H), 7.59 (t, J = 7.3 Hz, 1H), 7.55 (t, J = 7.4 Hz, 1H), 7.49 (d, J = 8.0 Hz, 2H), 7.44–7.25 (m, 10H), 7.08 (d, J = 7.6 Hz, 2H), 5.86 (d, J = 4.6 Hz, 1H), 5.28 (d, J = 7.4 Hz, 1H), 5.23 (s, 2H), 5.14 (s, 2H), 3.43 (dd, J = 3.3 and 13.4 Hz, 1H), 3.21 (dd, J = 6.2 and 13.1 Hz, 1H), 1.41 (s, 9H). ^{13}C NMR (150 MHz, $CDCl_3$): δ ppm 192.91, 163.83, 155.12, 153.94, 153.61, 153.48, 137.27, 136.60, 135.25, 134.47, 132.97, 130.05, 128.93, 128.77, 128.58, 128.43, 128.07 128.05, 127.98, 127.19, 126.86, 126.66, 125.88, 124.11, 122.46, 122.43, 122.06, 80.00, 68.51, 67.44, 57.53, 38.07, 29.72, 28.34. HRMS: $[M + H]^+$ calculated for $C_{38}H_{38}N_5O_7S^+$, 708.2486; found, 708.2497.

4.2.20. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-{[(tert-butoxy)carbonyl]amino}-3-oxopropyl]cyclohexyl}ami-no)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (19b)

This compound was afforded by typical procedure A with compound 18b and

benzothiazole in 85% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 11.79 (s, 1H), 8.20 (dd, J = 1.4 and 7.0 Hz, 2H), 7.98 (dd, J = 1.4 and 7.1 Hz, 1H), 7.63–7.50 (m, 2H), 7.43–7.27 (m, 10H), 5.64 (brs, 0.5H), 5.27 (d, J = 8.3 Hz, 0.5H), 5.15 (d, J = 10.7 Hz, 4H), 3.97 (m, 1H), 2.24-1.97 (m, 3H), 1.95–1.71 (m, 2H), 1.70–1.47 (m 3H), 1.45 (s, 9H), 1.31–1.05 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 194.50, 163.91, 163.86, 155.22, 153.88, 153.59, 137.23, 136.94, 134.69, 128.76, 128.69, 128.41, 128.39, 128.06, 127.95, 127.86, 127.11, 125.87, 122.34, 80.02, 68.06, 67.11, 54.75, 49.61, 40.19, 33.63, 32.54, 32.40, 32.08, 30.49, 29.70, 28.32. HRMS (ESI): $[M + H]^+$ calculated for $C_{38}H_{44}N_5O_7S^+$, 714.2956; found, 714.2955.

4.2.21. Benzyl N-[({4-[2-amino-3-(1,3-benzothiazol-2-yl)-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (20a)

To a solution of compound 19a (354 mg, 0.5 mmol) in CH_2Cl_2 (6.0 mL) was added TFA (2.0 mL), triethylsilane (0.2 mL), and water (0.1 mL). The reaction mixture was stirred at room temperature for 2 h and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 and evaporated several times until the residue was prepared in powder. The product was recrystallized by diethyl ether and CH_2Cl_2 (10:1, v/v) and afforded as orange powder of TFA salt form (307 mg, 0.4 mmol) in 85% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 8.19 (d, J = 7.7 Hz, 1H), 8.00 (d, J = 7.7 Hz, 1H), 7.65–7.56 (m, 2H), 7.48–7.22 (m, 14H), 7.18–7.11 (m, 1H), 5.44–5.34 (m, 1H) 5.26–5.14 (m, 2H), 5.09–5.00 (m, 2H), 3.83–3.76 (m, 1H), 3.21–3.13 (m, 1H). ^{13}C NMR (150 MHz, $CDCl_3$): δ ppm 189.01, 188.92, 161.94, 161.54, 154.23, 153.98, 153.68, 153.55, 153.33, 153.25, 152.97, 137.38, 136.80, 136.63, 135.82, 135.03, 134.27, 132.09, 131.66, 130.92, 130.75, 130.60, 130.21, 129.00, 128.79, 128.61, 128.52, 128.17, 128.10, 128.04, 127.54, 127.43, 126.88, 126.67, 126.34, 126.02, 125.36, 124.09, 123.94, 122.51, 122.37, 122.25, 122.14, 122.06, 121.90, 68.77, 68.66, 67.67, 57.81, 57.75,

36.03. HRMS (ESI): $[M + H]^+$ calculated for $C_{33}H_{30}N_5O_5S^+$, 608.1962; found, 608.1974.

4.2.22. Benzyl N-[({4-[2-amino-3-oxo-3-(1,3-thiazol-2-yl)propyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (20b)

This compound was afforded in 63% yield, following the same procedure described for synthesis of compound 20a with 19b instead of 19a. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 8.64 (d, J = 7.6 Hz, 1H), 8.29–8.22 (m, 1H), 8.19 (dd, J = 8.2 and 22.4 Hz, 0.6H), 7.99 (d, J = 7.6 Hz, 0.4H) 7.95–7.90 (m, 1H), 7.65–7.52 (m, 2H), 7.41–7.26 (m, 10H), 5.38–5.29 (m, 1H) 5.15–5.06(m, 4H), 4.33–4.25 (m, 0.7H), 4.02–3.94 (m, 0.3H), 2.37–1.58 (m, 8H), 1.48–1.05 (m, 3H). HRMS (ESI): $[M + H]^+$ calculated for $C_{33}H_{36}N_5O_5S^+$, 614.2432; found, 614.2401.

4.2.23. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamido-4-methylpentanamido]-3-oxopropyl]phe-nyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (21a)

This compound was afforded by typical procedure C with compound 20a in 65% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 8.24 (t, J = 7.7 Hz, 1H), 8.00 (dd, J = 5.0 and 7.7 Hz, 1H), 7.64–7.53 (m, 2H), 7.48 (d, J = 8.2 Hz, 1H), 7.42 (d, J = 8.4 Hz, 1H), 7.42–7.26 (m, 10H), 7.11 (t, J = 7.3 Hz, 2H), 6.96 (d, J = 8.0 Hz, 0.5H), 6.87 (d, J = 7.7 Hz, 0.5H), 6.12–6.00 (m, 1H), 5.91 (d, J = 8.3 Hz, 0.5H), 5.85 (d, J = 8.3 Hz, 0.5H), 5.24 (s, 2H), 5.17–5.06 (m, 2H), 4.51–4.39 (m, 1H), 3.54–3.43 (m, 1H), 3.25–3.11 (m, 1H), 1.96 (d, J = 16.7 Hz, 3H), 1.69–1.37 (m, 3H), 0.89–0.84 (m, 6H). HRMS (ESI): $[M + H]^+$ calculated for $C_{41}H_{42}N_6O_7S$, 736.2908; found, 736.2941.

4.2.24. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamido-4-methylpentanamido]-3-oxopropyl]cyclohexyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carba-mate (21b)

This compound was afforded by typical procedure C with compound 20b in 76% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 8.27–8.16 (m, 1H), 8.04–7.93 (m, 1H), 7.65–7.45 (m, 2H), 7.43–7.27 (m, 10H), 7.21–7.08 (m, 0.5H), 6.96–6.79 (m, 0.5H), 6.09 (dd, J = 8.3 and 22.4 Hz, 0.5H), 5.89–5.73 (m, 0.5H), 5.19–5.04 (m, 4H), 4.97–4.48 (m, 0.5H), 4.34–4.17 (m, 0.5H), 2.37–2.16 (m, 1H), 2.14–1.97 (m, 4H), 1.93–1.37 (m, 11H), 1.18–1.08 (m, 3H), 1.00–0.92 (m, 3H), 0.92–0.86 (m, 3H). HRMS (ESI): $[M + H]^+$ calculated for $C_{41}H_{48}N_6O_7S^+$, 769.3378; found, 769.3378.

4.2.25. (2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-aceta mido-4-methylpentanamide (22a)

This compound was afforded by typical procedure D with compound 21a in 31% yield as white powder. The mobile phase on semi-preparative RP-HPLC was consisted of 30% B at 2 mL/min flow rate. R_t = 16.58 and 20.28 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.25 (d, J = 8.1 Hz, 1H), 8.14 (d, J = 8.0 Hz, 1H), 7.70–7.59 (m, 2H), 7.42 (dd, J = 8.3 and 15.1 Hz, 2H), 7.21 (dd, J = 8.3 and 11.9 Hz, 2H), 5.94–5.85 (m, 1H), 4.38–4.26 (m, 1H), 3.56 (dt, J = 4.5 and 14.0 Hz, 1H), 3.11–3.03 (m, 1H), 1.92 (d, J = 14.3 Hz, 3H), 1.64–1.35 (m, 3H), 0.91–0.79 (m, 6H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 193.19, 193.00, 175.01, 174.88, 173.19, 173.11, 165.53, 165.51, 158.19, 158.16, 154.81, 154.79, 138.40, 138.31, 138.25, 134.79, 134.70, 132.13, 132.07, 129.40, 129.37, 128.56, 128.54, 126.77, 126.68, 126.55, 123.80, 58.05, 58.02, 53.34, 53.08, 41.73, 41.62, 37.79, 37.51, 25.80, 23.26, 23.22, 22.36, 22.29, 21.94.

HRMS: $[M + H]^+$ calculated for $C_{25}H_{31}N_6O_3S^+$, 495.2173; found, 495.2196.

4.2.26.

(2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidocyclohexyl)-1-oxopropan-2-yl]-2-acetamido-4-methylpentanamide (22b)

This compound was afforded by typical procedure D with compound 21b in 44% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 35% B at 2 mL/min flow rate. R_t = 8.42, 9.15, 9.83 and 11.17 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.17 (d, J = 7.7 Hz, 1H), 8.10 (d, J = 8.0 Hz, 1H), 7.68–7.53 (m, 2H), 5.81–5.64 (m, 1H), 4.51–4.36 (m, 1H), 3.70–3.65 (m, 0.5H), 3.40–3.25 (m, 0.5H), 2.19–1.99 (m, 2H), 1.98 (s, 3H), 1.89–1.50 (m, 9H), 1.48–1.20 (m, 3H), 0.99–0.85 (m, 6H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 194.55, 194.50, 194.29, 194.24, 175.27, 175.18, 174.96, 173.31, 173.27, 165.72, 165.68, 157.79, 157.71, 154.82, 154.80, 138.39, 129.31, 129.29, 128.48, 128.46, 126.42, 123.81, 123.79, 54.79, 54.70, 54.68, 53.32, 53.30, 53.20, 53.16, 51.98, 51.91, 42.00, 41.87, 39.09, 38.93, 34.88, 34.71, 33.58, 33.51, 33.41, 33.33, 33.00, 32.97, 31.31, 31.25, 30.08, 29.90, 29.30 HRMS (ESI): $[M + H]^+$ calculated for $C_{25}H_{37}N_6O_3S^+$, 501.2642; found, 501.2647.

4.2.27. Benzyl N-({[(benzyloxy)carbonyl]amino}({[4-(2-{[(tert-butoxy)carbonyl]amino}-3-oxo-3-(1,3-thiazol-2-yl)propyl)phenyl]amino})methylidene)carbamate (23a)

This compound was afforded by typical procedure A with compound 18a and thiazole in 90% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 11.89 (s, 1H), 10.22 (s, 1H), 8.06 (d, J = 3.0 Hz, 1H), 7.70 (d, J = 2.6 Hz, 1H), 7.48 (d, J = 8.2 Hz, 2H), 7.42–7.27 (m, 10H), 7.05 (d, J =

7.6 Hz, 2H), 5.70 (d, J = 5.4 Hz, 1H), 5.26 (d, J = 7.7 Hz, 1H), 5.22 (s, 2H), 5.14 (s, 2H), 3.36 (dd, J = 3.7 and 13.4 Hz, 1H), 3.12 (dd, J = 6.5 and 13.6 Hz, 1H), 1.40 (s, 9H). ^{13}C NMR (150 MHz, $CDCl_3$): δ ppm 191.33, 164.63, 163.83, 155.11, 153.94, 145.23, 136.59, 135.18, 134.46, 133.10, 130.00, 128.92, 128.76, 128.57, 128.43, 128.08, 127.98, 126.81, 122.47, 79.91, 68.51, 67.43, 57.45, 38.04, 29.72, 28.32. HRMS (ESI): $[M + H]^+$ calculated for $C_{34}H_{36}N_5O_7S^+$, 658.2330; found, 658.2332.

4.2.28. Benzyl N-({[(benzyloxy)carbonyl]amino}({[4-(2-{[(tert-butoxy)carbonyl]amino}-3-oxo-3-(1,3-thiazol-2-yl)propyl)cyclohexyl]amino})methylidene)carbamate (23b)

This compound was afforded by typical procedure A with compound 18b and thiazole in 84% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 11.77 (d, J = 5.8 Hz, 1H), 8.57 (d, J = 7.4 Hz, 0.5H), 8.19 (d, J = 8.0 Hz, 0.5H), 8.04 (dd, J = 3.0 and11.3 Hz, 1H), 7.70 (dd, J = 2.6 and 10.4 Hz, 1H), 7.42–7.26 (m, 10H), 5.49 (brs, 1H), 5.24 (t, J = 9.9 Hz, 1H), 5.17 (s, 2H), 5.12 (s, 2H), 4.28 (brs, 0.5H), 4.00–3.91 (m, 0.5H), 2.15–2.05 (m, 1H), 2.04–1.54 (m, 6H), 1.44 (s, 10H), 1.24–1.06 (m, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ ppm 192.90, 164.63, 163.91, 163.88, 155.49, 155.21, 155.08, 154.00, 153.88, 145.21, 145.17, 136.94, 136.92, 134.69, 134.62, 128.77, 128.70, 128.42, 128.40, 128.11, 128.07, 127.88, 126.68, 79.88, 68.06, 68.04, 67.11, 60.40, 54.69, 49.62, 40.19, 33.61, 32.56, 32.39, 32.09, 30.49, 29.71, 29.36, 29.11, 28.72, 28.33, 26.91, 21.06, 14.22. HRMS (ESI): $[M + H]^+$ calculated for $C_{34}H_{42}N_5O_7S^+$, 664.2799; found, 664.2770.

4.2.29. Benzyl

N-[({4-[2-amino-3-oxo-3-(1,3-thiazol-2-yl)propyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (24a)

This compound was afforded in 70% yield, following the same procedure described for synthesis of compound 20a with 23a instead of 19a. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 8.02 (d, J = 3.0 Hz, 1H), 7.73 (d, J = 2.9 Hz, 1H), 7.43–7.33 (m, 10H), 7.29 (t, J = 7.0 Hz, 2H), 7.25–7.21 (m, 3H), 5.28 (dd, J = 4.4 and 9.5 Hz, 1H), 5.19, (dd, J = 12.1 and 32.2 Hz, 2H), 5.06 (s, 2H), 3.64 (dd, J = 4.3 and 14.4 Hz, 1H), 3.16 (dd, J = 9.5 and 14.4 Hz, 1H). ^{13}C NMR (150 MHz, $CDCl_3$): δ ppm 187.35, 163.22, 162.70, 154.30, 153.75, 145.49, 135.99, 135.19, 135.12, 134.34, 131.92, 131.69, 130.51, 130.47, 128.97, 128.78, 128.58, 128.53, 128.16, 128.02, 126.52, 123.96, 122.05, 68.67, 67.54, 57.64, 57.54, 36.04. HRMS (ESI): $[M + H]^+$ calculated for $C_{29}H_{28}N_5O_5S^+$, 558.1806; found, 558.1830.

4.2.30. Benzyl N-[({4-[2-amino-3-oxo-3-(1,3-thiazol-2-yl)propyl]cyclohexyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (24b)

This compound was afforded in 92% yield, following the same procedure described for synthesis of compound 20a with 23b instead of 19b. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 11.75 (brs, 1H), 8.61 (d, J = 6.7 Hz, 0.4H), 8.26 (d, J = 7.2 Hz, 0.6H), 8.07–8.01 (m, 1H), 7.74–7.67 (m, 1H), 7.43–7.27 (m, 10H), 7.20 (dd, J = 8.3 and 16.9 Hz, 0.6H), 6.99–6.89 (m, 0.4H), 6.35–6.18 (m, 1H), 5.73–5.61 (m, 1H), 5.17 (s, 2H), 5.13 (s, 2H), 4.62–4.47 (m, 1H), 4.27 (s, 0.4H), 3.94 (s, 0.6H), 2.57 (brs, 1H), 2.08–1.96 (m, 5H), 1.87–1.46 (m, 8H), 1.44–1.30 (m, 1H), 1.24–1.05 (m, 3H), 1.00–0.87 (m, 6H). HRMS (ESI): $[M + H]^+$ calculated for $C_{29}H_{34}N_5O_5S^+$, 564.2275; found, 564.2258.

4.2.31. Benzyl N-({[(benzyloxy)carbonyl]amino}[(4-{2-[(2S)-2-acetamido-4-methylpentanamido]-3-oxo-3-(1,3-thiazol-2-yl)propyl}phenyl)amino]methylidene)carbamate (25a)

This compound was afforded by typical procedure C with compound 24a in 58% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 11.87 (s, 1H), 10.18 (d, J = 22.8 Hz, 1H), 8.04 (d, J = 5.9 Hz, 1H), 7.68 (d, J = 11.8 Hz, 1H), 7.47 (d, J = 7.9 Hz, 1H), 7.43–7.17 (m, 12H), 7.09 (dd, J = 7.8 and 18.3 Hz, 2H), 6.39–6.21 (m, 1H), 5.97–5.85 (m, 1H), 5.22 (s, 2H), 5.19–5.06 (m, 2H), 4.54–4.42 (m, 1H), 3.45–3.31 (m, 1H), 3.13–3.03 (m, 1H), 1.93 (d, J = 4.4 Hz, 3H), 1.63–1.32 (m, 3H), 0.84 (brs, 6H). HRMS (ESI): $[M + H]^+$ calculated for $C_{37}H_{41}N_6O_7S^+$, 713.2752; found, 713.2780.

4.2.32. N-({[(benzyloxy)carbonyl]amino}[(4-{2-[(2S)-2-acetamido-4-methylpentanamido]-3-oxo-3-(1,3-thiazol-2-yl)propyl}cyclohexyl)amino]methylidene)carbamate (25b)

This compound was afforded by typical procedure C with compound 24b in 80% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 8.61 (d, J = 6.7 Hz, 0.5H), 8.26 (d, J = 7.2 Hz, 0.5H), 8.07–8.22 (m, 1H), 7.74–7.66 (m, 1H), 7.42–7.26 (m, 10H), 7.24–7.17 (m, 0.5H), 6.99–6.89 (m, 0.5H), 6.35–6.18 (m, 1H), 5.74–5.62 (m, 1H), 5.17 (d, J = 2.3 Hz, 2H), 5.13 (s, 2H), 4.61–4.48 (m, 1H), 4.27 (brs, 0.5H), 3.94 (brs, 0.5H), 2.57 (brs, 1H), 2.09–1.94 (m, 4H), 1.89–1.46 (m, 9H), 1.44–1.27 (m, 1H), 1.23–1.05 (m, 3H), 0.99–0.87 (m, 6H). HRMS (ESI): $[M + H]^+$ calculated for $C_{37}H_{47}N_6O_7S^+$, 719.3221; found, 719.3207.

4.2.33.

(2S)-N-[3-(4-carbamimidamidophenyl)-1-oxo-1-(1,3-thiazol-2-yl)propan-2-yl]-2-acetamido-4-methylpentanamide (26a)

This compound was afforded by typical procedure D with compound 25a in 60% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 30% B at 2 mL/min flow rate. R_t = 5.76 and 6.32 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.17 (t, J = 2.5 Hz, 1H), 8.08 (d, J = 2.9 Hz, 1H), 7.40 (dd, J = 8.2 and 16.8 Hz, 2H), 7.22 (dd, J = 8.3 and 11.0 Hz, 2H), 5.83 (dt, J = 4.1 and 9.7 Hz, 1H), 4.40–4.25 (m, 1H), 3.56–3.47 (m, 1H), 3.04–2.96 (m, 1H), 1.95 (d, J = 23.2 Hz, 3H), 1.68–1.42 (m, 3H), 0.97–0.85 (m, 6H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 190.23, 190.05, 173.56, 173.44, 171.80, 164.52, 156.79, 156.76, 145.03, 144.92, 136.93, 133.37, 133.26, 130.70, 130.62, 130.56, 127.52, 127.39, 125.36, 125.27, 56.59, 56.54, 56.50, 52.03, 51.91, 51.71, 51.62, 36.62, 36.50, 36.43, 24.49, 24.38, 21.93, 21.87, 20.53. HRMS (ESI): $[M + H]^+$ calculated for $C_{21}H_{29}N_6O_3S^+$, 445.2016; found, 445.2030.

4.2.34.

(2S)-N-[3-(4-carbamimidamidocyclohexyl)-1-oxo-1-(1,3-thiazol-2-yl)propan-2-yl]-2-acetamido-4-methylpentanamide (26b)

This compound was afforded by typical procedure D with compound 25b in 35% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 30% B at 2 mL/min flow rate. R_t = 5.86, 6.09, 6.43, and 7.00 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.19–8.00 (m, 2H), 5.83 (brs, 2H), 5.67 (t, J = 10.3 Hz, 0.8H), 4.52–4.40 (m, 0.8H), 4.36 (t, J = 7.4 Hz, 0.1H), 4.29 (t, J = 7.4 Hz, 0.1H), 2.06 (brs, 1H), 2.00 (d, J = 7.6 Hz, 3H), 1.96 (s, 3H), 1.86–1.54 (m, 8H), 1.53–1.24 (m, 3H), 1.03–0.84 (m, 6H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 192.80, 192.77, 175.20, 175.01, 173.32, 173.28, 166.14, 157.79, 146.37, 146.21, 146.20,

128.59, 128.58, 54.66, 54.59, 54.56, 54.52, 53.35, 53.22, 53.19, 51.98, 51.90, 42.00, 41.86, 39.22, 39.13, 37.96, 37.77, 34.90, 34.70, 33.59, 33.52, 33.38, 33.30, 33.01, 31.14, 30.08, 29.83, 29.26, 29.24, 26.85, 26.01, 25.86. HRMS (ESI): $[M + H]^+$ calculated for $C_{21}H_{35}N_6O_3S^+$, 451.2486; found, 451.2476.

4.2.35. (2S)-3-{3-[({[(benzyloxy)carbonyl]amino}({[(benzyloxy)carbonyl]imino})methyl)amino]phenyl}-2-{[(tert-butoxy)carbonyl]amino}propanoic acid (27)

This compound was afforded in 60% yield, following the same procedure described for synthesis of compound 17a with N-(tert-butoxycarbonyl)-3-nitro-L-phenylalanine instead of N-(tert-butoxycarbonyl)-4-nitro-L-phenylalanine. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 7.53 (d, J = 7.9 Hz, 1H), 7.44–7.26 (m, 12H), 7.09 (d, J = 7.6, 1H), 5.22 (brs, 4H), 4.38–4.25 (m, 1H), 3.19 (dd, J = 4.6 and 13.9 Hz, 1H), 2.93 (dd, J = 8.9 and 13.9 Hz, 1H), 1.38 (s, 9H). HRMS (ESI): $[M + H]^+$ calculated for $C_{31}H_{35}N_4O_8^+$, 591.2449; found, 591.2467.

4.2.36. Benzyl N-({[(benzyloxy)carbonyl]amino}({3-[(2S)-2-{[(tert-butoxy)carbonyl]amino}-2-[methoxy(methyl)carbamoyl]ethyl]phenyl}amino)methylidene)carbamate (28)

This compound was afforded by typical procedure B with compound 27 in 58% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 11.90 (s, 1H), 10.24 (s, 1H), 7.53 (d, J = 7.8 Hz, 1H), 7.42–7.27 (m, 11H), 7.25 (t, J = 7.9 Hz, 1H), 6.97 (d, J = 7.8 Hz, 1H), 5.24 (s, 2H), 5.19–5.10 (m, 3H), 4.91 (brs, 1H), 3.63 (s, 3H), 3.13 (s, 3H), 3.04 (dd, J = 7.1 and 12.7 Hz, 1H), 2.84 (dd, J = 7.1 and 12.7 Hz, 1H), 1.39 (s, 9H). ^{13}C NMR (150 MHz, $CDCl_3$): δ ppm 172.09, 166.86,

155.19, 153.95, 153.58, 137.66, 136.53, 136.38, 134.49, 128.98, 128.93, 128.77, 128.60, 128.44, 128.16, 128.01, 126.45, 123.28, 121.09, 79.64, 68.51, 67.44, 61.56, 38.65, 28.35, 28.30. HRMS (ESI): $[M + H]^+$ calculated for $C_{33}H_{40}N_5O_8^+$, 634.2871; found, 634.2898.

4.2.37. Benzyl N-[({3-[3-(1,3-benzothiazol-2-yl)-2-{[(tert-butoxy)carbonyl]amino}-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (29)

This compound was afforded by typical procedure A with compound 28 and benzothiazole in 83% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 11.88 (s, 1H), 10.21 (s, 1H), 8.21 (d, J = 8.0Hz, 1H), 7.95 (d, = 7.9 Hz, 1H), 7.62–7.50 (m, 3H), 7.42–7.25 (m, 10H), 7.24–7.16 (m, 2H), 7.00–6.90 (m, 1H), 5.87 (d, J = 5.4 Hz, 1H), 5.40–5.29 (m, 1H), 5.23 (s, 2H), 5.12 (s, 2H), 3.44 (dd, J = 4.7 and 13.7 Hz, 1H), 3.15 (dd, J = 7.3 and 13.6 Hz, 1H), 1.40 (s, 9H). ^{13}C NMR (150 MHz, CDCl3): δ ppm 192.95, 163.83, 155.15, 153.93, 153.62, 153.57, 137.59, 136.98, 136.57, 136.35, 134.51, 129.30, 129.15, 128.90, 128.73, 128.53, 128.32, 128.14, 128.06, 127.93, 127.28, 127.06, 126.77, 126.45, 125.94, 125.82, 123.54, 123.39, 122.49, 122.32, 121.66, 121.45, 80.02, 68.53, 68.36, 67.44, 67.29, 67.11, 57.56, 57.36, 38.47, 28.60, 28.41, 28.21, 28.02. HRMS (ESI): $[M + H]^+$ calculated for $C_{38}H_{38}N_5O_7S^+$, 708.2486; found, 708.2496.

4.2.38. Benzyl N-[({3-[2-amino-3-(1,3-benzothiazol-2-yl)-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (30)

This compound was afforded in 86% yield, following the same procedure described

for synthesis of compound 20a with 29 instead of 19a. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 8.20 (d, J = 7.8 Hz, 1H), 7.96 (d, J = 7.5 Hz, 1H), 7.62–7.54 (m, 2H), 7.44–7.17 (m, 13H), 7.06 (d, J = 7.6 Hz, 1H), 5.51–5.45 (m, 1H), 5.20 (s, 2H), 5.03 (s, 2H), 3.76 (dd, J = 4.1 and 14.5 Hz, 1H), 3.38 (dd, J = 8.6 and 14.6 Hz, 1H). ^{13}C NMR (150 MHz, CDCl3): δ ppm 188.98, 163.72, 162.07, 154.27, 153.77, 153.28, 137.40, 136.65, 136.10, 129.97, 129.21, 128.98, 128.78, 128.64, 128.07, 127.57, 127.48, 126.02, 125.42, 123.59, 123.22, 122.50, 122.27, 68.57, 67.49, 57.91, 36.32. HRMS (ESI): $[M + H]^+$ calculated for $C_{33}H_{30}N_5O_5S^+$, 608.1962; found, 608.1976.

4.2.39. Benzyl N-[({3-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamido-4-methylpentanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (31)

This compound was afforded by typical procedure C with compound 30 in 46% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 8.23 (t, J = 7.9 Hz, 1H), 7.98 (t, J = 8.2 Hz, 1H), 7.62–7.53 (m, 2H), 7.52–7.26 (m, 14H), 7.06–6.97 (m, 2H), 6.16–6.05 (m, 1H), 6.04–5.95 (m, 1H), 5.26 (s, 2H), 5.14 (s, 2H), 4.46–4.31 (m, 1H), 3.64–3.48 (m, 1H), 3.22–3.08 (m, 1H), 1.90 (s, 1.5H), 1.80 (s, 1.5H), 1.59–1.42 (m, 2H), 1.41–1.31 (m, 1H), 0.85–0.81 (m, 6H). HRMS (ESI): $[M + H]^+$ calculated for $C_{41}H_{43}N_6O_7S^+$, 736.2908; found, 763.2945.

4.2.40. (2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(3-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamido-4-methylpentanamide (32)

This compound was afforded by typical procedure D with compound 31 in 60% yield

as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 35% B at 2 mL/min flow rate. R_t = 10.13 and 11.89 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.24 (d, J = 8.1 Hz, 1H), 8.13 (d, J = 7.9 Hz, 1H), 7.69–7.58 (m, 2H), 7.38 (q, J = 7.9 Hz, 1H), 7.33–7.22 (m, 2H), 7.17–7.10 (m, 1H), 5.97–5.89 (m, 1H), 4.36–4.25 (m, 1H), 3.60–3.52 (m, 1H), 3.05 (dt, J = 10.1 and 13.4 Hz, 1H), 1.90 (d, J = 3.7 Hz, 3H), 1.59–1.32 (m, 3H), 0.89–0.80 (m, 6H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 193.05, 192.87, 174.82, 173.21, 173.08, 165.53, 165.50, 158.08, 158.03, 154.77, 154.76, 140.66, 140.62, 138.39, 136.28, 136.15, 131.12, 131.06, 130.02, 129.93, 129.40, 129.37, 128.55, 128.54, 127.57, 127.39, 127.16, 126.51, 124.83, 124.77, 123.79, 57.83, 57.74, 53.24, 53.12, 41.66, 38.09, 37.93, 25.77, 23.22, 23.15, 22.36, 22.32, 22.04, 21.98. HRMS (ESI): $[M + H]^+$ calculated for $C_{25}H_{31}N_6O_3S^+$, 495.2173; found, 495.2190.

4.2.41. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamido-3-phenylpropanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33a)

This compound was afforded by typical procedure C with N-acetyl-L-phenylalanine and compound 20a in 62% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 11.86 (s, 1H), 10.18 (d, J = 6.9 Hz, 1H), 8.22 (dd, J = 3.1 and 8.0 Hz, 1H), 7.99 (dd, J = 8.0 and 11.6 Hz, 1H), 7.63–7.53 (m, 2H), 7.45–7.30 (m, 10H), 7.28 (t, J = 7.2 Hz, 2H), 7.22 (t, J = 7.3 Hz, 0.5H), 7.16 (d, J = 5.5 Hz, 2H), 7.11–7.06 (m, 0.5H), 6.97 (d, J = 8.4 Hz, 1H), 6.81 (d, J = 8.4 Hz, 1H), 6.62 (dd, J = 4.7 and 7.7 Hz, 1H), 6.12–5.90 (m, 2H), 5.23 (s, 2H), 5.13 (s, 1H), 5.13–5.02 (m, 1H), 4.71–4.62 (m, 1H), 3.44–2.92 (m, 4H), 1.93 (d, J = 2.4 Hz, 3H). HRMS (ESI): $[M + H]^+$ calculated for $C_{44}H_{41}N_6O_7S^+$, 797.2752; found, 797.2771.

4.2.42. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamido-3-(4-hydroxyphenyl)propanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylene]carbamate (33b)

This compound was afforded by typical procedure C with N-acetyl-L-tyrosine and compound 20a in 50% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 8.23 (d, J = 8.1 Hz, 1H), 8.00 (d, J = 8.0 and 11.7 Hz, 1H), 7.64–7.54 (m, 2H), 7.43–7.22 (m, 10H), 7.04 (d, J = 8.0 Hz, 1H), 6.97 (dd, J = 8.2 and 15.1 Hz, 2H), 6.92–6.85 (m, 2H), 6.82 (d, J = 7.9 Hz, 1H), 6.77 (d, J = 6.8 Hz, 1H), 6.69 (d, J = 8.3 Hz, 1H), 6.65 (brs, 1H), 6.62 (d, J = 8.3 Hz, 1H), 6.02–5.81 (m, 1H), 5.36–5.06 (m, 5H), 4.57–4.45 (m, 1H), 3.44–3.33 (m, 1H), 3.16–3.05 (m, 1H), 2.96–2.75 (m, 2H), 1.92 (s, 1.3H), 1.89 (s, 1.7H). HRMS (ESI): $[M + Na]^+$ calculated for $C_{44}H_{40}N_6NaO_8S^+$, 835.2521; found, 835.2529.

4.2.43. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-3-cyclohexyl-2-acetamidopropanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33c)

This compound was afforded by typical procedure C with N-acetyl-L-cyclohexlyalanine and compound 20a in 30% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 11.87 (d, J = 4.0 hz, 1H), 10.19 (d, J = 23.3 Hz, 1H), 8.24 (t, J = 8.9 Hz, 1H), 7.99 (t, J = 7.2 Hz, 1H), 7.64–7.53 (m, 2H), 7.48 (d, J = 8.5 Hz, 1H), 7.42 (d, J = 8.4 Hz, 1H), 7.40–7.26 (m, 10H), 7.10 (dd, J = 6.3 and 8.3 Hz, 2H), 6.98 (d, J = 8.3 Hz, 0.5H), 6.90 (d, J = 7.9 Hz, 0.5H), 6.10–6.00 (m, 1H), 5.93 (d, J = 8.2 Hz, 0.5H), 5.87 (d, J = 8.1 Hz, 0.5H), 5.23 (s, 2H), 5.16–5.06 (m, 2H), 4.52–4.43 (m, 1H), 3.53–3.42 (m, 1H), 3.26–3.12 (m, 1H), 1.95 (d, J = 20.2 Hz, 3H), 1.76–1.55 (m, 6H), 1.44–1.29 (m, 1H), 1.27–1.05 (m, 4H), 0.95–0.77 (m, 2H). HRMS (ESI): $[M + Na]^+$ calculated for $C_{44}H_{46}N_6NaO_7S^+$, 825.3041; found, 825.3032.

4.2.44. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamido-3-(1H-indol-3-yl)propanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33d)

This compound was afforded by typical procedure C with N-acetyl-L-tryptophan and compound 20a in 64% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 11.98 (s, 1H), 10.21 (s, 1H), 9.41 (s, 0.8H), 9.29 (s, 0.2H), 8.21 (d, J = 8.1 Hz, 1H), 7.98 (d, J = 8.0 Hz, 1H), 7.83 (d, J = 7.6 Hz, 1H), 7.66–7.50 (m, 2H), 7.46–7.33 (m, 7H), 7.25–7.15 (m, 6H), 7.12–7.03 (m, 2H), 6.94 (d, J = 8.3 Hz, 1H), 6.46 (d, J = 7.1 Hz, 1H), 6.23 (d, J = 1.7 Hz, 1H), 6.03–5.92 (m, 2H), 5.26 (d, J = 6.7 Hz, 2H), 5.19–5.06 (m, 2H), 4.86–4.77 (m, 1H), 3.43–3.12 (m, 2H), 2.97–2.85 (m, 1H), 2.71 (dd, J = 9.9 and 14.5 Hz, 1H), 2.02 (s, 1H), 1.97 (s, 2H). HRMS (ESI): $[M + H]^+$ calculated for $C_{46}H_{42}N_7O_7S^+$, 836.2861; found, 836.2901.

4.2.45. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamidohexanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33e)

This compound was afforded by typical procedure C with N-acetyl-L-norleucine and compound 20a in 77% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 8.24 (dd, J = 8.0 and 12.3Hz, 1H), 8.00 (t, J = 7.2 Hz, 1H), 7.64–7.53 (m, 2H), 7.45–7.27 (m, 13H), 7.24–7.18 (m, 1H), 7.15 (d, J = 7.8 Hz, 1H), 6.90 (d, J = 7.5 Hz, 0.5H), 6.76 (d, J = 7.6 Hz, 0.5H), 6.18–6.02 (m, 2H), 5.34–5.23 (m, 2H), 5.19–5.09 (m, 2H), 4.30 (brs, 0.5H), 4.10 (brs, 0.5H), 3.65–3.51 (m, 1H), 3.18–3.09 (m, 1H), 1.92 (s, 1.5H), 1.86 (s, 1.5H), 1.77–1.67 (m, 1H), 1.55–1.42 (m, 1H), 1.25–1.14 (m, 4H), 0.86–0.78 (m, 3H). HRMS (ESI): $[M + Na]^+$ calculated for $C_{41}H_{42}N_6NaO_7S^+$,

785.2728; found, 785.2727.

4.2.46. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamidopentanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33f)

This compound was afforded by typical procedure C with N-acetyl-L-norvaline and compound 20a in 72% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 8.24 (dd, J = 8.2 and 17.4 Hz, 1H), 8.00 (dd, J = 8.4 and 10.7 Hz, 1H), 7.64–7.53 (m, 2H), 7.47–7.27 (m, 14H), 7.18 (d, J = 8.1 Hz, 1H), 6.93 (d, J = 8.1 Hz, 0.5H), 6.78 (d, J = 8.7 Hz, 0.5H), 6.29 (d, J = 7.9 Hz, 0.5H), 6.23 (d, J = 6.5 Hz, 0.5H), 6.20–6.14 (m, 0.5H), 6.07–6.01 (m, 0.5H), 5.39–5.25 (m, 2H), 5.22–5.09 (m, 2H), 4.27 (q, J = 7.1 Hz, 0.5H), 3.98 (brs, 0.5H), 3.72–3.64 (m, 0.5H), 3.57 (dd, J = 4.9 and 14.1 Hz, 0.5H), 3.16–3.04 (m, 1H), 1.91 (s, 1.5H), 1.81 (s, 1.5H), 1.73–1.62 (m, 1H), 1.54–1.39 (m, 1H), 1.30–1.17 (m, 2H), 0.84 (dt, J = 3.1 and 7.3 Hz, 3H). HRMS (ESI): $[M + Na]^+$ calculated for $C_{40}H_{41}N_6NaO_7S^+$, 771.2571; found, 771.2550.

4.2.47. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamidobutanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33g)

This compound was afforded by typical procedure C with N-acetyl-L-2-aminobutyric acid and compound 20a in 44% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 11.86 (brs, 1H), 10.20 (brs, 1H), 8.24 (t, J = 7.5 Hz, 1H), 8.03–7.96 (m, 1H), 7.64–7.53 (m, 2H), 7.45 (d, J = 8.2 Hz, 1H), 7.43–7.27 (m, 12H), 7.15–7.08 (m, 2H), 6.91 (dd, J = 8.0 and 21. 6 Hz, 1H), 6.18 (t, J = 7.3 Hz, 1H), 6.13–6.03 (m, 1H), 5.24 (s, 2H), 5.13 (s, 2H), 4.33 (q, J = 6.8 Hz, 1H),

3.54–3.45 (m, 1H), 3.25–3.12 (m, 1H), 1.95 (s, 3H), 1.84–1.76 (m, 1H), 1.59–1.48 (m, 1H), 0.83 (dt, J = 7.4 and 20.8 Hz, 3H). HRMS (ESI): $[M + Na]^+$ calculated for $C_{39}H_{38}N_6NaO_7S^+$, 757.2415; found, 757.2419.

4.2.48. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamidopropanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33h).

This conpound was afforded by typical procedure C with N-acetyl-L-alanine and compound 20a in 64% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 8.25 (dd, J = 7.0 and 7.6 Hz, 1H), 8.00 (dd, J = 2.8 and 7.5 Hz, 1H), 7.64–7.54 (m, 2H), 7.46 (d, J = 8.4 Hz, 1H), 7.43–7.27 (m, 11H), 7.11 (dd, J = 8.4 and 14.8 Hz, 2H), 7.07 (d, J = 8.5 Hz, 0.5H), 6.93 (d, J = 8.0 Hz, 0.5H), 6.20 (dd, J = 7.5 and 15.8 Hz, 1H), 6.12–6.01 (m, 1H), 5.24 (s, 2H), 5.16–5.05 (m, 2H), 4.51–4.40 (m, 1H), 3.58–3.45 (m, 1H), 3.26–3.07 (m, 1H), 1.93 (d, J = 21.2 Hz, 3H), 1.28 (d, J = 7.1 Hz, 1.5H), 1.20 (d, J = 7.0 Hz, 1.5H). HRMS (ESI): $[M + H]^+$ calculated for $C_{38}H_{37}N_6O_7S^+$, 721.2439; found, 721.2464.

4.2.49. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-(2-acetamidoacetamido)-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33i)

This compound was afforded by typical procedure C with N-acetylglycine and compound 20a in 61% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 8.22 (d, J = 8.1 Hz, 1H), 7.96 (d, J = 8.0 Hz, 1H), 7.58 (t, J = 7.3 Hz, 1H), 7.53 (t, J = 7.6 Hz, 1H), 7.44–7.26 (m, 12H), 7.14 (d, J = 8.0 Hz, 1H), 7.09 (d, J = 8.2 Hz, 2H), 6.56 (t, J = 4.9 Hz, 1H), 6.06 (q, J = 5.2 and 7.6 Hz, 1H), 5.22 (s, 2H), 5.11 (s, 2H), 3.90 (dd, J = 5.4 and 16.4 Hz, 1H), 3.78 (dd, J = 5.2

and 16.4 Hz, 1H), 3.48 (dd, J = 4.9 and 14.1 Hz, 1H), 3.16 (dd, J = 7.7 and 14.1 Hz, 1H), 1.94 (s, 3H). HRMS (ESI): $[M + H]^+$ calculated for $C_{37}H_{35}N_6O_7S^+$, 707.2282; found, 707.2298.

4.2.50. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamido-4-(methylsulfanyl)butanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33j)

This compound was afforded by typical procedure C with N-acetyl-L-methionine and compound 20a in 66% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 8.24 (t, J = 8.9 Hz, 1H), 8.00 (dd, J = 6.1 and 7.2 Hz, 1H), 7.65–7.53 (m, 2H), 7.45 (d, J = 8.3 Hz, 1H), 7.43–7.25 (m, 12H), 7.19 (d, J = 8.1 Hz, 1H), 7.16 (d, J = 8.3 Hz, 1H), 7.11–6.97 (m, 1H), 6.41 (dd, J = 7.4 and 23.2 Hz, 1H), 6.12–6.02 (m, 1H), 5.32–5.22 (m, 2H), 5.18–5.08 (m, 2H), 4.56–4.43 (m, 1H), 3.55 (dt, J = 4.7 and 14.0 Hz, 1H), 3.17–3.08 (m, 1H), 2.52–2.44 (m, 1H), 2.43–2.37 (m, 0.5H), 2.34–2.27 (m, 0.5H), 2.03 (d, J = 5.5 Hz, 3H), 2.00–1.94 (m, 1H), 1.92 (d, J = 12.0 Hz, 3H), 1.88–1.78 (m, 1H). HRMS (ESI): $[M + Na]^+$ calculated for $C_{40}H_{40}N_6NaO_7S_2^+$, 803.2292; found, 803.2297.

4.2.51. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamido-3-methylpentanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33k)

This compound was afforded by typical procedure C with N-acetyl-L-isoleucine and compound 20a in 55% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 11.87 (s, 1H), 10.20 (s, 1H), 8.24 (dd, J = 4.3 and 7.4 Hz, 1H), 7.99 (t, J= 7.1 Hz, 1H), 7.62–7.50 (m, 2H), 7.49–7.27 (m, 12H), 7.16–7.06 (m, 2H), 6.88–6.66 (m, 1H), 6.18–6.01 (m, 2H), 5.23 (s, 2H), 5.13 (s, 2H),

4.47–4.26 (m, 1H), 3.51–3.41 (m, 1H), 3.27–3.14 (m, 1H), 1.97 (t, J = 6.8 Hz, 3H), 1.49–1.27 (m, 2H), 1.12–0.96 (m, 1H), 0.89–0.75 (m, 6H). HRMS (ESI): $[M + H]^+$ calculated for $C_{41}H_{43}N_6O_7S^+$, 763.2908; found, 763.2881.

4.2.52. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2S)-2-acetamido-3-methylbutanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33l)

This compound was afforded by typical procedure C with N-acetyl-L-valine and compound 20a in 57% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 11.87 (brs, 1H), 10.20 (s, 1H), 8.24 (dd, J = 5.6 and 7.9 Hz, 1H), 8.00 (dd, J = 4.6 and 7.7 Hz, 1H), 7.64–7.54 (m, 2H), 7.46 (dd, J = 8.4 and 12.2 Hz, 2H), 7.42–7.27 (m, 10H), 7.10 (dd, J = 8.3 and 13.4 Hz, 2H), 6.70–6.60 (m, 1H), 6.12–6.05 (m, 1H), 6.04 (dd, J = 8.6 and 13.1 Hz, 1H), 5.24 (s, 2H), 5.13 (s, 2H), 4.32–4.22 (m, 1H), 3.47 (dt, J = 5.0 and 14.3 Hz, 1H), 3.26–3.18 (m, 1H), 2.03–2.00 (m, 1H), 1.98 (d, J = 4.6 Hz, 3H), 0.83 (dd, J = 6.8 and 19.0 Hz, 6H). HRMS (ESI): $[M + Na]^+$ calculated for $C_{40}H_{40}N_6NaO_7S^+$, 771.2571; found, 771.2588.

4.2.53. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2R)-2-acetamido-4-methylpentanamido]-3-oxopropyl]phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33m)

This compound was afforded by typical procedure C with N-acetyl-D-leucine and compound 20a in 58% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 8.23 (t, J = 7.8 Hz, 1H), 7.99 (t, J = 7.2 Hz, 1H), 7.61–7.55 (m, 2H), 7.48 (d, J = 8.4Hz, 1H), 7.42–7.29 (m, 11H), 7.11 (dd, J = 1.8 and 8.4 Hz, 2H), 7.01–6.93 (m, 1H), 6.08–6.03 (m, 1H), 5.99–5.88 (m, 1H), 5.23 (s,

2H), 5.13 (s, 1H), 5.16–5.08 (m, 1H), 4.48–4.43 (m, 1H), 3.53–3.44 (m, 1H), 3.23–3.12 (m, 1H), 1.96 (d, J = 18.0 Hz, 3H), 1.63–1.48 (m, 2H), 1.44–1.32 (m, 1H), 0.87 (dd, J = 3.0 and 6.6 Hz, 3H), 0.85 (d, J = 6.0 Hz, 3H). HRMS (ESI): $[M + H]^+$ calculated for $C_{41}H_{43}N_6O_7S^+$, 785.2728; found, 785.2744.

4.2.54. Benzyl N-[({4-[3-(1,3-benzothiazol-2-yl)-2-[(2R)-2-acetamido-3-phenylpropanamido]-3-oxopropyl] phenyl}amino)({[(benzyloxy)carbonyl]amino})methylidene]carbamate (33n)

This compound was afforded by typical procedure C with N-acetyl-D-phenylalanine and compound 20a in 70% yield. ^{1}H NMR (600 MHz, CDCl3): δ ppm 8.22 (t, J = 8.4 Hz, 1H), 8.00 (t, J = 7.2 Hz, 1H), 7.63–7.54 (m, 2H), 7.44–7.38 (m, 6H), 7.37 (d, J = 8.3 Hz, 2H), 7.33–7.27 (m, 3H), 7.25–7.18 (m, 3H), 7.17–7.03 (m, 5H), 6.75–6.63 (m, 1H), 6.28 (brs, 0.5H), 6.11 (brs, 0.5H), 6.05–5.91 (m, 1H), 5.36–5.22 (m, 2H), 5.19–5.01 (m, 2H), 4.58–4.48 (m, 0.5H), 4.38 (brs, 0.5H), 3.47 (d, J = 12.8 Hz, 1H), 3.15–2.86 (m, 3H), 2.17 (s, 3H). HRMS (ESI): $[M + Na]^+$ calculated for $C_{44}H_{40}N_6NaO_7S^+$, 819.2571; found, 819.2582.

4.2.55. (2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamido-3-phenylpropanamide (34a)

This compound was afforded by typical procedure D with compound 33a in 33% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 35% B at 2 mL/min flow rate. R_t = 10.61 and 11.90 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.27 (t, J = 7.5 Hz, 1H), 8.18–8.15 (m, 1H), 7.72–7.62 (m, 2H), 7.41 (d, J = 8.2 Hz, 1H), 7.33 (d, J = 8.2

Hz, 1H) 7.26–7.13 (m, 7H), 5.98–5.89 (m, 1H), 4.63–4.57 (m, 1H), 3.55–3.46 (m, 1H), 3.12–3.01 (m, 2H), 2.86–2.74 (m, 1H), 1.87 (d, J = 13.4 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 192.97, 192.93, 173.79, 173.55, 173.11, 172.99, 165.52, 165.49, 158.14, 154.83, 138.50, 138.45, 138.36, 138.27, 138.11, 138.05, 134.81, 134.73, 132.15, 132.06, 130.17, 130.12, 129.45, 129.36, 128.60, 127.79, 127.71, 126.73, 126.68, 126.60, 126.57, 123.83, 57.90, 57.84, 56.07, 56.03, 38.76, 38.60, 38.01, 37.71, 22.33, 22.25. HRMS (ESI): $[M + H]^+$ calculated for $C_{28}H_{29}N_6O_3S^+$, 529.2016; found, 529.2016.

4.2.56.

(2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamido-3-(4-hydroxyphenyl)propanamide (34b)

This compound was afforded by typical procedure D with compound 33b in 75% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 30% B at 2 mL/min flow rate. R_t = 10.15 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.27 (dd, J = 3.4 and 8.1 Hz, 1H), 8.16 (dd, J = 3.7 and 7.8 Hz, 1H), 7.72–7.62 (m, 2H), 7.41 (d, J = 8.3 Hz, 1H), 7.30 (d, J = 8.3 Hz, 1H), 7.20 (dd, J = 2.2 and 8.4 Hz, 2H), 7.03–6.97 (m, 2H), 6.68 (d, J = 8.5 Hz, 1H), 6.60 (d, J = 8.5 Hz, 1H), 5.96–5.90 (m, 1H), 4.56–4.50 (m, 1H), 3.55–3.45 (m, 1H), 3.12–3.04 (m, 1H), 2.95 (dt, J = 6.1 and 13.4 Hz, 1H), 2.76–2.67 (m, 1H), 1.87 (d, J = 19.2 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 192.98, 192.91, 173.91, 173.67, 173.08, 165.50, 158.12, 157.32, 157.24, 154.80, 138.49, 138.43, 138.08, 137.98, 134.79, 134.72, 132.14, 132.01, 131.20, 131.12, 129.43, 128.90, 128.87, 128.58, 126.74, 126.66, 126.59, 126.55, 123.83, 123.81, 116.22, 116.12, 57.85, 56.38, 56.32, 38.00, 37.92, 37.73, 22.35, 22.26. HRMS (ESI): $[M + H]^+$ calculated for $C_{28}H_{29}N_6O_4S^+$, 545.1966; found, 545.1992.

4.2.57.

(2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-3-cyclohexyl-2-acetamidopropanamide (34c)

This compound was afforded by typical procedure D with compound 33c in 55% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 45% B at 2 mL/min flow rate. R_t = 6.40 and 6.80 min ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.23 (d, J = 8.2 Hz, 1H), 8.12 (d, J = 7.9 Hz, 1H), 7.67 (m, 2H), 7.40 (dd, J = 8.3 and 14.6 Hz, 2H), 7.20 (dd, J = 8.3 and 14.2 Hz, 2H), 5.93–5.83 (m, 1H), 4.38–4.26 (m, 1H), 3.57–3.48 (m, 1H), 3.10–3.01 (m, 1H), 1.91 (d, J =14.8 Hz, 3H), 1.70–1.57 (m, 5H), 1.51–1.34 (m, 2H), 1.24–1.07 (m, 4H), 0.90–0.78 (m, 2H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 193.13, 192.98, 175.04, 173.20, 173.07, 165.51, 158.18, 158.15, 154.79, 154.77, 138.41, 138.38, 138.24, 138.21, 134.78, 134.69, 132.17, 132.12, 132.03, 131.93, 129.40, 129.37, 128.55, 128.53, 126.76, 126.72, 126.64, 126.56, 126.54, 123.78, 57.99, 57.93, 52.64, 52.42, 40.38, 40.18, 37.74, 37.48, 35.19, 35.18, 34.68, 34.60, 33.52, 33.30, 27.49, 27.25, 27.22, 27.10, 27.05, 22.36, 22.29. HRMS (ESI): $[M + H]^+$ calculated for $C_{28}H_{35}N_6O_3S^+$, 535.2486; found, 535.2494.

4.2.58.

(2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamido-3-(1H-indol-3-yl)propanamide (34d)

This compound was afforded by typical procedure D with compound 33d in 20% yield as yellow power. The mobile phase on semi-preparative RP-HPLC was consisted of 35% B at 2 mL/min flow rate. R_t = 10.09 and 10.41 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.24 (t, J = 7.7 Hz, 1H), 8.14 (t, J = 9.3 Hz, 1H), 7.71–7.60 (m, 2H), 7.52 (dd, J = 7.8 and 14.9 Hz, 1H), 7.35–7.20 (m, 2H), 7.14 (d, J = 8.2 Hz, 2H), 7.11–6.97 (m, 4H), 5.95–5.80 (m, 1H), 4.68–4.58

(m, 1H), 3.48–3.32 (m, 1H), 3.24–3.15 (m, 1H), 3.07–2.96 (m, 2H), 1.88 (d, J = 10.9 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 192.92, 192.62, 174.14, 173.98, 173.15, 165.48, 158.10, 154.79, 138.43, 138.05, 137.99, 137.96, 137.80, 134.73, 134.68, 132.18, 132.04, 131.96, 129.43, 129.40, 128.76, 128.67, 128.59, 128.55, 126.68, 126.61, 126.57, 124.51, 124.48, 123.80, 123.78, 122.48, 122.31, 119.86, 119.70, 119.27, 119.12, 112.37, 112.28, 110.85, 110.79, 58.00, 57.77, 55.78, 55.72, 38.19, 37.81, 28.88, 28.76, 22.44, 22.36. HRMS (ESI): $[M + H]^+$ calculated for $C_{30}H_{30}N_7O_3S^+$, 568.2125; found, 568.2154.

4.2.59.

(2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamidohexanamide (34e)

This compound was afforded by typical procedure D with compound 33e in 37% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 35% B at 2 mL/min flow rate. R_t = 9.82 and 10.67 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.26 (d, J = 8.1 Hz, 1H), 8.14 (d, J = 7.9 Hz, 1H), 7.70–7.60 (m, 2H), 7.43 (dd, J = 8.4 and 10.5 Hz, 2H), 7.22 (dd, J = 8.3 and 12.2 Hz, 2H), 5.97–5.87 (m, 1H), 4.29–4.18 (m, 1H), 3.56 (dt, J = 4.3 and 13.7 Hz, 1H), 3.12–3.04 (m, 1H), 1.93 (d, J = 14.7 Hz, 3H), 1.74–1.62 (m, 1H), 1.60–1.46 (m, 1H), 1.33–1.15 (m, 4H), 0.87–0.81 (m, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 193.20, 193.02, 174.70, 174.56, 173.24, 173.13, 165.54, 165.52, 158.18, 158.16, 154.82, 154.79, 138.42, 138.41, 138.33, 138.23, 134.78, 134.70, 132.11, 132.05, 131.98, 129.41, 129.38, 128.56, 126.76, 126.69, 126.55, 126.53, 123.80, 58.00, 55.02, 54.80, 37.80, 37.53, 32.77, 32.52, 29.03, 28.91, 23.41, 23.35, 22.34, 22.26, 14.14. HRMS (ESI): $[M + H]^+$ calculated for $C_{25}H_{31}N_6O_3S^+$, 495.2173; found, 495.2175.

4.2.60.

(2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamidopentanamide (34f)

This compound was afforded by typical procedure D with compound 33f in 42% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 30% B at 2 mL/min flow rate. R_t = 11.58 and 13.19 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.27 (d, J = 8.1 Hz, 1H), 8.16 (d, J = 8.0 Hz, 1H), 7.72–7.62 (m, 2H), 7.45 (dd, J = 8.5 and 10.7 Hz, 2H), 7.23 (dd, J = 8.4 and 12.0 Hz, 2H), 5.99–5.89 (m, 1H), 4.32–4.20 (m, 1H), 3.58 (dt, J = 4.4 and 14.0 Hz, 1H), 3.13–3.03 (m, 1H), 1.94 (d, J = 14.6 Hz, 3H), 1.74–1.45 (m, 2H), 1.42–1.17 (m, 2H), 0.92–0.82 (m, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 193.23, 193.04, 174.72, 174.57, 173.25, 173.15, 165.53, 165.51, 158.18, 158.15, 154.81, 154.80, 138.42, 138.31, 134.78, 134.71, 132.19, 132.11, 132.05, 132.00, 129.41, 129.38, 128.57, 128.55, 126.78, 126.69, 126.57, 126.54, 126.53, 124.16, 123.80, 58.01, 58.00, 54.81, 54.58, 37.85, 37.58, 35.07, 34.86, 22.34, 22.26, 20.08, 19.96, 13.98, 13.96. HRMS (ESI): $[M + H]^+$ calculated for $C_{24}H_{29}N_6O_3S^+$, 481.2016; found, 495.2030.

4.2.61.

(2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamidobutanamide (34g)

This compound was afforded by typical procedure D with compound 33g in 50% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 30% B at 2 mL/min flow rate. R_t = 8.23 and 9.29 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.26 (d, J = 8.0 Hz, 1H), 8.15 (d, J = 8.0 Hz, 1H), 7.70–7.61 (m, 2H), 7.44 (dd, J = 8.2 and 10.7 Hz, 2H), 7.22 (dd, J = 8.3 and 10.5 Hz, 2H), 6.00–5.91 (m, 1H), 4.24–4.13 (m, 1H), 3.57 (dt, J = 4.3 and

14.3 Hz, 1H), 3.11–3.02 (m, 1H), 1.94 (d, J = 15.0 Hz, 3H), 1.81–1.67 (m, 1H), 1.65–1.52 (m, 1H), 0.94 (t, J = 7.4 Hz, 1.2H), 0.84 (t, J = 7.4 Hz, 1.8H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 193.26, 193.08, 174.55, 174.37, 173.33, 173.21, 165.53, 158.19, 154.83, 138.43, 138.31, 138.21, 134.80, 134.72, 132.10, 132.04, 129.43, 129.40, 128.57, 126.83, 126.77, 126.71, 126.54, 123.81, 57.98, 57.96, 56.42, 56.18, 37.91, 37.68, 26.25, 26.01, 22.35, 22.26, 10.66, 10.53. HRMS (ESI): $[M + H]^+$ calculated for $C_{23}H_{27}N_6O_3S^+$, 467.1860; found, 467.1882.

4.2.62.

(2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamidopropanamide (34h)

This compound was afforded by typical procedure D with compound 33h in 50% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 30% B at 2 mL/min flow rate. R_t = 7.29 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.23 (d, J = 8.2 Hz, 1H), 8.14–8.09 (m, 1H), 7.67–7.58 (m, 2H), 7.39 (dd, J = 8.4 and 10.0 Hz, 2H), 7.19 (dd, J = 8.5 and 9.8 Hz, 2H), 5.95–5.88 (m, 1H), 4.33–4.20 (m, 1H), 3.54 (dt, J = 4.4 and 13.8 Hz, 1H), 3.04 (dd, J = 13.9 Hz, 1H), 1.90 (d, J = 12.5 Hz, 3H), 1.27 (d, J = 7.2 Hz, 1.5H), 1.21 (d, J = 7.2 Hz, 1.5H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 193.22, 193.05, 175.26, 175.18, 173.09, 172.98, 165.49, 158.20, 158.15, 154.81, 138.41, 138.22, 138.10, 134.81, 134.73, 132.10, 132.05, 131.96, 129.42, 129.41, 128.58, 126.83, 126.76, 126.70, 126.65, 126.55, 123.80, 58.00, 57.92, 50.64, 50.28, 37.98, 37.79, 22.35, 22.26, 17.81, 17.75. HRMS (ESI): $[M + H]^+$ calculated for $C_{22}H_{25}N_6O_3S^+$, 453.1703; found, 453.1721.

4.2.63.

N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamido acetamide (34i)

This compound was afforded by typical procedure D with compound 33i in 42% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 30% B at 2 mL/min flow rate. R_t = 6.75 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.28 (d, J = 8.2 Hz, 1H), 8.16 (d, J = 7.8 Hz, 1H), 7.72–7.62 (m, 2H), 7.43 (d, J = 8.3 Hz, 1H), 7.24 (d, J = 8.4 Hz, 1H), 6.02 (dd, J = 4.4 and 9.5 Hz, 1H), 3.87 (d, J = 16.7 Hz, 1H), 3.80 (d, J = 16.7 Hz, 1H), 3.57 (dd, J = 4.4 and 14.0 Hz, 1H), 3.07 (dd, J = 9.6 and 14.0 Hz, 1H), 1.98 (s, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 191.75, 172.34, 170.27, 164.08, 156.74, 153.44, 137.06, 136.72, 133.40, 130.66, 130.46, 128.06, 127.20, 125.34, 125.21, 125.18, 122.42, 56.55, 41.99, 36.68, 20.93. HRMS (ESI): $[M + H]^+$ calculated for $C_{21}H_{23}N_6O_3S^+$, 439.1547; found, 439.1562.

4.2.64.

(2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamido-4-(methylsulfanyl)butanamide (34j)

This compound was afforded by typical procedure D with compound 33j in 8% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 35% B at 2 mL/min flow rate. R_t = 7.31 and 7.75 min. ^{1}H NMR (600 MHz, CD_3CN/D_2O = 1:1, v/v): δ ppm 8.80 (dd, J = 5.9 and 7.1 Hz, 1H), 8.70 (dd, J = 3.8 and 7.7 Hz, 1H), 8.29–8.17 (m, 2H), 7.90 (dd, J = 8.3 and 12.3 Hz, 2H), 7.74 (dd, J = 8.1 and 17.9 Hz, 2H), 6.43–6.34 (m, 1H), 4.91–4.85 (m, 1H), 4.09–3.97 (m, 1H), 3.68–3.55 (m, 1H), 3.00–2.79 (m, 2H), 2.51 (d, J = 7.3 Hz, 3H), 2.44 (d, J = 5.8 Hz, 3H), 2.41–2.55 (m, 2H). ^{13}C NMR (150 MHz, CD_3CN/D_2O = 1:1, v/v): δ ppm 192.22, 192.11, 172.39, 172.21, 163.85, 163.79, 161.32, 161.08, 155.96, 155.91,

152.81, 136.58, 136.20, 136.05, 132.74, 132.69, 130.70, 130.56, 128.36, 127.54, 125.51, 125.35, 125.02, 122.66, 56.33, 56.31, 52.36, 52.06, 36.11, 35.84, 30.49, 30.46, 28.96, 28.92, 21.41, 21.37, 13.82, 13.80. HRMS (ESI): $[M + H]^+$ calculated for $C_{23}H_{29}N_6O_3S_2^+$, 513.1737; found, 513.1749.

4.2.65.

(2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamido-3-methylpentanamide (34k).

This compound was afforded by typical procedure D with compound 33k in 22% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 35% B at 2 mL/min flow rate. R_t = 8.20 and 8.97 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.24 (dd, J = 2.6 and 7.8 Hz, 1H), 8.12 (d, J = 8.0 Hz, 1H), 7.69–7.58 (m, 2H), 7.46–7.40 (m, 2H), 7.24–7.17 (m, 2H), 5.98–5.87 (m, 1H), 4.32–4.12 (m, 1H), 3.61–3.49 (m, 1H), 3.09–3.00 (m, 1H), 1.96–1.89 (m, 3H), 1.83–1.69 (m, 1H), 1.48–1.17 (m, 1H), 1.16–1.03 (m, 1H), 0.89–0.72 (m, 6H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 193.22, 193.06, 174.14, 173.42, 165.54, 158.18, 154.80, 138.40, 138.38, 138.37, 138.25, 138.21, 134.77, 134.71, 132.10, 132.09, 132.00, 129.39, 129.36, 128.54, 126.80, 126.75, 126.64, 126.52, 123.79, 59.49, 59.13, 58.43, 58.41, 58.13, 58.03, 58.00, 57.89, 38.06, 37.87, 37.75, 37.72, 37.39, 27.18, 27.14, 25.80, 25.66, 22.39, 22.37, 22.31, 15.78, 14.86. HRMS (ESI): $[M + H]^+$ calculated for $C_{25}H_{31}N_6O_3S^+$, 495.2173; found, 495.2186.

4.2.66.

(2S)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamido-3-methylbutanamide (34l)

This compound was afforded by typical procedure D with compound 33l in 40% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 30% B at 2 mL/min flow rate. R_t = 11.40 and 12.69 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.25 (d, J = 8.2 Hz, 1H), 8.14 (d, J = 8.0 Hz, 1H), 7.70–7.59 (m, 2H), 7.44 (dd, J = 4.5 and 8.0 Hz, 2H), 7.21 (dd, J = 8.3 and 14.4 Hz, 2H), 6.00–5.90 (m, 1H), 4.16–4.08 (m, 1H), 3.61–3.50 (m, 1H), 3.10–3.01 (m, 1H), 2.02–1.96 (m, 1H), 1.93 (d, J = 13.3 Hz, 3H), 0.95–0.76 (m, 6H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 193.21, 193.10, 173.91, 173.84, 173.31, 173.15, 165.51, 158.15, 158.13, 154.78, 138.40, 138.27, 138.11, 134.79, 134.73, 132.18, 132.06, 132.02, 131.97, 129.39, 129.36, 128.55, 128.53, 126.77, 126.62, 126.52, 126.51, 123.77, 60.44, 60.24, 58.01, 57.92, 37.78, 37.48, 31.59, 31.44, 22.37, 22.30, 19.65, 19.63, 19.51, 18.73, 18.25. HRMS (ESI): $[M + H]^+$ calculated for $C_{24}H_{29}N_6O_3S^+$, 481.2016; found, 481.2039.

4.2.67.

(2R)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamido-4-methylpentanamide (34m)

This compound was afforded by typical procedure D with compound 33m in 20% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 35% B at 2 mL/min flow rate. R_t = 8.62 and 9.67 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.25 (d, J = 8.0 Hz, 1H), 8.14 (d, J = 8.0 Hz, 1H), 7.70–7.60 (m, 2H), 7.42 (dd, J = 8.3 and 11.1 Hz, 2H), 7.22 (dd, J = 8.2 and 12.3 Hz, 2H), 5.95–5.85 (m, 1H), 4.40–4.25 (m, 1H), 3.56 (dt, J = 4.4 and 15.1 Hz, 1H), 3.12–3.03 (m, 1H), 1.93 (d, J = 11.5 Hz, 3H) 1.64–1.38 (m, 3H), 0.91–0.80 (m,

6H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 193.20, 193.03, 175.00, 174.91, 173.19, 173.12, 165.53, 158.20, 158.17, 154.79, 138.41, 138.27, 138.21, 134.81, 134.72, 132.22, 132.12, 132.06, 129.40, 129.37, 128.55, 126.76, 126.67, 126.54, 123.79, 58.06, 58.03, 53.36, 53.09, 41.72, 41.62, 37.79, 37.50, 25.80, 23.26, 23.21, 22.35, 22.29, 21.94. HRMS (ESI): $[M + H]^+$ calculated for $C_{25}H_{31}N_6O_3S^+$, 495.2173; found, 495.2178.

4.2.68.

(2R)-N-[1-(1,3-benzothiazol-2-yl)-3-(4-carbamimidamidophenyl)-1-oxopropan-2-yl]-2-acetamido-3-phenylpropanamide (34n)

This compound was afforded by typical procedure D with compound 33n in 24% yield as white power. The mobile phase on semi-preparative RP-HPLC was consisted of 35% B at 2 mL/min flow rate. R_t = 10.11 and 11.68 min. ^{1}H NMR (600 MHz, CD_3OD): δ ppm 8.30–8.24 (m, 1H), 8.18–8.13 (m, 1H), 7.72–7.61 (m, 2H), 7.44–7.13 (m, 9H), 5.93 (dt, J = 4.8 and 9.3 Hz, 1H), 4.60 (dd, J = 5.9 and 8.7 Hz, 1H), 3.56–3.45 (m, 1H), 3.13–3.01 (m, 2H), 2.87–2.74 (m, 1H), 1.86 (d, J = 13.0 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 192.97, 192.92, 173.78, 173.55, 173.10, 172.99, 165.50, 165.47, 158.12, 154.80, 138.51, 138.48, 138.44, 138.33, 138.26, 138.06, 138.00, 134.81, 134.73, 132.13, 132.04, 130.15, 130.11, 129.44, 129.35, 128.58, 127.78, 127.70, 126.71, 126.66, 126.59, 126.56, 123.81, 57.90, 57.84, 56.07, 56.02, 38.74, 38.60, 38.00, 37.70, 22.33, 22.25. HRMS (ESI): $[M + H]^+$ calculated for $C_{28}H_{29}N_6O_3S^+$, 529.2016; found, 529.2018.

4.3. In vitro fluorescent inhibitor assays.

Hepsin inhibitors (0.1 nM–1 mM) were diluted in DMSO (2% final concentration in

reaction) and mixed with either activated Hepsin (#4776-SE-010, R&D Systems, Minneapolis, Minnesota) or Matriptase (#4735-SE-101, R&D Systems, Minneapolis, Minnesota) to a 96-well plate (REF 353219; BD Falcon). The final assay concentration for Hepsin was 0.3 nM and Matriptase 0.3 nM, respectively in TNC buffer (25 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Triton X-100, pH 8). After incubation for 30 min at 37 ℃, Boc-QAR-AMC substrate (#ES014, R&D Systems, Minneapolis, Minnesota) was added to the Hepsin and Matriptase assays. The final substrate concentration was 150 μM in final reaction volume of 100 μL. Changes in fluorescence (excitation at 380 nm and emission at 460 nm) were measured at room temperature over time (30, 60 and 120 min) in a Biotek Synergy 2 plate reader (Molecular devices). Using GraphPad Prism version 6.04 software program, (GraphPad Software, San Diego, CA, www.graphpad.com), a four parameter curve fit was used to determine the inhibitor IC_{50}s from a plot of the mean reaction velocity versus the inhibitor concentration. The IC_{50} values represent the average of three separate experimental determinations. K_i values were calculated using the Cheng and Prusoff equation ($K_i = IC_{50}/(1 + [S]/K_m)$ [66]. Measurements of enzymatic inhibitory activity of final compounds were performed in triplicate represent the mean ± SD (standard deviation) of at least three experiment sets.

Hepsin activation: Based upon the manufacturer's recommendations, recombinant Hepsin (#4776-SE-010, R&D Systems, Minneapolis, Minnesota) was diluted 5.5 fold in Tris buffer and incubated at 37 °C. After 24 hours, the hepsin was diluted in glycerol to 50%. This stock Hepsin (1.2 μM) was stored in a –20 °C freezer and diluted in Tris buffer for use in assays.

4.4. In silico docking studies

All compounds were generated as 2D and 3D structure by ChemDraw Ultra (ver. 12.0.2.1076) and Chem3D Pro (ver. 12.0.2), respectively. Ligand preparation and optimization

was performed by 'Sanitize' preparation protocol in SYBYL-X 2.1.1 (Tripos Inc., St Louis) to clean up of the structures. The group of ligands was saved as .sdf file. The protein structures of hepsin (PDB ID: 1O5E) and matriptase (PDB ID:3NCL) in PDB format were downloaded from RCSB protein data bank. SYBYL-X 2.1.1 program was employed for protein preparation including conflicted side chains of amino acid residues fixation. Water molecules were removed from 1O5E and 3NCL, and L chain of 1O5E was also removed. Hydrogen atoms were added under the application of AMBER7 FF09 for 1O5E and Tripos for 3NCL Force Field setting. Minimization process was performed by POWELL method, applying Fix Sidechain Bumps with AMBER7 FF09 setting at Ser195 of 1O5E. The initial optimization option of 1O5E and 3NCL were set to None. Termination gradient and max iteration for 1O5E and 3NCl were set 0.05 kcal/(mol*Å) and 100 times, respectively. Protonation type of His57 of 1O5E was set to Delta(HID). The docking studies of all prepared ligands were performed by Surflex-Dock GeomX module in SYBYL-X 2.1.1. Docking was guided by the Surflex-Dock protomol and docking site was defined by the 'Ligand' method with the complexed ligands 6-chloro-2-(2-hydroxy-biphenyl-3-yl)-1H-indole-5-carboxamidine with Threshold value 0.62 (for 1O5E) and phenyl (4-carbamimidoylbenzyl)phosphonate with Threshold value 0.50 (for 3NCL). Other parameters were applied with its default settings in all runs.

Appendix A. Supplementary data

The Supplementary data to this article can be found online at http://

The synthetic methods of N-acetylated amino acids, analytical data of final compounds including ^{1}H- and ^{13}C-NMR spectra, HPLC purity, and HRMS, schematic depiction of key interactions between hepsin and compounds 22a, 22b, and 32. (Bioorg_Supplementary data_1125.docx)

Docked pose of 22a in the active site of hepsin (1O5E_22a.pdb)

Docked pose of 22b in the active site of hepsin (1O5E_22b.pdb)

Docked pose of 32 in the active site of hepsin (1O5E_32.pdb)

Docked pose of 1 in the active site of matriptase (3NCL_1.pdb)

Docked pose of 22a in the active site of matriptase (3NCL_22a.pdb)

Docked pose of 22a, 22b, and 32 in the active site of hepsin (1O5E_22a_22b_32.pse)

Docked pose of 1 and 22a in the active site of matriptase (3NCL_1_22a.pse)

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Author Contributions

H.K., S.-H.S., and Y.B. designed the project. H.K. and H.H. synthesized and analyzed dipeptide analogs. H.K. performed in vitro enzymatic assays and in silico docking studies. H.J. performed PAMPA studies. H.K., H.H., J.J., S.-H.S., H.J., K.L., S.-K.P. and Y.B. analyzed the data and wrote the paper. All authors contributed to editing the final manuscript.

Declaration of competing interest

The authors declare that there is no conflict of interests

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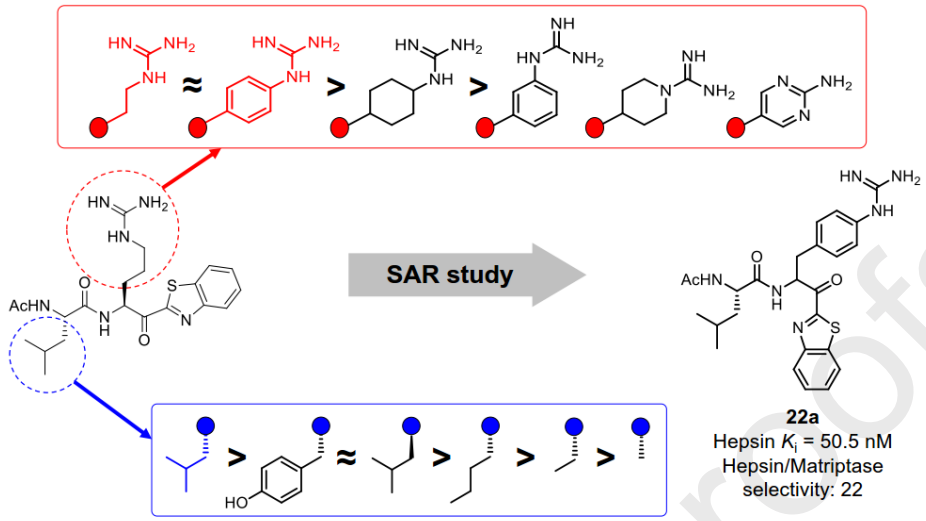
Graphical abstract



Highlights

- New hepsin inhibitors with an arginine bioisostere at the P1 position were designed and synthesized.
- p-Guanidinophenylalanine served as a promising bioisostere for arginine at the P1 position.
- Compound 22a showed strong hepsin inhibition and selectivity for hepsin over matriptase.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: