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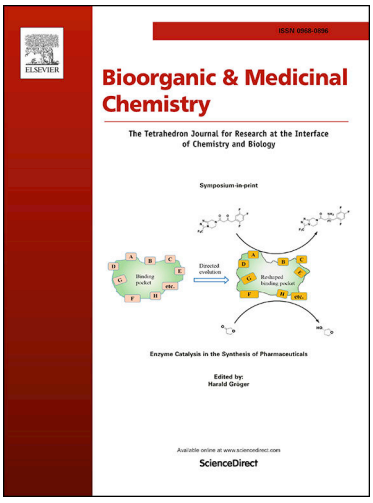

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PII: S0968-0896(20)30697-0
DOI: https://doi.org/10.1016/j.bmc.2020.115867
Reference: BMC 115867

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 31 August 2020
Revised Date: 11 October 2020
Accepted Date: 1 November 2020

Please cite this article as: Y. Zhao, L. Xu, J. Zhang, M. Zhang, J. Lu, R. He, J. Xi, R. Zhuang, J. Li, Y. Zhou, Optimization of piperidine constructed peptidyl derivatives as proteasome inhibitors, *Bioorganic & Medicinal Chemistry* (2020), doi: https://doi.org/10.1016/j.bmc.2020.115867

Optimization of piperidine constructed peptidyl derivatives as proteasome inhibitors

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Abstract: A series of non-covalent piperidine-containing peptidyl derivatives with various substituents at side chains of different residues were designed, synthesized and evaluated as proteasome inhibitors. After proteasome inhibitory evaluations of all the synthesized target compounds, selected ones were tested for their anti-proliferation activities against three multiple myeloma (MM) cell lines. 8 analogues displayed more potent activities than carfilzomib, and the most promising compound **24** showed IC_{50} values of 0.8±0.2 nM against 20S proteasome and 8.42±0.74 nM, 7.14±0.52 nM, 14.20±1.08 nM for RPMI 8226, NCI-H929 and MM.1S cell lines, respectively. Additionally, mechanisms of anti-cancer activity of representative compound **24** were further investigated. Apoptosis of RPMI-8226 cells were achieved through accumulating polyubiquitin and inducing the cleavage of caspase and PARP. Besides, half-life in rat plasma of compound **24** was prolonged after optimization, which would be helpful for increasing *in vivo* activities of this series of derivatives. All the studies confirmed that piperidine-containing non-covalent proteasome inhibitors can be potential leads for anti-MM drug development.

Keywords: Proteasome inhibitor, Piperidine, Peptidyl, Anti-cancer, SAR

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1. Introduction

The ubiquitin-proteasome pathway (UPP) plays a critical role in recognizing and degrading abnormal and misfolded proteins.[1-3] Proteins are targeted for proteasomal degradation via covalent attachment of a 8.5 kDa protein ubiquitin, and ubiquitination occurs with the assistance of three different enzymes. Firstly, ubiquitin is activated by ubiquitin-activating enzyme (E1), and subsequently transferred to ubiquitin-conjugating enzyme (E2) before it is finally coupled to the substrate protein by means of ubiquitin-protein ligase (E3). The ubiquitinated proteins are then flattened into the 26S proteasome and degraded in the 20s catalytic center.[4] In this pathway, the 26S proteasome is the main proteolytic component, which contains two ATP-dependent 19S regulatory particles (RPs) and one 20S core particle (CP). The 20S proteasome is the proteolytically active key element of the UPP that directs the majority of intracellular protein degradation in eukaryotic cells.[3] It is composed of four heptameric rings stacked in a α7-β7-β7-α7 arrangement and contains three proteolytic subunits β1, β2 and β5.[5-8] All of these components have been extensively investigated and validated as potential targets, and confirmed targeted drugs have played important roles for the treatment of multiple myeloma and other diseases.[9-10]

Currently, three proteasome inhibitors Bortezomib, Carfilzomib and Ixazomib have been approved by FDA for the treatment of multiple myeloma (Fig.**1**).[10-12] All three are covalent peptidyl inhibitors with an electrophilic moiety at the C-terminal end of the peptidyl backbone for covalent binding to the catalytic terminal Thr1 residue of the proteasome β5 subunit. Besides, various short peptides and pseudopeptides with these covalent binding groups were explored for cancer therapy.[12-17] However, the active electrophilic groups induce excessive reactivity, less specificity and instability of these inhibitors, and the stable covalent interaction is irreversible, which is believed to be the major cause for side effects during therapy.[12, 18] Meanwhile, most of these inhibitors failed in the treatment of solid cancers, which is mainly attributed to the quick covalent binding to the proteasome, thus limiting their widespread tissue distribution. [12, 19-20]

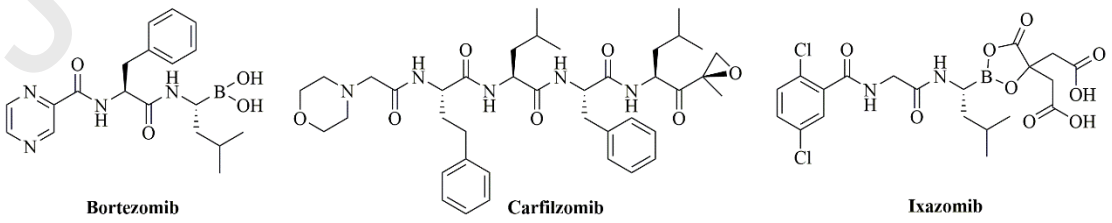


Figure **1**. Structures of FDA approved proteasome inhibitors for multiple myeloma

In contrast, non-covalent inhibitors with reversible binding mode ensure them with rapid binding and dissociation kinetics. These features endowed these analogues abilities for overcoming defects arising in

therapeutics of covalent combination drugs.[21-22] Although none of the non-covalent proteasome inhibitor has been approved, they have received more and more attention from researchers, and some of these compounds exhibited even more potent proteasome inhibitory activities than covalent ones.[12] Blackburn and colleagues reported a series of di- and tripeptidyl non-covalent proteasome inhibitors, among which ML 16 was the most promising one with IC_{50} values of 1.2 nM against constitutive proteasome and 1.1 nM against immunoproteasome (Fig.**2**). [19-20]

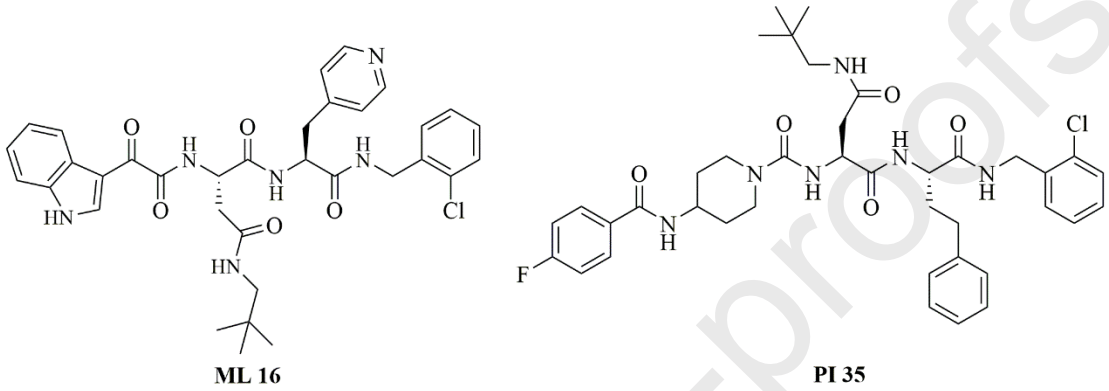


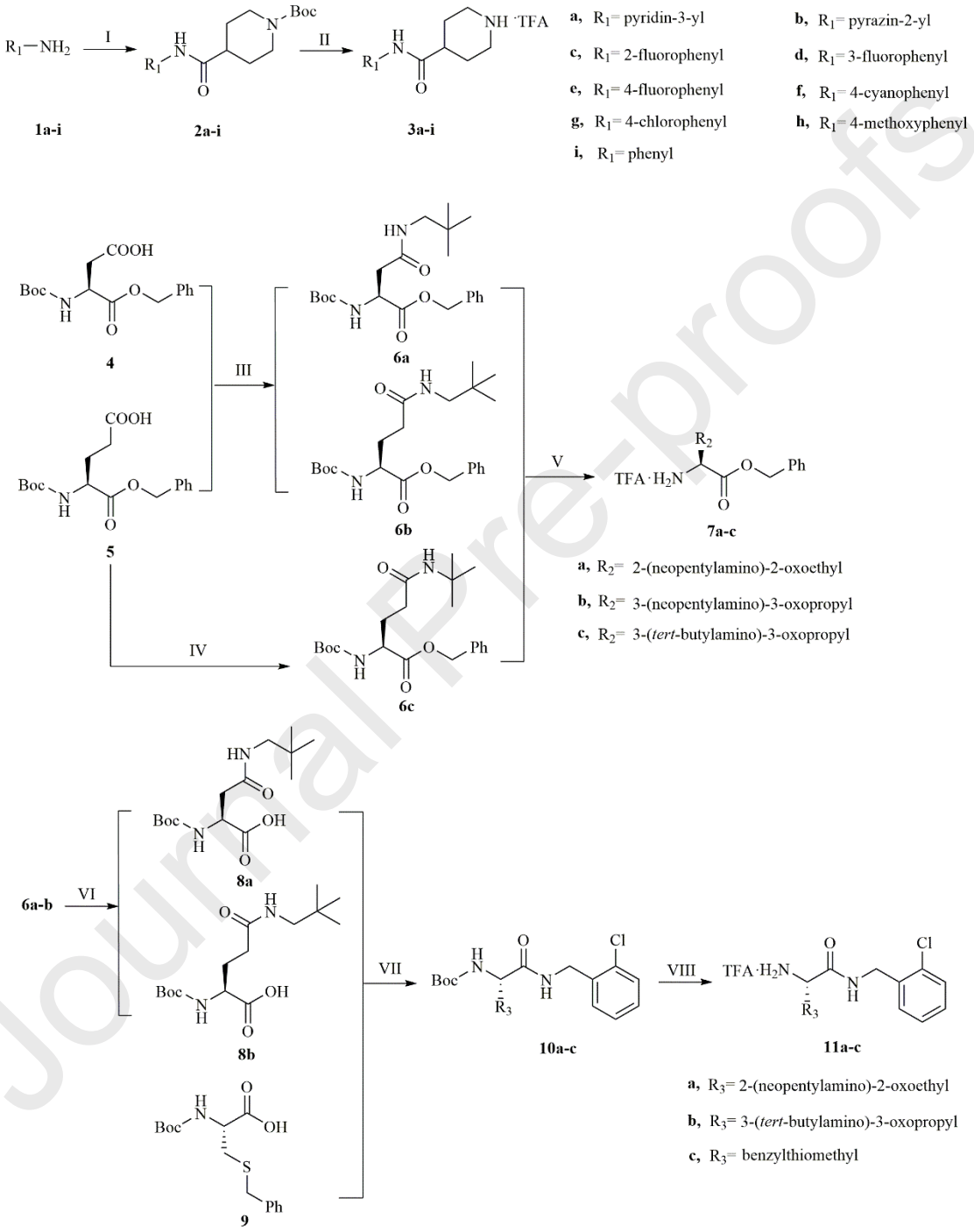
Figure **2**. Representative non-covalent proteasome inhibitors with potent enzymatic activities

Based on the previous studies, novel non-covalent peptidyl proteasome inhibitors containing piperidine or piperazine were developed in our group, among which the most promising analogue (**PI 35:** proteasome inhibitory activity, IC_{50}: 1.2 ± 0.1 nM) displayed potent anti-proliferation activities (IC_{50}: RPMI-8226: 8.4 ± 0.8 nM; MM-1S: 6.3 ± 0.8 nM).[12-13] Although the half-life of **PI 35** was improved by constructing a aliphatic heterocycle into the peptide skeleton, the blood cell proteasome inhibitory activity was not guaranteed. In this manuscript, further optimization of **PI 35** through various substitutions replaced at side chains of each residue with retained piperidine constructed skeleton was carried out. Promising derivatives with potent *in vitro* anti-cancer activities and improved stabilities were anticipated, thus make these analogues with potential *in vivo* activities.

2. Synthesis

The synthetic route for piperidine-containing fragments **3a-i** is summarized in Scheme **1**. An easy condensation of corresponding amine **1a-i** with protected piperidine fragment provided compounds **2a-i**. Afterwards, deprotection of **2a-i** with trifluoroacetic acid (TFA) afforded piperidine TFA salts (**3a-i**). As shown in Scheme **1**, compound **4** and **5** were treated with tert-butyl amine in the presence of EDCI and HOBt to give the key intermediates TFA salts protected amines (**6a-c),** which were then deprotected

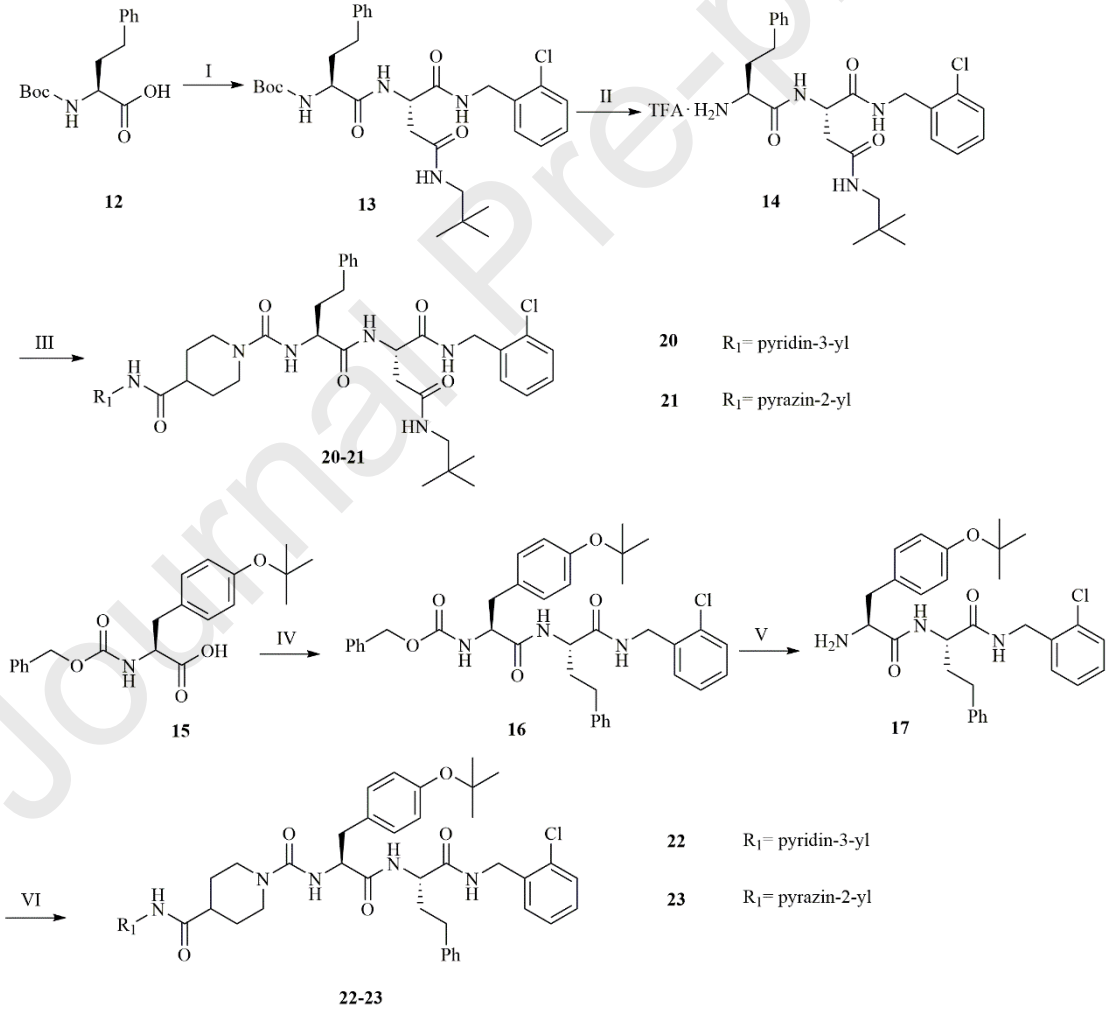
with TFA to afford TFA salts (**7a-c**). Reduction of fragments **6a-b** at the presence of Pd/C (10%) provided protected amino acid fragments **8a-b**. Compounds **8a-b** and **9** were then treated with 2-chlorobenzylamine, EDCI and HOBt to afford intermediates **10a-c**. Eventually, deprotection of **10a-c** with TFA afforded TFA salts **11a-c**.



Scheme **1**. Synthesis of piperidine-containing fragments (**3a-i**) and the TFA salts (**7a-c** and **11a-c**). Reagents and conditions: (I) HOBt, EDCI, corresponding amine, DIPEA, DCM, 0°C-rt; (II) TFA, DCM, 0°C-rt; (III) neopentylamine, HOBt, EDCI, DIPEA, DCM, 0°C-rt; (IV) tert-Butylamine, HOBt, EDCI,

DIPEA, DCM, 0°C-rt; (V) TFA, DCM, 0°C-rt; (VI) Pd/C (10%), H_2, methanol, rt; (VII) 2-chlorobenzylamine, HOBt, EDCI, DIPEA, DCM, 0°C-rt; (VIII) TFA, DCM, 0°C-rt.

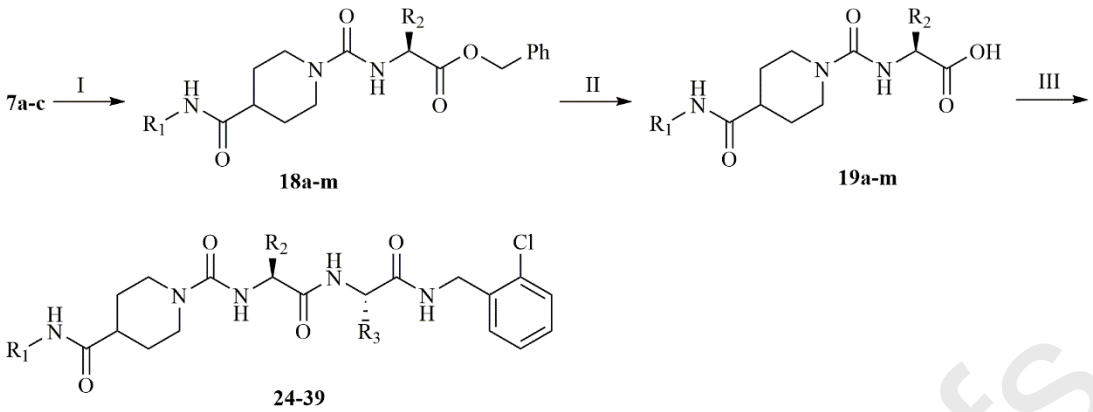
The synthetic route for target compounds **20-23** is outlined in **Scheme 2**. The piperidine TFA salt **12** was condensed with **11a** to afford compound **13**, which was then deprotected with TFA to afford piperidine TFA salt **14**. Subsequently, analogue **14** were transformed to corresponding isocyanate intermediates and reacted with **3a** or **3b** to obtain the target compounds **20-21**. As illustrated in **Scheme 2**, reaction of compound **15** with 2-amino-N-(2-chlorobenzyl)-3-phenylpropanamide furnished compound **16**, which was deprotected under the condition of Pd/C (10%) to afford compound **17**. Finally, compound **17** was transformed into corresponding isocyanate intermediates and then reacted with **3a** or **3b** to obtain the target compounds **22** and **23**.



Scheme **2**. Synthesis of piperidine-containing target compounds (**20-23**). Reagents and conditions: (I) **11a**, HOBt, EDCI, DIPEA, DCM, 0°C-rt; (II) TFA, DCM, 0°C-rt; (III) **3a** or **3b**, HOBt, EDCI, DIPEA,

DCM, 0°C-rt; (IV) 2-amino-N-(2-chlorobenzyl)-3-phenylpropanamide, HOBt, EDCI, DIPEA, DCM, 0°C-rt; (V) Pd/C (10%), H_2, methanol, rt; (VI) **3a** or **3b**, HOBt, EDCI, DIPEA, DCM, 0°C-rt.

The synthesis of target compounds **24-39** was summarized in Scheme **3**. Compounds **7a-c** were converted into corresponding isocyanate intermediates, which were unstable and were therefore prone to react with piperidine fragments **3a**-**i** to obtain key intermediates **18a**-**m**. Ultimately, the coupling of corresponding amine with various acids **18a**-**m** in the presence of EDCI, HOBt and DIPEA gave the target compounds **24-39**.



	R_1	R_2	R_3
24	pyridin-3-yl	3-(*tert*-butylamino)-3-oxopropyl	phenylethyl
25	pyrazin-2-yl	3-(*tert*-butylamino)-3-oxopropyl	phenylethyl
26	pyridin-3-yl	2-(neopentylamino)-2-oxoethyl	benzylthiomethyl
27	pyrazin-2-yl	2-(neopentylamino)-2-oxoethyl	benzylthiomethyl
28	pyridin-3-yl	2-(neopentylamino)-2-oxoethyl	2-(neopentylamino)-2-oxoethyl
29	pyrazin-2-yl	2-(neopentylamino)-2-oxoethyl	2-(neopentylamino)-2-oxoethyl
30	pyridin-3-yl	2-(neopentylamino)-2-oxoethyl	3-(*tert*-butylamino)-3-oxopropyl
31	pyrazin-2-yl	2-(neopentylamino)-2-oxoethyl	3-(*tert*-butylamino)-3-oxopropyl
32	pyridin-3-yl	3-(neopentylamino)-3-oxopropyl	phenylethyl
33	pyrazin-2-yl	3-(neopentylamino)-3-oxopropyl	phenylethyl
34	2-fluorophenyl	3-(*tert*-butylamino)-3-oxopropyl	phenylethyl
35	3-fluorophenyl	3-(*tert*-butylamino)-3-oxopropyl	phenylethyl
36	4-fluorophenyl	3-(*tert*-butylamino)-3-oxopropyl	phenylethyl
37	4-cyanophenyl	3-(*tert*-butylamino)-3-oxopropyl	phenylethyl
38	4-methoxyphenyl	3-(*tert*-butylamino)-3-oxopropyl	phenylethyl
39	phenyl	3-(*tert*-butylamino)-3-oxopropyl	phenylethyl

Scheme **3**. Synthesis of piperidine-containing target compounds (**24-39**). Reagents and conditions: (I) triphosgene, DIPEA, DCM, aqueous $NaHCO_3$, 0°C-rt.; (II) Pd/C (10%), H_2, methanol, rt; (III) corresponding amine, HOBt, EDCI, DIPEA, DCM, 0°C-rt.

3. Pharmacology

3.1 Proteasome inhibitory activities

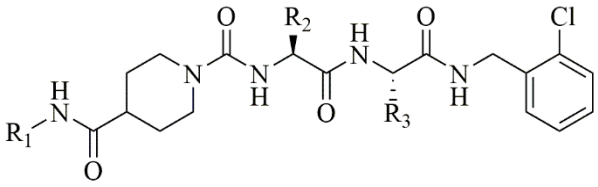
The obtained target compounds were screened for 20S proteasome chymotrypsin-like inhibitory activities *in vitro* with Carfilzomib employed as positive control. The results are shown in Table **1**. As summarized in Table **1**, the IC_{50} values of most target compounds were lower than 50 nM, among which 9 analogues were even lower than 10 nM, indicating that the activities of most compounds were reserved after different substituents introduced at R_1, R_2 and R_3 positions while retaining the dominant skeleton containing the piperidine ring.

Different substituents at R_2 position affected the activity obviously. Overall, 3-(tert-butylamino)-3-oxopropyl, 2-(neopentylamino)-2-oxopropyl and 3-(neopentylamino)-3-oxopropyl were all tolerable. By comparing activities of analogues **22**, **23**, **24**, **25**, **32** and **33**, decreasing of activities were observed in compounds with 4-tert-butoxybenzyl (**23** and **24**), and 3-(tert-butylamino)-3-oxopropyl substituted analogues displayed the most potent enzymatic activities (IC_{50}: **24**, 0.8±0.2 nM; **25**, 0.9±0.1 nM). Compounds **20** and **21** were obtained through exchanging R_2 and R_3 substituents based on our previous study, activities of which were not improved. Besides, **28** and **29** with 2-(neopentylamino)-2-oxopropyl at R_2 were also more potent than corresponding **20** and **21** with IC_{50} values of 5.2±0.3 and 19.9±0.3 nM, respectively, which was also an evidence for the inappropriateness of phenylethyl at R_2.

In order to investigate the influences of substitution at R_3, activities of analogues **26-31** were evaluated. All of these derivatives displayed comparable proteasome inhibitory activities with IC_{50} values ranging from 4.1 to 19.9 nM, which were all less potent than **24** and **25** with phenylethyl at R_3. Thus, 3-(tert-butylamino)-3-oxopropyl at R_2 and phenylethyl at R_3 were more preferable, which were further employed for the screening of R_1 groups.

The effects of various *N*-terminus groups (R_1) were studied. The results indicated that different substituted phenyl groups influenced proteasome inhibitory activities obviously. Electron-withdrawing groups at phenyl were beneficial for the activities with IC_{50} values of derivatives **34-37** ranging from 9.0 to 23.7 nM, while phenyl substituted compound **39** showed moderate activity with IC_{50} value of 338.3 nM. Analogue **38** only displayed weak activity with electron-donating group at R_1 (IC_{50}: 4134.0±794.8 nM). Besides, R_1 pyridine and pyrazine substituted derivatives (**24** and **25**) were much more potent than that of R_1 phenyl replaced compounds (**34-39**), through which more interactions might be formed with the target.

Table **1**. 20S proteasome chymotrypsin-like inhibitory activities of target compounds (**20-39**)



20-39

Compound	R_1	R_2	R_3	Proteasome inhibitory activities IC_{50} (nM)[a]
20	pyridin-3-yl	phenylethyl	2-(neopentylamino)-2-oxoethyl	13.8±2.4
21	pyrazin-2-yl	phenylethyl	2-(neopentylamino)-2-oxoethyl	42.1±2.1
22	pyridin-3-yl	4-tert-butoxybenzyl	phenylethyl	77.0±24.5
23	pyrazin-2-yl	4-tert-butoxybenzyl	phenylethyl	163.8±29.6
24	pyridin-3-yl	3-(tert-butylamino)-3-oxopropyl	phenylethyl	0.8±0.2
25	pyrazin-2-yl	3-(tert-butylamino)-3-oxopropyl	phenylethyl	0.9±0.1
26	pyridin-3-yl	2-(neopentylamino)-2-oxoethyl	benzylthiomethyl	4.1±0.4
27	pyrazin-2-yl	2-(neopentylamino)-2-oxoethyl	benzylthiomethyl	10.6±3.4
28	pyridin-3-yl	2-(neopentylamino)-2-oxoethyl	2-(neopentylamino)-2-oxoethyl	5.2±0.3
29	pyrazin-2-yl	2-(neopentylamino)-2-oxoethyl	2-(neopentylamino)-2-oxoethyl	19.9±0.3
30	pyridin-3-yl	2-(neopentylamino)-2-oxoethyl	3-(tert-butylamino)-3-oxopropyl	5.6±1.5
31	pyrazin-2-yl	2-(neopentylamino)-2-oxoethyl	3-(tert-butylamino)-3-oxopropyl	6.2±0.6
32	pyridin-3-yl	3-(neopentylamino)-3-oxopropyl	phenylethyl	1.4±0.1 2.7±0.7
33	pyrazin-2-yl	3-(neopentylamino)-3-oxopropyl	phenylethyl	11.0±0.7 16.5±4.0
34	2-fluorophenyl	3-(tert-butylamino)-3-oxopropyl	phenylethyl	9.0±0.2
35	3-fluorophenyl	3-(tert-butylamino)-3-oxopropyl	phenylethyl	23.7±1.5
36	4-fluorophenyl	3-(tert-butylamino)-3-oxopropyl	phenylethyl	4134.0±794.8
37	4-cyanophenyl	3-(tert-butylamino)-3-oxopropyl	phenylethyl	338.2±75.5

38	4-methoxyphenyl	3-(tert-butylamino)-3-oxopropyl	phenylethyl	8.4±0.9
39	phenyl	3-(tert-butylamino)-3-oxopropyl	phenylethyl	
Carfilzomib	-	-	-	

[a] The IC_{50} values were an average of three independent determinations.

3.2 Tumor cell growth inhibitory activities

Based on the obtained proteasome inhibitory activities, selected compounds with IC_{50} values lower than 10 nM were further screened for their *in vitro* anti-proliferative activities against three MM cell lines (RPMI 8226, NCI-H929 and MM.1S) by MTT-based assay with Carfilzomib used as positive control. As summarized in Table **2**, most of the tested compounds showed superior cytotoxic activities against three MM cell lines with IC_{50} values lower than 20 nM, which were consistent with the proteasome inhibitory activities. Overall, among all the tested compounds, **24** exhibited the most potent cytotoxicities with IC_{50} values of 8.42±0.74, 7.14±0.52 and 14.20±1.08 nM against RPMI 8226, NCI-H929 and MM.1S cell line, respectively. Thus, candidate **24** was then proceeded for further biological evaluation.

Table **2**. Anti-proliferative activities against three different MM cell lines of selected compounds

Compound	Cytotoxicity IC_{50} (nM) [a]		
	RPMI8226	NCI-H929	MM.1S
24	8.42±0.74	7.14±0.52	14.20±1.08
25	7.23±0.48	9.01±0.67	15.88±1.12
26	9.46±0.21	10.06±0.14	14.90±1.04
28	14.39±1.16	17.45±1.23	16.33±1.19
30	13.19±1.07	15.72±1.17	19.36±1.52
31	17.17±1.33	12.20±0.94	17.12±0.86
32	16.01±1.27	18.49±1.38	21.82±1.75
33	19.61±1.42	19.57±1.37	30.39±2.17
36	15.08±1.16	8.92±0.56	12.50±0.88
Carfilzomib	14.10±0.61	13.90±0.52	14.35±0.73

[a] The IC_{50} values were an average of three independent determinations.

3.3 DAPI staining analysis

To further investigate the mechanism of anti-cancer activity of compound **24**, cell apoptosis study by DAPI staining in the RPMI-8226 cells was performed. RPMI-8226 cells were treated with compound **24** or Carfilzomib for 48 h. As shown in Fig. **3**, the percentages of apoptotic RPMI-8226 cells were 3.10 ±

0.85% in control cells, 21.66 ± 5.25% with 1 nM of **24**, 58.18 ± 9.79% with 5 nM of **24**, 97.79 ± 3.51% with 10 nM of **24** and 72.42 ± 4.33% with 10 nM of Carfilzomib. Much more apoptotic RPMI-8226 cells were observed in **24** treatment group compared with Carfilzomib treatment group (10 nM of **24** vs. 10 nM of Carfilzomib, $p<0.05$) in dose dependent manner, indicating that analogue **24** possessed stronger anti-apoptotic abilities than Carfilzomib in RPMI-8226 cells at the concentration of 10nM. Since **24** significantly induced apoptosis, we sought to measure the expression of apoptotic related proteins in RPMI-8226 cells treated with **24** or Carfilzomib.

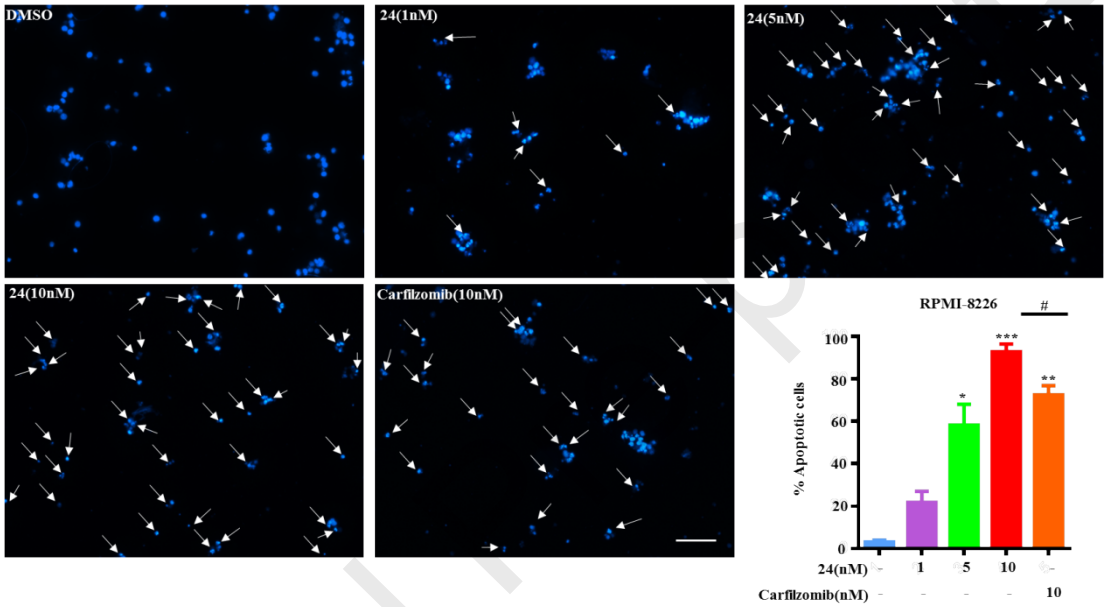


Figure **3**. Compound **24** induced apoptosis in RPMI-8226 cells. RPMI-8226 cells were incubated with **24** or Carfilzomib at indicated concentrations for 48 h, and cells were collected and detected by DAPI staining. Apoptotic nuclei were indicated by arrows, scale bar = 100 μm. **24** induced apoptosis in dose dependent manner on RPMI-8226 cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared with control. # $p<0.05$, 10 nM of **24** vs. 10 nM of Carfilzomib.

3.4 Western blot analysis

To determine whether proteasome inhibitory activity of compound **24** in a cell-free system can be recapitulated *in vitro*, we monitored the levels of polyubiquitinated proteins in treated cells by immunoblotting, using antibody specific towards free ubiquitin and ubiquitin conjugates to form a typical high molecular mass smear. As demonstrated in Fig. **4A** and **B**, we observed that treatment of RPMI 8226 cells with **24** for 48 h induced a significantly greater activation of PARP and capase-3 than Carfilzomib. To further investigate whether ubiquitin-proteasome system was involved in the anti-cancer

effect of **24**, we observed that the efficiency of **24** on accumulating ubiquitin was stronger than Carfilzomib, suggesting that **24** induced apoptosis via ubiquitin-proteasome pathway in RPMI-8226 cells. However, significant increase in PARP cleavage was observed at the concentration of 1 nM and 5 nM, while accumulation of ubiquitinated proteins was not obvious at the same concentrations, which indicated that the mechanism of cell death might not only attributed to proteasome inhibition. Further investigations in inducing apoptosis of this series of analogues are required.

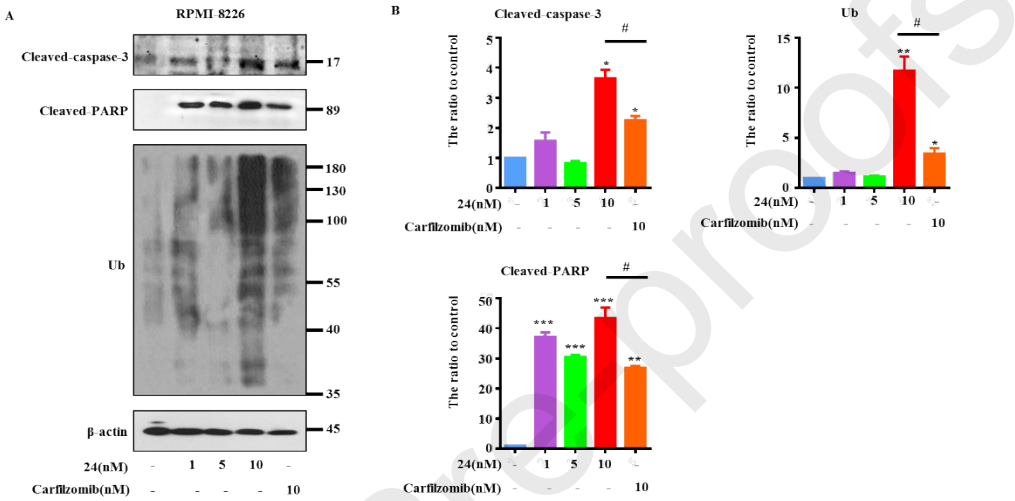


Figure **4**. Compound **24** affected apoptosis related proteins in RPMI-8226 cells. (**A**) RPMI-8226 cells were incubated with **24** or Carfilzomib at indicated concentrations for 24 h, and cells were collected and detected by western blotting. (**B**) Statistical analysis of the expression of cleaved-caspase-3, cleaved-PARP and Ub. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared with control. # $p<0.05$, 10 nM of **24** vs. 10 nM of Carfilzomib. The results are average of three independent experiments.

3.5 Plasma stability

Subsequently, 7 compounds with potent proteasome inhibitory activities and cytotoxicities were further tested for their stabilities by measuring the half lives in rat plasma. As illustrated in Tab. **3**, most of these analogues displayed comparable stabilities with **PI 35** discovered in our previous study. However, as expected, the most potent analogue **24** was most stable after optimization, which might be beneficial for improving *in vivo* anti-cancer activities of this promising analogue.

Table **3**. Rat plasma stabilities of selected compounds

Compound	Plasma half-life ($T_{1/2}$, min)
24	104.51
25	72.90

28	72.05
32	69.98
33	77.75
35	93.46
36	84.20
PI 35	91.55

3.6 Binding mode analysis

To further elucidate the binding modes of the most potent compound **24** with the active site of proteasome, docking simulation studies were carried out. The co-crystal structure of proteasome (PDB ID code: 3MG6) was selected as the docking template. As shown in Fig. **5**, the carbonyl and amino groups of the peptide skeleton of compound **24** formed several critical hydrogen bonds with residues Thr21, Ala49, Ala50 and Asp114 of the proteasome active site, respectively. These hydrogen bonds played important roles in the inhibitory potency against proteasome. Additionally, the 3-(tert-butylamino)-3-oxopropyl group occupied well defined S3 pocket, while C-terminus was located in the S1 binding pocket. The docking result gave a rational explanation for the potency of compound **24** and it is consistent with the previous biological results.

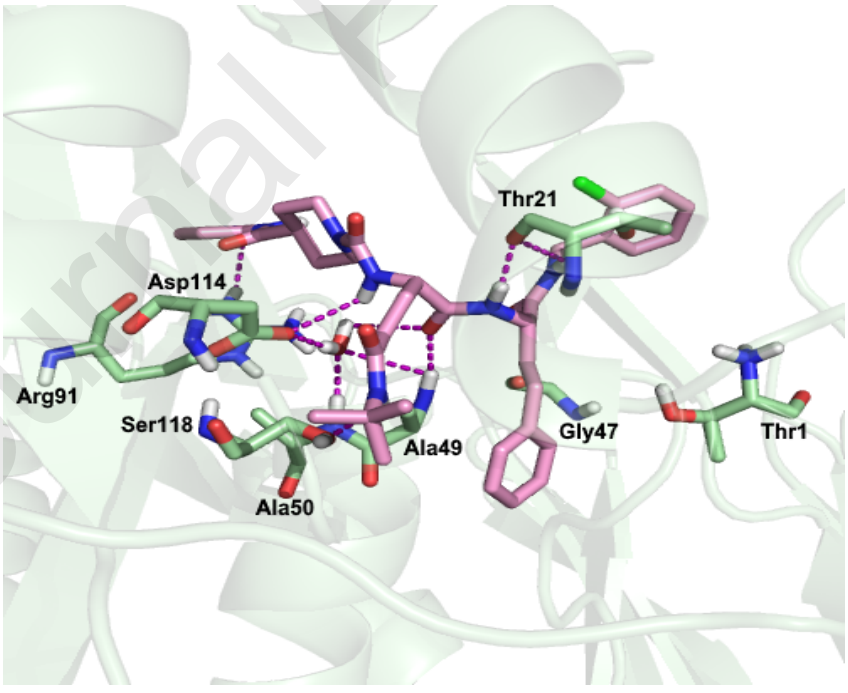


Figure **5**. The binding mode of compound **24** with chymotrypsin-like active site of 20S proteasome (PDB ID: 3MG6).

4. Conclusion

A series of piperidine-containing non-covalent peptidyl derivatives possessing various substituents at side chains of different residues were designed, synthesized and biologically evaluated for their proteasome inhibitory and anti-proliferation activities. Among all the tested compounds, 8 derivatives not only displayed proteasome inhibitory activities more excellent than the positive control Carfilzomib, but also possessed forceful anti-proliferation activities against three MM cell lines (RPMI 8226, NCI-H929 and MM.1S). Additionally, the most promising compound **24** showed stronger ability in inducing apoptosis than Carfilzomib, and promoted cell apoptosis via ubiquitin-proteasome pathway in RPMI-8226 cells. Besides, as anticipated, the most potent analogue **24** possessed the strongest stability in rat plasma. All of these studies suggested that compound **24** could be a potentially interesting lead molecule for further investigation.

5. Experimental procedures

5.1 Chemistry and general methods

Mass spectra (MS) were taken in the ESI mode on Esquire-LC-00075 spectrometer (Brüker Bioscience). ^{1}H and ^{13}C NMR spectra were obtained on Brüker 500/400MHz spectrometer (Brüker Bioscience, Billerica, MA, USA) with TMS as an internal standard and $CDCl_3$ or DMSO-d_6 as solvent. All yields are unoptimized and generally represent the result of a single experiment.

5.2 Synthetic procedures

5.2.1 General procedure for the synthesis of protected piperidine fragments 2a-i

N-Boc-4-piperidinecarboxylic acid (3.65g, 16.0mmol), CH_2Cl_2 (30.0mL) and pyridine (3.4mL, 40.0mmol) were added in the round-bottom flask in turn and then mixed and protected by N_2, $SOCl_2$ (1.5mL, 19.0mmol) was added slowly to the mixture and reacted for half an hour at room temperature. Then the solution of catalytic amount of DMAP, amine (**1a-i**, 18.0mmol) and Et_3N (8.0mL, 56.0mmol) in CH_2Cl_2 (30.0mL) was added dropwise sequentially and stirred overnight. Upon completion of the reaction, the organic phase was washed with 1N HCl (2×30mL) and aqueous $NaHCO_3$ (2×30mL), dried by Na_2SO_4. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (ethyl acetate: petroleum ether = 1:2~3:1) to give piperidine derivatives **2a-i**.

tert-Butyl 4-(Pyridin-3-ylcarbamoyl) piperidine-1-carboxylate (2a)

White solid; Yield: 63%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.61 (s, 1H, pyridine-H), 8.34 (d, 1H, J = 4.0

Hz, pyridine-H), 8.28 (d, 1H, *J* = 8.5 Hz, pyridine-H), 7.81 (s, 1H, NH), 7.32 (dd, 1H, *J* = 8.5, 5.0 Hz, pyridine-H), 4.19 (d, 2H, *J* = 13.0 Hz, CH_2), 2.90-2.71 (m, 2H, CH_2), 2.49-2.43 (m, 1H, CH), 1.91 (d, 2H, *J* = 12.0 Hz, CH_2), 1.77-1.73 (m, 2H, CH_2), 1.46 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 306.2 $[M+H]^+$.

tert-Butyl 4-(Pyrazin-2-ylcarbamoyl) piperidine-1-carboxylate (2b)

White solid; Yield: 78%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 9.55 (s, 1H, pyrazine-H), 8.35 (d, 1H, *J* = 2.0 Hz, pyrazine-H), 8.23 (s, 1H, pyrazine-H), 7.97 (s, 1H, NH), 4.24-4.15 (m, 2H, CH_2), 2.86-2.75 (m, 2H, CH_2), 2.51-4.46 (m, 1H, CH), 1.93 (d, 2H, *J* = 12.5 Hz, CH_2), 1.80-1.72 (m, 2H, CH_2), 1.47 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 307.2 $[M+H]^+$.

tert-butyl 4-(2-fluorophenylcarbamoyl)piperidine-1-carboxylate (2c)

White solid; Yield: 88%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.34 (s, 1H, *J* = 2.0 Hz, NH), 7.40 (s, 1H, Ar-H), 7.15-7.04 (m, 3H, Ar-H), 4.22 (d, 2H, *J* = 12.0 Hz, CH_2), 2.84-2.81 (m, 2H, CH_2), 2.45-2.42 (m, 1H, CH), 1.94-1.90 (m, 2H, CH_2), 1.79-1.73 (m, 2H, CH_2), 1.47 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 323.2 $[M+H]^+$.

tert-butyl 4-(3-fluorophenylcarbamoyl)piperidine-1-carboxylate (2d)

White solid; Yield: 65%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.71 (m, 1H, NH), 7.52 (d, 1H, *J* = 13.5 Hz, Ar-H), 7.29-7.23 (m, 1H, Ar-H), 7.14 (d, 1H, *J* = 10.0 Hz, Ar-H), 6.83-6.79 (m, 1H, Ar-H), 4.22-4.11 (m, 2H, CH_2), 2.82-2.76 (m, 2H, CH_2), 2.41-2.35 (m, 1H, CH), 1.91-1.88 (d, 2H, *J* = 9.5 Hz, CH_2), 1.75-1.69 (m, 2H, CH_2), 1.47 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 323.2 $[M+H]^+$.

tert-Butyl 4-(4-Fluorophenylcarbamoyl) piperidine-1-carboxylate (2e)

White solid; Yield: 75%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.50-7.42 (m, 2H, Ar-H), 7.20 (s, 1H, NH), 7.01 (t, 2H, *J* = 8.0 Hz, Ar-H), 4.18 (d, 2H, *J* = 12.0 Hz, CH_2), 2.80 (t, 2H, *J* = 12.5 Hz, CH_2), 2.39-2.32 (m, 1H, CH), 1.90 (d, 2H, *J* = 12.5 Hz, CH_2), 1.79-1.70 (m, 2H, CH_2), 1.47 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 323.3 $[M+H]^+$.

tert-butyl 4-(4-cyanophenylcarbamoyl)piperidine-1-carboxylate (2f)

White solid; Yield: 71%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.52 (d, 2H, *J* = 9.5 Hz, Ar-H), 7.34 (t, 2H, *J* = 10.0 Hz, Ar-H), 7.13 (t, 1H, *J* = 9.0 Hz, NH), 4.19 (s, 2H, CH_2), 2.82-2.79 (m, 2H, CH_2), 2.41-2.36 (m, 1H, CH), 1.79-1.69 (m, 2H, CH_2), 1.75-1.69 (m, 2H, CH_2), 1.47 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 330.3 $[M+H]^+$.

tert-butyl 4-(4-chlorophenylcarbamoyl)piperidine-1-carboxylate(2g)

White solid; Yield: 82%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.49 (d, 2H, *J* = 9.5 Hz, Ar-H), 7.29 (s, 1H,

N-H), 7.27 (d, 2H, J = 9.5 Hz, Ar-H), 4.19 (s, 2H, CH_2), 2.85-2.76 (m, 2H, CH_2), 2.41-2.35 (m, 1H, CH), 1.91-1.88 (m, 2H, CH_2), 1.79-1.68 (m, 2H, CH_2), 1.47 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 339.1 $[M+H]^+$.

tert-Butyl 4-(4-methoxyphenylcarbamoyl) piperidine-1-carboxylate (2h)

White solid; Yield: 85%; ^{1}H NMR (500 MHz,$CDCl_3$): δ = 7.39 (d, 2H, J = 8.5 Hz, Ar-H), 7.38 (s, 1H, NH), 6.83 (d, 2H, J = 7.5 Hz, Ar-H), 4.16 (d, 2H, J = 13.0 Hz, CH_2), 3.77 (s, 3H,CH_3), 2.81-2.69 (m, 2H, CH_2), 2.38-2.32 (m, 1H, CH), 1.86 (d, 2H, J = 12.0 Hz, CH_2), 1.76-1.71 (m, 2H, CH_2), 1.45 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 335.2 $[M+H]^+$.

tert-Butyl 4-(phenylcarbamoyl) piperidine-1-carboxy-late (2i)

White solid; Yield: 86%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.53 (d, 2H, J = 5.0 Hz, Ar-H), 7.39-7.30 (m, 3H, Ar-H+NH), 7.15-7.09 (m, 1H, Ar-H), 4.21 (d, 2H, J = 10.0 Hz, CH_2), 2.85-2.74 (m, 2H, CH_2), 2.44-2.38 (m, 1H, CH), 1.94-1.87 (m, 2H, CH_2), 1.77-1.69 (m, 2H, CH_2), 1.48 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 305.2$[M+H]^+$.

5.2.2 General procedure for the synthesis of deprotected piperidine contained fragments 3a-i

Trifluoroacetic acid (TFA, 5.0mL) was added to the solution of protected piperidine derivatives **2a-i** (5.0mmol) in CH_2Cl_2 (20.0mL) at 0^{o}C. The mixture was then stirred for 1h at room temperature. The volatiles were evaporated under reduced pressure and white solid was precipitated immediately after the addition of ether, filtered and dried, allowing you to move on to the next step without further purification.

5.2.3 General procedure for the synthesis of protected amino acid fragments 6a-c

Compound **4** or **5** (5.0 mmol), HOBt (0.75 g, 5.5 mmol) and EDCI (1.44 g, 7.5 mmol) were added in the round-bottom flask in turn and then mixed in CH_2Cl_2 (20.0 mL) at 0 ^{o}C and stirred for 30 min under 0 ^{o}C. A solution of corresponding amine (5.0 mmol) and diisopropylethylamine (1.85mL, 10.0 mmol) were then added sequentially at 0 ^{o}C. After stirred at room temperature for 3 hours, the solution was washed with aqueous $NaHCO_3$ solution (1 × 15 mL), brine (1 × 15 mL) and dried over Na_2SO_4. The organic phase was then concentrated in vacuo and purified by flash chromatography (ethyl acetate: petroleum ether = 1:3-1:2).

(S)-benzyl 2-(tert-butoxycarbonylamino)-4-(neopentylamino)-4-oxobutanoate (6a)

White solid; Yield: 99%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.41-7.30 (m, 5H, Ar-H), 5.80 (d, 1H, J = 6.5 Hz, NH), 5.66 (brs, 1H, NH), 5.17 (d, 2H, J = 5.0 Hz, CH_2), 4.55-4.52 (m, 1H, CH), 3.01 (d, 2H, J = 7.5 Hz, CH_2), 2.93 (dd, 1H, J = 18.0, 5.0 Hz, CH_2), 1.42 (s, 9H, $(CH_3)_3$), 0.87 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 393.3 $[M+H]^+$.

(S)-benzyl 2-(tert-butoxycarbonylamino)-5-(neopentylamino)-5-oxopentanoate (6b)

White solid; Yield: 95%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.35 (s, 5H, Ar-H), 6.13 (s, 1H, NH), 5.36 (d, 1H, J = 10.0 Hz, CH), 5.22 (dd, 2H, J = 31.5, 15.0 Hz, CH_2), 4.36-4.32 (m, 1H, NH), 3.06 (d, 2H, J = 7.5 Hz, CH_2), 2.26-2.18 (m, 3H, CH_2 + CH), 1.96-1.91 (m, 1H, CH) 1.43 (s, 9H, $(CH_3)_3$), 0.90 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 406.2 $[M+H]^+$.

(S)-benzyl 2-(tert-butoxycarbonylamino)-5-(tert-butylamino)-5-oxopentanoate (6c)

White solid; Yield: 98%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.36-7.33 (m, 5H, Ar-H), 5.57 (s, 1H, NH), 5.29 (d, 1H, J = 2.5 Hz, CH), 5.21 (dd, 2H, J = 33.5, 12.5 Hz, CH_2), 4.31-4.28 (m, 1H, NH), 2.13-2.08 (m, 3H, CH_2 + CH), 1.99-1.92 (m, 1H, CH) 1.43 (s, 9H, $(CH_3)_3$), 1.32 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 393.2 $[M+H]^+$.

5.2.4 General procedure for the synthesis of deprotected N-caped amino acids contained fragments 7a-c

Trifluoroacetic acid (TFA, 2.5mL) was added to a solution of protected amino acid fragments **6a-c** (2.5mmol) in CH_2Cl_2 (10.0mL) at 0 °C. The mixture was then stirred for 1h at room temperature. The volatiles were evaporated under reduced pressure and white solid was precipitated immediately after the addition of ether, filtered and dried, allowing you to move on to the next step without further purification.

5.2.5 General procedure for the synthesis of protected amino acids fragments (8a-b)

Amino acid fragments **6a-b** (5.0 mmol), 10% palladium on carbon (10 mol%) and methanol (10.0 mL) were added in the round-bottom flask in turn and then mixed together and the reaction mixture was stirred under an atmosphere of H_2 for 2 h. Filtrated to remove Pd/C and the solvent was then evaporated under reduced pressure to get compound **8a-b**. The crude product was used directly without further purification.

5.2.6 General procedure for the synthesis of protected Ortho chlorbenzamide contained fragments 10a-c

Compound **8a-b** or **9** (5.0 mmol), HOBt (0.75 g, 5.5 mmol) and EDCI (1.44 g, 7.5 mmol) were added in the round-bottom flask in turn and then mixed in CH_2Cl_2 (20.0 mL) at 0 °C and then stirred for 30 min. A solution of ortho chlorbenzamide (5.0 mmol) and diisopropylethylamine (1.85mL, 10.0 mmol) were then added sequentially at 0 °C. After stirred at room temperature for 3 hours, the solution was washed with aqueous $NaHCO_3$ solution (1 × 15 mL), brine (1 × 15 mL) and dried by Na_2SO_4. The organic phase was then concentrated under reduced pressure and purified by flash chromatography (ethyl acetate: petroleum ether = 1:4-1:3).

(S)-tert-butyl 1-(2-chlorobenzylamino)-4-(neopentylamino)-1,4-dioxobutan-2-ylcarbamate (10a)

White solid; Yield: 79%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.38 (s, 1H, NH), 7.35-7.30 (m, 2H, Ar-H), 7.22-7.20 (m, 2H, Ar-H), 6.30 (d, 1H, *J* = 6.5Hz, NH), 6.00 (s, 1H, NH), 4.54-4.51(m, 3H, CH_2 + CH), 3.05-3.01 (m, 2H, CH_2), 2.97-2.91 (m, 1H, CH), 2.59-2.55 (m, 1H, CH), 1.43 (s, 9H, $(CH_3)_3$), 0.87 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 426.3 $[M+H]^+$.

(*S*)-tert-butyl 5-(tert-butylamino)-1-(2-chlorobenzylamino)-1,5-dioxopentan-2-ylcarbamate(10b)

White solid; Yield: 81%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.39-7.36 (m, 2H, Ar-H), 7.25-7.23 (m, 2H, Ar-H), 7.18 (s, 1H, NH), 5.76 (s, 1H, NH), 5.64 (s, 1H, NH), 4.56 (d, 1H, *J* = 7.5Hz, CH_2), 4.56 (d, 1H, *J* = 6.5Hz, CH_2), 2.33-2.18 (m, 2H, CH_2), 2.11-1.94 (m, 2H, CH_2), 1.43 (s, 9H, $(CH_3)_3$), 1.36 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 426.2 $[M+H]^+$.

(*R*)-tert-butyl 3-(benzylthio)-1-(2-chlorobenzylamino)-1-oxopropan-2-ylcarbamate (10c)

White solid; Yield: 83%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.40-7.37 (m, 2H, Ar-H), 7.33-7.23 (m, 7H, Ar-H), 6.73 (brs, 1H, NH), 5.30 (brs, 1H, NH), 4.60-4.52 (m, 2H, CH_2), 4.28 (brs, 1H, CH), 3.72 (dd, 2H, *J* = 18.5, 13.5 Hz, CH_2), 2.82 (ddd, 2H, *J* = 81.0, 14.0, 6.0 Hz, CH_2), 1.50 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 435.1 $[M+H]^+$.

5.2.7 General procedure for the synthesis of deprotected Ortho chlorbenzamide contained fragments 11a-c

Trifluoroacetic acid (TFA, 2.5mL) was added to a solution of protected Ortho chlorbenzamide contained fragments (**10a-c**, 2.5mmol) in CH_2Cl_2 (10.0mL) at 0 °C. The mixture was then stirred for 1h at room temperature. The volatiles were evaporated under reduced pressure and white solid was precipitated immediately after the addition of ether, filtered and dried, allowing you to move on to the next step without further purification.

5.2.8 tert-butyl (*S*)-1-((*S*)-1-(2-chlorobenzylamino)-4-(neopentylamino)-1,4-dioxobutan-2-ylamino)-1-oxo-4-phenylbutan-2-ylcarbamate (13)

Compound **12** (6.0 mmol), HOBt (0.82 g, 6.0 mmol) and EDCI (2.25 g, 12.0 mmol) were added in the round-bottom flask in turn and then mixed at 0 °C in CH_2Cl_2 (20.0 mL) and then stirred for 30 min. A solution of corresponding protected amino acids fragments (6.0 mmol) and diisopropylethylamine (2.2mL, 12.0 mmol) were then added sequentially at 0 °C. After stirred for 3 hours at room temperature, the solution was washed with aqueous $NaHCO_3$ solution (1 × 20 mL), brine (1 × 20 mL) and dried by Na_2SO_4. The organic phase was then concentrated under reduced pressure and purified by flash

chromatography (ethyl acetate: CH_2Cl_2 = 1:5-1:1).

White solid; Yield: 85%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.32 (s, 1H, NH), 7.77 (s, 1H, NH), 7.32-7.29 (m, 4H, Ar-H), 7.24-7.18 (m, 5H, Ar-H), 6.24 (s, 1H, NH), 5.07 (s, 1H, NH), 4.81 (s, 1H, CH), 4.56-4.43 (m, 2H, CH_2), 4.15-4.04 (m, 2H, CH_2), 3.05-3.97(m, 3H, CH_2 + CH), 2.75-2.72 (m, 2H, CH_2), 2.55-2.52 (m, 1H, CH), 2.20-2.19 (m, 1H, CH), 1.39 (s, 9H, $(CH_3)_3$), 0.88 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 587.4 $[M+H]^+$.

5.2.9 General procedure for the synthesis of deprotected C-caped dipeptides fragments 14

Trifluoroacetic acid (TFA, 2.5mL) was added to a solution of protected C-caped dipeptides fragments **13** (10.0 mmol) in CH_2Cl_2 (40.0 mL) at 0 °C. The mixture was then stirred for 1h at room temperature. The volatiles were evaporated under reduced pressure and white solid was precipitated immediately after the addition of ether, filtered and dried, allowing you to move on to the next step without further purification.

5.2.10 benzyl (*S*)-3-(4-tert-butoxyphenyl)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-1-oxopropan-2-ylcarbamate (16)

Compound **15** (6.0 mmol), HOBt (0.82 g, 6.0 mmol) and EDCI (2.25 g, 12.0 mmol) were added in the round-bottom flask in turn and then mixed at 0 °C in CH_2Cl_2 (20.0 mL) and then stirred for 30 min. A solution of chlorobenzylamine (6.0 mmol) and diisopropylethylamine (2.2mL, 12.0 mmol) were then added sequentially at 0 °C. After stirred for 3 hours at room temperature, the solution was washed with aqueous $NaHCO_3$ solution (1 × 20 mL), brine (1 × 20 mL) and dried by Na_2SO_4. The organic phase was then concentrated under reduced pressure and purified by flash chromatography (ethyl acetate: CH_2Cl_2 = 1:2-1:1).

White solid; Yield: 73%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.36 (t, 1H, *J* = 12.0, 6.0 Hz, NH), 8.28 (d, 1H, *J* = 8.0 Hz, NH), 7.59 (d, 1H, *J* = 8.5 Hz, NH), 7.43-7.41 (m, 1H, Ar-H), 7.31-7.15 (m, 15H, Ar-H), 6.84 (d, 2H, *J* = 8.0 Hz, Ar-H), 4.97-4.91 (m, 2H, CH_2), 4.33-4.29 (m, 4H, CH_2 + CH + CH), 2.99-2.96 (m, 1H, CH), 2.75-2.71 (m, 1H, CH), 2.64-2.52 (m, 2H, CH_2), 2.02-1.95 (m, 1H, CH), 1.92-1.84 (m, 1H, CH), 1.24 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 656.3 $[M+H]^+$.

5.2.11 (*S*)-2-((*S*)-2-amino-3-(4-tert-butoxyphenyl)propanamido)-*N*-(2-chlorobenzyl)-4-phenylbutanamide (17)

Amino acid fragments **16** (5.0 mmol), 10% palladium on carbon (10 mol%) and methanol (10.0 mL) were added in the round-bottom flask in turn and then mixed together and the reaction mixture was stirred

under an atmosphere of H_2 for 2 h. Filtrated to remove Pd/C and the solvent was then evaporated under reduced pressure to get compound **17**. The crude product was used directly without further purification.

5.2.12 General methods for the preparation of compounds 18a-m

Corresponding fragment **7a, 7b** and **7c** (6.0 mmol) was dissolved in saturated aqueous $NaHCO_3$ (10.0 mL) and CH_2Cl_2 (10.0 mL) at 0 °C. Triphosgene (0.60 g, 2.0mmol) was added to the mixture in one portion and then stirred at 0 °C for 20 min. The aqueous layer was extracted with CH_2Cl_2 (2 × 20 mL) and the organic phase was combined and then dried by Na_2SO_4, evaporated under reduced pressure and used straightly. Diisopropylethylamine (1.6 mL, 8.0 mmol) was added to a solution of corresponding amine TFA salt (**3a-i**, 4.0 mmol). The previously obtained isocyanate was dissolved in CH_2Cl_2 (6.0 mL) and added to the reaction mixture. After stirring at room temperature for 3 hours, the mixture was washed with saturated $NaHCO_3$, NH_4Cl and brine, dried by Na_2SO_4. The organic phase was then concentrated under reduced pressure and purified by flash chromatography (dichloromethane: methanol=5:1-3:1).

(*S*)-benzyl 5-(tert-butylamino)-5-oxo-2-(4-(pyridin-3-ylcarbamoyl)piperidine-1-carboxamido)pentanoate(18a)

White solid; Yield: 52%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.97 (s, 1H, NH), 8.43 (d, 1H, *J* = 0.5 Hz, pyridine-H), 8.34 (dd, 1H, *J* = 4.5, 1.5 Hz, pyridine-H), 8.23 (d, 1H, *J* = 7.5 Hz, pyridine-H), 7.41-7.39 (m, 2H, Ar-H), 7.34-7.28 (m, 4H, Ar-H + pyridine-H), 5.33 (s, 2H, CH_2), 4.81(t, 1H, *J* = 8.5, 4.5 Hz, CH), 4.39 (s, 1H, NH), 3.88-3.82 (m, 2H, CH + CH), 3.45-3.41 (m, 2H, CH + CH), 2.59-2.54 (m, 1H, CH), 2.51(t, 2H, *J* = 11.0, 5.5 Hz, CH_2), 2.38-2.65 (m, 2H, CH + CH), 2.21-2.19 (m, 1H, CH), 1.89-1.79 (m, 2H, CH + CH), 1.35 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 524.1 $[M+H]^+$.

(*S*)-benzyl 5-(tert-butylamino)-5-oxo-2-(4-(pyrazin-2-ylcarbamoyl)piperidine-1-carboxamido)pentanoate(18b)

White solid; Yield: 71%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 9.96 (s, 1H, NH), 8.91 (s, 1H, pyridine-H), 8.39 (d, 1H, *J* = 7.5 Hz, pyridine-H), 8.34 (d, 1H, *J* = 7.5 Hz, pyridine-H), 7.42-7.39 (m, 2H, Ar-H), 7.34-7.27 (m, 3H, Ar-H), 5.42 (s, 2H, CH_2), 4.84(t, 1H, *J* = 8.5, 4.5 Hz, CH), 3.90-3.81 (m, 2H, CH + CH), 3.44-3.40 (m, 2H, CH + CH), 2.60-2.58 (m, 1H, CH), 2.52(t, 2H, *J* = 11.0, 5.5 Hz, CH_2), 2.36 (dd, 1H, *J* = 15.0, 5.0 Hz, CH), 2.31-2.25 (m, 1H, CH), 2.22 (dd, 1H, *J* = 15.0, 5.0 Hz, CH), 1.88-1.83 (m, 2H, CH + CH), 1.32 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 525.3 $[M+H]^+$.

(*S*)-benzyl 4-(neopentylamino)-4-oxo-2-(4-(pyridin-3-ylcarbamoyl)piperidine-1-carboxamido)butanoate(18c)

White solid; Yield: 62%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 9.41 (s, 1H, NH), 8.40 (d, 1H, *J* = 1.5 Hz, pyridine-H), 8.35 (dd, 1H, *J* = 4.5, 1.5 Hz, pyridine-H), 8.20-8.17 (m, 1H, pyridine-H), 7.35-7.29 (m, 6H, Ar-H + pyridine-H), 6.84 (s, 1H, NH), 5.72 (s, 1H, NH), 5.54(t, 1H, *J* = 12.5, 6.5 Hz, CH), 5.31 (s, 2H, CH_2), 3.79 (s, 1H, CH), 3.70-3.66 (m, 2H, CH + CH), 3.55-3.50 (m, 2H, CH + CH), 2.87-2.80 (m, 2H, CH + CH), 2.56-2.49 (m, 2H, CH + CH), 2.32-2.26 (m, 2H, CH + CH), 1.90-1.85 (m, 2H, CH + CH), 0.98 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 524.2 $[M+H]^+$.

(*S*)-benzyl 4-(neopentylamino)-4-oxo-2-(4-(pyrazin-2-ylcarbamoyl)piperidine-1-carboxamido)butanoate (18d)

White solid; Yield: 59%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 9.67 (s, 1H, NH), 9.08 (s, 1H, pyridine-H), 8.41 (d, 1H, *J* = 5.5 Hz, pyridine-H), 8.34 (dd, 1H, *J* = 4.5, pyridine-H), 7.34-7.25 (m, 5H, Ar-H), 7.17 (s, 1H, NH), 5.80 (s, 1H, NH), 5.41(t, 1H, *J* = 7.5, 4.0 Hz, CH), 4.05 (s, 1H, CH), 3.71-3.66 (m, 2H, CH + CH), 3.35-3.31 (m, 2H, CH + CH), 3.06 (s, 1H, CH), 2.92-2.89 (m, 2H, CH + CH), 2.69-2.65 (m, 1H, CH), 2.38-2.32 (m, 2H, CH + CH), 1.89-1.83 (m, 2H, CH + CH), 1.03 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 525.4$[M+H]^+$.

(*S*)-benzyl 5-(neopentylamino)-5-oxo-2-(4-(pyridin-3-ylcarbamoyl)piperidine-1-carboxamido)pentanoate(18e)

White solid; Yield: 69%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.69 (s, 1H, pyridine-H), 8.40 (d, 1H, *J* = 10.0 Hz, pyridine-H), 7.36-7.27 (m, 5H, Ar-H), 6.30 (t, 1H, *J* = 15.0, 7.5 Hz, NH), 6.25 (d, 1H, *J* = 10.5 Hz, NH), 5.21-5.08 (m, 2H, CH_2), 4.44-4.40 (m, 1H, CH), 4.08-4.02 (m, 1H, CH), 3.09-2.96 (m, 2H, CH_2), 2.88-2.79 (m, 2H, CH_2), 2.50-2.48 (m, 1H, CH), 2.34-2.14 (m, 3H, CH_2 + CH), 2.05-2.00 (m, 1H, CH), 1.91-1.88 (m, 2H, CH_2), 1.80-1.74(m, 2H, CH_2), 0.88 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 538.4 $[M+H]^+$.

(*S*)-benzyl 5-(neopentylamino)-5-oxo-2-(4-(pyrazin-2-ylcarbamoyl)piperidine-1-carboxamido)pentanoate(18f)

White solid; Yield: 55%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 9.55 (s, 1H, NH), 8.36 (d, 1H, *J* = 3.5 Hz, pyridine-H), 8.24 (s, 1H, pyridine-H), 8.06 (s, 1H, pyridine-H), 7.36-7.30 (m, 5H, Ar-H), 6.12 (d, 1H, *J* = 8.0 Hz, NH), 6.02 (t, 1H, *J* = 15.0, 7.5 Hz, NH), 5.24-5.12 (m, 2H, CH_2), 4.48-4.44 (m, 1H, CH), 4.11-4.06 (m, 1H, CH), 3.11-2.99 (m, 2H, CH_2), 2.93-2.87 (m, 2H, CH_2), 2.56-2.50 (m, 1H, CH), 2.33-2.10 (m, 4H, CH_2 + CH_2), 2.04-1.96 (m, 4H, CH_2 + CH_2), 0.89 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 539.2 $[M+H]^+$.

(*S*)-benzyl 5-(tert-butylamino)-2-(4-(2-fluorophenylcarbamoyl)piperidine-1-carboxamido)-5-oxopentanoate(18g)

White solid; Yield: 68%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.33 (t, 1H, *J* = 19.0, 9.5 Hz, NH), 7.36-7.34 (m, 8H, Ar-H), 7.15-7.10 (m, 1H, Ar-H), 7.07-7.04 (m, 1H, NH), 6.22 (d, 1H, *J* = 8.0 Hz, NH), 5.20-5.11 (m, 2H, CH_2), 4.46-4.40 (m, 1H, CH), 4.13-4.07 (m, 2H, CH_2), 2.92-2.85 (m, 2H, CH_2), 2.49-2.44 (m, 1H, CH), 2.23-2.10 (m, 4H, CH_2 + CH_2), 1.97-1.73 (m, 4H, CH_2 + CH_2), 1.32 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 541.3 $[M+H]^+$.

(*S*)-benzyl 5-(tert-butylamino)-2-(4-(3-fluorophenylcarbamoyl)piperidine-1-carboxamido)-5-oxopentanoate(18h)

White solid; Yield: 71%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.60 (s, 1H, NH), 7.53 (d, 1H, *J* = 14.0 Hz, Ar-H), 7.34-7.30 (m, 7H, Ar-H), 7.22-7.16 (m, 1H, Ar-H), 6.82-6.78 (m, 1H, NH), 6.29 (d, 1H, *J* = 7.5 Hz, NH), 5.23-5.09 (m, 2H, CH_2), 4.42-4.37 (m, 1H, CH), 4.15-4.08 (m, 2H, CH_2), 2.89-2.82 (m, 2H, CH_2), 2.45-2.40 (m, 1H, CH), 2.23-2.08 (m, 4H, CH_2 + CH_2), 1.92-1.73 (m, 4H, CH_2 + CH_2), 1.31 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 541.3 $[M+H]^+$.

(*S*)-benzyl 5-(tert-butylamino)-2-(4-(4-fluorophenylcarbamoyl)piperidine-1-carboxamido)-5-oxopentanoate(18i)

White solid; Yield: 57%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.46 (s, 1H, NH), 7.43-7.40 (m, 2H, Ar-H), 7.33-7.27 (m, 5H, Ar-H), 6.82-6.78 (d, 1H, *J* = 6.5 Hz, NH), 7.16 (t, 1H, *J* = 7.5, 15.5 Hz, Ar-H), 5.24-5.09 (m, 2H, CH_2), 4.35-4.33 (m, 1H, CH), 4.19-4.14 (m, 2H, CH_2), 2.92-2.84 (m, 2H, CH_2), 2.49-2.46 (m, 1H, CH), 2.23-2.12 (m, 4H, CH_2 + CH_2), 1.95-1.78 (m, 4H, CH_2 + CH_2), 1.32 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 541.3 $[M+H]^+$.

(*S*)-benzyl 5-(tert-butylamino)-2-(4-(4-cyanophenylcarbamoyl)piperidine-1-carboxamido)-5-oxopentanoate(18j)

White solid; Yield: 71%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.16 (s, 1H, NH), 7.73 (d, 2H, *J* = 10.5 Hz, Ar-H), 7.59 (d, 2H, *J* = 11.0 Hz, Ar-H), 7.37-7.31 (m, 5H, Ar-H), 6.82-6.78 (d, 1H, *J* = 6.5 Hz, NH), 5.22-5.06 (m, 2H, CH_2), 4.38-4.35 (m, 1H, CH), 4.13-4.08 (m, 2H, CH_2), 2.89-2.81 (m, 2H, CH_2), 2.47-2.45 (m, 1H, CH), 2.25-2.12 (m, 4H, CH_2 + CH_2), 1.92-1.76 (m, 4H, CH_2 + CH_2), 1.31 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 548.1 $[M+H]^+$.

(*S*)-benzyl 5-(tert-butylamino)-2-(4-(4-methoxyphenylcarbamoyl)piperidine-1-carboxamido)-5-oxopentanoate(18l)

White solid; Yield: 69%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.43-7.41 (m, 2H, Ar-H), 7.36-7.31 (m, 5H, Ar-H), 6.87-6.84 (d, 2H, *J* = 11.0 Hz, Ar-H), 6.22 (d, 1H, *J* = 8.0 Hz, NH), 5.58 (s, 1H, NH), 5.23-5.10

(m, 2H, CH_2), 4.42-4.37 (m, 1H, CH), 4.15-4.06 (m, 2H, CH_2), 2.90-2.82 (m, 2H, CH_2), 2.42-2.37 (m, 1H, CH), 2.21-2.00 (m, 4H, CH_2 + CH_2), 1.94-1.75 (m, 4H, CH_2 + CH_2), 1.66 (s, 3H, CH_3), 1.32 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 553.1 $[M+H]^+$.

(*S*)-benzyl 5-(tert-butylamino)-5-oxo-2-(4-(phenylcarbamoyl)piperidine-1-carboxamido)pentanoate(18m)

White solid; Yield: 55%. ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.53-7.51 (d, 2H, *J* = 9.5 Hz, Ar-H), 7.36-7.30 (m, 7H, Ar-H), 7.13 (t, 1H, *J* = 18.5, 9.5 Hz, Ar-H), 6.23 (d, 1H, *J* = 7.5 Hz, NH), 5.55 (s, 1H, NH), 5.24-5.11 (m, 2H, CH_2), 4.42-4.38 (m, 1H, CH), 4.13-4.06 (m, 2H, CH_2), 2.91-2.83 (m, 2H, CH_2), 2.45-2.39 (m, 1H, CH), 2.22-2.09 (m, 4H, CH_2 + CH_2), 1.95-1.78 (m, 4H, CH_2 + CH_2), 1.32 (s, 9H, $(CH_3)_3$); ESI-MS: m/z = 523.3 $[M+H]^+$.

5.2.13 General procedure for the synthesis of key intermediates 19a-m

The key intermediates **18a-m** (5.0 mmol), 10% palladium on carbon (10 mol%) and methanol (10.0 mL) were added in the round-bottom flask in turn and then mixed together and the reaction mixture was stirred under an atmosphere of H_2 for 2 h. Filtrated to remove Pd/C and the solvent was then evaporated under reduced pressure to get compound **19a-m**. The crude product was used straightly without further purification.

5.2.14 General procedure for the synthesis of target compounds 20-23

Corresponding fragment **14** or **17** (6.0 mmol) was dissolved in saturated aqueous $NaHCO_3$ (10.0 mL) and CH_2Cl_2 (10.0 mL) at 0 °C. Triphosgene (0.60 g, 2.0mmol) was added to the mixture in one portion and then stirred at 0 °C for 20 min. The aqueous layer was extracted with CH_2Cl_2 (2 × 20 mL) and the organic phase was combined and then dried by Na_2SO_4, evaporated under reduced pressure and used straightly. Diisopropylethylamine (1.6 mL, 8.0 mmol) was added to a solution of corresponding amine TFA salt (**3a** or **3b**, 4.0 mmol). The previously obtained isocyanate was dissolved in CH_2Cl_2 (6.0 mL) and added to the reaction mixture. After stirring at room temperature for 3 hours, the mixture was washed with saturated $NaHCO_3$, NH_4Cl and brine, dried by Na_2SO_4. The organic phase was then concentrated under reduced pressure and purified by flash chromatography (dichloromethane: methanol=100:1-50:1).

5.2.15 General procedure for the synthesis of target compounds 24-39

Corresponding acids fragments (**19a-m**, 10.0 mmol), HOBt (1.48 g, 12.0 mmol) and EDCI (2.88 g, 15.0 mmol) were added in the round-bottom flask in turn and then mixed at 0 °C in CH_2Cl_2 (40.0 mL) and then stirred for 30 min. A solution of Ortho chlorbenzamide (10.0 mmol) and diisopropylethylamine

(3.8mL, 24.0 mmol) were then added successively at 0 °C. After stirred for 3 hours at room temperature, the solution was washed with aqueous $NaHCO_3$ (1 × 50 mL), brine (1 × 50 mL) and dried by Na_2SO_4. The organic phase was then concentrated under reduced pressure and purified by flash chromatography (dichloromethane: methanol=50:1-20:1).

N^1-((S)-1-((S)-1-(2-chlorobenzylamino)-4-(neopentylamino)-1,4-dioxobutan-2-ylamino)-1-oxo-4-phenylbutan-2-yl)-N^4-(pyridin-3-yl)piperidine-1,4-dicarboxamide (20)

White solid; Yield: 37%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.10 (s, 1H, NH), 8.76 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.50 (d, 1H, J = 8 Hz, pyrazine-H), 8.36 (d, 1H, J = 6.0 Hz, pyrazine-H), 8.25-8.24 (m, 1H, NH), 8.07-8.04 (m, 1H, NH), 7.91 (d, 1H, J = 6.0 Hz, pyrazine-H), 7.40-7.39 (m, 1H, pyrazine-H), 7.35-7.27 (m, 4H, Ar-H), 7.22-7.19 (m, 5H, Ar-H), 6.86 (d, 1H, J = 6.0 Hz, NH), 4.53 (dd, 1H, J = 14.0, 6.0 Hz, CH), 4.39-4.34 (m, 1H, CH), 4.24-4.21 (m, 1H, CH), 4.04-3.99 (m, 2H, CH_2), 3.95 (dd, 1H, J = 14.0, 7.0 Hz, CH), 2.94-2.83 (m, 2H, CH_2), 2.80-2.70 (m, 4H, CH_2 + CH_2), 2.64-2.59 (m, 3H, CH_2+ CH), 1.95-1.92 (m, 2H, CH_2), 1.77-1.73 (m, 2H, CH_2), 1.62-1.52 (m, 2H, CH_2), 0.80 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.04, 173.44, 171.52, 170.66, 157,99, 144.47, 142.05, 141.27, 136.60, 136.42, 132.07, 129.28, 128.81, 128.72, 128.67, 127.40, 126.47, 126.25, 123.99, 55.77, 50.84, 50.10, 43.49, 43.47, 43.14, 36.64, 33.31, 32.35, 32.33, 28.47, 27.68; ESI-MS: m/z = 718.4 $[M+H]^+$.

N^1-((*S*)-1-((*S*)-1-(2-chlorobenzylamino)-4-(neopentylamino)-1,4-dioxobutan-2-ylamino)-1-oxo-4-phenylbutan-2-yl)-N^4-(pyrazin-2-yl)piperidine-1,4-dicarboxamide (21)

White solid; Yield: 41%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.76 (s, 1H, NH), 9.36 (d, 1H, J = 1.5 Hz, NH), 8.52 (d, 1H, J = 9.0 Hz, pyrazine-H), 8.41-8.40 (m, 1H, NH), 8.38-8.35 (m, 2H, pyrazine-H+ pyrazine-H), 7.92 (t, 1H, NH), 7.40-7.38 (m, 1H, J = 6.5 Hz, NH), 7.40-7.38 (m, 1H, Ar-H), 7.31-7.28 (m, 3H, Ar-H), 7.25-7.17 (m, 5H, Ar-H), 6.86 (d, 1H, J = 5.5 Hz, Ar-H), 4.52 (dd, 1H, J = 13.5, 5.5 Hz, CH), 4.39 (dd, 1H, J = 16.5, 6.5 Hz, CH), 4.24 (dd, 1H, J = 16.5, 6.5 Hz, CH), 4.04-3.99 (m, 2H, CH_2), 3.94 (dd, 1H, J = 13.5, 6.5 Hz, CH), 2.95-2.83 (m, 2H, CH_2), 2.79-2.76 (m, 1H, CH), 2.70-2.66 (m, 2H, CH_2 + CH_2), 2.62-2.57 (m, 2H, CH_2), 1.97-1.92 (m, 2H, CH_2), 1.79-1.73 (m, 2H, CH_2), 1.63-1.51 (m, 2H, CH_2), 0.80 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.61, 173.44, 171.52, 170.71, 157.99, 149.40, 143.02, 142.04, 140.02, 136.75, 136.60, 132.07, 129.27, 128.82, 128.72, 128.66, 127.39, 126.25, 55.80, 50.86, 50.09, 43.50, 43.45, 43.42, 42.58, 36.57, 33.27, 32.35, 28.35, 27.67; ESI-MS: m/z = 719.4 $[M+H]^+$.

N^1-((*S*)-3-(4-tert-butoxyphenyl)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-

1-oxopropan-2-yl)-N^4-(pyridin-3-yl)piperidine-1,4-dicarboxamide (22)

White solid; Yield: 45%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.07 (s, 1H, NH), 8.72 (s, 1H, pyrazine-H), 8.37 (t, 1H, J = 6.0 Hz, pyrazine-H), 8.22 (d, 1H, J = 4.5 Hz, pyrazine-H), 8.11 (d, 1H, J = 8.0 Hz, pyrazine-H), 8.02 (d, 1H, J = 7.5 Hz, NH), 7.31-7.14 (m, 12H, Ar-H), 6.84 (d, 2H, J = 9.0 Hz, Ar-H + NH), 6.68 (d, 1H, J = 9.0 Hz, NH), 4.27-4.22 (m, 4H, CH_2 + CH_2), 3.99-3.94 (m, 2H, CH_2), 2.98-2.95 (m, 1H, CH), 2.86-2.81 (m, 1H, CH), 2.71-2.52 (m, 4H, CH_2 + CH_2), 1.99-1.98 (m, 1H, CH), 1.89-1.85 (m, 1H, CH), 1.70 (d, 1H, J = 11.5 Hz, CH), 1.49-1.28 (m, 4H, CH_2 + CH_2), 1.21 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_1-$CDCl_3$): δ = 173.21,172.51, 171.32, 157.15, 157.11, 154.56, 144.76, 141.12, 140.83, 138.02, 135.25, 131.26, 131.22, 129.82, 129.61, 128.60, 128.56, 128.44, 127.67, 127.59, 127.38, 126.18, 124.55, 123.78, 78.72, 77.24, 56.52, 53.20, 43.43, 37.23, 33.53, 31.91, 29.71, 28.82, 28.07; ESI-MS: m/z = 750.3 $[M+H]^+$.

N^1-((*S*)-3-(4-tert-butoxyphenyl)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-1-oxopropan-2-yl)-N^4-(pyrazin-2-yl)piperidine-1,4-dicarboxamide (23)

White solid; Yield: 37%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.72 (s, 1H, NH), 9.33 (d, 1H, J = 1.5 Hz, NH), 8.30-8.38 (m, 1H, pyrazine-H), 8.37 (d, 2H, J = 3.5 Hz, pyrazine-H + pyrazine-H), 8.17 (d, 1H, J = 9.5 Hz, NH), 7.44 (m, 1H, Ar-H), 7.30-7.17 (m, 8H, Ar-H), 6.85 (d, 2H, J = 10.5 Hz, Ar-H + NH), 6.69 (d, 1H, J = 10.0 Hz, NH), 4.35-4.25 (m, 4H, CH_2 + CH_2), 4.00-3.94 (m, 2H, CH_2), 3.01-2.97 (m, 1H, CH), 2.89-2.83 (m, 1H, CH), 2.71-2.58 (m, 4H, CH + CH + CH_2), 2.56-2.54 (m, 1H, CH), 2.07-1.86 (m, 2H, CH_2), 1.72 (d, 1H, J = 13.0 Hz, CH), 1.50-1.32 (m, 3H, CH_2 + CH), 1.22 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.10, 172.73, 171.51, 157.16, 153.27, 148.87, 142.51, 141.35, 139.53, 136.23, 136.13, 133.22, 131.93, 129.86, 129.02, 128.61, 128.54, 128.30, 127.04, 125.78, 123.20, 77.48, 56.11, 52.38, 43.29, 43.04, 41.99, 36.07, 33.69, 31.26, 28.51, 27.58, 27.71; ESI-MS: m/z = 754.4 $[M+H]^+$.

N^1-((*S*)-5-(tert-butylamino)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-1,5-dioxopentan-2-yl)-N^4-(pyridin-3-yl)piperidine-1,4-dicarboxamide (24)

White solid; Yield: 35%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.10 (s, 1H, NH), 8.72 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.44 (m, 1H, J = 6.0 Hz, pyrazine-H), 8.36 (t, 1H, J = 4.8 Hz, pyrazine-H), 8.23-8.22 (dd, 1H, J = 4.0, 1.5 Hz, pyrazine-H), 8.04-8.01 (m, 1H, NH), 7.46 (s, 1H, NH), 7.41-7.40 (m, 1H, NH), 7.30-7.24 (m, 6H, Ar-H), 7.17-7.14 (m, 3H, Ar-H), 6.82 (d, 1H, J = 6.5 Hz, NH), 4.32 (d, 2H, J = 6.0 Hz, CH), 4.25-4.22 (m, 1H, CH), 4.01-3.98 (m, 3H, CH_2 + CH), 2.77-2.64 (m, 2H, CH_2), 2.62-2.51(m, 3H, CH_2 + CH), 2.19-2.15 (m, 2H, CH_2), 2.06-1.99 (m, 1H, CH_2), 1.92-1.84 (m, 3H, CH_2 + CH), 1.76-

1.73 (m, 2H, CH_2), 1.54-1.43 (m, 2H, CH_2), 1.22 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.12, 173.49, 172.28, 172.13, 157.85, 144.51, 143.71, 141.84, 141.27, 140.23, 136.63, 136.37, 132.40, 129.48, 128.81, 128.77, 127.55, 126.51, 126.29, 124.02, 55.60, 52.71, 50.41, 43.54, 43.46, 43.10, 33.85, 33.40, 31.85, 28.99, 28.53, 27.64; ESI-MS: m/z = 718.4 $[M+H]^+$.

N^1-((*S*)-5-(tert-butylamino)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-1,5-dioxopentan-2-yl)-N^4-(pyrazin-2-yl)piperidine-1,4-dicarboxamide (25)

White solid; Yield: 31%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.75 (s, 1H, NH), 9.31 (d, 1H, J = 1.0 Hz, NH), 8.43 (t, 1H, J = 6.0 Hz, pyrazine-H), 8.38 (t, 1H, J = 2.0 Hz, pyrazine-H), 8.33 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.44 (d, 1H, J = 13.0 Hz, NH), 7.45 (s, 1H, NH), 7.41-7.39 (m, 1H, Ar-H), 7.29-7.24 (m, 5H, Ar-H), 7.17-7.14 (m, 3H, Ar-H), 6.81 (d, 1H, J = 6.5 Hz, NH), 4.32 (d, 2H, J = 5.5 Hz, CH_2), 4.24-4.22 (m, 1H, CH), 4.01-3.97 (m, 3H, CH_2 + CH), 2.69-2.66 (m, 3H, CH_2 + CH), 2.62-2.52(m, 2H, CH_2), 2.19-2.15 (m, 2H, CH_2), 2.06-1.99 (m, 1H, CH), 1.92-1.82 (m, 3H, CH_2 + CH), 1.76-1.74 (m, 2H, CH_2), 1.53-1.42 (m, 2H, CH_2), 1.22 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.68, 173.49, 172.28, 172.13, 157.86, 149.36, 143.02, 141.84,140.06, 136.75, 136.63, 132.40, 129.47, 129.12, 128.98, 128.81, 128.77, 127.54, 126.28, 55.61, 52.71, 50.41, 43.53, 43.46, 42.51, 33.84, 33.40, 31.85, 28.98, 28.40, 27.62; ESI-MS: m/z = 719.4 $[M+H]^+$.

N^1-((*S*)-1-((*R*)-3-(benzylthio)-1-(2-chlorobenzylamino)-1-oxopropan-2-ylamino)-4-(neopentylamino)-1,4-dioxobutan-2-yl)-N^4-(pyridin-3-yl)piperidine-1,4-dicarboxamide (26)

White solid; Yield: 46%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.10 (s, 1H, NH), 8.72 (s, 1H, pyrazine-H), 8.64 (m, 1H, NH), 8.23 (d, 1H, J = 4.0 Hz, pyrazine-H), 8.20 (d, 1H, J = 9.0 Hz, pyrazine-H), 8.03 (d, 1H, J = 11.5 Hz, NH), 7.76 (m, 1H, pyrazine-H), 7.41-7.22 (m, 10H, Ar-H + NH), 6.73 (d, 1H, J = 7.0 Hz, NH), 4.48-4.46 (m, 1H, CH), 4.44-4.40 (m, 1H, CH), 4.36 (d, 2H, J = 5.5 Hz, CH_2), 3.99 (d, 2H, J = 13.5 Hz, CH_2), 3.74 (s, 2H, CH_2), 2.84-2.79 (m, 2H, CH_2), 2.75-2.64 (m, 4H, CH_2 + CH_2), 2.57-2.52 (m, 2H, CH_2), 1.89 (s, 1H, CH), 1.75-1.72 (m, 2H, CH_2), 1.50-1.45 (m, 2H, CH_2), 0.78 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.66, 172.87, 170.83, 170.46, 157.41, 149.37, 143.07, 140.09, 138.87, 136.73, 136.41, 132.35, 129.45, 129.43, 129.13, 129.01, 128.81, 127.53, 127.29, 53.16, 52.55, 50.17, 43.44, 42.47, 37.99, 35.68, 32.98, 32.34, 28.40, 28.33, 27.65; ESI-MS: m/z = 750.3 $[M+H]^+$.

N^1-((*S*)-1-((*R*)-3-(benzylthio)-1-(2-chlorobenzylamino)-1-oxopropan-2-ylamino)-4-(neopentylamino)-1,4-dioxobutan-2-yl)-N^4-(pyrazin-2-yl)piperidine-1,4-dicarboxamide (27)

White solid; Yield: 33%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.75 (s, 1H, NH), 9.32 (d, 1H, J = 1.0

Hz, NH), 8.65 (t, 1H, *J* = 5.5 Hz, pyrazine-H), 8.38-8.37 (m, 1H, NH), 8.33 (d, 1H, *J* = 3.5 Hz, pyrazine-H), 8.19 (d, 1H, *J* = 8.0 Hz, pyrazine-H), 8.65 (t, 1H, *J* = 6.0 Hz, NH), 7.42-7.20 (m, 9H, Ar-H), 6.73 (d, 1H, *J* = 7.5 Hz, NH), 4.50-4.40 (m, 2H, CH + CH), 4.34 (d, 2H, *J* = 6.0 Hz, CH_2), 4.02-3.93 (m, 2H, CH_2), 3.74 (d, 2H, *J* = 1.5 Hz, CH_2), 2.87-2.77 (m, 3H, CH_2 + CH), 2.72-2.65 (m, 2H, CH_2 + CH_2), 2.62-2.51 (m, 2H, CH_2), 1.74-1.73 (m, 2H, CH_2), 1.48-1.44 (m, 2H, CH_2), 0.78 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 175.19, 173.47, 171.34, 171.05, 158.62, 154.23 148.11, 145.19, 141.04, 139.77, 136.65, 135.21, 131.39, 130.78, 129.69, 129.36, 129.12, 128.42, 128.05, 127.58, 55.47, 53.17, 50.86, 45.98, 42.75, 38.74, 34.22, 32.79, 31.51, 28.58, 28.13, 27.66; ESI-MS: m/z = 751.3 $[M+H]^+$.

N^1-((*S*)-1-((*S*)-1-(2-chlorobenzylamino)-4-(neopentylamino)-1,4-dioxobutan-2-ylamino)-4-(neopentylamino)-1,4-dioxobutan-2-yl)-N^4-(pyridin-3-yl)piperidine-1,4-dicarboxamide (28)

White solid; Yield: 45%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.43 (s, 1H, NH), 8.54 (d, 1H, *J* = 7.5 Hz, pyrazine-H), 8.45 (m, 1H, NH), 8.30 (s, 1H, pyrazine-H), 8.10 (d, 1H, *J* = 10.0 Hz, pyrazine-H), 7.88 (s, 1H, NH), 7.81 (s, 1H, NH), 7.77 (t, 1H, *J* = 9.5 Hz, pyrazine-H), 7.41-7.22 (m, 4H, Ar-H), 7.09 (d, 1H, *J* = 5.5 Hz, NH), 6.81 (d, 1H, *J* = 6.0 Hz, NH), 4.53 (d, 1H, *J* = 7.5 Hz, CH), 4.38-4.23 (m, 3H, CH_2 + CH), 3.96 (d, 1H, *J* = 15.5 Hz, CH), 2.90-2.89 (m, 3H, CH_2 + CH), 2.79-2.69 (m, 4H, CH_2 + CH_2), 2.66-2.58 (m, 4H, CH_2 + CH_2), 1.76 (t, 2H, *J* =15.5 Hz, CH_2), 1.58-1.48 (m, 1H, CH_2), 0.81 (s, 18H, $(CH_3)_6$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 173.87，172.06, 171.01,170.11, 170.02, 156.92, 152.14, 147.85, 137.98, 136.10, 131.57, 128.78, 128.26, 128.18, 126.91, 119.18, 113.43, 52.58, 50.41, 49.68, 49.60, 42.89, 42.14, 37.17, 36.23, 31.88, 31.80, 27.94, 27.90, 27.20, 27.12; ESI-MS: m/z = 741.5 $[M+H]^+$.

N^1-((*S*)-1-((*S*)-1-(2-chlorobenzylamino)-4-(neopentylamino)-1,4-dioxobutan-2-ylamino)-4-(neopentylamino)-1,4-dioxobutan-2-yl)-N^4-(pyrazin-2-yl)piperidine-1,4-dicarboxamide (29)

White solid; Yield: 38%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.74 (s, 1H, NH), 9.35 (s, 1H, pyrazine-H), 8.56 (d, 1H, *J* = 10.0 Hz, pyrazine-H), 8.44-8.39 (m, 2H, NH + NH), 8.35 (d, 1H, *J* = 3.0 Hz, pyrazine-H), 7.89 (t, 1H, *J* = 7.5 Hz, NH), 7.83 (t, 1H, *J* = 7.5 Hz, NH), 7.41-7.24 (m, 4H, Ar-H), 6.83 (d, 1H, *J* = 8.0 Hz, NH), 4.54-4.49 (m, 1H, CH), 4.40-4.22 (m, 3H, CH_2 + CH), 3.97 (d, 2H, *J* =16.5 Hz, CH_2), 2.94-2.85 (m, 3H, CH_2 + CH), 2.80-2.76 (m, 1H, CH), 2.72-2.66 (m, 4H, CH_2 + CH_2), 2.61-2.55 (m, 3H, CH_2 + CH), 1.79-1.73 (m, 2H, CH_2), 1.61-1.48 (m, 2H, CH_2), 0.81 (s, 18H, $(CH_3)_6$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.07, 172.06, 171.01, 171.15, 170.01, 156.90, 148.88, 142.53, 139.53, 136.21, 136.08, 131.57, 128.78, 128.26, 128.17, 126.90, 52.61, 50.41, 49.68, 49.59, 42.87, 42.81, 41.97, 37.13, 36.16, 31.87, 31.80, 27.81, 27.17, 27.11; ESI-MS: m/z = 742.7 $[M+H]^+$.

N^1-((*S*)-1-((*S*)-1-(2-chlorobenzylamino)-4-(neopentylamino)-1,4-dioxobutan-2-ylamino)-4-(neopentylamino)-1,4-dioxobutan-2-yl)-N^4-(pyridin-3-yl)piperidine-1,4-dicarboxamide (30)

White solid; Yield: 39%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.45 (s, 1H, NH), 8.50 (t, 1H, J = 7.0 Hz, NH), 8.31 (d, 1H, J = 6.0 Hz, pyrazine-H), 8.22 (d, 1H, J = 10.0 Hz, pyrazine-H), 8.09 (d, 1H, J = 10.5 Hz, pyrazine-H), 7.83-7.74 (m, 2H, NH + pyrazine-H), 7.43-7.41 (m, 1H, NH), 7.29-7.26 (m, 4H, Ar-H), 7.10-7.07 (m, 1H, NH), 6.89 (d, 1H, J = 8.5 Hz, NH), 4.35-4.19 (m, 3H, CH_2 + CH), 4.22-4.19 (m, 1H, CH), 4.02-3.94 (m, 2H, CH_2), 2.84-2.56 (m, 7H, $3CH_2$ + CH), 2.10-1.75 (m, 6H, $3CH_2$), 1.49-1.44 (m, 2H, CH_2), 1.23 (s, 9H, $(CH_3)_3$), 0.79 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 173.96, 171.59, 172.37, 171.20, 170.08, 156.97, 152.10, 147.87, 138.02, 136.17, 131.80, 128.92, 128.46, 128.41, 127.01, 119.22, 113.45, 52.41, 52.24, 49.82, 49.67, 43.08, 42.96, 42.12, 37.60, 32.54, 31.77, 28.46, 28.01, 27.62, 27.13; ESI-MS: m/z = 741.5 $[M+H]^+$.

N^1-((*S*)-1-((*S*)-5-(tert-butylamino)-1-(2-chlorobenzylamino)-1,5-dioxopentan-2-ylamino)-4-(neopentylamino)-1,4-dioxobutan-2-yl)-N^4-(pyrazin-2-yl)piperidine-1,4-dicarboxamide (31)

White solid; Yield: 34%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.77 (s, 1H, NH), 9.34 (d, 1H, J = 1.5 Hz, NH), 8.49 (t, 1H, J = 8.0 Hz, pyrazine-H), 8.40-8.39 (m, 1H, NH), 8.35 (d, 1H, J = 3.5 Hz, pyrazine-H), 8.22 (d, 1H, J = 10.0 Hz, pyrazine-H), 7.83-7.80 (m, 1H, NH), 7.43-7.41 (m, 1H, NH), 7.33-7.26 (m, 4H, Ar-H), 6.70 (d, 1H, J = 8.5 Hz, NH), 4.39-4.28 (m, 3H, CH_2 + CH), 4.23-4.18 (m, 1H, CH), 4.03-3.95 (m, 2H, CH_2), 2.86-2.55 (m, 7H, $3CH_2$ + CH), 2.14-1.76 (m, 6H, $3CH_2$), 1.53-1.45 (m, 2H, CH_2), 1.23 (s, 9H, $(CH_3)_3$), 0.79 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.15, 172.36, 171.59, 171.22, 170.08, 156.97, 148.84, 142.54, 139.57, 136.23, 136.15, 131.80, 128.92, 128.46, 128.42, 127.01, 52.42, 52.23, 49.84, 49.67, 43.02, 42.92, 41.93, 37.59, 32.54, 31.76, 28.45, 27.90, 27.82, 27.64, 27.12; ESI-MS: m/z = 742.7 $[M+H]^+$.

N^1-((*S*)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-5-(neopentylamino)-1,5-dioxopentan-2-yl)-N^4-(pyridin-3-yl)piperidine-1,4-dicarboxamide (32)

White solid; Yield: 29%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.12 (s, 1H, NH), 8.74 (d, 1H, J = 3.0 Hz, NH), 8.47 (t, 1H, J = 7.0 Hz, pyrazine-H), 8.25 (d, 1H, J = 4.5 Hz, pyrazine-H), 8.08 (t, 1H, J = 10.0 Hz, NH), 7.80 (t, 1H, J = 7.5 Hz, pyrazine-H), 7.43-7.41 (m, 1H, Ar-H), 7.32-7.26 (m, 6H, Ar-H + NH), 7.19-7.17 (m, 3H, Ar-H), 6.82 (d, 1H, J = 8.0 Hz, NH), 4.34 (d, 2H, J = 7.5 Hz, CH_2), 4.29-4.24 (m, 1H, CH), 4.08-4.03 (m, 3H, CH_2 + CH), 2.93-2.83 (m, 2H, CH_2), 2.79-2.63(m, 3H, CH_2 + CH), 2.61-2.56 (m, 2H, CH_2), 2.28-2.26 (m, 1H, CH_2), 2.03-1.87 (m, 4H, CH_2 + CH_2), 1.78-1.76 (m, 2H, CH_2), 1.57-1.44

(m, 2H, CH_2), 0.82 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 173.61, 172.92, 172.27, 171.63, 157.33, 144.00, 141.33, 140.76, 136.11, 135.87, 131.89, 128.98, 128.60, 128.49, 128.31, 128.27, 127.05, 125.98, 125.79, 123.52, 54.99, 52.21, 49.63, 43.05, 42.98, 42.60, 33.37, 32.20, 31.88, 31.34, 28.01, 27.31, 27.22; ESI-MS: m/z = 732.4 $[M+H]^+$.

N^1-((*S*)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-5-(neopentylamino)-1,5-dioxopentan-2-yl)-N^4-(pyrazin-2-yl)piperidine-1,4-dicarboxamide (33)

White solid; Yield: 41%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.77 (s, 1H, NH), 9.33 (s, 1H, NH), 8.46 (t, 1H, *J* = 7.5 Hz, pyrazine-H), 8.39 (s, 1H, pyrazine-H), 8.35 (d, 1H, *J* = 3.0 Hz, NH), 8.07 (d, 1H, *J* = 10.0 Hz, NH), 7.80 (t, 1H, *J* = 7.5 Hz, pyrazine-H), 7.43-7.41 (m, 1H, Ar-H), 7.29-7.25 (m, 5H, Ar-H), 7.19-7.17 (m, 3H, Ar-H), 6.82 (d, 1H, *J* = 8.5 Hz, NH), 4.34 (d, 2H, *J* = 7.0 Hz, CH_2), 4.27-4.24 (m, 1H, CH), 4.08-4.03 (m, 3H, CH_2 + CH), 2.91-2.83 (m, 2H, CH_2), 2.77-2.64(m, 3H, CH_2 + CH), 2.62-2.56 (m, 2H, CH_2), 2.27-2.26 (m, 4H, CH_2), 2.03-1.87 (m, 4H, CH_2 + CH_2), 1.78-1.76 (m, 2H, CH_2), 1.56-1.46 (m, 2H, CH_2), 0.82 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.18, 172.93, 172.27, 171.63, 157.34, 148.86, 142.53, 141.33, 139.56, 136.24, 136.12, 131.89, 128.98, 128.60, 128.49, 128.31, 128.27, 127.05, 125.78, 55.00, 52.21, 49.62, 43.04, 42.98, 42.01, 33.36, 32.20, 31.88, 31.34, 27.89, 27.35, 27.22; ESI-MS: m/z = 733.4 $[M+H]^+$.

N^1-((*S*)-5-(tert-butylamino)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-1,5-dioxopentan-2-yl)-N^4-(3-fluorophenyl)piperidine-1,4-dicarboxamide (34)

White solid; Yield: 40%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.17 (s, 1H, NH), 8.52 (t, 1H, *J* = 6.5 Hz, NH), 8.12 (d, 1H, *J* = 10.0 Hz, NH), 7.69 (d, 1H, *J* = 14.5 Hz, NH), 7.53-7.48 (m, 3H, Ar-H), 7.39-7.32 (m, 5H, Ar-H), 7.26-7.23 (m, 4H, Ar-H), 6.94-6.88 (m, 2H, Ar-H + NH), 4.41-3.99 (m, 4H, CH_2 + CH_2), 4.33-4.29 (m, 1H, CH), 4.10-4.07 (m, 3H, CH_2 + CH), 3.32-3.23 (m, 1H, CH), 2.82-2.62 (m, 6H, $3CH_2$), 2.27-2.23 (m, 2H, CH_2), 1.82-1.79 (m, 2H, CH_2), 1.59-1.45 (m, 2H, CH_2), 1.30 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 175.66, 173.92, 172.26, 172.12, 161.38, 157.83, 142.45, 141.83, 136.95, 136.62, 132.96, 132.38, 129.54, 129.10, 128.75, 127.55, 126.29, 126.14, 115.29, 115.27, 106.46, 106.20, 55.58, 54.89, 52.69, 50.40, 43.24, 37.46, 33.84, 33.39, 31.97, 31.83, 28.98, 28.51; ESI-MS: m/z = 735.3 $[M+H]^+$.

N^1-((*S*)-5-(tert-butylamino)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-1,5-dioxopentan-2-yl)-N^4-(4-fluorophenyl)piperidine-1,4-dicarboxamide (35)

White solid; Yield: 31%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 7.92 (s, 1H, NH), 7.70 (d, 1H, *J* = 14.0

Hz, NH), 7.51-7.47 (m, 4H, Ar-H), 7.27-7.15 (m, 5H, Ar-H), 7.01-6.93 (m, 4H, Ar-H), 6.29 (d, 1H, J = 6.5 Hz, NH), 5.72 (d, 2H, J = 12.5 Hz, NH + NH), 4.53-4.45(m, 4H, CH_2 + CH_2), 4.33-4.30 (m, 1H, CH), 4.07-4.03 (m, 3H, CH_2 + CH), 2.89-2.77(m, 4H, CH_2 + CH_2), 2.42-2.24 (m, 3H, CH_2 + CH), 2.04-1.96 (m, 4H, CH_2 + CH_2), 1.79-1.64 (m, 2H, CH_2), 1.33 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_1-$CDCl_3$): δ = 173.78, 173.12, 173.07, 172.13, 171.18, 140.78, 129.30, 128.55, 128.40, 126.93, 126.23, 121.98, 121.91, 121.79, 121.71, 115.67, 115.62, 115.45, 115.40, 54.09, 52.29, 51.76, 51.45, 44.03, 43.54, 41.29, 34.07, 33.50, 32.11, 28.73, 28.68; ESI-MS: m/z = 734.7 $[M+H]^+$.

N^1-((*S*)-5-(tert-butylamino)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-1,5-dioxopentan-2-yl)-N^4-(2-fluorophenyl)piperidine-1,4-dicarboxamide (36)

White solid; Yield: 35%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 8.31-8.25 (m, 1H, NH), 7.70-7.67 (m, 1H, NH), 7.55-7.54 (m, 1H, NH), 7.46-7.42 (m, 1H, NH), 7.32-7.25 (m, 5H, Ar-H), 7.22-7.11 (m, 5H, Ar-H), 7.10-7.07 (m, 3H, Ar-H), 6.75 (d, 1H, J = 10.5 Hz, NH), 4.57-4.52 (m, 3H, CH_2 + CH), 4.46-4.42 (m, 2H, CH_2), 4.38-4.35 (m, 1H, CH), 4.19-4.10 (m, 2H, CH_2), 4.06-3.99 (m, 2H, CH_2), 2.90-2.88 (m, 2H, CH_2), 2.77-2.66 (m, 3H, CH_2 + CH), 2.45-2.43 (m, 2H, CH_2), 2.37-2.23 (m, 4H, CH_2 + CH_2), 1.34 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_1-$CDCl_3$): δ = 173.15, 172.85, 172.33, 171.50, 157.96, 140.91, 135.86, 133.40, 129.54, 129.25, 128.53, 128.43, 128.36, 126.89, 126.18, 124.64, 124.61, 122.00, 121.82, 114.86, 114.67, 54.03, 53.07, 51.81, 51.43, 41.25, 34.15, 33.54, 33.20, 32.19, 28.75, 28.70, 28.45; ESI-MS: m/z = 735.1 $[M+H]^+$.

N^1-((*S*)-5-(tert-butylamino)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-1,5-dioxopentan-2-yl)-N^4-(4-cyanophenyl)piperidine-1,4-dicarboxamide(37)

White solid; Yield: 26%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 8.46 (s, 1H, NH), 8.10 (s, 1H, NH), 7.53 (d, 1H, J = 10.5 Hz, NH), 7.39-7.37 (m, 4H, Ar-H), 7.28-7.22 (m, 5H, Ar-H), 7.20-7.18 (m, 4H, Ar-H), 7.02 (d, 1H, J = 10.0 Hz, NH), 6.43 (d, 1H, J = 7.5 Hz, NH), 4.56 (d, 2H, J = 7.5 Hz, CH_2), 4.48-4.34 (m, 3H, CH_2 + CH), 4.17-4.06 (m, 1H, CH), 3.50-3.47 (m, 2H, CH_2), 2.88-2.80(m, 4H, CH_2 + CH_2), 2.73-2.67 (m, 3H, CH_2 + CH), 2.48-2.45 (m, 4H, CH_2 + CH_2), 2.31-2.23 (m, 2H, CH_2), 1.34 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_1-$CDCl_3$): δ = 174.35, 173.76, 173.13, 172.16, 171.95, 157.39, 141.01, 135.75, 133.68, 133.21, 133.07, 130.06, 129.55, 128.89, 128.58, 128.51, 128.39, 128.37, 127.07, 126.12, 119.81, 119.63, 54.72, 54.14, 52.29, 51.82, 51.51, 41.17, 36.46, 33.49, 32.01, 28.73, 28.68, 28.22; ESI-MS: m/z = 741.5 $[M+H]^+$.

N^1-((*S*)-5-(tert-butylamino)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-1,5-

dioxopentan-2-yl)-N4-(4-methoxyphenyl)piperidine-1,4-dicarboxamide (38)

White solid; Yield: 39%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 7.76-7.69 (m, 1H, NH), 7.43-7.29 (m, 9H, Ar-H), 7.24-7.17 (m, 3H, Ar-H), 6.86 (d, 1H, J = 11.5 Hz, Ar-H), 6.21 (d, 1H, J = 8.0 Hz, NH), 5.58 (s, 1H, NH), 5.23 (d, 1H, J = 15.5 Hz, CH), 5.13 (d, 1H, J = 15.5 Hz, CH), 4.55 (d, 1H, J = 7.5 Hz, CH), 4.39-4.37 (m, 1H, CH), 4.13-4.05 (m, 2H, CH_2), 3.79 (s, 3H, CH_3), 2.89-2.82 (m, 2H, CH_2), 2.39-2.34 (m, 1H, CH), 2.24-2.09 (m, 4H, CH_2 + CH_2), 1.93-1.90 (m, 2H, CH_2), 1.85-1.74 (m, 6H, 3CH_2), 1.35 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_1-$CDCl_3$): δ = 174.49， 173.13， 172.51， 172.09， 157.35， 156.43，141.13，138.44，135.83，135.66，131.04，128.59，128.51，128.42，128.34，128.24，126.10，121.84，114.14，66.94，55.50，54.19，51.41，43.99，43.49，43.43，33.41，28.72，28.56，28.51，28.46，26.96; ESI-MS: m/z = 745.5 $[M+H]^+$.

N^1-((*S*)-5-(tert-butylamino)-1-((*S*)-1-(2-chlorobenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-1,5-dioxopentan-2-yl)-N4-phenylpiperidine-1,4-dicarboxamide (39)

White solid; Yield: 33%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 7.62-7.61 (m, 1H, NH), 7.58 (s, 1H, NH), 7.53-7.51 (m, 3H, Ar-H), 7.34-7.30 (m, 6H, Ar-H), 7.26-7.17 (m, 5H, Ar-H), 7.14 (m, 1H, NH), 6.85 (d, 1H, J = 11.0 Hz, NH), 5.62 (s, 1H, NH), 4.58-4.44 (m, 3H, CH_2 + CH), 4.06-4.03 (m, 3H, CH_2 + CH), 2.87-2.75 (m, 2H, CH_2), 2.71-2.65 (m, 2H, CH_2), 2.50-2.41 (m, 3H, CH_2 + CH), 2.38-2.27 (m, 2H, CH_2), 2.10-1.99 (m, 4H, CH_2 + CH_2), 1.93-1.81 (m, 2H, CH_2), 1.35 (s, 9H, $(CH_3)_3$); ^{13}C NMR (100MHz, d_6-DMSO): δ = 173.12, 172.94, 172.45, 171.60, 157.89, 140.85, 137.83, 135.68, 133.37, 129.53, 129.28, 128.99, 128.54, 128.45, 128.42, 126.93, 126.20, 124.40, 120.03, 57.34, 53.04, 51.79, 44.52, 43.46, 43.41, 41.31, 34.11, 33.42, 32.16, 28.70, 28.43, 26.08; ESI-MS: m/z = 717.2 $[M+H]^+$.

5.3. Biological evaluation

5.3.1 *In vitro* 20S proteasome chymotrypsin-like inhibition assay

Chymotrypsin-like enzyme activity assay was carried out in 50 μL volume and all the assay components were diluted in Tris-HCl buffer (100 mM Tris-HCl, pH 8.0). The reaction was carried out in black 384-well plates. 1 μL compound was added into 10 μL purified human proteasome (25 μg/mL), a gift from Dr. Jiang-ping Wu (Notre-Dame Hospital, Montreal, Quebec, Canada), incubated for 15 min, and then added with 39 μL synthesized substrate Suc-Leu-Leu-Val-Tyr-AMC (50□μM, GL Biochem Ltd., Shanghai, P.R. China) as the reference reported. And the AMC of probe was detected by monitoring the increase of fluorescence with Envision, at 355 nm excitation and 460 nm Emission. The IC_{50} data was

calculated using the software GraphPad Prism, and chosen the equation "sigmoidal dose-response (variable slope)" for curve fitting.

5.3.2 Tumor cell anti-proliferation assay

3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). Human multiple myeloma cell line (RPMI-8226) was obtained from Shanghai institute of biochemistry and cell biology (Shanghai, China) and was grown in RPMI-1640 medium plus 10% fetal bovine serum (FBS). All the cells were cultured in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C. DMSO is used to dissolve the indicated compounds, and the final concentration of DMSO in the culture medium is below 0.1%.

A 100 μL RPMI 8226 cells (0.5×10^4/well) were seeded into 96-well plates. After treated with tested compounds for 72h, cells were added with MTS at a final concentration of 0.5 mg/mL for 2 to 4 hours. Optical density was determined at 490 nm (background subtraction at 690 nm) by SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The growth inhibitory ratio was calculated as follows: Growth inhibitory ratio = $(A_{control}-A_{sample})/ A_{control}$. IC_{50} values were derived from a nonlinear regression model (curvefit) based on sigmoidal dose response curve (variable slope) and computed using Graghpad Prism version 5.02 (Graphpad Software).

5.3.3 DAPI staining

Cells were collected and washed with PBS for three times. Then, cells were fixed with 4% paraformaldehyde for 30 min, washed with PBS, and permeabilized with 0.4% Triton X-100 for 30 min. Finally, cells were then incubated with 0.1% DAPI and examined using a fluorescence microscope.

5.3.4 Western blotting

Cells were collected and extracted with protein lysate buffer, and the lysates were centrifuged at 10,000 g for 30 min at 4°C. Protein samples were electrophoresed on 10% Tris-glycine gels and then transferred to PVDF membrane. Then, fresh 5% nonfat milk was used to block the membrane for 1 h at room temperature, the membrane was incubated with primary antibody overnight at 4°C and secondary antibody for 1 h. Antibody binding was detected using a chemiluminescent substrate and observed on autoradiography film. The antibodies used for western blotting were obtained from different resources: anti-cleaved PARP antibody (cat. no. 9541) and anti-caspase-3 antibody (cat. no. 9661) were purchased from Cell Signaling Technology. β-actin (cat. no. sc-1616) and Ub (cat. no. sc-8017) were obtained from Santa Cruz Biotechnology.

5.3.5 Plasma stability assay

Rat plasma was prepared on sodium heparin. Stock solutions were prepared at 10 mM in DMSO for the test compounds. Aliquots of the stock solutions were diluted to 0.02 mM in 0.05 M sodium phosphate buffer containing 0.5% BSA as the dosing solution. Then 10 μL of the dosing solutions were dosed into 90 μL of pre-warmed plasma or blood (37 °C) in duplicates (n=2) in 96-well assay plates to reach a final test concentration of 2 μM. The plates were kept in a 37 °C water bath for the duration of the experiment. At each time point, 400 μL of acetonitrile was added into corresponding wells of the assay plates. After the final time point was quenched, the assay plates were ultrasonicated for 2 min, shaken at the vibrator (IKA, MTS 2/4) for 10 min (600 rpm/min) and then centrifuged at 5594 g for 15 min (Thermo Multifuge × 3R). Aliquots of the supernatant were taken, diluted 1:1 into distilled water, and analyzed by LC-MS/MS. The peak area response ratio to internal standard (PARR) of the compounds at different time point was compared to the PARR at time 0 to determine the percent of test compound remaining. Half-lives ($T_{1/2}$) were calculated using Excel software, fitting to a single-phase exponential decay equation.

5.3.6 Molecular modeling

Molecular Dock studies were performed on Maestro 10.5 embedded in Schrödinger software package (2016-1). Protein Preparation Wizard was utilized to prepare the structure of proteasome (PDB ID code: 3MG6). Protein energy was minimized with Root Mean Square Deviation (RMSD) value of 0.3 Å using OPLS-2005 force field. LigPrep was employed to prepare small molecules utilizing OPLS-2005 force field. The docking grid was centered by the centroid of ligand complexed with 3MG6. Then the Glide stand precision (SP) model was applied to dock small molecules to the active sites of proteasome for the investigation of potential interaction mechanisms.

Acknowledgements

The authors thank the National Natural Science Foundation of China (81803432) and the Public Welfare Technology Application Projects supported by Science Technology Department of Zhejiang Province (LGF18H300001) for financial support.

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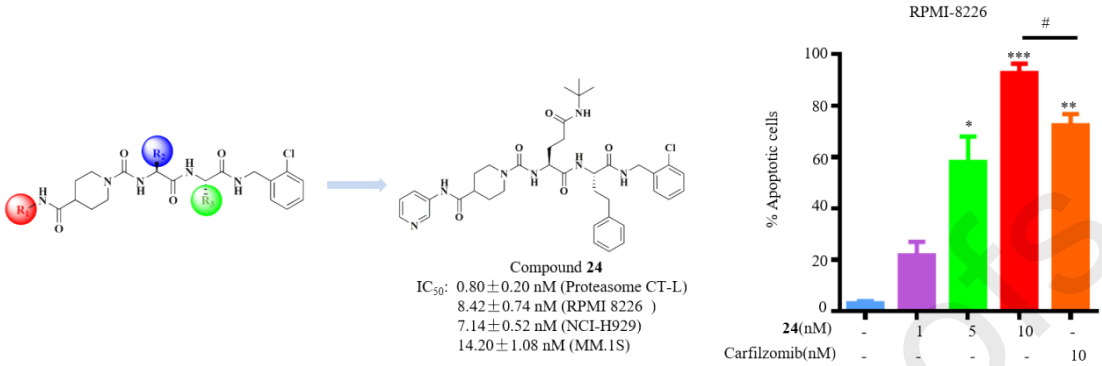
Graphical abstract



Conflict of interest

The authors confirm that this article content has no conflict of interest.