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Design, synthesis and biological evaluation of tripeptide boronic acid proteasome inhibitors

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ABSTRACT

A series of tripeptide boronate proteasome inhibitors were designed and synthesized on the basis of our previously built tripeptide aldehyde 3D-QSAR models. All the synthesized compounds were evaluated for their proteasome-inhibitory activities in an isolated 20S rabbit proteasome, and selected compounds were evaluated for their antitumor activities in vitro against four human cancer cell lines. Biological results showed bulky and negative substituents at P^2 position improved the proteasome-inhibitory potency obviously, which completely conformed to the theoretical models, while those at P^3 position thoroughly deviated from the 3D-QSAR model. Most of the screened compounds showed less than 1 nM inhibitory potency and high selectivity against 20S proteasome, of which **7f** is the most potent (IC_{50} = 0.079 nM) and twofold more active than bortezomib (IC_{50} = 0.161 nM). Cell viability indicated hydrophilic 4-hydroxyphenyl substituent at P^2 or P^3 position was not favorable to the cellular activities. Especially for the two hematologic cancer cell lines, HL-60 and U266, **7f** inhibited them at the level of less than 10 nM and was more potent than the control bortezomib. It is being considered a promising new lead to be developed for the treatment of various cancers.

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1. Introduction

The ubiquitin-proteasome pathway (UPP) in eukaryotes is considered to be critical to the intracellular protein homeostasis.[1] Many important biological processes[2–9] such as signal transduction, cell cycle control, transcriptional regulation, inflammation, and apoptosis are regulated by this pathway. The proteasome is a multicatalytic core that degrades the damaged or disfolded proteins. The 26S proteasome is composed of barrel-like 20S proteasome capped by the regulatory 19S proteasome at both ends. The 19S particles are responsible for the ubiquitinated substrate recognition, unfolding, and translocation of the proteins into the catalytic core. In fact, the 20S complex is the real catalytic core consisted of four stacks of subunits arranged as $(\alpha1–\alpha7, \beta1–\beta7)_2$, of which the β1, β2, and β5 subunits have the chymotripsin-like (CT-L) activity, trypsin-like (T-L), and post-glutamyl peptide hydrolysis activity (PGPH), respectively. Each active subunit utilizes the hydrophilic γ-hydroxyl group of the N-terminal threonine (Thr1) to hydrolyze the amide bond of protein substrates. The disruption of this degradation processing with small molecule inhibitors against one or more catalytic β-subunit has implications in some human diseases such as cancer, inflammation, and neurodegenerative diseases.

Several types of small molecule inhibitors of the proteasome had been developed and biologically assayed (Fig. 1). Among these inhibitors, a dipeptide boronic acid bortezomib (also named PS-341) showed high selectivity, potency, stability and safety, so in May 2003, it was approved by the FDA for the treatment of multiple myeloma (MM) patients who have received one prior therapy but failed.[10] And in April 2004 and in October 2006, the drug was permitted to be marketed in the EMEA and Japan, respectively. On December 8, 2006, the FDA granted full approval of bortezomib for the treatment of patients with relapsed mantle cell lymphoma (MCL). Nowadays, the drug is being investigated on other haematological maligancies and solid tumors, such as non-Hodgkin's lymphoma, prostate, breast and non-small-cell lung cancers in Phase I, Phase II, and Phase III. All these facts demonstrated the proteasome was a validated and reliable therapeutic target.

However, some clinical results showed that bortezomib led to some serious side-effects, including fatigue, nausea, sensory neuropathy, etc.[11] So it is an imperative task to develop the more potential proteasome inhibitor.

As a continuation of our program on proteasome inhibitors,[12] we had ever employed the three-dimensional quantitative structure–activity relationship (3D-QSAR) methods (including the Comparative Molecular Field Analysis (CoMFA) and Comparative Molecular Similarity Indices Analysis (CoMSIA) techniques) to

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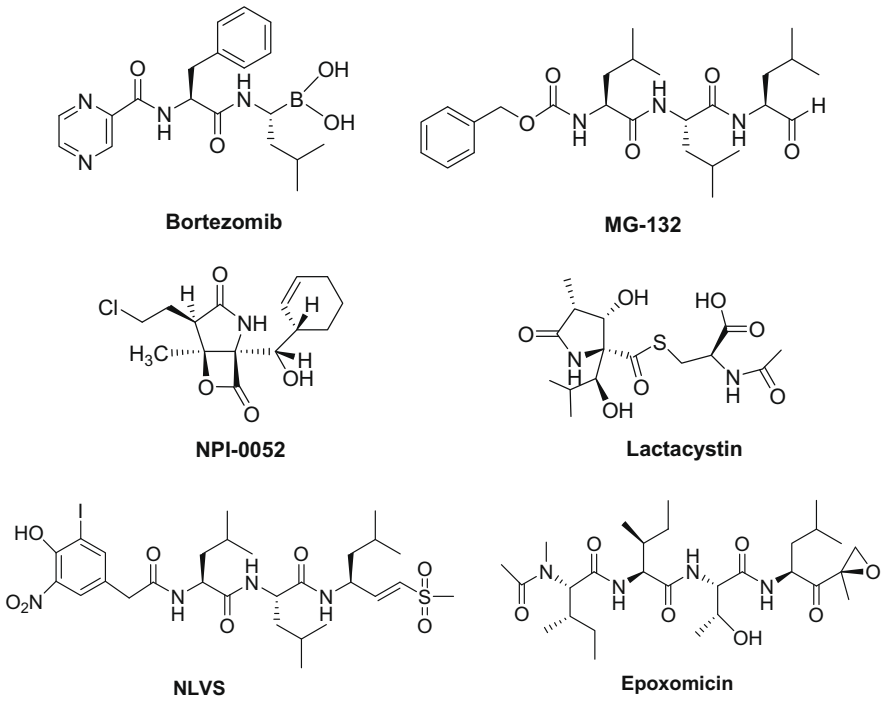


Figure 1. Major proteasome inhibitors.

analyze the interaction mode between the 20S proteasome and tripeptide aldehydes. The tripeptide aldehydes were much potent inhibitors of the 20S proteasome. But it is known that aldehyde group is too active so that the tripeptide aldehyde had too short half life in vivo. However, boronic acid group is comparatively stable and also a pharmacophore of bortezomib. So conjugation of the tripeptide moiety and boronic acid may provide a good opportunity to develop new inhibitors of the 20S proteasome. Based on our study of the special characteristics of the binding domain of the 20S catalytic core, some structurally novel tripeptide boronic acids were designed, synthesized, and biologically evaluated.

At the same time, a newly designed synthetic route shortened the synthetic steps and avoided the Boc group deprotection compared with bortezomib standard synthesis method. The biological results supported our previous calculation models in a certain extent and also offered some new aspects to understand the binding mode.

2. Results and discussion

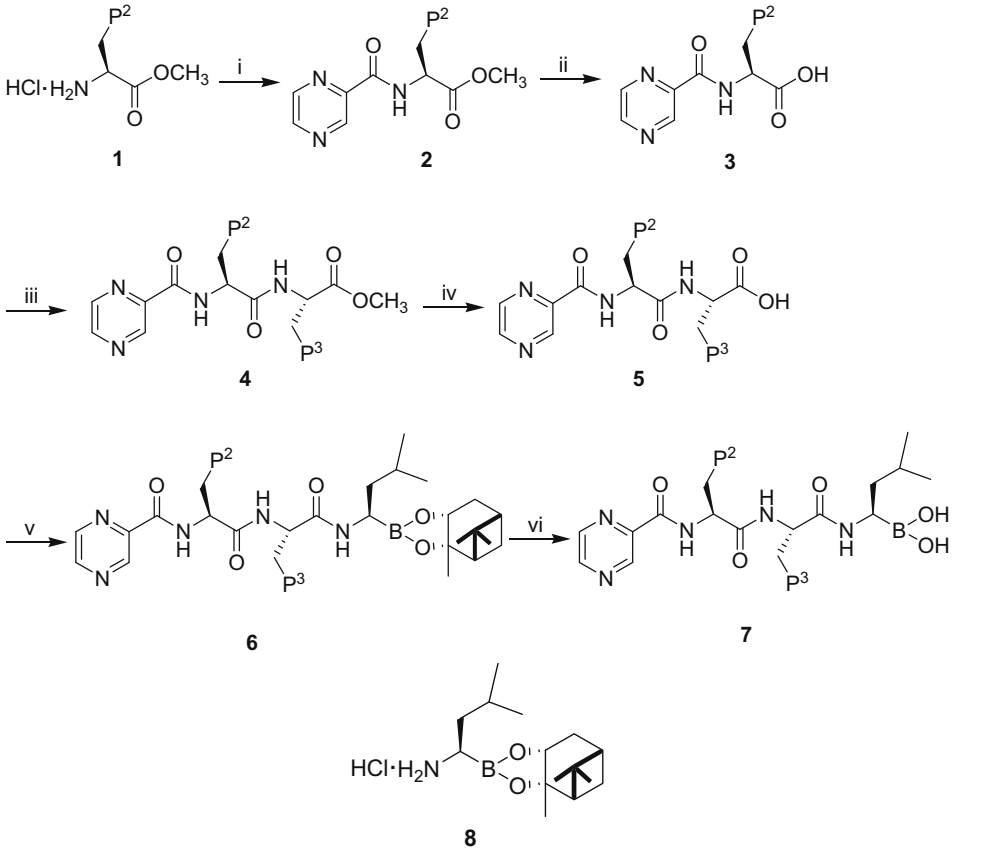
2.1. Synthesis of boronic acids

The generalized synthesis of the tripeptide boronic acids is illustrated in Scheme 1. The key intermediate dipeptides **5a–5m** were prepared according to the conventional liquid-phase synthesis.[13] The starting material L-configuration amino acid methyl esters **1a–1m** were coupled with pyrazine 2-carboxylic acid in the presence of DCC and HOBt to give methyl esters **2a–2m**, which were not separated and directly used for saponification to afford acids **3a–3m**. Another coupling of **3a–3m** with L-$P^3CH_2(HCl{\cdot}NH_2)COOCH_3$ and saponification was repeated to gain the important dipeptides **5a–5m** in high yield. The known amine boronate **8** was synthesized according to Matteson's procedure[14–17] and coupled with dipeptides 5a–**5m** in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)[18] to provide pure boronates **6a–6m** (listed in Table 1) in high yield (78–87%) after chromatographic purification. And then the protecting group pinanediol was removed by BBr_3 under mild conditions to give the target tripeptide boronic acids **7a–7m** (listed in Table 1). The bortezomib was also prepared according to the similar method.

2.2. Biological activity and SAR discussion

Proteasome inhibitory activity of the tripeptide boronates and boronic acids was evaluated in an isolated 20S mouse proteasome, including CT-L, T-L, and PGPH activities. The results were summarized in Table 1.

In our previous 3D-QSAR models of tripeptide aldehyde[12] (general structure was shown in Table 1), both CoMFA and CoMSIA contour maps had offered us the following conclusions: negative and hydrophobic substituents at P^1 and P^2 positions favored the activities, and bulky, positive and hydrophobic groups at P^4 position were essential to improve the activities, however, substituents at P^3 position had no any effect on the potency. Although the warhead of tripeptide aldehyde is different from tripeptide boronate or boronic acid, these two types of compounds inhibit 20S proteasome with the same mechanism by reacting with the hydroxyl group of the N-terminal threonine of the β5 subunit to form a tetrahedron, which had been confirmed by X-ray diffraction.[19,20] Due to the presence of pyrazin-2-carbonyl at N-terminal and isopropyl group at C-terminal in the marketed bortezomib, in this paper these two moieties were still, respectively remained at P^1 and P^4 positions in the designed inhibitors. Substituents at P^2 and P^3 positions were designed according to the calculated 3D-QSAR models. As demonstrated in Table 1, different amino acid screening of the P^2 and P^3 positions, including natural phenylalanine, leucine, tyrosine, tryptophan, and unnatural (2-naphthyl)-alanine was carried out in tripeptide boronates **6** and boronic acids **7**. The biological results showed that all the compounds had no T-L activities. However, most of the compounds inhibited the CT-L activities with IC_{50} values under 1 nM. For the PGPH activity, IC_{50} values lied beyond 2 nM level. The fact that the compounds inhibited the CT-L, T-L, and PGPH activities so differently reflected the designed inhibitors were quite selective. The following discussion was based on



	P^2		P^2	P^3
1a, **2a**, **3a**	phenyl	**4a**, **5a**, **6a**, **7a**	phenyl	phenyl
1b, **2b**, **3b**	2-naphthyl	**4b**, **5b**, **6b**, **7b**	2-naphthyl	phenyl
1c, **2c**, **3c**	phenyl	**4c**, **5c**, **6c**, **7c**	phenyl	4-hydroxylphenyl
1d, **2d**, **3d**	4-hydroxylphenyl	**4d**, **5d**, **6d**, **7d**	4-hydroxylphenyl	phenyl
1e, **2e**, **3e**	$(CH_3)_2CH$	**4e**, **5e**, **6e**, **7e**	$(CH_3)_2CH$	phenyl
1f, **2f**, **3f**	$(CH_3)_2CH$	**4f**, **5f**, **6f**, **7f**	$(CH_3)_2CH$	2-naphthyl
1g, **2g**, **3g**	phenyl	**4g**, **5g**, **6g**, **7g**	phenyl	2-naphthyl
1h, **2h**, **3h**	2-naphthyl	**4h**, **5h**, **6h**, **7h**	2-naphthyl	2-naphthyl
1i, **2i**, **3i**	3-indyl	**4i**, **5i**, **6i**, **7i**	3-indyl	phenyl
1j, **2j**, **3j**	phenyl	**4j**, **5j**, **6j**, **7j**	phenyl	3-indyl
1k, **2k**, **3k**	phenyl	**4k**, **5k**, **6k**, **7k**	phenyl	$(CH_3)_2CH$
1l, **2l**, **3l**	$(CH_3)_2CH$	**4l**, **5l**, **6l**, **7l**	$(CH_3)_2CH$	$(CH_3)_2CH$
1m, **2m**, **3m**	2-naphthyl	**4m**, **5m**, **6m**, **7m**	2-naphthyl	$(CH_3)_2CH$

Scheme 1. Synthesis of the tripeptide boronic acids. Reagents and conditions: (i) pyrazine 2-carboxylic acid, DCC, HOBt, NMM, THF; (ii) (1) 2 N NaOH, acetone, 0 °C; (2) 2 N HCl, ethyl acetate; (iii) L-$P^3CH_2(HCl{\cdot}NH_2)COOCH_3$, DCC, HOBt, NMM, THF, 0 °C; (iv) (1) 2 N NaOH, acetone, 0 °C; (2) 2 N HCl, ethyl acetate; (v) **8**, EDC, DIPEA, −20 °C; (vi) 1 N BBr_3, CH_2Cl_2.

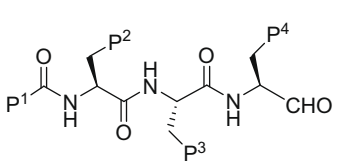
the CT-L activities for such kind of compounds mainly inhibited the β5 subunit.

It had been reported that the pinanediol ester of the boronic acid nearly had the same activity and selectivity as the free boronic acid.[21] This is the case for our synthesized series of inhibitors. Comparing pinanediol protected boronic acids **6a**, **6b**, and **6c** with their corresponding free acids **7a**, **7b**, and **7c** in inhibiting the CT-L activity, both of their IC_{50} were in the same level (Table 1). However, from the absolute value, the ester form was slightly more active than the acid one, which was concord with our calculation results and the hydrophobic characteristics of active residues in β5 subunit.[22]

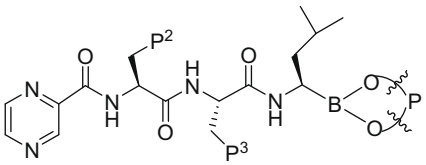
Based on our previously constructed CoMFA and CoMSIA contour maps of tripeptide aldehyde, more steric, negative, and hydrophobic substituents at P^2 position were introduced to increase the activities. Compounds **6b** (IC_{50} = 0.220 nM) and **7b** (IC_{50} = 0.417 nM) bearing a much more bulky and negative 2-naphthyl groups at P^2 positions were more active than compounds **6a** (IC_{50} = 0.402 nM) and **7a** (IC_{50} = 0.546 nM), both P^2 positions of which were substituted by phenyl groups. Comparison between compounds **7k** (IC_{50} = 0.173 nM) and **7l** (IC_{50} >20 nM) also accounted for this: when the negative phenyl group at P^2 position in **7k** was replaced by the positive isopropyl group (**7l**), the inhibition of the 20S proteasome decreased drastically.

However, to our great surprise, the calculation models were not consistent with experimental results on the substituents at P^3 position. Probably it was due to the less varieties of substituents at P^3 position of the compounds in the training set during we had ever carried out the 3D-QSAR calculations. The biological results showed different substituents at P^3 position significantly affected

Table 1
General structures of tripeptide aldehyde and boronate and biological activity of bortezomib, **6a–6m**, and **7a–7m** (IC_{50}, nM)



Tripeptide aldehyde



Tripeptide boronate

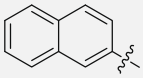
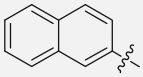
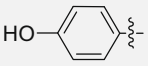
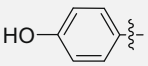
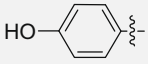
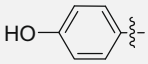
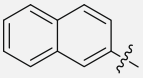
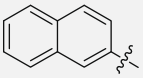
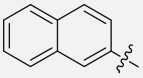
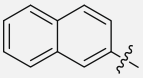
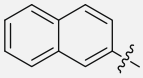
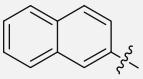
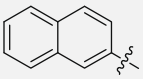
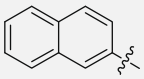
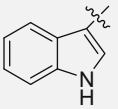
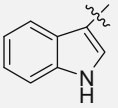
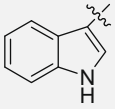
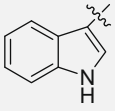
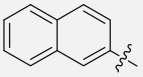
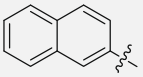
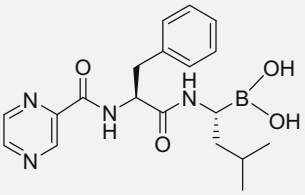
Compounds	P2	P3	P	CT-L	T-L	PGPH
6a				0.402 ± 0.024	>20	8.330 ± 2.493
7a			H, H	0.546 ± 0.077	>20	10.907 ± 3.206
6b				0.220 ± 0.106	>20	>20
7b			H, H	0.417 ± 0.070	>20	4.428 ± 1.407
6c				0.335 ± 0.031	>20	5.390 ± 0.368
7c			H, H	0.395 ± 0.035	>20	4.013 ± 1.472
6d				0.295 ± 0.049	>20	4.763 ± 1.308
7d			H, H	0.263 ± 0.081	>20	2.500 ± 1.485
6e				0.431 ± 0.086	>20	3.405 ± 0.021
7e			H, H	0.190 ± 0.017	>20	5.143 ± 2.166
6f				0.370 ± 0.141	>20	3.910 ± 1.718
7f			H, H	0.079 ± 0.011	>20	3.630 ± 1.669
6g				0.443 ± 0.081	>20	>20
7g			H, H	0.111 ± 0.050	>20	3.797 ± 0.983
6h				0.495 ± 0.092	>20	>20

Table 1 (*continued*)

Compounds	P2	P3	P	CT-L	T-L	PGPH
7h			H, H	0.335 ± 0.078	>20	>20
6i				>20	>20	>20
7i			H, H	>20	>20	>20
6j				>20	>20	>20
7j			H, H	>20	>20	>20
6k				0.287 ± 0.019	>20	2.725 ± 1.039
7k			H, H	0.173 ± 0.033	>20	5.663 ± 0.839
6l				>20	>20	>20
7l			H, H	>20	>20	>20
6m				0.345 ± 0.106	>20	14.145 ± ± 4.897
7m			H, H	0.220 ± 0.070	>20	12.750 ± 4.856
Bortezomib				0.161 ± 0.024	>20	>20

the activities. When the phenyl substituent at P^3 position of compound **7e** was replaced by 2-naphthyl (**7f**), the potency was greatly improved (IC_{50} changed from 0.190 nM to 0.079 nM). So increasing the amount and diversity of the substituents at P^3 position may result in a more reliable predictive model. From the data in hand, it seemed that steric factor instead of the electrostatic one influenced the activities in a larger extent (comparison between compounds **7a** and **7k**, among **7b**, **7h**, and **7m**). Such a deviation offered us useful information to deeply understand the interaction mode between the inhibitors and β5 subunit.

However, when bulky substituents, such as 2-naphthyl group (compound **7h**, IC_{50} = 0.335 nM), were employed at both P^2 and P^3 positions, the activity decreased in some extent. In fact, a proper combination of substituents at these two positions was beneficial to the potency. Employment of a positive and bulky isopropyl at P^2 position and a negative and steric 2-naphthyl group at P^3 position (compound **7f**, IC_{50} = 0.079 nM) gave an ideal result, twofold more potent than bortezomib.

Furthermore, introduction of a hydroxyl group on the phenyl ring whether at P^2 or P^3 position slightly enhanced the activity, presumably because of the formation of hydrogen bonds between the ligands and 20S proteasome. Both compounds **7c** (IC_{50} = 0.395 nM) and **7d** (IC_{50} = 0.263 nM) harboring a hydroxyl group at P^3 and P^2 position, respectively, were more active than **7a** (IC_{50} = 0.546 nM), the P^2 and P^3 positions of which were substituted with phenyl groups.

It is interesting to point out that inhibitors bearing indyl group at either P^2 or P^3 position, nearly had no effect on the 20S proteasome. Compounds **7i** and **7j** with indolyl group at P^2 and P^3 positions, respectively, had the IC_{50} value larger than 20 nM, which could be regarded as ineffective. The reason was being studied by using molecular modeling techniques.

From the screened results in enzyme, some interesting compounds, such as **7b**, **7c**, **7d**, **7f**, **7g**, **7h**, **7m** and bortezomib were selected to be further evaluated in vitro against four human cell lines, including two solid tumor cells, BGC-823 (human gastric carcinoma cell line) cell lines and BXPC-3 (human pancreatic cancer cell line), and two hematologic tumor cells, HL-60 (promyelocytic leukemia cell line) and U266 (multi myeloma cell line) human cell lines. Table 2 summarizes the cellular activity results. In general, the two hematologic tumor cell lines, HL-60 and U266, were more sensitive to such kind of inhibitors than the two solid tumors, BGC-823 and BXPC-3. So the following discussion was mainly based on these two cell lines unless otherwise stated. For P^3 position, the activity order is phenyl (**7b**) > isopropyl (**7m**) > 2-naphthyl (**7h**) for both cell lines. At the same time, the 2-naphthyl substituent (**7g**) at P^3 position was more potent than 4-hydroxyphenyl one (**7c**) due to its high hydrophobicity and cell permeability. However, for P^2 position, the situation varied in a certain extent. Isopropyl substituted **7f** was more active than phenyl **7g** and 2-naphthyl **7h** for all the cell lines. As described above, compared with hydrophobic 2-naphthyl substituent (**7b**) at P^2 position, hydrophilic 4-hydroxyphenyl group (**7d**) at the same position also decreased the cellular activity. However, increasing the sample volume is necessary for a comprehensive understanding of the SAR. Meanwhile, it is worthy to mention compound **7f**. This inhibitor not only showed the most active among all the investigated tripeptide boronic acids in enzyme (Table 1), but also in cellular aspects. Especially for the two hematologic tumor cell lines, **7f** inhibited both tumor cells with IC_{50} less than 10 nM, which was more potent than the standard bortezomib. So this compound was being selected as a promising candidate to be developed.

3. Conclusions

We have studied the structure–activity relationship of a series of tripeptide boronate and boronic acid proteasome inhibitors. The experimental results turned out the design of substituents at P^2 position could be guided by previously constructed 3D-QSAR model, while that of groups at P^3 position was incorrectly predicted. Additional structure modifications at P^3 position were needed to clearly elucidate the SAR. Among the screened proteasome inhibitors, the potency of **7f** was twofold more active than that of bortezomib. Cytotoxicity displayed that hydrophobic substituents instead of hydrophilic ones at P^2 or P^3 position were beneficial for higher cellular activities. It also turned out compound **7f** was more active than the marketed bortezomib and inhibited the two hemapotologic cancer cell lines with IC_{50} less than 10 nM. **7f** was being considered as a novel candidate to be developed for the treatment of different cancers.

4. Experimental

4.1. Chemistry

Commercially available reagents were used directly without any purification unless otherwise stated. Absolutely anhydrous solvents were obtained with the proper methods introduced in the literature. Reactions were monitored by thin-layer chromatography carried out on silica gel plates (60F-254) using UV light as a visualizing agent, 15% ethanolic phosphomolybdic acid and heat or ninhydrin and heat as developing agent. Column chromatography was performed on 200–300 mesh silica gel. Melting points were determined on an X4 melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature on a 500 Bruker AM-500, VXR 300, or AL 300 spectrometers. Chemical shifts were expressed in δ ppm and tetramethylsilane (TMS) was used as internal reference. Mass spectra were obtained using ZAB-2F instruments in electrospray positive and negative ionization modes. High-resolution mass spectra were recorded on a ZAB-HS instrument using an electrospray source (ESI). Elemental analyses were performed on a Vario EL III (Germany).

4.1.1. General procedure for the synthesis of *N*-pyrazin-2-yl amino acids 5a–5m

A mixture of methyl ester **1** (1 mmol) and pyrazine 2-carboxylic acid (1 mmol) in absolute THF (30 mL) at 0 °C was added DCC (1 mmol) and HOBt (1.2 mmol) and the reaction was allowed to performed until TLC showed the starting materials disappeared (2–4 h). After the reaction finished, the resulting solid was filtered and the solvent was removed under vacuo. The white solid was dissolved in 50 mL ethyl acetate and washed with 5% $NaHCO_3$, 10% citric acid, 5% $NaHCO_3$, and brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to provide crude methyl ester **2** without further purification and directly used in the next reaction.

The crude product **2** (1 mmol) was dissolved in 10 mL acetone and was added 2 N NaOH dropwise at 0 °C until TLC showed methyl ester **2** was saponified completely (2 h). The reaction solution was acidified to pH 2. The precipitate was filtered and washed with water until pH value reached 6–7. After dryness, the acid **3** was obtained with high purity and yield.

The process of the preparation of **2** and **3** was repeated to give *N*-pyrazin-2-yl dipeptide methyl ester **4** and acid **5**, respectively.

4.1.2. *N*-(2-Pyrazinecarbonyl)-L-phenylalanine-L-phenylalanine (5a)

Yield 90.5%, white solid, mp: 194–196 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.88 (s, 1H), 9.10 (s, 1H), 8.90–8.87 (m, 1H), 8.77–8.73 (m, 1H), 8.75–8.65 (m, 2H), 7.24–7.14 (m, 10H), 4.84–4.80 (m, 1H), 4.53–4.50 (m, 1H), 3.14–2.89 (m, 4H).

4.1.3. *N*-(2-Pyrazinecarbonyl)-L-(2-naphthyl)-alanine-L-phenylalanine (5b)

Yield 77%, white solid, mp:196–197 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.07 (s, 1H), 8.86 (d, J = 2.7 Hz, 1H), 8.70 (d, J = 1.2 Hz, 1H), 8.68–8.62 (m, 2H), 7.83–7.72 (m, 4H), 7.45–7.37 (m, 3H), 7.24–7.17 (m, 5H), 4.93–4.91 (m, 1H), 4.53–4.50 (m, 1H), 3.24–2.98 (m, 4H).

4.1.4. *N*-(2-Pyrazinecarbonyl)-L-phenylalanine-L-tyrosine (5c)

Yield 83%, foam solid. Used in the next step directly.

4.1.5. *N*-(2-Pyrazinecarbonyl)-L-tyrosine-L-phenylalanine (5d)

Yield 86%, foam solid. Used in the next step directly.

Table 2
Cell viability of tripeptide boronic acids and bortezomib (IC_{50}, nM)

Entry	BGC-823	BXPC-3	HL-60	U266
7b	2801	31.8	10	53.0
7c	5140	445.0	552.5	250.0
7d	2460	256.7	444.7	66.2
7f	600	21.2	4.6	9.9
7g	1040	28.7	7.4	15.8
7h	2450	33.3	19.6	195.0
7m	1720	43.5	15.0	120.0
Bortezomib	2890	11.8	6.9	12.2

4.1.6. *N*-(2-Pyrazinecarbonyl)-L-leucine-L-phenylalanine (5e)

Yield 95.6%, sticky solid; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.61 (s, 1H), 9.20 (s, 1H), 8.98–8.91 (m, 1H), 8.77 (s, 1H), 8.66–8.62 (m, 1H), 8.46–8.44 (m, 1H), 7.22–7.15 (m, 5H), 4.61 (s, 1H), 4.48–4.46 (m, 1H), 3.10–2.87 (m, 2H), 1.98–1.92 (m, 1H), 1.62–1.59 (m, 2H), 0.92–0.88 (m, 6H).

4.1.7. *N*-(2-Pyrazinecarbonyl)-L-leucine-L-(2-naphthyl)-alanine (5f)

Yield 83%, white solid, mp: 99–100 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.16 (s, 1H), 8.89 (d, J = 2.4 Hz, 1H), 8.70 (d, J = 1.5 Hz, 1H), 8.58 (d, J = 9.0 Hz, 1H), 8.46 (d, J = 7.8 Hz, 1H), 7.79–7.73 (m, 4H), 4.60–4.57 (m, 1H), 4.55–4.53 (m, 1H), 1.54–1.45 (m, 3H), 0.83–0.79 (m, 6H).

4.1.8. *N*-(2-Pyrazinecarbonyl)-L-phenylalanine-L-(2-naphthyl)-alanine (5g)

Yield 79%, white solid, mp: 191–193 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.07 (d, J = 1.2 Hz, 1H), 8.86 (d, J = 2.7 Hz, 1H), 8.68 (d, J = 1.5 Hz, 1H), 8.64–8.56 (m, 2H), 7.81–7.72 (m, 4H), 7.42–7.40 (m, 3H), 7.17–7.11 (m, 5H), 4.83–4.79 (m, 1H), 4.59–4.55 (m, 1H), 3.26–3.02 (m, 4H).

4.1.9. *N*-(2-Pyrazinecarbonyl)-L-(2-naphthyl)-alanine-L-(2-naphthyl)-alanine (5h)

Yield 72%, light yellow solid, mp: 208–209 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.04 (s, 1H), 8.84 (d, J = 2.4 Hz, 1H), 8.73–8.69 (m, 1H), 8.67–8.66 (m, 2H), 7.82–7.69 (m, 8H), 7.74–7.69 (m, 6H), 7.44–7.35 (m, 6H), 4.95–4.91 (m, 1H), 4.64–4.60 (m, 1H), 3.28–3.08 (m, H).

4.1.10. *N*-(2-Pyrazinecarbonyl)-L-tryptophan-L-phenylalanine (5i)

Yield 96.4%, white solid, mp: 74–76 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.79 (s, 1H), 9.13 (s, 1H), 8.86 (s, 1H), 8.68 (s, 1H), 8.62–8.59 (m, 2H), 7.54–6.84 (m, 10H), 4.84 (s, 1H), 4.58–4.50 (m, 2H), 3.20–3.09 (m, 4H).

4.1.11. *N*-(2-Pyrazinecarbonyl)-L-phenylalanine-L-tryptophan (5j)

Yield 93.7% light yellow solid, mp:106–108 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.59 (s, 1H), 10.87 (s, 1H), 9.16–9.12 (m, 1H), 8.89–8.87 (m, 1H), 8.75–8.72 (m, 1H), 8.66–8.59 (m, 2H), 7.56–6.96 (m, 10H), 4.88–4.85 (m, 1H), 4.59–4.56 (m, 1H), 3.22–3.05 (m, 4H).

4.1.12. *N*-(2-Pyrazinecarbonyl)-L-phenylalanine-L-leucine (5k)

Yield 93.9%, pale yellow solid, mp: 142–144 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.70 (s, 1H), 9.15–9.12 (m, 1H), 8.90–8.88 (m, 1H), 8.79–8.74 (m, 1H), 8.68–8.65 (m, 1H), 8.57–8.53 (m, 1H), 7.27–7.15 (m, 5H), 4.90–4.82 (m, 1H), 4.32–4.25 (m, 1H), 3.21–3.04 (m, 2H), 1.68–1.54 (m, 3H), 0.94–0.91 (m, 3H), 0.87–0.85 (m, 3H).

4.1.13. *N*-(2-Pyrazinecarbonyl)-L-leucine-L-leucine (5l)

Yield 83%, sticky solid. Used in next step directly.

4.1.14. *N*-(2-Pyrazinecarbonyl)-L-(2-naphthyl)-alanine-L-leucine (5m)

Yield 78%, white solid, mp: 98–100 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.09 (d, J = 1.2 Hz, 1H), 8.86 (d, J = 2.7 Hz, 1H), 8.71–8.70 (m, 2H), 8.57 (d, J = 7.8 Hz, 1H), 7.84–7.45 (m, 4H), 7.43–7.42 (m, 3H), 5.02–4.96 (m, 1H), 4.32–4.28 (m, 1H), 1.66–1.58 (m, 3H), 0.89 (d, J = 6.6 Hz, 3H), 0.83 (d, J = 6.3 Hz, 3H).

4.1.15. General procedure for the synthesis of the known salt (8)

The known salt **8** was synthesized completely according to the methods described in the literatures.[14–17]

Yield 72.5%, white solid, mp: 176–178 °C; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.12 (s, 3H), 7.45 (t, J = 50.7 Hz, 3H), 7.11 (q, J = 8.1 Hz, 5H), 4.35 (d, J = 8.9 Hz, 1H), 3.03–2.97 (m, 2H), 2.89–2.85 (m, 1H), 2.28–2.23 (m, 4H), 2.11–2.09 (m, 1H), 1.94 (t, J = 5.4 Hz, 1H), 1.84–1.81 (m, 1H), 1.67–1.63 (m, 2H), 1.27 (s, 3H), 1.23 (s, 3H), 1.08 (d, J = 10.9 Hz, 1H), 0.78 (s, 3H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 135.56, 133.80, 128.95, 128.90, 128.82, 128.77, 86.49, 77.50, 50.59, 38.72, 37.67, 34.65, 34.33, 27.93, 26.70, 25.72, 23.44, 20.50; MS (ESI) m/z 266.3 $(M+H)^+$.

4.1.16. General procedure for the synthesis of the tripeptide boronate (6)

To a solution of dipeptide **5** (1 mol) in CH_2Cl_2 (10 mL) at −20 °C was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (1.5 mmol) and 1-hydroxybenzotriazole monohydrate (HOBt) (1 mmol). Then the pinanediol leucine boronate hydrochloride **8** and DIPEA (1.2 mmol) was added. The mixture was allowed to warm to room temperature and stirred overnight. The insoluble material was filtered, the solvent removed under vacuum, and the residue dissolved in ethyl acetate (25 mL). The organic layer was washed with 5% $NaHCO_3$, 10% citric acid, 5% $NaHCO_3$, and saturated NaCl, finally with water. The organic layer was then dried over anhydrous Na_2SO_4, filtered, and the solvent evaporated to give a white solid. The crude product was purified by silica gel chromatography (eluted with proper ratio of ethyl acetate and petroleum ether) to afford the desired product **6**.

4.1.17. (+)-Pinanediol-(2-pyrazinecarbonyl)-L-phenylalanine-L-phenylalanine-L-leucine boronate (6a)

Yield 82%, white solid, mp: 78–80 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.31–9.26 (m, 1H), 8.82–8.77 (m, 1H), 8.58–8.54 (m, 1H), 8.23–8.19 (m, 1H), 7.32–6.99 (m, 10H), 6.48–6.42 (m, 1H), 5.72–5.67 (m, 1H), 4.80–4.77 (m, 1H), 4.59–4.55 (m, 1H), 4.36–4.33 (m, 1H), 3.19–3.09 (m, 4H), 2.90–2.88 (m, 1H), 2.36–2.22 (m, 2H), 2.08–2.03 (m, 1H), 1.94–1.86 (m, 2H), 1.47–1.39 (m, 3H), 1.30–1.28 (m, 3H), 1.29–1.21 (m, 3H), 0.90–0.83 (m, 9H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 169.86, 169.77, 163.21, 147.63, 144.33, 143.52, 142.69, 136.25, 135.94, 129.22, 129.13, 128.81, 128.45, 127.28, 126.72, 84.72, 54.73, 54.27, 51.50, 39.41, 39.37, 38.37, 38.03, 37.84, 37.64, 35.68, 28.53, 28.44, 27.05, 26.36, 26.32, 25.58, 23.96, 22.36; HRMS calcd for $C_{38}H_{49}BN_5O_5$ $(M+1)^+$ 666.3827, found 666.3823.

4.1.18. (+)-Pinanediol *N*-(2-pyrazinecarbonyl)-L-(2-naphthyl)-alanine-L-phenylalanine-L-leucine boronate (6b)

Yield 87%, white solid, mp: 98–100 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.13 (s, 1H), 8.74 (d, J = 2.4 Hz, 1H), 8.48 (d, J = 2.1 Hz, 1H), 8.23–8.20 (d, J = 7.5 Hz, 1H), 7.80–7.69 (m, 4H), 7.46–7.43 (m, 2H), 7.35 (d, J = 8.4 Hz, 1H), 7.02–6.95 (m, 5H), 6.53–6.45 (m, 1H), 4.65–4.57 (m, 1H), 4.29–4.27 (m, 1H), 3.40–3.26 (m, 2H), 3.13–3.02 (m, 1H), 2.95–2.84 (m, 1H), 2.35–2.27 (m, 1H), 2.20–2.15 (m, 1H), 2.04–1.99 (m, 1H), 1.89–1.80 (m, 1H), 1.39–1.23 (m, 12H), 0.87–0.83 (m, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.39, 169.85, 163.19, 147.57, 144.32, 143.59, 142.71, 136.13, 133.51, 133.46, 132.47, 129.22, 128.56, 128.35, 128.01, 127.66, 127.57, 127.51, 126.65, 126.24, 125.84, 54.55, 54.42, 53.64, 51.35, 39.70, 39.55, 38.19, 37.85, 37.74, 35.50, 28.61, 27.08, 26.24, 25.36, 25.21, 24.03, 22.97, 22.01; HRMS calcd for $C_{42}H_{51}BN_5O_5$ $(M+1)^+$ 716.3983, found 716.3986.

4.1.19. (+)-Pinanediol *N*-(2-pyrazinecarbonyl)-L-phenylalanine-L-tyrosine-L-leucine boronate (6c)

Yield 81%, light yellow solid, mp: 119–120 °C; ^{1}H NMR (CD_3OD, 300 MHz): δ 9.18 (s, 1H), 8.68 (d, J = 10.2 Hz, 1H), 8.48 (s, 1H), 8.26 (d, J = 7.5 Hz, 1H), 7.82–7.74 (m, 1H), 7.27–7.18 (m, 5H), 6.80–6.76 (m, 3H), 6.49–6.40 (m, 2H), 4.82–4.61 (m, 2H), 4.29–4.27 (d, J = 8.1 Hz, 1H), 3.13–3.06 (m, 2H), 2.95–2.84 (m, 3H), 2.35–2.27 (m, 2H), 2.04–2.02 (m, 1H), 1.85–1.80 (m, 2H), 1.43–1.26 (m, 9H), 0.88–0.83 (m, 9H); ^{13}C NMR (CD_3OD, 75 MHz): δ 170.17, 163.36, 155.62, 147.61, 143.96, 143.44, 142.83, 135.95, 130.37, 129.21, 128.78, 127.47, 127.23, 126.61, 126.44, 115.34, 85.30, 54.86, 54.74, 52.91, 51.54, 40.16, 39.82, 39.64, 38.10, 37.57, 36.97, 35.80, 35.69, 28.73, 27.10, 26.28, 25.41, 24.04, 23.06, 22.97, 22.03, 21.95; HRMS calcd for $C_{38}H_{49}BN_5O_6$ $(M+1)^+$ 682.3776, found 682.3767.

4.1.20. (+)-Pinanediol *N*-(2-pyrazinecarbonyl)-L-tyrosine-L-phenylalanine-L-leucine boronate (6d)

Yield 80%, light yellow solid, mp: 90–92 °C; ^{1}H NMR (CD_3OD, 300 MHz): δ 9.29 (s, 1H), 8.74 (d, J = 2.4 Hz, 1H), 8.50 (d, J = 2.1 Hz, 1H), 8.26 (d, J = 7.8 Hz, 1H), 7.11–6.99 (m, 8H), 6.74 (d, J = 8.4 Hz, 2H), 6.74–6.71 (m, 1H), 6.50–6.42 (m, 1H), 4.70–4.26 (m, 1H), 4.51–4.27 (m, 1H), 4.29–4.17 (m, 2H), 3.07–2.93 (m, 5H), 2.37–2.31 (m, 1H), 2.21–2.16 (m, 1H), 2.06–2.03 (m, 1H), 1.90–1.80 (m, 2H), 1.40–1.26 (m, 6H), 0.90–0.79 (m, 12H); ^{13}C NMR (CD_3OD, 75 MHz): δ 171.36, 170.18, 163.03, 155.47, 147.50, 144.29, 143.74, 142.73, 135.95, 130.29, 130.15, 129.31, 128.50, 127.51, 126.83, 116.47, 116.64, 85.33, 55.08, 53.19, 51.55, 39.73, 39.64, 39.40, 38.23, 38.16, 37.44, 37.14, 35.67, 28.73, 28.54, 27.14, 26.30, 25.44, 24.07, 22.98, 22.02; HRMS calcd for $C_{38}H_{49}BN_5O_6$ $(M+1)^+$ 682.3776, found 682.3795.

4.1.21. (+)-Pinanediol *N*-(2-pyrazinecarbonyl)-L-leucine -L-phenylalanine-L-leucine boronate (6e)

Yield 87%, white solid, mp: 63–65 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.32 (s, 1H), 8.78–8.67 (m, 1H), 8.55–8.21 (m, 1H), 8.10–7.95 (m, 1H), 7.15–6.99 (m, 6H), 6.04–5.96 (m, 1H), 4.65–4.34 (m, 2H), 4.30–4.15 (m, 1H), 3.22–3.01 (m, 2H), 2.37–2.22 (m, 2H), 2.12–2.04 (m, 1H), 1.93–1.90 (m, 2H), 1.76–1.63 (m, 3H), 1.48–1.39 (m, 1H), 1.28 (s, 3H), 1.29–1.22 (m, 3H), 0.94–0.90 (m, 6H), 0.86–0.83 (m, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 171.49, 170.91, 162.76, 147.12, 143.60, 141.48, 136.38, 128.13, 128.05, 127.98, 126.32, 85.18, 84.79, 53.19, 52.14, 51.22, 41.15, 41.04, 40.72, 39.78, 39.49, 35.82, 35.46, 35.28, 28.40, 26.83, 25.99, 25.40, 24.25, 23.75, 22.71, 21.78, 20.71; HRMS(ESI): calcd $C_{35}H_{51}BN_5O_5$ $(M+1)^+$ 632.3983, found 632.3984.

4.1.22. (+)-Pinanediol *N*-(2-pyrazinecarbonyl)-L-leucine-L-(2-naphthyl)-alanine -L-leucine boronate (6f)

Yield 85%, white solid, mp: 140–142 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.20 (d, J = 1.5 Hz, 1H), 8.71 (d, J = 2.4 Hz, 1H), 8.40 (t, J = 1.2 Hz, 1H), 7.93 (d, J = 8.1 Hz, 1H), 7.66–7.61 (m, 4H), 7.36–7.32 (m, 3H), 6.75 (d, J = 7.8 Hz, 1H), 6.00 (d, J = 4.8 Hz, 1H), 4.78–4.71 (m, 1H), 4.59–4.53 (m, 1H), 4.27 (d, J = 8.7 Hz, 1H), 3.23 (d, J = 6.6 Hz, 2H), 3.14–3.10 (m, 1H), 2.28–2.20 (m, 1H), 2.17–2.08 (m, 1H), 2.00–2.05 (m, 1H), 1.87–1.57 (m, 6H), 1.41–1.27 (m, 9H), 0.92–0.90 (m, 6H), 0.83–0.77 (m, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 171.05, 170.61, 163.13, 147.52, 144.24, 143.45, 142.59, 142.47, 133.933, 133.25, 132.22, 128.131, 127.99, 127.48, 127.26,125.95, 125.52, 85.83, 53.06, 51.98, 51.88, 51.32, 40.63, 39.68, 39.56, 38.19, 35.48, 28.56, 27.10, 26.23, 25.33, 25.22, 24.78, 24.04, 22.89, 21.80; HRMS calcd for $C_{39}H_{53}BN_5O_5$ $(M+1)^+$ 682.4140, found 682.4155.

4.1.23. (+)-Pinanediol *N*-(2-pyrazinecarbonyl)-L-phenylalanine-L-(2-naphthyl)-alanine-L-leucine boronate (6g)

Yield 86%, white solid, mp: 97–98 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.13–9.09 (m, 1H), 8.66 (d, J = 2.4 Hz, 1H), 8.34 (d, J = 1.2 Hz, 1H), 8.10 (d, J = 7.5 Hz, 1H), 7.62–7.46 (m, 3H), 7.41–7.21 (m, 9H), 6.53–6.45 (m, 1H), 5.99–5.78 (m, 1H), 4.76–4.72 (m, 1H), 4.25 (d, J = 8.7 Hz, 1H), 3.29–3.22 (m, 1H), 3.15 (d, J = 6.3 Hz, 2H), 3.11–3.04 (m, 1H), 2.29–2.26 (m, 1H), 2.17–2.12 (m, 1H), 2.02–1.98 (m, 1H), 1.86–1.67 (m, 2H), 1.38–1.25 (m, 12H), 0.87–0.81 (m, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.53, 169.85, 163.19, 147.50, 144.04, 143.28, 143.15, 142.63, 135.96, 135.89, 133.66, 133.54, 133.20, 132.19, 129.20, 128.78, 128.14, 127.96, 127.48, 127.28, 127.21, 125.92, 125.53, 54.56, 53.36, 53.01, 51.34, 39.88, 39.70, 39.57, 38.05, 37.60, 35.57, 28.57, 27.09, 26.22, 25.32, 25.16, 24.03, 22.91, 22.01, 21.92; HRMS calcd for $C_{42}H_{51}BN_5O_5$ $(M+1)^+$ 716.3983, found 716.3996.

4.1.24. (+)-Pinanediol*N*-(2-pyrazinecarbonyl)-L-(2-naphthyl)-alanine-L-(2-naphthyl)-alanine-L-leucine boronate (6h)

Yield 82%, white solid, mp: 136–137 °C; ^{1}H NMR (CD_3OD, 300 MHz): δ 9.10 (s, 1H), 8.63 (d, J = 2.4 Hz, 1H), 8.26 (d, J = 2.4 Hz, 1H), 8.12–8.02 (m, 1H), 7.81–7.57 (m, 6H), 7.53–7.32 (m, 8H), 7.18 (dd, J_1 = 1.8 Hz, J_2 = 8.4 Hz, 2H), 6.51–6.49 (m, 1H), 6.00 (d, J = 5.1 Hz, 1H), 4.87–4.72 (m, 2H), 4.26 (dd, J_1 = 1.5 Hz, J_2 = 8.7 Hz, 1H), 3.32 (d, J = 6.3 Hz, 2H), 3.24–3.22 (m, 1H), 3.07–3.00 (m, 1H), 2.33–2.25 (m, 1H), 2.18–2.13 (m, 1H), 2.04–2.00 (m, 1H), 1.87–1.67 (m, 2H), 1.36–1.25 (m, 8H), 0.87–0.74 (m, 12H); ^{13}C NMR (CD_3OD, 75 MHz): δ 170.46, 169.85, 163.22, 147.46, 144.00. 143.16, 142.61, 142.49, 133.58, 133.45, 133.34, 132.50, 132.13, 128.58, 127.97, 127.71, 127.57, 127.44, 126.31. 125.90, 125.52, 85.76, 54.41, 53.30, 51.35, 39.58, 38.19, 37.98, 37.59, 35.50, 28.59, 27.10, 26.24, 25.33, 25.18, 24.04, 21.96; HRMS calcd for $C_{46}H_{53}BN_5O_5$ $(M+1)^+$ 766.4140, found 766.4148.

4.1.25. (+)-Pinanediol *N*-(2-pyrazinecarbonyl)-L- tryptophan-L-phenylalanine-L-leucine boronate (6i)

Yield 78%, light yellow solid, mp: 127–129 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.24 (s, 1H), 8.76 (d, J = 2.4 Hz, 1H), 8.51 (d, J = 2.4 Hz, 1H), 8.50–8.46 (m, 1H), 8.22–8.14 (m, 1H), 7.40–7.38 (m, 5H), 7.01–6.88 (m, 4H), 6.78–6.58 (m, 1H), 6.09–5.97 (m, 1H), 5.75–5.71 (m, 2H), 4.82–4.75 (m, 2H), 3.51–3.45 (m, 1H), 3.31–3.01 (m, 4H), 1.25–1.23 (m, 10H), 0.99–0.89 (m, 8H), 0.88–0.85 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 171.77, 170.57, 163.21, 147.24, 143.48, 143.30, 142.48, 136.21, 135.38, 128.99, 128.09, 127.06, 126.38, 123.48, 121.93, 119.20, 118.45, 111.39, 109.29, 84.91, 54.22, 54.03, 52.42, 51.42, 39.95, 39.47, 37.99, 37.91, 37.19, 36.96, 35.56, 30.36, 28.61, 27.76, 27.53, 26.94, 26.19, 25.16, 22.86,21.83; HRMS(ESI): calcd $C_{40}H_{50}BN_6O_5(M+1)^+$ 705.3936, found 705.3946.

4.1.26. (+)-Pinanediol *N*-(2-pyrazinecarbonyl)-L-phenylalanine-L-tryptophan-L-leucine boronate (6j)

Yield 79%, light yellow solid, mp: 153–155 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.08 (s, 1H), 8.73–8.69 (m, 1H), 8.48 (s, 1H), 8.34 (s, 1H), 8.11–7.94 (m, 1H), 7.32–7.21 (m, 5H), 7.08–6.88 (m, 4H), 6.62–6.42 (m, 1H), 6.01 (s, 1H), 5.65–5.58 (m, 2H), 4.38–4.29 (m, 2H), 3.20–3.01 (m, 4H), 1.40–1.24 (m, 10H), 0.99–0.89 (m, 8H), 0.83–0.80 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 172.52, 170.09, 163.22, 147,23, 143.62, 143.06, 142.62, 135.90, 135.75, 129.13, 128.59, 127.13, 123.79, 121.34, 119.21, 117.82, 111.03, 108.08, 84,73, 54.69, 52.52, 51.55, 40.07, 39.74, 27.15, 38.03, 37.48, 35.79, 30.41, 28.70, 27.33, 27.00, 26.21, 25.18, 25.11, 23.92, 22.89, 21.86, 21.74; HRMS(ESI): calcd $C_{40}H_{50}BN_6O_5(M+1)^+$ 705.3936, found 705.3950.

4.1.27. (+)-Pinanediol *N*-(2-pyrazinecarbonyl)-L-phenylalanine-L-leucine-L-leucine boronate (6k)

Yield 81%, white solid, mp: 68–70 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.33 (s, 1H), 8.76–8.73 (d, 1H), 8.54 (s, 1H), 8.29–8.10 (m, 1H), 7.29–7.25 (m, 5H), 6.39–6.31 (m, 2H), 4.82–4.79 (m, 1H), 4.44–4.41 (m, 1H), 4.30–4.27 (m, 1H), 3.22–3.19 (m, 3H), 2.32–2.17 (m, 3H), 2.05–2.02 (m, 1H), 1.89–1.27 (m, 14H), 0.96–0.83 (m, 15H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 172.18, 170.33, 163.01, 147.34, 144.15, 143.84, 142.72, 135.91, 129.17,128.55, 126.99, 85.37, 84.38, 54.42, 51.38, 50,86, 40,53, 39.68, 39.48, 39.29, 38.25, 37.84, 35.60, 30.49, 28.45, 27.71, 26.95, 26.22, 25.50, 24.39, 23.86, 22.92, 22.50, 22.11, 20.98; HRMS calcd for $C_{35}H_{51}BN_5O_5$ $(M+1)^+$ 632.3983, found 632.3979.

4.1.28. (+)-Pinanediol *N*-(2-pyrazinecarbonyl)-L-leucine-L-leucine-L-leucine boronate (6l)

Yield 86.0%, sticky solid; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.41 (s, 1H), 8.76–8.65 (m, 1H), 8.56–8.45 (m, 1H), 8.18–8.10 (m, 1H), 5.43–5.38 (m, 1H), 4.71–4.50 (m, 1H), 4.38–4.32 (m, 1H), 4.07–3.97 (m, 2H), 3.86–3.84 (m, H), 2.52–2.08 (m, 3H), 2.04–1.90 (m, 6H), 1.67–1.60 (m, 2H), 1.41–1.25 (m, 10H), 0.99–0.83 (m, 21H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 172.47, 171.55, 162.96, 147.32, 144,21, 143.83, 142.62, 85.26, 85.04, 53.76, 51.71, 51.33, 51.26, 50.75, 41.25, 41.17, 40.67, 40.52, 39.23, 35.56, 29.47, 28.47, 27.69, 26.94, 26.17, 25.63, 25,19, 24.64, 23.88, 22.04, 21.88, 20,83; HRMS(ESI): calcd $C_{32}H_{53}BN_5O_5$ $(M+1)^+$ 598.4140, found 598.4144.

4.1.29. (+)-Pinanediol *N*-(2-pyrazinecarbonyl)-L-(2-naphthyl)-alanine-L-leucine–L-leucine boronate (6m)

Yield 81%, white solid, mp: 99–100 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.31 (s, 1H), 8.73 (s, 1H), 8.49 (d, J = 1.8 Hz, 1H), 8.33 (d, J = 7.5 Hz, 1H), 7.66–7.58 (m, 4H), 7.44–7.38 (m, 3H), 6.48–6.36 (m, 2H), 4.94–4.85 (m, 1H), 4.42–4.30 (m, 1H), 4.25–4.16(m, 1H), 3.38 (d, J = 6.3 Hz, 2H), 3.19–3.11 (m, 1H), 2.35–2.27 (m, 1H), 2.20–2.15 (m, 1H), 1.89–1.84 (m, 2H), 1.68–1.61 (m, 2H), 1.37–1.36 (m, 3H), 1.53–1.43 (m, 4H), 1.28–1.21 (m, 3H), 0.94–0.89 (m, 6H), 0.83–0.79 (m, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 171.81, 170.10, 163.298, 147.59, 144.34, 143.72, 142.77, 133.46, 132.47, 128.56, 128.12, 127.62, 127.16, 126.18, 125.80, 85.60, 54.70, 54.53, 51.40, 51.20, 50.96, 40.89, 40.08, 39.85, 39.56, 38.18, 38.09, 35.58, 28.59, 27.08, 26.25, 25.26, 25.46, 24.51, 24.03, 23.00, 22.71, 22.15, 21.94; HRMS calcd for $C_{39}H_{53}BN_5O_5$ $(M+1)^+$ 682.4140, found 682.4152.

4.1.30. General procedure for the synthesis of the tripeptide boronic acid (7)

To a stirred solution of the tripeptide boronates **6** (1 mmol) in anhydrous CH_2Cl_2 was added 1 N BBr_3 dissolved in CH_2Cl_2 (2.5 mL). The mixture was stirred at 0 °C for 2 h. The reaction was quenched with water and extracted with CH_2Cl_2 (4 × 15 mL). The combined organic layers were washed with water and brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The resulted crude product was crystallized with ether and CH_2Cl_2 to give tripetide boronic acid **7**.

4.1.31. *N*-(2-Pyrazinecarbonyl)-L-phenylalanine-L-phenylalanine-L- leucine boronic acid (7a)

Yield 72.1%, light yellow solid, mp:112–114 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.26–9.15 (m, 1H), 8.77 (s, 1H), 8.53 (s, 1H), 8.18 (s, 1H), 7.29–7.24 (m, 5H), 7.07–6.84 (m, 6H), 6.42–6.38 (m, 1H), 4.77–4.65 (m, 2H), 3.17–2.96 (m, 5H), 2.17–1.25 (m, 5H), 0.92–0.90 (m, 3H), 0.88–0.86 (m, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.01, 169.74, 163.58, 163.32, 147.75, 144.28, 143.32, 142.72, 135.89, 135.66, 129.20, 128.88, 128.54, 128.41, 127.41, 126.75, 54.96, 51.70, 37.54, 37.30, 25.66, 23.17, 22.51; HRMS calcd for $C_{28}H_{35}BN_5O_5$ $(M+1)^+$ 532.2731, found 532.2717.

4.1.32. *N*-(2-Pyrazinecarbonyl)-L-(2-naphthyl)-alanine-L-phenylalanine-L-leucine boronic acid (7b)

Yield 63.5%, light yellow solid, mp: 134–135 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.15 (s, 1H), 8.73 (d, J = 2.4 Hz, 1H), 8.46 (d, J = 2.1 Hz, 1H), 8.24–8.17 (m, 1H), 7.76–7.66 (m, 4H), 7.44–7.40 (m, 3H), 7.32–7.25 (m, 1H), 6.90–6.86 (m, 5H), 6.52–6.46 (m, 2H), 4.81–4.67 (m, 2H), 3.31–3.26 (m, 1H), 3.11 (d, J = 6.9 Hz, 2H), 2.93–2.89 (m, 1H), 1.92 (s, br, 2H), 1.55–1.21 (m, 3H), 0.85–0.82 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 172.96, 169.99, 163.61, 147.70, 144.24, 143.30, 142.70, 137.28, 135.61, 133.47, 133.19, 132.49, 129.13, 128.64, 128.35, 128.07, 127.64, 127.55, 127.11, 126.71, 126.39, 125.92, 54.88, 51.86, 39.85, 39.34, 37.43, 37.23, 36.88, 25.68, 23.12, 22.46. HRMS calcd for $C_{32}H_{36}BN_5O_5$ $(M-1)^-$ 580.2727, found 580.2736.

4.1.33. *N*-(2-Pyrazinecarbonyl)-L-phenylalanine-L-tyrosine-L-leucine boronic acid (7c)

Yield 56.3%, light yellow solid, mp: 298–300 °C; ^{1}H NMR (CD_3OD, 300 MHz): δ 9.13 (s, 1H), 8.78 (t, J = 2.4 Hz, 1H), 8.66 (dd, J_1 = 1.2 Hz, J_2 = 2.4 Hz, 1H), 7.23–7.16 (m, 5H), 7.16–7.01 (m, 2H), 6.66–6.18 (m, 2H), 4.80–4.73 (m, 2H), 3.20–3.18 (m, 1H), 3.08–3.02 (m, 3H), 2.68–2.56 (m, 1H), 1.62–1.54 (m, 2H), 1.33–1.26 (m, 2H), 1.14–1.04 (m, 2H), 0.89–0.83 (m, 6H); ^{13}C NMR (CD_3OD, 75 MHz): δ 177.69, 173.05, 165.13, 164.95, 157.77, 157.68, 148.79, 145.59, 144.96, 144.79, 144.49, 137.82, 131.47, 131.35, 130.34, 129.56, 127.97, 127.40, 127.16, 116.41, 56.11, 55.90, 52.87, 52.67, 40.70, 38.82, 38.49, 37.65, 37.30, 26.83, 26.61, 23.89, 23.69, 22.31, 21.83; HRMS calcd for $C_{28}H_{34}BN_5O_6$ $(M-1)^-$ 546.2529, found 546.2527.

4.1.34. *N*-(2-Pyrazinecarbonyl)-L-tyrosine-L-phenylalanine-L-leucine boronic acid (7d)

Yield 71.5%, light yellow solid, mp: 219–221 °C; ^{1}H NMR (CD_3OD, 300 MHz): δ 9.15 (s, 1H), 8.78 (d, J = 2.4 Hz, 1H), 8.66 (d, J = 2.1 Hz, 1H), 7.24–7.19 (m, 5H), 7.13–7.00 (m, 2H), 6.97–6.62 (m, 2H), 4.80–4.71 (m, 2H), 3.11–3.06 (m, 3H), 2.93–2.87 (m, 1H), 2.64–2.54 (m, 1H), 1.62–1.54 (m, 2H), 1.40–1.29 (m, 2H), 1.16–1.11 (m, 2H), 0.92–0.81 (m, 6H); ^{13}C NMR (CD_3OD, 75 MHz): δ 177.20, 173.18, 164.92, 157.51, 148.68, 145.62, 144.93, 144.73, 137.17, 136.96, 131.36, 130.46, 130.33, 129.69, 129.62, 128.25, 128.19, 116.32, 56.34, 56.17, 52.61, 40.77, 38.46, 38.11, 37.82, 26.62, 23.86, 21.91; HRMS calcd for $C_{30}H_{38}BN_5NaO_6$ $(M+Na+2CH_2)^+$ 598.2813, found 598.2815.

4.1.35. *N*-(2-Pyrazinecarbonyl)-L-leucine-L-phenylalanine-L-leucine boronic acid (7e)

Yield 68.4%, light yellow solid, mp: 113–115 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.25–9.16 (m, 1H), 8.72 (s, 1H), 8.47 (s, 1H), 8.02–7.96 (m, 1H), 7.25–7.19 (m, 1H), 7.02–6.92 (m, 6H), 4.76–4.61 (m, 1H), 4.41–4.28 (m, 1H), 3.09–2.89 (m, 3H), 1.95–1.92 (m, 1H), 1.57–1.19 (m, 7H), 0.82–0.78 (m, 13H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 172.04, 171.77, 163.55, 147.63, 144.25, 143.96, 142.84, 135.91, 129.95, 129.21, 128.40, 126.83, 53.29, 50.43, 40.32, 39.72, 37.06, 35.60, 25.64, 24.68, 24.19, 22.85, 22.59, 22.24, 21.73; HRMS (ESI): calcd For $C_{27}H_{40}BN_5NaO_5$ $(M+Na+2CH_2)^+$ 548.3020 found:548.3027.

4.1.36. *N*-(2-Pyrazinecarbonyl)-L-leucine-L-(2-naphthyl)-alanine-L-leucine boronic acid (7f)

Yield 67.0%, light yellow solid, mp: 157–159 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.13–9.10 (m, 1H), 8.67 (s, 1H), 8.36 (s, 1H), 7.98–7.94 (m, 2H), 7.59–7.29 (m, 7H), 7.22–7.19 (m, 2H), 4.98–4.84 (m, 1H), 4.41–4.37 (m, 1H), 3.46–3.35 (m, 2H), 3.24–3.10 (m, 1H), 1.71–1.25 (m, 8H), 0.88–0.78 (m, 12H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 171.48,

163,92, 147,65, 144,15, 143.31, 143.07, 142.56, 133.56, 133.22, 132.25, 129.16, 128.69, 128.24, 127.51, 127.10, 125.98, 125.58, 52.95, 52.77, 51.91, 41.12, 40.23, 36.76, 25.68, 24.72, 23.12, 22.83, 22.44, 21.69; HRMS calcd for $C_{29}H_{37}BN_5O_5$, $(M-1)^-$ 546.2888; found 546.2906.

4.1.37. *N*-(2-Pyrazinecarbonyl)-L-phenylalanine-L-(2-naphthyl)-alanine-L-Leucine boronic acid (7g)

Yield 53%, light yellow solid, mp: 137–138 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.90 (s, 1H), 8.61 (s, 1H), 8.26 (s, 1H), 8.09–7.98 (m, 1H), 7.50–7.23 (m, 12H), 7.19–7.07 (m, 1H), 6.73–6.56 (m, 2H), 4.88–4.76 (m, 1H), 4.72–4.67 (m, 1H), 3.33–3.28 (m, 1H), 3.13 (d, J = 6.3 Hz, 2H), 3.02–2.97 (m, 1H), 2.04 (s, br, 2H), 1.51–1.42 (m, 3H), 0.87–0.80 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 173.10, 170.06, 163.51, 147.59, 143.90, 142.95, 142.86, 142.64, 142.52, 135.90, 135.70, 133.45, 133.23, 133.07, 132.14, 129.29, 128.88, 128.24, 127.94, 127.46, 127.14, 125.90, 125.53, 54.94, 51.96, 42.92, 39.98, 37.26, 36.92, 25.69, 23.14, 22.56; HRMS calcd for $C_{32}H_{35}BN_5O_5$ $(M-1)^-$ 580.2731, found 580.2729.

4.1.38. *N*-(2-Pyrazinecarbonyl)-L-(2-naphthyl)-alanine-L-(2-naphthyl)-alanine-L-leucine boronic acid (7h)

Yield 72.1%, light yellow solid, mp: 145–146 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.95 (s, 1H), 8.54 (s, 1H), 8.16 (s, 1H), 8.17–8.11 (m, 1H), 7.74–7.16 (m, 14H), 7.08–6.95 (m, 2H), 6.87–6.50 (m, 2H), 4.95–4.87 (m, 1H), 4.79–4.68 (m, 1H), 3.28–3.26 (m, 2H), 3.00–2.86 (m, 1H), 2.33 (s, br, 2H), 1.38–1.35 (m, 3H), 0.81–0.79 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.18, 169.61, 163.56, 147.51, 143.79, 141.90, 142.55, 133.42, 133.126, 133.01, 132.46, 132.07, 128.58, 128.09, 127.39, 127.14, 127.01, 126.32, 125.94, 125.83, 125.49, 54.79, 51.52, 39.78, 37.49, 37.30, 36.95, 25.64, 23.09, 22.39, 22.19; HRMS calcd for $C_{36}H_{37}BN_5O_5$ $(M-1)^-$ 630.2893, found 630.2895.

4.1.39. *N*-(2-Pyrazinecarbonyl)-L-tryptophan-L-phenylalanine-L-leucine boronic acid (7i)

Yield 49.2%, sticky solid; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.72 (s, 1H), 9.28 (s, 1H), 9.17 (s, 1H), 8.64–8.56 (m, 1H), 8.06–7.97 (m, 1H), 7.19–7.16 (m, 9H), 6.89–6.78 (m, 1H), 5.05 (s, 1H), 4.98–4.87 (m, 1H), 3.98–3.75 (m, 1H), 3.58–3.53 (m, 1H), 3.19–3.15 (m, 1H), 2.13–1.97 (m, 10H), 1.60–1.40 (m, 3H), 0.79–0.75 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 171.82, 170.35, 164.19, 147.46, 143.99, 142.76, 136.29, 129.11, 128.42, 127.11, 126.68, 124.07, 122.33, 112.16, 109.91, 54.27, 53.07, 49.31, 42.29, 39.89, 39.21, 38.72, 34.16, 33.58, 29.69, 27.94, 25.61, 24.09, 30.57, 23.07; HRMS (ESI): calcd for $C_{32}H_{39}BN_6NaO_5$ $(M+Na+2CH_2)^+$ 621.2973, found 621.2963.

4.1.40. *N*-(2-Pyrazinecarbonyl)-L-phenylalanine-L-tryptophan-L-leucine boronic acid (7j)

Yield 42.4%, sticky solid; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.28–9.27 (m, 1H), 9.21–9.14 (m, 1H), 9.07–9.00 (m, 1H), 8.57–8.46 (m, 1H), 8.12–8.10 (m, 1H), 7.16–7.08 (m, 9H), 6.76–6.58 (m, 1H), 5.25–5.18 (m, 1H), 4.81–4.64 (m, 1H), 3.78–3.45 (m, 1H), 3.48–3.33 (m, 1H), 3.11–3.05 (m, 1H), 2.07–1.89 (m, 10H), 1.45–1.23 (m, 3H), 0.81–0.79 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.99, 169.94, 162.87, 147.61, 144.26, 142.78, 136.00, 135.92, 129.22,128.74, 127.16, 127.05, 54.59, 54.35, 51.63, 49.27, 46.08, 42.22, 41.26, 40.02, 38.26, 33.66, 26.83, 25.58, 24.62, 22.99, 22.74, 22.53, 22.16, 21.88; HRMS (ESI): calcd for $C_{32}H_{39}BN_6NaO_5$ $(M+Na+2CH_2)^+$ 621.2973 found: 621.2971.

4.1.41. *N*-(2-Pyrazinecarbonyl)-L-phenylalanine-L-leucine-L-leucine boronic acid (7k)

Yield 73.2%, light yellow solid, mp: 116–118 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.30–9.21 (m, 1H), 8.74 (s, 1H), 8.47 (s, 1H), 8.33–8.29 (m, 1H), 7.32–7.22 (m, 6H), 6.99–6.91 (m, 1H), 4.93–4.57 (m, 2H), 3.21 (s, 2H), 2.85–2.58 (m, 1H), 2.18–1.25 (m, 8H), 0.96–0.82 (m, 12H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.60, 169.97, 164.55, 147.49, 143.47, 135.72, 129.25, 128.96, 127.51, 124.86, 121.47, 119.49, 117.68, 111.50, 107.58, 55.78, 51.58, 50.94, 38.99, 37.06, 25.65, 25.52, 23.18, 22.61, 22.15; HRMS (ESI): calcd for $C_{27}H_{40}BN_5NaO_5$ $(M+Na+2CH_2)^+$ 548.3020, found 548.3033.

4.1.42. *N*-(2-Pyrazinecarbonyl)-L-leucine-L-leucine-L-leucine boronic acid (7l)

Yield 62.8%, sticky solid; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.30 (s, 1H), 8.71 (s, 1H), 8.50 (s, 1H), 8.28–8.06 (m, 1H), 7.16–7.09 (m, 1H), 6.99–6.85 (m, 1H), 4.86–4.69 (m, 1H), 4.53 (s, 1H), 2.91–2.85 (m, 1H), 2.82–1.19 (m, 11H), 0.97–0.81 (m, 18H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 171.77, 171.49, 163.10, 147.51, 144.36, 144.01, 143.88, 51.89, 51.36, 44.15, 40.55, 39.76, 38.73, 38.08, 30.55, 29.62, 28.55, 25.72, 24.78, 23.02, 21.89, 20.96; HRMS calcd for $C_{22}H_{37}BN_5O_5$ $(M-1)^-$ 462.2888, found 462.3825.

4.1.43. *N*-(2-Pyrazinecarbonyl)-L-(2-naphthyl)alanine-L-leucine-L-leucine boronic acid (7m)

Yield 66%, light yellow solid, mp: 135–136 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.29 (s, 1H), 8.70 (d, J = 2.1 Hz, 1H), 8.45 (d, J = 1.8 Hz, 1H), 8.40 (d, J = 7.5 Hz, 1H), 7.74–7.66 (m, 4H), 7.45–7.42 (m, 3H), 7.37 (d, J = 8.7 Hz, 1H), 5.05–4.93 (m, 1H), 4.53–4.45 (m, 1H), 3.38 (d, J = 8.7 Hz, 2H), 2.98–2.83 (m, 1H), 1.99 (s, br, 2H), 1.80–1.51 (m, 1H), 1.50–1.29 (m, 6H), 0.86–0.74 (m, 12H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.59, 170.45, 163.83, 147.74, 144.27, 143.61, 142.77, 133.46, 133.27, 132.47, 128.59, 128.12, 127.63, 127.05, 126.24, 125.84, 54.76, 39.88, 37.74, 25.78, 24.61, 24.48, 23.03, 22.82, 22.63, 21.60, 21.45; HRMS calcd for $C_{29}H_{37}BN_5O_5$ $(M-1)^-$ 546.2893, found 546.2903.

4.2. Biological testing

4.2.1. Assays for proteasome activities

The enzymatic activities of the proteasome were assayed using fluorogenic peptides: Suc-Leu-leu-Val-Tyr-AMC (Suc represents succinyl and AMC represents 7-amido-4-methyicoumarin, obtained from SIGMA) for chymotryptic-like (CT-L) activity, Z-Ala-Arg-Arg-AMC (Z represents benzyloxycarbonyl, obtained from Calbiochem) for trypsin-like (T-L) activity and Z-Leu-Leu-Glu- βNA (βNA represents β-naphtylamide, obtained from SIGMA) for peptidylglutamyl peptide-hydrolyzing activity (PGPH). One microgram of 20S proteasome purified from mouse liver was incubated with various concentrations of compounds and 50 μM fluorogenic peptides in 100 μl of 20 mM Tris–HCl pH 7.8, at 37 °C for 1 h, respectively. The fluorescence of released AMC and βNA reagents, was measured by a spectrofluorimeter (Fluostar OPTIMA, BMG Germany) at excitation/emission wavelengths of 380/440 nm and 335/410 nm, respectively. 0.1% DMSO was used as solvent control. Compared with the fluorescence of solvent control, an inhibition rate was calculated and thereafter the IC_{50} value was deduced.

4.2.2. Cell culture and cytotoxicity assays

HL-60 (promyelocytic leukemia cell line), U266 (multi myeloma cell line), and BXPC-3 (human pancreatic cancer cell line) human cell lines were obtained from the American Type Culture Collection (Manassas, VA). BGC-823 (human gastric carcinoma cell line) cell line was obtained from China Pharmaceutical University. HL-60 cell was cultured in IMDM supplemented with 20% fetal bovine serum at 37 °C in 5% CO_2. BGC-823 and BXPC-3 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. U266 cell was cultured in RPMI 1640 supplemented with 15% fetal bovine serum at 37 °C in 5% CO_2.

A standard MTT assay was used to measure cell growth. In brief, a suspension of 3000 cells/150 μL of medium was added to each well of 96-well plates and allowed to grow. Twenty-four hours later, drugs prepared in medium at 10 different concentrations were added to the corresponding plates at a volume of 50 μL per well, and the plates were incubated for 72 h with drugs. Then 20 μL of a solution of 5 mg/ml MTT were added to each well and incubated for another 4 h at 37 °C. Plates were then centrifuged at 1000 rpm at 4 °C for 5 min, and the medium was carefully discarded. The formazan crystals were dissolved in 100 μL of DMSO and absorbance was read on an Infinite M200 (Tecan, Austria) microplate reader at 540 nm. The result was expressed as the mean IC_{50} value, which is the average from at least three independent determinations.

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