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Structure-based design, synthesis, and biological evaluation of Leu-Arg dipeptide analogs as novel hepsin inhibitors

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ABSTRACT

Hepsin, a type II transmembrane serine protease, is an attractive protein as a potential therapeutic and diagnostic biomarker for prostate cancer because it is highly up-regulated in prostate cancer and promotes both progression and metastasis. Starting from the reported tetrapeptide hepsin inhibitor Ac-KQLR-ketothiazole (kt) (**1**), we investigated the minimal structural requirements for hepsin inhibitory activity by truncating amino acids at the N-terminus. The kt and ketobenzothiazole (kbt) dipeptide analogs Ac-LR-kt (**3**) and Ac-LR-kbt (**15**) were found to be potent hepsin inhibitors, exhibiting K_i values of 22 nM and 3 nM, respectively. The present work suggests that LR-containing dipeptide molecules could be useful as lead compounds for the development of novel hepsin inhibitors.

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among European and American men.[1–6] Localized prostate cancer can be considered a curable disease. However, once it progresses to the stage of metastasis, it is extremely difficult to treat and is almost uniformly fatal.[7,8] Unfortunately, there are currently no effective treatments that inhibit prostate cancer progression. Therefore, the development of an effective therapeutic strategy to prevent the progression and metastasis of prostate cancer is highly demanded.

Hepsin, a type II transmembrane serine protease,[9] is predominantly expressed in neoplastic prostate compared with benign prostate.[10–13] In addition, mRNA expression of hepsin was significantly elevated in more than 90% of prostate cancer specimens at levels that were 10-fold higher in metastatic prostate cancer compared with those in normal prostate or benign prostatic hyperplasia.[11,14–16] Hepsin expression is up-regulated in early-stage prostate cancer and expression continues at high levels as the cancer progresses to later stages and metastasis.[17] Moreover, hepsin is also highly expressed in ovarian and renal cell carcinomas.[18,19] Since hepsin expression is correlated with the progression and metastasis of prostate cancer, it is considered as to be attractive diagnostic biomarker and therapeutic target for metastatic prostate cancer.[11] Furthermore, previous studies have demonstrated that hepsin overexpression is involved in the regulation of prostate cancer progression and metastasis.[20] In a mouse model of non-metastasizing prostate cancer, overexpression of hepsin promoted the metastasis of primary prostate cancer to the bone, liver, and lung.[18,19,21,22] In addition, hepsin overexpression was associated with the disruption of the basement membrane, indicating that the degradation of basement membrane components are facilitated by hepsin activity.[20]

Therefore, we were interested in the discovery and development of potent and selective low molecular weight inhibitors of hepsin as pharmacological tools for better understanding the role of hepsin in cancer progression and as a potential target for the treatment and diagnosis of metastatic prostate cancer. Although attention has been focused on the development of synthetic hepsin inhibitors during the last decade, very few related studies have been reported to date. Recently, Janetka and co-workers reported that synthetic tetrapeptide (acetyl (Ac)-KQLR-ketothiazole (kt)) analogs exhibited strong inhibitory activities against hepsin with K_i value in the nanomolar range.[23] In this study, we designed and synthesized novel hepsin inhibitors by truncating amino acids at the N-terminus of the Ac-KQLR-kt (**1**) which was used as a template for structural modification (Fig. 1). We aimed to investigate three points: (1) effect of the truncation of N-terminal amino acids of Ac-KQLR-kt (**1**) on hepsin affinity, (2) the relationship between hepsin affinity and the absolute configuration of C-terminal Arg in the above-mentioned sequences, and (3) the effects of replacing C-terminal ketothiazole with other aromatic rings in the newly-identified peptides.

In order to identify the essential amino acids in Ac-KQLR-kt (**1**) required for hepsin inhibitory activity, we systematically designed three different truncated forms of Ac-KQLR-kt (**1**). In silico binding

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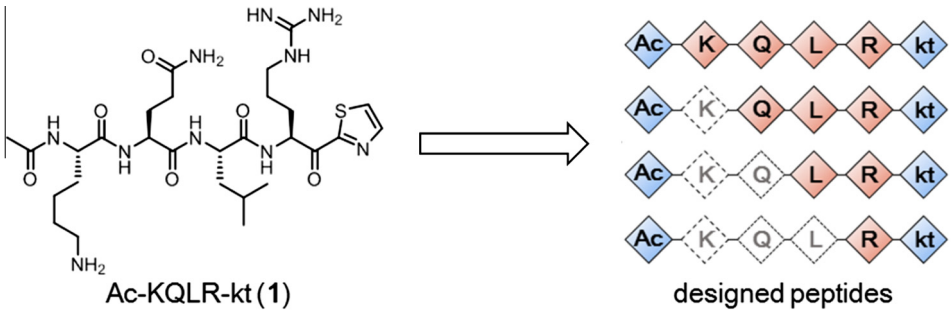


Figure 1. Design strategy of truncated peptides from Ac-KQLR-kt (**1**).

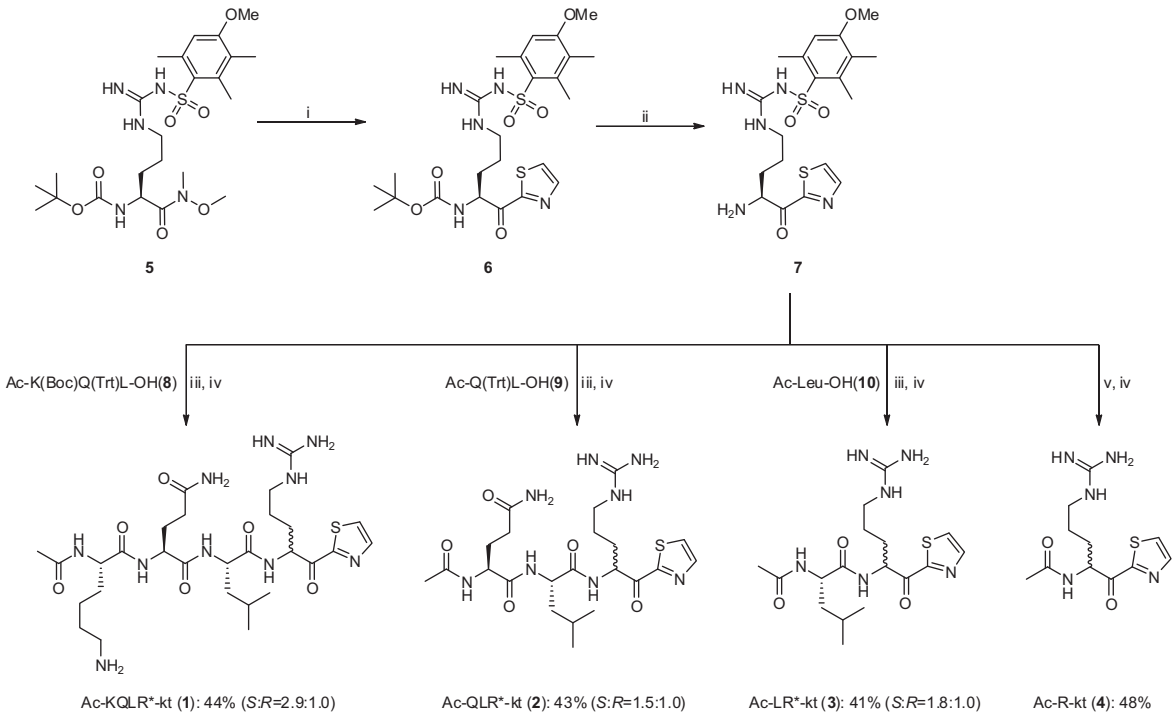
affinities for hepsin were evaluated using Surflex-Dock (Tripos Inc, NJ) protocols implemented in the SYBYL molecular docking module. Docking studies of the truncated Ac-KQLR-kt analogs (**2**–**4**) with the parent molecule Ac-KQLR-kt (**1**) were performed by using the available X-ray crystal structure of hepsin from Protein Data Bank (PDB code: 1O5E).[24] Docking results showed that the positions of the truncated Ac-KQLR-kt analogs (**2**–**4**) were similar to that of Ac-KQLR-kt (**1**). In particular, the side chain of the Arg residue in the truncated Ac-KQLR-kt analogs (**2**–**4**) was projected deeply into the S1 pocket, with its terminal guanidinium group forming a salt bridge with the carboxylate group of Asp189. The nitrogen atoms of the thiazole ring and the guanidine group interacted with Gly219. Although docking total scores of the truncated peptide analogs (**2**–**4**) were in the range of 7.0994–8.0906 compared with a score of 10.1733 for Ac-KQLR-kt (**1**), their binding modes were very similar to that of Ac-KQLR-kt (**1**). On the basis of extensive docking studies, we hypothesized that the Arg moiety and an additional hydrophobic interaction are the essential key structural features involved in the interaction between the ligand and hepsin. Furthermore, there is no reported information on hepsin affinity of the epimer of Ac-KQLR-kt (**1**) which has the (*R*)-configuration at the C_α atom of the Arg residue. Since peptides containing (D)-amino acids would be more resistant to protease degradation,[25,26] we endeavored to investigate the effect of the absolute configuration of Arg on hepsin binding affinity. Computational docking studies revealed that the Leu side chain in Ac-KQLR-kt (**1**) and its truncated analogs (**2** and **3**) were located on the hydrophobic surface of the hepsin active site. However, the relative orientation of the Leu side chain appeared to be determined by the chirality of the Arg residue. In the (*S*)-configuration of the Arg chiral center, the methyl group of Leu side chain were stabilized by an hydrophobic interaction with Gln192. Alternatively, the methyl group made an interaction with His57 when the absolute configuration of Arg is changed from (*S*) to (*R*) (see Supporting material).

To verify our hypothesis, we synthesized Ac-KQLR-kt (**1**) and its truncated analogs (**2**–**4**) by using a combination of Fmoc-strategy solid-phase peptide synthesis (SPPS) and solution-phase fragment assembly as outlined in Scheme 1. Ac-LR-ketobenzothiazole (kbt) (**15**) and Ac-LR-phenyl ketone (kPh) (**16**) were prepared according the synthetic routes shown in Scheme 2.

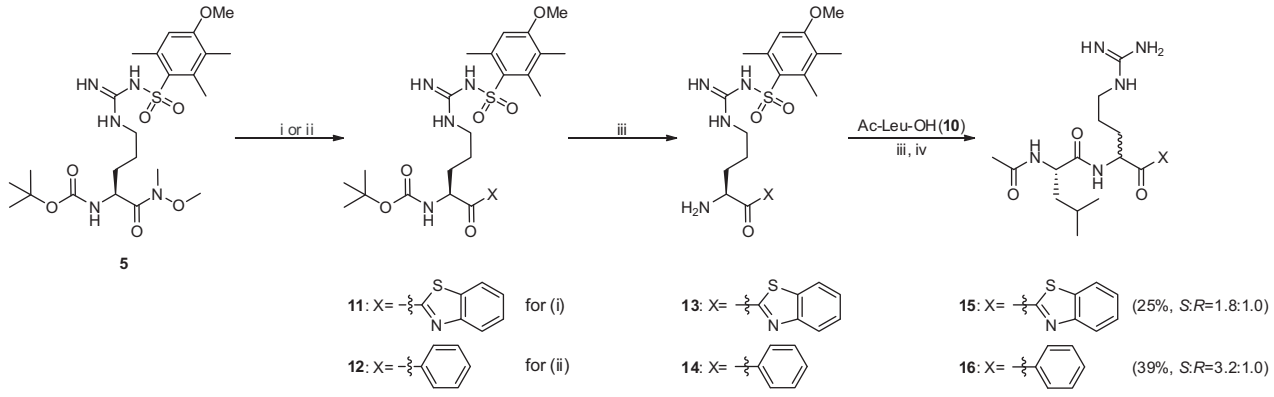
The protected peptides Ac-KQL (**8**), Ac-QL (**9**), and Ac-L (**10**) were prepared by SPPS using HBTU as a coupling reagent and Hünig's base. 2-Cl trityl resin was used as the starting material and was cleaved using 25% hexafluoroisopropanol (HFIP) in CH_2Cl_2. The key intermediates (**8**–**10**) were purified by column chromatography on silica gel with elution in CH_2Cl_2/MeOH (4:1, v/v). The Mtr-protected Arg-kt (**6**) and Arg-kbt (**11**) were prepared by reacting the corresponding Weinreb amide **5** with 2-lithiothiazole and 2-lithiobenzothiazole at −78 °C. In this synthetic process, we used an excess amount of *n*-BuLi to enhance the ratio of proton exchange at the chiral center of the Arg residue, thus resulting in an increased (D)-Arg epimer ratio as compared to that associated with previously reported method.[23] The Boc group of **6** and **11** was selectively removed by the treatment of 25% trifluoroacetic acid (TFA) in CH_2Cl_2 to afford **7** and **13** in nearly quantitative yields, which did not affect the side chain protecting group Mtr Intermediates **7** and **13** were used in the next coupling step without further purification. On the other hand, the Mtr-protected Arg-phenyl ketone (**12**) was synthesized by reacting the Weinreb amide **5** with Grignard reagent phenylmagnesium bromide (PhMgBr). Addition of PhMgBr to **5** in dry THF at 0 °C provided **12** in 70% yield. Selective removal of the Boc group from **12** was accomplished by 25% TFA in CH_2Cl_2 to afford the free amine **14** with enough purity to be used for the next step without further purification. The full-length Ac-KQLR-kt (**1**) and its truncated analogs **2**–**4** were synthesized by conjugating intermediates **8**–**10** with Mtr-protected Arg **7** under conventional peptide coupling conditions using HATU and DIPEA, followed by the deprotection of the global side chain with TFA/thioanisole/water (95:2.5:2.5, v/v/v).

The final products were purified by semi-preparative reversed-phase HPLC using a linear gradient of acetonitrile (ACN) and water containing 0.1% TFA. The detailed HPLC conditions are described in Supporting material. Under the optimized HPLC conditions, pure epimers of the diastereomeric mixtures with different configurations of the Arg chiral center were purified with the (*R*)-epimer eluting earlier than the corresponding (*S*)-epimer. The truncated peptide analogs (**2**–**4**) of Ac-KQLR-kt (**1**) were obtained in good overall yields and with diastereomeric ratio ranges from 2.9:1.0 to 1.5:1.0 (*S*:*R*). We compared the change of ^{1}H chemical shifts between the (*R*)-epimer and the (*S*)-epimer compounds (**1**–**3**) using 1D- and 2D-NMR spectroscopy. In all NMR spectra, each (*R*)-epimer consistently exhibited downfield chemical shifts for the Leu α proton and the Leu δ methyl groups relative to those of the corresponding (*S*)-epimer.

The hepsin inhibitory activity of the synthesized Ac-KQLR-kt (**1**) and its truncated analogs (**2**–**4**) was determined by applying the reported enzymatic assay, which used the fluorogenic substrate Boc-QAR-AMC with recombinant human hepsin.[23] The results of hepsin assay are summarized in Table 1. The Ac-KQLR-kt (**1***S*) showed strong hepsin affinity with a K_i value of 1.45 nM which is similar to the previously reported value,[23] while Ac-KQL-OH (**17**) without the Arg-kt moiety lost the hepsin activity completely. As expected from docking studies, the Arg-kt moiety was essential for binding to hepsin. Surprisingly, Ac-QLR-kt (**2***S*), which was obtained by deleting Lys at the N-terminus, showed strong hepsin inhibition with a K_i value of 1.04 nM. Although the truncation of Lys and Gln residues in Ac-LR-kt (**3***S*) decreased hepsin inhibition, resulting in a compound that was less potent than parent compound **1***S* with a K_i value of 22.4 nM, **3***S* was potent enough to be utilized as the lead compound for further structural modification. However, Ac-R-kt (**4**), in which the Lys-Gln-Leu tripeptide was removed, resulted in a dramatic loss of hepsin-binding affinity.



Scheme 1. Synthesis of Ac-KQLR-kt (**1**) and its truncated analogs (**2**–**4**). Reagents and conditions: (i) thiazole, TMEDA, *n*-BuLi, THF, −78 °C, 1 h; (ii) 25% TFA in CH_2Cl_2, 1.5 h; (iii) HATU, DIPEA, DMF, rt, 12 h; (iv) TFA/thioanisole/water (95/2.5/2.5), 4 h; (v) Ac_2O, DIPEA, DMF, rt, 1 h.



Scheme 2. Synthesis of benzothiazole- and phenyl-containing Ac-LR analogs. Reagents and conditions: (i) benzothiazole, TMEDA, *n*-BuLi, THF, −78 °C, 1 h; (ii) PhMgBr, THF, 0 °C to rt, 4 h; (iii) 25% TFA in CH_2Cl_2, 1.5 h; (iii) HATU, DIPEA, DMF, rt, 12 h; (iv) TFA/thioanisole/water (95/2.5/2.5), 4 h.

These results suggested that Ac-LR-kt (**3***S*) potentially represents a minimal structural element required for hepsin inhibitory activity with the Arg-kt considered the essential moiety. None of the compounds missing the Arg-kt moiety showed any hepsin inhibitory activity. Surprisingly, the compound with the (*R*)-configuration at the Arg C_α atom was as potent as the corresponding one with the (*S*)-configuration, indicating that the absolute configuration of the Arg C_α has a minimal effect on hepsin activity in vitro. All of the peptide analogs (**1**–**4**) were further evaluated with matriptase,[27] a known serine protease, to investigate the selectivity of the truncated peptides for binding to hepsin over matriptase (Table 1). The truncated analogs (**2** and **3**) were approximately 8- to 15-fold more selective for hepsin over matriptase.

Therefore, the dipeptide Ac-LR-kt (**3**) with minimal structural requirements was selected as the lead compound for structural modification and further development of hepsin inhibitors. The replacement of the C-terminal thiazole in **3** with benzothiazole affected the selectivity as well as the potency. Ac-LR-kbt (**15**) exhibited strong inhibition of hepsin activity with K_i value of 3.38 nM and 2.91 nM for the (*S*)-epimer and the (*R*)-epimer, respectively. Furthermore, both **15***S* and **15***R* showed increased selectivity for hepsin over matriptase, by >60-fold. However, the introduction of a phenyl group (Ac-LR-kPh, **16**) instead of a thiazole group in **3** resulted in a significant loss of inhibitory activity toward both hepsin and matriptase. As shown in Figure 2, in silico binding modes of **3***S*, **3***R*, **15***S* and **15***R* showed that Tyr146, Asp 189, and Gly 219 in the active site of hepsin are important residues for interactions with them. The benzothiazole analogs **15***S* and **15***R* made an additional tight hydrogen-bonding interaction with the backbone of Gly 193 (2.01 Å) or His57 (2.30 Å), explaining that their binding affinity for hepsin were 6-fold stronger than the thiazole analogs **3***S* and **3***R*.

In summary, we prepared a library of truncated Ac-KQLR-kt analogs to identify the minimal structural elements required for

Table 1
K_i values of inhibitors toward hepsin and matriptase

Sequence	Compound	Arg chirality	Hepsin K_i[a] (nM)	Matriptase K_i[a] (nM)
Ac-KQLR-kt	**1***S*	*S*	1.45 ± 0.07	10.4 ± 0.6
	1*R*	*R*	0.83 ± 0.02	8.0 ± 0.7
Ac-QLR-kt	**2***S*	*S*	1.04 ± 0.01	11.3 ± 1.0
	2*R*	*R*	2.88 ± 0.03	11.1 ± 0.4
Ac-LR-kt	**3***S*	*S*	22.4 ± 0.5	334 ± 62
	3*R*	*R*	21.5 ± 0.6	246 ± 37
Ac-R-kt	**4**[b]	*S*	>10,000	>10,000
Ac-LR-kbt	**15***S*	*S*	3.38 ± 0.21	227 ± 11
	15*R*	*R*	2.91 ± 0.11	177 ± 30
Ac-LR-kPh	**16***S*	*S*	>10,000	>10,000
	16*R*	*R*	>10,000	>10,000
Ac-KQL-OH	**17**	—	>10,000	>10,000

[a] Measurements of enzymatic activity were performed in triplicate and represent the mean ± SD of at least three experiment sets.
[b] A mixture of *R* and *S* was used for the assay although one enantiomer was enriched.

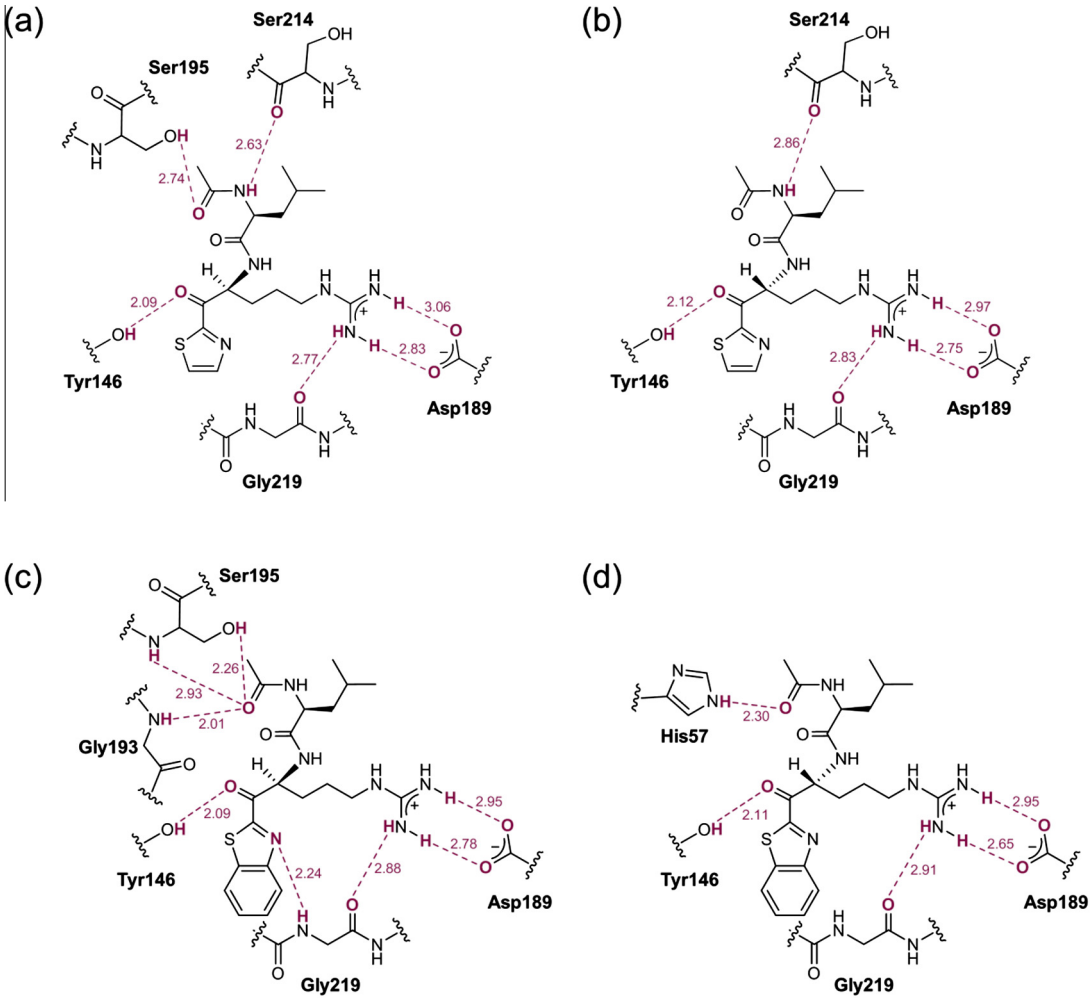


Figure 2. Schematic depiction of key interactions between a ligand and hepsin. (a) Ac-LR-kt (**3***S*), (b) Ac-LR-kt (**3***R*), (c) Ac-LR-kbt (**15***S*), and (d) Ac-LR-kbt (**15***R*).

hepsin inhibitory activity. The truncated Ac-QLR-kt (**2**) and Ac-LR-kt (**3**) showed strong hepsin inhibition with K_i values in the nanomolar range, while Ac-R-kt (**4**) revealed no activity. Furthermore, Ac-LR-kbt (**15**) exhibited a promising selectivity profile for hepsin over matriptase. These findings suggest that the dipeptides Ac-LR-kt (**3**) and Ac-LR-kbt (**15**) could serve as lead compounds for further structural modification toward hepsin inhibitors for clinical applications. We also investigated the relationship between hepsin affinity and the absolute configuration of the Arg C_α atom in the truncated analogs. The hepsin affinity of the (*R*)-epimer was similar to that of the corresponding (*S*)-epimer, indicating that epimerization of Arg did not make a significant difference for hepsin inhibition. The information from this study will be used as a basis for the development of structurally-simplified hepsin inhibitors.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.12.023.

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