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# Novel CADD-based peptidyl vinyl ester derivatives as potential proteasome inhibitors

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**Abstract**—A series of peptidyl vinyl ester derivatives bearing three different P1 substitutions as potential proteasome inhibitors were studied. The target molecules were designed based on CADD (computer aided drug design) protocol and synthesized. Their activities toward proteasome and four human cancer cell lines (including hepatoma cell line (Bel-7402), myeloid leukemic cell line (HL-60), gastric cancer cell line (BGC-823) and nasopharyngeal cancer cell line (KB)) were tested using fluorescence assay. Two compounds showed proteasome inhibitory activities, and four compounds showed weak antiproliferative activities toward HL-60 and BGC-823.


The ubiquitin–proteasome pathway (UPP) is one of the two ways cells utilize for protein degradation, in which the 26S proteasome complex makes a functional machinery where hydrolysis of targeted proteins takes place.[1] It consists of a 20S core catalytic cylindrical complex capped at both ends by 19S regulatory subunits. The 20S proteasome is formed by four stacked rings, with each of the two inner rings composed of seven different β subunits.[2–4] β1, β2, and β5 subunits contain the post-acidic-like (PGPH), trypsin-like (T-L), and chymotrypsin-like (ChT-L) active sites where peptide bonds are cleaved on the carboxyl side of acidic, basic and hydrophobic amino acid residues, respectively.[5–7] Substrates of 20S proteasome are intracellular proteins, including cyclins, caspases, BCL2 and nuclear factor of κB (NF-κB) , representative of mediators during cell-cycle progression and apoptosis. A large body of experiments have already proven that the effects of these proteins are regulated by 20S proteasome. Therefore, it is proposed to be a new target for antineoplastic and anti-inflammatory therapy.[8] To date, two compounds have entered the clinic as antineoplastic (PS-341, also known as bortezomib and Velcade, a peptidyl boronic acid approved for the treatment of multiple myeloma patients by FDA in 2003) and anti-inflammatory drugs (PS-519, also known as MLN519).[9,10]

Currently, the reported proteasome inhibitors included peptidyl aldehydes, α-ketoamides, peptidyl boronic acids, α′,β′-epoxyketones, and non-covalent binding inhibitors, etc.,[11–17] all of which targeted the chymotrypsin-like (β5) subunit because of its importance on protein hydrolysis.[18–20] Among them peptidyl boronic acids are the most potent and α′,β′-epoxyketones are the most selective.[21] However, Mauro Marastoni and coworkers[22] argued that the tripeptide vinyl ester derivatives also had the trypsin-like (β2) inhibitory activity. After modifications, Hmb-Val-Gln-Leu-VE (Hmb: 3-hydroxy-2-methylbenzoyl) was found to be the most potent compound toward the β2 subunit, and was about 500-fold less selective for the β5 subunit.

Peptides containing a Michael acceptor are known irreversible cysteine protease inhibitors.[23] They have already been proven to inhibit proteasome by forming a Michael adduct with the catalytic Thr1 of proteasome (Fig. 1) in an irreversible manner. We adopted the CADD methodology (SYBYL 7.1, DOCK 4.0 and Pymol 0.98) to design new peptidyl vinyl ester derivatives, hopefully with enhanced β5 subunit inhibitory potency. All binding modes are represented via Pymol 0.98.

First, we used *Z*-Leu-Leu-Leu-VE (designated as **M1**) as a model compound to investigate the binding mode.

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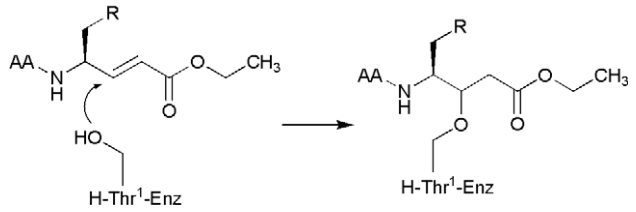



**Figure 1.** Vinyl ester inhibitors: potential interactions with proteasome 20S catalytic subunits.

Two major binding modes were observed (*mode a* and *mode b*). *Mode a* was the conventional binding mode (**M1** had two hydrogen bonds with Thr1 and Ser129, and the distance between the catalytic Thr1 and its reaction site was 5.24 Å, Fig. 2A), while *mode b* was a reversed one in which the P1 side chain and the C-terminal exchanged their pockets (**M1** had two hydrogen bonds with Thr1 and Asp125, and the distance was 4.58 Å, Fig. 2B), and this special binding mode was also seen in non-covalent inhibitors.[24] The binding energy of *mode a* was lower than that of *mode b*, suggesting its predominance in binding. Then, three larger groups, benzyl group (**C1**), 4-hydroxyl benzyl group (**C2**) and (3*S*)-2-oxo-3-pyrrolidinyl-methyl group (**C3**), were designed to replace the isobutyl group at the P1 position (Fig. 3), and another three model compounds were used in binding, *Z*-Leu-Leu-Phe-VE (**M2** with C1 group), *Z*-Leu-Leu-Tyr-VE (**M3** with C2 group) and *Z*-Leu-Leu-MK-VE (**M4** with C3 group) (the order of the size of C-terminal groups is: isobutyl group < C3 < C1 < C2). Interestingly, for **M4** and **M2** (with more bulky C-terminal groups), the conventional binding modes were observed (**M4** had two hydrogen bonds with Thr21 and

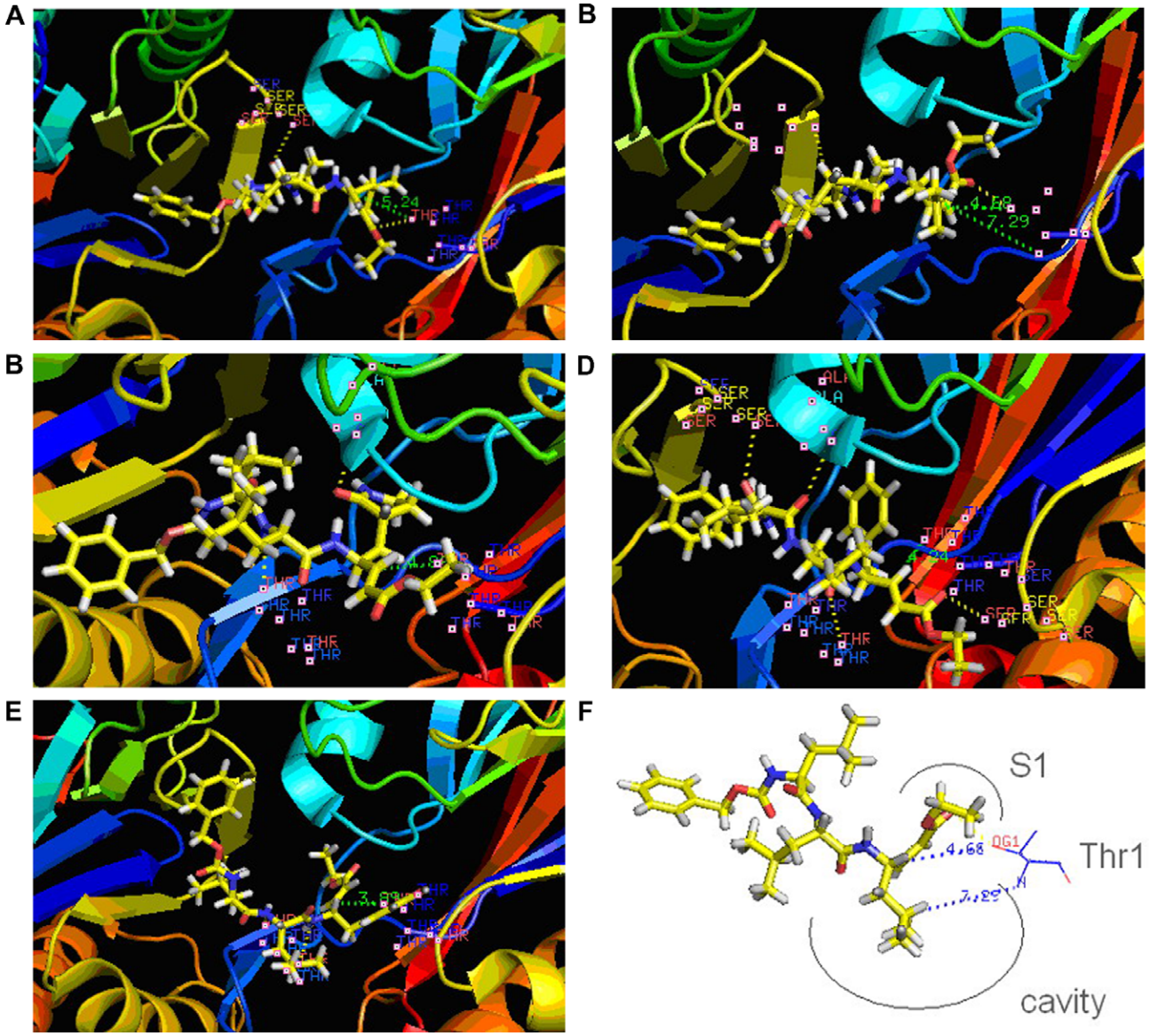



**Figure 2.** Binding modes of **M1** to **M4** docked into the β5 and β6 subunits of the bovine proteasome structure 1IRU using the AMBER_Parm99 force field in DOCK (UCSF, San Francisco, CA) as rendered in PyMOL (DeLano Scientific, South San Francisco, CA). (A) Binding mode of **M1**, *mode a*; (B) Binding mode of **M1**, *mode b*; (C) Binding mode of **M4**; (D) Binding mode of **M2**; (E) Binding mode of **M3**; (F) S1 pocket is composed by Met45, Gly47 and Ala49, and the adjacent cavity is composed by Thr1, Ala20 and Thr21.

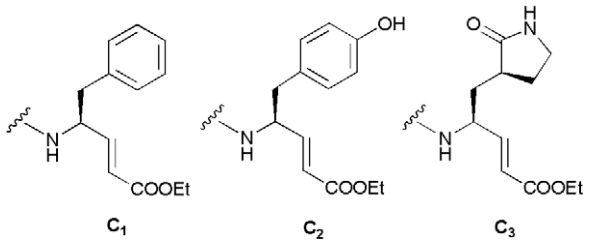



**Figure 3.** Newly designed C-terminus.

Ala49, and the distance was 4.96 Å, while **M2** had four hydrogen bonds with Thr21, Ala49, Ser129 and Ser130, respectively, and the distance was 4.24 Å, Fig. 2C and D); however, in case of **M3** (with the largest C-terminal group), the reversed mode was the only observation (**M3** had two hydrogen bonds with Thr21, and the distance was 3.89 Å, Fig. 2E).

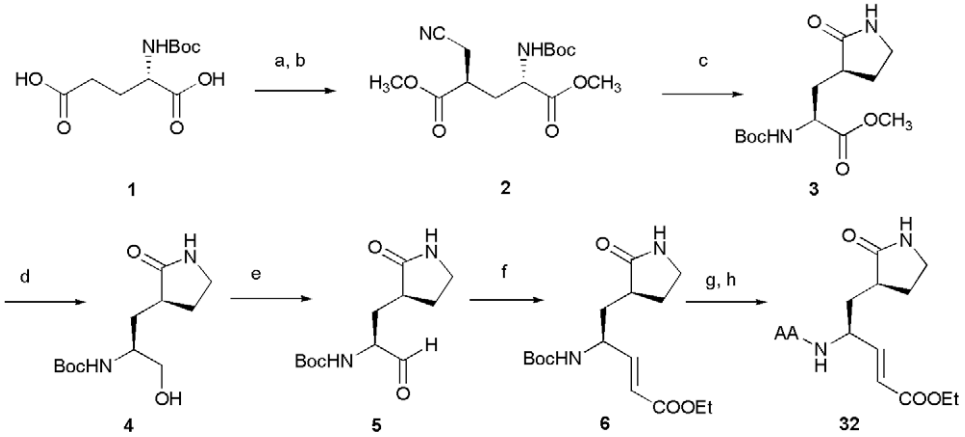
It was clear that S1 pocket is smaller than the cavity next to it (Fig. 2F), and when P1 side chain became larger, the reversed binding mode would be favored. In order to study the potency and selectivity of this special binding mode toward β5 subunit, a series of compounds with these C-terminus were synthesized and their inhibitory activities were compared (**C1** and **C3** represented the conventional binding mode while **C2** represented the reversed one).

Herein, 30 peptidyl vinyl ester derivatives with three different P1 side chains (Fig. 3) were synthesized according to standard solution-phase peptide chemistry. All amino acids used are of L configuration. Compound **32** was synthesized as an example shown in Scheme 1. Boc-protected Glutamic acid **1** was treated with $CH_3I$ under $NaHCO_3$ and DMF to give the dimethyl ester,[25] which was then reacted with $BrCH_2CN$ in the presence of lithium hexamethyldisilazide (LiHMDS) at −78 °C to afford the acetonitrile-substituted product **2** in a very high yield (crude yield for the first two steps is over 96%).[26] Compound **2** was then reduced by $NaBH_4$ and $CoCl_2{\cdot}6H_2O$ in methanol to give the cyclic product **3**,[27] followed by reduction with $NaBH_4$ and $CaCl_2$ in ethanol and THF (1:0.6) to give **4**.[25] One-pot reaction integrating oxidation of **4** by $Py{\cdot}SO_3$ to aldehyde **5** and subsequent reaction with the ylide $Ph_3P{=}CHCO_2Et$ afforded the vinyl ester **6** (yield for this one-pot reaction is about 76%).[26] At last, **6** was deprotected with 50% $TFA/CH_2Cl_2$ and then coupled with different amino acids, dipeptides and tripeptides under typical peptide coupling conditions using HOBt and EDC to obtain the final products.

The different peptidase activities of the proteasome were assayed using the following fluorogenic peptides: Suc-Leu-leu-Val-Tyr-AMC for chrymotryptic-like (ChT-L) activity; *Z*-Ala-Arg-Arg-AMC for trypsin-like (T-L) activity and *Z*-Leu-Leu-Glu-βNA for post-acidic-like (PGPH) activity (Suc for succinyl and AMC for 7-amido-4-methylcoumarin).

Cell growth inhibition effects of final products on four kinds of human cancer cell lines (including hepatoma cell line (Bel-7402), myeloid leukemic cell line (HL-60), gastric cancer cell line (BGC-823) and nasopharyngeal cancer cell line (KB)) were tested through a standard MTT or SRB-based colorimetric assay.

Structures of the final products and their inhibitory activities are listed in Table 1. The data revealed that tetrapeptides with **C3** terminus had better proteasome inhibitory activities: two compounds (**32** and **35**) had the best activities ($IC_{50}$ values were 48.31 and 63.41 μm toward the β5 subunit, respectively), but they showed poor inhibitory activities in the T-L active site. For cell proliferative activity, smaller inhibitors exhibited better effects: dipeptides had a mean antiproliferative rate of more than 20% toward HL-60, while tripeptides and tetrapeptides were about 10%, but one tripeptide, compound **27**, was an exception. By comparing the three terminus (compounds **17**–**31**), **C3** had the best activity (compounds **27** and **28** had antiproliferative rates of 36.97% and 31.04%), **C2** had a moderate activity (compounds **24** and **26** had antiproliferative rates of 26.73% and 26.50%), and **C1** had the lowest




**Scheme 1.** Reagents and conditions: (a) $NaHCO_3$, $CH_3I$, DMF; (b) $BrCH_2CN$, LiHMDS, −78 °C, 3–5 h; (c) $NaBH_4$, $CoCl_2{\cdot}6H_2O$, in methanol; (d) $NaBH_4$, $CaCl_2$ in ethanol and THF; (e) $Py{\cdot}SO_3$, TEA, DCM/DMSO; (f) $Ph_3P{=}CHCO_2Et$; (g) TFA, $CH_2Cl_2$; (h) Boc-Leu-Leu-Leu-OH, EDC, HOBt, NMM , DMF.

**Table 1.** Structure, proteasome inhibitory activity and antiproliferative rate of the peptidyl vinyl ester derivatives

| No. | Compound | Proteasome inhibitory activity ($IC_{50}$, μm) | | | Antiproliferative rate (% at 10 μm) | | | |
|---|---|---|---|---|---|---|---|---|
| | | ChT-L | T-L | PGPH | HL-60[a] | BGC-823[b] | Bel-7402[b] | KB[b] |
| **7** | Boc-Ile-C1 | >100 | >100 | >100 | 29.56 | 24.14 | −7.74 | −0.18 |
| **8** | Boc-Leu-C1 | >100 | >100 | >100 | 33.88 | 30.21 | 1.55 | −6.67 |
| **9** | Boc-Phe-C1 | >100 | >100 | >100 | 25.73 | 20.90 | −3.61 | −4.66 |
| **10** | Boc-Val-C1 | >100 | >100 | >100 | 27.13 | 21.27 | −3.57 | −1.97 |
| **11** | Boc-Ser-C1 | >100 | >100 | >100 | 19.98 | 19.11 | −3.14 | −11.24 |
| **12** | Boc-Ile-C2 | >100 | >100 | >100 | 21.66 | 23.06 | 3.32 | 5.27 |
| **13** | Boc-Leu-C2 | >100 | >100 | >100 | 5.87 | 17.01 | −10.27 | 1.72 |
| **14** | Boc-Phe-C2 | >100 | >100 | >100 | 23.66 | 33.88 | 12.68 | −0.70 |
| **15** | Boc-Val-C2 | >100 | >100 | >100 | 22.10 | 18.44 | 4.60 | 1.76 |
| **16** | Boc-Ser-C2 | >100 | >100 | >100 | 19.26 | 22.56 | −5.91 | −15.49 |
| **17** | Boc-Leu-Leu-C1 | >100 | >100 | >100 | 6.13 | 9.95 | −12.87 | −1.67 |
| **18** | Boc-Phe-Ser-C1 | >100 | >100 | >100 | 21.08 | 8.28 | −8.04 | 5.23 |
| **19** | Boc-Val-Ser-C1 | >100 | >100 | >100 | 0.36 | 0.04 | −11.09 | −6.79 |
| **20** | Boc-Leu-Phe-C1 | >100 | >100 | >100 | 8.65 | 13.32 | −4.56 | 4.33 |
| **21** | Boc-Ile-Thr-C1 | >100 | >100 | >100 | 8.17 | 13.62 | −1.79 | 10.75 |
| **22** | Boc-Leu-Leu-C2 | >100 | >100 | >100 | −2.64 | 0.02 | −6.95 | −5.99 |
| **23** | Boc-Phe-Ser-C2 | >100 | >100 | >100 | 13.37 | 10.28 | 3.39 | 7.44 |
| **24** | Boc-Val-Ser-C2 | >100 | >100 | >100 | 26.73 | 22.59 | 3.39 | 13.09 |
| **25** | Boc-Leu-Phe-C2 | >100 | >100 | >100 | 7.87 | 13.74 | −4.24 | −5.34 |
| **26** | Boc-Ile-Thr-C2 | >100 | >100 | >100 | 26.50 | 14.69 | 3.10 | 3.76 |
| **27** | Boc-Leu-Leu-C3 | >100 | >100 | >100 | 36.97 | 21.44 | −2.40 | −14.03 |
| **28** | Boc-Phe-Ser-C3 | >100 | >100 | >100 | 31.04 | 16.90 | 1.66 | −15.60 |
| **29** | Boc-Val-Ser-C3 | >100 | >100 | >100 | −1.94 | 5.52 | −4.98 | −3.45 |
| **30** | Boc-Leu-Phe-C3 | >100 | >100 | >100 | −7.61 | 13.89 | −1.63 | −1.56 |
| **31** | Boc-Ile-Thr-C3 | >100 | >100 | >100 | 0.71 | 0.69 | −16.67 | −10.29 |
| **32** | Boc-Leu-Leu-Leu-C3 | 48.31 | >100 | >100 | 17.17 | 16.14 | 12.02 | 15.73 |
| **33** | Boc-Phe-Ser-Leu-C3 | >100 | >100 | >100 | 4.94 | 14.25 | −0.39 | −4.17 |
| **34** | Boc-Val-Ser-Leu-C3 | >100 | >100 | >100 | −47.14 | 20.55 | −3.55 | 9.15 |
| **35** | Boc-Leu-Leu-Tyr-C3 | 63.41 | >100 | >100 | 17.73 | 28.90 | 7.64 | 6.64 |
| **36** | Boc-Ile-Thr-Leu-C3 | >100 | >100 | >100 | −2.32 | 19.98 | −0.31 | 4.30 |

activity (only compound **18** had an antiproliferative rate of 21.08%, whereas others were less than 9%) toward HL-60. Among the four human cancer cell lines, these inhibitors had better antiproliferative potencies toward HL-60 (the highest antiproliferative rate was 36.97%, compound **27**) and BGC-823 (the highest antiproliferative rate was 33.88%, compound **14**), but weaker effects toward Bel-7402 (the highest antiproliferative rate was 12.68%, compound **14**) and KB (the highest antiproliferative rate was 15.73%, compound **32**). Within these inhibitors, compounds **8**, **14**, **27** and **28** showed weak antiproliferative activities (antiproliferative rates were more than 30%) toward human cancer cell lines (HL-60 and BGC-823).

In summary, a virtual binding model of peptidyl vinyl ester derivatives with the β5 catalytic center of proteasome was proposed for the first time in this work. Based on this model, a series of potential proteasome inhibitors bearing vinyl ester as the C-terminal pharmacophore were designed and synthesized. Covalent Michael adducts can be formed between inhibitors and the catalytic Thr1, which might cause the inhibition of proteasome. The proteasome inhibitory and antiproliferative activities of these compounds toward four human cancer cell lines were tested. Tetrapeptides showed better proteasome inhibitory activities, while dipeptides showed better antiproliferative activities. The fact is that when the length of the peptide increased, more interactions could be expected between the inhibitor and the enzyme, which would lead to a better proteasome inhibitory activity, however, at the same time, membrane permeability decreased dramatically which might account for the relatively poor cell antiproliferative activity. In addition, low water solubility is another problem. Therefore, modifications of the N-terminal to increase the water medium solubility and replacement of the peptides with peptidomimetic structures to improve the membrane permeability are now in progress and will be reported in due course.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.12.077.

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