Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 1849–1854
Bioorganic & Medicinal Chemistry Letters

Vinyl ester-based cyclic peptide proteasome inhibitors

Anna Baldisserotto,[a] Mauro Marastoni,[a,*] Stella Fiorini,[a] Loretta Pretto,[b] Valeria Ferretti,[b] Riccardo Gavioli[c] and Roberto Tomatis[a]

[a]*Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, Via Fossato di Mortara 17-19, I-44100 Ferrara, Italy*
[b]*Chemistry Department and Center for Structural Diffractometry, University of Ferrara, I-44100 Ferrara, Italy*
[c]*Department of Biochemistry and Molecular Biology, University of Ferrara, I-44100 Ferrara, Italy*

Received 7 January 2008; revised 6 February 2008; accepted 7 February 2008
Available online 10 February 2008

Abstract—The 20S proteasome is a multicatalytic protease complex responsible for the degradation of many proteins in mammalian cells. Specific inhibition of proteasome enzymatic subunits represents a topic of great interest for the development of new drug therapies. Following our previous development of a new class of peptide-based inhibitors bearing a C-terminal vinyl ester residue as a pharmacophoric unit that are able to interact with the catalytic threonine, we report here the synthesis and biological properties of a new series of vinyl ester cyclopeptide analogues. Some of these derivatives were shown to inhibit the chymotrypsin-like activity of the proteasome at nanomolar concentration and their potency was found to depend on the size of the tetrapeptidic cyclic portion.

The proteasome, a multicatalytic protease complex,[1] is the essential component of the nonlysosomal protein degradation pathway in prokaryotes and eukaryotes. It is involved in many biological processes, including the removal of abnormal, misfolded or improperly assembled proteins, the stress response, and cell cycle control and differentiation, as well as metabolic adaptation and the generation of peptide antigens for presentation to $CD8^+$ cytotoxic T cells by major histocompatibility complex (MHC) class I molecules. These cellular functions are linked to an ubiquitin- and ATP-dependent protein degradation pathway involving the 26S proteasome (2.4 MDa), whose core and proteolytic chamber are formed by the 20S proteasome.[2]

The 20S proteasome consists of four stacked rings and each of the two inner rings is composed of seven different β subunits. Each β-ring contains three different proteolytically active sites: the β1 subunit contains a post-acidic (PGPH) active site, the β2 subunit is associated with trypsin-like (T-L) activity and the β5 subunit performs a chymotrypsin-like (ChT-L) proteolytic function. All its proteolytic sites utilize an N-terminal threonine residue of β subunits as nucleophile, via a catalytic mechanism similar to those of the serine proteases.[3]

The regulation of proteasomal activities by inhibition or modulation of its component molecules has considerable biological significance. Indeed, the development of specific and selective multicatalytic complex inhibitors into novel therapeutic agents represents a stimulating approach in the treatment of many disease states, including inflammation and cancer, as well as in the modification of immune responses. Thus, a variety of natural and synthetic products have been tested as inhibitors of the different proteasomal enzymatic subunits.[4]

In our earlier studies we synthesized and tested a series of peptide-based derivatives where vinyl ester moiety was considered as a potential substrate for catalytic threonine. The most efficacious compounds, with the general structure Hmb-Xaa-Xbb-Leu-VE, showed good inhibition, remarkable selectivity for the trypsin-like activity of the 20S proteasome and favorable pharmacokinetic properties.[5]

Several of these vinyl ester tripeptides were subjected to conformational studies in order to define the inhibitor–enzyme interaction subsite pocket, and through flexible alignment, our selected vinyl ester compounds demonstrated conformational similarities with the TMC95A

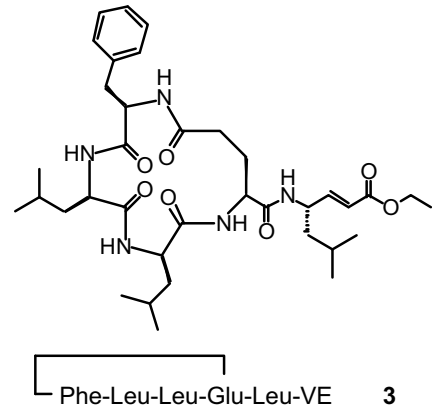
Keywords: Inhibitors; Proteasome; Chymotrypsin-like activity; Cyclic peptides.
* Corresponding author. Tel.: +39 532 291281; fax +39 532 291296; e-mail: mru@dns.unife.it

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.02.016

cyclic inhibitor.[6] As the availability of active inhibitors with a rigid conformation could provide much structural information, and could also indicate a target investigation into potency and selectivity,[7] we set out to develop molecules with a constrained conformation in the C-terminal of the vinyl ester pharmacophore.[8]

Here, we describe the synthesis and biological activity of cyclopeptides derived from our linear prototype inhibitors (Fig. 1). In this series, the endocyclic part of the molecules included the tetrapeptidic sequence Phe-AA_1-AA_2-Glu (or Asp); in derivatives **1**–**4**, the central dipeptide was Leu-Leu, while the more hydrophilic Val-Ser sequence was contained in the same position in analogues **5**–**8**. Cyclization was obtained acylating the N-terminal by the side chain carboxylic function of the acidic residue (**1**, **3**, **5**, and **7**) or head-to-tail in compounds **2**, **4**, **6**, and **8**. The exocyclic pharmacophoric unit Leu-VE was linked in the side chain or at the α-carboxy group of the acidic residue.

Vinyl ester cyclopeptides **1**–**8** were synthesized following the strategy reported in Scheme 1 by the classical solution method using C-terminal stepwise elongation. Starting from an acidic residue, monoprotected in the side chain or at the α-carboxy function, other N_α-Boc-protected amino acids of the sequence were condensed as succinimidyl esters. After each coupling step, Boc was removed by TFA. Cyclization of the linear tetrapeptides was obtained using the activating reagent DPPA (diphenylphosphorylazide) under conditions known to incur minor racemization and minimal oligomerization.[9] After saponification of the cyclic tetrapeptides the exocyclic leucine vinyl ester unit, prepared as described previously,[5a] was coupled using WSC/HOBt.



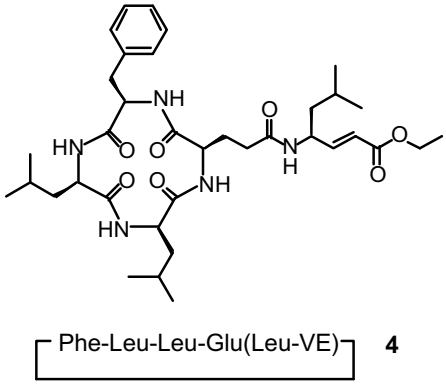


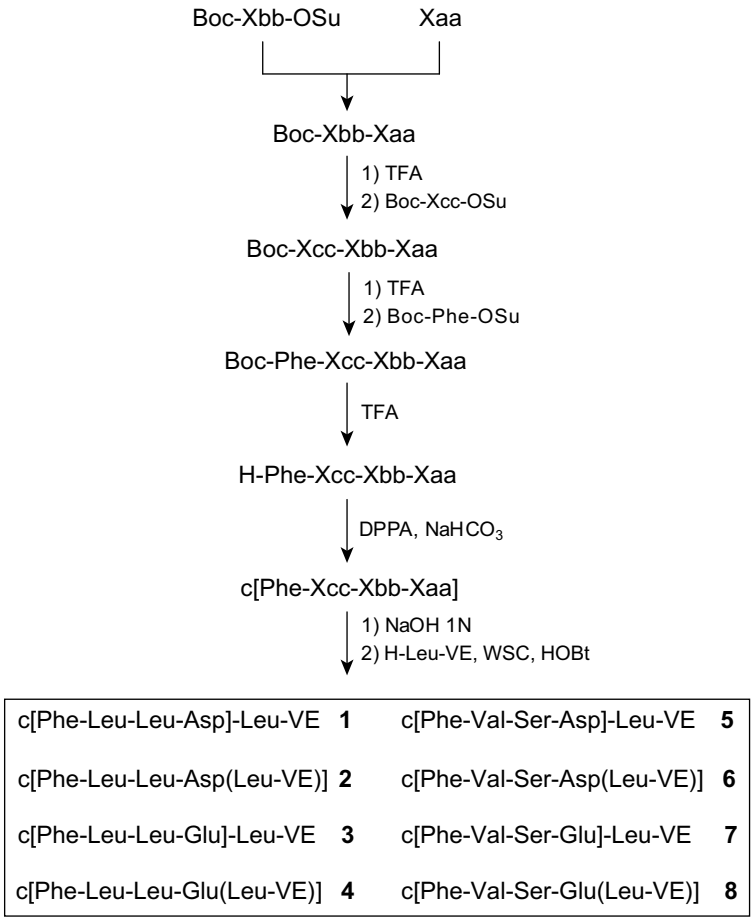
Figure 1.

All products were purified by preparative RP-HPLC, and the homogeneity of the purified products was accessed by HPLC. Structural characterization was then achieved by electrospray ionization (ESI) mass spectrometry (MICROMASS ZMD 2000) (Table 1) and ^{1}H NMR spectroscopy (Bruker AC 200).[10]

The activity of the vinyl ester cyclopeptides was tested to assess inhibition of the β1, β2, and β5 active sites of the 20S proteasome, previously purified from lymphoblastoid cell lines.[11,12] Suc-LLVY-AMC, Boc-LRR-AMC, and Ac-YVDA-AMC, specific fluorogenic substrates for the three main proteolytic activities of the enzymatic complex, were used to measure chymotrypsin-like, trypsin-like, and caspase-like proteasome activities, respectively. Substrates were incubated at 37 °C for 30 min with the proteasome, untreated or pre-treated with incremented concentrations (from 0.001 to 10 μM) of vinyl ester cyclopeptides with the reference inhibitors epoxomicin and the aldehydic tripeptide derivative MG132 in activity buffer. Fluorescence was determined in a fluorimeter (Spectrafluor plus, Tecan, Salzburg, Austria) using an excitation of 360 nm and an emission of 465 nm. Activity was evaluated in fluorescence units, and the inhibitory activity of the compounds is expressed here as IC_{50}. The data were then plotted as percentage control (the ratio of percentage conversion in the presence and absence of the inhibitor) versus the inhibitor concentration, and fitted with the equation $Y = 100/1 + (X/IC_{50})^A$, where IC_{50} is the inhibitor concentration at 50% inhibition and A is the slope of the inhibition curve.[13]

In general, the new cyclic analogues reversed the selectivity against the β2 and β5 proteasome catalytic subsites, as compared to the vinyl ester linear prototypes. Head-to-tail cyclized derivatives showed improved inhibitory capacity of the chymotryptic-like activity; in particular compounds **2** and **4** selectively inhibited the β5 subunit at a nM concentration. In our experiments, ChT-L inhibition seemed to depend on the cyclic dimensions; indeed, increasing the ring structure from 12 to 14 atoms, noticeably diminished activity. A more hydrophobic endocyclic tetrapeptidic sequence favoured β5 interaction, whereas the endocyclic dipeptidic sequence Val-Ser in derivatives **5**–**8** slightly improved inhibition of the proteasome's trypsin-like activity. The inhibitory capacity of the cyclopeptides against the β2 subunit was less influenced by ring size, and in the corresponding linear reference, the endocyclic hydrophilic sequence was shown to be more functional for inhibitory activity. Finally, all compounds were shown to be inactive against the 20S proteasome β1 catalytic subsite.

The cell membrane permeation of the most representative compounds, **2**, **4**, and **6**, was then tested in live cells. After cell treatment, proteasomes were purified and as-



Scheme 1. Synthesis of vinyl ester cyclopeptide derivatives **1**–**8**.

Table 1. Physicochemical data of vinyl ester cyclopeptides

Compound	HPLC[a]		P.f. (°C)	$[\alpha]_D^{20}$	MS
	K^I (a)	K^I (b)		(C = 1, MeOH)	$[M+H]^+$
1	11.71	9.90	220–223	−50.5	655.8
2	12.72	10.43	234–239	−43.7	655.7
3	11.85	9.98	198–201	−39.8	669.5
4	12.81	10.75	187–191	−42.1	669.6
5	9.60	8.38	108–112	−29.3	615.2
6	10.15	9.06	134–136	−30.9	615.2
7	9.74	8.67	123–126	−22.4	629.3
8	10.68	9.51	143–145	−25.6	629.3

[a] Capacity factor (K^I) of the cyclic peptides was determined by HPLC using two different solvent system gradients.

sayed for proteolytic activity using specific substrate for T-L, ChT-L, and PGPH activities as previously described.[13] The results obtained (Table 2) were comparable to those observed in the in vitro assay, thereby demonstrating that vinyl ester cyclopeptides are cell-permeable and able to inhibit the proteasome in vivo.

Resistance to proteolysis of the selected cyclic derivatives was studied in heparinized human plasma. The degradation kinetics were determined by incubation at 37 °C in human plasma for increments of time up to 360 min and the incubation was halted by addition of ethanol. After centrifugation, an aliquot of the clear supernatant was analyzed in HPLC.[13] The half-lives of the cyclopeptides were obtained by least-squares linear regression analysis of a plot of logarithmic compound concentrations versus time, using a minimum of five points. The data reported in Table 2, as expected on the basis of molecular features determined by the cyclization, show that all derivatives analyzed have great stability against human plasma protease with a half-live of over 6 h.

In order to obtain a deeper insight into the molecular basis of the enzyme–inhibitor recognition process, the molecule exhibiting the highest affinity, **4**, was chosen to perform a docking simulation to the 20S proteasome.[14] In Figure 2, a schematic view of the ligand-enzyme is shown. The binding pocket is delimited, besides some water molecules, by several residues, most of which corresponded to those found in the β5 active site of the proteasome-calpain crystallographic structure retrieved from PDB.[15] Only a small part of the inhibitor (shaded in blue in the figure) is placed towards the inner proteasome channel. The most remarkable result is that the vinyl ester moiety of the molecule was found to be located some 3–4 Å away from the Thr1 residue, in

Table 2. Proteasome inhibition and enzymatic stability of cyclopeptides **1**–**8** and reference inhibitors epoxomicin and MG132

No.	Compound	Isolated enzyme IC_{50}[a] (μM)			In vivo inhibition IC_{50}[a] (μM)			Half-life (min) plasma
		ChT-L	T-L	PGPH	ChT-L	T-L	PGPH	
1	c[Phe-Leu-Leu-Asp]-Leu-VE	0.206	0.447	>10				
2	c[Phe-Leu-Leu-Asp(Leu-VE)]	0.063	0.389	>10	0.081	0.436	>10	>360
3	c[Phe-Leu-Leu-Glu]-Leu-VE	0.310	0.424	>10				
4	c[Phe-Leu-Leu-Glu(Leu-VE)]	0.039	0.286	>10	0.052	0.473	>10	>360
5	c[Phe-Val-Ser-Asp]-Leu-VE	0.315	0.134	>10				
6	c[Phe-Val-Ser-Asp(Leu-VE)]	0.097	0.175	>10	0.110	0.197	>10	>360
7	c[Phe-Val-Ser-Glu]-Leu-VE	0.354	0.129	>10				
8	c[Phe-Val-Ser-Glu(Leu-VE)]	0.123	0.155	>10				
	Epoxomicin	0.005	0.284	4.560				
	MG132	0.002	1.077	>10				

[a] The values reported are the average of two independent determinations.

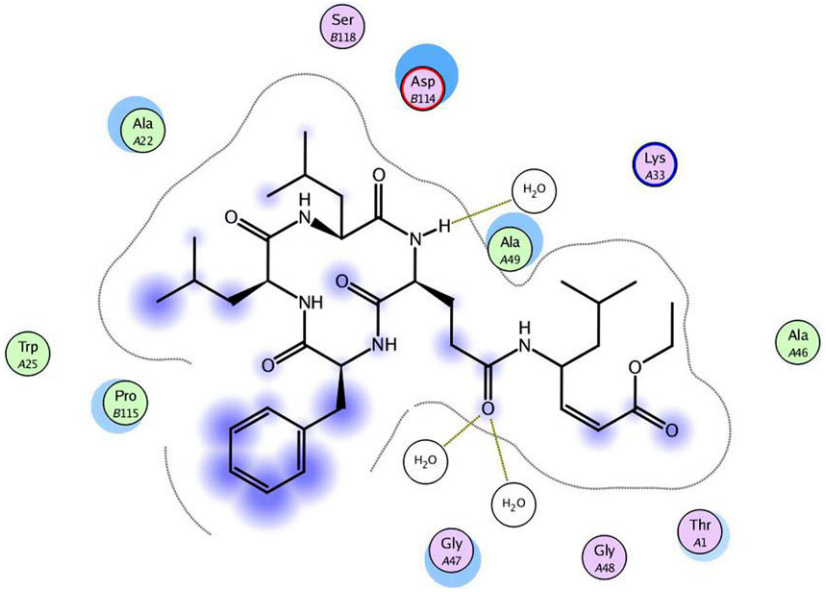


Figure 2.

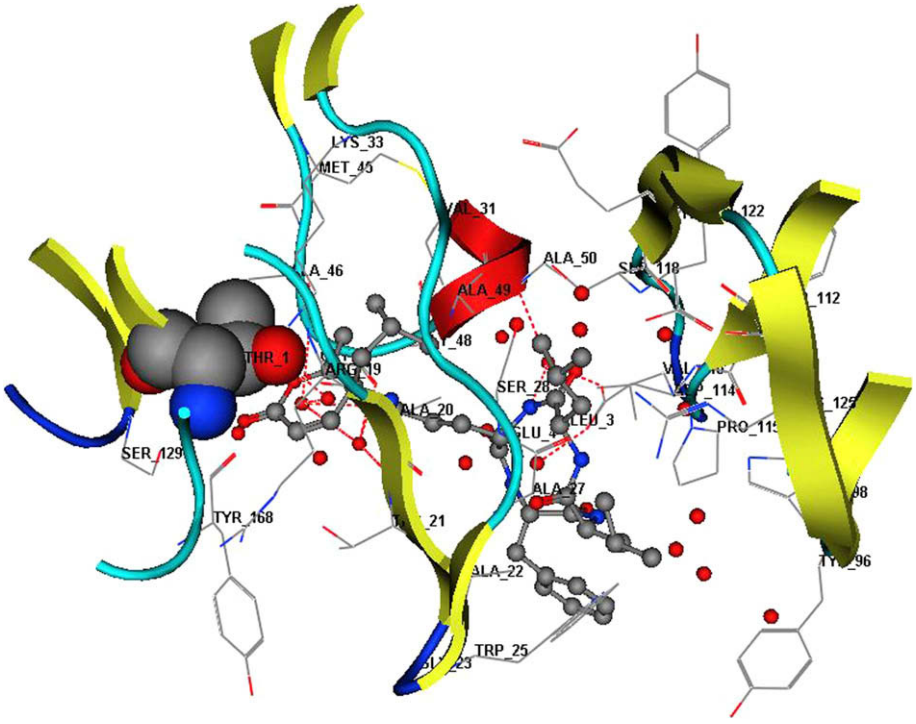


Figure 3.

accordance with the hypothesis that this molecular fragment can be considered the potential substrate for catalytic threonine. In Figure 3, the docked ligand is depicted in the active site.

In conclusion, we have presented here a new series of vinyl ester proteasome inhibitors. Cyclization of the linear prototype, achieved by insertion of an acidic residue into the sequence, changes the activity profile. In the resulting cyclic derivatives, the conformational restriction permits a better interaction with the β5 enzymatic subsite. The inhibition of proteasome ChT-L activity was also found to depend on the ring size. In some cases, head-to-tail cyclization, led to cyclopeptides with a potency in a nanomolar concentration.

With the aim of designing rigid molecules useful for close structural studies, we have obtained new inhibitors of the multicatalytic complex, selective for chymotrypsin-like activity and stable to enzymatic degradation, able to permeate cell membranes. These vinyl ester cyclopeptides represent an interesting springboard for the future development of new rigid structures with a better biological profile.

Acknowledgments

Financial support of this work by University of Ferrara, by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), the Associazione Italiana per la Ricerca sul Cancro (AIRC), and the Istituto Superiore di Sanità (progetto AIDS). English revision of the text was carried out by Anna Forster.

References and notes

1. (a) Ciechanover, A. *Cell* **1994**, *79*, 13; (b) Coux, O.; Tanaka, K.; Golberg, A. L. *Annu. Rev. Biochem.* **1996**, *65*, 801; (c) Hershko, A.; Ciechanover, A. *Annu. Rev. Biochem.* **1998**, *67*, 425.
2. (a) Coux, O.; Tanaka, K.; Golberg, A. L. *Annu. Rev. Biochem.* **1996**, *65*, 801; (b) Hershko, A.; Ciechanover, A. *Annu. Rev. Biochem.* **1998**, *67*, 425; (c) Orlowski, R. Z. *Cell Death Differ.* **1999**, *6*, 303, *1695*, 19; (d) Kisselev, A. F.; Songyang, Z.; Goldberg, A. L. *J. Biol. Chem.* **2000**, *275*, 14831; (e) Myung, J.; Kim, K. B.; Crews, C. M. *Med. Res. Rev.* **2001**, *21*, 245; (f) Boyo, M.; Wang, E. W. *Curr. Top. Microbiol. Immunol.* **2002**; (g) Adams, J.; Kauffman, M. *Cancer Invest.* **2004**, *22*, 304.
3. (a) Löwe, J.; Stock, D.; Zwickl, P.; Baumeister, W.; Huber, H. *Science* **1995**, *268*, 533; (b) Groll, M.; Ditzel, L.; Löwe, J.; Stock, D.; Bochtler, M.; Bartunik, H. D.; Huber, R. *Nature* **1997**, *386*, 463.
4. (a) Iqbal, M.; Chatterjee, S.; Kauer, J. C.; Das, M.; Messina, P. A.; Freed, B.; Biazzo, W.; Siman, R. *J. Med. Chem* **1995**, *38*, 2276; (b) Loidl, G.; Groll, M.; Musiol, H.-J.; Huber, R.; Moroder, L. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 5418; (c) Kisselev, A. F.; Goldberg, A. L. *Chem. Biol.* **2001**, *8*, 739; (d) Nazif, T.; Bogyo, M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2967; (e) Furet, P.; Imbach, P.; Noorani, M.; Koeppler, J.; Lumen, K.; Lang, M.; Guaniano, V.; Fuerst, P.; Roesel, J.; Zimmermann, J.; Garcia-Echevevarria, C. *J. Med. Chem.* **2004**, *47*, 4810; (f) Momose, I.; Umezawa, Y.; Hirosawa, S.; Iinuma, H.; Ikeda, D. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1867; (g) Vivier, M.; Jarrousse, A. S.; Bouchon, B.; Galmier, M. J.; Auzeloux, P.; Sauzieres, J.; Madelmont, J. C. *J. Med. Chem.* **2005**, *48*, 6731.
5. (a) Marastoni, M.; Baldisserotto, A.; Cellini, S.; Gavioli, R.; Tomatis, R. *J. Med. Chem.* **2005**, *48*, 5038; (b) Marastoni, M.; Baldisserotto, A.; Trapella, C.; Gavioli, R.; Tomatis, R. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3125; (c) Marastoni, M.; Baldisserotto, A.; Trapella, C.; Gavioli, R.; Tomatis, R. *Eur. J. Med. Chem.* **2006**, *41*, 978.
6. Baldisserotto, A.; Marastoni, M.; Trapella, C.; Gavioli, R.; Ferretti, V.; Pretto, L.; Tomatis, R. *Eur. J. Med. Chem.* **2007**, *42*, 586.
7. Borissenko, L.; Groll, M. *Chem. Rev.* **2007**, *107*, 687.
8. Baldisserotto, A.; Marastoni, M.; Lazzari, I.; Trapella, C.; Gavioli, R.; Tomatis, R. *Eur. J. Med. Chem.* **2007**, in press.
9. (a) Shiori, T.; Ninomiya, K.; Yamada, S. *J. Am. Chem. Soc.* **1972**, *94*, 6203; (b) Brady, S. F.; Freidinger, R. M.; Colton, C. D.; Homnick, C. F.; Whitter, W. L.; Curley, P.; Nutt, R. F.; Veber, D. F. J. *J. Org. Chem.* **1987**, *52*, 764.
10. HPLC analysis was performed by a Beckman System Gold with a Hypersil BDS C18 column (5 μm; 4.6 × 250 mm). Analytical determination and capacity factor (K') of the vinyl ester cyclopeptides were determined using HPLC conditions in the above solvent system (solvents A and B) programmed at flow rates of 1 mL/min using the following linear gradients: (a) from 0% to 100% B for 25 min and (b) from 30% to 90% B for 25 min. c[Phe-Leu-Leu-Glu(Leu-VE)] (**4**). Purified yield 36%; purity estimated by HPLC >98%; ^{1}H NMR ($CDCl_3$, 200 MHz): δ (ppm) 1.01 (d, 6H), 1.09 (m, 12H), 1.31 (t, 3H, J = 7.2), 1.44 (m, 2H), 1.70–1.81 (m, 7H), 2.09 (m, 2H), 2.21 (d, 2H), 2.98 (m, 2H), 4.13 (q, 2H, J = 7.3), 4.27–4.51 (m, 5H), 5.91 (d, 1H, J = 16.0), 6.98 (dd, 1H, J = 16.1), 7.19–7.33 (m, 5H), 8.05 (br s, 5H). c[Phe-Val-Ser-Asp]-Leu-VE (**5**). Purified yield 31%; purity estimated by HPLC >98%; ^{1}H NMR ($CDCl_3$, 200 MHz): δ (ppm) 0.99 (d, 6H), 1.13 (d, 6H), 1.29 (t, 3H, J = 7.3), 1.52 (m, 2H), 1.80 (m, 1H), 2.11 (s, 1H), 2.58–2.87 (m, 5H), 4.03 (m, 2H), 4.18 (q, 2H, J = 7.1), 4.32–4.60 (m, 5H), 5.93 (d, 1H, J = 16.2), 7.02 (dd, 1H, J = 16.1), 7.12–7.31 (m, 5H), 8.02 (br s, 5H).
11. Gavioli, R.; Vertuani, S.; Masucci, M. G. *Int. J. Cancer* **2002**, *101*, 532.
12. Hendil, K. B.; Uerkvitz, W. *J. Biochem. Biophys. Methods* **1991**, *22*, 159.
13. Purification of the proteasomes and enzyme assays in vitro and in live cells are described in "supporting information" of Ref. 5a.
14. The equilibrium structure of the molecule **4** was obtained by molecular mechanics optimization (MMFF94 force field: Halgren, T. A. The merck force field. *J. Comp. Chem.* **1996**, *17*, 490–512, 520–552, 553–586, 587–615, 616–641). The molecule was docked to the crystal structure of the yeast 20S proteasome (Groll, M.; Koguchi, Y.; Huber, R.; Kohno, J. *J. Mol. Biol.* **2001**, *311*, 543), retrieved from the Protein Data Bank (PDB) archive (www.pdb.org), using the MOE/Dock procedure integrated in the MOE system of programs (Chemical Computing Group Inc., MOE 2006.08). Before the simulation, hydrogen atoms were added to the enzyme and the energy of the structure was minimized using the Amber99 molecular mechanics forcefield (Ponder, J. W.; Case, D. A. *Adv. Prot. Chem.* **2003**, *66*, 27). During the first step of the docking application, the ligand was treated in a flexible manner by rotating routable bonds and a number of the ligand's configurations were generated and

scored in an effort to determine favorable binding modes by the application of the Alpha Triangle placement method. The ligand was docked restricting the search for binding modes to a specific, small region of the subunit called the β5 binding site, in which the terminal Thr1 is the active residue. Out of 34 poses obtained, the one having the highest score based on the estimate of the free energy of binding of the ligand from a given pose was retained.

15. Groll, M.; Brandstetter, H.; Bartunik, H.; Bourenkow, G.; Huber, R. *J. Mol. Biol.* **2003**, *327*, 75.