Substrate binding and sequence preference of the proteasome revealed by active-site-directed affinity probes

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Background: The proteasome is a multicatalytic protease complex responsible for most cytosolic protein breakdown. The complex has several distinct proteolytic activities that are defined by the preference of each for the carboxy-terminal (P1) amino acid residue. Although mutational studies in yeast have begun to define substrate specificities of individual catalytically active β subunits, little is known about the principles that govern substrate hydrolysis by the proteasome.

Results: A series of tripeptide and tetrapeptide vinyl sulfones were used to study substrate binding and specificity of the proteasome. Removal of the aromatic amino-terminal cap of the potent tripeptide vinyl sulfone proteasome inhibitor 4-hydroxy-3-iodo-2-nitrophenyl-leucinyl-leucinyl-leucine vinyl sulfone resulted in the complete loss of binding and inhibition. Addition of a fourth amino acid (P4) to the tri-leucine core sequence fully restored inhibitory potency. ^{125}I-labeled peptide vinyl sulfones were also used to examine inhibitor binding and to determine the correlation of subunit modification with inhibition of peptidase activity. Changing the amino acid in the P4 position resulted in dramatically different profiles of β-subunit modification.

Conclusions: The P4 position, distal to the site of hydrolysis, is important in defining substrate processing by the proteasome. We observed direct correlations between subunit modification and inhibition of distinct proteolytic activities, allowing the assignment of activities to individual β subunits. The ability of tetrapeptides, but not tripeptide vinyl sulfones, to act as substrates for the proteasome suggests there could be a minimal length requirement for hydrolysis by the proteasome. These studies indicate that it is possible to generate inhibitors that are largely specific for individual β subunits of the proteasome by modulation of the P4 and carboxy-terminal vinyl sulfone moieties.

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Introduction

The proteasome is a large, barrel-shaped multisubunit protease involved in most cytosolic proteolysis. It is made up of four stacked rings, each composed of seven α- or β-type subunits. Some of the β subunits are catalytically active; the α subunits appear to have mainly a structural role [1]. The eukaryotic proteasome contains three active β subunits repeated twice in the complex, whereas archaebacteria and eubacteria have only a single catalytic β subunit. The proteasome can degrade a wide variety of substrates that differ in the amino acid sidechain found at the site of hydrolysis [2]. The multiple peptidase activities have different preferences for the sidechain at the site of hydrolysis, and have been named accordingly as the chymotrypsin-like, trypsin-like, post-glutamyl peptide hydrolyzing (PGPH) and branched-chain amino-acid peptidase (BrAAP) activities. Although mutational studies in yeast have linked specific peptidase activities to individual β subunits, a direct correlation in mammalian cells is lacking.

The proteasome employs a rather unusual catalytic mechanism and is a member of the family of enzymes known as amino-terminal hydrolases. This class of enzyme utilizes an amino-terminal amino acid as the catalytic residue. The proteasome's amino-terminal threonine hydroxyl sidechain is activated by a single water molecule involving a charge relay system with other important internal amino acids such as lysine [1,3]. As a threonine protease, the proteasome does not fall into a standard category with other proteolytic enzymes in terms of its reactivity towards inhibitors. The sensitivity of the threonine protease to serine- as well as cysteine-protease inhibitors complicated its initial characterization [2]. Quite surprising is the variety of carboxy-terminally modified peptides that are reactive with the proteasome's amino-terminal catalytic residue [4–9].

The ability to modulate the activity of the proteasome experimentally using small molecule inhibitors is central to understanding the mechanism of action of this enzyme
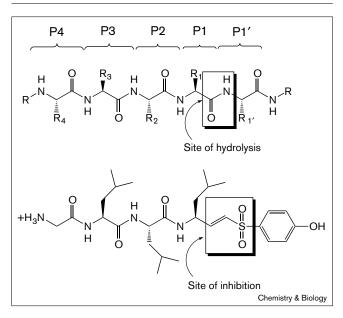
complex. The natural product lactacystin irreversibly inhibits the proteasome by covalent modification of the catalytic threonine hydroxyl group [10,11]. The lack of structural similarity between lactacystin and carboxy-terminally modified peptides suggests that these two classes of inhibitors may bind to the proteasome's active sites differently. This suggestion was verified with the determination of the crystal structure of the *Thermoplasma* 20S proteasome [12] and, more recently, the yeast 20S proteasome [13]. Crystals of proteasomes obtained in a complex with either the peptide aldehyde Cbz-leucinyl-leucinyl-norleucinal (LLnL or calpain I inhibitor) or lactacystin indicate that the two inhibitors do not interact with the proteasome equivalently. Unlike LLnL, which bound all three catalytically active β subunits (PUP1 or Z, PRE2 or X, and PRE3 or Y), lactacystin predominantly targeted the X β subunit [13]. Thus, although lactacystin may be a specific inhibitor of the proteasome it is unlikely to be of use in further studies of the proteasome's substrate specificity for polypeptide substrates.

Because of the proteasome's role in the production of antigenic peptides and in the activation of signaling molecules required for inflammation, inhibitors of the proteasome are being explored for use as potential anti-inflammatory agents and for treatment of auto-immune diseases. The usefulness of such inhibitors depends on their bioavailability and cell permeability, criteria that favor small hydrophobic, nonpeptide compounds. Although such compounds are potentially useful as therapeutic agents, they are unlikely to mimic a protein substrate accurately and therefore fail to provide information about catalytic mechanism and substrate specificity.

The yeast 20S proteasome crystal structure suggested the possibility of other active sites within the large multi-subunit complex that do not employ a catalytic amino-terminal threonine residue [13]. Multiple magnesium-binding sites create an acidic environment within the center of the proteasome's internal cavity. Thus, bound water molecules might serve as catalytic nucleophiles, in the same way as they do in hydrolysis by aspartyl proteases. Short peptide-based inhibitors and fluorogenic substrates might not access such a secondary binding pocket, and therefore might not accurately mimic substrate binding. It is therefore possible that additional proteasome–substrate interactions could be studied by using longer peptides.

We describe here the synthesis and characterization of a series of peptide vinyl sulfones of different amino acid sequences, lengths, and carboxy- and amino-terminal modification. These inhibitors are all equipped with a carboxy-terminal vinyl sulfone that covalently modifies the catalytic amino-terminal threonine residue of the proteasome [4]. Tripeptide vinyl sulfones containing a free amino terminus do not inhibit proteasomal proteolysis, but the addition of a

Figure 1

The P-notation used to identify sequence positions on a substrate or inhibitor relative to the site of amide-bond hydrolysis.
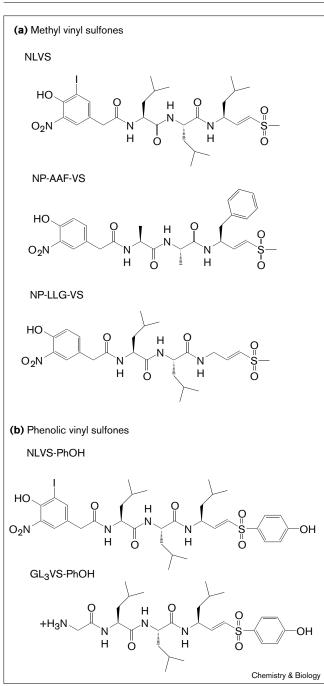
fourth amino acid (at position P4; Figure 1) results in a dramatic increase in inhibitor potency. When the inhibitors are labeled with ^{125}I, binding to proteasomal subunits can be assessed directly using electrophoretic methods. The amino acid sidechain in the P4 position of the inhibitor defines which subunits are modified by the inhibitor, and therefore which activities are blocked to the greatest extent. The P4 region of a substrate is a likely recognition element that helps to define the sequence of peptides produced by the proteasome. Labeling profiles obtained for different inhibitors also allow a correlation of inhibition of substrate hydrolysis with the subunit(s) modified.

Results
Synthesis of peptide vinyl sulfones

A series of tripeptide and tetrapeptide methyl vinyl sulfones were synthesized based on the core sequence Leu–Leu–Leu (LLL), previously described as a potent inhibitor of the proteasome [4] (Figure 2). The peptide vinyl sulfones inhibit the proteasome by specific, covalent modification of the amino-terminal threonine on the catalytically active β subunits [4]. This class of compounds has no reactivity against circulating free nucleophiles such as the thiol group of glutathione [14]. We also synthesized a related derivative in which the methyl group adjacent to the sulfone moiety was replaced with a phenolic group (these vinyl sulfones will be referred to as phenolic vinyl sulfones (VS-PhOH), whereas the methyl vinyl sulfones will be simply called vinyl sulfones (VS); Figure 2). The phenolic substituent allows attachment of ^{125}I at the carboxy-terminal end of the peptide vinyl sulfones.

Figure 2

(a) Methyl vinyl sulfones

NLVS

NP-AAF-VS

NP-LLG-VS

(b) Phenolic vinyl sulfones

NLVS-PhOH

GL₃VS-PhOH

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Structures of several peptide vinyl sulfones containing **(a)** a methyl group or **(b)** a phenolic group adjacent to the vinyl sulfone moiety. NLVS, 4-hydroxy-3-iodo-2-nitrophenyl-leucinyl-leucinyl-leucine vinyl sulfone; NP, 4-hydroxy-3-nitrophenyl acetyl; PhOH, phenolic group; all other letters correspond to single-letter amino-acid code.
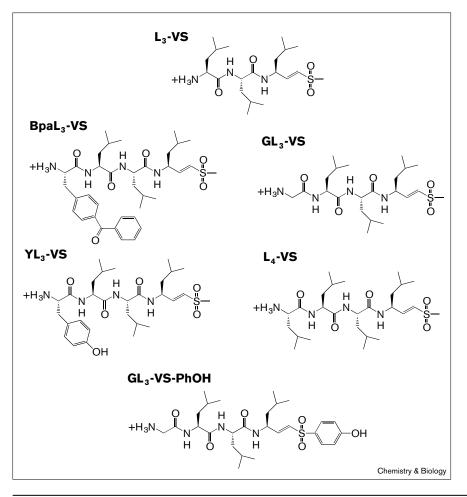
The L_3 tripeptide vinyl sulfone with a free amino terminus (Figure 3) was extended by addition of an amino acid at the P4 position. To examine the effects of the P4 amino acid on covalent modification we synthesized the

sequences GLLL (GL₃-VS), YLLL (YL₃-VS), YAAF (YAAF-VS) and LLLL (L₄-VS) as the corresponding peptide vinyl sulfones (single-letter amino-acid code is used to define the amino acids in the peptides). To examine whether the binding of the P4 amino acid was sensitive to chiral geometry, the D-isomer of tyrosine was also added at P4 ((D)YL₃-VS). We also introduced the non-natural amino acid benzoyl-phenylalanine (Bpa) in the P4 position (Bpa-L₃-VS) to examine whether this bulky sidechain could be accommodated by a secondary binding site (Figure 3).

The sequence LLG was synthesized to examine the importance of the P1 position and AAF was synthesized to determine the effects of a more substantial sequence change on binding. The lack of a sidechain at the P1 position in the LLG peptide resulted in the generation of a mixture of *cis* and *trans* geometric isomers of the vinyl sulfone group that could be separated by chromatography and used to examine steric effects at the site of hydrolysis in the active site.

P4 amino acid influences inhibition of fluorogenic substrate hydrolysis

Hydrolysis of the proteasome substrates Suc-LLVY-AMC (a substrate for the chymotrypsin-like activity; AMC, 7-amido 4-methylcoumarin), Boc-LRR-AMC (a substrate for the trypsin-like activity) and Ac-YVAD-AMC (a substrate for the PGPH activity) was measured. Ac-YVAD was chosen as a substrate rather than the classical PGPH substrate Boc-LLR-βNA, because it more closely matches the structure of the tetrapeptide inhibitors used in this study. Although it is difficult to determine whether both substrates are hydrolyzed by the same active sites, Ac-YVAD-AMC provides information about a proteasomal activity that is biochemically distinct from the chymotrypsin- and trypsin-like activities. As previously reported [4], the compound 4-hydroxy-3-iodo-2-nitrophenyl-leucinyl-leucinyl-leucine vinyl sulfone (NLVS) inhibited multiple proteasome activities with the fastest rate of inactivation of the chymotrypsin-like activity followed by the PGPH and trypsin-like activities. The omission of the amino-terminal carboxylbenzyl or nitro-iodo phenol yielded a free-amino compound that was only weakly inhibitory against all three substrates (Table 1). The free amino tetrapeptide vinyl sulfones YL₃-VS, BpaL₃-VS and L₄-VS, on the other hand, displayed inhibition kinetics similar to or better than those observed for the amino-capped tripeptide NLVS (Figure 4; Table 1). Of the three activities examined, inhibition of the trypsin-like activity was most dramatically increased by the addition of an aromatic, aliphatic, or bulky P4 amino acid. Further, the inhibition rate constants for the chymotrypsin-like and trypsin-like substrates differed, depending on the P4 amino acid. L₄-VS showed a sixfold reduction in activity against the chymotrypsin-like

Figure 3

Structures of the free amino tripeptide and tetrapeptide vinyl sulfones. Amino acids are designated using the single-letter amino-acid code. Bpa, benzoyl-phenylalanine.

activity and a threefold increase in activity against the trypsin-like activity when compared to YL_3-VS.

The related compound in which the P4 tyrosine was replaced with the D-isomer ((D)YL_3-VS) was less active than its diastereomeric equivalent (YL_3-VS), but showed increased reactivity compared to L_3-VS (Table 1). The inhibitory capacity of YL_3-VS against all three activities was reduced by replacement of the L-isomer for the D-isomer at P1. The trypsin-like activity was most affected (97% reduction) followed by the chymotrypsin-like activity (95% reduction), and finally the PGPH activity (75% reduction). Although the binding interaction of the P4 amino acid is sensitive to chiral geometry it does not exclude binding of a D-isomer at this position, as shown previously to be the case for the P1 position [4].

The tetrapeptide GL_3-VS, unlike the other tetrapeptides studied, has no sidechain at the P4 position. This compound showed dramatically reduced activity against hydrolysis of all three substrates when compared to YL_3-VS,

Bpa-L_3-VS and L_4-VS (Table 1). The rate constant for GL_3-VS inhibition of the PGPH activity most closely matched the rates observed for YL_3-VS, Bpa-L_3-VS, and L_4-VS (the GL_3-VS:YL_3-VS ratio was 11%, compared to 1.5% and 2% for the chymotrypsin- and trypsin-like activities respectively). Furthermore, upon prolonged incubation of proteasomes with GL_3-VS, appreciable inhibition was observed for only the PGPH activity (data not shown). These data indicate that the active sites for all three proteolytic activities prefer a hydrophobic or aliphatic P4 amino acid, but the requirements for this binding site may be different for different active sites.

A similar increase in inhibitory activity was observed when tyrosine was placed in the P4 position by addition to the tripeptide AAF-VS, indicating that P4 binding is not dependent on the sequences at P1–3, but rather is a more general phenomenon that leads to increased potency of inhibitors (data not shown). Again, the greatest enhancement of activity was observed against the trypsin-like activity.

Table 1

Kinetic constants for the peptide vinyl sulfones and lactacystin against hydrolysis of three proteasomal substrates.

	$k_{obs}/[I]$		
Inhibitor	Suc-LLVY-AMC	Boc-LRR-AMC	Ac-YVAD-AMC
Lactacystin	1,500 (0.2–0.5 µM)	110 (5–10 µM)	17 (25–100 µM)
NLVS-PhOH	13,400 (0.1–0.5 µM)	422 (25–50 µM)	100 (10–25 µM)
NLVS	13,500 (0.1–0.5 µM)	10 (50–100) µM)	24 (50–100 µM)
L_3-VS	2.7 (100–500 µM)	11 (50–250 µM)	0.21 (100–500 µM)
YL_3-VS	1,500 (2–6 µM)	560 (2–10 µM)	77 (825 µM)
(D)YL_3-VS	77 (5–25 µM)	20 (50–100 µM)	20 (50–100 µM)
$BpaL_3$-VS	960 (0.8–3.2 µM)	2,370 (0.5–1 µM)	63 (10–20 µM)
L_4-VS	240 (5–15 µM)	1,500 (0.5–2.5 µM)	29 (1550 µM)
GL_3-VS	21 (25–100 µM)	12 (25–100 µM)	8.0 (75–250 µM)
GL_3-VS-PhOH	57 (50–100 µM)	14 (50–100 µM)	23 (50–100 µM)
NP-AAF-VS	68 (2–15 µM)	14 (75–100 µM)	ND
NP-LLG-VS	28 (25–50 µM)	ND	ND

Hydrolysis of Suc-LLVY-AMC (chymotrypsin-like activity), Boc-LRR-AMC (trypsin-like activity), and Ac-YVAD-AMC (PGPH activity) was monitored after the addition of inhibitors listed above in the given concentration ranges (values in parentheses). Inhibition curves were plotted and fit to the equation for a slow binding covalent inhibitor as described in the Materials and methods section. Results are presented as a average of $k_{obs}/[I]$ values obtained for three different inhibitor concentrations. The kinetic rate constants for inhibition for several inhibitors could not be determined due to solubility problems and absorption of the nitro phenol group at high concentrations. These samples are listed as ND and are assumed to be quite small relative to the reported values for the other inhibitors.

Inhibition profiles for the chymotrypsin-like, trypsin-like and PGPH substrates obtained upon treatment of intact cells with several of the tripeptide and tetrapeptide vinyl sulfones agreed with the kinetic data described for sodium dodecyl sulfate (SDS)-activated 20S proteasomes (data not shown). Furthermore, kinetic rate constants obtained for NLVS using purified 26S proteasomes were similar to those obtained using SDS-activated 20S proteasomes (data not shown). These data suggest that although the presence of additional regulatory subunits may result in subtle changes to the pattern of inhibition by various peptide vinyl sulfones, the kinetic data obtained for SDS-activated 20S proteasomes is likely to be representative of the multiple physiologically relevant proteasome complexes that exist within a cell.

Labeling of proteasomes with tetrapeptide vinyl sulfones
The labeling pattern of proteasomes with the tripeptide ^{125}I-NLVS has been described [4]. This compound covalently labels multiple β subunits including the interferon-γ (IFN-γ) inducible LMP-2 and LMP-7 subunits. SDS–polyacrylamide gel electrophoresis (PAGE) analysis of ^{125}I-NLVS-labeled proteasomes revealed covalent modification of three subunits that overlap because of their nearly identical molecular weights, but which are easily resolved using two-dimensional isoelectric focusing (IEF)–SDS–PAGE.
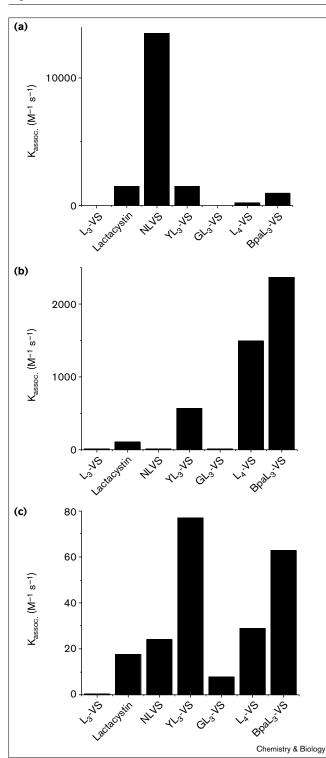
To further identify the subunits modified by the peptide vinyl sulfones, total cell lysates were labeled with the tetrapeptide vinyl sulfone ^{125}I-YL_3-VS. 20S and 26S proteasome complexes were immunoprecipitated with an antiserum that recognized the intact proteasome complex, denatured, and re-immunoprecipitated with β-subunit-specific antisera. All six catalytically active β subunits were modified by ^{125}I-YL_3-VS and the identity of each can now be confirmed on the basis of molecular weight and isoelectric point (Figure 5).

^{125}I-NLVS labeled a band of approximately 23 kDa with greatest intensity in total lysates. In human cells, this predominant labeled band was shown to be made up of three polypeptides upon two-dimensional gel analysis [4]. In mouse cells, the major 23 kDa species is accompanied by a second slightly smaller polypeptide of ~22 kDa. Results from the re-immunoprecipitation experiment confirmed the identity of the 22 kDa polypeptide as LMP-2 and the 23 kDa polypeptides as LMP-7, X and Y. Two much less intensely labeled polypeptides were observed at around 28 and 30 kDa and can now be identified as subunits Mecl-1 and Z respectively. Interestingly, in samples labeled with ^{125}I-YL_3-VS the Z subunit was modified to a much greater extent than observed for ^{125}I-NLVS (Figure 6). Thus, the addition of a bulky aromatic P4 amino acid resulted in increased binding specifically to the Mecl-1 and Z subunits.

The P4 position is important for β-subunit modification
Based on the finding that GL_3-VS is a poor inhibitor of the trypsin-like and chymotrypsin-like activities of the

Figure 4

Kinetics of inhibition of fluorogenic substrate hydrolysis by the free amino tetrapeptide vinyl sulfones as compared to lactacystin, NLVS and free amino tripeptide vinyl sulfone L_3-VS. $k_{assoc.}$ (k_{obs}/[I]) values are plotted for the indicated inhibitors against the three proteasomal fluorogenic substrates **(a)** Suc-LLVY-AMC (chymotrypsin-like activity), **(b)** Boc-LRR-AMC (trypsin-like activity), and **(c)** Ac-YVAD-AMC (PGPH activity).
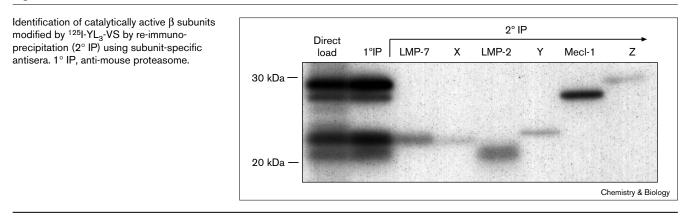
proteasome but a fairly good inhibitor of the PGPH activity, we next examined whether this difference in activity could be correlated with subunit modification. Partially purified preparations of proteasomes were labeled with ^{125}I-YL_3-VS in the simultaneous presence of increasing concentrations of NLVS, YL_3-VS, GL_3-VS, $BpaL_3$-VS and L_4-VS (Figure 7). The competition experiment indicated that YL_3-VS was able to compete for binding to all polypeptides labeled with ^{125}I-YL_3-VS, whereas GL_3-VS only blocked binding of ^{125}I-YL_3-VS to LMP-2. This result, combined with the data obtained for substrate hydrolysis, provides a direct correlation between the LMP-2 subunit and the PGPH activity of the mammalian proteasome.

Placement of the bulky non-natural amino acid benzoylphenylalanine at the P4 position resulted in an inhibitor with an activity profile similar to that of YL_3-VS. $BpaL_3$-VS competes for binding with ^{125}I-YL_3-VS to the Z and Mecl-1 subunits with greatest affinity, followed by the LMP-2 subunit and finally the LMP-7 and X subunits. Conversely, NLVS favors modification of the LMP-7 and X subunits, with only weak reactivity against the Z and Mecl-1 subunits. Placement of a leucine residue at the P4 position again increases the potency towards the Z and Mecl-1 subunits while reducing potency towards X and LMP-7. The amino acid composition at position P4 can thus help define the binding of a substrate to a particular active site.

Modifications at the carboxyl terminus of the peptide vinyl sulfones

The activities of peptide vinyl sulfones containing a phenol moiety adjacent to the sulfone group were compared to those of peptide vinyl sulfones containing a methyl group. These compounds allow the examination of binding interactions at the carboxy-terminal side of a predicted site of hydrolysis (likely to mimic the P1′ position). Interestingly, replacement of the methyl group in the compound NLVS with the phenol (NLVS-PhOH) resulted in no change in the potency of the compound against hydrolysis of LLVY, but resulted in a 40-fold increase in potency against the LRR substrate and a four-fold increase against the YVAD substrate (Table 1). Different active sites in the proteasome are therefore likely to have distinct specificities for the region carboxy-terminal to the site of hydrolysis.

The iodinated forms of the phenolic peptide vinyl sulfones were used to examine the effects of structural variation of the carboxy-terminal vinyl sulfone on subunit modification. Partially purified proteasome preparations were labeled with the methyl-vinyl-sulfone-containing NLVS, as well as with NLVS-PhOH (Figure 8). The labeling patterns obtained for these inhibitors were distinct, with the greatest difference in intensity of labeling observed for the LMP-2 subunit.

Figure 5

Identification of catalytically active β subunits modified by ^{125}I-YL₃-VS by re-immuno-precipitation (2° IP) using subunit-specific antisera. 1° IP, anti-mouse proteasome.

Synthesis of the tripeptide NP-LLG-VS resulted in the isolation of the *cis* and *trans* isomers of the carboxy-terminal vinyl sulfone. Interestingly, the ability of the isomers to inhibit substrate hydrolysis was quite different: the *cis* isomer had little or no inhibitory capacity against any of the substrates, whereas the *trans* isomer was merely less active when compared to tripeptides containing three leucine residues (data not shown). Even more surprising was the labeling pattern observed when these isomers were equipped with an ^{125}I moiety. The *trans* isomer, although a weaker inhibitor than the LLL equivalent, was still able to covalently tag proteasomal β subunits. The *cis* isomer, on the other hand, failed to label any polypeptides in the size range (20–30 kDa) expected for the proteasome, but efficiently labeled several other species of higher molecular weight (data not shown). The identity of these additional polypeptides was not established. Thus, unlike replacement of a methyl with a

Figure 6

Addition of a bulky, aromatic sidechain in the P4 position increases modification of the Mecl-1 and Z subunits of the proteasome. **(a)** Total lysates from EL-4 cells were labeled with ^{125}I-NLVS and ^{125}I-YL₃-VS and separated by one-dimensional SDS–PAGE either directly (left panel) or immunoprecipitated with a proteasome specific antiserum and then separated by SDS–PAGE (right panel). **(b)** Immunoprecipitates from (a) were also separated by two-dimensional SDS–PAGE. The doubling of spots in the ^{125}I-YL₃-VS samples (bottom panel) was due to the iodination of either *ortho-* or *meta-* to the phenolic hydroxyl group on tyrosine resulting in a minor change in the pK_a of this proton and therefore a slightly different isoelectric point of proteins modified by this inhibitor.
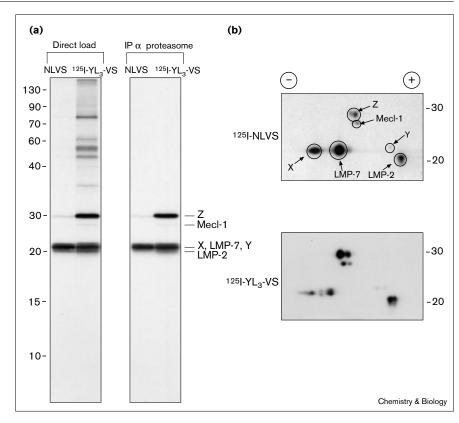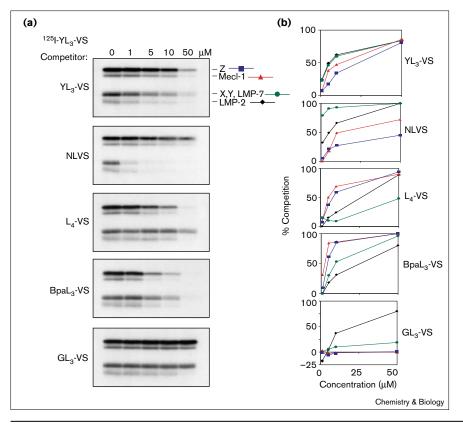
Figure 7

Changes in the P4 amino acid of the tetrapeptide vinyl sulfones results in changes in the pattern of β subunits modified. **(a)** NLVS and the tetrapeptide vinyl sulfones were added simultaneously with ^{125}I-YL$_3$-VS to EL-4 lysates and labeled polypeptides separated by SDS–PAGE as described in the Materials and methods section. **(b)** Densitometry profiles of the percent competition (y axis) versus inhibitor concentration (x axis) based on the data from (a).

phenol moiety, which resulted in only minor change in activity, the sharp kink induced in the carboxyl terminus by the *cis* double bond effectively precludes binding.

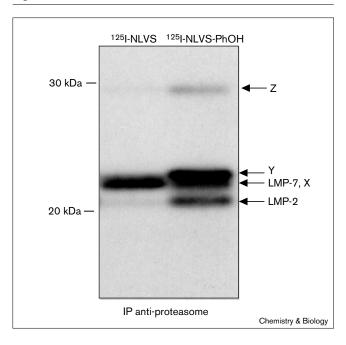### Inhibitor binding to β subunits is sequence specific

To investigate the effects of amino acid sequence on the binding of inhibitors to specific subunits, we examined the ability of several tripeptides to inhibit substrate hydrolysis and to label specific β subunits of the proteasome when converted to ^{125}I-labeled form. Results from analysis of inhibition of the three proteasome substrates by the tripeptide vinyl sulfones (NP-LLG-VS , NP-AAF-VS and NIP-L$_3$-VS) indicated that inhibition by these three compounds is nonequivalent (Table 1). Neither NP-LLG-VS nor NP-AAF-VS had a significant effect on the PGPH activity, although both were able to inhibit the chymotrypsin activity (NP-AAF-VS is about three times more potent than NP-LLG-VS).

Labeling of semi-purified proteasomes with ^{125}I-NIP-LLG-VS, ^{125}I-NIP-AAF-VS, and ^{125}I-NLVS revealed differences in the relative intensity of polypeptides labeled. NLVS predominantly labeled the β subunits X, LMP-2 and LMP-7 each with similar intensity. Labeling of Y, Mecl-1 and Z was also observed, but at a much lower intensity (Figure 9). NIP-AAF-VS showed predominant

labeling of LMP-7, with a reduction in the labeling of X and little or no labeling of the Mecl-1 and Y subunits. ^{125}I-NIP-LLG-VS did not directly modify the LMP-2 and Y subunits, but did label both Z and Mecl-1 with an intensity ratio that was similar to the ratio observed for ^{125}I-NLVS.

The tetrapeptide containing a glycine residue in the P4 position (GL$_3$-VS) was found to be a weak but specific tag for the LMP-2 subunit (Figure 7). Furthermore, replacement of the methyl group attached to the vinyl sulfone moiety with a phenol resulted in an increased propensity of NLVS to modify the LMP-2 subunit (Figure 8). We therefore reasoned that the GL$_3$ core sequence combined with the phenolic vinyl sulfone may represent a potentially LMP-2-specific reagent. Labeling of total lysates from mouse EL-4 cells with ^{125}I-GL$_3$-VS-PhOH followed by immunoprecipitation and two-dimensional electrophoresis showed a specific binding of the LMP-2 subunit with a minor amount of Y also modified (Figure 9). It could therefore be possible to generate subunit-specific inhibitors by modifying the P4 amino acid and carboxy-terminal sulfone group.

Discussion

The use of modified tripeptides that contain an active site-directed inhibitory functional group at the carboxyl
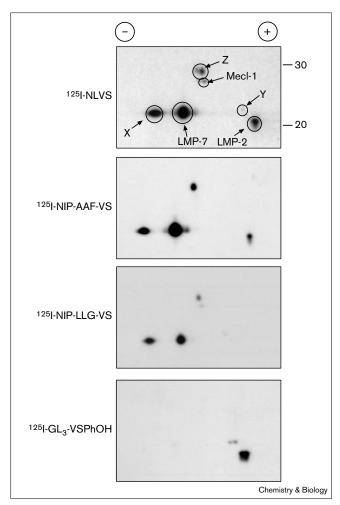
Figure 8

Replacement of the methyl group adjacent to the vinyl sulfone with a phenolic moiety results in an increase in modification of the LMP-2 subunit. Total lysates were labeled with ^{125}I-NLVS and ^{125}I-NLVS-PhOH. Proteasomes were isolated by immunoprecipitation and then separated by SDS–PAGE as described in the Materials and methods section.

Figure 9

Subunit binding is sequence specific and sensitive to changes at both sides of the site of hydrolysis. Total lysates were labeled with the indicated ^{125}I-tagged peptide vinyl sulfones. Proteasomes were isolated by immunoprecipitations and separated in one dimension using non-equilibrium pH gradient electrophoresis (NEPHGE) followed by SDS–PAGE in a second dimension. Note the change in relative intensities of subunit modified upon changes in the core amino acid sequence (LLL, AAF, LLG).

terminus has proven to be a valuable method for studying proteasome–substrate interactions. The synthesis of a new series of tetrapeptide vinyl sulfones has now uncovered the importance of the P4 amino acid position for binding and inhibition. Modifications at the carboxy-terminal portion of such compounds indicated some important differences in how individual proteasomal subunits bind substrates. In addition, direct labeling experiments using the inhibitors provided a link between substrate binding and hydrolysis. Labeling of proteasomes with peptide vinyl sulfones of different sequences revealed that binding of a substrate to individual β subunits is sequence dependent.

The potency of a free-amino tetrapeptide vinyl sulfone as a proteasome inhibitor is remarkable compared to the extremely poor activity of the corresponding free-amino tripeptide vinyl sulfone. The tripeptides capped with a bulky nitro phenol (NLVS) or benzyl (Z-L$_3$-VS) moiety are potent inhibitors, but removal of the cap resulted in severely diminished binding and inhibition. Several dipeptide aldehydes were described as potent inhibitors of the proteasome, but these inhibitors all have an amide-capped amino terminus (usually acetate) [15]. The presence of the amide cap is likely to result in substrates or inhibitors that mimic a longer peptide substrate. The inhibitors used in this study have the important feature of

containing a free amino terminus, as expected for a by-product of a prior proteolytic processing reaction. The very presence of a charged free amino group within the active site might, in fact, be required for length determination by the proteasome. Thus, our data suggest that there is a minimal length requirement for substrate binding and hydrolysis by the proteasome. If so, the possibility that the proteasome digests some substrates to single amino acids is not very likely.

The P4-binding site is unlikely to be as specific as that for the P1 amino acid. Several different bulky residues, including a nonnatural amino acid containing a benzophenone

sidechain, are accepted in the P4 position. In addition, unlike the P1 position where a D-isomer results in complete loss of binding [4], placement of a D-isomer at P4 resulted in an active inhibitor, albeit with reduced binding affinity. Thus, within the cavity of the proteasome, secondary binding sites are likely to exist that stabilize longer peptide substrates and can accommodate sidechains of varying sizes and shapes. This would be expected for a protease such as the proteasome, responsible for the degradation of most cytosolic proteins.

The structural requirements for binding of the P4 amino acid are distinct for different active sites and different β subunits. The trypsin-like and chymotrypsin-like activities of the proteasome seem to favor a bulky, aromatic or hydrophobic group in the P4 position, whereas the PGPH activity is less sensitive to changes in the P4 position.

The dramatic increase in the extent of modification by ^{125}I-YL$_3$-VS of the Mecl-1 and Z subunits when compared to NLVS correlated with the increase in activity against hydrolysis of the Boc-LRR-AMC substrate. Furthermore, the notable decrease in potency of L$_4$-VS relative to NLVS, YL$_3$-VS and BpaL$_3$-VS against hydrolysis of Suc-LLVY-AMC correlated with a decrease in the extent of modification of the X and LMP-7 subunits, as judged by competition experiments (Figure 7). Finally, the specific modification of the LMP-2 and Y subunits by GL$_3$-VS-PhOH and GL$_3$-VS correlated with increased activity against the Ac-YVAD-AMC substrate. These findings suggest that the Mecl-1 and Z subunits are primarily responsible for cleavage after basic amino acid residues, LMP-7 and X subunits predominantly for cleavage after hydrophobic residues and LMP-2 and Y for cleavage after acidic residues. These findings fit well with a recently reported study using ^{14}C-labeled dichloroisocoumarin (DCI) by Orlowski and co-workers [16]. They found that the X and LMP-2 subunits of pituitary and spleen proteasomes are preferentially modified by DCI, and that this modification correlates with inhibition of the chymotrypsin-like activity.

Surprisingly, the observed inhibition rate constants for GL$_3$-VS-PhOH are similar to those obtained for NP-AAF-VS, with respect to the chymotrypsin- and trypsin-like activities. The labeling patterns are quite different, however, indicating that the observed difference in inhibition of PGPH activity cannot be accounted for solely by the increased modification of LMP-2 and Y by GL$_3$-VS-PhOH. β subunits must therefore overlap, at least to some extent, in their substrate specificities. A single subunit is not likely to hydrolyze only a distinct subset of substrates containing a defined carboxyl terminus. This suggestion is supported by the finding that the BrAAP activity is the result of hydrolytic activity of multiple β subunits [17].

The functional group placed adjacent to the sulfone at the carboxy-terminal end of the inhibitor is likely to mimic the P1′ position of a protein substrate when bound in the active site. Our data support the notion that not all active sites have the same degree of specificity for the P1′ position. Addition of the phenol moiety in place of the methyl group adjacent to the vinyl sulfone results in increased affinity for the LMP-2 and Y subunits. These subunits may thus have a greater degree of sequence specificity for the portion of a substrate carboxy-terminal to the site of hydrolysis.

Finally, labeling of proteasomes with inhibitors of different sequences demonstrated that individual subunits have pronounced sequence preference. It is not surprising that by changing the amino acid sequence of a tripeptide inhibitor, it is possible to change the relative inhibitory capacity of that compound. By comparing the relative changes in binding affinity of a compound for particular subunits, however, it is possible to examine how these inhibitors interact with individual subunits of the proteasome. Of interest is the ability of a NIP-AAF-VS to bind to LMP-7, with greater affinity than to X, while not modifying Mecl-1 at all. This is in direct contrast to NLVS, which bound both LMP-7 and X nearly equally, and both Z and Mecl-1 to similar degrees. The LLG-containing tri peptide, on the other hand, failed to label the LMP-2 or Y subunits, suggesting that these β subunits have sequence requirements that define which substrates each will hydrolyze.

Fluorogenic substrates are often used to evaluate inhibitors of a proteolytic activity of an enzyme such as the proteasome. These tools, although useful, often fail to shed light on the underlying mechanisms by which a compound can cause inhibition. The data reported here show the usefulness of radiolabeled affinity reagents such as the vinyl sulfones, to study substrate binding. The results obtained for the tetrapeptide vinyl sulfone GL$_3$-VS-PhOH also suggest that, through a combinatorial approach, it may be possible to generate reagents that can selectively inhibit one active site or proteolytic activity of the proteasome. Such reagents would be of considerable use for further deconvolution of the proteasome's role in a wide range of biological processes.

Significance

The proteasome is a dynamic proteolytic complex that degrades a multitude of polypeptide substrates resulting in by-products of differing sequences, lengths and biological functions. Although the multiple proteolytic activities of the proteasome are usually defined according to the types of sidechains present immediately amino-terminal to the scissile bond (the P1 position), our results show that a considerable element of specificity is contributed by more distal sequence elements. These observations raise questions as to how substrates are threaded along

the proteasome's active sites. Furthermore, the use of 'suicide-substrate' inhibitors has allowed a correlation between inhibition of hydrolytic activity and subunit modification. The role of individual subunits in establishing a specific proteolytic activity can now be addressed. Finally, this work shows that it is possible to generate subunit-selective reagents that could be used to further define substrate specificity and to determine the significance of the multiple proteolytic activities of the proteasome in its many biological tasks.

Materials and methods

Inhibitor synthesis
NIP-L$_3$-VS, ^{125}I-NIP-L$_3$-VS, L$_3$-VS and Z-L$_3$-VS were synthesized as described previously [4]. The synthesis of all other inhibitors are described below. All inhibitors were dissolved in dimethyl sulfoxide (DMSO) and used in biological assays at the concentrations specified. Stock solutions were prepared such that the concentration of DMSO never exceeded 1%.

Synthesis of tetrapeptide vinyl sulfones containing the L$_3$ core sequence
The tripeptide free amine L$_3$-VS was used as a building block for the synthesis of the tetrapeptide vinyl sulfones containing the sequences: YL$_3$-VS, (D)YL$_3$-VS, BpaL$_3$-VS, GL$_3$-VS, and L$_4$-VS The synthesis of these tetrapeptides were carried out by the coupling of the desired Boc-amino acid to L$_3$-VS using the same protocol for all four derivatives. The Boc- protecting group was removed from the tetrapeptides by treatment with trifluoroacetic acid (TFA). All tetrapeptides were characterized as the free amine salts only and are described below.

Synthesis of Boc-protected tetrapeptide vinyl sulfones. The desired Boc protected amino acid (18.6 µmol) and PyBOP (18.6 µmol) were dissolved in 1 ml of dichloromethane. Diisopropylethyl amine (3.3 µl, 18.6 µmol) was then added. The reaction was stirred for 5 min and L$_3$-VS tosic acid salt (10 mg, 16.9 µmol) was added followed by diisopropylethyl amine (3.0 µl, 16.9 µmol). The reaction was stirred for 30 min at room temperature and solvent was removed by rotary evaporation. The resulting crude reaction mixture was purified by flash column chromatography using ethyl acetate:hexane as the mobile phase.

Synthesis of free amino tetrapeptide vinyl sulfone trifloroacetic acid salts. The desired Boc-protected tetrapeptide vinyl sulfone (13.2 µmol) was dissolved in 1 ml of dichloromethane. TFA (1 ml) was added and the reaction stirred at room temperature for 1 h. The reaction was quenched by the addition of 5 ml of toluene followed by removal of the solvent by rotary evaporation. A second portion of toluene was then added to the crude solid and again evaporated to dryness. The pure tetrapeptide was obtained by trituration of the solid residue in diethyl ether. Tyrosine-leucine-leucine-leucine vinyl sulfone (YL$_3$-VS): yield (9 mg, 13 µmol, 99%). ^{1}H-NMR (500 MHz, CD$_3$OD) δ 7.05 (2H, d), 6.80 (1H, dd), 6.70 (2H, d), 6.64 (1H, t), 4.7 (1H, m), 4.45 (1H, m), 4.20 (1H, dd), 3.25 (1H, m), 2.95 (3H, s), 2.95 (2H, ddd), 1.50-1.78 (9H, M), 0.95 (18H, m). FAB mass spectrum: [M+H] calc'd for C$_{29}$H$_{48}$N$_4$O$_6$S: 581.7; found: 581.4. (D)-tyrosine-leucine-leucine-leucine vinyl sulfone ((D)YL$_3$-VS): Yield (70%).^{1}H-NMR (500 MHz, CD$_3$OD) δ 7.05 (2H, d), 6.80 (1H, dd), 6.70 (2H, d), 6.64 (1H, t), 4.65 (1H, m), 4.45 (1H, m), 4.20 (1H, dd), 3.25 (1H, m), 2.95 (3H, s), 2.95 (2H, ddd), 1.50-1.78 (9H, M), 0.95 (18H, m). FAB mass spectrum: [M+H] calc'd for C$_{29}$H$_{48}$N$_4$O$_6$S: 581.7; found: 581.4. Glycine-leucine-leucine-leucine vinyl sulfone (GL$_3$-VS): yield (74%). 1H-NMR (500 MHz, CD$_3$OD) d 6.78 (1H, dd), 6.58 (1H, d), 4.65 (1H, m), 4.43 (1H, dd) 4.39 (1H, m), 3.71 (2H, s), 2.98 (3H, s), 1.43-1.71 (9H, m), 0.94 (18H, m). FAB mass spectrum: [M+H] calc'd for C$_{22}$H$_{42}$N$_4$O$_5$S: 475.6; found: 475.3. Leucine-leucine-leucine-leucine vinyl sulfone (L4-VS): yield (84%). 1H-NMR (500 MHz, CD$_3$OD) d 6.78 (1H, dd), 6.58 (1H, d), 4.65 (1H, m), 4.48 (1H, dd), 4.39 (1H, m), 2.97

(3H, s), 1.43-1.74 (12H, m), 0.91-1.17 (24H, m). FAB mass spectrum: [M+H] calc'd for C$_{26}$H$_{50}$N$_4$O$_5$S: 531.7; found 531.1. Benzoylphenylalanine-leucine-leucine-leucine vinyl sulfone (BpaL$_3$-VS) yield (Boc-Bpa-L$_3$-VS 92%, 96%).^{1}H-NMR (500 MHz, CD$_3$OD) δ 7.78 (4H, dd), 7.65 (1H, t), 7.55 (2H, t), 7.45 (2H, d), 6.79 (1H, dd), 6.59 (1H, d), 4.65 (1H, m), 4.45 (1H, dt), 4.18 (1H, t), 3.18 (1H, m), 2.98 (3H, s), 1.30-1.75 (9H, m), 0.95 (18H, m). FAB mass spectrum: [M+H] calc'd for C$_{36}$H$_{52}$N$_4$O$_6$S: 669.8; found: 669.5

Synthesis of NP-AAF
The NP-AAF-VS peptide vinyl sulfone was synthesized essentially as described for the synthesis of NLVS [4]. The synthetic details are described below.

Synthesis of Boc-phenylalanine vinyl sulfone (Boc-Phe-VS). Boc-phenylalanine vinyl sulfone was synthesized exactly as described for the synthesis of Boc-leucine vinyl sulfone [4], except that Boc-phenylalanine was used in place of Boc-leucine. The NMR data and typical yields obtained for each intermediate are listed. Boc-Phe-dimethyl hydroxyl amide: yield (97%), ^{1}H-NMR (300 MHz, CDCl$_3$) δ 7.18-7.38 (5H, m), 5.19 (1H, d), 4.98 (1H, m), 3.69 (3H, s), 3.20 (3H, s), 3.0 (2H, ddd), 1.40 (3H, s). Boc-Phenylalaninal: yield (73%), ^{1}H-NMR (300 MHz, CDCl$_3$) δ 9.65 (1H, s), 7.18-7.38 (5H, m), 5.05 (1H, d), 4.44 (1H, m), 3.15 (2H, d), 1.45 (9H, s). Boc-Phe-VS: yield (73%) ^{1}H-NMR (300 MHz, CDCl$_3$) δ 7.35 (3H, q), 7.19 (2H, d), 6.90 (1H, dd), 6.39 (1H, dd), 4.67 (1H, m), 4.58 (1H, d), 2.95 (2H, d), 2.93 (3H, s), 1.43 (9H, s).

Synthesis of phenylalanine-vinyl sulfone tosic acid salt (Phe-VS). Phenylalanine-vinyl sulfone was synthesized from Boc-Phe-VS by removal of the Boc-protecting group using tosic acid as described for Boc-leucine-vinyl sulfone [4]. Phe-VS tosic acid salt ^{1}H-NMR (300 MHz, CD$_3$OD) δ 7.74 (2H, d), 7.35 (3H, q), 7.25 (4H, d), 6.74 (2H, m), 4.29 (1H, q), 2.99-3.23 (2H, ddd), 2.89 (3H, s), 2.39 (3H, s).

Synthesis of Boc-alanine-alanine methyl ester (Boc-AA-OMe). To a flask was added Boc-alanine (2.0 g, 10.5 mmol), dicyclohexylcarbodiimide (DCC; 2.4 g, 11.6 mmol), and HOBt (1.57 g, 11.6 mmol) in 25 ml dimethyl formamide (DMF). The reaction was stirred for 30 min at room temperature. A white precipitate formed upon standing. Alanine-methyl ester hydrochloride salt (2.2 g, 15.8 mmol) was then added followed by diisopropylethyl amine (DIEA; 2.8 ml, 15.8 mmol). The reaction was stirred overnight at room temperature. Next, the crude reaction mix was filtered to remove precipitated solids and evaporated to a minimal volume by rotary evaporation. The resulting crude solution was diluted with 250 ml ethyl acetate and extracted with 3 × 75 ml portions of 0.1 N HCl and 3 × 75 ml portions of saturated sodium bicarbonate. The remaining organic phase was dried over MgSO$_4$ and dried to a solid by rotary evaporation. The pure di-peptide methyl ester was isolated by re-crystallization from ethyl acetate/hexane. yield (2.1 g, 7.6 mmol, 72%). Boc-AA-OMe ^{1}H-NMR (300 MHz, CDCl$_3$) δ 6.64 (1H, d), 5.01 (1H, d), 4.54 (1H, p), 4.15 (1H, q) 3.73 (3H, s), 1.42 (9H, s), 1.40 (3H, d). 1.38 (3H, d).

Synthesis of Boc-alanine-alanine (Boc-AA-OH). The methyl ester of Boc-AA was converted to the corresponding acid by hydrolysis using sodium carbonate and methanol. This compound was used in subsequent steps without further purification.

Synthesis of Boc-alanine-alanine-phenylalanine vinyl sulfone (Boc-AAF-VS). Boc-AAF-VS was synthesized by reaction of Boc-AA-OH with phenylalanine-vinyl sulfone tosic acid salt using the protocol described for the synthesis of Boc-protected tetrapeptide vinyl sulfones described above using PyBOP as the coupling agent. The product was purified by flash column chromatography using ethyl acetate/hexane (4:1 v:v) as the mobile phase. Yield (57%).

Boc-AAF-VS ^{1}H-NMR (300 MHz, CDCl$_3$) δ 7.25-7.38 (4H, m), 7.20 (2H, d), 7.12 (1H, d), 6.88 (1H, dd), 6.60 (1H, d), 6.56 (1H, d), 4.98 (1H, m), 4.37 (1H, t), 4.05 (1H, q), 2.95 (2H, dd), 2.90 (3H, s), 1.48 (9H, s), 1.36 (3H, d), 1.34 (3H, d).

Synthesis of alanine-alanine-phenylalanine vinyl sulfone tosic acid salt (AAF-VS). The Boc protecting group was removed from Boc-AAF-VS using the protocol described for removal of Boc from Boc-L$_3$-VS [1]. Yield (95%). AAF-VS ^{1}H-NMR (300 MHz, CD$_3$OD) δ 7.74 (2H, d), 7.20-7.38 (7H, m), 6.85 (1H, dd), 6.56 (1H, d), 4.82 (1H, q), 4.32 (1H, q), 3.95 (1H, q), 2.99 (2H, dd), 2.90 (3H, s), 2.39 (3H, s), 1.50 (3H, d), 1.32 (3H, d). FAB mass spectrum: [M+H] calc'd for C$_{17}$H$_{25}$N$_3$O$_4$S: 368.4; found: 368.3.

Synthesis of NP-AAF-VS. 4-hydroxy-3-nitrophenyl acetic acid was coupled to the free amino AAF-VS using PyBOP as described above for the Boc-tetrapeptide vinyl sulfones. Yield (78%). NP-AAF-VS 1H-NMR (300 MHz, CD3CN) δ 10.4 (1H, s), 8.08 (1H, s), 7.60 (1H, d), 6.95-7.29 (9H, m), 6.72 (1H, dd), 6.53 (1H, d), 4.69 (1H, t), 4.08 (2H, m), 2.85 (3H, s), 2.84 (1H, m), 2.65 (1H, m), 2.15 (2H, d), 1.35 (3H, d), 1.12 (3H, d). FAB mass spectrum: [M+H] calc'd for C$_{25}$H$_{30}$N$_4$O$_8$S: 547.5; found: 547.1.

Synthesis of LLG containing vinyl sulfones

Vinyl sulfones containing glycine at position P1 were synthesized as described for the synthesis of the tri-leucine vinyl sulfones except that the Boc-glycinal intermediate was prepared by the oxidation of Boc-3-amino-1,2-propane diol. The resulting aldehyde was converted to the corresponding vinyl sulfone as described for the synthesis of Boc-leucine vinyl sulfone.

Synthesis of Boc-3-amino-1,2-propane diol. 3-amino-1,2-propane diol (5.0 g, 55 mol) was dissolved in 20 ml of 1,4-dioxane and 10 ml of 3M NaOH. Di-*tert*-butoxy carbonyl ((Boc)$_2$O; 12 g, 55 mmol) was added and the reaction stirred for 4.5 h until the evolution of gas ceased. The solvent was removed by rotary evaporation and the resulting crude white solid dissolved in ethyl acetate. The ethyl acetate solution was washed with 3 × 50 ml portions of 5% potassium hydrogen sulfate (KHSO$_4$). The aqueous layers were combined and back-extracted with ethyl acetate. All organic layers were combined, dried over MgSO$_4$, and concentrated to an oil by rotary evaporation. The crude oil was used in subsequent steps without further purification.

Synthesis of Boc-glycinal (Boc-Gly-H). Boc-3-amino-1,2-propane diol (3.8 g, ~20 mmol) was dissolved as a crude oil in 30 ml of ethanol. Sodium periodate (NaIO$_4$; 12.8 g, 60 mmol) was added as a solution in 90 ml of 0.01M phosphate buffer (pH 7). The reaction was then stirred for 1.5 h. The reaction mix was washed with 3 × 50 ml portions of chloroform with the addition of 50 ml of saturate sodium chloride (to separate organic and aqueous phases). The organic phases were combined, dried over MgSO$_4$, and evaporated to dryness. The crude Boc-glycinal was used in subsequent steps without further purification.

Synthesis of Boc-glycine vinyl sulfone, cis *and* trans *isomers (Boc-G-cVS and Boc-G-tVS).* The *cis* and *trans* isomers of Boc-glycine vinyl sulfone were synthesized from the crude Boc-glycinal as described for the synthesis of Boc-leucine vinyl sulfone from Boc-leucinal [4]. This reaction resulted in two compounds that were separable by flash chromatography using hexane: ethyl acetate (2:1) as the mobile phase. Mass spectrum obtained for the two tripeptides isolated were identical (NP-LLG-tVS and NP-LLGcVS; see below) indicating that they were likely to be isomers. As enantiomers are not likely to be resolved by column chromatography, we concluded that the lack of a sidechain on glycine allowed for the formation of both the *cis* and *trans* isomers of the newly formed double bond. NMR data (see below) confirmed this hypothesis. The two compounds had nearly identical spectra with the largest difference observed for the vinyl resonances. The alpha carbon was also found to be slightly different for the two isomers. These data were consistent with *cis/trans* geometric isomers. Finally the *cis* and *trans* isomers were assigned using Z-factor calculations [18] to predict the chemical shift of the vinyl protons for the *cis* and *trans* isomers. The greatest difference in shifts between the two vinyl resonances was found for the *cis* isomer and was reflected in the NMR obtained for the slightly less polar

compound isolated. Yield (2:1 *trans:cis*, 43 % overall yield). Boc-glycine vinyl sulfone *cis* isomer ^{1}H-NMR (300 MHz, CDCl$_3$) δ 6.45 (1H, dd), 6.32 (1H, d), 4.98 (1H, m), 4.28 (2H, t), 3.04 (3H, s), 1.45 (9H, s). Boc-glycine vinyl sulfone *trans* isomer ^{1}H-NMR (300 MHz, CDCl$_3$) δ 6.94 (1H, dt), 6.52 (1H, dt), 4.78 (1H, m), 4.00 (2H, t), 2.96 (3H, s), 1.45 (9H, s).

Synthesis of glycine-vinyl sulfone tosic acid salt cis *and* trans *isomers (G-cVS, G-tVS).* The Boc protecting group was removed from the glycine vinyl sulfones as described for the removal of Boc from Boc-leucine vinyl sulfone described above. Yield (97%). Glycine vinyl sulfone *cis* isomer ^{1}H-NMR (300 MHz, CD$_3$OD) δ 7.72 (2H, d), 7.24 (2H, d), 6.78 (1H, dt), 6.43 (1H, dt), 4.21 (2H, d), 3.08 (3H, s), 2.38 (3H, s). Glycine vinyl sulfone *trans* isomer ^{1}H-NMR (300 MHz, CD$_3$OD) δ 7.72 (2H, d), 7.24 (2H, d), 6.95 (1H, d), 6.87 (1H, dt), 3.84 (2H, d), 3.02 (3H, s), 2.38 (3H, s).

Synthesis of Boc-leucinyl-leucinyl-glycine vinyl sulfone cis *and* trans *isomers (Boc-LLG-cVS, Boc-LLG-tVS).* Boc-LLG-VS *cis* and *trans* isomers were synthesized from the tosic acid salts of *cis* and *trans* glycine vinyl sulfone as described for the synthesis of Boc-L$_3$-VS . The compounds were purified by flash column chromatography using ethyl acetate as the mobile phase. Yield (95%). Boc-LLG-cVS ^{1}H-NMR (300 MHz, CDCl$_3$) δ 7.52 (1H, m), 6.88 (1H, dq), 6.63 (1H, t), 6.54 (1H, d), 5.09 (1H, dd), 4.44 (1H, m), 3.94-4.09 (2H, m), 2.93 (3H, s), 1.40-1.82 (6H, m), 1.42 (9H, d), 0.95 (12H, m). Boc-LLGtVS ^{1}H-NMR (300 MHz, CDCl$_3$) δ 7.52 (1H, m), 6.56 (1H, d), 6.30 (2H, m), 5.05 (1H, dd), 4.47 (1H, m), 4.34 (1H, t), 4.03 (1H, m), 3.06 (3H, s), 1.40-1.82 (6H, m), 1.42 (9H, d), 0.95 (12H, m).

Synthesis of leucinyl-leucinyl-glycine vinyl sulfone tosic acid salt cis *and* trans *isomers (LLG-cVS, LLG-tVS).* The Boc protecting group was removed as described for the removal of Boc from Boc-leucine-vinyl sulfone described above. Yield (52%)

Synthesis of 4-hydroxy-3-nitrophenyl acetyl-leucine-leucine-glycine vinyl sulfone cis *and* trans *isomers (NP-LLG-cVS, NP-LLG-tVS).* The hapten 4-hydroxy-3-nitrophenyl acetic acid was added to LLG-VS *cis* and *trans* isomers as described for the synthesis of NP-AAF-VS above. The *cis* and *trans* products were purified by flash column chromatography using ethyl acetate as the mobile phase. NP-LLG-cVS ^{1}H-NMR (300 MHz, DMSO-d$_6$) δ 8.29 (1H, d), 8.26 (1H, t), 7.96 (1H, d), 7.80 (1H, s), 7.44 (1H, d), 7.05 (1H, d), 6.53 (1H, dt), 6.18 (1H, dt), 4.13-4.36 (4H, m), 3.47 (2H, s), 3.10 (3H, s), 1.48-1.62 (6H, m), 0.75-0.90 (12H, m). FAB mass spectrum: [M+H] calc'd for C$_{24}$H$_{36}$N$_4$O$_8$S: 541.6; found: 541.3. NP-LLG-tVS ^{1}H-NMR (300 MHz, DMSO-d$_6$) δ 8.29 (1H, d), 8.22 (1H, t), 7.79 (1H, s), 7.42 (1H, d), 7.05 (1H, d),6.70 (1H, dt), 6.64 (1H, d), 4.28 (2H, m), 3.94 (1H, t), 3.89 (1H, t), 3.46 (2H, d), 2.98 (3H, s), 1.48-1.62 (6H, m), 0.75-0.90 (12H, m). FAB mass spectrum: [M+H] calc'd for C$_{24}$H$_{36}$N$_4$O$_8$S: 541.6; found: 541.3.

Synthesis of phenolic vinyl sulfones

The phenolic vinyl sulfones were synthesized using the same protocol as described for the methyl vinyl sulfones except the phenolic version of the corresponding methyl phosponate sulfone was used.

Synthesis of 4-Hydroxy-thiophenyl-methyl-diethylphosponate ((EtO)$_2$P(O)CH$_2$S-Ph-OH). To a dried flask flushed with argon was added 4-hydroxy-thiophenol (90% stock, 4.44 g, 31.7 mmol) in 20ml of diethyl ether. Sodium hydride (1.27 g, 31.7 mmol) was slowly added and the reaction stirred at room temperature for 30 min. A solid mass formed upon standing. Diethyl iodomethyl phosphonate (8.11 g, 29.17 mmol) was added as a solution in 15 ml of diethyl ether. The reaction was then stirred at room temperature for three hours. The reaction was quenched with 60 ml of 0.5 M citrate buffer (pH 4.0) and the resulting aqueous phase was extracted with 3 × 40 ml volumes of diethyl ether. The combined organic layers were dried over MgSO$_4$ and concentrated to an oil. The crude oil was oxidized as described below without further purification.

Synthesis of 4-Hydroxy-thiophenyl-methyl-diethylphosponate sulfone ((EtO)$_2$P(O)CH$_2$S(O)$_2$-Ph-OH). The crude oil of 3-hydroxy-thiophenyl-methyl-diethylphosponate was dissolved in 30 ml of 1,4-dioxane and chilled on ice. Peracetic acid (30 ml, 32% solution, 145 mmol) was slowly added while stirring the reaction mixture. The reaction was maintained on ice for 20 minutes and then warmed to room temperature for 4 h. Crushed platinum on carbon (2.8 g) was added and the reaction stirred overnight at room temperature. Potassium iodide was used to insure that no peracetic acid remained. The platinum carbon solids were removed by filtration and the remaining solution diluted with 250 ml of saturated sodium bicarbonate. The aqueous phase was extracted with 3 × 100 ml portions of ethyl acetate. The combined organic layers were dried over MgSO$_4$ and dried to a crude solid by rotary evaporation. The pure product was obtained by re-crystallization from hexane/ethyl acetate (1:2). Yield (7.1 g, 23.4 mmol, 80%). 4-hydroxy-thiophenyl-methyl diethylphosponate sulfone ^{1}H-NMR (300 MHz, CDCl$_3$) δ 9.25 (1H, s), 7.80 (2H, d), 6.75 (2H, d), 4.22 (4H, p), 3.81 (2H, d), 1.36 (6H, t).

Synthesis of leucine-leucine-leucine vinyl sulfone phenol tosic acid salt (L$_3$-VS-PhOH). L$_3$-VS was synthesized as described previously [4] except that for the conversion of Boc-leucinal to Boc-leucine vinyl sulfone 4-hydroxy-thiophenyl-methyl diethylphosponate sulfone was used instead of methyl-thiomethyl diethylphosponate sulfone. The yields obtained in a typical reaction were similar to those reported for the methyl derivative. L$_3$-VS-PhOH ^{1}H-NMR (300 MHz, CD$_3$OD) δ 7.68 (2H, d), 6.95 (2H, d), 6.78 (1H, dd), 6.45 (1H, d), 4.61 (1H, m), 4.42 (1H, t), 3.85 (1H, t), 1.40-1.78 (9H, m), 0.82-1.02 (18H, m). FAB mass spectrum: [M+H] calc'd for C$_{25}$H$_{41}$N$_3$O$_5$S: 496.6; found: 496.3

Synthesis of 4-hydroxy-5-iodo-3-nitrophenyl acetyl-leucine-leucine-leucine vinyl sulfone phenol and glycine-leucine-leucine-leucine vinyl sulfone phenol (NLVS-PhOH, GL$_3$-VS-PhOH). NLVS-PhOH was synthesized by addition of 4-hydroxy-5-iodo-3-nitrophenyl acetic acid to L$_3$-VS-PhOH as described above. GL$_3$-VS-PhOH was synthesized by coupling of Boc-glycine to L$_3$-VS-PhOH with PyBop followed by deprotection of the Boc group using trifluoroacetic acid as described above. Similar yields were obtained. NLVS-PhOH ^{1}H-NMR (300 MHz, DMSO-D$_6$) δ 8.30 (1H, d), 8.06 (2H, d), 8.00 (1H, s), 7.89 (1H, s), 7.61 (2H, d), 6.94 (2H, d), 6.60 (1H, dd), 6.50 (1H, d), 4.52 (1H, m), 4.22 (2H, m), 1.28-1.65 (9H, m), 0.73-0.98 (18H, m). FAB mass spectrum: [M+H] calc'd for C$_{33}$H$_{45}$N$_4$O$_9$SI: 801.7; found: 801.4. GL$_3$-VS-PhOH 1H-NMR (500 MHz, CD$_3$OD) δ 7.67 (2H, d), 6.92 (2H, d), 6.75 (1H, dd), 6.46 (1H, d), 4.61 (1H, m), 4.41 (1H, dd), 4.34 (1H, m), 3.69 (2H, s), 1.41-1.69 (9H, m), 0.86-1.00 (18H, m). FAB mass spectrum: [M+H] calc'd for C$_{27}$H$_{44}$N$_4$O$_6$S: 553.7; found: 553.3.

Cells and cell culture
The mouse lymphoblastoid cell line EL-4 was maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. The human EBV-transformed cell line HOM-2 was maintained as described previously [19].

Antibodies
The poly-clonal rabbit sera against whole proteasome, LMP-7, LMP-2, Mecl-1, X, Y and Z were kind gifts of Migel Cruez and John Monaco (University of Cincinnati, Cincinnati, OH). The sera were prepared using 20S proteasomes and purified, recombinant β subunits as immunogens.

Fluorogenic substrate hydrolysis assay
Hydrolysis of the fluorogenic substrates: Suc-LLVY-AMC (chymotrypsin-like substrate), Boc-LRR-AMC (trypsin-like substrate), and Ac-YVAD-AMC (PGPH substrate) was measured. In a typical assay 0.4 µg of purified rabbit muscle 20S proteasomes (gift of Fred Goldberg, Harvard Medical School) was diluted to a final volume of 500 µl in buffer A (20 mM HEPES pH 8.0, 0.5 mM EDTA and 0.01% SDS). Fluorogenic substrates were added to a final concentration of 5 µM and progress curves measured by monitoring methyl coumaric acid (MCA) fluorescence (em = 460 nm, ex = 380) at 37°C. Inhibitors were added in the concentration ranges indicated after hydrolysis reached steady

state. The reaction was allowed to proceed for an additional 45 min. at 37°C. Fluorescence data were collected as a function of time and processed using the Sigma Plot scientific graphing program. k$_{obs}$ values were obtained by a non-linear least squares fit of the data to the equation for time-dependent or slow-binding inhibition [20]:

$$\text{Fluorescence} = v_s t + [(v_o - v_s)/k_{obs}][1 - \exp(-k_{obs}t)] \qquad (1)$$

Where v$_o$ is the initial velocity that decays over time to a final velocity v$_s$ with a rate constant, k$_{obs}$. k$_{obs}$/[I] values are reported as an average of three independent experiments obtained with different inhibitor concentrations.

Preparation of lysates from EL-4 cells
Cells were lysed using a bead smashing technique. Briefly, cells were washed three times with cold PBS and then pelleted. A volume of glass beads (< 106 microns, acid washed; Sigma Chem. Co., St. Louis, MO) equivalent to the volume of the pellet were added followed by a similar volume of homogenization buffer (50 mM Tris pH 7.4, 1 mM DTT, 5 mM MgCl$_2$, 2 mM ATP, 250 mM sucrose). Cells were vortexed at high speed for 1 min. The beads and broken cell debris were removed by centrifugation at 10,000 g for 5 min. The resultant homogenate was centrifuged at 10,000 g for 20 min to remove unbroken cells and nuclei. Protein concentration was determined using the BCA protocol described by the manufacturer (Pierce Chem. Co., Rockford, IL).

Labeling of proteasomes with peptide vinyl sulfones
Radioactive concentrations of the stock solutions of ^{125}I-labeled inhibitors were determined so that equivalent amounts of radioactivity were used for all labeling experiments. It should be noted, however, that not all inhibitors may not have been modified to the same extent during iodination [4]. It is thus difficult to determine the amount of unlabeled inhibitor present in a given labeling stock. As any unlabeled inhibitor may serve to compete for the proteasome active site and thus, decrease labeling, these reagents cannot be used as quantitative measures of inhibitory activity. Relative inhibition of radiolabeling of specific β subunits by cold competition can be assessed reliably in this fashion. In labeling experiments, labeled inhibitors were added to a final concentration of 1.8 × 10^4 Bq/ml. Samples were labeled for 2 h at 37°C unless noted otherwise. Samples were quenched by dilution of 4 × SDS sample buffer to 1 × (for one-dimensional SDS−PAGE) or by dissolving urea to a final concentration of 9.5 M (for two-dimensional SDS−PAGE).

Competition experiments
GL$_3$-VS, YL$_3$-VS, Bpa-L$_3$-VS, NLVS, and L$_4$-VS (0, 1, 5 and 10 µM) were added simultaneously with ^{125}I-YL$_3$-VS (1.8 × 10^4 Bq/ml) to EL-4 lysates prepared as described above (100 µg total protein in 100 µl homogenization buffer; see above). Samples were incubated for 2 h at 37°C, the labeling reaction quenched by the addition of 30 µl 4 × SDS-sample buffer and heating to 95°C for 10 min. Samples were separated by 12.5% SDS−PAGE and then subjected to autoradiography. Labeled polypeptide bands were quantitated by densitometry using an AlphaImager 2000 (Alpha Innotech Corporation).

Gel electrophoresis
One-dimensional SDS−PAGE, two-dimensional, non-equilibrium pH gradient SDS-PAGE (NEPHGE) [21], and fluorography [22] were performed as described.

Immuno-precipitation
All immuno-precipitations were performed as described previously [23].

Acknowledgements

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