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Identification of a potent and rapidly reversible inhibitor of the 20S-proteasome

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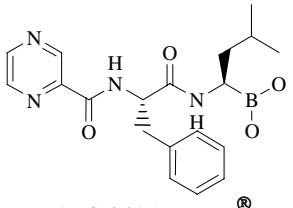
Abstract—Synthesis and in vitro characterization of novel, lactam boronic acid based, selective, and rapidly reversible inhibitor **14** of the 20S-proteasome is presented.

The proteasome, also referred to as the multicatalytic proteinase complex, is an unusually high molecular weight complex (about 700 kDa, 26S) that is found in both the cytoplasm and the nucleus of a wide variety of eukaryotic cell types.[1] The proteasome is comprised of the 20S central catalytic core, and two 19S regulatory caps.[2] The 19S regulatory caps are found at each end of the 20S cylinder-shaped complex, and regulate the entry of substrates into the central catalytic core. The 19S caps play a role in the recognition of substrates that have been targeted for degradation by the addition of multiple molecules of the 8.5 kDa polypeptide ubiquitin. The 19S caps facilitate the removal of the ubiquitin molecules from the substrate, and promote the unfolding of the substrate protein as it enters the central catalytic core. The catalytic core (20S) has a cylindrical shape with four stacked rings, each ring having seven subunits (α1-7β-1-7β1-7α1-7). The active site is an N-terminal threonine residue, which is deeply buried inside. The closest family member of this class of enzymes is the bacterial penicillin acylase. Early studies on the proteasome led to the delineation of three major different proteolytic activities (similar in specificity to chymotrypsin, trypsin, and peptidylglutamyl peptidase), each associated with a distinct component of the complex.

It is now well established that the proteasome is a major extralysosomal proteolytic system involved in the proteolytic pathways essential for diverse cellular functions such as cell division, antigen processing, and the degradation of short-lived regulatory proteins such as oncoproteins, transcription factors, and cyclins. Since the proteasome plays a key role in the orderly degradation of cyclins during the progression of the cell cycle, it plays a role in cell division. Additional studies demonstrated that disruption in any one of 12 out of 13 genes encoding yeast proteasome subunits results in an arrest in cellular proliferation, or an inability to degrade proteins, also suggesting a role for the proteasome in cell growth. Therefore, inhibition of the proteasome may be useful in the treatment of diseases resulting from aberrant cell division.[3] Further, the clinical proof of principal to demonstrate that proteasome inhibition may have utility in treating cancer was provided by the dipeptide boronic acid (**1**) (PS-341, bortezomib, Velcade®).[4] In addition, several other classes of proteasome inhibitors have been reported.[2,5] Herein we report the identification of a potent and rapidly reversible, inhibitor of the proteasome.

Focused screening of the company's compound collection identified **2** as a potent, lactam based boronate proteasome inhibitor for the chypotrypsin-like activity.[6]



1 PS-341 (Velcade®)

Keywords: : Proteasome inhibitor; Reversible.
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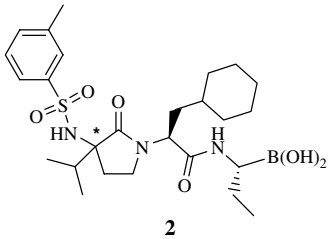
The compound was a 1:1 diastereomeric mixture at the quaternary center (indicated by * in the structure) at the ring junction.

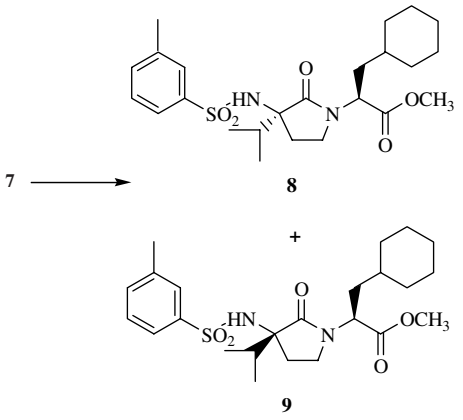


2

Proteasome IC_{50} 0.050 μM

At first, we set out to examine the stereospecificity of the inhibition. The required lactam was synthesized as shown in Scheme 1.

Intermediate **7** was resolved into two diastereomers (**8** and **9**) using reversed phase preparative HPLC.[7] The stereochemistry at the ring junction for these diastereomers was assigned based on X-ray crystallographic analysis.

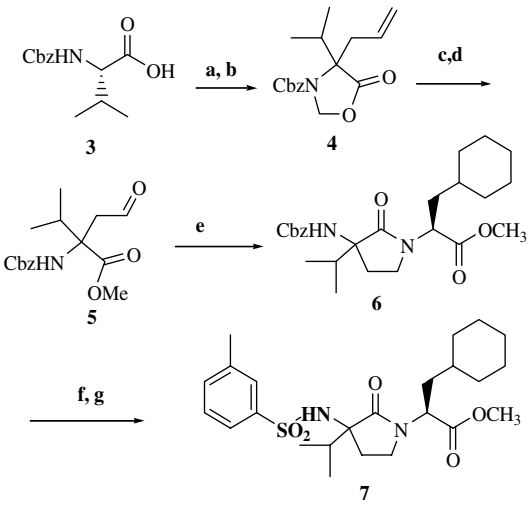


The required enantiomerically pure, protected, boronic acid component **13** was prepared using the modified literature procedure[8] as shown in Scheme 2. In a similar manner, the corresponding *S*-isomer was prepared from (−)-pinanediol.

8 and **9** were separately converted into the corresponding (*R*)- and (*S*)-diastereomers (**14** and **15**; respectively) (Scheme 3).

The diastereomer with *R*-stereochemistry at the ring junction (**14**) (IC_{50} 0.031 μM) was 12-fold more active than the corresponding *S*-isomer **15** (IC_{50} 0.4 μM), clearly indicating the preferential binding of one of the diastereomers.

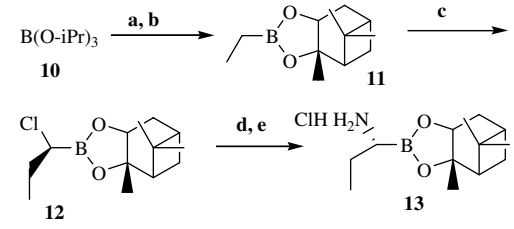
We further embarked on exploration of the boronate side chain. Since the methodology (shown in Scheme 2) was not suitable to explore substituted boronic acids, we investigated an alternative modular approach as shown in Scheme 4. In this modified approach, as demonstrated for *R*-leucine boronate ester **19**, the required substitution could be introduced at a common intermediate step, thereby providing flexibility and convenience.



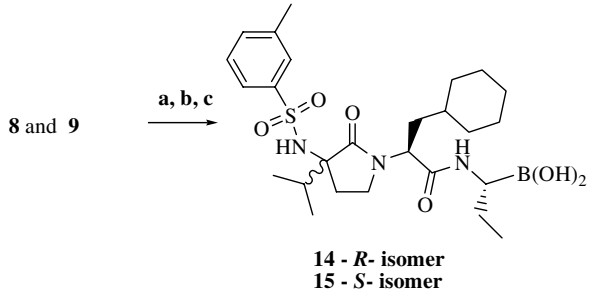
Scheme 1. Reagents and conditions: (a) paraformaldehyde, *p*-toluenesulfonic acid, toluene, 110 °C; (b) LiHMDS, allyl bromide, THF, −78 °C; (c) MeOH; (d) O_3, Me_2S, MeOH, −20 °C; (e) L-cyclohexyl alanine methyl ester, $NaHB(OAc)_3$, $ClCH_2CH_2Cl$, 0–80 °C; (f) H_2, 10%, Pd/C, MeOH; (g) *m*-CH_3–$PhSO_2Cl$, Et_3N, DMAP (cat), CH_2Cl_2.

Similarly, the corresponding *R*-valine boronate ester was synthesized. These boronate esters were converted into **20** and **21** using the methodology as described in Scheme 3. As seen in Table 1, the isobutyl side chain was preferred. The ethyl and isopropyl side chains were of similar activity.

Next, we examined the effect of replacement of the sulfonamide and cyclohexylmethyl alanine. These analogs

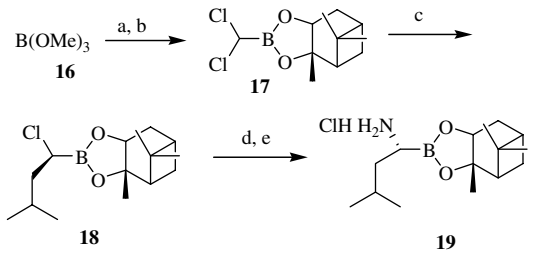


Scheme 2. Reagents and conditions: (a) EtMgBr, THF, −78 °C; (b) (+)-pinanediol; (c) CH_2Cl_2, LDA, $ZnCl_2$, THF, −78 °C to rt; (d) LiHMDS, THF, −78 °C to rt; (e) HCl, 1,4-dioxane.



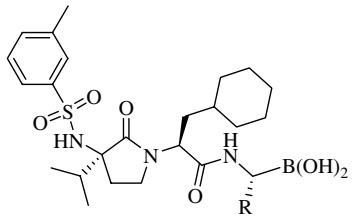
14 - *R*- isomer
15 - *S*- isomer

Scheme 3. Reagents and conditions: (a) LiOH, DME–H_2O; (b) **8** or **9**, PyOP, CH_2Cl_2; (c) $NaIO_4$, acetone–0.1 M aq NaOAc.



Scheme 4. Reagents and conditions: (a) CH_2Cl_2, *n*-BuLi, THF, −100 °C; (b) (+)-pinanediol; (c) *i*-BuMgBr, $ZnCl_2$, THF, −78 °C; (d) LiHMDS, THF, −78 °C to rt; (e) HCl, 1,4-dioxane.

Table 1.



Compd #	**R**	Proteasome IC_{50} μM
14	$-CH_2CH_3$	0.031 (±0.003)
20	$-CH(CH_3)_2$	0.028 (±0.002)
21	$-CH_2-CH(CH_3)_2$	0.008 (±0.001)

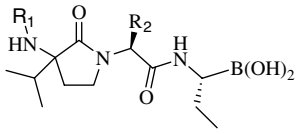
[a]The average of two independent IC_{50} determinations is reported, with the range between the two values in parantheses.

were synthesized from intermediate **5** using a sequence analogous to Schemes 1 and 3.

SAR from this exercise indicated that benzyloxy carbonyl is tolerated in place of *m*-tolylsulfonamide at $\mathbf{R_1}$. Interestingly, replacement with pyrazine amide, the side chain from PS-341, resulted in a loss of activity (**23**). Compound **24** having a phenyl group at $\mathbf{R_2}$ in place of cyclohexyl was active. Replacement of cyclohexyl, with a smaller group such as methyl (**25**), resulted in a loss of activity (Table 2).

In order to further characterize the proteasomal inhibition by these boronates, two of the most potent com-

Table 2.



Compd #	$\mathbf{R_1}$	$\mathbf{R_2}$	Proteasome IC_{50} μM
14	$-SO_2-$(*m*-Me)Ph	Cyclohexylmethyl	0.031 (±0.003)
22	$-CO-O-CH_2Ph$	Cyclohexylmethyl	0.032 (±0.0019)
23	–CO-pyrazine	Cyclohexylmethyl	>10
24	$-CO-O-CH_2Ph$	$-CH_2-Ph$	0.031 (±0.004)
25	$-CO-O-CH_2Ph$	–Me	1.00 (±0.17)

[a]The average of two independent IC_{50} determinations is reported, with the range between the two values in parantheses.

Table 3.

	Shift in IC_{50} upon dilution (fold)	
Compd #	20×	40×
14	16	35
21	2	5

pounds (**14** and **21**) were analyzed for reversibility using the following dilution method. Briefly, enzyme was pre-incubated with a wide range of inhibitor concentrations for 1 h, and then processed three different ways: (1) Dilution of the enzyme–inhibitor mixture 20-fold, and immediate addition of substrate, to follow enzyme activity for 1 h. (2) Dilution of the enzyme–inhibitor mixture 40-fold prior to the addition of substrate, to follow activity. (3) Confirmation of IC_{50} by addition of small aliquots of substrate directly to the enzyme–inhibitor mixture, to follow enzyme activity. A rapidly reversible inhibitor can be detected with this technique since the IC_{50} will shift approximately 20- to 40-fold after dilution due to the reduced free inhibitor concentration. Alternatively, an irreversible inhibitor would be expected to show little shift in the IC_{50} values after dilution.

The results from these experiments indicated that there was a significant difference in the reversibility of compounds **14** and **21** (Table 3).

The results for the dilution experiment with compound **14** are consistent with rapidly reversible inhibition, since the loss in inhibitor potency was similar to the extent of dilution of the enzyme–inhibitor mixture (i.e., 16-fold loss in potency after a 20-fold dilution). In contrast, the effects of dilution on the inhibitory potency of compound **21** were significantly reduced. A 20-fold dilution of enzyme–inhibitor produced only a 2-fold reduction in potency, whereas a 40-fold dilution reduced proteasomal inhibition just 5-fold. These results suggested that compound **21** was not a rapidly reversible inhibitor. Follow-up experiments with compound **21** indicated that there was a slow off-rate for dissociation of the compound from the proteasome (>5 h; data not shown). These results indicate that varying the **R** group, which is in the *alpha* position with respect to the boronate moiety, can alter not only the potency of inhibition of the proteasome, but also the reversible nature of the inhibition. Alteration of both the potency and reversibility of a proteasome inhibitor, as described in this report, would be predicted to significantly alter the therapeutic properties of these compounds.

Compound **14** also displayed >100-fold selectivity against chymotrypsin, trypsin, elastase, and several coagulation factors (Xa, XIa, VIIa).

In summary we have identified a potent, selective, and rapidly reversible, lactam boronic acid based inhibitor of the 20S-proteasome. Further characterization of this inhibitor and its cellular effects will be reported separately.[9]

Acknowledgements

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6. The proteolytic activity of the proteasome was determined by utilizing synthetic peptide substrates specific for the chymotrypsin-like activity of the proteasome. The activity of the proteasome toward the cleavage of succinyl-Leu-Leu-Val-Tyr-(7-amino-4-methyl coumarin) was measured by the increased fluorescence of the coumarin moiety after cleavage from the peptide. The sources of proteasomes were the proteasome enriched fraction from HL60 cells described above, and pure commercially available 20S human proteasomes (Affiniti Research Products, Mamhead, UK). The proteasome enriched fraction was assayed at 50 μg/mL concentration in a buffer containing 150 mM Tris–EDTA (pH 7.4). The pure 20S proteasomes were assayed at 3 μg/mL concentration in a buffer containing 25 mM hepes, 0.5 mM EDTA, 0.03% sodium dodecyl sulfate (pH 7.6). The activities of both preparations were monitored over time by measuring the increase in fluorescent signal (excitation at 360 nm; emission at 460 nm) in a Cytofluor Series 4000 multiwell platereader (Applied Biosystems). Compounds were evaluated for proteasome inhibition under steady state conditions where substrate concentration is approximately equal to the K_m, and the amount of substrate hydrolyzed during the reaction is less than 10% of the total substrate in the reaction. Appropriately diluted proteasome preparations were incubated with the inhibitor for 30 min at 37 °C prior to the initiation of the reaction by the addition of substrate. The substrate concentration for each assay was at, or below, the apparent K_m levels determined for each preparation (pure 20S proteasome, 10 μM; proteasome-enriched fraction, 50 μM). The reactions proceeded for 60 min at 37 °C before being read in the fluorescent platereader. Inhibition was quantitated by determination of the IC_{50} values for the given conditions. Two independent IC_{50} determinations is done. Values are reported as IC_{50} with range between the two values in paranthesis. There was less than 25% difference between the two IC_{50} determinations for all cases.
7. The diastereomers were separated using a chiral preparative column (chiral OJ column, 50 mm × 500 mm, flow rate 50 mL/min), eluting with ethanol/heptane (5/95).
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