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Proteasome inhibitors for cancer therapy

Mohamed Iqbal, Patricia A. Messina McLaughlin, Derek Dunn, Satish Mallya, Jean Husten, Mark A. Ator, Sankar Chatterjee *

Cephalon Inc., 145 Brandywine Parkway, West Chester, PA 19380, United States

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ABSTRACT

Proteasome, a large multicatalytic proteinase complex that plays an important role in processing of proteins, has been shown to possess multiple catalytic activities. Among its various activities, the 'chymotrypsin-like' activity of proteasome has emerged as the focus of drug discovery efforts in cancer therapy. Herein we report chiral boronate derived novel, potent, selective and cell-permeable peptidomimetic inhibitors **6** and **7** that displayed activity against various rodent and human tumor cell lines (in vitro).

1. Introduction

Proteasome is a large multicatalytic proteinase complex that plays an important role in several critical cellular functions including the processing of proteins involved in cell cycle progression and gene expression.[1,2] The 26S form of the proteasome, responsible for the ATP-dependent degradation of poly-ubiquitinated proteins, is composed of one copy of the catalytic core, known as the 20S subunit and two copies of the 19S regulatory subunit. The X-ray crystal structure of the 20S subunit from an archaebacterial proteasome revealed it to be a barrel-shaped structure made up of four stacked rings.[3] The two outer rings are each composed of seven identical α-subunits and the two inner rings are each composed of seven identical β-subunits. The catalytic nucleophile is the hydroxyl group of the N-terminal threonine (Thr) of the β-subunit; thus this enzyme is the first reported member of the threonine proteinase class. The proteasome from higher organisms has the same quaternary structure;[4] however the seven α and seven β subunits are distinct from one another. The 20S proteasome has been shown to possess multiple catalytic activities, including chymotryptic, tryptic and peptidylglutamyl-like activities; thus the complex is capable of cleaving most peptide bonds. However, among its various activities, the 'chymotrypsin-like' activity of the proteasome has emerged as the biological function of greatest interest and the focus of drug discovery efforts. Increased levels of this enzyme and subsequent protein breakdown have been implicated in many disease states including muscular dystrophy, cachexia accompanying cancer and malnutrition, lupus and cancers such as acute leukemia.[5] The proteasome also controls levels of proteins critical for cell cycle control, including p53, p27 and cyclin B, and is responsible for activation of the transcription factor NF-κB through the degradation of its regulatory subunit IκBα.[6] Thus, development of the proteasome inhibitors has emerged as an attractive target for cancer therapy.[7] Previously, we reported on novel, potent and selective peptidomimetic inhibitors of the chymotrypsin-like activity of proteasome containing an aldehyde enzyme reactive group at the P_1-position (Fig. 1, compounds **1** and **2**, IC_{50} values of 2 and 6 nM, respectively).[8,9] Compound **1**, subsequently, found utility as a tool in proteasome research.[10–14]

We also communicated the corresponding α-ketocarboxamide (**3**, IC_{50} 13 nM),[15] P′-extended ketoamide (**4**, IC_{50} 1 nM)[16] and pinacol-derived boronate ester (**5**, IC_{50} 6 nM)[15] as potent inhibitors of the chymotrypsin-like activity of proteasome (Fig. 1).

As a part of our continuous search for novel, potent, selective, and drug-like inhibitors of the proteasome, we explored additional enzyme reactive groups known to inhibit different classes of serine and cysteine proteases (unpublished). We also employed a chiral boronate derived from (+)-pinanediol as the enzyme reactive group. This research resulted in potent, selective and cell-permeable boronic ester inhibitors **6 and 7** (Fig. 2) that were active in vitro against various tumor cell lines (rodent and human).

Abbreviations: BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DMSO, dimethyl sulfoxide; HMPA, hexamethyl phosphoramide; HOBt, 1-hydroxybenzotriazole; NMM, *N*-methylmorpholine; DMF, *N*,*N*-dimethyformamide; LDA, lithium diisopropylamide; THF, tetrahydrofuran; TBTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate; HATU, 1-hydroxy-7-azabenzotriazole uranium salt; HOAt, 1-hydroxy-7-azabenzotriazole; AMC, 7-amido-4-methylcoumarin; AFC, 7-amido-4-trifluoromethylcoumarin; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

* Corresponding author.
E-mail address: Sankar.Chatterjee24@gmail.com (S. Chatterjee).

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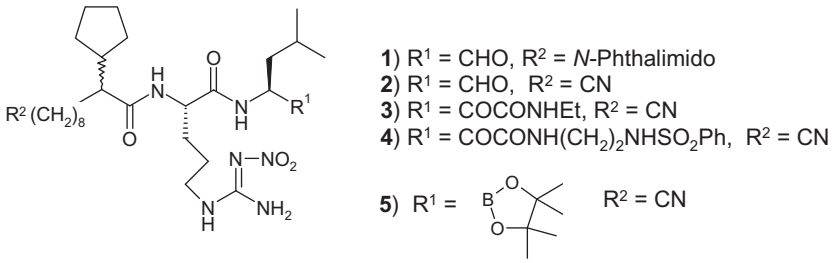


Figure 1. Chemical structures of compounds **1**–**5**.

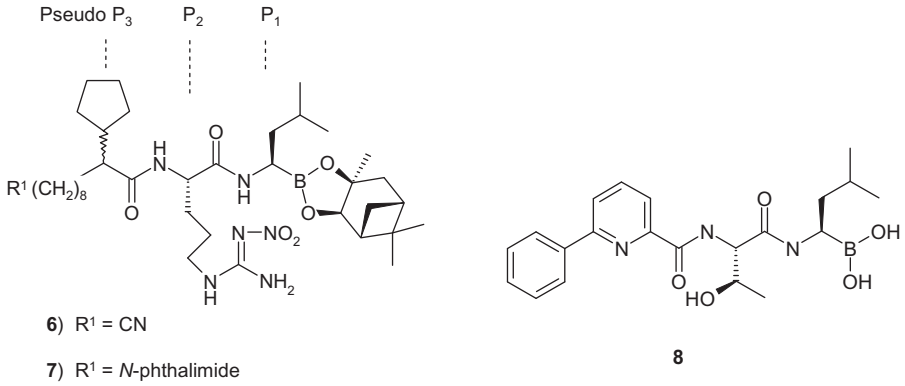


Figure 2. Chemical structures of compounds **6**–**8**.

Subsequently, compounds **6 and 7** provided the basis for additional synthetic exploration en route to compound **8**, a current clinical candidate in the oncology area.[17] In this report, we describe the characteristics of compounds **6 and 7** disclosing their proteasome inhibition data (against both isolated and intracellular enzyme) and selectivity profile. We also disclose their in vitro activity against a spectrum of tumor cell-lines.

While our effort was in progress, compound **9** (bortezomib, Fig. 3) was approved for the treatment of multiple myeloma and mantle cell lymphoma, validating the concept of targeted proteasome inhibition in cancer therapy.[18,19]

2. Chemistry

Syntheses of racemic acids **15 and 16** are shown in Scheme 1. Commercially available cyclopentyl acetic acid (compound **10**) was esterified with benzyl alcohol to generate compound **11** that was alkylated with 1,8-diiodooctane to produce compound **12**. Displacement of the iodo group in compound **12** with cyano and N-phthalimido groups generated compounds **13** and **14**, respectively. Debenzylation of compounds **13** and **14** produced acids **15** and **16**.

Scheme 2 depicts the synthesis of compound **22**. Commercially available boronic acid **17** was combined with (1*S*,2*S*,3*R*,5*S*)-(+)-pinanediol (compound **18**) to generate compound **19** that underwent a stereospecific chlorination reaction to generate compound **20**. Treatment of compound **20** with lithium hexamethyldisilazide produced compound **21** that was desilylated in presence of hydrochloric acid to generate the free amine as its hydrochloride salt **22**. The procedure was adapted from various reports from Matteson's laboratories.[20–22]

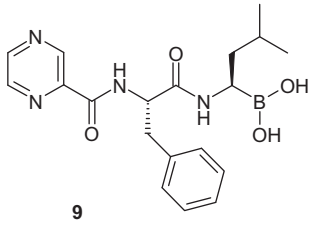


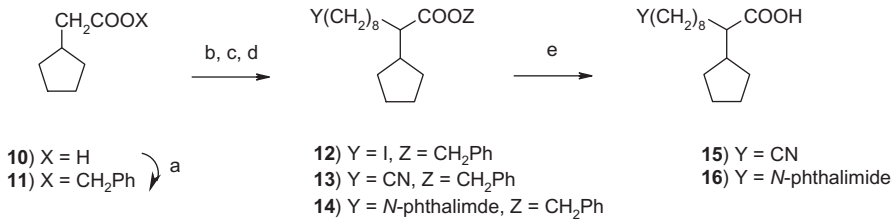
Figure 3. Chemical structure of compound **9**.

Scheme 3 displays the synthesis of compounds **6**, **7**, **26** and **27** respectively. Commercially available *t*Boc-Arg (NO_2)-OH (compound **23**) and boronate **22** (from Scheme 2) were coupled to afford compound **24**. Acidic treatment of compound **24** generated compound **25** as its hydrochloride salt that was combined with compounds **15** and **16** (from Scheme 1) to generate compounds **6** and **7**, respectively. Compounds **6** and **7** were subsequently converted to their corresponding boronic acid derivatives **26** and **27** via a boronate exchange reaction. All compounds were profiled as mixtures of diastereomers at pseudo-P_3 site (Fig. 2).

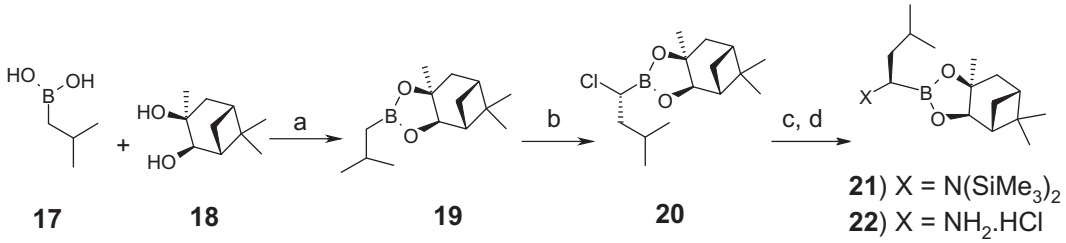
3. Biology

3.1. Enzyme inhibition

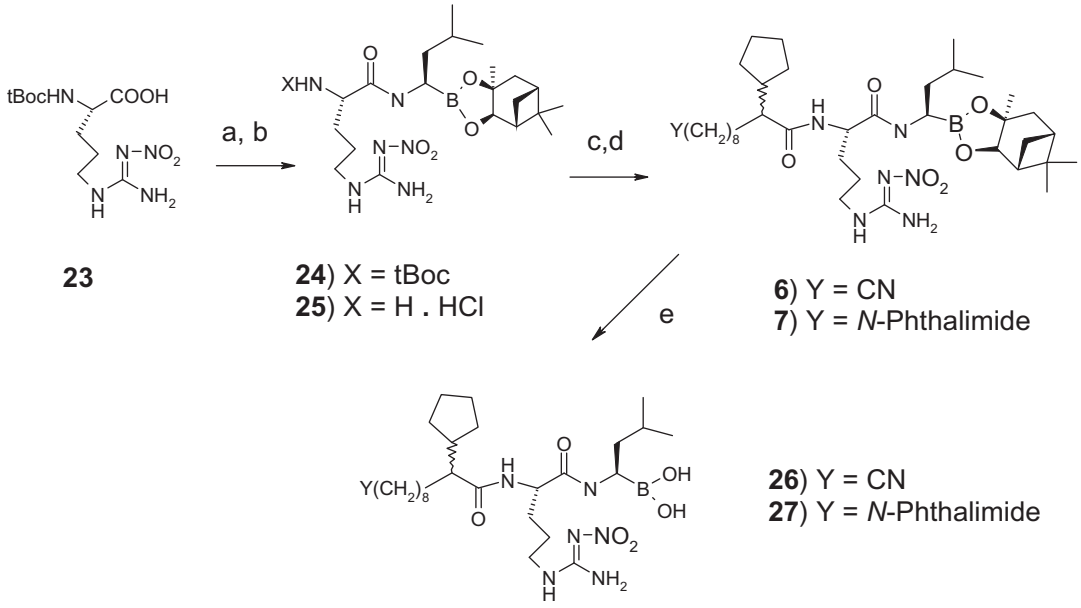
Partial purification of the proteasome from postmortem human liver was achieved by ion-exchange chromatography, ammonium sulfate precipitation and gel filtration according to published procedures.[23,24] Compounds were tested for their ability to inhibit the chymotrypsin-like (CT-like) activity of the 20S proteasome utilizing the fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-aminomethyl coumarin (Suc-LLVY-AMC).[25] They were also evaluated for their ability to inhibit intracellular proteasome activity in Molt-4 human T-cell leukemia lymphocytes.[10] The ratio of IC_{50} values for inhibition of enzyme in Molt-4 cells vs. isolated enzyme was calculated to offer a measure of the cell-permeability of an inhibitor (Table 1); a lower ratio reflected superior cell-permeability of an inhibitor. Compounds were also profiled against several related enzymes, for example, the proteasome's trypsin-like activity, bovine α-chymotrypsin (α-CT) and human calpain I, a cysteine protease (Table 1).



Scheme 1. Reagents and conditions: (a) Benzyl alcohol, *p*-toluenesulfonic acid, benzene, reflux (Dean–Stark), 2 h, quantitative; (b) compound **11**, LDA, 1,8-diiodooctane, THF–hexane, HMPA, −78 to 0 °C, 2.5 h; 45%; (c) for compound **13**: compound **12**, NaCN, DMSO, 70–75 °C; 80%; (d) for compound **14**: compound **12**, potassium phthalimide, DMF, 70–75 °C; 85%; (e) (i) for compound **15**: compound **13**, H_2, 10% Pd-C (50% water content); MeOH, 40 psi, 2 h, quantitative; (ii) for compound **16**: compound **14**, H_2, 10% Pd-C (50% water content); MeOH, 40 psi, 2 h, quantitative.



Scheme 2. Reagents and conditions: (a) Ether, room temp, 24 h; 94%; (b) (i) LDA in hexane–THF, compound **19** in THF–CH_2Cl_2, −70 °C, 1 h; (ii) 1.0 M $ZnCl_2$ in ether, −78 °C, 3 h; 89%; (c) for compound **21**: compound **20**, $LiN(SiMe_3)_2$, THF, −78 °C to room temperature, overnight; quantitative; (d) for compound **22**: compound **21**, 4 N HCl in dioxane, ether, 0 °C to room temperature 4 h; 73%.



Scheme 3. Reagents and conditions: (a) for compound **24**: compound **22** (Scheme 2), HATU, HOAt, NMM, DMF, 0 °C to room temperature, 5 h, 66% (b) for compound **25**: compound **24**, ether–dioxane, 4 N HCl in dioxane, 0 °C to room temperature, 4 h, 90%; (c) for compound **6**: compound **15** (Scheme 1), BOP, HOBt, NMM, DMF, 0 °C to room temp, 60%; (d) for compound **7**: compound **16** (Scheme 1), BOP, HOBt, NMM, DMF, 0 °C to room temp, 65%; (e) 2-methylpropylboronic acid, 2 N HCl, MeOH, hexane, 70–75%.

3.2. In vitro anti-tumor activity

The effects of the inhibitors **6 and 7** on in vitro viability of a panel of human and rodent cell lines representing different tumor types were investigated. The cell lines were AT-2 (rat prostatic carcinoma), DU-145 (human prostatic carcinoma), PANC-1 (human pancreatic carcinoma), SKMEL-5 (human melanoma), OVCAR-3 (human ovarian carcinoma), MCF-7 (human breast cell carcinoma), and Lewis lung (mouse lung carcinoma), respectively. The results of these studies are presented in Table 2.

4. Discussion

As shown in Table 1, both boronic esters **6 and 7** and boronic acids **26 and 27** were potent inhibitors of the chymotrypsin-like activity of isolated proteasome (IC_{50} ca. 4–9 nM). These compounds were uniformly unable to inhibit the trypsin-like activity of the enzyme complex at concentrations up to 10 μM, bovine α-chymotrypsin-like activity (IC_{50} >10 μM), and human calpain I, a cysteine protease at concentrations up to 10 μM, respectively. However, the boronic esters **6 and 7** displayed superior potency

Table 1
Enzyme inhibition data for compounds **6**, **7**, **26** and **27**

Compd	Prot. CT-like IC_{50}[a] (nM)	Prot. CT-like Molt-4 IC_{50}[a] (nM)	Ratio Molt-4 IC_{50}/enzyme IC_{50}	Prot. trypsin-like % inh @ 10 μM[b]	Bovine α-CT % inh @ 10 μM[b]	Human calpain I % inh @ 10 μM[b]
6	3.5 ± 0.5	240 ± 50	69	0	24	2
7	3.5 ± 1.3	70 ± 10	20	20	30	11
26	8.7 ± 3.2	7340 ± 1640	840	5	26	0
27	4.1 ± 1.6	2830 ± 1560	690	3	30	0

[a] Mean ± S.D., n = 3–6.
[b] Results are the averages of two independent experiments, each performed in triplicate.

Table 2
Effect of compounds **6** and **7** on viability of tumor cell lines[a]

Compd	AT-2 IC_{50} (nM)	DU-145 IC_{50} (nM)	PANC-1 IC_{50} (nM)	SKMEL-5 IC_{50} (nM)	OVCAR-3 IC_{50} (nM)	MCF-7 IC_{50} (nM)	Lewis lung IC_{50} (nM)
6	>1 μM	293	283	85	69	425	585
7	>1 μM	167	184	82	29	292	513

[a] Results are the averages of two independent experiments, each performed in triplicate.

in the Molt-4 cell assay (IC_{50} 240 and 70 nM, respectively) compared to the corresponding boronic acids **26 and 27** (IC_{50} 7340 and 2830 nM, respectively). This was reflective of the superior cell-permeability of compounds **6 and 7**. However, it should be noted that both bortezomib (compound **9**) and compound **8**, next generation inhibitor from follow-on work from our laboratories belong to the class of boronic acids. However, based on their superior cell activity, compounds **6 and 7** were advanced and profiled against a wide range of tumor types as shown in Table 2. Both compounds displayed significant activity against various tumor cell-lines except AT-2 (rat prostatic tumor) in the range of inhibitor concentrations tested, but were efficacious against the human prostate tumor line DU-145. The basis for this difference is unknown at this time. Based on their in vitro potency, intra-cellular inhibitory capability and in vitro anti-tumor activity, compounds **6 and 7** had emerged as the launching pad for additional exploration/modification in the series.

5. Conclusion

In this report, we disclosed compounds **6 and 7**, chiral boronate derived potent, selective and cell-permeable peptidomimetic inhibitors of the chymotrypsin-like activity of proteasome. These inhibitors displayed activity against various rodent and human tumor cell lines (in vitro). This research provided the impetus for additional exploration that led to potent, selective and orally bioavailable (rat and mouse) inhibitor **8** that entered into clinic for the treatment of myeloma.

6. Experimentals

6.1. Chemistry

6.1.1. General

Commercially available reagents and solvents were utilized without any further purification. ^{1}H NMR spectra were obtained at 300 or 400 MHz in the solvent indicated with tetramethylsilane (TMS) as an internal standard. Coupling constants (J) are reported in Hertz (Hz). The following abbreviations were used: s: singlet; d: doublet; dd: doublet of doublet; m: multiplet; q: quartet; t: triplet and b: broad. Column chromatography was performed on 230–400 mesh silica gel 60. Analytical reverse phase HPLC was performed using a Zorbax RX-C8, 5 × 150 mm column eluting with a mixture of acetonitrile and water containing 0.1% trifluoroacetic acid with a gradient of 10–100%.

6.1.2. Benzyl-2-cyclopentyl acetate (11)

A mixture of cyclopentylacetic acid (compound **10**, 10.02 g, 78.2 mmol), benzyl alcohol (8.45 g, 78.2 mmol) and *p*-toluenesulfonic acid monohydrate (1.48 g, 7.82 mmol) in benzene (60 mL) was refluxed under Dean–Stark condition for 2 h, cooled, concentrated under vacuum and redissolved in ether (60 mL). The organic layer was washed successively with water (1 × 25 mL), saturated aq $NaHCO_3$ solution (2 × 25 mL), water (1 × 25 mL), and brine (1 × 25 mL), dried ($MgSO_4$) and evaporated to generate compound **11** (15.40 g, quantitative) as a viscous oil that was utilized in next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.35 (m, 5H), 5.10 (s, 2H), 2.40 (d, J = 8 Hz, 2H), 2.26 (m, 1H), 1.81 (m, 2H), 1.6 (m, 4H), 1.18 (m, 2H).

6.1.3. 10-Iodo-2-[(*R*,*S*)-cyclopentyl]decanoic acid, benzyl ester (12)

A solution of compound **11** (3.96 g, 18 mmol) in anhydrous THF (10 mL) was added dropwise to a freshly prepared solution of lithium diisopropylamide (20 mmol) in hexane/THF (8 mL/20 mL) maintained at −78 °C. The mixture was stirred for additional 0.5 h and to it a solution of 1,8-diiodooctane (7.19 g, 20 mmol) in hexamethylphosphoramide (3.50 g, 20 mmol) was added. The reaction mixture was stirred at −78 °C for 0.5 h, slowly brought to 0 °C over a period of 2 h, quenched by slow addition of 12% aq NH_4Cl (50 mL) and extracted with ether (3 × 50 mL). The combined organic layer was washed with brine (2 × 25 mL), dried ($MgSO_4$) and concentrated to generate a crude product that was purified by flash chromatography (silica gel, 1% ethyl acetate in hexanes) to generate compound **12** (3.23 g, 45%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.35 (m, 5H), 5.15 (s, 2H), 3.20 (t, J = 8 Hz, 2H), 2.20 (m, 1H), 2.00 (m, 1H), 1.80–1.20 (a series of m, 22H).

6.1.4. 10-Cyano-2-[(*R*,*S*)-cyclopentyl]decanoic acid, benzyl ester (13)

A mixture of compound **12** (3.99 g, 8.7 mmol) and sodium cyanide (0.47 g, 9.6 mmol) in anhydrous DMSO (15 mL) was heated at 70–75 °C in a well-ventilated hood for 0.5 h, cooled and quenched with ice-water (~50 g). The aqueous layer was extracted into ether (3 × 25 mL) and the combined organic layer was successively washed with water (1 × 25 mL) and brine (1 × 25 mL), dried ($MgSO_4$) and evaporated to generate compound **13** (2.89 g, 80%) as a viscous oil that was utilized without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.35 (m, 5H), 5.15 (s, 2H), 2.30 (t, J = 8 Hz, 2H), 2.20 (m, 1H), 2.00 (m, 1H), 1.80–1.30 (m, 22H).

6.1.5. 10-(*N*-Phthalimido)-2-[(*R*,*S*)-cyclopentyl]decanoic acid, benzyl ester (14)

A mixture of compound **12** (1.21 g, 2.6 mmol) and potassium phthalimide (0.54 g, 3.0 mmol) in anhydrous DMF (8 mL) was heated at 70–75 °C for 0.5 h, cooled and quenched with ice-water (~40 g). The aqueous layer was extracted into ether (3 × 25 mL) and the combined organic layer was successively washed with water (1 × 25 mL) and brine (1 × 25 mL), dried ($MgSO_4$) and evaporated to generate compound **14** (1.24 g, 85%) as a viscous oil that was utilized directly in the next step. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.85 (m, 2H), 7.70 (m, 2H), 7.35 (m, 5H), 5.15 (s, 2H), 2.30 (t, J = 8 Hz, 2H), 2.20 (m, 1H), 2.00 (m, 1H), 1.80–1.30 (m, 22H).

6.1.6. 10-Cyano-2-[(*R*,*S*)-cyclopentyl]decanoic acid (15)

A mixture of compound **13** (2.88 g, 8.00 mmol) and 10% Pd-C (0.6 g, DeGussa, 50% H_2O content) in anhydrous methanol (35 mL) was hydrogenated for 2 h (42–26 psi), filtered through a pad of celite and concentrated to generate 2.02 g (quantitative) of compound **15** as a viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.35 (t, J = 8 Hz, 2H), 2.20 (m, 1H), 2.00 (m, 1H), 1.90–1.30 (a series of m and a broad, 23H).

6.1.7. 10-(*N*-Phthalimido)-2-[(*R*,*S*)-cyclopentyl]decanoic acid (16)

Following the same procedure as described above for the synthesis of compound **15**, 0.41 g of compound **14** was hydrogenated to afford compound **16** as viscous oil (0.31 g, quantitative). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.85 (m, 2H), 7.70 (m, 2H), 3.70 (t, J = 8.0 Hz, 2H), 2.15 (m, 1H), 1.95 (m, 1H), 1.85–1.10 (a series of m and a broad, 23H).

6.1.8. (2-(2-Methylpropyl)-(3a*S*,4*S*,6*S*,7a*R*)-hexahydro-3a,5,5-trimethyl-4,6-methano-1,3,2-benzodioxaborole (19)

A mixture of 2-methylpropylboronic acid (compound **17**,15.00 g, 0.147 mol) and (+)-pinanediol (compound **18**, 23.90 g, 0,140 mol) in ether (300 mL) was stirred at room temperature for 24 h, dried (Na_2SO_4) and evaporated to dryness. The crude residue was purified by flash column chromatography (silica gel, hexane–ethyl acetate: 9:1) to generate compound **19** as an oil (32.60 g, 94%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 4.28 (dd, J = 8.8 Hz, 2.0 Hz, 1H); 2.30 (m, 1H); 2.18 (m, 1H); 1.96 (t, J = 5.3 Hz, 1H); 1.86 (m, 1H); 1.78 (d, J = 6.8 Hz, 1H); 1.68 (m, 1H); 1.30 (s, 3H); 1.25 (s, 3H); 1.01 (d, 1H); 0.9 (d, J = 6.6 Hz, 6H); 0.81 (s, 3H); 0.69 (m, 2H).

6.1.9. 2-[(1*S*)-1-Chloro-3-methylbutyl]-(3a*S*,4*S*,6*S*,7a*R*)-hexahydro-3a,5,5-trimethyl-4,6-methano-1,3,2-benzodioxaborole) (20)

A freshly prepared cooled solution (−30 °C) of lithium diisopropylamide (LDA, 0.254 mol) in hexane–THF (1:2, 100 mL) was added slowly via canulation to a solution of compound **19** (50 g, 0.212 mol) in a mixture of THF (700 mL)–CH_2Cl_2 (50 mL), cooled to −70 °C and maintained at that temperature throughout the addition. After an additional stirring of 30 min, a solution of $ZnCl_2$ (1.0 M in ether, 340 mL, 0.34 mol) was slowly added to the reaction mixture that was stirred at −78 °C for an additional 3 h, slowly brought to room temperature, evaporated to dryness and partitioned between petroleum ether (1 L) and 10% aq NH_4Cl solution (800 mL). The separated aqueous layer was further extracted into petroleum ether (2 × 150 mL). The combined organic layers were dried (Na_2SO_4) and concentrated to generate compound **20** as an oil (59 g, 89%) that was utilized without further purification. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 4.43 (dd, J = 8.8 Hz, 1.8 Hz, 1H); 3.59 (m, 1H); 2.33 (m, 1H); 2.21 (m, 1H); 2.01 (m, 1H); 1.88 (m, 1H); 1.84–1.55 (m, 4.H); 1.34 (s, 3H); 1.26 (s, 3H); 1.09 (d, J = 10.1 Hz, 1H); 0.9 (d, J = 6.8 Hz, 3H); 0.87 (d, J = 6.4 Hz, 3H); 0.82 (s, 3H).

6.1.10. *N*,*N*-Bis(trimethylsilyl)-(1*R*)-1-[(3a*S*,4*S*,6*S*,7a*R*)-hexahydro-3a,5,5-trimethyl-4,6-methano-1,3,2-benzodioxaborol-2-yl]-3-methylbutylamine (21)

A solution of lithium bis(trimethylsilyl)amide in THF (1.0 M, 189 mL, 0.189 mol) was added dropwise to a solution of compound **20** (59.0 g, 0.189 mol) in anhydrous THF (580 mL) maintained at −78 °C. The reaction mixture was allowed slowly to return to room temperature, stirred overnight and concentrated. The crude residue was taken into dry hexane (800 mL), stirred for 2 h and filtered through a celite pad. Removal of solvent produced compound **21** (79 g, quantitative) as an oil that was immediately taken into the next step. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 4.33 (dd, J = 8.6 Hz, 1.5 Hz, 1H); 2.58 (m, 1H); 2.29 (m, 1H); 2.18 (m, 1H); 1.95 (t, J = 5.9 Hz, 1H); 1.85 (m, 1H); 1.9–1.55 (m, 3H); 1.31 (s, 3H); 1.24 (s, 3H); 1.17 (m, 1H); 1.01 (d, J = 10.6 Hz, 1H); 0.85 (d, J = 6.6 Hz, 3H), 0.83 (d, J = 6.6 Hz, 3H); 0.80 (s, 3H); 0.08 (s, 18H).

6.1.11. (1*R*)-1-[(3a*S*,4*S*,6*S*,7a*R*)-Hexahydro-3a,5,5-trimethyl-4,6-methano-1,3,2-benzodioxaborol-2-yl]-3-methylbutylamine hydrochloride salt (22)

A solution of 4 N HCl in dioxane (193 mL, 0.772 mol) was slowly added to a solution of compound **21** (79.00 g, 0.193 mol) in dioxane (100 mL) and ether (200 mL) maintained at 0 °C. The cooling bath was removed and the reaction mixture was stirred for 4 h at room temperature and concentrated. The residue was taken into anhydrous hexane (500 mL), cooled to 0 °C and treated with 2 M solution of HCl in diethyl ether (48 mL, 0.096 mol). The reaction mixture was stirred at 0 °C for 1 h, concentrated, diluted with anhydrous hexane and stirred at room temperature overnight. The solid that separated was filtered and dried under high vacuum to afford compound **22** (38.1 g, 73%). An additional crop of 4.13 g was obtained on concentration of the mother liquor. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.85 (br, 3H), 4.45 (t, J 9.2 Hz, 1H), 2.78 (m, 1H), 2.34 (m, 1H), 2.21 (m, 1H), 2.01 (t, J = 5.3 Hz, 1H), 1.89 (m, 1H), 1.82–1.65 (m, 3H), 1.49 (m, 1H), 1.38 (s, 3H), 1.27 (s, 3H), 1.12 (d, J = 1.1 Hz, 1H), 0.87 (d, J = 6.6 Hz, 6H), 0.83 (s, 3H).

6.1.12. Carbamic acid 1,1-dimethylethyl ester, *N*-[(1*S*)-1-[[[(1*R*)-1-[3a*S*,4*S*,6*S*,7a*R*)-hexahydro-3a,5,5-trimethyl-4,6-methano-1,3,2-benzodioxoborol-2-yl]-3-methylbutyl]amino]carbonyl]-4-[[imino(nitroamino)methyl]amino]butyl] (24)

A cooled (0 °C) solution of commercially available compound **23** (15.70 g, 49.30 mmol) in anhydrous DMF (100 mL) was successively treated with NMM (13.60 mL, 123 mmol), HOAt (6.71 g, 49.30 mmol) and HATU (18.70 g, 49.30 mmol). The mixture was stirred for an additional 15 min and then treated with compound **22** (12.40 g, 41.10 mmol). The cooling bath was removed and the mixture was stirred for 5 h, diluted with ethyl acetate (800 mL) and washed successively with 2% aq citric acid solution (2 × 150 mL), 2% aq $NaHCO_3$ solution (2 × 150 mL) and brine (2 × 100 mL). Each aqueous phase was re-extracted with ethyl acetate (1 × 100 mL). The combined organic layer was dried (Na_2SO_4), concentrated under high vacuum, dissolved in ether (150 mL) and triturated with hexane (600 mL). The separated solid was filtered and purified by flash column chromatography (silica gel, eluant: 1:1 ethyl acetate to ethyl acetate) to generate compound **24** (15.2 g, 66%); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.80 (br, 1H), 8.50 (br, 1H), 7.87 (br, 2H), 7.01 (br, 1H), 4.07 (dd, J = 7.9 Hz, 2.01 Hz, 1H), 4.00 (m, 1H), 3.12 (m, 2H), 2.55 (m, 1H), 2.20 (m, 1H), 2,01 (m, 1H), 1.83 (t, J = 5.1 Hz, 1H), 1.78 (m, 1H), 1.74–1.44 (m, 6H), 1.38 (s, 9H), 1.33 (m, 1H), 1.25 (s, 2H), 1.24 (s, 3H), 1.22 (s, 3H), 0.84 (d, J = 6.6 Hz, 6H), 0.81 (s, 3H).

6.1.13. (2*S*)-2-Amino-5-[[imino(nitroamino)methyl]amino] pentamide-*N*-[(1*R*)-1-[3a*S*,4*S*,6*S*,7a*R*)-hexahydro-3a,5,5-trimethyl-4,6-methano-1,3,2-benzodioxaborol-2-yl]-3-methyl butyl]-, hydrochloride salt (25)

A solution of compound **24** (4.04 g, 7.06 mmol) in a mixture of dioxane (40 mL) and ether (7 mL) maintained at 0 °C was treated with a 4 N solution of HCl in dioxane (15 mL). The cooling bath was removed and the reaction mixture was stirred for an additional 4 h, concentrated under high vacuum, and stirred in ether (50 mL) overnight to generate compound **25** (3.18 g, 90%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.56 (br, 2H), 8.22 (br, 3H), 7.97 (br, 2H), 4.28 (dd, J = 8.6 Hz, 2.0 Hz, 1H), 3.77 (m, 1H), 3.04 (m, 2H), 2.28 (m, 1H), 2.11 (m, 2H), 1.92 (t, J = 5.5 Hz, 1H), 1.83 (m, 1H), 1.79–1.59 (m, 4H), 1.31 (s, 4H), 1,19 (d J = 6.0 Hz, 1H), 1.24 (s, 3H), 1.22 (s, 3H), 0.84 (d, J = 6.6 Hz, 6H), 0.81 (s, 3H)

6.2. Compounds 6 and 7

General procedure: A cooled (0 °C) solution of compounds **15 or 16** (1 equiv) in anhydrous DMF was successively treated with NMM (3 equiv), HOBt (1.1 equiv) and BOP (1.1 equiv). The mixture was stirred for an additional 15 min and then treated with compound **25** (1.05 equiv). The cooling bath was removed and the mixture was stirred for 5 h, diluted with ethyl acetate and washed successively with 2% aq citric acid solution, 2% aq NaHCO3 solution and brine. Each aqueous phase was re-extracted with ethyl acetate. Combined organic layer was dried (Na_2SO_4), concentrated under high vacuum and purified by flash column chromatography (eluant: methylene chloride–methanol in increasing concentration) to generate compounds **6 or 7** (60–65%).

6.2.1. 2-[[(1*R*)-1-[[(2*S*-5-[[Imino(nitroamino)methyl]-2-[((*R*,*S*)-10-cyano-2-cyclopentyldecanoyl)amino-1-oxopentyl]amino]-3-methylbutyl]]-(3a*S*,4*S*,6*S*,7a*R*)-hexahydro-3a,5,5-trimethyl-4,6-methano-1,3,2-benzodioxaborole (6)

^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.50 (br, 2H), 8.00 (br, 3H), 4.40 (m, 1H), 4.15 (m, 1H), 3.20 (m, 2H), 2.70 (m, 1H), 2.50 (t, 2H), 2.20–1.25 (a series of overlapping d, t and m for aliphatic hydrogens, 37H), 1.24 (s, 3H), 1.22 (s, 3H), 0.84 (d, J = 6.6 Hz, 6H), 0.81 (s, 3H). MS m/e 714 (M+H). High resolution MS: calculated for $C_{37}H_{64}BN_7O_6$ 714.5090 (M+H); found 714.5095 (M+H).

6.2.2. 2-[[(1*R*)-1-[[(2*S*-5-[[Imino(nitroamino)methyl]-2-[((*R*,*S*)-10-*N*-phthalimido-2-cyclopentyldecanoyl)amino-1-oxopentyl] amino]-3-methylbutyl]]-(3a*S*,4*S*,6*S*,7a*R*)-hexahydro-3a,5,5-trimethyl-4,6-methano-1,3,2-benzodioxaborole (7)

^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.50 (br, 2H), 8.00 (br, 3H), 7.85 (m, 2H), 7.70 (m, 2H), 4.40 (m, 1H), 4.15 (m, 1H), 3.20 (m, 2H), 2.70 (m, 1H), 2.50 (t, 2H), 2.20–1.25 (a series of overlapping d, t and m for aliphatic hydrogens, 37H), 1.24 (s, 3H), 1.22 (s, 3H), 0.84 (d, J = 6.6 Hz, 6H), 0.81 (s, 3H). MS m/e 834 (M+H). High resolution MS: calculated for $C_{44}H_{68}BN_7O_8$ 834.5307 (M+H); found 834.5300 (M+H).

6.3. Compounds 26 and 27

General procedure: A mixture of compound **6** or **7** (1 equiv), 2-methylpropylboronic acid (2.5 equiv) and 2 N aqueous hydrochloric acid in a heterogeneous mixture of methanol and hexane was stirred overnight at room temperature, concentrated, diluted with EtOAc, washed with saturated $NaHCO_3$ solution and evaporated to dryness. Purification of residue by column chromatography (eluant: methylene chloride–methanol in increasing concentration) yielded compound **26** or **27** (70–75%).

6.3.1. [(1*R*)-1-[[(2*S*-5-[[Imino(nitroamino)methyl]-2-[((*R*,*S*)-10-cyano-2-cyclopentyldecanoyl)amino-1-oxopentyl]amino]-3-methylbutyl]boronic acid (26)

MS m/e 562 (M+H–H_2O). High resolution MS: calculated for $C_{27}H_{50}BN_7O_6$ 562.3891 (M+H–H_2O); found 562.3888 (M+H–H_2O).

6.3.2. [(1*R*)-1-[[(2*S*-5-[[Imino(nitroamino)methyl]-2-[((*R*,*S*)-10-*N*-phthalimido-2-cyclopentyldecanoyl)amino-1-oxopentyl] amino]-3-methylbutyl]boronic acid (27)

MS m/e 682 (M+H–H_2O). High resolution MS: calculated for $C_{34}H_{54}BN_7O_8$ 682.4103 (M+H–H_2O); found 882.4100 (M+H–H_2O).

6.4. Biology

6.4.1. Assay for the chymotrypsin-like activity of human liver proteasome

20S proteasome isolated from human liver was diluted to a final concentration of 6.7 μg/mL in 96-well U-bottom white plates (Dynex Technologies #6905) in buffer containing 20 mM Tris–Cl (pH 7.5), 0.04% SDS and 5% DMSO. Full activation of the enzyme was attained by pre-incubating 15 min at 27 °C. Reactions were started by addition of the fluorogenic substrate Succinyl-Leu-Leu-Val-Tyr-aminomethyl coumarin (Suc-LLVY-AMC; Bachem I-1395) to a concentration of 100 μM. The 100 μL assays were incubated at 27 °C and the release of AMC monitored by the increase in fluorescence (λ_{ex} 360 nm/λ_{em} 460 nm) every 1.5 min for 20 min (Cytofluor). The rate of product formation was evaluated at each inhibitor concentration. The IC_{50} values were calculated by non-linear regression using GraphPad Prism.

6.4.2. Cellular assay for the chymotrypsin-like activity of proteasome in Molt-4 cells

Protease inhibitors were tested for intracellular activity in Molt-4 human T-cell leukemia lymphocytes (ATCC#CRL-1582). Cells were washed and resuspended to a final concentration of 6×10^6 cells/mL in 5.4 mM KCl, 120 mM NaCl, 25 mM glucose, 1.5 mM $MgSO_4$, 1 mM Na pyruvate, 20 mM HEPES (pH 7.0) and dispensed to 96-well U-bottom white plates. Cells were treated with test compounds for 15 min at 37 °C prior to the addition of the lipophillic substrate MeOSuc-Phe-Leu-Phe-AFC (Enzyme Systems Products, AFC-088) to a concentration of 100 μM at a final concentration of 0.6% DMSO. The 100 μL assays were incubated at 37 °C and the release of AFC monitored by the increase in fluorescence (λ_{ex} 420 nm/λ_{em} 490 nm) every 1.5 min for 20 min (Cytofluor). Inhibition was evaluated as for the isolated enzyme.

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