N-Terminal peptidic boronic acids selectively inhibit human ClpXP†‡

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The synthesis and development of N-terminal peptidic boronic acids as protease inhibitors is reported. N-Terminal peptidic boronic acids interrogate the S′ sites of the target protein for selectivity and provide a new strategy that complements the currently known peptidic α-amino boronic acids (C-terminal boronic acids). After screening a series of N-terminal peptidic boronic acids, the first selective inhibitor of human ClpXP, an ATP-dependent serine protease present in the mitochondrial matrix, was discovered. This should facilitate the understanding of the physiological function of this protease

Introduction

An increasing number of boron-containing small molecules and peptides are being investigated for their medical potential in the treatment of various diseases.[1] For example, benzoxaborole AN2690, a compound with a novel mode of action of targeting leucyl-transfer RNA synthetase, is currently in phase III clinical trial for the treatment of onychomycosis.[2–4] Others incorporate α-aminoboronic acids into peptides to generate mechanism-based inhibitors of therapeutically relevant serine proteases including chymotrypsin,[5,6] thrombin,[7] dipeptidyl peptidase,[8] and HCV NS3 protease.[9]

Because peptidic boronic acids inhibit serine proteases, a series of dipeptidyl boronic acids were hypothesized to be potent and selective inhibitors of the proteasome, which contains a threonine residue in the active site.[10] This was confirmed when a reversible covalent bond with the N-terminal threonine residue was established.[11] Gratifyingly, the first boron-containing drug, Bortezomib (Velcade), was approved by the FDA for the treatment of multiple myeloma and mantle cell lymphoma.[12]

Peptidic α-amino boronic acids are excellent inhibitors of proteases because of the ability of Lewis basic residues located in the enzyme active site to bind to the empty p-orbital of boron forming a stable "ate" complex, which mimics the tetrahedral intermediate formed during amide bond hydrolysis.[5,6,13–15] When the protease substrate is known, this strategy becomes attractive because the P-site residues can readily be incorporated to provide a good starting point for inhibitor development. It is well-known, however, that substrate selectivity within the enzyme active site relies heavily on both the P/S- and P′/S′-site complementarity (Fig. 1). However, current methods incorporating the boronic acid moiety only take advantage of the binding pocket on P/S side of the scissile amide bond (C-terminal boronic acid). Herein,

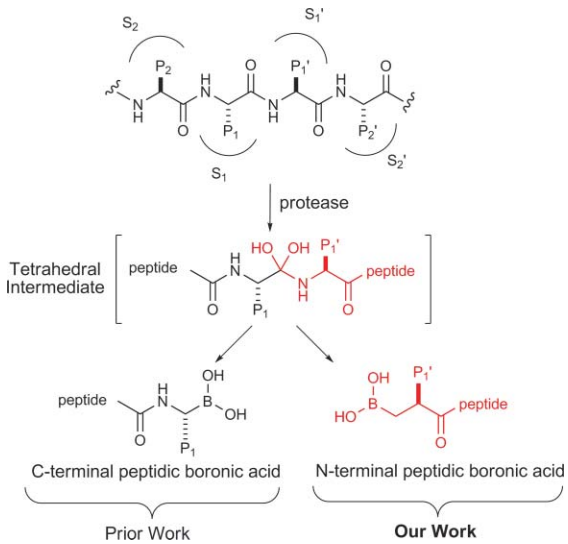

Fig. 1 Complementary peptidic boronic acid strategies.

we report the synthesis and development of a complementary approach, using N-terminal peptidic boronic acids, to harvest the potential of residues on the opposite side (P′-sites). We reason that the S′-sites may provide unique selectivity for a number of biologically important proteases. In particular, the S′-sites are attractive when the S-sites do not achieve the desired selectivity and inhibitory activity. In the design of N-terminal peptidic boronic acids, the carboxyl group is replaced with a boronic acid moiety and the nitrogen derived from the scissile amide bond is changed to carbon because the B–N bond is labile. The terminal boronic acid residue can be conveniently modified to incorporate both natural and unnatural amino acid functional groups.

To evaluate the utility of our approach, we compared the inhibition profiles of a series of N-terminal peptidic boronic acids toward proteases Lon and ClpXP, which are found in the matrix of human mitochondria. Both Lon and ClpXP are serine proteases but they only degrade proteins in the presence of ATP. Lon is a homo-oligomeric complex whereas ClpXP is a hetero-oligomeric complex.[16–18] Recently, it has been shown that ATP-dependent proteolytic activity can be up-regulated by cellular oxidative stress, especially during cardiac ischemia/reperfusion.[19,20] As Lon and ClpXP are both present in the mitochondrial matrix,[21] the

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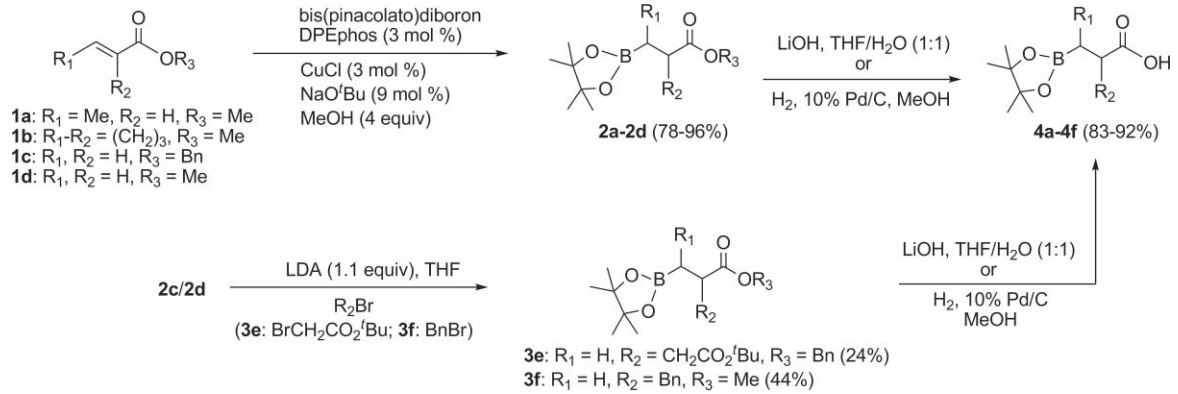
‡ Electronic supplementary information (ESI) available: Detailed synthesis for compounds **6g1/6g2**, ^{1}H and ^{13}C NMR spectra for all compounds synthesized, LC/MS of peptides used in this study and IC_{50} determination of **WLS6a**. See DOI: 10.1039/c004247a

observed up-regulation in ATP-dependent proteolytic activity could be attributed to one or both enzymes. The contribution of the respective protease in protecting mitochondria from oxidative stress is not known. Therefore, the development of selective inhibitors against the respective protease will provide valuable chemical tools to interrogate the physiological functions of the two mitochondrial proteases. Furthermore, several physiological protein substrates have been identified for human Lon (hLon) but not for human ClpXP (hClpXP). The availability of selective inhibitors should benefit the identification of physiological substrates of hClpXP when used in conjunction with proteomic studies.

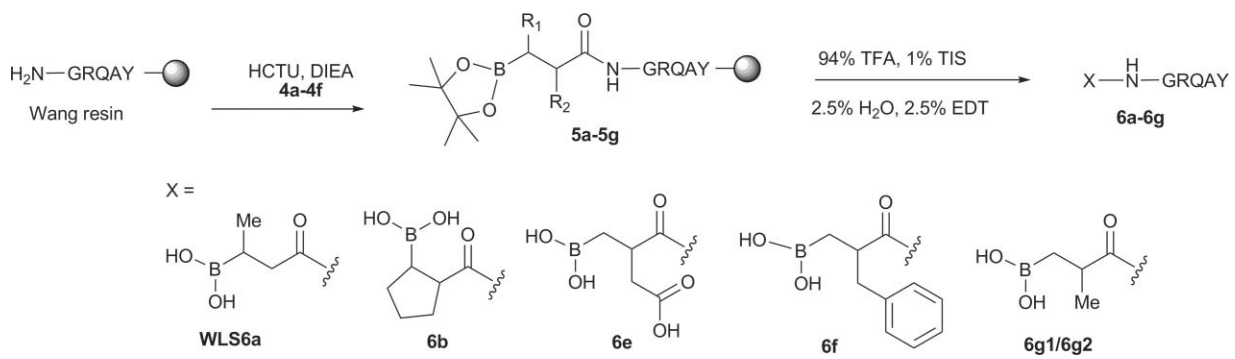
Results and discussion

Motivated by these goals, we set out to evaluate the extent to which N-terminal peptidic boronic acids can be exploited as inhibitors of hLon and hClpXP. Synthesis of N-terminal boronic acid building blocks commenced with the installation of the β-boronic ester moiety (Scheme 1). Treatment of α,β-unsaturated esters **1a–1d** with bis(pinacolato)diboron, CuCl, DPEphos, NaO^{t}Bu and MeOH[22–24] or a mixed diboron developed in our laboratory[25] readily provided β-boronic esters **2a–2d**. Functional groups on the α-position were introduced by enolization of **2c/2d** with LDA followed by alkylation with *tert*-butyl bromoacetate or benzyl bromide to yield **3e** and **3f**, respectively. Saponification with LiOH in THF–H_2O or catalytic hydrogenation provided the requisite β-boronic acids **4a–4f**. Using solid phase peptide synthesis, the sequence GRQAY[26,27] was attached to Wang resin and β-boronic acids **4a–4f** were smoothly attached using HCTU as the coupling agent generating peptides **5a–5g** (Scheme 2). Subsequent cleavage from the resin also achieved a concomitant removal of the pinacol ester protecting group affording **6a–6g**. Peptides **6g1** and **6g2** were synthesized as in Scheme 1 but enantiopure bis(pinanediolato)diboron was used.[28] HPLC purification using C18 column provided analytically pure material for subsequent assays.

Using a FRET assay previously developed for hLon,[29] we adapted the same protocol to determine inhibitors for hClpXP using FRETN 89–98 as the substrate. FRETN 89–98 is an 11 amino acid peptide that contains an anthranilamide/nitrotyrosine fluorescence donor/acceptor pair. In the intact peptide, the fluorescence is quenched, but upon cleavage, the fluorophore and quencher separate and an increase in fluorescence emission can be measured. Under the assay conditions used here, hClpXP shows the expected ATP-dependent activity as illustrated by an increase in fluorescence emission, which can be monitored in the absence or presence of modulators (data not shown). Initial screening of N-terminal peptidic boronic acids **WLS6a–6g** at 20 μM indicated that diasteromers **6g1** and **6g2** inhibited both hLon and hClpXP but **6b** showed no inhibition (Fig. 2A). In contrast, **WLS6a**, **6e** and **6f** inhibited hClpXP but not hLon. Encouraged by these results, we then tested these compounds at a higher concentration (1 mM) and discovered that after preincubation of the enzyme with the inhibitor for 30 min at room temperature, the proteolytic activity of hClpXP was almost completely inhibited (>90%) by N-terminal boronic peptide **WLS6a** while hLon was unaffected (Fig. 2B). **6e** and **6f** also inhibited hClpXP but to a lesser



Scheme 1 Synthesis of N-terminal boronic acid monomers.



Scheme 2 Solid-phase peptide synthesis of N-terminal peptidic boronic acids.

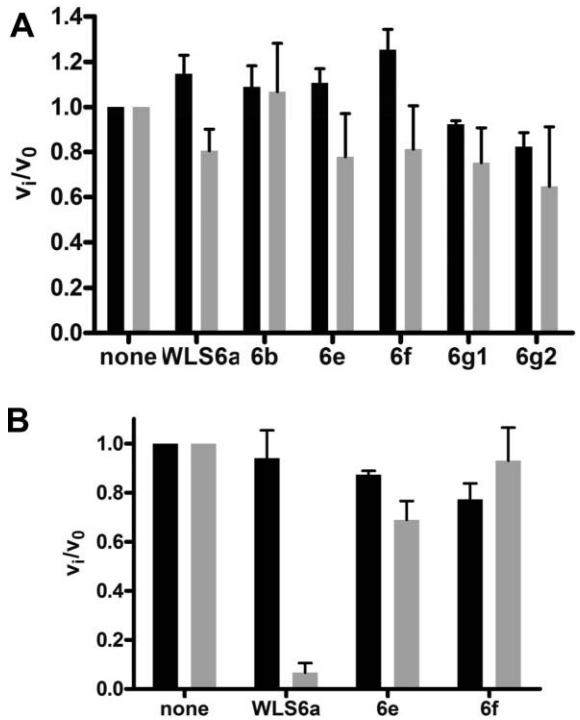


Fig. 2 Screening of N-terminal peptidic boronic acids using a FRET assay. 20 μM (A) or 1 mM (B) putative inhibitor was incubated with 300 nM hLon (black) or hClpXP (gray) at rt for 30 min prior to addition of ATP and FRETN 89–98 substrate. All values are the average of three experiments.

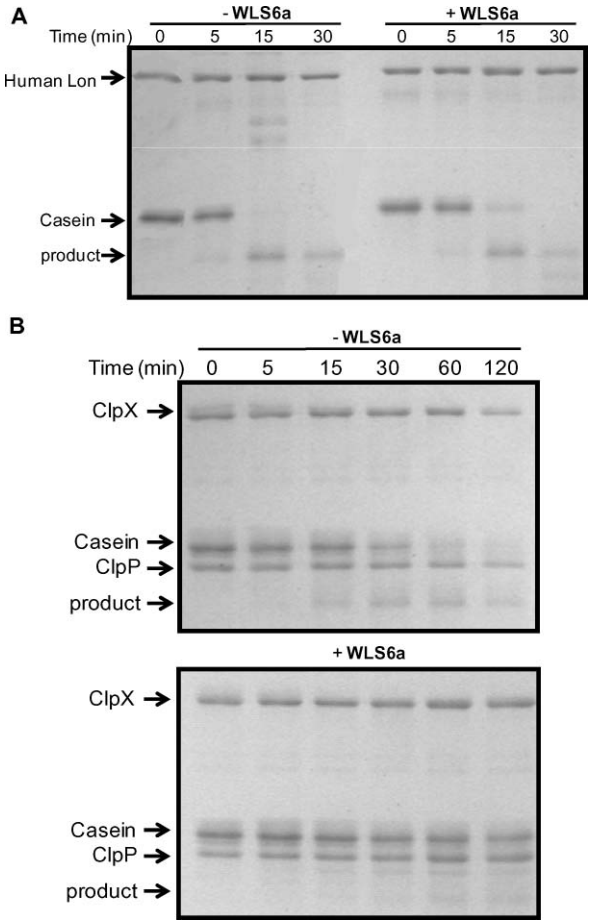


Fig. 3 Proteolytic cleavage of casein. Reactions containing 50 mM HEPES (pH 8.1), 15 mM $Mg(OAc)_2$, 5 mM DTT, and 1 μM hLon (A) or hClpXP (B) were incubated for 1 min at 37 °C in the absence and presence of 1 mM **WLS6a**. 5 mM ATP was added and the reactions were further incubated for 1 min at 37 °C. Reactions were initiated with 10 μM casein and aliquots were quenched at specific time points and run on a 12.5% SDS-PAGE.

extent. After identifying compound **WLS6a** as the most effective inhibitor of the series, we performed experiments under steady-state conditions to estimate its potency. An apparent IC_{50} value of 29 μM ± 9 was obtained.[28] Detailed kinetic assays to elucidate the mechanism of inhibition as well as the true inhibition constants for **WLS6a** are currently underway using an optimized peptidase assay.

To confirm that **WLS6a** is a selective inhibitor of hClpXP, we performed a secondary assay and investigated whether compound **WLS6a** inhibited hClpXP and hLon mediated protein degradation using SDS-PAGE analysis. We used casein as the substrate since it is the best known protein substrate for both hLon and hClpXP.[30,31] As expected, casein was completely degraded by hLon in the absence of inhibitor while minimal inhibition is observed in the presence of inhibitor as a small amount of casein persisted after 15 min of incubation (Fig. 3A). It is clear, however, that casein is completely hydrolyzed after 30 min. In the presence of hClpXP, proteolytic cleavage of casein was complete within 2 h in the absence of inhibitor but almost fully intact during the same time course of the experiment upon incubation with inhibitor **WLS6a** (Fig. 3B). These results are in complete agreement with the FRET assay and suggest that **WLS6a** is a selective inhibitor for hClpXP over hLon, even at a concentration of 1 mM. To the best of our knowledge, compound **WLS6a** is the first reported selective inhibitor for hClpXP. Although the exact mechanism of inhibition is currently unknown, this discovery should facilitate the understanding of the roles of hClpXP and hLon in the maintenance of mitochondrial integrity.

Conclusions

In conclusion, we demonstrated the potential broad utility of N-terminal peptidic boronic acids as protease inhibitors. For the first time, boron-containing peptidic probes that interrogate the S′-site of proteases are available. We have applied this strategy in the discovery of a selective inhibitor for hClpXP and provided a useful chemical biology tool to investigate its function. Current efforts along these lines as well as understanding the mechanism of action are underway.

Experimental

NMR spectra were recorded on a JEOL EclipsePlus 500 or a Varian INOVA 400. The peptides were purified on an Agilent 1200 semi-prep system using a Thermo Scientific Hyper Sil Gold C-18 column. Purity of the final peptides was verified by a Thermo TSQ ESI LC/MS. High resolution mass spectra were taken on an Agilent 6220 Accurate Mass TOF LC/MS. ^{1}H NMR chemical shifts are reported in ppm with the solvent resonance as the internal standard ($CDCl_3$: 7.26 ppm). Peak multiplicity is

described as follows: s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), m (multiplet) and coupling constant (J) values are given in Hz. ^{13}C NMR chemical shifts are reported in ppm with the solvent resonance as the internal standard ($CDCl_3$: 77.16 ppm). In all cases, except as otherwise noted, the signal for the carbon directly attached to boron was not observed in ^{13}C NMR because of quadrupolar relaxation. Bis(pinacolato)diboron was obtained from both Boron Molecular and Frontier Scientific. Sodium *tert*-butoxide and methyl methacrylate were obtained from Fluka. Methyl acrylate, benzyl methacrylate, and benzyl acrylate were obtained from Alfa Aesar. All amino acids and Wang resin were obtained from Novabiochem. HCTU was obtained from Peptides International. Anhydrous THF, DCM, and DMF were used from 18 L Pure-Pak™ tanks from Sigma Aldrich. All other chemicals were obtained from Sigma Aldrich. All chemicals were used without further purification.

General procedure A: β-boration of α,β-unsaturated esters

The following method was adapted from Yun *et al.*[32] Copper chloride (0.03 equiv), sodium *tert*-butoxide (0.09 equiv), and DPEphos (0.03 equiv) were weighed into a 2-neck flask and alternate cycles of nitrogen purge and vacuum were performed. Anhydrous THF was added to the reaction flask by needle and stirred for 45 min (the solution gradually darkened to a greenish suspension). Bis(pinacolato)diboron (1.1 equiv) dissolved in THF (1 ml) was added *via* syringe and stirred for 30 min (suspension rapidly darkened to a deep arterial red). The reaction flask was then cooled to 0 °C and the α,β-unsaturated ester (1 equiv) was added by syringe followed immediately by methanol (4 equiv). After the solution was allowed to warm to rt overnight, the suspension was filtered through Celite and dried under reduced pressure. A white suspension appeared after addition of hexanes, which was removed by filtration and the filtrate was purified by flash silica gel chromatography (0–20% ethyl acetate in hexanes). TLC analysis with 10% ethyl acetate/hexanes allowed visualization of the product by CAM or aqueous $KMnO_4$ stain.

General procedure B: α-alkylation of borylation products

LDA was prepared *in situ* by adding n-butyl lithium (1.1 equiv) drop-wise to diisopropylamine (1 equiv) dissolved in THF at −78 °C for 45 min. The solution was warmed to 0 °C for 10 min and again chilled to −78 °C before cannulating into a THF solution of the borylated compound at −78 °C. After stirring for 30 min, the alkyl halide was added and the temperature was maintained at −78 °C for 15 min before warming to 0 °C. The reaction was quenched with saturated aqueous ammonium chloride. The aqueous layer was extracted three times with EtOAc and the combined organic layers were washed with brine and dried over sodium sulfate. TLC analysis was performed with 10% ethyl acetate/hexanes and CAM stain. Flash silica gel chromatography (0–20% ethyl acetate in hexanes) provided the purified product.

Methyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)butanoate (2a)

Using general procedure A, beginning with methyl crotonate, the title product was isolated in 81% (1.856 g) yield as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.64 (s, 3H), 2.40 (dd, J = 16.4, 7.2 Hz, 2H), 1.37 (h, J = 7.5 Hz, 1H), 1.23 (s, 2H), 1.23 (s, 2H), 0.99 (d, J = 7.5 Hz, 3H).^{13}C NMR (126 MHz, $CDCl_3$) δ 174.17, 83.08, 51.24, 37.40, 24.68, 24.59, 15.03, (B–C) 13.54. HRMS-ESI (m/z): $[M + Na]^+$ calcd for $C_{11}H_{21}[^{11}B]NaO_4$, 251.14305; found, 251.14333.

Methyl 2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)cyclopentanecarboxylate (2b)

1,3-Dicyclohexylimidazolium chloride, (ICy)Cl, was prepared according to published literature procedure.[33] To a suspension of (ICy)Cl (5 mol%), CuCl (5 mol%) and NaO^{t}Bu (5 mol%) in THF (3 mL) stirring for 1.5 h was added an additional equiv of NaO^{t}Bu. After 1 h, bis(pinacolato)diboron in THF (1 mL) was added and the reaction mixture was stirred for another 10 min. Methyl 1-cyclopentene-1-carboxylate (194 mg), followed by methanol, was added and stirred at rt overnight. The product was filtered through Celite, dried, and purified by flash silica gel chromatography (0–20% ethyl acetate in hexanes) to provide the product in 96% yield (375 mg) as light yellow oil. This compound was isolated as an unresolved mixture of *cis*/*trans* diastereomers. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.64 (s, 0.63 H), 3.63 (s, 2.31 H), 3.04-2.96 (m, 0.68 H), 2.84-2.75 (m, 0.09 H), 1.95-1.35 (m, 7H), 1.23-1.20 (m, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 177.38, 83.11, 51.54, 46.83, 46.19, 30.97, 30.38, 28.87, 27.78, 26.50, 25.91, 25.00, 24.83, 24.72. HRMS-ESI (m/z): $[M + H]^+$ calcd for $C_{13}H_{23}[^{11}B]O_4$, 255.1762; found, 255.1763.

Benzyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)propanoate (2c)

Using general procedure A, beginning with benzyl acrylate, the title product was isolated in 79% (1.151 g) yield as a light yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35-7.28 (m, 5H), 5.10 (s, 2H), 2.49 (t, J = 7.5 Hz, 2H), 1.21 (s, 12H), 1.04 (t, J = 7.5 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.55, 136.33, 128.56, 128.16, 128.13, 83.32, 66.15, 28.92, 24.83. HRMS-ESI (m/z): $[M + H]^+$ calcd for $C_{16}H_{23}[^{11}B]O_4$, 291.17012; found, 291.17712.

Methyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)propanoate (2d)

Using general procedure A, beginning with methyl acrylate, the title product was isolated in 84% (1.789 g) yield as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.64 (s, 3H), 2.43 (t, J = 7.5 Hz, 2H), 1.23 (s, 12H), 1.01 (t, J = 7.5 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.15, 100.00, 83.31, 51.58, 28.68, 24.83. HRMS-ESI (m/z): $[M + Na]^+$ calcd for $C_{10}H_{19}[^{11}B]NaO_4$, 237.1274; found, 237.12754

1-Benzyl 4-*tert*-butyl 2-((4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)methyl)succinate (3e)

Using general procedure B, beginning with **2c** and alkylating with *tert*-butyl bromoacetate, the product was isolated in 24% yield (50 mg) as colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36-7.27 (m, 5H), 5.11 (q, J = 12.5 Hz, 2H), 3.08-2.99 (m, 1H), 2.64 (dd, J = 16.2, 8.1 Hz, 1H), 2.48 (dd, J = 16.2, 5.7 Hz, 1H), 1.39 (s, 9H), 1.19 (s, 6H), 1.18 (s, 6H), 1.17-1.13 (m, 1H), 0.99 (dd, J = 16.0, 7.0 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.37, 171.18,

136.25, 128.51, 128.12, 128.08, 100.00, 83.41, 80.65, 66.36, 39.19, 37.44, 28.10, 24.85, 24.78. HRMS-ESI (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{33}[^{11}B]O_6$, 405.2443; found, 405.2445.

Methyl 2-benzyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)propanoate (3f)

Using general procedure B, beginning with **2d** and alkylating with benzyl bromide, the title product was isolated in 44% yield (62 mg) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28-7.12 (m, 5H), 3.60 (s, $J = 0.6$ Hz, 3H), 3.01 (dd, $J = 13.1$, 6.8 Hz, 1H), 2.91-2.81 (m, 1H), 2.73 (dd, $J = 13.1$, 7.5 Hz, 1H), 1.21 (s, 6H), 1.20 (s, 6H), 1.03 (dd, $J = 15.8$, 8.8 Hz, 1H), 0.92 (dd, $J = 15.9$, 6.2 Hz, 1H). ^{13}C NMR (126 MHz, CD_2Cl_2) δ 176.30, 139.81, 129.14, 128.26, 126.23, 83.20, 51.32, 43.08, 40.14, 24.69, 24.51, 13.72. HRMS-ESI (*m/z*): $[M + H]^+$ calcd for $C_{17}H_{25}[^{11}B]O_4$, 305.1922; found, 305.1910.

3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)butanoic acid (4a)

Compound **2a** was dissolved in 2 ml THF and hydrolyzed with 4 equiv of LiOH dissolved in 2 ml H_2O at room temperature for 1 h. The product was isolated in 89% (48 mg) yield as colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.49-2.35 (m, 2H), 1.32 (s, 1H), 1.19 (s, 6H), 1.19 (s, 6H), 0.98 (d, $J = 7.5$ Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 180.40, 83.45, 37.61, 24.85, 24.76, 15.16, (B–C) 13.44. HRMS-ESI (*m/z*): $[M - H]^-$ calcd for $C_{10}H_{19}[^{11}B]O_4$, 213.1304; found, 213.1309.

2-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)cyclopentanecarboxylic acid (4b)

Compound **2b** was dissolved in 2 ml THF and hydrolyzed with 4 equiv of LiOH dissolved in 2 ml H_2O at room temperature for 1 h. The title product was isolated in 92% yield (112 mg) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.84 (q, $J = 8.6$ Hz, 1H), 1.99-1.81 (m, 3H), 1.73-1.62 (m, 2H), 1.61-1.47 (m, 2H), 1.25 (s, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 181.56, 83.52, 46.62, 30.37, 28.90, 26.60, 24.74. HRMS-ESI (*m/z*): $[M - H]^-$ calcd for $C_{12}H_{21}[^{11}B]O_4$, 239.146; found, 239.1458.

4-*tert*-Butoxycarbonyl-2-((4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)methyl)butanoic acid (4e)

Compound **3e** was dissolved in 2 ml MeOH in the presence of 10% Pd/C for 30 min under a large balloon of hydrogen. The crude mixture was filtered through Celite and purified by flash silica gel chromatography to provide the product in 83% yield (55 mg) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.02-2.91 (m, $J = 28.0$ Hz, 1H), 2.59 (dd, $J = 16.3$, 7.8 Hz, 1H), 2.47 (dd, $J = 16.3$, 5.5 Hz, 1H), 1.41 (s, 9H), 1.21 (s, 6H), 1.20 (s, 6H), 1.09 (dd, $J = 15.8$, 8.0 Hz, 1H), 0.94 (dd, $J = 15.9$, 7.1 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 181.40, 171.57, 83.25, 80.99, 39.02, 37.45, 28.09, 24.80, 24.76, (B–C) 14.19. HRMS-ESI (*m/z*): $[M - H]^-$ calcd for $C_{15}H_{27}[^{11}B]O_6$, 313.1901; found, 313.1812.

2-Benzyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)propanoic acid (4f)

Compound **3f** was dissolved in 1 ml THF and hydrolyzed with 4 equiv of LiOH dissolved in 1 ml H_2O at room temperature for 1 h. The product was isolated in 92% yield (7 mg) as slightly yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.29-7.26 (m, 2H), 7.21-7.16 (m, 3H), 3.07 (dd, $J = 13.4$, 6.5 Hz, 1H), 2.95-2.87 (m, $J = 8.7$ Hz, 1H), 2.76 (dd, $J = 13.4$, 7.7 Hz, 1H), 1.21 (s, 6H), 1.20 (s, 6H), 1.04 (dd, $J = 16.0$, 8.8 Hz, 1H), 0.93 (dd, $J = 16.0$, 6.1 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 181.23, 139.32, 129.29, 128.42, 126.42, 83.43, 42.74, 39.80, 24.88, 24.72. HRMS-ESI (*m/z*): $[M + H]^+$ calcd for $C_{16}H_{23}[^{11}B]O_4$, 289.1617; found, 289.1617.

Solid-phase peptide synthesis

To a cooled solution of Fmoc-Tyr(^{t}Bu)-OH (1241 mg, 2.70 mmol) in CH_2Cl_2 (15 ml) and minimum volume of DMF (1.5 ml) at 0 °C was added DIC in CH_2Cl_2 (1 ml). After 30 min, a gelatinous precipitate developed, which was redissolved in DMF (3 ml). After an additional 1 h, CH_2Cl_2 was removed under rotary evaporator, and the product was dissolved in minimum volume of DMF and saved for coupling onto Wang resin. In a Poly-Prep column, Wang resin (800 mg, 0.3 mmol g^{-1} loading) was swollen in CH_2Cl_2 (2 mL, 2 × 15 min) then suspended in DMF (2 mL, 15 min). To a suspension of the resin bubbling with argon, tyrosine anhydride (from above) and one small crystal of DMAP were added. After 45 min, the resin was filtered, washed with DMF (3×), CH_2Cl_2 (3×) and deprotected with 20% piperidine in DMF. Further couplings were performed through standard solid phase peptide synthesis using N-α-Fmoc protected L-amino acids, HCTU and DIEA in DMF. Solid phase synthesis was done on a vacuum manifold (Qiagen) outfitted with 3-way Luer lock stopcocks (Sigma) in either Poly-Prep columns or Econo-Pac polypropylene columns (Bio-Rad). The resin was mixed in solution by bubbling argon during all coupling and washing steps. Fmoc-Ala-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Gly-OH were coupled sequentially to provide Fmoc-Gly-Arg-Gln-Ala-Tyr-Wang resin. Beads were washed extensively with DMF between reactions, and the couplings were tested for completeness *via* Kaiser test.

Coupling of boronic ester monomers

Following the same procedure for SPPS as above, boronic ester monomers **4a–f** were each coupled to the above sequence. The couplings were tested for completeness *via* Kaiser test.

Side-chain deprotection and peptide cleavage

The peptides were cleaved and deprotected by a 6 hour treatment with 94 : 1 : 2.5 : 2.5 TFA/TIS/H_2O/EDT (v/v). The protocol also removed the pinacol protecting group. After peptide cleavage, TFA was removed under reduced pressure. The resulting yellow peptide was washed several times with cold diethyl ether, dried under reduced pressure and was purified on an Agilent 1200 semi-prep HPLC using a Thermo Scientific Hyper Sil Gold C-18 column.

Fluorescent peptidase assay

ClpXP peptidase activity was monitored using a previously designed assay[34] which utilizes an 11-amino acid peptide (FRETN 89–98) that contains an anthranilamide-nitrotyrosine donor/quencher pair on opposite termini. Fluorescent peptidase

assays were performed on a Fluoro-Max3 spectrophotometer at 37 °C using excitation and emission wavelengths of 320 nm and 420 nm, respectively. Reactions containing 50 mM HEPES (pH 8.1), 5 mM $Mg(OAc)_2$, 2 mM DTT, and 300 nM ClpXP were incubated in the absence and presence of 1 mM ATP for 1 min at 37 °C. Reactions were initiated by the addition of 500 μM FRETN 89–98 and fluorescence emission was monitored for 1200 s. In all assays, a mixed substrate was used (90% non-fluorescent 10% fluorescent) to avoid the inner filter effect.[35]

Screening for specific inhibitors against human Lon and ClpXP

To screen the effect of various compounds on the peptidase activity of Lon and ClpXP, reactions containing 50 mM HEPES (pH 8.1), 5 mM $Mg(OAc)_2$, 2 mM DTT, 300 nM human Lon or human ClpXP, and 20 μM various inhibitors were incubated for 30 min at room temperature. For the Lon assays, the reactions were then incubated for 1 min at 37 °C prior to initiation by the addition of 500 μM FRETN 89–98 and 1 mM ATP. For ClpXP assays, after incubation at room temperature for 30 min, 1 mM ATP was added and the reaction was further incubated at 37 °C for 1 min. The reaction was then initiated by the addition of 500 μM FRETN 89–98 and fluorescent emission was monitored for 1200 s at 420 nm with excitation at 320 nm. The amount of peptide cleaved was measured by determining the maximum fluorescence generated per micromolar peptide after complete digestion by trypsin. The steady-state rate of the reaction was determined from the tangent of the linear portion of the time course. This rate was converted to an observed rate constant (k_{obs}) by dividing the rate by the enzyme concentration used in the assay. These rate constants were normalized to 1 by dividing by the k_{obs} for the reaction in the absence of modulator. All experiments were performed in triplicate.

Human Lon steady-state inhibition of protein degradation

Reactions containing 50 mM HEPES (pH 8.1), 15 mM $Mg(OAc)_2$, 5 mM DTT, 1 μM human Lon, and 1 mM **WLS6a** were incubated at 37 °C for 1 min prior to addition of 5 mM ATP. Reaction aliquots were quenched in SDS loading dye at $t = 0$, 5, 15, 30 min. Each time point was loaded onto a 12.5% SDS-PAGE gel and stained by Coomassie Brilliant Blue.

ClpXP steady-state inhibition of protein degradation

Reactions containing 50 mM HEPES (pH 8.1), 15 mM $Mg(OAc)_2$, 5 mM DTT, 2 μM ClpXP, and 1 mM **WLS6a** were incubated at 37 °C for 1 min prior to the addition of 5 mM ATP. Following further incubation for 1 min at 37 °C, reactions were initiated with 10 μM casein. Reaction aliquots were quenched in SDS loading dye at $t = 0$, 5, 15, 30, 60, 120 min. Each time point was loaded onto a 12.5% SDS-PAGE gel and stained by Coomassie Brilliant Blue.

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