Chemical tools to probe the proteasome

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5 Mixing of peptides and electrophilic traps gives rise to potent, broad-spectrum proteasome inhibitors.

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5.1 Introduction

The development and use of proteasome inhibitors has found wide attention in recent years, both in fundamental and applied sciences.[1] The proteasome is a multicatalytic proteinase complex that is involved in many biological processes in man. Its primary function is the processing to oligopeptides of cytosolic and nuclear proteins, as well as *N*-linked glycoproteins that are rejected from the ER due to improper folding.[2] These substrates are marked for proteasomal degradation through the attachment of ubiquitin chains at specific sites. The ubiquitin modifications allow docking to one of the two 19S caps that, together with the inner catalytic 20S core, form the mammalian 26S proteasome. The approval of the peptide boronic acid PS341[3] (bortezomib, **1**, Figure 1) for the clinical treatment of multiple myeloma has led to a surge of activities in proteasome research. PS341 is a highly active proteasome inhibitor, but treatment with PS341 results in severe side effects. It is not clear yet whether this is the result of proteasome blockade or because of other factors that interact with the compound. The development of new and potentially more active or selective proteasome inhibitors may provide information on the mode of action of PS341 and open ways to develop more proteasome inhibitor based therapies in oncology.

Of interest also is the study of the role of the individual proteasomal proteolytic activities, which can be furthered greatly by the use of well-defined inhibitors. The mammalian proteasome differs from the corresponding prokaryotic particles in that the latter has only one type of catalytic activity. The overall shape of the prokaryotic 20S proteasome highly resembles the eukaryotic 20S proteasomes It has C2 symmetry and it

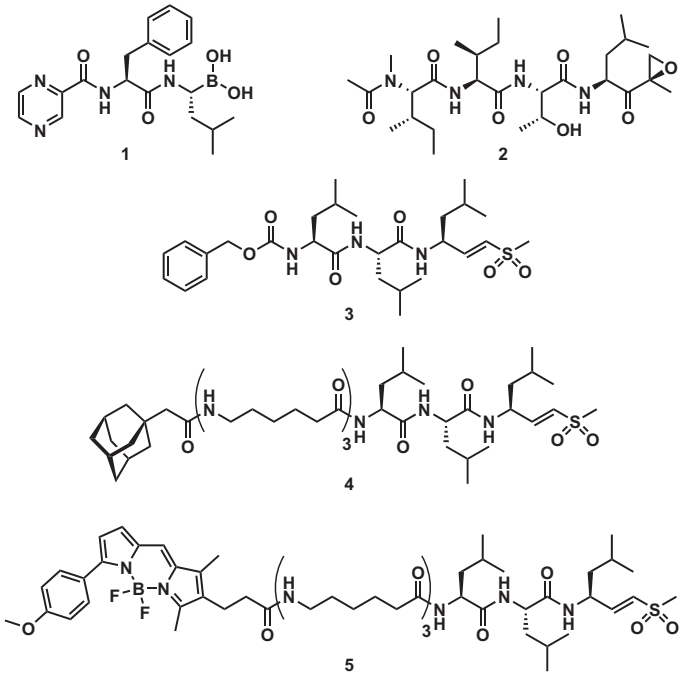


Figure 1. Proteasome inhibitors at the basis of this study.

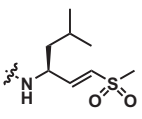
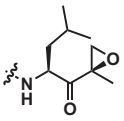
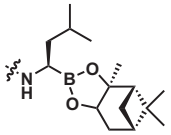
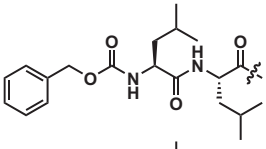
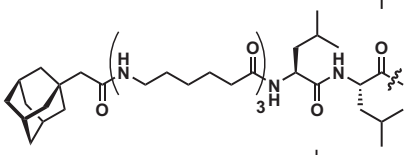
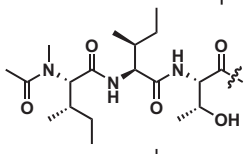
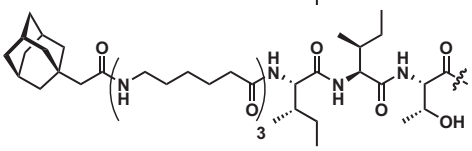
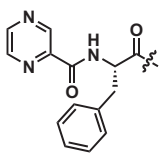
consists of two outer rings each containing seven identical α-subunits, and two inner seven-subunit rings containing identical catalytic β-subunits.[4] Eukaryotic proteasomes have evolved to such extent that each α-subunit in the outer ring has a unique sequence, as is the case with the inner β-rings. Remarkably, four of the β-subunits lost their proteolytic activities, whereas the three remaining subunits have a diverged substrate preference.[5] Based on fluorogenic substrate assays the substrate preference is loosely defined as caspase-like for the β1 subunit (cleaving after acidic residues), trypsin-like for β2 (cleaving after basic residues) and chymotrypsin-like for β5 (cleaving after neutral hydrophobic residues). However, many studies demonstrate that the subunits are much more promiscuous with respect to the amino acid residue at the cleavage site.

An important asset in proteasome research is the use of covalent and irreversible inhibitors such as the natural product epoxomicin (**2**),[6] the synthetic Michael acceptor ZL_3VS (**3**),[7] and their labelled (radio tag, affinity tag, fluorescent tag) counterparts.[7-10] Despite these studies the exact substrate preference of the individual catalytic activities, and the evolutionary benefit that results from the diversification, is not fully understood. The same holds true for the role of yet another proteasome particle, the immunoproteasome, which is formed upon challenge of the mammalian immune system and which contains three different catalytic subunits, namely β1i (LMP2), β2i (MECL1) and β5i (LMP7).[11] A much sought after research goal in immunology is to establish the impact of the immunoproteasome, in relation to the constitutively expressed proteasome, on the generation of specific oligopeptides that can be sequestered by the major histocompatibility complex class I pathway for presentation

to the immune system.[12] To aid these studies, several research groups are involved in the development of compounds that selectively target one catalytic subunit of either the constitutive proteasome or the immunoproteasome.

The pool of proteasome inhibitors reported to date encompasses numerous structurally diverse compounds. A large category within this pool has in common that they are peptide-based compounds equipped with an electrophilic trap at the *C*-terminus. *C*-terminal modifications include, next to boronic acid (as in **1**), epoxyketone (**2**), vinyl sulfone (**3**), aldehyde and other electrophiles.[1,13] The mechanism of inhibition is similar in all examples: the γ-hydroxyl of the *N*-terminal threonine within the active site of the catalytic subunits reacts with the electrophilic trap to form a covalent and (in most cases) irreversible bond. Some control over subunit specificity can be achieved by altering the nature of the amino acid residues. Several years ago, it was demonstrated that *N*-terminally extended, hydrophobic peptide vinyl sulfones, such as the adamantane containing compound **4**, are much more potent proteasome inhibitors than their truncated counterparts.[8] This gain in activity was accompanied by a loss in subunit selectivity. This finding was exploited by the development of the broad-spectrum cell permeable proteasome label MV151 (BODIPY TMR-Ahx$_3$L$_3$VS, Chapter 2, **5**).[10] One observation made is that the potency of peptide vinyl

Table 1. Panel of synthesized proteasome inhibitors.

			
	**3**	**9**	**13**
	**4**	**10**	**14**
	**6**	**2**	**15**
	**7**	**11**	**16**
	**8**	**12**	**17**

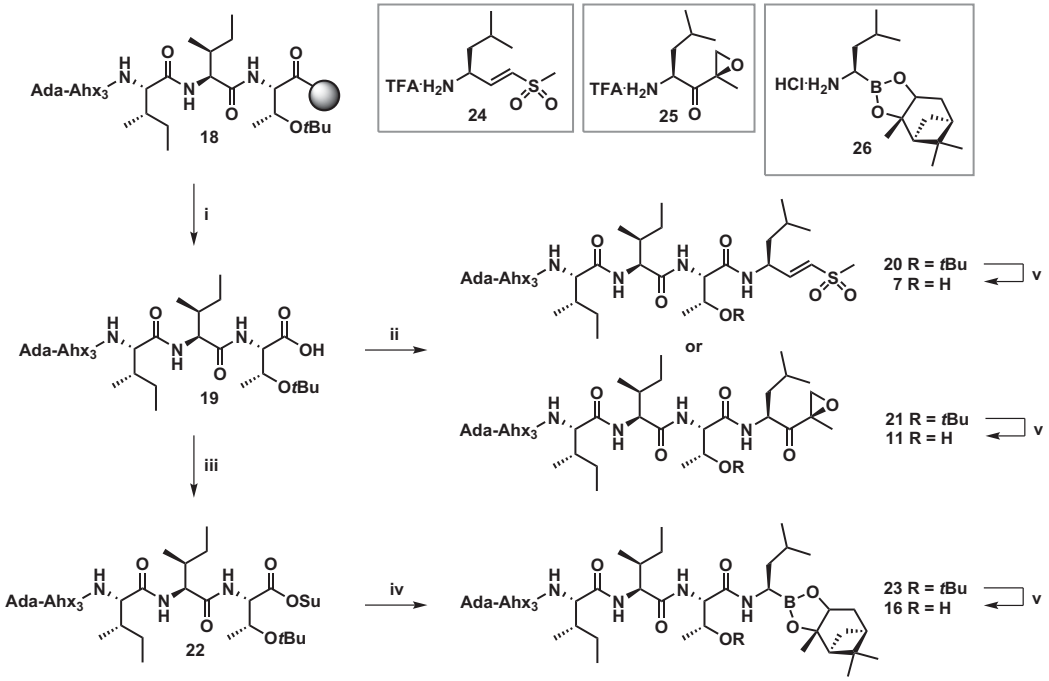
sulfone **4** to inhibit the proteasome approaches, or even surpasses that of bortezomib (**1**) and epoxomicin (**2**). This is remarkable, since the parent compound ZL_3VS (**3**) is a much weaker inhibitor. Indeed, in general oligopeptides containing epoxyketone or boronic acid warheads are found to be more potent proteasome inhibitors than their counterparts that have the same amino acid sequence but are equipped with a Michael acceptor. This observation raises the question whether recombining structural features of peptide-based proteasome inhibitors would lead to more potent compounds. To address this question, the structural characteristics of compounds **1**-**4** were scrambled to arrive at a number of *C*-terminally modified peptides, which were assessed on their proteasome inhibitory activity. Three distinct structural features were identified. These are a) the modified amino acid at the *C*-terminus, being boronic acid, vinyl sulfone and epoxyketone, b) the amino acid sequence, being the trileucine, the epoxomicin tetrapeptide and the bortezomib recognition element, and c) the presence (as in **4**) or absence (as in **3**) of the lipophilic *N*-terminal extension. These considerations led to the synthesis of the panel of 15 compounds listed in Table 1.

5.2 Results and discussion

In the design of the hybrid library a set of compounds that have the pyrazinoic acyl moiety in the bortezomib sequence replaced by the adamantane-spacer moiety was excluded. Such a set of compounds would likely resemble to a large extend the trileucine derivatives in their inhibition profile. Furthermore, it was decided to leave the pinanediol protection on the boronic ester, which stems from the enantioselective preparation of the leucine building block, in place. It has been reported that boronic esters of this nature have the same activity and specificity as their unprotected counterparts (Chapter 2).[10] Moreover, deprotection, for which there is no literature precedent (in fact there is no reliable literature synthesis of the drug bortezomib), proved to be detrimental in the course of the here described studies.

The preparation of all compounds follows the same general strategy: first the synthesis of the (*N*-terminaly extended) amino acid sequence and then coupling of these to the leucine derived warheads. As an example, the synthesis of compounds **7**, **11** and **16** is depicted in Scheme 1. Briefly, Fmoc-based solid phase peptide synthesis using HMPB functionalised MBHA resulted in **18**. Cleavage using 1% trifluoroacetic acid in dichloromethane gave partially protected oligopeptide **19**, which was condensed with either leucine vinyl sulfone **24**[7] or leucine epoxyketone **25**,[6] using BOP as the condensating agent. Acidic removal of the *t*Bu protecting group afforded the target compounds **7** and **11** after HPLC purification. Target compound **16** was obtained by coupling of succimidyl ester **22** with leucine boronic pinanediol ester **26** (synthesized as described in Chapter 2),[10] followed by acidic deprotection and HPLC purification.

Scheme 1. Synthesis of hybrid proteasome inhibitors **7**, **11** and **16**.



Reagents and conditions: i) 1% TFA in DCM, 3× 15 min., 73%. ii) **24** or **25** (1.1 equiv.), BOP (1.1 equiv.), DiPEA (2.2 equiv.), DMF, 12 hr., **21** 10%. iii) *N*-hydroxysuccinimide (1.7 equiv.), DIC (1.5 equiv.), DMF, 15 hr. iv) **26** (3.1 equiv.), DiPEA (24 equiv.), -80 °C to RT, 4 hr. v) TFA/DCM (1/1), 30 min., **7** 16% (2 steps), **11** 91%, **16** 4% (2 steps).

The inhibition potential of the panel of compounds was assessed in competition assays employing lysates of the murine EL4 cell line (expressing both the constitutive proteasome and the immunoproteasome, Figures 2 and 3) and the human HEK293T cell line (exclusively expressing the constitutive proteasome, Figure 4) in combination with fluorescent proteasome probe **5**. In a first set of experiments, EL4 cell lysates were incubated for one hour with each of the 15 compounds at 0, 0.1, 1, 10 and 100 μM final concentration, prior to treatment with 0.1 μM final concentration of MV151 (**5**). The samples were denatured and resolved by SDS-PAGE and the wet gel slabs were scanned on a fluorescence scanner (Figure 2). Lysates that have been exposed to the fluorescent label only display four bands corresponding to the six proteasome active sites (specified as depicted in the Figure 2).[10] The ability of the **15** compounds to inhibit the proteasome activities is reflected by disappearance of fluorescent labeling by MV151. Ten compounds from the panel of 15 (namely, **2, 4, 7, 9, 10, 11, 13** - **16**) proved to be potent proteasome inhibitors, with most or all labeling abolished at 1 μM. Vinyl sulfone derivatives **3, 6** and **8**, and epoxyketone **12** appear to be much weaker inhibitors. Boronic ester **17** is a weak inhibitor of the β2 and β2i subunits, while potently targeting the remaining subunits. As the next experiment the competition experiment was repeated, but with the difference that

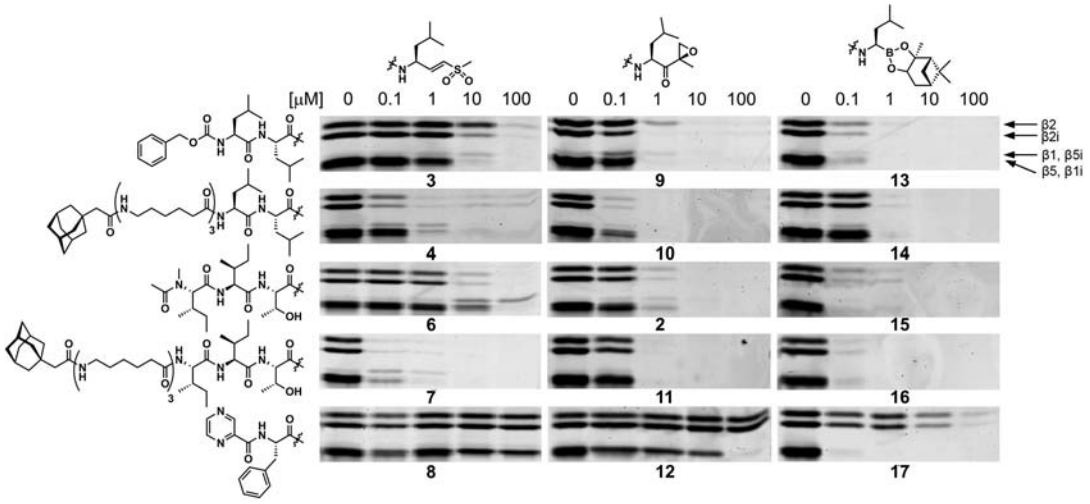


Figure 2. Competition assays of the hybrid library versus MV151 in EL4 cell lysates.
EL4 lysates (10 μg total protein) were incubated with the indicated concentrations of inhibitor for 1 hr. at 37 °C. Residual proteasome activity was labeled with 0.1 μM MV151 at 37 °C for 1 hr., prior to denaturation, SDS-PAGE and fluorescence scanning of the wet gel-slabs.

inhibitor concentrations were ranged from 0 to 500 nM (Figure 3, compounds **6**, **8** and **12** were excluded since these proved to be hardly effective between concentrations of 0 to 1000 nM in the first experiments). These results corroborate the earlier findings and allow the assessment of some more subtle differences between the different inhibitors. The most obvious result is that boronic ester **17** hardly targets β2 and β2i, a finding that corresponds to the reported specificity of the unprotected analogue, PS341 (**1**).[9] This selectivity is

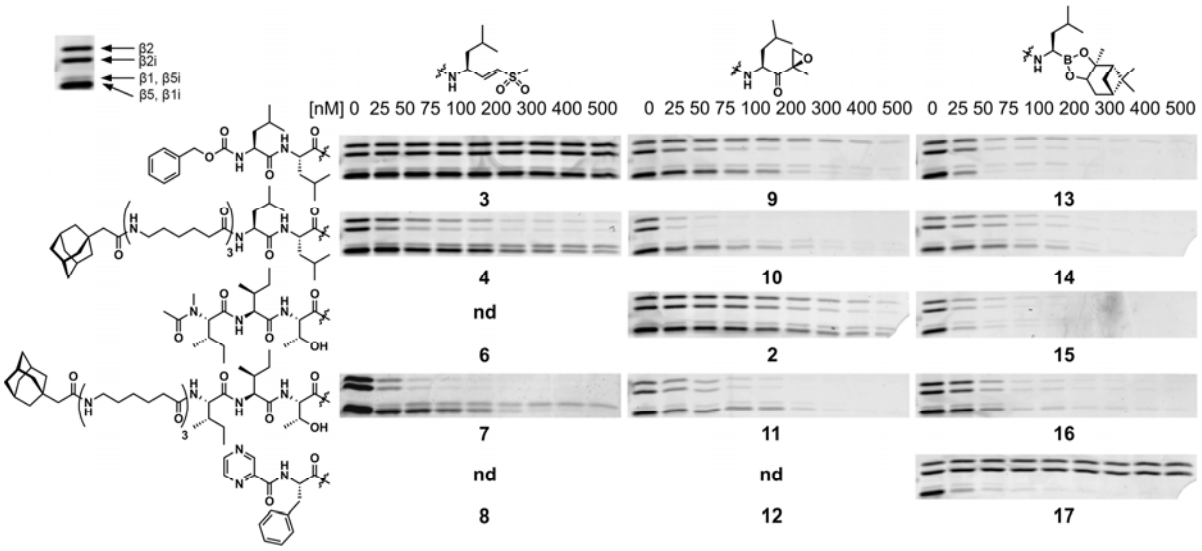


Figure 3. Competition assays of the hybrid library versus MV151 in EL4 cell lysates.
EL4 lysates (10 μg total protein) were incubated with the indicated concentrations of inhibitor for 1 hr. at 37 °C. Residual proteasome activity was labeled with 0.1 μM MV151 at 37 °C for 1 hr., prior to denaturation, SDS-PAGE and fluorescence scanning of the wet gel-slabs. nd = not determined.

abolished when keeping the boronic ester in place but substituting the peptide sequence, as in **13-16**. In general, the vinyl sulfone is the weakest electrophilic trap in each series, and the boronic ester the strongest, but there are some interesting differences. For instance, epoxyketone **10** is an equally potent inhibitor for each proteasome subunit as the boronic ester **14**, with a preference for the constitutive and immunoinduced β2 subunits. At the onset of the competition experiments it was assumed that elongation of a given proteasome inhibitor with the Ada(Ahx)$_3$ *N*-terminal cap leads to a more potent compound. This holds true to some extent, compare, for instance, vinyl sulfones **3** and **4**, vinyl sulfones **6** and **7**, epoxyketones **9** and **10**, and epoxyketones **2** and **11**. However the potency of ZL$_3$-boronic ester **13** (its unprotected counterpart has been described in the literature[3] and is known as MG262) belies the generality of this trend. Indeed, the potency of this compound is bested by boronic ester **15** only. Compound **15** is more potent than its *N*-terminally extended analogue **16** as well. In some cases, labeling of a specific active site increases as the other activities are inhibited. This finding, which could imply enhancement of a specific proteasome activity due to occupation of other active sites, has been noted before, however is not yet clearly understood.[14] When performing a similar competition experiment on human embryonic kidney HEK293T cell lysates (Figure 4), this effect is clearly visible in the case of the *N*-terminally extended epoxomicin sequence armed with the leucine vinyl sulfone warhead (**7**). This inhibitor potently inhibits β5 and β2, whereas β1 labeling increases dramatically. In general, the same trends are found as in the experiments in EL4 lysates.

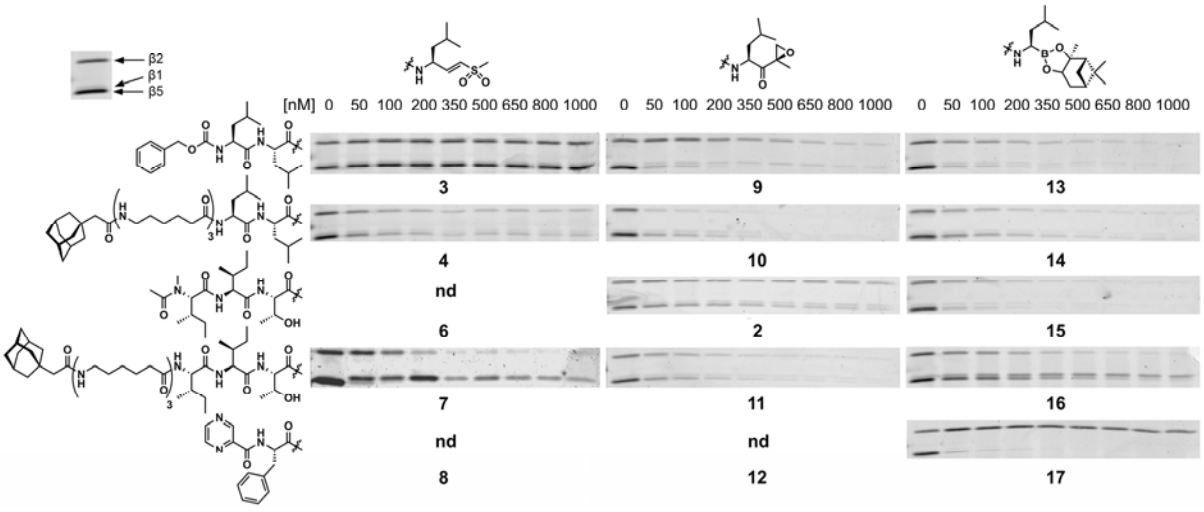


Figure 4. Competition assays of the hybrid library versus MV151 in HEK293T cell lysates.
HEK293T lysates (10 µg total protein) were incubated with the indicated concentrations of inhibitor for 1 hr. at 37 °C. Residual proteasome activity was labeled with 0.1 µM MV151 at 37 °C for 1 hr., prior to denaturation, SDS-PAGE and fluorescence scanning of the wet gel-slabs. nd = not determined.

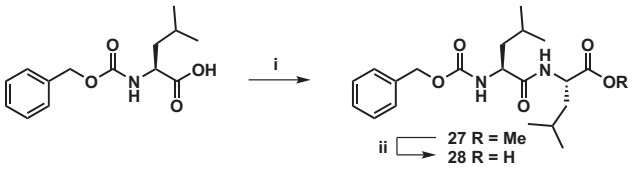
5.3 Conclusion

In conclusion, the work described in this Chapter demonstrates that scrambling of structural elements of known proteasome inhibitors is a viable strategy to arrive at potent new proteasome inhibitors. Prediction of the potency of a putative peptide-based inhibitor is not as straightforward as was considered at the onset of this study. For instance, while it is true that hydrophobic extension in most cases contributes to the potency, the most potent compound from the series tested in EL4 lysates presented here proved to be the boronic ester **15** bearing the epoxomicin tetrapeptide sequence without *N*-terminal extension. In the competition experiments performed in HEK293T lysates, inhibitor **10** proved to be at least equally potent as **15**. Although the potency of a certain inhibitor seems dependent on the cell type used or the type of proteasome (constitutive- or immunoproteasome) that is being expressed, compound **15** may well be the most potent peptide-based proteasome inhibitor reported to date. Possibly, such broad-spectrum proteasome inhibitors might find clinical application as an alternative for bortezomib. For instance, in cases where bortezomib resistance occurs, an event which is thought to be linked to upregulation of those proteasome activities that are left unmodified by bortezomib (bortezomib has a labeling profile similar to **17** and leaves the β2 and β2i subunits untouched at clinical doses).[9,10]

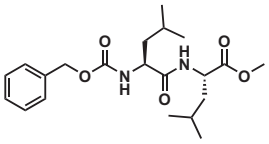
Experimental section

General: All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol..)(purum), ethyl acetate (EtOAc) (puriss.), diethyl ether (Et_2O) and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën and distilled prior to use. Dichloroethane (DCE), dichloromethane (DCM), dimethyl formamide (DMF) and dioxane (Biosolve) were stored on 4Å molecular sieves. Methanol (MeOH) and *N*-methylpyrrolidone (NMP) were obtained from Biosolve. Tetrahydrofuran (THF) (Biosolve) was distilled from $LiAlH_4$ prior to use. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm), spraying with 20% H_2SO_4 in ethanol followed by charring at ~150 °C, by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C or spraying with an aqueous solution of $KMnO_4$ (20%) and K_2CO_3 (10%). Column chromatography was performed on Screening Devices (0.040 – 0.063 nm). HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50), Bruker DPX-300 (300/75 MHz), Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory or a Bruker DMX-600 (600/150 MHz) with cryoprobe. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. UV spectra were recorded on a Perkin Elmer, Lambda 800 UV/VIS spectrometer. For RP-HPLC purifications a BioCAD "Vision" automated HPLC system (PerSeptive Biosystems, inc.) equipped with a semi-preparative Alltima C_{18} column was used. The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. Optical rotations were measured on a Propol automatic polarimeter (sodium D line, λ = 589 nm). ZL_3VS (**3**),[7] Ada-Ahx_3L_3VS (**4**),[8] bortezomib pinanediol ester (**8**),[10] epoxomicin (**2**),[6] Boc-leucine-vinyl-(methyl)-sulfone (**35**)[7] and (Boc-leucinyl)-methyloxirane (**37**)[6] were synthesised as described in literature.

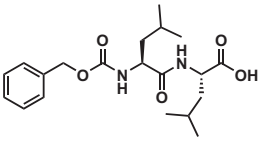
Scheme 2. Synthesis of Z-L_2-OH (**28**).



Reagents and conditions: i) HCl·Leu-OMe (1 equiv.), PyBOP (1 equiv.), DiPEA (2 equiv.), DCM, 3 hr., 68%. ii) NaOH (1.32 equiv.), dioxane/MeOH/H_2O, 3.5 hr., 92%.

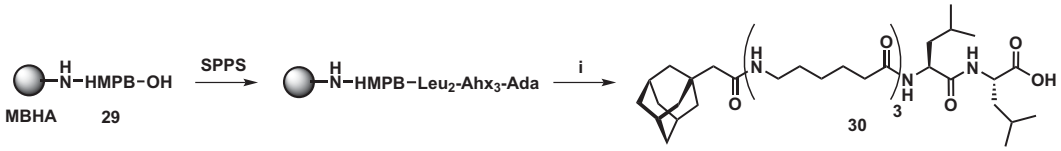


Z-leu$_2$-OMe (27). Z-Leu-OH (5.6 g, 21.5 mmol) and HCl·Leu-OMe (3.92 g, 21.5 mmol, 1 equiv.) were dissolved in 80 ml DCM and put under an Argon atmosphere. PyBOP (11.2 g, 21.5 mmol, 1 equiv.) and DiPEA (7.3 ml, 43 mmol, 2 equiv.) were added and the reaction mixture was stirred for 3 hr. The reaction mixture was washed with sat. aq. $NaHCO_3$, 1M HCl and brine, and the organic phase was dried over $MgSO_4$ and concentrated. Flash column chromatography (PetEt → 10% EtOAc/PetEt, v/v) gave the title compound (5.7 g, 14.5 mmol, 68%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.52 (d, *J* = 7.7 Hz, 1H), 7.32-7.15 (m, 5H), 6.31 (d, *J* = 8.4 Hz, 1H), 5.08-4.90 (m, 2H), 4.60-4.18 (m, 2H), 3.59 (s, 3H), 1.70-1.35 (m, 6H), 0.95 – 0.68 (m, 6H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ ppm 172.7, 172.5, 155.9, 136.0, 127.9, 127.5, 127.3, 66.2, 52.9, 51.5, 50.3, 41.1, 40.4, 24.3, 24.1, 22.2, 21.7, 21.4.



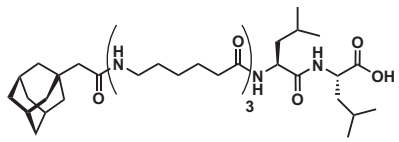
Z-Leu$_2$-OH (28). Z-Leu$_2$-OMe (**27**), (5.7 g, 14.5 mmol) was dissolved in a mixture of 61 ml dioxane, 21.7 ml MeOH and 4.78 ml 4M NaOH in H_2O (19.1 mmol, 1.32 equiv.) and stirred for 3.5 hr. The reaction mixture was acidified to pH 2 with 1M HCl and concentrated. The residue was dissolved in EtOAc, washed with H_2O. The water layer was extracted with EtOAc (2x), and the combined organic layers were dried over $MgSO_4$ and concentrated. Crystallisation from EtOAc/PetEt yielded the title compound (5.03 g, 13.3 mmol, 92%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.40-7.25 (m, 5H), 6.65 (d, *J* = 6.2 Hz, 1H), 5.48 (m, 1H), 5.1 (s, 2H), 4.65-4.50 (m, 1H), 4.38-4.19 (m, 1H), 1.81-1.43 (m, 6H), 1.05-0.80 (m, 6H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ ppm 175.8, 172.9, 156.5, 136.1, 128.4, 127.6, 127.8, 67.0, 53.3, 50.8, 41.0, 24.7, 24.4, 22.7, 21.7.

Scheme 3. Synthesis of Ada-Ahx$_3$-Leu$_2$-OH (**30**).



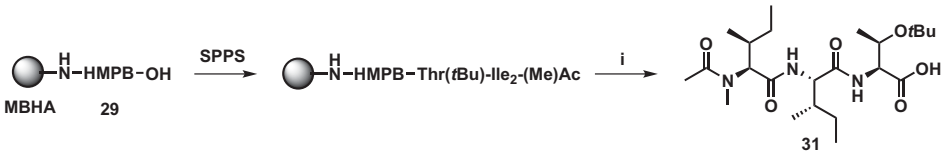
Reagents and conditions: i) 1% TFA in DCM, 3x 10 min.

HMPB-MBHA resin (29). 4-methylbenzhydrylamine (MBHA) functionalized polystyrene resin (0.56 g, 0.9 mmol/g, 0.5 mmol) was washed with NMP (3x) followed by addition of a preactivated mixture of 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) linker (0.36 g, 1.5 mmol, 3 equiv.), BOP (0.67 g, 1.5 mmol, 3 equiv.) and DiPEA (0.55 ml, 3 mmol, 6 equiv.) in NMP. After 2 hr. of shaking, the resin was washed with NMP (3x), MeOH (3x) and DCM (3x), dried and used as such.

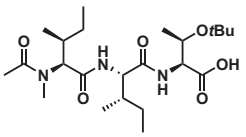


Ada-Ahx$_3$-Leu$_2$-OH (30). Resin **29** (0.5 mmol) was transferred to a flask, coevaporated with DCE (2x), and condensed with Fmoc-Leu-OH (0.53 g, 1.5 mmol, 3 equiv.) under the influence of DIC (0.26 ml, 1.67 mmol, 3.3 equiv.) and DMAP (10 mg, 0.075 mmol, 15 mol%) in DCM for 2 hr. The resin was filtered and washed with DCM (2x), followed by a second condensation cycle. The loading of the resin was determined to be 0.42 mmol/g (0.93 g, 0.39 mmol, 78%) by spectrophotometric analysis. The obtained resin was submitted to four cycles of Fmoc solid-phase synthesis with Fmoc-Leu-OH and Fmoc-Ahx-OH (3×), respectively, as follows: a) deprotection with piperidine/NMP (1/4, v/v, 20 min.), b) wash with NMP (3×), c) coupling of Fmoc amino acid (1.2 mmol, 3 equiv.) in the presence of BOP (0.53 g, 1.2 mmol, 3 equiv.) and DiPEA (0.4 ml, 2.3 mmol, 6 equiv.) in NMP and shaken for 2 hr., d) wash with NMP (3×) and DCM (3×). Couplings were monitored for completion by the Kaiser test. After deprotection of the resin bound pentapeptide, adamantylacetic acid (0.23 g, 1.2 mmol, 3 equiv.), PyBOP (0.63 g, 1.2 mmol, 3 equiv.), DiPEA (0.4 ml, 2.34 mmol, 6 equiv.) in NMP were added, and the resin was shaken for 2 hr. After washing with NMP (3×) and DCM (3×) the resin was subjected to mild acidic cleavage (TFA/DCM, 1/99 v/v, 10 min, 3x) and the collected fractions were coevaporated with Tol. (2x) to give the crude title compound, which was used without any further purification. ^{1}H NMR (200 MHz, CD_3OD): δ ppm 4.51-4.34 (m, 2H), 3.25-3.05 (m, 6H), 2.22-2.10 (m, 6H), 1.93 (s, 2H), 1.82-1.20 (m, 39H), 1.01-0.83 (m, 12H). ^{13}C NMR (50.1 MHz, CD_3OD): δ ppm 176.0, 175.7, 174.8, 173.8, 53.0, 51.9, 51.7, 43.7, 41.7, 40.2, 37.8, 36.9, 36.6, 33.8, 30.1, 30.0, 27.5, 26.7, 25.9, 22.1, 21.9.

Scheme 4. Synthesis of Ac(Me)-Ile$_2$-Thr(*t*Bu)-OH (**31**).

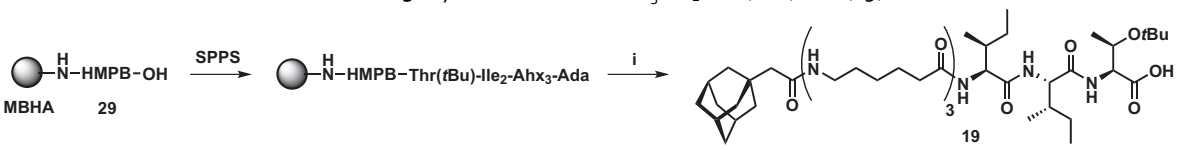


Reagents and conditions: i) 1% TFA in DCM, 3x 10 min.

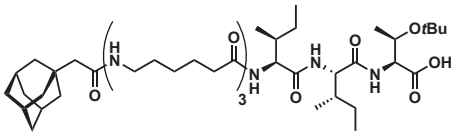


Ac(Me)-Ile$_2$-Thr(*t*Bu)-OH (31). Resin **29** (1 mmol) was transferred to a flask, coevaporated with DCE (2x), and condensed with Fmoc-Thr(*t*Bu)-OH (1.2 g, 3 mmol, 3 equiv.) under the influence of DIC (0.51 ml, 3.3 mmol, 3.3 equiv.) and DMAP (18 mg, 0.15 mmol, 15 mol%) in DCM for 2 hr. The resin was filtered and washed with DCM (2x), followed by a second condensation cycle. The loading of the resin was determined to be 0.55 mmol/g by spectrophotometric analysis. The obtained resin was submitted to two cycles of Fmoc solid-phase synthesis with Fmoc-Ile-OH and Fmoc(Me)-Ile-OH, respectively, as follows: a) deprotection with piperidine/NMP (1/4, v/v, 20 min.), b) wash with NMP (3×), c) coupling of Fmoc amino acid (2.5 mmol, 2.5 equiv.) in the presence of BOP (1.1 g, 2.5 mmol, 2.5 equiv.) and DiPEA (0.5 ml, 3 mmol, 3 equiv.) in NMP and shake for 2 hr., d) wash with NMP (3×) and DCM (3×). Couplings were monitored for completion by the Kaiser test. After Fmoc deprotection of the resin bound tripeptide, acetyl chloride (0.3 ml, 4 mmol, 4 equiv.) and DiPEA (0.66 ml, 4 mmol, 4 equiv.) in DCM were added, and the resin was shaken for 2 hr. After washing with DCM (3×) the resin was subjected to mild acidic cleavage (TFA/DCM, 1/99 v/v, 10 min, 3x) and the collected fractions were coevaporated with Tol. (2x) to give the crude title compound, which was used without any further purification.

Scheme 5. Synthesis of Ada-Ahx$_3$-Ile$_2$-Thr(tBu)-OH (19).

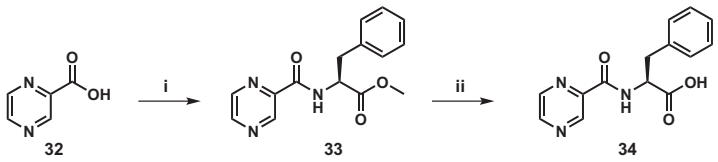


Reagents and conditions: i) 1% TFA in DCM, 3x 10 min.

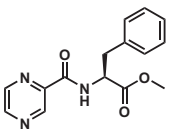


Ada-Ahx$_3$-Ile$_2$-Thr(tBu)-OH (19). HMPB-MBHA resin **29** (2 g, 0.75 mmol/g resin, 1.5 mmol) was coevaporated with DCE (2x) and condensed with Fmoc-Thr(tBu)-OH (1.8 g, 4.5 mmol, 3 equiv.) under the influence of DIC (0.7 ml, 4.5 mmol, 3 equiv.) and DMAP (10 mg, 75 µmol) for 2 hr. The resin was filtered, washed with DCM (3x) and subjected to a second condensation cycle. The loading of the resin was 0.67 mmol/g, as determined by spectrophotometric analysis. Next, the resin was subjected to five cycles of Fmoc solid-phase synthesis with Fmoc-Ile-OH (2x) and Fmoc-Ahx-OH (3x), respectively as follows: a) deprotection with piperidine/NMP (1/4, v/v, 15 min), b) washing with NMP (3x) and DCM (3x), c) coupling of the Fmoc amino acid (4.5 mmol, 3 equiv.) by shaking the resin for 2 hr. in the presence of HCTU (3 equiv., 4.5 mmol), DiPEA (6 equiv., 9 mmol) and NMP as solvent, d) washing with NMP (3x) and DCM (3x). Couplings were monitored by the Kaiser test for completion. This resin (1.7 g, 0.75 mmol) was deprotected using piperidine/NMP (1/4, v/v, 15 min), washed with NMP (3x) and DCM (3x) and condensed with Ada-OH (0.44 g, 2.25 mmol, 3 equiv.) using HCTU (0.93 g, 2.25 mmol, 3 equiv.) and DiPEA (0.75 ml, 4.5 mmol, 6 equiv.) in NMP for 2 hr. The resin was washed with NMP (3x) and DCM (3x), before being subjected to mild acidic cleavage (TFA/DCM, 1/99 v/v, 15 min, 3x). The fractions were collected and concentrated in the presence of Tol., yielding the title compound (505 mg, 0.55 mmol, 73 %). ^{1}H NMR (200 MHz, CD_3OD): δ ppm 4.48-4.23 (m, 4H), 3.25-3.08 (m, 6H), 2.35-2.12 (m, 7H), 2.04-1.10 (m, 52H), 1.00-0.83 (m, 12H). ^{13}C NMR (50.1 MHz, CD_3OD): δ ppm 175.8, 173.5, 173.6, 75.1, 68.8, 59.0, 51.8, 43.7, 40.1, 38.0, 37.9, 37.6, 36.9, 36.6, 33.7, 30.1, 30.0, 28.8, 27.5, 26.6, 26.0, 25.7, 21.1, 16.1, 15.9, 11.4, 11.2.

Scheme 6. Synthesis of pyrazine-2-carbonylphenylalanine (**34**).

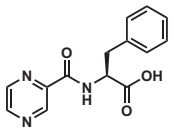


Reagents and conditions: i) H-Phe-OMe·HCl (1.1 equiv.), BOP (1.1 equiv.), DiPEA (3 equiv.), DMF, 1 hr., 70%. ii) NaOH (1.05 equiv.), dioxane/MeOH/H_2O, 1 hr., 69%.



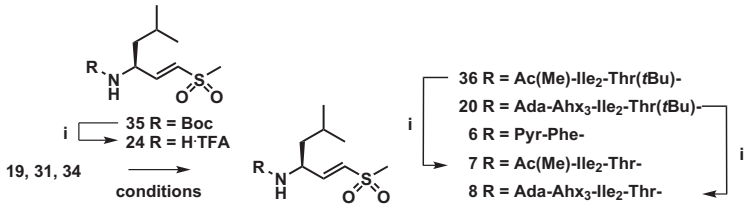
Pyrazine-2-carbonylphenylalanine methyl ester (33). Pyrazine-2-carboxylic acid (**32**) (0.37 g, 3 mmol) was dissolved in DMF (30 ml), put under an Argon atmosphere and preactivated with BOP (1.46 g, 3.3 mmol, 1.1 equiv.) and DiPEA (1.53 ml, 9 mmol, 3 equiv.) for 15 min. To this solution, phenylalanine methyl ester · HCl (0.712, 3.3 mmol, 1.1 equiv.) dissolved in 30 ml DMF was added, and the reaction mixture was stirred for 1 hr. Sat. aq. $NaHCO_3$ was added and the water layer was extracted with Et_2O (3x). The combined organic layers were dried over $MgSO_4$ and concentrated. The crude product was purified by column chromatography (Tol. → 40% EtOAc in Tol. v/v) to yield the title compound (0.6 g, 2.1 mmol, 70%). ^{1}H NMR (200 MHz, $CDCl_3$): δ

ppm 9.36 (d, *J* = 1.5 Hz, 1H), 8.71 (d, *J* = 2.6 Hz, 1H), 8.46 (m, 1H), 8.29 (d, *J* = 7.7 Hz, 1H), 7.25 (m, 5H), 5.14-5.04 (m, 1H), 3.74 (s, 3H), 3.35-3.15 (m, 2H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ ppm 171.2, 162.4, 147.2, 144.0, 143.7,142.5, 135.5, 128.9, 128.4, 126.9, 53.1, 52.2, 37.8.

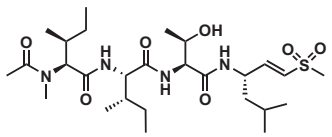


Pyrazine-2-carbonylphenylalanine (34). Methyl ester **33** (0.24 g, 0.85 mmol) was dissolved in 3 ml dioxane, 1.1 ml MeOH and 0.22 ml 4M NaOH. The reaction mixture was stirred for 1 hr., before 0.8 ml 1M $KHSO_4$ was added, and the solution was concentrated. The residue was taken up in H_2O/brine (1/1, v/v), and this solution was extracted with EtOAc (3x). The combined organic layers were dried over $MgSO_4$ and concentrated, yielding the title compound in 69% yield (0.16 gr, 0.59 mmol). LC/MS analysis: R_t 10.16 min (linear gradient 10 → 90% B in 20 min), *m/z* 272.0 $[M+H]^+$, 543.1 $[2M+H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 9.37 (s, 1H), 8.76 (s, 1H), 8.54 (s, 1H), 8.21 (d, *J* = 8.0 Hz, 1H), 7.3-7.19 (m, 5H), 5.18-5.07 (m, 1H), 3.42-3.20 (m, 2H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ ppm 174.0, 164.7, 148.7, 145.6, 144.7, 144.6, 137.9, 130.3, 129.5, 127.9, 54.9, 38.2.

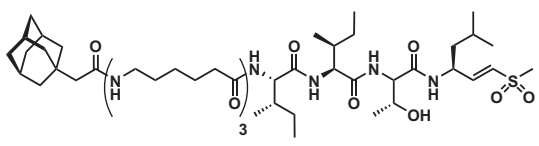
Scheme 7. Synthesis of the vinyl sulfone equipped hybrid inhibitors.



Reagents and conditions: i) TFA/DCM 1/1, 30 min.

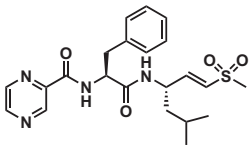


Ac(Me)-Ile$_2$-Thr-LeuVS (6). Boc-leucine-vinyl-(methyl)-sulfone (**35**)[7] (87 mg, 0.3 mmol) was stirred in TFA/DCM (1/1 v/v, 1 ml) until TLC analysis indicated complete deprotection. The reaction mixture was concentrated in presence of Tol. (2x) before being dissolved in DCM and put under an Argon atmosphere. Ac-(Me)-I$_2$-T(*t*Bu)-OH **31** (337 mg, 0.45 mmol, 1.5 equiv.), BOP (0.2 g, 0.45 mmol, 1 equiv.), DiPEA (0.12 ml, 0.76 mmol, 2.5 equiv.) were added, and the mixture was stirred for 12 hr., before being concentrated *in vacuo*. The crude vinyl sulfone was dissolved in TFA/DCM (1/1 v/v, 2 ml) and stirred at ambient temperature for 30 min., before being concentrated in presence of Tol. (2x), yielding Ac(Me)-Ile$_2$-Thr-LeuVS (**6**) as a mixture of two diastereomers. Semi-preparative RP-HPLC purification of the major product yielded the title compound as a white solid (1.2 mg, 2 µmol, 0.7% isolated yield). $[\alpha]_D^{20}$= -225 (c 0.024, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ ppm 6.81 (dd, J_1 = 14.6, J_2 = 4.0 Hz, 1H), 6.71 (d, *J* = 15.2 Hz, 1H), 4.78 (d, 11.2 Hz, 1H), 4.71 (m, 1H), 4.26 (m, 1H), 4.17 (m, 2H), 3.02 (s, 3H), 2.95 (s, 3H), 2.12 (s, 3H), 2.05 (m, 1H), 1.88 (m, 1H), 1.71 (m, 1H), 1.62-1.26 (m, 6H), 1.19 (m, 1H), 1.18 (d, *J* = 6.4 Hz, 3H), 1.02 (m, 1H), 0.92 (m, 18H). HRMS: calcd. for $[C_{27}H_{50}O_7N_4SH]^+$ 575.34730, found 575.34769.



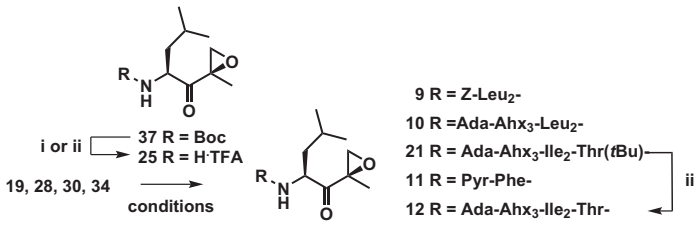
Ada-Ahx$_3$-Ile$_2$-Thr-LeuVS (7). Boc-leucine-vinyl-(methyl)-sulfone (**35**)[7] (76 mg, 0.26 mmol, 1.1 equiv.) was stirred in TFA/DCM (1/1 v/v, 1 ml) until TLC analysis indicated complete conversion of the starting material. The reaction mixture was concentrated in presence of Tol. (2x) before being dissolved in DMF and put under an Argon atmosphere. Ada-Ahx$_3$-I$_2$-T(*t*Bu)-OH **19** (217 mg, 0.24 mmol), BOP (117 mg, 0.26 mmol,

1.1 equiv.), DiPEA (90 µl, 0.53 mmol, 2.2 equiv.) were added, and the mixture was stirred for 12 hr., before being concentrated *in vacuo*. The crude vinyl sulfone was dissolved in TFA/DCM (1/1 v/v, 1 ml) and stirred at ambient temperature for 30 min., before being concentrated in presence of Tol. (2x), yielding Ada-Ahx$_3$-Ile$_2$-Thr-LeuVS (**7**) as a mixture of two diastereomers. Semi-preparative RP-HPLC purification of the major product yielded the title compound as a white solid (38.8 mg, 37.5 µmol, 16%). $[\alpha]_D^{20}$= -31 (c 0.2, MeOH). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 7.52 (m, 2H), 6.86 (dd, J_1 = 4.3, J_2 = 15 Hz, 1H), 6.65 (d, J = 15 Hz, 1H), 4.72 (m, 1H), 4.32 – 4.10 (m, 4H), 3.18 (t, J = 6.9 Hz, 6H), 2.98 (s, 3H), 2.29 (t, J = 7.5 Hz, 2H), 2.18 (t, J = 7.4 Hz, 4H), 1.97 (m, 3H), 1.93 (s, 2H), 1.83 (m, 2H), 1.73 – 1.45 (m, 30H), 1.39 – 1.15 (m, 10H), 0.97 – 0.89 (m, 18H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 175.2, 174.2, 172.7, 172.0, 171.7, 170.1, 147.4, 128.8, 66.5, 59.0, 58.7 (2x), 51.0, 47.8, 42.3, 42.2, 42.1, 38.8, 38.7, 36.4, 35.9, 35.8, 35.3, 32.4, 28.6 (2x), 28.5, 28.4, 26.0, 25.0, 24.9, 24.8, 24.6, 24.4, 22.5, 21.2, 19.2, 15.2, 15.1, 11.1, 10.6. HRMS: calcd. for $[C_{54}H_{95}N_7O_{10}SH]^+$ 1056.67533, found 1056.67658.

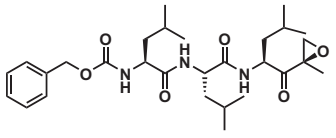


(Pyrazine-2-carbonylphenylalanyl)-leucine-vinyl-(methyl)-sulfone (8). Pyrazine-2-carbonylphenylalanine **34** (157 mg, 0.58 mmol) was preactivated with BOP (282 mg, 0.64 mmol, 1.1 equiv.) and DiPEA (0.3 ml, 1.7 mmol, 3 equiv.) in DCM. TFA·LeuVS **24** (212 mg, 0.7 mmol, 1.1 equiv.) was added, and the mixture was stirred for 4 hr. The reaction mixture was concentrated and subjected to flash column chromatography (Tol.. → EtOAc), yielding two epimers (both 82 mg, 0.18 mmol, 32%). Recrystallisation from PetEt/acetone yielded the title compound (50 mg, 0.113 mmol, 19%). $[\alpha]_D^{20}$= -12 (c 0.2, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 9.32 (s, 1H), 8.77 (d, J = 2.0 Hz, 1H), 8.59 (s, 1H), 8.50 (m, 1H), 7.43-7.22 (m, 5H), 7.16 (d, J = 8.0 Hz, 1H), 6.66 (dd, J_1 = 4.8, J_2 = 15.2 Hz, 1H), 5.99 (d, J = 15.2 Hz, 1H), 4.90-4.82 (m, 1H), 4.68-4.59 (m, 1H), 3.25-3.13 (m, 2H), 2.89 (s, 3H), 1.64-1.49 (m, 1H), 1.45-1.30 (m, 2H), 0.93-0.81 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 170.0, 163.0, 147.5, 147.2, 144.1, 143.8, 142.9, 136.0, 129.3, 128.9, 128.8, 127.3, 54.5, 47.9, 42.6, 42.5, 38.4, 24.5, 22.4, 21.7. HRMS: calcd. for $[C_{22}H_{28}O_4N_4SH]^+$ 445.19040 found 445.19031.

Scheme 8. Synthesis of the epoxyketone equipped hybrid inhibitors.

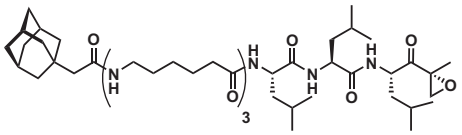


Reagents and conditions: i) TFA, 15 min. ii) TFA/DCM 1/1, 30 min.

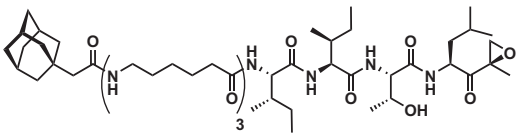


Z-Leu$_3$-2-methyloxirane (9). (Boc-leucinyl)-methyloxirane (**37**)[6] (67 mg, 0.25 mmol) was stirred in TFA (1 ml) until TLC analysis indicated complete consumption of the starting material. Tol.. was added, and the reaction mixture was concentrated. Z-Leu$_2$-OH **28** (104 mg, 0.275 mmol, 1.1 equiv.) was coevaporated with Tol.. (2x), dissolved in DCM and put under an Argon atmosphere. PyBOP (150 mg, 0.29 mmol, 1.16 equiv.) and DiPEA (0.13 ml, 0.75 mmol, 3 equiv.) were added, followed by the crude TFA·leucinyl-methyloxirane **25**. The reaction mixture was stirred for 1hr. The mixture was washed with H_2O and brine, dried over $MgSO_4$ and concentrated. Crystallisation from EtOAc/PetEt yielded crude title compound **9**, which was further purified by flash column chromatography (PetEt → 30%

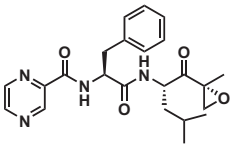
EtOAc/PetEt, v/v) yielding the title compound (18.7 mg, 44 µmol, 18%). $[\alpha]_D^{20}$ = -1 (c 0.2, MeOH). ^{1}H NMR (200 MHz, CD_3OD): δ ppm 7.35-7.25 (m, 5H), 5.08 (m, 2H), 4.62-4.38 (m, 2H), 4.22-4.05 (m, 1H), 3.24 (d, *J* = 5.1 Hz, 1H), 2.91 (d, 5.1 Hz, 1H), 1.77-1.25 (m, 9H), 0.95-0.83 (m, 18H). ^{13}C NMR (50.1 MHz, CD_3OD): δ ppm 209.5, 175.1, 174.5, 156.0, 129.5, 129.0, 128.8, 67.6, 60.1, 54.9, 53,1, 52.7, 51.9, 42.1, 41.9, 40.2, 26.2, 25.8, 25.7, 23.7, 23.4, 22.2, 22.0, 24.5, 17.0. HRMS: calcd. for $[C_{29}H_{45}O_6N_3H]^+$ 532.33811, found 532.33826.



Ada-Ahx$_3$-Leu$_2$-leucinyl-2-methyloxirane (10). (Boc-leucinyl)-methyloxirane (**37**)[6] (116 mg, 0.35 mmol) was stirred in TFA (1 ml) until TLC analysis indicated complete consumption of the starting material. The reaction mixture was concentrated in the presence of Tol.. (2x), dissolved in DCM/DMF (19/1, v/v) and put under an Argon atmosphere. Ada-Ahx$_3$-Leu$_2$-OH (**30**) (0.27 g, 0.35 mmol, 1 equiv.), PyBOP (0.2 g, 0.38 mmol, 1.1 equiv.) and DiPEA (0.17 ml, 1.1 mmol, 3 equiv.) were added and the mixture was stirred for 3 hr., before being concentrated and purified by flash column chromatography (DCM → 10% MeOH/DCM, v/v), yielding the title compound (130 mg, 0.14 mmol, 41%). $[\alpha]_D^{20}$= -1.5° (c 2, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ ppm 4.58-4.47 (m, 1H), 4.47-4.32 (m, 2H), 3.14 (t, *J* = 6.3 Hz, 6H), 3.24 (m, 1H), 2.92 (d, *J* = 5.1 Hz, 1H), 2.23 (t, *J* = 7.4 Hz, 2H), 2.17 (t, *J* = 7.4 Hz, 4H), 1.97-1.89 (m, 5H), 1.77-1.42 (m, 30H), 1.41-1.27 (m, 12H), 0.99-0.85 (m, 18H). HRMS: calcd. for $[C_{51}H_{88}N_6O_8H]^+$ 913.67390 found 913.67364



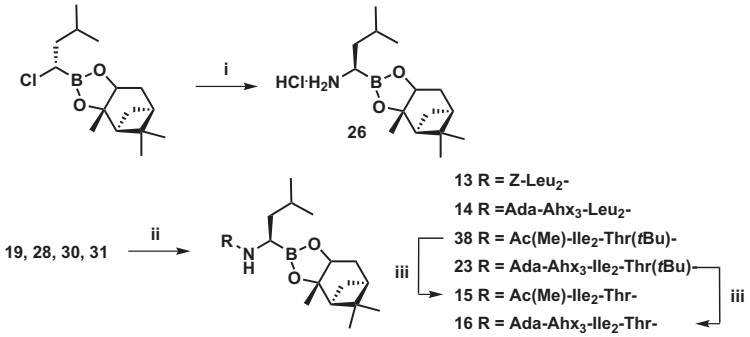
Ada-Ahx$_3$-Ile$_2$-Thr-leucinyl-2-methyloxirane (11). (Boc-leucinyl)-2-methyloxirane (**37**)[6] (78 mg, 0.29 mmol, 1.1 equiv.) was stirred in TFA/DCM (1/1 v/v, 1 ml) until TLC analysis indicated complete conversion of the starting material (20 min.). The reaction mixture was concentrated in presence of Tol. (2x) before being dissolved in DMF and put under an Argon atmosphere. Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-OH (**19**) (236 mg, 0.26 mmol), BOP (0.13 g, 0.29 mmol, 1.1 equiv.), DiPEA (95 µl, 1.1 mmol, 4.4 equiv.) were added, and the mixture was stirred for 3hr. The crude Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-leucinyl-2-methyloxirane (**21**) was precipitated with EtOAc yielding a mixture of diastereomers. The major product was purified by semi-preparative RP-HPLC yielding **21** (27.3 mg, 25.5 µmmol, 10%). Tert-butyl ether **21** was dissolved in TFA/DCM (1/1 v/v) and stirred for 30 min., before being evaporated in the presence of Tol. (2x) yielding the title compound as a colourless oil (23.6 mg, 23.3 µmol, 91%). $[\alpha]_D^{20}$= -17.5 (c 0.24, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ ppm 4.55 (dd, J_1 = 2.6, J_2 = 10.6 Hz, 1H), 4.33 (d, *J* = 5.2 Hz, 1H), 4.27-4.21 (m, 2H), 4.04 (m, 1H), 3.25 (d, *J* = 5.2 Hz, 1H), 3.14 (t, *J* = 7.0 Hz, 6H), 2.91 (d, *J* = 5.2 Hz, 1H), 2.24 (t, *J* = 7.2 Hz, 2H), 2.16 (t, *J* = 7.4 Hz, 4H), 1.94 (m, 3H), 1.91 (s, 2H), 1.82 (m, 2H), 1.74-1.56 (m, 22H), 1.55-1.47 (m, 7H), 1.46 (s, 3H), 1.39-1.11 (m, 11H), 0.94 – 0.86 (m, 18H). ^{13}C NMR (100.2 MHz, $CDCl_3$): δ ppm 209.4, 176.0, 175.9, 174.0, 173.7, 173.4, 172.1, 68.4, 60.0, 59.6, 59.3, 59.1, 53.0, 51.9, 51.8, 43.7, 40.3, 40.2 (2x), 40.1, 37.9 (2x), 37.6, 37.0, 36.6, 33.7, 30.1 (3x), 27.6, 27.5, 26.6, 26.2, 26.0, 25.9, 23.8, 19.9, 17.0, 16.0, 15.9, 11.4, 11.2. HRMS: calcd. for $[C_{55}H_{95}N_7O_{10}H]^+$ 1014.72132 found 1014.72270, calcd. for $[C_{55}H_{95}N_7O_{10}Na]^+$ 1036.70326, found 1036.70421.



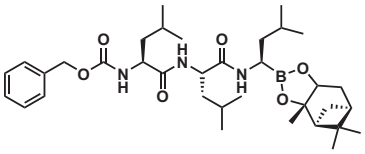
(Pyrazine-2-carbonylphenylalanyl)-leucinyl-2-methyloxirane (12). (Boc-leucinyl)-methyloxirane (**37**)[6] (103 mg, 0.38 mmol) was stirred in TFA (1 ml) until TLC analysis indicated complete consumption of the starting material. Tol. was added, and the reaction mixture was concentrated. Pyrazine-2-carbonylphenylalanine **34** (0.11 g, 0.4 mmol, 1.05 equiv.) was coevaporated with Tol. (2x) and dissolved in DCM (20 ml) and put under an Argon atmosphere. BOP (0.2 g, 0.44 mmol, 1.1 equiv.) and DiPEA (0.2 ml, 1.14 mmol, 3 equiv.) were added, followed by addition of the TFA·leucinyl-methyloxirane **25**. The

reaction mixture was stirred for 1hr., before washing with sat. aq. $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentrated. Flash column chromatography (PetEt → 25% EtOAc/PetEt) yielded the title compound (8.6 mg, 20 µmol, 5.3% isolated yield). $[\alpha]_D^{20}$= +12 (c 0.1, MeOH). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 9.35 (d, *J* = 1.1 Hz, 1H). 8.75 (d, *J* = 2.2 Hz, 1H), 8.53 (d, *J* = 1.5 Hz, 1H), 8.37 (d, *J* = 8.0 Hz, 1H), 7.31-7.20 (m, 5H), 6.36 (d, *J* = 7.7 Hz, 1H), 4.91 (dd, J_1 = 6.9, J_2 = 8.0 Hz, 1H), 4.60-4.50 (m, 1H), 3.28 (d, *J* = 5.1 Hz, 1H), 3.20-3.15 (m, 2H), 2.90 (d, *J* = 4.7 Hz, 1H), 1.60 – 1.30 (m, 6H), 0.89-0.82 (m, 6H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ ppm 207.9, 170.3, 162.9, 147.5, 144.3, 143.9, 142.7, 136.2, 129.3, 128.6, 127.1, 58.9, 54.3, 52.3, 50.2, 40.2, 38.4, 25.0, 24.8, 23.1, 16.6. HRMS: calcd. for $[C_{23}H_{28}O_4N_4H]^+$ 425.21833, found 425.21835.

Scheme 9. Synthesis of the boronic ester equipped hybrid inhibitors.

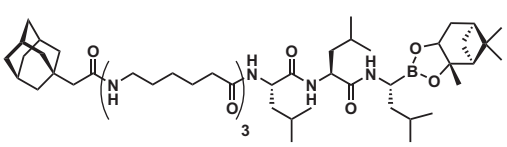


Reagents and conditions: i) (a) LiHMDS (1 equiv.), THF, -20 °C to RT, 12 hr. (b) HCl (5.7 equiv.), -90 °C to 0 °C, 2 hr. ii) (a) *N*-hydroxysuccinimide (1.4 equiv.), DIC (1.2 equiv.), DMF, 12 hr. (b) **26** (1 equiv.), DiPEA (1.02 equiv.), DMF/THF, -80 °C to RT, 4 hr. iii) TFA/DCM 1/1, 30 min.

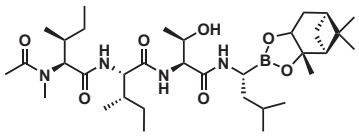


Z-Leu$_3$-boronic ester (13). Under argon atmosphere, Z-Leu$_2$-OH (**28**) (0.2 g, 0.53 mmol) and HOSu (85.1 mg, 0.73 mmol, 1.4 equiv.) were dissolved in DMF (1 ml) and DIC (100 µl, 0.64 mmol, 1.2 equiv.) was added. After a few min. a fine precipitate was formed. The mixture was stirred overnight at ambient temperature after which LC/MS analysis showed complete conversion of Z-Leu$_2$-OH (**28**) into Z-Leu$_2$-OSu. (1R)-4-(1-chloro-3-methyl(butyl)-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.o^{2,6}]decane[10] (152 mg, 0.53 mmol, 1 equiv.) was dissolved in THF (5 ml) in a flame dried two necked reaction flask which was put under an argon atmosphere. At -20 °C a LiHMDS solution (1M in THF, 600 µl, 0.60 mmol, 1.1 equiv.) was added dropwise. The mixture was allowed to warm to room temperature and stirred overnight. At -90 °C a HCl solution (2M in Et_2O, 1.50 ml, 3.0 mmol, 5.7 equiv.) was added drop wise. The mixture was slowly warmed to 0 °C (ca. 2 hr.) and re-cooled to -80 °C at which temperature the Z-Leu$_2$-OSu described above and DIPEA (0.90 ml, 5.4 mmol, 1.02 equiv.) were added. The mixture was slowly warmed to room temperature (ca. 2 hr.) and was stirred for another 2 hr. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silicagel column chromatography (10% EtOAc/PetEt → 25% EtOAc/PetEt) affording the title compound as a white solid (197 mg, 0.32 mmol, 59%). $[\alpha]_D^{20}$= -58 (c 1, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ ppm 7.37-7.28 (m, 5H), 6.79 (d, *J* = 8.1 Hz, 1H), 5.50 (d, *J* = 6.5 Hz, 1H), 5.12 (d, *J* = 12.0 Hz, 1H), 5.02 (d, *J* = 12.2 Hz, 1H), 4.58 (dd, J_1 = 13.4, J_2 = 7.5 Hz, 1H), 4.27 (dd, J_1 = 8.6, J_2 = 1.8 Hz, 1H), 4.22-4.13 (m, 1H), 3.10-2.97 (m, 1H), 2.36-2.27 (m, 1H), 2.15 (td, J_1 = 10.5, J_2 = 5.2 Hz, 1H), 2.01 (t, *J* = 5.4 Hz, 1H), 1.93-1.77 (m, 2H), 1.76-1.55 (m, 5H), 1.55-1.34 (m, 7H), 1.33-1.23 (m, 4H), 0.98-0.79 (m, 21H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 178.4, 175.3, 158.7, 138.0, 129.4(2x), 129.0, 128.8 (2x), 128.6, 84.0, 77.2, 67.7, 55.0, 53.5, 49.4, 42.0, 41.7,

41.3, 41.1, 39.1, 37.6, 29.9, 27.8, 27.4, 26.7, 25.8, 25.6, 24.6, 23.7, 23.5, 23.2, 22.5, 22.0, 21.8. HRMS: calcd. for $[C_{35}H_{56}BN_3O_6H]^+$ 626.43349, found 626.43408, calcd. for $[C_{35}H_{56}BN_3O_6Na]^+$ 648.41544, found 648.41578.

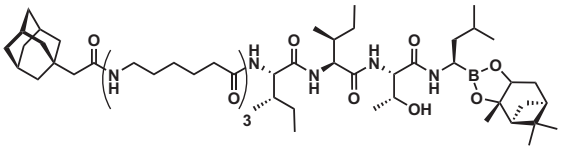


Ada-Ahx$_3$-Leu$_2$-leucinyl-boronic ester (14). Under argon Ada-Ahx$_3$-L$_2$-OH (**30**) (95 mg, 0.13 mmol) and HOSu (35 mg, 0.30 mmol, 2.3 equiv.) were dissolved in DMF (1 ml) and DIC (25 µl, 0.16 mmol, 1.2 equiv.) was added. After a few min. a fine precipitate was formed. The mixture was stirred overnight at ambient temperature after which LC/MS analysis showed complete conversion of Ada-Ahx$_3$-Leu$_2$-OH (**30**) into Ada-Ahx$_3$-Leu$_2$-OSu. (1R)-4-(1-chloro-3-methyl(butyl)-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane[10] (132 mg, 0.46 mmol, 3.5 equiv.) was dissolved in THF (4 ml) in a flame dried two necked reaction flask under an argon atmosphere. At -20 °C a LiHMDS solution (1M in THF, 460 µl, 0.46 mmol, 3.5 equiv.) was added drop wise. The mixture was allowed to warm to room temperature and stirred overnight. At -90 °C a HCl solution (2M in Et_2O, 1.00 ml, 2.0 mmol, 15.4 equiv.) was added drop wise. The mixture was slowly warmed to 0 °C (ca. 2 hr.) and re-cooled to -80 °C at which temperature the Ada-Ahx$_3$-Leu$_2$-OSu described above and DIPEA (0.60 ml, 3.6 mmol, 27.7 equiv.) were added. The mixture was slowly warmed to room temperature (ca. 2 hr.) at which it was stirred for another 2 hr. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silicagel column chromatography (PetEt/EtOAc/MeOH = 3/1/0 → 1/1/0 → 0/1/0 → 0/9/1 v/v/v) affording the title compound as a white solid (74 mg, 73 µmol, 59%). Further purification by RP-HPLC afforded **14** (20 mg, 20 µmol, 16%). $[\alpha]_D^{20}$= -21.7 (c 0.12, MeOH). ^{1}H NMR (600 MHz, CD_3OD): δ ppm 4.61 (dd, J_1 = 9.6, J_2 = 5.4 Hz, 1H), 4.37 (dd, J_1 = 9.6, J_2 = 5.4 Hz, 1H), 4.15 (dd, J_1 = 10.2, J_2 = 8.4 Hz, 1H), 3.17 (t, J = 7.2 Hz, 6H), 2.71 (t, J = 7.2 Hz, 1H), 2.36 (m, 1H), 2.27 (t, J = 7.2 Hz, 2H), 2.20 (t, J = 7.2 Hz, 4H), 2.15 (m, 1H), 1.97 (m, 4H), 1.94 (s, 2H), 1.88 (m, 1H), 1.81 (m, 1H), 1.79 – 1.61 (m, 23H), 1.58 (m, 2H), 1.56 – 1.50 (m, 6H), 1.48 (d, J = 10.2 Hz, 1H), 1.40 (s, 3H), 1.39 – 1.33 (m, 8H), 1.31 (s, 3H), 0.99 (s, 3H), 0.98 (s, 3H), 0.97 (s, 3H), 0.95 (s, 3H), 0.94 (s, 3H), 0.93 (s, 3H), 0.90 (s, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 178.5, 176.4, 175.8, 175.0, 173.6, 84.0, 77.2, 55.8, 53.5, 53.4, 51.9, 49.6, 43.8, 43.7, 41.8, 41.7, 41.4, 41.2, 40.2, 40.1, 39.2, 37.9, 37.7, 37.0, 36.6, 36.5, 33.8, 30.2, 30.1, 30.0, 29.9, 27.8, 27.6, 27.5, 27.4, 26.7, 26.6, 26.5, 26.3, 25.9, 25.7, 24.6, 23.6, 23.5, 23.4, 23.3, 23.2, 22.5, 22.1, 21.9, 21.8, 19.3, 18.8, 17.3, 13.2. HRMS: calcd. for $[C_{57}H_{99}BN_6O_8H]^+$ 1007.76722, found 1007.76902.



Ac(Me)-Ile$_2$-Thr-Leu-boronic ester (15). Under argon Ac(Me)-Ile$_2$-Thr(*t*Bu)-OH (**31**) (78 mg, 0.17 mmol) and HOSu (35 mg, 0.304 mmol, 1.8 equiv.) were dissolved in DMF (1 ml) and DIC (35 µl, 0.23 mmol, 1.4 equiv.) were added. The mixture was stirred overnight at ambient temperature after which LC/MS analysis showed complete conversion. (1R)-4-(1-chloro-3-methyl(butyl)-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane[10] (168 mg, 0.59 mmol, 3.5 equiv.) was dissolved in THF (5 ml) in a flame dried two necked reaction flask under an argon atmosphere. At -20 °C a LiHMDS solution (1M in THF, 550 µl, 0.55 mmol, 3.2 equiv.) was added drop wise. The mixture was allowed to warm to room temperature and stirred overnight. At -90 °C a HCl solution (2M in Et_2O, 1.50 ml, 3.0 mmol, 17.6 equiv.) was added drop wise. The mixture was slowly warmed to 0 °C (ca. 2 hr.) and re-cooled to -80 °C at which temperature the Ac(Me)-Ile$_2$-Thr(*t*Bu)-OSu described above and DIPEA (0.90 ml, 5.4 mmol, 31.8 equiv.) were added. The mixture was slowly warmed to room temperature (ca. 2 hr.) and was stirred for another 2 hr. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silicagel column chromatography (PetEt/EtOAc/MeOH = 3/1/0 → 1/1/0 →

0/1/0 → 0/9/1 v/v/v) affording Ac(Me)-Ile$_2$-Thr(*t*Bu)-Leu-boronic ester (**38**) as a white solid. This material was dissolved in TFA/DCM (1/1 v/v, 1 ml). After 30 min. Tol. (30 ml) was added and the solvents evaporated. Purification by RP-HPLC afforded the title compound (9 mg, 14 µmol, 8%). $[\alpha]_D^{20}$= -360 (c 0.04, MeOH). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.88 (s, 1H), 7.03 (d, *J* = 5.10 Hz, 1H), 6.77 (d, *J* = 8.0 Hz, 1H), 4.60-4.41 (m, 4H), 4.27 (d, *J* = 7.3 Hz, 1H), 4.09 (m, 1H), 3.82 (s, 1H), 2.94 (s, 3H), 2.87 (m, 1H), 2.32 (m, 1H), 2.14 (s, 3H), 2.02 (m, 2H), 1.96-1.71 (m, 4H), 1.41 (s, 3H), 1.34 (m, 3H), 1.28 (s, 2H), 1.18 (s, 1H), 1.14 (s, 1H), 0.96-0.81 (m, 27H). HRMS: calcd. for $[C_{34}H_{61}BN_4O_7H]^+$ 649.47061, found 649.47080, calcd. for $[C_{34}H_{61}BN_4O_7Na]^+$ 671.45255, found 671.45272.



Ada-Ahx$_3$-Ile$_2$-Thr-Leu-boronic ester (16). Under argon Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-OH (**19**) (134 mg, 0.15 mmol) and HOSu (29 mg, 0.25 mmol, 1.7 equiv.) were dissolved in DMF (2 ml) and DIC (35 µl, 0.23 mmol, 1.5 equiv.) were added. After a few min. a fine precipitate was formed. The mixture was stirred overnight at ambient temperature after which LC/MS analysis showed complete conversion of Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-OH (**19**) into Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-OSu. (1R)-4-(1-chloro-3-methyl(butyl)-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane[10] (132 mg, 0.46 mmol, 3.1 equiv.) was dissolved in THF (4 ml) in a flame dried two necked reaction flask which was put under an argon atmosphere. At -20 °C a LiHMDS solution (1M in THF, 460 µl, 0.46 mmol, 3.1 equiv.) was added drop wise. The mixture was allowed to warm to room temperature and stirred overnight. At -90 °C a HCl solution (2M in Diethyl ether, 1.00 ml, 2.0 mmol, 13.3 equiv.) was added drop wise. The mixture was slowly warmed to 0 °C (ca. 2 hr.) and re-cooled to -80 °C at which temperature the Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-OSu described above and DIPEA (0.60 ml, 3.6 mmol, 24 equiv.) were added. The mixture was slowly warmed to room temperature (ca. 2 hr.) and was stirred for another 2 hr. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silicagel column chromatography (PetEt/EtOAc/MeOH = 3/1/0 → 1/1/0 → 0/1/0 → 0/9/1 v/v/v) affording Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-Leu-boronic ester (**23**) as a white solid. This material was dissolved in TFA/DCM (1/1 v/v, 1 ml). After 30 min. Tol. (30 ml) was added and the solvents evaporated. Purification by RP-HPLC afforded the title compound (7 mg, 6.3 µmol, 4%). $[\alpha]_D^{20}$= -72.5 (c 0.08, MeOH). ^{1}H NMR (300 MHz, CD_3OD): δ ppm 4.52 (dd, J_1 = 7.2, J_2 = 4.5 Hz, 1H), 4.21-4.13 (m, 4H), 3.14 (t, *J* = 6.9 Hz, 6H), 2.72 (dd, J_1 = 8.4, J_2 = 6.9 Hz, 1H), 2.36 (m, 1H), 2.25 (t, *J* = 7.5 Hz, 2H), 2.17 (t, *J* = 7.5 Hz, 4H), 1.94 (m, 4H), 1.91 (s, 2H), 1.79 (m, 2H), 1.77-1.43 (m, 30H), 1.41-1.26 (m, 15H), 1.23-1.11 (m, 5H), 0.94 (s, 3H), 0.93 (s, 3H), 0.92 (s, 3H), 0.91 (s, 3H), 0.89 (s, 3H), 0.88 (s, 3H), 0.87 (s, 3H). HRMS: calcd. for $[C_{61}H_{106}BN_7O_{10}H]^+$ 1108.81670, found 1108.81820, calcd. for $[C_{61}H_{106}BN_7O_{10}Na]^+$ 1130.79864, found 1130.79987.

Competition experiments.

Whole cell lysates of EL4 or HEK293T were made by sonication (30 sec., 11 Watt) in lysis buffer containing 50 mM Tris pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 250 mM sucrose, 2 mM ATP. Protein concentration was determined by the Bradford assay. Cell lysates (10µg total protein) were exposed to the inhibitors for 1 hr. prior to incubation with MV151 (**5**, 0.1 µM) for 1 hr. at 37 °C. Reaction mixtures were boiled with Laemmli's buffer containing β-mercapto-ethanol for 3 min. before being resolved on 12.5% SDS-PAGE. In-gel detection of residual proteasome activity was performed in the wet gel slabs directly on the Typhoon Variable Mode Imager (Amersham Biosciences) using the Cy3/Tamra settings (λ_{ex} 532, λ_{em} 560).

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