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

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The synthesis and evaluation of hybrid proteasome inhibitors that contain structural elements of the known inhibitors bortezomib, epoxomicin and peptide vinyl sulfones is described. From the panel of 15 inhibitors some structure activity relationships can be deduced with regard to inhibitory activity in relation to peptide recognition element, inhibitor size and nature of the electrophilic trap. Further, the panel contains one of the most potent peptide-based pan-proteasome inhibitors reported to date.

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 (endoplasmatic reticulum) 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**, Fig. 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 PS341 and open the way to develop more proteasome inhibitor based therapies in oncology.

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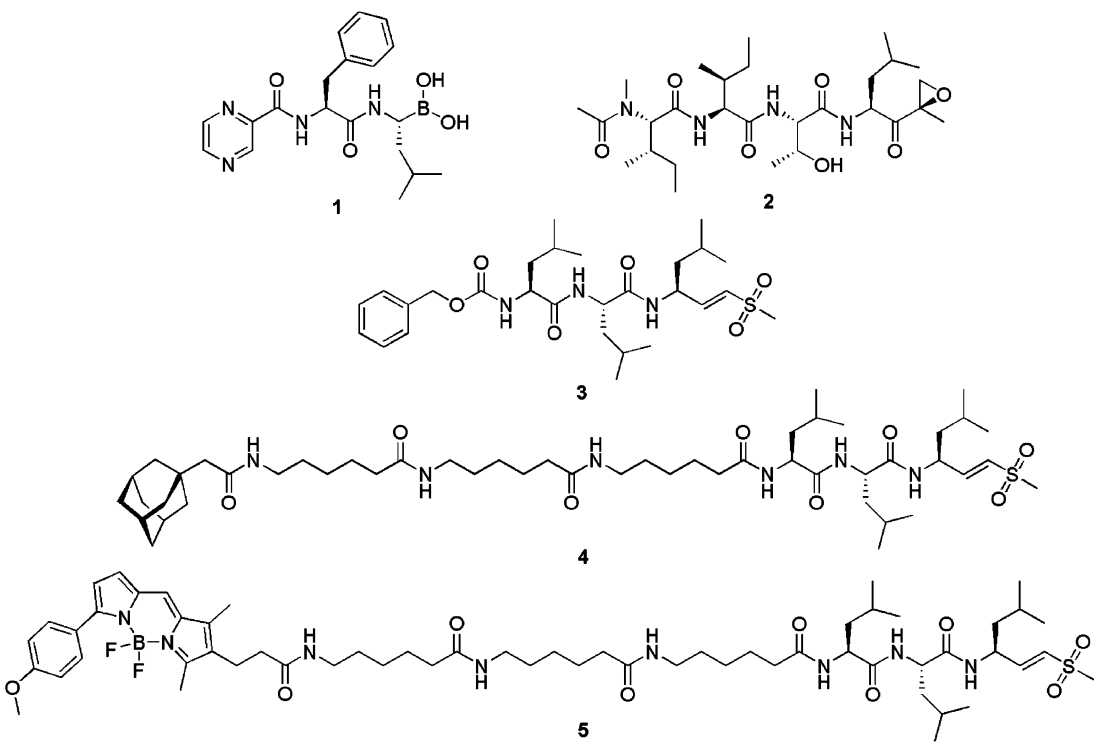

Fig. 1 Proteasome inhibitors at the basis of this study.

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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, such as the mammalian ones. It has C_2 symmetry and is assembled from two outer rings each containing seven identical α-subunits, and two inner seven-subunit rings containing identical and catalytic β-subunits.[4] Eukaryotic proteasomes have evolved to an extent that each α-subunit in one 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 these studies 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 we demonstrated that N-terminally extended, hydrophobic peptide vinyl sulfones, such as the adamantane containing compound **4**, are much more active proteasome inhibitors than their truncated counterparts (for instance, ZL_3VS (**3**)).[8] This gain in activity was accompanied by a loss in subunit selectivity, a finding we capitalized upon by the development of the broad-spectrum cell permeable proteasome label MV151 (Bodipy TMR-Ahx_3L_3VS, **5**).[10] One observation we made is that the potency of peptide vinyl 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, we set out to scramble compounds **1**–**4** to arrive at a number of new C-terminally modified peptides, and assessed these on their proteasome inhibitory activity. We identified three distinct structural features. 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 pharmacophore, and (c) the presence (as in **4**) or absence (as in **3**) of the lipophilic N-terminal extension. These considerations allowed us to synthesise the panel of 15 compounds listed in Table 1.

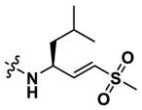
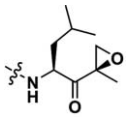
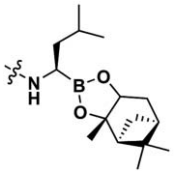
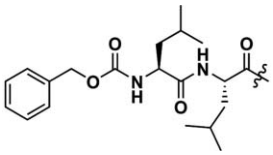
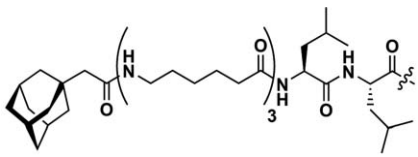
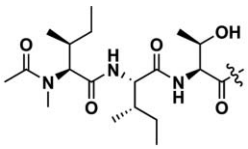
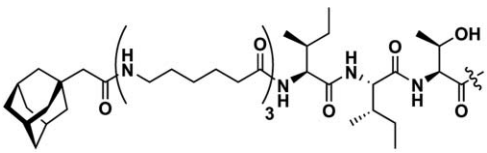
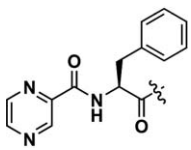
Results and discussion

The preparation of all compounds follows the same general strategy: first the synthesis of the (N-terminally 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 resin gained **18**, followed by cleavage using 1% trifluoroacetic acid in dichloromethane provides 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 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 ester **26**,[10] followed by acidic deprotection and HPLC purification.

In the design of our compounds we decided to exclude a set of compounds that have the azapyracyl moiety in the bortezomib sequence replaced by the adamantane-spacer moiety. Such a set of compounds would likely resemble to a large extent the trileucine derivatives in their inhibition profile. We further 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,[10] and 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 our hands.

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) in combination with fluorophore containing peptide vinyl sulfone **5**. In a first set of experiments, 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 (Fig. 2). Lysates that have been

Table 1 Panel of synthesized proteasome inhibitors

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

exposed to the fluorescent label only display four bands (three strong bands and one weaker band), that we have previously demonstrated to correspond to the six proteasome active sites (specified as depicted in Fig. 2).[10] The ability of the 15 compounds to inhibit the proteasome activities is reflected by disappearance of labelling. 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 labelling abolished at 1 μM.[14] 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 we repeated the competition experiment but with the difference that inhibitor concentrations were ranged from 0 to 500 nM (Fig. 3, compounds **6**, **8** and **12** were excluded since these proved to be hardly effective in the first experiments employing higher final concentrations). These results corroborate our 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 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 a more potent inhibitor for each proteasome subunit than the boronic ester **14**. At the onset of our experiments we 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 extend, 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 bettered by boronic ester **15** only. Compound **15** is more potent than its N-terminally extended analogue **16** as well. When we performed both competition assays on human embryonic kidney HEK293T cell lysates, which expresses the constitutive proteasome only, we found the same general trends (data not shown).

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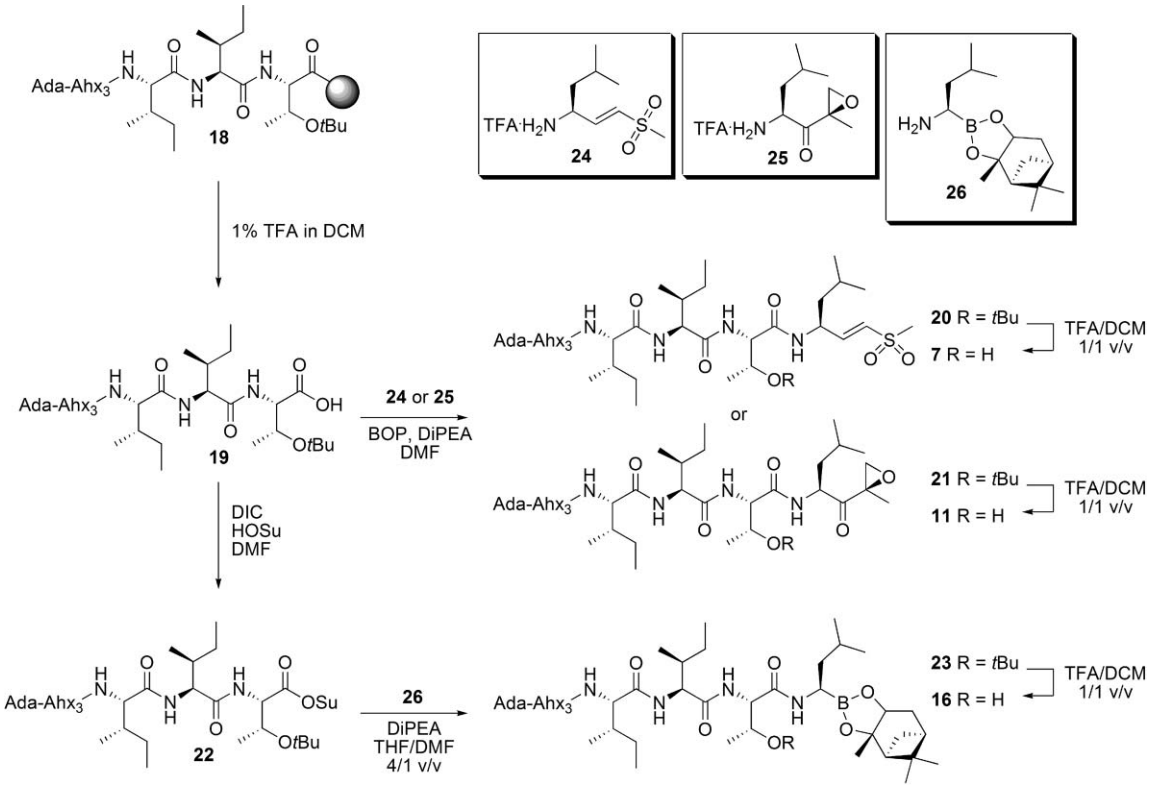


Scheme 1 Synthesis of compounds **7**, **11** and **16**.

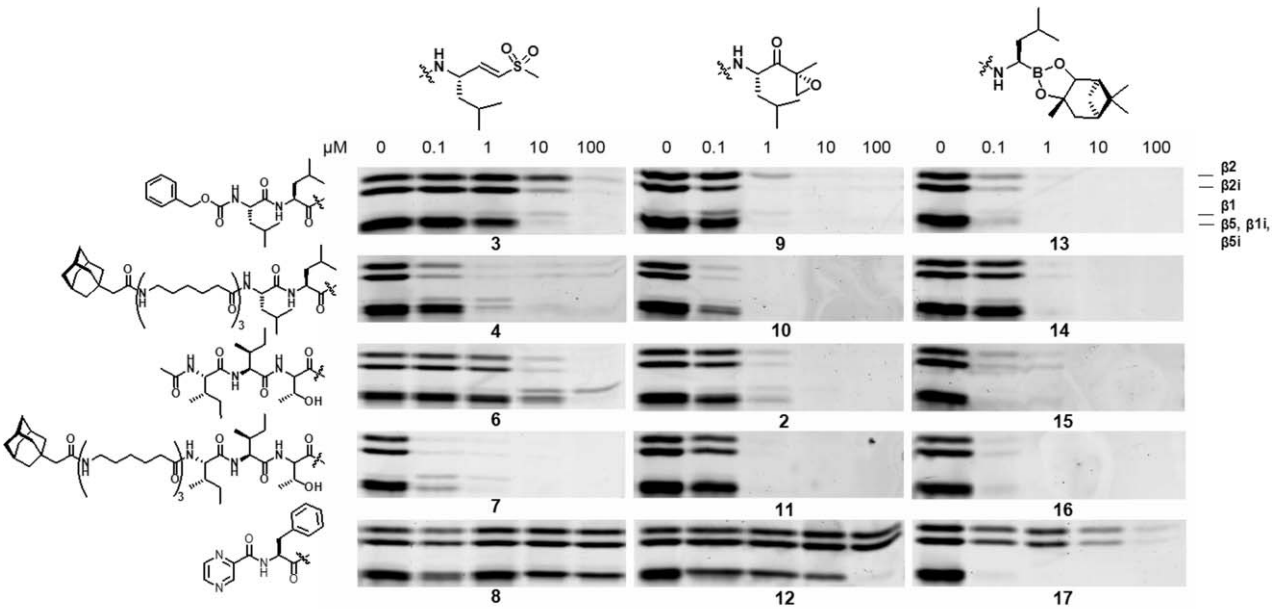


Fig. 2 Competition assays in EL4 lysates. Lysates were incubated for one hour at 0, 0.1, 1, 10 and 100 μM final concentration. Residual proteasome activity was labelled with 0.1 μM MV151 for one hour.

Conclusions

In conclusion, we have demonstrated 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 we considered at the onset of our study. For instance, while it is true that hydrophobic extension in most cases contributes to the potency, the most potent compound from the series presented here proved to be the boronic ester **15** bearing the epoxomicin tetrapeptide sequence without N-terminal extension. We believe compound **15** to be the most potent peptide-based proteasome inhibitor reported to date. Possibly, such broad-spectrum proteasome inhibitors might find clinical application

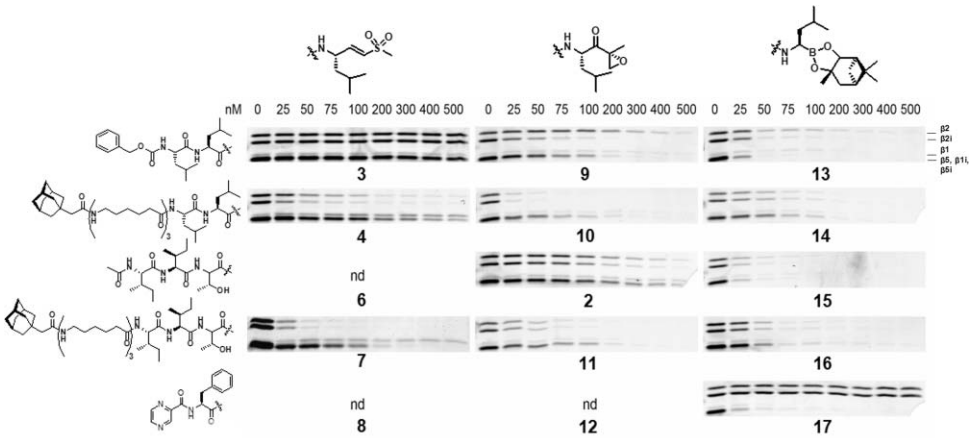
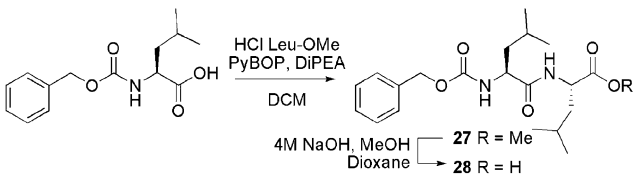


Fig. 3 Competition assays in EL4 lysates. Lysates were incubated for one hour at 0, 25, 50, 100, 200, 300, 400 and 500 nM final concentration. Residual proteasome activity was labelled with 0.1 μM MV151 for one hour. nd = not determined.

as an alternative for bortezomib. For instance in cases where bortezomib resistance occurs, an event which is thought to be linked to regulation in one way or another of those proteasome activities that are left unmodified by bortezomib (bortezomib has a labelling profile similar to **17** and leaves the β2 and β2i subunits untouched at clinical doses). Current investigations in our laboratory are aimed at further unravelling the subunit preferences of our panel of compounds, with the aim to arrive at subunit-specific inhibitors.

Experimental

All reagents were commercial grade and were used as received unless indicated otherwise. Tol (Tol)(purum), ethyl acetate (EA) (puriss.), diethyl ether (Et_2O) and light petroleum ether (PE) (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^{-1}) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g L^{-1}) in 10% sulfuric acid followed by charring at ~150 °C or spraying with an aqueous solution of $KMnO_4$ (7%) and KOH (2%). 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 the literature.



Scheme 2

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 h. The reaction mixture was washed with sat. aq. $NaHCO_3$, 1 M HCl and brine, and the organic phase was dried over $MgSO_4$ and concentrated. Flash column chromatography (PE → 10% EA/PE, v/v) gave the title compound (5.7 g, 14.5 mmol, 68%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.52 (d, 1 H, J = 7.7 Hz), 7.32–7.15 (m, 5 H), 6.31 (d, 1 H, J = 8.4 Hz), 5.08–4.90 (m, 2 H), 4.60–4.18 (m, 2 H), 3.59 (s, 3 H), 1.70–1.35 (m, 6 H), 0.95–0.68 (m, 6 H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ = 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 4 M NaOH in H_2O (19.1 mmol, 1.32 equiv.) and stirred for 3.5 h. The reaction mixture was acidified to pH 2 with 1 M HCl and concentrated. The residue

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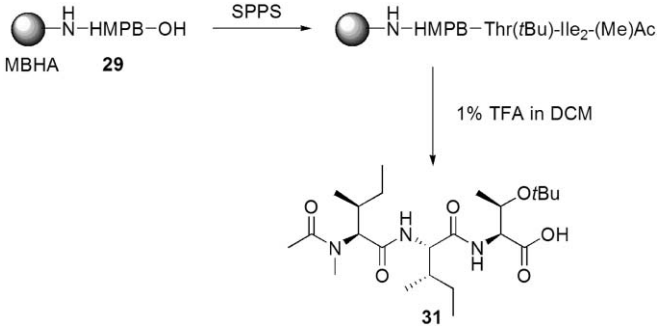
was dissolved in EA, and washed with H_2O. The water layer was extracted with EA (2×), and the combined organic layers were dried over $MgSO_4$ and concentrated. Crystallisation from EA/PE yielded the title compound (5.03 g, 13.3 mmol, 92%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.40–7.25 (m, 5 H), 6.65 (d, 1 H, J = 6.2 Hz), 5.48 (m, 1 H), 5.1 (s, 2 H), 4.65–4.50 (m, 1 H), 4.38–4.19 (m, 1 H), 1.81–1.43 (m, 6 H), 1.05–0.80 (m, 6 H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ = 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.

HMPB-MBHA resin (29)

4-Methylbenzhydrylamine (MBHA) functionalized polystyrene resin (0.56 g, 0.9 mmol g^{-1}, 0.5 mmol) was washed with NMP (3×) 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 h of shaking, the resin was washed with NMP (3×), MeOH (3×) and DCM (3×), dried and used as such.

Ada-Ahx$_3$-Leu$_2$-OH (30)

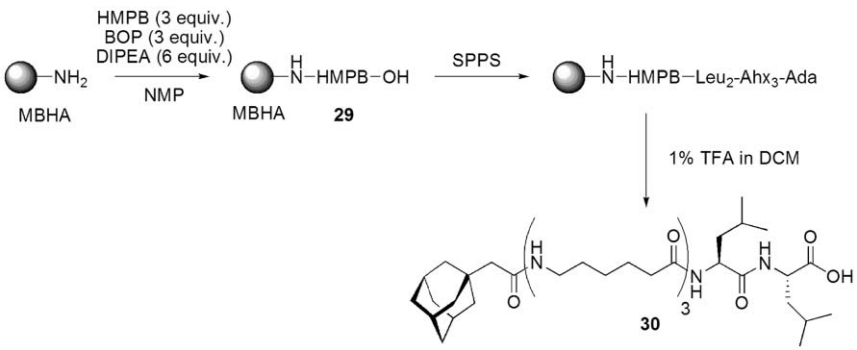
Resin **29** (0.5 mmol) was transferred to a flask, coevaporated with DCE (2×), and condensed with Fmoc-Leu-OH (0.53 g, 1.5 mmol, 3 equiv.) under the influence of DIC (0.26 mL, 1.67 mmol, 3.3 equiv.) and DMAP (10 mg, 0.075 mmol, 15 mol%) in DCM for 2 h. The resin was filtered and washed with DCM (2×), followed by a second condensation cycle. The loading of the resin was determined to be 0.42 mmol g^{-1} (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 h, (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 h. After washing with NMP (3×) and DCM (3×) the resin was subjected to mild acidic cleavage (TFA/DCM, 1/99 v/v, 10 min, 3×) and the collected fractions were coevaporated with Tol (2×) to give the crude title compound, which was used without any further purification. ^{1}H NMR (200 MHz, CD_3OD): δ = 4.51–4.34 (m, 2 H), 3.25–3.05 (m, 6 H), 2.22–2.10 (m, 6 H), 1.93 (s, 2 H), 1.82–1.20 (m, 39 H), 1.01–0.83 (m, 12 H). ^{13}C NMR (50.1 MHz, CD_3OD): δ = 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.



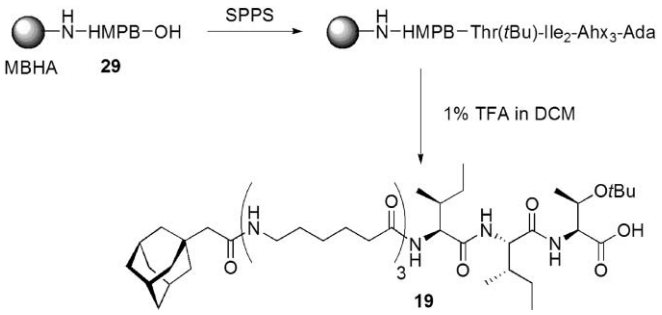
Scheme 4

Ac(Me)-Ile$_2$-Thr(*t*Bu)-OH (31)

Resin **29** (1 mmol) was transferred to a flask, coevaporated with DCE (2×), 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 h. The resin was filtered and washed with DCM (2×), followed by a second condensation cycle. The loading of the resin was determined to be 0.55 mmol g^{-1} 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 h, (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 h. After washing with DCM (3×) the resin was subjected to mild acidic cleavage (TFA/DCM, 1/99 v/v, 10 min, 3×) and the collected fractions were coevaporated with Tol (2×) to give the crude title compound, which was used without any further purification.



Scheme 3



Scheme 5

Ada-Ahx$_3$-Ile$_2$-Thr(tBu)-OH (19)

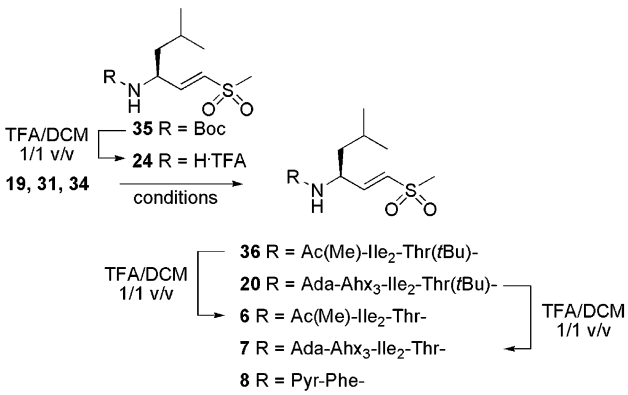
HMPB-MBHA resin **29** (2 g, 0.75 mmol g^{-1} resin, 1.5 mmol) was coevaporated with DCE (2×) and condensed with Fmoc-Thr(tBu)-OH (1.8 g, 4.5 mmol, 3 equiv.) under the influence of DIC (0.7 mL, 4.5 mmol, 3 equiv.) and DMAP (10 mg, 75 μmol) for 2 h. The resin was filtered, washed with DCM (3×) and subjected to a second condensation cycle. The loading of the resin was 0.67 mmol g^{-1}, as determined by spectrophotometric analysis. Next, the resin was subjected to five cycles of Fmoc solid-phase synthesis with Fmoc-Ile-OH (2×) and Fmoc-Ahx-OH (3×), respectively as follows: (a) deprotection with piperidine/NMP (1/4, v/v, 15 min), (b) washing with NMP (3×) and DCM (3×), (c) coupling of the Fmoc amino acid (4.5 mmol, 3 equiv.) by shaking the resin for 2 h in the presence of HCTU (3 equiv., 4.5 mmol), DiPEA (6 equiv., 9 mmol) and NMP as solvent, (d) washing with NMP (3×) and DCM (3×). 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 (3×) and DCM (3×) 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 h. The resin was washed with NMP (3×) and DCM (3×), before being subjected to mild acidic cleavage (TFA/DCM, 1/99 v/v, 15 min, 3×). 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 = 4.48–4.23 (m, 4 H), 3.25–3.08 (m, 6 H), 2.35–2.12 (m, 7 H), 2.04–1.10 (m, 52 H), 1.00–0.83 (m, 12H). ^{13}C NMR (50.1 MHz, CD_3OD): δ = 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.

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 h. Sat. aq. $NaHCO_3$ was added and the water layer was extracted with Et_2O (3×). The combined organic layers were dried over $MgSO_4$ and concentrated. The crude product was purified by column chromatography (Tol → 40% EA in Tol v/v) to yield the title compound (0.6 g, 2.1 mmol, 70%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 9.36 (d, 1H, J = 1.5 Hz), 8.71 (d, 1H, J = 2.6 Hz), 8.46 (m, 1H), 8.29 (d, 1H, J = 7.7 Hz), 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 = 171.2, 162.4, 147.2, 144.0, 143.7142.5, 135.5, 128.9, 128.4, 126.9, 53.1, 52.2, 37.8.

Pyrazine-2-carbonylphenylalanine (34)

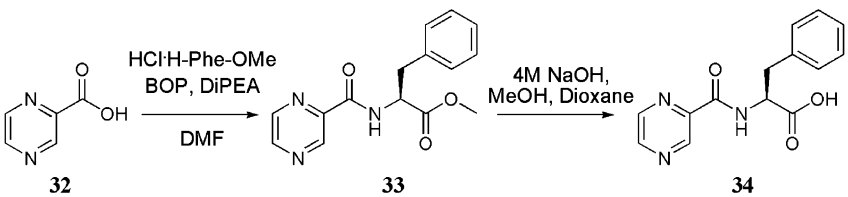
Methyl ester **33** (0.24 g, 0.85 mmol) was dissolved in 3 mL dioxane, 1.1 mL MeOH and 0.22 mL 4 M NaOH. The reaction mixture was stirred for 1 h before 0.8 mL 1M $KHSO_4$ was added, and the solution was concentrated. The residue was taken up in H_2O/brine (1/1, v/v), and this solution was extracted with EA (3×). The combined organic layers were dried over $MgSO_4$ and concentrated, yielding the title compound in 69% yield (0.16 g, 0.59 mmol). LC/MS analysis: R_f 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 = 9.37 (s, 1H), 8.76 (s, 1H), 8.54 (s, 1H), 8.21 (d, 1H, J = 8 Hz), 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 = 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

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 the presence of Tol (2×) before being dissolved in DCM and put



Scheme 6

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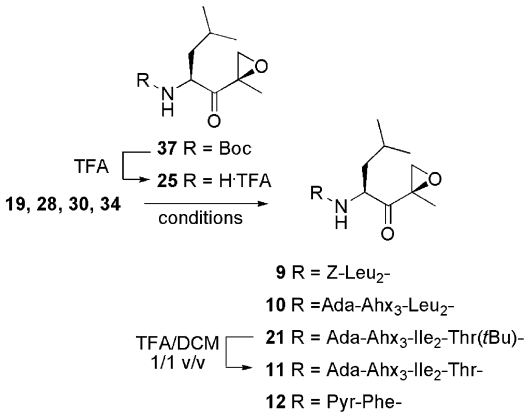
under an argon atmosphere. Ac-(Me)–I_2-T(*t*Bu)-OH **31** (337 mg, 0.45 mmol, 1.5 equiv.), BOP (0.2 g, 0.45 mmol, 1 equiv.), DiPEA (0.12 ml, 0.76 mmol, 2.5 equiv.) were added, and the mixture was stirred for 12 h, before being concentrated *in vacuo*. The crude was dissolved in TFA/DCM (1/1 v/v, 2 mL) and stirred at ambient temperature for 30 min before being concentrated in the presence of Tol (2×), 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). ^{1}H NMR (400 MHz, CD_3OD): δ 6.81 (dd, J = 14.6, 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). $[\alpha]_D^{20}$ −225° (c = 0.024, MeOH). 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 the presence of Tol (2×) before being dissolved in DMF and put under an argon atmosphere. Ada-Ahx_3-Ile_2-Thr(*t*Bu)-OH (**19**) (217 mg, 0.24 mmol), BOP (117 mg, 0.26 mmol, 1.1 equiv.), and DiPEA (90 μL, 0.53 mmol, 2.2 equiv.) were added, and the mixture was stirred for 12 h, before being concentrated *in vacuo.* The crude was dissolved in TFA/DCM (1/1 v/v, 1 mL) and stirred at ambient temperature for 30 min., before being concentrated in the presence of Tol (2×), 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%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.52 (m, 2H), 6.86 (dd, J = 4.3, 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 = 175.2, 174.2, 172.7, 172.0, 171.7, 170.1, 147.4, 128.8, 66.5, 59.0, 58.7 (2×), 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 (2×), 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. $[\alpha]_D^{20}$ −31° (c = 0.2, MeOH). 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 h. The reaction mixture was concentrated and subjected to flash column chromatography (Tol → EA), yielding two epimers (both 82 mg, 0.18 mmol, 32%). Recrystallisation from PE/acetone yielded the title compound (50 mg, 0.113 mmol, 19%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 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 = 4.8, 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 = 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. $[\alpha]_D^{20}$ −12° (c = 0.2, MeOH). HRMS: calcd for $C_{22}H_{28}O_4N_4SH^+$ 445.19040 found 445.19031.



Scheme 8

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 (2×), 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 1 h. The mixture was washed with H_2O and brine, dried over $MgSO_4$ and concentrated. Crystallisation from EA/PE yielded crude title compound **9**, which was further purified by flash column chromatography (PE → 30% EA/PE, v/v) yielding the title compound (18.7 mg, 44 μmol, 18%). ^{1}H NMR (200 MHz, CD_3OD): δ = 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 = 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. $[\alpha]_D^{20}$ −1° (c = 0.2, MeOH). 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 (2×), 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 h, 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%). ^{1}H NMR (400 MHz, CD_3OD): δ = 4.58-4.47

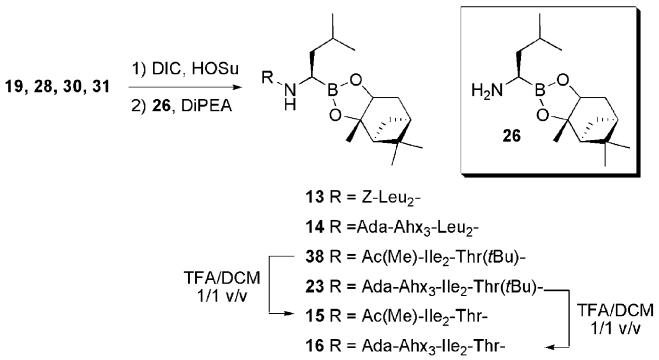
(m, 1H), 4.47-4.32 (m, 2H), 3.14 (t, $J = 6.3$ Hz, 6H), 3.24 (m, 1H), 2.92 (d, $J = 5.1$ Hz, 1H), 2.23 (t, $J = 7.4$ Hz, 2H), 2.17 (t, $J = 7.4$ Hz, 4H), 1.97–1.89 (m, 5H), 1.77–1.42 (m, 30H), 1.41-1.27 (m, 12H), 0.99-0.85 (m, 18H). $[\alpha]_D^{20}$ −1.5° ($c = 2$, MeOH). HRMS: calcd for $C_{51}H_{88}N_6O_8H^+$ 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 the presence of Tol (2×) 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.), and DiPEA (95 µL, 1.1 mmol, 4.4 equiv.) were added, and the mixture was stirred for 3 h. The crude Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-leucinyl-2-methyloxirane (**21**) was precipitated with EA yielding a mixture of diastereomers. The major product was purified by semi-preparative RP-HPLC yielding **21** (27.3 mg, 25.5 µmol, 10%). *tert*-Butyl ether **21** was dissolved in TFA/DCM (1/1 v/v) and stirred for 0.5 h, before being evaporated in the presence of Tol (2×) yielding the title compound as a colourless oil (23.6 mg, 23.3 µmol, 91%). ^{1}H NMR (400 MHz, CD_3OD): δ 4.55 (dd, $J = 2.6$, 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, $CDCl_3$): $\delta = 209.4$, 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 (2×), 40.1, 37.9 (2×), 37.6, 37.0, 36.6, 33.7, 30.1 (3×), 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. $[\alpha]_D^{20}$ −17.5° (c = 0.24, MeOH). 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 (2×) and dissolved in 20 mL DCM 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 1 h before washing with sat. aq. $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentrated. Flash column chromatography (PE → 25% EA/PE) yielded the title compound (8.6 mg, 20 µmol, 5.3% isolated yield). ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 9.35$ (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, $J = 8$ Hz, 1H), $\delta = 7.31$-7.20 (m, 5H), 6.36 (d, $J = 7.7$ Hz, 1H), 4.91 (dd, $J = 6.9$, 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, $CDCl_3$): $\delta = 207.9$, 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. $[\alpha]_D^{20}$ +12° ($c = 0.1$, MeOH). HRMS: calcd for $C_{23}H_{28}O_4N_4H^+$ 425.21833, found 425.21835.



Scheme 9

Z-Leu$_3$-boronic ester (13)

Under argon 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 minutes 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. A flame dried two necked reaction flask was put under an argon atmosphere in which (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] (152 mg, 0.53 mmol, 1 equiv.) was dissolved in THF (5 mL). At −20 °C a LiHMDS solution (1 M 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 (2 M in diethyl ether, 1.50 mL, 3.0 mmol, 5.7 equiv.) was added dropwise. The mixture was slowly warmed to 0 °C (*ca.* 2 h) 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 h) and was stirred for another 2 h. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silica gel column chromatography (10% EA/PE → 25% EA/PE) affording the title compound as a white solid (197 mg, 0.32 mmol, 59%). ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.37$–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, $J = 13.4$, 7.5 Hz, 1H), 4.27 (dd, $J = 8.6$, 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, $J = 10.5$, 5.2, 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): $\delta = 178.4$, 175.3, 158.7, 138.0, 129.4 (2×), 129.0, 128.8 (2×), 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. $[\alpha]_D^{20}$ −58° ($c = 1$, MeOH). 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.

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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 minutes 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 (1 M in THF, 460 µL, 0.46 mmol, 3.5 equiv.) was added dropwise. The mixture was allowed to warm to room temperature and stirred overnight. At −90 °C a HCl solution (2 M in diethyl ether, 1.00 mL, 2.0 mmol, 15.4 equiv.) was added dropwise. The mixture was slowly warmed to 0 °C (*ca.* 2 h) 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 h) at which it was stirred for another 2 h. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silica gel column chromatography (PE/EA/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%). ^{1}H NMR (600 MHz, CD$_3$OD): δ = 4.61 (dd, J = 9.6, 5.4 Hz, 1H), 4.37 (dd, J = 9.6, 5.4 Hz, 1H), 4.15 (dd, J = 10.2, 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$_3$OD): δ = 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. $[\alpha]_D^{20}$ −21.7° (c = 0.12, MeOH). 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.) was added. After a few minutes a fine precipitate was formed. 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 (1 M in THF, 550 µL, 0.55 mmol, 3.2 equiv.) was added dropwise. The mixture was allowed to warm to room temperature and stirred overnight. At −90 °C a HCl solution (2 M in diethyl ether, 1.50 mL, 3.0 mmol, 17.6 equiv.) was added dropwise. The mixture was slowly warmed to 0 °C (*ca.* 2 h) 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 h) and was stirred for another 2 h. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silica gel column chromatography (PE/EA/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%). ^{1}H NMR (200 MHz, CDCl$_3$): δ = 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). $[\alpha]_D^{20}$ −360° (c = 0.04, MeOH). 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.) was added. After a few minutes 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. A flame dried two necked reaction flask equipped was put under an argon atmosphere in which (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). At −20 °C a LiHMDS solution (1 M in THF, 460 µL, 0.46 mmol, 3.1 equiv.) was added dropwise. The mixture was allowed to warm to room temperature and stirred overnight. At −90 °C a HCl solution (2 M in diethyl ether, 1.00 mL, 2.0 mmol, 13.3 equiv.) was added dropwise. The mixture was slowly warmed to 0 °C (*ca.* 2 h) 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 h) and was stirred for another 2 h. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silica gel column chromatography (PE/EA/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%). ^{1}H NMR (300 MHz, CD$_3$OD): δ = 4.52 (dd, J = 7.2, 4.5 Hz, 1H), 4.21–4.13 (m, 4H), 3.14 (t, J = 6.9 Hz, 6H), 2.72 (dd, J = 8.4, 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). $[\alpha]_D^{20}$ −72.5° (c = 0.08, MeOH). 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 s, 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 (10 μg total protein) were exposed to the inhibitors for 1 h prior to incubation with MV151 (**5**, 0.1 μM) for 1 h at 37 °C. Reaction mixtures were boiled with Laemmli's buffer containing β-mercaptoethanol 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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