Bioorganic & Medicinal Chemistry 24 (2016) 3429–3435

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Proteasome inhibition by new dual warhead containing peptido vinyl sulfonyl fluorides

Arwin J. Brouwer [a,†], Natalia Herrero Álvarez [b,†], Adriano Ciaffoni [a], Helmus van de Langemheen [b], Rob M. J. Liskamp [a,b,*]

[a] Department of Medicinal Chemistry and Chemical Biology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands
[b] School of Chemistry, Joseph Black Building, University of Glasgow, University Avenue, Glasgow G12 8QQ, UK

ARTICLE INFO

Article history:
Received 4 April 2016
Revised 19 May 2016
Accepted 20 May 2016
Available online 24 May 2016

Keywords:
Drug design
Peptido vinyl sulfonyl fluoride
Proteasome
Irreversible inhibition
Dual warhead

ABSTRACT

The success of inhibition of the proteasome by formation of covalent bonds is a major victory over the long held-view that this would lead to binding the wrong targets and undoubtedly lead to toxicity. Great challenges are now found in uncovering ensembles of new moieties capable of forming long lasting ties. We have introduced peptido sulfonyl fluorides for this purpose. Tuning the reactivity of this electrophilic trap may be crucial for modulating the biological action. Here we describe incorporation of a vinyl moiety into a peptido sulfonyl fluoride backbone, which should lead to a combined attack of the proteasome active site threonine on the double bond and the sulfonyl fluoride. Although this led to strong proteasome inhibitors, in vitro studies did not unambiguously demonstrate the formation of the proposed seven-membered ring structure. Possibly, formation of a seven-membered covalent adduct with the proteosomal active site threonine can only be achieved within the context of the enzyme. Nevertheless, this dual warhead concept may provide exclusive possibilities for duration and selectivity of proteasome inhibition.

1. Introduction

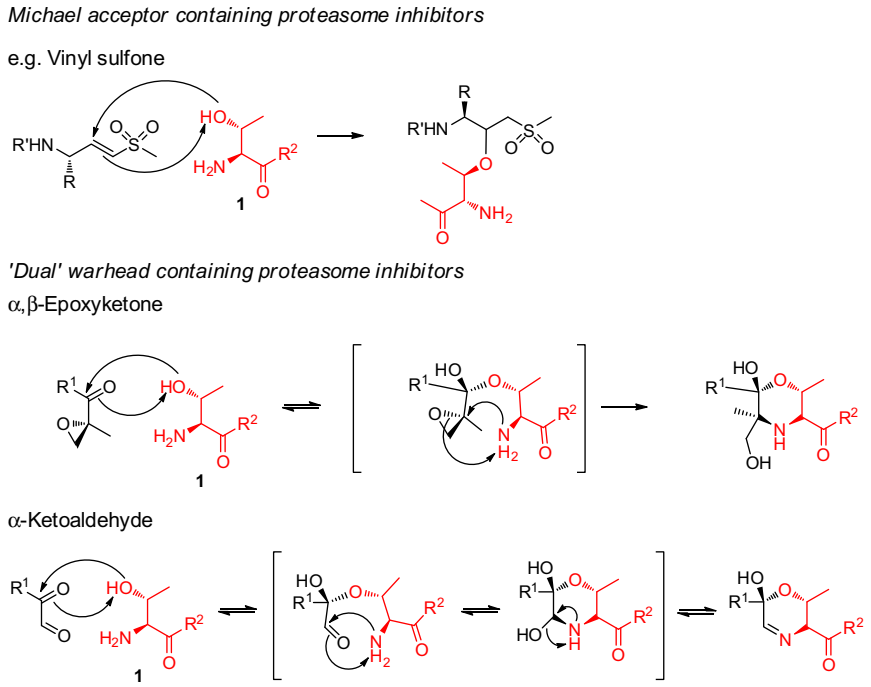
The development of proteasome inhibitors has been an outstanding case showing that irreversible inhibitors may provide unique advantages by forming long-lived ties with their target.[1] Depending on the degree of reversibility of this covalent interaction, the putative proteasome inhibitor may therefore display a prolonged interaction and biological action. A prolonged interaction may be beneficial when the undesired proteasome activity is manifest for an extended period.[2,3] Together with covalently reacting kinase inhibitors, which contain Michael acceptor moieties, proteasome inhibitors are part of the important arsenal of presently available crucial anti-cancer drugs. Inhibition of the protein degradation pathway in this manner is currently an effective approach for treatment of blood cancers.[4,5] Increasingly, established proteasome inhibitors are evaluated as anti-inflammatory immunoproteasome inhibitors leading to new therapeutic strategies for treatment of auto-immune diseases such as rheumatoid arthritis and multiple sclerosis[6,7] Recently, in collaboration with Groll et al., we have achieved selective inhibition of the immunoproteasome by crosslinking of the active site effected by a peptido sulfonyl fluoride ligand (PSF).[8]

Most proteasome inhibitors contain a single electrophilic moiety capable of covalently interacting with the threonine active site residue.[9] Especially the vinyl sulfone containing proteasome inhibitors have been subject of many investigations. (Scheme 1).[10] These contain a Michael acceptor as an electrophilic moiety.

However, in contrast to serine proteases in which the attacking nucleophile on the peptide-amide bond is solely the hydroxyl of the serine residue present as part of the catalytic triad, in the proteasome the amino acid involved in scission of the peptide-amide bond is an N-terminal threonine residue containing *two* nucleophiles. As a consequence, very effective and selective inhibition has been achieved by proteasome inhibitors having 'dual' warheads, that is containing two electrophilic sites. This is reflected by the treatment of multiple myeloma in patients with the proteasome inhibitor carfilzomib, containing both an epoxide

* Corresponding author.
E-mail addresses: r.m.j.liskamp@uu.nl, Robert.liskamp@glasgow.ac.uk (R.M.J. Liskamp).
† These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bmc.2016.05.042
0968-0896/



Scheme 1. Mechanisms of covalent inhibition of the proteasome by vinyl sulfones, α-β-epoxyketones and α-ketoaldehydes. The threonine depicted in red represents the N-terminal threonine of the proteasome.

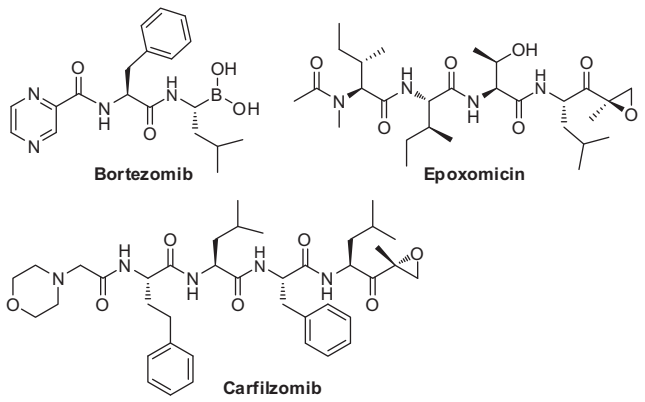


Figure 1. Structures of Bortezomib, Epoxomicin and Carfilzomib.

and carbonyl electrophilic site, after previous treatment with bortezomib, which contains just one electrophilic site (Fig. 1). In our opinion this justifies a quest for dual warhead containing inhibitors such as the one discussed in this research.

Inspired by the dual warhead approach we describe in this paper a new proteasome inhibitor concept in which a Michael electrophilic trap is combined with a sulfonyl fluoride electrophile incorporated into a peptide sequence leading to a peptido vinyl sulfonyl fluoride (PVSF). Both electrophilic traps may then interact with both nucleophilic amino and hydroxyl moieties of the N-terminal threonine residue present in the active site of the proteasome. Other covalently interacting proteasome inhibitors, having two electrophilic sites, including Epoxomicin (Fig. 1) and the alpha keto–aldehyde warhead containing inhibitors, show a similar molecular mechanism of action (Scheme 1).[11,12] However, in the sulfone Michael acceptor containing proteasome inhibitors only the four-position is reacting with the threonine hydroxyl nucleophile (Scheme 1).

2. Results and discussion

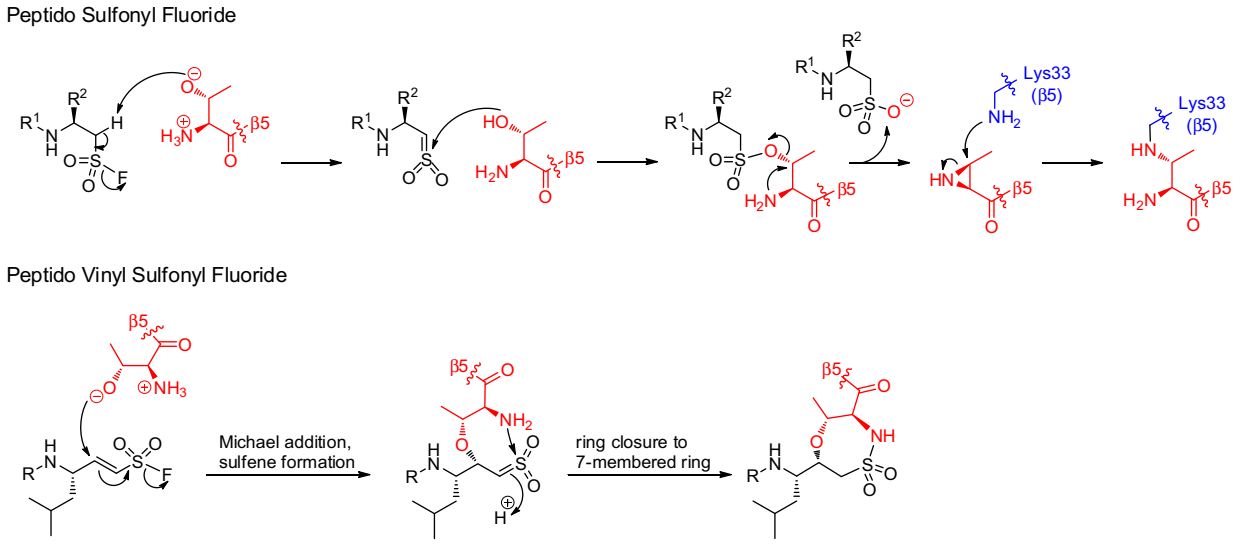
2.1. Chemistry

Here we propose the peptido vinyl sulfonyl fluoride (PVSF) as a new and promising *dual* warhead system. It was expected that its molecular structure would allow a Michael reaction leading to a sulfene intermediate followed by an intramolecular reaction of the second nucleophile in the threonine residue leading to a seven-membered ring covalent adduct (Scheme 2).

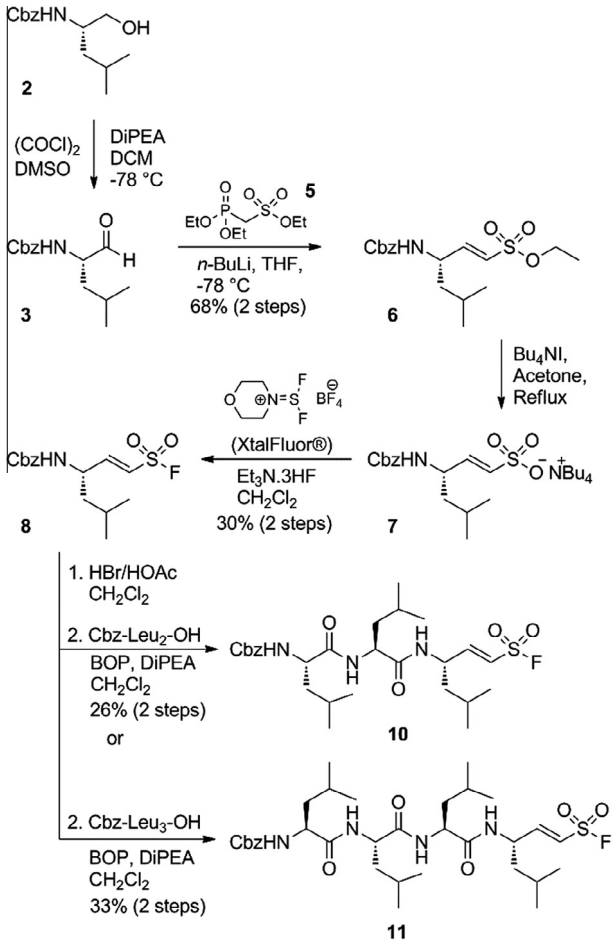
The synthesis of peptido vinyl sulfonyl fluorides involved employing vinylogous amino sulfonates, which are accessible from amino acid derived aldehydes as was described by Gennari et al. (Scheme 3).[13] Briefly, Cbz-protected leucinol (**2**) was converted into the corresponding amino aldehyde (**3**) by a Swern oxidation. A Wittig–Horner reaction with ethyl diethylphosphoryl methanesulfonate afforded vinyl sulfonate ester **6**, which was cleaved by Bu_4NI. The most efficient conversion of the resulting sulfonate salt (**7**) into the corresponding vinyl sulfonyl fluoride (**8**) was achieved by using XtalFluor-M®[14] in the presence of a catalytic amount of triethylamine trihydrofluoride acting as both a proton and fluoride source.[15] Two PVSF proteasome inhibitors (**10** and **11**, respectively) were obtained after cleavage of the Cbz-group from **8** followed by a coupling reaction with Cbz-Leu_2-OH and Cbz-Leu_3-OH using BOP.

2.2. Biological evaluation

Recently, we described and established the molecular mechanism of action of our peptido sulfonyl fluoride (PSF) proteasome protease inhibitors.[8] It was found that selective inhibition of the immunoproteasome occurred by ligand-induced cross-linking of the active site (Scheme 2). Although PSFs are capable of β5c inhibition, comparison with other warheads highlights the peptido sulfonyl fluoride as a promising motif for β5i targeting. The sequences of inhibitors **10** and **11** were chosen based on earlier results with our most potent PSF proteasome inhibitors **17** and



Scheme 2. Mechanism of covalent inhibition of the proteasome by peptido sulfonyl fluorides (PSF)[8] and proposed mechanism for inhibition by peptido vinyl sulfonyl fluorides (PVSF). The threonine depicted in red represents the N-terminal threonine of the proteasome.



Scheme 3. Synthesis of PVSF compounds **10** and **11**.

18 (IC_{50}-values 89 nM and 18 nM, respectively, Fig. 2).[16] Evaluation of the proteasome inhibitory activity gave IC_{50}-values of 218 nM and 99 nM for PVSF compounds **10** and **11**, respectively (Fig. 1). At first we were somewhat surprised by the diminished activity of the PVSF's as compared to PSF's **17** and **18**, respectively.

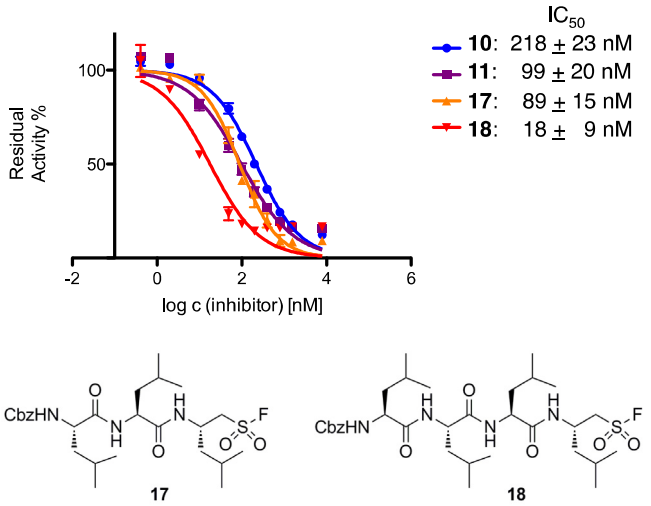
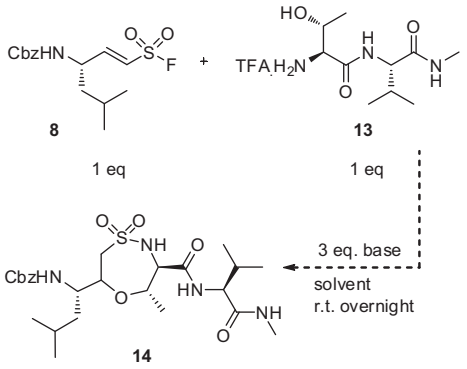


Figure 2. Inhibitory curves of human constitutive proteasome by PVSF's **10** and **11** and PSF's **17** and **18**.

Although a PVSF may be more reactive than a PSF, the sulfonyl fluoride warhead part may occupy a less favourable P1′ position because it is further positioned from the P1 side chain, leading to a reduced inhibition. Therefore, we believe that by evaluating different amino acid sequences with the vinyl sulfonyl fluoride dual warhead, as was done with the sulfonyl fluoride warhead,[16] even lower IC_{50}-values may be obtained.

To investigate whether the proposed formation within the enzyme of a seven-membered ring adduct could be observed by chemo-synthesis, in parallel, the reactivity of a simplified peptido vinyl sulfonyl fluoride (**8**) was studied with H-Thr-Val-N(H)Me (**13**) as a model of the threonine residue present in the catalytic site of the proteasome (Scheme 4). Since formation of a seven membered-ring is not a very favourable reaction and the threonine residue is an ambidextrous nucleophile, an entirely clean reaction was not expected. In addition, other residues of the catalytic site of the proteasome are absent, especially any basic residues, which may affect the relative nucleophilicity of the threonine nucleophiles and thereby the sequence of steps in the molecular



Scheme 4. Model reaction of a PVSF with a threonine containing dipeptide comprising the N-terminal proteasome site. The attempted base/solvent combinations were DBU, Et_3N or NMM in CH_2Cl_2 and DBU or Et_3N in CH_3CN.

mechanism of inhibition by this PVSF warhead. Thus our model compound **13** may not be an accurate representation of the proteasome active site, but it is best model we had in vitro.

It was possible to observe two small peaks at *m/z* 541.24 and 563.23, corresponding to the $[M+H]^+$ and $[M+Na]^+$ ions of the seven-membered ring containing molecule (**13**), (Scheme 4, for LCMS spectra see Supporting Information). However, even after several attempts, we were unable to isolate this adduct after (silica gel) column chromatography or preparative HPLC. In addition, attempts by varying the solvent (DCM or MeCN) of the reaction and base (DBU, Et_3N or NMM) were also unsuccessful to increase product formation and subsequent isolation of a seven-membered ring structure. Although the observed mass values are also in agreement with a non-cyclic structure without occurrence of a Michael reaction, these structures are unlikely since a Michael reaction is the preferred attack.[17,18]

Therefore, we felt that it was necessary to get some insight in the reactivity of the peptido vinyl sulfonyl fluorides and to what extent the proposed – 'in vivo', that is in the proteasome – seven-membered ring might be formed 'in vitro'.

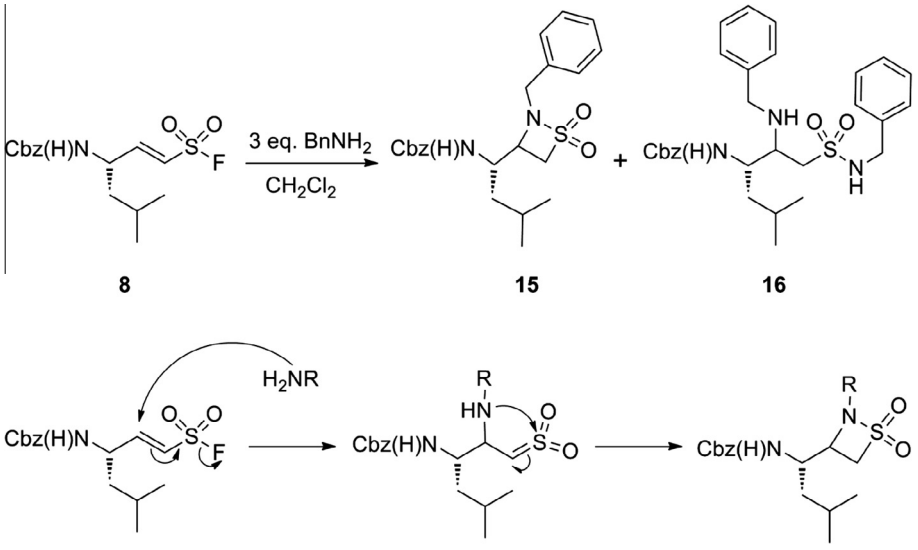
Instead of the amino-group nucleophile as present in the threonine dipeptide model, the much simpler benzylamine was used in excess. A disubstituted compound (**16**) resulting from a Michael reaction and substitution at the sulfonyl fluoride moiety was expected (Scheme 5). Unexpectedly, only traces of **16** were detected using ESI-MS, and instead β-sultam **15** was formed. A similar β-sultam system was also obtained by reaction of **8** with amino ethanol (data not shown). A plausible mechanism of formation is a Michael reaction followed by an intramolecular β-sultam formation. Indeed β-sultam compounds have been prepared conveniently in the past by reaction of ethenesulfonyl fluoride with various amines.[19] In agreement with the literature the first step is probably a Michael reaction of the amine (Scheme 5).[18]

3. Conclusions

We have introduced a peptido vinyl sulfonyl fluoride (PVSF) as a new dual warhead containing proteasome inhibitor, active in a concentration as low as 90 nM. In contrast to our recently described peptido sulfonyl fluoride inhibitors (Scheme 2), in which the inhibitor is released from the proteasome leaving a crosslinked proteasome active site behind, the peptido vinyl sulfonyl fluoride was proposed to give rise to the formation of a covalent seven-membered ring adduct. This adduct should result from reaction of both nucleophiles of the threonine active site residues with the electrophiles of the dual warhead. The presence of simultaneously two electrophilic sites, which can both react because of the 'combined effort' of the nucleophiles in the proteasome threonine residue, might be beneficial for the selectivity of these novel proteasome inhibitors, which were somewhat less active than the earlier developed PSF's. Although there was an indication of formation of the proposed seven-membered ring structure we were unable to isolate it and achieve its synthesis 'in vitro'. To our knowledge, no other more complex unsaturated sulfonyl fluorides, similar to the ones which are topic of this paper, have been described in the literature in reactions with nucleophiles leading to sultams. Clearly, elucidation of the mechanism of inhibition of the proteasome by these new dual warhead containing peptido vinyl sulfonyl fluorides awaits a crystallographic analysis of these inhibitors within the proteasome, which is an important aim for future research.

4. Experimental

All reagents were obtained from commercial sources and used without further purification. THF was distilled over $LiAlH_4$ or obtained using a Solv™ 500 Solvent Purification System. Petroleum



Scheme 5. Reaction and proposed mechanism of β-sultam formation of peptido vinyl sulfonyl fluoride **8** with benzylamine.

ether used for column chromatography was the 40–60 °C fraction. Peptide grade and HPLC grade solvents were purchased from Actu-All (Oss, The Netherlands). Solvents were evaporated under reduced pressure at 40 °C. The capping solution used was a mixture of 0.5 M acetic anhydride, 0.125 M D*i*PEA and 0.015 M HOBt in NMP. Reactions were carried out at ambient temperature unless stated otherwise. Reactions in solution were monitored by TLC analysis on Merck pre-coated silica gel 60 F-254 (0.25 mm) plates. Spots were visualised by UV light and by heating plates after dipping in a ninhydrine solution or in chlorine gas and TDM solution.[20] Column chromatography was performed on Siliaflash P60 (40–63 μm) from Silicycle (Canada). ^{1}H NMR data were acquired on a Varian Mercury 300 MHz spectrometer, an Agilent 400 MHz spectrometer or on Bruker Avance III 400 MHz and 500 MHz spectrometers in $CDCl_3$, DMSO-d_6 or acetone-d_6 as solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS (0.00 ppm) or to the solvent residual signal of DMSO-d_6 (2.50 ppm). Coupling constants (J) are reported in Hertz (Hz). Splitting patterns are designated as singlet (s), doublet (d), triplet (t), multiplet (m), and broad (br). ^{13}C NMR data were acquired on a Varian Mercury 300 MHz spectrometer at 75 MHz, an Agilent 400 MHz spectrometer at 100 MHz or on Bruker Avance III 500 MHz spectrometer at 126 MHz in $CDCl_3$, DMSO-d_6 or acetone-d_6 as solvent. Some of the ^{13}C NMR spectra were recorded using the attached proton test (APT) pulse sequence. Chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent residual signal, $CDCl_3$ (77.00 ppm), DMSO-d_6 (39.52 ppm), or acetone-d_6 (29.84 ppm). ^{19}F NMR data were acquired on an Agilent 400 MHz spectrometer at 376 MHz or on a Bruker Avance III 500 MHz spectrometer at 471 MHz. 2D NMR data (HSQC, COSY, and TOCSY) were acquired on Varian Mercury 300 MHz spectrometer, an Agilent 400 MHz spectrometer or on Bruker Avance III 400 MHz and 500 MHz spectrometers. High-resolution electrospray ionization (ESI) mass spectra were measured on a Bruker micrOTOF-Q II in positive or negative mode and calibrated with an ESI tuning mix from Agilent Technologies, or measured on a Jeol MStation JMS-700 instrument using positive chemical ionization (CI+) or positive ion impact (EI+). Proteasome Enzymatic Assays were performed using the VIVAdetect™ 20S Assay Kit PLUS (Viva bioscience, UK) and a Clariostar microplate reader (BMG LABTECH, Germany).

4.1. Cbz-Leucinal (3)

To a stirred solution of oxalyl chloride (5.45 mL, 63.0 mmol) in CH_2Cl_2 (100 mL), under N_2 atmosphere and cooled at −78 °C, were subsequently added dropwise a solution of DMSO (9.0 mL, 126 mmol) in CH_2Cl_2 (20 mL) and a solution of Cbz-Leucinol (38.2 mmol)[8] in CH_2Cl_2 (27 mL). After 10 min stirring at −78 °C a solution of D*i*PEA (40 mL, 230 mmol) in CH_2Cl_2 (100 mL) was added dropwise, and stirring was continued at −78 °C for 30 min. After warming up the mixture to rt, it was quenched with H_2O (13 mL) while severely stirring. Et_2O (300 mL) was added to the mixture and the organic layer was then washed with $KHSO_4$ (1.0 M, 2 × 100 mL). The water layer was extracted with Et_2O (1 × 100 mL) and the two organic layers were combined, dried over Na_2SO_4, and concentrated under reduced pressure, yielding Cbz-Leucinal (**3**) as a yellow oil (10.0 g, quantitative yield). The crude product was almost pure (TLC analysis) and was directly used in the Wittig–Horner reaction. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.90 (dd, J = 9.1, 6.7 Hz, 6H, 2× CH_3), 1.34 (ddd, J_{gem} = 13.5, J_{vic} = 9.6, 5.0 Hz, 1H, $CH^aCH(CH_3)_2$), 1.61 (ddd, J_{gem} = 13.5, J_{vic} = 8.5, 4.8 Hz, 1H, $CH^bCH(CH_3)_2$), 1.69 (m, 1H, $CH(CH_3)_2$), 4.26 (m, 1H, NCH), 5.05 (s, 2H, CH_2 (Cbz)), 5.18 (d, J = 6.4 Hz, 1H, NH), 7.21–7.32 (m, 5H, C_6H_5 (Cbz)), 9.52 (s, 1H, C(O)H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 21.9, 23.0 (CH_3), 24.6 ($CH(CH_3)_2$), 38.1 ($CH_2CH(CH_3)_2$), 58.8 (NCH), 67.1 (CH_2 (Cbz)), 128.1, 128.5, 136.1 (C_6H_5 (Cbz)), 156.1 (C=O (Cbz)), 199.7 (C(O)H). HRMS m/z calculated for $C_{14}H_{20}NO_3$ $[M+H]^+$: 250.1443, found: 250.1445.

4.2. Ethyl methanesulfonate (4)

Ethanol (6.40 mL, 110 mmol) was dissolved in dry CH_2Cl_2 (400 mL) and cooled in an ice bath. *N*-Methyl morpholine (22.0 mL, 200 mL) and methanesulfonyl chloride (7.70 mL, 100 mmol) were added and the mixture was stirred for 30 min. Then the ice bath was removed and the reaction was stirred overnight at room temperature. CH_2Cl_2 (200 mL) was added to the mixture and the organic layer was washed with an aqueous solution of $KHSO_4$ (1.0 M, 2 × 200 mL) and water (1 × 200 mL), dried over $MgSO_4$ and concentrated, resulting in ethyl methanesulfonate (10 g, 80 mmol, 81%) as a colorless oil. Characterization data were in agreement with the literature.[21]

4.3. Ethyl diethylphosphorylmethanesulfonate (5)

Ethyl methanesulfonate **4** (10 g, 80 mmol) was dissolved in dry THF (200 mL) and treated with a 2.5 M *n*-BuLi solution in hexanes (35 mL, 89 mmol) over 30 min at −78 °C. After 15 min, diethylchlorophosphate (6.5 mL, 45 mmol) was added and the solution was stirred for 30 min at −78 °C and allowed to stir for 1 h at −50 °C. The mixture was concentrated, the residue was diluted with water (100 mL) and extracted with CH_2Cl_2 (3 × 120 mL). The combined organic layers were dried over $MgSO_4$ and concentrated. Purification of the crude by silica column by petroleum ether/ethyl acetate (1:1) as eluents delivered **5** as a colorless oil (6.5 g, 25 mmol, 56%). Characterization data were in agreement with the literature.[22]

4.4. Cbz-vsLeu-OEt (6)

A stirring mixture of Wittig–Horner reagent **5** (6.5 g, 25 mmol) and anhydrous THF (100 mL) was cooled at −78 °C under N_2 atmosphere. A solution of *n*-Butyllithium in hexanes (2.5 M, 10.5 mL, 26.2 mmol) was added dropwise, and after 20 min Cbz-Leucinal **(3)** (7.5 g, 30 mmol) in anhydrous THF (25 mL) was slowly added. Stirring was continued for 45 min at −78 °C and overnight at rt. The reaction mixture was then concentrated in vacuo, quenched with H_2O (450 mL) and extracted with CH_2Cl_2 (3 × 450 mL). The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated to afford the crude **6**. Purification by silica gel chromatography (20% EtOAc in petroleum ether) yielded compound **6** as a yellowish oil (6.1 g, 17 mmol, 68% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.95 (d, J = 6.6 Hz, 6H, 2× CH_3), 1.36 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 1.44 (t, J = 7.3 Hz, 2H, $CH_2CH(CH_3)_2$), 1.63–1.76 (m, 1H, $CH(CH_3)_2$), 4.14 (q, J = 6.5 Hz, 2H, OCH_2CH_3), 4.45 (br s, 1H, NCH), 4.68 (br d, 1H, NH), 5.11 [s, 2H, CH_2 (Cbz)], 6.30 (dd, J_{AB} = 15.2 Hz, J_{AC} = 1.3 Hz, 1H, CH^CCH^B = CH^AS), 6.79 (dd, J_{BA} = 15.2 Hz, J_{BC} = 5.3 Hz, 1H, CH^CCH^B = CH^AS), 7.32–7.39 (m, 5H, C_6H_5); ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.8 (OCH_2CH_3), 21.9, 22.6 ($CH(CH_3)_2$), 24.6 ($CH(CH_3)_2$), 43.0 ($CH_2CH(CH_3)_2$), 49.9 (NCH), 67.0, 67.0 [OCH_2CH_3, CH_2 (Cbz)], 124.4 (CH=CHS), 128.0, 128.3, 128.5, 136.0 (C_6H_5 (Cbz)), 148.6 (CH=CHS), 155.5 (C=O). HRMS m/z calculated for $C_{17}H_{24}NO_5S$ $[M-H]^-$: 354.1381, found: 354.1366.

4.5. Cbz-vsLeu-ONBu$_4$ (7)

A solution of compound **6** (6.1 g, 17 mmol) and NBu_4I (6.3 g, 17 mmol) in acetone (400 mL) was stirred overnight under reflux. The reaction mixture was then concentrated in vacuo and coevaporated with $CHCl_3$ (3 × 50 mL), yielding compound **7** as a dense

yellow oil (11.3 g). TLC analysis showed that the crude product was pure enough for being used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (m, 6H, CH(CH_3)$_2$), 1.00 (t, J = 7.3 Hz, 12H, 4× CH_3 (NBu_4)), 1.42 (m, 10H, CH_2CH(CH_3)$_2$, 4× CH_2CH_3 (NBu_4)), 1.65 (m, 9H, 4× $CH_2CH_2CH_3$ (NBu_4), CH(CH_3)$_2$), 3.30 (m, 8H, 4× NCH_2 (NBu_4)), 4.37 (m, 1H, NCH), 4.61 (d, J = 9.1 Hz, 1H, NH), 5.05 (q, J = 12.3 Hz, 2H, CH_2 (Cbz)), 6.40 (dd, J = 15.3, 4.6 Hz, 1H, CH=CHSO$_3$), 6.48 (dd, J = 15.3, 1.1 Hz, 1H, CH=CHSO$_3$), 7.34 (m, 5H, C_6H_5 (Cbz)). ^{13}C NMR (126 MHz, $CDCl_3$): δ 13.7 (CH_3 (NBu_4)), 19.7 (CH_2CH_3 (NBu_4)), 22.2, 22.8 (CH(CH_3)$_2$), 24.1 ($CH_2CH_2CH_3$ (NBu_4)), 24.6 (CH(CH_3)$_2$), 44.4 (CH_2CH(CH_3)$_2$), 49.4 (NCH), 58.9 (NCH_2 (NBu_4)), 66.5 (CH_2 (Cbz)), 127.9, 128.4, 136.6 (C_6H_5 (Cbz)), 133.9 (CH=CHSO$_3$), 134.6 (CH=CHSO$_3$), 155.6 (C=O). HRMS m/z calculated for $C_{15}H_{20}NO_5S$ [M−NBu_4]$^-$: 326.1068, found: 326.1055.

4.6. Cbz-Leu-VSF (8)

To a solution of compound **7** (4.8 g, 7.2 mmol) in dry CH_2Cl_2 (170 mL) was added XtalFluor-M® (3.72 g, 15.3 mmol), under N_2 atmosphere. A catalytic quantity of $Et_3N{\cdot}3HF$ (59 μL, 360 μmol) was added to the mixture, which was stirred overnight under reflux. After destruction of residual XtalFluor-M® by addition of silica gel to the solution, the mixture was filtered and concentrated in vacuo. Purification by silica gel chromatography (eluent: CH_2Cl_2/petroleum ether (2:1)), afforded peptido vinyl sulfonyl fluoride **8** as a white solid (720 mg, 2.18 mmol, 30% yield). Mp = 120 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.96 (d, J = 6.6 Hz, 6H, CH(CH_3)$_2$), 1.47 [t, J = 7.4 Hz, 2H, CH_2CH(CH_3)$_2$], 1.71 (m, 1H, CH(CH_3)$_2$), 4.53 (m, 1H, NCH), 4.72 (br d, 1H, NH), 5.13 [s, 2H, CH_2 (Cbz)], 6.52 (d, J = 15.3 Hz, 1H, CH=CHS), 7.06 (dd, J_{AX} = 4.8 Hz, J_{AB} = 15.3 Hz, 1H, CH=CHS), 7.37 (s, 5H, C_6H_5). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.6, 22.6 (CH(CH_3)$_2$), 24.6 [CH(CH_3)$_2$], 42.5 (CH_2CH(CH_3)$_2$), 50.1 (NCH), 67.3 [CH_2 (Cbz)], 121.7 (d, J = 27.8 Hz, CH=CHS), 128.1, 128.3, 128.6 (C_5H_6), 135.8 (Ar-C), 153.6 (CH=CHS), 155.5 (C=O); ^{19}F NMR (471 MHz, $CDCl_3$): δ = 60.4 (s). HRMS m/z calculated for $C_{15}H_{19}FNO_4S$ [M−H]$^-$: 328.1024, found: 328.1017.

4.7. HCl·H-Leu-VSF (9)

A stirred solution of compound **8** (75.8 mg, 0.23 mmol) in CH_2Cl_2 (2.3 mL) was put under N_2 atmosphere. After addition of HBr in acetic acid (33% v/v, 1.4 mL) stirring was continued for 30 min at rt. Then the solvents were evaporated and the residue dissolved in H_2O (3.5 mL). Dowex-Cl (2 × 8, 200 mg) was added and the solution was stirred for 5 min at rt. and then filtrated. The water layer was washed with EtOAc (2 × 3.5 mL), then concentrated in vacuo and coevaporated with toluene (3 × 5 mL), yielding HCl·H-Leu-VSF (**9**) as a yellowish solid (53.7 mg, 0.23 mmol, quantitative yield). The crude **9** was used directly in the synthesis of **10** and **11**.

4.8. Cbz-Leu$_3$-VSF (10)

To HCl salt **9** (43.3 mg, 0.187 mmol) were subsequently added BOP (86.7 mg, 0.196 mmol), Cbz-Leu$_2$-OH[9] (70.8 mg, 0.187 mmol), CH_2Cl_2 (5 mL), and D*i*PEA (69 μL, 0.393 mmol). The mixture was stirred overnight at rt under N_2. During the reaction, the pH was monitored (pH indicator paper) and kept to approximately 9 by adding additional D*i*PEA, if necessary. After evaporation of the solvent, the residue was dissolved in EtOAc (15 mL) and was washed with $KHSO_4$ (1.0 M, 3 × 10 mL), and brine (10 mL). The organic layer was dried over Na_2SO_4 and concentrated. Purification with silica gel column chromatography (eluent: 26% EtOAc in hexanes) afforded Cbz-Leu^1-Leu^2-Leu^3-VSF (**10**) as a white solid (26.8 mg, 0.048 mmol, 26% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.85–1.01 [m, 18H, 3× CH(CH_3)$_2$], 1.41–1.86 [m, 9H, 3× CH_2CH(CH_3)$_2$], 4.14 [m, 1H, NCH (Leu^1)], 4.38 [m, 1H, NCH (Leu^2)], 4.74 [m, 1H, NCH (Leu^3)], 5.11 [2d, J = 12.2 Hz, 2H, CH_2 (Cbz)], 5.38 [d, J = 5.0 Hz, 1H, NH (Leu^1)], 6.60 [m, 2H, 2× NH, (Leu^2), CH=CHS], 7.00 [d, J = 8.1 Hz, NH (Leu^3)], 7.05 (dd, J_{AX} = 4.5 Hz, J_{AB} = 15.2 Hz, 1H, CH=CHS), 7.30–7.43 (m, 5H, C_6H_5); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.6, 21.7, 21.8, 22.8, 24.8, 25.0 (CH_2CH(CH_3)$_2$), 39.9, 40.8, 42.1 (CH_2CH(CH_3)$_2$), 48.0 (NCH^3), 52.3 (NCH^2), 54.3 (NCH^1), 67.3 [CH_2 (Cbz)], 121.7, 122.1 (d, J = 27.4 Hz, CH=CHS), 127.9, 128.4, 128.6, 135.7 (C_6H_5), 153.3 (CH=CHS), 156.7 [C=O (Cbz)], 171.5, 172.7 [C=O (Leu^{1,2})]; ^{19}F NMR (376 MHz, $CDCl_3$): δ = 59.2 (s); HRMS m/z calculated for $C_{27}H_{43}FN_3O_6S$ [M+H]$^+$: 556.2858, measured: 556.2857.

4.9. Cbz-Leu$_4$-VSF (11)

Cbz-Leu$_3$-OMe (550 mg, 1.0 mmol)[23] was dissolved in Tesser's base (12.5 mL, mixture of NaOH (2.0 M), MeOH and dioxane, in proportion 1:5:14 (v/v/v)). After the mixture was stirred overnight at rt, it was neutralized to pH 7 (pH indicator paper) with $KHSO_4$ (1.0 M). The dioxane was evaporated in vacuo and the mixture was acidified to pH 2 (pH indicator paper) with $KHSO_4$ (1.0 M). The water layer was extracted with EtOAc (2 × 30 mL). The organic layer was washed with H_2O (50 mL) and with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated in vacuo, yielding Cbz-leu$_3$-OH as a white solid (501 mg, 1.0 mmol, quantitative yield). Cbz-Leu$_3$-OH was coupled to HCl·H-Leu-VSF (**9**) using the procedure described in the synthesis of **10**. The scale of this reaction was 0.23 mmol. Purification with silica gel column chromatography (eluent: 36% EtOAc in hexanes) afforded Cbz-Leu^1-Leu^2-Leu^3-vsLeu^4-F (**11**) as a white solid (51.8 mg, 0.077 mmol, 33% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.77–1.03 (m, 24H, 4× CH(CH_3)$_2$), 1.38–1.90 (m, 12H, 4× CH_2CH(CH_3)$_2$), 3.93 [m, 1H, NCH (Leu^1)], 4.19, 4.38 [2× m, 2H, 2× NCH (Leu^{2,3})], 4.75 [m, 1H, NCH (Leu^4)], 5.14 [s, 2H, CH_2 (Cbz)], 5.20 [s, 1H, NH (Leu^1)], 6.40, 7.06 [2d, J = 4.6 Hz, J = 7.3 Hz, 2H, NH (Leu^{2,3})], 6.68 (dt, J_{AB} = 15.0 Hz, J_{AX} = 2.0 Hz, J_{AF} = 2.0 Hz, CH=CHS), 7.08 [m, 2H, CH=CHS, NH (Leu^4)], 7.29–7.43 (m, 5H, C_6H_5); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 21.2, 21.5, 21.6, 21.7, 22.9, 23.0, 24.2 (CH_2CH(CH_3)$_2$), 40.2, 40.6, 41.1 (CH_2CH(CH_3)$_2$), 47.6 [NCH (Leu^4)], 51.1, 51.4 [NCH (Leu^{2,3})], 53.3 [NCH (Leu^1)], 65.4 [CH_2 (Cbz)], 120.6 (d, J = 25.5 Hz, CH=CHS), 127.6, 127.8, 128.3, 137.0 (C_6H_5), 156.0 [C=O (Cbz)], 156.1 [CH=CHS], 171.7, 171.9, 172.5 [3× C=O (Leu^{1,2,3})]; ^{19}F NMR (376 MHz, $CDCl_3$): δ = 59.0 (s); HRMS m/z calculated for $C_{33}H_{54}FN_4O_7S$ [M+H]$^+$: 669.3698, measured: 669.3694.

4.10. TFA.H-Thr-Val-NHMe (13)

To a solution of Boc-valine (5.4 g, 23 mmol) was in CH_2Cl_2 (140 ml) was added BOP (10.2 g, 23 mmol). D*i*PEA (8.8 ml, 50 mmol) and subsequently methylamine (18.8 ml, 37.5 mmol, 2.0 M in THF) were added. After 2 h stirring at rt, the mixture was concentrated in vacuo. Ethyl acetate (400 mL) was added and washed two times with $KHSO_4$ (1.0 M, 200 mL), two times with $NaHCO_3$ (1.0 M, 200 mL) and with brine (100 mL). After drying over Na_2SO_4 and concentration in vacuo, column chromatography (ethyl acetate/hexane, 40/60) was performed to afford Boc-Val-NHMe (**12**) as a white solid (3.6 g, 65%). Boc-Val-NHMe (0.7 g, 3.0 mmol) was dissolved in CH_2Cl_2 (14 ml) and TFA was added (14 ml). The solution as stirred at rt for a half hour after which the mixture was concentrated in vacuo and coevaporated with chloroform (3× 200 mL). To the crude TFA.H-Val-NHMe was added CH_2Cl_2 (20 ml), BOP (1.0 g, 3.24 mmol), D*i*PEA (1.1 ml, 6.5 mmol) and Boc-Thr-OH (0.7 g, 3.0 mmol). After stirring at rt for 18 h, the solvent was evaporated and $KHSO_4$ (1.0 M, 250 mL)

was added. After extraction with ethyl acetate (3× 100 mL), the organic layers were combined, dried over Na_2SO_4 and concentrated in vacuo. Crystallization from ethyl acetate afforded Boc-Thr-Val-NHMe (**12**) as a white solid (346 mg, 32%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.94 (m, 6H, CH(CH_3)$_2$), 1.18 (d, J = 6.3 Hz, 3H, CH_3CHOH), 1.46 (s, 9H, C(CH_3)$_3$), 2.23 (m, 1H, CH(CH_3)$_2$), 2.82 (d, J = 4.4 Hz, 3H, CH_3NH), 3.32 (bs, 1H, OH), 4.08 (d, J = 7.5 Hz, 1H, CHCHOH), 4.21 (bt, 1H, CHCH(CH_3)$_2$), 4.32 (m, 1H, CHOH), 5.51 (d, J = 6.4 Hz, 1H, BocN$\underline{H}$), 6.16 (bd, 1H, NHCH$_3$), 6.96 (m, 1H, NHCHCH(CH_3)$_2$). ^{13}C NMR (300 MHz, $CHCl_3$): δ = 17.8, 18.5 (CH(CH_3)$_2$), 19.2 (CH_3CHOH), 26.0 (CH_3NH), 28.2 (C(CH_3)$_3$), 30.3 (CH(CH_3)$_2$), 58.6, 58.8 (NCH (Val and Thr)), 67.3 (CHOH), 80.2 (C(CH_3)$_3$), 156.3 (C=O (Boc)), 171.3, 171.9 ($CONHCH_3$, HOCHCHC=O). HRMS m/z calculated for $C_{15}H_{29}N_3NaO_5$ [M+Na]$^+$: 354.1999, found: 354.1986. Boc-Thr-Val-NHMe (346 mg, 0.96 mmol) was dissolved in CH_2Cl_2 (2 mL) and TFA (2 mL), and the solution was stirred for 30 min at rt. Concentration in vacuo and coevaporation with chloroform (3 × 20 mL) afforded the crude TFA.H-Thr-Val-NHMe (**13**), which was directly used in the next reaction.

4.11. β-Sultam 15

PVSF **8** (50 mg, 0.15 mmol) was dissolved in CH_2Cl_2 (3 mL) and treated with benzylamine (50 μL, 0.45 mmol) overnight at rt. Evaporation of the solvent and purification by silica gel column chromatography (eluent: gradient of hexanes/ethyl acetate (6:1 to 4:1)) afforded both diasteroisomers of β-sultam **15** as white solids (diastereoisomer 1: 10 mg, 24 μmol, 16%; diastereoisomer 2: 3 mg, 7.2 μmol, 5%). Major isomer: ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.76 (t, J = 7.8 Hz, 6H, CH(CH_3)$_2$), 1.11 (dd, J = 8.6, 4.4 Hz, 2H, CH_2CH(CH_3)$_2$), 1.47 (m, 1H, CH(CH_3)$_2$), 3.21 (m, 1H, SO_2NCH), 3.78 (m, 2H, CH^{a}SO$_2$, CHNCO$_2$), 3.95 (m, 1H, CH^{b}SO$_2$), 4.02 (d, J = 14.5 Hz, 1H, NCH^{a}Ar), 4.24 (bd, 1H, NH), 4.35 (d, J = 14.5 Hz, 1H, NCH^{b}Ar), 5.98 (d, J = 12.3 Hz, 1H, ArCH^a (Cbz)), 5.07 (dd, J = 12.3, 2.3 Hz, 1H, ArCH^b (Cbz)), 7.21–7.31 (m, 10H, 2× C_6H_5). ^{13}C NMR (126 MHz, $CDCl_3$) δ = 21.5, 23.2 (CH(CH_3)$_2$), 24.6 (CH(CH_3)$_2$), 39.6 (CH_2CH(CH_3)$_2$), 50.0 ($CHNCO_2$, NCH_2), 50.9 ($CHNSO_2$), 58.8 (CH_2SO_2), 66.9 (CH_2 (Cbz)), 127.9, 128.1, 128.2, 128.6, 128.7, 129.0, 134.9, 136.3 (C_6H_5), 156.2 (C=O (Cbz)). HRMS m/z calculated for $C_{22}H_{28}N_2NaO_4S$ [M+Na]$^+$: 439.1662, found: 439.1645.

4.12. Proteasome enzymatic assays for IC_{50} determination

Enzyme activity was determined by monitoring the hydrolysis of the fluorogenic substrate Suc-LLVY-AMC for 1 hour at room temperature. Fluorescence was measured at λ_{exc} = 360, λ_{em} = 460 nm. Point-measurements were performed with a 1 h incubation of the enzyme with the inhibitors prior to substrate addition. MG132 was used as reference inhibitor (included in the assay kit). The enzyme solution (25 nM) was prepared by dilution of the supplied 20S proteasome (1 mg/mL) in VIVA buffer. A 10 μM stock solution of the substrate was made by dissolving Suc-LLVY-AMC (500 μg) in DMSO, which was diluted with VIVA buffer resulting in a 1.0 mM substrate solution. For the inhibitor stock solution (500 μM), the inhibitor (1.0 mg) was dissolved in DMSO. DMSO was used for the inhibitor dilutions. In a typical assay to each well was added enzyme solution (5 μL), inhibitor solution (4 μL), substrate solution (5 μL) and buffer (36 μL). Final concentrations in the wells were: enzyme: 2.5 nM; substrate: 10 mM; inhibitor: 0.4, 2, 10, 50, 100, 200, 400, 800, 1600 and 8000 nM. For the no inhibitor controls DMSO was added instead of inhibitor solution, thereby maintaining a final concentration of 9% DMSO per well. The assays were performed in triplicate. The inhibitory activities of compounds were expressed as IC_{50} values. The values were obtained by plotting the percentage of enzymatic activity against the logarithm of the inhibitor concentrations and fitting the experimental data to the equation % Residual Activity = 100/(1 + 10^((LogIC_{50} − Log$c$ (inhibitor)) * Hill Slope)) using GraphPad Prism software.

Acknowledgements

We thank Thijs Nell for the synthesis of compound **12** and Javier Sastre Toraño for providing some of the HRMS spectra. This paper is dedicated to the memory of Johan Cruijff (1947–2016).

Supplementary data

Supplementary data (NMR data and IC_{50} determination data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.05.042.

References and notes

1. Guterman, L. *C&EN* **2011**, *89*, 19.
2. Mah, R.; Thomas, J. R.; Shafer, C. M. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 333.
3. Singh, J.; Petter, R. C.; Baillie, T. A.; Whitty, A. *Nat. Rev.* **2011**, *10*, 307.
4. Richardson, P. G.; Hideshima, T.; Anderson, K. C. *Cancer Control* **2003**, *10*, 361.
5. Demo, S. D.; Kirk, C. J.; Aujay, M. A.; Buchholz, T. J.; Dajee, M.; Ho, M. N.; Jiang, J.; Laidig, G. J.; Lewis, E. R.; Parlati, F.; Shenk, K. D.; Smyth, M. S.; Sun, C. M.; Vallone, M. K.; Woo, T. M.; Molineaux, C. J.; Bennet, M. K. *Cancer Res.* **2007**, *67*, 6383.
6. Muchamuel, T.; Basler, M.; Aujay, M. A.; Suzuki, E.; Kalim, K. W.; Lauer, C.; Sylvain, C.; Ring, E. R.; Shields, J.; Jiang, J.; Shwonek, P.; Parlati, F.; Demo, S. D.; Bennett, M. K.; Kirk, C. J.; Groettrup, M. *Nat. Med.* **2009**, *15*, 781.
7. Basler, M.; Mundt, S.; Muchamuel, T.; Moll, C.; Jiang, J.; Groettrup, M.; Kirk, C. J. *EMBO Mol. Med.* **2014**, *6*, 226.
8. Dubiella, C.; Cui, H.; Gersch, M.; Brouwer, A. J.; Sieber, S. A.; Kruger, A.; Liskamp, R. M. J.; Groll, M. *Angew. Chem., Int. Ed.* **2014**, *53*, 11969.
9. Verdoes, M.; Florea, B. I.; van der Linden, W. A.; Renou, D.; van den Nieuwendijk, A. M. C. H.; van der Marel, G. A.; Overkleeft, H. S. *Org. Biomol. Chem.* **2007**, *5*, 1416.
10. Kisselev, A. F.; Van der Linden, W. A.; Overkleeft, H. S. *Chem. Biol.* **2012**, *19*, 99.
11. Gräwert, M. A.; Groll, M. *Chem. Commun.* **2012**, *48*, 1364.
12. Huber, E. M.; Groll, M. *Angew. Chem., Int. Ed.* **2014**, *51*, 2.
13. Gennari, C.; Salom, B.; Potenza, D.; Williams, A. *Angew. Chem., Int. Ed.* **2014**, *53*, 11969.
14. L'Heureux, A.; Beaulieu, F.; Bennett, C.; Bill, D. R.; Clayton, S.; LaFlamme, F.; Mirmehrabi, M.; Tadayon, S.; Tovell, D.; Couturier, M. *J. Org. Chem.* **2010**, *75*, 3401.
15. The previously used fluorinating reagent DAST gave higher yields, but the reactions were less clean and purification of the sulfonyl fluoride was tedious. The use of XtalFluor-M® led to clean reactions and easy purifications by column chromatography.
16. Brouwer, A. J.; Jonker, A.; Werkhoven, P.; Kuo, E.; Li, N.; Gallastegui, N.; Kemmink, J.; Florea, B. I.; Groll, M.; Overkleeft, H. S.; Liskamp, R. M. J. *J. Med. Chem.* **2012**, *55*, 10995.
17. Dong, J.; Krasnova, L.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2014**, *53*, 9430.
18. Krutak, J. J.; Burpitt, R. D.; Moore, W. H.; Hyatt, J. A. *J. Org. Chem.* **1979**, *44*, 3847.
19. Champseix, A.; Chanet, J.; Etienne, A.; Le Berre, A.; Masson, J.; Napierala, C.; Vessiere, R. *Bull. Soc. Chim. Fr.* **1985**, *3*, 463.
20. von Arx, E.; Faupel, M.; Bruggen, M. J. *J. Chromatogr.* **1976**, *120*, 224.
21. Truce, W. E.; Vrencur, D. J. *J. Org. Chem.* **1970**, *35*, 1226.
22. Otrubova, K.; Cravatt, B. F.; Boger, D. L. *J. Med. Chem.* **2014**, *57*, 1079.
23. Tian, J.; Gao, W.-C.; Zhou, D.-M.; Zhang, C. *Org. Lett.* **2012**, *14*, 3020.