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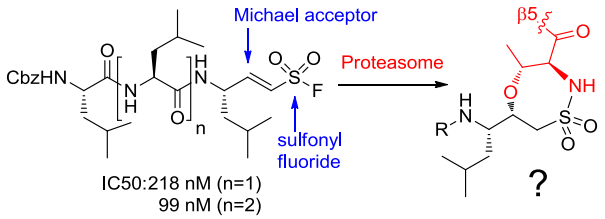

Proteasome Inhibition by New Dual Warhead Containing Peptido Vinyl Sulfonyl Fluorides

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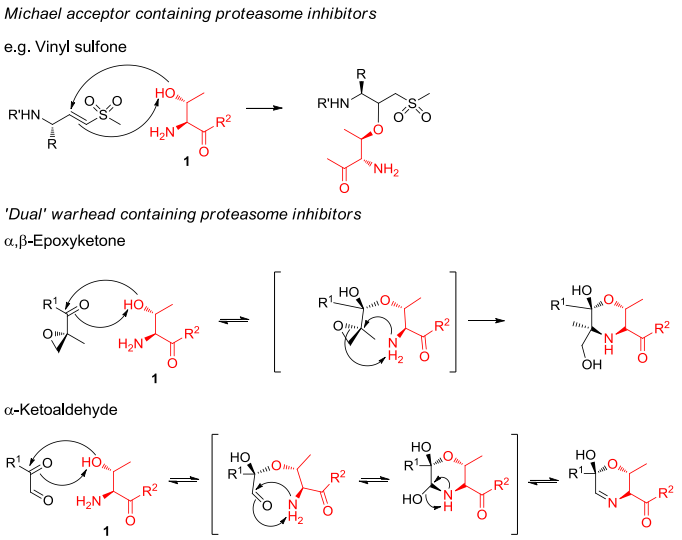
ABSTRACT

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 7-membered ring structure. Possibly, formation of a 7-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

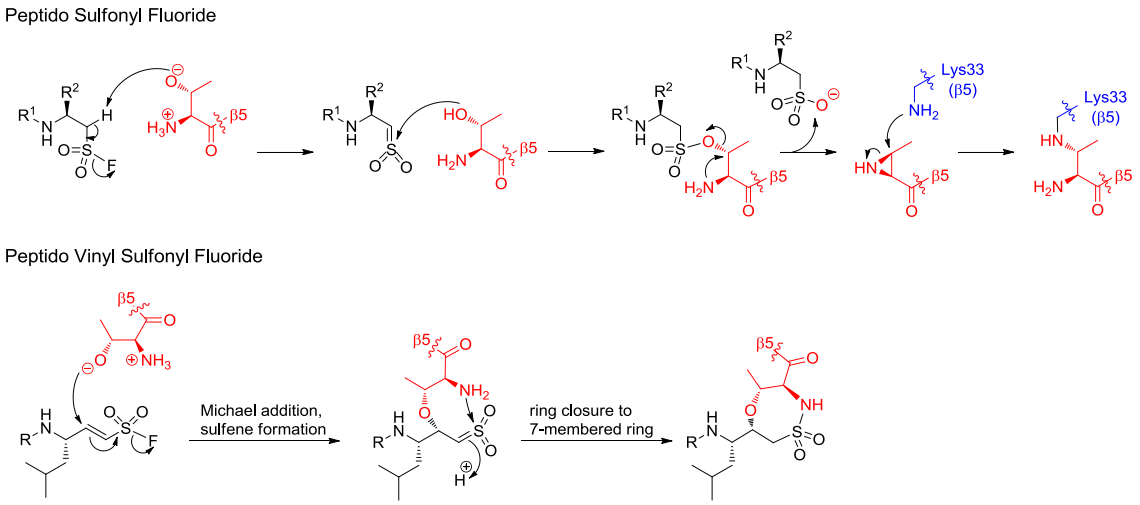
The development of proteasome inhibitors has been an outstanding case showing that irreversible inhibitors may provide unique advantages by forming long-lived ties with their target.[1] Depending on the degree of reversibility of this covalent interaction, the putative proteasome inhibitor may therefore display a prolonged interaction and therefore 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] For example, the Michael acceptor electrophile containing proteasome inhibitors, especially the vinyl sulfones containing ones have been subject of intense investigations (Scheme 1).[10] ***Rob, deze laatste zin vond reviewer 1 difficult to understand, dus moeten we herschrijven.***



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.

However, in contrast to serine proteases in which the attacking nucleophile on the peptide-amide bond is 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. This N-terminal threonine residue contains 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 and carbonyl electrophilic site, after previous treatment with bortezomib, which contains just one electrophilic site (Figure 1). In our opinion this justifies a quest for dual warhead containing inhibitors such as the one discussed in this research.



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

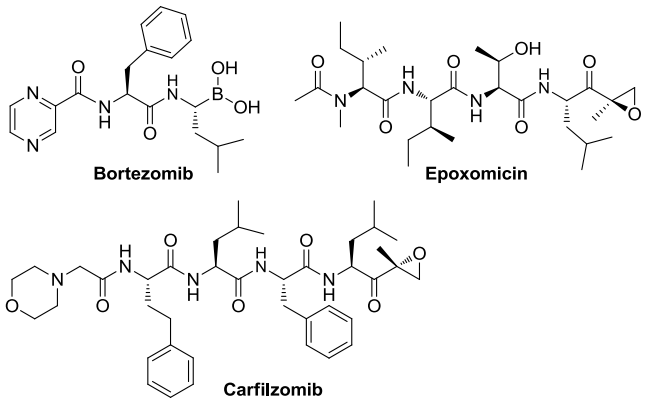


Figure 1. Structures of Bortezomib, Epxomicin and Carfilzomib.

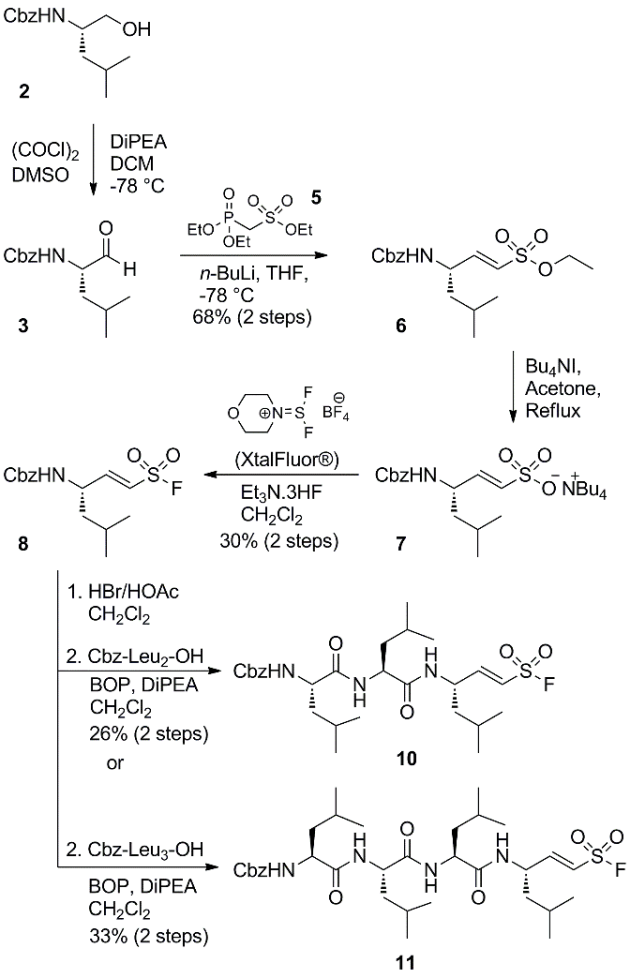
Inspired by the "dual' warhead approach we describe in this paper a new proteasome inhibitor concept in which a Michael electrophilic trap, is combined with a sulfonyl fluoride electrophile incorporated into a peptide sequence leading to a peptido vinylsulfonyl fluoride (PVSF). Both electrophilic traps 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 (Figure 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 4-position is reacting with the threonine nucleophile(s) (Scheme 1).

2. Results and discussion

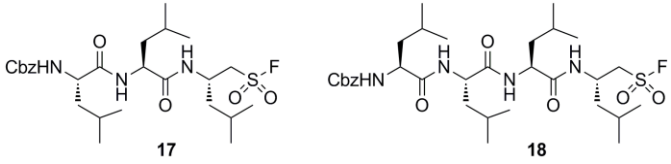
2.1 Chemistry

Here we propose the peptido vinylsulfonyl 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 vinylsulfonate ester **6**, which was cleaved by Bu_4NI. The most efficient conversion of the resulting sulfonate salt (**7**) into the corresponding vinylsulfonyl fluoride (**8**) was achieved by using talFluor-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.



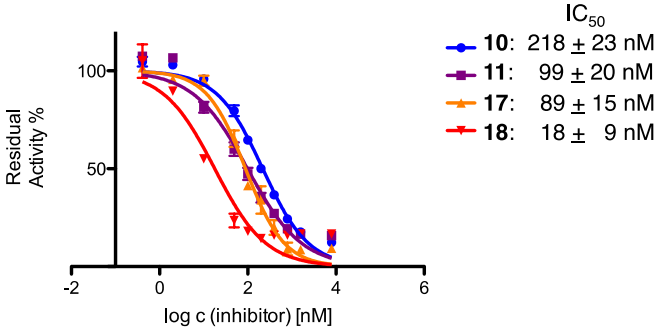
Scheme 3. Synthesis of PVSF compounds **10** and **11**.

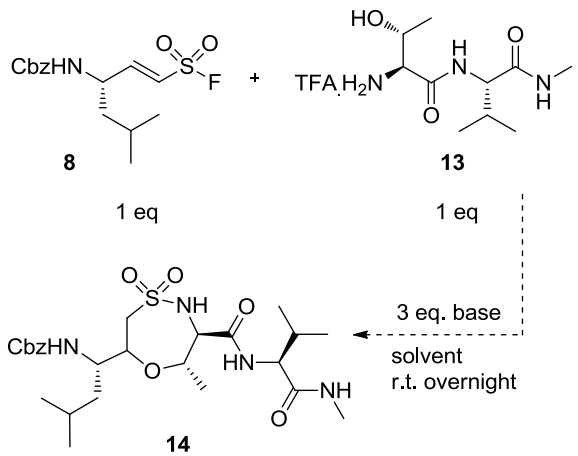
2.2 Biological evaluation

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). With respect to this, comparison with other warheads highlights the peptido sulfonyl fluoride as a promising motif for β5i targeting. **hier iets zeggen dat de PSF ook erg goed werkt voor β5c, nu lijkt het dat dit niet zo is*** The sequences of inhibitors **10** and **11** were chosen based on earlier results with our most potent PSF proteasome inhibitors **17** and **18** (IC_{50}-values 89 nM and 18 nM, respectively, Figure 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 (Figure 1). At first we were somewhat surprised by the diminished activity of the PVSF's as compared to PSF's **17** and **18**, respectively. 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 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.

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

To investigate whether the proposed formation within the enzyme of a 7-membered ring adduct could be observed by chemo-synthesis, in parallel, the reactivity of a simplified peptido vinylsulfonyl 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 mechanism of inhibition by this PVSF warhead.



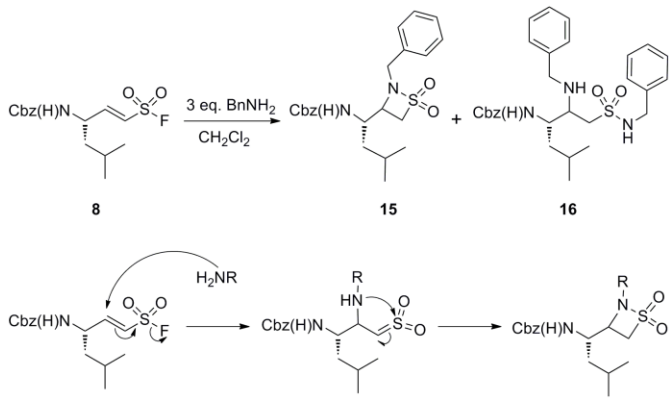


Scheme 4. Model reaction of a PVSF with a threonine containing dipeptide comprising the N-terminal proteasome site. The attempted base/ solvent combinations were DBU, Et_3N or NMM in CH_2Cl_2 and DBU or Et_3N in CH_3CN.

Although it was possible to observe two small peaks at m/z 541.44 and 563.49, corresponding to the $[M+H]^+$ and $[M+Na]^+$ ions of the 7-membered ring containing molecule (**13**), (Scheme 4, for LCMS spectrum see supporting information) ***Rob evt. Meer uitleg hier ivm laatste punt reviewer 5*** we were unable to isolate this adduct after (silica gel) column chromatography and preparative HPLC. 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 7-membered ring structure. ***hier korte uitleg dat de gevonden massa ook de open ring had kunnen zijn indien een Thr1 nucleofiel de fluor substitueert, en dat dit niet voor de hand ligt omdat uit eigen bevindingen en uit de literatuur[17+18] blijkt dat bij vinyl sulfonyl fluoriden het nucleofiel altijd eerst aanvalt op de Michael acceptor. Indien het tweede nucleofiel niet zou aanvallen om de 7-ring te vormen, dan zou de open ring nog een sulfonyl fluoride of sulfonaat bij eventuele hydrolyse hebben, wat een andere massa zou hebben.***

Therefore, we felt that it was necessary to get some insight in the reactivity of the peptido vinylsulfonyl fluorides and to what extent the proposed -"in vivo", that is in the proteasome - 7-membered ring might be formed "in vitro".

Instead of the amino-group nucleophile as present in the threonine dipeptide model, the much simpler benzyl amine 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 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] Since a 7-membered ring is in general not easily accessible, its proposed formation in vivo may be challenging to mimic in vitro.

Scheme 5. Reaction and proposed mechanism of β-sultam formation of vinylsulfonyl fluoride **8** with benzyl amine.

3. Conclusions

We have introduced a peptido vinylsulfonyl 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 vinylsulfonyl fluoride was proposed to give rise to the formation of a covalent 7-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 7-membered ring structure we were unable to isolate it and achieve its synthesis "in vitro", which instead led to formation of a β-sultam structure. 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 $LiAlH_4$. Petroleum 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). ^1H 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 (b). ^{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, 2x100 mL). The water layer was extracted with Et_2O (1x100 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, 2x 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, C$H(CH_3)_2$), 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 morpoline (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 x 200 mL) and water (1 x 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 hour at −50 °C. The mixture was concentrated, the residue was diluted with water (100 mL) and extracted with CH_2Cl_2 (3 x 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 x 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 x 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, C$H(CH_3)_2$), 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 $CHCl_3$ (3x50 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 x CH_3 (NBu_4)), 1.42 (m, 10H, $CH_2CH(CH_3)_2$, 4 x CH_2CH_3 (NBu_4)), 1.65 (m, 9H, 4 x $CH_2CH_2CH_3$ (NBu_4), $CH(CH_3)_2$), 3.30 (m, 8H, 4 x 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 $C_{15}H_{20}NO_5S$ $[M\text{-}NBu_4]^-$: 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 vinylsulfonyl 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 $C_{15}H_{19}FNO_4S$ $[M\text{-}H]^-$: 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 (2x3.5 mL), then concentrated *in vacuo* and coevaporated with toluene (3x5 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 x $CH(CH_3)_2$], 1.41-1.86 [m, 9H, 3 x $CH_2CH(CH_3)_2$], 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 x 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 (2x30 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 x $CH(CH_3)_2$), 1.38-1.90 (m, 12H, 4 x $CH_2CH(CH_3)_2$), 3.93 [m, 1H, NCH (Leu^1)], 4.19, 4.38 [2 x m, 2H, 2 x 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=*C*HS), 127.6, 127.8, 128.3, 137.0 (C_6H_5), 156.0 [C=O (Cbz)], 156.1 (*C*H=CHS], 171.7, 171.9, 172.5 [3 x 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 hours 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 (3x 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 hours, the solvent was evaporated and $KHSO_4$ (1.0 M, 250 mL) was added. After extraction with ethyl acetate (3x 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(C*H*$_3$)$_2$), 1.18 (d, *J* = 6.3 Hz, 3H, C*H*$_3$CHOH), 1.46 (s, 9H, C(C*H*$_3$)$_3$), 2.23 (m, 1H, C*H*(CH$_3$)$_2$), 2.82 (d, *J* = 4.4 Hz, 3H, C*H*$_3$NH), 3.32 (bs, 1H, OH), 4.08 (d, *J* = 7.5 Hz, 1H, C*H*CHOH), 4.21 (bt, 1H, C*H*CH(CH$_3$)$_2$), 4.32 (m, 1H, C*H*OH), 5.51 (d, *J* = 6.4 Hz, 1H, BocN<u>H</u>), 6.16 (bd, 1H, N*H*CH$_3$), 6.96 (m, 1H, N*H*CHCH(CH$_3$)$_2$). ^{13}C NMR (300 MHz, $CHCl_3$): δ = 17.8, 18.5 (CH(*C*H$_3$)$_2$), 19.2 (*C*H$_3$CHOH), 26.0 (CH$_3$NH), 28.2 (C(*C*H$_3$)$_3$), 30.3 (*C*H(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 (*C*ONHCH$_3$, HOCHCH*C*=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 minutes at rt. Concentration in vacuo and coevaporation with chloroform (3x 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(C*H*$_3$)$_2$), 1.11 (dd, *J* = 8.6, 4.4 Hz, 2H, C*H*$_2$CH(CH$_3$)$_2$), 1.47 (m, 1H, C*H*(CH$_3$)$_2$), 3.21 (m, 1H, SO$_2$NC*H*), 3.78 (m, 2H, CH^{a}SO$_2$, C*H*NCO$_2$), 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, 2x C_6H_5). ^{13}C NMR (126 MHz, $CDCl_3$) δ = 21.5, 23.2 (CH(*C*H$_3$)$_2$), 24.6 (*C*H(CH$_3$)$_2$), 39.6 (*C*H$_2$CH(CH$_3$)$_2$), 50.0 (*C*HNCO$_2$, NCH$_2$), 50.9 (CHNSO$_2$), 58.8 (CH$_2$SO$_2$), 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 IC50 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 hour 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 ul) and buffer (36 uL). 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 IC50 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^((LogIC50-Log c (inhibitor))*HillSlope))) using GraphPad Prism software.

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Supplementary Material

NMR data and IC50 determination data.