

Peptido Sulfonyl Fluorides as New Powerful Proteasome Inhibitors

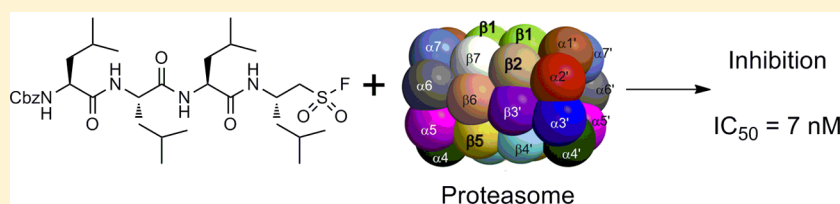
Arwin J. Brouwer,[†] Anika Jonker,[†] Paul Werkhoven,[†] Ethan Kuo,[‡] Nan Li,[‡] Nerea Gallastegui,[§] Johan Kemmink,[†] Bogdan I. Florea,[‡] Michael Groll,[§] Herman S. Overkleeft,[‡] and Rob M. J. Liskamp^{*,†}

[†]Department of Medicinal Chemistry and Chemical Biology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands

[‡]Bio-organic synthesis, Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA, Leiden, The Netherlands

[§]Center for Integrated Protein Science at the Department Chemie, Lehrstuhl für Biochemie, Technische Universität München, Lichtenbergstrasse 4, 85748, Garching, Germany.

S Supporting Information



ABSTRACT: A new class of potent proteasome inhibitors is described, of which the members contain an amino acid inspired sulfonyl fluoride as the electrophilic trap. In total, 24 peptido sulfonyl fluoride inhibitors have been designed and synthesized, which were inspired by the backbone sequences of the proteasome inhibitors bortezomib, epoxomicin, and Cbz-Leu₃-aldehyde. Nine of them were very potent proteasome inhibitors, the best of which had an IC₅₀ of 7 nM. A number of the peptido sulfonyl fluoride inhibitors were found to be highly selective for the $\beta 5$ proteasome subunit.

1. INTRODUCTION

Quality control of proteins is essential in normal cells but also in cancer cells and infected cells. The proteasome plays a crucial role in this process and is responsible for the proteolytic degradation of abnormal and damaged cellular proteins. The proteasome is a quaternary assembly of 28 subunit proteins that are arranged in two inner β -rings and two outer α -rings. In the β -rings the protease subunits are found and the $\beta 1$, $\beta 2$, and $\beta 5$ subunits are proteolytically active. Each subunit possesses a different substrate specificity; $\beta 1$ has a caspase-like (C-like), $\beta 2$ has a trypsin-like (T-like), and $\beta 5$ has a chymotrypsin-like (CT-like) activity.¹ Especially since the approval of bortezomib (PS341) for clinical treatment of multiple myeloma, research in the area of proteasome inhibition has been greatly intensified.^{2,3} Several other potent proteasome inhibitors have been described in the literature in the meantime, for example, epoxomicin, Cbz-Leu₃-aldehyde (MG132), and Cbz-Leu₃-VS (Figure 1).³

Like bortezomib, they are covalently binding inhibitors, each containing an electrophilic group nowadays often denoted as an electrophilic “trap” or “warhead”. Despite the availability of several different covalently interacting proteasome inhibitors, the number of electrophilic groups displaying the proper reactivity is still limited. This reactivity is very important for selectivity. In general, the reactivity should not be too high, to prevent reactions with abundantly present nucleophiles in proteins and other biomolecules in the aqueous environment.⁴

Recently, we have described a new class of serine protease inhibitors, containing an aliphatic *sulfonyl fluoride* as the electrophilic trap.⁵ Since these sulfonyl fluorides are derived

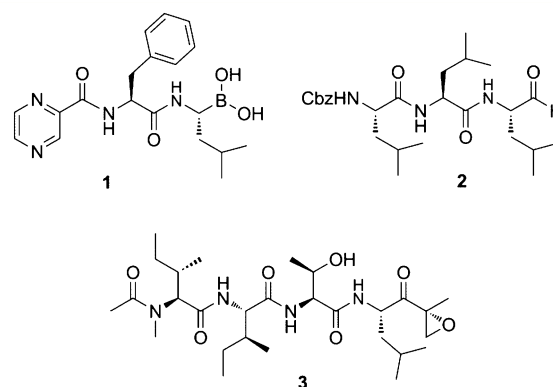


Figure 1. Structures of bortezomib (1), Cbz-Leu₃-aldehyde (2, MG132), and epoxomicin (3).

from amino acids, they contain a side chain that can interact with the S1 binding pockets of proteases. Evaluation of the inhibition of the serine protease chymotrypsin by a small set of amino acid derived and dipeptido sulfonyl fluorides showed the potential of this novel sulfonyl fluoride warhead.^{5b} Moreover, we demonstrated the importance of the side chain contribution to the binding affinity, holding great potential for tuning the selectivity of inhibitors to be developed. For example, a phenylalanine-derived sulfonyl fluoride inhibitor showed a K_i of

Received: October 5, 2012

Published: November 22, 2012

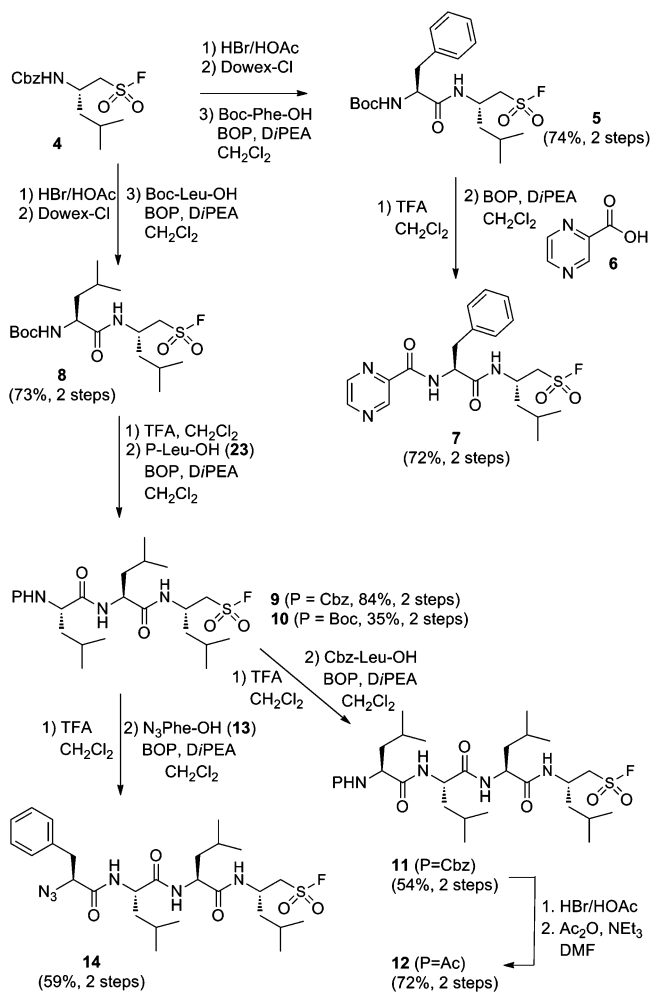
22 μM and a k_{inact} of 0.32 min^{-1} , which is significant considering the small size of this inhibitor. These very promising data on the reactivity and selectivity of our sulfonyl fluorides encouraged us to apply them to the challenging proteasome target. Several proteasome inhibitors that have been described so far thereby offer an excellent opportunity for comparing their activity to the activity of the peptido sulfonyl fluoride derived inhibitors to be obtained by replacement of the electrophilic group. Thus, in the bortezomib sequence **1** (pyrazine-Phe-Leu or pFL), the borate-containing amino acid derivative was replaced by a leucine-derived sulfonyl fluoride. Similarly, in the aldehyde-containing proteasome inhibitor **2** (Cbz-Leu₃ sequence), as well as in the inhibitor epoxomicin (**3**, Ac-IITL sequence), the electrophilic groups were replaced by the sulfonyl fluoride moiety.

2. RESULTS AND DISCUSSION

The syntheses of pFL peptido sulfonyl fluoride and trileucine-derived peptide sulfonyl fluorides are shown in Scheme 1.

Crucial in the syntheses of peptido sulfonyl fluorides was (a) the possibility to remove the Cbz-protective group and (b) coupling of the resulting amino deprotected amino acid derived sulfonyl fluorides largely without affecting the sulfonyl fluoride warhead.

Scheme 1. Synthesis of pFL (7**), Leu₃ (**9**, **10**, **14**), and Leu₄ (**11**, **12**) Peptido Sulfonyl Fluorides**



Thus, starting from the Cbz-protected leucine-derived sulfonyl fluoride **4** (Cbz-Leu-SF),⁵ pFL sulfonyl fluoride **7** was prepared in four reaction steps in an overall yield of 53% (Scheme 1). Starting from sulfonyl fluoride **4**, the Leu₃ peptido sulfonyl fluorides **9** and **10** were accessible in six reaction steps in decent (61% and 26%) overall yields. Azido derivative **14** was prepared to facilitate postinhibition attachment of fluorescent or affinity purification tags via a bio-orthogonal coupling reaction like the Bertozzi–Staudinger or the 1,3-dipolar Huisgen cycloaddition (click).^{6,7} The introduction of azidophenylalanine proceeded analogously after TFA-mediated removal of the Boc-group and BOP-coupling of azidophenylalanine to afford azido peptido sulfonyl fluoride **14** in 15% yield over eight reaction steps. Tetra-leucine peptido sulfonyl fluorides **11** and **12** were synthesized (Scheme 1), in reasonable yields [14% (six steps) and 10% (eight steps), respectively], because many other proteasome inhibitors described in the literature including epoxomicin (**3**) have a tetrapeptide backbone.

The synthesis of IITL peptido sulfonyl fluorides started from Cbz-protected leucine-derived sulfonyl fluoride **4** (Scheme 2). After HBr-induced cleavage of the Cbz-group, BOP-mediated coupling of Boc-Thr(OBn)-OH gave Boc-Thr(Bn)-Leu-SF (**15**) in high yield (81%). Two consecutive deprotection–coupling (with Boc-Ile-OH) cycles afforded tetrapeptido sulfonyl fluoride Boc-Ile-Ile-Thr(OBn)-Leu-SF (**17**) in good (43%, six steps) overall yield.

The N-terminal acetyl group, as is present in epoxomicin (**3**), was introduced after removal of the Boc-group and afforded Ac-Ile-Ile-Thr(OBn)-Leu-SF (**18**) in 78% yield. After the last step involving acidolytic (HBr/HOAc) cleavage of the benzyl protecting group on the threonine side chain, Ac-IITL peptido sulfonyl fluoride **19** was obtained in a modest yield of 34% (11% overall, nine steps), due to troublesome purification.

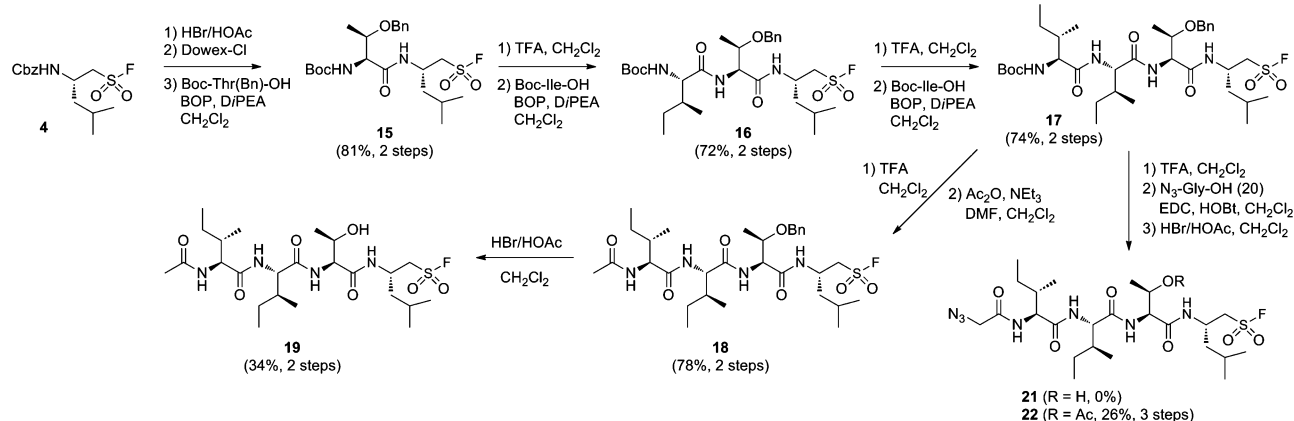
Although it was possible to synthesize a peptido sulfonyl fluoride stepwise from the “S”-terminus to the N-terminus in solution (Schemes 1 and 2), preventing destruction of the sulfonyl fluoride electrophile, an attractive alternative was first (solid phase) synthesis of the N-terminal peptide and then introduction of the amino acid derived sulfonyl fluoride as the last coupling step (vide infra). For example, solution-phase synthesis of azido-IITL peptido sulfonyl fluoride **21** was unsuccessful due to troublesome deprotection of the threonine side chain,⁸ so it was decided to first prepare tripeptide **27** by solid-phase peptide synthesis, followed by coupling to sulfonyl fluoride **28** (Scheme 3).

Peptide **27** with a free carboxylic acid terminus was obtained in three coupling steps starting from tritylresin **24** and used as such for coupling to peptido sulfonyl fluoride **28** using BOP in the presence of HOBt and *N*-methylmorpholine. After deprotection and purification by preparative HPLC, azido-IITL peptido sulfonyl fluoride **21** was obtained in 6% overall yield (10 steps, average yield per step: 76%).

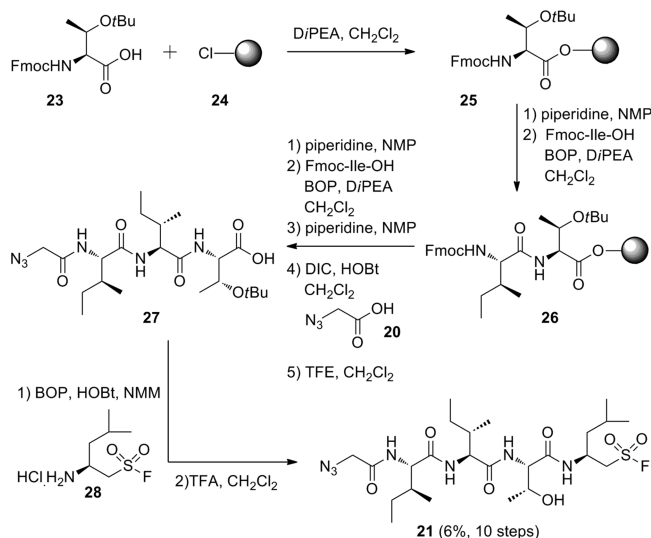
Nine additional peptido sulfonyl fluorides for biological evaluation (Figure 2) were obtained by (selective) cleavage of protecting groups from all thus far prepared peptido sulfonyl fluorides (Scheme 4).

pFL and Cbz-Leu₃ peptido sulfonyl fluorides **5**, **8**, **9**, and **11** were deprotected to give **29**, **30**, **31**, and **32**. IITL peptido sulfonyl fluorides **15**, **16**, and **17** were either completely deprotected to give **36** and **37** or only the N-protecting group was removed to give **33**, **34**, and **35**.

Scheme 2. Synthesis of IITL Sulfonyl Fluorides



Scheme 3. Solid-Phase-Assisted Synthesis of N-Terminal Azido IITL Sulfonyl Fluoride 21



Apart from characterization of the sulfonyl fluorides by ¹H, ¹³C, and 2D NMR spectroscopy and mass spectroscopy, the presence of the fluorine in the inhibitors was unambiguously demonstrated by the ¹⁹F NMR and was apparent from the ¹³C–F coupling in the ¹³C NMR spectra, visible in most spectra.

The inhibitory activity of the peptido sulfonyl fluorides was evaluated by determination of the residual proteasome activity in a competitive labeling reaction with a fluorescent proteasome probe.¹⁰ Decreased fluorescence labeling evident from less intensive proteasome subunit bands meant less residual proteasome activity and therefore more active peptido sulfonyl fluoride inhibitors.

After testing the 24 peptido sulfonyl fluorides, nine (9, 10, 11, 12, 14, 18, 19, 21, and 22) were capable of practically complete abolishment of labeling at 1 μM, indicating almost full inhibition for one or more subunits (Figures 3 and 4; for gel images of all tested sulfonyl fluorides with molecular weight markers see Supporting Information).

Eight peptido sulfonyl fluorides (16, 17, 31, 32, 34, 35, 36, and 37) showed weak to moderate proteasome inhibition. In general, no inhibition was found with the shorter dipeptide sulfonyl fluorides. Also peptido sulfonyl fluorides containing a deprotected N-terminal amine were clearly inferior to their

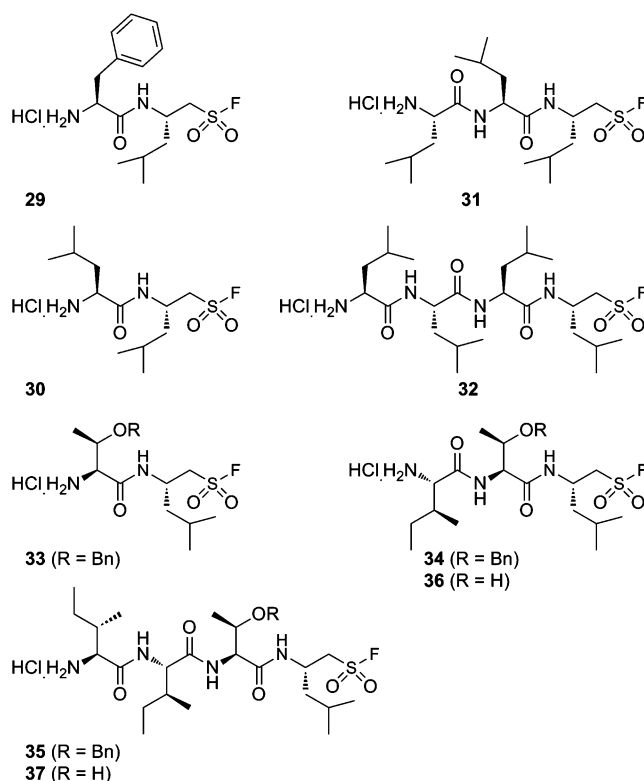
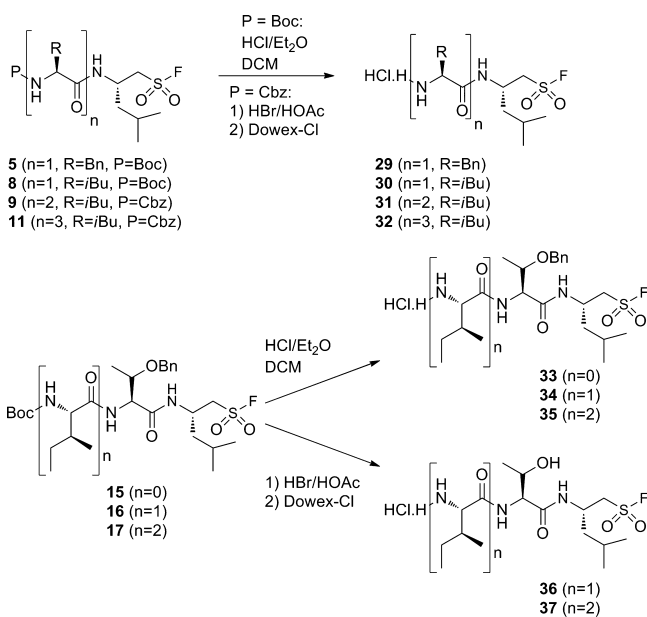


Figure 2. Structures of peptido sulfonyl fluorides 29–37.

protected analogs. No inhibition was found with the pFL peptido sulfonyl fluoride 7; this was also observed earlier with a bortezomib-inspired epoxy ketone.⁷ The best inhibitors were then evaluated in this assay, now using a smaller range of lower inhibitor concentrations varying from 0 to 10 μM (see Supporting Information).

The most potent inhibitor from the set of IITL peptido sulfonyl fluorides was 19. It shows inhibitory potency comparable to epoxomicin and some selectivity for the β5 subunit. Interestingly, peptido sulfonyl fluoride 18, containing a benzyl group protected threonine side chain, showed inhibition comparable to that of 19. Remarkably, the β5 selectivity disappeared in the N-terminal deprotected IITL peptido sulfonyl fluorides 35 and 37. Azido IITL peptido sulfonyl fluoride 21 was almost as active as 19, but more selective for the β5 subunit. The most potent peptido sulfonyl fluoride inhibitors were found within the set of Leu₃ peptido sulfonyl

Scheme 4. Synthesis of N-Terminally and Threonine Side Chain Deprotected Sulfonyl Fluorides



fluorides. Cbz-Leu₃-SF (**9**) was clearly a better inhibitor than its vinyl sulfone counterpart (Cbz-Leu₃-VS)⁹ and more selective for the β 1 and β 5 subunits. Its Boc-protected analog **10** was still potent, but slightly less. Elongation of the backbone by an additional leucine (Cbz-Leu₄-SF, **11**) boosted its potency and even its selectivity for the β 5 subunit. Replacement of the Cbz group by an acetyl group, leading to Ac-Leu₄-SF (**12**), reduced both its potency and selectivity. Azido peptido sulfonyl fluoride **14** was significantly more potent than Cbz-Leu₃-SF (**9**) and was clearly less selective for the β 1 and β 5 subunits.

For a better understanding of the inhibitory potencies, IC₅₀ values were determined for the best peptido sulfonyl fluoride fluorides (Table 1, Figure 5), using a previously described assay in which the separate enzyme activities (T-, CT-, or C-like) were determined as previously described by monitoring the hydrolysis of fluorogenic substrates selective for these in the presence of purified yeast proteasome (*S. cerevisiae*).¹¹ The inhibitory potency and subunit selectivity trends found with the competition assay above agreed in most cases with this fluorogenic substrate assay. The obtained IC₅₀ values varied between 7 nM and 1.7 μ M. As expected, **11** (Cbz-Leu₄-SF) was the most potent inhibitor with an IC₅₀ of 7 nM and high selectivity for the β 5 subunit. Its azide analog (**14**) was even more β 5 subunit selective but clearly less potent (IC₅₀ = 110 nM). IITL peptido sulfonyl fluoride **19** showed potent, panreactive potency in the fluorogenic assay (IC₅₀ = 250 nM). Azido IITL peptido sulfonyl fluoride **21** was more potent (IC₅₀ = 40 nM) and more β 5 subunit selective than was found in the competition assay. **37** was a panreactive inhibitor in the competition assay with reasonable potency, while in the fluorogenic substrate assay it was very selective for the β 5 subunit and much more potent. For comparison with the well-known potent proteasome inhibitor epoxomicin, its IC₅₀ value (261 nM) was determined using the same assay (Table 1, Figure 5). Three inhibitors (**11**, **14**, and **21**) clearly had lower IC₅₀ values than epoxomicin, proving the potency of these new peptido sulfonyl fluorides.

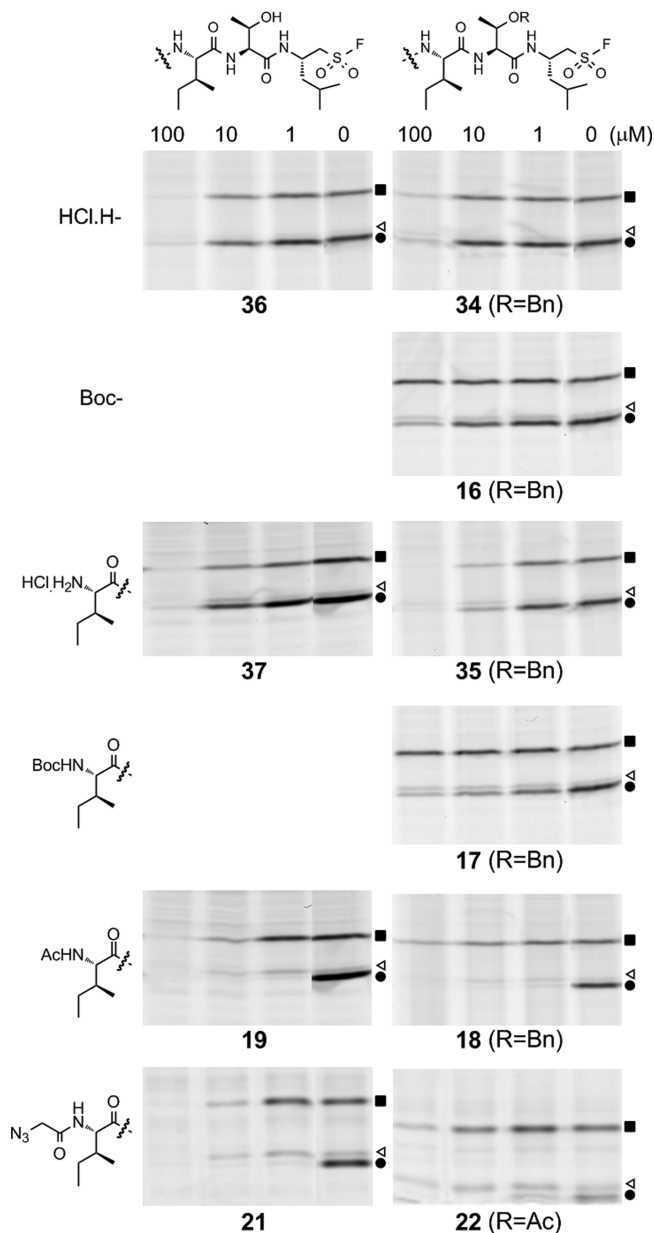


Figure 3. Competition assays in HEK lysates with IITL sulfonyl fluoride inhibitors. Lysates were incubated for 1 h at 0, 1, 10, and 100 μ M final concentration of inhibitor. Residual active proteasome was labeled with 0.1 μ M MV151 for 1 h. Upper band β 2 (solid box), middle band β 1 (open triangle), lower band β 5 (solid circle).

Three of most potent peptido sulfonyl fluorides (**11**, **14**, and **21**) were selected for evaluation of inhibition of the proteasome in living cells. To this end, HEK cells were treated for 1 h with the inhibitor, after which remaining active proteasome subunits were labeled with a bodipy label (MV151) for visualization on SDS-PAGE gels (Figure 6).¹⁰

Gratifyingly, our peptido sulfonyl fluorides were capable of penetrating into the cells, showing inhibition results comparable to the fluorogenic and competition assays. **11** inhibited the proteasome protease activity of the β 5 subunit almost completely at 5 μ M, while the β 1 subunit was only partly inhibited at 50 μ M. Slight inhibition of the β 2 subunit was observed at 50 μ M. **14** and **21** were capable of inhibition of all three different proteasome subunits, but were less active than **11**.

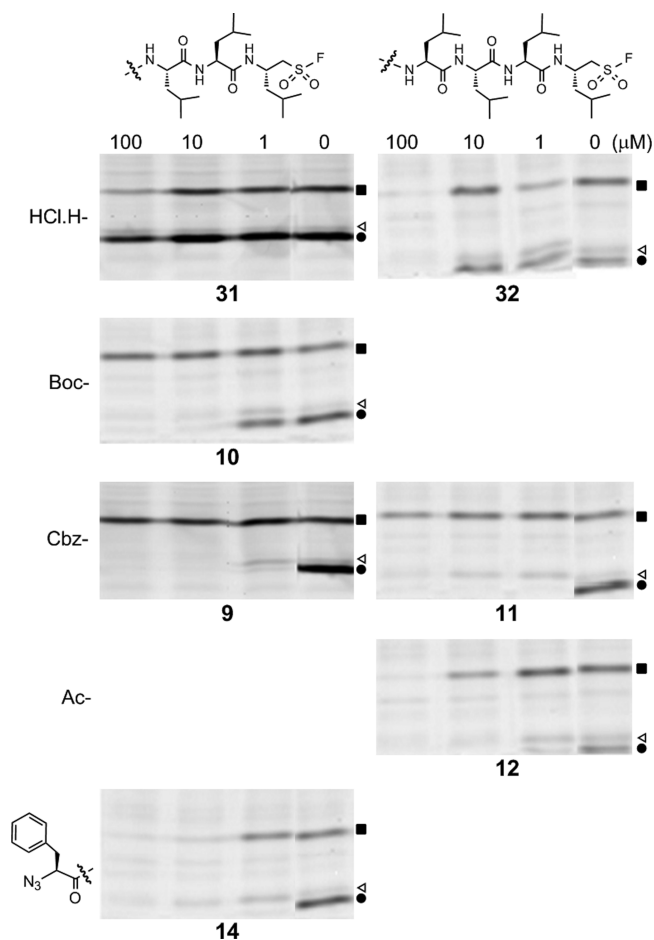


Figure 4. Competition assays in HEK lysates with (Leu)₃- and (Leu)₄-inspired sulfonyl fluoride inhibitors. Lysates were incubated for 1 h at 0, 1, 10, and 100 μM final concentration of inhibitor. Residual active proteasome was labeled with 0.1 μM MV151 for 1 h. Upper band β₂ (solid box), middle band β₁ (open triangle), lower band β₅ (solid circle).

Table 1. IC₅₀ Values for Sulfonyl Fluorides 9, 10, 11, 12, 14, 18, 19, 21, 22, 37, and Epoxomicin (3)

compound	IC ₅₀ (nM) ^a
epoxomicin (3)	261 ± 37
9	350 ± 70
10	1750 ± 48
11	7 ± 2
12	300 ± 40
14	110 ± 30
18	1150 ± 400
19	250 ± 70
21	40 ± 10
22	1570 ± 340
37	800 ± 200

^aCT-like active site IC₅₀ against yeast 20S proteasome

So far, the exact molecular mechanism of inhibition of the proteasome by these peptido sulfonyl fluorides is not completely understood. Analogous to the earlier described inhibition of chymotrypsin, at least the first step of inhibition of the proteasome may involve formation of a covalent bond.⁵ However, the special architecture of the proteasome with an N-terminal threonine residue in the active site may lead to

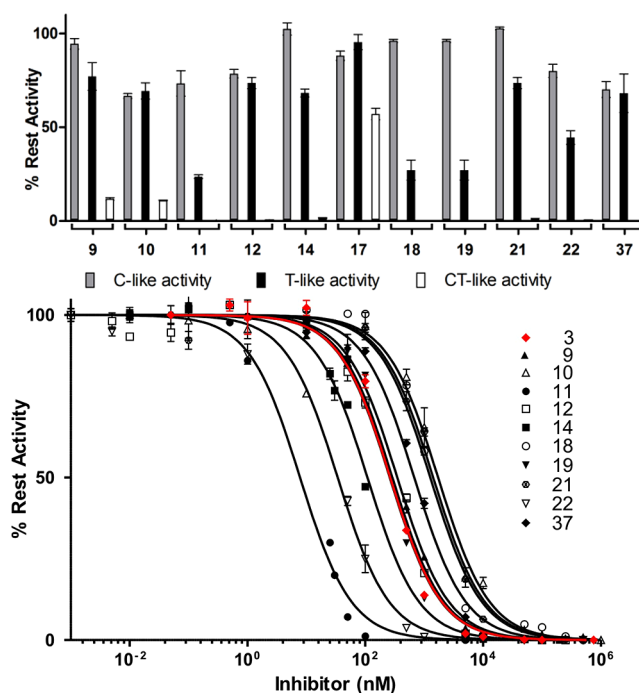


Figure 5. In vitro evaluation of compounds (top) using fluorogenic substrates selective for each of the proteasome activities. IC₅₀ values of CT-like activity of the proteasome (bottom). Assays were performed using yeast 20S proteasome (*Saccharomyces cerevisiae*).¹¹

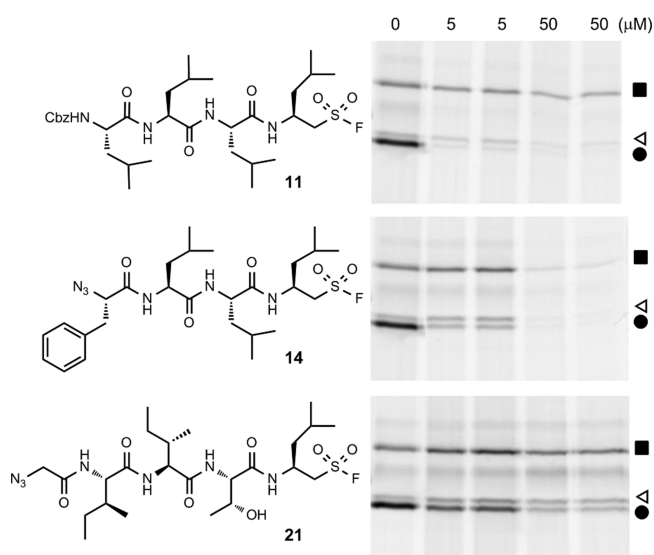


Figure 6. SDS-PAGE gels of lysed HEK cells treated with peptido sulfonyl fluorides 11, 14, and 21. Upper band β₂ (solid box), middle band β₁ (open triangle), lower band β₅ (solid circle).

subsequent reactions and even (partial) release of the inhibitor. Present investigations are underway to unravel the molecular events.

3. CONCLUSIONS

In conclusion, we have developed new potent proteasome inhibitors using the novel amino acid based sulfonyl fluoride electrophilic trap. Although the resulting peptido sulfonyl fluorides seemed to induce more β₅ subunit selectivity than other proteasome inhibitors, potent panreactive inhibitors were also found. Preparation of peptido sulfonyl fluorides containing

sequences of the known peptide-based proteasome inhibitors, that is, bortezomib, epoxomicin, and Cbz-Leu₃-aldehyde, afforded new inhibitors with comparable potencies as epoxomicin (Table 1). The highest potencies were observed with peptido sulfonyl fluorides containing new amino acid sequences of which Cbz-Leu₄-SF (**11**) was the best one with an IC₅₀ value of 7 nM, which was significantly lower than the IC₅₀ for epoxomicin (261 nM). On the basis of these promising results, we are convinced that amino acid based sulfonyl fluoride moieties as part of peptido sulfonyl fluorides will be widely used in the near future for targeting the proteasome but also other serine/threonine protease activities.

4. EXPERIMENTAL SECTION

Chemistry. Fmoc-protected amino acids and BOP were purchased from GL Biochem (Shanghai, China). Boc-protected amino acids were purchased from Fluka. Chemicals were purchased from Aldrich. Peptide grade solvents for synthesis were purchased from Biosolve. The Dowex ion-exchange resin (1 × 8, 200–400 mesh, Cl⁻) was purchased from Acros. Reactions were carried out at ambient temperature unless stated otherwise. TLC analysis was performed on Merck precoated silica gel 60 F-254 (0.25 mm) plates. Spots were visualized with UV light, ninhydrin, or Cl₂-TDM.¹² Solvents were evaporated under reduced pressure at 40 °C. Column chromatography was performed on Siliacflash P60 (40–63 μm) from Silicycle. For solid-phase synthesis, the Kaisertest was used for visualization of unprotected amines.¹³ Purities were determined using analytical HPLC, confirming ≥95% purity for all compounds except **34** and **37**. Analytical HPLC was performed on a Shimadzu HPLC using a C8 Alltima column at a flow rate of 1.0 mL/min. Buffers used: buffer A (0.1% TFA in CH₃CN/H₂O, 5/95, v/v) and buffer B ((0.1% TFA in CH₃CN/H₂O, 95/5, v/v). Runs started with an isocratic flow of buffer A (100%, 5 min), followed by a linear gradient of buffer B (100% in 20 min). Subsequently, an isocratic flow of buffer B (100%, 5 min) was performed followed by a linear gradient to buffer A (100%, 5 min). The run ended with an isocratic flow of buffer A (100%, 5 min). Lyophilization was performed on a Christ Alpha 1–2a apparatus. Electrospray mass spectra were recorded on a Shimadzu LCMS-QP-8000 spectrometer. Infrared spectra were recorded on a Bio-Rad FTS 6000 FTIR spectrometer. Melting points were measured on a Büchi melting point apparatus (according to the method of Dr. Tottoli) and are uncorrected. Elemental analyses were carried out at Kolbe Mikroanalytisches Laboratorium (Mülheim an der Ruhr, Germany). High-resolution mass spectrometry (HRMS) spectra were measured on an ESI-MS instrument. ¹H NMR (300 MHz), ¹³C NMR (75 MHz), COSY, and ¹⁹F NMR (282 MHz) spectra were recorded on a Varian M-300 spectrometer, and ¹H NMR (500 MHz), TOCSY, and HSQC spectra were recorded on a Varian Inova 500 spectrometer. Chemical shifts are reported in ppm relative to TMS (0 ppm), DMSO (2.5 ppm), or HDO (4.79 ppm) for ¹H NMR; to CDCl₃ (77 ppm) or DMSO (39.52 ppm) for ¹³C NMR; and to α,α,α-trifluorotoluene (–63.72 ppm) for ¹⁹F NMR as internal standards. ¹³C NMR spectra were recorded using the attached proton test (apt) pulse sequence. ¹³C NMR data for compounds **7**, **12**, **18**, **19**, **21**, **22**, **29**, and **31–37** were taken from the HSQC spectra. In the NMR assignments amino acids are numbered starting from the N-terminus, i.e., Ile¹ denotes an N-terminal isoleucine. During solid-phase peptide synthesis, washing of the resin was done by bubbling nitrogen gas for the stated amount of time. UV–vis measurements were done on an instrument from Thermo UV1 Electron Corp.

General Procedure of Boc-Deprotection Using TFA/CH₂Cl₂. The Boc-protected amino acid derived sulfonyl fluoride (1.00 mmol) was added to a 1:1 mixture of CH₂Cl₂ (15 mL) and TFA (15 mL). After stirring for 15 min at room temperature (rt) the solvents were evaporated. Coevaporation with chloroform (3×) yielded the crude TFA salt, which was directly used in the coupling reaction.

General Procedure for Benzyl- and/or Cbz-Deprotection Using HBr in Acetic Acid and Conversion to the HCl Salt. The Cbz-

protected compound (40 μmol) was dissolved in a 1:1 mixture of CH₂Cl₂ (1 mL) and HBr in acetic acid (33%, 1 mL). After stirring for 30 min at rt, the solvents were evaporated. The residue was dissolved in H₂O (1 mL), and Dowex-Cl (2 × 8, 24 mg) was added. The solution was stirred for 5 min at rt, followed by filtration. The water was removed by concentration in vacuo or by lyophilization, yielding the dry solid product.

General Procedure of Boc-Deprotection Using HCl/Diethyl Ether. The Boc-protected compound (40 μmol) was dissolved in a 1:1 mixture of CH₂Cl₂ (1 mL) and a saturated HCl in diethyl ether solution (directly used from –20 °C, 1 mL). After stirring for 30 min at rt, the product was concentrated in vacuo. The residue was dissolved in H₂O and lyophilized, yielding the Boc-deprotected product.

General Procedure Coupling Using BOP and DiPEA. To the crude HCl or TFA salt (1.00 mmol) were subsequently added BOP (1.05 mmol), Boc- or Cbz-protected amino acid (1.00 mmol), CH₂Cl₂ (40 mL), and DiPEA (2.21 mmol). The mixture was stirred for 2 h at rt. During the reaction, the pH was monitored (pH indicator paper) and adjusted to 8 with additional DiPEA, if necessary. After evaporation of the solvent, the residue was dissolved in EtOAc (100 mL) and was washed with 1.0 M KHSO₄ (3 × 50 mL) and brine (50 mL). Purification was performed using column chromatography.

Cbz-Leu¹-Leu²-Leu³-ψ[CH₂SO₂]-F (9**).** Compound **8**^{5b} (126 mg, 0.32 mmol) was Cbz-deprotected and coupled to Cbz-protected leucine (180 mg, 0.34 mmol). Column chromatography was performed using an eluent gradient of acetone:CH₂Cl₂ (2:98) followed by acetone:CH₂Cl₂ (5:95). Evaporation of the solvent yielded **9** as a white solid (146 mg, 84%). HPLC retention time: 25.2 min. *R*_f = 0.35 (acetone:CH₂Cl₂, 4:96). ¹H NMR (300 MHz, CDCl₃): δ = 0.88–0.95 [m, 18H, 3 × CH(CH₃)₂], 1.42–1.84 [m, 9H, 3 × CH(CH₃)₂, 3 × NCHCH₂], 3.49–3.57 (2t, *J*_{gem} = 14.8 Hz, *J*_{vic} = 4.8 Hz, ³*J*_{H-F} = 4.8 Hz, 1H, CH^aSO₂F), 3.83–3.90 (m, 1H, CH^bSO₂F), 4.08–4.15 [m, 1H, NCH (Leu¹)], 4.32–4.40 [m, 2H, 2 × NCH (Leu^{2,3})], 5.08–5.17 (m, 3H, CH₂C₆H₅, NHBoc), 6.34, 6.87 [2d, *J* = 7.7 Hz, *J* = 6.3 Hz, 2H, 2 × NH (Leu^{2,3})], 7.26–7.41 (m, 5H, Ar-CH). ¹⁹F NMR (282 MHz, DMSO): δ = –120.0 (s). ESI MS: *m/z* = 410.15 [M – Cbz + H]⁺, 819.03 [2M – Cbz + H]⁺. HRMS: calcd for C₂₆H₄₃FN₃O₆S [M + H] 544.2856, found 544.2831.

Boc-Leu-Leu-Leu-ψ[CH₂SO₂]-F (10**).** The coupling reaction was carried out starting from Boc-Leu-OH (342 mg, 1.48 mmol) and sulfonyl fluoride **8** (587 mg, 1.48 mmol), which was first Boc-deprotected. The product was purified using silica gel column chromatography (acetone:CH₂Cl₂, 2:98). Concentration in vacuo afforded **10** as a white solid (266 mg, 0.52 mmol, 35%). HPLC retention time: 25.1 min. ¹H NMR (300 MHz, CDCl₃): δ = 0.93 [m, 18H, 6 × CH₃ (Leu)], 1.45 [s, 9H, 3 × C(CH₃)₃], 1.42–1.84 [m, 9H, 3 × CHCH₂(CH₃)₂, 3 × CHCH₂(CH₃)₂], 3.49–3.59 (m, 1H, CH^aSO₂F), 3.78–3.90 (m, 1 H CH^bSO₂F), 4.00–4.05 [m, 1H, BocNHCH (Leu¹)], 4.26–4.39 [m, 2H, NHCH (Leu^{2,3})], 4.82 (d, *J* = 6.6 Hz, 1H, NHBoc), 6.45 [d, *J* = 7.4 Hz, 1H, NH (Leu³)], 6.84 [d, *J* = 6.9 Hz, 1H, NH (Leu²)]. ¹⁹F NMR (282 MHz, DMSO): δ = –116.4. HRMS: calcd for C₂₃H₄₄FN₄O₆S [M + H] 510.3013, found 510.3016.

Cbz-Leu-Leu-Leu-ψ[CH₂SO₂]-F (11**).** The deprotection and coupling reactions were performed starting from peptido sulfonyl fluoride **10** (188 mg, 0.37 mmol) and Cbz-Leu-OH (98 mg, 0.37 mmol). The product was purified using silica gel column chromatography (4% acetone:CH₂Cl₂) twice to afford the product as a white solid (132 mg, 0.20 mmol, 54%). HPLC retention time: 26.3 min. ¹H NMR (500 MHz, acetone-*d*₆): δ = 0.80 (m, 24H, 8 × CH₃), 1.29–1.78 [m, 12H, 4 × CH₂CH(CH₃)₂, 4 × CH₂CH(CH₃)₂], 3.89 (m, 2H, CH₂SO₂F), 4.13 [m, 1H, NHCH (Leu¹)], 4.33 [m, 2H, 2 × NHCH (Leu^{2,3})], 4.51 [m, 1H, NHCH (Leu⁴)], 5.10 [m, 2H, CH₂ (Cbz)], 6.81 [m, 1H, NHCH (Leu¹)], 7.28 [m, 1H, NHCH (Leu⁴)], 7.37, 7.73 [2m, 2H, NHCH (Leu^{2,3})], 7.43 (m, 5H, ArCH). ¹⁹F NMR (282 MHz, DMSO): δ = –118.9. HRMS: calcd for C₃₂H₅₄FN₄O₇S [M + H] 657.3697, found 657.3693.

Ac-Leu-Leu-Leu-ψ[CH₂SO₂]-F (12**).** Peptido sulfonyl fluoride **12** (19.8 mg, 35 μmol) was dissolved in DMF (30 mL), and acetic anhydride (5 μL, 53 μmol) and NEt₃ (10 μL, 71 μmol) were added.

The resulting mixture was stirred overnight at rt after which the solvents were evaporated, and the residue was taken up in EtOAc (100 mL). The organic layer was washed with KHSO_4 (1 M, 100 mL, 3 \times) and brine (100 mL). The organic layer was dried over Na_2SO_4 , and the solvents were evaporated. The product was purified using solid-phase extraction, using a C8 reversed phase column [eluting first with 1 column volume buffer A (0.1% TFA in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 5/95, v/v), followed by elution using buffer B (0.1% TFA in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 95/5, v/v)] (14.3 mg, 25 μmol , 72%). ^1H NMR (300 MHz, DMSO): δ = 0.86 [m, 24H, 8 \times CH_3 (4 \times Leu)], 1.25–1.62 [m, 12H, 4 \times $\text{CH}_2\text{CH}(\text{CH}_3)_2$, 4 \times $\text{CH}_2\text{CH}(\text{CH}_3)_2$], 1.85 [s, 3H, $\text{CH}_3\text{C}(\text{O})$], 3.88 (m, 1H, $\text{CH}^{\text{a}}\text{SO}_2\text{F}$), 4.11 (m, 1H, $\text{CH}^{\text{b}}\text{SO}_2\text{F}$), 4.30 (m, 4H, 4 \times NHCH), 7.94 (m, 4H, 4 \times NHCH). ^{19}F NMR (282 MHz, DMSO): δ = -120.1. ESI MS: m/z = 565.55 [M + H] $^+$; 587.40 [M + Na] $^+$; 603.15 [M + K] $^+$. HRMS: calcd for $\text{C}_{26}\text{H}_{30}\text{FN}_4\text{O}_6\text{S}$ [M + H] 565.3435, found 565.3440.

***N*₃Phe-Leu-Leu-Leu- ψ [CH_2SO_2]-F (14).** The deprotection and coupling reactions were performed starting from peptido sulfonyl fluoride **10** (48.1 mg, 82 μmol) and azido phenylalanine (**13**)¹⁴ (15.6 mg, 82 μmol). The purification by silica gel column chromatography was done with EtOAc:hexanes (1:1) as eluent. The product was obtained as a white solid (28.3 mg, 48 μmol , 59%). HPLC retention time: 25.7 min. ^1H NMR (500 MHz, CDCl_3): δ = 0.86 [m, 18H, 6 \times CH_3 (3 \times Leu)], 1.26–1.80 [m, 9H, 3 \times $\text{CH}_2\text{CH}(\text{CH}_3)_2$, 3 \times $\text{CH}_2\text{CH}(\text{CH}_3)_2$], 3.06 (dd, J_{gem} = 14.2 Hz, J_{vic} = 8.0 Hz, 1H, $\text{CH}^{\text{a}}\text{C}_6\text{H}_5$), 3.26 (dd, J_{gem} = 14.0 Hz, J_{vic} = 3.9 Hz, 1H, $\text{CH}^{\text{b}}\text{C}_6\text{H}_5$), 3.58 (m, 1 H $\text{CH}^{\text{a}}\text{SO}_2\text{F}$), 3.77 (m, 1 H $\text{CH}^{\text{b}}\text{SO}_2\text{F}$), 4.26 [m, 1H, NHCH (Leu¹)], 4.34 (br s, CHN_3), 4.46 [m, 2H, 2 \times NHCH (Leu^{1,2})], 6.95 (m, 2H, 2 \times NH (Leu^{2,3}), 7.11 [d, J = 8.0 Hz, 1H, NH (Leu¹)], 7.28 (m, 5H, Ar-CH). ^{19}F NMR (282 MHz, DMSO): δ = -115.5. IR (KBr): 2108 cm^{-1} (N_3). HRMS: calcd for $\text{C}_{27}\text{H}_{44}\text{FN}_6\text{O}_5\text{S}$ [M + H] 583.3078, found 583.3079.

***Boc*-Thr(Bn)-Leu- ψ [CH_2SO_2]-F (15).** The coupling reaction was performed using HCl salt **33** (330 mg, 1.5 mmol) and Boc-Thr(Bn)-OH (464 mg, 1.5 mmol). The product was purified by precipitation and crystallization instead of column chromatography. After extraction and concentration in vacuo, the crude product was dissolved in CH_2Cl_2 , which was followed by addition of hexanes. After filtration, the residue was crystallized from CH_2Cl_2 (50 mL) and hexanes (50 mL), which afforded **15** as a white solid (573 mg, 81%, two steps). HPLC retention time: 25.7 min. R_f = 0.52 (acetone: CH_2Cl_2 , 4:96). Mp = 158 $^\circ\text{C}$. ^1H NMR (300 MHz, CDCl_3): δ = 0.86 [2d, J = 3.03 Hz, 6H, $\text{CH}(\text{CH}_3)_2$], 1.21 [d, J = 6.05 Hz, 3H, CH_3 (Thr)], 1.46 [s, 9H, $(\text{CH}_3)_3$], 1.48–1.79 [m, 3H, $\text{CH}_2\text{CH}(\text{CH}_3)_2$], 3.55–3.71 (m, 2H, $\text{CH}_2\text{SO}_2\text{F}$), 4.21 (m, 2H, NCHCHCH₃), 4.46–4.63 [m, 3H, NCH (Leu), $\text{CH}_2\text{C}_6\text{H}_5$], 5.37 (br s, 1H, NHBoc), 6.87 [br s, 1H, NH (Leu)], 7.26–7.34 (m, 5H, Ar-CH). ^{19}F NMR (282 MHz, CDCl_3): δ = -114.8 (s). ESI MS: m/z = 375.15 [M - Boc + H] $^+$, 748.95 [2M - Boc + H] $^+$. HRMS: calcd for $\text{C}_{22}\text{H}_{36}\text{FN}_2\text{O}_6\text{S}$ [M + H] 475.2278, found 475.2277.

***Boc*-Ile-Thr(Bn)-Leu- ψ [CH_2SO_2]-F (16).** The coupling reaction was carried out starting from Boc-Ile-OH- $^{1/2}\text{H}_2\text{O}$ (233 mg, 0.97 mmol) and sulfonyl fluoride **15** (462 mg, 0.97 mmol), which was first Boc-deprotected. The resulting product was purified by column chromatography using an eluent gradient in three steps, first CH_2Cl_2 , second acetone: CH_2Cl_2 (1:99), and last acetone: CH_2Cl_2 (50:50). Concentration in vacuo afforded **16** as a white solid (409 mg, 72%). HPLC retention time: 26.5 min. R_f = 0.61 (acetone: CH_2Cl_2 , 10:90). ^1H NMR (300 MHz, CDCl_3): δ = 0.85–1.00 [m, 13H, $\text{CH}(\text{CH}_3)_2$, 2 \times CH_3 (Ile), $\text{CH}_3\text{CH}^{\text{a}}\text{CH}$ (Ile)], 1.10–1.29 [m, 4H, $\text{CH}_3\text{CH}^{\text{b}}\text{CH}$ (Ile), CH_3 (Thr)], 1.36 [s, 9H, $(\text{CH}_3)_3$], 1.43–1.74 [m, 3H, $\text{CH}_2\text{CH}(\text{CH}_3)_2$], 1.92–2.17 [m, 1H, NCHCH (Ile)], 3.48–3.57 (ddd, J_{gem} = 14.9 Hz, J_{vic} = 6.2 Hz, $^3J_{\text{HF}}^{\text{a}}$ = 5.5 Hz, 1H, $\text{CH}^{\text{a}}\text{SO}_2\text{F}$), 3.70–3.78 (ddd, J_{gem} = 14.9 Hz, J_{vic} = 5.5 Hz, $^3J_{\text{HF}}^{\text{b}}$ = 2.6 Hz, 1H, $\text{CH}^{\text{b}}\text{SO}_2\text{F}$), 3.92–3.96 [t, J = 5.0 Hz, 1H, NCH (Ile)], 4.32 [m, 1H, NCHCH (Thr)], 4.40–4.87 [m, 4H, NCH (Thr), NCH (Leu), $\text{CH}_2\text{C}_6\text{H}_5$], 4.86 (d, J = 5.0 Hz, 1H, NHBoc), 6.86, 7.20 [2d, J = 7.7 Hz, J = 7.2 Hz, 2H, NH (Leu), NH (Thr)], 7.23–7.36 (m, 5H, Ar-CH). ^{19}F NMR (282 MHz, CDCl_3): δ = -117.5 (s). ESI MS: m/z =

488.24 [M - Boc + H] $^+$, 975.10 [2M - Boc + H] $^+$. HRMS: calcd for $\text{C}_{28}\text{H}_{47}\text{FN}_3\text{O}_7\text{S}$ [M + H] 588.3119, found 588.3130.

***Boc*-Ile¹-Ile²-Thr(Bn)-Leu- ψ [CH_2SO_2]-F (17).** The coupling reaction was carried out starting from Boc-Ile-OH- $^{1/2}\text{H}_2\text{O}$ (130 mg, 0.54 mmol) and sulfonyl fluoride **16** (319 mg, 0.54 mmol), which was first Boc-deprotected. The resulting product was purified by column chromatography (acetone: CH_2Cl_2 , 6:94). Due to the low yield obtained, column chromatography was performed again on the impure fractions. Now an eluent gradient was used, acetone: CH_2Cl_2 (4:96) followed by acetone: CH_2Cl_2 (6:94). The combined pure fractions were concentrated in vacuo to obtain **17** as a white solid (279 mg, 74%). HPLC retention time: 27.1 min. R_f = 0.56 (acetone: CH_2Cl_2 , 10:90). ^1H NMR (300 MHz, CDCl_3): δ = 0.82–1.00 [m, 20H, $\text{CH}(\text{CH}_3)_2$, 4 \times CH_3 (Ile^{1,2}), 2 \times $\text{CH}_3\text{CH}^{\text{a}}\text{CH}$ (Ile^{1,2})], 1.14–1.26 [m, 5H, 2 \times $\text{CH}_3\text{CH}^{\text{b}}\text{CH}$ (Ile^{1,2}), CH_3 (Thr)], 1.42 [s, 9H, $(\text{CH}_3)_3$], 1.46–1.77 [m, 3H, $\text{CH}_2\text{CH}(\text{CH}_3)_2$], 1.90 [m, 1H, NCHCH (Ile¹)], 2.05 [m, 1H, NCHCH (Ile²)], 3.46–3.55 (ddd, J_{gem} = 14.9 Hz, J_{vic} = 6.2 Hz, $^3J_{\text{HF}}^{\text{a}}$ = 5.6 Hz, 1H, $\text{CH}^{\text{a}}\text{SO}_2\text{F}$), 3.71–3.79 (ddd, J_{gem} = 14.9 Hz, J_{vic} = 5.4 Hz, $^3J_{\text{HF}}^{\text{b}}$ = 2.8 Hz, 1H, $\text{CH}^{\text{b}}\text{SO}_2\text{F}$), 3.78 [m, 1H, NCH (Ile¹)], 4.15–4.19 [m, 1H, NCH (Ile²)], 4.33 [m, 1H, NCHCH (Thr)], 4.42–4.58 [m, 4H, NCH (Thr), NCH (Leu), $\text{CH}_2\text{C}_6\text{H}_5$], 4.80 (d, J = 5.8 Hz, 1H, NHBoc), 6.64 [s, 1H, NH (Ile²)], 6.86 [d, J = 8.0 Hz, 1H, NH (Thr)], 7.14 [d, J = 7.4 Hz, 1H, NH (Leu)], 7.25–7.30 (m, 5H, Ar-CH). ^{19}F NMR (282 MHz, CDCl_3): δ = -117.5 (s). ESI MS: m/z = 601.38 [M - Boc + H] $^+$, 623.44 [M - Boc + Na] $^+$, 1201.19 [2M - Boc + H] $^+$, 1223.10 [2M - Boc + Na] $^+$. HRMS: calcd for $\text{C}_{34}\text{H}_{58}\text{FN}_4\text{O}_8\text{S}$ [M + H] 701.3959, found 701.3969.

***Ac*-Ile¹-Ile²-Thr(Bn)-Leu- ψ [CH_2SO_2]-F (18).** To a stirring suspension of the TFA salt of compound **17** (174 mg, 0.25 mmol) in CH_2Cl_2 (2.5 mL) was added a solution of acetic anhydride in CH_2Cl_2 (10% v/v, 260 μL , 0.275 mmol) under N_2 atmosphere. After cooling to 0 $^\circ\text{C}$, triethylamine (104 μL , 0.75 mmol) was added dropwise. In order to improve the solubility, DMF (60 mL) was added. Additional acetic anhydride (52 μL , 0.55 mmol) and triethylamine (104 μL , 0.75 mmol) were added. The solution was stirred for 18 h at rt under N_2 atmosphere. The solvent was partially removed by evaporation. The remaining solution (~5 mL) was added to diethyl ether (~40 mL), which led to precipitation of the product. After centrifugation (10 min, 5000 rpm), the pellet was washed with diethyl ether (3 \times) and air-dried to yield **18** as a white solid (125 mg, 78%). HPLC retention time: 24.4 min. ^1H NMR (500 MHz, DMSO): δ = 0.75–0.82 (m, 18H, $\text{CH}(\text{CH}_3)_2$, 4 \times CH_3 (Ile^{1,2}), 1.02–1.10 [m, 5H, 2 \times $\text{CH}_3\text{CH}^{\text{a}}\text{CH}$ (Ile^{1,2}), CH_3 (Thr)], 1.30–1.35 [m, 1H, NCHCH^a (Leu)], 1.38–1.47 [m, 2H, 2 \times $\text{CH}_3\text{CH}^{\text{b}}\text{CH}$ (Ile^{1,2})], 1.48–1.54 [m, 1H, NCHCH^b (Leu)], 1.58–1.61 [m, 1H, $\text{CH}_2\text{CH}(\text{CH}_3)_2$], 1.65–1.76 [m, 2H, 2 \times NCHCH (Ile^{1,2})], 1.84 (s, 3H, $\text{CH}_3\text{C}=\text{O}$), 3.78–3.83 (m, 1H, $\text{CH}^{\text{a}}\text{SO}_2\text{F}$), 3.93 [m, 1H, NCHCH (Thr)], 4.08–4.13 (ddd, J_{gem} = 15.0 Hz, J_{vic} = 7.3 Hz, $^3J_{\text{HF}}^{\text{a}}$ = 4.3 Hz, 1H, $\text{CH}^{\text{a}}\text{SO}_2\text{F}$), 4.20–4.29 [2t, J = 8.2 Hz, 2H, 2 \times NCH (Ile^{1,2})], 4.37–4.52 [m, 4H, NCH (Thr), NCH (Leu), $\text{CH}_2\text{C}_6\text{H}_5$], 7.25–7.33 (m, 5H, Ar-CH), 7.79 [d, J = 9.2 Hz, 1H, NH (Thr)], 7.89–7.96 [m, 3H, NH (Leu), 2 \times NH (Ile^{1,2})]. ^{19}F NMR (282 MHz, DMSO): δ = -119.8 (s). HRMS: calcd for $\text{C}_{31}\text{H}_{52}\text{FN}_4\text{O}_7\text{S}$ [M + H] 643.3541, found 643.3538.

***Ac*-Ile¹-Ile²-Thr-Leu- ψ [CH_2SO_2]-F (18)** (50 mg, 77.8 μmol) in CH_2Cl_2 (10 mL) was added a solution of HBr in acetic acid (33%, 3.0 mL). After stirring for 2 h at rt, the mixture was concentrated in vacuo. Preparative HPLC was used for purification. The crude material was dissolved in a minimal amount of buffer A and loaded onto a C8 Alltima column. The product was eluted with a flow rate of 11.5 mL/min using a linear gradient to 100% buffer B (0.1% TFA in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 95/5, v/v) in 85 min from 100% buffer A (0.1% TFA in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 5/95, v/v). Fractions of 4.6 mL were taken. Epoxomicin-derived sulfonyl fluoride **19** was obtained as a white solid (15 mg, 34%). HPLC retention time: 30.5 min. ^1H NMR (500 MHz, DMSO): δ = 0.79–0.85 [m, 18H, $\text{CH}(\text{CH}_3)_2$, 4 \times CH_3 (Ile^{1,2})], 1.00–1.07 [m, 5H, 2 \times $\text{CH}_3\text{CH}^{\text{a}}\text{CH}$ (Ile^{1,2}), CH_3 (Thr)], 1.33 [m, 1H, NCHCH^a (Leu)], 1.42 [m, 2H, 2 \times $\text{CH}_3\text{CH}^{\text{b}}\text{CH}$ (Ile^{1,2})], 1.53 [m, 1H, NCHCH^b (Leu)], 1.64–1.75 [m, 3H, $\text{CH}_2\text{CH}(\text{CH}_3)_2$, 2 \times NCHCH (Ile^{1,2})], 1.85 (s, 3H, $\text{CH}_3\text{C}=\text{O}$), 3.85–3.93 [m, 2H,

CH^aSO₂F, NCHCH (Thr)], 4.11 [m, 2H, CH^bSO₂F + NCH (Thr)], 4.21 [m, 2H, NCH (Ile^{1,2})], 4.34 [m, 1H, NCH (Leu)], 4.67 (d, *J* = 3.7 Hz, 1H, OH), 7.72 [d, *J* = 7.9 Hz, 1H, NH (Thr)], 7.91 [m, 3H, NH (Leu), 2 × NH (Ile^{1,2})]. ¹⁹F NMR (282 MHz, DMSO): δ = -119.9 (s). HRMS: calcd for C₂₄H₄₆FN₄O₇S [M + H] 553.3071, found 553.3085.

*N*₃CH₂C(O)-Ile-Ile-Thr-Leu-ψ-[CH₂SO₂]-F (21). Crude 27 (303 mg, 0.63 mmol), BOP (305 mg; 0.69 mmol), HOBt (84.5 mg; 0.63 mmol), and crude deprotected peptido sulfonyl fluoride 28^{Sb} (191 mg, 0.87 mmol) were dissolved in CH₂Cl₂. To the resulting mixture was added NMM (233 μL; 2.12 mmol), and the resulting mixture was stirred overnight at room temperature. The mixture was washed with KHSO₄ (10 mL), NaHCO₃ (10 mL), and brine and was dried over Na₂SO₄. The solvents were evaporated, and 20 mL of TFA/CH₂Cl₂ (1:1) was added. The resulting mixture was stirred for 90 min, and the solvents were evaporated to yield the crude product. The product was purified using preparative HPLC, as was described for 19, to obtain 21 as a white solid (19.3 mg, 32 μmol, 6% yield over 10 steps). HPLC retention time: 22.6 min. ¹H NMR (300 MHz, DMSO): δ = 0.80 [m, 18H, 6 × CH₃ (2 × Leu, 4 × Ile)], 0.99–1.11 [m, 2H, CHCHCH^aCH₃ (Ile^{1,2})], 1.00 [d, *J* = 6.1 Hz, 3H, CH₃ (Thr)], 1.36–1.79 [m, 7H, CH₂CH(CH₃)₂, CH₂CH(CH₃)₂, CHCHCH^bCH₃ (Ile^{1,2})], CHCH₂CH₃ (Ile^{1,2})], 3.87 [m, 4H, N₃CH₂, CHOH (Thr), CH^aSO₂F], 4.14 [m, 2H, CH^bSO₂F, NHCH (Thr)], 4.29 [m, 3H, NHCH (Leu, Ile^{1,2})], 4.66 (d, *J* = 4.9 Hz, 1H, CHOH), 7.73 [d, *J* = 8.7 Hz, 1H, NHCH (Thr)], 7.92 [d, *J* = 8.6 Hz, 1H, NHCH (Leu)], 8.12 [2d, *J* = 8.2 Hz and *J* = 8.6 Hz, 2H, NHCH (Ile^{1,2})]. ¹⁹F NMR (282 MHz, DMSO): δ = -119.9. IR (KBr): 2105 cm⁻¹ (N₃). HRMS: calcd for C₂₄H₄₅FN₇O₇S [M + H] 594.3085, found 594.3075.

Biological Assays. Competitive Activity-Based Protein Profiling (ABPP). Whole cell lysates from Hek cells, expressing the constitutive β1, β2, and β5 proteasome subunits, were made in lysis buffer containing 50 mM phosphate buffer (pH 7.0), 5 mM MgCl₂, 250 mM sucrose. Protein concentration was determined by the colorimetric Bradford method. For the competitive ABPP, 25 μg of total protein was first incubated with increasing concentrations of the peptido sulfonyl fluoride proteasome inhibitors for 1 h at 37 °C and the remaining activity of the proteasome subunits was labeled with 1 μM MV151 for 1 h at 37 °C.¹⁰ Reaction mixtures were boiled with Laemmli's buffer containing β-mercaptoethanol for 3 min and proteins were resolved on 12.5% SDS-PAGE gels. In-gel visualization was performed on the wet gel slabs directly using the Cy3/Tamra settings (λ_{ext} = 530 nm, λ_{em} = 560 nm) on the Typhoon Variable Mode Imager (Amersham Biosciences) and the PMT set to 600 V. Decrease in signal intensity indicates that the inhibitors outcompete the MV151 probe for binding to the proteasome subunit. The dual color marker from Bio-Rad was used to visualize the 25 and 75 kDa marker bands. Incubation with 0 or 5 μM epoxomicin was used as positive and negative controls, respectively.

Evaluation in Living HEK Cells. Three of the most potent and/or selective inhibitors, 11, 14 and 21, were tested in a competitive ABPP assay in living HEK cells. The cells were first treated with increasing inhibitor concentrations for 3 h followed by a 3 h incubation with 2 μM of the panreactive proteasome activity-based probe MV151.⁷ After washing with PBS, the cells were harvested, lysed in 50 mM phosphate buffer pH 7.0, 5 mM MgCl₂, 250 mM sucrose, and sonicated for 30 s at 12 W on ice. Protein concentration was determined by the colorimetric Bradford method and 25 μg of total protein was resolved on 12.5% SDS-PAGE under reductive conditions. In-gel visualization was performed on the wet gel slabs directly, using the Cy3/Tamra settings (λ_{ext} = 530 nm, λ_{em} = 560 nm) on the Typhoon Variable Mode Imager (Amersham Biosciences) and the PMT was set to 600 V. A decrease in signal intensity indicates that the inhibitors outcompete the MV151 probe for binding to the proteasome subunit. The dual color marker from Bio-Rad was used to visualize the 25 and 75 kDa marker bands.

Proteasome Enzymatic Assays for IC₅₀ Determination.¹¹ Enzyme activity was determined by monitoring the hydrolysis of the fluorogenic substrates Suc-LLVY-AMC, ZLLR-AMC, and ZLLE-AMC (AMC-peptides were purchased from Bachem) for CT-, T-

and C-like activity for 1 h at rt ([E]₀ = 0.10 nM, [S]₀ = 250 μM). Reference inhibitor epoxomicin was obtained as a kind gift from Prof. Dr. S. A. Sieber (Technische Universität München, Garching, Germany). Assay buffer was 20 mM Tris pH 7.5, 0.01% (w/v) SDS. Fluorescence was measured at λ_{exc} = 360, λ_{em} = 460 nm using a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). Point-measurements were performed with a 15 min incubation of the enzyme with 200 μM concentration previous to substrate addition. Additionally, the inhibitory activities of compounds were expressed as IC₅₀ values (inhibitor concentrations giving 50% inhibition). The values were obtained by plotting the percent inhibition against inhibitor concentration and fitting the experimental data to the following equation: % inhibition = 100 × [I]₀ / (IC₅₀ + [I]₀).

■ ASSOCIATED CONTENT

📄 Supporting Information

Experimental procedures and NMR, HPLC, and biological assay data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

✉ Corresponding Author

*E-mail: r.m.j.liskamp@uu.nl

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank Cees Versluis for obtaining the HRMS spectra, and Prof. Dr. S. A. Sieber (Technische Universität München, Garching, Germany) for the kind gift of a sample of epoxomicin.

■ ABBREVIATIONS USED

BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; C-like, caspase-like; CT-like, chymotrypsin-like; DIC, *N,N'*-diisopropylcarbodiimide; DiPEA, *N,N'*-diisopropylethylamine; HOBt, *N*-hydroxybenzotriazole; NMM, *N*-methylmorpholine; T-like, trypsin-like; pFL, pyrazine-Phe-Leu; TFE, 2,2,2-trifluoroethanol; VS, vinyl sulfone.

■ REFERENCES

- (1) Dick, T. P.; Nussbaum, A. K.; Deeg, M.; Heinemeyer, W.; Groll, M.; Schirle, M.; Keilholz, W.; Stevanović, S.; Wolf, D. H.; Huber, R.; Rammensee, H-G; Hansjörg Schild, H. Contribution of proteasomal β-subunits to the cleavage of peptide substrates analyzed with yeast mutants. *J. Biol. Chem.* **1998**, *273*, 25637–25646.
- (2) (a) Adams, J.; Behnke, M.; Chen, S.; Cruickshank, A. A.; Dick, L. R.; Grenier, L.; Klunder, J. M.; Ma, Y.-H.; Plamondon, L.; Stein, R. L. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 333–338. (b) Adams, J.; Kauffman, M. Development of the proteasome inhibitor Velcade (bortezomib). *Cancer Invest.* **2004**, *22*, 304–311. (c) Ruschak, A. M.; Slassi, M.; Kay, L. E.; Schimmer, A. D. Novel proteasome inhibitors to overcome bortezomib resistance. *J. Natl. Cancer. Inst.* **2011**, *103*, 1007–1017. (d) Flemming, A. Therapeutics: Opening the door to a new class of proteasome inhibitors. *Nat. Rev. Cancer* **2012**, *12*, 5–5. (e) D'Arcy, P.; Brnjic, S.; Olofsson, M. H.; Fryknäs, M.; Lindsten, K.; De Cesare, M.; Perego, P.; Sadeghi, B.; Hassan, M.; Larsson, R.; Linder, S. *Nat. Med.* **2011**, *17*, 1636–1641.
- (3) (a) Kisselev, A. F.; van der Linden, W. A.; Overkleeft, H. S. Proteasome inhibitors: An expanding army attacking a unique target. *Chem. Biol.* **2012**, *19*, 99–115. (b) Huber, E. M.; Groll, M. Inhibitors for the immuno- and constitutive proteasome: Current and future trends in drug development. *Angew. Chem., Int. Ed.* **2012**, *51*, 8708–8720. (c) Gräwert, M. A.; Groll, M. Exploiting nature's rich source of proteasome inhibitors as starting points in drug development. *Chem. Commun.* **2012**, *48*, 1364–1378. (d) Mroczkiewicz, M.; Winkler, K;

Nowis, D.; Placha, G.; Golab, J.; Ostaszewski, R. Studies of the synthesis of all stereoisomers of MG-132 proteasome inhibitors in the tumor targeting approach. *J. Med. Chem.* **2010**, *53*, 1509–1518.

(4) Guterman, L. Firing up the \$1 trillion network. *Chem. Eng. News* **2011**, *89*, 19–26.

(5) (a) Brouwer, A. J.; Ceylan, T.; van der Linden, T.; Liskamp, R. M. J. Synthesis of β -aminoethanesulfonyl fluorides or 2-substituted taurine sulfonyl fluorides as potential protease inhibitors. *Tetrahedron Lett.* **2009**, *50*, 3391–3393. (b) Brouwer, A. J.; Ceylan, T.; Jonker, A. M.; van der Linden, T.; Liskamp, R. M. J. Synthesis and biological evaluation of novel irreversible serine protease inhibitors using amino acid based sulfonyl fluorides as an electrophilic trap. *Bioorg. Med. Chem.* **2011**, *19*, 2397–2406.

(6) Saxon, E.; Bertozzi, C. R. Cell surface engineering by a modified Staudinger reaction. *Science* **2000**, *287*, 2007–2010.

(7) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. Click chemistry: Diverse chemical function from a few good reactions. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004–2012.

(8) Instead some threonine acetylated product **22** was obtained (Scheme 2), which was included in the set of peptido sulfonyl fluorides for evaluation.

(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. Mixing of peptides and electrophilic traps gives rise to potent, broad spectrum proteasome inhibitors. *Org. Biomol. Chem.* **2007**, *5*, 1416–1426.

(10) Verdoes, M.; Florea, B. I.; Menendez-Benito, V.; Maynard, C. J.; Witte, M. D.; van der Linden, W. A.; van der Nieuwendijk, A. M. C. H.; Hofmann, T.; Berkers, C. R.; van Leeuwen, F. W.; Groothuis, T. A.; Leeuwenburgh, M. A.; Ovaa, H.; Neeffjes, J. J.; Filippov, D. V.; van der Marel, G. A.; Dantuma, N. P.; Overkleeft, H. S. A fluorescent broad-spectrum proteasome inhibitor for labeling proteasomes in vitro and in vivo. *Chem. Biol.* **2006**, *13*, 1217–1226.

(11) Gallastegui, N.; Groll, M. Analysing properties of proteasome inhibitors using kinetic and X-ray crystallographic studies. *Methods Mol. Biol.* **2012**, *832*, 373–390.

(12) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595–598.

(13) von Arx, E.; Faupel, M.; Brugger, M. Das 4,4'-tetramethyldiamino-diphenylmethan reagens (TDM): Eine modifikation der chlorotolidin farbereaktion für die dünnschicht-chromatographie. *J. Chromatogr.* **1976**, *120*, 224–228.

(14) (a) Goddard-Borger, E. D.; Stick, R. V. An efficient, inexpensive and shelf-stable diazotransfer reagent: Imidazole-1-sulfonyl azide hydrochloride. *Org. Lett.* **2007**, *9*, 3797–3800. (b) Goddard-Borger, E. D.; Stick, R. V. Erratum to: An efficient, inexpensive, and shelf-stable diazotransfer reagent: Imidazole-1-sulfonyl azide hydrochloride. *Org. Lett.* **2011**, *13*, 2514–2514.