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Solid-phase Synthesis and Application of a Clickable Version of Epoxomicin for Proteasome Activity Analysis

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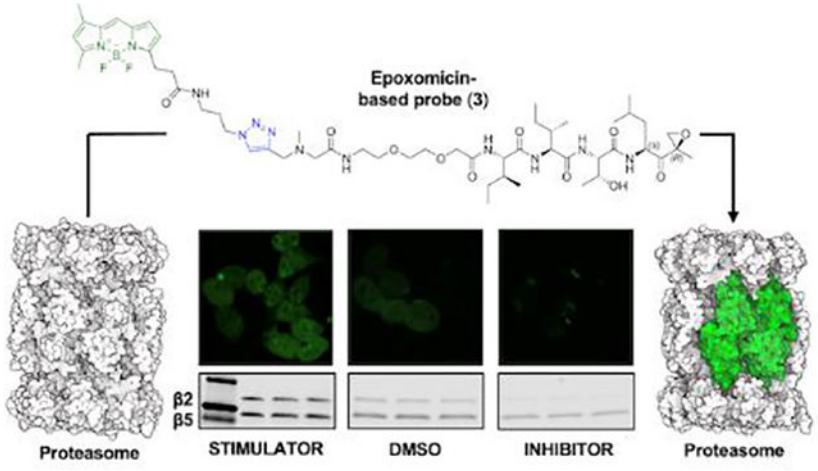
Abstract

Degradation of proteins by the proteasome is an essential cellular process and one that many wish to study in a variety of disease types. There are commercially available probes that can monitor proteasome activity in cells, but they typically contain common fluorophores that limit their simultaneous use with other activity-based probes. In order to exchange the fluorophore or incorporate an enrichment tag, the proteasome probe likely has to be synthesized which can be cumbersome. Here, we describe a simple synthetic procedure that only requires one purification step to generate epoxomicin, a selective proteasome inhibitor, with a terminal alkyne. Through a copper-catalyzed cycloaddition, any moiety containing an azide can be incorporated into the probe. Many fluorophores are commercially available that contain an azide that can be "clicked", allowing this proteasome activity probe to be included into already established assays to monitor both proteasome activity and other cellular activities of interest.

Graphical Abstract



The described epoxomicin-alkyne probe and be clicked to a variety of fluorophores. It can then be used in gel-based assays, microscopy, and flow cytometry to determine proteasome activity.

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Conflict of interest

Prof. Trader is a shareholder and consultant for Booster Therapeutics, GmbH. Other authors declare no conflict of interest.

It is sensitive enough to be able to detect both proteasome inhibition and stimulation by small molecules.

Introduction

Cells require a complex network of chaperones and proteolytic machineries to balance protein synthesis and degradation.[1] With the exception of intrinsically disordered proteins (IDPs), newly synthesized proteins require a defined tertiary and/or quaternary? Not sure if that sounds betterto achieve their purpose, and this conformational stability must be maintained to prevent a decline in cellular function.[1] Failure during protein synthesis or folding can yield faulty protein species which require proteolytic systems for their degradation. In eukaryotic cells, there are two major pathways, the ubiquitin-proteasome system (UPS) and the autophagy lysosomal pathway (ALP).[2] The UPS is responsible for degradation of more than80% of cellular proteins,[3] while the autophagy pathway is responsible for digesting long-lived proteins and cellular organelles.[1,2,4,5]

The proteasome is part of the UPS and exists as a 26S and 20S isoform in the cell. The multiunit ATP-dependent 26S isoform is comprised of the 19S regulatory particle (RP) and the 20S core particle (CP). Ubiquitinated proteins are recognized by the19S, unfolded by its ATPase ring, and subsequently translocated to the 20S for proteolytic cleavage. The β1-, β2-, and β5-subunits within the 20S are responsible for hydrolyzing amide bonds of the protein, and each active site recognizes a different type of amino acid. This generates of a variety of peptide products.[6–9] Given the essential proteolytic role of the 26S/20S proteasome isoforms, many small molecules have been developed to modulate their activity.[10,11] Three inhibitors, bortezomib, carfilzomib, and ixazomib, are commonly use therapies multiple myeloma treatment.[12]

Considering that the majority of these inhibitory molecules covalently attach to the catalytic residues within the active sites, a series of activity-based proteasome probes have been developed to monitor its activity by incorporating fluorophores.[13–15] One such molecule is epoxomicin, an epoxy-ketone peptide of bacterial origin first isolated in 1992. Its structure has previously been successfully derivatized to generate fluorescent probes.[16–18] However, the synthesis of these probes can be challenging, limiting their utility in the proteasome field. Additionally, generating probe derivatives that contain a different linker, fluorophore, or that could incorporate a biotin moiety are not typically commercially available, limiting the studies of proteasome activity.

Here, were describe a convenient multi-step synthesis of an alkyne-containing derivative of epoxomicin. Synthesis was achieved by a solid-phase methodology for the peptide region of the molecule, followed by a one-pot in-solution coupling for the epoxy-ketone moiety. The procedure conducted here represents an improvement over previous procedures given the fact that only one purification step is required to isolate the final compound, and the reactive group can be easily coupled in a one-pot fashion, thus saving time and simplifying the process. Additionally, our synthesis conveniently allows for the linker length or type to be readily changed, and the installment of the alkyne group provides a handle to perform click chemistry with any azide-containing molecule. We demonstrate the utility of this probe to

monitor proteasome activity in cells and that it can be used to discover or validate inhibitory and stimulatory small molecules using a variety of techniques.

Results and Discussion

Synthesis of Epoxomicin Probe.

The first total synthesis of epoxomicin was reported in 1999,[19] and since, different convergent methodologies have been established to achieve its synthesis using both solution- and solid-phase approaches.[14,20] Given the potency and great selectivity of this molecule, it has been widely used in proteasome research, and has inspired the development of fluorescently-labeled probes to visualize proteasome activity and intracellular distribution.[14] While there have been previous reports for the synthesis of fluorophore-conjugated epoxomicin or an alkyne-containing derivative, they require the use of highly reactive reagents, can take several days to complete, and require extensive purification procedures.[21–25] We sought to develop a more efficient approach that allowed us to obtain (**2**) in a high yield (greater than 70%) with fewer synthetic steps using a solid-phase approach, and only requiring a single purification process (Figure 1).

With the peptide backbone and the epoxy-ketone moiety at the *C*-terminus of epoxomicin (**1**), we aimed for an approach where the left portion of the molecule was synthesized via solid phase and the covalent warhead was subsequently attached in solution. Fmoc-Thr(tBu)-Wang resin was used as starting point for the synthesis and a PEG linker was installed after the addition of the remaining two amino acid residues. Given our interest in attaching a fluorescent dye at the *N*-terminus of the peptide structure, we opted for the incorporation of an alkyne group to promote the linkage via Copper(I)-catalyzed Azide-Alkyne Cycloaddition (CuAAC) reaction.[26] Inspired by the excellent purity and yields afforded after synthesis of peptoid compounds following the bromoacetic acid protocol,[27] we coupled a *N*-methyl propargyl amine monomer, to obtain (**1a**) without a purification step after resin cleavage. The *N*-methyl propargyl amine monomer was also selected to avoid any side-products that could be generated during the coupling of the epoxy-ketone fragment. This portion of the probe was cleaved from the resin using TFA and crashed out of solution using cold ether. It was subsequently used in the next step without purification.

Next, the coupling of the epoxy-ketone moiety was carried out in solution. We aimed for the development of a method that allowed us for a one-pot process using conventional amide coupling reagents. We envisioned that the free threonine hydroxyl group could be silyl protected in the presence of the molecule's free *C*-terminus using tert-butyldimethylsilyl chloride (TBS-Cl) under appropriate conditions (Figure 2);[28,29] additionally, we hoped the TBS group could be cleaved to recover the free hydroxyl of the threonine without opening of the epoxide ring during HPLC purification with TFA in the mobile phases. We tested a series of conditions and found that condition 4 yielded a 95% conversion of starting material (SM) to product over the shortest amount of time as evidenced by HPLC analysis (Table 1; Figure S1–13). After purification of this probe, it can be stored as a DMSO stock at 20 mM for 6+ months and still retain activity.

Comparison of inhibitory activity of (2) to unmodified epoxomicin:

To ensure that our modified epoxomicin did not affect its ability to bind the proteasome in cells, we performed a competition assay in which proteasome activity was monitored in HEK 293T cells. To accomplish this we used a previously developed proteasome activity reporter Me_4BodipyFLAhx$_3$L$_3$VS, based on a vinyl sulfone warhead developed by the Ovaa group (Figure S14A).[15] This reporter binds to the catalytic subunits of the 20S in living cells in an activity-dependent fashion; therefore, dosing cells with of the inhibitory molecules epoxomicin or (**2)** followed by treatment with the vinyl sulfone probe should lead to a decrease in fluorescent signal, which can be quantified by scanning a SDS-PAGE gel for the Bodipy signal (Figure S14B). Our results indicate that (**2)** was able to inhibit proteasome activity to a similar extent as epoxomicin at 1 μM or above, Figure 3. It was not as potent as epoxomicin but is well within the range that is acceptable for cell-based experiments.

We also performed a cell viability assay (CellTiter-Glo®) for 24 hours with epoxomicin or (**2)**. Again, using HEK-293T cells, epoxomicin and (**2)** had similar toxicity profiles, indicating the modification of the epoxomicin structure did not lead to additional toxicity (Figure S15). With these two positive results, we explored if we could monitor proteasome activity directly using (**2)** by clicking a BODIPY fluorophore to its scaffold, generating molecule (**3)** (Figure 4A). The BODIPY fluorophore was selected since its non-ionic character helps the probe to remain cell-permeable and its high quantum yield allows for direct gel scanning for fluorescence emission (as demonstrated with the reporter Me_4BodipyFLAhx$_3$L$_3$VS).[30] Since (**2)** contains a terminal alkyne, any moiety with an azide, such as many commercially available fluorophores and enrichment tags, could be easily incorporated.

Proteasome activity assays of positive and negative modulators using our epoxomicin probe:

We first aimed to establish the detection limit of (**3)** for its ability to interact with the β2 and β5 subunits of the proteasome in HEK-293T cells. Cells in a 24-well plate were dosed with (**3)** for one hour at decreasing concentrations starting at 2 μM. The cells were harvested, lysed, and the lysate subjected to SDS-PAGE. The gel was then scanned at the wavelength to detect the BODIPY fluorophore. We were excited to observe that only two bands were detected in the gel, one near the molecular weight for β5 subunit and the other to the β2 subunit of the proteasome, Figure 4B. A subsequent western blot analysis using the anti-β5 and anti-β2 antibody confirmed their identity (Figure S16). Signal for the β5 subunit was observed at all concentrations, including the 50 nM. The signal for the β2 subunit was easily observed until the 100 nM concentration, Figure 4B. Figure 4C shows the quantitation of the gel band density for each proteasome subunit. It was expected that more signal from the beta-5 subunit would be detected as this is the most active proteasome subunit in cells.[31]

We next aimed to test (**3)** for its ability to detect changes in proteasome activity in cells in the presence of proteasome stimulators or inhibitors. For the activity assays with modulators of the proteasome, we selected a set of previously established modulators of the multi-subunit complex in both biochemical and cellular environments.[32–35] Three activators and two inhibitors were used at two different concentrations for our studies, Figure 5A. Cells

were pre-treated with the small molecule inhibitor/stimulator for one hour, then (**3)** was added at 500 nM, and the cells were incubated for an additional hour. SDS-PAGE analysis of the lysate followed by fluorescence imaging of the gel indicated that all tested molecules modulated proteasome activity as anticipated, Figure 5B. Miconazole (MO), trifluoperazine (TFP) and AM-404 (stimulators) increased the activity of the proteasome in the HEK-293T cells using (**3)** as a reporter for activity. Proteasome labeling was increased by at least 40% over the DMSO treated samples at 25 μM, and MO and TFP elicited significant increase in activity at 5 μM. Both inhibitors, MG-132 and epoxomicin, prevented the binding of (**3)**, limiting the fluorescent signal observed, Figure 5C. This result highlights the utility of (**3)** for its ability to be used as a probe in drug discovery campaigns in live cells for proteasome modulators.

As an additional application of (**3)**, we used confocal microscopy to assess inhibition with MG-132 and stimulation with MO (Figure 6). Cells were plated on glass coverslip chambers coated with poly-*D*-lysine and were dosed with the small molecule modulators at 25 μM for one hour. (**3)** was subsequently added and incubated for another hour. A DMSO control without a proteasome stimulator or inhibitor, but including (**3)** to observe the basal level of proteasome activity was included. Cells treated with (**3)** showed an increase of fluorescent signal inside the cell, indicating the probe was able to enter the cells and bind to intracellular targets. Including MG-132 significantly decreased the signal, as this inhibitor prevents the interaction of (**3)** with the proteasome, indicating that the probe is highly selective for binding the proteasome. A remarkable increase in fluorescent signal inside the cells was observed when the cells were dosed with MO, a proteasome stimulator (Figure S17).

Besides confocal microscopy, we used flow cytometry to measure the changes in fluorescence upon addition of a proteasome modulator. Given the low throughput of the previous techniques, we aimed to assess the possibility of using (**3)** in a higher throughput setting. After dosing HEK-293T cells with MO, TFP or MG-132 for one hour, (**3)** was added and the samples were incubated for another hour. Cells were subsequently pelleted, rinsed with PBS to remove non-reacted (**3**), and analyzed by flow cytometry. Quantitation of the fluorescent signal showed an increase for MO and TFP treated samples, and a decrease for MG-132 samples over the DMSO treated samples (Figure S18).

Conclusion

Here, we described the synthesis and application of a clickable version of epoxomicin in a suite of proteasome activity assays. Our synthetic approach demonstrated the possibility of using a combination of solid- and solution-phase chemistry to generate a more time-efficient as well as lower complexity reaction scheme without sacrificing the overall yield. Using peptoid chemistry, we were able to efficiently install an alkyne moiety that can react with several commercially available fluorophores or enrichment tags that contain an azide.

To validate this modified epoxomicin we added a Bodipy fluorophore, which contained an azide, through a copper-catalyzed click reaction. We showed the utility of this probe to detect and quantify the activity of the proteasome in cells using a gel-based assay, confocal microscopy, and flow cytometry. Additionally, the probe was able to detect the change in

proteasome activity in the presence of a small molecule inhibitor or stimulator. We generated a second probe that incorporated a Cyanine 7 azide fluorophore to (**2**) to demonstrate the flexibility of our epoxomicin-alkyne scaffold's ability to incorporate different moieties via click chemistry, Figure S19A. This probe derivative has a similar detection limit as the Bodipy probe derivative (**Figure 19 B-C**) and was able to detect changes in proteasome activity in HEK-293T (Figure S20).

While epoxomicin activity probes for the proteasome have been previously reported, our method is simple enough that those not well experienced in organic synthesis can generate it. All the reagents are commercially available, and it only requires one purification step. Most significantly, the free alkyne allows for the addition of a wide variety of different fluorophores such as the ones here used, or enrichment tags. We envision that this probe will find utility with those interested in studying proteasome activity along with otherenzymes of interest by easily installing a fluorophore that would not overlap with other chemical probes. Additionally, a moiety such as biotin could be installed for those wishing to enrich the proteasome in their cell line or model of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Biographies

Andres graduated from the pharmacy program at Universidad Nacional de Colombia in 2017 and received an award for the best undergraduate thesis for his project titled 'Fragment Discovery for Nuclear Protein-Protein Interaction Hotspots'. He started his PhD training in the Medicinal Chemistry and Molecular Pharmacology Department at Purdue University in Professor Darci J. Trader's lab in 2018. His research interests are in protein degradation, chemoproteomics, and development of new chemical tools for proteasome activity analysis.

Dr. Trader received her PhD in 2013 under the direction of Prof. Erin E. Carlson. She then did a NIH-funded postdoc with Prof. Thomas Kodadek. Dr. Trader began her independent

career in 2016 as an assistant professor at Purdue University in the Department of Medicinal Chemistry and Molecular Pharmacology. Her lab is focused on studying the proteasome in a variety of disease types. They have developed a number of new activity-based probes that can be used in live cells to monitor proteasome activity and small molecule stimulators to increase the degradation rate of proteins through the ubiquitin-independent pathway.

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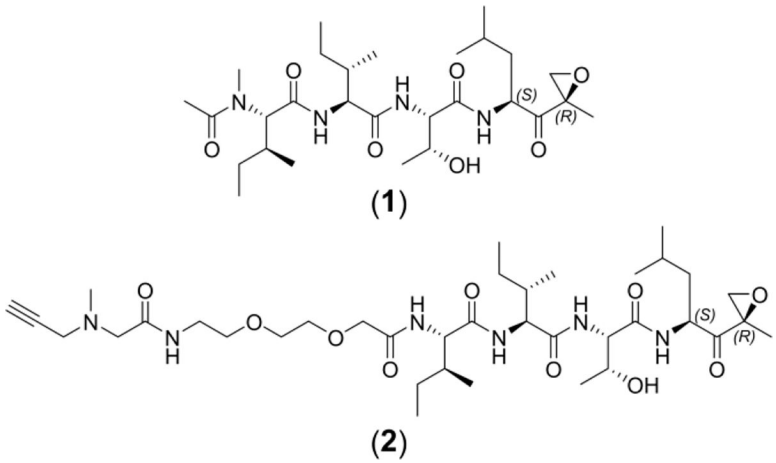

Figure 1.
Structures of epoxomicin (**1**) and a new clickable version of epoxomicin (**2**).

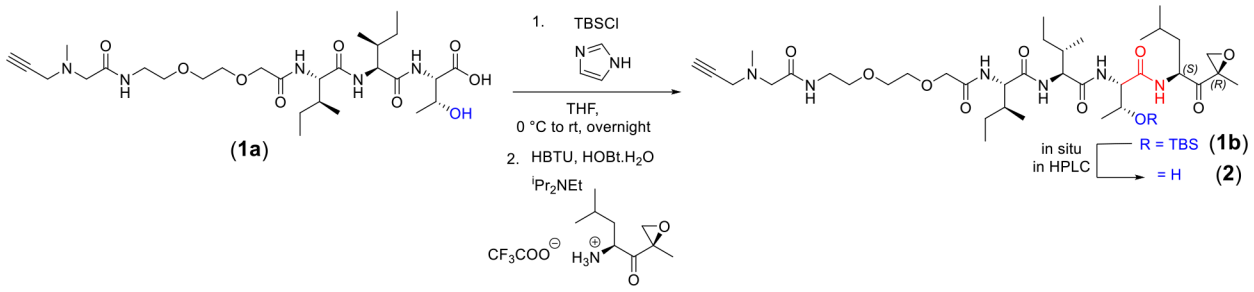

Figure 2:

One-pot reaction scheme conducted for the amidation of (**1a**) and the epoxy-ketone fragment.

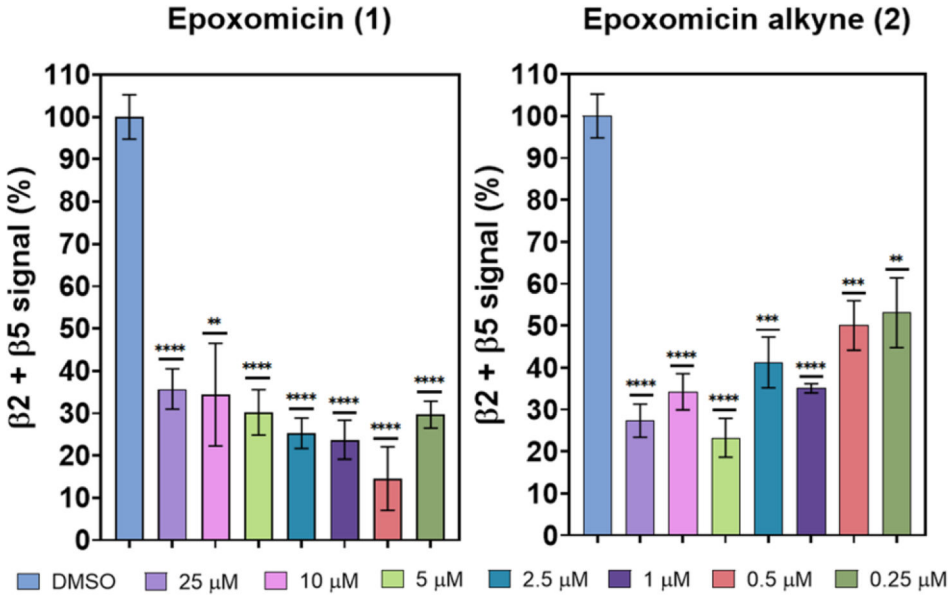

Figure 3:

HEK293T cells were dosed with DMSO, different concentrations of epoxomicin, or **2** and incubated for one hour, followed by addition of Me_4BodipyFLAhx$_3$L$_3$VS proteasome reporter at 0.5 μM, and incubated for another hour. SDS-PAGE analysis was subsequently conducted and fluorescent signal for each sample (which indicates how much of the proteasome was still active to be able to bind the Me_4BodipyFLAhx$_3$L$_3$VS proteasome reporter) for each sample was performed in triplicate and averaged. Quantitation of the fluorescent bands for the proteasome subunits, β2 and β5, were combined and data was normalized to DMSO.

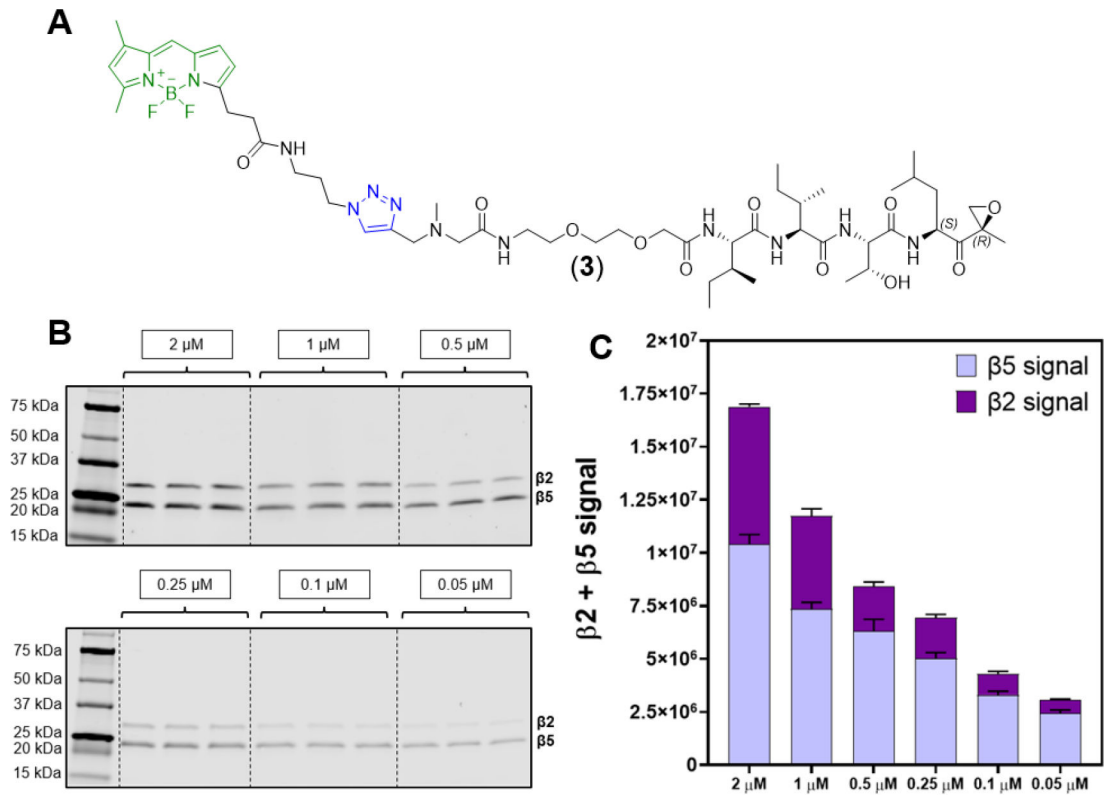

Figure 4:

(**A**) Structure of our BODIPY-epoxomixin probe, **3**. (**B**) SDS-PAGE analysis of HEK293T cells treated with **3** at different concentrations for 1 hour, in triplicate. (**C**) Quantification of fluorescent signal from the gels shown in **B**, which indicates proteasome activity. Error bars denote standard deviation.

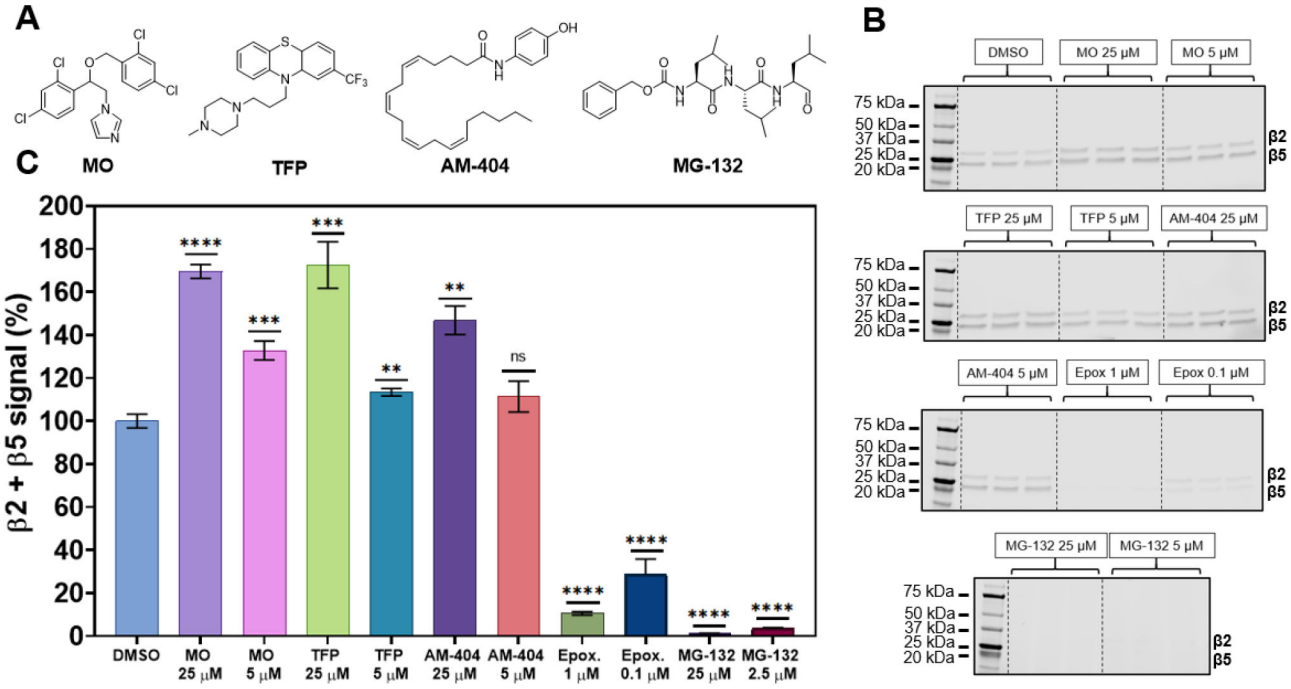

Figure 5:

(**A**) Structure of the small molecules used to modulate the activity of the proteasome in HEK293T cells. (**B**) SDS-PAGE analysis conducted with HEK293T cells dosed with DMSO or small molecule proteasome modulators at two concentrations (stimulators: MO, TFP, AM-404; inhibitors: Epoxomicin, MG-132) for one hour, followed by treatment with **3** at 500 nM for one hour (2-hour total time of incubation). (**C**) Quantification of the fluorescent signal obtained for the β2 and β5 subunits. Data was normalized to DMSO. Error bars denote standard deviation.

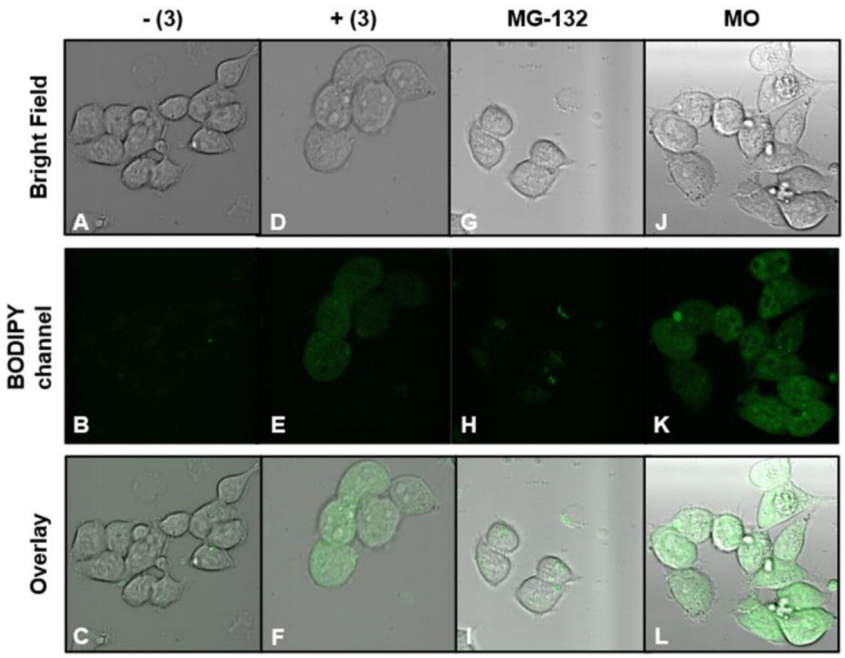

Figure 6:

Confocal microscopy images used to illustrate the intracellular location of (**3**) and the changes in fluorescence upon treatment with small molecule modulators of the proteasome. HEK-293T cells were treated with DMSO (**A–F**), MG-132 (**G–I**), or MO (**J–L**) for 1 hour and subsequently dosed with (**3**) at 500 nM (**D–L**) or DMSO (**A–C**) for another hour before a PBS wash was performed to remove extracellular or unbound (**3**). (**A, D, G, J**) Bright field. (**B, E, H, K**) BodipyFL (Green channel). (**C, F, I, L**) Overlay. These images demonstrate that there is no green fluorescence observed in cells not treated with (**3**); in contrast, cells treated with our probe exhibit intracellular fluorescence, and the intensity is decreased in MG-132 treated cells (**H, I**) and increased in MO treated samples (**K, L**).

Table 1:

Conditions tested for the one-pot synthesis. Conversion of **1a** to **2** was determined by HPLC isolation.

Condition #	Silyl group (eq.)	Solvent	Base (eq.)	Temp. (°C)	Time (h)	Conversion (%)
1	TBSCl (1.1)	THF	Imidazole (2.2)	rt	18	82
2	TBSCl (1.1)	THF	Imidazole (4)	rt	4	88
3	TBSCl (2)	THF	Imidazole (4)	rt	4	88
4	TBSCl (2)	THF	Imidazole (2.2)	rt	4	95
5	TBSCl (2)	THF	Imidazole (2.2)	rt	17	95