The proteasome is a multisubunit protein complex responsible for the degradation of proteins, making it essential in myriad cellular processes. Several reversible and irreversible peptide substrates inspired by known proteasome inhibitors have been developed to visualize it and monitor its activity; however, they have limited commercial availability or possess fluorophores that overlap with other known chemical probes, limiting their simultaneous use. The protocols presented here describe the synthesis of a clickable epoxomicin-based probe followed by the copper-catalyzed installment of an azide-containing fluorophore, and the application of the synthesized peptide in proteasome activity assays by SDS-PAGE and flow cytometry. © 2022 The Authors. Current Protocols published by Wiley Periodicals LLC.

**Basic Protocol 1:** Solid-phase synthesis of clickable peptide fragment (2)

**Basic Protocol 2:** In-solution coupling of epoxy-ketone moiety to fragment (2)

**Basic Protocol 3:** Copper-catalyzed click reaction of (3) with fluorophore of choice

**Basic Protocol 4:** Monitoring proteasome activity by SDS-PAGE in HEK-293T cells

**Alternate Protocol:** Monitoring proteasome activity by flow cytometry in HEK-293T cells

Keywords: cell-based assay • chemical probes • flow cytometry • in-gel fluorescence • proteasome

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**INTRODUCTION**

To maintain a functional proteome, an exhaustive network of regulators and chaperones is required to ensure an appropriate balance between protein synthesis, conformational maintenance, and degradation (Hipp, Kasturi, & Hartl, 2019). In eukaryotes, two major systems are responsible for degrading functional and faulty proteins alike, the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP) (Dikic, 2017;...
Hipp et al., 2019). The UPS is estimated to be responsible for \( \sim 80\% \) of protein turnover and mediates the degradation of mostly short-lived and damaged proteins (Dikic, 2017; Lee & Goldberg, 1998). The ALP is mainly responsible for the degradation of cellular organelles and long-lived proteins and is known to be a crucial adaptive mechanism in response to cellular stress. (Filomeni, De Zio, & Cecconi, 2015).

Given the key role of protein degradation in cellular processes, disruption of proteasome activity has been implicated in a variety of cancers and neurodegenerative diseases (Leestemaker et al., 2017; Orlowski & Dees, 2002; Orlowski et al., 1998; Trader, Simanski, & Kodadek, 2015; Zheng et al., 2016); therefore, modulation of proteasome activity has gained popularity due to its therapeutic potential. Several tools, from peptide-based fluorescent substrates to activity-based probes (ABPs) (Berkers et al., 2007; Coleman & Trader, 2018; Kisselev & Goldberg, 2005; Zerfas, Coleman, Salazar-Chaparro, Macatangay, & Trader, 2020), have been developed to visualize and monitor the activity of this enzyme; however, the structural complexity of these chemical tools represents a synthetic challenge, limiting their availability. Likewise, the lack of derivatives with different reporter tags can restrict their utility when used concomitantly with other chemical probes.

The protocols described here provide a simple and efficient route to the synthesis of a clickable version of epoxomicin (Basic Protocols 1 and 2), a highly potent and selective proteasome inhibitor, and the coupling of an azide-containing reporter tag (Basic Protocol 3). Additionally, SDS-PAGE and flow cytometry-based assays (Basic Protocol 4 and Alternate Protocol) are described for monitoring proteasome activity and the detection of positive and negative modulation in response to small molecules.

**SOLID-PHASE SYNTHESIS OF CLICKABLE PEPTIDE FRAGMENT (2)**

This protocol describes the process to obtain fragment (2), required for the synthesis of an epoxomicin-based probe amenable to click chemistry (Fig. 1). This fragment is synthesized using standard solid-phase conditions following Fmoc-based synthesis, from C- to N-terminus, using a high loading Wang-Thr resin, which proved to be high

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**Figure 1** (A) Structure of epoxomicin (1), a potent proteasome inhibitor; (B) structure of the clickable peptide fragment (2) synthesized via solid-phase; (C) structure of the designed clickable epoxomicin-based probe (3).
yielding, even at >1 g scale (Fig. 2). Following synthesis of the Thr-Ile-Ile peptide fragment, a PEG2 linker was attached and subsequent addition of an N-methyl propargyl amine was carried out via bromoacetic acid coupling. After cleavage from the resin, the crude material was precipitated using cold diethyl ether and dried. No purification is required at this stage.

**Materials**

- Fmoc-Threonine(tBu)-Wang resin (Fmoc-Thr(tBu)-Wang; Millipore Sigma catalog No. 856017)
- N-dimethylformamide (DMF)
- Anhydrous DMF
- Dichloromethane (DCM)
- Diethyl ether
- 20% (v/v) piperidine in DMF
- Fmoc-Isoleucine-OH (Fmoc-Ile-OH; Millipore Sigma catalog No. 852010)
- Kaiser reagents 1, 2, and 3 (see recipe)
- Cleavage solution (see recipe)
- Hexafluorophosphate benzotriazoole tetramethyl uronium (HBTU; Fisher catalog No. B16575G)
- N,N-Diisopropylethylamine (DIPEA)
- Bromoacetic acid (BAA)
- N,N-diisopropylcarbodiimide (DIC)
- N-Methylpropargylamine (Combi-Blocks catalog No. QJ-2154)
- [2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid (PEG2 linker) (Combi-Blocks catalog No. SS-2798)
- Fritted syringe with caps (Kamush catalog No. PPV006)
- Room-temperature shaker
Scale
Vortex
Vacuum Manifold (Promega catalog No. A7231)
Rotary evaporator
Vacuum pump
Centrifuge
Conical tube
Inert gas (argon/nitrogen)
Disposable Round-Bottom Rimless Glass Tubes
Disposable borosilicate glass Pasteur pipets (9 in.)
Heat gun or 95°C heating block/oven

All reactions must be carried out in a ventilated fume hood and wearing the appropriate personal protective equipment (PPE), such as reagent and solvent-suitable gloves and safety glasses.

1. Weigh Fmoc-Thr(tBu)-Wang resin in fritted syringe.

   The resin used in the described procedure is 0.61 mmol/g. The amount used can be as high as 1 g in a single syringe without detrimental effects on the final yield.

   Be mindful of the size of the syringe based on the scale, as the resin beads will grow when in contact with DMF.

2. Cap the bottom of the syringe and add 2 ml DMF to cover entirely the resin beads.

   The amount of DMF can vary depending on the amount of resin used. Typically, between 2 and 5 ml of solvent is sufficient.

3. Insert syringe plunger and push far enough to maximize contact between the resin beads and the solvent.

   Push gently. Be sure to remove the bottom cap first to avoid pressure buildup.

4. Incubate the syringe for 30 to 60 min at room temperature with shaking to allow the resin beads to swell.

5. Remove the plunger and place syringe on manifold to drain the solvent.

6. Cap the bottom of the syringe and add 2 ml of 20% piperidine in DMF to deprotect the Fmoc group.

7. Place syringe plunger back into the syringe and incubate for 30 min at room temperature with shaking. Put syringe on a manifold and drain the solution.

8. Wash resin three times with 2 to 5 ml of DMF and validate complete removal of the Fmoc group by performing a Kaiser test:
   a. Add 1 to 2 drops of each of the 3 Kaiser reagents into a small round-bottom rimless glass tube.
   b. Using a glass pipette, remove a small amount of resin beads from the syringe and transfer them to the glass tube.
   c. Heat the glass tube at ≥95°C for 5 min and check the color of the beads:

      A dark blue/purple coloration indicates a positive result (presence of primary amines). Slight to no change in color indicates the absence of primary amines, therefore, a negative result.

      If the amount of resin beads is too small to confidently observe, use a white-light microscope to examine them more closely.

      If the Kaiser test yields partial or no color change in the resin beads, repeat steps 6–8. If test is positive, proceed.
9. Weigh out 4 equivalents of Fmoc-Ile-OH and 4 equivalents of HBTU. Dissolve in 2 ml DMF then add 8 equivalents of DIPEA to the mixture.

*Coloration of the solution indicates activation of amino acid.*

10. Vortex mixture thoroughly and add solution to the resin. Incubate syringe for 1 hr at room temperature.

11. Repeat steps 5–10 using Fmoc-Ile-OH once again. This “double coupling” ensures complete coupling to the resin.

*The beads should be dark blue/purple after Fmoc cleavage, and no coloration should be observed after the addition of amino acid, indicating successful amide coupling.*

12. Repeat steps 5–10 using PEG$_2$ linker.

*Three equivalents of linker and 6 equivalents of DIPEA can also render successful coupling reactions when the prepared solution is concentrated (1–1.5 ml of DMF).*

13. Repeat steps 3–7. Wash resin with 2 ml anhydrous DMF and perform the Kaiser test.

*While this protocol describes the synthesis of a chemical probe containing only one PEG$_2$ linker fragment, additional couplings of the linker can be done as well. This will generate a more challenging substrate for the proteasome isoforms, thus providing a wider dynamic range and increasing the likelihood of identifying less potent proteasome modulators in drug discovery campaigns.*

14. Weigh out enough BAA to make 1 ml of a 2 M solution in dry DMF and prepare 1 ml of 1 M DIC in dry DMF.

*Make fresh solutions before each coupling. Flush the BAA bottle with argon/nitrogen before and after each use to ensure adequate storage.*

15. Mix BAA and DIC solutions and agitate in shaker until a white precipitate is observed.

*The precipitate is typically observed within <5 min of reaction time and it works as a visual indicator of BAA activation; however, it also means the conversion of the activated species to an inactive isomer. Therefore, the mixture should not be agitated after the precipitate is evident to the naked eye.*

16. Cap the bottom of the syringe and add the 2 ml mixture of activated BAA. Insert the plunger and incubate at 37°C for 20 min with shaking.

*Do not incubate longer or at higher temperatures. The alpha carbon on the carboxylic acid is highly reactive, and extending the time can generate unwanted side products.*

17. Drain solution, wash three times with 2 ml anhydrous DMF and perform the Kaiser test.

*The Kaiser test result should be negative since coupling of activated BAA to the free amine yields an amide bond. Do not let the resin sit too long before the next coupling as the bromine can become hydrolyzed by water in the atmosphere.*

18. Prepare 1.5 ml of a 1 M solution of N-methylpropargylamine in anhydrous DMF.

19. Add solution to the resin and incubate at 37°C for 1 hr with shaking.

20. Wash resin three times with 2 ml DMF and three times with DCM. Allow the resin to dry by pulling vacuum through the syringe.

*Using DCM after washing with DMF is key to remove the DMF, which is otherwise difficult to remove.*

21. Cleave peptide from resin by adding 3 ml of cleavage solution and incubating the syringe at room temperature for 2 hr.
22. Collect the resulting solution in a conical tube and wash beads three times with 3 ml DCM.

23. Carefully place a stream of argon/nitrogen over the top of the solution to evaporate the mixture of solvents until a viscous residue remains.

24. Add prechilled diethyl ether to the crude material and centrifuge at $4500 \times g$ for 10 min.

   *The amount of diethyl ether to be used varies depending on the reaction scale. Typically, 3 to 5 ml is adequate for washing away side products.*

25. Decant the diethyl ether and vacuum-dry the resulting product until a solid is observed.

   *A yield of 75%-85% of crude material should be obtained based on loading of the Fmoc-Thr(tBu)-Wang resin.*

26. Store product at $-20^\circ C$ until coupling of the epoxy-ketone fragment is conducted (Basic Protocol 2).

**BASIC PROTOCOL 2**

**IN-SOLUTION COUPLING OF EPOXY-KETONE MOIETY TO FRAGMENT (2)**

Once the clickable peptidomimetic fragment is synthesized, an in-solution amide linkage is performed after protection of the Thr hydroxyl group using conventional coupling reagents in tetrahydrofuran (THF). The crude material is dried under vacuum and resuspended in methanol (MeOH) for reverse-phase HPLC purification. The final compound is lyophilized overnight and resuspended in dimethylsulfoxide (DMSO) to be used in cell-based assays or stored at $-20^\circ C$ for subsequent click chemistry of a fluorophore of interest.

**Materials**

- Tetrahydrofuran (THF)
- Acetonitrile (ACN), HPLC grade (Fisher catalog No. AA22927K7)
- Water HPLC grade (Fisher catalog No. AA22934K2)
- Methanol (MeOH) optima (Fisher catalog No. A456-500)
- Trifluoroacetic acid (TFA) (Fisher catalog No. AAA1436518)
- 1-Hydroxybenzotriazole hydrate (HOBt) (Sigma-Aldrich catalog No. 54802)
- Hexafluorophosphate benzotriazole tetramethyl uronium (HBTU)
- tert-Butyldimethylsilyl chloride (TBSCI)
- Imidazole
- (S)-2-Amino-4-methyl-1-((r)-2-methyloxiran-2-yl)pentan-1-one 2,2,2-trifluoroacetate (Combi-Blocks catalog No. QN-6599)
- DMSO (molecular biology grade) (Fisher catalog No. ICN19481980)

- 8-ml glass vials with caps
- 0.31-inch stir bars
- Scale
- Stir plate
- Lyophilizer
- HPLC system: Agilent 1200 preparative system with Zorbax Eclipse XDB-C18 column (9.4 × 250 mm, 5-μm particle size) or comparable system
- LC/MS
- Vacuum pump
- Parafilm
- Glass container with rubber adaptor for lyophilizer
95°C oven

Inert gas (argon/nitrogen)

Rotary evaporator

1. Place a stir bar inside of the glass vial and place them in the oven the day before the experiment is to be conducted (Vial A). Repeat process for another vial and stir bar (Vial B).

2. Weigh out (2) into Vial A and dissolve material in 1 ml anhydrous THF.

   *Best results are obtained with new bottles of anhydrous THF (Sigma SureSeal bottle); otherwise, freshly distilled THF over Na/benzophenone is recommended if commercial dry THF is unavailable.*

3. Flush argon/nitrogen inside vial A and cap it.

4. Weigh out 2 equivalents of TBSCl and 2.2 equivalents of imidazole. Add them to vial A, flush with argon/nitrogen and cap.

5. Place vial A on stir plate and allow the mixture to stir for 18 hr.

   *The time required for the reaction was determined arbitrarily. The reagents were always allowed to react overnight; therefore, the reaction time can range from 12 to 18 hr.*

6. Weigh 1.05 equivalents of HBTU and 1.05 equivalents of HOBt and add them to vial A. Add 3 equivalents of DIPEA and flush the vial with argon/nitrogen.

   *While regular DIPEA can be used, the best results are obtained with dry DIPEA. This amine readily absorbs moisture from air; therefore, distilling it from CaH$_2$ under argon and then storing it with activated molecular sieves under argon provides higher yields. Alternatively, anhydrous DIPEA in septa sealed bottles can be purchased, if desired.*

7. Place vial A on stir plate and allow the mixture to react for 30 min.

8. Weigh 1.05 equivalents of (S)-2-Amino-4-methyl-1-((r)-2-methyloxiran-2-yl)pentan-1-2,2,2-trifluoroacetate and add them to oven-dried vial B. Dissolve the mixture in 0.5 ml anhydrous THF then add 3 equivalents of DIPEA.

9. Flush argon/nitrogen inside vial B and cap. Place vial on stir plate and allow the mixture to react for 5 to 10 min.

10. Transfer solution from vial B to vial A and allow the mixture to react at room temperature for 4 to 5 hr.

   *Transfer must be done under argon. Rinse vial B with additional 0.5 ml dry THF to ensure complete transfer of material.*

   *Synthesis of final product can be monitored by LC/MS. No significant conversion of starting material was observed beyond 5 hr; the generation of side products increases with longer reaction times.*

11. Concentrate crude material in rotary evaporator and redissolve in MeOH.

   *No work-up process is conducted in order to avoid water-mediated epoxide ring opening. Further, the TBS group comes off in situ during the HPLC purification when 0.1% TFA is present in solvents used as mobile phases.*

   *The amount of MeOH for dissolving the crude product depends on the scale of the reaction, the HPLC system, and the column used for purification.*

12. Purify crude solution by reverse-phase HPLC coupled to a UV-Vis detector at 210 nm, using the following mobile phases: (A) 95% Water/5% Acetonitrile/0.5% TFA (B) 95% Acetonitrile/5% Water and 0.5% TFA. Identify the peaks corresponding to the product material by LC/MS (Figure 3)
13. Combine the fractions containing the desired compound and concentrate them in a rotary evaporator to remove ACN and TFA.

14. Freeze the solution at $-80^\circ$C and lyophilize until a powder is obtained.

After HPLC purification, the purified yield should be between 70% and 80%. The amount obtained will depend on the amount of starting material, but we have successfully performed this reaction at up to 250 mg scale.

Store the powder at $-20^\circ$C as is or dissolved in DMSO.

**BASIC PROTOCOL 3**

**COPPER-CATALYZED CLICK REACTION OF (3) WITH BODIPY-FL AZIDE**

While the synthesized alkynyl fragment (2) possesses an intrinsic electrophile and a peptide recognition sequence for the proteasome in order for this probe to be useful for imaging and activity analyses, a reporter tag must be incorporated into the structure before or after in cellulo treatment (i.e., bioconjugation). This protocol details the copper-catalyzed ligation of an azide-containing cell permeable fluorophore with fragment (2) using copper sulfate as the copper source and sodium ascorbate as the reducing agent for generation of copper ions in the $+1$ oxidation state. The crude material from this protocol is purified using the same HPLC method as described above and lyophilized into a powder (Figure 4). Alternative azido fluorophores can be easily coupled as desired using the same protocol described below.

For information regarding the bioconjugation of alkyne- or azide-containing biomolecules see *Current Protocols in Chemical Biology*, 3: 153–162, December 2011.
Figure 4  (A) Structure of the synthesized epoxomicin-based probe coupled to a BodipyFL fluorophore (3). (B) Total ion chromatogram (TIC) of the synthesized chemical probe. >95% purity is obtained after purification via reverse-phase HPLC. (C) MS spectrum of the peak with retention time of 6.9 min. The observed mass (1127.6) matches the expected mass of the Bodipy clicked epoxomicin probe ([M+H]+ 1127.65 m/z). An additional mass observed in the spectrum correlates with doubly charged state (377.2 m/z), and [M+Na]+ adduct (1149.6 m/z).

Materials

Deionized water (DI water)
Fluorophore azide (e.g., Bodipy-FL azide, Lumiprobe catalog No. 11430)
Acetonitrile (ACN) HPLC grade
Water HPLC grade
Methanol (MeOH) optima
Trifluoroacetic acid (TFA)
Copper sulfate pentahydrate (CuSO₄) (Fisher catalog No. AC197730010)
Sodium ascorbate
Tris(benzyltriazolylmethyl)amine (THPTA) ligand (Optional) (Lumiprobe catalog No. F4050)

8-ml glass vials with caps
0.31-inch stir bars
Scale
Heat plate
Lyophilizer
HPLC system: Agilent 1200 preparative system with Zorbax Eclipse XDB-C18 column (9.4 × 250 mm, 5-μm particle size) or comparable system LC/MS Sonicator Glass container with rubber adaptor for lyophilizer Inert gas (argon/nitrogen) Rotary evaporator

1. Weigh out (2) and place it in glass vial. Dissolve in 0.5 ml of a 1:1 solution of DMSO:H₂O and cap vial.

2. Weigh 1 equivalent of a fluorophore that contains an azide (fluorophore selection will depend on what wavelengths are desired to be detected), 0.1 equivalents of copper sulfate pentahydrate, and 1 equivalent of sodium ascorbate and add it to the glass vial.

   Given that the mass of the solids might be too low to be accurately weighed, a concentrated solution of each of the solids can be prepared independently (Table 1) and subsequently added to the glass vial containing fragment (2).

   For example, assuming 1 equivalent equals 1 μmol, the following solutions should be prepared:

3. Place the stir bar inside the vial and flush with argon/nitrogen. Degas the mixture for 5 min by placing the vial in the sonicator bath.

4. Flush the vial once again with argon/nitrogen and cap it. Place the vial on a hot plate and allow the mixture to react at 80°C for 1 hr while stirring.

   If the fluorophore of choice is thermolabile, the reaction can be carried out in the presence of an accelerating ligand such as THPTA (0.1 equivalents) at 37°C.

5. Purify crude solution by reverse-phase HPLC coupled to a UV-Vis detector at 210 nm, using the previously described mobile phases. Identify the peaks corresponding to the product material by LC/MS.

6. Combine the fractions containing the desired compound and concentrate them in a rotary evaporator to remove ACN and TFA.

7. Freeze the solution at −80°C and lyophilize until a powder is obtained.

   Store the powder as is or dissolved in DMSO at −20°C for future testing.

**MONITORING PROTEASOME ACTIVITY BY SDS-PAGE IN HEK-293T CELLS**

The products of the previous steps can be used to assay proteasome activity in HEK-293T cells by SDS-PAGE. The model can be used to monitor proteasome activity in the presence and absence of candidate small molecule modulators in discovery activities. Once cells are cultured and plated, the activity-based probes are added, with or without
candidate modulators. After incubation, samples are prepared for separation and analysis by SDS-PAGE.

**Materials**

- Frozen vial of HEK-293T cells (ATCC® CRL-3216™).
- Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (Fisher catalog No. MT10027CV)
- M-PER™ Mammalian Protein Extraction Reagent (Fisher, catalog No. PI78501)
- Halt™ Protease Inhibitor Cocktail, 100× (ThermoFisher catalog No. 78429)
- 4×Laemmli sample buffer
- 2-mercaptoethanol
- 1× phosphate buffered saline (PBS)
- Coomassie Brilliant Blue stain (see recipe)
- Coomassie destaining solution (see recipe)
- Tris-HCl Glycine Running buffer (see recipe)
- 10-well, 50-μl/well gradient gel (4%–20%) Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad catalog No. 4561094)
- Protein ladder of choice (e.g., Dual Color, Kaleidoscope)

- Incubator with CO₂
- 37°C water bath
- Upright, bright-light microscope, with 4× and 20× lenses
- 15-ml sterile conical tubes
- T-75 cell culture flask
- 6-, 12-, or 24-well plate
- Hemocytometer with glass coverslip
- 1.5-ml Eppendorf tubes
- 0.6-ml Eppendorf tubes
- NanoDrop™ UV-Vis Spectrophotometer or comparable spectrophotometer, BCA assay kit (ThermoFisher catalog No. 23227), or protein quantitation kit of choice
- Deionized water
- Heating block
- Standard tabletop centrifuge
- Orbital shaker
- Biomolecular imager suitable for the selected fluorophore (e.g., Sapphire™ RGBNIR imager)
- Vacuum pump
- Vertical electrophoresis chamber and compatible power supply
- Plastic box with lid
- Optional: Poly-D-lysine hydrobromide (Sigma-Aldrich, catalog No. P6407)

All culture incubations are performed in a sterile humidified 37°C cell culture incubator with 5% CO₂.

A sterile laminar flow biosafety cabinet certified for the type of biosafety level should be used for cellular work. Sterilization of the hood is to be carried out by UV light irradiation 10 min prior to starting any work and by spraying down the surface using 70% (v/v) ethanol in Mili-Q water. All items must be wiped with the ethanol solution as well before placing them inside the hood.

All stock vials containing cells should be kept in a liquid nitrogen freezer until use.

1. Retrieve a frozen vial of HEK-293T cells from the freezer and thaw in a water bath at 37°C.
2. After thawing the vial transfer the suspension to a sterile 15-ml conical tube and gradually dilute it to 10 ml using DMEM with 10% FBS.

*Medium should be added slowly to minimize osmotic shock, which will have a detrimental effect on cell viability.*

3. Centrifuge the conical tube at 300 × g for 3 to 5 min to pellet the cells.

4. Carefully vacuum aspirate the medium and resuspend the pellet in 10 ml DMEM with 10% FBS.

*The medium change is important to remove residual DMSO from the cryopreservation medium.*

*We suggest warming the medium to at least room temperature 15 to 30 min prior to this step.*

5. Transfer solution to a T-75 culture flask and place it in the CO₂ incubator.

*The HEK-293T cell line is adherent. Therefore, a couple of hours after plating the cells will start to attach to the bottom surface. Longer incubation times yield a greater percentage of two-dimensional surface area that is covered with cells (confluency).*

*Check the culture flask periodically for cell health and density using an upright, bright-light microscope. Healthy cells remain attached to the bottom surface.*

6. Once 80% to 90% confluency is reached, remove the flask from the incubator and vacuum aspirate the medium.

7. Gently add 10 ml of 1× PBS to the culture flask to wash the cells and then vacuum aspirate the solution.

*Turn the culture flask upside down and add the PBS towards the now-bottom, cell-free surface. This is to prevent premature cell detachment.*

8. Add 1 ml of 0.25% trypsin-EDTA solution and return the flask to the incubator for 3 to 5 min.

*The presence of serum inactivates trypsin. Therefore, washing the cells prior to trypsin-mediated dislodgement is required to maximize the number of cells obtained.*

9. Visually inspect the culture flask to ensure complete detachment of the cells and then proceed to dilute the suspension with 9 ml DMEM with 10% FBS.

10. Transfer the cell suspension to a 15-ml conical tube and centrifuge at 300 × g for 5 min to pellet the cells.

11. Vacuum aspirate the supernatant and resuspend the cell pellet in 10 ml DMEM with 10% FBS by pipetting up and down.

12. Take 30 to 50 μl of the cell suspension and transfer to a 0.6-ml tube.

13. Place the glass coverslip on top of the hemocytometer and gently fill the chambers underneath, on both sides of the apparatus.

14. Place the hemocytometer under the bright-light microscope and count cells to determine the amount of cells per ml.

15. Prepare stock cell suspension based on the plate and number of samples to be used.

*The plate to be used depends on the amount of sample needed and the desired protein concentration. By following this protocol, at the end of sample processing, about 90 μl of lysate with a protein concentration of 3–5 mg/ml is usually obtained for each sample.*

16. Aliquot the stock cell suspension into the plate wells and place the plate back in the CO₂ incubator.
Table 2  Stock Cell Suspensions and Volumes Needed by Plate

| Plate   | Stock suspension (cells/ml) | Volume per well (μl) | Cells per well |
|---------|----------------------------|----------------------|----------------|
| 24-well | $40 \times 10^4$           | 500                  | 200,000        |
| 12-well | $50 \times 10^4$           | 1000                 | 500,000        |
| 6-well  | $1 \times 10^6$            | 2000                 | 2,000,000      |

Gently shake the stock cell suspension every 3–5 wells to make sure consistent suspension of cells as they start to settle with time.

Visually inspect the plate under the upright, bright-light microscope to ensure plating of the cells and consistency within wells.

17. Allow the plate to incubate until 90% to 95% confluency is achieved.

Typically, between 24 and 72 hr are required to achieve the desired cell density.

18. Remove the plate from the incubator and dose each well with the synthesized activity-based probe. Place the plate back in the CO$_2$ incubator for 1 hr.

A final probe concentration of 250–2000 nM is recommended; however, performing a dose-response curve is advised if a different cell line or fluorophore is used.

If a molecule is to be tested for its ability to modulate the activity of the proteasome isoforms, prepare stock solutions at the desired concentrations, and incubate them for at least 1 hr before adding the activity-based probe.

Replace the medium used to achieve the appropriate cell density with fresh medium containing the small molecules and/or the chemical probe to ensure an adequate supply of nutrients to the cells.

19. Remove the plate from the incubator and vacuum aspirate the medium from each well. Add M-PER$^\text{TM}$ lysis buffer with 100X Halt$^\text{TM}$ protease cocktail to each well and agitate the plate on an orbital shaker for 10 min.

The M-PER buffer is a convenient and mild lysis buffer suitable for a wide range of applications that allows the extraction of non-denatured proteins in high yields directly in plate. The amount of buffer recommended by the manufacturer is between 100 and 400 μl per well. For 24-well plate formats, 100 μl has been shown to be effective, yielding protein concentrations >3 mg/ml following the protocol here described.

Optional: If partial cell detachment is observed; therefore, if desired, coating the plate with poly-D-lysine can be done to ensure full adherence of the cells to the bottom of the plate.

a. Dissolve 5 mg of poly-D-lysine hydrobromide in 50 ml of autoclaved Mili-Q water (100 μg/ml).
b. Filter sterilize the resulting solution. Store excess at 4°C.
c. Add solution to each well. Volume is dependent of the plate used. Use values shown in Table 2.
d. Let the plate sit for 5 to 30 min at room temperature.
e. Vacuum aspirate the solution from each well and rinse the surface twice using the same volume of PBS.

20. Collect cell lysate in Eppendorf tubes and centrifuge at 14,000 $\times$ g for 10 min and 4°C to pellet cell debris.

21. Transfer the supernatant to fresh new tubes and measure the protein concentration following method of choice.

22. Based on the measured protein concentration, aliquot the samples in such a way that the total protein is equal amongst them.
Figure 5  (A) SDS-PAGE analysis conducted HEK-293T cells dosed with DMSO, miconazole (MO), a known 20S stimulator, and MG-132, a proteasome inhibitor, for 1 hr followed by the addition of the synthesized activity-based probe at 500 nM for 1 hr (2 hr total incubation time). (B) Quantification of the fluorescent signal obtained for the B2 and B5 proteasome subunits. Data were normalized to DMSO. Error bars denote standard deviation. A p-value \( \leq 0.05 \) was considered a statistically significant difference (* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \), **** \( p < 0.0001 \)).

For in-gel fluorescence of the beta subunits of the proteasome in HEK-293T cells, between 25 and 50 μg of total protein is desired. This might need to be adjusted based on the biological system used and the fluorophore tested.

23. Prepare loading buffer using 4× Laemmli sample buffer according to manufacturer instructions and add the necessary volume to each sample.

Briefly vortex the samples to mix the loading buffer with the lysates.

24. Heat samples in 95°C heat block for 5 to 10 min.

25. Briefly centrifuge the samples and load them in a 10-well, 50-μl/well gradient gel with compatible ladder.

It is recommended to not load more than 45 μl to each well to avoid overflowing into adjacent wells. Load samples slowly without disrupting the bottom of the wells.

26. Run the gel at 150 V and constant current for 55 min using 1× SDS Tris-HCl glycine running buffer and an electrophoresis cell with compatible power supply.

Time and voltage might need to be adjusted if using a gel with a different concentration of acrylamide. Check for manufacturer's recommendations before running.

27. Remove gel cassette from the electrophoresis cell and place the gel in a plastic box. Rinse three times with DI water.

28. Image gel using biomolecular imager suitable for the selected fluorophore.

If desired, it is feasible to distinguish all catalytic subunits of the proteasome by using gradient gels and high-resolution settings.

Band density can be quantified using Image Studio™, ImageJ, or other software programs.

29. Coomassie stain the gel for 30 to 60 min to ensure equal protein loading, and destain for 4 to 24 hr, or until the background is clear. See example in Figure 5.

**MONITORING PROTEASOME ACTIVITY BY FLOW CYTOMETRY IN HEK-293T CELLS**

Analysis of proteasome activity can be performed by other techniques such as confocal microscopy and flow cytometry. This Alternate Protocol is analogous to Basic Protocol 4 with the main difference being the sample preparation for the proteasome activity analysis.
Table 3 Minimum Experimental Controls Required for Successful Proteasome Activity Analysis by Flow Cytometry

| Controls            | Fluorescent probe | Viability dye |
|---------------------|-------------------|---------------|
| Unstained           | No                | No            |
| No probe            | No                | Yes           |
| No viability dye    | Yes               | No            |

by flow cytometry instead of SDS-PAGE. Once cells are cultured and plated, the live cell samples are rinsed and resuspended in PBS. A viability dye is used to discriminate live versus dead populations and fluorescence is measured by flow cytometry. Gating is performed sequentially by forward scatter versus side scatter to differentiate cells from debris based on size and granularity. Exclusion of the dead cell population is done based on the viability dye used.

**Materials**

- Frozen vial of HEK-293T cells (ATCC® CRL-3216™)
- Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS)
- 1× phosphate buffered saline (PBS)
- Incubator with CO₂
- Upright, bright-light microscope, with 4× and 20× lenses
- 15-ml sterile conical tubes
- T-75 cell culture flask
- 1.5-ml Eppendorf tubes
- TO-PRO™3 Ready Flow™ viability dye (ThermoFisher catalog No. R37170)
- Ice
- Ice bucket
- Cell scraper
- Standard tabletop centrifuge
- BD Accuri™ C6 Plus Flow Cytometer or compatible flow cytometer with fluorophore of choice

1. Incubation of cells as well as plating is to be carried out following the procedure described in Basic Protocol 4 (steps 1–17)

2. Remove the plate from the incubator and dose wells with the synthesized activity-based probe. Place the plate back in the CO₂ incubator for 1 hr.

   *A careful consideration of the appropriate controls is required for a successful experiment by flow cytometry (Table 3), therefore, make sure to have extra wells dedicated to the controls, as described in Table 3.*

   *If desired, when using the viability dye for the first time, preparing a control with only dead cell populations by heating a sample of cells in a 95°C heating block for 5 to 10 min could be useful when assessing the difference between live and dead cells.*

3. Remove the plate from the incubator and place on ice. Carefully vacuum aspirate the DMEM from each well.

4. Add 200 µl cold PBS to each well and gently dislodge the cells from the bottom of the plate using a cell scraper.

5. Collect the cell suspensions in a fresh tube and centrifuge at 4°C for 3 min at 300 × g.

6. Discard supernatant and resuspend in 200 µl of cold PBS. Centrifuge again as in Step 5.
Figure 6  (A) Forward versus side scatter (FSC vs. SSC) gating. (B) Viability gating. Alive populations are distinguished by a lower fluorescence in the APC channel. (C) Histogram showing the difference in fluorescence intensity between the unstained control (green) and samples treated with DMSO (red), a proteasome inhibitor (orange), and a proteasome activator (blue).

This washing process is necessary to remove unreacted probe present in the samples and thus avoid inaccurate fluorescence readings.

7. Remove supernatant and resuspend in another 200 μl of cold PBS. Add 20 μl of viability dye to all samples excepting the unstained and no viability dye controls. Incubate samples for 20 min at room temperature.

8. Proceed with flow cytometry analysis by recording 50,000 events per sample. See example in Figure 6.

Mix samples by pipetting up and down before analysis. Do not vortex. A sequential gating should be performed where a forward versus side scatter gating is used in the first level to differentiate cells from debris. A second level gating is performed in the channel corresponding to the excitation/emission wavelength of the dye, so that dead cells can be distinguish from live cells by the extent of fluorescence after incubation with the viability dye.

TO-PRO® 3 Ready Flow™ is a membrane-impermeable dye that binds to double-stranded DNA of dead cells; thus, dead populations can be distinguished alive ones by a shift in the fluorescence observed, in this case, the allophycocyanin (APC) channel.

REAGENTS AND SOLUTIONS

D.I. Water should be used for all preparations, unless otherwise noted.

Kaiser reagents

Reagent 1: 0.05 g/ml ninhydrin in ethanol (EtOH)
Reagent 2: 4 g/ml phenol in EtOH
Reagent 3: 0.2 mM KCN (aqueous) in pyridine
May be stored up to 6 months at room temperature.

Cleavage solution

95% (v/v) TFA
2.5% (v/v) DCM
2.5% (v/v) Triisopropylsilane (TIPS)
Use immediately after preparation.

Gel running buffer

25 mM Tris
192 mM glycine
Sodium dodecyl sulfate (SDS) 0.1% (w/v)
pH 8.3, adjust with 1 M HCl or 1 M NaOH
May be stored up to 2 years refrigerated.

**Coomassie staining solution**

3 g/L Coomassie brilliant blue R-250
45% (v/v) ml MeOH
45% (v/v) DI water
10% (v/v) glacial acetic acid
Dissolve Coomassie powder in MeOH and mix with DI water and acetic acid.
Filter before use.
May be stored up to 6 months at room temperature.

**Coomassie destaining solution**

50% (v/v) ml MeOH
40% (v/v) DI water
10% (v/v) glacial acetic acid
May be reused. Store at room temperature in a well-ventilated space.

**COMMENTARY**

**Background Information**

The proteasome is a highly conserved multisubunit complex responsible for the degradation of intracellular proteins in eukaryotic organisms (Dikic, 2017; Lee & Goldberg, 1998). It is essential for ensuring adequate protein levels by the controlled cleavage of functional proteins, as well as misfolded and oxidatively damaged species (Dikic, 2017). Given the pivotal role that such task entails, the proteasome regulates a myriad of cellular processes, including cell cycle progression and immune response (Kito et al., 2020; Zerfas, Maresh, & Trader, 2020).

Dysregulation of this machinery has been implicated in cancer and neurodegenerative diseases. Given the uncontrolled proliferation of cancerous cells, these abnormal cells rely on a robust proteasome system to prevent cell death; therefore, proteasome inhibitors can be valuable tools for their ability to induce apoptosis via different mechanisms (Almond & Cohen, 2002). On the other hand, a decline in proteasome function has been associated with age-related diseases (Hipp et al., 2019; Huang & Figueiredo-Pereira, 2010). Thus, the use small molecule activators of the various isoforms of the proteasome represent an alternative approach to treatment (Leestemaker et al., 2017).

Several fluorescent tools have been developed to monitor proteasome activity (Berkers et al., 2007; Capobianco, Lerner, & Goldman, 1992; Kisselev & Goldberg, 2005; Zerfas et al., 2020), but their synthesis can be challenging, and derivatives with different fluorophores and reporter tags are generally not commercially available. Here we describe an efficient synthesis method to obtain a clickable version of epoxomicin (Basic Protocols 1 and 2), a potent proteasome inhibitor, and the coupling of an azide-containing reporter tag via copper-catalyzed cycloaddition (Basic Protocol 3). Additionally, we describe their use for proteasome activity analysis by SDS-PAGE and flow cytometry (Basic Protocol 4 and Alternate Protocol).

**Critical Parameters**

**Anhydrous solvents**

The coupling of the epoxide fragment to the peptide backbone represents the key step towards the synthesis of the final molecule. Given the electrophilic nature of the 3-membered ring, the presence of moisture in the solvents can increase the amount of side products.

**Purity of activity-based probe**

The selectivity and potency of the epoxomicin-based probe lies on the peptide backbone of the molecule; therefore, impurities with different peptide sequences or stereochemistry can heavily impact these aspects. The purity of the final molecule should be ≥95% as determined by HPLC or LC/MS.

**Washing live cells with PBS**

In order to obtain accurate and reproducible results of proteasome activity by flow cytometry, live cells should be washed thoroughly to remove excess unreacted probe.
| Problem                                                                 | Possible cause                                  | Solution                                                                 |
|----------------------------------------------------------------------|------------------------------------------------|--------------------------------------------------------------------------|
| Little to no labeling of beta subunits                              | Cell impermeable fluorophore                     | Confirm permeability of the synthesized probe by confocal microscopy.    |
|                                                                     |                                                 | Cououple a different fluorophore to the alkyne-based epoxomicin core.  |
|                                                                     | Low concentration of chemical probe             | Increase the concentration of the chemical probe or incubate for a longer time. |
| Low protein concentration                                            | Insufficient incubation time                    | Increase the incubation time. Optimal protein concentrations are achieved between 24 and 72 hr of incubation after plating. |
| Poor yield                                                           | Non-anhydrous solvents                          | Freshly distill solvents before use. Use new bottles and store them properly by purging with an inert gas, taping the pierced septa, and parafilm around the bottle cap. |
|                                                                     | Low reaction time                               | The quality of the reagents might decrease over time; therefore, increasing the reaction time or the number of equivalents used can increase the reaction yields. |
| Little to no change in fluorescence upon treatment with proteasome modulator | Not a modulator of the proteasome isoforms      | Validate the ability of the compound to modulate the activity of the proteasome using purified proteasome (20S or 26S). |
|                                                                     | Insufficient incubation time                    | Increase the reaction time.                                             |
| Low potency                                                          |                                                | Increase the concentration of the molecule if the cell viability is not compromised. |
| Low dynamic range of the synthesized probe                          | If positive and negative controls are included in the experimental design, and they fail to elicit a change in labeling compared to the basal level, synthesize a new probe including a longer linker chain. A bigger molecule represents a more challenging substrate to the proteasome isoforms; therefore, the labeling rate will be lower, and the difference between an activated/inhibited proteasome versus a non-modulated proteasome will be more apparent. |

Possible causes and their corresponding potential solutions are included as well.

Troubleshooting
See Table 4 for problems that might be encountered, possible causes, and potential solutions.

Understanding Results
It is expected to obtain >80% yield for the synthesis of the peptide fragment (Basic Protocol 1) when conducting the reaction at 1 g scale or less. The amide coupling performed to obtain the alkyne-based epoxomicin core (Basic Protocol 2) is expected to yield 50% to 70% of the target molecule. The most common side products are obtained as a result of the epoxide opening. Coupling an azide-containing fluorophore to fragment (3) through click chemistry is expected to yield between 60% and 95% of the clicked product, with the purification process being critical to the amount obtained. The reaction typically proceeds without major side products, unless thermolabile or moisture-sensitive reagents are used.

In SDS-PAGE (Basic Protocol 4) and flow cytometry (Alternate Protocol) assays, a change in fluorescence is expected between untreated cells and cells incubated with the probe; likewise, when a proteasome inhibitor or stimulator is included, a shift in fluorescence in the channel corresponding to the fluorophore should be observed.

Time Considerations
Synthesis of fragment (2) is expected to take ~1 day with overnight concentration under high vacuum to remove residual moisture. Synthesis of fragment (3) followed by HPLC purification is expected to take 1 to 3 days depending on the reaction scale and the HPLC...
system used. Cell culture can take between 7 and 10 days depending on the concentration of cells in the frozen vial, the incubation time after plating, and the incubation time after dosing the small molecule modulator. Proteasome activity analysis by SDS-PAGE followed by in-gel fluorescence scanning is expected to take ~5 to 8 hr depending on the number of samples and the gel format used. Analysis by flow cytometry is expected to take between 2 and 5 hr.

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Author Contributions
Andres F. Salazar-Chaparro: Formal analysis, Investigation, Methodology, Original draft, review, and editing; Saayak Halder: Investigation, Review and editing; Darci J. Trader: Project administration, Review and editing.

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

Data Availability Statement
The data that support the findings of this study are available via https://doi.org/10.1002/cbic.202100710.

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