Fluorescent Probes

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Tunable Probes with Direct Fluorescence Signals for the Constitutive and Immunoproteasome

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Abstract: *Electrophiles are commonly used for the inhibition of proteases. Notably, inhibitors of the proteasome, a central determinant of cellular survival and a target of several FDA-approved drugs, are mainly characterized by the reactivity of their electrophilic head groups. We aimed to tune the inhibitory strength of peptidic sulfonate esters by varying the leaving groups. Indeed, proteasome inhibition correlated well with the pK_a of the leaving group. The use of fluorophores as leaving groups enabled us to design probes that release a stoichiometric fluorescence signal upon reaction, thereby directly linking proteasome inactivation to the readout. This principle could be applicable to other sulfonyl fluoride based inhibitors and allows the design of sensitive probes for enzymatic studies.*

Up to 2% of the human genome encodes for proteases, which reflects their indispensable importance for cell function.[1] Although a variety of proteolytic mechanisms have been discovered for these enzymes, they all follow common principles of catalysis: 1) direct (aspartic, glutamic, and metallo-proteases) or 2) indirect (serine, threonine, and cysteine proteases) activation of a water molecule to facilitate its nucleophilic attack on a peptide bond. As major intracellular proteolytic complexes, the constitutive 20S proteasome (cCP; CP = core particle) and immunoproteasome (iCP) exploit the basicity of an N-terminal threonine (Thr1) at the active site of their catalytic subunits β1c/i, β2c/i, and β5c/i.[2] In contrast to moderately nucleophilic water (pK_a = 15.7), the alkoxide of Thr1 ($pK_a \approx 9$)[3] readily attacks the carbonyl carbon atom of peptide bonds in protein substrates. This reactivity is exploited by antineoplastic agents such as bortezomib[4] and carfilzomib[5] (CFZ; Figure 1), which covalently bind to Thr1 with C-terminal electrophilic head groups (also called warheads). A systematic evaluation[6] revealed that CP inhibitors with various warheads are mainly characterized by the chemical reactivity of the electrophile.[7] However, this chemical reactivity is difficult to adjust for the majority of CP inhibitors.

In contrast, the recent use of sulfonyl fluorides in iCP and cCP inhibitors allows alterations in warhead reactivity.[8] For this purpose, we exchanged the fluoride leaving group (LG) of peptidic sulfonyl fluorides[8] (PSF; Figure 1) with phenolic LGs to generate sulfonate esters (PSE; Figure 1). This enables fine-tuning with electron-withdrawing groups on the phenol system, which results in differing pK_a values and thus variable LG ability. Similar studies were conducted using acyloxy or aryloxy methyl ketones to target cysteine proteases and showed a correlation between the LG pK_a (conjugated acid) and the inhibition rate.[9] Additionally, the evaluation of analogues of the CP inhibitor salinosporamide A with varying LGs suggested a correlation between LG ability and potency.[10] Considering this, we focused on phenols with pK_a values ranging from 6 to 10 to generate hydrolytically stable peptidic sulfonate esters (PSEs **1**–**3**).[11] Our probe design focused on fluorinated LGs that differ in their LG pK_a but have similar steric demands during binding. We therefore prepared a sulfonyl chloride precursor of L-leucine,[8] which was derivatized with phenol (pK_a = 9.9), 2,4,6-trifluorophenol (TFP; $pK_a \approx 7.6$), or pentafluorophenol (PFP; pK_a = 5.5).[12] In fact, PFP esters are substitutes for acid halides and succinimidyl esters, which are used in conjugation reactions due to their water stability and long shelf life.[13] The phenyl sulfonate ester precursors were fused through HATU-mediated amide coupling to a β5-specific CFZ peptide backbone prepared by Fmoc-based solid-phase peptide synthesis. This

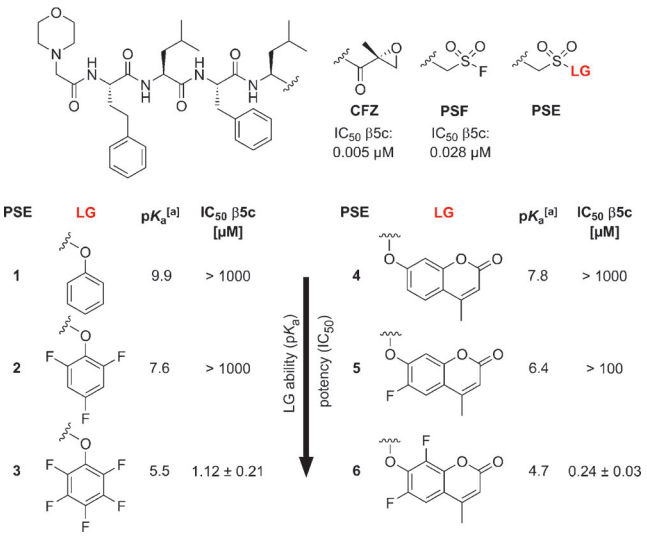

Figure 1. Carfilzomib (CFZ) and its peptidic sulfonyl fluoride (PSF), as well as sulfonate ester (PSE) counterparts. IC_{50} values were determined against human cCP in fluorogenic (PSEs **1**, **2**, **3**; 10 μg mL^{-1} cCP) and luminogenic substrate assays (PSEs **4**, **5**, **6**; 5 μg mL^{-1} cCP). [a] pK_a values are given for the conjugate acids of the leaving groups (LGs).

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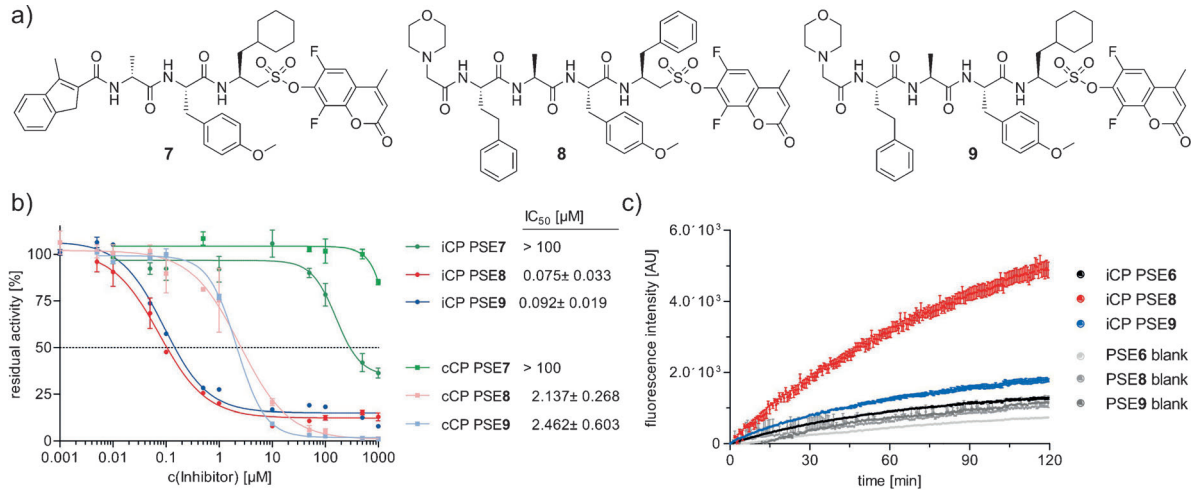

Figure 2. a) Probes optimized for iCP: PSEs **7**, **8**, and **9**. b) In vitro IC_{50} assays against the chymotrypsin-like (ChTL) β5 activity of human iCP and cCP after 1 h incubation with various concentrations of PSE **6**, **7**, **8,** and **9** using a luminogenic Suc-LLVY-aminoluciferin substrate assay. The data were normalized to DMSO-treated controls and are presented as relative activity with standard deviation. c) Time course of the fluorescence increase when using PSEs **6**, **8,** and **9** at 1 μM concentration at 30 °C in the presence of 50 $\mu g\,mL^{-1}$ iCP. It is of note that the reaction between iCP and the respective PSE does not saturate. The data shown in parts (b) and (c) are depicted as the mean ± SD of triplicates.

convergent synthesis strategy yielded the PSEs **1**, **2**, and **3** (Figure 1). The potency of PSEs **1**, **2**, and **3** against the chymotrypsin-like (ChTL) activity of subunit β5c was determined by using a fluorogenic 7-amino-4-methylcoumarin (AMC) substrate assay with purified human cCP. The non-fluorinated PSE **1** and the tri-fluorinated PSE **2** did not block ChTL activity ($IC_{50} > 1$ mM). Notably, PSE **3** substantially inhibited subunit β5c ($IC_{50} = 1.12$ μM, Figure 1 and Figure S1 in the Supporting Information), but with at least 40-fold decreased potency compared to its sulfonyl fluoride counterpart ($IC_{50} = 0.028$ μM). To investigate the binding mode of PSE **3** with CP, we soaked *Saccharomyces cerevisiae* CP crystals with the compound for X-ray analysis. Structure elucidation revealed a mechanism analogous to that of PSFs:[8a] 1) After adduct formation at Thr1 through sulfonylation, the inhibitor is displaced by intramolecular attack of the amino function in Thr1 to form an aziridine intermediate (2.5 Å resolution, $R_{free} = 21.9\%$, PDB ID: 5LAI, Figure S2 and Table ST1). 2) Subsequent nucleophilic aziridine-opening by the base Lys33 results in irreversible crosslinking of the β5 subunit (2.9 Å resolution, $R_{free} = 20.1\%$, PDB ID: 5LAJ, Figure S2 and Table ST1). The structural data thus confirm release of the PFP LG as an integral part of the inhibition mechanism. Since the steric requirements are similar for all of the phenyl systems used here, the increasing potency suggests a direct correlation between the pK_a value of the leaving groups and the reactivity of the electrophiles.

Since the release of the LG is an integral part of the inhibitory mechanism, this led us to design PSE probes that emit a signal upon inhibition. To this end, we employed easily detectable fluorophores as LGs for the quantification of CP (PSE **4**–**6**). In contrast to fluorescent assays, in which peptidic substrates are constantly cleaved, PSE probes would liberate a single fluorophore molecule per active site upon inhibition. As such, these probes could be used for the direct estimation of proteasome activity without downstream analysis. A similar mechanism is used by fluorescently-quenched activity-based probes, a variation of activity-based protein profiling.[14] In this case, a fluorescence-quenching LG is directly released after protease inactivation, thus retaining the fluorescent inhibitor bound to the target.[15]

Based on the aforementioned finding that a pK_a range between 4 and 6 is favorable, we utilized methylumbelliferones (MU) as small reporter LGs (PSE **4**–**6**, Figure 1). MUs are easily tuneable in their pK_a values through simple fluorination and are synthetically accessible through Pechmann condensation to yield 6-fluoro-4-methylumbelliferone (FMU; $pK_a = 6.4$) and 6,8-difluoro-4-methylumbelliferone (DiFMU; $pK_a = 4.7$).[16] Additionally, they feature favorable photophysical properties, including a high quantum yield ($\Phi_F = 0.89$), linear fluorescence over an appropriate range of concentrations (Figure S3), and high resistance to photobleaching[16]. Moreover, MUs and their derivatives are relatively small in size, which is favorable in view of the limited space at the proteasomal active site. Conveniently, DiFMU bound to sulfonate ester groups (DSE) is non-fluorescent and is widely used in substrate assays for several enzyme classes.[17] As a control, we prepared the non-fluorinated PSE **4** (MU; $pK_a = 7.8$;[16] Figure 1). The IC_{50} values against purified human cCP were determined by using a luminogenic aminoluciferin substrate assay, which is orthogonal in its readout to the released MU fluorophores. In line with our previous results, PSE **6** with a DiFMU LG was over 400-times more active ($IC_{50} = 0.236$ μM, Morrison $K_I = 0.062$ μM, K_I as a function of $K_{Obs} = 0.18$ μM; Figures S4–S6) than the monofluorinated PSE **5** ($IC_{50} > 100$ μM) and non-fluorinated PSE **4** ($IC_{50} > 1$ mM). No inactivation of β1c (caspase-like, $IC_{50} > 1$ mM), β2c (trypsin-like, $IC_{50} > 1$ mM), or several other proteases was observed (Figure S7). These findings demonstrate that the designed PSEs require LGs with a pK_a less than 5 for inhibition in the nanomolar range. To rank the DSE head group in comparison with other

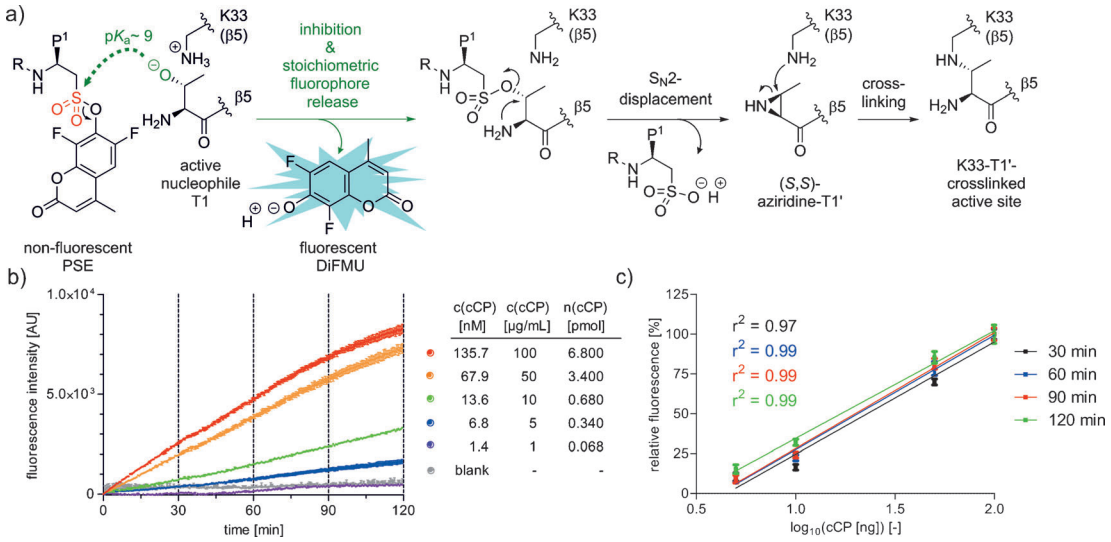

Figure 3. a) Proposed mechanism for the reaction of the PSEs with the proteasomal active site.[8a] The substituent R indicates the rest of the inhibitor backbone, and P^1 refers to the residue protruding into the S1 specificity pocket. b) Time course of the fluorescence increase with varying concentrations of cCP (1–100 ng mL^{-1} cCP) with 0.01 % SDS treated with 100 nM PSE **6** for 2 h at 30 °C and pH 7.5 (excitation: $\lambda = 360$ nm; emission: $\lambda = 460$ nm). It is of note that the reaction between PSE **6** and cCP does not saturate in the monitored time frame. c) Normalized fluorescence intensity at the indicated time points [vertical lines in (b)] depicted against the concentration of cCP [µg mL^{-1}] displays a linear correlation between cCP activity and fluorescence, irrespective of the chosen time point. r^2 = coefficient of determination. The data in parts (b) and (c) are shown as the mean ± SD of triplicates.

established electrophiles, we compared a set of proteasome inhibitors in a substrate assay. For this purpose, we evaluated the reactivity of different warheads (Figure S8) independently of the peptidic backbone by using inhibitors that feature the same sequence (Z-LLL).[6] In accordance with our structural data, the DSE electrophile displayed an irreversible binding mode and resided between epoxyketone and α-ketoaldehyde warheads in terms of reactivity.

Following our characterization of the DSE head group, we utilized β5i-optimized backbones for iCP probes. The iCP is a potential therapeutic target since it constitutes a key element in antigen presentation and cytokine production, thereby participating in immune responses.[18] Since iCP binding preferences differ from those of cCP, we assessed state-of-the-art inhibitor backbones (PSEs **7**–**9**, Figure 2a). The β5i-specific tripeptide of LU-035i (IC_{50} (β5i) = 0.011 µM)[19] served as a blueprint for PSE **7**, while the PSE **8** tetrapeptide is derived from ONX 0914 (IC_{50} (β5i) = 0.028 µM).[18a] These backbones were coupled to L-phenylalanine and 3-cyclohexyl-L-alanine DiFMU precursors.

To estimate inhibitor potency, we determined the IC_{50} values of PSE **7** and **8** against β5i versus β5c for human iCP and cCP (Figure 2b and Figure S9). Although the backbone of PSE **7** is identical to that of its potent epoxyketone equivalent LU-035i, PSE **7** only displays activity above a concentration of 100 µM. This is in agreement with findings that PSFs require at least tetrapeptidic backbones for sufficient stabilization at the active site.[8] Comparison of the DiFMU sulfonate ester electrophile with other warheads indicates that it reacts rather slowly with Thr1 (Figure S8). In contrast, PSE **8** exhibited inhibition of β5i in the low nanomolar range ($IC_{50} = 0.075$ µM) and moderate inhibition of β5c ($IC_{50} = 2.14$ µM), which represents a 25-fold selectivity comparable to that of PSF inhibitors.[8] In view of this, we measured the release of DiFMU by PSE **8** in the presence of iCP. Surprisingly, altering the backbone resulted in increased fluorescence signals in comparison to PSE **6** (Figure 2c). To estimate the reactivity of PSE **6** and **8** aside from proteasome binding, we evaluated the rates of hydrolysis at different pH values (Figure S10). Indeed, PSE **8** (P1-phenylalanine) was more susceptible to hydrolysis than PSE **5** (P1-leucine) despite having an identical warhead. This unexpected contribution of the P1 sidechain to the reactivity of the probe points towards an additional possibility for fine-tuning of the inhibitor. Our hypothesis was verified by synthesizing PSE **9**, which differs from PSE **8** only in its P1 site. Since we propose that the increased reactivity of PSE **8** originates from the electron-rich P1 aryl system, we exchanged this position with a saturated cyclohexyl residue in PSE **9**. While this exchange had no effect on β5i and β5c inhibition (IC_{50} (β5i) = 0.092 µM, IC_{50} (β5c) = 2.46 µM), PSE **9** displayed hydrolysis similar to PSE **6** in pH-dependent assays (Figure S10). This result indicates that the reactivity is indeed additionally influenced by properties of the P1 side chain. Notably, the high sensitivity given by the release of a single fluorophore upon inhibition enables detection of these minute changes in affinities, which are not visible in substrate assays.

Based on our proposed mechanism, every released DiFMU fluorophore correlates with a blocked proteasomal active site in a single-turnover reaction (Figure 3a). We investigated whether the fluorescence signal is proportional to the amount of inhibited CP and thus to the active CP in the sample. Since iCP-specific tissues are difficult to obtain in sufficient quantities, we purified human cCP from erythrocytes for quantitative assays with PSE **6** as a suitable probe. The fluorescence emission was determined at physiological

relevant concentrations (ca. 190 nM ≙ 140 μg mL^{-1} in living cells), while the concentration of PSE **6** was kept constant (100 nM: Figure 3b, or 1 μM: Figure S11).[20] We monitored the fluorescence over the course of 2 h and found that the signal intensified linearly with increasing amounts of cCP (Figure 3b). A quantitative estimation of cCP concentrations was possible with PSE **6** below its IC_{50} value (100 nM PSE; Figure 3c), where background fluorescence resulting from probe hydrolysis is negligible (Figure S12). Discernible signal resulting from the action of the inhibitor was found up to 5 μg mL^{-1} cCP, despite the lack of any signal amplification. This corresponds to an absolute protein amount of 340 fmol in a sample volume of 50 μL.

To verify that the fluorescence is not caused by unspecific reactions of PSE **6** with nucleophilic side chains in the protein, we used varying amounts of bovine serum albumin (BSA) as a negative control. The fluorescence in the presence of BSA was comparable to background hydrolysis (Figure S13). In order to confirm that PSE **6** derived fluorescence is caused by CP activity, we inactivated the latter with 50 μM of different, well-characterized proteasome inhibitors prior to the measurement (Figure S14). We were able to show that pre-incubated CP samples exhibited strongly reduced fluorescence (Figure S15). This confirms that fluorophore release from PSE **6** results directly from the inhibition mechanism and reflects the active concentration of CP in the sample. However, the applicability of PSEs as probes in cells or cell lysates is so far limited. This is in part due to side reactions occurring at physiological concentrations of other cellular components, for example glutathione (Figure S15).

In summary, we have described a new class of inhibitors for the constitutive proteasome and immunoproteasome, the efficiency of which can be fine-tuned through altering the properties of the LG. By using fluorescent probes as LGs, we were able to generate tools that allow direct detection of relative proteasomal activity and concentration in solution through a simple readout. The lack of linkers, spacers, or the need for downstream reactions for signal amplification avoids possible side reactions, thus rendering the system precise and sensitive. Our findings suggest that DiFMU sulfonate esters as electrophiles show a similar range of reactivity to the sulfonyl fluoride warhead. Therefore, this strategy for the design of fluorogenic probes as research tools could in principle be applied to a range of sulfonyl fluoride based inhibitors, which are widely used against a diverse set of targets.[21]

Acknowledgements

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Keywords: fluorescent probes · inhibitors · probe design · proteasome · structure–activity relationships

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Communications

Fluorescent Probes

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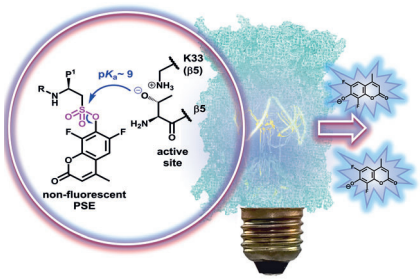
Tunable Probes with Direct Fluorescence Signals for the Constitutive and Immunoproteasome



A tunable light: The potency of peptidic sulfonate esters as proteasome inhibitors can be fine-tuned by altering the pK_a of the leaving group. The introduction of a fluorescent dye as a leaving group allows direct monitoring of proteasomal activity and can be used for quantification. This concept might inspire future work on other hydrolases.