Synthetic methodology towards ADP-ribosylation related molecular tools

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5 | *N*-Acylazetine as a Dienophile in Bioorthogonal Inverse-Electron-Demand Diels–Alder Ligation

Introduction

Bioorthogonal chemistry strives to chemoselectively modify biomolecules, such as proteins, carbohydrates and lipids, within the complex environments of cell-lysates, living cells and, ultimately, in living animals. Bioorthogonal chemistry has pushed forward various fields of research, such as activity-based protein profiling, *in vitro* and *in vivo* imaging and modified metabolite labeling.[1] Ideally, in a bioorthogonal process a reactive group incorporated into the biomolecule of interest reacts selectively with a complementary reactive group modified with a reporter moiety, such as a fluorescent label to enable visualization of the target or an affinity probe for post-labeling purification.[2] To enable this, a bioorthogonal tag should be inert to the broad spectrum of functional groups that reside in a biological system, while exerting fast enough reaction kinetics for conjugation to occur at nanomolar concentrations. Additives to facilitate the reaction between the tag and the reactive functionality of the reporter group are best avoided. Finally, the tag should be sterically compact to minimize unfavorable steric interactions with the biological system being studied and be synthetically accessible.

In the last decade, several bioorthogonal ligation strategies have been developed. Among these, prominent transformations are the Staudinger-Bertozzi ligation[3], the copper-catalysed[4] and the strain-promoted azide-alkyne [2+3] cycloaddition.[5] An important recent development is the inverse-electron-demand Diels-Alder reaction (IEDDA), which exhibits the fastest reaction kinetics among all commonly used bioorthogonal transformations, while being chemo-selective, efficient and additive-free.[6–8] In the most typical setup, IEDDA uses a strained alkene tag, while its reaction partner is a reporter-group functionalized tetrazine derivative. Well-established strained alkene handles are norbornene (Figure 1, A) and *trans*-cyclooctene (Figure 1, B). Although these alkenes react exceptionally fast, their steric bulk and

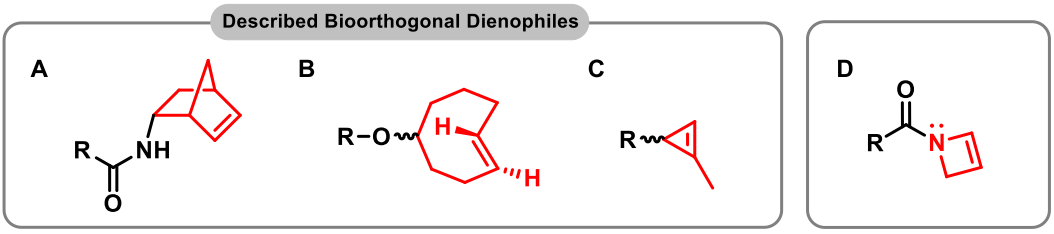


Figure 1: Known bioorthogonal dienophilic moieties (A-C) and the *N*-acylazetine tag (D) described in this chapter.

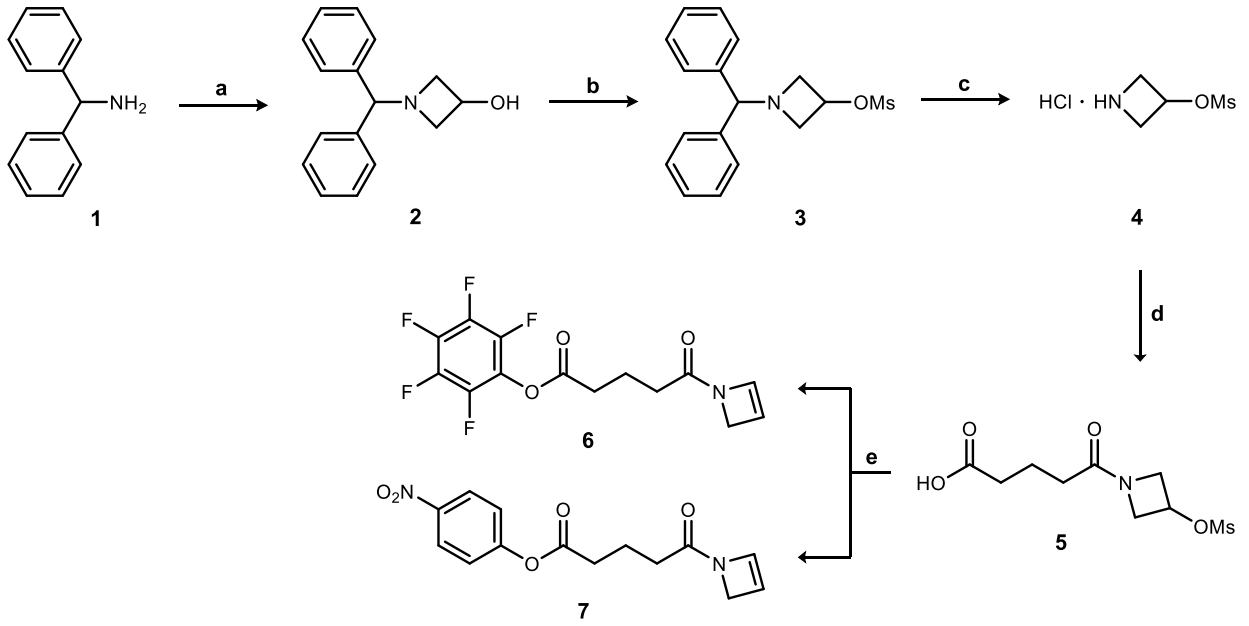
lipophilicity may induce a biological response, bringing a paradoxical problem, known as the observer effect, into chemical biology. Hence, there is room for the development of more compact dienophiles for tetrazine mediated bioorthogonal cycloadditions. Recently, Devaraj and co-workers introduced the methylcyclopropene core (Figure 1, C), as dienophile for IEDDA that combines small size with fast reaction kinetics.[9]

Currently, all contemporary dienophiles – norbornene, *trans*-cylcooctene and cyclopropene – used in IEDDA-based bioorthogonal chemistry rely on ring strain to attain the desired reactivity towards tetrazines. These were first described in the seminal 1990 report by Sauer and co-workers on the reactivity of cyclic dienophiles in IEDDA processes.[10] In the same paper, it was shown that alkenes activated by a single electron-donating heteroatom adjacent to the double bond, would exhibit a higher reactivity towards tetrazine relative to their non-conjugated analogues. Introduction of a heteroatom into the cyclobutene scaffold should therefore lead to a viable dienophile for IEDDA-based bioorthogonal chemistry. Following this reasoning, a 2-azetine would be the smallest viable core that could utilize this effect in addition to ring-strain, with the ring-nitrogen amenable for functionalization to yield a new and compact bioorthogonal tag. Although *N*-alkylazetines are theoretically the more electron-rich species, *N*-acylazetines (Figure 1, D) were considered more suitable candidates because of their reported stability.[11] This chapter describes the development and optimization of a synthetic route towards a bioorthogonal tag based on *N*-acylazetine moiety and a study towards its applicability in IEDDA-based bioorthogonal chemistry.

Synthesis Results and Discussion

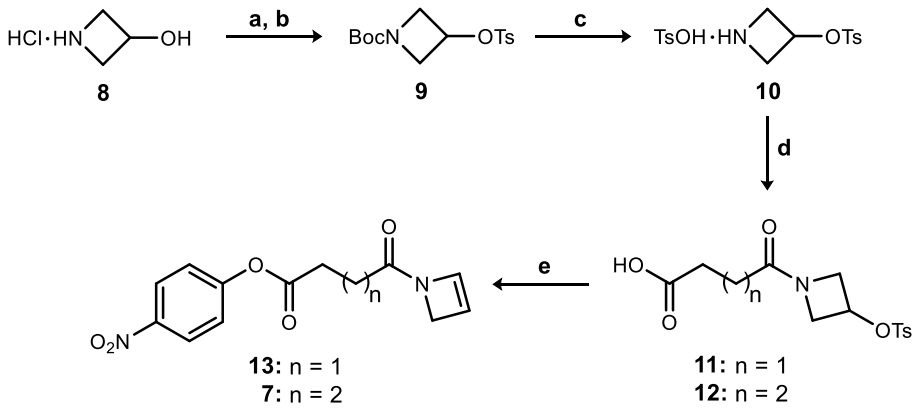
The synthesis of linkable *N*-alkyl-azetines **6** and **7**, equipped with an activated ester for further modification, (Scheme 1) commenced with the preparation of the azetidine core.[12,13] Epichlorohydrin was reacted with benzhydrylamine in a two-step one-pot procedure to afford protected azetidine **2**. The hydroxy group of **2** was mesylated, affording methanesulfonate **3**. Deprotection of the benzhydryl with chloroethyl chloroformate provided the acyl-intermediate, which was degraded by refluxing in methanol to give 3-mesylazetidine hydrochloride **4**. The route from **1** to **4** was optimized in such a way that each intermediate could be purified through crystallization and washing steps (in 48% yield over three steps). With free amine **4** in hand, the ring-opening of glutaric anhydride could commence. Initially this was carried out under the agency of triethylamine. However, during the reaction, the methane sulfonate was substituted by chloride originating from 3-mesylazetidine hydrochloride. Furthermore, the use of triethylamine complicated the column purification of the formed carboxylic acid. These problems were addressed by switching to potassium carbonate as the base, and premixing **4** with silver methanesulfonate, to precipitate the chloride as AgCl. After successful conversion, the free acid was regenerated from the potassium salt by treating with Amberlite-H^+, providing **5** in 79% yield. The following elimination-esterification sequence was carried out in a one-pot procedure. First, methanesulfonic acid was eliminated with KO*t*Bu to afford the *N*-acylazetine moiety. Next, the carboxylate was converted to either pentafluorophenol (PFP, **6**) or *p*-nitrophenol (PNP, **7**) esters via EDC mediated condensation with the appropriate alcohol. Both esters were readily obtained, however PFP-ester **6** proved to be unstable and could not be stored for extended periods of time. Another disadvantage was its gel-like consistency

at room temperature, making it difficult to handle. On the other hand, PNP-ester **7** was isolated as a crystalline solid that is stable at room temperature for at least three months.



Scheme 1: Synthesis of active ester functionalized *N*-acylazetine **6** and **7**. Reagents and conditions: [**a**] i: Epichlorohydrin, *i*PrOH, 30 ˚C, 16h. ii: $NaHCO_3$, MeCN, reflux, 30h, 83%. [**b**] MsCl, TEA, DCM, -40 ˚C, 0.5h, 80%. [**c**] i: Chloroethyl chloroformate, DCE, reflux, 1.5h. ii: MeOH, reflux, 2h, 66%. [**d**] i: MsOAg, MeCN, 15 min. ii: K_2CO_3, glutaric anhydride, reflux, 2h, 79%. [**e**] i: KO*t*Bu, DMF, 50 ˚C, 2h. ii: EDC·HCl, PFP/PNP, 1.5h, **6:** 63% **7**: 85%.

Besides the required physical properties, the applicability of a ligation handle is also dependent on its synthetic accessibility. The above described synthesis to acylazetine **7** consists of six reactions of which the ring-opening of glutaric anhydride by 3-mesylazetidine and the sequential one-pot elimination–esterification sequence are the key steps. Despite various attempts of optimization, these steps constrain the overall efficiency and scalability. Therefore another synthetic route towards **7** was explored (Scheme 2). Commercially available 3-hydroxyazetidine **8** was used as starting compound and the mesyl group was replaced by the tosyl group that is UV-detectable and a slightly better leaving group. The synthesis started with the sequential Boc-protection and tosylation of 3-hydroxyazetidine (**8**), providing protected azetidine **9** in 72% over two steps. To prevent potential tosyl displacement the Boc-group was cleaved using *p*-toluenesulfonic acid. Purification by crystallization from MeOH yielded **10** in 80%. Ring-opening of succinic and glutaric anhydride with **10** was carried out under the agency of potassium carbonate in refluxing acetonitrile, yielding the four- (**11**) and five-carbon spacer (**12**), respectively. Next, the key elimination was initiated by the addition of a KO*t*Bu solution in THF. The use of the tosyl improved solubility while the reaction now proceeded readily at room temperature. The respective *N*-acylazetine intermediates were treated *in situ* with bis(*p*-nitrophenyl)carbonate, to provide linkable handles **7** (80%) and **13** (69%) in good yields over two steps. Initially, the *p*-nitrophenol was introduced using DIC or EDC as coupling reagents. However, the use of bis(*p*-nitrophenol)carbonate resulted in cleaner and more consistent conversions.



Scheme 2: Improved synthesis of activated *N*-acylazetine handle **7** and the four-carbon variant **13**. Reagents and conditions: [**a**] i: Boc_2O, TEA, MeOH, 0 ˚C, 2h, used crude. [**b**] TsCl, TEA, DCM, 2h, 86% over 2 steps. [**c**] *p*TsOH, DCE, reflux, 16h, 79% [**d**] Succinic- (**11**) or glutaric (**12**) anhydride, K_2CO_3, MeCN, reflux, 6h, 50% **11**, 70% **12**. [**e**] KO*t*Bu, DMF, 2h. ii: bis(*p*-nitrophenol)carbonate, 16h, **13**: 80% **7**: 69%.

Reaction Kinetics

To investigate the rate of the IEDDA reaction of *N*-acylazetine with tetrazine, **7** was coupled with morpholine to make water soluble *N*-acylazetine **14** (Figure 2). A ten-fold excess of model compound **14** was reacted with tetrazine **15**.[14] The rate was determined by monitoring the absorbance of the tetrazine at 517 nm (Figure 2). The pseudo-first order rate constant k_1 was established to be $4.5 \cdot 10^{-3} \pm 1.6 \cdot 10^{-3}$ s^{-1} at 20 °C in a solution of 12% DMSO in water. In a separate experiment, the second-order rate constant k_2 was approximated to be 0.39 ± 0.1 s^{-1} M^{-1}.

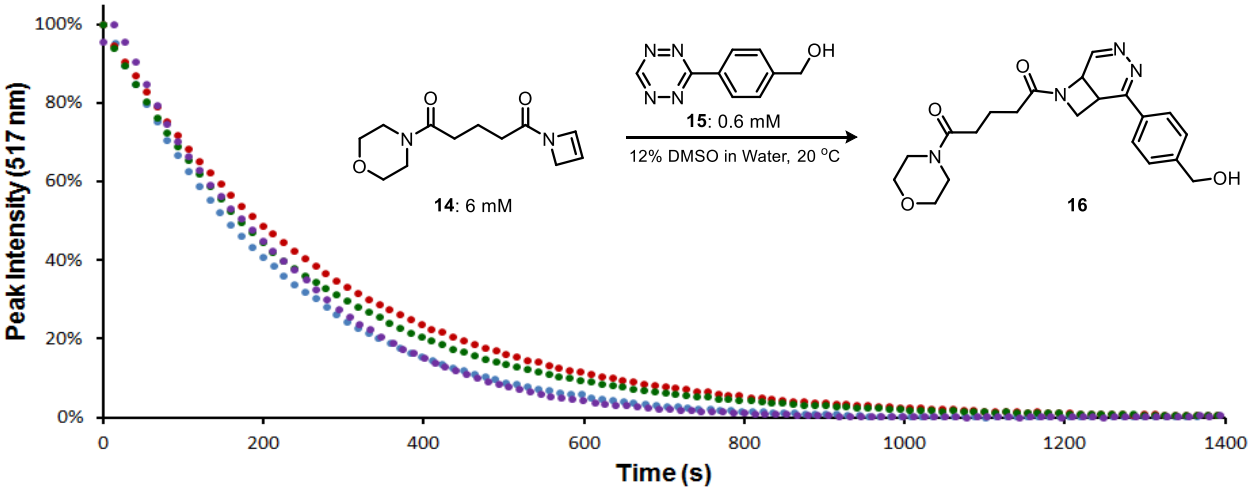
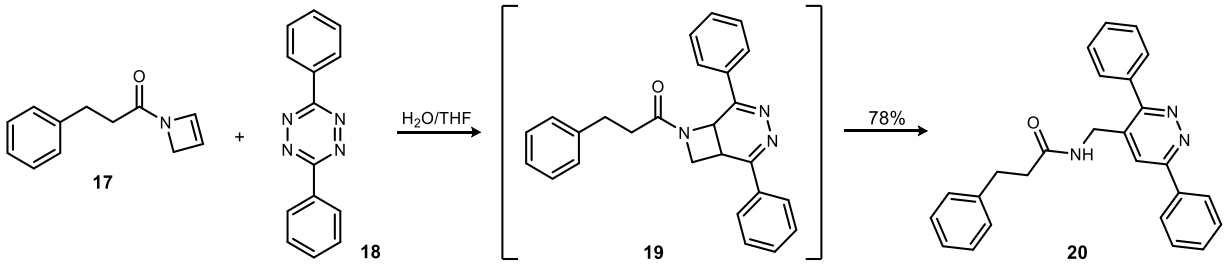


Figure 2: The graph consists of a data plot following the tetrazine absorption band over time in a reaction between *N*-acylazetine **14** (6 mM) and tetrazine **15** (0.6 mM) in 12% DMSO/water. The kinetics experiment was performed four times. Compound **16** is formed as a mixture of regioisomers and potentially as tautomers.

The mass-spectroscopic data of the reaction product, formed as a mixture of isomers, were fully consistent with putative structure **16**. However, characterization of **16** by NMR was complicated by the two tertiary amide bonds, which appeared to exist as rotamers on the NMR time scale. In order to obtain more conclusive spectroscopic data of the product, while simultaneously studying the course of the *N*-acylazetine cycloaddition in more detail, compound **17** was reacted with symmetrical tetrazine **18** (Scheme 2). During this experiment it was found that immediate IEDDA adduct **19** rapidly ring-opens to form compound **20** as the sole product. This process is likely thermodynamically driven by the restoration of aromaticity and relief of ring-strain, of which former is more favorable in higher conjugated systems. Trace amounts of product resulting from the same rearrangement were observed after the reaction of **14** with **15**, as evidenced by the resonance of the tertiary carbon of pyridazine at 123 ppm in the ^{13}C NMR spectrum of **16**.



Scheme 2: Model cycloaddition between *N*-acylazetine **17** and tetrazine **18**, shows that direct adduct **19** completely rearranges to open-ring isomer **20**.

Labeling Evaluation

In order to evaluate the applicability of the novel *N*-acylazetine ligation handle for biological labeling strategies, the tag was incorporated into an activity-based proteasome probe to enable two-step activity-based protein profiling through ligation with a fluorescently labeled tetrazine (**23**, Figure 3). As a model target enzyme the constitutive proteasome was selected. The proteasome is a multi-subunit protein complex containing three different catalytically active subunits (β1, β2 and β5). These β-subunits each have a different substrate preference and can be targeted by various subunit-selective[15] or broad-spectrum[16] activity based probes (ABPs). The designed ABP **21** is based on the broad-spectrum irreversible proteasome inhibitor epoxomicin, functionalized at the *N*-terminus with the acylazetine moiety.[17–19] Compound **21** (Figure 3) was readily prepared from protected peptide epoxyketone.

The ability of the *N*-acylazetine-functionalized ABP **21** to target the catalytically active proteasome β-subunits was confirmed by performing competition experiments against the fluorescent broad-spectrum proteasome ABP MV151 in human embryonic kidney (HEK) cell extracts. These experiments revealed that 3 μM of ABP **21** was required to completely block the fluorescent labeling by MV151. Therefore, for all ensuing two-step labeling experiments a probe concentration of 5 μM was used. The utility of the *N*-acylazetine tag for two-step labeling of proteasome activity in cell extracts was established by exposure of HEK cell extracts to ABP **21**, followed by ligation with varying concentrations of tetrazine **23**. As a control, the same procedure was performed using the previously reported norbornene-functionalized

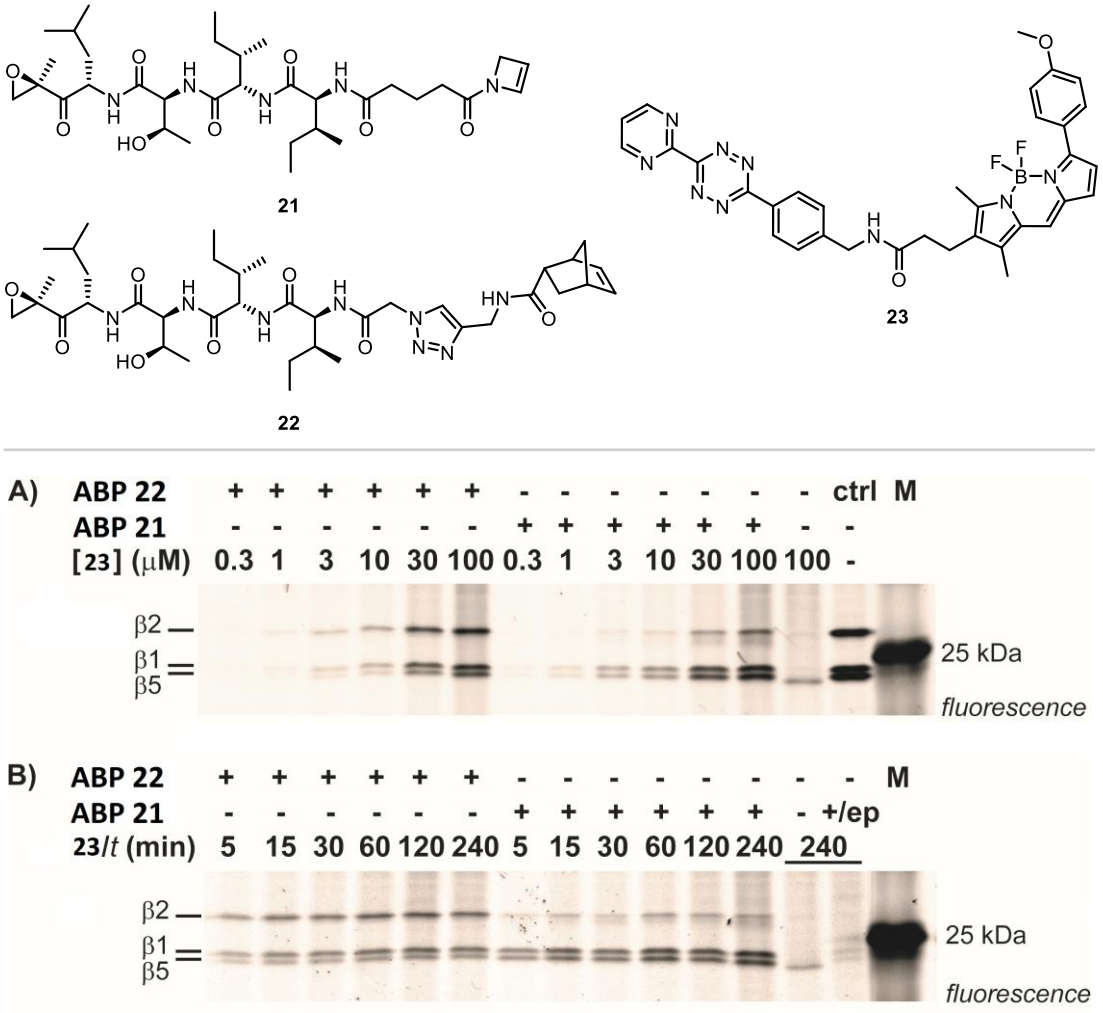


Figure 3: Structures of *N*-acylazetine- (**20**) and norbornene (**21**)-functionalized proteasome ABPs and tetrazine-BODIPY reporter reagent (**17**). [**A**] Labeling of proteasome activity in HEK cell extracts by exposure to 5 μM of ABP **21** or ABP **22** for 1 h followed by reaction with 0.3−100 μM of Bodipy-tetrazine **23** for 1 h. [**B**]: Labeling of proteasome activity in HEK cell extracts by exposure to 5 μM of ABP **21** or ABP **22** for 1 h followed by reaction with 10 μM of Bodipy-tetrazine **23** for 5−240 min. "ep": 100 μM epoxomicin added to incubation with ABP **21**.

ABP **22**.[20] Analysis of the labeled proteins on gel with fluorescent readout (Figure 3, A) revealed that tetrazine ligation of cell extracts treated with *N*-acylazetine-functionalized ABP **21** resulted in the specific fluorescent labeling of three bands, which correspond to the proteasome β-subunits labeled by fluorescent ABP MV151. The labeling is dependent on the concentration of tetrazine in a similar manner as for norbornene-functionalized ABP **22**. With both ABPs a comparable increase in fluorescent labeling was observed when the ligation step was performed at prolonged reaction times (Figure 3, B).

These results demonstrate that, in this experimental setup, the *N*-acylazetine moiety reacts with equal efficiency as the norbornene ligation handle in IEDDA reaction with tetrazine **23**. The absence of labeling in samples in which the proteasome activity was inhibited by an excess of epoxomicin, confirms that ABP **21** labels the catalytically active proteasome β-subunits. In order to determine whether *N*-acylazetine may cross-react with other commonly used ligation reagents, two-step proteasome labeling procedures were

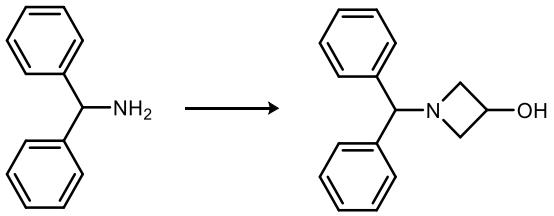
performed in HEK cell extracts using alkyne-, azide- and phosphine-functionalized reagents instead of tetrazine for the ligation step. No proteasome labeling was detected when using Bodipy-alkyne[20], Bodipy-azide[21], biotin-phosphine[22] and biotinylated dibenzocyclooctene[23] reagents, demonstrating that these do not react with the *N*-acylazetine-tagged probe. Together these results demonstrate the utility of the new compact *N*-acylazetine ligation handle for bioorthogonal labeling of proteins or other biomolecules via tetrazine ligation.

Conclusion

In conclusion, two *N*-acylazetine handles (**7** and **13**) for the tetrazine ligation strategy were designed and synthesized. After optimization, these handles have become accessible through an efficient four-step synthesis. Determination of the reaction kinetics between the *N*-acylazetine and tetrazine showed a reaction rate constant in line with that of the methylcyclopropene mini-tag. The applicability of the *N*-acylazetine was demonstrated in an ABPP experiment, where epoxomicin – a broad-spectrum proteasome inhibitor – was functionalized with **7** and successfully used to label the proteasome through tetrazine ligation.

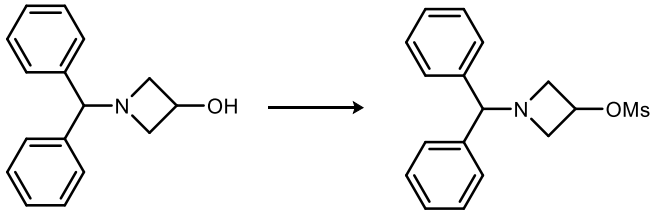
Experimental Section

General: Reactions were executed at ambient temperatures unless stated otherwise. All solvents used under anhydrous conditions were stored over 4Å molecular sieves, except for methanol which was stored over 3Å molecular sieves. Reactions were monitored by TLC-analysis using Merck aluminum DC Silicagel 60 F_{254}, using varying stains for visualization; an aqueous solution of cerium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ 25 g/L), an aqueous solution of potassium permanganate (5 g $KMnO_4$, 25 g K_2CO_3 per L) or an ethanolic solution bromocresol (0.4 g in 1 L, addition of 0.1M $NaOH_{(aq)}$ until the solution turns blue). Column chromatography was performed on silica gel (40-63 µm). Analysis by NMR and HRMS were performed as described in the experimental section of chapter 2.



1 2

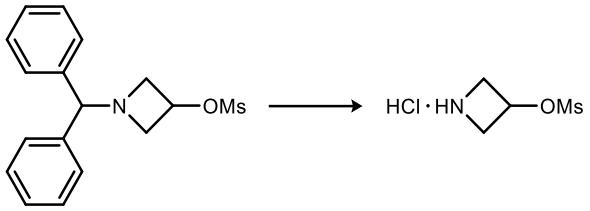
1-Benzhydrylazetidin-3-ol: Epichlorohydrin (10.75 ml, 138 mmol, 1.1 eq) was added to a solution of benzhydrylamine (21.55 ml, 125 mmol) in isopropanol (125 mL) and the reaction mixture was stirred overnight at 30 °C. LC/MS analysis showed full conversion into the open-chain intermediate. The reaction mixture was concentrated *in vacuo* and redissolved in MeCN (200 mL). Sodium bicarbonate (11.44 g, 188 mmol, 1.5 eq) was added and the reaction mixture was refluxed for 30 h. The reaction mixture was filtered and reduced *in vacuo*, providing a pale-yellow solid. The solid cake was thoroughly pulverized, suspended in an Et_2O/Pentane/EtOAc mixture (9:9:2, 50 mL) and sonicated for 15 minutes. The white residue was collected by filtration and dried under reduced pressure, yielding 1-benzhydrylazetidin-3-ol as a white solid (24.85 g, 104 mmol, 83%), which was used in the next step without further purification. R_f = 0.49 (1:1 ; EtOAc:PE). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.40 – 7.12 (m, 10H), 4.42 (p, *J* = 5.8 Hz, 1H), 4.34 (s, 1H), 3.55 – 3.47 (m, 2H), 3.17 (s, 1H), 2.96 – 2.83 (m, 2H). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 141.80, 128.42, 127.38, 127.14, 78.43, 63.30, 61.89.



2 3

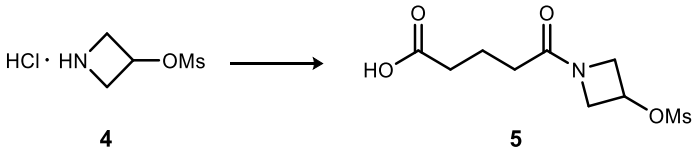
1-Benzhydrylazetidin-3-*O*-Mesyl: 1-benzhydrylazetidin-3-ol (24.65 g, 103 mmol) and triethylamine (21.53 ml, 155 mmol, 1.5 eq) were dissolved in DCM (80 mL) under argon atmosphere and cooled to -40 °C. Methanesulfonyl chloride (9.63 ml, 124 mmol, 1.2 eq) in DCM (20 mL) was slowly added dropwise, while

maintaining the temperature at -40 °C. The reaction mixture was stirred for 30 minutes, after which it was diluted with DCM (50 mL) and washed with water (2 x 100 mL). The organic phase was dried over magnesium sulfate, filtered and concentrated *in vacuo* (if the concentrate did not solidify, the oil was diluted with EtOAc and re-concentrated). The residual yellow solid was pulverized and thoroughly rinsed with cold Et_2O. The solid was suspended in a mixture of acetone/water (1:1, 50 mL) and sonicated for 15 minutes, filtered and vacuum dried, yielding product **3** as an off-white solid (26.3 g, 83 mmol, 80%). TLC R_f = 0.33 (1:3 ; EtOAc:PE). **^{1}H NMR:** (400 MHz, $CDCl_3$) δ = 7.53 – 7.25 (m, 10H), 5.16 (p, *J*=5.8, 1H), 4.50 (s, 1H), 3.73 – 3.66 (m, 2H), 3.31 – 3.23 (m, 2H), 2.93 (s, 3H). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 141.29, 128.46, 127.29, 127.19, 77.99, 67.86, 60.04, 37.91.



3 **4**

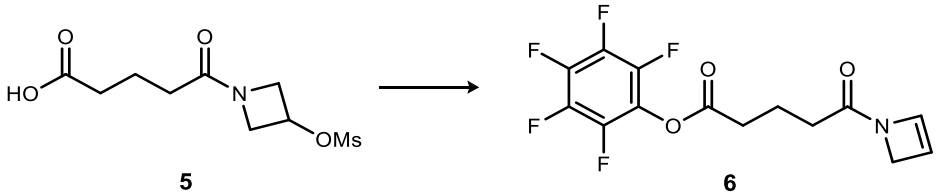
Azetidin-3-*O*-mesyl hydrochloride (4): A solution of 1-benzhydrylazetidin-3-yl methanesulfonate (26.3 g, 83 mmol) in DCE (150 mL) was charged with 1-Chloroethyl chloroformate (9.96 ml, 91 mmol, 1.1 eq) and heated to reflux. After 1.5 hour, TLC (Rf = 0.38 - 2:3 ; EtOAc:PE) indicated conversion of the starting material in a lower running product. The solution was concentrated *in vacuo*, redissolved in MeOH (150 mL) and refluxed for an additional 2 h. The reaction mixture was concentrated *in vacuo*. The residual yellow cake was suspended in a mixture of Et_2O:EtOH (2:1, 50 mL) and sonicated for 15 minutes. The remaining white solid was isolated by filtration, washed with Et_2O and dried *in vacuo*, yielding mesyl azetidine **4** (10.2 g, 54.4 mmol, 65.5 % yield) as a white solid. **^{1}H NMR:** (400 MHz, Methanol-d_4) δ 5.44 (ddd, *J* = 6.8, 4.8, 1.9 Hz, 1H), 4.52 (dt, *J* = 12.5, 4.4 Hz, 2H), 4.30 (dd, *J* = 12.6, 4.8 Hz, 2H), 3.23 (s, 3H). **^{13}C NMR:** (101 MHz, Methanol-d_4) δ 70.08, 54.51, 37.92.



4 **5**

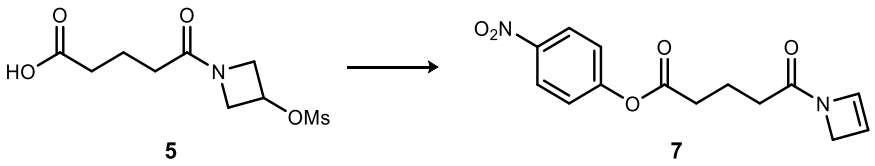
Glutaric Acylazetidine 5: A suspension of mesyl azetidine **4** (3.52 g, 18.8 mmol, 1.05 eq) and silver methanesulfonate (3.88 g, 19.1 mmol, 1.07 eq) in dry MeCN (25 mL) was prepared under argon atmosphere, and stirred vigorously at 40 °C for 15 minutes. Dry potassium carbonate (2.14, 35.7 mmol, 2 eq) and glutaric anhydride (2.04 g, 17.9 mmol, 1 eq) were added and the reaction was refluxed for 2 h. TLC indicated completion (10% EtOH in DCM), using bromocresol as a visualization agent. The reaction mixture was filtered over celite and carefully rinsed with water three times to remove product from the cake. The filtrate was diluted with 25% water in MeCN and acidified with Amberlite (IR120, H-form) to a pH below 3. After filtration, the solution was concentrated and purified by column chromatography (7% » 10% EtOH in DCM), yielding the title compound as a thick white solid (3.75 g, 14.1 mmol, 79%). **^{1}H NMR**

(400 MHz, $CDCl_3$) δ = 5.28 (tt, *J*=6.7, 4.1, 1H), 4.52 (ddd, *J*=10.1, 6.6, 1.5, 1H), 4.41 (ddd, *J*=11.5, 6.6, 1.4, 1H), 4.36 – 4.29 (m, 1H), 4.20 – 4.14 (m, 1H), 3.12 (s, 3H), 2.45 (t, *J*=7.0, 2H), 2.23 (t, *J*=7.3, 2H), 1.97 (p, *J*=7.1, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 177.36, 172.75, 66.53, 57.47, 55.14, 38.37, 32.87, 30.29, 19.60. **HRMS:** Calculated for $C_{14}H_{10}F_5NO_3$ 266.06928 $[M+H]^+$; found 266.06919



5 **6**

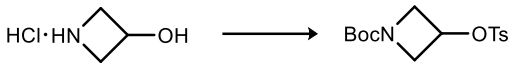
Pentafluorphenol Acylazetine 6: Potassium *tert*-butoxide in THF (20.5 mL, 1.6 M, 32.9 mmol, 2.3 eq) was added to a solution of mesylazetidine pentanoic acid spacer **5** in DMF (150 mL), under argon atmosphere. The reaction mixture was stirred at 50 °C for 2 h. After cooling to room temperature, EDC·HCl (8.23 g, 42.9 mmol, 3 eq.) and pentafluorophenol (4.58 mL, 42.9 mmol, 3 eq.) were added, and the reaction mixture was stirred for an additional 1.5 h. The mixture was poured into H_2O (150 mL) and extracted with DCM (150 mL). The organic layer was washed with brine (30 mL), dried over magnesium sulfate, filtrated and concentrated in *vacuo*. The crude product was purified by column chromatography (10% » 30% EtOAc in PE) to yield **6** as a colorless oil which solidified upon standing at -20°C (3 g, 9 mmol, 63%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ = 6.93 – 6.69 (d, *J*=95.6, 1H), 5.75 (d, *J*=9.6, 1H), 4.52 (d, *J*=37.4, 2H), 2.90 – 2.76 (m, 2H), 2.42 (dt, *J*=36.0, 7.2, 2H), 2.15 (p, *J*=7.1, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 177.36, 172.75, 66.53, 57.47, 55.14, 38.37, 32.87, 30.29, 19.60. **HRMS:** Calculated for $C_{14}H_{10}F_5NO_3$ 336.06536 $[M+H]^+$; found 336.06512.



5 **7**

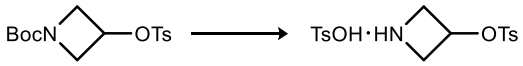
***p*-Nitrophenol Gluctaric Acylazetine 7:** A solution of mesylazetidine pentanoic acid spacer **5** (0.58 g, 2.186 mmol) in DMF (25 mL) was prepared and put under argon atmosphere. Next, potassium *tert*-butoxide (3.01 ml, 4.81 mmol) was slowly added and the reaction mixture was warmed to 50 °C and stirred vigorously for 2 hours. The reaction was allowed to cool to room temperature, after which *p*-nitrophenol (0.912 g, 6.56 mmol) was added. The reaction mixture was stirred until a clear yellow solution had formed. Then EDC·HCl (1.257 g, 6.56 mmol) was added and the reaction was stirred for additional 2 hours. The reaction mixture was poured into ether/EtOAc (2:1) and washed with water and brine. The organic layer was dried over magnesium sulfate, filtrated and concentrated in *vacuo*. The crude product was purified by column chromatography (30% » 80% EtOAc in PE) to yield **7** as a white crystalline substance (0.54 g, 1.86 mmol, 85%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ = 8.27 (d, *J*=9.1, 2H), 7.29 (d, *J*=9.1, 2H), 6.80 (dd, *J*=100.2, 1.7, 1H), 5.73 (d, *J*=6.9, 1H), 4.51 (d, *J*=35.3, 2H), 2.74 (t, *J*=7.2, 2H), 2.46 (t, *J*=7.1, 1H), 2.37 (t, *J*=7.1, 1H), 2.13 (p, *J*=7.2, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 170.72, 155.28, 145.20, 137.40, 136.68, 125.13, 122.37, 113.71, 113.41, 58.53, 56.56, 33.23, 30.60, 29.56, 19.96, 19.78.

Improved Synthesis



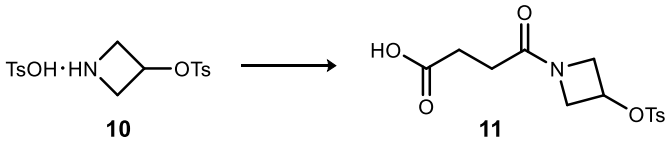
8 **9**

***N*-Boc-azetidin-3-*O*-mesyl (9):** A solution of commercially available 3-hydroxyazetidine hydrochloride (115 mmol, 10.55 g, 1 eq) and Et_3N (161 mmol, 22.5 mL, 1.4 eq) in MeOH (115 mL) was prepared at 0 ˚C. Boc_2O (126.5 mmol, 27.6 g, 1.1 eq) was added and the ice-bath was removed. After 5 hours of stirring, the reaction mixture was concentrated *in vacuo*, redissolved in DCM and washed twice with water. The water layers were combined and extracted twice with DCM. The organic layers were combined, dried with magnesium sulfate, filtered and concentrated *in vacuo*. The intermediate Boc-hydroxyazetidine was used without further purification. An ice-cooled solution of the crude **8** and Et_3N (172.5 mmol, 24 mL, 1.5 eq) in dry DCM (100 mL) was prepared under argon atmosphere. *p*-Toluenesulfonyl chloride (138 mmol, 26.3 g, 1.2 eq) was added in eight portions over 2 hours and the reaction mixture was stirred overnight. The reaction mixture was washed with water twice and the combined aqueous layers were extracted thrice with DCM. The organic layers were combined, dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (5% » 10% EtOAc in pentane), yielding tosylate **9** as a yellow oil. (82.6 mmol, 27.7 g, 72% over two steps). **^{1}H NMR:** (300 MHz, $CDCl_3$) δ 7.75 (d, *J* = 8.1 Hz, 2H), 7.34 (d, *J* = 8.1 Hz, 2H), 4.97 (ddd, *J* = 10.8, 6.6, 4.3 Hz, 1H), 4.14 – 4.01 (m, 2H), 3.97 – 3.82 (m, 2H), 2.43 (s, 3H), 1.38 (s, 9H). **^{13}C NMR:** (75 MHz, $CDCl_3$) δ 155.86, 145.61, 132.91, 130.17, 127.92, 80.23, 67.84, 56.28, 28.30, 21.74. **HRMS:** Calculated for 327.11404 $[M+H]^+$; found 328.12132.



9 **10**

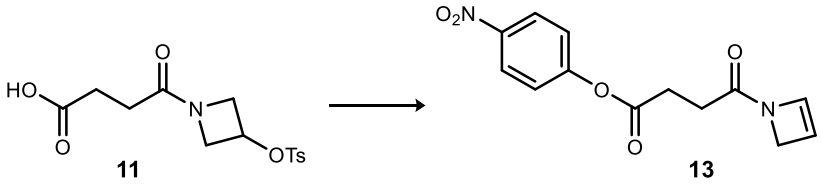
Azetidin-3-*O*-tosyl tosylate (10): A solution of compound **9** (82.6 mmol, 27.1g, 1 eq) in DCE (165 mL) was charged with *p*-toluenesulfonic acid (90.9 mmol, 17.3g, 1.1 eq) and refluxed for 20 hours. The reaction mixture was concentrated *in vacuo*. The crude product was crystalized from MeOH, yielding compound **20** as a white crystalline substance (65 mmol, 25.9 g, 79%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 9.00 (d, 2H), 7.70 (d, *J* = 8.2 Hz, 4H), 7.29 (d, *J* = 8.1 Hz, 2H), 7.19 (d, *J* = 7.8 Hz. **^{13}C NMR** (101 MHz, $CDCl_3$) δ 146.07, 141.26, 132.03, 130.38, 129.39, 128.17, 125.92, 67.85, 53.37, 21.86, 21.55.



10 **11**

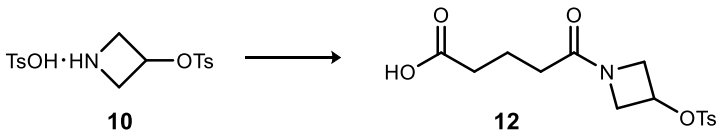
Succinic Acylazetidine 11: Compound **10** (20.0 mmol, 5.58 g, 1.1 eq) was co-evaporated with dioxane, redissolved in MeCN (200 mL), and put under argon atmosphere. Succinic anhydride (18.2 mmol, 1.82 g, 1 eq) was added to the reaction mixture, followed by potassium carbonate (45.5 mmol, 6.3 g, 2.5 eq) and the reaction mixture was refluxed for 6 hours. Reaction progress was monitored by TLC, using a bromocresol stain to visualize the produced carboxylic acid. The reaction mixture was diluted with water

(200 mL) and Amberlite-H^+ (IR120, ±70 g) was added until the pH fell below 3. The solution was filtered and the residual MeCN was removed *in vacuo*. The water layer was extracted twice with EtOAc. The organic layers were combined, dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (5% » 10% EtOH in DCM), yielding compound **11** as a white crystalline substance (9.1 mmol, 3.09 g, 50%). **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 8.3 Hz, 2H), 7.39 (d, *J* = 8.2 Hz, 2H), 5.08 (ddd, *J* = 11.1, 6.9, 4.3 Hz, 1H), 4.42 (dd, *J* = 9.4, 7.5 Hz, 1H), 4.30 – 4.08 (m, 2H), 3.94 (dd, *J* = 11.5, 4.0 Hz, 1H), 2.71 – 2.61 (m, 2H), 2.48 (s, 3H), 2.35 (t, *J* = 6.8 Hz, 2H). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 171.99, 145.99, 130.37, 128.07, 76.84, 67.22, 57.38, 55.23, 28.81, 26.20.



11 **13**

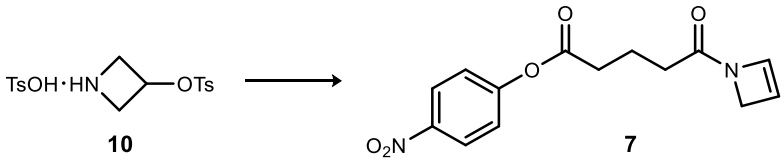
***p*-Nitrophenol Succinic Acylazetidine 13:** Compound **11** (9.1 mmol, 3.09 g, 1 eq) was co-evaporated with dioxane, redissolved in dry DMF (45.5 mL) and put under argon atmosphere. Next, a 1 M solution of potassium *tert*-butoxide in THF (20 mL, 2.1 eq) was added to the reaction mixture and the reaction was stirred for 1 hour. Subsequently the reaction mixture was charged with bis(para-nitrophenyl)carbonate (10 mmol, 3.01 g, 1.1 eq) and left stirring for an additional 3 hours. The reaction mixture was diluted with EtOAc and washed twice with 10% aqueous sodium bicarbonate, twice with water and once with brine. The combined organic layers were dried with $MgSO_4$, filtered and concentrated in *vacuo*. The crude product was purified with column chromatography (50% » 100% EtOAc in pentane), yielding compound **13** as a yellow crystalline substance (7.3 mmol, 2.0 g, 80%). **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 8.26 (d, *J* = 9.2 Hz, 2H), 7.31 (d, *J* = 9.1 Hz, 2H), 6.91 (s, 0.5H), 6.71 (s, 0.5H), 5.75 (d, *J* = 5.3 Hz, 1H), 4.61 (s, 1H), 4.48 (s, 1H), 2.97 (t, *J* = 6.6 Hz, 2H), 2.75 (t, *J* = 6.5 Hz, 1H), 2.66 (t, *J* = 6.5 Hz, 1H). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 170.76, 165.26, 164.88, 155.48, 145.32, 137.43, 136.64, 125.22, 122.55, 114.19, 113.93, 77.16, 58.67, 56.91, 29.13, 29.03, 26.74, 25.76.



10 **12**

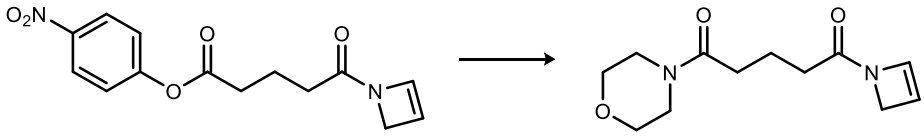
Succinic Acylazetidine 12: Compound **10** (14.0 mmol, 5.58g, 1.1 eq) was co-evaporated with dioxane, redissolved in MeCN (140 mL) and put under argon atmosphere. Glutaric anhydride (12.7 mmol, 1.45 g, 1 eq) was added to the reaction mixture, followed by potassium carbonate (31.8 mmol, 4.48 g, 2.5 eq) and the reaction mixture was refluxed for 6 hours. Reaction progression was monitored by TLC, using a bromocresol stain to visualize the produced carboxylic acid. The reaction mixture was diluted with water (200 mL) and Amberlite-H^+ (IR120, ±50 g) was added until the pH fell below 3. The solution was filtered and the residual MeCN was removed *in vacuo*. The water layer was extracted twice with EtOAc. The organic layers were combined, dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (3% » 5% EtOH in DCM), yielding compound **12** as

a white crystalline substance (8.9 mmol, 3.06 g, 70%). **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 7.81 (d, *J* = 8.3 Hz, 2H), 7.40 (d, *J* = 8.1 Hz, 2H), 5.08 (tt, *J* = 6.8, 4.2 Hz, 1H), 4.45 – 4.33 (m, 1H), 4.26 – 4.13 (m, 2H), 3.93 (dd, *J* = 11.5, 4.3 Hz, 1H), 2.49 (s, 3H), 2.42 (t, *J* = 7.0 Hz, 2H), 2.17 (t, *J* = 7.3 Hz, 2H), 1.92 (p, *J* = 7.2 Hz, 2H). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 177.76, 172.54, 145.84, 132.58, 130.24, 127.93, 67.11, 57.23, 54.93, 32.93, 30.29, 21.78, 19.57.



10 **7**

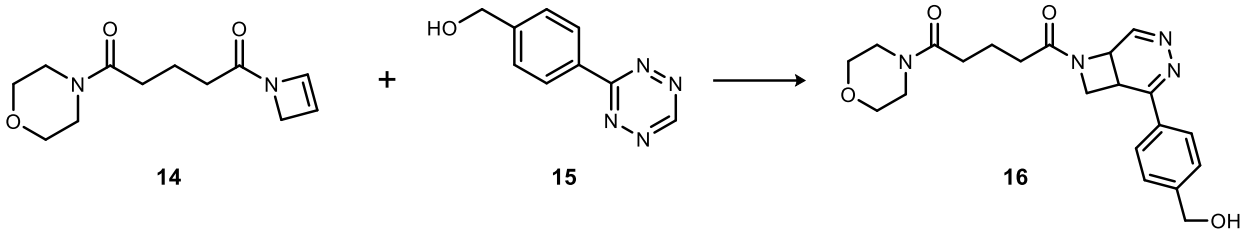
***p*-Nitrophenol Glutaric Acylazetine 7:** Compound **10** (8.9 mmol, 3.04 g, 1 eq) was co-evaporated with dioxane, redissolved in dry DMF (44.5 mL), and put under argon atmosphere. Next, a 1 M solution of potassium *tert*-butoxide in THF (18.7 mL, 2.1 eq) was added to the reaction mixture and left stirring for 1 hour. Subsequently the reaction mixture was charged with bis(para-nitrophenol)carbonate (9.8 mmol, 2.95 g, 1.1 eq) and left stirring for another 3 hours. The reaction mixture was diluted with EtOAc and washed twice with 10% aqueous sodium bicarbonate, twice with water and once with Brine. The combined organic layer were dried with magnesium sulfate, filtered and concentrated in *vacuo*. The crude product was purified with column chromatography (50% » 100% EtOAc in pentane), yielding compound **7** as a yellow crystalline substance (6.1 mmol, 1.78 g, 69%). **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 8.26 (d, *J* = 9.2 Hz, 2H), 7.31 (d, *J* = 9.1 Hz, 2H), 6.91 (s, 0.5H), 6.71 (s, 0.5H), 5.75 (d, *J* = 5.3 Hz, 1H), 4.61 (s, 1H), 4.48 (s, 1H), 2.97 (t, *J* = 6.6 Hz, 2H), 2.75 (t, *J* = 6.5 Hz, 1H), 2.66 (t, *J* = 6.5 Hz, 1H). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 170.76, 165.26, 164.88, 155.48, 145.32, 137.43, 136.64, 125.22, 122.55, 114.19, 113.93, 77.16, 58.67, 56.91, 29.13, 29.03, 26.74, 25.76.



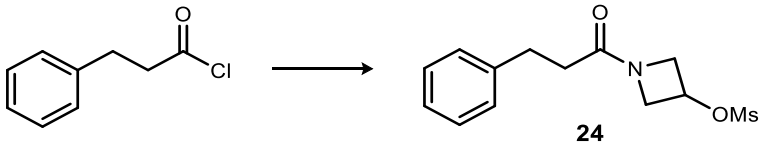
7 **14**

Morpholine Glutaric Acylazetine 14: Morpholine (0.099 ml, 1.137 mmol) was added to a solution of 4-nitrophenyl 5-(azet-1(2H)-yl)-5-oxopentanoate **7** (0.11 g, 0.379 mmol) in DCM (1 mL). After 1 hour, the reaction mixture was directly purified by column chromatography (DCM » DCM:Acetone:EtOH 80:20:1 » 50:45:5) to give **14** (88 mg, 0.369 mmol, 97%). **^{1}H NMR:** (400 MHz, $CDCl_3$) δ = 8.27 (d, *J*=9.1, 2H), 7.29 (d, *J*=9.1, 2H), 6.80 (dd, *J*=100.2, 1.7, 1H), 5.73 (d, *J*=6.9, 1H), 4.51 (d, *J*=35.3, 2H), 2.74 (t, *J*=7.2, 2H), 2.46 (t, *J*=7.1, 1H), 2.37 (t, *J*=7.1, 1H), 2.13 (p, *J*=7.2, 2H). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ = 170.72, 155.28, 145.20, 137.40, 136.68, 125.13, 122.37, 113.71, 113.41, 58.53, 56.56, 33.23, 30.60, 29.56, 19.96, 19.78. HRMS $[M+H]^+$ m/z calc. for $[C_{14}H_{14}N_2O_5]$ = 291.09755, found 291.09744. **HRMS:** Calculated for $C_{12}H_{18}N_2O_3$ 239.13902 $[M+H]^+$; found 239.13900.

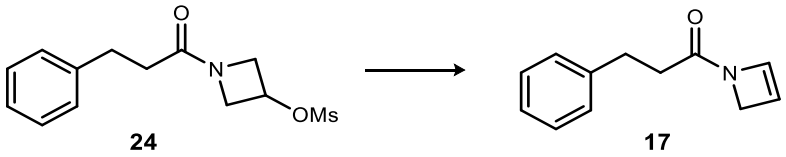
Synthesis of Model Compounds



IEDDA Adduct 16: Tetrazine **14** was added to a solution of acylazetidine **15** in a H_2O:THF mixture (1:1, 1 mL). The reaction was stirred for 0.5 hour, diluted with water and washed with EtOAc. The water layer was separated and reduced in volume and co-evaporated with dioxane. The residue was redissolved in DMSO (1 mL). Diethyl ether (10 mL) was added and the mixture was sonicated for 0.5 hour. The ether was decanted and the remaining off-yellow powder was dried in *vacuo* to give adduct **16**.

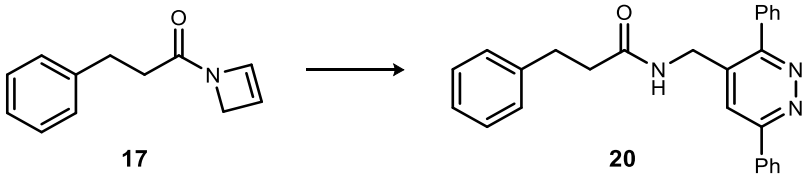


Model Acylazetidine 24: 3-Phenylpropionyl chloride (2.228 ml, 15.00 mmol) was added to a cooled solution (-78 °C) of azetidin-3-yl methanesulfonate hydrochloride (2.81 g, 15 mmol) and TEA (4,60 ml, 33,0 mmol) in DCM (60 mL), under argon atmosphere. The reaction mixture was stirred for 30 minutes, before being quenched with water. The aqueous layer was extracted twice with DCM (30 mL). The organic layer was isolated and dried over magnesium sulfate, filtered and concentrated in *vacuo*. The residual oil was purified by flash column chromatography (70% » 100% EtOAc in pentane), yielding **24** (3.04 g, 10.73 mmol, 72%) as a white crystalline substance. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.30 (dd, *J* = 8.1, 6.7 Hz, 2H), 7.24 – 7.16 (m, 3H), 5.12 (tt, *J* = 6.7, 4.1 Hz, 1H), 4.36 – 4.25 (m, 1H), 4.18 (ddd, *J* = 10.1, 6.6, 1.5 Hz, 1H), 4.11 – 3.97 (m, 2H), 3.03 (s, 3H), 2.93 (t, *J* = 7.6 Hz, 2H), 2.43 – 2.32 (m, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 172.36, 140.81, 128.60, 128.41, 126.42, 66.61, 57.17, 54.89, 38.35, 33.77, 31.04.



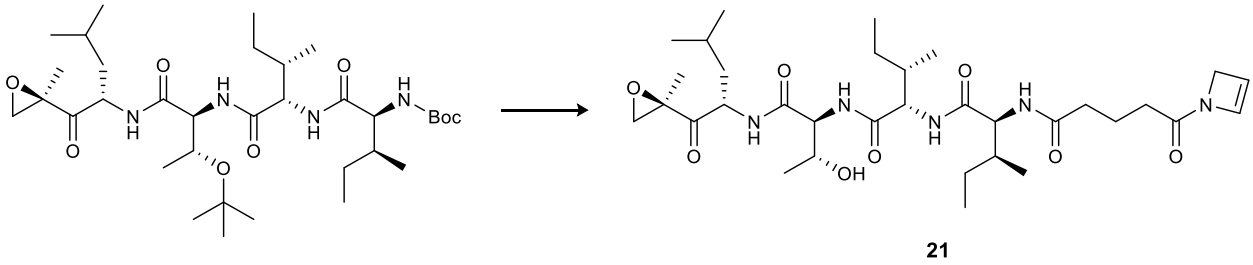
Model Acylazetine 17: Potassium tert-butoxide (1,459 g, 13.00 mmol) was added to a solution of **24** (2.83 g, 10 mmol) in *t*BuOH (30 mL), under argon atmosphere. The reaction mixture was stirred for 4h at 50 °C. The reaction mixture was poured into a diluted ammonium chloride solution and extracted with DCM. The organic layers were dried over magnesium sulfate, filtered and concentrated in *vacuo*. Purification by silica gel column chromatography (40% » 50% EtOAc in pentane) yielded **17** (1.09 g, 5.84 mmol, 58%) as a colorless oil that solidified upon standing. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.80 (dd, *J* = 7.1, 1.7 Hz, 2H), 7.62 (s, 1H), 7.53 (t, *J* = 6.0 Hz, 1H), 7.45 – 7.38 (m, 1H), 7.38 – 7.23 (m, 7H), 7.14 – 7.01 (m, 6H), 4.27 (d, *J* = 5.9 Hz, 2H), 2.88 (t, *J* = 7.6 Hz, 2H), 2.51 (t, *J* = 7.7 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 172.92, 159.03, 157.98,

140.49, 137.75, 135.72, 129.90, 129.07, 128.85, 128.72, 128.41, 128.27, 128.07, 126.92, 126.03, 122.63, 39.77, 37.48, 31.28.



17 20

IEDDA Adduct 20: 3,6-diphenyl-1,2,4,5-tetrazine (62.6 mg, 0.267 mmol) was added to a solution of 1-(azet-1(2H)-yl)-3-phenylpropan-1-one (50 mg, 0.267 mmol) in H_2O:THF (1:4, 0.5 mL). The reaction mixture was stirred overnight. TLC (20% EtOAc in DCM) showed conversion into a single product. The solution was concentrated in *vacuo* and purified by column chromatography, yielding adduct **20** as a pale yellow oil. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 7.1, 1.7 Hz, 2H), 7.62 (s, 1H), 7.53 (t, *J* = 6.0 Hz, 1H), 7.45 – 7.38 (m, 1H), 7.38 – 7.23 (m, 7H), 7.14 – 7.01 (m, 5H), 4.27 (d, *J* = 5.9 Hz, 2H), 2.88 (t, *J* = 7.6 Hz, 2H), 2.51 (t, *J* = 7.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.92, 159.03, 157.98, 140.49, 137.75, 135.72, 129.90, 129.07, 128.85, 128.72, 128.41, 128.27, 128.07, 126.92, 126.03, 122.63, 39.77, 37.48, 31.28.



21

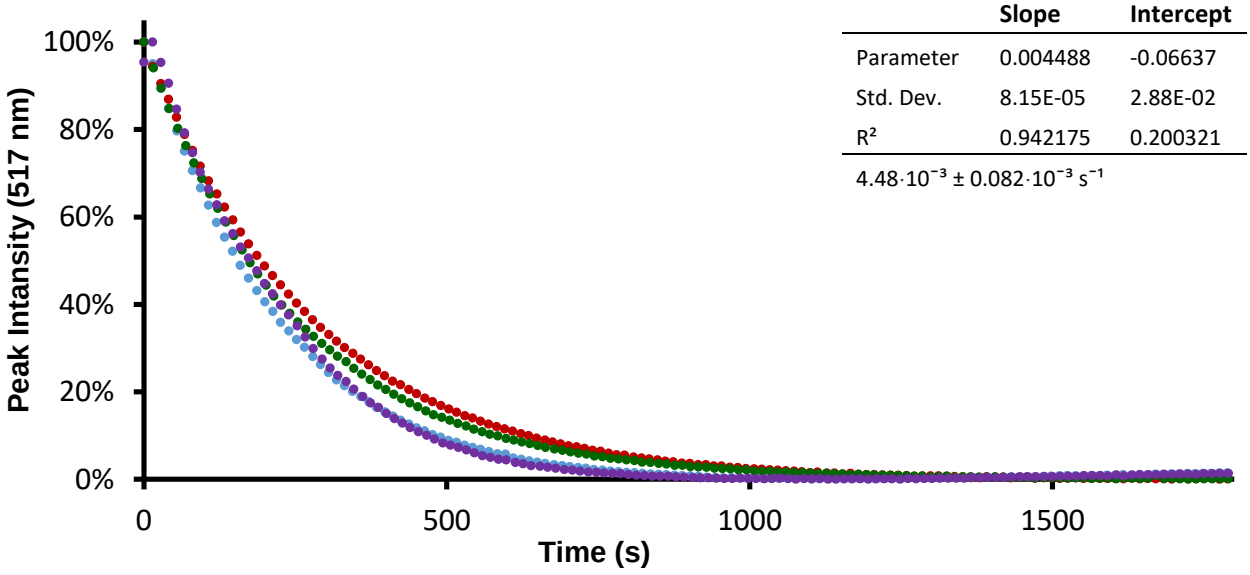
Acylazetine Epoxomicin 21: Epoxomicin-Thr(tBu)-Boc (0.05 g, 0.076 mmol) was dissolved in 0.5 ml TFA and stirred for 15 minutes. The reaction mixture was diluted with toluene and concentrated *in vacuo*. The residue was co-evaporated with toluene and redissolved in DMF (0.5 mL). DIPEA (0.053 ml, 0.305 mmol) was added, followed by perfluorophenyl 5-(azet-1(2H)-yl)-5-oxopentanoate (**6**) (0.026 g, 0.076 mmol). The reaction progress was followed by TLC-MS analysis. Upon completion, the reaction mixture was purified by HPLC to give **21** (15 mg, 0.023 mmol, 30%). **^{1}H NMR** (600 MHz, DMSO-d6) δ = 7.96 – 7.74 (m, 4H), 6.95 (d, *J*=20.1, 1H), 5.81 (d, *J*=48.3, 1H), 4.84 – 4.71 (m, 1H), 4.54 (d, *J*=4.9, 1H), 4.42 – 4.35 (m, 1H), 4.31 (s, 1H), 4.27 – 4.15 (m, 3H), 3.87 (h, *J*=5.7, 1H), 3.18 (d, *J*=5.3, 1H), 3.00 (d, *J*=5.3, 1H), 2.27 – 2.08 (m, 3H), 1.81 – 1.58 (m, 3H), 1.39 (s, 3H), 1.36 – 1.24 (m, 2H), 1.12 – 1.02 (m, 2H), 0.99 (d, *J*=6.3, 3H), 0.89 (d, *J*=6.7, 3H), 0.85 – 0.73 (m, 15H). **^{13}C NMR** (151 MHz, DMSO-d6) δ = 208.00, 171.63, 171.17, 170.85, 170.06, 138.49, 137.20, 113.98, 112.74, 66.43, 58.79, 58.36, 57.86, 56.86, 56.03, 51.55, 49.23, 43.30, 38.69, 36.44, 36.17, 34.69, 34.28, 32.55, 30.67, 29.53, 24.27, 23.16, 21.10, 21.05, 20.97, 19.70, 15.32, 15.19, 10.90, 10.84.

Kinetics Experiments

Determination of the pseudo-first order reaction rate constant

A 1.2 mM stock solution of tetrazine **15** in 12% DMSO:Water (v:v) and a 12 mM stock solution of acylazetine **14** in 12% DMSO:Water were prepared. 1 mL of the 1.2 mM tetrazine **15** stock solution was brought into a quarts cuvette (10 mm width, 2 mL volume), which was placed in the measurement chamber of a Cary 300 UV-Vis spectrophotometer (Agilant Technologies). Then, 1 mL of the 12 mM acylazetine **14** stock solution was added to the cuvette and the measurement was immediately started. Upon addition of the second solution, the concentration became 0.6 mM for **15** and 6 mM for **14**. The absorption decay was followed at 517 nm, measuring at 13 second intervals over 30 minutes. The experiments were conducted at uncontrolled room temperature (±20 ˚C).

The reaction rate was derived from four data sets using every measurement interval up to 600 seconds (absorption range from 100% to >10%). This gave 4 data points per value of time (x). This array was subjected to the LINEST function (Microsoft Excel 2013) to determine the slope and the standard deviation. The slope represents the pseudo first-order rate constant.

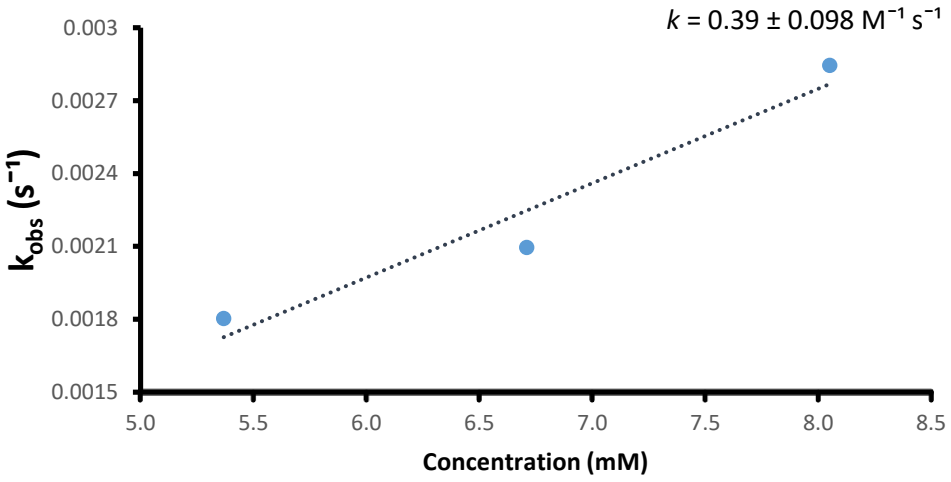


	Slope	Intercept
Parameter	0.004488	-0.06637
Std. Dev.	8.15E-05	2.88E-02
R^2	0.942175	0.200321

$4.48 \cdot 10^{-3} \pm 0.082 \cdot 10^{-3}\ s^{-1}$

Determination of the second order rate constant

The second order rate was derived from the observed pseudo-first order reaction rates at three different (excess) concentrations of **14**. These experiments were conducted at uncontrolled room temperature (approximately 20 ˚C). The slope of this plot represents the second order rate constant.



Biological Essays

Preparation of cell extracts

Human Embryonic Kidney (HEK) cell extracts were prepared from cultured HEK-293T cells by harvesting, washing with PBS (2x) and cell lysis in digitonin lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.025% digitonin) for 30 min on ice followed by sonication on ice for 3x 10 s. After centrifugation of the cells at 16,100 g for 15 min at 4 °C, the supernatants were collected and the protein concentration was determined by Bradford assay.

Competition assay versus MV151

HEK cell lysates (20 µg total protein per experiment) in lysis buffer (9 µL) were exposed to the indicated concentrations of **21** (1 µL 10x solution in DMSO) for 1 hr at 37 °C, after which the lysates were incubated with 1 µM MV151 (1.1 µL 10 µM in DMSO) for 1 hr at 37 °C. The reaction mixtures were then boiled at 100 °C for 5 minutes with 4 µL 4x Laemmli's sample buffer containing 2-mercaptoethanol and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using a Typhoon Variable Mode Imager (Amersham Biosciences) with Cy3/TAMRA settings (excitation wavelength 532 nm, emission wavelength 580 nm). As a loading control gels were stained with Coomassie Brilliant Blue. As a protein standard the PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was used.

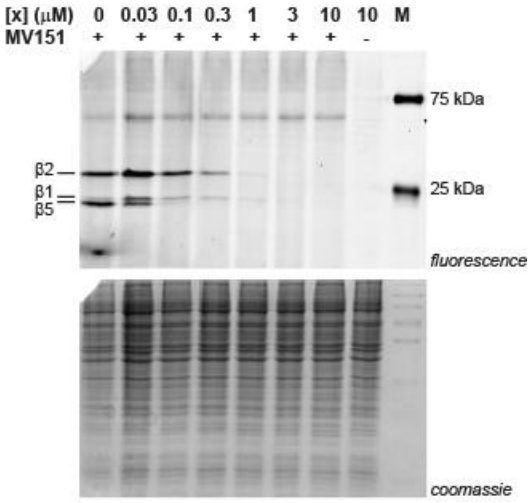


Figure E1. Competition experiment in HEK cell extracts. Cell extract were first exposed to 0 - 10 µM of azetidine-functionalized ABP **21** for 1 hr and then to fluorescent proteasome ABP MV151 for 1 hr. 12.5% SDS-PAGE with fluorescent readout followed by coomassie brilliant blue staining. Proteasome β-subunits are designated on the basis of reported labeling by MV151. 'M': protein marker.

Tetrazine ligation in cell extracts

HEK cell lysates (20 µg total protein per experiment) in lysis buffer (19 µL) were exposed to 5 µM of **12** or **11** (1 µL 100 µM in DMSO) for 1 hr at 37 °C. As a control, lysates were incubated with **11** in the presence of 100 µM epoxomicin (1 µL 2 mM in DMSO) or labeled with 1 µM MV151 (1 µL 20 µM in DMSO) for 1 hr at 37 °C. The cell extracts were then exposed to the indicated concentrations of **13** (1.1 µL 20x solution in DMSO) for the indicated time at 37 °C. After quenching by chloroform/methanol precipitation,[24] the proteins were taken up in 10 µL Laemmli's sample buffer containing 2-mercaptoethanol, boiled at 100 °C for 5 minutes and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using a Typhoon Variable Mode Imager (Amersham Biosciences) with Cy3/TAMRA settings (excitation wavelength 532 nm, emission wavelength 580 nm). As a loading control gels were stained with Coomassie Brilliant Blue. As a protein standard the PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was used.

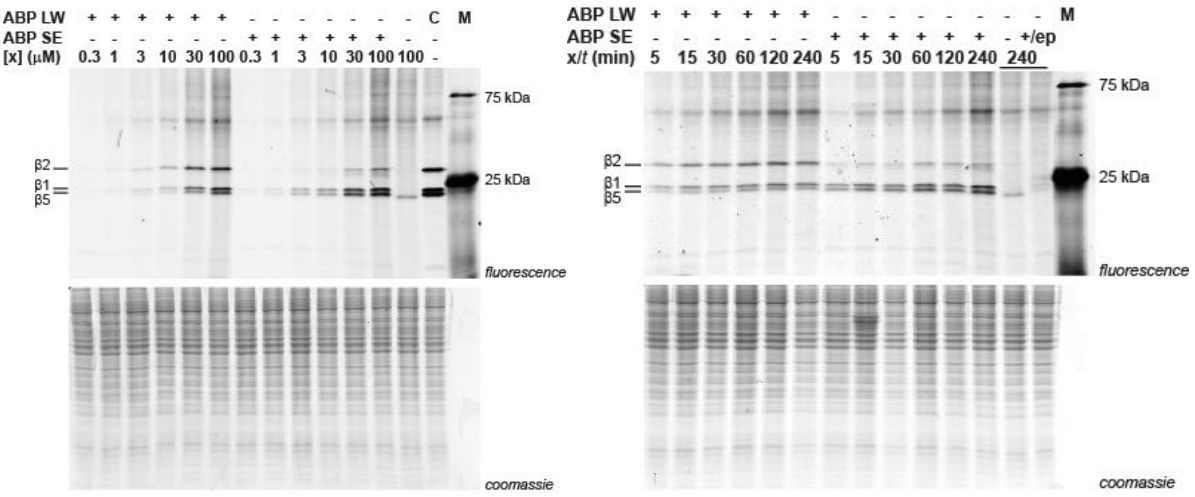


Figure E3: Full depiction of data presented in Figure 4.

Test of cross-reactivity in cell extracts

HEK cell lysates (20 µg total protein per experiment) in lysis buffer (19 µL) were exposed to 5 µM of **21** (1 µL 100 µM in DMSO) for 1 hr at 37 °C. The cell extracts were then exposed to 100 µM biotin-phosphine, 100 µM biotin-dibenzocyclooctyn, 50 µM azido-Bodipy, 50 µM Bodipy-alkyne or 50 µM tetrazine-Bodipy (each 1 µL 20x in DMSO) for 1 hr at 37 °C. After quenching by chloroform/methanol precipitation,[16] the proteins were taken up in 10 µL Laemmli's sample buffer containing 2-mercaptoethanol, boiled at 100 °C for 5 minutes and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using a Typhoon Variable Mode Imager (Amersham Biosciences) with Cy2/Blue Fam settings (excitation wavelength 488 nm, emission wavelength 520 nm). Next, the proteins were transferred onto a PVDF membrane for detection of biotinylated proteins. The membrane was blocked with 1% BSA in TBS-t(+) (0.1% Tween 20) for 1 hr at room temperature, hybridized with Streptavidin-HRP for 45 min at room temperature (1:10,000 in blocking buffer) (Molecular Probes, Life Technologies), washed with TBS-t(+) and TBS and then visualized using an ECL+ Western Blotting detection kit (Amersham Biosciences). As a loading control the membrane was stained with Coomassie Brilliant Blue.

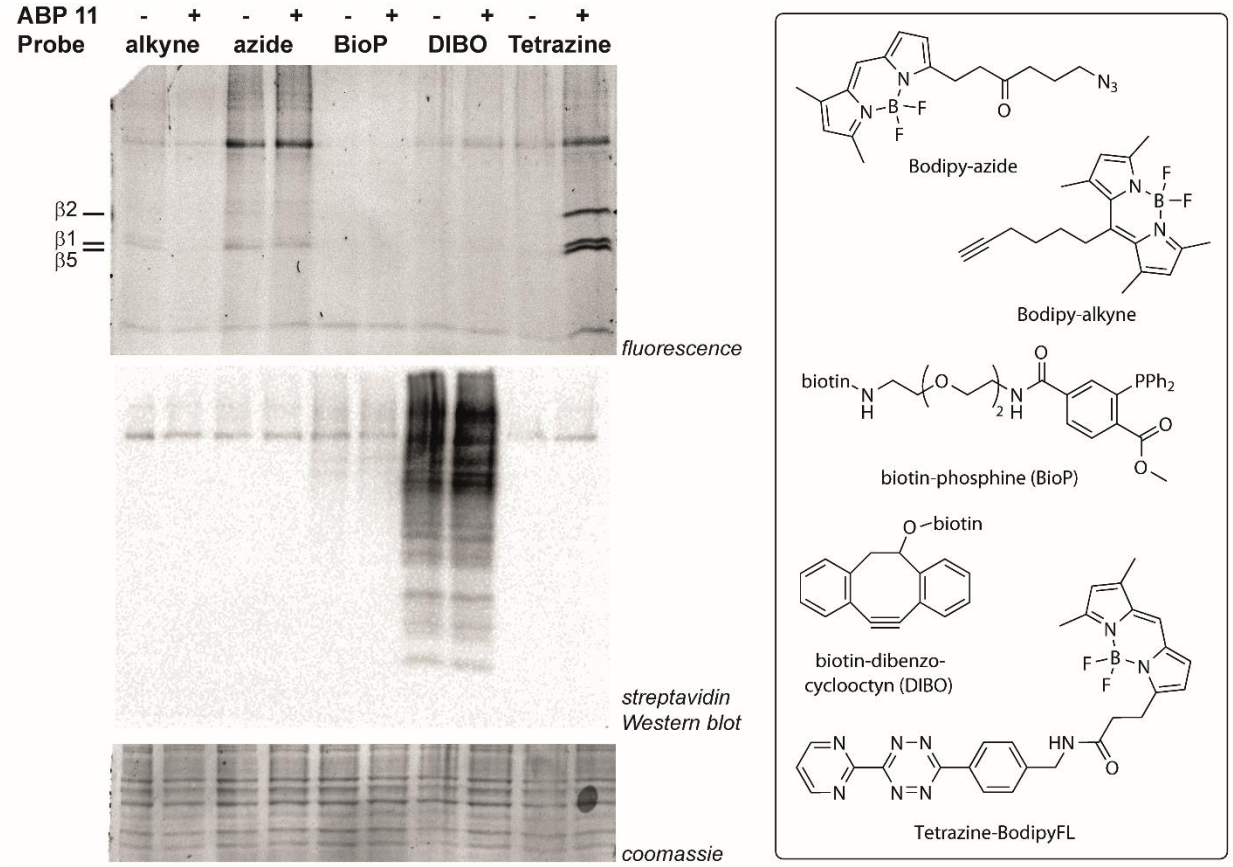


Figure E4. Test of cross-reactivity in HEK cell extracts. Cell extract were first exposed to 5 µM of azetidine-functionalized ABP **21** for 1 hr and then to Bodipy-azide (50 µM), Bodipy-alkyne (50 µM), tetrazine-BodipyFl (50 µM), biotin-phosphine ('BioP', 100 µM) or biotin-dibenzocyclooctyne ('DIBO', 100 µM) for 1 hr. 12.5% SDS-PAGE with fluorescent readout for detection of Bodipy-labeled proteins followed by streptavidin Western blotting to detect biotinylated proteins. Coomassie brilliant blue staining is shown as a loading control. 'M': protein marker.

References

1. D. M. Patterson, L. A. Nazarova, J. A. Prescher, **2014**.
2. L. I. Willems, W. A. Van Der Linden, N. Li, K. Y. Li, N. Liu, S. Hoogendoorn, G. A. Van Der Marel, B. I. Florea, H. S. Overkleeft, *Acc. Chem. Res.* **2011**, *44*, 718–729.
3. E. Saxon, C. R. Bertozzi, *Science (80-.).* **2000**, *287*, 2007–2010.
4. F. Himo, T. Lovell, R. Hilgraf, V. V. Rostovtsev, L. Noodleman, K. B. Sharpless, V. V. Fokin, *J. Am. Chem. Soc.* **2005**, *127*, 210–216.
5. N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047.
6. M. L. Blackman, M. Royzen, J. M. Fox, *J. Am. Chem. Soc.* **2008**, *130*, 13518–13519.
7. N. K. Devaraj, R. Weissleder, S. A. Hilderbrand, *Bioconjug. Chem.* **2008**, *19*, 2297–2299.
8. F. Liu, R. S. Paton, S. Kim, Y. Liang, K. N. Houk, *J. Am. Chem. Soc.* **2013**, *135*, 15642–15649.
9. J. Yang, J. Šečkute, C. M. Cole, N. K. Devaraj, *Angew. Chemie - Int. Ed.* **2012**, *51*, 7476–7479.
10. F. Thalhammer, U. Wallfahrer, J. Sauer, *Tetrahedron Lett.* **1990**, *31*, 6851–6854.
11. D. De Smaele, Y. Dejaegher, G. Duvey, N. De Kimpe, *Tetrahedron Lett.* **2001**, *42*, 2373–2375.
12. M. E. Jung, Y. M. Choi, *J. Org. Chem.* **1991**, *56*, 6729–6730.
13. V. V. R. M. Krishna Reddy, D. Udaykiran, U. S. Chintamani, E. Mahesh Reddy, C. Kameswararao, G. Madhusudhan, *Org. Process Res. Dev.* **2011**, *15*, 462–466.
14. J. Yang, M. R. Karver, W. Li, S. Sahu, N. K. Devaraj, *Angew. Chemie - Int. Ed.* **2012**, *51*, 5222–5225.
15. M. Verdoes, L. I. Willems, W. A. van der Linden, B. A. Duivenvoorden, G. A. van der Marel, B. I. Florea, A. F. Kisselev, H. S. Overkleeft, *Org. Biomol. Chem.* **2010**, *8*, 2719.
16. M. Verdoes, B. I. Florea, V. Menendez-Benito, C. J. Maynard, M. D. Witte, W. A. van der Linden, A. M. C. H. van den Nieuwendijk, T. Hofmann, C. R. Berkers, F. W. B. van Leeuwen, et al., *Chem. Biol.* **2006**, *13*, 1217–1226.
17. M. Hanada, K. Sugawara, K. Kaneta, S. Toda, Y. Nishiyama, K. Tomita, H. Yamamoto, M. Konishi, T. Oki, *Epoxomicin, a New Antitumor Agent of Microbial Origin.*, **1992**.
18. L. Meng, R. Mohan, B. H. B. Kwok, M. Elofsson, N. Sin, C. M. Crews, *Proc. Natl. Acad. Sci.* **1999**, *96*, 10403–10408.
19. N. Sin, K. B. Kim, M. Elofsson, L. Meng, H. Auth, B. H. B. Kwok, C. M. Crews, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2283–2288.
20. M. Verdoes, U. Hillaert, B. I. Florea, M. Sae-Heng, M. D. P. Risseeuw, D. V. Filippov, G. A. van der Marel, H. S. Overkleeft, *Bioorganic Med. Chem. Lett.* **2007**, *17*, 6169–6171.
21. L. I. Willems, N. Li, B. I. Florea, M. Ruben, G. A. Van Der Marel, H. S. Overkleeft, *Angew. Chemie - Int. Ed.* **2012**, *51*, 4431–4434.
22. M. Verdoes, B. I. Florea, U. Hillaert, L. I. Willems, W. A. van der Linden, M. Sae-Heng, D. V. Filippov, A. F. Kisselev, G. A. van der Marel, H. S. Overkleeft, *ChemBioChem* **2008**, *9*, 1735–1738.
23. X. Ning, J. Guo, M. A. Wolfert, G. J. Boons, *Angew. Chemie - Int. Ed.* **2008**, *47*, 2253–2255.
24. D. Wessel, U. I. Flugge, *Anal. Biochem.* **1984**, *138*, 141–143.