The versatility of asymmetric aminoethyl-tetrazines in bioorthogonal chemistry

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Chapter 2: Incorporation of tetrazine alanine as amino acid analogue in peptidase inhibitors for activity-based protein profiling

Introduction

Activity-based protein profiling (ABPP) is a well-established methodology to identify enzymes and visualize their activities within complex biological systems.[1] ABPP makes use of irreversible enzyme inhibitors termed activity-based probes (ABPs). These ABPs in general are substrate analogues featuring an electrophilic warhead strategically mimicking and replacing an essential part of the natural substrate and are furthermore equipped with a reporter tag extending from a non-essential part of the inhibitor. ABPP works especially well for enzymes that process their substrate through a covalent enzyme-substrate intermediate, such as the serine hydrolyses and cysteine proteases for which the technology was originally developed.[2] Key to the success of ABPP is the choice of the reporter tag, which should not interfere with enzyme recognition and its subsequent irreversible interaction with the enzyme's active site. Affinity tags (Biotin and a variety of fluorophores) can be, and have been, utilized as reporter tags for ABPP on enzymes that are generous to the addition of molecular bulk to the ABP[3], whereas smaller bioorthogonal tags (azide, alkyne, strained alkene) can be employed for a so called "two-step ABPP", where reporter tags are introduced as a second step after initial binding of the ABP to the enzymes, due to the more restrictive nature of the substrate binding site.[3, 4] The bioorthogonal tags are selected on their reactivity (fast, efficient, reactive only towards their orthogonal counterpart bearing a fluorophore or affinity tag), but also on their size, which should be as small and accessible as possible. In this respect - and this is the objective of this chapter - small tetrazine bearing amino acids are designed to mimic the structural and spatial properties of phenylalanine/tyrosine amino acids and are incorporated in model methylketone peptidase inhibitors[5] to explore their capability to act as an alternative bioorthogonal tag with excellent reactivity properties.

Synthesis of peptidase inhibitors containing tetrazine alanine

In order to prepare potential peptidase inhibitors containing (methyl-)tetrazine alanine (Tza or Tzm) as an analogue for phenylalanine (Phe) or tyrosine (Tyr) at the peptidases N-terminus, a synthetic approach was designed where both the N_α-protected tetrazine alanine building block (PG-Tzx-OH) and warhead building block (H-AA-warhead) could be synthesized separately, and condensed to each other to form the target inhibitor (**Figure 1**).

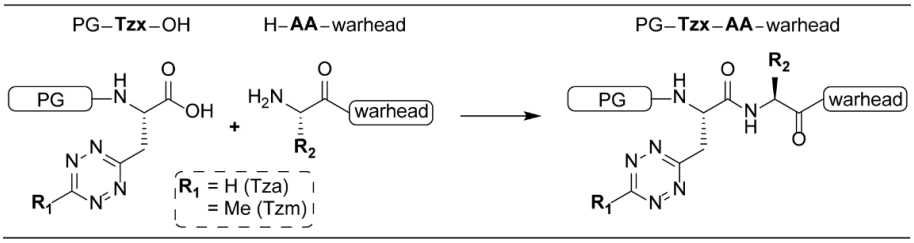
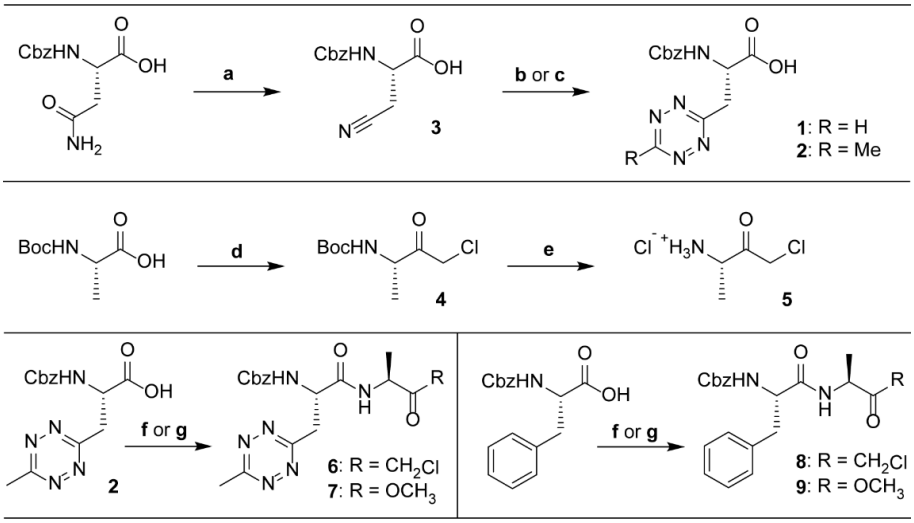


Figure 1: Synthesis approach for peptidase inhibitors containing a N-terminal protection and C-terminal warhead.

First, N_α-Cbz tetrazine alanine **1** and **2** were prepared in two steps (**Figure 2**). N_α-Cbz asparagine (Cbz-Asp-OH) was dehydrated using DCC to form N_α-Cbz cyanoalanine **3**. Compound **3** was then treated with either $Zn(OTf)_2$/formamidine or $Ni(OTf)_2$/acetonitrile in anhydrous hydrazine, after which the crude dihydrotetrazine intermediate was oxidized to yield tetrazine alanine **1** (Cbz-**Tza**-OH) or **2** (Cbz-**Tzm**-OH) respectively.[6] In parallel, N_α-Boc alanine (Boc-Ala-OH) was converted to Boc-Ala-CMK **4** using a diazomethane generation kit.[7] The N_α-Boc protective group was then removed using a HCl/Dioxane solution to obtain H-Ala-CMK **5** as a hydrochloride salt. The initial design was to incorporate Cbz-Tza-OH **1** instead of Cbz-Tzm-OH **2** in the peptidase inhibitor, however tetrazine alanine **1** was prone to degradation after synthesis. The synthesis was therefore continued using Cbz-Tzm-OH **2**. Cbz-Tzm-OH **2** and H-Ala-CMK **5** were condensed using isobutylchloroformate, obtaining the desired inhibitor Cbz-Tzm-Ala-CMK **6**. As control inhibitors Cbz-Tzm-Ala-OMe **7**, Cbz-Phe-Ala-CMK **8** and Cbz-Phe-Ala-OMe **9** were also synthesized using similar approaches. The N_α-Boc variants of each inhibitors were also prepared (**Figure 3**). The purpose of these inhibitors was to remove their N_α-Boc protective group to gain access to H-Tzm-Ala-CMK type inhibitors with a free N-terminus. N_α-Boc cyanoalanine **10** and N_α-Boc (methyl-)tetrazine alanine **11** and **12** were prepared using a synthetic strategy similar to that for compounds **6** – **9** yielding **11** (Boc-**Tza**-OH) and **12** (Boc-**Tzm**-OH). A test deprotection of the N_α-Boc protective group was successfully performed on both **11** and **12** yielded H-Tza-OH **13** and H-Tzm-OH **14** respectively in quantitative yield. This suggested the suitability of the approach for the synthesis of free N-terminus ABPs. Following the successful synthesis of **13** and

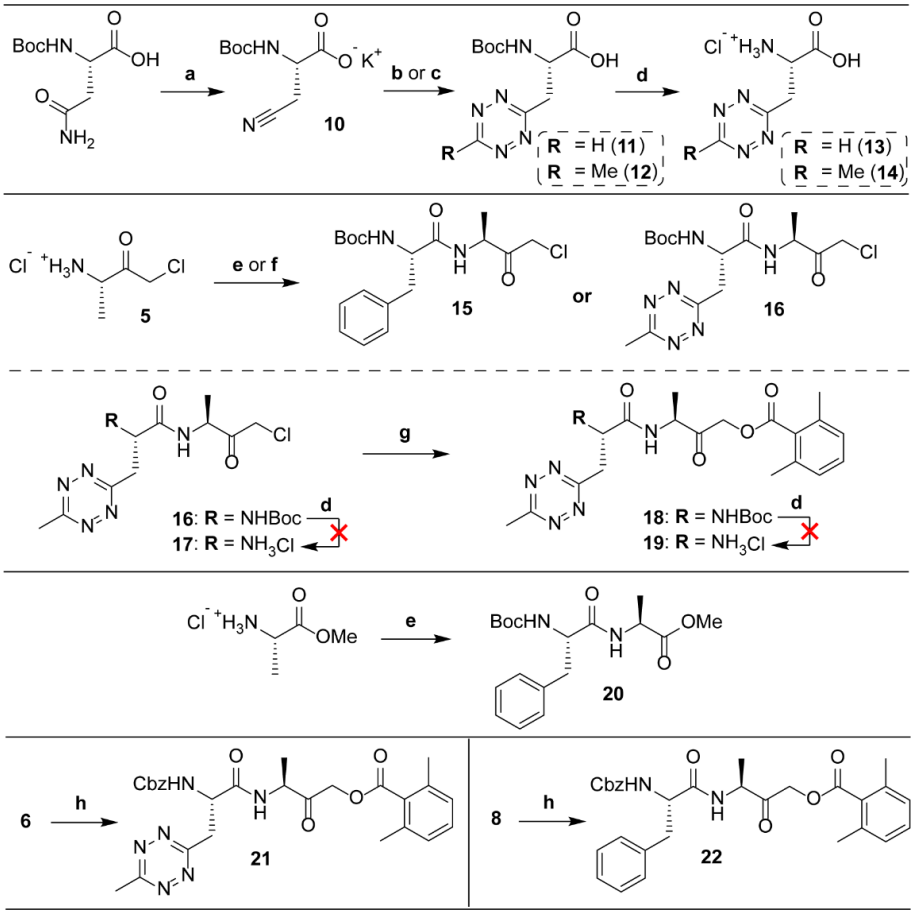
14, peptidase inhibitors Boc-Phe-Ala-CMK **15**, Boc-Tzm-Ala-CMK **16** and control inhibitor Boc-Phe-Ala-OMe **20** were also synthesized.



a) Cbz-Asp-OH, DCC, acetone/pyridine, r.t., 2 hrs, 93%. **b**) formamidine acetate, $Zn(OTf)_2$, dioxane/hydrazine, r.t., 3 days, 14%. **c**) acetonitrile, $Ni(OTf)_2$, dioxane/hydrazine, r.t., 3 days, 11%. **d**) First: Boc-Ala-OH, NMM, IBCF, THF, -25 °C to r.t., 60 min. Second: Diazomethane, Et_2O, 69%. **e**) 4M HCl/dioxane, dioxane, r.t., 2 hrs, quant. **f**) First: IBCF, TEA, THF, r.t., 45 min. Second: 5, TEA, DMF, r.t., 60 min, 21% (**6**), 47% (**8**). **g**) First: IBCF, TEA, THF, r.t., 45 min. Second: H-Ala-OMe*HCl, TEA, DMF, r.t., 50 min, 28% (**7**), 65% (**9**).

Figure 2: Synthesis of N_α-Cbz protected CMK peptidase inhibitors **6** and **8** and methyl ester control inhibitors **7** and **9**.

Next, to obtain H-Tzm-Ala-CMK **17**, compound **16** was treated with a 2M HCl/dioxane/DCM solution in an attempt to remove the N_α-Boc at the N-terminus. However, this resulted in total degradation of inhibitor **17**. The specific composition of the dipeptide was postulated to lead to undefined degradation inside the acidic environment. On the other hand, the disappearance of the distinct red color of the tetrazine, yielding a pale solid, was an indication that the tetrazine moiety was degraded under these conditions. In an attempt to solve this problem, the CMK warhead was substituted for the less reactive 2,6-dimethyl-benzyloxymethylketone (BOMK) warhead.[8a-d] Via a fairly straight forward substitution of the chloride for 2,6-dimethylbenzoate in the presence of excess potassium fluoride, Boc-Tzm-Ala-CMK **16** could be converted to the BOMK warhead functionalized inhibitor Boc-Tzm-Ala-BOMK **18**.



a) DCC, acetone/pyridine, r.t., 2 hrs, 96%. **b**) formamidine acetate, $Zn(OTf)_2$, dioxane/hydrazine, r.t., 3 days, 4%. **c**) acetonitrile, $Ni(OTf)_2$, dioxane/hydrazine, r.t., 3 days, 11%. **d**) 4M HCl/dioxane, DCM, r.t., 2 hrs, quant. **e**) First: Boc-Phe-OH, IBCF, TEA, THF, r.t., 60 min. Second: **5**, TEA, DMF, r.t., 60 min, 62%. **f**) First: **12**, IBCF, TEA, THF, r.t., 60 min. Second: **5**, TEA, DMF, r.t., 60 min, 80%. **g**) 2,6-dimethylbenzoic acid, KF, DMF, r.t., 2 hr, 86%. **h**) 2,6-dimethylbenzoic acid, KF, DMF, r.t., 1 day.

Figure 3: Synthesis N-terminal Boc/Cbz protected peptidase inhibitors containing CMK/BOMK warhead.

Unfortunately all efforts to deprotect the N_α-Boc protective group on inhibitor **18** resulted in degradation of the material as well. Because Boc-Tzm-Ala-BOMK **18** itself could potentially be an interesting inhibitor, the set was expanded to include N_α-Cbz BOMK inhibitors **21** and **22** explore the usefulness of the BOMK warhead. Inhibitors **21** and **22** were then synthesized successfully from chloromethylketone inhibitors **6** and **8** respectively.

ABPP of tetrazine-alanine modified peptidase inhibitors

To test the suitability of Cbz-Phe-Ala-CMK **8** and Cbz-Tzm-Ala-CMK **6** as ABPs for ABPP (**Figure 4**), their capacity to inhibit peptidases was first assessed in a competition study (**Figure 5**). For this study, lysates of mouse RAW 264.7 macrophages[9], and human-derived Jurkat T-cell line[10] were used and fluorescent activity based probe Cy5-DCG-04[11] was used as competitor ABP. This competitor ABP is a derivative of the naturally occurring epoxysuccinate E-64 papain-like cysteine protease inhibitor[12], and serves as a fluorescent marker for non-inhibited proteases. The competition study was performed by incubating the lysates of RAW 264.7 macrophages (**Figure 6 – assay 1**) or Jurkat T-cells (**Figure 6 – assay 2**) in a sodium acetate buffered solution (pH 5.5) with variable concentrations of inhibitor **8** ([C] = 0-500 nM, 30 min, 37 °C), followed by a second incubation with marker Cy5-DCG-04 ([C] = 1 µM, 30 min, 37 °C). The lysates were denatured by addition of mercaptoethanol and heat and Cy5-DCG-04 fluorescence was analyzed using SDS-PAGE. In control lane 1 (no inhibitor **8**), the labeling pattern of Cy5-DCG-04 shows a distinct pattern of strong and weakly visible bands, likely belonging to peptidases within the cathepsin and calpain family.[13] Increasing concentrations of Cbz-Phe-Ala-CMK **8** were able to inhibit a portion of the peptidases visualized by Cy5-DCG-04. To analyze characteristics of Cbz-Tzm-Ala-CMK **6** compared to Cbz-Phe-Ala-CMK **8** the competition study was repeated using Jurkat T-cells (**Figure 6 – assay 3 + 4**) including one of the two inhibitors ([C] = 0-10 µM, 30 min, 37 °C). The results showed near identical fluorescence patterns generated by the Cy5-DCG-04 marker.

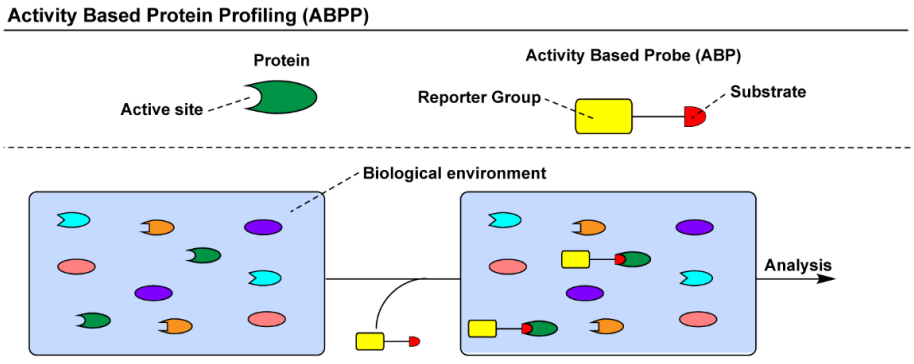


Figure 4: General representation of ABPP: The activity based probe (ABP) has a substrate that covalently binds with the active site of proteins bearing a certain type of active site. This ABP-bound protein can then be analyzed using its reporter group.

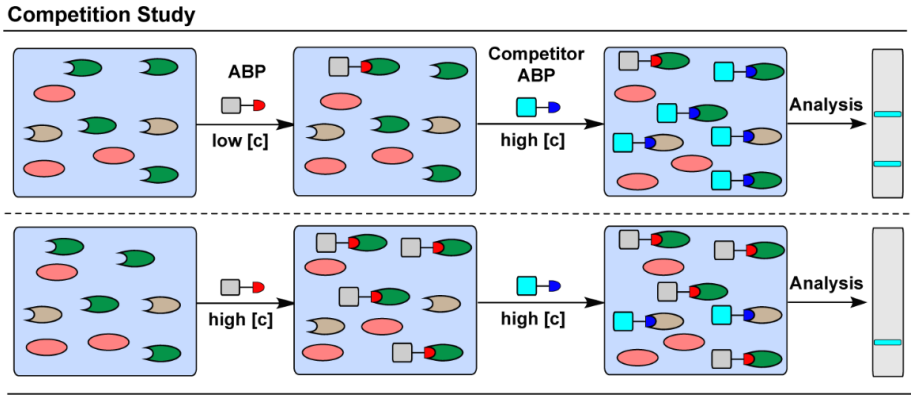


Figure 5: Competition study: The ABP's substrate binds covalently to the enzyme's active site. This is based on concentration, duration, temperature and reactivity. By varying one of the parameters, such as concentration, the amount of remaining enzyme available for a competitor probe changes and can be analyzed using the reporter groups of both probes. Probes can be introduced consecutively, or together.

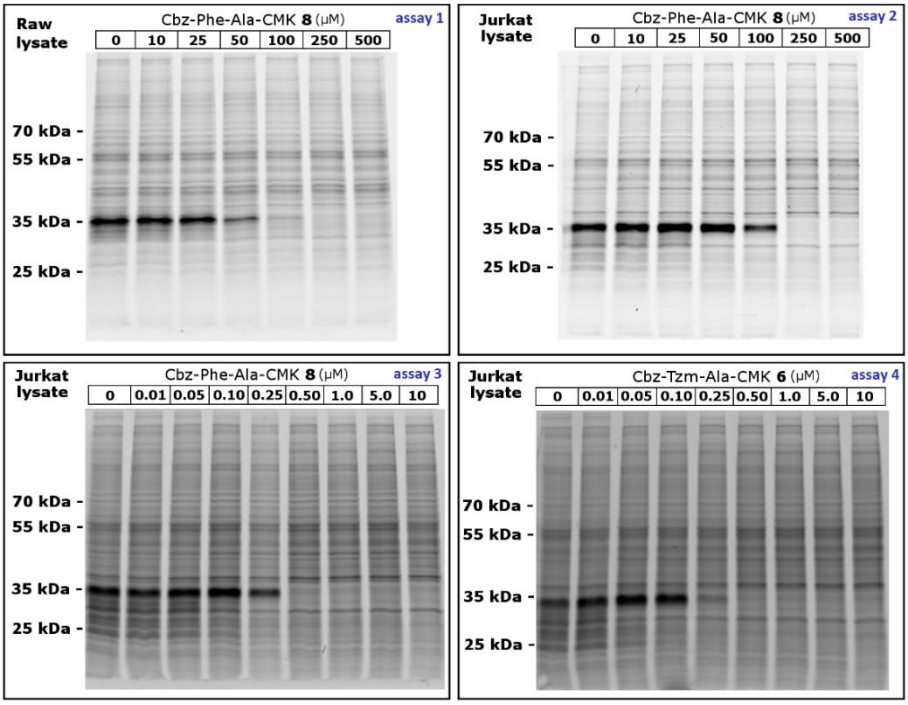


Figure 6: Lysates from RAW 264.7 macrophages (**assay 1**) or Jurkat T-cells (**assay 1-3**) were incubated with compound **8** or **6** at variable concentrations, followed by Cy5-DCG-04. Peptidase-bound Cy5-DCG-04 was visualized using SDS-PAGE fluorescence analysis (700/50 nm).

After the successful results of the competition study, a two-step ABPP experiment (**Figure 7**) was performed using Cbz-Tzm-Ala-CMK **6**, and the TCO-functionalized AF488 fluorophore. Jurkat T-cell lysates were incubated with varying concentrations of Cbz-Tzm-Ala-CMK **6** ([C] = 0-10 µM, 30 min, 37 °C), this was followed by a second incubation with the TCO-AF488 fluorophore, which attaches to the tetrazines through IEDDA reaction. Analysis was performed by denaturing the lysate followed by SDS-PAGE. The results from the SDS-PAGE analysis (**Figure 8 – assay 5**) showed many proteins labeled by inhibitor **6**. Interestingly, at low concentrations (0.1-1 µM), the expected labeling pattern (similar to figure **6**) was observed.

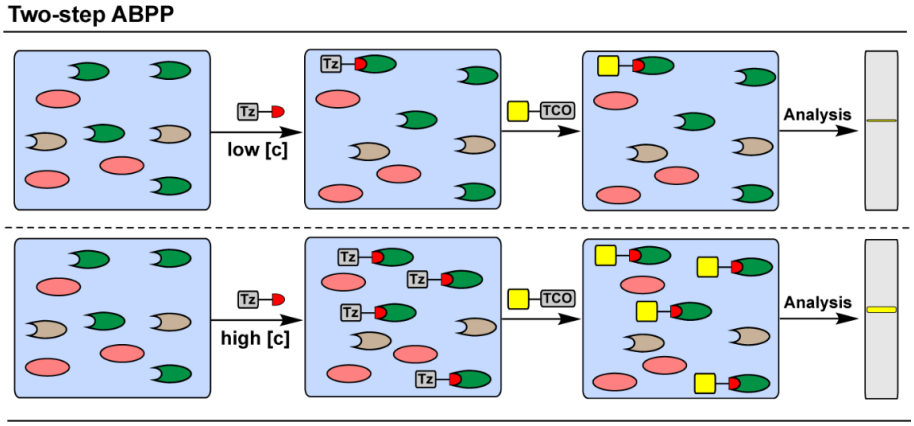


Figure 7: Two-step ABPP**:** The tetrazine-functionalized ABP binds to the enzyme's active site in varying amounts dependent on the ABP concentration. Treatment of the mixture with TCO-AF488, reacting with the tetrazine, results in AF488-labeled enzyme.

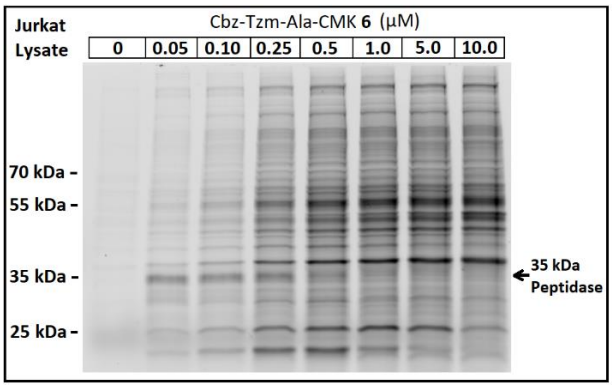


Figure 8: (**assay 5**) Jurkat T-cell lysate was incubated with **6** in a sodium acetate buffer solution at pH 5.5 at variable concentration, followed by incubation with AF488-TCO, denaturing and SDS-PAGE fluorescence analysis (532/28 nm).

At higher concentrations however, the labelling of the 35 kDa peptidase band disappeared, with the appearance of other protein bands. One possible explanation for this could be that, at high concentrations of **6**, proteases that are involved in formation of the 35kDa peptidase band are inhibited.

The next step was to determine if a two-step ABPP competition assay (**Figure 9**) was possible using Cbz-Tzm-Ala-CMK **6** and Cbz-Phe-Ala-CMK **8**, where by addition of **8** at various concentrations. First, Jurkat T-cell lysate was incubated with **8** and excess inhibitor was removed by various forms of precipitation (**Figure 10 – assay 6**), because the presence of unreacted inhibitor **8** during the incubation with AF488-TCO could influence the SDS-PAGE analysis through loss of signal, high background fluorescence or off-target labeling. From these, acetone precipitation worked best. It was then assessed whether further denaturing the proteins would influence the labelling with AF488-TCO, as steric constraints of the active site could limit accessibility of the AF488-TCO fluorophore (**Figure 10 – assay 7**). The tetrazine inhibitor was stable towards the denaturing conditions (25% SDS, 95 °C, 5 minutes), however this did not lead to improved labelling. Incubation at 20 °C for 45 minutes did lead to a change of signal. Finally, the concentration of AF488-TCO was optimized (**Figure 10 – assay 8**). Concentrations above 0.5 µM of AF488-TCO resulted in unwanted background fluorescence at the 20 – 30 kDa region, so the optimized concentration was set at 0.5 µM. With these conditions in hand, the two-step ABPP competition assay was performed to assess whether Cbz-Tzm-Ala-CMK **6** and Cbz-Phe-Ala-CMK **8** would have a specificity towards the same proteases. Lysates from Jurkat cells were incubated with varying concentrations of inhibitor **8** before incubation with inhibitor **6** (**Figure 10 – assay 9**) at 3.0 µM. Between 100-500 nM the 35 kDa peptidase signal disappeared exclusively, with other bands remaining visible. This indicated incomplete inhibition of the other proteases by **8** at the tested concentrations or a divergence in target specificity of **6** and **8**.

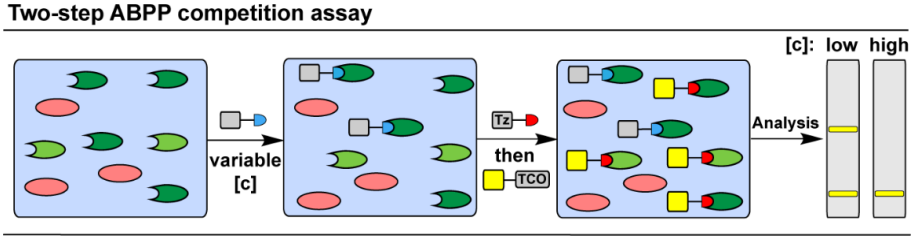


Figure 9: Two-step ABPP competition study: The ABP binds covalently to the enzyme's active site. By varying the concentration, the amount of remaining enzyme available for the tetrazine-functionalized ABP changes and can be analyzed through incubation with AF-488-TCO. Probes can be introduced consecutively, or together.

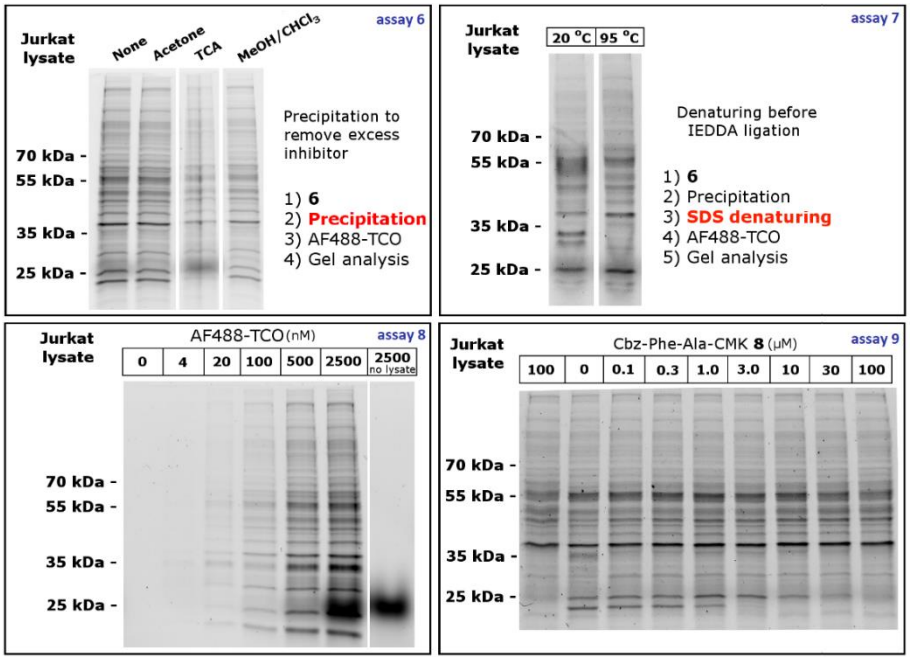


Figure 10: (**assay 6**) SDS-PAGE fluorescence analysis (532/28 nm) of acetone / trichloroacetic acid / methanol-chlorofom precipitation prior to incubation with AF488-TCO. (**assay 7**) SDS-PAGE fluorescence analysis (532/28 nm) of SDS denaturing at room temperature or 95 °C prior to incubation with AF488-TCO. (**assay 8**) SDS-PAGE fluorescence analysis (532/28 nm) of variable AF488-TCO concentrations. (**assay 9**) SDS-PAGE fluorescence analysis (532/28 nm) of variable concentrations inhibitor **8** prior to incubation with inhibitor **6**.

Conclusion

Cbz and Boc protected methyltetrazinealanine (Tzm) inhibitors **6**, **15** (CMK warhead), and **17**, **18** (BOMK warhead) were succesfully synthesized as peptidase inhibitors. A Cy5-DCG-04 competition assay used to compare methyltetrazinealanine inhibitor **6** to phenylalanine inhibitor **8** showed a high similarity in peptidases inhibited the 35kDa peptidase. Following these results the two-step ABPP labeling of peptidases and proteinases targeted by Cbz-Tzm-Ala-CMK **6** was also successful. It was shown that after incubation with **6** proteins can be precipitated with acetone and denatured using mercaptoethanol and heat before labeling with AF488-TCO, without affecting the test result. This validates the tetrazine/TCO two-step ABPP labeling technique as a versatile alternative candidate to other ABPP approaches for these proteins.

Compound synthesis

Procedure A: (Diazomethane generation) An Aldrich Mini Diazald® apparatus with Clear-Seal® joint funnel and receiver is used to add diazomethane. The "dry-ice condenser" is filled with dry ice and iso-propanol. To the "ether trap" a tube leading to an Erlenmeyer filled with a 1:1 solution of H_2O:AcOH is attached. A solution of KOH (5 g, 89 mmol) dissolved in 8 ml H_2O and 10 ml EtOH is placed in the "reaction vessel". The 100 ml Clear-Seal® joint receiving flask containing the pre-activated amino acid is attached and cooled in a dry-ice/EtOH bath. The Clear-Seal® joint separating funnel (containing 2.5 g (11.5 mmol) Diazald® dissolved in 25 mL Et_2O) is attached. On top of the separating funnel a nitrogen balloon is added to ensure a slight overpressure. To start the diazomethane generation the "reaction vessel" is heated to 65 °C and the Diazald® solution is dropwise added over 30-60 minutes. A slight reflux is observed in the reaction vessel, and a yellow condensed liquid is generated at the dry-ice condenser. After emptying the separating funnel, repeatedly, additional batches of 10 mL Et_2O are dropwise added to the reaction vessel until the condensed liquid turns nearly colorless.

Compound 1: 0.979 mmol (0.243 g) of compound **1**, 4.98 mmol (0.518 g) of formamidine acetate and 0.27 mmol (0.099 g) of $Zn(OTf)_2$ were added to a pressure tube. Then 1.5 mL of dry dioxane was added. The tube was sealed and 1.5 mL of anhydrous hydrazine was quickly injected under heavy stirring, while maintaining room temperature in a water bath. The reaction mixture was stirred for three days. The rubber seal was carefully punctured, slowly releasing the generated NH_3 gas. The reaction mixture was dissolved in 10 mL of 4M $NaNO_2$ (aq.) and 25 mL of 2M HCl (aq.) was added dropwise under heavy stirring until gas formation stops (pH = 2-3). Then 0.1M HCl (aq.) was added and the watery solution was extracted two times with EtOAc. The organic layers were combined, dried with $MgSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 2:2:96 AcOH/EtOAc/DCM eluent resulting in 0.040 g (0.132 mmol, 14%) of compound **1** as a thick red oil. **TLC**: Rf = 0.5, 2% AcOH in EtOAc. **^{1}H NMR** (400 MHz, Acetone) **δ** 10.42 (s, 1H), 7.52 – 7.18 (m, 5H), 6.87 (d, *J* = 8.1 Hz, 1H), 5.05 (s, 2H), 3.92 (ddd, *J* = 23.1, 15.0, 6.9 Hz, 2H). **^{13}C NMR** (101 MHz, Acetone) **δ** 172.29, 170.84, 159.22, 156.89, 137.86, 129.20, 128.68, 128.59, 66.95, 53.41, 38.33.

Compound 2: 0.979 mmol (0.243 g) of compound **1**, 4.79 mmol (0.25mL, 0.197 g) of acetonitrile and 0.26 mmol (0.094 g) of $Ni(OTf)_2$ were added to a pressure tube. Then 1.5 mL of dry dioxane was added. The tube was sealed and 1.5 mL of anhydrous hydrazine was quickly injected under heavy stirring, while maintaining room temperature in a water bath. The reaction mixture was stirred for three days. The rubber seal was carefully punctured, slowly releasing the generated NH_3 gas. The reaction mixture was dissolved in 10 mL of 4M $NaNO_2$ (aq.) and 25 mL of 2M HCl (aq.) was added dropwise under heavy stirring until gas formation stops (pH = 2-3). Then 0.1M HCl (aq.) was added and the watery solution was extracted two times with EtOAc. The organic layers were combined, dried with $MgSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 2:2:96 to 2:10:88 AcOH/EtOAc/DCM eluent resulting in 0.034 g (0.107 mmol, 11%) of compound **2** as a thick red oil. **TLC**: Rf = 0.5, 2% AcOH in EtOAc. **^{1}H NMR** (400 MHz, Acetone) **δ** 7.45 – 7.20 (m, 4H), 6.79 (d, *J* = 8.6 Hz, 1H), 4.98 (td, *J* = 8.4, 5.6 Hz, 1H), 4.00 – 3.70 (m, 2H), 2.96 (s, 3H). **^{13}C NMR** (101 MHz, Acetone) **δ** 172.29, 168.55, 167.82, 156.84, 137.92, 129.18, 128.64, 128.56, 66.87, 53.45, 37.69, 21.13.

Compound 3: 13.56 mmol (3.611 g) of Cbz-Asn-OH was dissolved in 35 mL of dry Acetone:Pyridine (1:1, v:v), 14.99 mmol of DCC (3.092 g) was dissolved in 35 mL of dry

Acetone and added to the solution. The reaction mixture was stirred at room temperature for 2 hours. Reaction completion was checked by TLC (Rf = 0.4, 25% MeOH in DCM). The reaction mixture was filtered and concentrated using rotary evaporation. The slurry was re-suspended in H_2O, adjusted to pH = 9 using an aqueous 1M k_2CO_3 solution, washed three times with $CHCl_3$, acidified to pH = 1 using an aqueous 1M HCl solution, extracted three times with $CHCl_3$, then dried using $MgSO_4$ and concentrated using rotary evaporation. 3.113 g (12.54 mmol, 93%) of compound **3** was obtained as a white solid. **^{1}H NMR** (400 MHz, MeOD) **δ** 7.54 – 7.11 (m, 5H), 5.10 (m, 4H), 4.62 – 4.41 (m, 1H), 2.98 (ddd, *J* = 25.1, 17.0, 6.6 Hz, 2H). **^{13}C NMR** (101 MHz, MeOD) **δ** 170.85, 156.86, 136.51, 128.16, 127.74, 127.47, 117.07, 66.60, 50.40, 20.02.

Compound 4: 5.28 mmol (0.998 g) of Boc-Ala-OH was dissolved in 25 mL of dry THF, cooled to -25 °C, and 6.82 mmol (0.75 ml, 0.69 g) of N-methylmorpholine (NMM) and 6.55 mmol (0.85 mL, 0.90 g) isobutyl chloroformate (IBCF) were added to the solution. The reaction mixture was stirred for 1 hour while warming to room temperature. Then, using an Aldrich Mini Diazald® apparatus 8.3 mmol diazomethane in Et_2O was added (**procedure A**). The reaction was quenched by dropwise addition of 10 ml of conc. HCl (aq) in AcOH (1:1, v:v) and then warmed to room temperature. Et_2O and sat. K_2CO_3 (aq.) were added before extracting the mixture and partitioning the layers. The organic layer was dried using Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 5-20% EtOAc in pentane eluent resulting in 0.764 g (3.45 mmol, 69%) of compound **4** as a colorless oil. **TLC**: Rf = 0.3, 20% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** δ 5.18 (s, 1H), 4.47 (p, *J* = 7.2 Hz, 1H), 4.27 (d, *J* = 3.7 Hz, 2H), 1.40 (s, 9H), 1.33 (d, *J* = 7.2 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 202.17, 155.32, 80.38, 77.48, 77.16, 76.84, 53.24, 46.35, 28.35, 17.42.

Compound 5: 1.8 mmol (0.40 g) of compound **4** was dissolved in 3.5 ml dry dioxane and 2.4 ml (9.6 mmol) 4M HCl in dioxane was dropwise over 1 minute to the solution. The reaction mixture was stirred for 2 hours before it was concentrated using rotary evaporation obtaining compound **5** in quantitative yield as a paly white solid. **^{1}H NMR** (400 MHz, DMSO) **δ** 8.31 (br, 3H), 4.80 (dd, *J* = 65.4, 17.2 Hz, 2H), 4.26 (q, *J* = 6.9 Hz, 1H), 1.41 (d, *J* = 7.3 Hz, 3H). **HR-MS**: [$C_4H_8ClNO+H^+$] found 122.0367, calculated 122.0294.

Compound 6: 0.11 mmol (34 mg) of compound **2** was dissolved in 1.6 ml dry THF, 0.11 mmol (14.6 mg, 200 µl of a 0.5 M solution in dry THF) IBCF and 0.10 mmol (10.9 mg, 200 µl of a 0.5 M solution in dry THF) TEA were added and the reaction mixture was stirred for 45 minutes at room temperature. An 1.8 ml dry DMF solution containing 0.11 mmol (17 mg) compound **5** and 0.13 mmol (13 mg, 17.9 µL) TEA was dropwise added to the reaction mixture and stirred for an additional 60 minutes at room temperature. Then, 50 ml EtOAc was added, and the organic layer was washed with 5% citric acid (aq.), water and brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20-40% EtOAc in pentane eluent resulting in 9.6 mg (0.023 mmol, 21%) of compound **6** as pink solid **TLC**: Rf = 0.5, 50% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.41 – 7.28 (m, 5H), 7.06 (d, *J* = 6.9 Hz, 1H), 6.03 (d, *J* = 8.6 Hz, 1H), 5.21 – 5.03 (m, 2H), 4.96 (q, *J* = 6.1 Hz, 1H), 4.72 (p, *J* = 7.1 Hz, 1H), 4.20 (s, 2H), 3.79 (qd, *J* = 16.0, 6.0 Hz, 2H), 3.04 (s, 3H), 1.35 (d, *J* = 7.2 Hz, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) **δ** 200.99, 170.04, 168.03, 166.90, 156.20, 135.88, 128.75, 128.55, 128.34, 67.69, 52.85, 52.39, 46.18, 36.91, 21.32, 17.17. **HRMS:** [$C_{18}H_{21}ClN_6O_4+H]^+$: found 421.1390, calculated 421.1313.

Compound 7: 25 µmol (7.9 mg) of compound **2** was dissolved in 300 µl dry THF, 25 µmol (3.4 mg, 100 µl of a 0.25 M solution in dry THF) IBCF and 25 µmol (2.5 mg, 100 µl of a 0.25 M

solution in dry THF) TEA were added and the reaction mixture was stirred for 45 minutes at room temperature. An 1.3 ml dry DMF solution containing 25 µmol (3.5 mg) alanine methyl ester hydrochloride and 30 µmol (3.0 mg, 4.1 µL) TEA was dropwise added to the reaction mixture and stirred for an additional 60 minutes at room temperature. Then, EtOAc was added, and the organic layer was washed with 5% citric acid (aq.), water and brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20-40% EtOAc in pentane eluent resulting in 2.8 mg (7 µmol, 28%) of compound **7** as pink solid **TLC**: Rf = 0.4, 50% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.44 – 7.28 (m, 5H), 6.88 (d, *J* = 7.3 Hz, 1H), 6.03 (d, *J* = 8.6 Hz, 1H), 5.15 – 5.03 (m, 2H), 4.95 (q, *J* = 6.7, 6.1 Hz, 1H), 4.50 (p, *J* = 7.3 Hz, 1H), 3.91 – 3.72 (m, 2H), 3.71 (s, 3H), 3.04 (s, 3H), 1.36 (d, *J* = 7.3 Hz, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) **δ** 172.90, 169.59, 167.93, 167.08, 156.12, 135.96, 128.73, 128.49, 128.32, 67.58, 52.73, 48.43, 37.16, 21.32, 18.27. **HRMS:** $[C_{18}H_{22}N_6O_5+H]^+$: found 403.1726, calculated 403.1652.

Compound 8: 0.10 mmol (30 mg) Cbz-Phe-OH was dissolved in 1.6 ml dry THF, 0.10 mmol (13.7 mg, 200 µl of a 0.5 M solution in dry THF) IBCF and 0.10 mmol (10.1 mg, 200 µl of a 0.5 M solution in dry THF) TEA were added and the reaction mixture was stirred for 1 hour at room temperature. An 1.8 ml dry DMF solution containing 0.10 mmol (16 mg) compound **5** and 0.12 mmol (12 mg, 16.7 µL) TEA was dropwise added to the reaction mixture and stirred for an additional 30 min at room temperature. Then, 50 ml EtOAc was added, and the organic layer was washed with 5% citric acid (aq.), 5% $NaHCO_3$ (aq.), water and brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20-30% EtOAc in pentane eluent resulting in 19.1 mg (0.047 mmol, 47%) of compound **8** as white solid **TLC**: Rf = 0.6, 50% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.43 – 7.23 (m, 8H), 7.19 (d, *J* = 6.8 Hz, 2H), 6.61 (d, *J* = 5.5 Hz, 1H), 5.45 (d, *J* = 7.3 Hz, 1H), 5.08 (s, 2H), 4.68 (p, *J* = 7.1 Hz, 1H), 4.46 (d, *J* = 7.1 Hz, 1H), 4.09 (s, 2H), 3.20 – 2.97 (m, 2H), 1.29 (d, *J* = 7.1 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 200.81, 170.96, 156.10, 136.08, 129.39, 128.93, 128.71, 128.43, 128.18, 127.41, 77.48, 77.16, 76.84, 67.37, 56.26, 52.11, 46.14, 38.50, 17.11. **HRMS**: $[C_{21}H_{23}ClN_2O_4+H]^+$: Found 403.1419, calculated 403.1346.

Compound 9: 0.10 mmol (30 mg) of Cbz-Phe-OH was dissolved in 1.6 mL dry THF, 0.10 mmol (13.7 mg, 200 µl of a 0.5 M solution in dry THF) IBCF and 0.10 mmol (10.1 mg, 200 µL of a 0.5 M solution in dry THF) TEA were added and the reaction mixture was stirred for 1 hour at room temperature. A 1.8 mL dry DMF solution containing 0.10 mmol (14 mg) alanine methyl ester hydrochloride and 0.12 mmol (12 mg, 16.7 µL) TEA was dropwise added to the reaction mixture and stirred for an additional 30 min at room temperature. Then, EtOAc was added, and the organic layer was washed with 5% citric acid (aq.), 5% $NaHCO_3$ (aq.), water and brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20-30% EtOAc in pentane eluent resulting in 24.8 mg (0.065 mmol, 65%) of compound **9** as white solid **TLC**: Rf = 0.5, 50% EtOAc in pentane **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.40 – 7.12 (m, 11H), 6.44 (d, *J* = 7.2 Hz, 1H), 5.40 (d, *J* = 8.1 Hz, 1H), 5.08 (s, 2H), 4.48 (dp, *J* = 14.2, 7.2 Hz, 2H), 3.70 (s, 3H), 3.19 – 2.99 (m, 2H), 1.32 (d, *J* = 7.2 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 172.93, 170.51, 156.02, 136.31, 136.23, 129.47, 128.78, 128.65, 128.32, 128.15, 127.17, 67.18, 56.10, 52.60, 48.25, 38.62, 18.38. **HRMS:** $[C_{21}H_{24}N_2O_5+H]^+$: found 385.1762, calculated 385.1685.

Compound 10: 19.27 mmol (4.470 g) of Boc-Asn-OH was dissolved in 50 mL of dry Acetone:Pyridine (1:1, v:v), 22.12 mmol of DCC (4.565 g) was dissolved in 40 mL of dry acetone and added to the solution. The reaction mixture was stirred at room temperature for 2 hours.

Reaction completion was checked by TLC (Rf = 0.5, 30% MeOH in DCM). The reaction mixture was filtered and concentrated using rotary evaporation. The slurry was redissolved in 10 mL (10 mmol) 1M k_2CO_3 solution, washed three times with $CHCl_3$ and concentrated using rotary evaporation. 4.680 g (18.57 mmol, 96%) of compound **10** was obtained as a white solid. **^{1}H NMR** (400 MHz, DMSO) **δ** 6.25 (d, *J* = 5.4 Hz, 1H), 3.65 (q, *J* = 5.2 Hz, 1H), 2.96 – 2.67 (m, 2H), 1.39 (s, 9H). **^{13}C NMR** (101 MHz, DMSO) **δ** 169.90, 154.91, 119.22, 78.02, 51.60, 28.16, 21.59.

Compound 11: 0.976 mmol (0.246 g) of compound **10**, 10.08 mmol (1.050 g) of formamidine acetate and 0.26 mmol (0.092 g) of $Zn(OTf)_2$ were added to a pressure tube. Then 1.5 mL of dry dioxane was added. The tube was sealed and 1.5 mL of anhydrous hydrazine was quickly injected under heavy stirring, while maintaining room temperature in a water bath. The reaction mixture was stirred for three days. The rubber seal was carefully punctured, slowly releasing the generated NH_3 gas. The reaction mixture was dissolved in 10 mL of 4M $NaNO_2$ (aq.) and 25 mL of 2M HCl (aq.) was added dropwise under heavy stirring until gas formation stops (pH = 2-3). Then 1M HCl (aq.) was added and the watery solution was extracted two times with EtOAc. The organic layers were combined, dried with $MgSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 2:2:96 AcOH/EtOAc/DCM eluent resulting in 11 mg (0.041 mmol, 4.2%) of compound **11** as a thick red oil. **TLC**: Rf = 0.5, 2% AcOH in EtOAc. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 10.25 (s, 1H), 8.41 (s, 1H), 5.63 (d, *J* = 7.9 Hz, 1H), 4.97 (m, 1H), 3.92 (ddd, *J* = 22.4, 15.5, 5.7 Hz, 2H), 1.38 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 174.60, 169.67, 158.27, 155.52, 81.00, 51.79, 37.91, 28.33.

Compound 12: 9.05 mmol (2.28 g) of compound **10**, 155 mmol (5.0 mL, 6.36 g) of acetonitrile and 2.40 mmol (0.872 g) of $Ni(OTf)_2$ were added to a pressure tube. The tube was sealed and 16 mL of anhydrous hydrazine was quickly injected under heavy stirring, while maintaining room temperature in a water bath. The reaction mixture was stirred for three days. The rubber seal was carefully punctured, slowly releasing the generated NH_3 gas. The reaction mixture was dissolved in 400 mL of DCM/AcOH (1:1, v:v) While stirring, the reaction mixture was added dropwise. Then 10 g solid $NaNO_2$ was added portion wise over 30 minutes. The mixture was concentrated using rotary evaporation, re-dissolved in EtOAc, washed with aqueous 2M HCl (check for pH = 1) and brine, dried using $MgSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 2:2:96 to 2:10:88 AcOH/EtOAc/DCM eluent resulting in 0.279 g (0.985 mmol, 11%) of compound **12** as a thick red oil. **TLC**: Rf = 0.5, 2% AcOH in EtOAc. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 8.20 (s, 1H), 5.63 (d, *J* = 7.6 Hz, 1H), 4.93 (d, *J* = 4.9 Hz, 1H), 3.85 (ddd, *J* = 22.1, 15.3, 5.3 Hz, 2H), 3.04 (s, 3H), 1.37 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 174.66, 167.90, 166.60, 155.52, 80.82, 51.86, 37.26, 28.32, 21.22.

Compound 13: Compound **11** was dissolved in DCM, cooled to 0 °C, and a 4M HCl in dioxane solution was added dropwise over 1 minute. The solution was stirred for 4 hours forming a suspension. The suspension was concentrated using rotary evaporation resulting in compound **13** in quantitative yield.

Compound 14: 0.071 mmol (20 mg) of compound **12** was dissolved in 0.4 mL DCM, cooled to 0 °C, and 0.4 mL of a 4M HCl in dioxane solution was added dropwise over 1 minute. The solution was stirred for 4 hours forming a suspension. The suspension was concentrated using rotary evaporation resulting in compound **14** in quantitative

yield. **^{1}H NMR** (400 MHz, D_2O) **δ** 4.68 – 4.62 (m, 1H), 3.92 (d, *J* = 6.2 Hz, 2H), 2.95 (s, 3H). **^{13}C NMR** (101 MHz, D_2O) **δ** 170.86, 168.27, 165.42, 50.98, 34.11, 20.18.

Compound 15: 0.40 mmol (106 mg) of Boc-Phe-OH was dissolved in 7 mL dry THF, 1.08 mmol (147 mg, 139 µL) IBCF and 0.49 mmol (50 mg, 69 µL) TEA were added and the reaction mixture was stirred for 1 hour at room temperature. An 7 mL dry DMF solution containing 0.49 mmol (78 mg) compound **5** and 1.2 mmol (122 mg, 168 µL) TEA was dropwise added to the reaction mixture and stirred overnight at room temperature. Then, 140 mL EtOAc was added, and the organic layer was washed with 5% citric acid (aq.), 5% $NaHCO_3$ (aq.), water and brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20-50% EtOAc in pentane eluent resulting in 91 mg (0.247 mmol, 62%) of compound **15** as white solid **TLC**: Rf = 0.5, 20% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.36 – 7.16 (m, 5H), 7.05 (s, 1H), 5.34 (d, *J* = 6.9 Hz, 1H), 4.66 (m, 1H), 4.44 (m, 1H), 4.08 (s, 2H), 3.05 (s, 2H), 1.41 (s, 9H), 1.30 (d, *J* = 7.1 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 200.88, 171.64, 155.63, 136.41, 129.35, 128.74, 127.17, 80.42, 55.78, 52.12, 46.28, 38.37, 28.32, 16.84.

Compound 16: 0.106 mmol (30 mg) of compound **12** was dissolved in 3 mL dry THF, cooled to 0 °C, 0.116 mmol (16 mg, 15 µL) IBCF and 0.115 mmol (12 mg, 16 µL) TEA were added and the reaction mixture was stirred for 30 minutes. A 2 mL dry THF solution containing 0.165 mmol (26 mg) compound **5** and 0.208 mmol (21 mg, 29 µL) TEA was dropwise added to the reaction mixture and stirred for 1 hour while warming to room temperature. Then, EtOAc was added, and the organic layer was washed water, brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 25-50% EtOAc in pentane eluent resulting in 33 mg (0.085 mmol, 80%) of compound **16** as a red solid. **TLC**: Rf = 0.5, 50% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.22 (d, *J* = 6.4 Hz, 1H), 5.81 (m, 1H), 4.85 (m, 1H), 4.79 – 4.67 (m, 1H), 4.23 (m, 2H), 3.80 – 3.71 (m, 2H), 3.03 (s, 3H), 1.41 – 1.35 (m, 12H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 201.05, 170.41, 167.89, 167.03, 79.23, 52.51, 52.33, 46.27, 46.20, 36.94, 28.31, 21.25, 17.09.

Compound 18: 49 µmol (19 mg) of compound **16** was dissolved in 1 mL of dry DMF and 153 µmol (23 mg) of 2,6-dimethylbenzoic acid was added before adding 517 µmol (30 mg) of solid KF and stirring the solution for 2 hours at room temperature. Reaction completion was checked by TLC (Rf = 0.55, 50% EtOAc in pentane). Then, 10 mL EtOAc was added, and the organic layer was washed two times with 10 mL 5% $NaHCO_3$ (aq.), 10 mL brine, dried with $MgSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 25-50% EtOAc in pentane eluent resulting in 21 mg (42 µmol, 86%) of compound **18** as a red solid. **^{1}H NMR** (500 MHz, $CDCl_3$) **δ** 7.20 (t, *J* = 7.6 Hz, 1H), 7.12 (s, 1H), 7.04 (d, *J* = 7.6 Hz, 2H), 5.74 (d, *J* = 7.4 Hz, 1H), 5.00 (m, 2H), 4.87 (dd, *J* = 13.7, 6.1 Hz, 1H), 4.67 (p, *J* = 7.0 Hz, 1H), 3.78 (d, *J* = 6.2 Hz, 2H), 3.03 (s, 3H), 2.37 (s, 6H), 1.43 (m, 12H). **^{13}C NMR** (126 MHz, $CDCl_3$) **δ** 201.69, 170.35, 169.08, 167.90, 167.12, 135.79, 132.45, 129.91, 127.82, 66.23, 52.49, 51.93, 51.84, 36.83, 28.35, 21.26, 20.02, 17.16.

Compound 20: 1.0 mmol (0.265 g) Boc-Phe-OH was dissolved in 4 ml of dry DMF, 1.2 mmol (0.167 g) alanine methyl ester hydrochloride, 1.2 mmol (0.162 g) HOBt and 1.3 mmol (0.131 g, 180 µL) TEA were added and stirred for 4 hours at room temperature. 100 mL of DCM was added and the organic layer was washed with sat. $NaHCO_3$ (aq.), 1M HCl (aq.), water and brine, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 30-50% EtOAc in pentane eluent resulting in 300 mg (0.856 mmol, 86%) of compound **20** as a white solid. **^{1}H NMR** (400 MHz,

$CDCl_3$) **δ** 7.34 – 7.10 (m, 5H), 6.44 (d, *J* = 7.1 Hz, 1H), 5.01 (s, 1H), 4.52 (p, *J* = 7.0 Hz, 1H), 4.36 (d, *J* = 5.9 Hz, 1H), 3.71 (s, 3H), 3.07 (t, *J* = 6.1 Hz, 2H), 1.40 (s, 9H), 1.34 (d, *J* = 7.2 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 172.97, 170.83, 136.60, 129.50, 128.77, 127.09, 55.68, 52.59, 48.23, 38.47, 28.37, 18.53.

Compound 21: 3.85 µmol (1.62 mg) of compound **6** was dissolved in 70 µL of dry DMF and 13 µmol (2 mg) of 2,6-dimethylbenzoic acid was added before adding 34 µmol (2 mg) of solid KF and stirring the solution for 1 day at room temperature. Reaction completion was checked by TLC (Rf = 0.6, 50% EtOAc in pentane). The reaction mixture was diluted with 1 mL EtOAc, washed with 1 mL sat. $NaHCO_3$ (aq.) and 1mL brine. The organic layer was dried using $MgSO_4$ and concentrated under rotary evaporation. Purification was performed with silica column chromatography using an 50:50 EtOAc/pentane eluent resulting in compound **21** as a red solid. **^{1}H NMR** (400 MHz, Chloroform) **δ** 7.39 – 7.29 (m, 5H), 7.24 – 7.18 (m, 1H), 7.05 (d, *J* = 7.6 Hz, 2H), 7.00 (s, 1H), 5.99 (d, *J* = 8.6 Hz, 1H), 5.20 – 4.86 (m, 5H), 4.65 (p, *J* = 6.8 Hz, 1H), 3.91 – 3.70 (m, 2H), 3.03 (s, 3H), 2.38 (s, 6H), 1.40 (d, *J* = 7.2 Hz, 3H).

Compound 22: 4.0 µmol (1.7 mg) of compound **8** was dissolved in 70 µL of dry DMF and 13 µmol (2 mg) of 2,6-dimethylbenzoic acid was added before adding 34 µmol (2 mg) of solid KF and stirring the solution for 1 day at room temperature. Reaction completion was checked by TLC (Rf = 0.4, 40% EtOAc in pentane). The reaction mixture was diluted with 1 mL EtOAc, washed with 1 mL sat. $NaHCO_3$ (aq.) and 1mL brine. The organic layer was dried using $MgSO_4$ and concentrated under rotary evaporation. Purification was performed with silica column chromatography using an 20:80 to 40:60 EtOAc/pentane eluent resulting in compound **22** as a red solid. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.46 – 7.19 (m, 11H), 7.10 (d, *J* = 7.7 Hz, 2H), 6.46 (d, *J* = 6.3 Hz, 1H), 5.30 (d, *J* = 7.2 Hz, 1H), 5.14 (s, 2H), 4.92 (dd, *J* = 39.8, 16.9 Hz, 2H), 4.69 (p, *J* = 7.1 Hz, 1H), 4.48 (d, *J* = 6.8 Hz, 1H), 4.16 (q, *J* = 7.1 Hz, 2H), 3.14 (ddd, *J* = 33.8, 13.8, 6.8 Hz, 1H), 2.43 (s, 6H), 1.40 (d, *J* = 7.1 Hz, 3H).

Biological Protocols

Cell Culturing

Jurkat T-cells were cultured in RPMI-1640 with 10% FBS, penicillin (100 U/mL), streptomycin (0.10 mg/mL) and Gibco GlutaMAX (2 mM). Cells were kept at a cell density between $1*10^5$ and $1*10^6$ cells/mL in T-25/T-75/T-150 flasks. Culture conditions were 95% air and 5% CO_2 at 37°C.

RAW 264.7 macrophages were cultured in DMEM with 10% FBS, penicillin (100 U/mL), streptomycin (0.10 mg/mL) and Gibco GlutaMAX (2 mM). Culture cell density was kept between $1*10^6$ and $2*10^6$ cells/mL in 10 cm Petri dishes. Culture conditions were 95% air and 5% CO_2 at 37°C.

Cell Harvesting

Cell harvest: Cells were collected (13 million / tube) and centrifuged at 300 RCF. The medium was decanted and the tubes were cooled to -20 °C before flash freezing in liquid N_2 for storage at -80 °C op to 3 months.

Cell Lysis

Lysis Buffer: A 50 mM NaOAc (aq.) solution was titrated to pH = 5.5 via dropwise addition of 1M NaOH (aq.) and supplemented with DTT (4 mM), $MgCl_2$ (10 mM) and 0.2% Triton-X 100, before diluting with water (1:1, v:v) and re-titrating to pH 5.5.

Cell Lysis: Frozen harvested cells were transferred to ice (-20 °C) and kept for 30 minutes to defrost. Excess liquid was removed before mixing the suspension with 25 µL lysis buffer for cell lysis (1 hour, -20 °C). The suspension was flash frozen three times (1 min liq. N_2 freeze, 7-10 min -20 °C thaw), centrifuged (30 min, 4°C, 30.000 RCF) and the lysate supernatant was collected to ice (-20 °C).

Bradford assay: Lysate supernatant (1 µL) was diluted with H_2O (39 µL), from which 1 µL was transferred to a 96 well plate containing 100 µL Bradford solution (1 part Bradford reagent, 4 parts H_2O) and measured at 595 nm absorption wavelength on a BioRAD iMark™ microplate reader. Lysates were measured triplo against a freshly prepared BSA in H_2O calibration line (2.0 mg/mL – 0.0625 mg/mL range).

Stock lysate: Lysate supernatant was diluted to a protein concentration of 1.8 µg/µL using 50 mM NaOAc (aq.) at -20 °C, separated into Eppendorf tubes (100 µL stocks, -20 °C) and flash frozen in liquid N_2 for storage at -80 °C op to 2 weeks.

Protein Precipitation

Acetone precipitation: Eppendorf tubes containing lysate in NaOAc buffered water were diluted with acetone (4x volume), cooled to -20 °C for 30 minutes, and centrifuged at 4 °C for 10 minutes before removing the supernatant. The remaining pellet (poorly visible) was redissolved in the original amount of NaOAc buffered water.

MeOH/$CHCl_3$ precipitation: Eppendorf tubes containing lysate in NaOAc buffered water were diluted with methanol (4x volume), chloroform (1x volume) and water (3x volume) respectively, vortexed, and centrifuged. The top and bottom layers were removed while keeping the precipitated protein at the interface. The protein was redissolved in the original amount of NaOAc buffered water.

SDS-PAGE

SDS-PAGE Protocol: 10 lane SDS-PAGE plates were prepared using: **(1)** Add 3 mL running gel (36% of H_2O, 25% of Buffer A, 33.5% of Acrylamide 30% (29:1) solution, 5% Glycerol, 0.5% APS, and 0.05% TEMED), followed by 60 µL iPrOH and 30 minutes polymerization. **(2)** Fill with stacking gel (60% H_2O, 24% Buffer B, 14.4% Acrylamide 30% (29:1) solution, 1.2% APS, 0.12% TEMED) before 30 minutes polymerization. Samples were denatured using 4 µL β-mercaptoethanol and heating to 95 °C for 5 minutes prior to loading. Samples were run at 90 volt for 15 minutes, followed by 150 volt for 80-90 minutes against a PAGE-ruler calibration ladder. Gel analysis was performed on a BioRAD ChemiDOC™ MP imaging system: Cy3-channel (602/50 nm) for PAGE-ruler lines (25kDa / 70 kDa), Cy5-channel (700/50 nm) for Cy5-DCG-04 labelled proteins and AF488-channel (532/28 nm) for AF488 labelled proteins. To verify protein concentration a Coomassie staining was performed. The Coomassie staining was performed on a BioRAD ChemiDOC™ MP imaging system: Coomassie (590/110 nm).

Biological Experimental

Assay 1 + 2: Stock RAW / Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 10 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Phe-Ala-CMK **8** (11x concentration, 1% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Then, per Eppendorf tube, 1.2 µL of Cy5-DCG-04 (12 µM, 6.5% DMSO) was added to reach 1 µM concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 1

			Lane Content							
			Ruler	Samples						
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7
-	RAW Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10
1 (30 min)	Cbz-Phe-Ala-CMK **8**	µL (range)	-	- (0)	1 (0.01)	1 (0.025)	1 (0.05)	1 (0.10)	1 (0.25)	1 (0.50)
2 (30 min)	Cy5-DCG-04	µL (20 µM)	-	1	1	1	1	1	1	1

Assay 2

			Lane Content							
			Ruler	Samples						
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10
1 (30 min)	Cbz-Phe-Ala-CMK **8**	µL (range)	-	- (0)	1 (0.01)	1 (0.025)	1 (0.05)	1 (0.10)	1 (0.25)	1 (0.50)
2 (30 min)	Cy5-DCG-04	µL (20 µM)	-	1	1	1	1	1	1	1

Assay 3 + 4: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 10 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Phe-Ala-CMK **8** / Cbz-Tzm-Ala-CMK **6** (11x concentration, 1% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Then, per Eppendorf tube, 1.2 µL of Cy5-DCG-04 (12 µM, 6.5% DMSO) was added to reach 1 µM concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 3

			Lane Content									
			Ruler	Samples								
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7	8	9
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10	10	10
1 (30 min)	Cbz-Phe-Ala-CMK **8**	µL (range)	-	- (0)	1 (0.01)	1 (0.05)	1 (0.10)	1 (0.25)	1 (0.50)	1 (1.0)	1 (5.0)	1 (10.0)
2 (30 min)	Cy5-DCG-04	µL (20 µM)	-	1	1	1	1	1	1	1	1	1

Assay 4

			Lane Content									
			Ruler	Samples								
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7	8	9
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10	10	10
1 (30 min)	Cbz-Tzm-Ala-CMK **6**	µL (range)	-	- (0)	1 (0.01)	1 (0.05)	1 (0.10)	1 (0.25)	1 (0.50)	1 (1.0)	1 (5.0)	1 (10.0)
2 (30 min)	Cy5-DCG-04	µL (20 µM)	-	1	1	1	1	1	1	1	1	1

Assay 5: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 10 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Tzm-Ala-CMK **6** (11x concentration, 1% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Then, per Eppendorf tube, 1 µL of AF488-TCO (6 µM, 10% DMSO) was added to reach 0.5 µM concentration, before incubating for 30 minutes at 37 °C. Then, per Eppendorf tube, 1.3 µL of Cy5-DCG-04 (13 µM, 6.5% DMSO) was added to reach 1

µM concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 5			Lane Content									
			Ruler	Samples								
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7	8	9
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10	10	10
1 (30 min)	Cbz-Tzm-Ala-CMK **6**	µL (range)	-	1 (0)	1 (0.01)	1 (0.05)	1 (0.10)	1 (0.25)	1 (0.50)	1 (1.0)	1 (5.0)	1 (10.0)
	Acetone precipitation											
2 (30 min)	AF488-TCO	µL (500 nM)	-	1	1	1	1	1	1	1	1	1
3 (30 min)	Cy5-DCG-04	µL (20 µM)	-	1	1	1	1	1	1	1	1	1

Assay 6: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 6 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Tzm-Ala-CMK **6** (5.5 µM, 1% DMSO) was added to reach 0.5 µM concentration, before incubating for 30 minutes at 37 °C. Then, the protein was precipitated using the acetone (lane 1+2), TCA (lane 3+4), or methanol/chloroform (lane 5+6) precipitation protocol. Then, per Eppendorf tube, 1 µL of AF488-TCO (6 µM, 10% DMSO) was added to reach 0.5 µM concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 6			Lane Content						
			Ruler	Samples					
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10
1 (30 min)	Cbz-Tzm-Ala-CMK 6	µL (500 nM)	-	1	1	1	1	1	1
	Precipitation			Acetone		TCA		$MeOH/CHCl_3$	
2 (30 min)	AF488-TCO	µL (500 nM)	-	1	1	1	1	1	1

Assay 7: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 2 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Tzm-Ala-CMK **6** (33 µM, 1% DMSO) was added to reach 3.0 µM concentration, before incubating for 30 minutes at 37 °C. Then, the protein was precipitated using the acetone precipitation protocol. Then, the lysates were denatured using an SDS solution at 20 or 95 °C for 45 minutes. Then, per Eppendorf tube, 1 µL of AF488-TCO (6 µM, 10% DMSO) was added to reach 0.5 µM concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol excluding denaturing step.

Assay 8: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 10 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Tzm-Ala-CMK **6** (5.5 µM, 1% DMSO) was added to reach 0.5 µM concentration, before incubating for 30 minutes at 37 °C. Then, the protein was precipitated using the acetone precipitation protocol. Then, per Eppendorf tube, 1 µL of AF488-TCO (12x concentration, 10% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 8			Lane Content									
			Ruler	Samples								
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7	8	9
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10	10	10
1 (30 min)	Cbz-Tzm-Ala-CMK **6**	µL (500 nM)	-	1	1	1	1	1	1	1	0	1
	Acetone precipitation											
3 (30 min)	AF488-TCO	µL (range)	-	1 (2500)	1 (500)	1 (100)	1 (20)	1 (4)	1 (1)	1 (0)	1 (2500)	1 (2500)

Assay 9: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 10 Eppendorf tubes (9 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Phe-Ala-CMK **8** (10x concentration, 1% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Then, per Eppendorf tube, 1.0 µL Cbz-Tzm-Ala-CMK **6** (33 µM, 1% DMSO) was added to reach 3.0 µM concentration, before incubating for 30 minutes at 37 °C. Then, the protein was precipitated using the acetone precipitation protocol. Then, per Eppendorf tube, 1 µL of AF488-TCO (12x concentration, 10% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 9			Lane Content									
			Ruler	Samples								
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7	8	9
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10	10	10
1 (30 min)	Cbz-Phe-Ala-CMK **8**	µL (range µM)	-	1 (100)	1 (0)	1 (0.1)	1 (0.3)	1 (1.0)	1 (3.0)	1 (10.0)	1 (30.0)	1 (100.0)
2 (30 min)	Cbz-Tzm-Ala-CMK **6**	µL (3.0 µM)	-	1	1	1	1	1	1	1	1	1
	Acetone precipitation											
3 (30 min)	AF488-TCO	µL (500 nM)	-	1	1	1	1	1	1	1	1	1

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[7] ***Caution:*** *Diazomethane is explosive. Use explosion-proof protective equipment (e.g. blast shield).*

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