Chemical tools for the study of proteolytic activities associated with antigen presentation

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Summary and future prospects

Organic synthesis has provided tools to study the proteasome and cysteine proteases of the cathepsin family. In this thesis, new tools are presented, extending the possibilities to study these proteolytic activities. In **Chapter 1** of this thesis, an overview is presented of chemical labeling of biomolecules in living cells. Incorporation of unnatural reactive handles into native proteins, followed by employing these reactivities for the installation of a label, allowed selective tagging of a single protein in a cellular system. Another strategy of labeling a single protein was found in the creation of fusion proteins between the protein of interest and either a special peptide sequence or an active protein. The fusion proteins are labeled *via* the unnatural part with a fluorescent dye reacting specifically with the special peptide sequence. The third strategy explored, uses cell permeable activity based probes to introduce a latent bioorthogonal reactivity on active proteins, and after cell lysis, the reporter group of choice can be attached to the latent reactivity.

In **Chapter 2**, an approach is described to target active proteasomes in living cells and detect the proteasomes after cell lysis *via* two-step labeling. After modifying active proteasome with an irreversible inhibitor that was equipped with an azide moiety, the Staudinger-Bertozzi ligation was employed to biotinylate the proteasome selectively. This is the first example in which all proteolytic activities of the proteasome in a cellular system

are visualized. In this way, labeling artifacts as associated with cell lysis and measuring enzymatic activities in cell lysates are precluded. The scope of this method could be extended by the use of different irreversible proteasome activity probes and different ligation methodology, such as the Huisgen cyclisation as applied by Cravatt and coworkers.[1]

In **Chapter 3**, the two-step labeling approach presented in Chapter 2 was used to assess the specificity of two subunit-specific cell permeable inhibitors of the caspase-like activity of the proteasome. Based on studies towards reversible subunit specific proteasome inhibitors, an irreversible and cell permeable inhibitor was designed. Modification of the newly synthesized inhibitor with an azide moiety yielded a probe for the caspase-like activity of the proteasome. The selectivity of the newly synthesized probes was assessed both by peptidase assays and Western blots. At a lower concentration of the inhibitor, selectivity towards the immunoproteasome was observed. To investigate the role of the caspase like subunit of immunoproteasome in antigen processing, the epoxomicin[2] (**1**, Figure 1) warhead and the peptide boronate known from bortezomib[3] (**2**) combined with the used peptide sequence, might lead to even more potent inhibitors **3** and **4**.

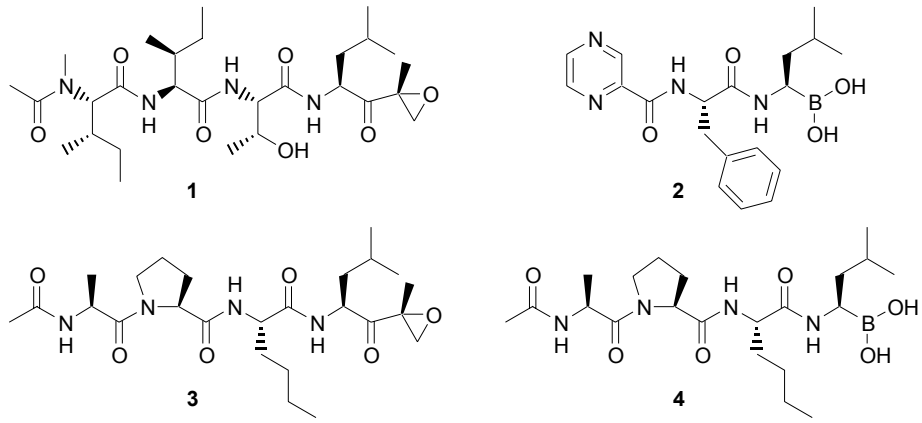
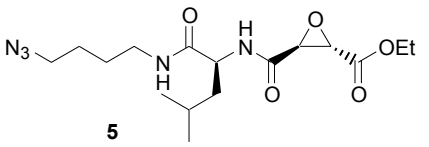


Figure 1. Possible immunoproteasome inhibitors based on other warheads

Furthermore, the technique of finding inhibitor targets by incorporation of a latent chemical group, like the azide moiety, in other irreversible inhibitors may contribute to elucidating the selectivity of the inhibitors and the identification of their minor targets. This could be of importance in recognizing side effects of drugs. In Figure 2, some azide-modified cysteine protease inhibitors are depicted, that might be used for two-step labeling. Compound **5** is based on E-64[4], a natural product that is an inhibitor of cysteine proteases

of the cathepsin family, whereas inhibitor **6** is an azide modification of ZVAD-FMK[5], an irreversible caspases inhibitor.



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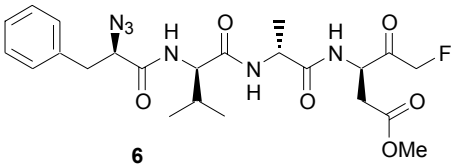

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Figure 2. Azide-modified irreversible protease inhibitors

Chapter 4 shows the synthesis of an isotope-coded activity based probe for cysteine proteases of the cathepsin class. A set of deuterium encoded spacer molecules suitable for incorporation by solid phase peptide synthesis were developed. Modification of a known broad class cysteine protease inhibitor with these isotope coded spacers did not impair the inhibitory profile. It was expected that by labeling two cysteine protease samples by an isotope coded inhibitor pair, the relative activity of cysteine proteases between two samples can be established by mass spectrometry. However, the detection of the expected isotope-labeled active site fragment of cathepsin B proved troublesome, and therefore an attempt was made to synthesise this fragment. The isotope coded linker developed in this chapter could also find their way into the proteasome inhibitor or biotinylation phosphine in presented in **Chapter 2** to allow quantitation of proteasomal activity in living cells. Furthermore, the methodology could be extended by the use of other isotope labels, such as ^{13}C, which is reported to have less effect of chromatographic separations.[6]

Chapter 5 deals with the synthesis of a new cysteine protease probe. This probe is based on a known probe, extended both with an activated ester functionality that is reactive towards amines and an photolabile linker. The active ester was employed to conjugate the cysteine protease probe covalently to a model antigen. Presentation of this modified antigen to a professional antigen presenting cell could result in activity profiling of cysteine proteases involved in the proteolytic processing of the model antigen. The photolabile linker could be of importance to remove the remained of the model antigen from the labeled cysteine proteases, thereby simplifying the identification of the labeled cysteine proteases. Initial experiments involving the cleavage of the photolabile linker, the conjugation to ovalbumin as a model antigen and labeling of cathepsin B proved encouraging. However, the purity and homogeneity of the ovalbumin-probe conjugate could not be established yet.

Some peptide sequences are known for their capacity to translocate through cell membranes. In **Chapter 6**, a cysteine protease probe was fused to such a peptide sequence,

rendering a construct that was actively transported into cells. Meanwhile, the cysteine protease probe sampled the pathway for active cysteine proteases. In a set of uptake experiments, it was shown that the uptake pathway has at least an energy-independent component. In future research, different cell penetrating peptides could be fused to a cysteine protease probe, in order to see if all peptide sequences dictate the same uptake mechanism.

References

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