Novel activity-based probes for serine proteases

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Contents

1 Abstract

Serine proteases are enzymes that cleave peptide bonds within proteins and play many roles ranging from digestion and protein turnover to signaling. They need to be tightly regulated and are therefore mostly expressed as inactive precursors called zymogens. A distortion of the balance of active and inactive protease can have severe consequences such as pancreatitis, allergies or even cancer.
Activity-based probes (ABPs) have become popular as tools for studying the activity of enzymes. These small molecules bind covalently to the enzyme only in its catalytically active form which is hence tagged allowing further analysis. Most ABPs react with a lot of different target enzymes and are therefore unselective. Especially for serine proteases, there are only two selective ABPs. Therefore, many target serine proteases remain so far unstudied.
In this thesis, we developed phosphoramidate peptides (PAPs) as novel ABPs for serine proteases. The phosphoramidate serves as reactive warhead and directs the probe to serine proteases. Next to this binding moiety, one to three amino acids make a recognition element to determine the selectivity for a subset of this class enzymes. They additionally carry either a biotin handle for streptavidin-mediated readout, or an alkyne which enables the attachment of diverse reporter tags via Cu(I)-catalyzed cycloaddition. The biotinylated PAPs were entirely synthesized by Fmoc-based solid phase peptide synthesis using commercially available building blocks. This way, they can be easily diversified upon need. We made a small library allowing to assess the influence of the different recognition elements on binding behaviour to the respective protease. We could demonstrate that their selectivity and potency is a function of the different recognition elements. After singling out

a biotinylated PAP with a lysine as recognition element, biotinK-PAP, we used it as a tools to enrich endogenous trypsin from rat pancreas lysate. Its identity was confirmed using MS analysis in combination with Swissprot database search.
Moreover, we diversified the already existing ABPs based on the 4-chloro-isocoumarin (IC) warhead. Therefore, we attached diverse substituents to either the 3- or the 7-position of the twomembered ring and could prove that they impact the compounds' affinity to the tested protease targets. We also show that these small molecules display a good selectivity towards purified serine proteases in a complex proteome background. We then demonstrate their good selectivity when reacting with endogenous targets in rat organ lysates. Furthermore, we prove that these ICs can be used for labeling via Cu(I)-catalyzed cycloaddition because of an alkyne handle, which is a valuable expansion of their applicability for complex systems.
In conclusion, in this work we show the power PAPs and ICs can provide to study active serine proteases and how their selectivity can be adjusted.

2 Zusammenfassung

Serinproteasen sind Enzyme die Peptidbindungen mithilfe eines Serinrestes in ihrem katalytischen Zentrum schneiden. Im Menschen sind Serinproteasen an vielen wichtigen Prozessen beteiligt, angefangen von Verdauung über Proteinturnover bis zu molekularer Kommunikation. Um diese Funktionen strikt regulieren zu können, werden die meisten Serinproteasen als inaktive Vorstufen exprimiert, sogenannte Zymogene. Wird die Balance zwischen aktivem und inaktivem Anteil einer Serinprotease gestört, kann dies schwere gesundheitliche Folgen haben wie Pankreatitis, Allergien oder sogar Krebs.
Aktivitätsbasierte Sonden (engl.: activity-based probes, ABPs) sind beliebte Werkzeuge bei der Untersuchung von Enzymen. Die kleinen Moleküle binden kovalent an das Enzym in seiner katalytisch aktiven Form, welches damit markiert ist und eine weitere Analyse erlaubt. Die meisten ABPs reagieren mit vielen verschiedenen Enzymen und sind damit nicht selektiv. Vor allem für Serinproteasen gibt es nur zwei Klassen selektiver ABPs. Deshalb konnten viele Serineproteasen noch nicht untersucht werden.
In dieser Arbeit wurden Phosphoramidatpeptide (PAPs) als neuartige ABPs für Serinproteasen entwickelt. Das Phosphoramidat dient als reaktive Kopfgruppe, die die Sonde zu Serinproteasen dirigiert. Eine bis drei Aminosäuren ergeben das Erkennungselement, das die Spezifität für eine Untergruppe dieser Enzymklasse bestimmt. Zusätzlich tragen sie entweder einen Biotinrest, den man direkt mit Hilfe von Streptavidin detektieren kann, oder ein Alkin, das das Anfügen diverser Markierungen mittels Cu(I)-katalysierter Cycloaddition ermöglicht. Die biotinylierten Sonden wurden vollständig durch Fmoc-basierte Festphasensynthese mit käuflich verfüg-

baren Bausteinen synthetisiert. Dadurch können sie je nach Bedarf sehr einfach diversifiziert werden. Wir haben eine kleine PAP-Bibliothek erzeugt, die es uns erlaubte, die Einflüsse der verschiedenen Erkennungselemente auf das Bindeverhalten an die jeweiligen Zielproteasen zu untersuchen. Wir konnten zeigen dass ihre Selektivität und Bindungsstärke von diesen Erkennungselementen abhängt. Das biotinyliertes PAP mit einem Lysinrest als Erkennungselement wurde dann als Werkzeug verwendet um endogenes Trypsin aus Rattenpankreaslysat zu isolieren. Die Identität dieser Protease wurde massensprektometrisch festgestellt.
Zusätzlich wurden ABPs, die auf der 4-Chlorisocoumarin (ICs)-Kopfgruppe basieren, weiter diversifiziert. Eine kleine Bibliothek mit IC wurde erstellt, die verschiedene Substituenten als Erkennungselemente tragen. Diese Moleküle sind selektiv sowohl für aufgereinigte als auch endogene Serinproteases vor dem Hintergrund eines komplexem Proteoms. Zusätzlich zeigen wir dass man sie wegen ihres Alkinsubstituenten für Cu(I)-katalysierte Cycloaddition verwenden kann.
Zusammenfassend stellt diese Arbeit die Stärken heraus, die PAPs und ICs für die Untersuchung von Serinproteasen haben und wie einfach die Selektivität dieser Moleküle angepasst werden kann.

Statement of authorship

I, Ute Haedke, confirm that the work presented in this report has been performed and interpreted solely by myself except where explicitly identified to the contrary. I confirm that this work is submitted in partial fulfilment to obtain the title Dr. rer. nat. and has not been submitted elsewhere in any other form for the fulfilment of any other degree or qualification.

3 Introduction

Proteolysis is the cleavage of peptide bonds in proteins resulting in smaller peptides or individual amino acids. It takes place in every living cell in biochemical pathways ranging from digestion and protein turnover to signaling. Proteolysis is catalyzed by enzymes, generally called proteases or peptidases [99]. Proteases can be classified based on their catalytic mechanism: In serine proteases as well as threonine, asparagine and cysteine proteases, the respective name giving residue is responsible for the first step within the catalysis. Aspartate, glutamic and metallo proteases are in contrast to that as it is a water molecule coordinated by the name giving residue which performs the first nucleophilic attack on the scissile bond [99]. In this work, we focus exclusively on serine proteases.

3.1 Serine proteases

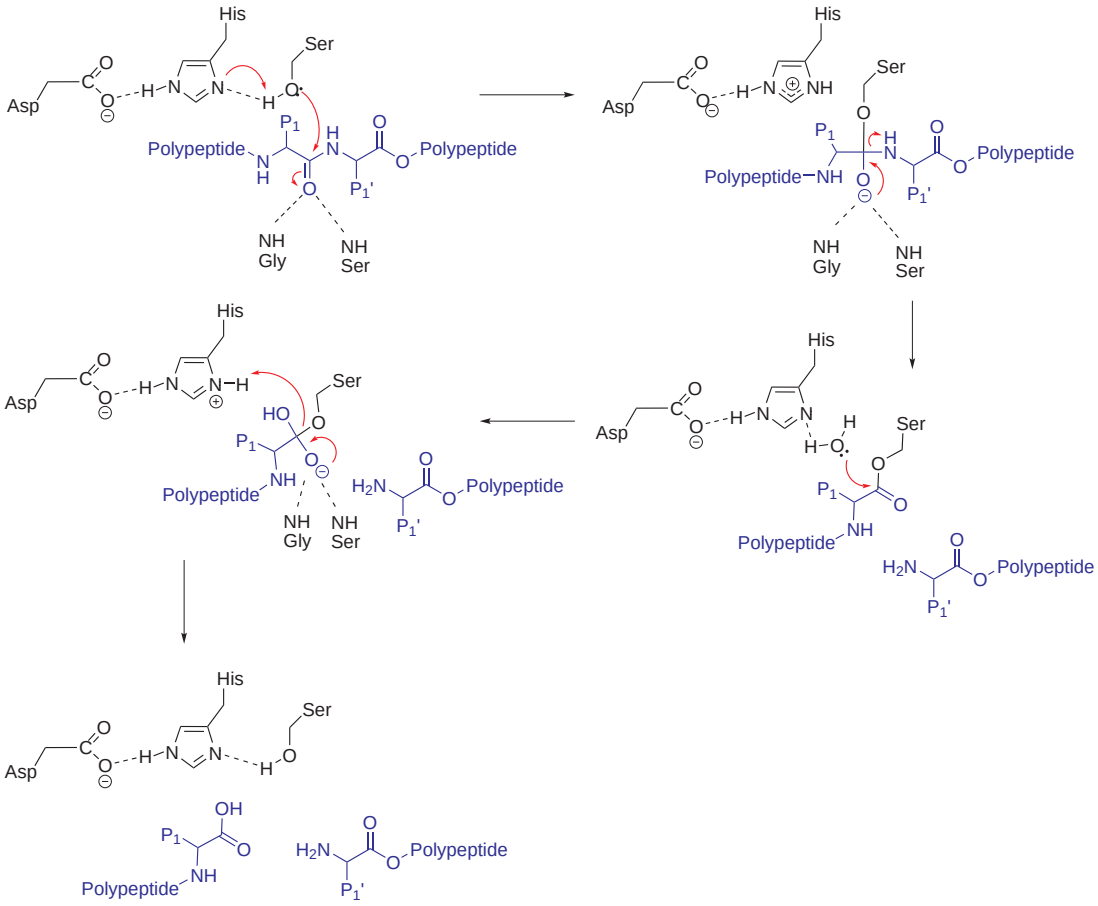
Serine proteases exist in all kingdoms of life. In humans, they are involved in processes such as apoptosis [89], inflammation and allergies [28, 40], digestion [61], cancer [5, 104], angiogenesis [104] and many more. Their total number is estimated between 100 and 200 [7, 57]. The physiological functions of many serine proteases are still unknown. This works aimed at the development of tools for their investigation. Beforehand, the main features common to most of them shall be outlined here.

3.1.1 Catalytic mechanism

Most serine proteases such as trypsin, chymotrypsin, subtilisin and pancreatic elastase use the classical Ser/His/Asp catalytic triad whose mechanism of action is sketched out in scheme 3.1. The cleavage of a peptide bond within a substrate starts with the nucleophilic attack at the carbonyl carbon by the hydroxyl oxygen of the serine side chain. The basic histidine supports this process by abstracting the hydroxyl hydrogen, thereby increasing the serines' nucleophilicity. The attack generates a tetrahedral intermediate from the former peptide bond which is stabilized by hydrogen bonds between the former carbonyl carbon and two backbone amides (glycine 193 and serine 195, chymotrypsin numbering), the so-called oxyanion hole [56]. The new N-terminus of the cleaved peptide bond is set free. The carbon terminus remains bound to the serine until being replaced by a hydroxyl from a water molecule. Again, the tetrahedral intermediate is stabilized through interactions with the nearby backbone of the enzyme. In this mechanism, the imidazole ring of the histidine clearly acts as a short-term repository for protons. The role of the aspartate residue is not as obvious, although its deletion results in a reduction of activity of up to four orders of magnitude [33]. Most probably, it supports the histidine both in spacial orientation and by neutralizing upcoming charges brought by transferred protons during the catalytic process [38].

3.1.2 Substrate selectivity

Most proteases have a cavity close to their catalytic machinery that is made to recognize their substrates [99]. Therefore, the residues surrounding the scissile bond are crucial for this recognition. For most serine proteases, the first side chain at the N-terminal side of the scissile bond is most important in this respect. A general terminology was introduced by Schechter and Berger, naming this position "P1" and counting upwards with each amino acid further away from the cleaved bond. After cleavage, the P1 residue forms the new carboxy terminus after hydrolysis. The opposite side of the scissile bond is denoted the prime side, hence the residues are numbered P1', P2' and so forth. The corresponding recognition sites within the cavity of the protease are named S1, S2 as well as S1', S2', respectively [109]. Ever since its introduction in 1967 to describe the specificity of papain, the nomenclature has found widespread use in the protease research community.



Scheme 3.1: **The Ser/His/Asp catalytic triad and its cleavage mechanism.** Most serine proteases possess a serine, a histidine and an aspartate residue in their active center which are spacially arranged to create a so-called catalytic triad. In this setup, they perform the actual cleavage of the peptide bond within the target protein. In the first step, the hydroxyl oxygen of the serine side chain attacks the carbonyl carbon within the cleavage bond of the target protein. The histidine supports this process by abstracting the hydoxyl hydrogen and therefore increasing the serines' nucleophilicity. This attack results in a tetrahedral intermediate which is stabilized by the oxyanion hole (glycine 193 and serine 195, chymotrypsin numbering). The new N-terminus of the cleaved peptide bond is set free. Next, a water molecule hydrolyzes the serine, which again takes place via a tetrahedral intermediate. The aspartate is believed to support the histidines' orientation and neutralize its charge during the catalytic process.

3.1.3 Expression as inactive zymogens

Most serine proteases are expressed as inactive precursors, named zymogens. Only certain triggers, such as the loss of a propeptide , lead to the finally active species. Zymogens are advantageous because they can be stored in large quantities without harming the cell due to unregulated activity. As a reaction to certain stimuli, the cell can provide high amounts of the active protease much faster than if it first had to be expressed. To limit the time frame for this activity, protease inhibitors such as serpins are present [57, 129]. This tight regulation allows very fine tuning according to each cell's needs.
Due to the zymogen concept, the activity of a given serine protease can not necessarily be inferred from its expression level. The activity can be much better investigated by activity-based probes as they only react with the active version of a given target enzyme.

3.1.4 Two examples: trypsin and β-tryptase

3.1.4.1 Trypsin from pancreas

Trypsin is one of the best studied serine proteases. It is structurally similar to chymotrypsin, and both enzymes complement each other in their digestive function [62]. Failure of activity regulation of trypsin can lead to several forms of pancreatitis [129]. The earliest publication listed in the MEROPS database about trypsin dates back to 1911 but already points at the large amount of literature about this topic [133]. Here, bovine trypsin as representative for the highly similar mammalian trypsins shall be described examplarily for the posttranslational processing a serine protease can undergo (see also figure 3.1A).
According to their relative isoelectric points and electrophoretic mobility, three expressed isoforms are distinguished: cationic trypsinogen, anionic trypsinogen and mesotrypsinogen, all subsumed here as trypsin. They are all expressed as proprecursors which are converted into the corresponding trypsinogen by removal of an N-terminal 16 amino acid signal peptide in the endoplasmic reticulum [74]. Trypsinogen consists of 229 amino acids and is stabilized in its tertiary structure by six disulfide bonds [139]. It is stored in zymogen granules of pancreatic acinar

cells [74] from where they are released as response to a number of stimuli into the duodenum. Once arrived, the enzyme enteropeptidase specifically removes the N-terminal propeptide Val-$[Asp]_4$-Lys, affording β-trypsin as the first active form [26, 59]. Limited autolysis after Lys^{131} yields the less active α-trypsin [82]. The disulfide bridges are maintained throughout the whole process. Other structural features include a Ca^{2+} binding site required to prevent unlimited autolysis and a surface loop of nine amino acids close to the active site serine which influences the specificity without directly interacting with the substrate [139].
Along with trypsin, a series of other zymogens are released from the pancreatic acinar cells: chymotrypsinogen, pancreatic proelastase, prolipases and procarboxypeptidases. Enteropeptidase initiates the proteolytic cascade illustrated in figure 3.1B, in which trypsin plays a key role in transforming the proenzymes into their active subforms [61].

3.1.4.2 β-Tryptase from mast cells

Tryptases are a large class of serine proteases with tryptic activity. They are expressed at high amounts by mast cells where they play decisive roles in inflammation [84], especially in tissues with direct contact to the environment [90, 28]. The most abundant tryptase subtypes are the highly identical βI, βII and βIII-tryptases, subsumed here as β-tryptase. The enzyme forms glycosylated homotetramers of about 140 kDa which seem to be stabilized by heparin [96]. The unusual structure, with each monomer having its own Ser/His/Asp catalytic triad pointing towards the inside of the central pore [96] is certainly one of the reasons for its resistance to endogenous inhibitors, which is why it remains active for longer than other secreted serine proteases [28, 102].
Unusually for serine proteases, β-tryptase is stored in its active form in intracellular granules. There, it can mount up to 90% of the total protein content, sometimes sufficiently dense to form semi-crystalline structures [28]. Its activity is muted only by the low pH inside the vesicles [96] and becomes active when exposed to the extracellular matrix pH 7.4. This exposure occurs when the mast cells degranulate, which means that they fuse the storage vesicles with their cell membrane as illustrated in figure 3.2. This process can occur as response to antigen recognition by an IgE antibody presented on the cell's surface. One antigen crosslinks two antibodies,

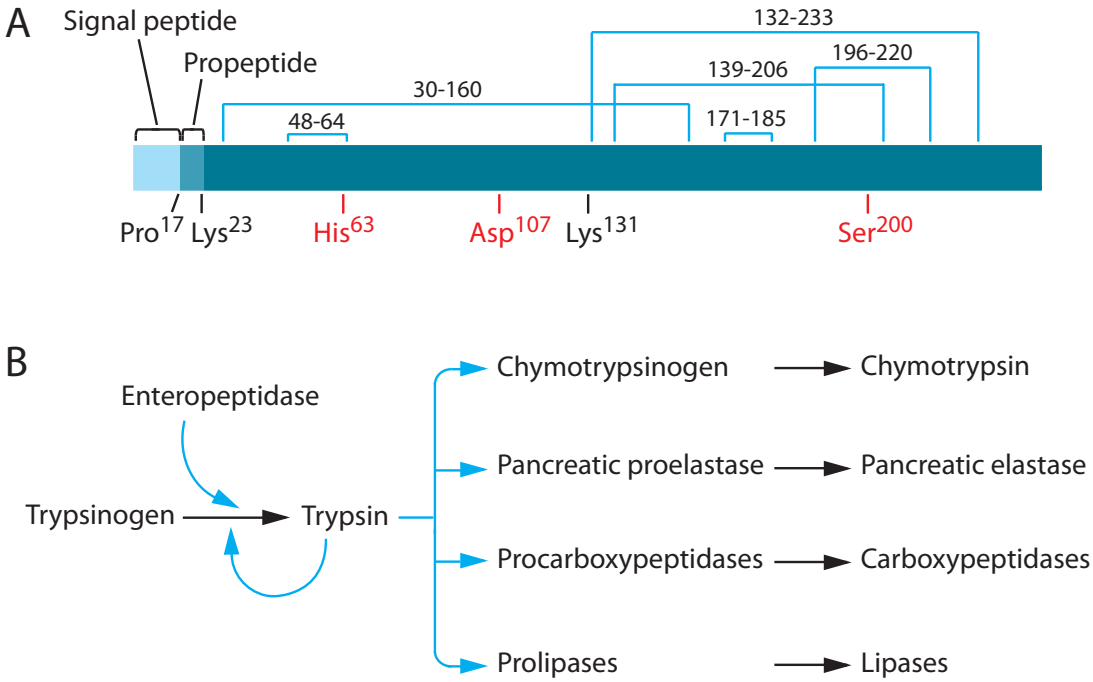


Figure 3.1: **Trypsinogen processing and activation of pancreatic enzymes. A:** Trypsin is expressed as inactive precursor carrying a signal peptide which is removed in the endoplasmatic reticulum. This affords trypsinogen, the zymogen form stored in granules in the pancreatic acinar cells. Certain stimuli trigger its release into the duodenum where it is transformed into its active form β-trypsin through specific removal of the propeptide by enteropeptidase. Autocleavage after Lys^{131} yields the less active isoform α-trypsin. All subforms have six disulfide bridges. The active site residues are indicated in red. (Modified according to [82]) **B:** The enteropeptidase-activated trypsin generates more trypsin and also converts other digestive enzymes into their active forms. (Modified according to [61].)

the impulse is transferred to the cytosol with the help of the antigen receptor FcεRI which in turn triggers a signal cascade involving Ca^{2+} [40, 97]. Aside from IgE, other mediators can provoke degranulation, for example endogenous proteases and proinflammatory signals but also diverse toxins [3]. To provoke this process *in vitro*, IgE is commonly used. Alternatively the cascade can be cut short with the help of ionophores such as A23187 that directly cause a Ca^{2+} influx [85]. The vesicules additionally release cathepsin G [100], granzyme B [57], carboxypeptidases, and caspases at varying amounts [3, 97, 119], as well as a number of non-proteinous pro-inflammatory agents [77, 84, 90, 119].

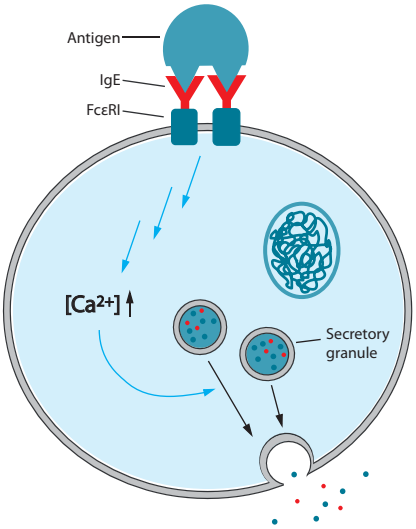


Figure 3.2: **Mast cell degranulation releasing β-tryptase.** Mast cells present IgE proteins on their surface which are bound to the high affinity receptor FcεRI. A bound antigen will crosslink two antibodies which triggers a receptor-mediated signal cascade in the cytosol involving the release of Ca^{2+}. At the end of this cascade, intracellular vesicles fuse with the cell membrane and unload their content into the extracellular space, a process called degranulation. Aside from IgE, other mediators can provoke degranulation, for example endogenous proteases and proinflammatory signals from other cells, but also diverse toxins. The secreted substances include β-tryptase and diverse other proteases as well as a number of non-proteinous proinflammatory agents.

3.2 Activity-based probes

Activity-based probes (ABPs) are small molecules that react with the catalytic center of a defined set of active target enzymes. The resulting covalent complex can be analyzed by means of the reporter tag within the ABP [48], which makes them powerful tools for proteomics. Figure 3.3 shows a general scheme of the ABP structure with its four elements: 1) the reactive warhead which forms a covalent bond with at least one active site residue. It additionally provides a first degree of selectivity for the target enzymes. 2) A recognition element refining the selectivity; 3) a spacer that creates a distance between the recognition element and the fourth constituent 4) a reporter tag which enables visualization and further analyses of the ABP-enzyme complex. The warhead does not need to be placed at the molecules' terminus but can be located between the recognition element an the spacer [145], or even between two potential recognition elements [4, 46, 126, 145].

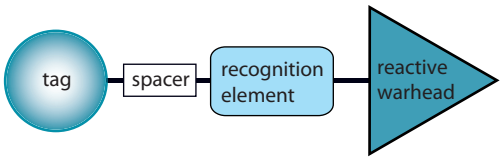


Figure 3.3: **General design of activity-based probes (ABPs).** ABPs consist of 1) a reactive warhead which provides a first degree of selectivity to a certain type of target enzyme; 2) a recognition element that refines the selectivity; 3) a spacer to create a distance between the recognition element and the 4) reporter tag which is used to visualize and analyze the enzyme-probe complex.

3.2.1 Protease-reactive warheads for serine proteases

Two factors determine which structure makes a suitable warhead: the enzymes' catalytic mechanism and the chemical properties of the active site nucleophile. A variety of structures have been reported for cysteine proteases like acyloxymethyl ketones [69], hydroxamates [127], α,β-unsaturated ketones [144], vinyl sulfones [21, 130, 145] and a number of epoxysuccinates [44]. The proteasome as the textbook example for threonine proteases has been studied using vinyl amides [32], epoxyketones [73] and vinyl sulfones [20, 42, 93].

The choice of warheads for serine proteases is rather small, we list those available to date in table 3.1. One reason for this certainly is that the hydroxyl moiety is a hard and not very reactive nucleophile, especially in comparison to the sulfhydryl in cysteine. Often serine protease-directed ABPs react unselectively: e.g. fluorophosphonates (FPs, diverse types of serine hydrolases [7, 72, 81, 95]); β-lactams and β-lactones (various enzymes including serine proteases [18, 17, 117, 118]) as well as sulfonyl fluorides [142] (lipases and serine proteases). When it comes to more specific targeting of serine proteases only, there are so far only two types: phosphonates [1, 54, 94, 105] and 4-chloro-isocoumarins (ICs) [4, 67, 136].

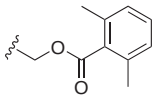
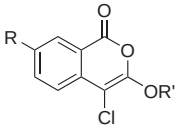
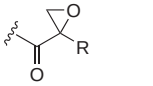
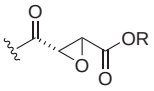
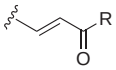
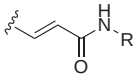
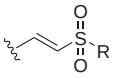
3.2.2 Affinity-based probes

Affinity-based probes such as photo-reactive probes and latent electrophiles are often mentioned together with ABPs in the scientific literature. Strictly speaking, they do not qualify as ABPs because the covalent link is formed independently of the target's catalytic mechanism. We here mention them very briefly for the purpose of completion only. The curious reader is referred to a recent review from our lab [48] or a more detailed one from the Overkleeft lab [42].

3.2.3 Probe selectivity

For the targeted detection of enzyme activity, the selectivity of a probe is an important feature. We define this term as a measure for the total number of targets an ABP binds to [47].
For most cases, high selectivity comes along with low reactivity [55, 71]. This seems intuitive, since enhanced reactivity usually also increases the likelihood for undesired side reactions. Therefore, the great challenge lies in the creation of ABPs that combine high degrees of both reactivity and selectivity.
The required selectivity level for an ABP depends on the scientific questions to be answered. Many applications use ABPs of low selectivity which enable the labeling and further analysis of many, often very different targets in parallel [7, 72, 81, 95]. This is welcomed if a general functional state of a proteome shall be monitored [35]. In contrast, more selective ABPs allow more detailed conclusions about a small subset of enzymes or even a single species and its functions.

Table 3.1: **Reactive ABP warheads targeting proteases**

Name	Structure	Target enzymes
acyloxymethyl ketones (AOMK)		cysteine proteases [69]
4-chloroisocoumarins		serine proteases [67, 136]
epoxyketones		proteasome [73, 125]
epoxisuccinates		cysteine proteases [44, 69]
fluoromethyl ketones		cysteine proteases [12]
fluorophosphonates (FPs)		serine hydrolases [7, 72, 81, 95]
α,β-unsaturated ketones		cysteine proteases [144]
β-lactams		various enzymes including serine proteases [17, 146]
β-lactones		various enzymes including serine proteases [18]
phosphonates		serine proteases [1, 54, 94, 105]
sulfonyl fluorides		serine proteases [142] lipases [120] proteasome [24]
vinyl amides		cysteine proteases [137] proteasome [32]
vinyl sulfones		cysteine protease [21, 130, 145] proteasome [19]

3.2.4 Recognition elements for enhanced selectivity

For protease-directed ABPs, the most common approach to increase selectivity is the introduction of amino acids or short peptides as recognition elements. When targeting serine proteases, already a single amino acid can reach strong discrimination, as this type of enzymes mainly recognizes P1 residues. Therefore, most of our PAPs have a single amino acid as recognition element which is located between the warhead and the spacer.

3.2.5 Reporter tags

The reporter tag of any ABP enables the visualization of the covalent enzyme-probe complex and determines which analytical procedures can be done. The two reporter tags used in this work are biotin and the fluorophore 5-(and 6-)carboxytetramethylrhodamine (TAMRA). For an optimization attempt, a His-tag was unsuccessfully used once.

Biotin offers a number of welcome features: first, it is heat stable, so the protein mix can be boiled for denaturation or to stop any residual enzymatic activity if desired. Second, it binds both highly specifically and tightly to streptavidin, allowing analysis via biotin blot and making the enrichment of tagged proteins a standard procedure. On the downside, any method involving streptavidin will also include endogenously biotinylated biomolecules. Furthermore, the disruption of the biotin-streptavidin interaction requires harsh conditions that likely denature the targets, and often the recovery is incomplete. Also, biotinylated peptides often cause detection problems in MS, either due to fragmentation or poor ionization. And also the labeling reaction itself can be impeded because biotinylated probes hardly cross cell membranes and can therefore not be used to investigate enzyme activities inside cells of even tissues. Taken together, despite its value as reporter tag, different substituents for this purpose are desirable.

Fluorophores are a good alternative to biotin. One major asset is that fluorophore-tagged proteins are immediately visible without any blotting procedure. Fluorophore-directed antibodies allow the affinity enrichment, avoiding the co-purification of endogenous proteins. However, unreacted probe also emits light, creating a high background when used in cells or tissues. To solve this problem, probes holding both a fluorescent moiety as well as a quencher have been developed. Upon re-

action with the enzyme, the quencher is cleaved off and the fluorescence becomes detectable [15].

3.2.6 Two-step labeling reactions

Aside from the reporter tags discussed above, chemical biology has provided two-step labeling as alternative to readily tagged ABPs. A small moiety being part of the ABP allows the attachment of a reporter tag in a second step following the reaction with the enzyme. That way, any label can be introduced according to the experimenters needs as long as it reacts with the corresponding handle on the probe. Such chemical ligation strategies have advantages that weigh out the slightly longer protocol compared to one-step labeling. First, the enzyme-binding probes are small. The lack of bulky substituents avoids problems with sterical hindrance during the interaction and additionally improves the molecules' cell permeability. Furthermore, the researcher is no longer restricted to one reporter tag but can decide which one to pick according to the analytical method. For ABPs derived from inhibitors, another aspect comes into play: small substituents are only minor changes of the overall structure so the probe is likely to hit the same target as the parent compound. We make use of this fact in our *T. gondii* study. The protocols for one-step and two-step labeling are sketched in figure 3.4.
The two reaction partners for such tandem labelings must fulfill a number of requirements, e.g. both are inexistent in nature and react specifically with each other under aqueous conditions [22, 110]. From the different reaction types available to connect the reporter tag to the probe, Cu(I)-catalyzed "click chemistry" is probably the most popular one. This Cu^{+}-aided connection of an alkyne and an azide to form a 1,4-substituted triazole was improved by Sharpless and coworkers, based on Huisgen's 1,3-dipolar cycloaddition [58].
Other two-step labeling methods include "strain-promoted click chemistry" which avoids the often harmful Cu^{+} [11, 66, 124], the Staudinger ligation [108] as well as the Diels-Alder ligation [135, 134].

3.2.7 ABPs investigated in this thesis

In this work, two types of ABPs were explored: diphenyl phosphoramidates (PAs) and 4-chloroisocoumarins ICs.

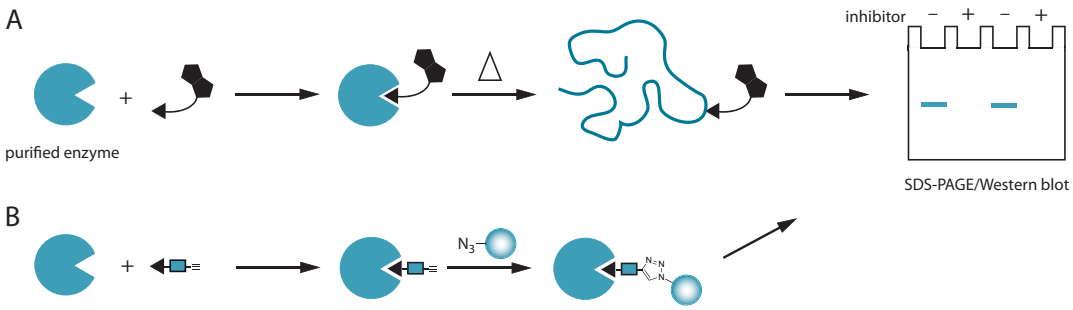
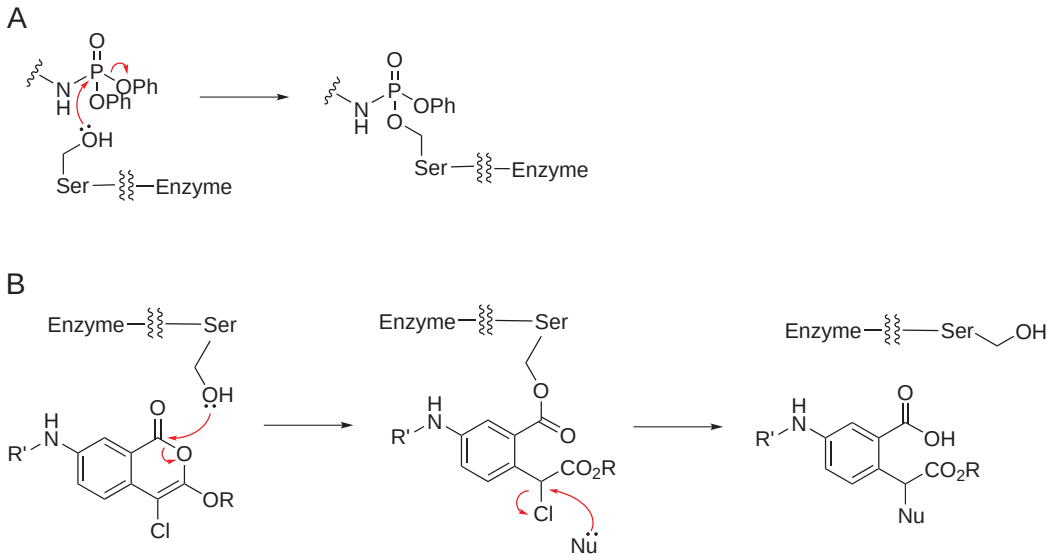


Figure 3.4: **Strategies for labeling of purified enzymes. A:** The purified enzyme is incubated with the biotinylated ABP to form a covalent bond. Subsequently, the sample is heat-denatured, separated via SDS-PAGE and blotted onto a nitrocellulose membrane. Then, the labeled proteins are visualized with the help of luminescence generated by horseradish peroxidase conjugated to streptavidin. **B:** Two-step labeling: in the first step, the purified enzyme is incubated with the alkynylated ABP to form a covalent bond. Secondly, a reporter tag (usually a fluorophore) is coupled using Cu(I)-catalyzed click chemistry. Subsequently, the sample is heat-denatured and separated on an SDS-PAGE which can be read out directly with a fluorescence scanner.

PAs contain an electrophilic phophorous atom which attracts the nucleophilic serine side chain. The two diphenyl subsituents are good leaving groups, so one of them can be substituted by that very serine. That way, PAs are supposed to undergo a similar reaction mechanism as diphenyl phosphonates (see scheme 3.2 A).
While the PA warhead is thought to guide the probe towards serine proteases in general, a vicinal recognition element provides more selectivity for certain enzyme subtypes. For example, basic residues as recognition element will have a much higher affinity to tryptic serine proteases than hydrophobic ones. To explore how much the selectivity of a PA can be tuned, we synthesized a small library of probes with different amino acids next to the warhead. Their peptidic nature gave rise to the name "phosphoramidate peptides" (PAPs).
Most of the PAPs synthesized in this work are biotinylated. At this early stage of development, biotin is a useful reporter tag which is easy to handle. For a broader applicability, we additionally made versions holding an alkyne handle instead of a reporter tag.
Moreover, we investigated ICs as ABPs for serine proteases. The binding mechanism of ICs to the target enzyme, sketched in scheme 3.2 B, starts with a nucleophilic attack of the active site serine to the carbonyl carbon within the two-membered ring. In this



Scheme 3.2: **Putative binding mechanism of phosphoramidates (PAs) and 4-chloro-isocoumarins (ICs). A:** PA binding mechanism: the hydroxyl moiety of the active site serine nucleophilically attacks the phosphorous electrophile within the PA warhead. This leads to the formation of a covalent bond and the substitution of one phenoxy group. **B:** IC binding mechanism: the hydroxyl moiety of the active site serine nucleophilically binds to the carbonyl carbon, which opens one of the two isocoumarin rings. This system tends to hydrolyze within the range of hours, but nearby nucleophiles such as the active site histidine can trap the inhibitor by substituting the chloride ion.

open form, the probe remains bound covalently to the enzyme but is susceptible to hydrolysis within hours. Nearby free nucleophiles such as the active site histidine can trap the probe by substituting the chloride atom [128].
Previously these probes were constrained to biotinylated versions, limiting their applicability in complex systems [67, 136]. We therefore synthesized a set of IC-based ABPs holding alkyne handles which allow chemical ligation via Cu(I)-catalyzed click. In addition to the 3-position, we explored the 7-position of the two-membered ring for its suitability to hold recognition elements.

3.2.8 Applications for ABPs

The simple ABP concept opens an ever-growing number of applications. Gel-based techniques have always been popular as they are well-established and reliable. They have been used to characterize many target enzymes as well as ABPs themselves,

either through direct fluorescent readout or in combination with Western blotting [64, 65, 72, 81, 92, 95]. On the downside, gel-based assays can not be scaled up and have limited sensitivity [92].

MS-based technologies have the potential for higher resolution and can be combined with ABPs that help to identify and even quantify the target enzymes. On the scientific forefront is activity-based protein profiling using the multidimensional protein identification technology (ABPP-MudPIT). Here, a labeled proteome is first digested using a proteolytic enzyme with defined cleavage sites. The resulting peptides are separated by two chromatography columns (usually a strong ion exchanger followed by a reversed phase column) and subsequently detected by an MS (either simple or tandem). Comparison of these masses with a database using the SEQUEST algorithm allows the identification of the labeled targets [79, 131]. This methodology is especially interesting for low-abundance enzymes because they can be enriched by means of the ABPs' reporter tag [92]. ABPP-MudPIT is often combined with techniques that use isotope labeling to study enzyme activity. This involves stable isotope labeling of amino acids in culture (SILAC) [83] and isotope-doded isotope-coded affinity tagging (ICAT) [110]. Another MS technique is matrix-assisted laser desorption ionisation (MALDI) MS. It uses rather mild ionization conditions and was developed to detect large peptides and whole proteins, which complements most other ionization methods suitable for smaller molecules.

Aside from MS, techniques based on the detection of fluorescence have been investigated with growing interest, as fluorescent ABPs enable direct imaging and especially non-invasive tumor localization in tissues or even live animals [15, 16]. A rather young technology is FluoPol-ABPP that measures the change in fluorescence polarisation of an ABP upon binding to its target [6, 8].

Yet another field where ABPs have found more and more use are microarrays, where one reaction partner (either the enzyme or the ABP) is immobilized and then exposed to the other in solution [30, 60, 113, 141]. That way, a whole array of analytes can be read out simultaneously.

A multitude of findings have been made with the help of the technologies mentioned above. Among them are the development and optimization of more sophisticated ABPs and small molecule inhibitors [6, 8, 29, 64]. New targets have been characterized [31], and the characteristic proteolytic states of whole proteomes or tissues can be monitored [35, 141]. These are just a few examples to underline the broad appli-

cability and usefulness of ABPs for both fundamental and applied research. A more detailed description of all these technologies and findings is beyond the scope of this manuscript. The interested reader is referred to the respective literature mentioned in this paragraph.

3.3 A case study: *Toxoplasma gondii*

3.3.1 A dormant threat

Toxoplasma gondii is an eukaryotic parasite which infects virtually any nucleated cell. Humans usually acquire it accidentially either trough contact with infected cats or when eating raw meat from infected cattle. After ingestion, the cysts that encapsulate the dormant form named bradyzoites are disrupted, enabling infection of the intestinal epithelium. There, they differenciate into tachyzoites, the form responsible for rapid multiplication and dispersal within the body. New dormant cysts are mainly found in nervous and muscle tissues, where they often remain throughout the person's life without posing any threat. However, toxoplasmosis can break out and lead to severe brain damage in unborn babies of mothers undergoing their first infection as well as in immunodeficient people (e.g. AIDS patients). The current medicines available have strong unwanted side effects. They only affect trachyzoites, while the dormant bradyzoites are left undisturbed, making long-term treatment unavoidable [13]. Better pharmacological treatment is therefore desirable. In order to develop drugs, the molecular mechanisms involved in *T. gondii* host cell infection need to be better understood.

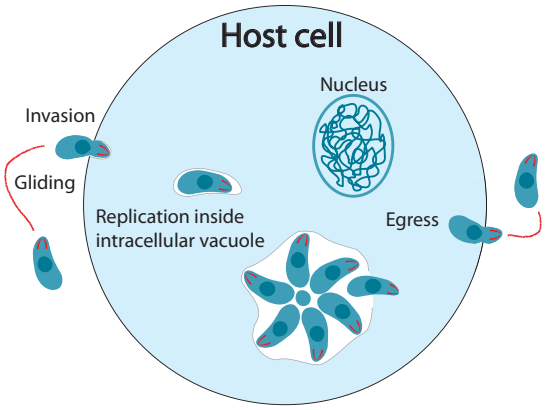


Figure 3.5: **The lytic cycle of *T. gondii*.** The parasites approach their host cells by gliding towards them, a process requiring short term adhesion that leaves protein traces on the respective surface. The actual invasion takes about 20-30 s and ends with the parasite residing in a vacuole inside the cell. There, it replicates and forms rosettes until the vacuole bursts open to release the daughter parasites into the cytosole. The host cell dies and a new infectious cycle starts.

3.3.2 Host cell invasion involves serine proteases

When infecting a cell, *T. gondii* "glides" towards the host cell requiring short term adhesion to the respective surface. Once arrived, the parasites invades the cell and ends

up in an intracellular vacuole where it replicates and forms rosettes. The vacuole bursts open, releases the parasites which then egress into the cell's surroundings to start a new cycle of infection [13]. This lytic cycle is illustrated in Figure 3.5.
To date there is evidence that the invasion involves serine proteases such as rhomboids [23, 27, 112] as well as the subtilisin protease *Tg*SUB1 [78]. An inhibitor study from the Bogyo lab in Stanford, California supports this, as it identified three ICs which reduced *T. gondii* infection of human foreskin fibroblasts (HFF) in culture [50]. Within frame of this manuscript we present a case study in which we tested alkyne analogs of these compounds for their usability as ABPs against serine proteases crucial for infection. Once established, these probes could be used to investigate the roles their targets play during host cell invasion by *T. gondii*.

3.4 Aim of this work

The reactive warheads available nowadays for serine proteases are often unable to distinguish between different subsets of this enzyme class. Therefore the work presented here aimed at developing a new type of warhead that would overcome this problem: diphenyl phosphoramidates (PAs). They are the first ABPs for serine proteases which are entirely synthesized on solid support, a procedure that needed to be adapted to our conditions. This synthetic approach is advantageous because it is rapid and makes their diversification easy. A small library of PAs with peptidic structures as recognition element and spacer was to be made in order to characterize them as ABPs. Subsequently, they were supposed to be used for targeted enzyme enrichment from endogenous sources.
A second scope of this thesis was to investigate a library of in-house made ICs as selective ABPs for serine proteases, especially concerning their suitability for Cu(I)-catalyzed click chemistry. That way, and by using two substituents as recognition elements, we hoped to pave the way for a broader applicability of ICs as ABPs.
During the course of this thesis, we had the opportunity for a collaboration with the Bogyo lab at the Stanford University in Stanford, California. The aim was to investigate a different set of ICs for their potential to prevent the infection of host cells by the *T. gondii* parasites. If successful, these compounds could be used as ABPs to study the proteases involved in the process of infection.

4 Materials and Methods

4.1 Chemistry

All reactants were purchased from Sigma-Aldrich (Germany) with purity of 90% or higher. Solvents for solution phase synthesis were bought from Applichem (Germany). Deuterated solvents, with purity greater than 99% containing 0.03% TMS were purchased from Roth (Germany). Fmoc-protected L-amino acids, rink amide resin and coupling reagents for SPPS were obtained from Creosalus (USA), all other SPPS material was acquired from ERC GmbH (Germany). Salts for workup solutions were purchased from Applichem (Germany).

4.1.1 Solid phase peptide synthesis

The PAPs were synthesized on rink amide resin starting with the C-terminal amino acid. For the biotinylated PAPs, readily biotinylated lysine (1.5 equiv) was coupled using DIEA/HBTU (1.5 equiv each). Complete coupling was checked by a Kaiser test (ninhydrin reaction) which generates a blue colour in presence of free primary amines [107]. Subsequently, Fmoc was deprotected with 20% piperidine for about 15 min and the next amino acid was coupled using HOBt/DIC (3 equiv) in DMF. These two steps were repeated until yielding the desired peptide sequence. The last coupling step formed the PA warhead using diphenyl chlorophosphate (DCP, 5 equiv) and N,N-diisopropylethylamine (DIEA, 3 equiv) in dichloromethane (DCM) . The amount of DIEA was increased when the solution had become acidic. Cleavage with 95% trifluoro acetic acid (TFA), 2,5% triisopropylsilane (TIS) and 2,5% H_2O

Kaiser test solutions

Solution A:	2% KCN/H_2O (v/v) 98% Pyridine (v/v)	Solution B:	5% Ninhydrin (w/v) in Ethanol
Solution C:	4:1 Phenol (w/v) in Ethanol		

yielded a peptide carrying a PA at its N- and an amide at the C terminus. The TFA solution was triturated in cold ether, dried under air flow and purified by HPLC (yield 3-51%). Purity was verified using LC-MS.

4.1.2 Synthesis of alkyne phosphoramidate peptides in solution

Truncated PAPs carrying an alkyne tag instead of the linker and biotin handle were synthesized in solution. First, an Fmoc-protected amino acid (1 eq) was coupled to propargylamine (1.1 eq) using 2.5 eq DIEA and 1.1 equiv HBTU in DMF. After 2 h, the solution was washed with 1 M $KHSO_4$, water, saturated $NaHCO_3$ and brine. The organic layer was dried using $MgSO_4$, filtered and evaporated under reduced pressure. As a next step, the crude material (250 mg) was Fmoc-deprotected using 20% piperidine in DMF for approx. 15 min. The piperidine/DMF was evaporated under reduced pressure; repeated coevaporation with dry DMF removed traces of water. Next, the PA warhead was coupled using 1.1 eq DCP and 1.1 eq TEA. Cleanup using a silica column yielded 180 mg crude product. Basic amino acids needed to be Boc-deprotected with 6-7 eq TFA, 1 eq TIS in DCM for ~1 h. The compound was HPLC purified and purity was verified using LC-MS.
AlkyneF-PAP was purified by HPLC as a white solid and an exemplary ^1H NMR (400 MHz, DMSO) was measured. d 8.47 (t, J = 5.4 Hz, 1H), 7.31 (q, J = 16.2, 8.7 Hz, 4H), 7.25 - 7.11 (m, 7H), 7.06 (d, J = 8.6 Hz, 2H), 6.96 (d, J = 8.6 Hz, 2H), 6.21 (dd, J = 13.3, 10.6 Hz, 1H), 3.83 (qdd, J = 17.5, 5.4, 2.5 Hz, 2H), 3.15 (t, J = 2.5 Hz, 2H), 2.92 - 2.82 (m, 1H), 2.69 (dd, 1H). ESI-HRMS: $[M+H]_+$ m/z 435.14 (found), $C_{24}H_{23}N_2O_4P$ requires 435.14.

4.1.3 Synthesis of 4-chloro-isocoumarins

4-Chloroi-socoumarins were kindly provided by Dr. Steven Verhelst who had synthesized them as previously described in [46].

4.1.4 Chromatography

4.1.4.1 Thin layer chromatography

Thin layer chromatography (TLC) plates (ALUGRAM sil G/UV254 from Roth, Germany) were used to monitor the course of the synthetic reactions in solution. Spots were detected with UV light and the staining solutions in table 4.1:

Table 4.1: **Staining solutions for thin layer chromatography**

Staining solution	Composition	Stained moiety	Stain
Cerium ammonium molybdate (CAM)	1 g $Ce(SO_4)_2 \cdot 4H_2O$ 5 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 90 ml H_2O 10 ml H_2SO_4	universal	blue spots on light background
Ninhydrin	1.5 g ninhydrin 100 ml n-butanol 3 ml acetic acid	amines	pink to blue spots on light background
Potassium permanganate, basic ($KMnO_4$)	1.5 g $KMnO_4$ 10 g K_2CO_3 1.25 ml 10% NaOH 200 ml H_2O	olefins, oxidizable groups	yellow spots on purple background

4.1.4.2 Silica column purification

Solvents used for silica columns were purchased at technical grade from Applichem without further purification. Compounds were separated using silica gel (grain distribution of 0.04-0.063 mm, pore size of 60 Å, silica gel 60, Roth, Germany) and a solvent gradient.

4.1.4.3 HPLC

Solvents used for HPLC were purchased at HPLC grade from Applichem. Before use, they were filtered (pore size 0.45 μm) and degassed. HPLC purification was done using a Waters system with two 515 HPLC pumps, a FlexInject injector, a 2487 Dual Wavelength Absorbance Detector and a Fraction Collector III. For separation on analytical scale, a Waters Xbridge C18, 5 μm (4.6 x 150 mm) column was used, for preparative scale a Waters Xbridge BEH130 Prep C18 5 μm (19 x 150 mm) column for preparative scale runs. Separation was carried out with gradients of solvent A (100% ACN + 0.1% TFA) and B (100% H_2O + 0.1% TFA).

4.1.4.4 LC-MS

All synthesized compounds were tested for purity using an Agilent 1100 Series LC system coupled to an Agilent 6210 electrospray ionisation-time of flight (ESI-ToF) mass spectrometer. Measurements were carried out in positive ion mode and the resulting spectra analyzed with Masshunter Software B.03.01. Samples were separated on a Zorbax SB C18 5 μm (0.5 x 150 mm) capillary column at RT using a gradient of solvents A (5% ACN/H_2O + 0.1% FA) and B (95% ACN/H_2O + 0.1% FA) with a flow rate of 20 μl/min and with an increase of 2.57% ACN/min starting with solvent A for 35 min. All solvents used for LC-MS were of HPLC grade or filtered before use (pore size 0.45 μm).

4.1.4.5 NMR spectroscopy

^{1}H NMR spectra were measured on a Bruker 400 MHz DRX (400; 100 MHz) in DMSO-d_6. Chemical shift δ [ppm] was calibrated on the proton peak of TMS (0 ppm), a supplement of deuterated solvents used for NMR. Signal multiplicity is characterized as s (singlet), d (doublet) and m (multiplet) and their combinations.

4.2 Biochemistry

4.2.1 Buffers and solutions

All solutions were made with deionized water, stored at 4°C and used at room temperature (RT) if not indicated differently. Many buffers were prepared at a higher concentration which is indicated (e.g. 10x) and were diluted with the respective amount of deionized water to afford 1x. Organic solvents were purchased from Applichem, Germany, salts and chemicals for buffers and the solutions listed here were purchased from Roth, Germany.

Biotin elution buffer pH 8	4 mM biotin 50 mM Tris-HCl 150 mM NaCl 1 mM EDTA 0.1% Triton X-100 (v/v)
Coomassie destaining solution	50% H_2O 40% Ethanol 10% Acetic acid
Developing solution (Silver stain)	18 g Na_2CO_3 300 mL H_2O 150 mL 37% formaldehyde 6 mL of Fixing solution 4 mL of Pretreat solution
Fixing solution (Silver stain)	500 mL Methanol 120 mL Acetic acid 500 mL 37% Formaldehyde bring to 1 L with H_2O
HEPES pH 7.4	50 mM HEPES 100 mM NaCl

Impregnation solution	4 mL of 20% (w/v) $AgNO_3$ stock solution
(Silver stain)	400 mL H_2O
	300 mL 37% formaldehyde
MS suitable buffer	25 mM $(NH_4)_2CO_3$
pH 7.4	
Organ lysis buffer	50 mM NaH_2PO_4
pH 7.4	150 mM NaCl
	0.5% nonidet P-40 substitute
PBST	101 mM Na_2HPO_4
pH 7.4	17.6mM KH_2PO_4
(10x)	1.37 M NaCl
	27 mM KCl
	add 0.1% Tween (v/v) after dilution
Phosphate buffer	50 mM NaH_2PO_4
pH 7.4	150 mM NaCl
Pretreat solution	80 mg $Na_2S_2O_3 \cdot 5\ H_2O$
(Silver stain)	400 mL H_2O
Running buffer (mini gels)	620 mM Tris, pH 8.3
(5x)	4.8 M Glycine
	87 mM SDS
	add to 5 L
Running buffer (3x wide gels)	200 mM Tris, pH 8.3
(10x)	1.9 M Glycin
	35 mM SDS

Sample buffer (4x)	500 mM Tris-HCl, pH 6.8 12% SDS (w/v) 40% Glycerol (v/v) 20% β-Mercaptoethanol 0.04% Bromophenol blue
Separating gel buffer (4x)	1.5 M Tris, pH 8.8
Stacking gel buffer (4x)	500 mM Tris, pH 6.8 0.04% (w/v) Bromophenol blue
Stop solution (Silver stain)	50% Methanol 12% Acetic acid (=Fixing solution without formaldehyde)
Urea washing buffer pH 8	50 mM Tris-HCl 4 M urea 150 mM NaCl 1 mM EDTA 0.1% Triton X-100 (v/v)

4.2.2 Gel electrophoresis

Gel-based assays were performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All SDS-gels used at the Technische Universität München were prepared in house and stored under moist conditions at 4°C for later use. SDS-gels used for all *Toxoplasma gondii* (*T. gondii*) assays were gradient gels (4-20% acrylamide, BioRad).

For in-house preparation, polyacrylamide gels were prepared according to the recipes in table 4.3. Ten mini gels or two triple wide gels were always made at

Table 4.3: Recipes for SDS gels

	10 Mini gels		2 Triple wide gels	
Stock solution	Stacking gel (5% acrylamide)	Separating gel (15% acrylamide)	Stacking gel (5% acrylamide)	Separating gel (12/15% acrylamide)
30% acrylamide	5 mL	30 mL	3 mL	20/25 mL
Stacking gel buffer (4x, blue)	7.5 mL	-	4.5 mL	-
Separating gel buffer (4x, colourless)	-	15 mL	-	12.5 mL
H_2O	17.5 mL	15 mL	10.5 mL	17.5/12.5 mL
10% APS/H_2O (w/v)	300 μL	450 μL	120 μL	250 μL
TEMED	30 μL	45 μL	12 μL	25 μL

the same time. Mini gels were clamped into a PerfectBlue Dual Gel System Twin S (Peqlab, Germany) and submitted to 180 V; triple-wide gels were fastened in a Triple Wide Gel Unit (C.B.S. Scientific, USA) and ran at 120 V. As molecular weight standards, either the pre-stained protein marker 'Seeblue Plus2' (Invitrogen, Germany) for subsequent Western blotting was used or the ECL Plex Fluorescent Rainbow Marker for fluorescence scanning. In-gel fluorescence was measured on a Typhoon TRIO+ fluorescence scanner (GE Healthcare, Switzerland) and images were viewed by ImageQuant version 5.2.

4.2.3 Gel staining

Following electrophoresis, SDS gels could be stained to check for the protein contents of each lane. Coomassie staining is more convenient as it only requires incubation with the staining solution for at least one hour at RT. However, silver staining is

more sensitive and the band intensity is tunable. Therefore, we used either method depending on the total amount of protein loaded per lane.

4.2.3.1 Coomassie staining

The coomassie staining solution was prepared fresh according to the vendor's recommendations: 20 mL 5x stock solution (Roti-Blue, Carl Roth) diluted with 20 mL methanol and 60 mL H_2O. This solution was used to incubate the gels for at least one hour at RT on a horizontal shaker, or over night to improve contrast. Afterwards, the gel was washed briefly 1-2 times with H_2O and then with coomassie destaining solution until bands became visible (usually after 15-20 min). The gels were scanned using a CanoScan LIDE 100 scanner (Canon, Germany).

4.2.3.2 Silver staining

Silver staining was done for low amounts of protein. It has the advantage that the band intensities can be tuned by shortening or extending the incubation time with the developing solution. On the other hand, silver staining is more elaborate than coomassie staining, the solutions always need to be fresh and require special waste treatment. Effectively, we used this method only if coomassie staining was expected to be insufficient.
The gels were incubated on a horizontal shaker with the following solutions for the amounts of time indicated: 1) Fixing solution (1 h), 2) methanol/water 1/1 (2x 15 min), 3) Pretreat solution (1 min), 4) water (3x 20 s), 5) Impregnation solution (20 min), 6) water (2x 30 s), 7) Developing solution until bands became visible, 8) Stop solution (2x 10 min).

4.2.4 Biotin blot

Proteins labeled with biotinylated ABPs were analyzed by biotin blotting, a procedure that works like classical Western blot except that horseradish peroxidase conjugated to streptavidin (S-HRP) is used for detection. After separation via SDS-PAGE, proteins were transferred onto a nitrocellulose membrane (Roth, Germany) with a semi-dry blotter (V20 SDB; Scie-Plas, UK) at 1.5 mA/cm^2 of gel surface for 1.5 h. The membrane was blocked with 3% milk powder in PBST for 1 h and subsequently incubated with 0.3 mg/mL S-HRP for 45 min. The membrane was washed

three times for 5 min and then, the luminescence was detected using the ECL plus western blotting detection system (GE healthcare, Germany) and Kodak X-Omat LS films (VWR, Germany). Exposure of the films varied from 1 s to 2 min.

4.2.5 Preparation of organ lysates

A piece of the respective rat organ was forced through a 70 μM cell strainer and suspended in cold organ lysis buffer. After 1 h incubation on ice, the sample was spun briefly in a tabletop centrifuge (Eppendorf, Germany) at 15,000 rpm for 10 min to remove cell debris. The supernatant was snap-frozen in liquid nitrogen and stored at -80°C until usage. For labeling assays, the organ lysates were diluted to 1 mg/mL total protein with phosphate buffer.

4.2.6 Determination of protein concentration

For normal labeling assays, the protein concentration of cell and organ lysates was adjusted to 1 mg/mL. Protein quantification was performed largely according to the vendor's recommendations using DC Protein Assay Kit II (Bio-Rad). 10 μL lysate (plain or diluted with the respective buffer according to the expected protein concentration) were pipetted in triplicates into the wells of a 96-well polystyrene microtiter plate (VWR). A BSA standard in H_2O ranging from 0.1 to 1.5 mg protein per mL was used for calibration. 10 μL H_2O were added to each sample, while 10 μL of the lysate buffer were added to the BSA standard. When the lysate contained detergents, 25 μL of reagent A' (a mix of solutions S and A, 1:50 (v/v)) was added to the samples. For detergent-free samples, plain reagent A was used. Subsequently, 200 μL solution B were added, the microtiter plate was shaken and left to equilibrate for about 15 min. The absorbances were read at 750 nm in a Versamax microplate reader using the software Softmax Pro, version 5.0.1 (both from Molecular Devices, Germany), and the sample protein concentrations were calculated.

4.2.7 Enzyme labeling

All purified enzymes were purchased from the vendors indicated in table 6.3 in the appendix. Preparation of lyophilized enzymes and storage of stock solutions was done according to the vendor's recommendations. Most enzyme reactions were

performed in phosphate buffer, only for cathepsin G HEPES buffer was used. When labeling of purified enzymes was to be followed by MS analysis, MS suitable buffer was used instead.
All inhibitors were purchased from the vendors indicated in table 6.3 in the appendix. Inhibitor stock solutions were prepared in DMSO at 100x the desired reaction concentrations and stored at -20°C. Only the PMSF stock solutions were in ethanol.

4.2.7.1 Standard procedure

If not indicated otherwise, the standard procedure, also sketched in figure 3.4A was as follows: 1) preblocking of the enzyme by an active-site inhibitor, negative controls were treated with the same volume of DMSO as used for the inhibitor treatment; 2) labeling: incubation with ABP; 3) optional: Cu(I)-catalyzed click chemistry in case alkyne-PAPs were used as probes (see two step-labeling in section 4.2.7.4); 4) addition of sample buffer and heating to 95° C for 3 min for protein denaturation.

4.2.7.2 Labeling of purified enzymes in complex proteome backgrounds

The soluble fraction of a cell lysate provides a fairly representative mixture of proteins a soluble protease would naturally occur in. This background gives a general idea about the likelyhood of the probe binding to off-targets. We used EL4 lysate with a protein concentration of 1 mg/mL in which the purchased purified protease was spiked in at a given concentration, ususally specified in percentage with respect to the total amount of protein. The ABP was added and labeling was performed according to the standard procedure for enzyme labeling.

4.2.7.3 Labeling of endogenous proteases in rat pancreas

The pancreas contains inactive precursors of serine proteases at high concentrations. Their activation cascade (described in section 3.2 in the introduction part of this manuscript) can be mimicked by addition of active enteropeptidase [111]. Therefore, the pancreas lysate was incubated with 1 U enteropeptidase per mg of total protein for 2 h on ice. Afterwards, probe labeling was done according to the standard procedure.

4.2.7.4 Two step-labeling using Cu(I)-catalyzed click chemistry

Biotin or TAMRA (custom-made) as a fluorophore conjugated to an azide moiety was coupled to enzymes previously treated with alkyne-PAPs (see figure 3.4B) using Cu(I)-catalyzed click chemistry. Therefore, the following reagents were added: 50 μM TAMRA, 25 μM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), from 5 mM DMSO stocks, 1 mM $CuSO_4$ (from 50 mM H_2O stock) and 0.5 mM Na^+ ascorbate (from 25 mM H_2O stock). The $CuSO_4$ and ascorbic acid stocks were prepared freshly after about 4-5 freeze thaw-cycles. If not indicated otherwise, the reaction was allowed to proceed for 30 min. Subsequently, the procedure went on as described in the standard procedure.

4.2.8 Target enrichment experiments using biotinK-PAP

For pulldown assays, the protein concentration was not diluted in order to have available as much protein as possible. Following labeling with biotinK-PAP, the solution was desalted using a Zeba spin column (moleculer weight cutoff: 7 kDa, Perbio/Pierce, Germany) to remove unreacted probe. The eluate was subjected to streptavidin-coated agarose beads (Merck/Calbiochem, Germany) for 1-2 h in presence of 50 μM EDTA and an EDTA-free protease inhibitor cocktail. Subsequently, the beads were washed with urea washing buffer and then heated to 95° C for 10 min in biotin elution buffer to denature the streptavidin for release of bound target protein. This procedure, including the following digest by glutamyl endopeptidase C (GluC) and MS identification is sketched in figure 5.5.

4.2.9 Reduction, alkylation and digestion of enriched proteins

The eluted protein samples were separated on an SDS-PAGE. The bands containing the expected targets were cut out, chopped into small pieces and the contained proteins were reduced with about 50 μL 10 mM DTT in MS suitable buffer for 45 min. The supernatant was removed and 55 mM IAA in the same buffer was added for alkylation of free cysteines for at least 30 min in the dark. After discarding the supernatant, the gel pieces were incubated for 15 min in 100 μL MS suitable buffer as a washing step. After removal of the buffer, the gel pieces were exposed to 200 μL ACN until they became white as a sign of dryness. ACN was removed in a vacuum

concentrator (Univapo 100H, UniEquip, Germany) and the proteins were digested with glutamyl endopeptidase (GluC, Promega, Germany) in MS suitable buffer at 37°C over night.

4.2.10 Liquid chromatography mass spectrometry of enriched peptides

The protein identifications using liquid chromatography mass spectrometry (LC-MS) were done in collaboration with H. Hahne and S. Lemeer in the Küster lab, largely as previously described [49]: MS was carried out using an LTQ Orbitrap Velos or an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Germany) connected to a nanoLC Ultra 1D+ LC system (Eksigent, CA) equipped with an in-house packed precolumn (20 mm x 75 μm ReproSil-Pur C18, Dr. Maisch, Germany) followed by an analytical column (400 mm x 50μm ReproSil-Pur C18, Dr. Maisch, Germany). Ionization took place in a nanoelectrospray ion source (Proxeon Biosystems, DK), applying the electrospray voltage via a liquid junction. Measurements were accomplished in positive ion mode. Mass spectra of intact peptides were obtained with a resolution of 60,000 (at m/z 400) (target value of automatic gain control (AGC) 10^6). For the most intense ions, collision-induced or higher energy-collision induced dissociation (CID; LTQ Orbitrap XL or HCD; LTQ Orbitrap Velos, respectively) was used for fragmentation. Profile mode was used for full scans and centroid mode for tandem mass spectra. CID was carried out for up to 15 MS/MS (2 h gradient) or 8 MS/MS (4 h gradient) per full scan with 35% normalized collision energy (NCE) (AGC target value 5000). HCD was performed for up to 10 MS/MS per full scan with 40% NCE (AGC target value 35,000). Ions without assigned charge state as well as singly charged ions were excluded from fragmentation. Fragmented precursor ions were excluded in a dynamic manner (2 h gradient, 10 s; 4 h gradient, 30 s). The mass spectrometers were calibrated internally with the polysiloxane ion signal at m/z 445.1200 present in laboratory air at RT.

4.2.11 Analysis of LC-MS data for protein identification

All MS/MS samples were analyzed using Mascot Distiller (Matrix Science, London, UK; version 2.4.0). Carbamidomethylation of cysteine residues, oxidation of methionine and bound biotinK-PAP to serine were specified in Mascot as variable

modifications. Enzyme specificity was set to GluC with up to two missed cleavage sites. The target-decoy option of Mascot was enabled and peptide mass tolerance was set to 10 ppm and fragment mass tolerance to 0.50 Da and a mass tolerance of 10 ppm. The peak list files were then searched with Mascot 2.3.0 against the SwissProt database (version 57_15, selected for Rodentia, 25305 sequences), combined with sequences of common contaminants. Search results were imported into Scaffold (version 3.6.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Mascot identifications required at least ion minus identity scores of greater than -40 and ion scores of greater than 15. Protein identifications were accepted if they contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

4.3 Bioinformatics

To calculate the pore depth of the β-tryptase tetramer, the freeware Visual Molecular Dynamics (VMD, Version 1.9 for Windows, Uiversity of Illinois, IL) was used.

4.4 Cell culture

EL4 cells were a kind gift from Matthew Bogyo, human foreskin fibroblasts (HFF) were a kind gift from John Boothroyd, both at Stanford University School of Medicine, Stanford, CA. The HMC-1 line was a kind gift from the Mayo Clinic US. All buffers, media and assays involving HFF cells are described in section 4.5. A cell lines used in this work are listed in table 6.3 in the appendix. All cells were cultured at 37°C , 5% CO_2 and 95% humidity.

4.4.1 Buffers and media

All cell culture media were purchased from Invitrogen, supplemented as listed, stored under sterile conditions at 4°C and used at 37°C if not stated differently. Supplements were bought from Invitrogen as well, except for β-mercaptoethanol (from Applichem).

HEPES lysis buffer (pH 8)	50 mM HEPES 150 mM NaCl
MES lysis buffer (pH 6.5)	50 mM MES 150 mM NaCl
NP40 lysis buffer (pH 8)	50 mM Tris-HCl 150 mM NaCl 1 mM EDTA 0.1% nonidet P-40 substitute (v/v)
RIPA buffer (pH 7.4)	50 mM Tris-HCl 150 mM NaCl 0.1% SDS 0.5% sodium deoxycholate 1% Triton X-100 (v/v)
Triton lysis buffer (pH 8)	50 mM Tris-HCl 150 mM NaCl 1 mM EDTA 0.1% Triton X-100 (v/v)
Tween lysis buffer (pH 8)	50 mM Tris-HCl 150 mM NaCl 1 mM EDTA 0.1% Tween (v/v)

4.4.2 Cultivation and passaging

Cryostocks were thawed, suspended in 10 mL medium per vial and centrifuged to remove the DMSO which serves as cryoprotectant but is harmful under liquid conditions. The pellet was resuspended and the cells were counted under a micro-

scope using a hemocytometer. Their number was adjusted by addition of medium to approximately $5{\cdot}10^5$cells/mL and transferred into culture flasks. The medium was exchanged every other day or when it began to turn yellow. When about 80% confluent, the cells were passaged using trypsin (trypsin-EDTA 1x, Invitrogen) and distributed into fresh culture flasks. Suspension cells were grown to a density of around 1-1.5$\cdot10^6$ cells/mL as determined by counting in a hemocytometer. For storage, 5% DMSO (v/v) was added to the medium and between 20-30$\cdot10^6$ cells were pipetted into one vial and slowly frozen to -80°C with the help of a freeing container. 12-24 h later, they could be stored under liquid N_2.

4.4.3 Cell lysates

Cells were grown for about 6-8 days of culturing after defrosting. Subsequently, they were washed once with HBSS, resuspended in lysis buffer (Triton lysis buffer for HMC-1 cells as well as RIPA for all other cells) at 4°C and kept on ice with occasional vortexing for 30 min (HMC-1) or 2 min (other cell lines). The lysate was centrifuged at 21390 g, cell debris was discarded and the supernatant was frozen in liquid nitrogen and stored at -80 °C until usage. For labeling assays, the protein concentration was diluted to 1 mg/mL unless stated differently.
To test alternative lysis methods for HMC-1 cells, buffers with different buffering agents and pH values instead of Triton lysis buffer were used in combination with glass bead lysis: HEPES lysis buffer, MES lysis buffer. Additionally, Triton was exchanged against alternative detergents (NP40 lysis buffer, RIPA buffer as well as Tween lysis buffer).

4.5 *Toxoplasma gondii*

All experiments involving *T. gondii* were performed in the Bogyo lab at the Department of Pathology, Stanford University School of Medicine, Stanford, CA and essentially followed the procedures previously described in [50].

4.5.1 Buffers and media

All buffers were made with deionized water, stored at 4°C and used at 37°C if not stated differently. All media were purchased from Invitrogen and supplemented as

described. The culture media for *T. gondii* in HFF cells are listed in table 6.3 in the appendix.

Endo synchronization buffer	20 mM Tris-H_2SO_4 44.7 mM K_2SO_4 10 mM $MgSO_4$ 106 mM sucrose 5 mM glucose 3.5 mg/mL BSA
Hank's buffered salt solution (HBSS)	no supplementation
Invasion medium	DMEM 1% FBS
Phosphate buffered saline (PBS)	no supplementation
Toxo lysis buffer	50 mM Tris pH 7.5 200 mM NaCl 5 mM EGTA 2 mM DTT 0.1% SDS 1% nonidet P-40 substitute

4.5.2 *T. gondii* culture maintenance and harvest

T. gondii is an obligate intracellular parasite and therefore needs a host cell culture to grow in. We used human foreskin fibroblasts (HFF, see table 6.3) in DMEM at confluence at 37°C , 5% CO_2 and 95% humidity. To harvest the parasites, a culture in one T25 cell culture flask was scraped and forced through a canula (27 G 1/2), which disrupts the host cells but leaves the parasites intact. When used for invasion or plaque assays, the cells were centrifuged twice with intermediate washing by cold (4°C) HBSS to remove cell debris. Approximately 10^6 parasites, determined

by a hemocytometer, were used to infect one T25 flask of confluent HFFs. For experimental use, the parasites were used within 24 h after infection to ensure they would all be in the same infectious and proliferative stage.

4.5.3 Invasion assay

The parasites from one T25 flask were used to infect the confluent HFF culture in one 24-well plate. The cells were PBS-washed for three times before changing the medium to Endo synchronization buffer. In parallel, one flask of *T. gondii* was harvested, aliquoted and treated with the compounds (DMSO for negative control) in invasion medium for 10 min at 25°C. The parasites were centrifuged for 5 min at 1200 x g to remove excess compound with the supernatant. After resuspension in Endo buffer, equal numbers of parasites were distributed onto the HFFs and incubated at 37°C, 5% CO_2 and 95% humidity for 15 min. *T. gondii* takes approximately 30 s for the actual invasion process after the first contact with its host cell. We reasoned that a total of 15 min should be enough to let them sink to the bottom of the well and infect. Cells were washed once with PBS, fixed for 10 min at RT in 3.5% formaldehyde (capsules containing 16% methanol-free formaldehyde in ultrapure H_2O, electron microscopy grade, from Polysciences, PA; supplemented with fresh PBS) and stored at 4°C. Extracellular parasites were antibody-stained for 15 min at 25°C (primary: mouse anti *T. gondii* 11-132 dilution 1:1,000, secondary: FITC-conjugated goat anti-mouse IgG (Invitrogen) 1:5000 dilution, both in PBS supplemented with 3% BSA and filtered). Plates were preserved in 75% glycerol in PBS (v/v) and stored at 4°C until imaging.

4.5.4 Imaging of invaded *T. gondii* parasites

4.5.4.1 Automated quantitative imaging

Imaging of fixed and preserved cells was carried out with an ImageXpress 5000A automated image capture system (Molecular Devices; part of the Stanford University High Throughput Biosciences Center) equipped with a CFP/YFP/HcRed-3X3M-A Triple-band "Sedat" Filter Set for HcRed and YFP (FITC) fluorescence. The Images were monitored by the MetaXpress image analysis software. Merged images were scored automatically with the help of the freeware ImageJ 1.45s (running with Java

1.6.0_20, 32 bit; NIH, USA). Initially intended yellow spots indicating extracellular parasites (overlay of red and green) as well as green spots could not be counted due to high background noise.

4.5.4.2 Manual qualitative imaging on coverslips

Qualitative imaging was performed essentially as quantitative imaging with the exception that the HFF host cells were cultured on glass coverslips.
After the invasion assay as described above, the glass coverslips were mounted on glass slides with 3μL VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, USA) and stored at 4°C until imaging. Therefore, ten randomly selected fields were selected and scored manually for the number of extracellular and intracellular parasites, visible as green and red spots through an Olympus BX60 microscope, respectively. Phase and fluorescence images were captured by a Hamamatsu Orca100 CCD camera and were processed using Image-Pro Plus 2.0 (Media Cybernetics) and Photoshop CS4 (Adobe Systems).

4.5.5 Plaque assay

T. gondii parasites were harvested, resuspended in invasion medium and treated with compound or DMSO for 15 min at RT. 200 parasites were pipetted into each well of a six-well plate containing confluent HFFs. The cells were left undisturbed for 6 days at 37°C , 5% CO_2 and 95% humidity. Subsequently, they were washed once with PBS, incubated for 5 min in methanol and then stained for 30 min in the same coomassie solution as used for SDS gels. Subsequent washing with H_2O revealed wells covered with blue cells and cell-free colorless spots which were counted manually.

4.5.6 Gel-based competition assay in *T. gondii* lysate

T. gondii lysate was incubated with 50 μM of the alkynylated analogs of the parent compounds identified as inhibitors in [50] and subsequently with 2 μM with a rhodamine-tagged FP (FP-rhodamine) for 20 min on ice.

5 Results and Discussion

ABPs are versatile tools for the functional and spacial analysis of enzyme activity. For serine proteases, only two selective types existed when we started this project: diphenyl phosphonates [1, 54, 94] and 4-chloro-isocoumarins [67]. We therefore set out to develop diphenyl phosphoramidates (PAs) as a new type of highly selective ABP warhead for serine proteases. In addition, we investigated ICs for their suitability for two-step-labeling as well as their tunable selectivity towards certain subsets of serine proteases.

5.1 Phosphoramidates as ABPs for serine proteases

5.1.1 Structure and reaction mechanism of the warhead

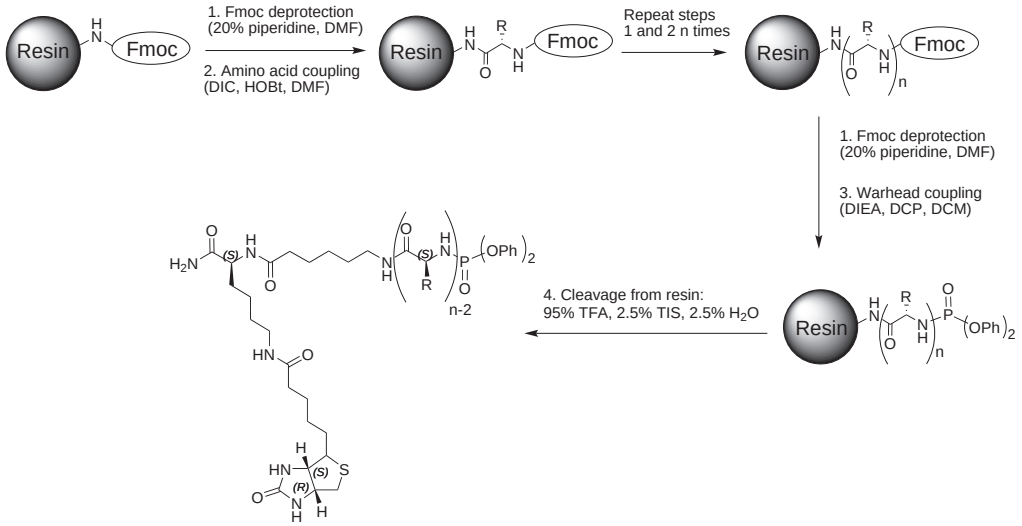
The PA warhead is structurally similar to the diphenyl phosphonate group previously reported to selectively bind to serine proteases [1, 54, 94]. In PAs, a nitrogen is directly bound to the phosphorous atom, linking it to the carbon atom that carries the substituent serving as recognition element.
The postulated reaction mechanism of PAs with their target enzyme is shown in scheme 3.2. It starts with a nucleophilic attack on the phosphorous atom by the active site serine hydroxyl. One of the phenoxy moieties acts as leaving group and the warhead is linked covalently to the enzyme.

5.1.2 Synthesis on solid support

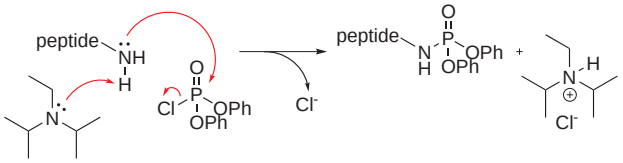
In contrast to phosphonates, phosphoramidates can easily be synthesized on solid support. This enables the synthesis by simple solid-phase peptide synthesis (SPPS) which is advantageous in view of the option to easily diversify the probes by introducing different recognition elements. To our knowledge, phosphoramidate peptides (PAPs) are the first type of ABPs for serine proteases which can be entirely synthesized on solid support using commercially available building blocks.
Fmoc-based SPPS is a standard method for the production of large numbers of peptides with defined or randomized sequences [88]. As solid support, beads usually made of polymer resins with a functionalized surface are used. The synthetic procedure consists of consecutive repetitions of 1) Fmoc deprotection and 2) coupling of a building block. Each attached unit must comprise an electrophile, usually a carboxylic acid, to react with the previously deprotected and activated amine on the solid support. They furthermore need an Fmoc-protected amine themselves to allow subsequent coupling of the next segment. This way, diverse building blocks can be incorporated, ranging from standard amino acids to those with modified structures and non-peptidic compounds.
We used SPPS to synthesize ABPs for serine proteases according to the procedure in scheme 5.1. The synthesis starts with coupling an ϵ-biotinylated lysine as the C-terminal reporter tag to a rink amide resin. Next, an aminohexanoic acid (AHX) is attached as a spacer, followed by up to three amino acids. The synthesis of the His-tagged probes is analogous but uses different building blocks. To couple the warhead, DCP is used which undergoes a nucleophilic substitution of the chloride according to the postulated mechanism in scheme 5.2. The finished PA is cleaved off the resin under highly acidic conditions.

5.1.3 Structures of biotin-PAPs

The collection of all biotin-PAPs with different P1 recognition elements synthesized on solid support is shown in scheme 5.4, their LC-MS traces are shown in the appendix. Right next to the PA warhead, they contain a P1 amino acid as recognition element for their target enzymes. In two cases, this recognition element is extended by either one or two alanines. The recognition element is linked to the reporter tag by a spacer, alternatively AHX or two diethylene glycols. The reporter tag is usually



Scheme 5.1: **Solid-phase peptide synthesis of phosphoramidate peptides (PAPs).** The procedure consists of iterations of Fmoc-deprotection (1.) and subsequent coupling of an Fmoc-protected amino acid (2.). It starts with the carboxy terminal ϵ-lysine. Next, the spacer is coupled, followed by the residues considered as recognition element (RE). For the PAPs carrying a His-tag, the procedure is analogous with different building blocks. After coupling of the last residue, the PA head group is attached (3., postulated mechanism in scheme 5.2). The peptide is cleaved from the resin using TFA (4.), yielding an ABP with a PA warhead, a C-terminal amide and either a biotin handle (biotin-PAP as shown here) or a His-tag (H_6K-PAP). The set of ABPs synthesized using this method is shown in scheme 5.4.

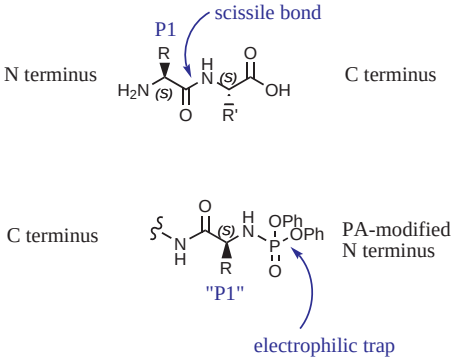


Scheme 5.2: **Putative mechanism of the warhead coupling reaction.** The primary amine of the deprotected amino acid is deprotonated by the base DIEA. The resulting nucleophile attacks the electrophilic phosphorous within DCP, thereby substituting the chloride ion. The reaction is considered an S_N2 mechanism as described in [9, 122].

biotin, bound to the side chain of a lysine; only one probe carries a His-tag. Instead of a terminal carboxyl function, these probes have an amide cap to avoid a negative charge which would make it even harder for the probe to cross cell membranes.
Our synthetic approach leads to a reversed peptide backbone orientation resulting in an inversed stereochemistry of the amino acids compared to natural substrates (see scheme 5.3). We initially had concerns that this orientation might have a negative impact on recognition by the target enzyme. Therefore, we synthesized biotin(D)K-PAP and hypothesized that this enatiomer would make up for the stereochemical switch.
The inversed backbone polarity also means that our P1 recognition element is located where the P1' would be in a natural substrate (ignoring that the scissile bond is now replaced by the PA). However, we reasoned that because of our electrophile being at the head of the probe, the peptide would be sufficiently flexible to still fit into the S1 pocket.

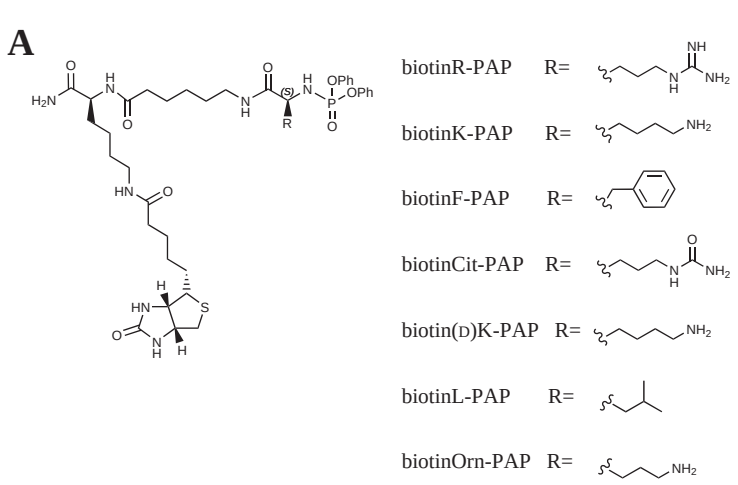
5.1.4 Synthesis of alkynylated PAPs in solution

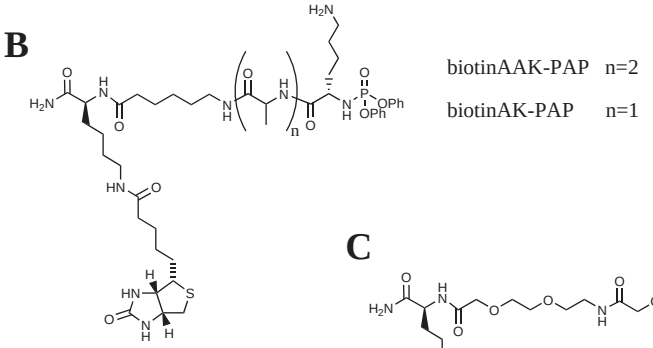
Biotinylated ABPs have poor cell penetration capabilities, which limits their applicability in systems beyond lysates [106]. Additionally, the purification of proteins labeled with biotin-tagged probes involves the use of streptavidin immobilized on a column and will always co-purify endogenously biotinylated proteins which increases the number of false positives. The elution conditions are very harsh and usually denature the targets [106], a fact that needs to be avoided for certain downstream applications.
One alternative to biotinylation is the introduction of a moiety that allows attachment of any group carrying an azide function via Cu(I)-catalyzed click chemistry (also called "two-step labeling", see section 3.2.6). That way, several reporter tags including fluorophores can be introduced according to the requirements of the individual experiment. Furthermore, the reaction with the enzyme is independent from the click chemistry, enabling intermediate sample storage.
To synthesize alkynylated versions of our PAPs, we chose a procedure in solution because it afforded higher yields than on solid support. In order to test their functionality as ABPs for serine proteases with different P1 preferences, we made a set of four alkyne-PAPs covering both hydrophobic and hydrophilic P1s (see scheme 5.5).

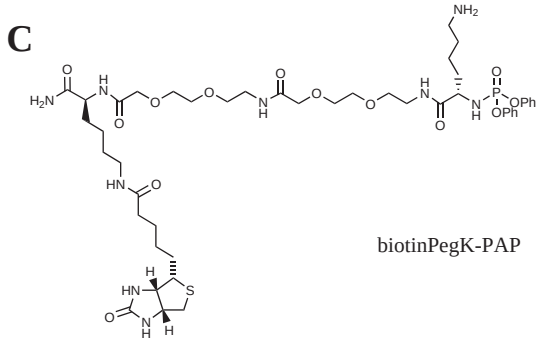


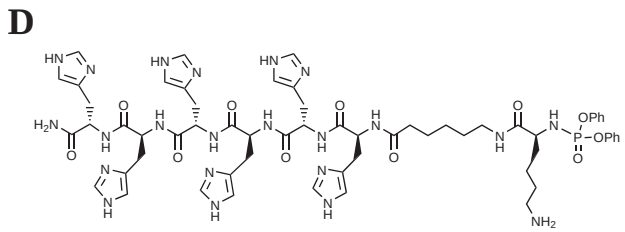
Scheme 5.3: **PAPs have an inversed backbone orientation compared to natural peptides.** The upper structure shows a natural dipeptide in N to C-orientation. The P1 amino acid is N terminal to the cleavage bond. In PAPs, as shown in the lower structure, the cleavage bond is mimicked by the PA warhead that carries a phosphorous atom as electrophilic trap. The nitrogen links it to the amino acid that is treated as P1 despite its reversed stereochemistry with respect to the peptide backbone.

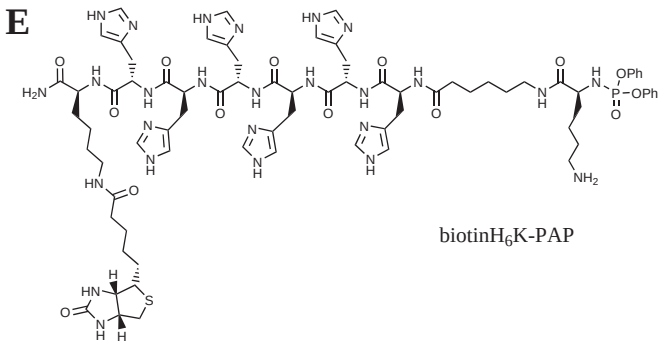
Scheme 5.4: **Overview of phosphoramidate peptides (PAPs) made on solid support.** All biotinylated and His-tagged PAPs were synthesized using Fmoc-based solid phase peptide synthesis on rink resin (see scheme 5.1). **A**: the set of biotinylated PAPs (biotin-PAPs) with one amino acid as recognition element. For biotin(D)K-PAP, the configuration of the substituent is (*R*). **B**: the two biotin-PAPs with P1 lysine and extended recognition elements (biotinAK-PAP and biotinAAK-PAP); **C**: biotin-PAP with a P1 lysine and an extended linker of two diethylene glycol units (biotinPegK-PAP); **D**: His-tagged PAP without biotin and with a P1 lysine (H_6K-PAP); **E**: biotin-PAP with an additional His-tag (biotinH_6K-PAP)

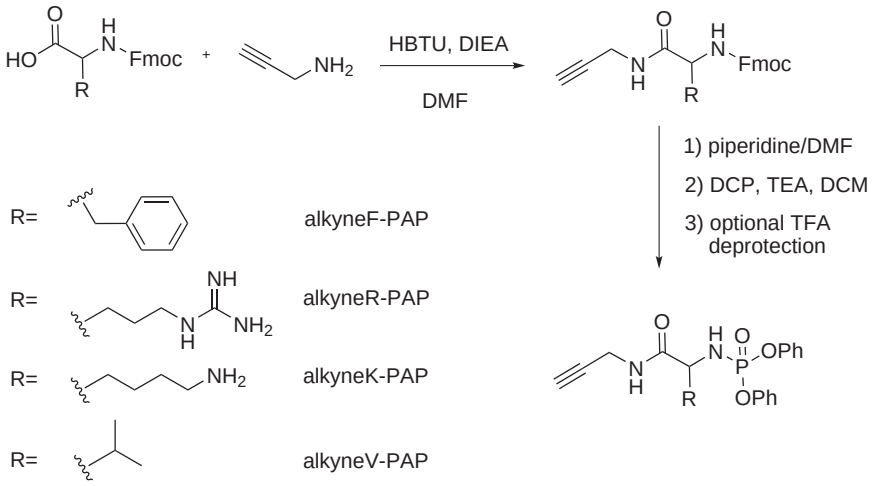












Scheme 5.5: **Synthesis and structures of alkynylated phosphoramidates (alkyne-PAPs) made in solution.** Truncated PAPs were synthesized in solution and carry an alkyne handle instead of a linker and a biotin. This structure improves cell penetrability and enables the attachment of any reporter tag holding an azide moiety via Cu(I)-catalyzed click chemistry. Step 1: the Fmoc-protected amino acid at ~2.5 mM and 1.1 eq propargylamine were coupled for 2 h using 1.1 eq HBTU and 2.5 eq DIEA. After workup, Fmoc was deprotected with 20% piperidine/DMF followed by coupling of the warhead using 1.1 eq DCP and 1.1 eq TEA in DCM. Basic amino acids needed to be Boc-deprotected with 6-7 eq TFA, 1 eq TIS in DCM.

5.1.5 Proof of the PAP concept using purified proteases

5.1.5.1 Probe selectivity

The degree of a probe's target selectivity is an important feature that determines its use for either broadband or highly selective applications [48]. PAPs are novel structures in this field that needed to be characterized in order to be established as ABPs. To this end, we chose certain serine proteases with different P1 selectivities which are commercially available in purified form: trypsin for its preference to cleave after basic, chymotrypsin after large hydrophobic and type I pancreatic elastase (PE) after small hydrophobic residues [99].

Figure 5.1 A shows the result of the labeling assays using biotin- and alkyne-PAPs. Both types of PA probes react with trypsin according to its expected preference for basic P1 residues [99]. This preference is even stricter than expected as the enzyme

accepts neither ornithine nor (D)-lysine in the P1 position, which are both structurally very similar analogs of lysine. Not surprisingly, it also does not recognize citrulline, an uncharged analog of arginine. For the biotin-PAPs, arginine is a weaker P1 recognition element than lysine, independent of the peptide length, whereas for the alkyne-PAPs, this effect is reversed.
The outcome of PE labeling is rather unexpected as this enzyme is known to prefer small hydrophobic P1 amino acids. However, the only hydrophobic recognition element it accepts is the comparatively big phenylalanine, for both alkynylated and biotinylated PAPs. Biotin-PAPs with both lysine enantiomers bind PE, whereas alkyneK-PAP is not accepted. A P2 alanine enhances the affinity, an additional P3 alanine even more so, showing that these positions also influence the reaction with the enzyme. The differences in labeling between the alkyne- and the biotin-PAPs show that even the plain size of an ABP can affect its target affinity. Unfortunately, we can not offer any satisfying hypothesis for this recognition behaviour.
The impact of overall probe structure and size is underlined by the results found for chymotrypsin. While this protease recognizes hydrophobic but not basic alkyne-PAPs, it reacts promiscuously with all biotin-PAPs. We observed a similar phenomenon when labeling chymotrypsin with ICs (see figure 5.7 in section 5.2) Although chymotrypsin is known for its specific cleavage after large hydrophobic residues, it has been reported to also accept different amino acids [52]. This is quite a common observation since most enzymes catalyze reactions with substrates beyond their known operating range [71]. We speculate that structures distant from the cleavage site could play a role for the affinity of biotin-PAPs to chymotrypsin. Then, the alkyne-PAPs would be too small for this, leaving only the P1 residues as a means of distinction.
It is especially important to show this selectivity in view of the reversed backbone orientation and the inversed stereochemistry of the biotin-PAPs. To exclude any negative effect of this stereochemistry on the affinity of the probes to the proteases, we synthesized a biotin-PAP with a D-lysine in the P1 (biotin(D)K-PAP). We reasoned that this enantiomer of biotinK-PAP could make up for the flipped peptide polarity and therefore increase both the selectivity of the probe as well as its potency. Additionally, we tested P1 ornithine as another close relative of lysine holding one methylene less. Our concerns were unfounded: L-lysine as recognition element is efficiently recognized and D-lysine and ornithine are not. Apparently, the biotin-PAP

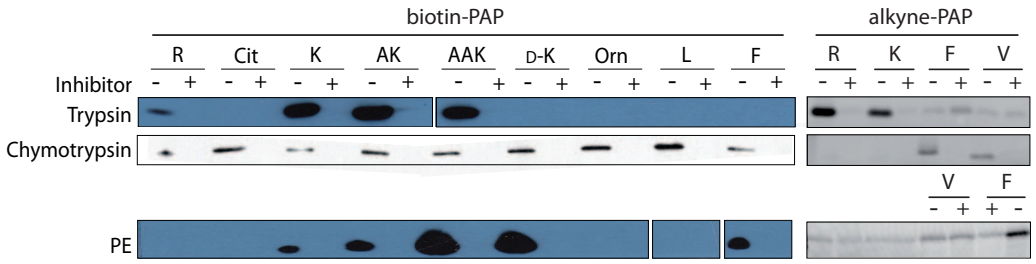


Figure 5.1: **Specific protease labeling by phosphoramidate peptides (PAPs).** Each enzyme was pre-blocked with an active site inhibitor (1 mM DCI for trypsin and pancreatic elastase (PE), 100 μM DAP22c for chymotrypsin; control: DMSO (-)) and subsequently treated with 50 μM biotin- or alkyne-PAP. The capital letters indicate the amino acid used as recognition element. Afterwards, the samples labeled with biotin-PAPs were boiled for 5 min with sample buffer and analyzed using SDS-PAGE and biotin blot. Detection was done with the help of horseradish peroxidase (generating chemiluminescence) conjugated to streptavidin and a light-sensitive film (trypsin and PE) or read out using a chemiluminescence scanner (chymotrypsin). The samples labeled with alkyne-PAP underwent Cu(I)-catalyzed click chemistry to attach a fluorophore before fluorescence scanning.

structure is flexible enough to fit into the S1 pocket and active site of trypsin.

5.1.5.2 Activity dependence

The second crucial requirement for ABPs, as implied in their name, is that they bind their target in an activity-dependent manner. That means that an inactive enzyme will not react with the probe. Therefore, as a standard activity control, we pre-blocked the proteases with an active-site inhibitor before incubation with the probe, which prevented reaction with the ABP (figure 5.1).
Lack of activity can also be due to the enzyme being in its inactive zymogen form. We used trypsinogen as a representative to ensure that PAPs do not react with zymogens. As expected, trypsinogen was not labeled by biotinK-PAP (figure 5.2), the probe which most intensely labels the active trypsin in figure 3.1. Together, these data prove that PAPs label their targets in an activity-dependent way.

5.1.5.3 Probe potency

The potency of an ABP is a measure for its target affinity: a probe is considered more potent in comparison to another if it can be applied in lower concentrations to label the same amount of enzyme. The relative potency of biotin-PAPs was investigated

Figure 5.2: **Labeling of trypsin versus trypsinogen.** Either trypsin (Try) or trypsinogen (TG) was incubated with 10 μM biotinK-PAP and subsequently analyzed via SDS-PAGE and biotin blot.

using a dose-response assay shown in figure 5.3 using trypsin as our representative protease and three biotin-PAPs which had labeled trypsin robustly in the previous assay (figure 5.1). At 100 μM of all three probes saturated 15 ng enzyme. For biotinK-PAP, the detection goes down to 0.8 μM, while a recognition element extended by one alanine decreased the probes potency about 5 fold. This stands in contrast to our hypothesis that a P2 alanine could enhance the probe's affinity to trypsin as suggested by the specificity matrix in the MEROPS database for bovine trypsin [99]. For biotinR-PAP, the detection limit was just below 20 μM. This concentration is about 25 times higher than for biotinK-PAP and supports previous findings showing that trypsin rather cleaves after lysine than arginine [99]. However, according to the MEROPS database, this preference is less pronounced than our results suggest.

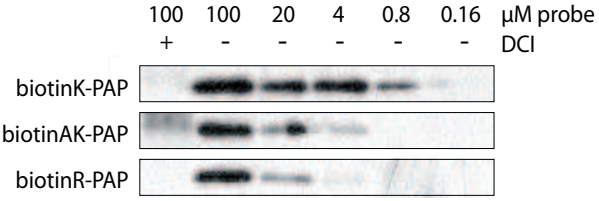


Figure 5.3: **Labeling purified trypsin in a dose-response assay with selected biotin-PAPs.** Three biotin-PAPs were used in decreasing concentrations to label 15 ng of purified trypsin after preblocking with DFP (+) or DMSO (-).

5.1.5.4 Summary of the establishment of PAP-ABPs for serine proteases

Taken together, we could show that PAPs bind their target enzymes with different affinities depending on their recognition elements which can largely be predicted according to the known P1 preferences of the respective protease. However, there are some exceptions, best demonstrated by the promiscuous behavior of chymotrypsin: this protease recognizes all biotin-PAPs despite its preference for large hydrophobic P1 amino acids. With the alkyne-PAPs, chymotrypsin sticks to its reported selectivity. The different labeling behavior of alkyne- and biotin-PAPs demonstrate that not

only the warhead and the recognition element determine whether a probe binds to an enzyme. Features like probe size, hydrophobicity or side chain flexibility may play a role. Without crystal structures, we can not offer any reliable explanation. But we reason that as long as it is selective, an ABP may turn out to have a recognition element deviating from preconceived expectations.

5.1.6 Labeling of endogenous targets

After demonstrating that PAPs fulfill all requirements for ABPs, we set out to investigate their applicability for endogenous targets in more natural environments. Therefore, rat pancreas tissue and a human mast cell line (HMC-1) were chosen because they are known to contain high concentrations of serine proteases [111]. Rat pancreas was used as lysed tissue in which the zymogens needed to be activated by enteropeptidase (see figure 3.1) [111]. The mast cells were available as a cell line which were also lysed prior to labeling.

5.1.6.1 Labeling in activated rat pancreas and mast cell lysate

In a first gel-based screen, all biotin-PAPs were tested for selective activity-based labeling in activated rat pancreas (aRPL, figure 5.4 B) and human mast cell (HMC-1) lysate (figure 5.4 A). In aRPL, all three probes holding a P1 lysine reacted intensely, yielding one (biotinK- and biotinAAK-PAP) or two protein bands (biotinAK-PAP). The labeling is very selective, as no other labeled proteins are visible.
While proceeding with protein enrichment of the target bands, we also tested the alkyne-PAPs in aRPL (figure 5.4 C). That way, we showed that these probes react with the same targets as judged by the molecular weight of the labeled bands. Additionally, we can show that these reactions are entirely activity-dependent as they do not take place without activation by enteropeptidase nor upon inhibition by an active-site inhibitor. Both basic probes afforded two bands that were suppressed by treatment with 1 mM DCI. AlkyneF-PAP labeled one band that could be outcompeted by DAP22c, whereas the faint band yielded by alkyneV-PAP was DFP-inhibitable.
Biotin-PAPs also yielded intense and broad protein bands in HMC-1 lysate. This labeling is activity-dependent as their intensity is reduced when pre-incubated with 1 mM DFP as active-site inhibitor. The fact that the bands do not completely disap-

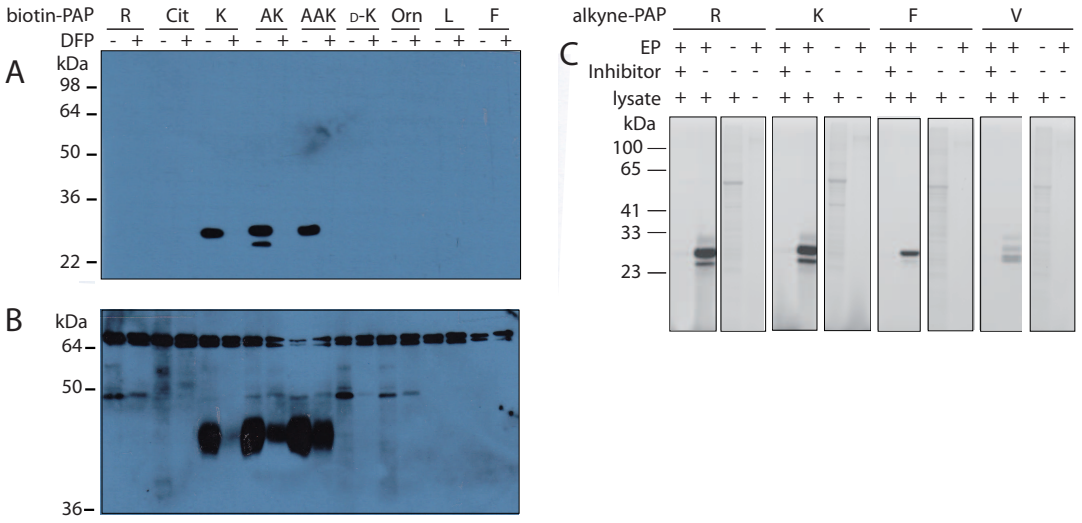


Figure 5.4: **Labeling of endogenous targets with different PAPs.** biotin-PAPs were used at 10 μM, alkyne-PAPs at 100 μM, capital letters indicate the amino acids in the recognition elements; structures see scheme 5.4A-C. **A:** Activated rat pancreas lysate (aRPL) labeled with biotinylated PAPs, preblocking was done with 1 mM DFP. **B:** Human mast cell (HMC-1) lysate, treated as in A. **C:** Activated rat pancreas lysate labeled with alkyne-PAPs. Active site blocking was performed with 1 mM DCI for alkyneR- and alkyneK-PAP, 100 μM DAP22c for alkyneF-PAP and 1 mM DFP for alkyneV-PAP. EP: enteropeptidase.

pear after pre-blocking can be due to incomplete inactivation or off-target binding independent from activity. Some basic biotin-PAPs label a band of approximately 48 kDa. This seemingly selective and activity-based labeling (especially in the case of (D)-lysine) could not be reproduced (see figure 6.3.1). Although this seemed specific and activity-dependent for (D)-lysine, in other blots these bands could not be reproduced and were therefore not considered for further analysis. The bands at approximately 80 kDa are endogenously biotinylated proteins which serve as control for equal protein loading.

5.1.7 PAPs as tools for protein enrichment and identification

In the previous section we demonstrated that biotinK-PAP labels distinct target enzymes in both aRPL and HMC-1 lysate. With these results at hand, we aimed to enrich these proteins in order to identify them using LC-MS according to the procedure sketched in figure 5.5. The biotin handle within the probe should selectively

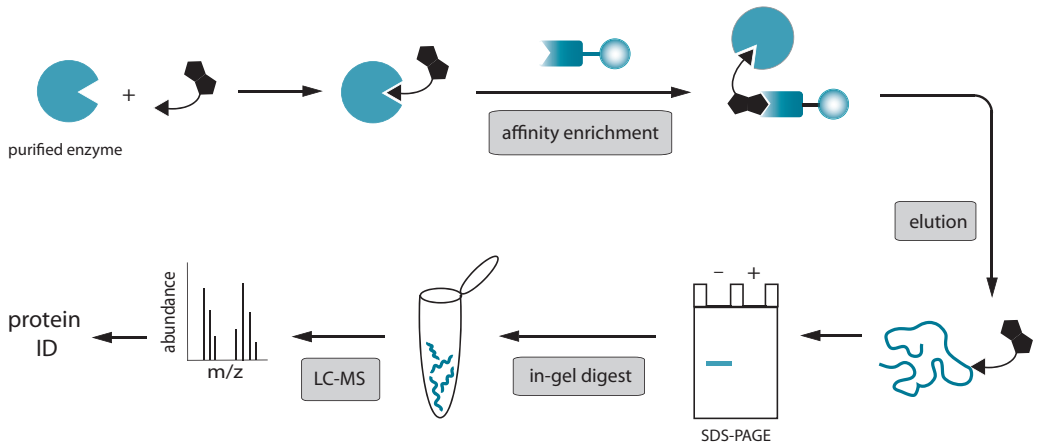


Figure 5.5: **Strategy for target enrichment and subsequent identification using PAPs.** The sample (e.g. activated pancreas lysate) was incubated with biotinK-PAP to form a covalent bond. Subsequently, the labeled proteins were affinity-enriched using streptavidin-coated agarose beads. For elution, the beads were heated in biotin-containing buffer and the eluate was separated via SDS-PAGE. The bands were excised from the gel, and prepared for protein identification via LC-MS.

and tightly bind to streptavidin. The latter being immobilized on agarose beads will consequently retain the probe-bound enzyme onto the beads during several washing steps to clear off any unspecifically bound molecules. Subsequent elution using heat should yield the pure targets that, after digestion into peptides, can be analyzed via LC-MS.

Using this approach, we enriched the proteins labeled by biotinK-PAP from aRPL. Each step was monitored on a biotin blot which clearly shows that all proteins labeled by an ABP are efficiently bound and subsequently eluted from the beads (figure 5.6 A). Interestingly, one additional band around 10 kDA was detected. We reasoned that it was due to on-bead digestion of the captured proteins by leftover proteolytic activity in the lysate despite addition of a protease inhibitor cocktail.

Separation using an SDS-gel and subsequent coomassie staining showed a clean enrichment result: there was only one band present which had previously been shown in the biotin blot (figure 5.6). In contrast, the supernatant was loaded with proteins which had not bound to the streptavidin beads. The enriched bands were excised from the gel and prepared for peptide identification via LC-MS. Table 5.1 lists three trypsins which were identified from the excised gel band. Their molecular weight matches their position in the gel. All other proteins found (listed in the

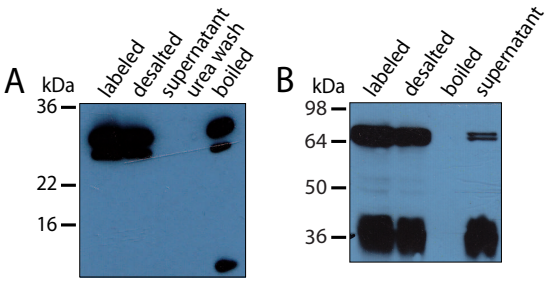


Figure 5.6: **Enrichment of endogenous proteases using biotinK-PAP.** The lysates were labeled using 10 μM biotinK-PAP for 1 h, followed by a desalting step to remove unbound probe. Protease inhibitor cocktail and 50 μM EDTA was added and the labeled protein was enriched for 2 h using streptavidin-coated beads. After removal of the supernatant and washing of the beads with 4 M urea, bound protein was eluted by "boiling" for 10 min to 95°C. A sample of each step was loaded on an SDS-gel and analyzed by biotin blot. **A:** Activated rat pancreas lysate. **B:** human mast cell lysate.

appendix) are either common contaminants introduced during sample preparation (keratins, bovine casein) or can not be identified with high certainty (99% identification probability or higher). This proves that our biotinK-PAP-based enrichment is highly specific and pure. Disappointingly, the identified peptides do not include the labeling site. According to the postulated mechanism in figure 3.2 A, the PA warhead should bind to the active site serine. Detection problems with inhibitor-modified peptides are common, mainly for two reasons: first, it is one peptide with a single modification which needs to be detected which requires a sufficiently high concentration and ionization. Second, biotin and peptide bonds within the ABP tend to fragment so that many different ions are detected for the same peptide. That way, the signal intensity drops to noise level and makes reliable mass assignment impossible. We therefore can not provide ultimate proof for the binding site.

The same approach for target enrichment using biotinK-PAP in HMC-1 lysate was less successful. Fig. 5.6 B shows that despite the intensely labeled band at 36 kDa, the targets remained in the supernatant over the streptavidin-coated beads. To overcome this, we tested a number of modification of the protocol which shall be described here.

1) Using an extended linker: the putative target β-tryptase is a homotetramer which forms a central tunnel. Each of the units has its own active site oriented towards the tunnel [96]. The distance between one of these active sites and the protein surface was calculated to be around 40 Å. We speculated that despite efficient labeling of the target, the probes' biotin handle might not protrude sufficiently from the protein

Table 5.1: **Enriched and identified trypsin from activated rat pancreas lysate (aRPL).** Labeled proteins in aRPL were enriched on streptavidin-coated beads using the biotin handle of the ABP. The eluate was separated via SDS-PAGE, in-gel digested with glutamyl endopeptidase C and analyzed via LC-MS. Listed are the number of unique peptides and the sequence coverage (in brackets) of all trypsin targets of biotinK-PAP identified from aRPL. A list with all identified proteins (99% identification probability or higher) is given in the appendix.

Protein name	Molecular weight	Accession number	Number of unique peptides	Sequence coverage	Protein identification probability
Anionic trypsin-1	26 kDa	TRY1_RAT	5	27%	100%
Anionic trypsin-2	26 kDa	TRY2_RAT	3	15%	100%
Trypsin V-A	27 kDa	TRYA_RAT	2	13%	100%

pore and therefore be unreachable for the streptavidin on the beads. Admittedly, denaturation using urea should disrupt the tetramer and expose the bound probe to the solvent. However, the unfolding might have been incomplete. Taking advantage of the simple and fast PAP synthesis, we considered it worth trying to extend the linker between the recognition element and the biotin handle. Two diethylene glycol units were chosen for this purpose because together they span the required length and additionally made the probe more hydrophilic in comparison to the previously used spacer. Our modified probe, named biotinPegK-PAP (see scheme 5.4 C), labeled the target in HMC-1 lysate as desired. However, it was not more potent in enriching the target than biotinK-PAP.

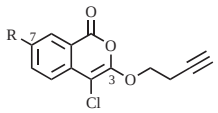
2) Use of a His-tag as alternative detection method. Two PAPs were synthesized holding a His-tag either in addition or instead of the biotin as reporter tag (scheme 5.4D and E). This would enable detection of the labeled targets via an anti-His-tag antibody (Roche, Germany) and pulldown using a Ni-NTA column. Disappointingly, this probe failed to react with the target protein altogether.

3) Altered sample preparation: a) different lysis buffers (pH values 6, 7.4, and 8; the buffering agents HEPES, MES and Tris-HCl as well as the addition of the glycosaminoglycan heparin as stabilizing agent for β-tryptase); b) alternative cell lysis methods (detergent-versus glass beads-mediated lysis); c) enrichment using streptavidin-coated beads from different providers - none of which succeeded in pulling down the labeled proteins.

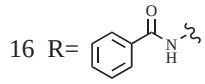
Apparently, an unknown factor in the HMC-1 lysate counteracts the enrichment. But the efficient pulldown from aRPL shows that this problem is not intrinsic of biotinK-PAP and proves that our PAPs are well-suited for protein enrichment.

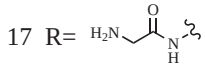
5.2 4-Chloro-isocoumarins as ABPs for serine proteases

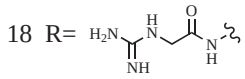
ICs had been investigated as ABPs for different serine hydrolases before [4, 67, 136]. However, they were biotinylated on the 7-position of the two-membered ring. We set out to diversify the selectivity of IC probes by attaching either an recognition element or an alkyne handle in the 3- and 7-position. With this small library at hand (see scheme 5.6), we hoped to broaden the applicability to different targets and refine the selectivity of this ABP type.

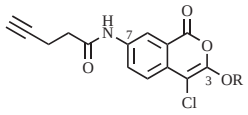


12 R= NO_2
13 R= H
14 R= NH_2

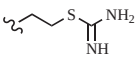








5 R= Me



Scheme 5.6: **Overview of all 4-chloro-isocoumarins (ICs) used for *in vitro* labeling.** ICs holding the alkyne handle in the 3-position of the two-membered ring (numbers 12 - 18) on the left and ICs holding the alkyne substituent in position 7 (numbers 5 - 8) on the right.

5.2.1 Proof of the enhanced IC concept using purified proteases

5.2.1.1 Probe selectivity and activity-dependence

As a proof of concept analogous to the tests performed for PAPs in section 5.1.5, the IC-library was tested for its reactivity with purified serine proteases of different P1 preferences which afforded figure 5.7.
IC 13 reacted with all enzymes in an activity-dependent manner, probably because

of the absence of a 7-substituent which would make the probe selective. Without a 7-recognition element, IC 13 has a structure very similar to DCI, a broadband inhibitor for virtually all types of serine proteases [51].
Cathepsin G reacted with ICs holding small to medium size hydrophobic substituents at either position (probes 5, 7, 14 and 16) which is in accordance with its known chymotrypsin-like activity. In contrast to our expectations, it did not display any trypsin-like activity as it did not react with ICs 8, 17 and 18.
Pancreatic elastase (PE) was also labeled by probes with small and medium-sized hydophobic recognition elements. In addition, IC 18 with its bulky, charged arginine mimic yielded a strong band. But most outstanding for PE is that none of the bands is completely inhibited away, which can have two potential reasons: one could be that the probes bind to residues outside the active side, which would disqualify them as ABPs for this type of enzyme. However, we consider the second reason to be more likely: DCI has repeatedly been shown to hydrolyze from the active site, which results in reactivation of the enzyme [51, 128]. The probe can therefore react with previously inhibited enzyme and result in a weaker, but clearly visible band.
Trypsin and urokinase plasminogen activator (uPA) show labeling patterns that largely resemble each other. This is not surprising as they are known to mainly recognize big, basic, charged substituents in both positions of the IC core. Like PE, trypsin activity was not fully inhibited by pre-blocking which we again attribute to the hydrolysis of DCI from the active center.
Chymotrypsin reacted rather promiscuously. We hypothesized that the hydrophobic alkyne was responsible for this: it could either act as recognition element, or undergo an undesired reaction, for example with a cysteine residue [39]. To test the role the alkyne might play in the enzyme recognition, we modified the order of the labeling protocol: either the standard procedure, in which the enzyme first reacts with the probe and the fluorophore is subsequentially attached by Cu(I)-catalyzed click chemistry; or alternatively performing the click chemistry prior to the reaction with the IC. The second way, we reasoned, the pre-clicking would make the alkyne unavailable due to transformation into a triazole with a bulky fluorophore substituent. We picked probes 8 and 18 to test this because they both have comparatively big, charged recognition elements as 7- or 3-substituents that should be unfavourable for chymotrypsin. IC 16 was chosen as representative probe for hydrophobic recognition elements which should react with the same potency in both

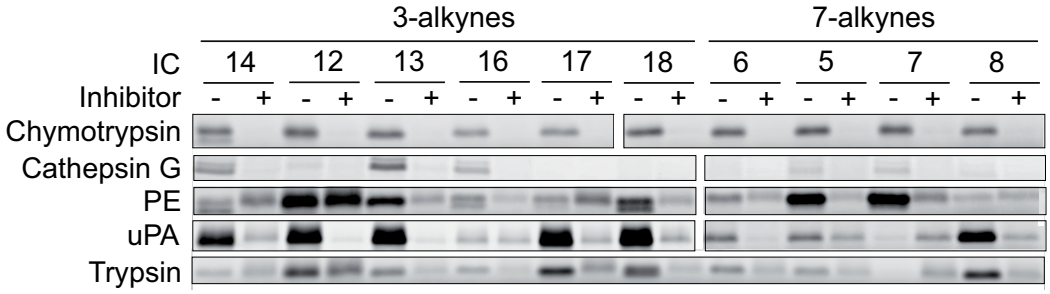


Figure 5.7: **Labeling of purified enzymes with different P1 selectivities using 4-chloro-isocoumarins (ICs)**. 100 ng enzyme of purified enzyme per lane were incubated with (+) or without (-) an active site-directed inhibitor (100 μM DAP22c for chymotrypsin, 1 mM PMSF for cathepsin G, 100 μM DFP for uPA and 1 mM dichloro-isocoumarin for trypsin and elastase) before labeling with 2 μM of the indicated IC. The enzyme-probe complexes were coupled to a fluorophore by Cu(I)-catalyzed click chemistry and detected using fluorescence scanning.

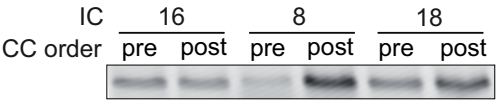


Figure 5.8: **Does the alkyne substituent serve as recognition element?** 100 ng of purified chymotrypsin per lane were labeled with the indicated ICs. The standard protocol for Cu(I)-catalyzed click chemistry (CC) also used in figure 5.7 ("post" for CC after reaction with the enzyme) was compared to CC before reaction with the enzyme ("pre").

orders in case our hypothesis held true. The result is shown in figure 5.8. For IC 16 and 8, it did hold true. In contrast, probe 18 labeled the enzyme with nearly the same efficiency in both protocols. We conclude that not only the position but also both size and charge of the substituents play a role for recognition by an enzyme. In the PAPs experiment we observed a similar pattern when labeling chymotrypsin (figure 5.1). Further investigation would require more probes which only slightly differ from each other to systematically find out the crucial functions for recognition by chymotrypsin. An extended peptide chain including P2 to P4 residues would probably help to direct these ABPs specifically to chymotrypsin [98].
Overall, our ICs mainly label the selected serine proteases according to the enzyme's expected selectivity and depending on the structure of the 3- and 7-substituents. Our library provides a good start for the design of more selective yet potent ICs as ABPs for serine proteases.

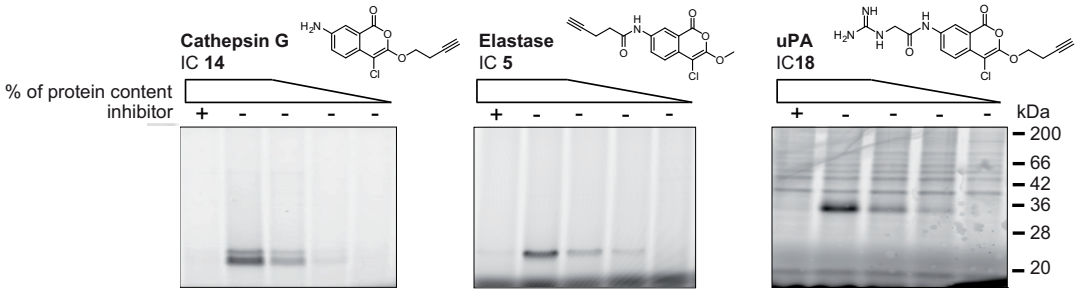


Figure 5.9: **ICs label their targets sensitively in complex proteome background.** Three proteases that showed strong and activity-based labeling by a certain IC in figure 5.7 were spiked in purified form at decreasing concentrations into a mammalian cell lysate (EL4 mouse lymphoma, human cathepsin G and uPA at 0.7%, 0.2%, 0.03% and 0.007% of total protein content, and 0.2%, 0.03%, 0.007% and 0.001% for pancreatic elastase (PE).) They were labeled with 2 μM of the indicated probe. Detection took place by Cu(I)-catalyzed click chemistry using a fluorophore-azide. + and - indicate the presence or absence of an active site-directed inhibitor (1 mM PMSF for cathepsin G and PE, 100 μM DFP).

5.2.1.2 Probe sensitivity

After demonstrating the selective and activity-based reaction of ICs with purified enzymes, we proceeded to determine the detection limit and the potential of the ABPs to react with off-targets within a mammalian proteome background. A low detection limit is important for live applications of ABPs, because it enables the visualization of very low concentrations of active enzyme within a tissue which is often the case when expressed endogenously. Also, low amounts of a probe are less likely to cause toxic effects in tissues or animal models.

Three protease-probe pairs were picked according to their labeling intensities (absolute and in comparison to the other enzymes tested with the same probe) as well as activity dependence: IC 5 for PE, IC 14 for cathepsin G and IC 18 for uPA. Each enzyme was spiked into a mammalian cell lysate (EL4 mouse lymphoma at 1 mg/mL) at decreasing concentrations: 0.2%, 0.03%, 0.007% and 0.001% of total protein content for PE and 0.7%, 0.2% 0.03% and 0.007% for human cathepsin G and uPA. PE was detected down to a concentration as low as 0.007% (which corresponds to 40 fmol), whereas cathepsin G and uPA could be made visible down to 0.03% (figure 5.9). We consider this result indicative for the good selectivity and sensitivity of the ICs for their protease targets.

5.2.2 Labeling endogenous targets

We had shown that ICs work as potent and selective ABPs for serine proteases. In a next step we chose rat kidney and liver lysate to test labeling of endogenously expressed enzymes.
In rat liver lysate, probes 13, 14 and 16 yielded distinct labeling patterns with little background (figure 5.10). For IC 13, this was rather surprising because in figure 5.7, we had observed its promiscuous reactivity with all purified proteases and therefore expected it to label more enzymes or at least create a lot of background. We think that the abundance of other proteases is too low so the probe is reacted away by the enzyme(s) in the visible band. IC 16 was most selective, labeling one band and almost no background. Both probes 13 and 16 could be outcompeted by TPCK, which hints at a chymotrypsin-like substrate preference. This is in contrast to IC 14, which generated the highest background labeling and could not be blocked by TPCK. Activity inhibition by the more broadband inhibitor PMSF only confirmed that the target was a serine protease but could not give more detailed information about the P1 specificity. Rat kidney lysate generally created a higher background than liver lysate and ICs 5 and 14 were the only probes affording distinct and activity-dependent labeling (figure 5.10). IC 14 gave rise to three bands: two around 65 kDa whose inhibitability by both PMSF and TLCK hints at a trypsin-like activity. The band at around 27 kDa could only be suppressed by PMSF, speaking for a general serine hydrolase of undefined specificity. IC 5 weakly labeled a protein with tryptic activity of about 50 kDa. Interestingly and similarly to our findings in rat liver lysate (figure 5.10 A), probe 13 neither labeled any specific targets nor led to much background although it had been very reactive to all purified proteases (figure 5.7). In sum, we show that the selectivity of the IC scaffold for different target serine proteases can be tuned by substituents in the 3- or 7-position. Further investigations would include enrichment and identification of the targets labeled in figure 5.10 using the same procedure as for the biotin-PAPs in section 5.1.6.

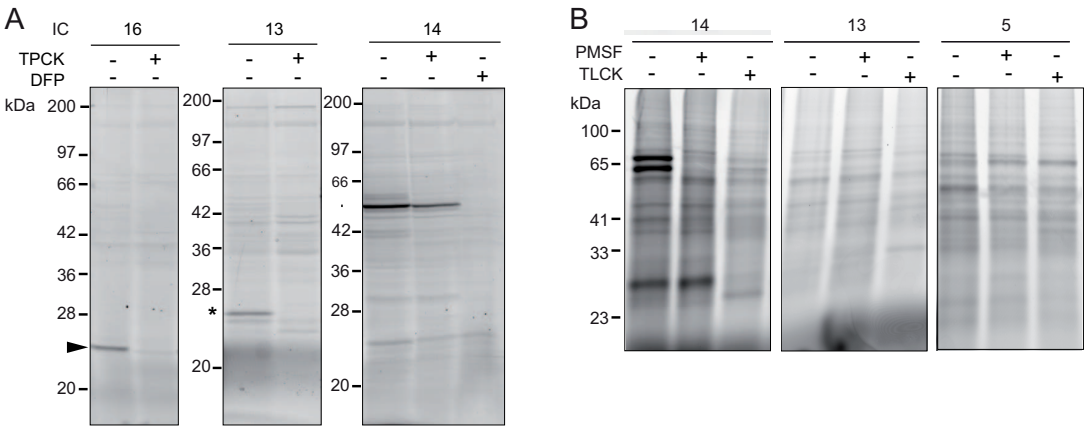


Figure 5.10: **Labeling of endogenous targets in lysates of rat liver and kidney.** The lysates were pre-blocked with the indicated active-site inhibitor (+) or DMSO (-) and subsequently labeled with the indicated IC for 30 min each. Detection took place by Cu(I)-catalyzed click chemistry using a fluorophore-azide. **A:** Rat liver lysate. **B:** Rat kidney lysate.

5.3 *Toxoplasma gondii*

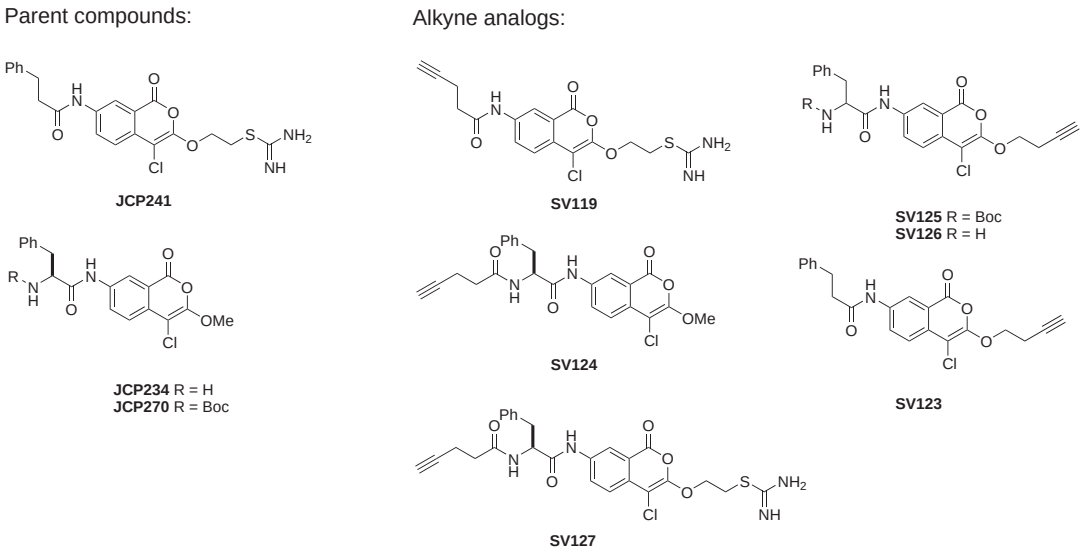
In 2011, Bogyo et al. published a library screen for compounds that inhibit the invasion of *T. gondii* parasites into cultured host cells [50]. Together with other studies, these findings suggest that serine proteases are involved in the infection process [27]. Our own data show that compounds based on the IC scaffold bind to soluble and intramembrane serine proteases [128]. Based on these findings, we hypothesized that alkynylated versions of the inhibitors found by the Bogyo lab could be used as ABPs for two-step labeling to identify the serine proteases involved in the infection process. In this section, we present the results that were obtained in a preliminary study.

5.3.1 Probe structures

A set of six alkynylated ICs was synthesized, structurally very close to three anti-invasion hits identified by Hall et al. [50]. They all carry a phenylmethyl function as part of their 7-substituents. We hypothesized the aryl moiety to be important for selectivity because it is a common feature of all hits from the library screen. In figure 5.7, all IC probes used in our *T. gondii* experiments and their parent compounds are shown.

5.3.2 Quantitative analysis of parasite invasion

At the heart of the *T. gondii* case study stands a series of assays for the quantification of attached and invaded parasites to host cells in culture. The parasites were first incubated with the compounds and subsequently added to the cell culture. Reduced numbers per counted field indicate that the respective compound counteracts infection. As to be seen in figure 5.11 left, *T. gondii* that had fully invaded into the hosts autofluoresce red. Parasites which had attached but not invaded were reached by the antibody, visible as yellow spots due to overlaid green antibody and red autofluorescence.
Unfortunately, many images were very noisy making especially the counting of FITC-stained spots unreliable. Therefore we only took into consideration the total parasite numbers. The three compounds SV 119, 124 and 126 caused an invasion defect, their normalized numbers are shown in a bar graph (figure 5.11 right). Clearly,



Scheme 5.7: **Overview of 4-chloro-isocoumarins (ICs) used for *T. gondii* assays** The three parent compounds (JCP234, JCP241, JCP270) prevented *T. gondii* parasites from invading a host cell culture of HFFs [50]. The alkyne analogs SV119 and SV123-SV127 were tested for the same effect, a prerequisite for their use as activity-based probes for serine proteases involved in the invasion process.

the numbers of attached parasites decrease with increased compound dosage. All probes have the same anti invasion effect without any significant differences which we assign to the close structural resemblance to their parent compounds. However, we can not exclude a general toxicity of the probes for the parasites and are aware that further experiments are needed to elucidate this. Additionally, the test should be optimized to deliver results with less background. Taken together, we still consider our probes useful for the identification of serine proteases involved in *T. gondii* invasion into host cells.

5.3.3 Gel-based competition assay in *T. gondii* lysate

To test whether ICs SV119-SV127 react with any active serine hydrolase of *T. gondii*, we added them to lysed parasites and subsequently labeled the mixture with FP-rhodamine. The IC probes were expected to outcompete the FP-rhodamine labeling of selected bands, resulting in the absence of individual fluorescent bands on an SDS-gel shown in figure 5.12.

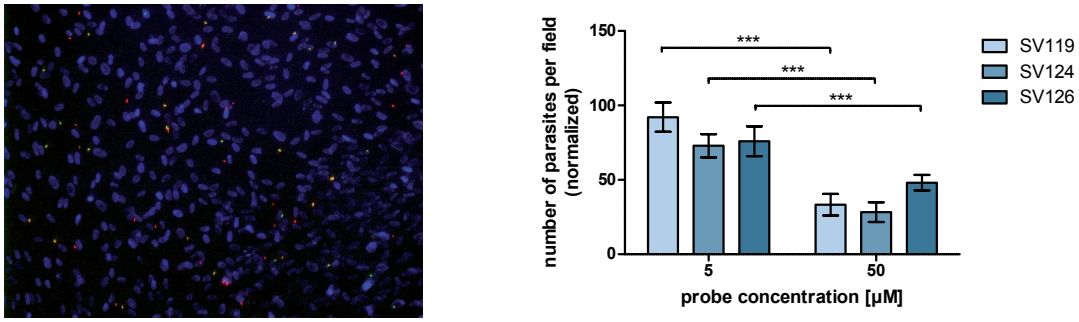


Figure 5.11: **Host cell invasion by *T. gondii*.** Human foreskin fibroblasts were cultured to confluence and then infected with an autofluorescent *T. gondii* strain which had been pretreated with one of the compounds. The culture was fixed and subsequently stained with an anti *T. gondii* antibody and a FITC-labeled secondary antibody. **Left:** Fluorescence image of the invaded cells. Parasites which had invaded the host cells are seen in red, those which had attached but remained outside cells are seen in yellow due to overlayed red and green fluorescence. **Right:** *T. gondii* numbers of the different treatments counted with the help of an automized procedure in ImageJ . Due to high noise, only red spots reflecting total parasite numbers delivered reliable numbers.

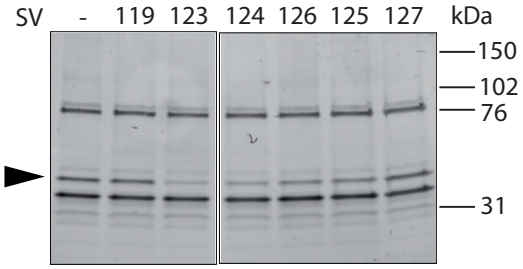


Figure 5.12: **Gel-based competition assay with alkynylated 4-chloro-isocoumarins (ICs) against FP-rhodamine.** Samples of *T. gondii* lysate were incubated with the indicated IC and subsequently with FP-rhodamine. The protein bands were detected using a fluorescence scanner.

SV 123 and 124 hit a band of approximately 40 kDa (see arrowhead in figure 5.12), proving that there is a protease for which these two compounds can be used as ABPs for. However, cell lysis has a strong impact on the biochemical conditions any potential target is exposed to. For example, disruption of cellular compartments brings together molecules that would never come into contact in the live system. Consequently, protease reactivity can be modified and thus the labeled lysate does not necessarily reflect the situation *in vivo*. We therefore consider the results from this experiment as preliminary and set out for a competition assay and the invasion experiments in culture described in the following sections.

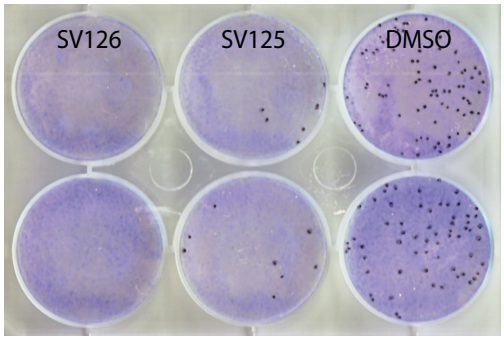


Figure 5.13: **Plaque assay for determination of infectivity.** 200 *T. gondii* parasites per well were treated with the indicated probe, washed and added to confluent HFFs in a six well-plate. The culture was left undisturbed for six days to allow for several cycles of infection. This leads to the formation of small plaques which were Coomassie-stained and marked with a pen (black dots).

5.3.4 Determination of *T. gondii* infectivity

In order to support the data from the invasion assays in the previous section, we performed a plaque assay. 200 parasites were first incubated with a compound and then added to a well of confluent host cells. The cultures were left undisturbed for 6 days. Infected cells would burst open to pass on the parasites to their close neighbors, creating small plaques after a number of cycles. We reasoned that if the compounds inhibit invasion, less plaques would be generated. As shown in figure 5.13, all compounds largely reduced the number of plaques compared to the DMSO controls. The probes obviously harm the parasites' infectivity. However, this assay does not rule out their potential to be unspecifically toxic to *T. gondii*. These findings should encourage future research about the proteases involved in *T. gondii* infection of host cells with the help of IC-based ABPs.

6 Conclusion

The goal of this work was the synthesis and characterization of PAPs and ICs as ABPs for serine proteases. That way, we aimed to contribute a research tool to the scientific community that will allow further elucidation of the functions of serine proteases.

We synthesized a small library of either biotinylated or alkynylated PAPs and showed that the biotinylated versions can be made entirely on solid support. The alkynylated version were made in solution to enable the production of greater amounts for potential use *in viso*. We could demonstrate that PAPs selectively react with serine proteases in an activity-based manner. The selectivity can be tuned to a great degree with different REs so that they are directed to certain serine proteases and away from others. We found out that despite carrying the same RE, the biotinylated PAPs show slightly different labeling patterns than the truncated versions. Obviously, also the overall probe size and structure influence this selectivity. Our results also give evidence that the alkynylated PAPs are compatible with Cu(I)-catalyzed click chemistry. We could furthermore demonstrate that one library member, biotinK-PAP, selectively labels endogenous trypsin from rat pancreas lysate which was subsequently enriched with the help of the ABP and identified using LC-MS and a database search. This application illustrates that PAPs are compatible with MS-based target identification.

Second, we evaluated a set of ICs with enhanced features. Although this type of ABPs had been established as serine protease-targeting ABPs in the beginning of the 1990s [67], they had been used, to our knowledge, only in very few studies [4, 136]. The reported ICs all carried a biotinylated substituent at the 7-position of

the two-membered ring. In this work, we tested a library of in-house made ICs with two additional features: first, both positions 7 and 3 of the core structure were made avaiable as recognition elements. Second, the probes carry an alkyne moiety at the opposite position, respectively. To charatcerise these probes, we show that they selectively bind serine proteases in an activity-dependent way. Their selectivity depends on the two variable substituents at the 3- and the 7-position. We demonstrate that these ABPs can be used for Cu(I)-catalyzed CC, as we affixed a TAMRA fluorophore. This feature broadens their applicability through the use of different reporter tags. With this study, we were able to demonstrate that even established compounds can be further developed and refined for more distinguished applications.
In this chapter, the results of the developed ABPs described in the results and discussion part shall be put into persepective of the current state of research.

6.1 The model systems used to study serine proteases

Serine proteases are a large group of enzymes which play crucial roles in diverse functions of life. Most of them are expressed as inactive precursors, so-called zymogens [70]. The advantage of this expression form is that they can be stored and quickly activated upon need. To prevent the activity from getting out of hand after this transformation, the cells or tissues hold antagonists ready, ensuring a short time window of action and providing several points of regulation for their function.
For the proof of concept, we used endogenously expressed serine proteases from model systems such as cultured mast cells or different rat organ lysates because they reflect the broad range of this type of soluble enzymes existing in mammals. The first system is rat pancreas, pieces of which were lysed to set free its digestive proteases. *In vivo*, they must be released at high amounts to efficiently digest the foodstuff arriving in the duodenum which makes them a showcase example for the usefulness of expression as zymogens. Furthermore, they need to be rather insensitive towards their environment in order to cope with changing food compositions from one meal to the next. The three major endopeptidases released from the pancreas together display a wide range of P1 selectivities that in combination with unspecific carboxypeptidases ensures efficient digestion of proteins contained

in food. With trypsin aiming for basic side chains, chymotrypsin for large and PE for small hydrophobic residues, they are an excellent starting point for the evaluation of ABPs with different selectivities. Also from a medical point of view, the pancreas as an organ with its proteolytic capacities is relevant as it is involved in diseases such as pancreatitis [41]. ABPs could be of diagnostic use, as increased levels of pancreatic enzymes in serum of people suffering from abdominal pain have been reported [129]. Therefore, highly specific probes would be beneficial to avoid false positives, because other serine proteases such as thrombin are present in serum also in its healthy state [36].

Our second study object were mast cell tryptases which are both relevant for medical purposes. Despite being stored in their active form, which is an exception compared to the majority of serine proteases, they are kept inactive by the low pH present in the granules they are accumulated in. Activation comes by a change of the surrounding pH upon release into the extracellular matrix. This release takes place as a response to the recognition of an IgE-bound antigen to the Fc receptor at the cellular surface (Figure 3.2). This generally being a healthy inflammatory response [28], an overreaction causes symptoms involving allergic asthma [91].

Third, we prepared liver and kidney lysates to test the activity of potential target enzymes contained in these organs. We were indeed able to demonstrate that also these systems display hydrolytic activity.

There is valid criticism among scientists about the usefulness of *in vitro* and *ex vivo* enzyme activity data [10]. Enzymes which have been isolated from their natural environment may react differently, and potential influences may be left unnoticed. The same is true for so-called spike-in experiments, where isolated enzymes are added at a defined concentration to a proteome such as a cell lysate (as we have done in 5.9). Even when using lysed organs, the environment of the studied proteases changes significantly. For example, the pancreatic acinar cells hold ready inhibitory agents in their cytoplasm that form a secondary barrier to prevent harm from accidentially released and activated proteases: the pancreatic secretory trypsin inhibitor (SPINK1) and a number of unspecific antiproteases such as α-1-antitrypsin, α-1- intertrypsin and α-2-macroglobulin [129]. In our own lab, reduced activity of cysteine cathespins upon cell lysis was found [143]. These factors can be a problem because of two aspects: first, homogenizastion fuses all tissue compartments and therefore brings together all components which would not come into contact under natural

conditions. Second, all the regulated cellular changes occuring during enzyme release are circumvented by the lysis. Although we acknowledge these remarks, our view on the topic is a little milder: the mentioned inhibitors did not prevent the robust and intense labeling as well as the isolation of pancreatic proteases by PAPs which we demonstrate in this work. In general, particularly digestive proteases are released into the duodenum, where the food composition changes regularly. We are therefore confident that are active under a broad range of conditions. For the other systems used here, our argument may be a little weaker, as the involved proteases are usually released into tissues and fluids with defined conditions which may be only poorly mimicked by our buffer systems. However, it is favourable to decrease the complexity of the studied system which means leaving out most influences that would occur in nature. After demonstrating that an ABP does indeed react with its target, the complexity must be increased and a probes' applicability must be proved under more difficult conditions.

6.2 APBs as key tools to study protease activity

As there are more catalytic types of enzymes than proteases, let alone serine proteases, there are also ABPs for many of these enzymes. However, in order to stick to the focus of this work, we restrict ourselves exclusively to ABPs for serine proteases and often compare them to those directed to cysteine proteases.
Depending on the application, ABPs with different levels of selectivitiy are required. There are comparatively broadband probes, a term which shall describe probes that hit many, if not all, enzymes within one catalytic class. In 2000, Cravatt et al. even defined ABPs as small molecules that react with a broad range of active enzymes from a particular class in complex proteomes [34]. The most famous example certainly is the FP warhead which reacts with all hydrolases carrying a serine residue in their catalytic center. Using FP-ABPs in combination with an elaborate MS-based analysis, Cravatt and coworkers identified around 100 enzymes. They beautifully demonstrated for the example of KIAA1363 hydrolase, that mRNA expression levels as determined by cDNA microarrays do not necessarily correlate with the catalytic activity of the expressed enzyme [64]. Almost a decade later, the involvement of KIAA1363 in prostate cancer pathogenesis could be proved using an approach involving ABPs [29].

ABPs with a wide reactivity have a couple of disadvantages. For example, enzymes of low abundance are likely to be missed because the fluorescent singals are too weak for detection or they tend to disappear in the noise level during MS analyses. A more specific probe would enable selective enrichment of a low number of target proteases, avoiding signal saturation by peptides derived from more abundant enzymes. Another drawback of broadband ABPs is that although they give insight into overall proteolytic states of a cell or tissue, the precise role of small enzymatic subsets or even individual targets can not be determined - a problem that can be overcome with highly selective ABPs.
The ABPs developed in this thesis have a medium degree of selectivity. That means, the do not only distinguish serine proteases from others enzymes, but also differentiate between subsets within this catalytic class. The selectivity could be tuned further by refining the different substituents we regard as recognition elements. For PAPs, we consider this tunability limited, mainly becausehigher selectivity often come with lower reactivity. With the PA warhead already not being overly reactive, target binding would probably become inefficient. For ICs, we see more potential because their core already displays an elevated reactivity compared to PAPs.
Despite the existence of ABPs for many proteases and several types of downstream analyses, and despite the methodology being conceptually established, many target serine proteases have not yet been hit. The development of ABPs which are selective for serine proteases and especially for certain subtypes within this class is more difficult than for cysteine proteases, mainly because of the reduced reactivity of the hydroxyl moiety in comparison to the sulfhydryl group [46]. Here, the structural and functional parameters an ABP must fulfill shall be outlined and discussed again. For visualization, we refer to Figure 3.3 in the introduction of this manuscript.

6.2.1 The reactive warhead

The warhead is the first crucial element composing an ABP. It must have some intrinsic reactivity strong enough to bind to residues displaying enhanced nucleophilicity. That way, the binding is restricted to individual residues such as the active site serine in serine proteases; whereas any other hydroxyl moieties on the proteins' surface are avoided [47]. For cysteine side chains, Weerapana et al. demonstrated this phenomenon: IAA, when applied at low concentrations, would bind

only to those cysteines with increased nucleophilicity, e.g. the active site cysteine [132]. In contrast, higher concentrations of IAA can be used to saturate all cysteines independent of their molecular environment - a method widely used as preparation for MS analyses, including this work (section 4.2.9). Apart from the enhanced nucleophilicity, the type of target side chain and therefore for the class of proteases, e.g. serine, cysteine proteases or any other proteolytic class, is decisive for the type of warhead to be chosen. The proteases' catalytic nucleophile is the residue to react with electrophiles. Both reaction partners should match in their hardness (respectively softness). For example, hydroxyl groups, as found in the serine side chain, are hard nucleophiles and therefore require equally hard electrophiles to efficiently interact with. For cysteine proteases, both reaction partners are comparatively soft [46]. In table 3.1, we list all types of warheads that are currently available for serine proteases. Apart from such considerations, the structure of the overall warhead obviously influences the affinity to a given protease, a phenomenon we observed for PAPs and ICs: PE bound to IC 18, which carries a charged arginine mimic as recognition element. In contrast, it ignored biotinR-PAP as well as alkyneR-PAP although they both carry an arginine. Of course, the position of the substituents have a great impact on the affinity, and one should be careful to draw definite conclusions from such observations.

6.2.2 The recognition element

As indicated above, the second element of an ABP is the recognition element which further tunes an ABPs' selectivity. That way, the number of potential targets is reduced from virtually all enzymes of one catalytic class (e.g. the general FP-based ABPs for serine hydrolases) to a subset of targets (e.g. only serine proteases displaying trypsin-like activity). Some approaches use natural inhibitors [18, 117] of which the ABP DCG-04 derived from the natural cysteine protease inhibitor E-64 probably is the most well-known one [44]. In some cases such as the cysteine protease-directed eposuccinates, a recognition element is even obligatory to create sufficient affinity to the enzyme [44]. There are other ways to develop recognition elements, including the use of natural small molecule inhibitors as lead structures. Staub et al. used a library of alkyne-modified β-lactams to target active enzymes in bacteria. One compound bound to ClpP, a serine protease which belongs to the

category of penicillin-binding proteins [117].
Peptides are the most obvious structures to use for this purpose because to a great extent, they resemble any proteases' natural substrates. Despite the fact that proteases all cleave peptide bonds and recognize proteins, they all display different target preferences. A study from the Craik lab even suggests that much more than the sequence, it is the length of the peptide recognition element, as well as the sterics and stability of the leaving group influencing the affinity of an ABP to its target [25]. Therefore, the construction of a recognition element, especially one that sits close to the warhead, should be inspired by the molecular structure around the cleavage. Other structures distant from this bond within the substrate may as well influence the interaction.
For our PAPs, we used the preferred P1 residues for the design of the recognition element, as this position is most important for serine proteases. Moreover, we complemented this set with some noncanonical side chains possessing slightly different properties, such as the non-charged yet basic citrulline and the basic but short ornithine. The reconition elements of the ICs do not resemble peptides but still exhibit substituents that mimic P1 residues.
When assessing the influence of the different recognition elements, we saw our probe design strategy confirmed: both ICs and especially PAPs often reacted according to our expectations based on known cleavage preferences [99]. We could especially confirm the affinity of ICs carrying small 3-substituents such as a methoxy or a 1-bromoethoxy to PE with previous findings made using biotinylated ICs [67, 136]. Yet, a number of unexpected labeling behaviors were also detected. For example, PE reacted with all PAPs holding a P1 lysine although it is known to avoid this side chain in natural substrates. Chymotrypsin reacted in a promiscuous manner as well, as it bound to all tested probes alike (Figures 5.1 and 5.7). So far, we do not have a satisfying explanation for this phenomenon. But it underlines the fact that prediction of binding behaviour always needs to be of a given ABP confirmed experimentally. Unfortunately, these factors render probe design a bit arbitrary.

6.2.3 The reporter tag

Last but not least, the analytical method must be taken into account for the choice of a reporter tag. The most popular moieties used for this purpose are biotin and fluorophores. The former has a number of advantages including heat stability and high affinity to streptavidin and is therefore among the most popular tags used for ABPs [106]. It was used for the initial characterisation of ICs as ABPs [67] and we used it to establish our set of PAPs as well. However, biotin as a tag has some shortcomings: first, endogenously biotinylated proteins can not be distinguished from artificially tagged ones. This can be problematic for profiling of complex proteomes, as these often contain endogenously biotinylated proteins (figure 5.4). Second, biotin tends to fragment in MS and therefore increases the complexity of the resulting spectra. And third, biotinylated probes have poor cell penetration properties which makes them unsuitable for *in situ* and *in vivo* experiments [106].
For these reasons, we created a set of PAPs with modified structures by replacing the tag by a primary alkyne moiety altogether. This group permits the attachment of a reporter tag in a second step using bioorthogonal CC. This reaction relies on the selective fusion of an alkyne with an azide to a triazole ring, aided by Cu(I) ions. Other bioorthogonal reactions classified as CC include the following: Cu-free CC, in which a secondary alkyne is part of an eight-membered ring whose strain promotion drives the formation of the triazole [2]; Bertozzi-Staudinger ligation where an azide and a phosphane react to an aza-ylide which forms a stable amide bond through intramolecular rearrangement [75, 108]; and the Diels-Alder ligation which is a cycloaddition using trans-cyclooctenes and tetrazines [14]. This two-step labeling obviously adds flexibility to the analysis as it is up to the experimentors choice which type of reporter tag to introduce. Therefore, mainly the alkyne-azide cycloaddition has found widespread use [76, 114, 116, 117] after its first introduction to the scientific community by Sharpless and coworkers [103] and refinement in the Cravatt lab [115].
It should be noted that the distinction between the recognition element and the reporter tag can oversimplify reality. For example, an ABP designed for glucocerebrosidase based on its natural inhibitor cyclophellitol showed increased affinity to the enzyme after being appended by a BODIPY, although this group was initially supposed to function as reporter tag only [138]. Our own data provide another ex-

ample: PAPs with the same recognition elements but different reporter tags labeled chymotrypsin disparately. From the alkynylated PAPs, only those with a hydrophobic side chain could bind, whereas it the protease did not differentiate between any of the biotin-PAPs. This underlines that in the end, the entire probe interacts with its target. The individual parts of a probe are categorized mainly to improve our understanding as the main functions we consider them to fulfill are pointed out.

6.2.4 Potency considerations

The potency of PAPs and ICs to react with their respective target enzymes is another quality which ought to be discussed. As a measure for this, we titrated either the probe or the protease concentrations while leaving the other reaction partner constant. That way, we were able to determine the minimal probe concentration required for visualisation.
For ICs, probe 18 is most potent as 0.016 μM detected 100 ng of purified trypsin in buffer, whereas all other ICs tested need at least twice the concentration [46]. Also uPA was labeled most efficiently with IC 18 as demonstrated after being spiked into a complex proteome background at decreasing amounts (Figure 5.3). PAPs turned out a little less potent because at least 0.8 μM biotinK-PAP was the minimal concentration to detect 15 ng of trypsin.
The numbers presented above are difficult to compare by reason of two factors: first, different amounts of enzymes were used. And second, some of the inhibited enzyme tagged with alkynylated probes may not actually be visible because the CC could suffer from limited efficiency and therefore failed to put a tag on the inhibited enzyme. For a direct comparison, we would have had to investigate the potency of our truncated PAPs. But the goal of this work was not to compare the relative affinities of the developed probes but the characterisation of their selective and activity-dependent reaction with the respective targets proteases. These data stilll provide a rough idea about the affinity of our compounds to serine proteases.
The potency data of our PAPs and ICs puts them in line with established ABPs. There are β-lactam-based probes which need to be applied at 3 - 50 μM in lysed bacterial proteome and even at 250 μM for intact bacteria [117]. At the high affinity end of the ladder is BODIPY-conjugated analogue of cyclophellitol, a known covalent inhibitor of the glutamic acid hydrolase glucocerebrosidase [80, 101]. The derived

probe is able to detect recombinant glucocerebrosidase when used at low attomolar amounts [138]. To our knowledge, this ultrahigh affinity has not been reached to date by protease-directed ABPs.

6.2.5 Synthetic considerations

Apart from the functional criteria to be fulfilled by an ABP, they must be synthetically available, favourably in a simple and inexpensive manner. The potential for diversification of the probes is an aspect crucial for structural refinement of identified lead compounds. In this work, we present the first serine protease-directed set of ABPs whose synthesis, to our knowledge, entirely takes place on solid support. ABPs for example for cysteine proteases have been synthesized with this method by Bogyo and coworkers [69, 68, 126].
SPPS is a synthetic method that enables both the generation of compounds at large scale and their diversification. Initially invented by Merrifield in the early 1960s, it led to a Nobel Prize in chemistry in 1984. SPPS has some advantages compared to solution phase chemistry: it is simple, does not require the workup and purification of intermediates such as leftover reagents. Side products can simply be washed out without loss of compound. After the completion of each step, the dry, resin-bound compounds can be stored until further proceeding the synthesis. Furthermore, it allows to incorporate unnatural amino acids which can add potential for extra diversity over *in vitro* expression [86]. Due to its iterative nature, the process can easily be scaled up and automized with the help of synthesis robots. Unfortunately, the synthetic progress is hard to monitor under automized conditions. Full coupling is usually ensured by the application of excess reagents.
SPPS relies on so-called building blocks - compounds which are coupled to the growing molecular chain. A wide range of such building blocks are commercially available (e.g. Creosalus), and their variety is growing. In the standard procedure which was also used to make the biotin-PAPs within frame of this thesis, the Fmoc group is first deprotected followed by the HOBt/DIC-mediated coupling of a building block. Depending on the reaction conditions, the cheap HOBt may be replaced by other uronium reagents such as HBTU or the highly reactive HATU (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate). Alternatives for DIC are carbodiimides with different sub-

stituents such as EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) or DCC (dicyclohexylcarbodiimide) depending on the solubilitiy properties of the byproducts.
The reactive PA warhead was coupled at last. We consider this succession advantageous because it avoids potential degradation or side reactions caused by an undesired nucleophilic attack of the warhead during following coupling reactions. This synthetic order had proven successful in a previous study [69].
SPPS has afforded the synthesis of many peptides with different properties. Some outstanding examples certainly include the total synthesis of fully active enzymes such as Rnase A [45] or HIV-1 protease [87, 121]. Many more challenges that are both impressive and interesting but would be out of the focus of this work are summarized in the cited articles [63, 88].
Despite all its advantages, SPPS can only be scaled up to a very limited extent. In order to be prepared for potential mouse injections, we synthesized the alkynylated PAPs in solution.

6.3 Future applications and challenges for the developed probes

We have characterized the two types of ABPs central to this work in their labeling selectivity in complex proteome systems. For biotinylated PAPs, we have successfully shown that they can islolate their target proteins, for ICs this remains to be accomplished. But also beyond that, both types of probes have potential for further use. Here, we briefly outline what follow-up projects could look like.
A potential diagnostic systems for the detection of certain diseases could for example be constructed as a "small molecule microarray" inspired by Wu et al. [141] (figure 6.1). A library of biotinylated PAPs would be immobilized on a streptavidin-coated surface, which would then be exposed to fluorescently labeled proteomes of cells or tissues from different origins. A similar architecture could be designed for the alkynylated probes, both PAPs and ICs, where the immobilization would be performed using an azide-functionalized surface. Alternatively, synthesized and pruified PAPs could be printed on polylysine coated glass surfaces as demonstrated by Ellman and coworkers [43]. The most elegant version would be their direct

synthesis on microarrays functionally coated to enable the attachment of primary amines [123]. Independent of the immobilization method, such an array would then be exposed to proteolytically active samples. Wherever proteases would bind to an immobilized ABP, a fluorescent signal could be read out. The position of the fluorescent spot would give information about the preferred recognition element, and the whole array would inform about the general activity of serine proteases within the sample. Comparison with known characteristic activity fingerprints of purified proteases [43] or of diseased cells stored in a database could then serve as diagnosis about diseases. One such example has already been reported from Yao and coworkers, who showed different fluorescent patterns on their microarray from the different stages of malaria-infected blood cells [140].

We have provided evidence that PAPs can be used as tools for target enrichment

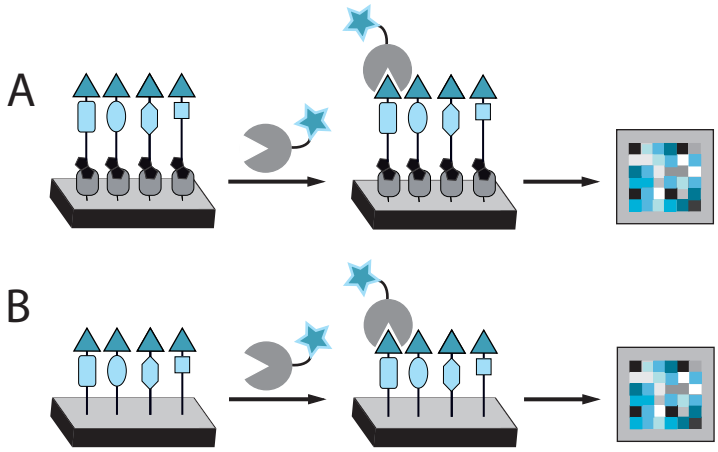


Figure 6.1: **Potential biotin-PAP microarray designs. A:** Immobilized streptavidin on the array serves to bind the biotin-PAPs. The array would then be exposed to a sample of fluorescence-tagged serine proteases (e.g. from a tissue) leading to a characteristic binding pattern. **B:** Instead of streptavidin immobilization, the probes could be directly synthesized on the array. Sample exposure and readout analoguous to A.

from natural sources. However, the enrichment attemps from HMC-1 lysate shows that this procedure may be rather challenging at times, despite reproducible labeling. We hypothesize that the amount of labeled enzyme in HMC-1 cells is too low to be enriched at amounts sufficient to generate a robust signal above noise level in MS analyses. Although this cell line is known to express high amounts of β-tryptase,

it does not reach the levels of the digestive proteases in the pancreas. To make up for this problem, an increased affinity of the PAPs using different or elongated recognition elements as well as unnatural amino acids should be a promising route. However, we are concious that the PAP warhead itself is not highly reactive which may limit the potential for enhancement of affinity. On the other hand, low reactivity can be an advantage in diagnostic use because those probes are less likely to lead to false positives.

Of course we hope that our probes can be used for *in vivo* studies, as this would give much more relevant data about serine protease activities. Although highly toxic compounds such as fluophosphonates have found widespread use in proteomics research, animal studies obviously require nontoxic agents. Concerning PAPs, their closest structural relatives that have been used as ABPs are diphenyl phosphonates which apparently do not show any toxic effect *in vivo* [54]. This came as a surprise because the reaction with a nucleophile sets free a phenol both in diphenyl phosphonates and PAPs that is generally considered to be highly toxic. Taken together, the toxicity of each PAP and IC needs to be carefully evaluated.

Another obstacle is cell permeability. Many probes do not easily penetrate cells, which limits their applicability to lysed systems or extracellular fluids. This is especially true for biotinylated compounds and therefore excludes all biotin-PAPs. The truncated ABPs however could be appended with permeability-enhancing moities. Fluorophores such as BODIPY for example have been reported to have such an effect [106]. Also the plain attachment of hydrophobic substituents can improve cellular uptake as reported for cysteine protease-directed AOMK probes [68]. However, increased hydrophobicity may easily lead to decreased solubility in aqueous physiological fluids. To overcome such problems, researchers have integrated cell penetrating peptides into their probes which mediate the probes membrane crossing. These peptides either consist of positively charged residues which easily adhere to the cell surface or hydrophobic side chains facilitating the interaction with the fatty acid chains within the memnbrame bilayers.[37, 53] The advantage of such penetrating enhancers would be again their ease of synthesis on solid support.

List of Abbreviations

A	alanine
ABP	activity-based probe
ABPP	activity-based protein profiling
AGC	automatic gain control
AIDS	acquired immunodeficiency syndrome
aRPL	activated rat pancreas lysate
BSA	bovine serum albumin
CC	Cu(I)-catalyzed click chemistry
CFP	cyan fluorescent protein
CID	energy collision-induced dissociation
Cit	citrulline
DAP22c	biotin-Phe-Ala-Ala-diphenylphosphonate
ESI	electrospray ionization
F	phenylalanine
FcεRI	antigen receptor
GluC	glutamyl endopeptidase
HFF	human foreskin fibroblast line
H	histidine
HMC-1	human mast cell line 1
HPLC	high performance liquid chromatography
IC	4-chloro-isocoumarin
ICAT	isotope-coded affinity tagging
IgE	immunoglobuline E
K	lysine

L	leucine
LC	liquid chromatography
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
MudPIT	multidimensional protein identification technology
NCE	normalized collision energy
NMR	nuclear magnetic resonance spectroscopy
Orn	ornithine
PA	phosphoramidate
PAGE	polyacrylamide gel electrophoresis
PAP	phosphoramidate peptide
Peg	polyethylene glycol
R	arginine
RE	recognition element
RFP	red fluorescent protein
RT	room temperature
SILAC	stable isotope labeling by amino acids in culture
SPPS	solid phase peptide synthesis
*Tg*SUB1	subtilisin protease 1 of *T.gondii*
TLC	thin layer chromatography
V	valine
YFP	yellow fluorescent protein

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Appendix

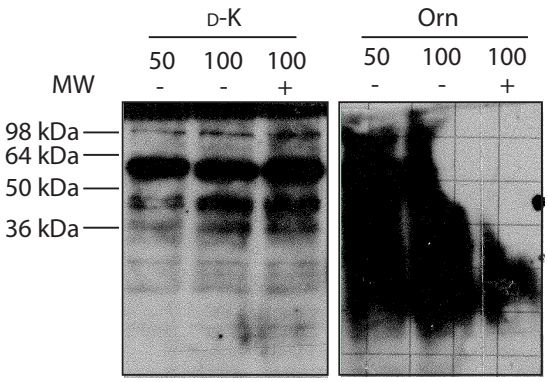

Figure 6.3.1: **Labeling of HMC-1 lysate by biotin(D)K-PAP and biotinOrn-PAP.** biotin-PAPs were used at 10 μM to label HMC-1 lysate, capital letters indicate the amino acids in the recognition elements, preblocking was done with 1 mM DFP.

Proteins from activated rat pancreas lysate identified with >99% probability

Activated rat pancreas lysate (aRPL) was reacted with biotinK-PAP, labeled proteins were pulled down using streptavidin-coated agarose beads and the enriched proteins were separated using SDS-PAGE. The only coomassie-stainable band was excised and its content was prepared for protein identification via LC-MS/MS. The digestion was performed using GluC to avoid confusion with endogenous proteases from the sample. This list includes all proteins that were identified with 99% or higher probability using the SwissProt database. Unique peptides only occur in the very identified proteins and are therefore not found in its isotopes. Keratins and bovine casein are common laboratory contaminants introduced into the sample during manipulation.

Protein name	Protein accession numbers	Protein MW (Da)	Identification probability	Number of unique peptides	sequence coverage
Anionic trypsin-1 OS=Rattus norvegicus GN=Prss1 PE=1 SV=1	TRY1_RAT	25.959,1	100,00%	5	26,80%
Anionic trypsin-2 OS=Rattus norvegicus GN=Prss2 PE=1 SV=2	TRY2_RAT	26.227,4	99,90%	3	15,00%
Anionic trypsin-2	TRY2_RAT	26.227,4	100,00%	3	15,00%
CAS1_BOVIN	CAS1_BOVIN	0	99,50%	2	0,00%
Glutamyl endopeptidase	SSPA_STAAU	36.325,4	100,00%	18	41,70%
Keratin, type I cytoskeletal 10	K1C10_HUMAN	58.828,8	100,00%	9	13,00%
Keratin, type I cytoskeletal 42 OS=Rattus norvegicus GN=Krt42 PE=2 SV=1	K1C42_RAT	50.214,0	100,00%	3	7,96%
Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	K1C9_HUMAN	62.065,9	100,00%	3	4,49%
Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	K2C1_HUMAN	66.040,3	100,00%	10	13,40%
Keratin, type II cytoskeletal 2 epidermal	K22E_HUMAN	65.433,9	100,00%	5	10,80%
SSPA_STAAU-R	SSPA_STAAU-R	0	99,70%	2	0,00%
Trypsin V-A OS=Rattus norvegicus 0,44%	TRYA_RAT,	26.900,3	99,80%	2	13,40%

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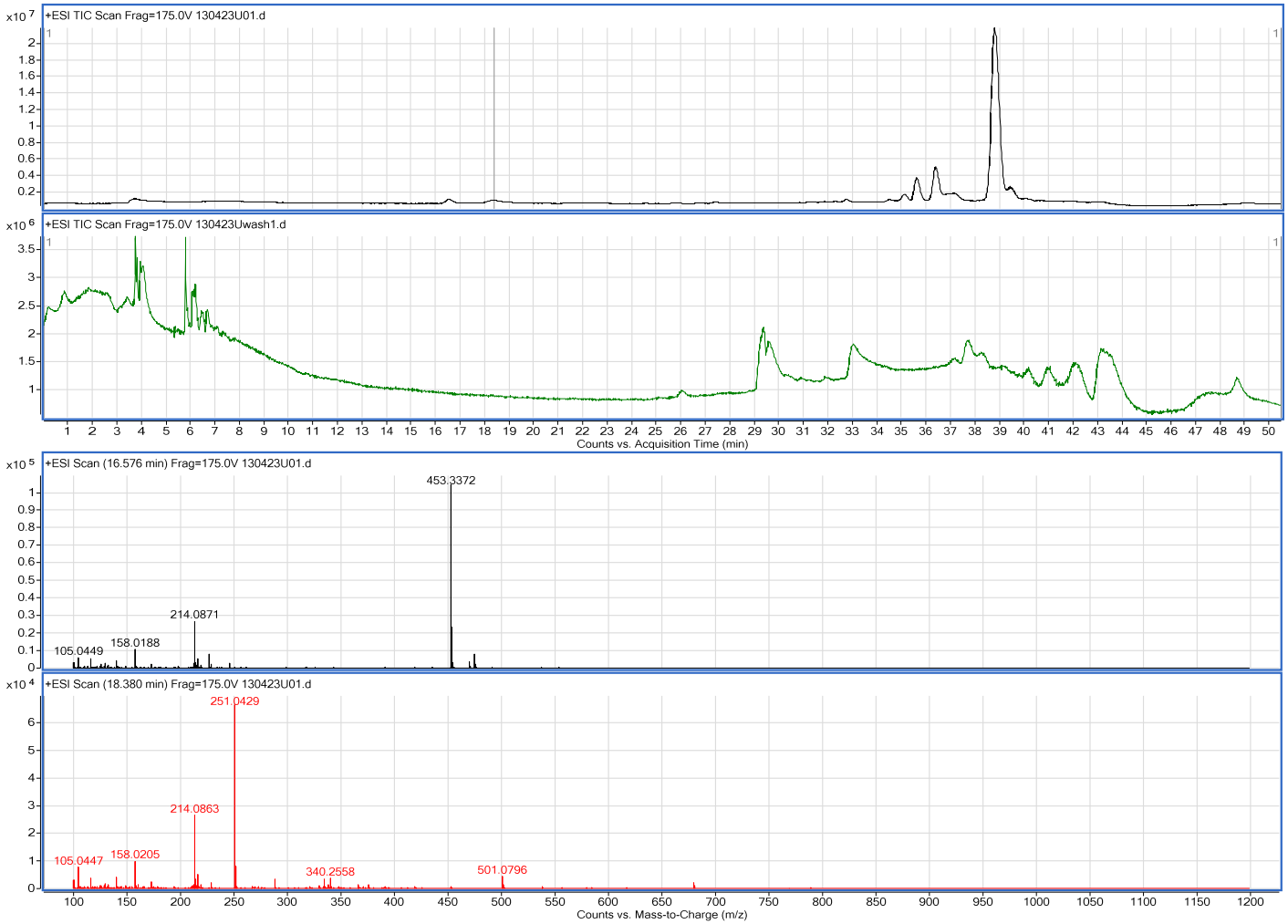

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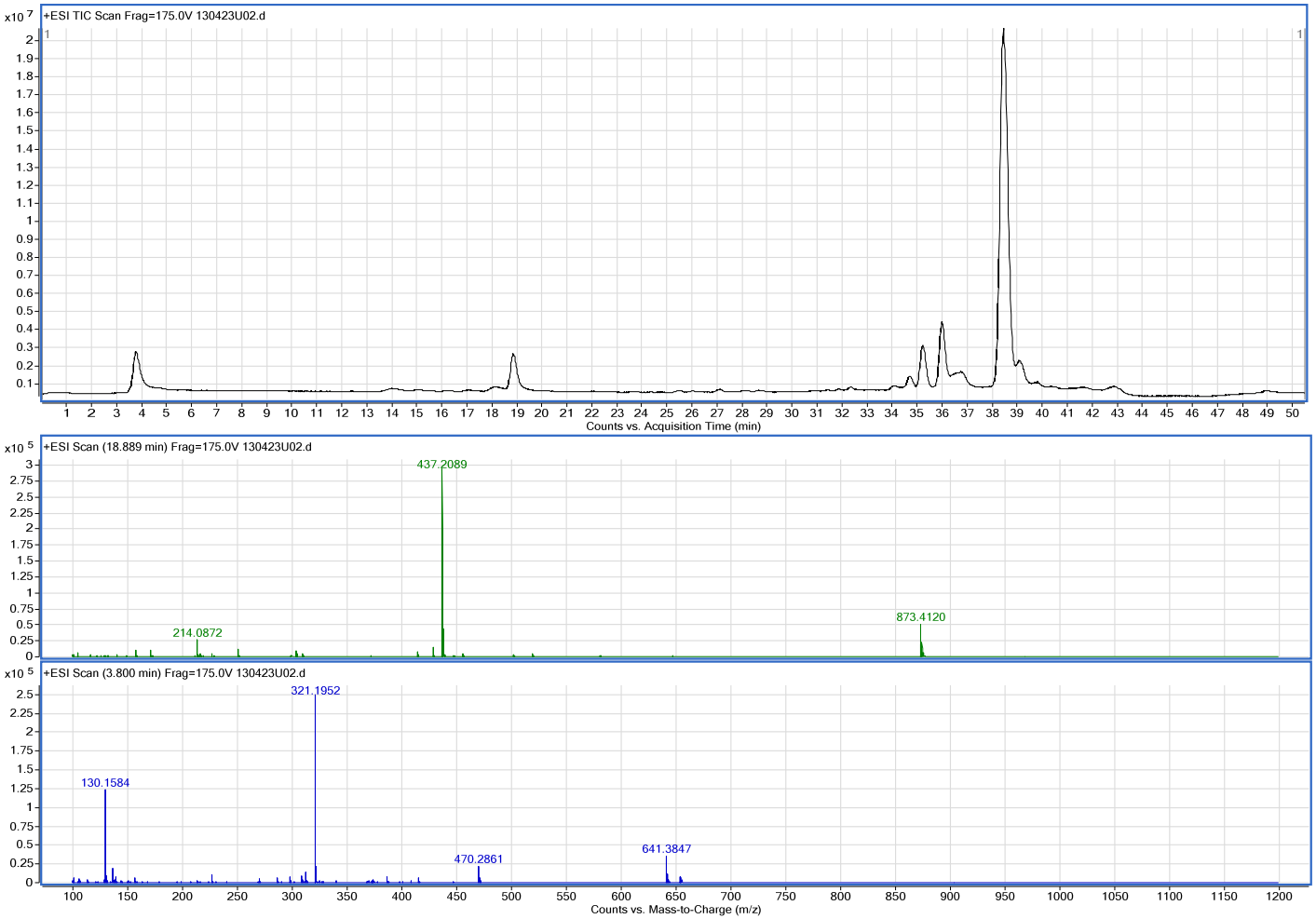

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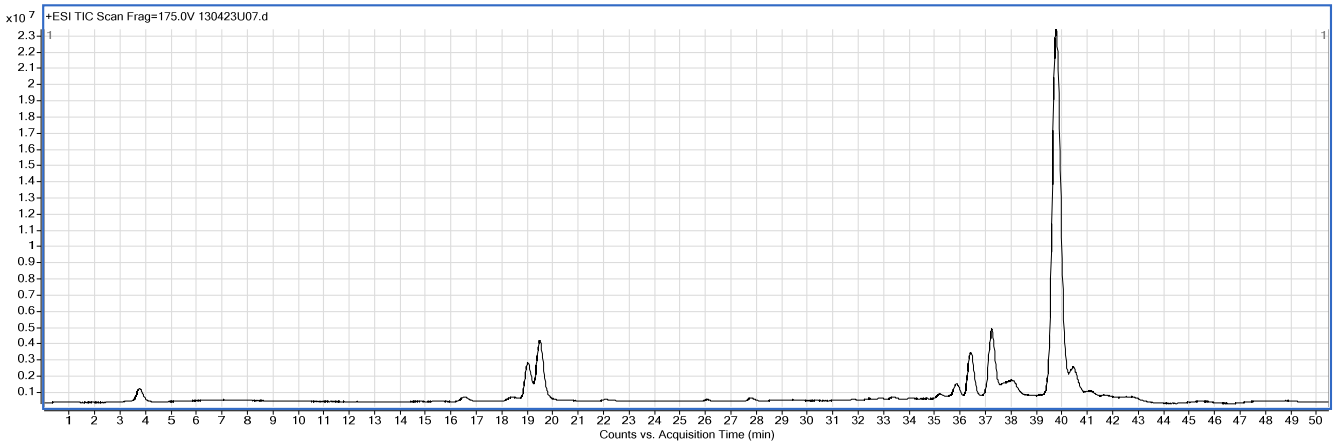

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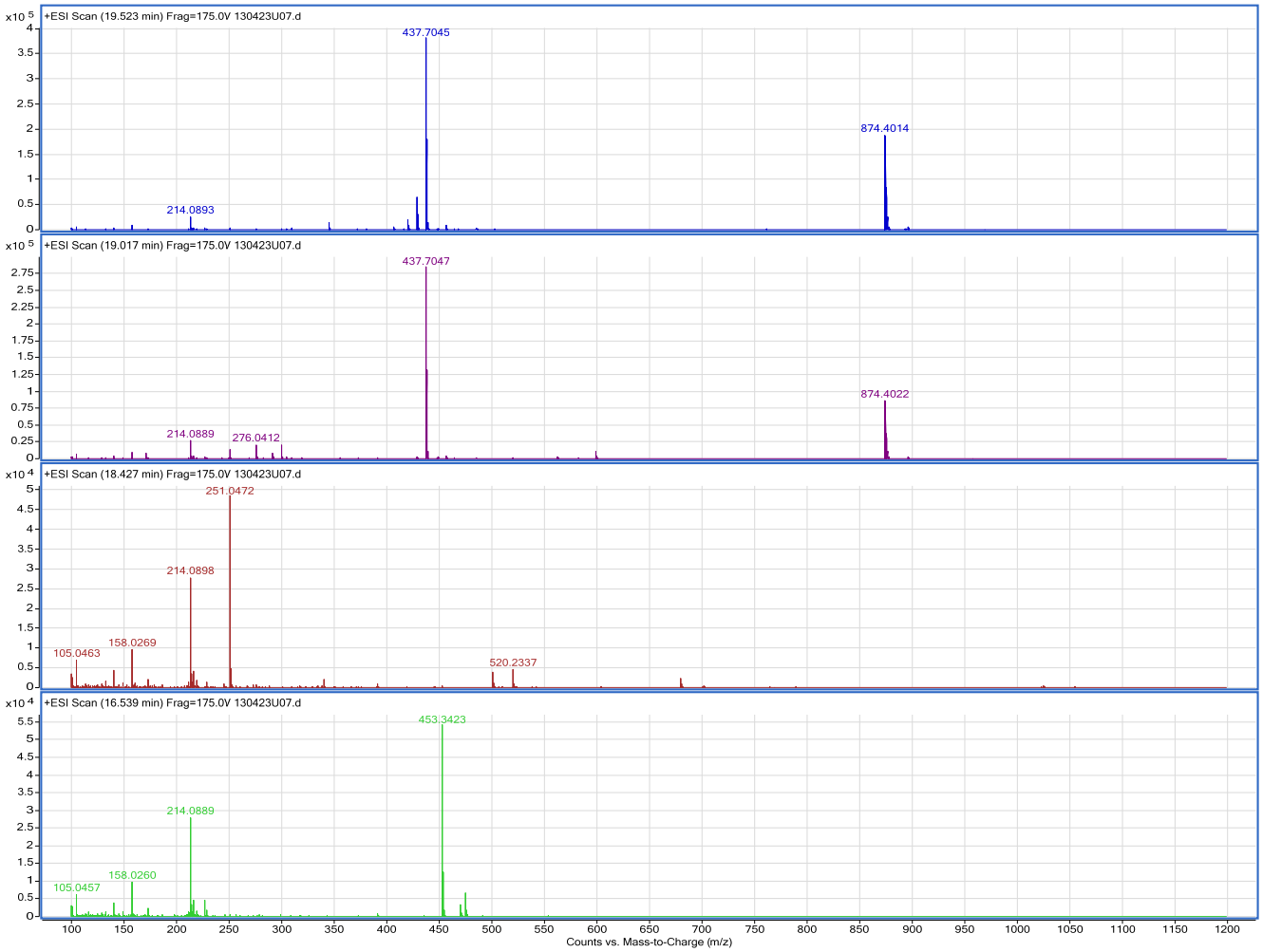

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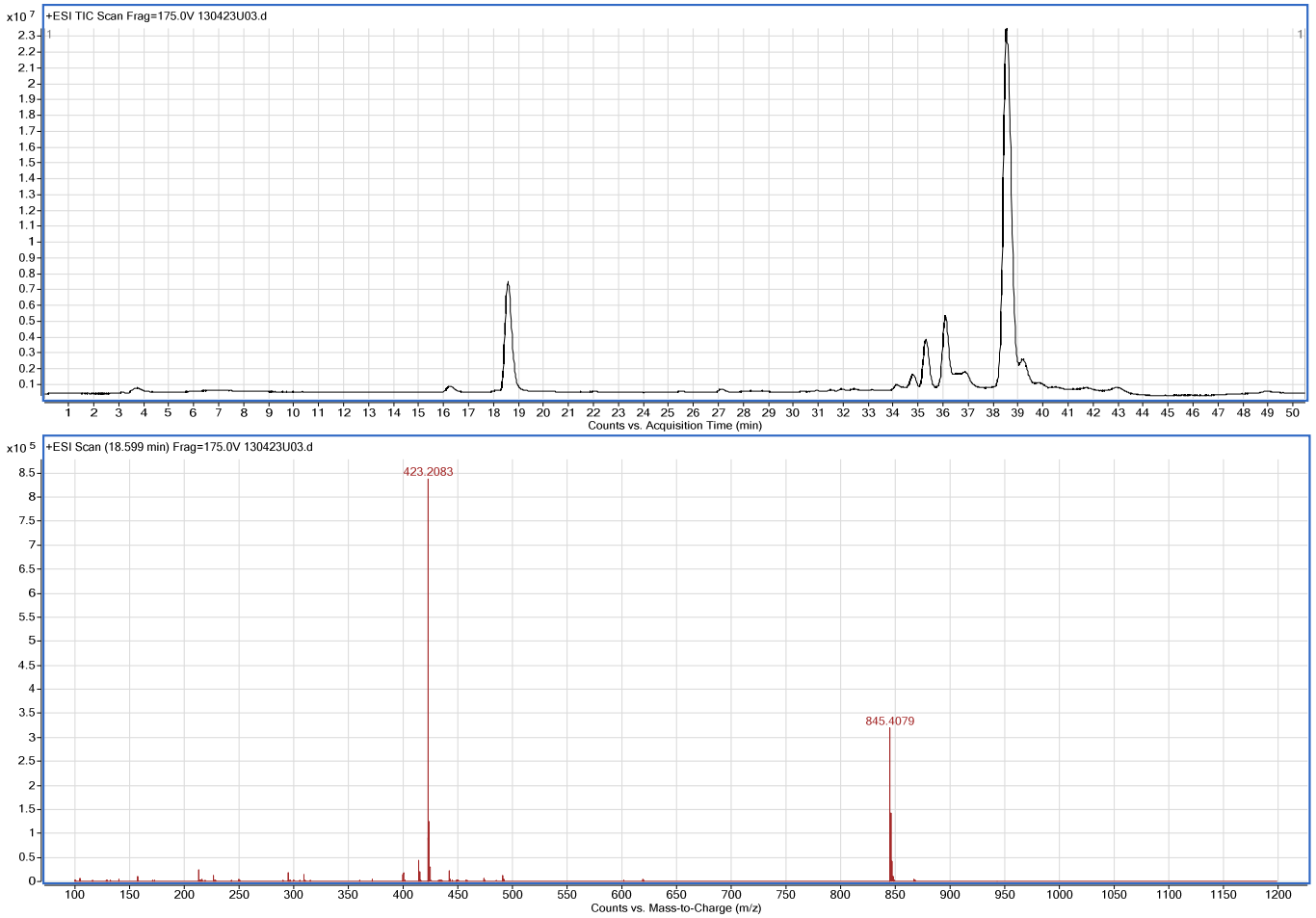

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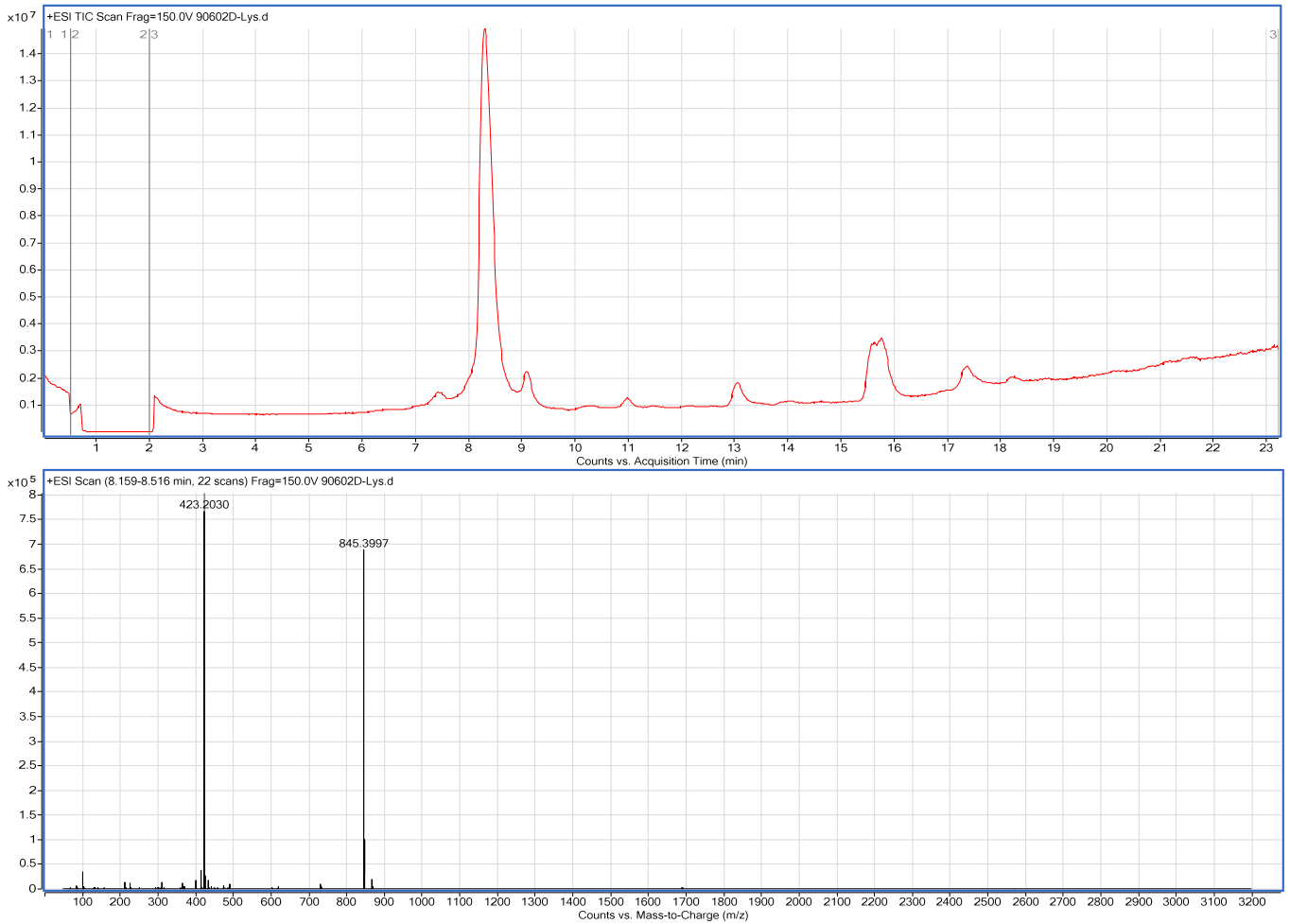

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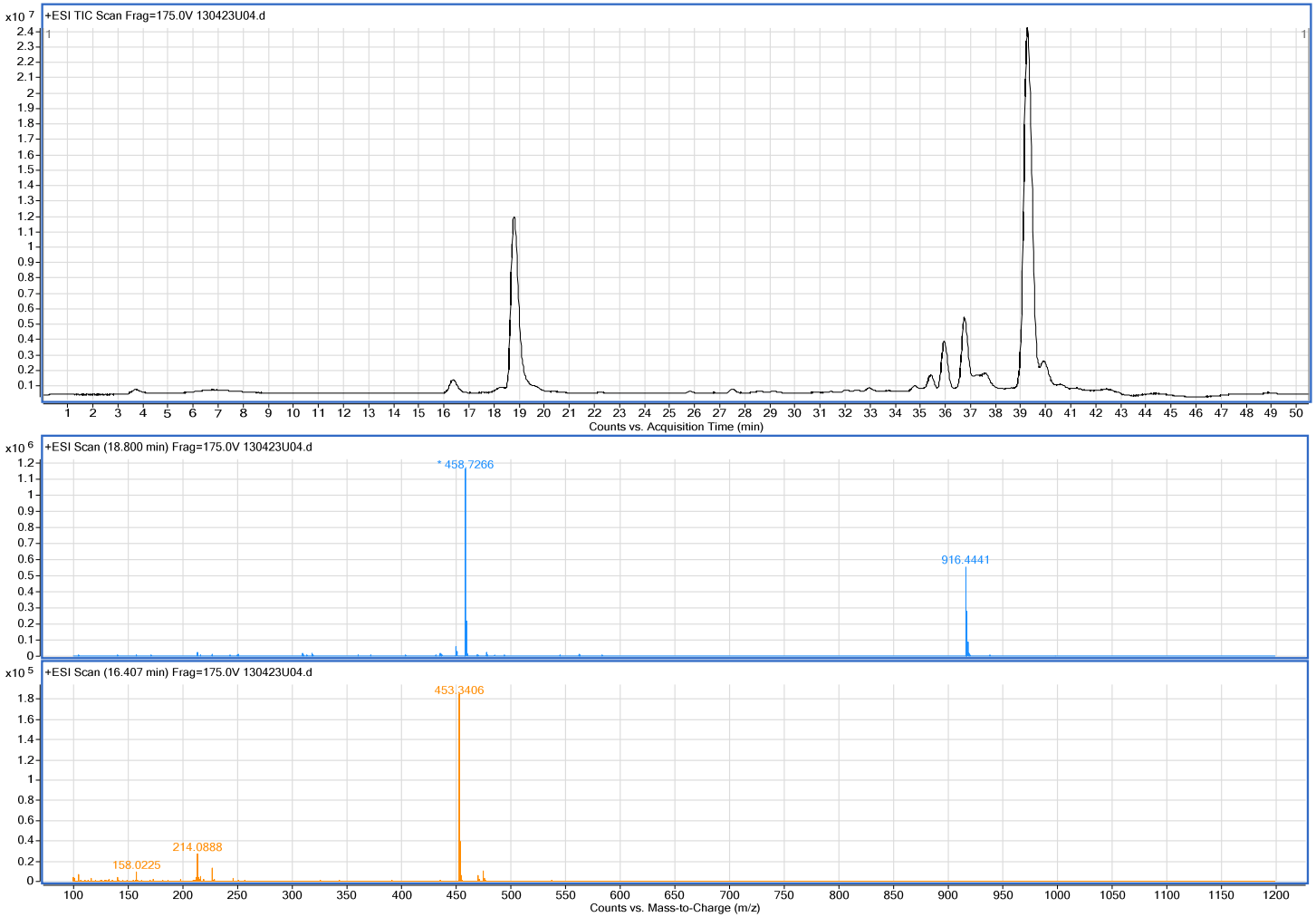

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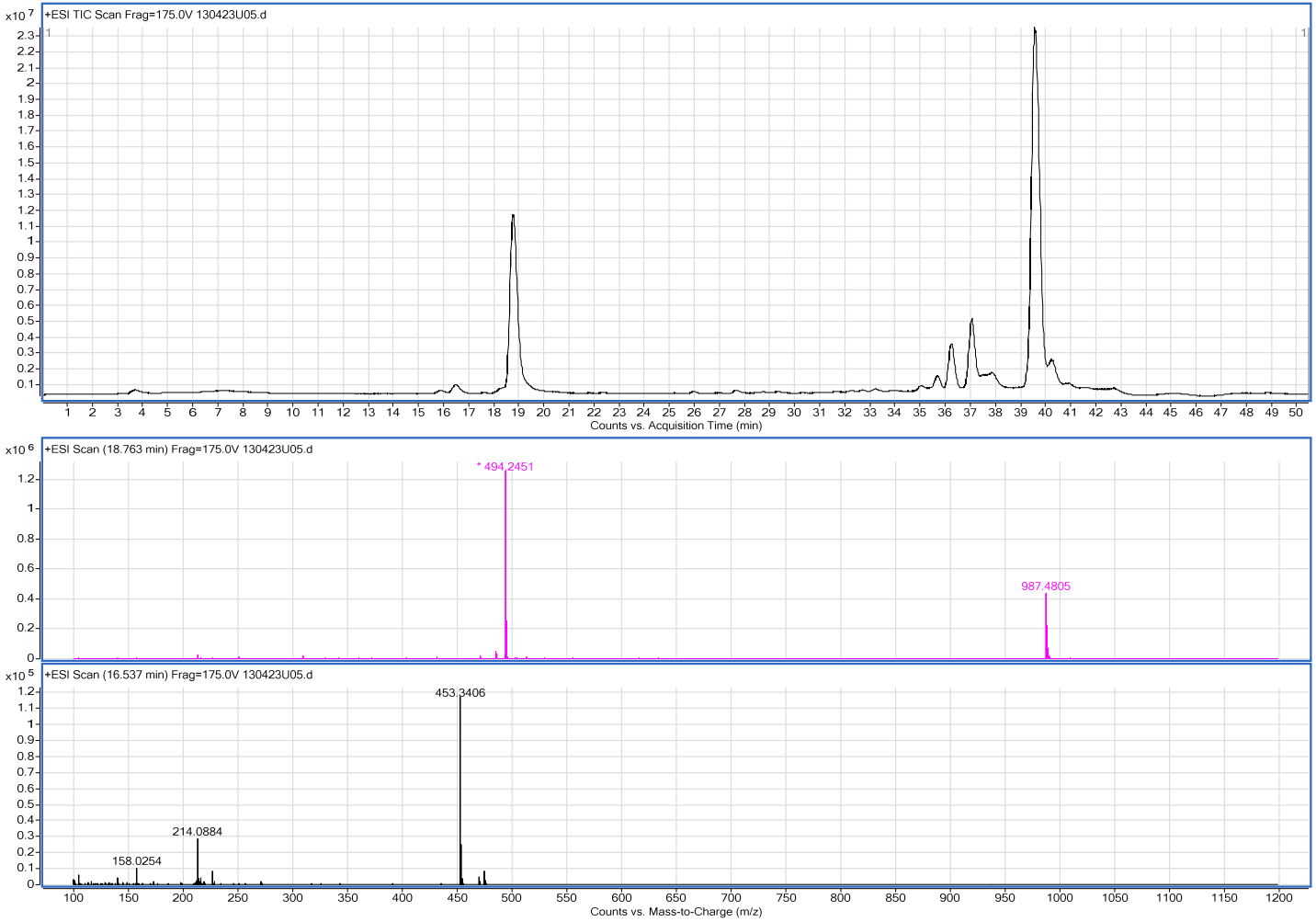

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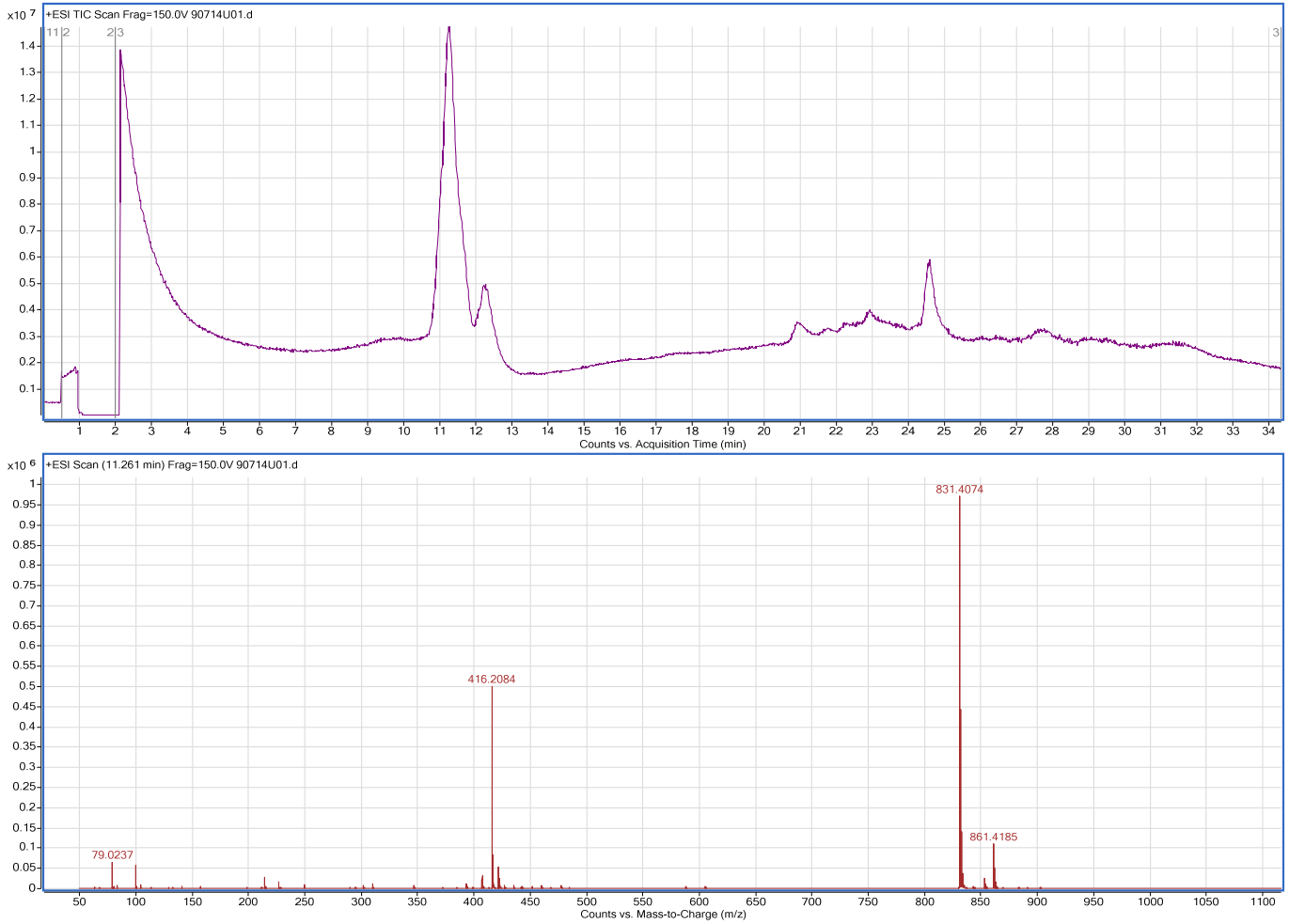

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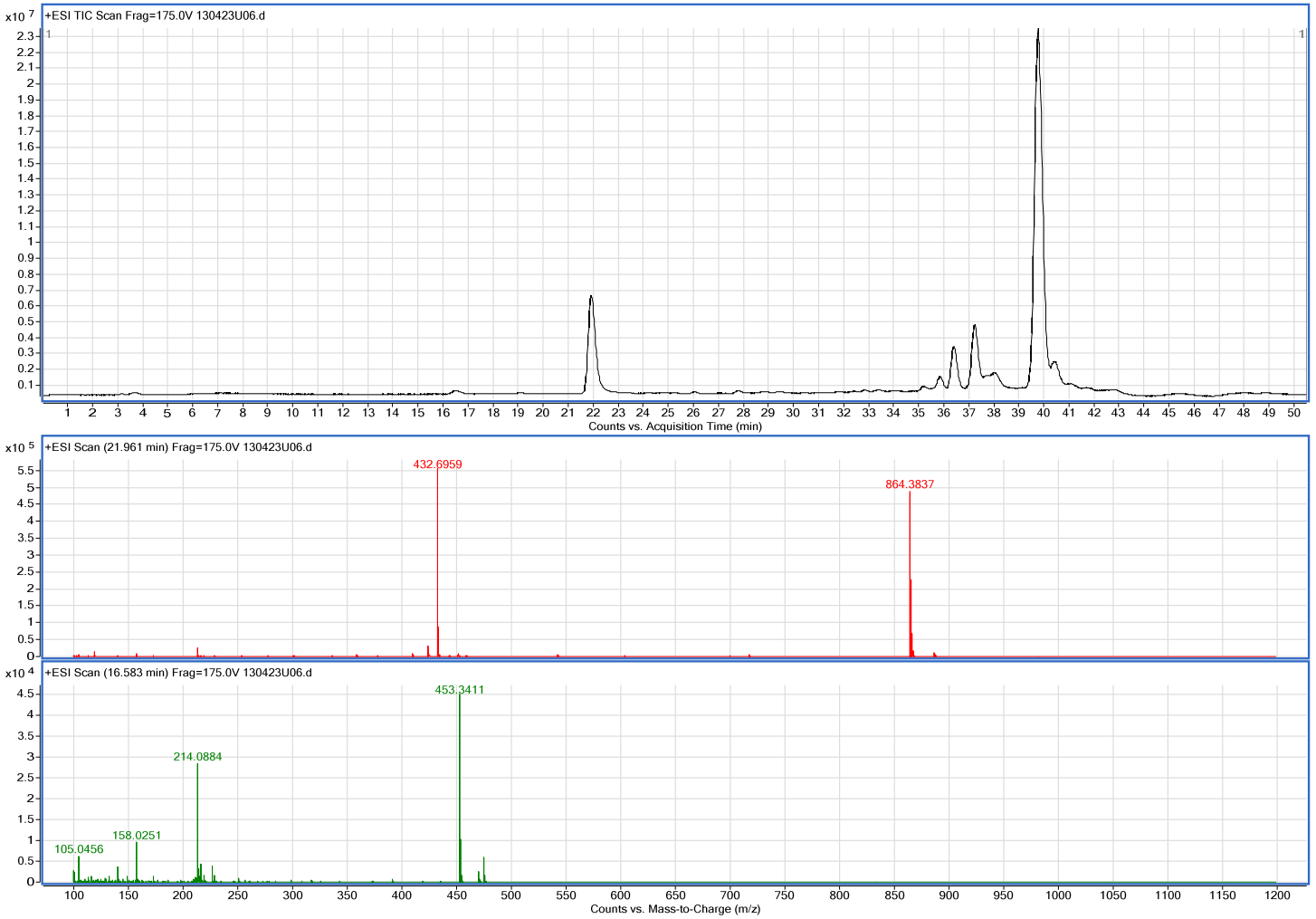

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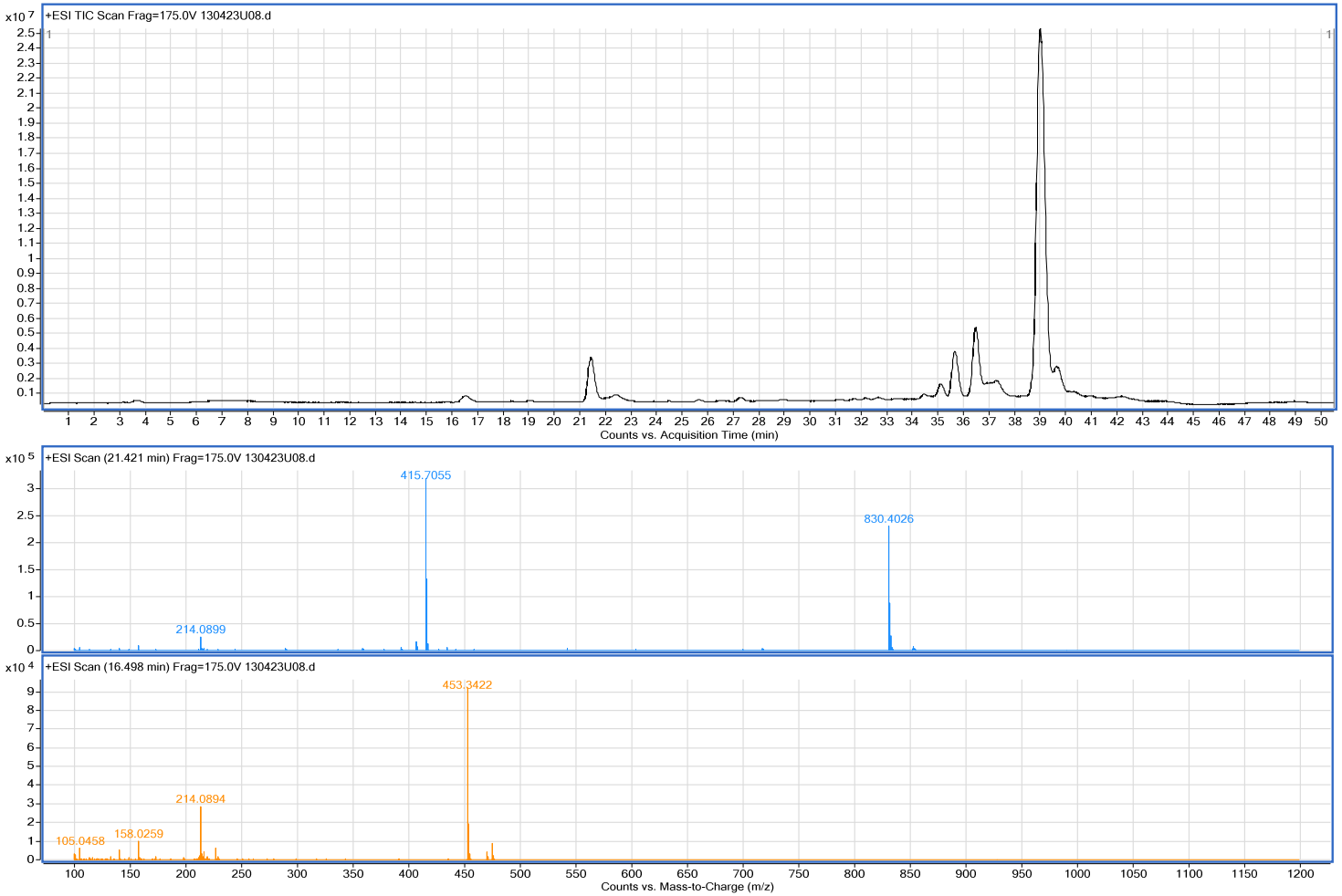

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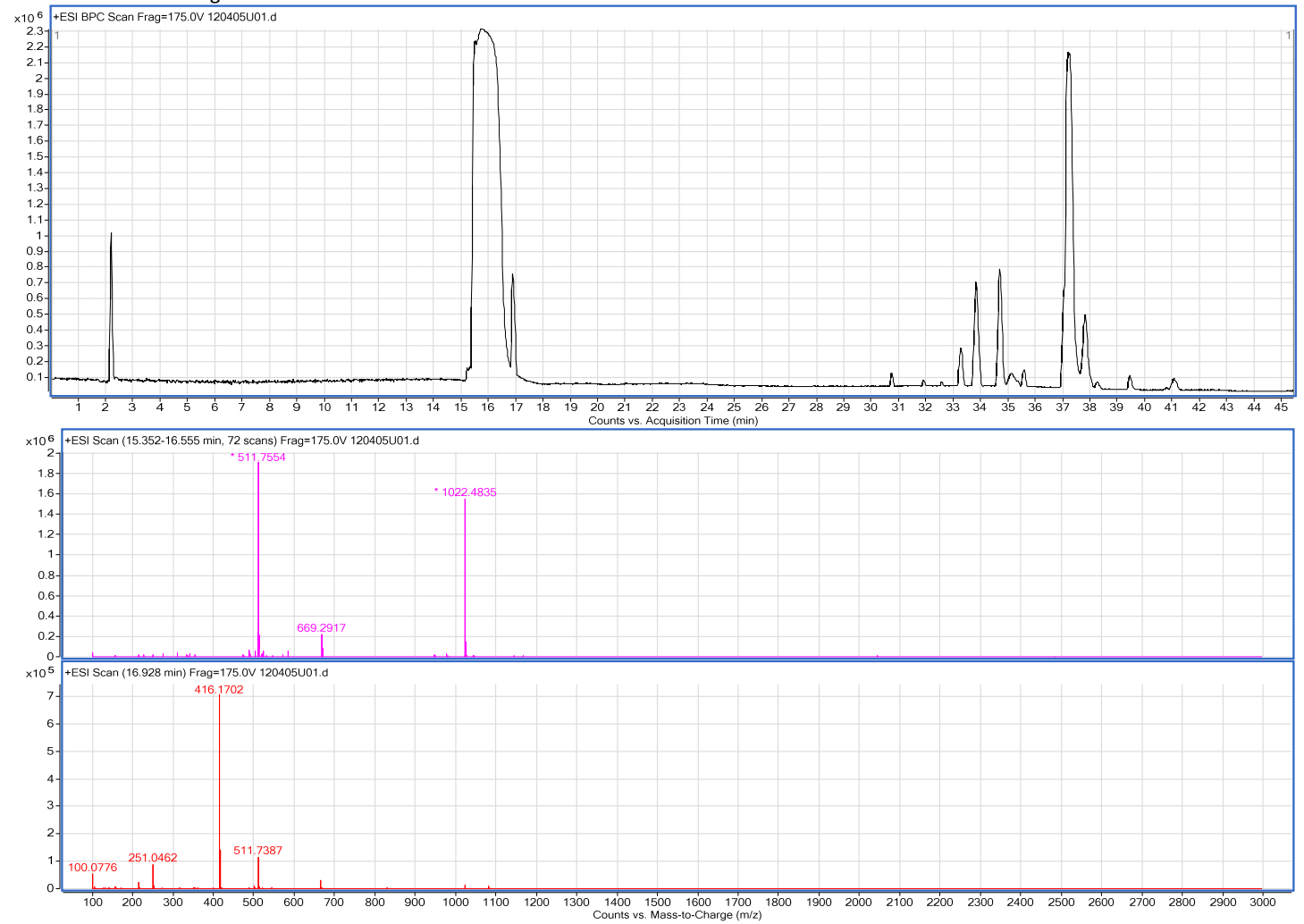

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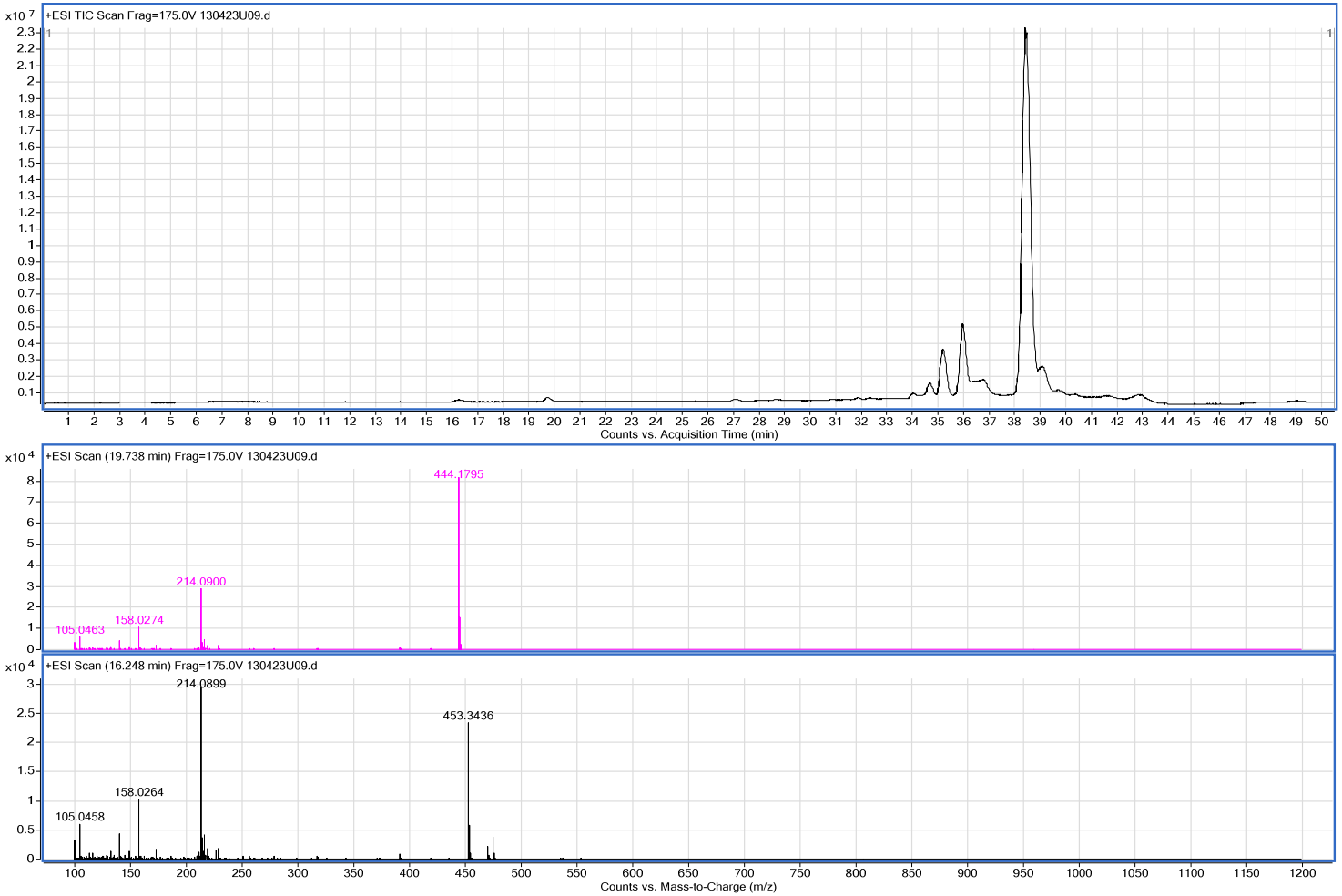

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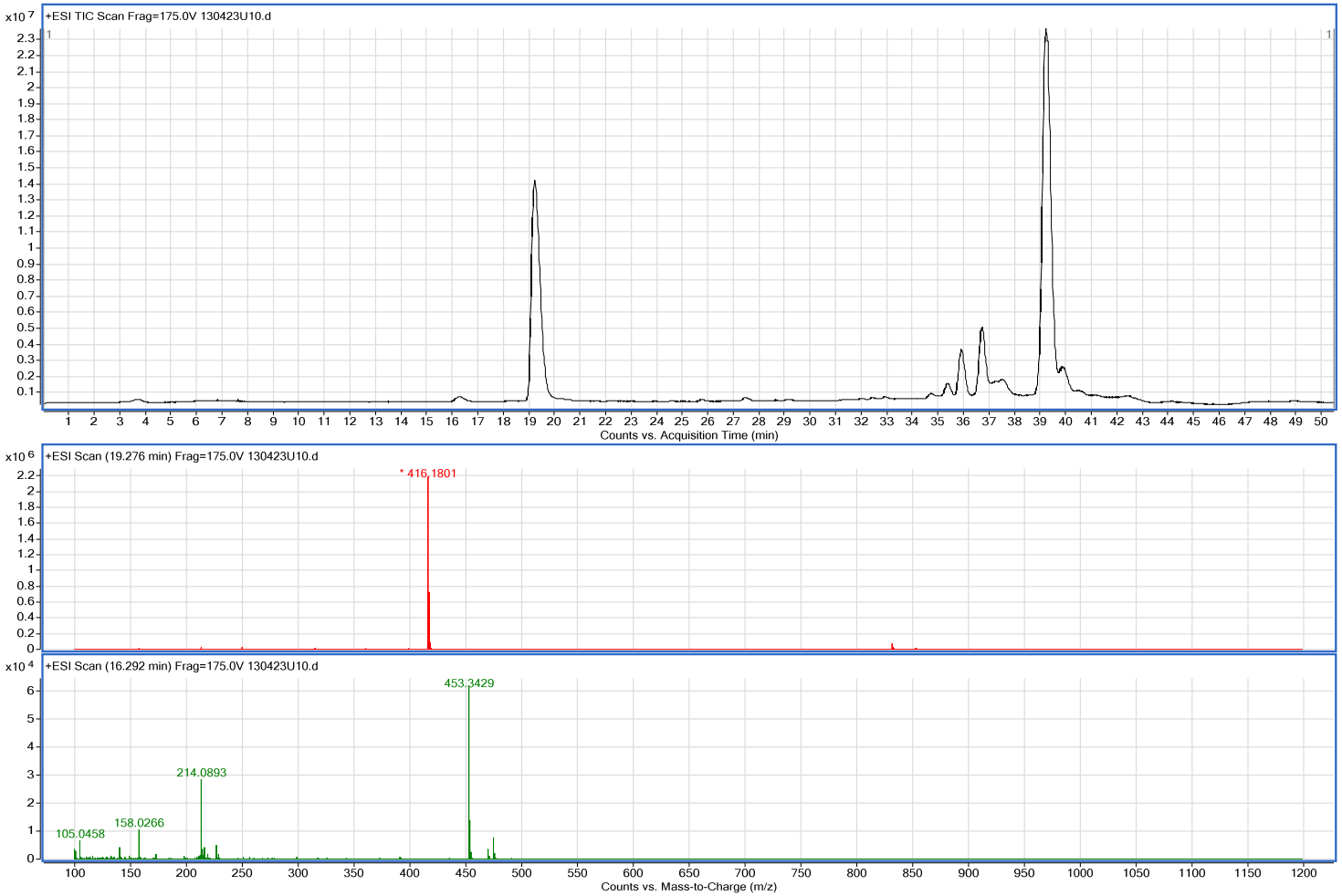

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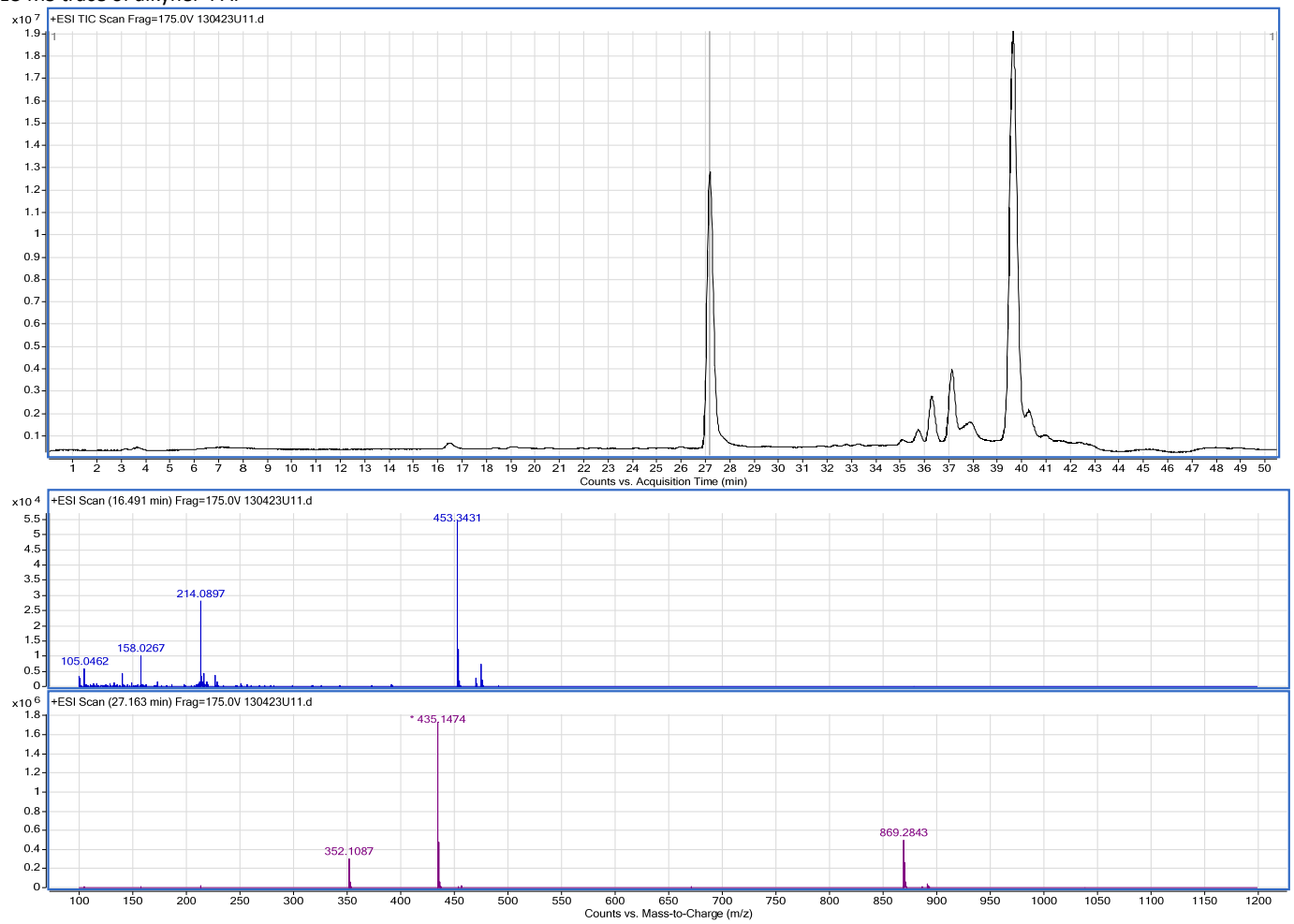

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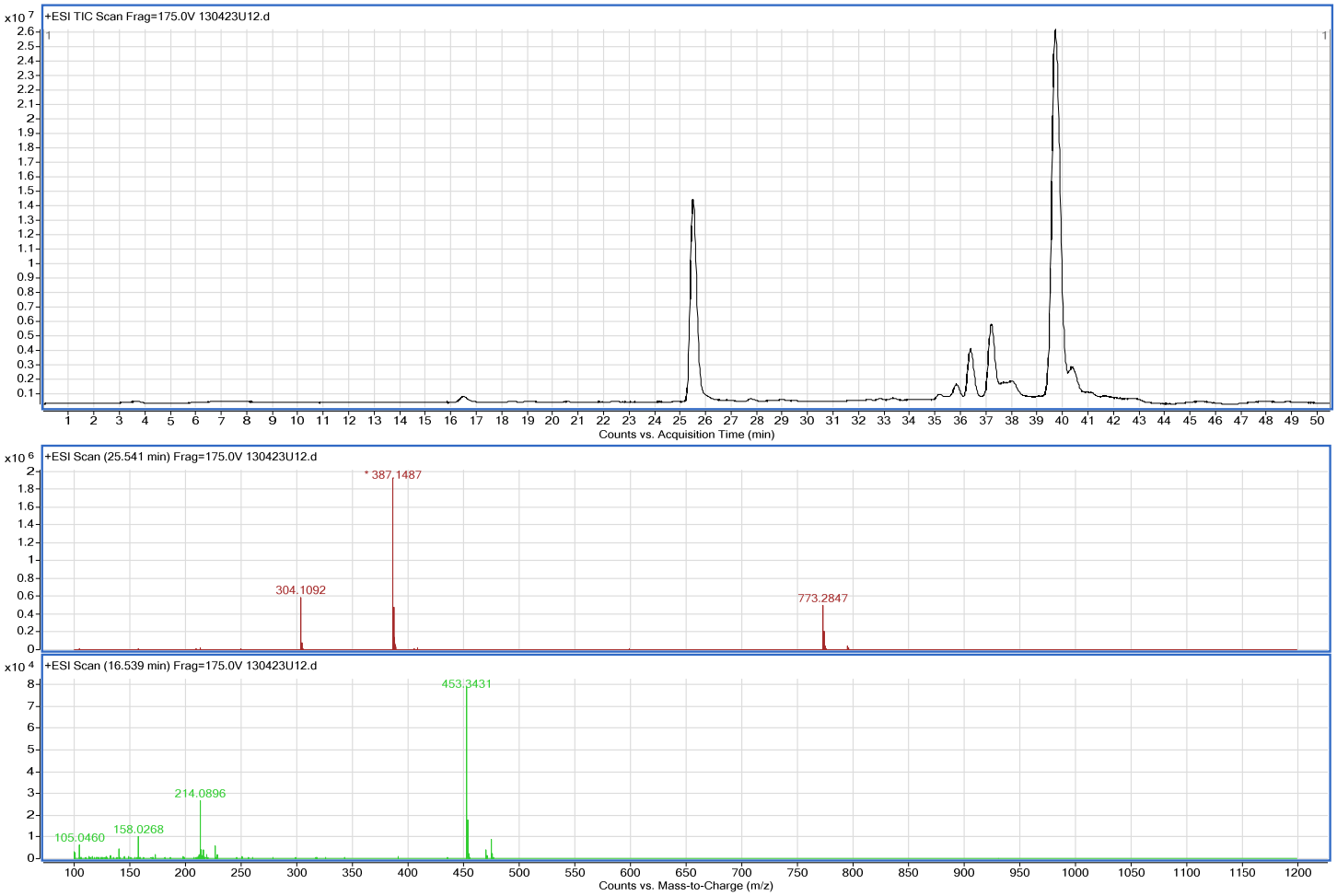

Table 3: **Calculated and measured masses of the ABPs synthesized in this work**

Probe name	calculated mass	measured mass ([M+H$^+$])
biotinR-PAP	872.41	873.4120
biotinCit-PAP	873.40	874.4014
biotinK-PAP	844.41	845.4079
biotin(D)K-PAP	844.41	845.2007
biotinAK-PAP	915.44	916.4441
biotinAAK-PAP	986.48	987.4805
biotinOrn-PAP	830.39	831.4074
biotinF-PAP	863.38	864.3837
biotinL-PAP	829.40	830.4026
biotinPegK-PAP	1022.16	1022.4836
alkyneR-PAP	443.17	444.1795
alkyneK-PAP	415.17	416.1801
alyneF-PAP	434.14	435.1474
alkyneV-PAP	386.14	387.1487

List of cell lines

Cell line	Cell type	Growth	Culture medium	Gift from
EL4	mouse lymphoma	suspension	DMEM (high glucose) 10% FBS 2 mM GlutaMax $5{\cdot}10^4$ U penicillin $5{\cdot}10^4$ μg streptomycin	Bogyo lab
Human foreskin fibroblast (HFF)	human fibroblast	adherent	DMEM (high glucose) 10% FBS 2 mM GlutaMax $5{\cdot}10^4$ U penicillin $5{\cdot}10^4$ μg streptomycin	Boothroyd lab
Human mast cell line-1 (HMC-1)	human mast cells	suspension	IMDM 10% FBS 1.8μL β-mercapto-ethanol 2 mM GlutaMax $5{\cdot}10^4$ U penicillin $5{\cdot}10^4$ μg streptomycin	Mayo Clinic
Toxoplasma gondii	parasite	intracellular	in HFF culture	Boothroyd lab

List of purified enzymes

Enzyme	Source	Vendor
Cathepsin G	human sputum	Sigma-Aldrich
Chymotrypsin type II	bovine pancreas	Sigma-Aldrich
Pancreatic elastase (PE)	porcine pancreas	VWR
Enteropeptidase	porcine intestine	Sigma-Aldrich
Glutamyl endopeptidase	source	Promega
Trypsin, type I	bovine pancreas	Sigma-Aldrich
Trypsin, sequ. grade	porcine pancreas	Promega
Trypsinogen	bovine pancreas	Sigma-Aldrich
β-Tryptase	mouse	R&D systems
Urokinase-type plasminogen activator (uPA)	human urine	Sigma-Aldrich

List of inhibitors

Inhibitor	Specificity	Vendor
4-(2-Aminoethyl)benzene-sulfonylfluoride (AEBSF)	trypsin-like	Applichem
biotin-Phe-Ala-Ala-diphenylphosphonate DAP22c	trypsin- and chymotrypsin-like	made in-house
Diisopropyl fluorophosphate (DFP)	broadband	Sigma-Aldrich
3,4-Dichloro isocoumarin (DCI)	broadband	Sigma-Aldrich
Phenylmethylsulfonyl fluoride (PMSF)	trypsin- and chymotrypsin-like	Sigma-Aldrich
Tosyl-L-lysine chlormethyl ketone (TLCK)	trypsin-like	Sigma-Aldrich
Tosyl phenylalanyl chlormethyl ketone (TPCK)	chymotrypsin-like	Sigma-Aldrich

List of chemicals

Chemical	description	vendor
AcOH	acetic acid	Applichem
Acrylamid	4K solution (30%)	
	mix 37,5:1	Applichem
Acetone	solvent	Applichem
ACN	acetonitrile	Applichem
AEBSF	4-(2-Aminoethyl)benzene-sulfonylfluoride	Applichem
$AgNO_3$	silver nitrate	Applichem
Anti-His_6-peroxidase	peroxidase-conjugated antibody	Roche
APS	ammonium persulfate	Applichem
Na^+ ascorbate		Applichem
β-mercaptoethanol		Applichem
BSA	albumin fraction V, blotting grade	Applichem
bromophenol blue		Applichem
BuOH	n-butanol	Applichem
$Ce(SO_4)_24H_2O$	Cerium(IV) sulfate	Applichem
$CuSO_4$	copper(II) sulfate	Sigma-Aldrich
DCI	3,4-dichloroisocoumarin	Sigma-Aldrich
DCM	dichlormethan	Applichem
DFP	diisopropyl fluorophosphate	Sigma-Aldrich
DIEA	N,N-diisopropylethylamine	Sigma-Aldrich
DMEM	Dulbecco's Modified Eagle Medium	Invitrogen
DMF	N,N-dimethylformamid	Applichem
DMSO	dimethyl sulfoxide	Applichem
DMSO D_6	with TMS	Roth
DTT	dithiothreitole	Roth
EDTA	ethylenediamine-	Sigma-Aldrich

Chemical	description	vendor
	tetraacetic acid	
EtAc	ethylacetate	Applichem
EtOH	ethanol	Applichem
FA	formic acid	Applichem
FBS	fetal bovine serum, south american	Invitrogen
Fmoc-Ala-OH·H_2O	alanine building block	Creosalus
Fmoc-6-Ahx-OH	aminohexanoic acid building block	Creosalus
Fmoc-Arg(Pbf)-OH	arginine building block	Creosalus
Fmoc-Cit-OH	citrulline building block	Creosalus
Fmoc-His(Trt)-OH	histidine building block	Creosalus
Fmoc-Leu-OH	leucine building block	Creosalus
Fmoc-(D)-Lys(Boc)-OH	D-lysine building block	Creosalus
Fmoc-Lys(Biotin)-OH	biotinylated lysine building block	Creosalus
Fmoc-Lys(Boc)-OH	lysine building block	Creosalus
Fmoc-Orn(Boc)-OH	ornithine building block	Creosalus
Fmoc-Phe-OH	phenylalanine building block	Creosalus
Fmoc-Val-OH	valine building block	Creosalus
formaldehyde	formaldehyde 37% solution	Applichem
glucose		Invitrogen

Chemical	description	vendor
Glutamax	media supplement	Invitrogen
glycerol		Applichem
glycine		Applichem
goat anti-mouse IgG	antibody	Invitrogen
HBTU	O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate	Creosalus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Applichem
HOBt	1-hydroxybenzotriazole	Creosalus
H_2SO_4	sulfuric acid	Applichem
IAA	iodoacetamide	Sigma-Aldrich
IMDM	Iscove's Modified Dulbecco's Media	Invitrogen
KCN	potassium cyanide	Sigma-Aldrich
K_2CO_3	potassium carbonate	Applichem
NaH_2PO_4	potassium dihydrogen phosphate	Roth
KOH	potassium hydroxide	Applichem
MeOH	methanol	Applichem
$MgSO_4$	magnesium sulfate	Roth
MES·H_2O	2-(N-morpholino)-ethanesulfonic acid monohydrate	Applichem
milk powder	blotting grade	Roth
mounting medium	with DAPI	Vector Laboratories
mouse anti *T. gondii*	antibody	Invitrogen
NaCl	sodium chloride	Roth
NaH_2PO_4	sodium dihydrogen phosphate	Roth
Na_2HPO_4	disodium dihydrogen phosphate	Roth
Na_2CO_3	sodium carbonate	Applichem
Na deoxycholate		Applichem

Chemical	description	vendor
NaOH	sodium hydroxide	Roth
$Na_2S_2O_3 \cdot 5\ H_2O$	sodium thiosulfate pentahydrate	Applichem
$(NH_4)_2CO_3 \cdot H_2O$	ammonium carbonate	Roth
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	ammonium molybdate	Applichem
ninhydrin		Sigma-Aldrich
nitrocellulose	Roti-NC, transfer membrane	Roth
NMP	1-methyl-2-pyrrolidone	Applichem
NP-40	nonidet P-40	Applichem
penicillin		Invitrogen
petroleum ether	solvent	Applichem
phenol		VWR
piperidin		VWR
PMSF	Phenylmethylsulfonyl fluoride	Sigma-Aldrich
protease inhibitor	cocktail tablets (EDTA-free)	Roche
pyridin		Applichem
rink aimde resin		Creosalus
SDS	detergent	Applichem
S-HRP	horseradish peroxidase-conjugated streptavidin	Sigma-Aldrich
silica gel	Kieselgel 60, (230-400 mesh)	Roth
streptomycin		Invitrogen
sucrose		Invitrogen
TAMRA	5-(and 6-)carboxy-tetramethylrhodamine, succinimidyl ester	Invitrogen
TEA	triethyl amine	Sigma-Aldrich
TFA	trifluoro acetic acid	Applichem
THF	tetrahydrofuran	Applichem
THPTA	tris(3-hydroxypropyl-triazolylmethyl)amine	Applichem

Chemical	description	vendor
TIS	triisopropylsilane	VWR
TLCK	tosyl-L-lysine chloromethyl ketone	Sigma-Aldrich
Toluene	solvent	Applichem
TPCK	tosyl-phenylalanyl chloromethyl ketone	Sigma-Aldrich
Tris	tris-(hydroxymethyl)-aminomethane	Applichem
Triton X-100	detergent	Applichem
Trypan blue solution	live cell staining agent	Sigma-Aldrich
trypsin-EDTA		Invitrogen
Tween	detergent	Applichem

Bibliography

[1] A. S. Abuelyaman, D. Hudig, S. L. Woodard, and J. C. Powers. Fluorescent derivatives of diphenyl [1-(n-peptidylamino)alkyl]phosphonate esters: synthesis and use in the inhibition and cellular localization of serine proteases. *Bioconjug. Chem.*, 5:400–405, 1994.

[2] N. J. Agard, J. A. Prescher, and C. R. Bertozzi. A strain-promoted [3+ 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *Journal of the American Chemical Society*, 126(46):15046–15047, 2004.

[3] K. Amin. The role of mast cells in allergic inflammation. *Respir. Med.*, 106(1):9–14, 2012.

[4] S. Arastu-Kapur, E. L. Ponder, U. P. Fonovic, S. Yeoh, M. F. Fang Yuan, M. Grainger, C. I. Phillips, J. C. Powers, and M. Bogyo. Identification of proteases that regulate erythrocyte rupture by the malaria parasite *Plasmodium falciparum*. *Nat. Chem. Biol.*, 4(3):203–213, 2008.

[5] M. Avgeris, K. Mavridis, and A. Scorilas. Kallikrein-related peptidases in prostate, breast, and ovarian cancers: from pathobiology to clinical relevance. *Biol. Chem.*, 393(5):301–317, 2012.

[6] D. A. Bachovchin, S. J. Brown, H. Rosen, and B. F. Cravatt. Identification of selective inhibitors of uncharacterized enzymes by high-throughput screening with fluorescent activity-based probes. *Nat. Biotechnol.*, 27:387–394, 2009.

[7] D. A. Bachovchin, T. Ji, W. Li, G. M. Simon, J. L. Blankman, A. Adibekian, H. Hoover, S. Niessen, and B. F. Cravatt. Superfamily-wide portrait of serine hydrolase inhibition achieved by library-versus-library screening. *Proc. Natl. Ac. Sci. USA*, 107(49):20941–20946, 2010.

[8] D. A. Bachovchin, M. R. Wolfe, K. Masuda, S. J. Brown, T. P. Spicer, V. Fernandez-Vega, P. Chase, P. S. Hodder, H. Rosen, and B. F. Cravatt. Oxime esters as selective, covalent inhibitors of the serine hydrolase retinoblastoma-binding protein 9 (RBBP9). *Bioorg Med Chem Lett*, 20:2254–2258, apr 2010. 10.1016/j.bmcl.2010.02.011.

[9] H. R. Barai and H. W. Lee. Kinetics and mechanism of the pyridinolysis of bis (2, 6-dimethylphenyl) chlorophosphate in acetonitrile. *Bull. Korean Chem. Soc*, 32(12):4179, 2011.

[10] A. Baruch, D. A. Jeffery, and M. Bogyo. Enzyme activity–it's all about image. *Trends in cell biology*, 14(1):29–35, 2004.

[11] J. M. Baskin, J. a. Prescher, S. T. Laughlin, N. J. agard, P. V. Chang, I. a. Miller, a. Lo, J. a. Codelli, and C. R. Bertozzi. Copper-free click chemistry for dynamic in vivo imaging. *Proc. Natl. Ac. Sci. USA*, 104(43):16793–16797, 2007.

[12] E. Bedner, P. Smolewski, P. Amstad, and Z. Darzynkiewicz. Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. *Exp. Cell Res.*, 259(1):308–313, Aug 2000.

[13] M. W. Black and J. C. Boothroyd. Lytic cycle of *Toxoplasma gondii*. *Microbiol. Mol. Biol. Rev.*, 64(3):607–623, 2000.

[14] M. L. Blackman, M. Royzen, and J. M. Fox. Tetrazine ligation: fast bioconjugation based on inverse-electron-demand diels- alder reactivity. *Journal of the American Chemical Society*, 130(41):13518–13519, 2008.

[15] G. Blum, S. R. Mullins, K. Keren, M. Fonovic, C. Jedeszko, M. J. Rice, B. F. Sloane, and M. Bogyo. Dynamic imaging of protease activity with fluorescently quenched activity-based probes. *Nat. Chem. Biol.*, 1(4):203–209, Sep 2005.

[16] G. Blum, G. von Degenfeld, M. J. Merchant, H. M. Blau, and M. Bogyo. Noninvasive optical imaging of cysteine protease activity using fluorescently quenched activity-based probes. *Nat. Chem. Biol.*, 3(10):668–677, 2007.

[17] Boettcher and S. A. Sieber. beta-lactams and beta-lactones as activity-based probes in chemical biology. *MedChemComm*, 3(4):408–417, 2012.

[18] T. Boettcher and S. A. Sieber. β-lactones as privileged structures for the active-site labeling of versatile bacterial enzyme classes. *Angew. Chem. Int. Ed. Engl.*, 47(24):4600–4603, 2008.

[19] M. Bogyo, J. S. McMaster, M. Gaczynska, D. Tortorella, A. L. Goldberg, and H. Ploegh. Covalent modification of the active site threonine of proteasomal beta subunits and the escherichia coli homolog hslv by a new class of inhibitors. *Proc. Natl. Acad. Sci. U. S. A.*, 94(13):6629–6634, Jun 1997.

[20] M. Bogyo, S. Shin, J. S. McMaster, and H. L. Ploegh. Substrate binding and sequence preference of the proteasome revealed by active-site-directed affinity probes. *Chemistry & Biology*, 5(6):307–320, 1998.

[21] M. Bogyo, S. Verhelst, V. Bellingard-Dubouchaud, S. Toba, and D. Greenbaum. Selective targeting of lysosomal cysteine proteases with radiolabeled electrophilic substrate analogs. *Chem. Biol.*, 7(1):27–38, 2000.

[22] R. Breinbauer and M. Koehn. Azide-alkyne coupling: a powerful reaction for bioconjugate chemistry. *Chembiochem*, 4:1147–1149, Nov 2003. 10.1002/cbic.200300705.

[23] F. Brossier, G. L. Starnes, W. L. Beatty, and L. D. Sibley. Microneme rhomboid protease *Tg*rom1 is required for efficient intracellular growth of *Toxoplasma gondii*. *Eukaryot. Cell*, 7:664–674, apr 2008. 10.1128/EC.00331-07.

[24] A. J. Brouwer, A. Jonker, P. Werkhoven, E. Kuo, N. Li, N. Gallastegui, J. Kemmink, B. I. Florea, M. Groll, H. S. Overkleeft, et al. Peptido sulfonyl fluorides as new powerful proteasome inhibitors. *J. Med. Chem.*, 55(24):10995–11003, 2012.

[25] C. M. Brown, M. Ray, A. a. Eroy-Reveles, P. Egea, C. Tajon, and C. S. Craik. Peptide length and leaving-group sterics influence potency of peptide phosphonate protease inhibitors. *Chem. Biol.*, 18(1):48–57, Jan 2011.

[26] T. Bugge, T. Antalis, and Q. Wu. Type ii transmembrane serine proteases. *J. Biol. Chem.*, 284, aug 2009. 10.1074/jbc.R109.021006.

[27] J. S. Buguliskis, F. Brossier, J. Shuman, and L. D. Sibley. Rhomboid 4 (ROM4) affects the processing of surface adhesins and facilitates host cell invasion by *Toxoplasma gondii*. *PLoS Pathog.*, 6(4):e1000858, 2010.

[28] G. H. Caughey. Mast cell tryptases and chymases in inflammation and host defense. *Immunol. Rev.*, 17:141154, 2007.

[29] J. W. Chang, D. K. Nomura, and B. F. Cravatt. A potent and selective inhibitor of KIAA1363/AADACL1 that impairs prostate cancer pathogenesis. *Chemistry & Biology*, 18(4):476–484, 2011.

[30] G. Y. Chen, M. Uttamchandani, Q. Zhu, G. Wang, and S. Q. Yao. Developing a strategy for activity-based detection of enzymes in a protein microarray. *Chembiochem*, 4(4):336–339, 2003.

[31] K. P. Chiang, S. Niessen, A. Saghatelian, and B. F. Cravatt. An enzyme that regulates ether lipid signaling pathways in cancer annotated by multidimensional profiling. *Chemistry & biology*, 13(10):1041–1050, 2006.

[32] J. Clerc, B. I. Florea, M. Kraus, M. Groll, R. Huber, A. S. Bachmann, R. Dudler, C. Driessen, H. S. Overkleeft, and M. Kaiser. Syringolin A Selectively Labels the 20 S Proteasome in Murine EL4 and Wild-Type and Bortezomib-Adapted Leukaemic Cell Lines. *Chembiochem*, 10(16):2638–2643, 2009.

[33] C. S. Craik, S. Roczniak, C. Largman, W. J. Rutter, et al. The catalytic role of the active site aspartic acid in serine proteases. *Science*, 237(4817):909–913, 1987.

[34] B. F. Cravatt and E. J. Sorensen. Chemical strategies for the global analysis of protein function. *Current opinion in chemical biology*, 4(6):663–668, 2000.

[35] B. F. Cravatt, A. T. Wright, and J. W. Kozarich. Activity-based protein profiling: From enzyme chemistry to proteomic chemistry. *Annu. Rev. Biochem.*, 77:383–414, Jun 2008.

[36] E. W. Davie. A brief historical review of the waterfall/cascade of blood coagulation. *J Biol Chem*, 278(51):50819–50832, 2003.

[37] L. Edgington, A. Berger, G. Blum, V. Albrow, M. Paulick, N. Lineberry, and M. Bogyo. Non-invasive optical imaging of apoptosis by caspase-targeted activity-based probes. *Nat. Med.*, 15:967–973, aug 2009. 10.1038/nm.1938.

[38] O. D. Ekici, M. Paetzel, and R. E. Dalbey. Unconventional serine proteases: variations on the catalytic Ser/His/Asp triad configuration. *Protein Sci.*, 17:2023–2037, Dec 2008.

[39] B. D. Fairbanks, T. F. Scott, C. J. Kloxin, K. S. Anseth, and C. N. Bowman. Thiol- yne photopolymerizations: Novel mechanism, kinetics, and step-growth formation of highly cross-linked networks. *Macromolecules*, 42(1):211–217, 2008.

[40] L. Fiorucci and F. Ascoli. Mast cell tryptase, a still enigmatic enzyme. *Cell. Mol. Life Sci.*, 61(11):1278–1295, 2004.

[41] J.-L. Frossard, S. M. L, and P. C. M. Acute pancreatisis. *The Lancet*, 371:143–152, 2008.

[42] P. P. Geurink, W. A. van der Linden, A. C. Mirabella, N. Gallastegui, G. de Bruin, A. E. Blom, M. J. Voges, E. D. Mock, B. I. Florea, G. A. van der Marel, et al. Incorporation of non-natural amino acids improves cell permeability and potency of specific inhibitors of proteasome trypsin-like sites. *J. Med. Chem.*, 2013.

[43] D. N. Gosalia, C. M. Salisbury, D. J. Maly, J. A. Ellman, and S. L. Diamond. Profiling serine protease substrate specificity with solution phase fluorogenic peptide microarrays. *Proteomics*, 5:1292–1298, 2005.

[44] D. Greenbaum, K. F. Medzihradszky, A. Burlingame, and M. Bogyo. Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. *Chemistry & biology*, 7(8):569–581, 2000.

[45] B. Gutte and R. Merrifield. The synthesis of ribonuclease a. *J Biol Chem*, 246(6):1922–1941, 1971.

[46] U. Haedke, M. Goetz, P. Baer, and S. H. L. Verhelst. Alkyne derivatives of isocoumarins as clickable activity-based probes for serine proteases. *Bioorg Med Chem.*, 20:633640, 2012.

[47] U. Haedke, E. V. Kttler, O. Vosyka, Y. Yang, and S. H. L. Verhelst. Tuning probe selectivity for chemical proteomics applications. *Curr. Opin. Chem. Biol.*, 17(1):102–109, Feb 2013.

[48] U. Haedke, E. V. Kuttler, O. Vosyka, Y. Yang, and S. H. Verhelst. Tuning probe selectivity for chemical proteomics applications. *Curr. Opin. Chem. Biol.*, 2012.

[49] H. Hahne, N. Sobotzki, T. Nyberg, D. Helm, V. Borodkin, D. van Aalten, B. Agnew, and B. Kuster. Proteome wide purification and identification of O-GlcNac modified proteins using click chemistry and mass spectrometry. *J. Proteome Res.*, 12:927936, Jan 2013.

[50] C. Hall, M. Reese, E. Weerapana, M. Child, P. Bowyer, V. Albrow, J. Haraldsen, M. Phillips, E. Sandoval, G. Ward, B. Cravatt, J. Boothroyd, and M. Bogyo. Chemical genetic screen identifies *Toxoplasma* DJ-1 as a regulator of parasite secretion, attachment, and invasion. *Proc. Natl. Ac. Sci. USA*, 108:1056810573, Jun 2011.

[51] J. W. Harper, K. Hemmi, and J. C. Powers. Reaction of serine proteases with substituted isocoumarins: discovery of 3,4-dichloroisocoumarin, a new general mechanism based serine protease inhibitor. *Biochemistry (Mosc.)*, 24:1831–1841, apr 1985.

[52] J. L. Harris, B. J. Backes, F. Leonetti, S. Mahrus, J. a. Ellman, and C. S. Craik. Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. *Proc Natl Acad Sci U S A*, 97:7754–7759, Jul 2000.

[53] J. Hawiger. Noninvasive intracellular delivery of functional peptides and proteins. *Curr Opin Chem Biol*, 3:89–94, 1999.

[54] S. Hawthorne, R. Hamilton, B. J. Walker, and B. Walker. Utilization of biotinylated diphenyl phosphonates for disclosure of serine proteases. *Anal. Biochem.*, 326(2):273–275, Mar 2004.

[55] W. P. Heal, T. H. T. Dang, and E. W. Tate. Activity-based probes: discovering new biology and new drug targets. *Chem. Soc. Rev.*, 40(1):246–257, Jan 2011.

[56] L. Hedstrom. Serine protease mechanism and specificity. *Chem. Rev.*, 102:4501–4524, Nov 2002.

[57] K. M. Heutinck, I. J. M. Ten Berge, C. E. Hack, J. Hamann, and A. T. Rowshani. Serine proteases of the human immune system in health and disease. *Mol. Immunol.*, 47:1943–1955, Jul 2010.

[58] F. Himo, T. Lovell, R. Hilgraf, V. V. Rostovtsev, L. Noodleman, K. B. Sharpless, and V. V. Fokin. Copper(I)-catalyzed synthesis of azoles. DFT study predicts unprecedented reactivity and intermediates. *J. Am. Chem. Soc.*, 127(1):210–216, Jan 2005.

[59] J. D. Hooper, J. A. Clements, J. P. Quigley, and T. M. Antalis. Type II transmembrane serine proteases insights into an emerging class of cell surface proteolytic enzymes. *J. Biol. Chem.*, 276(2):857–860, 2001.

[60] K. Horiuchi and H. Ma. Innovative chemical compound microarrays for drug screening. *DrugsPlus Int*, Jun/Jul 2006.

[61] R. Horton, L. A. Moran, G. Scrimgeour, M. Perry, and D. Rawn. *Biochemie, 4., aktualisierte Auflage*. Pearson Education, Inc, 2008.

[62] R. Huber and W. Bode. Structural basis of the activation and action of trypsin. *Acc. Chem. Res.*, 11(3):114–122, 1978.

[63] I. W. James. Linkers for solid phase organic synthesis. *Tetrahedron*, 55(16):4855–4946, 1999.

[64] N. Jessani, Y. Liu, M. Humphrey, and B. F. Cravatt. Enzyme activity profiles of the secreted and membrane proteome that depict cancer cell invasiveness. *Proc. Natl. Acad. Sci. U. S. A.*, 99:10335–10340, Aug 2002.

[65] N. Jessani, J. a. Young, S. L. Diaz, M. P. Patricelli, A. Varki, and B. F. Cravatt. Class assignment of sequence-unrelated members of enzyme superfamilies by activity-based protein profiling. *Angew Chem Int Ed*, 44(16):2400–2403, 2005.

[66] J. C. Jewett and C. R. Bertozzi. Cu-free click cycloaddition reactions in chemical biology. *Chem. Soc. Rev.*, 39:1272–1279, apr 2010.

[67] C. M. Kam, A. S. Abuelyaman, Z. Li, D. Hudig, and J. C. Powers. Biotinylated isocoumarins, new inhibitors and reagents for detection, localization, and isolation of serine proteases. *Bioconjug. Chem.*, 4(6):560–567, 1993.

[68] D. Kato, K. M. Boatright, A. B. Berger, T. Nazif, G. Blum, C. Ryan, K. A. Chehade, G. S. Salvesen, and M. Bogyo. Activity-based probes that target diverse cysteine protease families. *Nat Chem Biol*, 1(1):33–38, 2005.

[69] D. Kato, S. H. L. Verhelst, K. B. Sexton, and M. Bogyo. A general solid phase method for the preparation of diverse azapeptide probes directed against cysteine proteases. *Org. Lett.*, 7:5649–5652, Dec 2005.

[70] A. R. Khan and M. N. James. Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. *Protein Science*, 7(4):815–836, 1998.

[71] O. Khersonsky and D. S. Tawfik. Enzyme promiscuity: a mechanistic and evolutionary perspective. *Annu. Rev. Biochem.*, 79:471–505, 2010.

[72] D. Kidd, Y. Liu, and B. F. Cravatt. Profiling serine hydrolase activities in complex proteomes. *Biochemistry*, 40(13):4005–4015, 2001.

[73] K. B. Kim, J. Myung, N. Sin, and C. M. Crews. Proteasome inhibition by the natural products epoxomicin and dihydroeponemycin: insights into specificity and potency. *Bioorg Med Chem Lett*, 9(23):3335–3340, 1999.

[74] O. Kiraly, L. Guan, E. Szepessy, M. Toth, Z. Kukor, and M. Sahin-Toth. Expression of human cationic trypsinogen with an authentic N terminus using intein-mediated splicing in aminopeptidase P deficient E *coli*. *Protein Expr Purif*, 48(1):104–111, 2006.

[75] M. Koehn and R. Breinbauer. Die staudinger-ligation–ein geschenk fuer die chemische biologie. *Angew Chem*, 116(24):3168–3178, 2004.

[76] H. C. Kolb, M. G. Finn, and K. B. Sharpless. Click chemistry: Diverse chemical function from a few good reactions. *Angew. Chem. Int. Ed. Engl.*, 40:2004–2021, Jun 2001.

[77] S. Kumar, A. K. Verma, M. Das, and P. D. Dwivedi. Molecular mechanisms of IgE mediated food allergy. *Int Immunopharmacol*, 2012.

[78] V. Lagal, E. M. Binder, M.-H. Huynh, B. F. Kafsack, P. K. Harris, R. Diez, D. Chen, R. N. Cole, V. B. Carruthers, and K. Kim. *Toxoplasma gondii* protease *Tg*SUB1 is required for cell surface processing of micronemal adhesive complexes and efficient adhesion of tachyzoites. *Cell. Microbiol.*, 12(12):1792–1808, 2010.

[79] A. Link, J. Eng, D. Schieltz, E. Carmack, G. Mize, D. Morris, B. Garvik, and J. Yates. Direct analysis of protein complexes using mass spectrometry. *Nat Biotech*, 17:676–682, Jul 1999.

[80] B. Liou and G. A. Grabowski. Participation of asparagine 370 and glutamine 235 in the catalysis by acid β-glucosidase: The enzyme deficient in gaucher disease. *Mol Genet Metab*, 97(1):65–74, 2009.

[81] Y. Liu, M. P. Patricelli, and B. F. Cravatt. Activity-based protein profiling: The serine hydrolases. *Proc. Natl. Acad. Sci. U. S. A.*, 96:14694–14699, Dec 1999.

[82] S.-A. C. LLC. Trypsin in vivo processing, 2013.

[83] M. Mann. Functional and quantitative proteomics using silac. *Nat. Rev. Mol. Cell Biol.*, 7(12):952–958, 2006.

[84] J. S. Marshall. Mast-cell responses to pathogens. *Nat. Rev. Immunol.*, 4(10):787–799, 2004.

[85] H. L. Meier, L. W. Heck, and J. D. W. Schulman, E. S. MacGlashan. Purified Human Mast Cells and Basophils Release Human Elastase and Cathepsin G by an IgE-Mediated Mechanism. *Int Arch Allergy Immunol*, 77, Aug 1985.

[86] R. Merrifield. Solid phase peptide synthesis. i. the synthesis of a tetrapeptide. *J. Am. Chem. Soc.*, 85:2149–2154, Jul 1963. 10.1021/ja00897a025.

[87] R. d. Milton, S. Milton, and S. Kent. Total chemical synthesis of a d-enzyme: the enantiomers of hiv-1 protease show reciprocal chiral substrate specificity [corrected]. *Science*, 256(5062):1445–1448, 1992.

[88] A. R. Mitchell. Studies in solid-phase peptide synthesis: A personal perspective. *Peptide Science*, 90(3):215–233, 2008.

[89] K. Moffitt, B. Walker, and S. Martin. Chymotrypsin-like serine proteinases are involved in the maintenance of cell viability. *Biochimie*, 94:2582–2589, 2012.

[90] G. Nilsson, T. Blom, M. Kusche-Gullberg, L. Kjellen, J. H. Butterfield, C. Sundstrom, K. Nilsson, and L. Hellman. Phenotypic characterization of the human mast-cell line HMC-1. *Scand J Immunol*, 39:489–498, May 1994.

[91] S.-W. Oh, C. I. Pae, D.-K. Lee, F. Jones, G. K. S. Chiang, H.-O. Kim, S.-H. Moon, B. Cao, C. Ogbu, K.-W. Jeong, G. Kozu, H. Nakanishi, M. Kahn, E. Y. Chi, and W. R. J. Henderson. Tryptase inhibition blocks airway inflammation in a mouse asthma model. *The Journal of Immunology*, 168:1992–2000, Feb 2002.

[92] E. S. Okerberg, J. Wu, B. Zhang, B. Samii, K. Blackford, D. T. Winn, K. R. Shreder, J. J. Burbaum, and M. P. Patricelli. High-resolution functional proteomics by active-site peptide profiling. *Proc. Natl. Acad. Sci. U. S. A.*, 102(14):4996–5001, 2005.

[93] H. Ovaa, P. F. van Swieten, B. M. Kessler, M. A. Leeuwenburgh, E. Fiebiger, A. M. van den Nieuwendijk, P. J. Galardy, G. A. van der Marel, H. L. Ploegh, and H. S. Overkleeft. Chemistry in living cells: Detection of active proteasomes by a two-step labeling strategy. *Angew Chem Int Ed*, 115(31):3754–3757, 2003.

[94] Z. Pan, D. A. Jeffery, K. Chehade, J. Beltman, J. M. Clark, P. Grothaus, M. Bogyo, and A. Baruch. Development of activity-based probes for trypsin-family serine proteases. *Bioorg Med Chem Lett*, 16:2882–2885, Jun 2006.

[95] M. P. Patricelli, D. K. Giang, L. M. Stamp, and J. J. Burbaum. Direct visualization of serine hydrolase activities in complex proteomes using fluorescent active site-directed probes. *Proteomics*, 1:1067–1071, Sep 2001.

[96] P. J. B. Pereira, A. Bergner, S. Macedo-Ribeiro, R. Huber, G. Matschiner, H. Fritz, C. Sommerhoff, and W. Bode. Human beta-tryptase is a ring-like tetramer with active sites facing a central pore. *Nature*, 392:306–311, Mar 1998.

[97] C. Prussin and D. D. Metcalfe. 4. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol*, 111(2):S486–S494, 2003.

[98] a. W. Puri, P. Broz, a. Shen, D. M. Monack, and M. Bogyo. Caspase-1 activity is required to bypass macrophage apoptosis upon salmonella infection. *Nat. Chem. Biol.*, 2012.

[99] N. D. Rawlings, A. J. Barrett, and A. Bateman. Merops: the peptidase database. *Nucleic Acids Res.*, 38(suppl 1):D227–D233, 2010.

[100] W. W. Raymond, N. N. Trivedi, A. Makarova, M. Ray, C. S. Craik, and G. H. Caughey. How immune peptidases change specificity: cathepsin G gained tryptic function but lost efficiency during primate evolution. *J Immunol*, 185(9):5360–5368, 2010.

[101] B. P. Rempel and S. G. Withers. Covalent inhibitors of glycosidases and their applications in biochemistry and biology. *Glycobiology*, 18(8):570–586, 2008.

[102] S. Ren, K. Sakai, and L. Schwartz. Regulation of Human Mast Cell beta-Tryptase: Conversion of Inactive Monomer to active Tetramer at acid pH. *J Immunol*, 160, May 1998.

[103] V. V. Rostovtsev, L. G. Green, V. V. Fokin, and K. B. Sharpless. A stepwise huisgen cycloaddition process: copper(i)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angewandte Chemie International Edition*, 41:2596–2599, Jul 2002. 10.1002/1521-3773(20020715)41:14<2596::aID-aNIE2596>3.0.CO;2-4.

[104] R. Roy, B. Zhang, and M. A. Moses. Making the cut: protease-mediated regulation of angiogenesis. *Exp. Cell Res.*, 312(5):608–622, 2006.

[105] E. Sabido, T. Tarrago, S. Niessen, B. F. Cravatt, and E. Giralt. Activity-based probes for monitoring postproline protease activity. *Chembiochem*, 10:2361–2366, Sep 2009.

[106] A. M. Sadaghiani, S. H. Verhelst, M. Bogyo, et al. Tagging and detection strategies for activity-based proteomics. *Curr. Opin. Chem. Biol.*, 11(1):20–28, 2007.

[107] V. K. Sarin, S. B. Kent, J. P. Tam, and R. B. Merrifield. Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem.*, 117:147–157, Oct 1981.

[108] E. Saxon and C. R. Bertozzi. Cell surface engineering by a modified staudinger reaction. *Science*, 287(5460):2007–2010, 2000.

[109] I. Schechter, A. Berger, et al. On the size of the active site in proteases. I. Papain. *Biochem Biophys Res Commun*, 27(2):157, 1967.

[110] S. Serim, U. Haedke, and S. H. Verhelst. Activity-based probes for the study of proteases: Recent advances and developments. *ChemMedChem*, 7(7):1146–1159, 2012.

[111] D. A. Shannon, C. Gu, C. J. McLaughlin, M. Kaiser, R. a. L. van der Hoorn, and E. Weerapana. Sulfonyl fluoride analogues as activity-based probes for serine proteases. *Chembiochem*, 13(16):2327–2330, Nov 2012.

[112] L. D. Sibley. How apicomplexan parasites move in and out of cells. *Curr Opin Biotechnol*, 21(5):592–598, 2010.

[113] S. A. Sieber, T. S. Mondala, S. R. Head, and B. F. Cravatt. Microarray platform for profiling enzyme activities in complex proteomes. *J. Am. Chem. Soc.*, 126(48):15640–15641, 2004.

[114] S. A. Sieber, S. Niessen, H. S. Hoover, and B. F. Cravatt. Proteomic profiling of metalloprotease activities with cocktails of active-site probes. *Nat. Chem. Biol.*, 2(5):274–281, 2006.

[115] A. E. Speers, G. C. Adam, and B. F. Cravatt. Activity-based protein profiling in vivo using a copper (i)-catalyzed azide-alkyne [3+ 2] cycloaddition. *Journal of the American Chemical Society*, 125(16):4686–4687, 2003.

[116] A. E. Speers and B. F. Cravatt. Profiling enzyme activities in vivo using click chemistry methods. *Chem Biol*, 11:535–546, apr 2004. 10.1016/j.chembiol.2004.03.012.

[117] I. Staub and S. A. Sieber. β-lactams as selective chemical probes for the in vivo labeling of bacterial enzymes involved in cell wall biosynthesis, antibiotic resistance, and virulence. *J. Am. Chem. Soc.*, 130(40):13400–13409, Oct 2008.

[118] I. Staub and S. A. Sieber. β-lactam probes as selective chemical-proteomic tools for the identification and functional characterization of resistance associated enzymes in MRSA. *J Am Chem Soc*, 131(17):6271–6276, May 2009.

[119] J. Swedenborg, M. I. Mayranpaa, and P. T. Kovanen. Mast cells important players in the orchestrated pathogenesis of abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*, 31(4):734–740, 2011.

[120] J. Tam, M. Henault, L. Li, Z. Wang, A. W. Partridge, and R. A. Melnyk. An activity-based probe for high-throughput measurements of triacylglycerol lipases. *Anal. Biochem.*, 414(2):254–260, Jul 2011.

[121] V. Y. Torbeev and S. B. Kent. Convergent chemical synthesis and crystal structure of a 203 amino acid "covalent dimer" hiv-1 protease enzyme molecule. *Angewandte Chemie*, 119(10):1697–1700, 2007.

[122] M. E. Ul Hoque and H. W. Lee. Kinetics and mechanism of the anilinolysis of diisopropyl chlorophosphate in acetonitrile. *Bull. Korean Chem. Soc.*, 32:3245 – 3250, 2011.

[123] M. Uttamchandani, C. H. Lu, and S. Q. Yao. Next generation chemical proteomic tools for rapid enzyme profiling. *Acc Chem Res*, 42:1183–1192, 2009.

[124] W. A. van der Linden, N. Li, S. Hoogendoorn, M. Ruben, M. Verdoes, J. Guo, G.-J. Boons, G. A. van der Marel, B. I. Florea, and H. S. Overkleeft. Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: a comparative study. *Bioorg. Med. Chem.*, 20(2):662–666, Jan 2012.

[125] M. Verdoes, U. Hillaert, B. I. Florea, M. Sae-Heng, M. D. P. Risseeuw, D. V. Filippov, G. A. van der Marel, and H. S. Overkleeft. Acetylene functionalized BODIPY dyes and their application in the synthesis of activity based proteasome probes. *Bioorg Med Chem Lett*, 17(22):6169–6171, Nov 2007.

[126] S. H. Verhelst and M. Bogyo. Solid-phase synthesis of double-headed epoxysuccinyl activity-based probes for selective targeting of papain family cysteine proteases. *Chembiochem*, 6(5):824–827, 2005.

[127] S. H. Verhelst, M. D. Witte, S. Arastu-Kapur, M. Fonovic, and M. Bogyo. Novel aza peptide inhibitors and active-site probes of papain-family cysteine proteases. *Chembiochem*, 7(6):943–950, 2006.

[128] O. Vosyka, K. R. Vinothkumar, E. V. Wolf, A. J. Brouwer, R. M. Liskamp, and S. H. Verhelst. Activity-based probes for rhomboid proteases discovered in a mass spectrometry-based assay. *Proc Natl Acad Sci*, 110(7):2472–2477, 2013.

[129] A. Waldthaler, K. Schutte, and P. Malfertheiner. Causes and mechanisms in acute pancreatitis. *Dig. Dis.*, 28(2):364–372, 2010.

[130] G. Wang, U. Mahesh, G. Y. Chen, and S. Q. Yao. Solid-phase synthesis of peptide vinyl sulfones as potential inhibitors and activity-based probes of cysteine proteases. *Org. Lett.*, 5(5):737–740, 2003.

[131] M. P. Washburn, D. Wolters, and J. R. Yates. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol*, 19(3):242–247, 2001.

[132] E. Weerapana, C. Wang, G. M. Simon, F. Richter, S. Khare, M. B. Dillon, D. A. Bachovchin, K. Mowen, D. Baker, and B. F. Cravatt. Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature*, 468(7325):790–795, 2010.

[133] G. F. White and W. Crozier. Comparative proteolysis experiments with trypsin. *J. Am. Chem. Soc.*, 33(12):2042–2048, 1911.

[134] 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, and H. S. Overkleeft. Bioorthogonal chemistry: applications in activity-based protein profiling. *Acc Chem Res*, 44(9):718–729, 2011.

[135] L. I. Willems, M. Verdoes, B. I. Florea, G. a. van der Marel, and H. S. Overkleeft. Two-step labeling of endogenous enzymatic activities by Diels-Alder Ligation. *Chembiochem*, 9999:Na, xx 2010. 10.1002/cbic.201000280.

[136] U. Winkler, N. J. Allison, S. L. Woodard, R. A. Gault, G. R. Ewoldt, C. M. Kam, A. Abuelyaman, J. C. Powers, and D. Hudig. Characterization, application and potential uses of biotin-tagged inhibitors for lymphocyte serine proteases (granzymes). *Mol Immunol*, 33(7-8):615–623, 1996.

[137] N. Winssinger, S. Ficarro, P. G. Schultz, and J. L. Harris. Profiling protein function with small molecule microarrays. *Proc Natl Acad Sci U S A*, 99(17):11139, 2002.

[138] M. D. Witte, W. W. Kallemeijn, J. aten, K.-Y. Li, a. Strijland, W. E. Donker-Koopman, a. M. C. H. van den Nieuwendijk, B. Bleijlevens, G. Kramer, B. I. Florea, B. Hooibrink, C. E. M. Hollak, R. Ottenhoff, R. G. Boot, G. a. van der Marel, H. S. Overkleeft, and J. M. F. G. Aerts. Ultrasensitive in situ visualization of active glucocerebrosidase molecules. *Nat. Chem. Biol.*, 6(12):907–913, Dec 2010.

[139] Worthington. Elastase - worthington enzyme manual.

[140] H. Wu, J. Ge, M. Uttamchandani, and S. Q. Yao. Small molecule microarrays: the first decade and beyond. *Chem. Commun.*, 47(20):5664–5670, 2011.

[141] H. Wu, J. Ge, P.-Y. Yang, J. Wang, M. Uttamchandani, and S. Q. Yao. A peptide aldehyde microarray for high-throughput profiling of cellular events. *J. Am. Chem. Soc.*, 133(6):1946–1954, 2011.

[142] X. Yan, Y. Luo, Z. Zhang, Z. Li, Q. Luo, L. Yang, B. Zhang, H. Chen, P. Bai, and Q. Wang. Europium-Labeled Activity-Based Probe through Click Chemistry: Absolute Serine Protease Quantification Using 153Eu Isotope Dilution ICP/MS. *Angew Chem Int Ed*, 124(14):3414–3419, 2012.

[143] Y. Yang, H. Hahne, B. Kuster, and S. H. L. Verhelst. A simple and effective cleavable linker for chemical proteomics applications. *Mol Cell Proteomics*, 12:237–44, 2013.

[144] Z. Yang, M. Fonović, S. H. Verhelst, G. Blum, and M. Bogyo. Evaluation of α, β-unsaturated ketone-based probes for papain-family cysteine proteases. *Bioorg Med Chem*, 17(3):1071–1078, 2009.

[145] F. Yuan, S. H. Verhelst, G. Blum, L. M. Coussens, and M. Bogyo. A selective activity-based probe for the papain family cysteine protease dipeptidyl peptidase I/cathepsin C. *J Am Chem Soc*, 128(17):5616–5617, 2006.

[146] a. M. Zuhl, J. T. Mohr, D. a. Bachovchin, S. Niessen, K.-L. Hsu, J. M. Berlin, M. Dochnahl, M. A. P. Lo Pez-Alberca, G. C. Fu, and B. F. Cravatt. Competitive activity-based protein profiling identifies aza-β-lactams as a versatile chemotype for serine hydrolase inhibition. *J Am Chem Soc*, 134(11):5068–5071, 2012.

Publications

Parts of this thesis have been or will be published in international, peer-reviewed journals:

1) Ute Haedke, Markus Götz, Philipp Baer, Steven H. L. Verhelst. Alkyne derivatives of isocoumarins as clickable activity-based probes for serine proteases. *Bioorg Med Chem*, Jan 15;20(2):633-40, 2012

2) Sevnur Serim, Ute Haedke, Steven H. L. Verhelst. Activity-Based Probes for the Study of Proteases: Recent Advances and Developments. *ChemMedChem*, Jul;7(7):1146-59, 2012

3) Ute Haedke, Eliane Viola Küttler, Oliver Vosyka, Yinliang Yang, Steven H. L. Verhelst. Tuning probe selectivity for chemical proteomics applications. *Curr Opin Chem Biol* Feb;17(1):102-9 2013

4) Ute Haedke et al., Phosphoramidates as novel activity-based probes for serine proteases. (Manusript in preparation)

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