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Alkyne derivatives of isocoumarins as clickable activity-based probes for serine proteases

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ABSTRACT

Activity-based probes (ABPs) have found increasing use in functional proteomics studies. Recently, ABPs that can be employed in combination with click chemistry gained particular attention due to their flexible application in vitro and in vivo. Moreover, there is a continuous need for new ABPs that target small subsets of enzymes. We here report novel clickable ABPs based on the 4-chloro-isocoumarin (IC) electrophile, a mechanism-based inhibitor scaffold that covalently binds serine proteases. We describe the synthesis of a small library of IC ABPs containing an alkyne function and a set of diverse selectivity elements. The different substituents on the IC structure determine which proteases are bound, showing good correlation with the preferred substrate preferences. The IC ABPs can detect their target proteases in a proteome background in a sensitive manner (down to 0.007% of total protein). Furthermore, we show activity-dependent and selective labeling of endogenous proteases in a tissue proteome. These ICs therefore represent a valuable extension to already existing ABPs for serine proteases and may be instrumental in future elucidation of serine protease functions.

1. Introduction

In the post-genomic era, with genome sequences of numerous organisms available, most proteomics studies are aimed at quantification of the level of expressed proteins. However, the mere abundance of a protein does not address its functional state. The difference between abundance and activity is especially crucial for enzymes, whose activity is under tight post-translational control. In order to determine the active partition of expressed enzymes, small molecule activity-based probes (ABPs) have become a valuable tool in functional proteomics. Most ABPs consist of a certain detection tag conjugated to a mechanism-based inhibitor (often termed warhead) that forms a covalent bond with the target enzymes.[1]

In the last decade, ABPs have proven particularly useful for the functional analysis of proteases.[2–4] The proteasome, for example, has been studied using ABPs based on vinyl sulfones,[5,6] vinyl amides[7] and epoxyketones.[8] For cysteine proteases, a wide variety of warheads have been reported based on mild electrophiles that selectively react with the active site cysteine. Examples include acyloxymethyl ketones,[9] *O*-acyl hydroxamates,[10] unsaturated ketones[11,12] and sulfones,[13,14] and several derivatives of epoxysuccinates.[15–17] For serine proteases, the activity-based proteomics toolbox is less well equipped. Serine proteases require different types of warheads, because of the lower reactivity and the increased hardness of the serine active site nucleophile. Up to date, the main serine protease ABPs comprise fluorophosphonates[18] and peptidyl diphenyl phosphonates.[19–21] We aimed to expand the ABP toolbox for serine proteases with non-peptidic reagents based on the 4-chloro-isocoumarin scaffold.

4-Chloro-isocoumarins (ICs) have been investigated as inhibitors for soluble serine proteases,[22,23] rhomboids[24] (intramembrane serine proteases) and cholesterol esterase.[25] The mechanism of inactivation involves an attack of the nucleophilic serine residue in the active site of the protease on the carbonyl group of the isocoumarin. Depending on the substituent at the 7-position, the chloride is eliminated, giving rise to a quinone imine methide, which can in turn trap a second nucleophile, such as the active site histidine or a solvent molecule (Fig. 1).[26]

ICs do not closely mimic a protease substrate. Hence, it is hard to predict which substituents on the isocoumarin ring are being recognized by the S1 pocket, which is the primary specificity pocket in serine proteases. Interestingly, crystal structures of proteases in covalent complex with 3,7-disubstituted ICs have shown binding in different conformations, depending on the inhibitor structure and their mode of inhibition (i.e., only bound to the active site serine or doubly bound: to the active site serine and histidine). A substituent on the 3-position can, for example, dock into the S1 or the S3 pocket of porcine pancreatic elastase,[27,28] whereas a recent crystal structure of the *Escherichia coli* rhomboid GlpG shows that a substituent at the 7-position can dock into the S1 pocket.[24] Previous studies of ICs with positively charged binding elements at

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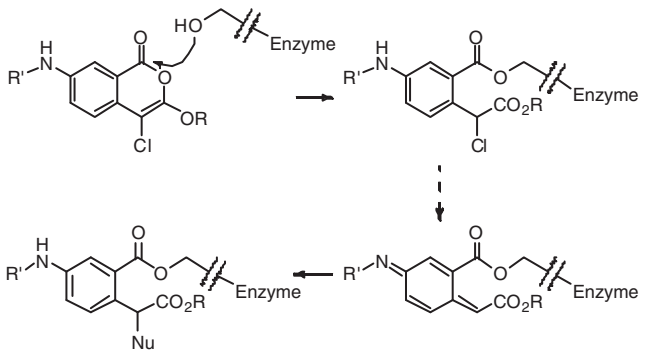


Figure 1. The mechanism of covalent modification of proteases by 4-chloro-isocoumarins.

either the 3- or the 7-position have shown inhibition of proteases with tryptic activity.[29] These results suggest that elements in both positions can potentially dock into the S1 pocket.

Despite their application as protease inhibitors, ICs have only been used as ABPs to a very limited extent. The few IC ABPs reported to date carry a biotin tag at the 7-position and a low variety of functional groups at the 3-position.[30,31] Recently, one of these biotinylated ICs has been used to identify a protease involved in host cell rupture by the malaria parasite *Plasmodium falciparum*,[32] illustrating that IC ABPs are promising tools for the functional characterization of serine protease activities.

In order to exploit ICs to their full potential, we decided to synthesize a set of IC ABPs with a variety of recognition elements and a versatile alkyne handle amenable for the introduction of detection tags by click chemistry (Fig. 2). Two versions of IC ABPs were designed – either with a selectivity element on the 3-position and an alkyne handle on the 7-position (**A**), or the other way around (**B**). We here report the synthesis of these probes and show that they react with trypsin-like, elastase-like and chymotrypsin-like serine proteases depending on the substituents on the IC scaffold. Furthermore, these reagents selectively label proteases in complex proteomes, demonstrating the potential of alkyne IC ABPs in future functional proteomics studies.

2. Results and discussion

2.1. Synthesis

Scheme 1 outlines the synthesis of the 7-alkyne series. The reaction sequence starts with an acid-catalyzed mono-esterification of 4-nitrohomophthalic acid using slight modifications from known procedures.[26,33] The mono-esters **2a–c** were subsequently converted into the ICs **3a–c** upon reaction with PCl_5.[26] In order to allow further modification, the 7-nitro substituent was reduced to an aniline by catalytic hydrogenation. Due to the low reactivity of the aniline function, amide-bond formation with carboxylic acids proved to be cumbersome using standard peptide coupling reagents such as DIC/HOB*t* or HATU/DIEA. However, the mixed anhydride method with the condensation reagent isobutyl chloroformate could be used to successfully introduce pentynoic acid as a click-tag, yielding the desired target probes **5–7**. At the 3-position, these probes have either a small or a hydrophobic selectivity element. The formation of a positively charged group at the 3-position was accomplished by nucleophilic substitution of the bromide in compound **7** by thiourea, yielding an isothioureido group, which mimics the arginine side chain.

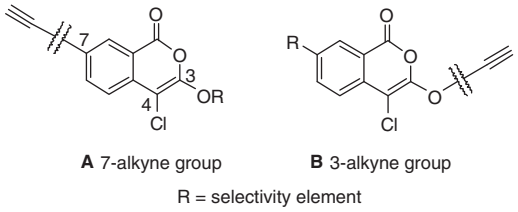


Figure 2. General structure of the target clickable isocoumarin ABPs.

Ideally, compound **7** could serve as a starting point for broader diversification at the 3-position through substitution of the bromide by different nucleophiles. Unfortunately, when we tested this out on a model compound, it turned out that the use of nucleophiles other than thiourea led to degradation of the isocoumarin scaffold, presumably by attack on the electrophilic carbonyl.[34]

At this point, we turned our attention to the synthesis of the 3-alkyne series (Scheme 2). Selective esterification of 4-nitrohomophthalic acid with 3-butyn-1-ol was accomplished using TsOH as a catalyst,[35] after which reaction with PCl_5 yielded the IC **12**. The same reaction sequence for homophthalic acid gave probe **13**. Selective reduction of the nitro-group in **12** in the presence of the alkyne function was achieved by reaction with iron powder in acetic acid. The resulting free amino group now allowed the introduction of different substituents at the 7-position, either small (**15**), hydrophobic (**16**) or basic (**17** and **18**).

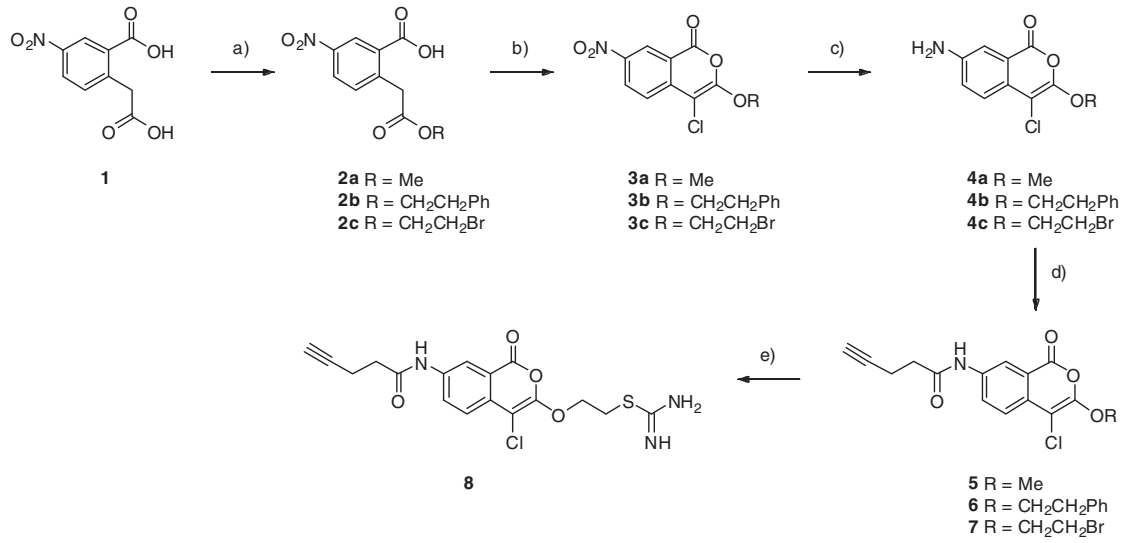
2.2. Labeling specificity

With the desired molecules at hand, we first tested out their labeling efficiency using members from different subclasses of serine proteases: bovine chymotrypsin, human cathepsin G and porcine pancreatic elastase, known for their specificity for different types of hydrophobic residues, and trypsin and urokinase-type plasminogen activator (uPA) as examples of trypsin-like proteases. ICs were used at a concentration of 2 μM (see Supplementary Fig. 1) and visualized using Cu(I)-mediated click chemistry with a tetramethylrhodamine (TAMRA) derivative carrying an azide function.

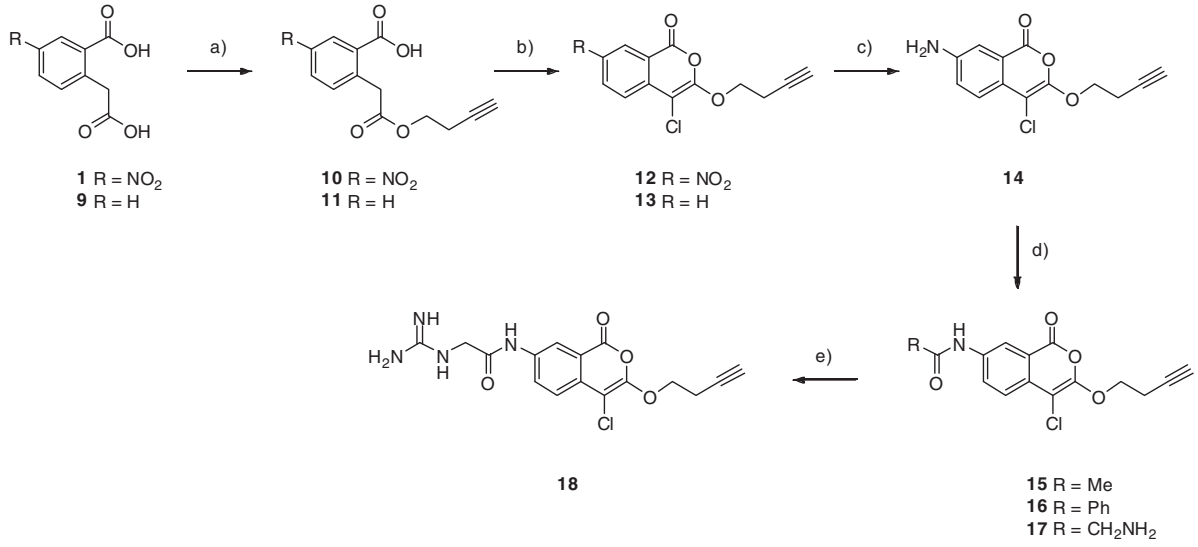
Probe **13** (Fig. 3a) reacted with all tested proteases. We attribute this to the lack of a 7-substituent that would induce selectivity.

Bovine chymotrypsin is labeled by all probes with approximately the same band intensity, including by the ICs with positively charged substituents (Fig. 3a). The reaction occurs in an activity-dependent manner, since pretreatment with an active site-directed covalent inhibitor abrogates labeling. We hypothesized that the alkyne function, which was intended to serve solely as a tagging handle, docks into the hydrophobic S1 pocket of chymotrypsin, which may explain its promiscuous behavior. To test this hypothesis, we modified the labeling experiment by performing the azide–alkyne click reaction before adding the enzyme. We assumed that once modified to a triazole ring holding a bulky fluorophore, the former alkyne group would no longer fit into the S1 pocket. As shown in Figure 3b, this holds true for compound **8**: in contrast to the alkyne, its 'clicked' version does not label chymotrypsin (lanes 3 and 4). IC **16** binds both before and after modification, probably because the 3-alkyne as well as the 7-benzoyl group can serve as recognition site for chymotrypsin (lanes 1 and 2). The bands resulting from compound **18**, which carries a positively charged 7-substituent, show that the hypothesized binding mode may not be true for all tested probes (lanes 5 and 6).

Human cathepsin G reacted with fewer probes than chymotrypsin (Fig. 3a). 3-Alkynes with small and hydrophobic substituents at the 7-position (**14** and **16**) labeled as well as 7-alkynes with small to medium-sized hydrophobic substituents at the 3-position (**5** and **7**). This finding is consistent with the chymotrypsin-like activity of cathepsin G. No reactivity towards probes with positively charged substituents was observed, although human cathepsin G also displays tryptic activity.[36]



Scheme 1. Synthesis of the 7-alkyne IC ABPs. Reagents and conditions: (a) R-OH, H_2SO_4 (cat), toluene, 70 °C; (b) PCl_5, toluene, 70 °C; (c) H_2, Pd–C, THF/$CHCl_3$; (d) 4-pentynoic acid, isobutyl chloroformate, *N*-methyl morpholine, THF, −20 °C → rt; (e) thiourea, THF, 65 °C.



Scheme 2. Synthesis of the 3-alkyne IC ABPs. Reagents and conditions: (a) 3-butyn-1-ol, *p*-TsOH, toluene, 70 °C; (b) PCl_5, toluene, 70 °C; (c) Fe(s), AcOH, 70 °C; (d) Ac_2O/*N*-methyl morpholine/DMAP (cat), or BzCl/pyridine/THF, or Boc-Gly-OH, isobutylchloroformate, *N*-methyl morpholine, THF, −20 °C → rt, then 25% TFA in DCM; (e) (1) di-Boc-guanidine-triflate, *N*-methyl morpholine, (2) 25% TFA in DCM.

Elastase was strongly labeled by the 7-alkynes with small and medium-sized hydrophobic 3-substituents (**5** and **7**), in accordance with the substrate preference of this protease for small hydrophobic amino acids. Compound **18**, with a positively charged 7-substituent, also reacted with elastase. All these labeling events could be blocked by inhibitor pretreatment, demonstrating the reaction's activity-dependency. In contrast, probes **14** and especially **12** label regardless of the presence of an inhibitor. Apparently, binding to a residue distant from the active site takes place, likely due to the high reactivity caused by the electron withdrawing nitro-substituent.

As expected, uPA, a serine protease with tryptic activity, is strongly labeled by probes **8**, **17** and **18**, which all have positively charged 3- or 7-substituents. The same pattern is seen for trypsin. This observation suggests that both the 3- and the 7-position can display a positively charged selectivity element to the S1 pocket of tryptic proteases, in line with previous data.[29] Labeling of uPA and trypsin was achieved by probes **12**–**14**, although only for uPA the reaction occured in an activity-dependent manner.

Overall, the majority of alkyne IC ABPs display activity-dependent labeling of their target proteases. The reaction reflects in most cases the P1 selectivity of the labeled protease. However, there are also some exceptions, making it difficult to rationally design and optimize IC ABPs for specific proteases without a crystal structure available.

2.3. Labeling in proteomes

At this stage, we aimed to find out what the labeling capacity of the IC APBs is in the context of a complex proteome. Therefore, we titrated decreasing amounts of selected proteases into a lysate of mammalian cells (EL4 mouse lymphoma) and added the ICs that displayed strong activity-based labeling in the previous experiments.

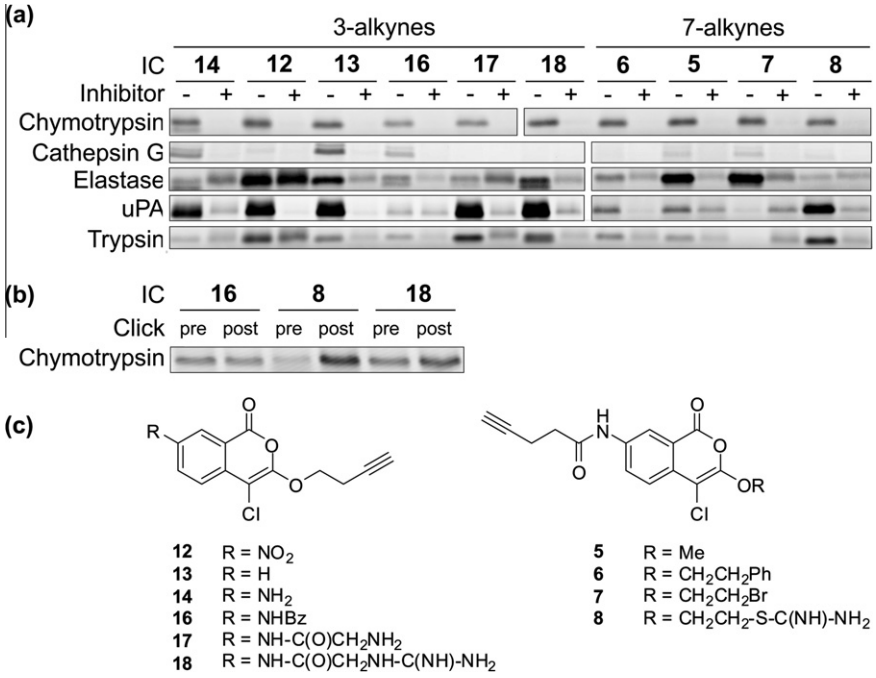


Figure 3. Labeling of representative members of serine protease subclasses using isocoumarin (IC) activity-based probes (ABPs). (a) 100 ng of protease per lane were incubated for 30 min with (+) or without (−) an active site-directed inhibitor (100 μM Ala-Ala-Phe-diphenylphosphonate for chymotrypsin, 1 mM PMSF for cathepsin G, 100 μM DFP for uPA and 1 mM dichloroisocoumarin for trypsin and elastase) and subsequently labeled with 2 μM of the indicated IC. The enzyme-probe complexes were detected by Cu(I)-catalyzed click chemistry and fluorescence scanning. (b) The Cu(I)-catalyzed click reaction of selected ICs with a fluorophore azide was performed before (pre) and after (post) reaction with chymotrypsin. Note that labeling by IC **8** is strongly diminished when functionalized with a fluorophore prior to labeling, indicating that the alkyne function is involved in recognition by chymotrypsin. (c) Overview of the used IC ABPs.

The protease uPA can be visualized by IC **18** at a concentration of 0.03% of total protein content (Fig. 4, right panel). IC **18** yielded some additional weak bands, which could not be blocked by DFP pretreatment indicating that these are off-target proteins that do not represent serine protease activities. IC **5** detected elastase without any background labeling at an amount as low as 1 ng (40 fmol) or 0.007% of total protein. Labeling of cathepsin G with IC **14** did also not lead to any off-target reactions, but was slightly less efficient, with a detection limit of 0.03% of total protein content.

To verify that the IC ABPs can be utilized to detect endogenously expressed, active proteases, a selection of IC ABPs with different substituents at the 7-position was reacted with a rat liver proteome, followed by click chemistry-mediated fluorescent detection. Compound **16** shows an exquisitely selective labeling of a band of approximately 25 kDa (Fig. 5, left panel, arrowhead). Pretreatment of the lysate with *N*-tosyl-phenylalanine-chloromethyl ketone (TPCK), a known mechanism-based inhibitor of chymotrypsin-like proteases, completely prevents detection of this band confirming the activity-dependent nature of the labeling. Although compound **13** reacted with all tested purified proteases (see Fig. 3) it displays a remarkable selectivity in the context of the rat liver proteome, where it labels one major band at 26 kDa (Fig. 5, middle panel, open arrowhead). Interestingly, this band can be competed by pretreatment with TPCK, but is distinct from the band tagged by **16** (see Supplementary Fig. 2), suggesting that the two IC probes can distinguish between different chymotryptic activities. Treatment of the rat liver proteome with IC **14** resulted in labeling of an intense band around 50 kDa, which could not be competed with TPCK, but disappeared by pretreatment with

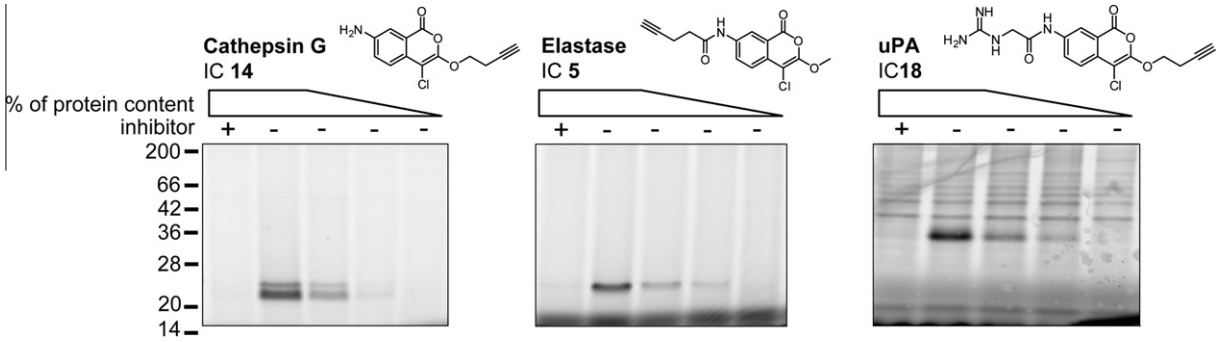


Figure 4. Labeling of serine proteases in a proteome background. Selected IC ABPs were used to determine the detection limits of three representative serine proteases. Decreasing amounts of pure proteases (0.7%, 0.2%, 0.03% and 0.007% of total protein content for human cathepsin G and uPA, and 0.2%, 0.03%, 0.007% and 0.001% for porcine pancreatic elastase) were spiked into a mammalian cell lysate (EL4 mouse lymphoma, 1 mg/mL). After labeling with the indicated IC ABPs, 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. Note that elastase was detected down to a concentration of 0.007%, cathepsin G and uPA down to 0.03% of total protein content.

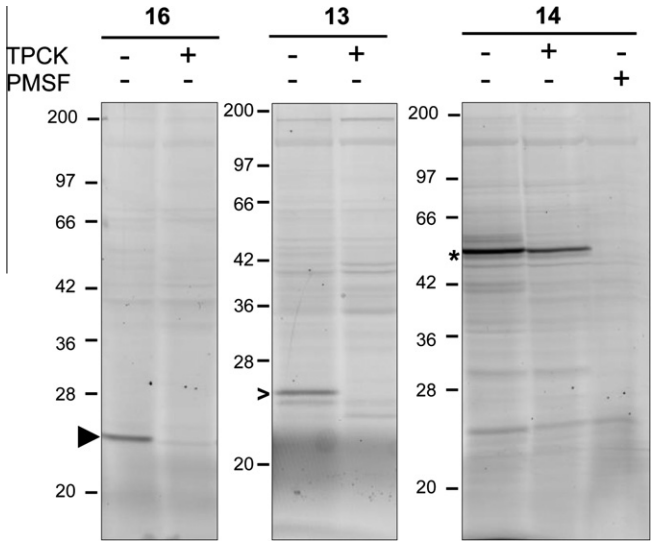


Figure 5. In vitro labeling of a rat liver proteome at pH 7.4 with ICs **13**, **14** and **16**. Target proteins were detected by click chemistry and fluorescent scanning. All proteome samples were pretreated with DMSO (−) or the indicated active-site directed serine protease inhibitor (+).

the more broad spectrum serine protease inhibitor PMSF (Fig. 5, right panel, star). Overall, these experiments show that the IC ABPs can label endogenous proteins in a selective and competable manner, dependent on the substituent at the 7-position.

3. Conclusion

In this study, we describe the synthesis and evaluation of alkyne derivatives of ICs as ABPs for serine proteases. We demonstrate that these ABPs can react with the different subclasses of serine proteases dependent on the substituents displayed on the IC scaffold. However, rational design of IC ABPs for specific proteases remains a challenge due to the lack of a clear structure–activity relationship.

Our ICs can detect target proteases in a whole proteome background down to 40 fmol or 0.007% of total protein content. These sensitivities are in the same order of magnitude as previously reported click ABPs.[37,38] Furthermore, experiments in a rat tissue proteome show clean, selective, activity-dependent labeling.

The alkyne handle on the described IC ABPs offers advantages over previously reported biotinylated ICs: the versatile choice of tags creates flexibility in the use of detection methods. Moreover, biotinylated molecules generally have poor cell permeability. Alkyne versions may circumvent this unfavorable property. Overall, we think that the clickable IC ABPs represent valuable compounds for the use in future functional proteomics studies of serine proteases.

4. Experimental

4.1. General

4-Nitrohomophthalic acid was synthesized as described in Ref. 33. All other reagents were obtained from commercial suppliers and used without further purification, unless noted otherwise. All solvents were of analytical grade, except for solvents used in silica column chromatography. Reactions were monitored by TLC (silica-gel 60) using UV light and/or treatment with cerium ammonium molybdate followed by heating. Preparative HPLC purification was performed on a Waters 515 HPLC system using an X-bridge C_{18} column. High resolution mass spectrometry analysis was performed on an Agilent 6210 LC–MS equipped with an electrospray TOF. NMR data were recorded on a Bruker 500 MHz Avance III.

4.2. Synthesis

4.2.1. Esterification of homophthalic acid and 4-nitrohomophthalic acid

A suspension of the homophthalic acid and the appropriate alcohol (3 equiv) in toluene (2 mL/mmol) was heated to 70 °C. Several drops of 98% sulfuric acid were added, except for the alcohol 3-butyn-1-ol, for which 0.1 equiv pTsOH was used. The mixture was stirred for 4–6 h when TLC indicated complete consumption of the starting acid. After cooling to room temperature, the reaction mixture was quenched with diluted sodium bicarbonate and extracted twice with ethyl acetate. Next, the aqueous layer was acidified using concentrated HCl and extracted twice with ethyl acetate. The resulting organic layer was dried ($MgSO_4$), filtered and concentrated under reduced pressure. The material was used in the next step without further purification.

4.2.2. Formation of the 3-alkoxy-4-chloro-isocoumarin scaffolds

PCl_5 (2.5 equiv) was added to a solution of the mono-esterified homophthalic acid derivatives in toluene (10 mL/mmol) and the resulting solution was stirred overnight at 70 °C. The reaction mixture was cooled down, diluted with ethyl acetate and extracted with sodium bicarbonate. Water layers were backextracted with ethyl acetate and the combined organic layers were washed with sodium bicarbonate and brine, dried ($MgSO_4$), concentrated under reduced pressure and subjected to silica column chromatography (50–0% petroleum ether in toluene).

4.2.3. Coupling of pentynoic acid to the aniline function

Pentynoic acid (dried by coevaporation of water with dioxane) was pre-activated in THF (2 mL/mmol) at −20 °C for 30 min with isobutyl chloroformate (1.1 equiv) and *N*-methylmorpholine (1.2 equiv with respect to pentynoic acid). Next, 2 equiv of activated pentynoic acid were added to a stirred solution of the aniline in THF (final concentration 0.2–0.25 M) and the reaction was allowed to warm up to room temperature. After TLC analysis revealed dissapearance of the starting material, the reaction mixture was diluted with EtOAc and subsequently washed with 1 M $KHSO_4$, saturated $NaHCO_3$ and brine. The organic layer was dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude material was purified by HPLC.

4.2.4. 4-Chloro-3-methoxy-7-nitro-isocoumarin (3a)

The title compound was isolated as a yellow solid in 51% yield from its mono-esterified nitro-homophthalic acid precursor. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.05 (d, 1H, J = 2.4 Hz), 8.53 (dd, 1H, J = 2.4 Hz, J = 9.0 Hz), 7.84 (d, 1H, J = 8.9 Hz), 4.18 (s, 3H). ^{13}C DEPT-NMR (126 MHz, $CDCl_3$): δ 129.8, 126.4, 123.4, 57.3. ESI-HRMS: $[M+H]^+$ m/z 255.9904 (found), $C_{10}H_7ClNO_5^+$ requires 256.0007.

4.2.5. 4-Chloro-3-(2-phenyl-ethoxy)-7-nitro-isocoumarin (3b)

The title compound was isolated as a yellow solid in 38% yield from its mono-esterified nitro-homophthalic acid precursor. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.71 (d, 1H, J = 2.4 Hz), 8.59 (dd, 1H, J = 2.5 Hz, J = 8.9 Hz), 7.81 (d, 1H, J = 8.9 Hz), 7.37–7.19 (m, 5H), 4.67 (t, 2H, J = 6.6 Hz), 3.11 (t, 2H, J = 6.6 Hz). ^{13}C DEPT-NMR (126 MHz, DMSO-d_6): δ 129.6, 128.8, 128.1, 126.3, 124.7, 122.8, 71.0, 34.4.

4.2.6. 4-Chloro-3-(2-bromo-ethoxy)-7-nitro-isocoumarin (3c)

The title compound was isolated as a yellow solid in 52% yield from its mono-esterified nitro-homophthalic acid precursor. ^{1}H

NMR (500 MHz, $CDCl_3$) δ 9.07 (d, 1H, J = 2.3 Hz), 8.57 (dd, 1H, J = 2.3 Hz, J = 8.9 Hz), 7.89 (d, 1H, J = 8.9 Hz), 4.77 (t, 2H, J = 6.1 Hz), 3.71 (t, 2H, J = 6.1 Hz). ^{13}C DEPT-NMR (126 MHz, $CDCl_3$): δ 129.8, 126.3, 123.9, 69.6, 27.7. ESI-HRMS: $[M+H]^+$ m/z 347.9303 (found), $C_{11}H_8BrClNO_5^+$ requires 347.9269.

4.2.7. 7-Amino-4-chloro-3-methoxy-isocoumarin (4a)

A mixture of **3a** in THF/$CHCl_3$ 4:1 and palladium on carbon was stirred under an atmosphere of hydrogen until TLC revealed complete disappearance of the starting material. The reaction mixture was filtered and concentrated in vacuo. The title compound was isolated quantitatively as a yellow powder. ^{1}H NMR (500 MHz, $CDCl_3$ + methanol-d_4) δ 7.56 (d, 1H, J = 8.6 Hz), 7.48 (d, 1H, J = 2.5 Hz), 7.22 (dd, 1H, J = 2.5 Hz, J = 8.6 Hz), 4.05 (s, 3H). ^{13}C DEPT-NMR (126 MHz, $CDCl_3$ + methanol-d_4): δ 124.8, 124.0, 113.9, 57.6. ESI-HRMS: $[M+H]^+$ m/z 226.0173 (found), $C_{10}H_9ClNO_3^+$ requires 226.0265

4.2.8. 7-Amino-4-chloro-3-(2-phenyl-ethoxy)-isocoumarin (4b)

A mixture of **3b** in THF/$CHCl_3$ 4:1 and palladium on carbon was stirred under an atmosphere of hydrogen until TLC revealed complete disappearance of the starting material. The reaction mixture was filtered and concentrated in vacuo. The title compound was isolated as a yellow powder in 87% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.40 (d, 1H, J = 8.6 Hz), 7.34–7.19 (m, 6H), 7.15 (dd 1H, J = 2.5 Hz, J = 8.6), 4.47 (t, 2H, J = 6.8 Hz), 3.05 (t, 2H, J = 6.8 Hz). ^{13}C DEPT-NMR (126 MHz, DMSO-d_6): δ 128.7, 128.0, 126.2, 122.9, 122.7, 110.7, 71.1, 34.8. ESI-HRMS: $[M+H]^+$ m/z 316.0674 (found), $C_{17}H_{15}ClNO_3^+$ requires 316.0735.

4.2.9. 7-Amino-3-(2-bromoethoxy)-4-chloro-isocoumarin (4c)

A mixture of **3c** in THF/AcOH 2:1 and palladium on carbon was stirred under an atmosphere of hydrogen until TLC revealed complete disappearance of the starting material. The reaction mixture was filtered, concentrated under reduced pressure and purified by flash column chromatography (10–50% EtOAc in toluene), yielding the title compound as a yellow powder in 56% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.44 (d 1H, J = 8.6 Hz), 7.29 (d, 1H, J = 2.4 Hz), 7.17 (dd, 1H, J = 2.5 Hz, J = 8.6 Hz), 4.56 (t, 2H, J = 5.5 Hz), 3.80 (t, 2H, J = 5.5 Hz). ^{13}C DEPT-NMR (126 MHz, DMSO-d_6): δ 123.1, 122.7, 110.8, 70.3, 30.5. ESI-HRMS: $[M+H]^+$ m/z 317.9637 (found), $C_{11}H_{10}BrClNO_3^+$ requires 317.9527.

4.2.10. 4-Chloro-3-methoxy-7-(4-pentynoylamino)-isocoumarin (5)

The title compound was isolated as a fluffy, slightly yellow solid in 35% yield after HPLC purification. ^{1}H NMR NMR (500 MHz, DMSO-d_6) δ 10.37 (s, 1H), 8.50 (d, 1H, J = 2.2 Hz), 7.99 (dd, 1H, J = 2.3 Hz, J = 8.8 Hz), 7.64 (d, 1H, J = 8.7 Hz), 4.02 (s, 3H), 2.81 (t, J = 2.6 Hz, 1H), 2.59–2.55 (m, 2H), 2.52–2.48 (m, 2H). ^{13}C DEPT-NMR (126 MHz, DMSO-d_6): δ 126.8, 122.2, 117.8, 83.2, 71.3, 57.0, 39.7, 39.5, 39.3, 34.9, 13.7.

4.2.11. 4-Chloro-7-(4-pentynoylamino)-3-(2-phenylethoxy)-isocoumarin (6)

The title compound was isolated as a fluffy, slightly yellow solid in 59% yield after HPLC purification. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.37 (s, 1H), 8.48 (d, 1H, J = 2.2 Hz), 7.99 (dd, 1H, J = 2.3 Hz, J = 8.8 Hz), 7.63 (d, 1H, J = 8.7 Hz), 7.36–7.26 (m, 4H), 7.25–7.18 (m, 1H), 4.55 (t, 2H, J = 6.7 Hz), 3.07 (t, 2H, J = 6.7 Hz), 2.80 (t, 1H, J = 2.6 Hz), 2.56 (d, 2H, J = 6.6 Hz), 2.53–2.46 (m, 2H). ^{13}C DEPT-NMR (126 MHz, DMSO-d_6): δ 128.7, 128.0, 126.8, 126.2, 122.4, 117.83 83.2, 71.3, 70.8, 34.9, 34.7, 13.7. ESI-HRMS: $[M+H]^+$ m/z 396.0912 (found), $C_{22}H_{19}ClNO_4^+$ requires 396.0997.

4.2.12. 3-(2-Bromoethoxy)-4-chloro-7-(4-pentynoylamino)-isocoumarin (7)

The title compound was isolated as a fluffy, slightly yellow solid in 30% yield after HPLC purification. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.39 (s, 1H), 8.51 (d, 1H, J = 2.2 Hz), 8.01 (dd, 1H, J = 2.3 Hz, J = 8.7 Hz), 7.68 (d, 1H, J = 8.7 Hz), 4.64 (t, 2H, J = 5.5 Hz), 3.85 (t, 2H, J = 5.5 Hz), 2.81 (t, 1H, J = 2.6 Hz), 2.57 (t, 2H, J = 6.9 Hz), 2.53–2.46 (m, 2H).). ^{13}C DEPT-NMR (126 MHz, DMSO-d_6): δ 126.8, 122.6, 117.9, 83.2, 71.3, 69.9, 34.9, 30.4, 13.7. ESI-HRMS: $[M+H]^+$ m/z 397.9796 (found), $C_{16}H_{14}BrClNO_4^+$ requires 397.9795.

4.2.13. 4-Chloro-7-(4-pentynoylamino)-3-(2-isothioureidoethoxy)-isocoumarin (8)

A solution of compound **7** (6.62 mg) and thiourea (2.6 mg, 2 equiv) in THF/DMF (1:1, 350 μL) was stirred at 65 °C for 2 d. The reaction mixture was purified by HPLC and the title compound was isolated as a fluffy, slightly yellow solid in 30% yield. ^{1}H NMR (500 MHz, DMSO-d_6 + methanol-d_4) δ 8.54 (d, 1H, J = 2.0 Hz), 8.04–7.97 (m, 1H), 7.69 (d, 1H, J = 8.7 Hz), 4.57 (t, 2H, J = 5.8 Hz), 3.63 (t, 2H, J = 5.8 Hz), 2.80 (t, 1H, J = 2.6 Hz), 2.60–2.56 (m, 2H), 2.53–2.48 (m, 2H). ^{13}C DEPT-NMR (126 MHz, DMSO-d_6 + methanol-d_4): δ 126.8, 122.6, 117.9, 83.2, 71.3, 68.1, 34.9, 29.5, 13.7. ESI-HRMS: $[M+H]^+$ m/z 394.0725 (found), $C_{16}H_{14}BrClNO_4^+$ requires 394.0623.

4.2.14. 3-(3-Butynoxy)-4-chloro-7-nitro-isocoumarin (12)

The title compound was isolated as a yellow solid in 59% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.03 (d, 1H, J = 2.4 Hz), 8.54 (dd, 1H, J = 2.4 Hz, J = 8.9 Hz), 7.86 (d, 1H, J = 8.9 Hz), 4.59 (t, 2H, J = 6.8 Hz), 2.78 (dt, 2H, J = 2.7 Hz, J = 6.8 Hz), 2.08 (t, 1H, J = 2.7 Hz). ^{13}C DEPT-NMR (126 MHz, $CDCl_3$): δ 129.8, 126.3, 123.7, 78.6, 71.1, 68.3, 19.7. ESI-HRMS: $[M+H]^+$ m/z 294.0155 (found) $C_{13}H_9ClNO_5^+$ requires 294.0164.

4.2.15. 3-(3-Butynoxy)-4-chloro-isocoumarin (13)

The title compound was isolated as an off-white solid in 42% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.12 (dd, 1H, J = 0.9 Hz, J = 7.9 Hz), 7.91 (ddd, 1H, J = 1,3 Hz, J = 7.3 Hz, J = 8.5 Hz), 7.74–7.64 (m, 1H), 7.52 (ddd, 1H, J = 1.1 Hz, J = 7.4 Hz, J = 8.2 Hz), 4.43 (t, 2H, J = 6.4 Hz), 2.92 (t, 1H, J = 2.7 Hz), 2.70 (dt, 2H, J = 2.7 Hz, J = 6.4 Hz). ^{13}C DEPT-NMR (126 MHz, DMSO-d_6): 135.8, 129.4, 126.4, 121.6, 80.1, 72.6, 68.0, 18.8. ESI-HRMS: $[M+H]^+$ m/z 294.0155 (found) $C_{13}H_9ClNO_5^+$ requires 294.0164.

4.2.16. 7-Amino-3-(3-butynoxy)-4-chloro-isocoumarin (14)

Powdered iron (9 equiv) was added to solution of compound **12** (130 mg) in acetic acid (2.5 mL) and stirred at 70 °C until TLC revealed complete conversion of the starting material to a lower running spot (30 min). The reaction mixture was filtered over a layer of sand, which was subsequently washed with methanol. The filtrate was concentrated under reduced pressure and the residue was purified by flash column chromatography (0–50% EtOAc in toluene). The title compound was isolated as a yellow solid (82 mg, 71% yield). ^{1}H NMR (500 MHz, $CDCl_3$ + methanol-d_6): δ 7.54 (d, 1H, J = 8.6 Hz), 7.43 (d, 1H, J = 2.5 Hz), 7.10 (dd, 1H J = 2.5 Hz, J = 8.6 Hz), 4.39 (t, 2H, J = 7.0 Hz), 2.69 (dt, 2H, J = 2.7 Hz, J = 6.9 Hz), 2.02 (t, 1H J = 2.7 Hz). ^{13}C DEPT-NMR (126 MHz, $CDCl_3$ + methanol-d_6): δ 124.3, 124.2, 113.0, 70.7, 69.0, 19.9. ESI-HRMS: $[M+H]^+$ m/z 264.0409 (found) $C_{13}H_9ClNO_5^+$ requires 264.0427.

4.2.17. 7-Acetylamino-3-(3-butynoxy)-4-chloro-isocoumarin (15)

To a solution of compound **14** (10.1 mg) in THF (250 μL) were added acetic anhydride (9.6 μL, 2.5 equiv), *N*-methylmorpholine (4.3 μL, 1.2 equiv) and a catalytic amount of dimethylamino–pyridine. After TLC revealed complete disappearance of the starting

material, the reaction mixture was diluted with acetonitrile/water/TFA 50:50:0.1 and purified by HPLC. Peak fractions were pooled and lyophilized, yielding the title compound as a fluffy, slightly yellow solid in 53%. ^{1}H NMR (500 MHz, $CDCl_3$ + methanol-d_4): δ 8.28 (d, 1H, J = 8.8 Hz), 8.09 (d, 1H, J = 1.9 Hz), 7.69 (d, 1H, J = 8.8 Hz), 4.46 (t, 2H, J = 6.9 Hz), 2.77–2.71 (m, 2H), 2.19 (s, 3H), 2.07 (t, 1H, J = 2.6 Hz). ^{13}C DEPT-NMR (126 MHz, $CDCl_3$ + methanol-d_4): δ 128.1, 123.6, 119.3, 79.0, 70.7, 68.5, 24.1, 19.8. ESI-HRMS: $[M+H]^+$ m/z 306.0509 (found) $C_{15}H_{13}ClNO_4^+$ requires 306.0528.

4.2.18. 7-Benzoylamino-3-(3-butynoxy)-4-chloro-isocoumarin (16)

Benzoyl chloride (9 μL, 2 equiv) and pyridine (9.2 μL, 3 equiv) were added to a solution of compound **14** (10 mg, dried by coevaporation of water with dioxane) in THF (100 μL). After 30 min, the reaction mixture was diluted with acetonitrile and water, and purified by HPLC. Peak fractions were pooled and lyophilized, yielding the title as a fluffy, slightly yellow solid in 46%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.63 (s, 1H), 8.69 (d, 1H, J = 2.2 Hz), 8.30 (dd, 1H, J = 8.8 Hz, J = 2.3 Hz), 8.00 (dd, 2H, J = 5.3, J = 3.3 Hz), 7.72 (d, 1H, J = 8.7 Hz), 7.65–7.53 (m, 3H), 4.43 (t, 2H, J = 6.4 Hz), 2.93 (t, 1H, J = 2.6 Hz), 2.71 (dt, 2H, J = 6.4 Hz, J = 2.7 Hz). ^{13}C DEPT-NMR (126 MHz, DMSO-d_6): δ 132.6, 131.6, 129.0, 128.3, 128.2, 127.9, 127.4, 122.3, 119.2, 80.1, 72.6, 68.2, 18.8.

4.2.19. 3-(3-Butynoxy)-4-chloro-7-glycylamino-isocoumarin (17)

Boc-glycine (88 mg, dried by coevaporation of water with toluene) was activated at −20 °C with isobutyl chloroformate (72 μL, 1.1 equiv) and *N*-methylmorpholine (60 μL, 1.2 equiv) in THF (0.5 mL) and stirred for 30 min. To a solution of compound **14** (dried by coevaporation of water with toluene) and *N*-methylmorpholine (8.4 μL) in THF (0.1 mL) was added 0.3 mL of the activated glycine mixture (2 equiv with respect to compound **14**), and the final solution was allowed to warm up to room temperature. After 1 h, the reaction was diluted with EtOAc, washed with 1 M $KHSO_4$, water, saturated $NaHCO_3$ and brine. The organic layer was dried ($MgSO_4$), filtered and evaporated under reduced pressure. The crude material was then treated with 25% TFA in DCM for 1 h and subjected to HPLC purification. Peak fractions were pooled and lyophilized, giving the title compound as a fluffy, slightly yellow solid in 39% yield over two steps. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.50 (d, 1H, J = 2.2 Hz), 8.24 (s, 1H), 7.99 (dd, 1H, J = 2.3 Hz, J = 8.7 Hz), 7.73 (d, 1H, J = 8.7 Hz), 4.42 (t, 2H, J = 6.4 Hz), 3.84 (s, 2H), 2.92 (d, 1H, J = 2.2 Hz), 2.7 (dt, 2H, J = 2.7 Hz, J = 6.4 Hz). ^{13}C DEPT-NMR (126 MHz, DMSO-d_6): δ 126.8, 122.9, 118.1, 80.1, 72.6, 68.3, 40.7, 18.8. ESI-HRMS: $[M+H]^+$ m/z 321.0658 (found) $C_{15}H_{14}ClN_2O_4^+$ requires 321.0637.

4.2.20. 3-(3-Butynoxy)-4-chloro-7-[guanidinoacetyl]amino-isocoumarin (18)

To a solution of compound **17** (7.95 mg) in DCM/DMF 1:1 (0.4 mL) were added *N, N′*-di-Boc-guanidine-*N″*-triflate (9.7 mg, 1 equiv) and *N*-methylmorpholine (2.7 μL, 1 equiv). After 30 min, the reaction was diluted with EtOAc and subsequently washed with 1 M $KHSO_4$, water, dilute $NaHCO_3$ and brine. The organic phase was dried on $MgSO_4$, filtered and concentrated under reduced pressure. The crude material was treated with 25% TFA in DCM for 1 h and HPLC purified, giving the title compound as a fluffy, slightly yellow solid in 29% yield. ^{1}H NMR (500 MHz, methanol-d_4): δ 8.48 (bs, 1H), 7.97 (d, 1H, J = 8.7 Hz), 7.71 (d, 1H, J = 8.7 Hz), 4.42 (t, 2H, J = 6.6 Hz), 4.11 (s, 2H), 2.68 (t, 2H, J = 6.6 Hz), 2.32 (bs, 1H). ^{13}C DEPT-NMR (126 MHz, methanol-d_4): δ 126.8, 122.9, 118.1, 80.1, 72.6, 68.3, 40.7, 18.8. ESI-HRMS: $[M+H]^+$ m/z 363.0684 (found) $C_{16}H_{16}ClN_4O_4^+$ requires 363.0855.

4.3. Click chemistry

We used Cu(I)-catalyzed click chemistry to visualize the complexes of IC ABPs bound to their target enzymes. After the labeling reactions, the following reagents were added: 25 μM TAMRA-PEG-N_3, 50 μM tris(3-hydroxypropyltriazolyl-methyl)amine (THPTA) ligand[39] (both fluorophore and ligand from 5 mM DMSO stocks), 1 mM $CuSO_4$ (from a freshly prepared 50 mM stock in H_2O) and 500 μM Na^+ ascorbate (from a freshly prepared 25 mM stock in H_2O). The reaction was allowed to proceed for 30 min at room temperature (for pure proteases) or on ice (for lysates) and was stopped by addition of 4× sample buffer and heating for 3 min at 95 °C. Protein samples were separated on 12% or 15% SDS polyacrylamide gels. Fluorescent bands were detected by scanning with a Typhoon TRIO + fluorescent scanner (excitation at 532 nm, emission at 580 nm).

4.4. Labeling of purified proteases

Cathepsin G was diluted in HEPES buffer (50 mM pH 7.4 containing 100 mM NaCl), all other proteases in phosphate buffer (50 mM, pH 7.4), or EL4 cell lysate (1 mg/mL protein in 50 mM phosphate buffer, pH 7.4). Lysates were prepared by incubating EL4 mouse lymphoma cells in RIPA buffer on ice for 30 min and separating insoluble cell debris by centrifugation). Where indicated, proteases were pre-blocked with active site-directed inhibitors: 1 mM 3,4-dichloroisocoumarin for elastase and trypsin, 100 μM DFP for urokinase, 100 μM of Ala-Ala-Phe-phosphonate[40] for chymotrypsin and 1 mM PMSF for cathepsin G. Subsequently, 2 μM IC ABP was added and the mixtures were allowed to react for 15 min at room temperature (for pure proteases) or on ice (for lysates). Click chemistry was then performed for visualization as described under 4.3. The amount of 100 ng of purified proteases or 15 μg of total protein (for proteases in a proteome background) were loaded per gel lane.

4.5. Labeling of rat liver proteome

Part of a rat liver was passed through a 70 μM cell strainer, 0.5% nonidet P-40 substitute in PBS was added (~2 mL/g of tissue) and and cells were incubated on ice. After 1 h, cell debris was spun down in a tabletop eppendorf centrifuge at 15,000 rpm for 10 min. The supernatant was collected, snap-frozen in liquid nitrogen and stored at −80 °C until usage.

Rat liver proteome (diluted to 1 mg/mL total protein with 50 mM phosphate buffer) was pretreated with TPCK (100 μM), PMSF (1 mM) or DMSO for 30 min on ice. Then, proteomes were treated with the IC ABP (1 μM for **13** and **16**; 0.5 μM for **14**) for 30 min on ice. Click chemistry with *N*-TAMRA-3-azido-propylamine was performed as described in section 4.3.

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Supplementary data

Supplementary data (two extra figures and copies of all ^{1}H NMR spectra) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.03.014.

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