ACCOUNTS
of chemical research

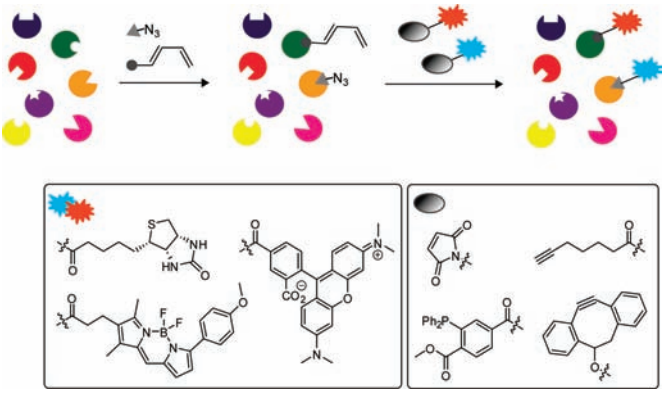
Bioorthogonal Chemistry: Applications in Activity-Based Protein Profiling

LIANNE I. WILLEMS, WOUTER A. VAN DER LINDEN, NAN LI, KAH-YEE LI, NORA LIU, SASCHA HOOGENDOORN, GIJS A. VAN DER MAREL, BOGDAN I. FLOREA,* AND HERMAN S. OVERKLEEFT*

Leiden Institute of Chemistry and The Netherlands Proteomics Centre, Einsteinweg 55, 2300 RA Leiden, The Netherlands

RECEIVED ON MAY 2, 2011

CONSPECTUS



The close interaction between organic chemistry and biology goes back to the late 18th century, when the modern natural sciences began to take shape. After synthetic organic chemistry arose as a discipline, organic chemists almost immediately began to pursue the synthesis of naturally occurring compounds, thereby contributing to the understanding of their functions in biological processes. Research in those days was often remarkably interdisciplinary; in fact, it constituted chemical biology research before the phrase even existed. For example, histological dyes, both of an organic and inorganic nature, were developed and applied by independent researchers (Gram and Golgi) with the aim of visualizing cellular substructures (the bacterial cell wall and the Golgi apparatus).

Over the years, as knowledge within the various fields of the natural sciences deepened, research disciplines drifted apart, becoming rather monodisciplinary. In these years, broadly ranging from the end of World War II to about the 1980s, organic chemistry continued to impact life sciences research, but contributions were of a more indirect nature. As an example, the development of the polymerase chain reaction, from which molecular biology and genetics research have greatly profited, was partly predicated on the availability of synthetic oligonucleotides. These molecules first became available in the late 1960s, the result of organic chemists pursuing the synthesis of DNA oligomers primarily because of the synthetic challenges involved.

Today, academic natural sciences research is again becoming more interdisciplinary, and sometimes even multidisciplinary. What was termed "chemical biology" by Stuart Schreiber at the end of the last century can be roughly described as the use of intellectually chemical approaches to shed light on processes that are fundamentally rooted in biology. Chemical tools and techniques that are developed for biological studies in the exciting and rapidly evolving field of chemical biology research include contributions from many areas of the multifaceted discipline of chemistry, and particularly from organic chemistry. Researchers apply knowledge inherent to organic chemistry, such as reactivity and selectivity, to the manipulation of specific biomolecules in biological samples (cell extracts, living cells, and sometimes even animal models) to gain insight into the biological phenomena in which these molecules participate.

In this Account, we highlight some of the recent developments in chemical biology research driven by organic chemistry, with a focus on bioorthogonal chemistry in relation to activity-based protein profiling. The rigorous demands of bioorthogonality have not yet been realized in a truly bioorthogonal reagent pair, but remarkable progress has afforded a range of tangible contributions to chemical biology research. Activity-based protein profiling, which aims to obtain information on the workings of a protein (or protein family) within the larger context of the full biological system, has in particular benefited from these advances. Both activity-based protein profiling and bioorthogonal chemistry have been around for approximately 15 years, and about 8 years ago the two fields very profitably intersected. We expect that each discipline, both separately and in concert, will continue to make important contributions to chemical biology research.

Published on the Web 07/28/2011 www.pubs.acs.org/accounts
10.1021/ar200125k

Introduction

In a bioorthogonal ligation process, two reactants that are added to a biological sample react with each other in a chemoselective manner, which means that they are inert to any other chemical entity present. At the same time, the chemistry needs to be compatible with living systems. Ideally, the reactants that participate in a bioorthogonal ligation reaction should also react fast, in a 1:1 ratio and in quantitative yield with respect to each other, conditions that are commonly seen as typical features of "click" chemistry. Such bioorthogonal reactions are related to bioconjugation processes with one important difference, namely that the reaction between for example a biomolecule and a small organic entity takes place *in the presence of all reactivities present in the biological system at hand*. Given that a single, quantitative process of two isolated reactants in a 1:1 ratio is difficult to achieve in synthetic organic chemistry practice, it is not surprising that to date a truly bioorthogonal reagent pair has not been developed. In recent years, however, remarkable progress has been made and bioorthogonal chemistry has matured such that it makes tangible contributions to chemical biology research. From an organic chemical point of view, biological samples in which bioorthogonal reactions are intended to take place increase in complexity ranging from a cell extract (tissue lysate) to a cell (tissue culture) to an animal model. Obviously, the more complex the biological mixture, the more difficult it is to achieve a selective chemical transformation. The actual complexity of a biological sample is also dependent on the type of experiment: whereas labeling of a biomolecule in cell extracts has to occur in the presence of (nearly) all possible biomolecules, in a cell-surface labeling experiment "only" those molecules present at the outer cell membrane may interfere. From a biological point of view, the bioorthogonal ligation process should obviously not interfere with the integrity of the tissue/organism at hand. The development of bioorthogonal chemistry is further hampered by the fact that nature does not set guidelines on which direction to take. This is in contrast to other areas of interdisciplinary organic chemistry/biochemistry research, in which biological processes often are a source of inspiration. Chemical reactants that can partake in a successful bioorthogonal reaction need to have a high intrinsic reactivity toward each other, whereas at the same time they need to be inert toward any other biological functionality. Nature has solved the problem of selectivity by evolving catalysts (enzymes, ribosomes) that recognize intrinsically relatively inert functionalities and cause these to react, often in a highly controlled and selective manner. Bioorthogonal chemistry aims to develop reactant pairs that, in an ideal situation, react in a biological environment without the assistance of a (biological) catalyst.

Bioorthogonal Chemistry: Scope and Limitations

The concept of bioorthogonal chemistry was introduced first by Bertozzi and co-workers in their seminal work on cell surface *N*-glycoprotein labeling. In a first installment, they revealed that *N*-levulinoyl-mannosamine is a valid substrate of the CMP-sialic acid biosynthesis machinery of mammalian cells.[1] The resulting modified CMP-sialic acids are in turn transferred by sialic acid transferases to *N*-glycoprotein chains to eventually end up on the cell surface. Next, the ketone moiety of the levulinoyl groups can react with a tagged (fluorophore, affinity tag) hydrazide in a bioorthogonal fashion, as neither of the two reactants is present in naturally occurring cell surface biomolecules. In a subsequent study that revolutionalized the field, the Bertozzi group demonstrated that the same objective can be achieved with much higher efficiency by metabolic incorporation of an azide (in *N*-azidoacetylmannosamine, or ManNAz) followed by reaction with a biotin phosphane in what has become known as the Staudinger ligation or Staudinger–Bertozzi ligation.[2] Dating from the same period is the advent of the copper(I)-catalyzed azide–alkyne cycloaddition ("click" reaction), which has many applications in synthetic organic chemistry, bioorganic chemistry, and in particular chemical biology.[3−5] This process requires the installation of either an azide or an alkyne in a biomolecule, for instance, via engineering of ribosomal protein synthesis to introduce either propargylglycine or azidohomoalanine in a nascent polypeptide. The complementary reactant is part of a reporter molecule, for instance, a biotinylated or fluorescent azide or alkyne. Over the past decade, "strain-promoted" click reagents have been developed that take away the need of a copper catalyst.[6]

For a chemical transformation to be of use to synthetic organic chemistry, it needs to be both efficient, in the sense that at least the most elaborate or expensive reaction partner is transformed in (near) quantitative fashion, as well as clean, so that unwanted side reactions are limited. Yields, the nature of (side) products, and reaction kinetics are the

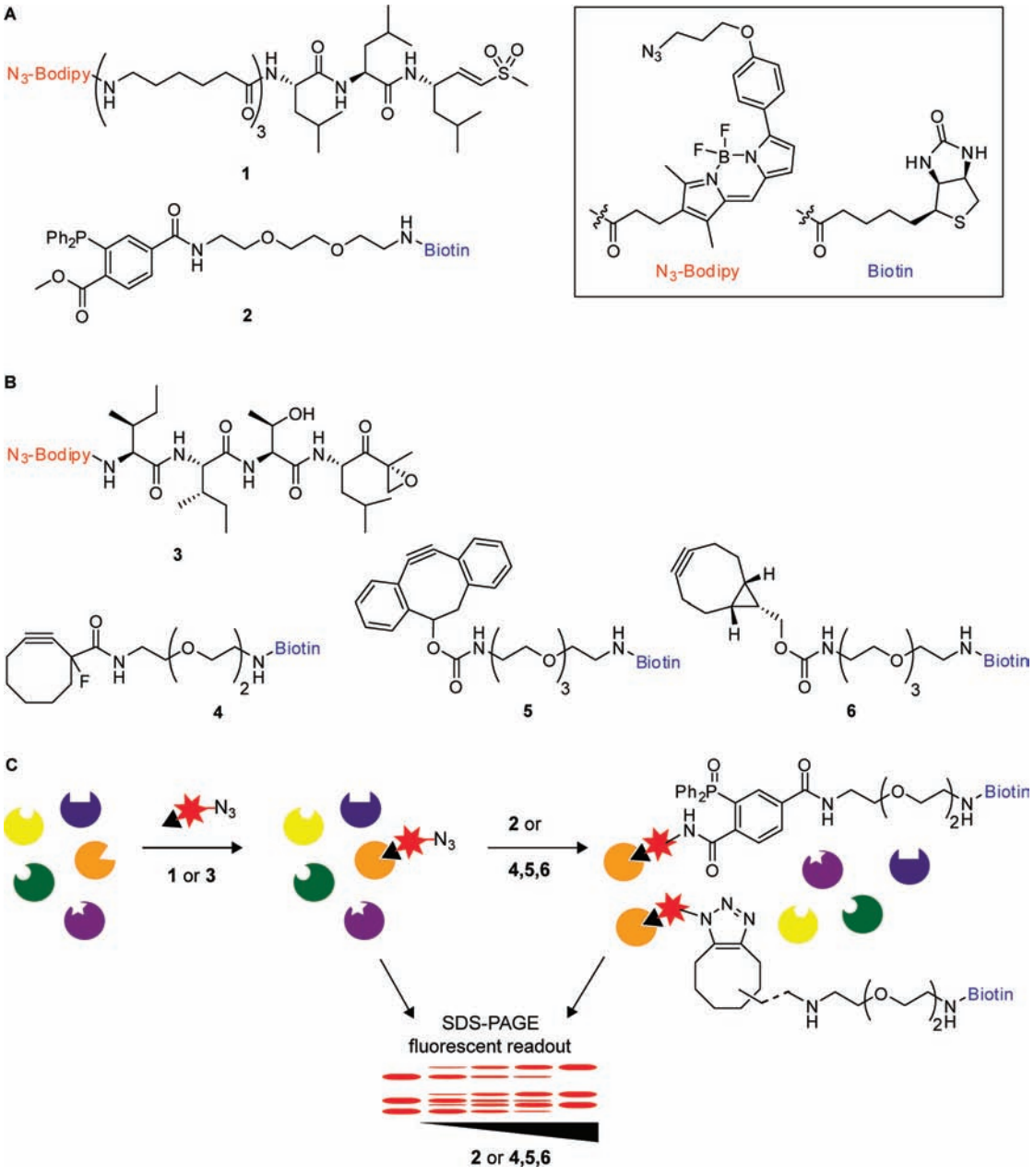


FIGURE 1. Bioorthogonal chemistry in profiling of active proteasome subunits. (A, B) Azide-functionalized fluorescent activity-based proteasome probes (**1**, **3**), biotin-phosphane (**2**) for Staudinger–Bertozzi ligation, and biotin-cyclooctynes (**4**–**6**) for "strain-promoted" azide–alkyne cycloaddition. (C) Ligation efficiency can be determined from a gel-shift of the fluorescent protein bands.

parameters on which a synthetic transformation is judged, and these should be considered also in evaluating the merits of a bioorthogonal ligation. With respect to the Staudinger–Bertozzi ligation, these aspects have been studied in some detail (Figure 1). Making use of a fluorescent activity-based proteasome probe equipped with an azide (Figure 1A, compound **1**), we could reveal that Staudinger–Bertozzi ligation in cell extracts can be pushed to completion, provided that an excess of biotin-phosphane **2** is added.[7] In this experiment, we first treated cultured cells with the pan-reactive proteasome inhibitor **1**. After cell lysis, SDS-PAGE separation of the protein content and fluorescence readout, protein bands corresponding to the catalytically active subunits of the murine proteasome (β1, β2, and β5 and their immunogenic counterparts) appear. The efficiency of the Staudinger–Bertozzi ligation is apparent from a shift

of the fluorescently labeled proteins on gel after exposure of the inhibitor-treated lysates to the biotin-phosphane, the molecular weight of which is roughly 1 kDa, inducing a detectable gel-shift of the proteasome activities at 25−35 kDa (Figure 1C). Complete ligation could be achieved by applying 100−200 equiv of biotin-phosphane **2** relative to the activity-based probe **1** with relatively little background labeling (that is, little cross-reactivity of **2** to endogenous functionalities). The yield in terms of conversion of reactant **2** is therefore poor, which is most likely due to the inherent instability of the trivalent phosphine. This issue was the subject of detailed studies by Bertozzi and co-workers. They revealed that oxidation of the phosphine in **2**, and reactions ensuing from this event, is at the basis of this complication.[8]

In recent years, a wide and rapidly growing number of cyclooctynes was put forward that can undergo what is known as a "strain-promoted" azide−alkyne cycloaddition.[9] Recent years have witnessed a trend toward the use of functionalized cyclooctynes in addressing cell surface azides in in situ and in vivo experiments instead of the Staudinger−Bertozzi ligation strategy. In these settings, cyclooctyne reagents appear superior compared to biotin-phosphane, and the ease with which (some of) these reagents are prepared and handled is likely at the basis of this trend. With the aim to establish whether strained cyclooctynes are also the reagent of choice in addressing azide-modified biomolecules in cell extracts, we applied biotinylated cyclooctynes **4**−**6** in a two-step proteasome profiling assay using activity-based probe **3** (Figure 1B) and compared the results with those obtained after Staudinger ligation with **2**.[10] We found that cyclooctynes give a quantitative transformation (with respect to the azide) at lower amounts of reagent than the phosphane. In particular, dibenzocyclooctyne **5**[11] provided rapid and quantitative transformation at reagent concentrations some 40-fold lower than **2**. On the downside, all three reagents suffered from considerable cross-reactivity resulting in the appearance of numerous background protein bands. Similar observations of high background labeling with the dibenzocyclooctyne reagents in cell lysates were reported by Tate and co-workers.[12]

Of the various bioorthogonal reactions alternative to click chemistry and Staudinger ligation, those that are based on Diels−Alder chemistry are probably the most promising. This holds true in particular for inverse-electron-demand Diels−Alder ligations. Weissleder and co-workers were the first to use the potential of this approach for live cell imaging.[13] After treatment of cancer cells with either norbornene (**7**)- or *trans*-cyclooctene (**8**)-modified antibodies, the cells were labeled through a very fast and selective cycloaddition with tetrazine-fluorophore **9** (Figure 2A). In the following years, the scope of this strategy was further expanded by the application of a radiolabeled tetrazine reagent for pretargeted tumor imaging in live mice[14] and the labeling of microtubule structures inside living cells.[15] In the latter study, Weissleder and co-workers treated cultured cells with a *trans*-cyclooctene-modified taxol derivative, followed by treatment with tetrazine-BODIPY-FL **10** to visualize microtubule structures, the subcellular target of taxol. The cellular images in these experiments are strikingly clean, and one explanation put forward by the authors is the weak intrinsic fluorescence of tetrazine-modified BODIPY dye **10**, which turns brightly fluorescent after reaction with the dienophile. An inherent advantage of inverse-electron-demand Diels−Alder reactions in comparison with "normal" Diels−Alder reactions is the propensity of the dienophile in the latter to undergo 1,4-conjugate addition. This necessitates masking of the free thiols in the biological sample at hand in order to prevent cross-reactivity, and essentially precludes in situ applications. Even so, we used a standard Diels−Alder ligation to demonstrate another aspect of bioorthogonal chemistry.[16] While both Staudinger−Bertozzi and click ligations rely on an azide in one of the reaction partners, the availability of bioorthogonal chemistry with reagents that are orthogonal to azides opens up a means to perform two individual bioorthogonal reactions at the same time. Whereas it has been shown that samples tagged with both azide and alkyne groups can be efficiently labeled by stepwise click ligation,[17,18] the cross-reactivity between the reagents still precludes the performance of both ligation reactions simultaneously and also necessitates the use (and efficient removal) of large excesses of ligation reagents. Diels−Alder reactions (both standard and inverse-electron-demand) provide a means of solving the orthogonality issue, as is exemplified in Figure 2.[16] We equipped the pan-reactive proteasome activity-based probe epoxomicin with a diene (**11**) and prepared a β1-specific proteasome inhibitor equipped with an azide (**12**) (Figure 2B). Treatment of cell extracts with **12** and **11**, respectively, was followed by exposure to biotin-phosphane **2**. The extracts were then denatured and treated with BODIPY-TMR-maleimide **13**. Streptavidin blotting revealed a single protein band corresponding to the proteasome β1 activity labeled by Staudinger ligation, whereas the two remaining catalytic sites became fluorescently tagged by Diels−Alder ligation (Figure 2C).

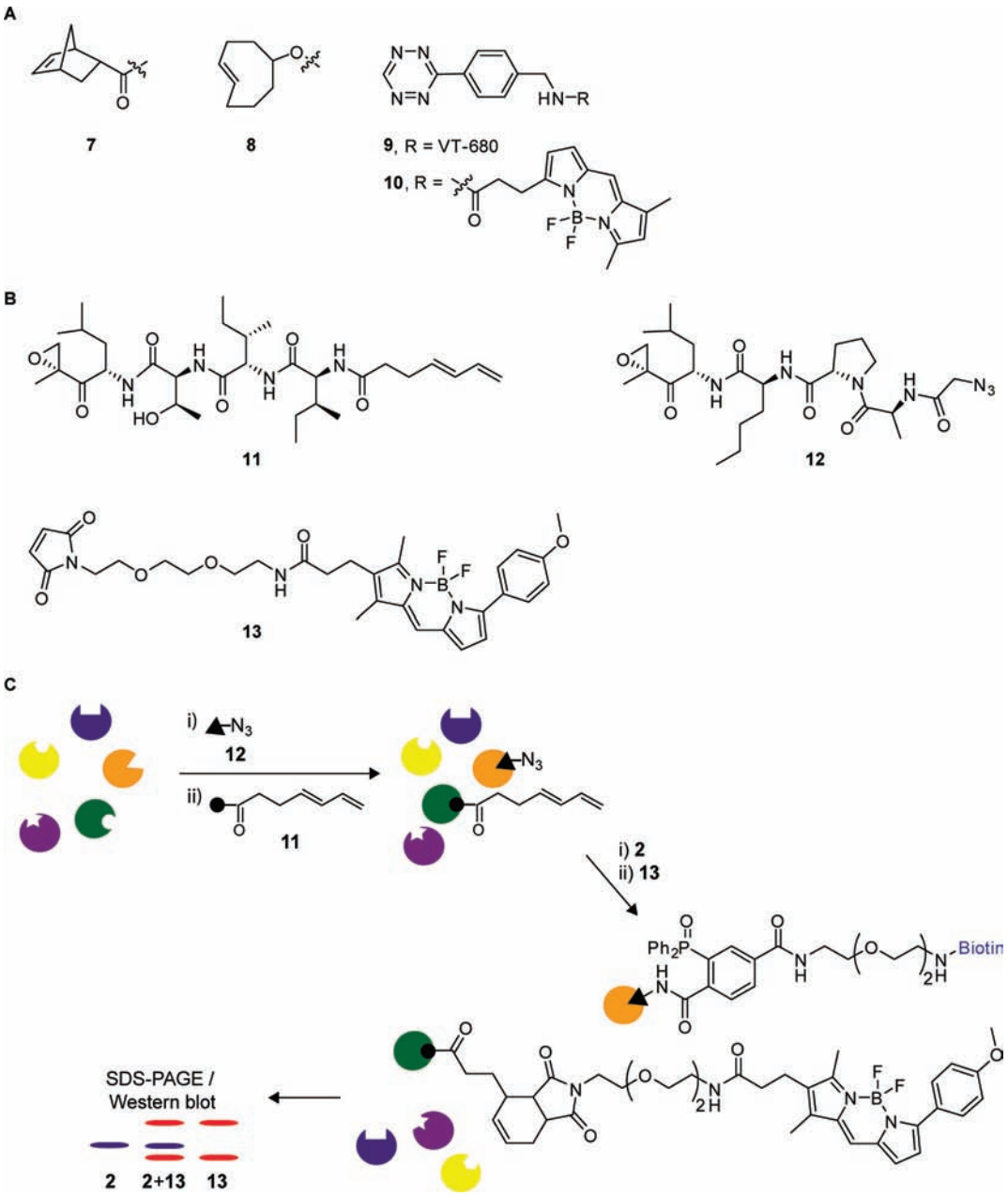


FIGURE 2. Bioorthogonal ligations based on Diels–Alder chemistry. (A) Norbornene (**7**) and *trans*-cyclooctene (**8**) as dienophiles in inverse-electron-demand Diels–Alder reactions with fluorescently labeled tetrazines (**9**, **10**). (B) Activity-based probes functionalized with a diene (**11**) or azide (**12**) for Diels–Alder ligation with BODIPY-TMR-maleimide (**13**) or Staudinger–Bertozzi ligation, respectively. (C) Diels–Alder reaction and Staudinger ligation can be performed in the same sample.

Activity-Based Protein Profiling: Direct versus Two-Step Labeling

Activity-based protein profiling (ABPP) aims to obtain information on the functioning of a protein, or protein family, in the context of a biological system.[19] In most reports that have appeared to date, the subject of such ABPP studies is an enzyme, or enzyme family, although recently some interesting reports have appeared on the use of this strategy to identify the target of natural products with druglike properties that bind covalently to a (nonenzyme) protein.[20] ABPP strategies generally make use of activity-based probes (ABPs) that are designed to be recognized by the target enzyme(s) and react in a covalent and irreversible manner with an active site residue, hence labeling only active enzymes. An ABP is equipped with a means to identify its target protein. This reporter entity can be a biotin, a fluorophore, an epitope tag, or a combination of these. From a proteomics point of view, ABPP provides attractive methodology for the simplification

of a complex proteome, while at the same time it reports on functional enzymes, rather than polypeptide expression levels.

Activity-based probes have been applied in bioorganic chemistry for many decades. Originally, most ABPs were applied on isolated enzymes and it was only in the nineties of the last century that the potential to use ABPs in a proteome-wide setting was recognized. Two seminal reports that opened up the ABPP field describe the application of serine hydrolase ABPs and cysteine protease ABPs in complex biological samples. Cravatt and co-workers made use of the intrinsic reactivity of serine hydrolases toward fluorophosphonates in their design of the ABP FP-biotin (**14**, Figure 3), with which many serine hydrolases can be selected from cell extracts.[21] In a related strategy, Bogyo and co-workers revealed that the biotinylated peptide epoxysuccinate DCG-04 (**15**) reacts, again in cell extracts, specifically with a number of cysteine proteases of the cathepsin family.[22]

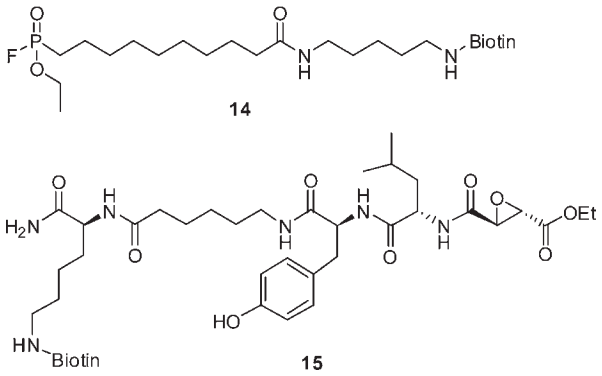


FIGURE 3. Biotin-functionalized fluorophosphonate (**14**) and epoxysuccinate (**15**) probes for activity-based profiling of serine hydrolases and cysteine proteases, respectively.

Bioorthogonal chemistry comes to play in a specific type of ABPP studies, namely those that make use of two-step ABPs. Covalent attachment of biotin or a fluorophore to an ABP may have several disadvantageous consequences. First, the ABP might not inhibit the intended enzyme(s) or have limited activity, even though the parent compound on which the ABP was based is highly potent. Second, the ABP might not be cell-permeable or suffer from low bioavailability due to the hydrophobic nature of the tag. Furthermore, the resemblance a given inhibitor possesses to an enzyme substrate is negated by attachment of biotin or a fluorophore. Bioorthogonal chemistry provides an attractive alternative in case any of these issues apply. Building on their previous ABPP work, Cravatt and co-workers reported on the copper(I)-catalyzed alkyne–azide cycloaddition mediated two-step labeling of active enzymes in cell extracts.[23] In this study, azide-functionalized sulfonate **16** (Figure 4A) was applied to tissue lysate, followed by rhodamine-alkyne **17** and copper(I) as the catalyst. Coinciding with

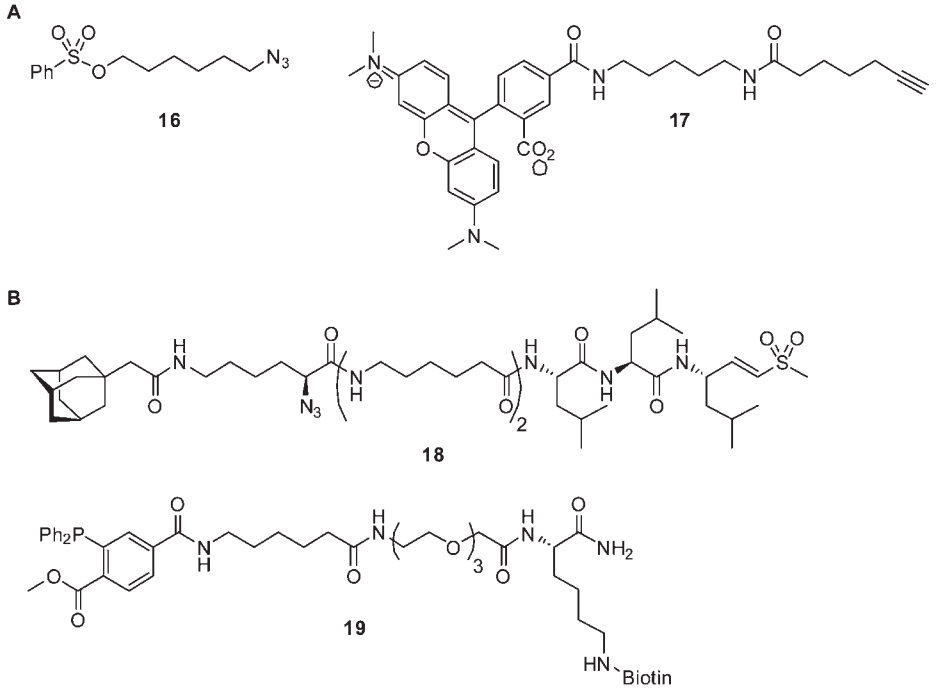


FIGURE 4. Bioorthogonal chemistry in two-step activity-based protein profiling. (A) Azide-functionalized sulfonate (**16**) and rhodamine-alkyne (**17**) for azide–alkyne cycloaddition. (B) Azide-functionalized vinyl sulfone (**18**) and biotin-phosphane (**19**) for Staudinger–Bertozzi ligation.

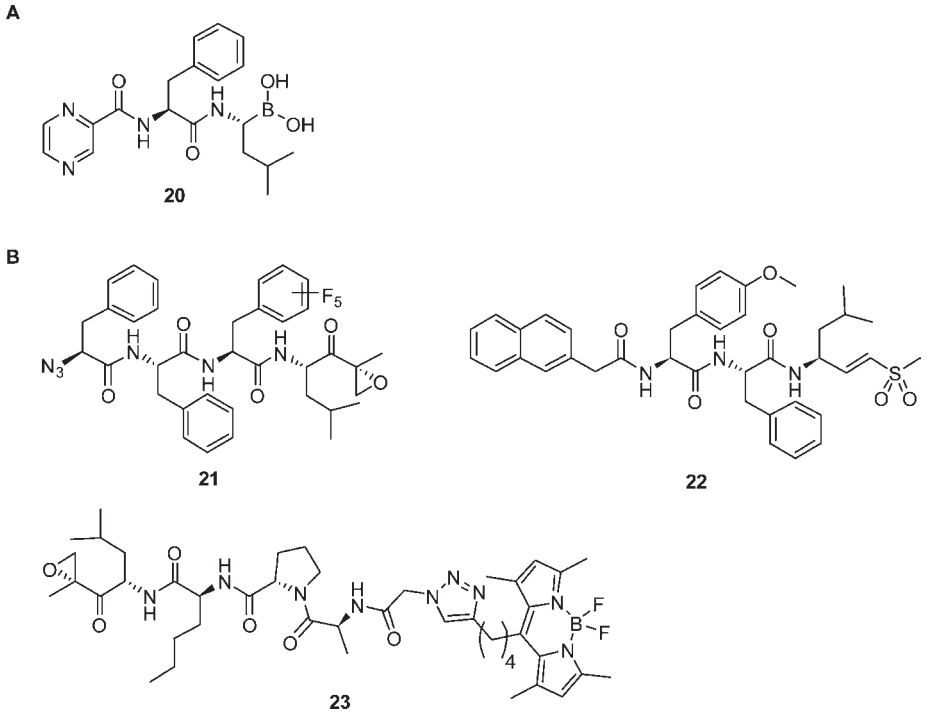


FIGURE 5. (A) Proteasome inhibitor bortezomib (**20**). (B) Inhibitors specific for the β5 subunit of the proteasome (**21**, **22**) and a fluorescent probe targeting the β1 and β5 subunits (**23**).

the work by Cravatt and his colleagues, we demonstrated the versatility of the Staudinger–Bertozzi ligation in the two-step ABPP of the proteasome active sites.[24] Figure 4B represents the first steps we took in bioorthogonal chemistry. Azide-modified peptide vinyl sulfone **18** proved to be a potent, broad-spectrum proteasome inhibitor targeting the catalytic subunits of both the constitutive proteasome (β1, β2, and β5) and the immunoproteasome (β1i, β2i, and β5i). Treatment of murine EL4 cells with **18** was followed by cell lysis and exposure of the cell extracts to biotin-phosphane **19**, resulting in labeling of all six subunits. Following a related strategy, we demonstrated that *O*-GlcNAzylated peptide epoxyketones are recognized by proteasomes,[25] indicating that the proteasome might also be able to turn over *O*-GlcNAcylated proteins, with possible consequences for antigen presentation processes.

A major advantage of the use of ABPs in studying proteasome active sites is related to the pharmaceutical relevance of this major cytosolic and nuclear protein degradation machinery. Screening of enzyme inhibitors using ABPP as a readout is one of the attractive applications of the methodology. The activities of the proteasome active sites cannot be studied individually and in isolated form, since they work in concert with the integrity of the 28-protein subunit large, inner catalytic core called the 20S proteasome. However, changes in the individual activities of the catalytic subunits can be revealed by competing a specific inhibitor against a broad-spectrum proteasome ABP, so that only those subunits that are not targeted by the inhibitor are visualized. Such experiments have demonstrated that the proteasome inhibitor bortezomib **20** (Figure 5A), which is used in the clinic for the treatment of multiple myeloma and mantle cell lymphoma and was originally developed as a β5-specific inhibitor, in fact also targets β1.[26] There is currently much debate about the issue to which extent each of the three proteasome subunits should be downregulated for optimal clinical activity. We have contributed to the field by the development of cell-permeable, subunit-specific inhibitors of two of the constitutive proteasome active sites, β1 (**12**, Figure 2B)[27] and β5 (**21**, **22**, Figure 5B).[28] In the design of our inhibitors, which react covalently and irreversibly with the *N*-terminus threonine residues within the active sites, we include the installment of an azide as the minimal deviation of the parent compound. By this means, we have the option to create a direct ABP by the installment of a fluorophore, but also to perform two-step ABPP. In the case of β1-specific inhibitor **12**, we have done both, and after two-step ABPP using biotin-phosphane **2** we found that **12** is indeed highly β1-specific.[27] In contrast, its direct ABP counterpart **23** shows cross-reactivity toward other proteasome active sites.[28]

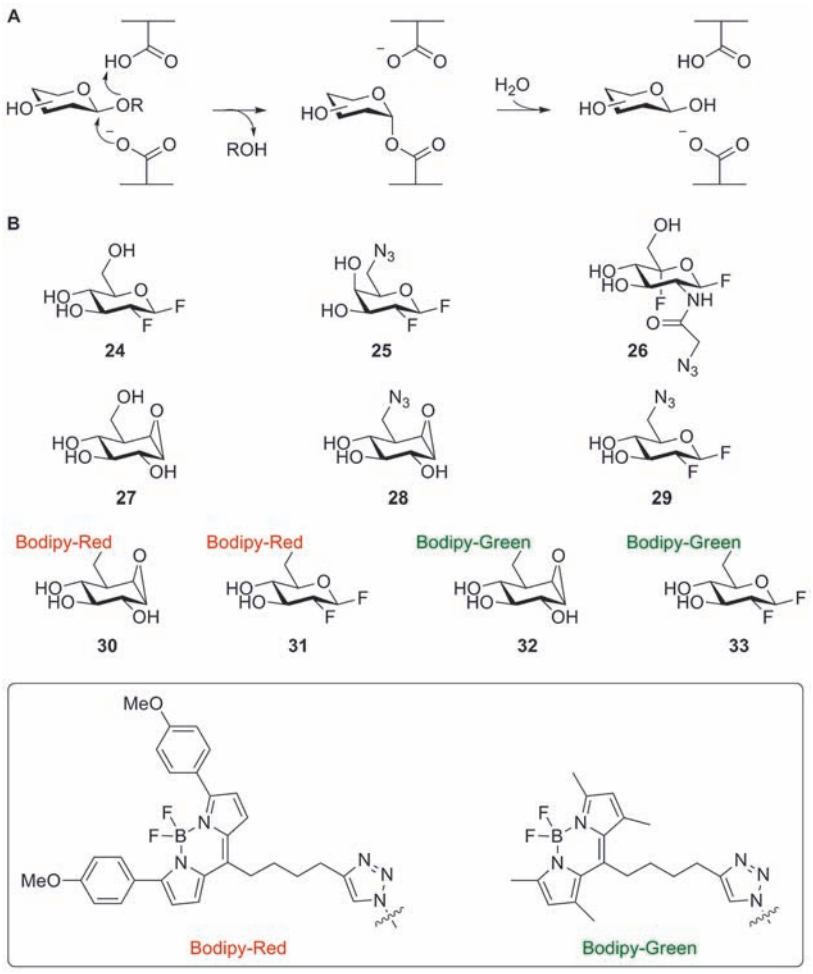


FIGURE 6. Activity-based profiling of β-glycosidases. (A) Mechanism of β-glucosidic bond cleavage by retaining β-glucosidases. (B) Structures of two-step probes for retaining β-glucosidases (**29**), β-galactosidases (**25**), and hexosaminidases (**26**) derived from retaining β-glucosidase inhibitor **24**; cyclophellitol (**27**) and a derivative (**28**) for two-step activity-based β-glucosidase profiling; and fluorescently labeled activity-based β-glucosidase probes (**30**–**33**).

Activity-Based Protein Profiling: Going for the Less Obvious Enzymes

Activity-based protein profiling has met with most success in the assessment of esterase and protease activities, in particular those that employ an amino acid (serine, threonine, cysteine) side chain functionality as the active site nucleophile and that can be made to react with an ABP. Obviously there are many more enzyme families, taking on different substrates and making use of quite different chemical processes. The future of the ABPP field is therefore bright, with many openings for the development of new classes of ABPs, but also for the application of bioorthogonal chemistry since many enzyme activities are highly particular with respect to their substrate. Besides tight substrate specificity, two other obstacles in ABP development are low enzyme expression levels and the absence of an amino acid side chain as the active site nucleophile, which prevents the use of traditional ABPs. Instead, affinity-based probes (AfBPs) may be employed that are able to bind available binding sites on target enzymes independent of the actual enzymatic activity. Quite some progress has been made in the design of probes incorporating a photoreactive group as the species responsible for creating a covalent bond with the enzyme, for instance, with matrix metalloproteases[29] and kinases.[30]

An enzyme class that has remained rather resistant to the development of general ABPP protocols is that of the glycosidases. Yet at least some glycosidases employ a mechanism involving a covalent enzyme-adduct intermediate for the cleavage of an interglycosidic linkage; a prerequisite for direct ABP development. Figure 6A depicts the hydrolysis of a β-glucosidic bond as effected by retaining β-glucosidases. Upon protonation of the aglycon, the leaving group is expulsed in an Sn2-type displacement and an enzyme-acyl glycoside intermediate is formed with inversion of configuration.

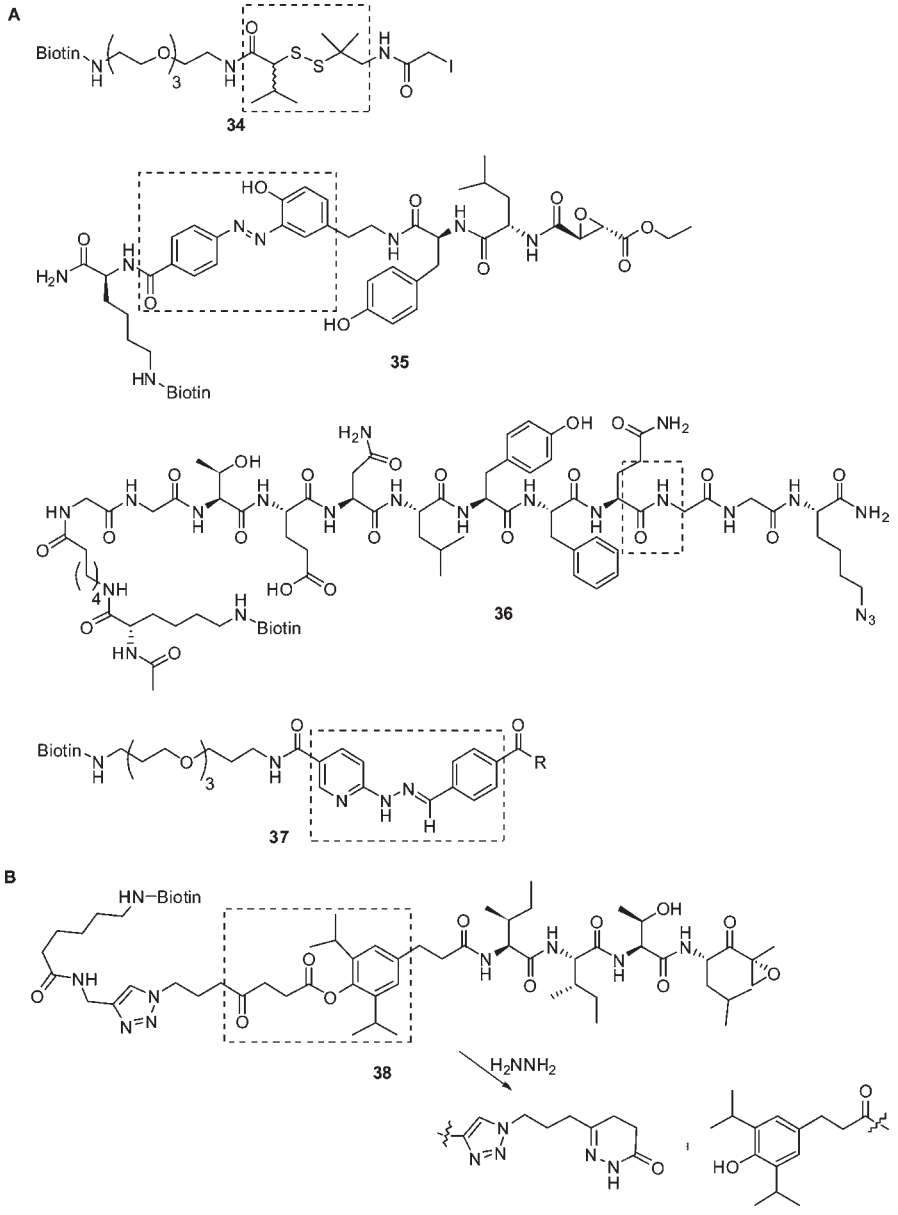


FIGURE 7. Cleavable linkers in activity-based protein profiling. (A) Probes incorporating disulfide (**34**), diazobenzene (**35**), TEV-cleavable (**36**), and hydrazone (**37**) linkers. (B) Proteasome probe incorporating a Lev linker susceptible to hydrazine cleavage (**38**).

In the next step, the acylal is hydrolyzed with regeneration of the enzyme active site and formation of β-glucose, thus giving overall retention of configuration of the anomeric substituent. Vocadlo and Bertozzi capitalized on pioneering work of Withers and co-workers who used 2-deoxy-2-fluoroglycosides (for instance, **24**, Figure 6B) in structural studies on retaining β-glucosidases.[31] The electron-withdrawing group at C2 of the glucopyranose ring ensures stabilization of the enzyme acylal intermediate to the extent that Röntgen diffraction data on this key intermediate can be gathered. In two separate studies Vocadlo and co-workers applied this concept in the development of a two-step retaining β-galactosidase ABP (**25**)[32] and a two-step retaining hexosaminidase ABP (**26**).[33] We felt, however, that the Withers type of inhibitors, though well suited for structural studies on transition states, are not the ideal starting point for glycosidase ABP development. They are rather poor glycosidase inhibitors, and we realized that cyclophellitol **27** or its analogues, potent mechanism-based inhibitors of retaining glycosidases, might make more impact in this direction. In a comparative study we applied cyclophellitol derivative **28** and 2-deoxy-2-fluoroglucoside **29**, both with an azide at C6 (glucose numbering), to human acid glucosylceramidase

and almond β-glucosidase.[34] We also included direct ABPs **30**–**33** based on both scaffolds to establish the merits of direct and two-step ABPP of retaining β-glucosidases. Cyclophellitol derivatives indeed proved to be the superior inhibitor class. Copper(I)-catalyzed azide–alkyne cycloaddition ligation proved feasible for the two-step labeling of recombinant enzyme; however, the combined processes of inhibition (with **28**) and click ligation proved to be of too low efficiency to detect endogenous human acid glucosylceramidase. To our surprise, we found that BODIPY-modified cyclophellitol derivatives **30** and **32** are highly potent and selective ABPs of the human acid glucosylceramidase. We were able to capitalize on this fortuitous finding by monitoring acid β-glucosidase activity in healthy and Gaucher cells, the latter disease caused by partially deficient acid β-glucosidase activity.[35] To make glycosidase ABPP more generally applicable, there are quite a few steps that need to be taken. Photoreactive ABPs may be a solution for those glycosidases that do not proceed through a covalent glycosyl-enzyme intermediate.[36]

Bioorthogonal Chemistry and Protein Enrichment

Recent years have witnessed the development of a conceptually new application of selective organic chemistry in chemical biology research: linker systems that allow mild cleavage under conditions ideally orthogonal to functionalities present in the biological system at hand. A major application of these linkers is in the purification of proteins tagged with a biotinylated ABP. Biotinylated ABPs are often used for enrichment of captured enzymes, for instance, by pull-down with streptavidin-coated beads. The main disadvantages of this approach, however, are that the conditions to liberate the captured proteins from the beads are harsh (boiling of the sample, all or not in the presence of unmodified biotin) and that, beside the target proteins, both endogenously biotinylated proteins and (denatured) streptavidin can end up in the sample. Several groups have developed linker systems that can be incorporated in the ABP, or alternatively in a bioorthogonal reagent for two-step ABPP, and that can be cleaved in a chemoselective manner after affinity pull-down. Examples include the disulfide **34**,[37] diazobenzene **35**,[38] bisaryl hydrazone **37**,[39] and the enzyme cleavable linker **36**[40] (Figure 7A).[41,42] Our own contribution to this field is based on a protective group often employed in synthetic organic chemistry, the levulinoyl (Lev) group.[43] While the Lev group is both hydrazine labile and base-labile, we designed a Lev derivative that is stable under basic conditions (by creating an ester that is sterically congested and electron-rich) but still susceptible to intramolecular nucleophilic attack during hydrazine cleavage. Altogether we came to proteasome ABP **38** (Figure 7B), with which we enriched for proteasome active sites by (1) treatment of cell extracts with **38**, (2) capture of biotinylated proteins by streptavidin coated magnetic beads, (3) treatment with hydrazine, and (4) resolution of the released polypeptides by SDS-PAGE. Future research will determine whether our Lev linker, or one of the other systems depicted in Figure 7A, will be of use in wide application in chemical proteomics research. It is at the same time clear that there is much room for development in the direction of new cleavable linkers.

Outlook

Both activity-based protein profiling and bioorthogonal chemistry, the two areas of chemical biology research discussed in this Account, have been around for about 15 years. Since 8 years they have been connected, and it is our expectation that both fields, both separately and in combined studies, will continue to make an impact on chemical biology research. New enzymes (or indeed other protein families) will become subject to ABPP protocols, new and (even) more efficient bioorthogonal chemistry will be developed, and new linker systems will see the light. The field will merge further with general proteomics sciences, and concepts such as stable isotope labeling and reporter groups for easy mass spectrometry detection will be incorporated in future direct and two-step ABP design. One issue that is in need of specific attention is the identification, in a chemical sense, of the nature of adducts derived from applying a bioorthogonal reaction to a biological system. At one point, we hope to be able to avoid the term "background labeling" or at least to be able to specify the nature of the undesired side products. The future will also see a further combination of aspects found in the various ABPs known today. One example from our own work in which we combined several features is in the design of a number of proteasome inhibitors equipped with an electrophilic trap at the C-terminus, an azide for two-step bioorthogonal ligation and a photoreactive group at the N-terminus for cross-linking to nearby protein binding sites.[44] With these probes, we were able to perform three consecutive organic reactions in a biological system: (1) suicide inhibition of the proteasome active sites, (2) photoactivation followed by cross-linking (to sites other than the active site, opening up the way for mapping the position the inhibitor assumes in the 20S cavity), and (3)

Staudinger−Bertozzi ligation for identification of the obtained fused proteasome subunits. Finally, very recent work from Cravatt and colleagues introduces a new concept of chemical selectivity in biology.[45] Rather than tuning an electrophile such that it only reacts with the most reactive nucleophiles (as found in enzyme active sites), they made use of highly reactive electrophiles, geared to react with any cysteine residue, which they applied only in substoichiometric amounts. Cysteines activated for a specific purpose (such as in an enzyme active site) will react fastest and will deplete the ABP pool before reaction with general cysteines can take place. By comparing the results with those obtained after reaction with an excess of the ABP, they were able to map with remarkable accuracy the proteins possessing an activity that relies on an activated cysteine residue. This last example bodes well for the future, in which creative thinking from an organic chemistry background will continue to produce new concepts, both in bioorthogonal chemistry and in activity-based protein profiling, for furthering chemical biology research.

BIOGRAPHICAL INFORMATION

Lianne Willems (18-01-1986) received her Master's degree in Biopharmaceutical Sciences "Cum Laude" at Leiden University in 2009. She is currently pursuing her Ph.D. research at Leiden University in the Bioorganic Synthesis group on the development of new activity-based probes and bioorthogonal ligation strategies for application in activity-based protein profiling.

Wouter van der Linden (18-02-1983) received his Master's degree in Chemistry "Cum Laude" at Leiden University in 2007. Currently he is doing his Ph.D. research at Leiden University in the Bioorganic Synthesis group, where he conducts research on the development of new proteasome inhibitors and probes.

Nan Li (08-10-1984) received his Bachelor's degree in Chemical Biology at Xiamen University (2007) and his Master's degree in Biological Chemistry at Leiden University in 2009. Currently he is doing his Ph.D. research in the Bioorganic Synthesis group at Leiden University. His research interests include activity-based protein profiling, proteasome biology, and chemical biology on post-translational modifications.

Kah-Yee Li (21-10-1985) graduated from Leiden University in 2009 with a Master's degree in Biopharmaceutical Sciences. Currently she is a Ph.D. student in the group of Bioorganic Synthesis at Leiden University. Her research includes the development of inhibitors and tools for glycosidase profiling.

Nora Liu (04-07-1986) received her Master's degree in Biopharmaceutical Sciences "Cum Laude" at Leiden University in 2009. She is currently a Ph.D. student at Leiden University in the group of Bioorganic Synthesis. Her research interests include the development of activity-based probes and potent kinase inhibitors.

Sascha Hoogendoorn (01-08-1984) received her Bachelor's degree in Chemistry (2007) and Biopharmaceutical Sciences (2008) and her Master's degree in Organic Chemistry (2008) "Cum Laude" from Leiden University. Currently, she is working as a Ph.D. student in the group of Bioorganic Synthesis at Leiden University on the synthesis of fluorescently labeled receptor ligands and activity-based probes.

Gijs van der Marel (03-04-1952) performed his Ph.D. research in the group of the late Professor Jacques van Boom on the subject of synthetic nucleic acid chemistry. Upon graduating in 1982, he pursued his independent career at Leiden University where he was appointed Professor in Organic Chemistry in 2004. His current research interests are in organic synthesis with a focus on nucleic acids and carbohydrates.

Bogdan Florea (14-10-1973) received his Ph.D. from Leiden University in 2003 (supervisor Professor Hans Junginger). After 2 years of postdoctoral research in the Genetics and Cell Biology group of Professor Hoeijmakers and Professor Grosveld at the Erasmus University in Rotterdam, he joined the Bioorganic Synthesis group at Leiden University in 2005. His current research interests are activity-based protein profiling, LC-MS-based proteomics, and biochemistry.

Herman Overkleeft (12-04-1969) performed his graduate research at the University of Amsterdam under the guidance of Professor Upendra Pandit. After postdoctoral research at Leiden University (Professor Jacques van Boom, 1997-1999) and Harvard Medical School (Professor Hidde Ploegh, 1999-2001), he was appointed Professor in Bioorganic Chemistry at Leiden University. He is currently pursuing chemical biology research in relation to glycolipid metabolism and antigen processing.

The Netherlands Organization for Scientific Reseach (NWO) and The Netherlands Genomics Initiative (NGI) are kindly thanked for financial support.

FOOTNOTES

*To whom correspondence should be addressed. E-mail: b.florea@chem.leidenuniv.nl (B.I.F.); h.s.overkleeft@chem.leidenuniv.nl (H.S.O.).

REFERENCES

1 Mahal, L. K.; Yarema, K. J.; Bertozzi, C. R. Engineering chemical reactivity on cell surfaces through oligosaccharide biosynthesis. *Science* **1997**, *276*, 1125–1128.

2 Saxon, E.; Bertozzi, C. R. Cell surface engineering by a modified Staudinger reaction. *Science* **2000**, *287*, 2007–2010.

3 Huisgen, R. 1,3-Dipolar cycloaddition — introduction, survey, mechanism. In *1,3- Dipolar Cycloaddition Chemistry*; Padwa, A., Ed.; Wiley-Interscience: New York, 1984; Vol. 1, pp 1−176.

4 Tornøe, C. W.; Christensen, C.; Meldal, M. Peptidotriazoles on solid phase: [1,2,3]- triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem.* **2002**, *67*, 3057–3064.

5 Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J. Am. Chem. Soc.* **2003**, *125*, 3192–3193.

6 Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047.

7 Verdoes, M.; Florea, B. I.; Hillaert, U.; Willems, L. I.; Van der Linden, W. A.; Sae- Heng, M.; Filippov, D. V.; Kisselev, A. F.; Van der Marel, G. A.; Overkleeft, H. S. Azido-bodipy-acid

reveals quantitative Staudinger-Bertozzi ligation in two-step activity-based proteasome profiling. *ChemBioChem* **2008**, *9*, 1735–1738.

8 Agard, N. J.; Baskin, J. M.; Prescher, J. A.; Lo, A.; Bertozzi, C. R. A comparative study of bioorthogonal reactions with azides. *ACS Chem. Biol.* **2006**, *1*, 644–648.

9 Debets, M. F.; Van der Doelen, C. W. J.; Rutjes, F. P. J. T.; Van Delft, F. L. Azide: a unique dipole for metal-free bioorthogonal ligations. *ChemBioChem* **2010**, *11*, 1168–1184 and references cited therein.

10 Van der Linden, W. A.; Li, N.; Hoogendoorn, S.; Ruben, M.; Verdoes, M.; Guo, J.; Boons, G.-J.; Van der Marel, G. A.; Florea, B. I.; Overkleeft, H. S. Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: A comparative study. *Bioorg. Med. Chem.* 2011, DOI: 10.1016/j.bmc.2011.06.037.

11 Ning, X.; Guo, J.; Wolfert, M. A.; Boons, G. J. Visualizing metabolically labeled glycoconjugates of living cells by copper-free and fast Huisgen cycloadditions. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253–2255.

12 Berry, A. F.; Heal, W. P.; Tarafder, A. K.; Tolmachova, T.; Baron, R. A.; Seabra, M. C.; Tate, E. W. Rapid multilabel detection of geranylgeranylated proteins by using bioorthogonal ligation chemistry. *ChemBioChem* **2010**, *11*, 771–773.

13 Devaray, N. K.; Weissleder, R.; Hilderbrand, S. A. Tetrazine-based cycloadditions: application to pretargeted live cell imaging. *Bioconjugate Chem.* **2008**, *19*, 2297–2299.

14 Devaray, N. K.; Upadhyay, R.; Haun, J. B.; Hilderbrand, S. A.; Weissleder, R. Fast and sensitive pretargeted labeling of cancer cells through a tetrazine/trans-cyclooctene cycloaddition. *Angew. Chem., Int. Ed.* **2009**, *48*, 7013–7016.

15 Devaray, N. K.; Hilderbrand, S.; Upadhyay, R.; Mazitschek, R.; Weissleder, R. Bioorthogonal turn-on probes for imaging small molecules inside living cells. *Angew. Chem., Int. Ed.* **2010**, *49*, 2869–2872.

16 Willems, L. I.; Verdoes, M.; Florea, B. I.; Van der Marel, G. A.; Overkleeft, H. S. Two- step labeling of endogenous enzymatic activities by Diels-Alder ligation. *ChemBioChem* **2010**, *11*, 1769–1781.

17 Zhang, M. M.; Tsou, L. K.; Charron, G.; Raghavan, A. S.; Hang, H. C. Tandem fluorescence imaging of dynamic S-acylation and protein turnover. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 8627–8632.

18 Heal, W. P.; Jovanovic, B.; Bessin, S.; Wright, M. H.; Magee, A. I.; Tate, E. W. Bioorthogonal chemical tagging of protein cholesterylation in living cells. *Chem. Commun.* **2011**, *47*, 4081–4083.

19 Evans, M. J.; Cravatt, B. F. Mechanism-based profiling of enzyme families. *Chem. Rev.* **2006**, *106*, 3279–3301.

20 Rizvi, S. A.; Courson, D. S.; Keller, V. A.; Rock, R. S.; Kozmin, S. A. The dual mode of action of bistramide A entails severing of filamentous actin and covalent protein modification. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 4088–4092.

21 Liu, Y.; Patricelli, M. P.; Cravatt, B. F. Activity-based protein profiling: the serine hydrolases. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14694–14699.

22 Greenbaum, D.; Medzihradszky, K. F.; Burlingame, A.; Bogyo, M. Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. *Chem. Biol.* **2000**, *7*, 569–581.

23 Speers, A. E.; Adam, G. C.; Cravatt, B. F. Activity-based protein profiling in vivo using a copper(I)-catalyzed azid-alkyne [3 + 2] cycloaddition. *J. Am. Chem. Soc.* **2003**, *125*, 4686–4687.

24 Ovaa, H.; Van Swieten, P. F.; Kessler, B. M.; Leeuwenburgh, M. A.; Fiebiger, E.; Van den Nieuwendijk, A. M. C. H.; Galardy, P. J.; Van der Marel, G. A.; Ploegh, H. L.; Overkleeft, H. S. Chemistry in living cells: detection of active proteasomes by a two- step labeling strategy. *Angew. Chem., Int. Ed.* **2003**, *42*, 3626–3630.

25 Witte, M. D.; Florea, B. I.; Verdoes, M.; Adeyanju, O.; Van der Marel, G. A.; Overkleeft, H. S. O-GlcNAc peptide epoxyketones are recognized by mammalian proteasomes. *J. Am. Chem. Soc.* **2009**, *131*, 12064–12065.

26 Berkers, C. R.; Verdoes, M.; Lichtman, E.; Fiebiger, E.; Kessler, B. M.; Anderson, K. C.; Ploegh, H. L.; Ovaa, H.; Galardy, P. J. Activity probe for in vivo profiling of the specificity of the proteasome inhibitor bortezomib. *Nat. Methods* **2005**, *2*, 357–362.

27 Britton, M.; Lucas, M. M.; Downey, S. L.; Screen, M.; Pletnev, A. A.; Verdoes, M.; Tokhunts, R. A.; Amir, O.; Goddard, A. L.; Pelphrey, P. M.; Wright, D. L.; Overkleeft, H. S.; Kisselev, A. F. Selective inhibitor of proteasome's caspase-like sites sensitizes cells to specific inhibition of chymotrypsin-like sites. *Chem. Biol.* **2009**, *16*, 1278–1289.

28 Verdoes, M.; Willems, L. I.; Van der Linden, W. A.; Duivenvoorden, B. A.; Van der Marel, G. A.; Florea, B. I.; Kisselev, A. F.; Overkleeft, H. S. A panel of subunit- selective activity-based proteasome probes. *Org. Biomol. Chem.* **2010**, *8*, 2719–2727.

29 Chan, E. W. S.; Chattopadhaya, S.; Panicker, R. C.; Huang, X.; Yao, S. Q. Developing photoactive affinity probes for proteomics profiling: hydroxamate-based probes for metalloproteases. *J. Am. Chem. Soc.* **2004**, *126*, 14435–14446.

30 Hagenstein, M. C.; Mussgnug, J. H.; Lotte, K.; Plessow, R.; Bockhinke, A.; Kruse, O.; Sewald, N. Affinity-based tagging of protein families with reversible inhibitors: a concept for functional proteomics. *Angew. Chem., Int. Ed.* **2003**, *42*, 5635–5638.

31 Vocadlo, D. J.; Davies, G. J.; Laine, R.; Withers, S. G. Catalysis by hen egg-white lysozyme proceeds via a covalent intermediate. *Nature* **2001**, *412*, 835–838.

32 Vocadlo, D. J.; Bertozzi, C. R. A strategy for functional proteomic analysis of glycosidase activity from cell lysates. *Angew. Chem., Int. Ed.* **2004**, *43*, 5338–5342.

33 Stubbs, K.; Scaffidi., A.; Debowski, A. W.; Mark, B. L.; Stick, R. V.; Vocadlo, D. J. Synthesis and use of mechanism-based protein profiling probes for retaining beta-D- glucosaminidases facilitate identification of Pseudomonas aeruginosa NagZ. *J. Am. Chem. Soc.* **2008**, *130*, 327–335.

34 Witte, M. D.; Walvoort, M. T. C.; Li, K.-Y; Kallemeijn, W. W.; Donker-Koopman, W. E.; Boot, R. G.; Aerts, J. M. F. G.; Codée, J. D. C.; Van der Marel, G. A.; Overkleeft, H. S. Activity-based profiling of retaining β-glucosidases: a comparative study. *ChemBioChem* **2011**, *in press*.

35 Witte, M. D.; Kallemeijn, W. W.; Aten, J.; Li, K.-Y; Strijland, A.; Donker-Koopman, W. E.; Van den Nieuwendijk, A. M. C. H.; Bleijlevens, B.; Kramer, G.; Florea, B. I.; Hooibrink, B.; Hollak, C. E. M.; Ottenhoff, R.; Boot, R. G.; Van der Marel, G. A.; Overkleeft, H. S.; Aerts, J. M. F. G. Ultrasensitive in situ visualization of active glucocerebrosidase molecules. *Nat. Chem. Biol.* **2010**, *6*, 907–913.

36 Gandy, M. N.; Debowski, A. W.; Stubbs, K. A. A general method for affinity-based proteomic profiling of exo-alpha-glycosidases. *Chem. Commun.* **2011**, *47*, 5037–5039.

37 Gartner, C. A.; Elias, J. E.; Bakalarski, C. E.; Gygi, S. P. Catch-and-release reagents for quantitative proteomics analyses. *J. Proteome Res.* **2007**, *6*, 1482–1491.

38 Verhelst, S. H. L.; Fonovic, M.; Bogyo, M. A mild chemically cleavable linker system for functional proteomics applications. *Angew. Chem., Int. Ed.* **2007**, *46*, 1284–1286.

39 Dirksen, A.; Yegneswaran, S.; Dawson, P. E. Bisaryl hydrazones as exchangeable biocompatible linkers. *Angew. Chem., Int. Ed.* **2010**, *49*, 2023–2027.

40 Speers, A. E.; Cravatt, B. F. A tandem orthogonal proteolysis strategy for high-content chemical proteomics. *J. Am. Chem. Soc.* **2005**, *127*, 10018–10019.

41 For more examples, see: Yang, Y. Y.; Grammel, M.; Raghavan, A. S.; Charron, G.; Hang, H. C. Comparative analysis of cleavable azobenzene-based affinity tags for bioorthogonal chemical proteomics. *Chem. Biol.* **2010**, *17*, 1212–1222 and references cited therein.

42 See also Szychowski, J.; Mahdavi, A; Hodas, J. J.; Bagert, J. D.; Ngo, J. T.; Landgraf, P.; Dieterich, D. C.; Schuman, E. M.; Tirrell, D. A. Cleavable biotin probes for labeling of biomolecules via azide-alkyne cycloaddition. *J. Am. Chem. Soc.* **2010**, *132*, 18351–18360.

43 Geurink, P. P.; Florea, B. I.; Li, N.; Witte, M. D.; Verasdonck, J.; Kuo, C. L.; Van der Marel, G. A.; Overkleeft., H. S. A cleavable linker based on the levulinoyl ester for activity-based protein profiling. *Angew. Chem., Int. Ed.* **2010**, *38*, 6802–6905.

44 Geurink, P. P.; Florea, B. I.; Van der Marel, G. A.; Kessler, B. M.; Overkleeft, H. S. Probing the proteasome cavity in three steps: bioorthogonal photo-reactive suicide substrates. *Chem. Commun.* **2010**, *47*, 9052.

45 Weerapana, E.; Wang, C.; Simon, G. M.; Richter, F.; Khare, S.; Dillon, M. B. D.; Bachovchin, D. A.; Mowen, K.; Baker, D.; Cravatt, B. F. Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* **2010**, *468*, 790–795.