Proteomics/Metabolomics

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Natural Products and Their Biological Targets: Proteomic and Metabolomic Labeling Strategies

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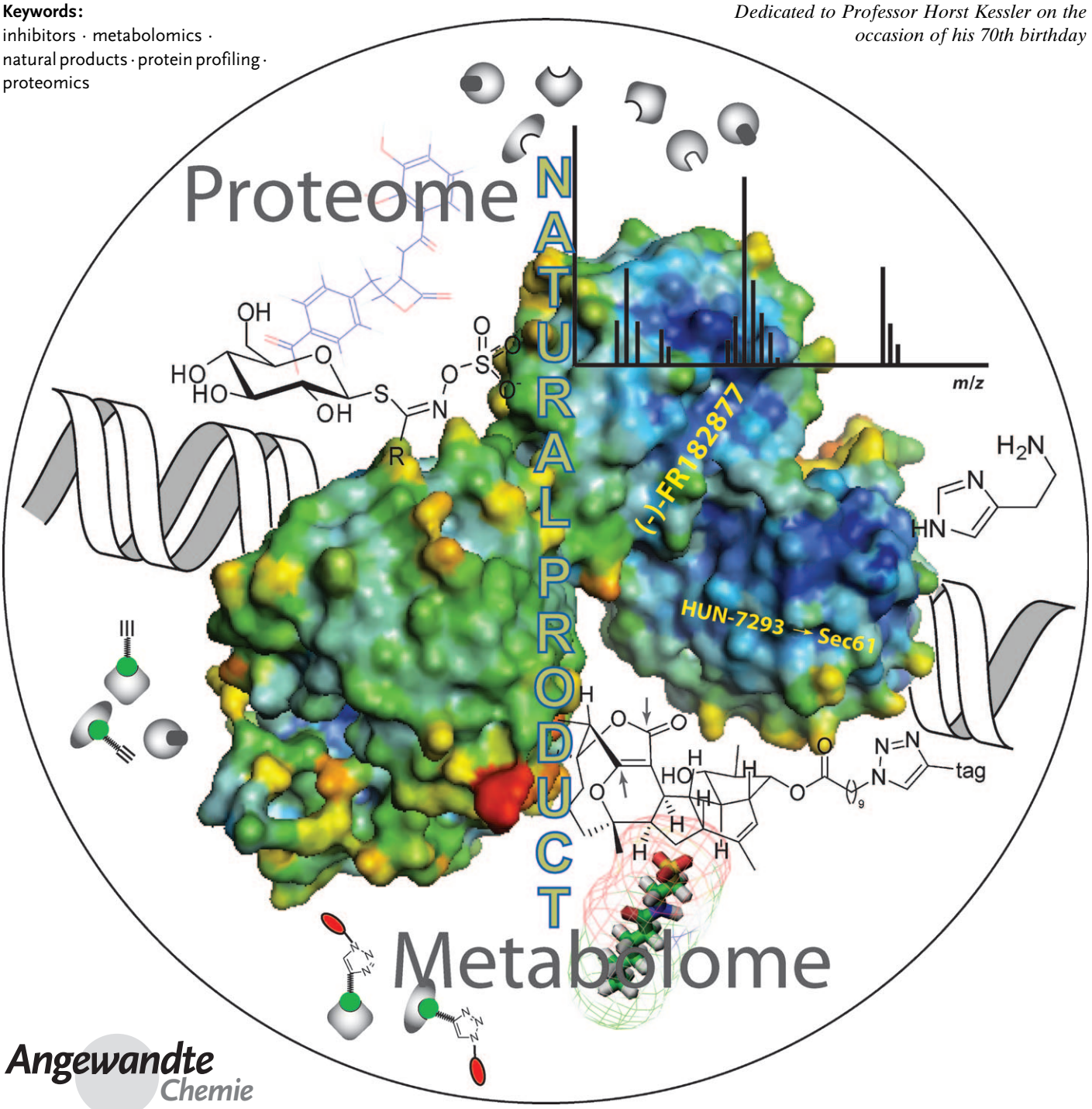
Dedicated to Professor Horst Kessler on the occasion of his 70th birthday



Activity-based protein profiling (ABPP) has matured into a standard method for the fast, sensitive, and selective identification of enzyme activity and inhibitors in proteomes. By using natural product based probes, the targets of many uncharacterized molecules can be easily identified in complex proteomes, and their exact function and mechanism of action understood. Natural products and their derivatives can also serve as pharmaceutical lead structures that impede essential components in the cell and their effects can be studied in biological assays. Since the complex regulatory processes in a cell go beyond mere transcription, translation, and activation, it is imperative to also identify the products of the active proteome—the metabolites and binding partners of individual enzymes and proteins. Therefore, methods by which the chemically complex metabolome can be characterized are necessary. A series of interesting approaches have become available in recent years that enable the global investigation of enzyme–metabolite pairs.

From the Contents

1. Introduction

The complexity and diversity of life is reflected by the molecular blueprint of every single cell. Hereby, the important functions are carried out by proteins which are encoded in the deoxyribonucleic acid (DNA) sequence of the genes. The DNA is first rewritten into messenger ribonucleic acid (mRNA) in the cell (transcription), which then serves as a template for the synthesis of proteins (translation). The great success of many genome sequencing projects has led to the decoding of the genomes of numerous eukaryotes and prokaryotes, including the human genome and those of the most important pathogens. The challenge now facing the dawning postgenomic era is to assign a function to the many uncharacterized genes and enable an understanding of their potential role in the development of diseases.[1] A series of sophisticated methods are necessary for this ambitious plan, and these have only become possible through the recent advances in chemistry, biology, and analytics. Since it is not the genes, but the encoded proteins that execute the relevant physiological functions in the cell, their misguided expression and regulation is the direct cause of disease development. To understand what role individual genes or proteins play in these processes, one compares the expression patterns (that is, the amount of an mRNA (genomics) or a protein (proteomics)) between diseased and healthy cells.[2–6]

Since in many cases proteins are subject to downstream regulatory processes, the amount of a given protein occasionally does not correlate with its activity or its related physiological or pathological function (Figure 1). This is particularly important for enzymes, the degradative action of which, such as proteolysis, can pose a threat to the function of a cell. The activity of proteases, and many other enzymes, is therefore regulated through a series of posttranslational processes (Figure 1).[7] In diseased cells, however, such regulatory process can get out of control. Proteases are, for example, thought to play an important role in the development of cancer and Alzheimer's disease.[8–11] However, because proteomics methods can only determine the amount, that is, how much of a given enzyme is in a cell, but not its activity, it is not possible to draw any direct conclusions regarding protein function with these techniques. To overcome this limitation a new chemical proteomics approach, called activity-based protein profiling (ABPP), was established by the research groups of Cravatt[12,13] and Bogyo[7,14] about ten years ago. Studies by the research groups of Walker[15] and Powers[16,17] had already prepared the way for the ABPP technique.

One particularly powerful application of ABPP is in the elucidation of the cellular targets of natural products.[18] Natural products offer a large spectrum of biological activity and are the basis for numerous approved drugs. In addition to their isolation, structural determination, and total synthesis, it is above all the knowledge of their molecular targets and mechanism of action that is of central interest in current natural product research. Should a natural product be approved as a drug, it is important to determine its exact site of action and also its other possible cellular targets, which might lead to side effects. The techniques used to date have mainly involved in vitro studies on the interactions of a given natural product with a range of isolated standard enzymes, for example, kinases. This approach has the disadvantage that, on the one hand, only a selection of proteins present in a cell can interact with the compound, and on the other, the physiological conditions in an intact cell (for example, activity regulation) cannot be imitated. Since an increasing number of natural products have been used in ABPP to determine their targets in recent years, this exciting research area will be one of the main focuses of this Review.

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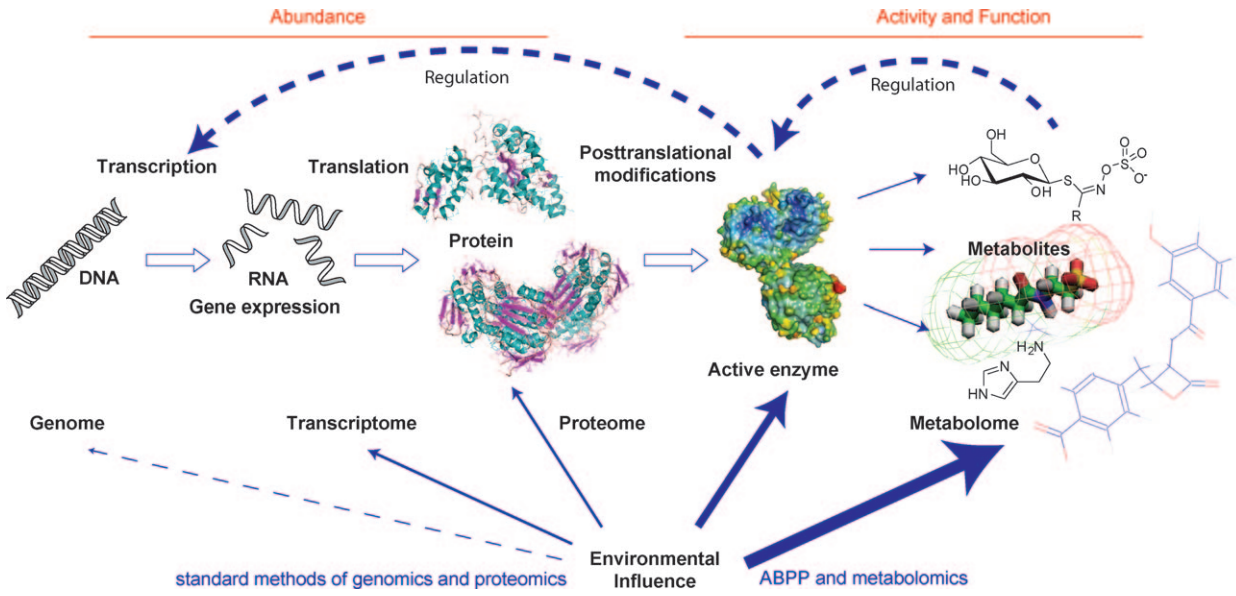

Figure 1. Cellular interactions through the regulation of proteins and metabolites. The essential information regarding the activity and function of these biomolecules cannot be determined with classical molecular biology techniques and require new strategies, such as ABPP and metabolite profiling.

To fully comprehend the complexity of a cell, its regulation, and also the development of diseases, the critical role played by DNA, RNA, and proteins as well as by small molecules or metabolites needs to be studied. The metabolome (the complete collection of metabolites in a cell) is the product of the networks of enzymatic reactions in cells and is mirrored by a high chemical diversity, in, for example, peptides, sugars, lipids, and nucleosides. Therefore, the metabolome corresponds to a snapshot of the products of the finely tuned network of enzymatic activity in an organism, which is in turn the result of the exchange that takes place with the organism's immediate environment (Figure 1). The metabolome plays an essential role in many signal transduction cascades. Therefore, the exact structural determination of all the metabolites, as well as the comprehension of their function in cellular networks, is a fundamental focus of current research. In contrast to RNA and proteins, metabolites are structurally diverse molecules that do not have a direct connection with the genetic code, and are not built from a defined set of monomeric building blocks. Consequently, the development of a universal method for the characterization of the metabolome—in contrast to the genome and proteome—is all the more difficult. The development of new methods in this area have now enabled the determination of the metabolic substrates of individual enzymes, thus leading to a more precise knowledge of their functions.[19] In this way a series of enzyme–metabolite pairs were discovered, which have provided a powerful supplement to ABPP for elucidating the physiological and pathological processes at the interface between the proteome and the matabolome.[19,20] This will be the second theme of this Review.

2. Principles of Activity-Based Protein Profiling (ABPP)

2.1. Probe Design and Proteome Labeling

ABPP has gradually advanced to a complementary proteomic technique, with which it is possible to study the

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Maximilian Pitscheider was born in 1983 in Munich and received his MSc in 2008 in chemistry at the Ludwig-Maximilians-Universität München (LMU), where he conducted his research under the supervision of Dr. S. A. Sieber. He is currently working on his dissertation in the same group, where he is engaged in the development of Michael acceptor based probes and the investigation of natural products for use in activity-based profiling.

activity, function, and regulation of enzymes in vitro as well as in vivo. The key to this technique has been an interdisciplinary approach involving chemical, biological, and analytical methods. With this combination, small molecules that have a high affinity for active sites of individual enzymes can be equipped with a tag either for the purpose of visualization (fluorescent dye) or enrichment (biotin). These small probes then bind in the free active site of the target enzyme and provide a facile means for the visualization and identification by gel electrophoresis (SDS-PAGE) and fluorescence detection. Enzymes, the active centers of which are blocked and therefore not accessible to small molecules, are not bound by the probes, and so cannot be visualized; this presents an important distinction to classical proteomics techniques.

In recent years, a series of groundbreaking discoveries were reported relating to the activity of pathogenosis–associated enzymes, the functional characterization of which is required for the development of possible inhibitors. A probe is basically composed of three elements (Figure 2A). The first element comprises the reactive group, which is specially designed for reaction with a specific active site. The fluorophosphonate probes (FP probes) introduced by Cravatt and co-workers are an excellent example of selective inhibitors that specifically recognize enzymes of the serine hydrolase family—an enzyme family that represents over 1% of proteins expressed by eukaryotic cells.[21–24] Bound to the reactive group is a spacer (the second element of a probe), which is usually a short hydrophilic polyethylene glycol chain that separates the reactive group from the marker (tag). The hydrophobicity of the spacer should take the characteristics of the target enzymes into account, and through derivatization can be tailored to satisfy its various substrate-binding preferences. The third element of a probe is the marker, which serves for the enrichment, identification, and visualization of the target proteins. The originally used radioactive markers are difficult to use and dangerous and they have now mostly been replaced by biotin and fluorescent molecules, such as tetramethylrhodamine (TAMRA). Additionally, biotin aids labeling through binding to avidin, and thus enables characterization by mass spectrometry through enrichment with avidin beads.

In a typical labeling experiment, the selected probe is incubated with proteomes from, for example, two different stages of a particular pathogen. During the incubation, the fluorescent probes bind directly at the active sites of the desired target proteins. Analytical techniques, such as electrophoresis combined with fluorescent scanning, allow the fast and comparative analysis of the status of the cells or tissues, and can indicate disease-associated enzyme activity (Figure 2B). These pathogenesis-associated enzymes can then be investigated more thoroughly and can later serve as therapeutic and diagnostic targets. The interaction of probes with their target enzymes has enabled many important targets for cancer, infectious diseases, and metabolic disorders to be identified (see Section 3.2).

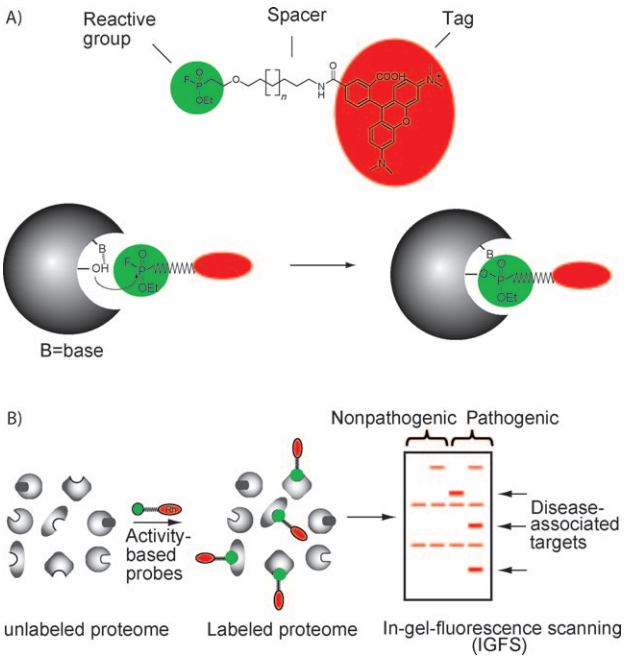

Figure 2. A) Structure of a probe, and B) the operation of a classical ABPP experiment. Rh = Rhodamine.

2.2. Bioorthogonal Chemistry for In Vivo Experiments

The use of covalently bound, sterically demanding markers have restricted the application scope of ABPP. Their low cell permeability has generally restricted application to cell lysates. Cell lysates have the disadvantage that activators and inhibitors are often released when the cellular organelles and various compartments are disrupted, which influences the enzyme activity.[25] This problem has been solved through the introduction of bioorthogonal reactions, such as the copper(I)-catalyzed Huisgen-[3 + 2]-azide-alkyne cycloaddition ("click" chemistry, CC; Scheme 1A) or the Staudinger ligation (SL, Scheme 1B) between azides and methyl ester modified triphenylphosphines.[26–31] Such orthogonal reactions show low reactivity towards other biomolecules such as DNA or unlabeled proteins, and can be easily conducted in aqueous media. Therefore, it is possible to label proteins in living cells by using cell-permeable small molecules, and following cell disruption, tag them with the respective marker.[32–34]

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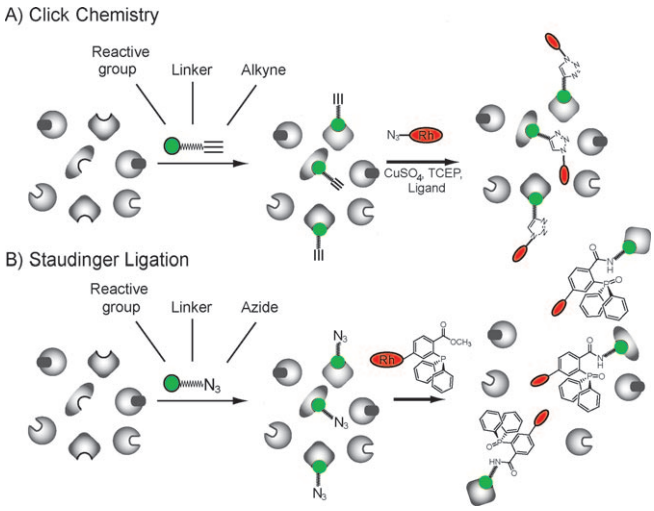


Scheme 1. The different principles of the click reaction (A) and the Staudinger ligation (B) in ABPP. TCEP = tris(2-carboxyethyl)phosphine.

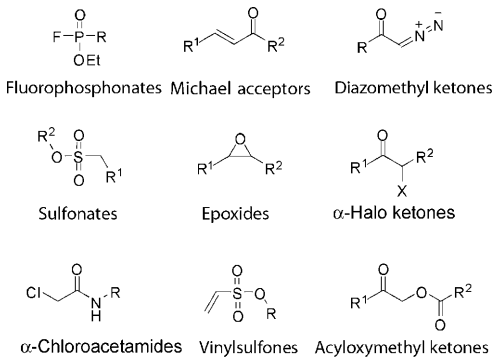


Figure 3. Examples of reactive groups for ABPP probes.

2.3. Analytical Methods in ABPP

Many methods have been developed for the detection and identification of tagged proteins which should be regarded as being complementary. One of the most mature and frequently used analytical methods for ABPP is in-gel fluorescence scanning (IGFS). This approach enables proteomes labeled with fluorescent ABPP probes to be resolved by SDS-PAGE according to size. Affinity enrichment through biotin–avidin binding is often used to identify the fluorescent bands. The bands are subsequently cut out from the gel, and after tryptic digestion are analyzed by using liquid chromatography/mass spectrometry (LC-MS). In recent years a series of gel-free methods have been developed that meet different demands, such as higher sensitivity or simultaneous determination of the binding position of a probe in an enzyme.[35–37] Among these, tandem orthogonal proteolysis (TOP),[36] antibody-based methods,[35,38] as well as two-dimensional LC-MS/MS protein identification technology (MudPIT)[39] should be mentioned.

2.4. Reactive Groups and the Specificity of ABPP Probes

Numerous reactive groups have been deployed as the central motifs for the development of ABPP probes, including fluorophosphonate,[21–23] sulfonate ester,[40] α-chloroacetamide,[41,42] Michael acceptors,[43–45] as well as epoxide, vinylsulfone, diazomethyl ketone, α-haloketones, and acyloxymethyl ketone.[46–52] Probes that contain these reactive groups bind covalently in the active site of enzymes, and the various enzyme classes can be classified and differentiated on the basis of their mechanism of action (Figure 3). While fluorophosphonates react highly specifically with the enzymes of the serine hydrolase family, vinylsulfone, diazomethyl ketone, α-haloketones, and acyloxymethyl ketone show a clear preference for cysteine proteases. Sulfonate ester, α-chloroacetamide, and Michael acceptors largely react unspecifically, and can, depending on the structure and side chains of the probe, react with various enzyme classes that have an active center with appropriate nucleophilicity. Chemical modification enables the reactivity to be confined and the probes optimized according to the requirements of specific target enzyme classes. In this way, reactive Michael acceptors, such as cinnamaldehyde[44,45] or aryl vinyl sulfonate and aryl sulfone,[53] can be applied successfully for the labeling of phosphatases.

Probes with a hydroxamate group can provide a highly specific interaction by chelating with bivalent zinc ions in the active centers of metalloproteases. Although this interaction is reversible, hydroxamates can still be used as ABPP probes. By using a photoreactive cross-linker, such as benzophenone or diazirine, the probes can be covalently bound to their target enzymes after UV irradiation, thereby achieving permanent labeling of the proteins.[54,55] The metalloprotease enzyme family, which is involved in many cancers, has been studied by using such probes.[55,56]

These examples demonstrate the wide range of potential reactive groups and their application to the specific labeling of proteins. Many natural products already display such reactive groups, such as, for example, epoxide or Michael acceptors. Fine adjustment through chemical modification is usually necessary to achieve specific biological effects. However, reversibly binding natural products can also be converted into ABPP probes through the preparative introduction of one of the previously mentioned reactive groups or a photoreactive cross-linker, which can enable the subsequent detection of the target protein of a compound or its localization in the complex proteome of an organism. Other articles in the literature can be referred to for a detailed overview of ABPP probes that are not based on natural products and which are therefore not elaborated on extensively in this Review.[13,57–60]

3. Natural Products and Privileged Structures

Although natural products constitute the bulk (approximately 60%) of all approved pharmacologically active substances, many of their target locations and mechanisms

of action are so far unknown.[61–63] Natural products normally display a finely attuned reactivity for their natural molecular targets; they bind highly specifically and selectively to a few selected structures, such as proteins, nucleic acids, or their complexes. The interaction of natural products with their target proteins can range from relatively weak protein–ligand interactions to very stable covalent modifications. Some natural products contain highly reactive groups, which at first glance appear to be barely compatible with the many possible reaction partners present in a living organism. However, the reactivity of these compounds can be appropriately adjusted through modification so that they inhibit fewer enzymes and thereby achieve a more-specific effect in the target organism.[64]

Natural products have become optimized for their corresponding effects over millions of years of evolution, and thus their reactive or high affinity central elements (for example, lactone ring or penam scaffold) represent privileged structures, the application of which as molecular tools or pharmacological agents is of particular relevance.[18,61–63] The finely tuned reactivity of these compounds presents a very promising basis for their systematic investigation and modulation through the synthesis of derivatives. This makes it possible to carry out tailored changes to their reactivity or make them accessible to entirely new target proteins. In this section ABPP probes that are based on natural products and their derivatives or those that have been developed by the exploitation of their privileged structures will be introduced, and their possible biological applications discussed. Some of the subsequently discussed structures show a specific affinity for a particular enzyme or enzyme class; however, others label protein targets throughout different enzyme classes. The binding preferences of natural products is as versatile as their application spectrum, which spans from their use as lead structures in the design of active agents, to the identification of target structures of known compounds, and to their use as molecular tools.

3.1. ABPP Probes Based on Natural Products and Privileged Structures

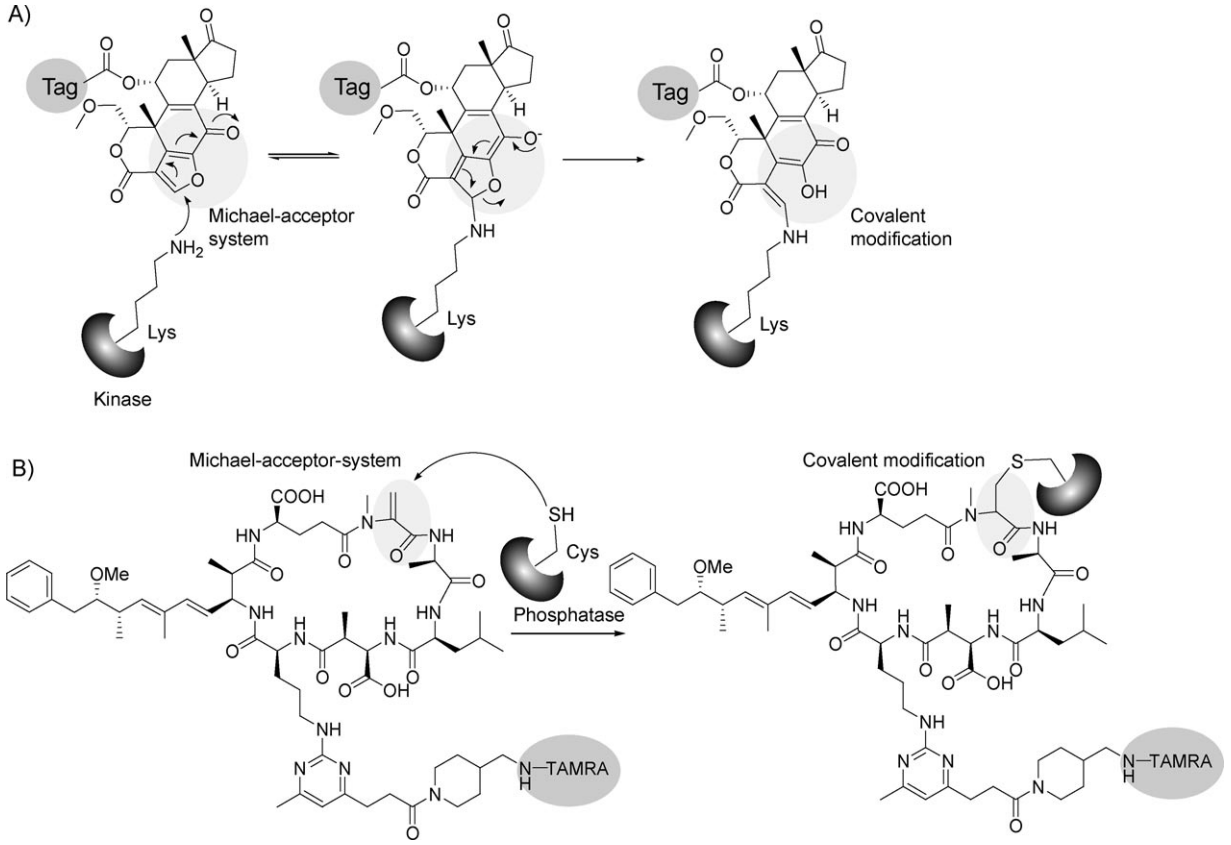
Two conditions have to be fulfilled when converting a natural product or a compound with a privileged structure into an ABPP probe. The natural product should either already display an intrinsic reactivity that makes it possible to bind the probe covalently to the target protein, or it should be possible to artificially introduce such a reactivity through a cross-linking moiety (Sections 2.1 and 2.4). The second essential requirement is the presence of a reporter group (for example, a fluorescent label; see Section 2.1) for detection.

3.1.1. Wortmannin

Wortmannin is a natural product that belongs to the furanosteroid group of compounds and is a metabolite produced by the fungus *Penicillium wortmannii*. Numerous studies suggest that Wortmannin is a potential inhibitor of the phosphoinositide-3-kinase (PI-3K) enzyme superfamily,[65,66] and at high concentrations also of related enzymes such as mTOR and DNA-PK.[67] The inhibition of these enzymes is achieved through covalent modification of the catalytic lysine residue in their active site by the electrophilic Michael acceptor system of Wortmannin. Since the regulation of the activity of the PI-3K enzyme family is disordered in many forms of cancer and they then become constitutively active, the antitumor activity of Wortmannin and its derivatives has been ascribed to their inhibition of PI-3 kinases.[68] The potential of Wortmannin as a pharmacological lead structure and for investigating cellular-regulation processes has inspired the development of Wortmannin-derived ABPP probes (Scheme 2 A). Attachment of fluorescent labels (BODIPY and rhodamin) to the Wortmannin scaffold through esterification of a deacetylated alcohol side chain at C11 have enabled the binding partners of Wortmannin and its derivatives to be studied in lysates and in living cells. In this way, Yee et al. were able to confirm the reactivity of Wortmannin against
PI-3 kinases and their related enzymes.[69] By using a similar activity-based approach, Liu et al. identified Polo-like kinase 1 (Plk1) and Polo-like kinase 3 (Plk3) as cellular targets of Wortmannin.[70,71] Here, the covalent binding was also achieved through attack of the electrophilic scaffold of Wortmannin on a conserved lysine; however, in this case the residue was in the ATP-binding pocket of the Polo-like kinases.[71] Since there is also a defective over-activation of Polo-like kinases in cancer cells, it is likely that Wortmannin is in part able to exert its pharmacological effect alongside PI-3 kinases through these enzymes. Such results highlight the value of ABPP in investigating molecular targets, and, from a pharmacological point of view, for identifying secondary targets of potent natural products in the proteome of organisms.

3.1.2. Microcystins

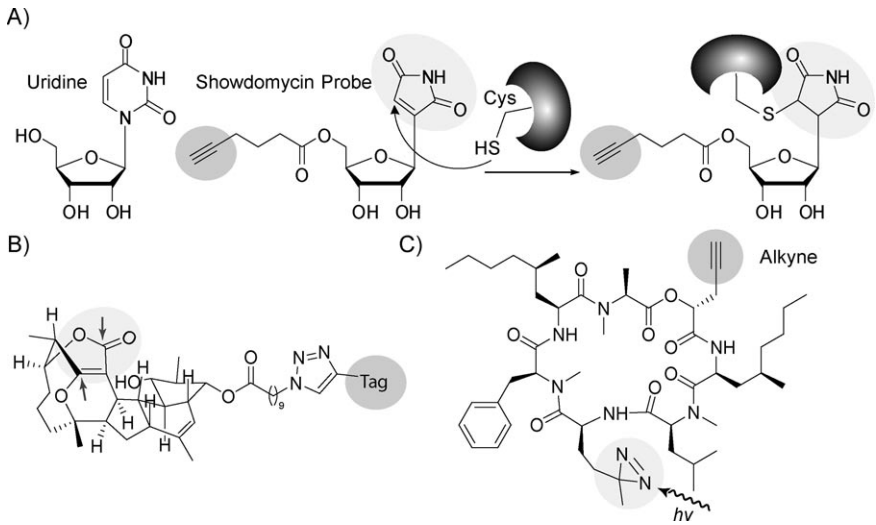
Similar to Wortmannin, microcystins also have a reactive Michael-acceptor system. Microcystins are nonribosomally synthesized cyclic heptapeptides, which are produced by cyanobacteria during algal bloom and are toxic to humans and animals. Microcystins are known inhibitors of the serine/threonine protein phosphatase families PP1 and PP2A, and show potential as anticancer agents.[72] The enzymes can attack the Michael-acceptor system of microcystins through nucleophilic attack by a conserved cysteine in their active site; this leads to the covalent binding of the inhibitor (Scheme 2 B).[73] By condensation of a 1,3-diketone with an arginine residue, which was not essential for affinity against phosphatases, Shreder et al. were able to introduce rhodamine as a fluorescent label in the microcystin (microcystins-LR), and thereby establish a microcystin probe for ABPP.[74] Specific binding of microcystins-LR to various protein phosphatases could be demonstrated in Jurkat cell (immortalized T lymphocyte cell line) lysates by using this probe. Furthermore, the method could be successfully used to investigate changes in the activity of phosphatases in cells pretreated with the phosphatase inhibitor calyculin A.



Scheme 2. Natural product based probes with a Michael acceptor as the reactive group. Mechanisms for labeling kinases with a Wortmannin probe (A) and phosphatases with a microcystin probe (B).

3.1.3. ***Showdomycin***

The natural product and nucleoside analogue showdomycin is an active antibiotic isolated from the actinobacterium *Streptomyces showdoensis*.[75] The structural similarity of showdomycin to uridine and pseudouridine makes it a potential reaction partner for corresponding uridine- or pseudouridine-binding enzymes.[76,77] Instead of a pyrimidine in the 1′-position of ribose, showdomycin has a reactive maleimide unit. Nucleophilic thiols, such as those of cysteine residues in the active site or substrate-binding pockets of various enzymes, could be added to this unit (Scheme 3 A). Böttcher and Sieber developed a showdomycin probe by modifying its free primary 5′-OH group with a hexanoyl moiety.[78] The application of this probe to living Gram-positive and Gram-negative pathogenic bacteria and subsequent identification of its molecular targets resulted in the detection of a series of essential enzymes of the oxidoreductase and transferase families. The sum of all essential target enzymes might explain the antibacterial effects of showdomycin.[78] The detection of the molecular targets MurA1 and MurA2, which are essential enzymes for cell-wall biosynthesis, is of particular interest. Furthermore, some important differences in the activity of virulence-associated enzymes between pathogenic and non-



Scheme 3. A) The showdomycin probe as a uridine nucleoside analogue reacts with proteins by the addition of a mercapto group. B) Probe based on FR182877 with two reactive groups (arrows), and C) probe based on HUN-7293 with a diazirine, which can be photoactivated for covalent binding to the target protein, and an alkyne group, for subsequent click reactions.

pathogenic strains were uncovered. These results highlight the value of this probe as a tool for the investigation of the process involved in pathogenesis.

3.1.4. FR182877

Cyclostreptin (−)-FR182877 is a complex natural product with a series of interesting pharmacological effects, including towards cancer cells. The structure of this compound provides two electrophilic groups—a Michael acceptor with a strained olefin on its bridgehead atom and a lactone unit—for reaction with its targets in the proteome. The effect of cyclostreptin in the disruption of the cell cycle has been traced to its interaction with microtubules, the protein filaments that ensure the correct segregation of chromosomes during cell division.[79,80] To identify further targets Adam et al. synthesized (−)-FR182877 and modified it through a free alcohol group with rhodamine and a rhodamine–biotin linker for enrichment of its binding partners (Scheme 3B).[81] By using this probe, carboxylesterase-1 was identified as the main target of (−)-FR182877 in the proteome derived from a variety of mouse tissues. This enzyme was found to be completely bound with a 100-fold lower concentration of the compound than reported with microtubules. Moreover, it was shown that only the natural cyclostreptin (−)-FR182877, but not (+)-FR182877 inhibits carboxylesterase-1. Since carboxylesterases have been shown to be incorrectly regulated in many forms of cancer, cyclostreptin and its derived probes are simultaneously interesting pharmacological lead structures and diagnostic tools. By using ABPP it was thus possible to identify an additional binding partner for this natural product, which probably contributes significantly to its pharmacological effects.

3.1.5. HUN-7293

HUN-7293 is a fungal cyclodepsipeptide that inhibits the expression of vascular cell adhesion molecule (VCAM). In contrast to the aforementioned inhibitors, HUN-7293 binds reversibly and thus does not become covalently linked to its targets, which impedes their identification. Therefore, MacKinnon et al. synthesized a photoaffinity probe by using photoleucine instead of leucine in the scaffold of the cyclodepsipeptide. This probe is able to covalently bind to HUN-7293 binding partners through the photoreactive diazirine group of the photoleucine after UV irradiation (Scheme 3C).[82] A propargyl group was introduced for visualization, linked through a click reaction to rhodamine azide after UV irradiation of the probe with the endoplasmic reticulum proteome fraction. Sec61α was identified as the probe's binding partner; this protein forms the structural core of the translocation channel at the start of the secretion pathway for all proteins in the endoplasmic reticulum. Thus, the molecular target of HUN-7293 could be directly detected by using ABPP, and an explanation for its mode of action attained.

3.1.6. β-Lactones

Other smaller, more moderate types of electrophilic heterocycles that have been used as ABPP probes are β-lactones. Their electrophilic ring systems can be attacked at the C2- or C4-positions by corresponding nucleophilic groups in the active site of enzymes, and create a covalent bond with their target protein under ring opening. Although some β-lactones, such as obafluorin[83] or hymeglusin,[84] have been reported to exhibit antibiotic activity, and lipstatin[85] and tetrahydrolipstatin,[86] which act as inhibitors of pancreatic lipase, have medicinal applications, relatively little is known about the natural spectrum of the target enzymes of β-lactones in bacterial and eukaryotic proteomes. To address this, Böttcher and Sieber synthesized a small biomimetic library of activity-based *trans*-β-lactone probes with a variety of side chains in the C3-position and an alkyne at the C4-position, which could then be modified with a fluorescent reporter group by using a click reaction after labeling the proteome with the probes.[87] Aromatic and aliphatic side groups of varying length and degrees of substitution were applied to obtain naturally inspired β-lactones (Figure 4A).

In vitro experiments with bacterial proteomes resulted in the selective labeling of over twenty enzymes from four different enzyme classes with different preferences depending on the level of the substitution of the probe. Although the natural substrates of these enzymes and their catalytic reactions are very different, all the labeled enzymes share either a nucleophilic cysteine or serine residue in their active sites. Some of the identified targets, which include resistance- and virulence-associated enzymes, such as the bacterial caseinolytic peptidase (ClpP), are of particular interest and will be described in more detail in the section on biological applications (Section 3.2.1.2).

Activity-based probes consisting of a peptide scaffold, a β-lactone ring as the reactive unit, and biotin as the reporter group have been used to investigate the proteome of *Arabidopsis thaliana*. The use of these probes in vitro led to the identification of a peptide ligase (RD21) that was found to be responsible for N-terminal labeling of PsbP, a protein from the photosystem II.[88]

In another study clasto-lactacystin (omuralide), a β-lactone that is produced in situ as the active metabolite of the potent proteasome inhibitor lactacystin,[89] was used as a [^{3}H] probe. This led to the α chain of the proteasome being identified as the target of this natural product.[90]

3.1.7. β-Lactams

Besides β-lactones, the structurally closely related β-lactams are also privileged structures with a reactive electrophilic unit. Since the discovery of penicillin by Alexander Fleming, they represent the most important antibiotically active natural products in both pharmacology and applied medicine. Their antibacterial effects are based on the inhibition of penicillin-binding proteins (PBPs), which are essential constituents of the cell wall biosynthesis machinery and catalyze the linkage of the cell wall building blocks, the peptidoglycans. Different PBPs show a range of affinities for

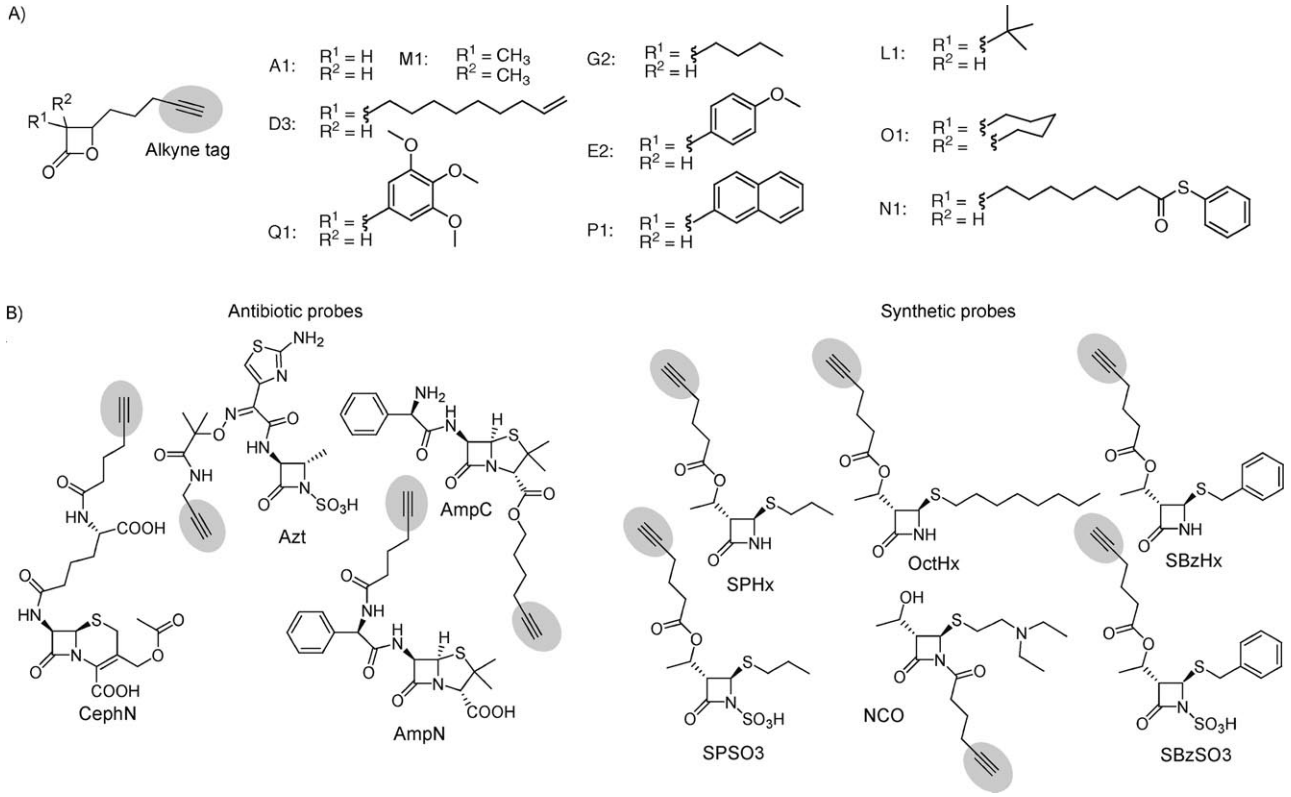
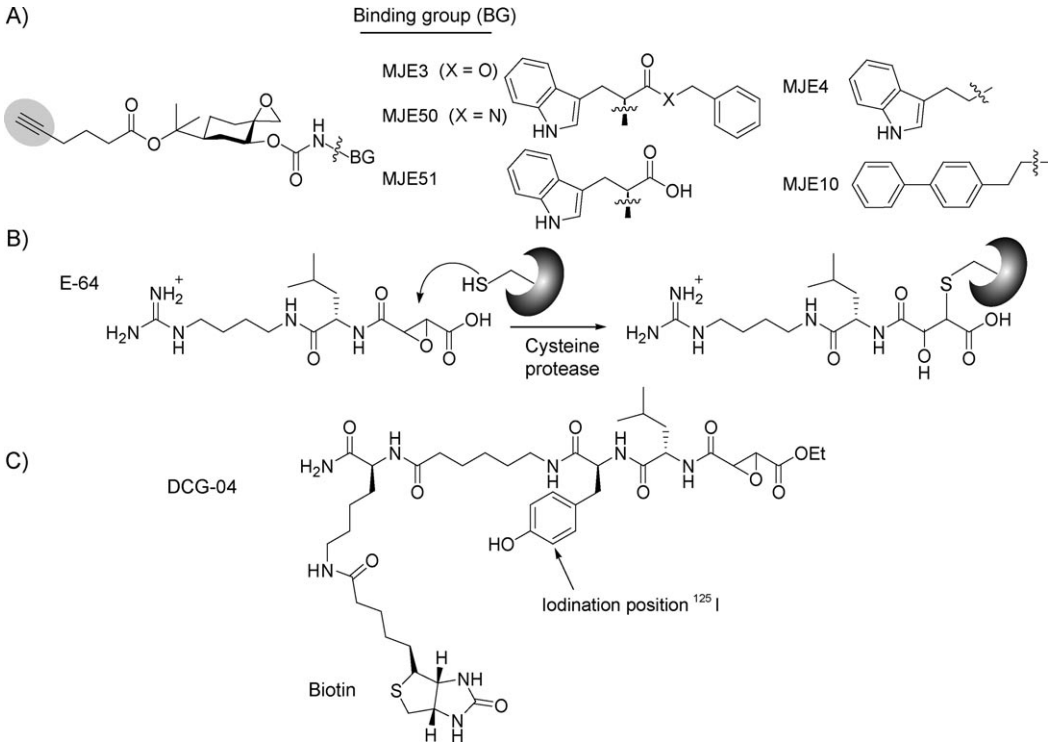


Figure 4. A) Biomimetic library of β-lactone probes. B) Probes obtained from the known antibiotics ampicillin (AmpC and AmpN), cephalosporin (CephN), and aztreonam (Azt) as well as some synthetic derivatives thereof.

particular β-lactams and provide a starting point for the evolutionary adaptation of bacteria in response to the enormous selection pressure applied by antibiotic treatment.[91] Research on PBPs is, therefore, of central importance for understanding the processes that lead to resistance and for developing new effective β-lactam antibiotics. β-Lactam probes with either radioactive or fluorescent reporter groups were used early on for the visualization of active PBPs in bacterial membrane fractions.[92,93] The significant disadvantages of these methods are, in the case of radioactive reporter groups, the time and technical requirements, and in the other case, the large fluorescent appendages, which adversely affect both the specific binding of the probes in active sites and their cell permeability in in situ studies. Staub and Sieber thus used β-lactams with a short alkyne group to detect and identify to date unknown PBPs and target enzymes in situ in living bacterial cells.[94] By using a probe library, which was derived from naturally occurring conventional antibiotics, they uncovered a variety of PBPs that were labeled by probes with differing preferences. At the same time, synthetic β-lactam probes revealed additional target structures, including a resistance-associated β-lactamase (Figure 4B). In a follow-up study, significant differences could be demonstrated between the enzyme activities of the pathogens *Staphylococcus aureus* and a multiresistant strain (methicillin-resistant *Staphylococcus aureus*, MRSA).[95] Hence, besides the already known resistance-associated enzymes, other to-date-unknown proteins were identified as binding partners, which were later characterized as having β-lactamase activity. Such results underscore the value of activity-based probes as tools for the identification and characterization of new target enzymes, which, particularly with regard to pathogenic bacteria, could be useful for the development of new strategies in the search for lead structures for novel drugs.

3.1.8. ***Spiroepoxides***

Some natural products have an electrophilic spiroepoxide unit as the reactive group in their molecular structure. These include the inhibitors and antitumor substances fumagillin,[96] luminacin D,[97] and FR901464.[98] The use of a biotinylated probe has already resulted in methionine aminopeptidase 2 (MetAP-2) being identified as a target for fumagillin in the proteome of eukaryotic cells.[99] On the basis of these natural products Evans et al. have produced a 1-oxaspiro[2.5]octan probe with an alkyne linker, which could be modified through a bioorthogonal click reaction with rhodamine and rhodamine/biotin reporter groups and used for the visualization and enrichment of target enzymes.[100] The scaffold of the probes was modified by the addition of various side groups, which were then used to uncover the different steric and electronic preferences of the substrate-binding pockets of their target enzymes (Scheme 4A). One probe (MJE3) was found to display particularly outstanding activity during pharmacological activity testing of the probe library against a breast cancer cell line. Live cells were thus treated with the probe library in situ to investigate the molecular target of this probe. Following cell disruption and a click reaction, the



Scheme 4. Probes with epoxy groups as the reactive motif: A) natural product based spiroepoxide probes with various substituents. B) Mechanism of inhibition of cysteine proteases with E-64; C) an E-64-derived probe with biotin and a radioactive ^{125}I marker.

targets of the probe were visualized on a fluorescence (SDS-PAGE) gel. The results showed that a band associated with a 26 kDa protein was only obtained with probe MJE3. This band was absent with all other structurally related probes, and all other bands labeled by MJE3 were also covered by other compounds from the probe library. Mass spectrometric analysis of the band resulted in identification of the enzyme phosphoglycerate mutase 1 (PGAM1), which, however, can only be labeled in living cells. This finding suggests that in living cells other factors must be involved in the binding of spiroepoxide probes that are not available in the in vitro proteome; this underlines the importance of ABPP in in situ studies. Preliminary studies already suggest a possible role of PGAM1 in the proliferation of cancer cells.[101] In this particular case, ABPP could be used to establish a new pharmacologically active inhibitor together with its target in the proteome.

3.1.9. Epoxides

Alongside spiroepoxides, non-anellated epoxides are also known to act as reactive groups in natural products. These electrophiles react preferentially with nucleophilic mercapto groups in the active centers of cysteine proteases (Scheme 4B). Epoxysuccinyl peptide E-64 (L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane), which was isolated from the mold *Aspergillus japonicus*,[102] also belongs to this class of compound. This natural product is a known covalent inhibitor of various cysteine proteases, including cathepsins B, H, and L, which play an essential role in programmed cell death (apoptosis) as well as proliferation and invasion of cancer cells.[46,103] Other protease families, however, such as the serine proteases or metalloproteases, are not inhibited by E-64. Greenbaum et al. have established ABPP probes based on E-64 with different reporter groups, such as the radionuclide ^{125}I, biotin, and the fluorophore BODIPY (Scheme 4C).[104,105] These probes have proved to be valuable analytical tools for the functional analysis of cathepsins.

Epoxomicin, another epoxypeptide with anti-inflammatory and antitumor activity, has also been modified with biotin linkers to produce a probe, which was used for the investigation of its binding partners in the proteome of mouse cell lines. Four catalytically active subunits of the proteasome have been identified as its molecular targets in living cells.[106,107]

Trapoxin is also a natural epoxide; however, in contrast to the linear epoxypeptides, such as epoxomicin and E-64, trapoxin is a cyclotetrapeptide. It was isolated from the fungus *Helicoma ambiens*, and has been described to be an antitumor agent and efficient inhibitor of histone deacetylation.[108] The combination of isotope-labeled [^{3}H]- and [^{125}I]-trapoxin probes and trapoxin derivatives immobilized on solid supports led to the detection and characterization of a histone deacetylase as the target of this natural product.[109]

3.2. Biological Applications

Natural products have a wide spectrum of biological effects and pharmacological application opportunities. As ABPP probes, they have enormous potential, on the one hand, as molecular biological tools, and on the other, as

instruments and lead structures for research on pharmaceutically active compounds. The graduated reactivity of natural products offers the possibility for the selective labeling of active enzymes in complex proteomes. Thus, pathogenesis-associated enzyme activities can be investigated in proteome lysates and in living cells, or diagnostic profiles of diseases or their causative agents can be determined. As demonstrated with the various β-lactone,[87] β-lactam,[94] and the showdomycin[78] probes, pathogenic and nonpathogenic bacteria can be differentiated by the activity profiles of their proteomes. Thereby, some tremendous differences in the activities of virulence- and resistance-associated enzymes could be determined, which may represent a new strategy for the identification and characterization of pathogenic and multiresistant bacterial strains on the basis of their proteome signature.

Since activity-based labeling implies the binding of a probe in the active site of an enzyme, a potential inhibitor is simultaneously discovered with every labeled target structure. A successful probe could, therefore, be used in pharmaceutical research either directly or after further structural optimization. Hence, there is a smooth transition from the use of ABPP probes as tools in molecular biology to their application as agents in pharmacological research.

Classical methods used in the search for pharmaceutically active compounds usually test a library of natural products and their derivatives against individual pharmacologically interesting target enzymes or against pathological model systems (for example, cancer cells), and promising candidates are then selected from the library. This approach, however, has significant disadvantages. In the first case, many positive results are obtained with compounds which have either bound unspecifically to an enzyme and/or show preference for entirely different target proteins in the complex proteome of an organism. In the second case, the molecular targets are initially unknown, and it is possible that the observed effect is achieved through an indirect activity or toxicity, which then disqualifies the compound from therapeutic applications. Furthermore, promising lead structures can also be completely overlooked if they have been used without preceding pharmacokinetic optimization, as they might not show any effects if they have low cell permeability or are quickly degraded in the organism.

Activity-based probes circumvent these problems. They facilitate the determination of the molecular targets of natural products either in complex proteomes or even directly in living organisms, and thereby enable the characterization of both the primary and secondary targets. This approach is particularly appealing for site-directed structure optimization for the binding of a particular protein.[110] It should, however, be noted that this technique is primarily effective for natural products that bind covalently, whereas many reversibly binding pharmacologically active compounds that are of interest for the pharmaceutical industry have limited applicability, since they require a cross-linking reagent. Nevertheless, the use of ABPP probes eases the search for interactions of the compound under study with proteins in the human organism, which is essential for the early detection and prevention of potential side effects during the development of pharmaceuticals.

The application of natural product based ABPP probes both as tools and pharmaceutical agents will be discussed in this section. As both aspects are tightly interlinked, they will be detailed in the following according to the given subheadings.

3.2.1. *Human Diseases*

3.2.1.1. *Cancer*

The vast potential of ABPP probes for research into the development of cancer can already be impressively demonstrated with fully synthetic fluorophosphonate and hydroxamate probes, which have been used for the selective analysis of the activity of serine hydrolases[39,111] and metalloproteases, respectively.[55] Natural product probes have also made significant contributions in this respect. Probes based on E-64 (Section 3.1.9) have been used to investigate the role of the cysteine cathepsin family in pancreatic islet cell tumors of transgenic RIP1-Tag2 mice. These studies were motivated by the discovery by Joyce et al. that the expression of the genes for these cysteine proteases, particularly for cathepsins B, C, L, and Z, are up-regulated during tumorigenesis.[8] The activity of cathepsins could be directly confirmed by labeling the proteome with E-64 probes. In vivo application of a fluorescent (BODIPY), cell-permeable E-64 probe enabled the localization of the cathepsin activity in tissues and tumor cells by confocal microscopy. Particularly high activity was found in the angiogenic vasculature and the invasive front of carcinomas. The combination of chemical knockouts with an E-64-derived broad-spectrum inhibitor for cathepsins enabled the crucial role of these enzymes during angiogenesis, proliferation, and invasion of tumor growth to be determined. Cathepsins could, therefore, serve as potential pharmacological targets, and E-64 could be used as a potential lead structure in the search for new drugs in cancer treatment.

Other natural products, such as FR182877, microcystins, and Wortmannin, which have been implicated in cancer research, have already been discussed Section 3.1.

3.2.1.2. *Infectious Diseases*

It is not only in cancer that proteases play an essential role. Numerous individual proteases are also involved in the life cycle of the protozoan *Plasmodium falciparum*, the causative agent of malaria, and they present attractive targets for medical treatment of this infection.[112] Greenbaum et al. used a protease-specific probe based on E-64 to carry out a detailed investigation of one of these protease classes (Section 3.1.9).[113] A systematic activity-based study of the various life-cycle stages of the parasite showed falcipain 1 to be the only active cysteine protease in the merozoite stage during cell invasion of erythrocytes. The activities of falcipain 2/3 were, however, found to be strongly increased in other stages (trophozoite and schizont). Small epoxysuccinyl peptides were used in a competitive selection assay as inhibitors of falcipain 1, and their affinities and selectivities were categorized according to the competition of the inhibitors with the E-64 probe. The most potent inhibitor of falcipain 1 could prevent the invasion of *Plasmodium* into

erythrocytes and thereby disabled an essential step in the life cycle of the parasite.

In another study, an azide-modified E-64 probe was used by Hang et al. to investigate the activity of cathepsin B in eukaryotic cells during infection with *Salmonella typhimurium*.[114] Here, the E-64 probe was used to label cathepsin B in the proteome of primary macrophages and was then visualized following modification by Staudinger ligation (Section 2.2 and Scheme 1 B). As a result, it could be shown that the vacuoles of host cells that had internalized *Salmonella* no longer displayed cysteine protease cathepsin B activity, whereas nonpathogenic *E. coli* did not impair protease activity in the vacuoles and were digested. These results suggest that the inhibition of cathepsin B plays a possible role in the survival and virulence of the bacteria during intracellular infection of *S. typhimurium*.

The emergence of multiresistant strains has resulted in pathogenic bacteria such as *Staphylococcus aureus* posing an increasing threat to human health. The search for new targets in pathogenic bacteria, as well as the development of novel antibacterial agents is, therefore, of paramount importance in the struggle against infectious diseases.

By using a library of biomimetic β-lactone probes, Böttcher and Sieber identified inhibitors of caseinolytic peptidase (ClpP), a conserved serine protease that is essential for the virulence of many pathogenic bacteria.[87,115] Although it was already known from knockout mutants that ClpP plays a central role in infection by regulating the release of toxins and other virulence factors, and had, therefore, been put forward as a possible target for the treatment of infectious diseases, specific inhibitors of the protein were not known.[116] The treatment of living *S. aureus* cultures with a structurally optimized β-lactone led to the complete elimination of extracellular hemolytic and proteolytic activities, and also a considerable reduction in the production of the life-threatening PTSA toxins, even in multiresistant and virulent strains.[110,117]

This procedure was recently extended to pathogenic *Listeria monocytogenes*, which showed a significant reduction in virulence and intracellular growth of the bacteria in mouse macrophages.[110]

The deactivation of ClpP as a central regulator of the virulence of pathogenic bacteria offers an attractive method that enables the disarming of pathogens, and would then give the immune system of the host the opportunity to eliminate the bacteria. Biomimetic β-lactones could in this respect serve as potent lead structures, and their corresponding ABPP probes could serve as important tools in the development of antibacterial drugs.

4. Metabolite Profiling

In the event of disease, such as cancer or bacterial infections, it is the precisely balanced regulation of the activity of all the enzymes in the proteome or its disruption that is important. This complex regulation is not accessible to classical proteomics. ABPP probes based on natural products could help to close this gap. In addition to the regulation of enzyme activity, the products that form during the catalysis of biochemical reactions are also of fundamental relevance. Besides the usual products of primary metabolism, these comprise metabolites that include mediators of inflammation pathways, signaling molecules, toxins, and antibiotics, as well as numerous therapeutically and diagnostically interesting small molecules. Ultimately, the natural products from which ABPP probes are derived are themselves metabolites obtained from particular organisms. As the products of the entire network of interrelated biochemical reactions in an organism, they are the direct consequence of the cooperation of the active proteome. Although proteomic analysis by using ABPP probes with the currently available genomic and proteomic databases is now well-established, investigation of the metabolome—metabolomics—is still in its infancy. Nevertheless, important successes have already been achieved, and they will be outlined in this section.

4.1. Functional Characterization of ABPP-Identified Enzymes

Since the activity of enzymes often has a direct influence on the regulation and diversity of the metabolome, the identification of their substrates is an important prerequisite for the understanding, diagnosis, and treatment of disease. The identification of enzyme substrates has so far been carried out with in vitro investigations on compound libraries. The disadvantages of this approach are the limited diversity of the libraries, which only contain already known structures, and the neglect of important regulatory factors that only occur in living cells, such as, for example, posttranslational modifications, which are not taken into account by in vitro assays.[20] Although the in vivo interaction of metabolites with enzymes is intrinsically complex and methodologically challenging to detect, there have been a series of highly promising advances in recent years.[1,19,20,118–121]

Similar to ABPP, research on the metabolome is often carried out in a comparative fashion. In this way it is very easy to establish the profile of small-molecule metabolites, the occurrence of which can be correlated with the deactivation of particular enzymes. It has, thereby, already been possible to obtain meaningful information about the function of important enzymes previously identified by ABPP. In principle, the method of metabolite profiling can be divided into two approaches (Figure 5). The targeted approach is restricted to the characterization of specific metabolite classes, for which knowledge of enzyme function is a prerequisite. In this way, unique chemical characteristics, such as NMR-active atoms (^{31}P), molecular mass, and fragmentation pattern, are drawn together; this brings with it an increase of sensitivity in detection and quantification.[120,122] The second approach is untargeted, that is, metabolites are examined in their full breadth, but of course forfeits sensitivity.[1,19,20,118] We will limit the following discussion to untargeted discovery metabolite profiling (DMP), because of the larger methodological breadth that it offers, which in principle allows the determination of all the metabolite substrates of a particular target enzyme.

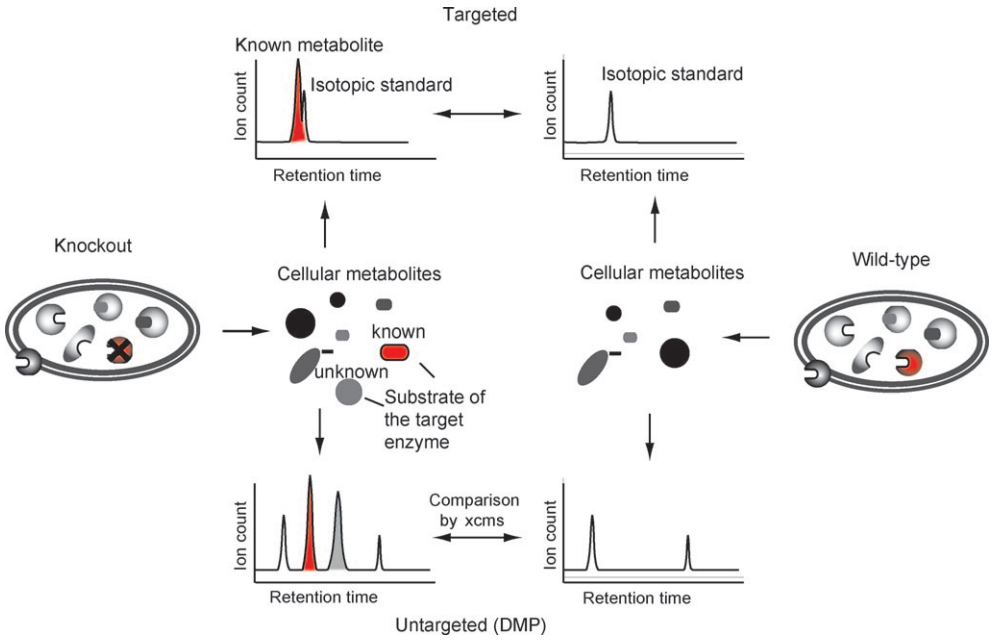


Figure 5. Principles of targeted and untargeted (DMP) metabolome analysis. While it is only possible to identify known metabolite substrates for individual enzymes by mass spectrometry with the targeted approach, unknown metabolites can also be detected with untargeted DMP.

4.2. Metabolite Profiling for the Elucidation of Enzyme Function

In contrast to other LC-MS-based metabolite analysis procedures in which isotope-labeled molecules are used, DMP operates on a global, untargeted level and enables the quantification of metabolites based on the absolute intensities of their mass ions over a broad mass range, and therefore does not require the use of internal standards.[1,19,20,118] To determine the masses and relative abundance of metabolites that arise as a consequence of enzyme inhibition and can thus provide information about the substrate and function of a given enzyme, software especially designed for this purpose, called XCMS, is used.[123] XCMS aids the detection, comparison, quantification (based on signal area), and assignment of statistical mass signals through the pairwise comparison of different LC-MS chromatograms (mutant enzyme versus wild-type). Thus, DMP allows the identification of known as well as unknown metabolites, which makes this method particularly interesting for subsequent experiments for the characterization of enzymes identified by ABPP, but for which the exact function is not clear.

DMP was first employed by Cravatt and co-workers for the investigation of metabolites in the central nervous system (CNS; constitutes the brain and spinal cord). In this study the metabolite substrates of the enzyme fatty acid amide hydrolase (FAAH) were investigated.[118] It is known from genetic and chemical inhibition studies that inactivation of FAAH is associated with antidepressive, anti-inflammatory, and sleep-promoting effects.[124–126] Hence, FAAH is a highly interesting target for therapeutic purposes, which is why the consequences of its inactivation need to be adequately investigated. Previous studies had shown that FAAH metabolizes lipids involved in signal transduction, for example, cannabinoid *N*-arachidonoylethanolamine (anandamide; Figure 6). Whether other metabolites of the CNS also acted as substrates for FAAH was, however, not known. Therefore, DMP was used to directly compare the metabolite extracts from the brain and spinal cords of mice with functional FAAH (wild-type) with those in which the enzyme was genetically deactivated (knockout).[118] The metabolome was obtained by extraction of the CNS tissue with organic solvents and separation by HPLC. The masses of its constituents were subsequently determined over the range 200–1200 kDa by using electrospray ionizations mass spectrometry (ESI-MS) in the positive and negative modes. In the case of the FAAH knockouts, a series of lipids were identified, including free fatty acids, phospholipids, and ceramides, the amounts of which were

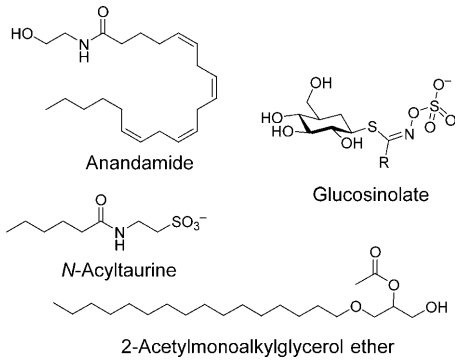


Figure 6. Molecular structures of some metabolites studied.

unchanged compared to the wild-type. As expected, however, a drastic change was observed in the known FAAH substrate anandamide and other *N*-acylethanolamines (NAEs). Interestingly, the amount of a previously uncharacterized class of metabolite was also found to be drastically reduced in knockout tissue. The real challenge of DMP indeed lies in the determination of the identity of such unknown substrates. It is, therefore, very helpful if clues already exist regarding the composition and characteristics of the molecule, for example, the biological context in which it occurs. In the case of the unknown FAAH substrate, the molecule was isolated by using preparative HPLC, and its molecular formula predicted on the basis of high-resolution MS. Together with mass spectrometric fragmentation studies, a pattern was identified that suggested the presence of a taurine unit and a very long fatty acid chain. To determine its exact identity it was ultimately necessary to synthesize many derivatives of different fatty acids and compare them with the natural metabolite. Indeed, the retention times and fragmentation patterns of both

natural and synthetic *N*-acyltaurines (NATs) were found to match; this clearly confirmed the identity of the matabolite (Figure 6). Further investigation with DMP also showed a high level of NATs in mouse liver and kidney tissue as a consequence of genetic or chemical FAAH knockouts; these results highlighted for the first time the importance of FAAH in the regulation of NATs in peripheral organs.[127] Subsequent investigation of the physiological importance of NATs showed that these small signaling molecules are able to activate the transient receptor potential (TRP) calcium channel.

It has long been suspected that the composition of the metabolome of cancer cells is significantly altered compared to that of healthy cells, which would then support their malignant characteristics. In previous ABPP investigations with FP probes (Section 2.4), Cravatt and co-workers found enzyme activities that occur in aggressive human cancer cell lines and primary tumors.[111,128,129] Among these, one particular enzyme (KIAA1363), which was active in the cell lines of a number of aggressive tumors, such as breast, skin, and ovarian cancers, particularly stood out. A potent and selective inhibitor was found by using competitive ABPP that would allow the precise function of this enzyme to be studied. By the addition of the inhibitor it was possible to deactivate the enzyme in subsequent experiments and thereby create a chemical knockout.[128] A comparative DMP analysis between KIAA1363-inhibited and uninhibited cancer cell lines showed that this uncharacterized enzyme regulates an unusual class of neutral lipids, the monoalkylglycerol ethers (MAGEs; Figure 6).[130] Further investigations then confirmed that the enzyme is a 2-acetyl-MAGE-hydrolase that produces large amounts of MAGEs in cancer cells. Moreover, the inactivation of KIAA1363 led to reversal of the migration and growth capabilities of cancer cells; these results make this enzyme an interesting pharmacological target. In this way, DMP was successfully used as a supplement to ABPP for the functional characterization of an important, pathologically relevant enzyme.

Sagathelian and co-workers recently looked closely at the function of the enzyme dipeptidyl peptidase 4 (DPP4) and demonstrated that the use of DMP is not only limited to the investigation of small hydrophobic molecules, such as lipids or steroids.[131] DPP4 regulates peptide hormones and plays a role in the occurrence of diabetes. To identify all the possible peptide substrates of this enzyme, metabolite extracts from the kidneys of knockout mice were compared with those of wild-type animals. Two peptides with the characteristic DPP4 cleavage site were found in higher amounts in the metabolome of knockout mice, and their corresponding cleavage products, resulting from activity of DPP4, were shown to arise in wild-type mice. These results confirmed that MS-based methods can also be used effectively for the identification of peptide metabolites and the elucidation of their physiological function.

As impressively documented by these examples, DMP is a powerful technological platform that enables the assignment of substrates to individual enzymes, the physiological and pathological importance of which has been investigated by ABPP, and therefore facilitates the elucidation of their exact cellular function. As an addition to DMP, Saito et al. have established another untargeted procedure for the determination of enzyme substrates.[119] The main difference between DMP and their method, which has the name metabolic enzyme and reaction discovery by metabolite profile analysis and identification (MERMAID), is that capillary electrophoresis is placed upstream of the mass spectrometer (CE-MS). This provides better separation and higher sensitivity compared to LC-based methods. Since only charged molecules can be separated by capillary electrophoresis, this method is unsuitable for uncharged or non-ionizable metabolites. MERMAID has hitherto only been applied to the in vitro characterization of so far unknown *E. coli* enzymes. For this purpose, recombinant enzymes were incubated with a large mixture of commercially available metabolites, and the subsequent changes arising in the metabolome composition were detected by CE-MS. Metabolites that decreased in concentration are probably substrates of the target enzyme, whereas metabolites that increased in concentration are likely products of the enzyme reaction. In this way, the uncharacterized *E. coli* enzymes YbhA, YbiV, and YihU were successfully assigned as a phosphotransferase, phosphatase, and dehydrogenase, respectively. These promising results raise the expectations for the in vivo application of this approach.

Undirected metabolome investigation can also be combined with the results of genome-expression profiles, and so enable the identification of protein–metabolite pairs. In this manner, Hirai et al. succeeded in correlating temporal changes in the transcriptome, as determined by DNA microarrays, with alterations in the metabolome, as obtained with mass spectrometry, in sulfur-starved *Arabidopsis*.[132] Under these sulfur-deficient conditions, it was possible to demonstrate that a class of sulfotransferases is involved in the meatbolism of glucosinolate (GLS); this was later confirmed with recombinant sulfotransferases and GLS (Figure 6). In addition to the above-discussed examples, these results illustrate how the integration of metabolome and transcriptome data can be used to elucidate the function of uncharacterized enzymes.

Apoptosis can be used as an example to illustrate that, in addition to small molecules, proteins can also act as substrates for particular enzymes, and can, therefore, play an important role in signal transduction. The specific challenge here lies in the identification of such proteins, which are degraded by the caspases responsible for the apoptosis proteolyic cascade. By using a novel process called PROTOMAP, which combines SDS-PAGE and LC-MS/MS, Cravatt and co-workers have also succeeded in taking into account the topographical information of proteolysis; this could not be done with previous methods (Figure 7).[133,134] The process begins with comparative gel elctrophoresis with apoptotic and normal proteomes. The various gel lanes are then cut into uniform fragments and, after tryptic digestion, the soluble proteins are analyzed by LC-MS/MS. Given that a good sequence coverage is achieved with this mass spectrometric sequencing method, conclusions can be drawn regarding the topographical cleavage sites of the proteases. This approach has resulted in 91 characterized and 170 uncharacterized protein sub-

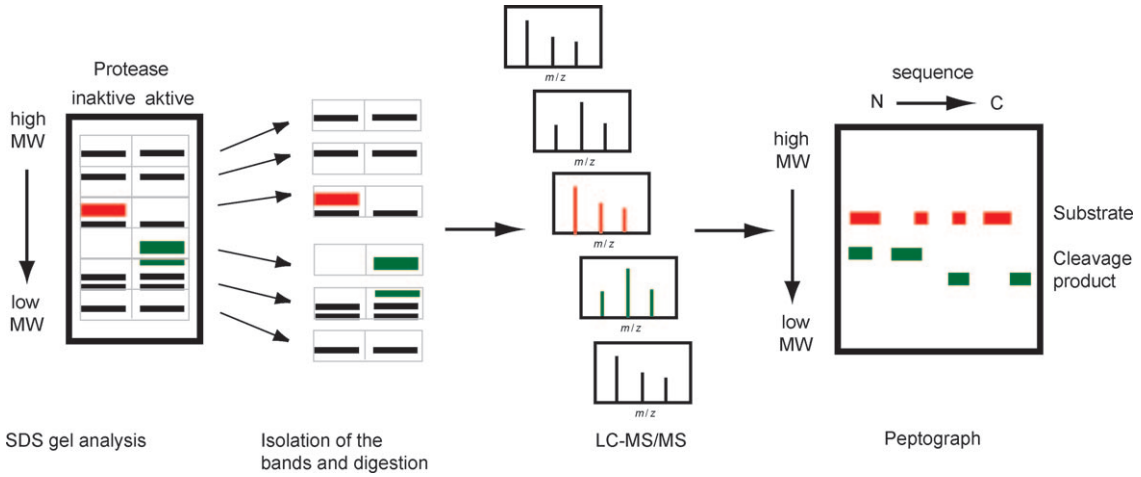


Figure 7. Principles of PROTOMAP technology. The method begins with comparative SDS gel electrophoresis of, for example, apoptotic and normal proteomes. The various gel lanes are then cut into uniform fragments. After tryptic digestion, the soluble peptides are analyzed by LC-MS/MS. This mass spectrometric sequencing of the total protein content enables, provided there is a good sequence coverage, conclusions to be drawn regarding the cleavage sites of proteases.

strates, which are degraded during apoptosis, being uncovered. Since this method is generally suitable for the investigation of proteolytic degradation, its application is not limited to apoptosis, but can be applied to the examination of protein substrates of a given protein, by comparison, for example, of a knockout proteome (chemically or genetically inhibited enzyme) with that of the wild-type.

The examples discussed so far have been concerned with the characterization and elucidation of enzymes that convert specific substrates in a catalytic process, and so, in a versatile way, are responsible for essential functions of the cell, such as, for example, signaling cascades and apoptosis. Beside these enzymes, however, there are also further sets of proteins in the cell that bind metabolites but do not process them. Saghatelian and co-workers have established a new approach to study such protein–metabolite interactions more closely, and through this they have identified the binding partners of various lipid-binding proteins (Figure 8).[135] For this purpose, the protein to be characterized is bound to a solid phase and incubated with the relevant metabolite extract. The protein–metabolite complex is subsequently cleaved from the solid phase and the purified protein is identified through DMP. The interaction partners of the fatty acid binding protein 2 (FABP2), which are associated with metabolic and cardiovascular diseases, and that of the lipid transfer domain StarD3 were successfully elucidated in this way. The advantage of this approach is not only that it can be used to identify metabolite–protein interactions, but metabolites that can have very low concentrations in the cell can also be isolated and enriched with this approach, which then facilitates their identification and structural assignment.

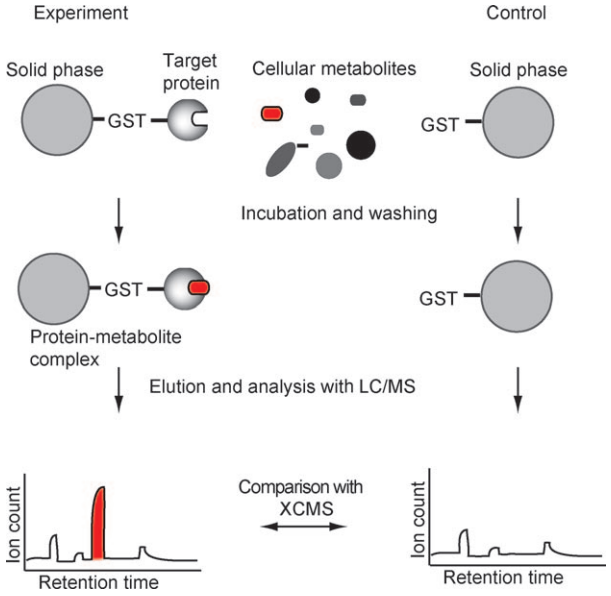
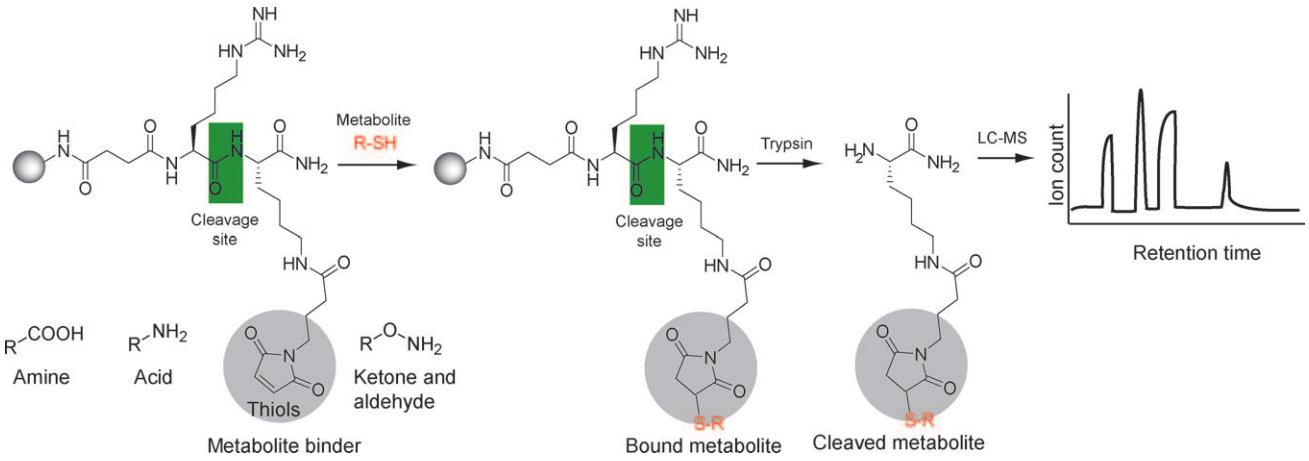


Figure 8. Identification of binding partners for lipid-binding proteins. The protein to be characterized is bound to a solid support and incubated with the relevant cellular metabolome extract. Protein–metabolite complexes are subsequently released from the support and the enriched metabolite is identified by using DMP.

4.3. Chemoselective Probes for the Targeted Enrichment and Identification of Metabolites

As already mentioned in the introduction, metabolites constitute a heterogeneous class of molecules that differ strikingly in their physiological properties, such as polarity, mass, and stability. Estimates indicate that between 4000 and 20000 different metabolites occur in the cell. It would be extremely useful to develop a directed approach for the targeted labeling, enrichment, and identification of individual metabolite classes—similar to that of ABPP discussed earlier—to elucidate the identity and function of these metabolites. The first step in this direction has been taken by Cravatt and Carlson, who have developed specific probes that allow the binding of metabolites based on a common chemical motif. This technique has been termed metabolite enrichment by tagging and proteolytic release (METPR;



Scheme 5. The principle of the METPR procedure. A solid support is derivatized with a series of different metabolite binders. These metabolite binders are a part of the linker, which combines two further essential functionalities. One of these is a recognition sequence, which after the addition of the protease trypsin, allows the release of the bound metabolites from the solid support and makes them available for detection by MS. An appendage is bound to the molecule through the reactive group to enable detection of metabolites that are normally too small for direct detection by MS by increasing the molecular weight of the metabolite to be identified.

Scheme 5).[136,137] For this purpose a solid support is derivatized with a series of metabolite binders, such as maleimide for the binding of thiol-containing metabolites, an alkoxyamine to bind ketones and aldehydes, a primary amine for the binding of preactivated acids in the metabolome, and an *N*-hydroxysuccinimide-activated acid to bind amines. These four groups already provide an extensive coverage of metabolites with suitable chemical functionalities. The metabolite binders are part of a linker that connects two further essential functionalities. One of these is a recognition sequence, which, after the addition of the protease trypsin, enables the removal of the metabolite from the solid phase and makes it available for MS detection. A molecular appendage was attached to the reactive group of metabolites that are normally too small for direct identification with MS so as to increase the molecular weight of the metabolite of interest.[136] After the method had been established with a series of test substances, the authors demonstrated the capability of their approach in a complex cellular metabolome. The sum of all the metabolite binders used was able to provide coverage for a substantial number of approximately 300 different molecules. Furthermore, the composition of the metabolome of a breast cancer cell line in the presence and absence of the antioxidant *N*-acetyl-L-cysteine was compared by using the METPR method. *N*-Acetyl-L-cysteine captures reactive oxygen in the cell by increasing the concentration of small thiol-containing molecules. Consequently, maleimide appeared to be the most suitable type of binder for this investigation. Analysis of the results of this comparative study with the aid of the software XC-MS showed a striking increase in the concentrations of cysteine and glutathione in antioxidant-treated cells. In addition, 17 other compounds were found to also have highly increased concentrations. The remaining challenge for this and other metabolite-profiling technologies as a whole lies in the rapid and reliable structural determination of the identified metabolites on the basis of their mass data.

5. Summary and Outlook

Over the past 10 years, ABPP has developed into a mature, standard technology for the rapid, sensitive, and selective identification of enzyme activity in complex proteomes. Today, a wide range of chemical probes exist that can be used in the investigation of many important enzyme classes and thereby make a significant contribution to the functional characterization of many diseases, for example, cancer, infection, and metabolic disorders. Natural products play a prominent role in ABPP technology. The examples outlined here highlight how probes based on natural products can easily be used to elucidate the targets of many so far uncharacterized molecules in complex proteomes and thereby can lead to the comprehension of their exact function and mechanism of action. Furthermore, natural products and their derivatives serve as pharmacological lead structures that can inhibit important targets in the cell and their efficacy can be demonstrated in biological assays. One future challenge is the expansion of the breadth of chemical probes to be able to study other protein classes, such as receptors, ion channels, and structural proteins.

Since the complex regulatory processes of a cell go beyond transcription, translation, and activation, it is crucial to also identify the substrates and binding partners of individual enzymes and proteins. For this, methods are necessary that allow characterization of the chemically complex metabolome, which is estimated to comprise up to 20000 different molecules. In recent years a series of interesting approaches have emerged for this purpose, one being, for example, DMP, which presents a suitable mass spectrometric platform for the untargeted global investigation of enzyme–metabolite pairs. One of the largest methodological challenges is the structural characterization of the masses of the metabolite obtained, which in many cases requires a high degree of intuition as opposed to standardized approaches that would be possible with protein sequences.

One of the challenges in this field, therefore, is the construction of databases, the mass and fragmentation patterns in which would allow faster clarification of the identity of individual metabolites.

In more recent years, further methods have been introduced that complement the more established genomic, proteomic, and metabolomic techniques, and which allow the investigation of other aspects of cellular regulation. One aspect that has been neglected to date is the breadth of the modification potential of transfer-RNA (tRNA), which in addition to the four standard nucleosides adenosine, cytidine, guanosine, and uridine can access approximately 95 additional modified bases. To understand how these modified nucleosides influence the translation and function of tRNA, Carell and co-workers have developed a chemical mass spectrometric method, during which the modified bases are first synthesized and labeled with a heavy atom.[138] These bases are then added to the prepared tRNA extracts and serve as internal standards for mass spectrometric quantification. The potential of this method has already been demonstrated in human cell lines and animal tissue with a variety of modified bases. Interestingly striking variations were observed in the pattern of tRNA–nucleoside pairs between cancerous and normal cell lines. These results open up new prospects for further intensive investigation in this, until now, overlooked area.

With such a breadth of established and new methods, one can speculate as to the extent and speed with which the remaining secrets of cell regulation and function will be uncovered in the future. The technological advancements in recent years, particularly in the field of mass spectrometry, have accelerated these developments and will in the future also be relevant for the success of this chemical biology approach.

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