

# Activity-Based Protein Profiling for Natural Product Target Discovery

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**Abstract** Natural products represent an important treasure box of biologically active molecules, from which many drug candidates have been sourced. The identification of the target proteins addressed by these natural products is a foremost goal for which new techniques are required. Activity-based protein profiling (ABPP), exploiting protein-reactive functional groups present in many natural products, offers unseen opportunities in this respect. This review article describes the current status of this field. Many examples are given for the annotation of biological target proteins of natural products containing epoxides, lactones, lactams, Michael acceptors, and other electrophilic groups. In addition, the development of probe molecules identified from biomimetic natural product libraries is discussed.

**Keywords** Activity-based protein profiling · Chemical biology · Molecular probes · Natural products · Target profiling

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## Abbreviations

|                  |                                                    |
|------------------|----------------------------------------------------|
| ABPP             | Activity-based protein profiling                   |
| ADPKD            | Autosomal dominant polycystic kidney disease       |
| AGT              | Alkylguanine DNA alkyltransferase                  |
| Asn              | Asparagine                                         |
| ATP              | Adenosine triphosphate                             |
| BG               | Benzylguanine                                      |
| BIOS             | Biology-oriented synthesis                         |
| BODIPY           | Boron-dipyrromethane fluorescent dye               |
| Cys              | Cysteine                                           |
| DNA              | Deoxyribonucleic acid                              |
| DOS              | Diversity-oriented synthesis                       |
| FAS              | Fatty acid synthase                                |
| FDA              | Food and Drug Administration                       |
| GT               | Glutamyl transpeptidase                            |
| HSP              | Heat shock protein                                 |
| HUVEC            | Human umbilical venous endothelial cells           |
| IC <sub>50</sub> | Half maximal inhibitory concentration              |
| Lys              | Lysine                                             |
| MALDI            | Maxtix-assisted laser desorption ionization        |
| MIC              | Minimal inhibitory concentration                   |
| MRSA             | Methicillin-resistant <i>Staphylococcus aureus</i> |
| MS               | Mass spectrometry                                  |
| NES              | Nuclear export sequence                            |
| PAGE             | Polyacrylamide gel electrophoresis                 |
| PBP              | Penicillin binding protein                         |
| PIKK             | Phosphoinositide 3-kinase related kinase           |
| PP               | Protein phosphatase                                |
| RNA              | Ribonucleic acid                                   |
| S                | Svedberg unit                                      |
| SDS              | Sodium dodecylsulfate                              |
| Ser              | Serine                                             |
| TAMRA            | Tetramethyl-5(6)-carboxyrhodamine dye              |
| THL              | Tetrahydrolipstatin                                |
| Thr              | Threonine                                          |
| UDP              | Uridine diphosphate                                |
| UMP              | Uridine monophosphate                              |

## 1 Introduction

Natural products are a distinguished class of organic molecules, which have turned out to be an incredible source of biologically active molecules. In contrast to synthetic compounds, they are privileged by their biosynthetic origin and their evolutionary purpose to address (in most cases) protein targets to exert their biological function. The evolutionary relationship between proteins of different species and even different functions might contribute to the explanation of why a natural product such as discodermolide, which is produced by a marine sponge, is also active against breast cancer in humans [1]. In an important analysis, Newman estimated that 47% of the drugs that received clinical approval over the last 25 years are either natural products, derivatives of natural products, or are based on a natural product lead [2].

The current innovation deficit in the pharmaceutical industry has initiated various programs to meet the challenges imposed by new and more difficult drug targets, more complex biology, and the tougher requirements for registration. Although in the recent literature some authors have claimed that for certain drug targets natural products do not perform as well as compound libraries arising from DOS (diversity oriented synthesis) [3], the general view is that natural products have a privileged status due their biosynthetic origin and remain an important source of future drug candidates [4]. As a consequence, two lines of research are pursued in this respect. The first involves inclusion of isolated natural products or natural product extracts in the compound collections used in high-throughput screening campaigns of pharmaceutical companies or academic consortia. The second involves the systematic scientific investigation of traditional folk medicine (traditional Chinese medicine, Ayurveda, ethnomedicine, etc.). The application of modern analytical methods to isolate the active principles of herbal medicines will identify new natural products as drug candidates.

## 2 Techniques for Identification of Protein Targets

These opportunities for drug discovery are complemented by the significant challenges imposed by the interest in and need for identification of the drug target(s) of these natural products. As the established paradigm of “specific compounds which bind to a single drug target” is now challenged by the insight that many drugs are effective because they modulate the function of several proteins at the same time (“polypharmacology”) [5], the problem of target annotation has become relevant for any small molecule drug, independent of its origin (synthetic or natural product) [6–8]. For the identification of drug targets of natural products, the approaches outlined in Fig. 1 are pursued.

**Qualified Guess**

- ⊖ is not systematic
- ⊖ requires a significant amount of available literature information
- ⊖ will not lead to a comprehensive annotation
- ⊕ can lead to a result very fast

**Identification in Specific Protein Assays**

- ⊖ will not lead to comprehensive annotation
- ⊕ simple to use; can be performed in a high-throughput format

**Identification via Matching Phenocopies**

- ⊖ will not lead to comprehensive annotation
- ⊖ immature, but emergent technique
- ⊖ probably not feasible for compounds addressing multiple targets
- ⊕ is designed for *in vivo* systems

**Affinity Based Approach (Chemical Proteomics)**

- ⊖ requires modification of the molecule to introduce an affinity tag
- ⊖ location of affinity modification might affect biological activity
- ⊖ cannot be used in *in vivo* systems, but only with cell lysates
- ⊖ limited to ligands with strong affinity to protein targets ( $K_d < 1\mu\text{M}$ )
- ⊕ will give a rather comprehensive annotation
- ⊕ in principle usable for any natural product which binds reversibly to a protein

**Activity Based Protein Profiling**

- ⊖ limited to natural products which bind irreversibly to a protein target
- ⊖ requires modification of the molecule to introduce a labeling tag
- ⊖ location of affinity modification might affect biological activity
- ⊕ will give a rather comprehensive annotation
- ⊕ can be used *in vivo*

**Fig. 1** Overview of the different approaches used for target identification of natural products

## **2.1 *Qualified Guesses***

Several natural products have been identified as the active principle of herbs or other medicines that have been used against certain diseases in traditional folk medicine. Starting from established mechanistic hypotheses about a certain disease, a natural product active against an illness can be screened for activity against proteins involved in the particular disease mechanism.

## **2.2 *Identification in Specific Protein Assays***

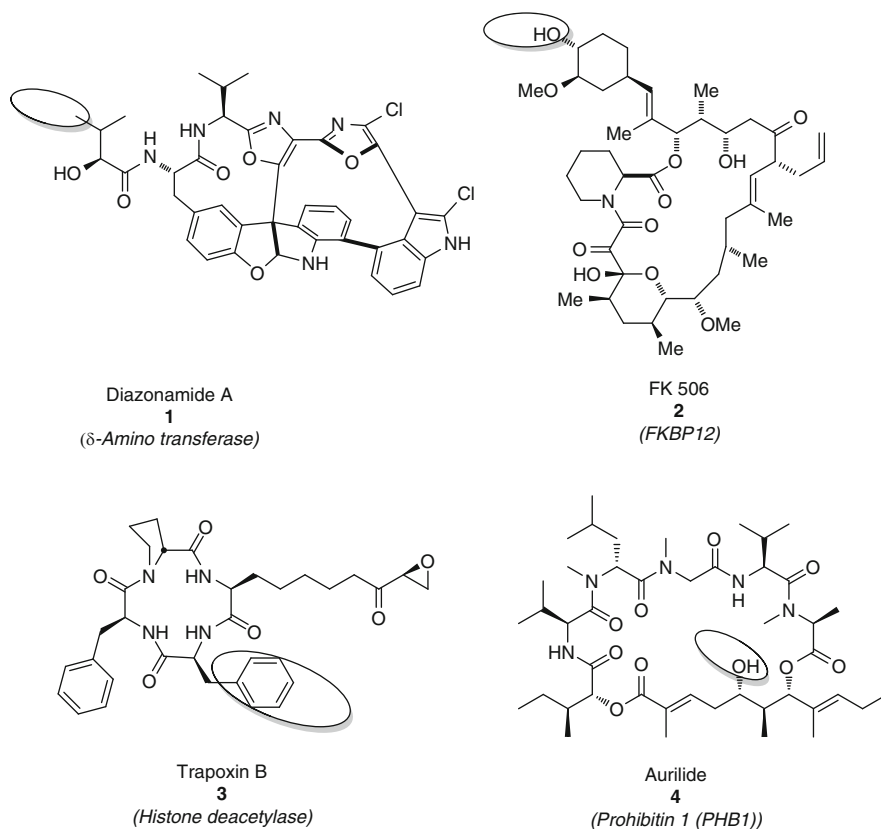
High-throughput screening techniques allow the unbiased screening of compound collections (up to three million compounds) in protein or cell-based assays. Routinely, natural products are also part of such screening libraries. Biological data gained from such screens on the one hand allow identification of new tool compounds for new drug targets and, on the other hand, contribute to the annotation of the biological activity of the screened compounds. For example, the public database PUBCHEM (<http://pubchem.ncbi.nlm.nih.gov/>) makes the screening data of hundreds of performed bioassays available, and increasingly the picture of the protein target profile of chemical compounds and natural products is emerging with higher resolution.

## **2.3 *Identification via Matching Phenocopies***

The protein target of compounds can be assigned by comparing the observed phenotype induced by this compound with known phenotypes from comprehensive phenotype collections achieved via gene knockout, RNA interference or by other chemicals [9]. This technique is currently far from maturity, but with increasing progress in automated microscopy, image analysis, etc. it promises a lot for the future. Nevertheless, one should keep in mind that the complexity of a compound that addresses several targets and gives unclear phenotypes will be difficult to resolve.

## **2.4 *Affinity Chromatography of Immobilized Natural Products***

This approach involves the immobilization of a natural product (at a site that is not responsible for its biological activity) on an insoluble support (or through a tag such as biotin, which can be used for subsequent immobilization) to form a probe. A cell lysate is brought into contact with this probe, so that any protein that binds to the natural product sticks to the support. After intensive washing of the affinity matrix,



**Fig. 2** Examples of natural products whose targets have been identified by an affinity-based approach. The *ellipse* indicates the location used for immobilization on an affinity matrix

the bound proteins are released, e.g., by addition of a chaotropic reagent. The proteins are then analyzed using standard techniques such as sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectroscopy (MS). Control experiments are necessary to distinguish between unspecific and specific binding to the affinity matrix [10].

This technique has led to the target identification of many natural products, such as Diazonamide A (**1**) [11], FK 506 (**2**) [12], Trapoxin (**3**) [13], Aurilide (**4**) [14] and many others (Fig. 2). Taking advantage of the impressive progress in MS has made this approach very popular for annotating the protein targets of drug molecules [15, 16]. This approach requires quite strong binders (typically a dissociation constant,  $K_d < 1 \mu\text{M}$  is necessary) to produce reliable data, but a modification in which a photoaffinity label is introduced at the probe molecules to enable a covalent linkage has expanded the range of molecules for which this approach is accessible.

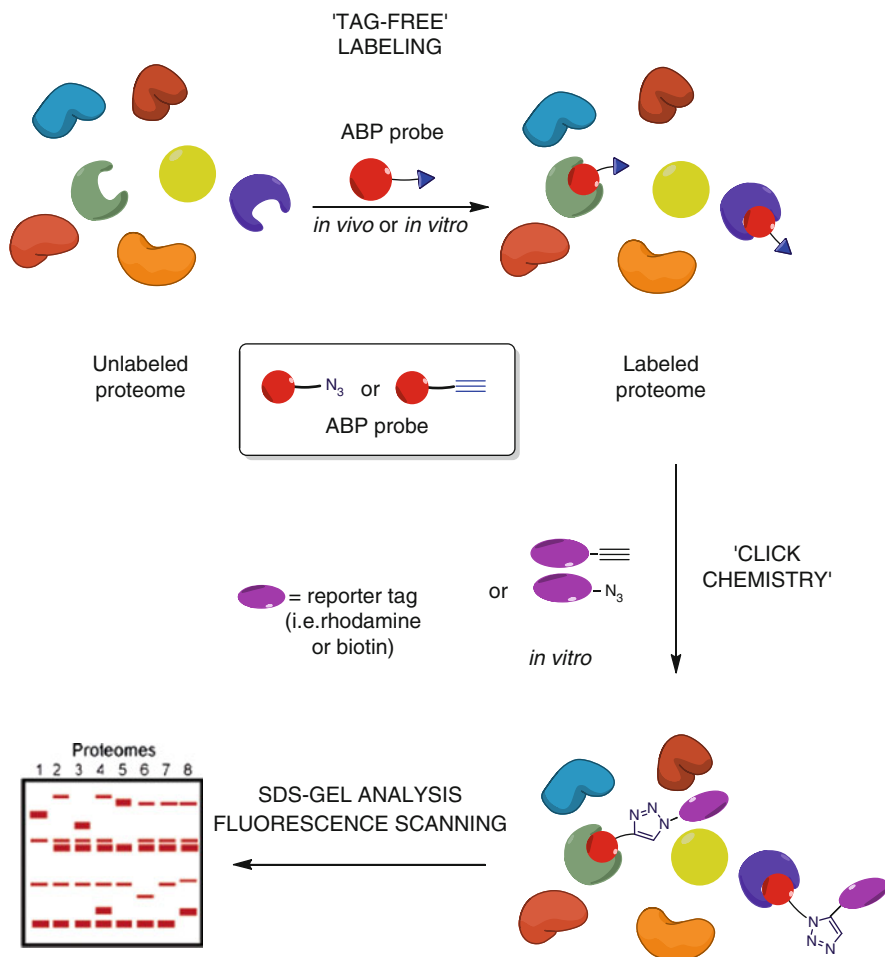


Fig. 3 General workflow in ABPP

## 2.5 Activity-Based Protein Profiling

Activity-based protein profiling (ABPP) is a technique that builds on a covalent linkage formed between the probe molecule and its protein target (Fig. 3). This technique has been pioneered by Bogoy, Cravatt and others, and has been summarized in several excellent reviews [17, 18]. Typically, the probe molecule contains an electrophilic group, which reacts with a nucleophilic amino acid close to the binding pocket of this substrate. To allow the identification within the complex proteome samples, the probe molecule should contain a label (radioactivity, affinity tag, fluorescence dye) or a group (azide, alkyne, etc.) to which such a label can be introduced via a bioorthogonal ligation reaction.

### 3 ABPP of Natural Products

Of the approaches listed above the last two (chemical proteomics and ABPP) are currently receiving the most interest and have complementary fields of applications. The affinity-based approach is perfectly suitable for reversible inhibitors, but is limited to quite strong binders. The focus of this chapter will be on the other of these two approaches, namely the activity-based approach to natural product identification. An essential requirement for this approach is that the natural product of investigation contains a reactive functional group that reacts with the protein target, forming a covalent bond. Fortunately, a considerable number of natural products contain such reactive groups [19]. Mostly, electrophilic moieties such as epoxides, Michael acceptors, disulfides, lactones,  $\beta$ -lactams, quinones, etc. can be found.

As described in the Introduction, natural products are privileged by their biosynthetic origin and biological purpose. Waldmann, Danishefsky, and others have suggested that natural products are ideal starting points for the synthesis of probe molecules. The concepts of “biology-oriented synthesis (BIOS)” [20] or “diverted total synthesis” [21] advertise that not necessarily a known natural product but some of its structural analogs will give a tool compound with improved specificity and affinity, or a tool compound that addresses new targets. As described in Sect. 4.3.3, the combination of BIOS around reduced natural product scaffolds and ABPP offers new opportunities for identifying molecular probes against new protein targets.

This chapter discusses several case studies of successful ABPP approaches in annotating the target proteins of natural products. The examples are classified according to the nature of the protein-reactive electrophilic group within the natural products. This article builds on the recent excellent review by Sieber et al. [22].

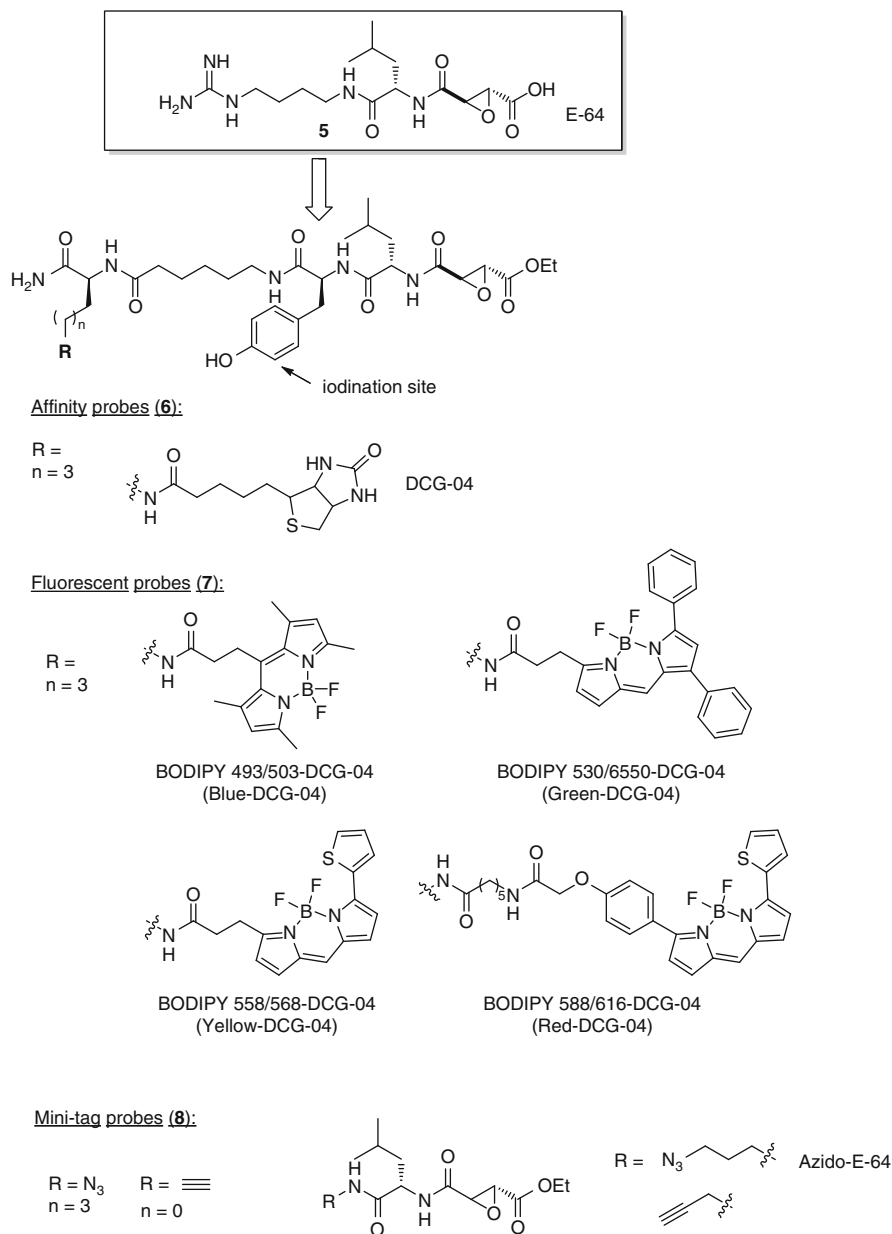
## 4 Examples of Natural Product Target Identification by ABPP

### 4.1 Natural Products Containing an Epoxide Group

#### 4.1.1 E-64

E-64 (1-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane) **5** was one of the first natural products used as a core for the development of an efficient activity-based probe (Fig. 4). It was isolated from a fungus *Aspergillus japonicus* [23] and its structure comprises an electrophilic epoxysuccinyl motif involved essentially in the inhibition by an active site Cys residue, whereas a Leu unit, which mimics the P2 amino acid of a substrate, is responsible for target recognition, and an agmatine moiety resides in the S3 binding pocket [24]. **5** was found to be a specific irreversible inhibitor of the broad papain family of cysteine proteases [25]. Some of these proteases are involved in physiological processes such as antigen presentation [26], bone remodeling [27], and prohormone processing [28]. Also, several of these





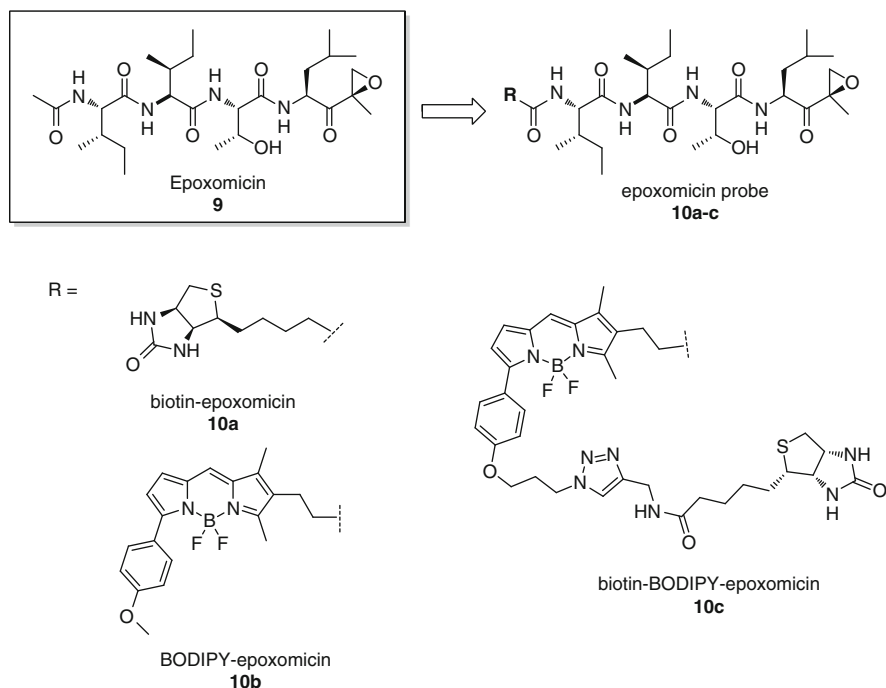
**Fig. 4** E-64 and probe derivatives used in ABPP

enzymes have been identified as playing an important role in many pathological conditions, such as osteoporosis [27], asthma [29], rheumatoid arthritis [30], or cancer invasion and metastasis [31, 32], making them a potentially important class

of enzymes for drug development. However, the papain family contains many closely related family members whose functions are poorly elucidated [27]. Therefore, efficient small molecule-based tools are appreciated in defining their molecular targets as well as their exact cellular function.

Bogyo et al. [33] developed a set of activity-based probes inspired by the natural product E-64 for global analysis of activity patterns of the papain family of cysteine proteases. Instead of the agmatine moiety, the first generation of ABPP probes (**6**) comprised a Tyr structure, which can be easily iodinated to introduce a radioactive tag. Also, a biotinylated Lys was introduced via an amino hexanoic acid spacer to furnish an affinity tag. The probes were used to profile activity of several cysteine proteases such as cathepsins B, H, and L in dendritic cell lysate and multiple tissue types (brain, kidney, liver, prostate, testes) as well as to follow their activity changes during the progression of skin cancer. The second library of probes (**7**) incorporated four cell-permeable boron-dipyrromethane (BODIPY) fluorescent dyes with nonoverlapping excitation and emission spectra, allowing for probe multiplexing [34]. It could be demonstrated that all fluorescent probes labeled the same four predominant proteases in dendritic cell lysates, as previously described for probes with a biotin or a radioactive tag. However, their remarkable advantage over the other probes was the cell permeability, which allowed in situ labeling in intact cells as well as imaging of labeled enzymes by fluorescence microscopy. The fluorescent probes also created a simple basis for rapid screening of a small molecule inhibitor library for selected targets in crude tissue extracts. This allowed the identification of a new cathepsin B-selective inhibitor from screening of a relatively broad library in crude liver extracts.

Van der Hoorn et al. [35] used a biotinylated version of E-64 to profile plant proteases in *Arabidopsis thaliana* and was able to identify six papain-like cysteine proteases enclosing the vacuolar *Arabidopsis* aleurain-like protease (AALP) [36] and the vesicle-localized desiccation-induced protease RD21 [37]. In a further study, Kashani et al. [38] used minitagged derivatives of E-64 (**8**) which contained a small innocent alkyne or azide group instead of a bulky fluorescent dye or a biotin. These tags are compatible with a two-step labeling procedure and click chemistry approach for appending a reporter tag. Importantly, the minitagged probes displayed a superior potency (tenfold) in labeling the above-mentioned enzymes in vivo (i.e., on detached leaves, cell cultures, or seedlings) due to their ability to penetrate cell membranes. The idea of using a small azide chemical handle was also used by Hang et al. [39]. They used their cell-permeable azido-E-64 probe **8** to profile the activity of cathepsin B during infection of primary macrophages with *Salmonella typhimurium*, a facultative intracellular bacterial pathogen. For visualization of the labeled cathepsin B residing in endocytic compartments, the Staudinger ligation was employed to attach a biotin tag. Interestingly, it could be demonstrated that the active cathepsin B is specifically excluded from *Salmonella*-containing vacuoles in infected cells, leading to the conclusion that the inhibition of endocytic proteases may give rise to the survival and virulence of intracellular bacterial pathogens.



**Fig. 5** Epoxomicin and probe derivatives used in ABPP

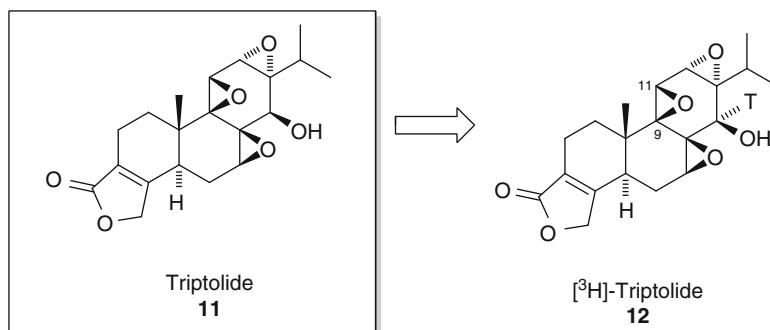
#### 4.1.2 Epoxomicin

Epoxomicin (**9**) is a natural product comprising an epoxy- $\beta$ -aminoketone moiety and tripeptide side chain and was isolated from an *Actinomycetes* strain [40]. It exhibits a strong antitumor activity against B16 mouse melanoma in vivo. To clarify the mode of action leading to this biological effect, the research group of Crews prepared a synthetic biotinylated derivative **10a** of epoxomicin (Fig. 5) to reveal its molecular targets [41, 42]. Incubation of the epoxomicin probe with the murine lymphoma EL4 cell line led to the detection of four catalytically active  $\beta$  subunits of the 20S proteasome: LMP7, LMPX, LMPZ, and MECL1. Thus, epoxomicin inhibits primarily the chymotrypsin-like activity of the proteolytic machinery of the 20S proteasome. It was also found that epoxomicin efficiently inhibited the nuclear factor- $\kappa$ B (NF- $\kappa$ B) regulator of inflammation and was able to reduce significantly a cutaneous inflammation in a mouse model. In further ABPP studies, BODIPY-derived epoxomicin probes **10b** and **10c** confirmed that epoxomicin inhibits effectively all three  $\beta$  subunits of the constitutive proteasome ( $\beta$ 1,  $\beta$ 2,  $\beta$ 5) as well as of the immunoproteasome ( $\beta$ 1i,  $\beta$ 2i,  $\beta$ 5i) in mammalian cells [43] and in plants (*A. thaliana*) [44]. Importantly and interestingly, it was demonstrated that epoxomicin, in contrast to other proteasome inhibitors (i.e., vinyl sulfones, lactacystin, bortezomib, NC005), was able to block the activity of the  $\beta$ 5t

subunit of murine thymoproteasome [45]. The thymoproteasome was found to be a third 20S proteasome particle, next to the constitutive proteasome and immunoproteasome, expressed in the epithelial cells of thymus cortex [46]. It comprises  $\beta 1i$  and  $\beta 2i$  subunits, exactly like the immunoproteasome, as well as a unique subunit  $\beta 5t$  of unexplained activity [47]. Using epoxomicin probes, it could be revealed that  $\beta 5t$  exhibits an intrinsic proteasome activity, exclusively present in young thymus, and that this unique subunit plays an active role in positive T cell selection, which is crucial for efficient functioning of the mammalian immune system.

### 4.1.3 Triptolide

Triptolide (**11**) is a diterpene triepoxide (Fig. 6) isolated from the *Tripterygium wilfordii*, which is a medicinal plant whose extracts have been used in traditional Chinese medicine as *lei gong teng*. Triptolide exhibits a whole range of interesting biological activities, such as potent antiproliferative and immunosuppressive activities. The proven medicinal track record in traditional medicine for important diseases has raised questions about the protein targets of this natural product. Crews prepared a tritium-labeled version ( $[^3\text{H}]$ triptolide, **12**) (Fig. 6) that they used for incubation studies of cells. After “extensive chromatographic protein fractionation, SDS-PAGE separation, MALDI-MS analysis” they identified, among other proteins of the membrane fraction, PC2 (calcium channel polycystin-2), whose cellular function they studied further. They could demonstrate that triptolide induces  $\text{Ca}^{2+}$  release by a PC2-dependent mechanism. Interestingly, they could show in a murine model of autosomal dominant polycystic kidney disease (ADPKD), an inherited disease that ultimately leads to renal failure, that triptolide attenuates overall cyst growth by restoring  $\text{Ca}^{2+}$  signaling in these cells [48]. In the same year, a research group at Merck using a similar approach but focusing on the anti-inflammatory effect of triptolide reported that this natural product covalently binds to a 90-kDa nuclear protein [49]. Recently, this protein was identified

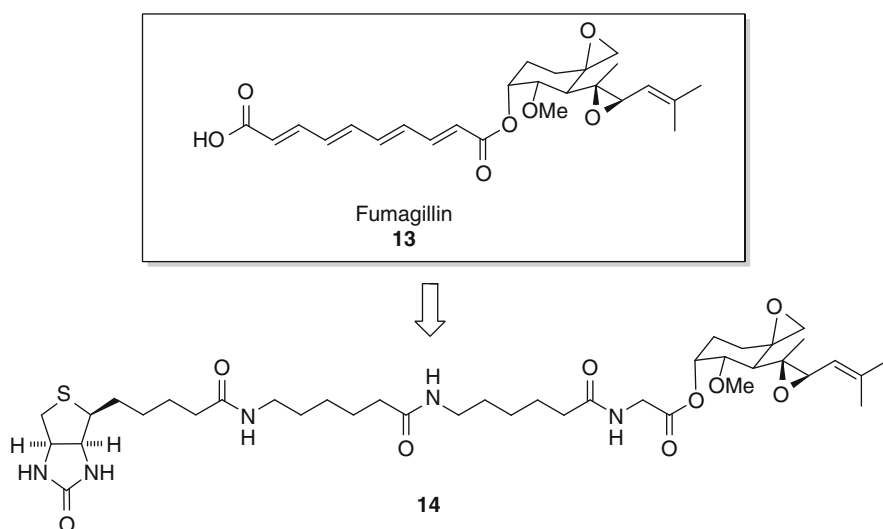


**Fig. 6** Triptolide

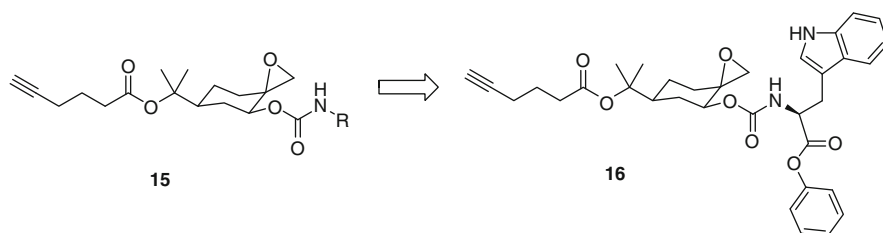
as XPB, a subunit of the transcription factor TFIIH. Triptolide inhibits its DNA-dependent ATPase activity, which results in inhibition of RNA polymerase II-mediated transcription and nucleotide excision repair [50]. Triptolide features several potentially reactive groups, such as an  $\alpha,\beta$ -unsaturated  $\gamma$ -lactone and three consecutive epoxides. The studies related to XPB indicate that the 9,11-epoxide is responsible for the covalent binding. Further studies will be necessary to give a full description of the interesting biological functions of this molecule.

#### 4.1.4 Fumagillin

The antiangiogenic natural product fumagillin (**13**) inhibits neovascularization via endothelial cell cycle arrest in the late G1-phase. Crews et al. speculated that the epoxide groups in this molecule might result in a covalent interaction with its target protein (Fig. 7) [51]. Having an activity-based approach in mind, they converted natural fumagillin with a few straightforward transformations into the biotinylated probe **14**, which retained cytostatic activity toward human umbilical vein endothelial cells (HUVECs). Incubation of HUVECs with **14**, followed by adsorption to streptavidin agarose, SDS-PAGE and blotting with avidin–horse radish peroxidase revealed a newly biotinylated 67-kDa band. Considerable efforts were necessary to isolate sufficient protein material for Edman sequencing, but this was ultimately rewarded by the identification of human methionyl aminopeptidase (metAP-2) as the cellular target of fumagillin [51].



**Fig. 7** Fumagillin and biotinylated probe derivative



**Fig. 8** Spiroepoxide library

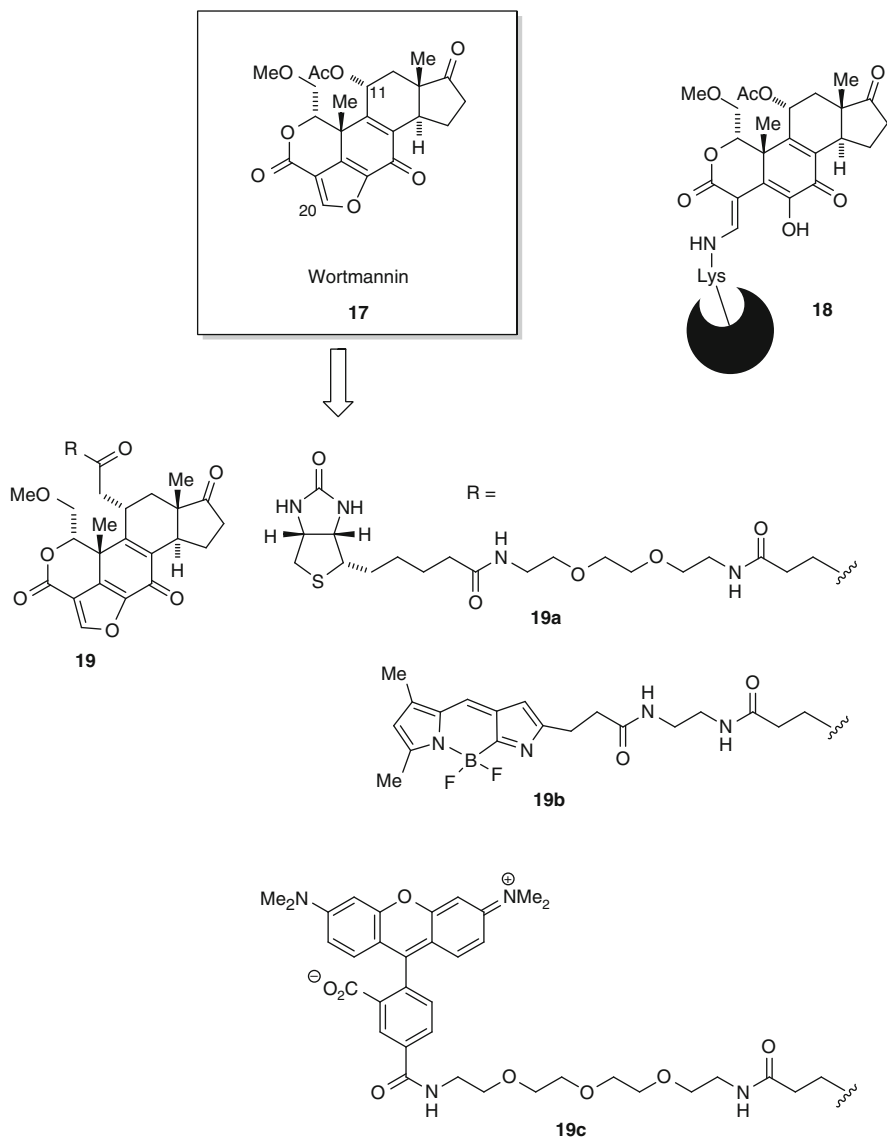
#### 4.1.5 Biomimetic Spiroepoxide Library

Inspired by the spiroepoxide structure of natural products such as fumagillin (**13**), lumanicin D, and FR901464, the groups of Cravatt and Sorensen designed a ~50-member library of spiroepoxides (**15**) containing an amino-derived recognition unit and clickable alkyne tag (Fig. 8) [52]. This library was screened for antiproliferation activity against the human breast cancer line MDA-MB-231. Compound **16** showed the highest activity and was also the compound that was used in an ABPP approach to identify its protein targets. Notably, brain-type phosphoglycerate mutase 1 (PGAM1) was identified as a protein target of **16**. This protein is a key enzyme in glycolysis and converts 3-phosphoglycerate to 2-phosphoglycerate. Interestingly, labeling and inhibition of PGAM1 with **16** was observed exclusively in intact cells [52].

### 4.2 Natural Products Containing a Michael Acceptor

#### 4.2.1 Wortmannin

The steroid-derived fungal metabolite wortmannin (**17**) was originally found as a potent and selective inhibitor of phosphoinositide 3-kinase (PI3K) family members. Members of this kinase family phosphorylate the 3'-hydroxyl position of the inositol headgroup of phosphoinositides lipids. The mode of action of **17** has been associated with irreversible binding with a crucial Lys residue conserved in the PI3K family. The  $\epsilon$ -amino group of the Lys attacks the furan ring and forms **18** (Fig. 9) [53]. Wortmannin has been used as a tool compound to study cell signaling processes involving PI3Ks and PI3K-related kinases (PIKK), as it is considered to be a selective kinase inhibitor. In 2005, two groups independently reported an ABPP approach for studying the protein targets of wortmannin. The group of Cimprich used the biotin-labeled probe **19a** to perform the pull-down experiments and the BODIPY derivative **19b** as a cell-permeable probe for labeling of proteins within cells. They could confirm certain members of the PI3K and PIKK families, such as ATR, ATM, DNA-PKc, and a protein entitled p110 as targets of



**Fig. 9** Wortmannin and probe derivatives used in ABPP

wortmannin [54]. In parallel, the group of Liu used the TAMRA probe **19c** for similar purposes [55]. Interestingly, in addition to the expected PI3Ks, they identified members of the polo-like kinase (Plk1) family as protein targets potentially inhibited by wortmannin. This finding is quite important as, on the one hand, it documents the broader range of kinase families addressed by wortmannin and, on the other hand, provides with **19c** a tool compound that allows the monitoring of Plk1 expression during the cell cycle [55].

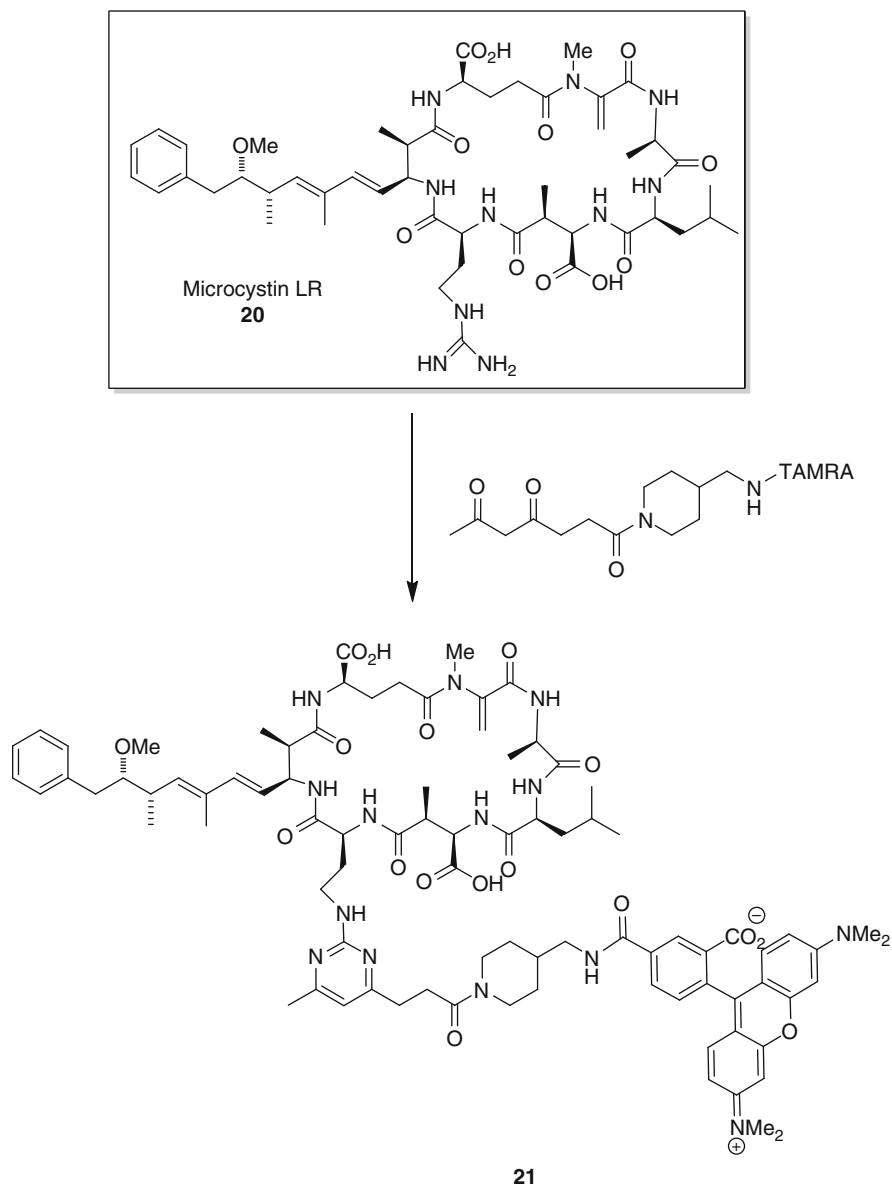
### 4.2.2 Microcystins

Microcystins are a group of cyclic heptapeptide hepatotoxins produced by cyanobacteria and are potent inhibitors of Ser/Thr protein phosphatases. While the hydrophobic 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic (ADDA) moiety and the *N*-methyldehydroalanine (Mdha) unit are essential for the biological activity and have been observed to remain constant in different microcystins, the Arg residue in microcystin LR (**20**) is exchanged for Val, Met, Phe, Leu, or Ala in other natural microcystins (Fig. 10). The  $\alpha,\beta$ -unsaturated Mdha unit of microcystins has been identified to serve as a Michael acceptor towards thiol nucleophiles. Surprisingly, adducts of microcystins with aminoethanethiol remain very potent inhibitors of protein phosphatases PP1 and PP2A, suggesting that the hydrophobic ADDA group is a key contributor to ligand binding. To shed further light into the inhibition mechanism of this class of natural products, two groups independently prepared [ $^{125}\text{I}$ ]-labeled microcystin derivatives. With these probes and by mutation experiments they could show that microcystin covalently binds to the Cys273 of PP1 and Cys266 of PP2A [56, 57]. This information set the stage for a group from ActivX Biosciences to synthesize activity-based probe compound **21** by attaching a TAMRA fluorophore onto the Arg residue, which had been identified as not crucial for ligand binding. For probe **21**, an  $\text{IC}_{50}$  of 4.0 nM for PP1 (compared to an  $\text{IC}_{50}$  of 0.3 nM for microcystin **20**) was determined, confirming that the attachment of the fluorophore did not harm the biological function of the probe molecule. In order to test the use of probe **21** in a complex proteome, soluble fractions of Jurkat cells were incubated with **21**. Protein separation by SDS-PAGE followed by MS protein identification confirmed, as expected, PP1 and PP2A to be protein targets, but also identified PP4 and PP6 (which are closely related to PP2A) as new covalent targets of microcystin [58].

### 4.2.3 Parthenolide and $\gamma$ -Lactones

The sesquiterpene parthenolide (**22**) from the anti-inflammatory medicinal herb Feverfew (*Tanacetum parthenium*) embodies a  $\alpha$ -methylene- $\gamma$ -lactone moiety shared by several other sesquiterpene lactones (Fig. 11). The interesting biological activity of **22** motivated the group of Crews to reveal its biological target [59]. Parthenolide possesses two electrophilic moieties, namely an internal epoxide and an  $\alpha,\beta$ -unsaturated lactone. Reduction of the exocyclic double bond of **22** furnished reduced parthenolide **23**, which showed none of the anti-inflammatory activity characteristic of the parent compound. Having confirmed the importance of the  $\alpha$ -methylene- $\gamma$ -lactone, the Crews group set out to use the biotinylated parthenolide probe **24** for an affinity-based pull-down experiment. Affinity purification of biotinylated proteins using streptavidin resin, followed by SDS-PAGE and western blotting demonstrated that parthenolide formed a covalent adduct with I $\kappa$ B kinase  $\beta$  (IKK $\beta$ ), a protein that is involved in the cascade activating the NF- $\kappa$ B. Tryptic

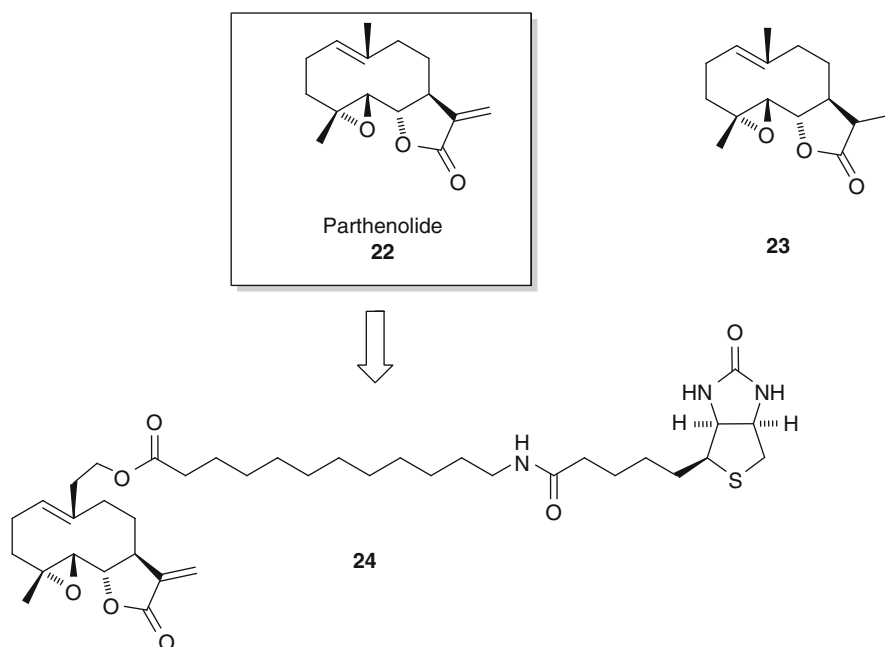




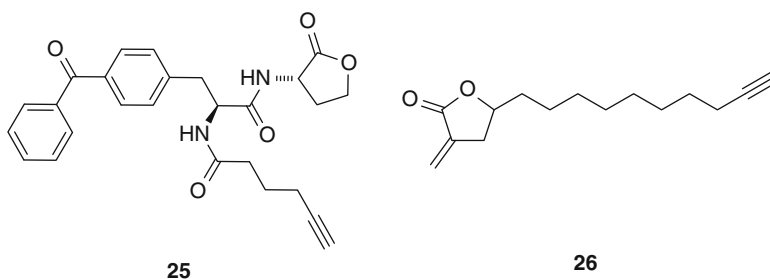
**Fig. 10** Microcystin LR

digest and MS analysis complemented by mutation studies clearly showed that the biological action of parthenolide results from a Michael-type addition reaction of Cys179 of IKK $\beta$  at the exocyclic methylene group of the lactone ring [59].

Very recently, Sieber et al. investigated in an important study the general suitability of  $\gamma$ -lactones (being present in almost 10% of all natural products) and



**Fig. 11** Parthenolide and biotinylated derivative



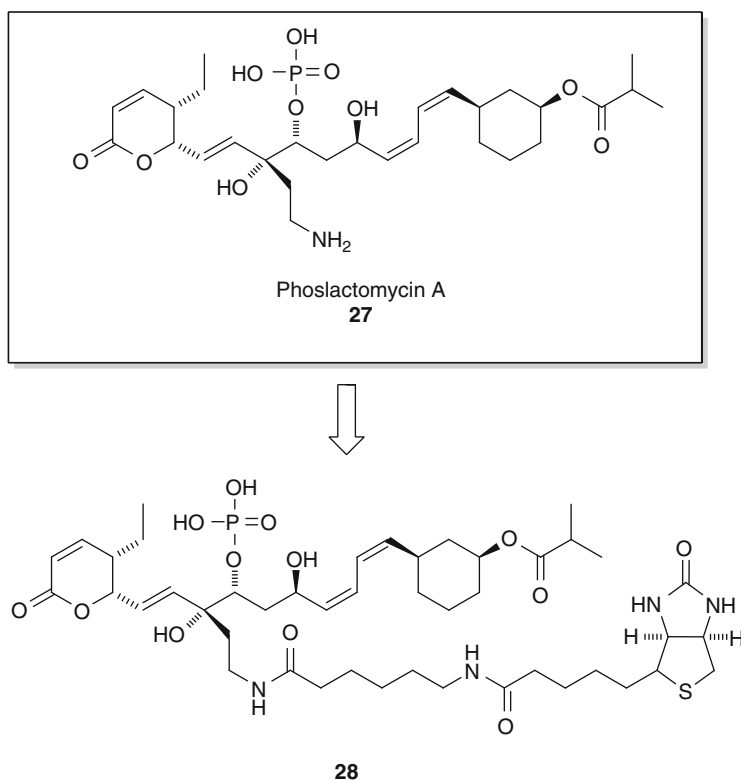
**Fig. 12**  $\gamma$ -Lactones used in ABPP

$\gamma$ -thiolactones to serve as activity-based probes [60]. From the proteomic analysis of their small, but well-chosen set of minimal  $\gamma$ -lactones possessing an alkyne handle for labeling purposes, they could conclude that these molecules showed only little reactivity with proteomic samples. Equipping their probe **25** with a benzophenone unit suitable for photocrosslinking allowed them to identify protein targets that noncovalently interact with lactone molecules (Fig. 12). Incubating bacterial proteomes with **25** and subsequent photocrosslinking at 366 nm presented trigger factor, a protein that has peptidylprolyl isomerase activity, as a target of this molecule. In contrast, the  $\alpha$ -methylene- $\gamma$ -lactone moiety, which is present in 3% of all natural products, serves as an excellent electrophilic trap and makes these

molecules suitable for ABPP analysis, as discussed for parthenolide (Sect. 4.2.3). Sieber and coworkers used the minimal scaffold probe **26** to search for covalently addressed targets of  $\alpha$ -methylene- $\gamma$ -lactones in bacterial proteomes. Formate acyltransferase, dihydrolipoamide dehydrogenase, 6-phosphofructokinase, MurA1, and MurA2 (the latter two enzymes being essential for bacterial cell wall biosynthesis) were identified via MS analysis [60].

#### 4.2.4 Phoslactomycin

Phoslactomycins A–F are antifungal antibiotics isolated from *Streptomyces* sp. and reported to be potent protein Ser/Thr phosphatase 2A (PP2A) inhibitors in vitro. The  $\alpha,\beta$ -unsaturated  $\delta$ -lactone moiety should be sufficiently electrophilic to serve as an anchor point for covalent attachment to the target proteins of phoslactomycin. The group of Simizu tested this hypothesis by attaching to phoslactomycin A (**27**) a biotin label furnishing probe molecule **28** (Fig. 13) [61]. Human fibrosarcoma HT1080 cells were incubated with probe **28** for 30 min, lysed, and the

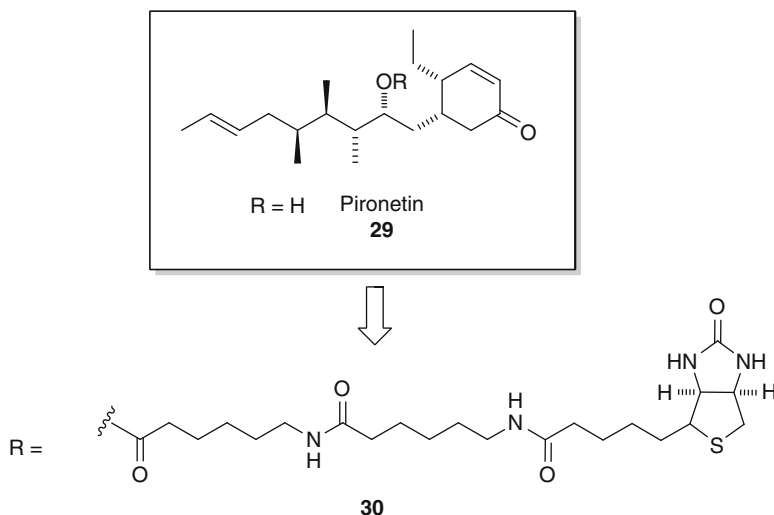


**Fig. 13** Phoslactomycin and biotinylated derivative

biotin-containing complexes isolated by the use of streptavidin-conjugated agarose beads. The **28**-bound proteins were analyzed by SDS-PAGE followed by silver staining. Compared with appropriate control probes, five additional bands were found in the gel (120 kDa, 65 kDa, and three 36 kDa bands). Western blotting analysis confirmed that the bands at 36 kDa account for the catalytic subunits PP2Ac, PP2Ac $\beta$  and PP6 of the PP2A family of proteins. Further investigation revealed that the Cys269 of the PP2A catalytic subunit is responsible for the covalent binding to the  $\alpha,\beta$ -unsaturated lactone of phoslactomycin A via a Michael-type addition mechanism. The 65 kDa protein was PP2A and the identity of the 120 kDa protein has not yet been determined but the authors speculate that it might be a protein that associates with one of the PP2A isoforms as a substrate or regulator [61]. Interestingly, the same group later used phoslactomycin as a test case to demonstrate the applicability of a general strategy to immobilize biologically active molecules for pull-down assays using a site-nonselective carbene addition/insertion upon irradiation of diazirine-modified agarose beads [62]. This interesting approach promises to solve one of the greatest bottlenecks of preparing affinity matrixes: providing a fast and easy access to an immobilized version of a natural product without destroying its pharmacological function.

#### 4.2.5 Pironetin

Small molecules that interact with microtubules are not only useful as antitumor agents (such as paclitaxel, vinblastin, epothilone, etc.) but also as tool compounds for understanding the wide variety of the cellular functions of microtubules. Pironetin (**29**) is a natural product that disrupts the cell cycle by inhibiting the M phase and exhibits antitumor activity by apoptosis induction via microtubule disassembly. The characteristic  $\alpha,\beta$ -unsaturated  $\delta$ -lactone moiety suggested that it could interact with its target protein in a covalent fashion. This proposal was supported by the observation that its saturated analog showed much weaker biological activity. The group of Osada et al. used an activity-based approach to search for the molecular target of **29** [63]. Attachment of a biotin label at the central hydroxy group of pironetin furnished probe **30** (Fig. 14). Despite its close vicinity to the lactone warhead, this structural modification did not alter the biological activity and induced cell cycle arrest at 1  $\mu$ M concentration. Using the standard workflow for using biotinylated probe compounds as described above, Osada et al. observed that **30** specifically and covalently binds to a 50 kDa protein. Western blotting identified  $\alpha$ -tubulin as the single cellular target of **29**. During the course of the binding assay, the authors noticed that pironetin binding to tubulin is labile under acidic (pH < 4.5) and basic (pH > 8.0) conditions, making liquid chromatography-MS characterization of the binding site difficult. But, partial digestion of **30**-treated tubulin with several proteases narrowed the binding site down to being between residues 270 and 370 of  $\alpha$ -tubulin. As the addition of a Cys or Lys nucleophile across the  $\alpha,\beta$ -unsaturated  $\delta$ -lactone moiety seemed to be a plausible mode of action, an Ala exchange with all Cys and Lys residues within this region was performed. From 12

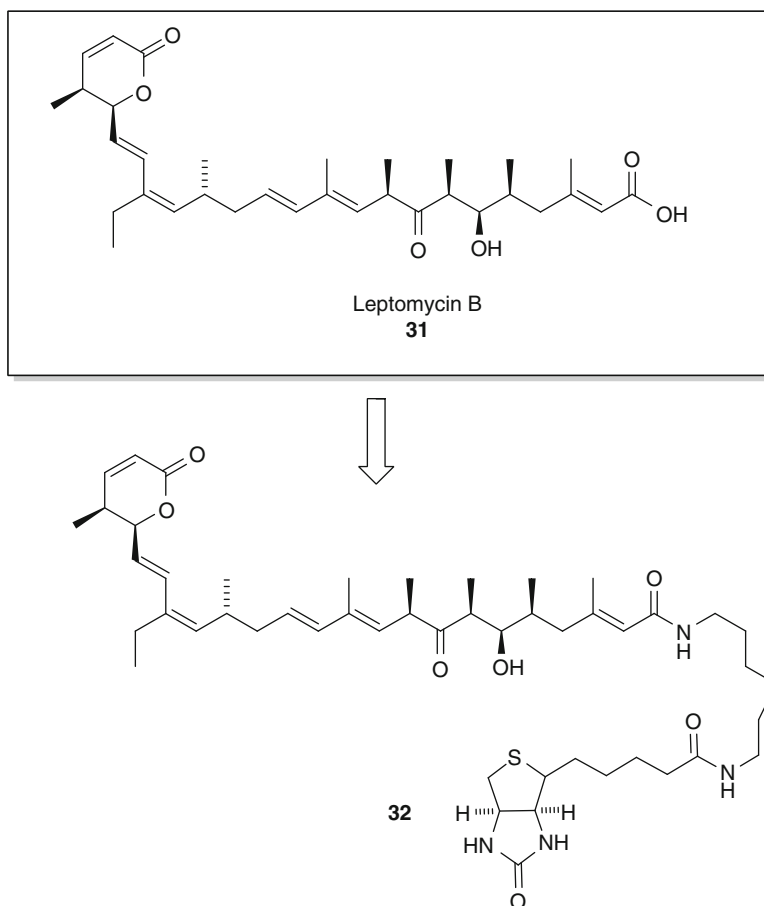


**Fig. 14** Pironetin and biotinylated derivative

such mutants only Lys352 emerged as the crucial amino acid responsible for the binding of pironetin to  $\alpha$ -tubulin, although five Cys residues with intrinsically higher nucleophilicity were also found in this protein region [63]. This finding emphasizes that not only the reactive group but also the remaining structure of the natural product are instrumental in achieving selectivity in the binding of the probe to its protein targets.

#### 4.2.6 Leptomycin B

The transport of cellular proteins between nucleus and cytoplasm across the nuclear envelope is mediated by energy-dependent transport. Specific sequences within a protein contain the information necessary for the nucleocytoplasmic transport. In lower and higher eukaryotes, CRM1/exportin 1 is the receptor for short nuclear export sequences (NES), which are rich in Leu. The polyketide natural product leptomycin B (**31**) was discovered as a potent antifungal antibiotic that blocks the eukaryotic cell cycle (Fig. 15). Kudo et al. discovered that leptomycin B exerts its function by directly binding to CRM1 and therefore blocking the nuclear export of proteins [64]. After preparing the biotinylated probe **32** from **31** they cultured HeLa cells in the presence of 10 nM **32** for 2 h. The biotin-containing complexes were isolated by streptavidin-conjugated agarose beads and identified by SDS-PAGE and western blotting. Only a single protein band at 102 kDa was identified as being selectively bound by **32**. This protein was identified as CRM1. Further analysis revealed that Cys529 covalently binds to leptomycin B via nucleophilic 1,4-addition across the  $\alpha,\beta$ -unsaturated  $\delta$ -lactone unit of this natural product [65]. The covalent modification of CRM1 by **31** apparently induces a major change in its

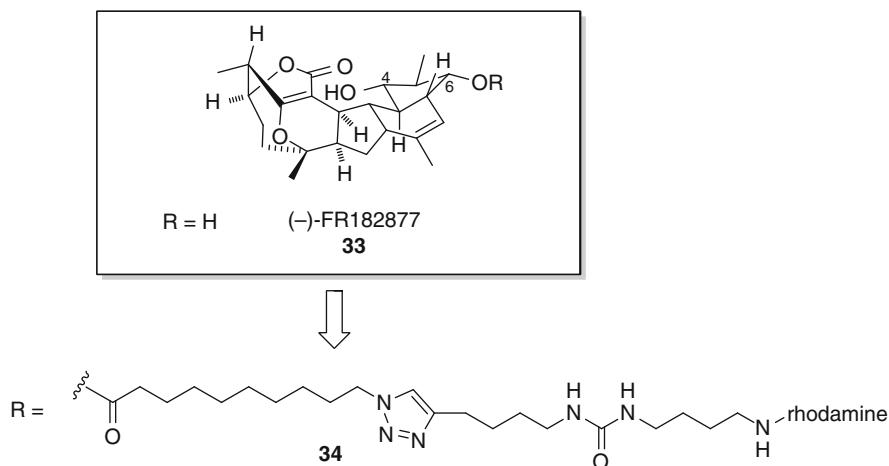


**Fig. 15** Leptomycin B and biotinylated derivative

three-dimensional structure in a native gel, and a significant mobility shift is observed. It is fascinating that despite the high *in vivo* reactivity of **31** with thiol nucleophiles, in the environment of a cell leptomycin B exerts its biological function even at low concentration. The authors speculate that the large hydrophobic part of **31** might prevent access of the more abundant *L*-cysteine or glutathione to the warhead. Leptomycin has because of its high selectivity seen many applications as a tool compound to study the nuclear export of proteins.

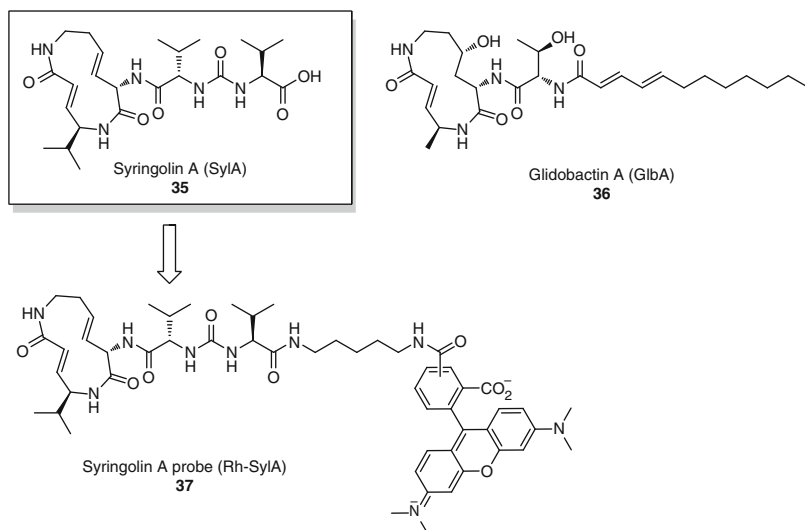
#### 4.2.7 Cyclostreptin [(–)-FR182877]

Because of its complex and unprecedented structure (–)-FR182877 (**33**), which has been renamed cyclostreptin, has attracted the attention of synthetic chemists.



**Fig. 16** Cyclostreptin [(-)-FR182877]

Sorensen et al. presented the first total synthesis of this fascinating molecule [66] and later joined forces with the group of Cravatt to search for its protein targets. **33** displays two potential electrophilic sites at which protein nucleophiles could react: the strained bridgehead olefin and the carbonyl group of a lactone (Fig. 16). Sorensen and Cravatt exploited the better accessible 6-OH group to attach a  $\omega$ -azido alkanolic acid. The azido group allowed the flexible introduction of different labels (rhodamine dye, biotin, etc.) via copper-catalyzed Huisgen–azide alkyne coupling. Rhodamine-labeled probe **34** was used for an ABPP search for the protein target. Proteomic analysis of different mouse tissues identified only one major protein band at 70 kDa as specific for **33**, as compared with other control experiments featuring notably the enantiomer of **33**, which was accessible through the previous total synthesis efforts. Tryptic digestion and MS identification presented carboxylesterase-1 (CE1) as the molecular target of **33** [67]. At this stage, the authors could not explain the very large concentration difference between the inhibition of CE1 ( $\text{IC}_{50} = 34 \text{ nM}$ ) by **33** and the reported effects on tubulin polymerization ( $\sim 100 \text{ }\mu\text{M}$ ), and suggested that it would require more detailed investigations. A few years later, Diaz et al. used an MS-based approach to search for protein fragments containing cyclostreptin and demonstrated that cyclostreptin covalently binds to Thr220 and Asn228 of  $\beta$ -tubulin [68]. Their carefully carried out study demonstrated that cyclostreptin favors microtubules (and not unpolymerized tubulin) as the target of the compound, and that the strained olefin is required for cyclostreptin activity [68]. This different outcome using two different activity-based proteomic techniques allows the humble conclusion that neither technique represents a panacea for obtaining comprehensive binding data and that the observed results are also probably influenced by the concentration of compounds and by the state of the investigated cellular systems.

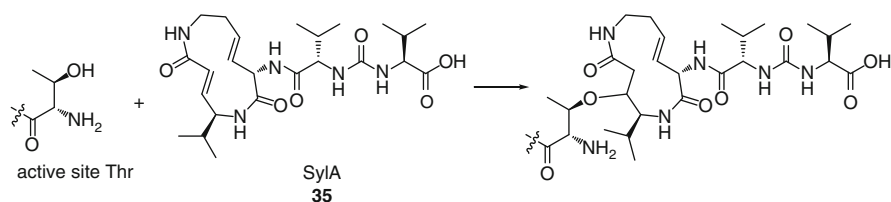


**Fig. 17** Syringolin A and glidobactin A

#### 4.2.8 Syringolin A

Syringolin A (SylA, **35**) is a plant effector produced by the phytopathogenic bacterium *Pseudomonas syringae* pv. *Syringae* (*Pss*) [69]. Along with the structurally related natural product glidobactin A (GlbA, **36**) isolated from unknown species of the order *Burkholderiales*, it belongs to the family of the syrbactins. SylA is an unusual peptide product of mixed nonribosomal peptide/polyketide synthetase that contains a 12-membered macrocycle consisting of two nonproteinogenic amino acids 5-methyl-4-amino-2-hexenoic acid and 3,4-dehydrolysine, as well as a dipeptide tail linked through an ureido bond (Fig. 17). To shed light on the role of SylA in the plant–pathogen interaction, a SylA-deletion mutant was generated by targeted gene disruption in a *Pss* strain, which causes brown spot disease on bean. Infection experiments on bean plants showed a significant reduction of virulence in mutant strain, thus identifying SylA as a potent virulence factor [70]. Strikingly, in the same study, the research group of Groll demonstrated that SylA leads to the efficient and irreversible inhibition of eukaryotic 20S proteasome, a proteolytic complex machinery regulating numerous cellular processes [70]. The chymotrypsin-like activity was the most sensitive towards SylA, although trypsin- and caspase-like activities were diminished as well. The crystal structure of yeast 20S proteasome with SylA revealed its covalent binding to the hydroxyl group of the active site Thr via a Michael-type 1,4-addition of the ThrO<sup>γ</sup> to the α, β-unsaturated amide moiety of the 12-membered ring system (Fig. 18) [70]. Those results strongly supported previous work by Coleman et al. [71] in which it was shown that SylA (**35**) exhibited a strong antiproliferative activity in human neuroblastoma and ovarian cancer cell lines in a dose-dependent manner, with an IC<sub>50</sub> in the range of 20–25 μM. It also induced cell



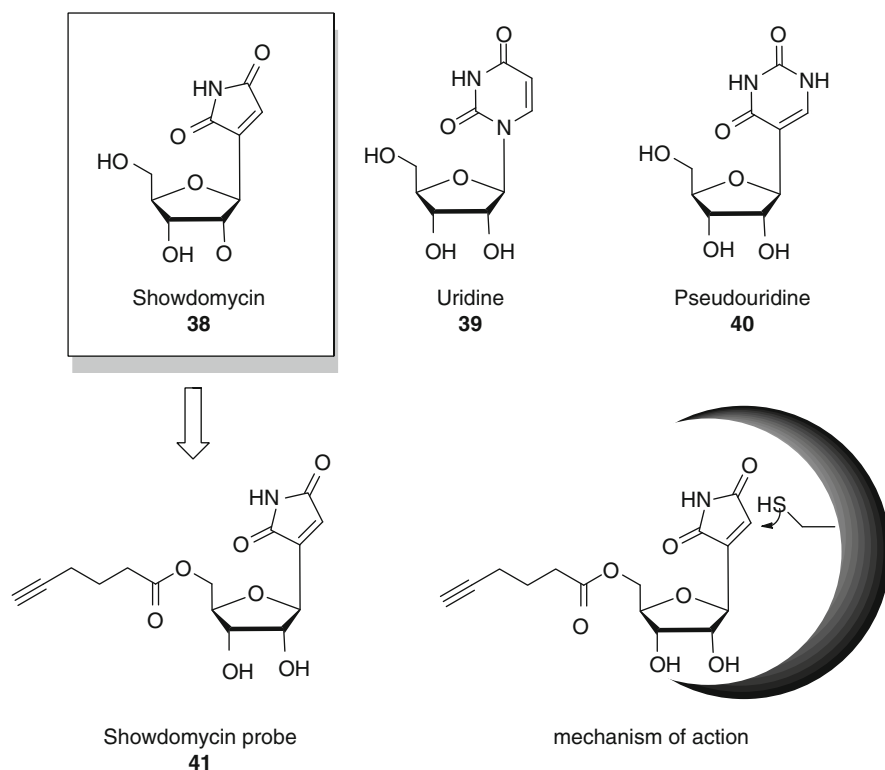


**Fig. 18** Covalent inhibition mechanism of syringolin A

apoptosis; however, the cellular mechanism underlying those effects was not elucidated. Taken together, the results show that SylA exerts a promising anticancer activity due to the proteasome inhibition, thus expanding the repertoire of structural diversity of such therapeutic agents (i.e., bortezomib). Nevertheless, further development of a specific and effective anticancer agent requires in-depth investigation on side effects caused, for example, by cross-reactivity with other nucleophilic enzymes or reactivity in resistant cells due to rapid mutations of the proteasome active site. To answer these important questions, Clerc et al. [43] created SylA-based chemical probe **37** for ABPP. To facilitate an easy fluorescent read-out of cellular labeling events, a rhodamine tag was appended at the free carboxylic acid moiety of the dipeptide tail (Fig. 17). Rh-SylA (**37**) was examined in murine cell line EL4 and was able to efficiently label all  $\beta$ -subunits of both the constitutive proteasome and the immunoproteasome in a dose-dependent manner, with the strongest binding toward the  $\beta 2$  and  $\beta 5$  subunits. In contrast, bortezomib, a FDA-approved proteasome inhibitor used in cancer therapies, preferentially blocks  $\beta 1$  and  $\beta 5$  subunits. Interestingly, even at the concentration of 10  $\mu\text{M}$ , which is far above cellular concentrations of anticancer drugs, no off-targets could be detected, although the reactive  $\alpha,\beta$ -unsaturated carbonyl moiety could in principle also cross-react with more reactive cysteine proteases. This high specificity is presumably associated with the unusual structure of SylA and the accommodation of a side-chain cyclized inhibitor in S2 and S4 binding pockets. Also, **37** showed a significant labeling potency in clinically important leukemia cell lines, including bortezomib-adapted cells. Noticeably, active-site mutations originating from bortezomib treatment did not seem to influence preferential binding of SylA toward  $\beta 2$  and  $\beta 5$  subunits, thus creating an alternative for the next generation of potent proteasome inhibitors for the treatment of cancer. Most recently, the SylA-based ABPP probe was also used for fluorescence imaging and profiling of plant proteasome activity in vivo in *A. thaliana* [44], setting optimal conditions for studying proteasome activity in vivo in different organisms.

#### 4.2.9 Showdomycin

The C-glycosyl nucleoside antibiotic showdomycin (**38**) was first isolated from *Streptomyces showdoensis* [72]. Its structure has striking similarity to uridine (**39**) and pseudouridine (**40**), but is distinguished by exhibiting a maleimide moiety (Fig. 19). The known high electrophilicity of this group towards S-nucleophiles



**Fig. 19** Showdomycin and its mechanism of action

provoked early suggestions that this molecule exerts its biological function by covalent inhibition of active Cys residues of its protein targets. The structural similarity to uridine inspired, several years after its discovery the study of the effect of showdomycin on uridine metabolism. In this effort, the inhibitory effect of **38** against UMP kinase, uridine phosphorylase, and UDP-glucose dehydrogenase was reported and its mechanism of action by covalent binding confirmed [73].

More recently, Böttcher and Sieber undertook an ABPP approach for identifying the target proteins of **38** [74]. Recognizing the ideal situation of a built-in chemical warhead, they synthesized a showdomycin analog **41**, which featured an alkyne group suitable for click chemistry labeling. In an antibacterial assay, **38** and **41** showed similar values for minimum inhibitory concentrations (MIC) for *Staphylococcus aureus*, demonstrating that introduction of the label did not affect the antibiotic properties of the probe molecule. In another experiment, they compared the labeling pattern of the labeled showdomycin probe **41** with that of an N-labeled fluorescent maleimide probe. While the maleimide probe promiscuously labeled almost all proteins in the lysate, the showdomycin probe revealed selectivity for only a limited number of proteins. This important result substantiates the general view that, in

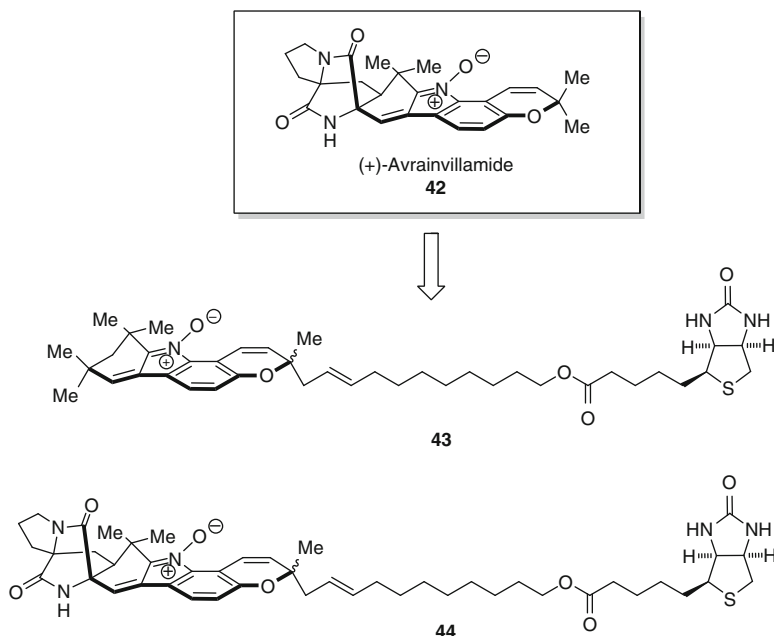
protein-reactive natural products, the otherwise promiscuous reactive warhead is tamed by the sophisticated three-dimensional structures of these molecules and thus selectivity is gained. With **41** in hand, Böttcher and Sieber performed a comprehensive analysis of protein targets using the combined SDS-PAGE/MS identification approach, which revealed 13 different enzymes. These included enzymes that are crucial for oxidative stress resistance in *S. aureus*, such as alkylhydroperoxide reductases C (AhpC) and F (AhpF) and thioredoxin reductase (TrxB). The flexibility of their ABPP approach allowed them to compare the unique protein signature patterns of different strains of pathogenic and nonpathogenic bacteria. One important result from this investigation produced the insight that apparently the UDP-*N*-acetylglucosamine 1-carboxyvinyltransferases 1 (MurA1) and 2 (MurA2), which are involved in cell wall biosynthesis, are signature proteins for the MRSA strain Mu50. Although the expression levels of the corresponding genes *murA1* and *murA2* are comparable in all strains, the different amounts of active protein indicated that the difference in the in situ labeling is due to posttranslational regulation of enzyme activity [74].

#### 4.2.10 Avrainvillamide

Under cellular conditions, the dimeric alkaloid stephacidin B was identified to be in equilibrium with its monomeric building block avrainvillamide (**42**), which is the true active species responsible for its known biological activity, namely the inhibition of tumor growth. Having mastered the total synthesis of both compounds, the Myers group attempted the annotation of the biological targets of **42** (Fig. 20) [75]. In their initial effort, they used a biotinylated activity-based probe **43** that incorporated a structurally less complex analog of avrainvillamide. Treatment of a LNCaP cell lysate with **43**, followed by affinity separation with streptavidin-agarose and SDS-PAGE, allowed the separation of several proteins that were addressed by **42**. MS identification showed that several Cys-containing proteins such as heat shock protein 60 (HSP60), exportin 1 (XPO1), glutathione reductase (GR), and peroxiredoxin 1 (PRX1) were protein binders of **42** [75]. In a subsequent study using the probe **44**, which is structurally more related to **42**, in a similar activity-based setup, they could demonstrate that avrainvillamide irreversibly binds to Cys275 of nucleophosmin, a known regulator of tumor suppressor protein p53 [76]. Depletion of nucleophosmin by RNA silencing led to increased sensitivity of HeLa cells towards apoptotic cell death in the presence of **42**, suggesting that the interaction of **42** with cellular nucleophosmin may play a role in the observed antiproliferative effects of this natural product [76].

#### 4.2.11 Withaferin A

Extracts from the medicinal plant *Withania somnifera* have been used in Ayurvedic medicine for the treatment of chronic human diseases such as arthritis and human



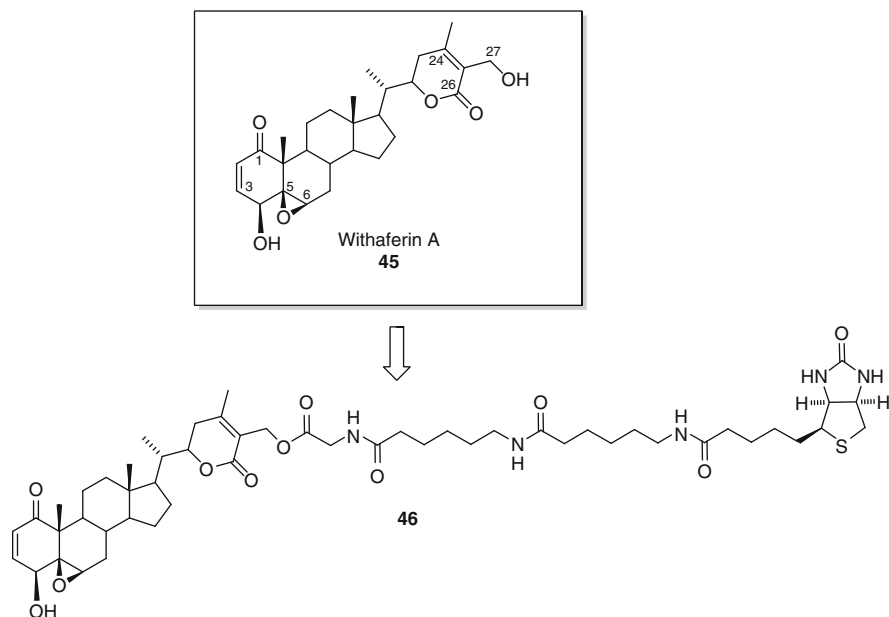
**Fig. 20** (+)-Avrainvillamide and probe molecules

bleeding. Withaferin A (**45**) is a highly oxygenated steroid lactone found in this plant and has reported antitumor cytotoxic activities. Previous structure–activity studies, which indicated the importance of the  $4\beta$ -hydroxy- $5\beta,6\beta$ -epoxy-2-en-1-one moiety and the unsaturated lactone side chain for biological activity, guided Mohan et al. in preparation of the biotinylated analog **46**, which retained the biological activity of the parent compound **45** (Fig. 21) [77]. Affinity-based protein profiling identified 180 and 56 kDa proteins as covalent binding partners and suggested that another protein with 70 kDa might bind **45** reversibly [78]. In a subsequent publication about the nature of the 56 kDa protein, the group reported that it is the intermediate filament (IF) protein vimentin. **45** binds to this protein at Cys328 in the  $\alpha$ -helical coiled coil 2B rod domain, probably via its A-ring electrophilic pharmacophore, consequently causing endothelial cell apoptosis [77].

### 4.3 Natural Products Containing a Lactone

#### 4.3.1 Tetrahydrolipstatin

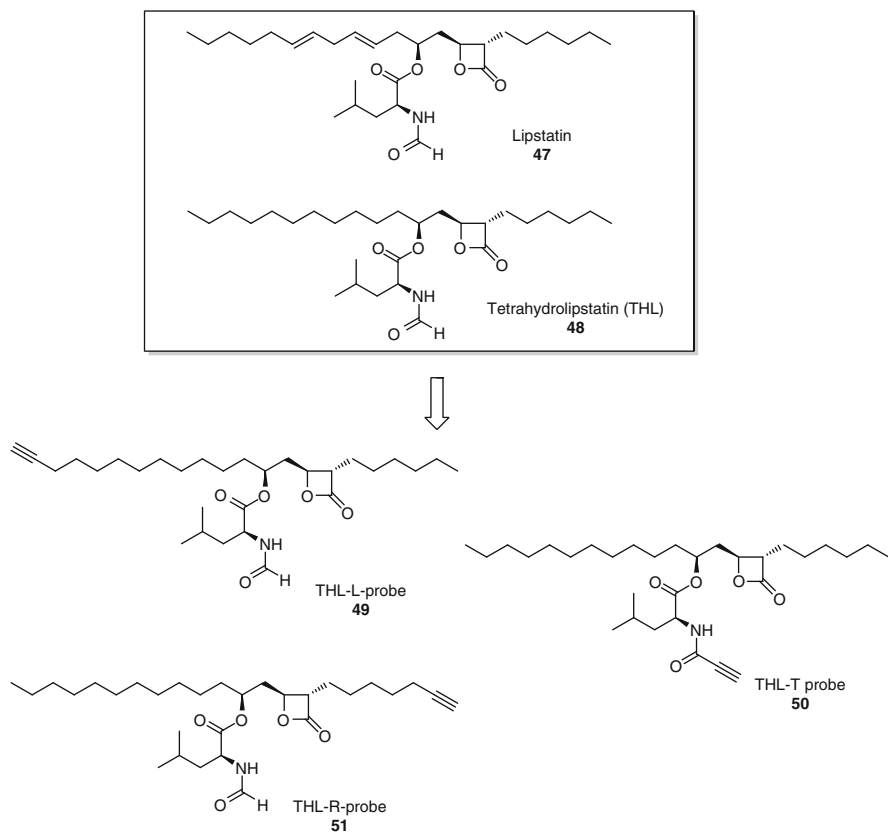
The  $\beta$ -lactone moiety represents a ubiquitous structural motif present in natural products and its moderately electrophilic heterocyclic ring reacts with nucleophilic residues of many proteins active sites. For example, esterastin is a known pancreatic



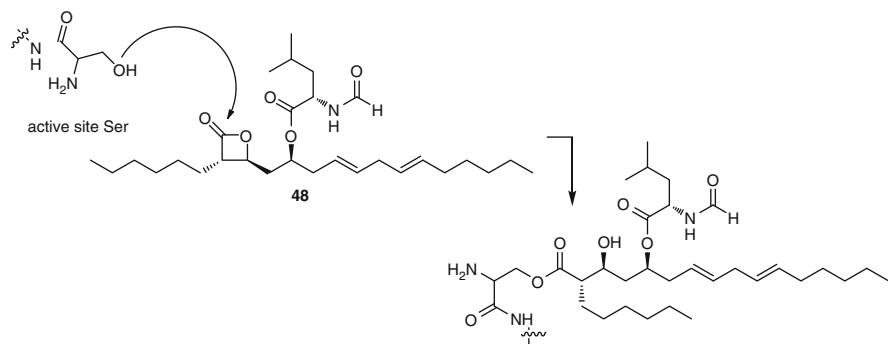
**Fig. 21** Withaferin A and biotinylated derivative

esterase inhibitor produced by *Streptomyces lavendulae* [79], while its structurally related analog lipstatin (47) isolated from *Streptomyces toxytricini* [80] is a potent and selective inhibitor of pancreatic lipase. Other members belonging to this  $\beta$ -lactone family of inhibitors of pancreatic enzymes include ebelactones A and B [81], valilactone [82] and panclicins A–E [83]. On the other hand, hymeglusin [84] and obafluorin [85] are  $\beta$ -lactones displaying antifungal and antimicrobial activities. However, we still lack a comprehensive knowledge about the whole spectrum of cellular targets of  $\beta$ -lactones occurring in nature.

The most is known about lipstatin (47) and its close synthetic derivative tetrahydrolipstatin (THL, 48) (Fig. 22). Lipstatin encompasses a  $\beta$ -lactone ring with two linear carbon chains. The longer carbon chain has two double bonds and an *N*-formylleucine ester side chain. THL (48) results from catalytic hydrogenation of unsaturated double bonds in lipstatin [86, 87]. Both compounds inhibit pancreatic lipase and other gastric lipases within the gastrointestinal tract by the nucleophilic attack of the active site Ser at the carbonyl carbon C1 of the  $\beta$ -lactone ring to form a  $\beta$ -hydroxyl serine ester (Fig. 23). THL is used as an anti-obesity drug under the trade name Orlistat to help obese patients lose weight by blocking dietary fat absorption [88, 89]. Interestingly, 48 was also recently found to inhibit the thioesterase domain of fatty acid synthase (FAS), an enzyme involved in the proliferation of cancer cells but not normal cells [90]. Orlistat (48) induces stress in tumor cells, blocks their growth and angiogenesis and, as a consequence, leads to a delay of tumor progression in various cancer types including prostate, breast,



**Fig. 22** Lipstatin and tetrahydrolipstatin



**Fig. 23** Covalent inhibition mechanism of tetrahydrolipstatin

ovary, and melanoma. However, potential side effects of Orlistat, such as inhibition of other cellular off-targets or poor bioavailability, might hamper its application as an effective antitumor agent. To this end, the research group of Yao undertook an ABPP approach to look for new cellular targets of Orlistat, including its off-targets [91]. They prepared three THL-like chemical probes **49–51** (Fig. 22) by introducing very conservative modifications (alkyne tag) to the parent structure at different molecule locations. The modifications maintained its native properties but allowed target identification by conjugation of a reporter tag (rhodamine or biotin) via the bioorthogonal click chemistry reaction. First, all three probes were evaluated with respect to their ability to block cell proliferation, induce phosphorylation of the translation initiation factor eIF2 $\alpha$ , and induce cell apoptosis by activation of caspase-8. These three cellular assays were able to compare the biological activity of the probes with that of the parent THL molecule. Collectively, the assays showed that introduction of the alkyne handle did not affect their biological activity, which was comparable with that of Orlistat. Next, all probes were incubated with live HepG2 cells (human hepatocellular carcinoma cell line) and, after downstream processing, the cellular targets were detected. Apart from the expected FAS enzyme, several other bands were also observed that were removed by treatment with an excess of Orlistat as competition. Enrichment of protein extracts, separation by SDS-PAGE, and MS/MS analysis led to the identification of eight new proteins, one of which was an unnamed protein. Two of the proteins, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and  $\beta$ -tubulin are housekeeping proteins expressed in most cells and responsible for cell division and energy production, respectively, but known to be expressed at elevated levels in tumor cells. Three other proteins, RLP7a, RLP14, and RPS9 are ribosomal proteins involved in protein synthesis and control of tumor growth, aggressiveness, and metastasis. The last two proteins, Annexin A2 and Hsp90AB1, play an important role in cell proliferation/division and protein degradation, respectively. The identity of all listed proteins labeled by the THL probes were also validated by western blot analysis with their respective antibodies, thus confirming that they are true cellular targets of **48**. This profiling study showcased unambiguously the importance of identification of other targets and elucidation of their role in connection to the potent drug candidate. In order to search for Orlistat derivatives of improved specificity towards FAS and decreased number of off-targets, another proteomic profiling was performed by the same research group [92]. They prepared a library of THL analogs diversified through a differently substituted triazole ring that was furnished by click chemistry reaction between alkyne-THL and various azides. Four derivatives with aromatic or sulfonamide residues displaying similar inhibitory activity towards FAS were identified; nevertheless, they also labeled most of the Orlistat off-targets, but to a lesser extent. Very recently [93], an expanded library built-up on Orlistat, encompassing modifications with regard to the position of the alkyne tag, identity of the incorporated amino ester, and chirality of a probe, was evaluated with the goal of improving the cellular activity of the probes. Strikingly, the authors could demonstrate that even very subtle changes in the structure caused a significant difference in cellular potency and target profile of the probes and allowed the

identification of both common and unique protein targets. In this study, a cell-permeable, benzylguanine (BG)-containing Orlistat version was also implemented within an AGT/SNAP-tag strategy to deliver the probe to particular cell organelles in living cells with the aim of enhancing the cellular activity of Orlistat.

### 4.3.2 Lactacystin

Lactacystin (**52**) is a *Streptomyces* metabolite that inhibits cell cycle progression and induces neurite outgrowth in a murine neuroblastoma cell line. Although of low molecular weight, **52** embodies significant structural complexity exhibiting a [3.2.0] bicyclus containing a five-ring lactam and a four-ring lactone, and four consecutive stereogenic centers (Fig. 24). In order to investigate the origin of its interesting biological activity, Schreiber et al. prepared a tritium-labeled version of **52** by oxidizing the HO-group at the 9-position followed by reduction with tritiated sodium borohydride [94]. Incubation of crude extracts from Neuro-2a-cells or bovine brain with [ $^3\text{H}$ ]lactacystin followed by SDS-PAGE and fluorography revealed the presence of an intensely labeled protein band of 24 kDa. Enrichment studies and Edman sequencing of tryptic fragments identified the 20S proteasome as its specific cellular target. The electrophilic lactone carbonyl of lactacystin (**52**) can covalently modify the highly conserved N-terminal Thr of the mammalian proteasome subunit X/MB1, thereby exhibiting considerable selectivity compared to other proteasome inhibitors [94].

### 4.3.3 Biomimetic $\beta$ -Lactone Library

Recognizing the potential of  $\beta$ -lactones as a warhead for probe molecules, Böttcher and Sieber designed a small library of *trans*- $\beta$ -lactones **53** (Fig. 25), which they screened against several bacterial strains [95]. Several targets of medicinal interest were identified, among them  $\beta$ -ketoacyl acyl carrier protein synthase II (KAS II), proline iminopeptidase (PIP), and caseinolytic protein protease (ClpP) [95]. ClpP, which is a phylogenetically highly conserved serine protease and an important virulence factor, warranted further investigation [96]. Chemical knockout treatment with

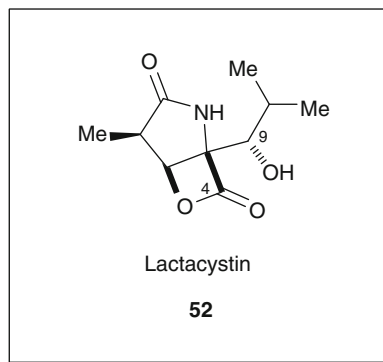
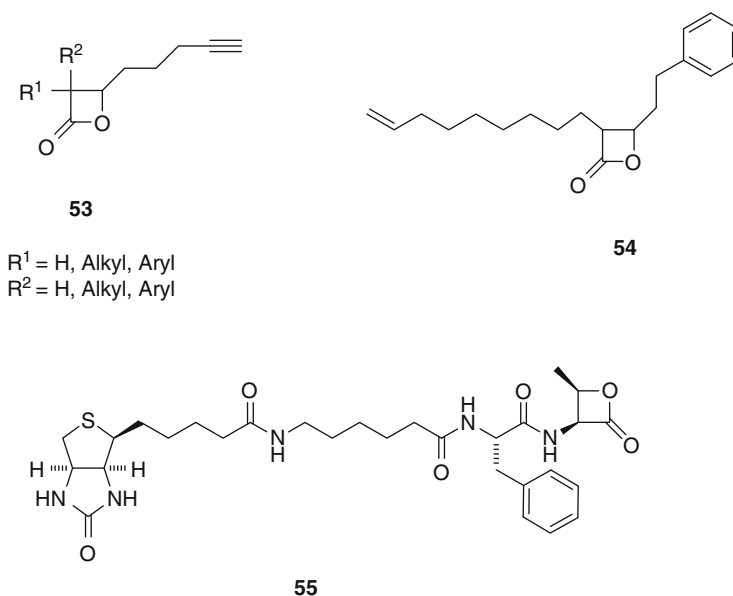


Fig. 24 Lactacystin





**Fig. 25**  $\beta$ -Lactone library and tool compounds

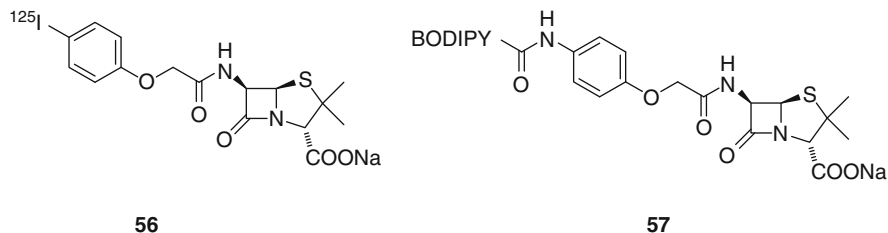
improved ClpP inhibitor **54** resulted in significantly reduced secreted protein levels of important toxins [97], and downregulation of the expression of listeriolysin L (LLO) and phosphatidylinositol-specific phospholipase C (PI-PLC), which in consequence led to a decreased virulence of *Listeria monocytogenes* [98].

In a parallel effort, van der Hoorn et al. synthesized a library of biotinylated  $\beta$ -lactones for the ABPP monitoring of protease activity of plant extracts [99]. SDS-PAGE revealed several biotinylated compounds, from which MS analysis identified the 23 kDa band as protein P of the oxygen-evolving complex of photosystem II (PsbP). Remarkably, for this protein no nucleophilic Ser or Cys residues have been reported. Careful investigation of the labeling mechanism revealed that probe **55** reacts first with Cys-protease RD21, forming a thioester which then reacts with the N-terminus of PsbP. This impressive study teaches that initial ABPP results always need careful secondary screening and that further investigation of unexpected labeling sites can lead to intriguing molecular mechanisms [99].

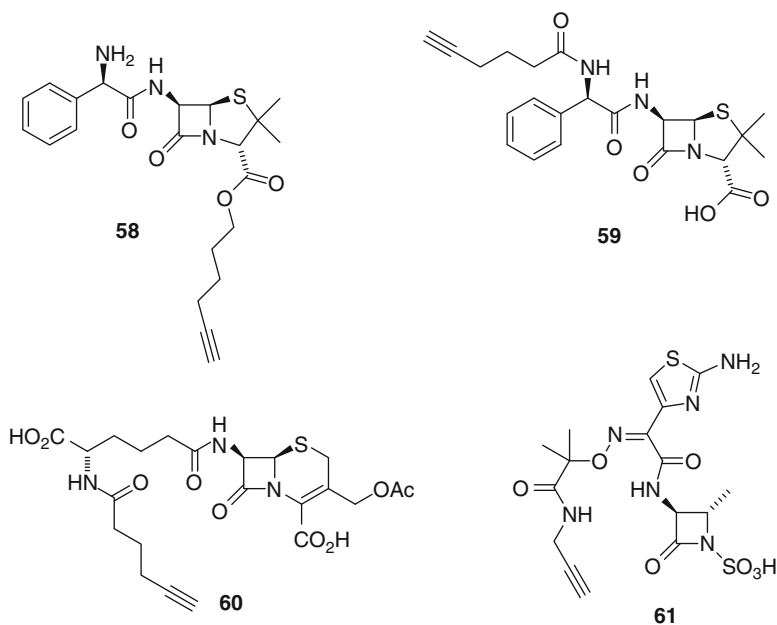
## 4.4 Natural Products Containing a Lactam

### 4.4.1 Penicillin and $\beta$ -Lactams

Penicillins are natural or semisynthetic antibiotics containing a reactive  $\beta$ -lactam functional group, which irreversibly reacts with proteins involved in cell wall biosynthesis. By definition, its target proteins are called penicillin-binding proteins



**Fig. 26** Penicillin derivatives containing labels

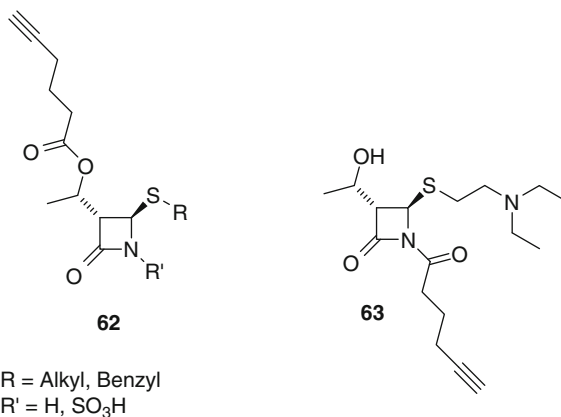


**Fig. 27**  $\beta$ -Lactam activity-based probes inspired by commercial drugs

(PBPs). A group at Lilly has introduced the radiolabeled probe **56** and fluorescence-labeled probe **57** as tool compounds to profile the PBPs in different bacterial strains using SDS-PAGE (Fig. 26) [100, 101]. At this time, no MS analysis of the protein bands has been performed.

The group of Sieber has used an improved ABPP approach to look again at the targets of certain  $\beta$ -lactam antibiotics and prepared derivatives **58–61** by attaching an alkyne tag to clinically used parent compounds (Fig. 27). With these probes, they were able to perform in vivo experiments and identify a series of PBPs [102].

In a second line of research, they prepared a new set of synthetic  $\beta$ -lactam probes **62** around the monocyclic aztreonam structure (Fig. 28). Interestingly, none of these modified  $\beta$ -lactam probes labeled any PBPs but had preference for other enzymes such as  $\beta$ -ketoacyl acyl carrier protein III (KAS III), a  $\beta$ -lactamase,

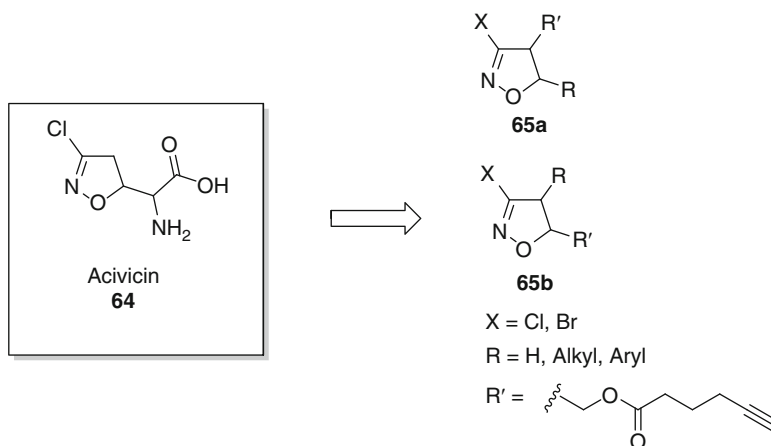
**Fig. 28**  $\beta$ -Lactam library

a lipase acylhydrolase, an antioxidant (AhpC), and the virulence-associated protein ClpP [102]. In a following publication, Staub and Sieber used one probe molecule **63** for the in situ profiling of antibiotic-sensitive and resistant *S. aureus* strains. With this probe they could identify five proteins unique for the MRSA strain. MS analysis revealed their identities as PBP2, PBP2', the serine protease SPD<sub>0</sub>, esterase E28, and a dipeptidase. These results offer not only a convenient way to monitor the activity of these enzymes important for the resistance of MRSA, but also a new lead structure for therapeutic intervention against these new protein targets [103].

## 4.5 Others

### 4.5.1 Acivicin

The natural product acivicin (**64**) is an amino acid containing a 3-chlorodihydroisoxazole ring. Its mode of action as an antibiotic is thought to occur via covalent modification of active site Cys or Thr of enzymes such as L-glutamine-dependent amidotransferases, glutamate synthase, and  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT). Sieber et al. recognized the potential of the 3-halogenodihydroisoxazole warhead to serve as a scaffold for a small library of activity-based probe molecules [104]. Using efficient [3+2]-cycloaddition reactions they prepared an ensemble of regioisomeric compounds **65a** and **65b**, each containing an alkyne handle as a tag for their modification with rhodamine and biotin azides via Huisgen–azide–alkyne coupling (Fig. 29). Screening these probes against several different bacterial strains revealed the potential of these molecules. They identified pyrroline-5-carboxylase dehydrogenase, aldehyde dehydrogenase, bifunctional aldehyde-CoA/alcohol dehydrogenase, and 3-oxoacyl-[acyl-carrier-protein] synthase III (FabH) as targets covalently modified by their probes. Strikingly, the individual probes showed



**Fig. 29** Acivicin and a biomimetic library

considerable selectivity between these targets, and none of them labeled  $\gamma$ -GT, probably because of the missing amino acid recognition element. This study demonstrates that natural products serve as excellent starting points for probes against targets and can go beyond the target range of the original natural product.

## 5 Conclusions

In this review, we have collected several examples of natural products that react with their protein targets in a covalent manner. By introduction of an appropriate label onto these natural products, researchers were able to identify the target proteins responsible for the biological function. In the 1990s only one or two proteins could be annotated, but since then the impressive progress made in proteomic techniques, especially in MS instrumentation and data analysis, has opened a new dimension to more complete target annotation of natural products. We expect that ABPP will in the future be increasingly used in natural product research, and that the design of ABPP libraries based on natural product scaffolds, as pioneered by Cravatt and Sieber, will become a very important source of new probe molecules against new and biologically relevant targets.

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