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Protein Reactivity of Natural Product-Derived γ-Butyrolactones[†]

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ABSTRACT: The discovery of novel and unique target−drug pairs for the treatment of human diseases such as cancer and bacterial infections is an urgent goal of chemical and pharmaceutical sciences. Natural products represent an inspiring source of compounds for designing chemical biology methods with applications in target identification and characterization. Inspired by the huge structural diversity of γ-butyrolactones, which constitute up to 10% of all known compounds of natural origin, we extended the "activity-based protein profiling" (ABPP) target identification technology to this promising and so far unexplored natural compound class. We designed and synthesized a comprehensive set of natural product-derived γ-lactones and thiolactones that varied in protein reactivity. Several important bacterial enzymes that are involved in diverse cellular functions such as metabolism (dihydrolipoyl dehydrogenase and 6-phosphofructokinase), cell wall biosynthesis (MurA1 and MurA2), and protein folding (trigger factors) were obtained. Especially protein folding in bacteria could represent a novel strategy for antibiotic intervention and requires chemical tools for characterization and inhibition. Future studies that extend structural modifications to protein reactive α-methylene-γ-butyrolactone as well as to reversible binding γ-lactones and thiolactones will reveal if this premise holds true.

Electrophilic entities such as epoxides, β-lactam, β-lactone, and Michael acceptor systems constitute a large fraction of natural products with a huge diversity of biological activities. In many cases, the biological potency is directly linked to the intrinsic electrophilic reactivity that leads to covalent modifications of nucleophilic active site residues such as serine, cysteine, and lysine of many important enzyme classes, including dehydrogenases, hydrolases, and transferases (*1*, *2*). These enzymes play crucial roles in diseases such as cancer and diabetes as well as bacterial infections and are of major interest as novel drug targets. However, especially in the case of bacterial infections, new potent and selective drugs and their corresponding novel and resistance-free targets are lacking, which has led to a renaissance of untreatable infectious diseases caused by multiresistant bacteria. For the identification of these urgently needed unique target−drug pairs, natural products represent an inspiring source of compounds for designing chemical biology methods for target identification (tools) and characterization (*3*−*6*). Initial studies with β-lactones already demonstrated the power of this approach by the identification of a central regulator of virulence as a novel concept in combatting pathogenic bacteria (*5*, *7*). Inspired by this approach, we extended the "activity-based protein profiling" (ABPP) (*2*, *8*, *9*) target identification technology to the γ-butyrolactones, a structural motif found in up to 10% of all known compounds of natural origin (*10*). The high abundance and structurally diverse decoration of this motif already suggest that evolution has selected γ-butyrolactones for a variety of important biological applications that could give rise to novel lead structures for potent drugs (*11*). Structurally closely related γ-butyrothiolactones also exhibit interesting biological activities, for example, the natural product antibiotic thiolactomycin (*12*).

To explore the full reactivity profile of five-membered cyclic lactones and thiolactones, we synthesized a diverse set of γ-butyrolactones and thiolactones as well as their Michael acceptor-activated derivatives α-methylene-γ-butyrolactones and α-butenolides. All core scaffolds were equipped with an alkyne handle that serves as a benign tag for the modification with rhodamine azide and rhodamine-biotin-azide for visualization (fluorescence scanning) and identification (mass spectrometry) of labeled proteins, respectively, via the Huisgen, Sharpless, Meldal click chemistry (CC) reaction (Figure 1) (*13*−*16*).

The advantages of this approach are the small size and the less perturbed structure of the original lactone motif that is particularly suitable to labeling proteins in living cells (in situ). We here show that this approach with several five-membered lactones reveals important insights into the reactivity and binding preferences of these natural product inspired probes. While regular γ-butyrolactones and thiolactones exhibited only little reactivity with proteomic samples, the Michael acceptor-based α-methylene-γ-butyrolactones exhibited a strong tendency to covalently label a series of important bacterial enzymes. To compensate for the lack of labeling for the majority of γ-butyrolactones, we synthesized an affinity binding probe with a photocrosslinking moiety (*17*−*20*) (benzophenone) that revealed a remarkable selectivity for the binding of bacterial cis−trans prolyl isomerases (Figure 1).

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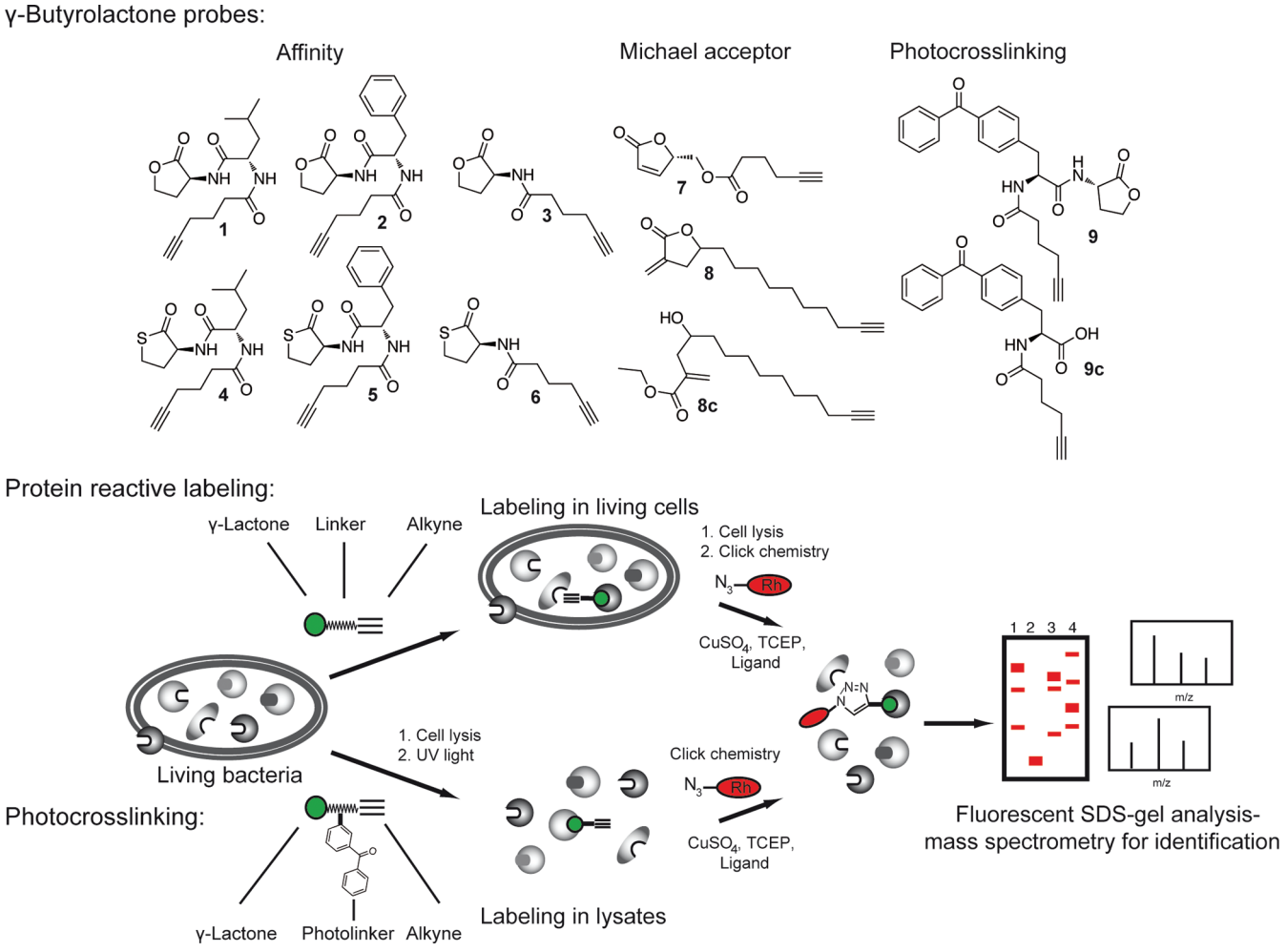

FIGURE 1: Design of γ-butyrolactone and thiolactone probes and their application in bacterial proteome profiling. Probes are directly incubated with living bacteria to penetrate the cells and label dedicated protein targets in situ. The cells are subsequently lysed, and the labeled proteins are "clicked" to a fluorescent rhodamine azide dye for visualization by SDS gel electrophoresis and identification by MS. In the case of photocrosslinking, the lactone binds its target in cellular extracts and becomes covalently attached by UV irradiation at 366 nm.

MATERIALS AND METHODS

Syntheses of γ-Lactones. γ-Lactones were prepared as described in the Supporting Information.

Preparation of Bacterial Proteomes. Proteomes were prepared from liquid cultures harvested 1 h after transition in the stationary phase by centrifugation. *Escherichia* and *Pseudomonas* strains were grown in LB (Luria-Bertani broth) medium, *Burkholderia* strains in CASO (casein−soybean broth) medium, and *Staphylococcus* strains in BHB (brain−heart broth) medium. The bacterial cell pellets were washed with PBS, resuspended in PBS, and lysed with a French press.

In Vitro Labeling of Bacterial Proteomes. Proteome samples were adjusted to a final concentration of 1 mg of protein/mL by dilution in PBS prior to probe labeling. Experiments for visualization by one-dimensional sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were conducted in a total volume of 50 μL and those for affinity enrichment in a total volume of 2 mL. Reactions were initiated by the addition of probe followed by incubation for 60 min at room temperature. For labeling with the photocrosslinking probes **9** and **9c**, the reaction mixture (43 μL) was preincubated with the probe for 10 min at room temperature and then irradiated in open polystyrene micro well plates for 60 min at 0 °C under 366 nm UV light (Benda UV hand lamp NU-15 W). For heat controls, the proteome was denatured with 1% SDS at 95 °C for 6 min and cooled to room temperature before the probe was applied. Following incubation, reporter-tagged azide reagents (100 μM rhodamine azide for analytical or 15 μM rhodamine-biotin-azide for preparative scale) were added followed by TCEP (20 or 5 μM) and ligand (6 or 1.5 μM). The cycloaddition was initiated by the addition of $CuSO_4$ (20 or 5 μM). The reaction mixtures were incubated at room temperature for 1 h. For analytical gel electrophoresis, SDS loading buffer was added, the sample was applied on the gel, and the resulting fluorescence was recorded.

Reactions for enrichment were conducted together with a control lacking the probe to compare the results of the biotin−avidin-enriched samples with the background of unspecific protein binding on avidin−agarose beads. After CC, proteins were precipitated using an equal volume of prechilled acetone. Samples were stored on ice for 20 min and centrifuged. The supernatant was discarded and the pellet washed with prechilled methanol and resuspended by sonication. Subsequently, the pellet was dissolved in a PBS/0.2% SDS mixture by sonication and incubated with avidin−agarose beads at room temperature. The beads were washed three times with a PBS/0.2% SDS mixture, twice with 6 M urea, and three times with PBS. SDS loading buffer was added, and the proteins were released for preparative SDS−PAGE by incubation at 95 °C. Gel bands were isolated, washed, and tryptically digested.

In Situ Labeling of Bacterial Proteomes. Bacteria were grown in BHB, CASO, or LB medium, and 2 mL of cells derived from a culture with an OD_{600} of 2 were harvested 1 h after reaching stationary phase by centrifugation for analytical studies. The volume was increased 5-fold for preparative studies. After being washed with PBS, the cells were resuspended in 100 μL of PBS. Unless indicated otherwise, bacteria were incubated for 2 h with varying concentrations of the probe at room temperature. Subsequently, the cells were washed with PBS and lysed by sonication. Membrane and cytosol were separated by centrifugation, and the membrane fraction was resuspended in PBS, followed by click chemistry as described above. Reactions for enrichment were conducted as described for the in vitro experiments.

In Situ Labeling of Bacterial Proteomes with Probe ***9***. *Pseudomonas putida* was grown to stationary phase in LB medium. Then, 2 mL of the culture were centrifuged, and the pellet was washed with PBS and resuspended in 100 μL of PBS. Probe **9** was added (final concentration of 200 μM) and the mixture incubated for 1 h at room temperature followed by UV exposure (366 nm) for 1 h for photoreactive probes. The cells were washed with PBS, and the pellet was resuspended in PBS. Lysis was performed by sonication as described previously, and the cytosolic and membrane fractions were subjected to click chemistry.

Mass Spectrometry and Bioinformatics. Tryptic peptides were loaded onto a Dionex C18 Nano Trap Column (100 μm) and subsequently eluted and separated by a Dionex C18 PepMap 100 (3 μm) column for analysis by tandem MS followed by high-resolution MS using a coupled Dionex Ultimate 3000 LC-Thermo Finnigan LTQ-Orbitrap MS system. The mass spectrometry data were searched using the SEQUEST algorithm against the corresponding databases via the software "bioworks". The search was limited to tryptic peptides, two missed cleavage sites, monoisotopic precursor ions, and a peptide tolerance of < 10 ppm. Filters were set to further refine the search results. The X_{corr} versus charge state filter was set to X_{corr} values of 1.5, 2.0, and 2.5 for charge states +1, +2, and +3, respectively. The number of different peptides has to be at least two, and the peptide probability filter was set to < 0.001.

Competitive in Vitro Labeling with Reversible Binding γ*-Lactones*. In competitive assays, a 100-, 50-, or 10-fold excess of a reversible binding γ-lactone was added to the proteome 15 min prior to addition of the photocrosslinking probe **9**.

Recombinant Expression. The major hits of MS analysis were recombinantly expressed in *Escherichia coli* as an internal control of the MS results by using the Invitrogen Gateway Technology. Target genes were amplified from the corresponding genomes by polymerase chain reaction (PCR) with an AccuPrime *Pfx* DNA Polymerase kit with 65 ng of genomic DNA, prepared by standard protocols (Supporting Information).

Identification of the Binding Site of Probe ***8*** *in Dehydrolipoamide Dehydrogenase*. To a solution of recombinantly expressed and purified *Pseudomonas aeruginosa* PAO1 dihydrolipoamide dehydrogenase (3.5 μg/μL in PBS) was added probe **8** (50 μM), and the mixture was incubated for 30 min at room temperature. The buffer was exchanged with 25 mM aqueous NH_4HCO_3 to wash away unbound probe. $CaCl_2$ (1 mM) and a chymotrypsin solution were added, and the solution was incubated at room temperature overnight. Formic acid (2 μL, 1% in H_2O) was added to 18 μL of the chymotryptic digest. The sample was measured by mass spectrometry as specified in the Mass spectrometry and bioinformatics section of the Supporting Information.

IC$_{50}$ *Measurement*. The Pro−Phe bond of the *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide substrate can exist in a trans or cis (higher absorption coefficient) form. The substrate was dissolved in a solution of 0.5 M LiCl in trifluoroethanol [TFE (~50% cis peptide)], and the re-equilibration of the cis:trans ratio during dilution into Hepes buffer (~10% cis peptide) can be measured by monitoring the absorption at 330 nm. Because traces of water in the LiCl/TFE solution decrease the cis content dramatically, LiCl was dried over P_4O_{10} under vacuum and TFE was dried over 4 Å molecular sieves. A solution of lactone **1**, **2**, **4**, or **5** (0−10 mM in DMSO, 20 μL) was added to a solution containing Hepes (50 mM, pH 7.8, 1950 μL) and the *E. coli* trigger factor (100 μM, 10 μL). After incubation for 10 min at 10 °C, the substrate (20 μL of 6 mM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide in 0.5 M LiCl in trifluoroethanol) was added and the change in absorption subsequently monitored at 330 nm for 10 min at 10 °C. The data of each measurement were fitted with $A = y_0 A_0 e^{-kt}$ as the exponential function leading to a kinetic constant k (s^{-1}). The mean average kinetic constants (each of three independent experiments) versus lactone concentration were plotted, and the concentration of 50% inhibition (IC_{50}) was determined.

RESULTS AND DISCUSSION

For our initial reactivity studies with cyclic five-membered ring systems, we designed several probes that were based on a lactone or thiolactone ring equipped with hexynoyl, leucyl-hexynoyl, or phenylalanyl-hexynoyl peptidic side chains at position 3 (probes **1**−**6**). Synthesis of these probes was achieved by the modification of L-homoserine lactone and L-homocysteine thiolactone (Scheme S1 of the Supporting Information). In addition, the initial probe selection was complemented by the synthesis of a butenolide probe **7**. We rationalized that this compound could have an elevated electrophilicity because of the presence of an internal Michael acceptor system. The probe was prepared by coupling of hexynoic acid to commercially avialable (*S*)-(−)-5-(hydroxymethyl)-2(5*H*)-furanone (Scheme S1 of the Supporting Information).

To test these probes with respect to their protein reactivities, we incubated them with intact bacterial cells as well as bacterial lysates derived from nonpathogenic strains such as *B. thailandensis*, *E. coli*, and *P. putida* as well as pathogenic strains such as *B. cenocepacia*, *P. aeruginosa*, and *S. aureus* for 1 h at room temperature. Upon incubation, cells were lysed and a fluorescent rhodamine azide tag was attached via CC. In all cases, almost no or only weak labeling intensities were observed, indicating that the unactivated γ-lactones and thiolactones, including the internal Michael acceptor system, are not sufficiently reactive to be attacked by nucleophilic enzyme active site residues (Figure S1 of the Supporting Information). This result clearly indicates that these motifs are not suitable for direct proteome profiling and suggests that most likely many natural products that contain these structural motifs such as homoserine lactones that are involved in bacterial quorum sensing most likely exhibit their biological activities via a reversible binding mode of their dedicated targets (*21*). We therefore adjusted our initial strategy and designed two additional γ-lactone-based probes that contained either a benzophenone photocrosslinker (**9**) in the side chain to monitor reversible binding interactions or an α-methylene-γ-butyrolactone probe (**8**) with elevated

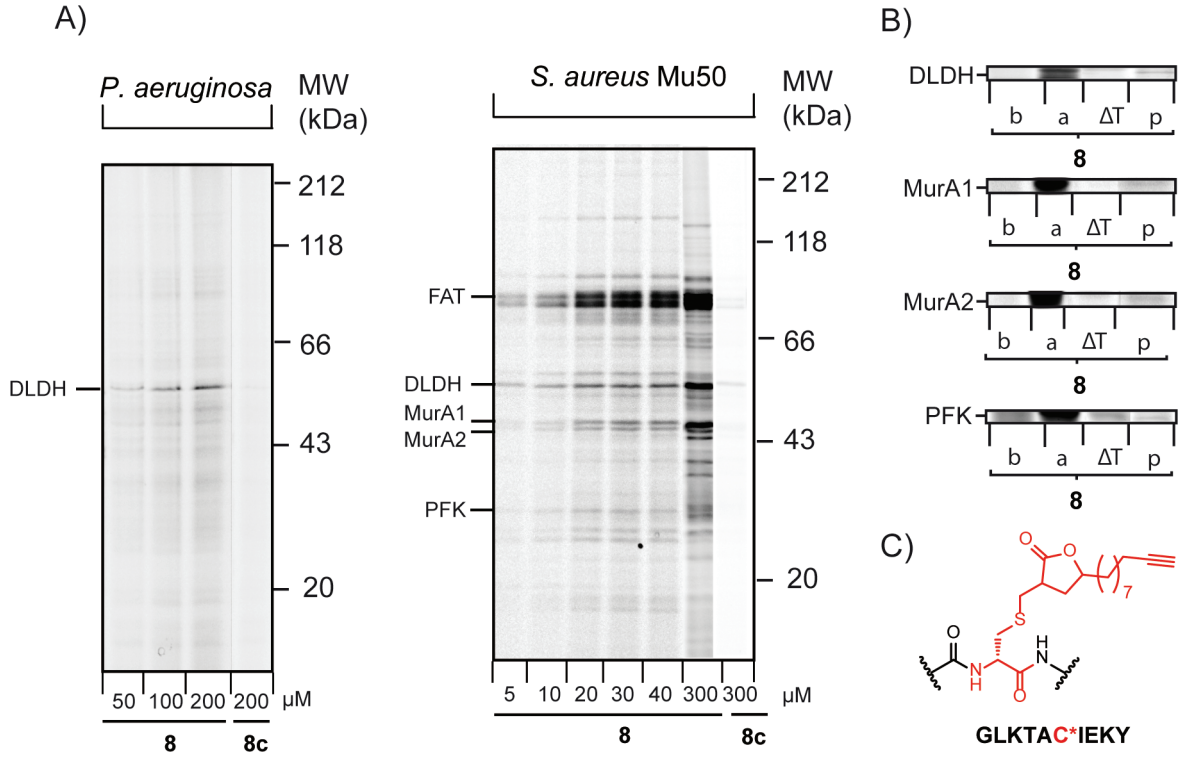

FIGURE 2: In situ labeling of bacterial proteomes with the α-methylene-γ-butyrolactone probe (**8**). (A) Fluorescent SDS gels with labeled proteins. The protein identity is abbreviated next to the gels: DLDH, dihydrolipoamide dehydrogenase; FAT, formate acyltransferase; MurA1 and -2, UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase; PFK, 6-phosphofructokinase; MW, molecular weight marker. (B) Recombinant expression and labeling of target proteins (b, before induction; a, after induction; ΔT, heat control of induced recombinant protein; p, native protein band in the corresponding proteome). (C) Site of modification of *P. aeruginosa* DLDH.

electrophilicity because of an external Michael acceptor system. In the following, we will first discuss the profiling results for the α-methylene-γ-butyrolactone probe and then compare these studies with those done with the photocrosslinking probe.

The α-methylene-γ-butyrolactone group is present in many bioactive compounds of natural as well as synthetic origin. Remarkably, ~3% of all known natural products contain this functional group, which emphasizes its potential role in its parent compounds that display a diverse range of bioactivities such as anticancer, antimalarial, antiviral, and antibacterial activity (*10*). To test the proteome reactivity and dedicated target preferences of this structural motif, we designed and synthesized an alkyne-substituted α-methylene-γ-butyrolactone probe for global proteome analysis. The synthesis started with oxidation of undecynol by Dess−Martin periodinane, leading to the corresponding aldehyde undecynal. The aldehyde was subsequently coupled to ethyl 2-(bromomethyl)acrylate via a Reformatsky reaction, which led to 4-hydroxy-2-methylenetetradec-13-ynoic acid ethyl ester (**8c**). The cyclization of **8c** was initiated by addition of a catalytic amount of 4-toluenesulfonic acid, leading to 5-dec-9-ynyl-3-methylenedihydrofuran-2-one (**8**) (Scheme S2 of the Supporting Information). The precursor molecule (**8c**) of the cyclized α-methylene-γ-butyrolactone probe (**8**) contains a linear Michael acceptor system that was used as a control to investigate the influence of the intact cyclic γ-lactone motif for reactivity and selectivity compared to the open form.

Proteome profiling experiments were initiated by incubation of probes **8** and **8c** with several pathogenic bacterial organisms such as *P. aeruginosa* and *S. aureus* under in situ conditions with intact cells. Subsequent CC, SDS gel analysis, and fluorescent scanning revealed a distinct set of specific protein bands in all proteomes investigated, emphasizing that the probe displays suitable properties for in situ studies. Concentrations of 200 μM for Gram-negative *P. aeruginosa* and 40 μM for Gram-positive *S. aureus* as well as an incubation time of 1 h turned out to be optimal for saturated labeling (Figure 2 and Figure S2 of the Supporting Information). Some targets could be labeled even at significantly lower concentrations (≥5 μM), indicating an increased sensitivity and specificity. In addition, the control probe **8c** with the open Michael acceptor displays significantly less labeling, indicating that the cyclic γ-lactone motif is required for the obtained target preferences.

Subsequent target identification was conducted by mass spectrometry using a quantitative enrichment strategy for labeled enzymes (*5*). In brief, labeled proteomes were incubated with a trifunctional rhodamine-biotin-azide tag under CC conditions, enriched and purified on avidin beads, separated by SDS gel electrophoresis, and fluorescent bands were isolated, digested, and subjected to mass spectrometric (MS) analysis with an orbitrap mass spectrometer. Fragmentation data were analyzed with the SEQUEST algorithm, and several protein hits were obtained that correlated at their molecular weight with the sizes of the corresponding gel bands (Table S1 of the Supporting Information). Several proteins that play important roles in the primary metabolism of bacteria were identified, such as 6-phosphofructokinase (PFK) in *S. aureus* that is a key enzyme in glycolysis as well as dihydrolipoamide dehydrogenase (DLDH) in both *S. aureus* and *P. aeruginosa* that is also important for glycolysis and the citrate cycle. Moreover, MurA1 and MurA2, two essential enzymes for cell wall biosynthesis in *S. aureus*, were identified in multiresistant *S. aureus* cells (MRSA). Interestingly, these enzymes have been previously labeled by a showdomycin

probe, which also represents a Michael acceptor natural product emphasizing a similar reactivity and affinity profile (*3*).

To confirm the results of mass spectrometry by additional independent experiments, we recombinantly expressed several of our observed targets and labeled them subsequently with the corresponding probes. In fact, in all cases, the probes labeled their targets in an activity-dependent manner as confirmed by heat denaturation controls (Figure 2B). As an example of the mode of binding of the α-methylene-γ-butyrolactone probes to their targets, we investigated the site of modification of *P. aeruginosa* DLDH with probe **8** by MS. One cysteine residue was found to be modified by the probe, while no other cysteine and none of the 19 serine residues reacted with the Michael acceptor (Figure 2 and Figure S6 of the Supporting Information).

These results emphasize the fine-tuned reactivity of the α-methylene-γ-butyrolactone probe, demonstrate the value of this reactive motif for proteome profiling experiments, and further suggest that the full potential of this motif can be explored by an increasing number of structurally diverse molecules that bind a vast array of enzymes based on dedicated and customized affinity features.

To broaden our knowledge of the binding preferences of γ-lactones that are not activated by an electrophilic Michael acceptor system, we designed and synthesized a photo-cross-linker-functionalized lactone probe with a benzophenone moiety and the alkyne handle embodied in a peptidic side chain [probe **9** (Figure 1)]. Because benzophenone represents a large structural element, it could influence and direct the binding of the probe to additional, γ-lactone-unrelated, targets. To identify only those targets that specifically bind the γ-lactone motif, we synthesized a control probe **9c**, in which only the benzophenone and alkyne-containing peptidic side chain without the γ-lactone moiety are present. All targets that are labeled by both probes are presumably bound predominantly by the benzophenone peptide, whereas targets that are labeled by only the γ-lactone probe represent proteins with specific recognition elements for this motif. Because we are interested in the biological targets of reversible binding γ-lactones, we will only focus on the identification of their corresponding protein bands.

The synthesis of probes **9** and **9c** followed standard peptide coupling procedures and is described in more detail in Scheme S3 of the Supporting Information. Labeling experiments were conducted in bacterial lysates of several pathogenic and nonpathogenic organisms such as *P. aeruginosa*, *P. putida*, *B. cenocepacia*, *B. thailandensis*, and *E. coli*. Probe **9** (20 μM) and control probe **9c** (20 μM) were incubated with each bacterial proteome on ice and irradiated at 366 nm for 1 h. Proteomes were then subject to CC and separated and visualized by SDS gel electrophoresis and fluorescent scanning as described above. Several fluorescent bands were observed on the gels, but only one strong band that was present in all proteomes correlated uniquely to γ-lactone-containing probe **9** (Figure 3A and Figure S3 of the Supporting Information).

The band was investigated by mass spectrometry and identified as a trigger factor (TF). Trigger factors are essential bacterial enzymes for the cis−trans isomerization of peptidyl−prolyl bonds and represent a molecular switch for the regulation of enzyme activity (*22*). The trigger factors like those found in *E. coli* K12 belong to the FK506 binding protein family of peptidyl−prolyl cis−trans isomerases and assist the initial folding steps during bacterial protein synthesis (*23*−*25*). They consist of the N-domain, which mediates binding to the ribosomal tunnel exit protein, the PPIase (peptidyl−prolyl isomerase) domain, and the C-domain with chaperone activity. The folding is assisted downstream by the ATP-dependent DnaK/J and GroEL/ES.

The results from mass spectrometric identification were again independently verified by recombinant expression of several trigger factor enzymes from *B. thailandensis*, *B. cenocepacia*, *P. aeruginosa*, and *E. coli* (Figure 3B and Figure S3 of the Supporting Information). All recombinant enzymes were specifically labeled by the corresponding lactone probe **9**, whereas heat-denatured enzymes did not reveal any labeling, indicating that only the native and folded protein can interact with the probe. Moreover, we were able to demonstrate trigger factor binding of probe **9** in living cells of *P. putida*, emphasizing the relevance and great utility of this probe as a tool in proteomics (Figure S5 of the Supporting Information).

Because the labeling of the trigger factors by probe **9** is UV-dependent, a reversible binding mode of the γ-lactone is most likely. We therefore tested all our other reversible binding γ-lactones and thiolactones that we used for initial screens (see above, compounds **1**−**7**) in competitive assays to investigate whether they could compete for the same binding site, which would further confirm that the γ-lactone moiety, as a close mimic of proline, is the central recognition element of trigger factors. Interestingly, several γ-lactones and thiolactones were able to compete for binding to various degrees. Probe **5**, for instance, revealed strong competition at 50- and 100-fold excesses, while all other compounds such as structurally closely related probes **1**, **2**, and **4** exhibited much weaker competition for the binding site, indicating that a phenyl-substituted thiolactone is the most promising motif from our initial library for binding to and inhibiting trigger factors (Figure 3 and Figure S4 of the Supporting Information). To evaluate its potency for the inhibition of PPIase (peptidyl−prolyl isomerase) activity, we used an assay that is based on the solvent-dependent cis−trans re-equilibration of the tetrapeptide *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Supporting Information) (*26*). The peptide was dissolved in 0.5 M LiCl in TFE, which led to a cis content of the Pro−Phe bond of ∼50%. After dilution into aqueous buffer [50 mM Hepes (pH 7.8)], the cis content decreased to ∼10%. Because the cis peptide has a higher absorption coefficient, the decrease during dilution into aqueous buffer can be measured by monitoring the absorption at 330 nm at 10 °C for 10 min. The mean average kinetic constant (each of three independent experiments) versus lactone concentration was plotted, and the concentration of 50% inhibition (IC_{50}) was determined. Figure 4 shows the IC_{50} determination of the *E. coli* trigger factor by lactones **1**, **2**, **4**, and **5**. Interestingly, an IC_{50} value of 50 μM emphasizes that the compound directly targets the PPIase domain of trigger factors and leads to a potent inhibition. On the other hand, compounds **1**, **2**, and **4** exhibited weaker binding in the competition assay and showed almost no inhibition in the assay. Although compounds **1**−**7** were initially designed as reactive probes, their conserved structural lactone motifs can be utilized in combination with cross-linking groups to identify reversible binding inhibitors for dedicated targets.

In summary, we designed and synthesized an initial set of γ-lactones and thiolactones that varied in their protein reactivity. Our initial selection of nonactivated compounds did not reveal significant protein reactivities, but one individual member turned out to be a reversible inhibitor of the trigger factor. This result emphasizes the potency of our chemical proteomic strategy that utilizes protein reactive probes in combination with photoreactive

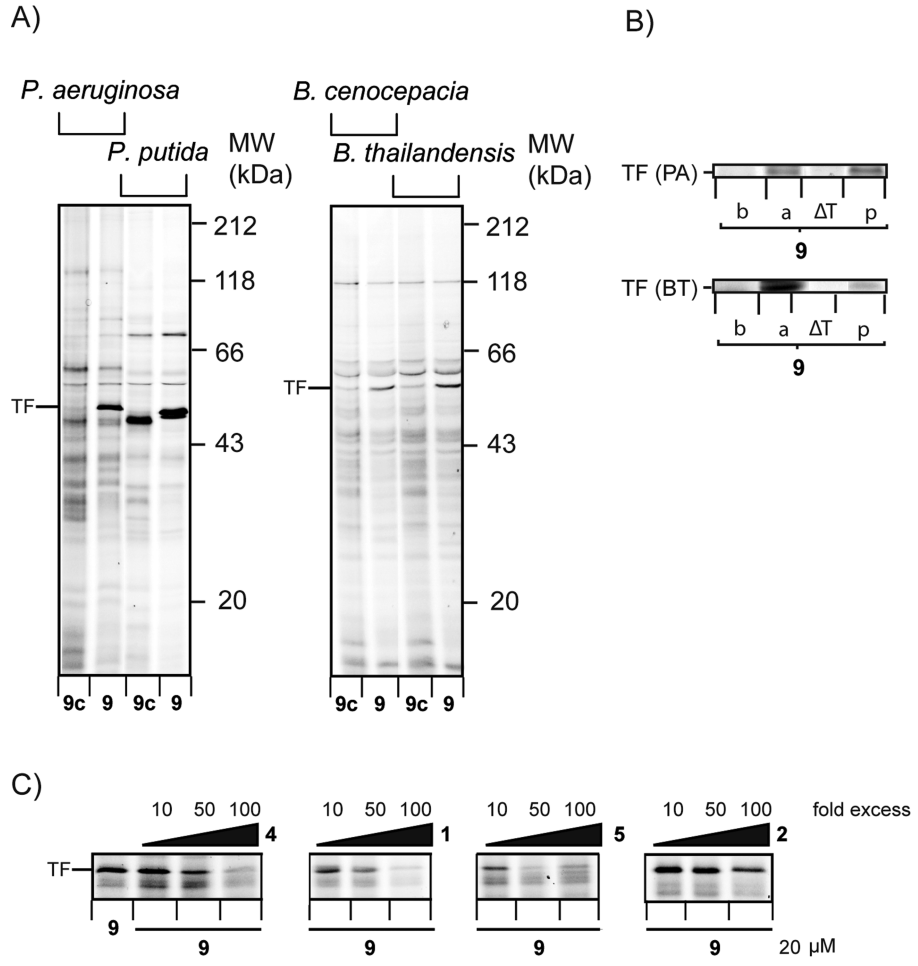

FIGURE 3: Labeling of bacterial lysates with a γ-lactone photocrosslinking probe. (A) Fluorescent SDS gels (TF, trigger factor; MW, molecular weight marker). (B) Recombinant expression of trigger factor and labeling (b, before induction; a, after induction; ΔT, heat control of induced recombinant protein; p, native protein band in the corresponding proteome). (C) Competitive profiling with reversible binding γ-lactones **4**, **1**, **5**, and **2**. Lactones were preincubated in 10-, 50-, and 100-fold excess with the *P. aeruginosa* proteome and subsequently labeled with photocrosslinking probe **9**.

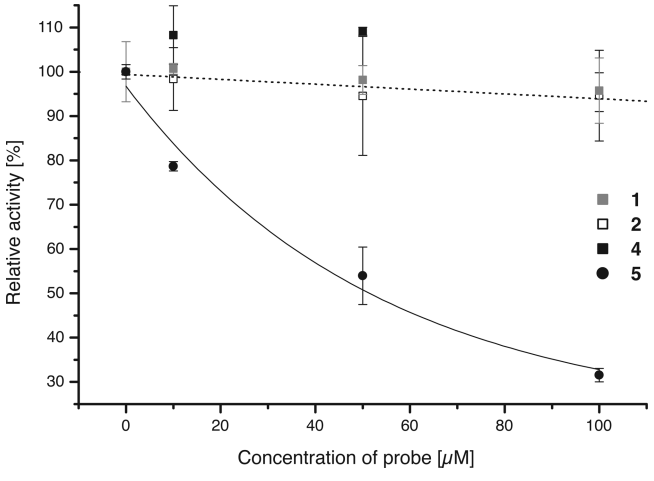

FIGURE 4: Determination of the IC_{50} values of the *E. coli* TF with lactones **1**, **2**, **4**, and **5**.

affinity probes to identify the biological targets of γ-lactones. We obtained several important bacterial enzymes that are involved in diverse cellular functions such as metabolism, cell wall biosynthesis, and protein folding. Protein folding in bacteria could particularly represent a novel strategy for antibiotic intervention and requires chemical tools for characterization and inhibition. In fact, it has already been demonstrated that a dual knockout of trigger factor and a complementary chaperone folding machinery, DnaK and DnaJ, is lethal (*27*, *28*). We here introduce novel tools for studying trigger factors and present novel lead structures based on the γ-lactone structural moiety for inhibition and potential therapeutic application. Future studies that extend structural modifications to protein reactive α-methylene-γ-butyrolactone as well as to reversible binding γ-lactones and thiolactones will reveal if this premise holds true.

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SUPPORTING INFORMATION AVAILABLE

Synthesis of the probes, more detailed experimental procedures, mass spectrometric results, and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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