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β-Lactones as Privileged Structures for the Active-Site Labeling of Versatile Bacterial Enzyme Classes**

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Evolution of multiresistant bacterial strains has meant that infectious diseases once again pose a major threat to public health. Since many antibiotics still target only a limited set of cellular functions, it is a desirable goal to expand the number and breadth of therapeutic targets as well as to gain a deeper understanding of the molecular mechanisms responsible for pathogenesis.[1] To approach this goal, a chemical proteomic strategy (activity-based protein profiling, ABPP), developed by Cravatt and co-workers,[2] that uses active-site-directed probes was directly applied to bacterial proteomes. ABPP probes consist of at least two general elements: 1) a reactive group for binding and covalently modifying the active site of a certain enzyme class, and 2) a reporter tag for the detection, enrichment, and identification of probe-labeled proteins.[3]

Many ABPP probes have so far utilized electrophilic reactive groups,[2] including fluorophosphonates,[4] sulfonate esters,[5] and epoxides,[6] which exhibit preferences for nucleophilic groups in the active site of several distinct enzyme classes. For bacterial ABPP, we selected a new reactive group based on the β-lactone (2-oxetanone) structure derived from natural products. β-Lactones represent promising biologically active privileged structures that can react covalently with the active sites of certain enzymes.[7] Although β-lactones such as obafluorin[8] and hymeglusin[9] have been proven to exhibit antibiotic activity, their molecular targets remain largely unknown (see Figure S1 in the Supporting Information). Here, we apply ABPP with β-lactones to prokaryotes to identify dedicated target enzymes, with special emphasis on those which are crucial for bacterial viability and virulence.

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Supporting information for this article (details on the synthesis and characterization of probes as well as proteome preparation and labeling) is available on the WWW under http://www.angewandte.org or from the author.

To maximize the number of labeled enzymes we synthesized a small library of probes with an alkyne tag at the C-4-position and with diversity introduced at the C-3-position of the 2-oxetanone ring (Figure 1 A).[10] The modification of the alkyne tag through a 1,3-dipolar Huisgen cycloaddition (click chemistry) allows a fluorophor reporter group to be appended for visualization of the target enzyme by SDS-gel electrophoresis after proteome labeling,[11] as reported by Cravatt and co-workers (Figure 1 B).

Most β-lactones derived from natural products are biologically active in the *trans* configuration,[8,9,12] but do not show a clear preference for the absolute configuration within the *trans* stereochemistry.[13] Therefore, we focused our attention on developing a synthetic strategy to yield *trans*-β-lactones as racemic mixtures, which show similar labeling profiles as the corresponding *cis* isomers (see Figure S2 in the Supporting Information). Inspired by naturally occurring β-lactones, the biomimetic library comprised 10 compounds with aliphatic or aromatic substitutions of different length and branching. To evaluate the selectivity for the target enzymes, the library was screened against the proteomes of several Gram-positive and Gram-negative bacteria—*Pseudomonas putida*, *Listeria welshimeri*, *Bacillus licheniformis*, *Bacillus subtilis*, and *Escherichia coli*—which are phylogenetically related to pathogenic strains. Mouse liver cytosol was also included in the study as an eukaryotic reference proteome. Initial labeling experiments were carried out by adding individual probes at a concentration of 50 μM to the proteome; this concentration is sufficient to achieve full saturation of most target enzymes. Interestingly, individual members of the probe library showed highly distinct reactivity profiles against the native proteomes investigated (see Figure S3 in the Supporting Information), thus indicating that the substitution at the C-3-position exerted a strong influence over specific probe–protein interactions. As an example, the labeling of *L. welshimeri* cytosolic and membrane proteomes as well as of *B. subtilis* cytosolic proteome with a subset of the most complementary probes is shown in Figure 2 A and B. Only one unspecific binding event was observed in the heat-denatured control proteome of *L. welshimeri*, which emphasizes the predominant preference of β-lactones for native proteins (Figure 2 C, and see Figure S4 in the Supporting Information). Most of the targeted proteins were of low abundance, as shown by direct comparison of the relative intensities observed with coomassie staining and fluorescence scanning (see Figure S5 in the Supporting Information).

Subsequent identification of the labeled target enzymes by LC-MS analysis (see the Supporting Information) revealed the labeling of about 20 different enzymes (Table 1). The MS

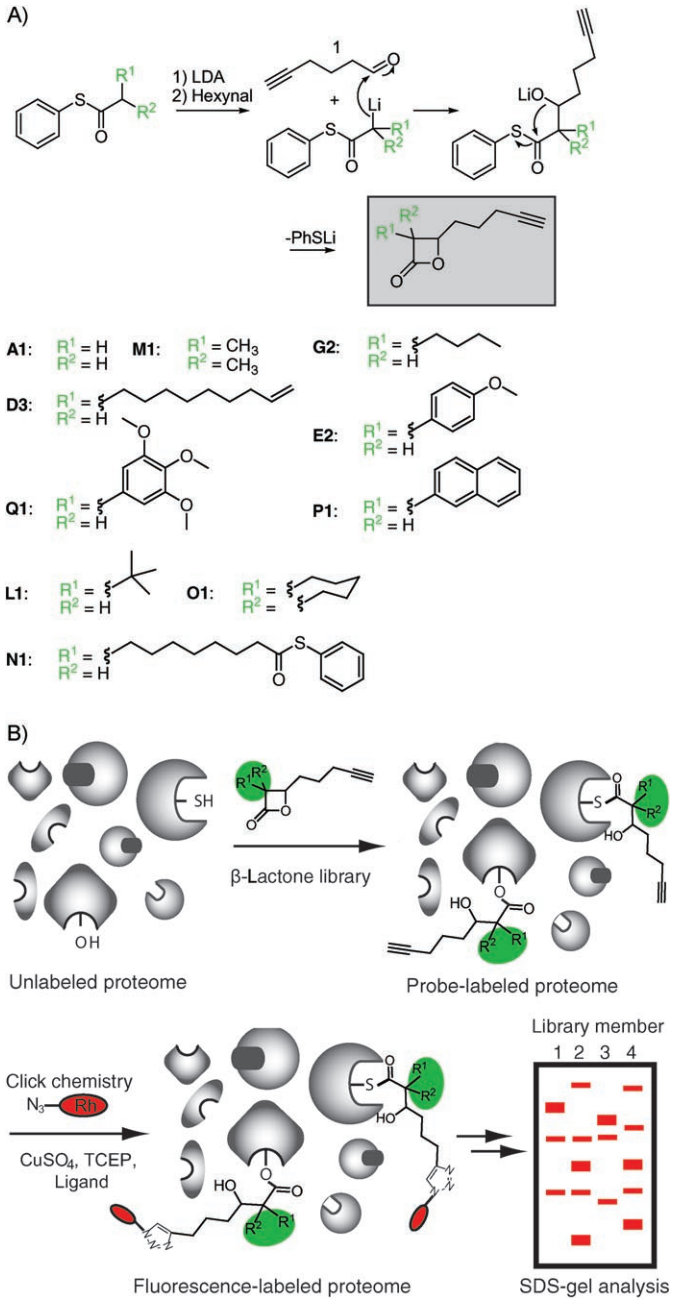

Figure 1. β-Lactones as privileged structures for bacterial ABPP. A) Synthetic route and biomimetic structures of the β-lactone library. LDA = lithium diisopropylamide B) Proteomes are first treated with the β-lactone library and subsequently appended with a fluorescent dye by click chemistry. Labeled proteomes were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorescence scanning.

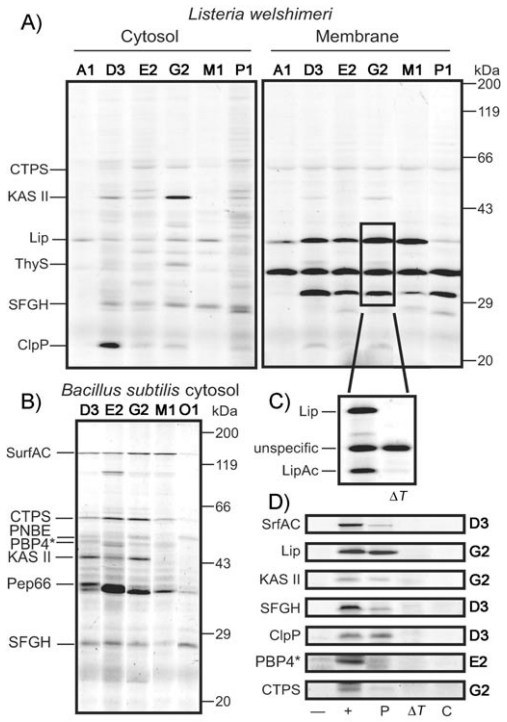

Figure 2. Labeling profiles of the β-lactone library against bacterial proteomes. A) Fluorescent gel of *L. welshimeri* cytosol and membrane proteomes after treatment with selected library members. The enzyme identities are assigned to the corresponding gel band (for a list of abbreviations, see Table 1). B) Fluorescent gel of *B. subtilis* cytosol proteome showing the identified enzyme targets. C) All reactions were carried out with a heat control (Δ*T*) to identify unspecific labeling. An example is shown for **G2** in the membrane proteome of *L. welshimeri* (boxed). D) Examples of recombinantly expressed enzymes (−: before induction, +: after induction, P: native proteome, Δ*T*: after induction/heat control, C: after induction/no probe).

results were confirmed by recombinant expression of all major hits and subsequent labeling by the corresponding probes (Figure 2D, and see Figure S6 in the Supporting Information). The identified enzymes belong to four major families comprising ligases, oxidoreductases, hydrolases, and transferases (Table 2). All of these families require a nucleophilic residue in their active site for catalysis (Cys or Ser), with this residue likely attacking the electrophilic β-lactone ring. Indeed, preincubation of several proteins with phenylmethylsulfonyl fluoride (PMSF) and cerulenin, which are known active-site inhibitors of serine proteases[14] and β-ketoacyl acyl carrier protein synthases (KAS),[15] respectively, prevented subsequent labeling with the probe (see Figure S7 in the Supporting Information). In addition, substrate inhibition assays with S-formylglutathione hydrolase (SFGH) demonstrated probe-mediated inhibition of enzyme activity ($IC_{50} = 5\ \mu M$). The inhibition of enzyme activity and the broad coverage of mechanistically distinct enzyme classes emphasizes the unprecedented utility of β-lactones as new proteomic tools for ABPP.

Interestingly, a large number of the identified enzymes are involved in important cellular functions, such as primary (KAS I and II) and secondary metabolism (surfactin A synthetase C, SurfAC), nucleotide synthesis (CTP synthase), detoxification (SFGH), antibiotic resistance (penicillin-binding protein (PBP) 4*) as well as in virulence (ATP-dependent caseinolytic protease, ClpP). With the exception of SFGH, no homologue of these enzymes was detected in the mouse liver proteome.

Several targets are of special medicinal interest: KAS II, an essential component of the fatty acid biosynthetic path-

Table 1: Enzymes identified (clustered by function).

Function	Enzyme (abbreviation)	Proteomes identified
primary metabolism		
	acetylCoA hydrolase (ACoAH)	*P. putida*
	aldehyde dehydrogenase B (ADB)	*E. coli*
	formate-C-acetyltransferase (FCA)	*B. licheniformis*
	lipase (Lip)	*L. welshimeri*
	lipase/acylhydrolase (LipAc)	*L. welshimeri*
	lysophospholipase (LPL)	*Mus musculus*
	β-ketoacyl acyl carrier protein synthase I (KAS I)	*E. coli*, *P. putida*
	β-ketoacyl acyl carrier protein synthase II (KAS II)	*B. subtilis*, *B. licheniformis*, *E. coli*, *L. welshimeri*
	β-ketoacyl acyl carrier protein synthase III (KAS III)	*B. licheniformis*
	β-ketothiolase (BKT)	*P. putida*
Secondary metabolism		
	surfactin A synthetase subunit C (SrfAC)	*B. subtilis*
nucleotide synthesis		
	CTP synthase (CTPS)	*B. subtilis*, *B. licheniformis*, *E. coli*, *L. welshimeri*
	thymidylate synthase (ThyS)	*L. welshimeri*
resistance/cell-wall biosynthesis		
	penicillin-binding protein 4* (PBP4*)	*B. subtilis*
virulence associated		
	ATP-dependent Clp protease (ClpP)	*P. putida*, *L. welshimeri*
	proline iminopeptidase (PIP)	*P. putida*
detoxification		
	S-formylglutathione hydrolase (SFGH)	*B. subtilis*, *E. coli*, *L. welshimeri*, *Mus musculus*
	dienelactone hydrolase (DLH)	*P. putida*
unknown function		
	AB hydrolase (ABH)	*Mus musculus*
	para-nitrobenzyl esterase (PNBE)	*B. subtilis*
	peptidase S66 (Pep66)	*B. subtilis*
	putative ATP dependent protease (PADP)	*P. putida*
	putative esterase (PutE)	*B. licheniformis*

way,[16] is highly conserved among key pathogens,[17] and was labeled by the aliphatic β-lactone probes **D3** and **G2** (Figure 2A and B). In addition, PBP4*, an enzyme which is reported to exhibit β-lactamase activity,[18] was labeled in *B. subtilis* (Figure 2B). Although we used nonpathogenic bacterial proteomes for our screens, two virulence-associated enzymes, ClpP and proline iminopeptidase (PIP), which play crucial roles in many pathogenic strains were detected in *L. welshimeri* and *P. putida*. In particular, ClpP has attracted much attention because of its fundamental role for stress tolerance and virulence in many pathogenic bacteria, including *S. aureus*, *L. monocytogenes*, and *P. aeruginosa*.[19–21]

To estimate the strength of probe–protein interactions we compared the reactivity profiles of selected β-lactones across a broad concentration range from 10 μM to 5 nM. Several target enzymes displayed a very robust labeling behavior. For example, the aliphatic β-lactone **G2** is a very sensitive probe for lipase (Lip, down to 20 nM probe concentration), but does not exhibit a strong interaction with lipase/acylhydrolase (LipAc) in *L. welshimeri* (Figure 3A). In contrast, the aromatic β-lactone **P1** shows the opposite selectivity (down to 160 nM for LipAc), thus illustrating the sensitivity and target enzyme selectivity of the β-lactones **G2** and **P1**. Interestingly, ADB shows a strong preference only for aliphatic β-lactones (**D3, G2**) at 100 nM concentration in *E. coli* cytosol (Figure 3B). Similarly, several other enzymes could be clustered on the basis of their preferred binding partners (Figure 3C). This information is useful to gain insight into the native substrate preferences of all labeled enzymes, especially for those which have been less investigated and are uncharacterized. In this context, it is interesting to note that unsubstituted β-lactones (**A1**) seem to be poor at labeling enzymes. Steric constraints in probes such as **O1** and **L1** also decreased the number of specific labeling events. Therefore, β-lactones with aliphatic and aromatic moieties seem to be the most potent probes, which might further help to guide the design of specific inhibitors in the future.

Table 2: Enzyme classes labeled by β-lactone probes.

Enzyme class	EC	Active site	Example
ligase	EC 6.3.4.	Cys, His, Glu	CTP synthase
oxidoreductases	EC 1.2.1.	Cys, Glu	aldehyde deyhdrogenase B
hydrolases	EC 3.1.2.	Ser, Asp, His	S-formylglutathione hydrolase
	EC 3.1.1.	Ser, Glu, His	*para*-nitrobenzyl esterase
	EC 3.4.11.	Ser, Asp, His	proline iminopeptidase
	EC 3.4.21.	Ser, His	ATP-dependent Clp protease
transferases	EC 2.3.1.	Cys, His, Asn	β-Ketoacyl-ACP synthase II
	EC 2.3.1.	Gly, Cys, Cys	formate-C-acetyltransferase
	EC 2.3.1.	Cys, His, Cys	β-Ketothiolase
	EC 2.1.1.	Cys	thymidylate synthase

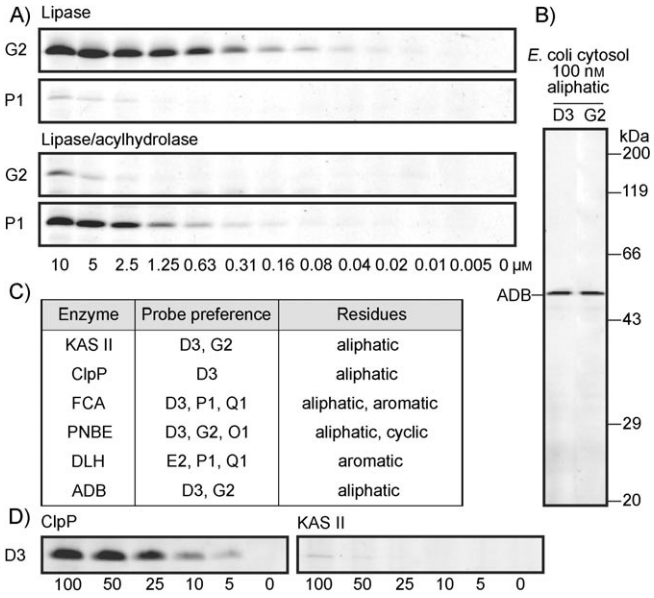

Figure 3. Sensitivity and specificity of the β-lactone library for individual enzymes. A) Concentration series of selected probes in the *L. welshimeri* proteome. B) ADB is the only enzyme in *E. coli* cytosol which is labeled by **D3** and **G2** at 100 nM. C) List of selected enzymes with their corresponding β-lactone probe preferences. D) In vivo labeling of *L. welshimeri* with β-lactone **D3** at various concentrations.

The active-site labeling of essential enzymes such as KAS II raised the question of a possible antibacterial effect. Several probes were tested for growth inhibition, but no antibiotic activity could be observed. One possible reason could be a limited cellular uptake of probes by intact bacteria. To clarify this, we incubated *L. welshimeri* with various concentrations of probes in vivo. These experiments revealed that KAS II, the most important target for viability, was only weakly labeled at high concentrations (100 μM) and, therefore, probably did not reach saturation for full inhibition. In contrast, strong labeling occurred for the virulence-associated enzyme ClpP with probe concentrations as low as 5 μM (Figure 3D). This specific and sensitive in vivo labeling of a common virulence factor, which is not essential for viability but indispensable for bacterial pathogenesis, shows that probes are applicable to in vivo studies and represents an attractive strategy for the inhibition of new target enzymes from bacteria in the future. Inhibitors of virulence-associated enzymes display many advantages over conventional antibiotics, such as preserving the host endogenous microbiome and exerting less-selective pressure, which may result in decreased resistance and lead to longer lasting drugs.[22]

In conclusion, side-chain-modified β-lactones represent promising privileged structures for the design of novel ABPP probes which can be utilized to screen and compare enzyme activity of different bacterial proteomes. We were able to identify a variety of mechanistically distinct target enzymes that show sensitive labeling as well as inhibition by the probe molecules. In view of the labeling of several important bacterial enzymes, this approach might represent a promising starting point for the identification of novel antibacterial targets together with their corresponding inhibitors.

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