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β-Sultams exhibit discrete binding preferences for diverse bacterial enzymes with nucleophilic residues†

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β-Sultams are potent electrophiles that modify nucleophilic residues in selected enzyme active sites. We here identify and characterize some of the specific bacterial targets and show a unique inhibition of the azoreductase family.

Four-membered rings such as β-lactams and β-lactones inhibit diverse enzyme classes by covalently modifying nucleophilic active site residues.[1,2] Interestingly, although β-lactams and β-lactones are structurally related and present in many bioactive natural products they bind and inhibit a complementary set of enzymes pointing to a fine-tuned reactivity towards different active sites. Although not of natural origin β-sultams represent a third class of four-membered ring scaffolds that exhibit discrete reactivity towards nucleophiles.[3–6] The structural similarity to β-lactams raised interest in the use of these compounds as novel antibacterials that overcome resistance development. Thus the binding of β-sultams to β-lactamases and penicillin binding proteins (PBPs), the dedicated targets of β-lactams, has been previously studied.[7–9] It was shown that the nucleophilic serine active site attacks the sulfonyl center and displaces the amine as a leaving group resulting in a stable covalent attachment and corresponding enzyme inhibition (Fig. 1).[9] Alternatively, it has been observed that the sulfonate ester can undergo an elimination reaction leading to dehydroalanine (Dha).[7] β-Sultams exhibit a smaller intramolecular resonance stabilization compared to the β-lactam analogues that in combination with ring strain contribute to their elevated reactivity.[4] The proteome wide binding of β-lactams has been investigated previously and a high preference of natural product derived compounds for several PBPs was observed.[2,10] Similar studies with related β-sultam scaffolds have not been performed up to now. We thus designed a small collection of diverse β-sultams with differing side chain decorations as well as an alkyne handle which allows us to detect irreversibly bound targets of these compounds in living bacterial systems *via* activity based protein profiling (ABPP)

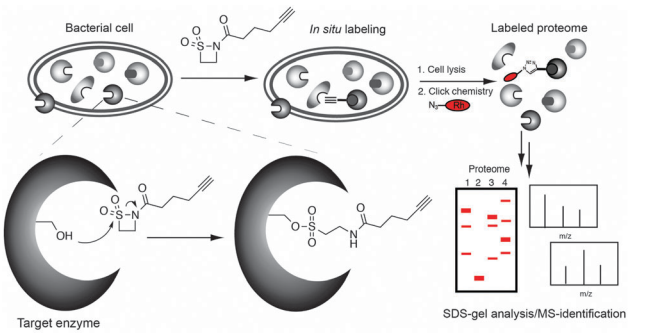

Fig. 1 Principle reaction of β-sultams with serine nucleophiles in proteins and target identification *via* activity based protein profiling.

(Fig. 1 and Fig. S1, ESI†).[11–15] We incubated these probe scaffolds with living *Burkholderia cenocepacia*, *B. thailandensis*, *Listeria welshimeri* and *L. monocytogenes* and analyzed the corresponding target profiles *via* gel-based and gel-free mass spectrometry (MS). Interestingly, the β-sultams under investigation revealed a discrete labeling pattern of several different enzymes. One protein, an azoreductase which is an important enzyme class for azo dye removal in biotechnological applications, was found in several investigated bacterial systems. Subsequent activity studies with methyl red revealed that this enzyme is able to break azo bonds and gets inhibited by selected β-sultams *via* an unprecedented labeling of a conserved threonine residue.

Based on previous studies of *N*-acylated β-sultams as peptidase inhibitors[9,16,17] we designed and synthesized a comprehensive collection of diverse alkynylated derivatives (Scheme 1 and Fig. S1, ESI†). These compounds thus serve as proteomic tools for the identification of irreversibly bound protein targets in living bacterial cells and extend the initial work on isolated enzymes to the diversity of cellular proteins.

The collection of β-sultam probes can be divided into three categories. The first category comprises molecules that are *N*-acylated β-sultams lacking additional substituents at the core structure (**S02** and **S05**). The alkyne handle for target identification is part of the acyl substituent. In the second category the *N*-acyl chain varies and an additional phenone moiety is

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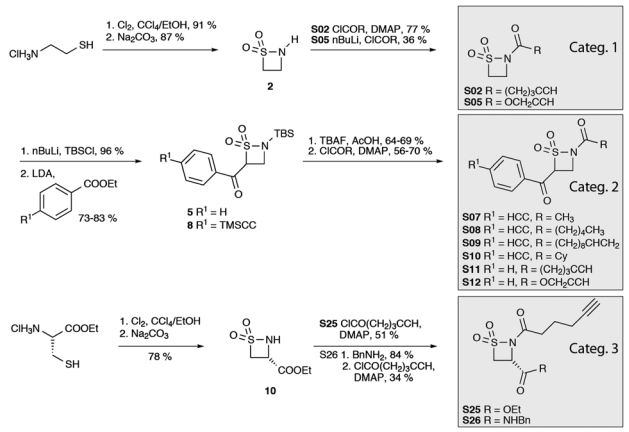

Scheme 1 Synthesis of category 1, 2 and 3 β-sultams. For synthetic details please refer to the ESI.†

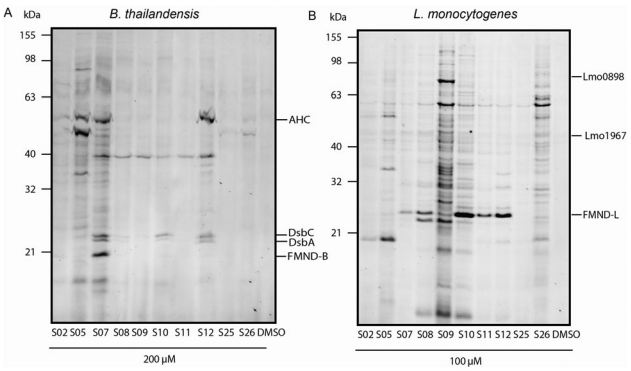

Fig. 2 β-Sultam profiling. Fluorescent gel of labeled proteins in the cytosolic fractions of *B. thailandensis* (A) and *L. monocytogenes* (B). Identified protein targets are indicated on the right. For unabbreviated forms of protein names please refer to Table S1 (ESI†).

attached in the 2-position (**S07–S12**). The alkyne is either incorporated in the *para* position of the phenone or as a terminal part of the *N*-acyl chain. The third category has an amide or ester substituent in the 3-position (**S25** and **S26**). Details of the synthesis are listed in Scheme 1; Schemes S1 and S2 (ESI†).

To test the reactivity of β-sultams with full proteomes we first incubated intact cells of *B. thailandensis* and *L. monocytogenes* with various concentrations of probes **S07** and **S10**, respectively. After 2 h of incubation the cells were washed, lysed and treated with rhodamine azide by a copper catalysed click chemistry reaction to append the fluorescent tag to compound labeled proteins.[18–21] Subsequent separation of the proteome by SDS-PAGE and fluorescent scanning revealed discrete bands that differed in intensity depending on the compound concentration (Fig. S2, ESI†). Based on this pattern a concentration of 100–200 μM was selected in order to obtain the best signal to noise ratio. However, two low molecular weight proteins could be visualized down to 20 μM in both bacterial strains emphasizing high binding affinity. Profiling of the entire compound collection *in situ* with pathogenic *B. cenocepacia* and non-pathogenic *B. thailandensis* revealed a highly diverse labeling pattern in the cytosol emphasizing that the compounds are cell-permeable and that the different structural decorations directly bind in diverse active sites (Fig. 2A, Fig. S3 and S5, ESI†). The membrane fraction showed a similar but weaker labeling (Fig. S4 and S6, ESI†).

Although no differences in probe binding preferences between the two *Burkholderia* strains could be observed the comparison of the two *Listeria* strains revealed a pronounced labeling of additional proteins in pathogenic *L. monocytogenes* (Fig. 2B and Fig. S5, ESI†). We focused our analysis on *B. thailandensis* as well as *L. monocytogenes* in order to identify characteristic protein targets of β-sultams. Interestingly, all members of the small sultam library exhibited binding to several proteins in either of the two bacterial species. A significant labeling of proteins in the 21 to 60 kDa range of *B. thailandensis* was obtained for category 2 compound **S07** with a short *N*-acetyl substitution. Category 2 compound **S12** with a *N*-acetoxy side chain revealed a similar binding pattern but lacks the 21 kDa protein. Profiling of the compound collection in *L. monocytogenes* revealed a strong preference of category 2 probes **S10**, **S11** and **S12** for a 24 kDa protein.

In order to identify the proteins that prominently associate with selected β-sultams we utilized a biotin and rhodamine functionalized azide linker and enriched labeled proteins *via* avidin bead binding.[22,23] After SDS-PAGE analysis selected fluorescent protein bands were isolated, trypsinized and analyzed *via* HPLC-MS/MS. In addition, we performed gel-free MS experiments to get a comprehensive list of putative targets. Peptide masses were searched with the SEQUEST algorithm against available databases and the corresponding protein hits are listed in Tables S1–S3 (ESI†). The prominent 50 kDa protein in *B. thailandensis* was identified as adenosylhomocysteinase (AHC) and the lower bands around 22 kDa as thiol disulfide interchange proteins (DsbC and DsbA) and FMN-dependent NADH-azoreductase (FMND-B) (Fig. 2A). Interestingly, a related azoreductase (FMND-L) was also identified in *L. monocytogenes* (Fig. 2B) emphasizing that selected β-sultams prefer binding to this enzyme class. In addition, Lmo0898 was identified as an uncharacterized protein, which exhibited sequence similarity to a transcription associated protein for toxin expression.[24]

The two azoreductases exhibit only 32% sequence identity suggesting that the enzymes may recognize different substrates. This is also supported by the broader labeling of *Listeria* azoreductase by several probes and the exclusive labeling of *Burkholderia* azoreductase only by **S07**. Importantly, a comparison of the labeling pattern between pathogenic *L. monocytogenes* and non-pathogenic *L. welshimeri* reveals that the azoreductase is predominantly present in the pathogenic strain which may implicate a so far undiscovered role of this enzyme in disease development (Fig. S5, ESI†). It was previously suggested that bacteria need azoreductases for the detoxification of quinones and thus could be reclassified as NAD(P)H quinone oxidoreductases.[25–27] However, the focus of azoreductase research so far has been on its application as a biotechnological tool for the removal of azo dyes from waste water.[27–30]

In order to verify the results of MS we cloned identified proteins into expression vectors and incubated cells that overexpressed the protein with the corresponding probe molecules. Heat denaturation of the proteome prior to labeling resulted in a lack of binding suggesting that the molecules require an intact and folded enzyme for interaction (Fig. S7, ESI†).

To elucidate the mechanism of azoreductase specific labeling we first determined how many molecules are attached per protein.

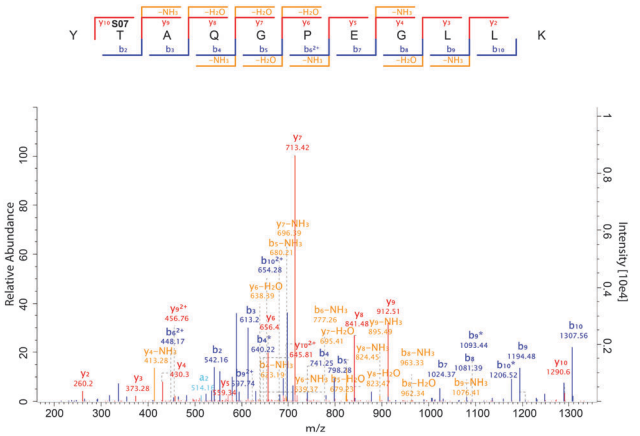

Fig. 3 MS/MS analysis of the modified Thr122 in FMND-B treated with **S07**. For all other spectra please refer to Fig. S10 (ESI†).

Intact protein MS of FMND-B with compound **S07** resulted in the addition of one molecule to the enzyme (Fig. S8, ESI†). The site of modification was determined by digestion of the protein with trypsin or chymotrypsin after **S07** labeling. The resulting peptides were investigated by MS/MS fragmentation. Proteome Discoverer (PD) and MaxQuant (MQ) software revealed the selective modification of Thr122 in a sequence region that is conserved in several azoreductases (Fig. 3 and Fig. S9, ESI†).

Based on the crystal structure of the related enzyme from *E. coli* this residue is outside the active site and does not coordinate to the FMN cofactor.[28] A direct or indirect role of this residue in catalysis has not been proposed so far. The same threonine (Thr128) was modified in FMND-L with **S10** emphasizing a conserved nucleophilic reactivity across species (Fig. S10, ESI†). Interestingly, MS/MS sequencing and binding site localization analysis with PD and MQ revealed an additional Ser152 binding site (Fig. S10, ESI†). Although both probes are members of category 2 the difference in the alkyl chain (methyl *vs.* cyclohexyl) seems to significantly influence their binding in the respective enzyme pockets.

We finally evaluated if binding of azoreductase directed probes would lead to a corresponding inhibition of enzyme activity. This was monitored by the enzyme catalyzed cleavage of methyl red. Probes **S10** and **S07** that both labeled the azoreductases in *Listeria* and *Burkholderia*, respectively, revealed a concentration dependent inhibition with IC_{50} values of about 30 μM in both cases (Fig. S11, ESI†). In contrast probes **S05** and **S02** that did not label both enzymes also revealed no inhibition (Table S4, ESI†). Based on the important role of azoreductases for biotechnological applications as well as their yet undiscovered role in many bacteria our compounds represent active site directed tools to study their activity and function in living cells.

β-Sultams have attracted attention in the past as substitutes for β-lactam antibiotics which suffer from bacterial resistance development. Studies with recombinant PBPs, elastase and β-lactamase indeed suggested inhibition of these targets by covalent binding.[9,16,17] Our *in situ* cellular profiling did not indicate interaction with these enzymes by a covalent interaction. Correspondingly, we did not observe any antibacterial activity of all compounds used here up to a concentration of 1000 μM which is in line with previous literature reports (Table S5, ESI†).[31] The β-sultam targets discovered here are different to those observed by β-lactams and β-lactones (Fig. S12, ESI†).[2,23] One of these targets are azoreductases that are important enzymes in biotechnological application for the removal of azo dyes as well as in pathogenic bacteria with a yet undefined role. Among other applications, the β-sultam probes introduced here represent customized tools for the discovery and study of azoreductase activity across different pathogenic and non-pathogenic bacterial strains.

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