The biological targets of acivicin inspired 3-chloro- and 3-bromodihydroisoxazole scaffolds†

Ronald Orth, Thomas Böttcher and Stephan A. Sieber*

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Target analysis of acivicin derived 3-halodihydroisoxazoles scaffolds in living non-pathogenic and pathogenic bacteria.

Nature's repertoire of bioactive compounds has provided a rich source of potent drugs against many human diseases[1] and a diverse molecular toolkit for biochemical research. Due to the tremendous success of protein reactive natural products such as beta-lactams,[2] beta-lactones[3,4] and Michael acceptor[5] systems to inhibit key enzymes involved in viability and pathogenesis we here extend our efforts to unravel the targets of additional privileged electrophilic natural products. We report the synthesis of acivicin inspired 3-chloro- and 3-bromo-dihydroisoxazole probes and their application in target profiling in non-pathogenic bacteria as well as in pathogenic bacteria such as *S. aureus* and multiresistant *S. aureus* (MRSA). Acivicin is produced by *Streptomyces sviceus*[6] and was first structurally characterized by Hanaka *et al.* in 1973.[6] It is reported to be a glutamine antimetabolite antibiotic[7] that in addition exhibits anti-tumor activity. Although no detailed target analysis has been carried out so far, *in vitro* analysis showed that several important enzymes such as L-glutamine-dependent amidotransferases,[8] glutamate synthase (GltS)[9] as well as γ-glutamyl transpeptidase (g-GT) could be inhibited by a covalent modification of the active site cysteine and threonine residues.[10]

As a total proteome analysis of the acivicin derived privileged core structure 3-halo-dihydroisoxazoles in living bacteria will likely reveal a more complete picture of the full complement of dedicated targets, we designed and synthesized structurally diverse 3-chloro- and 3-bromo-dihydroisoxazole probes for activity based protein profiling (ABPP).[11–13] All probes contain an alkyne handle as a benign tag for the modification with rhodamine and biotin azides *via* the Huisgen cycloaddition (click chemistry, CC)[14–16] after cell penetration and lysis to visualize and identify target proteins *via* fluorescent SDS-gel analysis and mass spectrometry (MS), respectively (Fig. 1).

In order to explore the protein binding preferences of 3-halodihyroisoxazoles we designed several probes which were fine tuned towards binding and reactivity by substitution of the dihydroisoxazole ring with chloro- and bromo substituents in 3 position and structurally diverse residues in 4 and 5 positions. Halogen substituted dihydroisoxazoles are protein

Center for Integrated Protein Science Munich CIPS^M, Department of Chemistry, Technische Universität München, Lichtenbergstr. 4, 85747 Garching, Germany. E-mail: Stephan.sieber@tum.de; Fax: +49 289 13302; Tel: +49 289 13319

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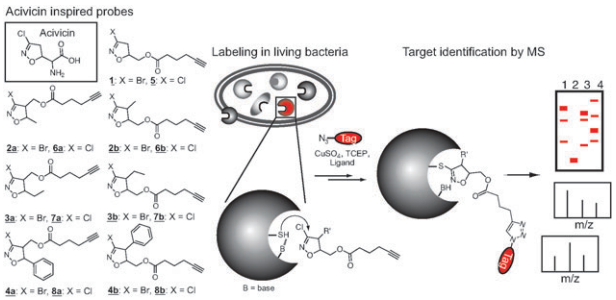


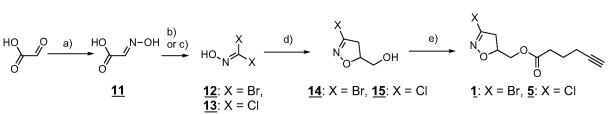
Fig. 1 Identification of target proteins *via* ABPP. Modification of cysteine by the 3-chlorodihydroisoxazole probe.[17]

reactive compounds that are prone to react with enzyme active sites containing residues of elevated nucleophilicity such as cysteines and serines. Nucleophilic attack *via* an addition–elimination mechanism (Fig. 1) most likely leads to a halogen displacement and a resulting stable covalent bond.[17] As this intrinsic reactivity of the 3-halodihydroxyisoxazole is a crucial parameter for their inhibition capability we utilized a bromo substituent (probes **1** to **4**) in addition to the naturally occurring chloro substituent (probes **5** to **8**) to investigate and compare protein reactivity profiles (Fig. 1).

We equipped the core scaffold with a comprehensive set of diverse substituents ranging from unsubstituted to small aliphatic (methyl and ethyl) and aromatic (phenyl) residues to explore different target protein selectivities.

The synthesis of probes **1** to **8** started by an established procedure. Glyoxylic acid reacted with hydroxyl amine to give glyoxylic acid aldoxime[18] that was subsequently converted to dibromo- or dichloroformaldoxime by treatment with Br_2 or *N*-chloro succinimide, respectively (Scheme 1 and Scheme S1, ESI†).[19] Both formaldoximes were used immediately for the cycloaddition reaction, in which they reacted with unsaturated alcohols to give a 3-halo-5-(hydroxymethyl)-dihydroisoxazole.

Although synthetically very useful, this *syn*-cycloaddition was not enantio- and regioselective and generated racemic mixtures of 3-halodihydroisoxazoles for compounds **14** and **15** as well as *cis*-substituted regioisomers (variation of substitution in ring positions 4 and 5) for compounds **16–21**. As the preferences of dedicated targets were unknown, we rationalized



Scheme 1 Synthesis of the probes **1** and **5**. For other probes please refer to ESI.† (a) NH_2OH, H_2O, 16 h, rt, (b) for dibromo: Br_2, H_2O, 3 h, 0 °C to rt, (c) for dichloro: *N*-chloro succinimide, DME, 10 min, 110 °C to rt, (d) allyl alcohol, K_2CO_3, EtOAc, 24 h, rt, (e) hexynoic acid chloride, DCM, TEA, 16 h, rt.

that for initial screens multiplexed regioisomeric mixtures of our probes would likely increase the chance of productive binding in proteome profiling studies. If specific binding is observed for a regioisomeric mixture, deconvolution by HPLC based separation and subsequent individual analysis will reveal the binding preferences of each individual compound with their dedicated targets. All 3-halo-5-(hydroxymethyl)-dihydroisoxazoles were easily converted to their corresponding activity probes by esterification with hex-5-ynoic acid chloride in high yield (ESI†).

We initiated our proteome screening with 3-chloro- and 3-bromo-dihydroisoxazol probes (**1** to **8**) against intact cells of *B. licheniformis*, *P. putida*, *L. welshimeri* as well as pathogenic *S. aureus* and a resistant MRSA strain. These initial screens were carried out with regioisomeric probe mixtures (**2–4** and **6–8**) to speed up the discovery process and identify promising probe scaffolds and ring substitutions which, in case of promising candidates, were subsequently separated and again investigated by repeated proteome profiling to unravel the structure of the active species (see below). Cells were grown into stationary phase and incubated with varying probe concentrations for 2 h. Upon incubation, cells were lyzed and a fluorescent rhodamine azide attached *via* CC. Subsequent SDS-gel analysis and fluorescent scanning revealed a distinct set of specific protein bands in all proteomes investigated, emphasizing that the probes display suitable properties for *in situ* studies. A concentration of 150 μM and an incubation time of 2 h turned out to be optimal for saturated labeling (Fig. 2a and b and Fig. S1a,b, ESI†).

Interestingly, a comparison of the labeling profiles of compounds with different ring substitutions revealed a highly diverse and distinct pattern of enzyme binding preferences (Fig. 2a and Fig. S1a and S2, ESI†).

In *S. aureus* for example, probe **1** with an unsubstituted scaffold revealed the labeling of two different enzymes, while in contrast probe isomers **3** with an ethyl substituent in 5 (isomer **3a**) or in 4 position (isomer **3b**) exhibited a binding preference for a protein of lower molecular weight (Fig. 2a). Interestingly, probe mixture **2** with a shorter methyl side chain in 5 (isomer **2a**) or in 4 position (isomer **2b**) revealed again a different pattern that represents a combination of the other probes, emphasizing that the targeted enzymes exhibit very distinct active site geometries.

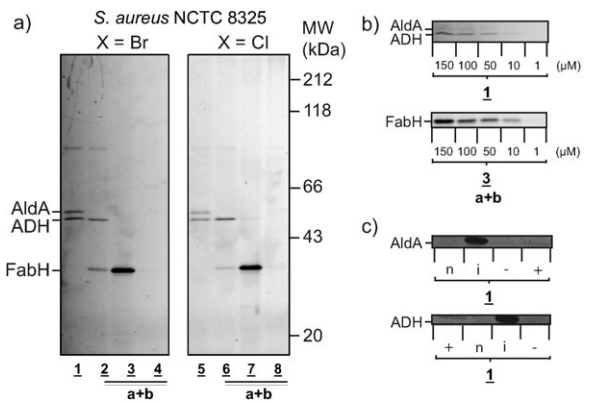


Fig. 2 (a) *In situ* labeling (fluorescent images) of non-pathogenic *S. aureus* NCTC 8325 using probes **1** to **8** (1 h incubation). Aldehyde dehydrogenase (AldA, 53.6 kDa), aldehyde dehydrogenase (ADH, 51.7 kDa) and 3-oxoacyl-[acyl-carrier-protein] synthase III (FabH, 33.9 kDa) were detected in *S. aureus* NCTC 8325. (b) Titration of probes **1** and **3** in *in situ* labeling experiments. (c) *In vitro* labeling of recombinant, overexpressed target enzymes (+: positive control using native proteome, n: not induced, i: induced, −: heat control using induced proteome).

Phenyl-substituted probe isomers **4** showed significantly less labeling events in the majority of proteomes (Fig. 2 and Fig. S1 and S2, ESI†). Comparison of all proteome labeling profiles further revealed that chloride substituted dihydroisoxazoles led to a slightly weaker labeling compared to the bromide substituted counterparts, but not to a change in target preferences.

Target identification was carried out by mass spectrometry (MS) using a quantitative enrichment strategy for labeled enzymes (ESI†).[3] Among the identified enzymes there was a surprisingly large fraction of six members of the dehydrogenase (DH) enzyme family including pyrroline-5-carboxylate DH (PCDH), aldehyde DH (ADH) and bifunctional aldehyde-CoA/alcohol dehydrogenase (AADH) emphasizing that the probe structures and reactivities are well suited to interact with this important enzyme class (Table S1, ESI†). As the substrate of PCDH, pyrroline-5-carboxylate (Fig. S3, ESI†), is structurally related to probes **1** and **5**, the observed labeling of this enzyme by probe **1** seems to be based on similar recognition elements.

To confirm the results of MS 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 (Fig. 2c and Fig. S1c, ESI†).

To investigate the mode of binding in more detail, we incubated AADH, a so far uncharacterized DH, with probe **1** and applied the labeled enzyme to MS analysis after trypsin digestion to search for the peptide fragment and residue with the attached probe. Among the peptide fragments, one sequence occurred in which Cys 255 was covalently modified with the dihydroisoxazole scaffold (Fig. S4 and Table S2, ESI†) supporting our initial hypothesis that nucleophilic residues attach to the electrophilic 3-halo-dihydroisoxazole ring by an addition–elimination mechanism. The observed acylation of cysteine corresponds very well to the oxidation mechanism of many aldehyde dehydrogenases in which a cysteine residue attacks the aldehyde moiety in the first step of catalysis.[20]

All identified enzymes are important for the primary metabolism of bacteria and involved in multiple cellular functions such as glutamate and proline metabolism in case of PCDH[21] or alcohol metabolism for energy production in case of AADH[22] (Table S1, ESI†). Moreover, FabH, the only identified enzyme that does not belong to the dehydrogenase enzyme family, is an important acyltransferase that is crucial for bacterial fatty acid biosynthesis.[23]

All previous screens revealed that unsubstituted (probe **1**) as well as methyl (probes **2a** + **b**) and ethyl (probes **3a** + **b**) substituted bromine dihydroisoxazole probes represent a minimal probe set for an almost complete coverage of all observed biological targets. As this initial screen was designed to rapidly identify promising scaffold decorations by the application of

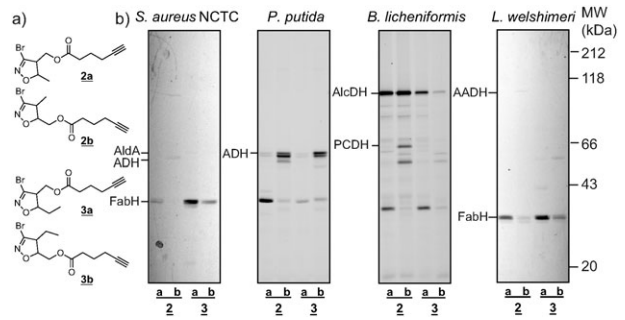


Fig. 3 Labeling studies by isolated isomers. (a) Structural composition of individual isomers that constitute a minimal probe set for an almost complete target coverage. (b) Labeling profiles of the individual isomers with several bacterial proteomes (MW = molecular weight marker).

multiplexed regioisomeric samples, we next deconvoluted this information in order to unravel the labeling preferences of the most valuable individual isomers **2(a + b)** and **3(a + b)** (Fig. 3a). Preparative HPLC was applied to separate regioisomers (racemic) which were subsequently characterized by 2D NMR (COSY, NOESY, HMQC and HMBC). In brief, probes **2a**/**3a** were assigned as methyl substituted in 5 position (ESI†) and *vice versa* probe **2b**/**3b** are methyl substituted in 4 position. All probes were *cis* configured.

Subsequent labeling studies with bacteria under *in situ* conditions revealed indeed a remarkable selectivity of individual probes for certain enzyme targets depending on the ring substitution pattern (Fig. 3b). For instance, in *S. aureus* probe **2a** with the methyl group in 5 position labels the FabH enzyme but once the methyl group exchanges with the hexynoic ester group (probe **2b**) ADH is labeled instead. A similar pattern can be obtained in *P. putida* as well as in *L. welshimeri* where FabH is predominantly labeled by **2a** and **3a** but AADH exclusively by **2b**.

This unique selectivity emphasizes the great utility of the dihydroisoxazole scaffold as a tool for proteome profiling experiments in order to identify biologically relevant targets and study their activity and regulation in the context of complex proteomic mixtures.

Since we were able to confirm the covalent binding mode of probe **1** with AADH we continued to investigate the capability of this probe to inhibit enzyme activity. AADH showed activity in a substrate turnover assay with acetaldehyde and NAD^+ in which enzyme catalyzed NAD^+ reduction to $NADH/H^+$ could be followed *via* UV-vis at 340 nm. Upon addition of the best AADH labeling probe **1**, the enzyme was effectively inhibited with an IC_{50} of 11 μM (Fig. S5, ESI†). All other probes, which exhibited less or little labeling of AADH such as probes **3** and **4**, also showed only weak inhibition properties with IC_{50}s > 100 μM (Fig. S6, ESI†).

The good cell permeability, active site labeling and inhibition of several enzymes raised the question if the compounds exhibit any antibacterial effect. Although the targeted enzymes are involved in important cellular processes we were not able to detect any antibacterial activity of probes **1**, **2**, **3**, **5** and **6** up to a concentration of 500 μM in *S. aureus*, *Listeria welshimeri*, *Bacillus licheniformis* and *Pseudomonas putida*. Despite the structural similarity of probe **1** with acivicin, which was reported to be an irreversible inhibitor of γ-GT,[10] no labeling of the purified recombinant enzyme could be observed by probe **1** at concentrations at which recombinant AADH and PCDH showed strong signals (Fig. S7 and S8, ESI†). This emphasizes that small variations at the dihydroisoxazole scaffold may significantly alter target preferences.

In conclusion we designed and synthesized a library of natural product inspired 3-chloro- and 3-bromodihydroisoxazole probes for bacterial proteome analysis and obtained a unique preference of the probes to label and inhibit members of the DH enzyme family *via* an addition–elimination mechanism on a nucleophilic cysteine residue. The probe selectivity for a certain enzyme target is remarkable as small structural changes *e.g.* methyl *vs.* ethyl substitution already discriminate binding. Although the probes did not reveal antibiotic effects, their application for the functional analysis and annotation of uncharacterized DH enzymes is a useful addition to the proteomic tool box and enlarges our knowledge on how nature utilizes electrophilic scaffolds for directed protein inhibition.

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