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Identification of a Selective, Activity-Based Probe for Glyceraldehyde 3-Phosphate Dehydrogenases**

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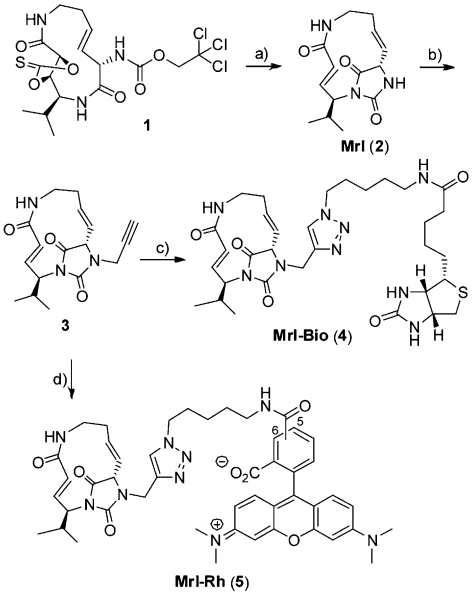
Bioreactive small molecules are powerful tools for biological research.[1] They have been used, for example, to profile chemically reactive sites in proteins, thereby allowing the identification of posttranslational modifications on a proteome-wide scale.[2] In activity-based protein profiling (ABPP), however, bioreactive small molecules known as activity-based probes (ABPs) are employed to covalently modify the active site of active enzymes. ABPP, therefore, can be used to measure enzyme activity states under various physiological conditions and in different living systems.[3] Despite the continuous development of novel probe types for distinct enzyme classes,[4] the feasibility of this approach is limited by the availability of suitable probes. This aspect is of particular importance for ABPP of "difficult" proteomes such as those found in plants. These proteomes are characterized by the occurrence of a major protein species (i.e. RuBisCO which accounts for more than 80% of the protein content in plant leaves) that far outweighs the low levels of other cellular proteins. Also, proteins in these proteomes are often post-translationally modified, leading to highly heterogenous protein mixtures.

In order to identify suitable selective chemical probes, irreversible mechanism-based enzyme inhibitors are often used as starting structures.[3a,4a,c] A complementary approach is the screening of chemically reactive compounds. This unbiased approach allows, for example, the identification of probes for enzyme classes for which no covalently acting mechanism-based inhibitors have yet been described.[5] It suffers, however, from the often low selectivity of bioreactive compounds in chemical libraries, resulting in non-activity-dependent nonspecific labeling. Finally, bioreactive probes based on natural product derived scaffolds have also been shown to be suitable starting points for the development of activity-based probes.[1,6]

During our studies on the synthesis of syringolins,[7] we observed an unusual side reaction that led to a bicyclic hydantoin derivative with a potentially reactive α,β-unsaturated Michael system (**Mrl**, compound **2** in Scheme 1). Hydantoins are often integral substructures in bioactive



Scheme 1. Synthesis of the hydantoin-based probes **Mrl-Bio** (**4**) and **Mrl-Rh** (**5**). a) $P(OMe)_3$, 150°C, 2 h, 47%; b) propargyl bromide (6 equiv), K_2CO_3 (5 equiv), acetone, reflux, 10 h, 82%; c) biotin-N_3 (1.1 equiv), TBTA (0.1 equiv), ascorbic acid (0.08 equiv), $NaHCO_3$ (0.08 equiv), $CuSO_4$ (0.02 equiv), *t*BuOH/H_2O 2:1, RT, 10 h, 95%; d) Rh-N_3 (1.1 equiv), TBTA (0.1 equiv), ascorbic acid (0.08 equiv), $NaHCO_3$ (0.08 equiv), $CuSO_4$ (0.02 equiv), *t*BuOH/H_2O 2:1, RT, 10 h, 75%. TBTA = tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine.

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compounds and have therefore been recognized as privileged structures for compound library synthesis.[8] Impressed by its molecular structure and driven by our interest in developing novel small-molecule probes for plant biology research, we tested whether this hydantoin can be used as a bioreactive probe.

In order to determine whether the hydantoin **Mrl** (**2**) labels any enzyme in the plant proteome, we first devised a synthesis for a suitably tagged derivative (Scheme 1). We decided to incorporate a terminal alkyne moiety because it can be easily modified by click chemistry with various reporter groups such as biotin and rhodamine.[9] Before synthesizing the probe, we optimized the yield of the hydantoin formation by increasing the reaction temperature of the Corey–Winter elimination to 150 °C. The terminal alkyne group was attached by reaction of compound **2** with propargyl bromide and potassium carbonate, resulting in the desired click probe **3** in 82 % yield. To obtain probes that could be directly used for target identification studies, **3** was subsequently converted with biotin-azide or rhodamine-azide (for their synthesis, see the Supporting Information) into the tagged probes **Mrl-Bio** and **Mrl-Rh** with 95 % and 75 % yield, respectively.

The bioreactivity of the two probes was evaluated by labeling experiments with Arabidopsis cell culture extracts. First, the labeling pattern of **Mrl-Rh** was determined at different pH values (Figure 1 A). The probe showed little reactivity towards plant proteins at low pH values. At pH values higher than pH 7.5, however, a single 40 kDa signal was visible. The maximum intensity for this signal was reached at pH 9.

A further experiment carried out with a heat-treated cell culture extract corroborated that the observed **Mrl-Rh** labeling requires a functional enzyme (Figure 1 B, lane 1). In order to characterize **Mrl-Rh** labeling, we performed a reaction in the absence of DTT, a reducing agent often used to activate enzymes with a cysteine residue in the catalytic pocket, and we noticed a much weaker labeling efficiency (Figure 1 B, lane 6). In addition, preincubation with a protease inhibitor cocktail (PIC) almost completely abolished labeling (Figure 1 B, lane 5), indicating that a component of this mixture reacts with **Mrl-Rh**'s target protein. In contrast, labeling experiments in the presence of E-64 (a broad-band PLCP inhibitor) did not have any effect on signal intensity (Figure 1 B, lane 2), which excludes the possibility that the observed 40 kDa band corresponds to a papain-like cysteine protease (PLCP).[10]

Competition of labeling with a 20-fold excess of **Mrl-Bio** (Figure 1 B, lane 4) or **Mrl** (**2**) (see Figure S1 A in the Supporting Information) did not completely abolish labeling with the probe **Mrl-Rh**. Despite this unexpected result, we noted that the intensity of the 40 kDa signal continuously increased in a dose-dependent manner (up to 250 μM of **Mrl-Rh**, see Figure S1 B in the Supporting Information) with background signals arising only at concentrations of more than 200 μM. These experiments indicate that the target of **Mrl** is either highly abundant or that labeling with the probe is a quite slow reaction. We also tested the labeling pattern of **Mrl-Bio** and found it to be similar to that of **Mrl-Rh**. A single biotinylated band was detected on Western blots in the 40 kDa region which is accompanied by a weaker band at higher **Mrl-Bio** concentrations (Figure S2 A in the Supporting Information).

After these initial experiments, we next aimed to identify the direct target of **Mrl-Bio** by using affinity purification on avidin beads. The concentrated target proteins were then separated by SDS PAGE and subjected to in-gel digestion (Figure S2 B in the Supporting Information).[11] The resulting peptides were analyzed by LC-MS/MS and the obtained spectra were analyzed with MASCOT and an Arabidopsis database. Identified proteins were accepted as **Mrl** targets if at least two unique peptides were found in each independent probe-treated sample and if the number of identified peptides (spectral counts) surpassed a value of 20 peptides. The analysis revealed two highly homologous glyceraldehyde 3-phosphate dehydrogenases (GAPDHs), GAPC-1 (At3g04120) and GAPC-2 (At1g13440), as the major targets of **Mrl-Bio** in the 40 kDa region. For both proteins, unique peptides were identified in addition to a large number of shared peptides (Figure S3 in the Supporting Information).

Besides GAPC-1/2, the Arabidopsis genome also encodes two other phosphorylating glycolytic GAPDHs, GAPCp-1 (AT1G79530) and GAPCp-2 (AT1G16300) (Figure S4 in the Supporting Information), localized in plastids, as well as three nonphosphorylating GAPDHs that participate in the photosynthetic reductive carbon cycle, GAPA1, GAPA2, and GAPB.[12] The GAPCps evolved from cytosolic GAPCs by gene duplication and their catalytic mechanism

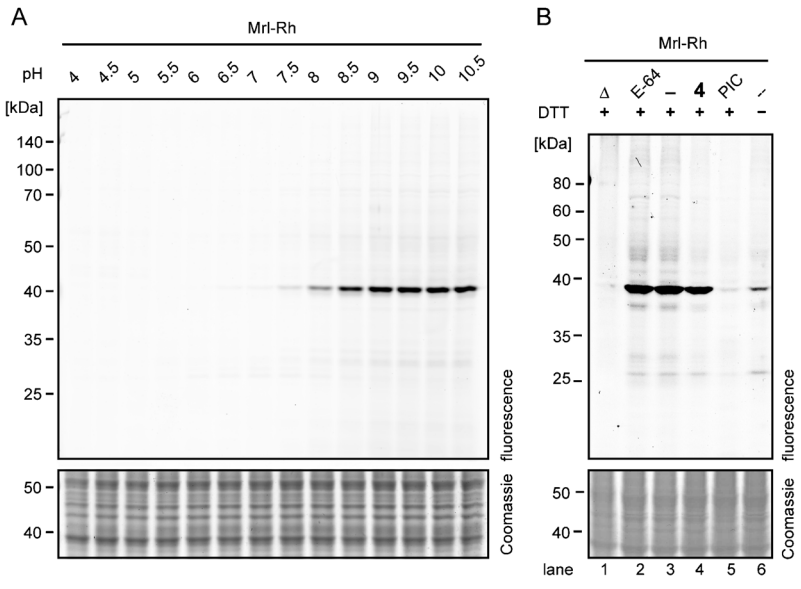

Figure 1. Labeling of Arabidopsis cell culture extracts with **Mrl-Rh** is pH and activity dependent. A) The labeling pattern of **Mrl-Rh** is pH dependent and maximal at pH 9.0. B) Labeling with **Mrl-Rh** requires a functional enzyme. See text for details.

is identical to that of cytosolic GAPCs.[13] Interestingly, we did not find any significant peptides for GAPCp-1/2 or the nonphosphorylating GAPDHs in our MS data. We cannot, however, exclude that GAPCp-1/2 are also targets of **Mrls** at high probe concentrations. In **Mrl-Bio**-treated extracts, some weakly labeled, additional signals are visible that might correspond to GAPCp-1/2 (Figure S2A in the Supporting Information).

We next confirmed that GAPC-1/2 are the targets of **Mrl-Rh** by labeling crude *E. coli* extracts containing heterologously overexpressed GAPC-1/2.[14] As expected, labeling with **Mrl-Rh** resulted in both cases in a single fluorescent band at 40 kDa. Interestingly, we did not observe labeling of bacterial GAPDH under these conditions. In order to assess the GAPDH detection limit, we next added increasing concentrations of purified, recombinant AtGapC-2 to *E. coli* proteome (Figure S5A,C in the Supporting Information), revealing a detection limit of approximately 20 ng μL^{-1} under these conditions. However, the labeling efficiency for the recombinant AtGapC-2 is quite low in the *E. coli* proteome, indicating a potentially lower detection limit of GAPCs in Arabidopsis extracts. Labeling experiments carried out with Arabidopsis Col0 wildtype extracts and extracts obtained from a *gapC-1* knock-out line (Figure 2B) corroborated the overexpression results.[15] We observed that the signal intensities in the 40 kDa region were reduced by 25% when compared to the signals of the wildtype Arabidopsis extracts. The partial activity reduction is probably a result of a feedback overexpression of GapC-2 to counteract loss of GapC-1 activity.[15]

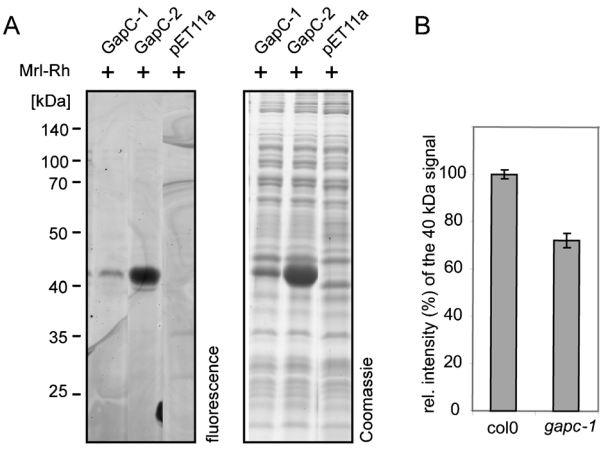

Figure 2. The targets of **Mrl** in Arabidopsis plant leaf cultures are the two highly homologous enzymes GapC-1 and GapC-2. A) Overexpression of GapC-1 and GapC-2 in *E. coli* and subsequent labeling of crude extracts with **Mrl-Rh**. B) The intensity of the 40 kDa signal is reduced in the *gapc-1* mutant by 25% upon labeling with probe **Mrl-Rh**. The error bars indicate the standard error of the mean of six independent quantifications.

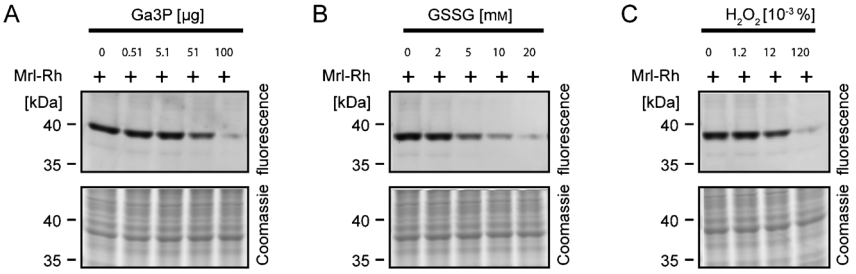

Figure 3. **Mrl-Rh** labeling of GapC-1/2 is inhibited by Ga3P and the oxidizing agents GSSG and H_2O_2. A) Increasing concentrations of DL-glyceraldehyde 3-phosphate (Ga3P) inhibit labeling of GapC-1/2 with **Mrl-Rh**. B) and C) Increasing concentrations of the oxidizing agents GSSG (B) and H_2O_2 (C) prevent labeling of GapC-1/2 with **Mrl-Rh**.

We also labeled cell culture extracts in the presence of the natural substrate Ga3P (Figure 3A), the mildly oxidizing reagent glutathione disulfide (GSSG, Figure 3B), and the harsh oxidizing agent H_2O_2 (Figure 3C). All three treatments resulted in a concentration-dependent decrease of GAPDH labeling, concurrent with previously published data that reported a decline in GAPDH activity upon these treatments and indicating activity-dependent labeling.[14]

Finally, we tested whether **Mrl-Rh** also labels mammalian GAPDHs (Figure S5B in the Supporting Information). To this end, commercially available human GAPDH was added to *E. coli* proteome. A subsequent profiling experiment revealed labeling of human GAPDH, however with lower efficiency than that observed for plant GAPDHs. Nevertheless, this experiment suggests a complementary use for **Mrl**-based probes outside plant research.

GAPDHs play a key role during glycolysis and are essential for the energy balance and carbon supply in all organisms. They catalyze the conversion of glyceraldehyde 3-phosphate (Ga3P) to D-glycerate 1,3-bisphosphate, whereby NAD^+ is reduced to $NADH/H^+$. Besides its well-established role in primary metabolism, recent studies in mammalian organisms demonstrated that GAPDHs are also implicated in several additional nonglycolytic functions, turning this long-known enzyme into a key factor for diverse biological processes.[16] GAPDHs have furthermore been found as the targets of several biologically active natural products, among them the anticancer agent saframycin and the insulin mimic demethylasterriquinone B1.[17]

So far, the function of GAPDHs in plants is much less clear and has been studied mostly by genetic approaches because of the lack of biochemical and cell biological approaches. However, genetic studies alone cannot deliver insights into their cellular function. We anticipate that the small-molecule probe **Mrl** and its fluorophore- (**Mrl-Rh**) or biotin-tagged (**Mrl-Bio**) derivatives will be useful tools to investigate GAPDH function. Their predominant labeling of the cytosolic GAPDHs GAPC-1/2 in *Arabidopsis thaliana* cell culture allows efficient enzyme activity state monitoring. The **Mrl** probes represent, therefore, the first fully characterized chemical tools for this important enzyme class. The probes might be particularly useful for studying the role of GAPDHs during plant pathogen infections. On the one hand, it has been long known that plants respond to pathogen

infection with transcriptional upregulation of GAPDH expression.[18] On the other hand, pathogen infections trigger oxidative defense mechanisms that result in inhibition of plant GAPDH activity.[19] Furthermore, some plant pathogens like the fungus *Trichoderma koningii* secrete endogenous GAPDH inhibitors such as koningic acid (heptelic acid), which also interferes with GAPDH activity.[20] Thus, to assess the biological role of GAPDH in pathogenesis, information on the overall enzyme activity levels is required.[21] In order to demonstrate the value of **Mrl**-based probes, we performed a profiling experiment on an established Bortrytis-plant infection model (Figure S6 in the Supporting Information).[11] These studies revealed differential GAPDH activities in pathogen-susceptible and nonsusceptible plant species and underline the utility of **Mrl** as a useful tool to study, for the first time, the role of GAPC-1/2 during plant-pathogen interactions.

Taken together, we have reported the first selective, activity-based probes for investigating GAPDH functions. These probes might represent powerful chemical tools to determine GAPDH activities in diverse biological processes, including plant-pathogen interactions.

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