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Photoaffinity SAM analogues for the identification of SAM-binding proteins†

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S-Adenosylmethionine (SAM) serves as an important substrate in a variety of biochemical reactions, and it is important to identify unknown SAM-binding proteins to fully understand the biological functions of SAM. Previous studies on SAM-binding proteins used S-Adenosylhomocysteine (SAH)-analogues, which mainly identified SAM dependent methyltransferases. Here, we developed and validated three SAM photoaffinity probes to label and enrich SAM-binding proteins. These probes efficiently labeled the known SAM-binding protein Dph2 involved in diphthamide biosynthesis from cell lysates. Using these probes, we enriched SAM-binding proteins from the cell lysates of *Burkholderia gladioli* and *Saccharomyces cerevisiae*. In addition, we validated five SAM binders and revealed the SAM cleavage activities of three of them, including the radical SAM enzyme ArsL, which cleaves SAM to generate methylthioadenosine (MTA), and AcnA and EDD84_07545, which generate S-adenosyl-L-homocysteine (SAH). Therefore, our SAM-based photoaffinity probes are promising tools for the identification of SAM-binding proteins.

Introduction

S-Adenosylmethionine (SAM), a sulfonium-containing compound formed by the condensation of ATP and methionine, is widely involved in various biochemical reactions in living organisms. SAM is one of the most frequently used substrates in enzymatic reactions. As SAM has three labile C–S bonds, its functions are mostly accompanied by the cleavage of these C–S bonds. SAM is best known as the methyl donor of SAM-dependent methyltransferase (MTase), which transfers a methyl group from SAM to various substrates, such as DNA, RNA, proteins and small metabolites.^{1–3} SAM can also be cleaved by radical SAM enzymes to generate a 5'-deoxyadenosyl radical (5'-da[•]) and subsequently catalyze numerous important reactions.^{4–6} Additionally, the 3-amino-3-carboxypropyl (ACP) group of SAM is involved in many biochemical reactions.^{7–13} In addition to SAM cleavage reactions, SAM has been found to be a prosthetic group without any covalent changes in many enzymes, such as LepI¹⁴ and SpnF¹⁵ for enzymatic cycloaddition reactions. Therefore, the discovery of SAM-binding proteins is highly important for the study of the unknown biological functions of SAM. It is difficult to predict SAM-binding proteins on the basis of sequence and structural specificity. Methyltransferases generally have a Rossmann-fold structure,^{16–18}

which can also bind to other nucleoside compounds, such as NAD^{19,20} and FAD.^{21,22} Furthermore, many SAM-dependent enzymes do not exhibit sequence features of methyltransferases.²³ Although radical SAM enzymes can be predicted by the CXXXCXXC sequence, proteins such as Dph2 do not contain such a sequence.⁸ Therefore, identifying new SAM-binding proteins with unusual functions is difficult.

In recent years, molecular probes have been used as efficient tools for studying interactions between small molecules and proteins. Probes of a variety of cofactors, such as ATP,^{24,25} 2',3'-cGAMP²⁶ and LCFA-CoA,²⁷ have been used for the identification of unknown binding proteins. However, the development of small molecule probes for the study of SAM-binding proteins has focused mainly on radioisotope labeling of methyltransferases, such as [methyl-³H] AdoMe,^{28–31} [³⁵S]AdoMet,²⁹ 8-N₃AdoMet³² and 8-N₃Ado[⁷⁵Se]SeMet.³³ Photoaffinity probes of S-adenosyl-L-homocysteine (SAH) analogues were previously developed for identifying SAM-binding proteins. These SAH-based probes displayed remarkable specificity for SAM-dependent MTs. Among the comprehensive probe-identified SAM-associated proteins, nearly all of them were MTs, along with a small number of MT-associated proteins and proteins previously shown to bind SAH, such as 5'-methylthioadenosine (MTA)/SAH nucleosidase (MtnN).^{34–36} This is not very surprising as SAH is a product of MT-catalyzed reactions and binds with high affinity to many MTs. However, other SAM-related enzymes, such as ACP transferases and radical SAM enzymes, have not been identified. This highlights the limitations of these SAH photoaffinity probes. SAM probes with the characteristic sulfonium moiety, which could be very important for binding proteins, are highly desirable for the comprehensive identification of SAM-binding proteins.

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It is very important to determine the unbiased SAM interactome to clarify the new biological functions of SAM. Here, we synthesized and developed three SAM-based photoaffinity probes that are capable of labeling and identifying unknown SAM-binding proteins.

Results and discussion

Design and synthesis of SAM photoaffinity probes

We designed photoaffinity probes with a photocrosslinkable diazirine group and a clickable terminal alkyne group on SAM (probes 1–3 in Fig. 1A). As the position of the functional group attached to cofactors interferes with the interaction profiles,³⁷ we functionalized SAM at different positions, such as the N6 position of adenine and the carboxyl and amino groups of methionine. The corresponding *S*-adenosyl-*L*-homocysteine (SAH) moieties of these probes were easily synthesized, as described in the supplemental material. The SAH analogues were subsequently reacted with methyl iodide to produce probes 1–3. The selection of a small diazirine and a terminal alkyne modified as a clickable photocrosslinking group to bind with potential SAM-binding proteins would reduce the effects of structural changes and presumably retain the same biological activity as SAM. Once the target proteins bind with these probes, they can be covalently crosslinked to the probes upon UV-irradiation (365 nm). The

binding proteins could be subsequently labeled with a fluorescence tag (Rh-N₃) for visualization *via* in-gel fluorescence imaging or enriched with an affinity tag (biotin-N₃) for target protein identification *via* click chemistry (Fig. 1B).

Labeling of the known SAM-binding protein *PhDph2*

To test whether the probes we developed could crosslink known SAM-binding proteins, we initially applied them to *Pyrococcus horikoshii* Dph2 (*PhDph2*), a known SAM-binding protein involved in diphthamide biosynthesis.³⁸ Each probe was incubated with the purified *PhDph2* protein for 1 h and subjected to UV-irradiation (at 365 nm). Conjugation with Rh-N₃ was performed *via* click chemistry, and the samples were then resolved *via* SDS-PAGE and visualized *via* fluorescence scanning. The labeling efficiency was indicated by the intensity of the fluorescence signal. As shown in Fig. 2, probes 1–3 all efficiently labeled the purified *PhDph2*. This labeling was dependent on UV-irradiation, and was also dose-dependent on the probes (Fig. 2B). Samples without UV-irradiation or probes were not labeled. Among these probes, probe 2 had the most significant labeling effect (Fig. 2A), which suggested better binding to *PhDph2*. When SAM was used as a competitor in the labeling experiment, the fluorescence intensity of the labeled protein decreased significantly for all three probes, indicating the specificity of the labeling (Fig. 2C and D). Therefore, the significant

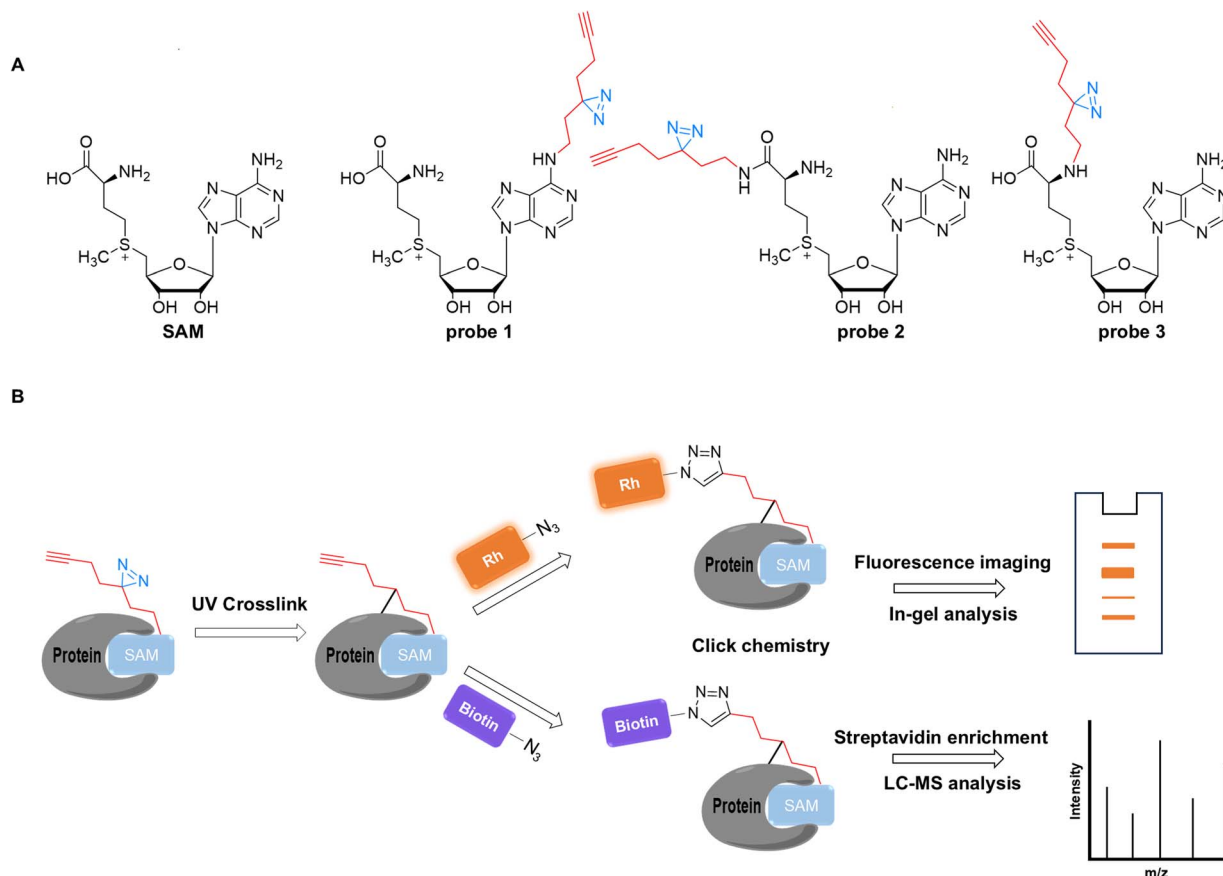


Fig. 1 (A) Structures of SAM and the photoaffinity probes. (B) Workflow of photoaffinity labeling and enrichment of SAM-binding proteins with the probes.



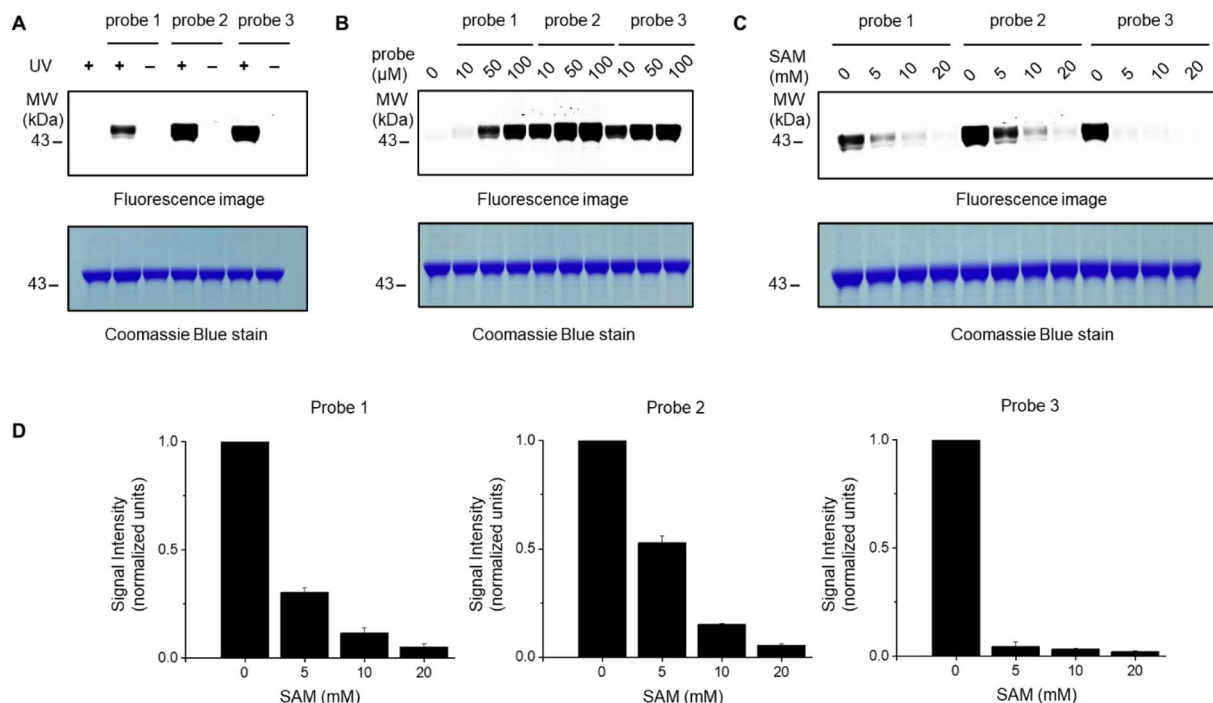


Fig. 2 (A) In-gel fluorescence analysis of *PhDph2* treated with probes 1–3. *PhDph2* (10 μM) was incubated with 100 μM probes 1–3 under UV light irradiation (at 365 nm) before click chemistry. (B) Fluorescence in-gel analysis of *PhDph2* with increased concentrations of probes 1–3. *PhDph2* (10 μM) was incubated with increasing concentrations (0, 10, 50, 100 μM) of probes 1–3 then applied to UV light irradiation (365 nm) and click chemistry. (C) Different concentrations of SAM were used as the competitors. *PhDph2* (10 μM) was pre-incubated with increasing concentrations of SAM (0, 5, 10, or 20 mM) and then incubated with 100 μM of probes 1–3. (D) Fluorescence intensity of SAM competition with probes 1–3 in C. The fluorescence intensity was normalized to the value of the no competition band (SAM at 0 mM).

specific labeling of *PhDph2* confirms the feasibility of these probes. Since the three probes showed significant labeling efficiency, we used all three probes for subsequent verification.

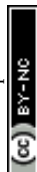
We then applied these probes to the cell lysates of *E. coli* overexpressing *PhDph2*. The results revealed that different probes in the lysate had similar labeling patterns but with different intensities (Fig. 3A). In particular, there was a clear protein band near 44 kDa labeled with probes 1–3. The labeling depended on UV-irradiation and was competed by SAM. The labeling intensity increased with increasing concentrations of the probes (Fig. 3B). We extracted the 44 kDa protein from the gel and identified it as *PhDph2* by mass spectrometry (MS) (Table S2†). Thus, we successfully demonstrated the feasibility of these probes for labeling SAM-binding proteins from cell lysates.

Labeling of SAM-binding proteins from cell lysates

After successful labeling of the overexpressed protein in the cell lysate of *E. coli* with probes 1–3, we attempted to identify unknown SAM-binding proteins in different cell lysates. We applied these probes to two different cell lysates: *Saccharomyces cerevisiae* (yeast) and *Burkholderia gladioli*. The experimental results showed that the same probe in different cell lysates had different labeling patterns and intensities, which means that the potential SAM-binding proteins vary in different cells. With the same cell lysates, different probes exhibited diverse labeling patterns and intensities, which indicated that modifications at

different sites of SAM may affect the interaction between probes and target proteins (Fig. 4A and B). In both cell lysates, UV-irradiation caused significant enrichment of different protein bands. These proteins should all be SAM-binding proteins, as the labeling almost completely disappeared when SAM was used as a competitor. Because probe 2 had the greatest enrichment in both cell lysates, we first selected probe 2 for subsequent pulldown experiments.

Pulldown experiments in *B. gladioli* lysate using probe 2 were performed *via* UV-irradiation and then conjugation with biotin. Avidin-based enrichment was performed with streptavidin beads. The pulldown proteins were then digested with trypsin, and analyzed *via* MS. In particular, MS analysis revealed 52 and 45 specific SAM interactors respectively in the cell lysate of *B. gladioli* with probe 2 compared with the SAM competition experiment and the control experiment (Fig. 5A and B). MS analysis revealed 28 specific SAM interactors in the cell lysate of *B. gladioli* with probe 2 from three replicates (Fig. 5C). To identify more specific proteins, we also analyzed protein enrichment with probe 1 and probe 3. Nine proteins overlapped among the three probes (Fig. 5D). According to UniProt and NCBI database analyses, 8 of these proteins were predicted to potentially recognize SAM or some structural elements of SAM, such as sugar, purine and amino acid. These proteins included 2 SAM, 2 NADH/NADPH, 3 ATP and 1 RNA binding proteins (data S1). In addition, 1 protein was not predicted to be SAM binders or SAM-related binders. For the cell lysate of *S. cerevisiae*, 89 specific proteins were identified



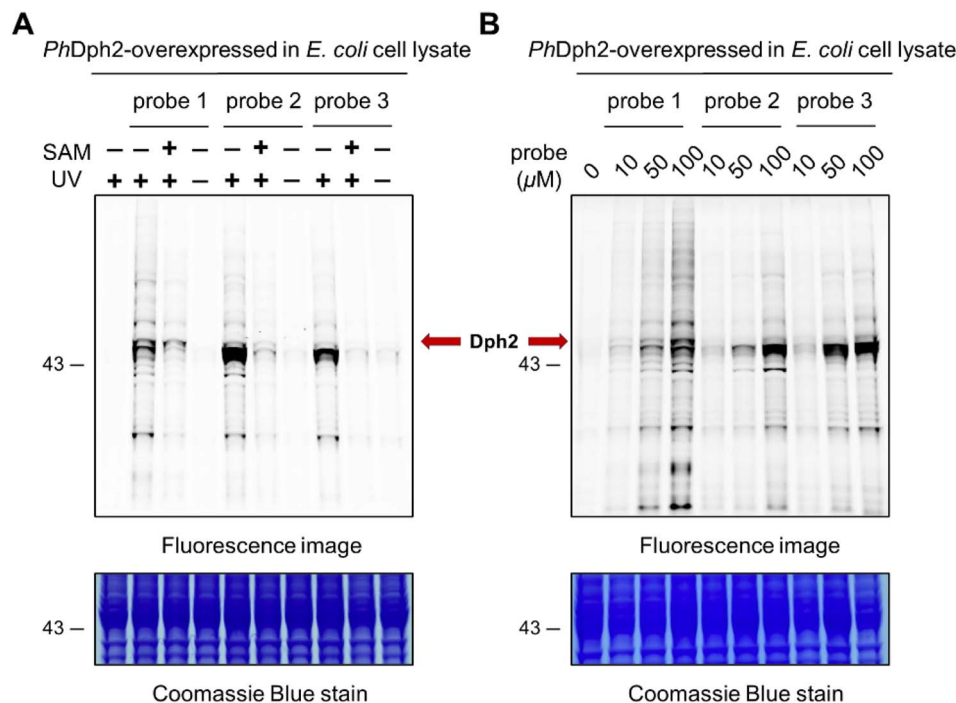


Fig. 3 (A) In-gel fluorescence analysis of *E. coli* cell lysate overexpressing *PhDph2* treated with probes 1–3. *E. coli* cell lysate was incubated with 100 μM probes 1–3 under UV light irradiation (at 365 nm) before click chemistry. SAM was used as the competitor. (B) Increasing concentrations of probes 1–3. *E. coli* cell lysate was incubated with increasing concentrations of probes 1–3 (0, 10, 50, 100 μM) then applied to UV light irradiation (365 nm) and click chemistry.

by probe 2 (Fig. S7A†). Fifty proteins overlapped among the three probes (Fig. S7B†). We filtered proteins according to the Contaminant Repository for Affinity Purification (CRAPome)³⁹

and 32 high-confidence proteins remained. Based on Gene Ontology and UniProt analyses, 21 of these proteins were annotated as potentially recognizing SAM or some structural elements

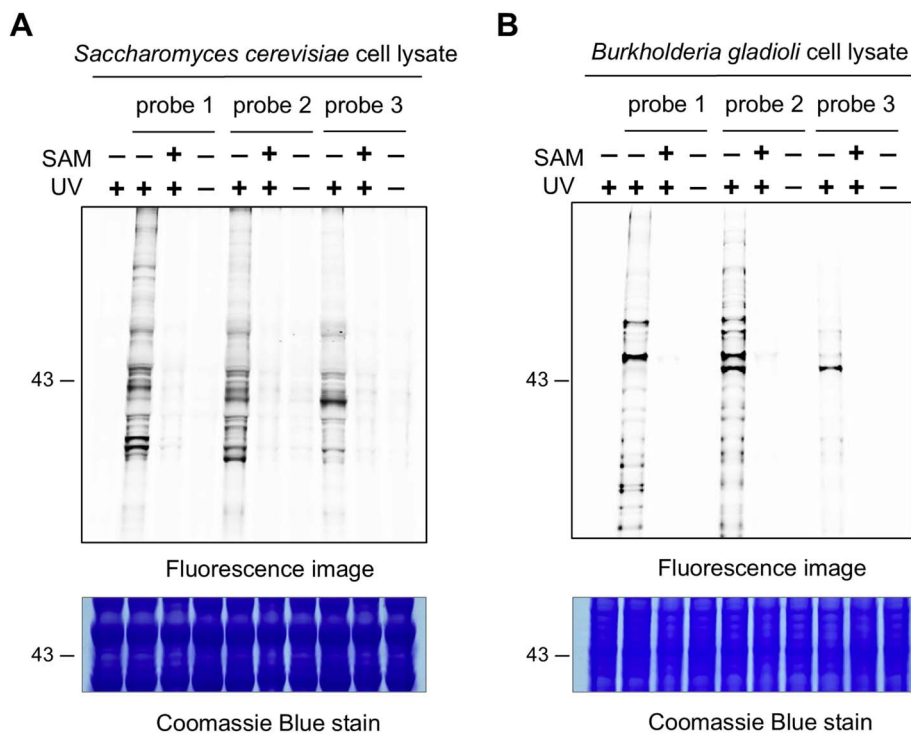
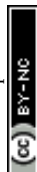


Fig. 4 In-gel fluorescence analysis of *Saccharomyces cerevisiae* (A) and *Burkholderia gladioli* (B) cell lysates treated with probes 1–3. The cell lysates were incubated with 100 μM probes 1–3 under UV light irradiation (at 365 nm) before click chemistry. SAM was used as the competitor.



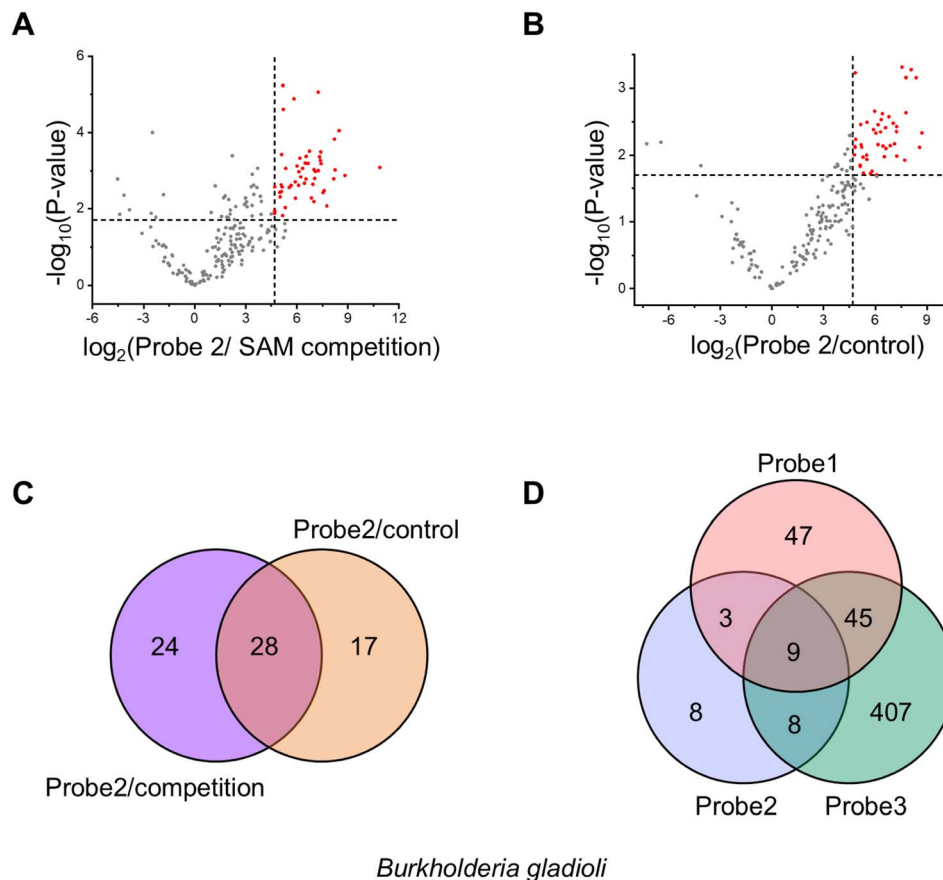


Fig. 5 (A) Volcano plots showing the quantification results of enriched proteins with 100 μM probe 2 compared with the 10 mM (SAM) of *B. gladioli*. The ratios of $\log_2(\text{FC}) > 4.7$ and $p\text{-value} < 0.02$ were considered high-confidence proteins in all three independent replicates. (B) Volcano plots showing the quantification results of enriched proteins with 100 μM probe 2 compared with the control (no probe) of *B. gladioli*. The ratios of $\log_2(\text{FC}) > 4.7$ and $p\text{-value} < 0.02$ were considered high-confidence proteins in all three independent replicates. (C) Venn diagram showing the overlap high-confidence proteins labeled with probe 2. (D) Venn diagram showing the overlap between the sets of experiments for proteins in *B. gladioli* labeled with probes 1–3.

of SAM, such as sugar, purine and amino acid. These proteins included 2 SAM (Dph1-2), 1 dcSAM (SPE3), 1 homocysteine, 7 NADH/NADPH, 8 ATP and 2 DNA/RNA binding proteins (data S1). In addition, 11 proteins were unannotated as SAM binders or SAM-related binders in the cell lysate of *S. cerevisiae*.

Select candidate validation

To further validate specific proteins enriched by these probes, we selected one that was predicted as a SAM-binding protein (ArsL), two that were predicted as SAM-related binders (ORFs EDD84_07545 and MET6) and two that were not annotated as SAM or SAM-related binders (AcnA and TPI1) for validation. BSA was included as a control protein that has no interaction with SAM. ArsL, EDD84_07545 and AcnA are from *B. gladioli*, and MET6 and TPI1 are from yeast. ArsL was proved to be an arsinothricin biosynthetic radical SAM protein.⁴⁰ The protein whose gene ORF was named EDD84_07545 was predicted to be a ribonucleoside-diphosphate reductase protein from homology and to catalyze the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides.⁴¹ AcnA was predicted to be an aconitate hydratase from homology and to catalyze the isomerization of citrate to isocitrate *via cis*-aconitate.⁴² MET6 belongs to the vitamin-B12

independent methionine synthase family and catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine, resulting in methionine formation.⁴³ TPI1 belongs to the triosephosphate isomerase family and catalyzes the inter-conversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate.⁴⁴ TPI1 is involved in carbohydrate biosynthesis, gluconeogenesis and glycolysis.⁴⁵ These proteins were expressed and purified *via* an *E. coli* expression system. We subsequently employed the same labeling strategy for each protein with probe 2. The results showed that probe 2 had significant labeling efficiency for each protein, and its labeling depended on UV-irradiation and competed with SAM (Fig. 6). The control protein BSA did not show any labeling with probe 2. Therefore, these five proteins were identified as SAM-binding proteins. However, the apo form of AcnA functions as an RNA-binding regulatory protein.⁴⁶ EDD84_07545 binds ATP, which serves as an allosteric activator.⁴⁷ Met6 binds the product methionine.⁴⁸ TPI1 physically interacts with several ATP hydrolysis enzymes.⁴⁹ These suggest that the SAM binding affinity could imply some new SAM-related functions, but could also be because the binders of these proteins, RNA, ATP and methionine, share a common moiety with SAM. We further investigated whether these proteins have any SAM-cleavage activity

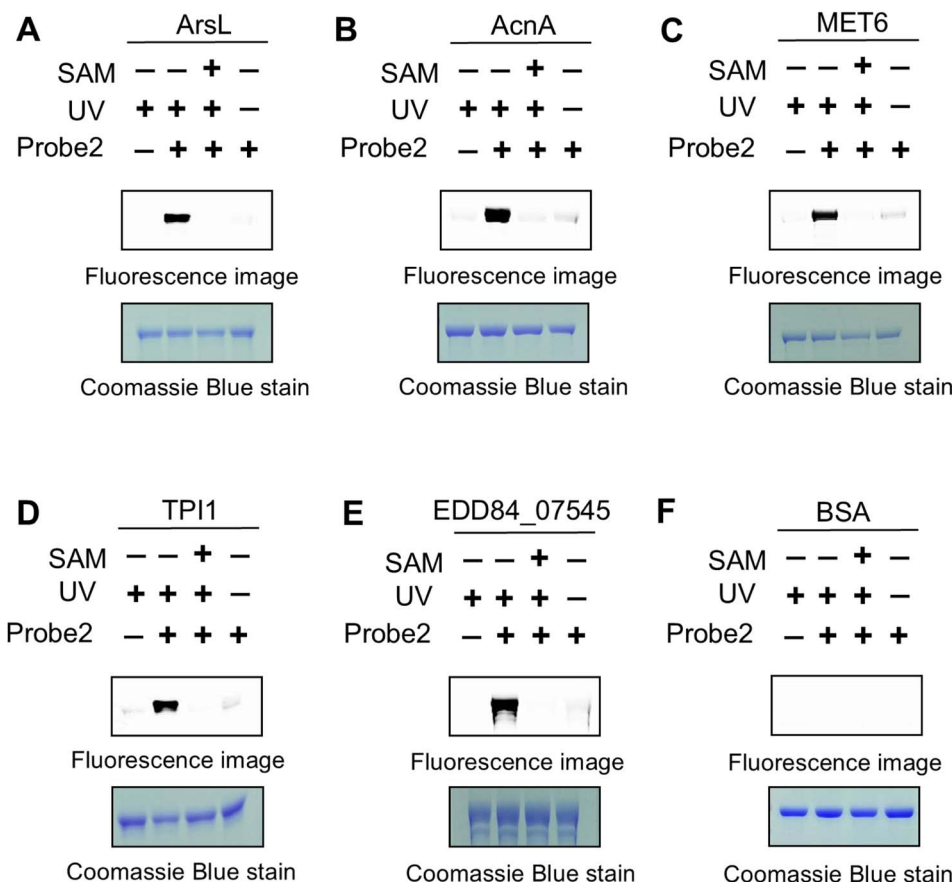


Fig. 6 Verification of five SAM-binding proteins (A–E) and BSA (F) by fluorescence in-gel analysis. Proteins (10 μ M) were incubated with 100 μ M probe 2 under UV light irradiation (at 365 nm) before click chemistry. SAM was used as the competitor.

via high-performance liquid chromatography (HPLC) and MS. Three out of these five proteins exhibited SAM cleavage activity: ArsL, a crucial enzyme in arsinthricin biosynthesis, noncanonically cleaves SAM to produce MTA, which we characterized in detail recently.⁵⁰ Interestingly, both AcnA and EDD84_07545 can cleave SAM to produce SAH in the presence of dithiothreitol. (Fig. S4–S6†), whether this activity is physiologically relevant to a new function or artifact effect caused by dithiothreitol awaits further detailed study.

The SAM analogue photoaffinity probes that we developed successfully labeled SAM-dependent methyltransferases identified by the previously reported SAH analogue probes. Our probes also successfully enriched radical SAM enzymes such as Dph2 and ArsL. Therefore, the probes that we developed have broader usage for the identification of SAM-related proteins. The way we analyzed the data from the proteins enriched by all three probes may exclude some SAM binding proteins that cannot tolerate modifications on one SAM probe. In addition, the SAM analogue probes also labeled some NAD, ATP or methionine binding proteins, whose cofactors have moieties common to SAM.

Conclusions

In summary, we successfully developed three novel SAM photoaffinity probes and validated their feasibility for covalently

labeling of SAM-binding proteins. We successfully used these SAM photoaffinity probes in labeling and identifying potential SAM-binding proteins from *B. gladioli* and yeast cell lysates. We identified five SAM-binding proteins in yeast and *B. gladioli* cell lysates via *in vitro* biochemical validation. We further demonstrated that three SAM-binding proteins from *B. gladioli* cell lysate, ArsL, AcnA and EDD84_07545, could cleave SAM to generate MTA or SAH. This study marks the first time that SAM photoaffinity probes have been developed and used in the study of SAM-related proteins. Our work highlights the ability and usefulness of SAM photoaffinity probes for the identification and validation of SAM-binding proteins other than methyltransferases that cannot be identified by the reported SAH analogue probes.

Data availability

All experimental procedures and characterization data are available in the ESI.†

Author contributions

X. W. performed all the experiments, interpreted the data and wrote the initial manuscript. M. D. supervised the project, and reviewed and edited the manuscript.



Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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