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Novel biotin linker with alkyne and amino groups for chemical labelling of a target protein of a bioactive small molecule

Tomoaki Anabuki [a], Miu Tsukahara [a], Masanori Okamoto [b], Hideyuki Matsuura [a], Kosaku Takahashi [a,*]

[a] Research Faculty of Agriculture, Hokkaido University, Kita9 Nishi9, Kita-ku, Sapporo 060-8589, Japan
[b] Center for Bioscience Research and Education, Utsunomiya University, 350 Mine-cho, Utsunomiya 321-8505, Japan

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ABSTRACT

We synthesized a novel linker (**1**) with biotin, alkyne and amino groups for the identification of target proteins using a small molecule that contains an azide group (azide probe). The alkyne in the linker bound the azide probe via an azide-alkyne Huisgen cycloaddition. A protein cross-linker effectively bound the conjugate of the linker and an azide probe with a target protein. The covalently bound complex was detected by western blotting. Linker **1** was applied to a model system using an abscisic acid receptor, RCAR/PYR/PYL (PYL). Cross-linked complexes of linker **1**, the azide probes and the target proteins were successfully visualized by western blotting. This method of target protein identification was more effective than a previously developed method that uses a second linker with biotin, alkyne, and benzophenone (linker **2**) that acts to photo-crosslink target proteins. The system developed in this study is a method for identifying the target proteins of small bioactive molecules and is different from photo-affinity labelling.

Photo-affinity probes have been applied to identify target proteins using small bioactive molecules with photophores and tags for detection.[1,2] Aryl azide, benzophenone and 3-aryldiazirine are well-known photophores that can be photo-activated for cross-linking between small bioactive molecules and target proteins via UV irradiation. Tags such as biotin or fluorescent groups are used to detect proteins that are cross-linked with photo-affinity probes.[3] Photo-affinity probes are useful for identifying proteins bound to small bioactive molecules; however, the introduction of photophores and tags into small molecules often inhibits both their affinity for the target protein and the bioactivity of the small molecule. Additionally, the cross-linking reactions between photophores and proteins were generally less than 10%.[2] Therefore, a more efficient method is required for target protein identification of small bioactive molecules.

To overcome the bulkiness of photo-affinity probes, a novel method to identify target proteins using an azide probe, which is derived from a small bioactive molecule, was previously reported.[4] The outline is depicted in Fig. 1A. This probe is synthesized by introduction of an azide group into a small bioactive molecule, which minimizes the loss of both the affinity for the target protein and the bioactivity of the original molecule. A linker containing alkyne, biotin, and a photophore group (linker **2**, Fig. 2) connects the azide probe with the target protein. The alkyne in the linker is used to bind an azide probe through the Huisgen [3+2] cycloaddition known as the click reaction.[5–7] A photophore connects a conjugate of the azide probe and a linker with a target protein through UV irradiation. Biotin functions as a tag for western blot analysis.[8,9] The linker thus performs the function of binding the azide probe to the target protein and labelling the protein for detection. This method was shown to effectively identify a target protein.[4]

To solve the insufficient photo-crosslinking reactivity that is often found with photo-affinity probes, we focus on the high reactivity of protein cross-linkers to bind two individual amino acids. The current study provides a new method for identifying target proteins using an azide probe, a biotin linker containing alkyne and amino groups (linker **1**, Fig. 2), and a protein cross-linker (Fig. 1B). Instead of photophores, the protein cross-linker is utilized to effectively connect a target protein and an azide probe conjugate of linker **1**. Therefore, the photo-crosslinking reaction is not necessary to bind the target protein and the azide probe. To demonstrate that the proposed method is feasible, linker **1** was synthesized, and the suberic acid bis(3-sulfo-*N*-hydroxysuccinimide ester) sodium salt (BS3, **3**) was used as a protein-crosslinker because its sulfate groups increase its hydrophilicity, making it suitable for aqueous reactions (Fig. 2). The method in this study was then used to detect a RCAR/PYR/PYL protein (PYL),[10] which is an abscisic acid receptor, using abscisic acid (ABA, **4**) and 3′-azide-ABA (**5**) (Fig. 2).[11] ABA is a plant hormone that regulates various plant physiological events such as stress adaptation and development.[8]

* Corresponding author.
E-mail address: kosaku@chem.agr.hokudai.ac.jp (K. Takahashi).

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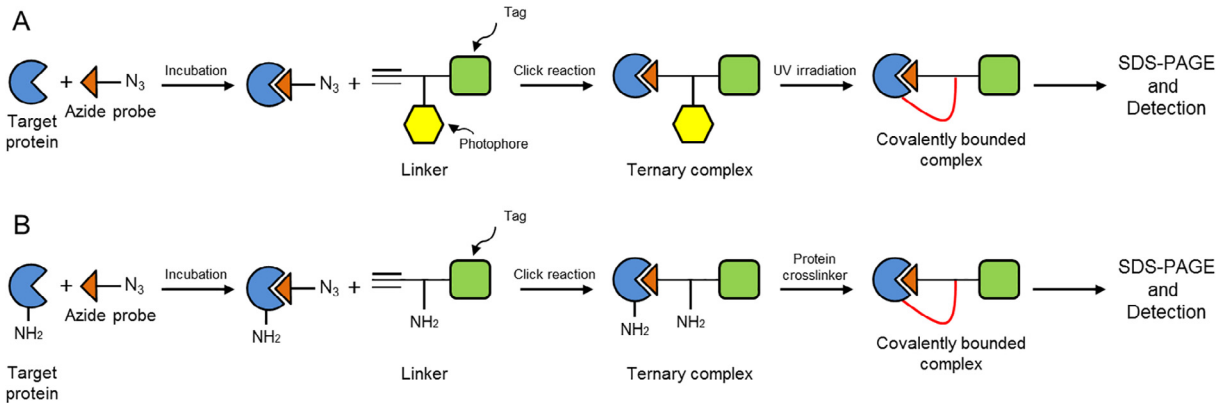

Fig. 1. Illustration of the methods used for identifying target proteins using a probe with an azide group. A: previous reported method[4]; B: the method in this study.

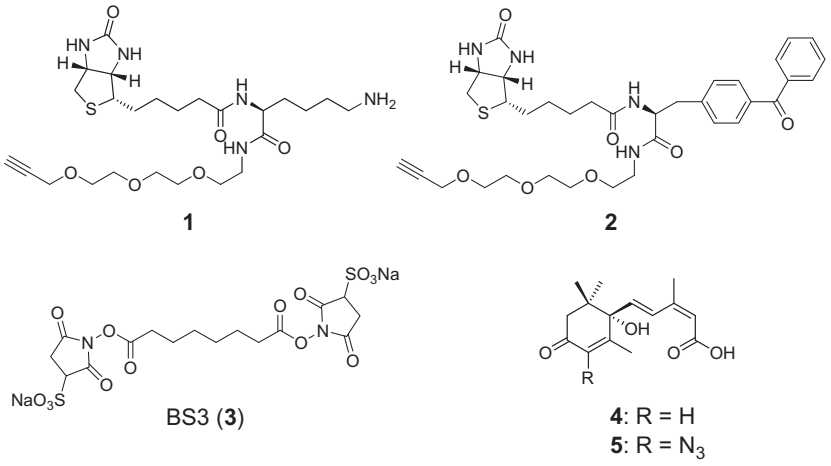

Fig. 2. Compounds used in this study.

The framework for the method proposed in this study is depicted in Fig. 1B: (1) the azide probe is incubated with the target protein; (2) the linker is added into the mixture of the azide probe and the target protein, and Huisgen cycloaddition conjugates the linker and the azide probe; (3) the addition of the protein cross-linker binds the target protein and the conjugate of the azide probe and the linker through the amino groups; (4) after SDS-PAGE analysis, the complex composed of the target protein, the azide probe and the linker is identified by western blotting. Therefore, the azide probe does not need to contain a photophore, which could prevent access to the binding site of the target protein. Instead of a photophore, a protein cross-linker plays an important role in forming a covalently bound complex that includes the azide probe, the linker, and the target protein. Thus, our proposed method aims to effectively identify target proteins.

In order to accomplish the purpose described above, linker **1** was synthesized (scheme 1). Biotin is commonly used as a tag for protein detection via western blotting. An amino alkyl group was introduced into the linker using lysine. Polyether is commonly used as a hydrophilic spacer, and an alkyne is necessary for Huisgen cycloaddition.

To determine whether our strategy can identify a protein targeted with a small molecule, an ABA receptor of *Arabidopsis thaliana*, AtPYL2, was selected as the target protein and ABA (**4**) and 3′-azide ABA (**5**) were selected as the small molecules for a model experiment (Fig. 2). ABA is a plant hormone that functions in many developmental processes and environmental stress responses in plants.[12,13] Binding of ABA to PYL proteins inhibits the activity of protein phosphatase 2C (PP2C), allowing the activation of SNF1-related protein kinase 2 (SnRK2s) and the phosphorylation of ABA-responsive element binding factors (ABFs) to activate ABA-responsive genes.[14,15] The inhibitory activity of **5** in lettuce seedling was almost the same as that of **4**.[11]

Azide probe **5** was incubated with AtPYL2, and linker **1** was then conjugated with **5** via the click reaction using Cu (I) as a catalyst. The AtPYL2 and a conjugate of **1** and **5** were cross-linked through each amino group in **1** and AtPYL2 using BS3 (**3**). The complex containing AtPYL2, **1** and **5** was analyzed by SDS-PAGE. To detect the biotinylated components, the complex was electrophoretically transferred onto a membrane surface, and the blot was successfully observed via chemiluminescence using streptavidin and horseradish peroxidase (Fig. 3A). As a result, the detection of AtPYL2 using azide probe **5** and linker **1** was successful (Fig. 3A). Additionally, no band was detected without linker **1** (Fig. 3A). To prove that **5** specifically bound AtPYL2, AtPYL2 was replaced with PpAOC2, which is a protein responsible for the biosynthesis of 12-oxo-phytodienoic acid (OPDA).[5] No signal was detected when PpAOC2 was used, showing that the binding of **5** to AtPYL2 was selective (Fig. 3A). To examine whether the intensity of the chemiluminescence signal varied with AtPYL2 concentration, the chemiluminescence signal of AtPYL2 was monitored as the AtPYL2 concentration was varied from 1.3 to 10 μM (Fig. 3B). The intensity of the chemiluminescence signal dose-dependently increased until 10 μM AtPYL2 was reached. The dependence of