Synthesis of Staudinger-type Molecular Probe for Catch-and-release Purification of the Binding Protein for Potassium Isolespedezate, a Leaf-closing Substance of Leguminous Plant

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We synthesized azide-containing photoaffinity probe **1** based on the structure of potassium isolespedezate. This probe can be used for catch-and-release-mechanism purification of binding protein for **1**: photo-crosslinking with **1** gave azide-labeled receptor which can be captured by phosphane-linked gel matrix by the Staudinger ligation. After washing the gel, the caught binding protein can be released by the reductive cleavage of disulfide bond in **1**. This process can be used as a convenient method for the purification of binding protein for bioactive natural product.

Circadian rhythmic leaf-closing and leaf-opening movements called nyctinasty are widely observed in legumes. It is well known that Charles Darwin is a pioneer in this field.[1] We identified chemical factors controlling this movement, and found that their binding proteins are involved in the control of nyctinasty.[2,3] The bioorganic studies on this binding protein needs its supply. Thus, an efficient method for the purification of the binding protein is required.

The Staudinger ligation is used as truly chemoselective reaction for the preparation of bioconjugates which can be used even in the complex environment of living cell.[4] The azide group reacts with phosphane to form phospha-aza-ylide, which is trapped by ester to give a stable amide bond. The azide group is small and can be introduced into a bioactive molecule without decrease in bioacitivity. And it is more important that an azide group can be used in a highly chemoselective modification of target protein because an azide group is absent in nature. We designed azide-containing photoaffinity probe **1** for chemical modification on the binding protein of potassium isolespedezate, a leaf-opening factor of *Cassia mimosoides* L.[2] An azide group is smaller than the widely used biotin group which can be trapped by streptoavidin, thus probe **1** is expected to have higher bioactivity than biotin-labeled probes. After photo-crosslinking, probe **1** covalently binds to the corresponding binding protein to give azide-labeled binding protein which can be trapped by gel matrix **3** by the Staudinger ligation (Figure 1). After washing, a disulfide bond in the linker moiety of **1** can be dissociated by mild reduction to release biding protein. This catch-and-release process can be used as a convenient method for the purification of binding protein.

On the design of **1**, we used an α-azideacetamide unit as a highly reactive azide unit in the Staudinger ligation. Before designing the probe, we compared the reactivities of several azide compounds in the Staudinger ligation reaction. We compared the time until the disappearance of each azide in the reaction mixture for the comparison of reactivity of each azide. As shown in Table 1, an alkyl azide **4** was almost inert in the Staudinger reaction, whereas α-azideacetate **5**, β-azideacetamide **8**, and *N*-methyl α-azideacetamide **9** were moderately reactive. α-Azideacetamides **6** and **7** were found to be highly reactive in the Staudinger ligation and quantitatively gave corresponding amide at rt within 2.5 h. This result strongly suggested that hydrogen atom in an amide would be important for the acceleration of the Staudinger ligation.

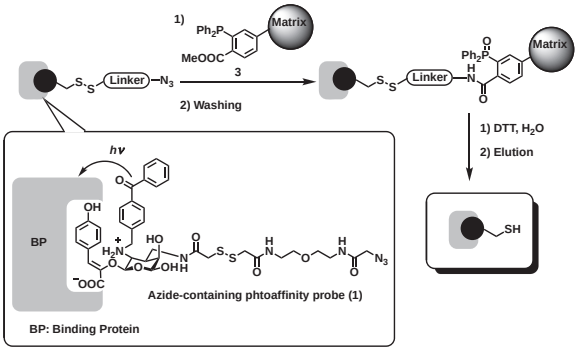
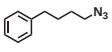
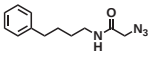
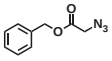
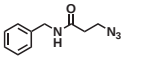
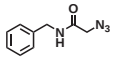
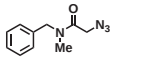


Figure 1. Catch-and-release purification of binding protein using probe **1**.

Table 1. Comparison of the reactivity of several azides in the Staudinger ligation

Entry	Azide	Reaction time/h	Entry	Azide	Reaction time/h
1	4: 	>26	4	7: 	2.5
2	5: 	9	5	8: 	11
3	6: 	2.5	6	9: 	9.5

DFT calculation (B3LYP/6-31G*) on the transition state of **6** strongly suggested this hypothesis (Figure 2).[5] A five-membered ring system was formed between an amide hydrogen and the azide nitrogen.

Thus, we designed and synthesized probe **1** containing an α-azideacetamide group at the terminal of linker moiety (Scheme 1). Properly protected azide compound **10**[3] was reduced and coupled with linker moiety containing an α-azideacetamide group at the terminal **11**.[6] Introduction of benzophenone as a photoaffinity labeling group and successive deprotection gave probe **1**.[7] Probe **1** have a leaf-opening activity against a leaf

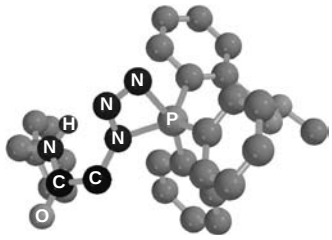
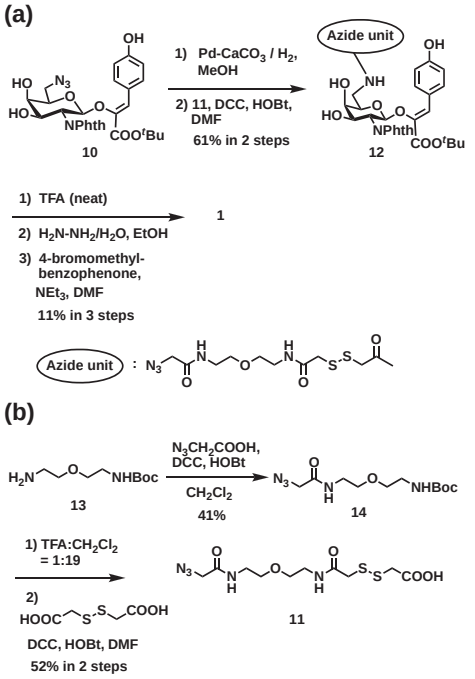


Figure 2. Proposed transition state of **6** in the Staudinger reaction at the B3LYP/6-31G* level.

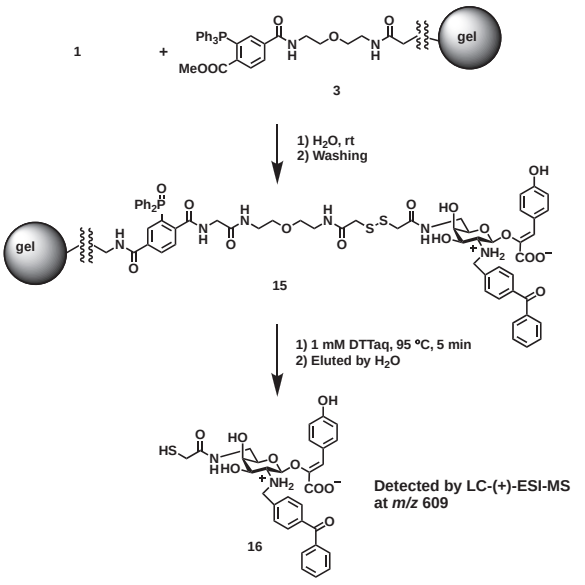


Scheme 1. (a) Synthesis of azide-containing photoaffinity probe **1**; (b) Synthesis of the azide unit containing an α-azideacetamide group.

of *Cassia mimosoides* at 5×10^{-5} M which is twice as strong as the previously reported biotin-linked photoaffinity probe.[3]

We carried out a model examination of catch-and-release process using **1** (Scheme 2). We synthesized gel matrix **3** using corresponding phosphane and activated agarose gel (Affi-gel 10, Bio-Rad Co., Ltd.). This gel matrix **3** (1×10^{-3} M equivalence) was mixed with aqueous solution of **1** (1×10^{-5} M) at rt. Time course change in the level of remaining probe **1** was quantitatively analyzed by LC-ESI MS (Bruker esquire 4000, Bruker Daltonics Co., Ltd.). About 82% of probe **1** was consumed and would be linked with gel **3** within 4 h under these conditions, and almost all of **1** was consumed within 8 h. No degradation product of probe **1** was detected by LC-MS/MS analyses of reaction mixture. And we also confirmed that probe **1** which is covalently linked to gel **3** can be released by 1 mM DTT(aq) treatment (95 °C, 5 min) to give thiol **16**. This result clearly demonstrated the success in catch-and-release process using the Staudinger ligation and successive reductive cleavage of disulfide bond.

Our results showed that the Staudinger ligation between



Scheme 2. Model experiments of catch-and-release.

1×10^{-5} M of **1** and gel **3** can proceed in high yield in an aqueous solution, and probe **1** can be released by mild reductive conditions. Thus, probe **1** would be a powerful tool for the purification of binding protein of **1**, and a similar method can be widely applicable for the cases of other bioactive natural products.

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References and Notes

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5 *Gaussian 03, Revision D.02*, Gaussian, Inc., Wallingford CT, **2004**.
6 **1**: ^{1}H NMR (300 MHz, CD_3OD, rt): δ 7.81–7.59 (9H, m), 7.48 (2H, d, $J = 8.8$ Hz), 7.18 (1H, s), 6.73 (2H, d, $J = 8.8$ Hz), 5.19 (1H, d, $J = 8.4$ Hz), 4.74 (2H, m), 4.51 (1H, d, $J = 13.2$ Hz), 4.02 (1H. dd, $J = 3.2$, 11.0 Hz), 3.86 (3H, s), 3.69 (1H, m), 3.62 (2H, s), 3.60–3.39 (14H, m) ^{13}C NMR (150 MHz, CD_3OD, rt): δ198.5, 172.5, 172.0, 171.3, 161.2, 140.5, 139.2, 138.0, 135.0, 134.8, 132.3, 131.7, 130.4, 128.9, 126.2, 117.1, 102.4, 76.0, 72.3, 72.2, 71.1, 70.5, 62.5, 53.8, 44.0, 43.4, 42.3, 41.6, 41.1. HR-ESI-MS (positive) m/z: $[M + Na]^+$ calcd for $C_{39}H_{45}O_{12}N_7NaS_2$, 890.2460; found 890.2464, IR (film) ν: 3297, 2113, 1656, 1606 cm^{-1}; $[\alpha]_{23}^{D}$ $-6.8°$ (c 0.5, MeOH).

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