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Detection of potential membrane receptor proteins concerning circadian rhythmic leaf movement of legumes using novel photoaffinity probe compounds

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Abstract—Circadian rhythmic plant leaf movement, called nyctinasty, is controlled by a time-course change in the internal concentration of the leaf-movement factor in the plant body. We report that specific binding protein (210 and 180 kDa) for a leaf-movement factor, potassium lespedezate, is contained in the plasma membrane of the plant motor cell. These proteins would be potential receptors for leaf-movement factor to control the leaf movement.
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Receptor proteins are the key molecules which connect the bioorganic study using low-molecular weight natural products with molecular biology within the cell. Thus, the detection of a receptor protein for a biologically active natural product is one of the central issues of bioorganic studies.

We have studied the biologically intriguing phenomena of leguminous plants, called nyctinasty: most leguminous plants close their leaves in the evening, as if to sleep, and open them early in the morning according to the circadian rhythm controlled by the biological clock. It was Charles Darwin, well known for his theory of evolution, who established the basis of this field in the 19th century.[1] Our extensive studies on nyctinasty led to the isolation of a variety of leaf-closing and leaf-opening substances that control nyctinasty.[1] Recently, we revealed that some receptor for this leaf-movement factor is located in motor cells,[2] which plays a key role in nyctinastic leaf movement.[3] The most important issue of this field that we must work on is the detection and isolation of a receptor molecule for the leaf-movement factor.

Photoaffinity labeling is a powerful tool for the detection of these receptors;[4] however, successful results cannot be obtained in many cases. We thought that this difficulty in the labeling experiment would be attributable to the unsuitable molecular design of the probe molecule. In the past photolabeling studies, the molecular design of the probe requires the cost of either high binding affinity or high biological activity because these two factors are never compatible. This is the most important problem in the photolabeling studies using a probe compound. However, no example has been reported to deal with this issue to evaluate the effectiveness of these two factors.

We succeeded in synthesizing biologically active photoaffinity probes (**1**–**3**) to look for the native factor receptor based on potassium lespedezate (**4**),[5] which is a leaf-opening substance of *Cassia mimosoides*. These probes are efficient tools for the biotinylation of the receptor proteins for **4**. Novel probe **1** has a benzophenone group[6] and probes **2** and **3** have trifluoromethydiazirine as photoaffinity groups.[7] Probes **1** and **2** were designed for high labeling yield with the receptor molecule. These probes bear a photolabeling group on the 2′-position of the sugar moiety, which is adjacent to the *p*-coumaroyl group, a potential binding site to the receptor molecule. On the other hand, probe **3**, which bears a photoaffinity group on the 6′-position of the sugar moiety, was designed for high affinity with

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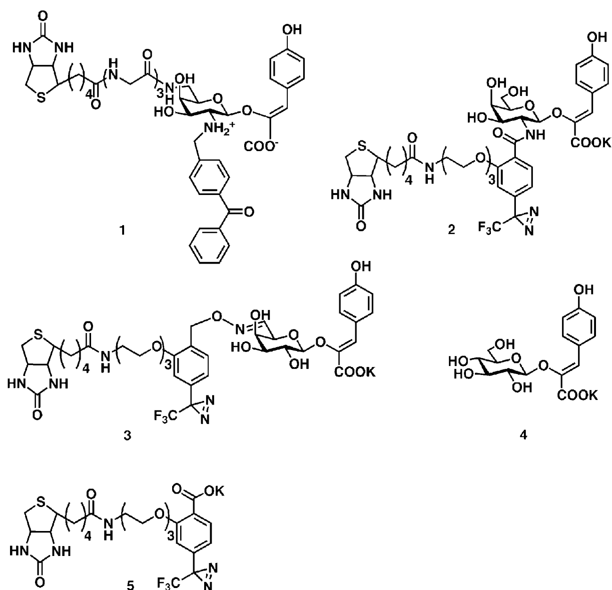
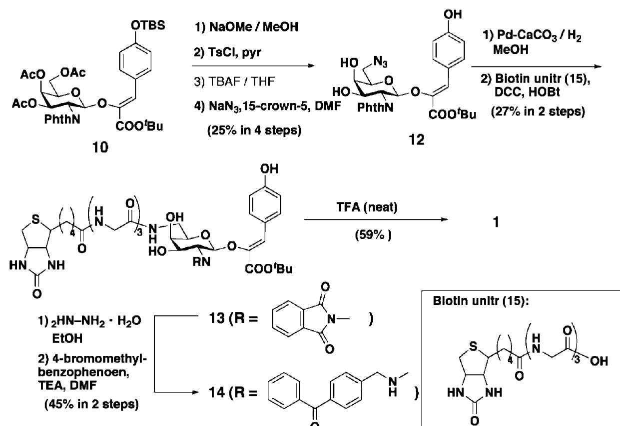
the receptor by reducing the steric hindrance by a large photolabeling group. We examined the difficulty in the photoaffinity labeling by using two photoaffinity probes designed on different concepts. In this paper, we report the detection of a receptor molecule for the leaf-movement factor using these three probes, and advance a guideline for the molecular design of probe compounds for successful photoaffinity labeling.



Synthesis of novel probe **1**[8] was carried out according to the route in Scheme 1. In this probe, a large benzophenone group and a large biotin unit were separately introduced on the 2′- and 6′-position of the sugar moiety, respectively. *tert*-Butyl ester in **10** was essential for the synthesis of **14**.[9] In the synthesis of probe **2**, which was reported in Ref. 9, we experienced that the use of methyl ester instead of **10**, lactam was obtained quantitatively in the following deprotection of the amino group on the 2′-position. The *tert*-Butyl group would prevent the formation of lactam by its steric hindrance.



Scheme 1. Synthesis of probe **1**.

Thus, we synthesized diamine from **12** as intermediate, and then, biotin coupled with a triglycine linker was introduced on the 6′ position of the glycon moiety using DCC, and then 4-bromomethylbenzophenone was introduced on its 2′ position. Removal of *tert*-butyl group by using neat TFA gave probe **1**. Probe **1** was effective for the leaf opening of *C. mimosoides* at 1×10^{-4} M that is 100 times as weaker as the native factor (**4**). Probes **2** (bioactivity: 8×10^{-5} M) and **3** (bioactivity: 5×10^{-5} M) were also synthesized according to Ref. 9.

Photolabeling examination needed a large amount of motor cells containing the receptor because of its extremely low content. We collected a large amount of plant motor cells by cutting a large number of sections of plant pulvini (size: ca. 1 mm × 1 mm) containing the motor cell one by one under a stereoscopic microscope. One cross-linking experiment needed about 1000 plant sections. Successive homogenization in extraction buffer (0.25 M sucrose, 3 mM EDTA, 2.5 mM DTT, 25 mM Tris–MES, pH 7.8) at 4 °C, filtration with nylon mesh, and twice ultracentrifugation (1st: 3000 × g, 15 min, 4 °C, 2nd: 100,000 × g, 60 min, 4 °C) gave a pellet of the crude membrane fraction. The content of protein in that fraction was determined to be 134 μg by the Bradford method with BSA as a reference. The membrane ATPase activity, which is a reference for the purity of the plasma membrane was determined to be 0.29 μmol/mg min by Sandstom's method.[10] The crude membrane fractions were suspended and incubated with 3 μM aqueous solution of probes **1**–**3** for 20 min at rt, respectively. After cross-linking by irradiation of UV-light (365 nm) for 6 min, the suspended membrane fraction was solubilized by the addition of an electrophoresis buffer containing SDS. The membrane fraction was analyzed by SDS–PAGE (7.5%T). After Western blotting, detection of the bands of the potential receptor for **4** was carried out by chemiluminescence detection with ECL Advance Western Blotting Detection Kit (Amersham Bioscience Co. Ltd.), which is a method for the detection of bands of biotinylated proteins. Photolabeling experiments with probes **1** and **2** gave two bands corresponding to binding proteins for **4**. One is due to a protein of 210 kDa molecular weight, and the other to a protein of 180 kDa (lane 2 in Fig. 1). The molecular weight was estimated from comparison with a biotinylated molecular weight marker (Amersham Bioscience Co. Ltd.).

Specific bindings of the probes were confirmed by the disappearance of the corresponding bands in the cross-linking in the presence of 1000-fold molar excess of nonlabeled leaf-opening substance **4** (lane 3 in Fig. 1). The bands for proteins smaller than 100 kDa that were observed in both lanes 2 and 3 in Figure 1 were concluded to be nonspecific bands due to the biotin unit, because they were also detected in the cross-linking examination using photolabeling unit **5**, which was not connected with **4**. The reproducibility was checked by labeling experiments repeated 10 times. On the other hand, no specific band was detected in the labeling experiments with probe **3** (Fig. 2). These results suggested that the close arrangement of the photolabeling group and the

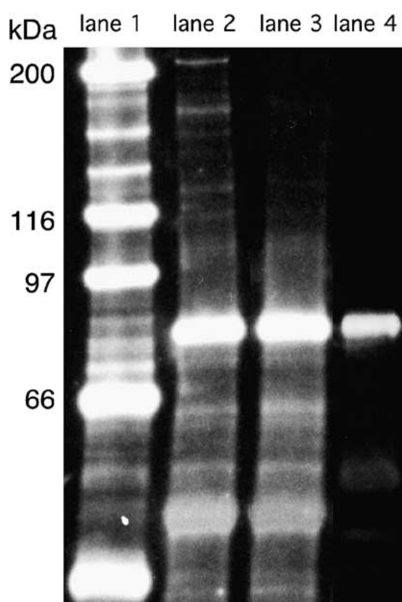


Figure 1. SDS–PAGE analyses of potential membrane receptor for **4** by the chemiluminescence detection (lane 1: molecular weight marker, lane 2: membrane protein of motor cell incubated with **1**, lane 3: membrane protein of motor cell incubated with **1** in the presence of 1000-fold molar excess of **4**, and lane 4: membrane protein of leaf cell incubated with **1**).

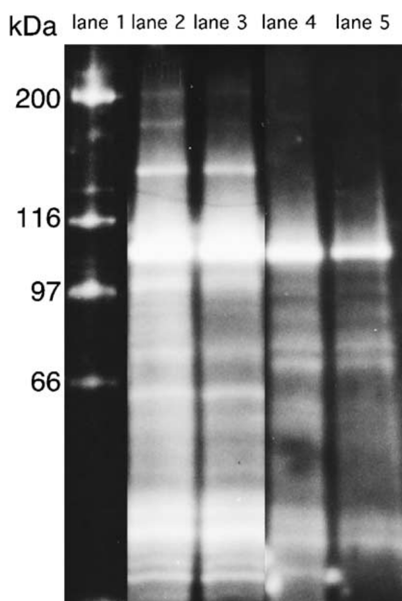


Figure 2. SDS–PAGE analyses (10–20%T gradient gel) of potential membrane receptor using probes **2** and **3** by the chemiluminescence detection (lane 1: molecular weight marker, lane 2: membrane protein of motor cell incubated with **2**, lane 3: membrane protein of motor cell incubated with **2** in the presence of 5000-fold molar excess of **4**, lane 4: membrane protein of leaf cell incubated with **3**, and lane 5: membrane protein of motor cell incubated with **3** in the presence of 5000-fold molar excess of **4**).

potential binding site is most important for the design of a photoaffinity probe even at the cost of strong bioactivity.

Next, we examined the localization of these binding proteins. In the fluorescence study with a fluorescent probe,[2] it was revealed that leaf-opening substance **4** exclusively binds with the motor cell in the plant body. If these binding proteins were the genuine receptor for the leaf-movement factor, they would be localized in the motor cell. Photolabeling experiment with a crude membrane fraction prepared from the section of plant leaves, which contain no motor cell gave no specific band on the chemiluminescence detection for biotinylated proteins. From these results, we proposed that potential receptor proteins for **4** (210 and 180 kDa) that have specific binding activity for **4** are contained in the plasma membrane of the motor cell (lane 4 in Fig. 1).

These results are an important advance in the bioorganic studies of nyctinasty, and would be an important clue for the molecular mechanism of nyctinasty that has been a historical mystery since the era of Darwin. Potential receptor proteins reported in this paper are key molecules connecting the bioorganic study using low-molecular weight natural products with molecular biology within the cell. Moreover, our result showed an advantage of the chemical approach using synthetic probes over the molecular biological approach to the issue of cell biology because direct detection of such large proteins (210 and 180 kDa) is very difficult by the normal molecular genetic approach. Trials for the cloning of this receptor are now in progress. The chemical approach could be compatible with the molecular biological approach, and it will become more important in the field of cell biology.

Acknowledgements

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