Protein Detection

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# Enantiodifferential Approach for the Detection of the Target Membrane Protein of the Jasmonate Glycoside that Controls the Leaf Movement of *Albizzia saman***

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The mode of action of bioactive natural products has attracted much attention. Consequently, bioorganic studies of the target proteins of natural products are one of the most important topics in organic chemistry and chemical biology.[1] Synthetic photoaffinity probes designed to mimic natural products are powerful tools employed in such studies.[2]

Unfortunately, many difficulties usually accompany the molecular identification of a target protein by means of a probe. The living organism or cell contains many proteins, with which a ligand can have multiple, complex interactions. Therefore, nonspecific binding between a probe and multiple proteins is usually observed along with the specific recognition of its true target protein. Thus, clear differentiation between specific and nonspecific binding is required to detect the true target protein of a bioactive natural product. Herein, we report an enantiodifferential approach to detect the target protein of a bioactive natural product in a highly complex living system.

Nonspecific binding is due to the noncovalent association of the probe with a protein and largely arises for two reasons:[3,4] the hydrophobicity of the probe, and an electrostatic interaction between the probe and the protein as a result of the acid-dissociation properties of their carboxylate and ammonium groups (Figure 1). Competitive inhibition is usually used to confirm specific binding in an experiment involving probes: The binding of the probe to the target protein is inhibited competitively in the presence of excess unlabeled ligand. However, when a ligand has carboxylate or ammonium groups that are easily dissociated, competitive-binding experiments yield a misleading result. Any nonspecific binding between the probe and proteins due to electrostatic interactions are also inhibited competitively by the unlabeled ligand. This phenomenon is well known in affinity chromatography with charged ligands.[3] Thus, a more reliable method is necessary to confirm the specificity of binding between the probe and the target protein.

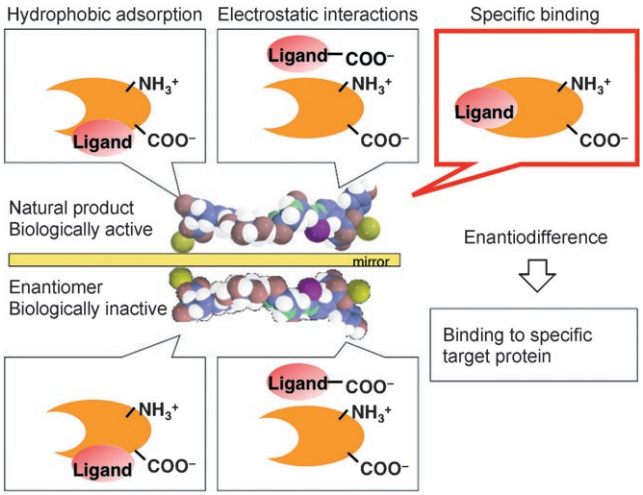


***Figure 1.*** Interactions of a bioactive natural product and its enantiomer with proteins in a living organism.

Enantiomeric pairs of chiral natural products have almost identical physical properties, with the exception of their optical rotation and affinity for chiral molecules, such as proteins. Both enantiomers undergo the same nonspecific binding to proteins through noncovalent or electrostatic interactions (Figure 1). A clear difference would be observed, however, in any specific binding based on the stereospecific molecular recognition of a ligand by its target protein. We have used an enantiomer of a chiral natural product as a control in bioorganic studies with probe compounds. We applied this enantiodifferential approach to the identification of the target protein of **1**, a chemical factor that controls the nyctinastic leaf movement of the leguminous plant *Albizzia saman* (Figure 2).

***Figure 2.*** Nyctinastic leaf movement of *A. saman* (left: daytime, right: nighttime).

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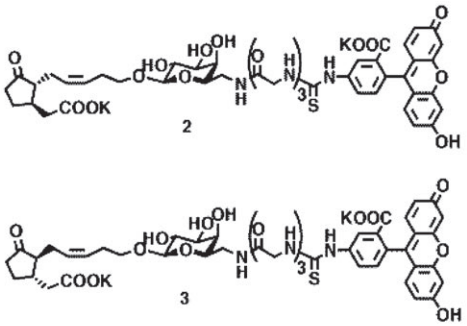
Nyctinasty is a leaf-folding and leaf-opening movement that is controlled by the circadian rhythm and observed in all leguminous plants.[5] We revealed previously that nyctinasty is controlled by endogenous chemical factors.[6] A jasmonate glucoside, **1**, was isolated as a leaf-closing factor of *A. saman*.[7] We used an enantiomeric pair of fluorescent probes **2** and **3** as a molecular probe designed for **1** to confirm specific recognition between **1** and its target protein. We reported that the target protein of **1**, which recognizes the configuration of **1**, is involved in the motor cells that control nyctinasty in *A. saman*.[8] No other part of *A. saman* plants was involved in stereospecific binding with probe **2** (Figure 3). Herein, we describe the expansion of this enantiodifferential approach to the photoaffinity labeling of the target protein involved in motor cells of *A. saman*.



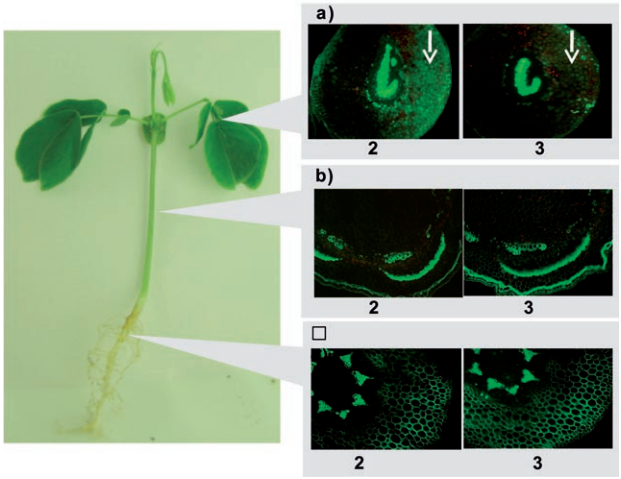
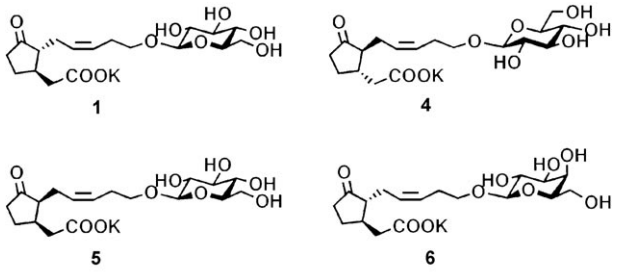




***Figure 3.*** Enantiodifferential fluorescence detection of target cells of **1** in a) pulvini, b) stems, c) roots: A difference in fluorescence was observed in cells located on the right-hand side of the images in (a), in the area indicated by the arrow.

We designed a photoaffinity probe on the basis of the results of a structure–activity-relationship study on **1**, in which we used **4** (the enantiomer of **1**), a *cis* analogue **5**, and a D-galactoside derivative **6** (Figure 4). The leaf-closing activity of **6** for *A. saman* leaves was as strong as that of **1** ($5 \times 10^{-4}$ M); however, **4** and **5** did not show any leaf-closing activity, even at a concentration of $1 \times 10^{-3}$ M. These results showed that the aglycone moiety of **1** is important for the leaf-closing activity and must be recognized accurately by the target protein; they



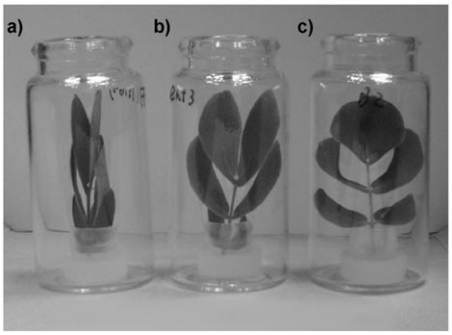
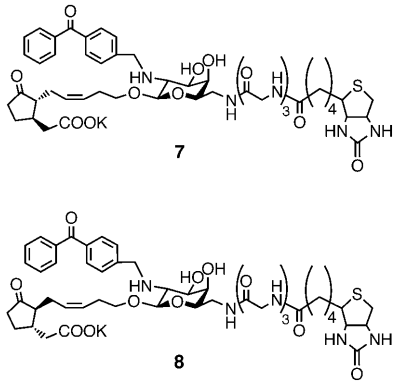




***Figure 4.*** Structure–activity-relationship studies of **1**: a) Compounds **1** and **6** were biologically active and caused *A. saman* leaves to fold; b) compounds **4** and **5** were biologically inactive; c) untreated leaf remained open.

also suggested that structural modification of the sugar moiety of **1** would not affect its bioactivity.

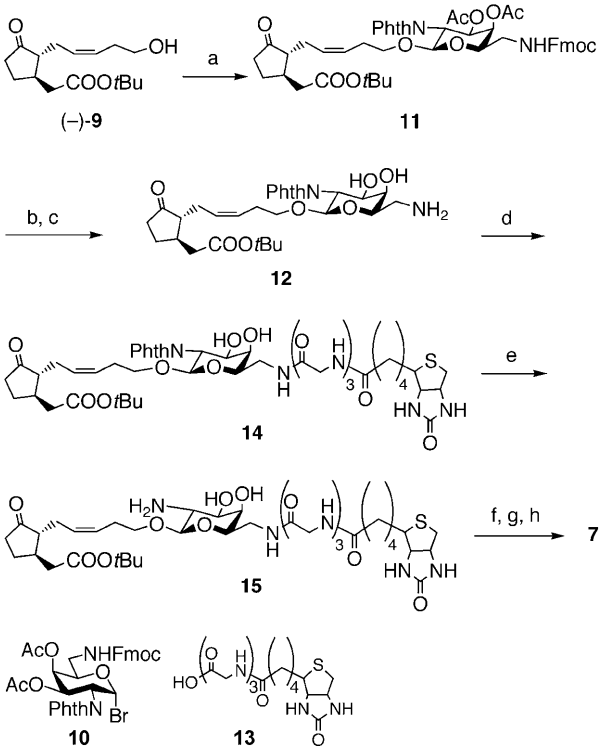
Thus, we designed and synthesized the photoaffinity probe **7** with a benzophenone and a biotinyl group in the




sugar moiety. To confirm the results obtained with **7**, we also used the diastereomeric probe **8**. The probes were designed as D-galactosides to circumvent enzymatic hydrolysis by endogenous β-glucosidase.[8] The reason for selecting a pair of probes, **7** and **8**, in which each enantiomeric aglycone unit is connected to the D-galactose moiety, is that proteins that recognize the configuration of a galactose moiety, such as membrane transporters[9] or glycosidases (e.g. galactosidase), would also be detected through a difference in the binding of the two probes with aglycone units of opposite configuration.

The optically pure *tert*-butyl aglycones (−)-**9** and (+)-**9** were synthesized according to previously reported procedures.[8,10] Scheme 1 shows the synthesis of the photoaffinity




***Scheme 1.*** Synthesis of the photoaffinity probe **7** with the natural configuration: a) **10**, AgOTf, $Ag_2CO_3$, 4 Å molecular sieves, $CH_2Cl_2$, toluene, 10 °C, 90%; b) NaOMe, MeOH; c) 5% piperidine, DMF, 67% over 2 steps; d) **13**, HBTU, $Et_3N$, DMF, 84%; e) ethylenediamine, MeOH, 40 °C, 73%; f) 4-bromomethylbenzophenone, Hünig base, DMF, 49%; g) TFA (neat), 84%; h) aqueous $KHCO_3$, quantitative. DMF = *N*,*N*-dimethylformamide, Fmoc = 9-fluorenylmethoxycarbonyl, Phth = phthalimidyl.

probe **7**, with the natural configuration. The aglycone (−)-**9** was coupled with the sugar moiety **10** to give **11** in high yield.[11] Compound **11** was deprotected to give **12**, which was coupled with the biotin unit **13**[12] by using HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate).[13] Subsequently, **14** was treated with ethylenediamine,[14] coupled with 4-bromomethylbenzophenone, and deprotected with neat trifluoroacetic acid (TFA) to give the photoaffinity probe with natural configuration, **7**, which showed leaf-closing activity at a concentration of $1 \times 10^{-3}$ M. The photoaffinity probe **8** with the non-natural configuration was synthesized in the same manner from (+)-**9** (see Scheme 2 in the Supporting Information), and was not effective in promoting leaf closing at $1 \times 10^{-3}$ M.

Next, we carried out an enantiodifferential photoaffinity-labeling experiment with **7** and **8** (Figure 5). Enantiodifferential photoaffinity labeling was conducted with protoplasts of motor cells that were prepared from *A. saman* leaves according to the method reported by Satter and co-workers.[15,16] We excised and collected approximately 200 leaflet pulvini. Protoplasts were prepared from the collected pulvini and incubated with probe **7** or **8** ($1 \times 10^{-4}$ M) at 4 °C for 5 min. Photo-cross-linking on the cell surface was carried out by irradiation with UV light (365 nm) at 4 °C for 20 min. Labeled protoplasts were washed with buffer to remove excess probe,

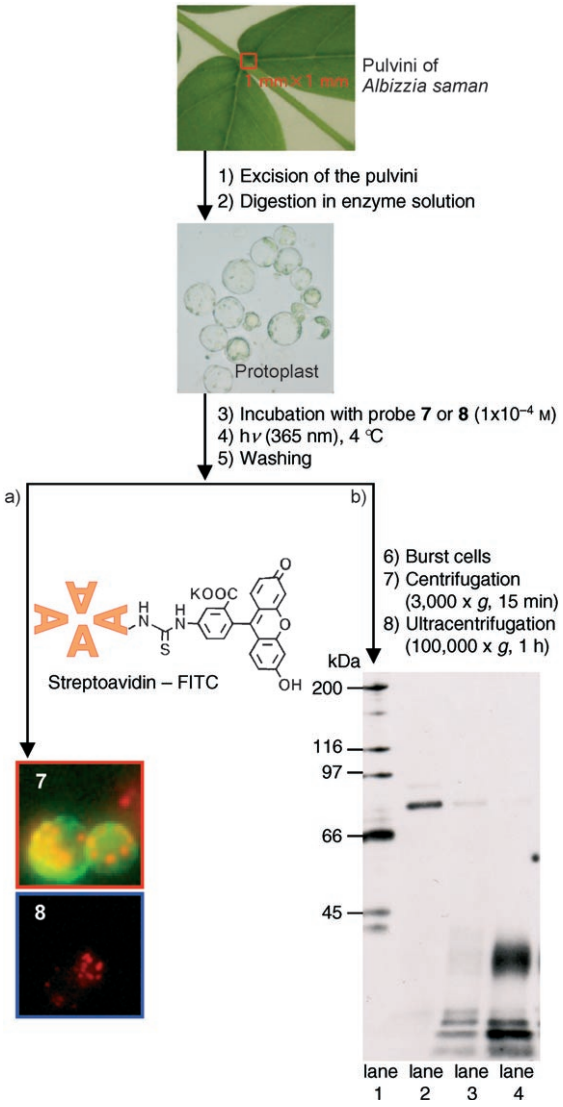



***Figure 5.*** Enantiodifferential photoaffinity labeling of protoplasts with probes **7** and **8**: a) Fluorescence detection of protoplasts after treatment with streptavidin–FITC; b) SDS-PAGE analysis of membrane fractions prepared from photolabeled protoplasts (lane 1: molecular-weight marker, lane 2: untreated membrane fraction, lane 3: membrane fraction treated with **8**, lane 4: membrane fraction treated with **7**).

treated with streptavidin–FITC (fluorescein isothiocyanate) conjugate, and then monitored under a fluorescence microscope. Green fluorescence due to fluorescein was observed only on the plasma membrane of protoplasts that had been incubated with the biologically active probe **7**. This result suggests strongly that the target protein that recognizes the configuration of the aglycone unit in probe **2** is associated with the plasma membrane of motor cells.

We carried out SDS-PAGE analysis of membrane proteins of motor-cell protoplasts incubated with each probe. After photo-cross-linking, a membrane fraction of the motor cells was prepared by using a Potter-type homogenizer and subjecting the samples to successive centrifugation steps (Figure 5). The pellet containing the crude plasma membrane was analyzed by SDS-PAGE, with chemiluminescence detec-

tion carried out after Western blotting. In Figure 5, lane 2 contained the crude membrane fraction, which had not been incubated with a probe, lane 3 contained the membrane fraction incubated with probe **8**, and lane 4 contained the membrane fraction incubated with probe **7**. The appearance of several bands below 30 kDa in both lanes 3 and 4 indicated nonspecific binding of the probe. However, one difference between probes **7** and **8** was evident at around 38 kDa and indicated that this protein showed stereospecific recognition of the aglycone unit of the probe. The difference between probes **7** and **8** could be detected when their concentration was $5 \times 10^{-6}$ M (see Figure 7 in the Supporting Information). Additionally, the binding of probe **7** by this protein was inhibited competitively in a photoaffinity-labeling experiment in the presence of an excess amount of **1** ($1 \times 10^{-3}$ M; see Figure 8 in the Supporting Information). Our enantiodifferential approach clearly discriminated specific from nonspecific binding of the probe. The observation that only the biologically active stereoisomer was recognized by this protein suggests strongly that this membrane protein is the true target protein of **1** involved in the control of nyctinasty in *A. saman*.

An enantiodifferential approach was also applied successfully to phyllanturinolactone, a leaf-closing factor of *Phyllanthus urinaria*, to detect its target cell type.[17] Applications of this enantiodifferential approach to other chiral natural products with biological activity are now in progress. The enantiodifferential approach is a powerful method for the detection of target proteins of biologically active natural products, as such a large number of natural products are chiral, and their bioactivity is inherently related to their stereostructure.

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