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Enantio-differential approach using fluorescence-labeled phyllanthurinolactone, a leaf-closing factor of *Phyllanthus urinaria* L.

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Abstract—We report here the synthesis of enantio-pair-type fluorescence-labeled probes based on phyllanthurinolactone (**1**), which is a leaf-closing substance of *Phyllanthus urinaria* L. Moreover, the application of an enantio-differential approach to distinguish between specific and nonspecific binding of ligands and receptors was performed. Fluorescence studies using a natural-type probe **6** and a nonnatural-type probe **7** revealed that the target cell for **1** is a motor cell. Furthermore, our results lead to the suggestion that some receptors, which recognize the stereochemistry of the aglycon of **1**, exist on the plasma membrane of the motor cell, as do leaf-opening substances.

It is well known that many small molecules operate within living systems to maintain homeostasis.[1] These molecules are thought to express their bioactivities through interaction with receptors. Although identification of the receptors is complicated, detection of the target cell of a ligand and the corresponding target protein is the first step in bioorganic studies. Moreover, studies that focus on a target cell or protein of endogenous bioactive molecules attract the attention of many scientists. Fluorescence-labeled probes are widely used for the identification of target cells in living systems. However, a considerable amount of nonspecific binding is commonly observed, due to increased lipophilicity and the electrostatic charge of the probe, compared with the original bioactive compounds. This can hamper the identification of minuscule amounts of target protein, therefore an appropriate control experiment is required to distinguish specific from nonspecific binding. For this purpose, we developed an enantio-differential approach for identification of the target cell for glucosyl jasmonate-type leaf-closing factor.[2] In general, vital activities in the living system are operated by the receptor's recognition of a corresponding chiral compound with natural stereochemistry. A comparison of the results using a biologically active 'positive' probe and a biologically inactive 'negative' probe would clarify a genuine ligand–receptor interaction (Fig. 1). A 'negative' probe must be highly similar in nature to a 'positive' probe, apart from binding protein affinity. Therefore, the enantiomer of a biologically active natural product is an ideal 'negative' probe since all physical properties, except optical rotation and affinity to binding proteins, are identical between a pair of enantiomers. An enantio-differential approach, which allowed comparison of the results using a pair of probes prepared from a pair of enantiomers, would be an ideal method to discriminate between specific ligand–receptor interaction and nonspecific binding of the probe.

Phyllanthurinolactone (**1**) is a leaf-closing substance involved in the nyctinastic leaf-movement of *Phyllanthus urinaria* L. (Fig. 2).[3] The structure of **1** is large enough to undergo the structural modification required for a molecular probe. The synthesis of **1** was completed by Audran and Mori in 1998,[4] and their study shows that the stereochemistry of natural **1** is essential for its bioactivity. We have previously reported on the enantioselective total synthesis of **1**, and our structure–activity-relationship study shows that changing the sugar moiety of **1**, for example to L-glucoside or D-galactoside, does

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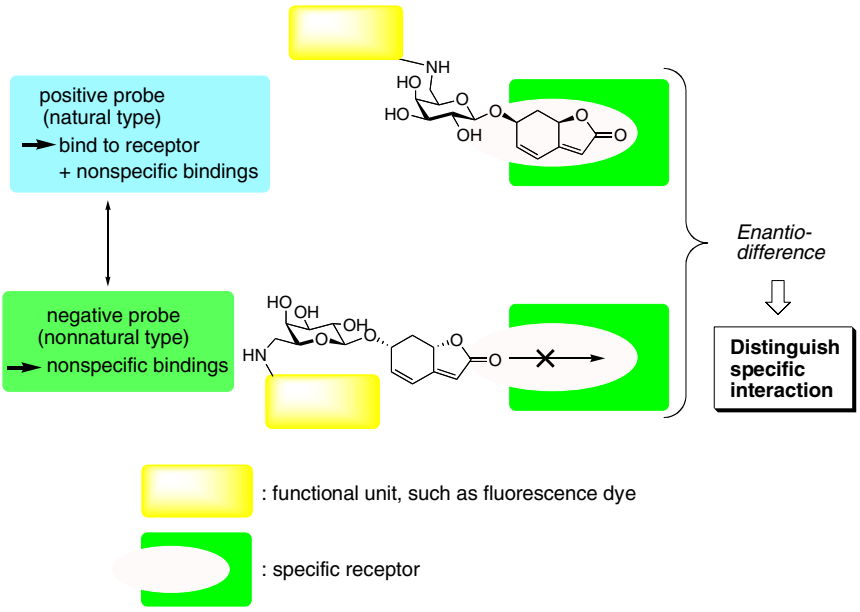


Figure 1. Enantio-differential approach using an enantio-pair of molecular probes.

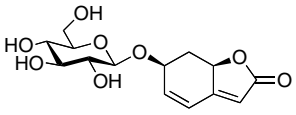


Figure 2. Structure of phyllanthurinolactone (**1**).

not affect its bioactivity.[5] Based on this result, we designed a fluorescence-labeled probe for **1**, and fluorescence was observed specifically bound by target motor cells, as well as nonspecifically bound by xylem and epidermis.[6,7] Thus, the fluorescence study was considered insufficient to confirm the target cells. A reliable method was therefore required to detect only specific binding signals resulting from ligand–receptor interactions. In this study, we synthesized probe **2** and its congener **3**, by using a pair of enantiomerically pure aglycons of phyllanthurinolactone, prepared by optical resolution. We then carried out fluorescence studies using **2** and **3** to identify the target cell of **1** (Fig. 3).

Although we previously synthesized phyllanthurinolactone (**1**),[5] we have since investigated a more efficient method of achieving this. Improved syntheses of aglycons **8** and *ent*-**8** are illustrated in Scheme 1. Monoepoxidation of 1,4-cyclohexadiene (**4**) followed by ring opening with cyanomethillithium in acetonitrile/THF provided alcohol **5**. Acetylation of the hydroxy group of **5**, followed by lipase-mediated kinetic resolution of the resulting racemic acetate (±)-**6**, gave (−)-**5** and (+)-**6** in 42% yield, 97% ee, and 40% yield, 97% ee, respectively (ee was determined by chiral HPLC after conversion to the corresponding benzoate (for **5**: BzCl, pyridine; for **6**: (1) K_2CO_3, MeOH; (2) BzCl, pyridine). Analytical condition is as follows; wave length: 260 nm, column: SHISEIDO Ceramospher Chiral RU-1, eluent: MeOH/MeCN = 9/1, flow rate: 0.5 mL/min, retention time: 17.9 and 21.6 min). Hydrolysis of the nitrile group of (−)-**5**, followed by acid treatment afforded γ-lactone **7** in good yield. According to Mori's procedure,[4] **7** was converted to aglycon **8** in six steps. On the other hand, (+)-**6** was also transformed to aglycon *ent*-**8**.

The 'positive' and 'negative' probes **2** and **3** were synthesized according to our previous report (Scheme 2).[7] Since probes **2** and **3** were available, we examined their bioactivities using the method previously reported.[7] Probe **2** induced effective leaf-closing of *P. urinaria* L. at 1×10^{-5} M, thus it was one-hundredth as effective as natural product **1**. On the other hand, probe **3**, which has the enantiomeric aglycon moiety of **2**, was biologically inactive ($>1 \times 10^{-4}$ M).

We used fluorescence-labeled probes to identify the target cell for **1**, and the fluorescence study was performed as previously reported.[7] The sections of pulvini, which are organs containing the motor cells,[6] were immersed in a solution containing 1×10^{-4} M of the probe (**2** or **3**), and left in the dark at room temperature for 6 h to allow staining. The stained sections were incubated for 15 min in equilibration buffer (Slow Fade™ Gold Antifade Reagent, Molecular Probes, Inc.) to remove excess

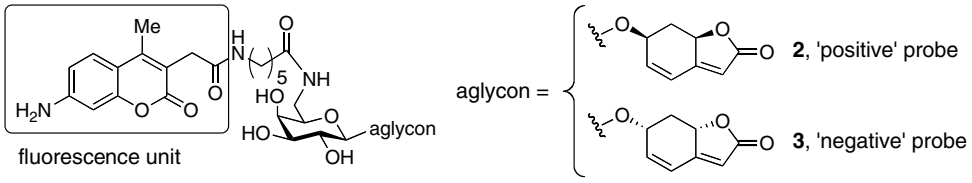
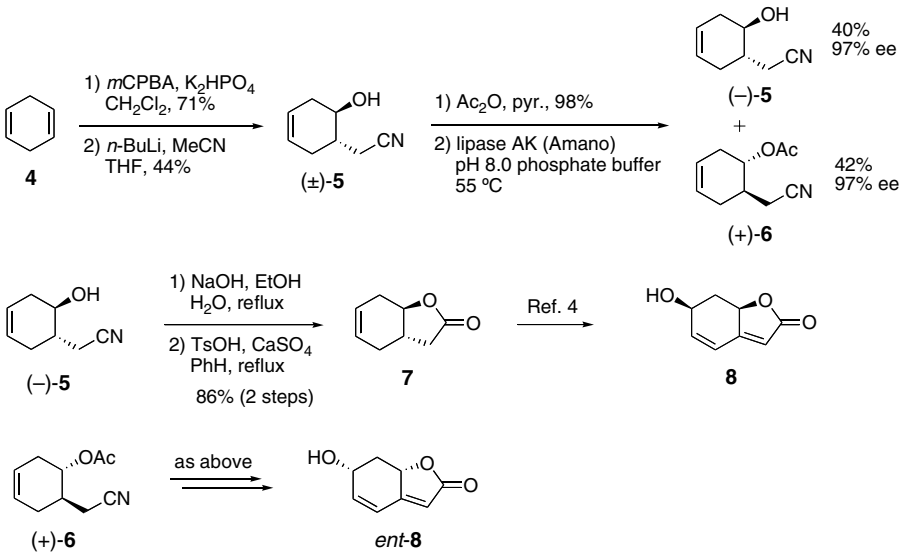
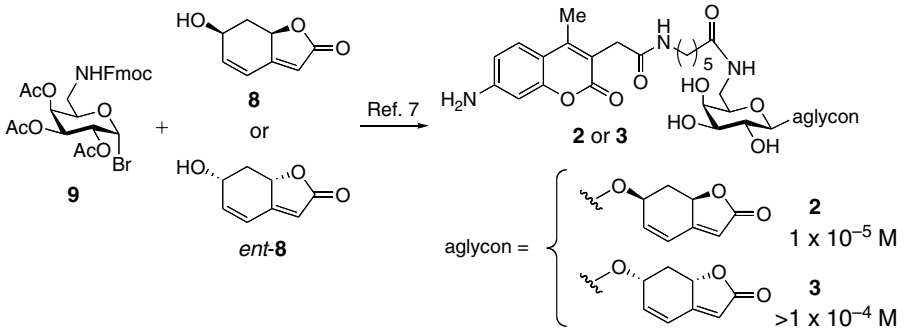


Figure 3. Structures of fluorescence-probe of phyllanthurinolactone.



Scheme 1. Syntheses of aglycons **8** and *ent*-**8**.



Scheme 2. Syntheses of fluorescence-labeled 'positive' and 'negative' probes **2** and **3**.

fluorescence probes. These sections were observed by using a fluorescence microscope (ECLIPSE E800, Nikon Co., Ltd) with an appropriate filter (UV-2A, Nikon Co., Ltd; excitation wavelength 330–380 nm).

Figure 4 shows photographs of plant pulvini taken under a fluorescence microscope. Red fluorescence observed on the fluorescence images is due to porphyrin in the plant cells. The blue fluorescence staining pattern

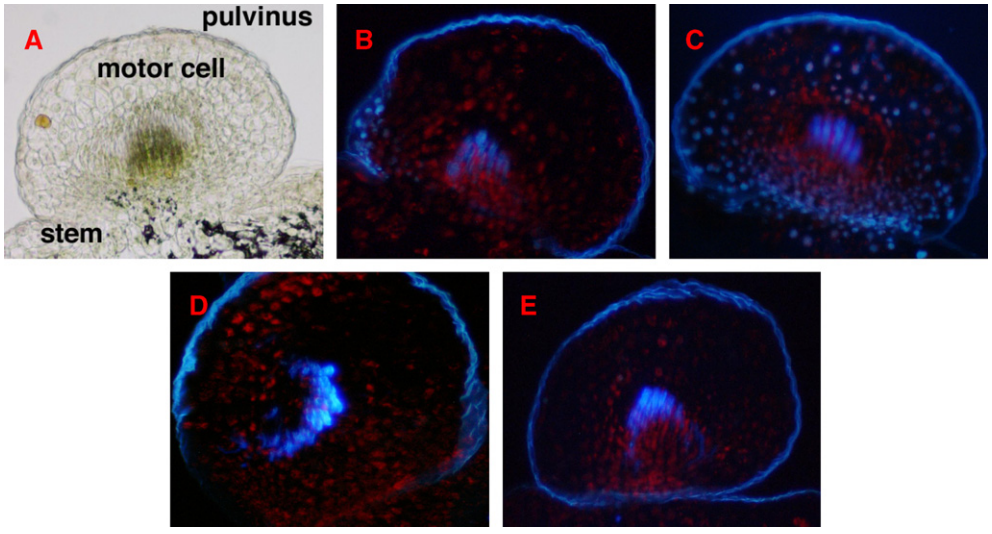


Figure 4. Fluorescence study using plant pulvini containing motor cells with fluorescence-labeled probes; (A) Nomarski image of a plant section; (B) fluorescence image of a control section; (C) fluorescence image of a section treated with **2**; (D) fluorescence image of a section treated with **3**; (E) fluorescence image of a section treated with **2** in the presence of 100-fold molar excess of phyllanthurinolactone (**1**).

of the biologically active probe **2** was observed on the motor cells, xylem, and epidermis (**C**). However, blue fluorescence was observed only on the xylem and epidermis in the control sections and in the biologically inactive probe **3** (**B** and **D**). These nonspecific bindings would be due to the lipophilicity and electrostatic charge of the fluorescence moiety and the linker of **2** and **3**.[8] On comparing these two results we strongly suggest that fluorescence on the motor cells can be attributed to specific binding between probe **2** and the receptor.

In conclusion, we improved the methods for the synthesis of phyllanthurinolactone (**1**), and applied an enantio-differential approach to detect the receptor of **1**, a leaf-closing substance of *P. urinaria* L. We demonstrated that *P. urinaria* L. has a receptor for **1** in pulvini motor cells, and that the receptor recognizes the stereochemistry of the ligand. An enantio-differential approach allows a distinction to be made between the specific binding of a ligand to its receptor and nonspecific binding; indistinction is one of the main problems of using molecular probes in bioorganic studies. Thus, an enantio-differential approach can be widely used for bioorganic studies of biological phenomena. More recently, we have succeeded in expanding the enantio-differential approach to include photoaffinity labeling of a specific receptor protein, and the results will be published in due course.

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