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Tetrahedron Letters 46 (2005) 5537–5541
Tetrahedron Letters

Direct observation of a target cell of the leaf-closing factor by using novel fluorescence-labeled phyllanthurinolactone

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Received 5 May 2005; revised 7 June 2005; accepted 9 June 2005
Available online 1 July 2005

Abstract—We report the synthesis of the fluorescence-labeled probe **2** based on phyllanthurinolactone **1**, which is a leaf-closing substance of *Phyllanthus urinaria* L. Bioorganic studies using probe **2** showed leaf-closing activity at 1×10^{-5} M, which was one-hundredth of that of the natural product **1**. The fluorescence study using **2** revealed that the target cell for **1** is a motor cell and suggested that some receptors for **1** exist on the plasma membrane of the motor cell as with leaf-opening substances.

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 a biological clock.[1] Nyctinastic leaf movement is induced by the swelling and shrinking of motor cells in the pulvini, a small organ located in the joint of the leaf to the stem. Motor cells play a key role in plant leaf-movement. Flux of potassium ions across the plasma membranes of the motor cells is followed by massive water flux, which results in swelling and shrinking of these cells.[2] We have revealed that nyctinasty is controlled by a pair of leaf-movement factors: leaf-opening and leaf-closing substances.[3] Recently, we revealed that the target cell of the leaf-opening substance is a motor cell,[4] which plays a key role in nyctinastic leaf-movement.[2] On the other hand, no attempt has been carried out to clarify the target cell of the leaf-closing substances because the structure of most leaf-closing substances is so simple[3] that structural modification toward a molecular probe, such as a fluorescence-labeled one, seems to be difficult. This is because large fluorescence dye or a photoaffinity unit would cause serious decrease in the bioactivity of the synthetic probe.

Phyllanthurinolactone (**1**), a leaf-closing substance of *Phyllanthus urinaria* L., is a glycoside-type leaf-closing substance (Fig. 1).[5] The structure of **1** is comparatively

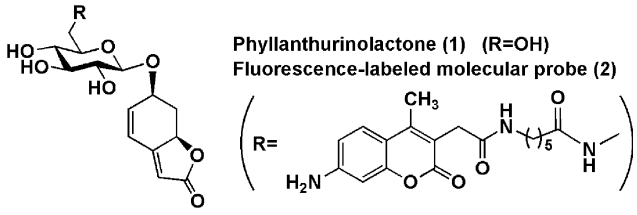

Figure 1.

large enough for the structure modification that is essential for a synthetic probe. The synthesis of **1** was completed by Audran and Mori in 1998.[6] They also showed that the stereochemistry of natural **1** is essential for its bioactivity. We also carried out structure–activity relationship studies by using synthetic analogs of **1** with sugars other than D-glucose, and it was revealed that L-glucose-type and D-galactose-type analogs were as effective as natural **1**.[7] This result showed that we can develop a biologically active molecular probe based on **1** when a large functional unit such as a fluorescence dye or a photoaffinity labeling unit is introduced into the sugar moiety. In this letter, we report a synthesis of fluorescence-labeled **1** (**2**) and the direct observation of its target cell in the plant body by using **2**.

We already reported the enantioselective synthesis of **1** and its sugar analogs.[7] However, total yield to **1** was too low to prepare an adequate quantity of the probe. Thus, we tried to improve yields in the synthesis of **1**; especially low yields in the synthesis of the aglycon

Keywords: Nyctinasty; Leaf-closing substance; Fluorescence; Probe compound; Motor cell; Mitsunobu reaction; Glycosidation.
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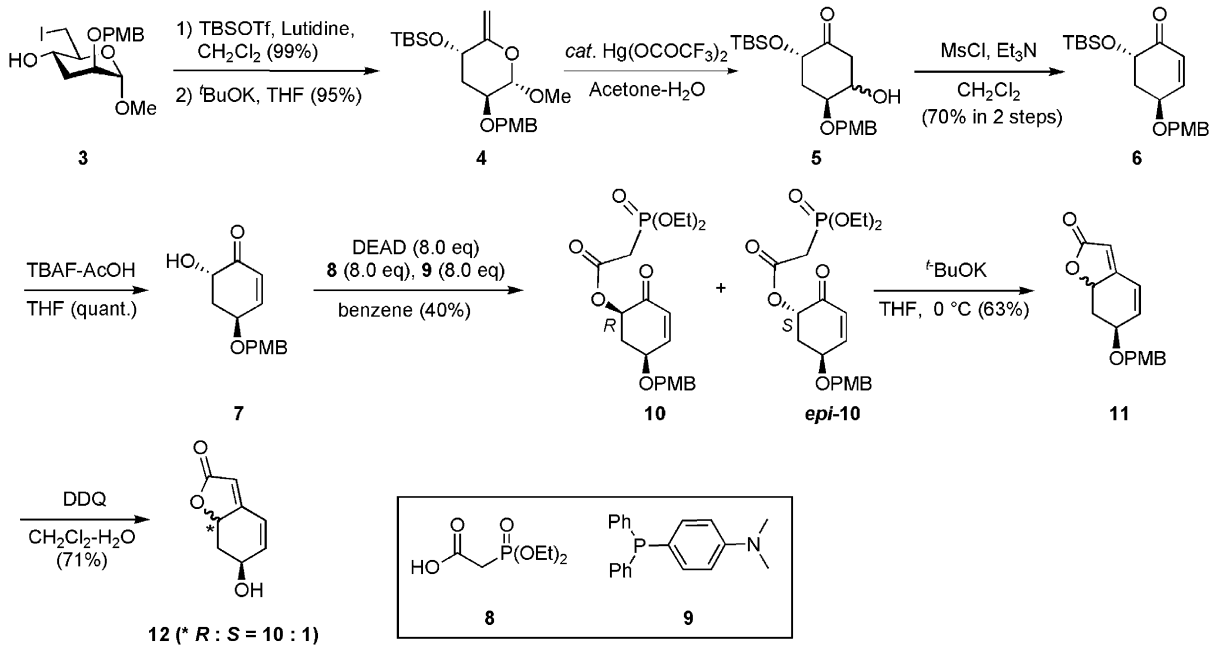
doi:10.1016/j.tetlet.2005.06.044

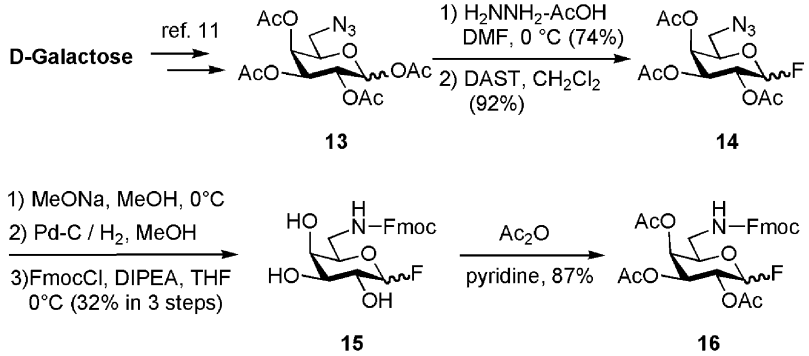


Scheme 1. Synthesis of aglycon **12**.

moiety from D-glucose and glycosidation reaction incurred serious problems.

First, we improved the synthesis of aglycon by using the Mitsunobu reaction.[8] The improved synthesis of aglycon is shown in Scheme 1. Intermediate **3** that was prepared according to the previous letter,[7] was protected by the TBS group, and then subjected to elimination of iodide to afford the enol ether **4**. A catalytic amount of $Hg(OCOCF_3)_2$-mediated Ferrier's carbocyclization[9] of **4** and followed by treatment of the alcohol **5** with mesylchloride and triethylamine, that is a one-pot reaction involving mesylation and β-elimination, afforded the cyclohexanone derivative **6** in 70% overall yield in two-steps. Deprotection of the TBS group and the obtained alcohol **7** was coupled with diethyl phosphonoacetate by the Mitsunobu reaction.[8] In general, the Mitsunobu reaction is known to be difficult to proceed in a cyclohexanol system.[10] However, in this case, the Mitsunobu product was obtained as a mixture of stereoisomers (R:S = 10:1) in moderate yield by using excess amount of the Mitsunobu reagents. When stoichiometric amount of Mitsunobu reagent was used, the Mitsunobu product was hardly obtained. However, the desired **10** was obtained as a major product. Additionally, despite our best efforts, the epimerization could not be suppressed. Intramolecular Hornor–Emmons reaction using *t*-BuOK as the base afforded the cyclized product **11**. Finally, deprotection of the PMB group by DDQ gave the aglycon **12** as a 10:1 diastereomixture. Total yield of **12** from D-glucose was improved from 0.25% to 2.0% compared with our previous enantioselective synthesis.[7]

Next, we synthesized 1-fluorosugar. The synthesis of 1-fluorosugar **16** is shown in Scheme 2. The acetate **13**, prepared from D-galactose according to the procedure in Ref. 11, was treated with hydrazine monohydrate



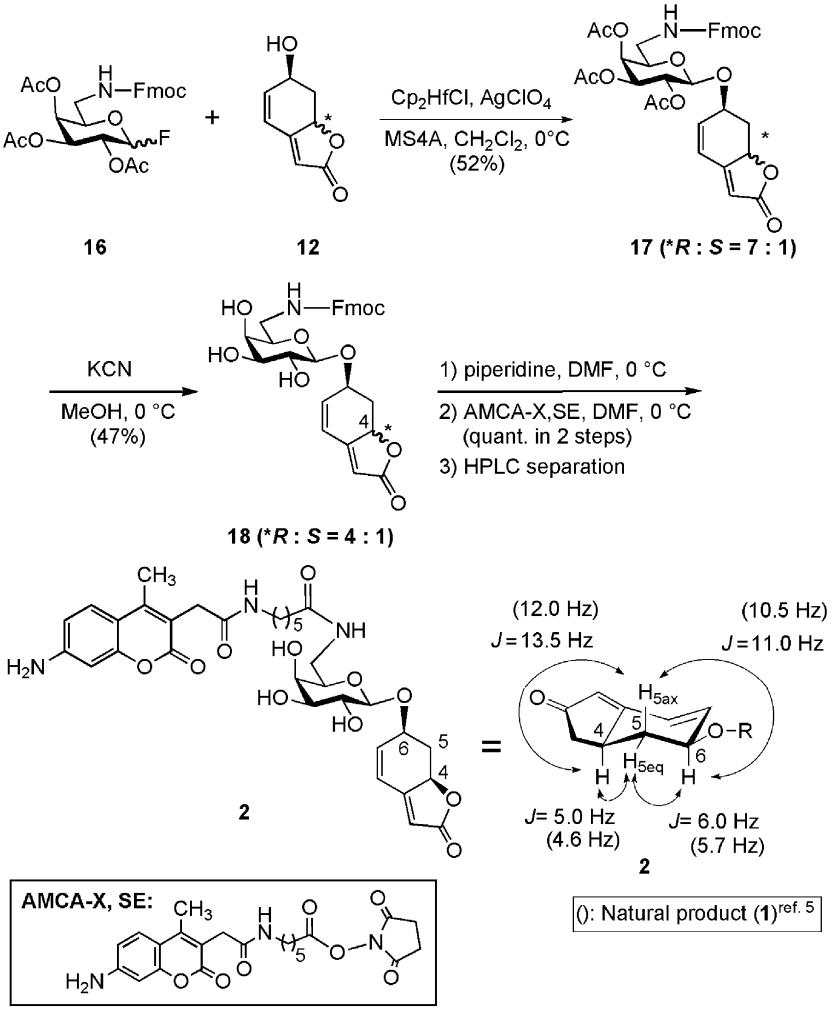
Scheme 2. Syntheses of 1-fluorosugar **16**.

and fluorination of the resulting hydroxyl group afforded the fluoride **14**. Removal of acetates with sodium methoxide, reduction of azide, and followed by protection of amine with Fmoc group, gave a triol **15**. Finally, acetylation of the triol yielded the 1-fluorosugars **16**.

Synthesis of fluorescence-labeled probe **2** was then examined (Scheme 3). Glycosidation of 1-fluorosugar **16** via the Suzuki method[12] gave the glycoside **17** in moderate yield. Acetyl groups of the glycoside **17** were deprotected with KCN to give the triol **18** as a 4:1 mixture of stereoisomers. At this stage, further epimerization occurred. Because the C4 proton in **18** would be easily abstracted by KCN and the resulting stable furan-type intermediate gave a mixture of **1**and its epimer by protonation. Deprotection of the Fmoc group and introduction of AMCA-X, which is a fluorescence group gave the fluorescence-labeled probe **2** as a 4:1 diastereomixture. Diastereomerically pure **2**[13] and its epimer *epi*-**2**[14] were obtained after purification by HPLC (COSMOSIL 5C18-AR, 20% CH_3CN aq, 260 nm). The desired epimer **2** was confirmed by coupling constants in the ^1H NMR spectra (Scheme 3). The relation of H4 and H6 was determined to be *syn* on the basis of the observation of the coupling constants between H4 and $H5_{ax}$, H4 and $H5_{eq}$, $H5_{ax}$ and H6 and $H5_{eq}$ and H6.

With the fluorescence-labeled probe **2** in hand, we investigated the bioactivity of **2**. The young leaves detached from the stem of the plant *P. urinaria* with a sharp razor blade were used for bioassay. One leaf was placed in H_2O (ca. 1.0 mL) using a 20 mL glass tube in the greenhouse kept at 25–35 °C and allowed to stand overnight. The leaves, which closed again in the evening were used for the bioassay. The test solution was carefully poured into test tubes with a microsyringe around 10:00 a.m. The bioactive fraction was judged by the leaf-closing after the leaf-opening of the plant leaf in the blank solution. The probe **2** was effective for the leaf-closing of *P. urinaria* at 1×10^{-5} M that is one-fiftieth as effective as natural product **1**. And the epimer of **2** was biologically inactive.

We used probe **2** to seek the target cell for **1**. For this purpose, the binding experiment using a plant section was carried out. The leaf of *P. urinaria* opening in the daytime was cut in an appropriate size and fixed in agar. The agar was sliced perpendicular to the petiole by a microslicer (Dousaka EM CO., Ltd.) to a thickness of



Scheme 3. Syntheses of fluorescence-labeled probe **2**.

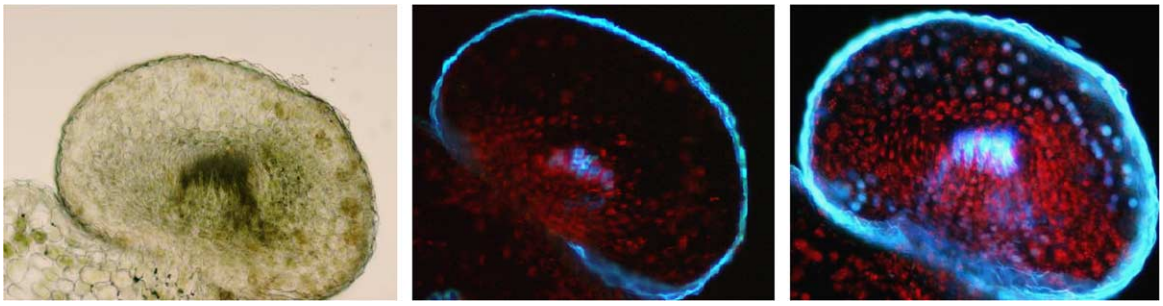


Figure 2. Fluorescence study using *Phyllanthus pulvini* containing motor cell with a probe **2**; left: Nomarskii image of plant section, center: fluorescence image of control section, right: fluorescence image of a section treated with **2**.

thirty micrometers and the sections containing the pulvini were floated on distilled water. The sections were immersed in a solution containing 7×10^{-6} M of **2** and allowed to stand overnight under shielded condition at room temperature for staining. After staining, the stained section was incubated for 30 min with equilibrium buffer (Amersham CO., Ltd.) to remove excess fluorescence probes. Then, the stained section was placed on a slide glass and covered by a cover glass after adding a drop of antifadant reagent (Slow Fade™ Antifade Kits, Molecular Probes Inc.). The observation of these sections was carried out by a fluorescence microscope (ECLIPSE E800, Nicon CO., Ltd.) with an appropriate filter (B-2A, Nicon CO., Ltd; excitation wavelength 450–490 nm). At this time, the use of an antifadant reagent was essential to prevent photobleaching (fading of fluorescence). Figure 2 shows photographs of plant pulvini, which contains motor cell, under a fluorescence microscope. The staining pattern for the fluorescence of probe **2** was observed on the surface of the motor cell (Fig. 2). No stain was observed in the control section, which was treated with an aqueous solution containing no **2** (Fig. 2). These results revealed that the target cell for **1** is a motor cell, which plays a central role in the plant leaf movement,[15] as with leaf-opening substances.[16]

The binding of probe can be strongly correlated with leaf-closing activity. Biologically inactive *epi*-**2** did not bind with motor cell at all. This result strongly suggested that some receptor for **1**, which is located in the motor cell and specifically recognizes the stereochemistry of probe **2**, would be involved in the leaf-closing movement of *P. urinaria*. In conclusion, we have succeeded in visualization of the target cell of the leaf-closing substance in the plant body by using novel probe **2**. Some receptor for **1** would be involved in the leaf-closing movement in *P. urinaria*. To reveal a receptor protein of **1**, the syntheses of photoaffinity labeling probes based on **1** are now in progress.

Acknowledgments

This work was supported by the Ministry of Education, Science, Sports and Culture (Japan) Grant-in-Aid for Scientific Research (No. 15681013) and Grant-in-Aid for the 21st Century COE program of Tohoku University from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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13. Compound **2**: ^{1}H NMR (400 MHz, D_2O, rt): 6.75 (1H, dd, J = 10.0, 2.4 Hz), 6.47 (1H, br d, J = 10.0 Hz), 5.95 (1H, br s), 5.15 (1H, ddd, J = 13.6, 5.2, 2.0 Hz), 4.85 (1H, dddd, J = 10.4, 5.2, 2.4, 2.0 Hz), 4.69 (1H, d, J = 8.0 Hz), 3.91 (1H, dd, J = 12.4, 2.4 Hz), 3.72 (1H, dd, J = 12.4, 5.6 Hz), 3.50 (1H, t, J = 8.8 Hz), 3.48 (1H, ddd, J = 8.8, 5.6, 2.4 Hz), 3.39 (1H, t, J = 8.8 Hz), 3.27 (1H, dd, J = 8.8, 8.0 Hz), 3.03 (1H, dt, J = 10.4, 5.2, Hz), 1.78 (1H, dt, J = 13.6, 10.4 Hz); ^{13}C NMR (100 MHz, D_2O, rt) 178.3, 167.1, 142.1, 122.1, 112.1, 103.5, 80.9, 77.5, 77.1, 75.7, 74.9, 71.1, 62.6, 39.5 ppm; HR ESI-MS (positive): $[M+H]^+$. Found: m/z 315.1054, $C_{14}H_{19}O_8$ requires m/z 315.1080; IR (film) ν: 3388, 1735, 1637, 1585, 1387 cm^{-1}; $[\alpha]_D^{25}$ −30.0 (c 0.11, H_2O); $[\alpha]_D^{21}$ −6.0 (c 0.20, H_2O) in naturally occurring **1**.
14. *epi*-**2**: ^{1}H NMR (400 MHz, D_2O, rt): 6.75 (1H, dd, J = 10.0, 2.4 Hz), 6.47 (1H, br d, J = 10.0 Hz), 5.95 (1H, br s), 5.15 (1H, ddd, J = 13.6, 5.2, 2.0 Hz), 4.85 (1H, dddd,

J = 10.4, 5.2, 2.4, 2.0 Hz), 4.69 (1H, d, J = 8.0 Hz), 3.91 (1H, dd, J = 12.4, 2.4 Hz), 3.72 (1H, dd, J = 12.4, 5.6 Hz), 3.50 (1H, t, J = 8.8 Hz), 3.48 (1H, ddd, J = 8.8, 5.6, 2.4 Hz), 3.39 (1H, t, J = 8.8 Hz), 3.27 (1H, dd, J = 8.8, 8.0 Hz), 3.03 (1H, dt, J = 10.4, 5.2, Hz), 1.78 (1H, dt, J = 13.6, 10.4 Hz); HR ESI-MS (positive): $[M+H]^+$. Found: *m/z* 315.1054, $C_{14}H_{19}O_8$ requires *m/z* 315.1080.

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