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Syntheses of novel photoaffinity probes for bioorganic studies on nyctinasty of leguminous plants

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Abstract—Novel and non-radioactive photoaffinity probes (**1** and **2**) for the bioorganic study of nyctinasty are designed and synthesized based on potassium isolespedezate (**3**), which induce leaf-opening against the leaf of *Cassia mimosoides* L. These probes bear a trifluoromethyldiazirine or diazophenyl group for photoaffinity and a biotin subunit for affinity chromatography and chemiluminescent detection. Probes (**1** and **2**) showed leaf-opening activity at 5×10^{-5} mol/L with leaves of *C. mimosoides*; thus, they would be an important tool for the identification of a receptor protein for **3**.

Most leguminous plants close their leaves in the evening, as if to sleep, and open them early in the morning. This circadian rhythm is known to be controlled by the biological clock of such plants. We have identified several bioactive substances that regulate this leaf-movement, and our recent studies revealed the mechanism for the control of nyctinasty by the biological clock.[1] The next issue is to explore how these compounds induce leaf-movement.

A recent fluorescence study using fluorescence labeled probe compound designed on the structure of potassium isolespedezate (**3**), which induces leaf-opening against the leaf of *Cassia mimosoides* L., revealed that the target cell for **3** is motor cells.[2] Motor cells, which are contained in pulvini, are most important for leaf-movement because their change in volume induces leaf-movement. Leaf-opening substance **3** is supposed to induce the swelling and shrinking of motor cells by direct interaction with them to control leaf-movement. Because of its hydrophobicity, **3** is supposed not to be able to pass through the plasma membrane of a motor cell. Thus, it was assumed that some receptors for **3** would exist on a plasma membrane of the motor cell of *C. mimosoides*.

The next issue of our research is focused on investigation of the role of leaf-movement factors in leaf-movement, especially, the perception of the leaf-movement factor by its receptor and the following signal transduction. Exploration of receptor molecule is the starting point for a better understanding of how leaf-movement factors function. Thus, it was strongly desired to develop novel probe compounds for the identification and characterization of receptor protein for the leaf-movement factor. Photoaffinity labeling can provide important information about the binding protein for bioactive substances of low molecular weight.[3] In the photoaffinity labeling experiments, a trifluoromethyldiazirine or phenyl azide group is usually used as a photoaffinity group.[3] Now, we have synthesized two photoaffinity probes (**1** and **2**) designed on the structure of **3** for the detection and identification of a receptor protein for **3**.

Both of our synthetic photoaffinity probes have a photoaffinity group and a biotin unit in the molecule: one has a trifluoromethyldiazirine group and the other has a phenyl azide group for photoaffinity labeling, and both of them have a biotin subunit for purification of the binding protein by affinity chromatography and chemiluminescent detection. These biotinyl photoaffinity probes enable a non-radiochemical detection of labeled protein on a polyvinylidene difluoride (PVDF) membrane.[4]

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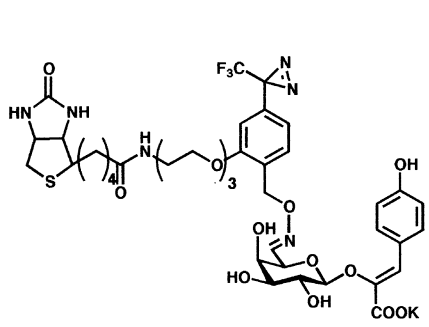
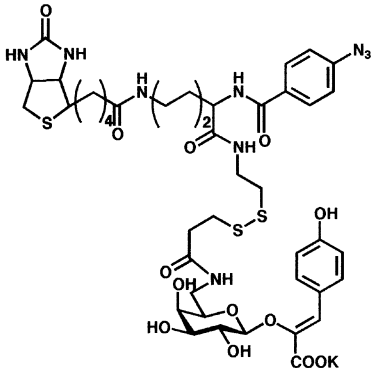


1



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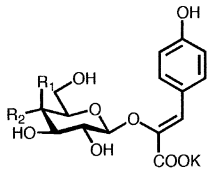
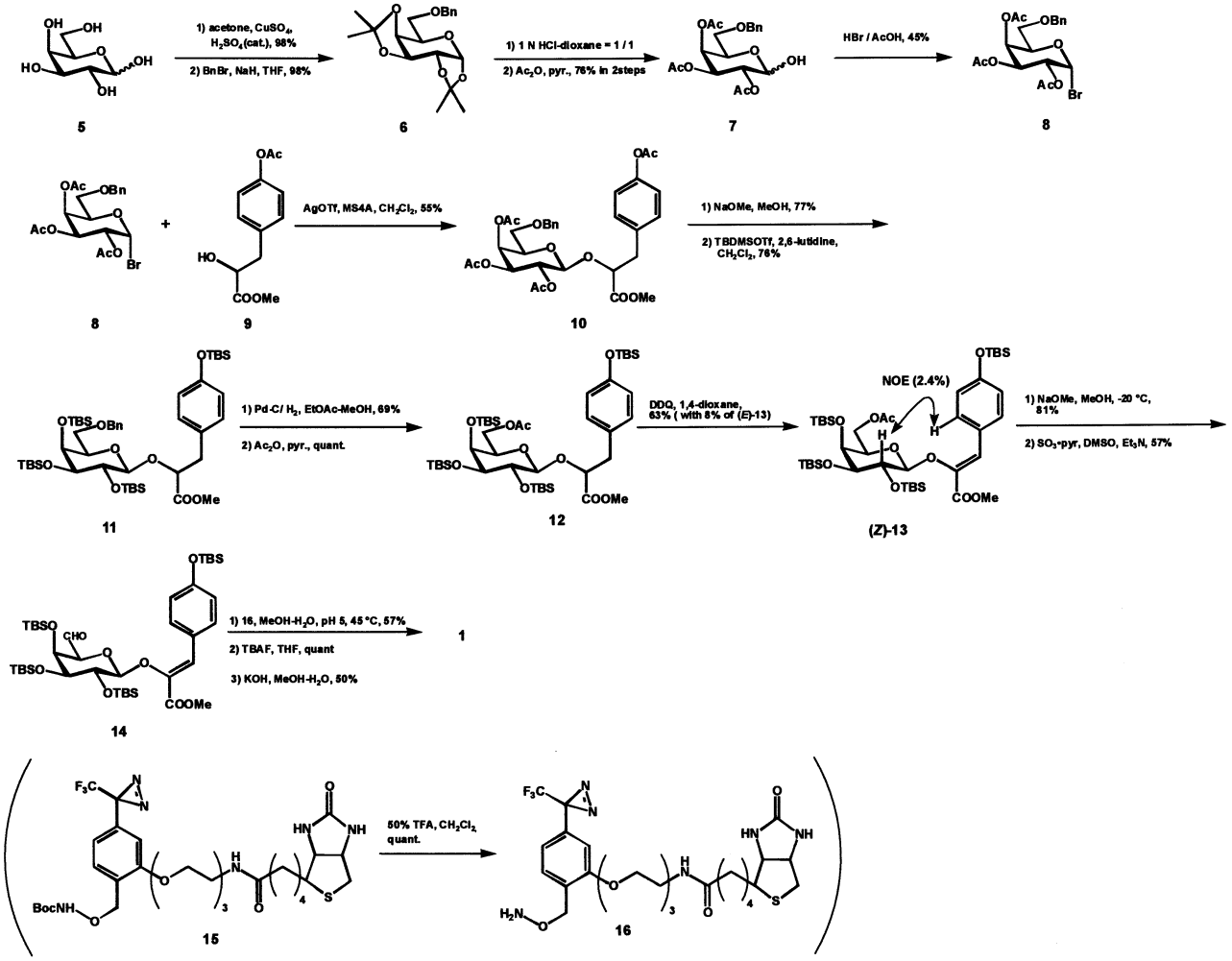


3 : R_1 = H, R_2 = OH
4 : R_1 = OH, R_2 = H

The most important issue is the introduction of large photoaffinity and biotin groups with retention of bioactivity. The molecular design of photoaffinity probes followed that of previously reported fluorescence-labeled probes. From the structure–activity relationship study of **3**, it was revealed that structure modification in the sugar moiety of **3** did not diminish its bioactivity; thus, we introduce these large functionalities into the 6-position of the sugar moiety. Also, we used galactoisolespedezate (**4**) instead of **3** to circumvent enzymatic hydrolysis of the glycosidic linkage in the plant body.[2]

Synthesis of photoaffinity probe **1** was carried out according to the synthetic route in Scheme 1. For the sake of later oxidation, the primary hydroxy group of galactose should be protected by a different protective group from secondary hydroxy groups. Thus, 6-*O*-ben-



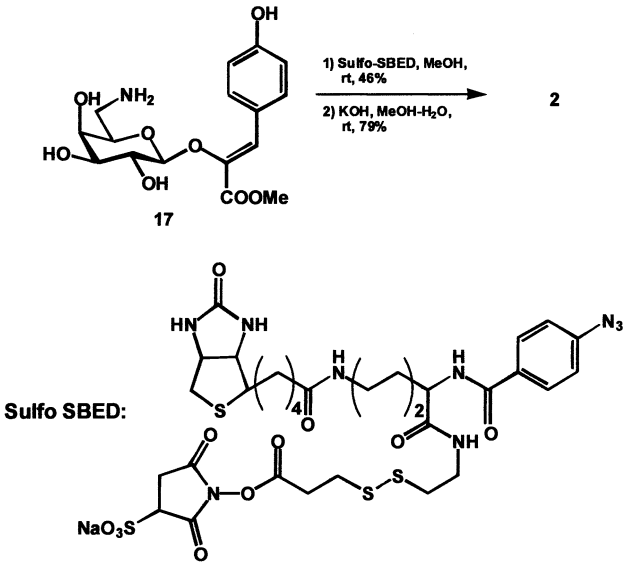
Scheme 1. Synthesis of photoaffinity probe **1**.

zyl diacetonegalactose (**6**) was synthesized by selective acetonide formation between secondary hydroxy groups in **5** using copper sulfate with catalytic sulfuric acid[5] and following protection of the residual primary hydroxy group with a benzyl group. After acid treatment and following acetylation, compound **7**, which has different protective groups on primary and secondary hydroxy groups, was obtained. Coupling of **8** with **9** gave **10**, and then the product was subjected to deacetylation, and the resulting secondary hydroxy groups were protected with TBSOTf to give **11**. After conversion of the protective group on the 6-position of the galactose moiety in **11** from the benzyl to an acetyl group, resulting **12** was treated with excess amount of DDQ (7 equiv.) for 2 days to give **13**. This oxidation gave an 8:1 mixture of (*Z*)- and (*E*)-**13**. The stereochemistry was assigned by NOE correlation observed in (*Z*)-**13** (Scheme 1). These stereoisomers were separable by column chromatography. The selective deacetylation of **13** was achieved by carrying out the reaction at −20°C. When the reaction was carried out at higher temperature, the TBS group on the phenolic hydroxy group was deprotected simultaneously. Then, the resulting primary hydroxy group was oxidized to give aldehyde (**14**). Coupling reaction of aldehyde (**14**) with **16** which has a trifluoromethydiazirine and biotin group[4] was carried out in aqueous methanol, and the following deprotections and purification by HPLC using Develosil C8 UG-5 column gave the two isomers on the oxime moiety of photoaffinity probe **1** (0.8 mg of (*E*)-**1** and 0.8 mg of (*Z*)-**1**, respectively).[6]

Photoaffinity probe **2** was synthesized according to the procedure reported in Ref. 2. Coupling of amine (**17**)[2] with sulfosuccinimidyl [2-6-(biotinamido)-2-(*p*-azidobenz-amido)-hexanoamido]ethyl-1,3′-dithiopropionate (sulfo SBED)[7] which has phenyl azide and biotin groups gave 2.4 mg of **2** (Scheme 2).[8]

Both of the resulting photoaffinity probes **1** and **2** showed leaf-opening activity against the leaf of *C. mimosoides* at 5×10^{-5} M (Table 1). (*E*)-**1** was as effective as (*Z*)-**1** on the bioassay. The bioactivity was about one-tenth of that of natural product (**3**). Thus, we have succeeded in the development of bioactive photoaffinity probes for the bioorganic studies of nyctinasty.

In previous fluorescence studies,[2] our fluorescence-labeled probes bound to motor cells selectively. This result suggests that no false binding to the protein contained in neighboring cells was observed. This highly selective binding will be also expected in our photoaffinity probes that were synthesized according to the same molecular design as fluorescent probes. Thus, our bioactive photoaffinity probes would be highly useful for the detection and identification of a receptor protein for **3** on the motor cell. With a large quantity of plasma membrane fraction collected by ultracentrifugation of plant extract, the exploration of a receptor molecule for **3** will be carried out using these photoaffinity probes.



Scheme 2. Synthesis of photoaffinity probe **2**.

Acknowledgements

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Table 1. Time-course change of the status of the leaves treated with 5×10^{-5} M of **1**, **2**, and **3**

	Status of the leaves						
	16:00	17:00	18:00	19:00	20:00	21:00	22:00
1	++	++	++	++	++	++	++
2	++	++	++	++	++	++	++
3	++	++	++	++	++	++	++
Blank	++	++	+−	−−	−−	−−	−−

Status of the leaf was represented by the following marks: ++ completely open; + nearly open; +− at random; − nearly closed; −− completely closed.

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6. (*Z*)-**1**: ^{1}H NMR (400 MHz, CD_3OD, rt): 7.76 (2H, d, $J=8.8$ Hz), 7.33 (1H, d, $J=7.8$ Hz), 6.85 (1H, s), 6.82 (1H, d, $J=7.8$ Hz), 6.74–6.67 (3H, m), 5.13 (2H, s), 4.65 (1H, dd, $J=1.4$, 4.9 Hz), 4.44 (1H, dd, $J=4.4$, 7.3 Hz), 4.25 (1H, dd, $J=4.4$, 7.8 Hz), 4.17–4.10 (3H, m), 3.87–3.78 (3H, m), 3.70–3.54 (3H, m), 3.51 (2H, t, $J=5.8$ Hz), 3.15 (1H, m), 2.88 (1H, dd, $J=4.9$, 12.7 Hz), 2.66 (1H, d, $J=12.7$ Hz), 2.18 (2H, t, $J=7.3$ Hz), 1.75–1.48 (4H, m), 1.44–1.31 (2H, m) ppm; HR FAB MS (negative): $[M-K]^-$ Found m/z 925.2872, $C_{40}H_{48}O_{14}F_3N_6S$ requires m/z 925.2901; IR (film) ν: 3309, 1685, 1650, 1608, 1578 cm^{-1}; $[\alpha]_D^{22}$ +66.1 (*c* 0.62, MeOH); (*E*)-**1**: ^{1}H NMR (400 MHz, CD_3OD, rt): 7.76 (2H, d, $J=8.8$ Hz), 7.49 (1H, d, $J=6.3$ Hz), 7.32 (1H, d, $J=7.8$ Hz), 6.84 (1H, s), 6.77 (1H, d, $J=7.8$ Hz), 6.71 (2H, d, $J=8.8$ Hz), 6.70 (1H, s), 5.12 (1H, d, $J=13.7$ Hz), 5.07 (1H, d, $J=13.7$ Hz), 4.59 (1H, br), 4.44 (1H, dd, $J=4.4$, 7.8 Hz), 4.25 (1H, dd, $J=4.4$, 7.4 Hz), 4.14–4.09 (3H, m), 3.85–3.78 (3H, m), 3.68–3.55 (5H, m), 3.50 (2H, t, $J=4.9$ Hz), 3.14 (1H, m), 2.89 (1H, dd, $J=4.4$, 12.2 Hz), 2.67 (1H, d, $J=12.2$ Hz), 2.17 (2H, d, $J=6.8$ Hz), 1.74–1.50 (4H, m), 1.42–1.34 (2H, m) ppm.; HR FAB MS (negative): $[M-K]^-$ Found m/z 925.2906, $C_{40}H_{48}O_{14}F_3N_6S$ requires m/z 925.2901; IR (film) ν: 3309, 1687, 1650, 1645, 1608, 1579 cm^{-1}; $[\alpha]_D^{22}$ +82.8 (*c* 0.53, MeOH).
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8. **2**: ^{1}H NMR (400 MHz, CD_3OD, RT): 7.95 (2H, d, $J=8.8$ Hz), 7.75 (2H, d, $J=8.8$ Hz), 7.15 (2H, d, $J=8.8$ Hz), 6.79 (1H, s), 6.73 (2H, d, $J=8.8$ Hz), 4.53–4.43 (2H, m), 4.26 (1H, dd, $J=4.4$, 7.8 Hz), 3.78 (3H, t, $J=7.8$ Hz), 3.73 (1H, d, 3.4 Hz), 3.44–3.55 (6H, m), 2.90 (1H, dd, $J=4.9$, 12.7 Hz), 2.83–2.75 (4H, m), 2.67 (1H, d, $J=12.7$ Hz), 2.39 (2H, t, $J=7.3$ Hz), 2.17 (2H, t, $J=7.8$ Hz), 1.79–1.98 (2H, m), 1.24–1.78 (10H, m) ppm; HR FAB MS (negative): $[M-K]^-$ Found m/z 1002.3184, $C_{43}H_{56}N_9O_{13}S_3$ requires m/z 1002.3160; IR (film) ν: 3296, 2126, 1644, 1604 cm^{-1}; $[\alpha]_D^{23}$ +68.4 (*c* 0.80, MeOH).