

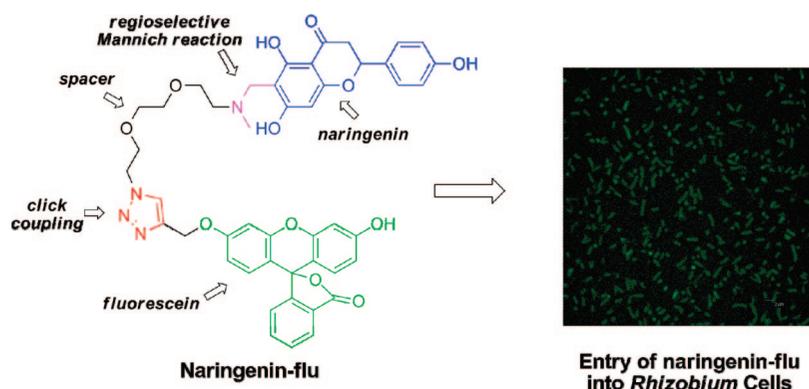
Site-Specific Fluorescent Labeling Approaches for Naringenin, an Essential Flavonone in Plant Nitrogen-Fixation Signaling Pathways

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In search of an appropriate position for the fluorescent labeling, six chemically available positions of the flavonone core of naringenin have been examined. A number of azido-containing naringenin derivatives were accordingly prepared in various site-specific fashions, and the mild Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition successfully served as the common “Click” labeling tool in the final steps. On the basis of the biological activities of the first batch of labeled compounds, further optimization at the C-6 position of naringenin finally afforded naringenin-flu (**27**), which acquired 20% of the potency of naringenin and presented good optical properties. Entry of naringenin-flu into living *Rhizobium* cells was demonstrated by in vitro fluorescent imaging experiments.

Introduction

Flavonoids (phenylbenzopyrones) are widely present in all vascular plants and are associated with quite a number of biological properties, including antioxidant, anti-inflammatory, and antiviral activities.^{1,2} Some have been found to play very important roles in nitrogen fixation in a variety of legume species.³ Naringenin (**1**, Figure 1) is a representative in this family of natural products. Many studies have shown that it is

a key signaling molecule in the control of root nodulation, a prerequisite for the plant nitrogen fixation.⁴ Genetics evidence^{5,6} also indicates that the binding of naringenin to gene product nod-D initiates the production of NodRm-IV,⁷ a bacterial nodulation signal molecule, and NodRm-IV, in turn, affects the

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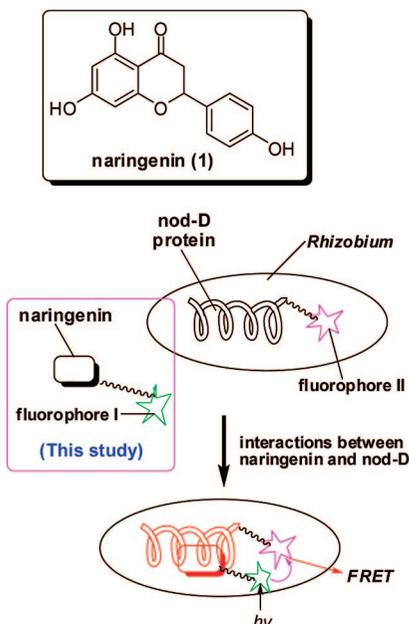


FIGURE 1. A FRET protocol proposed for detecting the interactions between naringenin and nod-D.

changes in legume root cells that lead to root hair deformation.⁸ To prove the binding of the flavonoids to nod-D proteins and to reveal the details of the nature of putative binding site, an organometallic IR probe was designed by Anson and co-workers.⁹ The interactions were tracked by detecting a distinct wavelength shift of the tricarbonyliron flavonone derivatives. Though those organometallic flavonoid derivatives were able to induce nod gene expression, the corresponding FTIR investigations in the presence of nod-D-containing fractions of lysed *Rhizobium* cells could not provide definite conclusions.¹⁰

Fluorescent labeling is the process of covalently attaching a fluorophore to the studied molecule, providing the optical information by fluorescence reading instruments such as a fluorescence microscope. It is thus a suitable protocol for detecting the bindings of naringenin to nod-D after its entry into *Rhizobium* cells. In contrast to the infrared methods, it usually presents much higher sensitivity even at very low concentrations. This would be more advantageous in the biological experiments. More importantly, fluorescent compounds would be able to provide the real-time and visible information inside the living cells. To demonstrate the interactions between naringenin and nod-D protein, utilization of fluorescent visualization techniques including fluorescence resonance energy transfer (FRET)^{11–13} would be a predominant choice (Figure 1). For such a purpose, a biologically active

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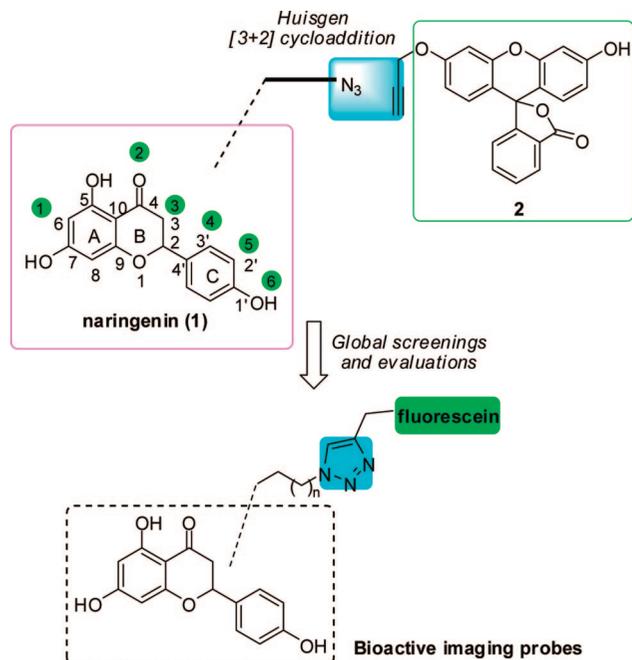


FIGURE 2. Representative positions of naringenin (1) for Click fluorescent labeling.

naringenin derivative labeled with a qualified fluorophore is thus requested. In this article, we report our efforts in achieving a qualified fluorescent probe of naringenin, including the synthesis and biological assessment of different labeled compounds, further optimizations to a final qualified probe, and the application of this probe to the fluorescent imaging in living *Rhizobium* cells.

Since few structure–activity relationships are known, it is very difficult to predict a suitable position for the fluorophore attachment on naringenin. In addition, many frequently used fluorophores are a comparative size to naringenin. Their attachments might result in decreasing or losing the biological activities. Acquisition of a qualified labeled naringenin derivative with satisfactory biological and optical properties is therefore very challenging work. No matter how this process is complicated, a suitable labeling position should be determined at first. Six chemically available positions on the A-, B-, or C-ring of naringenin were then selected for the first round of fluorophore attachment (Figure 2, positions 1–6 labeled in green color). The corresponding biological evaluation of labeled naringenin derivatives would help us judge the direction for the next endeavor. Because multiple functional groups in the substrates (naringenin derivatives) would be exposed to the reaction media, a milder reaction would be preferred for the final labeling step. Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition^{14,15} was thus chosen to fulfill this task. Based on such a design, azido-containing naringenin derivatives and a fluorophore containing terminal acetylene functionality are requisite.

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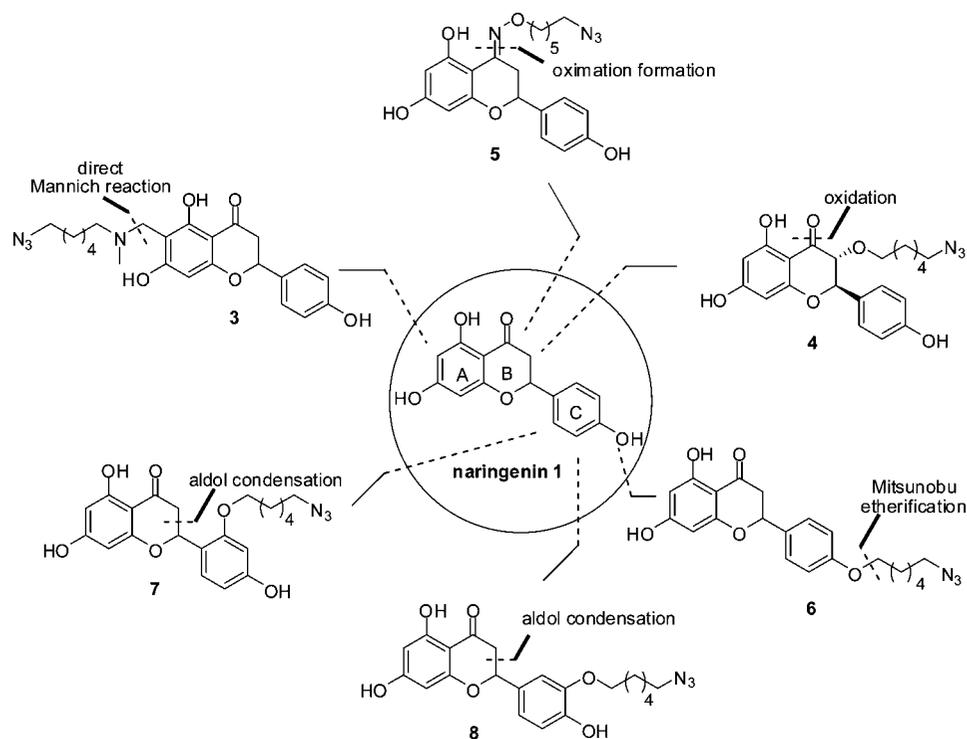


FIGURE 3. Six azido-containing derivatives and their major synthetic protocols.

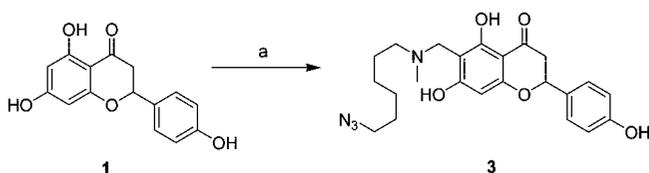
In biology, nod-D gene induction by the labeled compounds could be used as the activity assessment protocol. The biological data comparison would enable us to determine a better position for further modification on the skeleton of naringenin. Further amendments would include adjustment of the nature and length of the linker, and the linkage formations between naringenin and the fluorophore, until a qualified fluorescent probe is achieved.

Results and Discussion

Synthesis of Azido-Containing Naringenin Derivatives As Labeling Precursors. The first batch of flavonoid derivatives were designed as the labeling precursors bearing an azido functionality on the A-, B-, or C-ring of naringenin via the same hydrocarbon spacer (Figure 3). Regioselective formation of a new C–C bond at the C-6 position of the A-ring of naringenin was accomplished in our previous study¹⁶ using a protecting-group-free direct Mannich reaction. Treatment of the commercially available (\pm)-naringenin (**1**)¹⁷ with paraformaldehyde and *N*-methyl-6-azidohexane-1-amine in the presence of 10 mol % of ZnCl₂ in EtOH at 60 °C afforded only adduct **3** in an excellent yield (Scheme 1).

On the contrary, achieving a similar direct modification on the B- or C-ring of naringenin is impossible. The total syntheses

SCHEME 1^a



^a Reagents and conditions: (a) (HCHO)_n, N₃CH₂(CH₂)₅NHCH₃, ZnCl₂, 60 °C, 90%.

were adopted instead. Afzelechin derivative **12** was readily prepared from chalcone **9** in three steps (Scheme 2). Etherification of the *trans*-3-flavanol **12** with 1-azido-6-bromohexane under basic conditions afforded the corresponding azido-afzelechin derivative **13**. Successive oxidations with DDQ and PDC furnished the chromanone **15**. Global deprotection of *O*-methoxymethyl protecting groups was carried out in 2 N HCl solution in MeOH under reflux, providing the azido-precursor **4** in 93% yield.

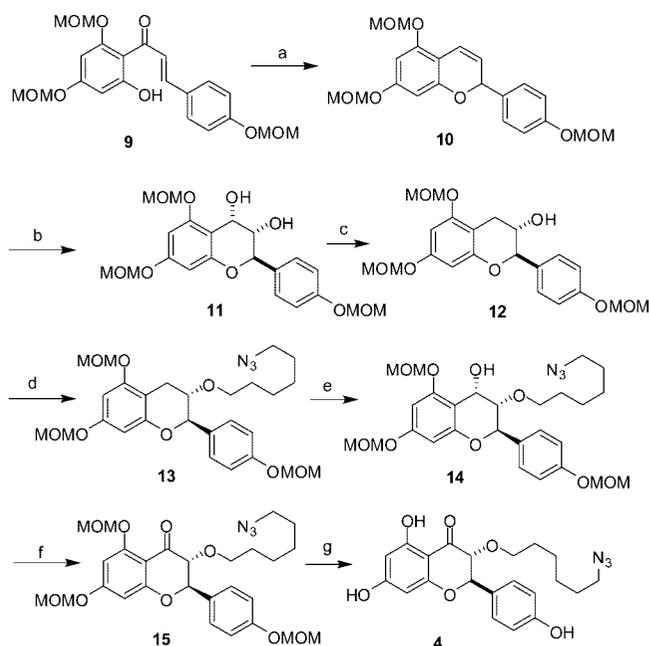
Facile oximido chemistry was applied in the synthesis of another B-ring azido-precursor **5**. This compound was conveniently prepared in 83% yield by a single step condensation of naringenin with *O*-(6-azidohexyl)hydroxylamine in ethanol under reflux (Scheme 3).

Three options are available for the modification of the C-ring of naringenin, including the C-1' phenol functionality (via *O*-alkylation) and the C-2' and C-3' C–H bonds (via new C–O bond formations). The *O*-alkylation of the C-1' phenol hydroxyl group of naringenin was carried out in an indirect way. Treatment of naringenin with *tert*-butylchlorodiphenylsilane in the presence of imidazole and a catalytic amount of DMAP in DMF afforded the mono *O*-TBDPS ether **16**, whose C-1' phenol hydroxyl group was then condensed with 6-azidohexan-1-ol under Mitsunobu's conditions. Final removal of the *O*-TBDPS protecting group with TBAF in THF afforded the azido-compound **6** in 85% yield (Scheme 4).

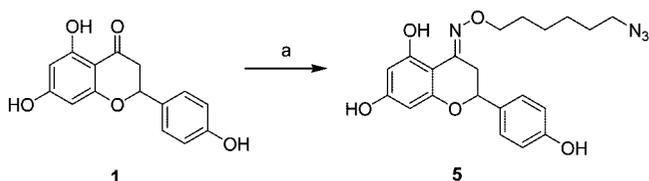
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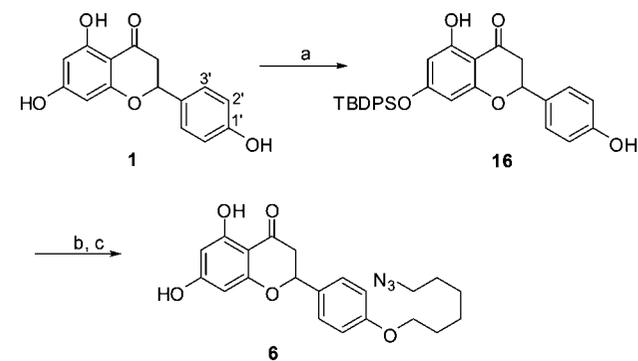
(17) In all reported works, the commercially available (\pm)-naringenin was used in the nod-D gene induction experiments and other related studies in plant nitrogen fixation.

SCHEME 2^a

^a Reagents and conditions: (a) NaBH₄, THF, EtOH, 94%; (b) OsO₄, 4-methylmorpholine 4-oxide, *t*-BuOH, H₂O, 95%; (c) NaBH₃CN, AcOH, 92%; (d) KH, BrCH₂(CH₂)₅N₃, THF, 98%; (e) DDQ, CH₂Cl₂, 75%; (f) PDC, CH₂Cl₂, 80%; (g) 2N HCl, MeOH, 93%.

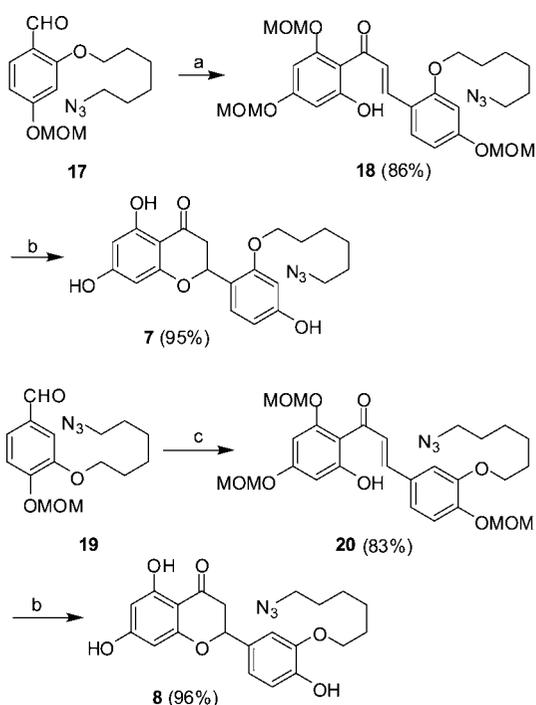
SCHEME 3^a

^a Reagents and conditions: (a) NH₂OCH₂(CH₂)₅N₃, pyridine, ethanol, reflux, 83%.

SCHEME 4^a

^a Reagents and conditions: (a) TBDPSCl, imidazole, DMF, 90%; (b) DEAD, PPh₃, HOCH₂(CH₂)₅N₃, THF, 92%; (c) TBAF, THF, 85%.

Two other C-ring azido-precursors **7** and **8** were prepared by traditional dihydroflavone synthesis (Scheme 5). Reaction of benzaldehyde **17** with 4',6'-bis(methoxymethoxy)-2'-hydroxyacetophenone in KOH solution in EtOH–H₂O yielded the chalcone **18** in 86% yield. Deprotection of MOM ethers and intramolecular cyclization were accomplished in one pot with 2 N HCl–MeOH under reflux, providing the azido-compound **7** in 95% yield. Accordingly, another C-ring azido-compound

SCHEME 5^a

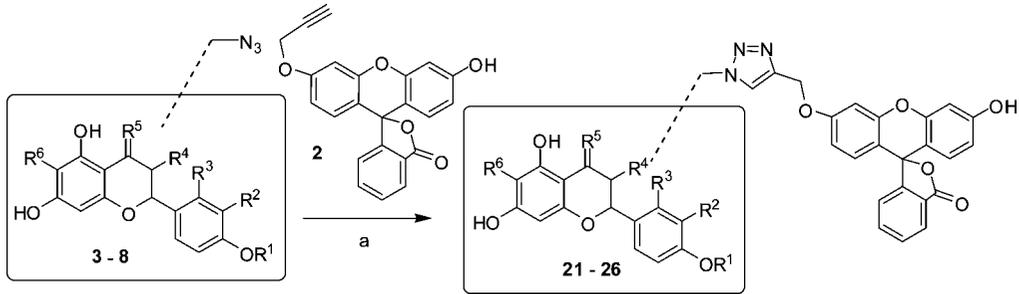
^a Reagents and conditions: (a) 4',6'-bis(methoxymethoxy)-2'-hydroxyacetophenone, KOH, EtOH–H₂O; (b) 2N HCl, MeOH, reflux, 1 h; (c) 50% a. NaOH–dioxane (v/v, 1:1).

8 was prepared in 73% overall yield through a similar procedure from the benzaldehyde **19**.¹⁸

“Click” Couplings with the Fluorophore and Evaluations of the Labeled Compounds. Attachments of the fluorophore to azido derivatives **3**–**8** were accomplished by Cu(I)-catalyzed Huisgen reaction under mild conditions. Parallel 1,3-dipolar cycloadditions of these azido compounds with acetylene-fluorescein **2**,¹⁶ an easily prepared and economic labeling reagent, were carried out in *t*-BuOH–H₂O in the presence of catalytic amounts of CuSO₄·5H₂O and ascorbic acid. Six fluorescently labeled naringenin derivatives **21**–**26** were afforded in satisfactory isolated yields (Table 1).

The physical property characterizations show that all six labeled compounds **21**–**26** exhibit the characteristic absorption and emission of fluorescein (Table 2). In respect to the optical properties, our labeling approach for naringenin is successful. Biological activities of these derivatives were also examined. Labeled compound **21** bearing a fluorophore at the C-6 position (A ring) remains approximately 9% of the bioactivity of naringenin, while five other compounds **22**–**26** are almost inactive (Table 2). Therefore, the C-6 position of the A-ring of naringenin would be the most favorable site for further modifications. Using a newly formed C–C bond, this modification well preserved the parent skeleton and retains all the original functionalities in naringenin. Further endeavor toward a qualified fluorescent probe was thus decided to focus on the C-6 position of naringenin.

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TABLE 1. Parallel “Click” Couplings of Azido Precursors 3–8 with the Fluorophore 2^a


azides	triazoles	labeled position ^b	other positions	yields (%)
3	21	R ⁶ = CH ₂ N(CH ₃)(CH ₂) ₄ CH ₂ -X	R ¹ = R ² = R ³ = R ⁴ = H, R ⁵ = O	67
4	22	R ⁴ = OCH ₂ (CH ₂) ₃ CH ₂ -X	R ¹ = R ² = R ³ = R ⁶ = H, R ⁵ = O	71
5	23	R ⁵ = NOCH ₂ (CH ₂) ₃ CH ₂ -X	R ¹ = R ² = R ³ = R ⁴ = R ⁶ = H	92
6	24	R ¹ = OCH ₂ (CH ₂) ₃ CH ₂ -X	R ² = R ³ = R ⁴ = R ⁶ = H, R ⁵ = O	63
7	25	R ³ = OCH ₂ (CH ₂) ₃ CH ₂ -X	R ¹ = R ² = R ⁴ = R ⁶ = H, R ⁵ = O	84
8	26	R ² = OCH ₂ (CH ₂) ₃ CH ₂ -X	R ¹ = R ³ = R ⁴ = R ⁶ = H, R ⁵ = O	81

^a Reagents and conditions: 2, CuSO₄·5H₂O (cat.), ascorbic acid, *t*-BuOH, H₂O, rt. ^b X = Click reaction site, from azides (reactants) to triazoles (products).

TABLE 2. Optical Properties^{a,b} and Enzyme Activities^c of the Labeled Naringenin Derivatives 21–26

compd no.	λ _{ex1} (nm)	ε ₁ (cm ⁻¹ M ⁻¹)	λ _{em1} (nm)	Φ ₁	λ _{ex2} (nm)	ε ₂ (cm ⁻¹ M ⁻¹)	λ _{em2} (nm)	Φ ₂	enzyme activity index ^{d,e}
21	454	24132	539	0.11	480	19628	530	0.13	50.7
22	453	4488	539	0.09	480	3724	533	0.18	16.4
23	453	6081	538	0.08	479	4810	533	0.16	1.1
24	454	11180	544	0.03	479	9156	530	0.08	17.2
25	453	11103	538	0.07	481	9405	530	0.11	17.8
26	453	11288	534	0.04	479	9138	541	0.05	17.7
1 ^f									588.3

^a All were measured in methanol. ^b Quantum yields were measured and calculated relative to quinine in 1 M H₂SO₄ as a standard (excited at 478 nm). ^c The biological activities were measured by β-galactosidase activity assays¹⁹ at the same cell density (OD₆₀₀ of 0.4). ^d Naringenin fluorescent derivatives (8 μM) were used in the experiments. ^e Enzyme activity index is the average of results of at least 3 assays and is corrected by the control values. ^f Commercially available (±)-naringenin¹⁷ (Sigma) was used as the control.

Further Optimizations. Our previous imaging study²⁰ suggests that the improvement of water solubility of the probe might enhance the bioactivity. Therefore, naringenin derivative **27** with an embedded hydrophilic ethylene–glycol–ether spacer was designed (Figure 4). To exclude the uncertain biological roles of 1,2,3-triazoles in the bindings,^{21–24} another naringenin derivative **28** with a thiocarbamide linkage was also listed in our plan.

Azide intermediate **27a** prepared from the direct Mannich reaction of (±)-naringenin with N₃CH₂CH₂(OCH₂CH₂)₂NHCH₃ and paraformaldehyde was subjected to fluorescein derivative **2** in the presence of CuSO₄·5H₂O (cat.) and ascorbic acid, giving the labeled compound **27** (termed as naringenin-flu in

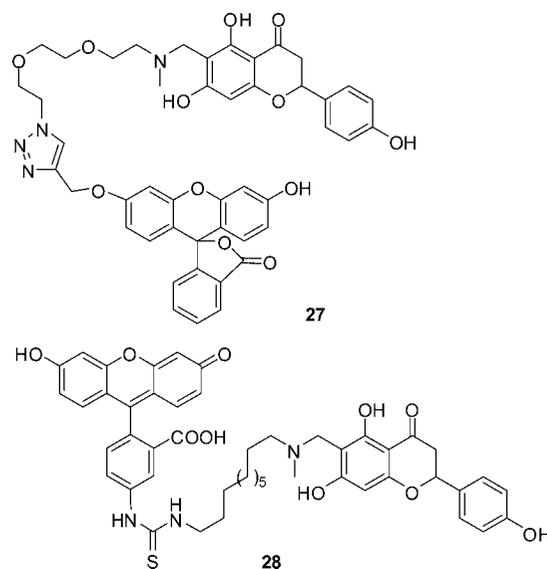


FIGURE 4. Two additional derivatives **27** and **28** designed for further evaluations.

the following text) in a satisfactory yield (Scheme 6). In parallel, removal of *N*-Boc functionality of another Mannich adduct **29** followed by coupling with commercially available fluorescein isothiocyanate (FITC) provided the corresponding thioamide compound **28** (Scheme 6).

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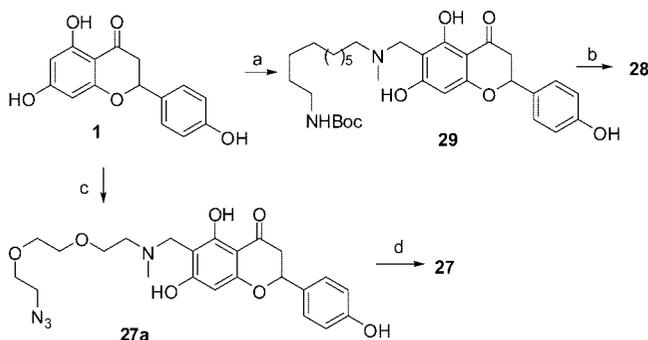
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TABLE 3. Optical Properties and Bioactivities of the Labeled Compounds **27** and **28**^{a,b,c}

compd no.	λ_{ex1} (nm)	ϵ_1 (cm ⁻¹ M ⁻¹)	λ_{em1} (nm)	Φ_1	λ_{ex2} (nm)	ϵ_2 (cm ⁻¹ M ⁻¹)	λ_{em2} (nm)	Φ_2	enzyme activity index ^{d,e,f}
27	453	39950	537	0.09	479	30693	536	0.12	116.5
28	481	12339	526	0.07					31.6

^a All optical properties were measured in methanol. ^b Quantum yields were measured and calculated relative to quinine in 1 M H₂SO₄ as a standard (excited at 478 nm). ^c Biological activities of the compounds were measured by β -galactosidase activity assays¹⁹ at the same cell density (OD600 of 0.4). ^d Naringenin derivatives (8 μ M) were used in the experiments. ^e Enzyme activity index is the average results of at least 3 assays and is corrected by the control values. ^f (\pm)-Naringenin was used as the control with an enzyme activity index of 588.3.

SCHEME 6^a

^a Reagents and conditions: (a) BocNHCH₂(CH₂)₈CH₂NHCH₃, HCHO, EtOH, 81%; (b) TFA, CH₂Cl₂, then FITC, DIPEA, THF, 62%; (c) (HCHO)_n, N₃CH₂CH₂(OCH₂CH₂)₂NHCH₃, ZnCl₂, 65 °C, 76%; (d) **2**, CuSO₄·5H₂O (cat.), ascorbic acid, *t*-BuOH, H₂O, rt, 84%.

Both compounds **27** and **28** were found to exhibit satisfactory optical properties according to the corresponding characterizations (Table 3). To our delight, naringenin-flu (**27**) acquires approximately 20% of the activity of naringenin, though the other thiocarbamide derivative **28** is inactive (Table 3). With its satisfactory bioactivity and optical properties, naringenin-flu (**27**) should be qualified for further biological experiments in living *Rhizobium* cells.

Validation of the Use of a Racemic Probe. Though all known reports in nod-D signalings and plant nitrogen-fixation used the commercially available racemic naringenin (Sigma),¹⁷ it is still of importance to validate the rationality of employing a racemic probe in further biological experiments. A straightforward way is to examine the difference of the biological effects of (*R*)- and (*S*)-naringenin in regulating the nod-D signaling. Both samples of (*R*)- and (*S*)-naringenin (>99% ee by HPLC) were carefully prepared by chiral HPLC separation of the racemic material.²⁵ Their enzyme activities were then examined under normal conditions.²⁶ To our surprise, the results show that (*S*)-naringenin, (*R*)-naringenin, and (\pm)-naringenin exhibited no observable difference in potency after 18 h of induction (see the Supporting Information for details). To the best of our knowledge, this is the first time such an unusual situation has been experimentally demonstrated, though its cause remains unknown. With these data, we thought the use of an enantiopure probe of naringenin would be unnecessary in further applications.

Cell Permeability of Naringenin-Flu. Entry of naringenin-flu (**27**) into *Rhizobium* cells was unambiguously confirmed by visible evidence under a confocal microscope. Fluorescent

imaging²⁷ clearly shows that naringenin-flu (**27**) permeated into the *Rhizobium* cells, which retained livability and unceasing random movements (see the Supporting Information for the imaging picture). Since the size of *Rhizobium* cells is hundreds of times smaller than that of mammalian cells, further imaging-based studies on living *Rhizobium* cells are still technically challenging with the current instrumental accuracy. Application of naringenin-flu (**27**) to the FRET experiments and other biological topics associated with nod-D protein and plant nitrogen fixation is underway in this laboratory.

Conclusion

A bioactive naringenin derivative, naringenin-flu (**27**), has been acquired after global modification of the available positions of natural product and further focused optimization. The mild catalytic Huisgen 1,3-dipolar cycloaddition successfully served as the common “Click” labeling tool in the last step. Various methodologies developed for the site-specific modifications of naringenin and for the facile syntheses of new naringenin derivatives will be helpful in the biological applications of naringenin and other flavonones. Naringenin-flu (**27**) was found to acquire approximate 20% of the activity of naringenin, and its entry into the living *Rhizobium* cells was unambiguously observed in the corresponding fluorescent imaging experiment.

Experimental Section

Mannich Adduct 27a. To a solution of (\pm)-naringenin (157 mg, 0.58 mmol) and paraformaldehyde (35 mg, 1.17 mmol) in ethanol (5 mL) was added the secondary amine (MeNH-(CH₂CH₂O)₂CH₂CH₂N₃) (130 mg, 0.69 mmol) at room temperature. The mixture was then heated to 65 °C and stirred at this temperature until the reactants were consumed. EtOAc (30 mL) and diluted aqueous HCl (30 mL, pH 3) were added. The aqueous phase was adjusted to pH 7 by adding solid NaHCO₃, and then extracted with EtOAc. The combined extracts were dried over anhydrous Na₂SO₄ and concentrated. The crude product was purified by flash column chromatography (CH₂Cl₂/MeOH = 50/1 to 15/1) to afford azide **27a** (208 mg, 76%) as a white foam. IR (KBr) ν_{max} 2878, 2110, 1679, 1638, 1519, 1459, 1344, 1303, 1170, 136, 1087, 836 cm⁻¹. ¹H NMR (CDCl₃, 300 MHz) δ 7.15 (2H, d, *J* = 8.4 Hz), 6.67 (2H, d, *J* = 8.1 Hz), 5.59 (1H, s), 5.18 (1H, dd, *J* = 12.6, 3.0 Hz), 3.61 (2H, s), 3.50–3.31 (8H, m), 3.15 (2H, t, *J* = 4.8 Hz), 2.91 (1H, dd, *J* = 16.8, 12.6 Hz), 2.59 (2H, t, *J* = 5.4 Hz), 2.48 (1H, dd, *J* = 17.4, 3.0 Hz), 2.17 (3H, s). ¹³C NMR (CDCl₃, 75 MHz) δ 196.0, 169.3, 162.5, 160.9, 157.7, 130.0, 128.1, 115.2, 101.2, 100.8, 95.5,

(27) Competent 8401/pKT230 cells (100 μ L) were mixed with naringenin-flu **27** (1 μ L, 4 μ M). The mixture was electroschocked at 1000 V for 4.5 ms with a micropulser (wave shape PL5). The mixture was then incubated at 28 °C for half an hour in darkness and centrifuged at 12 000 rpm for 10 min. The sediments were washed with TEG buffer three times and re-solved in TEG buffer (50 μ L). Sample (5 μ L) was dropped to the microplate and photos taken with a confocal microscope. The excitation wavelength is 488 nm. The scale bar is 2 μ m (for details, see Figure S-1 in the Supporting Information).

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78.9, 70.3, 69.8, 67.7, 55.9, 52.4, 50.5, 42.6, 40.6. HRMS calcd for $C_{23}H_{29}N_4O_7$ ($M + H$)⁺ 473.2031, found 473.2023.

Naringenin-Flu (27). To a mixture of azide **27a** (50 mg, 0.11 mmol), fluorescein derivative **2** (43 mg, 0.11 mmol) in *t*-BuOH (2 mL), and H₂O (2 mL) were added CuSO₄·5H₂O (2 mg, 0.008 mmol) and ascorbic acid (5 mg, 0.011 mmol). The reaction was stirred at room temperature for 24 h. The mixture was diluted with EtOAc (50 mL). The organic phase was washed with brine, dried over anhydrous Na₂SO₄, and concentrated. The residue was purified by silica gel column chromatography (CH₂Cl₂/MeOH = 25/1 to 10/1) to afford naringenin-flu (**27**, 75 mg, 84%). IR (KBr) ν_{\max} 3385, 2944, 1752, 1707, 1597, 1501, 1459, 1369, 1250, 1182, 1110, 1024, 836 cm⁻¹. ¹H NMR (acetone-*d*₆, 300 MHz) δ 8.12 (1H, s), 7.99 (1H, d, *J* = 7.2 Hz), 7.78–7.71 (2H, m), 7.35 (2H, d, *J* = 8.7 Hz), 7.26 (1H, d, *J* = 7.5 Hz), 7.00 (1H, d, *J* = 2.1 Hz), 6.88 (2H, d, *J* = 8.4 Hz), 6.79–6.77 (1H, m), 6.74–6.69 (2H, m), 6.64 (2H, br), 5.78 (1H, s), 5.35 (1H, dt, *J* = 12.9, 3.0 Hz), 5.23 (2H, s), 4.58 (2H, t, *J* = 4.8 Hz), 3.89 (2H, t, *J* = 4.8 Hz), 3.83 (2H, s), 3.66–3.55 (6H, m), 3.09 (1H, dd, *J* = 17.1, 12.9 Hz), 2.83 (2H, t, *J* = 5.4 Hz), 2.66 (1H, dd, *J* = 17.1, 3.0 Hz), 2.39 (3H, s). ¹³C

NMR (acetone-*d*₆, 75 MHz) δ 195.7, 170.4, 168.6, 162.5, 161.2, 160.2, 159.7, 157.8, 153.0, 152.4, 152.4, 142.6, 135.2, 130.0, 129.9, 129.2, 129.1, 128.1, 126.9, 124.7, 124.5, 124.1, 115.3, 112.7, 112.4, 112.0, 110.5, 102.5, 101.7, 100.9, 100.4, 95.7, 82.7, 78.8, 70.1, 69.1, 67.2, 61.8, 55.7, 52.6, 49.9, 42.5, 40.3, 29.2. HRMS calcd for $C_{46}H_{42}N_4O_{12}$ ($M + H$)⁺ 843.2872, found 843.2897.

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Supporting Information Available: Detailed procedures and characterizations of new compounds, confocal imaging of *Rhizobium* cells, chiral HPLC separation of (*R*)- and (*S*)-naringenin, determination of the enzyme activities, and NMR copies of new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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