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Synthesis of fluorescent molecular probes based on *cis*-cinnamic acid and molecular imaging of lettuce roots

Hiroshi Fukuda [b], Keisuke Nishikawa [a], Yukihiro Fukunaga [a], Katsuhiro Okuda [a], Kozue Kodama [a], Kenji Matsumoto [a], Arihiro Kano [a], Mitsuru Shindo [a,*]

[a] Institute for Materials Chemistry and Engineering, Kyushu University, 6-1, Kasuga-koen, Kasuga 816-8580, Japan
[b] Interdisciplinary Graduate School of Engineering Sciences, Kyushu University, 6-1, Kasuga-koen, Kasuga 816-8580, Japan

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ABSTRACT

We synthesized azo dye- and fluorescence-labeled *cis*-cinnamic acid analogues possessing inhibitory activity against lettuce root growth and a *trans*-isomer without bioactivity as a control probe. The radicles incubated with the azo dye-labeled analogue were stained red, with their tips especially deeply dyed. The fluorescent images of the radicles incubated with each of these molecular probes depicted that the root cap was fluorescence-stained. However, images of the control radicles prepared by staining with the *trans*-isomer fluorescent probe did not show emission at the root cap. These contrasts suggest specific localization of the *cis*-cinnamate analogue at the columella cells.

1. Introduction

Allelopathy is an important self-defense system in higher plants, which is based on the production of secondary metabolites that show inhibitory or stimulatory interactions with other plants, including microorganisms.[1] Allelochemicals would provide insights into the molecular mechanisms of bioactivity and enable the design of useful bioactive compounds, especially agrochemicals.[2] While numerous plant ecological and plant physiological studies on allelopathy have been reported, studies on the underlying molecular mechanisms still remain unexplored.

1-*O*-*cis*-Cinnamoyl-β-D-glucopyranose (**1**) (Fig. 1), isolated by Hiradate and Fujii from *Spiraea thunbergii* as a potent allelochemical, shows growth-inhibitory activity on root elongation of germinated seedlings of lettuce (*Lactuca sativa* L.).[3] A crucial structure responsible for the bioactivity of **1** is *cis*-cinnamic acid (*cis*-CA, *cis*-**2**), which also works as an effective inhibitor of lettuce root growth similar to **1**, whereas *trans*-cinnamic acid (*trans*-CA, *trans*-**2**), which would be a *cis*-**2** precursor as well as a common plant metabolite, is not effective for such inhibition.[4] While *trans*-**2** is considered as a weak antagonist of auxin,[5–7] *cis*-**2** has an auxin-like activity.[8] While mechanistic studies based on molecular biology have been stated,[9–12] the molecular mechanisms of the inhibitory activity as well as target molecules of *cis*-**2** have not yet been explored. Recently, we published the structure–activity relationship (SAR) studies on *cis*-CA growth inhibition,[13] including the substituent effect of *cis*-**2** as well as more potent synthetic analogues.[14] We also found *cis*-**2** selective suppressors, the bioactivities of which were distinct from those of both auxin and anti-auxin, suggesting mechanistic insights of auxin-independent signaling pathways.[10b,15] The subsequent issues for mechanistic investigations are to clarify the plant target sites for *cis*-**2**. Fluorescent molecular probes are a powerful tool for visualization of target or localized sites of bioactive compounds.[16] We report herein the synthesis of fluorescence-labeled *cis*-**2** as a molecular probe as well as its molecular imaging, which indicates the localization of *cis*-**2** in a lettuce radicle.

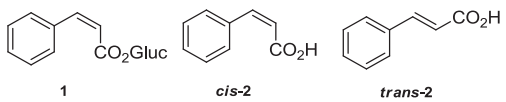

Fig. 1. Cinnamates.

* Corresponding author. E-mail address: shindo@cm.kyushu-u.ac.jp (M. Shindo).

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2. Results and discussion

2.1. Design of molecular probes for bioimaging

Our previous SAR studies revealed that the essential structural components for bioactivity were the *cis*-configuration of the alkene, a carboxylate, and a planar ring. Furthermore, the substituent effect on the aromatic ring of *cis*-**2** also disclosed that *para*- and *ortho*-substitution tended to decrease its potency for the inhibition, but *meta*-substituted *cis*-**2** analogues were more likely to be potent. Development of reliable molecular fluorescent probes requires that they should show bioactivity corresponding to the original bioactive compounds. Additionally, in order to avoid false conclusions on target sites by non-specific binding of the probes to biopolymers, control experiments using biologically inactive fluorescence-labeled analogues are required.[17] Based on this concept, we designed the fluorophore-possessing *cis*-**2** analogues as a molecular probe, and a biologically inactive *trans*-isomer (*trans*-**2**) as a control probe. Our previous SAR study indicated that the *meta*-substitution would be less negatively effective for the bioactivity described above, thereby suggesting that the *meta* position of the aromatic ring would be a suitable connecting position of the linker having a fluorophore at another terminal (Figs. 2 and 3). Although *m*-methoxy and *m*-ethoxy analogues (*cis*-**3a** and *cis*-**3b**) showed similar bioactivity to *cis*-**2**, sterically bulky *m*-alkoxy analogues (*cis*-**3c** and *cis*-**3d**) reduced the bioactivity (Table 1, entries 1–4). This tendency demonstrated that substitutions, even at the *meta* position, were sensitive to steric effects on the bioactivity. We thus concluded that alkyl ethers were not suitable as a connector between *cis*-**2** and a dye.

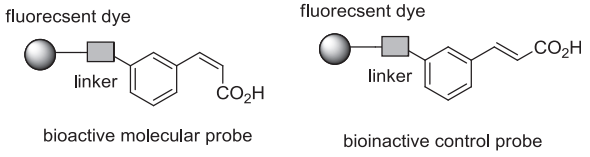


Fig. 2. Design of fluorescent probe for *cis*-**2**.

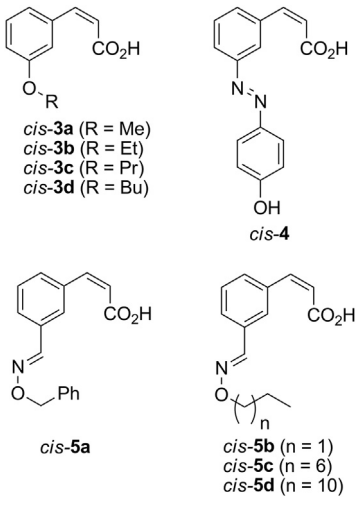


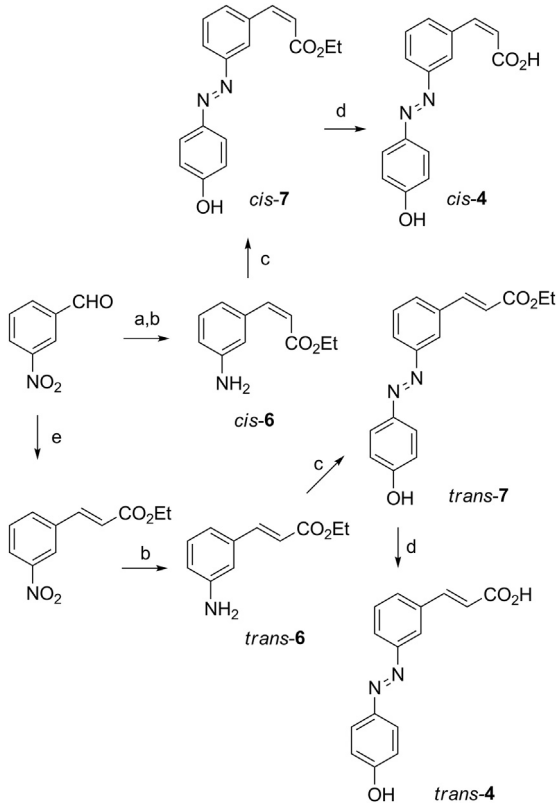
Fig. 3. *meta*-Substituted *cis*-**2** analogues.

After screening of the other functional groups at the *meta* position as a connector, we found a azo group as a potential candidate that could be easily prepared. Azo analogues **4** were prepared as shown in Scheme 1. *m*-Nitrobenzaldehyde was olefinated with the modified Horner–Wadsworth–Emmons reagent,[18] followed by reduction to afford *cis*-**6**, which in turn was subjected to a diazo-coupling reaction with phenol to give *cis*-**7**. Finally, *cis*-**7** was hydrolyzed to provide *cis*-**4**. The *trans*-isomer (*trans*-**4**) was also prepared via the same procedure.

Table 1
Growth inhibitory activities of *meta*-substituted *cis*-**2** analogues

Entry	Compounds	EC_{50} (μM)[a]
1	*cis*-**3a**	1.1
2	*cis*-**3b**	9.3
3	*cis*-**3c**	150
4	*cis*-**3d**	110
5	*cis*-**4**	20
6	*trans*-**4**	>500

[a] EC_{50} values are the effective concentration required to induce a half-maximum effect against root-growth of lettuce (*L. sativa* cv.).



Scheme 1. Synthesis of diazo analogues *cis*-**4** and *trans*-**4**: (a) ethyl 2-[bis(2-isopropylphenoxy)phosphoryl]acetate, Triton B, THF, −78 °C: 83%, (b) $SnCl_2$, THF/H_2O, reflux, 92% for *cis*-**6**, 64% for *trans*-**6**, (c) $NaNO_2$, concd HCl aq, THF, 0 °C then phenol, NaOAc, DMF/MeOH, 87% for *cis*-**7**, 68% for *trans*-**7**, (d) 10% NaOH, EtOH, rt, 56% for *cis*-**4**, 56% for *trans*-**4**, (e) ethyl 2-(diethoxyphosphoryl)acetate, Triton B, THF, −78 °C, 60%.

The bioactivity of *cis*-**4** (EC_{50} 20 μM) was expected to be potent enough as a probe, because the corresponding *trans*-**4** was inactive (EC_{50} >500 μM) (Table 1, entries 5 and 6). Since the phenylazo compounds (*cis*- and *trans*-**4**) displayed a red color, they were expected to be visible molecular probes for molecular imaging without the use of a fluorescent microscope. Figure 4 demonstrates the lettuce radicles stained by 1000 μM of *cis*-**4** and *trans*-**4** after incubation for 48 h. The radicles with their growth inhibited by *cis*-**4** were stained red as a whole, with especially their tips deeply dyed, while radicles treated with *trans*-**4** grew normally and were hardly stained. This contrast of staining suggested that *cis*-**4** would likely be incorporated into the radicles and inhibited their growth by localizing there; however, *trans*-**4** would be taken up only

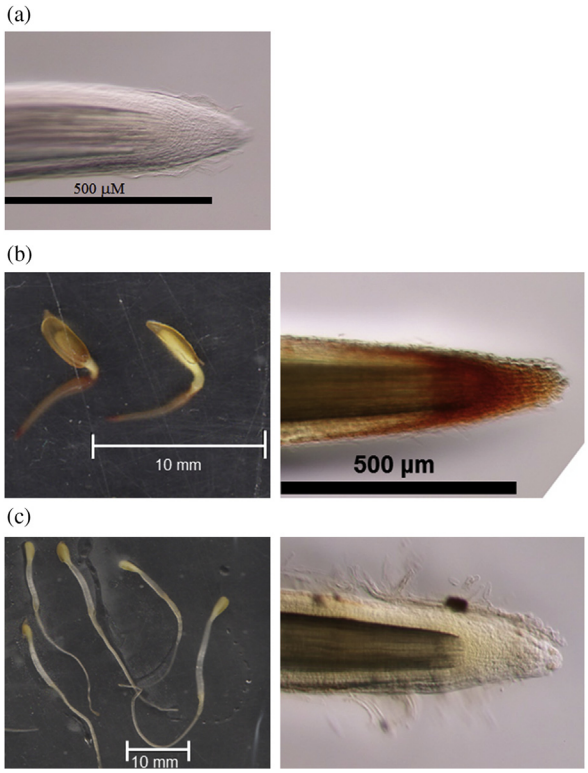


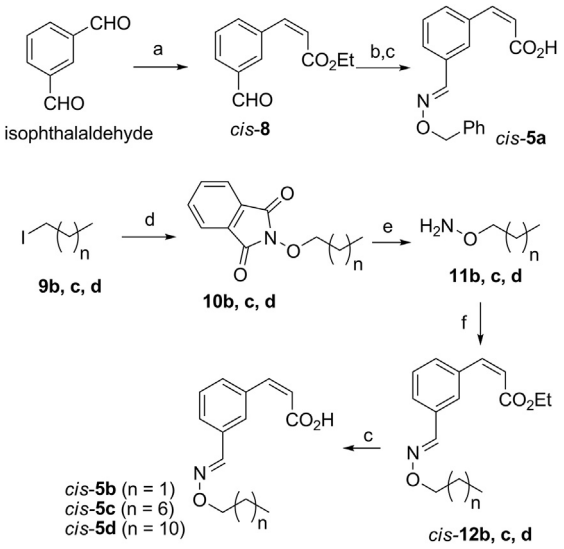
Fig. 4. The lettuce radicles stained by (a) no compound, (b) *cis*-**4**, and (c) *trans*-**4** after incubation for 48 h at 1000 μM of each compounds.

slightly or would be metabolized rapidly, resulting in no effects on their growth. These experiments can be regarded as concise molecular imaging which can be performed without the use of expensive facilities and can be visualized by the naked eye.

2.2. Synthesis of fluorescent probes

In order to detect more precise sites of localization of the inhibitors with higher sensitivity and contrasts, we decided to prepare fluorescent probes. Judging from the azo probe experiments described above, we deduced that sp^2 hybridized atoms were important as a connector unit, without the loss of bioactivity. We thus adopted oxime ethers as an alternative sp^2-hybridized atom connector, because they can be easily constructed.[19] As shown in Scheme 2, isophthalaldehyde was mono-olefinated with the modified Horner–Wadsworth–Emmons reagent to give *cis*-**8**, which was then condensed with *O*-benzylhydroxylamine to afford an oxime ether *cis*-**5a**. The bioactivity of *cis*-**5a** was 4.0 μM despite having sterically hindered benzyl ether (Table 2, entry 1). This may be owing to the planar sp^2 carbon linkage of oxime moiety, which is less sterically hindered in the vicinity of the *meta*-position on the aromatic ring. Encouraged by this result, we further examined the length of the carbon-chain linker moiety. *O*-Alkoxyamines (**11b**–**d**) were prepared from *N*-alkoxyphthalimides (**10b**–**d**), which were derived from the alkylation of *N*-hydroxyphthalimide. The alkoxyamines (**11b**–**d**) were condensed with *cis*-**8**, followed by hydrolysis, to afford oxime ethers (**5b**–**d**). Although propyl (*cis*-**5b**) and dodecyl (*cis*-**5d**) oxime ethers were not potent, the bioactivity of octyl oxime ether (*cis*-**5c**) was similar to that of *cis*-**2** (Table 2). These results suggested dependence of bioactivity on the length of the carbon-chain linker.

We then synthesized various fluorescent probes with different linker lengths and fluorophores. After fluorophore screening, we selected fluorescein as a fluorophore.[20] The fluorescein-labeled *cis*-



Scheme 2. (a) Ethyl 2-[bis(2-isopropylphenoxy)-phosphoryl]acetate, Triton B, THF, −78 °C, 91%, (b) *O*-benzylhydroxylamine hydrochloride, pyridine, EtOH, reflux, 92%, (c) 10% NaOH aq, EtOH, rt, *cis*-**5a**: 93%, *cis*-**5b** (n=1): 66%, *cis*-**5c** (n=6): 85%, *cis*-**5d** (n=10): 53%, (d) NaH, *N*-hydroxyphthalimide, DMF, 70 °C, *cis*-**10b** (n=1): 89%, *cis*-**10c** (n=6): 92%, *cis*-**10d** (n=10): 85%, (e) $NH_2NH_2 \cdot H_2O$, EtOH, rt, (f) *cis*-**8**, EtOH, 50 °C to rt, 89% for *cis*-**12b** (n=1), 79% for *cis*-**12c** (n=6), 89% for *cis*-**12d** (n=10).

Table 2
Growth inhibitory activities of *cis*-**5**

Entry	Compounds	EC_{50} (μM)[a,b]
1	*cis*-**5a**	4.0
2	*cis*-**5b**	170
3	*cis*-**5c**	2.4
4	*cis*-**5d**	>500

[a] EC_{50} values are the effective concentration required to induce a half-maximum effect against root-growth of lettuce (*L. sativa* cv.).
[b] All the corresponding *trans*-**5** analogues were inactive (EC_{50} >500 μM).

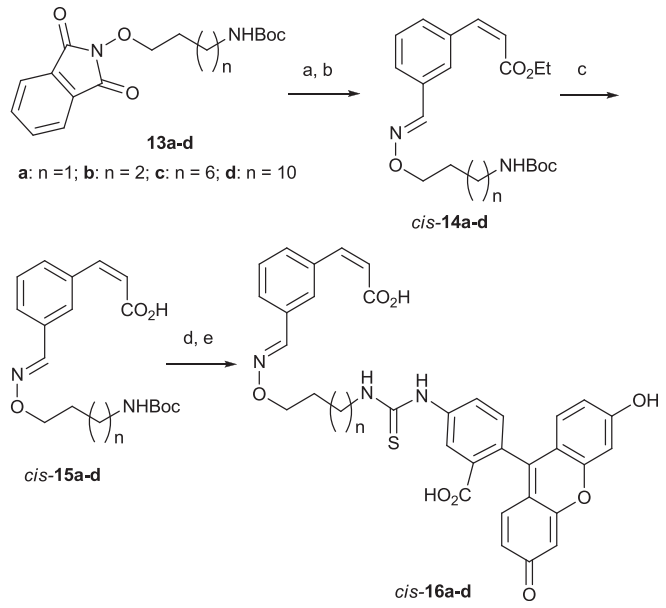
cinnamic acids were prepared as shown in Scheme 3. The compounds **13a**–**d** were treated with hydrazine for deprotection of phthalimide moiety, followed by formation of oxime ethers *cis*-**14a**–**d** by addition of the aldehyde *cis*-**8**. After hydrolysis of ethyl ester, the resulting carboxylic acids *cis*-**15a**–**d** were deprotected by treatment with trifluoroacetic acid (TFA) to afford the amino acids, which were treated with FITC to provide the fluorescent probes *cis*-**16a**–**d**. In the same manner, *trans*-**16c** was also prepared as a negative control as shown in Scheme 4.

2.3. Inhibitory activity of fluorescein-labeled *cis*-cinnamic acids

We examined the inhibitory activity of the fluorescein-labeled *cis*-CA analogues against the root-growth of lettuce (Table 3). Among the four *cis*-analogues, *cis*-**16c** was seen to be most potent. Although the EC_{50} value (58 μM) was less than that of *cis*-**2**, it would be suitable for bioimaging, because *trans*-**16c** was observed to have lesser bioactivity (Table 3, entry 5). Based on these results, we decided to use *cis*-**16c** and *trans*-**16c** as fluorescent and control probes, respectively.

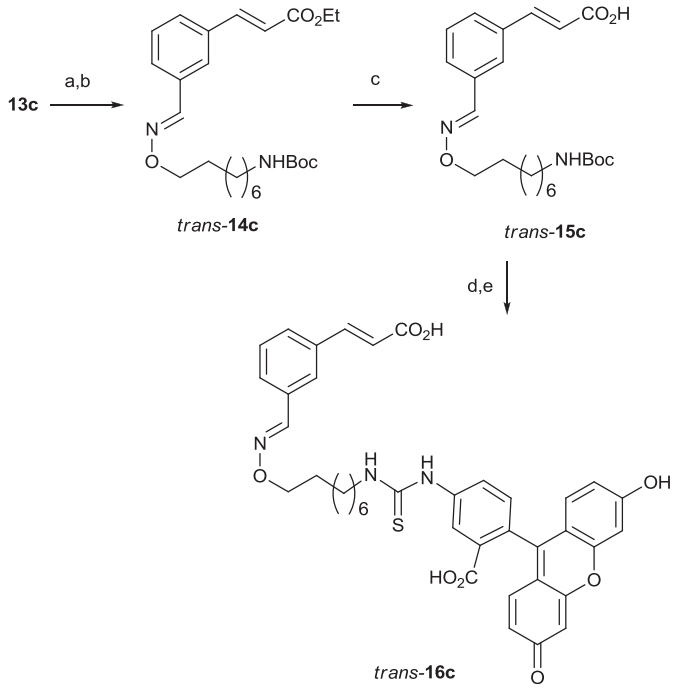
2.4. Molecular imaging

With the biologically active fluorescent probe and the control probe, we then examine molecular imaging using lettuce roots for investigating the distribution of the bioactive compounds in the



Scheme 3. Synthesis of fluorescent probes: (a) $NH_2NH_2 \cdot H_2O$, EtOH, rt, (b) *cis*-**8**, EtOH, rt, 90% for *cis*-**14a**; 95% for *cis*-**14b**; 74% for *cis*-**14c**; 84% for *cis*-**14d**, (c) 10% NaOH, EtOH, rt, 87% for *cis*-**15a**, 67% for *cis*-**15b**, 87% for *cis*-**15c**, 98% for *cis*-**15d**, (d) TFA, CH_2Cl_2, rt, (e) FITC, Et_3N, MeOH, reflux, 54% for *cis*-**16a**, 43% for *cis*-**16b**, 65% for *cis*-**16c**, 65% for *cis*-**16d**.

radicles. The probes *cis*- and *trans*-**16c** were dissolved in water at concentrations of 50 μM each. Pregerminated seedlings (at 25 °C in water in the dark) of lettuce (*L. sativa* cv. Great Lakes 366) were stained by incubation with these fluorescent-labeled compound solutions for 48 h at 25 °C in the dark. After being washed with ethanol for 30 s, the radicles were placed on a glass slide and monitored by a fluorescent microscope. We focused on the tip of the radicles, where the images (200×) showed the fluorescence emission by *cis*-**16c** (Fig. 5B), but no emission was seen by the unstained control (Fig. 5A) and *trans*-**16c** (Fig. 5B). This contrast obviously indicated the specific emission by *cis*-**16c**.



Scheme 4. Synthesis of *trans*-**16c**: (a) $NH_2NH_2 \cdot H_2O$, EtOH, rt, (b) *trans*-**8**, EtOH, rt, quant., (c) 10% NaOH, EtOH, rt, 97%, (d) TFA, CH_2Cl_2, rt, (e) FITC, Et_3N, MeOH, reflux, 21%.

Table 3
Inhibitory activity of the fluorescent probes on the root-growth of lettuce seedlings (*L. sativa* cv.)

Entry	Compounds	*N*	EC_{50} (μM)
1	*cis*-**16a**	1	>500
2	*cis*-**16b**	2	260
3	*cis*-**16c**	6	58
4	*cis*-**16d**	10	>500
5	*trans*-**16c**	6	>500

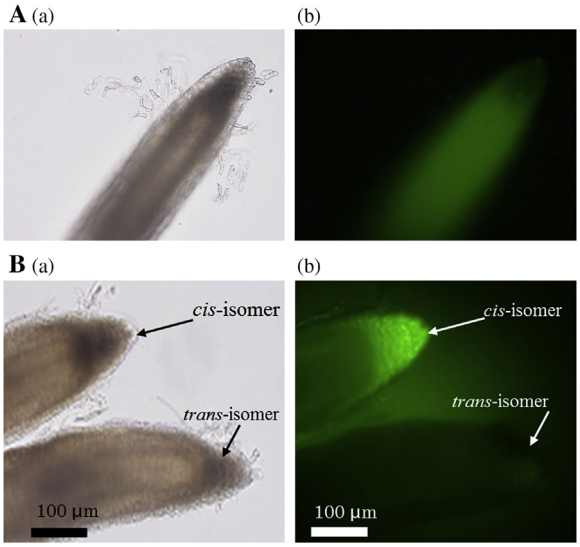


Fig. 5. Images of the tips of radicles. **A**: control (200×): (a) bright; (b) fluorescent image, **B**: stained by *cis*-**16c** and *trans*-**16c** (200×): (a) bright; (b) fluorescent image. Conditions: 200× (exposure time: left 1/230, right 1/35).

To obtain more precise images and to determine the sites of localization, we observed the radicles treated by the fluorescent probe *cis*-**16c** using confocal fluorescence microscopy. As shown in Figure 6a, the fluorescence was observed at the region of the root cap. The magnified photo (Fig. 6b) indicated that the fluorescence could be traced to the granules, the diameter of which was approximately 2 μm. Judging from these fluorescence images, we speculated that amyloplasts in the columella cells may be stained by the fluorescent probe.

2.5. Competition experiments

To confirm that the localizing site of *cis*-**16c** in the radicles was identical to that of *cis*-**2**, competitive inhibition between *cis*-**2** and

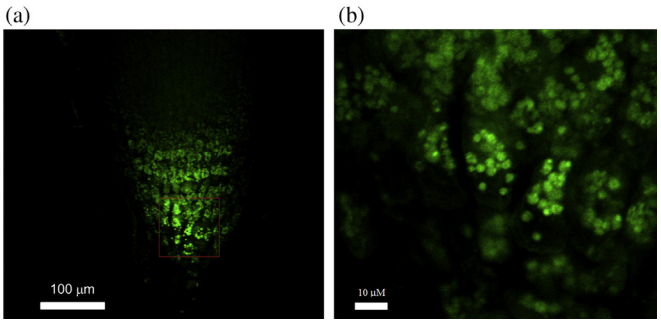


Fig. 6. Fluorescent images of a radicle stained by *cis*-**16c** with a confocal fluorescence microscope.

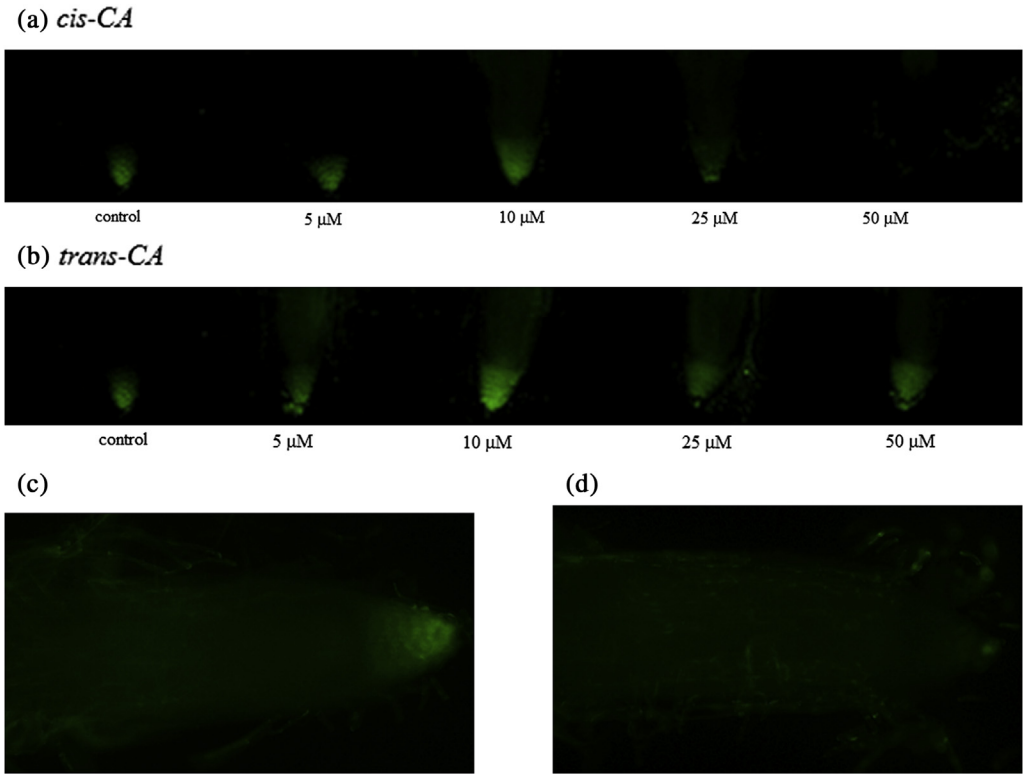


Fig. 7. Competitive inhibition. Fluorescent images of lettuce radicle treated with (a) a mixture of *cis*-**16c** (50 μM) and *cis*-**2** (5–50 μM), (b) a mixture of *cis*-**16c** (50 μM) and *trans*-**2** (5–50 μM). Fluorescent images of (c) the *cis*-**16c** (50 μM)-stained lettuce radicle, (d) the *cis*-**16c** (50 μM)-stained radicle followed by treatment with *cis*-**2** (50 μM).

cis-**16c** was carried out. Germinated lettuce seeds were treated with a mixture of *cis*-**16c** (50 μM) and *cis*-**2** (0–50 μM). After 24 h of incubation at 25 °C, the surfaces of the radicles were washed with ethanol, and fluorescence was observed. As shown in Figure 7a, the fluorescence reduced at 25 μM of *cis*-**2** and disappeared with 50 μM of *cis*-**2**, whereas *trans*-**2** did not affect the fluorescence of *cis*-**16c**.

For the same purpose, the germinated lettuce seeds were incubated with *cis*-**16c** (50 μM) for 24 h at 25 °C, and then *cis*-**2** was added at a concentration of 50 μM. After incubation for 2 h, surfaces of radicles were washed with ethanol. Figure 7d shows the disappearance of fluorescence by addition of *cis*-**16c**, suggesting that *cis*-**16c** was competitively excluded by *cis*-**2**.

3. Discussion

Fluorescent probes with fluorescein connected by a linker were synthesized. Their bioactivities were dependent on the carbon-chain linker length as well as the functional group, which is an oxime ether in this case, connecting the linker to cinnamic acid. Among the several fluorescent *cis*-cinnamic acid analogues prepared, *cis*-**16c** with an eight-carbon chain linker showed the highest bioactivity. A hydrophobic carbon chain linker would increase non-specific binding to proteins, but also improve the uptake of the compounds into plant tissues. A balance of these effects would therefore be important. We considered *cis*-**16c** to be an effective fluorescent probe, because the corresponding fluorescent *trans*-analogue *trans*-**16c** was biologically inactive. The *trans*-analogue is useful as a control probe, which could enable discrimination between non-specific binding of the probes. The tip of the radicles was stained only with *cis*-**16c**, and not with *trans*-**16c**. Confocal microscopy revealed the root cap, most likely the columella cells, to be stained. The competitive inhibition studies clearly demonstrated that *cis*-**16c** competed with *cis*-**2** in the root cap, but not with *trans*-**2**. Consequently, these contrasts suggested that *cis*-**16c** specifically localized in the columella cells, whereas biologically inactive *trans*-**13c** did not. If *cis*-**2** works in the columella cells which are considered to be responsible for gravity sensing of the root,[21] it might affect gravitropism of roots, either negatively or positively. While the confocal microscopy may only shed light on the surface layers of the radicle,[22] these molecular imaging studies would be very useful in elucidating the mechanisms of *cis*-**2**.

4. Conclusions

We synthesized the first fluorescein-labeled *cis*-cinnamic acid analogue possessing lettuce root growth inhibitory activity, and its *trans*-isomer with no bioactivity as a control probe. The fluorescent images of the radicles which were incubated with each of those molecular probes depicted that the root cap, presumably columella cells, was fluorescence-stained while those of control radicles treated with or without *trans*-isomer fluorescent probes did not show the fluorescence at the root cap. These contrasts suggested specific localization of the *cis*-cinnamate analogue at this site. Although we demonstrated that the columella cells are a possible target of *cis*-cinnamic acid, the underlying route of localization is still unknown. Furthermore, it is not clear if the localization of *cis*-**2** in columella cells is directly related to the growth inhibition of radicles. For further clarifications regarding the target site and the inhibitory mechanism, biochemical and molecular biological studies are additionally required. This chemical probe study thus provides a useful foundation to further explore the biological mechanisms of *cis*-**2**.

5. Experimental

5.1. General

^{1}H NMR, ^{13}C NMR were measured in $CDCl_3$ solution using JEOL JNM-AL-400 spectrometer (^{1}H NMR at 400 MHz, ^{13}C NMR at

100 MHz) and JNM-ECA-600 spectrometer (^{1}H NMR at 600 MHz, ^{13}C NMR at 150 MHz) as the referenced standard (^{1}H NMR at 0.00 ppm (TMS), ^{13}C NMR at 77.0 ppm ($CDCl_3$)). Chemical sifts are reported in ppm. Peak multiplicities are used the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; sept, septet; m, multiplet; br, broadend. IR spectra were recorded on Shimadzu FTIR-8300 and IR Prestige-21 spectrometers. Mass spectra and high resolution mass spectra were obtained on a JMS-K9 (GC–MS), JEOL JMS-700 and Shimadzu LCMS-2010EV mass spectrometers. Melting points were measured with a Yanaco MP-500D apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on precoated plates (0.25 mm, silica gel Merck 60F245). Column chromatography was performed on silica gel (Kanto Chemical Co., Inc.). Preparative HPLC was performed on a system utilizing a system utilizing a JASCO PU-2087 Intelligent Pump with Dynamic Mixer MX-2080-32 and UV detector UV-2075 and RI detector RI-2031. All reactions were performed under an air atmosphere unless otherwise noted, and dry dichloromethane (CH_2Cl_2), diethyl ether (Et_2O), and tetrahydrofuran (THF) were purchased from Kanto Chemical Co., Inc., and other solvents were distilled. Unless otherwise noted, reagents were obtained from chemical sources and without further purification.

5.2. Synthesis of fluorescein-labeled probes

*5.2.1. Synthesis of (Z)-ethyl 3-(3-formylphenyl)acrylate (cis-**8**).* To a solution of isophthalaldehyde (1.0 g, 7.4 mmol) and ethyl 2-[bis(2-isopropylphenoxy)phosphoryl]acetate (1.0 g, 2.5 mmol) in THF (140 mL), a solution of Triton B (40% in MeOH, 1.3 mL, 3.2 mmol) in THF (30 mL) was added dropwise at −78 °C under an argon atmosphere. After 14 h, the mixture was quenched with saturated aqueous NH_4Cl and extracted with EtOAc. The organic layer was washed with H_2O, saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by silica gel column chromatography (EtOAc/hexane 5%, then 8%) to give *cis*-**8** (91%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.25 (t, J=7.2 Hz, 3H), 4.18 (q, J=7.2 Hz, 2H), 6.06 (d, J=12.8 Hz, 1H), 7.02 (d, J=12.8 Hz, 1H), 7.53 (t, J=7.6 Hz, 1H), 7.84 (d, J=7.6 Hz, 1H), 7.86 (d, J=7.6 Hz, 1H), 8.08 (s, 1H), 10.03 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 14.0 (q), 60.4 (t), 121.5 (d), 128.6 (d), 129.7 (d), 131.0 (d), 135.3 (d), 135.7 (s), 136.1 (s), 141.6 (d), 165.6 (s), 191.9 (d); IR (NaCl Neat) 1703 cm^{-1}; MS (EI) m/z 204 (M^+), 175 (M^+-CHO), 159 (M^+−OEt, 100%); HRMS (EI) m/z calcd for $C_{12}H_{12}O_3$(M^+) 204.0786, found 204.0783.

*5.2.2. General procedure of synthesis of cis-**14**.* Hydrazine monohydrate (5.1 mmol) was added to a solution of **13** (1.7 mmol) in EtOH (9 mL) at room temperature under an argon atmosphere. After 12 h, the solvent was removed in vacuo, diluted with Et_2O, filtered and concentrated. The resultant oil was submitted to the next reaction without further purification. A solution of (*Z*)-ethyl 3-(3-formylphenyl)acrylate (*cis*-**8**) (1.71 mmol) in dry EtOH (3.5 mL) was slowly added to a solution of the alkoxyamine in dry EtOH (3.5 mL) at room temperature. After 12 h, the solvent was removed in vacuo. The crude product was purified by silica gel column chromatography (EtOAc/hexane, 10:90) to give *cis*-**14a**–**d**.

*5.2.3. (Z)-Ethyl 3-((E)-((8-(tert-butoxycarbonylamino)-octyloxy)imino)methyl)phenylacrylate (cis-**14c**).* 74% as a colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.23 (t, J=7.2 Hz, 3H), 1.32–1.45 (m, 19H), 1.67–1.70 (m, 2H), 3.07–3.11 (m, 2H), 4.14–4.19 (m, 4H), 5.98 (d, J=12.8 Hz, 1H), 6.94 (d, J=12.8 Hz, 1H), 7.34 (t, J=8.0 Hz, 1H), 7.54 (d, J=8.0 Hz, 1H), 7.57 (d, J=8.0 Hz, 1H), 7.73 (s, 1H), 8.07 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 14.0 (q), 25.7 (t), 26.6 (t), 28.4 (q), 29.0 (t), 29.1 (t), 29.3 (t), 30.0 (t), 40.5 (t), 60.3 (t),74.3 (t), 78.8 (s), 120.7 (d), 127.2 (d), 128.0 (d), 128.2 (d), 130.6 (d), 132.2 (s), 135.3 (s), 142.0 (d), 147.7 (d), 155.9 (s), 165.9 (s); IR (KBr) 3454, 1713, 1631 cm^{-1}; MS (FAB) m/z 447 ([M+H]$^+$); HRMS (FAB) m/z calcd for $C_{25}H_{38}N_2O_5$ 447.2859, found 447.2859 ([M+H]$^+$).

5.2.4. General procedure of hydrolysis of ethyl cis-cinnamate analogues. To a solution of *cis*-**14c** in EtOH was added 10% NaOH at room temperature. After 12 h, the mixture was adjusted to pH 1.0 with 1 M HCl, and then extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by recrystallization to afford a *cis*-cinnamic acid derivative.

*5.2.5. (Z)-3-((E)-((8-(tert-Butoxycarbonylamino)octyloxy)-imino)methyl)phenylacrylic acid (cis-**15c**).* 87% as colorless needles. Mp 46–50 °C (EtOAc/hexane=10%); ^{1}H NMR (CD_3OD, 600 MHz) δ: 1.32–1.45 (m, 19H), 1.69 (quintet, J=7.2 Hz, 2H), 3.01 (t, J=7.2 Hz, 2H), 4.13 (t, J=7.2 Hz, 2H), 6.00 (d, J=12.6 Hz, 1H), 6.97 (d, J=12.6 Hz, 1H), 7.34 (t, J=7.8 Hz, 1H), 7.56 (d, J=7.8 Hz, 1H), 7.59 (d, J=7.8 Hz, 1H), 7.76 (s, 1H), 8.07 (s, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 26.9 (t), 27.8 (t), 28.8 (q), 30.2 (t), 30.3 (t), 30.4 (t), 30.9 (t), 41.3 (t), 75.3 (t), 79.7 (s), 122.0 (d), 128.0 (d), 129.3 (d), 129.4 (d), 131.8 (d), 133.8 (s), 137.0 (s), 142.5 (d), 149.1 (d), 158.5 (s), 169.6 (s); IR (KBr) 1697, 1631 cm^{-1}; MS (FAB) m/z 419 ([M+H]$^+$); HRMS (FAB) m/z calcd for $C_{23}H_{35}N_2O_5$ 419.2546, found 419.2550 ([M+H]$^+$).

*5.2.6. General procedure for synthesis of fluorescein-labeled probes (cis-**16**).* To a solution of **15** (0.28 mmol) in CH_2Cl_2 (8 mL) was added TFA (2.3 mL, 29.5 mmol) dropwise at room temperature under an argon atmosphere. After 1.5 h, the solvent was removed in vacuo. The resultant crude oil (TFA salt) was used in the next reaction without further purification. To a solution of the crude TFA salt in CH_2Cl_2–MeOH (25:1, 42 mL) were added fluorescein isothiocyanate (FITC, 0.28 mmol) and Et_3N (4.7 mmol) at room temperature under an argon atmosphere. The mixture was refluxed for 23 h. After being cooled to room temperature, the solvent was removed in vacuo. The residue was purified by reverse-phase HPLC (Nacalai Tesque COSMOSIL 5C_{18}-AR-II, 250 mm×20 mm) to give *cis*-**16**. In the ^{13}C spectra, several peaks due to quaternary carbons were missed, probably because fluorescein would be in equilibrium with the lactone form. *cis*-**16c**: 65% as yellow needles (reverse-phase HPLC, MeCN–H_2O containing 0.5% TFA, 55:45). Mp 108–109 °C (toluene); ^{1}H NMR (CD_3OD, 600 MHz) δ: 1.38–1.45 (m, 8H), 1.66–1.73 (m, 4H), 3.50 (m, 2H), 4.04 (t, J=7.2 Hz, 2H), 5.90 (d, J=12.6 Hz, 1H), 6.45 (d, J=8.4 Hz, 2H), 6.58 (m, 4H), 6.86 (d, J=12.6 Hz, 1H), 7.03 (d, J=8.4 Hz, 1H), 7.24 (t, J=7.8 Hz, 1H), 7.45 (d, J=7.8 Hz, 1H), 7.48 (d, J=7.8 Hz, 1H), 7.65 (m, 2H), 7.97 (s, 1H), 8.02 (s, 1H); ^{13}C NMR (CD_3OD, 150 MHz) δ: 26.9 (t), 27.9 (t), 29.9 (t), 30.2 (t), 30.36 (t), 30.42 (t), 49.9 (t), 75.2 (t), 103.5 (d), 111.5 (s), 113.6 (d), 113.7 (d), 120.3 (d), 122.0 (d), 125.6 (s), 128.0 (d), 129.3 (d), 129.4 (d), 130.3 (d), 131.8 (d), 133.8 (s), 137.8 (s), 142.5 (d), 149.1 (d), 150.1 (s), 153.2 (s), 154.2 (s), 171.2 (s), 173.0 (s); IR (KBr) 3448, 1749, 1730, 1608 cm^{-1}; MS (FAB) m/z 706 (M-H$^+$); HRMS (FAB) m/z calcd for $C_{39}H_{36}N_3O_8S$ 706.2223, found 706.2234 (M-H$^+$).

5.3. Root growth inhibition assay

The assay was performed according to Hiradate's method[3] with minor modifications. A ϕ27 filter paper was placed in a glass Petri dish. Methanol solution of test compounds and growth inhibitors were added into the dish simultaneously (70 μL each, ×10 concentration for desired concentration). The solvent was removed from filter paper under reduced pressure. After addition of 700 μL distilled water, six pre-germinated Lettuce seedlings (*L. sativa* cv. Great Lakes 366) were placed on the filter paper. The pre-germination was induced with distilled water at 25 °C, 60% relative humidity for 24 h in a dark. Two dishes were prepared for each

concentration (n=12). The dishes were incubated at 25 °C, 60% relative humidity for 48 h in a dark. The root growth was evaluated by measuring the length of each root and compared to that of untreated control.

5.4. Molecular imaging experiments with fluorescence and confocal microscopy

Seeds of Lettuce (*L. sativa* cv. Great Lakes 366) were surface-sterilized by immersing them in 2% bleach for 10 min and then rinsing in distilled water four times. The seeds were then pre-germinated in a Petri dish at 23 °C for 3 h.[23] The pre-germinated Lettuce seedlings were then grown in darkness at 23 °C for 24 h. Four of the seedlings were placed in a well of a 12-well plate, and then 1 mL of distilled water and a fluorescent probe solution (5 mM in DMSO) were added to the each well, where the concentration of the fluorescent probe was 50 μM. The seedlings were then incubated at 23 °C for 24 h. After washing the seedlings with 0.1% TritonX-100 in distilled water, the radicles were mounted on slides with distilled water. The images were obtained by Olympus fluorescence microscope system with a green fluorescence protein filter (excitation 450–490 nm) and Zeiss laser confocal imaging system.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2016.08.060.

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