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

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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.

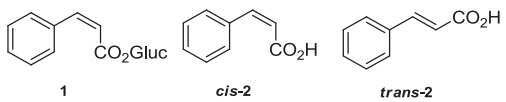

Fig. 1. Cinnamates.

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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.

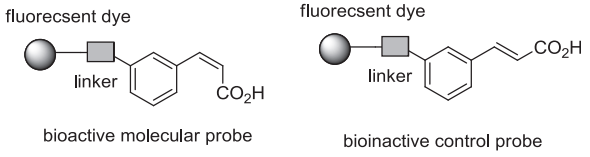


Fig. 2. Design of fluorescent probe for *cis*-**2**.

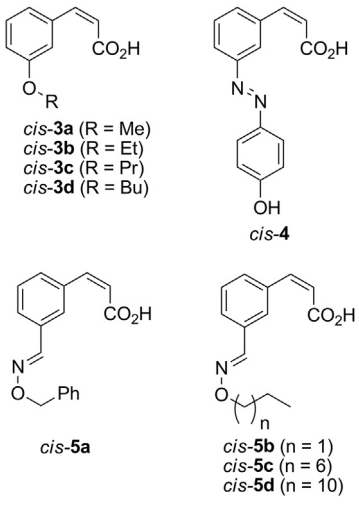


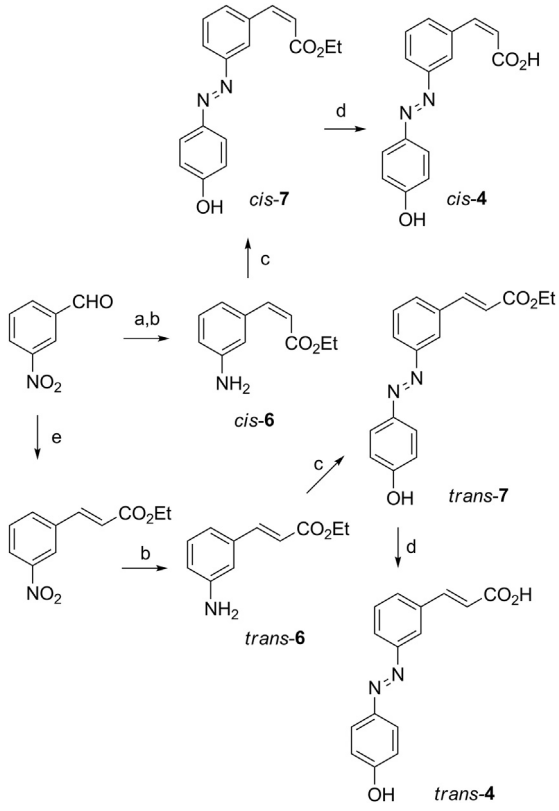
Fig. 3. *meta*-Substituted *cis*-**2** analogues.

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