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Induction of 4-Hydroxycinnamate Decarboxylase in *Klebsiella oxytoca* Cells Exposed to Substrates and Non-substrate 4-Hydroxycinnamate Analogs

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The 4-hydroxycinnamate decarboxylase (4-HCD)-inducing activity of several substrate analogs toward *Klebsiella oxytoca* was investigated. Four *E*-cinnamate-class compounds, *E*-4-hydroxycinnamic acid (1), caffeic acid (2), ferulic acid (3) and *E*-2,4-dihydroxycinnamic acid (4), all of which were accepted as substrates, all of which were accepted as substrates of 4-HCD, enable *K. oxytoca* cells to induce the decarboxylase at a 2.0 mM concentration, while five non-substrate compounds of the *E*-cinnamate class so far tested were completely inactive. However, 6-hydroxy-2-naphthoic acid (11) and 7-hydroxycoumarin 3-carboxylic acid (14), both of which are non-cinnamate-class analogs of the substrate, acted as strong 4-HCD inducers, even at a 0.5 mM concentration. The 4-HCD-inducing activities of compounds 11 and 14 at 0.5 mM were 10–12-fold higher than that of substrate 1. Compound 11 maintained its 4-HCD-inducing activity toward cultured cells through the late-log and stationary phases, unlike 1 that induced 4-HCD only in the early log phase. SDS-PAGE electrophoresis of protein mixtures from the cultured cells exposed to any 4-HCD inducer indicated that the 21.5 kDa protein was always present.

Key words: *Klebsiella oxytoca*; 4-hydroxycinnamate decarboxylase inducer; 6-hydroxy-2-naphthoic acid; 7-hydroxycoumarin 3-carboxylic acid; substrate analog

When exposed to exogenous toxic chemicals, many microorganisms express a dynamic action to detoxify the chemicals, in association with the metabolic enzyme induced by the substrate. Not only pesticides⁽¹⁾ and chemical pollutants,^(2,3) but also naturally-occurring defensive compounds of higher plants, including many phytoalexins and phytoanticipins,⁽⁴⁾ trigger such a dynamic response in the microorganisms. In respect of plant-microbe interaction, the enzymes induced by phytoalexins in association with their detoxification have attracted many researchers.⁽⁵⁾ In

particular, the pisatin-inducible enzymes produced by *Nectria haematococca*, which is known as a phytopathogen of some legumes, has been well investigated.^(6,7) In this case, pisatin, a phytoalexin of the garden pea (*Pisum sativum*), is regarded as the host-specific substrate toward the induction of the pisatin-degrading enzymes.

In respect of *Klebsiella oxytoca*⁽⁸⁾ and some other Gram-positive and -negative bacteria,^(8–14) 4-hydroxycinnamate decarboxylases (4-HCD, also called *p*-coumarate decarboxylases or phenylacrylic acid decarboxylases) which catalyze the decarboxylation of some phenylpropanoid-class carboxylic acids, are also enzymes associated with the dynamic response of the bacterium toward exogenous chemicals. *K. oxytoca*, which is widely known as a bacterium of phylloplane microflora of plants,⁽¹⁵⁾ performed the decarboxylation reaction on the leaves of host plants that had accumulated chlorogenic acid and its related compounds.⁽⁸⁾ Mechanical damage caused the host leaves to exude from the wounded part conjugated and free *E*-4-hydroxycinnamic acid (1) and caffeic acid (2). When *K. oxytoca* on the leaf surface senses the resulting phenylpropanoic acids, the bacterium starts to induce 4-HCD that decarboxylatively converts these compounds into the corresponding vinylphenol derivatives. We have demonstrated that, while 4-hydroxystyrenes were less toxic to a wild strain of *K. oxytoca*, the decarboxylated compounds were highly toxic to phytopathogenic fungi compared with the precursory 4-hydroxylated cinnamic acids.⁽⁸⁾ This fact suggested that the decarboxylation products acted as allelochemicals to regulate epiphytic microflora on those leaves that had been mechanically damaged. Interestingly, *K. oxytoca* positively hydrolyzed exogenous chlorogenic acid (Hashidoko, unpublished data), even though the hydrolysis of chlorogenic acid and its related compounds would lead to a disadvantageous accumulation of 4-hydroxycinnamic acids that are toxic toward the bacterium.

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Abbreviations: CM, corn meal broth; DMSO, dimethylsulfoxide; EtOAc, ethyl acetate; 4-HCD, *E*-4-hydroxycinnamate decarboxylase; MeOH, methanol; PD, potato-dextrose broth; SD, standard deviation; TLC, thin-layer chromatography

4-HCD induction in *K. oxytoca* is, therefore, regarded as a significant response that is more than just simple detoxification of the phenylpropanoid acids. The rapid response of *K. oxytoca* to sense 4-HCD substrates and to express the 4-HCD gene is probably involved in the survival strategy of phylloplane microflora.

It has been found in our previous study that 4-HCD induced in *K. oxytoca* cells with *E*-4-hydroxycinnamic acid (**1**) or *E*-3,4-dihydroxycinnamic acid (**2**) was accompanied by the active cell division only in the early logarithmic phase.⁸⁾ The 4-HCD activity per cell with these substrate inducers reached the maximal level in the early logarithmic phase and then quickly decreased as the culture grew older. The dependence of this 4-HCD induction on the cell growth curve was probably due to acceptance of the inducers as substrates of induced 4-HCD. In fact, the amounts of compounds **1** and **2** soon diminished in the culture media in the mid-logarithmic phase, because they were quickly decarboxylated by the enzyme. This observation led to the speculation that non-substrate compounds possessing 4-HCD-inducing activity allowed the cells to produce 4-HCD through all the phases of cell growth. Since it has been demonstrated that compound **1** was the primary substrate of 4-HCD (Hashidoko, unpublished data), we screened non-substrate 4-HCD inducers from *E*-cinnamic

acid-class and non-*E*-cinnamic acid-class compounds that are analogous to **1**. We described here the results of screening the 4-HCD inducers and an evaluation of their 4-HCD-inducing activities. The metabolic behavior of *K. oxytoca* toward the non-substrate 4-HCD inducer, 6-hydroxy-2-naphthoic acid (**11**), whose activity was newly found in the present study, is also reported.

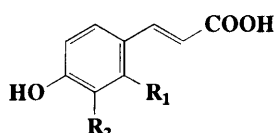
Materials and Methods

General. TLC plates (Merck Kieselgel 60F₂₅₄, 0.25 mm) were used for preparative and analytical TLC. A Hitachi G 5000 gas chromatograph connected to an OV-1 glass-capillary column (TC-1, GL Science, 30 m × 0.32 mm i.d.; 2 ml/min of helium as the carrier gas) was used under the following analytical conditions: oven temp., 60°C for 1 min and then programmed from 60°C to 150°C at a rate of 7.5°C/min.

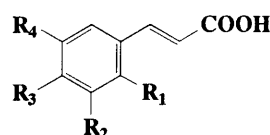
Chemicals. Commercially available chemicals were used in this study. *E*-4-hydroxycinnamic acid (**1**), *E*-3,5-dimethoxy-4-hydroxycinnamic acid (**5**), *E*-3-hydroxycinnamic acid (**7**) and coumarin 3-carboxylic acid (**15**) were purchased from Aldrich Chemical Co. Caffeic acid (**2**), 3-(4-hydroxyphenyl)propionic acid (**10**) and 3,4-dihydroxybenzoic acid were products of

Cinnamate Class

Substrates of 4-HCD

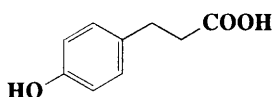


- 1:** R₁=R₂=H, *E*-4-hydroxycinnamic acid
2: R₁=H, R₂=OH, caffeic acid
3: R₁=H, R₂=OCH₃, ferulic acid
4: R₁=OH, R₂=H, *E*-2,4-dihydroxycinnamic acid

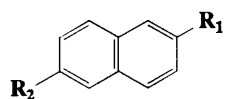


- 5:** R₁=H, R₂=R₄=OCH₃, R₃=OH, *E*-3,5-dimethoxy-4-hydroxycinnamic acid
6: R₁=R₂=R₃=R₄=H, *E*-cinnamic acid
7: R₁=R₃=R₄=H, R₂=OH, *E*-3-hydroxycinnamic acid
8: R₂=R₃=R₄=H, R₁=OH, *E*-2-hydroxycinnamic acid
9: R₁=R₂=R₄=H, R₃=OCH₃, *E*-4-methoxycinnamic acid

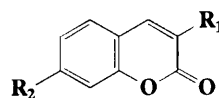
Non-cinnamate Class



- 10:** 3-(4-hydroxyphenyl)propionic acid



- 11:** R₁=COOH, R₂=OH, 6-hydroxy-2-naphthoic acid
12: R₁=COOH, R₂=H, 2-naphthoic acid
13: R₁=H, R₂=OH, 2-naphthol



- 14:** R₁=COOH, R₂=OH, 7-hydroxycoumarin 3-carboxylic acid
15: R₁=COOH, R₂=H, coumarin 3-carboxylic acid
16: R₁=H, R₂=OH, 7-hydroxycoumarin

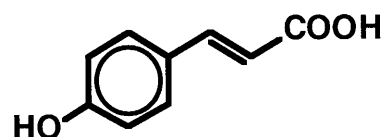
Fig. 1. Chemical Structures of the Cinnamate and Non-cinnamate Classes of Compounds to Examine their 4-HCD-inducing Activities.

Wako Pure Chemical Industries Co. Ferulic acid (**3**), *E*-2-hydroxycinnamic acid (**8**), *E*-4-methoxycinnamic acid (**9**), 6-hydroxy-2-naphthoic acid (**11**), 2-naphthoic acid (**12**), mimosine and dealkaline lignine were from Tokyo Chemical Industry Co. *E*-Cinnamic acid (**6**) and 2-naphthol (**13**) were from Kanto Chemical Co. 7-Hydroxycoumarin 3-carboxylic acid (**14**) and 7-hydroxycoumarin (**16**) were from Sigma and Dojindo, respectively.

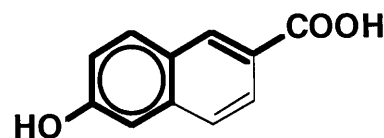
Chemical preparation of *E*-2,4-dihydroxycinnamic acid (4**).** *E*-2,4-Dihydroxycinnamic acid (**4**) was chemically prepared from **16**. Powder of the starting material (320 mg) was dissolved in 5 ml of a 2 M KOH solution and kept at 80°C for 1 h, before UV light (365 nm) was radiated on the solution over a glass tube for 15 min. The resulting solution was diluted with *ca.* 100 ml of deionized water and then acidified to below pH 4.0 with HCl. The acidic compounds were extracted with ethyl acetate (EtOAc). Crude **4** in the organic layer was eventually purified by preparative TLC developed in 20% methanol (MeOH)/chloroform to yield 222 mg of a pale yellow amorphous compound (62% yield). *E*-2,4-Dihydroxycinnamic acid (**4**). EI-MS (*m/z*, %): 180 (M^+ , 11), 162 (33), 136 (100), 134 (40), 121 (27), 107 (26) and 44 (29). $^1\text{H-NMR}$ δ (acetone- d_6): 7.95 (d, $J=16.2$ Hz, 7-H), 7.46 (d, $J=8.6$ Hz, 6-H), 6.49 (d, $J=2.3$ Hz, 3-H), 6.44 (d, $J=16.2$ Hz, 8-H), and 6.43 (dd, $J=8.6$ and 2.3 Hz, 5-H).

Preparation of the test compound-containing media. The chemicals tested during the screening of 4-HCD inducers were initially prepared as a 1.0 M solution in DMSO, the stock solution being added to each media before autoclaving. Potato-dextrose broth (PD, Difco Laboratories) dissolved in deionized water was used as the basal liquid medium for *K. oxytoca* cultures. In most cases, 100 μl of a 1.0 M solution of the test compound was added to 200 ml of the basal medium in a 500-ml shaking flask to a final concentration of 0.5 mM. In the case of the 2.0 mM concentration, 400 μl of the stock solution was added to 200 ml of the PD medium. The final concentrations of DMSO in the media were 0.05–0.2% (v/v), and in any particular cases, the DMSO concentration in each medium was less than 0.5%.

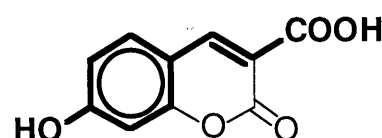
Bacterium and its culture conditions. *Klebsiella oxytoca* JCM 1665 stored at Japan Institute of Physical and Chemical Research (Wako-shi, Japan) was continuously sub-cultured on corn meal (CM) agar slants in our laboratory. It has previously been demonstrated that the bacterial strain had no constitutive 4-HCD. To *K. oxytoca* pre-cultured overnight on CM- or PD-agar slants at 20°C, 2 ml of sterile water was added to obtain a bacterial cell suspension that was used as the inoculum. To each



1: *E*-4-hydroxycinnamic acid



11: 6-hydroxy-2-naphthoic acid



14: 7-hydroxycoumarin 3-carboxylic acid

Fig. 2. Structures of Non-substrate Inducers 6-Hydroxy-2-naphthoic Acid (**11**) and 7-Hydroxycoumarin 3-Carboxylic Acid (**14**) Analogous to Primary Substrate *E*-4-Hydroxycinnamic Acid (**1**).

500-ml shaking flask containing 200 ml of the basal medium, 100 μl of the cell suspension was inoculated, before setting the flask on a rotary shaker (80 rpm/min at 25°C in the dark).

Preparation of the protein mixture extracted from *K. oxytoca* cells. The bacterial cells were incubated in each medium containing a 2.0 mM concentration of a test compound until the culture exhibited turbidity of over 0.7 absorbance at 660 nm. The cells were then centrifuged at $10,000 \times g$ and washed twice with 50 ml each of a 50 mM Tris-HCl buffer (pH 7.2). The resulting cells were re-suspended in 25 ml of the buffer and sonicated with a Branson 250 Sonifier (10% pulsed) for 2 min in an ice bath, after which the cell suspension was centrifuged at $6,000 \times g$ for 5 min to obtain the supernatant containing a solubilized protein mixture. The supernatant transferred into a glass vial was then saturated with an excess of ammonium sulfate to allow precipitation of the protein at 4°C. Since the 4-HCD activity in the protein mixture was completely stable in the saturated ammonium sulfate-containing buffer (pH 6.9), the protein mixture that had been precipitated was collected by centrifugation and re-dissolved in a small volume of a 50 mM buffer just before the 4-HCD activity was measured. After part of the solution had been used

for the 4-HCD assay, the remaining solution of the protein was assayed with a Bio Rad[®] Protein Assay[®] kit, using bovine serum albumin as the standard.

Whenever it was necessary to process a large number of samples for the enzyme assay at the same time, we used a modified method for preparing the protein mixture. Cells that had been incubated for 24 or 48 hours were centrifuged, washed and re-suspended in a 50 mM Tris-HCl buffer saturated with ammonium sulfate (pH 6.8, 20 ml), before the cell suspension was sonicated for 5 min under the conditions already described. The resulting solution was left overnight or for longer at 4°C in the dark to allow precipitation of the protein, after which part of the solution (1.5 ml) was centrifuged at $10,000 \times g$ to recover the protein and cell residue. The resulting pellet was immediately dissolved in 200 μ l of 100 mM Tris-HCl (pH 7.2) in an ice bath and spun briefly to remove the cell residue. The supernatant was transferred to a 0.5-ml Eppendorf tube to use in the 4-HCD and protein assays. In parallel with re-examining the 4-HCD-inducing activity of each test compound, we checked the reproducibility of 4-HCD extraction from bacterial cells. The cells of the bacterial culture were put into three tubes and separately processed as just described. Each sample was then subjected to the 4-HCD and the protein assays.

Properties of 4-HCD obtained from *K. oxytoca* JCM 1665. Crude 4-HCD showed the following enzymatic properties: K_m value of 2.4 mM in a 100 mM Tris-HCl buffer (pH 7.2); optimum pH 7.2, and not active at pH lower than 5.5 and higher than 8.5; tolerant of 1.7 M NaCl and 2.0 M $(\text{NH}_4)_2\text{SO}_4$ maintaining over 90% of the enzyme activity; highly susceptible to organic solvents, and almost inactive in a buffer containing 3% EtOAc and 10% EtOH. When the protein mixture in a 40% glycerol/50 mM Tris-HCl buffer was kept overnight at room temperature, it completely lost its enzymatic activity. Even if the 40% glycerol stock solution was kept at -20°C , half of the activity in the crude enzyme was often lost within several weeks. In contrast, protein precipitated in an ammonium sulfate-saturated 50 mM Tris-HCl buffer and stocked at 4°C maintained its activity for over 3 years (Hashidoko, unpublished data).

Preliminary investigation of the conditions to induce 4-HCD. In a preliminary examination, the concentration of a test compound in the medium was initially set at 2.0 mM, simply because *K. oxytoca* JCM 1665 exposed to compound **1**¹⁾ at this concentration still yielded an acceptable cell culture, despite the antibacterial activity of **1**. To investigate the most appropriate assay conditions for screening the 4-HCD inducers, a simple 4-HCD assay combined with a TLC analysis of the decarboxylation product was performed,¹⁶⁾ using **2** as the positive control. The bac-

terial cell culture (10 ml) was centrifuged, and the resulting cells re-suspended in a Tris-HCl buffer were briefly sonicated and centrifuged to obtain a protein mixture. Approximately 1 ml of the supernatant was transferred into a 1.5-ml Eppendorf tube, and *ca.* 1 mg of compound **1** was added as the substrate. The tube was kept at room temperature for 30 min, at which point 200 μ l of EtOAc was added and then the tube was vortexed. 4-Hydroxystyrene produced by decarboxylation in the reaction mixture was transferred into the upper organic layer which was directly analyzed on silica gel TLC plates of 0.25 mm thickness. The size and depth of the color toward Gibbs reagent¹⁷⁾ on the spot of the decarboxylation product was used to determine the volume of the protein solution for a precise 4-HCD assay by gas-chromatography.

Evaluation of 4-HCD-inducing activity and screening of the 4-HCD inducers. The 4-HCD-inducing activity of a test compound was evaluated by the 4-HCD activity in a protein mixture prepared from cells cultured in the test compound-containing medium. The 4-HCD activity was evaluated by the released molar amount of 4-hydroxystyrene per gram of protein per second ($\mu\text{mole/g.s}$) in 10 ml of 10 mM **1**-containing 100 mM Tris-HCl (pH 7.2). Quantitative analyses of the 4-hydroxystyrene produced were performed as described in the Materials and Methods section and in our previous paper.¹⁶⁾ The enzyme reaction was allowed to run for 10 min at 25°C and then stopped by adding 2 ml of 2.0 M Tris (2-amino-2-hydroxymethyl-1,3-propanediol, Wako Pure Chemical Industries Co.). To the resulting solution, 4 ml of EtOAc and 100 μ l of the internal standard solution were added, and the mixture vortexed for 10 seconds.

To screen the 4-HCD inducers, *K. oxytoca* was cultured in a PD medium containing a test compound (**1–12**, **14** or **16**) at a 2.0 mM concentration for 24–72 hours, according to the advance of growth. As compounds **1–2** were already known as 4-HCD inducers from our previous observations, they were used as the reference compounds in the assay. In each treatment, a protein mixture was prepared from the cultured cells as already described, and the 4-HCD activity of the protein mixture was examined.

Determination of the 4-HCD activity in a protein mixture prepared from *K. oxytoca* cultured in a medium containing each test compound. The standard curve for 4-hydroxystyrene toward naphthalene as an internal standard gave a primary regular correlation in the range from 0.0 to 1.0 mg of the decarboxylated product. We therefore conducted the 4-HCD reaction to not give more than 1.0 mg of 4-hydroxystyrene by adjusting the volume of the protein mixture.¹⁶⁾ The amounts of protein in 10 ml of

the buffer were 27–84 μg and 240–270 μg for the positive and trace/negative samples, respectively.

Time-course experiment on 4-HCD activity. A cell suspension of *K. oxytoca* that had been pre-cultured overnight on a CM agar slant was inoculated as already described. The cells were then shake-cultured in a PD broth medium (200 ml) containing 1.0 mM each of non-substrate **11** and reference compound **1** for 7, 8.5, 11, 24 and 48 hours to monitor the cell growth by measuring the turbidity at 660 nm. The cultured cells were centrifuged, and the collected cells were washed with a 50 mM Tris-HCl buffer (pH 7.2) and then sonicated to give a protein mixture. The 4-HCD activity of the protein mixture was measured by the method already described, and a protein assay of the stock solution was also conducted.

The time-course experiment through the stationary and death phases used the PD medium containing 2.0 mM of each test compound. The 4-HCD level in *K. oxytoca* cells was investigated at 24-h intervals up to 96 h of incubation.

Results and Discussion

Caffeic acid (**2**), ferulic acid (**3**) and *E*-2,4-dihydroxycinnamic acid (**4**), all of which are substrates of 4-HCD, showed 4-HCD-inducing activity at a 2.0 mM concentration, as did the primary 4-HCD substrate, *E*-4-hydroxycinnamic acid (**1**). In contrast, non-substrate compounds of the cinnamate class, *E*-3,5-dimethoxy-4-hydroxycinnamic acid (**5**), *E*-cinnamic acid (**6**), *E*-3-hydroxycinnamic acid (**7**), *E*-2-hydroxycinnamic acid (**8**) and *E*-4-methoxycinnamic acid (**9**), all disabled *K. oxytoca* cells from inducing any 4-HCD activity. The analogous compounds possessing the *E*-cinnamic acid skeleton thus exhibited clear differentiation between being active (**1–4**, substrates) and inactive (**5–9**, non-substrates) compounds for 4-HCD induction. Acceptance as the 4-HCD substrate and activity as a 4-HCD inducer were thus inseparable among *E*-cinnamate-class compounds (Figs. 1 and 3). This fact suggested that *K. oxytoca* sensed 4-HCD substrates that were only able to induce the decarboxylase. This substrate-sensing system that is likely to restrict and bio-rationalize in respect of *K. oxytoca* was certainly involved in the ability of the bacterium to survive in phylloplane microflora.⁸⁾

In our preliminary screening for non-substrate-type 4-HCD inducers, we tested 3,4-dihydroxybenzoic acid (at 2.0 mM), mimosine (2.0 mM) and lignine (100 mg/100 ml), but they were all inactive. We next examined 3-(4-hydroxyphenyl)propionic acid (**10**) which is analogous to **1** with the C-7:C-8 double bond of **1** replaced by a single bond. This compound displayed 4-HCD-inducing activity; however, the activity of compound **10** was relatively low, even at a

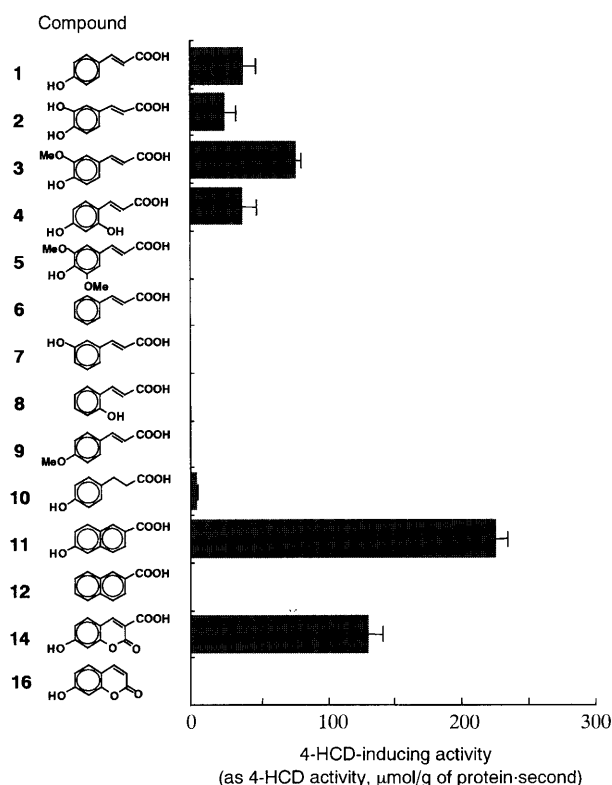


Fig. 3. Evaluation of the 4-HCD-Inducing Activity of Compounds **1–12**, **14** and **16** at a Concentration of 2.0 mM.

K. oxytoca JCM 1665 was cultured until the cell growth reached 0.7 in absorbance at 660 nm. Error bars represent the standard deviation (\pm SD). To visualize the 4-HCD-inducing activity of a test compound, the bacterium was shake-cultured in glass flasks each filled with the medium containing a test compound ($n = 3–5$ per compound).

2.0 mM concentration (4.3 $\mu\text{mol/g}$ s averaged from $n = 4$, approximately 0.1-fold the activity of **1** under the same conditions). In further searching for more powerful 4-HCD inducers of the non-substrate type, we tested 6-hydroxy-2-naphthoic acid (**11**), since *trans*-cinnamate 4-hydroxylase (C4H, EC 1.14.13.11) that catalyzes the hydroxylation of *E*-cinnamic acid at the C-4 to yield *E*-4-hydroxycinnamic acid (**1**) is also known to accept 2-naphthoic acid (**12**) as the substrate.¹⁸⁾ We also examined 7-hydroxycoumarin 3-carboxylic acid (**14**) which was another analog of **1** structurally related to **11**. We found that neither compound **11** nor **14** was accepted as a substrate of 4-HCD, despite their part structures completely matching the skeleton of *E*-4-hydroxycinnamic acid (**1**) (Fig. 2).

At a 2.0 mM concentration, both compounds **11** and **14** induced 4-HCD in *K. oxytoca* cells with 3–5-fold higher activity than that of **1** or **2** (Fig. 3). Since these non-substrate **1**-analogues possessed carboxyl and hydroxyl groups corresponding to those of **1**, we conducted a 4-HCD-inducing assay to test 2-naphthoic acid (**12**), 2-naphthol (**13**), coumarin 3-carboxylic acid (**15**) and 7-hydroxycoumarin (**16**), together with

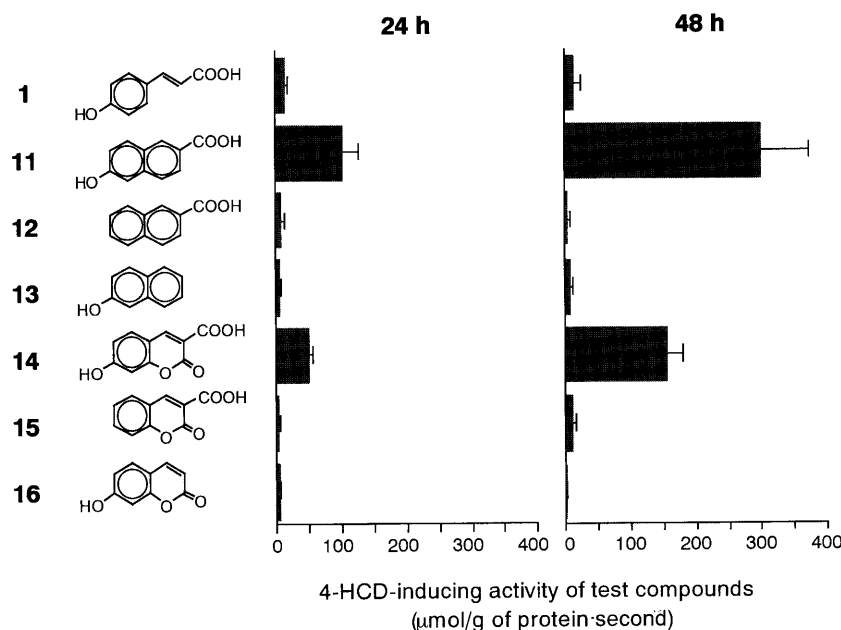


Fig. 4. Evaluation of the 4-HCD-Inducing Activity of Compounds 11–16 and 1 at a Concentration of 0.5 mM.

K. oxytoca JCM 1665 showed almost normal growth in any medium. Error bars represent \pm SD. Compounds 11 and 14 were evaluated for $n=6$ and the others for $n=3$.

11 and 14. However, 2.0 mM of compound 13 did not allow the bacterium to grow in the medium, and compounds 14 and 15 also severely retarded the cell growth at this concentration. We therefore preliminarily examined the dose-response characteristic of *K. oxytoca* cells toward 11. In bacterial cells cultured in a PD medium containing a 0.1, 0.2, 0.3, 0.5, 1.0, 1.5, 2.0, 3.0 or 4.0 mM concentration of 11 (as 0.5% DMSO in v/v), 4-HCD induction was clearly apparent within 24 h in the range from 0.5 to 3.0 mM. Accordingly, the concentration of test compounds 11, 12, 13, 14, 15, 16 and 1 was determined to be at 0.5 mM.

At this concentration, the turbidity of the cultured bacteria was almost the same as that with the control after a 24-h and 48-h incubation (0.2–0.5 and 0.3–0.6 at 660 nm, respectively). As shown in Fig. 4, compounds 12, 13, 15 and 16 were eventually found to be inactive as 4-HCD inducers. This finding suggested that not only the part structure based on *E*-4-hydroxycinnamic acid (1) but also the functional groups corresponding to the C-4-hydroxyl and C-9-carboxyl groups of 1 would be required for 4-HCD inducers of the non-cinnamate class. Although compound 1 showed trace activity at this concentration, compounds 11 and 14 led to substantial 4-HCD induction, particularly after a 48-h incubation, with 10- to 12-fold higher than that of substrate inducer 1.

As shown in our previous paper,⁸⁾ a protein mixture extracted from cells cultured in the 2-containing medium showed the highest 4-HCD activity per cell in the early log phase, which then rapidly fell in the mid-log phase and remained at almost the same level

throughout the late log and stationary phases. This dynamic decrease in 4-HCD activity was probably because *K. oxytoca*, which induced 4-HCD in the early log phase, rapidly converted 2 into 3,4-dihydroxystyrene with induced 4-HCD. On the other hand, it was speculated that 4-HCD induction by non-substrate 4-HCD-inducer 11 would have resulted in a continuously increasing curve through the log and early stationary phases. We therefore investigated the pattern of 4-HCD induction in the bacterium cultured in 1.0 mM of the 1- and 11-containing PD-medium. Both cultures of *K. oxytoca* in the respective medium showed a typical sigmoidal curve (Fig. 5(a)). In parallel with monitoring the culture growth by measuring the turbidity at 660 nm, the 4-HCD activity in the protein mixture extracted from the cells was also recorded. The patterns of the 4-HCD-induction curves with 1 and 11 were completely different from each other (Fig. 5(b)), demonstrating sustainable induction of 4-HCD led by the treatment with non-metabolized inducer 11 in *K. oxytoca* cells. With long-term incubation, the 4-HCD activity in *K. oxytoca* induced by 2.0 mM of 11 reached its maximal level after 72 h of incubation and then dramatically decreased, whereas the activity induced by the same concentration of 1 remained at a lower level throughout the stationary and death phases (Fig. 6).

Polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide) of the protein mixtures from *K. oxytoca* cells showed a protein band at 21.5 kDa that was apparent only in the samples prepared from the cells exposed to the substrate and non-substrate 4-HCD inducers (Fig. 7). An induced protein of

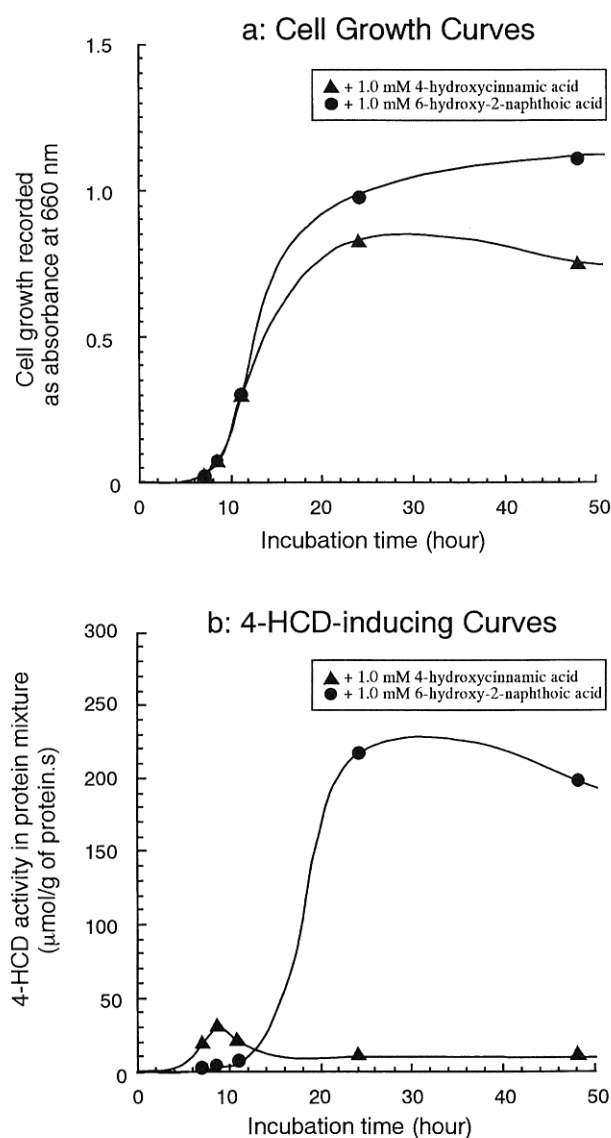


Fig. 5. Cell-growth and 4-HCD Induction Curves of *K. oxytoca* Cultured in a Medium Containing 1.0 mM *E*-4-Hydroxycinnamic Acid (**1**) or 1.0 mM 6-Hydroxy-2-naphthoic Acid (**11**) through the Exponential and Logarithmic Growth Phases.

To investigate the 4-HCD-inducing patterns of test compounds **1** and **11**, the bacterium was shake-cultured in glass flasks half-filled with the **1**- or **11**-containing medium. At 24-h intervals, the incubation of the cell culture in one flask was stopped, absorbance recorded at 660 nm, and the bacterial cells collected by centrifugation to extract a protein mixture.

21.5 kDa was apparent in the lanes for **B** (cultured in 2.0 mM 6-hydroxy-2-naphthoic acid, **11**), **C** (in 7-hydroxycoumarin 3-carboxylic acid, **14**), **D** (in ferulic acid, **3**) and **E** (*E*-4-hydroxycinnamic acid, **1**). It is to be noted that the thickness of the induced band closely matched the 4-HCD activity of the protein mixture as shown in Fig. 3. The size of the induced protein in the SDS-PAGE gel is about the same as that of the purified 4-HCD proteins from *Pseudomonas fluorescens* (a homodimer consisting of two 20.4-kDa subunits)¹¹ and from *Bacillus pumilus* (a homodimer consisting of two 23-kDa subunit).¹³ The 21.5-kDa

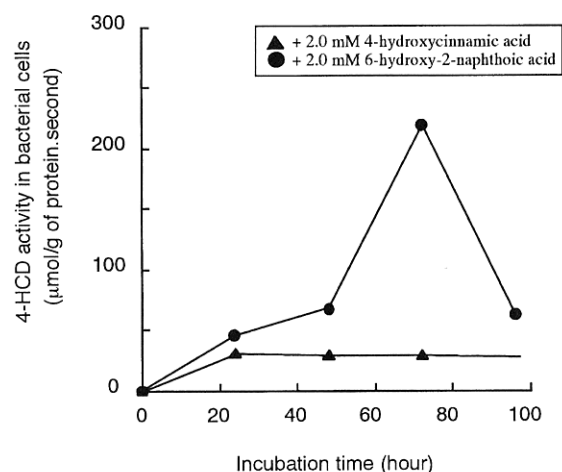


Fig. 6. 4-HCD Induction Curves of *K. oxytoca* Cells Cultured in a Medium Containing 2.0 mM *E*-4-hydroxycinnamic acid (**1**) or 2.0 mM 6-Hydroxy-2-naphthoic Acid (**11**) through the Stationary Phase.

Bacterial cells were also separately cultured in the compound-containing medium. At 24-h intervals, the cultured cells from one shaking flask were sonicated, and protein mixture was obtained to examine its 4-HCD activity. The bacterial culture exposed to 2.0 mM of **11** showed the maximal level of 4-HCD induction 72 h after the beginning of incubation, this 4-HCD level being reproducible with those from the 72-h incubation shown in Fig. 3.

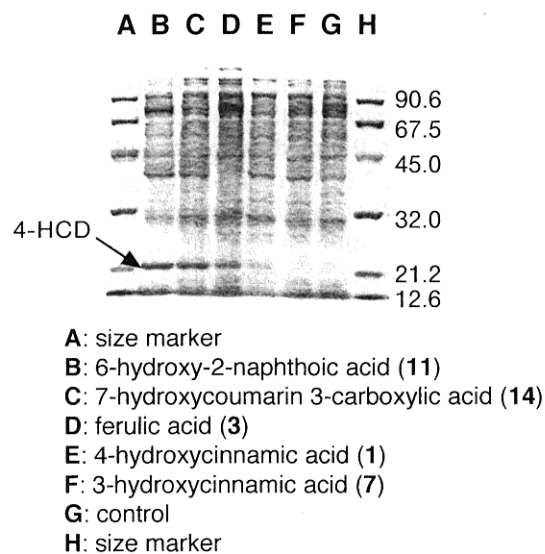


Fig. 7. SDS-PAGE Electrophoresis of Solubilized Protein from *K. oxytoca* Cells Cultured in the Test Compound-containing Media.

Each protein mixture extracted from bacterial cells that had been exposed to 2.0 mM of a test compound was diluted to 250 μ g of protein/100 μ l with 50 mM Tris-HCl (pH 7.2), and desalted in a spin column of Sephadex G-25. After mixing with an equal volume of the SDS-PAGE buffer, the sample was boiled for 3 min, and 8 ml of the PAGE sample was mixed with 2 ml of a loading buffer. The resulting samples (10 μ g/10 μ l) were separately loaded into each lane of 10% polyacrylamide gel. The electrophoresed gel was stained with the Gelcode® Blue Stain reagent (Pierce).

protein induced in *K. oxytoca* exposed to the inducers was probably a subunit of the 4-HCD enzyme.

The 4-HCD genes of *B. pumilus*,¹⁹⁾ *B. subtilis*²⁰⁾ and *Lactobacillus plantarum*²¹⁾ have been successfully characterized, and *PADI*, another family of the cinnamate decarboxylase (PAD, phenylacrylic acid decarboxylase,) gene of *Saccharomyces cerevisiae* has been cloned.²²⁾ We have also obtained a 9.6-kb DNA fragment carrying the 4-HCD gene from a wild variety of *K. oxytoca*.²³⁾ In the present study, it is likely that *K. oxytoca* had a certain substrate-sensing system that was involved in the biochemical response of 4-HCD induction; however, no information is available concerning the molecular mechanism for sensing the substrate and activating the 4-HCD gene. We hypothesize the presence of a substrate-sensing protein that binds with 4-HCD inducers. Since both compounds **11** and **14** emitted characteristic blue fluorescence under irradiation of UV 365 nm, it is likely that these compounds could be used as fluorescence-labeling reagents in the search for a hypothetical substrate-sensing protein.

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