Phytochemistry 75 (2012) 14–20

Contents lists available at SciVerse ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Conversions of deoxyradicinin to radicinin and of radicinin to 3-*epi*-radicinin in the phytopathogenic fungus *Bipolaris coicis*

Masanobu Suzuki [a], Emi Sakuno [a], Atsushi Ishihara [a], Jun-ichi Tamura [b], Hiromitsu Nakajima [a,*]

[a] Department of Agricultural Chemistry, Faculty of Agriculture, Tottori University Koyama, Tottori 680-8553, Japan
[b] Department of Environmental Sciences, Faculty of Education and Regional Sciences, Tottori University, Koyama, Tottori 680-8551, Japan

ARTICLE INFO

Article history:
Received 19 April 2011
Received in revised form 5 November 2011
Available online 16 December 2011

Keywords:
Radicinin
Deoxyradicinin
3-*epi*-Radicinin
Bipolaris coicis
Conversion
Biosynthesis
Monooxygenase
Epimerase
Fungus

ABSTRACT

Radicinin is a phytotoxic and antibiotic metabolite produced by some phytopathogenic fungi. Precursor administration and cell-free experiments with deoxyradicinin and radicinin were carried out in *Bipolaris coicis* H13-3. When deoxyradicinin was administered to the fungus, radicinin and 3-*epi*-radicinin formed. When radicinin administered, 3-*epi*-radicinin was formed. Their formation was confirmed by cell-free experiments. Deoxyradicinin 3-monooxygenase which catalyzes conversion of deoxyradicinin to radicinin showed the best activity at 35 °C and pH 7.0, and required NAD^+ as co-enzyme. Its molecular weight was determined to be 130–184 kDa. Radicinin epimerase catalyzing the reaction of radicinin to 3-*epi*-radicinin was purified from a cell-free extract. Radicinin epimerase is a homodimer of a 28 kDa subunit, and its highest activity was achieved at 30–35 °C and pH 7.0–9.0.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Radicinin (**3**) (Fig. 1) is a phytotoxic and antibiotic metabolite produced by some phytopathogenic fungi. It was first isolated from *Stemphylium radicinum* in the 1950s (Clarke and Nord, 1953), and since then has been reported to be produced by several fungal species, *Cochliobolus lunatus* (Nukina and Marumo, 1977), *Alternaria chrysanthemi* (Robeson and Strobel, 1982), *Alternaria helianthi* (Tal et al., 1985), *Phoma andina* (Noordeloos et al., 1993), *Curvularia* sp. (Kadam et al., 1994), *Alternaria radicina* (Pryor and Gilbertson, 2002) and *Alternaria petroselini* (Pryor and Gilbertson, 2002). It shows phytotoxicity, for example, killing roots of *Lepidium sativum* (Hansen, 1954), browning and causing a loss of viability in *Nicotiana tabacum* (Canning et al., 1992), as well as producing necrotic lesions in *Coix lachryma-jobi* (Nakajima et al., 1997) and inhibiting root growth in carrot seedlings (Solfrizzo et al., 2004).

The structure of radicinin (**3**) except for its stereochemistry, was determined by Grove (1964) on the basis of chemical and spectroscopic evidence. Its absolute stereochemistry was inferred from the CD spectrum of the 3,4-bis-*O*-*p*-chlorobenzoyl derivative of radicinol by Nukina and Marumo (1977) based on the dibenzoate exciton chirality rule, and this was supported by an X-ray crystallographic experiment with its 4-*O*-*p*-bromobenzoyl ester by Robeson et al. (1982). The β-oxygenated α-pyrone moiety of radicinin (**3**) is not unusual among natural products. Its biosynthesis has been studied by some researchers. Radioactive tracer experiments by Grove (1970) demonstrated that it is synthesized from two different polyketide chains originating from acetate and malonate. Other research groups confirmed this using ^{13}C labeled compounds (Tanabe et al., 1970; Seto and Urano, 1975). Although it has been assumed that the direct precursor of radicinin (**3**) is deoxyradicinin (**1**), there has been no experimental data to support this until now. Formation of deoxyradicinin (**1**) through an uncommon condensation of two polyketide chains, cyclization and ring-cleavage was demonstrated by incorporation studies with ^{13}C-labeled acetates and a ^{2}H-labeled one (Tal et al., 1988).

The *Bipolaris coicis* H13-3 used in this study is a plant pathogen causing serious leaf blight on Job's tears (*Coix lachryma-jobi* L.), and it was reported that radicinin (**3**), 3-*epi*-radicinin (**4**), 3-*epi*-radicinol (**5**) and its epoxide (**6**) were produced by this fungus (Nakajima et al., 1997). Their structures suggested a biosynthetic relationship between these metabolites shown in Fig. 1. In this scheme, both radicinin (**3**) and 3-*epi*-radicinin (**4**) are synthesized from deoxyradicinin (**1**), which was isolated from the plant pathogen *A. helianthi* together with radicinin (**3**) (Robeson and Strobel, 1982); 3-*epi*-radicinin (**4**) is then reduced to 3-*epi*-radicinol (**5**), which is oxidized to 3-*epi*-radicinol epoxide (**6**).

* Corresponding author. Tel./fax: +81 857 31 5342.
E-mail address: nakajima@muses.tottori-u.ac.jp (H. Nakajima).

0031-9422/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2011.11.010

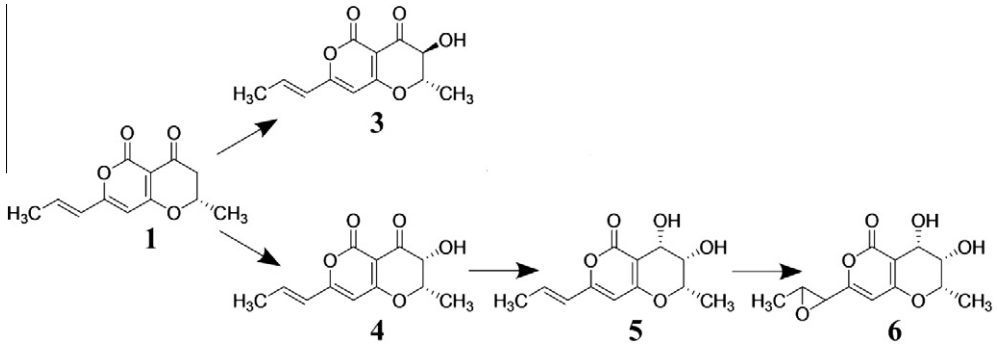

Fig. 1. Proposed processes for formation of radicinin (**3**) and 3-*epi*-radicinin (**4**) from deoxyradicinin (**1**) and transformation of 3-*epi*-radicinin (**4**) to metabolites (**5** and **6**) by *Bipolaris coicis* H-13-3.

In this work, the hypothesis shown in Fig. 1 was examined by a precursor administration experiment and a cell-free approach with deoxyradicinin (**1**) and radicinin (**3**). The former was not commercially available, and a sufficient amount of deoxyradicinin (**1**) could not be obtained from *B. coicis* H13-3. We thus synthesized deoxyradicinin (**1**) according to the reported methods (Kato et al., 1969; Suzuki et al., 1975).

2. Results and discussion

2.1. Feeding experiment

To confirm the conversion of deoxyradicinin (**1**) to radicinin (**3**) and the latter to 3-*epi*-radicinin (**4**), deoxyradicinin (**1**) and radicinin (**3**) were administered to the fungus separately. The deoxyradicinin (**1**) was synthesized from 4-methoxy-6-methyl-2*H*-pyran-2-one (**7**) according to the reported literature (Kato et al., 1969; Suzuki et al., 1975), and the synthetic scheme is shown in Fig. 2. Since the final product was a mixture of deoxyradicinin (**1**) and its C-2 epimer (**2**), they were separated via chiral HPLC. The overall yield of compounds **1** and **2** from compound **7** was 1.2% and 1.5%, respectively. To determine which compound has the same stereochemistry as the natural product, the optical rotation of each compound was measured as −82° for compound **1** and +90° for compound **2**. To our knowledge, optical rotation of deoxyradicinin (**1**) isolated from the filamentous fungus has not been reported, and we therefore compared the optical rotation of the synthetic compounds with $[\alpha]_D$ −125° of radicinin (**3**) (Hansen, 1954), $[\alpha]_D$ −105° of 3-*epi*-radicinin (**4**) (Nakajima et al., 1997) and $[\alpha]_D$ −19° of 3-*epi*-radicinol (**5**) (Nakajima et al., 1997). From its negative optical rotation, (−)-deoxyradicinin (**1**) has the same stereochemistry as radicinin (**3**) produced by this fungus. The optical purity of compounds **1** and **2** was determined to be 94.4% *ee* and 96.6% *ee*, respectively, using chiral HPLC. The radicinin (**3**) used in the precursor administration experiment was isolated from the culture filtrate of the fungus *B. coicis* H13-3 grown on malt extract medium. Its optical purity was confirmed by analysis of the NMR spectrum of its (−)-MTPA ester, in which no resonance due to the (−)-MTPA ester of the enantiomer was detected. Thus, it was concluded that radicinin (**3**) isolated from the fungus was optically pure.

Compounds **1** and **2** were administered to the fungus separately, and the conversion products were analyzed by HPLC. As shown in Fig. 3B, the amount of radicinin (**3**) (27.6 nmol/l) detected, when compound **1** was administered, was about eight times more than that of the control (3.4 nmol/l). Additionally, the amount of 3-*epi*-radicinin (**4**) also increased as compared to the control. There are two possible explanations for the increase in the amount of 3-*epi*-radicinin (**4**). One is that (−)-deoxyradicinin (**1**) was hydroxylated to be 3-*epi*-radicinin (**4**) directly and the other is that radicinin (**3**) produced from (−)-deoxyradicinin (**1**) was epimerized at C-3 to be 3-*epi*-radicinin (**4**). By contrast, administration of (+)-deoxyradicinin (**2**) caused no significant increase in either the amounts of radicinin (**3**) or of 3-*epi*-radicinin (**4**) compared with the control (Fig. 3C). This supports the fact that compound **1** has the same stereochemistry at C-2 as radicinin (**3**) produced by *B. coicis*, but compound **2** does not. To examine the biogenetical origin of 3-*epi*-radicinin (**4**), radicinin (**3**) was administered to the fungus, and the conversion products were analyzed by HPLC. As shown in Fig. 4, when radicinin (**3**) was administered, there was about a 4-fold increase in the amount of 3-*epi*-radicinin (**4**) (3.5 nmol/l) detected by HPLC compared with the control (0.9 nmol/l). These results indicated that (−)-deoxyradicinin (**1**), not (+)-deoxyradicinin (**2**), is a direct precursor of radicinin (**3**) and also that the fungus have an epimerizing enzyme which catalyzes the conversion of radicinin (**3**) to 3-*epi*-radicinin (**4**).

2.2. Enzyme activity

To confirm the conversion of deoxyradicinin (**1**) to radicinin (**3**) as indicated by precursor administration, the experiment using a

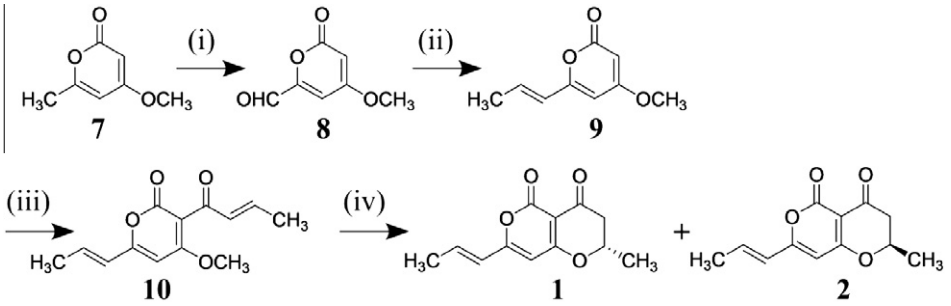

Fig. 2. Synthesis of deoxyradicinin (**1** and **2**). Reagents and conditions: (i) SeO_2, dioxane, 160 °C; (ii) ethyl triphenyl phosphonium bromide, sodium bis(trimethylsilyl) amide, DMF; (iii) $TiCl_4$, crotonoyl chloride, CH_2Cl_2; (iv) $TiCl_4$, CH_2Cl_2.

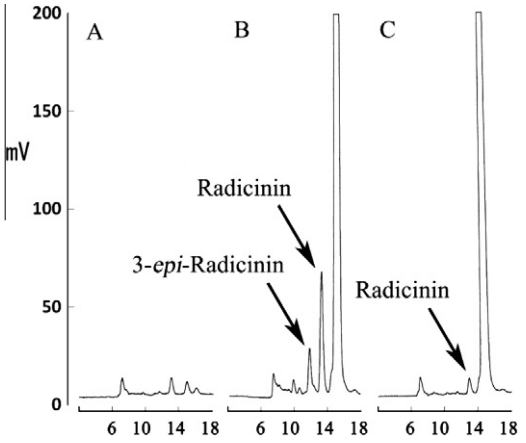

Fig. 3. HPLC profiles of products converted from deoxyradicinin by *Bipolaris coicis* H13-3. (A) control, (B) (−)-deoxyradicinin (**1**), (C), (+)-deoxyradicinin (**2**).

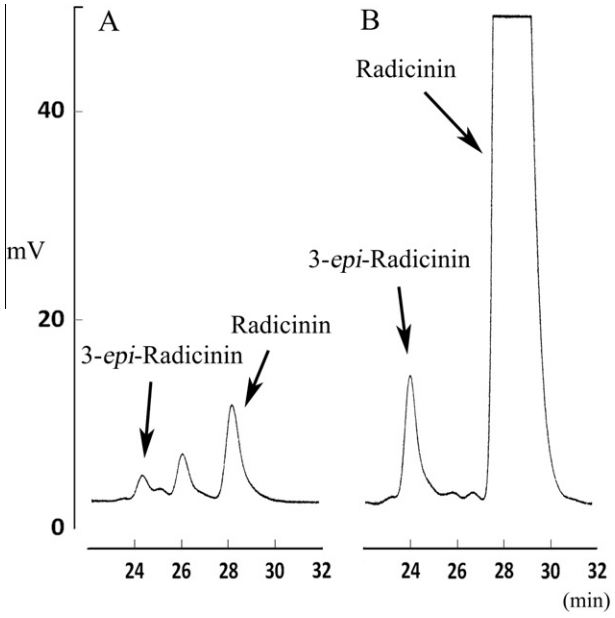

Fig. 4. HPLC profiles of the products converted from radicinin by *Bipolaris coicis* H13-3. (A) control, (B) radicinin (**3**).

cell-free system prepared from *B. coicis* H13-3 was carried out as follows. Incubation of (−)-deoxyradicinin (**1**) with the crude cell free extract, in the presence of co-enzymes (NAD^+, $NADP^+$, NADH and NADPH 20 mM respectively), for 2 h gave rise to the enzymatic formation of radicinin (**3**) (Fig. 5). Next, the crude cell free extract was divided into cytosolic and microsomal fractions. When (−)-deoxyradicinin (**1**) was incubated with the cytosolic fraction in the presence of co-enzymes (NAD^+, $NADP^+$, NADH and NADPH), the amount of radicinin (**3**) after 2-h incubation increased remarkably, but when incubated with the microsomal fraction, no significant formation of radicinin (**3**) was detected. (+)-Deoxyradicinin (**2**) was also incubated with the cytosolic fraction or the microsomal fraction in the presence of co-enzymes (NAD^+, $NADP^+$, NADH and NADPH), but, in both cases, no remarkable increases in the amount of radicinin (**3**) in the 2-h incubation extracts were observed. Deoxyradicinin 3-monooxygenase activity was measured at various conditions with the cytosolic fractions. The optimum temperature for the enzyme activity was determined by comparing the reaction rates at 25–45 °C at pH 7.0. The optimum pH for the

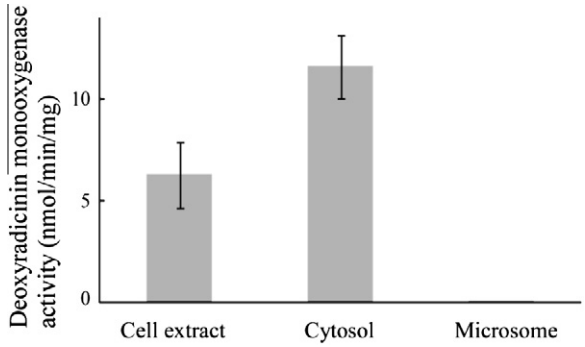

Fig. 5. Deoxyradicinin monooxygenase activity to convert (−)-deoxyradicinin (**1**) to radicinin (**3**) in the cell-free extract, cytosolic and microsomal fractions. Data presented is mean of three replicates and SD.

enzyme activity was determined by comparing the reaction rates at pH 4–9 at 35 °C. SD1 demonstrates that the reaction was catalyzed most effectively by the monooxygenase at 35 °C, pH 7.0. The monooxygenase prefers NAD^+ to other co-enzymes (Fig. 6). The molecular weight of the monooxygenase was determined to be 130–184 kDa by gel filtration column chromatography. Although we suggested in a previous paper (Nakajima et al., 1997) that the enzyme catalyzing this reaction was cytochrome P450 monooxygenase, the enzyme activity for the conversion of deoxyradicinin (**1**) to radicinin (**3**) was distributed in the cytosolic fraction and not in the microsomal fraction. The monooxygenase is therefore, a soluble protein present in the cytoplasm. The enzyme that catalyzes the hydroxylation like this was classified as oxidoreductase, and is activated in the presence of NAD^+. Thus deoxyradicinin 3-monooxygenase belongs to a monooxygenase group such as EC 1.14.13.

To investigate the metabolism of radicinin (**3**) and the origin of 3-*epi*-radicinin (**4**), radicinin (**3**) was incubated with the cytosolic or microsomal fraction in the presence of co-enzymes (NAD^+, $NADP^+$, NADH and NADPH) for 30 min (Fig. 7). Incubation of radicinin (**3**) with the cytosolic fraction caused an increase in 3-*epi*-radicinin (**4**), but no increase of 3-*epi*-radicinin (**4**) was observed when incubating with the microsomal fraction. No 3-*epi*-radicinin (**4**) was detected when incubating radicinin (**3**) with sterile water in place of the cytosolic or the microsomal fraction, indicating that radicinin (**3**) does not racemize at detectable rate without enzyme. The radicinin epimerase that catalyzes the reaction of radicinin to 3-*epi*-radicinin (**4**) was purified with ammonium sulfate fractionation and several chromatographic processes by monitoring enzyme activity (Table 1). To characterize the epimerase, radicinin (**3**) was incubated with this purified enzyme under several conditions, demonstrating that the highest activity of the epimerase

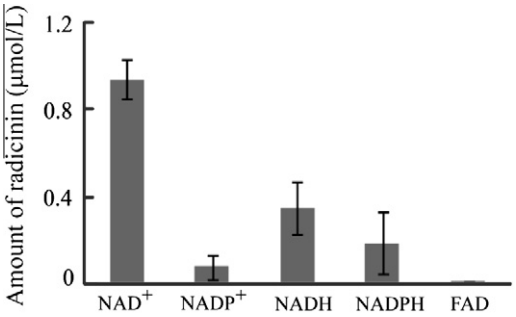

Fig. 6. Effect of addition of co-enzyme to the enzyme assay solution on formation of radicinin (**3**) from (−)-deoxyradicinin (**1**). The cytosolic fraction was used at 35 °C, pH 7.0. Data presented is the mean of three replicates and SD.

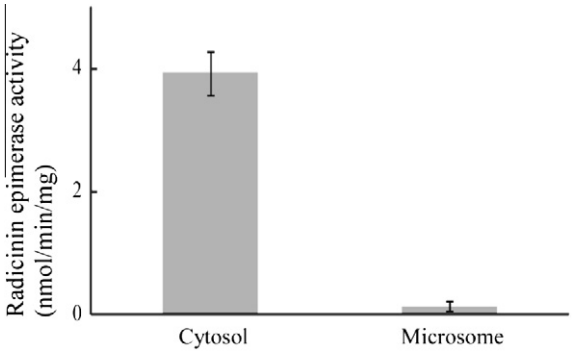

Fig. 7. Radicinin epimerase activity to epimerize radicinin (**3**) to 3-*epi*-radicinin (**4**) in the cytosolic and microsomal fractions. Data presented is the mean of three replicates and SD.

Table 1
Purification of radicinin epimerase from *Bipolaris coicis* H 13-3. Total protein was measured by Bradford protein assay. Total activity was calculated from the reaction product monitoring by HPLC.

Purification step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Purification fold	Recovery (%)
Cytosol	262	1035	4	1	100
DE-52	40.6	230	5.7	1.4	22.3
Phenyl Sepharose	13.9	140	10.1	2.6	14
Superdex	2.12	38.7	18.3	4.5	3.7
1st MonoQ	0.22	13.7	62.3	15.8	1.3
2nd MonoQ	0.04	9.1	228	57.7	0.9

was found at 30–35 °C and pH 7.0–9.0 (SD2), and that the epimerase did not require any co-enzyme for this conversion. The molecular weight of the epimerase was determined to be 52 kDa based on its gel filtration chromatographic behavior. The fractions from the 2nd Mono Q HR 5/5 column chromatography were characterized by SDS–PAGE analysis and enzyme assay. A major band corresponding to 28-kDa on SDS–PAGE is in accordance with the enzyme activity, indicating that the epimerase is homodimeric in its native condition (SD3). To ascertain whether the epimerase catalyzes the inverse reaction, the isolated epimerase was incubated with 3-*epi*-radicinin (**4**). HPLC analysis of the products indicated formation of radicinin (**3**) from 3-*epi*-radicinin (**4**) (data not shown), demonstrating that this reaction was reversible. The enzyme activity was inhibited by copper sulfate and iodoacetic acid (data not shown), suggesting that radicinin epimerase is a SH enzyme. Most epimerization enzymes that have been discovered so far utilize carbohydrates, amino acids, hydroxyl acids and their derivatives as substrates (Mazumder et al., 1962; Dahm et al., 1968; van der Drift et al., 1975; Schmitz et al., 1994). However, no epimerization enzymes involved in secondary metabolism have been found until now. The epimerase did not need any co-enzymes for the reaction, indicating that the reaction proceeds through keto-enol tautomerization. Thus, radicinin epimerase should belong to the tautomerase group such as the EC number of 5.3.2. The toxicity of radicinin (**3**) for *Coix lachryma-jobi* L. was ten times higher than 3-*epi*-radicinin (**4**) (Nakajima et al., 1997), and hence it was assumed that radicinin epimerase regulated the pathogenicity of the fungus to the plants.

3. Conclusions

In our previous report (Nakajima et al., 1997), a biosynthetic relationship was proposed between radicinin (**3**) and its analogues based on their structural features. The present result obtained from the feeding and cell-free experiments indicates that radicinin (**3**) is synthesized from an anticipated precursor, deoxyradicinin (**1**). Furthermore, a small amount of deoxyradicinin (**1**) was produced by *B. coicis*, suggesting that the deoxyradicinin (**1**) biosynthesized is rapidly converted to radicinin (**3**) or to the following biosynthetic product, and thus ostensible amount of deoxyradicinin (**1**) is extremely small in amount at any time. Previously, we proposed that the same enzyme catalyzes the conversions of deoxyradicinin (**1**) to radicinin (**3**) and also to 3-*epi*-radicinin (**4**). Actually, the precursor administration experiment with deoxyradicinin (**1**) showed formation of not only radicinin (**3**), but also 3-*epi*-radicinin (**4**). At the same time, however, we established that the enzyme in the cytosolic preparation from *B. coicis* H13-3 catalyzes reaction of radicinin (**3**) to 3-*epi*-radicinin (**4**). This epimerization was reversible and a new metabolic fate of radicinin (**3**). From these results, a biosynthesis and metabolism scheme for radicinin (**3**) was deduced, as shown in Fig. 8. First, deoxyradicinin (**1**) is converted to radicinin (**3**) by stereospecific hydroxylation at C-3. Then, radicinin epimerase catalyzes epimerization of radicinin (**3**) at C-3 to 3-*epi*-radicinin (**4**) reversibly. The direct conversion of deoxyradicinin (**1**) to 3-*epi*-radicinin (**4**) could not be confirmed in this study. Finally, 3-*epi*-radicinin is probably converted to 3-*epi*-radicinol (**5**) by stereospecific reduction at C-4, followed by epoxidation of the side chain in 3-*epi*-radicinol (**5**). In this study, the key enzyme that catalyzes the reaction of deoxyradicinin (**1**) to radicinin (**3**) could not be isolated, probably because of enzyme instability.

4. Experimental

4.1. Experimental procedures

NMR spectra were recorded in $CDCl_3$ on a JEOL JNM-ECP 500 spectrometer. NMR chemical shifts were referenced to $CDCl_3$ (δ_H

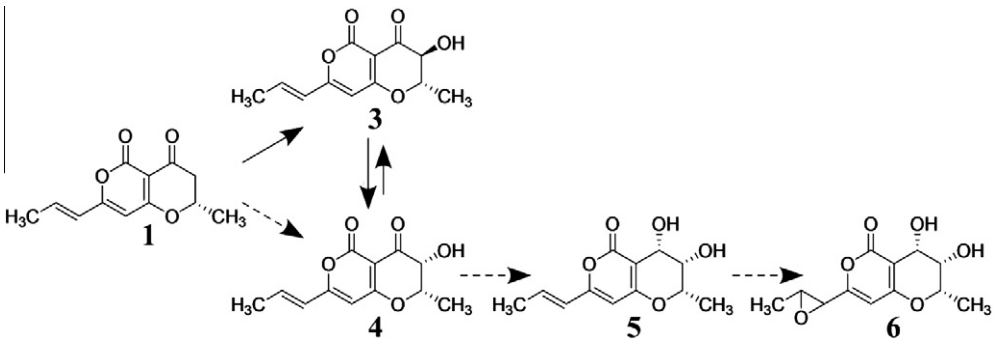

Fig. 8. Proposel biosynthesis of radicinin (**3**) from (−)-deoxyradicinin (**1**) and conversion of radicinin to 3-*epi*-radicinin (**4**). Solid arrows show the pathways established in this research, and dashed arrows show the unproven pathways.

7.26, δ_C 77.0). Mass spectra were obtained with a JEOL AX-505 spectrometer. Optical rotations were determined with a Horiba SEPA-200 high sensitive polarimeter. A Shimadzu LC-6A liquid chromatography system was used for HPLC analysis. In the precursor administration experiment and the enzyme assay for the conversion of deoxyradicinin (**1**) to radicinin (**3**), HPLC was performed using a DAISOPAK SP-120-5-ODS-AP column (DAISO Co., Ltd., 150 × 6 mm), MeOH–H_2O–AcOH (100:99:1, v/v/v) as solvent at a flow rate of 0.5 ml/min, monitoring at 280 nm. In the enzyme assay for the conversion of radicinin (**3**) to 3-*epi*-radicinin (**4**), HPLC was carried out using a DAISOPAK SP-250-10-ODS-AP column (DAISO Co., Ltd., 250 × 10 mm), MeOH–H_2O–AcOH (100:99:1, v/v/v) as solvent at a flow rate of 0.5 ml/min, and monitoring at 280 nm. Silica gel flash column chromatography (CC) was carried out by use of Wakogel FC-40 (Wako Pure Chemical Industries, Ltd.). Protein concentrations were determined with Bradford reagent (Sigma–Aldrich) using BSA as a standard.

4.2. Fungal strain

The strain H13-3 of *B. coicis* was used in the experiments (Nakajima et al., 1997). *B. coicis* H13-3 was maintained on potato dextrose agar slants.

4.3. Isolation of radicinin

The fungus was grown without shaking at 24 °C for 14 days in the dark in a 500 ml conical flask containing liquid medium (200 ml × 5) made up of glucose (30 g/l), peptone (3 g/l) and an extract from 50 g/l of malt and H_2O. The culture filtrate was acidified to pH 2.0 with HCl, and the metabolites in the culture filtrate were extracted with EtOAc (500 ml × 3). The EtOAc extract was dried over Na_2SO_4 and evaporated. The residue (270 mg) was applied to a silica gel column (Daisogel IR-60, DAISO, Co., Ltd., 180 × 18 mm), and the column was washed with 1500 ml of Me_2CO–*n*-hexane (1:9,v/v), then developed successively with 750 ml each of Me_2CO–*n*-hexane (2:8, 3:7 and 4:6, v/v). Fractions 3 and 4 eluted by Me_2CO–*n*-hexane (3:7, v/v) were combined and evaporated. Recrystallization of the residue (34 mg) from MeOH afforded radicinin (**3**) as colorless needles (6 mg): δ_H 6.93 (1H, dq, *J* = 15.5, 7.0 Hz, 2′-H), 6.02 (1H, dq, *J* = 15.5, 1.7 Hz, 1′-H), 5.83 (1H, s, 8-H), 4.36 (1H, dq, *J* = 12.4, 6.0 Hz, 2-H), 3.97 (1H, d, *J* = 12.4 Hz, 3-H), 1.96 (3H, dd, *J* = 7.0, 1.7 Hz, 3'-H), 1.64 (3H, d, *J* = 6.0 Hz, 2-Me); δ_C ($CDCl_3$) 188.7, 176.0, 164.4, 156.8, 141.0, 122.6, 98.1, 97.2, 80.0, 72.0, 18.8, 18.1, CI-MS (*iso*-butane, probe), 200 eV, *m*/*z* 237 ([M+H]$^+$, 100%).

4.4. Synthesis of deoxyradicinin

4.4.1. 6-Formyl-4-methoxy-2H-pyran-2-one (*8*)

The mixture of 4-methoxy-6-methyl-2*H*-pyran-2-one (**7**; 500 mg, Sigma–Aldrich, Inc.), SeO_2 (1.2 g, Kanto Chemical Co., Inc.) and anhydrous dioxane (5 ml) in a sealed tube was heated at 160 °C (outside) and stirred vigorously. After 3 h, the precipitate was removed by filtration and washed with dioxane. The latter was removed by evaporation in vacuo to afford a residue, which was poured into brine (100 ml). The resulting product was extracted with EtOAc (100 ml × 3) with the EtOAc solubles combined. After drying over Na_2SO_4, the extract was evaporated to dryness. The residue was purified by Si gel flash CC. The column (150 × 20 mm) was developed successively with 300 ml of Me_2CO–*n*-hexane (3:7, 4:6 and 1:1 v/v), respectively. Compound **8** (362 mg) was eluted with Me_2CO–*n*-hexane (4:6, v/v)**:** δ_H 9.48 (1H, s, 7-H), 6.63 (1H, d, *J* = 2.3 Hz, 5-H), 5.70 (1H, d, *J* = 2.3 Hz, 3-H), 3.82 (3H, s, 8-H); EI-MS, 70 eV, *m*/*z* 154 (M$^+$, 22%), 125 (100), 69 (20), and 59 (15).

4.4.2. 4-Methoxy-6-[(E)-1-propenyl]-2H-pyran-2-one (*9*)

Ethyltriphenylphosphonium bromide (1.2 g, Wako Pure Chemical Industries, Ltd.) was dissolved into DMF (11 ml), and then bis(trimethylsilyl) amide (3 ml, Sigma–Aldrich, Inc.) was added. Compound **8** (120 mg) in DMF (4 ml) was added and the mixture was stirred vigorously at r.t. for 3 h under N_2. The reaction mixture was poured into brine (70 ml) and extracted with EtOAc (70 ml × 3). The combined EtOAc solubles were dried (Na_2SO_4) and evaporated in vacuum to dryness. The residue was subjected to Si gel flash CC (150 × 20 mm), which was developed successively with 200 ml each of Me_2CO–*n*-hexane (5:95,1:9,2:8 and 3:7,v/v), respectively. Compound **9** and its *Z*-isomer were eluted with Me_2CO–*n*-hexane (1:9 and 1:4, v/v) fractions (61 mg). Heating of the crystalline mixture at 130 °C in a sealed tube under N_2 caused isomerization to give 57 mg of compound **9**: δ_H 6.68 (1H, dq, *J* = 15.5, 7.0 Hz, 8-H) 5.97 (1H, dq, *J* = 15.5, 1.5 Hz, 7-H), 5.74 (1H, d, *J* = 2.2 Hz, 5-H), 5.43 (1H, d, *J* = 2.2 Hz, 3-H), 3.80 (3H, s, 10-H), 1.88 (3H, dd, *J* = 7.0, 1.5 Hz, 9-H); EI-MS, 70 eV, *m*/*z* 166 (M$^+$, 63%), 138 (100), and 69 (38).

4.4.3. 3-[(E)-2-butenoyl]-4-methoxy-6-[(E)-1-propenyl]-2H-pyran-2-one (*10*)

To a mixture of compound **9** (80 mg) and $TiCl_4$ (260 μl, Wako Pure Chemical Industries, Ltd.) in CH_2Cl_2 (1 ml), crotonoyl chloride (70 μl, Wako Pure Chemical Industries, Ltd.) was added slowly. The solution was stirred at r.t. for 20 min, and then at 45 °C for 6 h. It was then poured into brine (30 ml), and extracted with EtOAc (30 ml x 3). The EtOAc solubles were combined, dried over Na_2SO_4, and evaporated to dryness. The resulting residue was purified by Si gel flash CC (150 × 20 mm) developed successively with 100 ml each of Me_2CO in *n*-hexane (1:4,1:3,3:7 and 35:65 v/v). Compound **10** (40 mg) was eluted with Me_2CO–*n*-hexane (1:4, v/v) whereas compounds **1** and **2** were eluted using Me_2CO–*n*-hexane (3:7, v/v). Separation of compounds **1** and **2** by chiral HPLC used a CHIRALPAK OD column (Daicel Chemical Industries, Ltd., 4.6 × 250 mm), eluted with EtOH-*n*-hexane (1:1, v/v) at a flow rate of 0.5 ml/min, and monitoring at 280 nm to afford compounds **1** (2.5 mg) and **2** (3.0 mg). The retention times of compounds **1** and **2** were 26.2 and 30.8 min, respectively. Compound **10:** δ_H 1.85 (3H, dd, *J* = 7.0, 1.4 Hz, 9-H), 1.87 (3H, dd, *J* = 7.0, 1.4 Hz, 14′-H), 3.82 (3H, s, 10-H), 5.94 (1H, s, 5-H), 5.97 (1H, dq, *J* = 15.5, 1.4 Hz, 7-H), 6.40 (1H, dq, *J* = 15.5, 1.4 Hz, 12-H), 6.82 (2H, m, 8, 13-H); EI-MS, 70 eV, *m*/*z* 234 (M$^+$, 100%), 193 (80), 165 (48), and 138 (32).

4.4.4. Deoxyradicinin (*1*)

A mixture of compound **10** (40 mg), $TiCl_4$ (90 μl) and CH_2Cl_2 (1 ml) was stirred at 45 °C for 3 h. It was then poured into brine (30 ml), extracted with EtOAc (30 ml × 3). The organic layer was washed with brine (100 ml × 3). The combined EtOAc extracts were dried (Na_2SO_4) and evaporated to dryness, with the residue was subjected to Si gel flash CC (150 × 20 mm) developed successively with 100 ml each of Me_2CO-*n*-hexane (1:4,1:3,3:7 and 35:65,v/v). Compounds **1** and **2** were eluted with Me_2CO-*n*-hexane (3:7, v/v), and separated by chiral HPLC as above to afford compounds **1** (1.8 mg) and **2** (2.5 mg). Compound **1**, **2**: δ_H 6.86 (1H, m, 2′-H), 5.95 (1H, dq, *J* = 15.5, 1.8 Hz, 1′-H), 5.76 (1H, s, 8-H), 4.67 (1H, m, 2-H), 2.59 (2H, m, 3–H), 1.88 (3H, dd, *J* = 7.3, 1.4 Hz, 3′-H), 1.46 (3H, d, *J* = 5.6 Hz, 2-Me); δ_C 186.2, 176.6, 163.8, 156.7, 138.9, 124.0, 100.7, 99.1, 77.6, 44.3, 20.4, 18.6; EI-MS, 70 eV, *m*/*z* 220 (M$^+$, 100%), 205 (50), and 177 (59). Compound **1**: $[\alpha]_D$ +90°(*c* 0.1, $CHCl_3$). Compound **2**: $[\alpha]_D$ −82° (*c* 0.1, $CHCl_3$).

4.5. Precursor administration

The fungus was grown on medium (500 μl) containing 15 g/l of malt extract broth (Difco Laboratories Inc.) in a test tube

(75 × 8 mm) without shaking at 24 °C for 7 days in the dark. Then the medium was removed aseptically from the tube with fungal mats washed with 100 mM K-Pi buffer, pH 7.0. The fungus was incubated in 500 μl of the same buffer at 24 °C for 3 days in the dark. After incubation, the buffer was removed from the tube aseptically and the fungal mats were washed with buffer. Then, new buffer (500 μl) was introduced into the tube and DMSO (50 μl) containing precursor (0.05 mg) was added to the buffer. After 5 days incubation, the fungal mat was removed and the remaining buffer was extracted with EtOAc (100 μl × 3). The EtOAc extracts were combined, air-dried overnight, redissolved in MeOH and analyzed by HPLC as described in Section 4.1. Retention times of compounds **1**, **3** and **4** were 15.8, 13.5 and 11.8 min, respectively.

4.6. Preparation of cell-free extract

The grown mycelia were homogenized with sea sand, a mortar and pestle in 50 mM K-Pi buffer, pH 7.0 at 4 °C. The homogenate was centrifuged at 2500*g* for 10 min at 4 °C. The supernatant was centrifuged at 19,000*g* for 15 min at 4 °C to yield the cell-free extract. The extract thus obtained was then subjected to ultracentrifugation at 30,000*g* for 180 min at 4 °C to afford cytosolic and microsomal fractions. The microsomal precipitates were suspended in 50 mM K-Pi buffer, pH 7.0, and used for the experiments. Glycerol was added to each fraction up to 15% (v/v), and fractions were maintained at −80 °C until use.

4.7. Enzyme activity

The following method was used to detect deoxyradicinin monooxygenase or radicinin epimerase activity in the cell extract, cytosolic and microsomal fractions. The crude enzyme preparation (10 μl) was incubated at 35 °C for 2 h or 30 min with 2 μl of 20 mM substrate in MeOH, 3 μl of co-factor solution (containing 20 mM each of NAD^+, $NADP^+$, NADH and NADPH), 35 μl of 70 mM K-Pi buffer, pH 7.0. During purification of epimerase, 3 μl of buffer was used in place of 3 μl of co-factor solution in the enzyme assay. After incubation, the reaction mixture was extracted with EtOAc (100 μl × 3). The combined EtOAc solution was air-dried overnight, dissolved in the MeOH, and analyzed by HPLC described in Section 4.1. In the HPLC analysis for the conversion of deoxyradicinin (**1**) to radicinin (**3**), their retention times were 15.8 and 13.5 min and in the HPLC analysis for conversion of radicinin (**3**) to 3-*epi*-radicinin (**4**), those were 28.5 and 24.0 min. The crude enzyme solution usually contained radicinin (**3**) and 3-*epi*-radicinin (**4**). Thus EtOAc was added first to the enzyme cocktail prior to substrate and then substrate was added. The mixture was extracted with EtOAc without incubation. The EtOAc extract was analyzed by HPLC to afford the initial amount of the products in the enzyme solution. The true amount of the product formed by the enzyme preparation was obtained by subtracting initial amount from amount after enzyme reaction. To determine the effect of pH on enzyme activity and stability, 0.2 M NaOAc buffer was used for pH 4.0 to 5.0, 0.2 M K-Pi buffer for pH 6.0 to 8.0 and 0.2 M Tris–HCl buffer for pH 7.0 to 9.5. One milli molar of phenylmethylsulfonyl fluoride in 50 mM K-Pi buffer (pH 7.0) was used for the optimum temperature determination of the monooxygenase enzyme (SD1), and 0.4 mM EDTA, 10 mM $MgCl_2$, 10% glycerol in 20 mM Tris–HCl buffer (pH 8.0) was used for the optimum temperature determination of the epimerase enzyme (SD2).

4.8. Purification of radicinin epimerase

All procedures were conducted at 4° unless otherwise stated. The cytosolic fraction was loaded onto a DE52 (Whatman) column (100 × 38 mm) equilibrated with 50 mM Tris–HCl buffer (pH 7.0). The column was washed with 80 ml of buffer, followed by a linear gradient elution of 0–0.5 M NaCl in the buffer, at a flow rate of 0.6 ml/min, and each 3.0 ml was collected as one fraction. Active fractions eluted between 0.19 and 0.31 M NaCl were combined, and an equivalent amount of buffer containing 1.6 M $(NH_4)_2SO_4$ was added. The solution was loaded onto a Phenyl Sepharose CL-4B (Sigma–Aldrich) column (90 × 9 mm). The column was washed with 0.8 M $(NH_4)_2SO_4$ buffer (80 ml), followed by a linear gradient elution of 0.8–0 M $(NH_4)_2SO_4$ in the buffer, at a flow rate of 0.6 ml/min, and each 2.0 ml fraction was collected. Active fractions eluting between 0.56 and 0.32 M were combined, loaded onto a Superdex 200 10/300 GL (GE Healthcare) column, eluted with 20 mM Tris–HCl buffer (pH 8.0) at a flow rate of 0.6 ml/min, and each 0.5 ml was collected as one fraction. Active fractions were combined and loaded onto a Mono Q HR 5/5 (Amersham) column (100 × 38 cm) equilibrated with 20 mM Tris–HCl buffer (pH 8.0). The column was washed with 80 ml of the same buffer, followed by a linear gradient elution of 0–0.4 M NaCl in the buffer, at a flow rate of 0.5 ml/min, and each 0.3 ml was collected as one fraction. The active fractions eluted between 0.18 and 0.23 M NaCl were combined, and the same purification with a Mono Q HR 5/5 (Amersham) column was repeated. Radicinin epimerase was eluted at around 0.21 M NaCl (see Table 1 and SD3B).

4.9. Molecular weights of deoxyradicinin monooxygenase and radicinin epimerase

To determine the molecular weights of monooxygenase and epimerase, Superdex 200 10/300 GL CC as described above was performed with the standard proteins, thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B-12 (1.35 kDa).

4.10. SDS–PAGE

After reduction with 2-mercaptoethanol, the relative molecular mass of the purified enzyme was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% polyacrylamide gels at 75 mA per slab with Tris–glycine, pH 8.3, using 0.1% SDS as running buffer. Coomassie brilliant blue stain solution (CBB R-25 1 g, MeOH 100 ml, AcOH 30 ml, in D.W. 400 ml) was used to stain the enzyme. After decolorization, the gel was stained with a silver staining kit (Silver Staining II kit, Wako Pure Chemical Industries, Ltd.). LMW Marker Kit (GE Healthcare) was used as molecular marker (SD3A).

Acknowledgments

We would like to thank Ms. N. Inoue and Mr. S. Matsunaga for technical assistance. This work was supported by a Global COE Program (J-09) from the Japan Society for the Promotion of Science.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.11.010.

References

Canning, A.-M., Hook, I., Sheridan, H., James, J.P., Kelly, D.R., 1992. Bisradicinin: a novel dimer elicited in cultures of *Alternaria chrysanthemi*. J. Nat. Prod. 55, 487–490.

Clarke, D.D., Nord, F.F., 1953. Radicinin: a new pigment from *Stemphylium radicinum*. Arch. Biochem. Biophys. 45, 469–470.

Dahm, K., Lindlau, M., Breuer, H., 1968. Steroid epimerase-a new enzyme of oestrogen metabolism. Biochim. Biophys. Acta 159, 377–389.

Grove, J.F., 1964. Metabolic products of *Stemphylium radicinum*. Part I. Radicinin. J. Chem. Soc., 3234–3239.

Grove, J.F., 1970. Metabolic products of *Stemphylium radicinum*. Part III. Biosynthesis of radicinin and pyrenophorin. J. Chem. Soc. C: Organic. 13, 1860–1865.
Hansen, R.O., 1954. Stemphylone, a root-killing substance from *Stemphylium radicinum*. Acta Chem. Scand. 8, 1332–1334.
Kadam, S., Poddig, J., Humphrey, P., Karwowski, J., Jackson, M., Tennent, S., Fung, L., Hochlowski, J., Rasmussen, R., McAlpine, 1994. Citrinin hydrate and radicinin: human rhinovirus 3C-protease inhibitors discovered in target-directed microbial screen. J. Antibiot. 47, 836–839.
Kato, K., Hirata, Y., Yamamura, S., 1969. Syntheses of (±)-radicinin and (±)-dihydroradicinin. J. Chem. Soc. (C) 15, 1997–2002.
Mazumder, R., Sasakawa, T., Kaziro, Y., Ochoa, S., 1962. Metabolism of propionic acid in animal tissues. IX. Methylmalonyl coenzyme A racemase. J. Biol. Chem. 237, 3065–3068.
Nakajima, H., Ishida, T., Otsuka, Y., Hamasaki, T., Ichinoe, M., 1997. Phytotoxins and related metabolites produced by *Bipolaris coicis*, the pathogen of Job's tears. Phytochemistry 45, 41–45.
Noordeloos, M.E., De Gruyter, J., Van Eijl, G.W., Roeijmans, H.J., 1993. Production of dendritic crystals in pure cultures of *Phoma* and *Ascochyta* and its value as a taxonomic character relative to morphology, pathology and cultural characteristics. Mycol. Res. 97, 1343–1350.
Nukina, M., Marumo, S., 1977. Radicinol, a new metabolite of *Cochliobolus lunata*, and absolute stereochemistry of radicinin. Tetrahedron Lett. 37, 3271–3272.
Pryor, B.M., Gilbertson, R.L., 2002. Relationships and taxonomic status of *Alternaria radicina*, *A. Carotiincultae*, and *A. petroselini* based upon morphological, biochemical, and molecular characteristics. Mycologia 94, 49–61.
Robeson, D.J., Gary, G.R., Strobel, G.A., 1982. Production of the phytotoxins radicinin and radicinol by *Alternaria chrysanthemi*. Phytochemistry 21, 2359–2362.
Robeson, D.J., Strobel, G.A., 1982. Deoxyradicinin, a novel phytotoxin from *Alternaria helianthi*. Phytochemistry 21, 1821–1823.
Schmitz, W., Fingerhut, R., Conzelmann, E., 1994. Purification and properties of an α-methylacyl-CoA racemase from rat liver. Eur. J. Biochem. 222, 313–323.
Seto, H., Urano, S., 1975. Revised assignment of the ^{13}C NMR spectrum of radicinin. Agric. Biol. Chem. 39, 915–916.
Solfrizzo, M., Vitti, C., De Girolamo, A., Visconti, A., Logrieco, A., Fanizzi, F.P., 2004. Radicinols and radicinin phytotoxins produced by *Alternaria radicina* on carrots. J. Agric. Food Chem. 52, 3655–3660.
Suzuki, E., Hamajima, R., Inoue, S., 1975. A facile synthesis of 6-conjugated 2-pyrones. Synthesis 192, 194.
Tal, B., Goldsby, G., Burke, B.A., Aasen, A.J., Robeson, D.J., 1988. Studies on the mechanism of polyketide-derived biosynthesis of deoxyradicinin and related metabolites of Alternaria helianthi. J. Chem. Soc. Perkin Trans. I, 1283–1287.
Tal, B., Robeson, D.J., Burke, B.A., Aasen, A.J., 1985. Phytotoxins from *Alternaria helianthi*: radicinin, and the structures of deoxyradicinol and radianthin. Phytochemistry 24, 729–731.
Tanabe, M., Seto, H., Johnson, L., 1970. Biosynthetic studies with carbon-13. Carbon13 nuclear magnetic resonance spectra of radicinin. J. Am. Chem. Soc. 92, 2157–2158.
van der Drift, L., Vogels, G.D., van der Drift, C., 1975. Allantoin racemase: a new enzyme from *Pseudomonas* species. Biochim. Biophys. Acta 391, 240–248.