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ISOLATION AND METABOLISM OF 3′-HYDROXY-γ-IONYLIDENEACETIC ACIDS IN *CERCOSPORA CRUENTA*

HIROSHI YAMAMOTO, TAKAYUKI ORITANI, TAKESHI MORITA and AKIRA TANAKA

Department of Applied Biological Chemistry, Faculty of Agriculture, 1-1 Tsutumidori-Amamiyamachi, Tohoku University, Aoba-ku, Sendai 981, Japan

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Key Word Index—*Cercospora cruenta*; Hyphomycetes; sesquiterpene; abscisic acid; positional isomer; hydroxylation; biosynthesis; germination inhibitor.

Abstract—Four isomers of 3′-hydroxy-γ-ionylideneacetic acid were isolated from *Cercospora cruenta* cultures. The ratio of isomers was found to be 44:24:21:11 for (2*Z*,4*E*)-(1′*S*,3′*S*)-, (2*Z*,4*E*)-(1′*S*,3′*R*)-, (2*E*,4*E*)-(1′*S*,3′*S*)- and (2*E*,4*E*)-(1′*S*,3′*R*)-3′-hydroxy-γ-acid, respectively. Feeding experiments with [^{14}C] labelled (2*Z*, 4*E*)-(1′*S*, 3′*S*)-3′-hydroxy-γ-acid showed high incorporation into 1′,3′-dihydroxy-γ-acid and 1′-hydroxy-3′-oxo-γ-acid, but not into 4′-hydroxy-γ-acids. This result suggested that 3′-hydroxy-γ-acids were not involved in the ABA biosynthetic pathway. The biological activities of these new metabolites were compared with those of known ABA biosynthetic intermediates.

INTRODUCTION

The biosynthetic pathway to the plant hormone, abscisic acid (ABA), has been studied in fungi by several groups. The main similarity among fungal pathways is the existence of unfunctionalized simple ionylidene ethanols [1, 2] as precursors attributable to the C-15 direct pathway, which differs from the C-40 carotenoid pathway in plants. Each fungus is, however, distinct in its production of ABA precursors. For example, *Cercospora cruenta* is the sole strain known to produce an analogue of γ-ionylideneacetic acid (**1**) [2].

Another remarkable feature of *C. cruenta* is its loose selectivity in the hydroxylation step (Fig. 1). In addition to both epimeric 4′-hydroxy-γ-acids (**2** and **3**), a mixture of 3′-hydroxy-γ-acids (**4** and **5**) was also isolated on administering (±)-[2-^{14}C]-**1** to this organism [3]. Thus, besides the known ABA biosynthetic intermediates, **2**, **3** and 1′,4′-dihydroxy-γ-acid (**6**), the high radioactivity was also detected in the new monohydroxy-γ-acids **4** and **5**, which seemed to be outside the ABA biosynthetic pathway.

The natural production of 3′-hydroxy-γ-acids by *C. cruenta* remains to be proven. In this study, efforts were made to isolate a reasonable amount of **4** and **5** without exogenous addition of any precursor, and to investigate the biological significance of 3′-γ-acid. To further trace its metabolism, [^{14}C]-labelled 3′-hydroxy-γ-acid was synthesized and adiminstered to the culture of *C. cruenta*. Four stereoisomers of 3′-hydroxy-γ-acids (**4**, **5**, **7** and **8**) had already been synthesized [4]. The syntheses of 1′,3′-dihydroxy-γ-acid (**9**) and 1′-hydroxy-3′-oxo-γ-acid (**10**) have also been achieved recently [5] to provide authentic specimens for feeding experiments and samples for biological testing.

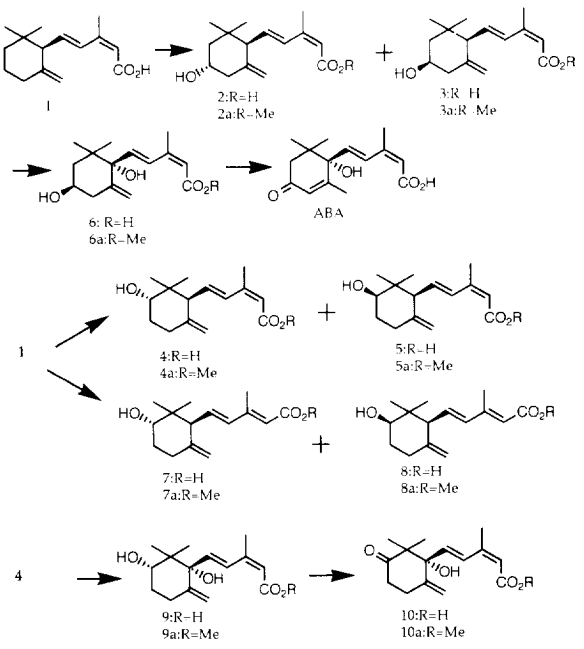

Fig. 1. Metabolism of γ-ionylideneacetic acid analogues in *Cercospora cruenta*.

RESULTS AND DISCUSSION

Isolation of 3′-hydroxy-γ-acids

Preliminary work using synthetic samples [4] paved the way for smooth isolation of the desired metabolites. Thus, a crude mixture of 3′-hydroxy-γ-acids (fraction A, 190 mg) was efficiently obtained from a large-scale cul-

ture (69 l) of *C. cruenta* without exogenous precursors under normal conditions. The existence of four isomers **4**, **5**, **7** and **8** was established by ^{1}H NMR spectroscopy (100 MHz), where the ratio of (2*Z*,4*E*)- and (2*Z*,4*E*)-isomers was roughly estimated to be 7:3 following integration of the olefinic methyl proton resonances. At this stage, a more detailed assignment was impossible. 3′-Hydroxy-γ-acids were next converted into their methyl esters as a prelude to separation. In spite of extensive examination of various TLC solvent systems, the R_f values of its four isomers were still very close. Finally, repeated preparative TLC provided the separation of a small amount of each isomer for spectral analysis. Subsequent comparison with authentic specimens unequivocally established the structure of the naturally occurring metabolites. Accordance was also observed in the direction of optical rotation. In particular, natural **4a** showed a positive Cotton effect on ORD, thus establishing the (1′*S*)-configuration. The exact isomeric ratio of natural 3′-hydroxy-γ-acids was determined by a combination of GLC and HPLC as follows: (2*Z*,4*E*)-(1′*S*,3′*S*)-**4a**: (2*Z*,4*E*)-(1′*S*,3′*R*)-**5a**: (2*E*,4*E*)-(1′*S*,3′*S*)-**7a**: (2*E*,4*E*)-(1′*S*,3′*R*)-**8a** = 44:24:21:11, in contradiction to our previous reports (**4a**:**5a** = 2:8) [3].

In summary, C. cruenta *produces relatively high levels of 3′-hydroxy-γ-acids* as a mixture of four isomers. It is presumed that the culture conditions such as the exogenous addition of a precursor and the culture period affects the level of 3′-hydroxy-γ-acids, as well as the isomer ratios.

Administration of 3′-hydroxy-γ-acids to C. cruenta *cultures*

Like the transformation of the 4′-hydroxy-γ-acids, **2** and **3**, to ABA, it was possible that *C. cruenta* could convert 3′-hydroxy-γ-acid **4** to **10** via **9**. Those putative metabolites have not been isolated presumably due to their very small quantities. A substantial amount of **4** and **5** was isolated in this study. On the other hand, the cyclization mechanism of farnesyl pyrophosphate on the ABA biosynthesis has not been completely clarified to explain the C-15 direct pathway in fungi [6]. The finding of 3′-hydroxy-γ-acid is reminiscent of epoxide-mediated cyclization of squalene to lanosterol via 2,3-oxidosqualene [7]. Presumable deoxygenation of 3′-hydroxy-γ-acid to **1** might play the role of a cyclized intermediate in ABA biosynthesis. A similar discussion has been made for the biosynthesis of cryptoporic acid, a bicyclic sesquiterpenoid [8]. Therefore, it was instructive to establish whether 3′-hydroxy-γ-acid can be converted into ABA and/or its intermediates e.g. **2**, **3** and **6**.

First, 1′,3′-dihydroxy-γ-acid **9** was isolated as a primary metabolite from the major isomer of 3′-hydroxy-γ-acid **4**. Unlabelled (1′*S*,3′*S*)-**4** was fed to the early culture of *C. cruenta*, and the acidic metabolites were extracted after additional culturing. Analysis of acidic fractions revealed that **9** had the same R_f as that of 1′,4′-dihydroxy-γ-acid **6** by TLC. The olefinic protons of **9** and **6** on ^{1}H NMR spectra (270 MHz) were well-separated, and the level of **9** was estimated nearly equal to endogenous **6**. The methyl esters, prepared from the acidic fraction, were separated by TLC to yield **9a** which was identical to an authentic specimen [5]. An extensive search for 1′-hydroxy-3′-oxo-γ-acid **10** was unsuccessful.

Similarly, [2-^{14}C]-labelled **4** was administered to *C. cruenta* to examine further the metabolism of **9**, and to establish its metabolic fate. Horner reaction using [2-^{14}C]-ethyl diethylphosphonoacetate [3] was adopted for the previous synthetic route [4] to afford labelled substrates. In the case of [2-^{14}C]-(2*Z*,4*E*)-isomer **4**, a significant amount of radioactivity was incorporated into **9** (19.8%) and **10** (4.8%) [Table 1]. Besides partial isomerization of (2*Z*,4*E*)-**4** to (2*E*,4*E*)-**7**, no substantial incorporation into known ABA biosynthetic intermediates was observed. High recovery of **7** suggested the endogenous accumulation of this compound in the culture. These results suggest no actual participation of 3′-hydroxy-γ-acid in the ABA biosynthetic pathway.

Biological activity of new compounds derived from 3′-hydroxy-γ-acid

Although several ABA analogues possessing strong biological activity have been found, a positional isomer of ABA like **10** has not been reported. It might be convenient to designate **10** tentatively γ-pseudoABA [5] to express its structural importance. The biological activity of **1** and related compounds is of great interest. Growth inhibitory activity on rice seedlings was examined (Fig. 2). On each part of the plant, there appeared to be a tendency whereby the inhibitory activity increased in order of **4**, **9** and **10**. In particular, **10** was relatively potent. This observation was reminiscent of the biological activity of ABA biosynthetic intermediates produced by *C. cruenta*. It has been reported that there was a **10**-fold increase in activity between **3** and ABA [9], or between **6** and ABA [10]. Germination inhibitory activity was examined on lettuce and radishes (Fig. 3). Lettuce is

Table 1. Incorporation of ^{14}C-labelled 3′-hydroxy-γ-acids by *C. cruenta*

Metabolites	From **4**	From **7**
ABA	0.3%	0.2%
1′-DeoxyABA	0.2	0.2
trans-4′-Hydroxy-γ-acid, **2**	0.5	0.6
cis-4′-Hydroxy-γ-acid, **3**	0.5	0.3
(2*Z*,4*E*)-3′-Hydroxy-γ-acid, **4**	29.3 (recovery)	2.0
1′,4′-Dihydroxy-γ-acid, **6**	0.6	0.4
(2*E*,4*E*)-3′-hydroxy-γ-acid, **7**	8.0	53.3 (recovery)
1′,3′-Dihydroxy-γ-acid, **9**	19.8	6.7*
1′-Hydroxy-3′-oxo-γ-acid, **10**	4.8	0.7
Neutral fraction	1.7	0.5
Aqueous fraction	3.6	2.2

*This fraction consists of **9** and its (2*E*,4*E*)-isomer (9:91).

known as one of the most sensitive species for the assay, while radishes are rather insensitive, but **9** and **10** were more effective on radishes than on lettuce. High activity of γ-pseudoABA **10** might be expected for certain plant species. Unfortunately, the Japanese azuki bean (*Phaseolus angularis* cv Dainagon), the host plant of *C. cruenta*, was not suitable for the germination assay because of its stout hull. Though biological activities of new compounds were rather inferior to those of ABA and ABA biosynthetic intermediates, it was notable that **10** was a more effective inhibitor than ABA on radish germination.

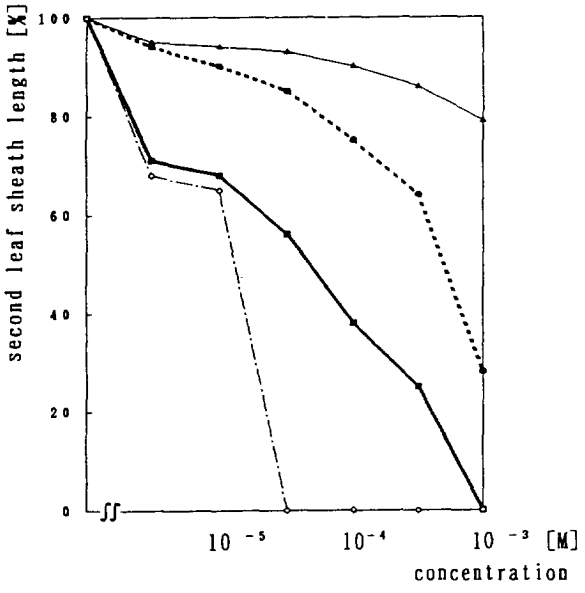

Fig. 2. Growth inhibitory activity on rice seedlings. 3'-Hydoxy-γ-acid (**4**,—▲), 1',3'-dihydroxy-γ-acid (**9**,– – –●), 1'-hydroxy-3'-oxo-γ-acid (**10**, –■), ABA (– · – ◇).

EXPERIMENTAL

Mps: uncorr. ^{1}H NMR (100 MHz) was measured using TMS as int. standard. MS spectra were measured on a Hitachi M-52 instrument (13.5 eV and 80°), for a JEOL JMS MX-105 (70 eV and 180–200°). GLC analysis was performed on a JEOL JGC-20K using 2 × 3 mm glass column packed with 5% SE-30. HPLC analysis was performed on a JASCO Trirotor instrument using ERC-silica-1181 with CH_2Cl_2–*iso*-PrOH (97:3) as eluent. Radioactivity was measured on a scintillation spectrometer, using a liquid scintillation cooktail (toluene–methanol–POP–A–POPOPP = 150 ml: 150 ml: 1.5 g: 30 mg). Analyt. (0.25 mm thickness, 10 × 2 cm) and prep. TLCs (0.70 mm, 40 × 20 cm) were performed on silica gel (Merck PF_{254} 60H) with the following solvent systems; A: benzene–*iso*-propyl ether (10:3), developed twice. B: benzene–EtOAc–HOAc (60:40:1), developed once. C: *n*-hexane–EtOAc (50:15), developed twice.

Isolation of 3'-hydroxy-γ-ionylidenaecetic acids from the culture broth of C. cruenta *IFO* 6164. On 1 l of modified potato medium (20 g of glucose, 4 g of yeast extract and 1.5 g of agar in 1 l of potato extract, pH 6.8), *C. cruenta* was cultured under shaking (120 rpm) and lighting (4000 lux) at 26° for 8–10 days. The filtrate of the culture broth was adjusted to pH 3.0 and then extracted with EtOAc to yield acidic metabolites (typically 0.25 g). This procedure was repeated on 0.5–1.0 l scale. Finally, 21.3 g

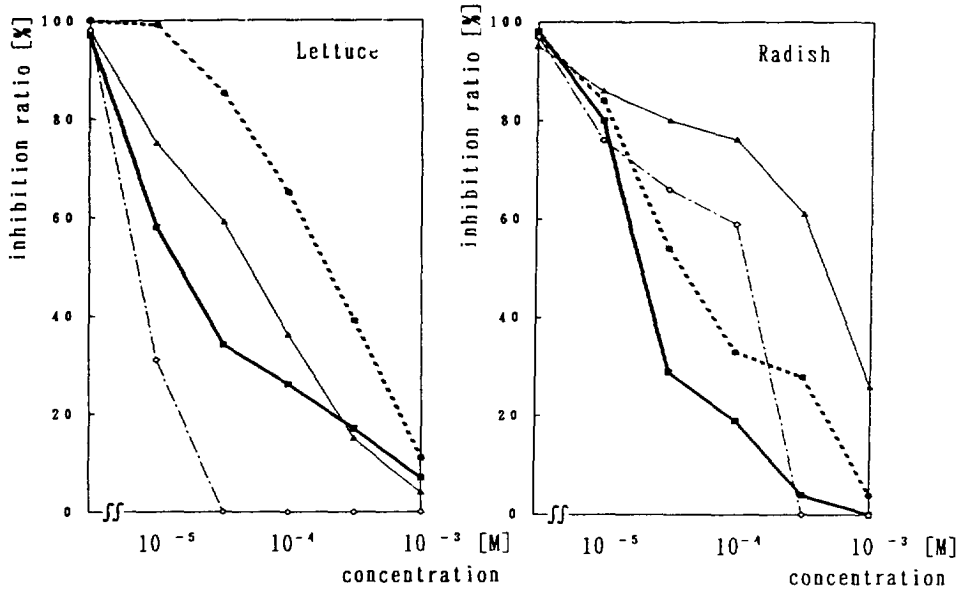

Fig. 3. Germination inhibitory activity on lettuce and radish. 3'-Hydroxy-γ-acid (**4**, –▲), 1',3'-dihydroxy-γ-acid (**9**, – – –●), 1'-hydroxy-3'-oxo-γ-acid (**10**, –■), ABA (– · – ◇).

of acidic metabolities was obtained from 69.3 l of total culture vol. The crude acids were subsequently methylated with ethereal CH_2N_2, washed with 5% aq. $NaHCO_3$ soln, and chromatographed over silica. The crude methyl esters (2.8 g) so obtained were further purified by prep. TLC (*ca* 50 plates) to yield purified esters (240 mg, R_f 0.35–0.40, solvent system A). Alkaline hydrolysis followed by TLC sepn yielded fr. A (190 mg, R_f 0.44–0.45, solvent system B) and *p*-hydroxyphenylacetic acid (mp 149°, 21 mg, R_f 0.42–0.43). Comparison of the ^{1}H NMR (100 MHz) spectra of authentic **4** revealed that fr. A consisted of a mixt. of **4**, **5**, **7** and **8**.

Separation of four isomers (**4a**, **5a**, **7a** *and* **8a**). Fr. A was again methylated for further sepn. A small portion (16 mg) was sepd by prep. TLC (solvent system A) to yield **4a** (R_f 0.49, 4.9 mg), **5a** (R_f 0.52, 2.0 mg), and the mixt. of **7a** and **8a** (65:35 from ^{1}H NMR, R_f 0.58, 4.0 mg). The exact ratio of 4 isomers was determined as follows. GLC (200°, N_2 at 1.0 kg cm^{-2}): 65% (R_f 10.8 min for **4a** and **5a**) and 35% (R_f 12.9 min for **7a** and **8a**). HPLC (25° 1.0 ml min^{-1}): 68%(R_f 10.8 min for **4a** and **7a** and 32% (R_f 12.9 min for **5a** and **8a**). The ^{1}H NMR (100 MHz), EI-MS (13.5 eV), and IR (film) spectra of each sample were almost identical to those of authentic specimens [4]. ORD of **4a** (MeOH; *c* 0.0098) $[\alpha]_D^{20}$: + 70° (350), + 920° (285), 0° (272), − 4700° (242).

Isolation of (1′R,3′S)-(2Z,4E)-1′,3′-*dihydroxy*-γ-*ionylideneacetic acid* (**9**) *from the precursor-fed broth of* C. cruenta. *Cercospora cruenta* was subcultured on modified potato medium (1 l). When the culture became light grey after *ca* 5 days, a soln of (1′*S*,3′*S*)-**4** (24.0 mg dissolved in 1.0 ml of 5% $NaHCO_3$ aq. soln) was added. After incubation for another 5 days, the acidic metabolites (*ca* 0.2 g) were extracted in the usual manner. These were subsequently sepd by prep. TLC (solvent system B). A main broad band of dihydroxy-γ-acids (R_f 0.46) was submitted to ^{1}H NMR spectral analysis. Although almost all ^{1}H NMR (100 MHz) signals of **9** and endogenous **6** overlapped with each other, for each set of olefinic protons the signal was well-discriminated by use of a 270 MHz instrument (**9**:**6** = 46:54). This mixt. was methylated and further sepd by prep. TLC (solvent system D) to give 2 frs; a less polar methyl ester **9a** was obtained as a yellow oil (red brown spot by 5% H_2SO_4 spray, R_f 0.31). The exact amount of **9a** was calcd at 33 mg (13% from exogenous **4**) from log ε value (4.30) at 265 nm. Treatment with *n*-hexane–EtOAc (1:1) gave a crystal, mp 117–120°, $[\alpha]_D^{20}$ + 76° ($CHCl_3$; *c* 0.062) [lit. mp 119–120°, $[\alpha]_D^{20}$ + 77.9° ($CHCl_3$; *c* 0.296)] [5]. IR ν_{max}^{film} cm^{-1}: 3360, 3080, 1710, 1640, 1600, 1435, 1360, 1265, 1230, 1160. EIMS (GC) 70 eV, *m/z* (rel. int.) 281 (1), 280 $[M]^+$ (2), 262 $[M - H_2O]^+$ (51), 244 $[M - 2H_2O]^+$ (51), 197 (58), 171 (100), 128 (44). ^{1}H NMR (270 MHz) 0.96 (3H, *s*), 1.05 (3H, *s*), 1.69–2.73 (4H, *m*), 2.05 (3H, *d*, *J* = 1.0 Hz), 3.66 (1H, *br s*), 3.71 (3H, *s*), 4.84 (1H, *s*), 4.97 (1H, *s*), 5.73 (1H, *d*, *J* = 1.0 Hz), 6.38 (1H, *d*, *J* = 15.7 Hz), 7.85 (1H, *d*, *J* = 15.7 Hz). These data were almost identical with those of synthetic specimen [5]. The more polar methyl ester **6a** was obtained as a brown oil (purple spot, R_f 0.28). Spectral data of **6a** were identical with those of a synthetic specimen [11]. The exact amount of **6a** was calcd at 4.3 mg from log ε value (4.32) at 266 nm.

Preparation of [2-^{14}C]-(1′S,3′S)-(2Z,4E)-3′-*hydroxy*-γ-*ionylideneaecetic acid* (**4** *and* **7**). According to the reported procedure [4], (1′*S*,3′*S*)-3′-tetrahydropyranyloxy-γ-ionone (101 mg) was reacted with [2-^{14}C]-ethyl diethylphosphonoacetate [3] and converted to the labelled substrates, [2-^{14}C]-**4** (14.0 mg, 1.40×10^4 dpm mg^{-1}) and [2-^{14}C]-**7** (19.7 mg, 1.40×10^4 dpm mg^{-1}). These were finally purified by prep. TLC (R_f 0.45 for **4** and R_f 0.48 for **7**, solvent system C), and no impurity was observed on each ^{1}H NMR (100 MHz) spectra.

Metabolism of [2-^{14}C]-((1′S,3′S)-(2Z,4E)- *and* (2E,4E)-3′-*hydroxy*-γ-*ionylidenaecetic acid* (**4** *and* **7**). *Cercospora cruenta* was subcultured (1 l). When the cultures became light grey after *ca* 5 days, a soln of [^{14}C]-labelled substrates (*ca* 2.0×10^4 dpm dissolved in 1.0 ml of 5% $NaHCO_3$ eq. soln) was added. The culture always turned black the day after. After incubation for another 5 days, the acidic and neutral metabolites were extracted in the usual manner. The crude acidic metabolites were sepd by prep TLC (solvent system A). In cases of incomplete sepn, frs were again sepd by prep. TLC (solvent system C) and checked by ^{1}H NMR (100 MHz). Incorporation ratio based on the fed amount of **4** or **7** was measured by scintillation counter in duplicate. The feeding experiment was repeated again in the same conditions for each sample, and the total recovery of radioactivity always ranged within 67–70%. R_f values (solvent system B and C) of each compound were **6** (0.25 and 0.16), **9** (0.25 and 0.21) **10** (0.29 and 0.28), ABA (0.32 and 0.32), **2** (0.38 and 0.43), **3** (0.42 and 0.45), **4** (0.46 and 0.45), **7** (0.46 and 0.48), and 1′-deoxyABA (0.45 and 0.51). The fr. containing **9** and **7** was methylated with CH_2N_2 and further sepd by prep. TLC (solvent system D) to give **9a** (9%, R_f 0.31) and its (2*E*,4*E*)-isomer (91%, R_f 0.33).

Biological assay. Synthetic (2*Z*,4*E*) (1′*S*,3′*S*)-**4**, (2*Z*,4*E*)-(1′*R*,3′*S*)-**9** and (1′*R*)-**10** [4, 5] were employed for all assays in duplicate. Racemic (2*Z*,4*E*)-ABA was used as a standard. Growth inhibitory activity was measured on young seedlings of rice (*Oryza sativa* cv Sasaminori) as previously described [12]. After growing at 28° under 5000 lux for 7 days, the length of the second leaf sheath was measured as a percentage of that of the control. Plant height and root length were also measured, and a similar tendency was observed for each part of the plant. Germination inhibitory activity was measured on lettuce (*Lactiva sativa* cv Great Lake) or radish (*Raphanus sativas* cv Akamaru). Germination was carried out in a humid box at 24° in the light (2000 lux) for 4–5 days and the number of seeds which germinated was counted. The activities were expressed as a crude percentage and not compared with control values (96–100%). Methyl ester **9a** was also examined on each assay and showed almost equal activity to that of free acid **9**.

REFERENCES

1. Neil, S. J., Horgan, R., Walton, D. C. and Mercer, C. A. M. (1987) *Phytochemistry* **26**, 2515.

2. Oritani, T., Niitsu, M., Kato, T. and Yamashita, K. (1985) *Agric. Biol. Chem.* **49**, 2819.
3. Kato, T., Oritani, T. and Yamashita, K. (1987) *Agric. Biol. Chem.* **51**, 2695.
4. Yamamoto, H., Oritani, T. and Yamashita, K. (1988) *Agric. Biol. Chem.* **52**, 2203.
5. Yamamoto, H., Oritani, T., Heald, J. A. and Horgan, R. *Phytochemistry* (submitted).
6. Bennett, R. D., Norman, S. M. and Maier, V. P. (1990) *Phytochemistry* **29**, 3473.
7. Corey, E. J. and Virgil, S. C. (1991) *J. Am. Chem. Soc.* **113**, 4025.
8. Hirotani, M., Ino, C. and Furuya, T. (1993) *Phytochemistry* **32**, 891.
9. Sassa, T., Mitobe, H. and Haruki, E. (1988) *Agric. Biol. Chem.* **52**, 1652.
10. Oritani, T., Ichimura, M. and Yamashita, K. (1984) *Agric. Biol. Chem.* **48**, 1677.
11. Yamamoto, H., Oritani, T. and Yamashita, K. (1990) *Agric. Biol. Chem.* **54**, 1923.
12. Oritani, T. and Yamashita, K. (1974) *Agric. Biol. Chem.* **38**, 801.