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Metabolism of (2*Z*,4*E*)-γ-Ionylideneethanol and (2*Z*,4*E*)-γ-Ionylideneacetic Acid in *Cercospora cruenta*

Taku KATO, Takayuki ORITANI and Kyohei YAMASHITA

Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai 980, Japan

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[2-^{14}C]-(2*Z*,4*E*)-γ-Ionylideneethanol and [2-^{14}C]-(2*Z*,4*E*)-γ-ionylideneacetic acid were converted by *Cercospora cruenta* to [2-^{14}C]-(2*Z*,4*E*)-1′,4′-dihydroxy-γ-ionylideneacetic acid and [2-^{14}C]-(2*Z*,4*E*)-4′-hydroxy-γ-ionylideneacetic acid, which are intermediates of ABA biosynthesis in *C. cruenta*.

It has been reported that abscisic acid (ABA, **1**), a plant hormone, is produced by some phytopathogenic fungi.[1～4] We had found that *Cercospora cruenta* could produce (+)-ABA[2] and its analogs: (2*Z*,4*E*)-4′-oxo-α-ionylideneacetic acid (**6**),[2] (2*Z*,4*E*)-1′,4′-dihydroxy-γ-ionylideneacetic acid (**5**),[5] (2*Z*,4*E*)-4′-hydroxy-γ-ionylideneacetic acid (**4**),[6] (2*Z*,4*E*)-γ-ionylideneethanol (**2**),[7] and a trace of (2*Z*,4*E*)-γ-ionylideneacetic acid (**3**).[7] Thus, we presented the biosynthetic pathway[6] of (+)-ABA in *C. cruenta*, on the assumption that **2** and **3** were precursors of **4** from farnesyl pyrophosphate (FPP) (Fig. 1). In this paper, we describe how γ-alcohol (**2**) and γ-acid (**3**) are converted to ABA (**1**) and its analogs (**4**, **5** and **6**) in ABA biosynthesis by *C. cruenta*.

The metabolism of cold substrates, (2Z,4E)-γ-ionylideneethanol (**2**) *and (2Z,4E)-γ-ionylideneacetic acid* (**3**) *by C. cruenta*

First, to follow the microbial conversion of substrates **2** and **3** by spectral analyses of their products, the cold substrates were metabolized by *C. cruenta*. Subcultured mycelium was incubated with **2** for 24 hr, and neutral and acidic metabolite fractions were obtained from the medium. Both fractions were very similar to those of the control, and did not show any other new metabolites on TLC, although **2** disappeared.

Similarly, the acid **3** was fed to the suspension of mycelium. After incubation for 24 hr, the acidic metabolites were extracted from the filtrate of culture broth and then compared with those of a control on TLC. The acid **3** had disappeared, and two spots with *Rf* 0.40 and 0.45 were detected. The compound with *Rf* 0.40 was identified as (2*Z*,4*E*)-4′-hydroxy-γ-ionylideneacetic acid (**4**) on the basis of its *Rf* value on TLC, melting point (215.0～217.5°C) and spectral data of the corresponding methyl ester. The optical purity (O.P.) of the separated **4** was very low, 19% O.P. after

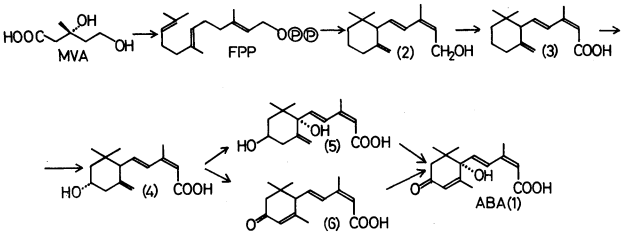
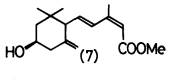
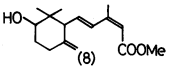


FIG. 1.

incubation for 24 hr, and 5% O.P. after 48 hr. The compound with *Rf* 0.45 was methylated with diazomethane and separated by TLC to give two components. One of them was a stereoisomer of 4′-hydroxy-γ-methyl ester (**7**, *Rf* 0.43).[11] The other (*Rf* 0.40) was further separated by TLC to give two components (**8**: *Rf* 0.46, and **9**: *Rf* 0.40). **8** was estimated to be (2*Z*,4*E*)-methyl-3′-hydroxy-γ-ionylideneacetate on the basis of spectral data as follows. IR spectrum of **8** indicated the presence of a hydroxyl group at 3400 cm^{-1} and a methoxycarbonyl group at 1720 cm^{-1}. The ^{1}H-NMR spectrum of **8** (in $CDCl_3$) showed the presence of a (2*Z*,4*E*)-methyl 3-methyl-2,4-pentadienoate moiety at δ: 2.03 (3H, d, J=1.2 Hz), 3.69 (3H, s), 5.65 (1H, s), 6.18 (1H, dd, J=9 and 16 Hz), 7.61 (1H, d, J=16 Hz) and a methylene group at δ: 4.73 (1H, m), 5.02 (1H, m). MS of **8** revealed peaks at *m/z*: 264 (M^+, $C_{16}H_{24}O_3$), 246 ($M^+ - H_2O$) and 125 (ABA like chain). The product, obtained by oxidation of **8** with pyridinium chlorochromate, showed the presence of an unconjugated carbonyl group at ν_{max} (film): 1720 cm^{-1}. In addition, **8** was resistant to oxidation with active manganese dioxide. From the spectral data of crude **9**; MS (*m/z*: 264 (M^+), 246 ($M^+ - H_2O$) and 125 (ABA like chain)), IR (ν_{max} (film): 3420, 1720 cm^{-1}) and ^{1}H-NMR ($CDCl_3$) δ: 4.54 (1H, m), 4.81 (1H, m), 5.66 (1H, br), 6.25 (1H, dd, J=10 and 16 Hz), 7.57 (1H, d, J=16 Hz)), **9** was estimated to be another epimer of the 3′-hydroxy-γ-acid methyl ester (**8**) or 5′-hydroxy-γ-acid methyl ester. A small amount of a stereoisomeric mixture of 3′-hydroxy-γ-ionylideneacetic acids has been separated from the culture broth of *C. cruenta* and their stereochemistry is now being studied.



(7)



(8)

Incorporation of [2-^{14}*C*]-(2*Z*,4*E*)-γ-*ionylideneethanol* (**2***) *and* [2-^{14}*C*]-(2*Z*,4*E*)-γ-*ionylideneacetic acid* (**3***) *into ABA and its analogs by C. cruenta*

[2-^{14}C]-Methyl bromoacetate was heated with triethylphosphite[8] to give [2-^{14}C]-methyl diethylphosphonoacetate. The Wittig reaction[10] of γ-ionone[9] with the above radioactive phosphonoacetate gave a mixture of [2-^{14}C]-(2*Z*,4*E*)- and (2*E*,4*E*) methyl γ-ionylideneacetates. Purified (2*Z*,4*E*)-methyl ester was hydrolyzed with alkali to give [2-^{14}C]-γ-ionylideneacetic acid (**3***). The methyl ester of radioactive γ-acid (**3***) was reduced with sodium bis(2-methoxyethoxy)aluminum hydride to give [2-^{14}C]-(2*Z*,4*E*)-γ-ionylideneethanol (**2***).

Radioactive alcohol (**2***) was fed to a culture of *C. cruenta*. After incubation, the neutral metabolites and acidic metabolites were extracted from the culture broth. The former were separated by preparative TLC to give recovered γ-alcohol (**2**). The latter gave ABA (**1**) and its analogs **4**, **5**, **6**, **7**, **8**, and **9** by separation with TLC. The distribution of radioactivity and incorporation ratio are shown in Table I. Thus it became obvious that γ-alcohol (**2**) was converted to ABA (**1**) with a low incorporation ratio (0.2%).

Similarly, radioactive acid (**3***) was fed to the culture broth of *C. cruenta* and labelled acidic metabolites (80.6% recovery of total radioactivity) were extracted from culture broth. The metabolites were separated by TLC to give ABA (**1**) and its analogs **4**, **5**, **6**, **7**, **8**, and **9**. The incorporation ratios of each compound are shown in Table I. γ-Acid (**3**) was converted to ABA (**1**) and its analogs.

From these results, it was indicated that γ-alcohol (**2**) and γ-acid (**3**) are intermediates of ABA biosynthesis in *C. cruenta*. As the incorporations of the substrates **2** and **3** into 1′,4′-dihydroxy-γ-acid (**5**) were higher than those into 4′-oxo-α-acid (**6**) as shown in Table I, it was confirmed that the biosynthetic pathway of ABA *via* 1′,4′-dihydroxy-γ-acid (**5**) from γ-alcohol (**2**) in *C. cruenta* could be the main route rather than that *via* 4′-oxo-α-acid as demonstrated in our previous paper.[6] The incorporation ratios of 4′-hydroxy-γ-acids (**4**, **5** and **7**) from γ-alcohol (**2**) were lower than those from γ-acid (**3**). This may come from the instability of **2**, and may indicate the possibility that **2** exists as the pyrophosphate in the

TABLE I. INCORPORATION OF ^{14}C-LABELLED γ-ALCOHOL **2** AND γ-ACID **3** INTO ABA ANALOGS BY *C. cruenta*

Substrate	γ-Alcohol **2** (6.4×10^4 dpm)		γ-Acid **3** (5.58×10^4 dpm)	
	Radioactivity (dpm)	Incorporation ratio (%)	Radioactivity (dpm)	Incorporation ratio (%)
ABA (**1**)	1.39×10^2	0.2	1.89×10^2	0.3
1′,4′-Dihydroxy-γ-acid (**5**)	3.66×10^3	5.7	1.41×10^3	2.5
(4′*R*)-4′-Hydroxy-γ-acid (**4**)	6.13×10^3	9.6	2.30×10^4	41.2
(4′*S*)-4′-Hydroxy-γ-acid (**7**)[a]	1.12×10^3	1.8	3.02×10^3	5.4
3′-Hydroxy-γ-acid (**8**)	1.43×10^3	2.2	5.45×10^3	9.8
Monohydroxy-γ-acid (**9**)	2.60×10^3	4.1	5.16×10^3	9.2
4′-Oxo-α-acid (**6**)	4.10×10^2	0.6	1.93×10^2	0.3
γ-Alcohol (**2**, recovery)	7.87×10^1	0.1		
Aqueous Fraction	6.97×10^2	1.1		

[a] Purified and counted as methyl ester.

biosynthetic pathway of ABA, and it was considered that 3′-hydroxy-γ-acid (**8**), derived from γ-acid (**3**), may not be a biosynthetic intermediate of ABA due to the 3′-position of hydroxyl group. Further, it was suggested that the enzyme which oxidizes γ-acid (**3**) to 4′-hydroxy-γ-acid (**4**) would recognize the chirality at the 1′-carbon to a lesser extent, because racemic γ-acid (**3**) fed to the culture broth of *C. cruenta*, disappeared promptly and formed racemic 4′-hydroxy-γ-acid (**4**).

EXPERIMENTAL

Melting points were measured with a Yanagimoto micro hot stage apparatus and are uncorrected. IR spectra were recorded on a JASCO IR-810 spectrometer. ^{1}H-NMR (100 MHz) spectra were measured on a JEOL JNM FX-100 spectrometer with TMS as an internal standard. UV spectra were measured on a Hitachi Model 124 spectrometer. Preparative TLC was done using silica gel PF_{254} (Merck), 0.5 mm thickness. GLC analyses were done with a Yanagimoto G-3800 instrument with a flame ionization detector, using a 2 m × 3 mm glass column packed with 5% Silicone Gum SE-30. Radioactivity was counted on a scintillation spectrometer, using a scintillator (toluene–methanol–PPO–POPOP = 150 ml : 150 m : 1.5 g : 30 mg).

1) Preparation of (2Z,4E)-γ-ionylideneethanol (**2**) *and (2Z,4E)-γ-ionylideneacetic acid* (**3**). γ-Alcohol (**2**) and γ-acid (**3**) were prepared as described in our previous paper,[7] except for the Wittig reaction for elongation of acetate residue. γ-Ionone (147 mg) was added to the ylid formed from ethyl diethylphosphonoacetate (0.32 ml) and NaH (62 mg) in THF (7 ml). The mixture was heated at 60°C for 20 hr, poured into brine, and extracted with ethyl acetate twice. The organic layer was evaporated and the residue was chromatographed on silica gel and purified with TLC (solvent–benzene) to give ethyl γ-ionylideneacetates (89 mg, 51%) as an oil, which consisted of 25% of (2*Z*,4*E*)-isomer and 75% of (2*E*,4*E*)-isomer by GLC analysis (column temp. 190 C, N_2 flow rate: 20 ml/min).

2) Preparation of [2-^{14}*C]-(2Z,4E)-γ-ionylideneethanol* (**2***) *and [2-*^{14}*C]-(2Z,4E)-γ-ionylideneacetic acid* (**3***). [2-^{14}C]-bromoacetic acid (250 μCi, Amersham Co., Ltd. Code CFA 18) was methylated with ethereal diazomethane and diluted with methyl bromoacetate (2.5 g), then 2.8 ml of triethylphosphite was added. The mixture was heated (120°C) for 24 hr, and evaporated. The residue was used for the Wittig reaction without further purification. γ-Ionone (190 mg) in benzene (5 ml) was added to the ylid formed from the above ^{14}C-labelled Wittig reagent (0.1 ml) and non-labelled ethyl diethylphosphonoacetate (0.22 ml) and NaOMe (184 mg) in methanol (1.7 ml).[10] The mixture was heated at 60°C and stirred for 18 hr, poured into water and then extracted with ethyl acetate. The organic layer was washed with water and dried over $MgSO_4$, and then evaporated. The residue was diluted with cold marker **2** and then purified on TLC (solvent–benzene) twice to give [2-^{14}C]-(2*Z*,4*E*) methyl-γ-ionylideneacetate. The radioactive methyl ester was hydrolyzed with 5% ethanolic KOH and recrystallized from acetonitrile twice to give [2-^{14}C]-(2*Z*,4*E*)-γ-ionylideneacetic acid (**3***, 5.31×10^4 dpm/mg). The radioactive acid **3*** (5.3 mg) and γ-acid **3** (20 mg) was methylated with ethereal diazomethane and then added to RedAl (14% solution in benzene, 0.2 ml). The mixture was stirred for 45 min at 0°C and extracted with ethyl acetate after the addition of 10% ethereal acetic acid. The organic layer was washed with water and dried over $MgSO_4$ before evaporation. The residue was diluted with cold marker **2** and purified on TLC (solvent system;

benzene–ethyl acetate = 4 : 1) to give [2-^{14}C]-(2*Z*,4*E*)-γ-ionylideneethanol (**2***, 12 mg, 1.12×10^4 dpm). The reactions were monitored on TLC, with non-labelled compounds as standards.

3) Metabolism of (2Z,4E)-γ-ionylideneethanol (**2**) *by C. cruenta.* After *C. cruenta* was subcultured on 100 ml of modified potato medium (2.0 g of glucose, 0.4 g of yeast extract, and 0.15 g of agar in 100 ml of potato extract) for 6 days under shaking and lighting (about 4500 lux) at 27°C, the mycelium was centrifuged at 9000 rpm, washed with 1/30 M phosphate buffer (pH 7.3) and then suspended in 100 ml of Czapek-Dox medium without glucose. γ-Alcohol (**2**, 0.2 ml of ethanolic solution) was added to the above suspension and incubated for 24 hr under the same conditions. The culture broth was then filtered and the filtrate was extracted with ethyl acetate at pH 2.5. The extract was further extracted with 5% aqueous $NaHCO_3$. The organic layer (neutral metabolites) was washed with brine and dried over $MgSO_4$. The aqueous layer was adjusted to pH 2 with 1 N-HCl and extracted with ethyl acetate. The organic layer (acidic metabolites) was washed with brine and dried over $MgSO_4$. The neutral metabolites (6 mg) and acidic metabolites (9 mg) were developed on TLC (solvent system; benzene–EtOAc = 8 : 1 and benzene–EtOAc–AcOH = 60 : 40 : 1, respectively).

4) Incorporation of [2-^{14}C]-(2Z,4E)-γ-ionylideneethanol (**2***) *into ABA and its analogs by C. cruenta.* [2-^{14}C]-(2*Z*,4*E*)-γ-ionylideneethanol (**2***, total 6.40×10^4 dpm, ethanolic solution) was added to 100 ml of the culture broth of *C. cruenta*, which had been grown for 5 days under the conditions described in 3). After incubation for 48 hr, the metabolites were extracted from the culture broth and fractionated into acidic and neutral fractions as described in 3). The recoveries of the radioactivities were 38.8% (2.48×10^4 dpm) for the acidic fraction and 15.2% (9.70×10^3 dpm) for the neutral fraction. The neutral metabolites were diluted with γ-alcohol (**2**), and **2*** was recovered by preparative TLC (solvent system; benzene–EtOAc = 4 : 1). The acidic metabolites were methylated with ethereal diazomethane and separated by TLC (solvent system; benzene–EtOAc = 4 : 1) to give methyl ester of ABA (**1**), 4′-hydroxy-γ-acid (**4**), 1′,4′-dihydroxy-γ-acid (**5**), 4′-oxo-α-acid (**6**) and **7**, **8** and **9**. The aqueous fraction was concentrated and extracted with butanol.

5) Metabolism of (2Z,4E)-γ-ionylideneacetic acid (**3**) *by C. cruenta.* After *C. cruenta* was subcultured for 5 days and suspended in 100 ml of Czapek-Dox medium in the same manner as 3). γ-acid **3** (potassium salt in 0.2 ml of ethanol) was added to the above suspension. After incubation for 24 hr, the acidic metabolites were extracted in the same manner as 3) from the culture broth and purified by TLC (solvent system; benzene–EtOAc–AcOH = 60 : 40 : 1). 4′-Hydroxy-γ-acid (**4**,[6] *Rf* 0.40, $[\alpha]_D = -4.5°$ (c = 0.133, EtOH), UV λ_{max} = 261 nm, ε = 13700, mp 215~217.5°C) was methylated with ethereal diazomethane to give the methyl ester of **4**, IR ν_{max} (film) cm^{-1}: 3400, 2960, 1720, 1645, 1605, 1250, 1160, 1050, 990. ^{1}H-NMR ($CDCl_3$) δ: 0.83 (3H, s), 0.95 (3H, s), 1.20~1.80 (2H, m), 2.04 (3H, d, J = 1.2 Hz), 2.56~2.81 (2H, m), 3.69 (3H, s), 4.58 (1H, m), 4.95 (1H, m), 5.66 (1H, br.), 6.12 (1H, dd, J = 10 and 16 Hz), 7.59 (1H, d, J = 16 Hz). GLC (column temp. 200°C, N_2 flow rate: 20 ml/min) t_R = 9.4 min. Compounds with *Rf* 0.45 were methylated with ethereal diazomethane and separated by TLC (solvent system; benzene–EtOAc = 4 : 1) to give two components. One of them was a stereoisomer of the 4′-hydroxy-γ-acid methyl ester (**7**, *Rf* 0.43),[11] IR ν_{max} (film) cm^{-1}: 3400, 2920, 2850, 1720, 1630, 1600, 1155, 1030, 990, 890. ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, s), 0.93 (3H, s), 1.20~1.85 (4H, m), 2.00 (3H, d, J = 1.2 Hz), 2.65 (1H, d, J = 9 Hz), 3.69 (3H, s), 3.90 (1H, m), 4.83 (1H, s), 4.84 (1H, s), 5.65 (1H, br.), 6.43 (1H, dd, J = 9 and 16 Hz), 7.63 (1H, d, J = 16 Hz). The other (*Rf* 0.40) was further separated by TLC (solvent system; hexane–THF = 4 : 1) to give two components (**8** and **9**). Spectral data of **8** (*Rf* 0.46) are shown below. IR ν_{max} (film) cm^{-1}: 3420, 2950, 2850, 1720, 1630, 1605, 1160, 1050, 900. ^{1}H-NMR ($CDCl_3$) δ: 0.84 (3H, s), 0.94 (3H, s), 2.03 (3H, d, J = 1.2 Hz), 3.03 (3H, d, J = 9 Hz), 3.69 (3H, s), 4.30 (1H, m), 4.73 (1H, m), 5.02 (1H, m), 5.65 (1H, s), 6.18 (1H, dd, J = 9 and 16 Hz), 7.61 (1H, d, J = 16 Hz). MS *m/z* (rel. int.): 265 ($M^+ + 1$, 2), 264 (M^+, 5), 246 ($M^+ - H_2O$, 9), 232 (12), 214 (11), 190 (13), 187 (16), 125 (ABA-like chain, 100). **9** (*Rf* 0.40) was still a mixture on the basis of ^{1}H-NMR data as follows. δ: 0.82 (3H, s), 0.98 (3H, s), 2.05 (3H, d, J = 1.2 Hz), 3.69 (3H, s), 4.54 (1H, m), 4.81 (1H, m), 5.66 (1H, br), 6.25 (1H, dd, J = 9 and 16 Hz), 7.57 (1H, d, J = 16 Hz) for major compound, and 0.91 (3H, s), 0.94 (3H, s), 2.02 (3H, d, J = 1.2 Hz), 7.62 (1H, d, J = 16 Hz) for minor compound (about 20% estimated from ^{1}H-NMR spectrum). IR and MS data of **9** were as follows. ν_{max} (film) cm^{-1}: 3430, 3075, 2930, 2850, 1718, 1635, 1600, 1155, 890. *m/z* (rel. int.): 265 ($M^+ + 1$, 2), 264 (M^+, 3), 246 ($M^+ - H_2O$, 5), 231 (4), 214 (5), 187 (19), 126 (20), 125 (ABA-like chain 100).

6) Incorporation of [2-^{14}C]-(2Z,4E)-γ-ionylideneacetic acid (**3***) *into ABA and its analogs by C. cruenta.* After *C. cruenta* was subcultured on 100 ml of modified potato medium for 5 days, radioactive γ-acid (**3***, 5.58×10^4 dpm, potassium salt, solution in 0.2 ml of ethanol) was added to the culture flask, and incubated for 24 hr. Acidic metabolites were extracted from the culture broth as same manner as 3), and then developed on TLC (solvent system; benzene–EtOAc–AcOH = 60 : 40 : 1) to give ABA (**1**), and its analogs. Each metabolite was methylated with ethereal diazomethane and further purified in the same manner as 5), except for the 4′-hydroxy-γ-acid (**4**).

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