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# Early Biosynthetic Pathway to Abscisic Acid in *Cercospora cruenta*

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**A new biosynthetic intermediate of ABA, (2*Z*,4*E*)-γ-ionylideneacetaldehyde, was isolated from young mycelia of *Cercospora cruenta*. Under an $^{18}O_2$ atmosphere, an oxygen atom of this endogenous aldehyde was exclusively labeled. Similarly, three $^{18}O$ atoms were incorporated into the ABA molecule recovered after prolonged incubation; selectively labeled were one of the carboxyl oxygen atoms and the two on the ring portion of ABA. A feeding experiment with [1-$^{13}C$]glucose proved the exclusive operation of the mevalonate pathway for the formation of both ABA and β-carotene. These results suggest that (2*Z*,4*E*)-γ-ionylideneacetaldehyde can be a key ABA biosynthetic intermediate formed by the oxidative cleavage of a carotenoid precursor.**

**Key words:** abscisic acid; (2*Z*,4*E*)-γ-ionylideneacetaldehyde; carotenoid pathway; mevalonate pathway; *Cercospora cruenta*

Two independent routes have been proposed for the biosynthesis of the plant hormone, abscisic acid (ABA, **1**; Fig. 1 and 2).[1,2] In higher plants, crucial evidence for the carotenoid pathway (indirect pathway or $C_{40}$ pathway) has come from ABA-deficient mutant *vp14* of maize[3] and from the cloning of a series of related genes encoding 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) of tomato, maize, *Arabidopsis*, and bean.[4] In the case of fungi, both (2*Z*,4*E*)-α-ionylideneethanol (**2**)[5] and (2*Z*,4*E*)-γ-ionylideneethanol (**3**)[6,7] have been isolated from *Cercospora rosicola* and *C. cruenta*, respectively, and proved to be biosynthetic precursors of **1**. These $C_{15}$ alcohols were structurally related to the direct cyclization of farnesyl diphosphate (FDP, **4**) derivatives, thus inferring the direct pathway ($C_{15}$ pathway). However, the fungal $C_{15}$ pathway is merely based on the *in vivo* metabolism of putative precursors[8] and is still controversial.

Extensive $^{18}O_2$ feeding studies on **1** in higher plants have strongly suggested that stress-induced **1** was derived *via* oxidative cleavage of a double bond of xanthophylls,[9–12] although no alternative study has been reported for fungi so far.

Although the biosynthesis of **1** from mevalonic acid (MVA) was once advocated in higher plants, recent studies have revealed that isoprenoids in higher plant chloroplasts could be generally formed by the MVA- independent pathway (non-MVA pathway),[13] and it has been suggested that **1** in avocado fruit mesocarp was the case.[14] While [1,2-$^{13}C_2$]acetic acid (AcOH) was converted to **1** in *C. rosicola* and its labeling pattern was accordance with the isoprenoid pathway,[15] it is not certain whether the MVA and/or non-MVA pathway operates for the formation of three isoprenyl units of **1** in ABA-producing fungi.

In this paper, we report the first isolation of (2*Z*,4*E*)-γ-ionylideneacetaldehyde (**5**) as a key ABA biosynthetic intermediate in *C. cruenta*. In order to deduce the unknown precursor just prior to **5**, endogenous **5** as well as **1** were recovered from the cultures under an $^{18}O_2$ atmosphere and analyzed by GC-MS. A feeding experiment with [1-$^{13}C$]glucose[16] on **1** and β-carotene (**6**) was also carried out to determine the origin of the $C_5$ units of these $C_{15}$ and $C_{40}$ isoprenoids by $^{13}C$-NMR.

## Material and Methods

*General notes. Cercospora cruenta* IFO6134 was cultured on a modified potato medium containing 2 % glucose as reported previously.[17] Shaking was continued at 30°C. $^1H$-NMR (400 MHz) and $^{13}C$-NMR (100 MHz) spectra were measured with a Bruker DPX 400 spectrometer at 27°C. $^{13}C$-NMR signals were assigned by DEPT 45, DEPT 90, DEPT135, and HMQC. GC-EI-MS, HR-EI-MS, and GC-NCI-MS spectra were measured by a Jeol SX102 spectrometer (ion source temperature at 200°C, ionization voltage of 70 eV) with a DB-1 column (30 m × 0.32 mm, J&W Scientific), and 40 ml/min of He

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*Abbreviations*: ABA, abscisic acid; FDP, farnesyl diphosphate; IDP, isopentenyl diphosphate; MVA, mevalonic acid; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NCI, negative chemical ionization; PCR, polymerase chain reaction flow. The oven temperature for GLC was programmed from 120°C to 250°C at 3°C/min for **1a** and 80–150°Cat 3°C/min for **3**, **5**, **7**, **8a** and **9a**. Methane was used as the reagent gas for NCI, (the source pressure was $5\times10^{-6}$ torr). HPLC was performed with a Hitachi L-7100 pump, Hitachi L-7400 UV detector and Hitachi D-2500 chromato-integrator with a $\mu$-Porasil column (30 cm × 3.9 mm, Waters), and 1.0 ml/min of solvent flow at 20°C. Preparative TLC was performed on silica gel (Merck 60 $PF_{254}$, 0.75 mm thickness).

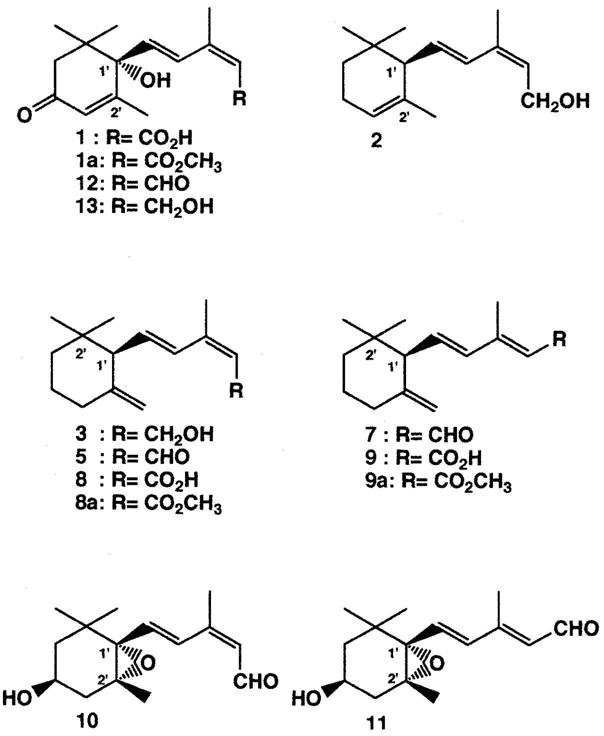

**Fig. 1.** ABA-Related Metabolites.

*Isolation and identification of naturally occurring* **5**. The culture (10 liter in total) was filtered off after 5 d. Fresh grayish white mycelia (678 g) were harvested, rinsed by water, and briefly lyophilized. The resulting material was dipped in MeOH and homogenated in the presence of sea sand. The homogenate was further extracted with MeOH. The combined MeOH solution (2.3 liter) was evaporated *in vacuo* to give a residue (5.2 g) which was repeatedly precipitated by diethyl ether at 0°C to recover oily material (1.6 g). Purification by preparative TLC (*n*-hexane:EtOAc = 5:1) gave a mixture of two aldehydes (**5** and 7, 2.4 mg, $R_f$ 0.31–0.41). This mixture was separated by preparative HPLC (*n*-hexane:EtOAc = 95:5) to give **5** (0.77 mg, $t_R$ 8.1 min) and **7** (1.63 mg, $t_R$ 10.6 min). Data for **5**: $\lambda_{max}$ (EtOH) nm ($\varepsilon$): 282.0 ($1.57\times10^4$). $^1$H-NMR (acetone-$d_6$) $\delta$: 0.86 (3H, s, H-8′), 0.92 (3H, s, H-7′), 1.42 (2H, m, H-4′), 1.56 (2H, m, H-3′), 2.04 (1H, m, H-5′), 2.31 (1H, m, H-5′), 2.14 (3H, d, $J$ = 0.8 Hz, H-6), 2.71 (1H, $J$ = 9.9 Hz, H-1′), 4.60 (1H, s, H-9′), 4.76 (1H, s, H-9′), 5.78 (1H, d, $J$ = 8.0 Hz, H-2), 6.45 (1H, dd, $J$ = 9.9 and 15.2 Hz, H-5), 7.28 (1H, d, $J$ = 15.2 Hz, H-4), 10.2 (1H, d, $J$ = 8.0 Hz, H-1). GC-EI-MS ($t_R$ 24.4 min) *m*/*z* (rel. int.): 218 ($M^+$, 12), 203 (20), 185 (10), 175 (9), 159 (12), 149 (18), 147 (22), 122 (15), 105 (17), 95 (100), 91 (20). HR-EI-MS: found, 218.1671; calcd. for $C_{15}H_{22}O$, 218.1671. Data for 7, $\lambda_{max}$ (EtOH) nm ($\varepsilon$): 283.5 ($1.57\times10^4$). $^1$H-NMR (acetone-$d_6$) $\delta$: 0.85 (3H, s, H-8′), 0.90 (3H, s, H-7′), 1.36 (2H, m, H-4′), 1.57 (2H, m, H-3′), 2.03–2.09 (2H, m, H-5′), 2.34 (3H, s, H-6), 2.65 (1H, d, $J$ = 9.7 Hz, H-1′), 458 (1H, s, H-9′), 4.75 (1H, s, H-9′), 5.84 (1H, d, $J$ = 8.0 Hz, H-2), 6.29 (1H, d, $J$ = 16.0 Hz,

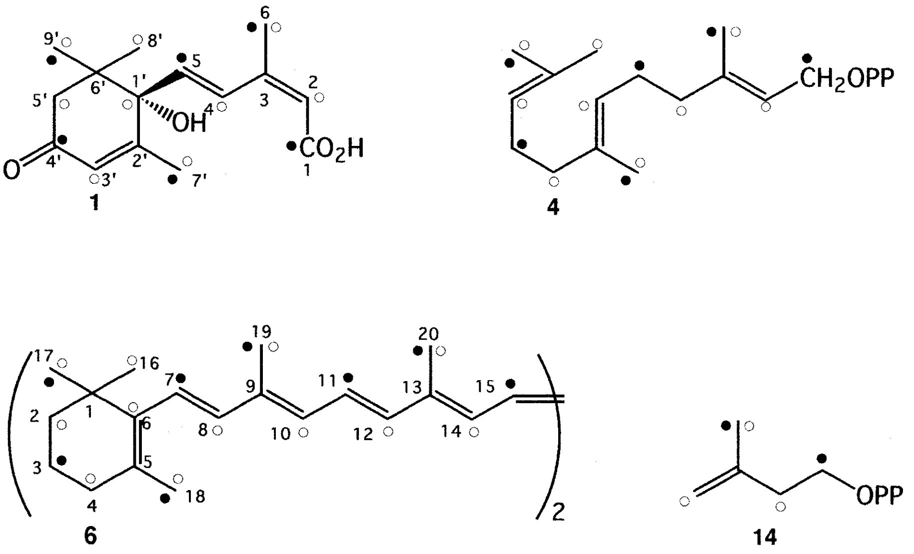

**Fig. 2.** Expected Labeling Patterns of ABA (**1**), FDP (**4**), $\beta$-Carotene (**6**), and IDP (**14**) from [1-$^{13}$C] Glc *via* the MVA (○) and Non-MVA (●) Pathways.

H-4), 6.54 (1H, dd, $J=9.7$ and 16.0 Hz, H-5), 10.1 (1H, d, $J=8.0$ Hz, H-1). GC-EI-MS: ($t_R$ 25.7 min) $m/z$ (rel. int.): 218 ($M^+$, 52), 203 (37), 185 (8), 175 (17), 161 (11), 149 (26), 147 (31), 135 (27), 121 (39), 107 (59), 96 (76), 95 (100), 93 (43), 91 (64). HR-EI-MS: found, 218.1670; calcd. for $C_{15}H_{22}O$, 218.1671. These data were identical with those of authentic **5** and **7**, respectively.

*Conversion of* **5** *to* **8a**. To avoid the artificial isomerization of **5** after its extraction from the mycelia, a portion of the MeOH solution (230 ml) already prepared was carefully purified as reported previously.[18] A broad HPLC fraction ($t_R$ 7.5–11.0 min) corresponding to both **5** and **7** was collected and reduced to dryness at 0°C. According to the reported procedure,[19] HPLC-purified aldehyde in dry MeOH (5.0 ml) was added to a mixture prepared from powdered NaCN (200 mg) and absolute AcOH (200 mg) in dry MeOH (10 ml). After stirring for 60 min at room temperature, the mixture was further stirred overnight with additional $MnO_2$ (2.0 g of activated powder). The resultant methyl ester was recovered by the usual workup and analyzed by GC-EI-MS to identify **8a** ($t_R$ 26.9 min, rel. area on GC of more than 95%) as the dominant isomer; $m/z$ (rel. int.): 248 ($M^+$, 4), 233 (5), 217 (7), 201 (9), 189 (21), 173 (10), 159 (4), 147 (18), 145 (8), 125 (100), 119 (23), 105 (20), 91 (17). A trace peak for 9a ($t_R$ 28.8 min, rel. area on GC of less than 5%) was also detected; $m/z$ (rel. int.): 248 ($M^+$, 79), 233 (22), 217 (22), 201 (19), 189 (100), 173 (44), 159 (31), 147 (53), 145 (41), 125 (77), 119 (78), 105 (70), 91 (57). These data were identical with those of authentic **8a** and **9a**, respectively.

*Preparation of authentic specimens and $^{13}C_2$ internal standards.* Authentic **3**, **5**, and **8** were prepared from γ-ionone as reported previously.[6] In the usual manner, shaking **3** with $MnO_2$ in $CHCl_2$ for 1 h gave **5**. For GC-EI-MS quantification, [1,2-$^{13}C_2$]methyl diethylphosphonoacetate[7] was prepared from [1,2-$^{13}C_2$]bromoacetic acid (Aldrich, more than 99 atom %) and used to synthesize the [1,2-$^{13}C_2$] internal standards.

*Quantification of the endogenous products.* Time-course data for the endogenous levels of **3**, **5**, and **8** in young mycelia were measured by GC-EI-MS with the [1,2-$^{13}C_2$] internal standards. From the large culture volume (1.0 liter), an aliquot (20 ml) was sampled every 12 h. After the aliquot had been separated into the mycelia and broth by filtration, a known amount of internal standard was added to each part. The MeOH extract from the mycelia was evaporated, and the residue was again fractionated with EtOAc. The filtered broth was extracted by EtOAc. During the extraction procedure, **3** ($R_f$ 0.16) and **5** ($R_f$ 0.37) were recovered in the neutral fraction and further separated by preparative TLC (*n*-hexane:EtOAc$=5:1$), while **8** was found in the acidic fraction and converted to **8a** by $CH_2N_2$. GC-EI-MS data enable the endogenous levels to be calculated from the following peak intensity ratios: $m/z$ 222/220 for **3** ($t_R$ 24.3 min), $m/z$ 220/118 for **5** ($t_R$ 24.4 min), and $m/z$ 127/125 for **8a** ($t_R$ 26.9 min) (Fig. 3).

*Feeding experiments with $^{18}O_2$.* To a shaking flask (500 ml) containing a liquid medium (50 ml) was fitted a plastic bag (5 liter) containing sterilized $^{18}O_2$ (Isotec Inc., more than 97 atom%, 500 ml) and $N_2$ (2 liter). The air in the flask was completely purged by bubbling the medium with the artificial air in the bag (500 ml). After shaking for 5 d, an MeOH extract was obtained from the young mycelia and analyzed by GC-EI-MS in the same manner as already described to identify $^{18}O$-labeled **5**, $m/z$ (rel. int.): 220 ($M^+$, 12), 205 (32), 187 (15), 175 (9), 160 (10), 151 (11), 149 (11), 137 (11), 133 (11), 123 (13), 122 (12), 107 (16), 106 (15), 98 (15), 97 (100), 93 (12), 92 (15), 81 (11), 80 (13). HR-EI-MS: found, 220.1715; calcd. for $C_{15}H_{22}{}^{18}O$, 220.1713. Authentic **5** was also measured under the same conditions (Fig. 4). The artificial air containing $^{18}O_2$ (500 ml) was similarly supplied to the culture while shaking for 15 d. The black mycelia were filtered off, and the broth was extracted with EtOAc after its acidification with 20% $H_3PO_4$ at pH 3. After treating with $CH_2N_2$, $^{18}O$-labeled **1a** was preliminary purified by preparative HPLC (*n*-hexane:EtOAc$=3:1$). A broad fraction ($t_R$ 11.0–12.0 min) was collected for **1a** ($t_R$ 11.4 min) and measured by GC-NCI-MS ($t_R$ 24.6 min) to identify $^{18}O$-labeled **1a**, $m/z$ (rel. int.): 316 ($^{18}O_3$-$M^-+O_2$, 16), 299 ($^{18}O_3$-$M^-+O_2-CH_3$, 3), 284 ($^{18}O_3$-$M^-$, 100), 282 ($^{18}O_2$-$M^-$, 14), 280 ($^{18}O$-$M^-$, 3), 278 ($^{16}O_3$-$M^-$, 0.8), 264 ($^{18}O_3$-$M^-$-$^{18}H_2O$, 8), 249 ($^{18}O_3$-$M^-$-$^{18}H_2O$-$CH_3$, 6), 156 (ring of $^{18}O_3$-$M^-$, 21), 147 (side chain of $^{18}O_3$-$M^-$, 57). Authentic **1a** was also measured under the same conditions (Fig. 5), $m/z$ (rel. int.): 310 ($M^-+O_2$, 16), 299 ($M^-+O_2$-$CH_3$, 2), 278 ($M^-$, 100), 260 ($M^-$, 8), 245 ($M^-$-$H_2O$-$CH_3$, 6), 152 (ring of $M^-$, 21), 141 (side chain of $M^-$, 57).

*Feeding experiments with [1-$^{13}C_1$] glucose.* A liquid medium (150 ml) containing Glc (2.0 g) and [1-$^{13}C_1$] glucose (Aldrich, more than 99 atom%, 1.0 g) was prepared in a shaking flask (2 liter). After shaking for 5 d, the culture was filtered off. Fresh grayish white mycelia (13 g) were harvested, dipped in MeOH, and homogenated in the presence of sea sand. The homogenate was further extracted with MeOH. The combined MeOH solution (200 ml) was extracted by *n*-hexane (50 ml × 4 times) and evaporated *in vacuo*. The residue was purified by preparative HPLC (*n*-hexane only) to recover [U-$^{13}C$]-**6** (0.31 mg, $t_R$ 3.6 min); $\lambda_{max}$ (*n*-hexane) nm: 425, 449, 475.[20] $^{13}C$-NMR data were measured and compared with that of authentic **6** (Table 2).[21] A liquid medium (30 ml) containing glucose (400 mg) and [1-$^{13}C_1$] glucose (200 mg) was prepared in a shaking flask (500 ml). In the same manner as mentioned above, [U-$^{13}$C]-**1** was isolated after shaking for 15 days. After its conversion with $CH_2N_2$ and purification by preparative HPLC, [U-$^{13}$C]-**1a** (0.37 mg) was measured on $^{13}$C-NMR (Table 1).

*Feeding experiments with [1,2-$^{13}C_2$]-5.* The culture (1.0 liter) was filtered off after 5 d. The fresh mycelia (68 g) were rinsed with water and suspended in a phosphate buffer (0.1 M, 50 ml) containing Triton X-100 (100 mg) and [1,2-$^{13}C_2$]-**5** (10 mg). The suspension was incubated at 35°C in the vessel of an ultrasonicator (Sine Sonic 100, 36 kHz)[8] for 2 h and then poured into a fresh medium (1.0 liter). In the same manner as that already mentioned, an aliquot (20 ml) was sampled daily to trace the incorporation of [1,2-$^{13}C_2$]-**5** into **1** and **3** by GC-EI-MS (Fig. 6). The incorporation ratio was calculated by the combination of isotopic composition and quantification. Quantification of **1** was based on the HPLC peak area of recovered **1a**, while that of **3** and **5** was estimated from the GC peak area compared with authentic **2** and **7** as internal standards, respectively.

## Results and Discussion

### *Isolation and identification of new fungal alde hyde* 5

During the course of our extensive search for a new ABA biosynthetic intermediate in young mycelia of *C. cruenta*, we could find no $C_{15}$ putative alcohol to explain the link between **3** and **4**.[8] Instead, we obtained a trace amount of a less polar oily material that was found to be a mixture (*ca.* 7:3) of two aldehydes by $^1$H-NMR. Further purification gave the geometrical isomers, (2*E*,4*E*)-γ-ionylideneacetaldehyde (**7**) as the major component and its (2*Z*,4*E*) isomer **5** as the minor one. Their $^1$H-NMR spectra and GC-MS data were identical with those of authentic specimens prepared fromγ-ionone. However, the original ratio of these isomers is still obscure at this stage. The artificial isomerization almost exclusively from **5** to **7** is thought to have been plausible during the conventional extraction, purification, and analytical procedures as reported for xanthoxin isomers (**10** and **11**) in higher plants.[18] While (2*Z*,4*E*) isomer **10** is now known to be selectively produced *in vivo*, (2*E*,4*E*) isomer **11** has been typically identified as the dominant isomer.[22] To overcome these problems, we next adopted an extremely careful extraction[18] procedure for **5** and/or **7** and their conversion[19] to the corresponding methyl ionylideneacetates (**8a** and **9a**). A similar procedure has already given successful results for the identification and quantification of natural **10** and **11**. Light, heat, and concentration in the presence of contaminating pigments were strictly avoided, and the resulting crude extract of **5** and/or **7** was directly subjected to NaCN/AcOH and then to an $MnO_2$ treatment in MeOH. An analysis of the converted products by GC-EI-MS revealed that the ratio of **8a**/**9a** was always more than 95/5. It is concluded that the isomer of γ-ionylideneacetaldehyde found in the young mycelia of *C. cruenta* was exclusively (2*Z*,4*E*)-**5** in accordance with the geometry of natural (2*Z*,4*E*)-**1**. Although the endogenous level of **5** was very low, a small accumulation of **5** was detected at the early growth stage of the mycelia (Fig. 3). It was also found that alcohol **3** and aldehyde **5** were retained in the mycelia, while acid **8** could be secreted into the medium.

### *Incorporation of $^{18}O_2$ into* 5 *and* 1

Aldehyde **5** can be presumed to be a biosynthetic intermediate not only in the $C_{15}$ but also in the $C_{40}$ pathway for *C. cruenta.* While **5** is presumed to be an intermediate between alcohol **3** and acid **8** in the $C_{15}$ pathway, oxidative cleavage of a putative carotenoid precursor is assumed to explain the role of **5** in the $C_{40}$ pathway. The origin of an oxygen atom of **5** is respectively ascribable to glucose and molecular oxygen in the cases of the $C_{15}$ and $C_{40}$ pathways. Extensive $^{18}O_2$ feeding studies have been reported to reveal the $C_{40}$ pathway in higher plants,[9-12] and this prompted us to examine the incorporation of molecular $^{18}O_2$ into **5** and 1 produced by *C. cruenta*. Constant feeding of $^{18}O_2$ during the fungal growth stage was found essential to achieve substantial incorporation. This is in contrast to the experimental advantages with water-stressed plants whereby typical incubation with $^{18}O_2$ for 48 h was enough to obtain satisfactory results.[9-12] It is deduced that the biological role of **1** was merely as one of the minor secondary metabo-

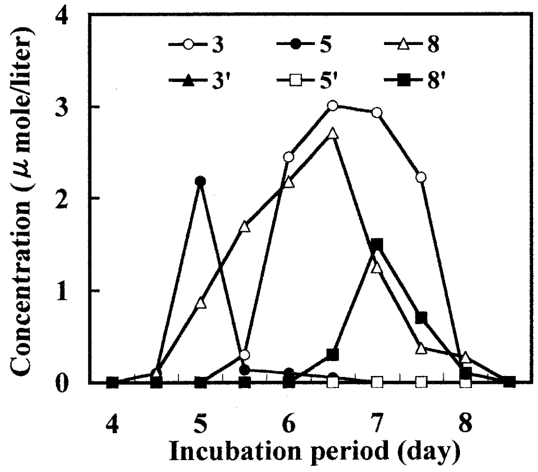

**Fig. 3.** Time Course Plots for the Endogenous Levels of Alcohol **3**, Aldehyde **5**, and Acid **8** Found in the Mycelia of *Cercospora cruenta.*

Time-course data for **3′**, **5′**, and **8′** indicate the corresponding levels found in the medium.

lites in *C. cruenta*, and the production of **1** should be neither rapid nor drastic compared with the selective induction of **1** in water-stressed plants. The required amount of the total $^{18}O_2$ supplied was almost equal to the volume estimated from the complete aerobic consumption of total glucose in the medium. Thus, more than 6 molar equivalents of $^{18}O_2$ to glucose in total was necessary for normal fungal growth without retardation, and the production of **1** was completely inhibited by an insufficient $^{18}O_2$ supply of less than 4 molar equivalent. We first tried to investigate the incorporation of $^{18}O$ into **5** isolated from young mycelia grown under an $^{18}O_2$ atmosphere. The GC-EI-MS spectra of **5** identified in the crude MeOH extract indicates the exclusive incorporation of one $^{18}O$ atom into the aldehyde group (Fig. 4). Besides the molecular ion peak at $m/z$ 220, which corresponds to $m/z$ 218 of authentic **5**, a parent ion peak originating from a 3-methyl-2,4-pentadienal side chain was dominantly observed at $m/z$ 97 instead of $m/z$ 95 for authentic **5**. This result is in contrast with the previous identification of $^{18}O$-labeled ABA-aldehyde (**12**) from apple fruit in which the lifetime of **12** was estimated to be long enough to cause substantial loss of $^{18}O$ from the aldehyde group by the exchange of $H_2{}^{16}O$ molecules in the tissue.[10] Contrary to **12**, which is water-soluble and found in the chloroplast cytoplasm of plants, less polar **5** is a hydrophobic molecule and its formation and distribution may be events on plasma lemma in *C. cruenta*. Water was also avoided during the extraction of **5** from desiccated mycelia. It was deduced that these conditions prevented the loss of $^{18}O$ from **5** in our experiments. Alternatively, $^{18}O$-labeled 1 was identified as one of the acidic metabolites from the culture broth after a prolonged incubation. In this case, GC-NCI-MS of methyl ester 1a was performed to unequivocally obtain the molecular ion peaks (Fig. 5). The highest relative abundance was found at $m/z$ 284, indicating three $^{18}O$ atoms in the molecule of **1a** besides $m/z$ 282 and $m/z$ 280 as minor peaks. No peak was found at $m/z$ 286. In a previous fragmentation analysis of authentic 1a, it has been well discussed that minor peaks at $m/z$ 141 and $m/z$ 152 were fragments of the side chain and ring of **1a**, respectively.[23] In our case, fragment peaks were observed at $m/z$ 143 and $m/z$ 156, indicating one and two $^{18}O$-atoms in the side chain and ring, respectively. These results suggest the involvement of some oxidative enzymes; a putative dioxygenase to give **5** and two hydroxylating enzymes[17] such as P-450 monooxygenase to add two functional groups to the ring portion of **1**. High incorporation in the side chain of **1** also suggests that the biosynthetic conversion from **5** to **8** must be rapid enough to maintain the $^{18}O$ atom without exchange.

### *Incorporation of* $^{13}C_2$-**5** *into* **1** *and* **3**

When [1,2-$^{13}C_2$]-**5** was fed to the young mycelia of *C. cruenta*, a small incorporation into alcohol **3** was observed in the initial stage of the feeding experiments (Fig. 6). It is suggested that naturally occurring **3** originated not from a $C_{15}$ alcohol derivative

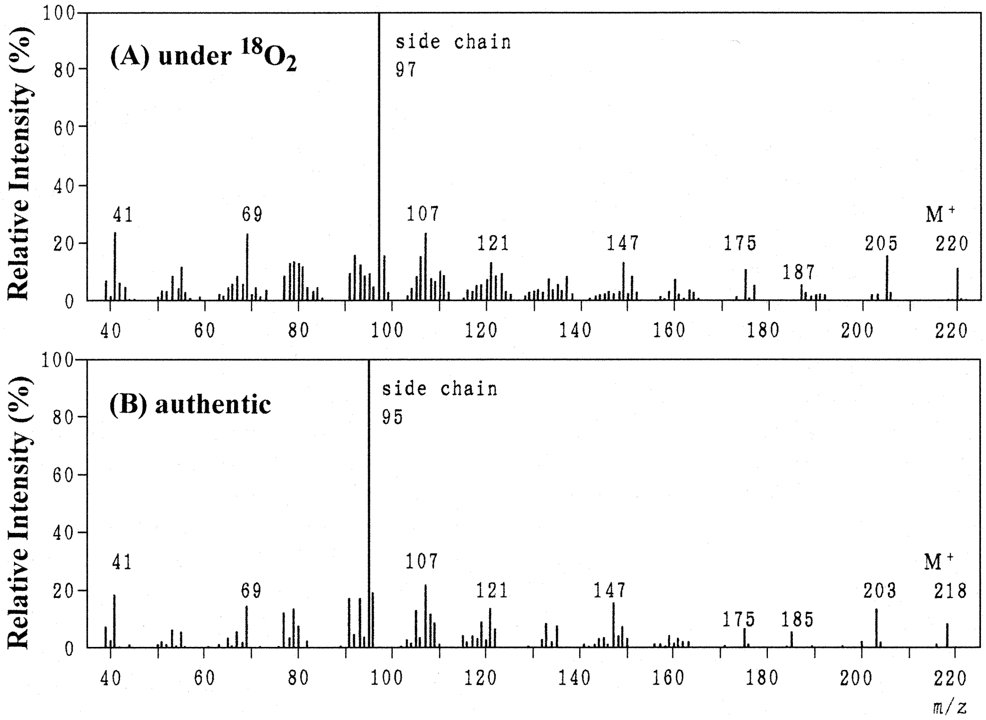

**Fig. 4.** GC-EI-MS Spectra of Aldehyde **5**.
a) Isolated from the young mycelia of *C. cruenta* (5 days old) under a 20 v/v% $^{18}O_2$ atmosphere. b) Authentic.

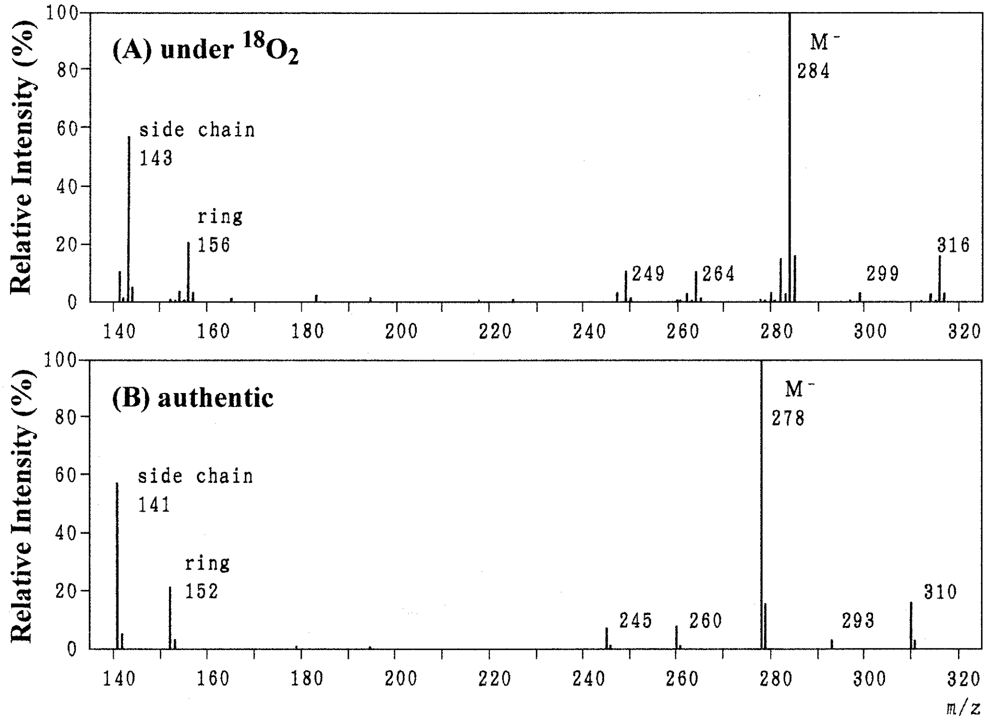

**Fig. 5.** GC-NCI-MS Spectra of ABA Methyl Ester **1a**.

a) Isolated from the culture broth of *C. cruenta* (15 days old) under a 20 v/v% $^{18}O_2$ atmosphere. b) Authentic.

such as **4** but from aldehyde **5**. From the results of previous feeding experiments with [2-$^{14}$C]-**3**,[7] it is certain that the conversion of **3** into **1** must be *via* **5**. Thus, the accumulation of **3** is regarded as being a temporary pool of biochemically unstable **5**. This phenomenon is reminiscent of the shunt pathway for aldehyde **12** to ABA-alcohol (**13**) in ABA-deficient plants.[11]

### *Feeding experiments using $^{13}$C-glucose*

In the isoprenoid biosynthesis, [1-$^{13}$C] glucose is converted to [2-$^{13}$C] AcOH and then incorporated into isopentenyl diphosphate (IDP, **14**) *via* the MVA or/and non-MVA pathway.[16] Consequently, the possible incorporation patterns from [1-$^{13}$C] glucose are predictable for both **1** and **6** (Fig. 2). When one third of the glucose in the medium was substituted by [1-$^{13}$C] glucose, [U-$^{13}$C]-**1** was successfully isolated. The isotopic composition measured by GC-EI-MS was $^{13}C_0$: $^{13}C_1$: $^{13}C_2$: $^{13}C_3$-**1a** = 100:68:24:2. The $^{13}$C-NMR spectrum of [U-$^{13}$C]-**1a** was compared with that of an authentic specimen (Table 1). While no signals assigned to C-1, 3, 5, 2′, 4′, and 6′ could be detected, the relative intensity of signals for C-2, 4, 6, 1′, 3′, 5′, 7′, 8′, and 9′ was almost identical with that of an authentic specimen. This result indicates the unequivocal incorporation of $^{13}$C atoms into the carbons that has been predicted for the MVA pathway and no or negligible incorporation to explain the biosynthesis of **1** *via* the non-MVA pathway. Although a putative biosynthetic precursor of **5** has not so far been found, typical carotenoid **6** is known to exist in young mycelia of *C. cruenta*.[6] To investigate the origin of the $C_{40}$ carotenoid in this fungus, endogenous [U-$^{13}$C]-**6** was also isolated and analyzed by $^{13}$C-NMR (Table 2). Again, the incorporation of $^{13}$C atoms was unequivocally found in the carbons predicted for the MVA pathway such as C-2, 4, 6, 8, 10, 12, 14, 16, 17, 18, 19, and 20, while no C-1, 3, 5, 7, 9, 11, 13, and 15 could be detected. These results are in contrast to the recent report for the biosynthesis of **1** in higher plants.[14] However, it is now generally accepted that most fungal isoprenoids and chlo-

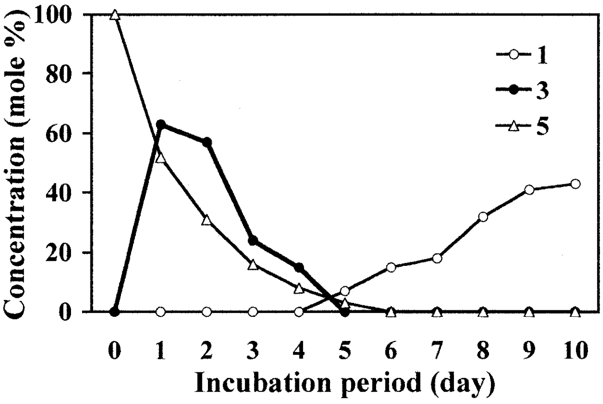

**Fig. 6.** Incorporation of (±)-[1,2-$^{13}C_2$]-Aldehyde **5** into Alcohol **3** and ABA **1** in *C. cruenta*.

Fresh mycelia (5 days old) were harvested and treated with **5** under Triton X and sonication at 0 day before further incubation.

roplast-related ones tend to be derived from the MVA and the non-MVA pathway, respectively.[13] The biosyntheses of **1** here can be the same case.

**Table 1.** $^{13}$C-NMR Chemical Shifts and Relative Peak Height of ABA Methyl Ester **1a**

| Carbon number[a] | Chemical shift[b] $\delta$ [ppm] | Authentic [%][c] | From [1-$^{13}$C]glucose [%][c] | Isotopic abundance [%][d] |
|---|---|---|---|---|
| 1 | 166.6 | 34 | —[f] | —[f] |
| 2 | 117.9 | 93 | 100 | 10.0 |
| 3[e] | 151.3 | 45 | —[f] | —[f] |
| 4 | 128.5 | 69 | 71 | 9.7 |
| 5 | 138.8 | 69 | —[f] | —[f] |
| 6 | 21.2 | 100 | 100 | 9.2 |
| 1′ | 80.0 | 28 | 19 | 6.1 |
| 2′[e] | 163.0 | 21 | —[f] | —[f] |
| 3′ | 127.3 | 86 | 84 | 9.1 |
| 4′ | 197.2 | 35 | —[f] | —[f] |
| 5′ | 50.3 | 85 | 68 | 7.5 |
| 6′ | 42.2 | 60 | —[f] | —[f] |
| 7′ | 19.1 | 69 | 65 | 8.7 |
| 8′ | 24.9 | 91 | 111 | 11.1 |
| 9′ | 23.5 | 94 | 116 | 11.4 |
| $CH_3$ | 51.0 | 67 | 8[f] | 1.1 |

[a] See Figure 2.

[b] Measured in acetone-$d_6$ and almost identical with previous data in $CDCl_3$,[15] except for C-3 and C-2′.

[c] The peak height of C-6 is assigned as 100%.

[d] A very weak signal of $CH_3$ (51.0 ppm, isotopic abundance of 1.1%) was used as the reference.

[e] The previous assignment[15] was revised by DEPT, HMQC, and HMBC data.

[f] Hardly detectable.

**Table 2.** $^{13}$C-NMR Chemical Shifts and Relative Peak Heights of $\beta$-Carotene **6**

| Carbon number[a] | Chemical shift[b] $\delta$ [ppm] | Authentic [%][c] | From $^{13}$C-Glc [%][c] |
|---|---|---|---|
| 1 | 34.6 | 93 | —[e] |
| 2 | 39.9 | 98 | 95 |
| 3 | 19.7 | 81 | —[e] |
| 4 | 33.3 | 89 | 81 |
| 5 | 129.4 | 81 | —[e] |
| 6 | 138.4 | 75 | 63 |
| 7 | 126.9 | 80 | —[e] |
| 8 | 138.0 | 47 | 32 |
| 9 | 136.0 | 63 | —[e] |
| 10 | 131.9 | 81 | 77 |
| 11 | 125.5 | 75 | —[e] |
| 12 | 138.7 | 57 | 35 |
| 13 | 136.7 | 52 | —[e] |
| 14 | 133.3 | 71 | 88 |
| 15 | 130.7 | 78 | —[e] |
| 16 + 17[d] | 29.2 | 199 | 190 |
| 18 | 22.0 | 96 | 98 |
| 19 | 12.8 | 100 | 100 |
| 20 | 12.9 | 72 | 75 |

[a] See Figure 2.

[b] Measured in $C_6D_6$ and almost identical with previous data in $CDCl_3$.[21]

[c] The peak height of C-19 is assigned as 100%.

[d] Not separable.

[e] Not detected.

### *Conclusions*

It is concluded that the MVA pathway almost exclusively operated for the formation of both $C_{15}$ **1** and $C_{40}$ **6** in *C. cruenta*. Like other isoprenoids, the origin of **14** seems different between fungi and higher plants in spite of the same biosynthetic goal for **1**. However, the incorporation of molecular oxygen into a key aldehyde **6** strongly suggests the possibility that oxidative cleavage of a precursor larger than $C_{15}$ isoprenoids could be also involved in ABA biosynthesis in fungi. The finding of partial conversion of **5** into **3** seems to have reduced the significance of **3** as a key biosynthetic intermediate to support the $C_{15}$ pathway in this fungus. When the hexane-soluble fraction from the young mycelia was analyzed, some minor pigments other than 6 were also detected by HPLC and identified as carotenoids from UV data. We assume that the putative precursor of **5** can be a carotenoid possessing a terminal double bond on the ring.[24] It is premature at this stage to conclude the $C_{40}$ pathway in *C. cruenta*. However, we expect that more direct evidence will be delivered by the unequivocal identification of a putative $C_{40}$ precursor and by molecular cloning of the putative biosynthetic gene by the PCR technique based on the DNA sequence similarity among a series of known dioxygenases including plant NCEDs. This work is now in progress.

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