We examined the biosynthetic pathway of abscisic acid (ABA) after isopentenyl diphosphate in a fungus, *Cercospora cruenta*. All oxygen atoms at C-1, -1, -1, and -4' of ABA produced by this fungus were labeled with $^{18}$O from $^{18}$O$_2$. The fungus did not produce the 9Z-carotenoid possessing γ-ring that is likely a precursor for the carotenoid pathway, but produced new sesquiterpenoids, 2E,4E-γ-ionylideneethane and 2Z,4E-γ-ionylideneethane, along with 2E,4E,6E-allofarnesene. The fungus converted these sesquiterpenoids labeled with $^{13}$C to ABA, and the incorporation ratio of 2Z,4E-γ-ionylideneethane was higher than that of 2E,4E-γ-ionylideneethane. From these results, we concluded that *C. cruenta* biosynthesized ABA by the direct pathway via oxidation of ionylideneethane with molecular oxygen following cyclization of allofarnesene. This direct pathway via ionylideneethane in the fungus is consistent with that in *Botrytis cinerea*, except for the positions of double bonds in the rings of biosynthetic intermediates, suggesting that the pathway is common among ABA-producing fungi.

A sesquiterpenoid, abscisic acid (ABA, 1), is a plant hormone which regulates seed dormancy and induces dehydration tolerance by reducing the stomatal aperture.\(^1\) ABA is biosynthesized by some phytopathogenic fungi in addition to plants,\(^2\) but the biosynthetic origin of isopentenyl diphosphate (IDP) for fungal ABA is different from that for plant ABA (Fig. 1). Fungi use IDP derived from the mevalonate pathway for ABA, while higher plants biosynthesize IDP for ABA by the non-mevalonate pathway.\(^3\) For the biosynthetic pathway of ABA after IDP, the carotenoid pathway involving cleavage of 9Z-xanthophyll has been elucidated for plant ABA.\(^4\) In contrast to ABA of higher plants, two pathways, the direct and carotenoid pathways after IDP, have been proposed for fungal ABA. The direct pathway is supposed to contain oxidation of ionylideneethanol by oxidase after cyclization of farnesyl diphosphate.
(FDP),5–7 the classical direct pathway in Fig. 1, and the carotenoid pathway might be similar to that of plant ABA.8,9 Sufficient evidence appears to be lacking for occurrence of the direct pathway over the biosynthesis of ABA. This paper reports the labeled compounds in C. cruenta and sesquiterpenoids, and did feeding experiments of 18O from FDP,5–7 the classical direct pathway in Fig. 1, and the biosynthetic gene.13 This finding is consistent with labeling of ABA with 18O that the fungus formed O-label at C-4 of 4 was labeled with 18O from 18O2. Methyl ester of 5 showed molecular ions corresponding to [M+2]+ and [M]+ at m/z 222 and 220 respectively, indicating that the oxygen atom at C-1 of 4 was labeled with 18O from 18O2. Methyl ester of 5 showed molecular ions corresponding to [M+8]+, [M+6]+, [M+4]+, [M+2]+, and [M]− at m/z 288, 286, 284, 282, and 280 respectively. This showed that all four oxygen atoms of 5 at maximum were labeled with 18O from 18O2 also. In B. cinerea cultured under 18O2, 18O-label at C-4' of ABA was lost by exchange with 18O from H216O due to the acidity of the medium. The remaining 18O-label at C-4' in C. cruenta is probably due to the pH of the medium, which was kept between 5.8 and 7.5 during the culture. Exchange of 4',18O with 16O of H216O was less than 7% in this pH range.12 The 18O labels incorporated into compounds 4, 5, and ABA might be derived from H218O that the fungus formed from 18O2 by respiration during culture. This possibility appears to be small, since no oxygen atom from water was incorporated into ABA in the labeling experiment with H218O in B. cinerea.12 Thus all oxygen of 4, 5, and

Table 1. Relative Intensities9 and Compositions8 of Molecular Ions of 4 and Methyl Esters of 5 and ABA Isolated from C. cruenta Cultured under 18O2

<table>
<thead>
<tr>
<th>Compound 4</th>
<th>Methyl ester of 5</th>
<th>Methyl ester of ABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z</td>
<td>ion species</td>
<td>rel. int. (%)</td>
</tr>
<tr>
<td>222</td>
<td>[M+2]+</td>
<td>3.0</td>
</tr>
<tr>
<td>220</td>
<td>[M]⁺</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

9Relative intensities were corrected by natural 13C, 2H- and 17O-isotopic abundance.
8Composition of relative intensities of each ion group.

Results and Discussion

Labeling of 4, 5, and ABA with 18O

18O-Labeling of the intermediates 4 and 5 in addition to ABA was examined. The period of accumulation and the location were different between compound 4 on the one hand and compound 5 and ABA on the other. Compound 4 was isolated from the mycelia of C. cruenta cultured under 18O2 for 7 d, and compound 5 and ABA were isolated from the medium of the fungus cultured under 18O2 for 16 d. Compound 6 was not detected in the mycelia of the fungus during the culture for 16 d.

Compound 4 and methyl esters of 5 and ABA were analyzed by EIMS to determine their labeled positions with 18O from 18O2. The relative intensities and compositions of molecular ions of 4 and methyl esters of 5 and ABA are summarized in Table 1. Compound 4 gave molecular ions corresponding to [M+2]+ and [M]+ at m/z 222 and 220 respectively, indicating that the oxygen atom at C-1 of 4 was labeled with 18O from 18O2. Methyl ester of 5 showed molecular ions corresponding to [M+8]+, [M+6]+, [M+4]+, [M+2]+, and [M]− at m/z 288, 286, 284, 282, and 280 respectively. This showed that all four oxygen atoms of 5 at maximum were labeled with 18O from 18O2 also. In B. cinerea cultured under 18O2, 18O-label at C-4' of ABA was lost by exchange with 18O from H216O due to the acidity of the medium. The remaining 18O-label at C-4' in C. cruenta is probably due to the pH of the medium, which was kept between 5.8 and 7.5 during the culture. Exchange of 4',18O with 16O of H216O was less than 7% in this pH range.12 The 18O labels incorporated into compounds 4, 5, and ABA might be derived from H218O that the fungus formed from 18O2 by respiration during culture. This possibility appears to be small, since no oxygen atom from water was incorporated into ABA in the labeling experiment with H218O in B. cinerea.12 Thus all oxygen of 4, 5, and
ABA produced by C. cruenta is derived from molecular oxygen, but not from water.

The result of $^{18}$O-labeling of ABA was different from that reported by Yamamoto et al.83 This difference can be explained by the metabolic rate of 6 to ABA. Accumulation of compound 6 in their cultural condition suggests slow turnover of 6 to ABA. The $^{18}$O-label at C-1 of 6 would exchange easily with $^{16}$O from H$_2$O in a medium via a hydrate of the aldehyde group at C-1. Slow turnover of 6 to ABA should allow this exchange, resulting in a decrease or loss of 1-$^{18}$O of 6. The temperature (30°C) for the culture used by Yamamoto et al. was higher than the optimum temperature for fungal growth and might affect the biosynthetic rate of ABA. We also observed that all four oxygen atoms of ABA were not always labeled with $^{18}$O. When the fungus began to produce ABA at a late period, day 22 after inoculation, two oxygen atoms at C-1 and -4' were labeled with $^{18}$O from $^{18}$O$_2$, but two oxygen atoms at C-1 and -4' were not (data not shown).

Our finding is consistent with the direct pathway via ionylideneethane. But incorporation of two $^{18}$O atoms into C-1 of ABA from $^{18}$O$_2$ might be possible for the carotenoid pathway in which oxidation at C-1 of a putative C$_{15}$-aldehyde intermediate was catalyzed by monooxygenase with molecular oxygen. If the carotenoid pathway occurs in C. cruenta, the fungus could produce the 9Z-carotenoid possessing γ-ring as well as the C$_{15}$-intermediates 4–6. We carefully searched mycelia extract of the fungus for ABA-related carotenoids and sesquiterpenoids to distinguish between the carotenoid pathway and the direct pathway via ionylideneethane.

**Isolation and identification of ABA-related compounds**

First, the extract from the mycelia of the fungus cultured in a flask capped with a cotton plug was analyzed by HPLC to detect carotenoids. One carotenoid-like compound was eluted at $t_R$ 13.6 min, and identified as all-β-carotene (7). The content of 7 was 17 μg/g fresh weight of mycelia. No other carotenoid was detected. No detection of precursor carotenoids in the mycelia might be due to the rapid conversion of them to ABA. The oxidative cleavage of precursor carotenoids is inhibited under anaerobic conditions. We cultured C. cruenta in a flask capped with a silicone plug, which passes air with more difficulty than a cotton plug. The fungus produced 0.2 mg/l of ABA at maximum at day 11 after inoculation, while the fungus cultured in a flask capped with a cotton plug produced 4 mg/l of ABA at maximum at day 13, suggesting that precursor carotenoids had accumulated in the mycelia of the fungus cultured in the flask capped with a silicone plug. The extract from the mycelia of the fungus cultured in the flask capped with a silicone plug was analyzed by HPLC with a photodiode array detector at a detection limit of 0.3 ng for 7. Compounds 8–13 possessing characteristic absorption spectra for carotenoids were detected at $t_R$s 9.0, 11.7, 14.3, 14.9, 16.9, and 21.4 min respectively, along with compound 7. These compounds were identified as 15,15’Z-phytoene (8), 15,15’Z-phytofluene (9), all-E-β-zeacarotene (10), all-E-ζ-carotene (11), all-E-7,8-dihydro-β-carotene (12), and all-E-neurosporene (13) (Fig. 2). These carotenoids appeared to have been accumulated by the inhibition of desaturation of acyclic carotene in the biosynthesis of 7 under anaerobic conditions. Carotenoid 12 has been known as the product of carotenogenesis genes expressed in Excherichia coli,15 but was first isolated from a natural organism. The contents of 7–13 were 3, 4, 0.1, 0.1, 0.4, 0.8, and 0.1 μg/g fresh weight of mycelia respectively. 9Z-Carotenoids possessing γ-rings were not found even under anaerobic conditions, suggesting the absence of precursor carotenoids for ABA. We tried to detect C$_{15}$ hydrocarbons for the intermediate for ABA biosynthesis.

The HPLC analysis of extract from the mycelia at an early growth stage showed two peaks at $t_R$s 10.2 min and 14.1 min the absorption spectra of which were similar to those of 2Z,4E-α-ionylideneethane and 2 respectively. The GC–MS analysis of the material eluted at $t_R$ 10.2 min showed that it consisted of two compounds, 14 and 15, with a ratio of 86:14, having molecular ions at $m/z$ 204. $^1$H- and $^{13}$C-NMR spectra of the mixture of 14 and 15 suggested that compound 14 was 2Z,4E-γ-ionylideneethane. Identification of 14 as 2Z,4E-γ-ionylideneethane was confirmed by comparison of the spectral data with those of the chemically synthesized 2Z,4E-γ-ionylideneethane. Compound 15 was identified
as 2E,4E-γ-ionylideneethane also by comparison of the \( t_R \) in GC and mass spectrum with those of the chemically synthesized 2E,4E-γ-ionylideneethane. Compounds 14 and 15 are new compounds. The material eluted at \( t_R \) 14.1 min was identified as 2 by spectral analysis.12) The contents of 2, 14, and 15 were 0.5, 15, and \( \frac{3}{22} \) g/g fresh weight of mycelia respectively.

The absence of precursor carotenoids and the occurrence of C\(_{15}\) compounds 2, 14, and 15 strongly suggested that this fungus biosynthesized ABA by the direct pathway via ionylideneethane as well as \( B. \) cinerea. This pathway contains two possible routes: FDP is reduced at C-1 and desaturated at C-4 to give 2, isomerization at C-2 of 2 before cyclization of 3 or cyclization of 2 before isomerization at C-2 of 15 to give 14, and oxidation at C-1, -1', and -4' of that with molecular oxygen to form ABA (Fig. 3). A feeding experiment with \( [2-\text{13C}] \)-2, 3, 14, and 15 was performed to confirm the occurrence of the direct pathway via ionylideneethane, and to distinguish the two routes from 2 to 14.

**Feeding experiment of \([2-\text{13C}] \)-2, 3, 14, and 15**

2-\text{13C}-Labeled 2, 3, 14, and 15 were synthesized and fed to \( C. \) cyanota. The incorporation ratios of these compounds into 2, 3, 14, 15, and ABA are summarized in Table 2. The conversion of \([2-\text{13C}] \)-2, 3, 14, and 15 to 5 and ABA indicated that these compounds were biosynthetic precursors for ABA. The incorporation ratio of 14 to ABA was clearly higher than that of 15, while that of 2 was almost the same as that of 3. This result suggests that isomerization of 2 to 3 proceeds before cyclization of 2 to 15. Failure to detect 3 and 6 might be due to the rapid conversion of 3 to 14 and of 6 to 5 respectively. Compounds 2 and 3 gradually decompose upon exposure to oxygen, water, and acid, so this instability in addition to volatility might cause low incorporation of these compounds. It is unknown at present whether desaturation at C-4 forming 2 precedes reduction at C-1.

The stereospecificity of oxygenase catalyzing hydroxylation at C-1' of 14 was evaluated by the enantiomeric excess of ABA that was formed by the fungus fed with \( (\pm) -[2-\text{13C}] \)-14. The incorporation ratio of \([2-\text{13C}] \)-14 into the ABA was 3.3%. If both enantiomers of \([2-\text{13C}] \)-14 are converted to ABA, the enantiomeric excess of the ABA should be 97.87%, and if \((S) -[2-\text{13C}] \)-14 is selectively converted to ABA, the enantiomeric excess of the ABA should be 95.73%. HPLC analysis of the

![Fig. 3. The Direct Pathway via Ionylideneethane for ABA Biosynthesis in \( C. \) cyanota.](image)

**Table 2. Incorporation Ratios (%) of 2-\text{13C} Labeled 2, 3, 14, and 15 to the Products, and 13C-Isotopic Abundance (%) of the Products (shown in parentheses)**

<table>
<thead>
<tr>
<th>Products</th>
<th>Substrates</th>
<th>([2-\text{13C}] )-2</th>
<th>([2-\text{13C}] )-3</th>
<th>([2-\text{13C}] )-15</th>
<th>([2-\text{13C}] )-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Not tested</td>
<td>1.2 (11.6)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>0.4 (26.8)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>0.1 (1.3)</td>
<td>0.1 (1.5)</td>
<td>0.2 (1.6)</td>
<td>ND(^a)</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>0.5 (1.8)</td>
<td>0.4 (1.7)</td>
<td>0.3 (1.6)</td>
<td>6.9 (3.4)</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>0.1 (1.1)</td>
<td>0.1 (1.1)</td>
<td>0.3 (1.6)</td>
<td>3.3 (3.2)</td>
<td>–</td>
</tr>
<tr>
<td>ABA</td>
<td>0.1 (1.2)</td>
<td>0.2 (1.2)</td>
<td>0.2 (1.6)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^a\)Not tested.  
\(^b\)Not detected.
ABA with a chiral column showed that it was a natural (S) isomer with 98.91 ± 0.04% ee, indicating that 1'-hydroxylase of 4 recognizes the chirality at C-1', although the selectivity was not high.

These findings strongly suggest that C. cruenta biosynthesizes ABA by the direct pathway via ionylidene-ethane. In this pathway, dehydrogenation at C-4, 5 and reduction at C-1 of FDP gives 2, cyclization of 3 after isomerization at C-2 of 2 forms (R)-14, and then C-1, -1’, -1, and -4’ of that is oxidized by monoxygenase to give (S)-ABA via 4, 6, and 5 (Fig. 3). This direct pathway in C. cruenta corresponds to that in B. cinerea except for the positions of double bonds in the rings of their intermediates. The direct pathway via ionylideneethane appears to be common among ABA-producing fungi. The gene encoding P450 monoxygenase corresponding to the bcaA of B. cinerea might be present in C. cruenta. The biosynthetic pathway of ABA not only before but also after IDP in fungi is different from that in higher plants. Fungi probably acquired the genes for ABA biosynthesis independently of higher plants.

**Experimental**

**General experimental procedures.** 1H- and 13C-NMR, NOESY, HMQC, and HMBC spectra were measured with a Bruker ARX500 instrument (500 MHz for 1H and 125 MHz for 13C). Chemical shifts were given with TMS (δ 0.00 ppm) as the internal standard for 1H- and 13C-NMR analysis with CDCl3, and with a signal of benzene-d6 (δ 7.27 ppm) as the standard for 1H-NMR analysis with benzene-d6. Direct EIMS was carried out with a JEOL JMS-600H mass spectrometer, the temperature of the direct probe being increased from 50 °C to 450 °C at a rate of 128 °C/min. The instrument was operated at a chamber temperature of 250 °C, accelerating voltage of 3 kV, ionization voltage of 70 eV, and ionization current of 300 μA in the positive ion mode, the resolution being 1,000 during the measurement. GC–EI mass spectra were recorded on the above mass spectrometer equipped with a Hewlett Packard 6890 instrument, using an HP-5 column (30 m length × 0.32 mm i.d., 5% diphenyl–95% dimethylpolysiloxane, film thickness 0.25 μm, Hewlett Packard Co., Wilmington, DE, U.S.A.), and a He flow of 1.0 ml/min. The parameters of the mass spectrometer were the same as those of direct EIMS. The oven temperature for GC was programmed from 120 °C to 270 °C at a rate of 5 °C/min for methyl esters of ABA and 5, and from 100 °C to 190 °C at a rate of 3 °C/min for 2, 3, 4, 14, and 15. The 18O contents were calculated after correction by natural 13C-, 2H-, and 17O-isotopic abundance. IR spectra were obtained with a Shimadzu FTIR-8100AI spectrometer. UV and VIS spectra, and optical rotation were measured with a Shimadzu UV 2200AI and a JASCO DIP-1000 polarimeter respectively. HPLC was performed with a Hitachi L-7100 pump, Hitachi L-7400 UV detector, and Hitachi D-7500 chromato-integrator. For analysis of carotenoids, HPLC was performed with a Waters 600E multisolvod delivery system using a photodiode array detector Waters 991J with a detection range of 200–600 nm. The HPLC columns used were a YMC-Pack ODS-AQ 311 column (100 mm length × 6.0 mm i.d., YMC Co., Ltd., Kyoto, Japan), a YMC Carotenoid column (250 mm length × 4.6 mm i.d., YMC Inc., Wilmington, NC, U.S.A.), and a Chiralcel OD column (250 mm length × 4.6 mm i.d., Daicel Chemical Industries Ltd., Niigata, Japan). Column chromatography was carried out on Wakogel C-200 (silica gel; particle size, 0.075–0.15 mm, Wako Pure Chemical Industries Ltd., Osaka, Japan) or aluminium oxide 90 (alumina; particle size, 0.063–0.20 mm, Merck KGaA, Darmstadt, Germany).

**Materials.** The phytopathogenic fungi Cercospora cruenta, IF06134, which was used by Yamamoto et al.,39 was kindly provided by Professor Emeritus Takayuki Oritani of Tohoku University, Japan. (±)-ABA and β-carotene were purchased from Wako Pure Chemical Industries Ltd., and 18O2 (99 atom % 18O) was purchased from ISOTEC, Inc., Miamisburg, OH, U.S.A. [1-15]Bromoethane (99 atom % 13C) was purchased from Aldrich Chemical, Co., Inc., Milwaukee, WI, U.S.A.

**Labeling of 4, 5, and ABA with 18O2.** Ten milliliters of a modified potato-dextrose medium containing 0.2 g (1.1 mmol) of d-glucose, 0.04 g of yeast extract and 0.015 g of agar was added to a 300 ml-sized Erlenmeyer flask. For the labeling experiment of compound 4 and 5, and ABA, a flask with a side arm sealed with a silicone rubber stopper was used to collect the medium using a syringe. After sterilization by autoclave and inoculation of C. cruenta, the flask was sealed with a stopcock. The flask was immediately evacuated and then filled with N2 to purge the air. This procedure was repeated two more times. An air bag (5-liter-size) containing 0.25-liter (10 mmol, 25 °C) of 18O2 and 1.0-liter of N2 was fitted to the flask, and C. cruenta was cultured on a rotary shaker (110 rpm) at 25 °C under a fluorescent light. The atmosphere in the air bag was not replenished with 18O2 during the culture. The culture periods were 7 d for 4, and 16 d for 5 and ABA. The medium was collected every two days from day 10 after inoculation, and analyzed by HPLC with a YMC-Pack ODS-AQ 311 column (solvent, 0.1% AcOH–45% MeOH–55% H2O; flow rate, 1.0 ml/min; detection, 254 nm; tR of ABA, 12.9 min).

**Isolation of 4.** After culture of C. cruenta for 7 d in an 18O2 atmosphere, the mycelia were recovered and washed with 30 ml of distilled water. The mycelia (1.6 g) were homogenized in 10 ml of MeOH with sea sand and extracted with 60 ml of EtOAc–MeOH (1:1) solution. The extract was filtered and the filtrate was concentrated to give 10 ml of a yellow aqueous solution.
After the addition of 40 ml of distilled water to the solution, this aqueous solution was partitioned three times with 20 ml of EtOAc. The organic layers were combined, washed with distilled water, dehydrated with Na₂SO₄, filtered, and concentrated to give a yellow oil (18 mg). The oil was subjected to silica gel (5 g) column chromatography with mixtures of n-hexane and EtOAc as the eluent. The material (5 mg) eluted with 15–20% EtOAc was subjected to preparative HPLC with a YMC-Pack ODS-AQ 311 column (solvent, 75% MeOH–25% H₂O; flow rate, 1.0 ml/min; detection, 254 nm). The material eluted at tᵣ 19.3 min was collected and concentrated to give 4 (0.5 μg). GC–EIMS (tᵣ 15.0 min) m/z (rel. int.): 222 [M + 2]⁺ (3), 220 [M]⁺ (2), 207 [M + 2 – CH₃]⁺ (6), 205 [M – CH₃]⁺ (3), 202 (13), 189 (53), 187 (52), 159 (24), 133 (51), 121 (43), 105 (100), 91 (71), 81 (75), 69 (54), 55 (39). The total ¹⁸O content was calculated from the relative intensities of [M + 2]⁺ and [M]⁺ ions to be 58%.

Isolation of 5 and ABA. After the culture of C. cruenta for 16 d under ¹⁸O₂ was filtered, the mycelia were washed with 40 ml of distilled water. The filtrate and the washing (50 ml, in total) were combined, acidified with 25% H₃PO₄ to pH 3, and partitioned three times with 20 ml of EtOAc. The organic layers were combined, dehydrated with Na₂SO₄, filtered, and concentrated to give a yellow oil (5 mg). The oil was subjected to silica gel (2 g) column chromatography using mixtures of toluene and EtOAc containing 1% AcOH as the eluent. The material (0.2 mg) eluted with 30–40% EtOAc was subjected to preparative HPLC using a YMC-Pack ODS-AQ 311 column (solvent, 40% to 100% MeOH in H₂O containing 0.1% AcOH over a period of 30 min; flow rate, 1.0 ml/min; detection, 254 nm). Materials eluted at tᵣ 7.9 min and 10.3 min were collected and concentrated to give 5 (19 μg) and ABA (10 μg) respectively. To the ABA dissolved in MeOH ethereal CH₂N₂ was added, and the solution was left at room temperature for 1 h. The solution was concentrated to give a methyl ester of ABA (10 μg). Methyl ester of ABA. GC–EIMS (tᵣ 14.7 min) m/z (rel. int.): 286 [M + 8]⁺ (0.5), 284 [M + 6]⁺ (0.9), 282 [M + 4]⁺ (2), 280 [M + 2]⁺ (1), 278 [M]⁺ (0.8), 266 (1), 264 (4), 262 (5), 260 (3), 196 (23), 194 (76), 192 (100), 190 (53), 164 (35), 162 (26), 129 (15), 127 (35), 125 (26). The total ¹⁸O content was calculated from the relative intensities of the molecular ions to be 44%. Compound 5 was methylated by the same method as that for ABA to give a methyl ester of 5 (19 μg). Methyl ester of 5. GC–EIMS (tᵣ 14.9 min) m/z (rel. int.): 288 [M + 8]⁺ (1), 286 [M + 6]⁺ (4), 284 [M + 4]⁺ (8), 282 [M + 2]⁺ (7), 280 [M]⁺ (3), 268 (4), 266 (12), 264 (18), 262 (8), 129 (70), 127 (100), 125 (81). The total ¹⁸O content was calculated from the relative intensities of the molecular ions to be 45%.

Detection and identification of 7. C. cruenta was cultured on a reciprocal shaker (160 rpm) with six 2 liter-sized flasks capped with cotton plugs, each containing 700 ml of a modified potato-dextrose medium at 25 °C under a fluorescent light for 8 d. After filtration, the mycelia (400 g) were washed with distilled water, homogenized in 100 ml of MeOH with sea sand, and extracted three times using 2-liter of EtOAc–MeOH (1:1) solution. These extracts were combined and filtered, and the filtrate was concentrated to give 290 ml of orange aqueous solution. After the addition of 1.7-liter of MeOH to the solution, this 85% MeOH solution was partitioned five times with 500 ml of n-hexane. The n-hexane layers were combined, washed with 100 ml of 1% NaHCO₃ and distilled water successively, dried over Na₂SO₄, filtered, and concentrated to give an orange oil (9 g). The oil was analyzed by HPLC with a photodiode array detector (200–600 nm): column, YMC Carotenoid column; solvent, 15% to 85% tert-butyl methyl ether in MeOH over a period of 30 min; flow rate, 1.0 ml/min; tᵣ of compound 7, 13.6 min. The content of 7 was calculated from the calibration curve between the peak area in HPLC and the amount of authentic 7. The orange oil was subjected to silica gel (200 g) column chromatography using mixtures of n-hexane and toluene as the eluent. The materials (10 mg) eluted with 10–20% toluene were subjected to alumina (20 g, 6% water) column chromatography with mixtures of n-hexane and toluene as the eluent. The materials (6 mg) eluted with 1–10% toluene were subjected to preparative HPLC with a YMC Carotenoid column (solvent, 70% MeOH–30% tert-butyl methyl ether; flow rate 1.0 ml/min; detection, 450 nm). The material eluted at tᵣ 17.1 min was collected and concentrated to give 7 (4 mg, red solid). For spectral data of 7, see Britton et al. and Englert.

Quantification of ABA produced under the two culture conditions. C. cruenta was cultured on a reciprocal shaker (160 rpm) with 700 ml of a modified potato-dextrose medium in a 2-liter-sized flask capped with a cotton plug at 25 °C under a fluorescent light. After 4 d, 20 ml of aliquot containing both mycelium and medium was collected from the culture, homogenized in 10 ml of MeOH with sea sand, and extracted with 50 ml of EtOAc–MeOH (1:1) solution. The extract was filtered, and the filtrate was concentrated. Twenty-five milliliters of 1% NaHCO₃ was added to the residue, and the solution was partitioned three times with 30 ml of EtOAc. The aqueous layer was acidified to pH 3 with 25% H₃PO₄ and partitioned three times using 30 ml of EtOAc. The aqueous layer was acidified to pH 3 with 25% H₃PO₄ and partitioned three times using 30 ml of EtOAc. The organic layers were combined, dehydrated with Na₂SO₄, filtered, and concentrated to give a yellow oil (33 mg). ABA in this oil was quantified by HPLC using a YMC-Pack ODS-AQ 311 column (solvent, 0.1% AcOH–55% H₂O–45% MeOH; flow rate, 1.0 ml/min; detection 254 nm; tᵣ of ABA, 12.6 min). The content of ABA was calculated from the calibration curve between the peak area in HPLC and the amount of authentic
ABA. ABA from 5 to 14 d was quantified every day. ABA produced by the fungus cultured in a flask capped with a silicone plug was quantified every day from 4 to 14 d by the methods described above.

Detection and identification of 7–13. C. crucenta was cultured on a reciprocal shaker (160 rpm) with six 2-liter-sized flasks capped with silicone plugs, each containing 700 ml of a modified potato-dextrose medium at 25°C under a fluorescent light. After 8 d, the mycelia (270 g) were washed with distilled water, homogenized in 100 ml of MeOH with sea sand, and extracted three times using 2-liter of EtOAc–MeOH (1:1) solution. The combined extracts were filtered, and the filtrate was concentrated to give 240 ml of a yellow aqueous solution. After the addition of 1.6-liter of MeOH to the residue, this 85% MeOH solution was partitioned five times with 300 ml of n-hexane. The n-hexane layers were combined, washed with 100 ml of 1% NaHCO₃ and distilled water successively, dried over Na₂SO₄, filtered, and concentrated to give an orange oil (1.5 g). The oil was analyzed by HPLC with a photodiode array detector (200–600 nm); column, YMC Carotenoid column; solvent, 10% toluene and with 5% toluene were applied. The orange oil was subjected to HPLC under the same condition as that described for the first fraction. The materials eluted at Rₜ 17.7 min and 29.3 min were collected and concentrated to give 7 (0.4 mg, red solid) and 12 (0.2 mg, yellow solid) respectively.

The materials eluted with 15% toluene were subjected to alumina (15 g, 6% water) column chromatography with mixtures of n-hexane and toluene as the eluent, and the materials eluted with 0% toluene and with 5% toluene were collected. The material (0.1 mg) eluted with 0% toluene was subjected to preparative HPLC with a YMC carotenoid column (solvent, 15% to 85% tert-butyl methyl ether in MeOH over a period of 30 min; flow rate, 1.0 ml/min; detection 350 or 450 nm). The materials eluted at Rₜs 11.7 min and 14.3 min were collected and concentrated to give 9 (12 μg) and 10 (4 μg) respectively. The material (0.1 mg) eluted with 5% toluene was purified with HPLC under the same condition as that described for the material eluted with 0% toluene, except that the detection was 400 or 450 nm. The materials eluted at Rₜs 14.9 min and 21.4 min were collected and concentrated to give 11 (50 μg) and 13 (14 μg) respectively.

Compound 9. NMR δₙ (benzene-d₆): 1.68 (9H, br.s, H-16, 18, 16′), 1.72 (6H, s, H-19, 18′), 1.80 (6H, s, H-17, 17′), 1.81 (3H, s, H-20), 1.84 (3H, s, H-19′), 1.99 (3H, s, H-20′), 2.29 (20H, m, H-3, 4, 7, 8, 11, 12, 3′, 4′, 7′, 8′), 5.38 (5H, m, H-2, 6, 10, 2′, 6′), 6.25 (2H, m, H-10′, 15′), 6.42 (1H, m, H-15), 6.54 (1H, d, J = 15.0 Hz, H-12′), 6.72 (3H, m, H-14, 11′, 14′). Compound 10. NMR δₙ (benzene-d₆): 1.27 (6H, s, H-16, 17), 1.61 (2H, m, H-2), 1.68 (3H, s, H-16′), 1.72 (3H, s, H-18′), 1.80 (3H, s, H-17′), 1.85 (3H, s, H-19′), 1.93 (3H, s, H-18), 1.97 (3H, s, H-19), 2.00 (3H, s, H-20′), 2.05 (3H, s, H-20), 2.09 (2H, m, H-4′), 2.27 (8H, m, H-3′, 4′, 7′, 8′), 5.35 (1H, m, H-2′), 5.40 (1H, m, H-6), 6.26 (1H, m, H-10), 6.46 (4H, m, H-8, 10, 14, 14′), 6.51 (1H, d, J = 16.1 Hz, H-7), 6.54 (1H, d, J = 17.0 Hz, H-12′), 6.60 (1H, d, J = 14.4 Hz, H-12), 6.79 (3H, m, H-15, 11′, 15′), 6.89 (1H, m, H-11). The signal at H-3′ could not be detected by superimposition with impurity peaks. Compound 11. NMR δₙ (benzene-d₆): 1.68 (6H, s, H-16, 16′), 1.72 (6H, s, H-18, 18′), 1.80 (6H, s, H-17, 17′), 1.85 (6H, s, H-19, 19′), 1.99 (6H, s, H-20, 20′), 2.28 (16H, m, H-3, 4, 7, 8, 3′, 4′, 7′, 8′), 5.35 (2H, m, H-2, 2′), 5.40 (2H, m, H-6, 6′), 6.26 (2H, m, J = 11.0 Hz, H-10, 10′), 6.43 (2H, br.d, J = 9.8 Hz, H-14, 14′), 6.54 (2H, d, J = 15.0 Hz, H-12, 12′), 6.77 (2H, m, H-11, 11′), 6.78 (2H, m, H-15, 15′). Compound 12. UV and VIS λₘₐₓ (n-hexane) nm (ε): 405 (73,600), 427 (111,000), 453 (101,000). NMR δₙ (CDCl₃): 1.01 (6H, s, H-16, 17), 1.03 (6H, s, H-16, 17′), 1.42 (2H, m, H-2), 1.47 (2H, m, H-2′), 1.59 (4H, m, H-3, 3′), 1.62 (3H, s, H-18), 1.72 (3H, s, H-18′), 1.86 (3H, s, H-19), 1.92 (2H, t, J = 6.4 Hz, H-4), 1.95 (3H, s, H-20), 1.97 (6H, s, H-19′, 20′), 2.02 (2H, t, J = 6.0 Hz, H-4′), 2.12 (4H, m, H-7, 8), 5.98 (1H, d, J = 10.9 Hz, H-10), 6.13 (1H, d, J = 14.6 Hz, H-8′), 6.15 (1H, m, H-10′), 6.17 (1H, m, H-7′), 6.20 (2H, d, J = 10.7 Hz, H-14), 6.24 (1H, d, J = 7.4 Hz, H-14′), 6.26 (1H, d, J = 15.0 Hz, H-12), 6.35 (1H, d, J = 14.9 Hz, H-12′), 6.50 (1H, dd, J = 10.9, 15.0 Hz, H-11), 6.61 (2H, m, H-15, 15′), 6.65 (1H, m, H-11′); EIMS m/z (rel. int.): 538 [M⁺] (100), 446 [M-92]⁺ (3), 401 [M-137]⁺ (29), 309 (18), 256 (10), 211 (11), 157 (13), 145 (13), 137 (27), 133 (21), 119 (25), 95 (30), 69 (34). HR-EIMS m/z [M⁺]: Calcd. for C₉₃H₇₃: 538.4538. Found 538.4535. This is the first report of the UV, El mass, and ¹H-NMR spectral data of 12. Compound 13. NMR δₙ (benzene-d₆): 1.68 (6H, s, H-16, 16′), 1.76 (3H, s, H-18), 1.78 (3H, s, H-17), 1.80 (3H, s, H-17), 1.85 (6H, s, H-19, 18′), 1.97 (3H, s, H-
19', 2.00 (3H, s, H-20), 2.03 (3H, s, H-20'), 2.29 (12H, m, H-3, 4, 7, 8, 3', 4'), 5.35 (2H, m, H-2, 2'), 5.41 (1H, m, H-6), 6.28 (2H, m, H-10, 6'), 6.44 (1H, m, H-14), 6.46 (2H, m, H-10', 14'), 6.55 (2H, d, \( J = 14.9 \) Hz, H-12, 8'), 6.60 (1H, d, \( J = 14.9 \) Hz, H-12'), 6.79 (4H, m, H-11, 15, 7', 15'), 6.87 (1H, m, H-11'). See Ebenezer and Pattenden for the spectral data of 8, and see the literature for the UV and El mass spectral data of 9,10,11, and 13.16

Detection and identification of 2, 14, and 15. C. cruenta was cultured on a reciprocal shaker (160 rpm) with six 2-liter-sized flasks capped with cotton plugs, each containing 700 ml of a modified potato-dextrose medium at 25 °C under a fluorescent light for 5 d. After filtration of the culture, the mycelia (310 g) were washed with distilled water, homogenized in 100 ml of acetone with sea sand, and extracted three times with 1-liter of acetone. The extracts were combined and filtered, and the filtrate was concentrated to give 100 ml of an orange aqueous solution. After the addition of 500 ml of 1% NaHCO₃ to the residue, the solution was partitioned five times using 200 ml of n-hexane. The n-hexane layers were combined, washed with distilled water, dried over Na₂SO₄, filtered, and concentrated to give an orange oil (3 g). The oil was analyzed by HPLC with a photodiode array detector (200–600 nm): column, YMC Carotenoid column; solvent, 10% H₂O–90% MeOH; flow rate, 1.0 ml/min; and by GC–MS, which was set at the condition described above in General experimental procedures. Ig8 of 2, 14, and 15 in GC were 12.6 min, 7.4 min, and 8.1 min respectively. The content of 2 was calculated from the calibration curve between the peak area in HPLC and the amount of authentic 2. The total content of compounds 14 and 15 was calculated from the calibration curve between the peak area in HPLC and the amount of authentic 14. The content of 14 and that of 15 were calculated by multiplying the total content and the ratio of the peak areas in GC. Chemically synthesized 2 and 14, described below, were used as the authentic samples. The orange oil was subjected to silica gel (50 g) column chromatography using mixtures of n-hexane and toluene as the eluent. The materials (18 mg) eluted with 5% toluene was subjected to alumina (15 g, 0% water) column chromatography using mixtures of n-hexane and toluene as the eluent. The materials (16 mg) eluted with 0–5% toluene were subjected to preparative HPLC using a YMC Carotenoid column (solvent, 10% H₂O–90% MeOH; flow rate, 1.0 ml/min; detection, 254 nm). The materials eluted at Ig8 10.2 min and 14.1 min were separately collected and partitioned three times with 10 ml of n-hexane, and the n-hexane layers were combined and concentrated to give a mixture of 14 and 15 (3 mg, colorless oil), and 2 (1 mg, colorless oil) respectively. Compound 14. NMR δH1 (CDCl₃): 0.81 (3H, s, H-7'), 0.90 (3H, s, H-8'), 1.33 (1H, m, H-3'), 1.50 (1H, m, H-3'), 1.59 (2H, m, H-4'), 1.71 (3H, d, \( J = 7.0 \) Hz, H-1'), 1.83 (3H, br.s, H-6'), 2.05 (1H, m, H-5'), 2.29 (1H, m, H-5'), 2.51 (1H, d, \( J = 9.6 \) Hz, H-1'), 4.58 (1H, s, H-9'), 4.74 (1H, s, H-9'), 5.35 (1H, q, \( J = 7.0 \) Hz, H-2), 5.78 (1H, dd, \( J = 9.6, 15.5 \) Hz, H-5), 6.43 (1H, d, \( J = 15.5 \) Hz, H-4). NMR δC (CDCl₃): 13.0 (C-1), 20.8 (C-6), 23.5 (C-4', 7'), 29.6 (C-8'), 34.7 (C-5'), 35.5 (C-2'), 39.2 (C-3'), 58.3 (C-1'), 108.3 (C-9'), 123.0 (C-2'), 128.7 (C-4'), 128.9 (C-5), 132.8 (C-3), 150.6 (C-6'). GC–EI-MS m/z (rel. int.): 204 [M]+ (93), 189 (54), 175 (10), 161 (24), 148 (22), 135 (87), 119 (70), 107 (100), 94 (76), 79 (40), 69 (57), 55 (35). Compound 15. GC–EI-MS m/z (rel. int.): 204 [M]+ (93), 189 (50), 175 (11), 161 (24), 147 (21), 135 (84), 119 (69), 107 (100), 94 (73), 79 (42), 69 (53), 55 (36). For the spectral data of 2, see Inomata et al.12

Synthesis of 14 and 15. At room temperature, 1.0 mmol THF solution of potassium tert-butoxide (0.4 ml, 0.4 mmol) was added dropwise to a stirred suspension of ethyl triphenylphosphonium bromide (148 mg, 0.4 mmol) in 5 ml of THF under N₂. After 30 min stirring, y-iodine (39 mg, 0.2 mmol) was added to the orange solution, and the mixture was stirred for 30 min at room temperature under N₂. Distilled water (0.5 ml) was added to the mixture and the solution was stirred for 1 h at room temperature. After the addition of another 50 ml of distilled water to the mixture, the solution was partitioned three times with 20 ml of Et₂O. The organic layers were combined, washed with distilled water, dried over Na₂SO₄, filtered, and concentrated to give a yellow oil (230 mg). The oil was subjected to AgNO₃-silica gel (1:20, 10 g) column chromatography using mixtures of n-hexane and toluene as the eluent. The materials eluted with 5–10% and 10% toluene were concentrated to give compounds 14 (18 mg, 42% yield) and 15 (10 mg, 22% yield) respectively as a colorless oil. Compound 14 (2Z,4E-5-(2',2'-dimethyl-6'-methylenecyclohexyl)-3-methyl-2,4-pentadiene). UV \( \lambda_{\text{max}} \) (n-hexane) nm (ε): 237 (24,900). IR: \( \nu_{\text{max}} \) (KBr) cm⁻¹: 3080, 3040, 2965, 2930, 2865, 1645, 1455, 1345, 1385, 970, 890. GC–HR–EI-MS m/z [M]+: Calcd. for C₁₅H₂₄: 204.1878. Found: 204.1880. For other spectral data, see 14 isolated from C. cruenta. Compound 15 (2E,4E-5-(2',2'-dimethyl-6'-methylenecyclohexyl)-3-methyl-2,4-pentadiene). UV \( \lambda_{\text{max}} \) (n-hexane) nm (ε): 233 (22,700). IR: \( \nu_{\text{max}} \) (KBr) cm⁻¹: 3080, 3030, 2930, 2865, 1645, 1460, 1435, 1385, 970, 890. NMR δH1 (CDCl₃): 0.81 (3H, s, H-7'), 0.89 (3H, s, H-8'), 1.32 (1H, m, H-3'), 1.49 (1H, m, H-3'), 1.57 (2H, m, H-4'), 1.71 (3H, d, \( J = 6.9 \) Hz, H-1), 1.77 (3H, s, H-6), 2.03 (1H, m, H-5'), 2.28 (1H, m, H-5'), 2.44 (1H, d, \( J = 9.6 \) Hz, H-1'), 4.57 (1H, s, H-9'), 4.72 (1H, s, H-9'), 5.47 (1H, q, \( J = 6.9 \) Hz, H-2), 5.65 (1H, dd, \( J = 9.6, 15.5 \) Hz, H-5), 6.04 (1H, d, \( J = 15.5 \) Hz, H-4). NMR δC (CDCl₃): 12.3 (C-6), 13.7 (C-1), 23.5 (C-4', 7'), 29.6 (C-8'), 34.6 (C-5'), 35.5 (C-2'), 39.2 (C-3'), 57.9 (C-1'), 108.2 (C-9'), 125.0 (C-2), 125.8 (C-5), 134.5 (C-3), 136.5 (C-4), 150.7 (C-6'). GC–HR–EI-MS m/z [M]+: Calcd. for C₁₅H₂₄: 204.1878. Found
204.1880. For GC–EI mass spectral data, see 15 isolated from C. cruenta.

*Synthesis of [2-13C]-2, 3, 14, and 15.* [1-13C]Ethyl triphenylphosphonium bromide, and [2-13C]-2 and [2-13C]-3, were synthesized by the same methods as those described in the literature. In the same manner as that described for the synthesis of 14 and 15, γ-ionone (100 mg, 0.5 mmol) was reacted with [1-13C]ethyl triphenylphosphonium bromide (390 mg, 1.0 mmol) and potassium tert-butoxide (1.0 ml of 1.0 M THF solution, 1.0 mmol) in 5 ml of THF, and the reaction mixture was purified to give [2-13C]-14 (37 mg, 36% yield) and [2-13C]-15 (28 mg, 27% yield) respectively. The 13C contents of these compounds were calculated from the relative intensities of the molecular ions in their mass spectra to be 99%. [2-13C]-14, GC–EIMS (řR 7.7 min) m/z (rel. int.): 205 [M]+ (100), 190 (56), 175 (8), 162 (25), 148 (19), 136 (85), 120 (61), 108 (71), 95 (87), 80 (25), 69 (43), 55 (23). [2-13C]-15, GC–EIMS (řR 8.0 min) m/z (rel. int.): 205 [M]+ (100), 190 (52), 175 (9), 162 (27), 148 (20), 136 (86), 120 (68), 108 (82), 95 (99), 80 (36), 69 (60), 55 (36).

*Feeding of 2-13C labeled 2, 3, 14, and 15 to C. cruenta.* C. cruenta was sub-cultured on a reciprocal shaker (160rpm) with three 2-liter-sized flasks capped with cotton plugs, each containing 500 ml of a modified potato-dextrose medium at 25°C under a fluorescent light for 6 d. The mycelia (435 g) were harvested by centrifugation at 6000 x g for 10 min and then suspended in sterile water. This suspension was centrifuged again at 6000 x g for 10 min, and the supernatant was removed by decantation. The mycelia were suspended in 6000 ml of sterile water. This suspension was centrifuged at 16000 rpm in 5 ml of THF, and the reaction mixture was purified to give [2-13C]-14 (37 mg, 36% yield) and [2-13C]-15 (28 mg, 27% yield) respectively. The 13C contents of these compounds were calculated from the relative intensities of the molecular ions in their mass spectra to be 99%. [2-13C]-14, GC–EIMS (řR 7.7 min) m/z (rel. int.): 205 [M]+ (100), 190 (56), 175 (8), 162 (25), 148 (19), 136 (85), 120 (61), 108 (71), 95 (87), 80 (25), 69 (43), 55 (23). [2-13C]-15, GC–EIMS (řR 8.0 min) m/z (rel. int.): 205 [M]+ (100), 190 (52), 175 (9), 162 (27), 148 (20), 136 (86), 120 (68), 108 (82), 95 (99), 80 (36), 69 (60), 55 (36).

The incorporation ratio was calculated using the following equation: Incorporation ratio (% of the metabolite biosynthesized from the administered substrate) = PA/SB x 100%; P = mole of the product isolated, A = % of 13C-isotopic abundance of the product – 1.1 (% of natural abundance of 13C), S = mole of the substrate administered, and B = 99 (% of 13C-isotopic abundance of the substrate). The 13C-isotopic abundance of 2, 3, 14, and 15 was calculated from their GC–EI mass spectra, and that of 5 and ABA was evaluated by the 13C-NMR spectra of their methyl esters.

*Optical purity of ABA produced by the fungus fed with (±)-[2-13C]-14.* ABA (5 mg) produced by C. cruenta fed with (±)-[2-13C]-14 was analyzed by HPLC with a Daicel Chiralcel OD column (solvent, 0.1% AcOH–80% n-hexane–20% 2-ProOH; flow rate, 1.0 ml/min; detection, 254 nm; řR of (+)- and (−)-ABA, 6.8 min and 8.3 min respectively). The optical purity of ABA was calculated from peak areas and shown as percent of enantiomeric excess. Data is the mean value ± SE of three measurements.

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