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BIOSYNTHESIS OF ABSCISIC ACID FROM α-IONYLIDENEETHANOL IN *CERCOSPORA PINI-DENSIFLORAE*

MASAHIKO OKAMOTO, NOBUHIRO HIRAI and KOICHI KOSHIMIZU

Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

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Key Word Index—*Cercospora pini-densiflorae*, Deuteromycetes, biosynthesis; abscisic acid, α-ionylideneethanol, α-ionylideneacetic acid, 1′,4′-*trans*-diol of abscisic acid, 1′-deoxyabscisic acid; 4′-hydroxy-α-ionylideneacetic acid; 1′-hydroxy-α-ionylideneacetic acid

Abstract—^{2}H-Labelled α-ionylideneethanol was efficiently incorporated into the 1′,4′-*trans*-diol of ABA via (1′*R*)-4′*S*-hydroxy-α-ionylideneacetic acid by *Cercospora pini-densiflorae*, and ^{2}H-labelled α-ionylideneacetic acid was incorporated into 1′-deoxyABA via (1′*R*)-4′*R*-hydroxy-α-ionylideneacetic acid The results of the feeding of these and other putative intermediates suggested that ABA was biosynthesized in this fungus from α-ionylideneethanol via (1′*R*)-4′*S*-hydroxy-α-ionylideneacetic acid to the 1′,4′-*trans*-diol of ABA and hence to ABA

INTRODUCTION

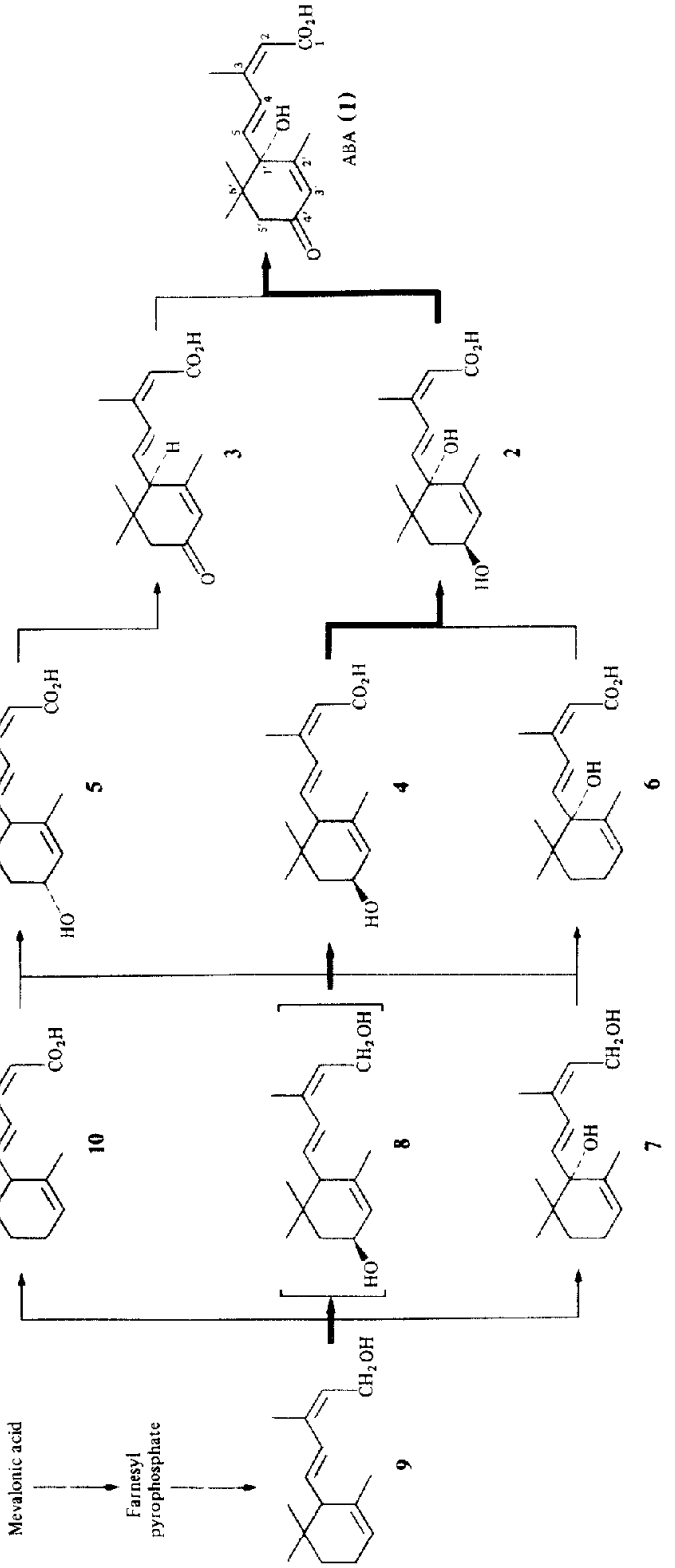
Cercospora pini-densiflorae produces large quantities of the 1′,4′-*trans*-diol of abscisic acid (1′,4′-*t*-diolABA, **2**) and small amounts of 4′-hydroxy-α-ionylideneacetic acids (4′-OH-α-ionylideneacetic acids, **4** and **5**) and 1′-deoxyabscisic acid (1′-deoxyABA, **3**) together with (+)-abscisic acid (ABA, **1**). The fungus converted 1′,4′-*t*-diolABA into ABA more easily than 1′-deoxyABA into ABA [1] This shows that the biosynthetic pathway of ABA via 1′,4′-*t*-diolABA is the major route in this fungus, as in *Botrytis cinerea* [2], rather than that via 1′-deoxyABA. The 1′,4′-*t*-diolABA is metabolized into ABA easily in plants [3–6], and it occurs endogenously in peas and avocados [3], suggesting that the pathway of ABA in fungi may be similar to that in plants. We report here the details of the conversion of ABA-related metabolites and other putative intermediates into ABA by *C. pini-densiflorae*.

RESULTS AND DISCUSSION

Our previous results [1] suggested that ABA biosynthesis in *C. pini-densiflorae* proceeds via the successive oxidation of α-ionylidyl intermediates such as α-ionylideneethanol (**9**) or α-ionylideneacetic acid (**10**). These compounds would arise via cyclization, desaturation, and oxidation of farnesol or farnesyl derivatives [7–9]. These putative intermediates were synthesized to examine their effectiveness as precursors of ABA in this fungus.

^{2}H-Labelled **9** and **10** were fed individually to the fungus ABA and its related metabolites were purified from the ethyl acetate-soluble part to detect ^{2}H-incorporation by GC/MS and ^{2}H NMR. Labelled **10** was metabolized to at least six compounds, 4′-OH-α-ionylideneacetic acids (**4**, **5**), 1′-deoxyABA, 1′,4′-*t*-diolABA, ABA, and one unknown metabolite (**6**). When feeding was with the labelled **9**, **10** and 1′-OH-α-ionylideneethanol (**7**) seemed to be present in addition to these six compounds, to judge from the GC and HPLC analysis (see Experimental). The unknown metabolite, **6**, showed a positive Cotton effect with extrema at 286 nm ($[\phi]^{27}$ +25 800°) and 249 nm ($[\phi]^{27}$ −25 300°) in the ORD curve, and had UV maximum in ethanol at 265 nm arising from an ABA-like side chain. The GC/MS of **6**-Me had a $[M]^+$ at *m/z* 267 and significant ions at *m/z* 249, 234, 211, 179, and 128 A strong peak at *m/z* 128 immediately suggested the incorporation of three ^{2}H into the side-chain methyl group, because the side chain with no ^{2}H shows a fragment ion at *m/z* 125 [10] The ions at *m/z* 267, 249 and 234 would correspond to those of $[M+d_3]^+$, $[M-d_3-H_2O]^+$, and $[M+d_3-H_2O-Me]^+$, respectively. The ions at *m/z* 211 $[M+d_3-C_4H_8]^+$ and 179 $[M+d_3-C_4H_8-MeOH]^+$ were presumed to have been formed via retro-Diels–Alder reaction, indicating that **6** was an α-apocarotenoid [11] These results suggested that **6** was a monohydroxy-derivative of α-ionylideneacetic acid· either 1′-OH- or 4′-OH-α-ionylideneacetic acid. The possibility that **6** was either 3′-OH- or 5′-OH-α-ionylideneacetic acid could be excluded because there were ions formed via a retro-Diels–Alder reaction (numbered as in ABA). It co-chromatographed on GC-ECD and HPLC (system A) with synthetic 1′-OH-α-ionylideneacetic acid and the spectral data of **6** coincided with those of 1′-OH-α-ionylideneacetic acid. Thus, **6** was identified as 1′-OH-α-ionylideneacetic acid.

The deuterium content and incorporation ratio of the six metabolites are summarized in Table 1 Similar results were obtained in three different experiments with labelled **9** and **10**. The fungus converted **9** more easily into 1′,4′-*t*-diolABA (7.4%) than 1′-deoxyABA (4 7%), but **10** more easily into the latter (20 4%) rather than the former (0.2%) More of the labelled **9** was incorporated into (1′*R*)-4′*S*-OH-α-ionylideneacetic acid (**4**, 3.7%) than into (1′*R*)-4′*R*-OH-α-ionylideneacetic acid (**5**, 0.7%), but **10** was converted into **5** (7.1%) in a higher yield than into **4** (0.5%). If α-ionylideneethanol was oxidized to these endogenous metabolites via α-ionylideneacetic acid, the incorporation pattern of **9** into these metabolites should be the same as that of **10**, however, it was different. This meant that **9** was metabolized not via α-ionylideneacetic



Scheme 1. Proposed biosynthetic pathway of ABA in *Cercospora pini-densiflorae*. Thick arrows represent the major route confirmed by feeding experiments.

Table 1 Deuterium content and incorporation ratio of ^{2}H-labelled intermediates into ABA and its related metabolites by *Cercospora pini-densiflorae*

	α-Ionylidene ethanol (**9-d**)		α-Ionylidene acetic acid (**10-d**)		(1′*R*)-4′*S*-OH-α-Ionylidene acetic acid (**4-d**)		(1′*R*)-4′*R*-OH-α-Ionylidene acetic acid (**5-d**)		1′-OH-α-Ionylidene acetic acid (**6-d**)		1′-OH-α-Ionylidene ethanol (**7-d**)	
	%D	%I R	%D	%I R	%D	%I R	%D	%I R	%D	%I R	%D	%I R
ABA (**1**)	40	2 3	55	1 5	—*	—	- *	--	66	1 6	—*	—
1′,4′-*t*-DiolABA (**2**)	22	7 4	8	0 2	33	23 2	-		49	3 2	8	0 3
1′-DeoxyABA (**3**)	72	4 7	100	20 4	0	0	100	2 8	—	—	—	—
(1′*R*)-4′*S*-OH-α-Ionylideneacetic acid (**4**)	32	3 7	22	0 5	—		-	--	—	—	—	—
(1′*R*)-4′*R*-OH-α-Ionylideneacetic acid (**5**)	100	0 7	97	7 1	-	--	--	—	—	—	—	—
1′-OH-α-Ionylidene-acetic acid (**6**)	100	0 5	100	0 9	--	-	--	-	100	—	—	—

%D Deuterium content (%) These values were corrected, as the substrates were 100% labelled

%I R Incorporation ratio = % of biosynthesized metabolite synthesized from labelled precursor = DT/B × 100, where D = %^{2}H incorporation was calculated from GC/MS, T = total amount of metabolite and B = amount of administered labelled precursor

— Not tested

* Not detected due to its low concentration

acid but via 1′-OH-α-ionylideneethanol (**7**) or (1′*R*)-4′*S*-OH-α-ionylideneethanol (**8**). Compound **7** cannot be a precursor for 4′-OH-α-ionylideneacetic acids and 1′-deoxyABA, so the fungal oxidation of **9** may proceed via **8**. This means that α-ionylideneethanol was mainly hydroxylated *cis* to the C-1′ side chain to form **8**. This putative intermediate appeared to be rapidly oxidized to **4** α-Ionylideneacetic acid was directly hydroxylated *trans* to the C-1′ side chain to give **5**, as has been reported for *C cruenta* [12]. These results suggested that the immediate precursor of 1′,4′-*t*-diolABA was **4** and that of 1′-deoxyABA was **5**. To test this hypothesis, ^{2}H-labelled epimeric 4′-OH-α-ionylideneacetic acids (**4-d**, **5-d**) were fed individually to the fungus Other possible precursors, 1′-OH-α-ionylideneethanol (**7**) and 1′-OH-α-ionylideneacetic acid (**6**), for 1′,4′-*t*-diolABA were also tested The labelled **4** was converted to 1′,4′-*t*-diolABA with a higher incorporation ratio (23.2%) than that of the labelled **7** (0.7%) and **6** (3.2%) The **6** isolated after feeding of **9-d** and **10-d** was 100% labelled with deuterium, indicating the absence of endogenous **6** 1′-DeoxyABA was formed not from (1′*R*)-4′*S*-OH-α-ionylideneacetic acid but exclusively from (1′*R*)-4′*R*-OH-α-ionylideneacetic acid in 2.8% yield (see Table 1). These results confirmed the above hypothesis The conversion of **9** and **10** to metabolites **2**–**5** seemed to be a natural metabolic event *in vivo*, as these metabolites are endogenous to this fungus [1]

The ABA from feeds of **6**, **9**, and **10** has a higher deuterium content than 1′,4′-*t*-diolABA as shown in Table 1 Possible explanations for this are as follows. When **6** was fed to the fungus, we cannot exclude the possibility that 1′,4′-*cis*-diolABA was formed in the medium. The *cis*-diol, not endogenous to this fungus, was metabolized into ABA in higher yield than 1′,4′-*t*-diolABA [1]. It might be converted immediately to ABA and not accumulate, since we could not detect the *cis*-diol in the medium This result suggests that, in addition to **2**, the *cis*-diol also contributed to the deuterium content of ABA. In the feed of **9**, the observed deuterium content of ABA must be contributed by not only **2** but **3**, because both **2** and **3** were converted into ABA [1]. In the feed of **10**, the pathway **10**→**5**→**3** functions exclusively, and the pathway **9**→**4**→**2** which dominates *in vivo* hardly operates in the fungus. In this case, most of the ABA is formed from not **2**, with a deuterium content of 8% but **3** with that of 100%, resulting in a deuterium content of ABA higher than that of **2**.

1′,4′-*t*-DiolABA has been shown to be the major immediate precursor of ABA rather than 1′-deoxyABA in *C. pini-densiflorae* [1] Therefore, the major pathway for ABA biosynthesis from **9** in this fungus was probably that **9** was converted to 1′,4′-*t*-diolABA via **4**, and the 1′,4′-*t*-diolABA was oxidized to ABA. In *C rosicola*, both **9** and **10** are converted to 1′-deoxyABA via **5** [13]. The difference in the results might be caused by the absence of endogenous 1′,4′-*t*-diolABA in *C. rosicola*. In the feeding of **9** and **10**, the ABA, 1′,4′-*t*-diolABA, and 1′OH-α-ionylideneacetic acid were very pure optically compared to 1′-deoxyABA and 4′-OH-α-ionylideneacetic acids (**4**, **5**), (Table 2) The 4′-hydroxylation and oxidation enzymes seem unable to recognize the chirality at the 1′-carbon very well.

Norman *et al* [14] and Neill *et al.* [10] reported that the 1′-hydroxylation of **10** and 1′-deoxyABA (**3**) occurs easily in *C rosicola*. However, the incorporation of **10** and 1′-deoxyABA into ABA does not occur in many plants although **10** is mainly converted to 1′-deoxyABA [13–15] The compounds with a 1′-hydroxyl group, 1′-OH-α-ionylideneacetic acid (**6**) and 1′,4′-*t*-diolABA (**2**), are converted to ABA easily by plants [3–6, 15]. The available data [3–6, 15–17] show that the metabolic fate of four compounds (**2**, **3**, **6**, **10**) added exogenously to

Table 2 Optical purity of metabolites isolated from feeds of racemic ^{2}H-labelled α-ionylideneethanol (**9-d**) and α-ionylideneacetic acid (**10-d**)

Metabolites	α-Ionylidene ethanol (**9-d**) (%)	α-Ionylideneacetic acid (**10-d**) (%)
ABA (**1**)	100	100
1′,4′-*t*-DiolABA (**2**)	100	100
1′-DeoxyABA (**3**)	70	60
(1′*R*)-4′*S*-OH-α-Ionylideneacetic acid (**4**)	80	90
(1′*R*)-4′*R*-OH-α-Ionylideneacetic acid (**5**)	40	50
1′-OH-α-Ionylidene acetic acid (**6**)	100	100

higher plants was very similar to that in *C. pini-densiflorae* This suggests that the proposed biosynthetic pathway for this fungus (Scheme 1) operates in a similar way to plants However, the presence of α-ionylideneethanol as an endogenous metabolite in higher plants remains to be confirmed

EXPERIMENTAL

Chromatography HPLC systems A YMC-Pack A311 ODS column (6 × 100 mm), eluted with MeOH–H_2O–HOAc (70 30·0 1), 1 5 ml/min, detection UV_{254}, B YMC-Pack A311 ODS column eluted with MeOH–H_2O–HOAc (60 40 0 1), 1 5 ml/min, detection UV_{254} GC 2% OV-1 column (1 m × 3 mm) at 165° for **6**-Me and 160° for **7**, detector temp, 270° (ECD), N_2 60 ml/min TLC was done on silica gel in systems (A) toluene–EtOAc–HOAc (80 20 1), and (B) toluene–EtOAc–HOAc (18 12 1)

Preparation of ^{2}H-*labelled* α-*ionone* α-Ionone (0 5 g) was dissolved in **7** ml of 1 N NaOD (D_2O–CD_3OD, 9 4) The mixt was stirred at room temp for 24 hr Further handling caused the α-ionone to deteriorate The mixt was neutralized with 1 N DCl and extracted with EtOAc The EtOAc extract was chromatographed on silica gel with *n*-hexane and toluene to give the labelled α-ionone (350 mg) as a pale yellow oil Its deuterium content was 88%, estimated by GC/MS (EI) after correction for isotopic contributions

Syntheses of ^{2}H-*labelled* α-*ionylideneethanol* (**9-d**) *and* α-*ionylideneacetic acid* (**10-d**) Labelled α-ionone (350 mg) and carbethoxymethylenetriphenylphosphorane (CETP, 1 2 g) were mixed together and heated to 180–200° for 3 5 hr under fusion conditions and then dil with Et_2O and filtered The filtrate was chromatographed on a silica gel column eluted with *n*-hexane–toluene (19 1) to give the Et ester of **10-d** (87 mg) and its 2-*trans*-isomer (92 mg) separately The Et ester of **10-d** (55 mg) was hydrolysed with 5 5 ml of 0 5 N KOH (EtOH–H_2O, 10 1) at room temp for 2 days The acidic EtOAc extract was purified by prep TLC (system A) to give pure **10-d** (30 mg) **10-d** ^{1}H NMR (90 MHz, $CDCl_3$) δ0 79 (3H, *s*), 0 89 (3H, *s*), 1 00–1 49 (2H, *m*), 1 60 (3H, *d*, *J* = 2 Hz), 1 85–2 00 (2H, *m*), 2 32 (1H, *d*, *J* = 9 Hz), 5 45 (1H, *br s*), 5 62 (1H, *br s*), 5 92 (1H, *dd*, *J* = 9 and 16 Hz), 7 52 (1H, *d*, *J* = 16 Hz) **10-d**-Me EIMS (GC/MS), 70 eV, *m/z* (rel int) 251 $[M+d_3]^+$ (0 5), 249 $[M+d_1]^+$ (0 5), 248 $[M]^+$ (0 5), 195 (8), 194 (6), 193 (1), 136 (54), 135 (37), 134 (21), 133 (12), 128 (100), the deuterium content was 71% The Et ester of **10-d** (50 mg) was reduced with $LiAlH_4$ (40 mg) in 4 ml of Et_2O at room temp for 2 hr After decomposition of excess $LiAlH_4$ with satd NH_4Cl, the labelled α-ionylideneethanol (**9-d**, 50 mg) was extd into EtOAc **9-d**. ^{1}H NMR (90 MHz, $CDCl_3$) δ0 80 (3H, *s*), 0 89 (3H, *s*), 1 10–1 14 (2H, *m*), 1 56 (3H, *br s*), 1 70–2 00 (2H, *m*), 2 15 (1H, *d*, *J* = 11 Hz), 4 30 (2H, *d*, *J* = 7 Hz), 5 30–5 60 (2H, *m*), 5 53 (1H, *d*, *J* = 11 and 14 Hz), 6 38 (1H, *d*, *J* = 14 Hz) EIMS (GC/MS), 70 eV, *m/z* (rel int) 223 $[M+d_3]^+$ (2 1), 222 $[M+d_2]^+$ (1 0), 221 $[M+d_1]^+$ (1 2), 220 $[M]^+$ (0 9) 206 (19), 205 (51), 204 (37), 203 (11), 202 (3), 162 (19), 148 (100), 133 (95), 132 (57), 131 (53), the deuterium content was 60%

Preparation of ^{2}H-*labelled* 1′-OH-α-*ionone* 1′-OH-α-Ionone was synthesized by the method of ref [18] SeO_2 (1 1 g) in 95% EtOH (400 ml) was added dropwise over 6 hr to a refluxing soln of α-ionone (2 5 g) in 95% EtOH (100 ml) After refluxing for a further 20 hr, the reaction mixt was filtered and evapd to yield a dark brown oil (3 2 g), which was chromatographed on silica gel with *n*-hexane–EtOAc (9 1) A colourless oil was recrystallized from *n*-hexane to give 1′-OH-α-ionone (0 7 g) as colourless needles, mp 88–89° (lit 89–90°) Labelled 1′-OH-α-ionone (0 15 g, mp 88–89°) was prepared from 1′-OH-α-ionone (0 2 g) in the same way The deuterium content was 75%

Syntheses of ^{2}H-*labelled* 1′-OH-α-*ionylideneacetic acid* (**6-d**) *and* 1′-OH-α-*ionylideneethanol* (**7-d**) Labelled 1′-OH-α-ionone (82 mg) and CETP (400 mg) were refluxed in 2 ml of xylene for 10 hr Chromatography on silica gel (*n*-hexane–EtOAc, 97 3) gave 61 mg of the Et ester of **6-d** and 40 mg of its 2-*trans*-isomer The Et ester of **6-d** (16 mg) was hydrolysed with 0 5 N KOH in 90% EtOH at room temp and then sepd by prep HPLC (system A) to give pure **6-d** (11 mg) **6-d** ^{1}H NMR (400 MHz, $CDCl_3$) δ0 90 (3H, *s*), 1 00 (3H, *s*), 1 44 (1H, *ddd*, *J* = 4, 10, 14 Hz), 1 62 (4H, 2′-Me and 5′-H overlapping, *d* and *m*, *J* = 2 Hz), 2 04 (2H, *m*), 5 54 (1H, *s*), 5 70 (1H, *s*), 6 18 (1H, *d*, *J* = 16 Hz), 7 68 (1H, *d*, *J* = 16 Hz) **6-d**-Me EIMS (GC/MS), 70 eV, *m/z* (rel int) 267 $[M+d_3]^+$ (2 7), 266 $[M+d_2]^+$ (3 2), 265 $[M+d_1]^+$ (2 5), 264 $[M]^+$ (1 2), 252 (1), 251 (2), 250 (9), 249 (32), 248 (52), 247 (50), 246 (13), 234 (22), 233 (21), 232 (19), 231 (6), 202 (46), 201 (100), 200 (85), 128 (32), the deuterium content was 57% Labelled 1′-OH-α-ionyli-

deneethanol (**7-d**, 11 mg) was prepd from the Et ester of **6-d** (15 mg) by methods analogous to those used for **9-d**. **7-d** ^{1}H NMR (400 MHz, $CDCl_3$): δ0 89 (3H, *s*), 0.98 (3H, *s*), 1 45 (2H, *m*), 1.61 (3H, *d*, $J=2$ Hz), 2 03 (2H, *m*), 4 31 (2H, *d*, $J=7$ Hz), 5.51 (1H, *br s*), 5 55 (1H, *t*, $J=7$ Hz), 5 78 (1H, *d*, $J=16$ Hz), 6 61 (1H, *d*, $J=16$ Hz) EIMS (GC/MS), 70 eV, *m/z* (rel int.) 221 $[M+d_3-H_2O]^+$ (12), 220 $[M+d_2-H_2O]^+$ (14), 219 $[M+d_1-H_2O]^+$ (22), 218 $[M-H_2O]^+$ (15), 202 (44), 201 (54), 200 (63), 199 (47), 143 (100), 129 (55), the deuterium content was 50%.

Preparation of ^{2}H-*labelled* (1'R)-4'S- *and* (1'R)-4'R-OH-α-*ionylideneacetic acid* (**4-d** and **5-d**). These compounds were prepared by biotransformation from **10-d** by *C pini-densiflorae* No. 26. The fungus was sub-cultured on 600 ml (200 ml × 3) of the medium in the laboratory for 30 days Mycelia were removed from the original medium, carefully washed with sterile H_2O, and refloated on a new medium Labelled α-ionylideneacetic acid (4.5 mg) was fed to the mycelia as a MeOH soln (150 μl) and incubated for a further 14 days The medium was filtered and extracted with EtOAc (150 ml × 4) at pH 3 The EtOAc extract (45 mg) was chromatographed on a silica gel (11 g) column eluted with toluene–EtOAc containing 0.1% HOAc The fraction eluted with 10% EtOAc was purified by prep TLC (system B, ×6 developments) to give a fraction with R_f 0 45. This fraction was further purified by prep HPLC (system B). The peaks at R_t 6 7 min (**4-d**) and 7 7 min (**5-d**) were collected and concd to afford **4-d** (300 μg) and **5-d** (300 μg). Their ^{1}H NMR and MS were identical to those previously reported [1] The deuterium contents of **4-d** and **5-d** were 32 and 75%, respectively.

Administration of ^{2}H-*labelled compds to cultures. C. pini-densiflorae* No. 26 was subcultured on 200 ml of Czapek–Dox medium (pH 5) in a 1 l Roux flask The labelled compds were added individually to the culture as in the prepn of **4-d** and **5-d** The amount added to the medium (200 ml) was between 1 5 and 2.0 mg for **9-d** and **10-d**, and *ca* 200 μg for **4-d**, **5-d** and **6-d** After incubation for another 14–20 days, an EtOAc ext (5–50 mg) of the culture medium was obtained and purified as described previously [1] to give each metabolite The purified metabolites were methylated and analysed by GC/MS, ^{2}H NMR, and ORD ^{2}H-Incorporation was detected by a full MS taken across the GC peak and calculated after correction for isotopic contributions. When the proportion of the labelled compds was less than 20%, ^{2}H NMR (60 MHz, $CHCl_3$) studies were used to confirm the incorporation of ^{2}H The incorporation ratio was calculated as described elsewhere [1] The enantiomeric ratio of the purified compds was calculated from the ORD curves compared with those reported for each compd [1, 2, 19–22] 1'-OH-α-Ionylideneethanol (**7**) and α-ionylideneacetic acid (**10**) were identified by their R_t on HPLC (system A) and GC after feeding of **9-d**. A distinct peak for **7** without derivatization was observed, but the peak was slightly broad, indicating some dehydration on the column The incorporation of **7** and **10** was 0.1% and 0.01%, respectively, if the compds were converted from **9-d** only

Stability of ^{2}H-*labelled compds* Each synthetic compd (40 μg) used for feeding expts was dissolved in buffers of pH 2–8 and left under the same conditions as in the feeding expt for 14 days. No conversion of any of these compds into ABA was observed. The 1'-OH-α-ionylideneethanol is very labile at acidic pH and the concn of **7** in the presence of 0 1% HOAc in MeOH caused this alcohol to deteriorate.

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