Biosci. Biotechnol. Biochem., **64** (10), 2089–2098, 2000

Changes in the Content and Biosynthesis of Phytoalexins in Banana Fruit

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Received March 24, 2000; Accepted June 5, 2000

Changes in the phytoalexin content in unripe fruit of banana, *Musa acuminata*, were analyzed after various treatments. The results show that level of hydroxyanigorufone started to increase 1–2 day after either wounding or inoculation with conidia of *Colletotrichum musae*. Inoculation followed by wounding induced the formation of many other phenylphenalenones. The accumulation of hydroxyanigorufone decreased, after its transient maximum, on ripening by exposure of the wounded fruit to ethylene. The level of production of hydroxyanigorufone in ripe fruit treated by wounding and/or by inoculation was much lower than that in unripe fruit. 2-Aminooxyacetic acid, an inhibitor of phenylalanine ammonia-lyase (PAL), inhibited the accumulation of hydroxyanigorufone in wounded fruit, and the PAL activity increased after wounding and ethylene treatment, respectively. Feeding experiments with [1-^{13}C] and [2-^{13}C]cinnamic acids, and [2-^{13}C]malonate show that two molecules of cinnamic acid and one of malonate were incorporated into each molecule of hydroxyanigorufone. The phytoalexins isolated from fruit to which deuterated hydroxyanigorufone and irenolone had been administered revealed that 2-(4′-hydroxyphenyl)-1,8-naphthalic anhydride was biosynthesized from hydroxyanigorufone rather than from irenolone.

Key words: *Musa acuminata*; banana; phytoalexin; phenylphenalenone; phenylalanine ammonia-lyase (PAL)

Unripe fruit of banana, *Musa acuminata*, resist infection by *Colletotrichum musae*, and the pathogen is essentially quiescent until the fruit ripens.1) Brown and Swinburne have shown that unripe banana fruit inoculated with conidia of *C. musae* after wounding produced phytoalexins which inhibited fungal growth.2) Accelerated ripening by exposing inoculated unripe fruit to ethylene decreased the antifungal activity in fruit tissues,2) suggesting that ripening suppressed the accumulation of phytoalexins. We have isolated seventeen presumed phytoalexins from unripe fruit of *M. acuminata* [AAA] cv. Buñgulan and *M. balbisiana* [BBB] cv. Saba sa Hapon, and identified them as phenylphenalenone derivatives: hydroxyanigorufone (**1**), anigorufone (**2**), 2-(4′-hydroxyphenyl)-1,8-naphthalic anhydride (**3**), irenolone (**4**), 2-hydroxy-4-(4′-methoxyphenyl) phenalen-1-one (**5**), (+)-*cis*-2,3-dihydro-2,3-dihydroxy-4-(4′-hydroxyphenyl)phenalen-1-one (**6**), (+)-*cis*-2,3-dihydro-2,3-dihydroxy-4-(4′-methoxyphenyl)phenalen-1-one (**7**), (−)-*trans*-2,3-dihydro-2,3-dihydroxy-9-phenylphenalen-1-one (**8**), 2-phenyl-1,8-naphthalic anhydride (**9**) and 2-(4′-methoxyphenyl)-1,8-naphthalic anhydride (**10**), as well as seven others (Fig. 1).3) These compounds could not be detected in the extract from untreated fruit peel. Phenylphenalenones were also identified in infected banana rhizomes, and it was proposed that they were also phytoalexins since they could not be detected in healthy tissues.4) However, changes in the phenylphenalenone content in unripe fruit after wounding and/or inoculation and the effects of ripening on the phenylphenalenone content have not previously been examined. A quantitative analysis of the changes in phenylphenalenone levels is essential to confirm that they are phytoalexins, this being the first step towards understanding their putative roles in the quiescent fungal infection of banana fruit.

Biosynthetic studies on phenylphenalenones of the Haemodoraceae plants have shown that phenylphenalenones were formed from two molecules of a phenylpropanoid derivative and one molecule of acetate, probably *via* a diarylheptanoid, and that one molecule of malonate was also incorporated into phenylphenalenones to a lesser extent than that of acetate.5–10) Recent taxonomic studies with the gene encoding the large subunit of ribulose-1,5-

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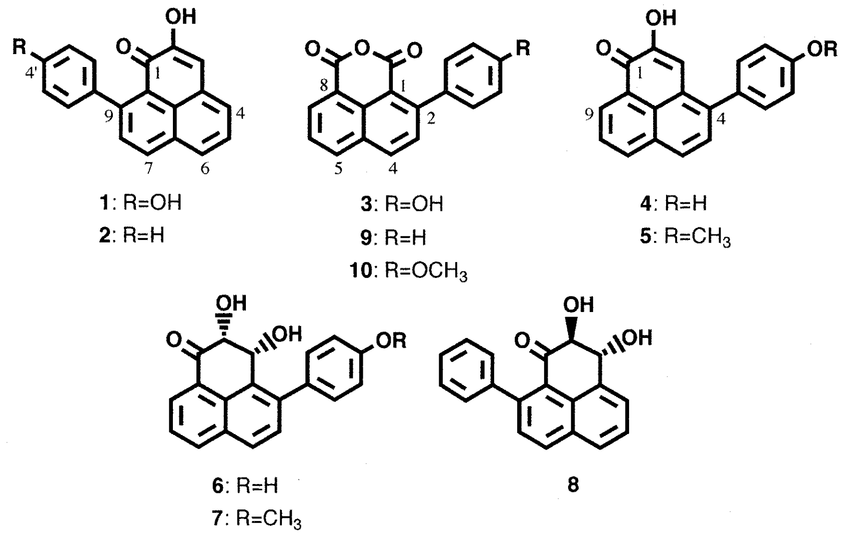

Fig. 1. Phenylphenalenone-type Phytoalexins in Banana Fruit. Relative configurations are shown.

bisphosphate carboxylase, *rbc*L, have suggested that Haemodoraceae, a family originally classified as belonging to Liliales,[11] should be classified into the new order, Haemodorales, close to Zingiberales.[12] Since the Musaceae, including *Musa*, belong to the Zingiberales, phenylphenalenones in banana fruit are probably biosynthesized along the same pathway as that present in Haemodoraceae plants, based on their close botanical relationship. However, the precursors for curcumin, a diarylheptanoid of the Zingiberales plant, *Curcuma longa*, have been reported to be derived from one phenylpropanoid and five malonyl-CoA moieties based on the results of ^{14}C-administration experiments.[13] Therefore, we cannot exclude the possibility that banana phenylphenalenones are biosynthesized along a pathway similar to that of curcumin.

In this study, we examined changes in the contents of phenylphenalenones in banana fruit, and the effect of ripening on these changes. Biosynthetic studies on banana phenylphenalenones, on the effects of 2-aminooxyacetic acid (AOA) on phenylphenalenone levels, and on the change in PAL activity are also reported.

Materials and Methods

^{1}H- and ^{13}C-NMR spectra were recorded with TMS as an internal standard by a Bruker ARX500 spectrometer. Mass spectra were obtained with a Jeol JMS-DX300/DA5000 mass spectrometer, and UV spectra were measured with a Shimadzu UV-2200AI spectrometer. Column chromatography was carried out on Wakogel C-200 (Wako Pure Chemical Industries), and HPLC was performed with an ODS column (YMC, AQ-311, 6 × 100 mm) at a flow rate of 1.0 ml/min with detection at UV 254 nm.

Plant material. M. acuminata [AAA] cv. Buñgulan cultivated on Negulos Island in the Philippines was used for all experiments. Unripe fruit, which had not been treated with fungicides, were imported by Alter Trade Japan, Tokyo, Japan. These unripe fruit were kept at 17°C under 100% humidity with 1000 ppm of ethylene gas for 24 h, at 15°C under 80% humidity without ethylene for 48 h, and then at 21°C under 50% humidity for a further 48 h for ripening.

Fungus. C. musae (Berk. & Curt.) Arx. strain no. 1679 was obtained from the Department of Scientific and Industrial Research, Mount Albert Research Centre, Auckland, New Zealand, and maintained on a potato-sucrose-agar medium in the dark at 23°C. Suspensions of conidia were prepared from 5- to 9-day-old cultures. Ten ml of sterile water was poured on to the surface of the medium, which was then rubbed with a glass bar. The suspension was passed through filter paper (Wipers S-200, Crecia Corp.) and diluted to 6.0×10^6 conidia/ml with sterile water.

Treatment and incubation. All unripe and ripe fruit were washed with water and wiped with 70% EtOH prior to the experiments. Fruit was wounded by rubbing with sandpaper (G-60) on only one side. Sterile gauze (3 × 15 cm) was attached to the wounded surface of each fruit, and was wet with sterile water (2 ml). Inoculation was accomplished by applying a suspension (2 ml) of conidia of *C. musae* to gauze attached to fruit which had not been wounded. Wounding and inoculation (i.e. inoculation just after wounding) involved the application of a suspension (2 ml) of conidia to gauze attached to the surface of fruit which had been wounded with sandpaper. Sterile water (2 ml) was applied to the gauze attached to the surface of intact fruit as a control. The treated fruit samples were placed in plastic boxes (24 width × 31 depth × 10 cm height) and kept in the dark at 17°C for 6 d. Wet cotton was placed in the boxes to maintain a high level of humidity. The gauze was removed 3 d after the treatment. Every 24 h, one fruit was picked for analysis. Fruit incubated for 4 d was partly used for ripening experiments with ethylene gas.

Application of AOA. Fruit was wounded with sandpaper (G-60), and gauze was attached to the surface. AOA (carboxymethoxylamine hemihydrochloride, Wako Pure Chemical Industries) solutions were adjusted to pH 7 with aqueous NaOH, and diluted to concentrations of 0.01–10 mM. For each fruit, 4, 3 and 3 ml of an AOA solution was applied 0, 12 and 24 h after wounding, respectively.

Extraction and HPLC analysis. The peel of fruit that had been incubated after each treatment was cut into 1 × 1 cm pieces, and samples of 8–12 g were extracted with *ca.* 15 ml of EtOAc for 3 d at room temperature. Aliquots of 5 μl of the EtOAc extracts were analyzed by HPLC, eluting with CH_3CN-TFA-H_2O (30:0.1:70–70:0.1:30; a linear gradient for 25 min). Phenylphenalenones were identified by comparing their t_R values with those of authentic samples by the HPLC anaysis. The t_R values of phenylphenalenones and their derivatives were as follows: **1**, 17.6 min; **2**, 24.4 min; **3**, 15.0 min; **4**, 16.3 min; **5**, 24.3 min; **6**, 9.4 min; **7**, 15.0 min; **8**, 14.0 min; **9**, 21.0 min; and **10**, 21.2 min. The EtOAc extracts were also analyzed with MeOH-AcOH-H_2O (65:0.1:35–80:0.1:20; a linear gradient for 10 min). The t_R values of these compounds in the extracts were as follows in the same order as that just described: 8.0 min, 11.5 min, 5.0 min, 8.2 min, 12.8 min, 4.0 min, 6.9 min, 6.9 min, 7.7 min and 8.2 min, respectively. Calibration curves for the amounts and peak areas of these compounds were constructed by using authentic samples. The amount of each compound was calculated by comparing its peak area on the chromatogram to the calibration curve. The phenylphenalenone content in each extract was quantified three times by the method described, and the average was calculated (standard deviation was ±5%). Experiments were carried out independently at least three times to confirm the reproducibility, and the representative data that were closest to the averages are shown.

Preparation of the crude enzyme solution. Samples (10 g) of fruit peel that had been incubated after wounding were cooled on an ice-chilled aluminum sheet and then grated by using an ice-chilled ceramic grater with 20 ml of a pre-chilled borate-HCl buffer (0.1 M, pH 8.8, containing 30 mM of 2-mercaptoethanol). The material was homogenized in an ice-chilled mortar for 5 min with the addition of quartz sand (1.88 g), Polyclar SB-100 (0.25 g) and another 20 ml of the buffer. The slurry was centrifuged for 10 min at 3,000 × *g*, and the supernatant re-centrifuged for 30 min at 30,000 × *g*. Centrifugation was performed at 4°C. The final supernatant was used as the crude enzyme solution.

Measurement of PAL activity. The measurements were performed by using the method reported by Carver *et al.* with some modifications.[14] The reaction mixture in each test tube contained 200 μl of L-phenylalanine (0.1 M) and 1.3 ml of a borate-HCl buffer (0.1 M, pH 8.8, containing 30 mM of 2-mercaptoethanol), the tubes being preincubated in a water bath at 40°C for 10 min. As a reference, D-phenylalanine was used as the substrate for the reaction. The reaction was started by adding 500 μl of the crude enzyme solution, and the tubes were incubated at 40°C for 4 h without shaking. After the incubation, 80-μl aliquots of the mixture were diluted to 2.0 ml with 0.05 N NaOH, and the absorbance at 268 nm was immediately measured. The concentration of cinnamic acid ($\varepsilon = 19{,}000$) was calculated after subtracting the absorbance of the reference from that of the reaction mixture containing L-phenylalanine. The PAL activity of each enzyme solution was measured three times by the method described, and the average was calculated (standard deviation was ±5%). All experiments were carried out independently three times to confirm the reproducibility, and the representative data that was closest to the average is shown.

Synthesis of [1-^{13}C] and [2-^{13}C]cinnamic acids. To a solution of [1,3-$^{13}C_2$]malonic acid (Isotec, 99.0 atom%, 300 mg, 2.83 mmol) in pyridine (0.3 ml) was added benzaldehyde (0.30 ml, 2.9 mmol). The reaction mixture was stirred at 100°C for 30 min, brought to 15 ml with water, and then adjusted to pH 10 with 1 N NaOH. The solution was extracted with EtOAc (5 ml × 3), and the aqueous layer was acidified with 1 N HCl to pH 2 before being extracted again with EtOAc (5 ml × 3). The acidic organic layer was washed with water (5 ml × 3), dried (Na_2SO_4), filtered and concentrated to dryness. The residue (276 mg) was dissolved in hot water (40 ml) and allowed to stand overnight at room temperature. The resulting crystals were desiccated to yield [1-^{13}C]cinnamic acid (99.0 atom%, 200 mg, 47.4% yield) as colorless needles, mp 131-133°C. HREIMS *m/z* (M^+): calcd. for $^{12}C_8{}^{13}C_1H_8O_2$, 149.0558; found, 149.0496. EIMS *m/z* (%): 149 (M^+, 100), 148 ($M\text{-}H^+$, 87), 132 ($M\text{-}OH^+$, 16), 103 ($M\text{-}^{13}CO_2H^+$, 33). NMR δ_H ($CDCl_3$): 6.46 (1H, dd, $J = 16.0$ Hz and 2.7 Hz, 2-*H*), 7.40–7.56 (5H, m, 2′-6′-*H*), 7.80 (1H, dd, $J = 16.0$ Hz and 6.9 Hz, 3-*H*). NMR δ_C ($CDCl_3$): 117.4 (d, $J = 73.9$ Hz, C-2), 128.4 (C-2′ and 6′), 129.0 (C-3′ and 5′), 130.8 (C-4′), 134.1 (d, $J = 7.3$ Hz, C-1′), 147.1 (C-3), 172.6 (C-1). [2-^{13}C]Cinnamic acid was synthesized by a similar method to that used for [1-^{13}C]cinnamic acid, except that [2-^{13}C]malonic acid (Isotec, 99.0 atom%) was used instead of [1,3-$^{13}C_2$]malonic acid. Colorless needles resulted, mp 131–133°C. HREIMS m/z (M^+): calcd. for $^{12}C_8{}^{13}C_1H_8O_2$, 149.0558; found, 149.0551. EIMS m/z (%): 149 (M^+, 96), 148 (M-H^+, 100), 132 (M-OH^+, 23), 104 (M-CO_2H^+, 39). NMR δ_H ($CDCl_3$): 6.45 (1H, dd, J=162.8 Hz and 16.0 Hz, 2-*H*), 7.40–7.56 (5H, m, 2′-6′-*H*), 7.80 (1H, dd, J= 16.0 Hz and 3.0 Hz, 2-*H*). NMR δ_C ($CDCl_3$): 117.4 (C-2), 128.4 (d, J=5.0 Hz, C-2′ and 6′), 129.0 (C-3′ and 5′), 130.8 (C-4′), 134.1 (C-1′), 147.1 (d, J=70.9 Hz, C-3), 172.6 (d, J=73.8 Hz, C-1).

Administration of [1-^{13}C] and [2-^{13}C]cinnamic acids, [2-^{13}C]acetate, and [2-^{13}C]malonate. Ninety-four ripe fruit were wounded and wrapped in gauze (15 × 15 cm), and an H_2O-EtOH (97:3) solution (4 ml) of [1-^{13}C]cinnamic acid (0.3 mM) was applied to each fruit. The solution (3 ml) was applied again to each fruit after 12 and 24 h, before incubating for 5 d at 17°C in the dark. Sixty-four ripe fruit were used for the administration of [2-^{13}C]cinnamic acid (0.3 mM), sodium [2-^{13}C]acetate (99.0 atom%, 0.3 mM), and sodium [2-^{13}C]malonate (99.0 atom%, 0.3 mM), respectively, in the same manner as that already described.

Isolation of hydroxyanigorufone (1). The peel (3.0 kg) of wounded fruit that had been treated with [1-^{13}C]cinnamic acid was cut into pieces and soaked in EtOAc (2 l) at room temperature for 3 d. The EtOAc layer was washed with water (300 ml × 3), dried (Na_2SO_4), and concentrated to dryness, yielding a crude extract (4.0 g). An HPLC analysis showed that 5.1 mg of **1** was contained in this extract. The extract was then applied to a silica gel (150 g) column and eluted with toluene-EtOAc. The material eluted with 20% EtOAc was next subjected to chromatography on a silica gel TLC plate (G 1500/LS 254, Schleicher & Schuell), using *n*-hexane-$CHCl_3$-MeOH (10:10:1). The material with R_f 0.22 was extracted with acetone (30 ml) and concentrated to give an orange powder (3.0 mg). This powder was further purified by preparative HPLC, eluting with MeOH-H_2O (70:30). Collection of the material at t_R 7.0 min yielded **1** (1.3 mg). EtOAc extracts of the peel (2.8 kg) that had been treated with [2-^{13}C]cinnamic acid, [2-^{13}C]acetate and [2-^{13}C]malonate contained 1.9 mg, 1.3 mg and 1.3 mg of **1**, respectively. The purification procedure just described gave 1.6 mg, 1.0 mg, and 1.0 mg of **1**, respectively.

Administration of [1-^{13}C]cinnamic acid and isolation of 2-(4′-hydroxyphenyl)-1,8-naphthalic anhydride (3). In a manner similar to that just described, [1-^{13}C]cinnamic acid was administered to the peel (1.8 kg) of wounded and inoculated fruit. After 4 d of incubation, the peel yielded 2.3 g of an EtOAc extract containing 3.3 mg of **3**. This crude extract was applied to a silica gel (75 g) column eluted with toluene-EtOAc. The material eluted with 20% EtOAc was chromatographed on a silica gel TLC plate (G 1500/LS 254, Schleicher & Schuell), using *n*-hexane-EtOAc-MeOH (15:5:1). The material with R_f 0.35 was extracted with acetone, and the resulting solution was concentrated to yield a yellow powder (3.7 mg). This material was purified again by preparative HPLC with CH_3CN-H_2O (50:50). Collection of the material at t_R 7.0 min yielded **3** (2.5 mg).

Synthesis of [2,3,5,6-$^2H_4$]-4-bromoanisole. To a stirred solution of phenol-d_6 (Isotec, 99% atom, 2.5 g, 25 mmol) in dioxane (25 ml) was slowly added Br_2 (4.0 mg, 25 mmol) that had been previously dissolved in 25 ml of dioxane at room temperature. The solution was then made up to 80 ml with water and extracted with EtOAc (20 ml × 3). The organic layer was washed with water (20 ml × 3), dried on sodium sulfate, and concentrated to dryness. The residue was dissolved in an aqueous solution of NaOH (1.0 g, 25 mmol), evaporated, and then dissolved in THF (30 ml). To this stirred solution was added CH_3I (3.6 g, 25 mmol), and the solution was allowed to stand at room temperature for 24 h. The volume was brought to 50 ml with water, and the mixture was extracted with EtOAc (15 ml × 3). The organic layer was washed with H_2O (15 ml × 3), dried (Na_2SO_4), and concentrated to dryness. The residual oil (4.6 g) was purified by column chromatography on silica gel (100 g) with *n*-hexane-EtOAc to give [2,3,5,6-$^2H_4$]-4-bromoanisole (3.8 g, 79% yield) as a colorless oil. HREIMS m/z (M^+): calcd. for $C_7{}^1H_3{}^2H_4OBr$, 189.9927; found, 189.9891. EIMS m/z (%): 192 ($M+2^+$, 99), 190 (M^+, 100), 177 (36), 175 (40), 149 (30), 147 (30). NMR δ_H ($CDCl_3$): 3.77 (3H, s, O*Me*). NMR δ_C ($CDCl_3$): 55.4 (C-O*Me*), 112.6 (C-4), 115.4 (t, J=24.6 Hz, C-2 and 6), 131.8 (t, J=25.3 Hz, C-3 and 5), 158.7 (C-1).

Synthesis of [2′,3′,5′,6′-$^2H_4$]-1 and [2′,3′,5′,6′-$^2H_4$]-4. These compounds were synthesized from perinaphthenone (Aldrich) and [2,3,5,6-$^2H_4$]-4-bromoanisole according to the method reported by Luis *et al.*[4,15)] [2′,3′,5′,6′-$^2H_4$]-**1**: An orange powder. HREIMS m/z (M^+): calcd. for $C_{19}{}^1H_8{}^2H_4O_3$, 292.1033; found, 292.0950. EIMS m/z (%) 292 (M^+, 44), 291 (M-H^+, 34), 290 (M-$^2H^+$, 100), 275 (26). NMR δ_H (acetone-d_6): 7.15 (1H, s, 3-*H*), 7.62 (1H, d, J=8.3 Hz, 8-*H*), 7.65 (1H, dd, J=8.2 Hz and 7.1 Hz, 5-*H*), 7.79 (1H, brs, 2-O*H*), 7.84 (1H, dd, J= 7.1 Hz and 0.6 Hz, 4-*H*), 8.04 (1H, dd, J=8.2 Hz and 0.6 Hz, 6-*H*), 8.35 (1H, d, J=8.3 Hz, 7-*H*), 8.45 (1H, brs, 4′-O*H*). NMR δ_C (acetone-d_6): 113.2 (C-3), 125.0 (C-9a), 126.4 (C-9b), 128.1 (C-5), 130.3 (C-3a), 130.6 (C-6), 131.4 (C-4), 132.8 (C-6a), 133.0 (C-8),

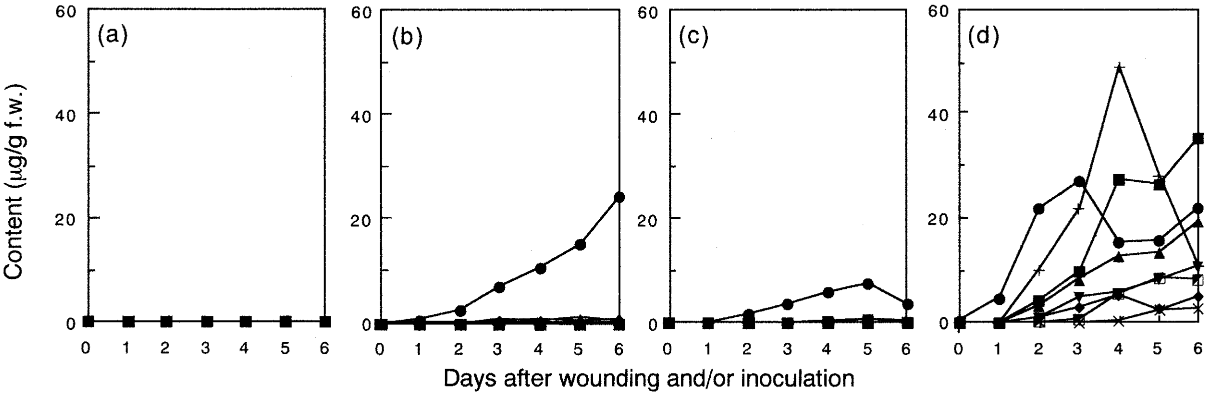

Fig. 2. Changes in the Contents of Phenylphenalenones (**1**, ●; **2**, ◆; **3**, ▼; **4**, ■; **5**, ×; **6**, +; **7**, ▲; **8**, ▨) in Wounded and/or Inoculated Unripe Fruit.

(a) Control (b) Wounded (c) Inoculated (d) Wounded and inoculated

134.6 (C-1′), 136.6 (C-7), 150.1 (C-9), 151.8 (C-2), 158.4 (C-4′), 181.1 (C-1). [2′,3′,5′,6′-$^{2}H_4$]-**4**: A red powder. HREIMS *m* / *z* (M^+): calcd. for $C_{19}{}^{1}H_8{}^{2}H_4O_3$, 292.1033; found, 292.0900. EIMS *m*/*z* (%): 292 (M^+, 100), 291 ($M-H^+$, 22), 275 (25), 273 (38), 245 (23). NMR δ_H (acetone-d_6): 7.22 (1H, s, 3-*H*), 7.62 (1H, d, *J* = 8.5 Hz, 5-*H*), 7.88 (1H, dd, *J* = 8.0 Hz and 7.5 Hz, 8-*H*), 8.07 (1H, d, *J* = 8.5 Hz, 6-*H*), 8.09 (1H, s, 2-O*H*), 8.42 (1H, dd, *J* = 8.0 Hz and 1.0 Hz, 7-*H*), 8.69 (1H, dd, *J* = 7.5 Hz and 1.0 Hz, 9-*H*), 8.71 (1H, brs, 4′-O*H*). NMR δ_C (acetone-d_6): 113.4 (C-3), 126.1 (C-9b), 126.2 (C-3a), 127.8 (C-8), 129.5 (C-9a), 130.6 (C-6), 131.3 (C-5), 131.9 (C-9), 132.0 (C-1′), 132.8 (C-6a), 137.4 (C-7), 144.6 (C-4), 151.4 (C-2), 159.0 (C-4′), 180.7 (C-1).

Administration of [2′,3′,5′,6′-$^{2}H_4$]-1 and [2′,3′,5′,6′-$^{2}H_4$]-4. Ripe fruit were wounded and inoculated, wrapped with gauze (15 × 15 cm), and incubated for 4 d at 17°C in the dark. H_2O-EtOH (97:3) solutions (3 ml) of [2′,3′,5′,6′-$^{2}H_4$]-**1** and [2′,3′,5′,6′-$^{2}H_4$]-**4** (100 μM) were applied to 40 fruit each 36 h after the treatment. The fruit peel (1.3 kg) was soaked in EtOAc (1 l) and allowed to stand for 3 d at room temperature. Each resulting extract was washed with water (300 ml × 3), dried (Na_2SO_4), and concentrated to give a residue (2 g, respectively). The residue from fruit treated with [2′,3′,5′,6′-$^{2}H_4$]-**1** contained 7.0 mg of **3**, giving 1.4 mg of **3** after purification. The residue from fruit treated with [2′,3′,5′,6′-$^{2}H_4$]-**4** contained 10.2 mg of **3**, giving 2.5 mg of **3** by purification. The methods for purification and the HPLC analysis of **3** were the same as those previously described. The mass spectrum of each sample was measured three times, the relative intensity of the $[M+4]^+$ ion being expressed as the average (± standard deviation).

Results and Discussion

Changes in the phenylphenalenone content of unripe fruit

Figure 2 shows changes in the phenylphenalenone content of unripe fruit which had been wounded and /or inoculated with conidia of *C. musae*. No phenylphenalenones were detected in untreated fruit over the time period examined (Fig. 2a). Wounding induced an accumulation of **1** which was detected 1 d after wounding and continued to increase, reaching 22.7 μg/g f.w. after 6 d of incubation (Fig. 2b). Inoculation also induced the formation of **1**, although to a lower level than that observed in the wounded fruit (Fig. 2c). When fruit were inoculated just after wounding, the formation of compounds **2**–**8** was induced in addition to that of **1** (Fig. 2d). Compounds **9** and **10** were also produced in wounded and inoculated fruit, but their contents remained less than 1.0 μg/g f.w. during 6 d of incubation (data not shown). The level of compound **6** decreased rapidly after reaching the maximum of 49.0 μg/g f.w. 4 d after wounding and inoculation, whereas that of compound **4** continued to increase, reaching 35.4 μg/g f.w. after 6 d of incubation.

The induction of the formation of phenylphenalenones in wounded and/or inoculated fruit, and the lack of similar induction in healthy fruit, confirmed that the phenylphenalenones were phytoalexins in banana fruit. Compound **1** was the major phenylphenalenone that was induced more in wounded fruit than in inoculated fruit. This suggests that it could be a stress metabolite rather than a phytoalexin. Inoculation just after wounding would have more severe consequences for the tissues than either inoculation or wounding alone, and correspondingly provoked a greater response from the peel. This change might be advantageous for the fruit, since **3** and **4** showed 10- and 3-fold higher antifungal activities, respectively, than **1**.[3] 4-Phenylphenalenones are thought to be

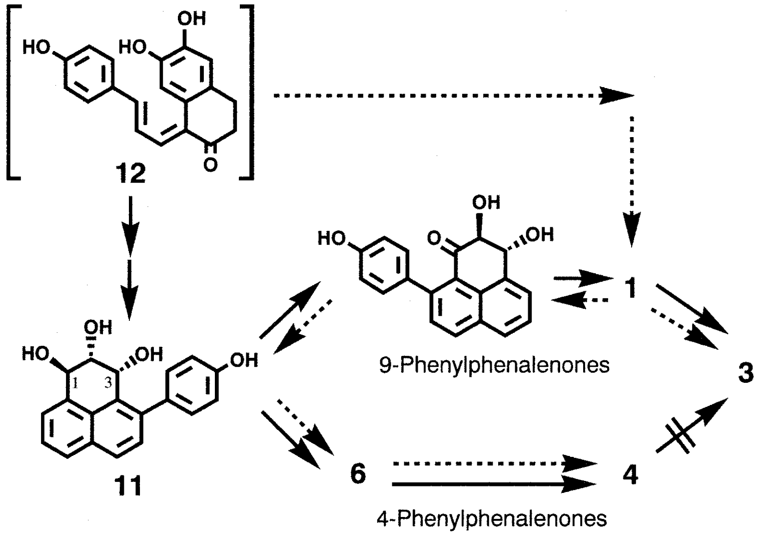

Fig. 3. Putative Biosynthetic Pathway for 4- and 9-Phenylphenalenones.
The pathway proposed by Luis *et al.* is shown by solid arrows, and an alternative pathway is indicated by dashed arrows.

biosynthesized by oxidation at C-1 of a putative intermediate (**11**), whereas 9-phenylphenalenones are generated by oxidation at C-3 of **11** (Fig. 3).[15] An alternative pathway, in which 9-phenylphenalenones such as **1** are directly biosynthesized from a diarylheptanoid intermediate (**12**), is also possible. The changes in levels of **4** and **6** are consistent with the presumption that 4-phenylphenalenones are biosynthesized from the hydrates *via* dehydration.[3,15]

Effect of ripening on the phenylphenalenone content

Upon exposure to ethylene gas for ripening 4 d after wounding, the fruit were incubated for a further 8 d. At the end of this incubation period, the color of the ethylene-treated fruit had fully changed to yellow, whereas the unripened fruit remained green. Figure 4 shows the change in content of **1** in banana fruit after the ethylene treatment. Other phenylphenalenones, which were detected at levels lower than 1.0 μg/g f.w., are not shown. The content of **1** in the ethylene-treated fruit reached a transient maximum of 31.7 μg/g f.w. after 6 d, and decreased rapidly to 12.6 μg/g f.w. 8 d after wounding, while that in the untreated fruit continued to increase, reaching 49.5 μg/g f.w. 12 d after wounding. The level of **1** in ethylene-treated fruit 5–6 d after wounding was higher than that in the untreated fruit. These results show that ethylene treatment of the wounded tissues accelerated the accumulation of **1**, and that the level decreased quickly as the fruit ripened. The level of **1** in ripened fruit peel after 12 d of incubation was 22% of that in unripe fruit peel. This could explain why banana fruit loses its antifungal activity during ripening. The phytoalexins in grape berry, *Vitis vinifera* and *V. labrusca*, and in red pepper, *Capsicum annuum*, also decreased with ripening.[16,17]

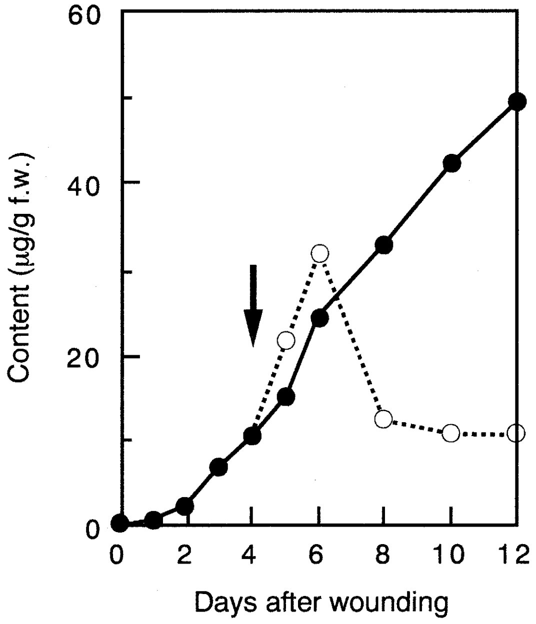

Fig. 4. Change in the Content of **1** in Unripe Fruit.
Fruit were treated or not with ethylene 4 d after wounding (ethylene-treated, dashed line, ○; untreated, solid line, ●). The arrow indicates the point of ethylene treatment.

The exposure of intact unripe fruit to ethylene gas did not induce the production of phenylphenalenones (data not shown), confirming that ethylene gas alone could not elicit phenylphenalenone biosynthesis in banana fruit.

Ripe fruit were treated by the same method as that used for unripe fruit to compare their phenylphenalenone-producing abilities. In wounded fruit (Fig. 5a), the phenylphenalenone content was low and the maximum content of **1** was less than 1.0 μg/g f.w., indicating that ripe fruit did not retain the ability to produce phenylphenalenones. In inoculated and

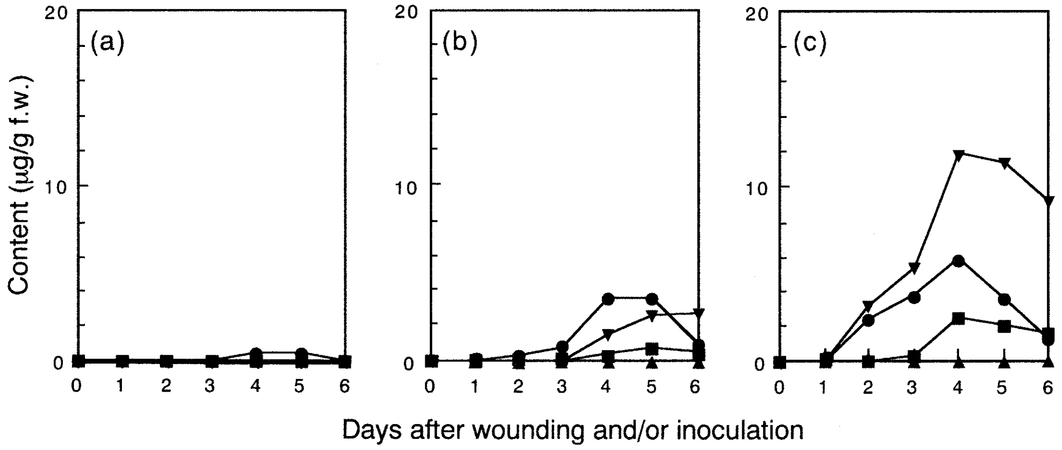

Fig. 5. Changes in the Contents of Phenylphenalenones (**1**, ●; **3**, ▼; **4**, ■; **7**, ▲) in Wounded and/or Inoculated Ripened Fruit.
(a) Wounded (b) Inoculated (c) Wounded and inoculated

in wounded and inoculated fruit (Figs. 5b and 5c, respectively), the contents of phenylphenalenones **1**, **2** and **4**–**8** were lower than those of unripe fruit, but the content of **3** increased to 11.9 μg/g f.w. 4 d after treatment in the wounded and inoculated fruit, equivalent to that present in the unripe fruit. This result indicates that ripe fruit retained the ability to biosynthesize **3**, probably by decarboxylation of **1** and/or **4**, although the biosynthesis of phenylphenalenones was reduced in ripe fruit. The formation of **3** from **1** and **4** will be described later.

Effects of AOA on the phenylphenalenone content

Phenylalanine is converted to cinnamic acid by phenylalanine ammonia-lyase (PAL) in plants.[18] PAL would be involved in phenylphenalenone biosynthesis, whether one or two phenylpropanoid(s) are used for the formation of each molecule of phenylphenalenone. To confirm this, 2-aminooxyacetic acid (AOA), an inhibitor of PAL,[19] was applied to wounded fruit.

The contents of phenylphenalenones **1**, **3** and **7** in unripe fruit 5 d after wounding and following the application of AOA are shown in Fig. 6. No other phenylphenalenones were detected in this experiment due to the low levels present. The content of **1** decreased to 12.1 μg/g f.w. in fruit treated with 0.1 mM AOA, while that in control fruit was 27.6 μg/g f.w., indicating that AOA application suppressed the accumulation of **1**. This result suggests that cinnamic acid, which is formed from phenylalanine by PAL, was the precursor of **1**. When AOA solutions at a concentration higher than 1.0 mM were applied, compounds **3** and **7**, which were hardly present in wounded fruit, could be detected. The application of a high concentration of AOA might not have inhibited, but rather increased phenylphenalenone biosynthesis in banana fruit. AOA solutions were applied to intact unripe fruit and, after incubation, the peel extracts were analyzed. Although no phenylphenalenones could be deteded in the peel by application of 0.01–0.1 mM AOA, compounds **1**, **3** and **7** showed a slight accumulation after the application of 1 mM AOA, and 6.3 μg/g f.w. of **1**, 6.2 μg/g f.w. of **3**, and 1.2 μg/g f.w. of **7** were detected in the extract of fruit treated with 10 mM AOA. The application of 1 and 10 mM AOA changed the color of the fruit peel to pale green, whereas no visible change was apparent in fruit treated with AOA at a concentration less than 0.1 mM. AOA application at a high concentration might have caused serious physiological damage to the fruit peel, resulting in an abnormal increase in phenylphenalenone biosynthesis.

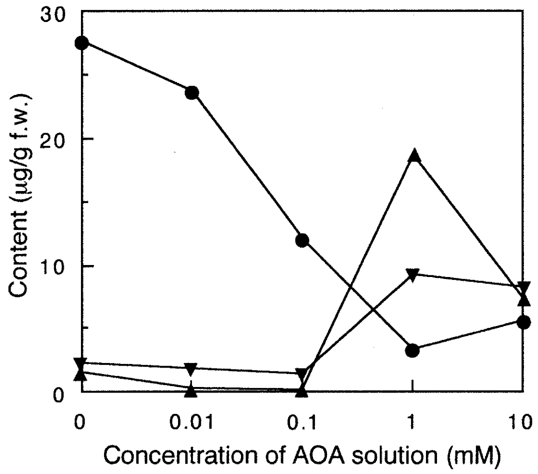

Fig. 6. Effect of AOA on Phenylphenalenone Contents.
The contents of phenylphenalenones (**1**, ●; **3**, ▼; **7**, ▲) in unripe fruit which had been wounded and then treated with an AOA solution (10^{-2} mM – 10 mM) are shown.

Change in PAL activity

Figure 7 shows the PAL activity of crude enzyme preparations obtained from fruit peel after wound-

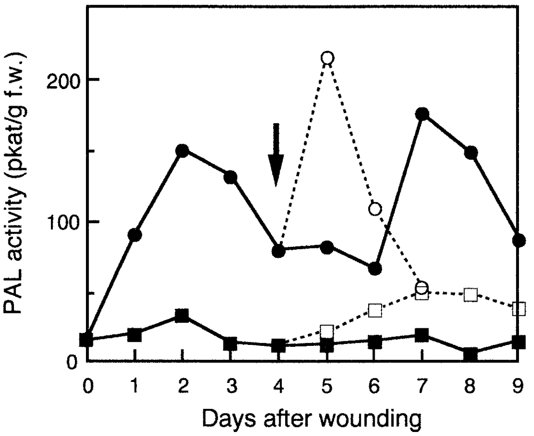

Fig. 7. Changes in PAL Activities.

The PAL activities of ethylene-treated fruit are shown by the dashed line (wounded, ○; control, □); untreated by the solid line (wounded, ●; control, ■). The arrow indicates the point of ethylene treatment.

ing. The PAL activity of wounded fruit increased rapidly to reach a maximum of 151 pkat/g f.w. 2 d after wounding, while no change was apparent in the control fruit, whose PAL activity remained at a low level during the time period examined. PAL activity increased prior to the accelerated accumulation of **1** in the wounded peel (Fig. 2b), this being consistent with the assumption that phenylphenalenones would be formed *via* a phenylpropanoid pathway in banana fruit.

The change in PAL activity of wounded fruit upon exposure to ethylene gas was correlated with the change in phenylphenalenone content (Fig. 4). PAL activity rapidly increased to 217 pkat/g f.w. 24 h after exposure to ethylene, while that of unripened fruit hardly changed, remaining at 80–83 pkat/g f.w. A similar change was apparent in the control fruit, and the PAL activity of the ethylene-treated fruit increased to 53 pkat/g f.w. 7 d after wounding, a level 3-fold higher than that in untreated intact fruit. These results show that the PAL activity in banana fruit peel was increased by the ethylene treatment, as has been previously reported for other fruits.[20–22] The PAL activity of wounded fruit which had not been treated by ethylene increased to 177 pkat/g f.w. 7 d after wounding. This change might have been due to the level of ethylene produced upon maturation that was accelerated by the ethylene produced by the wounding treatment, since wounding generally triggers ethylene production,[23,24] and the ethylene produced autocatalycally enhanced its biosynthesis.[25] These findings suggest that the biosynthetic precursors of the phenylphenalenones were phenylpropanoids, but that the PAL activity increased for many secondary metabolic pathways when plant tissues were wounded.[18] Further investigations are required to determine the incorporation of C_6-C_3 unit(s) into phenylphenalenones.

Biosynthetic precursors of 1 and 3

To establish the possible precursor(s) of phenylphenalenones in banana fruit, [1-^{13}C]cinnamic acid was administered to wounded unripe fruit, but little incorporation of the label into **1** was apparent by ^{13}C-NMR spectroscopy (data not shown). Unripe fruit produced higher levels of phenylphenalenones than ripe fruit, and thus, even if exogenously applied cinnamic acid was partly incorporated into phenylphenalenones, it could be diluted with endogenously biosynthesized cinnamic acid to a greater extent in unripe than in ripe fruit. The relative ratio of labeled phenylphenalenones was expected to increase when the labeled precursors were administrated to ripe fruit, if the amount incorporated into the phenylphenalenones was constant. Thus, ripe fruit were used, and **1** was isolated from the peel after the administration of [1-^{13}C]cinnamic acid. The signal intensities at δ 130.6 (C-6) and 136.6 (C-7) ppm in its ^{13}C-NMR spectrum were enhanced by 61% and 57%, respectively, in comparison with those of natural **1** (Fig. 8a). The percentage of ^{13}C-labeled molecules derived from [1-^{13}C]cinnamic acid in isolated **1** was 0.60%, and the percentage incorporation of the labeled precursor into isolated **1** was 0.074%. [2-^{13}C]Cinnamic acid was also administered to wounded fruit to confirm the incorporation of two molecules of cinnamic acid into **1**. The ^{13}C-NMR spectrum of **1** isolated from fruit treated with [2-^{13}C]cinnamic acid showed signal enhancement of C-5 at δ 128.1 and of C-8 at δ 133.0 ppm by 71% and 88%, respectively (data not shown, 0.80% of ^{13}C-labeled molecules derived from [2-^{13}C]cinnamic acid in isolated **1** and 0.054% incorporation of the labeled precursor into isolated **1**), indicating that two molecules of cinnamic acid had obviously been incorporated into the molecule of **1**. The origin of C-6a was examined by the administration of [2-^{13}C]acetate and [2-^{13}C]malonate to ripe fruit. In the ^{13}C-NMR spectra of **1** isolated from fruit treated with [2-^{13}C]malonate, a 69% enhancement of the signal was observed at δ 132.8 (C-6a) ppm (Fig. 8b, 0.70% of ^{13}C-labeled molecules derived from [2-^{13}C]malonate in isolated **1** and 0.016% incorporation of the labeled precursor into isolated **1**), while no incorporation of the label was apparent into **1** isolated from fruit treated with [2-^{13}C]acetate (data not shown). These results show that **1** was biosynthesized from two C_6-C_3 units and one malonate in banana fruit.

To examine whether **3** was also biosynthesized by a phenylpropanoid pathway, **3** was isolated from fruit that had been treated with [1-^{13}C]cinnamic acid. Its ^{13}C-NMR spectrum showed 56% and 62% enhancement of the signals at δ 135.5 (C-4) and 136.7 (C-5) ppm, respectively (Fig. 9a, 0.61% of ^{13}C-labeled molecules derived from [1-^{13}C]cinnamic acid in iso-

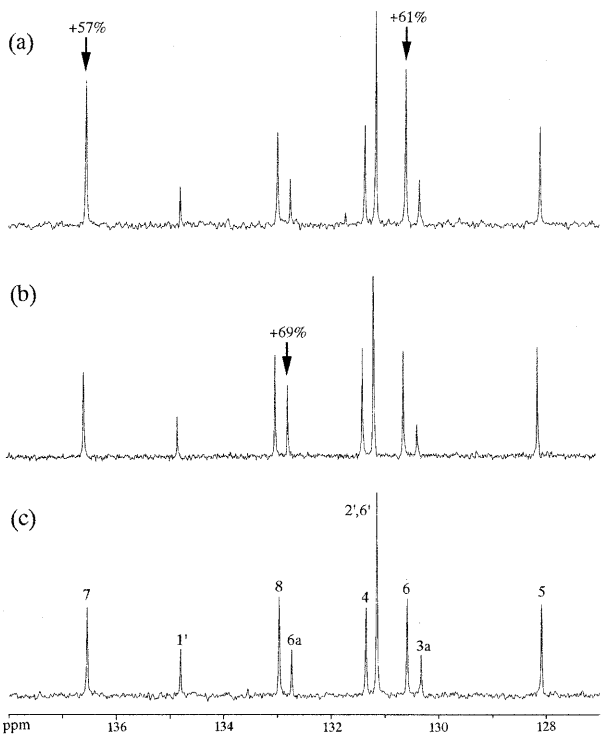

Fig. 8. ^{13}C-NMR Spectra of **1** (Acetone-d_6, 125 MHz).
(a) Isolated from fruit treated with [1-^{13}C]cinnamic acid. (b) Isolated from fruit treated with [2-^{13}C]malonate. (c) Natural abundance.

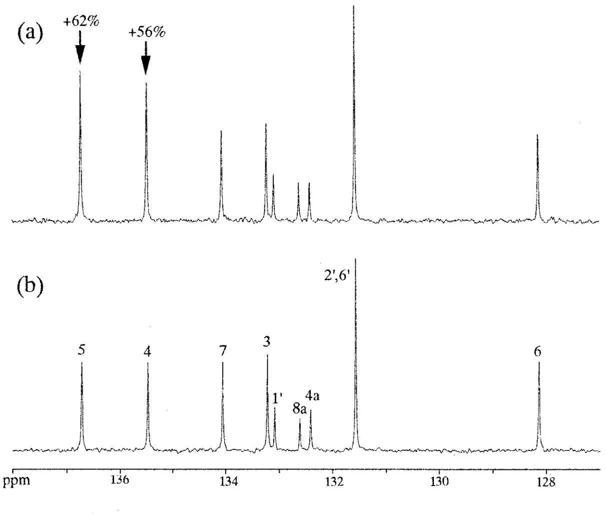

Fig. 9. ^{13}C-NMR Spectra of **3** (Acetone-d_6, 125 MHz).
(a) Isolated from fruit treated with [1-^{13}C]cinnamic acid. (b) Natural abundance.

lated **3** and 0.078% incorporation of the labeled precursor into isolated **3**). This result indicates that **3** was also biosynthesized from two C_6-C_3 units, suggesting that **3** was formed from **1** and/or **4**. Labeled compounds [2′,3′,5′,6′-$^2H_4$]-**1** and [2′,3′,5′,6′-$^2H_4$]-**4** were synthesized and administered to ripe fruit after wounding and inoculation.[4,15] Compound **3** isolated from fruit treated with [2′,3′,5′,6′-$^2H_4$]-**1** showed a relative intensity of the $[M+4]^+$ ion in the mass spectrum of 0.44% (±0.012), whereas the relative intensity of the $[M+4]^+$ ion of natural **3** was 0.08% (±0.014). The percentage of $^2H_4$-labeled molecules derived from [2′,3′,5′,6′-$^2H_4$]-**1** in isolated **3** was 0.36%, and the percentage incorporation of the labeled precursor into isolated **3** was 0.70%. This result indicates that **1** had been incorporated into the peel and converted to **3** (see Fig. 3). On the other hand, the mass spectrum of **3** isolated from fruit treated with [2′,3′,5′,6′-$^2H_4$]-**4** showed little enhancement of the $[M+4]^+$ ion, the relative intensity of which was 0.12% (±0.009), suggesting that **1** had been metabolized to **3** to a greater extent than **4** was. Compound **3** could have been formed from **1** through decarboxylation catalyzed by an unidentified decarboxylase, and the putative enzyme decarboxylating 2-C of phenylphenalenones seemed to prefer 9-phenylphenalenones to 4-phenylphenalenones as substrates. The involvement of decarboxylase in the biosynthesis of 2-phenyl-1,8-naphthalic anhydrides from 9-phenylphenalenones has also been suggested in Haemodoraceae plants.[7]

These results show that phenylphenalenone biosynthesis in banana is almost the same as that in Haemodoraceae plants, supporting the close relationship between Haemodoraceae and Musaceae as suggested by an analysis of the *rbc*L gene,[12] and providing chemotaxonomic evidence for this new classification. Phenylphenalene metabolites occurring in one of the Pontederiaceae plants in Pontederiales, *Eichhornia crassipes*,[26,27] could be expected to be biosynthesized *via* the same pathway as that in these plants, since Pontederiales is close to Zingiberales and Haemodorales in its botanical classification.[12]

Several researchers have proposed that phenylphenalenones are biosynthesized *via* diarylheptanoid.[6,7,9] Cyclic diarylheptanoids have been isolated from *Musa* rhizomes and fruit, and are regarded as intermediates for phenylphenalenones.[15,28] Curcumin, therefore, also seems to be biosynthesized from two molecules of phenylpropanoid and one molecule of acetate or malonate. The oxygenation pattern of curcumin could not be explained by the formation of common polyketide compounds. In addition, a diarylheptanoid in *Acer nikoense*, acerogenin A, has been shown to be formed from two molecules of phenylpropanoid and one molecule of malonate.[29] The biosynthetic precursor of curcumin should thus be re-examined.

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