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Conversion of [19-$^2H_2$]fusicocca-2,10(14)-diene into its 8β-ol and fusicoccins by *Phomopsis* (*Fusicoccum*) *amygdali*

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Abstract

Feeding experiments of (+)-[19-$^2H_2$]fusicocca-2,10(14)-diene, a deuterated derivative of a new candidate for the initially-forming fusicoccane-hydrocarbon in the fusicoccin biosynthesis, with the fusicoccin-producing fungus *Phomopsis* (*Fusicoccum*) *amygdali* have revealed its incorporation into fusicoccins J and A through (+)-fusicocca-2,10(14)-dien-8β-ol, whose occurrence in the mycelial extract has been confirmed by direct comparison of its ^1H NMR and GC–MS data with those of the synthetic sample, proving the early biosynthetic route leading to the aglycons of fusicoccins. © 2000 Elsevier Science Ltd. All rights reserved.

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Fusicoccins A (**1**)[1,2] and J (**2**)[3,4] are the main metabolites produced by the plant pathogenic fungus *Phomopsis* (*Fusicoccum*) *amygdali* (Del.) Tuset and Portilla (Fig. 1).[5] They and the structurally-related cotylenins[6–8] [cotylenin A (**3**) is shown as a representative], isolated from *Cladosporium* sp. 501-7W, display a number of phytohormone-like effects for all higher plants via the activation of the plasma membrane H^+-ATPase.[9] Currently, their action is attracting much attention as the fusicoccin-binding 14-3-3 proteins are regarded to be the key proteins in intracellular signal transductions in both plant[10] and animal cells.[11] It has been an accepted belief since the 1970s that fusicocca-1,10(14)-diene (**4**) is an intermediary hydrocarbon at an early stage of the biosynthesis of fusicoccins.[12,13] However, recently, we have isolated and synthetically determined (+)-fusicocca-2,10(14)-diene (**5**), a double bond isomer of **4**, as the main hydrocarbon constituent from the mycelial extract of *P. amygdali*.[14] Furthermore, the intermediacy of **5** in the fusicoccin biosynthesis is strongly supported by the transformation of fusicocca-2,10(14)-dien-8β-ol (**6**), provided synthetically as a deuterium-labeled derivative, into **2** by the fungus.[15] Since this bio-transformation from **6** to **2** proceeds very efficiently, we suspected that the conversion of **5** into **6** is the real process in vivo as the first hydroxylation step leading to fusicoccins. Here, as direct

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proof of this postulation, we report the isolation of **6** from the mycelial extract of *P. amygdali* and the transformation of **5** by this fungus into **2** and **1** via **6** using synthetic deuterium-labeled **5-*d*$_2$** as a feeding substrate.

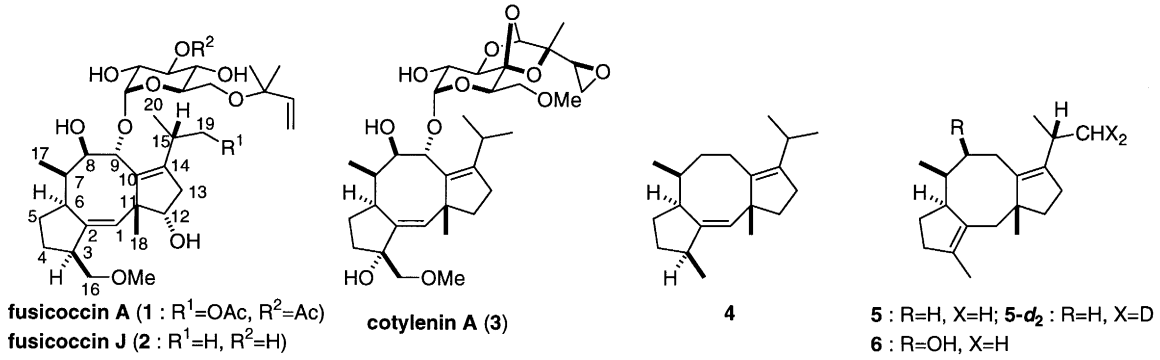
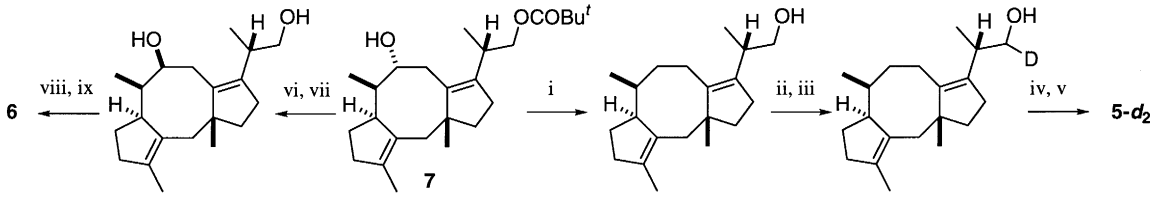


Fig. 1.

The feeding substrate, [19-^{2}H$_2$]fusicocca-2,10(14)-diene (**5-*d*$_2$**), and an authentic sample of fusicocca-2,10(14)-dien-8β-ol (**6**) were synthesized from the previously reported fusicocca-2,10(14)-diene derivative **7**[14] (Scheme 1); **5-*d*$_2$** [a colorless oil, *m/z* (%) 274 ($C_{20}H_{30}D_2$, M^+, 14), 273 (M^+-1, 5.8), and 135 (100); carbon signals distinguishable from those of non-deuterated **5**[14] are δ_C (100 MHz, $CDCl_3$) 20.40 (quintet, deuterated C-19, $\Delta\delta$ −0.58), 21.21 (C-20, $\Delta\delta$ −0.03), and 26.89 (C-15, $\Delta\delta$ −0.15)] and **6** [a colorless oil, $[\alpha]_D^{20}$ +75 (*c* 0.048, $CHCl_3$); *m/z* (%) 288 (M^+, 7.5), 245 (10), 227 (12), 151 (52), 122 (46), and 95 (100) by GC–MS (t_R 10.779–10.818 min);† ^{1}H NMR is broadened at ambient temperature because of the conformational mobility, but there are several diagnostic signals at δ_H (400 MHz, $CDCl_3$) 0.91 (3H, s, H-18), 0.92 (3H, d, *J* 6.8, H-19), 1.00 (3H, br d, *J* 6.8, H-20), 1.62 (3H, br s, H-16), 2.35 (dm, *J* 10.8, H-9), and 3.82 (dm, *J* 12.0, H-8)].



Scheme 1. Reagents and conditions. (i) Ref. 14; (ii) $(ClCO)_2$, DMSO then Et_3N, CH_2Cl_2; (iii) $LiAlD_4$, THF (2 steps, 80%); (iv) MsCl, Py; (v) $LiEt_3BD$, THF (two steps, 88%); (vi) same as (ii); (vii) excess DIBAL-H, toluene (two steps, 79%); (viii) TsCl, Py; (ix) $LiEt_3BH$, THF (two steps, 71%)

At first, the natural occurrence of **6** in mycelia of *P. amygdali* F6[14] was investigated in detail. A pale yellow hexane extract (ca. 4.0 g) of mycelia obtained from 200 culture flasks, each containing 100 cm^3 of the culture medium (8.0% commercial sugar, 1.0% corn steep liquor, and 0.5% peptone in deionized water),‡ was carefully separated by silica gel flash chromatography. From the fraction (1.63 g) eluted with a mixture of hexane:EtOAc (10:1), a colorless oil {0.36 mg, $[\alpha]_D^{20}$ +50 (*c* 0.033, $CHCl_3$)}, of which the R_f value is consistent with that of authentic **6**, was obtained by repeated chromatography on silica gel using mixtures of $CHCl_3$:EtOH (40:1), hexane:acetone (50:1), and $CHCl_3$:EtOAc (50:1). Its entity

† Shimadzu GC-17A/QP-5000; DB-1 capillary column, 30 m; flow rate of the carrier (He), 1.9 cm^3/min; chamber temp., 60–170°C (30°C/min) then 170–280°C (15°C/min).

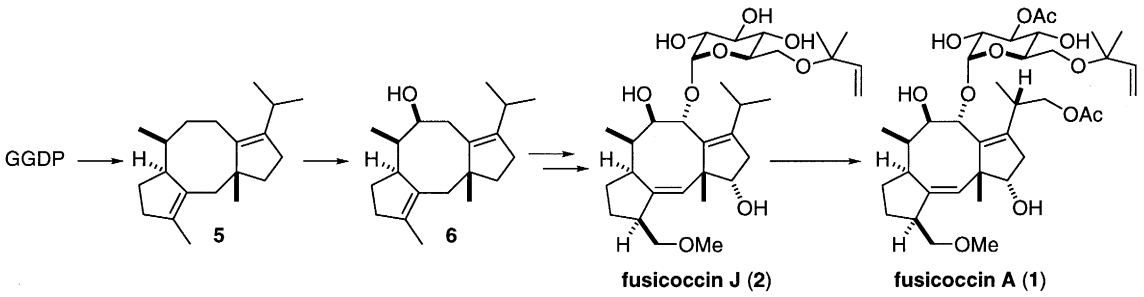
‡ The fungus strain F6 grown for 5 days produced 120 μg/cm^3 of fusicoccin J (**2**) as the main metabolite.

was unambiguously established by direct comparisons of full-scanned GC–MS (t_R 10.772–10.808 min) and ^{1}H NMR (400 MHz, $CDCl_3$) data with those of authentic **6** (see above). Thus, **6**, used as the feeding substrate in the previous paper,[15] is the real metabolite existing in mycelia of *P. amygdali*.

Then the feeding experiments were carried out using **5-*d*$_2$** to get direct proof for the intermediacy of **5** as well as **6** in the biosynthesis of fusicoccins. Each EtOH solution of **5-*d*$_2$** (3.0 mg in 0.2 cm^3) was added to six cultures (each 100 cm^3 of a medium containing 2.0% glucose, 0.2% corn steep liquor, and 0.2% peptone in deionized water[§]) of the fungus strain F6 which had been grown for 3 days at 25°C. After additional cultivation for 37 h, mycelia were obtained by filtration and treated as above. The hexane extract (82 mg) obtained was flash-chromatographed with a mixture of $CHCl_3$:EtOH (80:1) to give a **6**-containing fraction (ca. 0.8 mg). GC–MS data of the **6**-containing fraction and its mixture with synthetic **6-*d*$_1$**[15] as an internal standard clearly demonstrated that the fed **5-*d*$_2$** was incorporated efficiently into **6**; the former *m/z* (%) 95 (100), 288 {[M(**6**)]$^+$, 4}, 290 {[M(**6-*d*$_2$**)]$^+$, 8}; and the latter *m/z* (%) 95 (100), 288 {[M(**6**)]$^+$, 2}, 289 {[M(**6-*d*$_1$**)]$^+$, 7}, 290 {[M(**6-*d*$_2$**)]$^+$, 5.5}.

Fusicoccins, **1** and **2**, in the feeding experiment were chromatographically separated from the EtOAc extract (11.4 mg from one culture flask described above) of the culture filtrate with the additional cultivation for 48 h. The **1**-containing fraction (0.8 mg) and the **2**-containing fraction (1.1 mg) were analyzed by FAB-MS; *m/z* (rel. int.%, the former//natural-**1**) 703 [(M+Na)$^+$, 100//100], 704 [(M+1+Na)$^+$, 50.5//39.3], 705 {[M+2+Na and M(***d*$_2$**)+Na]$^+$, 46.3//10.6} and *m/z* (rel. int.%, the latter//natural-**2**) 603 [(M+Na)$^+$, 100//100], 604 [(M+1+Na)$^+$, 41.8//36.1], 605 {[M+2+Na and M(***d*$_2$**)+Na]$^+$, 47.5//9.0}. The incorporation of **5-*d*$_2$** is estimated as ca. 30% in **2**.

These and the previously reported (the Δ^1 isomer of **6** was not converted into natural fusicoccins)[15] results lead to the conclusion that the fusicoccane hydrocarbon leading to the aglycons of fusicoccins should now be revised to **5** from **4**. The results also reveal that the first hydroxylation of **5** occurs on the non-allylic 8β-position to afford **6** in the early metabolic process (Scheme 2).

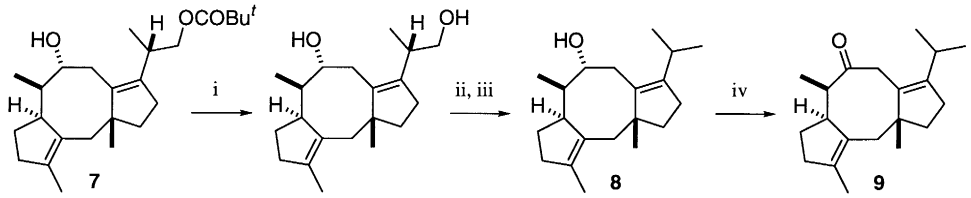


Scheme 2. The metabolic process from geranylgeranyl diphosphate (GGDP) to fusicoccins in *Phomopsis* (*Fusicoccum*) *amygdali*

The direct formation of **6** from **5** was further supported from the feeding experiment using the 8-epimer of **6** (**8**), which was synthesized easily from **7**. The bio-transformation of **8** by the fungus afforded only the corresponding ketone **9** (Scheme 3). This result clearly rules out the possibility of intervention of either **8** or **9** in the formation of **6**.

Consequently, as exemplified also by the case of the taxadiene biosynthesis,[16] the structure of the initially forming hydrocarbon proposed only on the basis of structure–similarity to that of the final metabolites should not be accepted blindly without corroborative proof even for the skeleton which obeys the 'isoprene rule'. The conclusion on the fusicoccane-hydrocarbon is of primary importance for

§ In this medium, the production of fusicoccin J (**2**) dropped to 10–20 μg/cm^3.



Scheme 3. Reagents and conditions. (i) $LiAlH_4$, THF (100%); (ii) TsCl, Py; (iii) $LiEt_3BH$, THF (two steps, 20%);[¶] (iv) *P. amygdali*, 3 days (ca. 30%)

our current efforts to isolate a gene encoding the 'fusicoccane synthase' as a fungal novel diterpene cyclase.[17]

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[¶] The low yield of the reductate (**8**) was due to the elimination of the tosylate to form fusicocca-2,10(14),15(19)-trien-8α-ol.