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Biosynthesis of Diterpenoid Aphidicolin: Isolation of Intermediates from P-450 Inhibitor Treated Mycelia of *Phoma betae*

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Abstract: Treatment of *Phoma betae* with P-450 inhibitors caused accumulation of biosynthetic precursors **2**, **3** and **4** of aphidicolin (**1**). Their structures were elucidated by spectroscopic analysis and they were confirmed by chemical transformations from **1**. Isotopically labeled precursors were synthesized by either reductive deoxygenation of aphidicolin derivatives or microbial conversion of [1-^{14}C]acetate in the presence of the P-450 inhibitor. Feeding experiments of the labeled precursors confirmed late biosynthetic pathway of **1**.

Key Words: Terpenes and terpenoids; Biosynthesis

Rapid progress on molecular biology makes it possible to identify the biosynthetic genes by sequence homology-based gene amplification. The technique, however, is rather complicated and the function of genes which involves in biosynthesis of complex molecules cannot be always clarified by gene disruption and overexpression of the corresponding gene. Alternatively, a specific inhibitor of an enzyme has been used to identify the intermediate and to elucidate the biosynthetic pathway. The inhibitor methodology is very simple and sometimes provides invaluable information on the biosynthetic pathway. Recently, we applied P-450 inhibitor experiments to elucidate biosynthesis of many natural products.[1] Since cytochrome P-450 plays an important role in terpenoid biosynthesis on the conversion of hydrocarbon to bioactive highly oxygenated natural product such as gibberellic acid[2] and trichothecene.[3] The inhibitor experiments would provide a detailed information of terpenoid biosynthesis.

Diterpenoid aphidicolin (**1**) was first isolated as an antiviral and antitumor agent isolated from *Cephalosporium aphidicola*[4] and later from three other fungi.[5] It shows specific inhibitory activity against DNA polymerase a which plays a major role of DNA replication.[6] Thus, **1** is now commercially available and becomes a useful tool to study eucaryotic DNA synthesis, DNA repair and cell cycle. The unique molecular

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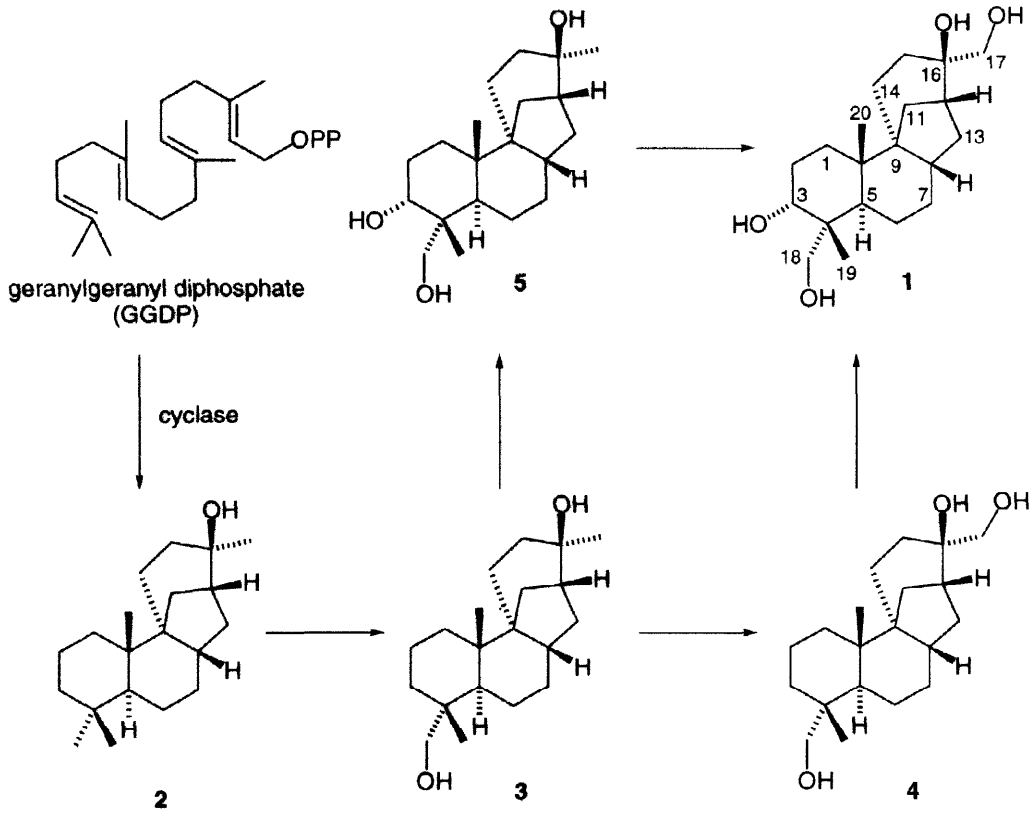
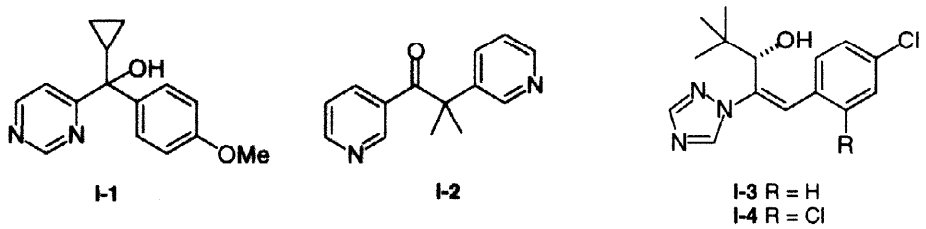
skeleton and the interesting bioactivity prompted a number of the synthetic studies.[7] The biosynthetic study of **1** was initiated by Adams and Bu'Lock[8] who proposed construction mode of aphidicolane skeleton and the proposal of the unique biosynthesis was confirmed by Hanson's groups.[9] The extensive studies were continued by Hanson's group who elucidated post cyclization sequence by incorporation of synthetic labeled compounds (Scheme 1).[10]



Scheme 1. The biosynthetic pathway of aphidicolin (**1**).

The proposed biosynthetic pathway of **1** implies that **1** could be synthesized by cloning and expressions of four enzymes such as a cyclase producing **2** from geranylgeranyl diphosphate (GGDP), and three hydroxylases which convert **2** to **1**. This extremely simplifies synthetic procedure of **1** than the chemical syntheses and seems attractive for a practical large-scale synthesis of **1**. Cyclization enzymes which convert GGDP to diterpene hydrocarbons casbene,[11] *ent*-kaurene,[12] abietadiene[13] and taxadiene[14] have been cloned and characterized. These provided several useful motifs for sequence homology-based gene amplification. However, none of data on the hydroxylase in the biosynthesis of **1** was available. For our aim to clone the biosynthetic genes and express them in heterologous host, we investigate late stage of aphidicolin biosynthesis to obtain preliminary information of the hydroxylases. In this paper, we describes a full account of our work[15] on isolation of biosynthetic intermediates by use of the P-450 inhibitors and efficient preparations of labeled precursors from **1**.

Isolation of Biosynthetic Precursors from P-450 Inhibitors Treated Mycelia



I-1 I-2 I-3 R = H I-4 R = Cl

Fig. 1. Cytochrome P-450 inhibitors.

The inhibitor (Fig. 1), ancymidol (**I-1**)[16] (final concentration, 1.04 mM) was added at the sixth day after inoculation to the culture of *Phoma betae* PS-13 which produces a phytotoxin aphidicolin (**1**) and its analogs.[5c] After an additional ten days of fermentation, the phytotoxins were extracted from the culture broth and the mycelium. Compared with non-treated samples, the accumulation of two metabolites was found on TLC in less polar fractions of the extract. These metabolites were isolated by repeated chromatography to provide two constituents, **2** and **4**. The accumulation of these compounds increased in proportion to the amount of **I-1**(0.26. 0.52 and 1.04 mM) but the addition of more than 1.04 mM ancymidol did not affect the production yields of these metabolites.

The polar compound **4** was identified as 3-monodeoxy analog of **1**, which was isolated from a culture broth of *P. betae* as a minor component.[5c] On the other hand, a molecular formula $C_{20}H_{34}O$ (unsaturation 4) of the less polar compound **2**, and the presence of a oxygenated quaternary carbon signal at 72.91 ppm in the ^{13}C-NMR spectrum suggested that **2** is a trideoxy analog of **1**, aphidicolan-16β-ol. Although signals in the ^{1}H-NMR spectrum of **2** were concentrated at 1.15 - 2.05 ppm, all proton signals were reasonably separated and all proton and protonated carbon signals were assigned by analyses of COSY and HSQC spectra (Table). HMBC data allowed us to elucidate proton-carbon connectivities and these data allowed us to propose the gross structure of **2** as shown in Fig. 2. The stereochemistry of **2** was determined by means of NOESY data (Fig. 2). The structure was later confirmed by chemical correlation with **1**. With this authentic sample, we found that the mycelium which was not treated with the inhibitor also contains trace amount of **2**.

Table NMR data of aphidicolan-3β-ol (**2**) in $CDCl_3$.

	^{13}C-NMR (125 MHz) ppm	^{1}H-NMR[§] (500 MHz) ppm		^{13}C-NMR (125 MHz) ppm	^{1}H-NMR[§] (500 MHz) ppm
C-1	34.17 (t)	1.19 (m), 1.48 (m)	C-11	32.96 (t)	1.31 (m), 1.57 (m)
C-2	19.41 (t)	1.38 (m), 1.61 (m)	C-12	46.82 (d)	1.95 (m)
C-3	42.76 (t)	1.11 (m), 1.36 (m)	C-13	32.14 (t)	0.92 (m), 1.81 (m)
C-4	33.43 (s)	—	C-14	25.20 (t)	1.72 (m), 1.79 (m)
C-5	46.55 (d)	1.47 (m)	C-15	33.00 (t)	1.31 (m), 1.69 (m)
C-6	23.79 (t)	1.21 (m), 1.63 (m)	C-16	72.91 (s)	—
C-7	27.15 (t)	1.23 (m), 1.66 (m)	C-17	28.00 (q)	1.12 (s)
C-8	40.06 (d)	1.99 (m)	C-18	34.48 (q)	0.84 (s)
C-9	40.08 (s)	—	C-19	21.84 (q)	0.83 (s)
C-10	48.16 (s)	—	C-20	14.71 (q)	0.93 (s)

[§] The *J* values could not obtained due to severe overlapping of the signals.

Inhibitor treatment sometimes causes accumulation of a number of less oxidized metabolites. This makes it hard to identify a desired biosynthetic intermediate. In our case, *P. betae* PS-13 which produces other different type of metabolites including a polyketide phytotoxin betaenone[17] accumulated a complex mixture of less oxidized metabolites when it was treated with the inhibitor. Since we expected that **1** would be biosynthesized via three successive P-450 dependent hydroxylations (Scheme 1), we further explored inhibition conditions to obtain a dideoxy analog **3** in the mycelial extracts treated with the inhibitors. In a preliminary study, we found that S-3307D[1b] (**I-3**) and S-3308L[2b] (**I-4**) strongly inhibited the first hydroxylation whereas treatment with **I-1** and metyrapone (**I-2**)[1b] effectively caused accumulation of **4** and effect of **I-2** was moderate. In order to detect small amount of **3**, other aphidicolin producing strains (*P. betae*) were screened. Among the strains (ATCC 6504, 24635, 24797) tested, we found that the strain ATCC 24797 produced only **1** as a major metabolite. This greatly simplified a TLC pattern of the extract. Using this strain and the inhibitor **I-2**, another biosynthetic intermediate **3** was isolated from the extracts of the inhibitor-treated mycelia. The compound **3** possessed a molecular formula ($C_{20}H_{34}O_2$, unsaturation 4), and the presence of hydroxymethyl signals at 3.05 and 3.39 ppm in the ^{1}H-NMR spectrum and the resistance against periodate oxidation suggested that **3** is the dideoxy analog of **1**. The structure was later established by chemical transformation from **1**.

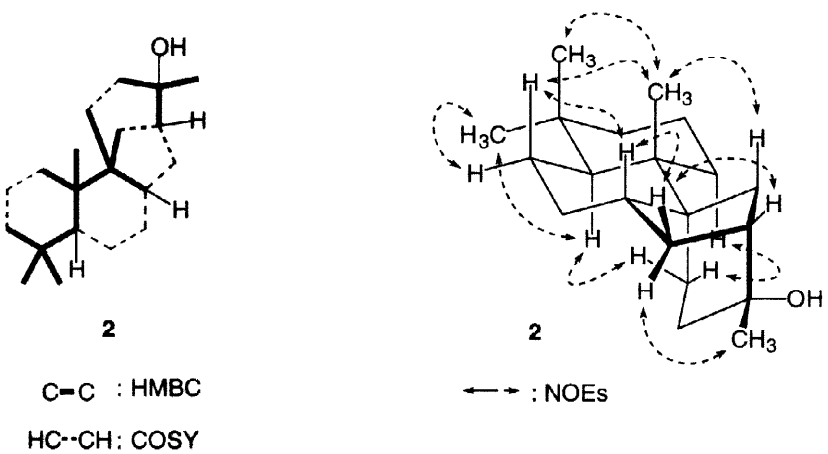


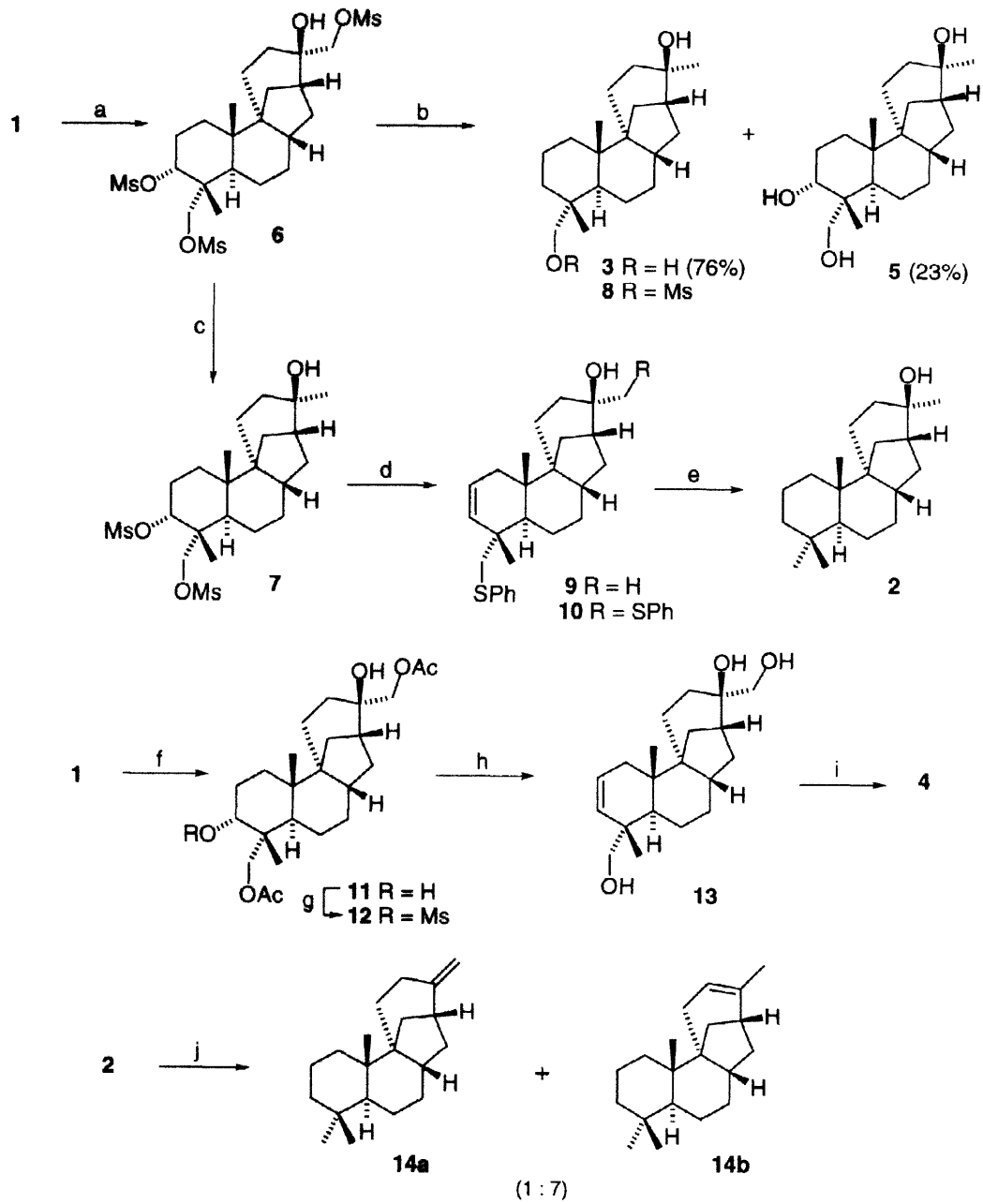
Fig. 2. Carbon connectivities and stereochemical relationship of **2** deduced from HMBC, COSY and NOESY spectra.

Synthesis of Non-labeled and [^{2}H]-Labeled Biosynthetic Precursors

Previously, Hanson's group reported the synthesis of deoxy analogs **2**-**5** starting from **1**.[18] The route, however, required rather long steps. In order to confirm the structures of the accumulated compounds and to prepare their labeled forms, we developed a new route which incorporates selective deoxygenations of trimesylate **6** (Scheme 2).

Mesylation of **1** gave trimesylate **6** which was reduced under various conditions. Reduction with $LiAlH_4$ gave a complex mixture whereas $LiBH(Et)_3$ reduction cleanly proceeded to afford dimesylate **7** in quantitative yield. Reduction of **6** with Li in liquid ammonia afforded diol **3** and triol **5** in 76 and 23% yield, respectively, along with trace amount of monool **2**. Observation that reduction of mesylate **8** derived from **3** with $LiAlH_4$

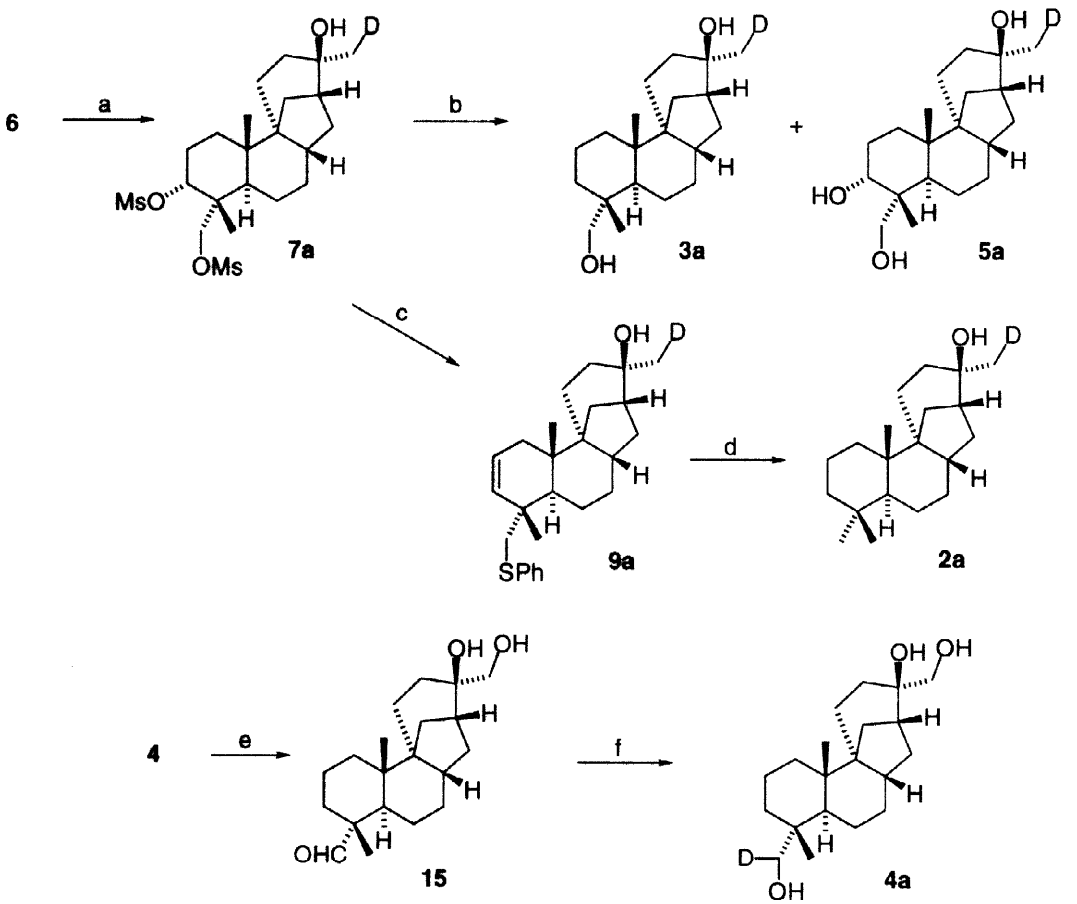
recovered **3** suggested severe steric congestion around C-18. We next explored substitution conditions of mesyloxy group to reductively removable group because direct transformation of **6** to trideoxy analog **2** is not practical. Treatment of **7** with thiophenoxide caused substitution at C-18 and concomitant elimination of 3-mesyloxy group to afford **9** in 73% yield. Direct conversion of **6** to **10**, however, resulted in low yield (13%). Desulfurization and concomitant hydrogenation of thioether **9** with Raney Ni were achieved to afford **2** in quantitative yield. The intermediate **14a** in the minor biosynthetic pathway of **1** was obtained as a 1:7 mixture of **14a** and **14b** by dehydration of **2**.



Scheme 2. (a) MsCl, Py (77%); (b) Li, liq.NH_3-THF, -78°C; (c) $LiBH(Et)_3$, THF (quant.); (d) NaH, PhSH, DMF (73%); (e) Raney Ni (W-4), H_2, EtOH (quant.); (f) Ac_2O, Py (29%, isomer 30%); (g) MsCl, Py, –20 ~ –5°C (51%); (h) $LiAlH_4$, THF (52%); (i) PtO_2, H_2, EtOH (77%); (j) MsCl, Et_3N, THF, -25°C, 84%.

Monodeoxy analog **4** was synthesized via different route (Scheme 2). Partial acetylation of **1** gave a 1:1 mixture of diacetate **11** and its regioisomer in 59% yield. Mesylation of **11** followed by reduction with $LiAlH_4$ unexpectedly afforded **1** and elimination product **13**. Hydrogenation of **13** with PtO_2 under hydrogen atmosphere furnished **4** in 77% yield.

Deuterium labeled precursors which would be useful for incorporation study and differentiation of products formed in reaction with crude enzymes were prepared (Scheme3). Reduction of trimesylate **6** with $LiBD(Et)_3$ afforded deuterated dimesylate **7a** which was converted to deuterated **3a** and **5a** with dissolving metal reduction. Thiophenoxide treatment of **7a** followed by reduction with Raney Ni afforded trideoxy analog **2a**. On the other hand, selective oxidation of **4** with Dess-Martin periodinane gave **15** which was then reduced with $NaBD_4$ to afford **4a**.



Scheme 3. (a) $LiBD(Et)_3$, THF (quant.); (b) Li, liq.NH_3-THF, –78°C; (c) NaH, PhSH, DMF (26%); (d) Raney Ni (W-4), H_2, EtOH (quant.); (e) Dess-Martin periodinane, CH_2Cl_2 (65%); (h) $NaBD_4$, MeOH (62%).

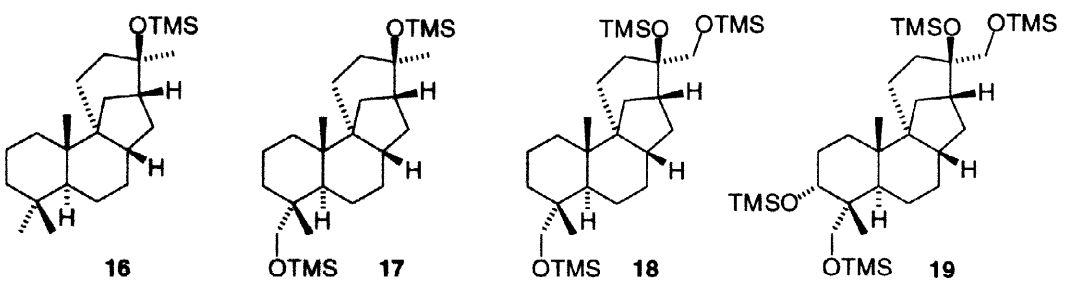
Incorporation Study of [^{14}C]-Labeled Biosynthetic Precursors

In the previous work, we reported preparation of isotopically labeled precursors with simple labeled precursors such as methionine and acetate in the presence of the P-450 inhibitors, and its successful

incorporation in the study of the biosynthesis at late stage.[1a,1b] Using 1.04 mM of the inhibitor **I-1**, we prepared ^{14}C-labeled **2** and **4** by feeding with sodium [1-^{14}C]acetate. Re-incorporation of ^{14}C-labeled **2** showed relatively high conversion (8.3%) to **1**. When similar experiment was employed in the presence of the inhibitor **I-1**, **2** was incorporated into **1** (1.3%) and **4** (0.5%). These results showed that the deoxy analogs **2** and **4** are intermediates of **1** and the inhibitor experiment is useful to study successive hydroxylations due to incomplete inhibition in each steps.

Enzymatic Conversion of [^{2}H]-Labeled Precursors

Since Hanson *et al.* established intermediacy of the less oxidized precursors **2**, **3**, **4** and **5**, we examined enzymatic conversions of these biosynthetic precursors using a microsomal fraction in which cytochrome P-450 is commonly found. In order to analyze all aphidicolin analogs in capillary gas chromatography, **2**, **3**, **4** and **1** were silylated with 1-(trimethylsilyl)imidazole to give the corresponding TMS ethers **16**, **17**, **18** and **19** in quantitative yields. [^{2}H]-Labeled precursors **2a**, **3a** and **4a** were incubated with a microsomal fraction prepared from the mycelia in the presence of NADPH overnight. After silylation, GC-MS analysis of the products in the reaction using **2a** showed a new peak corresponding to **17** and the conversion from **2a** to **3a** was estimated as 9.4%. In the MS spectrum of the new GC peak, characteristic fragment peaks shifted by 1 mass unit due to retention of deuterium were observed at m/z 145, 218 and 436 (M^+–CH_3). In the case of incubation with **3a** and **4a**, contamination of **1** and **4** (more than 1/10 of substrates added) in the microsomal fraction made it difficult to detect the labeled fragment peaks. In our hand, the reaction products were not detected within experimental error possibly due to weak activities of other hydroxylases. Further examination on optimizing enzyme preparation is necessary for detection of all hydroxylase activities.



Conclusion

We successfully blocked hydroxylation steps in the later stage of the biosynthesis of aphidicolin (**1**) by the P-450 inhibitor experiments and this resulted in the accumulation of the intermediates. The structures of the intermediates **2**-**4** were established by spectroscopic methods and chemical transformations which were developed for efficient preparations of the labeled precursors. Simple incorporation experiments which were carried out under either non-blocked or partially blocked conditions demonstrated stepwise conversion of the precursors and proved the intermediacy of **2** and **4**. Thus, we showed that the P-450 experiment would be useful for the biosynthetic study of highly oxygenated metabolites.

We believe that similar inhibitor experiment can be applied to biosynthetic study of the structurally related

tetracyclic diterpenoids stemodine,[20] stemarin,[21] oryzalexin S[22] and thysiflorin A[23, 24] in which three cyclic systems are branched from C-9. Identification of hydroxylation enzyme with the specific inhibitors could facilitate gene amplification with homology-based PCR and its expression. In several fungi producing secondary metabolites, it is known that genes encoding enzymes responsible for their biosynthesis exist as a cluster.[25] This indicates that various hydroxylase genes involved in the late biosynthetic pathway can be easily characterized once the gene cluster is identified.

Experimental

IR spectra were measured on a Perkin-Elmer 2000 FT-IR spectrometer, ^{1}H-, and ^{13}C-NMR spectra on JEOL EX-270 and Bruker AM-500 spectrometers for solutions in $CDCl_3$, mass spectra on JEOL DX-300, 01SG-2 spectrometers and optical rotation on a Jasco DIP-4 polarimeter. GC-MS analysis was conducted with Thermo Quest GCQ. Column chromatography used Merck Kieselgel 60 (0.04-0.063 mm), and TLC was performed on Merck Kieselgel 60 F_{254}. Solvents were dried shortly before use with an appropriate drying agent. Anhydrous reactions were carried out under argon. Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. Sodium [1-^{14}C]-acetate (1 mCi; 58.0 mCi/mmol) was purchased from New England Nuclear. [^{2}H]-$NaBH_4$ (98 atom% ^{2}H) and [$^2H_3$]-$LiBH(Et)_3$ (99 atom% ^{2}H) were from Aldrich.

Treatment of *P. betae* with cytochrome P-450 inhibitors. *P. betae* PS-13 was inoculated to the cultures, in two 500 ml flasks containing 200 ml of 2 % potato-sucrose medium. On the fifth day after inoculation, the DMSO solution (2 ml) of 80 mg of ancymidol (**I-1**) was equally distributed into two 500 ml flasks (the amount of inhibitor, 1.04 mM/flask). After further incubation (10 days), mycelium was collected by filtration and extracted with acetone. This extract was concentrated *in vacuo* and the resultant aqueous layer was extracted with EtOAc. The filtrate was extracted in a similar way and the organic layers were combined. Drying over Na_2SO_4, the EtOAc extract was concentrated *in_vacuo* to afford the oily residue which was chromatographed over SiO_2 column eluting sequentially with hexane/EtOAc (4:3) – $CHCl_3$/MeOH (9:1). The fractions containing **1**, **2** and **4** were chromatographed with PTLC i) cyclohexane/EtOAc (2:1), ii) $CHCl_3$/MeOH (4:1) to yield **2** (6.1 mg), **4** (1.7 mg) and **1** (6.1 mg). **2**: $[\alpha]_D^{22.5}$ +16.3° (*c* 1.3, $CHCl_3$) mp 138-139°C. HRMS m/z 290.2622 (M^+, $C_{20}H_{34}O$ calcd. for 290.2610. EI MS m/z 290 (M^+), 272. IR ν_{max} (KBr) cm^{-1} 3400. **4**: $[\alpha]_D^{22.5}$ +21.6° (*c* 0.1, EtOH) mp 138-140°C.

Isolation of aphidicolan-16β,18-diol (3). Metyrapone (**I-2**) (160 mg) was dissolved into 8 ml of DMSO. At the fifth day after inoculation of *P. betae* (ATCC 24797), this solution was distributed equally to four flasks (the amount of inhibitor, 0.88 mM/flask). After further incubation (12 days), extraction was made in a similar way shown above. The extracts were chromatographed over SiO_2 column eluting sequentially with hexane/EtOAc (4:3) – $CHCl_3$/MeOH (9:1). The fraction containing **3** was further chromatographed by PTLC with $CHCl_3$/MeOH (95:5) to give **3** (1 mg) as an oil. At the same time, **2** (trace), **4** (9.4 mg) and **1** (11.4 mg)

were also isolated. **3:** gum: $[\alpha]_D^{24}$+17.1° (*c* 1.0, $CHCl_3$); IR (film) ν_{max}: 3374, 2946, 1046, 757 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 3.37 (d, 1H, J = 10.9 Hz), 3.05 (d, 1H, J = 10.9 Hz), 1.12 (s, 3H), 0.97 (s, 3H), 0.77 (s, 3H), 0.79-1.97 (m, 23H) ; EIHRMS m/z 275.2397 (M^+-CH_2OH, $C_{19}H_{31}O$ requires 275.2375)

Preparations of the isotopically labeled deoxy analogs 2 and 4 by feeding with sodium [1-^{14}C]acetate. On the eighth day after inoculation of *P. betae* PS-13, [1-^{14}C] sodium acetate (100 mCi) and non-labeled sodium acetate (200 mg) in 4 ml of water, and ancymidol (**I-1**) (80 mg) in 4 ml of DMSO were added to the two flasks. After a further twelve days of fermentation, radioactive **2** (9.5 mg, 1.0 x 10^6 dpm, 3.1 x 10^7 dpm/mmol) was obtained from the mycelial EtOAc extracts as described above .

On the sixth day after inoculation, **2** (4.4 mg, 4.8 x 10^5 dpm) in MeOH-DMSO (5:3, v/v, 4 ml) was distributed into four flasks. To two of them was added **I-1** (80 mg) in 4 ml of DMSO at the same time. After a further ten days of fermentation, **1** (16.7 mg, 2.0 x 10^4 dpm, 4.0 x 10^5 dpm/mmol, 8.3% incorporation) was isolated from EtOAc extracts of the non-treated fermentation broth. From the inhibitor-treated broth, **1** (6.1 mg, 2948 dpm, 1.6 x 10^5 dpm/mmol, 1.3% incorporation) and **4** (1.7 mg, 1229 dpm, 2.3 x 10^5 dpm/mmol, 0.5% incorporation) were obtained.

3α,17,18-Trimesyloxyaphidicol-16β-ol (6). To a solution of **1** (50.5 mg, 0.149 mmol) in pyridine (0.75 ml) was added MsCl (0.25 ml, 3.23 mmol). After stirring at ambient temperature for 45 min, the mixture was quenched with 2M HCl and extracted with $CHCl_3$ The combined organic extracts were washed with sat. NaCl, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by silica gel flash chromatography ($CHCl_3$/MeOH, 95:5) gave trimesylate **6** (65.4 mg, 77%) and 17,18-dimesylate (4.7 mg, 6%). **6:** gum; $[\alpha]_D^{24}$ -16.2° (*c* 1.0, $CHCl_3$); IR (film) ν_{max}: 3533, 2942, 1351, 1174, 959, 900, 843, 756, 529 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 4.76 (br.d, 1H, J = 2.3 Hz), 4.12 (d, 1H, J = 10.2 Hz), 4.04 (d, 1H, J = 10.2 Hz), 4.03 (s, 1H), 3.08 (s, 3H), 3.07 (s, 3H), 3.05 (s, 3H), 2.23 (m, 1H), 1.08 (s, 3H), 1.02 (s, 3H), 0.90-2.18 (m, 19H); EIHRMS m/z 284.2120 (M^+-MsOH x 3, $C_{20}H_{28}O$ requires 284.2141)

3α,18-Dimesyloxyaphidicol-16β-ol (7). To a solution of trimesylate **6** (20 mg, 0.035 mmol) in THF (1 ml) was added $LiBH(Et)_3$ (0.04 ml, 1 M in THF, 0.04 mmol). After stirring at ambient temperature for 15 min, the mixture was quenched with water. The mixture was extracted with ether and the combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. Purification by silica gel flash chromatography ($CHCl_3$/MeOH, 95:5) gave dimesylate **7** (12.6 mg, quant.) as gum. $[\alpha]_D^{24}$ -15.7° (*c* 1.0, $CHCl_3$); IR (film) ν_{max}: 2939, 1351, 1173, 960, 900 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 4.76 (br.d, 1H, J = 2.3 Hz), 4.04 (s, 3H), 3.08 (s, 3H), 3.07 (s, 3H), 1.12 (s, 3H), 1.08 (s, 3H), 1.01 (s, 3H), 0.92-2.17 (m, 20H) ; EIHRMS m/z 478.2071 (M^+, $C_{22}H_{38}O_2S_2$ requires 478.2059).

Aphidicol-16β,17-diol (3) and aphidicol-3α,16β,18-triol (5). To a solution of lithium (100 mg, 0.014 g-atom) in liquid NH_3 (10 ml) was added dropwise trimesylate **6** (8.5 mg, 0.015 mmol) in THF (1 ml). After stirring at ambient temperature for 5 h, the mixture was quenched with solid NH_4Cl and the bulk of liquid NH_3 was evaporated and then diluted with saturated NH_4Cl. The mixture was extracted with ether and the combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by PTLC (hexane/EtOAc, 1:2) gave diol **3** (3.5 mg, 76%) and triol **5** (1.1 mg, 23%). **3:** gum; $[\alpha]_D^{24}$ +17.1° (*c* 1.0, $CHCl_3$). **5:** gum; $[\alpha]_D^{24}$ -2.8° (*c* 1.0, $CHCl_3$); IR (film) ν_{max}: 3376, 2929, 1032, 756 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 3.67 (br.s, 1H), 3.47 (d, 1H, J = 11.2 Hz), 3.37 (d, 1H, J = 11.6 Hz), 2.42 (dd, 1H, J = 12.5 and 3.3 Hz), 1.13 (s, 3H), 0.98 (s, 3H), 0.70 (s, 3H), 0.92-2.04 (m, 21H) ; EIHRMS m/z 304.2410 (M^+-H_2O, $C_{20}H_{32}O_2$ requires 304.2402)

18-Phenylthioaphidicol-2-en-16β-ol (9). To a suspension of NaH (60% in mineral oil, 4 mg, 0.098 mmol) in DMF (0.15 ml) was added thiophenol (0.01 ml, 0.098 mmol) and the mixture was stirred for 1 h at 90℃. A solution of dimesylate **7** (17.9 mg, 0.037 mmol) in DMF (0.5 ml) was added dropwise. After stirring at 90℃ for 13 h, the mixture was quenched with ice-water and was extracted with EtOAc and the combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by silica gel flash chromatography (hexane/EtOAc, 5:1) gave thioether **9** (10.7 mg, 73%) as gum. $[\alpha]_D^{24}$ +0.4° (*c* 0.5, $CHCl_3$); IR (film) ν_{max}: 3419, 2929, 1654, 1584, 1457, 1480, 1439, 1090, 737 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 7.10-7.39 (m, 5H), 5.71 (ddd, 1H, J = 10.2, 6.3 and 2.0 Hz), 5.29 (dd, 1H, J = 10.2 and 2.6 Hz), 3.10 (d, 1H, J = 12.2 Hz), 2.84 (d, 1H, J = 12.5 Hz), 2.33 (br.d, 1H, J = 16.8 Hz), 2.26 (dd, 1H, J = 12.9 and 4.0 Hz), 1.11 (s, 3H), 1.01 (s, 3H), 0.98 (s, 3H), 0.88-2.00 (m, 16H); EIHRMS m/z 396.2465 (M^+, $C_{26}H_{36}OS$ requires 396.2487).

Aphidicol-16β-ol (2). To a solution of thioether **9** (3.6 mg, 0.009 mmol) in EtOH (2 ml) was added Raney Ni (W-4) (ca. 3 mg). The resultant suspension was stirred vigorously under reflux and hydrogen atmosphere. After 2 h, the reaction mixture was filtered through a pad of Celite and concentration of the filtrate to afford **2** (2.6 mg, quant.) as white crystalline. mp 134-135℃; $[\alpha]_D^{24}$ -18.0° (*c* 0.1, $CHCl_3$).

17,18-Diacetoxyaphidicol-3α,16β-diol (11). To a solution of **1** (140 mg, 0.414 mmol) in pyridine (0.9 ml) was added acetic anhydride (0.3 ml, 2.72 mmol). After stirring at ambient temperature for 20 min, the mixture was poured into ice-water, and extracted with EtOAc. The combined organic extracts were washed with sat. $NaHCO_3$, sat. NaCl successively, and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by silica gel flash chromatography ($CHCl_3$/acetone, 93:7) gave diacetate **11** (50 mg, 29%) and the other diacetate (53 mg, 30%). **11:** white crystalline; mp 164-166℃ (from EtOAc); IR (KBr) ν_{max}: 3450, 2950, 1750, 1720, 1270, 1240, 1060 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 4.11 (d, 1H, J = 11.2 Hz),

3.97 (s, 2H), 3.91 (d, 1H, J = 10.3 Hz), 3.59 (br.s, 1H), 2.09 (s, 6H), 0.99 (s, 3H), 0.91 (s, 3H) ; EIHRMS m/z 404.2571 (M^+-H_2O, $C_{24}H_{36}O_5$ requires 404.2561)

17,18-Diacetoxy-3α-mesyloxyaphidicol-16β-ol (12). To a solution of **11** (50 mg, 0.118 mmol) in pyridine (0.3 ml) was added MsCl (0.1 ml, 0.590 mmol) at -20℃. After stirring at -25 – -5℃ for 3.5 h, the mixture was poured into ice-water, and extracted with EtOAc. The combined organic extracts were washed with sat. $NaHCO_3$, sat. NaCl successively, and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by silica gel flash chromatography ($CHCl_3$/acetone, 95:5) gave mesylate **12** (30 mg, 51%) as gum. IR (film) ν_{max}: 3550, 2950, 1735, 1330, 1250, 1170, 960 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 4.85 (br.s, 1H), 3.97 (s, 2H), 3.98 (s, 2H), 3.80 (s, 2H), 2.98 (s, 3H), 2.10 (s, 3H), 2.09 (s, 3H), 1.04 (s, 3H), 1.01 (s, 3H) ; EIHRMS m/z 482.2359 (M^+-H_2O, $C_{25}H_{38}SO_7$ requires 482.2339)

Aphidicol-2-en-16β,17,18-triol (13). To a solution of **12** (9.0 mg, 0.018 mmol) in THF (1.5 ml) was added $LiAlH_4$ (10 mg, 0.263 mmol). After stirring at ambient temperature for 2 h, the mixture was quenched with adding EtOAc and then water. The resultant mixture was acidified with 2M HCl and extracted with EtOAc. The combined organic extracts were washed with sat. $NaHCO_3$, sat. NaCl successively, and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by silica gel flash chromatography ($CHCl_3$/acetone, 3:1) gave olefin **13** (3.0 mg, 52%) and **1** (1.8 mg, 30%). **13**: gum; IR (film) ν_{max}: 3350, 2925, 1720, 1450, 1040, 760 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 5.91 (ddd, 1H, J = 10.3, 6.3 and 2.0 Hz), 5.21 (dd, 1H, J = 10.3 and 2.9 Hz), 3.47 (d, 1H, J = 11.2 Hz), 3,33-3.39 (m, 2H), 3.09 (d, 1H, J = 10.7 Hz), 1.01 (s, 3H), 0.83 (s, 3H) ; EIHRMS m/z 320.2378 (M^+, $C_{20}H_{32}O_3$ requires 320.2351)

Aphidicol-16β,17,18-triol (4). To a solution of olefin **13** (3.0 mg, 0.009 mmol) in EtOH (0.5 ml) was added PtO_2 (9 mg). The resultant suspension was stirred vigorously under hydrogen atmosphere. After 5.5 h, the reaction mixture was filtered through a pad of Celite and the filtrate was concentration *in vacuo*. Purification by silica gel flash chromatography ($CHCl_3$/acetone, 3:1) gave **4** (2.3 mg, 77%) as white crystalline. mp 138-141℃ (MeOH); $[\alpha]_D^{24}$ (*c* 0.1, EtOH).

Aphidicol-16-ene (14a) and aphidicol-15-ene (14b). To a solution of monool **2** (3.7 mg, 0.013 mmol) in THF (0.25 ml) were added Et_3N (0.1 ml, 0.717 mmol) and MsCl (0.1 ml, 1.292 mmol) at -20℃. After stirring at -25℃ for 1 h, the mixture was diluted with ether, and washed with sat. $NaHCO_3$, sat. NaCl successively. The combined organic extracts were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by PTLC ($CHCl_3$) gave a 1:7 mixture of olefin **14a** and **14b** (2.5 mg, 72%) and starting material (0.4 mg, 12%). **14a** and **14b:** gum; IR (film) ν_{max}: 2933, 2866, 1464 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$) (**14b**): δ 4.99 (d, 1H, J = 1.0Hz), 1.25 (s, 3H), 1.00 (s, 3H), 0.84 (m, 2H), 0.83 (s, 3H); ^{1}H-NMR

(270 MHz, $CDCl_3$) (**14a**): δ 4.47 (m, 1H), 4.39 (m, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$) (**14b**): δ 147.36, 118.09, 48.23, 45.09, 42.57, 41.96, 40.15, 39.12, 38.60, 35.42, 34.09, 33.74, 33.10, 29.69, 27.96, 25.85, 22.94, 21.70, 19.37, 15.68; ^{13}C-NMR (125 MHz, $CDCl_3$) (**14a**): δ 159.19, 102.36, 45.66, 43.50, 43.23, 42.68, 41.16, 39.40, 34.44, 34.21, 33.36, 31.92, 29.44, 28.67, 28.18, 26.49, 23.53, 21.80, 19.37, 14.82; EIHRMS m/z 272.2500 (M^+, $C_{20}H_{32}$ requires 272.2504).

[17-^{2}H]-3α,18-Dimesyloxyaphidicol-16β-ol (7a). The title compound was synthesized as described for non-labeled compound. **7a**: IR (film) ν_{max}: 2939, 1351, 1173, 960, 900 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 4.76 (br.d, 1H, J = 2.3 Hz), 4.04 (s, 2H), 3.08 (s, 3H), 3.07 (s, 3H), 1.12 (s, 2H), 1.08 (s, 3H), 1.01 (s, 3H), 0.92-2.17 (m, 20H) ; EIHRMS m/z 479.2090 (M^+, $C_{22}H_{37}DO_2S_2$ requires 479.2121).

[17-^{2}H]-Aphidicol-16β,18-diol (3a) and [17-^{2}H]-aphidicol-3α,16β,18-triol (5a). The title compounds were synthesized as described for non-labeled compound. **3a:** IR (film) ν_{max}: 3346, 2927, 1043, 758 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 3.39 (d, 1H, J = 10.9 Hz), 3.05 (d, 1H, J = 10.9 Hz), 1.11 (s, 2H), 0.98 (s, 3H), 0.77 (s, 3H), 0.84-1.98 (m, 23H) ; EIHRMS m/z $C_{19}H_{30}DO$ (276.2451, M^+-CH_2OH, requires 276.2437). **5a:** IR (film) ν_{max}: 3355, 2927, 1045, 733 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 3.68 (br.s, 1H), 3.48 (d, 1H, J = 11.2 Hz), 3.37 (d, 1H, J = 11.2 Hz), 2.42 (dd, 1H, J = 12.5 and 3.0 Hz), 1.12 (s, 2H), 0.98 (s, 3H), 0.70 (s, 3H), 0.92-2.04 (m, 21H) ; EIHRMS m/z 305.2450 (M^+-H_2O, $C_{20}H_{31}DO_2$ requires 305.2464)

[17-^{2}H]-18-Phenylthioaphidicol-2-en-16β-ol (9a). The title compound was synthesized as described for non-labeled compound. **9a**: IR (film) ν_{max}: 3419, 2929, 1654, 1584, 1457, 1480, 1439, 1090, 737 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 7.10-7.39 (m, 5H), 5.71 (ddd, 1H, J = 10.2, 6.3 and 2.0 Hz), 5.29 (dd, 1H, J = 10.2 and 2.6 Hz), 3.10 (d, 1H, J = 12.2 Hz), 2.84 (d, 1H, J = 12.5 Hz), 2.33 (br.d, 1H, J = 16.8 Hz), 2.26 (dd, 1H, J = 12.9 and 4.0 Hz), 1.11 (s, 2H), 1.01 (s, 3H), 0.98 (d, J = 1.0 Hz, 3H), 0.88-2.00 (m, 16H); EIHRMS m/z 397.2518 (M^+, $C_{26}H_{35}DOS$ requires 397.2549).

[17-^{2}H]-Aphidicol-16β-ol (2a). The title compound was synthesized as described for non-labeled compound. **2a**: white crystalline; IR (KBr) ν_{max}: 3424, 2944 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 1.11 (s, 2H), 0.94 (s, 3H), 0.85 (d, 3H, J = 2.3 Hz), 0.88-2.00 (m, 25H) ; EIHRMS m/z 291.2671 (M^+, $C_{20}H_{33}DO$ requires 291.2671).

Aphidicol-16β,17-diol-18-one (15). To a solution of triol **4** (8.7 mg, 0.027 mmol) in CH_2Cl_2 (1.5 ml) was added Dess-Martin periodinane (113 mg, 0.267 mmol). After stirring at ambient temperature for 5 min, the mixture was quenched with sat. $Na_2S_2O_3$ and sat. $NaHCO_3$ and extracted with $CHCl_3$ (10 ml). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by silica gel flash chromatography ($CHCl_3$/MeOH, 95:5) gave aldehyde **15** (5.6 mg, 65%) and starting material (3.0 mg, 35%). **15**:$[\alpha]_D^{24}$ +10.0° (*c* 1.0, $CHCl_3$); IR (film) ν_{max}: 3415, 2918, 2850, 1723, 1451, 756 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 9.20 (s, 1H), 3.47 (d, 1H, J = 10.9 Hz), 3.38 (d, 1H, J = 10.9 Hz), 2.18 (m, 1H), 1.09 (s, 3H), 1.02 (s, 3H), 0.93 (m, 1H), 1.04-2.08 (m, 21H) ; EIHRMS m/z 289.2160 (M^+-CH_2OH, $C_{19}H_{29}O_2$ requires 289.2167)

[18-^{2}H]-Aphidicol-16β,17,18-triol (4a). To a solution of aldehyde **15** (2.9 mg, 9.1 mmol) in MeOH (0.5 ml) was added $NaBD_4$ (5.0 mg, 0.132 mmol). After stirring at ambient temperature for 8 h, the reaction mixture was quenched with acetone (2.0 ml) and diluted with water and extracted with ether. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by PTLC ($CHCl_3$/MeOH, 9:1) gave triol **4a** (1.8 mg, 62%) and starting material (0.4 mg, 12%). **4a:** gum; IR (film) ν_{max}: 3211, 2926, 1288, 1100 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 3.46 (d, 1H, J = 10.9 Hz), 3.370 (d, 1H, J = 10.9 Hz), 3.366 (s, 0.7H), 3.04 (s, 0.3H), 2.16 (m, 2H), 0.99 (s, 3H), 0.91 (m, 2H), 0.78 (s, 3H), 1.17-2.03 (m, 19H) ; EIHRMS m/z 292.2400 (M^+-CH_2OH, $C_{19}H_{30}DO_2$ requires 292.2386).

Enzymatic Conversion of [^{2}H]-Labeled Precursors 2a, 3a and 4a.

Mycelia (15 g) of *P. betae* were homogenized with 60 ml of degassed buffer (pH 7.5) consisting of 50 mM potassium phosphate, 10 mM EDTA and 20% (v/v) glycerol by Polytron homogenizer at 4℃. The homogenate was centrifuged at 1,100 x *g* for 10 min and the resultant supernatant was further centrifuged at 18,000 x *g* for 20 min. The supernatant obtained was ultracentrifuged at 126,000 x *g* for 120 min. The precipitates were suspended with 20 mM potassium phosphate buffer (pH 7.5, 1.5 ml) and the suspension was used as a microsomal fraction. After addition of [^{2}H]-labeled precursors in methanol (20 μl, final concentration 60 mM), the assay mixture consisting of the microsomal fraction (150 μl), 1 M Tris-HCl (pH 8.0, 14 μl), 1 M $MgCl_2$ (10 μl) and water (870 μl) was preincubated at 37℃ for 5 min. After addition of 0.5 M NADPH (10 μl), the resultant mixture was incubated at 30℃ overnight. The reaction was terminated by vigorous mixing with ethyl acetate (1 ml). The extracts were concentrated *in vacuo* and the residue was silylated by treatment with a mixture of pyridine (0.1 ml) and 1-(trimethylsilyl)imidazole (0.1 ml) for 3 hr. The reaction mixture (0.5 μl) was directly analyzed by GS-MS on a RTX-5MS capillary column (ϕ0.25 mm x 30 m, Restek). Injection port was set at 250℃ and the GC oven was held at 150℃ for 1 min followed by heating to 280℃ at 10℃/min and holding at the final temperature for 5 min. Under these conditions, retention times (t_R) of authentic samples were as follows; **16** 11:44, **17** 13:15, **18** 15:05, **19** 16:35. Control experiment was carried out in the absence

of the substrates and methyl stearate (t_R 9:56) was used as an internal standard.

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References

1. (a) Oikawa, H.; Ichihara, A.; Sakamura, S. *J. Chem. Soc., Chem. Commun.* **1990**, 908-909; (b) Oikawa, H.; Murakami, Y.; Ichihara, A. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2949-2953; (c) Oikawa, H.; Aihara, Y.; Ichihara, A.; Sakamura, S. *Biosci. Biotech. Biochem.* **1992**, *56*, 684; (d) Tabuchi, H.; Oikawa, H.; Ichihara, A. *J. Chem. Soc., Perkin Trans. 1* **1994**, 2949-2953.
2. (a) Izumi, K.; Kamiya, Y.; Sakurai, A.; Oshio, H.; Takahashi, N. *Plant Cell Physiol.* **1985**, *26*, 821-827; (b) Takano, H.; Oguri, Y.; Kato, T. *J. Pesticide Sci.* **1983**, *8*, 575.
3. Zamir, L. O.; Devor, K. A.; Sauriol, F. *J. Biol. Chem.* **1991**, *266*, 14992-15000.
4. Dalziel, W.; Hesp, B.; Stevenson, K. M.; Jarvis, J. A. J. *J. Chem. Soc., Perkin Trans I.* **1973**, 2841-2851.
5. (a) Starratt, A. N.; Loschiavo, S. R. *Can. J. Microbiol.*, **1974**, *20*, 416-7; (b) Kawada, K.; Kimura, Y.; Katagiri, K.; Suzuki, A.; Tamura, S. *Agric. Biol. Chem.* **1978**, *42*, 1611-1612; (c) Ichihara, A.; Oikawa, H.; Hayashi, K.; Hashimoto, M.; Sakamura, S.; Sakai, R. *Agric. Biol. Chem.* **1984**, *48*, 1687-1689.
6. Ikegami, S.; Taguchi, T.; Ohashi, M.; Oguro, M.; Nagano, H.; Mano, Y. *Nature* **1978**, *275*, 458-60.
7. (a) McMurry, J. E.; Andrus, A.; Ksander, G. M.; Musser, J. H.; Johnson, M. A. *J. Am. Chem. Soc.* **1979**, *101*, 1330; (b) Trost, B. M.; Nishimura, Y.; Yamamoto, K. *J. Am. Chem. Soc.* **1979**, *101*, 1328; (c) Corey, E. J.; Tius, M. A.; Das, J. *J. Am. Chem. Soc.* **1980**, *102*, 1742-1744; (d) Ireland, R. E.; Godfrey, J. D.; Thaisrivongs, S. *J. Am. Chem. Soc.* **1981**, *103*, 2446-2448; (e) van Tamelen, E. E.; Zawacky, S. R.; Russell, R. K.; Carlson, J. G. *J. Am. Chem. Soc.* **1983**, *105*, 142-143; (f) Bettollo, R. M.; Tagliaesta, P.; Lupi, A.; Bravetti, D. *Helv. Chem. Acta* **1983**, *66*, 1922; (g) Holton, R. A.; Kennedy, R. M.; Kim, H.-B.; Krafft, M. E. *J. Am. Chem. Soc.* **1987**, *109*, 1597-1600.
8. Adams, M. R.; Bu'Lock, J. D. *J. Chem. Soc., Chem. Commun.* **1975**, 389-391.
9. Ackland, M. J.; Hanson, J. R.; Ratcliffe, A. H.; Sadler, I. H. *J. Chem. Soc., Chem. Commun.* **1982**, 165-166.
10. (a) Ackland, M. J.; Gordon, J. F.; Hanson, J. R.; Yeoh, B. L.; Ratcliffe, A. H. *J. Chem. Soc., Chem. Commun.* **1987**, 1492-1493; (b) Ackland, M. J.; Gordon, J. F.; Hanson, J. R.; Ratcliffe, A. H. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2009-2012.
11. Mau, C. J.; West, C. A. *Proc. Natl. Acad. Sci. USA*, **1994**, *91*, 8497-8501.
12. Yamaguchi, S.; Saito, T.; Abe, H.; Yamane, H.; Murofushi, N.; Kamiya, Y. *Plant J.* **1996**, *10*, 203-213.
13. Wildung, M. R.; Croteau, R. *J. Biol. Chem.* **1996**, *271*, 9201-9204.
14. Vogel, B. S.; Wildung, M. R.; Vogel, G.; Croteau, R. *J. Biol. Chem.* **1996**, *271*, 23262-23268.
15. Our preliminary work, see: Oikawa, H.; Ichihara, A.; Sakamura, S. *Agric. Biol. Chem.* **1989**, *53*, 299-300.
16. Oikawa, H.; Ichihara, A.; Sakamura, S. *J. Chem. Soc., Chem. Commun.* **1988**, 600-602.
17. (a) Ichihara, A.; Oikawa, H.; Hayashi, K.; Sakamura, S.; Furusaki, A.; Matsumoto, T. *J. Am. Chem. Soc.* **1983**, ***105***, 2907-2908; (b) Ichihara, A.; Oikawa, H.; Hashimoto, M.; Sakamura, S.; Haraguchi, T.; Nagano, H. *Agric. Biol. Chem.* **1983**, *47*, 2965-2967.
18. Ackland, M. J.; Gordon, J. F.; Hanson, J. R.; Yeoh, B. L.; Ratcliffe, A. H. *J. Chem. Soc., Perkin Trans. 1* **1988**, 1477-1480.
19. (a) Chu, M.; Coates, R. M. *J. Org. Chem.* **1992**, *57*, 4590-4597; (b) Mohan, R. S.; Yee, N. K.; Coates, R. M.; Ren, Y. Y.; Stamenkovic, P.; Mendez, I.; West, C. A. *Arch. Biochem. Biophys.*, **1996**, *330*, 33-47.
20. Manchand, P. S.; White, J. D.; Wright, H.; Clardy, J. *J. Am. Chem. Soc.* **1973**, *95*, 2705.
21. Manchand, P. S.; Blount, J. F. *J. Chem. Soc., Chem. Commun.* **1975**, 894-895.
22. Tamogami, S.; Mitani, M.; Kodama, O.; Akatsuka, T. *Tetrahedron* **1993**, *49*, 2025-2032.
23. Chamy, M. C.; Piovano, M.; Garbarino, J. A.; Miranda, C.; Gambaro, V.; Rodriguez, M. L.; Ruiz-Perez, C.; Brito, I. *Phytochem.* **1991**, *30*, 589-592.
24. Chamy, M. C.; Piovano, M.; Garbarino, J. A.; Gambaro, V. *Phytochem.* **1991**, *30*, 3365-3368.
25. Keller, N. P.; Hohn, T. M. *Fung. Genet. Biol.* **1997**, *21*, 17-29.