Cloning and Functional Expression of cDNA Encoding Aphidicolan-16β-ol Synthase: A Key Enzyme Responsible for Formation of an Unusual Diterpene Skeleton in Biosynthesis of Aphidicolin

Hideaki Oikawa,*,† Tomonobu Toyomasu,§ Hiroaki Toshima,† Satoshi Ohashi,† Hiroshi Kawaide,‡ Yuji Kamiya,‡ Minoru Ohtsuka,§ Shoko Shinoda,§ Wataru Mitsuhashi,§ and Takeshi Sassa*,§

Department of Applied Bioscience
Graduate School of Agriculture
Hokkaido University, Sapporo 060-8589, Japan
Institute of Physical and Chemical Research (RIKEN)
Wako, Saitama 351-0198, Japan
Department of Bioresource Engineering
Faculty of Agriculture, Yamagata University
Tsuruoka, Yamagata 997-8555, Japan

Received March 2, 2001
Revised Manuscript Received April 23, 2001

A tetracyclic diterpene, aphidicolin (**1**), was first isolated as an antiviral agent against Herpes simplex type 1.[1] Later, it was found that **1** shows a variety of biological activity such as antitumor[2a] and phytotoxic[2b] and specific inhibition of DNA polymerase α.[2c] Because of this latter property, **1** is a commercially available agent for studying cell cycles. Recently, it has been reported that **1** specifically damages a fragile site of the manmalian genome.[2d] Besides its remarkable bioactivity, its unique molecular skeleton has attracted synthetic chemists. This prompted numerous synthetic studies and, to date, more than 10 groups have achieved the total synthesis of **1**.[3]

Based on an incorporation study with doubly isotope labeled precursors, Bu'Lock et al. proposed that the molecular skeleton of **1** is constructed by a stepwise cyclization of geranylgeranyl diphosphate (GGDP, **2**) to aphidicol-16-ene (**5a**) via an unusual intermediate *syn*-copalyl diphosphate (*syn*-CDP, **3**) as outlined in Scheme 1.[4] According to incorporation studies with the plausible intermediates, Hanson et al. established that post-cyclization conversion to **1** occurs by two routes: a major route via aphidicolan-16β-ol (**4**) and a minor route via **5a**.[5] Accumulation of less oxidized intermediates in mycelia treated with P-450 inhibitors led us to propose cytochrome P-450 dependent sequential hydroxylations from **4** to **1**.[6] These data indicate that **4** is a major cyclization product of the corresponding diterpene cyclase. Herein, we report the cDNA cloning and functional expression

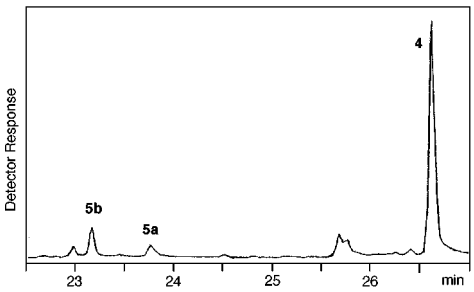
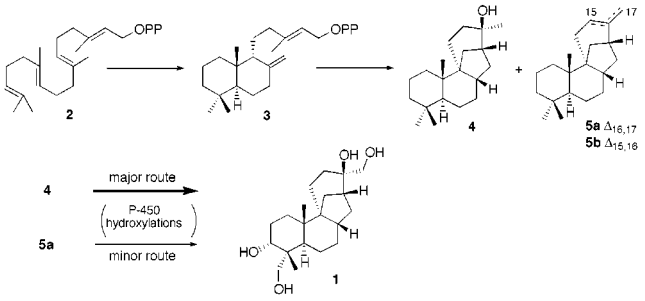


Figure 1. GC chart of the products of incubation of **2** with (GST)-ACS. DB-1 capillary column (ϕ 0.25 mm × 30 m, J&W Scientific); 100−280 °C, 5 °C/min. Numbers on the top of peaks correspond to compound numbers in the text.

Scheme 1. The Biosynthetic Pathway of Aphidicolin (**1**)



of aphidicolan-16β-ol synthase that is a key enzyme in aphidicolin biosynthesis in the fungus *Phoma betae* PS-13.[2b]

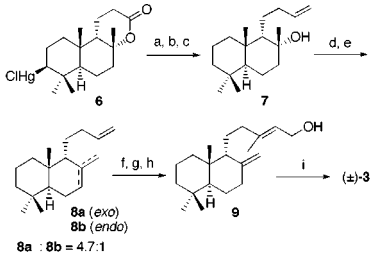
Reverse transcription-polymerase chain reaction (RT-PCR) with mRNA from *P. betae* and degenerate primers[7] based on the conserved amino acid sequences of plant and fungal diterpene cyclases allowed us to amplify the 1100-bp band that showed a significant similarity to fungal *ent*-kaurene synthases (FKS).[7] The nucleotide sequence of the full-length cDNA was determined by 5′ rapid amplification of the cDNA ends (5′-RACE) and 3′-RACE by using gene-specific primers. This contained the predicted 2997-bp open reading frame, encoding a product of 998 amino acids that was named aphidicolan-16β-ol synthase (ACS).[8] Homology searches indicate that the derived amino acid sequence of ACS shows good identity (36−37%)[9] with FKS and contains aspartate/glutamate rich motifs (DXDD and DD(E)XXD(E)). A full-length cDNA was ligated into a pGEX 4T-3 vector for a protein-expression analysis and the glutathione *S*-transferase (GST)-ACS fusion protein was expressed in *Escherichia coli* JM109. Purification of a cell-free extract with affinity chromatography for GST gave reasonably pure (GST)-ACS. The hexane extracts of the reaction mixture obtained by incubation of **2** with (GST)-ACS afforded three products **4**, **5a**, and **5b** (Figure 1). These products of the (GST)-ACS were identified, by comparison of retention time and mass spectra with those of synthetic standards,[6b] as aphidicolan-16β-ol (**4**, 87%), aphidicol-16-ene (**5a**, 5%), and aphidicol-15-ene (**5b**, 8%). Since all products are found in the mycelial extracts of *P. betae*, it is confirmed that these products are produced by a single enzyme. Recently, Croteau et al. reported that abietadiene synthase also produces multiple products.[10]

* Address correcpondence to these authors. E-mail: hoik@chem.agr.hokudai.ac.jp and tsassa@tds1.tr. yamagata-u.ac.jp.
† Hokkaido University.
§ Yamagata University.
‡ RIKEN.

(1) Dalziel, W.; Hesp, B.; Stevenson, K. M.; Jarvis, J. A. J. *J. Chem. Soc., Perkin Trans. 1* **1973**, 2841−2851.
(2) (a) Douros, J.; Suffness, M. *New Anticancer Drugs*; Carter, S., Sakurai, Y., Eds.; Springer-Verlag: Berlin, 1980. (b) Ichihara, A.; Oikawa, H.; Hayashi, K.; Hashimoto, M.; Sakamura, S.; Sakai, R. *Agric. Biol. Chem.* **1984**, *48*, 1687−1689. (c) Ikegami, S.; Taguchi, T.; Ohashi, M.; Oguro, M.; Nagano, H.; Mano, Y. *Nature* **1978**, *275*, 458−460. (d) Palin, A. H.; Critcher, R.; Fitzgerald, D. J.; Anderson, J. N.; Farr, C. J. *J. Cell Sci.* **1998**, *111*, 1623−1634.
(3) For a review on the synthesis of aphidicolanes, see: Toyota, M.; Ihara, M. *Tetrahedron* **1999**, *55*, 5641−5679.
(4) Adams, M. R.; Bu'Lock, J. D. *J. Chem. Soc., Chem. Commun.* **1975**, 389−391.
(5) (a) Ackland, M. J.; Hanson, J. R.; Yeoh, B. L.; Ratcliffe, A. H. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2705−2707. (b) Ackland, M. J.; Gordon, J. F.; Hanson, J. R.; Yeoh, B. L.; Ratcliffe, A. H. *J. Chem. Soc., Perkin Trans. 1* **1988**, 1477−1480.
(6) (a) Oikawa, H.; Ichihara, A.; Sakamura, S. *Agric. Biol. Chem.* **1989**, *53*, 299−300. (b) Oikawa, H.; Ohashi, S.; Ichihara, A.; Sakamura, S. *Tetrahedron* **1999**, *55*, 7541−7554.
(7) (a) Kawaide, H.; Imai, R.; Sassa, T.; Kamiya, Y. *J. Biol. Chem.* **1997**, *272*, 21706−21712. (b) Toyomasu, T.; Kawaide, H.; Ishizaki, A.; Shinoda, S.; Otsuka, M.; Mitsuhashi, W.; Sassa, T. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 660−664.
(8) The nucleotide sequence reported in this paper was deposited in the GenBank/EBI Data Bank with accession number AB049075.
(9) See Supporting Information for sequence alignments for ACS and FKS.
(10) Peters, R. J.; Flory, J. E.; Jetter, R.; Ravn, M. M.; Lee, H.-J.; Coates, R. M.; Croteau, R. B. *Biochemistry* **2000**, *39*, 15592−15602.

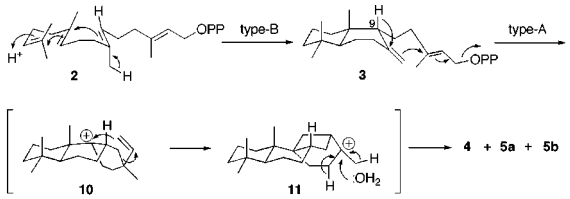
10.1021/ja015747j CCC: $20.00
Published on Web 05/05/2001

Scheme 2.[a] Synthesis of *syn*-Copalyl Diphosphate (**3**)



[a] Conditions: (a) $NaBH_4$/3 M NaOH, EtOH−$CHCl_3$, 4 °C to room temperature, 30 min, 92%; (b) DIBAH/THF, −78 °C, 30 min; (c) Ph_3PCH_3Br, NaH/DMSO, room temperature to 70 °C, 2 h, 82%, 2 steps; (d) Ac_2O, Et_3N, DMAP/toluene, 70 °C, 2 d, 91%; (e) 2,4,6-collidine, reflux, 8 h, 93%; (f) O_2, $PdCl_2$, CuCl/DMF−H_2O, room temperature, 5 h, *endo*:*exo* = 4.2:1,[14] 82%; (g) $(EtO)_2P(O)CH_2CO_2Me$, NaH/THF, 4 °C to room temperature, overnight, *E*:*Z* =6.4:1, 96%; (h) DIBAH/Et_2O, 4 °C to room temperature, 1 h, 99%; (i) ref 13.

Scheme 3. Enzymatic Formation of Aphidicolan-16β-ol (**4**)



With (GST)-ACS in hand, the intermediacy of *syn*-copalyl diphosphate (**3**) was next examined. Synthesis of **3** was started from racemic lactone **6**[11] reported by Nishizawa et al. (Scheme 2). Sequential reductions with $NaBH_4$ and DIBAH followed by Wittig olefination afforded alcohol **7**. Dehydration via the corresponding acetate afforded regioselectively a 4.7:1 mixture of olefins **8a** and **8b**. The olefins were converted into **9** by essentially the same protocol[12] (Wacker oxidation, Horner−Emmons olefination, and DIBAH reduction) reported previously. After separation of the minor regioisomer derived from **8b** by reverse phase HPLC, **9** was further converted to racemic **3**.[13] Under conditions identical to those used for incubation of **2**, **3** was incubated with (GST)-ACS. GC-MS analysis of the hexane extract of the reaction mixture showed essentially the same pattern as that of **2**. Thus, the result provided the conclusive evidence that the enzymatic transformation from **2** to **4** proceeds via intermediate **3**. Coates et al. previously reported that cell-free conversion of **3** to stemar-13-ene,[13] which is structurally related to **5a** and proposed to be a precursor of a phytoalexin in rice.

A proposed mechanism for the reaction catalyzed by aphidicolan-16β-ol synthase is shown in Scheme 3. The proton-initiated type B cyclization[15] of **2** provides **3** via an unusual chair-boat transition state. The second diphosphate-induced type A cyclization[15] is initiated by formation of an allylic cation followed by *si*-face attack of the olefin which is accompanied by a hydride shift from C-9 to C-8 to afford 8β-pimarenyl cation **10**. Electrophilic attack of the vinyl group and subsequent migration of the alkyl group give aphidicolenyl cation **11**. Final quenching of the carbocationic intermediate **11** proceeds in two ways: (1) deprotonation of either 15-H or 17-H and (2) stereoselective capture of water. Efficient quench of the cation with water is relatively unusual in terpene synthases.[16] Since the active sites of terpene synthase are usually hydrophobic to avoid improper quenching of the cationic intermediate with an external nucleophile such as water, the stereoselective delivery of water is an interesting character of this enzyme. *P. betae* produces a number of diterpene hydrocarbons including stemar-13-ene.[17] Investigation on the enzyme−product relationship is currently under way.

Despite a remarkable sequence similarity between ACS and FKS,[9] *pseudo*-enantiotopic and diastereotopic cyclizations of GGDP **2** with these enzymes afford the 6,6-bicyclic products (+)-*syn*-CDP **3** (ACS) and (−)-CDP (FKS), respectively. It suggests that a relatively small change in amino acid sequence of a cyclase is enough to promote the formation of a different diterpene carbon skeleton. Although the sesquiterpene synthases from microorganisms do not share high homology with each other,[18] our results reveal the significant homology among the fungal diterpene synthases, suggesting that a homology-based PCR strategy could be useful for cDNA cloning of other fungal diterpene synthases such as plant terpene synthases. Clustering for the biosynthetic genes of fungal natural products has been recently recognized.[19] This indicates that identification of the aphidicolan-16β-ol synthase gene would allow us to find a gene cluster for the aphidicolin biosynthesis by chromosome walking.

Acknowledgment. We are grateful to Prof. M. Nishizawa, Tokushima Bunri University, for providing information on preparation of **6** and Mr. K. Watanabe and Dr. E. Fukushi in our department for measuring MS spectra. This work was supported by grants (No. 10556024 to H.O. and No. 11460053 to T.S.) from the Ministry of Education, Science, Sports and Culture of Japan.

Supporting Information Available: GC-MS data on the authentic samples **4**, **5a**, and **5b** and the reaction products of **2** and **3** with (GST)-ACS, and sequence alignments of ACS and FKS (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA015747J

(11) Nishizawa, M.; Takao, H.; Iwamoto, Y.; Yamada, H.; Imagawa, H. *Synlett* **1998**, 79−80.

(12) Toshima, H.; Oikawa, H.; Ohashi, S.; Toyomasu, T.; Sassa, T. *Tetrahedron* **2000**, *56*, 8443−8450.

(13) 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.

(14) Selective formation of a byproduct from the *exo* isomer reduced an isomer ratio.

(15) MacMillan, J.; Beale, M. H. In *Isoprenoids Including Carotenoids and Steroids*; Cane, D., Ed.; Elsevier: Amsterdam, 1999; Vol. 2, pp 217−243.

(16) (a) Squalene-tetrahymaol synthase: Zander, J. M.; Greig, J. B.; Caspi, E. *J. Biol. Chem.* **1970**, *245*, 1247−1254. (b) Patchoulol synthase: Munck, S. L.; Croteau, R. *Arch. Biochem. Biophys.* **1990**, *282*, 58−64. (c) Epicubenol synthase: Nabeta, K.; Fujita, M.; Komuro, K.; Katayama, K.; Takasawa, T. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2065−2070. Cane, D. E.; Ke, N. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 105−107.

(17) Oikawa, H.; Toshima, H.; Ohashi, S.; König, W. A.; Kenmoku, H.; Sassa, T. *Tetrahedron Lett.* **2001**, *42*, 2329−2332.

(18) (a) Cane, D. E. *Chem. Rev.* **1990**, *90*, 1089−1103. (b) Caruthers, J. M.; Kang, I.; Rynkiewicz, M. J.; Cane, D. E.; Christianson, D. W. *J. Biol. Chem.* **2000**, *275*, 25533−25539.

(19) (a) Keller, N. P.; Hohn, T. M. *Fung. Genet. Biol.* **1997**, *21*, 17−29. (b) Tudzynski, B. *Appl. Microbiol. Biotechnol.* **1999**, *52*, 298−310.