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Diversity of diterpene hydrocarbons in fungus *Phoma betae*

Hideaki Oikawa,[a,*] Hiroaki Toshima,[a] Satoshi Ohashi,[a] Wilfried A. König,[b] Hiromichi Kenmoku[c] and Takeshi Sassa[c,*]

[a]*Department of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan*
[b]*Institute für Organische Chemie, Universität Hamburg, D-20146 Hamburg, Germany*
[c]*Department of Bioresource Engineering, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997-8555, Japan*

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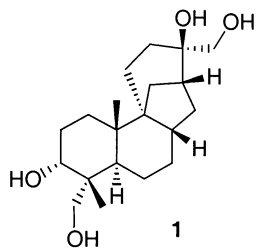
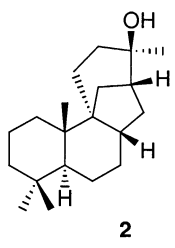
Abstract—Isolation and examination of diterpene hydrocarbons produced by the fungus *Phoma betae* allowed us to determine the structures of eight compounds. Analysis by capillary GC with a cyclodextrin derived chiral stationary phase allowed us to determine the absolute configuration of the diterpenes. The occurrence of aphidicolenes, stemar-13-ene, pimara- and isopimaradienes demonstrates the diversity of diterpenes found in this fungus.

To date a number of natural diterpene hydrocarbons have been found in plants,[1] whereas only a few studies on structure elucidation of diterpene hydrocarbons in fungi have been reported. Although several fungal strains producing aphidicolin (**1**), a specific inhibitor of DNA polymerase α, are known to yield more than six diterpene hydrocarbons,[2] no study was conducted to determine their structures and absolute configuration. During our study on the cloning of the aphidicolan-16β-ol synthase gene, we found that a phytopathgenic fungus *Phoma betae*[3] produces a number of diterpene hydrocarbons. In order to clone and to characterize the corresponding diterpene cyclase genes, we decided to determine their structures. Herein, we report the diversity of diterpene molecular skeletons produced by *P. betae*.

GC–MS analysis of the less polar fraction from the mycelial extracts of *P. betae* revealed that a major product aphidicolan-16β-ol (**2**)[3] is accompanied by at least 11 minor diterpene hydrocarbons (Fig. 1). The mass spectra of these hydrocarbons showed characteristic fragment peaks such as m/z 272 (M^+) and 257 (M^+–CH_3). Compared with authentic samples[3] two of them were identified as aphidicol-15-ene (**3**) and aphidicol-16-ene (**4**) which was proven to be a precursor of **1** in a minor biosynthetic pathway.[4] In order to determine the structures of the remaining hydrocarbons, large scale fermentation (21 L) was employed. The obtained mycelial extract was separated on SiO_2 column flash chromatography to afford a hydrocarbon fraction which was further chromatographed by reversed phase HPLC to give hydrocarbons **3**–**10** (ca. 10–460 μg).





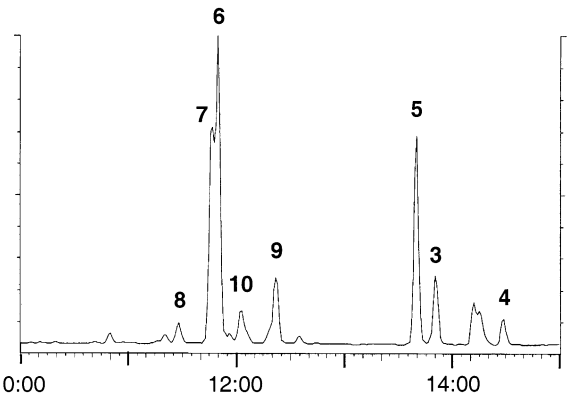


Figure 1. GC chart of *P. betae* hydrocarbon fraction. RTX-5MS capillary column (ϕ 0.25 mm×30 m, Restek); 150–280°C, 5°C/min. Numbers on the top of peaks correspond to compound numbers in the text.

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* Corresponding authors.

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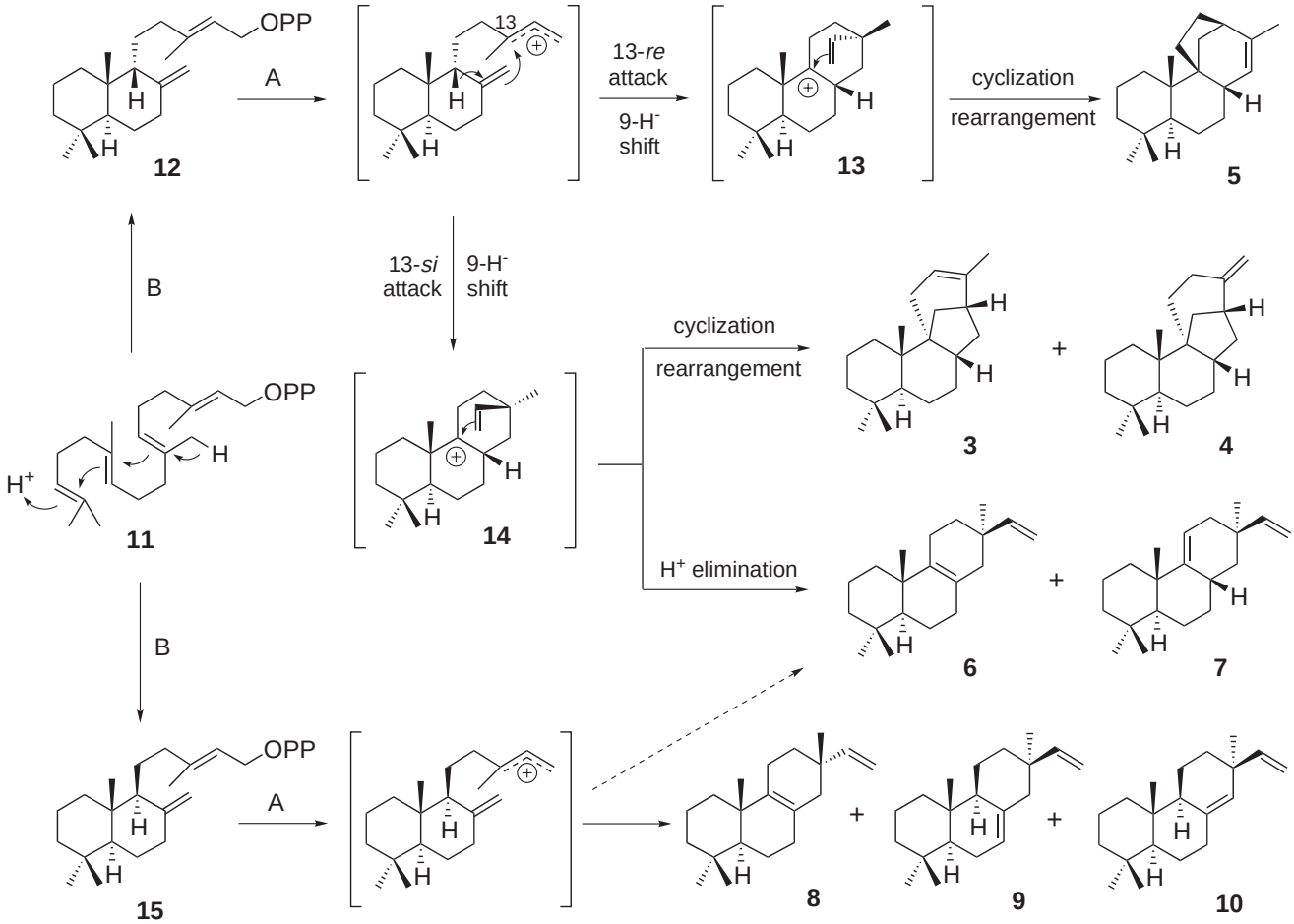

Scheme 1. The biogenetic pathway of diterpene hydrocarbons produced by *P. betae*. A and B represent two modes of cyclization catalyzed by diterpene cyclases.[15]

^{1}H NMR data of a relatively abundant constituent **5**[5] showed four singlet methyl groups and an olefin proton at 4.96 ppm which is coupled with the allylic methyl group at 1.62 ppm. These data suggested that this hydrocarbon is a tetracyclic monoolefin stemar-13-ene[6] (Scheme 1). Compound **5** has not been isolated from natural sources but is proposed to be a biosynthetic precursor of stemarin.[7] Optical rotation and all spectral data of **5** were identical to those of a synthetic reference material.[8]

Two abundant compounds **6**[5] and **7**[5] were isolated in sufficient amounts for spectroscopic analysis. Optical rotation and all spectral data revealed that **6** is identical to (+)-pimara-8,15-diene[9] (Scheme 1). ^{1}H NMR data of **7** showed four singlet methyl groups, three mutually coupled olefin signals and an olefinic signal at 5.29 ppm. These data suggested **7** to be a pimaradiene analog. Extensive NMR analysis including COSY, HSQC and HMBC allowed us to elucidate the planar structure of **7** (Fig. 2). The relative stereochemistry of **7** was deduced from coupling constants, NOED and NOESY experiments, as shown in Fig. 2. Although 8-epimer of **7** was isolated from a plant source,[10] **7** has not been reported in literature before.

Based on the occurrence of **6** and **7**, the other minor constituents **8**, **9** and **10** were speculated to be members of the pimaradiene family. Since isolation of sufficient amounts of diterpenes was impractical, we prepared a mixture of pimaradienes by acid treatment[9] of copalol. Comparison of the authentic pimaradienes with GC–MS analysis allowed us to identify **8**, **9** and **10** as isopimara-8,15-diene,[9] pimara-7,15-diene,[9] pimara-8(14),15-diene,[9] respectively (Scheme 1). The structures of the pimaradienes **7**, **9** and **10** were further confirmed by acid-catalyzed isomerization[11] to more stable **6**.

Separations of enantiomeric diterpene hydrocarbons with capillary GC with chiral stationary phase are limited due to the fact that only in a few cases are both

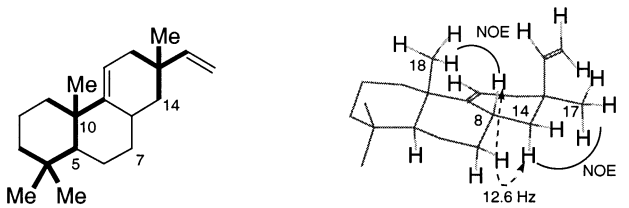

Figure 2. HMBC correlations and relative stereochemistry of 8β-pimara-9(11),15-diene (**7**).

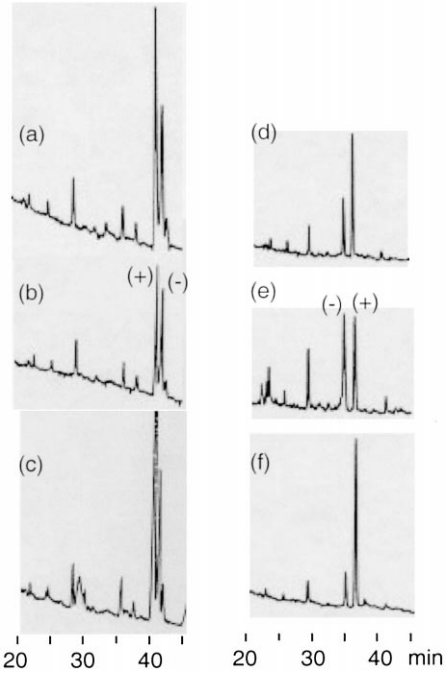

Figure 3. Enantiomer separation of diterpene hydrocarbons **6** and **8**. (a) (±)-**6**+**6** from isomerization of **9**; (b) (±)-**6**; (c) (±)-**6**+(+)-**6**; (d) (±)-**8**+**8** isolated from *P. betae*; (e) (±)-**8**; (f) (±)-**8**+(+)-**8**. 25 m fused silica capillary with heptakis(6-*O*-*t*-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin (50% in OV 1701, w/w) at 150°C.

enantiomers present in a single natural source.[12] We recently developed an efficient synthesis[13] of both enantiomers of copalol via optical resolution of synthetic intermediates. Thus, both racemic and enantiomerically pure **6** and **8** were prepared by acid treatments[9] of the synthetic copalols. Co-injection of the racemate and (+)-enantiomers of **6** and **8** in chiral GC analysis under the conditions[14] reported allowed us to assign the order of elution of the enantiomers, as shown in Fig. 3. Pimaradienes **6** and **8**, as prepared by the isomerization of **7**, **9** and **10** were analyzed under the same conditions. The resultant data indicated that the absolute configuration of the diterpene hydrocarbons was as shown in Scheme 1. Importantly, all hydrocarbons isolated from *P. betae* have 5*S*, 10*S* configuration.

On the basis of the structures of the hydrocarbons their general biogenetic pathway can be drawn as shown in Scheme 1. Type-B cyclization[15] of geranylgeranyl diphosphate (GGDP, **11**) provides 9,10-*syn*-copalyl diphosphate (*syn*-CDP, **12**)[15] which further undergoes second type-A cyclization[15] from either *re*- or *si*-face attack of C-13 olefin and hydride shift from C9 to C8 to afford carbocations **13** and **14**. Deprotonation of either 8-H or 11-H in **14** would provide **6** and **7**, whereas sequential rearrangement of **13** and **14** provided **5** and **4**, respectively. Alternative type-B cyclization of **11** would afford copalyl diphosphate (CDP, **15**) from which type-A cyclization proceeds to yield various pimaradiene analogs **6**, **8**, **9** and **10**. Although the involvement of **12** in the biosynthesis of aphidicolane, stemodane and stemarane diterpenes[15] is proposed according to the labeling pattern of **1** after incorporation of doubly labeled acetate[2] and cell-free conversion of **12** to oryzalexin S,[6] isolation of **7** can be regarded as the first direct evidence of the intermediacy of **12** and 8-epipimarenyl cation **14**, which has the correct stereochemistry at C-13 to afford **2**, in the biosynthesis of aphidicolin (**1**). The co-occurrence of structurally related aphidicol-16-ene (**4**) and stemar-13-ene (**5**) is especially noteworthy since it has not been reported before and the stemarane skeleton diterpene has been found only in plants.

It has been reported that single mono- and sesquiterpene cyclases produce multiple products.[16] This implies that a single diterpene cyclase possibly produces several diterpene hydrocarbons in the second cyclization of **12** or **15**. Croteau et al. recently reported that pseudomature abietadiene synthase produces three major and three minor diterpene hydrocarbons.[17] On the other hand, five known diterpene synthases and aphidicolan-16β-ol synthase,[18] which we have recently cloned, catalyze predominant formation of a single product. Thus, it is an interesting question how many enzymes are involved in the formation of the diterpene hydrocarbons found in the mycelial extract of *P. betae*. This enzyme–product relationship could be answered by cloning and expression of the gene encoding diterpene cyclases. On this point of view, *P. betae* is an interesting system for studying the diterpene cyclases.

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References

1. (a) Glasby, J. S. *Encyclopaedia of the Terpenoids*; Wiley: New York, 1982; (b) For periodical reviews, see: Hanson, J. R. *Nat. Prod. Rep.* **2000**, *17*, 165–174.
2. (a) Ackland, M. J.; Hanson, J. R.; Yeoh, B. L.; Ratcliffe, A. H. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2705–2707; (b) Adams, M. R.; Bu'Lock, J. D. *J. Chem. Soc., Chem. Commun.* **1975**, 389–391; (c) Dalziel, W.; Hesp, B.; Stevenson, K. M.; Jarvis, J. A. J. *J. Chem. Soc., Perkin Trans. 1* **1973**, 2841–2851.
3. (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.
4. (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.

5. Data for **5**, **6** and **7** are as follows, **5**: $[\alpha]_D^{27}$ +49.0 (*c* 0.01, $CHCl_3$); synthetic **5**:[8] $[\alpha]_D^{21}$ +55.9 (*c* 0.56, $CHCl_3$). **6**: $[\alpha]_D^{27}$ +21.6 (*c* 0.019, hexane); lit.[9] $[\alpha]_D$ +57.9 (*c* 0.59, hexane). **7**: $[\alpha]_D^{27}$ +16.1 (*c* 0.038, $CHCl_3$); δ_H (400 MHz, $CDCl_3$), 5.77 (dd, $J=11.2$, 17.2 Hz, 1H), 5.29 (m, 1H), 4.85–4.93 (m, 2H), 0.975 (s, 3H), 0.950 (s, 3H), 0.838 (s, 3H×2).
6. 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.
7. Manchand, P. S.; Blount, J. F. *J. Chem. Soc., Chem. Commun.* **1975**, 894–895.
8. Berettoni, B.; Bettolo, R. M.; Montanari, V.; Prencipe, T.; Romeo, S. *Helv. Chim. Acta* **1991**, *74*, 1671–1678.
9. Hall, S. F.; Oehlschlager, A. C. *Tetrahedron* **1972**, *28*, 3155–3173.
10. Knudsen, F. S.; Vilegas, W.; Oliveira, F.; Roque, N. F. *Phytochemistry* **1986**, *25*, 1240–1242.
11. McCreadie, T.; Overton, K. H. *J. Chem. Soc. (C)* **1971**, 312–316.
12. Pietsch, M.; Konig, W. A. *Phytochem. Anal.* **1999**, *11*, 99–105.
13. Toshima, H.; Oikawa, H.; Ohashi, S.; Toyomasu, T.; Kawaide, H.; Sassa, T. *Tetrahedron* **2000**, *56*, 8443–8450.
14. (a) König, W. A. *Chirality* **1998**, *10*, 499–504; (b) Pietsch, M.; König, W. A. *J. High Resolut. Chromatogr.* **1997**, *20*, 257–260.
15. MacMillan, J.; Beale, M. H. In *Isoprenoids Including Carotenoids and Steroids*; Cane, D., Ed. Diterpene Biosynthesis. Elsevier: Amsterdam, 1999; Vol. 2, Chapter 8, pp. 217–243.
16. (a) Colby, S. M.; Alonso, W. R.; Katahira, E. J.; McGarvey, D. J.; Croteau, R. *J. Biol. Chem.* **1993**, *268*, 23016–23024; (b) Steele, C. L.; Crock, J.; Bohlmann, J.; Croteau, R. *J. Biol. Chem.* **1998**, *273*, 2078–2089; (c) Schmidt, C. O.; Bouwmeester, H. J.; Bulow, N.; König, W. A. *Arch. Biochem. Biophys.* **1999**, *364*, 167–177.
17. 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.
18. Oikawa, H.; Toyomasu, T.; Toshima, H.; Ohashi, S.; Kawaide, H.; Kamiya, Y.; Ohtuka, M.; Shinoda, S.; Mitsuhashi, W.; Sassa, T., unpublished results.