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Proposed mechanism for diterpene synthases in the formation of phomactatriene and taxadiene†

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Received 9th May 2005, Accepted 1st June 2005
First published as an Advance Article on the web 21st June 2005

To obtain insight into how the cyclization pathway is controlled, the mechanism of diterpene synthase reactions (the putative phomactatriene synthase and taxadiene synthases) involving the same intermediate was investigated in detail. The mechanism of the initial transformation of GGDP to verticillen-12-yl cation ($\mathbf{A}^+$) was proposed based on the labelling pattern of phomactatriene (**9a**) obtained in the feeding experiments with ^{13}C-labelled acetates. To obtain information on the reaction pathway of $\mathbf{A}^+$ to **9a** and taxadiene (**10**), reactions of verticillol (**5**) with various acids were conducted. Structural determination of products **9b, 9c, 13a, 13b** and **14** allowed us to propose a reaction pathway *via* cations $\mathbf{A}^+$, $\mathbf{D}^+$, $\mathbf{E}^+$, $\mathbf{F}^+$ and $\mathbf{G}^+$. Identification of hydrocarbons **6, 9b, 13a** and **13b** in mycelial extracts of phomactin-producing fungus supported the proposed reaction mechanism. Based on the results of *ab initio* calculations for highly flexible cation intermediates, a mechanism is proposed.

Introduction

Bicyclic diterpene phomactins represented by phomactin B (**2**, Fig. 1) are known to be potent platelet-activating factor (PAF) antagonists.[1] PAF is a mediator of anaphylaxis released by a number of stimulated cells and causes platelet aggregation, chemotaxis, smooth muscle contraction and hypotension. Since PAF is proposed to be involved in inflammatory respiratory and cardiovascular diseases,[2] specific antagonist phomactins could be attractive agents for preventing these allergic and non-allergic inflammatory diseases. To date, eleven phomactins have been isolated from a marine fungus, *Phoma* sp.[1] Phomactins, including the recently found phomactin H (**4**)[3], possess a structurally unique bicyclo[9.3.1]pentadecane ring system. This unique structure and the pharmacological interest prompted the synthetic study of phomactins. The first total synthesis of the most potent PAF antagonist phomactin, phomactin D (**3**), was achieved by Miyaoka *et al.*[4] Recently, the total synthesis of the most structurally complex phomactin, phomactin A (**1**), was completed independently by two groups.[5]

Diterpenes with a bicyclo[9.3.1]pentadecane skeleton are found in various sources, but are relatively rare in nature. Verticillol (**5**) was isolated from a constituent of the evergreen wood of the conifer *Sciadopitys vertilillata*.[6] Its enantiomer and the corresponding hydrocarbons were found in Japanese liverwort *Jackiella javanica*.[7] The essential oil of *Boswellia carterii* contains verticilla-4(20),7,11-triene (**6**).[8] Recently, the cytotoxic diterpene cespitularin A (**7**) was found in the formosan soft coral, *Cespitularia hypotentaculata*.[9] Cleomeolide (**8**) from

† Electronic supplementary information (ESI) available: computational results for carbocations (energies and structures of conformers found by CONFLEX search), ^{1}H NMR and ^{13}C NMR spectra for compounds **9b,9c,13a,13b** and **14**. See http://dx.doi.org/10.1039/b506411b

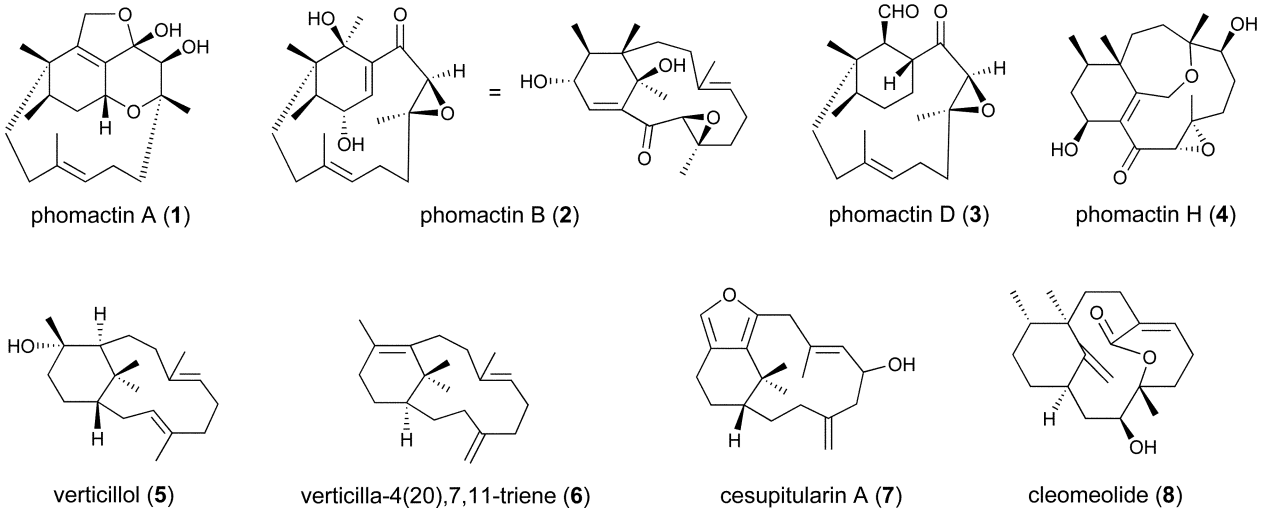
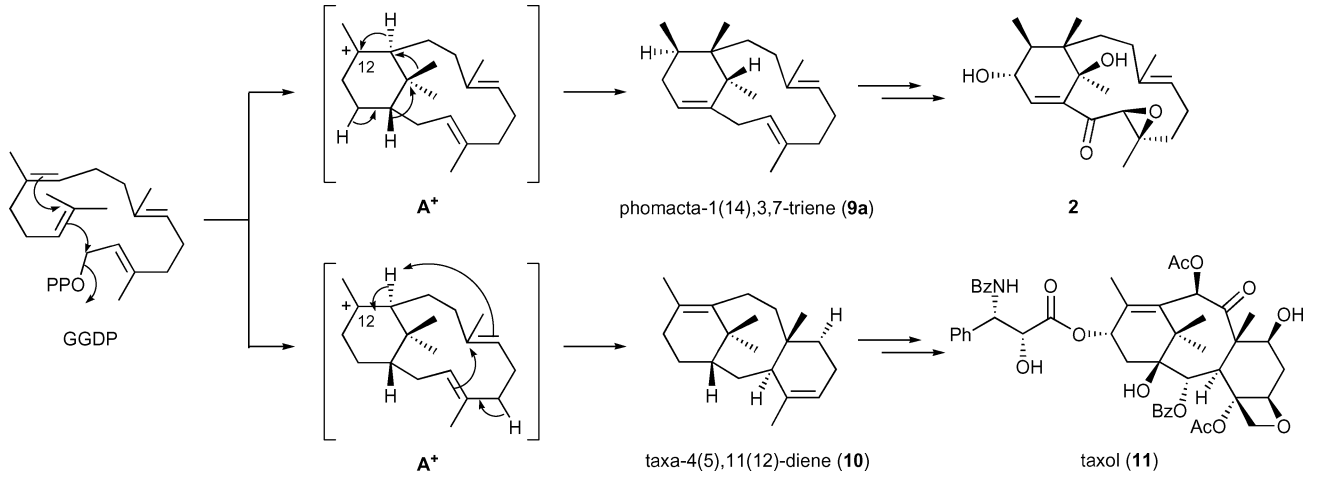


Fig. 1 Phomactins and other diterpenes with a bicyclo[9.3.1]pentadecane skeleton.

DOI: 10.1039/b506411b



Scheme 1 Proposed cyclization pathways of phomactane and taxane diterpenes *via* a common intermediate ($\mathbf{A}^+$).

Cleome icosandra Linn. is the sole example possessing the same molecular skeleton as those of phomactins although the absolute configuration of **8** is enantiomeric to that of phomactins.[10]

Considering the carbon framework of phomacta-1(14),3,7-triene (**9a**), a plausible precursor of phomactins, we proposed the cyclization pathway of **9a** from geranyl geranyl diphosphate (GGDP) involving the verticillen-12-yl cation intermediate ($\mathbf{A}^+$) as shown in Scheme 1.[11] A similar biogenetic proposal was made by Pattenden and co-workers.[12] Croteau and co-workers have investigated a detailed enzymatic cyclization pathway of taxa-4(5),11(12)-diene (**10**), a precursor of the antitumor agent taxol (**11**).[13,14a] They proposed that **10** was formed *via* $\mathbf{A}^+$ by an unusual intramolecular 1,5-proton transfer and further cyclization in a reaction with taxadiene synthase.[14b] Since the same intermediate $\mathbf{A}^+$ is involved in the biosynthesis of phomactin and taxol, we became interested in how key enzymes responsible for constructing these molecular skeletons control the reaction pathways to give individual products *via* intermediates having a highly flexible 12-membered ring. Recently, we suggested a detailed cyclization mechanism for the formation of phomactatriene (**9a**) *via* $\mathbf{A}^+$.[11] Herein we propose the cyclization mechanism of GGDP toward diterpene hydrocarbons based on experimental data from incorporation of isotopically labelled precursors, non-enzymatic reactions of verticillol (**5**) *via* $\mathbf{A}^+$ and *ab initio* calculations of carbocation intermediates.

Results

Mechanism of enzymatic cyclization of GGDP to verticillen-12-yl cation

We isolated phomacta-1(14),3,7-triene (**9a**) from *Phoma* sp. and determined the relative configuration by NOE experiments (Fig. 2).[11] Chu *et al.* previously reported Sch 49026 which is the epimer of **9a** at C15.[15] Since the structure was proposed without any experimental data and the spectral data are consistent with those of **9a**, the structure of Sch 49026 should be identical to that of **9a**. To investigate the stereochemical course in the formation of **9a** from GGDP, incorporation experiments were performed with *Phoma* sp. using [1-^{13}C]- and [1,2-$^{13}C_2$]acetates.[11] The ^{13}C NMR analysis of labelled **9a** exhibited typical labelling patterns for the isoprene unit derived from the mevalonate pathway as summarized in Table 1 and Fig. 3.[16] In the ^{13}C-NMR spectra derived from [1,2-$^{13}C_2$]acetate, the coupled signal at 37.69 ppm (C15) was observed as shown in Fig. 4. These results established that 11-Me migrated from C15 without loss of ^{13}C–^{13}C coupling, indicating that 11-Me originated from **a** in the corresponding dimethylallyl diphosphate (Scheme 2). No significant decrease in the intensity of the coupled signal at 37.69 ppm (C15) was observed on comparison with that at 41.60 ppm (C12) in the

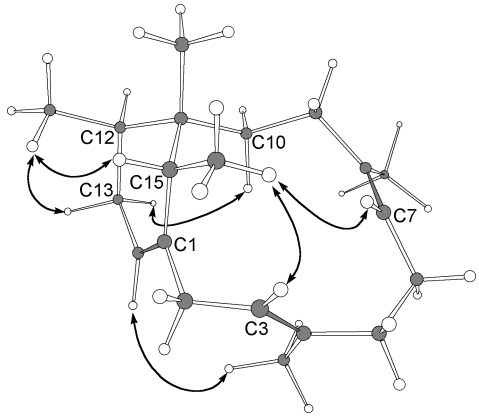


Fig. 2 NOE correlations of phomacta-1(14),3,7-triene (**9a**).

^{13}C-NMR spectrum (Fig. 4). This finding indicated that the free rotation of the C1–C15 bond, *e.g.* from $\mathbf{B}^+$ to $\mathbf{C}^+$ (Scheme 2), is restricted by the prefixed conformation in the active site of the plausible terpene synthase, phomactatriene synthase (PMTS).

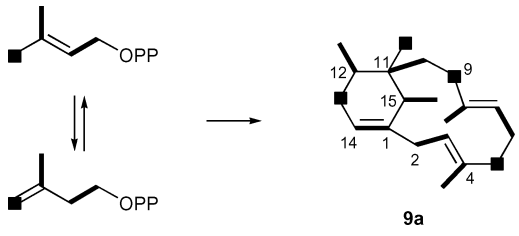


Fig. 3 Incorporation of [1,2-$^{13}C_2$]-acetate into **9a**.

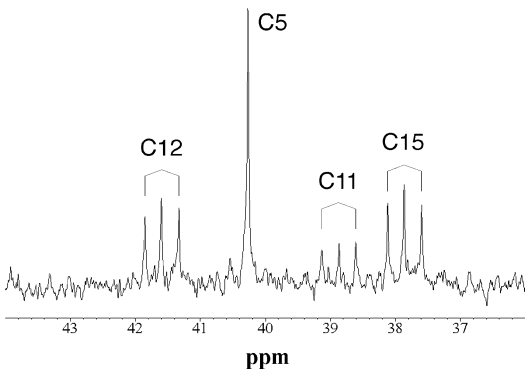
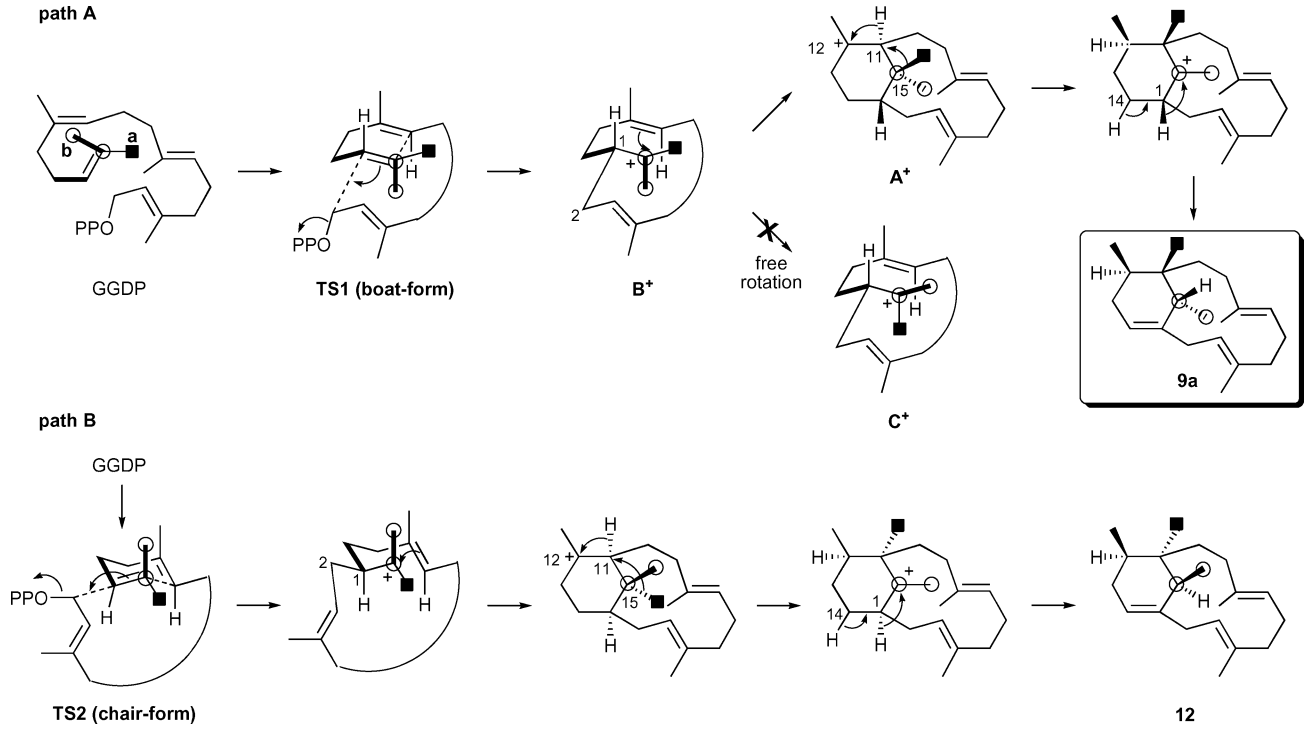


Fig. 4 Part of the ^{13}C NMR spectrum of **9a** from feeding with [1,2-$^{13}C_2$]-acetate.



Scheme 2

Table 1 ^{13}C NMR data of **1** derived from ^{13}C-labelled acetates[a]

			[1,2-$^{13}C_2$]Acetate	
	δ_C/ppm	[1-^{13}C]Acetate enrichment[b]	Enrichment[b]	J_{C-C}/Hz
1	139.08	—	3.0	71
2	35.44	19.8	1.6	43
3	127.21	—	2.6	43
4	136.10	20.8	2.4	42
5	40.27	—	2.2	—
6	25.07	19.1	3.0	45
7	123.08	—	2.6	45
8	134.07	14.9	4.2	42
9	33.79	—	2.5	—
10	34.34	15.8	2.6	35
11	38.86	—	3.0	35
12	41.60	11.7	2.7	36
13	31.63	—	2.4	—
14	121.58	19.2	2.7	71
15	37.87	13.9	2.6	35
4-Me	15.64	—	3.5	42
8-Me	17.18	—	4.0	42
11-Me	22.32	—	2.2	—
12-Me	17.74	—	3.1	36
15-Me	14.11	—	2.8	35

[a] The signals were assigned by NMR analyses including HMQC and HMBC.[11] [b] The values for enrichments were determined by comparison of the relative peak intensities of the corresponding carbons in labelled and unlabelled spectra.

Only two biosynthetic pathways are possible which result in coupled signals at C15 and 15-Me (Scheme 2). Two-step cyclization of GGDP can occur *via* boat- and chair-like transition states (**TS1** and **TS2**, respectively). Since 1,2-shifts would occur in a stepwise manner, and be possible from both the α- and β-faces, the stereochemistry of the product is simply determined by the stereogenic centers attached to the migrating groups. The first C–C bond formation in path A affords a 1*S*-configuration. The subsequent migration of 11-H, followed by a 1,2-rearrangement of 15β-Me/1β-H and deprotonation of 14-H afford phomactatriene (**9a**). On the other hand, similar C–C bond formation in path B gives 1*R*-configuration, and the sequential rearrangements including 15α-Me/1α-H provide **12**, a diastereomer of **9a**. Since the correct configuration of the product **9a** is 11*S*, 12*R*, 15*S*, the actual biosynthetic route should be path A *via* a boat-like transition state.

The detailed cyclization mechanism of the taxadiene synthase (TXDS) reaction was investigated with a partially purified enzyme using various deuterium-labelled GGDP.[13, 14a] However, the stereochemical course from GGDP to cationic intermediate $\mathbf{A}^+$ was not reported. Based on successful conversion of verticillol (**5**) to taxadiene (**10**) described later, we assume that the proposed cyclization mechanism in the reaction of the plausible phomactatriene synthase can be applied to that of the taxadiene synthase reaction.

Lewis acid-mediated rearrangements of verticillol

To explore the enzymatic reaction mechanism of PMTS and TXDS *via* intermediate $\mathbf{A}^+$, we next investigated biomimetic reactions of verticillol (**5**) with various acids. The results are summarized in Table 2. Treatment of **5** with $BF_3{\cdot}OEt_2$ at −78 °C for 10 min afforded three products, **9b, 13a** and **13b**, in the ratio 37 : 32 : 31 (Scheme 3). No starting material was detected under these conditions. Two of the isomeric compounds, **13a** and **13b**, were identified as *exo*- and *endo*-dehydration products which were reported as synthetic hydrocarbons derived from acid treatment of **5**.[17] Enantiomers of verticillenes (**13a** and **13b**) were also found as constituents of the Japanese liverwort.[18] Isolation of **13a** and **13b** strongly suggested formation of a key cationic intermediate $\mathbf{A}^+$ in this reaction. An NMR spectrum of the hydrocarbon **9b** was closely related to that of phomactatriene (**9a**) except for the presence of a tetrasubstituted olefin [δ_C 138.5 (C13), 123.6 (C14)]. Its planar structure was determined to be phomacta-1(15),3,7-triene by extensive NMR analysis including COSY, HSQC and HMBC. The stereochemistry of **9b** was determined by NOESY data as shown in Fig. 5. The structure of **9b** was further confirmed by isomerization of **9a** isolated from *Phoma* sp. into **9b** with $BF_3{\cdot}OEt_2$ at −30 °C (Table 2, entry 8). The isomerization did not proceed at −78 °C.

Non-enzymatic reactions with other protic (CH_3CO_2H, CF_3CO_2H, TsOH, HBF_4) and Lewis acids (Me_3B, Me_3Al, $SnCl_2$, $TiCl_2$, $TiCl_2(OiPr)_2$, $Cu(OTf)_3$, $Zn(OTf)_3$, $Sn(OTf)_3$, $Yb(OTf)_3$)

Table 2 Acid treatment of **5** and other diterpenes in CH_2Cl_2

				Product yield[b](%)						
				Verticillenes		Phomactatrienes				
Entry	Substrate	Acid	Temp./°C	**13a**	**13b**	**9a**	**9b**	**9c**	**14**	Others[c]
1	**5**	$BF_3{\cdot}OEt_2$	−78	32	31	—	37	—	—	—
2	**5**	$BF_3{\cdot}OEt_2$	−30	2	36	—	62	—	—	—
3	**5**	$BF_3{\cdot}OEt_2$	−20	<1	—	25	—	46	6	22
4	**13a**	$BF_3{\cdot}OEt_2$	−78	no reaction	—	—	—	—	—	—
5	**13a**	$BF_3{\cdot}OEt_2$	−30	3	32	—	—	65	—	—
6	**13b**	$BF_3{\cdot}OEt_2$	−78	—	no reaction	—	—	—	—	—
7	**13b**	$BF_3{\cdot}OEt_2$	−30	—	83	—	17	—	—	—
8	**9a**	$BF_3{\cdot}OEt_2$	−30	—	—	63	37	—	—	—
9	**9b**	$BF_3{\cdot}OEt_2$	−78	—	—	—	no reaction	—	—	—
10	**9b**	$BF_3{\cdot}OEt_2$	−30	—	—	—	97	3	—	—
11	**9b**	$BF_3{\cdot}OEt_2$	−20	—	—	—	74	8	—	18
12	**5**	HBF_4	−78	<1	18	—	44	—	—	37
13	**5**	*p*-TsOH	25	12	88	—	—	—	—	—
14	**5**	$La(OTf)_3$[a]	40	—	—	—	—	35	—	65[d]
15	**5**	$TiCl_4$	−95	—	—	—	—	—	10	90[e]

[a] In $(CH_2Cl)_2$. [b] Yields were calculated by peak areas in the GC chart. [c] Diterpene products. [d] Containing 32% of alcohols. [e] Containing 8% of chlorides.

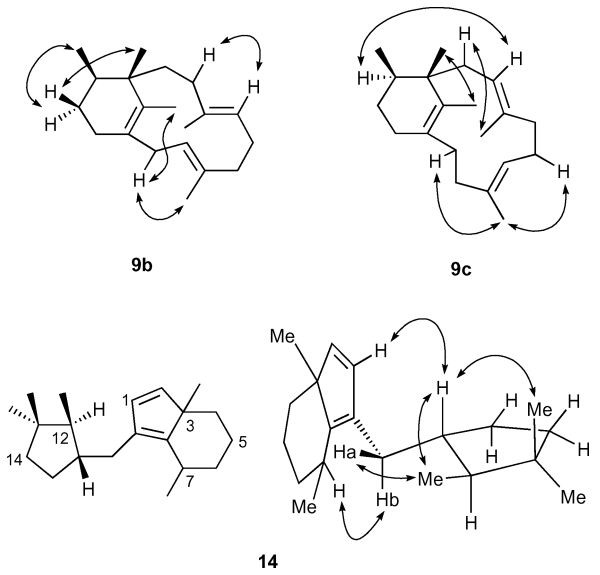


Fig. 5 NOE correlations of **9b, 9c** and **14**. The relative stereochemistry of **14** at C3 and C7 has not been determined.

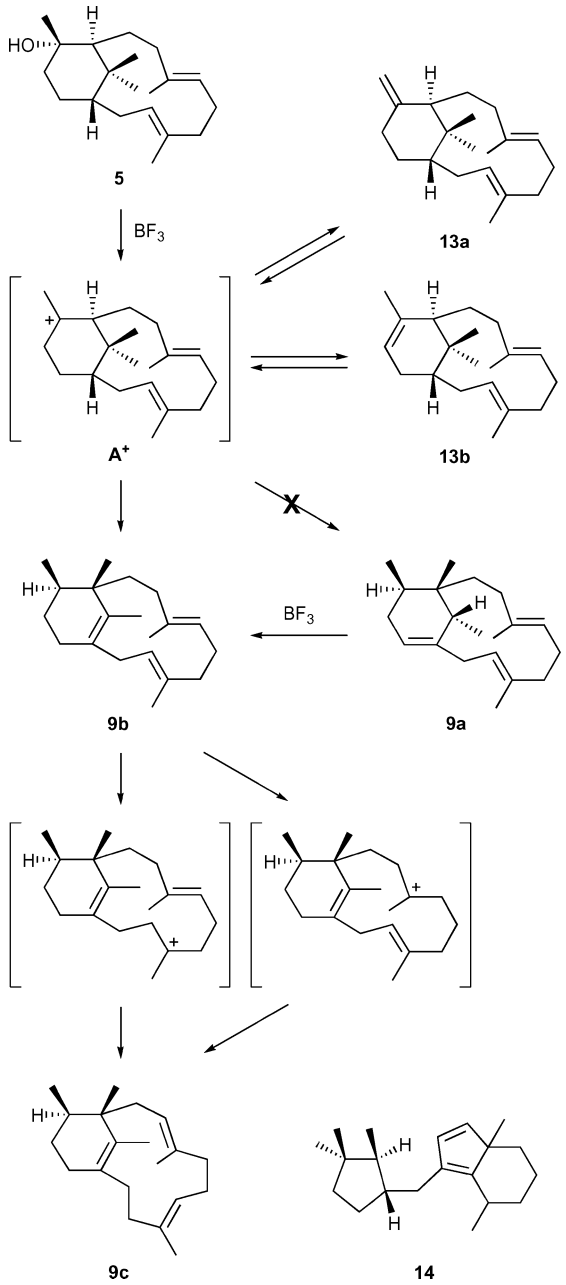
gave essentially the same results as those with BF_3 (Table 2). However, in the treatment of verticillol (**5**) with $La(OTf)_3$, the novel hydrocarbon **9c** was obtained as a major constituent of the reaction mixture. Since its NMR data were essentially same as those of phomacta-1(15),3,7-triene (**9b**), it is suggested that **9c** is an isomeric form of **9b**. This was confirmed by the BF_3-catalyzed conversion of **9b** to a product which showed the same retention time and spectra to those of **9c** in GC-MS analysis. The structure of **9c** including the relative stereochemistry was determined by extensive NMR analysis (Fig. 5).

Treatment of **5** with $TiCl_4$ afforded a novel diterpene hydrocarbon **14** as a major constituent with a number of minor diterpene chlorides which were not isolated in sufficient amounts to allow structure elucidation. This hydrocarbon **14** showed a different NMR pattern to those of other 12-membered hydrocarbons obtained in the reaction of **5**. In the UV spectra, absorption at 259.7 nm showed the presence of a conjugated diene. In the ^{1}H-NMR spectrum, olefin protons at 6.20 and 6.28 ppm had a relatively small coupling constant (5.2 Hz) for the *cis*-double bond, indicating that the double bond was in a five-membered ring. The structure of **14** including its partial stereochemistry was determined by extensive NMR analysis (Fig. 4). The proposed structure was further supported by the observation that the characteristic fragment ion peaks at *m/z* 148 ($M^+ - C_{11}H_{16}$) and 161 ($M^+ - C_{12}H_{17}$) were detected in the MS spectrum (Scheme 4). Transformation of **5** to **14** requires complex rearrangements including six- to five-membered ring conversion and opening of a 12-membered ring. The possible reaction mechanism affording **14** is shown in Scheme 4.

Treatment of verticillol **5** with BF_3 was conducted at various temperatures to examine the effect of reaction temperature, and the results are shown in Table 2. Predominant formation of phomacta-1(15),3,7-triene (**9b**) accompanied by a decrease of verticillenes (**13a, 13b**) was observed when the reaction temperature was raised from −78 °C to −20 °C. A new product which was identical to phomacta-1(15),4,8-triene (**9c**) produced in the reaction with $La(OTf)_3$ was observed to be formed at −20 °C, and the amount of **9c** further increased at 0 °C. At temperatures above −20 °C, many minor diterpene hydrocarbons were formed and the amounts of these products increased when the reaction was done at room temperature. Although the data obtained were inconclusive, the isolation of ring-opened product **14** suggested that various ring-opening reactions of the 12-membered ring of cation intermediates could occur affording a complex mixture of hydrocarbons in the BF_3 treatments at higher temperature.

Isolated hydrocarbons **9b, 13a** and **13b** were resubjected to the BF_3 treatments to evaluate their thermodynamic stability. *Exo*-isomer **13a** was efficiently converted into **9b** and **13b** at −30 °C while conversion of *endo*-isomer **13b** into **9b** was less effective. These results indicated that equilibrium existed among **13a, 13b** and **9b**, and the predominant formation of **9b** showed that this is the most thermodynamically stable isomer below −30 °C. Most of the reactions took place on the six-membered ring at below −30 °C. On the other hand, isomerization of the double bonds in the macrocycle of **9b** occurred at above −20 °C to give **9c**, probably *via* cation intermediates as shown in Scheme 3. Although isomerization of the 3,4- and 7,8-olefins occurred on the 12-membered ring in the reaction at −20 °C, no further reaction took place at these positions.

Pattenden and co-workers reported that all their efforts to transform verticillene and its derivatives into taxadiene derivatives with Lewis acid treatments failed.[17] To re-examine the possibility for the conversion of verticillol (**5**) to taxadiene (**10**), we tried to detect **10** in Lewis acid treatments of **5** under various



Scheme 3

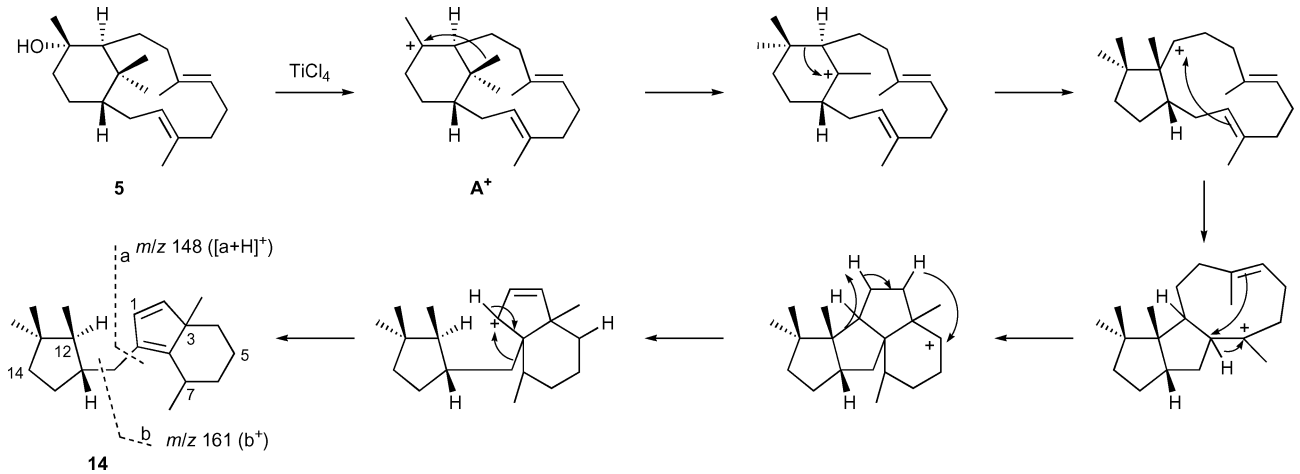
conditions. Among the complex mixtures obtained in the BF_3 treatment of **5** at room temperature, we identified a very small amount of **10** (0.004%) after repeated HPLC (Fig. 6). This was confirmed by a GC co-injection experiment with an authentic sample. Taxadiene was not observed in any other experiments which we have undertaken.

Diterpene hydrocarbons from *Phoma* sp.

It is common for terpene synthases to produce a small amounts of minor products which are derived from carbocation intermediates.[19] For example, abietadiene synthase gives pimara-8(4),15-diene, sandaracopimaradiene and palustradiene,[20] and taxadiene synthase affords taxa-4(20),11,12-diene and verticilla-3(4),7(8),11(12)-triene.[14a] During our studies on aphidicolan-16β-ol synthase (ACS), we found that the aphidicolin-producing fungus yielded various diterpene hydrocarbons which we proposed as minor products of the ACS reaction.[21] This observation prompted us to explore identification of the minor hydrocarbon constituents from the phomactin-producing fungus *Phoma* sp. From the mycelial acetone extracts, we identified phomactatriene (**9a**) and four other components by HPLC and GC-MS (Fig. 6). On comparison of MS spectral data with those of materials synthesized in the acid treatments of verticillol (**5**), three of them were identified as the 12-membered hydrocarbons **9b, 13a** and **13b**. The mass spectrum of the remaining compound, which was not obtained by the acid treatments of **5**, was identical to that of verticilla-4(20),7,11-triene (**6**)[8] from the essential oil of *Boswellia carterii*. GC co-injection experiments with the authentic samples **6, 9b, 13a** and **13b** confirmed their structures (Fig. 7). Since all diterpene hydrocarbons from *Phoma* sp. have a 12-membered ring similar to verticillane, these can be regarded as minor reaction products of the putative phomactatriene synthase. Interestingly, the acid treatments of verticillol (**5**) provided most of the diterpenes found in the extract of *Phoma* sp.

With authentic samples available, absolute configurations of the isolated hydrocarbons were examined. Since the yields of these compounds were limited (*ca.* 20–50 μg from 1 L culture broth), optical rotations did not give reliable data. On the other hand, CD-spectra of natural hydrocarbons **9b, 13a** and **13b** were identical to those of synthetic samples, determining their absolute configurations as shown in Scheme 5.‡

‡ Our conclusion on the absolute configurations of **9b, 13a** and **13b** contradicted that reported for verticillol (**5**). The proposed absolute configurations (ref. 1*b*) of phomactins A (**1**) and D (**3**) have been established unambiguously by their total synthesis (refs. 4, 5). Thus, the absolute configuration of phomactatriene (**9a**) is proposed as shown in Scheme 5. On the other hand, the absolute configuration of **5** was determined by the CD data of a derivative of **5** applying the empirical octant rule (ref. 6). Since the asymmetric total synthesis of **5** has not been achieved yet, the absolute configuration is possibly opposite to that originally proposed.



Scheme 4

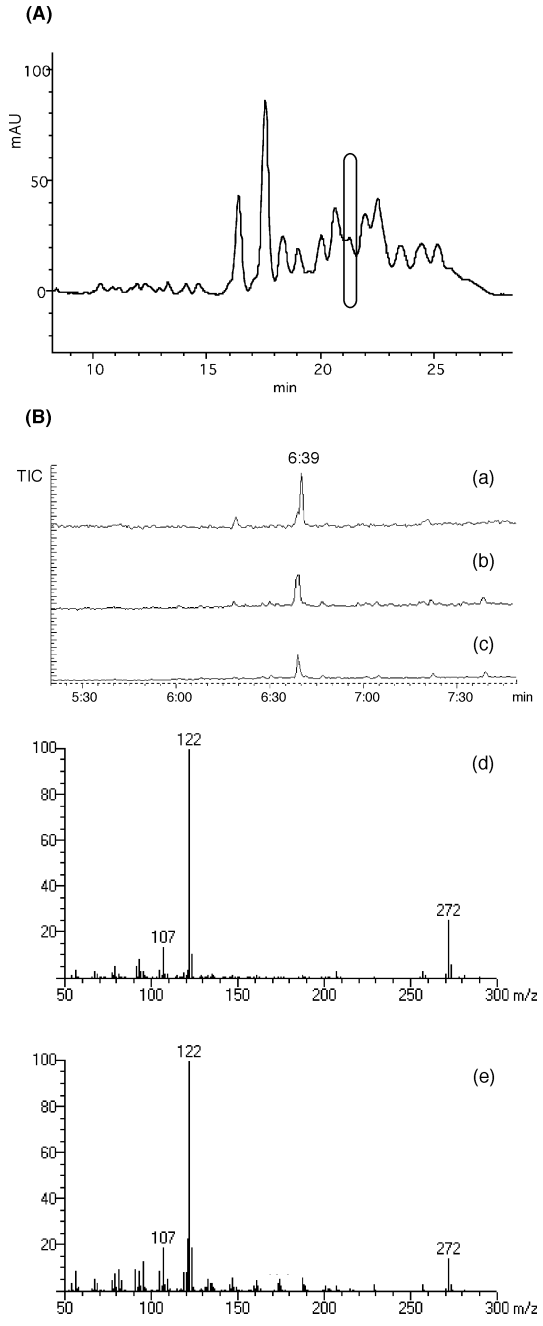


Fig. 6 (A) Reverse-phase HPLC chromatogram of products in the $BF_3{\cdot}OEt_2$-catalyzed reaction of **5** at room temperature; the area in the frame is the fraction containing taxadiene. (B) GC-MS analysis: (a) chromatogram of **10** purified repeatedly by HPLC; (b) chromatogram of the same sample as (a) co-injected with authentic **10**; (c) chromatogram of authentic **10**; (d) MS spectrum of the peak (t_R 6 : 39) in the chromatogram (a); (e) MS spectrum of authentic **10**.

Possible cyclization pathways of verticillane, phomactane and taxane diterpenes

There are several biosynthetic cyclization pathways to generate verticillane, phomactane and taxane diterpenes as shown in Scheme 5. Considering the stereochemistry of these diterpenes, we can propose that all such compounds are derived by macrocyclization of GGDP to verticillen-12-yl cation ($\mathbf{A}^+$) *via* a boat-like transition state as proposed in the case of phomactatriene (**9a**). From cation $\mathbf{A}^+$, simple deprotonation or capture of H_2O would give verticillenes (**13a,13b**) or verticillol (**5**). In the case of taxadiene synthase, Croteau established that an unusual intramolecular 1,5-proton transfer from C11 to C7 in $\mathbf{A}^+$ to give the verticillen-8-yl cation $\mathbf{D}^+$ which further cyclized to afford

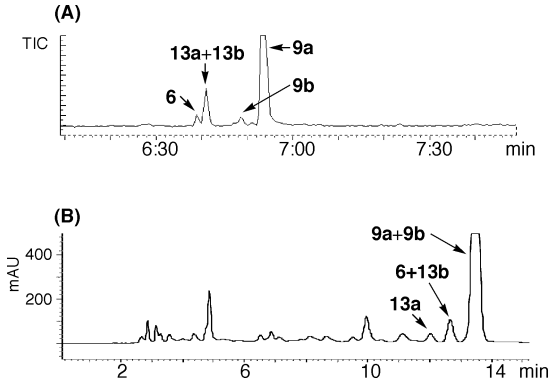


Fig. 7 GC-MS (A) and HPLC (B) analyses of diterpene hydrocarbons isolated from *Phoma* sp.

taxadiene (**10**).[14b] In the most stable conformer of $\mathbf{A}^+$ (described later), the H11 extrudes inside the 12-membered ring, and this proton is located close to both 3,4- and 7,8-double bonds. Thus, alternative 1,5-proton transfer from C11 to C3 would give verticillen-4-yl cation $\mathbf{E}^+$ and deprotonation of H_2O from $\mathbf{E}^+$ would afford verticillatriene (**6**). In the reaction of the putative PMTS, sequential 1,2-shifts of $\mathbf{A}^+$ could afford phomactan-15-yl cation $\mathbf{F}^+$ which is converted either to phomacta-1(15),3,7-triene (**9b**) or to core skeleton **9d** of cleomeolide (**8**) by deprotonation at 15-CH_3 or at H1. A hydride shift of H1 from $\mathbf{F}^+$ and the subsequent deprotonation of H14 would yield **9a**.

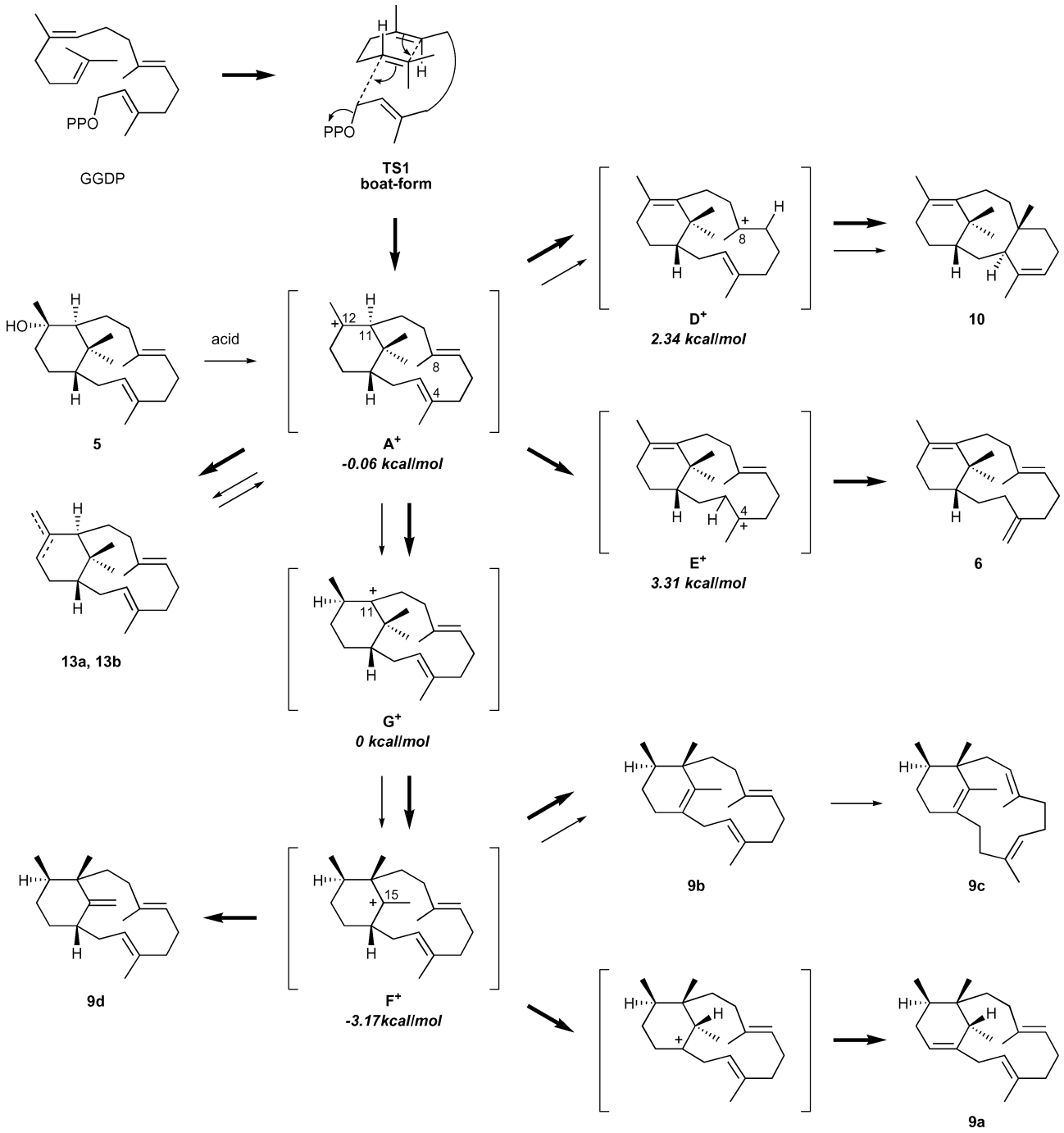
Ab initio calculations on the carbocationic intermediates

In order to evaluate the energy levels of the plausible cations, the relative energies of the cationic stationary structures were estimated by *ab initio* calculation[21c,22] using GAUSSIAN 03.[23] For this purpose, conformational searches on flexible bicyclo[9.3.1]pentadecane cation intermediates were necessary. Thus, conformational searches of the cations $\mathbf{A}^+$, $\mathbf{D}^+$, $\mathbf{E}^+$, $\mathbf{F}^+$, $\mathbf{G}^+$ were performed by using the CONFLEX5 program[24] with the MMFF94 force field[25] as the structural optimizer. The top five stable conformers of each cation found by CONFLEX were subjected to re-optimization at the HF/6-31G(d) level of *ab initio* molecular orbital theory.[23] *Ab initio* energies for the most stable conformers of carbocations are shown in Table 3.[26] The theoretical results show that the most stable cation is $\mathbf{F}^+$, and energy differences for the conversions from $\mathbf{A}^+$ to $\mathbf{G}^+$, and $\mathbf{G}^+$ to $\mathbf{F}^+$ are 0.06 and −3.17 kcal/mol, respectively, which explain most of our experimental results of acid treatments for verticillol (**5**) at various temperatures (see Table 2). Large energy differences between $\mathbf{A}^+$ and $\mathbf{F}^+$ strongly support the extremely facile transformation from verticillol **5** to phomacta-1(15),3,7-triene (**9b**) in the BF_3 treatment of **5** at −78 °C, and also the smooth conversion from verticillenes (**13a** and **13b**) to **9b** on increasing the reaction temperature. Additionally, theoretical prediction that the conversions from $\mathbf{A}^+$ to $\mathbf{D}^+$ and to $\mathbf{E}^+$ require higher energies, about 2.3 and 3.3 kcal mol^{-1}, respectively, than the conversion energy of $\mathbf{A}^+$ to $\mathbf{G}^+$ (0.06 kcal mol^{-1}), satisfactorily explains the inefficient conversion from **5** to taxadiene (**10**) in the BF_3 treatment at room temperature.

Table 3 *Ab initio* (HF/6-31G(d)) calculations of the most stable conformers of five cations

	$\mathbf{A}^+$	$\mathbf{D}^+$	$\mathbf{E}^+$	$\mathbf{F}^+$	$\mathbf{G}^+$
E [a]	−776.2916	−776.2877	−776.2862	−776.2965	−776.2915
ΔE [b]	−0.06	2.34	3.31	−3.17	0.00

[a] In au. [b] In kcal mol^{-1}.

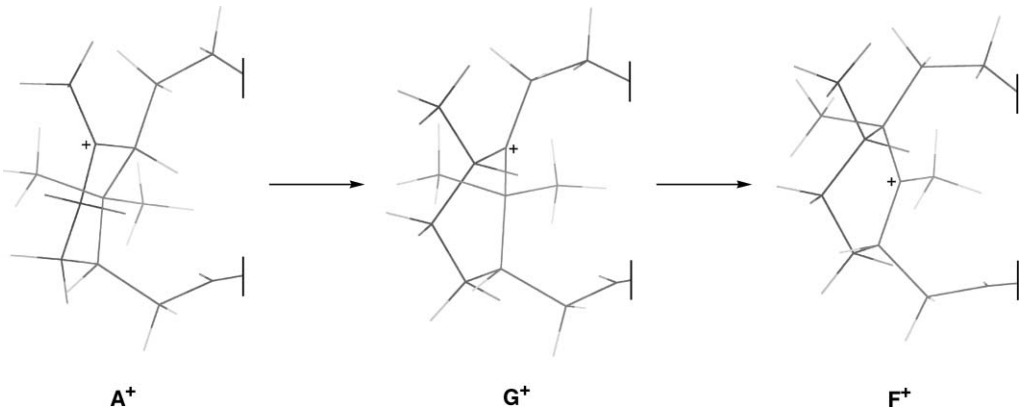


Scheme 5 Proposed enzymatic and non-enzymatic reactions of verticillene-12-yl cation ($\mathbf{A}^+$); the bold arrow indicates a route in the plausible enzymatic reaction.

Discussion

From the series of experimental results described above, we proposed a detailed cyclization mechanism of a plausible phomactatriene synthase (PMTS) as shown in Scheme 5. The results of feeding experiments with ^{13}C-labelled acetates suggested that PMTS incorporates GGDP and stabilizes it as a suitable boat-like transition state in which the terminal allylic pyrophosphate group and two olefins are located at proper positions for cyclization. Enzyme-assisted ionization of the diphosphate group and production of the resultant allylic cation trigger sequential C–C bond formations to give verticillen-12-yl cation ($\mathbf{A}^+$). Based on the incorporation study with ^{13}C-labelled acetate, these reactions proceed in a highly restricted space of the active site. Recently, experimental evidence has been accumulated on the preorganization of a polyene substrate in the active site of terpene synthases. Crystal structures of bornyl diphosphate synthase[27] and squalene–hopene cyclase[28] complexed with aza analogues of cation intermediates have been reported. In the active sites of these enzymes, highly flexible intermediates adopt suitable orientations and conformations for cyclization by a number of hydrophobic contacts with amino acid residues.

Experimental results on the acid treatment of verticillol (**5**) provide useful information on the reactivity of carbocationic intermediates and on the reaction pathways forming diterpene hydrocarbons. The result that BF_3 treatment of **5** at room temperature gave a number of products suggests the high reactivity of the initial intermediate $\mathbf{A}^+$ under standard conditions for the enzymatic reaction, and the existence of many reaction pathways yielding diterpene hydrocarbons. This suggests that the plausible PMTS freezes the conformation of cation $\mathbf{A}^+$ as the most stable



Scheme 6

one to avoid premature quenching to the products **13a** and **13b**. Facile transformation of **5** to phomacta-1(15),3,7-triene (**9b**) under mild conditions ($BF_3{\cdot}OEt_2$ at −78 °C) implies that conversion from the most stable conformer $\mathbf{A}^+$ to phomactadien-15-yl cation ($\mathbf{F}^+$) would proceed in a non-enzymatic manner. Since phomacta-1(14),3,7-triene (**9a**) was not obtained in the BF_3-treatment, the H1-hydride shift of $\mathbf{F}^+$ is an energy-requiring process which needs enzyme catalysis.

The transformation of $\mathbf{A}^+$ to $\mathbf{G}^+$ requires a conformational change from chair to boat (Scheme 6). Data on both non-enzymatic reactions and *ab initio* calculations suggested that this conversion is slightly endothermic with a very small barrier. Based on these results, we assume that the conformation of $\mathbf{A}^+$ derived from enzymatic cyclization of GGDP would be similar to a transition state in the rearrangement of $\mathbf{A}^+$ to $\mathbf{G}^+$.

Isolation of **9b, 13a** and **13b** from mycelial extracts of phomactin-producing fungus strongly suggests involvement of the two distinct intermediates $\mathbf{A}^+$ and $\mathbf{F}^+$ in the enzymatic reaction with PMTS. Thus, in the complex transformation of GGDP to **9a**, the role of PMTS appears rather straightforward. Apart from the formation and termination of carbocation intermediates, this enzyme may control conformations of intermediates and chaperon these short-lived intermediates with rich aromatic amino acid residues at the active site to prevent premature quenching. The inefficiency of 1,5-proton transfer in the production of taxadiene (**10**) indicates that conversion from $\mathbf{A}^+$ to $\mathbf{D}^+$ requires an enzyme-assisted conformational change and this is a key branching point between PMTS and TXDS. It should be noted that PMTS possibly promotes an alternative endothermic 1,5-proton transfer to provide verticillatriene (**6**), suggesting that the ability for 1,5-proton transfer may be attributed to a minute differences in the active site of the diterpene cyclases. This suggests that a mutation changing the volume of the active site could alter the reaction pathway of these diterpene synthases from **9a** to **6** or **10**. Cane and co-workers have reported the importance of active site volume in a sesquiterpene synthase for specific product formation on the basis of X-ray crystallographic analysis of mutants affording aberrant products.[29]

In conclusion, we have proposed a plausible cyclization mechanism for phomactatriene synthase based on the results of feeding experiments with ^{13}C-labelled acetates and an acid-catalyzed biomimetic reaction with verticillol (**5**). This proposal is supported by the isolation of minor products from the mycelial extracts of phomactatriene-producing fungus and the stability of carbocation intermediates estimated by *ab initio* calculation. The simple experiments described in this report provide information on enzyme catalysis. Although the computational approach for elucidating the reaction mechanism of terpene cyclases has been limited to model cationic intermediates (lanosterol synthase)[22] and the conformationally rigid intermediates (aphidicolan-16β-ol synthase),[21c] we showed that the *ab initio* calculations considering possible conformations of highly flexible intermediates provide useful information for discussion of an enzymatic terpene cyclization.

Experimental

General

Unless otherwise noted, nonaqueous reactions were carried out under an argon atmosphere. Dichloromethane (CH_2Cl_2) and 1,2-dichloroethane were distilled from calcium hydride. Sodium [1-^{13}C]-(99 atom% ^{13}C) and [1,2-$^{13}C_2$]-acetates (99 atom% ^{13}C) were purchased from the Cambridge Isotope Lab. All other commercially supplied reagents were used as received. Optical rotations were recorded on a JASCO DIP-360 digital polarimeter. NMR spectra were obtained on a JEOL ECP-300, Alpha-400 and a Bruker AM-500 spectrometer for solutions of $CDCl_3$ and C_6D_6. ^{1}H and ^{13}C chemical shifts were referenced to the solvent signals: 7.26 and 77.0 ppm for $CDCl_3$, 7.15 and 128.0 ppm for C_6D_6. Resolution of the ^{13}C spectra is 1.01 Hz point^{-1}. GC-MS analysis was conducted with a HP5890 gas chromatograph and a JMS-600H mass spectrometer (JEOL) under the following conditions: a fused silica capillary column (DB-1, 30 m × 0.25 mm id.; J & W Scientific); injection port temperature, 280 °C; initial temperature of the GC oven, 100 °C for 2 min, followed by heating to 280 °C at 30 °C min^{-1} and holding at the final temperature for 2 min. GC analysis was conducted with a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector (FID) and the amounts of products were determined by injecting with methyl stearate as an internal standard.

Computational method

Conformation searches of five carbocations with CONFLEX5 Revision A.2[24a] were performed as follows: the bicyclic ring-atoms were subject to perturbations with both "corner flap" and "bond flip" to generate new ring conformers,[24b–d] and the MMFF94 force field[25] was used as a structural optimizer. The chirality of the asymmetric carbon atoms was maintained during the conformation search. Restrictions were imposed to keep the starting configurations about the double bonds. A gradual increase to a search limit of 15.0 kcal mol^{-1} determined the energy range from which the initial structures for the search were selected.[24b–d] The default values for the other options were used. Finally, 20, 30, 26, 18, 26 conformers of $\mathbf{A}^+$, $\mathbf{D}^+$, $\mathbf{E}^+$, $\mathbf{F}^+$, $\mathbf{G}^+$, respectively, were found within relative energies from the each global minimum of less than 10.0 kcal mol^{-1}.

Ab initio molecular orbital calculations with Gaussian03 Revision B.4[23] were performed as follows: five of the most stable conformers of each of the carbocations were optimized using the

HF/6-31G(d) level molecular orbital theory. Their optimized structures were also subject to vibrational frequency analyses to examine their existence at the stationary points on the potential energy hypersuface (energy minima).

General procedure of acid-catalyzed rearrangement of verticillol (5)

To a solution of **5** (100 μg, 0.345 μmol) in CH_2Cl_2 (1 ml) was added acid (80 μmol). After stirring for 10 min, the reaction mixture was quenched with saturated aqueous $NaHCO_3$ (2 ml) and the aqueous layer was extracted with hexane. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The reaction products were directly analyzed by GC and GC-MS.

$BF_3{\cdot}OEt_2$-catalyzed rearrangement of 5

To a solution of **5** (5.1 mg, 19 μmol) in CH_2Cl_2 (1 ml) was added a solution of $BF_3{\cdot}OEt_2$ (10 μl, 80 μmol) in CH_2Cl_2 (1 ml) at −78 °C. After stirring for 10 min, the reaction mixture was worked up in a similar way to that described in the general procedure. The reaction products were separated by reverse-phase HPLC (Inertsil ODS-2, ϕ 4.6 × 250 mm, GL Science; eluent, 100% CH_3CN; flow rate, 0.5 ml min^{-1}; UV 195 nm) to afford **9b** (660 μg, 14%), **13a** (245 μg, 5.7%), and **13b** (507 μg, 11%).

Data for phomacta-1(15)-3,7-triene (9b). Colorless oil; $[\alpha]_D^{25}$ +259° (*c* 0.25, $CHCl_3$); CD (CH_3CN) λ_{ext} 218.0 nm ($\Delta\varepsilon$ +1.7); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 5.00 (1H, d, J = 12.0 Hz, H3), 4.79 (1H, d, J = 10.3 Hz, H7), 3.06 (1H, t, J = 12.0 Hz, H2a), 2.38–2.28 (2H, m, H6a, H14a), 2.13 (1H, dd, J = 12.2, 13.8 Hz, H9a), 2.02–1.94 (4H, m, H2b, H5, H6b), 1.84–1.74 (3H, m, H9b, H12, H14b), 1.65 (1H, dd, J = 12.2, 15.0 Hz, H10a), 1.61 (3H, s, 4Me), 1.54 (3H, s, 8Me), 1.45–1.40 (2H, m, H13), 1.30 (1H, H10b), 1.30 (3H, s, 15Me), 0.80 (3H, d, J = 6.9 Hz, 12Me), 0.74 (3H, s, 11Me); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 133.7 (C15), 133.1 (C8), 131.6 (C1), 130.8 (C4), 127.0 (C7), 124.4 (C3), 41.0 (C11), 38.5 (C5), 34.5 (C9), 34.3 (C2), 33.3 (C12), 32.5 (C14), 30.9 (C10), 27.6 (C13), 26.6 (C6), 21.8 (11Me), 16.4 (12Me), 16.1 (4Me), 15.6 (8Me), 15.0 (15Me); EI-HR-MS calcd. for $C_{20}H_{32}$ 272.2504 (M^+), found m/z 272.2501.

Data for 13a. Colorless oil; $[\alpha]_D^{25}$ +206° (*c* 0.27, $CHCl_3$); CD (CH_3CN) λ_{ext} 206.6 nm ($\Delta\varepsilon$ +33.4); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.61 (1H, br d), 4.81 (1H, q, J = 1.9 Hz), 4.70 (1H, br d), 4.53 (1H, q, J = 1.6 Hz), 2.89 (1H, d, J = 9.6 Hz), 2.72 (1H, m), 2.43 (1H, m), 2.32–2.17 (2H, m), 2.10–1.25 (17H, m), 0.85 (3H, s), 0.73 (3H, s); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 149.6, 133.9, 132.9, 128.4, 127.4, 105.2, 44.8, 42.6, 41.3, 37.8, 37.7, 36.1, 33.5, 30.3, 27.3, 26.4, 24.4, 19.4, 15.6, 15.0; EI-HR-MS calcd. for $C_{20}H_{32}$ 272.2504 (M^+), found m/z 272.2504.

Data for 13b. Colorless oil; $[\alpha]_D^{25}$ +235° (*c* 0.17, $CHCl_3$); CD (CH_3CN) λ_{ext} 206.8 nm ($\Delta\varepsilon$ +25.9); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.39 (1H, br), 5.28 (1H, br d, J = 11.8 Hz), 4.82 (1H, br d, J = 9.1 Hz), 2.95 (1H, br), 2.64 (1H, ddd, J = 14.9, 12.0, 4.5 Hz), 2.52–2.38 (3H, m), 2.17–1.25 (9H, m), 1.76 (3H, s), 1.58 (3H, s), 1.50 (3H, s), 0.80 (3H, s), 0.74 (3H, s); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 135.9, 132.9, 132.7, 29.8, 124.8, 121.6, 42.4, 40.9, 39.6, 38.1, 35.7, 34.1, 30.7, 27.1, 26.7, 23.7, 23.0, 21.4, 15.7, 15.2; EI-HR-MS calcd. for $C_{20}H_{32}$ 272.2504 (M^+), found m/z 272.2455.

$La(OTf)_3$-catalyzed rearrangement of 5

To a suspension of $La(OTf)_3$ (50.1 mg, 85.4 μmol) in 1,2-dichloroethane (0.5 ml) was added a solution of **5** (1.7 mg, 6.3 μmol) in 1,2-dichloroethane (0.5 ml) at 28 °C. The reaction mixture was stirred at 28 °C for 10 min, and worked up in a similar way to that described in the general procedure. The resultant residue was purified by reverse-phase HPLC under the same conditions described above to afford **9c** (246 μg, 14%). The amount of **9c** was calculated from the integration of ^{1}H NMR signals for H9 and C_6D_5H (2.4% in 90 μl of C_6D_6). **9c**: colorless oil; $[\alpha]_D^{25}$ −321° (*c* 0.11, $CHCl_3$); ^{1}H-NMR (500 MHz, C_6D_6) δ 5.01 (1H, t, J = 8.1 Hz, H9), 4.76 (1H, d, J = 10.3 Hz, H5), 2.59 (1H, dt, J = 2.4, 13.2 Hz, H2a), 2.28 (1H, m, H6a), 2.14–2.06 (3H, m, H3a, H7a, H14a), 2.03 (2H, d, J = 8.1 Hz, H10), 2.00–1.98 (3H, m, H3b, H6b, H7b), 1.64–1.62 (3H, m, H2b, H12, H14b), 1.56 (3H, s, 4Me), 1.52 (3H, s, 8Me), 1.41 (3H, s, 15Me), 1.38 (2H, m, H13), 0.91 (3H, d, J = 6.9 Hz, 12Me), 0.89 (3H, s,11Me); ^{13}C-NMR (125 MHz, C_6D_6) δ 133.3 (C15), 132.9 (C8), 132.1 (C4), 131.2 (C1), 128.0 (C5), 123.5 (C9), 43.2 (C11), 38.9 (C7), 37.9 (C3), 34.6 (C12), 34.3 (C10), 32.8 (C2), 31.3(C14), 27.9 (C13), 25.6 (C6), 20.0 (11Me), 17.3 (12Me), 16.9 (8Me), 16.3 (4Me), 14.5 (15Me); EI-HR-MS calcd. for $C_{20}H_{32}$ 272.2504 (M^+), found m/z 272.2492.

$TiCl_4$-catalyzed rearrangement of 5

To a solution of **5** (6.3 mg, 22 μmol) in CH_2Cl_2 (1 ml) was added a solution of $TiCl_4$ (10 μl, 91 μmol) in CH_2Cl_2 (1 ml) at −90 °C. After stirring for 10 min, the reaction mixture was worked up in a similar way to that described in the general procedure. The resultant residue was purified by reverse-phase HPLC under the same conditions described above to afford **14** (176 μg, 2.9%). The amount of **14** was calculated from the integration of ^{1}H NMR signals of H1 and C_6D_5H (2.4% in 90 μl of C_6D_6). **14**: colorless oil; $[\alpha]_D^{25}$ 0° (*c* 0.10, $CHCl_3$); UV (CH_3CN) λ_{max} 259.7 nm ($\Delta\varepsilon$ 2675); ^{1}H-NMR (500 MHz, C_6D_6) δ 6.28 (1H, d, J = 5.2 Hz, H1), 6.20 (1H, d, J = 5.2 Hz, H2), 3.12 (1H, dq, J = 7.4, 7.4 Hz, H7), 2.41 (1H, dd, J = 13.5, 5.1 Hz, H10a), 2.13 (1H, dd, J = 13.5, 8.4 Hz, H10b), 1.88 (1H, br d, J = 13.2 Hz, H4), 1.78 (1H, m, H15), 1.72 (1H, m, H11), 1.60 (1H, br H6), 1.45–1.30 (2H, m, H14), 1.35 (2H, m, H5), 1.30 (1H, m, H6), 1.27 (1H, m, H15), 1.19 (3H, s, 3Me), 1.18 (3H, d, J = 7.4 Hz, 7Me), 1.10 (1H, m, H12), 1.08 (1H, ddd, J = 13.8, 13.2, 3.4 Hz, H4), 0.85 (3H, d, J = 6.9 Hz, 12Me), 0.96 (3H, s, 13Meβ), 0.74 (3H, s, 13Meα); ^{13}C-NMR (125 MHz, C_6D_6) δ 150.08 (C8), 145.64 (C2), 134.12 (C9), 131.04 (C1), 53.83 (C3), 50.49 (C12), 45.90 (C11), 41.47 (C13), 40.51 (C14), 37.04 (C4), 34.57 (C6), 33.06 (C10), 30.16 (C5), 29.96 (C7), 29.56 (C15), 28.72 (13Meβ), 22.64 (13Meα), 20.71 (3Me), 18.40 (7Me), 13.27 (12Me); EI-LR-MS m/z 272 (M^+, 32), 161 (88), 148 (100); EI-HR-MS calcd. for $C_{20}H_{32}$ 272.2504 (M^+), found m/z 272.2509, calcd. for $C_{12}H_{17}$ 161.1330, found m/z 161.1343, calcd. for $C_{11}H_{16}$ 148.1252, found m/z 148.1334.

$BF_3{\cdot}OEt_2$-catalyzed reaction of 5 to taxa-4(5),11(12)-diene (10)

To a solution of **5** (5.8 mg, 20 μmol) in CH_2Cl_2 (1 ml) was added a solution of $BF_3{\cdot}OEt_2$ (20 μl, 160 μmol) in CH_2Cl_2 (1 ml) at 25 °C. After stirring for 10 min, the reaction mixture was worked up in a similar way to that described in the general procedure. The resultant residue was fractionated by reverse-phase HPLC. The fraction containing taxadiene was purified by normal-phase HPLC (LiChrosorb Si60, Ø 10 × 250 mm, Merck; eluent, 100% hexane; flow rate, 1.5 ml min^{-1}; UV 195 nm) affording taxa-4(5),11(12)-diene (223 ng, 0.004%) estimated by GC-MS analysis.

Isolation of diterpenes from *Phoma* sp.

Fermentation of *Phoma* sp. was carried out at 24 °C on a rotary shaker at 180 rpm for 14 days in five 500 ml Erlenmeyer flasks containing 100 ml of a medium which consisted of sucrose 2.0%, peptone 1.0%, and K_2HPO_4 0.5% (pH 8.5). Mycelium was collected by filtration and extracted with acetone. The extract was concentrated *in vacuo*, and the residual aqueous layer was extracted with ethyl acetate. The organic layers were dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography, and the hexane fractions gave the mixture of hydrocarbon products (1.4 mg).

This mixture was further separated by reverse- and normal-phase HPLC under the same conditions described above. The amounts of products were determined by GC (FID) analysis; **6** (15 μg), **9a** (596 μg), **9b** (13 μg), **13a** (11 μg), and **13b** (26 μg). Retention times (min) of products in GC-MS analysis were as follows: **6**, 6:40; **13a** and **13b**, 6:41; **9b**, 6:49; **9a**, 6:54. CD (CH_3CN): **6**, λ_{ext} 226.4 nm ($\Delta\varepsilon$ +3.2); **9b**, λ_{ext} 216.8 nm ($\Delta\varepsilon$ +3.9); **13a**, λ_{ext} 207.2 nm ($\Delta\varepsilon$ +21.9); **13b**, λ_{ext} 210.8 nm ($\Delta\varepsilon$ +11.3).

Feeding experiments with isotopically labelled compounds

On the second day after inoculation, the aqueous solution (8.8 mL) of 400 mg of isotopically labelled compounds filtered through sterilized microfilter (0.2 μm) was equally distributed into four 500 mL flasks. After further incubation for 14 days, **9a** was isolated as described above. The yield of **9a** was 1.0 mg for sodium [1-^{13}C]-acetate and 1.6 mg for sodium [1,2-$^{13}C_2$]-acetate.

Acknowledgements

We are grateful to Prof. T. Norin of the Royal Institute of Technology in Sweden for supplying a generous amount of natural verticillol, and to Prof. R. Croteau of the Washington State University for an authentic sample of taxa-4(5),11(12)-diene and to Prof. W. A. König of the Universität Hamburg for an authentic sample of verticilla-4(20),7,11-triene and MS spectra. This work was supported by grants (No. 13306009 and 14360061) from the Ministry of Education, Science, Sports and Culture of Japan and, in part, from the Yamada Science Foundation.

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