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Proposed Mechanism for the Reaction Catalyzed by a Diterpene Cyclase, Aphidicolan-16β-ol Synthase: Experimental Results on Biomimetic Cyclization and Examination of the Cyclization Pathway by ab Initio Calculations

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Abstract: To examine the mechanism of the cyclization reaction catalyzed by aphidicolan-16β-ol synthase (ACS), which is a key enzyme in the biosynthesis of diterpene aphidicolin, a specific inhibitor of DNA polymerase α, skeletal rearrangement of **2a** and biomimetic cyclization of **4b** were employed. The structures of the reaction products, which reflect penultimate cation intermediates, allowed us to propose a detailed reaction pathway for the Lewis acid-catalyzed cyclizations and rearrangements. Isolation of these products in an aphidicolin-producing fungus led us to speculate that the mechanism of the ACS-catalyzed cyclization reaction is the same as that of a nonenzymatic reaction. Ab initio calculations of the acid-catalyzed reaction intermediates and the transition states indicate that the overall reaction catalyzed by ACS is an exothermic process though the reaction proceeds via an energetically disfavored secondary cation-like transition state. In conjunction with the solvent effect in the acid-catalyzed reactions, this indicates that the actual role of ACS is to provide a template which enforces conformations of the intermediate cations leading to the productive cyclization although it has been believed that the cation$-\pi$ interaction between cation intermediates and aromatic amino acid residues in the active site is important for the enzymatic catalysis. This study provided important information on the role of various cationic species, especially secondary cation-like structures, in both nonenzymatic and enzymatic reactions.

Introduction

Aphidicolin (**1**) was isolated from a fungus, *Cephalosporium aphidicola* (*Verticillium lecanii*), as an antiviral agent against Herpes simplex type 1.[1] Later, it was found that **1** is a specific inhibitor of DNA polymerase α[2] and shows biological activity such as phytotoxic[3] and antitumor.[4] Recently, it has been reported that **1** specifically damages a fragile site of the mammalian genome.[5] Besides its remarkable bioactivity, its unique molecular skeleton has prompted numerous synthetic studies, and to date, more than 10 groups have achieved the total synthesis of **1**.[6]

Recent studies on diterpene synthases[7] indicated that a single enzyme catalyzes conversion from geranylgeranyl diphosphate (GGDP, **3**) to aphidicolan-16β-ol (**2a**), which is a biosynthetic intermediate of **1**. The first cloning of a diterpene synthase, *ent*-kaurene synthase from the fungus *Phaeosphaeria* sp.,[7a] prompted us to clone the cDNA encoding aphidicolan-16β-ol synthase (ACS), a key enzyme in aphidicolin biosynthesis, from the pathogenic fungus *Phoma betae*, which produces **1** together with less oxidized intermediates.[8] Recently, we have succeeded in

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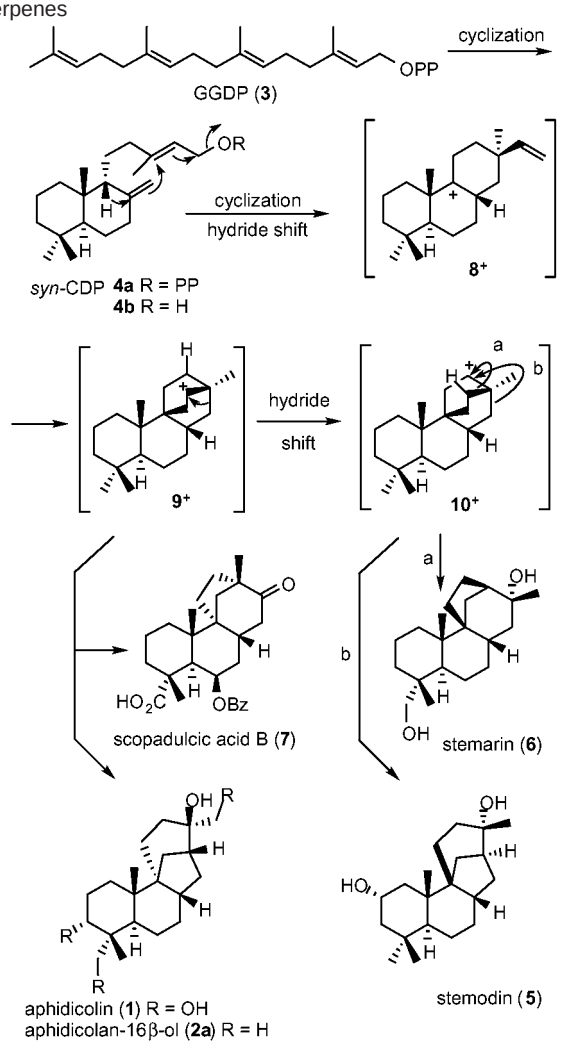
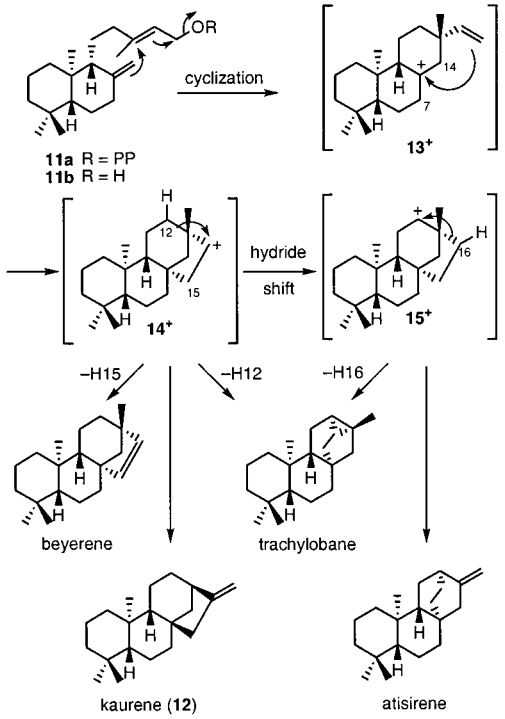
Scheme 1. Proposed Biosynthetic Pathway of C9-Ethano-Bridged Diterpenes



Scheme 2. Proposed Biosynthetic Pathway of C8-Ethano-Bridged Diterpenes



overexpression of the cDNA in *Escherichia coli*.[9] ACS can convert **3** and the anticipated bicyclic intermediate *syn*-copalyl diphosphate (*syn*-CDP, **4a**) to **2a**.[9]

Diterpenes possessing a unique bicyclooctane skeleton in the C/D ring are divided into two classes, namely, C8-ethano-bridged diterpene (C8EBD) and C9-ethano-bridged diterpene (C9EBD). C9EBD includes **1**, stemodin (**5**),[10] stemarin (**6**),[11] and a potent antitumor agent, scopadulcic acid B (**7**) (Scheme 1),[12] whereas C8EBD includes kaurene (**12**), beyerene, trachylobane, and atisirene (Scheme 2).[13] In 1955, Wenkert proposed that C8EBD is produced by a number of skeletal rearrangements (Scheme 2).[14] After this proposal, interconversions[15] of C8EBD were extensively studied, and the observations in these studies further supported the proposed biosynthetic pathway for C8EBD (Scheme 2) although a number of attempts to convert bicyclic or tricyclic intermediate analogues to C8EBD failed.[16] On the other hand, C9EBDs are proposed to be biosynthesized via **4a**.[17] Formation of **4a** as an intermediate is explained by a mechanism whereby cyclization to the A/B ring proceeds via the chair−boat transition state of **3**.[17] Interestingly, all C9EBDs possess a hydroxy group at the position of the penultimate carbocation, while most diterpene synthases produce olefins.[13] The second cyclization requires several carbocations such as $\mathbf{8}^+$, $\mathbf{9}^+$, and $\mathbf{10}^+$, but details of this cyclization mechanism still remain to be clarified.

Enzyme-catalyzed cationic cyclizations of linear polyenes in the biosynthesis of terpenes is a fascinating process involving complex C−C bond formation in a highly stereocontrolled manner,[18] and they involve many reaction steps and mutiple cationic intermediates. Among these cyclizations, there are cases that involve energetically disfavored secondary cation intermediates in the reactions catalyzed by terpene synthases such as bornyl diphosphate synthase (monoterpene),[19] pentalenene and trichodiene synthases (sesquiterpene),[20,21] *ent*-kaurene and abietadiene synthases (diterpene),[22,23] and hopene and lanosterol

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Table 1. Acid Treatments of Tetracyclic Diterpenes in CH_2Cl_2 at 0 °C

			product yield[e] (%)										
entry	substrate	acid	**2b**	**2c**	**16a**	**16b**	**18**	**2a**	**17a**	**17b**	**19**	**25**	**26**
1	**2a**	BF_3			67	3	5		3	3	5		
2	**2a**	BF_3[a]			28	3	25		<1	<1	2		
3	**2a**	BF_3[b]	no reaction										
4	**2a**	BF_3[c]			68	12			<1	<1	12		
5	**16a**	BF_3			60	3	<1		5	5	<1		
6	**17a**	BF_3			53	18	5		<1	<1	2		
7	**19**	BF_3			5	<1			<1	<1	73		
8	**2a**	$SnCl_2$	3	5	<1	2		10	5			37	35
9	**2a**	$TiCl_4$	3	5	6	8		18	2			44	5
10	**2a**	$Yb(OTf)_3$[a]	no reaction										
11	**2a**	$La(OTf)_3$[a]	no reaction										
11	**2a**	TsOH[a,d]	no reaction										

[a] Room temperature. [b] −78 °C. [c] In hexane. [d] In dioxane−H_2O (2:1). [e] Yields were calculated by peak areas in GC charts.

synthases (triterpene).[24,25] However, studies on the energy profiles of these biosynthetically important reactions are limited.[26] For studying such issues, the second cyclization step in the biosynthesis of C8-ethano-bridged diterpene (i.e., **11a** to **12**) and C9-ethano-bridged diterpene (i.e., **4a** to **2a**) provides a suitable system which is proposed to involve several secondary cations, $\mathbf{9}^+$, $\mathbf{10}^+$, $\mathbf{14}^+$, and $\mathbf{15}^+$.

Herein, we report a biomimetic conversion of a bicyclic intermediate analogue, **4b**, to tetracyclic diterpenes structurally related to **1** and isolation of the quenched products of plausible intermediates, and propose a mechanism for the ACS-catalyzed cyclization. The relationship between the biosynthetic pathway and the nonenzymatic reaction is discussed in conjunction with experimental results and data from ab initio calculations.

Results and Discussion

To examine the cationic rearrangement observed in the biosynthesis of C9-ethano-bridged diterpene, aphidicolan-16β-ol (**2a**) was treated with various acids shown in Table 1. Treatment of **2a** with $BF_3{\cdot}Et_2O$ (1.5 equiv) at 0 °C for 10 min afforded a number of products, **16a**−**19** (Scheme 3). The reaction products were repeatedly separated with reversed-phase HPLC to afford one major hydrocarbon, **16a** (52%), minor hydrocarbons **16b** (1%) and **18** (3%), and alcohols **17a** (11%), **17b** (12%), and **19** (3%). Interestingly, no starting material nor simple dehydration products **2b** and **2c** were detected under these conditions.

Major product **16a** possessed a trisubstituted olefin [δ_H 4.95 (br s, 14-H); δ_C 138.5 (C13), 123.6 (C14)]. Its planar structure was determined by extensive NMR analysis including COSY, HSQC, and HMBC (Figure 1). Minor product **16b** possessed a methylene [δ_H 4.31 (br s, 17-Ha), 4.37 (br s, 17-Hb); δ_C 154.2 (C13), 102.8 (C14)]. Alcohols **17a** and **17b** have a tertiary alcohol moiety [(**17a**) δ_H 1.14 (s, 17-CH_3); δ_C 72.8 (C13); (**17b**) δ_H 1.26 (s, 17-CH_3); δ_C 72.6 (C13)]. The structures of these minor products were determined by NMR analysis and the chemical correlation illustrated in Scheme 4. Treatment of *endo*-olefin **16a** with AcOH−H_2SO_4−H_2O (87:3:10) afforded a 1.8:1 mixture of alcohols **17a** and **17b**. Acid treatment of *exo*-isomer **16b** under similar conditions provided essentially the same products. Epoxidation of **16a** proceeded diastereoselectively to

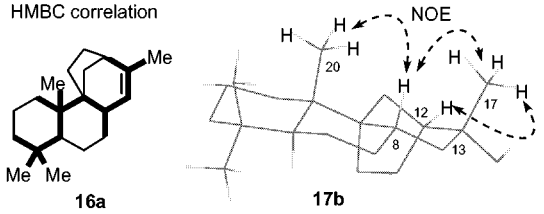
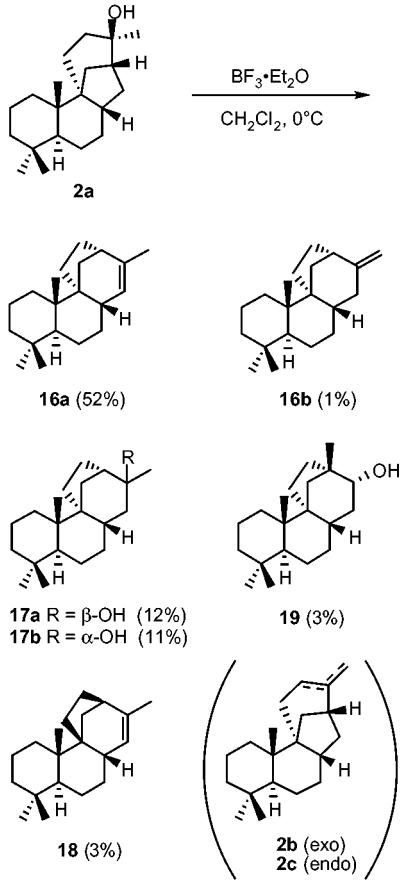


Figure 1. NMR data for the products **16a** and **17b** obtained by BF_3 treatment.

Scheme 3. BF_3 Treatment of Aphidicolan-16β-ol (**2a**)



give **20** as a single isomer. Reduction of **20** with $LiAlH_4$ afforded a tertiary alcohol which is identical to **17a** in all respects. In the NOESY spectrum of **17b**, the observed NOE correlation between 8-H and 17-CH_3 confirmed the 13*S* configuration (Figure 1). Thus, the stereochemistry of **16a**, **16b**,

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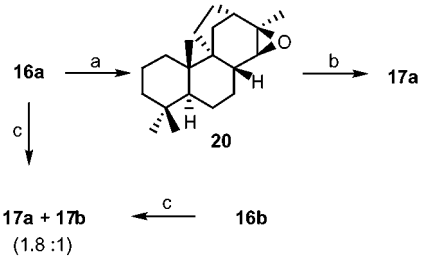
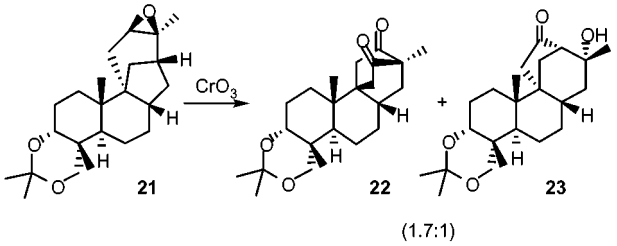
Scheme 4 [a]



[a] Reagents and conditions: (a) *m*CPBA, CH_2Cl_2, −20 °C; (b) $LiAlH_4$, THF; (c) $AcOH-H_2SO_4-H_2O$ (87:3:10), room temperature.

Scheme 5. Rearrangement Similar to That in BF_3 Treatment of **2a**

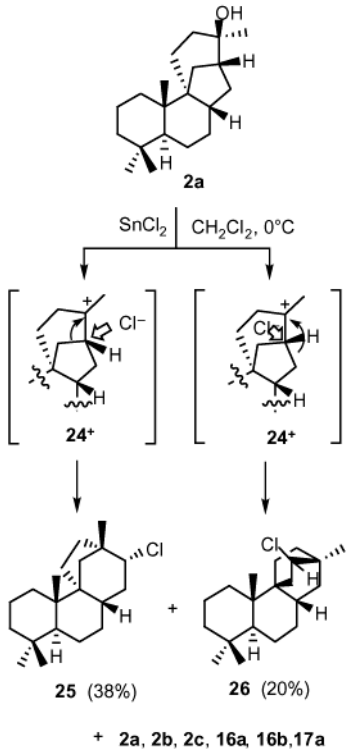


and **17a** was unambiguously assigned as shown in Scheme 3. Compounds **16b**, **17a**, and **17b** were also isolated from mycelia of the aphidicolin-producing fungus *P. betae*.[27] Since these had not been reported before, we named this novel diterpene skeleton betaerane.

In addition to betaerane diterpenes, two minor compounds, **18** and **19**, were also isolated. Compared with the reported data, their structures were determined as stemar-13-ene[28] and scopadulan-13α-ol (demalonylthyrsiflorin A[29]), respectively (Scheme 3). The former is known as a biosynthetic intermediate of a phytoalexin in rice[30] and was recently isolated from *P. betae*.[31] Interestingly, acid treatment of **2a** provided all diterpene skeletons of C9-ethano-bridged diterpene except the stemodane skeleton. Hanson et al. has reported an unusual skeletal rearrangement of epoxide **21** in CrO_3 oxidation to afford **22** and **23**, the latter of which possesses the betaerane skeleton (Scheme 5).[32] In this reaction, CrO_3 acted as both Lewis acid and oxidant. The cationic species corresponding to compound **22** can be regarded as an intermediate in the skeletal rearrangement from **21** to **23**.

BF_3 treatment of **2a** was carried out at different temperatures (Table 1). At −20 °C, the reaction proceeded in essentially the same way as at 0 °C, whereas no rearrangement product was detected at −78 °C, indicating that sufficient energy for the rearrangements is provided at over −20 °C. The reaction at room temperature yielded a 1:1 mixture of **16a** and **18**. Prolonged reaction caused decomposition of these products. These results show that a high-energy barrier exists in a 1,3-hydride shift from C12 to C16. To examine the solvent effect in this reaction, the acid treatment was conducted in less polar solvents. The product

Scheme 6. $SnCl_2$ Treatment of **2a**



ratio was essentially the same in toluene, whereas the ratios of *exo*-olefin **16b** and secondary alcohol **19** were increased in hexane.

The reaction products obtained were resubjected to the reaction conditions. Betaer-13-ene (**16a**) afforded olefins **16a**, **16b**, and **18** (60% + 3% + <1%) and alcohols **17a**, **17b**, and **19** (5% + 5% + <1%), while betaeran-13β-ol (**17a**) was converted to the same products in similar ratios. Under the same conditions, scopadulan-13α-ol (**19**) slowly converted to olefins **16a** and **16b** (<6%) and alcohols **17a** and **17b** (<2%). These data indicated that there is equilibrium among the olefins and alcohols under the reaction conditions and that secondary alcohol **19** is more stable than the tertiary alcohols **17a** and **17b**. Interconversion of aphidicolane, stemodane, and stemarane diterpenes was previously proposed by White et al.[33] We experimentally demonstrated these conversions.

Various acids were tested in the skeletal rearrangement of **2a**, and the results are shown in Table 1. No product was detected in the case of protic acid TsOH and Lewis acids $Yb(OTf)_3$ and $La(OTf)_3$. Treatment of **2a** with $TiCl_4$ and $SnCl_2$ provided chlorinated compounds **25** and **26** as major products along with small amounts of **16a**, **16b**, and **17a** (Scheme 6). This result was different from other cases wherein the starting material **2a** and its dehydration products **2b** and **2c** were obtained in these reactions. The molecular ion peak (*m*/*z* 308) and characteristic signals [(**25**) δ_H 3.84 (ddd, 13-H); δ_C 70.11 (C13); (**26**) δ_H 3.98 (ddd, 12-H); δ_C 67.82 (C12)] of the major products indicated that these are secondary chlorides. The spectral pattern of **25** was similar to that of **19**, whereas the spectrum of **26** was totally different from those of other compounds. Extensive NMR analysis of each compound established the structures as shown in Scheme 6. The relative stereochem-

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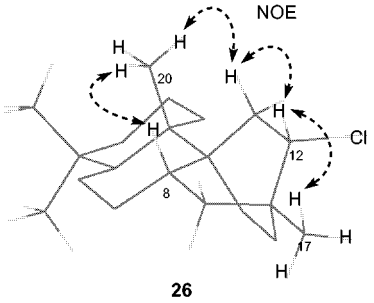
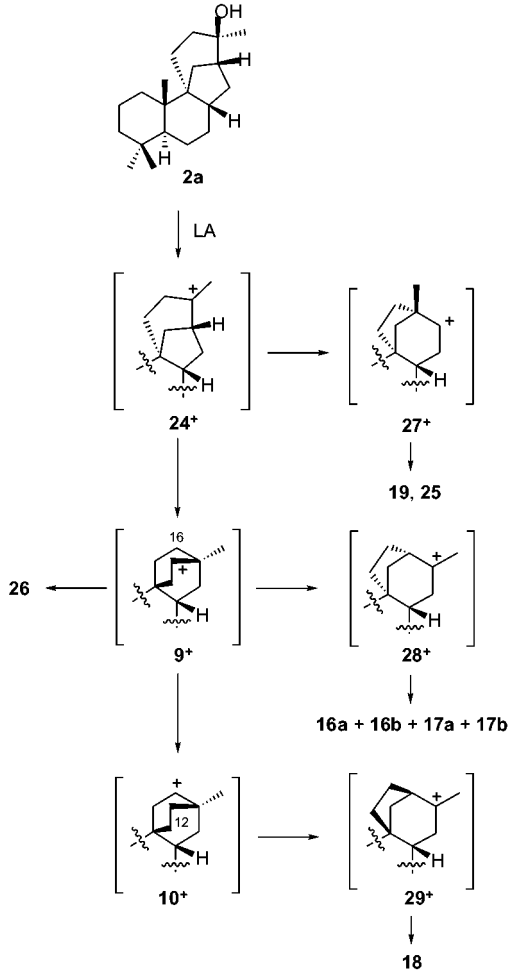


Figure 2. NOE data for the product **26** obtained by $SnCl_2$ treatment.

Scheme 7. Proposed Rearrangement Pathway Catalyzed by Lewis Acids



istry of the chlorides was determined by NOE analysis (Figure 2). The C12 stereochemistry of **25** is the same as that of the rearranged product reported previously.[32] Since these are derived from energetically disfavored cations and obtained as a single isomer, they can be regarded as kinetic products. The stereochemistry of the chlorine-substituted carbons indicated that the chloride ion attacked from the face opposite the breaking bond in an either inter- or intramolecular manner, suggesting that this reaction may proceed in a concerted manner (Scheme 6).

The experimental results of Lewis acid treatment can be explained as shown in Scheme 7: tertiary cation $\mathbf{24}^+$ initially formed was converted into secondary cation-like structure $\mathbf{27}^+$, which undergoes trapping with the nucleophilic oxygen of water to give the scopadulane-type product **19**. On the other hand, alternative rearrangement from $\mathbf{24}^+$ to another unstable cationic

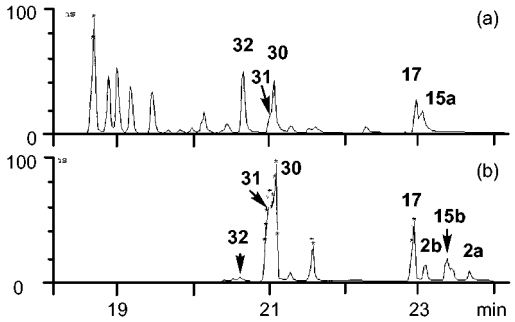
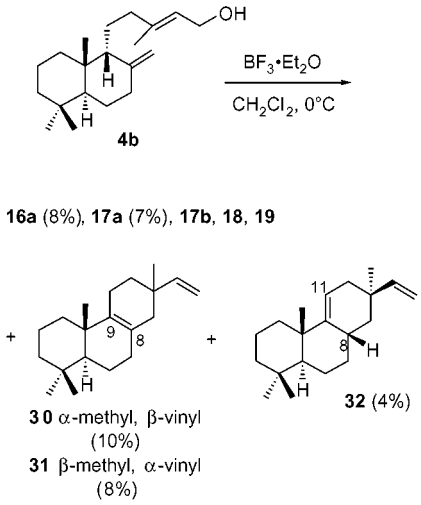


Figure 3. GC charts of diterpene hydrocarbons: (a) reaction products from BF_3 treatment of **4b**; (b) metabolites isolated from *P. betae*.

Scheme 8. BF_3 Treatment of **4b**



structure, $\mathbf{9}^+$, triggered formation of carbocation $\mathbf{28}^+$, which provided the betaerane skeleton of **16** and **17** via either proton elimination or water trapping. 1,3-Hydride shift from C16 to C12 furnished a third secondary cation-like structure, $\mathbf{10}^+$, which afforded the stemarane skeleton via cation $\mathbf{29}^+$. Diastereoselective formation of alcohol **19** and chloride **25** suggested that trapping of $\mathbf{27}^+$ took place in a concerted manner.[34]

As mentioned above, numerous attempts to convert a bicyclic alcohol such as (+)-copalol (**11b**) to C8-ethano-bridged diterpene were unsuccessful. It was interesting to know whether similar cyclization of *syn*-copalol (**4b**)[9,35] could take place. Thus, we performed this biomimetic cyclization of **4b** to tetracyclic diterpenes under the conditions as in the case of **2a**. BF_3 treatment of **4b** afforded a mixture of diterpenes (Scheme 8 and Figure 3). Comparison with the authentic samples[31] allowed us to determine their structures as tricyclic pimaradienes **30**−**32** and tetracyclic compounds **16a** and **18**, together with trace amounts of alcohols **17a**, **17b**, and **19**. To our knowledge, this is the first example of biomimetic formation of C9-ethano-bridged diterpene from a bicyclic intermediate analogue. Among these diterpenes, 8β-pimara-9(11),15-diene (**32**),[31] which is presumably derived from the characteristic carbocation $\mathbf{8}^+$ in the formation of C9-ethano-bridged diterpene, is especially noteworthy.

(34) Stereoselective capture of secondary cations in bicyclo[2.2.2]octane and bicyclo[3.2.1]octane was reported. In the papers, the stereoselectivity was explained by involvement of a nonclassical carbocation intermediate. See: Goering, H. L.; Fickes, G. N. *J. Am. Chem. Soc.* **1968**, *90*, 2848−2856. Goering, H. L.; Fickes, G. N. *J. Am. Chem. Soc.* **1968**, *90*, 2856−2862.
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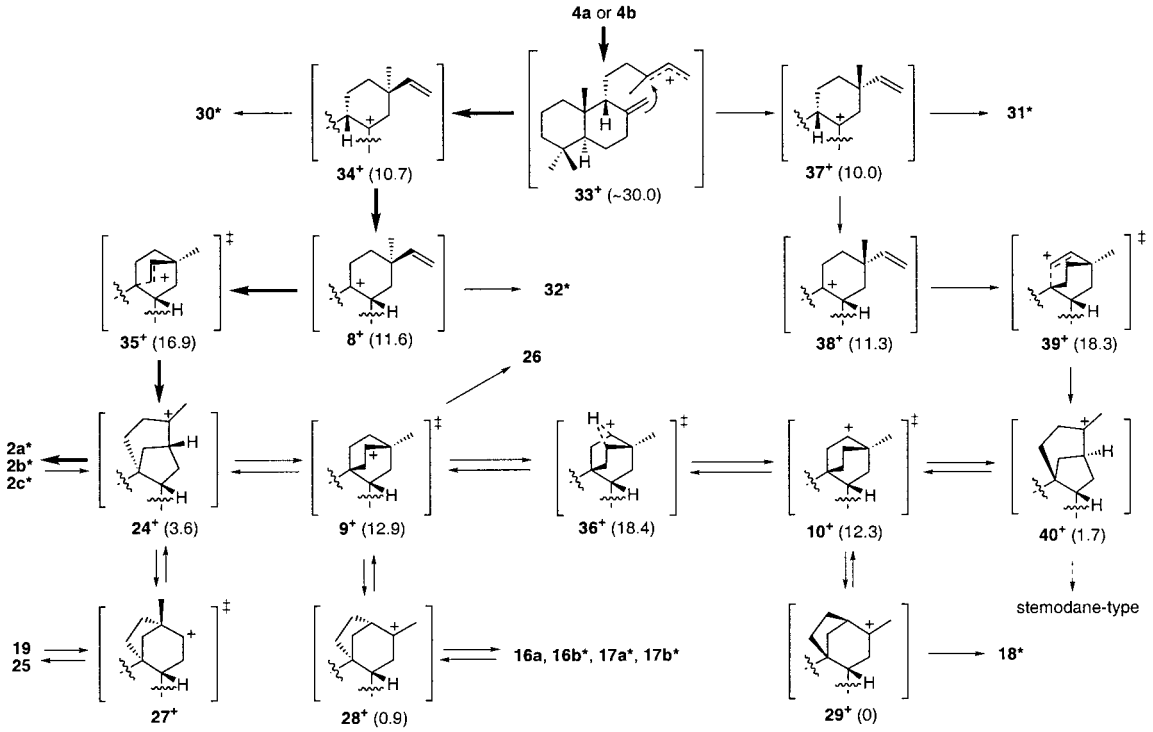
Scheme 9. Proposed Cyclization Pathway Catalyzed by Aphidicolan-16β-ol Synthase and Pathway of Nonenzymatic Cationic Rearrangements[a]



[a] The bold arrow indicates a major route of the enzymatic reaction. Asterisks indicate compounds isolated from *P. betae*. The values in parentheses are relative energies (kcal/mol) obtained by ab initio calculations (B3LYP/6-31G(d,p)).

On the basis of the structures of the products, the mechanism of this reaction is proposed as shown in Scheme 9. Elimination of the hydroxy group to give allyl cation **33**$^+$, followed by cyclization with an *exo*-methylene, affords **34**$^+$, which subsequently provides tertiary carbocation **8**$^+$ by a 1,2-hydride shift from C9 to C8. Formation of the tricyclic pimaradienes **30**−**32** is readily explained by quenching of the cations **34**$^+$, **8**$^+$, and **37**$^+$. The carbocation **8**$^+$ then cyclizes with the vinyl group to give a secondary cation-like structure, either **9**$^+$ or **35**$^+$. Rearrangement of **9**$^+$ or **35**$^+$ provides tertiary cations **24**$^+$, **28**$^+$, and **29**$^+$, which afford various tetracyclic compounds, **16a**, **17a**, **17b**, **18**, and **19**. The first cyclization step would also provide the diastereomeric cation **37**$^+$, which would undergo alternative cyclization (via **37**$^+$, **38**$^+$, **10**$^+$ or **39**$^+$, and **29**$^+$) to yield the stemarane skeleton **18**. Isolation of these hydrocarbons and alcohols provides direct support for the involvement of the corresponding carbocation intermediates.

From the mycelial extracts of *P. betae*, we isolated a number of diterpenes, **2a**, **2b**, **2c**, **18**, and **30**−**32**,[31] and detected other diterpenes, **16b**, **17a**, and **17b**, by GC−MS analysis[27] (Figure 3). These metabolites are expected to be derived from the corresponding cation intermediates, and their structures are essentially the same as those of the reaction products in the biomimetic cyclization. On the basis of these observations, it is reasonable to assume that the fungal hydrocarbons and alcohols are products of the ACS-catalyzed cyclization.[36] The finding that the recombinant ACS did not produce a detectable amount of these diterpenes may be due to the low yields of these minor products and to the low level of expression of the ACS gene. Actually, the fungus *P. betae* produced a trace amount of the diterpene hydrocarbons and the alcohols (<0.06% **1**). Thus, we propose that cyclization catalyzed by ACS proceeds as shown in Scheme 9 (bold arrows). Croteau et al. recently reported that pseudomature abietadiene synthase produces abietadienes and two minor pimaradienes,[23] which presumably derived from the premature quenching of cation intermediates similar to the ACS reaction. We are currently working on improving the expression level of the ACS gene to support the reaction mechanism proposed.

The smooth cyclization of the bicyclic compound **4b** to tetracyclic diterpenes was somewhat surprising, since the rearrangements from tertiary cations **8**$^+$ and **38**$^+$ to secondary cation-like structures **9**$^+$ and **10**$^+$ are considered to be energetically disfavored processes, and effective stabilization of these cations cannot be expected in the nonenzymatic cyclization. Thus, we became interested in the energy profile of the rearrangements in Scheme 9. To evaluate the energy levels of the plausible cations, the relative energies of the cationic stationary structures were calculated with ab initio density functional calculation using GAUSSIAN98 (B3LYP/6-31G(d,p)).[37] The energy values calculated for the carbocations are shown in Scheme 9.

It is clear that in the gas phase the conversion from **33**$^+$ to **8**$^+$ is exothermic with small barriers. Transformation from **8**$^+$ to **24**$^+$ does not involve any stable intermediate but proceeds via a transition state, **35**$^+$, and is an exothermic process by 8.0 kcal/mol. In the cases of BF_3 treatment of **2a** and **4b**, the rearrangement from **24**$^+$ and **28**$^+$ involves an energetically disfavored process for the tertiary cation **24**$^+$ to a secondary cation-like structure, **9**$^+$. The gas-phase activation energy for the conversion from **24**$^+$ to **9**$^+$ is 9.3 kcal/mol. The ab initio calculations show that **9**$^+$ is not an energy minimum and is actually a transition state. Reflecting the energy difference (2.7 kcal/mol), **24**$^+$ is converted to **28**$^+$, predominantly affording **16a**,

16b, **17a**, and **17b**. The observation that this conversion occurred even in hexane suggests that solvation of the cation centers is not essential in the rearrangement. Formation of **19** in BF_3 treatment of **4b** suggests that there is a minor route via $\mathbf{9}^+$, $\mathbf{24}^+$, and $\mathbf{27}^+$ to yield **19**.

Alternative cyclization of $\mathbf{33}^+$ provides a diastereomeric carbocation, $\mathbf{37}^+$, which is converted into a tertially cation, $\mathbf{40}^+$, via $\mathbf{38}^+$ and $\mathbf{39}^+$. The relative energies of $\mathbf{37}^+$ and $\mathbf{40}^+$ indicate that this transformation is also an exothermic process by 8.3 kcal/mol. Rearrangement of $\mathbf{40}^+$ similar to that of $\mathbf{24}^+$ afforded a tetracyclic diterpene, **18**, via $\mathbf{10}^+$ and $\mathbf{29}^+$.

Isolation of **18** in BF_3 treatment of **2a** indicates that the 1,3-hydride shift from $\mathbf{9}^+$ to $\mathbf{10}^+$, which is more energetically demanding than the rearrangement from $\mathbf{24}^+$ to $\mathbf{28}^+$, took place via transition state $\mathbf{36}^+$ at 0 °C. The ratio of the products **16a** and **18** increased from 0.06 to 0.9 when the reaction temperature was raised from 0 °C to room temperature. This suggests that the increase of temperature supplies sufficient energy for the hydride shift. The energy change from $\mathbf{9}^+$ to $\mathbf{36}^+$ is 5.5 kcal/mol, and the activation energy from $\mathbf{24}^+$ to $\mathbf{36}^+$ is calculated to be 14.8 kcal/mol. The actual barrier could be smaller than the value calculated due to stabilization by a counteranion.

Data obtained from the ab initio calculations agree well with the experimental results. Nonenzymatic reactions of **2a** and **4b** proceeded rapidly at lower temperature and even in a nonpolar solvent, indicating that solvation to stabilize intermediary carbocations is not an essential factor in the cyclizations or rearrangements. In conjunction with the result that most of the rearranged products in the nonenzymatic reaction were detected in mycelial extracts of the aphidicolin-producing fungus, we reasonably speculate that ACS catalyzes formation of an allylic cation and an exothermic process from $\mathbf{33}^+$ to $\mathbf{24}^+$ by providing a template to enforce cyclization and rearrangement ($\mathbf{8}^+$ to $\mathbf{24}^+$). The solvent effects in the nonenzymatic reaction suggest that this enzymatic conversion does not require a significant cation$-\pi$ interaction with aromatic amino acid residues, which are proposed to stabilize the cationic intermediates[38] and which are abundant in the active sites of the terpene synthases. We assume that other diterpene synthases yielding C9-ethano-bridged diterpene and C8-ethano-bridged diterpene adopt the same strategy on formation of tetracyclic ring systems. To date, it has been believed that the cation$-\pi$ interaction between aromatic amino acid residues and cation intermediates is important for the enzymatic catalysis.[39] Our data, however, suggest that at least in reactions of the diterpene synthases affording C9-ethano-bridged diterpene such interaction is not essential even when a secondary cation-like structure is involved.

Cyclization of **4b** and skeletal rearrangement of **2a** provided important information on the various cationic intermediates, especially secondary cation-like structures in both nonenzymatic and enzymatic reactions. Our study demonstrates that the minor constituents found in terpene-producing sources can provide information on carbocation species involved in the multistep cyclization catalyzed by terpene synthases.

Experimental Section

General Methods. Unless otherwise noted, nonaqueous reactions were carried out under an argon atmosphere. Tetrahydrofuran (THF) was distilled from sodium metal/benzophenone ketyl. Benzene, toluene, hexane, and CH_2Cl_2 were distilled from calcium hydride. All other reagents commercially supplied were used as received. Melting points are uncorrected. GC−MS analysis was conducted with a Thermo Quest GCQ under the following conditions: a fused silica capillary column (DB-1, ∅ 0.25 mm × 30 m, J & W Scientific); temperature of the injection port, 260 °C; initial temperature of the GC oven, 100 °C for 2 min, followed by heating to 280 °C at 5 °C/min and holding at the final temperature for 2 min.

Computational Method. All of the geometries presented were fully optimized with density functional calculations (B3LYP) with 6-31G-(d,p) basis sets. Some of the structures were calculated at the RHF/6-31G(d) and RHF/3-21G levels to estimate the potential energy profile. All of the quantum chemical calculations were performed using the GAUSSIAN98 program package.[37]

BF_3-Catalyzed Rearrangement of 2a. To a solution of **2a** (43.6 mg, 0.150 mmol) in CH_2Cl_2 (3 mL) was added $BF_3{\cdot}OEt_2$ (29 μL, 0.229 mmol). After being stirred at 0 °C for 30 min, the mixture was quenched with saturated sodium bicarbonate solution and extracted with CH_2Cl_2. 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 (100% hexane and then 100% $CHCl_3$) gave hydrocarbon and alcohol fractions. The fractions were repeatedly separated with reversed-phase HPLC (100% CH_3CN) to afford **16a** (21.3 mg, 52.1%), **16b** (0.6 mg, 1.5%), **18** (1.3 mg, 3.2%), and alcohols **17a** (4.9 mg, 11.2%), **19** (1.6 mg, 3.7%), and **17b** (5.0 mg, 11.5%). Retention times (t_R, min) of the products in GC−MS analysis were as follows: **18**, 23:09; **16a**, 23:15; **16b**, 23:37; **17a**, 26:35; **19**, 26:43; **2a**, 26:46; **17b**, 26:52.

Data for Betaer-13-ene (16a): colorless rods; mp 68−69 °C (from CH_3CN); $[\alpha]_D^{25}$ +9.0° (*c* 1.14, $CHCl_3$); IR (film) 2931, 1447, 973, 845 cm^{-1}; ^1H NMR (500 MHz, C_6D_6) δ 4.92 (s, 1H, H14), 2.63 (br d, 1H, J = 11.8 Hz, H8), 2.14 (m, 1H, H12), 1.70 (m, 1H, H11a), 1.66 (s, 3H, 17-H_3), 1.64 (m, 1H, H2a), 1.53 (m, 1H, H6a), 1.51−1.71 (m, 2H, H16), 1.51 (m, 1H, H7a), 1.50 (m, 1H, H11b), 1.48 (m, 2H, H15), 1.45 (m, 1H, H2b), 1.42 (m, 1H, H7b), 1.40 (m, 1H, H3a), 1.34 (m, 2H, H1), 1.45 (m, 1H, H2b), 1.28 (m, 1H, H6b), 1.15 (dt, 1H, J = 3.9, 13.8 Hz, H3b), 1.05 (dd, 1H, J = 3.0, 12.8 Hz, H5), 0.99 (s, 3H, 20-H_3), 0.87 (s, 3H, 19-H_3), 0.85 (s, 3H, 18-H_3); ^{13}C NMR (125 MHz, C_6D_6) δ 138.5 (C13), 123.6 (C14), 51.3 (C9), 48.5 (C5), 42.3 (C8), 42.3 (C3), 41.8 (C12), 38.6 (C10), 36.2 (C15), 34.1 (C11), 33.5 (C18), 33.2 (C4), 31.8 (C1), 27.9 (C7), 24.4 (C16), 22.0 (C17), 21.8 (C6), 21.6 (C19), 18.8 (C2), 17.2 (C20); EI-MS *m*/*z* 272 (M^+, 34), 257 (100), 229 (16), 213 (16), 201 (16), 187 (32), 175 (23), 161 (41),105 (27); EI-HR-MS *m*/*z* calcd for $C_{20}H_{32}$ 272.2506 (M^+) found *m*/*z* 272.2508.

Data for Betaer-13(17)-ene (16b): colorless oil: $[\alpha]_D^{25}$ −28.0° (*c* 0.03, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 4.45 (t, 1H, J = 2.0 Hz, H17a), 4.38 (t, 1H, J = 2.0 Hz, H17b), 2.61 (t, 1H, J = 4.9 Hz, H12),

(36) The mycelial extracts of another aphidicolin-producing fungus, *C. aphidicola*, contains essentially the same hydrocarbons in a similar ratio. Using the homology-based PCR used in cloning of the ACS gene, we failed to clone cDNA encoding other terpene synthases which possibly provide the hydrocarbons found in *P. betae*. These results provided circumstantial evidence for the production of minor hydrocarbons by ACS.

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2.14 (dd, 1H, J = 4.4, 13.9 Hz, H8), 0.95−1.85 (m, 19H), 0.99 (s, 3H, 20-H_3), 0.83 (s, 3H, 19-H_3), 0.81 (s, 3H, 18-H_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 154.2 (C13), 102.8 (C17), 52.6 (C9), 48.6 (C5), 44.5 (C12), 42.3 (C3), 39.8 (CH_2), 39.7 (C8), 38.8 (C10), 37.2 (C14), 33.7 (C18), 33.3 (C4), 32.5 (C1), 31.0 (CH_2), 30.7 (CH_2), 23.2 (C7), 22.2 (C6), 21.7 (C19), 18.9 (C2), 17.6 (C20); EI-MS m/z 272 (M^+, 96), 257 (56), 243 (24), 231 (94), 187 (100), 159 (58), 145 (42), 131 (28), 91 (36); EI-HR-MS m/z calcd for $C_{20}H_{32}$ 272.2506 (M^+), found 272.2510.

Data for Stemar-13-ene (18): colorless oil; $[\alpha]_D^{25}$ 45.3° (c 0.10, $CHCl_3$) (lit. $[\alpha]_D^{25}$ 55.9° (c 0.56, $CHCl_3$)).

Data for Betaeran-13β-ol (17a): colorless oil; $[\alpha]_D^{25}$ −29.2° (c 0.13, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.98 (m, 1H, H8), 1.87 (br t, 1H, J = 4.9 Hz, H12), 1.68 (br d, 1H, J = 11.8 Hz), 1.23−1.66 (m, 15H), 1.14 (s, 3H), 0.96−1.14 (m, 3H), 1.04 (s, 3H), 0.93 (dd, 1H, J = 3.0, 12.8 Hz, H5), 0.818 (s, 3H), 0.812 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 72.8 (C), 51.5 (C), 48.5 (CH), 46.8 (CH), 42.3 (CH_2), 41.9 (CH_2), 38.5 (C), 34.8 (CH), 33.6 (CH_3), 33.2 (C), 32.5 (CH_2), 32.4 (CH_2), 30.3 (CH_2), 28.6 (CH_3), 28.1 (CH_2), 22.1 (CH_3), 21.93 (CH_2), 21.86 (CH_2), 18.8 (CH_2), 17.6 (CH_3); EI-MS m/z 290 (M^+, 29), 275 (20), 272 (43), 257 (26), 232 (31), 187 (100), 159 (43); EI-HR-MS m/z calcd for $C_{20}H_{34}O$ 290.2611 (M^+), found 290.2594.

Data for Betaeran-13α-ol (17b): colorless oil; $[\alpha]_D^{25}$ −9.8° (c 0.13, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.84 (br t, 1H, J = 4.9 Hz, H12), 1.65−1.90 (m, 2H), 1.20−1.60 (m, 15H), 1.25 (s, 3H), 1.00−1.19 (m, 3H), 1.00 (s, 3H), 0.94 (dd, 1H, J = 3.0, 12.8 Hz, H5), 0.82 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 72.6 (C13), 52.1 (C9), 48.5 (C5), 47.2 (C12), 43.0 (C14), 42.2 (C3), 38.5 (C10), 36.2 (C8), 35.0 (CH_2), 33.7 (C18), 33.2 (C4), 32.7 (C1), 30.5 (CH_2), 27.8 (C17), 26.1 (CH_2), 22.5 (CH_2), 22.2 (C19), 22.00 (CH_2), 18.9 (C2), 17.6 (C20); EI-MS m/z 290 (M^+, 83), 257 (100), 272 (36), 257 (40), 232 (77), 187 (70), 137 (33); EI-HR-MS m/z calcd for $C_{20}H_{34}O$ 290.2611 (M^+), found 290.2596.

Data for Scopadulan-13α-ol (Demalonyl Thyrsiflorin A, 19): colorless oil; $[\alpha]_D^{25}$ −46° (c 0.3, $CHCl_3$) (lit. $[\alpha]_D^{25}$ −38° (c 1.0, $CHCl_3$)).

13β,14β-Epoxybetaerane (20). To a solution of **16a** (1.4 mg, 3.68 mmol) in CH_2Cl_2 (0.2 mL) was added *m*-chloroperbenzoic acid (50%, 5.1 mg, 0.015 mmol). After being stirred at −20 °C for 70 min, the mixture was quenched with 5% sodium thiosulfate solution and extracted with hexane. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give **20** (1.5 mg, quantitative). The crude material thus obtained was used without further purification: colorless oil; IR (KBr) 1252, 1203, 1112, 1072, 979, 856, 806 cm^{-1}; ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.37 (s, 1H), 2.23 (m, 1H), 2.13 (m, 1H), 0.92−1.81 (m, 17H), 1.29 (s, 3H), 0.93 (s, 3H), 0.83 (s, 3H), 0.80 (s, 3H); EI-MS m/z 288 (M^+); EI-HR-MS m/z calcd for $C_{20}H_{32}O$ 288.2454 (M^+), found 288.2432.

$LiAlH_4$ Reduction of 20. To a suspension of $LiAlH_4$ (9.3 mg, 0.245 mmol) in THF (0.5 mL) was added epoxide **20** (1.5 mg, 3.68 mmol) at ambient temperature. After being stirred overnight, the mixture was quenched with 2 M HCl and extracted with hexane. 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 (100% hexane) gave **17a** (1.1 mg, 72.8%).

BF_3-Catalyzed Equilibration of 16a, 17a, and 19. To the compound (ca. 100 mg, ca. 3.6 mmol) was added a solution of BF_3·OEt_2 (10 μL, 0.079 mmol) in CH_2Cl_2 (1 mL). After being stirred at 0 °C for 30 min, the mixture was quenched with saturated sodium bicarbonate solution and extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The reaction products were directly analyzed by GC−MS.

Hydrations of 16a and 16b. To compound **16a** (2.5 mg, 9.19 μmol) was added a solution of AcOH−H_2SO_4–H_2O (83:7:10, 2.5 mL) at ambient temperature. After being stirred for 6 h, the mixture was extracted with hexane. The combined organic extracts were washed with saturated sodium bicarbonate solution and then with brine. The extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Purification by preparative TLC (hexanes−EtOAc, 3:1) gave alcohols **17a** (1.4 mg, 52.5%) and **17b** (0.8 mg, 30.0%), which were identical to the products of the BF_3 treatment with respect to MS and ^{1}H NMR analysis. Compound **16b** (0.1 mg) was treated under the same conditions to give a mixture of alcohols. GC−MS analysis of this mixture indicated that it contained **17a** and **17b** in a ratio of 1.6:1.

$SnCl_2$-Catalyzed Rearrangement of 16a. To a solution of **16a** (10.0 mg, 0.037 mmol) in CH_2Cl_2 (0.7 mL) was added $SnCl_2$ (10.4 mg, 0.056 mmol). After being stirred at 0 °C for 10 min, the mixture was quenched with saturated sodium bicarbonate solution and extracted with CH_2Cl_2 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 (100% hexane and then 100% $CHCl_3$) gave chloride and alcohol fractions. The fractions were repeatedly separated with reversed-phase HPLC (100% CH_3CN) to afford chlorides **25** (4.3 mg, 37.7%), **26** (2.3 mg, 20.2%), and a mixture of hydrocarbons (0.9 mg, 9.6%) and alcohols (0.7 mg, 6.5%). Retention times (t_R, min) of the products in GC−MS analysis were as follows: **2b**, 23:48; **2c**, 24:25; **25**, 28:37; **26**, 28:46.

Data for 13α-Chloroscopadulane (25): colorless oil; $[\alpha]_D^{25}$ −9.9° (c 0.46, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.84 (ddd, 1H, J = 1.7, 4.4, 10.3 Hz, H13), 2.02 (td, 1H, J = 5.2, 13.3 Hz, H14a), 1.84 (m, 1H, H16a), 1.81 (m, 1H, H8), 1.75 (m, 1H, H15a), 1.64 (d, 1H, J = 11.8 Hz, H11a), 1.55 (m, 1H, H2a), 1.51 (m, 1H, H7a), 1.48 (m, 1H, H6a), 1.46 (m, 1H, H15a), 1.43 (m, 1H, H2b), 1.37−1.43 (m, 2H, H1), 1.36 (m, 1H, H3a), 1.35 (m, 1H, H14b), 1.28 (m, 1H, H6b), 1.18 (m, 1H, H16b), 1.17 (m, 1H, H7b), 1.09 (m, 1H, H3b), 1.07 (s, 3H, 17-H_3), 1.04 (m, 1H, H11b), 0.96 (s, 3H, 20-H_3), 0.89 (dd, 1H, J = 2.5, 12.3 Hz, H5), 0.81 (s, 3H, 18-H_3), 0.80 (s, 3H, 19-H_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 70.1 (C13), 52.5 (C9), 48.0 (C5), 46.1 (C11), 45.3 (C12), 42.2 (C3), 40.6 (C14), 39.2 (C8), 38.6 (C10), 33.7 (C18), 33.2 (C4), 32.6 (C1), 31.5 (C16), 29.9 (C7), 25.5 (C17), 24.5 (C15), 22.1 (C19), 21.8 (C6), 18.8 (C2), 17.4 (C20); EI-MS m/z 308 (M^+, 87), 293 (77), 279 (82), 273 (83), 257 (100), 223 (36), 189 (42), 161 (35),123 (69), 81 (31); EI-HR-MS m/z calcd for $C_{20}H_{33}Cl$ 308.2273 (M^+), found 308.2276.

Data for 12α-Chloro-13-methyl-9α,13α-ethano-9 α-podocarpane (26): colorless oil; $[\alpha]_D^{25}$ +41° (c 0.23, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.98 (ddd, 1H, J = 2.5, 5.6, 9.9 Hz, H12), 2.14 (ddd, 1H, J = 3.6, 10.2, 14.2 Hz, H11a), 1.89 (m, 1H, H6a), 1.82 (m, 1H, H16a), 1.76 (s, 1H, H8), 1.72 (dd, 1H, J = 3.4, 14.5 Hz, H11b), 1.61−1.66 (m, 1H, H2a), 1.62 (m, 1H, H1a), 1.57 (m, 1H, H6b), 1.55 (m, 1H, H14a), 1.50 (m, 1H, H15a), 1.41 (m, 1H, H2b), 1.36 (m, 1H, H3a), 1.33 (m, 1H, H5), 1.22−1.31 (m, 2H, H7), 1.23 (m, 1H, H1b), 1.18 (m, 1H, H15b), 1.16 (m, 1H, H16b), 1.09 (m, 1H, H3b), 0.99 (dd, 1H, J = 4.0, 13.2 Hz, H14b), 0.891 (s, 3H, 20-H3), 0.869 (s, 3H, 17-H3), 0.856 (s, 3H, 18-H3), 0.835 (s, 3H, 19-H3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 67.8 (C12), 46.2 (C5), 42.42 (C14), 42.25 (C3), 40.0 (C11), 39.4 (C10), 39.0 (C9), 34.3 (C18), 33.5 (C8), 33.3 (C13), 33.17 (C7), 33.14 (C4), 32.9 (C1), 27.9 (C16), 25.5 (C17), 22.5 (C15), 22.4 (C19), 21.1 (C6), 18.9 (C2), 15.8 (C20); EI-MS m/z 308 (M^+, 52), 273 (100), 257 (21), 224 (36), 223 (41), 189 (17), 161 (14), 133 (13), 105 (12); EI-HR-MS m/z calcd for $C_{20}H_{33}Cl$ 308.2273 (M^+), found 308.2263.

BF_3-Catalyzed Rearrangement of 4b. To a solution of *syn*-copalol (**4b**; 12.1 mg, 0.042 mmol) in CH_2Cl_2 (1 mL) was added BF_3·OEt_2 (8 mL, 0.063 mmol). After being stirred at 0 °C for 10 min, the mixture was quenched with saturated sodium bicarbonate solution and extracted with CH_2Cl_2. 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 (100% hexane and then

100% $CHCl_3$) gave hydrocarbon and alcohol fractions. The fractions were repeatedly separated with reversed-phase HPLC (100% CH_3CN) to afford **30** (1.1 mg, 9.6%), **31** (0.9 mg, 7.9%), **32** (0.4 mg, 3.5%), **16a** (0.9 mg, 7.9%), **18** (0.8 mg, 7.0%), and a mixture of alcohols (1.3 mg, 10.7%) containing **17a**, **17b**, and **19**. Retention times (t_R, min) of the products in GC−MS analysis were as follows: **31**, 20:45; **32**, 21:03; **30**, 21:07.

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Supporting Information Available: Transition structures optimized at B3LYP/6-31G(d,p) and ^1H NMR and ^{13}C NMR spectra for compounds **16a**, **16b**, **17a**, **17b**, **20** (only ^1H NMR), **25**, and **26** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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