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A New Approach for the Investigation of Isoprenoid Biosynthesis Featuring Pathway Switching, Deuterium Hyperlabeling, and ^{1}H NMR Spectroscopy. The Reaction Mechanism of a Novel *Streptomyces* Diterpene Cyclase

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Recent methodology for the investigation of isoprenoid biosynthesis featuring pathway switching and hyperdeuteration has shown significant advantages in elucidating the reaction mechanism of a novel *Streptomyces* diterpene cyclase with use of precise atom-level analysis. Insight into the cyclization mechanism involved in the conversion of geranylgeranyl diphosphate (GGPP) into a clerodane hydrocarbon terpentetriene was obtained by heterologous expression in doubly engineered *Streptomyces lividans* of a diterpene cyclase gene derived from *Streptomyces griseolosporeus*, a producer of an unique diterpenoid cytotoxic antibiotic terpentecin, and by in vivo labeling with mevalonate-d_9. The cyclization involved electrophilic protonation, cationic ring closure, Wagner−Meerwein-type rearrangements, and deprotonation. A key feature was that the labeled metabolite as a mixture of predominantly deuterated mosaic molecules provided sufficient information that close analysis of the labeling pattern for each individual isoprene unit was achieved primarily by ^{1}H NMR spectroscopy. The cyclization of GGPP into the clerodane skeleton catalyzed by the cyclase appears to involve *Si*-face specific protonation, intermediates with A/B chair-boat conformation, and specific methyl and hydride migrations to give an intermediary C-4 carbocation. Subsequent collapse of the cation through specific removal of the initiating proton and final elimination of diphosphate gives rise to the terpentetriene hydrocarbon.

Introduction

Isoprenoids are among the most diverse natural products in terms of chemical structure and biological properties.[1] Substantial efforts have been devoted to elucidating the origin of the diversity found in nature and the ways it is generated.[2] Key elements appear to be various similarities and differences in chemical reactions and biosynthetic machinery. A major divergence is that, depending upon organisms and cellular organella, the biosynthesis of isoprenoids proceeds through either the mevalonate pathway[3] or the nonmevalonate pathway.[4] However, the key C_5 intermediates, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), are common in both cases. IPP is used as the elongating C_5 donor in terpene biosynthesis. Thus the condensation of IPP with a variety of allylic diphosphates yields new

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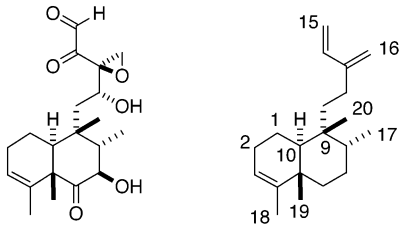

FIGURE 1. Structures of terpentecin (**1**) and terpentetriene (**2**).

polyprenyl diphosphates. In many cases, the polyprenyl diphosphates are commited to a range of cyclization reactions catalyzed by so-called "cyclases" to form diverse arrays of carbon skeletons. The resulting hydrocarbon skeletons are primarily subjected to subsequent modifications, including oxidation, dehydration, hydrogenation, acylation, etc., to give a large number of different isoprenoid metabolites. Many isoprenoid cyclases have been purified and the responsible genes cloned.[5] Three-dimensional structures and cyclization mechanism of relevant cyclases have been extensively discussed to obtain insight into the precise enzyme−substrate interaction. In this context, close analysis by means of isotope-tracer methodology has been indispensable for elucidating the fine structural changes and behavior of each atom of substrate taking place inside the enzyme active site in in vivo biosynthetic systems as well as in in vitro purified enzymes.

Recently, two genes encoding terpene cyclase-like proteins, which function in the biosynthesis of an antibiotic terpentecin (**1**),[6] were identified from *Streptomyces griseolosporeus* and the genes were heterologously expressed in *Streptomyces lividans*. A transformant produced a novel cyclic diterpenoid, *trans*-cleroda-3,13(16),-14-triene, named terpentetriene **2**, which appears to be a precursor of **1** and was obviously derived from geranylgeranyl diphosphate (GGPP) (Figure 1).[7] Apparently, one of the two foreign genes was responsible for the production of this particular isoprenoid cyclase in this heterologous expression system, making this the first eubacterial diterpene cyclase identified. These advances prompted us to study the precise mechanism of the cyclase reaction to gain insight into the dynamics of the molecular transformation in the enzyme active site and to compare the enzymology with those of other organisms. Since this heterologously expressed cyclase can function independently of the enzymes of the *S. lividans* host, we exploited this transformant to study the fate of substrate hydrocarbons in in vivo whole cell expression systems. We recently developed a straightforward analytical method for isoprenoid biosynthesis using labeling with perdeuterated mevalonate and ^{1}H NMR spectroscopy,[8] particularly, in combination with pathway switching.[9] This paper describes the precise mechanism of a novel eubacterial diterpene cyclase reaction employing the above-mentioned hyperdeuteration technique and engineered *S. lividans*.

Materials and Method

Expression of the Cyclase Genes in *S. lividans*. The HindIII−HindIII fragment of pWHM-MEV1[10] carrying the genes of the mevalonate pathway and GGPP synthase, and the XbaI-HindIII fragment of pWHM-TER1[7] were inserted into the same sites of pWHM860 (a gift from C. R. Hutchinson, Kosan Bioscience Inc., Hayward, CA) to give pWHM-TER2, in which these genes were expressed under the control of the *erm*E* promoter. The transformation protocol used for *S. lividans* was essentially the same as that described by Dairi et al.[11]

Isolation of Perdeuterated Terpentetriene 2 Produced by an *S. lividans* Transformant. *S. lividans* harboring pWHM-TER2 was grown in 300-mL Erlenmeyer flasks containing SK-No. 2 medium[12] and thiostrepton (10 μg/mL) with supplementation of mevalonolactone-d_9 (0.5 g/L), which had been synthesized as described previously.[8] Fermentation was carried out for 7 days at 30 °C with agitation (200 rpm). The culture broth (total 1 L) was centrifuged, and the precipitated mycelial cake was suspended in 1 L of acetone. After vigorous shaking, the suspension was filtered and the acetone filtrate was concentrated to dryness in vacuo. The residue was dissolved in 50 mL of chloroform and water (1:1). After centrifugation to separate the emulsion, the organic layer was recovered. The aqueous layer was extracted twice with chloroform. The combined organic layer was evaporated to dryness under reduced pressure. The dried material was dissolved in a small volume of chloroform and acetone (1:1) and subjected to thin-layer chromatography (silica gel 60F_{254}; Merck), using hexane as developing solvent. A suitable band (R_f, 0.47 to 0.68) was extracted with acetone, and the extract was filtered and concentrated to dryness. The dried material was dissolved in a small volume of acetonitrile and then fractionated by preparative HPLC (Merck Mightisil RP-8 column, 250 × 20 mm; mobile phase, acetonitrile; flow rate, 5 mL/min; UV detection wavelength, 210 nm) to give perdeuterated **2** (ca. 2 mg).

Results and Discussion

Since *S. lividans* produces its essential isoprenoids via the nonmevalonate pathway, a switch to the mevalonate pathway was first pursued. Thus, *S. lividans* TK23 was transformed with pWHM-TER2 carrying the two genes of cyclases, GGPP synthase, and the mevalonate pathway enzymes. It should be pointed out here that, in this particular transformed *S. lividans*, both the intrinsic nonmevalonate pathway and the foreign mevalonate pathway are operative, since the former had not been disrupted. Subsequently, the resulting engineered *S. lividans* was cultured as already described[7] with supplementation with synthetic (±)-mevalonolactone-d_9, which had been synthesized as described previously.[8] After growing for 7 days at 30 °C, the culture (1 L) was

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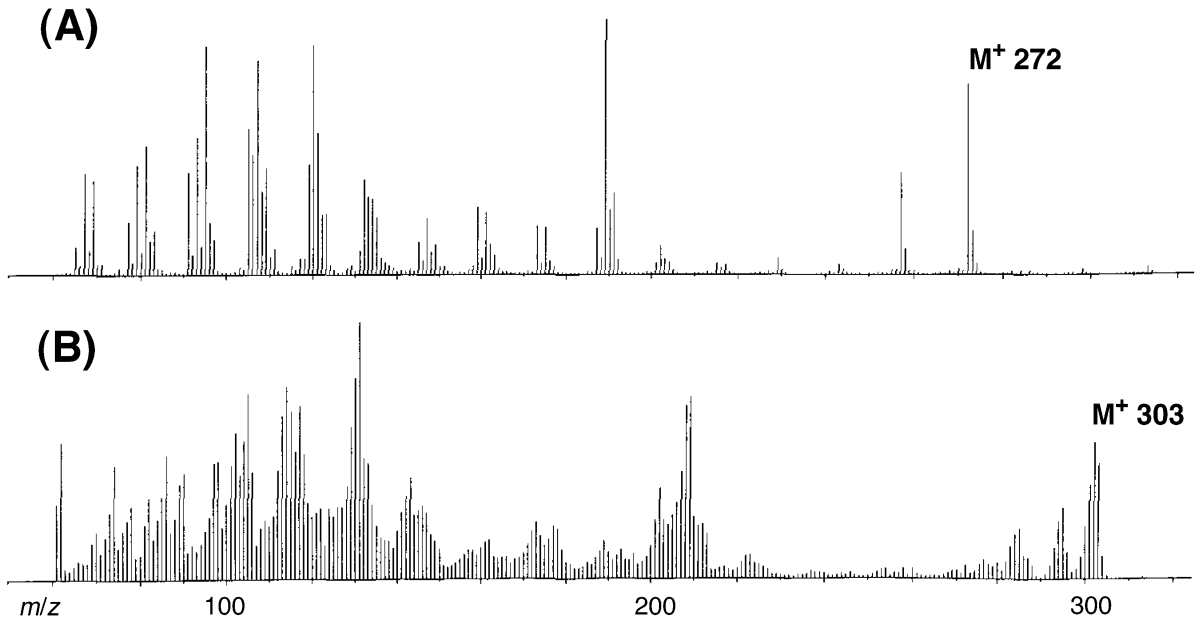

FIGURE 2. Mass spectra of (A) nonlabeled **2** and (B) perdeuterated **2**.

centrifuged and the precipitated mycelial cake was extracted with acetone. The isoprenoid product was then purified according to the literature[7] to afford ca. 2 mg of **2**.

First, mass spectrometric analysis of **2** was undertaken. If the mevalonate pathway genes were predominantly involved in the biosynthesis of the isoprene unit, the supplemented mevalonate-d_9 would be highly incorporated and eight deuterium atoms of the supplemented mevalonate-d_9 should remain in each isoprene unit of terpentetriene **2**. On the other hand, if the native nonmevalonate pathway genes were responsible, deuterium incorporation would be poor. The resulting mass spectrum of the labeled **2** is shown in Figure 2. While the molecular ion of the nonlabeled **2** was observed at *m*/*z* 272 in its EI-MS spectrum, the spectrum of the deuterated **2** showed signal clusters at a much higher molecular ion region than *m*/*z* 272. The same was true for the fragment ions as well. Since four isoprene units should be incorporated into **2**, one would expect that the most highly deuterated isotopomer would have an atomic mass 31 units higher than the parent ion of the corresponding nonlabeled **2**, taking into account the involvement of dehydration or dephosphorylation reaction. This was indeed the case; the most isotopically abundant molecular ion was observed at *m*/*z* 303. Further, a series of molecular ions having up to 4 labeled isoprene units were observed together with the weak ions of the nonlabeled **2**. From this spectrum, the average enrichment of each isoprene unit in a hydrocarbon molecule **2** was estimated to be as high as about 80%. These results clearly suggested that under these conditions, the foreign mevalonate pathway was predominantly operative in the biosynthesis of the isoprene units compared to the nonmevalonate pathway. Conversely, 20% of each isoprene unit was not deuterated. This is important because most of the product molecules appear to be transparent to ^{1}H NMR spectroscopy, yet all the observable ^{1}H NMR signals must be influenced by the deuterium isotope effect both in spin−spin coupling and in chemical shifts. It should be pointed out further that the location of nonlabeled units was random in each molecule of **2**, and the biosynthetically deuterated **2** is expected to be a mixture of heterogeneous "mosaic" isotopomers.

The clerodane class of diterpenes is biosynthesized from GGPP through enzymatic cyclization initiated by electrophilic protonation, followed by a series of methyl and hydride shifts.[13,14] In the case of the *trans*-clerodane cyclase, the overall transformation involves electrophilic protonation to the 14,15-double bond of GGPP, nucleophilic attack of C-10 on C-15, and then C-7 on C-11, followed by 1,2-methyl and hydride migration processes and ultimate deprotonation. First to be elucidated was the cyclization pathway. Two cyclization modes are possible, either through a chair-boat conformation or through a boat-boat conformation, to give the same final product as depicted in Figure 3. Although both pathways would give rise to the same intermediary 8-carbenium ion, differentiation between them should be possible based on the stereoelectronic effect of the Wagner−Meerwein-type rearrangement. In the pathway through the chair-boat conformation, the C-18 methyl group in **2** should be derived from the 16-position of GGPP, originating from the C-2 position of mevalonate (path a). In contrast, in the boat-boat conformation pathway, the C-18 methyl group must originate from the 17-position of GGPP (path b). Further, if major changes from the initial conformation have not taken place throughout the cyclase reaction, the electrophilic trigger proton should be retained in **2** in both cases.

Since the average deuterium enrichment at each isoprene unit was as high as 80%, the observed ^{1}H NMR signals of the biosynthesized **2** should be derived from the isoprene units produced by de novo synthesis and from the protons incorporated from an aqueous medium during the biosynthesis. The ^{2}H decoupled ^{1}H NMR

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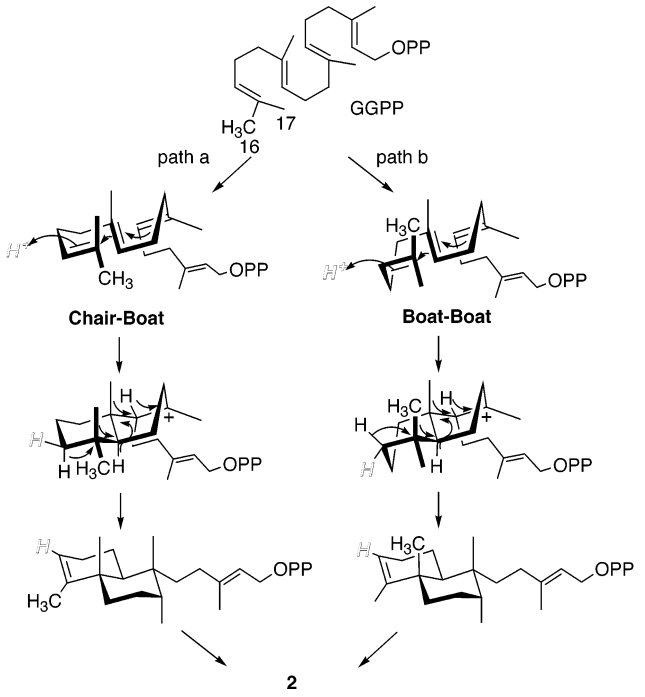

FIGURE 3. Hypothetical mechanism of cyclase reaction to form **2**.

spectrum of biosynthesized **2** is shown in Figure 4.[15] The C-18 methyl signal was observed as a cluster of three singlets at δ 1.552 (CHD_2), 1.568 (CH_2D), and 1.585 (CH_3), and a signal having long-range coupling at δ 1.588 (CH_3). Apparently, the three singlets can be attributed to a methyl group involved in the hydrogen-exchange equilibrium reaction between the deuterated IPP and DMAPP catalyzed by IPP isomerase.[9] Therefore, the C-18 methyl group of **2** must be derived from the 16-position of GGPP, but not from the 17-position, based on the well-documented GGPP biosynthesis.[16] The long-range coupled signal (δ 1.588) appears to be derived from a nonlabeled isoprene unit.

Further, the singlet methyl signals of C-19 and -20 of the labeled **2** were shifted upfield by ca. 0.05 ppm. These shifts were apparently caused by deuterium isotope effects, which clearly confirms the involvement of a 1,2-migration of the C-19 and C-20 methyl groups from a nonlabeled isoprene unit to a deuterated isoprene unit within the same GGPP molecule. Without the heterogeneously labeled mosaic molecules of **2**, these methyl shifts would not have been clearly defined. Since it would be difficult for conventional ^{2}H-tracer methodology to detect these group migrations, this appears to be a significant advantage of the pathway switching approach. These results clearly indicate that the cyclization proceeded through the chair-boat conformation.

Clear evidence of the final deprotonation was obtained by inspecting the C-3 hydrogen of **2**. The fact that the ^{1}H NMR spectrum showed no significant increase at the C-3 hydrogen indicated that the initially incorporated

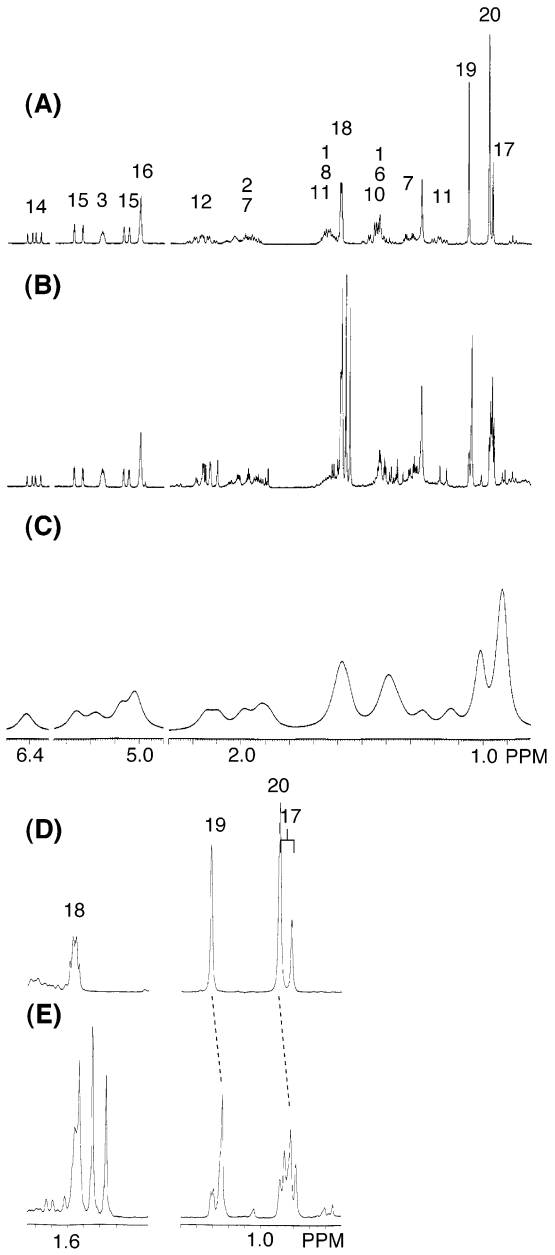

FIGURE 4. ^{1}H NMR spectra (500 MHz, $CDCl_3-CD_3OD$, 10:1) of (A) nonlabeled **2**, (B) perdeuterated **2** (^{2}H decoupled), (C) ^{2}H NMR spectrum (76.8 MHz, $CHCl_3$) of perdeuterated **2**, (D) expansion of a pertinent region of (A), and (E) expansion of a pertinent region of (B).

proton (derived from the medium) was lost during the cyclase reaction. If this proton was retained, an extremely strong signal should have been observed. This was in turn verified by the ^{2}H NMR spectrum, as shown in Figure 4C, in which a broad signal was observed at 5.18 ppm in the olefinic region. Apparently, the initial triggering proton was lost and the hydrogen or deuterium at the 14-position of GGPP was retained throughout the cyclization. Further proof of this was obtained by ^{13}C NMR analysis. The labeling pattern at C-3 in the deuterated **2** was analyzed by ^{13}C{^{1}H,^{2}H} NMR spectroscopy. As shown in Figure 5, a pair of ^{13}C signals (120.20 and 119.71 ppm) was observed for C-3. The signal

(15) ^{1}H and ^{13}C NMR of assignments for terpenetetriene **2** have been rigorously established. See ref 7.

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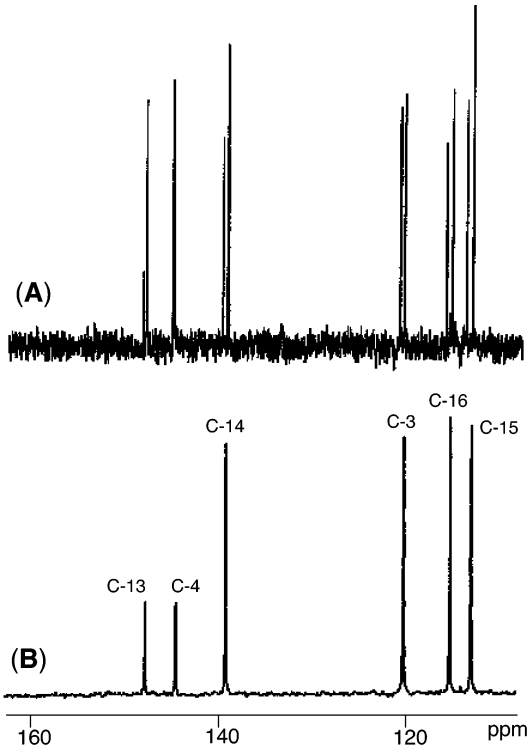

FIGURE 5. (A) $^{13}C\{^{1}H,^{2}H\}$ NMR spectrum (125 MHz, $CDCl_3$) of perdeuterated **2** and (B) ^{13}C NMR spectrum of nonlabeled **2**.

intensity of the paired ^{13}C signal for C-3 does not correspond to the abundance of deuterium. As one would expect, a carbon attached to ^{1}H is obviously enhanced in its intensity by NOE, whereas a carbon attached to ^{2}H is decreased in its intensity by a prolonged relaxation time. Therefore, the observation of the upfield signal is important here. Thus, one part of the paired signal appears to be due to the deuterium isotope shift, which definitely shows a deuterium atom retention at the C-3 position.

The results described above demonstrate that (1) the cyclization proceeded through the chair-boat conformation and (2) the initial triggering proton was lost at the end of the cyclization reaction. The third issue established was the elimination mechanism of the initial triggering proton. A simple explanation is that the penultimate C-4 cation is a relatively stable intermediate with a significant lifetime and the major conformational change in the A-ring at this stage leads to an axial orientation of the initial triggering proton from its original equatorial position. Accordingly, stereospecific deprotonation may be possible on the basis of stereoelectronic effect. To gain further insight into this problem, we undertook theoretical studies on the mechanism of the cyclization reaction, especially the 1,2-hydride and methyl shift steps. These studies were carried out with the semiempirical PM3 method.[17]

Stationary structures of the possible C-8, -9, -10, -5, and -4 cation intermediates were calculated with use of a dephosphorylated model molecule as shown in Figure 6. The corresponding heats of formation are also indicated in Figure 6. Obviously, a particular conformation should be required for the 1,2-migration step, i.e., the migrating σ-bond should be perpendicular to the plane of the sp^2 cation carbon. In each calculated cation structure, the dihedral angle composed of the migrating σ-bond and the plane of the sp^2 cation carbon is within the range 86−106°, which is in good accordance with the molecular orbital demand. Although all the intermediary cations are tertiary, the processes starting from the C-8 cation to the C-5 cation through the C-9 and C-10 cations turned out to be exothermic, probably due to the increase of C−C hyperconjugative stabilization in the cation intermediates and the release of the unfavorable 1,3-diaxial interaction between the C-4 and C-10 methyl groups. This result appears to suggest that these 1,2-migration reactions may proceed in a smooth almost barrierless manner. However, the 1,2-methyl migration leading to the final C-4 cation intermediate (**i**) from the C-5 cation appears to be energetically unfavorable by ca. 5 kcal/mol mostly due to the steric repulsive 1,3-diaxial interaction between the C-9 and C-5 methyl groups in the C-4 cation (**i**). Thus, significant conformational change should be involved in this step. Since, as observed, the initial triggering proton was lost in the enzyme reaction, the C-4 intermediary cation must be generated at the end of the rearrangement cascade. Therefore, stabilization of this cation by the enzyme should be required, most probably through cation-π interactions with aryl groups of side chains or through electrostatic interactions with negatively charged residues. This fascinating enzymatic process will be the subject of future studies.

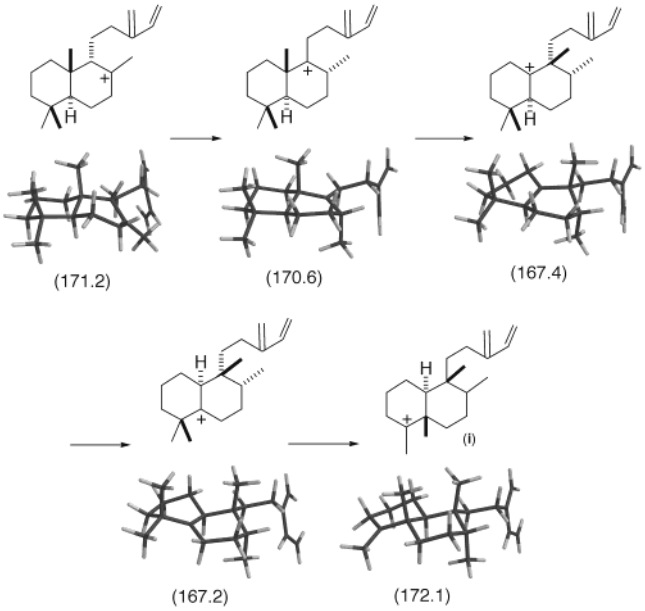

FIGURE 6. Calculated structures of the intermediary cation models in the diterpene cyclase reaction. The values in parentheses are the heat of formation (kcal/mol) obtained by semiempirical PM3 calculation.

In summary, the precise mechanism of the diterpene cyclase reaction in the biosynthesis of *Streptomyces* antibiotic terpentecin was investigated by pathway switching, labeling with mevalonolactone-d_9, and ^{1}H NMR

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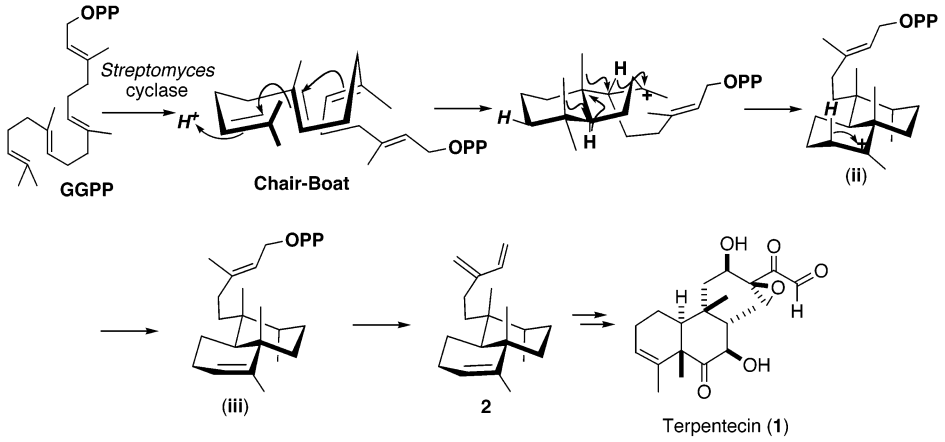

FIGURE 7. Precise reaction mechanism of the first diterpene cyclase of *Streptomyces* sp.

spectroscopy. In addition, the mechanism of the 1,2-hydride and methyl shifts in the cyclase reaction was investigated by using the PM3 semiempirical method. As depicted in Figure 7, GGPP cyclizes through a chair-boat conformation, followed by a series of methyl and hydride shifts to form an intermediary C-4 cation (**ii**). The cyclase reaction terminated by stereospecific deprotonation to form an allylic diphosphate intermediate (**iii**). Conversion to give the final terpentetriene **2** is effected by the other cyclase-like enzyme, which catalyzes the elimination of diphosphate.[18]

The clerodane diterpenes, all having the same relative stereochemical skeleton, were previously isolated from *Ajuga* sp. (*Labiatae*),[19] *Compositae*,[20] fungi,[21] and *Streptomyces* sp.[22] Although the detailed biosynthetic mechanism of these diterpenes has not yet been clarified, the same cyclization reaction may be possible. Further, the present study clearly demonstrates the precise analysis of the simplified proton spectra of highly deuterated isoprenoids, especially under deuterium-decoupled conditions. This approach appears to be beneficial for mechanistic enzymology, particularly for the key transformations involving proton attack, methyl and hydride shifts, and deprotonation as observed in the terpene cyclase reactions.

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Supporting Information Available: Cartesian coordinates and total energies of the compounds in Figure 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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