

Biosynthetic Formation of the 6/7/5-Odyverdiene Diterpenes via a 6/10-*trans*-Eunicellanyl Intermediate

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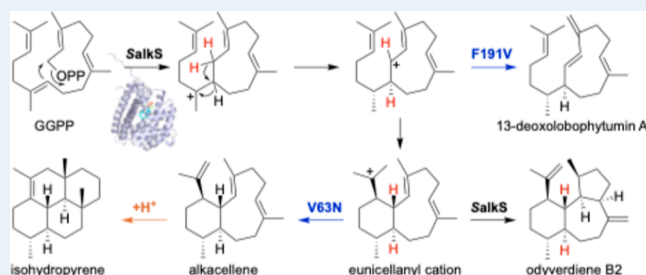
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ABSTRACT: Odyverdiene is a rare class of bacterial diterpenes possessing 6/8/4- or 6/7/5-tricyclic skeletons, and the cyclization mechanism underlying their formation remains unknown. Based on their skeletons, we hypothesized that their tricyclic skeletons pass through a neutral or cationic 6/10-eunicellane intermediate. Here, we investigated the mechanism of odyverdiene B2 synthase, SalkS from *Streptomyces alkaliterrae*, using site-directed mutagenesis, isotopic labeling experiments, and quantum chemical calculations. Two key shunt products, the 10-membered monocyclic 13-deoxolobophytumin A and a *trans*-eunicellane named alkacellene, isolated from SalkS variants, and quantum chemical calculations supported the proposed mechanism to odyverdiene B2 via initial 1,10-cyclization and a cationic eunicellanyl intermediate. Deuterium labeling studies also supported the occurrence of two sequential 1,2-hydride shifts that set distinctive stereocenters during eunicellanyl formation. Alkacellene was also shown to undergo acid-catalyzed cyclization to the 6/6/6/6-tetracyclic isohydromyrene, an isomer of the bacterial diterpene synthase product hydromyrene. This mechanistic study of SalkS highlights the frequent formation and use of eunicellanyl intermediates in diversifying bacterial diterpene biosynthesis and provides a model system to address fundamental questions involving 6/10-bicyclic ring systems.

KEYWORDS: diterpene synthase, terpenoid, quantum chemical calculations, cyclization, mechanism, vibrational circular dichroism



INTRODUCTION

Terpene synthases (TSs) are captivating enzymes that convert acyclic and achiral oligoprenyl diphosphate precursors into complex polycyclic hydrocarbons or alcohols with multiple stereocenters.¹ TSs are categorized into two canonical types based on their mechanism of initial ionization: diphosphate abstraction (type I) or protonation of an olefin or epoxide (type II);^{2,3} there are also several families of noncanonical TSs.⁴ After initial carbocation formation, cyclization ensues via a multistep cascade including intramolecular C–C bond formations, rearrangements such as the Wagner–Meerwein rearrangement, hydride or proton migrations, and ultimately cation quench by deprotonation or water attack.^{1,5}

Eunicellane diterpenoids represent a distinctive family of natural products found in marine organisms, plants, and bacteria and possess impressive biological activities.⁶ The eunicellane skeleton is a 6/10-bicyclic framework that can be divided into two main classes, *cis* and *trans*, based on the configuration of their ring fusion at C-1 and C-10 (Figure 1).⁷ The first eunicellane synthase, a bacterial TS named Bnd4 from *Streptomyces* sp. CL12–4, was reported in 2021. A total of 11 eunicellane synthases, nine from bacteria and two from corals,^{8–15} have been reported. Most of these TSs do not share significant sequence similarity with each other and two distinct pathways are experimentally supported to form the

eunicellane skeleton.⁷ In many of the bacterial TSs, the 10-membered ring is formed prior to the second cyclization to yield the 6/10-bicyclic structure;^{9,12,16} in the coral TSs and one bacterial TS, a 14-membered cembrenyl cation is first formed before the second cyclization splits the macrocycle into the eunicellane skeleton.^{13–15} Both mechanisms can yield *cis* or *trans* ring fusions, and the four stereocenters are set according to substrate conformations and hydride shifts.

We recently discovered the odyverdiene B2 synthase, SalkS from *Streptomyces alkaliterrae*,¹⁰ which produces the naturally rare 6/7/5-tricyclic skeleton. Odyverdiene B2 (1) is a diastereomer of previously discovered odyverdiene B (2), one of the two major products of the diterpene synthase (di-TS) ND90 from *Streptomyces* sp. ND90; the other is the 6/8/4-tricyclic odyverdiene A (3) and neither the relative nor the absolute configurations of 2 and 3 were previously determined (Figure 1).¹⁷ The cyclization mechanism of odyverdiene formation is unknown; however, we hypothesized that their

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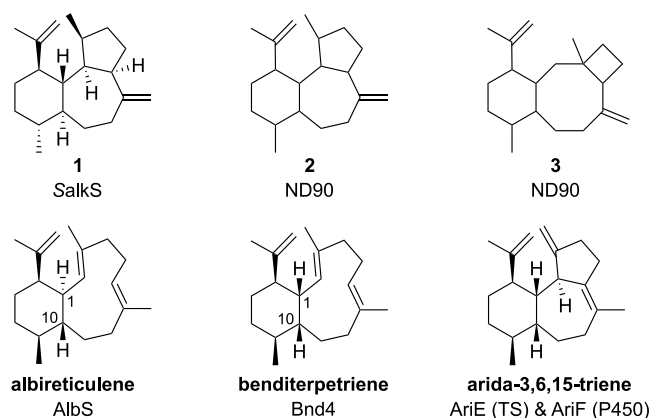


Figure 1. Structures of odyverdiene and representative eunicellane diterpenes known prior to this study. The TSs that form these skeletons are listed below the compound numbers or names; arida-3,6,15-triene is formed by a two-enzyme reaction sequence, a eunicellane synthase AriE and cytochrome P450 AriF. The structural similarities of the eunicellanes and odyverdiene suggested similar cyclization mechanisms.

tricyclic skeletons likely pass through a neutral or cationic eunicellanyl intermediate. A parallel can be drawn with the formation of the tetracyclic venezuelaene A by the di-TS VenA, where the eunicellanyl cation serves as a crucial intermediate.^{18,19} Interestingly, the 6/7/5-tricyclic aridacins, produced by *Amycolatopsis arida*, are formed through sequential cyclization reactions catalyzed by AriE, a eunicellane synthase, and AriF, a cytochrome P450 (Figure 1).¹¹

In this study, we investigated the cyclization mechanism for the biosynthesis of odyverdiene B2 (1) catalyzed by SalkS using a combination of mutagenesis, structural elucidation of shunt pathway products, deuterium labeling studies, and quantum chemical calculations. Given identical planar skeletons, we

propose a mechanism similar to that for the formation of 2 and 3 by ND90. Because the absolute configurations of 2 and 3 were previously unreported, we determined their configurations through NMR analysis and vibrational circular dichroism (VCD). We also explored the chemical reactivity of a new *trans*-eunicellane by forming the 6/6/6/6-tetracyclic isohydro-pyrene using acid catalysis and surreptitiously forming a new eunicellane alcohol.

RESULTS AND DISCUSSION

Stereochemical Determination of the Odyverdiene

We previously determined the structure and absolute configuration of odyverdiene B2 (1);¹⁰ however, the absolute configurations of odyverdiene B (2) and odyverdiene A (3) from *Streptomyces* sp. ND90 were never determined.²⁰ Given the structure of 1 and the possible biosynthetic relationship with the eunicellanes, we sought to determine the absolute configurations of 2 and 3. Already having a codon-optimized gene for *E. coli* expression for SalkS (UniProt ID: A0ASP0YYU2),¹⁰ we similarly obtained a synthetic gene for ND90 (UniProt ID: A0A097ZQD1) from *Streptomyces* sp. ND90 (Tables S1–S3). We first produced and purified SalkS and ND90 in *E. coli* (Figure S1) and individually tested each TS for cyclization activity with geranyl diphosphate (GPP; C10), farnesyl diphosphate (FPP; C15), and geranylgeranyl diphosphate (GGPP; C20). According to GC-MS analysis (Figure S2), SalkS produced two products in the presence of GGPP, 1 and a minor unknown diterpene, 4. Consistent with previous literature,¹⁷ ND90 produced its two major diterpenes, 2 and 3, when incubated with GGPP. No products were seen when either SalkS or ND90 was incubated with GPP or FPP.

The three major diterpenes 1–3 were isolated from our engineered *E. coli* GGPP overproduction system²¹ and their structures were confirmed by 1D and 2D NMR spectroscopy

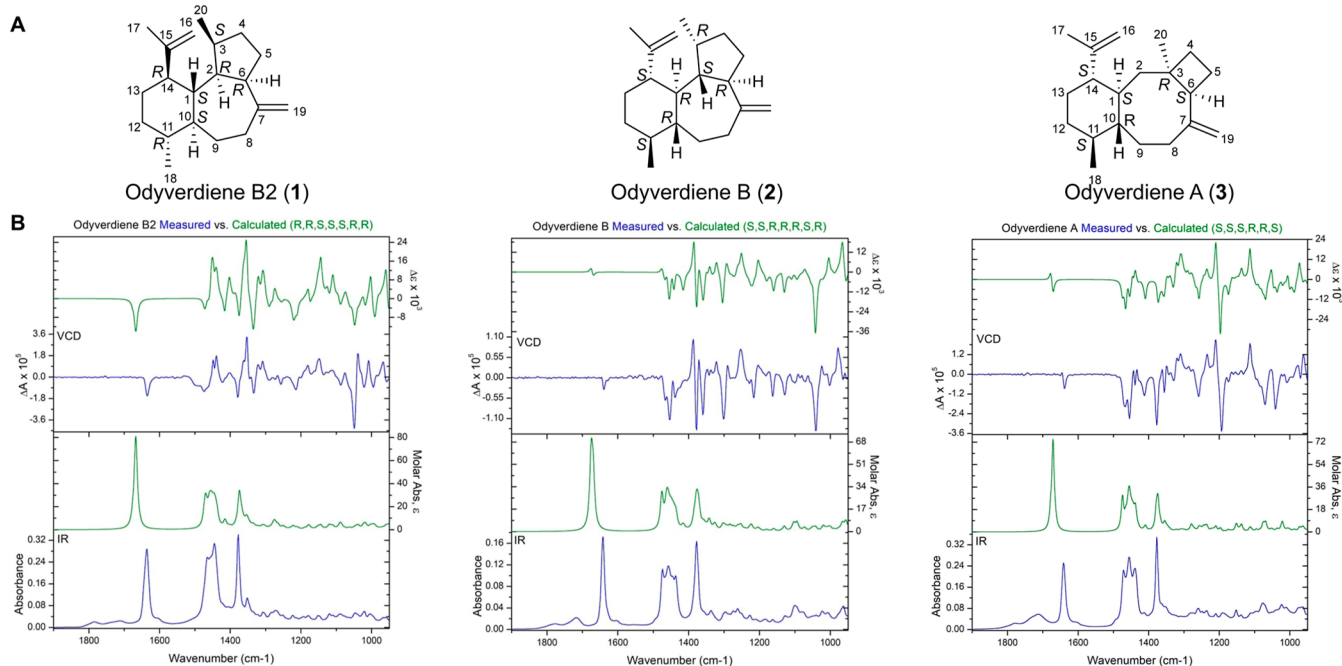


Figure 2. Structures and IR and VCD spectra of the odyverdiene diterpenes. (A) Structures of odyverdiene B2 (1), odyverdiene B (2), and odyverdiene A (3). (B) IR (lower frame) and VCD (upper frame) spectra observed for 1–3 (blue) compared with Boltzmann-averaged spectra of the calculated conformations (green); the VCD of 1 was previously reported¹⁰ but shown here for comparison.

and EI spectrometry (Tables S4–S6 and Figures S3–S20); initial isolation of **4** failed from native SalkS due to its low production. We determined the absolute configurations of **2** and **3** for comparison to that of **1**. Using 2D NOESY NMR analysis (Figures S12 and S20) and vibrational circular dichroism (VCD),^{22,23} we assigned **2** as 1*R*,2*S*,3*R*,6*R*,10*R*,11*S*,14*S* and **3** as 1*S*,3*R*,6*S*,10*R*,11*S*,14*S* (Figure 2). Similar to **1**, both **2** and **3** have *trans* ring fusions between their 6- and 7- or 8-membered rings, suggesting these may originate from *trans*-eunicellanes. Interestingly, **2** is nearly the enantiomer of **1**, with only one (C-6) of its seven stereocenters not being flipped.

Alteration of the Product Profile of SalkS by Mutagenesis Supports an Eunicellanyl Intermediate via an Initial 10-Membered Cyclization

To investigate the cyclization mechanism of odyverdiene formation, we chose to utilize SalkS as it only produces a single major product, **1**, and had a higher enzyme activity than ND90 *in vitro*. Based on the structures of **1**–**3** and our hypothesis that eunicellane is a key intermediate in odyverdiene biosynthesis, we hypothesized that cyclization toward the odyverdiene follows the same early steps as most of the eunicellane syntheses from bacteria (Figure 3).^{7,9,16} Initial 1,10-cyclization of the ionized

in diterpene cyclization mechanisms.^{5,24–27} A final 2,6-cyclization occurs to form **F**⁺ and deprotonation at C-19 yields **1**.

Site-directed mutagenesis is a useful technique to investigate mechanisms of TSs because TS variants may improve yields of minor products or alter product profiles. These alternate products often expose on-pathway skeletons as the native enzymatic template, i.e., how the active site shapes the substrate and stabilizes carbocation intermediates, is disrupted. SalkS contains the highly conserved motifs of class I TSs: the Asp-rich motif (D⁹³DLAAD), the NSE triad (N²³²DVLSLRKE), the pyrophosphate sensor (R187), and the R³¹⁷Y pair (Figure S21). We also generated a model of SalkS using AlphaFold3 and docked GGPP into the active site using AutoDock Vina to identify residues of potential interest (Figure 4A).^{28,29} While AlphaFold structures sometimes fail to capture key details for TSs, they are generally reliable for identifying active site residues. All mutations were constructed using site-directed mutagenesis, and SalkS variants were tested *in vitro*. First, we made the variants D93N, D93E, and D94N to test the requirement of the DDxxx motif. TS activity was completely abolished in D93N and D93E while D94N showed increased activity (170 ± 5%, Figure 4B and Table S7), supporting the notion that the second Asp in the DDXXX motif is not essential for activity. Similar results were seen in several native TSs from bacteria that do not include the second Asp such as Bnd4 (D⁹⁴Nxxx) and VenA (D¹¹⁵SFVSD).^{16,18}

Several aromatic (Y66, Y86, F161, F191, F195, F311, and Y318 from the RY motif) and hydrophobic residues (V63, V89, I196, and I229) line the active pocket of SalkS. We mutated these amino acids to test their effects on the formation of **1** (Figure 4B). The Y318F variant showed increased production of **1** (120 ± 2%) and significant production of unknown diterpene **4** (Figure 4C). Isolation, EIMS, and NMR analysis of **4** led us to identify it as a 10-membered monocyclic system with an *E*-configured C-1/C-2 alkene (Figure 4D), which was determined by its large coupling constant ($J_{\text{H1,H2}} = 15.8$ Hz) (Table S8 and Figures S22–S29). This diterpene, which we named 13-deoxolobophytumin A based on its similarity to the coral diterpenoid,³⁰ was recently reported from a variant of the coral biflorane synthase PcTS1.³¹ Based on its biosynthetic relationship with **1**, we tentatively propose that **4** has the same two stereocenters at C-10 (*S*) and C-11 (*R*). The structure of **4** supports that SalkS initially catalyzes 1,10-cyclization in the pathway to **1** as deprotonation of **C**⁺ at C-18 leads to **4** (Figure 3). F191 appears to play a significant role in the formation of **1**, perhaps through the stabilization of the monocyclic cationic intermediate as two variants (F191A and F191V) abolished the production of **1** while increasing the yield of **4**; three others (F191D, F191E, and F191Y) negatively impacted protein solubility. The π -systems of Y66 and Y86 were also found to be important, as the activities and product distribution of Y66F and Y86F were essentially unaffected compared to native SalkS, but Y66A and Y86A severely inhibited the formation of **1**. Because Y66A produces β -springene (**6**) and (–)-cembrene A (**7**) (Figures 4 and S30–S33, Tables S9 and S10), it is likely that the π -system of Y66 plays a critical role in providing an appropriately shaped active site to fold GGPP for the initial 1,10-cyclization. Major changes at various positions including V89W, I196Y, and F311A dramatically decreased TS activity, while several variants (V63N, F161A, F195A, and I196L) had only minor effects on the production of **1** (Figure 4). Although F161A did not affect the production of **1**, a new polar diterpene,

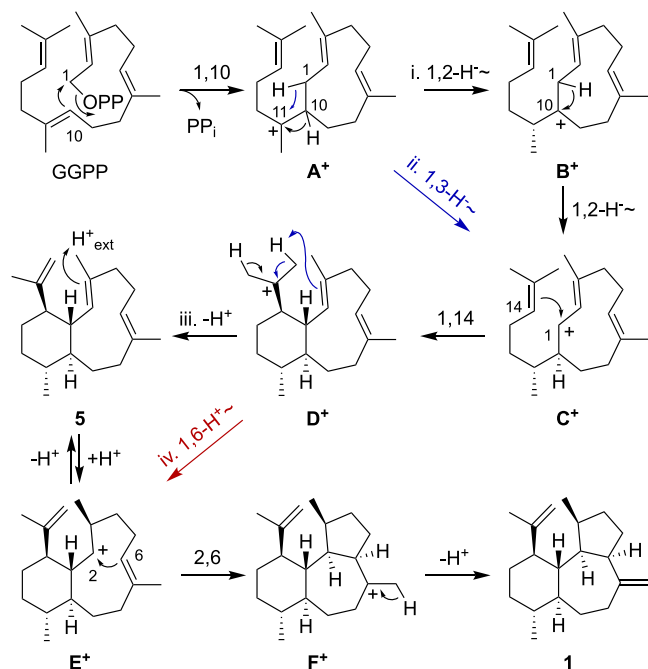


Figure 3. Proposed cyclization mechanism from GGPP to **1** by SalkS. The mechanism has two different possible branchpoints. First, two sequential 1,2-hydride shifts (pathway i, black) or a direct 1,3-hydride shift (pathway ii, blue) is possible for the formation of **C**⁺. Second, the formation of **E**⁺ from **D**⁺ can occur via the neutral intermediate **5** (iii, black) or via direct 1,6-proton transfer (iv, red). In pathway iv, **5** may be a shunt product formed from deprotonation of **D**⁺ or **E**⁺.

GGPP precursor leads to **A**⁺, followed by two sequential 1,2-hydride shifts (pathway i) or a single 1,3-hydride shift (pathway ii) to **C**⁺, and 1,14-cyclization to the eunicellanyl **D**⁺. After the formation of **D**⁺, we proposed two possible pathways to form **E**⁺: pathway iii deprotonates **D**⁺ at C-16 to give the neutral *trans*-eunicellane intermediate **5**, which is then reprotonated at C-3 to yield the secondary carbocation **E**⁺; pathway iv forms **E**⁺ directly through a 1,6-proton transfer. Both pathways seem feasible as neutral intermediates and long-range proton transfers are known

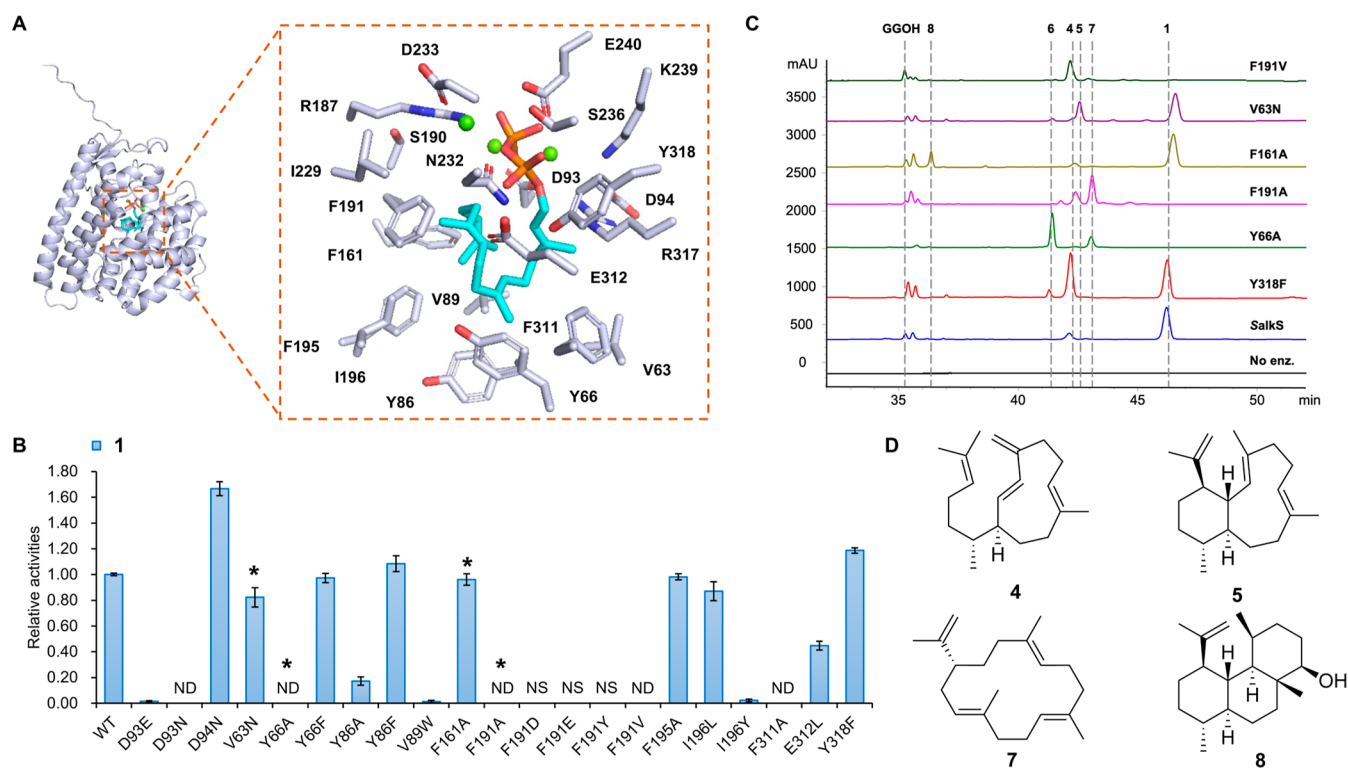


Figure 4. Structural and mutational analysis of SalkS. (A) AlphaFold model of SalkS displaying key active site residues (lavender) with a docked model of GGPP (cyan). Three Mg^{2+} ions (green spheres) from selinadiene synthase (PDB: 4OKZ) were overlaid to show their approximate positions. (B) Relative cyclization activities of SalkS and mutants forming **1**. Values are the mean of three independent experiments with error bars representing standard deviations. NS and ND denote not soluble and no detectable activity, respectively. Variants marked with asterisks produced other diterpene products. (C) HPLC-UV analysis of the *in vitro* activity of SalkS or selected variants with GGPP. Products were detected using absorbance at 210 nm. Geranylgeraniol (GGOH), the hydrolyzed form of GGPP, is commonly present in the *E. coli* MK14 system. (D) Structures of **4**, **5**, **7**, and **8**.

8, was detected. Isolation, EIMS, and NMR analysis of **8** led to the elucidation of a 6/6/6-tricyclic alcohol (Figures 4 and S34–S41, and Table S11). The absolute configuration of this new compound, named gersemiol D (**8**) after the structurally similar gersemols from soft corals,³² was determined as 1R,2R,3S,6S,7R,10S,11R,14R by 1D NOE and VCD analysis (Figures S42–S47). The loss of the phenyl side chain in F161A must perturb the active site enough to allow a minor competing 2,7-cyclization step at E⁺ to provide the 6/6/6 skeleton prior to the water quench at C-6.

We proposed that the formation of the odyverdienes went through a eunicellane intermediate, whether a neutral or a cationic one. Gratifyingly, the V63N variant produced **5** (Figure 4C), which was identified by EIMS and NMR analysis as a *trans*-eunicellene isomer we named alkacellene (Table S12 and Figures S48–S54); the absolute configuration of **5** is proposed below. The identification of **5** supports the proposed 1,14-cyclization of intermediate C⁺ to yield D⁺ (Figure 3). In many TS mechanisms, deprotonation and reprotonation sequences through neutral intermediates are proposed and experimentally supported.^{1,5,24,25} We attempted to ascertain whether **5** is a neutral intermediate on the pathway to **1** or a shunt product by conducting the SalkS reaction in D₂O.³³ However, EIMS of **1** produced in this reaction showed no incorporation of a deuterium atom (Figure S55) suggesting that **5** is a shunt product from D⁺ in the pathway to **1**. An alternative explanation may be that **5** is indeed an intermediate and that the proton that is removed from D⁺ is transferred indirectly through a proton relay to C-3 to form E⁺.

Deuterium Labeling Confirms Sequential 1,2-Hydride Shifts Prior to the Second Cyclization

Because C-1 forms two new bonds during cyclization from GGPP to the 6/10-eunicellanyl intermediate, the carbocation initially at C-1 after diphosphate abstraction must return to C-1. In eunicellane cyclization, TSs that first form the 10-membered ring have been shown to catalyze direct 1,3-hydride shifts (i.e., A⁺ to C⁺) while TSs that form the 14-membered cembrenyl cation have been shown to use sequential 1,2-hydride shifts to regenerate the cation at C-1. This sequence is partially responsible for setting the stereocenters at C-10 and C-11. To determine how SalkS reforms the C-1 cation (C⁺), we incubated 1,1-²H₂-GGPP with SalkS and isolated the deuterated enzymatic product for characterization by NMR analysis. The ¹H NMR spectrum of ²H₂-**1** was nearly identical to that of **1**, with the exception of the absence of signals for H-1 (δ_H 1.45 ppm) and H-10 (δ_H 1.06 ppm); in addition, H-2 and H-14 of ²H₂-**1** displayed reduced splitting patterns as those seen in **1** (Figures S56–S58). A comparison of the HSQC spectra of **1** and ²H₂-**1** confirmed that the deuterium atoms were on C-1 and C-10 (Figure 5). These results unambiguously indicated that both deuterium atoms were retained in **1** via two sequential 1,2-hydride shifts, first from H-10 to H-11 in A⁺ and then from H-1 to H-10 in B⁺. This is different than the direct 1,3-hydride shifts seen in the eunicellane synthases Bnd4 and AlbS,^{9,16} instead, following the hydride shift pattern seen in microeunicellene and klysimplexin R formation by MicA and EcTPS1, respectively.^{13,14}

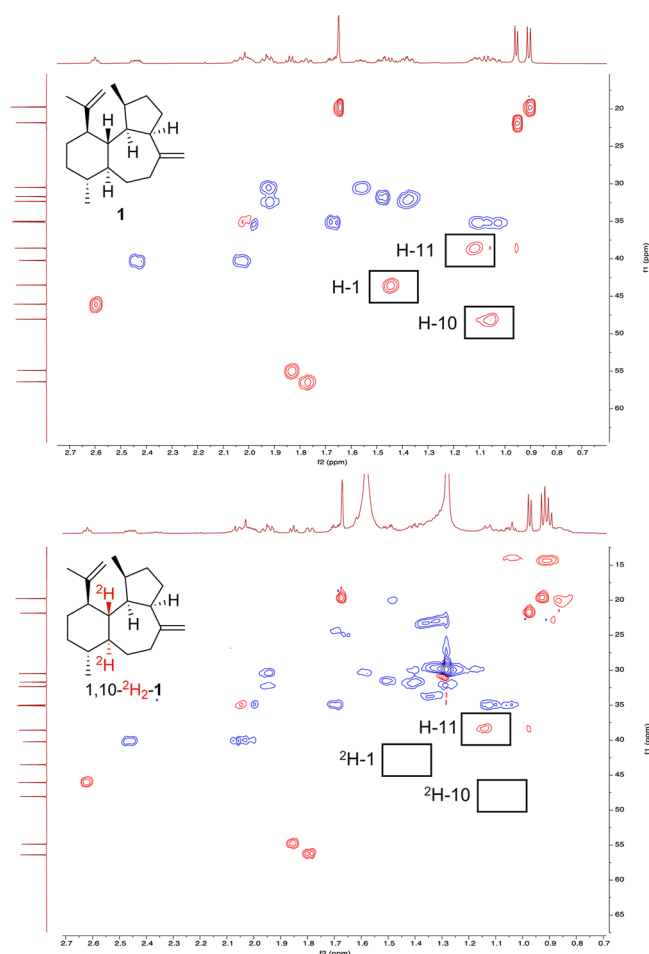


Figure 5. Deuterium labeling support for the cyclization of GGPP to **1** by SalkS. ^1H – ^{13}C HSQC spectra of **1** (top) and **1,10- $^2\text{H}_2$ -1** (bottom) from reactions with unlabeled GGPP and **1,1- $^2\text{H}_2$ -GGPP**.

Quantum Chemical Calculations Validate the Feasibility of the Proposed Mechanism and Reveal a More Thermodynamically Favorable, but Unused, Pathway

To evaluate the energetic viability of our proposed mechanism and assess the two hypothesized pathways for the formation of **E**⁺, we conducted DFT calculations utilizing the mPW1PW91/6-31+G(d,p) method^{25,34–38} to calculate the relative free energies of cationic intermediates and transition structures (Figures 6 and S59 and S60 and Table S13). The first 1,2-hydride shift, converting intermediate **A**⁺ to cation **B**⁺, is facile and nearly barrierless. Conformational changes of **B**⁺ to **B'**⁺ to **B''**⁺ (Table S14 and Figure S61) allow a second 1,2-hydride shift converting **B**⁺ to **C**⁺ with an overall 1.6 kcal mol^{−1} barrier. This pathway is feasible and supported by the deuterium labeling experiment described above; however, we also computationally investigated the alternate 1,3-hydride shift to directly convert **A**⁺ to **C'**⁺ (TS_{A⁺/C'⁺}), but the 4.3 kcal mol^{−1} barrier is less favorable than the sequential 1,2-hydride shifts.

We next investigated whether **A**⁺ is the 10*S* or 10*R* enantiomer; previous studies supported that the eunicellane synthases Bnd4 and AlbS navigate through the 10*S* enantiomer.^{9,16} We first assessed the pathway starting with the 10*R* configuration as it can yield the expected 10*S*,11*R* configurations of **C**⁺ (and of **1**) via two suprafacial 1,2-hydride shifts without significant bond rotations (Figure 6). Alternatively, the conversion of ent-**A**⁺ (i.e., 10*S*) to **B''**⁺ requires rotation of the

C-10/C-11 bond and the barrier (0.86 kcal mol^{−1}; Table S15 and Figure S62) is slightly higher than that of **A**⁺ to **B**⁺ (or ent-**A**⁺ to ent-**B**⁺), although this is within the error of DFT calculations. Another pathway from ent-**A**⁺ could be envisaged via two 1,2-hydride shifts of the antiperiplanar-like positioned H-10 and pro-S (designated based on **B''**⁺) H-1 ultimately providing **C**⁺ (Figure S62). However, the TS_{B''⁺/C⁺} barrier (8.5 kcal mol^{−1}) is significantly higher than the barrier for the TS_{B''⁺/B'⁺} conformational change (2.8 kcal mol^{−1}). Based on these results, we cannot definitively conclude which enantiomer of **A**⁺ is formed by SalkS as **B'**⁺ can reasonably be accessed from either enantiomer of **A**⁺.

After a series of conformational changes of **C**⁺ (**C**⁺ to **C'**⁺ to **C''**⁺, overall barrier of 7.2 kcal mol^{−1}), a favorable conformation can be achieved to cyclize into the eunicellanyl intermediate **D**⁺ with a low barrier of 0.5 kcal mol^{−1} through TS_{C''⁺/D⁺}. The range of conformational changes required for **C**⁺ is unusual and may suggest that the active site of SalkS is somewhat spacious or that precursors to **C**⁺ are formed in less-than-ideal conformations. The odysseydiene skeleton can then be formed via a 1,6-proton transfer and concomitant cyclization, forming the 5,7-fused rings of **F**⁺ through TS_{D⁺/F⁺} at a barrier of 4.7 kcal mol^{−1}. Overall, this proposed pathway to 6/7/5-tricyclic **1** via the *trans*-eunicellanyl cation **D**⁺ is very energetically feasible.

During computational investigation, we fortuitously discovered an alternative, hypothetical, double cyclization transition structure from **B'**⁺ to the 5/5/10-tricyclic **G**⁺ (TS_{B'⁺/G⁺}) ultimately leading to a series of unique skeletons. From **G**⁺, a possible 13.9 kcal mol^{−1} 1,5-proton transfer and cyclization leads to the 5/5/7/5-fused tetracycle **H**⁺ followed by a subsequent 1,2-alkyl shift leading to the 5/5/6/6-tetracyclic **I**⁺; a boat-to-chair conformational change yields the low energy conformer **I'**⁺. It is notable that the free energy barrier minorly favors TS_{B'⁺/C⁺} relative to TS_{B'⁺/G⁺} by 0.3 kcal mol^{−1} but that TS_{B'⁺/G⁺} is slightly favored enthalpically (numbers in parentheses) by 1.1 kcal mol^{−1}. We posit that this entropic factor could be leveraged within SalkS to guide the pathway toward **F**⁺ instead of the much more thermodynamically favorable **B'**⁺ to **I**⁺ cascade. SalkS could, of course, also provide selective electrostatic stabilization to TS_{B'⁺/C⁺} or be an impediment (electronic or steric) to reaching TS_{B'⁺/G⁺}. Structure-based searches of **G**⁺, **H**⁺, and **I**⁺ revealed that there are no diterpenes known with these polycyclic skeletons; however, it is not unreasonable to envisage another TS capable of guiding GGPP to these unique structures.³⁹

Chemical Reactivity of Alkacellene (5) Produces a New Eunicellane Alcohol and a 6/6/6/6-Tetracyclic Skeleton

Eunicellanes, like germacrenes, are often conformationally dynamic due to their 10-membered rings.^{8,9,12,41,42} Alkacellene (**5**) was also found to undergo slow chemical exchange as we observed doubled resonances in both the ^1H and ^{13}C spectra as well as chemical exchange crosspeaks in the 2D NOESY spectrum (Figures S49–S54). Some eunicellanes are prone to thermal Cope rearrangements or are easily converted into 6/6/6-tricycles in mildly acidic conditions.^{9,11,43–45} In acid, **5** is converted into a new 6/6/6/6-tetracyclic diterpene **9** (Table S16 and Figures S63–S70). Structural elucidation of **9** by NMR and VCD analysis (Figure S71) indicated a few differences between **9** and two known 6/6/6/6-tetracyclic TS products hydropyrene and hydropyrenol.^{17,20} The alkene in **9** was located at C-14/C-15 and the configuration of **9** is nearly enantiomeric of hydropyrene, except for the stereocenter at C-10 and the lack of the stereocenter at C-14; thus, we named **9** isohydropyrene.

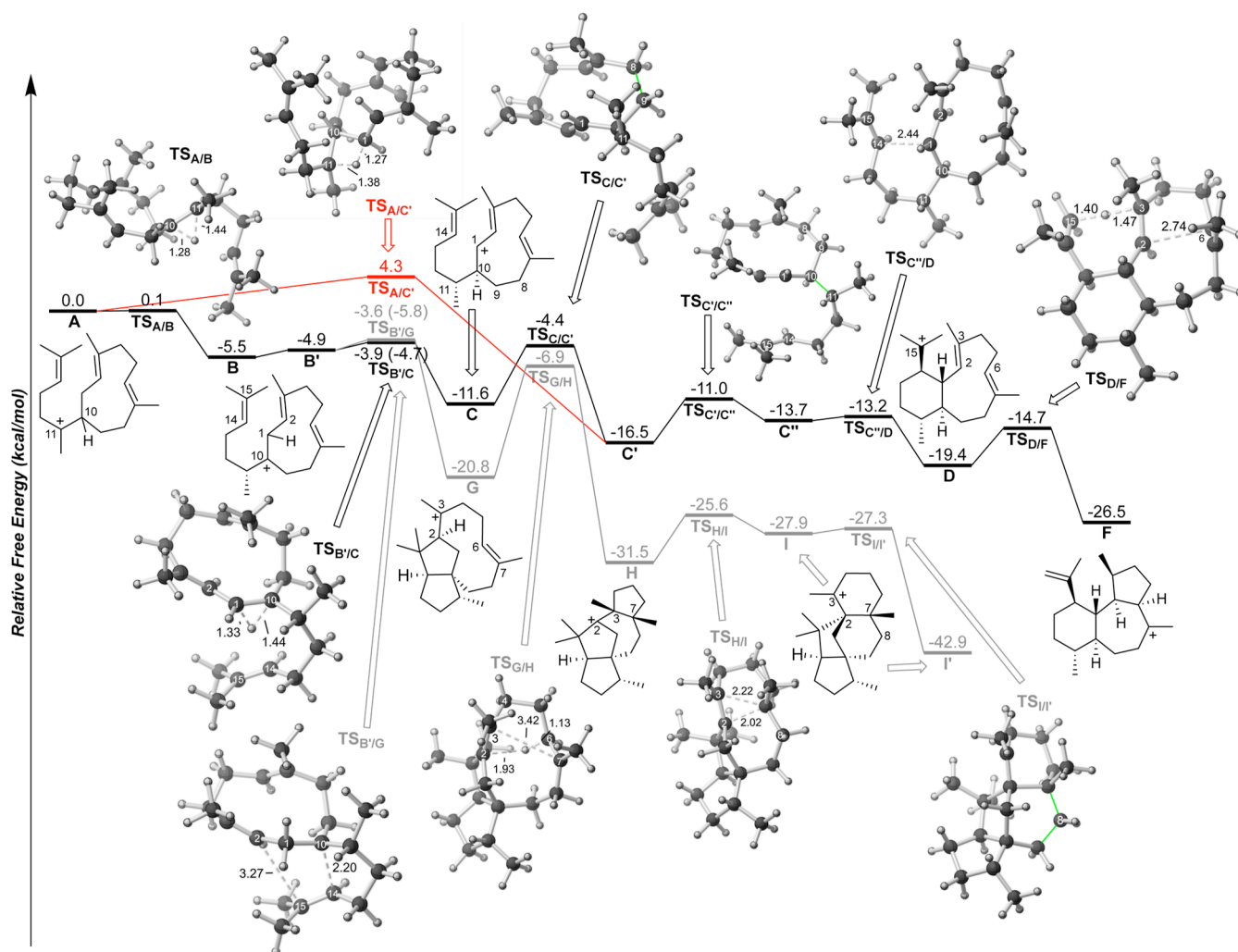


Figure 6. Relative free energies of intermediates and transition structures in kcal mol⁻¹, calculated at the mPW1PW91/6-31+G(d,p) level of theory. The black pathway is the mechanistically and computationally supported mechanism for the formation of **1**. The red pathway shows an alternative 1,3-hydride shift. The gray pathway is a theoretical alternative pathway that is thermodynamically favored but not catalyzed by SalkS; the parenthetical numbers for TS_{B/G} and TS_{B/C} are relative enthalpies. Computed structures were deposited in the IoChem-BD repository,⁴⁰ and see Figure S59 for computed intermediates. Bond distances (in Å) for key steps are listed beside the bond. In this figure, TS denotes transition structures; for TSs of conformational changes, the rotating bonds are colored green. For simplicity, the cationic (+) indicators were removed from the labels.

Hydropyrene and the related hydropyrenol were previously found to be downstream TS products from the *cis*-2*E*,6*E* eunicellene neutral intermediate prehydropyrene (Figure 7).⁴⁶ We also evaluated whether **5** undergoes [3,3]-sigmatropic rearrangement at 120 °C but were unable to identify any major Cope rearrangement products, although there were unknown products in the reaction mixture. Instead, we identified and isolated an air oxidation product, **10**, with an M⁺ peak at *m/z* 288.2435 (Figure S72), supporting a molecular formula of C₂₀H₃₂O. NMR analysis of **10**, named alkacellenol, revealed the *trans*-eunicellane core with alkenes at C-2/C3, C-7/C-8, and C-15/C-16 and a hydroxyl at C-6 (Table S17 and Figures S73–S78). The configuration of the C-7/C-8 alkene was determined to be *Z* based on the strong NOESY correlation between H-8 and H-19 and the absolute configuration of **10** was confirmed as 1*S*, 6*S*, 10*S*, 11*R*, and 14*R* by VCD analysis (Figures S79 and S80). Given the synthetic considerations of converting **5** into **9** and **10**, as well as the biosynthetic relationship between **5** and **1**, the absolute configuration of **5** is proposed to be 1*S*, 10*S*, 11*R*, and 14*R*.

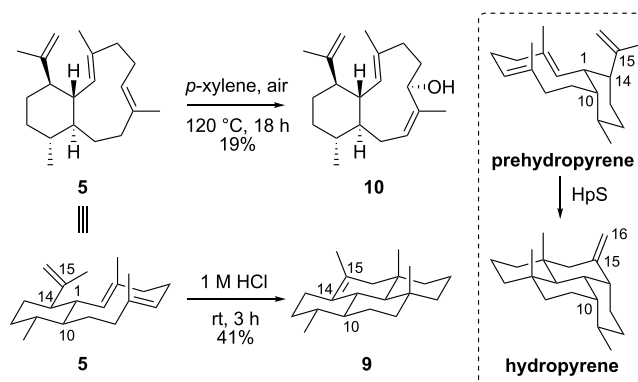


Figure 7. Chemical reactions with **5**. The oxidation product **10** was identified after an attempted [3,3]-sigmatropic rearrangement at 120 °C and the tetracyclic **9** was formed under mild acidic conditions. The structurally similar and hydropyrene synthase product hydropyrene that originates from the neutral intermediate prehydropyrene is shown in the dotted box.

CONCLUSIONS

In this study, we investigated the mechanism of formation of the 6/7/5-tricyclic odyverdiene diterpenes using SalkS, a recently discovered diterpene synthase, as the model system. Using site-directed mutagenesis, congener isolation and structure elucidation, isotopic labeling experiments, and DFT calculations, SalkS forms and uses a eunicellanyl cationic intermediate to generate the tricyclic skeleton. In addition, we determined the absolute configurations of the previously discovered odyverdiene A and B. Although the TSs that form the three odyverdiene are different, we propose that ND90 follows a similar mechanistic pathway (Figure S81). In fact, the proposed eunicellanyl intermediates in SalkS and ND90 are enantiomers and may follow the same (enantiomeric) pathway until the final cyclization. In conclusion, there is growing evidence that the eunicellane skeleton plays an important role in bacterial diterpene biosynthesis, whether as a product of a TS or as a neutral or cationic intermediate on the pathway to a more complex polycyclic skeleton. This mirrors the influence that germacrene plays in sesquiterpene biosynthesis.⁴⁷ Understanding the principles of how TSs control eunicellane formation or use it as a template will allow the biocatalytic community to access these skeletons, find new natural versions to expand the bacterial terpenome,⁴⁸ or engineer cationic cascades to develop novel diterpenes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Chemicals

Strains, plasmids, and PCR primers used in this study are listed in Tables S1–S3. PCR primers were obtained from Sigma-Aldrich. Q5 high-fidelity DNA polymerase and restriction endonucleases were purchased from NEB and used by following the protocols provided by the manufacturers. DNA gel extraction and plasmid preparation kits were purchased from Omega Bio-Tek. DNA sequencing was conducted by Genewiz. ²H₂-1 was enzymatically synthesized (see the Supporting Information for the detailed methods). Other common chemicals and biochemical and media components were purchased from standard commercial sources.

General Experimental Procedures

All 1D (¹H and ¹³C) and 2D (¹H–¹³C HSQC, ¹H–¹H COSY, ¹H–¹³C HMBC, and ¹H–¹H NOESY) NMR experiments were performed in CDCl₃ or C₆D₆ on Bruker AVANCE III Ultrashield 600, Bruker AVANCE III HD 600, or Bruker AVANCE III 800. All NMR chemical shifts were referenced to residual solvent peaks or to Si(CH₃)₄ as an internal standard. Terpene production was monitored by high-performance liquid chromatography (HPLC). HPLC was performed on an Agilent 1260 Infinity LC equipped with a Restek Roc-C18 column (150 mm × 4.6 mm, 5 μm). Preparative HPLC was carried out on an Agilent 1260 Infinity LC equipped with an Agilent Eclipse XDB-C18 column (250 mm × 21.2 mm, 7 μm). GC-MS analysis was run using a Thermo Scientific Orbitrap Exploris spectrometer with a Rxi-SMS column (Restek Corp, 30 m × 0.25 mm i.d. and 0.251 μm film). Optical rotations were measured using a JASCO P-2000 polarimeter. IR data were measured by a PerkinElmer Spectrum Two FT-IR spectrometer. VCD experimental spectra were collected on a ChiralIR-2XTM Dual PEM FT-VCD spectrometer (BioTools, Inc., West Palm Beach, FL).

General Cloning

Primers were designed for the T5 exonuclease-dependent assembly (TEDA) method.⁴⁹ For site-directed mutagenesis of SalkS, overlap PCR was used with the salks gene from pJR4013 as a template with the Q5 DNA polymerase. The PCR products were purified by gel extraction and cloned into pET28a, which was linearized with BamHI

and HindIII, using TEDA to afford plasmids pJR1101–pJR1122. The sequences of all of the genes were confirmed by DNA sequencing.

Protein Production and Purification

Plasmids harboring each gene were transformed into *E. coli* BL21 Star (DE3). *E. coli* strains harboring plasmids were grown in lysogeny broth (LB) containing 50 mg mL^{−1} kanamycin for antibiotic selection. Each strain was grown in 3 × 1 L of LB at 37 °C with shaking at 200 rpm until an optical density at 600 nm (OD₆₀₀) of 0.6 was reached. Gene expression was induced with the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cells were further cultured around 18 h at 16 °C with shaking. After the cells were harvested by centrifugation at 5000g for 10 min at 4 °C, the pellet was resuspended in cold lysis buffer (50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl). Cells were lysed by sonication (Sonic Dismembrator Model 500) and centrifuged at 40,000g for 30 min at 4 °C. Target proteins were purified by nickel-affinity chromatography by adding the supernatant to a column packed with HisPur Ni-NTA Resin (Thermo Scientific), washing with wash buffer (lysis buffer containing 20 mM imidazole), and eluting with elution buffer (lysis buffer containing 500 mM imidazole). The resultant protein was immediately desalted using a PD-10 column (GE Healthcare Biosciences) and concentrated using an Amicon Ultra-15 concentrator (Millipore) in 50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl. Protein purities were assessed by SDS-PAGE analysis, and protein concentration was determined at 280 nm with an Eppendorf 6136 Kinetic BioSpectrometer. The extinction coefficients of SalkS and ND90 were calculated as 38600 M^{−1} cm^{−1} and 77510 M^{−1} cm^{−1}, respectively, via an AAT Bioquest protein calculator. Individual aliquots of each protein were flash-frozen in liquid nitrogen and stored at −80 °C until use.

Enzyme Assays

The assays were performed in 50 mM Tris–HCl, pH 7.5, containing 10 mM GGPP, FPP, or GPP, 10 mM MgCl₂, and 50 μM ND90 and SalkS in a total volume of 100 μL. The reactions were initiated by the addition of enzyme and incubated for 1 h at 30 °C. The reactions were quenched with 100 μL of ice-cold acetonitrile and saturated with solid NaCl. After the two phases were separated by vortexing, the organic phase was analyzed by HPLC and/or GC-MS. For HPLC and GC-MS analysis, the methods described above were used. Triplicates were performed for every enzyme variant.

Chromatography

For GC-MS analysis, purified diterpenes were dissolved in hexanes at a concentration of 0.1 mg mL^{−1}. The source, transfer line, and injection port were set to 250 °C, respectively, and the carrier gas flow rate was set at 1.2 mL min^{−1}. Products were measured with an electron ionization of 70 eV and the mass scan range was from *m/z* 30 to 500 @ 30000 resolution with a temperature gradient as follows: 50 °C (0–3 min), ramp to 280 °C @ 10 °C min^{−1} (hold 5 min). Kovats retention index (RI) values were calculated for all products in this study using the GC-MS conditions described above in comparison with C7–C30 saturated alkanes (Sigma).

For HPLC analysis, products were detected at 210 nm using a linear gradient with a flow rate of 1 mL min^{−1}: 5% acetonitrile/water (0–5 min); 5% to 95% acetonitrile/water (5–35 min at 5% min^{−1}); 95% acetonitrile/water (hold 35 min) at 35 °C.

Isolation and Purification of Diterpene Products

E. coli cells harboring pCDF-MK14 were transformed with pET28a-ND90, -SalkS or -SalkS variant and single transformants were into LB medium containing kanamycin (50 μg mL^{−1}) and streptomycin (50 μg mL^{−1}). Overnight cultured cells were then inoculated into 12 × 1 L of fresh terrific broth (TB) media. IPTG (0.5 mM) and isoprenol (4 mM) were added when the cultures reached OD₆₀₀ = 0.8 and further shaking was carried out at 20 °C (for ND90) and 28 °C (for SalkS and variants) for 48 h. Cells were collected by centrifugation at 5000g for 10 min at 4 °C and transferred to a glass beaker. The cell pellets were extracted with acetone (2× volume of the pellet), and the organic phase was extracted with the same volume of hexanes three times. The hexane extractions were combined and concentrated in vacuo at room temperature. The

resulting extract was redissolved in hexanes and purified by silica chromatography, employing an isocratic hexanes mobile phase. Fractions containing the target products were combined and subjected to further purification using preparative HPLC. For the isolation of 2 and 3, 10% (w/v) silver nitrate-treated silica gel was used for chromatography, employing a solvent system of *n*-pentane/dichloromethane (10:1 to 1:1).

Chemical Synthesis of 9 and 10

Compound 5 (10 mg, 0.037 mmol) was dissolved in 1 M HCl (1 mL) and the mixture was stirred for 3 h at room temperature. The reaction mixture was extracted with hexanes three times, and the combined organic extract was concentrated *in vacuo*. Diterpene 9 was purified from the resulting mixture by preparative HPLC as described above to afford 9 as a colorless oil (4 mg, 41% yield).

Compound 5 (10 mg, 0.037 mmol) was dissolved in *p*-xylene (5 mL), heated to 120 °C, and stirred for 18 h overnight. The reaction mixture was concentrated *in vacuo*. The resulting mixture was subjected to preparative HPLC as described above to afford pure 10 as a colorless oil (2 mg, 19% yield).

VCD Measurements and Calculations

See the SI for detailed methods. Briefly, 1, 3, and 4 were individually dissolved in CDCl₃ and transferred to a liquid IR cell, and IR and VCD data were collected. Quantitative comparisons of the experimental and DFT-calculated IR and VCD spectra resulted in very high confidence level assignments.

Quantum Chemical Calculations

Geometry optimization and vibrational analysis were performed with the Gaussian16 Revision C.01 suite of programs⁵⁰ utilizing the Adamo and Barone's modified Perdew–Wang exchange and PW91 correlation hybrid functional (mPW1PW91)⁵⁴ in conjunction with the Pople 6-31+G(d,p) basis set^{35–38} for all atoms in the gas phase. Transition structures were verified to process only one vibrational mode with imaginary frequency and by subsequent Intrinsic Reaction Coordinate calculations^{51,52} or optimization following displacement along the imaginary vibrational mode. Free energy corrections at 1 M and 298.15 K with quasi-harmonic approximation proposed by Grimme⁵³ with 100 cm^{−1} cutoff were calculated with the GoodVibes package.⁵⁴ The enthalpy reported is the sum of the electronic energy and thermal enthalpy correction. Data sets of optimized geometries are available at the ioChem-BD repository.^{40,55}

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscatal.6c00111>.

Methods and spectroscopic data and supporting references; strains, plasmids, and primers (Tables S1–S3); summary of NMR data for 1–10 (Tables S4–S6, S8–S12, and S16–S17); relative activities of SalkS and variants (Table S7); calculated energies (Tables S13–S15); SDS-PAGE analysis (Figure S1); GC-MS chromatograms (Figure S2); spectroscopic data for 1–10 (Figures S3–S20, S22–S58, and S63–S80); sequence alignment of SalkS and related TSs (Figure S21); computed structures, plots of calculated intrinsic reaction coordinates, and additional plots of calculated energies (Figures S59–S62); proposed mechanism for ND90 (Figure S81); and VCD calculations (PDF)

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Notes

The authors declare no competing financial interest.

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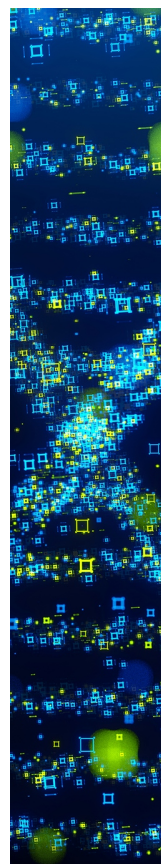
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