Cryptic isomerization in diterpene biosynthesis and the restoration of an evolutionarily defunct P450

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ABSTRACT: Biosynthetic modifications of the 6/10-bicyclic hydrocarbon skeletons of the eunicellane family of diterpenoids are unknown. We explored the biosynthesis of a bacterial *trans*-eunicellane natural product, albireticulone A (**3**), and identified a novel isomerase that catalyzes a cryptic isomerization in the biosynthetic pathway. We also assigned functions of two cytochromes P450 that oxidize the eunicellane skeleton, one of which was a naturally evolved non-functional P450 that when genetically repaired, catalyzes allylic oxidation. Finally, we describe the chemical susceptibility of the *trans*-eunicellane skeleton to undergo Cope rearrangement to yield inseparable atropisomers.

The eunicellane diterpenoids are a structurally unique class of natural products, each possessing or originating from a 6/10-fused bicyclic skeleton (Fig. 1A). Based on the ring fusion, eunicellanes can be found in *cis* or *trans* configuration. The *cis* ring fusion is the dominant form as over 98% of all known eunicellane-derived diterpenoids are *cis*-fused.[1] *cis*-Eunicellanes are found in soft corals, the most prevalent producers of these natural products, and most recently bacteria; few *trans*-eunicellane natural products are known.[1–5] Many of these diterpenoids have potent biological activities with promising clinical applications,[2] including the tubulin inhibitor eleutherobin.[6,7]

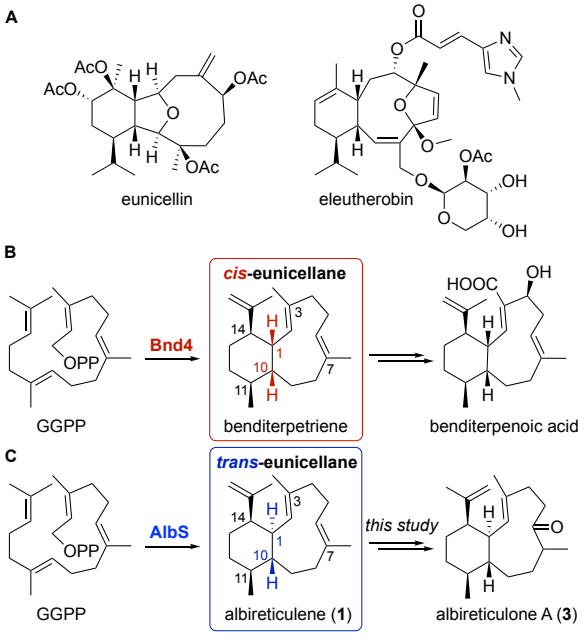

Figure 1. Eunicellane diterpenoids. (A) Representative eunicellane natural products. The *cis*-eunicellane synthase Bnd4 (B) and *trans*-eunicellane synthase AlbS (C) are bacterial terpene synthases responsible for forming the eunicellane skeletons.

Biosynthetically, not much is known about eunicellane diterpenoids. By genome mining for novel terpene synthases (TSs) in bacteria, the first *cis*- and *trans*-eunicellane synthases in nature were identified.[4,5,8] The *cis*-eunicellane synthase, Bnd4, was found in the biosynthetic gene cluster (BGC) responsible for the production of benditerpenoic acid (Fig. 1B).[4] *cis*-Eunicellane synthases were later identified in coral genomes as part of eukaryotic BGCs,[9,10] but their protein sequences and mechanisms are distinct from those in bacteria. Very recently, the aridacins from *Amycolatopsis arida* were discovered.[11] The aridacins are 6/7/5-tricyclic diterpenoids originating from the *cis*-eunicellane skeleton. At least three cytochrome P450s, one of which catalyzes diterpene cyclization to form the 6/7/5-fused tricycle from the 6/10 scaffold, act to produce the highly oxygenated natural products.[11] The *trans*-eunicellane synthase, AlbS (Fig. 1C), was encoded within a BGC from *Streptomyces albireticuli* NRRL B-1670 and produced albireticulene (**1**).[5] We postulated that this rare *trans*-eunicellane skeleton is a precursor of a more functionalized natural product.

In a search for *trans*-eunicellane natural products, we fermented *S. albireticuli* in several media but did not detect albireticulene (**1**) or any diterpenoids; however, we identified several teleocidins[12] suggesting that *S. albireticuli* produces terpene precursors (Fig. S1). We turned to heterologous expression to activate the *alb* BGC. We cloned the full *alb* BGC (Table S4, Fig. 2A, *albG–albP2*), which was predicted to encode a geranylgeranyl diphosphate (GGPP) synthase (*albG*), a truncated terpene precursor gene (*albM)*, albireticulene synthase (*albS*), an unknown protein (*albU*), and two cytochromes P450 (*albP1* and *albP2*), into pSET152 under the control of the sp44 promoter with an sr40 ribosome binding site.[13] Introduction of the *alb* BGC into *Streptomyces lividans*, *S. albus*, and *S. venezuelae*, as well as the native producer *S. albireticuli*, yielded three strains that produced **1** and two other new products (**2** and **3**) as detected by HPLC (Figs. 2B and S1). Therefore, we fermented *S. albireticuli* UFJR1004 on a 12-L scale to isolate **2** and **3** for structural elucidation.

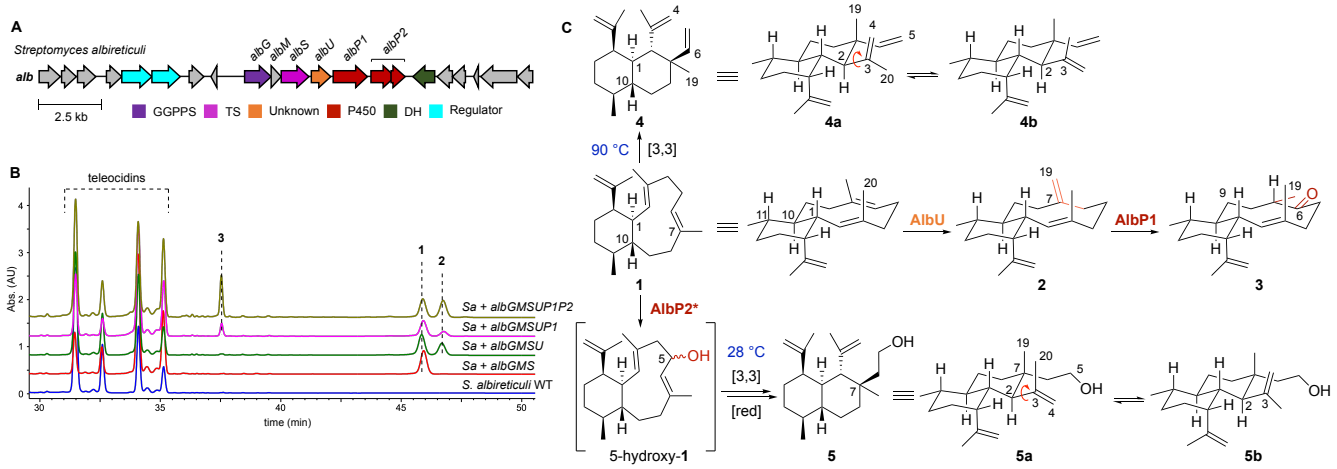

Figure 2. Biosynthesis of **3** and **5**. (A) Genetic organization of the *alb* biosynthetic gene cluster from *S. albireticuli* NRRL B-1670. (B) Metabolite profiles of selected *S. albireticuli* strains expressing *alb* genes upon HPLC-UV analysis (absorbance was detected at 210 nm). The detection and isolation of **2**, **3**, and **4** from strains expressing partial *alb* support the enzyme functions of AlbS, AlbU, and AlbP1. AlbP2 is nonfunctional. (C) Proposed biosynthetic scheme of **3** and **5**. The *trans*-eunicellane isomer **1** and its putative C5-hydroxy congener undergo Cope rearrangement. The Cope products **4** and **5** were determined to be atropisomers with limited rotational freedom around the C2-C3 bond (red arrows). See Fig. S45 for detailed proposals for the formations of **3** and **5**.

iso-Albireticulene (**2**) and albireticulone A (**3**) had the molecular formulas $C_{20}H_{32}$ and $C_{20}H_{32}O$, respectively, as supported by EI-MS ions at *m/z* 272.2 (calcd 272.2499 for M^+) and *m/z* 288.2 (calcd 288.2448 for M^+, Fig. S2). Both **2** and **3** share the 6/10-fused *trans*-eunicellane skeleton based on NMR analysis (Tables S5 and S6, Figs. S3–S20). In both **2** and **3**, and as seen for **1**,[5] strong NOE correlations of H-1 to H-11 and Me-20 and H-2 to H-10 and H-4, as well as the large ^3J coupling constant between H-1 and H-10 (~11 Hz), support that H-1 is *trans* to H-10 and the C-2 alkene is *E* configuration. Albireticulone A (**3**) was reported during the preparation of this manuscript;[14] however, the configuration at C-7 was unclear. NOE correlations between H-1_{ax}, H-11_{ax}, and H-9_{ax}, along with H-9_{ax} with H-7 suggest that H-7 prefers to be axial in the relatively rigid pseudo-cyclohexane ring system. The ^3J coupling constants of H-7, 10.1 Hz with H-8_{ax}, 6.8 Hz with Me-19, and 2.6 Hz with H-8_{eq}, also support this assignment. Therefore, C-7 is *S* configuration.

The *trans*-eunicellane isomer **2** is significantly more stable than **1**, which was previously shown to rapidly convert into a 6/6/6-tricyclic system under acidic conditions,[5] and here shown to undergo [3,3]-sigmatropic rearrangement[15] at 90 °C to form the 6/6-bicyclic triene **4** (Fig. 2C). This Cope product was found as two inseparable atropisomers (Fig. 2C, Table S7, Figs. S21–S41). NOE correlations between H-1_{ax} and Me-19, and H-2_{ax} with H-6 suggested that the methyl (Me-19) and vinyl groups on C-7 were axial and equatorial, respectively, in both atropisomers **4a** and **4b**. NOESY analysis revealed the isopropylene moiety on C-2 is found in two different orientations, with NOE correlations from H-4 to H-1_{ax} and Me-19 in **4a** and between Me-20 and Me-19 in **4b**. The highly congested space around C-2, C-7, and C-14 limits rotation of the isopropylene moiety on C-2 giving rise to atropisomerism. Atropisomerism in natural products is typically found in biaryl systems or large ring systems,[16,17] not in aliphatic terpenoids. At 25 °C, atropisomers **4a**/**4b** were found in a ratio of 2:1 in benzene-d_6 and 1.7:1 in chloroform-*d*. Relocation of the alkene from C-6 in **1** to C-7(19) in **2** prevents both acid-catalyzed cyclization and Cope rearrangement.

To investigate the biosynthetic pathway of **3** and probe the importance of **2** as an intermediate, we constructed a series of heterologous expression constructs each with a partial *alb* BGC (i.e., *albG*–*albS*, *albG*–*albU*, *albG*–*albP1*) and introduced these constructs into *S. albireticuli* (Fig. 2B) and *S. venezuelae* (Fig. S42). As each of the strains possessed GGPP synthase and AlbS, they each produced the hydrocarbon **1**, exemplified by the *albG*–*albS* strain. Addition of *albU*, in the *albG*–*albU*-expressing strain, resulted in the production of **2**, indicating that AlbU isomerizes **1** into **2**. When only *albP2* was missing, as in the *albG*–*albP1* strain, **1**–**3** were each produced, implicating that AlbP1 is responsible for C-6 oxidation and that AlbP2 is not functional.

We first turned our attention to AlbU, the putative eunicellane isomerase. Bioinformatically, AlbU is obscure. There is not a PFAM or InterPro designation for AlbU, sequence similarity searches returned only hypothetical proteins (<15 sequences with >30% identity and >50% coverage; Table S9), with most of these homologues found in BGCs similar, but not identical, to that of *alb* (Fig. S43). To confirm the function of AlbU, we produced AlbU in *E. coli* and subsequent incubation of AlbU with **1** produced **2** (Fig. S44), albeit slowly (<100% conversion after 4 h in a **1**:AlbU ratio of 50:1). The optimum temperature and pH were determined to be 37 °C at pH 7 (Fig. S44). AlbU was unable to catalyze the reverse reaction, **2** to **1**, when incubated with **2**; the *cis*-eunicellane Bnd4 product, benditerpetriene, was also not a viable substrate for isomerization (Fig. S44). As we saw enzymatic conversion without the addition of any cofactors, the AlbU mechanism is proposed to be an acid-base isomerization, somewhat akin to type 1 isopentenyl diphosphate isomerase.[18]

With the production of **2** confirmed to be genetically encoded by the *alb* BGC, we next sought to characterize *albP1*, the P450 proposed to oxidize C-6. In comparison to *albG*–*albP1*, which produces **1**–**3**, expression of *albG*–*albP1* without *albU* only produced **1** supporting the intermediacy of **2** and that AlbP1 acts only on **2** (Fig. 3). Allylic oxidation[19] of **2** to form the C-6 ketone appears to be a likely pathway to **3**, although this would require an additional step to reduce the C-7(19) alkene, perhaps

via AlbU, to form a transient enol prior to tautomerization (Fig. S45).

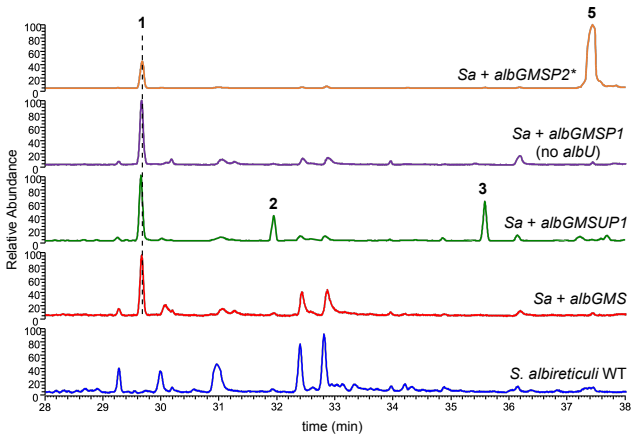

Figure 3. Metabolite profiles of selected *S. albreticuli* strains upon GC-MS analysis. In the absence of *albU*, only **1** is formed supporting **2** as an intermediate in the biosynthesis of **3**. Diterpene **5** is produced when *albP2** is expressed with *albS*, supporting **1** as its precursor.

Finally, we investigated the lack of function of *albP2*. Upon closer examination of the *albP2* gene sequence (Fig. 2A), we recognized that it has a premature stop codon at position 967 resulting in a truncated P450 that does not have the axial Cys for heme binding. Using the homologous *alb* BGCs for comparison (Fig. S43), as their second encoded P450s appeared to be full-length variants with the conserved Cys, we traced the mutation in *albP2* to a single base deletion after position 812 (Fig. S46). Thus, it appears that *S. albireticuli* NRRL B-1670 evolved to eliminate the function of AlbP2 and instead produce **3**. This mutation may have recently evolved since the other homologues appear to be intact (Fig. S47). We decided to engineer *albP2* back into its, presumably, active form by cloning *albP2**, the full-length P450 gene with a cytosine insertion at 813 (Fig. S46). The closest homologue of AlbP2* is from *Streptomyces* sp. WAC06614 and shows 63% identity and 77% similarity; other top hits (up to 47% identities) are from TS-encoding BGCs from a variety of *Streptomyces* (Fig. S43). Alignment of AlbP2* to the second P450 in the homologous *alb* BGCs revealed <40% identity, suggesting that these P450s likely have different functions to that of AlbP2.

Inclusion of *albP2** with *alb*G–*alb*P1 in *S. albireticuli* and *S. venezuelae* satisfyingly produced the minor new product **5** as detected by HPLC (Fig. S48) and GC-MS analysis (Figs. 3 and S42). Isolation of **5**, from a 24-L culture of *S. albireticuli* UFJR1007 allowed its structural determination (Tables S10 and S11, Fig. S21, 49–68). Diterpene **5**, like that of **4**, was also found as two inseparable atropisomers, although the major isomer **5a** rotates its isopropyl moiety in a similar orientation as **4b** (Fig. 2C). Cope rearrangement of the proposed intermediate 5-hydroxy-**1** likely yields the 6/6-bicyclic framework of **5**, although a reduction of the enol (or keto) is required to form **5** (Fig. S45). Therefore, AlbP2* hydroxylates the C-5 allylic position of **1** and diverts biosynthesis away from **2** and **3** (Fig. 2C). Molecular docking results of AlbP2* with **1** and AlbP1 with **2** support the regioselective distinctions between these two P450s (Fig. S69). Interestingly, **5** was isolated from *Streptomyces* grown at 28 °C, whereas **4** was only formed when **2** was heated above 90 °C, suggesting the hydroxyl group at C-5 lowers the activation energy required for oxy-Cope rearrangement. No Cope products of *cis*-eunicellanes are known, even for the C-4 hydroxy-containing benditerpenoic acid, implicating the importance of the *trans* ring fusion for this transformation. While we propose a non-enzymatic Cope rearrangement of 5-hydroxy-**1**, we cannot rule out the possibility of an enzyme-catalyzed transformation.

The discovery of the *trans*-eunicellane diterpenoids **3** and **5** have exposed new chemical, genetic, and enzymological insights into diterpenoid biosynthesis. The reactivity of **1**, a potential liability in biosynthesis, is resolved via an isomerization to yield the stable isomer **2**. The exocyclic 7(19)-alkene of **2** is a functionality commonly seen in coral *cis*-eunicellane diterpenoids (Fig. S70)[1] and while AlbU did not isomerize benditerpetriene, it is intriguing to consider a similar diterpene isomerase performing an analogous reaction in coral. Given the sequence novelty of AlbU, further investigation into its mechanism and substrate scope is needed. The *alb* BGC appears to be in a state of evolutionary flux. AlbM, an IspH homologue is truncated (Table S4) and likely non-functional. AlbP2 evolved to be non-functional by a single base pair deletion and resultant frame shift, a change that is not present in homologous BGCs. Restoring its activity led to **5**, a new-to-nature terpene framework. Finally, we anticipate additional *trans*-eunicellane diterpenoids are produced by bacteria and those natural products will expand the bacterial terpenome.[20,21]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures, strains, plasmids, and primers, bioinformatics, spectroscopic data, and supporting HPLC and GC-MS chromatograms (PDF).

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Notes

The authors declare no competing financial interest.

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