Exploring and expanding the natural chemical space of bacterial diterpenes

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ABSTRACT: Terpenoids are the largest family of natural products but are relatively rare in bacteria. Genome mining reveals widespread prevalence of terpene synthases, the enzymes responsible for constructing the hydrocarbon skeletons, in bacteria. Using an engineered *E. coli* diterpene production system, we screened 334 diverse terpene synthases from 8 phyla, 17 classes, and 83 genera of bacteria and found that 125 (37%) were active as diterpene synthases. Isolation and structural elucidation of 28 bacterial diterpenes from 31 TSs revealed three new terpene skeletons previously unseen in nature, skeletons of known natural products from other organisms (e.g., coral, sponge, algae) with unknown biosynthetic pathways, diterpenes that are known in other organisms (e.g., fungi, plants) but have not been previously seen in bacteria, and new structural and stereochemical isomers of diterpenes. This study will lead to novel natural products, advances in terpenoid biosynthesis and enzymology, and provide model systems to probe the ecological roles of these natural products.

INTRODUCTION

Terpenoids are the largest and most structurally diverse family of natural products, consisting of over 100,000 known compounds, and are widely used as pharmaceuticals, herbicides, flavors, fragrances, and biofuels.[1–5] Plants, fungi, and marine organisms are well known producers of terpenoids; however, less than 2% of all known terpenoids are of bacterial origin.[6] This disparity is particularly true for diterpenoids, the C20 subclass of natural products famous for the anticancer drug Taxol and the plant hormone gibberellin, as less than 1% of diterpenoids are from bacteria (<200 out of ~25,000).[1]

Terpene synthases (TSs), the enzymes that begin specialized terpene biosynthesis by converting acyclic achiral prenyl diphosphates into diverse polycyclic skeletons with numerous stereocenters, are prevalent throughout nature.[7,8] Despite the relatively small numbers of bacterial terpenoids, TSs are widespread in bacterial genomes. In one of the early genome mining studies for bacterial TSs, over 120 putative TSs were identified, mostly from 20 actinomycete genomes.[9] A few years later, a hidden Markov model (HMM) identified 262 presumptive TSs from a variety of bacteria.[10] Now, with the ever-growing microbial genomic libraries, a simple search on the UniProt database for the type I terpene cyclase-like 2 family (IPR034686) reveals over 5000 bacterial proteins; ~80% of these are from Actinomycetota. This number does not include other subfamilies of TSs, such as the type II TSs[11] or any of the noncanonical TSs.[12] Overall, this highlights the enormous potential to use TSs to characterize new terpene skeletons, discover new terpenoid natural products, investigate terpene enzymology and evolution, and develop biocatalysts to produce structurally and stereochemically complex hydrocarbons.

In this study, we explore and expand the natural chemical space of diterpenes in bacteria by screening 334 uncharacterized TSs for activity. Isolation and structural elucidation of 28 diterpenes from 31 prioritized TSs revealed impressive product diversity. We identified novel diterpene skeletons previously unseen in nature, skeletons of natural products from other organisms (e.g., coral, sponge, algae) with unknown biosynthetic pathways, diterpenes that are known in other organisms but have not been previously seen in bacteria, and new structural and stereochemical isomers of diterpenes. We also found that different phyla of bacteria encode TSs, without significant sequence identities to each other, that make identical diterpene products. We additionally confirmed that the in vitro activities of six TSs matched the products from heterologous expression in *E. coli*.

RESULTS AND DISCUSSION

Genome mining for bacterial terpene synthases

TSs act as molecular chaperones that use carbocation chemistry to catalyze complex cyclization reactions.[7,8] They are differentiated into subclasses based on how they form the initial cation.[8,11,12] Type I TSs use a trinuclear divalent cation cluster to abstract the diphosphate moiety of the substrate and initiate cyclization.[8] Although type I TSs, found in plants, fungi, bacteria, and recently corals, have conserved protein folds, they often have little sequence conservation outside of two metal-binding Asp-rich motifs.[7] Due to the complexities of terpene cyclization, it is currently impossible to predict the substrate (e.g., C15, C20, C25 prenyl diphosphate) and products of TSs based on protein sequence alone. An additional complicating factor of TS sequence–function relationships is that a single amino acid

change, in a well-positioned site, can dramatically alter the product profile of a TS.[13]

We set out to map the natural chemical space of bacterial diterpenes by screening a library of putative TSs. In bacteria, there are 141 characterized type I TSs: >62% (88) are from the actinomycetota phylum, 40 are diterpene synthases (di-TSs), and only 26 are associated with known natural products. Using a combination of genome mining, phylogenetics, sequence similarity networking, and biosynthetic gene cluster (BGC) analysis, we selected 334 TSs from 8 phyla, 17 classes, and 83 genera of bacteria for functional analysis. The phylogenetic distribution of selected TSs was intentionally broad (Figs. 1 and S1). To expedite functional characterization, we used an *E. coli* heterologous expression system to detect products without the need for protein purification or substrate synthesis. We synthesized the 334 TSs as codon-optimized genes and cloned them into an engineered *E. coli* strain that overproduces geranylgeranyl diphosphate (GGPP).[14] Terpene products were initially detected via TLC or HPLC of organic extracts from the TS library in *E. coli.* In a first-pass screen, with no optimization of the genetic system or the expression and culture conditions, we identified 125 positive hits (37%, Fig. 1). After the success of this initial screen, we prioritized 31 TSs for large-scale fermentations for diterpene isolation and structural elucidation (Fig. S2). TSs were chosen based on sequence diversity, genetic source, location in a unique BGC, and/or high initial yields. We isolated 28 diterpenes and determined their structures using NMR, GC-MS, and vibrational circular dichroism (VCD). We organized the bacterial diterpenes into four categories below, although several diterpenes fit into more than one category.

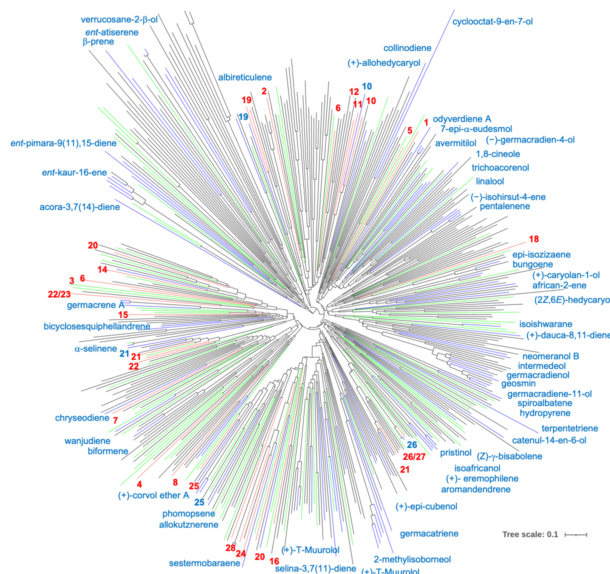

Figure 1. Phylogenetic analysis of 475 type I TSs from bacteria showing broad distribution of di-TSs. The blue lines represented previously characterized TSs (141), red lines are the 31 TSs characterized in this study, green lines are the other 94 TSs that showed di-TS activity but not studied here, and black lines are the remaining 209 TSs in our bacterial TS library. A larger tree is shown in Fig. S1.

Discovery and structural elucidation of three novel diterpene skeletons

Tetraisoquinene (**1**), a novel diterpene with a 5/5/5/5-fused tetracyclic skeleton, was produced by a TS (TiqS) from the myxobacterium *Melittangium boletus* (Fig. 2). Myxobacteria are well-established producers of natural products but only a few diterpenoids have been isolated from the phylum.[6] The GC-MS of **1** showed a molecular ion peak at *m/z* 272.2499 (Fig. S3), supporting a molecular formula of $C_{20}H_{32}$ and a diterpene with five degrees of unsaturation. The NMR spectra of **1** (Table S5, Figs. S4–S10) revealed a single double bond with no olefinic protons, as well as five methyl groups, six methylene carbons, five methine carbons, and two sp^3 quaternary carbons; thus, **1** was tetracyclic. ^{1}H-^{1}H COSY analysis revealed three spin systems connecting C-14-C-1(C-20)-C-2-C-3, C-6-C-7-C-8, and C-10-(C-15-C-16/C-17)-C-11-C-12. Several key correlations from the ^{1}H-^{13}C HMBC spectra connected these fragments into an angular tetraquinane skeleton: H_3-18 to C-8, C-9, C-10, and C-13; H_2-12 to C-9, C-13, and C-14; H-6 to C-4, C-5, C-9, C-13, and C-14; H_3-19 to C-3, C-4, C-5, and C-6; and H-14 to C-3, C-4, C-5, C-6, C-9, and C-13. The relative configuration of **1** was determined by NOESY correlations from H-14 to H_3-18 and H_α-8, H_β-8 to H_3-20, and H_β-12 to H-6, H-10, and H_3-20. As diterpenes are particularly well suited for analysis by vibrational circular dichroism (VCD), a powerful technique for the determination of absolute configuration in solution phase without derivatization,[15–17] we used VCD to determine the absolute configuration of **1** to be 1*S*,6*S*,9*S*,10*S*,13*S*,14*S* (Fig. S11). The only other tetraquinane diterpenes from nature are the crinipellins, basidiomycete fungal diterpenoids with antibacterial, antifungal, anticancer, and anti-inflammatory properties.[18] However, the connections between the C and D rings of **1** and the crinipellins are not the same (Fig. 2). The TS responsible for tetraquinane formation is unknown and no biosynthetic studies are reported; only chemical synthetic methods to access the crinipellin skeleton are known.[19]

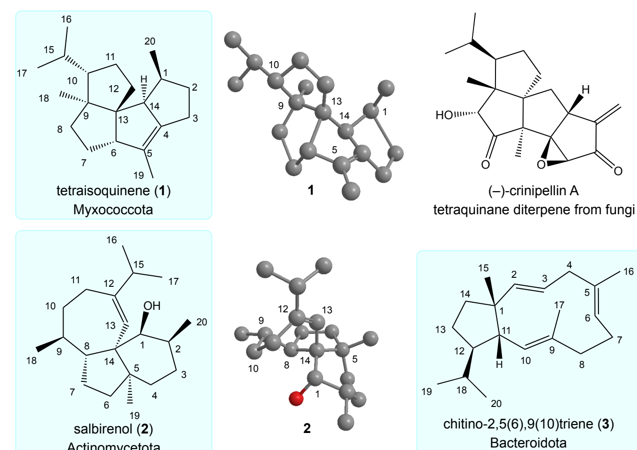

Figure 2. Novel skeletons (**1**–**3**) isolated from three different phyla of bacteria. 3D representations of **1** and **2** are shown. The phyla encoding the responsible TSs are listed. The fungal crinipellins are structurally the closest known skeleton to **1**.

Salbirenol (**2**), isolated from *E. coli* expressing SalS from *Streptomyces albireticuli*, is a novel diterpene alcohol with a 7/5/6 tricyclic skeleton (Fig. 2). The GC-MS of **2** showed a molecular ion peak at *m/z* 290.2607 (Fig. S11), supporting a molecular formula of $C_{20}H_{34}O$ and a diterpene with four degrees of unsaturation. The NMR spectra of **2** (Table S6, Figs. S12–S19) revealed a single double bond with one olefinic proton, as well as five methyl groups, six methylene carbons, five methine carbons, one of which was oxygenated, and two sp^3 quaternary

carbons. ^1H-^1H COSY correlations supported fragments of C-20-C-2-C-3-C-4, C-6-C-7-C-8-C-9, C-10-C-11, and C-16-C-15-C-17. Extensive ^1H-^{13}C HMBC correlations supported a 7/5/6 skeleton: H-13 to C-8, C-11, C-12, and C-14; H-1 to C-2, C-3, C-5, C-13, and C-14; H_3-20 to C-1, C-2, and C-3; H_3-19 to C-4, C-5, C-6, and C-14; H_3-18 to C-8, C-9, and C-10; and H-15 to C-11, C-12, and C-13. Although several NOESY correlations were detected for **2**, due to peak overlap we could not assign the relative configuration of **2** by NMR. Using VCD in comparison with the calculated spectra of nearly all 32 possible stereoisomers, the absolute configuration of **2** was determined to be 1*S*,2*S*,5*R*,8*R*,9*S*,14*S* (Fig. S20).

Chitino-2,5(6),9(10)-triene (**3**), a 5/11 bicyclic diterpene, was the major product of ChtS from *Chitinophaga japonensis* (Bacteroidota; Fig. 2). The GC-MS of **3** showed a molecular ion peak at m/z 272.2498 (Fig. S21), supporting a molecular formula of $C_{20}H_{32}$ and a diterpene with five degrees of unsaturation. The NMR spectra of **3** (Table S7, Figs. S22–S28) revealed three double bonds with four olefinic protons, as well as five methyl groups, five methylene carbons, three methine carbons, two sp^2 and one sp^3 quaternary carbons. ^1H-^1H COSY analysis revealed connected fragments of C-2-C-3-C-4, C-7-C-8, and C-10-C-11-C-12(C-18-C-19/C-20)-C-13-C-14. Key correlations from the ^1H-^{13}C HMBC spectra connected these fragments into a bicyclic skeleton: H-2 to C-1; H_2-14 to C-1; H_3-15 to C-1, C-2 and C-14; H-18 to C-11 and C-13; H_3-16 to C-4, C-5 and C-6; and H_3-17 to C-8, C-9, and C-10. The relative configuration of **3** was determined by NOESY correlations from H-11 to H_3-15, H-11 to H_3-20, and H_3-15 to H_3-19. The absolute configuration of **3** was determined to be 1*R*,11*S*,12*R* by VCD (Fig. S29). At first glance, **3** appears to be a dolabellatriene; however, the positions of its methyl groups on the 11-membered ring confirms it is a novel skeleton, one that has no corresponding natural products in nature, and is likely formed via a different cyclization mechanism than the dolabellatrienes.

Bacteria produce diterpene skeletons found in other organisms

The next five diterpenes are all known skeletons but have not been previously seen in bacteria and either do not have characterized TSs responsible for their formation, associated natural products, or both. Peysonnosene (**4**) was identified as the major product of a TS (PeyS) from the chloroflexota bacterium *Anaerolineaceae* sp. Named after the peyssonnosides, unusual diterpene glycosides isolated from the marine red alga *Peyssonnelia* sp., **4** shares its 5/6/3/6-fused tetracyclic skeleton with these algal natural products (Fig. 3). The GC-MS of **4** showed a molecular ion peak at m/z 272.2498 (Fig. S30), supporting a molecular formula of $C_{20}H_{32}$ and a diterpene with five degrees of unsaturation. De novo structural elucidation by 1D and 2D NMR (Table S8, Figs. S31–S37) revealed four rings consisting of five methyl groups, six methylene carbons, five methine carbons, and four quaternary carbons. ^1H-^1H COSY correlations supported connected fragments of C-1-C-2, C-4-C-5, C-19-C-7-C-8-C-9, and C-12-C-13-C-14-C-15-C-16/C-17. ^1H-^{13}C HMBC correlations from H_3-20 to C-2, C-3, and C-4, as well as from H-1 to C-3, C-6, C-10, C-11, and C-12 built the C/D ring system. Additional HMBC correlations from H-18 to C-9, C-10, C-11, and C-14 and from H-19 to C-6, C-7, and C-8 confirmed the 5/6/3/6 planar structure of **4**. The relative configuration of **4**, which was supported by limited NOESY correlations, was proposed to be the same as that of the peyssonnosides. We confirmed its absolute configuration, 1*S*,6*S*,7*R*,10*S*,11*S*,14*S*, by VCD (Fig. S38). The peyssonnosides have low μM activities against MRSA, the malarial parasite *Plasmodium berghei*, and the marine fungus *Dendryphiella salina*, with no cytotoxicity against human keratinocytes.[20] While the total synthesis of the peyssonnosides has been completed,[21,22] there are no biosynthetic reports of these natural products. Interestingly, *Anaerolineaceae* bacteria are known to occur in marine sediments,[23] providing a potential link between their diterpene production and agal natural products.

Clavulara-1(15),17-diene (**5**) is a 6/7/5 tricycle, produced by CvdS from *Nocardia vulneris* (Fig. 3). The GC-MS of **5** showed a molecular ion peak at m/z 272.2502 (Fig. S39), supporting a molecular formula of $C_{20}H_{32}$ and a diterpene with five degrees of unsaturation. The NMR spectra of **5** (Table S8, Figs. S39–S46) revealed two double bonds with four olefinic protons, as well as three methyl groups, eight methylene carbons, three methine carbons, two sp^2 and two sp^3 quaternary carbons. ^1H-^1H COSY analysis revealed three spin systems connecting C-2-C-3-C-4, C-13-C-14, and C-7-C-8-C-9-C-10-C-11. Key correlations from the ^1H-^{13}C HMBC spectra connected these fragments into a tricyclic skeleton: H_2-7 to C-12; H-9 to C-12; H_3-19 to C-9; H_2-18 to C-9; H_3-16 to C-8, C-11, C-12, and C-13; H_3-15 to C-1, C-2, and C-14; and H_3-20 to C-4, C-5, C-6, and C-14. The relative configuration of **5** was determined by NOESY correlations from H_3-16 to H_3-19, H-14 to H_3-16, and H-8 to H_3-20. The absolute configuration of **5** was determined to be 5*R*,8*R*,9*R* 12*S*,14*S* by VCD (Fig. S47). We named **5** after a small family of coral diterpenoids, the clavularanes, that share the same planar skeleton (Fig. 3).[24] However, the presumed biosynthesis of the third ring in the clavularanes, and related dolastanes, stems from oxidized dolabellanes (5/11 bicyclic skeletons)[25,26] and no coral TSs are yet known to produce either the dolabellane or clavularane skeletons.[27]

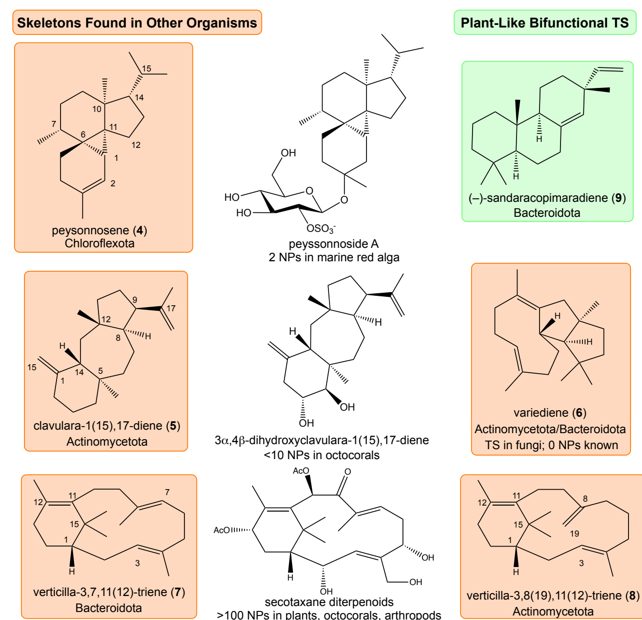

Figure 3. Diterpene skeletons (**4**–**9**), known in other organisms, now identified in bacteria. Bacterial TSs are the first known to form **4** and **5**; TSs in fungi and plants were known to produce **6** and **9**. The phyla encoding the responsible TSs are listed.

Variediene (**6**), a 5/5/9 tricyclic diterpene, was found from two distinct bacterial TSs (Fig. 3, Table S10, Figs. S48–S54). *Ps*VS from *Prauserella shujinwangii* (Actinomycetota, order

Pseudonocardiales) and *Od*VS from *Olivibacter domesticus* (Bacteroidota, order Sphingobacteriales) share only 20% sequence identity. Three fungal chimeric TSs, containing both polyprenyl synthase and cyclase domains, from ascomycetes are known to produce variediene.[28–30] This is the first report of a bacterial variediene synthase and they share essentially no sequence identity (<20%) with any of the fungal versions. Interestingly, the genuine natural products from the fungal variediene BGCs are still unknown.

The verticillenes are 6/12-bicyclic diterpenes that share a biosynthetic relationship with the taxane and phomactin families of natural products.[31] Verticillene natural products are known in plants, corals, and insects, and verticillene isomers have been isolated from taxadiene synthase engineered variants.[32,33] Here, we charactered two verticillene synthases, *Cn*VrtS from *Chitinophaga niabensis* and *S*VrtS from *Streptomyces* sp. TLI_235 produce 1*S*-verticilla-3,7,11(12)-triene (**7**) and 1*S*-verticilla-3,8(19),11(12)-triene (**8**), respectively (Fig. 3, Tables S11 and S12, Figs. S55–S62 and S63–S70). The NMR of **7** matched previous literature, confirming its structure. The NMR of **8**, in comparison with that of **7**, clearly showed its exocyclic C-8-C-19 olefin. The absolute configuration of **8** was confirmed by VCD (Fig. S70).

A tridomain, bifunctional terpene synthase from bacteria

While genome mining for unique TSs from bacteria, we identified a putative bifunctional TS from *Chitinophaga japonensis*. TSs with both type I and II TS activity, are prominent in plants and fungi,[34] and while they have been evolutionarily proposed in bacteria,[35] there were no known examples when we began this study. The longer than typical length, 785 amino acids, piqued our interest in this TS, which was previously annotated as a prenyltransferase/squalene oxidase-like protein but showed similarity to plant bifunctional TSs. Expression in *E. coli* yielded (-)-sandaracopimaradiene (**9**, also known as 8(14),15-isopimaradiene; Fig. 3, Table S13, Figs. S71–S77), clearly indicating type II activity forming *n*-copalyl diphosphate from GGPP and subsequent type I cyclization to **9**. There are known sets of discrete type II and type I TSs from Actinomycetota that form (iso)pimaradienes,[36] and at least one bacterial diterpenoid, gifhornenolone B,[37] formed from the exact skeleton of **9**. At the time of our discovery, this was the first natural bifunctional TS identified in bacteria. During preparation of this manuscript, the function of this TS, named *Chj*DCS was reported.[38] This finding opens the door to understanding TS evolution in terrestrial organisms.

New isomers of known skeletons are prevalent in bacteria

We identified several diterpenes with known planar skeletons but have alkenes at different positions or are diastereomers of previously known diterpenes. (Fig. 4). Two *Streptomyces* spp. TSs, *Sa*VenA and *Ss*VenA, produce the 5/5/6/7-tetracyclic scaffold seen in venezuelaene A (**10**).[39] With sequence identities of 59% and 57% to venezuelaene A synthase (VenA), it is not particularly surprising that *Sa*VenA forms **10** (Table S14, Figs. S78–S81) and *Ss*VenA forms the nearly identical venezuelaene A2 (**11**; Table S15, Figs. S82–S90). Tetracyclic **11** has its single alkene at C-2/C-6 rather than the C-6/C-7 in **10**, and an absolute configuration of 1*R*,3*S*,7*S*,10*S*,11*R*,14*S* as determined by VCD analysis (Fig. S90). Interestingly, another homologous TS from *Streptomyces alkaliterrae*, *Salk*S, with 49% sequence identity to VenA, forms the 5/7/6-tricyclic odyverdiene B2 (**12**; Table S16, Figs. S91–S99). Similar diterpenes were seen in *Streptomyces* sp. ND90,[40] but that di-TS is less similar with only 24% identity to *Salk*S. The absolute configuration of **12**, which was determined by VCD (Fig. S99), is likely not the same as the previously reported odyverdiene B given differences in their NMR spectra; the absolute configuration of odyverdiene B was not reported.[40]

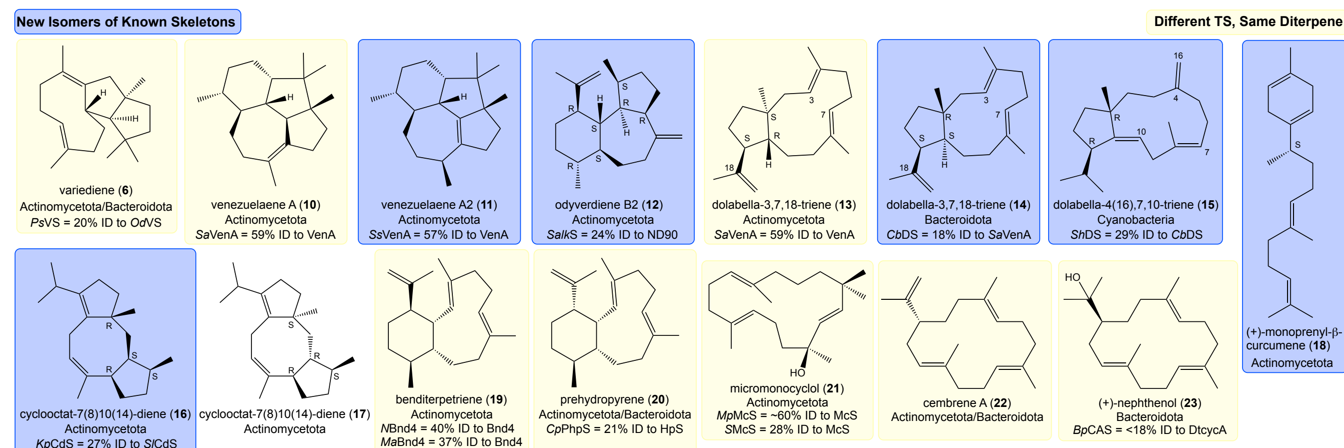

Figure 4. New (blue boxes) and known (yellow boxes) structural and stereochemical isomers of bacterial diterpenes (**10**–**27**). The phyla encoding the responsible TSs and protein sequence identities to known TSs or TSs in this study are listed.

Dolabellatrienes, the 5/11-bicyclic precursors to the dolabellane family of natural products known in algae, fungi, marine invertebrates,[25] are rare in bacteria. Only a few are known and are often implicated as intermediates in more complex cyclization cascades.[41] *Sa*VenA, the venezuelaene A (**10**) synthase described above also yields 1*S*,3*E*,7*E*,11*R*,12*S*-dolabella-3,7,18-triene (**13**; Table S17, Figs. S99–S101), previously reported as a shunt product from several VenA mutated variants.[42] *Cb*DS, from *Chitinophaga barathri*, forms **14** (Table S18, Figs. S103–S110), a diastereomer of **13**; a relative configuration of 1*R**,11*S**,12*S** for **14** is supported by NOESY correlations between H-11 and H-12, and H_3-19 and H_3-15. Finally, a cyanobacterial TS, *Sh*DS from *Scytonema hofmannii*, was shown to form 1*R*,7*E*,10*E*,12*R*-dolabella-4(16),7,10-triene (**15**; Table S19, Figs. S110–S117), a possible precursor to the coral diterpenoids stolonitriene and stolonidiol.[43]

*Kp*CdS from *Kibdelosporangium phytohabitans* (Actinomycetota, order Pseudonocardiales), with 19%–28% identities to the dolabellatriene synthases above, formed a 5/8/5-tricyclic skeleton that likely originates from a dolabellyl intermediate. The structure of this diterpene (**16**; Table S20, Figs. S119–S127) matched that of the previously reported cyclooctat-7(8),10(14)-diene (**17**) from *Streptomyces lactacystinaeus* OM-6159,[40] but its NMR was not identical. Since the absolute configuration of **17** was not reported, we obtained the gene (*slt18_1078*) that encodes this di-TS (here named *Sl*CdS) and isolated **17** (Table S21, Figs. S128–S135). Comparison of the NOESY data and VCD analysis concluded their absolute configurations: 2*S*,3*S*,6*R*,7*Z*,10*Z*,11*R*-**16** from *Kp*CdS and 2*R*,3*S*,6*R*,7*Z*,10*Z*,11*S*-**17** from Slt18_1078. The fact that these two enzymes share 27% identity yet construct diastereomeric 5/8/5-tricyclic diterpenes reiterates the challenges of using sequence to predict TS function.

(+)-Monoprenyl-β-curcumene (**18**) was produced by *St*MpcS from *Saccharopolyspora terrae* (Table S22, Figs. S136–S142). Monocyclic **18** is the C20 version of β-curcumene produced by plant sesqui-TSs and mutants of *epi*-isozizaene synthase[44,45] and tetraprenyl-β-curcumene from YtpB in *Bacillus*.[46] *St*MpcS shares 30% sequence identity with *epi*-isozizaene synthase, but no significant sequence similarity with the plant sesqui-TSs or the non-canonical "large TS" YtpB. Tetraprenyl-β-curcumenes are often precursors for a 2nd cyclization reaction, but the BGC encoding *St*MpcS does not have a type II TS nearby suggesting different downstream tailoring modifications.

Distinct TSs construct the same diterpenes

As described above for the variediene (**6**) synthases from different bacterial phyla and fungi, it is not unprecedented for TSs with disparate sequences, often from different phyla or genera, to form identical products. Here, we list several examples of known diterpenes isolated in this study (Fig. 4).

Benditerpetriene (**19**), a 2*E*,6*E*-*cis*-eunicellane, which was first identified from benditerpenoic acid biosynthesis in *Streptomyces* sp. (CL12-4)[47] and later in aridacin biosynthesis from *Amycolatopsis arida*,[48] was produced by two additional TSs (*N*Bnd4, *Ma*Bnd4) from *Nocardia* sp. SYP-A9097 and *Mycobacteroides abscessus* (Table S23, Figs. S143–S145). *N*Bnd4 and *Ma*Bnd4 share 40% and 37% sequence identity with Bnd4, respectively, and while *N*Bnd4 is found in a BGC similar to that of benditerpenoic acid and the aridacins,[47] it is intriguing to consider why the pathogenic *M. abscessus* encodes a eunicellane synthase.

Prehydropyrene (**20**), a diastereomer of benditerpetriene (**19**), was found to be the major product of *S*PhpS and *Cp*PhpS (Table S24, Figs. S146–S152). Although **20** is known as a neutral intermediate in the biosynthesis of hydropyrene,[49] this is the first report of a prehydropyrene synthase. *S*PhpS, from *Streptomyces* sp. TLI_053, shares no significant sequence identity with hydropyrene synthase (HpS) from *Streptomyces clavuligerus*; *Cp*PhpS, from *Chryseobacterium populi* (Bacteroidota, order Flavobacteriales), shares 21% sequence identity with HpS.

Micromonocyclol (**21**), a diterpene alcohol with a rare 15-membered ring originally identified from McS from *Micromonospora marina*,[50] was rediscovered from three TSs (Table S25, Figs. S153–S158). *Mp*McS and *Mb*McS are from two *Micromonospora* spp. with 62% and 61% sequence identities to McS; *S*McS is from *Streptomyces* sp. CB01580 and only shares 28% identity.

Several 14-membered diterpenes, were identified from a variety of bacterial TSs. *Mp*McS and *Mb*McS, the micromonocyclol synthases, as well as *Ml*CAS from *Micromonospora* sp. Liam0 and *Bp*CAS from *Belliella pelovolcani* (Bacteroidota, order Cytophagales) produced cembrene A (**22**; Table S26, Figs. S159–S165); *Bp*CAS also formed (+)-nephthenol (**23**; Table S27, Figs. S166–S168) but does not show any significant identity (<18%) to DtcycA, a producer of **23**.[51] *Ny*ICS, from *Nocardia yunnanensis*, forms isocembrene C (**24**; Table S28, Figs. S169–S174).

Spiroviolene (**25**), an interesting spirocyclic triquinane diterpene from *Streptomyces violens* and known in fungi,[52–56] was also identified from *S130*SvS from a related *Streptomyces* sp. (Table S29, Figs. S175–S181). Spata-13,17-diene (**26**) and cneorubin Y (**27**) were found from a TS (SpS) with high homology to the known spatadiene synthase (Tables S30 and S31, Figs. S182–S186).[57]

In some cases, we identified the GGPP elimination product β-springene (**28**), such as from the *Streptomyces* sp. Tü2975 *Stü*TS (Table S32, Figs. S187–S192), and attribute this to the possibility that these are not di-TSs and should be screened for preference to other prenyl diphosphates. In support of this conclusion is the fact that the closest characterized TS in bacteria to *Stü*TS is SmTS1 (42% identity), a sester-TS that makes several polycyclic sestermobaraenes.[58]

Diterpene products verified by in vitro characterization

To verify that the TSs are catalyzing the same reactions in vitro as they do in our heterologous expression system, we subcloned TS genes encoding TiqS (**1**), SalS (**2**), PeyS (**3**), *Od*VS (**6**), *Salk*S (**12**), and *S*PhpS (**20**) with N-terminal His_6 tags and purified them from *E. coli* (Fig. S190). Each purified recombinant TS was incubated with GGPP and analyzed by HPLC in comparison to the extracts from the heterologous expression system. For each of the six TSs, the major products matched with those produced in *E. coli* (Fig. 5), supporting that the isolated products are in fact genuine bacterial diterpenes. It remains to be seen if expression in their native hosts yields identical compounds; although recent characterization of AlbS, a *trans*-eunicellane synthase from *Streptomyces albireticuli*, in vitro and via heterologous expression in both *E. coli* and *Streptomyces albireticuli* confirmed the same enzymatic product.[59,60]

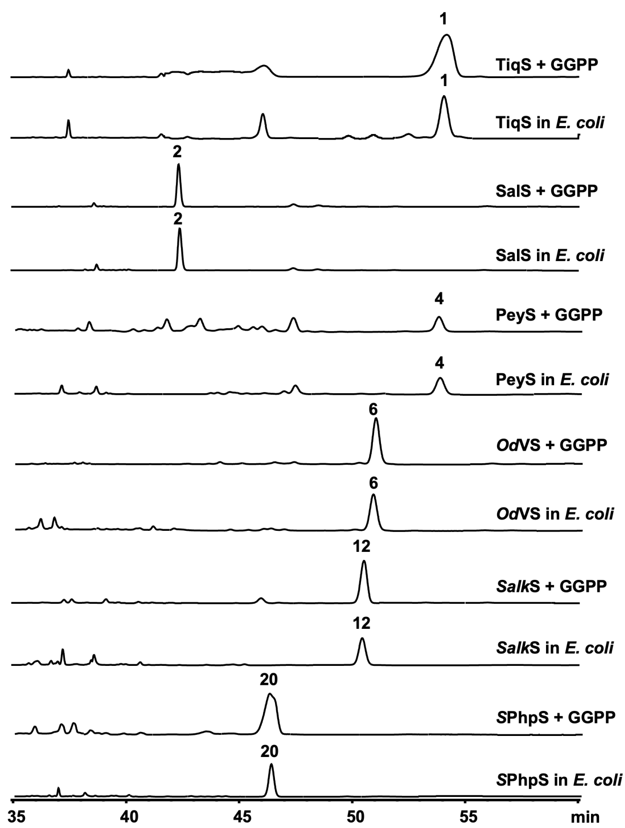

Figure 5. HPLC analysis of in vitro reactions of six TSs with GGPP in comparison to extracts obtained from expressing these genes in our GGPP-production system in *E. coli*. Absorbance was measured at 210 nm.

CONCLUSION

In conclusion, we used genome mining to identify and screen a large library of bacterial TSs for diterpene activity. Of the 334 TSs tested, 125 produced diterpenes and 31 were functionally characterized. This screen nearly doubles the number of characterized bacterial diTSs. It is highly probable that many of the remaining TSs are active but are selective for different prenyl lengths (i.e., monoterpene, sesquiterpene, or sesterterpene synthases); these studies are being conducted now. It is evident that the bacterial terpenome,[6] the collection of genomically-encoded terpenoid natural products, is much larger than previously thought!

From an enzymology perspective, questions abound about how these enzymes spatially and stereochemically control cyclization. How do totally disparate sequences form presumably (nearly) identical active sites that ionize GGPP and guide it through several reactive intermediates to yield identical products, and how these enzymes evolved? How can these TSs, or later engineered versions, be used as biocatalysts to efficiently produce complex hydrocarbons? Why do some TSs selectively yield diterpenes that other TSs use as neutral intermediates on pathways to more complex polycyclic skeletons? Libraries like this one will be essential for the investigation of these questions and for current and future machine-learning approaches to predict enzyme function.

The future impact of this study is immense. Diterpenes that are either completely novel or those seen for the first time in bacteria, are likely precursors to more complex diterpenoid natural products. These natural products are also likely to be novel and any downstream biosynthetic enzymes will also be new. It is tempting to speculate the possibility that bacterial symbionts may be producing some of the known terpenoids in eukaryotic systems;[61] however, the recent discovery of coral TSs support that at least some, if not all, are genuine eukaryotic natural products.[62,63] We also identified functional TSs in organisms, including human pathogens, that were not known to produce terpenoids. What are the ecological roles of these molecules? Very little is known about why bacteria make terpenoids, although recent studies suggest volatile terpenes enable interspecies communication.[64,65] Finding similar compounds in multiple phyla or organisms may provide insights into some of these difficult-to-answer questions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge online. Experimental procedures, strains, plasmids, and primers, bioinformatics, supporting HPLC and spectroscopic data, SDS-PAGE analysis, and VCD calculations (PDF).

The raw NMR data of **xx**–**xx** were deposited in the Natural Product Magnetic Resonance Database (np-mrd.org) under accession numbers NPxxx–NPxxx.

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Notes

The authors declare no competing financial interest.

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