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Sesquarterpenes (C_{35} Terpenes) Biosynthesized via the Cyclization of a Linear C_{35} Isoprenoid by a Tetraprenyl-β-curcumene Synthase and a Tetraprenyl-β-curcumene Cyclase: Identification of a New Terpene Cyclase

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ABSTRACT: In this study, mono- and pentacyclic C_{35} terpenes from *Bacillus subtilis* were biosynthesized via the cyclization of C_{35} isoprenoid using purified enzymes, including the first identified new terpene cyclase that shows no sequence homology to any of the known terpene cyclases. On the basis of these findings, we propose that these C_{35} terpenes should be called the new family of "sesquarterpenes."

Terpenoids make up a very large family of natural products containing more than 50 000 structurally diverse compounds. They are categorized by the number of C_5 isoprene units as follows: hemi- (one C_5 unit), mono- (C_{10}; two C_5 units), sesqui- (C_{15}; three C_5 units), di- (C_{20}; four C_5 units), sester- (C_{25}; five C_5 units), tri- (C_{30}; six C_5 units), and tetraterpenes (C_{40}; eight C_5 units).[1] In contrast, little is known about C_{35} terpenes. To the best of our knowledge, only 18 cyclic and 8 linear C_{35} terpenes have been identified to date,[2−8] and no family name has yet been assigned to this terpene class. Eight of the compounds in this class, **1**−**8**, have been found in *Bacillus subtilis* (Scheme 1).[2−4] Compound **1** is known to be synthesized by a heterodimeric enzyme, heptaprenyl diphosphate synthase (HepS and HepT) (Scheme 1).[2] However, no enzymes have been identified as producing the other seven compounds **2**−**8**. In this study, we have unveiled the biosynthetic pathway of C_{35} terpenes, which was found to be catalyzed by two unique enzymes. One is a new terpene cyclase, tetraprenyl-β-curcumene synthase (TS) (Scheme 1). This enzyme shows no sequence homology with any of the known terpene cyclases, and it cyclizes an unusually long prenyl diphosphate chain (C_{35}), whereas geranyl (C_{10}), farnesyl (C_{15}), and geranylgeranyl (C_{20}) diphosphate are the known precursors for the diphosphate-triggered type of terpene cyclases.

A number of genes responsible for the biosynthesis of terpenoids form clusters in microbial genomes.[9] Although we searched for *ts* among the genes around *hepS*, *hepT*, and a putative tetraprenyl-β-curcumene cyclase (TC) gene (*sqhC*) in the *B. subtilis* genome, we could not find a candidate. Thus, we investigated 2514 gene-disrupted strains of *B. subtilis* provided by the National BioResource Project (NIG, Japan) to isolate *ts*. The candidates for *ts* were narrowed down to 49 strains by the following two screenings. First, "functional unknown proteins" and "conserved hypothetical proteins" consisting of more than 180 and 240 amino acids, respectively, were selected as the candidates because all known terpene cyclases have >240 amino acids. Second, we analyzed the production of C_{35} terpenes in several bacteria for which genomic analyses had been completed. Four *Bacillus* species (*B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, and *B. thuringiensis*) biosynthesized **2** and **3**, whereas *Cytophaga hutchinsonii*, *Flavobacterium johnsoniae*, *Pseudoalteromonas atlantica*, and *Pseudoalteromonas haloplanktis*, which belong to the same genus as the marine bacteria that produce **2** and **3**,[5] unexpectedly did not. The genes found in the genomes of C_{35} terpene producers but not in those of the nonproducers were selected as the candidates.

TLC and GC−MS analyses of the lipid fractions of 49 gene-disrupted strains revealed that only the *ytpB* disruptant produced no detectable amounts of **2** and **3**. To demonstrate that the YtpB protein was TS, the *ytpB* gene was introduced into the pColdTF vector, expressed as a soluble form in *Esherichia coli*, and purified by nickel-chelating affinity column chromatography (Figure 1). As a substrate for the TS assay, **1** was biosynthesized by the incubation of farnesyl diphosphate (FPP) and isopentenyl diphosphate (IPP) with HepS and HepT, which were purified by a procedure similar to that for YtpB (Figure 2A). The purified YtpB successfully converted **1** to **2** (Figure 2C). The ratio of **3** to **2** detected in the GC−MS chromatogram of the reaction products synthesized by the incubation of **1** with YtpB was almost the same as that in the chromatogram for pure **2** isolated from *B. subtilis*, suggesting that **3** could be formed by autooxidation during the measurements (Figure 2B,C). The results from the mutant strain and the in vitro enzymatic reaction indicate that *ytpB* encodes TS. These results are the first to demonstrate at both the gene and enzyme levels that C_{35} terpenes are biosynthesized via the cyclization of the linear C_{35} isoprenoid.

TS homologues exist as proteins of unknown function in various bacterial species in addition to the *Bacillus* ones. Sequence alignments of TS with its homologues indicated the presence of conserved amino acids. However, the active sites of TS could not be determined because the primary structure of TS

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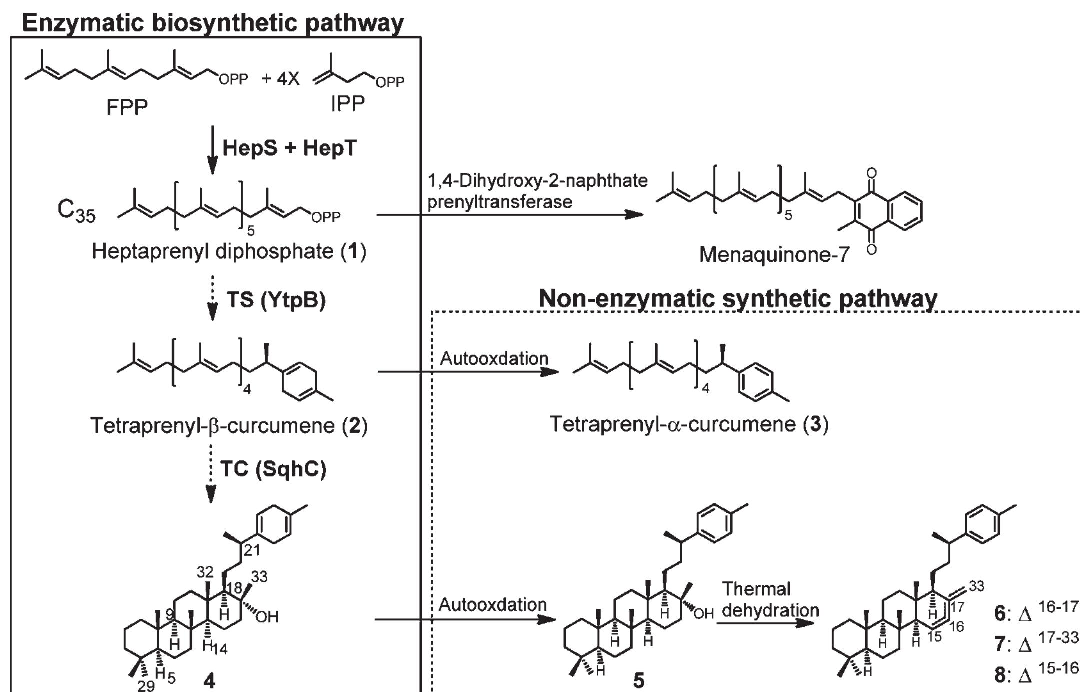
Scheme 1. Proposed Pathway for the Biosynthesis of C_{35} Terpenes in *B. subtilis*[a]



[a] The heterodimeric heptaprenyl diphosphate synthase (HepS and HepT), which converts FPP and IPP to **1**, was identified by previous studies of menaquinone biosynthesis.[2] The nonenzymatic synthetic pathways **2** → **3** and **4** → **5** → **6**–**8** were identified by Takigawa et al.[4]

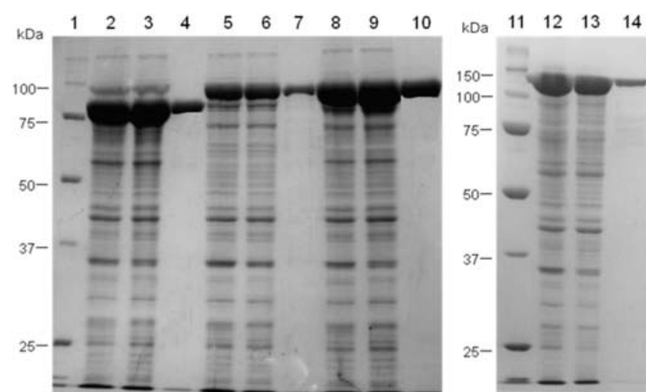


Figure 1. SDS-PAGE of the overproduced and purified recombinant enzymes. The deduced molecular weights of HepS, HepT, YtpB, and SqhC, which were expressed as fusion proteins with a trigger factor (52 kDa), were 81.1, 91.5, 94.8, and 123.2 kDa, respectively. Lanes 1 and 11, molecular weight marker; lanes 2, 5, 8, and 12, crude extract from BL21(DE3)/pColdTF-hepS, BL21(DE3)/pColdTF-hepT, BL21(DE3)/pColdTF-ytpB, and BL21(DE3)/pColdTF-sqhC, respectively; lanes 3, 6, 9, and 13, total soluble proteins from BL21(DE3)/pColdTF-hepS, BL21(DE3)/pColdTF-hepT, BL21(DE3)/pColdTF-ytpB, and BL21-(DE3)/pColdTF-sqhC, respectively; lanes 4, 7, 10, and 14, purified HepS, HepT, YtpB, and SqhC, respectively.

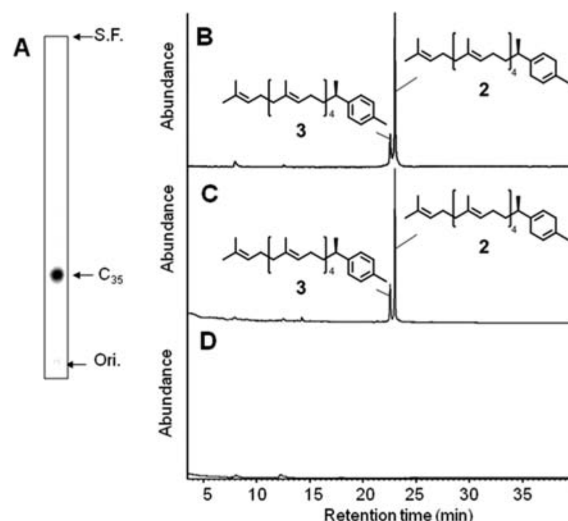


Figure 2. Analyses of products biosynthesized by purified heterodimeric heptaprenyl diphosphate synthase (HepS and HepT) and purified TS (YtpB). (A) TLC analysis of radiolabeled products synthesized by the incubation of FPP and [1-^{14}C]IPP with HepS and HepT. (B) GC–MS analysis of pure **2** isolated from *B. subtilis*. (C) GC–MS analysis of reaction products synthesized by the incubation of **1** with TS (YtpB). (D) GC–MS analysis of reaction products synthesized by the incubation of **1** with heat-denatured TS (YtpB).

has no similarity to that of the known terpene cyclases. Future biochemical and structural characterizations of TS will elucidate the catalytic mechanism. Previously, our study of a nonpathogenic *Mycobacterium* species revealed that the cell-free extracts converted a *Z*-type C_{35} isoprenoid to a cyclic compound other than **2**–**8**.[6] However, no TS homologue could be found in the mycobacterial genome, indicating that at least one more novel terpene cyclase remains unidentified.

A mutant *B. subtilis* strain lacking SqhC, which had similarity to squalene-hopene cyclase,[10] did not produce detectable amounts of

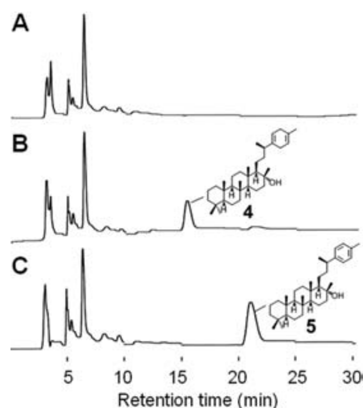


Figure 3. HPLC analyses of products biosynthesized by purified TC (SqhC). (A) Reaction products synthesized by the incubation of no substrate with TC (SqhC). (B) Reaction products synthesized by the incubation of **2** with TC (SqhC). (C) Reaction products synthesized by the incubation of **3** with TC (SqhC).

6−**8**.[3a] On the basis of this result, it had been proposed that the *sqhC* gene encodes TC.[3a] However, the enzymatic reaction has not been clearly demonstrated in vitro. In this study, the *sqhC* gene was expressed in *E. coli* using pColdTF (Figure 1), and the structures of the enzymatic products **4** and **5**, which were isolated from the reaction mixture of **2** with the cell-free extracts that included recombinant SqhC, were determined by NMR (^{1}H, ^{13}C, DEPT, COSY, HOHAHA, NOESY, HMQC, and HMBC) and MS (ESI and EI) analysis. The molecular formula of **4** was determined to be $C_{35}H_{58}O$ on the basis of HR-ESI-MS. The chemical shifts of **4** in the ^{13}C NMR spectra assigned by us were largely different from those by Takigawa et al.[4] This may be a result of mistyping by them, because our spectrum seems to be same as theirs. In addition, the relative stereochemistry of the tetracyclic skeleton in **4** was determined for the first time by observing the NOE correlations (Me29/H5, H5/H9, H9/H14, H14/H18, and Me32/Me33), as shown in Scheme 1. The stereochemistry at C21 of **4** is assumed to be same as that of substrate **2** (Scheme 1). The structure of **5** was also confirmed by NMR and MS analysis. The purified SqhC (Figure 1) successfully converted **2** and **3** to **4** and **5**, respectively, as confirmed by HPLC (Figure 3) and GC−MS (see the Supporting Information). The amount of **5** formed by autoxidation of **4** was negligible (Figure 3B), which may be the result of our careful preparation. In particular, the sample was not evaporated to complete dryness. Therefore, the cyclization of **2** to **4** was clearly demonstrated by the in vitro reaction, indicating that the *sqhC* gene encodes TC. In addition, the conversion of **3** to **5** by TC may also have occurred in *B. subtilis* cells.

In conclusion, the enzymatic biosynthetic pathway **1** → **2** → **4** was clearly shown using purified enzymes. Moreover, the results indicate that the C_{35} terpenes **2**−**8** were biosynthesized via the cyclization of C_{35} isoprenoid **1**. We propose that these C_{35} terpenes should be called "sesquarterpenes". The prefix *sesquar* means half (*se*mis in Latin) to four (*quar*tus in Latin) (i.e., three and a half), just as the prefix *ses*ter means half (*se*mis in Latin) to three (*ter*tius in Latin). The sesquarterpenes may be widely distributed in bacteria, as the TS homologues exist in various bacterial species. The physiological functions of **2** and **3** remain unknown, while it has been proposed that **6**−**8** (thermally dehydrated compounds formed from **5**) increase the resistance of spores to reactive oxygen species.[3] The *ts* gene (*ytpB*) forms an operon with a lysophospholipase gene (*ytpA*), which is responsible for the biosynthesis of the antibiotic bacilysocin in *B. subtilis*,[11] and many bacterial species with TS homologues possess no TC homologue. Therefore, the role of **2** and **3** may not be only to serve as the precursors of **4** and **5**. On the other hand, our previous studies of mycobacteria revealed that unique enzymes, such as a bifunctional *Z*-prenyltransferase that preferentially synthesizes C_{35} or C_{50} products and a prenyl reductase that can reduce both *E*- and *Z*-prenyl residues, were also responsible for the sesquarterpene biosynthesis in addition to the terpene cyclase.[6] Thus, biosynthetic studies of the sesquarterpenes could be one promising approach for the discovery of novel enzymes. The studies of sesquarterpenes promise to be an attractive field for expanding our understanding of the terpene world.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Experimental details, sequence data of enzymes, MS spectra, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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