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Identification of Novel Sesterterpene/Triterpene Synthase from *Bacillus clausii*

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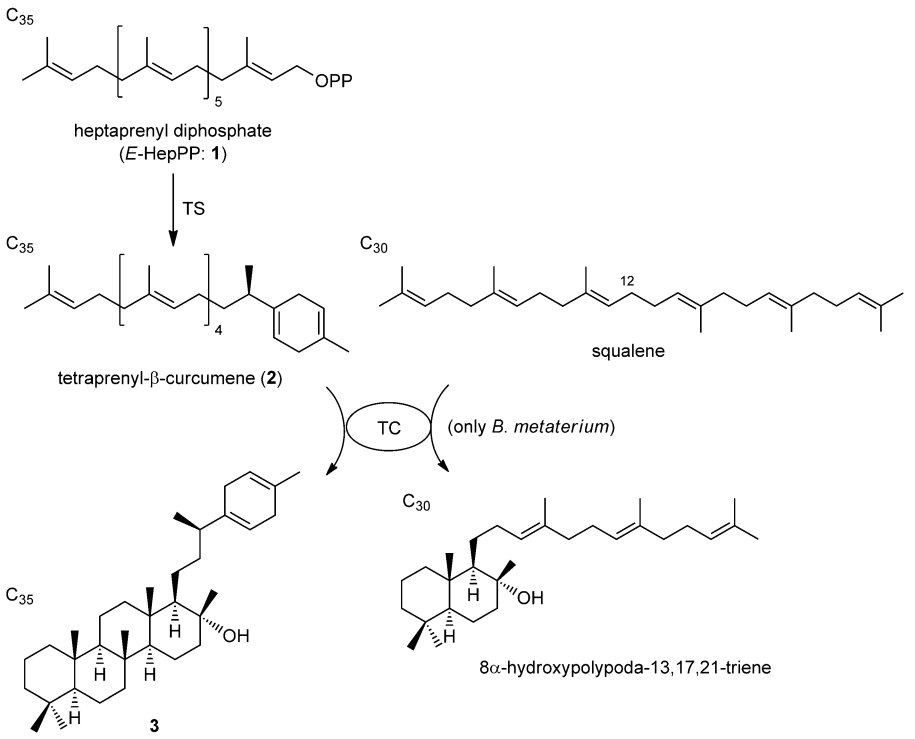
Terpenoids make up a very large family of natural products (more than 50000 structurally diverse compounds). They are categorised by the number of C_5 isoprene units: hemi- (one C_5 unit), mono- (C_{10}; two C_5 units), sesqui- (C_{15}; three C_5 units), di- (C_{20}; four C_5 units), sester- (C_{25}; five C_5 units), tri- (C_{30}; six C_5 units) and tetraterpenes (C_{40}; eight C_5 units).[1] Recent studies have revealed a unique biosynthetic pathway for sesquarterpenes, the new family name for C_{35} terpenes.[2–5] Tetraprenyl-β-curcumene synthase (TS), which catalyses the conversion of linear C_{35} isoprenoid **1** into monocyclic isoprenoid **2** in *Bacillus subtilis* (Scheme 1), is a new type of terpene cyclase; it has no sequence homology to any known terpene synthase.[3] In contrast, tetraprenyl-β-curcumene cyclase (TC), which forms pentacyclic **3** from **2** (Scheme 1), has a primary structure similar to that of squalene–hopene cyclase.[4] We have previously demonstrated that the *Bacillus megaterium* TC is a bifunctional terpene cyclase that also catalyses the conversion of squalene (C_{30}) into the bicyclic triterpene, 8α-hydroxypolypoda-13,17,21-triene, in vivo (Scheme 1).[4] Although TS homologues exist in various bacterial species, the catalytic functions of all TS homologues remain unknown. In the present study, we found that a TS homologue from the alkalophilic bacterium *Bacillus clausii* is a promiscuous enzyme that uses C_{25} or C_{30} as substrate (i.e., a sesterterpene/triterpene synthase).

First, we performed GC-MS analysis of terpenoids produced by *B. clausii*, for which genome sequencing has been completed (Figure 1 A). We assumed that sesquarterpene **2** would be detected because *B. clausii* has a TS homologue. However, **2** was not found. Instead, we found two unknown lipids, **6** and **7**, with putative molecular ions of *m/z* 340 and 408, respectively (Figure 1 A, Figure S1 in the Supporting Information). No terpenoid with a molecular weight lower than those of **6** and **7** was detected by GC-MS at lower column temperatures (30–280 °C, 3 °C min^{-1}). To determine the structures of **6** and **7**, *B. clausii* was cultured in 30 flasks (1 L of medium each), and compounds **6** and **7** were isolated by silica gel column chromatography and SiO_2 HPLC, to yield 22.0 and 14.2 mg of purified **6** and **7**, respectively.



Scheme 1. Proposed pathways for the biosynthesis of sesquarterpenes and triterpenes in *B. subtilis* and *B. megaterium*. Dehydrosqualene has a double bond at C-12 of squalene.

By using NMR (^{1}H, ^{13}C, DEPT, COSY, HOHAHA, NOESY, HMQC and HMBC) and EI-MS, the structures of **6** and **7** were determined (Scheme 2). The molecular formulae of **6** and **7** were determined to be $C_{25}H_{38}$ and $C_{30}H_{46}$, respectively, on the basis of the HR-EI-MS results. The methylidene protons of **6** at $\delta_H = 5.33$

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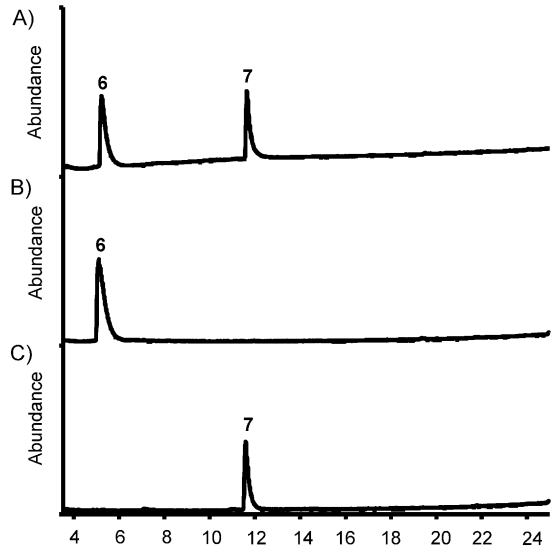
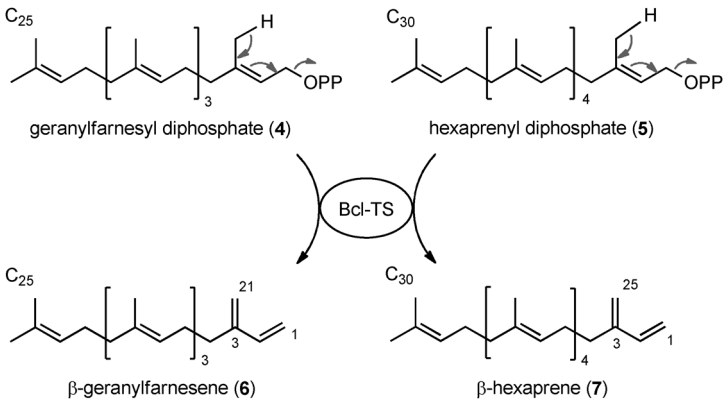


Figure 1. GC-MS analysis of terpenoids. A) *n*-Hexane extracts from *B. clausii* cells. B) Enzymatic product from incubation of substrate **4** with purified Bcl-TS. C) Enzymatic product from incubation of substrate **5** with purified Bcl-TS. Compound **2** was detected at 22.3 min under the same conditions.



Scheme 2. Proposed pathways for the biosynthesis of sesterterpene and triterpene in *B. clausii*.

(d, $J=17.4$ Hz) and 5.10 ppm (d, $J=10.7$ Hz) exhibited strong HMBC correlation with C-2 ($\delta_C=139.42$ ppm, d) and C-3 ($\delta_C=146.45$ ppm, s), thus indicating that these protons are assignable to H-1. H-2 ($\delta_H=6.49$ ppm) exhibited a dd splitting pattern ($J=17.6$ and 10.7 Hz) because of spin-spin couplings with H-1a (*trans*) and H-1b (*cis*). The methylidene protons at $\delta_H=5.12$ (s) and 5.11 ppm (s) were assigned to H-21, because of the clear cross-peak with C-2, C-3 and C-4 ($\delta_C=31.86$ ppm, t) in the HMBC spectrum. Thus, **6** contains an isoprene moiety. No NOE was observed for H-6 ($\delta_H=5.36$–5.42 ppm)/H-22 ($\delta_H=1.69$ ppm, s), H-10 ($\delta_H=5.36$–5.42 ppm)/H-23 ($\delta_H=1.73$ ppm, s) and H-14 ($\delta_H=5.36$–5.42 ppm)/H-24 ($\delta_H=1.73$ ppm, s), thus indicating that all prenyl residues in **6** are arranged in the *E* configuration. Comparison of the chemical shifts of C-8 ($\delta_C=40.15$ ppm), C-12 ($\delta_C=40.18$ ppm) and C-16 ($\delta_C=40.20$ ppm) with those of ficaprenol[6] (*Z*, ~32.0 ppm; *E*, ~40.0 ppm) further supports the *E* geometry. The NMR data for **7** were similar to those for **6**. The complete assignments of ^{1}H and ^{13}C NMR data for **6** and **7** unambiguously supported the structures of **6** and **7**. Whereas β-myrcene (C_{10}), β-farnesene (C_{15}) and β-springene (C_{20}) have been identified as acyclic terpene hydrocarbons containing a similar isoprene moiety, to the best of our knowledge, **6** and **7** are novel terpenoids. We propose the name β-geranylfarnesene and β-hexaprene for **6** and **7**, respectively.

The *B. clausii* genome has only one terpene synthase homologue: a TS homologue (Bcl-TS). Thus, we postulated the biosynthetic pathways for **6** and **7** on the basis of the biosynthetic pathway of sesquarterpene (Scheme 1): Bcl-TS converts **4** and **5** into **6** and **7**, respectively (Scheme 2). The subsequent cyclisation step for **6** and **7** might not exist in *B. clausii*, because it does not contain a TC homologue. To demonstrate our putative pathway (**4**→**6** and **5**→**7**; Scheme 2), we introduced the *bcl-ts* gene (ABC1218) from *B. clausii* into the vector pColdTF, expressed it as a soluble form in *Escherichia coli* and purified the protein by nickel-chelating affinity column chromatography (Figure 2). In order to synthesise substrates **4** and **5**, geranylfarnesol **S-6** and hexaprenol **S-12** were chemically synthesised (see the Supporting Information), and both **S-6** and **S-12** were diphosphorylated by using the method described by Davisson et al.[7] Purified Bcl-TS successfully converted **4** and **5** into **6** and **7**, respectively (Figure 1 B and C).

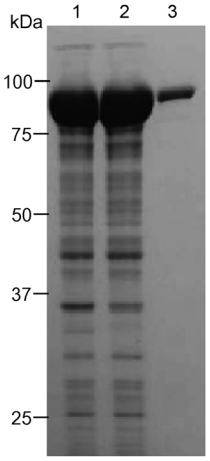


Figure 2. SDS-PAGE of overexpressed and purified recombinant Bcl-TS. The deduced molecular weights of Bcl-TS, expressed as fusion protein with a trigger factor (*E. coli* chaperone, 52 kDa), was 93.4 kDa. Lane 1, crude extract from BL21(DE3)/pColdTF-Bcl-TS; lane 2, total soluble protein; lane 3, purified Bcl-TS.

It has recently been reported that changing the pH of the reaction dramatically changes the product profile of sesquiterpene cyclase.[8] However, no products other than **6** or **7** were produced by Bcl-TS in reaction mixtures at different pH values (Figures S16 and S17). Thus, Bcl-TS was confirmed to play a role in the synthesis of **6** and **7** in vivo; it is sesterterpene/triterpene synthase. The reaction mechanism for **4** or **5** would be similar to that for common acyclic terpenes such as isoprene.[9] However, the catalytic mechanism of Bcl-TS might differ from that of the usual terpene synthases, because Bcl-TS has no sequence homology to any known terpene synthase. The *B. clausii* genome includes only one prenyltransferase homologue (heterodimeric *E*-HepPP synthase homologue: ABC1885 and ABC1889) that biosynthesises a medium-chain-length compound. We assumed that this compound was a substrate for Bcl-TS, **4**, and **5**. We are now performing a functional analysis of the prenyltransferase homologue, and will report the results in the near future.

Four alkalophilic *Bacillus* species, *Bacillus alcalophilus*, *Bacillus firmus* and *Bacillus* spp. OF1 and OF4, have been reported to produce squalene and dehydrosqualene.[10] However, the present study revealed that alkalophilic *B. clausii* biosynthesises compounds **6** and **7** but not squalene or dehydrosqualene. To review the terpenoids produced by alkalophilic *Bacillus* species, we analysed ten additional species: *Bacillus alcalophilus*, *Bacillus akibai*, *Bacillus cohnii*, *Bacillus halodurans*, *Bacillus horti*, *Bacillus krulwichiae*, *Bacillus marmarensis*, *Bacillus polygoni*, *Bacillus pseudofirmus* and *Bacillus wakoensis*. *B. alcalophilus* was used in a previous study by Clejan et al.[10] Compound **7** (M_w = 408 kDa) can be distinguished from dehydrosqualene (M_w = 408 kDa) by GC-MS analysis, because the fragmentation pattern of **7** differs from that of dehydrosqualene; in particular, a *m/z* 203 fragment was found for compound **7** (Figure S1) but not for dehydrosqualene.[11] As a result, these ten species were categorised on the basis of their production of sesterterpene, triterpene and sesquarterpene (Table 1). Compounds **6** and **7** were produced by *B. alcalophilus*, *B. akibai*, *B. cohnii*, *B. halodurans*, *B. marmarensis*, *B. polygoni* and *B. pseudofirmus*, as well as by *B. clausii*, (type I), whereas both squalene and **2** were detected in *B. horti*, *B. krulwichiae* and *B. wakoensis* (type II). Surprisingly, the product patterns (Table 1) for all species (including *B. alcalophilus*) differed from those described in the previous report, in which production of both squalene and dehydrosqualene was confirmed.[10] In particular, dehydrosqualene was not detected in any species. Thus, **7** might have been wrongly assigned as dehydrosqualene in the previous study.[10]

Table 1. Production of terpenes in alkalophilic *Bacillus* species.[a]

Species	C_{25}	C_{30}		C_{35}			Type
	6	Squalene	Dehydrosqualene	**7**	**2**	**3**	
B. clausii	+	−	−	+	−	−	I
B. alcalophilus	+	−	−	+	−	−	I
B. akibai	+	−	−	+	−	−	I
B. cohnii	+	−	−	+	−	−	I
B. halodurans	+	−	−	+	−	−	I
B. marmarensis	+	−	−	+	−	−	I
B. polygoni	+	−	−	+	−	−	I
B. pseudofirmus	+	−	−	+	−	−	I
B. horti	−	+	−	−	+	−	II
B. krulwichiae	−	+	−	−	+	−	II
B. wakoensis	−	+	−	−	+	−	II

[a] +: detected; −: not detected.

In addition, among the *Bacillus* species for which genome sequencing has been completed, seven *Bacillus* species that produce **2** and three that produce **6** and **7** were identified (Figure S19), as found in previous studies.[3,4] Mutational analysis targeting residues that differ in the TS homologues of the *Bacillus* groups (Figure S19) should reveal the mechanisms controlling acyclic and cyclic terpene synthesis in the future.

In conclusion, we have discovered novel head-to-tail types of acyclic sesterterpene **6** and triterpene **7** in *B. clausii*, and revealed that **6** and **7** are biosynthesised by Bcl-TS from **4** and **5**, respectively (Scheme 2). Bcl-TS is the first example of a head-to-tail triterpene synthase to be identified. Because little is known about the biosynthesis of sesterterpene and head-to-tail triterpenes (nonsqualene derivatives), our results might promote interest in the study of these compounds. In addition, our study revealed that TS homologues are a new family of terpene synthases that form not only sesquarterpene but also sesterterpene and triterpene. Genome mining of the new terpene synthase family should lead to the discovery of novel terpene synthases and terpenoids.

Experimental Section

General procedure and materials: NMR spectra were recorded on a DMX 600 spectrometer (Bruker Daltonics) at 600 MHz (^{1}H) and 125 MHz (^{13}C). GC-MS was performed on a JMS-T100GCV spectrometer (JEOL, Tokyo, Japan) equipped with a DB-1 capillary column (30 m × 0.25 mm × 0.25 μm; J&W Scientific/Agilent) or on a JMS-Q1000 GC spectrometer (JEOL) equipped with a ZB-5 capillary column (30 m × 0.25 mm × 0.25 μm; Zebron/Phenomenex, Torrance, CA) with EI mode at 70 eV. *B. clausii* JCM9138, *B. alcalophilus* JCM5262, *B. akibai* JCM9157, *B. cohnii* JCM12300, *B. halodurans* JCM9148, *B. horti* JCM9943, *B. krulwichiae* JCM11691, *B. marmarensis* JCM15719, *B. polygoni* JCM14604, *B. pseudofirmus* JCM9141 and *B. wakoensis* JCM9140 were used. *E. coli* JM109 was used for sequence analysis.

GC-MS analyses of *n*-hexane extracts from alkalophilic *Bacillus* species: Eleven alkalophilic *Bacillus* species were cultured by shaking at 37 °C for 4 days in 1 L of HORIKOSHI-I medium, as recommended by JCM (http://www.jcm.riken.go.jp/). Cells were then collected by centrifugation (6000 *g*, 4 °C, 20 min). After lyophilisation, cell extracts were made in MeOH (3 × 200 mL), and the MeOH fractions were partitioned by using *n*-hexane (3 × 600 mL). The GC-MS analysis (injection temperature 290 °C, oven temperature 220–300 °C, 3 °C min^{-1}) of *n*-hexane extracts from eight alkalophilic *Bacillus* species revealed **6** and **7** (Table 1; EI-MS spectra in Figure S1). The EI-MS spectrum of dehydrosqualene has no *m/z* 203 ion fragment.[11]

Isolation and identification of compounds 6 and 7 from *B. clausii*: *B. clausii* was cultured in medium (30 × 1 L) at 30 °C for 4 days. Lyophilised cells (dry weight 37 g) were extracted in MeOH (3 × 800 mL), and the MeOH fractions were concentrated to 800 mL and partitioned by using *n*-hexane (3 × 800 mL). The *n*-hexane layer was partially purified by silica gel (20 g) column chromatography with *n*-hexane and EtOAc. The fraction eluted with *n*-hexane (68.7 mg) was subjected to further silica gel (20 g) column chromatography with *n*-hexane and SiO_2 HPLC with *n*-hexane, thereby yielding 22.0 and 14.2 mg of purified **6** and **7**, respectively. The structures of **6** and **7** were determined by MS and NMR (Figures S2 and S3; NMR spectra in Figures S4–S15).

β-Geranylfarnesene (6): Colourless oil; ^{1}H NMR (600 MHz, C_6D_6): δ = 1.69 (s, 6H, Me-22, Me-25), 1.73 (s, 6H, Me-23, Me-24), 1.80 (s, 3H, Me-20), 2.20–2.24 (m, 6H, H-8, H-12, H-16), 2.29–2.35 (m, 6H, H-9, H-13, H-17), 2.38 (m, 2H, H-4), 2.39 (m, 2H, H-5), 5.10 (d, J = 10.7 Hz, 1H, H-1a), 5.11 (s, 1H, H-21), 5.12 (s, 1H, H-21), 5.33 (d, J = 17.4, 1H, H-1b), 5.36 (br t, 1H, H-18), 5.36–5.42 (m, 3H, H-6, H-10, H-14), 6.49 ppm (dd, J = 17.6, 10.7 Hz, 1H, H-2); ^{13}C NMR (150 MHz, C_6D_6): δ = 16.10 (q, C-22, C-23, C-24), 17.71 (q, C-25), 25.81 (q, C-20), 27.06 (t, C-5), 27.08 (t, C-9), 27.13 (t, C-13), 27.24 (t, C-17), 31.86 (t, C-4), 40.15 (t, C-8), 40.18 (t, C-12), 40.20 (t, C-16), 113.08 (t, C-1), 115.95 (t, C-21), 124.60 (d, C-6), 124.80 (d, C-10, C-14), 124.95 (d, C-18), 131.08 (s, C-19), 134.98 (s, C-11), 135.01 (s, C-15), 135.39 (s, C-7), 139.42 (s, C-2), 146.45 ppm (s, C-3). The following ^{13}C NMR sig-

nals were indistinguishable because of the close chemical shifts: C-5/C-9/C-13/C-17, C-6/C-10/C-14/C-18, C-8/C-12/C-16 and C-11/C-15. EI-MS: *m/z* (%): 69 (100), 81 (52), 93 (27), 107 (10), 135 (5), 161 (10), 203 (8), 271 (3), 340 ($[M]^+$, 5). HR-EI-MS: *m/z* calcd for $C_{25}H_{38}$: 340.31300 $[M]^+$; found: 340.31389.

β-Hexaprene (7): Colourless oil; ^{1}H NMR (600 MHz, C_6D_6): $\delta = 1.69$ (s, 6H, Me-26, Me-30), 1.73 (s, 9H, Me-27, Me-28, Me-29), 1.80 (s, 3H, Me-24), 2.20–2.24 (m, 8H, H-8,H-12, H-16, H-20), 2.29–2.35 (m, 8H, H-9,H-13, H-17, H-21), 2.37 (m, 2H, H-4), 2.39 (m, 2H, H-5), 5.10 (d, $J = 10.7$ Hz, 1H, H-1a), 5.11 (s, 1H, H-25), 5.12 (s, 1H, H-25), 5.33 (d, $J = 17.7$ Hz, 1H, H-1b), 5.36 (brt, 1H, H-22), 5.36–5.42 (m, 4H, H-6, H-10, H-14, H-18), 6.49 ppm (dd, $J = 17.6$, 10.6 Hz, 1H, H-2); ^{13}C NMR (150 MHz, C_6D_6): $\delta = 16.11$ (q, C-26, C-27), 16.13 (q, C-28, C-29),17.72 (q, C-30), 25.82 (q, C-24), 27.07 (t, C-5), 27.09 (t, C-9), 27.14 (t, C-13), 27.16 (t, C-17), 27.25 (t, C-21), 31.86 (t, C-4), 40.15 (t, C-8, C-12), 40.20 (t, C-16, C-20), 113.07 (t, C-1), 115.95 (t, C-25), 124.60 (d, C-6), 124.80 (d, C-10), 124.83 (d, C-14, C-18),124.96 (d, C-22), 131.07 (s, C-23), 134.96 (s, C-7), 134.99 (s, C-11), 135.01 (s, C-15), 135.39 (s, C-19), 139.42 (s, C-2), 146.44 ppm (s, C-3). The following ^{13}C NMR signals were indistinguishable because of the close chemical shifts: C-5/C-9/C-13/C-17/C-21, C-6/C-10/C-14/C-18/C-22, C-8/C-12/C-16/C-20 and C-11/C-15/C-19. EI-MS: *m/z* (%): 69 (100), 81 (69), 93 (30), 107 (16), 135 (10), 161 (10), 203 (14), 271 (5), 339 (4), 408 ($[M]^+$, 4). HR-EI-MS: *m/z* calcd for $C_{30}H_{46}$: 408.37560 $[M]^+$; found: 408.37539.

Expression, purification and enzyme assays for Bcl-TS: Primers (Bcl-TS-S, 5′-TCGTTT GGGTTA GCCTTT CTC-3′; Bcl-TS-A, 5′-CTGCCA CCTGTT CATCTG CTC-3′) and *B. clausii* JCM9138 genomic DNA were used for PCR amplification of the Bcl-TS gene (ABC1218), including approximately 200 bp upstream and downstream of the open reading frame. Amplified DNA fragment was inserted into pGEM-T vector (Promega), and the construct was used for sequence analysis. The DNA sequence of *bcl-ts* was deposited in the DNA Data Bank of Japan database under accession number AB735674. The amino acid sequence of *B. clausii* JCM9138 Bcl-TS (Figure S18) showed 98% identity to that of *B. clausii* KSM-K16, for which complete genome sequence data are available (Figure S18). The following primer pair and the pGEM-T derivative of *bcl-ts* were used to add restriction sites into the gene by PCR: TF-Bcl-TS-S (XhoI), 5′-GGAGGA CTCGAG ATGGGC ACAGTA CCTGCC AAC-3′; TF-Bcl-TS-A (XbaI), 5′-TTTGCC GGATCC TTATGA AATTTT ACGTTT AAAAATC-3′. Amplified DNA fragments were inserted into pGEM-T. The XhoI/XbaI fragment was inserted into the XhoI/XbaI fragment of the pColdTF vector (Takara Bio, Shiga, Japan). *E. coli* BL21 (DE3) harbouring the pColdTF construct (pColdTF-Bcl-TS) was grown at 37 °C in lysogeny broth containing ampicillin (100 μg mL^{-1}). Expression of the recombinant protein was induced by addition of IPTG (0.1 mM) when OD_{600} reached approximately 0.6. Cells were grown for a further 24 h at 15 °C, then harvested by centrifugation and resuspended in buffer containing Tris·HCl (20 mM, pH 7.9), imidazole (10 mM) and NaCl (300 mM). Cells were disrupted by sonication in a UP200S sonicator (4–10 °C, 15 min; Hielscher Ultrasonics, Teltow, Germany), and the resulting suspension was centrifuged (10000 *g*, 20 min). The supernatant containing soluble His-tagged fusion enzyme was purified according to the manufacturer's manual (Takara). Expression and purification of Bcl-TS were analysed by SDS-PAGE on 10% gels (Figure 2). In order to synthesise substrates **4** and **5**, geranylfarnesol **S-6** and hexaprenol **S-12** were chemically synthesised as described in the Supporting Information; both **S-6** and **S-12** were diphosphorylated by using the method described by Davisson et al.[7] For Bcl-TS enzymatic assays, the reaction mixture contained Tris·HCl buffer (25 mM, pH 8.5), dithiothreitol (10 mM), $MgCl_2$ (1 mM), NH_4Cl (25 mM), **4** or **5** (65.5 μM) and purified Bcl-TS (2.0 μM) in a total volume of 2 mL. The reaction was carried out at 37 °C for 1 h. KOH/MeOH (15%, 2.4 mL) was added to terminate the reaction, and the mixture was then heated (80 °C, 30 min). The lipophilic product was extracted from the reaction mixture by using *n*-hexane (3×4 mL) and analysed by GC-MS (injection temperature, 300 °C; oven temperature, 220–300 °C; 3 °C min^{-1}; Figure 1B and 1C). Products **6** and **7** were identified by both comparisons of mass spectra and coinjection experiments with authentic samples. To measure the influence of pH on the reaction product profiles of Bcl-TS, we used MES buffer (3 mL, 200 mM, pH 6.5), Tris·HCl (200 mM, pH 8.5) or CAPS (200 mM, pH 10.5) in the reaction mixture. The results are shown in Figures S16 and S17.

Acknowledgements

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Keywords: biosynthesis · enzymes · sesterterpenes · terpene synthases · triterpenes

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