# $C_{35}$-Terpenes from *Bacillus subtilis* KSM 6-10

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Four $C_{35}$-terpenes (**1**−**4**) were isolated from a liquid culture of *Bacillus subtilis* KSM 6-10. Compounds **1** and **2**, tetraprenyl-$\beta$-curcumene and tetraprenyl-α-curcumene, respectively, were previously isolated from a spore preparation of the same species, whereas **3** and **4** are new $C_{35}$-terpenols. The $C_{35}$-terpenols (**3** and **4**) possess polycyclic skeletons. We propose that the $C_{35}$-terpenols (**3** and **4**) are formed by cyclization of the acyclic $C_{35}$-terpenes (**1** and **2**). Because only trace amounts of $C_{35}$-terpenols **3** and **4** were found in the spore in contrast to the vegetative cells of *B. subtilis* KSM 6-10, it is assumed that the expression of terpene cyclase is not related to sporulation, as has been observed by other researchers in a different strain.

Characteristic components of archaeal cell membranes comprise hydrophobic aliphatic isoprenoid chains.[1] The deep-sea methanogen *Methanococcus jannaschii* produces mainly $C_{30}$- or $C_{35}$-isoprenoids,[2,3] and *Methanosarcina barkeri* produces mainly $C_{25}$-isoprenoids.[4] These isoprenoid chains are extremely stable over time and have been used as biomarkers of ancient bacterial activity during geological periods.[5] Recent studies demonstrated that a ubiquitous bacterium such as *Bacillus subtilis* produces $C_{35}$-isoprenoids in spores.[6,7] The strain was also shown to produce three pentacyclic terpenoids (sporulenes) via the enzyme squalene cyclase.[7] In this report, we describe the characterization of new $C_{35}$-terpenols with the same polycyclic skeleton as the sporulenes reported by Kontnik et al.[6,7] A possible formation route for these $C_{35}$-terpenes is suggested.

## Results and Discussion

To identify the hydrophobic metabolites of *B. subtilis* KSM 6-10, whole culture broth or culture supernatants were extracted with EtOAc, leading to four $C_{35}$-terpenoids (**1**−**4**). Compound **1**, with the molecular formula $C_{35}H_{56}$ as determined by GC-MS, was obtained as a colorless oil. The MS fragmentation pattern was identical to a bacterial metabolite reported by Bosak et al.[6] $^1$H and $^{13}$C NMR analyses were also consistent with the chemical shift data of tetraprenyl-$\beta$-curcumene reported by Bosak et al.[6] and Boroczky et al.[8] Compound **2**, with the molecular formula $C_{35}H_{54}$ as determined by GC-MS, was also obtained as a colorless oil. The mass spectrum and $^1$H and $^{13}$C NMR spectra were identical to the spectra of tetraprenyl-α-curcumene reported previously.[6,8] Purified compound **1** in *n*-hexane was converted to compound **2** within 3 days at room temperature without chemical catalysis (data not shown). It is possible that compound **2** is a chemical conversion product of **1**, but enzymatic formation cannot be excluded.

Compound **4** was isolated as a colorless solid with positive specific rotation ($[\alpha]^{20}_D$ +41.6 in $CDCl_3$). The LC-MS of **4** showed a molecular ion peak at *m*/*z* 515.6 $[M + Na]^+$ (Figure S1, Supporting Information). Signals in the $^1$H NMR spectrum of **4** were assigned to six tertiary methyl [$\delta$ 0.79, 0.83, 0.78, 0.75, 0.66, 1.09 (3H each, all s $H_3$-28 to 33)], one aromatic methyl [$\delta$ 2.32 (3H, s $H_3$-35)], one secondary methyl [$\delta$ 1.20 (3H, d $H_3$-34)], five methine [$\delta$ 0.75 (d, H-5), 0.84 (d, H-9), 0.72 (d, H-14), 0.99 (t, H-18), 2.62 (d, H-21)], and four aromatic protons [$\delta$ 7.08 (t, H-23), 7.09 (t, H-24), 7.09 (t, H-26), 7.08 (t, H-27)]. The $^{13}$C NMR spectrum showed 35 carbon signals, which were assigned as eight methyl, 11 methylene, nine methine, and seven quarternary carbons, and one quarternary carbon bearing an oxygen function. The molecular formula was deduced as $C_{35}H_{54}O$. The structure of **4** was also investigated by 2D NMR studies including COSY, HMQC, and HMBC correlation experiments (Table 1). Compound **4** appeared to possess the same tetracyclic skeleton as that of sporulenes,[6,7] except for an additional hydroxy group at C-17 ($\delta$ 74.3) and the disappearance of a methylene carbon (C-33) signal at $\delta$ 105.8 in the spectrum of sporulene B.[7] An NOE association observed between Me-32 ($\delta_H$ 0.66, s) and Me-33 ($\delta_H$ 1.09, s) suggested a *cis* relationship between these two groups (Figure S15, Supporting Information). NMR signals found for ring E were similar to those for **2**, and the signals of the tetracyclic terpene moiety were similar to those of the sesterterpene scalaranes,[9−11] respectively. Thus, the structure of **4** was established as shown in Figure 1.

**Table 1.** $^1$H and $^{13}$C NMR Data of Compound **4** in $CDCl_3$

| no. | $\delta_C$ | $\delta_H$ | COSY | HMBC |
|---|---|---|---|---|
| 1 | 39.9 | 1.66, m; 0.76, m | | 2 |
| 2 | 18.7 | 1.33, m; 1.08, m | | 1 |
| 3 | 42.1 | 1.33, m; 1.13, dd (13.2, 3.8) | | 1 |
| 4 | 33.3 | | | |
| 5 | 56.6 | 0.75, dd (2.4) | | 1,6,30 |
| 6 | 18.3 | 1.49, m | 7 | |
| 7 | 41.9 | 1.73, dt (12.4, 3.2); 1.29, m | 6 | 5,6,8,9,14 |
| 8 | 37.4 | | | |
| 9 | 61.0 | 0.84, dd (2.1) | | 8,10,12 |
| 10 | 37.7 | | | |
| 11 | 16.9 | 1.42, m; 1.23, m | 9, 12 | 8,10 |
| 12 | 41.3 | 1.50, m; 0.91, m | 11 | 14 |
| 13 | 39.3 | | | |
| 14 | 61.2 | 0.72, dd (12.1, 2.0) | | 8,32 |
| 15 | 19.3 | 1.58, m; 1.19, m | 16 | 16,17 |
| 16 | 44.3 | 1.82, dt (12.4, 3.2); 1.29, m | 15 | 14,15,17,18,33 |
| 17 | 74.3 | | | |
| 18 | 62.8 | 0.99, t (3.8) | | 12,13,14,17 |
| 19 | 23.6 | 1.32, m; 1.18, m | 20 | |
| 20 | 42.2 | 1.66, dd (16.7, 7.3) | 19 | 18,19,21,22 |
| 21 | 40.5 | 2.62, dd (13.9, 6.8) | 20,34 | 20,22,23,27,34 |
| 22 | 144.7 | | | |
| 23 | 126.9 | 7.08, t (8.1) | 24 | 21,25 |
| 24 | 129.0 | 7.09, t (8.1) | 23 | 22,35 |
| 25 | 135.1 | | | |
| 26 | 129.0 | 7.09, t (8.1) | 27 | 22,35 |
| 27 | 126.9 | 7.08, t (8.1) | 26 | 21,25 |
| 28 | 21.3 | 0.79, s | | 3,5 |
| 29 | 33.3 | 0.83, s | | 4,5 |
| 30 | 16.2 | 0.78, s | | 1,5,9,10 |
| 31 | 17.4 | 0.75, s | | 7,8,9,14 |
| 32 | 16.5 | 0.66, s | | 12,13,14,18 |
| 33 | 23.8 | 1.09, s | | 16, 17, 18 |
| 34 | 23.0 | 1.20, d (6.8) | 21 | 20,21,22 |
| 35 | 21.0 | 2.32, s | | 24, 25, 26 |

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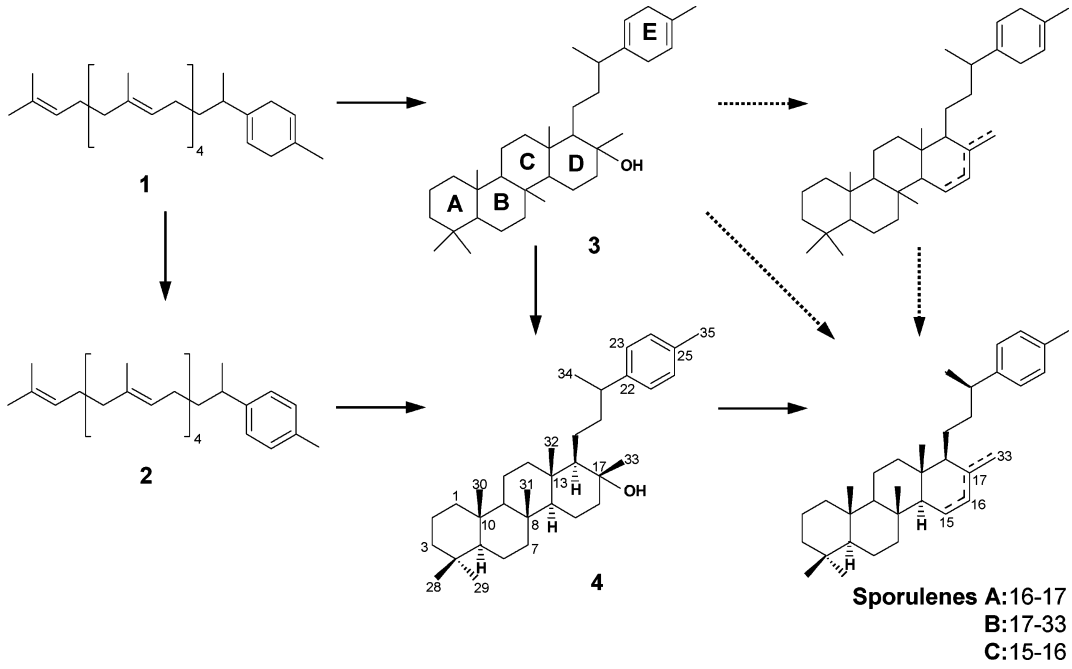


**Figure 1.** Possible biosynthetic pathways for $C_{35}$-terpenes isolated from *Bacillus subtilis* KSM 6-10.

Compound **3** was isolated as a colorless solid. The LC-MS of **3** showed a molecular ion peak at *m/z* 517.6 $[M + Na]^+$ (Figure S2, Supporting Information). The molecular formula of **3** could be deduced as $C_{35}H_{56}O$ from LC-MS and NMR data. The NMR spectrum of **3** is similar to that of **4**, except for ring E. HMBC and COSY correlations defined the $\gamma$-terpinene moiety to be terminal (Table 2). The planar structure of **3** was thus determined and is shown in Figure 1. In the same manner as compounds **1** and **2**, **3** was converted to **4** at room temperature without any chemical catalysis (data not shown).

A typical GC of **3** is presented in Figure S16 (Supporting Information). Though isolated as a single peak, its GC comprises three major peaks. Unexpectedly, the GC of **4** presented in Figure S13 (Supporting Information) is similar to that of **3**, with a small difference in the proportion of the three major peaks. Both **3** and **4** seemed to be heat-labile. MS data of the three peaks in Figures S13 and S16 are presented in Figures S14 and S17 (Supporting Information), respectively. The GC-MS of **4** showed a molecular ion peak at *m/z* 474.4 $[M - H_2O]^+$ like **3** (Figures S14 and S17, Supporting Information), which represents the thermal loss of a water molecule. Interestingly, the data were identical to those of the pentacyclic terpenoids known as sporulenes.[6,7] It is likely that both **3** and **4** decomposed to generate three sporulenes under the GC conditions (heating conditions described in the Experimental Section).

According to the procedure of Kontnik et al.,[6,7] pentacyclic terpenoids were detected from spores acidified and heated at 80 °C for 36 h after harvesting. In our study, $C_{35}$-terpenoids **1**−**4** were isolated from the culture broth at room temperature without heating. Moreover, in contrast to the vegetative cells, only trace amounts of the $C_{35}$-terpenoids were recovered in the spore of *B. subtilis* KSM 6-10 prepared under standard sporulation conditions.[6] Though the three sporulenes[6,7] could be produced by thermal dehydration from our isolated $C_{35}$-terpenoids **3** and **4**, their contents in the spore could not be detected without heating. Thus, we could not establish whether compounds **3** and **4** were contained in the spores of the isolates by Kontnik et al.[6,7] It may be speculated that the expression of the terpene cyclizing enzyme is not related to sporulation in our isolate, even though our strain was identified as a strain of *B. subtilis* just as the isolate by Kontnik et al.

*B. subtilis* is closely related to *Alicyclobacillus acidocaldarius*,[13] and some strains were separated from the genus *Bacillus* and reclassified as the new genus *Alicyclobacillus*.[14] Nevertheless, a striking dissimilarity in cyclization enzymes between the two bacteria is found concerning the steric hindrance of a methyl group in the sequential ring-forming reaction. As shown in the structures of **3** and **4**, a $\beta$-oriented C-13 methyl group (Figure 2) does not block the cyclization reaction by steric hindrance in the strains[6,7] of *B. subtilis*, including our isolated KSM 6-10. In contrast, a $\beta$-oriented C-13 methyl group was found to block the cyclization

**Table 2.** $^1H$ and $^{13}C$ NMR Data of Compound **3** in Benzene-$d_6$

| no. | $\delta_C$ | $\delta_H$ | COSY | HMBC |
|---|---|---|---|---|
| 1 | 40.1 | 1.62, m; 0.74, m | | |
| 2 | 19.1 | 1.48, m | | |
| 3 | 42.5 | 1.40, m | | |
| 4 | 33.4 | | | |
| 5 | 56.7 | 0.74, m | | 6,9,10 |
| 6 | 18.7 | 1.32, m | | |
| 7 | 42.0 | 1.62, m; 0.75, m | | |
| 8 | 37.9 | | | |
| 9 | 61.1 | 0.62, m | 11 | 7,8,10,31 |
| 10 | 37.7 | | | |
| 11 | 17.2 | 1.43, m; 0.79, m | 9 | |
| 12 | 41.7 | 1.74, m | | |
| 13 | 39.4 | | | |
| 14 | 61.0 | 0.74, m | | 8,9,15 |
| 15 | 19.6 | 1.46, m; 1.05, m | | |
| 16 | 44.9 | 1.77, m; 1.36, m | | |
| 17 | 73.7 | | | |
| 18 | 62.8 | 1.02, m | | 13,14,17,19,20 |
| 19 | 23.9 | 1.20, m | 18,20 | |
| 20 | 39.4 | 1.63, m | | 18,19,21,22 |
| 21 | 42.3 | 2.23, m | 34 | 19,20,22,23,27,34 |
| 22 | 139.5 | | | |
| 23 | 27.1 | 2.71, m | 24 | 21,22,24 |
| 24 | 119.3 | 5.50, m | 23 | 22,23,25 |
| 25 | 131.3 | | | |
| 26 | 32.0 | 2.59, m | 27 | 25,27 |
| 27 | 118.2 | 5.57, m | 26 | 22,23,26 |
| 28 | 21.5 | 0.87, s | | 3,4,5 |
| 29 | 33.5 | 0.93, s | | 4,5,28 |
| 30 | 16.4 | 0.81, s | | 1,5,9,10 |
| 31 | 17.6 | 0.73, s | | 7,9,14 |
| 32 | 16.7 | 0.69, s | | 12,14,18 |
| 33 | 24.2 | 1.09, s | | 16,17,18 |
| 34 | 20.2 | 1.14, br s | 21 | 20,21,22 |
| 35 | 23.2 | 1.35, s | | 23,24,25,26 |

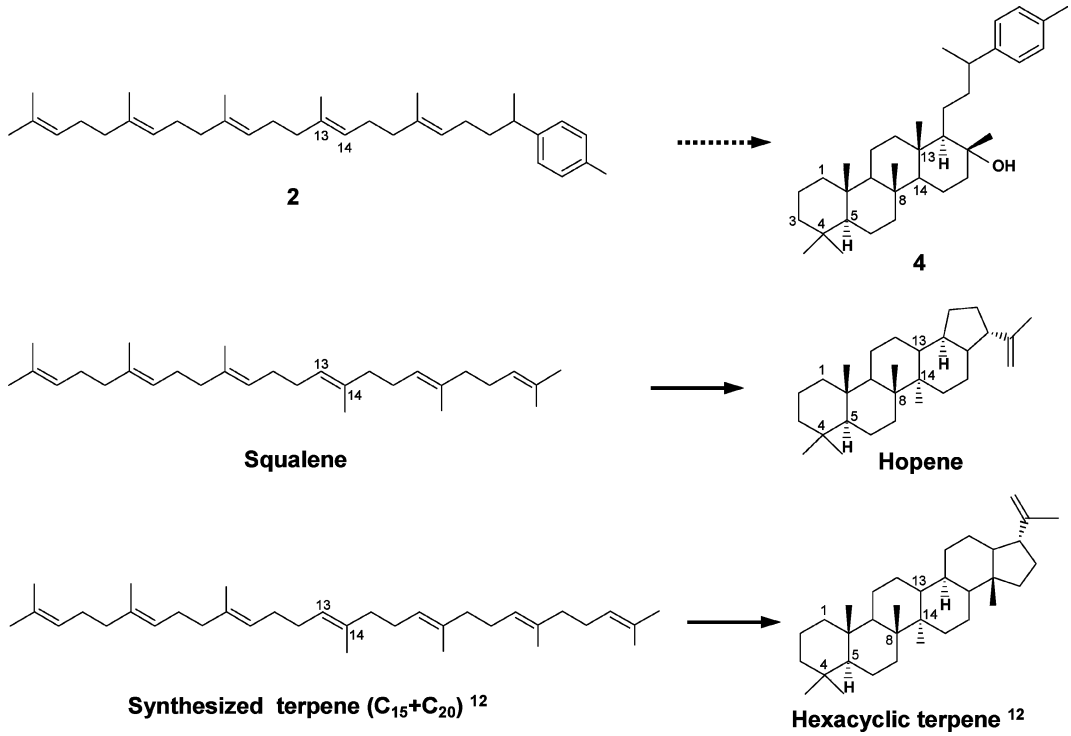


**Figure 2.** Structures of selected polycyclic terpenes.

reaction by steric hindrance in *A. acidocaldarius*.[12,13] When an unnatural hexacyclic $C_{35}$ polyprenoid was formed from a $C_{35}$ analogue in which a farnesyl $C_{15}$ unit was connected in a head-to-head fashion to a geranylgeranyl $C_{20}$ unit, it was initiated from the $C_{15}$ end, not from the $C_{20}$ end, suggesting that α-orientation of the C-14 methyl group is crucial,[12] just as in the case of cyclization of squalene into hopene (Figure 2).

We are presently attempting to estimate the biological role of polycyclic $C_{35}$-terpenoids **3** and **4** and the key factor that initiates the expression of terpene cyclizing enzymes. Because cyclic terpenes without a γ-terpinene or *p*-cymene moiety on the opposite terminal position were not found, it is not clear whether a γ-terpinene or *p*-cymene moiety on the opposite terminal position is essential for reactions with the terpene cyclizing enzyme.

## Experimental Section

**General Experimental Procedures.** Optical rotations were measured with a JASCO P-1020 polarimeter (Easton, MD). $^1$H and $^{13}$C NMR spectra were recorded on a JEOL JNM-ECP 600 spectrometer (Penbody, MA) using TMS as an internal standard, and chemical shifts were recorded as δ values. GC-MS analysis was carried out on a GC system coupled with a quadrupole mass spectrometer (GCMS-QP2010, Shimadzu Corporation, Tokyo, Japan). The compounds were separated on a DB-5 capillary column (Agilent Technologies, Santa Clara, CA; 30 m × 0.25 mm, 0.25 μm) using He as carrier gas (1.2 mL/min). The GC oven temperature program utilized an initial temperature of 250 °C and an initial holding time of 1 min, then increased at 8 °C/min to 320 °C. The final temperature was held for 20 min. The injection and ion source temperatures were both 320 °C. LC-MS analysis was carried out on an Agilent 1100 Series (Agilent Technologies GmbH, Waldbronn, Germany) consisting of a BinPump, an 1100 Series injector, and a mass spectrometer (Bruker Esquire 3000plus, Bruker-Franzen Analytik GmbH, Bremen, Germany) with an atmospheric source. The ESIMS interface was operated in the positive mode under the following conditions: gas temperature in the nebulizer, 450 °C; nebulizing gas pressure, 50 psig.

**Cultivation.** The bacterium *Bacillus subtilis* KSM 6-10 was grown in K medium (17 g/L *Mieki* (soybean protein hydrolysate; Ajinomoto), 5 g/L D-glucose, 7.2 g/L yeast extract, 0.25 g/L $KH_2PO_4$, 0.25 g/L $K_2HPO_4$, 0.1 g/L $MgSO_4$, 5 mg/L $MnCl_2$, 5 mg/L $FeSO_4$; pH 7) for 2 days at 30 °C and 120 rpm on a rotary shaker in 10 2-L baffled flasks, each containing 0.5 L of liquid medium. The large-scale fermentation was also performed in a 30-L fermentor (working volume, 15 L; space velocity, 0.35 vvm; agitation speed, 300 rpm; air pressure, 0.04 MPa).

**Extraction and Isolation.** Without adjusting the pH of the broth (7.5−8.0), the whole broth was exhaustively extracted at RT with EtOAc (2 × 5 L) to yield 50 mg of a pale brownish material. The EtOAc extract was separated by liquid−liquid extraction (50/50; *n*-hexane/90% aqueous MeOH). The *n*-hexane extract (1.5 g) was fractionated using a High-Flash column (16 × 60 mm, Yamazen, Osaka, Japan) employing gradient elution from *n*-hexane to EtOAc to yield four fractions (20 mL each). Fraction 2 (38 mg) was further fractionated by the same conditions to yield three fractions. Fraction 2-1 gave 5 mg of pure compound **1**, and fraction 2-2 gave 18 mg of pure compound **2**. Fraction 3 (1420 mg) was further fractionated under the same conditions to yield three fractions. Fraction 3-3 (1340 mg) was further fractionated to yield seven fractions. Fraction 3-3-2 (240 mg) was further fractionated by gradient preparative HPLC (20% to 100% $CH_3CN$ in $H_2O$) to yield three fractions. Fraction 3-3-2-1 (9 mg) gave 1 mg of pure compound **3** and 5 mg of pure compound **4**.

**Isolation and Identification of the Bacterium Strain.** *B. subtilis* KSM 6-10 was isolated from a Japanese traditional pickle made from Chinese cabbage (*tsukemono*) using SCD agar (Difco, Detroit, MI). The strain was identified as the genus *Bacillus* on the basis of physiological tests. The sequence of 16S rDNA was compared directly to all known sequences deposited in GenBank databases using the basic local alignment search tool (BLAST). The isolate showed approximately 99.8% sequence homology to the standard strain of *B. subtilis*.

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**Supporting Information Available:** Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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