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Cyclization of Squalene from Both Termini: Identification of an Onoceroid Synthase and Enzymatic Synthesis of Ambrein

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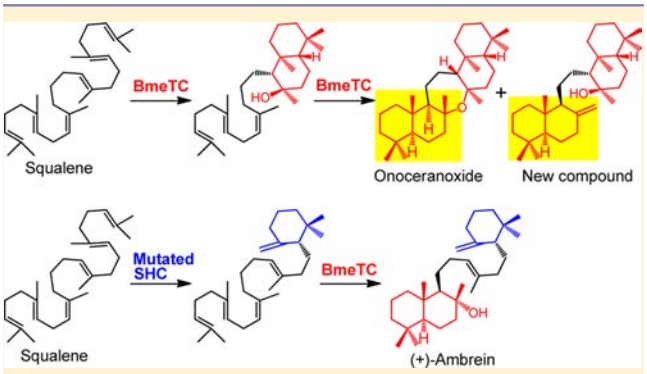
Supporting Information



ABSTRACT: The onoceroids are triterpenoids biosynthesized from squalene or (3*S*)-2,3-oxidosqualene by cyclization from both termini. We recently revealed that tetraprenyl-*β*-curcumene cyclase from *Bacillus megaterium* (BmeTC) is a bifunctional triterpene/sesquarterpene cyclase that converts head-to-tail tetraprenyl-*β*-curcumene and tail-to-tail squalene into pentacyclic and bicyclic products, respectively, in vivo. Here, we reveal that BmeTC has an unprecedented catalytic function in cyclizing squalene from both termini and is the first onoceroid synthase. We also report the first onoceroids from bacterial origin. Our discoveries suggest that symmetric and asymmetric onoceroids could be biosynthesized by a single enzyme via an intermediate cyclized at one terminus of squalene. Furthermore, the new function of BmeTC enabled the synthesis of (+)-ambrein, a major constituent of ambergris that is difficult to obtain naturally, via a mutated squalene-hopene cyclase−catalyzed reaction from easily available squalene.

The onoceroids are a group of triterpenes which are biosynthesized from squalene (**3**) or (3*S*)-2,3-oxidosqualene by cyclization from both termini. They include compounds having onocerane, serratane, ambrane, and colysane skeletons (Figure 1)[1a] and have been found in ferns,[1] higher plants,[2,3] and animals.[4] The onoceroids have exhibited several types of biological activities.[3,4] In particular, (+)-ambrein (**8**) is a major constituent of ambergris, which is a metabolite of the sperm whale and one of the most valuable animal perfumes.[4a] Compound **8** has been suggested as the possible active component of ambergris that produces its supposed aphrodisiac effects.[4b−d] However, the supply of ambergris has become very limited due to international agreements to avoid whaling.

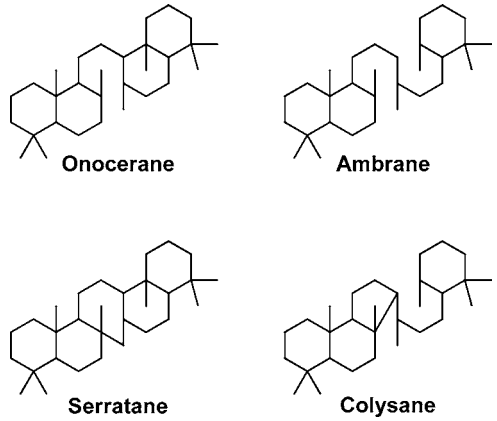


Figure 1. Cyclic skeletons of onoceroids found in nature.

Many triterpene synthases, including *seco*-type triterpene synthases[5] which afford nonfused cyclic skeletons by Grob fragmentation, have been identified.[6] However, an onoceroid synthase has never been found. Thus, it has not been elucidated whether a single enzyme produces onoceroids or two, and an intermediate formed by a one-terminus cyclization has not been identified.[7]

Biosynthetic studies on the sesquarterpenes, a family of C_{35} terpenes,[8] in *Bacillus subtilis* have identified tetraprenyl-*β*-curcumene synthase, which lacks sequence homology to any known terpene synthases,[8] and tetraprenyl-*β*-curcumene cyclase (TC), which shows a primary structure similar to squalene-hopene cyclase (SHC) (ca. 30% identity with SHC).[9] We recently revealed that *Bacillus megaterium* TC (BmeTC) is a bifunctional triterpene/sesquarterpene cyclase that converts head-to-tail C_{35} **1** and tail-to-tail C_{30} **3** into pentacyclic **2** and bicyclic **4**, respectively, in vivo (Scheme 1).[8,10] Here, we reveal that BmeTC has an unprecedented catalytic function in cyclizing **3** from both termini and is the first onoceroid synthase. Furthermore, the new function of BmeTC enabled the synthesis of **8** via a mutated SHC−catalyzed reaction from easily available **3**. Several reports have described the total synthesis of **8**; however, all required numerous operational steps.[11]

In the present study, two new products, **5** and **6**, were detected in addition to **4** from the incubation of **3** with *Escherichia coli* cell-free extracts containing overexpressed

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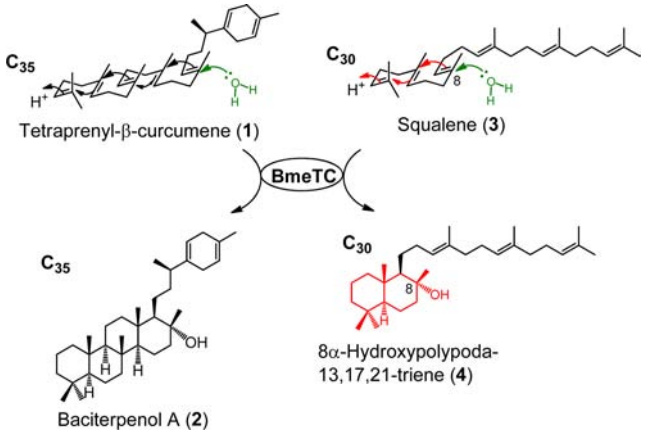
Scheme 1. Cyclization of Sesquarterpene (1) and Triterpene (3) Catalyzed by BmeTC[a]



[a]The numbering of C-8 in **3** is that of a bicyclic intermediate.

recombinant BmeTC (Figure 2A). The incubation of substrate **4** with BmeTC also afforded **5** and **6** (Figure 2B). These results revealed that BmeTC produced two new products (**5** and **6**) through consecutive reactions: (**4** → **5** + **6**) after (**3** → **4**). Our previous report described the formation of only **4** from **3** using purified BmeTC.[10] We consider that the amount of compound **4** produced in the previous study was insufficient for conversion into **5** or **6** because only ∼10% of the purified BmeTC was used in the earlier work compared to the present conditions. Since peaks of **5** and **6** were detected in addition to **1**−**4** in the GC−MS of the *n*-hexane extracts from *B. megaterium* cells, these compounds are natural products (Figure 2C).

To determine the structures of **5** and **6**, products **4**−**6** were synthesized by the incubation of **3** (48 mg) with *E. coli* cell-free extracts containing overexpressed recombinant BmeTC. Compound **4** was also used as a substrate for further reaction with BmeTC to produce **5** and **6**, as described in the Supporting Information. *n*-Hexane extracts from the reaction mixture were purified by silica gel column chromatography and SiO_2 HPLC to yield pure **5** (5.8 mg) and **6** (2.8 mg). The proportions of the products **5** and **6** were estimated as 64% and 36%, respectively, by comparing their GC peak areas. The structures of **5** and **6** were determined by NMR (^{1}H, ^{13}C, DEPT, COSY, HOHAHA, NOESY, HSQC, and HMBC) and MS (ESI and EI). Although substrates **3** and **4** are C_{30} compounds having eight methyl groups, there were only 15 signals in the ^{13}C NMR spectrum of **5** and only four methyl signals in its ^{1}H NMR spectrum. The molecular formula of **5** was determined to be $C_{30}H_{52}O$ on the basis of HR-ESI-MS. These results indicated that compound **5** has two symmetric C_{15} structures. As shown in Scheme 2, the structure of **5** was determined to be a 6/6/7/6/6-fused pentacycle possessing a heterocyclic 7-membered C-ring. In contrast, the number of ^{13}C-signals was 30 for **6**, suggesting that compound **6** was structurally asymmetric. As shown in Scheme 2, **6** was determined to be a 6/6 + 6/6-tetracyclic compound having an exomethylene group at the C8−C26 position. Compound **5** was an onoceranoxide, which was previously isolated from a fern,[1a] and compound **6** was a novel triterpene, 14*β*-hydroxyonocera-8(26)-ene. This was the first discovery of an onoceroid of bacterial origin.

The structures of **5** and **6** suggested that the BmeTC-catalyzed reaction of substrate **4** was initiated by the protonation of a terminal isopropylidene moiety, triggering a series of cyclization events (Scheme 2). The polycyclizations of **4** into **5** and **6** are terminated by the nucleophilic addition of a hydroxyl group (path *a*) or the deprotonation of H-26 (path *b*) at the resulting C-8 carbocation, respectively (Scheme 2). These results suggest that onoceroids are synthesized from **3** via an intermediate cyclized from one terminus, such as **4**, by cyclization at both termini. We speculate that BmeTC may have single active site: BmeTC starts with the initial cascade leading to the bicyclic intermediate **4** from **3**, then turns molecule **4** outside of the active site cavity, and again uptake **4** to catalyze the formation of additional bicyclic and tricyclic structures of **5** and **6** on the other side of **4**. BmeTC is not only a bifunctional terpene cyclase which converts **1** and **3** into **2** and **4** but also an onoceroid synthase that catalyzes the reaction (**4** → **5** + **6**). This is the first identification of an onoceroid synthase.

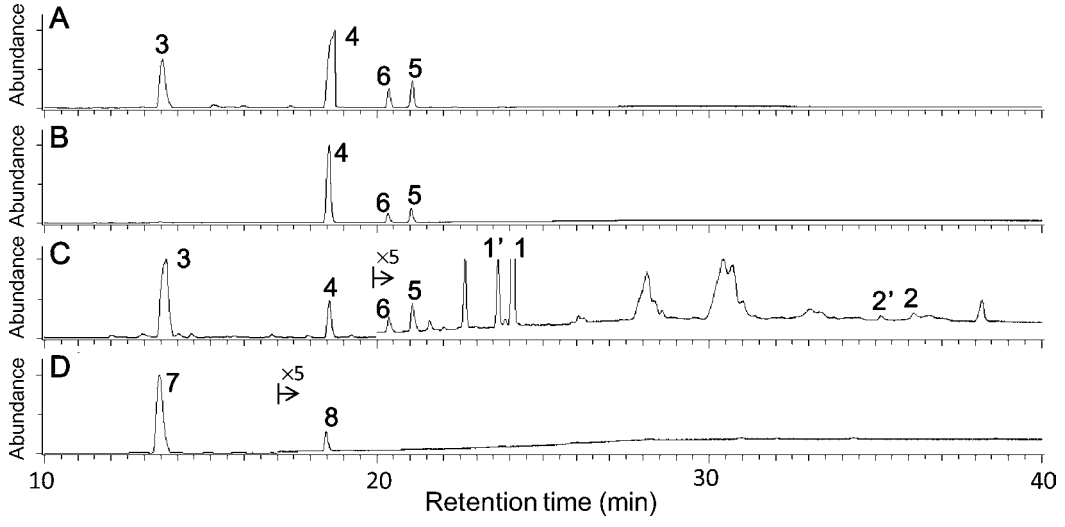


Figure 2. GC−MS total ion chromatograms used to analyze the enzymatic products of BmeTC with substrates **3** (A), **4** (B), and **7** (D) and the *n*-hexane extract from *B. megaterium* cells (C). Compounds **1**′ and **2**′, tetraprenyl-*α*-curcumene and baciterpenol B, are produced by the autoxidation of **1** and **2**, respectively, during GC−MS measurement.[8]

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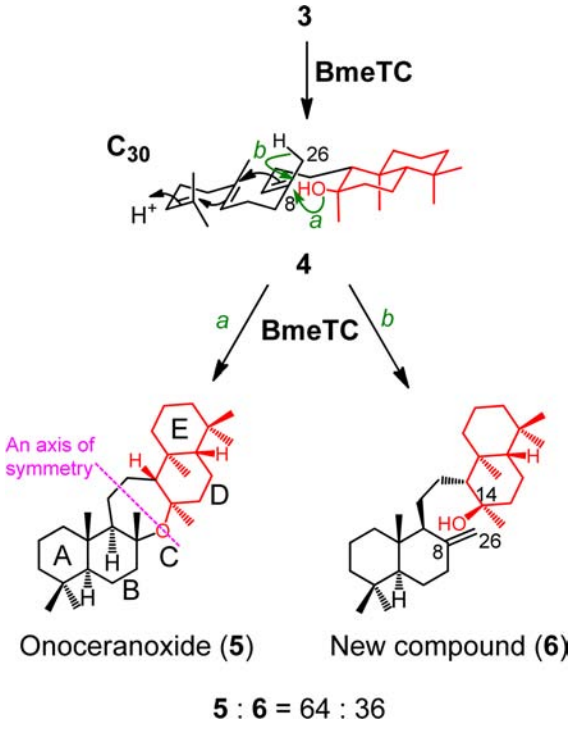
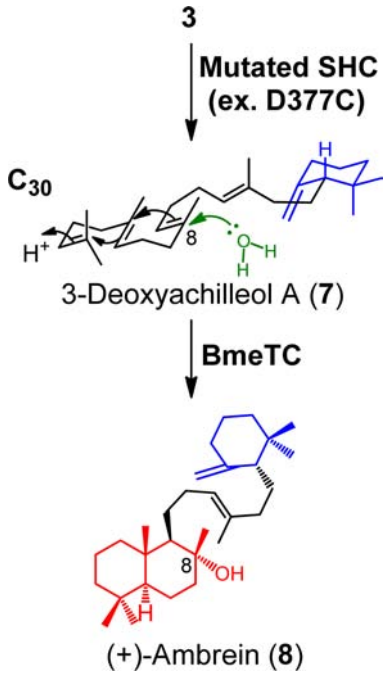
Scheme 2. Cyclization of Bicyclic Triterpene (4) into Onoceroids 5 and 6 Catalyzed by BmeTC[a]



[a]The numbering of C-8 and C-26 in **4** is that of a bicyclic intermediate. The new compound **6** should be named 14*β*-hydroxyonocera-8(26)-ene.

Scheme 3. Cyclization of 3-Deoxyachilleol A (7), Which was Produced by the Incubation of 3 with Mutated SHC, into (+)-Ambrein (8) Catalyzed by BmeTC[a]



[a]The numbering of C-8 in **7** is that of a bicyclic intermediate.

BmeTC can form not only the symmetric structure (**5**) but also the asymmetric one (**6**), indicating that this enzyme creates two different ring systems from the same linear substructure of substrate **3**. Other asymmetric onoceroids found in nature may also be biosynthesized by a single enzyme. On the other hand, the methyl group orientations of the D- and E-rings in compounds **5** and **6** are directly opposite from those of the head-to-tail 6/6/6/6/6-fused pentacyclic triterpenes identified in our previous study.[12] This promiscuous product formation and substrate acceptance suggest that the active site cavity of BmeTC around the D- and E-rings of the product is flexible and/or not compact. In addition, we previously reported that *B. subtilis* TC (BsuTC) synthesized compound **4** from **3** and did not convert **4** into any products. Mutations of BmeTC targeted at the amino acids different from those of BsuTC, which has 50% identity with BmeTC, and the 3-D structure of BmeTC would help elucidate the uptake mechanism of the intermediate cyclized at one terminus.

An unnatural triterpene (**7**) shares the same monocyclic substructure at one terminus with **8** (Scheme 3). Compound **7** can be synthesized from **3** using mutated SHCs from *Alicyclobacillus acidocaldarius*.[6,13] Compound **7** (42.2 mg) was obtained from **3** (50 mg) incubated with *E. coli* cell-free extracts containing overexpressed recombinant D377C SHC from 6 L culture.[13a] In order to confirm whether BmeTC produces **8** from **7**, we incubated **7** with BmeTC. As shown in Figure 2D, one product peak (**8**) was detected by GC–MS.

To determine the structure of **8**, enzymatically synthesized **7** (35 mg) was incubated with *E. coli* cell-free extracts containing overexpressed recombinant BmeTC. The synthesized **8** (1.4 mg) was isolated by silica gel column chromatography and SiO_2 HPLC. The structure of **8** was confirmed as (+)-ambrein by NMR (^{1}H, ^{13}C, DEPT, COSY, HOHAHA, NOESY, HSQC, and HMBC), MS (ESI and EI), and specific rotation analysis. The developed enzymatic synthesis of **8** from **3** required only two enzymes, although the reported total synthesis required 19–35 steps.[11] The calculated yield of **8** (3.4%) was comparable with that by total synthesis (1.3–3.8%)[11] and could possibly be improved by mutations of BmeTC.

In conclusion, BmeTC, which was originally identified as a sesquarterpene cyclase that constructed the 6/6/6/6-tetracyclic scaffold (**2**) from the head-to-tail monocyclic **1**, also formed the 6/6/7/6/6-pentacyclic **5** and the 6/6 + 6/6-tetracyclic **6** from the tail-to-tail acyclic triterpene **3** via the 6/6-bicyclic intermediate **4** in *B. megaterium* cells (Scheme 2). The elucidation of the multifunctional reaction mechanism for the construction of a variety of polycyclic skeletons by BmeTC from head-to-tail and tail-to-tail substrates will be an attractive project in the future. We also identified BmeTC as the first onoceroid synthase and demonstrated that symmetric and asymmetric onoceroids could be biosynthesized by a single enzyme via an intermediate cyclized at one terminus of **3**. This was also the first report of an onoceroid from bacterial origin. In addition, (+)-ambrein **8**, which is difficult to obtain from nature, could be synthesized from **3** by the combination of mutated SHC and BmeTC. The use of other abnormal products (e.g., mono-, bi-, and tricycles) formed by mutated SHCs[6] as substrates of BmeTC, and mutations of BmeTC targeted at the amino acid residues around the active site cavity and its entrance, will facilitate high-yielding syntheses of a greater number of natural and unnatural onoceroids in the future.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental details and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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