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Analysis of the Catalytic Mechanism of Bifunctional Triterpene/Sesquarterpene Cyclase: Tyr167 Functions To Terminate Cyclization of Squalene at the Bicyclic Step

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Onoceroids are a group of triterpenes biosynthesized from squalene or dioxidosqualene by cyclization from both termini. We previously identified a bifunctional triterpene/sesquarterpene cyclase (TC) that constructs a tetracyclic scaffold from tetraprenyl-β-curcumene (C_{35}) but a bicyclic scaffold from squalene (C_{30}) in the first reaction. TC also accepts the bicyclic intermediate as a substrate and generates tetracyclic and pentacyclic onoceroids in the second reaction. In this study, we analyzed the catalytic mechanism of an onoceroid synthase by using mutated enzymes. TC^{Y167A} produced an unnatural tricyclic triterpenol, but TC^{Y167L}, TC^{Y167F}, and TC^{Y167W} formed small quantities of tricyclic compounds, which suggested that the bulk size at Y167 contributed to termination of the cyclization of squalene at the bicyclic step. Our findings provide insight into the unique catalytic mechanism of TC, which triggers different cyclization modes depending on the substrate. These findings may facilitate the large-scale production of an onoceroid for which natural sources are limited.

Onoceroids are a group of triterpenes that are biosynthesized from squalene (**3**) or (3*S*,22*S*)-2,3,22,23-dioxidosqualene by cyclization from both termini.[1] They include compounds with onocerane, serratane, ambrane, and colysane skeletons.[1] Onoceroids exhibit various biological activities.[1] In particular, ambrein (**8**), a chief source of volatile compounds in ambergris, is a metabolic product of the sperm whale that accumulates as concretions in the gut, and it is one of the most valuable scents in fine perfumery.[1–3] Oxidative degradation of **8** around its central double bond produces volatile compounds.[1–3] Furthermore, ambergris has been used as a traditional medicine to cure migraine headaches, common colds, constipation, and rheumatism and as an aphrodisiac.[1–3] However, the bioactivity of **8** is unclear. Sperm whales are currently a protected species under the Convention on International Trade in Endangered Species of Wild Fauna and Flora, and the supply of ambergris from nature is highly limited.[1–3]

In addition to ferns, higher plants, and animals, we previously identified the first bacterial onoceroid and onoceroid synthase, a bifunctional triterpene/sesquarterpene cyclase (TC), in *Bacillus megaterium*.[4,5] TC constructs a tetracyclic scaffold from tetraprenyl-β-curcumene (**1**; C_{35}) but a bicyclic scaffold from squalene (**3**; C_{30}) in the first reaction (Scheme 1).[4] A recent study revealed that TC could also accept bicyclic intermediate **4** as a substrate, which was converted into tetracyclic and pentacyclic onoceroids **5** and **6** in the second reaction (Scheme 1).[1] In addition, successful enzymatic synthesis of **8** was achieved by using mutated squalene-hopene cyclase (SHC) and TC (Scheme 1).[1] However, the yield of **8** was too low for industrial applications and for detailed analyses of its bioactivity. In this study, we analyzed the catalytic mechanism of onoceroid synthase by using mutated enzymes. This analysis provides insight into the unique catalytic mechanism of TC, which catalyzes different cyclization modes toward substrates **1** and **3**. Elucidation of the catalytic mechanism of TC may enable significant increases in the production of **8**.

SHC has 30% identity with TC (Figure S1 in the Supporting Information) and catalyzes consecutive pentacyclization to form hopene (**9**; Scheme 2A).[4,6] The catalytic mechanism of SHC was previously investigated extensively by X-ray crystal structure analyses and site-directed mutagenesis.[6] In this study, we performed site-directed mutagenesis of TC targeting Y167, which corresponds to the W169 position near the *pre*-C ring of **3** in SHC (Scheme 2A, Figures S1 and S2). It has been proposed that W169 may function as a binding site of **3**.[6] We analyzed TC^{Y167A} products generated from **3** and **4** (Figure 1 and Table 1). TC^{Y167A} produced unknown compound **10** with

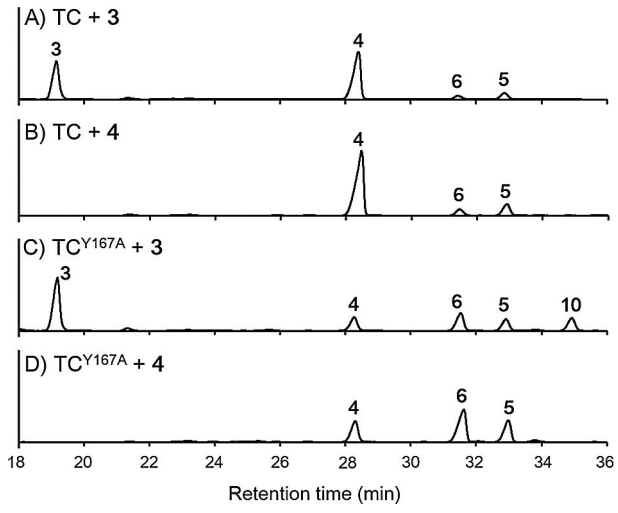

Figure 1. GC analysis of products formed from substrates **3** and **4** by enzymes TC and TC^{Y167A}.

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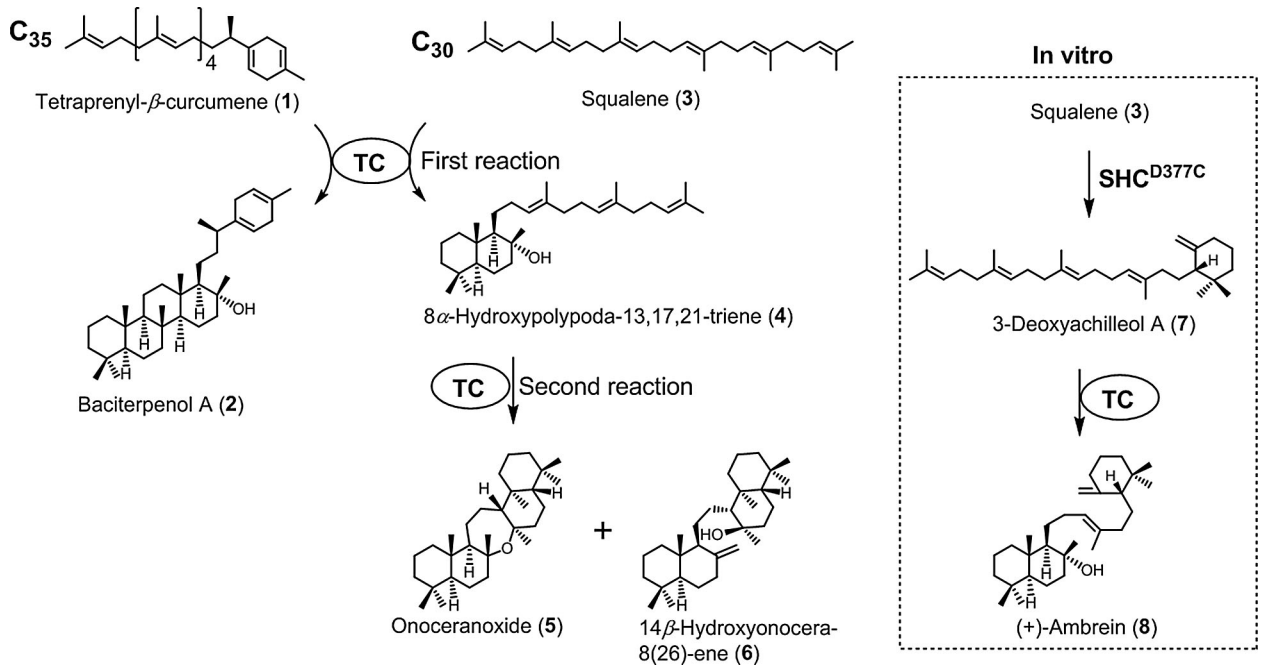
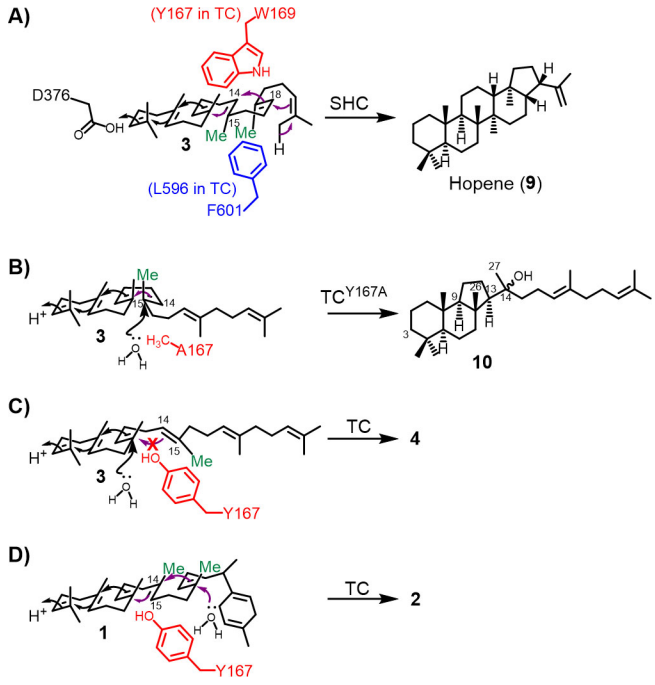


Scheme 1. Cyclization of **1** and **3** catalyzed by TC, and enzymatic synthesis of **8** by using SHC^{D377C} and TC.



Scheme 2. Proposed catalytic mechanisms of SHC, TC, and TC^{Y167A} towards substrate **1** or **3**.

Table 1. Products formed by the incubation of **3** with wild-type and mutated TCs.

Enzyme	Ratio [%]			
	4	**5**	**6**	**10**
wild-type	85.8	9.5	4.7	n.d.[a]
Y167A	24.2	20.2	32.1	23.6
Y167L	80.3	9.6	8.1	2.0
Y167F	57.2	29.6	12.4	0.8
Y167W	88.0	7.9	2.5	1.6
Y167A/L596F	84.5	n.d.[a]	n.d.[a]	15.5

[a] n.d.: not detected.

normal products from **3** but not from **4** (Figure 1 and Table 1), which indicated that **10** was directly synthesized from **3** in the first reaction. Compound **10** was isolated from the mixture, and its structure was determined by MS and NMR spectroscopy. Compound **10** has three vinylic methyl groups (δ_H = 1.70–1.83 ppm), which suggests that two isoprene units might remain unreacted. Compound **10** has a tertiary alcoholic carbon atom (δ_C = 74.5 ppm, s) at the C-14 position, as evidenced by the apparent HMBC cross peaks of Me-27 (δ_H = 1.37 ppm, s, 3H) and H-13 (δ_H = 1.42 ppm, br t, J = 10.0 Hz) for the alcoholic carbon atom. A strong NOE (H-9–H-13 and Me-26–Me-27) clearly supports the α orientation of H-13. Detailed analysis by 2D NMR spectroscopy revealed that **10** is a tricyclic triterpenol (Scheme 2B and Figure S7). The 3β-hydroxylated derivative of **10** is an arabidiol, which was produced by an oxidosqualene cyclase (At4g15340) cloned from the *Arabidopsis thaliana* genome and by mutated SHC ($SHC^{\Delta G600}$).[7,8] However, **10** has never been found in nature and, thus, is a novel unnatural triterpene, 3-deoxyarabidiol.

TC^{Y167A} produced tricyclic **10**. Thus, Y167 would function to terminate the cyclization of **3** at the bicyclic step. Given that TC^{Y167L}, TC^{Y167F}, and TC^{Y167W} formed only small amounts of **10** (Table 1), the bulk size at the Y167 position is expected to be functionally important. Wild-type and mutant TCs all produced only normal pentacyclic product **2** from **1** (Figure S3). The position of the methyl residue of the *pre*-C ring in **3** (C-15) is

different from that in **1** (C-14; Scheme 2C and D). We propose that Y167 may interact with the methyl residue at C-15 of **3** to prevent cyclization of the C ring (Scheme 2C) and may not interact with the methyl residue at C-14 of **1** (Scheme 2D). The *pre*-C ring of **3** to form **10** is boat form (Scheme 2B), which suggests that A167 of TC^{Y167A} interacts with the methyl residue at C-15 to prevent the formation of the stable chair form in cyclization of the C ring. This also supports our hypothesis.

As W169 is located at the opposite side of the methyl residue of C-15 of **3** in SHC (Scheme 2A and Figure S2), it is not expected to prevent the cyclization of **3** at the bicyclic step. In the modeled structure, Y167 is positioned near W169 and at the opposite side toward the methyl residue of C-15 (Figure S2). However, the present study proposes that the actual position of Y167 may be near the methyl residue of C-15. TC^{Y167W}, which is similar to wild-type SHC, stopped cyclization at the bicyclic step in the first reaction (Table 1), which supports the hypothesis that the location of Y167 in TC is different from the location of W169 in SHC in the active-site cavity.

Whereas active-site residues of SHC are highly conserved in TC, F601 of SHC is not conserved and corresponds to L596 in TC (Scheme 2A, Figures S1 and S2), which is a characteristic of TC according to Bosak et al. and our previous work.[4,9] Given that F601 may function to stabilize the secondary carbocation (C-14 and C-18) of tricyclic and tetracyclic intermediates through cation–π interactions in SHC (Scheme 2A),[6] it is possible that the lack of π electrons at position 596 might stop cyclization at the tricyclic step in TC^{Y167A}. According to a previous study, SHC^{F601A} produces tricyclic products,[10] similar to TC^{Y167A}. Thus, we prepared $TC^{Y167A/L596F}$, which is similar to wild-type SHC, and analyzed products formed by incubation with **3**. However, no new product possessing a tetra- or pentacyclic skeleton, except for **10**, was detected (Table 1), which indicated that F596 could not stabilize the secondary carbocation (C-14) in the mutant. Whereas the position of L596 was almost identical to that of F601 in the modeled structure of TC (Figure S2), the actual location of L596 may be different from that in the model.

Recently, new onoceroid synthases were identified in ferns.[11,12] Ferns utilize two enzymes for onoceroid biosynthesis.[11,12] One fern onoceroid synthase, pre-α-onocerin synthase (LCC), catalyzes the formation of a bicyclic compound. Araki et al. pointed out that its sequence (^{254}MNIHG^{258}) was notably different from that of lanosterol synthase. H257 of LCC corresponded to Y167 of TC (Figure S4). Although Araki et al. speculated that the sequence was responsible for recognizing the terminal epoxide of the substrate,[11] H257 of LCC might also function to terminate cyclization at the bicyclic step, similar to Y167 of TC.

In conclusion, this is the first analysis of the catalytic mechanism of an onoceroid synthase that makes use of mutated enzymes. The bulk size at the Y167 position was found to be important for termination of the cyclization of **3** at the bicyclic step. The active-site structure of TC would be slightly different from that of SHC. Analysis of the 3D structure of TC is necessary to elucidate the catalytic mechanism of TC and will facilitate the efficient biosynthesis of bioactive onoceroids, such as **8**.

Experimental Section

General procedure and materials: NMR spectra were recorded by using a Bruker DPX 400 spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C). GC–MS was performed with 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, Inc., Folsom, CA, USA) in the EI mode operated at 70 eV. HRMS was performed by using a JMS-T100LP spectrometer (JEOL) in ESI mode. GC analyses were performed by using a Shimadzu GC-2014 chromatograph equipped with a flame-ionization detector and a DB-1 capillary column (30 m × 0.25 mm × 0.25 μm; J&W Scientific, Inc.). Compound **1** was previously isolated from *B. subtilis*.[5a] Compound **3** was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Site-directed mutagenesis: The QuikChange Site-directed Mutagenesis Kit (Stratagene) was used to introduce the desired mutations (Y167A) by following the manufacturer's instructions by using pairs of mutagenic primers (Y167A fwd: 5′-GAATT AAGCA CTGCT GCCAG AATTC ACTTC GTTCC GATG-3′, Y167A rev: 5′-CATCG GAACG AAGTG AATTC TGGCA GCAGT GCTTA ATTC-3′). Y167F, Y167L, Y167W, and Y167A/L596F were synthesized by GENEWIZ, Inc. (Suzhou, China).

Enzymatic assay for wild-type and mutant TCs: *Escherichia coli* BL21(DE3) harboring pColdTF-BmeTC[1] was grown at 37 °C in lysogeny broth (LB; 1 L) with ampicillin (100 μg mL^{-1}). Expression of the recombinant protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM) once the OD_{600} reached approximately 0.6. Further cultivation of BL21(DE3) recombinants was performed for 12 h at 15 °C. *E. coli* cells expressing recombinant TC were harvested by centrifugation and were resuspended in buffer A (30 mL) containing Tris·HCl (50 mM, pH 7.5), dithiothreitol (2.5 mM), EDTA (1 mM), and 0.1% Triton X-100. The cells were disrupted by sonication with UP200s (Hielscher Ultrasonics GmbH, Teltow, Germany) at 4–10 °C for 15 min. The resulting suspension was centrifuged at 10000 *g* for 20 min. The pellet was discarded, and the resulting supernatant was used as a cell-free extract. The mixture for analyzing the enzymatic activity of TC contained substrate (**1**: 0.05 mg and **3**: 0.1 mg) emulsified with Triton X-100 (2 mg) in buffer A (1 mL) and cell-free extract (4 mL) containing TC in a total volume of 5 mL. The reaction was performed at 30 °C for 64 h and was terminated by using a 15% KOH/MeOH solution (6 mL). The lipophilic enzymatic product was extracted from the incubation mixtures with *n*-hexane (3 × 5 mL). Triton X-100 detergent was removed by passing the extract through a short SiO_2 column (*n*-hexane/EtOAc = 100:20) and then subjecting the eluent to GC and GC–MS. GC conditions for the products from substrate **3** were as follows: injection temperature = 300 °C, column temperature = 220–280 °C (1 °C min^{-1}), flow rate (He gas) = 1.30 mL min^{-1}. GC conditions for the products from substrate **1** were as follows: injection temperature = 300 °C, column temperature = 220–300 °C (3 °C min^{-1}), flow rate (He gas) = 1.30 mL min^{-1}. Enzyme assays for mutated TCs were performed following the same methods as those used for TC.

Isolation of product 10 synthesized from 3 by TC^{Y167A}: The cell-free extracts (720 mL) were prepared in a manner similar to that described above from *E. coli* BL21(DE3) harboring pColdTF-TC^{Y167A} cultured in LB (24 L). To isolate product **10** formed by TC^{Y167A}, compound **3** (45 mg) was emulsified with Triton X-100 (900 mg) in buffer A containing Tris·HCl (50 mM, pH 7.5), dithiothreitol (2.5 mM), EDTA (1 mM), and 0.1% Triton X-100 and was then incubated with the cell-free extracts (720 mL) at 30 °C for 64 h. After 15% KOH/MeOH solution (1.08 L) was added to the mixture, the lipophilic

products were extracted with *n*-hexane (3×1.98 L) and concentrated. The *n*-hexane extract (63.0 mg) was partially purified by silica gel (6.3 g) column chromatography (*n*-hexane and *n*-hexane/EtOAc = 100:20). The fraction (15.3 mg) eluted with *n*-hexane/EtOAc (100:20) containing product **10** was subjected to SiO_2 HPLC (Inertsil PREP-SIL, 6.0×250 mm; GL Sciences, Tokyo, Japan) with *n*-hexane/THF (100:2), and pure **10** (oil; 2.2 mg; t_R = 48.1 min) was obtained.

Structural analyses of 10: The structure of **10** was determined by MS (Figure S5) and NMR spectroscopy (Figures S6–S13). $[\alpha]_D^{25} = -0.03$ (c = 0.22 in EtOH); MS (EI): *m/z* (%): 69 (100), 81 (51), 95 (48), 109 (49), 123 (44), 137 (27), 149 (19), 163 (15), 177 (20), 191 (65), 204 (13), 217 (10), 231 (85), 259 (8), 273 (5), 341 (6), 395 (4), 410 [M^+−OH, 5]; HRMS (ESI): *m/z*: calcd for $C_{30}H_{52}NaO$ (**10**): 451.39158 [M+Na]$^+$, found: 451.39153.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biosynthesis · cyclization · enzymes · onoceroids · terpenoids

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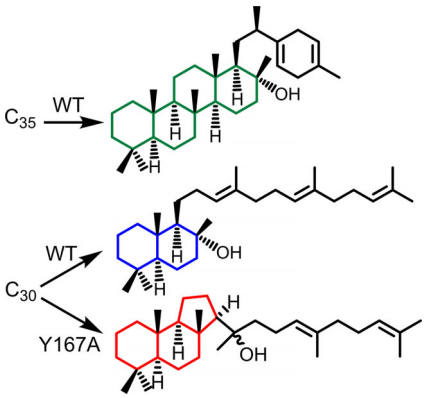
COMMUNICATIONS

Size matters: The catalytic mechanism of onoceroid synthase (bifunctional C_{30}/C_{35} terpene cyclase) is analyzed for the first time by using mutant enzymes. The mutant enzyme Y167A produces tricyclic triterpene from C_{30} squalene. The bulk size at the Y167 position is significant for termination of the cyclization of squalene at the bicyclic step.



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