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An Unusual Terpene Cyclization Mechanism Involving a Carbon–Carbon Bond Rearrangement**

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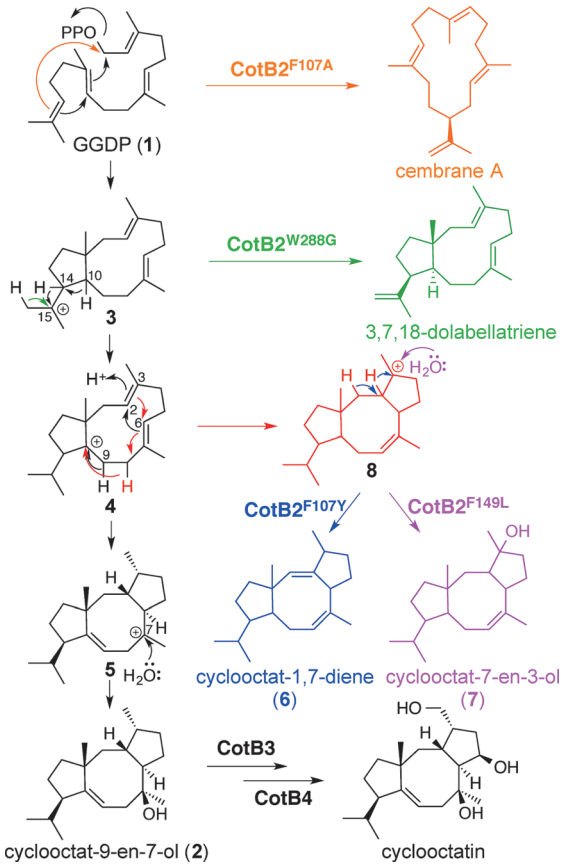
In memory of Haruo Seto

Abstract: *Terpene cyclization reactions are fascinating owing to the precise control of connectivity and stereochemistry during the catalytic process. Cyclooctat-9-en-7-ol synthase (CotB2) synthesizes an unusual 5-8-5 fused-ring structure with six chiral centers from the universal diterpene precursor, the achiral C_{20} geranylgeranyl diphosphate substrate. An unusual new mechanism for the exquisite CotB2-catalyzed cyclization that involves a carbon–carbon backbone rearrangement and three long-range hydride shifts is proposed, based on a powerful combination of in vivo studies using uniformly ^{13}C-labeled glucose and in vitro reactions of regiospecifically deuterium-substituted geranylgeranyl diphosphate substrates. This study shows that CotB2 elegantly demonstrates the synthetic virtuosity and stereochemical control that evolution has conferred on terpene synthases.*

Cyclooctatin, a diterpene with a unique tricyclic diterpene skeleton characterized by a 5-8-5 fused-ring system, is a potent inhibitor of lysophospholipase, which catalyzes the hydrolysis of the fatty acid ester bonds of lysophospholipids.[1] This inhibitor was originally isolated from the culture broth of *Streptomyces melanosporofaciens* MI614-43F2 while screening for lead compounds for the development of anti-inflammatory drugs that target lysophospholipase.[1] Recently, we revealed that the minimal cyclooctatin biosynthetic genes encode geranylgeranyl diphosphate (GGDP) synthase (CotB1), a diterpene cyclase (CotB2), and two cytochrome P450 hydroxylases (CotB3 and CotB4).[2] CotB2 catalyzes the cyclization of GGDP to produce cyclooctat-9-en-7-ol, the parent tricyclic diterpene alcohol intermediate in cyclooctatin biosynthesis (Scheme 1).

CotB2 has the unique ability to synthesize the characteristic 5-8-5 fused-ring system of cyclooctat-9-en-7-ol having six chiral centers by cyclization of the universal diterpene precursor, the achiral C_{20} allylic diphosphate GGDP.[2] The acyclic substrate GGDP is well known to undergo cyclization



Scheme 1. Hypothetical mechanism originally proposed for the formation of cyclooctat-9-en-7-ol (**2**) from GGDP (**1**) catalyzed by CotB2.[2,4,5] OPP = pyrophosphate.

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in many different ways to yield a large variety of structurally diverse diterpenes with a wide spectrum of biological activities that have been isolated from a broad range of natural sources.[3] As with all terpene cyclizations, the ability of CotB2 to synthesize only a single product with the correct C–C bond connectivity and stereochemistry requires the diterpene synthase to be able to precisely control the conformation of the substrate, GGDP, and of all reactive intermediates held in the active-site pocket. Given the complexity of its characteristic product, CotB2 is clearly a highly refined biocatalyst. Understanding the biochemical reaction mechanism could lead to the development of novel biocatalysts useful for the production of a diverse range of complex compounds.

We have previously proposed a plausible mechanism for the CotB2-catalyzed cyclization reaction (Scheme 1).[2] According to this proposal, initial ionization of GGDP (**1**) is followed by a successive pair of intramolecular electrophilic reactions to give bicyclic carbocation intermediate **3**. Next, **3** is converted into carbocation intermediate **4** by two consecutive 1,2-hydride shifts, from the C10 to the C14 and from the C14 to the C15 carbon atoms. Deprotonation of **4** at the C9 position to give a neutral olefinic intermediate is then followed by reprotonation at the C3 position leading to bond formation between the C6 and C2 carbon atoms with generation of the tricyclic carbocation intermediate **5**. Finally, **5** would be quenched by water as the nucleophile to yield the characteristic tricyclic CotB2 reaction product, cyclooctat-9-en-7-ol (**2**). Consistent with this proposed mechanism, two aberrant products, cyclooctat-1,7-diene (**6**) and cyclooctat-7-en-3-ol (**7**), have been shown to be formed by the action of the CotB2 mutant enzymes CotB2^{F107Y} and CotB2^{F149L}, respectively.[4] The additional derailment products (*R*)-cembrane A and (1*R*,3*E*,7*E*,11*S*,12*S*)-3,7,18-dolabellatriene have been shown to be synthesized by the CotB2^{F107A} and CotB2^{W288G} mutants, respectively.[5] To date, however, no direct experimental evidence has been provided to validate the proposed mechanism for the CotB2-catalyzed cyclization reaction. We now propose a new mechanism involving an unusual carbon backbone rearrangement and a series of long-range hydride shifts that account for the CotB2-catalyzed cyclization of GGDP to the unusual tricyclic fused-ring system of cyclooctat-9-en-7-ol, by combining results of in vivo feeding experiments and in vitro enzyme incubations of specifically labeled substrates.

As a first step in investigating the mechanism of the enzymatic formation of **2**, we determined the absolute configuration of the closely related co-metabolite 16-hydroxycyclooctatin (Supporting Information, Table S1 and Figures S1–S3), which was isolated from the broth of cyclooctatin-producing *S. melanosporofaciens* MI614-43F2. Using a modified version of the Mosher method (Figures S4–S10),[6] the absolute configuration of **2** was established as 2*R*,3*R*,6*R*,7*S*,11*R*,14*R*.

To investigate the assembly of the carbon skeleton catalyzed by CotB2 during cyclooctatin biosynthesis, we administered uniformly ^{13}C-labeled glucose ([U-$^{13}C_6$]glucose) to the culture broth of a cyclooctatin-producing *Streptomyces albus* transformant. The resulting ^{13}C enrichments and the ^{13}C–^{13}C coupling patterns in the derived cyclooctatin were determined by a combination of one- and two-dimensional ^{13}C NMR spectroscopy. *S. albus* exclusively uses the methylerythritol phosphate (MEP) pathway to form the fundamental five-carbon isoprenoid precursor units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which serve as the precursors of **1** (Figure S11).[7] The ^{13}C labeling pattern of **1** resulting from the incorporation of [U-$^{13}C_6$]glucose is thus well understood and can therefore be used to infer the expected labeling pattern in the derived cyclooctatin (Figure 1 a, left).[8]

For the efficient heterologous production of cyclooctatin, we constructed the plasmid pSE101-cot-lac by sub-cloning

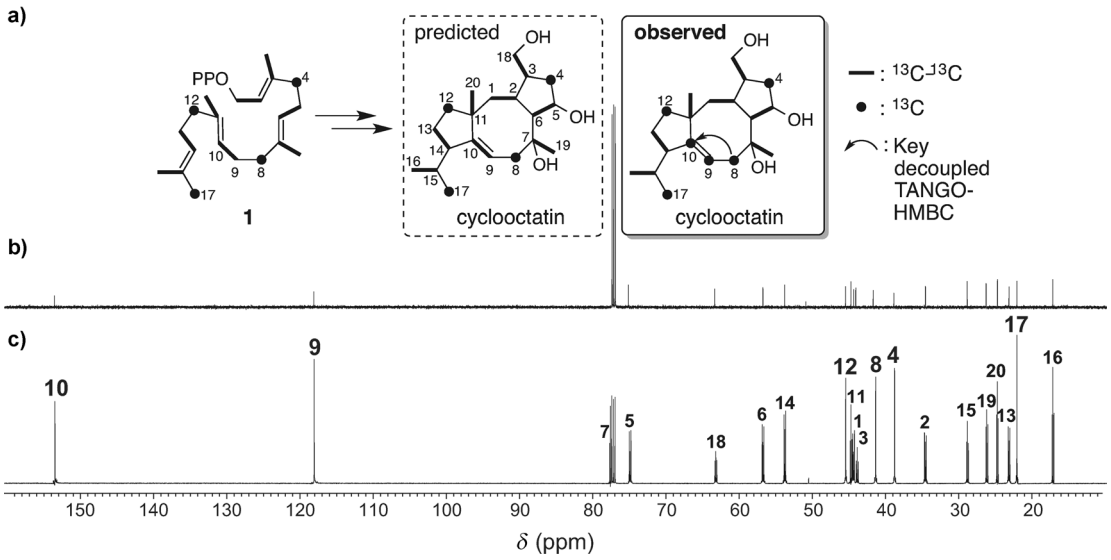
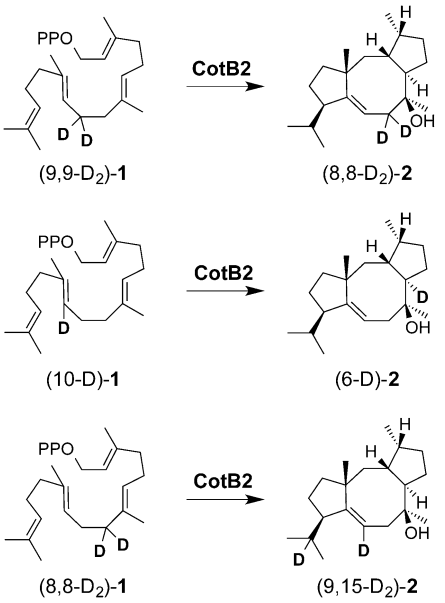


Figure 1. Isotope tracing experiments for the biosynthesis of cyclooctatin. a) Predicted (left) and observed (right) labeling patterns for cyclooctatin. b) ^{13}C NMR spectrum of natural-abundance cyclooctatin. c) ^{13}C NMR spectrum of cyclooctatin obtained in the tracer experiment with [U-$^{13}C_6$]glucose.

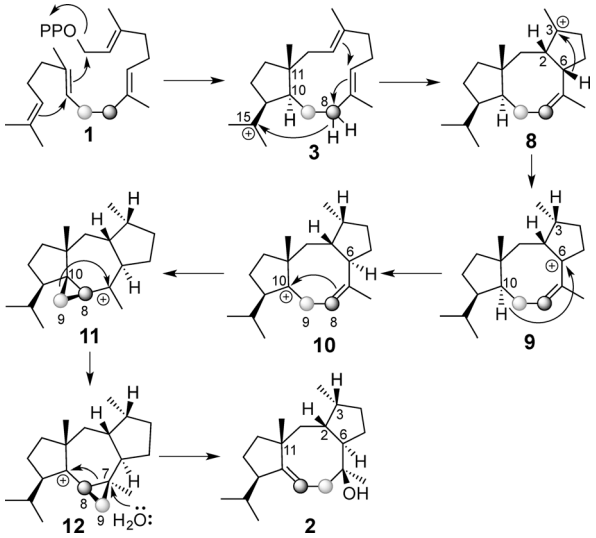
DNA fragments containing the *cotB* genes, which were obtained from pCOT104,[2] downstream of the *lac* promoter of the *Streptomyces–E. coli* shuttle vector pSE101.[9] This was done because gene expression is more effective with the *lac* promoter than with the *ori* promoter.[10] An *S. albus* transformant harboring the plasmid pSE101-cot-lac (*S. albus*/pSE101-cot-lac) was cultured in the presence of [U-$^{13}C_6$]glucose. The resulting cyclooctatin was purified and analyzed by ^{13}C NMR spectroscopy (Figure 1c; see also Table S2 and Figure S12). The ^{13}C NMR spectrum revealed efficient ^{13}C incorporation into all twenty carbon positions of the cyclooctatin molecule, as established by comparison with the ^{13}C NMR spectrum of natural-abundance cyclooctatin (Figure 1b, Figure S13). Unexpectedly, although seven of the predicted C–C couplings, C1–C2, C3–C18, C5–C6, C7–C19, C11–C20, C13–C14, and C15–C16, were clearly observed, the expected C9–C10 coupling that would be observable if the cyclization mechanism of Scheme 1 was correct was absent (Figure 1a, right). Furthermore, a decoupled TANGO (testing for adjacent nuclei with a gyration operator) HMBC spectrum,[11] which is a useful NMR technique for the detection of carbon atoms that are derived from the same precursor molecule and that also permits the virtual observation of 1,3 long-range ^{13}C–^{13}C couplings that are normally too small ($J_{C1–C3}$ = 3–10 Hz) to be observable by ^{13}C NMR spectroscopy, revealed that the C8 and C10 carbon atoms were derived from the same glucose molecule, whereas C9 and C10 were not (Figure S14). The observed ^{13}C labeling pattern in cyclooctatin strongly suggests that a carbon–carbon bond rearrangement must occur between the original C8 and C9 carbon atoms of **1** during the CotB2-catalyzed conversion into **2** (Figure S15).

To study the mechanism of the CotB2-catalyzed cyclization of **1** in more detail, we next carried out individual incubations of recombinant CotB2 with three regiospecifically deuterated samples of GGDP, (9,9-D_2)-**1**, (10-D)-**1**, and (8,8-D_2)-**1**, which were synthesized from (9,9-D_2)-, (10-D)-, and (8,8-D_2)-geranylgeraniol, respectively, as previously described.[12,13] Comparing the ^{1}H NMR spectra of the deuterated samples of cyclooctat-9-en-7-ol (**2**) generated in each reaction with that of natural-abundance **2** (Figures S16–S18) allowed for the assignment of the deuteration sites in each product, thereby establishing that (9,9-D_2)-**1**, (10-D)-**1**, and (8,8-D_2)-**1** were converted into (8,8-D_2)-**2**, (6-D)-**2**, and (9,15-D_2)-**2**, respectively (Scheme 2). The formation of (8,8-D_2)-**2** from (9,9-D_2)-**1** is fully consistent with a carbon–carbon bond rearrangement between the C8 and C9 carbon atoms during the CotB2 reaction cascade, as suggested by the results of the feeding experiment with [U-$^{13}C_6$]glucose (Figure S15). The formation of (6-D)-**2** from (10-D)-**1** also indicates that a 1,5-hydride shift from the C10 to the C6 carbon atom occurs during the cyclization reaction cascade. Moreover, the observed conversion of (8,8-D_2)-**1** into (9,15-D_2)-**2** also supports both the C8/C9 carbon–carbon bond rearrangement and a 1,5-hydride shift from the C8 to the C15 carbon atom.

We now propose a new mechanism for the CotB2-catalyzed reaction cascade that fully accounts for the results of the isotopic labeling experiments (Scheme 3). The cyclization process begins with dissociation of the pyrophosphate



Scheme 2. Cyclization reaction of deuterium-substituted **1** catalyzed by CotB2.



Scheme 3. Proposed mechanism for the formation of **2** catalyzed by CotB2.

leaving group of **1** and formation of an allylic carbocation. Two successive electrophilic cyclizations will generate the bicyclic cationic intermediate **3**, which will then undergo a 1,5-hydride shift from the C8 to the C15 carbon atom coupled with electrophilic attack of the transiently generated allylic cation on the C2−C3 double bond to form the tricyclic 5-8-5 fused-ring structure **8** with a positive charge at the C3 position. A similar cyclization mechanism has previously been proposed for the biosynthesis of ophiobolin (Figure S19).[14,15] Next, cationic intermediate **8** likely undergoes a 1,3-hydride shift from the C6 to the C3 position followed by another 1,5-hydride shift from the C10 to the C6 carbon atom to generate homoallylic cation **10** via allylic cation **9**. Alternatively, intermediate **9** could be obtained by two successive non-

concerted 1,2-hydride shifts: A first hydride shift from the C2 to the C3 carbon atom yielding a cation at the C2 position followed by a second hydride shift from the C6 to the C2 carbon atom. Collapse of intermediate **10** to yield cyclopropylcarbinyl cation **11**, which features a C8/C9/C10 cyclopropyl ring, would be followed by the formation of isomeric cyclopropylcarbinyl cation **12**. A similar transformation has also been proposed for the biosynthesis of verrucosan-2β-ol (Figure S20).[16] Finally, cyclopropyl ring opening by cleavage of the C7–C8 bond with concomitant capture of water by the homoallylic cation results in reformation of the monounsaturated eight-membered ring to form **2**. During the conversion of **10** into the characteristic 5-8-5 fused-ring compound **2**, the C8 and C9 carbon atoms are interchanged. The proposed CotB2-catalyzed reaction cascade, which involves a 1,3-hydride shift, two 1,5-hydride shifts, and a cyclopropylcarbinyl cation rearrangement, accounts for all of the experimental results that were obtained in the present study.

The proposed cyclization mechanism makes it possible to infer the stereochemistry of each reactive intermediate as well as to rationalize the configuration of the final product **2** (Scheme 3). The β-configuration of both the isopropyl side chain and the C11 methyl group must be set in the formation of **3** and is then preserved in all derived intermediates (**8**→**2**). The α-configuration of the C3 methyl group in **2** is initially set by the suprafacial 1,3-hydride shift that generates intermediate **9** and is preserved in all subsequent intermediates, thereby requiring that the C6 hydrogen atom in **8** has the β-configuration. It is also evident from the β-configuration of the C11 methyl group in **3** and in all derived intermediates that the H10 hydrogen atom in intermediates **3**, **8**, and **9** must adopt α-configuration, given that the vicinal substituents are derived from the same *E*-configured double bond of GGDP, from which it is readily apparent that the suprafacial 1,5-hydride shift from the C10 to the C6 carbon atom necessarily sets the H6 α-configuration in **10** and all derived intermediates, including the final product **2**. Finally, the β-configuration of the H2 hydrogen atom in **8** and all derived intermediates is set by electrophilic attack of the transiently generated C6 cation on the *Re* face of the C2–C3 double bond of **3**. Given the close analogy between the formation of **8** and the mechanism of formation for ophiobolin F,[14] which is identical in stereochemistry to **8** at the C2, C6, C10, C11, and C14 positions (Figure S19), it is likely that the 1,5-hydride shift that generates the isopropyl side chain of **8** also involves the H-8α atom.

The postulated interconversion of cyclopropylcarbinyl cations has precedence in the reactions of the tricyclic C_{15} sesquiterpene thujopsene under mildly acidic conditions (Figure S21).[17] The latter abiological mechanism, however, lacks regioselectivity, thus resulting in the formation of a mixture of homoallylic alcohols. By contrast, the precise control of the regioselectivity in the CotB2-catalyzed reaction during the carbon–carbon bond rearrangement (**10**→**11**→**12**→**2**) results in the formation of only one homoallylic alcohol, namely **2**, with retention of the configuration at the C7 position (Scheme 3; see also Figure S21).

In conclusion, CotB2 has been shown to catalyze an unusually complex regiospecific and stereospecific cyclization that involves a unique carbon–carbon bond rearrangement and multiple hydride shifts, which all take place at a single active site. CotB2 thus elegantly demonstrates the synthetic virtuosity and stereochemical control that evolution has conferred on terpene synthases. Structural studies on CotB2 complexed with **1** or its analogues would provide further insight into the catalytic mechanisms and how CotB2 precisely controls connectivity and stereochemistry during catalysis. Improving our understanding of the biochemical reaction mechanisms of other diterpene cyclases would lead to the development of novel biocatalysts useful for the production of a diverse range of complex compounds.

Keywords: biosynthesis · enzyme catalysis · reaction mechanism · terpenes

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