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Migrated Hopene Synthases from *Colysis pothifolia* and Identification of a Migration Switch Controlling the Number of 1,2-Hydride and Methyl Shifts

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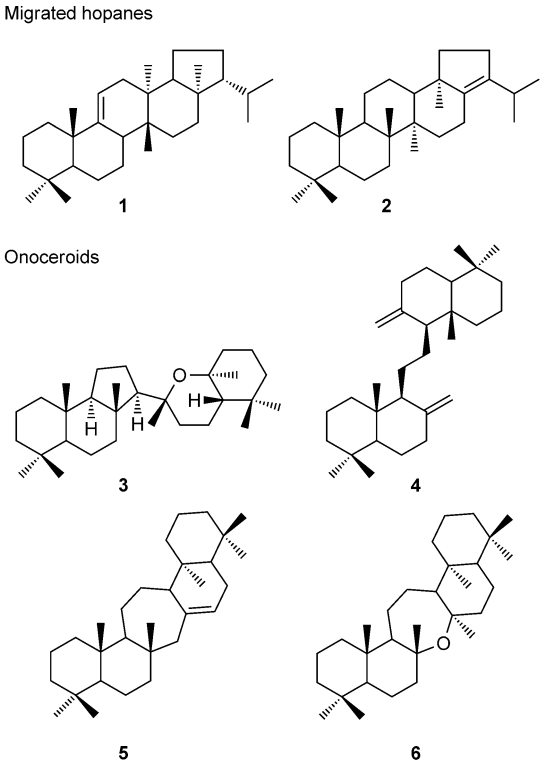
Ferns are known to produce triterpenes, derived from squalene, that are synthesized by squalene cyclases (SCs). Among these, *Colysis* species produce onoceroids, the bis-cyclic skeleton of which can be cyclized from both termini of squalene. To gain insight into the molecular basis of triterpene structural diversity, cDNA cloning of SCs from *C. elliptica* and *C. pothifolia* was performed; this leads to the isolation of five SC cDNAs. Functional analysis of these clones revealed their enzymatic products to be hop-22(29)-ene, α-polypodatetraene, and hop-17(21)-ene. One of these clones (*CPF*) is a transcribed pseudogene with a 22-nucleotide deletion causing a nonsense mutation. To predict the inherent function of CPF, we constructed an insertion mutant of *CPF* that successfully converted inert CPF to the active SC, the product of which is fern-9(11)-ene. Subsequent mutations identified active-site residues that control the number of 1,2-hydride and methyl shifts.

Introduction

Triterpenes are a large group of structurally diverse natural products. Cyclic triterpenes are constructed by the cyclization of acyclic precursors, leading to mono- to pentacyclic skeletons.[1] Cyclic carbocation intermediates can be rearranged by 1,2-hydride and methyl shifts, thereby expanding the structural diversity of the triterpene skeletons.[2] Ferns are known to produce triterpene hydrocarbons with structurally diverse skeletons (Scheme 1),[3] for example, the migrated hopane fern-9(11)-ene (**1**), which was initially isolated from *Dryopteris crassirhizoma*.[4] This triterpene hydrocarbon was the first example of a migrated hopane, and is one of the most widely distributed triterpenes in ferns. Another example of a migrated hopane is hop-17(21)-ene (**2**), which has a potent anti-tumor-promoting activity.[5]

The bis-cyclic triterpenes, also known as onoceroids, include ambrane, onocerane, serratane, and colysane skeletons; they can be biosynthesized from (oxido)squalene by cyclization from both termini.[2] Among these onoceroids, ferns produce all types of the triterpene skeletons, except ambrane, although the triterpenes' distribution in ferns is quite limited. Several *Colysis* species produce colysanoxide (**3**) and α-onoceradiene (**4**), whereas serratene (**5**) and onoceraneoxide (**6**)—and **4**—have been isolated from *Lemmaphyllum microphyllum* (Scheme 1).[6]

The enzymes responsible for construction of cyclic triterpene hydrocarbons are the squalene cyclases (SCs). The genes encoding SCs are widely distributed in bacteria, such as the well-studied squalene-hopene cyclases (SHCs) from *Alicyclobacillus acidocaldarius*[7] and *Zymomonas mobilis*.[8] Using a recombinant SHC from *A. acidocaldarius* has allowed us to gain insight into the structural and mechanistic features of this enzyme. The SHC from *Z. mobilis* has been shown to have a unique substrate specificity compared to that from *A. acidocaldarius*; the



Scheme 1. Representative migrated hopanes and onoceroids. Fern-9(11)-ene (**1**), hop-17(21)-ene (**2**), colysanoxide (**3**), α-onoceradiene (**4**), serratene (**5**), onoceraneoxide (**6**).

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Zymomonas SHC (ZMO1548) has been demonstrated to act as a general Brønsted acid biocatalyst with various substrates that contain a functionalized, shortened polyisoprene backbone. Ferns have also been shown to be a source of SCs with more variable catalytic properties than those derived from bacteria. The SCs from ferns characterized thus far produce 22-hydroxyhopane,[9] dammara-18(28),21-diene,[10] germanicene,[11] and tirucalla-7,21-diene,[11] whereas all the functionally characterized SCs from bacteria are SHCs, the product of which is hop-22(29)-ene (**7**).

No genetic information on the migration reaction responsible for skeletal rearrangements in SC-catalyzed reactions is available, despite rigorous efforts to isolate and biochemically characterize SCs. The tirucalladiene synthase from the fern *Polypodiodes niponica* (PNT),[11] which produces the migrated dammarane tirucalla-7,21-diene, is the only SC. SCs yielding migrated hopane triterpenes, which are characteristic metabolites of ferns, remain to be isolated. However, Sato and colleagues recently showed that a tetraprenyl-β-curcumene cyclase from *Bacillus megaterium* (BmeTC) has an onoceroid synthase activity.[12] BmeTC has a catalytic function in cyclizing squalene (**8**) to **6** and 14β-hydroxyonocer-8(26)-ene.

Herein, we report the cloning and characterization of three SCs from the fern *Colysis elliptica* and two from *Colysis pothifolia*, including a hopene synthase, α-polypodatetraene synthase, hop-17(21)-ene synthase, and fern-9(11)-ene synthase. One of these clones (*CPF*) was a transcribed pseudogene with a 22-nucleotide deletion that causes a nonsense mutation. We successfully constructed an insertion mutant of *CPF* (designated *CPFa*), which resulted in the conversion of nonfunctional CPF to the active SC. In addition, site-directed mutagenesis experiments on two migrated hopene synthases (CPH and CPFa) identified a migration switch that controls the number of 1,2-hydride and methyl shifts. Furthermore, the finding that the CPH product was not detected in *C. pothifolia* is the first example of an inconsistency between phenotype (triterpene profile) and genotype (gene function) in ferns.

Results

Cloning and sequence analysis of *SC*s from two *Colysis* ferns

In order to obtain a partial sequence, nested PCR was performed with a set of degenerate primers designed from conserved SC amino acid sequences[9,10] and cDNAs isolated from the above-ground part of *C. elliptica* and *C. pothifolia*. PCR products of the expected size (ca. 450 bp) were isolated, cloned, and propagated in *Escherichia coli*. Positive transformants were selected, and the inserted fragments were sequenced. Sequence analysis of the PCR fragment derived from *C. elliptica* revealed the presence of three independent clones (the corresponding full-length clones were named *CEH1*, *CEH2*, and *CEP*); the PCR fragment from *C. pothifolia* comprised two clones: *CPH* and *CPF*. To determine the full-length sequences of these five clones, we conducted 5′- and 3′-RACE (rapid amplification of cDNA ends) reactions with specific primers.

All the cloned *Colysis SC*s except *CPF* seemed to be canonical *SC*s, based on the presence of the characteristic motifs for SCs, and the nucleotide length. The five clones possess the DXDD motif[13,14] implicated in protonating the terminal double bond of the substrate to initiate the cyclization reaction. Other motifs, the so-called QW motifs[15,16] that contribute to stabilization of the overall protein structure are also found in these clones. The ORFs of the *Colysis SC*s range from 2064 to 2109 nucleotide in length, sharing nucleotide identities of between 83 and 95%, and amino acid identities of between 76 and 93%. It is noteworthy that in the nucleotide sequences of *CPF*, there is a 22-nucleotide deletion of with a frameshift mutation, when compared to known *SC*s from ferns (Supporting Information), thus suggesting that the protein encoding *CPF* has no catalytic function.

Functional analysis of *Colysis* SC cDNAs in yeast

In order to characterize the putative SCs from *Colysis* plants, their ORFs were heterologously expressed in the methylotrophic yeast *Pichia pastoris* under the control of methanol-inducible AOX1 promoter. All five cDNAs were subcloned into the expression vector, pPIC3.5, and the resulting plasmids were transformed into *P. pastoris* strain GS115 or KM71. The transformants were cultured, and the hexane extracts from the yeast cells were analyzed by GC (Figure 1). This revealed that the extracts harboring the cDNA encoding the putative SCs contained a product other than squalene (**8**), a substrate of SCs. The extracts of the transformants were separated by silica gel column chromatography with *n*-hexane to yield a triterpene hydrocarbon-enriched fraction, then analyzed by ^{1}H NMR spectroscopy. Based on the NMR analysis and comparison with the literature, we identified hop-22(29)-ene (**7**)[17] as a CEH1 and CEH2 product, α-polypodatetraene (**9**)[18] as a CEP product, and hop-

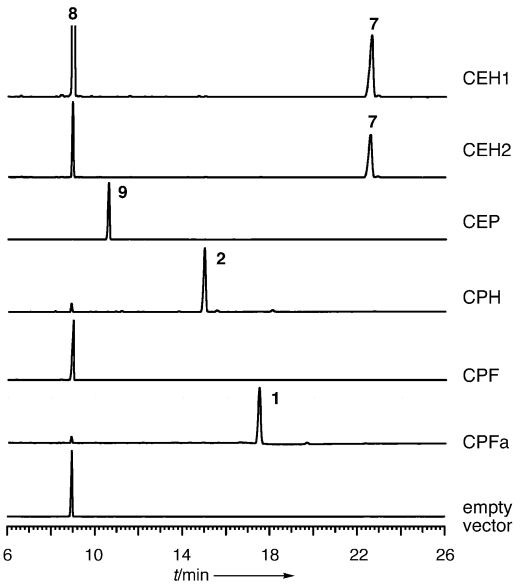


Figure 1. Gas chromatograms of the products of *Colysis* squalene cyclases expressed in *P. pastoris*.

17(21)-ene (**2**)[19] as a CPH product. As anticipated, no compound other than **8** was detected in the extract from yeast harboring *CPF*, as described above.

Apart from the deletion region, the nucleotide and amino acid sequences of *CPF* share relatively high homology with known *SC*s from ferns. Therefore, we investigated whether a *CPF* mutant created by introducing corresponding sequences to the deletion region reverts the squalene cyclization activity of inherent CPF. Because *CPF* has a 22-nucleotide deletion at the 452nd position when compared to *CPH* (Supporting Information), the 452A of *CPF* was replaced with the 23-nucleotide fragment from *CPH* to yield an insertion mutant designated *CPFa*. Yeast cells overexpressing *CPFa* accumulated a single product that was not detected in the yeast cells carrying *CPF* and the empty vector pPIC3.5 (Figure 1). The CPFa product was identified as fern-9(11)-ene (**1**)[19] by comparing the ^{1}H NMR data with the literature. Taken together, these results clearly indicate that *CEH1* and *CEH2* encode hopene synthases, *CEP* encodes α-polypodatetraene synthase, *CPH* encodes hop-17(21)-ene synthase, and *CPF* is the transcribed pseudogene with a inherent fern-9(11)-ene synthase activity.

Identification of the crucial amino acid residue involved in the rearrangement

Having successfully isolated and characterized the cDNAs encoding migrated hopene synthases (*CPH* and *CPFa*), we then attempted to identify the amino acid residue(s) governing the number of 1,2-hydride and methyl shifts. We compared the putative active-site residues of CPH and CPFa with that of the SHC from *A. acidocaldarius* (Supporting Information). Based on mutational experiments with the bacterial SHC, we selected 17 amino acid residues[13,20] for which substitutions to other amino acids altered the product profile. Of these residues, six were different between the two fern SCs and the SHC. CPH and CPFa share five of the six residues, so we finally identified one amino acid residue (Q276 in CPH, V281 in CPFa, and I261 in SHC) that the three SCs do not share with each other.

In order to verify the role of these amino acids in controlling the number of rearrangement reactions, site-directed mutagenesis experiments were carried out, followed by functional analysis of the resulting mutated SCs. We constructed CPH Q276V, CPFa V281Q, and SHC I261Q and I261V mutants. The cDNAs of the mutated SCs were independently expressed in *P. pastoris*, then the hexane extracts from the cultured yeast cells were analyzed by ^{1}H NMR spectroscopy and GC (Figure 2). The results showed that CPH Q276V produced **1** and CPFa V281Q produced **2**, thus clearly demonstrating that only a single substitution is necessary to convert hop-17(21)-ene synthase to fern-9(11)-ene synthase and vice versa. In contrast, SHC I261Q formed several products with tetra- and pentacyclic skeletons (**7**, **10**–**12**), including **2**, although this was a minor product. No alteration of the product profile compared to the wild type was observed for SHC I261V (Supporting Information). The results from the mutated SHCs indicated that I261, or its corresponding position of Q276 in CPH and V281 in CPFa, plays a role in constructing the E ring during hopane skeleton formation; this is consistent with a previous study.[20f]

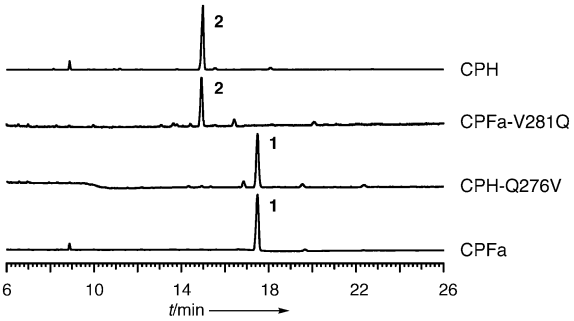


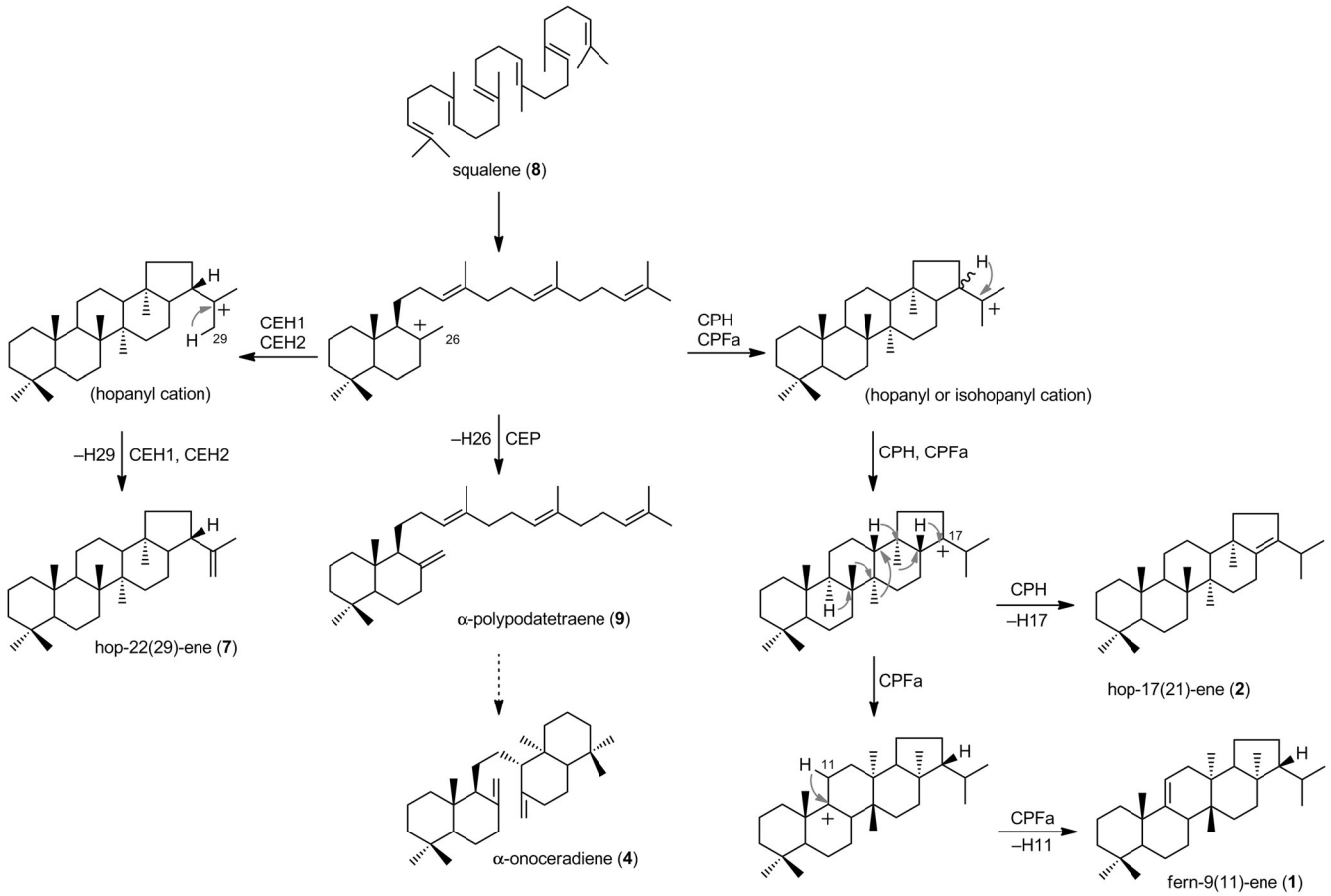
Figure 2. Gas chromatograms of the products of mutated and wild-type CPH and CPFa.

Discussion

Mechanisms of *Colysis* squalene cyclase reactions

We characterized migrated hopene (CPH and CPFa) and hopene (CEH1 and CEH2) synthases as well as α-polypodatetraene synthase (CEP). Subsequent mutation experiments revealed "the migration switch", the amino acid residues responsible for controlling the number of 1,2-hydride and methyl shifts: Q276 in CPH and V281 in CPFa. The corresponding residue of I261 in the bacterial SHC, however, did not work as a migration switch, thus implying a catalytic difference between these two types of enzyme during cyclization. In the CPH- and CPFa-catalyzed reactions, cyclization of **8** is initiated by proton attack on a terminal double bond; this leads to a C-22 pentacyclic cation intermediate, hopanyl (21βH) or isohopanyl (21αH). The cation undergoes a 1,2-hydride shift with proton elimination from H-17 to yield **2**; alternatively, seven 1,2-hydride and methyl shifts with proton elimination from H-11 give **1** (Scheme 2). In contrast, the CEH1-, CEH2-, and SHC-catalyzed reactions proceed from polycyclization of **8** to give a hopanyl C-22 cation (21βH), followed by proton elimination from H-29 to yield **7**. Considering that a series of 1,2-hydride and methyl shifts can proceed in an antiperiplanar fashion, it is likely that the pentacyclic intermediate is an isohopanyl cation (21αH) in the migrated hopane biosynthesis; conversely, in the hopane biosynthesis without an rearrangement reaction, the intermediate is a hopanyl cation (21βH) that has the same isoprenyl side-chain stereochemistry as the final product, **7**. The differences between the pentacyclic intermediates in migrated-hopane and hopane biosynthesis could cause discrete interactions between the intermediates and the enzyme active site. Thus, the role of Q276 in CPH and V281 in CPFa for cyclization is inconsistent with that of I261 in the SHC; this indicates that, with respect to the molecular evolution of SCs, these two types of enzymes appear to be significantly distant from each other, despite yielding products with closely related skeletons.

The novel SC of CEP is a monofunctional α-polypodatetraene synthase that yields a bicyclic scaffold. The CEP-catalyzed reaction consists of three steps: 1) formation of the A-



Scheme 2. Proposed reaction mechanisms of *Colysis* squalene cyclases. The broken arrow indicates a deduced pathway.

ring by proton attack on the terminal double bond of **8**; 2) a second ring closure to yield a B-ring; 3) deprotonation at H-26 of the resulting bicyclic cation intermediate to give **9** (Scheme 2). The CEP product is an immature compound during hopane biosynthesis, thus suggesting that the *CEP* gene might come from an ancestral hopene synthase gene through duplication and subfunctionalization. This scenario seems to be supported by the phylogenetic analysis (Supporting Information) that CEP falls into the species-specific clade including CEH1 and CEH2.

Biosynthesis of onoceroids in *Colysis*

Isolation of *CEP* from an onoceroid-producing fern is noteworthy because the product, **9**, is a postulated intermediate of **4**, one of the onoceroids (Scheme 2). Sato and colleagues have shown that the *Bacillus* triterpene/sesquaterpene synthase (BmeTC) catalyzes cyclization of **8** from both termini to yield two onoceroids, **6** and 14β-hydroxyonocer-8(26)-ene as well as a bicyclic triterpene, 8α-hydroxypolypoda-13,17,21-triene (**13**); additionally, incubation of **13** as a substrate with BmeTC also yields these two onoceroids.[12] In our study, no onoceroids were detected by GC analysis in the hexane extract from the yeast carrying CEP, and no new products were observed when **9** was added to the CEP-overexpressing yeast culture. Neither were onoceroid products detected when **9** was added to the CEH1- and CEH2-harboring yeast culture. Furthermore, when **9** was incubated with the cell-free extract of an *E. coli* transformant carrying the SHC from *A. acidocaldarius*, no reaction was observed (data not shown). Based on these results, we conclude that CEP as well as CEH1 and CEH2 are genuine SCs that use **8** as sole substrate but, not onoceroid synthases. A previous phylogenetic analysis showed that the SC-like enzymes of *Bacillus* species, including BmeTC, formed an outgroup between canonical SCs and eukaryotic oxidosqualene cyclases.[21] Recent studies[12,21] together with the results of our study indicate that enzymes with squalene cyclization activity may be divided into two classes of enzymes: canonical SCs distributed in ferns and most bacteria, and *Bacillus* cyclases, including BmeTC. It is, however, likely that, in addition to canonical SCs, *Colysis* ferns possess unidentified BmeTC-like onoceroid synthases.

Conclusion

We isolated and characterized three new *SC* clones that code for enzymes that catalyze the conversion of squalene into hop-17(21)-ene (CPH), fern-9(11)-ene (CPFa), and α-polypodatetraene (CEP), as well as two cDNAs encoding hop-22(29)-ene synthase (CEH1 and CEH2) from the ferns, *C. elliptica* and *C. po-*

thifolia. Mutation experiments on CPH and CPFa identified a migration switch that controls the number of 1,2-hydride and methyl shifts. To the best of our knowledge, this is the first study to isolate a transcribed pseudogene with a 22-nucleotide deletion (*CPF*), and the first demonstration of the restoration of its function by addition of appropriate nucleotides. Our study also identified an inconsistency between genotype and chemotype in *C. pothifolia*; this fern produces only colysanoxide and α-onoceradiene, although it possesses the functional hop-17(21)-ene synthase (CPH). This finding prompted us to propose a hypothesis that *SC* genes are widely distributed throughout ferns whether or not they produce triterpene hydrocarbons; whether this hypothesis is valid is the subject of ongoing research.

Experimental Section

Plant material and cDNA synthesis: The above-ground parts of *C. elliptica* and *C. pothifolia* were collected at the medicinal plant garden of Showa Pharmaceutical University (Tokyo). They were immediately frozen in liquid N_2 and stored at −80 °C. Extraction of total RNA from the ferns and purification of mRNA from total RNA were performed as reported in our previous paper.[9] Single-strand cDNA was synthesized by using SuperScript III (Invitrogen) and RACE32 primer[22] according to the manufacturer's protocol. Adaptor-ligated double-stranded cDNA was prepared by using a SMARTer RACE cDNA Amplification Kit (Clontech).

Cloning of squalene cyclase cDNAs: Homology-based nested PCR was performed with degenerate oligonucleotide primers (SC-306S-1, SC-306S-2, SC-358S, SC-494A, and SC-538A),[9,10] under the conditions described previously.[9] The gel-purified PCR products were ligated into pT7Blue T-Vector (Novagen), propagated in *E. coli*, and sequenced by using an ABI PRISM 3130-Genetic Analyzer (Applied Biosystems) with BigDye Terminator v3.1 Cycle Sequencing Kit. Sequence analysis revealed the presence of three clones (designated *CEH1*, *CEH2*, and *CEP*) in *C. elliptica* and two clones (designated *CPH* and *CPF*) in *C. pothifolia*.

For 3′- and 5′-end amplification, two specific primers were designed based on the obtained sequences (Supporting Information). 3′- and 5′-RACE was performed by using a SMARTer RACE cDNA Amplification Kit according to the product manual. The nucleotide sequences of *Colysis* squalene cyclases obtained in this study have been deposited in the GenBank/EMBL/DDBJ databank (accession nos. CEH1: LC074708; CEH2: LC074709, CEP: LC074710, CPH: LC074711, CPF: LC074712).

Expression of *Colysis* squalene cyclases in *P. pastoris*: Full-length ORFs were cloned in pPIC3.5 (Invitrogen) under the control of the AOX1 promoter, then transformed into *P. pastoris* GS115 or KM71 by using the method reported in our previous paper.[11]
For the GS115 recombinant strains, the transformant was grown in yeast extract peptone dextrose (YPD) medium (50 mL; 1% yeast extract, 2% peptone, and 2% glucose) at 30 °C for 24 h with shaking. Cells were collected by centrifugation (2380 *g*, 20 min) and resuspended in YP medium (200 mL; YPD without glucose) supplemented with 1% methanol, and cultured at 30 °C for 48 h with shaking and periodic addition of methanol (final concentration of 1%, every 24 h). Cells were collected by centrifugation (2380 *g*, 20 min) and resuspended in potassium phosphate (0.1 M, 200 mL; pH 7.0) supplemented with 3% glucose and terbinafine (10 μg mL^{-1}).[23] The mixture was further cultured at 30 °C for 24 h with shaking. Cells were collected by centrifugation (2380 *g*, 20 min), refluxed with 20% KOH in 50% ethanol (40 mL), and extracted twice with the same volume of *n*-hexane.

For the KM71 recombinant strains, the transformant was grown in YPD medium (200 mL) at 30 °C for 48 h with shaking. Cells were collected by centrifugation (2380 *g*, 20 min) and resuspended in YP medium (80 mL) supplemented with 1% methanol, and cultured at 30 °C for 48 h with shaking and periodic addition of methanol (final concentration of 1%, every 24 h). Cells were collected by centrifugation (2380 *g*, 20 min) and resuspended in potassium phosphate (0.1 M, 80 mL; pH 7.0) supplemented with 3% glucose and terbinafine (10 μg mL^{-1}). The mixture was further cultured at 30 °C for 24 h with shaking.

GC and NMR analysis of *Colysis* squalene cyclase products: Concentrated hexane extracts were loaded onto a BondElut-Si minicolumn (500 mg, 3 mL; Varian) and eluted with *n*-hexane to yield triterpene hydrocarbon fractions. These fractions were analyzed by GC, which was performed on a Hitachi G-6000 instrument with a DB-5HT column (30 m × 0.25 mm, Agilent Technologies) under the same conditions as in our previous paper.[11]

Again, the triterpene hydrocarbon fractions were loaded onto a minicolumn and eluted with *n*-hexane. All fractions containing compounds less polar than squalene (according to the TLC profile) were combined into one fraction and concentrated. ^{1}H NMR spectra were measured in $CDCl_3$ (Bruker AV600) with tetramethylsilane (TMS) as an internal standard. NMR analysis and comparison with the published data revealed hop-22(29)-ene (**7**)[17] as a product of CEH1 and CEH2, α-polypodatetraene (**9**)[18] from CEP, and hop-17(21)-ene (**2**)[19] from CPH. Notable ^{1}H NMR spectra signals for the products were follows:

Hop-22(29)-ene (7): ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 0.72$ (s, 3H; H-28), 0.79 (s, 3H; H-24), 0.81 (s, 3H; H-25), 0.84 (s, 3H; H-23), 0.94 (s, 3H; H-27), 0.96 (s, 3H; H-26), 1.75 (s, 3H; H-30), 4.78 ppm (s, 2H, H-29).

α-Polypodatetraene (9): ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 0.66$ (s, 3H; H-25), 0.80 (s, 3H; H-24), 0.87 (s, 3H; H-23), 1.56 (s, 3H; H-27), 1.60 (s, 6H; H-28 and H-29), 1.68 (s, 3H; H-30), 4.54 (s, 1H; H-26), 4.82 (s, 1H; H-26), 5.09–5.14 ppm (m, 3H; H-13, H-17, H-21).

Hop-17(21)-ene (2): ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 0.79$ (s, 3H; H-24), 0.82 (s, 3H; H-25), 0.83 (s, 3H; H-28), 0.85 (s, 3H; H-23), 0.92 (d, $J = 6.6$ Hz, 3H; H-30), 0.93 (s, 3H; H-26), 0.98 ppm (d, $J = 6.6$ Hz, 3H; H-29).

Construction and functional analysis of the insertion mutant CPFa: From the pPIC3.5 harboring *CPF* as a template, the insertion mutant *CPFa* was constructed by using a KOD -Plus- Mutagenesis Kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. (The primer set for construction of the insertion mutant is shown in the Supporting Information.) The mutation was confirmed by sequence analysis. The resultant plasmid was linearized and transformed in *P. pastoris* strain KM71 by using Frozen-EZ Yeast Transformation II (Zymo Research, Orange, CA, USA). Subsequently, functional analysis was performed according to the method described above. The CPFa product was identified as fern-9(11)-ene (**1**),[19] the ^{1}H NMR signals of which were as follows:

Fern-9(11)-ene (1): ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 0.73$ (s, 3H; H-26), 0.76 (s, 3H; H-28), 0.82 (s, 3H; 27), 0.83 (d, $J = 6.6$ Hz, 3H; H-30), 0.85 (s, 3H; H-23), 0.89 (s, 3H; H-24), 0.89 (d, $J = 6.6$ Hz, 3H; H-29), 1.05 (s, 3H; H-25), 5.29 ppm (ddd, $J = 5.4$, 2.4, 2.4 Hz, 1H; H-11).

Site-directed mutagenesis of CPH, CPFa, and bacterial SHC: With pPIC3.5 harboring *CPH*, *CPFa*, and *SHC* as a template, *CPH Q276V*, *CPFa V281Q*, *SHC I261Q*, and *SHC I261V* were constructed, as described above. The synthetic oligonucleotides designed to produce the desired point mutations are shown in the Supporting Information. The mutants were subjected to sequence analysis to confirm the desired mutations. Yeast transformation and functional analysis of mutated SCs were the same as described above. Based on ^{1}H NMR analysis, the products of CPH Q276V and CPF V281Q were fern-9(11)-ene (**1**)[19] and hop-17(21)-ene (**2**),[19] respectively. ^{1}H NMR spectral data for the products of SHC I261Q showed the presence of hop-22(29)-ene (**7**),[17] hop-17(21)-ene (**2**),[19] (17*E*)-dammara-17,21-diene (**10**),[24] (17*Z*)-dammara-17,21-diene (**11**),[24,25] and (18*R*)-dammara-13(17),21-diene (**12**).[26] Selected ^{1}H NMR spectra signals of **10**, **11**, and **12** were follows:

(17*E*)-Dammara-17,21-diene (10): ^{1}H NMR (600 MHz, $CDCl_3$): $\delta =$ 0.81 (s, 6H; H-24, H-27), 0.85 (s, 6H; H-23, H-25), 0.97 (s, 3H; H-26), 1.60 (s, 3H; H-29), 1.67 (s, 3H; H-30), 1.69 (s, 3H; H-28), 5.12 ppm (m, 1H; H-21).

(17*Z*)-Dammara-17,21-diene (11): ^{1}H NMR (600 MHz, $CDCl_3$): $\delta =$ 0.81 (s, 6H; H-24, H-27), 0.85 (s, 6H; H-23, H-25), 0.98 (s, 3H; H-26), 1.59 (s, 3H; H-28), 1.61 (s, 3H; H-29), 1.69 (s, 3H; H-30), 5.11 ppm (m, 1H; H-21).

(18*R*)-Dammara-13(17),21-diene (12): ^{1}H NMR (600 MHz, $CDCl_3$): $\delta =$ 0.80 (s, 3H; H-24), 0.85 (s, 9H; H-23, H-25, H-26), 0.92 (d, $J =$ 6.8 Hz, 3H; H-28), 1.07 (s, 3H; H-27), 1.59 (s, 3H; H-29), 1.69 (s, 3H; H-30), 5.13 ppm (m, 1H; H-21).

Substrate specificity of CEP: Compound **9** (ca. 4 mg) was obtained from large-scale (1 L) preparation of the recombinant yeast carrying *CEP*. The transformants carrying *CEP* were grown in YPD medium (200 mL), then YP medium (80 mL) supplemented with 1% methanol, as described above. Cells were resuspended in potassium phosphate (0.1 M, 80 mL; pH 7.0) supplemented with **9** (ca. 4 mg), and further cultured at 30 °C for 24 h with shaking. After work-up, the cells were extracted with *n*-hexane. The extract was subjected to TLC (developed with *n*-hexane) and GC analysis.

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