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Protostadienol synthase from *Aspergillus fumigatus*: Functional conversion into lanosterol synthase

Miki Kimura, Tetsuo Kushiro, Masaaki Shibuya, Yutaka Ebizuka, Ikuro Abe *

Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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ABSTRACT

Oxidosqualene:protostadienol cyclase (OSPC) from the fungus *Aspergillus fumigatus*, catalyzes the cyclization of (3*S*)-2,3-oxidosqualene into protosta-17(20)*Z*,24-dien-3β-ol which is the precursor of the steroidal antibiotic helvolic acid. To shed light on the structure–function relationship between OSPC and oxidosqualene:lanosterol cyclase (OSLC), we constructed an OSPC mutant in which the C-terminal residues ^{702}APPGGMR^{708} were replaced with ^{702}NKSCAIS^{708}, as in human OSLC. As a result, the mutant no longer produced the protostadienol, but instead efficiently produced a 1:1 mixture of lanosterol and parkeol. This is the first report of the functional conversion of OSPC into OSLC, which resulted in a 14-fold decrease in the V_{max}/K_M value, whereas the binding affinity for the substrate did not change significantly. Homology modeling suggested that stabilization of the C-20 protosteryl cation by the active-site Phe701 through cation-π interactions is important for the product outcome between protostadienol and lanosterol.

Introduction

Oxidosqualene cyclases (OSCs) are pivotal enzymes in the biosynthesis of sterols and triterpenes [1–4]. Oxidosqualene:lanosterol cyclase (OSLC) catalyzes the cyclization of (3*S*)-2,3-oxidosqualene into lanosterol, which is the key step in the biosynthesis of sterols in animals and fungi (Fig. 1). The formation of lanosterol is initiated by the oxirane ring opening of oxidosqualene folded in the *chair–boat–chair* conformation; the sequential ring forming reaction first produces the tetracyclic protosteryl C-20 cation, which then undergoes backbone rearrangement (H-17α → 20, H-13α → 17α, CH_3-14β → 13β, CH_3-8α → 14α) followed by removal of H-9 to generate the Δ^8 double bond of lanosterol. On the other hand, oxidosqualene:protostadienol cyclase (OSPC) (AfuOSC3 or *Afu4g14770*) from the fungus *Aspergillus fumigatus* is a recently reported novel OSC which is involved in the biosynthesis of the fusidane-type steroidal antibiotic helvolic acid [5,6]. OSPC is an *M*r 83,086 membrane bound protein with 735 amino acids, sharing 40% amino acid identity with human OSLC. A recombinant OSPC heterologously expressed in an OSLC-deficient mutant GIL77 strain of *Saccharomyces cerevisiae* catalyzed the cyclization of (3*S*)-2,3-oxidosqualene into a 3:1 mixture of protosta-17(20)*Z*,24-dien-3β-ol and (20*R*)-protosta-13(17),24-dien-3β-ol (Fig. 1) [5]. In this case, the cyclization of (3*S*)-2,3-oxidosqualene proceeds *without* the backbone rearrangement, and the C-20 protosteryl cation immediately undergoes proton elimination from H-17α or H-13α to form a double-bond between C-17/C-20 or C-13/C-17, respectively.

To clarify the structure–function relationship between OSLC and OSPC, and to understand the intimate structural details that govern the stabilization of the C-20 protosteryl cation and the backbone rearrangement reactions, we constructed a series of *A. fumigatus* OSPC mutants and investigated the effects of the mutagenesis on the enzyme activity. Here we report the functional conversion of *A. fumigatus* OSPC into OSLC by replacing the C-terminal residues ^{702}APPGGMR^{708}, located just downstream of the active-site Phe701, with ^{702}NKSCAIS^{708}, as in human OSLC (Fig. 2). The active-site Phe701 is considered to be important for stabilization of the positive charges at the C-20 protosteryl cation through cation-π interactions, which would be crucial for the formation of lanosterol *via* the backbone rearrangement reaction [7]. The results suggested that the swapping of the residues neighboring the active-site Phe701 caused changes in the position of Phe701, thereby affecting the cation-π interactions and the stabilization of the C-20 cation, which led to the production of lanosterol by the OSPC mutant.

Materials and methods

PCR and sequence analysis. The oligo DNAs were synthesized by Nihon Bioservice (Saitama, Japan) and Invitrogen. PCR was performed with a PTC-200 Peltier Thermal Cycler (MJ Research). Sequencing was accomplished with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystem).

* Corresponding author. Fax: +81 3 5841 4744.
E-mail address: abei@mol.f.u-tokyo.ac.jp (I. Abe).

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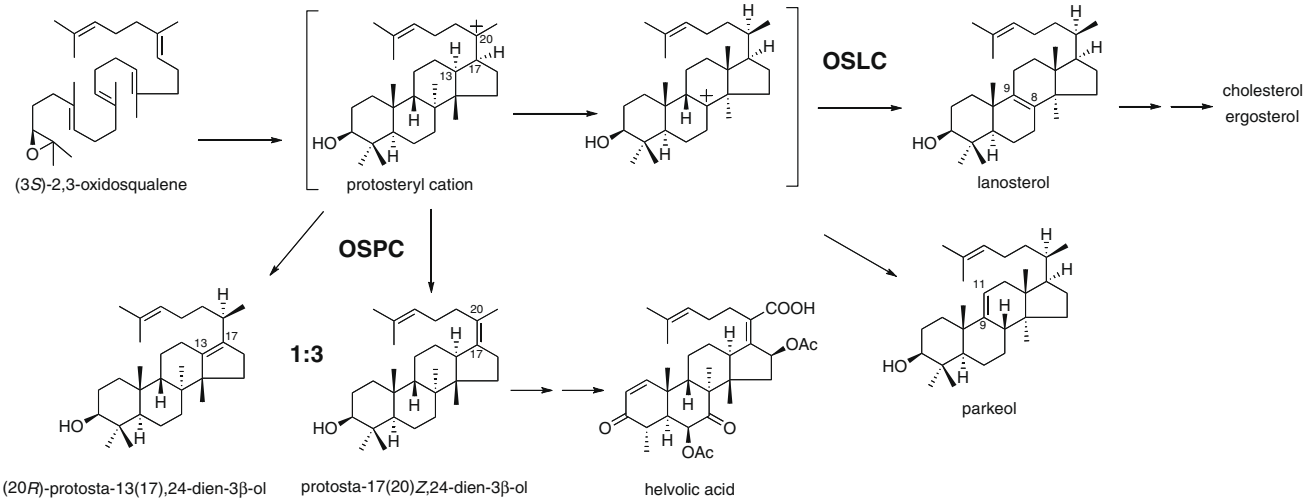

Fig. 1. Proposed mechanism of cyclization of (3*S*)-2,3-oxidosqualene into lanosterol by OSLC and protosta-17(20)*Z*,24-dien-3β-ol and (20*R*)-protosta-13(17),24-dien-3β-ol by OSPC.

Site-directed mutagenesis. The *A. fumigatus* OSPC mutant in which the residues ^{702}APPGGMR^{708} were replaced with ^{702}NKSCAIS^{708}, was constructed by a PCR method. Thus, the first PCR was performed with 1 μL (20 pmol) of a C-terminal primer (AfuOSPC-C-BgI: 5′-GTACCAAGATCTTTATATAGCTAGACATTCATTTCCATGG-3′) and 1 μL (20 pmol) of a mutation primer (AfuOSPC-NKSCAIS-S: 5′-CCGCTGGAAGGAGTGTTTAATAAGTCTTGCGCGATCAGCTATCCAAATTAC-3′) with 1 μL of plasmid DNA containing the full length cDNA encoding *A. fumigatus* OSPC [5] as the template. Phusion DNA polymerase (Finnzymes) was used with dNTP (0.2 mM) in a final volume of 50 μL, according to the manufacturer's protocol. The reaction proceeded for 30 cycles with the following program: 98 °C, 10 s, 60 °C, 24 s, 72 °C, 30 s, and final extension at 72 °C, 10 min. The resulting 120 bp fragment was separated by agarose gel (2%) electrophoresis and purified using a MonoFas DNA purification kit (GL Sciences). The second PCR was performed with 10 μL of this fragment as an anti-sense primer, and 1 μL (20 pmol) of the N-terminal primer (AfuOSPC-N-Spe, 5′-CTTGCCTACTAGTATGGCGACAGACAGCAGCATG-3′) with 1 μL of plasmid DNA containing the full length cDNA encoding *A. fumigatus* OSPC as the template. The reaction was performed for 30 cycles with the following program: 98 °C, 10 s, 60 °C, 24 s, 72 °C, 30 s, and final extension at 72 °C, 5 min. The resulting 2.3 kb band, corresponding to the full length cDNA fragment was digested with SpeI and BglII and ligated into the yeast expression plasmid pESC-URA (Stratagene), which was previously digested with the same restriction enzymes.

For the construction of point mutants of *A. fumigatus* OSPC, the following primers were used. AfuOSPC-A702N, 5′-CCGCTGGAAGGAGTGTTTAATCCTCCTGGGGGGATGCGGTATCCAAATTAC-3′; AfuOSPC-P703 K/P704S, 5′-CCGCTGGAAGGAGTGTTTGCTAAGTCTGGGGGGATGCGGTATCCAAATTAC-3′; AfuOSPC-G705C, 5′-CCGCTGGAAGGAGTGTTTGCTCCTCCTTGCGGGATGCGGTATCCAAATTAC-3′; AfuOSPC-M707I, 5′-CCGCTGGAAGGAGTGTTTGCTCCTCCTGGGGGGATCCGGTATCCAAATTAC-3′.

Enzyme expression. After confirmation of the sequence, the plasmid was transformed into the OSLC-deficient yeast mutant strain GIL77 (*erg7, ura3-167, hem3-6, gal2*) using Frozen-EZ Transformation II kit (Zymo Research), and the cells were plated onto synthetic complete medium without uracil (SC-U), containing ergosterol (20 μg/mL), hemin chloride (13 μg/mL), and Tween 80 (5 mg/mL). The cells harboring the plasmid were incubated at 30 °C for 2 days for selection of the desired transformants. The culture of the transformed yeast, the protein expression, and the isolation, purification and GC-MS analysis of the products were performed as described previously [5].

Enzyme preparation. A 2 L culture of GIL77 harboring each gene was induced with galactose for 1 day and harvested. The collected cells were suspended in 20 mL of 0.1 M KPB (pH 7.4) and the cells were disrupted with a French Press (25,000 psi). After centrifugation (10,000*g*) to remove the cell debris, ultracentrifugation (100,000*g*) was performed for 1 h to collect the microsome fraction which was then suspended in 2 mL of 0.1 M KPB (pH 7.4) with 0.2% Triton X-100. Enzyme solubilization was accomplished by vortexing for 1 h and then ultracentrifugation (100,000*g*) for 1 h. The resulting supernatant was then passed through a hydroxylapatite column (1 mL) equilibrated with 5 mM KPB (pH 7.4) and the flow through fraction was collected and used for the enzyme reaction.

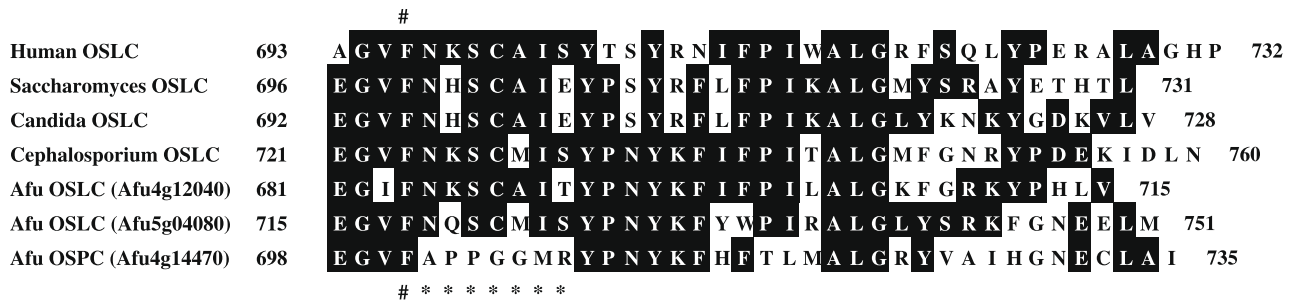

Fig. 2. Comparison of the amino acid sequences of the C-terminal regions of the *A. fumigatus* OSPC and the OSLCs from a mammal, yeast, and fungi: Human, *Homo sapiens*; Saccharomyces, *Saccharomyces cerevisiae*; Candida, *Candida albicans*; Cephalosporium, *Cephalosporium caerulens*; Afu, *Aspergillus fumigatus*. The active-site Phe701 (numbering in *A. fumigatus* OSPC) is marked with #. The residues ^{702}APPGGMR^{708} are marked with ∗.

Enzyme assay. For the steady-state kinetic analysis, 100 μL of the solubilized enzyme solution was incubated with [^{14}C]-(3*S*)-2,3-oxidosqualene (10,000 dpm/5 μL EGME solution) at various concentrations for 1 h at 30 °C. The reaction was quenched by the addition of 20% KOH/EtOH, extracted with hexane and spotted onto a TLC plate (Merck #11798), which was developed with benzene:acetone = 19:1. Radioactivities were quantified by an imaging plate (Fuji Film) and analyzed by a BAS-1500 system (Fuji Film). Lineweaver–Burk plots of the data were utilized to derive the apparent K_M and V_{max} values (average of triplicates ± standard deviation).

GC-MS analysis. The 4,4-dimethyl sterol fractions were applied to a GC-MS system (Shimadzu, GCMS-QP2010) equipped with a Restec Rtx-5MS glass capillary column (30 m in length, 0.25 mm in diameter, 0.25 μm film thickness) and using He as a carrier gas (45 cm/min) with the following program: maintained at 240 °C for 2 min, followed by temperature increase at the rate of 10 °C/min up to 330 °C. The temperature of the ionization chamber was 250 °C, with electron impact ionization at 70 eV.

Results and discussion

A comparison of the primary sequences of the *A. fumigatus* OSPC and OSLC enzymes from a mammal, yeast and fungi revealed that the moderately conserved C-terminal residues of human OSLC, ^{697}NKSCAIS^{703}, located just downstream of the active-site Phe696 (numbering in human OSLC), are characteristically replaced with ^{702}APPGGMR^{708} in *A. fumigatus* OSPC (Fig. 2) [5]. In the X-ray crystal structure of human OSLC, the active-site Phe696 (which corresponds to Phe701 in *A. fumigatus* OSPC) is located near the C-13/C-20 of the bound lanosterol. This Phe residue is considered to be important for stabilization of the positive charges at the C-13 anti-Markovnikov tricyclic cation and also at the C-20 tetracyclic protosteryl cation through cation-π interactions, thereby guiding the stereochemical course of the enzyme reaction [7]. Indeed, the F696T mutant of *S. cerevisiae* OSLC (ERG7) (numbering in human OSLC) was recently shown to produce a truncated rearrangement product, protosta-13(17),24-dien-3β-ol, which was formed by hydride shift from C-17 to C-20 followed by proton elimination of H-13α [8,9]. We therefore surmised that the residues located around Phe701 might be participating in the fate of the C-20 cation, and the difference between OSLC around this region might be responsible for the different product outcomes between the two enzymes. Thus, we first constructed a mutant of *A. fumigatus* OSPC in which the residues ^{702}APPGGMR^{708} were replaced with ^{702}NKSCAIS^{708}, as in human OSLC, and investigated the effects of the mutagenesis on the enzyme activity.

As in the case of the wild-type *A. fumigatus* OSPC, the gene encoding the mutant enzyme was cloned into the yeast expression vector pESC(Ura) and heterologously expressed in the OSLC-deficient sterol auxotrophic yeast mutant strain GIL77 (*erg7, ura3-167, hem3-6, gal2*) under the control of the *GAL10* promoter [5]. After induction, the transformed cells were harvested and the recombinant membrane-bound enzyme was solubilized with Triton X-100 and partially purified by hydroxylapatite column chromatography as described previously [10].

An *in vitro* enzyme assay revealed that the *A. fumigatus* OSPC mutant in which the residues ^{702}APPGGMR^{708} were replaced with the conserved ^{702}NKSCAIS^{708} sequence no longer produced protostadienols, but instead efficiently produced a 1:1 mixture of lanosterol and parkeol (Fig. 3). Indeed, the mutant complemented the *erg7* deficiency of the yeast GIL77 strain, since the transformed cells could grow on media lacking ergosterol, in sharp contrast to the wild-type OSPC. This is the first demonstration of the functional conversion of OSPC into OSLC and the formation of lanosterol by the OSPC mutant. Here the proposed mechanism of (3*S*)-2,3-oxidosqualene cyclization to parkeol is essentially the same as that for lanosterol; the only difference is the final proton elimination from H-11 to produce the double-bond between C-9/C-11 of parkeol, whereas proton elimina-

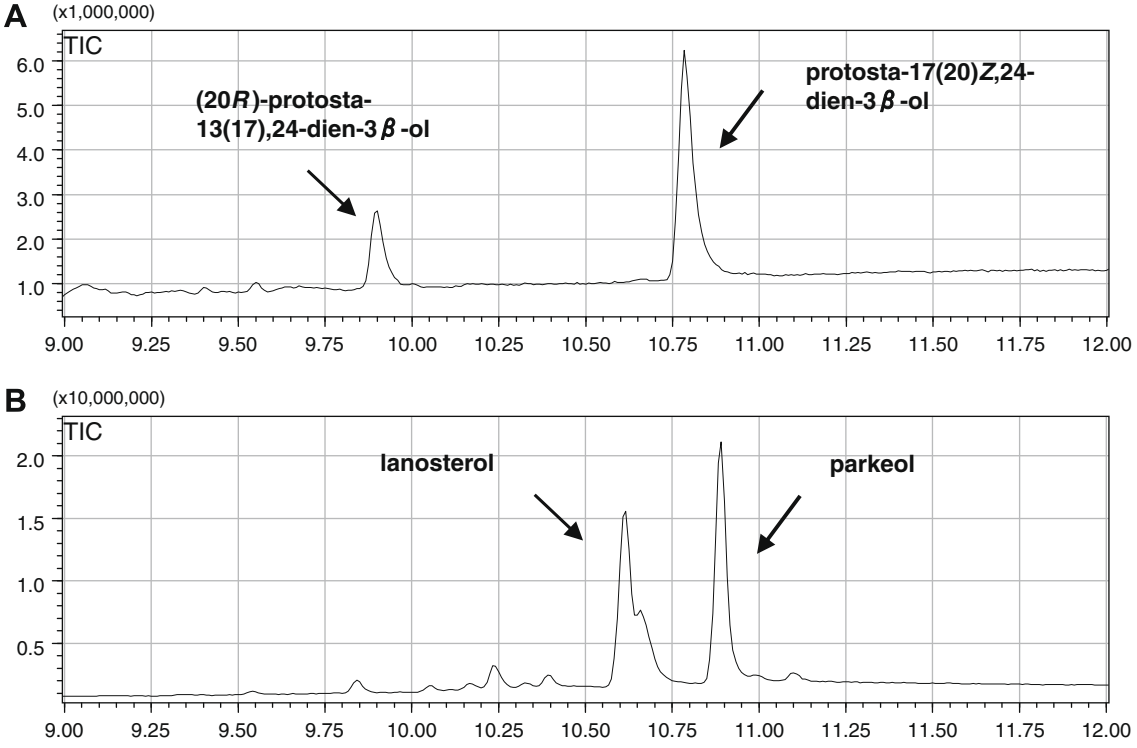

Fig. 3. GC-MS trace of the enzyme reaction products of (A) the wild-type *A. fumigatus* OSPC and (B) the OSPC mutant in which the residues ^{702}APPGGMR^{708} were replaced with the conserved ^{702}NKSCAIS^{708}.

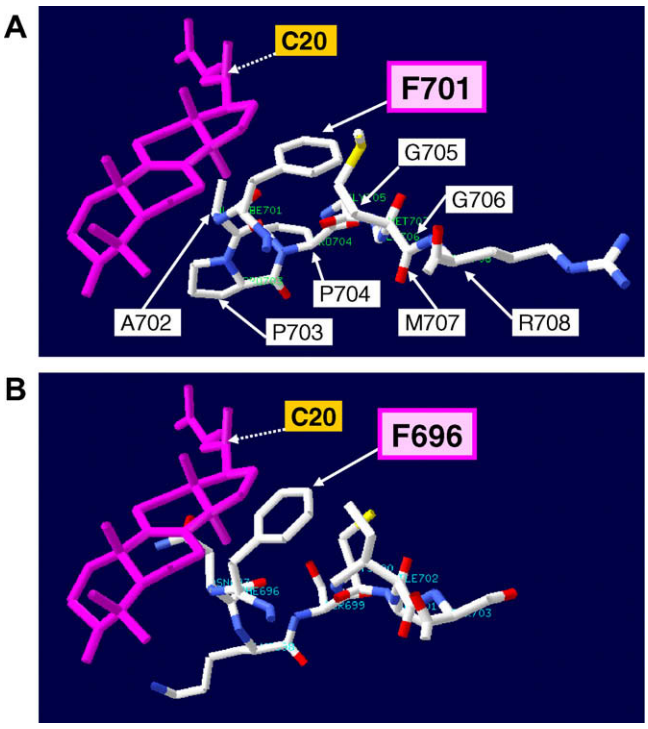

Fig. 4. (A) A homology model of *A. fumigatus* OSPC complexed with lanosterol. (B) The X-ray crystal structure of human OSLC complexed with lanosterol. Only the active-site Phe701 and the residues ^{702}APPGGMR^{708} (numbering in *A. fumigatus* OSPC) are indicated. Lanosterol is shown in magenta and the position of the C-20 indicated.

tion from H-9 generates the double-bond between C-8/C-9 of lanosterol (Fig. 1). The steady-state kinetic analysis revealed that the mutant had an apparent K_M = 16.5 μM, V_{max} = 0.32 μM min^{-1}, and V_{max}/K_M value of 0.019 min^{-1}, which are comparable to the values of the wild-type OSPC, which showed an apparent K_M = 14.4 μM, V_{max} = 4.08 μM min^{-1}, and V_{max}/K_M value of 0.283 min^{-1}. Thus, the swapping of the residues resulted in a 14-fold decrease in the V_{max}/K_M value whereas the binding affinity for the substrate did not change significantly. To further evaluate the importance of the conserved residues, we also created a set of four point mutations (A702N, P703K/P704S, G705C, and M707I) in *A. fumigatus* OSPC. However, none of the three mutants (A702N, G705C, and M707I) affected the enzyme reaction product pattern while the P703K/P704S mutant lost its activity, which clearly demonstrated that a single amino acid substitution is not sufficient for the functional conversion of OSPC into OSLC.

It was thus confirmed that these residues located just downstream of Phe696 of human OSLC play important roles for the formation of lanosterol *via* the stabilization of the C-20 protosteryl cation and the backbone rearrangement reaction. Presumably, the swapping of the residues neighboring the active-site Phe701 caused changes in the position of Phe701, thereby affecting the cation-π interactions and the stabilization of the tetracyclic C-20 cation, which led to the formation of lanosterol and parkeol by the OSPC mutant. Indeed, a homology model based on the human OSLC crystal structure [7] suggested that in *A. fumigatus* OSPC, the active-site Phe701 is located a bit farther from the presumed C-20 cationic center of the protosteryl cation as compared with that in human OSLC (Fig. 4). This increased distance would reduce both the cation-π interactions and the stabilization of the C-20 cation, and hence would interrupt the backbone rearrangement, resulting in termination of the enzyme reaction by proton elimination of H-17α or H-13α to produce a 3:1 mixture of protosta-17(20)*Z*,24-dien-3β-ol and (20*R*)-protosta-13(17),24-dien-3β-ol (Fig. 1). Alternatively, an activated water molecule might be located in a cavity formed between the C-20 cation and Phe701, and abstract a proton from C-17 or C-13 to terminate the reaction at the protosteryl cation stage. The relocation of the Phe701 residue in the mutant might have prevented the water molecule from entering this cavity, thus precluding the early termination of the reaction to produce lanosterol and parkeol.

In conclusion, this is the first report of the functional conversion of OSPC to OSLC by changing the conserved C-terminal residues ^{702}APPGGMR^{708} into ^{702}NKSCAIS^{708}. The results suggested that stabilization of the C-20 protosteryl cation by the active-site Phe701 through cation-π interactions is important for the product outcome between protostadienol and lanosterol. To further clarify the structural details of the enzyme reactions, crystallization trials of both the wild-type and mutant *A. fumigatus* OSPCs are now in progress in our laboratories.

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References

[1] I. Abe, M. Rohmer, G.D. Prestwich, Enzymatic cyclization of squalene and oxidosqualene to sterols and triterpenes, Chem. Rev. 93 (1993) 2189–2206.
[2] I. Abe, Enzymatic synthesis of cyclic triterpenes, Nat. Prod. Rep. 24 (2007) 1311–1331.
[3] K.U. Wendt, G.E. Schulz, E.J. Corey, D.R. Liu, Enzyme mechanisms for polycyclic triterpene formation, Angew. Chem. Int. Ed. 39 (2000) 2812–2833.
[4] T. Kushiro, Y. Ebizuka, Triterpene, in: L.N. Mander, H.-w. Liu (Eds.), Comprehensive Natural Products II: Chemistry and Biology, vol. 1. Elsevier, Oxford, in press.
[5] H. Mitsuguchi, Y. Seshime, I. Fujii, M. Shibuya, Y. Ebizuka, T. Kushiro, Biosynthesis of steroidal antibiotic fusidanes: functional analysis of oxidosqualene cyclase and subsequent tailoring enzymes from *Aspergillus fumigatus*, J. Am. Chem. Soc. 131 (2009) 6402–6411.
[6] S. Lodeiro, Q. Xiong, W.K. Wilson, Y. Ivanova, M.L. Smith, G.S. May, S.P.T. Matsuda, Protostadienol biosynthesis and metabolism in the pathogenic fungus *Aspergillus fumigatus*, Org. Lett. 11 (2009) 1241–1244.
[7] R. Thoma, T. Schulz-Gasch, B. D'Arcy, J. Benz, J. Aebi, H. Dehmlow, M. Hennig, M. Stihle, A. Ruf, Insight into steroid scaffold formation from the structure of human oxidosqualene cyclase, Nature 432 (2004) 118–122.
[8] T.-K. Wu, H.-Y. Wen, C.-H. Chang, Y.-T. Liu, Protein plasticity: a single amino acid substitution in the *Saccharomyces cerevisiae* oxidosqualene-lanosterol cyclase generates protosta-13(17), 24-dien-3β-ol, a rearrangement product, Org. Lett. 10 (2008) 2529–2532.
[9] T.-K. Wu, C.-H. Chang, Y.-T. Liu, T.-T. Wang, *Saccharomyces cerevisiae* oxidosqualene-lanosterol cyclase: a chemistry–biology interdisciplinary study of the protein's structure–function–reaction mechanism relationships, Chem. Rec. 8 (2008) 302–325.
[10] I. Abe, M. Bai, X.-y. Xiao, G.D. Prestwich, Affinity labeling of vertebrate oxidosqualne cyclases with a tritiated suicide substrate, Biochem. Biophys. Res. Commun. 187 (1992) 32–38.