

Purification, kinetics, inhibitors and CD for recombinant β -amyrin synthase from *Euphorbia tirucalli* L and functional analysis of the DCTA motif, which is highly conserved among oxidosqualene cyclases

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Keywords

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β -Amyrin, a natural triterpene, is widely distributed in the plant kingdom, and its pentacyclic skeleton is produced by oxidosqualene cyclase (OSC). OSC enzymes are classified as membrane proteins, and they catalyze the polycyclization reaction of (3*S*)-2,3-oxidosqualene to yield nearly 150 different cyclic triterpene skeletons. To date, no report has described the successful purification and characterization of plant β -amyrin synthase. The β -amyrin synthase from *Euphorbia tirucalli* (EtAS) was expressed as a poly-histidine-tagged protein in *Saccharomyces cerevisiae* GIL77, which lacks the lanosterol synthase gene. The expression yield, determined by western blotting analysis, was 5–7 mg. By Ni²⁺–nitrilotriacetic acid affinity column chromatography and careful selection of the proper imidazole concentration during the purification processes of washing and elution, a single band was successfully obtained on SDS/PAGE. We then tested the effects of four detergents on the enzyme activity. Supplementation with Triton X-100 at a concentration of 0.05% yielded the highest activity. The optimal pH and temperature were 7.0 and 30 °C, respectively. The kinetic parameters, K_m and k_{cat} , were determined to be $33.8 \pm 0.53 \mu\text{M}$ and $46.4 \pm 0.68 \text{ min}^{-1}$, respectively. To the best of our knowledge, there are no reports describing both K_m and k_{cat} for OSCs except for two examples of rat and bovine lanosterol synthases. The β -amyrin synthase purified in this study showed a significantly higher catalytic efficiency (k_{cat}/K_m) ($\sim 10^3$ -fold) than those of the two reported lanosterol synthases. Gel-filtration HPLC indicated that the OSC exists as a monomer, and the eluted OSC retained its activity. Furthermore, the inhibition constants K_i and IC_{50} and types of inhibition by iminosqualene, Ro48-8071 and U18666A were determined, and indicated that iminosqualene and Ro48-8071 are potent inhibitors. Additionally, this is the first report of the kinetic data of the mutated enzymes targeted for the DCTAE (485–489) motif, which is a putative initiation site for the polycyclization reaction. No activity of the D485N variant and significantly decreased activity of the C564A variant were found, definitively demonstrating that the acidic carboxyl residue Asp485 serves as a proton donor to initiate the polycyclization reaction, and that Cys564 is involved in hydrogen bond formation with the carboxyl residue Asp458 to enhance the acidity. The CD spectrum is the first to be reported for OSCs, and the CD spectra of the wild-type and the mutated EtASs were almost the same, indicating that the protein architecture was not altered by these mutations.

Abbreviations

EtAS, *Euphorbia tirucalli* β -amyrin synthase; OSC, oxidosqualene cyclase; SHC, squalene-hopene cyclase; THF, tetrahydrofuran; β -OG, β -octylglucoside.

Introduction

Naturally occurring cyclic triterpenes are biosynthesized by an enzymatic reaction with squalene or (3*S*)-2,3-oxidosqualene (**1**) as the substrate. The structural diversity of cyclic triterpenes is remarkable; ~150 additional triterpenoid scaffolds have been identified to date [1]. Lanosterol and cycloartenol can serve as precursors for membrane sterol and steroid hormones. Many genes encoding squalene-hopene cyclases (SHCs) [2,3] and oxidosqualene cyclases (OSCs) [4,5] have been identified, and investigations of OSCs from *Arabidopsis thaliana* have provided substantial insights into plant OSCs. OSCs are generally classified into the following three categories: accurately monofunctional OSCs (giving a single or dominant product) [5,6], multifunctional OSCs (producing a variety of triterpene skeletons, none of which are dominant) [5,6], and a *seco*-type triterpene synthase OSC [C–C bond cleavage(s), i.e. Grob fragmentation]. Only three examples of *seco*-type triterpene synthases have been identified to date: marnerial synthase (At5 g42600, PEN5) from *A. thaliana* [7], *seco*- β -amyrin synthase or *seco*- α -amyrin synthase (At1 g78500, PEN6) [8], and achilleol B synthase from *Oryza sativa* [9].

β -Amyrin (**2**) is widely distributed in the plant kingdom, and its scaffold is constructed by the OSC enzyme, which folds **1** in a *chair–chair–chair–boat–boat* conformation in the reaction cavity (Fig. 1) [10]. Proton attack on the epoxide ring initiates a C–C bond-forming reaction with regioselectivity and stereospecificity to give a 6/6/6/5-fused tetracyclic dammarenyl cation, followed by ring expansion to give a 6/6/6/6-fused tetracyclic baccharenyl cation. Further cyclization gives a 6/6/6/6/5-fused pentacyclic lupenyl cation, which undergoes ring expansion to give a 6/6/6/6/6-fused pentacyclic oleanyl cation. The subsequent reactions of hydride shift and deprotonation in an antiperiplanar fashion give the β -amyrin skeleton. Many β -amyrin synthase genes have been identified from dicots – *Panax ginseng* (PNY) [11], *Pisum sativum* (PSY) [12], *Glycyrrhiza glabra* (GgbAs1) [13], *Medicago truncatula* [14], tomato [15], and *Gentiana straminea* Maxim [16] – and from a monocot, *Avena strigosa* [17], and so on. A variety of other plant OSCs have also been cloned and characterized [5].

Very little is known of the enzymatic properties of OSCs, owing to the difficulties that are frequently encountered in purifying membrane proteins. To date, among the many known OSCs, only two lanosterol synthases, from human [18] and bovine liver [19], have

been completely purified. There is no report describing the complete purification and the enzyme characterization of purified plant OSCs. Recently, Kajikawa *et al.* cloned the gene encoding β -amyrin synthase (EtAS; EC 5.4.99.39) from *Euphorbia tirucalli* [20]. We achieved the first complete purification of this plant OSC, which retained remarkably high activity. Here, we report the detailed characterization of the OSC, including the kinetic parameters for **1** and for three potent inhibitors. Moreover, the CD spectrum of native EtAS is also reported here for the first time. The DCTAE(485–489) motif, which is highly conserved among OSCs, has been presumed to be responsible for the proton attack on the epoxide ring, but no kinetic data for the site-directed mutated OSCs have been reported. Comparison of the CD spectra between the wild type and the mutants allowed us to determine whether alteration of the protein architecture occurred because of the mutations. We also report here the kinetic data for the mutated EtASs targeted for the motif.

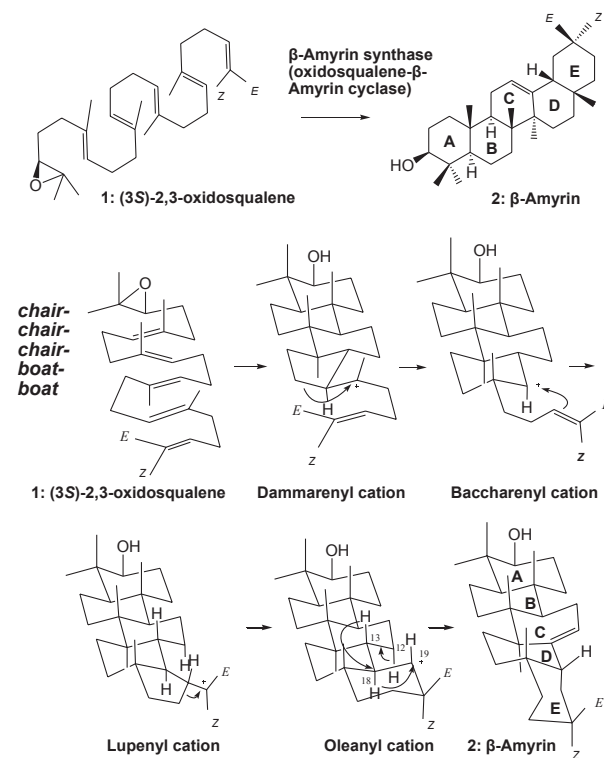


Fig. 1. The biosynthetic pathway of (3*S*)-2,3-oxidosqualene (**1**) into β -amyrin (**2**).

Results and Discussion

Expression of β -amyrin synthase in *Saccharomyces cerevisiae* and the cyclization products **3** and **4**

The pPICZ A plasmid harboring the EtAS coding sequence [20] was used as the template for PCR amplification of the gene. The amplified gene was ligated into the yeast expression vector pYES2, and then transformed into *S. cerevisiae* GIL77 lacking the lanosterol synthase gene. The grown yeast cells were collected by centrifugation, and then subjected to heat treatment with 15% KOH/MeOH at 80 °C. Hexane was added to the MeOH solution, and the hexane extract was analyzed with a GC instrument (Fig. 2). As reported by Kajikawa *et al.* [20], we also detected β -amyrin by GC/MS. In addition to β -amyrin (**2**), very small amounts of other two triterpenes, **3** and **4** (m/z 426, M^+), were found, although Kajikawa *et al.* did not refer to the production of **3** and **4**. The two products were isolated as follows. The hexane extract was first fractionated by SiO₂ column chromatography [hexane/tetrahydrofuran (THF) = 100 : 1] to collect the triterpene fraction, which was acetylated with Ac₂O/pyridine. The triterpene acetate mixtures were subjected to repeated separation by normal-phase HPLC with a mixture of hexane and THF (100 : 0.15), leading to the isolation of pure β -amyrin (**2**). However, separation of the two minor triterpene acetates was unsuccessful, despite the use of RP-HPLC (C₁₈). Complete separation was attained by using a

chiral column (CHIRALPACK IB, Daicel; hexane/THF = 100 : 0.05). The former peak (**3**) on GC was identified as butyrospermol [eupha-7(8),24-diene-3 β -ol] and the latter peak (**4**) was tirucall-7(8),24-diene-3 β -ol by the detailed NMR analyses, including two-dimensional NMR spectra (Figs S1 and S2). The structural difference between **3** and **4** is found only in the stereochemistry at C20. The stereochemistry was determined to be 20*R* for **3**, but 20*S* for **4**, on the basis of the reported chemical shifts of Me-21 in ¹H-NMR [21]. The detailed NMR analyses of **3** and **4** are described in Figs S1 and S2. The **2/3/4** product ratio was determined to be 96.6 : 1.6 : 1.8 by GC analysis. Formation of the tetracyclic **3** and **4**, albeit in negligible amounts, suggests that the polycyclization reaction stopped at the premature cyclization stage. Folding of the *chair-chair-chair-chair* conformation could give **3**, whereas folding of the *chair-chair-chair-boat* conformation could give **4**, according to the reaction scheme of Fig. 3. Formation of the tetracyclic compounds **3** and **4** indicates that the β -amyrin synthase is somewhat loosely packed around D-ring and E-ring formation sites [1–6], but the amounts of **3** and **4** produced were negligible, indicating that this amyrin synthase is a monofunctional OSC.

Purification of the recombinant β -amyrin synthase (pYES2/CT-EtAS)

The EtAS gene was ligated into the pYES2/CT vector, in order to purify the EtAS with an Ni²⁺-nitrilotriacetic acid column. The construct pYES2/CT-EtAS was transformed into *S. cerevisiae* GIL77. This strain also produced the two minor triterpenes, **3** and **4**, in addition to a large quantity of **2**. The product ratio was the same as that of pYES2-EtAS. The β -amyrin synthase is expressed as polyhistidine-tag fusion protein (His₆, C-terminus), which enabled complete purification in a single step by use of an Ni²⁺-nitrilotriacetic acid column (Fig. 4A). The yeast cells from a 0.5-L culture were suspended in 30 mL of binding buffer (20 mM Tris/HCl, pH 7.9, containing 10 mM imidazole, 300 mM NaCl, and 0.1% Triton X-100), and then disrupted with glass beads (60 g). The supernatants obtained by centrifugation were pooled after repeated extractions, and then loaded onto the Ni²⁺-nitrilotriacetic acid resin column. The bound protein was washed with the buffer containing 40 mM imidazole, and finally eluted with the same buffer solution, which also contained 250 mM imidazole. As shown in Fig. 4A (lane 5), the eluted β -amyrin synthase resulted in a single band on 10% SDS/PAGE. The molecular mass estimated from the SDS/PAGE gel mobility was 77–79 kDa (Fig. S3), and the apparent

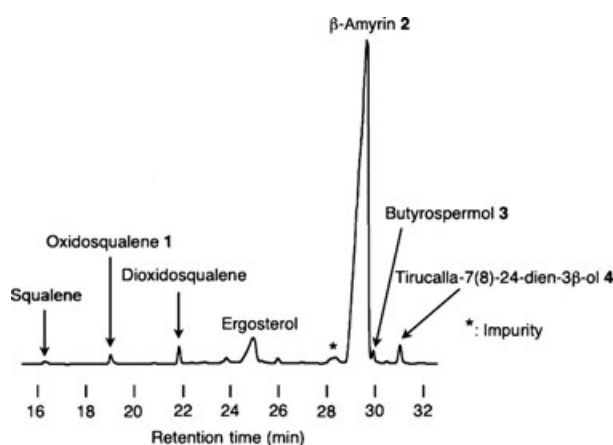


Fig. 2. GC trace of the hexane extract from the cultured *S. cerevisiae* GIL77 harboring pYES2-EtAS. GC conditions: injection, 270 °C, column temperature, 220–270 °C (rate, 2 °C·min^{−1}); GC column, J&D, DB-1, capillary (length, 30 m; internal diameter, 0.32 mm; film thickness, 0.25 μm).

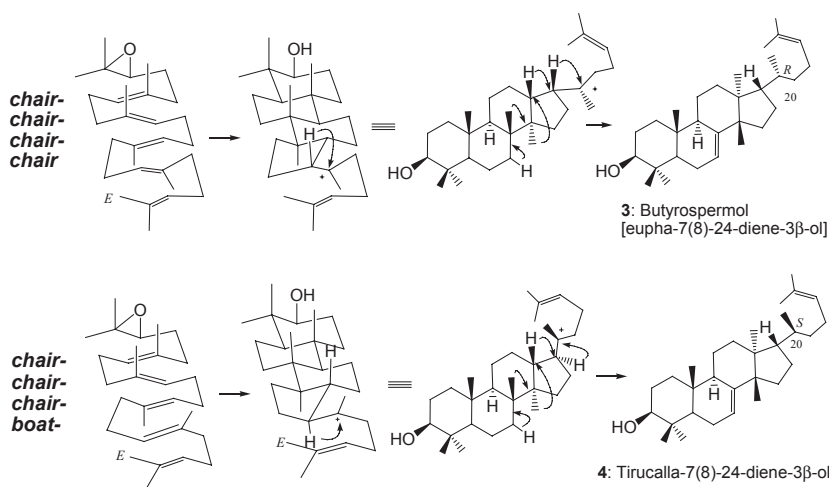


Fig. 3. The polycyclization pathway of (3*S*)-2,3-oxidosqualene into butyrospermol (**3**) and tirucalla-7(8)-24-diene-3 β -ol (**4**).

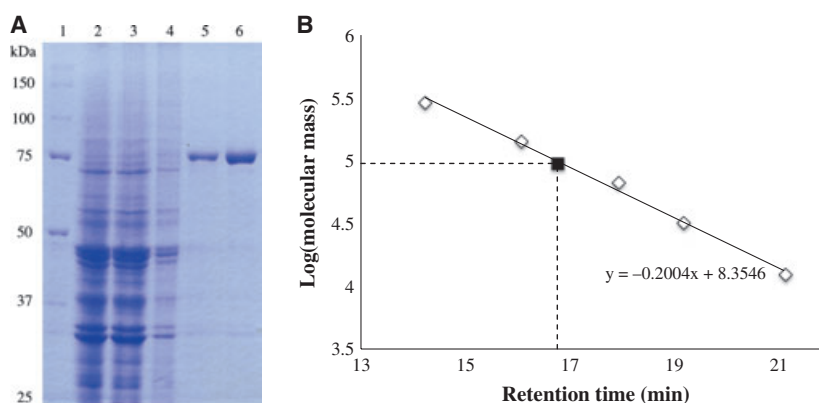


Fig. 4. (A) SDS/PAGE of the expressed EtAS. Lane 1: molecular mass marker. Lane 2: total protein. Lane 3: soluble protein. Lane 4: insoluble protein. Lane 5: purified EtAS [washed with Tris/HCl buffer (20 mM, pH 7.9) including 40 mM imidazole, 300 mM NaCl, and 0.1% Triton X-100, and then eluted with Tris/HCl buffer solution (20 mM, pH 7.9) composed of 250 mM imidazole, 300 mM NaCl, and 0.1% Triton X-100. Lane 6: purified EtAS [washed with Tris buffer (20 mM, pH 7.9) composed of 80 mM imidazole, 300 mM NaCl, and 0.01% Brij35, and then eluted with Tris buffer (20 mM, pH 7.9) containing 250 mM imidazole, 300 mM NaCl, and 0.01% Brij35. Lanes 5 and 6 differ in type of detergent employed. (B) Molecular mass calibration curve based on the retention times of standard proteins obtained by gel-filtration HPLC. The conditions were as follows. Column: TSKgel G3000SW_{XL}. Mobile phase: 50 mM potassium phosphate buffer (pH 7.0), 0.005% Brij35, and 0.2 M KCl. Flow rate: 0.5 mL·min⁻¹, detected at 280 nm. Standard proteins: glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142.0 kDa), enolase (67 kDa), myokinase (32.0 kDa), and cytochrome *c* (12.4 kDa). The molecular mass of EtAS was determined to be 94.8 kDa).

mass was smaller than the calculated one (91.1 kDa). A significant difference between the calculated and apparent molecular masses is frequently encountered; for example, for fission yeast proteins, the observed difference was reported to be at the level of 10–30% [22]. We also found that the molecular mass of the Rv3378c enzyme from *Mycobacterium tuberculosis* estimated with SDS/PAGE was significantly lower (~20%) than the calculated one [23], but MALDI-TOF MS analyses of the proteolytic digestion fragments showed that the expressed protein was actually of full length [23]. A gel filtration experiment showed that the actual size of this triterpene synthase was 94.8 kDa (Fig. 4B), indicating

that EtAS exists as a monomer, as does human lanosterol synthase [18]. In contrast, bovine liver lanosterol synthase was reported to form a dimeric structure [19]. The amount of EtAS expressed by pYES2/CT was estimated by western blotting using an SDS/PAGE gel. The amount of EtAS expressed from 1 L of culture was estimated to be 5.5–6.7 mg. The purified protein was obtained in ~50% yield. To measure the CD spectrum of EtAS, Triton X-100 was replaced by Brij35, because Triton X-100 and Tween-80 have benzene rings and double bonds in the molecules that electronically absorb in the UV region and interfere with the CD spectrum, whereas Brij35 has no such functional groups. The

bound EtAS on the Ni^{2+} -nitrilotriacetic acid column, described above, was washed with a buffer (80 mL) containing a mixture of 0.01% Brij35 and 80 mM imidazole, and then eluted with a buffer (4 mL) including 250 mM imidazole (Fig. 4A, lane 6). After dialysis, the CD spectrum was measured (Fig. 5), and showed that the enzyme architecture was not altered at temperatures $< 30^\circ\text{C}$. The enzymatic activity was significantly lower in Brij35 detergent (Fig. 6A). However, substantial molecular ellipticity was observed, suggesting that a higher-order protein structure was maintained in Brij35 solution. Furthermore, the CD spectrum was quite similar to that of SHC, and was superimposable on that measured in a different type of detergent, β -octylglucoside (β -OG) (Fig. S4A).

Optimal catalytic conditions and kinetic parameters

Figure 6 shows the determination of optimal incubation conditions. The enzymatic activities with respect

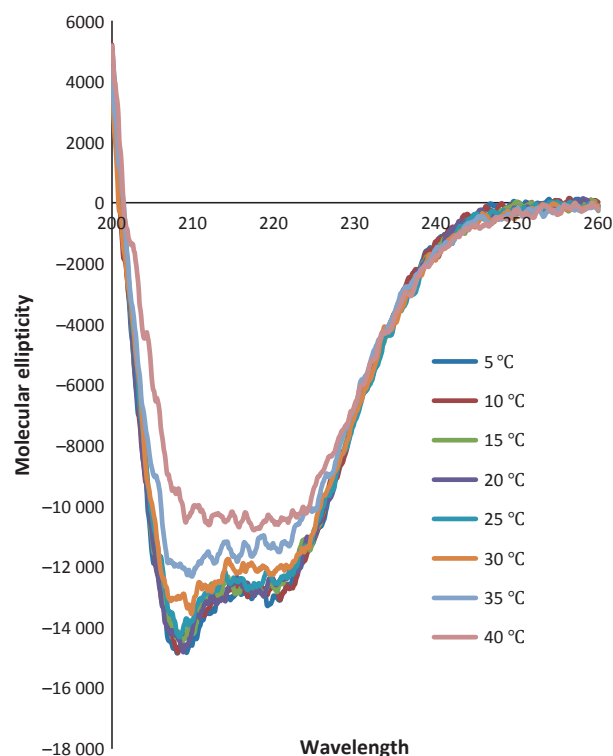


Fig. 5. CD spectra of the wild type measured at 5–40 $^\circ\text{C}$. The CD spectra remained unchanged at temperatures of 5–30 $^\circ\text{C}$, but decreased molecular ellipticity and an altered wave shape were observed with increasing temperatures, indicating that the protein architecture was altered at temperatures $> 30^\circ\text{C}$, which is consistent with the results shown in Fig. 6B (optimum catalytic temperature: 30 $^\circ\text{C}$).

to the detergent concentrations are shown in Fig. 6A. The following four different detergents were utilized: Triton X-100, Tween-80, β -OG, and Brij35. The highest activity was seen for Triton X-100, at a concentration of 0.05% (w/v). The activity in Tween-80 was nearly equal to that in Triton X-100. In the case of β -OG, reasonably high activities were observed at concentrations less than the critical micelle concentration (CMC, 0.6%), but the activities gradually decreased at higher concentrations. In Brij35, the activities were significantly low, as shown in Fig. 6A. Brij35 (0.01%) was replaced with Triton X-100 (0.05%) by use of a His-tag column, and the activity was then assayed. No decrease in the enzymatic activity was found (Fig. S4B). Thus, there was no protein denaturation by Brij35. No effect of dithiothreitol on the enzymatic activity was observed in the concentration range of 0–10 mM (Fig. S5). In contrast, in the case of the cycloartenol synthase from *Ochromonas malhamensis*, supplementation with 5 mM dithiothreitol gave an approximately two-fold enhancement of the activity [24]. The optimal temperature and pH for the catalysis were 30 $^\circ\text{C}$ and 7.0, respectively (Fig. 6B,C). The kinetic parameters relating to enzymatic activity were measured under optimal catalytic conditions. Figure 6D shows that the enzymatic activities were linearly increased for up to 30 min after the incubation. Kinetic parameters were obtained by estimating the amounts of β -amyrin produced after incubation for 20 min. The specific activity of the OSC was estimated to be $352 \pm 11.8 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. The Michaelis–Menten plot is shown in Fig. 7. Steady-state parameters were determined by fitting the curve to $v = V_{\text{max}}[\text{S}]/(K_{\text{m}} + [\text{S}])$, with the SOLVER program of Microsoft Excel. The values of K_{m} and k_{cat} for (3S)-2,3-oxidosqualene (**1**) were determined to be $33.8 \pm 0.53 \mu\text{M}$ and $46.4 \pm 0.68 \text{ min}^{-1}$, respectively, and $k_{\text{cat}}/K_{\text{m}}$ was $1.37 \pm 0.21 \mu\text{M}^{-1}\cdot\text{min}^{-1}$ (Table 1). Table 1 lists the kinetic data of other OSCs [19,25–28] and SHCs [27,29,30] that have been reported to date. The difference in K_{m} values between the OSCs was not significant, but the k_{cat} and specific activity of EtAS were appreciably higher than those of the other three OSCs, indicating that the enzymatic activity was retained during the purification process. The k_{cat} and specific activity of SHCs are remarkably higher than those of OSCs, but the reported kinetic data differed appreciably between the three research groups [27,29,30]. This may have been caused by the use of different assay methods and different detergents [30]. It is difficult to determine whether the substrate inhibition occurs at higher concentrations, because oxidosqualene substrate **1** was not dissolved at concentrations $> 112.7 \mu\text{M}$.

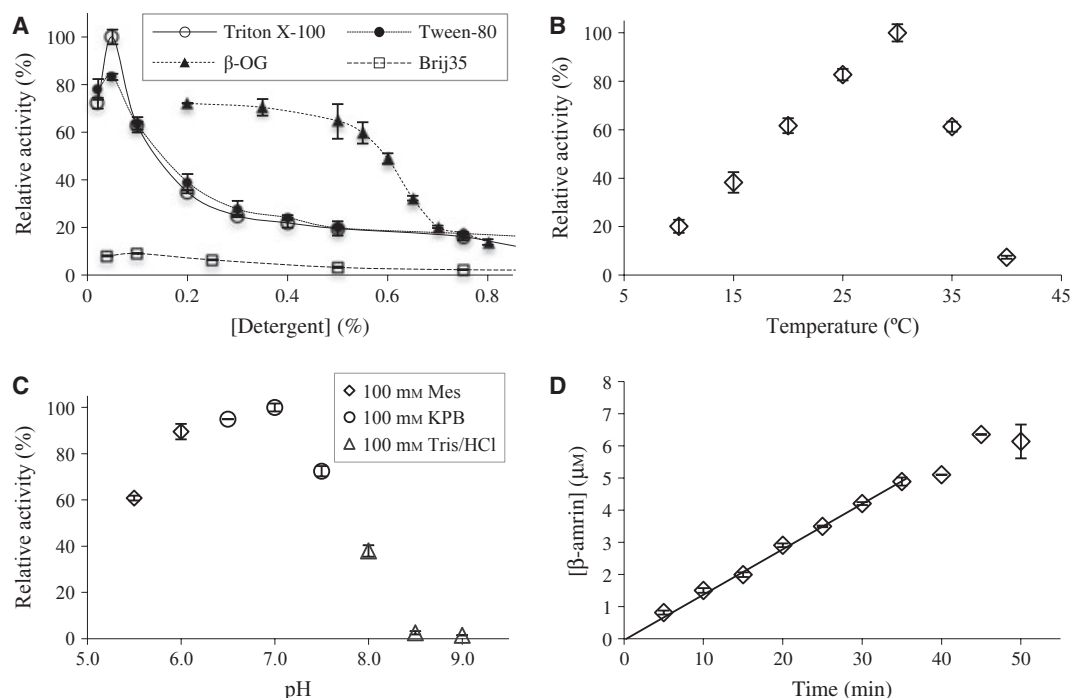


Fig. 6. (A) Effect of detergent concentrations on the specific activities. The relative activities are plotted. Four different detergents were employed: 0.02–0.75% (w/v) for Triton X-100; 0.02–0.75% (w/v) for Tween-80; 0.2–0.8% (w/v) for β -OG; and 0.04–0.75% (w/v) for Brij35. The (3*S*)-oxidosqualene concentration used in these experiments was 47 μ M. To prepare a solution of oxidosqualene (47 μ M) dissolved in β -OG, concentrations > 0.2% were required. (B) Determination of optimal temperature. (C) Determination of optimal pH. The buffer solutions used in the experiment were as follows: 0.1 M Mes buffer for pH 5.5–6.0; 0.1 M potassium phosphate buffer (KPB) for pH 6.5–7.5; and 0.1 M Tris/HCl buffer for pH 8.0–9.0. (D) Determination of incubation times. On the basis of these data, the enzymatic reactions were conducted for 20 min to determine the values of K_m and k_{cat} . The (3*S*)-substrate concentration was 113 μ M for (B)–(D), where Triton X-100 (0.05%, w/v) was included in the incubation mixture (highest activity; Fig. 6A). The error bars show the difference between two independent experiments. The specific activity corresponding to 100% relative activity was 352 nmol·min^{−1}·mg^{−1}.

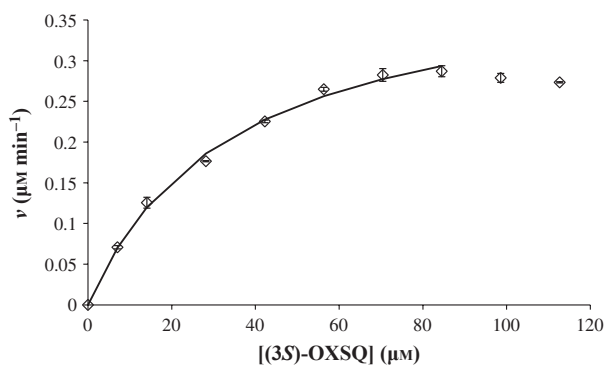


Fig. 7. Michaelis–Menten plot of β -amyrin synthase with (3*S*)-2,3-oxidosqualene. Kinetic parameters were obtained by fitting the data to the Michaelis–Menten equation by using the SOLVER function in Excel (Microsoft, Redmond, WA, USA) as follows: $K_m = 33.8 \pm 0.53$ μ M; $k_{cat} = 46.4 \pm 0.68$ min^{−1}; $k_{cat}/K_m = 1.37 \pm 0.21$ min^{−1}· μ M^{−1}. The error bars show the differences between two independent experiments. The two values at the high concentrations of substrate 98.6 and 112.7 μ M were omitted, owing to the occurrence of substrate inhibition.

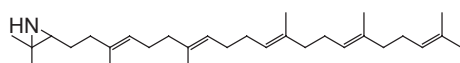
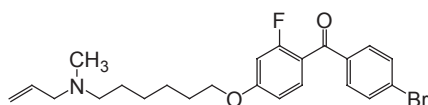
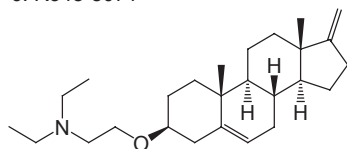
However, slight inhibition was observed at concentrations > 98.6 μ M (Fig. 7), suggesting that substrate inhibition for EtAS had occurred. SHC, one of the triterpene cyclases, also showed substrate inhibition in our experiment (unpublished result). No change in EtAS activity was observed for at least 2 months when it was stored at -80 °C.

Kinetics of inhibition of EtAS by iminosqualene (5), Ro48-8071 (6), and U18666A (7)

Figure 8 shows the structure of known OSC inhibitors (5–7). The inhibitory effects of these three compounds on the activities of EtAS were examined (Table 2; Fig. 9). Inhibition constants were determined with Dixon plots [31]. The (3*R,S*)-racemic mixture of iminosqualene (5) was chemically synthesized according to the reported method [32–34]. The synthetic method for 5 is described in Fig. S6. Compound 5 strongly inhibited the activity of EtAS: IC₅₀ of 30.9 nM, and K_i of 13.4 nM. Corey *et al.* first reported the potent

Table 1. Kinetic data for EtAS, lanosterol cyclases and SHCs that have been reported to date. NR, not reported.

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1}\cdot\text{min}^{-1}$)	Specific activity ($\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)
EtAS [this study]	33.8 ± 0.53	46.4 ± 0.68	1.37 ± 0.21	352 ± 11.8
Bovine lanosterol synthase [19]	11	1.6×10^{-2}	1.45×10^{-3}	1.747
Rat lanosterol synthase [25–27]	15 [25]	3.0×10^{-2} [25]	2×10^{-3} [25]	8.8×10^{-2} [25]
	55 [26]	NR	NR	26.16 [26]
	86 [27]	NR	NR	NR
<i>S. cerevisiae</i> lanosterol synthase [28]	18	NR	NR	40.8
SHC [27,29,30]	16.7 [29]	289 [29]	17.3 [29]	1910 [29]
	1.6 [27]	2.4 [27]	1.5 [27]	NR
	38 [30]	72 [30]	1.89 [30]	NR

**5:** 2,3-iminosqualene**6:** Ro48-8071**7:** U18666A**Fig. 8.** Chemical structures of the inhibitors employed in this study.

inhibition of hog liver lanosterol cyclase by **5** [32]. Duriatti *et al.* reported that **5** also inhibited pea seedling β -amyrin synthase (IC_{50} of $0.3 \mu\text{M}$; K_i of $0.2 \mu\text{M}$) and rat liver lanosterol synthase (IC_{50} of $0.3 \mu\text{M}$; K_i of $0.4 \mu\text{M}$), and that the inhibition of both enzymes occurred in a noncompetitive fashion [35], which differed from our result; Fig. 9A indicates that **5** exhibited competitive and potent inhibition of EtAS (IC_{50} of 30.9 nM ; K_i of 13.4 nM), but less potent inhibition of pea seedling β -amyrin synthase and rat liver lanosterol synthase (Table 2). No other report has described the inhibition of OSCs by **5**. The nitrogen atom of **5** is protonated under physiological pH to yield an aziridinium ion, which mimics the protonated oxirane ring (transition-state structure). Thus, the inhibition appeared to be competitive but not noncompetitive.

Ro48-8071 (**6**) was also a potent inhibitor (IC_{50} of 10.7 nM ; K_i of 7.0 nM). As shown in Table 2, **6** is a potent inhibitor of the lanosterol synthases from

bovines [19], rats [27,35], and humans [18,36,37], and of the SHC from *Alicyclobacillus acidocaldarius* [27,30]. The IC_{50} values for EtAS ($10.7 \mu\text{M}$) were determined as shown in Fig. S7, and they were nearly the same as those for the lanosterol synthases and SHC. Lineweaver–Burk plots (Fig. 9B) indicated that the inhibition was mixed noncompetitive. Abe *et al.* concluded that this inhibitor appeared to be a noncompetitive inhibitor of rat lanosterol synthase, although their experimental data in Lineweaver–Burk plots clearly indicate mixed noncompetitive inhibition [27]. This mode of inhibition indicates that there is a second binding site in addition to the active site. However, X-ray analyses of cocrystals of **6** with human OSC [36] and with SHC [30] revealed that this inhibitor is located only in the reaction cavity, suggesting that **6** should behave as a competitive inhibitor. Fluorescence titration experiments showed a 1 : 1 binding ratio for human OSC and **6** [18], further supporting competitive inhibition. These findings for human OSC and SHC imply that the plant EtAS also undergoes competitive inhibition by **6**, i.e. that a secondary binding site is not involved. To explain this inconsistency between the kinetic and the X-ray data, Ruf *et al.* [18] and Lenhart *et al.* [30] suggested that the biphasic state needed for the enzymatic activity measurements could have influenced the kinetics as a result of altered accessibility of the substrate and inhibitor in mixed micelles. X-ray analysis of the complex of **6** with human lanosterol synthase revealed the following findings [36]: (a) Asp455 is hydrogen-bonded to the nitrogen atom of the amine group; (b) the fluorophenyl group is stacked between the side chains of Phe696 and His232; and (c) the terminal bromophenyl group interacts with Trp192, Trp230, and Phe521. These Asp, Trp and Phe residues are highly conserved among EtAS, bovine OSC, rat OSC, and SHCs (Table S1). These highly conserved residues would have significantly contributed to the potent inhibition of various triterpene cyclases by **6**.

Table 2. Comparison of the inhibitory activities against the OSCs by **5–7** and inhibition types. K_i values were determined by Dixon plots [31]. The K_i value of **5** was calculated as the [3*R,S*]-racemic mixture, because it is likely that both the *R*-configuration and the *S*-configuration behave as the inhibitors. NR, not reported.

	IminoSQ (5)			Ro48-8071 (6)			U18666A (7)		
	IC ₅₀ (nM)	K_i (nM)	Inhibitory type	IC ₅₀ (nM)	K_i (nM)	Inhibitory type	IC ₅₀ (nM)	K_i (nM)	Inhibitory type
EtAS (this study)	30.9	13.4 ± 1.3	Competitive	10.7	6.98 ± 0.42	Mixed noncompetitive	142	102 ± 4.4	Noncompetitive
Pea β -amyrin synthase [35]	300	200	Non-competitive	NR	NR	NR	250	NR	NR
Bovine lanosterol synthase [19]	NR	NR	NR	11	NR	NR	NR	NR	NR
Rat lanosterol synthase [27,35]	300 [35]	400 [35]	Non-competitive	40 [27]	22 [27]	Noncompetitive [35] Mixed noncompetitive [18]	800 [35]	NR	NR
Human lanosterol synthase [18,36]	NR	NR	NR	6.5 [36] (cf. 8.0 [18] for recombinant)	NR	Competitive [18]	NR	NR	NR
Squalene-hopene cyclase [27,30]	NR	NR	NR	9.0 [27] 61.0 [30]	6.6 [27] NR [30]	Noncompetitive [27] Mixed noncompetitive [18,30]	NR	NR	NR

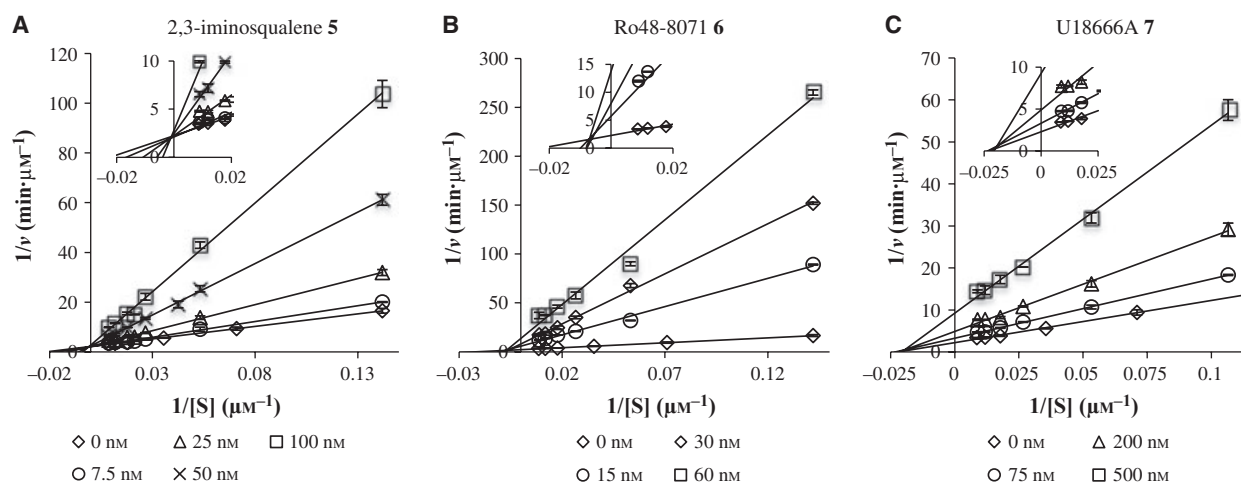


Fig. 9. Lineweaver–Burk plots for determination of the type of inhibition exerted by inhibitors **5**, **6** and **7** towards EtAS. The completely purified wild-type enzymes were used. The double reciprocal plots indicated that their inhibitory types were as follows: competitive for **5**; mixed noncompetitive for **6**; and noncompetitive for **7**. The error bars show the differences between two independent experiments.

It has been reported that U18666A (**7**) inhibits rat liver lanosterol synthase and pea seedling β -amyrin synthase at IC₅₀ levels of 0.8 and 0.25 μ M, respectively, but this inhibition type has not been reported previously [35]. The IC₅₀ and K_i for EtAS were determined

to be 0.14 μ M and 102 nM, respectively. The inhibition appeared to be noncompetitive (Fig. 9C), so **7** might bind to the outer surface area of EtAS, leading to a loss of enzymatic activity because of the altered protein architecture. The inhibitory activity of **7** was rela-

Table 3. The enzymatic activities of the D485, C486 and C564 variants. ND: the enzyme activity was not detected and was completely lost. The enzyme amounts of the C486A and C564A variants used for obtaining the kinetic data were 5 μ g (22 nM) and 10 μ g (44 nM), respectively. These kinetic data were determined by incubation at 30 °C. Other incubation conditions were the same as those for the wild type (see Experimental procedures).

	K_m (μ M)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1}\cdot\text{min}^{-1}$)	Specific activity ($\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	Activity relative to the specific activity (%)
Wild	33.8 ± 0.53	46.4 ± 0.68	1.37 ± 0.21	352 ± 11.8	100
D485N	ND	ND	ND	ND	0
D485E	ND	ND	ND	ND	0
C486A	34.8 ± 3.4	20.4 ± 1.0	0.59 ± 0.03	163 ± 5.7	46
C564A	51.1 ± 4.1	$(0.77 \pm 3.6) \times 10^{-3}$	$(0.015 \pm 6.9) \times 10^{-5}$	5.79 ± 0.085	1.6

tively moderate as compared with those of **5** and **6** (Table 2).

Enzymatic activities of site-directed mutated EtAS targeted for the DCTAE motif

The DCTAE motif and the DXDD motif are highly conserved in eukaryotic OSCs and prokaryotic SHCs, respectively (Fig. S8). We have previously reported the enzyme kinetic data for the mutated SHCs targeted for the DXDD motif, and have demonstrated that the DXDD motif plays a crucial role in the initiation of the polycyclization reaction by a proton attack on the terminal double bond of the squalene molecule [29]. It has been predicted that the DCTAE motif corresponding to the DXDD motif works to initiate the polycyclization reaction. The attack of the proton, which is released from the acidic Asp, on the terminal epoxide ring of **1** triggers a cascade of the ring-forming reaction [36,38]. However, kinetic studies on the site-directed mutated OSCs targeted for the DCTAE motif have not been reported thus far. Corey *et al.* proposed that Asp456 of the DCTAE motif of *S. cerevisiae* lanosterol synthase is responsible for initiating the cyclization, on the basis of the result of the viability assay of the D456N variant [38], but no kinetic data have been provided. On the basis of X-ray crystallographic analysis of human lanosterol synthase, Thoma *et al.* proposed that the acidity of Asp455 of the DCTAE motif is enhanced by the hydrogen bond formed with Cys456 and Cys533 [36], but no kinetic evidence for the role of the Cys residues has been reported. To verify the functions of these three residues of EtAS, four variants (D485N, D485E, C486A, and C564A) were constructed. Figure 10 shows the relative activities versus incubation temperatures. The optimum temperatures of the mutants were slightly changed, but were little different from that of the wild type. The enzymatic activities of the D485N and D485E variants were completely lost at all tempera-

tures examined. The activity of the C486A variant was somewhat retained ($\sim 50\%$ of the wild type), whereas that of the C564A variant was almost completely lost. The kinetic data are shown in Table 3. The absence of activity of the D485N variant indicates that the acidic carboxylic residue Asp serves as a proton donor to initiate the polycyclization reaction, but the Glu variant also showed no activity, despite the acidic residue, indicating that the extra CH_2 moiety attached to Asp had interfered with the access of the carboxyl group to the epoxide ring. Thus, an appropriate distance of the carboxyl moiety from the epoxide ring must be present for cyclization to be initiated. Cys564 is more strongly hydrogen-bonded with the carboxyl residue Asp458 than with Cys486, because the C564A variant had significantly decreased activity (1.6% of the wild type), whereas the Cys486-mutated EtAS retained $\sim 50\%$ activity (Fig. 10; Table 3). The CD spectra of the four mutated EtASs were the same as that of the wild type (Fig. 11), indicating that the protein architecture was

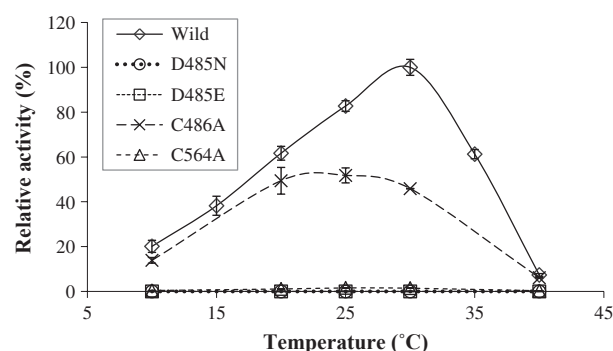


Fig. 10. Specific activities of the wild type and the mutated EtASs plotted against incubation temperature. The completely purified wild-type enzymes were used. The maximum specific activity of the wild type was estimated to be $352 \pm 11.8 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. The optimum temperatures of the mutated EtASs were slightly changed, but with very little difference from that of the wild type. The error bars were determined in two separate experiments.

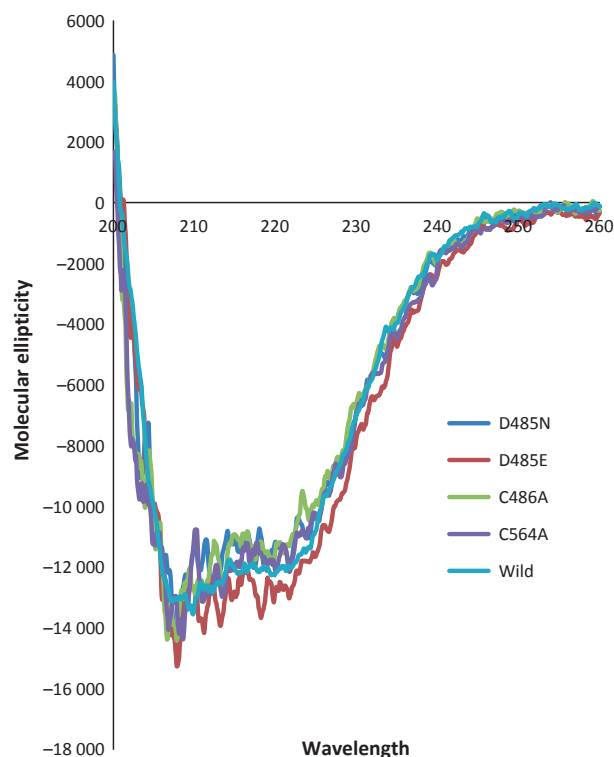


Fig. 11. CD spectra of the wild type and the site-directed mutated EtASs (measured at 30 °C). The CD intensities and wave shapes remained unchanged by the mutations. Structural alteration did not occur.

not altered or was only slightly altered by these mutations.

Estimation of *in vivo* enzymatic activities of mutants

The expression of mutated OSCs is frequently found to be absent or significantly decreased. To analyze *in vivo* the functions of the site-directed variants, it is crucial to determine both the amounts of the expressed OSCs and the quantities of the triterpenes produced by the mutants; the relative activities must then be compared with that of the wild type. The enzymatic activities are determined by dividing the amounts of product produced *in vivo* by the amount of EtAS expressed in the mutants and the wild type. Figure 12 shows the relative activities estimated by *in vivo* experiments. The amounts of protein expressed were estimated by western blot analysis (approximately 5.5–6.7 mg for both the wild type and mutants per liter of culture; Fig. S9), and the quantities of β -amyrin were determined by GC analysis. No other triterpene was found to be produced by these variants. The vertical

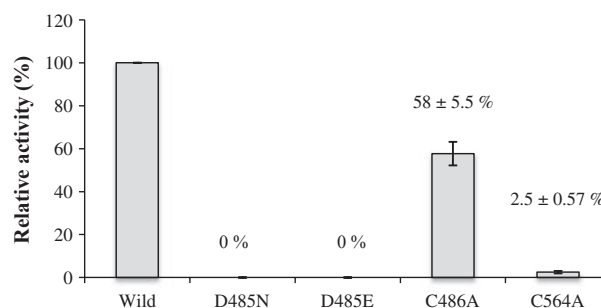


Fig. 12. The activities of the mutants relative to that of the wild type, estimated by *in vivo* experiments. The amounts of EtAS expressed were measured by western blotting: 5.99 ± 0.58 mg·L⁻¹ (per liter of culture) for the wild type; 6.70 ± 1.96 mg·L⁻¹ for D485N; 5.55 ± 0.25 mg·L⁻¹ for D485E; 6.30 ± 0.62 mg·L⁻¹ for C486A; and 6.22 ± 0.36 mg·L⁻¹ for C564A. These protein amounts were determined in three independent experiments. The amounts of β -amyrin produced by the different strains were estimated by GC, and the relative activity was determined to be $58 \pm 5.5\%$ for C486A and $2.5 \pm 0.57\%$ for C564A.

line indicates the activity of the mutants relative to that of the wild type. No activity was observed for the D485N and D485E variants, as was also observed in the *in vitro* experiments with the purified enzymes (Fig. 10; Table 3). The C486A variant showed ~58% activity relative to the wild type, and the C564A variant only 2.5% activity. These results of the *in vivo* experiments (Fig. 11) are in agreement with the results of the *in vitro* experiments (Fig. 10; Table 3). Thus, it can be concluded that the *in vivo* assay could be an alternative to the *in vitro* assay in cases where the enzymatic activities of the mutated OSCs are too weak for measurement of the K_m and k_{cat} and/or the expression levels of the mutated OSCs are significantly low.

There are presently several reports identifying the functions of the active site residues of some OSCs. This is especially true for *S. cerevisiae* lanosterol and plant cycloartenol synthases, the functions of which were inferred mainly from the product ratios of each of the triterpenes produced *in vivo* by the mutants [39–42]. However, no report describing the OSC expression level of the mutants has since been published. We achieved the first complete purification of a β -amyrin synthase. Furthermore, we report that the enzymatic activities of the mutated β -amyrin synthases can be estimated by *in vitro* and/or *in vivo* experiments. CD measurements are necessary for probing of the protein architecture. However, the CD spectrum of any OSC has never been reported to date. We successfully obtained the first CD spectrum of a β -amyrin synthase. On the basis of these studies, we propose a method with which to determine the exact function of active site residues. We are now

identifying the functions of the active site residues through site-directed mutagenesis experiments, followed by enzyme activity analysis. These results will be reported in the near future.

Experimental procedures

Instruments and chemicals

NMR spectra were recorded in CDCl_3 with a Bruker DMX 600 spectrometer. GC analyses were performed on a Shimadzu GC-2014 chromatograph fitted with a flame ionization detector (J&W DB-1 capillary column, 0.32 mm $\times$ 30 m). GC/MS spectra were obtained with a JMS-Q1000 GC K9 (JEOL) instrument under electronic impact at 70 eV, with a Zebron ZB-5 ms capillary column (0.25 mm $\times$ 30 m), and the oven temperature being elevated from 220 to 270 $^{\circ}\text{C}$ (3 $^{\circ}\text{C}\cdot\text{min}^{-1}$). CD spectra were measured on a Jasco J-725 with a 0.1-cm quartz cell (Jasco; Tachikawa, Tokyo, Japan). EtAS was dissolved in 50 mM potassium phosphate buffer containing 0.01% Brij35 (0.1 $\text{mg}\cdot\text{mL}^{-1}$). Compounds **1** and **5** were synthesized as previously described [29,32–34]. Compounds **6** and **7** were from Sigma (St Louis, MO, USA) and Merck Calbiochem (Darmstadt, Germany), respectively. β -OG and Tween-80 were from Kanto Kagaku (Tokyo, Japan). Triton X-100 and Brij35 were from Wako Pure Chemical Industries (Osaka, Japan). Restriction enzymes were from Toyobo Co. (Osaka, Japan).

Cloning, expression in yeast, and protein purification

Construction of pYES2–EtAS, expression of EtAS in *S. cerevisiae* GIL77, and analysis of triterpene products

The pPICZ A plasmid harboring the EtAS gene was kindly provided by K. Ohya (Ishikawa Prefectural University, Japan). The EtAS gene was amplified by PCR with the following primers: as sense primer, 5'-CACGTTGAAAAG CTTATGTGGAAGCTGAAG-3' (the *Hind*III site is underlined); and as antisense primer, 5'-GGCTGGGCGG CCGCTTAAAGAGTAG-3' (the *Not*I site is underlined). PCRs were conducted with a 30-cycle program: 98 $^{\circ}\text{C}$ for 1 min, 60 $^{\circ}\text{C}$ for 1 min, 68 $^{\circ}\text{C}$ for 2 min, 68 $^{\circ}\text{C}$ for 2 min, and a final extension at 68 $^{\circ}\text{C}$ for 7 min. KOD-plus DNA polymerase (Toyobo) was used with dNTPs (0.2 mM) in a final volume of 50 μL , according to the manufacturer's protocol. The PCR product was digested with *Hind*III and *Not*I (Toyobo). The resulting 2.3-kbp fragment was ligated into the yeast expression vector pYES2 (Life Technologies, Carlsbad, CA, USA), which was previously digested with the same restriction enzyme. The constructed plasmid was named pYES2–EtAS. The integrity of the gene was verified by sequencing. The expression plasmid pYES2–EtAS was

transformed into *S. cerevisiae* GIL77 lacking lanosterol synthase (*erg7*, *ura3-167*, *hem3-6*, *gal2*), by use of a Frozen-EZ Transformation II Kit (Zymo Research, Irvine, CA, USA). The transformants were plated onto synthetic complete medium without uracil (SC-U), supplemented with ergosterol (20 $\mu\text{g}\cdot\text{mL}^{-1}$), hemin chloride (13 $\mu\text{g}\cdot\text{mL}^{-1}$), and Tween-80 (5 $\text{mg}\cdot\text{mL}^{-1}$), and then incubated at 30 $^{\circ}\text{C}$ for 3 days. Expression of EtAS in the yeast was conducted as reported by Kushiro *et al.* [11]. A 100-mL culture of *S. cerevisiae* GIL77 harboring pYES2–EtAS was grown in SC-U supplemented with ergosterol (20 $\mu\text{g}\cdot\text{mL}^{-1}$), hemin chloride (13 $\mu\text{g}\cdot\text{mL}^{-1}$), and Tween-80 (5 $\text{mg}\cdot\text{mL}^{-1}$) at 30 $^{\circ}\text{C}$ with shaking (150 r.p.m.). After being grown for 2 days, cells were collected and resuspended in SC-U without glucose, supplemented with ergosterol, hemin chloride, and Tween-80, and 2% galactose was then added to induce EtAS expression at 30 $^{\circ}\text{C}$ for 24 h. The yeast cells were collected and resuspended in 0.1 M potassium phosphate (pH 7.0) supplemented with 3% glucose and hemin chloride, and further incubated at 30 $^{\circ}\text{C}$ for 24 h. KOH/MeOH (15%) was added to the grown cells collected, and the suspension was refluxed by heating to disrupt the cells. The lipophilic materials including the cyclization products were extracted with hexane, and the extract was subjected to GC/MS and GC analysis.

Construction and expression of polyhistidine-tagged EtAS

The pYES2–EtAS construct was used as template. The following primers were employed for PCR amplification: 5'-GAATATTAAGCTTGGTACCATGTGGAAGC-3' (the *Kpn*I site is underlined) as sense primer; and 5'-CTCG AGCCTCGAGTAAAAGAGTAGTGGAAG-3' (the *Xba*I site is underlined) as antisense primer, which was designed to delete the stop codon. PCR conditions were the same as described above. The amplified PCR product was digested with *Kpn*I and *Xba*I (Toyobo). The resulting fragment was ligated into the yeast expression vector pYES2/CT (Life Technologies), which was previously digested with the same restriction enzymes. The constructed plasmid was named pYES2–EtAS/CT, and was introduced into *S. cerevisiae* GIL77. His-tagged EtAS (His₆) was expressed by culture of the transformed yeast and by induction with galactose, added according to the same protocols described above. The cultured cells collected by centrifugation (10 000 g) were disrupted with glass beads, and purified on an Ni^{2+} –nitrilotriacetic acid column according to the manufacturer's protocol (Qiagen, Valencia, CA, USA); the purity was checked by SDS/PAGE on 10% gels. The detailed purification procedure is described in the text; the imidazole concentration in the elution buffer is crucial for obtaining high purity. The protein concentration was measured by the Bradford method with a Bio-Rad protein assay solution with BSA as a standard.

CD measurements

EtAS was dissolved in potassium phosphate buffer (pH 7.0, 50 mM) containing 0.01% Brij35, and the final protein concentration was adjusted to 0.1 mg·mL⁻¹ for CD measurements. The temperatures were varied in the range 5–40 °C. The data were expressed as molar ellipticity.

In vitro enzyme assays

A reaction mixture (2.5 mL, pH 7.0, 100 mM potassium buffer) consisting of Triton X-100 (0.05%, w/v), **1** (120 μ g), BSA (1 mg·mL⁻¹), dithiothreitol (1 mM) and purified His-tagged EtAS (2 μ g, 8.8 nm) was incubated at 30 °C for 20 min. The reaction was quenched by heating at 100 °C for 3 min. To the reaction mixture, 3 mL of 15% KOH/MeOH, including squalene (25 μ g) as an internal standard, was added. The lipophilic materials were extracted with hexane, and the extract was subjected to GC analysis to measure the quantities of β -amyrin produced.

Quantitative western blotting for estimation of in vivo protein expression levels

Purified EtAS or the yeast cell lysates were dissolved or suspended in SDS/PAGE sample buffer (62.5 mM Tris/HCl pH 6.8, 2% SDS, 5% β -mercaptoethanol, 10% glycerol, 0.2 mg·mL⁻¹ bromophenol blue) and boiled for 5 min, and 2 μ L per lane was applied to 7.5% polyacrylamide gels. The amounts of purified EtAS (10–80 ng) were used to make a standard curve. The gels were electrophoresed at 40 mA for 60 min, and the proteins then were transferred to a poly(vinylidene difluoride) membrane by semidry electroblotting at 144 mA for 60 min. Blots were blocked with blocking buffer [20 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween-20, 5% (w/v) skimmed milk] overnight at room temperature, and then washed three times for 10 min each with NaCl/Tris-Tween [20 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween-20]. Blots were incubated with antibody against V5 (1 : 5000 dilution; Life Technologies) for 1 h at room temperature, and then washed three times in NaCl/Tris-Tween for 10 min each. Blots were then incubated with goat anti-(mouse IgG_{2a}) human ads-fluorescein isothiocyanate (1 : 1250 dilution; Beckman Coulter, Brea, CA, USA) in NaCl/Tris-Tween for 1 h at room temperature, and then washed three times in NaCl/Tris-Tween for 10 min each. Signal detection and imaging were performed with A Luminescent Image Analyzer LAS3000 (Fujifilm, Akasaka, Tokyo, Japan), and protein band densities were analyzed WITH MULTI GAUGE software (Fujifilm). The details are given in Fig. S9.

Mutagenesis experiments

Mutagenesis of Asp485, Cys486 and Cys564 in wild-type pYES2–EtAS/CT was performed with the QuickChange

site-directed mutagenesis method. The following oligonucleotide primers were used; substitutions are underlined.

For the D485N variant: sense primer, 5'-CATGGT TGGCAAGTTTCTAATTGCACTGCTGAAGG-3'; and antisense primer, 5'-CCTTCAGCAGTGCAATTAGAAA CTTGCCAACCATG-3'.

For the D485E variant: sense primer, 5'-GG TTGGCAAGTTTCTGAGTGCCTGCTGAAGG-3'; and antisense primer, 5'-CCTTCAGCAGTGCACTCAGAAAAC TTGCCAACCC-3'.

For the C486A variant: sense primer, 5'-GATCATGG TTGGCAAGTTTCTGATGCCACTGCTGAAGG-3'; and antisense primer, 5'-CCTTCAGCAGTGGCATCAGAAAAC TTGCCAACCATGATC-3'.

For the C564A variant: sense primer, 5'-GAACATG AATATGTAGAGGCCCACAGCATCTGCAATCCATG-3'; and antisense primer, 5'-CATGGATTGCAGATGCTGTG GCCTCTACATATTCATGTTTC-3'.

PCRs were conducted with a 16-cycle program: 98 °C for 0.5 min, 60 °C for 1 min, 68 °C for 9 min, and a final extension at 68 °C for 9 min. KOD-plus DNA polymerase (Toyobo) was used with dNTPs (0.2 mM), dimethylsulfoxide (5%) and MgSO₄ (0.1 mM) in a final volume of 50 μ L. Mutations were confirmed by DNA sequencing with the dideoxy chain-termination method and a Beckman Coulter CEQ8000 (Beckman Coulter).

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Structure determination of product **3** acetate by the detailed NMR analyses.

Fig. S2. Structure determination of product **4** acetate by the detailed NMR analyses.

Fig. S3. Determination of molecular mass by SDS/PAGE.

Fig. S4. (A) CD spectra measured in the solutions of Brij 35 and beta-OG and (B) the enzyme activity recovered after removing Brij35.

Fig. S5. Effect of dithiothreitol on enzymatic activity.

Fig. S6. Synthetic scheme of (3*R*,*S*)-iminosqualene (**5**).

Fig. S7. Determination of IC₅₀ values of inhibitors **5**–**7**.

Fig. S8. Amino acid alignment of various oxidosqualene cyclases.

Fig. S9. The method for determining the expressed amounts of EtAS proteins.

Table S1. Active site residues of human lanosterol synthase that interact with the inhibitor Ro48-8071 (**6**).