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A Putative Precursor of Isomalabaricane Triterpenoids from Lanosterol Synthase Mutants

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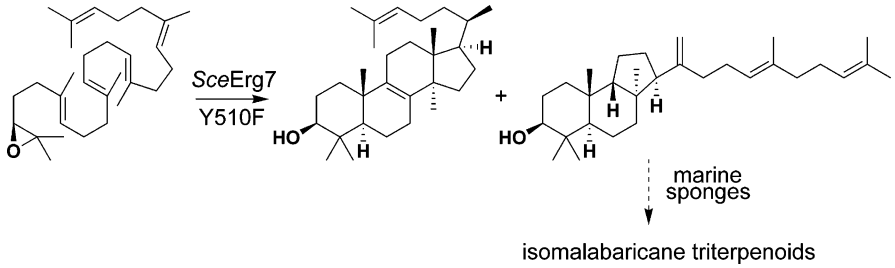
ABSTRACT



Known lanosterol synthase mutants produce monocyclic or tetracyclic byproducts from oxidosqualene. We describe Erg7 Tyr510 mutants that cause partial substrate misfolding and generate a tricyclic byproduct. This novel triterpene, (13αH)-isomalabarica-14(27),17*E*,21-trien-3*β*-ol, is the likely biosynthetic precursor of isomalabaricane triterpenoids in sponges. The results suggest the facile evolution of protective triterpenoids in sessile animals.

Lanosterol synthase[1] cyclizes (*S*)-2,3-oxidosqualene (**1**) to lanosterol (**4**). The complex cationic mechanism (Scheme 1) consists of epoxide protonation, four cation−olefin annulations to generate a *trans-syn-trans*[2] tetracycle, a series of Wagner−Meerwein rearrangements, and a highly specific deprotonation to form the olefinic product.[3] Cycloartenol synthase (CAS1) employs a similar *trans-syn-trans* mechanism to make cycloartenol (**6**). The mechanistic process has been illuminated through point mutations that modify the product profile.[4] Reported mutants disrupt substrate folding or modify cation quenching to generate monocyclic or tetracyclic byproducts. However, no characterized mutant of lanosterol synthase or CAS1 generates any tricycles, which are the putative precursors of the *trans-syn-trans* isomalabaricane triterpenoids[5] in sponges. We now report two lanosterol synthase single mutants that generate the missing biosynthetic intermediate, the novel tricyclic triterpene **3**. This result suggests the facile evolution of tricyclic terpenoids as secondary metabolites in sponges.

The catalytic importance of Tyr510[6] was initially established in directed evolution experiments with cycloartenol synthase (CAS1) from *Dictyostelium discoideum*[7] (*Ddi*CAS1) and *Arabidopsis thaliana*[8] (*Ath*CAS1). Mutating the corre-

(1) Corey, E. J.; Russey, W. E.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1966**, *88*, 4750−4751.

(2) *trans* AB ring junction, 9,10-*syn* ABC ring fusion, and *trans* BC ring junction.

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(6) Protein residue numbering corresponds to *Sce*Erg7. Triterpene numbering (Figure 1) is used for cationic intermediates and products.

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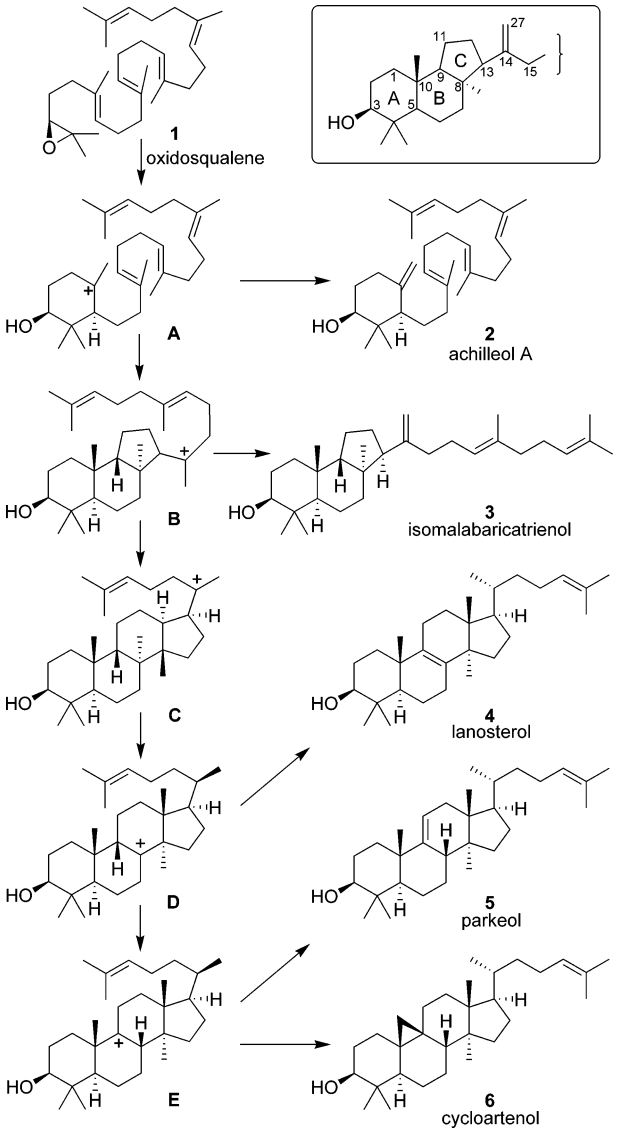
Scheme 1. Cyclization of Oxidosqualene by Lanosterol Synthase, CAS1, and Mutants Described Herein



sponding Tyr in *Ath*CAS1 to His abolishes cycloartenol biosynthesis and results in a mixture of the monocycle achilleol A (24%) and two tetracycles, lanosterol (45%) and parkeol (31%).[9] This catalytic outcome is consistent with a homology model that predicts this Tyr residue to be part of a hydrogen-bond network that facilitates cation deprotonation.[4b] The human lanosterol synthase crystal structure indicates a different role for Tyr510; its phenol is hydrogen bonded directly to the proposed active-site base His234. The proximity of the phenolic oxygen to the C9 proton suggests that Tyr510 is the initial proton acceptor and transfers its phenolic proton to His234.[10] To investigate experimentally the roles of Tyr510, we generated the Tyr510His and Tyr510Phe[11] mutants of lanosterol synthase from *Saccharomyces cerevisiae* (*Sce*Erg7).[12]

We used site-specific mutagenesis to generate the *Sce*Erg7 Tyr510 mutants in the integrative galactose-inducible yeast expression vector pRS305GAL. These mutant enzymes were expressed in the yeast strain RXY6,[13] which has two gene deletions that facilitate accurate quantitation of oxidosqualene cyclase products. The lanosterol synthase deletion abolishes background cyclase activity, and the squalene epoxidase deletion prevents the biosynthesis of the precursor (*S*)-2,3-oxidosqualene and consequently ensures that RXY6 homogenates lack triterpene alcohols.

In vitro assays were conducted by incubating racemic oxidosqualene (1 mg/mL) with homogenates obtained from 1-L cultures of recombinant yeast. After extraction and partial purification, GC, GC−MS, and NMR analyses were performed, and known compounds (**2**, **4**, and **5**) were identified by comparison with spectral data for authentic standards. As shown in Table 1, the Tyr510His triterpene fraction contained

Table 1. % Product Composition of Oxidosqualene Cyclases[a]

mutant	**2**	**3**	**4**	**5**
*Ath*CAS1 Tyr532His	24	0	45	31
*Sce*Erg7	0	0	100	0
*Sce*Erg7 Tyr510Phe	0	5	95	0
*Sce*Erg7 Tyr510His	45	4	42	9

[a] Ratios from GC-FID analyses of products from in vitro reactions with mutant enzymes expressed in RXY6. *Ath*CAS1 Tyr532 corresponds to *Sce*Erg7 Tyr510.

achilleol A (**2**), lanosterol (**4**), parkeol (**5**), and an unidentified $C_{30}H_{50}O$ triterpene alcohol (**3**) with a mass spectral fragmentation suggesting incomplete cyclization. The Tyr510Phe reaction gave lanosterol and the same unknown **3** in a 95:5 ratio.

*Sce*Erg7 Tyr510His and *Sce*Erg7 Tyr510Phe were also expressed in SMY8,[14] a yeast strain that lacks lanosterol synthase and cannot synthesize ergosterol. Both mutants genetically complemented SMY8 and showed growth comparable to wild-type *Sce*Erg7 expressed under identical conditions. In SMY8 cultures expressing *Sce*Erg7 Tyr510Phe, the lanosterol was largely converted to ergosterol, whereas **3** was evidently not metabolized. Consequently, the triterpene alcohols that accumulated in vivo were dramatically enriched

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(8) Corey, E. J.; Matsuda, S. P. T.; Bartel, B. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11628−11632.
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(10) Thoma, R.; Schulz-Gasch, T.; D'Arcy, B.; Benz, J.; Aebi, J.; Dehmlow, H.; Hennig, M.; Stihle, M.; Ruf, A. *Nature* **2004**, *432*, 118−122.
(11) *Sce*Erg7 Tyr510Phe was previously constructed and shown to complement SMY8, but no product characterization was done. Corey, E. J.; Cheng, H.; Baker, C. H.; Matsuda, S. P. T.; Li, D.; Song, X. *J. Am. Chem. Soc.* **1997**, *119*, 1289−1296.
(12) Corey, E. J.; Matsuda, S. P. T.; Bartel, B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2211−2215.
(13) Fazio, G. C.; Xu, R.; Matsuda, S. P. T. *J. Am. Chem. Soc.* **2004**, *126*, 5678−5679.
(14) Corey, E. J.; Matsuda, S. P. T.; Baker, C. H.; Ting, A. Y.; Cheng, H. *Biochem. Biophys. Res. Commun.* **1996**, *219*, 327−331.

in the unknown. These SMY8 cultures showed a distorted triterpene product ratio relative to the RXY6 ratios (Table 2).[15] The results illustrate the pitfalls of using in vivo

Table 2. Comparison of in Vivo and in Vitro Product Ratios[a]

conditions	**3**	**4**
RXY6 (in vitro)	5	95
SMY8 (in vivo)	90	10

[a] Ratios from GC-FID analyses of crude products.

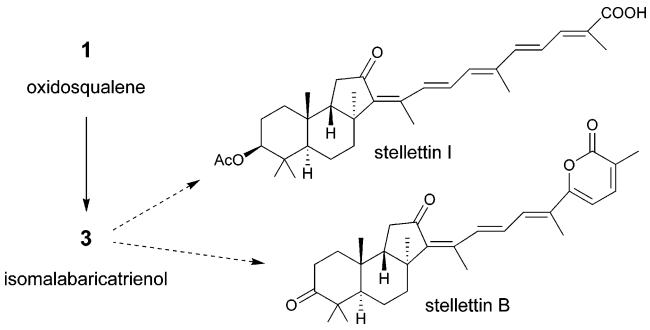
accumulation to estimate the product profile of a triterpene synthase.[16]

We exploited the distorted product profile of SMY8 [*Sce*Erg7 Tyr510Phe] to obtain enough **3** for structure elucidation. A 1-L culture gave after saponification and partial purification a triterpene alcohol fraction containing predominantly **3**, an analytical sample of which was obtained by HPLC.[17] Compound **3** was identified by NMR spectroscopy (^{1}H, ^{13}C, DEPT, COSYDEC, HSQC, HMBC, NOE) as (13α*H*)-isomalabarica-14(27),17*E*,21-trien-3β-ol. Details are given in the Supporting Information.

The novel tricycle **3** is the parent skeleton of isomalabaricanes, found in nature only in certain Asian sponges.[5] These secondary metabolites are tricyclic triterpenoids that display the distinctive *trans-syn-trans* ring fusion found in lanosterol biosynthesis. Sponges synthesize sterols from lanosterol,[18] and the enzyme that constructs the isomalabaricatrienol skeleton probably evolved from a lanosterol synthase. Sponges could cyclize oxidosqualene to **3** and produce the isomalabaricane triterpenoids by additional desaturation and specific oxidation (Scheme 2).[19] These sessile marine animals produce numerous secondary metabolites for a variety of purposes.[20]

Among the many 6−6−5 tricycles isolated from nature or from experiments with substrate analogues and cyclase

Scheme 2. Proposed Biosynthesis of Isomalabaricane Triterpenoids via Tricycle **3**



mutants, nearly all have a *trans-anti-trans* stereochemistry. The sole characterized oxidosqualene cyclase that forms tricycles is thalianol synthase,[13] a plant enzyme that generates a 6−6−5 malabaricatrienol from a *trans-anti-trans* all-chair cation. Interestingly, thalianol synthase shares close phylogenetic affinity to plant β-amyrin and lupeol synthases which generate tetracyclic all-chair intermediates. These observations are consistent with the hypothesis that cyclases rarely evolve from a B-ring chair to B-ring boat mechanism or vice versa.[21]

Some reported cyclase mutants generate 6−6−5 *trans-anti-trans* tricycles.[22] Malabarica-14(27),17*E*,21-trien-3β-ol, a *trans-anti-trans* isomer of **3**, has been produced from 2,3 oxidosqualene by a squalene-hopene cyclase mutant[22b] and nonenzymatically under mildly acidic[23] or free-radical[24] conditions. Other biomimetic reactions also produce 6−6−5 tricycles, all with the *trans-anti-trans* stereochemistry.[25] The only reported cyclizations to isomalabaricane skeletons involve reaction of oxidosqualene substrate analogues with lanosterol synthase.[26] However, none of these reactions produce the novel isomalabaricatrienol **3**.

The product profiles described herein provide some insight into the cyclization mechanism, although interpretation of the results is necessarily speculative in the absence of

(15) In vivo SMY8 product ratios varied from 80:20 to 90:10. This variablity is unsurprising because time of harvest, amount of ergosterol in the medium, degree of aeration, and other culture conditions could affect the extent of lanosterol metabolism. By contrast, in vitro RXY6 product ratios are not affected by such conditions and are reproducible.

(16) This concern applies chiefly to cyclases that generate lanosterol, parkeol, cycloartenol, and other products that are metabolized by yeast.

(17) SMY8[*Sce*Erg7 Tyr510Phe] was cultured in inducing medium (YPGH, 1 L). Saponification of the cell pellet (10% KOH in 80% EtOH) followed by hexanes extraction furnished the nonsaponifiable lipids (NSL). Removal of ergosterol by silica gel column chromatography gave a triterpene alcohol fraction containing mainly **3**, with some lanosterol, 4,4-dimethylcholest-8-en-3β-ol (T-MAS), traces of sterol intermediates and possibly unidentified triterpenes.

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(19) The Δ13(14) isomer of **3** could alternatively be a precursor of isomalabaricanes: Xu, R.; Fazio, G. C.; Matsuda, S. P. T. *Phytochemistry* **2004**, *65*, 261−290. However, **3** should be easier to generate enzymatically since a C14 methyl proton is more readily abstracted than the more hindered C13 methine proton. Also, abstraction of the C13 proton would probably require rotation of the side chain to align the cationic 2p orbital with the C−H bond; see discussion of horizontal and vertical cations in: Matsuda, S. P. T.; Wilson, W. K.; Xiong, Q. *Org. Biomol. Chem.* **2006**, in press, DOI: 10.1039.

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(25) Bartlett, P. A. In *Asymmetric Synthesis*; Morrison, J. D., Ed.; Academic Press: New York, 1984; Vol. 3, pp 341−409. The *trans-syn-trans* stereochemistry has been detected in minor products of biomimetic reactions: Nishizawa, M.; Yadav, A.; Iwamoto, Y.; Imagawa, H. *Tetrahedron* **2004**, *60*, 9223−9234 and ref therein; however these reactions did not generate 6−6−5 tricycles. A *trans-syn-cis* tetracycle was made biomimetically with a specially designed substrate: Corey, E. J.; Wood, H. B., Jr. *J. Am. Chem. Soc.* **1996**, *118*, 11982−11983.

(26) (a) Van Tamelen, E. E.; Sharpless, K. B.; Hanzlik, R.; Clayton, R. B.; Burlingame, A. L.; Wszolek, P. C. *J. Am. Chem. Soc.* **1967**, *89*, 7150−7151. (b) Krief, A.; Schauder, J. R.; Guittet, E.; Herve du Penhoat, C.; Lallemandt, J. Y. *J. Am. Chem. Soc.* **1987**, *109*, 7910−7911. (c) Guittet, E.; Herve du Penhoat, C.; Lallemand, J. Y.; Schauder, J. R.; Krief, A. *J. Am. Chem. Soc.* **1987**, *109*, 7911−7913. (d) Krief, A.; Pasau, P.; Guittet, E.; Shan, Y. Y.; Herin, M. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 365−368.

crystallographic studies of mutants. Considering the critical role of the Tyr510 hydroxyl in deprotonating H-9 of the C-8 cation (**D**) to form lanosterol,[10] it is interesting that the Tyr510Phe mutant makes predominantly lanosterol despite loss of the deprotonating hydroxyl. The mutant may have a similar active-site geometry except that the native phenolic OH is replaced by an ordered water. This water could accept the H-9 proton and transfer a proton to its hydrogen-bonding partner His234.

Alternatively, the new hydrogen-bond network may locate His234 close enough to H-9 for direct deprotonation. As shown in Figure 1, the aryl ring of Tyr510 does not face the active-site cavity and thus affects folding only through steric interactions. The occasional misfolding that leads to tricycle **3** in Tyr510Phe may result from mobility of the aryl ring, which is no longer anchored to the hydrogen-bond network. Alternatively, steric changes caused by a modified hydrogen-bond network may slightly retard folding for D-ring formation, and the delay could allow some deprotonation of intermediate **B** at the C-14 methyl. This methyl is on the β face of the intermediate near H-9β, and deprotonation by His234 could be mediated by ordered waters in the active site. Molecular modeling and the absence of tricycles with alternate deprotonation positions (0.1% detection limit) suggest that steric constraints block all Wagner−Meerwein rearrangments other than the ring expansion en route to the tetracyclic cation (see the Supporting Information).

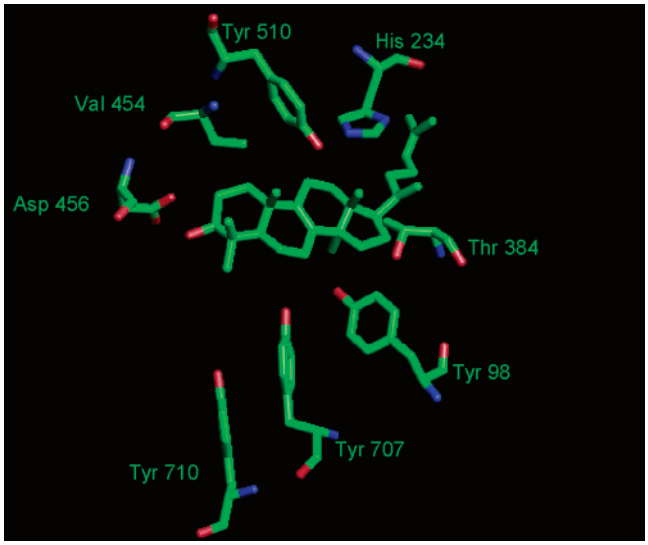

Figure 1. Tyr 510 and other selected residues in the active site of lanosterol synthase. Human lanosterol synthase crystal structure coordinates are from ref 10.

The Tyr510His mutation has substantial effects on cyclization, reducing the proportion of molecules that undergo tetracyclization to 50%. The imidazole ring is only slightly smaller than a phenol, but the dramatically different electronic properties could result in extensive rearrangement of the hydrogen-bond network and induce the significant misfolding that is apparent. Minor change in location of His234 could alter deprotonation to form parkeol. Likewise, the His510 may be suitably located for facile deprotonation at C-11 to form parkeol or at the C-10 methyl to form achilleol A.

Similar considerations could account for the extensive misfolding reported recently for Tyr510Ala, Tyr510Lys, and Tyr510Trp mutants.[4c] However, these reports should be interpreted cautiously; the mutants were characterized by analysis of the products accumulated in vivo, and our results in Table 2 indicate that such product ratios can severely underestimate lanosterol content.

In summary, lanosterol synthase mutants produce a tricyclic alcohol that is the putative biosynthetic precursor of cytotoxic[5] isomalabaricane triterpenoids in sponges. Isomalabaricatrienol synthase may have evolved from lanosterol synthase. The ability of simple lanosterol synthase mutants to generate **3** supports this hypothesis. Further evidence will await the cloning and characterization of sponge cyclases. Animals produce only three different triterpene skeletons: lanosterol, parkeol, and isomalabaricatrienol. Notably, the Tyr510His mutant makes all three of these.

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Supporting Information Available: Details of experimental procedures, molecular modeling, and NMR and GC−MS spectra of **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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