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Steric Bulk at Cycloartenol Synthase Position 481 Influences Cyclization and Deprotonation

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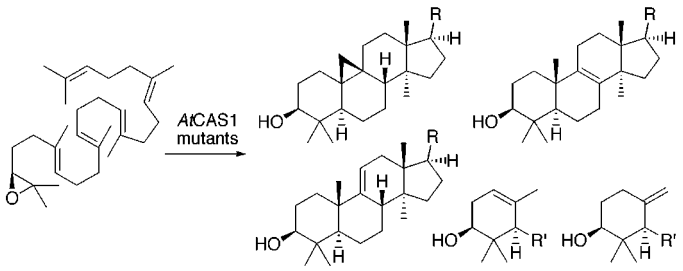
ABSTRACT



Cycloartenol synthase converts oxidosqualene to the pentacyclic sterol precursor cycloartenol. An *Arabidopsis thaliana* cycloartenol synthase Ile481Val mutant was previously shown to produce lanosterol and parkeol in addition to its native product cycloartenol. Experiments are described here to construct Phe, Leu, Ala, and Gly mutants at position 481 and to determine their cyclization product profiles. The Phe mutant was inactive, and the Leu mutant produced cycloartenol and parkeol. The Ala and Gly mutants formed lanosterol, cycloartenol, parkeol, achilleol A, and camelliol C. Monocycles comprise most of the Gly mutant product, showing that an alternate cyclization route can be made the major pathway by a single nonpolar mutation.

Terpene synthases catalyze cationic cyclization and rearrangement reactions that convert acarbocyclic precursors to carbocyclic products. Four enzyme families provide hundreds of terpene hydrocarbon structures: the higher plant TPS enzymes (which form monoterpenes, sesquiterpenes, and diterpenes),[1] fungal[2] sesquiterpene synthases, bacterial sesquiterpene synthases,[3] and a prokaryotic and eukaryotic triterpene synthase family that cyclizes either squalene[4] or oxidosqualene.[5] How terpene synthases mediate these reactions has recently begun to be investigated by mutagenesis. Sesquiterpene synthases[6] and squalene-hopene cyclases[7] have been modified to make new compounds. We describe here

(1) Bohlmann, J.; Meyer-Gauen, G.; Croteau, R. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4126−4133.
(2) Hohn, T. M.; Beremand, P. D. *Gene* **1989**, *79*, 131−138.
(3) (a) Cane, D. E.; Sohng, J. K.; Lamberson, C. R.; Rudnicki, S. M.; Wu, Z.; Lloyd, M. D.; Oliver, J. S.; Hubbard, B. R. *Biochemistry* **1994**, *33*, 5846−5857. (b) Proctor, R. H.; Hohn, T. M. *J. Biol. Chem.* **1993**, *268*, 4543−4548.
(4) Kannenberg, E. L.; Poralla, K. *Naturwissenschaften* **1999**, *86*, 168−176.
(5) (a) Abe, I.; Rohmer, M.; Prestwich, G. D. *Chem. Rev.* **1993**, *93*, 2189−2206. (b) Matsuda, S. P. T. In *Biochemical Principles and Mechanisms of Biosynthesis and Biodegradation of Polymers*; Steinbüchel, A., Ed.; Wiley-VCH: Weinheim, 1998; pp 300−307.
(6) (a) Cane, D. E.; Xue, Q. *J. Am. Chem. Soc.* **1996**, *118*, 1563−1564. (b) Cane, D. E.; Xue, Q.; Van Epp, J. E.; Tsantrizos, Y. S. *J. Am. Chem. Soc.* **1996**, *118*, 8499−8500. (c) Seemann, M.; Zhai, G.; Umezawa, K.; Cane, D. *J. Am. Chem. Soc.* **1999**, *121*, 591−592. (d) Rising, K. A.; Starks, C. M.; Noel, J. P.; Chappell, J. *J. Am. Chem. Soc.* **2000**, *122*, 1861−1866.
(7) (a) Merkofer, T.; Pale-Grosdemange, C.; Wendt, K. U.; Rohmer, M.; Poralla, K. *Tetrahedron Lett.* **1999**, *40*, 2121−2124. (b) Sato, T.; Abe, T.; Hoshino, T. *Chem. Commun.* **1998**, *23*, 2617−2618. (c) Pale-Grosdemange, C.; Merkofer, T.; Rohmer, M.; Poralla, K. *Tetrahedron Lett.* **1999**, *40*, 6009−6012. (d) Hoshino, T.; Sato, T. *Chem. Commun.* **1999**, *1999*, 2005−2006. (e) Sato, T.; Hoshino, T. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 2189−2198. (f) Full, C.; Poralla, K. *FEMS Microbiol. Lett.* **2000**, *183*, 221−224.
(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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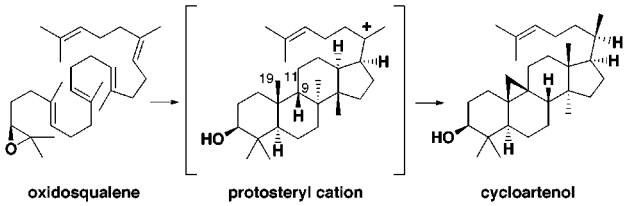
experiments to determine the role of steric bulk at position 481 of *Arabidopsis thaliana* cycloartenol synthase (*At*-CAS1).[8]

Cycloartenol synthase cyclizes oxidosqualene to the protosteryl cation, which is then rearranged and deprotonated to give cycloartenol (Scheme 1).[9] Isoleucine 481 (*A. thaliana*

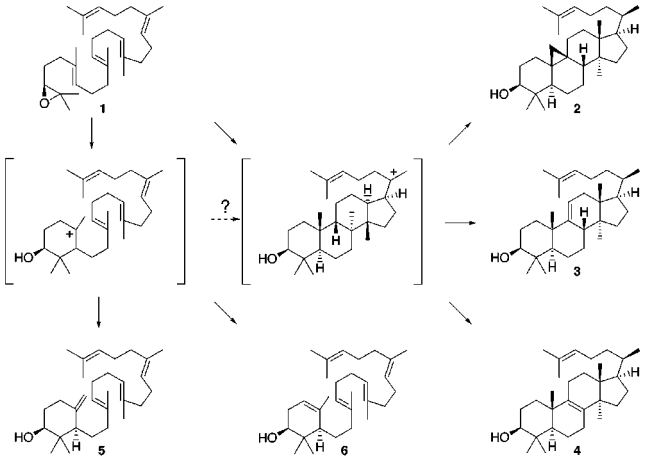
Scheme 1. Reaction Catalyzed by Cycloartenol Synthase



numbering) is strictly conserved in the known cycloartenol synthases.[8,10] We recently described an *At*CAS1 Ile481Val mutant that converts oxidosqualene to cycloartenol (55%), lanosterol (24%), and parkeol (21%).[11] To investigate further the importance of sterics at this position, we constructed *At*CAS1 mutants bearing phenylalanine, leucine, alanine, or glycine at position 481. The *At*CAS1 cDNA was subjected to oligo-directed mutagenesis in vector pRS305GAL.[12] The mutants were subcloned into the high-copy yeast expression vector pRS426GAL[11] and expressed in the yeast strain LHY2, from which both squalene synthase and lanosterol synthase had been deleted.[11] Typically, cells from 1-L cultures prepared as described[13] were suspended in 100 mM sodium phosphate buffer at pH 6.4 and lysed in a French Press. Racemic oxidosqualene was added to 1 mg/mL from a 20 mg/mL solution in 20% Triton X-100. The reaction was quenched after 24 h, and triterpene alcohol products were isolated as described.[14]

Reactions were monitored by TLC (silica gel, 1:1 hexane/ether), and products were acetylated (acetic anhydride/pyridine) and identified by GC, GC/MS, and ^{1}H, and/or ^{13}C NMR. *At*CAS1 Ile481Phe was inactive, but the remaining mutants metabolized oxidosqualene. The *At*CAS1Ile481Leu products were indistinguishable from cycloartenol by TLC, which cannot resolve most triterpene alcohols because their 3β-hydroxy groups are in sterically similar environments. The products were acetylated, and NMR and GC revealed multiple compounds. The ^{13}C NMR spectrum displayed all 32 cycloartenyl acetate signals,[15] establishing the major product as cycloartenol. Cycloartenyl acetate signals were also dominant in the ^{1}H NMR, but signals at δ 5.22 (C-11), 0.64 (C-18), and 0.73 ppm (C-30) and ^{13}C NMR signals at δ 44.27, 46.99, and 148.11 were characteristic of parkeyl acetate.[11] The ^{1}H NMR spectrum revealed a trace of lanosterol, with characteristic lanosteryl acetate signals at δ 0.68 and 1.00 ppm corresponding to C18 and C19, respectively.[16] GC/MS analyses confirmed the NMR results; the unknowns had retention times (relative to cholesteryl acetate) and fragmentation patterns identical to those of acetylated cycloartenol, parkeol, and lanosterol standards (Scheme 2).

Scheme 2. Cycloartenol Synthase Ile481 Mutants Convert Oxidosqualene (**1**) to a Variety of Polycyclic and Monocyclic Structures Including Cycloartenol (**2**), Parkeol (**3**), Lanosterol (**4**), Achilleol A (**5**), and Camelliol C (**6**)



GC and GC/MS analyses of the acetylated Ile481Ala and Ile481Gly products also revealed cycloartenol, lanosterol, and parkeol. However, two additional compounds were observed to have the appropriate mass for triterpenyl acetates. These novel compounds were separable (silica gel 2:1 hexane/ether) from a fraction shown by ^{1}H NMR to be a mixture of lanosterol, cycloartenol, and parkeol. Preparative argentic TLC (SiO_2/$AgNO_3$, 3 developments in 1:1 methylene chloride/hexane)[17] provided from the Ile481Gly mutant pure samples shown to be achilleol A[18] acetate and camelliol C[19] acetate by ^{1}H NMR analysis (Scheme 2). Similar analyses showed that the Ile481Ala mutant produced the same five compounds.

(9) (a) Rees, H. H.; Goad, L. J.; Goodwin, T. W. *Biochem. J.* **1968**, *107*, 417−426. (b) Seo, S.; Uomori, A.; Yoshimura, Y.; Takeda, K.; Seto, H.; Ebizuka, Y.; Noguchi, H.; Sankawa, U. *J. Chem. Soc., Perkin Trans. 1* **1989**, 261−263.

(10) (a) Morita, M.; Shibuya, M.; Lee, M.-S.; Sankawa, U.; Ebizuka, Y. *Biol. Pharm. Bull.* **1997**, *20*, 770−775. (b) Kushiro, T.; Shibuya, M.; Ebizuka, Y. *Eur. J. Biochem.* **1998**, *256*, 238−244. (c) Hayashi, H.; Hiraoka, N.; Ikeshiro, Y.; Yazaki, K.; Tanaka, S.; Kushiro, T.; Shibuya, M.; Ebizuka, Y. *Plant Physiol.* **1999**, *121*, 1384. (d) Hayashi, H.; Hiraoka, N.; Ikeshiro, Y.; Kushiro, T.; Morita, M.; Shibuya, M.; Ebizuka, Y. *Biol. Pharm. Bull.* **2000**, *23*, 231−234. (e) Godzina, S. M.; Lovato, M. A.; Meyer, M. M.; Foster, K. A.; Wilson, W. K.; Gu, W.; de Hostos, E. L.; Matsuda, S. P. T. *Lipids* **2000**, *36*, 249−255.

(11) Hart, E. A.; Hua, L.; Darr, L. B.; Wilson, W. K.; Pang, J.; Matsuda, S. P. T. *J. Am. Chem. Soc.* **1999**, *121*, 9887−9888.

(12) Corey, E. J.; Matsuda, S. P. T.; Baker, C. H.; Ting, A. Y.; Cheng, H. *Biochem. Biophys. Res. Commun.* **1996**, *219*, 327−331.

(13) Herrera, J. B. R.; Bartel, B.; Wilson, W. K.; Matsuda, S. P. T. *Phytochemistry* **1998**, *49*, 1905−1911.

(14) Joubert, B. M.; Hua, L.; Matsuda, S. P. T. *Org. Lett.* **2000**, *2*, 339−341.

(15) Radics, L.; Kajtar-Peredy, M.; Corsano, S.; Standoli, L. *Tetrahedron Lett.* **1975**, *48*, 4287−4290.

(16) Emmons, G. T.; Wilson, W. K.; Schroepfer, G. J., Jr. *Magn. Res. Chem.* **1989**, *27*, 1012−1024.

(17) Pascal, R. A., Jr.; Farris, C. L.; Schroepfer, G. J., Jr. *Anal. Biochem.* **1980**, *101*, 15−22.

(18) Barrero, A. F.; Alvarez-Manzaneda, E. J.; Alvarez-Manzaneda, R. *Tetrahedron Lett.* **1989**, *30*, 3351−3352.

(19) Akihisa, T.; Arai, K.; Kimura, Y.; Koike, K.; Kokke, W. C. M. C.; Shibata, T.; Nikaido, T. *J. Nat. Prod.* **1999**, *62*, 265−268.

Table 1. Percentage Product Composition of *A. thaliana* Cycloartenol Synthase Ile481 Mutants[a]

481 residue	side chain	cycloartenol (**2**)	parkeol (**3**)	lanosterol (**4**)	achilleol A (**5**)	camelliol C (**6**)
isoleucine (wild-type)[11]	*sec*-butyl	99	1	0[b]	0[b]	0[b]
leucine	isobutyl	83	16	1	0[b]	0[b]
valine[11]	isopropyl	55	21	24	0[b]	0[b]
alanine	methyl	12	15	54	13	6
glycine	hydrogen	17	4	23	44	12

[a] Products were quantitated from crude mixtures by GC-FID after acetylation. [b] Not detected; detection limit ≤0.2%.

The products (percent composition determined by GC) of these enzymes are summarized in Table 1. Even minor steric changes at position 481 impair cycloartenol production. Forming the strained cyclopropane ring is apparently readily compromised. Mutating Ile481 to Leu maintains total steric bulk but shifts a methyl group from the β- to the γ-carbon. This isomeric side chain allows deprotonation from C-11 to yield parkeol, but only minimal deprotonation from C-9 or C-8 to form lanosterol. In contrast, mutating to the methyl side chain (Ile481Ala) generates an enzyme that forms lanosterol as its major product. Furthermore, Ala promotes ring B formation less efficiently than Ile, Leu, or Val. The monocyclic cation can deprotonate to either achilleol A or camelliol C. Ile481Gly forms primarily monocycles, and the ratio of achilleol A to camelliol C formation increases relative to that of the Ile481Ala products.

S. cerevisiae lanosterol synthase (*Sc*ERG7) mutants were recently described in which Val454 (which corresponds to *At*CAS1 Leu481) was mutated to Phe, Leu, Ile, Ala, and Gly.[14] Like *At*CAS1, *Sc*ERG7 lost activity upon mutating the target residue to Phe. The *Sc*ERG7 Val454 Leu and Ile mutants remained accurate lanosterol synthases. The Ala and Gly mutants were lanosterol synthases that also produced achilleol A (5% and 12%, respectively). Decreasing steric bulk at *Sc*ERG7 position 454 compromised ring B formation, although to a lesser extent than at the analogous *At*CAS1 position 481. Moreover, *Sc*ERG7Val454 mutants did not form measurable amounts of the alternative deprotonation products cycloartenol, parkeol, and camelliol C (<0.5%). Lanosterol synthase is evidently less sensitive than cycloartenol synthase to steric alterations at this position. Perhaps less precise positioning of active site electron density and steric bulk is required to form the energetically favorable tetrasubstituted olefin than to close the strained cyclopropane ring.

These experiments establish that cycloartenol synthase can be mutated to make different tetracyclic or monocyclic compounds in high yield. The mutants described herein may mimic naturally occurring enzymes. Achilleol A and camelliol C have been isolated from plants, but the enzymes that catalyze their formation remain undiscovered. Several organisms[20] produce achilleol A without camelliol C, as do the *Sc*ERG7 Val454 Ala and Gly mutants. Sasanqua oil[21] contains camelliol C and achilleol A, as in the products of *At*CAS1 Ile481 Ala and Gly mutants. None of these mutants produced camelliol C without achilleol A, and similarly, camelliol C has never been isolated from a natural source unaccompanied by achilleol A. It is tempting to speculate that camelliol C may be produced in nature by a cycloartenol synthase Ile481 Ala or Gly mutant, and achilleol A from a similar mutant of a different oxidosqualene cyclase.

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(20) (a) Barrero, A. F.; Alvarez-Manzaneda, E. J.; Alvarez-Manzaneda, R. *Tetrahedron Lett.* **1989**, *30*, 3351−3352. (b) Barrero, A. F.; Haidour, A.; Munoz-Dorado, M.; Akssira, M.; Sedqui, A.; Mansour, I. *Phytochemistry* **1998**, *48*, 1237−1240. (c) Barrero, A. F.; Alvarez-Manzaneda, E. J.; Herrador, M. M.; Alvarez-Manzaneda, R.; Quilez, J.; Chahboun, R.; Linares, P.; Rivas, A. *Tetrahedron Lett.* **1999**, *40*, 8273−8276.

(21) Akihisa, T.; Arai, K.; Kimura, Y.; Koike, K.; Kokke, W. C. M. C.; Shibata, T.; Nikaido, T. *J. Nat. Prod.* **1999**, *62*, 265−268.