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Alteration of the Substrate's Prefolded Conformation and Cyclization Stereochemistry of Oxidosqualene-Lanosterol Cyclase of *Saccharomyces cerevisiae* by Substitution at Phenylalanine 699

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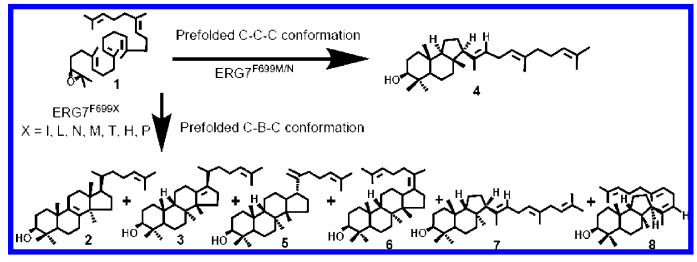
ABSTRACT



The S*accharomyces cerevisiae* ERG7^{Phe699} mutants produced one chair−chair−chair (C−C−C) and two chair−boat−chair (C−B−C) truncated tricyclic compounds, one tetracyclic 17α-exocyclic unrearranged intermediate, and two 17*β*-exocyclic truncated rearranged intermediates. These results provided direct evidence for the importance of the residue in affecting mechanistic transitions between C−B−C and C−C−C substrate conformation and between the 17α- and 17*β*-exocyclic side chain stereochemistry as well as in stabilizing the 6−6−5 tricyclic and the protosteryl C-17 cations.

The advent of contemporary protein engineering techniques, including mutation, recombination, and selection, has paved the way to understand the structure−function−mechanism relationships of enzyme catalysis and to tailor enzymes with new catalytic activity and selectivity.[1] Identification of plasticity residues in target proteins with diverse product profiles provides the starting point to understand how proteins acquire novel or altered functions and how plasticity residues contribute to the natural evolution process as well as allow an efficient design methodology for new enzymes to be formulated. Plasticity has been studied in a number of enzymes and recent findings have shown a divergent strategy for shaping cyclization of (3*S*)-2,3-oxidosqualene (**1**, OS), wherein mutational steps alter the biosynthetic properties of enzymes. Interestingly, the fascinating enzymes, the oxidosqualene cyclases (OSC or ERG7), cyclize acyclic OS into diverse polycyclic sterols and triterpenoids with two funda-

(1) (a) Bolon, D. N.; Voigt, C. A.; Mayo, S. L. *Curr. Opin. Chem. Biol.* **2002**, *6*, 125–129. (b) Yoshikuni, Y.; Ferrin, T. E.; Keasling, J. D. *Nature* **2006**, *440*, 1078–1082. (c) Glasner, M. E.; Gerlt, J. A.; Babbitt, P. C. *Adv. Enzymol. Relat. Areas Mol. Biol.* **2007**, *75*, 193–239.

(2) (a) Abe, I.; Rohmer, M.; Prestwich, G. D. *Chem. Rev.* **1993**, *93*, 2189–2206. (b) Wendt, K. U.; Schulz, G. E.; Corey, E. J.; Liu, D. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 2812–2833. (c) Xu, R.; Fazio, G. C.; Matsuda, S. P. T. *Phytochemistry* **2004**, *65*, 261–291. (d) Abe, I. *Nat. Prod. Rep.* **2007**, *24*, 1311–1331.

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mental biosynthetic routes, based either on chair−boat−chair (C−B−C) or chair−chair−chair (C−C−C) prefolded substrate conformation.[2−8] In addition, cyclase-catalyzed reactions can proceed in either an accurate or multifunctional fashion to achieve catalytic perfection or to generate diverse product profiles.[3,5] The isolated product profiles reveal the catalytic function of the given amino acids and support the idea that subtle changes in individual residues can have electronic effects and/or can sterically alter the active site cavity structure to generate diverse product profiles. However, the functional residues involved in determining substrate specificity for squalene/oxidosqualene, C−B−C/C−C−C substrate conformation, and 17α/17*β* stereochemical control of exocyclic side chain, remain unsolved.

To explore whether a single-site mutation in enzymes possessing the cyclase structure is sufficient to alter substrate conformation and protosteryl 17α/17*β* stereochemistry, amino acids spatially near the substrate B/C/D ring positions were targeted. We have previously characterized a functional role of *Saccharomyces cerevisiae* ERG7^{F699T} mutation in influencing protosteryl C-17 cation stabilization.[3h] A more complex interaction between Phe699 and prefolded substrate conformation or cationic intermediates is expected and provokes further investigation into the effects of substitution of other amino acids on the catalytic activity and capacity to traverse mechanistic barriers between C−B−C and C−C−C substrate conformation and between 17α and 17*β* stereochemistry of tetracyclic intermediates.

The Phe699 position of ERG7 was exchanged with other amino acids using the QuikChange Site-Directed Mutagenesis Kit and transformed into the *S. cerevisiae* TKW14 and YTL4 strains, as previously described.[3h] The genetic selection results showed that TKW14[pERG7^{F699X}] site-saturated mutants allowed for ergosterol-independent growth, with the exception of Leu, Ile, His, Met, Pro, and the previously reported Thr substitutions. Next, the product profiles generated by the ERG7 mutant enzymes were analyzed, using the YTL4[pERG7^{F699X}] strain, and characterized by GC-MS and NMR (^{1}H, ^{13}C NMR, DEPT, ^{1}H−^{1}H COSY, HMQC, HMBC, and NOE) spectroscopies.[3f]

Different ERG7^{F699X} mutants produced diverse product profiles, ranging from none to as many as seven compounds with molecular mass of 426 Da. Among them, four compounds were indistinguishable from authentic lanosterol (**2, LA**), protosta-13(17)-dien-3*β*-ol (**3**), (13α*H*)-isomalabarica-14*E*,17*E*,21-dien-3*β*-ol (**7**), and (13α*H*)-isomalabarica-14*Z*,17*E*,21-dien-3*β*-ol (**8**) standards by ^{1}H and ^{13}C NMR, as well as GC-MS.[3j] The first novel new compound, independently isolated from ERG7^{F699N} and ERG7^{F699M} mutants, was characterized with NMR and confirmed as malabarica-14*E*,17*E*,21-trien-3*β*-ol (**4**), a 6−6−5 tricyclic product with chair−chair conformation and $\Delta^{14,17,21}$ double bonds (see Supporting Information). This work describes the first truncated intermediate of the oxidosqualene cyclases-catalyzed cyclization/rearrangement cascade that alters the substrate conformation from a ring-B boat conformation to a ring-B chair conformation. The second novel compound identified from ERG7$^{F699M/N}$ mutants was characterized as 17α-protosta-20,24-dien-3*β*-ol (**5**), a product with $\Delta^{20,24}$ double bonds and a C-17α hydrocarbon side chain configuration. Interestingly, this is the first reported product derived from the protosteryl cation containing a C-17α-side chain conformation, abrogating its further rearrangement reaction, in ERG7 mutants. The third acetylated compound was characterized as protosta-17(20),24-dien-3*β*-acetate. This acetylated derivative was then hydrolyzed to yield its native nonsaponifiable lipid, (17*Z*)-protosta-17(20),24-dien-3*β*-ol (**6**). Recently, Lodeiro et al. also reported the characterization of compound **3** and **6** from the oxidosqualene cyclases of *Aspergillus fumigates*.[5t]

Table 1 shows the product profiles produced from the ERG7^{F699X} site-saturated mutants. No product was observed for the nonviable mutants, except for the ERG7^{F699N} mutant, indicating the abolished cyclization function of these mutated ERG7 cyclases. The ERG7^{F699N} mutant required exogenous ergosterol to maintain its viability and produced **3**, **4**, **5**, and **6** in the ratio of 55:5:24:16 without any compound **2**. In the case of the viable mutants, the ERG7^{F699L}, ERG7^{F699I}, and ERG7^{F699P} mutants produced **2** as their sole product, whereas the

(3) (a) Wu, T. K.; Chang, C. H.; Liu, Y. T.; Wang, T. T. *Chem. Rec.* **2008**, *8*, 302–325. (b) Wu, T. K.; Griffin, J. H. *Biochemistry* **2002**, *41*, 8238–8244. (c) Wu, T. K.; Chang, C. H. *ChemBioChem* **2004**, *5*, 1712–1715. (d) Wu, T. K.; Liu, Y. T.; Chang, C. H. *ChemBioChem* **2005**, *6*, 1177–1181. (e) Wu, T. K.; Yu, M. T.; Liu, Y. T.; Chang, C. H.; Wang, H. J.; Diau, E. W. G. *Org. Lett.* **2006**, *8*, 1319–1322. (f) Wu, T. K.; Liu, Y. T.; Chang, C. H.; Yu, M. T.; Wang, H. J. *J. Am. Chem. Soc.* **2006**, *128*, 6414–6419. (g) Wu, T. K.; Liu, Y. T.; Chiu, F. H.; Chang, C. H. *Org. Lett.* **2006**, *8*, 4691–4694. (h) Wu, T. K.; Wen, H. Y.; Chang, C. H.; Liu, Y. T. *Org. Lett.* **2008**, *10*, 2529–2532. (i) Wu, T. K.; Wang, T. T.; Chang, C. H.; Liu, Y. T. *Org. Lett.* **2008**, *10*, 4959–4962. (j) Wu, T. K.; Li, W.-H.; Chang, C.-H.; Wen, H.-Y.; Liu, Y.-T.; Chang, Y.-C. *Eur. J. Org. Chem.* **2009**, 5731–5737.

(4) (a) Abe, I.; Sankawa, U.; Ebizuka, Y. *Chem. Pharm. Bull.* **1989**, *37*, 536–538. (b) Kelly, R.; Miller, S. M.; Lai, M. H.; Kirsch, D. R. *Gene* **1990**, *87*, 177–183. (c) Buntel, C. J.; Griffin, J. H. *J. Am. Chem. Soc.* **1992**, *114*, 9711–9713. (d) Shi, Z.; Buntel, C. J.; Griffin, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7370–7374.

(5) (a) Corey, E. J.; Matsuda, S. P.; Bartel, B. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11628–11632. (b) Corey, E. J.; Matsuda, S. P. T.; Bartel, B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2211–2215. (c) Baker, C. H.; Matsuda, S. P. T.; Liu, D. R.; Corey, E. J. *Biochem. Biophys. Res. Commun.* **1995**, *213*, 154–160. (d) Herrera, J. B.; Bartel, B.; Wilson, W. K.; Matsuda, S. P. *Phytochemistry* **1998**, *49*, 1905–1911. (e) Segura, M. J.; Meyer, M. M.; Matsuda, S. P. *Org. Lett.* **2000**, *2*, 2257–2259. (f) Corey, E. J.; Virgil, S. C. *J. Am. Chem. Soc.* **1991**, *113*, 4025–4026. (g) Corey, E. J.; Virgil, S. C.; Cheng, H.; Baker, C. H.; Matsuda, S. P. T.; Singh, V.; Sarshar, S. *J. Am. Chem. Soc.* **1995**, *117*, 11819–11820. (h) Corey, E. J.; Cheng, H.; Baker, C. H.; Matsuda, S. P. T.; Li, D.; Song, X. *J. Am. Chem. Soc.* **1997**, *119*, 1289–1296. (i) 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. (j) Joubert, B. M.; Hua, L.; Matsuda, S. P. T. *Org. Lett.* **2000**, *2*, 339–341. (k) Meyer, M. M.; Segura, M. J. R.; Wilson, W. K.; Matsuda, S. P. T. *Angew. Chem., Int. Ed.* **2000**, *39*, 4090–4092. (l) Lodeiro, S.; Segura, M. J.; Stahl, M.; Schulz-Gasch, T.; Matsuda, S. P. T. *ChemBioChem* **2004**, *5*, 1581–1585. (m) Phillips, D. R.; Rasbery, J. M.; Bartel, B.; Matsuda, S. P. *Curr. Opin. Plant Biol.* **2006**, *9*, 305–314. (n) Herrera, J. B.; Wilson, W. K.; Matsuda, S. P. T. *J. Am. Chem. Soc.* **2000**, *122*, 6765–6766. (o) Xiong, Q.; Wilson, W. K.; Matsuda, S. P. *Angew. Chem., Int. Ed.* **2006**, *45*, 1285–1288. (p) Lodeiro, S.; Xiong, Q.; Wilson, W. K.; Kolesnikova, M. D.; Onak, C. S.; Matsuda, S. P. *J. Am. Chem. Soc.* **2007**, *129*, 11213–11222. (q) Corey, E. J.; Ortiz de Montellano, P. R.; Yamamoto, H. *J. Am. Chem. Soc.* **1968**, *90*, 6245–6255. (r) Corey, E. J.; Lin, K.; Yamamoto, H. *J. Am. Chem. Soc.* **1969**, *91*, 2132–2134. (s) Corey, E. J.; Virgil, S. C.; Sarshar, S. *J. Am. Chem. Soc.* **1991**, *113*, 8171–8172. (t) Lodeiro, S.; Xiong, Q.; Wilson, W. K.; Ivanova, Y.; Smith, M. L.; May, G. S.; Matsuda, S. P. *Org. Lett.* **2009**, *11*, 1241–1244.

(6) (a) Connolly, J. D.; Hill, R. A. *Nat. Prod. Rep.* **2002**, *19*, 494–513. (b) Kushiro, T.; Shibuya, M.; Ebizuka, Y. *Eur. J. Biochem.* **1998**, *256*, 238–244. (c) Husselstein-Muller, T.; Schaller, H.; Benveniste, P. *Plant Mol. Biol.* **2001**, *45*, 75–92. (d) Schulz-Gasch, T.; Stahl, M. *J. Comput. Chem.* **2003**, *24*, 741–753.

Table 1. Product Profiles of *S. cerevisiae* ERG7^{F699X} Mutants[a]

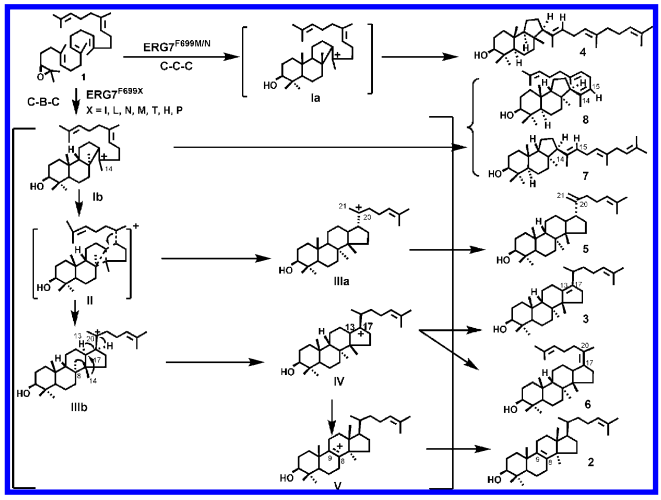
amino acids substitution	2	3	4	5	6	7	8
^{wt}Phe	100	–	–	–	–	–	–
Leu	100	–	–	–	–	–	–
Ile	100	–	–	–	–	–	–
Asn	–	55	5	24	16	–	–
His	13	70	–	–	17	–	–
Thr	<0.2	>99.8	–	–	–	–	–
Met	1	46	7	1	10	17	18
Pro	100	–	–	–	–	–	–

[a] Neither cell viability nor product was characterized for the rest of the ERG7^{F699X} mutants.

ERG7^{F699H} mutant produced **2**, **3**, and **6** in the relative ratio of 13:70:17. To our surprise, the ERG7^{F699M} mutant generated many different products including **2**, **3**, **4**, **5**, **6**, **7**, and **8**. Notably, for the viable ERG7^{F699T} mutant, compound **3** accounted for nearly one hundred percent (>99.8%) of its products, with lanosterol **(2)** accounting for <0.2% as previously described.[3h]

On the basis of the product characterization, two alternative cyclization/rearrangement pathways were proposed for the ERG7^{F699X} site-saturated mutants (Scheme 1). In the ERG7^{F699X}

Scheme 1. Proposed Cyclization/Rearrangement Pathways of Oxidosqualene within *S. cerevisiae* ERG7^{F699X} Mutants



mutants, with the exception of the Asn and Met substitutions, **OS** enters the enzyme active site cavity with a prefolded C−B−C conformation. In the ERG7$^{F699M/N}$ mutants, although most of the substrate prefolds with the C−B−C conformation, some of the substrate forms an enforced C−C−C conformation before the initial electrophilic epoxide activation. In one way, the C−C−C substrate conformer is cyclized to a C−C 6−6−5 tricyclic Markonikov C-14 cation **Ia**, that is followed by direct abstraction of proton from C-15 to yield **4** as the end product. On the other hand, the C−B−C substrate conformer is cationic cyclized to a C−B 6−6−5 tricycic Markonikov C-14 cation (**Ib**) as the first stopping point. In the ERG7^{F699M} mutant, a small portion of this intermediate directly abstracts the C-15 proton, with varying predisposition, and results in the production of **7** and **8**. Alternatively, the cation **Ib** proceeds with a C-ring expansion and is followed by D-ring annulations (**II**) to generate the protosteryl C-20 cation with different stereochemical control at the C-17 position (**IIIa** and **IIIb**). Notably, in the ERG7^{F699M} and ERG7^{F699N} mutants, **IIIa** with a 17α- exocyclic hydrocarbon side chain proceeds the deprotonated termination to produce **5**. Conversely, in most viable mutants, the protosteryl C-20 cation is formed with a 17β- side chain conformation **IIIb**. Subsequently, a backbone rearrangement of H-17α to H-20α via a 1,2 hydride shift generates the protosteryl C-17 cation **IV**. Elimination of a proton at C-13 or C-20 yielded **3** and **6**, respectively, as the major end products in most of the mutants. Then, subsequent skeletal rearrangements of a hydride shift (H13α→H17α), two methyl-group shifts (Me14β→Me-13β and Me-8α→Me-14α), and another hydride shift (H-9β→H8β), generate the lanosteryl C-8/C-9 cation (**V**), which undergoes deprotonation at C-9 or C-8 to form **2**, a normal biosynthetic product.

Although it is difficult to rationalize the effect of a single amino acid substitution on the production of diverse product patterns, the illustration of the structure−function−mechanism relationships through sequence alignment and homology modeling might provide suitable insights for examining the experimental results (Figure 1). First, multiple sequences alignment results revealed that Phe699 of ERG7 is highly conserved among all known triterpene cyclases. Nevertheless, a single residue deletion was observed between Gly697 and Val698 of ERG7, as compared with the corresponding region of SHC (Figure 1a). Second, the homology structural model of the ERG7 complexed with cation **IV** suggested that the Phe699 of ERG7 is spatially positioned below the molecular plane of the substrate with a distance of ~4.8 Å between the observed phenyl side chain and the C-17 position of **IV** (Figure 1b).[3h] The π-electron rich phenyl group is optimal for the stabilization of the electron-deficiency of **IV**, although

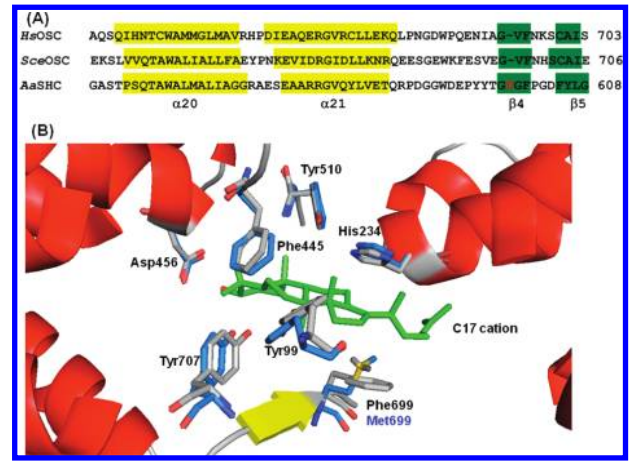


Figure 1. (A) Partial sequences alignment among *Hs*OSC, *Sce*ERG7, and *Aa*SHC. (B) The superimposed homology models of *Sce*ERG7 and *Sce*ERG7^{F699M} complexed with protosteryl C-17 cation.

Thorma et al. have proposed that the Phe699 side chain is positioned to stabilize the anti-Markovnikov C-13 cation during the C-ring formation.[7a] In addition, the His234 is hydrogen bonded to Tyr510 and located spatially above the molecular plane near the C-13 and C-20 positions of the substrate.[3d,f,7a] Coupling with the Tyr99 positioned spatially in the middle side wall proximal to the C-ring and the Tyr707 affecting the B-ring, enables these residues to form a restricted girdle around the B/C/D ring region and exocyclic side chain.[3i] Third, the structure comparison between ERG7 and SHC showed that the β-sheet domain of the ERG7 Gly697-Cys703 region is more loosely packed than the corresponding region of SHC.[6d] The one amino acid deletion between Gly697 and Val698 of ERG7 might promote the position of Phe699 closer to the methyl group during the B/C ring formation and also result in less space between Phe699 and Tyr707, or between Phe699 and Tyr99, for the orientation of the resulting methyl group. Consistent with the observation is the isolation of a bicyclic intermediate from the ERG7^{Y707X} mutants, supporting the functional role of Tyr707 in stabilizing both the bicyclic C-8 cation and the final lanosteryl C-8/C-9 cation intermediates.[3i] Nevertheless, genetic analysis and product characterization of both ERG7$^{F699M/Y707H}$ and ERG7$^{F699M/Y707Q}$ double mutants showed that both mutants cause yeast nonviability and no product formation. In parallel, an Ala substitution of Phe601 from SHC (corresponding to Phe699 of ERG7) also interrupted the polycyclization cascade at either the tricyclic or tetracyclic ring stage, resulting in the isolation of two premature 6/6/5 malabarica-14(27),17,21-triene derivatives with different stereochemically controlled exocyclic side-chain and a 6/6/6/5-fused tetracyclic 17-isodammara-20(21),24-diene, consistent with our results from the ERG7^{F699X} mutants.[9,10] Taken together, these results indicated that Phe699, in conjunction with Tyr99, His234, and Tyr707, may play a functional role in restricting the C−B−C conformation and/or side chain rotation as well as in stabilizing the protosteryl C-17 cation. The substitution of Phe699 with Asn, Met, His, and Thr might cause inappropriate positioning of the side chain and/or interrupt steric or electrostatic interaction within the active site cavity, just allowing the unconstrained folding of the substrate. Specifically, substitution of Phe699 to Asn or Met might partially disrupt the transient dipole interactions between the carbocationic intermediate and the hydroxyl group of Tyr99 and/or of Tyr707, which in turn affects the substrate folding and cyclization, resulting in the accumulation of C−C−C or C−B−C conformor-induced tricyclic, stereochemically inverted tetracyclic, and truncated rearranged tetracyclic products. Or, mutant enzymes may have more capacity to tolerate energetically unfavorable conformers than wild-type due to the perturbance of their cyclization energies prior to or during D-ring closure, resulting in the isolation of intermediates arrested at tricyclic stage with C−C−C conformation and a 17α epimer. Subsequent misfolding of the exocyclic side chain might terminate the subsequent rearrangement steps of the protosteryl C-20 cation **IIIa** and result in the production of **5**. However, other factors, including steric or electrostatic effects from other residues of the enzyme, which might be involved in the alternation of the prefolded substrate conformation and subsequent cyclization to diverse product profiles, could not be excluded.

In conclusion, our study demonstrate, for the first time, that a single amino acid mutation at the 699 position of *S. cerevisiae* ERG7 can change the course of the oxidosqualene cyclization/rearrangement process on traversing the mechanistic barriers between the prefolded C−B−C and C−C−C substrate conformation, and between the 17α and 17β stereochemistry of exocyclic hydrocarbon side chain. The isolation of various tricyclic compounds with opposite B-ring conformation demonstrates the possibility of traversing the catalytic specificity between two distinct yet evolutionarily related triterpene cyclases through molecular directed evolution or site-specific mutation of critical residues surrounding the active site cavity. Finally, products with tailored specificity or expanded diversity could only be simultaneously obtained via the substitution of all proteinogenic amino acids at some specific positions.

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Supporting Information Available: Experimental procedures and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

OL902694Y

(7) (a) 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. (b) Wendt, K. U. *Angew. Chem., Int. Ed.* **2005**, *44*, 3966–3971.

(8) (a) Eschenmoser, A.; Ruzicka, L.; Jeger, O.; Arigoni, D. *Helv. Chim. Acta* **1955**, *38*, 1890–1904. (b) Cornforth, J. W. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 903–911.

(9) (a) Hoshino, T.; Kouda, M.; Abe, T.; Ohashi, S. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 2038–2041.

(10) Merkofer, T.; Pale-Grosdemange, C.; Rohmer, M.; Poralla, K. *Tetrahedron Lett.* **1999**, *40*, 2121–2124.