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Enzymatic cyclizations of squalene analogs with threo- and erythro-diols at the 6,7- or 10,11-positions by recombinant squalene cyclase. Trapping of carbocation intermediates and mechanistic insights into the product and substrate specificities[†]

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In order to trap the carbocation intermediates formed during the squalene cyclization cascade, squalene analogs with threo- and erythro-diols at the 6,7- and 10,11-positions were incubated with the recombinant squalene cyclase from Alicyclobacillus acidocaldarius, leading to the construction of the triterpenes with tetrahydropyran, octahydrochromene, decahydronaphthalene with a carbonyl group, dodecahydrobenzo[f]chromene, tetradecahydronaphtho[2,1-b]oxepine and malabaricane skeletons, almost of which are novel compounds. These products indicate that 6-membered monocyclic, 6/6-fused bicyclic and 6/6/5-fused tricyclic cations were involved in the cyclization reaction in addition to acyclic cation. All the trapped cations were the stable tertiary cation, but not the secondary one, indicating that the polycyclization reaction proceeds with a Markovnikov closure. The product profiles revealed that the cyclization reactions proceeded with the product and substrate specificities in addition to enantioselectivity. Mechanistic insight into the observed stereochemical specificities indicated that the pre-organized chair-conformation of squalene-diols is tightly constricted by the cyclase and a free motion or a conformational change is not allowed in the reaction cavity, thus, the substrate and product specificities are dominantly directed by the least motion of the nucleophilic hydroxyl group toward the intermediary carbocation; a small rotation of the hydroxyl group afforded the cyclization products in a good yield, but a large rotation of the hydroxyl group gave a marginal or no detectable amount of products.

Introduction

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The enzymatic cyclizations of acyclic squalene 1 and 2,3oxidosqualene to form polycyclic triterpenes have fascinated chemists and biochemists for over half a century.^{1,2} X-Ray crystallographic analysis of prokaryotic squalene-hopene cvclase (SHC) was reported in 1997.³ More recently, the three-dimensional structure of human lanosterol synthase was successfully elucidated.⁴ SHC catalyses the conversion of 1 into the pentacyclic triterpenes of hopene 2 and hopanol 3, the ratio being ca. 5: 1, respectively (Scheme 1).5,6 This polycyclization cascade is attained by a single enzyme. 1 is folded in all pre-chair conformation inside the reaction cavity and cyclized in a regio- and stereospecific manner through a series of carbocationic intermediates, leading to the formation of new five C-C bonds and nine chiral centers. Recent studies on the SHC from Alicyclobacillus acidocaldarius have revealed that the polycyclization reaction consists of 8 reaction steps (Scheme 1)5: (1) 1st cyclization to form A-ring 4 by proton attack on the terminal double bond, donated by the DXDD motif,7 (2) 2nd ring closure to give the B-ring (6/6-fused A/B ring system 5),^{8,9} (3) 3rd cyclization to yield 5-membered C-ring (6/6/5-fused A/B/C-tricyclic ring system 6) by Markovnikov closure,^{9,10} (4) which then undergoes ring expansion to form the 6-membered C-ring (6/6/6-fused tricyclic ring system 7),¹⁰ (5) 5th cyclization to give the thermodynamically favored 5-membered D-ring (6/6/6/5-fused A/B/C/D ring system 8, 17-epi-dammarenyl cation),¹¹⁻¹³ (6) followed by the second ring enlargement process to form the 6-membered D-ring (6/6/6/6-fused A/B/C/D-ring system, prohopanyl cation 9),^{11–14} (7) the last ring closure process to construct the 6/6/6/6/5-fused A/B/C/D/E-ring system





В

14 16

30

8

(10, hopanyl cation),¹⁵ and (8) the final deprotonation reaction from 10 to introduce the double bond. Recently, Rajamani and Gao have given deeper insight into the polycyclization mechanism of 1; they proposed that formation of 10 from 5 is achieved by a highly asynchronous concerted reaction and occurs by a kinetical preference.¹⁶ The transient carbocations formed during the polycyclization cascade are stabilized by cation- π -interaction.¹⁷ Previously, we clarified the roles of all the Trp residues conserved among prokaryotic SHCs,18 and also demonstrated that cation- π interaction between π -electrons of aromatic amino acids and the transient carbocations made a great contribution to the acceleration of the polycyclization reaction at lower incubation temperatures. The replacement of Phe365,⁸ Phe601¹⁰ and Phe605^{15a} by Tyr greatly enhanced the reaction velocity due to the elevated π -electron density of Tyr. In addition, a few tyrosine residues were shown to intensify the cation- π interaction by being placed at correct positions in the reaction cavity.¹⁹⁻²¹ The steric bulk size of active site residues is likely to be responsible for stereochemical control during the polycyclization reaction; the substitution of Ile with smaller Ala or Gly at position 261 afforded false intermediates having 13 α -H in the 6/6/5-fused tricyclic and 17 α -H in the 6/6/6/5fused tetracyclic cation,14 the configurations of which are opposite to those of true intermediates 6 (13 β -H) and 8 (17 β -H) (Scheme 1). Replacement of Tyr420 or Leu607 by the bulkier Trp afforded unnatural monocyclic triterpene having (5R, 6R)-1,5,6-trimethylcyclohexene ring, named neoachillapentaene;²² this cyclization proceeded via a constrained boat structure. This is the first example that 1 was folded into a boat structure despite the SHC adopting all chair structure. These findings indicate that the steric bulk size of active site residues direct the folding conformation of 1, i.e., the stereochemical destiny, during the polycyclization cascade.⁵ Recently, we have clarified the catalytic mechanism for the final deprotonation reaction $10 \rightarrow$ 2.^{23,24} Based on the X-ray analysis of A. acidocaldarius SHC,^{3a} the catalytic base responsible for the deprotonation reaction has been suggested to be a water molecule (named a "front water"), the polarization of which is enhanced by other waters (named "back waters") that construct the hydrogen-bonding network by a combination of seven residues T41, E45, E93, R127, Q262, W133 and Y267.^{3a,25} The "front water" thus polarized can store the proton generated from either Me-29 or Me-30 of hopanyl cation 10 to form 2 (Scheme 6, top), but 3 is produced if the "front water" adds as hydoxyl to the C-22 cation of 10 instead of accepting the proton.^{3a,6,25} Recently, we have validate this proposal by the site-directed mutagenesis experiments.²³ The site-directed mutagenesis, targeted for the amino acids surrounding the "front water" such as Q262 and P263, resulted in a greatly enhanced production of 3 along with the decreased formation of 2. A high production of 3 could be explained as follows. The point mutations could give rise to the perturbation around the "front water". This disordered "front water" can not act correctly as the catalytic base for the deprotonation reaction to form 2, and in turn could be placed near to the final hopanyl cation 10, leading to a high production of 3 without forming 2.23 The detailed polycyclization mechanism of squalene, described above, has been inferred from many site-directed mutagenesis experiments. Prokaryotic SHC is of particular note from the aspect of molecular evolution; deletion of Gly600 from the SHC altered the substrate specificity into that of eukaryotic oxidosqualene cyclases (OSCs), which is limited to (3S)-oxidosqualene.²⁶

In addition, studies on the substrate analogs also have provided some important information for the reaction mechanism and the substrate recognition.^{24,27-32} Previously, we reported the trapping experiments of the cationic intermediates. The truncated C₂₂-11 and C₂₇-analogs 13 having an alcoholic group were efficiently cyclized into the carbocyclic skeleton with 6/6/5 + tetrahydrofuran (THF) ring 12^{5,10} and 6/6/6/5 + THF ring 14,^{5,12} respectively (Scheme 2), supporting that carbocationic



Scheme 2 Trappings of cationic intermediates 6 (6/6/5-fused tricycle) and 8 (6/6/6/5-fused tetracycle) by employing truncated squalene analogs 11 and 13 having a highly nucleophilic hydroxyl group.



Scheme 3 Synthetic scheme of *threo* squalenediols **15–18** and *erythro* diols **19–22**. (10R, 11R)-**15** and (6R, 7R)-**16** were prepared with the chiral ligand of $(DHQD)_2$ -PHAL, while (10S, 11S)-**17** and (6S, 7S)-**18** were with that of $(DHQ)_2$ -PHAL. (3R)- and (3S)-2,3-squalenediols, which are produced in the reactions, are omitted in this scheme. To obtain the *erythro* diols, squalene **1** was treated with *m*-chloroperbenzoic acid (MCPBA) to afford oxidosqualenes having the epoxide at the (2,3)-, (6,7)- and (10,11)-positions, which are then subjected to hydrolysis with perchloric acid, giving the desired analogs. The *erythro* diols thus obtained are the racemic mixture of (10R, 11S)-**19** and (10S, 11R)-**20**, and that of (6R, 7S)-**21** and (6S, 7R)-**22**.

intermediates 6 and 8 are involved in the polycyclization reaction of 1. To extend the trapping experiments of cationic intermediates, we synthesized threo-squalene diols, e.g., 6,7dihydroxy-16 and 18, 10,11-dihydroxysqualenes 15 and 17 in addition to the corresponding erythro-diols 19-22 (Scheme 3). By using the diol analogs, cationic intermediates 4, 5 and 6 were successfully trapped. Moreover, these enzymic reactions were more or less accompanied by product and substrate specificities. The stereochemical analyses of the enzymic products demonstrated that the prefolded chair conformation of the analogs is rigidly constricted by squalene cyclase, thus a free motion or a conformational change of the folded chair structure is prohibited in the reaction cavity, once the polycyclization reaction starts, leading to the stereospecific construction of the triterpene skeleton. Herein, we describe the structures of the enzymic products and discuss the mechanism for the formation

of the enzymic products that were generated by incubating *threo*diols **15–18** and *erythro*-diols **19–22** with squalene cyclase.

Results

Syntheses of threo-diols 15-18 and erythro-diols 19-22

The synthetic method is shown in Scheme 3. The asymmetric dihydroxylation of 1 was carried out using chiral ligands of (DHQD)₂PHAL and (DHQ)₂PHAL, which were devised by Crispino and Sharpless.³³ The former ligand gives 15 and 16 having the (R, R)-stereochemistry, while the latter one affords 17 and 18 with the stereochemistry of (S, S). Isolation of 15– 18 was carried out as follows. 2,3-Dihydroxysqualenes were concomitantly produced by the asymmetric dihydroxylation reactions. A SiO₂ column chromatography eluting with hexane-EtOAc (100: 20) gave unreacted 1 and 2,3-dihydroxysqualenes in a pure state. A mixture of 15 and 16 was successfully separated with 5% AgNO₃-SiO₂ column chromatography by eluting with hexane-EtOAc (100 : 50). Isolation yields of 2,3-dihydroxysqualene, 15 and 16 were ca. 4, 7 and 6.5%, respectively. Analogs 17 and 18 were also obtained in similar yields. The enantiomeric excess (ee) of each diol was determined to be 88-95% based on the published values of optical rotation of each compound.³⁴ The erythro-analogs 19–22 were prepared as follows. 1 was treated with MCPBA and the epoxide rings was hydrolyzed with aqueous HClO₄, giving the racemic mixture of (10R, 11S)-19 and (10S, 11R)-20, those of (6R, 7S)-21 and (6S, 7R)-22, and (3R, S)-2,3-dihydroxysqualene, the separation of these erythro-diols was done with a similar method to that of the threo-diols.

Incubations of 15-22 with wild-type SHC and product profiles

Each of diols 15-22 (1 mg) was incubated with 2 cm³ of the cellfree homogenates, which were prepared from an E. coli clone encoding the wild-type SHC. The incubation was carried out at optimal catalytic conditions (pH 6.0 and 60 $^{\circ}\mathrm{C})$ for 16 h and terminated by adding methanolic KOH (5%). The hexane extracts from the reaction mixture were subjected to a short SO₂ column chromatography eluting by hexane-EtOAc (100 : 30) in order to remove an excess of Triton X-100, which was included in the incubation mixture. Fig. 1 shows GC traces of the hexane extracts. As depicted in Fig. 1a, four products 23, 24, 25 and 26 were detected from threo-diol 15 in the distribution ratio of 2.2, 9.2, 61.3 and 19.3%, respectively, and the recovered 15 was 8.0%. Fig. 1b shows that diol 17 gave three products 27, **28** and **29**, the product distribution being 61.9, 3.9 and 10.4%, respectively, along with the recovered 17 (23.8%). The GC peaks of 17 and 27 were overlapped, thus the distribution ratios were estimated by the HPLC separation. As seen in Fig. 1c, two compounds 30 (21.8%) and 31 (8.2%) were obtained from the reaction mixture of analog 16 in a moderate reaction yield (70% of 16 recovered). The cyclization reaction of diol 18 was poor (Fig. 1d) to afford one product 32 (10% yield), almost of 18 remained unchanged. The reaction yield was estimated by the separation of 32 and 18 with a SiO_2 column chromatography, because their GC peaks were overlapped (Fig. 1d). A mixture of erythro-diols 19 and 20 afforded 33 and 34 in distribution ratios of 15.2 and 29.8%, respectively, and the amount of the recovered analogs was 55.0% (Fig. 1e); as described in the Discussion section, 33 and 34 were produced only from diol 20 and no cyclization reaction occurred for diol 19 (Scheme 6). A racemic mixture of 21 and 22 afforded 35 and 36 in yields of 9.3 and 9.2%, respectively, and the recovered racemic mixture of 21 and 22 were 81.5%. As discussed later, this reaction was active only to 21, but inert to 22 (Scheme 6).

Purification and structures of enzymic products 23-36

All the hexane extracts from the incubation mixture were passed through a short SiO_2 column in order to remove an excess



Fig. 1 Gas chromatograms of the hexane extracts from the incubation mixture of analogs **15–22** with the wild-type SHC. Triton X-100 included in the incubation mixture was removed by a short SiO₂ column eluting with hexane–EtOAc (100 : 30). (a) **15**, (b) **17**, (c) **16**, (d) **18**, (e) a racemic mixture of **19** and **20**, (f) a racemic mixture of **21** and **22**. The quantities of enzyme and the analogs used in each experiment (a–f) were identical: 2 cm³ of the cell-free homogenates (equivalent to 0.4 mg of the pure SHC) and 1.0 mg of **15–22**. The asterisk symbols indicate the impurities. As shown in (b), (d) and (e), one of the enzymic products was inseparable from the diol substrates, thus the relative amounts of the products and the unreacted analogs were estimated by separating with SiO₂ column chromatography or HPLC.

amount of Triton X-100. Products 23–26 from diol 15 were partially purified by step-wise gradient elution (hexane–EtOAc, $100: 3 \rightarrow 100: 10$). For the complete separation of products 23, 24 and 25, HPLC was carried out eluting with hexane–2-PrOH (100: 0.1). Product 26 was purified in a pure state by a



reversed phase HPLC (THF- $H_2O = 3 : 2$). Products from other diol substrates 16-18 were purified by a combination of normal and reversed phase HPLC, the composition and the ratio of the mixed solvent for the HPLC being hexane-2-PrOH (100 : 0.1) and THF-H₂O = 3 : 2, respectively. Separation of 27 and 29 was attained by the normal phase HPLC, but 28 was still impure. The complete purification of 28 was done by the reversed phase HPLC. Pure 31 was obtained by the normal HPLC, and the repetitive reversed phase HPLC gave pure 30. Separation of product 32 and diol 18 was performed by the normal phase HPLC. Products 33 and 34, from a racemic mixture of 19 and 20, were purified as follows. 33 was isolated by the repetitive SiO₂ column chromatography (hexane-EtOAc = 100 : 1) and then the residues were subjected to the reversed phase HPLC (THF- $H_2O = 1 : 1$) to afford pure 34. Products 35 and 36 obtained from diols 21 and 22 were homogeneously purified by the normal (hexane-2-PrOH = 100 : 0.05) and reverse phase HPLC (THF- $H_2O = 1 : 1$).

Structures of all the enzymic products were determined by detailed NMR analyses including DEPT, COSY 45, HOHAHA. NOESY, HMQC and HMBC. All the substrate analogs 15-22 contain five olefinic protons and seven allylic methyl groups. The ¹H NMR spectra of 23 showed the presence of three olefinic protons ($\delta_{\rm H}$ 5.51, 5.44, 5.37, each 1H, t, J 6.4 or 7.0) and four allylic methyl groups ($\delta_{\rm H}$ 1.69, 1.74, 1.81, 1.84, each 3H, s), while four methyl groups appeared at higher filed ($\delta_{\rm H}$ 0.77, 0.90, 1.29 and 1.32, each 3H, s). In the HMBC spectrum, Me-25 ($\delta_{\rm H}$ 1.32, 3H, s) and Me-26 ($\delta_{\rm H}$ 1.29, 3H, s) had a correlation with C-9 ($\delta_{\rm C}$ 75.4, s) and C-8 ($\delta_{\rm C}$ 76.0, s), respectively, which suggested that a tetrahydropyran ring is involved. A clear NOE was observed between Me-26 and H-5 ($\delta_{\rm H}$ 1.43, m), but no NOE of Me-25/Me-26, indicating the equatorial orientation of Me-26. Thus, 23 was determined to have bicyclic octahydrochromene structure as shown in Fig. 2. Product 27 also had a similar bicyclic structure, but the stereochemistry at C-8 was opposite to that of 23; Me-25 ($\delta_{\rm H}$ 1.27, 3H, s) of 27 had a strong NOE cross peak for Me-26 ($\delta_{\rm H}$ 1.25, 3H, s). Interestingly, product 24 had a carbonyl group ($\delta_{\rm C}$ 214.3, s), the position of which

same bicyclic skeleton with a carbonyl group as 24, but the stereochemistry at C14 of 28 should be S in contrast to R of 24, because (10S,11S)-17 was used as the substrate. Tricyclic structures 25 and 29 were produced as the enzyme products from 15 and 17, respectively. For 25, an ether bond was found between C-8 ($\delta_{\rm C}$ 74.9, s) and C-13 ($\delta_{\rm C}$ 74.5, d), the assignments of which were determined by apparent HMBC cross peaks from Me-26 ($\delta_{\rm H}$ 1.21, 3H, s) and Me-27 ($\delta_{\rm H}$ 1.33, 3H, s), respectively. Detailed NMR analyses indicated that 25 had the 6/6/6-fused A/B/C tricyclic ring system, where the C-ring consists of a tetrahydropyran structure, thus dodecahydrobenzo[f]chromene skeleton was proposed as shown in Fig. 2. Detailed NMR analyses of product 29 also indicated the same tricyclic system as 25. However, the stereochemistry at C-13 of 25 was opposite to that of **29**. A strong NOE between Me-26 ($\delta_{\rm H}$ 1.21, 3H, s) and H-13 ($\delta_{\rm H}$ 3.52, dd, J 11.6, 2.4) was observed for 25, suggesting the axial orientation of H-13 in the chair structure. On the other hand, no observation of the NOE between them (Me-26, $\delta_{\rm H}$ 1.18, 3H, s; H-13, $\delta_{\rm H}$ 3.76, 1H, dd, J 12.4, 2.8) for 29 suggested the equatorial orientation for H-13 of 29, but the large coupling constant (J 12.4) of the H-13 of 29 does not support the equatorial disposition. Molecular dynamics by Chem3D (Ver. 8.0, CambridgeSoft) indicated a boat structure for the C-ring, in which the dihedral angles between H-12 and H-13 were estimated to be about 170 and 53°, these values being consistent with the observed coupling constants (J 12.4, 2.8). Product 26 had a quite interesting structure consisting of the 6/6/7-fused A/B/C tricyclic ring system having the tetradecahydronaphtho[2,1-b]oxepine skeleton (Fig. 2). The ¹H NMR spectrum of 26 in acetone- d_6 clearly showed the presence of alcoholic proton ($\delta_{\rm H}$ 3.37, d, J 6.0), which had the COSY cross peaks with H-13 ($\delta_{\rm H}$ 3.67, t, J 6.0). An ether linkage was

was determined to be at C-13 by a clear HMBC from Me-

27 ($\delta_{\rm H}$ 1.32, 3H, s). An apparent NOE between Me-25 ($\delta_{\rm H}$

0.926, 3H, s) and Me-26 ($\delta_{\rm H}$ 0.975, 3H, d, J 7.2) indicated the axial orientation of Me-26. The detailed analyses of HMBC

spectrum established that 24 is composed of a 6/6-fused bicyclic

skeleton having a carbonyl group (Fig. 2). Product 28 had the

found between C-8 (δ_c 80.28, s) and C-14 (δ_c 81.33, s). In the HMBC spectrum, Me-27 (δ_H 1.616, 3H, s) had cross peaks with C-14 and C-13 (δ_c 75.59, d), indicating that C(11)-OH of diol substrate **15** was employed for the ether bridge instead of C(10)-OH. Thus, **26** has the 7-membered oxepane skeleton. Indeed, the clear connectivity of H-12/H-11/H-9 was found in the COSY and HOHAHA spectra. Protons of H-13 and Me-27 were arranged in α -disposition, because strong NOEs were observed between OH at C-13 and Me-26 (δ_H 1.336, 3H, s) and between H-7 (δ_H 1.57, m) and Me-27, but no NOE between Me-26 and Me-27. Further detailed NMR analyses proved that the complete structure of **26** is shown in Fig. 2. It should be noted that the enzyme product having the 7-membered C-ring was constructed only from (*R*, *R*)-**15**, but not from the antipode (*S*, *S*)-**17**.

Compounds 30 and 31 were produced from (6R, 7R)-16 as shown in Fig. 1. The ¹H NMR spectrum of **30** dissolved in C₆D₆ showed four olefinic protons ($\delta_{\rm H}$ 5.55, 5.45, 5.42, 5.37) and five allylic methyl protons ($\delta_{\rm H}$ 1.79, 3H, s; 1.73, 6H, s; 1.69, 3H, s; $\delta_{\rm H}$ 1.80, 3H, s), suggesting that one double bond of 16 was used for the cyclization reaction to afford a monocyclic skeleton. The chemical shift values of C-4 ($\delta_{\rm C}$ 71.5, s) and C-5 ($\delta_{\rm C}$ 75.8, s) verified that an ether linkage is intervenient between them. The alcoholic proton ($\delta_{\rm H}$ 2.76, d, J 4.0) exhibited doublet pattern due to the spin coupling with H-6 ($\delta_{\rm H}$ 3.48, m). Both C-5 and C-6 of 30 must be R-configuration, because (6R, 7R)-16 was employed as the substrate. Thus, the complete structure of 30 is depicted in Fig. 2. Product **31** had only one double bond ($\delta_{\rm H}$ 5.45, t, J 6.8, $\delta_{\rm C}$ 125.7, d; $\delta_{\rm C}$ 130.9, s). An ether linkage was found between C-14 ($\delta_{\rm C}$ 89.95, s) and C-17 ($\delta_{\rm C}$ 82.03, d), the assignment of the carbon signals having been determined by the HMBC cross peaks from Me-27 ($\delta_{\rm H}$ 1.29, 3H, s) and Me-28 ($\delta_{\rm H}$ 1.22, 3H, s), respectively. A strong HMBC correlation was observed between the hydroxyl proton ($\delta_{\rm H}$ 2.13, s) and C-28 ($\delta_{\rm C}$ 21.99, q). These data strongly support that **31** possesses 5-membered tetrahydrofuran (THF) ring, but does not involve the 6-membered tetrahydropyran ring. Detailed analyses of the HMBC spectrum clearly verified that both 6/6/5-fused tricyclic ring system and THF ring are involved in 31. A strong NOE between Me-26 ($\delta_{\rm H}$ 1.00, 3H, s) and H-13 ($\delta_{\rm H}$ 1.78, m) indicated the β -orientation of H-13. Stereochemistry of the THF ring was determined as follows. The chiral center at C-17 must have Rconfiguration, because diol substrate 16 used in this experiment has (R, R)-stereochemistry. A clear NOE between Me-27 and H-17 ($\delta_{\rm H}$ 3.72, dd, J 8.4, 6.4) proved that these protons are arranged in the same geometry, leading to α -orientation of Me-27 (*i.e.* 14S-stereochemistry) due to α -orientation of H-17 in the THF ring (i.e. 17R-configuration), as shown in Fig. 1. From (6S, 7S)-diol 18, only one product 32 was obtained (Fig. 1d). Product 32 also consisted of both a 6/6/5-fused tricyclic ring system and a THF ring, which was revealed by detailed analyses of the HMBC spectrum. A clear NOE between Me-26 ($\delta_{\rm H}$ 1.06. 3H, s) and H-13 ($\delta_{\rm H}$ 1.87, m) indicated that H-13 of **32** is arranged in the same β -orientation as that of **31**. However, the orientation of Me-27 ($\delta_{\rm H}$ 1.23, 3H, s) is opposite to that of H-17 ($\delta_{\rm H}$ 3.71, dd, J 10.4, 4.6), because no NOE was found between them. Since the diol substrate used in this experiment was (6S, 7S)-18, the chiral center of C-17 must have S-stereochemistry. Thus, the stereochemistry at C-14 of 32 was determined to be S as shown in Fig. 2. When the spin-spin coupling constant of H-17/H-16 was compared between 31 and 32, the following idea can be proposed: the C(17)-S configuration gives rise to a pair of large and small coupling constants (J 10.4, 4.6) as shown in the ¹H NMR spectrum of **32**, whereas the C(17)-*R* stereochemistry provides a set of nearly equivalent values (J 8.4, 6.4) as seen in that of **31**, often leading to triplet-like splitting pattern.³⁵

From the racemic mixture of *erythro*-diols of (10*R*, 11*S*)-19 and (10*S*, 11*R*)-20, products 33 and 34 were produced. Detailed analyses of the HMBC spectrum of 33 led to the bicyclic octahydrochromene structure like product 27. Furthermore, the EI-MS spectrum of 33 was superimposable onto that of 27 (refer to the electronic supplementary information). A clear NOE between Me-25 ($\delta_{\rm H}$ 1.31, 3H, s) and Me-26 ($\delta_{\rm H}$ 1.23, 3H, s) indicated β -orientation of Me-26, verifying that the chiral center of C-8 has S-configuration. The proton chemical shift of H-10 of 33 was significantly different from that of 27, compared to other proton signals; $\delta_{\rm H}$ 3.43 for 27, while $\delta_{\rm H}$ 3.63 for 33, indicating that 33 has C(10)R-configuration in contrast to that of 27. Thus, product 33 was produced only from (10S, 11R)-20, not from the corresponding enantiomer (10R, 11S)-19. Product 34 showed the same EI-MS spectrum as 25 including the fragment ions (see the supplementary information). Detailed analyses of the NMR spectra in C_6D_6 verified that 34 has the 6/6/6-fused tricyclic skeleton (dodecahydrobenzo[f]chromene structure) like **25**. A strong NOE between Me-26 ($\delta_{\rm H}$ 1.21, 3H, s) and H-13 $(\delta_{\rm H} 3.51, dd, J 11.8, 2.2)$ showed the axial disposition of H-13, verifying the same C(13)-R-stereochemistry as that of 25. This further indicates that the C(14)-stereochemistry must be S, because the substrate used for the incubation is *erythro*-diol. Thus, product 34 was obtained only from 20, but not from 19. It is inferable that the polycyclization reaction enantioselectively occurred; diol 20 only was active, but inert to the corresponding enantiomer 19.

Products 35 and 36 were obtained by the incubation of a mixture of erythro-diols, (6R, 7S)-21 and (6S, 7R)-22. EI-MS spectra of 35 and 36 were identical to those of 30 and 32, respectively, suggesting that 35 and 36 are monocycle and tetracycle (6/6/5-fused tricycle + THF ring), respectively. Detailed analyses of the NMR spectra are in full accord with the proposed structures depicted in Fig. 2. In the NOESY spectrum of 35, H-2 ($\delta_{\rm H}$ 1.48, m, ax.) had clear cross peaks with Me-25 ($\delta_{\rm H}$ 1.20, 3H, s) and Me-24 ($\delta_{\rm H}$ 1.216, 3H, s), indicating that the Me-24 and Me-25 are arranged in axial orientation like 30. Thus, the stereochemistry at the C-5 of 35 is R, leading to S-configuration at the C-6, because erythro-diol was used as the substrate. In the ¹H NMR spectrum of **36** in acetone- d_6 , the signal of H-17 $(\delta_{\rm H} 3.31)$ showed double-doublet splitting pattern (J 11.1 and 3.3), that is, consisted of a pair of large and small coupling constants. This indicates C(17)-S configuration, as described in a comparison of 31 with 32, which is further indicative of C(18)-R, because erythro-diol was used as the substrate. No NOE was observed between Me-27 ($\delta_{\rm H}$ 1.37, s) and H-17, suggested that H-17 and Me-27 was placed in an opposite direction. Thus, the stereochemistry of the THF ring of 36 was determined to be 14S and 17S as shown in Fig. 2.

It is noticeable that some signals of the cyclopentane and the THF rings involved in 31, 32 and 36 did not appear in the ¹³C NMR spectrum, suggesting that there was little tumbling motion of these rings (see spectroscopic data in the Experimental section and supporting information). Products 31, 32 and 36 are classified as malabaricane triterpenes with a 6/6/5-fused tricyclic ring system. The structure of malabaricol,³⁶ which was isolated from the wood of Ailanthus malabarica, is quite similar to that of 31; the structural differences are found only in the following two points: malabaricol has a carbonyl group at C(3) of **31** and the stereochemistry of C(18)is opposite to that of **31** (see the structures shown in Fig. 2). van Tamelen et al. reported the chemical synthesis of the 6/6/5fused tricyclic malabaricatriene from 2,3-oxidosqualene by using a Lewis acid.³⁷ Sharpless also reported the first nonenzymatic synthesis of malabaricanediol (3β-hydoxymalabaricol); erythro-18,19-dihydroxysqualene-2,3-oxide was treated with picric acid in CH₃NO₂ and then allowed to stand for 22 h at ambient temperature, giving a malabaricanediol in a yield of 7%.³⁸ The structure of malabaricanediol has the similar fundamental skeleton as those of 31, 32 and 36, but the configurations of C-17 and C-18 have not been elucidated. Furthermore, malabaricanediol was reported to have 14S-stereochemistry by Sharpless, which was opposite to 14R configuration of malabaricol, 31, 32 and 36.

Discussion

Mechanistic insight into the formation of enzymic products 23–32 from *threo*-diols 15–18

By using threo-diols 15–18, cationic intermediates 4, 5 and 6 were successfully trapped. Products 23 and 27 indicate that monocyclic cation 4 is involved during the polycyclization cascade. Products 24-26, 28 and 29 demonstrated the intermediacy of bicyclic cation 5. The presence of tricyclic cation 6 is verified by production of 31 and 32. Furthermore, the production of 30 showed that acyclic C2-cation 42 (Scheme 5) is involved in the cyclization reaction, which has not been demonstrated before. Thus, the trappings of the stable tertiary cations 4-6 and 40 were successful, but that of secondary cation 7 failed, suggesting a significantly longer lifetime of the tertiary cations than that of the secondary one. A ring expansion from 5- to 6-membered ring ($6 \rightarrow 7$) is likely to occur through cation- π -interaction for the complete polycyclization cascade,^{8,15a,17} although another cyclization mechanism has been proposed that the polycyclization reaction occurs under a concerted manner without violation of Markovnikov's rule.^{16,39} The following results obtained from this study should be noted.

(1) The conversion yields of squalene-diols 15-18 were different. 10,11-Dihydroxysqualenes 15 and 17 gave high conversion ratios of 92 and 76.2%, respectively, but those of 6,7-dihydroxylsqualenes 16 and 18 were significantly low (10–30%).

(2) The 6/6/7-fused tricycle (**26**: 6/6-fused bicycle + oxepane ring, *i.e.*, tetradecahydronaphthooxepine skeleton) was generated from (10*R*, 11*R*)-diol **15**, but not from the corresponding enantiomer (10*S*, 11*S*)-diol **17**. In addition, the monocycle **30** with a tetrahydropyran ring was constructed from (6R, 7R)-diol **16**, but not from the antipode (6S, 7S)-diol **18**. Thus, the formation of these products was enantioselective.

(3) Both enantiomers of (10R, 11R)-15 and (10S, 11S)-17, and those of (6R, 7R)-16 and (6S, 7S)-18 gave enzymic products with the same heterocyclic skeletons, but the product distribution markedly differed between their enantiomers. The cyclic skeletons produced from both enantiomers 15 and 17 were as follows: octahydrochromene skeleton (23 and 27), decahydronaphthalene ring having an additional carbonyl group (24 and 28), and dodecahydrobenzo[f]chromene (25 and 29). On the other hand, 16 and 18 gave 3-deoxymalabaricol skeleton (31 and 32) consisting of both carbocyclic 6/6/5-fused tricycle and heterocyclic THF ring. However, the product distribution of 15 was quite different from that of the corresponding enantiomer 17. The production ratio of 25, 26, 24 and 23 from 15 was estimated to be 27.9 : 8.8 : 4.2 : 1, while that of 27, 29and 28 from 17 was 15.9: 2.7: 1. Diol 16 gave monocycle 30 and tricycle 31 in a ratio of 2.7 : 1 (higher yield of monocycle 30), while the corresponding enantiomer 18 afforded only tricycle 32. Thus, it is inferable that the cyclization reactions of 15-18 exhibited the product and substrate specificities, which are accompanied by stereochemical control.

Mechanistic insights into why and how these specificities occurred provide a better understanding of squalene cyclization mechanism. Acyclic **1** is folded in all pre-chair conformation in the reaction cavity, thus substrate analogs **15–18** should be folded as depicted in Schemes 4 and 5. Previously, we reported that the isopropylidene moiety is essential to the initiation of the polycyclization reaction.^{24,27} Analogs **15–18** have the isopropylidene double bond at both terminal sides, thus the cyclization reactions can start from both left side (a) and right side (b), as shown in Schemes 4 and 5. Scheme 4A shows the mechanisms for the formations of products **23–26** from **15**. According to Scheme 1, **15** underwent the cyclization reaction to afford both bicyclic cation **37** (like **5**) from **15a** and monocyclic cation **38** (like **4**) from **15b**. Both 10- and 11-OH of **15** could attack on cation **37** gave **25** (path *a*), while the attack of 10*R*-OH

afforded product 26 (path b). The hydride shift of H-11 to the C15-cation of **37**, followed by the deprotonation from 11*R*-OH, afforded a carbonyl group (path c) to yield 26. To the C6-cation of 38, 10R-OH attacked to give 23 (path d). As described above, the yield of 25 was significantly higher than that of 23 (ca. 28fold), suggesting that the nucleophilic attack of axial OH to cation 37 was more favorable than that of equatorial OH. This product specificity would be explained in terms of the rotation angle between the OH and the p-orbital of carbocation. The equatorial OH of cation 38 must be largely rotated through C11–C12 axis in the cyclase cavity to form 23 (180°), whereas, in case of the axial OH, only a small motion (60°) is enough for the production of 25 from cation 37, resulting in a significantly higher production of 25 than that of 23. Thus, the findings strongly indicate that a free motion or large conformational change of the pre-organized chair structures is not allowed in the reaction cavity due to the tight binding of the folded chair structures to the cyclase enzyme. A rotation angle of 10R-OH of 37 for the formation of 26 is also smaller compared to that for the production of 23 and the equatorial OH is close to the C-15 cation, leading to higher production of 26 by ca. 9-fold than that of 23. However, the yield of 26 having the 7-membered ring was less by 3-fold than that of 25 with the 6-membered ring, possibly due to the more favorable formation of 6-membered ring than that of 7-membered ring. Further evidence that the least motion directs the product specificity was given also by the cyclization reaction of (10S, 11S)-diol 17 (Scheme 4B); the yield of bicyclic 27 was highest (61.9%) among all the products 27-29, due to the least motion (60°). Both enantiomers of 15 and 17 afforded the similar octahydrochromene skeleton (23, 27), but the yields were significantly different; the yield of 23 was negligible (2.2%) and less by 30-fold than that of 27. This product specificity can be explained also in terms of the least motion of the OH group; only a small rotation (60°) of the axial 10S-OH of cation 40 is required for the formation of 27 (path h), but a large rotation (180°) of 10*R*-OH of **38** for the formation of **23**. The production of 29 from cation 39 (path e) was not high (10.4%), due to the large rotation of 11S-OH. Path g led to the formation of 28 via hydride shift of H-11 to the C15-cation of 39 in a way similar to the formation of 24. Formation of naphthooxepine skeleton from 39 is expected to occur via path f, but no detectable amount of this skeleton was found in the incubation mixture, due to the requirement of a large motion of the axial 10-OH of 39; 10R-OH of 37 is close to the C15-cation to give 26, but 10S-OH of 39 is distant from the C15-cation.

A tetrahydropyran ring 30 was constructed from (6R, 7R)-diol 16, but not from the corresponding enantiomer (6S, 7S)-18 (see Scheme 5A-B). This enantioselectivity also can be explained in terms of the degree of motion of the OH group; only a small motion (60°) toward C2-cation of acyclic cation 42 (path j), but a large rotation (180°) toward C2-cation of cation 44 (path l), thus, no detectable amount of the monocyclic product with a tetrahydropyran ring was found in the reaction mixture of 18. The production ratio of 30 to 31 was 2.7 : 1. As shown in Scheme 5A, 7R-OH of cation 41 must rotate more largely (180°, path i) to produce 31 than 6*R*-OH of cation 42 (60°, path j), leading to a less amount of **31**. (6S, 7S)-Diol **18** also afforded product 32 via the least motion (60°) of 7S-OH of cation 43. It should be noted that both products 31 and 32 had 14S-stereochemistry (the numbering according to Fig. 2), strongly indicating that the C10-Me of tricyclic cations 41 and 43 is in the α -orientation during the polycyclization cascade (Scheme 5).

Insight into the formation of enzymic products 33–36 from *erythro*-diols 19–22

Next, we discuss the cyclization mechanism of *erythro*-diols 19– 22. The formation mechanism of 33 and 34 from the racemate (19 + 20) is shown in Scheme 6A. The product structures of 33 and





Ratio of Products 27 and 29 to 28=15.9:2.7:1

Ĥ

C5 Ĥ

40

Scheme 4 (A) Mechanisms for the formation of products 23–26 from (10*R*, 11*R*)-diol 15 and (B) those of products 27–29 from (10*S*, 11*S*)-diol 17.

OH

3

2

Me

C10

39

Ĥ

C16 Ĥ

OH

C19



Scheme 5 (A) Mechanism for the formation of products 30 and 31 from (6R, 7R)-diol 16 and (B) that of 32 from (6S, 7S)-diol 18.

34 (Fig. 2) clearly indicate that 20 only underwent the cyclization reaction, but the corresponding antipode 19 was not active, thus this reaction enantioselectively proceeded. Monocyclic 45 and bicyclic cations 46 could be converted to 33 and 34 via paths m and n, respectively, involving a least motion (60°), but no product from 19 was found due to the requirement of large rotation of the OH, as illustrated in Scheme 6A. The amount of 34 produced was ca. two-fold higher than that of 33, indicating that bicyclic cation 46 was more easily formed than monocyclic cation 45. The reason is not clear, but the steric bulk size of the large OH group may have affected the conversion yield, as discussed in the next section. The enantioselective cyclization was also found for the incubation of a racemic mixture of 21 and 22, 21 was selectively converted to give 35 and 36 due to a small rotation (60°) of the OH (Scheme 6B), but 22 underwent no reaction due to the large rotation (180°). Thus, it is inferable that a motion degree of the nucleophilic OH group, irrespective of *threo-* and *erythro-*diols, dominantly determines the product and substrate specificities.

Effect of the bulk size of diols on the cyclization yields

Previously, we have demonstrated that the introduction of methyl group(s) at position(s) of C-7 and C-11 (the numbering starts from the initiation site of cyclization reaction) of the squalene backbone strongly prevents the formation of the chair-folding conformation inside the reaction cavity, leading to no reaction and/or to the early truncation of the polycyclization cascade.^{29,32} This could possibly arise from the disorganization of the folded chair conformations as a result from the repulsive interaction of the methyl group(s) with the corresponding recognition site(s) of the cyclase.^{29,32} Conformations **15b** and

20 (10S, 11R)-diol (50%)



B) Racemic mixture of 21 and 22



Scheme 6 (A) Cyclization of racemic mixture of erythro-diols 19 and 20 and (B) that of 21 and 22.

18b have a bulky hydroxyl group at 11- or 7-position, which is arranged in an axial disposition. In addition to the large rotation angle of the OH (180°), which is required for the formation of 23 from 15b and a terahydropyran ring from 18b, the steric repulsive interaction of the bulky hydroxyl group with the cyclase may have disordered the pre-folded chair structure, resulting in a further decrease of the cyclization yields (2.2% for 15b and 0% for 18b). When the cyclization reactions of erythro-diols 20 and 21 were compared, the production of 34 was higher than that of 36 (ca. 3-fold), despite both products having been produced via a least motion of the OH (60°) (see Scheme 6), indicating that bicyclic cation 5 was more easily formed than tricyclic cation 6. A more preferable formation of 5 was also found in a comparison of threo-diols of 15 and 18; the production amount of 25 was remarkably larger (ca. 6fold) than that of 32, despite both products being generated via a least motion (60°) of the OH (see Schemes 4 and 5). These facts indicate that the cyclization yields depended not only on the rotation angle of the OH, but also on the steric bulk size of the substrate analog. The repulsive interaction of the bulky OH group at C7 of 43 and 48 with the cyclase would be larger compared to that of C11 of 37 and 46, resulting in higher production of the bicyclic cation 5 than that of the tricyclic cation 6. As described above, 10,11-diols 15 and 17 were more favorably accepted as the substrates than 6,7-diols 16 and 18. On the other hand, 6,7-epoxysqualene afforded significantly higher conversion yield compared to 10,11-epoxysqualene (results to be published), indicating that, in the case of epoxysqualene, the formation of tricyclic cation 6 is more preferable than that of bicyclic cation 5. This opposite feature between epoxide and diol would be due to the difference of their steric bulk sizes; the epoxide ring is smaller than that of diol. This finding also indicates that the bulkier hydroxyl groups more or less affected the formation of the rigidly held chair conformation, resulting in decreased cyclization yield. However, we can unambiguously conclude that the rotation angle of the nucleophilic OH group substantially directs the substrate and product specificities, as discussed in the previous sections.

In conclusion, we succeeded in trapping the intermediates of acyclic C2-cation, monocyclic 4, bicyclic 5 and tricyclic cations 6 by using threo- and erythro-diols of squalene, demonstrating the significantly longer lifetime of these tertiary cations (Markovnikov cations) formed during the polycyclization cascade of 1. The cyclization reactions proceeded with the product specificity and/or substrate specificity, which can be explained in terms of the motion degree of the nucleophilic hydroxyl group toward the intermediary carbocation. Thus, we have now the strong evidence that all the prefolded chair conformation is tightly constricted by the cyclase enzyme, thus, a free motion or a large conformational change of the folded chair conformation is not allowed inside the reaction cavity. Previously, Cornforth proposed that the protosterol cation has 17α -oriented side chain,⁴⁰ but in order to produce the 20*R*stereochemistry of lanosterol, a 120° rotation of the side chain through the C17-C20 bond is required prior to the hydride shift from C17 to C20. In contrast, Corey proposed, based on studies of the substrate analogs, e.g. 20-oxa analog and (20E)-20,21-dehydro-analog of oxidosqualene, that the side chain at C17 of protosterol cation must be arranged in β orientation, in which only a small rotation ($<60^{\circ}$) around the C17-C20 bond is required,^{41,42} thus Cornforth's rationalization of the conformational change to explain C20-R configuration is no longer necessary. Corey's idea indicates that the preorganized chair-boat-chair conformation is tightly bound to the oxidosqualene cyclase and the conformational change is not allowed in the reaction cavity, which is in good accordance with the inference from the present investigations of squalene diols. Moreover, through these trapping experiments, novel triterpenes were created that have the skeletons of octahydrochromene, dodecahydrobenzo[f]chromene and tetradecahydronaphtho[2,1b]oxepine, in addition to the production of natural triterpene skeleton of 3-deoxymalabaricol.

Experimental

Analytical methods

NMR spectra were mainly recorded in C₆D₆ on a Bruker DMX 600 or DPX 400 spectrometer, the chemical shifts being relative to the solvent peak $\delta_{\rm H}$ 7.280 and $\delta_{\rm C}$ 128.0 ppm as the internal reference for ¹H- and ¹³C NMR spectra, respectively. Some synthetic intermediates were measured in CDCl₃. The chemical shifts in CDCl₃ solution were given according to the internal solvent peaks of $\delta_{\rm H}$ 7.26 and $\delta_{\rm C}$ 77.0 ppm. To further validate the proposed structures, the NMR data of some enzymic products were measured in acetone- d_6 , the solvent peak being referred to be $\delta_{\rm H}$ 2.04, $\delta_{\rm C}$ 29.8 ppm. The coupling constants J are given in Hz. GC analyses were done on a Shimadzu GC-8A chromatograph equipped with a flame ionization detector (DB-1 capillary column, 0.53 mm \times 30 m). GC-MS spectra were on a JEOL SX 100 spectrometer under electronic impact at 70 eV with a DB-1 capillary column (0.32 mm \times 30 m), the oven temperature being elevated from 220 to 270 °C (3 °C min⁻¹). HR-EIMS was measured by direct inlet system. Specific rotation values were measured at 25 °C with a Horiba SEPA-300 polarimeter. FABMS (positive) including HRMS was measured using glycerol matrix.

Incubation condition and purification of enzymatic products

Standard incubation conditions were performed according to the published protocols.^{7*a*,24} The cell-free homogenates were prepared as follows. A one liter culture of *E. coli* encoding the native SHC was harvested by centrifugation and to the collected pellets was added 50 cm³ of citrate buffer solution (pH 6.0), and then subjected to ultrasonication to disrupt the cells. The supernatant was used for the incubations after removing the cell debris by centrifugation. One cm³ of the supernatant contains *ca.* 200 µg of the pure SHC. The incubation mixtures were lyophilized, and then the products and the unreacted substrate analogs were extracted three times with a mixture of hexane and EtOAc (100 : 10). An excess of Triton X-100 detergent was removed by passing a short SiO₂ column eluting with hexane– EtOAc (100 : 20). Each product was purified by a combination of the normal and the reverse phase HPLC, as described in the text.

Syntheses of threo-squalene diols 15-18

These compounds were synthesized according to the published protocol. The flask (500 cm3) containing 10 cm3 of 'BuOH-H₂O was well cooled to 0 °C. To the well-stirred solution, (DHQD)₂PHAL (40 mg, 5.0 mol%), K₃Fe(CN)₆ (0.99 g, 3 mmol), K₂CO₃ (0.42 g, 3 mmol), CH₃SO₂NH₂ (96 mg, 1 mmol), OsO₄ (2.54 mg, 1.0 mol%) were added and further stirred for 30 min until almost of the reagents was dissolved. To the mixture was added squalene 1 (410 mg, 1 mmol), dissolved in a small amount of 'BuOH. The reaction was further continued by stirring at 0 °C for 24 h, followed by addition of Na₂S₂O₅ (1.5 g, 7.9 mmol), and then warmed to room temperature. The diol mixture was extracted with EtOAc (50 cm³ \times 3). The organic layer was washed with 6 N aq. NaOH and dried over anhydrous Na₂SO₄ and stood overnight. After evaporating EtOAc under reduced pressure, the residues were subjected to a column chromatography over SiO2. To isolate the desired materials, the following solvents were used. First, hexane was used to remove the unreacted starting material 1. Second, by eluting with hexane-EtOAc (100 : 10), 2,3(R)-squalene diol was obtained in pure state, but the separation of 15 and 16 failed. AgNO₃-impregnated (5%) SiO₂ column chromatography led to the successful separation, giving the desired products in the following yields: 16.9 mg (3.8%) for 2,3(R)-squalene diol, 9.0 mg (2.0%) for 15 and 20.2 mg (4.6%) for 16. Sufficient amounts of 16 and 17 for the present experiments were prepared by repeated and large-scale experiments. The specific rotations $([a]_{D}^{25})$, of **15** and **16** were +10.44° (c 2.14, CHCl₃) and +10.23 (c 16.96, CHCl₃), respectively, indicating that the optical purities were 94.9 and 87% ee, respectively.³⁴ To prepare the *threo-(S, S)*diols, (DHQ)₂PHAL was used instead of (DHQD)₂PHAL, but the synthetic and isolation methods were essentially the same as those of the enantiomers. The specific rotations ($[a]_{D}^{25}$), for 18 and 17 were $+10.33^{\circ}$ (c 8.51, CHCl₃) and +9.75 (c 4.02, CHCl₃), respectively. Thus, the optical purities of 18 and 17 were 88.9 and 87.5% ee, respectively. The NMR and MS spectral data were indistinguishable between the (R, R)- and the (S, S)-forms, because of the enantiomeric relationship between them. NMR data of 15–18 are given in the ESI.

Syntheses of a mixture of *erythro*-(6R, 7S)-21 and (6S, 7R)-22, and that of *erythro*-(10R, 11S)-19 and (10S, 11R)-20

To the cooled solution of squalene (101.3 mg, 0.25 mmol) in CH₂Cl₂ (2.5 cm³) at 0 °C, MCPBA (62.9 mg, 0.365 mmol) was added and stirred for 2 h. This reaction mixture was washed with 20% NaHCO₃ (10 cm³ × 3) and with brine (10 cm³ × 2), and the organic layer was dried over anhydrous Na₂SO₄. To remove the unreacted **1**, the reaction mixture was passed through a short SiO₂ column to afford 6,7-oxido- and 10,11-oxido- and 2,3-oxidosqualenes (total yield, 71.7%). To a mixture of the three oxidosqualenes (1.0 g) dissolved in THF (17.5 cm³),

was added 2 cm³ of 3% perchloric acid and stirred for 3 h at ambient temperature. The reaction mixture was poured into the ice-water, then the products were extracted with hexane (500 cm³ × 3), which was washed with 10% NaHCO₃ and with brine, then dried over anhydrous Na₂CO₃. The three squalene diols were partially separated by SiO₂ column chromatography. Complete separation was accomplished with 5% AgNO₃–SiO₂ column chromatography eluting with hexane–EtOAc (100 : 20). The yields were as follows: 0.28 g for the mixture of (10*R*, 11*S*)and (10*S*, 11*R*)-squalene diol, 0.24 g for the mixture of (6*R*, 7*S*)and (6*S*, 7*R*)-squalene. NMR data of the racemic mixture of (6*R*, 7*S*)-**21** and (6*S*, 7*R*)-**22** and that of (10*R*, 11*S*)-**19** and (10*S*, 11*R*)-**20** are given in the ESI.

Spectroscopic data of enzymic products 23-36

Product 23 (oil). NMR data in C₆D₆ (400 MHz): $\delta_{\rm H}$ 0.775 (3H, s, H-24), 0.903 (3H, s, H-23), 1.21 (dd, J 12.8, 4.8, H-3), 1.29 (3H, s, H-26), 1.32 (3H, s, H-25), 1.34 (2H, m, H-2 & H-3), 1.39 (m, H-6), 1.43 (1H, m, H-5), 1.48 (m, H-2), 1.49 (m, H-7), 1.50 (2H, m, H-6 & H-11), 1.67 (m, H-11), 1.69 (3H, s, H-29), 1.74 (3H, s, H-28), 1.75 (m, H-1), 1.81 (3H, s, H-30), 1.84 (3H, s, H-27), 1.89 (m, H-7), 2.21 (2H, t, J 7.2, H-19), 2.22 (2H, t, J 7.2, H-15), 2.27 (2H, t, J 7.2, H-20), 2.33 (2H, t, J 7.2), 2.48 (m, H-1), 2.51 (m, H-12), 2.72 (m, H-12), 3.81 (d, J 10.0, H-10), 5.37 (t, J 6.4, H-21), 5.44 (t, J 6.4, H-17), 5.51 (t, J 7.0, H-13); $\delta_{\rm C}$ 16.07 (C-6), 16.11 (C-28), 16.19 (C-27), 17.72 (C-29), 20.34 (C-2), 20.68 (C-24), 23.57 (C-25), 24.29 (C-26), 25.84 (C-30), 26.08 (C-12), 27.23 (C-16), 27.30 (C-20), 31.87 (C-7), 32.14 (C-23), 32.52 (C-11), 33.54 (C-4), 40.21 (C-15), 40.33 (C-19), 41.72 (C-3), 42.54 (C-1), 51.57 (C-5), 75.39 (C-9), 75.68 (C-10), 76.03 (C-8), 124.88 (C-17), 124.93 (C-21), 125.2 (C-13), 131.1 (C-22), 134.9 (C-18), 135.6 (C-14). The assignments of C-16 and C-20, and those of C-15 and C-19 are interchangeable. EIMS m/z(%): 69 (39), 81 (15), 95 (13), 109 (8), 137 (15), 177 (74), 195 (100), 444 (M⁺, 1). HRFABMS (glycerol) m/z (M + H): calcd. for $C_{30}H_{53}O_2$, 445.4046; found 445.4122. $[a]_D^{25} = +26.1$ (c 0.11, CHCl₃).

Product 24 (oil). NMR data in C₆D₆ (400 MHz): $\delta_{\rm H}$ 0.905 (m, H-5), 0.926 (3H, s, H-25), 0.930 (m, H-1), 0.950 (3H, s, H-24), 0.975 (d, J 7.2, H-26), 1.01 (3H, s, H-23), 1.12 (m, H-9), 1.25 (ddd, 12.8, 12.8, 3.6, H-3), 1.32 (3H, s, H-27), 1.41 (m, H-2), 1.47 (2H, m, H-3 & H-6), 1.47 (m, H-3), 1.55 (m, H-2), 1.64 (2H, m, H-6 & H-7), 1.65 (m, H-11), 1.69 (3H, s, H-29), 1.70 (3H, s, H-28), 1.75 (m, H-7), 1.80 (m, H-1), 1.803 (3H, s, H-30), 1.86 (m, H-15), 2.03 (m, H-11), 2.07 (m, H-16), 2.18 (2H, t, J 6.8, H-19), 2.26 (m, H-15), 2.29 (2H, t, J 6.8, H-20), 2.30 (m, H-12), 2.39 (m, H-16), 2.43 (m, H-12), 5.31 (t, J 6.8, H-17), 5.34 (t, J 6.8, H-21); $\delta_{\rm C}$ 15.44 (C-26), 16.04 (C-28), 16.45 (C-25), 17.71 (C-29), 17.76 (C-6), 18.76 (C-2), 20.52 (C-11), 21.79 (C-24), 22.70 (C-16), 25.75 (C-27), 25.82 (C-30), 27.08 (C-20), 29.82 (C-8), 33.45 (C-4), 33.70 (C-23), 34.45 (C-12), 34.99 (C-7), 38.75 (C-10), 39.72 (C-1), 40.04 (C-19), 40.16 (C-15), 42.34 (C-3), 53.03 (C-9), 56.87 (C-5), 78.61 (C-14), 124.1 (C-17), 124.8 (C-21), 131.3 (C-22), 135.9 (C-18), 214.3 (C-13). EIMS m/z (%): 69 (100), 81 (33), 95 (29), 109 (31), 137 (26), 177 (63), 294 (37), 444 (M⁺, 1). HRFABMS (glycerol) m/z (M + H): calcd. for $C_{30}H_{53}O_2$, 445.4046; found: 445.4127. $[a]_D^{25} = +8.18$ (c 0.275, CHCl₃).

Product 25 (oil). NMR data in C_6D_6 (400 MHz): δ_H 0.738 (3H, s, H-25), 0.780 (ddd, *J* 12.8,12.8,3.2, H-1), 0.888 (3H, s, H-24), 0.900 (m, H-5), 0.956 (3H, s, H-23), 1.14 (bd, *J* 12.0, H-9), 1.21 (3H, s, H-26), 1.22 (m, H-3), 1.25 (m, H-11), 1.27 (m, H-6), 1.33 (3H, s, H-27), 1.46 (m, H-3), 1.48 (m, H-2), 1.49 (m, H-12), 1.51 (m, H-7), 1.55 (m, H-1), 1.60 (m, H-11), 1.62 (m, H-12), 1.63 (m, H-6), 1.67 (m, H-2), 1.69 (3H, s, H-29), 1.80 (m, H-7), 1.81 (2H, m, H-15), 1.811 (3H, s, H-30), 1.82 (3H, s, H-28), 2.24 (2H, t, *J* 7.2, H-19), 2.32 (2H, t, *J* 7.2, H-20), 2.48

(2H, m, H-16), 2.69 (s, O*H*), 3.52 (dd, *J* 11.6, 2.4, H-13), 5.38 (t, *J* 6.8, H-21), 5.50 (t, *J* 6.6, H-17); $\delta_{\rm C}$ 15.67 (C-25), 16.10 (C-28), 17.73 (C-29), 18.34 (C-11), 18.91 (C-2), 20.11 (C-6), 20.80 (C-26), 21.46 (C-24), 21.80 (C-27), 22.77 (C-16), 25.85 (C-30), 27.09 (C-12), 27.22 (C-20), 33.33 (C-4), 33.46 (C-23), 36.79 (C-10), 39.08 (C-1), 39.76 (C-15), 40.24 (C-19), 42.06 (C-7), 42.34 (C-3), 56.24 (C-5), 57.52 (C-9), 73.04 (C-14), 74.51 (C-13), 74.91 (C-8), 125.0 (C-21), 125.7 (C-17), 131.1 (C-22), 134.7 (C-18). EIMS *m*/*z* (%): 69 (100), 81 (35), 95 (31), 109 (37), 137 (32), 191 (90), 231 (51), 276 (38), 426 (M⁺ – H₂O, 3). HRFABMS (glycerol) *m*/*z* (M + H): calcd. for C₃₀H₅₃O₂, 445.4046; found: 445.4070. [$al_{\rm D}^{25} = -12.9$ (*c* 0.458, CHCl₃).

Product 26 (oil). NMR data in C₆D₆ (400 MHz): $\delta_{\rm H}$ 0.791 (3H, s, H-25), 0.840 (m, H-1), 0.866 (3H, s, H-24), 0.870 (m, H-5), 0.958 (3H, s, H-23), 1.21 (m, H-3), 1.23 (m, H-6), 1.357 (6H, s, H-26 & H-27), 1.44 (m, H-3), 1.47 (m, H-2), 1.51 (m, H-11), 1.59 (m, H-15), 1.61 (m, H-9), 1.64 (m, H-2), 1.65 (m, H-12), 1.68 (m, H-6), 1.70 (3H, s, H-29), 1.72 (m, H-7), 1.74 (m, H-11), 1.79 (m, H-1), 1.81 (3H, s, H-30), 1.85 (3H, s, H-28), 1.98 (m, H-7), 2.00 (m, H-12), 2.11 (d, J 6.0, OH), 2.16 (dd, J 13.2, 13.2, 4.8, H-15), 2.26 (2H, t, J 6.8, H-19), 2.31 (m, H-16), 2.33 (2H, t, J 6.8, H-20), 2.41 (m, H-16), 3.62 (t, J 5.4, H-13), 5.39 (t, J 6.8, H-21), 5.51 (t, J 7.0, H-17),; $\delta_{\rm C}$ 16.14 (C-25), 16.16 (C-28), 17.39 (C-11), 17.75 (C-29), 19.11 (C-2), 21.48 (C-6), 21.50 (C-24), 23.21 (C-16), 24.20 (C-27), 24.69 (C-26), 25.85 (C-30), 27.26 (C-20), 32.97 (C-12), 33.50 (C-4), 33.55 (C-23), 39.39 (C-10), 40.24 (C-19), 40.43 (C-1), 41.71 (C-15), 42.25 (C-3), 44.00 (C-7), 56.64 (C-5), 59.88 (C-9), 74.73 (C-13), 80.44 (C-8), 80.73 (C-14), 125.0 (C-21), 125.7 (C-17), 131.1 (C-22), 134.6 (C-18). ¹H NMR data in acetone- d_6 (400 MHz): δ 0.794 (3H, s, H-24), 0.807 (3H, s, H-25), 0.880 (3H, s, H-23), 0.910 (m, H-5), 0. 920 (m, H-1), 1.16 (m, H-3), 1.202 (3H, s, H-27), 1.27 (ddd, J 12.4, 12.4, 3.2, H-6), 1.34 (3H, s, H-26), 1.35 (m, H-15), 1.35 (m, H-3), 1.38 (m, H-9), 1.42 (m, H-2), 1.47 (m, H-11), 1.57 (m, H-7), 1.58 (3H, s, H-29), 1.62 (3H, s, H-28), 1.62 (m, H-6), 1.645 (3H, s, H-30), 1.65 (m, H-2), 1.69 (m, H-12), 1.72 (m, H-11), 1.77 (m, H-7), 1.83 (2H, m, H-1 & H-15), 1.85 (m, H-12), 1.96 (2H, t, J 6.8, H-19), 2.02 (m, H-16), 2.08 (m, H-16), 2.33 (2H, t, J 7.2, H-20), 3.37 (d, J 6.0, OH), 3.67 (t, J 6.0, H-13), 5.10 (bt, J 6.8, H-21), 5.16 (t, J 6.8, H-17). EIMS m/z (%): 69 (100), 81 (47), 95 (42), 109 (51), 136 (47), 191 (90), 231 (18), 235 (13), 250 (11), 276 (18), 426 (M⁺ – H₂O, 1). HRFABMS (glycerol) m/z (M + H): calcd. for $C_{30}H_{53}O_2$, 445.4046; found: 445.4113. $[a]_D^{25} = -22.9$ (c 0.142, CHCl₃).

Product 27 (oil). NMR data in C₆D₆ (400 MHz): $\delta_{\rm H}$ 0.768 (3H, s, H-24), 0.910 (3H, s, H-23), 1.16 (dd, J 12.0, 2.0, H-5), 1.20 (m, H-3), 1.255 (3H, s, H-26), 1.274 (3H, s, H-25), 1.33 (m, H-3), 1.34 (m, H-1), 1.40 (m, H-6), 1.46 (m, H-7), 1.48 (2H, m, H-2), 1.50 (m, H-6), 1.59 (m, H-11), 1.69 (3H, s, H-29), 1.71 (2H, m, H-1 & H-11), 1.73 (3H, s, H-28), 1.74 (m, H-7), 1.81 (3H, s, H-30), 1.83 (3H, s, H-27), 2.21 (2H, m, H-19), 2.25 (2H, m, H-15), 2.30 (2H, m, H-20), 2.33 (2H, m, H-16), 2.49 (m, H-12), 2.69 (m, H-12), 2.76 (d, J 5.0, OH), 3.43 (ddd, J 10.4, 5.0, 2.0, H-10), 5.37 (t, J 7.0, H-21), 5.42 (t, J 6.8, H-17), 5.53 (t, J 6.8, H-13); $\delta_{\rm C}$ 16.11 (C-28), 16.19 (C-27), 16.34 (C-6), 17.73 (C-29), 20.26 (C-2), 20.87 (C-24), 22.39 (C-26), 23.54 (C-25), 25.62 (C-12), 25.84 (C-30), 27.11 (C-16), 27.23 (C-20), 31.28 (C-11), 32.20 (C-23), 33.39 (C-4), 34.83 (C-7), 40.21 (C-15), 40.26 (C-19), 41.62 (C-3), 41.92 (C-1), 54.37 (C-5), 75.11 (C-9), 75.73 (C-8), 79.63 (C-10), 124.9 (C-17), 125.0 (C-21), 125.3 (C-13), 131.1 (C-22), 134.9 (C-18), 135.2 (C-14). The assignments of C-16 and C-20 and those of C-15 and C-19 are interchangeable. EIMS m/z (%): 69 (22), 81 (10), 95 (9), 109 (7), 137 (12), 177 (61), 195 (100), 444 (M⁺, 3). HRFABMS (glycerol) m/z (M + H): calcd. for C₃₀H₅₃O₂, 445.4046; found: 445.4120. $[a]_{D}^{25} = -9.01$ (*c* 0.233, CHCl₃).

Product 28 (oil). NMR data in C₆D₆ (400 MHz): $\delta_{\rm H}$ 0.90 (dd, J 12.8, 3.0, H-5), 0.91 (m, H-1), 0.951 (3H, s, H-24), 0.951 (3H, s, H-25), 0.990 (3H, d, J 7.2, H-26), 1.01 (3H, s, H-23),

1.10 (m, H-9), 1.26 (dd, J 12.4, 3.2, H-3), 1.32 (3H, s, H-27), 1.44 (m, H-6), 1.48 (m, H-2), 1.49 (m, H-3), 1.59 (m, H-6), 1.60 (m, H-7), 1.64 (m, H-11), 1.65 (m, H-2), 1.69 (3H, s, H-29), 1.70 (3H, s, H-28), 1.73 (m, H-7), 1.79 (m, H-1), 1.806 (3H, s, H-30), 1.81 (2H, m, H-15), 1.88 (m, H-8), 2.08 (m, H-16), 2.09 (m, H-11), 2.18 (2H, t, J 6.8, H-19), 2.25 (m, H-12), 2.27 (2H, m, H-20), 2.40 (m, H-16), 2.46 (m, H-12), 4.06 (s, OH), 5.32 (t, J 6.6, H-17), 5.35 (t, J 6.8, H-21); $\delta_{\rm C}$ 15.43 (C-26), 16.02 (C-28), 16.44 (C-25), 17.71 (C-29), 17.75 (C-6), 18.76 (C-2), 20.44 (C-11), 21.78 (C-24), 22.69 (C-16), 25.78 (C-27), 25.82 (C-30), 27.07 (C-20), 29.79 (C-8), 33.44 (C-4), 33.71 (C-23), 34.38 (C-12), 34.98 (C-7), 38.71 (C-10), 39.68 (C-1), 40.03 (C-15), 40.15 (C-19), 42.35 (C-3), 52.95 (C-9), 56.86 (C-5), 78.60 (C-14), 124.1 (C-17), 124.8 (C-21), 131.3 (C-22), 135.9 (C-18), 214.2 (C-13). EIMS m/z (%): 69 (70), 81 (23), 95 (22), 109 (31), 137 (26), 177 (100), 294 (97), 444 (M⁺, 1). HRFABMS (glycerol) m/z (M + H): calcd. for $C_{30}H_{53}O_2$, 445.4046; found: 445.4115. $[a]_{D}^{25} = +32.1$ (c 0.42, CHCl₃).

Product 29 (oil). NMR data in C₆D₆ (400 MHz): $\delta_{\rm H}$ 0.792 (3H, s, H-25), 0.820 (m, H-1), 0.888 (3H, s, H-24), 0.89 (m, H-5), 0.958 (3H, s, H-23), 1.13 (m, H-6), 1.18 (3H, s, H-26), 1.22 (m, H-3), 1.29 (3H, s, H-27), 1.36 (m, H-11), 1.46 (m, H-3), 1.47 (m, H-2), 1.51 (m, H-1), 1.53 (m, H-9), 1.54 (m, H-11), 1.57 (m, H-7), 1.58 (m, H-6), 1.60 (m, H-12), 1.66 (m, H-2), 1.69 (m, H-12), 1.69 (3H, s, H-29), 1.80 (3H, s, H-30), 1.81 (3H, s, H-28), 1.84 (2H, m, H-15), 1.89 (m, H-7), 2.24 (2H, t, J 7.2, H-19), 2.32 (2H, m, H-20), 2.51 (2H, m, H-16), 3.76 (dd, J 12.4, 2.8, H-13), 5.38 (t, J 6.8, H-21), 5.52 (t, J 6.8, H-17); δ_C 14.98 (C-25), 15.82 (C-11), 16.12 (C-28), 17.73 (C-29), 18.82 (C-2), 20.42 (C-6), 21.31 (C-12), 21.63 (C-24), 21.74 (C-27), 22.83 (C-16), 25.84 (C-30), 26.54 (C-26), 27.24 (C-20), 33.18 (C-4), 33.50 (C-23), 37.24 (C-10), 39.16 (C-1), 39.75 (C-15), 40.24 (C-19), 42.23 (C-3), 43.40 (C-7), 50.69 (C-9), 56.30 (C-5), 72.98 (C-14), 74.72 (C-13), 74.91 (C-8), 125.0 (C-21), 125.7 (C-17), 131.1 (C-22), 134.8 (C-18). EIMS m/z (%): 69 (51), 81 (22), 95 (21), 109 (25), 137 (34), 149 (54), 191 (93), 231 (100), 249 (30), 276 (18), 279 (16), 444 (M⁺, 10). HRFABMS (glycerol) m/z (M + H): calcd. for C₃₀H₅₃O₂, 445.4046; found: 445.4070. $[a]_{D}^{25} = +14.7 (c \ 0.16, \text{CHCl}_3).$

Product 30 (oil). NMR data in C₆D₆ (400 MHz): $\delta_{\rm H}$ 1.19 (6H, s, H-23 & H-24), 1.23 (3H, s, H-25), 1.26 (m, H-3), 1.29 (m, H-1), 1.35 (m, H-3), 1.40 (m, H-8), 1.45 (m, H-2), 1.49 (m, H-1), 1.58 (m, H-2), 1.69 (2H, m, H-7), 1.69 (3H, s, H-29), 1.73 (3H, s, H-27), 1.731 (3H, s, H-28), 1.79 (3H, s, H-26), 1.80 (3H, s, H-30), 2.21 (2H, bt, J 6.8, H-19), 2.21 (2H, m, H-15), 2.27 (4H, m, H-11 & H-12), 2.30 (2H, m, H-16 & H-20), 2.72 (m, H-8), 3.48 (bd, J 10.6, H-6), 5.37 (bt, J 6.8, H-21), 5.42 (m, H-17), 5.45 (m, H-13), 5.55 (bt, J 6.8, H-10); $\delta_{\rm C}$ 16.10 (C-27), 16.15 (C-28), 16.30 (C-26), 16.45 (C-2), 17.73 (C-29), 21.22 (C-25), 25.85 (C-30), 27.10 (C-20), 27.22 (C-16), 28.09 (C-24), 28.77 (C-12), 28.79 (C-11), 29.79 (C-7), 31.77 (C-1), 32.79 (C-23), 36.62 (C-3), 37.34 (C-8), 40.21 (C-15), 40.21 (C-19), 71.47 (C-4), 75.81 (C-5), 78.94 (C-6), 124.8 (C-10), 124.8 (C-13), 124.9 (C-17), 124.9 (C-21), 131.1 (C-22), 134.9 (C-14), 135.1 (C-18), 135.6 (C-9). The following assignments are interchangeable due to the very close values: C-10/C-13/C-17/C-21, C-11/C-12, C-14/C-18, C-16/C-20, C-27/C-28. EIMS m/z (%): 69 (32), 81 (16), 109 (56), 127 (100), 444 (M⁺, 3). HRFABMS (glycerol) m/z (M + H): calcd. for C₃₀H₅₃O₂, 445.4046; found; 445.4029. $[a]_{D}^{25} = +5.80 \ (c \ 0.50, \text{CHCl}_3).$

Product 31 (solid). NMR data in C_6D_6 (400 MHz): δ_H 0.957 (3H, s, H-25), 0.960 (m, H-5), 0.980 (3H, s, H-24), 0.999 (3H, s, H-26), 1.02 (m, H-1), 1.05 (3H, s, H-23), 1.22 (3H, s, H-28), 1.29 (3H, s, H-27), 1.32 (m, H-3), 1.41 (m, H-11), 1.46 (m, H-2), 1.49 (m, H-6), 1.53 (2H, m, H-3 and H-15), 1.55 (m, H-9), 1.59 (m, H-1 & H-11), 1.68 (m, H-16), 1.70 (m, H-6), 1.74 (m, H-2), 1.77 (3H, s, H-29), 1.78 (m, H-13 & H-19), 1.79 (2H, m, H-7), 1.82 (m, H-15), 1.83 (3H, s, H-30), 1.93 (m, H-19), 1.96 (2H, m, H-12), 1.99 (m, H-16), 2.13 (1H, bs, OH), 2.35 (m, H-20), 2.47 (m,

H-20), 3.72 (dd, *J* 8.4, 6.4, H-17), 5.45 (t, *J* 6.8, H-21); $\delta_{\rm C}$ 16.25 (C-25), 17.68 (C-29), 18.84 (C-2), 20.03 (C-6), 21.33 (C-11), 21.52 (C-24), 21.99 (C-28), 23.27 (C-20), 24.50 (C-12), 24.50 (C-27), 25.86 (C-30), 26.30 (C-26), 26.45 (C-16), 33.18 (C-4), 33.73 (C-23), 37.40 (C-10), 38.00 (C-7), 38.49 (C-15), 41.32 (C-1), 41.58 (C-19), 42.75 (C-3), 44.66 (C-8), 57.46 (C-5), 59.23 (C-9), 60.83 (C-13), 72.48 (C-18), 82.03 (C-17), 85.95 (C-14), 125.7 (C-21), 130.9 (C-22). The following carbon signals were particularly small and did not clearly appear: C-7, C-8, C-9, C-10, C-12, C-13, C-16, C-17, C-19 and C-27, thus, the chemical shifts ($\delta_{\rm C}$) were determined by the cross peaks of HMQC or HMBC spectra. EIMS *m*/*z* (%): 69 (56), 81 (32), 109 (31), 191 (43), 211 (100), 231 (39), 317 (33), 344 (27), 444 (M⁺, 10). HREIMS: *m*/*z* (M⁺), calcd. for C₃₀H₅₂O₂, 444.3967; found, 444.3971. [*a*]_D²⁵ = +1.94 (*c* 0.283, CHCl₃).

Product 32 (solid). NMR data in C_6D_6 (400 MHz): δ_H 0.960 (m, H-5), 0.985 (3H, s, H-25), 0.998 (3H, s, H-24), 1.02 (m, H-1), 1.05 (3H, s, H-23), 1.06 (3H, s, H-26), 1.21 (3H, s, H-28), 1.23 (3H, s, H-27), 1.33 (m, H-3), 1.46 (m, H-11), 1.47 (m, H-9), 1.49 (2H, m, H-2 and H-6), 1.53 (2H, m, H-3 & H-15), 1.60 (m, H-16), 1.62 (2H, m, H-1 & H-11), 1.70 (m, H-15), 1.73 (m, H-6), 1.74 (m, H-19), 1.765 (3H, s, H-29), 1.77 (m, H-2), 1.79 (2H, m, H-7), 1.83 (3H, s, H-30), 1.87 (m, H-13), 1.91 (m, H-19), 1.96 (2H, m, H-12), 2.02 (m, H-16), 2.20 (bs, OH), 2.40 (2H, m, H-20), 3.71 (dd, J 10.4, 4.6, H-17), 5.44 (t, J 6.8, H-21); $\delta_{\rm C}$ 16.37 (C-25), 17.72 (C-29), 18.86 (C-2), 20.04 (C-6), 21.41 (C-11), 21.54 (C-24), 21.92 (C-28), 23.29 (C-20), 24.50 (C-12), 25.19 (C-27), 25.86 (C-30), 26.53 (C-26), 26.54 (C-16), 33.20 (C-4), 33.75 (C-23), 37.41 (C-10), 37.87 (C-7), 39.10 (C-15), 41.16 (C-1), 41.75 (C-19), 42.70 (C-3), 44.26 (C-8), 57.46 (C-5), 59.56 (C-9), 60.31 (C-13), 71.61 (C-18), 83.96 (C-17), 85.84 (C-14), 125.7 (C-21), 130.9 (C-22). The carbon signals of C-7, C-8, C-12, C-13, C-14, C-15, C-16, C-17 and C-27 did not appear, thus, the $\delta_{\rm C}$ was determined by the cross peaks of HMBC and/or HMQC spectra. EIMS m/z (%): 69 (37), 81 (20), 95 (21), 109 (31), 191 (32), 211 (100), 231 (30), 317 (25), 344 (15), 444 (M⁺, 5). HREIMS: *m*/*z* (M+), calcd. for C₃₀H₅₂O₂, 444.3967; found, 444.3978. $[a]_{D}^{25} = +4.35 (c \ 0.09, \text{CHCl}_3).$

Product 33 (oil). NMR data in C₆D₆ (600 MHz): $\delta_{\rm H}$ 0.761 (3H, s, H-24), 0.902 (3H, s, H-23), 1.13 (m, H-3), 1.14 (dd, J 12.1, 1.5, H-5), 1.23 (m, H-1), 1.236 (3H, s, H-26), 1.30 (m, H-3), 1.309 (3H, s, H-25), 1.37 (dt, J 4.8, 13.1, H-7), 1.40 (m, H-6), 1.44 (2H, m, H-2), 1.53 (bd, J 12.3, H-6), 1.61 (2H, m, H-11), 1.694 (3H, s, H-29), 1.72 (m, H-1), 1.729 (3H, s, H-28), 1.812 (3H, s, H-30), 1.823 (3H, s, H-27), 1.99 (dt, J 4.8, 13.1, H-7), 2.23 (2H, m, H-19), 2.25 (2H, m, H-15), 2.32 (2H, m, H-20), 2.33 (2H, m, H-16), 2.53 (m, H-12), 2.73 (m, H-12), 3.19 (bs, OH), 3.63 (dd, J 9.9, 2.0, H-10), 5.38 (t, J 6.9, H-21), 5.43 (t, J 6.5, H-17), 5.51 (t, J 6.8, H-13); $\delta_{\rm C}$ 16.08 (C-6), 16.10 (C-27), 16.16 (C-28), 17.75 (C-29), 20.22 (C-2), 20.93 (C-24), 23.49 (C-25), 24.02 (C-26), 25.88 (C-30), 25.91 (C-12), 27.06 (C-16), 27.22 (C-20), 30.63 (C-11), 30.77 (C-7), 32.18 (C-23), 33.35 (C-4), 40.23 (C-15), 40.25 (C-19), 41.36 (C-3), 41.84 (C-1), 54.60 (C-5), 75.51 (C-9), 76.47 (C-8), 77.33 (C-10), 124.9 (C-17), 124.9 (C-21), 125.2 (C-13), 131.1 (C-22), 134.9 (C-18), 135.4 (C-14). The assignments of C-16 and C-20 and those of C-15 and C-19 are interchangeable. The major fragments and their peak intensities in the EIMS spectrum were superimposable to those of 27. HRFABMS (glycerol) m/z (M + H): calcd. for C₃₀H₅₃O₂, 445.4046; found: 445.4122. $[a]_{D}^{25} = 12.7 (c \ 0.525, \text{CHCl}_3).$

Product 34 (oil). NMR data in C₆D₆ (400 MHz): $\delta_{\rm H}$ 0.735 (3H, s, H-25), 0.77 (m, H-1), 0.889 (3H, s, H-24), 0.89 (m, H-5), 0.957 (3H, s, H-23), 1.14 (dt, 2.0, 12.4, H-9), 1.210 (3H, s, H-26), 1.23 (m, H-3), 1.25 (m, H-11), 1.27 (m, H-6), 1.380 (3H, s, H-27), 1.45 (m, H-3), 1.48 (m, H-2), 1.49 (m, H-12), 1.51 (2H, m, H-1 and H-7), 1.56 (m, H-15), 1.60 (m, H-11), 1.64 (m, H-6), 1.67 (m, H-2), 1.682 (3H, s, H-29), 1.69 (m, H-12), 1.80 (m, H-7), 1.802 (3H, s, H-30), 1.811 (3H, s, H-28), 1.96 (td, *J* 12.7, 4.9, H-15),

2.24 (2H, m, H-19), 2.32 (2H, m, H-20), 2.39 (m, H-16), 2.49 (bs, OH), 2.60 (m, H-16), 3.51 (dd, J 11.8, 2.2, H-13), 5.37 (bt, 6.8, H-21), 5.52 (t, J 7.0, H-17); $\delta_{\rm C}$ 15.67 (C-25), 16.12 (C-28), 17.72 (C-29), 18.41 (C-11), 18.91 (C-2), 20.11 (C-6), 20.78 (C-26), 21.46 (C-24), 22.58 (C-16), 23.58 (C-27), 25.83 (C-30), 26.96 (C-12), 27.22 (C-20), 33.33 (C-4), 33.45 (C-23), 36.79 (C-10), 37.40 (C-15), 39.10 (C-1), 40.24 (C-19), 42.08 (C-7), 42.35 (C-3), 56.24 (C-5), 57.60 (C-9), 73.04 (C-14), 74.94 (C-8), 75.91 (C-13), 125.0 (C-21), 125.8 (C-17), 131.1 (C-22), 134.8 (C-18). The major fragments and their ion intensities in the EIMS spectrum were superimposable on those of **25**. HRFABMS (glycerol) m/z (M + H): calcd. for C₃₀H₅₃O₂, 445.4046; found: 445.4068. $[a]_{\rm D}^{25} = 2.20$ (*c* 0.25, CHCl₃).

Product 35 (oil). NMR data in C₆D₆ (600 MHz): $\delta_{\rm H}$ 1.153 (3H, s, H-23), 1.200 (3H, s, H-25), 1.216 (3H, s, H-24), 1.23 (m, H-1), 1.24 (m, H-3), 1.35 (m, H-3), 1.48 (m, H-2), 1.63 (m, H-7), 1.64 (m, H-2), 1.693 (3H, s, H-29), 1.735 (3H, s, H-27), 1.735 (3H, s, H-28), 1.75 (m, H-7), 1.77 (m, H-1), 1.788 (3H, s, H-26), 1.805 (3H, s, H-30), 2.24 (2H, m, H-15), 2.24 (2H, m, H-19), 2.27 (2H, m, H-12), 2.27 (2H, m, H-11), 2.32 (2H, H-16), 2.32 (2H, H-20), 2.40 (m, H-8), 2.71 (m, H-8), 2.92 (bs, OH), 3.57 (bd, J 10.4, H-6), 5.37 (bt, J 4.8, H-21), 5.42 (m, H-17), 5.45 (m, H-13), 5.55 (bt, J 4.8, H-10); $\delta_{\rm C}$ 16.11 (C-27), 16.17 (C-28), 16.25 (C-2), 16.25 (C-26), 17.72 (C-29), 22.67 (C-25), 25.83 (C-30), 27.13 (C-16), 27.24 (C-20), 27.42 (C-24), 27.88 (C-1), 28.75 (C-11), 28.78 (C-12), 29.00 (C-7), 33.32 (C-23), 36.66 (C-3), 37.52 (C-8), 40.22 (C-15), 40.22 (C-19), 71.73 (C-4), 76.38 (C-5), 77.80 (C-6), 124.87 (C-21), 124.92 (C-13), 124.92 (C-17), 124.97 (C-10), 131.1 (C-22), 134.9 (C-14), 135.1 (C-18), 135.5 (C-9). The following assignments are interchangeable due to the close values: C-10/C-13/C-17/C-21, C-11/C-12, C-14/C-18, C-16/C-20, C-27/C-28. The major fragments and their ion peak intensities in the EIMS spectrum were superimposable on those of **30**. HRFABMS (glycerol) m/z (M + H): calcd. for C₃₀H₅₃O₂, 445.4046; found; 445.4032. $[a]_{D}^{25} = -3.03$ (*c* 0.33, CHCl₃).

Product 36 (solid). NMR data in C₆D₆ (600 MHz): $\delta_{\rm H}$ 1.014 (3H, s, H-24), 1.032 (3H, s, H-25), 1.066 (3H, s, H-23), 1.100 (3H, s, H-26), 1.17 (m, H-5), 1.20 (m, H-1), 1.271 (3H, s, H-28), 1.371 (very broad singlet, H-27), 1.40 (m, H-3), 1.44 (m, H-11), 1.45 (m, H-16), 1.46 (m, H-13), 1.54 (m, H-2), 1.56 (m, H-3), 1.59 (m, H-6), 1.63 (dt, J 5.3, 12.9, H-19), 1.68 (2H, m, H-1 & H-9), 1.70 (2H, m, H-15), 1.70 (m, H-16), 1.70 (m, H-1), 1.71 (m, H-11), 1.73 (2H, m, H-12), 1.78 (m, H-2), 1.80 (m, H-6), 1.825 (3H, s, H-29), 1.846 (3H, s, H-30), 1.89 (dt, J 5.3, 12.9, H-19), 1.99 (very broad, H-7), 2.41 (m, H-20), 2.54 (m, H-20), 3.27 (very broad, H-17), 5.45 (t, J 7.0, H-21); in acetone-d₆, the signal of H-17 clearly appeared as dd (J 11.1 and 3.3), indicating the stereochemistry of C17 is S, and OH signal was clearly found at 3.83 (brs); $\delta_{\rm C}$ 16.77 (C-25), 18.11 (C-29), 19.05 (C-2), 19.66 (C-28), 20.43 (C-6), 21.50 (C-24), 22.13 (C-11), 22.35 (C-20), 25.13 (C-16), 25.84 (C-30), 26.38 (C-27), 27.32 (C-26), 30.25 (C-12), 33.21 (C-4), 33.78 (C-23), 34.10 (C-15), 37.46 (C-10), 38.94 (C-7), 41.25 (C-1), 42.79 (C-3), 43.00 (C-19), 45.26 (C-8), 56.97 (C-5), 60.28 (C-9), 61.95 (C-13), 73.74 (C-17), 76.01 (C-18), 76.38 (C-14), 126.2 (C-21), 130.7 (C-22). The assignments of C-11 and C-12 are exchangeable. The carbon signals of C-7, C-8, C-12, C-13, C-15, C-16, C-27 and C-28 was significantly small and some peaks did not appear, thus, the δ_{C} was determined by the cross peaks of HMBC and/or HMQC spectra. ¹H NMR data in acetone-d₆ (600 MHz): 0.807 (3H, s, H-24), 0.823 (3H, s, H-23), 0.832 (3H, s, H-25), 0.92 (m, H-5), 0.920 (3H, s, H-26), 0.97 (m, H-1), 1.13 (m, H-3), 1.15 (3H, s, H-28), 1.24 (m, H-11), 1.324 (s, H-27), 1.33 (m, H-3), 1.35 (m, H-2), 1.38 (m, H-13), 1.42 (m, H-11), 1.42 (m, H-19), 1.43 (m, H-6), 1.47 (m, H-1), 1.52 (m, H-9), 1.55 (m, H-16), 1.58 (m, H-6), 1.62 (2H, m, H-2 & H-6), 1.62 (3H, s, H-29), 1.63 (m, H-12), 1.65 (3H, s, H-30), 1.67 (m, H-19), 1.70 (2H, m, H-15), 1.72 (m, H-12), 1.79 (2H, m, H-7), 2.13 (m, H-20), 2.23 (m, H-20), 3.31 (dd, J 11.1, 3.3, H-17), 3.83 (brs, OH), 5.11 (bt, J 6.8, H-22). The proton signal of Me-27 was significantly small. $\delta_{\rm C}$ in acetone- d_{6} , 16.86 (C-25), 18.09 (C-29), 19.24 (C-2), 20.24 (C-28), 20.25 (C-6), 21.54 (C-24), 22.35 (C-11), 22.56 (C-20), 25.46 (C-16), 25.81 (C-30), 26.50 (C-12), 27.10 (C-27), 27.37 (C-26), 33.53 (C-4), 33.84 (C-23), 34.12 (C-15), 37.82 (C-10), 39.30 (C-7), 41.53 (C-1), 43.09 (C-3), 43.37 (C-19), 45.64 (C-8), 57.29 (C-5), 60.56 (C-9), 62.50 (C-13), 73.59 (C-17), 76.72 (C-18), 76.89 (C-14), 126.7 (C-21), 130.9 (C-22). The carbon signals of C-7, C-8, C-11, C-12, C-13, C-15, C-16, C-27 and C-28 was significantly small and some peaks did not appear, thus, the $\delta_{\rm C}$ was determined by the cross peaks of HMBC and/or HMQC spectra. EIMS m/z (%): 69 (52), 81 (32), 109 (31), 191 (62), 211 (32), 231 (100), 317 (12), 344 (24), 444 (M⁺, 4). HREIMS: m/z (M⁺), calcd. for C₃₀H₃₂O₂, 444.3967; found, 444.3974. $[a]_{\rm D}^{\rm 25} = +29.5$ (*c* 0.78, CHCl₃).

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