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Squalene–hopene cyclase: final deprotonation reaction, conformational analysis for the cyclization of (3*R*,*S*)-2,3-oxidosqualene and further evidence for the requirement of an isopropylidene moiety both for initiation of the polycyclization cascade and for the formation of the 5-membered E-ring

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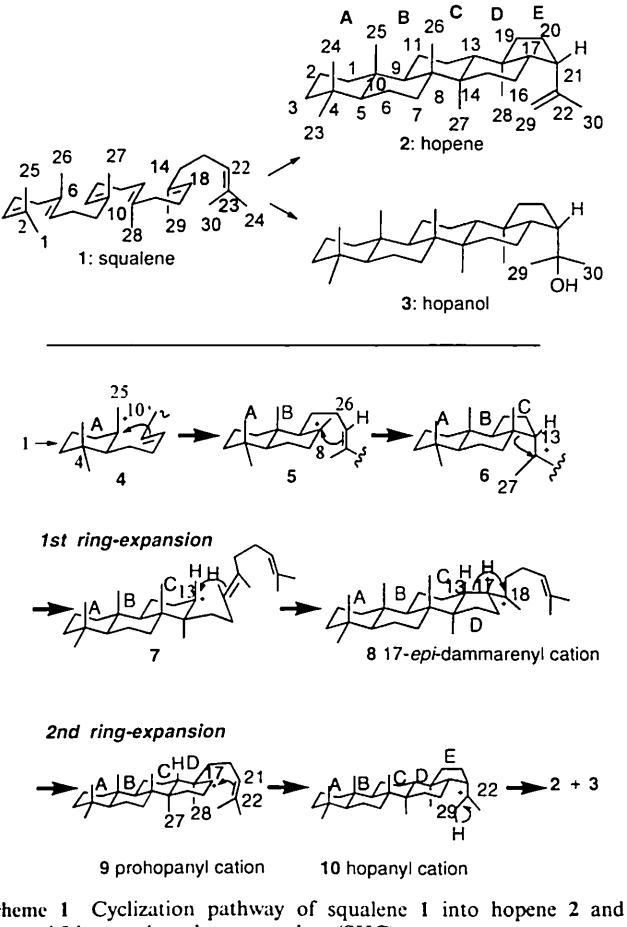
To provide insight into the polycyclization mechanism of squalene by squalene–hopene cyclase (SHC) from *Alicyclobacilus acidocaldarius*, some analogs of nor- and bisnorsqualenes were synthesized including the deuterium-labeled squalenes and incubated with the wild-type SHC, leading to the following inferences. (1) The deprotonation reaction for the introduction of the double bond of the hopene skeleton occurs exclusively from the *Z*-methyl group on the terminal double bond of squalene. (2) 3*R*-Oxidosqualene was folded in a boat conformation for the A-ring construction, while the 3*S*-form was in a chair structure. (3) The terminal two methyl groups are indispensable both for the formation of the 5-membered E-ring of the hopene skeleton and for the initiation of the polycyclization cascade, but the terminal *Z*-methyl group has a more crucial role for the construction of the 5-membered E-ring than the E-methyl group. (4) Some of the novel terpene skeletons, **36**, **37**, **39** and **40**, were created from the analogs employed in this investigation.

Introduction

Squalene–hopene cyclase (SHC) from prokaryotes catalyses the conversion of the acyclic molecule of squalene **1** into the pentacyclic triterpenes of hopene **2** and hopanol **3**, the ratio being *ca.* 5 : 1, respectively (Scheme 1). This polycyclization reaction is attained by a single enzyme.[1,2] **1** is folded in an 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 five new C–C bonds and nine chiral centers. The polycyclization mechanism is analogous to that of eukaryotic oxidosqualene cyclases.[1,2] Recent studies on the SHC from *Alicyclobacillus acidocaldarius* have revealed that the polycyclization reaction consists of 8 reaction steps (Scheme 1) [1a]: (1) 1st cyclization to form A-ring **4** by proton attack on the terminal double bond, donated by the DXDD motif, [3] (2) 2nd ring closure to give the B-ring (6/6-fused A/B ring system **5**), [4,5] (3) 3rd cyclization to yield 5-membered C-ring (6/6/5-fused A/B/C-tricyclic ring system **6**) by Markovnikov closure,[5,6] (4) which then undergoes ring expansion to form the 6-membered C-ring (6/6/6-fused tricyclic ring system **7**).[6] (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), [7-9] (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**), [7-10] (7) the last ring closure process to construct the 6/6/6/6/5-fused A/B/C/D/E-ring system (**10**, hopanyl cation),[11a] and (8) the final deprotonation reaction from **10** to introduce the double bond. Very recently, Rajamani and Gao have given deeper insight into the polycyclization mechanism of **1**;[12] they proposed that formation of **10** from **5** is achieved by a highly asynchronous concerted reaction and occurs by a kinetic preference. The transient carbocations formed during the polycyclization cascade are



Scheme 1 Cyclization pathway of squalene **1** into hopene **2** and hopanol **3** by squalene–hopene cyclase (SHC).

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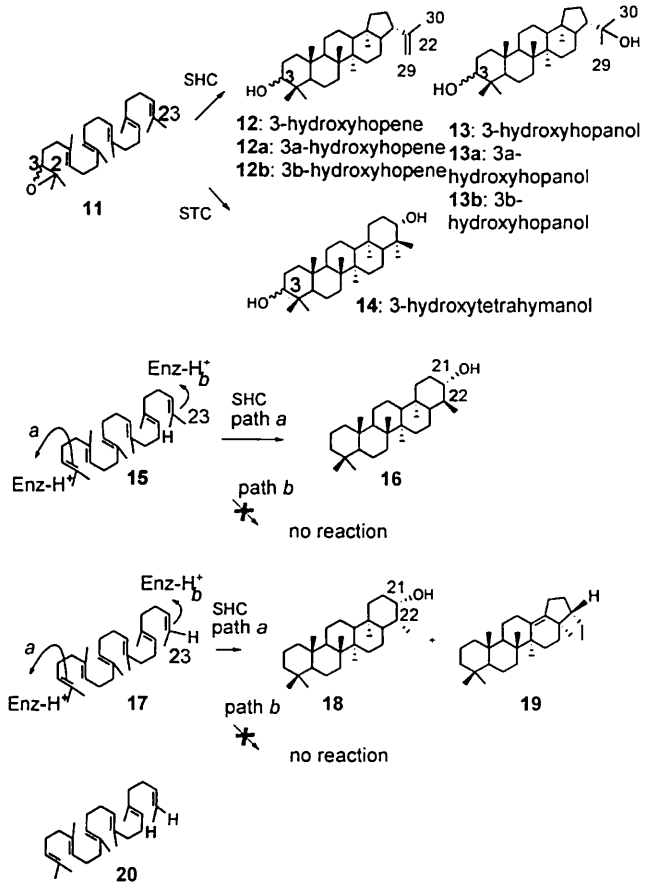
stabilized by a cation/π-interaction.[13] Previously, we clarified the roles of all the Trp residues conserved among prokaryotic SHCs,[14] and also demonstrated that cation–π interaction between π-electrons of aromatic amino acids and the transient carbocations made a great contribution to the acceleration of the polycyclization reaction at lower incubation temperatures. The replacement of Phe365,[4] Phe601[6] and Phe605[11a] 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.[15–17] 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 13α-H in the 6/6/5-fused tricyclic and 17α-H in the 6/6/6/5-fused tetracyclic cation,[10] the configurations of which are opposite to those of true intermediates **6** (13β-H) and **8** (17β-H) (Scheme 1). Replacement of Tyr420 or Leu607 by the bulkier Trp afforded an unnatural monocyclic triterpene having a (5*R*,6*R*)-1,5,6-trimethylcyclohexene ring, named neoachillapentaene;[18] 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 the all chair structure. These findings indicate that the steric bulk of the active site residues direct the folding conformation of **1**, *i. e.* the stereochemical destiny, during the polycyclization cascade.[1a]

As described above, there have been remarkable advances in understanding the polycyclization mechanism of **1**, but some questions have remained unresolved. (1) From which terminal methyl group, *i.e.* either the *Z*- or *E*-methyl group at 23-position of **1**, does the final deprotonation reaction occur to introduce the double bond at C22–C29? (2) It is known that the SHCs from *A. acidocaldarius*,[14,19] *Acetobacter pasteurianum*[20] and *Methylococcus capsulatus*,[21] accept both enantiomers of (3*S*)- and (3*R*)-2,3-oxidosqualene **11**, giving 3-hydroxyderivatives **12** and **13** (Scheme 2). Tetrahymanol cyclase (STC) from eukaryotic protozoon *Tetrahymena pyriformis* also mediates the cyclization of **11** to produce 3-hydroxyderivatives of tetrahymanol **14**.[22] However, the folding conformations of **11** for the A-ring formation of **12** and **13** by *A. acidocaldarius* SHC are still ambiguous, although those by the STC have been established.[22] (3) In a preliminary report,[23] we have demonstrated that the two methyl groups on the terminal double bond (isopropylidene moiety) are indispensable for initiation of the polycyclization reaction, based on the structures of the enzymic products from norsqualenes **15** and **17** lacking one of two methyl groups at C(23)-position of **1**. Both **15** and **17** were converted into the tetrahymanol-like skeletons **16** and **18** having 6/6/6/6/6-fused ring system, but only from the norsqualene **17** having the *Z*-methyl group at C(23) a significantly large amount of neohopane skeleton **19** (6/6/6/6/5-fused ring system) was formed. Thus, we suggested that the *Z*-methyl group at C(23) is more crucial for the construction of the 6/6/6/6/5-fused pentacyclic ring system than the *E*-methyl group. Small amounts of products also were detected other than major products **16**, **18** and **19**, but the structures have remained unidentified due to their isolation difficulty.[23]

To understand the role of the terminal methyl group(s) in more detail, we have tried to isolate and identify the several minor products from **15** and **17**, and also planned the enzymatic reaction of C(23);C(23)-bisnorsqualene **20**. To give insight into the three unresolved issues described above, the following compounds were synthesized and incubated with the native SHC: deuterium-labeled squalenes **26** and **27**, deuterium-labeled (3*R*,*S*)-oxidosqualene **29**, bisnorsqualenes **20**, **30** and **31**, and norsqualenes **15** and **17**. We describe here the results obtained from the incubation experiments of these substrates and discuss in detail the important role of the methyl group(s) involved in **1** for the polycyclization cascade.



Scheme 2 Cyclization products of (3*R*,*S*)-oxidosqualene and squalene analogs lacking methyl group(s) at the terminal side.

Results

Syntheses of deuterium-labeled compounds: [1,1,1-$^2H_3$]squalene **26**, [25,25,25-$^2H_3$]squalene **27** and (2*E*,22*E*)-[1,1,1,24,24,24-$^2H_6$]-2,3-oxidosqualene **29**

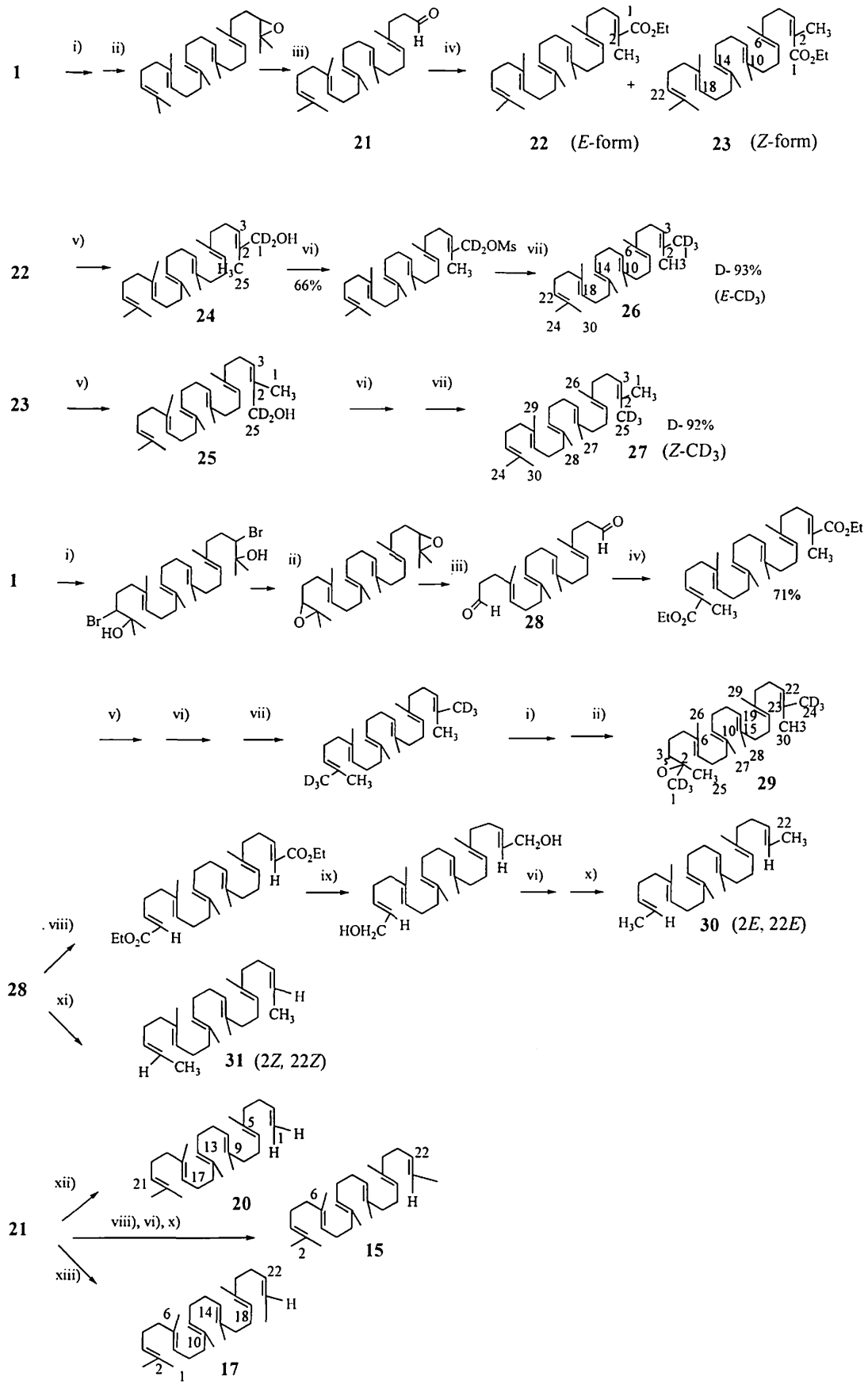
To determine which of the *Z*- or *E*-methyl groups at C(2) of **1**, *i.e.* at C(23), undergoes the deprotonation reaction, compounds **26** and **27** having *E*- and *Z*-trideuteriomethyl groups at C(2), respectively, were synthesized. The synthetic procedures are shown in Scheme 3. The synthetic intermediates **22** and **23** were separated by HPLC. Deuterium contents of **26** and **27** (*m/z* 413, M^+) were estimated to be 93% and 92%, respectively, by EIMS analyses. The ^2H NMR spectra of **26** and **27** in $CHCl_3$ showed δ_D 1.66 and 1.62 relative to the solvent signal of $CHCl_3$ (δ_D 7.26 ppm), which corresponded to Me-1 (*E*-Me) and Me-25 (*Z*-Me) of **1**, respectively. In order to analyse the folding conformation of **11** during the polycyclization cascade, hexadeuterium-labeled oxidosqualene **29** (*m/z* 432, M^+, D content 92%) having two *E*-trideuteriomethyl groups was also prepared from dialdehyde **28** by a similar method as for those of **26** and **27** (Scheme 3). The two *E*-methyl signals of Me-1 and Me-24 of **11**, δ_H 1.27 and δ_H 1.79, respectively, were missing in the ^1H NMR spectrum of **29** in C_6D_6.

Syntheses of 15, 17, 20, 30 and 31

Aldehyde **21** was used for the preparation of **15**, **17** and **20**, while dialdehyde **28** was used for those of **30** and **31**. The synthetic plan is shown in Scheme 3. Selective synthesis of **17** with *Z*-olefin was done under a lithium-salt free condition, *i.e.*, by using $NaN(SiMe_3)_2$ as base.

Incorporation experiments of 26, 27 and 29

To identify the deuterium-labeled position(s), ^1H and ^{13}C NMR signals of **2**, **3**, 3α- **12a** and 3β-hydroxyhopene **12b**, and 3α- **13a**



Scheme 3 Synthetic scheme for deuterated squalenes and squalene analogs. *Reagents and conditions*: i) NBS, THF/H_2O. ii) $K_2CO_3/MeOH$. iii) H_5IO_4/Et_2O. iv) Ph_3P^+-$CH_3CHBr^-COOC_2H_5$, *n*-BuLi/THF. v) $LiAlD_4/Et_2O$. vi) MsCl, Et_3N/CH_2Cl_2. vii) $LiAlD_4$/THF. viii) $(EtO)_2P(O)CH_2COOEt$ in NaH/THF. ix) $(C_4H_9)_2AlH/Et_2O$. x) $LiAlH_4$/THF. xi) Ph_3P^+-$CH_2CH_3Br^-$, *n*-BuLi/THF. xii) Ph_3P^+-CH_3Br^-, *n*-BuLi/THF. xiii) Ph_3P^+-$CH_2CH_3Br^-$, $NaN(SiMe_3)_2$/THF, −78 °C.

and 3β-hydroxyhopanol **13b** were assigned by the detailed analyses of DEPT, ^1H–^1H COSY 45, HOHAHA, NOESY, HMQC and HMBC spectra (see Experimental). The complete assignments of ^{13}C NMR data of **2** have been published before,[24] but those of C-11 and C-14 should be exchanged. Cell-free homogenates of the cloned *E. coli* encoding the wild-type

SHC[25] were incubated separately with trideuterated squalene **26** and **27** at the catalytic optimum conditions (pH 6.0 and 60 °C) for 16 h. The enzymic reaction was terminated by boiling for 5 min after adding 5% KOH/MeOH. The hexane extract from the reaction mixture was subjected to SiO_2 column chromatography by eluting with hexane to obtain pure **2**. Fig. 1 shows the ^{1}H NMR spectrum of natural **2** (Fig. 1A) and the ^{2}H NMR spectra of deuterated **2** prepared by incubating **26** and **27**. The deuterium atoms were placed selectively at the Me-30 and Me-23 of **2** (Figs. 1C), which was obtained by the incubation of **26**. In the case of **27** bearing a *Z*-deuteriomethyl group, vinyl protons at C(29) and methyl protons at C(24) were labeled (Fig. 1B), definitively demonstrating that the deprotonation reaction of the final reaction step occurs exclusively from the *Z*-methyl group at C(23) of **1**, but not from the *E*-methyl group. The deuterium-labeled positions of **2** and **3** are shown in Scheme 4. The integration of the ^{1}H NMR spectra of deuterium-labeled **2**, prepared from **26** and **27**, showed that the signal intensities corresponding to the labeled positions were nearly half those of natural **2**, indicating that the polycyclization reaction occurred from both the left and right sides of **1** (Scheme 4). This is consistent with the fact that **1** is a symmetrical molecule. These incorporation experiments also allowed us to differentiate the ^{1}H signals of Me-29 of **3** from that of Me-30. The ^{1}H NMR spectrum of **3** in C_6D_6 prepared by the incubation of **26** showed that the signal intensities of δ_H 1.22 (3H, s) and Me-23 (δ_H 1.05, 3H, s) were half those of natural **3**, indicating that the signal δ_H 1.22 (3H, s) was originated from the *E*-methyl at C(23) of **1** (data not shown). As for the deuterium-labeled **3** obtained from **27**, the signals of δ_H 1.27 (3H, s) and Me-24 (δ_H 0.997, 3H, s) significantly decreased, thus the signal of δ_H 1.27 was assigned to be derived from the *Z*-methyl at C(23) of **1**.

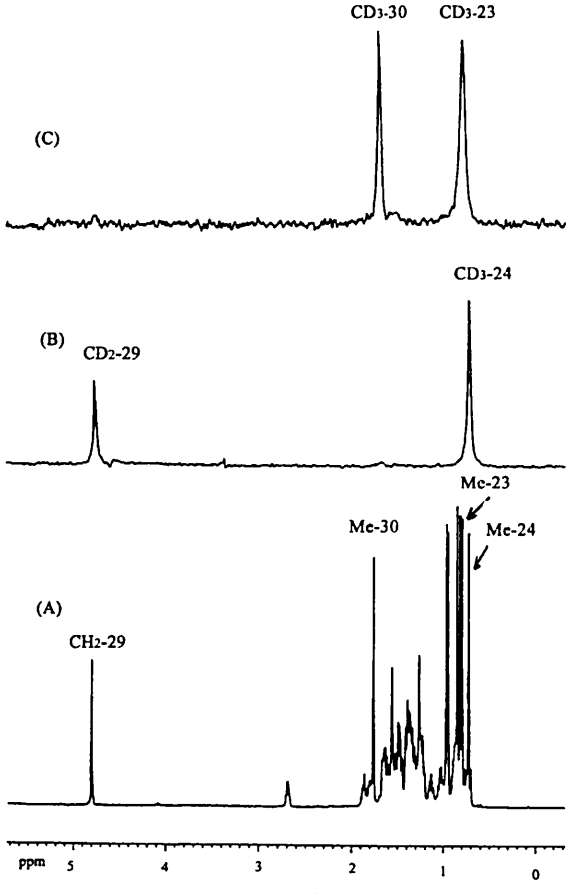
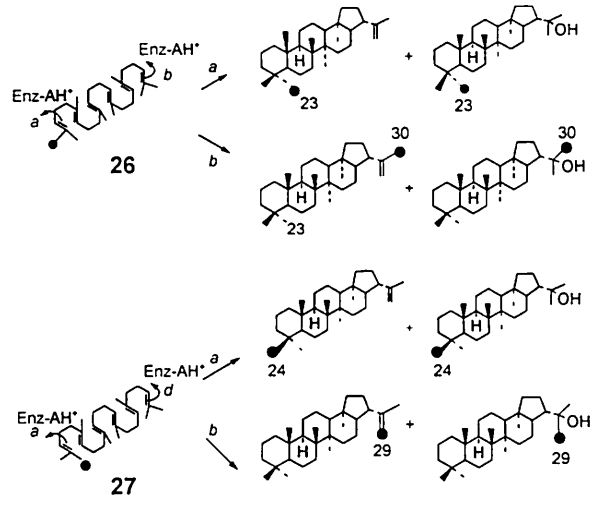


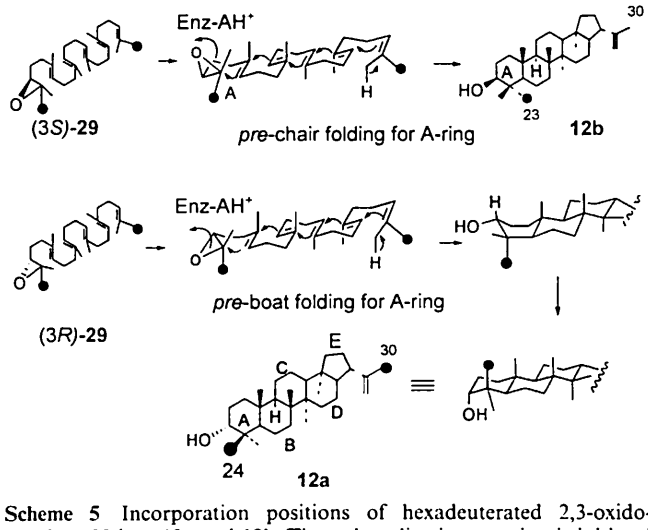
Fig. 1 ^{1}H- (600.13 MHz, $CDCl_3$) and ^{2}H-NMR (92.124 MHz, $CHCl_3$) spectra of hopene **2**. (A) natural **2**; (B) deuterated **2** obtained by incubating **27** having a *Z*-trideuteriomethyl group; (C) deuterated **2** prepared by incubating **26** having *E*-trideuteriomethyl group.



Scheme 4 Deuterium-labeled positions of **2** and **3** prepared by separately incubating **26** and **27**. Squalene **1** is a symmetrical molecule, thus the polycyclization of **1** occurs from both the left and right sides. **26** and **27** have the trideuteriomethyl group at one terminal side, but no deuterium atom at the alternative terminal side. Thus, two labeled species of **2** can be produced for which the labeled positions are different. The symbol • shows the deuterium-labeled position.

To analyse the folding conformation of oxidosqualene **11** during the polycyclization reaction, (3*R*,*S*)-oxidosqualene **29** bearing (2*E*,23*E*)-hexadeuteriomethyl groups was incubated with the native SHC for 16 h. The following four compounds were produced: 3α- **12a**, 3β-hydroxyhopene **12b** and 3α- **13a**, 3β-hydroxyhopanol **13b**. The production ratio of **12a** to **12b** and that of **13a** to **13b** were *ca.* 1 : 1 by the GC analysis, while that of **12a** (**12b**) to **13a** (**13b**) was *ca.* 5 : 1 with the same ratio of **2** to **3**. The 3β-hydroxy derivatives more strongly adsorb on SiO_2 than the 3α-hydroxy derivatives, thus both derivatives could easily be separated by a SiO_2 column chromatography by eluting with hexane/EtOAc (100 : 20). The presence of a β-oriented hydroxyl group in **12b** was verified by the splitting pattern of H-3 (δ_H 3.16, dd, *J* 10.4, 5.4), while **12a** showed broad singlet for H-3 (δ_H 3.30) due to the small spin coupling constants, which indicates α-orientation of the hydroxyl group. Fig. 2 illustrates the ^{1}H NMR spectra (600 MHz) of natural **12a** and **12b** and the isotopically-labeled ones obtained by incubating **29**. The proton signals of Me-30 and Me-23 of **12b** markedly deceased. In contrast, those of Me-30 and Me-24 of **12a** significantly decreased.

The labeled positions of **12a** and **12b** are shown in Scheme 5. This incorporation experiment clearly demonstrated that the



Scheme 5 Incorporation positions of hexadeuterated 2,3-oxidosqualene **29** into **12a** and **12b**. The polycyclization reaction is initiated exclusively from the epoxide-ring. (3*S*)-**29** is folded in a pre-chair conformation, whereas (3*R*)-**29** is in a pre-boat structure for the A-ring formation. The symbol • shows the deuterium-labeled position.

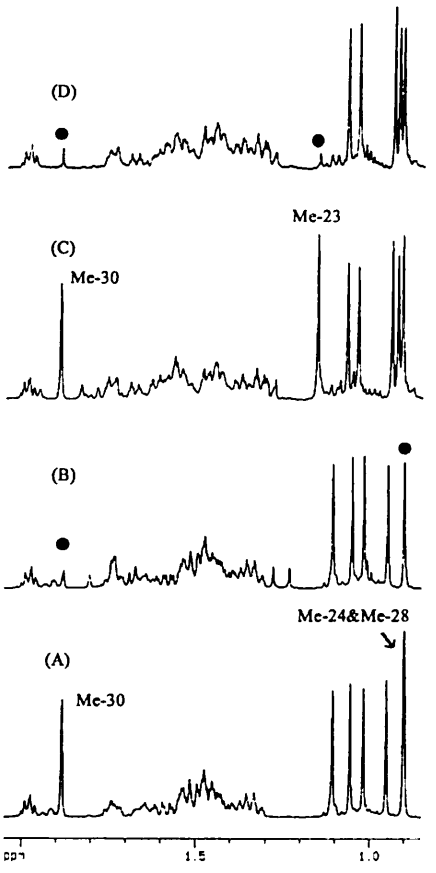


Fig. 2 ^{1}H NMR spectra (600.13 MHz, C_6D_6) of natural 3α-, 3β-hydroxyhopene (**12a, 12b**) and the isotopically-labeled ones obtained by incubating oxidosqualene **29** having (2*E*,22*E*)-hexadeuteriommethyl groups. (A) natural 3α-hydroxyhopene **12a**; (B) deuterium-labeled **12a**; (C) natural **12b**; (D) deuterium-labeled **12b**.

(3*S*)-epoxide was folded in a chair structure, while the (3*R*)-form was folded in a boat conformation for the A-ring formation. The signal intensity of vinyl protons at C(29) of **12a** and **12b** remained unchanged, indicating that the final deprotonation reaction occurs exclusively from the Z-methyl at C(23) of **11**. This finding coincides with the inference from the cyclization of **26** and **27** as described above (Scheme 4). The labeled positions of 3-hydroxyhopanol **13a** and **13b** were the same as those of **12a** and **12b** (data not shown).

Enzymic reaction of C(2);C(23)-bisnorsqualenes 30 and 31

We have previously reported that the terminal isopropylidene moiety is indispensable for initiating the polycyclization reaction.[23] This inference was drawn from the incubation experiments of **15** and **17**. The enzymic products of **16**, **18** and **19** were found showing that the polycyclization reaction started from the terminal isopropylidene moiety, but no product was found that showed that the cyclization had started from the methyl-deficient side. To further confirm this idea, compounds **30** and **31** lacking each methyl group at both terminal sides, were synthesized according to Scheme 3. No detectable amount of the enzymic products was found on TLC and GC despite a large amount of the SHC having been employed. This finding unequivocally demonstrates that the two terminal methyl groups are essential for initiating the polycyclization reaction of **1**.

Enzymatic products of C(23);C(23)-bisnorsqualene 20†

The incubation was performed at the catalytic optimal conditions (pH 6.0 and 60 °C) for 16 h. GC analysis of the hexane extract from the enzymic reaction mixture is shown in Fig. 3A. A good conversion was observed with a small amount of **20** being recovered (7.7%); under identical incubation conditions (the same amounts of the SHC and the substrate), **1** was quantitatively converted into **2** and **3**. To isolate the enzymic products in a sufficient amount for spectroscopic analysis, 40 mg of **20** was incubated with a large amount of the cell-free homogenates (250 cm^3), prepared from a 5 L culture of the *E. coli* clone encoding the wild-type SHC. Seven products **32a–38** (Fig. 3A) were successfully isolated. The purification procedure was as follows. The hexane extract from the reaction mixture was subjected to a SiO_2 column chromatography by eluting with *n*-hexane to give a mixture of **32a–37**. Product **38** (solid) was isolated in a pure state by eluting with a mixed solvent of EtOAc/*n*-hexane (5 : 100). A SiO_2 column chromatography (impregnated with 5% $AgNO_3$) with a step-wise gradient elution of EtOAc/*n*-hexane (0–5%) afforded pure **36** (solid), **34a** (oil) and **35** (oil) according to the elution order, but the separation of **32a**, **33a** and **37** was unsuccessful. A careful re-chromatography (5% $AgNO_3$–SiO_2) afforded pure **35** (solid), but the separation of **32a** and **33a** failed. A SiO_2 column chromatography (10% $AgNO_3$) enabled us to separate **32a** and **33a**, **33a** being followed by **32a**.

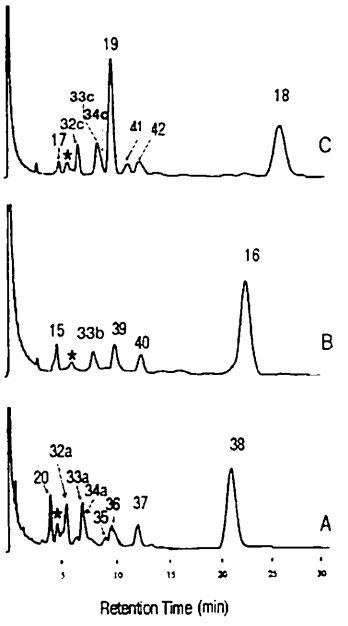


Fig. 3 Gas chromatograms of the reaction mixture obtained by separately incubating **20** (A) and **15** (B) and **17** (C) with the wild-type SHC. Triton X-100 was removed with a short SiO_2 column chromatography by eluting with a mixture of *n*-hexane and EtOAc (100 : 20). Identical incubation conditions were done to compare each of the product distribution patterns from the substrates: substrate 1.0 mg, cell-free extract 1.0 cm^3 as the enzyme source, Triton X-100 20 mg, optimal pH 6.0, optimal temperature 60 °C, incubation time 16 h, total volume 5 cm^3. The products represented by the symbol (*) have possibly the 6/6/5-fused tricyclic podiodatriene skeleton (see Text). The minor tricyclic product(s) was not taken into consideration for the discussion of product distribution ratio. Product distribution of tetracyclic products was *ca.* 17% for **17**, *ca.* 5% for **15** and *ca.* 32% for **20**. The 6/6/6/6/6-fused pentacyclic products from **17**, **15** and **20** were *ca.* 37%, 90% and 60%, respectively. The hopane skeleton having 6/6/6/6/5-fused pentacyclic ring system was produced only from **17** in the highest yield (*ca.* 45%) among all the products from **17**.

The structures of **32a–38** (Fig. 4) were determined by the detailed analyses of 2D NMR spectra in C_6D_6. Products **32a–35** still had the original vinyl moiety of **20** (δ_H 5.81, 1H, m; δ_H 5.01, 1H, dd, *J* 17.0, 1.7; δ_H 4.93, 1H, bd, *J* 10.1 in C_6D_6). The detailed NMR analyses revealed that **32a**, **33a** and **35** have a dammarene-type skeleton and that **34a** possesses an euphane-

† The numbering of the triterpene skeleton used in the text differs in part from the ordinary one. For example, C(18) described here corresponds to the C(20) of the commonly used numbering.

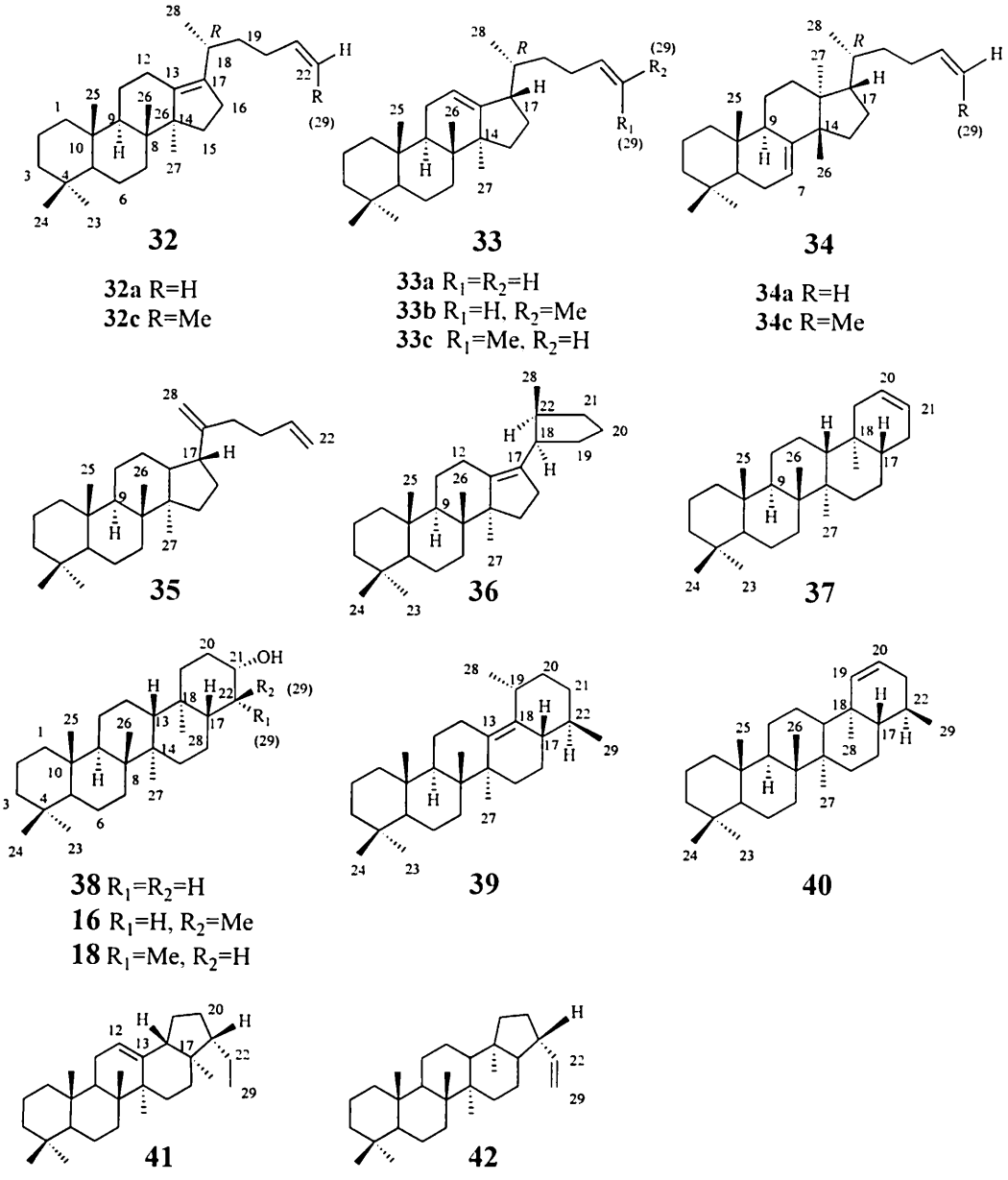


Fig. 4 Structures of the enzymatic products of **15**, **17** and **20** by the wild-type SHC.

like structure having C_{28}. The double bond position of **32a** was determined to be at C13–C17 by the HMBC cross peaks between Me-28 (δ_H 1.11, d, J 7.2) and C-17 (δ_C 134.7), and between Me-27 (δ_H 1.29, s) and C-13 (δ_C 139.8). The double bond position of **33a** was also inferred from the HMBC cross peak between Me-27 (δ_H 1.17, s) and C-13 (δ_C 149.1). As for **35**, one of 6 methyl groups involved in **20** was missing, but in turn methylidene protons appeared (δ_H 5.18, 1H, s; δ_H 5.11, 1H, s) that had the HMBC cross peak with C-17 (δ_C 44.32). No NOESY cross peak between H-17 and Me-27 for **33a** and **35**, but a clear NOE of H-17 with Me-26 for **34a**, indicated β-orientation for H-17 of **33a**, **34a** and **35**. The stereochemistry at C-18 of **32a–34** was determined to be the *R*-configuration according to the published ^{1}H NMR data in $CDCl_3$.[26] The chemical shifts of Me-28 of **32a** (δ_H 0.924, d, J 6.8), **33a** (δ_H 0.799, d, J 6.8) and **34a** (δ_H 0.843, d, J 6.8) in $CDCl_3$ were very close to those of authentic dammar-13(17)-ene (δ_H 0.910), dammar-12(13)-ene (δ_H 0.784) and euph-7-ene (δ_H 0.835) having *R*-configuration at the corresponding position, respectively (*cf.* δ_H 0.951 for that of dammar-13(17)-ene having *S*-stereochemistry).[26] The vinyl protons of **20** were missing in the ^{1}H NMR spectra of **36** and **37**, indicating that the terminal vinyl group underwent a further cyclization. Compound **36** had a novel carbocyclic skeleton with a 6/6/6/5 + 5 ring system. No NOE was observed between H-18 (δ_H 2.10, m) and Me-28 (δ_H 1.18, d, J 7.3), indicating that H-18 and Me-28 were situated in *trans* geometry. A strong NOE was observed between H-12eq. (δ_H 2.65, m) and H-22 (δ_H 2.86, m), but no NOE between H-18 and H-12eq.. A clear NOE was also observed between H-12ax (δ_H 2.08, m) and Me-27 (δ_H 1.33, s). These findings indicated that the relative stereochemistry at C-18 and C-22 of the cyclopentane ring of **36** could be depicted as shown in Fig. 4. Products **37** and **38** consisted of a 6/6/6/6/6-fused pentacyclic ring system. Product **38** had a tetrahymanol-like skeleton (C_{28}) in which a hydroxyl group was attached to C-21 (δ_C 71.0) in an equatorial direction; H-21 (δ_H 3.52) showed the multiplicity of dddd (J 11, 11, 5.1, 5.1) as results of spin–spin couplings of H-21 with four protons at C-20 and C-22. The splitting pattern (δ_H 5.74, 1H, m; δ_H 5.84, 1H, m) of the double bond protons involved in **37** was clearly different from those of the vinyl protons of **20**. The double bond position of **37** was determined to be at C20–C21 by observing the HMBC cross peak of H-20 (δ_H 5.74) with C-18 (δ_C 35.3) and that of H-21 with C17 (δ_C 42.2). It should be noted that the hopane skeleton having the 6/6/6/6/5-fused ring system was *never* detected from the enzymatic products from **20**. Product distribution ratio of **32a–38** and the remaining starting **20** was estimated to be 9.7 : 9.7 : 3.3 : 1.8 : 7.0 : 7.5 : 52.3 : 8.7, respectively, by GC analysis (Fig. 3A).

Enzymatic products of C(23)-norsqualene 15 having an *E*-methyl group at C(23)

Previously, we have isolated 6/6/6/6/6-fused tetrahymanol skeleton **16** as a major product from the incubation mixture of **15**, but minor products could not be separated due to nearly equal polarity between each product.[23] Fig. 3B shows the GC pattern of the reaction mixture obtained by incubating **15** under the same conditions as **20**. The four products of **33b**, **39**, **40** and **16** were clearly found besides the unreacted **15**. A highly polar product **16** was isolated by using SiO_2 chromatography with a mixture of *n*-hexane and EtOAc. A low polar fraction containing the minor products was subjected to careful SiO_2 chromatography (5% $AgNO_3$) with step-wise elution (*n*-hexane–5% EtOAc/*n*-hexane), affording pure products in the following elution order: **39** (solid), **40** (solid), and **33b** (oil). The structures were determined by the spectroscopic analyses including 2D NMR. The ^1H NMR spectrum of **40** indicated the presence of disubstituted *Z*-olefin: δ_H 6.16 (1H, bd, *J* 10) and δ_H 5.60 (1H, m). The double bond position was determined to be at C19–C20 by the clear HMBC cross peak between Me-28 (δ_H 1.08, s) and C-19 (δ_C 138.7). The observation of the doublet methyl (δ_H 1.00, 3H, d, *J* 6.4) due to the spin–spin coupling with H-22 (δ_H 1.81, m) and a strong HMBC of the doublet methyl with C-17 (δ_C 51.4) permitted the assignment of Me-29. A clear NOE of Me-28 with H-22 indicated the β-orientation of Me-29. Product **39** had two doublet methyl groups (δ_H 1.03, d, *J* 6.4, 3H, and δ_H 1.20, d, *J* 7.6, 3H), and one tetrasubstituted olefin (δ_C 134.2, s, and δ_C 136.4, s). An apparent HMBC of Me-27 (δ_H 1.32, s) with δ_C 134.2 and that of H-20 (δ_H 1.58, m) with δ_C 136.4 indicated that the double bond position was at C13–C18, which was further supported by a strong HMBC of H-12 (δ_H, 2.10, m) with the two sp^2 carbons. The ^1H–^1H COSY 45 spectrum showed that H-19 (1H, δ_H 2.87, m) was coupled with both H-20 (δ_H 1.58, m) and one of the two doublet methyls (δ_H 1.20, 3H, d, *J* 7.6), which had a strong HMBC cross peak for one of the sp^2 carbon (δ_C 136.4, s). These findings indicated that this doublet methyl (Me-28) was placed at C-19 (δ_C 30.6). The HMBC cross peak of H-17 (δ_H 1.83) with C-13 (δ_C 134.2) and that of another doublet methyl (δ_H 1.03 3H, d, *J* 6.4) with C-17 (δ_C 41.1) clarified that this methyl group (Me-29) was positioned at C-22 (δ_C 31.3). A clear NOE of H-22 with Me-27 suggested the β-orientation of Me-29. Strong NOEs of H-17 with Me-29 and with H-19 verified that H-17 and H-19 had a β-disposition, indicating that Me-28 was arranged in the α-orientation. The detailed NMR analyses of **33b** showed that this product had a 17-*epi*-dammarane skeleton having a double bond at C12–C13, which also was found in the enzymic products from **20**. The double bond position was established by the HMBC cross peak between Me-27 (δ_H 1.18, 3H, s) and C-13 (δ_C 149.2). The stereochemistry at C-18 was determined to be *R*-configuration by measuring the proton chemical shift of Me-28 (δ_H 0.783, 3H, d, *J* 6.6) in $CDCl_3$, which was close to that of the literature value of the C_{30} skeleton (δ_H 0.784).[26] Product distribution ratio of **33b**, **39**, **40** and **16** and the remaining unreacted **15** was estimated to be 5.4 : 10.7 : 7.9 : 71.3 : 4.7, respectively, by GC analysis (Fig. 3B). It is of particular interest that all the pentacyclic products had a 6/6/6/6/6-fused ring system, but no product having 6/6/6/6/5-fused pentacyclic skeleton like **2** was found.

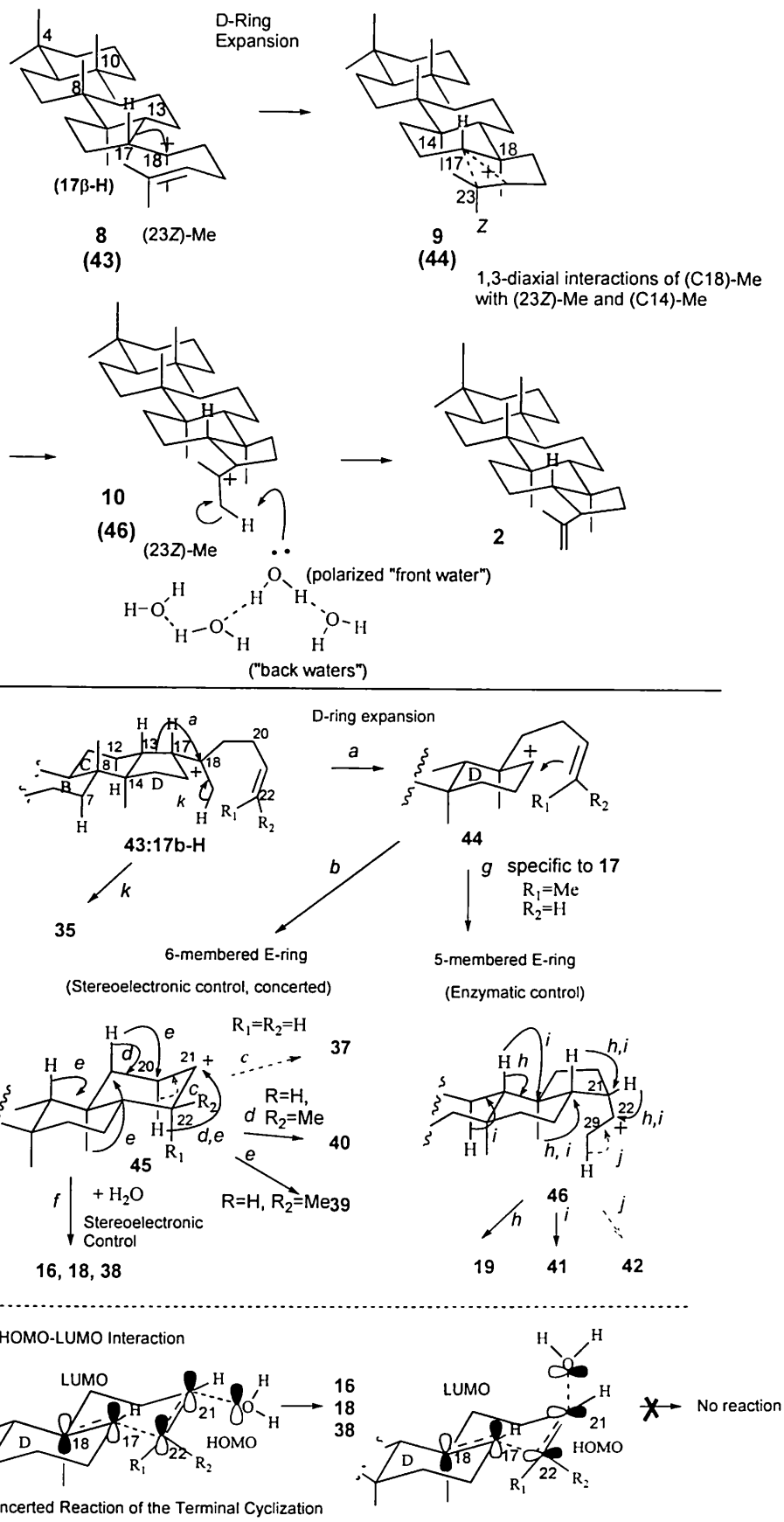
Enzymatic products of C(23)-norsqualene 17 having a *Z*-methyl group at C(23)

The previous paper showed that the 6/6/6/6/6-fused tetrahymanol skeleton **18** and 6/6/6/6/5-fused neohopane skeleton **19** as major products from the incubation mixture of **17** with the cyclase, [23] but isolation of minor products has not been successful. Fig. 3C shows the gas chromatogram of the incubation mixture of **17**. After removing a highly polar product **18** with SiO_2 column chromatography, a mixture of low polar products was subjected to repeated SiO_2 column chromatography impregnated with 5% and/or 10% $AgNO_3$, affording **32a–38** in a pure state. These structures (Fig. 4) were also determined by the detailed NMR analyses. Products **32c** and **33c** had a dammarene-like skeleton, the double bond positions of which were placed at C13–C17 and C12–C13, respectively. **34c** was a euphane-like compound. All the stereochemistry at C-18 of **32c**, **33c** and **34c** was determined to be the *R*-configuration by comparing the proton chemical shifts of the Me-28 in $CDCl_3$ with the literature values: δ_H 0.921 (0.910) for **32c**, δ_H 0.804 (0.784) for **33c** and δ_H 0.855 (0.835) for **34c**, the parentheses showing the published values of the C_{30} triterpenes.[26] These three tetracyclic skeletons all were found also in the enzymic products of **20**. Compound **41** also had a neohopane skeleton as well as **19**, but the double bond positions were different: C12–C13 for **41** and C13–C17 for **19**. Product **42** had a hopene skeleton similar to **2**. These double bond positions were clearly determined by the HMBC spectra. The product distribution ratio of **32c**, **33c**, **34c**, **19**, **41**, **42**, **18** and the remaining starting material **17** was determined by the GC analysis to be 5.9, 8.1, 2.8, 32.3, 4.8, 7.3, 37 and 1.8. The ratio of tetra- to pentacyclic products was 17 : 81. It is of particular importance that the pentacyclic **19**, **41** and **42** with the 5-membered E-ring was produced from **17**, but *never* from **15** (see Figs. 3B and 3C). This finding allowed us to infer that the terminal *Z*-methyl group is indispensable for the construction of hopane skeleton **2**. In addition, all the cyclization products from **15**, **17** and **20** including the minor products had the two methyl groups at the C-4 position, but no product was found which had one methyl group or two hydrogen atoms at C-4, indicating that the polycyclization reaction never started from the methyl-deficient side. It should be also noted that the ratio of the fused tetracyclic products to the fused pentacyclic ones increased with the loss of the terminal methyl groups; 31.5 to 59.8 for **20**, 16.8 to 81.4 for **17** and 5.4 to 89.9 for **15**. Compounds **32–36** are classified into tetracyclic products, while **37–42** are into pentacyclic ones. The production amount of the tetracyclic products from **20** was two times higher than that from **17**, indicating that the two methyl groups at the terminal position are necessary for the ring enlargement process (**8** ⟶ **9**) during the polycyclization cascade of **1**.

The asterisk symbol (*) of Fig. 3A–C shows negligibly small amounts of unidentified products, the structures of which were not established due to the purification difficulty. However, these are likely to be podioda-8,17,21-triene and/or podioda-7,17,21-triene (with C_{28} or C_{29}) having the 6/6/5-fused tricyclic ring system, because the GC-MS analyses showed the *m/z* 231 (base peak) characteristic to a podiodatriene skeleton.[11a,27,28] The terpene skeletons with C_{30} were previously isolated from the reaction mixture obtained by incubating **1** with the mutants Phe605A and Phe605G.[11a]

Discussion

The polycyclization of **1** to **2** consists of sequential ring-forming reaction steps. The polycyclization cascade is initiated by the electrophilic attack of the acidic proton, donated by the DXDD motif, to one of the two terminal double bonds.[3] The polycyclization reaction is quenched by proton elimination from the alternative terminal methyl group of **1**. Based on the X-ray analysis of *A. acidocaldarius* SHC, 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. [29,30] 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 hydroxyl to the C-22 cation of **4** instead

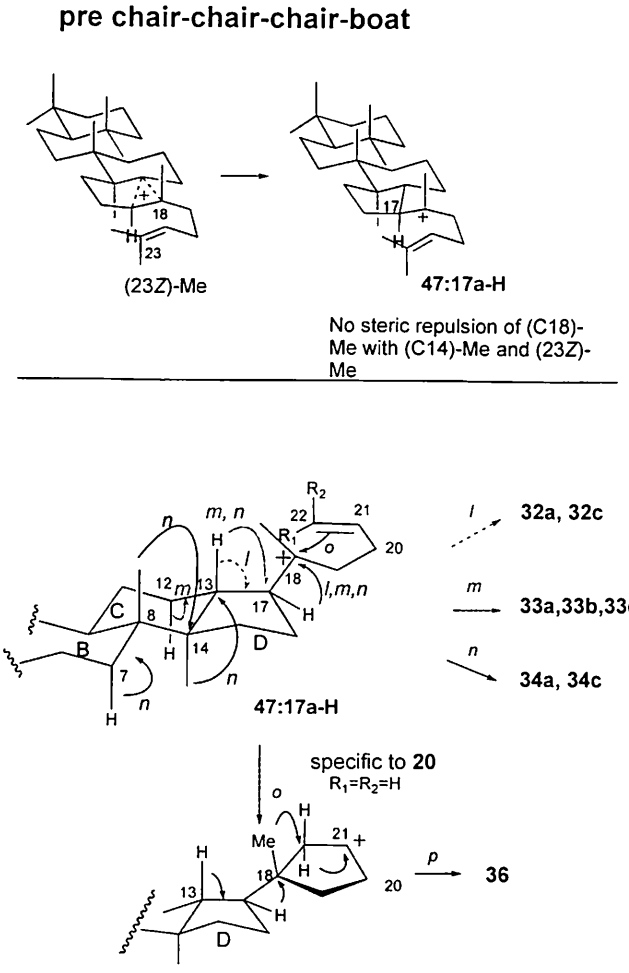


Scheme 6 Cyclization mechanism of **15**, **17** and **20** *via* a tetracyclic cation having 17β-H.

of accepting the proton.[2a, 29, 30] Recently, we have validated this proposal by site-directed mutagenesis experiments.[31] 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** would be explained as follows. The point mutations could give rise to the perturbation around the "front water". This disordered "front water" cannot correctly

act 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**.[31] However, a question has remained unresolved as to which terminal methyl group, *i.e.* either the *Z*- or *E*-methyl at C(23) of **1** is responsible for the final deprotonation reaction. The deuterated squalenes **26** and **27** with *E*-trideuteriomethyl or *Z*-trideuteriomethyl group at the terminal side, respectively, were synthesized to answer the question. Fig. 1 and Scheme 4 clearly demonstrate that the final deprotonation step, responsible for the formation of **2** from **10**, occurs exclusively from the *Z*-Me at C-23 of **1**, not from the *E*-Me. It can be concluded that the strongly polarized "front water" acts as the catalytic base to eliminate the proton of 23*Z*-Me of **1** (Me-29 of **10**). 2,3-Oxidosqualene **11** also could be converted into 3-hydroxy derivatives of **1** and **2**. Analyses of labeling positions obtained by the incorporation experiment of 3*S*- and 3*R*-**29** (Fig. 2) shows that the polycyclization proceeded as shown in Scheme 5. 3*S*-**29** was folded in a chair structure, while the 3*R*-**29** was in a boat form. This conclusion coincided with that from squalene-tetrahymanol cyclase.[22] Tetrahymanol synthase also accepts **29** to produce 3α- and 3β-hydroxytetrahymanols *via* a boat structure from 3*R*-**29** and *via* a chair form from 3*S*-**29**, respectively.[22] Schemes 4 and 5 show that the final deprotonation reactions for the formation of **12a** and **12b** occur exclusively from the terminal *Z*-methyl group as well as that of **2**. Scheme 2 shows that products **16**, **18** and **19** were formed according to path *a*, but not from path *b*. Thus, we have inferred that the two methyl groups at the terminal side are necessary for starting the polycyclization reaction.[23] To validate this idea, **30** and **31** were synthesized and incubated with the native SHC. No conversion occurred even by using a 5-fold larger amount of the cell-free extracts than that used for the standard incubation condition. An investigation of analog **20** also gave additional evidence for the essentiality of an isopropylidene moiety, because no product was detected that the polycyclization started from the methyl-deficient side, but large amounts of products were obtained that the cyclization had started from the terminal isopropylidene moiety (Figs. 3 and 4). Why is the isopropylidene moiety essential for initiating the polycyclization? One possible answer may be as follows. A specific binding or accepting site may be involved in the cyclase that the isopropylidene moiety is strongly captured. Once the squalene molecule has been inside the enzyme cavity, the isopropylidene moiety may tightly associate with the binding site, which would be located near the DXDD motif responsible for initiating the cyclization, thus resulting in a closer proximity of the proton-donating DXDD motif to the π-electron of the terminal double bond of **1**. On the other hand, the methyl-deficient terminal side of **15**, **17**, **20**, **30** and **31** was loosely captured by the specific binding site, thus the access of the DXDD motif to the π-electrons of the terminal double bond failed, resulting in no cyclization.

The squalene analogs **15**, **17** and **20** lacking methyl group(s) could undergo the polycyclization to afford various enzymic products (Fig. 4). As shown in Scheme 6 (top), squalene cyclization proceeds in the all pre-chair structure.[2b,26] Schemes 6 (bottom) and 7 summarize the formation mechanism of these products. According to Scheme 1 and Scheme 6 (top), tetracyclic cation **43** (with C_{28} or C_{29} like **8**) having 17β-H was formed, which is a common intermediate for the formation of pentacyclic products. Intermediate **43** underwent a ring expansion to form intermediate **44** having the 6-membered D-ring (path *a*), which was subjected to a further cyclization to afford cationic intermediate **45** with 6/6/6/6/6-fused pentacyclic ring system (path *b*). To quench C-21 cation, the proton elimination at C-20 occurred to give **37** (path *c*). The hydride shift of H-20 to C-21 cation and the deprotonation of H-19 gave **40** (path *d*), and this hydride shift also gave rise to a sequential 1,2-shift reactions of hydride and methyl group in antiparallel manner to quench the C-21 cation, leading to novel skeleton **39** (path *e*). To cation **45**,



Scheme 7 Production mechanism of **32-34** and **36** *via* the folding conformation of chair-chair-chair-boat to give the stereochemistry of 17α-H.

a water molecule attacked to afford **16**, **18** and **38**, all of which had a hydroxyl group in an equatorial disposition. The (23*E*)-Me of **15** (squalene numbering) was placed in equatorial disposition at C-22 of **16**, while the (23*Z*)-Me of **17** was arranged in the axial direction at C-22 of **18**, as shown in Scheme 2. In addition, **39** and **40**, which were produced from **15**, had the equatorial-oriented methyl group at C-22 as well as **16**. Thus, the formation of **16**, **18** and **37–40** having the 6-membered E-ring (**44**⟶ **45**) would proceed under stereoelectronic control and possibly be explained in terms of HOMO–LUMO orbital interaction (Scheme 6, bottom). Given that a water molecule attacks in the axial direction, a more hindered interaction would occur between the LUMO at C17–C18 and the HOMO at C21–C22 due to the constrained overlapping.

This orbital interaction also indicates that the (23*E*)-Me of **1** is arranged in the equatorial orientation, while the (23*Z*)-Me is placed in the axial direction, which is in good agreement with the experimental results described above.

Intermediate cation **44** could undergo the alternative cyclization pathway to give cation **46** having the 5-membered E-ring (path *g*). Formation of the 5-membered E-ring occurred only from **17**, but never did from **15**, indicating that the (23*Z*)-Me of **1** was strongly captured by the binding site responsible for the deprotonation from Me-29 of **10**. The deprotonation from Me-29 of **46** gave **42** (path *j*) having hopene skeleton. The hydride shift of H-21 to C-22 cation gave sequential 1, 2-shift reactions of hydrides and methyl group in an antiparallel manner, affording **19** and **41** (path *h* or *i*). It should be noted that both (23*Z*)- and (23*E*)-methyl groups are necessary for the complete building of the 5-membered E-ring, since the 6-membered species **19**, **18** and **37–40** and the tetracyclic ones **32–36** were

produced in significantly large amounts when one of the two methyl groups was absent. The tetracyclic product **35** from **20** was formed just by the deprotonation from Me-28 of **43** having 17β-H (path *k* of Scheme 6), but the production amount was negligible (*ca.* 1.8%).

The tetracyclic products **32–34** had 18*R*-stereochemsitry. The similar tetracyclic skeletons of dammar-13(17)-ene and dammar-12(13)-ene having 18*R*-configuration were also isolated by incubating 2,3-dihydrosqualene with the cell-free extracts from *A. acidocaldarius* and the folding conformation has been discussed by Abe and Rohmer.[26] Substrates **17** and **20** are likely to be folded in the pre chair–chair–chair–boat conformation (Scheme 7, top). If the analogs are folded in the all the pre chair conformation (Scheme 6, top), a large rotation (120°) through the C17–C18 axis must occur to gain the *R*-configuration, whereas only a small rotation (60°) occurs for the folding conformation of the pre chair–chair–chair–boat (Scheme 7, top).[26] Thus, **32–34** would have been produced *via* tetracyclic intermediate **47** having 17α-H configuration, which was formed by the folding conformation of the pre chair–chair–chair–boat. The hydride shift of 17α-H to C-18 cation, followed by the deprotonation of H-13, afforded **32a** and **32c** (path *l*). **33a**, **33b** and **33c** was produced as follows. The hydride shift of H-17 to C-18 and that of H-13 to C-17, followed by deprotonation from H-12 gave introduction of the double bond at C12–C13 (path *m*). All the processes occurred in an anti-periplanar fashion. The formation mechanisms of **34a** and **34c** were as follows. A series of 1,2-shift reactions of H-17 to C-18, H-13 to C-17 and C(14)-Me to C-13 and C(8)-Me to C-14, followed by deprotonation at C-7, gave the double bond at C7–C8 (path *n*), all the processes of which also proceeded in an antiparallel manner. A nucleophilic attack of the vinyl group of **47** to the C-18 cation gave intermediate **48** having the novel carbocyclic ring system of 6/6/6/5 + 5 (path *o*). A series of 1,2-shifts of hydride and methyl group, followed by the deprotonation reaction at C-13, gave a novel compound **36** (path *p*). These antiparallel processes lead to the stereochemistry of **36**. Formation of a boat conformation for the D-ring would be also explained in terms of steric repulsion (1,3-diaxial interactions). When analogs **17** and **20** are folded in the all pre-chair conformation, a large steric hindrance occurs among C(14)-Me, C(18)-Me and (23*Z*)-Me (Scheme 6, top), but the interaction is small for the boat conformation as shown in Scheme 7 (top).

Product distribution ratios were estimated from Fig. 3. The distribution ratio of tetracyclic products were *ca.* 17% for **17**, *ca.* 5% for **15** and *ca.* 32% for **20**. The 6/6/6/6/6-fused pentacyclic products from **17**, **15** and **20** possessed *ca.* 37%, 90% and 60%, respectively. A hopane skeleton having the 6/6/6/6/5-fused pentacyclic ring system was produced only from **17** in a highest yield (*ca.* 45%) among all the products from **17**. From **20**, large amounts of the tetracyclic products were produced *via* **47** (*ca.* 32%). In contrast, the production amounts of tetracyclic species from **15** and **17** significantly decreased (*ca.* 5% for **15**, *ca.* 17% for **17**), in turn the formation of intermediate **44** (anti-Markovnikov cation) markedly increased, leading to a high production of the 6/6/6/6/6-fused and/or the 6/6/6/6/5-fused ring systems (pentacyclic products: *ca.* 90% for **15**, *ca.* 81% for **17**, *cf.* 60% for **20**). These findings suggest that one of the two terminal methyl groups (23*Z*- or 23*E*-Me) may work to some extent, though not perfectly, to pose a chair structure for the D-ring (*i.e.* a ring enlargement process **43**→**44**) against the unfavorable 1,3-diaxial interaction between C(14)-Me and C(18)-Me (Scheme 6, top), but this inference is uncertain at the present time. Production of the tetracyclic species from **17** was remarkably higher than those from **15**. One possible answer to this remarkable difference may be explained as follows. When **15** and **17** adopt pre chair conformation for D/E-rings (Scheme 6, top), steric repulsion from 1,3-diaxial interaction is greater for **17** than that for **15**, since *three* methyl groups of C(14)-Me, C(18)-Me and (23*Z*)-Me are arranged in axial orientation for **17**, but *two* methyls of C(14)-Me and C(18)-Me for **15**. The greater repulsion for **17** may have led to higher production of intermediate **47**, compared to that for **15**. A next cyclization reaction of **44** led to a branching into two pathways. Norsqualene **17** having the (23*Z*)-Me was converted into both 6/6/6/6/5-fused (*ca.* 45%) and 6/6/6/6/6/6-fused pentacyclic products (*ca.* 37%). As shown in Scheme 6 (bottom), the 6-membered E-ring was constructed under stereoelectronic control (no enzymatic control), but the formation of the 5-membered E-ring is strictly controlled by the cyclase enzyme. The *Z*-Me may be strongly captured by the cleft constructed by some amino acid residues surrounding the "front water". As described above, the "front water" acts as the catalytic base for introduction of the double bond to give **42**, but the binding force became looser to give **19** and **41** having different double bond positions, which would have occurred due to a release of the *Z*-Me from the "front water". When the *Z*-Me was entirely free of the binding, **18** was produced under no enzymic control. Analogs **15** and **20** do not have the *Z*-Me, thus converted into the 6/6/6/6/6-fused pentacyclic skeleton (**16**, **39** and **40** from **15**, and **37** and **38** from **20**) in a similar way as the formation of **18** from **17**.

Recently, we have reported that the methyl group at C(10) of **1** strongly affects the polycyclization destiny. When the methyl group is deficient, unusual carbocyclic skeletons with 6/5+5/5+(5) ring system(s) were formed. [32] We have proposed that the methyl groups of **1** are strongly captured by the squalene cyclase for the normal cyclization pathway. The two terminal methyl groups (isopropylidene moiety) are required for starting the polycyclization reaction. The central methyl group at C(10) is important in acquiring the desired product-like chair conformation to construct the fused B/C/D-ring system. In addition, the alternative terminal *E*- and *Z*-methyl groups are both necessary for completion of the five-membered E-ring without forming aberrant cyclization products. Our studies on the cyclization of the substrate analogs, which have the methyl group at a position different from that of **1**, have revealed that the methyl position of **1** also is important for the normal polycyclization cascade to construct the hopane skeleton (unpublished results). The cyclizations of 2,3-oxidosqualene analogs by lanosterol synthase have also showed the importance of precise steric bulk size of the substrate for the normal polycyclization pathway.[2b,33–35] To date, many reports have appeared demonstrating that alteration of the steric bulk size by point mutations of squalene and 2,3-oxidosqualene cyclases significantly affected the polycyclization pathways.[1a,10,18,36–42] It appears that precise steric interactions between the substrates and the side chains at active sites enforce the product-like conformation upon squalene and oxidosqualene substrates.

Conclusion

The following results were obtained through this investigation. (1) The final deprotonation reaction for introducing the double bond occurs exclusively from the *Z*-methyl at C(23) of **1**. (2) (3*S*)-2,3-Oxidosqualene is folded in a pre-chair structure to form the A-ring of 3β-hydroxyhopene **12b**, whereas the 3*R*-form is folded in a boat structure for the building of the A-ring of 3α-hydroxyhopene **12a**. (3) A large amount of a 6/6/6/6/6-fused pentacyclic ring skeleton was produced from **20** (*ca.* 60%). However, hopane skeleton (a 6/6/6/6/5-fused ring system) was not produced, neither was the product of the polycyclization reaction starting from the methyl-deficient side. In addition, **30** and **31**, which lack each methyl group at both of the terminal sides of **1**, were never cyclized. These findings definitively demonstrated that the isopropylidene moiety is required both for constructing a hopane skeleton and for initiating the polycyclization reaction. The remaining other products (*ca.* 32%) had the 6/6/6/5-fused tetracyclic ring skeletons. Significantly larger

amounts of the tetracyclic compounds from **20**, compared with those from **15** and **17**, indicated that the isopropylidene moiety also plays a crucial role for the folding of the all chair conformation. When the terminal methyl groups are absent, the substrate is folded in the chair–chair–chair–boat conformation, leading to tetracyclic products having 18*R*-stereochemistry. (4) Small amounts of products from **15** and **17** were successfully isolated: no product having a 5-membered E-ring was found from the incubation mixture of **15**, whereas significantly large amounts (*ca* 45%) of the products with the 5-membered E-ring were obtained from that of **17**. This supports unequivocally that the Z-methyl group has a more crucial role for the formation of the 5-membered E-ring than the *E*-methyl group. (5) Unnatural terpenoid skeletons **36**, **37**, **39**, **40** were created through this investigation.

Experimental

Analytical methods

NMR spectra were recorded in C_6D_6 on a Bruker DMX 600 or DPX 400 spectrometer, the chemical shifts being relative to the solvent peak δ_H 7.280 and δ_C 128.0 ppm as the internal reference for ^1H and ^{13}C NMR spectra, respectively. The chemical shifts in $CDCl_3$ solution were given according to the internal solvent peaks of δ_H 7.26 and δ_C 77.0 ppm. The coupling constants (*J* values) are given in Hz. GC analyses were performed on a Shimadzu GC-8A chromatograph equipped with a flame ionization detector (DB-1 capillary column (0.53 mm × 30 m); injection temperature, 290 °C; column temperature, 270 °C; flow rate of N_2 carrier, 1.0 kg cm^{-2}). GC-MS spectra were recorded on a JEOL SX 100 spectrometer under electronic impact at 70 eV with a DB-1 capillary column (0.32 mm × 30 m), the oven temperature being elevated from 220 to 270 °C (3 °C min^{-1}). HR-EIMS was performed using a direct inlet system. Specific rotation values were measured at 25 °C with a Horiba SEPA-300 polarimeter.

Incubation conditions

Standard incubation conditions were performed according to the published protocols.[3a,4,25] The cell-free extract was prepared as follows. One litre culture of *E. coli* encoding the native SHC was harvested by centrifugation and to the collected pellets was added 50 cm^3 of citrate buffer solution (pH 6.0), and then subjected to the ultrasonication to disrupt the cells. The supernatant was used for the incubations after removing the cell debris by centrifugation. One cm^3 of the supernatant contains *ca.* 200 μg of the pure SHC.

Preparation of (2*E*)-[1,1,1-$^2H_3$]squalene 26, (2*Z*)-[25,25,25-$^2H_3$]squalene 27 and (2*E*,22*E*)-[1,1,1,24,24,24-$^2H_6$]-2,3-oxidosqualene 29

The synthetic method is shown in Scheme 3. 2,3-Oxidosqualene was synthesized in a similar manner as that published previously.[43] To a stirred suspension of $H_5IO_6 \cdot 2H_2O$ (9.49 g, 35.9 mmol) in Et_2O (200 cm^3), was added dropwise 100 cm^3 of the ethereal solution of oxidosqualene (11.88 g, 27.88 mmol). After being stirred for 2.5 h at room temperature, the reaction mixture was poured into 250 cm^3 of brine. The organic phase was washed with 2 × 250 cm^3 brine and dried over anhydrous sodium sulfate, and evaporated. The residues were chromatographed on a silica gel column in 5 : 1 hexane/EtOAc to afford 7.25 g (84.8%) of aldehyde **21**. EIMS fragments *m/z* (%): 69 (100), 384 (M^+, 15). To a suspension of (1-ethoxycarbonyl-ethyl)-triphenylphosphonium bromide (1.3 g), Ph_3P^+-$CH_3CHBr^-CO_2C_2H_5$, in dry THF (25 cm^3), which was prepared by refluxing a benzene solution of triphenylphosphine and 2-bromopropionic acid ethyl ester for 3 h, was added a solution of 2 cm^3 of *n*-BuLi (1.6 mol l^{-1} in hexane) and stirred for 20 min at 0 °C under a nitrogen atmosphere. To the solution of phosphorous ylide thus obtained was added a solution of 25 cm^3 of **21** (500 mg) in THF and further stirred for 1 h at room temperature. The reaction mixture was carefully poured into ice water and the product was extracted with hexane and dried over anhydrous sodium sulfate. Evaporation of solvent followed by HPLC chromatography (a prepacked column: Mightysil Si 60, 5 μm, from Kanto Chemical Co., Inc.) by eluting with 100 : 0.08 hexane/2-propanol afforded **22** and **23** in a pure state, yielding 430 mg (71%) and 30 mg (5%), respectively. **22**: (2*E*,6*E*,10*E*,14*E*,18*E*)-2,6,10,15,19,23-hexamethyl-tetracosa-2,6,10,14,18,22-hexaenoic acid ethyl ester: *m/z* (%) 69 (100), 81 (93), 121 (83), 468 (M^+, 32); δ_H (in $CDCl_3$, 400 MHz) 6.74 (1H, bt, *J* 6.0), 5.09 (5H, m), 4.19 (2H, q, *J* 6.8), 2.26 (2H, dt, *J* 7.6, 7.6), 2.10–1.93 (18H, m), 1.81 (3H, s), 1.67 (3H, s), 1.60 (3H, s), 1.58 (9H, s), 1.57 (3H, s), 1.27 (t, *J* 6.8). **23**: (2*Z*,6*E*,10*E*,14*E*,18*E*)-2,6,10,15,19,23-hexamethyl-tetracosa-2,6,10,14,18,22-hexaenoic acid ethyl ester; *m/z* (%) 468 (M^+, 32). Compound **22** was reduced with $LiAlD_4$ to give the deuterated alcohol derivative **24**, (2*E*,6*E*,10*E*,14*E*,18*E*)-[1-$^2H_2$]-2,6,10,15,19,23-hexamethyl-tetracosa-2,6,10,14,18,22-hexaen-1-ol. To the ethereal solution of **22** (120 mg, 0.25 mmol), was slowly added a powder of $LiAlD_4$ (Aldrich Co. 98% D) at 0 °C under a nitrogen atmosphere until **22** disappeared on TLC.

The reaction was quenched by addition of EtOAc saturated with water and stirred overnight followed by filtration of the precipitates. Evaporation of solvent and chromatography on SiO_2 column by eluting with 100 : 2 hexane/EtOAc gave the deuterated alcohol **24** (*E*-CD_2OH, 75 mg) in 82% yield. 94% D content; *m/z* (%) 69 (100), 81 (100), 95 (100), 137 (85), 428 (M^+, 10); δ_H (in $CDCl_3$, 400 MHz) 5.37 (1H, bt, *J* 6.8, H-3), 5.10 (5H, m), 2.20–1.93 (20H, m), 1.66 (3H, s, Me-25), 1.65 (3H, s, Me), 1.59 (15H, s, 5 × Me) and no NOE between H-3 and Me-25. To a solution of **24** (75 mg) dissolved in CH_2Cl_2 (25 cm^3), was added 0.15 cm^3 of dried triethylamine at −15 °C under a nitrogen atmosphere and then a freshly distilled methanesufonyl chloride (15 μl) was slowly added and stirred for 1.5 h. The reaction mixture was transferred into the separatory funnel, washed with cold water, cold 5% HCl, saturated $NaHCO_3$ and finally with brine. The organic solvent was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*, and subjected to the next reaction without further purification (66% yield). The crude mesylate (32 mg) was dissolved in dry THF (3 cm^3) and 2.5 mg of $LiAlD_4$ was added in small portions. The reaction was terminated by addition of EtOAc saturated with water and further stirred overnight. Filtration of the precipitates and chromatography on SiO_2 in 100 : 5 hexane/EtOAc gave the desired deuterated squalene **26** (20 mg) in 70% yield from **24**. **26**: D content 93%; *m/z* (%) 413 (M^+); δ_H (in $CDCl_3$, 400 MHz) 5.11 (5H, m), 2.10–1.93 (20H, m), 1.66 (3H, s, Me-24), 1.58 (18H, s, 6 × Me). The synthesis of **25** from **23** was according to the same procedure as described for that of **26** from **24**. **25**: 93% D content; *m/z* (%) 428 (M^+, 10); δ_H (in $CDCl_3$, 600 MHz) 5.28 (1H, bt, *J* 6.8, H-3), 5.11 (5H, m), 2.13 (2H, dt, *J* 7.6, 7.6), 2.10–1.95 (18H, m), 1.77 (3H, s, Me-1), 1.66 (3H, s, Me), 1.58 (15 H, s, 5 × Me), a strong NOE between H-3 and Me-1. **27**: D content 92%, *m/z* (%) 413 (M^+); δ_H (in $CDCl_3$, 400 MHz) 5.11 (5H, m), 2.10–1.93 (20H, m), 1.66 (6H, s, Me-1–24), 1.58 (15H, s, 5 × Me). Preparation of hexadeuterated **29** was essentially the same as that of **26**, but the amount of *N*-bromosuccinimide (NBS) used was 2-fold higher for the synthesis of 2,3;22,23-dioxidosqualene, compared to that for 2,3-oxidosqualene. The epoxide rings were cleaved to give dialdehyde **28**: 4,8,13,17-tetramethyl-eicosa-4,8,12,16-tetraenedial. δ_H (in $CDCl_3$, 400 MHz) 9.74 (2H, bs), 5.14 (4H, bt, *J* 6.0), 2.50 (4H, bt, 7.6), 2.31 (4H, bt, *J* 7.6), 2.06 (2H, bt, 7.2), 1.98 (4H, m), 1.61 (6H, s, 2 × Me), 1.59 (6H, s, 2 × Me). **29** (*m/z* 432, M^+, D content 92%).

Preparation of (2*E*,22*E*)- 30 and (2*Z*,22*Z*)-6,10,15,19-tetramethyl-tetracosa-2,6,10,14,18,22-hexaene 31

To the stirred solution of ethyl diethylphosphonoacetate (800 mg, 3.57 mmol) in dry THF 10 cm^3, was added NaH (75 mg, 3.13 mmol) at 0 °C under a nitrogen atmosphere followed by slow addition of **28** in THF (Horner–Wadsworth–Emmons reaction). After being stirred for 1 h at 0 °C and further stood at room temperature for 2 h, the reaction mixture was poured into ice water. The product was extracted with hexane and dried over anhydrous Na_2SO_4 and subjected to a column chromatography on SiO_2 by eluting with 100 : 1 hexane/EtOAc, giving the diethyl ester 170 mg in 30% yield. *m/z* (%) 81 (92), 93 (100), 498 (M^+, 2). The ethereal solution of the diethyl ester (40 mg, 0.08 mmol), 0.6 cm^3 of 0.95 mol l^{-1} diisobutylaluminium hydride (DIBAL-H) in hexane was added in small portions over 2 h at −40 °C under a nitrogen atmosphere. The reaction was quenched by adding EtOAc saturated with water and the mixture was stirred overnight. Filtration of the white precipitates and evaporation of the solvent *in vacuo* followed by chromatography on SiO_2 column in 10 : 1 hexane/EtOAc gave the corresponding diols (32 mg, 97% yield). *m/z* (%) 69 (100), 81 (100), 414 (M^+, 12). The mesylation of the diols and the removal of the mesyl group by $LiALH_4$ were done with the same protocols as described in the preparation of **26** and **27**, affording the desired **30**. *m/z* (%) 94 (78), 124 (85), 127 (90), 327 (100), 382 (M^+, 76). δ_H (in $CDCl_3$, 400 MHz) 5.44 (4H, bs), 5.12 (4H, m), 2.2–1.8 (20H, m), 1.61 (3H, s, Me), 1.609 (3H, s, Me), 1.58 (6H, s, 2 × Me), 1.57 (6H, s, 2 × Me). To obtain **31**, triphenylethylphosphonium bromide was subjected to a Wittig reaction with **28** by using *n*-BuLi as a base. Chromatography on SiO_2 column by eluting with hexane afforded pure (*Z*,*Z*)-**31**: *m/z* (%) 81 (100), 95 (56), 123 (100), 327 (28), 382 (M^+,15); δ_H (in $CDCl_3$, 400 MHz) 5.40 (2H, m), 5.11 (4H, m), 2.2–2.8 (20H, m), 1.57 (12H, s, 6 × Me).

Preparation of (5*E*,9*E*,13*E*,17*E*)-5,9,14,18,22-pentamethyl-tricosa-1,5,9,13,17,21-hexaene 20

To a solution of *n*-BuLi (0.3 cm^3 of 0.95 mol l^{-1} hexane) in 5 cm^3 of THF, was added triphenylmethylphosphonium iodide (100 mg) and stirred for 20 min at 0 °C under a nitrogen atmosphere, resulting in an orange color. To a solution of the ylide, a 2 cm^3 of the solution of **21** (50 mg, 0.13 mmol) in THF was slowly added and stirred for 1 h. After being poured into ice water, the product was extracted with hexane and dried over anhydrous $MgSO_4$ and then purified with SiO_2 column by eluting with hexane, giving 32 mg of **20**: *m/z* (%) 69 (100), 81 (95), 109 (80), 149 (35), 382 (M^+, 11); δ_H (in $CDCl_3$, 400 MHz) 5.83 (1H, m), 5.13 (5H, m), 5.01 (1H, bdd, *J* 17.2, 3.0), 4.98 (1H, bd, *J* 10.0), 2.2–2.0 (20H, m), 1.69 (3H, s, Me), 1.61 (15H, s, 5 × Me).

Preparation of (2*E*,6*E*,10*E*,14*E*,18*E*,22*Z*)-2,6,10,15,19-pentamethyl-tetracosa-2,6,10,14,18,22-hexaene 17

To the stirred suspension of triphenylethylphosphonium iodide in 5 cm^3 of THF, 0.5 cm^3 of 1 M $NaN(SiMe_3)_2$ in THF was slowly added at 0 °C and stood for 30 min under a nitrogen atmosphere; the solution color changed into orange, showing production of the ylide. The reaction temperature was further lowered to −78 °C and a solution of 20 mg of aldehyde **21** (0.05 mmol) in THF (5 cm^3) was dropwise added. After being poured into ice water, the product was extracted with hexane and dried over anhydrous Na_2SO_4 and then purified with SiO_2 column by eluting hexane, giving 16 mg of **17**: *m/z* (%) 55 (45), 69 (100), 81 (92), 123 (43), 396 (M^+, 3). δ_H (in $CDCl_3$, 600 MHz) 5.44 (1H, m), 5.38 (1H, m), 5.13 (5H, m), 2.15 (2H, t, *J* 7.5), 2.09 (6H, m), ~2.0 (12H, m), 1.69 (3H, s), 1.61 (18H, s). The ^1H NMR spectrum showed little contamination of **15**.

Preparation of (2*E*,6*E*,10*E*,14*E*,18*E*,22*E*)-2,6,10,15,19-pentamethyl-tetracosa-2,6,10,14,18,22-hexaene 15

The synthetic procedure is shown in Scheme 3 and described above. δ_H (in $CDCl_3$, 400 MHz) 5.42 (2H, bs), 5.13 (5H, m), 2.09 (6H, m), ~1.99 (20H, m), 1.69 (3H, s), 1.64 (3H, s), 1.61 (12H, s), 1.59 (3H, s). The MS spectrum of **15** was indistinguishable from that of **17**.

Spectral data of Compounds 2, 3, 12a, 12b and 32–42

2 (solid) in $CDCl_3$. δ_H 0.68 (m, H-5), 0.704 (3H, s, Me-28), 0.73 (m, H-1), 0.773 (3H, s, Me-24), 0.797 (3H, s, Me-25), 0.825 (3H,s, Me-23), 0.926 (3H, s, Me-27), 0.940 (3H,s, Me-26), 1.10 (m, H-19), 1.11 (m, H-3), 1.21 (m, H-7), 1.24 (m, H-9), 1.25 (m, H-16), 1.29 (m, H-6), 1.33 (2H, m, H-3, H-19), 1.34 (m, H-2), 1.35 (3H, m, H-13, H-15), 1.37 (m, H-17), 1.38 (m, H-12), 1.45 (2H, m, H-6, H-12), 1.46 (2H, m, H-7, H-11), 1.50 (m, H-16), 1.55 (m, H-2), 1.63 (m, H-1), 1.64 (m, H-11), 1.73 (3H, s, Me-30), 1.78 (m, H-20), 1.84 (m, H-20), 2.66 (ddd, *J* 8.5, H-21), 4.76 (2H, s, CH_2-29). δ_C 15.83 (C-25), 16.07 (C-28), 16.69 (C-26), 16.74 (C-27), 18.69 (C-6), 18.71 (C-2), 20.93 (C-16), 21.58 (C-24), 21.67 (C-11), 24.00 (C-12), 24.96 (C-30), 27.40 (C-20), 33.24 (C-4), 33.28 (C-7), 33.39 (C-23), 33.65 (C-15), 37.41 (C-10), 40.34 (C-1), 41.91 (C-3), 41.91 (C-8), 42.09 (C-14), 42.12 (C-19), 44.79 (C-18), 46.50 (C-21), 49.47 (C-13), 50.41 (C-9), 54.92 (C-17), 56.13 (C-5), 110.0 (C-29), 148.7 (C-22). Assignments are exchangeable between C26 and C27, between C2 and C6, and between C-8 and C-14. $[a]_D^{25}$ ($CHCl_3$) +15.43 (c = 0.055).

3 (solid) in C_6D_6. δ_H 0.88 (bd, *J* 12.5, H-5), 0.90 (m, H-1), 0.997 (3H, s, Me-24), 0.99 (3H, s, Me-25), 0.935 (3H, s, Me-28), 1.35 (2H, m, H-7, H-15), 1.40 (m, H-12), 1.42 (m, H-9), 1.45 (m, H-15), 1.47 (3H, m, H-2, H-6, H-17), 1.49 (m, H-13), 1.52 (m, H-3), 1.58 (2H, m, H-11), 1.60 (m, H-20), 1.62 (m, H-7), 1.64 (2H, m, H-6, H-12), 1.73 (m, H-2), 1.76 (m, H-20), 1.78 (2H, m, H-1, H-19), 1.02 (m, H-19), 1.05 (3H,s, Me-23), 1.09 (3H, s, Me-27), 1.11 (3H, s, Me-26), 1.22 (3H,s, Me-30), 1.27 (3H, s, Me-29), 1.30 (m, H-3), 2.05 (2H, bd, *J* 11.2, H-16), 2.15 (ddd, *J* 9, H-21) δ_C 16.08 (C-25), 16.32 (C-28), 16.99 (C-26), 17.27 (C-27), 19.09 (C-2, C-6), 21.83 (C-24), 22.30 (C-16), 24.50 (C-11, C-12), 26.79 (C-20), 29.27 (C-30), 31.30 (C-29), 33.42 (C-4), 33.63 (C-7, C-23), 34.71 (C-15), 37.67 (C-10), 40.59 (C-1, C-19), 40.59 (C-19), 42.12 (C-8), 42.16 (C-14), 42.40 (C-3), 44.29 (C-18), 50.23 (C-13), 50.76 (C-9), 51.19 (C-21), 54.38 (C-17), 56.50 (C-5), 73.10 (C-22). Assignments of C8 and C14 are exchangeable. $[a]_D^{25}$ ($CHCl_3$) +23.49 (c = 0.048).

12a (solid) in C_6D_6. δ_H 0.90 (6H, s, Me-24, Me-28), 0.95 (3H, s, Me-25), 1.06 (3H, s, Me-23), 1.08 (m, H-19), 1.09 (3H, s, Me-27), 1.11 (3H, s, Me-26), 1.33 (m, H-15), 1.34 (2H, m, H-7, H-17), 1.38 (m, H-16), 1.43 (m, H-13), 1.45 (2H, m, H-6, H-7), 1.47 (m, H-5), 1.50 (m, H-1), 1.51 (m, H-9), 1.55 (3H, m, H-12, H-6), 1.59 (m, H-2), 1.60 (m, H-11), 1.62 (2H, m, H-15, H-16), 1.64 (m, H-1), 1.72 (m, H-2), 1.73 (m, H-11), 1.74 (m, H-19), 1.88 (3H, s, Me-30), 1.97 (2H, m, H-20), 2.73 (ddd, *J* 8.8, H-21), 3.30 (1H, bs, H-3), 5.06 (2H, s, Me-29). δ_C 15.95 (C-25), 16.35 (C-28), 16.88 (C-27), 16.91 (C-26), 18.67 (C-6), 21.21 (C-16), 22.02 (C-2, C-11), 22.31 (C-24), 22.32 (C-12), 25.24 (C-30), 27.66 (C-20), 28.54 (C-23), 33.50 (C-1), 33.57 (C-7), 33.89 (C-15), 37.44 (C-10), 37.65 (C-4), 42.09 (C-14), 42.19 (C-19), 42.29 (C-8), 44.97 (C-18), 46.77 (C-21), 48.98 (C-5), 49.78 (C-13), 50.40 (C-9), 55.04 (C-17), 75.81 (C-3), 110.7 (C-29), 148.4 (C-22). $[a]_D^{25}$ (EtOH) −7.76 (c = 0.067).

12b (solid) in C_6D_6. δ_H 0.74 (bd, *J* 12.0, H-5), 0.89 (m, H-1), 0.91 (3H, s, Me-25), 0.94 (3H, s, Me-24), 1.07 (3H, s, Me-26), 1.07 (m, H-19), 1.15 (3H, s, Me-23), 1.30 (m, H-9), 1.33 (m, H-7), 1.34 (m, H-11), 1.36 (m, H-17), 1.40 (m, H-13), 1.44 (2H, m, H-15), 1.47 (m, H-6), 1.52 (m, H-7), 1.54 (m, H-11), 1.55 (2H, m, H-12), 1.58 (2H, m, H-2), 1.62 (2H, m, H-6, H-16), 1.68 (m, H-1), 1.73 (m, H-19), 1.76 (m, H-16), 1.97 (2H, m, H-20), 2.73 (ddd, *J* 8.4, H-21), 3.16 (dd, *J* 10.4, 5.4, H-3). δ_C 15.71

(C-24), 16.07 (C-25), 16.38 (C-28), 16.85 (C-26), 16.91 (C-27), 18.73 (C-5), 18.73 (C-6), 18.73 (C-7), 21.33 (C-11), 22.03 (C-16), 23.69 (C-12), 25.23 (C-30), 27.69 (C-20), 27.90 (C-2), 28.77 (C-23), 33.95 (C-15), 37.31 (C-10), 38.99 (C-1), 39.08 (C-4), 41.92 (C-8), 42.20 (C-19), 42.26 (C-14), 44.98 (C-18), 46.78 (C-21), 49.82 (C-13), 50.66 (C-9), 55.05 (C-17), 78.54 (C-3), 110.7 (C-29), 148.4 (C-22). Assignments are exchangeable between C-26 and C-27, and between C8 and C14. $[\alpha]_D^{25}$ (EtOH) +6.63 (c = 0.090).

32a (oil) in C_6D_6. δ_H 0.90 (m, H-1), 0.93 (dd, J 12, 1.8, H-5), 0.97 (3H, s, Me-24), 0.99 (3H, s, Me-25), 1.48 (m, H-7), 1.03 (3H, s, Me-23), 1.09 (3H, s, Me-26), 1.11 (d, J 7.2, Me-28), 1.26 (m, H-3), 1.29 (3H, s, Me-27), 1.40 (m, H-12), 1.43 (m, H-15), 1.44 (2H, m, H-2, H-6), 1.48 (m, H-3), 1.52 (m, H-19), 1.58 (m, H-9), 1.62 (m, H-19), 1.65 (m, H-6), 1.66 (m, H-7), 1.67 (m, H-12), 1.70 (m, H-2), 1.77 (m, H-1), 2.03 (m, H-11), 2.09 (m, H-15), 2.12 (m, H-20), 2.22 (m, H-20), 2.59 (m, H-11), 2.68 (m, H-18), 5.15 (bd, J 10.4, H-22), 5.24 (dd, J 16.8, 1.6, H-22), 5.96 (m, H-21). δ_C 16.65 (C-25), 17.62 (C-26), 18.90 (C-2), 19.03 (C-6), 20.08 (C-28), 21.88 (C-24), 22.05 (C-12), 23.03 (C-27), 23.26 (C-11), 29.31 (C-16), 30.97 (C-15), 31.81 (C-18), 32.65 (C-20), 33.48 (C-4), 33.66 (C-23), 34.95 (C-19), 35.79 (C-7), 37.96 (C-10), 40.81 (C-1), 41.46 (C-8), 42.29 (C-3), 52.07 (C-9), 56.90 (C-14), 57.26 (C-5), 114.5 (C-22), 134.7 (C-17), 139.4 (C-21), 139.8 (C-13). Assignments of ^1H and ^{13}C at C-2 and C-6 are interchangeable. EIMS m/z 191 (100%), 205 (39), 299 (16), 382 (M^+, 61).

32c (oil) in C_6D_6. δ_C 12.95 (C-29), 16.64 (C-25), 17.62 (C-26), 18.91 (C-2), 19.04 (C-6), 20.13 (C-28), 21.89 (C-24), 22.11 (C-12), 23.10 (C-27), 23.33 (C-11), 29.42 (C-16), 30.17 (C-15), 31.02 (C-18), 32.21 (C-20), 33.49 (C-4), 33.66 (C-23), 35.68 (C-19), 35.79 (C-7), 37.9 8 (C-10), 40.84 (C-1), 41.53 (C-8), 42.31 (C-3), 52.12 (C-9), 56.90 (C-14), 57.26 (C-5), 123.8 (C-22), 131.2 (C-21), 134.9 (C-17), 139.7 (C-13). EIMS m/z 191 (100), 205 (38), 341 (37), 396 (M^+, 36).

33a (oil) in C_6D_6. δ_H 0.92 (2H, m, H-1, H-5), 1.00 (3H, s, Me-24), 1.01 (d, J 6.9, Me-28), 1.02 (3H, s, Me-26), 1.04 (3H, s, Me-23), 1.07 (3H, s, Me-25), 1.17 (3H, s, Me-27), 1.27 (m, H-3), 1.30 (m, H-15), 1.45 (m, H-19), 1.47 (m, H-3), 1.48 (2H, m, H-2, H-6), 1.61 (2H, m, H-16, H-19), 1.65 (m, H-1), 1.68 (2H, m, H-2, H-6), 1.70 (m, H-7), 1.78 (m, H-15), 1.82 (2H, m, H-9, H-7), 1.93 (m, H-18), 1.94 (m, H-11), 2.15 (m, H-11), 2.17 (m, H-20), 2.23 (m, H-20), 2.53 (m, H-17), 5.15 (bd, J 10.1, H-22), 5.21 (dd, J 17.0, 1.7, H-22), 5.39 (dd, J 3.5, 3.1, H-5), 5.97 (m, H-21). δ_C 15.35 (C-28), 15.9 (C-25), 17.09 (C-26), 18.87 (C-2), 19.08 (C-6), 21.95 (C-24), 22.86 (C-27), 23.93 (C-11), 24.44 (C-16), 32.04 (C-15), 32.19 (C-20), 33.34 (C-4), 33.65 (C-23), 34.57 (C-18), 35.13 (C-7), 35.33 (C-19), 37.84 (C-10), 38.53 (C-8), 40.58 (C-1), 42.29 (C-3), 48.66 (C-9), 48.77 (C-17), 50.61 (C-14), 57.26 (C-5), 114.4 (C-22), 116.2 (C-12), 139.4 (C-21), 149.1 (C-13). Assignments of C-2 and C-6 are interchangeable. EIMS m/z 190 (100%), 367 (13), 382 (M^+, 29)

33b (oil) in C_6D_6. δ_H 5.61 (2H, m, H-21 and H-22), 1.76 (3H, d, J 5.0, Me-29), 1.04 (3H, s, J 6.4, Me-28). δ_C 15.44 (C-28), 15.91 (C-25), 17.10 (C-26), 18.13 (C-29), 18.88 (C-2), 19.08 (C-6), 21.95 (C-24), 22.88 (C-27), 23.93 (C-11), 24.50 (C-16), 31.05 (C-20), 32.05 (C-15), 33.34 (C-4), 33.65 (C-23), 34.61 (C-18), 35.13 (C-7), 36.07 (C-19), 37.84 (C-10), 38.53 (C-8), 40.57 (C-1), 42.29 (C-3), 48.67 (C-9), 48.86 (C-17), 50.61 (C-14), 57.26 (C-5), 116.1 (C-12), 124.7 (C-22), 132.1 (C-21), 149.2 (C-13). EIMS, m/z 191 (100%), 204 (95), 396 (M^+, 52)

33c (oil) in C_6D_6. δ_H 5.66 (m, H-21), 5.64 (m, H-22), 1.73 (3H, d, J 6.6 Hz, H-29). δ_C 15.44 (C-28), 15.91 (C-25), 17.10 (C-26), 18.13 (C-29), 18.88 (C-2), 19.08 (C-6), 21.95 (C-24), 22.88 (C-27), 23.93 (C-11), 24.50 (C-16), 31.05 (C-20), 32.05 (C-15), 33.34 (C-4), 33.65 (C-23), 34.61 (C-18), 35.13 (C-7), 36.07 (C-19), 37.84 (C-10), 38.53 (C-8), 40.57 (C-1), 42.29 (C-3), 48.67 (C-9), 48.86 (C-17), 50.61 (C-14), 57.26 (C-5), 116.1 (C-12), 124.7 (C-22), 132.1 (C-21), 149.2 (C-13). Assignments of C-2 and C-6 may be interchangeable. EIMS m/z 191 (100%), 204 (93), 396 (M^+, 55).

34a (oil) in C_6D_6. δ_H 0.96 (6H, s, Me-24 and Me-25), 0.99 (3H, d, J 6.4, Me-28), 1.03 (3H,s, Me-23), 1.03 (3H, s, Me-27), 1.05 (m, H-2), 1.06 (m, H-1), 1.09 (m, H-6), 1.17 (3H,s, Me-26), 1.23 (m, H-19), 1.28 (m, H-3), 1.38 (m, H-16), 1.48 (m, H-5), 1.51 (m, H-2), 1.52 (m, H-3), 1.55 (m, H-15), 1.57 (m, H-18), 1.61 (m, H-17), 1.62 (2H, m, H-11), 1.65 (m, H-15), 1.74 (m, H-1), 1.78 (m, H-12), 1.86 (m, H-19), 1.92 (m, H-12), 2.03 (m, H-16), 2.26 (m, H-6), 2.30 (m, H-20), 2.43 (m, H-9), 5.15 (bd, J 9.1, H-22), 5.23 (bdd, J 17, 1.8, H-22), 5.47 (dd, J 6.4, 3.1, H-7), 5.99 (m, H-21). δ_C 13.36 (C-25), 18.49 (C-11), 18.63 (C-28), 19.45 (C-2), 21.52 (C-23), 22.40 (C-27), 24.71 (C-6), 27.60 (C-26), 28.76 (C-16), 31.47 (C-20), 33.15 (C-24), 33.30 (C-4), 34.22 (C-12), 34.66 (C-19), 35.40 (C-10), 35.85 (C-18), 39.31 (C-1), 42.69 (C-3), 43.82 (C-13), 49.45 (C-9), 51.66 (C-5), 51.71 (C-15), 53.58 (C-17), 114.4 (C-22), 118.5 (C-7), 139.6 (C-21), 146.0 (C-8). EIMS m/z 367 (100%), 382 (M^+, 18)

34c (oil) in C_6D_6. δ_H 5.66 (m, H-22), 5.67 (m, H-21), 1.75 (3H, bd, J 5.8, Me-29), 1.04 (3H, m, Me-28). δ_C 12.9 (C-29), 13.4 (C-25), 18.5 (C-6), 18.7 (C-28), 19.4 (C-2), 21.5 (C-24), 22.4 (C-27), 24.6 (C-12), 24.7 (C-11), 27.6 (C-26), 28.8 (C-20), 36.0 (C-17), 33.1 (C-23), 33.3 (C-4), 34.3 (C-16), 34.4 (C-15), 35.2 (C-19), 35.4 (C-10), 39.3 (C-1), 42.7 (C-3), 43.8 (C-13), 49.5 (C-9), 51.7 (C-5), 51.7 (C-14), 53.7 (C-18), 118.5 (C-7), 123.7 (C-22), 131.4 (C-21), 146.0 (C-8). EIMS m/z 381 (100%), 382 (M^+, 16)

35 (oil) in C_6D_6. δ_H 0.89 (m, H-5), 0.92 (m, H-1), 0.989 (3H,s, Me-24), 1.01 (3H, s, Me-25), 1.02 (3H, s, Me-23), 1.07 (3H, s, Me-26), 1.11 (3H,s, Me-27), 1.22 (m, H-12), 1.25 (m, H-3), 1.38 (m, H-15), 1.39 (m, H-7), 1.45 (m, H-3), 1.48 (3H, m, H-2, H-6, H-9), 1.62 (m, H-11), 1.63 (2H, m, H-2, H-6, H-15), 1.68 (m, H-12), 1.70 (m, H-7), 1.75 (m, H-1), 1.78 (m, H-11), 1.87 (m, H-16), 1.98 (m, H-16), 2.05 (m, H-13), 2.17 (m, H-19), 2.28 (m, H-19), 2.32 (2H, m, H-20), 2.65 (m, H-17), 5.11 (bs, H-28), 5.14 (bd, J 10.4, H-22), 5.18 (bs, H-28), 5.21 (bd, J 15.7, H-22), 5.98 (m, H-21). Assignments of H-11 and H-12 may be exchangeable. δ_C 16.13 (C-26), 16.46 (C-25), 17.50 (C-27), 19.06 (C-2, C-6), 21.75 (C-24), 22.14 (C-12), 25.38 (C-11), 28.65 (C-16), 33.32 (C-20), 33.37 (C-15), 33.55 (C-4), 33.57 (C-23), 35.37 (C-7), 37.50 (C-10), 38.26 (C-19), 40.88 (C-1), 41.29 (C-8), 42.42 (C-3), 44.32 (C-17), 44.93 (C-13), 50.19 (C-14), 51.33 (C-9), 57.29 (C-5), 109.6 (C-28), 114.6 (C-22), 138.9 (C-21), 146.6 (C-18). Assignments of C-11 and C-12 or C-15 and C-20 are exchangeable. EIMS m/z 191 (100%), 205 (37), 299 (28), 341 (37), 396 (M^+, 36).

36 in C_6D_6. δ_H 0.94 (dd, J 12, 1.7, H-5), 0.95 (m, H-1), 0.99 (3H, s, Me-24), 1.02 (3H, s, Me-25), 1.05 (3H, s, Me-23), 1.12 (3H, s, Me-26), 1.18 (3H, d, J 7.3, Me-28), 1.30 (m, H-2), 1.33 (m, H-16), 1.33 (3H, s, Me-27), 1.38 (m, H-15), 1.40 (m, H-19), 1.41 (2H, m, H-6, H-11), 1.52 (m, H-2), 1.55 (2H, m, H-7), 1.58 (m, H-21), 1.60 (m, H-11), 1.63 (2H, m, H-9, H-16), 1.65 (2H, m, H-6, H-19), 1.74 (m, H-20), 1.80 (m, H-1), 1.84 (2H, m, H-20, H-21), 1.86 (m, H-15), 2.08 (m, H-12), 2.10 (m, H-18), 2.65 (dd, J 14.5, 3.3, H-12), 2.86 (m, H-22). δ_C 16.47 (C-25), 17.96 (C-20, C-26), 19.03 (C-2, C-6), 20.71 (C-28), 21.84 (C-11), 21.90 (C-24), 22.48 (C-27), 25.11 (C-12), 26.38 (C-21), 27.44 (C-19), 29.20 (C-16), 30.33 (C-22), 31.12 (C-15), 33.39 (C-4), 33.67 (C-23), 34.70 (C-18), 34.97 (C-7), 37.87 (C-10), 40.60 (C-1), 41.50 (C-8), 42.31 (C-3), 44.63 (C-14), 51.00 (C-9), 56.76 (C-5), 133.83 (C-13), 136.52 (C-17). EIMS m/z 176 (100), 191 (60), 231 (26), 243 (26), 367 (21), 382 (M^+, 50). $[\alpha]_D^{25}$ ($CHCl_3$) +37.6 (c = 0.012).

37 (solid) in C_6D_6. δ_H 0.88 (dd, J 12.0, 1.9, H-5), 0.93 (m, H-1), 0.94 (3H,s, Me-28), 0.99 (6H, s, Me-24, Me-25), 1.03 (3H, s, Me-23), 1.09 (3H, s, Me-26), 1.24 (3H,s, Me-27), 1.27 (m, H-17), 1.30 (2H, m, H-3, H-15), 1.37 (m, H-16), 1.38 (m, H-7), 1.40 (m, H-13), 1.43 (4H, m, H-6, H-9, H-11), 1.45 (m, H-16), 1.50 (m, H-2), 1.51 (m, H-3), 1.55 (m, H-15), 1.57 (m, H-7), 1.63 (m, H-6), 1.65 (2H, m, H-12), 1.69 (m, H-19), 1.73 (m, H-2), 1.78 (m, H-1), 1.83 (m, H-22), 1.92 (m, H-22), 2.07 (dd, J 17, 3

Hz, H-19), 5.74 (m, H-20), 5.84 (m, H-21). δ_C 13.34 (C-28), 16.27 (C-25), 16.29 (C-27), 16.64 (C-26), 19.05 (C-2), 19.07 (C-6), 21.46 (C-12), 21.82 (C-24), 21.94 (C-11), 25.96 (C-16), 30.38 (C-22), 31.45 (C-15), 33.42 (C-7), 33.59 (C-23), 33.59 (C-4), 35.30 (C-18), 37.63 (C-10), 40.63 (C-1), 42.09 (C-14), 42.12 (C-19), 42.22 (C-17), 42.22 (C-8), 42.40 (C-3), 47.86 (C-13), 50.85 (C-9), 59.58 (C-5), 126.18 (C-21), 126.41 (C-20). Assignments are interchangeable between C-25 and C-27, between C-2 and C-6, and between C-8 and C-14. EIMS *m/z* 161 (88%), 191 (100), 367 (55), 382 (M^+, 36). $[\alpha]_D^{25}$ ($CHCl_3$) +2.32 (c = 0.04).

38 (solid) in C_6D_6. δ_H 0.82 (m, H-19), 0.83 (3H, s, Me-28), 0.87 (dd, J 12.0, 1.6, H-5), 0.89 (m, H-1), 0.91 (m, H-17), 0.98 (3H, s, Me-25), 0.99 (3H,s, Me-24), 1.03 (3H, s, Me-23), 1.09 (6H, s, Me-26, Me-27), 1.22 (m, H-16), 1.27 (m, H-3), 1.28 (m, H-13), 1.31 (m, H-20), 1.36 (m, H-15), 1.37 (2H, m, H-11), 1.38 (m, H-9), 1.44 (2H, m, H-2), 1.46 (m, H-16), 1.48 (m, H-22), 1.49 (m, H-6), 1.51 (m, H-3), 1.52 (m, H-20), 1.57 (m, H-15), 1.63 (m, H-6), 1.64 (2H, m, H-12), 1.67 (m, H-19), 1.76 (m, H-1), 1.80 (m, H-22), 3.52 (dddd, J 11, 11, 5.1, 5.1, H-21). The chemical shifts of H-2 and H-6 may be interchangeable. δ_C 13.09 (C-28), 16.11 (C-25), 16.79 (C-26), 16.79 (C-27), 19.05 (C-6), 19.07 (C-2), 21.40 (C-12), 21.79 (C-24), 21.95 (C-11), 26.32 (C-16), 31.63 (C-22), 32.27 (C-7), 33.38 (C-15, C-4), 33.57 (C-23), 36.03 (C-18), 37.63 (C-10), 38.39 (C-20), 38.60 (C-19), 40.62 (C-1), 42.40 (C-3), 42.44 (C-8), 42.31 (C-14), 45.54 (C-17), 48.22 (C-13), 50.71 (C-9), 56.53 (C-5), 71.06 (C-21). Assignments of C-2 and C-6 or C-8 and C-14 are interchangeable. EIMS *m/z* 161, 385 (17%), 400 (M^+, 29). $[\alpha]_D^{25}$ (EtOH) +11.4 (c = 0.028).

39 (solid) in C_6D_6. δ_H 0.92 (m, H-5), 0.96 (m, H-1), 1.00 (3H, s, Me-24), 1.03 (3H, s, Me-25), 1.03 (3H, d, J 6.0, Me-29), 1.06 (3H, s, Me-23), 1.12 (3H, s, Me-26), 1.20 (3H, d, J 7.6, Me-28), 1.24 (m, H-21), 1.30 (m, H-3), 1.32 (3H, s, Me-27), 1.35 (m, H-16), 1.38 (m, H-15), 1.41 (2H, m, H-2, H-11), 1.42 (m, H-22), 1.51 (m, H-6), 1.53 (m, H-3), 1.55 (2H, m, H-7), 1.58 (m, H-20), 1.60 (m, H-11), 1.62 (m, H-9), 1.68 (m, H-6), 1.74 (m, H-16), 1.75 (m, H-15), 1.77 (m, H-2), 1.83 (2H, m, H-1, H-17), 1.88 (m, H-20), 1.99 (m, H-21), 2.10 (m, H-12), 2.67 (bdd, J 13, 3.5, H-12), 2.87 (m, H-19). δ_C 16.50 (C-25), 17.97 (C-26), 19.05 (C-2), 19.05 (C-6), 20.90 (C-29), 20.96 (C-28), 21.85 (C-11), 21.91 (C-24), 22.37 (C-27), 25.30 (C-12), 26.35 (C-20), 27.68 (C-21), 30.59 (C-19), 30.94 (C-15), 30.95 (C-16), 31.27 (C-22), 33.41 (C-4), 33.68 (C-23), 35.01 (C-7), 37.88 (C-10), 40.63 (C-1), 41.08 (C-17), 41.48 (C-8), 42.33 (C-3), 44.13 (C-14), 51.00 (C-9), 56.80 (C-5), 134.2 (C-13), 136.4 (C-18). EIMS *m/z* 175 (28%), 191 (100), 204 (36), 396 (M^+, 59). $[\alpha]_D^{25}$ ($CHCl_3$) +58.18 (c = 0.022).

40 (solid) in C_6D_6. δ_H 0.88 (dd, J 12.0, 2.0, H-5), 0.94 (m, H-1), 0.95 (3H,s, Me-25), 0.99 (3H, s, Me-24), 1.00 (3H, d, 6.4, Me-29), 1.03 (3H, s, Me-23), 1.06 (3H, s, Me-26), 1.08 (3H, s, Me-28), 1.14 (3H, s, Me-27), 1.15 (m, H-17), 1.28 (m, H-3), 1.30 (m, H-16), 1.35 (3H, m, H-7, H-15), 1.40 (m, H-2), 1.42 (m, H-11), 1.44 (dd, J 12.8, 2.4, H-9), 1.50 (2H, m, H-3, H-6), 1.52 (m, H-15), 1.55 (m, H-16), 1.62 (2H, m, H-12), 1.64 (m, H-2), 1.72 (m, H-21), 1.77 (m, H-6), 1.78 (m, H-1), 1.81 (m, H-22), 1.86 (m, H-11), 2.20 (m, H-21), 5.60 (m, H-20), 6.16 (bd, J 10.0, H-19). δ_C 16.12 (C-25), 16.76 (C-26), 17.15 (C-27), 18.46 (C-28), 19.06 (C-2), 19.06 (C-6), 20.03 (C-29), 20.82 (C-16), 21.42 (C-12), 21.79 (C-24), 21.88 (C-11), 27.86 (C-22), 32.76 (C-15), 33.32 (C-7), 33.40 (C-4), 33.60 (C-23), 36.20 (C-21), 37.59 (C-10), 38.63 (C-18), 40.54 (C-1), 42.18 (C-14), 42.31 (C-8), 42.40 (C-3), 44.40 (C-13), 50.80 (C-9), 51.42 (C-17), 56.55 (C-5), 123.5 (C-20), 138.7 (C-19). Assignments of C-2 and C-6 are interchangeable. EIMS *m/z* 175 (54), 191 (100%), 231 (23), 381 (28), 396 (M^+, 31). $[\alpha]_D^{25}$ ($CHCl_3$) +12.50 (c = 0.012).

41 (solid) in C_6D_6. δ_H 0.918 (3H, s, Me-28), 0.92 (m, H-1), 0.97 (m, H-5), 0.98 (3H, s, Me-26), 1.00 (3H, s, Me-24), 1.03 (3H, s, Me-25), 1.05 (3H, s, Me-23), 1.06 (t, J 7.4, Me-29), 1.16 (dd, J 12.1, 6.4, H-15), 1.28 (m, H-3), 1.31 (m, H-20), 1.35 (3H, s, Me-27), 1.38 (m, H-7), 1.42 (m, H-21), 1.51 (2H, m, H-2, H-6), 1.52 (m, H-3), 1.53 (m, H-7), 1.56 (m, H-19), 1.58 (m, H-22), 1.62 (m, H-1), 1.68 (m, H-16), 1.70 (2H, m, H-2, H-6), 1.82 (dd, J 12.1, 6.4, H-15), 1.86 (2H, m, H-16, H-19), 1.86 (m, H-19), 1.88 (m, H-22), 1.95 (m, H-11), 1.96 (m, H-9), 2.00 (m, H-20), 2.23 (m, H-11), 2.25 (m, H-18), 5.33 (bs, H-12). δ_C 13.60 (C-29), 15.46 (C-25), 16.77 (C-26), 18.75 (C-2), 18.79 (C-28), 19.31 (C-6), 21.79 (C-24), 22.45 (C-27), 23.26 (C-19), 23.69 (C-22), 23.92 (C-11), 25.08 (C-15), 28.14 (C-20), 32.68 (C-16), 33.08 (C-7), 33.26 (C-4), 33.62 (C-23), 38.19 (C-10), 39.83 (C-1), 39.88 (C-8), 40.08 (C-17), 42.39 (C-3, C-14), 48.61 (C-9), 52.68 (C-18), 55.22 (C-21), 56.92 (C-5), 118.4 (C-12), 145.3 (C-13). EIMS *m/z* 175 (62%), 190 (50), 204 (100), 341 (12), 368 (10), 381 (11), 396 (M^+, 18). $[\alpha]_D^{25}$ ($CHCl_3$) +17.35 (c = 0.017).

42 in C_6D_6. δ_H 0.847 (3H, s, Me-28), 0.87 (bd, J 12.2, H-5), 0.90 (m, H-1), 0.985 (3H, s, Me-25), 0.992 (3H, s, Me-24), 1.04 (3H, s, Me-23), 1.05 (m, H-19), 1.06 (3H, s, Me-27), 1.11 (3H, s, Me-26), 1.24 (m, H-17), 1.28 (m, H-3), 1.35 (2H, m, H-7, H-15), 1.38 (m, H-11), 1.40 (m, H-9), 1.46 (m, H-6), 1.47 (m, H-15), 1.48 (m, H-13), 1.49 (m, H-2), 1.52 (3H, m, H-16, H-3), 1.54 (2H, m, H-12), 1.58 (m, H-7), 1.63 (2H, m, H-6, H-11), 1.68 (m, H-19), 1.71 (m, H-2), 1.78 (2H, m, H-1, H-20), 2.07 (m, H-20), 2.71 (m, H-21), 5.09 (bd, J 8.4, H-29), 5.11 (bd, J 16.8, H-29). δ_C 16.10 (C-25), 16.13 (C-28), 16.95 (C-27), 16.97 (C-26), 19.07 (C-2, C-6), 21.09 (C-16), 21.22 (C-11), 21.83 (C-24), 24.37 (C-12), 29.14 (C-20), 33.43 (C-4), 33.46 (C-15), 33.62 (C-23), 33.67 (C-7), 37.65 (C-10), 40.59 (C-1), 42.04 (C-19), 42.17 (C-14), 42.38 (C-3), 42.45 (C-8), 44.59 (C-18), 44.92 (C-21), 49.32 (C-13), 50.76 (C-9), 54.94 (C-17), 56.47 (C-5), 113.55 (C-29), 143.6 (C-22). Assignments are interchangeable between C-25 and C-28, between C-26 and C-27, and between C-8 and C-14. EIMS *m/z* 175 (94%), 191 (100), 381 (14), 396 (M^+, 23). $[\alpha]_D^{25}$ ($CHCl_3$) +32.86 (c = 0.028).

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