Production of epoxydammaranes by the enzymatic reactions of (3R)- and (3S)-2,3-squalene diols and those of 2,3:22,23-dioxidosqualenes with recombinant squalene cyclase and the mechanistic insight into the polycyclization reactions[†]

Tsutomu Hoshino,* Yukie Yonemura, Takamasa Abe and Yumi Sugino

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The enzymatic cyclizations of (3R)- and (3S)-2,3-squalene diols by squalene cyclase afforded bicyclic compounds and epoxydamamranes in a *ca.* 3 : 2 ratio. Formation of the epoxydammarane scaffold indicates that a 6/6/6/5-fused tetracyclic cation is involved as the intermediate in the polycyclization reaction. 2,3:22,23-Dioxidosqualenes also afforded an epoxydammarane skeleton, *i.e.*, 3α - or 3β -hydroxyepoxydammaranes, but the amount of bicyclic compounds produced was markedly lower than that of the squalene diols, indicating that the larger steric bulk of the diols had a more significant influence on the polycyclization pathway than the smaller bulk of the expoxide. All the epoxydammaranes had 17R,20*R* stereochemistry except for one product, demonstrating that these analogs were folded into an *all-chair* conformation in the reaction cavity. The mechanistic insight into the observed stereochemical specificities indicated that the organized *all-chair* conformation is rigidly constricted by squalene cyclase and, thus, free conformational change is not allowed inside the reaction cavity; a small rotation of the hydroxyl group or the epoxide toward the intermediary cation gave a high yield of the enzymatic products, while a large rotation led to a low yield of the product. The stereochemistries of the generated epoxydammaranes are opposite to those from natural sources, and thus almost all of the enzymatic products described here are novel.

Introduction

Triterpenes are abundant in nature and confer important biological functions. Polycyclic triterpenes and steroid scaffolds are biosynthesized by ring-forming reactions (polycyclization) of the linear C_{30} molecules squalene 1 or 2,3-oxidosqualene.¹ The structural diversity of triterpene skeletons is remarkable;² lanosterol from vertebrates and fungi, cycloartenol and a, \beta-amyrin from plants, and hopene from prokaryotes are well known. The polycyclization reactions proceed with complete regio- and stereospecificity, leading to the formation of new C-C bonds and chiral centers; seven chiral centers and four C-C bonds for lanostane skeleton, and nine stereocenters and five C-C bonds for hopanoids. Site-directed mutations of squalene-hopene cyclase (SHC) from Alicyclobacillus acidocaldarius led to the early truncation of the polycyclization cascade^{1a,b} and/or to aberrant cyclization products whose stereochemistry was opposite to that of the normal cyclization intermediates, strongly indicating that the stereochemical result of the polycyclization cascade is directed by the steric bulk of the active site residues.³ We have isolated many

truncated cyclization products from various site-directed mutants in which the electronic and steric environments were altered.^{1b} Based on the number of rings of the isolated enzymatic products, *i.e.*, mono-, bi-, tri- and tetracyclic skeletons, we have proposed that the cyclization pathway of **1** to pentacyclic hopene **2** and hopanol **3** is as shown in Scheme 1.^{1b}

Carbocationic intermediates are involved in these multi-step reactions, and ring expansion processes occur $(7 \rightarrow 8 \text{ and } 9 \rightarrow 10)$ during hopene biosynthesis. By incorporating unnatural amino acids (fluorophenylalanines) into the catalytic sites,⁴ we have recently succeeded in providing strong evidence that the intermediary cations are stabilized by the π -electrons of the aromatic residues and that the ring enlargement process $(9 \rightarrow 10)$ takes place as a result of stabilization of the secondary cation 10 by the π -electrons of the Phe605 residue, that is, a cation- π interaction plays a key role in the catalytic mechanism.

Prokaryotic squalene cyclase (SHC) is of particular note from the aspect of molecular evolution, because it is believed that eukaryotic cyclases evolved from the prokaryotic SHC.⁵ Recently, we demonstrated that the substrate specificity of prokaryotic cyclase can be successfully altered into that of the eukaryotic type, which is specific to (3S)-2,3-oxidosqualene.⁶

In addition to the mutagenesis experiments, numerous studies on substrate analogs also have provided important information on the reaction mechanism and substrate recognition.^{7–15} The methyl group(s) on the squalene backbone have a crucial role in the normal polycyclization reaction.^{7–10} SHC tolerates a variety of carbon chain lengths (C_{15} – C_{35}) and analogs with aromatic

Department of Applied Biological Chemistry, Faculty of Agriculture, and Graduate School of Science and Technology, Niigata University, Ikarashi, Niigata, 950-2181, Japan. E-mail: hoshitsu@agr.niigata-u.ac.jp; Fax: +81-25-262-6854

[†] Electronic supplementary information (ESI) available: Analytical methods, incubation conditions, GC and HPLC details, syntheses of **18–22**, purification procedures for the enzymatic products, detailed 2D NMR analyses, and assignments of the minor peaks found in the reaction mixture of **20** and **21**. See DOI: 10.1039/b615897h



Scheme 1 Cyclization pathway of squalene 1 to pentacyclic hopene 2 and hopanol 3.

rings.¹¹⁻¹⁶ Previously, we reported trapping experiments of the cationic intermediates by using squalene analogs with a highly nucleophilic hydroxyl group (Fig. 1). The truncated analogs 12 (C_{22}) and 13 (C_{27}) were efficiently cyclized into heterocyclic skeletons with a 6/6/5 + tetrahydrofuran (THF) ring $23^{1b,3b}$ and a 6/6/6/5 + THF ring 24,^{1b,16} respectively (Fig. 2), demonstrating that carbocations 7 and 9 are involved in the polycyclization reaction of 1. In the preceding paper,¹⁷ we reported the enzymatic reactions of threo-squalene diols, e.g. 6,7-dihydroxysqualenes 14 and 15, and 10,11-dihydroxysqualenes 16 and 17 (Fig. 2). By employing diols 16 and 17, the monocyclic cation 5 and the bicyclic cation 6 were successfully trapped to give 26, 27, 28 and 29. Compound 26 has an octahydrochromene core, i.e., a 6/THP-fused bicycle (THP: tetrahydropyran), and 28 has a dodecahydrobenzo[*f*]chromene core (a 6/6/THP-fused tricycle). Through enzymatic experiments with 14 and 15, acyclic 4 and tricyclic cation 7 were trapped, affording 25 and 30; 30 has a 3deoxymalabaricol nucleus (acyclic cation 4 had never been trapped before). Thus, it can be now proposed that hopene biosynthesis consists of nine steps including acyclic cation 4, as shown in Scheme 1. Moreover, the enzymatic reactions of diols 14–17 were more or less product- and substrate-specific.17

Next, we examined the enzymatic reactions of (3S)-18, (3R)-2,3-diols 19 and dioxidosqualenes 20–22 in a series of trapping experiments of carbocation intermediates. These analogs were converted into 6/6-fused bicyclic compounds and/or epoxydamamaranes (these are pentacycles, *viz.* a 6/6/6/5-fused tetracycle and a THF or THP ring), which indicates that carbocation intermediate 9 is generated during hopene biosynthesis. The 17R,20R stereochemistry of epoxydamamranes produced from **18–22** was opposite to those from plant sources. Herein, we discuss the formation mechanisms of the epoxydammaranes by squalene cyclase.

Results and discussion

Preparation of squalene diols 18 and 19 and dioxidosqualenes 20–22

Methods for the synthesis of **18** and **19** are described in a previous paper.¹⁷ Treatment of **1** with the chiral ligand (DHQ)₂PHAL gave **14**, **16** and **18**, while treatment with (DHQD)₂PHAL afforded **15**, **17** and **19**. Isolation of **18** and **19** was easily achieved by SiO₂ column chromatography (eluting with a mixture of hexane and EtOAc), because the R_f values of **18** or **19** were lower than those of **14–17**. Reaction of **1** with an excess amount of NBS in THF gave a dibromohydrin, which, followed by treatment with K₂CO₃ in MeOH, gave rise to diastereomeric mixtures of diepoxides **20–22** (see ESI[†]).

Incubation of 18-23 with the wild-type SHC, and product profiles

Diols 18 and 19 (1.0 mg each) were separately incubated with the cell-free homogenates, which were prepared from an *E. coli* clone encoding the wild-type SHC. The incubations were carried out at optimal catalytic conditions (under which 1 was fully converted)





Fig. 1 Structures of squalene diols and dioxidosqualenes employed in the cation-trapping experiments.

into 2 and 3) and then terminated by adding methanolic KOH. The hexane extracts from the reaction mixture were subjected to short SiO_2 column chromatography with hexane-EtOAc (100 : 30) to remove an excess of Triton X-100, which was included in the incubation mixture. The lipophilic fraction thus prepared was subjected to GC analysis (Fig. 3). Four major products, 31, 32, 33 and 34, were detected from the reaction mixture of (3S)-diol 18, the yields being estimated as 22, 21, 5 and 25%, respectively, by GC analysis (Fig. 3A). The diol 18 was recovered in 27% yield. Fig. 3B shows that (3R)-diol 19 gave four products, 35, 36, 37 and 38, in yields of 33, 15, 18, and 12%, respectively, with unreacted 19 being recovered in 22% yield. After removing Triton X-100 from the incubation mixtures of diepoxides 20-22, the lipophilic materials were acetylated with Ac₂O/Py, and then submitted to GC analysis (Fig. 4A), which showed two major peaks, but HPLC analysis indicated that four acetate products, 39-42, were actually involved (Fig. 4B). Many small peaks (total amount 15%) were observed in the retention time region 14-28 min (Fig. 4A), but the amount of each product was small. The yields of 39, 40, 41 and 42, the total amount of minor products and recovered 20-22 was 13, 13, 23, 24, 15 and 12%, respectively. It should be noted that the conversions of diepoxides 20-22 were higher (88%, Fig. 4A) than those of diols 18 and 19 (ca. 73-78%, Fig. 3A and 3B).



Fig. 2 Enzymatic products of squalene analogs with highly nucleophilic hydroxyl group(s). Products **23** and **24** were generated from the truncated C_{22} -analog and the C_{27} -analog with a hydroxyl group, respectively.^{16,36,16} Products **25–30** were isolated from the reactions of *threo*-diols of squalene **14–17** with the wild-type SHC.¹⁷

Structures of enzymatic products 32-43

Products 31–42 were purified by SiO₂ column chromatography together with HPLC (see ESI[†]). The structures of the enzymatic products were determined by detailed NMR analyses, including DEPT, COSY, HOHAHA, NOESY, HMQC and HMBC. Diols 18 and 19 have eight methyl groups. For product 31, one methyl group was missing, and two vinyl protons appeared ($\delta_{\rm H}$ 5.10 and 4.84, each 1H, s). The terminal diol moiety remained unchanged. The presence of two allylic methyl groups and the terminal diol moiety, in conjunction with the detailed analyses of HMBC and NOESY spectra, indicated that 31 has the 6/6-fused bicyclic skeleton as shown in Fig. 5. The ¹H, ¹³C NMR and MS spectra were identical between 35 and 31 and between 32 and 36 (ESI⁺), indicating that the structures of 31 and 35 and those of 32 and 36 are identical except for the C-21 stereochemistry. The detailed HMBC analyses of 32 and 36 supported the 6/6-fused bicyclic nucleus, and the allylic methyl group at $\delta_{\rm H}$ 1.92 (Me-26) had HMBC cross-peaks with C-9, C-8 and C-7, verifying that a double bond is introduced between C-7 and C-8. The complete structures of 32 and 36 are illustrated in Fig. 5. Products 31 and 35 have an α -polypodatetraene core,¹⁸⁻²⁰ while those of **32** and **36** have a γ -polypodatetraene skeleton.^{18–20}

Products 33, 34, 37 and 38 had no olefinic proton or sp² carbon, suggesting that a full polycyclization reaction had occurred.





Fig. 3 GC traces of the reaction mixtures of diols 18 (A) and 19 (B) with the wild-type SHC. An excess of Triton X-100 included in the incubation mixture was removed by short SiO₂ column chromatography.

Detailed HMBC analyses confirmed that all the products had a 6/6/6/5-fused tetracycle. Products **33**, **34** and **37** had strong NOEs for Me-18/H-13/H-17, indicating a 17β-H orientation (17*R*). In contrast, product **39** had no NOE between H-13 and H-17, but a clear NOE between Me-30 and H-17, and thus an 17α-H (17*S*) stereochemistry was inferred. All products **33**, **34**, **37** and **38** showed three C–O carbons in the ¹³C NMR spectra, despite substrates **18** and **19** having only two C–O carbons, suggesting that the secondary or tertiary alcohol of **18** and **19** participated in the polycyclization reaction to afford either THF or THP rings.

Product **33** had an OH proton ($\delta_{\rm H}$ 2.73, br s, in acetone- $d_{6,0}$) that had no COSY cross-peak with any other proton, suggesting that the tertiary alcohol of **18** did not participate in the cyclization reaction, and that the secondary alcohol was responsible for the oxygen bridge, affording a five-membered THF ring. The HMBC cross-peak between Me-21 and C-17 supported that the THF ring was linked to C-17. The clear NOEs of Me-21/H-24 proved the *cis*-orientation between Me-21 and H-24, indicating that the C-24 stereochemistry of **33** must be *S*, because of the *S* stereochemistry of C-3 in **18**, thus leading to an assignment of 20*R* for **33**. Therefore, detailed 2D NMR analyses unambiguously showed that **33** is (17*R*,20*R*,24*S*)-20,24-epoxydammarane-25-ol (Fig. 5).

As for product **34**, the OH proton appeared as a broad doublet ($\delta_{\rm H}$ 3.78, br d, J 4.4) in acetone- d_6 , which correlated with H-24 in the COSY spectrum, strongly suggesting that the secondary alcoholic OH at C-3 of **18** remained intact; thus **35** possesses a six-membered THP ring. The coupling constant (ddd, J 11.5, 4.4, 4.4) of H-24 indicated an axial orientation. The strong NOEs of



Fig. 4 (A) Gas chromatogram of the reaction mixture obtained by incubating diastereomers 20–22, which was obtained after the lipophilic materials were acetylated with Ac₂O/Py. In addition to major two peaks, many small peaks appeared between 14 and 28 min. HPLC analysis (B) revealed that the peak at 34.6 min was a mixture of the acetates of 40 and 42, while the peak at 44.5 min was a mixture of the acetates of 39 and 41 (see Fig. 4B). Compounds 20–22 were recovered in 12% yield. The total amount of the minor products between 14 and 28 min was 15%. The amounts of the former and the latter major peaks were estimated to be 37% and 36%, respectively. (B) Normal phase HPLC profile of the enriched fraction of the acetates of 39–42. The distribution ratio of the acetates was as follows: 39 : 40 : 41 : 42 and recovered 20-22 = 1.08 : 1.92 : 1.08 : 2.0 : 1.



Fig. 5 Structures of all the enzymatic products from 18–22.

Me-27/Me-21, Me-21/H-23ax and H-24ax/H-22ax showed the axial disposition of Me-21, implying a 20*R*-stereochemistry. Thus, the complete structure of **34** was proposed to be (17R,20R,24S)-20,25-epoxydammarane-24-ol (Fig. 5). A 20,25-epoxydammarane nucleus involving a six-membered THP ring is very rare in nature. To the best of our knowledge, only two examples has been reported,^{21a-c} but the C-17 and C-20 stereochemistries are opposite to that of **34**, which is thus novel.

The involvement of THF ring in **37** was confirmed by the absence of a COSY correlation between the OH proton ($\delta_{\rm H}$ 2.79, br s) and H-24 in acetone- d_6 . In addition, the absence of an NOE between H-24 and Me-21 (implying a *trans* arrangement), indicated a 20*R*-stereochemistry for **37** due to the defined configuration (3*R*) of substrate **19**. The structure of **37** is therefore (17*R*,20*R*,24*R*)-20,24-epoxydammarane-25-ol (Fig. 5).

The involvement of THF ring in **38** was also confirmed by the absence of a COSY correlation between the OH proton ($\delta_{\rm H}$ 3.29, br s) and H-24. A strong NOE between Me-21 and H-24, in conjunction with the 3*R* stereochemistry of substrate **19**, supported the proposal for 20*S*,24*R* stereochemistry. Thus, the complete structure of **38** was determined to be (17*S*,20*S*,24*R*)-20,24-epoxydammarane-25-ol (Fig. 5), *i.e.*, 3-deoxyocotillol, which has been isolated from *Pyrrosia lingua*.²² The ¹H and ¹³C NMR data of **38** were in full accordance with those published in the literature²² (see ESI[†]).

The acetates of products **39–42** had one acetyl group ($\delta_{\rm H}$ 1.98– 2.00, 3H, acetone- d_6) and a 6/6/6/5-fused tetracyclic skeleton, which was revealed by HMBC analysis. The C-17 stereochemistries of all the **39–42** acetates were shown to be *R* (17β-H) due to a clear NOE between H-13 and H-17. The H-3 signal ($\delta_{\rm H}$ 4.48, acetone d_6) of the acetates of **39** and **41** was a double doublet (*J* 11.1, 5.1), indicating the β-orientation of 3-OAc, while H-3 of the acetates of **40** and **42** was a broad singlet ($\delta_{\rm H}$ 4.56, acetone- d_6); thus 3-OAc of **40** and **42** was in an α -disposition. Three C–O carbon signals other than 3-OAc were detected in the ¹³C NMR spectra of all the 39-42-acetates. The OH protons of the 39-42-acetates were detected as broad singlets in acetone- d_6 , and showed no correlation with any other proton in the COSY spectra, in contrast to 34. Thus, the 39-42-acetates contain a tertiary alcohol (i.e., a THF ring), which agrees with the fact that attempted acetylation of a tertiary alcohol with Ac₂O/Py at room temperature does not occur. As shown in Table 1, Me-21 and H-24 had the same chemical shifts in the **39–42**-acetates ($\delta_{\rm H}$ 1.18–1.19 and 3.71–3.72, respectively), but the splitting pattern of H-24 was different and was classified into two categories: t, J 7.3 for 39-acetate and 40acetate; and dd, J 10.5, 5.2 for 41 and 42 acetates. The former is identical to that of 33, while the latter is the same as that of 37. A strong NOE for H-24/Me-21 for 39-acetate and 40-acetate indicated a cis-orientation between them, while the absence of an NOE for H-24/Me-21 for 41-acetate and 42-acetates revealed a trans-geometry. When the ¹³C chemical shifts of 39-42-acetates were compared with each other, the $\delta_{\rm C}$ difference of C-24 was remarkable (Table 1): $\delta_{\rm C}$ 84.6 for the acetates of 33, 39and 40, but $\delta_{\rm C}$ 88.2–88.3 for the acetates of **37**, **41** and **42**. These findings strongly indicate that the THF rings of 39-acetate and 40-acetate have the same 20R,24S stereochemistry as that of 33, while 41acetate and 42-acetate possessed the same 20R,24R-configuration as 37. The structures of 39-42 are depicted in Fig. 5: (17R,20R, 24S)-20,24-epoxydammarane-3β,25-diol for 39; (17R,20R,24S)-20,24-epoxydammarane-3α,25-diol for **40**; (17*R*,20*R*,24*R*)-20,24epoxydammarane-3β,25-diol for 41; and (17R,20R,24R)-20,24epoxydammarane-3α,25-diol for 42.

EI-MS spectra of all the epoxydammaranes showed ion m/z 143 as a base peak (see ESI†) corresponding to the 2-(5-methyltetrahydrofuran-2-yl)propan-2-ol moiety,^{21b,23} which is characteristic of the epoxydammarane nucleus (see ESI). The minor products, which appeared at a retention time of 14–28 min in the GC (Fig. 4A), were presumed to mainly consist of 6/6-fused bicyclic and 6/6/5-fused tricyclic compounds, because the EI-MS showed fragment ion m/z 189 and 229 suggestive of the bi- and tricyclic compounds (see ESI).

Table 1 Chemical shifts of products 33, 34 and 37–42 in acetone- d_6^a

Position	33 (20 <i>R</i> ,24 <i>S</i>)	34 (THP ring)	37 (20 <i>R</i> ,24 <i>R</i>)	38 (20 <i>S</i> ,24 <i>R</i>)	39 (20 <i>R</i> ,24 <i>S</i>)	40 (20 <i>R</i> ,24 <i>S</i>)	41 (20 <i>R</i> ,24 <i>R</i>)	42 (20 <i>R</i> ,24 <i>R</i>)
C-13	44.36	44.83	44.53	43.56	44.41	44.36	44.63	44.59
C-14	49.95	49.74	49.80	50.78	49.89	49.94	49.77	49.83
C-15	33.13	33.11	33.08	32.09	33.17	33.13	33.14	33.11
C-16	27.28	27.71	28.25	26.33	27.27	27.25	28.27	28.23
C-17	49.20	51.41	49.30	50.56	49.19	49.16	49.33	49.31
C-20	85.75	75.83	86.01	86.68	85.75	85.75	86.01	86.03
C-21	25.21	27.53	28.87	23.45	25.19	25.23	28.89	28.90
C-22	38.45	35.05	38.65	36.36	38.43	38.44	38.64	38.68
C-23	26.20	25.89	26.37	26.33	26.18	26.19	26.36	26.38
C-24	84.60	75.36	88.16	84.21	84.59	84.63	88.24	88.28
C-25	71.62	75.83	70.44	71.49	71.60	71.61	70.39	70.43
C-26	26.66	22.49	25.84	26.73	26.71	26.71	25.82	25.79
C-27	26.33	30.49	27.28	26.29	26.32	26.34	27.36	27.34
H-21(Me)	1.183	1.284	1.188	1.222	1.183	1.191	1.189	1.193
H-24	3.72 (t, J =	$3.28 (\mathrm{ddd}, J =$	$3.71 (\mathrm{dd}, J =$	3.71 (t, J =	3.72 (t, J =	3.72 (t, J =	3.71 (dd, J =	$3.72 (\mathrm{dd}, J =$
	7.3 Hz)	11.5, 4.4, 4.4 Hz)	10.5, 5.2 Hz)	7.3 Hz)	7.3 Hz)	7.2 Hz)	10.6, 5.2 Hz)	10.6, 5.3 Hz)

^{*a*} The ¹³C chemical shift differences between 33, 39 and 40, and those between 37, 41 and 42 are negligible, indicating that the C-20 and C-24 stereochemistries of 39 and 40 are the same as that for 33, while those for 41 and 42 are identical to that for 37. In contrast, the chemical shifts of 38 are different from those of 33, 37 and 39–42, reflecting the different stereochemistries, (20R, 24S) and (20R, 24R). The ¹H and ¹³C NMR data of 34 are markedly different from 33 and 37–42 (which have a THF ring), especially with respect to the $\delta_{\rm C}$ of C-20 and C-24, suggesting the involvement of a THP ring.

Mechanism of formation of products 32-43

Diols 18 and 19 gave bicyclic products via cation 43 in yields of 43% and 48%, respectively (Fig. 3A and B), but diepoxides 20-22 afforded very small or marginal amounts of bi- and tricyclic products (Fig. 4A). This difference could be ascribed to the steric bulk difference of the substituents, which had an additional influence on the conversion ratios of the substrates; the recovered yield of diols 18 and 19 (22-27%) were higher than those of epoxides 20-22 (12%) (compare Fig. 3 with Fig. 4A). In previous work,10,17 we demonstrated that the cyclization yields depends on the bulk sizes of the substituents on the squalene backbone. The somewhat larger bulk would have led to termination of the polycyclization cascade at the bicyclic stage (6, see Scheme 1), possibly due to poor positioning of 18 or 19 inside the reaction cavity. As shown in Scheme 2A, deprotonation of Me-26 in cation 43 could give 31 and 35 (path a), while that of H-7 could afford **32** and **36** (path b). A portion of the diol substrates (30%) underwent further cyclization reactions to give the 6/6/6/5-fused tetracyclic cations 44-46 (like 9), which were then trapped by the highly nucleophilic hydroxyl group, leading to pentacycles 33 and 34 from 18, and to pentacycles 37 and 38 from 19. An all-chair conformation of 18 in the reaction cavity led to cationic intermediate 44, with 17β -H (17*R*) configuration (Scheme 2B). According to Baldwin's rule,²⁴ formation of the five-membered THF ring is generally preferred to that of the six-membered THP ring, but production of 34 (THP ring) was higher than 33 (THF ring) by a factor of 5 (Fig. 3A). The explanation for this inconsistency is as follows. The equatorial C-3 OH of 44 must rotate (by ca. 180°) through the C-3-C-4 bond to afford 33 (path a), while the C-2 OH of 45 could readily access cation 44 to give 34 by a smaller motion of C-2 OH through the C-2-C-3 bond ($<60^{\circ}$) (path b), thus leading to higher production of 34. It should be highlighted that the C-20 and C-24 stereochemistries of 33 and 34 predicted from the C-C bond rotation (Scheme 2B) were in good accordance with those determined independently by NMR analyses. Scheme 2C depicts the cyclization pathway of 19 into pentacycles 37 and 38. Intermediary cation 45 (like 9) was produced through the folding of an *all-chair* structure, leading to a 17β-H configuration. The re-face attack of axially oriented 3*R*-OH upon the cation by a small motion (*ca.* 60°) through the C-3-C-4 bond afforded 37 (18%) with 20R,24R stereochemistry (path a). On the other hand, the folding of **19** into a *chair-chair*chair-boat conformation with an extended side chain could give cation 46 with the 17a-H stereochemistry (12%). The si-face attack of 3R-OH on 46 could give 38 with 20S,24R stereochemistry (path b). In conformation 46, the axially oriented OH had a repulsive interaction with the E-ring formation site of the cyclase cavity, which could guide the disorganization of a chair structure for the D-ring to some extent, thus giving rise to the formation of an extended side chain to prevent the repulsive interaction. Furthermore, the unfavorable 1,3-diaxial interactions of Me-30/Me-21/Me-27 occur in the all-chair conformation 45, but there is little repulsion in 46.4,7,10 Therefore, the organization of chairchair-chair-boat conformation 46 would be more favorable than that of all-chair conformation 45, but the amount of 37 produced was ca. 1.5 times higher than that of 38. SHC cyclase could still confer an *all-chair* structure to 19 despite these unfavorable interactions. In the case of 44, where the OH is equatorial, the



Scheme 2 Cyclization mechanisms and folding conformations of squalene diols 18 and 19. (A) Formation mechanism of the 6/6-fused bicyclic products 31 and 32 from 18, and that of the bicycle 35 and 36 from 19. (B) The cyclization mechanisms of 18 into 33 and 34. (C) The cyclization pathways of 19 into 37 and 38. Plain carbon labels: squalene diol numbering. Underlined carbon labels: dammarane numbering. An *all-chair* conformation leads to the formation of (17*R*)-epoxydammaranes 33, 34 and 37, while a *chair-chair-chair-boat* conformation leads to (17*S*)-epoxydammarane 38.

repulsive interaction with the cyclase cavity would be minimal, and thus epoxydammarane with 17α -H (like **38**) was not produced from **18**.

(3S)-2,3-Oxidosqualene is converted into 3 β -hydroxyhopene through an *all-chair* conformation by SHC, while the 3*R* form

is converted into 3a-hydroxyhopene through a *boat-chair-chair*chair-chair conformation.7 Each of the diastereomers 20-22 also underwent the polycyclization reaction to afford the 6/6/6/5fused tetracyclic cation, with a 17β-H configuration. Scheme 3 shows the polycyclization mechanism of 20-22. Diepoxides (3S,22S)-20 and (3S,22R)-21 could be organized into an all-chair conformation by SHC. The re-face attack of the 22-epoxide on to the tetracyclic cation could provide the new tertiary cation after the THF ring formation. A water molecule attacked the cation to afford 39 and 41 in yields of 13% and 23%, respectively. A large rotation of the (22S)-epoxide (ca. 180°) is required to form **39**, but a small motion (*ca.* 60°) of the (22*R*)-epoxide is enough to produce 41, thus leading to ca. two-fold higher production of 41. Diepoxides (3R,22R)-22 and (3R,22S)-21 were folded into a boat-chair-chair-chair conformation to give 42 (24%) and 40 (13%), respectively. A higher production of 42 could be explained in terms of the smaller motion of the (22R)-epoxide of 22.



Scheme 3 Cyclization mechanism and folding conformations of dioxidosqualenes 20–22.

It is likely that diol and epoxide groups donate or accept hydrogen atoms to form hydrogen bonds with the cyclase, and this may have an influence on the conformations of substrates adopted in the reaction cavity. However, the distribution of yields and the stereochemistries of the products can be best explained in terms of the angles of motion of the nucleophilic oxygen atoms and/or the conformation of the substrates, as discussed above. Thus the steric factor (*viz.* the repulsive interaction with the cyclase), would be the dominant effect on the polycyclization pathway; the electronic effect (hydrogen bond formation) on the conformational structure would be small in this case. This idea is in good agreement with our earlier paper.¹⁷

Conclusions

In this study, carbocation intermediate 9 was successfully trapped by the nucleophilic oxygen atom of squalene diols and diepoxides, resulting in the production of epoxydammaranes. In the incubation mixtures of 18-22, dammarene-type triterpenes (such as 20(21)-, 13(17)- and 12(13)-dammarenes and 7(8)-euphene that are generated from deprotonation reactions at the corresponding positions) were not detected. This finding strongly indicates that the folding of the side chain of 9 into a chair structure quickly occurs prior to the 1,2-hydride shift of 17β-H to the C-20 cation to give these dammarene skeletons, resulting in the proximity of the oxygen atom(s) to the C-20 cation of 9, preferentially affording epoxydammaranes. Previous experiments with 6,7- and 10,11-diols have provided the definitive evidence that cations 4, 5, 6, 7 are involved as intermediates during the polycyclization cascade of 1.17 Thus, we have now succeeded in trapping all the tertiary cations 4, 5, 6, 7 and 9 shown in Scheme 1. The trapping of secondary cations 8 and 10 have failed, suggesting that the lifetime of these secondary cations, formed after the ring-expansion of 7 and 9, is short. A recent study on Arabidopsis lupeol synthase showed that the 6/6/6/6-fused secondary baccharenyl cation is actually involved in the polycyclization reaction;²⁵ (3S, 22S)-2,3;22,23-dioxidosqualene was converted into the following three products: (17R,20R,24S)-17,24-epoxybaccharane-3β,25diol. (17S, 20S, 24R)-20,24-epoxydammarane-3 β ,25-diol and (17S,20S,24S)-20,24-epoxydammarane-3β,25-diol in a 3 : 4 : 2 ratio.²⁵ This simultaneous trapping of the secondary and the tertiary cations in a high yield unambiguously demonstrates that lupeol biosynthesis occurs by a ring enlargement process. It should be noted that all of the enzymatic products, except for **38**, have 17β -H (17*R*) and 20*R* stereochemistry. Plant triterpenes are usually biosynthesized via a 17a-H (17S) dammarenyl cation, formed by the folding into a *chair-chair-chair-boat* conformation. This conformation usually leads to 20S stereochemistry, as depicted in 46 (Scheme 3C). Some papers have referred to the isolation of (20R)-epoxydamamranes from natural sources, but it was recently suggested that there is no documented evidence for the 20R stereochemistry,²⁵ implying that the C-20 stereochemistry of natural epoxydammaranes is actually S (see the Supporting Information of ref. 25). On the other hand, prokaryotic hopene is generated via a 17-epi-dammarenyl cation 9 (with 17β -H (17R) stereochemistry), which is formed by the folding of an *all-chair* conformation, leading to a (17R,20R)-epoxydammaranes, as seen in 33, 34, 37 and 39-42 produced from cations 44 and 45. Thus, all the epoxydammaranes described here are novel except for 3-deoxyocotillol 38. Squalene cyclase has a great potential for creating unnatural natural terpenoids, in addition to one-pot syntheses of natural products, by the enzymatic reactions of numerous squalene analogs.

Experimental

Please see the ESI for additional experimental data.†

NMR and EI-MS spectroscopic data of enzymatic products 31-42

Products 33, 34, 37 and 38 were measured in both C_6D_6 and acetone- d_6 . NMR data of 39–42-monoacetates were acquired in both CDCl₃ and acetone- d_6 .

Product 31. Oil. ¹H NMR (C₆D₆, 600.13 MHz), δ 5.46 (2H, br s, H-17 and H-13), 5.10 (br s, H-26), 4.84 (br s, H-26), 3.35 (dd, 10.4, J 1.8, H-21), 2.52 (m, H-7), 2.45 (m, H-12), 2.44 (m, H-19), 2.34 (2H, dt, J 7.6, 7.6, H-16), 2.26 (2H, t, J 7.8, H-15), 2.21 (m, H-19), 2.15 (m, H-12), 2.11 (m, H-7), 1.83 (m, H-1), 1.78 (m, H-9), 1.770 (3H, s, H-27), 1.75 (m, H-6), 1.725 (3H, s, H-28), 1.69 (m, H-11), 1.66 (m, H-2), 1.62 (m, H-11), 1.61 (m, H-20), 1.55 (m, H-2), 1.50 (m, H-20), 1.49 (m, H-3), 1.41 (m, H-6), 1.27 (m, H-3), 1.154 (3H, s, H-30), 1.148 (3H, s, H-29), 1.14 (m, H-5), 1.08 (m, H-1), 0.965 (3H, s, H-23), 0.921 (3H, s, H-24), 0.868 (3H, s, H-25). ¹³C NMR (C₆D₆, 150.9 MHz), δ 148.87 (s, C-8), 135.29 (s, C-18), 134.95 (s, C-14), 125.80 (d, C-13), 125.13 (d, C-17), 106.66 (t, C-26), 78.28 (d, C-21), 72.56 (s, C-22), 56.52 (d, C-9), 55.65 (d, C-5), 42.43 (t, C-3), 40.21 (t, C-15), 39.31 (t, C-1), 39.84 (s, C-10), 38.73 (t, C-7), 37.24 (t, C-19), 33.66 (s, C-4), 33.72 (q, C-23), 30.13 (t, C-20), 27.43 (t, C-12), 27.13 (t, C-16), 26.40 (q, C-29), 24.76 (t, C-6), 24.24 (t, C-11), 23.58 (q, C-30), 21.89 (q, C-24), 19.76 (t, C-2), 16.16 (q, C-27), 16.06 (q, C-28), 14.75 (q, C-25). The assignments of H-29, H-30, C-29 and C-30 are exchangeable. EI-MS *m/z* (%): 69 (50), 81 (100), 95 (53), 109 (40), 135 (34), 137 (42), 153 (30), 189 (13), 191 (23), 411 (11), 426 (10), 444 (5, M⁺). HR-EI-MS: m/z (M⁺), calcd. for C₃₀H₅₂O₂, 444.3967; found, 444.3972. [a]_D²⁵ = +79.6 (c 0.40, EtOH).

Product 32. Oil. ¹H NMR (C₆D₆, 600.13 MHz), δ 5.58 (br s, H-7), 5.46 (br t, J 6.8, H-17), 5.45 (br t, J 6.8, H-13), 3.34 (br d, J 9.5, H-21), 2.45 (m, H-19), 2.42 (m, H-12), 2.34 (2H, m, H-16), 2.25 (2H, t, J 7.4), 2.21 (2H, m, H-19 and H-12), 2.06 (m, H-6), 1.99 (m, H-6), 1.98 (m, H-1), 1.916 (3H, s, H-26), 1.79 (m, H-9), 1.773 (3H, s, H-27), 1.731 (3H, s, H-28), 1.65 (2H, m, H-2 and H-11), 1.61 (m, H-20), 1.55 (m, H-2), 1.51 (m, H-3), 1.50 (m, H-20), 1.44 (m, H-11), 1.32 (dd, J 11.9, 4.9, H-5), 1.27 (ddd, J 14,13.9, 3.9, H-3), 1.150 (3H, s, H-30), 1.145 (3H, s, H-29), 1.08 (m, H-1), 1.000 (3H, s, H-24), 0.980 (3H, s, H-23), 0.955 (3H, s, H-25). The assignments of H-29 and H-3 are exchangeable. ¹³C NMR (C_6D_6 , 150.9 MHz), δ 135.39 (s, C-8), 135.34 (s, C-18), 134.89 (s, C-14), 125.52 (d, C-13), 125.07 (d, C-17), 122.54 (d, C-7), 78.28 (t, C-21), 72.54 (s, C-22), 54.57 (d, C-9), 50.37 (d, C-5), 42.64 (t, C-3), 40.17 (t, C-15), 39.49 (t, C-1), 37.23 (t, C-19), 37.02 (s, C-10), 33.35 (q, C-23), 33.08 (s, C-4), 30.76 (t, C-12), 30.14 (t, C-20), 27.74 (t, C-11), 27.09 (t, C-16), 26.39 (q, C-29), 24.20 (t, C-6), 23.58 (q, C-30), 22.46 (q, C-26), 22.03 (q, C-24), 19.21 (t, C-2), 16.24 (q, C-27), 16.06 (q, C-28), 13.77 (q, C-25). The assignments of C-8 and C-18 and those of C-29 and C-30 are exchangeable. EI-MS m/z (%): 69 (50), 81 (100), 95 (53), 109 (58), 135 (34), 153 (42), 189 (42), 191 (54), 204 (38), 411 (6), 426 (7), 444 (5, M⁺). HR-EI-MS: *m*/*z* (M⁺), calcd. for $C_{30}H_{52}O_2$, 444.3967; found, 444.3966. $[a]_D^{25} = -33.3$ (c 0.41, EtOH).

Product 33. Oil. ¹H NMR (C₆D₆, 600.13 MHz), δ 3.74 (t, J 7.3, H-24), 2.16 (m, H-17), 2.07 (m, H-13), 1.93 (m, H-12), 1.91

(m, H-23), 1.82 (m, H-16), 1.75 (m, H-1), 1.74 (2H, m, H-23 and H-22), 1.74 (m, H-22), 1.68 (m, H-7), 1.66 (2H, m, H-6 and H-11), 1.61 (m, H-16), 1.58 (m, H-15), 1.55 (m, H-12), 1.52 (m, H-3), 1.50 (2H, m, H-2 and H-6), 1.48 (m, H-22), 1.47 (m, H-9), 1.448 (3H, s, H-27), 1.38 (2H, m, H-2 and H-7), 1.30 (m, H-3), 1.276 (3H, s, H-26), 1.273 (3H, s, H-21), 1.22(m, H-15), 1.20 (m, H-11), 1.151(3H, s, H-30), 1.15 (3H, s, H-18), 1.039 (3H, s, H-28), 1.01 (3H, s, H-19), 0.998 (3H, s, H-29), 0.919 (br d, J 12.5, H-5), 0.87 (m, H-1). The assignments of H-2 and H-6 and those of H-26 and H-27 are exchangeable. ¹³C NMR (C₆D₆, 150.9 MHz), δ 85.31 (s, C-20), 83.98 (d, C-24), 71.28 (s, C-25), 57.29 (d, C-5), 51.15 (d, C-9), 49.43 (s, C-14), 48.66 (d, C-17), 43.81 (2C, t for C-3 and d for C-13), 41.17 (s, C-8), 40.81 (t, C-1), 38.22 (t, C-22), 37.69 (s, C-10), 35.63 (t, C-7), 33.63 (q, C-28), 33.54 (s, C-4), 32.72 (t, C-15), 27.78 (q, C-27), 27.08 (t, C-16), 26.21 (t, C-12), 25.79 (t, C-23), 25.29 (q, C-26), 25.23 (q, C-21), 22.65 (t, C-11), 21.76 (q, C-29), 19.12 (t, C-2), 19.08 (t, C-6), 17.20 (q, C-30), 16.47 (q, C-19), 16.13 (q, C-18). The assignments of C-2 and C-6 and those of C-26 and C-27 are exchangeable. EI-MS m/z (%): 69 (10), 81 (11), 95 (11), 125 (15), 143 (100), 191 (10), 385 (10), 429 (3, M⁺ – Me). HR-EI-MS: m/z (M⁺ – Me), calcd. for C₂₉H₄₉O₂, 429.3733; found, 429.3730. $[a]_{D}^{25} = -72.5$ (c 0.090, EtOH).

Product 34. Solid. ¹H NMR (acetone- d_6 , 600.13 MHz) δ 3.78 (very broad d, J 4.4, OH), 3.28 (ddd, J 11.5, 4.4, 4.4, H-24), 2.04 (m, H-13), 2.02 (m, H-12), 1.93 (ddd, J 9.3, 8.5, 7.8, H-17), 1.78 (m, eq, H-23), 1.68 (m, H-22 ax), 1.64 (m, H-1), 1.62 (m, H-2), 1.60 (m, H-23 ax), 1.59 (2H, m, H-7 and H-16), 1.56 (2H, m, H-11 and H-12), 1.51 (m, H-6), 1.44(m, H-9), 1.41 (m, H-2), 1.40 (m, H-16), 1.39 (m, H-6), 1.38 (m, H-15), 1.35(m, H-3), 1.32 (m, H-22eq.), 1.284 (3H, s, H-21), 1.23 (m, H-7), 1.179 (3H, s, H-27), 1.17 (m, H-11), 1.151 (3H, s, H-26), 1.15 (m, H-3), 1.034 (3H, s, H-30), 1.03(m, H-15), 0.946 (3H, s, H-18), 0.87 (m, H-1), 0.853 (3H, s, H-19), 0.845 (3H, s, H-28), 0.82(m, H-5), 0.813(3H, s, H-29). ¹³C NMR (acetone- d_6 , 150.9 MHz), δ 75.83 (2C, s, C-20 and C-25), 75.36 (d, C-24), 57.80 (d, C-5), 51.62 (d, C-9), 51.41 (d, C-17), 49.74 (s, C-14), 44.83 (d, C-13), 42.89 (t, C-3), 41.61 (s, C-8), 41.33 (t, C-1), 38.14 (s, C-10), 36.07 (t, C-7), 35.05 (t, C-22), 33.93 (s, C-4), 33.75 (q, C-28), 33.11 (t, C-15), 30.49 (q, C-27), 27.71 (t, C-16), 27.53 (q, C-21), 26.95 (t, C-12), 25.89 (t, C-23), 23.22 (t, C-11), 22.49 (q, C-26), 21.83 (q, C-29), 19.21 (t, C-2), 19.12 (t, C-6), 17.29 (q, C-30), 16.61 (q, C-19), 16.28 (q, C-18). The assignments of C-2 and C-6 are exchangeable. ¹H NMR (C₆D₆, 600.13 MHz) δ 3.22 (dd, J 11.5, 4.4, H-24), 2.18(m, H-12), 2.14 (m, H-13), 1.91 (m, H-17), 1.78 (3H, m, H-1, H-6 and H-7), 1.77 (m, H-12), 1.75 (m, H-2), 1.74 (2H, m, H-11 and H-16), 1.68 (2H, m, H-22 and H-23eq), 1.66 (m, H-16), 1.62 (m, H-9), 1.58 (m, H-14), 1.55 (m, H-6), 1.52 (m, H-3), 1.50 (m, H-2), 1.46 (m, H-23ax), 1.44 (m, H-7), 1.419 (3H, s, H-27), 1.35 (m, H-11), 1.336 (3H, s, H-21), 1.32 (3H, s, H-26), 1.30 (m, H-14), 1.29 (3H, s, H-30), 1.28 (m, H-3), 1.22 (m, H-22), 1.123 (3H, s, H-18), 1.03 (6H, s, H-19 and H-28), 1.004 (3H, s, H-29), 0.973 (dd, J 12.0, 2.4, H-5), 0.94 (m, H-1). The assignments of H-2 and H-6 in C₆D₆ are exchangeable. 13 C NMR (C₆D₆, 150.9 MHz) δ 13 C NMR (C₆D₆, 150.9 MHz), δ 75.32 (s for C-20, d for C-24), 75.07 (s, C-25), 57.42 (d, C-5), 51.86 (d, C-17), 51.17 (d, C-9), 49.38 (s, C-14), 44.33 (d, C-13), 42.54 (t, C-3), 41.23 (s, C-8), 40.89 (t, C-1), 37.74 (s, C-10), 35.78 (t, C-7), 34.63 (t, C-22), 33.65 (q, C-28), 33.56 (s, C-4), 32.84 (t, C-15), 30.11 (q, C-27), 27.49 (t, C-16), 27.25 (q, C-21), 26.58 (t, C-12), 25.57 (t, C-23), 22.88 (t, C-11), 22.02 (q, C-26), 21.78 (q, C-29), 19.21 (t, C-2), 19.12 (t, C-6), 17.14 (q, C-30), 16.49 (q, C-19), 16.19 (q, C-18). The assignments of H-2 and H-6 in C₆D₆ are exchangeable. EI-MS: m/z (%): 69 (10), 81 (11), 95 (11), 125 (15), 143 (100), 191 (10), 345 (5), 386 (4), 429 (4, M⁺ – Me). HR-EI-MS: m/z (M⁺ – Me), calcd. for C₂₉H₄₉O₂, 429.3733; found, 429.3741. $[a]_D^{25} = +55.3$ (c 0.09, EtOH).

Product 35. Oil. The NMR signals in C_6D_6 were identical to those of product **31**. EI-MS m/z (%): 69 (58), 81 (100), 95 (53), 109 (41), 135 (34), 137 (43), 153 (30), 189 (13), 191 (28), 411 (12), 426 (10), 444 (5, M⁺). HR-EI-MS: m/z (M⁺), calcd. for $C_{30}H_{52}O_2$, 444.3967; found, 444.3961. $[a]_D^{25} = +22.8$ (*c* 0.28, EtOH).

Product 36. Oil. The NMR signals were the same as those of product **32**. EI-MS: m/z (%): 69 (52), 81 (100), 95 (47), 109 (53), 135 (33), 153 (33), 189 (35), 191 (62), 204 (33), 411 (5), 426 (5), 444 (4, M⁺). HR-EI-MS: m/z (M⁺), calcd. for C₃₀H₅₂O₂, 444.3967; found, 444.3979. $[a]_{D}^{25} = +4.08$ (*c* 0.29, EtOH).

Product 37. Oil. ¹H NMR (C_6D_6 , 600.13 MHz), δ 3.67 (dd, J 10.5, 5.0, H-24), 2.17(m, H-17), 2.10 (ddd, J 11.5, 10.8, 3.2, H-13), 1.96 (2H, m, H-12 and H-23), 1.85 (m, H-22), 1.82 (m, H-16), 1.78 (m, H-7), 1.77 (m, H-1), 1.72 (m, H-2), 1.70 (m, H-11), 1.63 (m, H-6), 1.61 (m, H-16), 1.57 (2H, m, H-9 and H-15), 1.56 (2H, m, H-12 and H-23), 1.54 (m, H-3), 1.52 (2H, m, H-2 and H-6), 1.48 (m, H-22), 1.43(m, H-7), 1.405 (3H, s, H-27), 1.31(m, H-3), 1.26(2H, m, H-11 and H-15), 1.231 (6H, s, H-21 and H-26), 1.196 (3H, s, H-30), 1.110 (3H, s, H-18), 1.048 (3H, s, H-28), 1.027(3H, s, H-19), 1.008 (3H, s, H-29), 0.96 (2H, m for H-1; br d, J 12.4 for H-5). The assignment of H-2 and H-6 and that of H-26 and H-27 are exchangeable. ¹³C NMR (C_6D_6 , 150.9 MHz), δ 87.59 (d, C-24), 85.51 (s, C-20), 69.83 (s, C-25), 57.36 (d, C-5), 51.15 (d, C-9), 49.36 (s, C-14), 48.73 (d, C-17), 44.03 (d, C-13), 42.48 (t, C-3), 41.20 (s, C-8), 40.89 (t, C-1), 38.28 (t, C-22), 37.74 (s, C-10), 35.73 (t, C-7), 33.68 (q, C-28), 33.55 (s, C-4), 32.76 (t, C-15), 28.65 (q, C-21), 28.36 (q, C-27), 27.99 (t, C-16), 26.48 (t, C-23), 25.80 (t, C-12), 24.64 (q, C-26), 22.76 (t, C-11), 21.78 (q, C-29), 19.16 (t, C-2), 19.10 (t, C-6), 16.65 (q, C-30), 16.49 (q, C-19), 16.15 (q, C-18). The assignments of C-2 and C-6 and those of C-26 and C-27 are exchangeable. EI-MS m/z (%): 69 (10), 81 (11), 95 (11), 125 (15), 143 (100), 191 (8), 385 (10), 429 (2, M⁺ – Me). HR-EI-MS: m/z $(M^+ - Me)$, calcd. for $C_{29}H_{49}O_2$, 429.3733; found, 429.3717. $[a]_D^{25} =$ -22.8 (c 0.38, EtOH).

Product 38 (3-deoxycotillol). Solid. ¹H NMR (C₆D₆, 600.13 MHz), δ 3.75 (t, J 7.3, H-24), 1.98 (m, H-12), 1.94 (m, H-17), 1.92 (m, H-23), 1.91 (m, H-16), 1.79 (m, H-22), 1.77 (m, H-13), 1.73 (m, H-2), 1.72 (m, H-23), 1.70 (m, H-1), 1.68 (m, H-7), 1.66 (m, H-16), 1.64 (2H, m, H-6 and H-11), 1.60 (m, H-15), 1.55 (m, H-22), 1.52 (m, H-2), 1.50 (m, H-3), 1.48 (2H, m, H-6 and H-9), 1.434 (3H, s, H-27), 1.38 (m, H-7), 1.33 (m, H-12), 1.30 (m, H-11), 1.28 (m, H-3), 1.263 (3H, s, H-26), 1.222 (3H, s, H-21), 1.18 (m, H-15), 1.111 (3H, s, H-18), 1.029 (3H, s, H-28), 0.987 (3H, s, H-30), 0.982 (3H, s, H-29), 0.972 (3H, s, H-19), 0.909 (dd, J 13.4, 2.6, H-5), 0.873 (m, H-1). The assignments of H-26 and H-27 and those of H-29 and H-30 are exchangeable. ¹³C NMR (C_6D_6 , 150.9 MHz), δ 86.27 (s, C-20), 83.69 (d, C-24), 71.08 (s, C-25), 57.31 (d, C-5), 51.30 (d, C-9), 50.37 (s, C-14), 50.04 (d, C-17), 43.25 (d, C-13), 42.43 (t, C-3), 40.85 (2C, t for C-1; s for C-8), 37.70 (s, C-10), 36.19 (t, C-22), 35.64 (t, C-7), 33.63 (q, C-28), 33.52 (s, C-4), 31.80

(t, C-15), 27.83 (t, C-12), 27.77 (q, C-27), 26.26 (t, C-16), 26.02 (t, C-23), 25.04 (q, C-26), 23.66 (q, C-21), 21.76 (2C, t for C-11; q for C-29), 19.05 (t, C-2), 18.97 (t, C-6), 16.70 (q, C-30), 16.45 (q, C-19), 15.69 (q, C-18). The NMR data for product **39** in CDCl₃ are described in the ESI and are identical to those in the literature.^{22a} EI-MS m/z (%): 69 (16), 81 (15), 95 (15), 125 (15), 143 (100), 191 (13), 385 (20), 429 (3, M⁺ – Me). HR-EI-MS: m/z (M⁺ – Me), calcd. for C₂₉H₄₉O₂, 429.3733; found, 429.3717. [a]_D²⁵ = +35.9 (c 0.04, EtOH). cf. lit.^{21a} [a]_D = +19.3 (c 0.04, EtOH).

Product 39-acetate. Solid. ¹H NMR (acetone- d_6 , 600.13 MHz), δ 4.43 (dd, J 11.3, 5.0, H-3), 3.72 (t, J 7.3, H-24), 2.73 (br s, OH), 2.25 (m, H-17), 2.11 (m, H-13), 1.979 (3H, s, CH₃CO), 1.90 (m, H-12), 1.86 (m, H-23), 1.81 (m, H-23), 1.73 (m, H-16), 1.71 (m, H-22), 1.68 (m, H-1), 1.63 (m, H-16), 1.62 (2H, m, H-2), 1.61 (m, H-7), 1.57 (m, H-11), 1.55 (m, H-22), 1.52 (m, H-6), 1.47 (m, H-6), 1.46 (2H, m, H-12 and H-9), 1.45 (m, H-15), 1.26 (m, H-7), 1.21 (m, H-11), 1.183 (3H, s, H-21), 1.13 (3H, s, H-27), 1.09 (3H, s, H-26), 1.08 (m, H-15), 1.06 (m, H-1), 1.030 (3H, s, H-30), 0.971 (3H, s, H-18), 0.91 (dd, J 11.7, 2.5, H-5), 0.887 (3H, s, H-19), 0.852 (3H, s, H-29), 0.841 (3H, s, H-28). ¹³C NMR (acetone-d₆, 150.9 MHz), δ 170.9 (s, CH₃CO), 85.75 (s, C-20), 84.59 (d, C-24), 80.99 (d, C-3), 71.60 (s, C-25), 55.63 (d, C-5), 51.39 (d, C-9), 49.89 (s, C-14), 49.19 (d, C-17), 44.41 (d, C-13), 41.43 (s, C-8), 39.33 (t, C-1), 38.53 (s, C-10), 38.43 (t, C-22), 37.79 (s, C-4), 35.96 (t, C-7), 33.17 (t, C-15), 28.24 (q, C-28), 27.27 (t, C-16), 26.71 (q, C-26), 26.58 (t, C-12), 26.32 (q, C-27), 26.18 (t, C-23), 25.19 (q, C-21), 24.42 (t, C-2), 23.22 (t, C-11), 21.04 (q, CH₃CO), 18.98 (t, C-6), 17.37 (q, C-30), 16.83 (q, C-19), 16.65 (q, C-29), 16.19 (q, C-18). The assignments of C-19 and C-29 and those of C-26 and C-27 are exchangeable. The NMR data in CDCl₃ are given in the ESI. EI-MS *m*/*z* (%): 125 (38), 143 (100), 189 (13), 191 (35), 383 (45, M⁺ – $C_5H_{11}O_3$), 443 (10, M⁺ – C(Me)₂OH), 487 (5, M⁺ – Me). HR-EI-MS: m/z (M⁺ – C₅H₁₁O₃), calcd. for C₂₇H₄₃O, 383.3314; found, 383.3304. $[a]_{D}^{25} = -5.92$ (*c* 0.17, EtOH).

Product 40-acetate. Oil. ¹H NMR (acetone- d_6 , 600.13 MHz), δ 4.56 (br s, H-3), 3.72 (t, J 7.2, H-24), 2.79 (br s, OH), 2.25 (m, H-17), 2.11 (m, H-13), 2.00 (3H, s, CH₃CO), 1.90 (3H, m, H-2, H-12 and H-23), 1.82 (m, H-23), 1.74 (2H, m, H-16 and H-22), 1.63 (m, H-16), 1.62 (m, H-7), 1.58 (2H, m, H-11 and H-22), 1.50 (2H, m, H-2 and H-9), 1.46 (m, H-12), 1.45 (3H, m, H-6 and H-15), 1.43 (m, H-1), 1.31 (m, H-5), 1.27 (m, H-7), 1.23 (m, H-1), 1.21 (m, H-11), 1.191 (3H, s, H-21), 1.133 (3H, s, H-27), 1.08 (m, H-15), 1.054 (3H, s, H-26), 1.044 (3H, s, H-30), 0.980 (3H, s, H-18), 0.898 (3H, s, H-29), 0.893 (3H, s, H-19), 0.832 (3H, s, H-28). The chemical shifts of H-19 and H-29 and those of H-26 and H-27 are exchangeable. ¹³C NMR (acetone- d_6 , 150.9 MHz), δ 170.5 (s, CH₃CO), 85.75 (s, C-20), 84.63 (d, C-24), 78.42 (d, C-3), 71.61 (s, C-25), 51.63 (d, C-5), 51.41 (d, C-9), 49.94 (s, C-14), 49.16 (d, C-17), 44.36 (d, C-13), 41.59 (s, C-8), 38.44 (t, C-22), 37.89 (s, C-10), 37.41 (s, C-4), 35.92 (t, C-7), 34.99 (t, C-1), 33.13 (t, C-15), 28.27 (q, C-28), 27.25 (t, C-16), 26.71 (q, C-26), 26.58 (t, C-12), 26.34 (q, C-27), 26.19 (t, C-23), 25.23 (q, C-21), 23.53 (t, C-2), 23.04 (t, C-11), 22.05 (q, C-29), 21.10 (q, CH₃CO), 19.40 (q, C-19), 18.89 (t, C-6), 17.50 (q, C-30), 16.23 (q, C-18). The signals of C-26 and C-27 are exchangeable. The NMR data in CDCl₃ are given in the ESI. EI-MS m/z (%): 125 (35), 143 (100), 189 (10), 191 (24), 383 $(40, M^{+} - C_5H_{11}O_3), 443 (5, M^{+} - C(Me)_2OH), 487 (5, M^{+} - Me).$ HR-EI-MS: m/z (M⁺ – C₃H₁₁O₃), calcd. for C₂₇H₄₃O, 383.3314; found, 383.3314. $[a]_{D}^{25} = -13.27$ (*c* 0.11, EtOH).

Product 41-acetate. Solid. ¹H NMR (acetone- d_6 , 600.13 MHz), & 4.43 (dd, J 11.4, 5.0, H-3), 3.71 (dd, J 10.6, 5.2, H-24), 2.78 (br s, OH), 2.25 (m, H-17), 2.08 (m, H-13), 1.980 (3H, s, CH₃CO), 1.94 (m, H-12), 1.92 (m, H-22), 1.83 (m, H-23), 1.77 (m, H-16), 1.74 (m, H-23), 1.70 (m, H-1), 1.64 (m, H-7), 1.61 (2H, m, H-2), 1.57 (m, H-22), 1.56 (m, H-11), 1.55 (m, H-6), 1.53 (2H, m, H-12 and H-16), 1.48 (m, H-6), 1.47 (m, H-9), 1.43 (m, H-15), 1.28 (m, H-7), 1.21 (m, H-11), 1.189 (3H, s, H-21), 1.134 (3H, s, H-27), 1.08 (m, H-1), 1.07 (m, H-15), 1.049 (3H, s, H-26), 1.031 (3H, s, H-30), 0.974 (3H, s, H-18), 0.915 (dd, J 11.6, 2.3, H-5), 0.889 (3H, s, H-19), 0.855 (3H, s, H-29), 0.843 (3H, s, H-28). The assignments of H-26 and H-27 are exchangeable. ¹³C NMR (acetone-d₆, 150.9 MHz), δ 170.67 (s, CH₃CO), 88.24 (d, C-24), 86.01 (s, C-20), 80.99 (d, C-3), 70.39 (s, C-25), 55.65 (d, C-5), 51.39 (d, C-9), 49.77 (s, C-14), 49.33 (d, C-17), 44.63 (d, C-13), 41.46 (s, C-8), 39.35 (t, C-1), 38.64 (t, C-22), 38.53 (s, C-4), 37.79 (s, C-10), 35.99 (t, C-7), 33.14 (t, C-15), 28.89 (q, C-21), 28.27 (t, C-16), 28.25 (q, C-28), 27.36 (q, C-27), 26.75 (t, C-12), 26.36 (t, C-23), 25.82 (q, C-26), 24.44 (t, C-2), 23.33 (t, C-11), 21.05 (q, CH₃CO), 19.00 (t, C-6), 16.88 (q, C-29), 16.84 (q, C-30), 16.66 (q, C-19), 16.21 (q, C-18). The chemical shifts of C-26 and C-27 and those of C-29 and C-30 are exchangeable. The NMR data in CDCl3 are given in the ESI. EI-MS m/z (%): 125 (55), 143 (100), 189 (13), 191 (35), 383 (48, M⁺ – $C_5H_{11}O_3$, 443 (10, M⁺ – C(Me)₂OH), 487 (5, M⁺ – Me). HR-EI-MS: m/z (M⁺ – C₅H₁₁O₃), calcd. for C₂₇H₄₃O, 383.3314; found, 383.3309. $[a]_{D}^{25} = +11.38 (c \ 0.27, EtOH).$

Product 42-acetate. Oil. ¹H NMR (acetone- d_6 , 600.13 MHz), δ 4.56 (br s, H-3), 3.72 (dd, J 10.6, 5.3, H-24), 2.73 (br s, OH), 2.25 (m, H-17), 2.11 (m, H-13), 2.00 (3H, s, CH₃CO), 1.94 (m, H-12), 1.92 (m, H-22), 1.90 (m, H-2), 1.85 (m, H-23), 1.76 (2H, m, H-16 and H-23), 1.63 (m, H-7), 1.58 (m, H-11), 1.57 (m, H-22), 1.54 (m, H-16), 1.53 (m, H-12), 1.51 (2H, m, H-2 and H-9), 1.45 (2H, m, H-6), 1.44 (m, H-15), 1.43 (m, H-1), 1.31 (m, H-5), 1.27 (m, H-7), 1.22 (2H, m, H-1 and H-11), 1.193 (3H, s, H-21), 1.139 (3H, s, H-27), 1.07 (m, H-15), 1.056 (3H, s, H-26), 1.046 (3H, s, H-30), 0.981 (3H, s, H-18), 0.898 (3H, s, H-29), 0.895 (3H, s, H-19), 0.832 (3H, s, H-28). The assignments of H-26 and H-27 are exchangeable. ¹³C NMR (acetone- d_6 , 150.9 MHz), δ 170.5 (s, CH₃CO), 88.28 (d, C-24), 86.03 (s, C-20), 78.43 (d, C-3), 70.43 (s, C-25), 51.64 (d, C-5), 51.40 (d, C-9), 49.83 (s, C-14), 49.31 (d, C-17), 44.59 (d, C-13), 41.63 (s, C-8), 38.68 (t, C-22), 37.89 (s, C-10), 37.41 (s, C-4), 35.95 (t, C-7), 34.99 (t, C-1), 33.11 (t, C-15), 28.90 (q, C-21), 28.27 (q, C-28), 28.23 (t, C-16), 27.34 (q, C-27), 26.76 (t, C-12), 26.38 (t, C-23), 25.79 (q, C-26), 23.54 (t, C-2), 23.14 (t, C-11), 22.06 (q, C-29), 21.10 (q, CH₃CO), 18.91 (t, C-6), 17.00 (q, C-30), 16.41 (q, C-19), 16.25 (q, C-18). The assignments of C-26 and C-27 are exchangeable. The NMR data in CDCl_3 are given in the ESI. EI-MS m/z (%): 125 (63), 143 (100), 189 (13), 191 (30), 383 (52, M⁺ – $C_5H_{11}O_3$), 443 (5, M⁺ – C(Me)₂OH), 487 (3, M⁺ – Me). HR-EI-MS: m/z (M⁺ – C₅H₁₁O₃), calcd. for C₂₇H₄₃O, 383.3314; found, 383.3306. $[a]_{D}^{25} = -17.3$ (*c* 0.36, EtOH).

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