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

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The enzymatic cyclizations of (3*R*)- and (3*S*)-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 17*R*,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 α,β-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** → **8** and **9** → **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** → **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 (3*S*)-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

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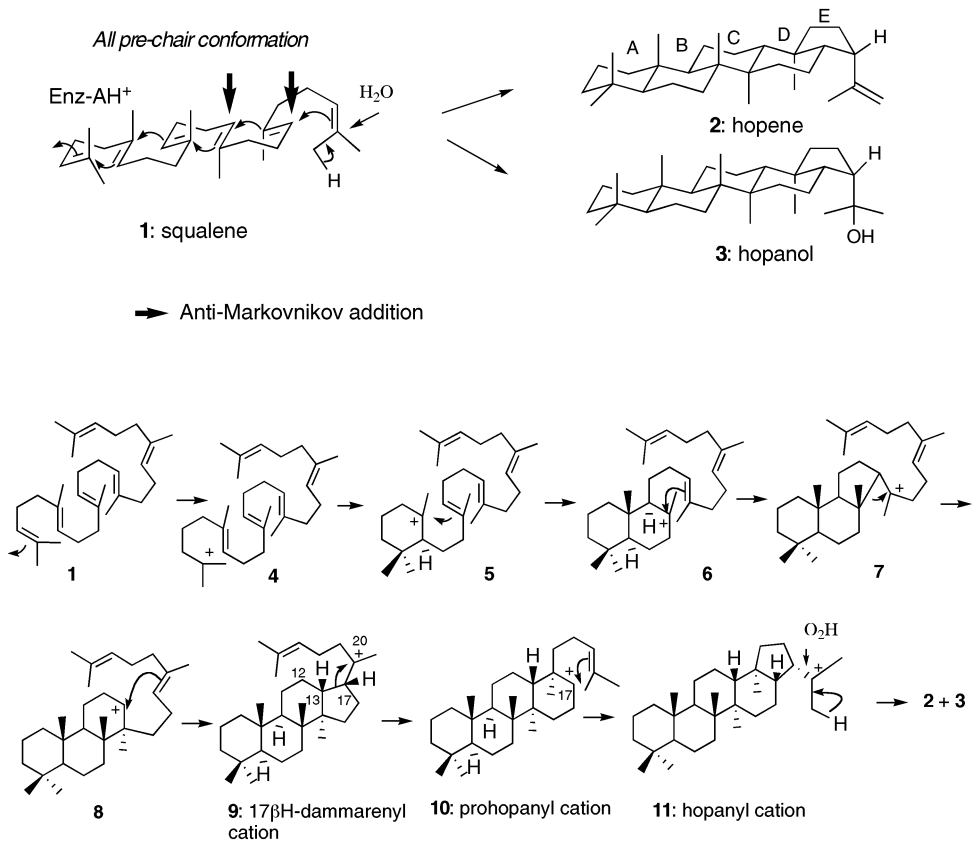
† Electronic supplementary information (ESI) available: Analytical methods, incubation conditions, GC and HPLC details, syntheses of **18**–**22**, purification procedures for the enzymatic products, detailed 2D NMR analyses, and assignments of the minor peaks found in the reaction mixture of **20** 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 3-deoxymalabaricol 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 (3*S*)-**18**, (3*R*)-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 17*R*,20*R* 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 SiO_2 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 K_2CO_3 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

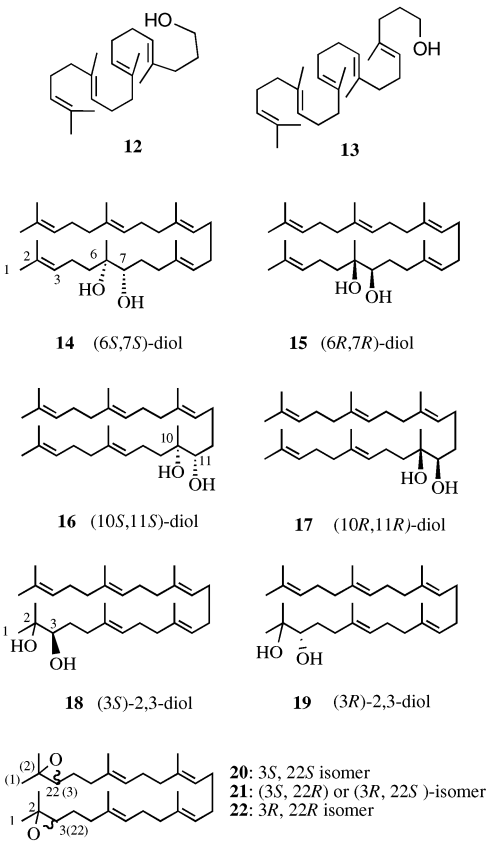


Fig. 1 Structures of squalene diols and dioxidosqualenes employed in the cation-trapping experiments.

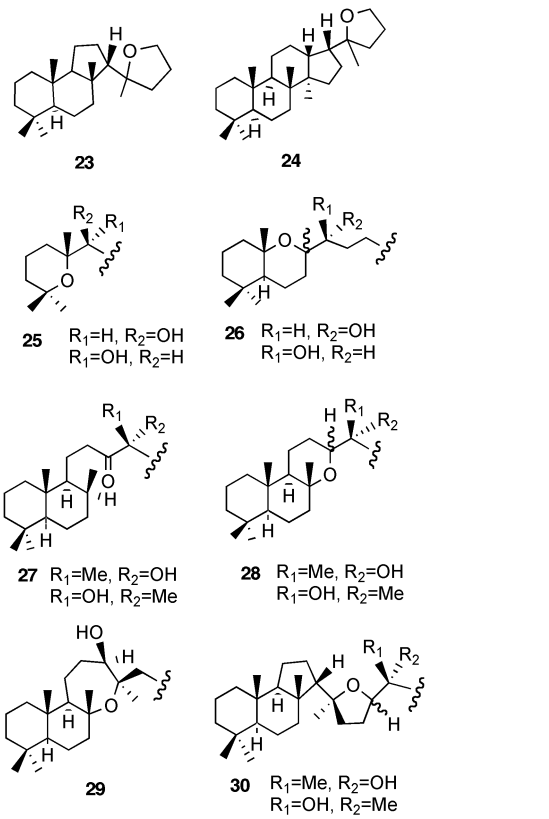


Fig. 2 Enzymatic products of squalene analogs with highly nucleophilic hydroxyl group(s). Products **23** and **24** were generated from the truncated C_{22}-analog and the C_{27}-analog with a hydroxyl group, respectively.[1b,3b,16] Products **25–30** were isolated from the reactions of *threo*-diols of squalene **14–17** with the wild-type SHC.[17]

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 (3*S*)-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 (3*R*)-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 Ac_2O/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).

Structures of enzymatic products 32–43

Products **31–42** were purified by SiO_2 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 (δ_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 ^{1}H, ^{13}C 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 δ_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 sp^2 carbon, suggesting that a full polycyclization reaction had occurred.

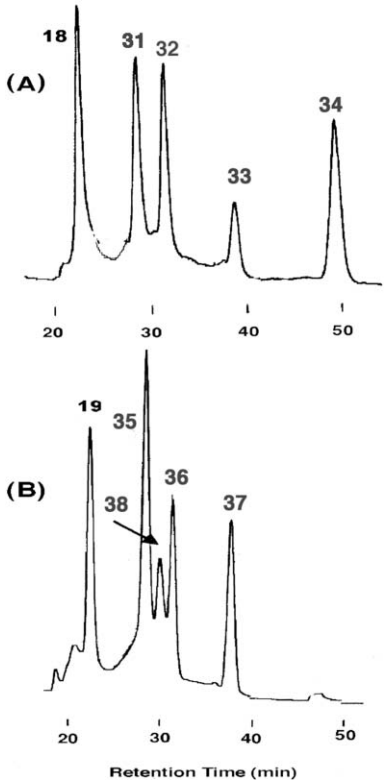


Fig. 3 GC traces of the reaction mixtures of diols **18** (A) and **19** (B) with the wild-type SHC. An excess of Triton X-100 included in the incubation mixture was removed by short SiO_2 column chromatography.

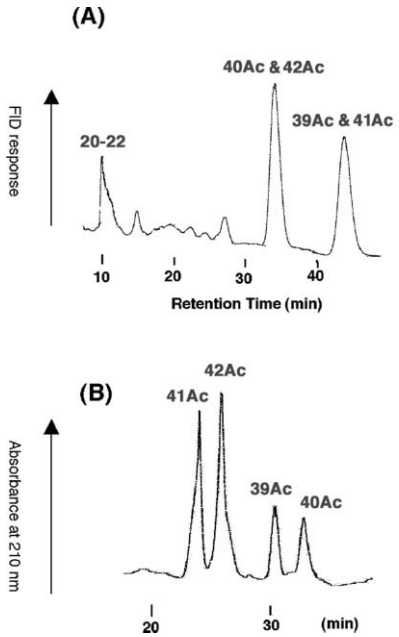


Fig. 4 (A) Gas chromatogram of the reaction mixture obtained by incubating diastereomers **20–22**, which was obtained after the lipophilic materials were acetylated with Ac_2O/Py. In addition to major two peaks, many small peaks appeared between 14 and 28 min. HPLC analysis (B) revealed that the peak 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.

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 ^{13}C 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 O*H* proton (δ_H 2.73, br s, in acetone-d_6,) 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 O*H* proton appeared as a broad doublet (δ_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

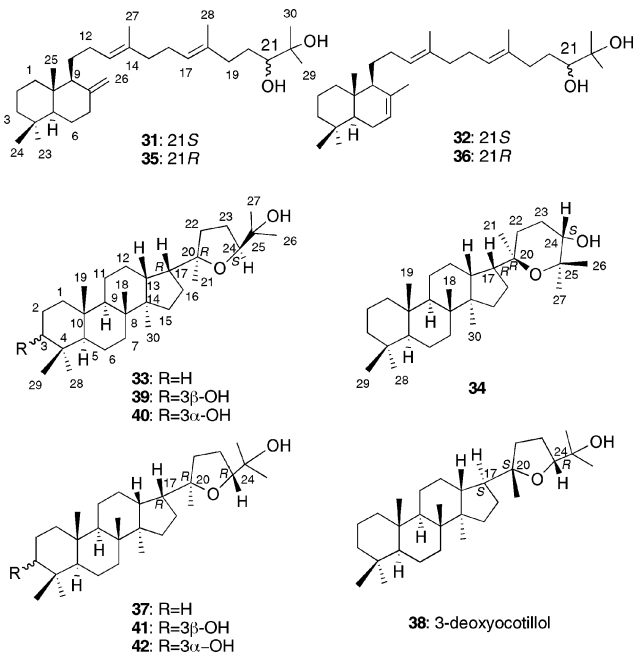


Fig. 5 Structures of all the enzymatic products from **18–22**.

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 (17*R*,20*R*,24*S*)-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 O*H* proton (δ_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 O*H* proton (δ_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 ^{1}H and ^{13}C NMR data of **38** were in full accordance with those published in the literature[22] (see ESI†).

The acetates of products **39–42** had one acetyl group (δ_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 (δ_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 (δ_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 ^{13}C NMR spectra of all the **39–42**-acetates. The O*H* 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 Ac_2O/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 (δ_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 **40**-acetate; 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 ^{13}C chemical shifts of **39–42**-acetates were compared with each other, the δ_C difference of C-24 was remarkable (Table 1): δ_C 84.6 for the acetates of **33**, **39**and **40**, but δ_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 20*R*,24*S* stereochemistry as that of **33**, while **41**-acetate and **42**-acetate possessed the same 20*R*,24*R*-configuration as **37**. The structures of **39–42** are depicted in Fig. 5: (17*R*,20*R*,24*S*)-20,24-epoxydammarane-3β,25-diol for **39**; (17*R*,20*R*,24*S*)-20,24-epoxydammarane-3α,25-diol for **40**; (17*R*,20*R*,24*R*)-20,24-epoxydammarane-3β,25-diol for **41**; and (17*R*,20*R*,24*R*)-20,24-epoxydammarane-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).

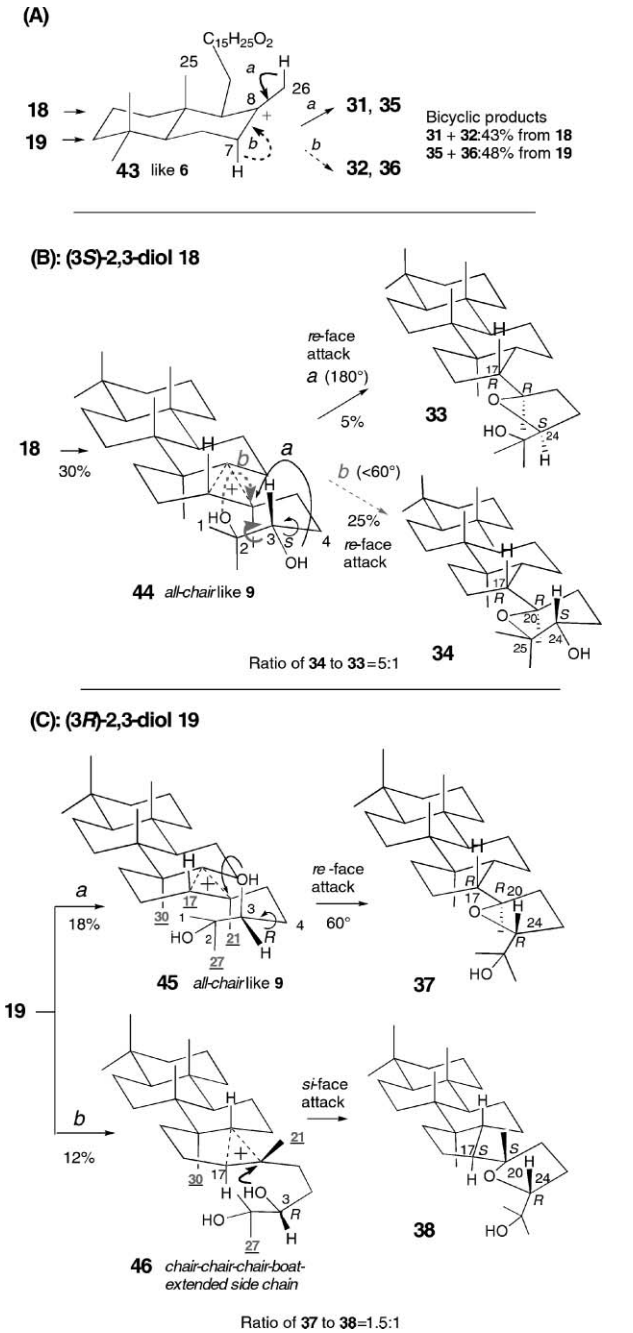
Table 1 Chemical shifts of products **33**, **34** and **37–42** in acetone-d_6[a]

Position	**33** (20*R*,24*S*)	**34** (THP ring)	**37** (20*R*,24*R*)	**38** (20*S*,24*R*)	**39** (20*R*,24*S*)	**40** (20*R*,24*S*)	**41** (20*R*,24*R*)	**42** (20*R*,24*R*)
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* = 7.3 Hz)	3.28 (ddd, *J* = 11.5, 4.4, 4.4 Hz)	3.71 (dd, *J* = 10.5, 5.2 Hz)	3.71 (t, *J* = 7.3 Hz)	3.72 (t, *J* = 7.3 Hz)	3.72 (t, *J* = 7.2 Hz)	3.71 (dd, *J* = 10.6, 5.2 Hz)	3.72 (dd, *J* = 10.6, 5.3 Hz)

[a] The ^{13}C 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, (20*R*,24*S*) and (20*R*,24*R*). The ^{1}H and ^{13}C NMR data of **34** are markedly different from **33** and **37–42** (which have a THF ring), especially with respect to the δ_C of C-20 and C-24, suggesting the involvement of a THP ring.

Mechanism of formation of products 32–43

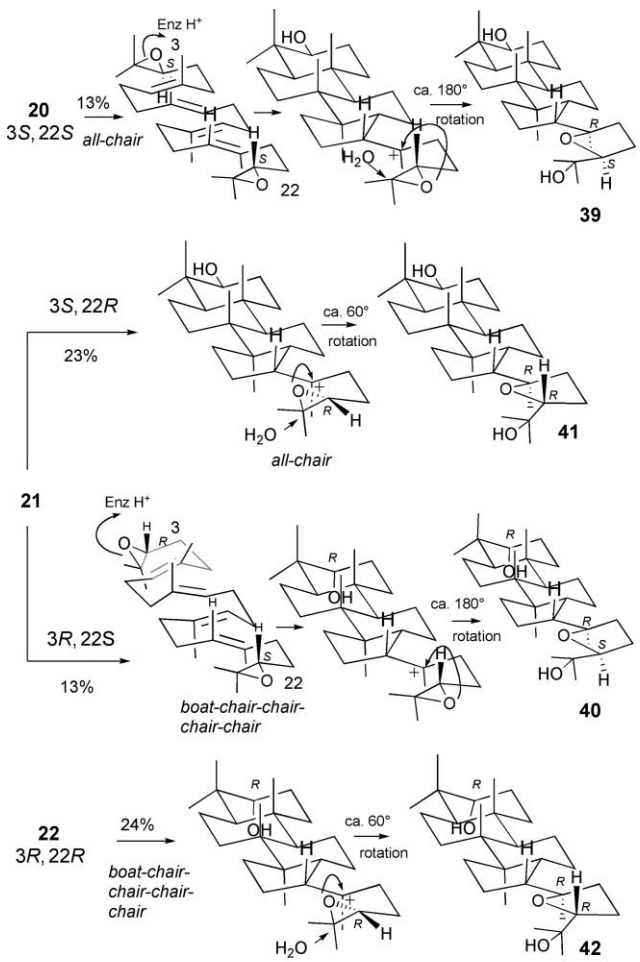
Diols **18** and **19** gave bicyclic products *via* cation **43** in yields of 43% and 48%, respectively (Fig. 3A and B), but diepoxides **20–22** afforded very small or marginal amounts of bi- and tricyclic products (Fig. 4A). This difference could be ascribed to the steric bulk difference of the substituents, 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°) (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 20*R*,24*R* 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 17α-H stereochemistry (12%). The *si*-face attack of 3*R*-OH on **46** could give **38** with 20*S*,24*R* 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 *chair-chair-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 repulsive interaction with the cyclase cavity would be minimal, and thus epoxydammarane with 17α-H (like **38**) was not produced from **18**.



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**.

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

is converted into 3α-hydroxyhopene through a *boat-chair-chair-chair-chair* conformation.[7] Each of the diastereomers **20–22** also underwent the polycyclization reaction to afford the 6/6/6/5-fused tetracyclic cation, with a 17β-H configuration. Scheme 3 shows the polycyclization mechanism of **20–22**. Diepoxides (3*S*,22*S*)-**20** and (3*S*,22*R*)-**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 (22*S*)-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 (3*R*,22*R*)-**22** and (3*R*,22*S*)-**21** were folded into a *boat-chair-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 (22*R*)-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] (3*S*,22*S*)-2,3;22,23-dioxidosqualene was converted into the following three products: (17*R*,20*R*,24*S*)-17,24-epoxybaccharane-3β,25-diol, (17*S*,20*S*,24*R*)-20,24-epoxydammarane-3β,25-diol and (17*S*,20*S*,24*S*)-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 17α-H (17*S*) dammarenyl cation, formed by the folding into a *chair-chair-chair-boat* conformation. This conformation usually leads to 20*S* stereochemistry, as depicted in **46** (Scheme 3C). Some papers have referred to the isolation of (20*R*)-epoxydamamranes from natural sources, but it was recently suggested that there is no documented evidence for the 20*R* 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 (17*R*) stereochemistry), which is formed by the folding of an *all-chair* conformation, leading to a (17*R*,20*R*)-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 $CDCl_3$ and acetone-d_6.

Product 31. Oil. ^{1}H NMR (C_6D_6, 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). ^{13}C NMR (C_6D_6, 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 $C_{30}H_{52}O_2$, 444.3967; found, 444.3972. $[a]_D^{25} = +79.6$ (*c* 0.40, EtOH).

Product 32. Oil. ^{1}H NMR (C_6D_6, 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. ^{13}C 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. ^{1}H NMR (C_6D_6, 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. ^{13}C NMR (C_6D_6, 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 $C_{29}H_{49}O_2$, 429.3733; found, 429.3730. $[a]_D^{25} = -72.5$ (c 0.090, EtOH).

Product 34. Solid. ^{1}H NMR (acetone-d_6, 600.13 MHz) δ 3.78 (very broad d, *J* 4.4, O*H*), 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-22*eq.*), 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). ^{13}C 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. ^{1}H NMR (C_6D_6, 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-23*eq*), 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-23*ax*), 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 C_6D_6 are exchangeable. ^{13}C NMR (C_6D_6, 150.9 MHz) δ ^{13}C NMR (C_6D_6, 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 C_6D_6 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 $C_{29}H_{49}O_2$, 429.3733; found,429.3741. $[\alpha]_D^{25} = +55.3$ (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. $[\alpha]_D^{25} = +22.8$ (*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 $C_{30}H_{52}O_2$, 444.3967; found, 444.3979. $[\alpha]_D^{25} = +4.08$ (*c* 0.29, EtOH).

Product 37. Oil. ^{1}H 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. ^{13}C 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. $[\alpha]_D^{25} = -22.8$ (*c* 0.38, EtOH).

Product 38 (3-deoxycotillol). Solid. ^{1}H NMR (C_6D_6, 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. ^{13}C 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 $CDCl_3$ 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 $C_{29}H_{49}O_2$, 429.3733; found, 429.3717. $[\alpha]_D^{25} = +35.9$ (*c* 0.04, EtOH). *cf.* lit.[21a] $[\alpha]_D = +19.3$ (*c* 0.04, EtOH).

Product 39-acetate. Solid. ^{1}H 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, O*H*), 2.25 (m, H-17), 2.11 (m, H-13), 1.979 (3H, s, CH_3CO), 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). ^{13}C NMR (acetone-d_6, 150.9 MHz), δ 170.9 (s, CH_3CO), 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, CH_3CO), 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 $CDCl_3$ 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* ($M^+ - C_5H_{11}O_3$), calcd. for $C_{27}H_{43}O$, 383.3314; found, 383.3304. $[\alpha]_D^{25} = -5.92$ (*c* 0.17, EtOH).

Product 40-acetate. Oil. ^{1}H 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, O*H*), 2.25 (m, H-17), 2.11 (m, H-13), 2.00 (3H, s, CH_3CO), 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. ^{13}C NMR (acetone-d_6, 150.9 MHz), δ 170.5 (s, CH_3CO), 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, CH_3CO), 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 $CDCl_3$ 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 ($M^+ - C_5H_{11}O_3$), calcd. for $C_{27}H_{43}O$, 383.3314; found, 383.3314. $[a]_D^{25} = -13.27$ (c 0.11, EtOH).

Product 41-acetate. Solid. ^{1}H NMR (acetone-d_6, 600.13 MHz), δ 4.43 (dd, J 11.4, 5.0, H-3), 3.71 (dd, J 10.6, 5.2, H-24), 2.78 (br s, O*H*), 2.25 (m, H-17), 2.08 (m, H-13), 1.980 (3H, s, CH_3CO), 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. ^{13}C NMR (acetone-d_6, 150.9 MHz), δ 170.67 (s, CH_3*C*O), 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, *C*H_3CO), 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 $CDCl_3$ 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 ($M^+ - C_5H_{11}O_3$), calcd. for $C_{27}H_{43}O$, 383.3314; found, 383.3309. $[a]_D^{25} = +11.38$ (c 0.27, EtOH).

Product 42-acetate. Oil. ^{1}H 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, O*H*), 2.25 (m, H-17), 2.11 (m, H-13), 2.00 (3H, s, CH_3CO), 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. ^{13}C NMR (acetone-d_6, 150.9 MHz), δ 170.5 (s, CH_3*C*O), 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, *C*H_3CO), 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 ($M^+ - C_5H_{11}O_3$), calcd. for $C_{27}H_{43}O$, 383.3314; found, 383.3306. $[a]_D^{25} = -17.3$ (c 0.36, EtOH).

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