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Reviewing the Polyolefin Cyclization Reaction of the C_{35} Polyprene Catalyzed by Squalene-Hopene Cyclase

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Abstract: Squalene-hopene cyclase (SHC) catalyzes the polycyclization of squalene (C_{30}) to the pentacyclic hopene with regio- and stereochemical specificity. In this study, we reviewed the polycyclization reaction of the C_{35} polyprenoid catalyzed by SHC. Surprisingly, our results completely disagreed with a previous publication in which it was reported that a hexacyclic skeleton was constructed as the single product in 10% yield (I. Abe, H. Tanaka, H. Noguchi, *J. Am. Chem. Soc.* **2002**, *124*, 14514–14515). Our experimental results showed that many tri- and tetracyclic products, up to 12, including novel carbocyclic cores, were generated in a high conversion ratio (97%), but no detectable amounts of the penta- and hexacycle were produced. The mechanisms for the formation of the C_{35} polyprene products isolated by us are discussed in this paper. The following four conformations were generated during the polycyclization cascade: chair-chair-boat, chair-chair-chair, chair-chair-chair-boat, and chair-chair-chair-chair. Larger amounts of the false intermediates with 13α-H (tricycle) and 17α-H (tetracycle) were produced compared with the true intermediates (13β-H and 17β-H), which indicates that the C_{35} polyprene cannot fold correctly in the enzyme cavity due to the extra C_5 unit appended to squalene. This would have promoted the formation of the aborted cyclization products with tri- and tetracycles. In addition, the fact that no penta- or hexacyclic products were formed further indicates that SHC does not have sufficient space to accommodate the entire carbon framework of C_{35}.

Keywords: alkenes · cyclization · enzyme catalysis · polycycles · terpenoids

Introduction

Acyclic squalene (**1**) is cyclized into pentacyclic triterpenes hop-22(29)-ene (**2**) and hopan-22-ol (**3**) (in a ratio of ca. 5:1). The polycyclization reaction is mediated by the squalene-hopene cyclase (SHC) enzyme obtained from prokaryotic species (Scheme 1).[1] The reaction proceeds with regio- and stereochemical specificity under precise enzyme control to form a 6/6/6/6/5-fused ring system with nine new chiral centers. SHC folds **1** into an all prechair conformation (a product-like conformation) inside the enzyme cavity and subsequently forms the final hopanyl cation **11** through sequential ring-forming reactions. In the formation of **2**, a proton is eliminated exclusively from the (23*Z*)-methyl group, but not from the (*E*)-methyl group.[1a,2] On the other hand, **3** is produced by the nucleophilic attack of water on cation **11**. The polycyclization reaction is analogous to that with eukaryotic oxidosqualene cyclase.[1] We have previously reported extensive site-directed mutagenesis experiments[3] in which the electronic and steric environments of active-site residues were altered. These alterations led to the isolation of products from the early truncation of the polycyclization cascade (mono-, bi-, tri-, and tetracyclic scaffolds) and/or to aberrant cyclization products, the stereochemistries of which were opposite to those of normal cyclization intermediates. These findings indicated that the stereochemical destiny of a polycyclization cascade is directed by the steric bulk of the active-site residues. On the basis of the number of rings in the enzymatic products isolated from various mutants, we have proposed a mechanism for the cyclization process (Scheme 1).[1a] X-ray crystallographic analysis revealed that the reaction cavity is lined with aromatic amino acid residues.[4] The carbocation intermediates involved in the polycyclization reaction are stabilized by the π electrons of aromatic amino acid residues. We very recently succeeded in providing unambiguous evidence for the cation–π interaction by replacing phenylalanine with an un-

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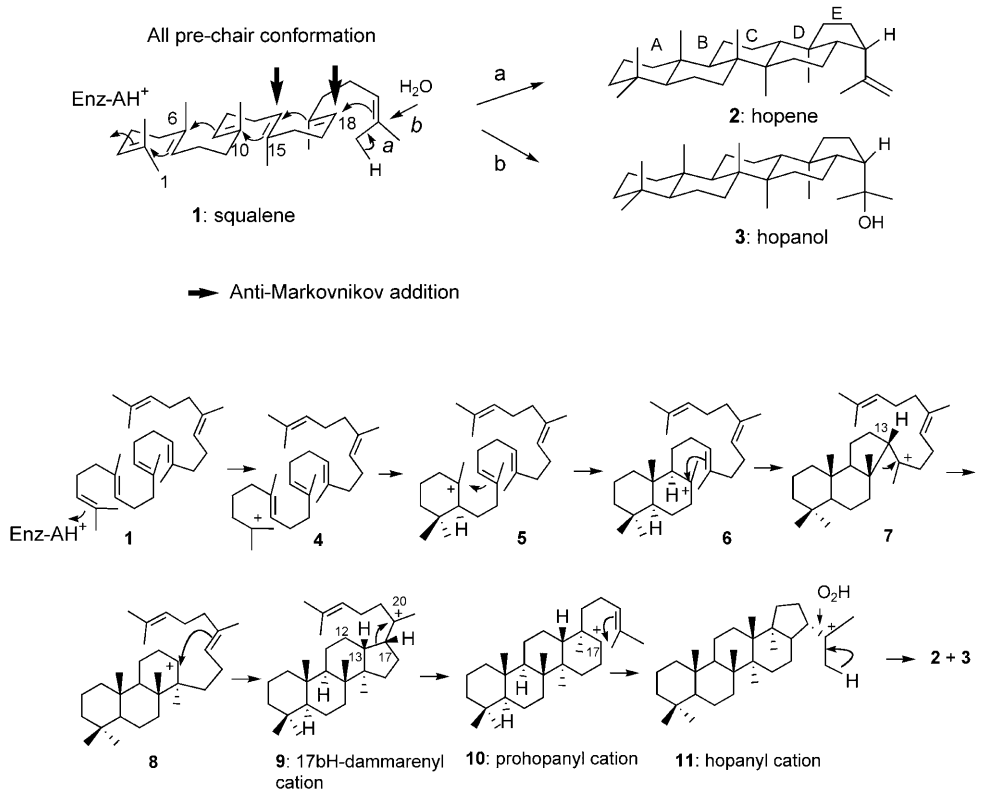
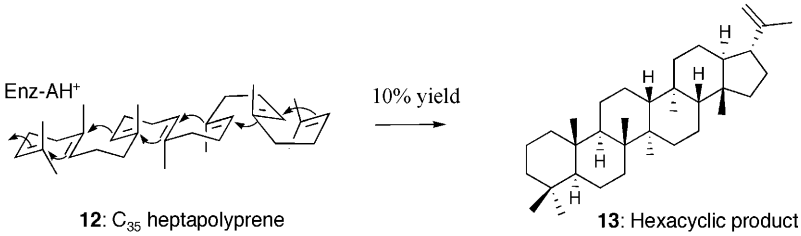


Scheme 1. Cyclization pathway for the transformation of squalene (**1**) into pentacyclic hopene (**2**) and hopanol (**3**).

natural amino acid (*p*-fluorophenylalanine) at catalytic sites.[5] The ring-expansion process from a five- to six-membered ring (**7**→**8** and **9**→**10**) is achieved by the stabilization of the secondary cation with the aid of a π electron from the aromatic amino acid.[5] These cationic intermediates were successfully trapped by the enzymatic reactions of squalene analogues with highly nucleophilic hydroxy group(s).[3b,e,6,9e] Recently, we succeeded in altering the substrate specificity to that of a eukaryotic-type cyclase, which is specific to (3*S*)-2,3-oxidosqualene.[7] This finding provided an interesting insight into the evolution of eukaryotic cyclases from prokaryotic SHC suggested by Ourisson and coworkers. [8]

Numerous studies of squalene substrate analogues[2,6,9] have provided important information on substrate recognition and for understanding of the reaction mechanism. The substrate specificity of SHC is remarkably broad. C(10)-Norsqualene gave abnormal cyclization products with a 6/5+5/5+(6) ring system, which indicates that the methyl group at C-10 is indispensable for a normal polycyclization reaction.[9b] The terminal methyl group on the squalene backbone is crucial for the initiation of a polycyclization reaction and for the formation of a five-membered E ring.[2,9a] Furthermore, the positions of methyl groups on the squalene backbone influence the polycyclization pathway.[9c] The analogues with indole and pyrrole rings were recognized to afford enzymatic products.[10] We have shown that SHC tolerates truncated analogues with a variety of carbon chain lengths (C_{15}–C_{25}), such as farnesol and geranylgeraniol.[9d] In addition, Abe et al. reported that the elongated squalene analogue **12** (C_{35}-heptaprenoid) can be accepted as a substrate to afford the 6/6/6/6/6/5-fused hexacyclic ring system **13** as a single product in 10% yield (Scheme 2).[11] This result is very attractive because a hexacyclic terpene skeleton has never been found in nature, which motivated us to synthesize further cyclized terpenoids, such as a heptacyclic scaffold. During our study,

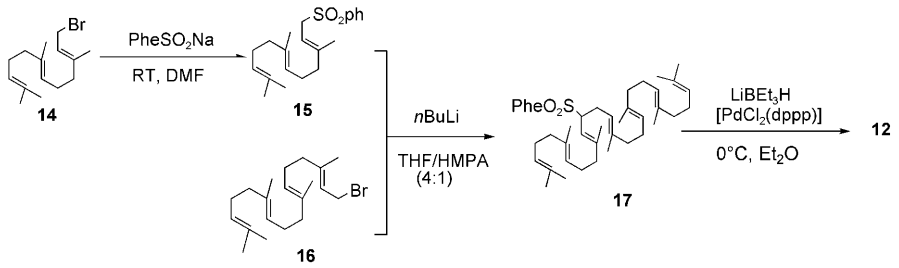


Scheme 2. Cyclization pathway for the transformation of C_{35} **12** ($=C_{15}+C_{20}$) to the hexacyclic polyprenoid **13** reported by Abe et al.[11]

we reviewed the cyclization reaction of **12** and isolated all the enzymatic products from **12** in a pure state by employing SHC. In contrast to the study conducted by Abe et al., we were unable to detect **13**, even in a negligibly small amount. Instead, many tri- and tetracyclic products, up to 12, were found. Herein we report in detail our experimental results for the enzymatic reaction of **12** and revise the cyclization pathway reported by Abe et al. Our results indicate that the reaction cavity of the bacterial SHC does not have sufficient space to accommodate the entire carbon framework of the C_{35}-heptaprenoid **12**.

Results and Discussion

Preparation of C_{35} polyprene 12: The synthesis was performed according to the method of Abe et al.[11] (Scheme 3, see the Supporting Information). Farnesyl phenyl sulfone (**15**) was prepared from farnesyl bromide (**14**), which was obtained from farnesol by treatment with PBr_3. The coupling reaction of **15** with geranylgeranyl bromide (**16**) yielded the C_{35}-containing phenyl sulfone **17** and subsequent desulfonation with $LiBEt_3H$ in the presence of a catalytic amount of $[PdCl_2(dppp)]$ (dppp = 1,3-bis(diphenylphosphino)propane) afforded the desired **12**.



Scheme 3. Synthesis of **12**.

Incubation of 12 with wild-type SHC and the product profile: A wild-type *shc* gene was cloned into the *Nde*I/*Bam*HI site of pET3a[3a] and the *Nde*I/*Hind*III site of pET26b.[5] Each of the recombinant enzymes was expressed in *E. coli* BL21(DE3). The gene product expressed by pET26b is identical to that expressed by pET22b, which was used by Abe et al.[11] The C_{35} polyprene **12** (1.0 mg) was incubated with 2 mL of cell-free homogenates, which were prepared from the *E. coli* clones. The incubations were carried out under the optimal catalytic conditions (pH 6.0 and 60 °C) for 16 h, in which **1** was fully converted into **2** and **3**, and then terminated by adding 5 % methanolic KOH. The hexane extracts from the reaction mixture were subjected to a short SiO_2 column chromatography with hexane/EtOAc (100:20) as the eluent to remove the excess Triton X-100 in the incubation mixture. Figure 1 shows the GC traces of the hexane extracts obtained from the incubation mixture with the cell-free extract of *E. coli* (pET3aSHC). It was found that many

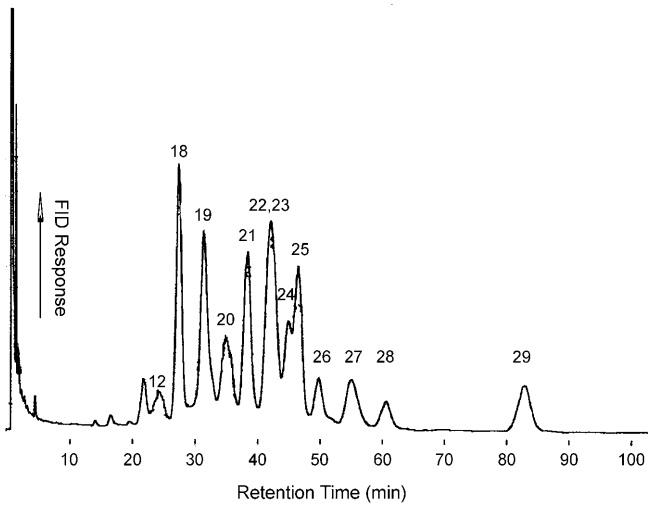


Figure 1. GC trace of the reaction mixture of **12** with wild-type SHC (pET3a). An excess of Triton X-100 included in the incubation mixture was removed by short column chromatography on SiO_2. Note that this GC profile is identical to that obtained with native SHC (pET26b or pET22b).

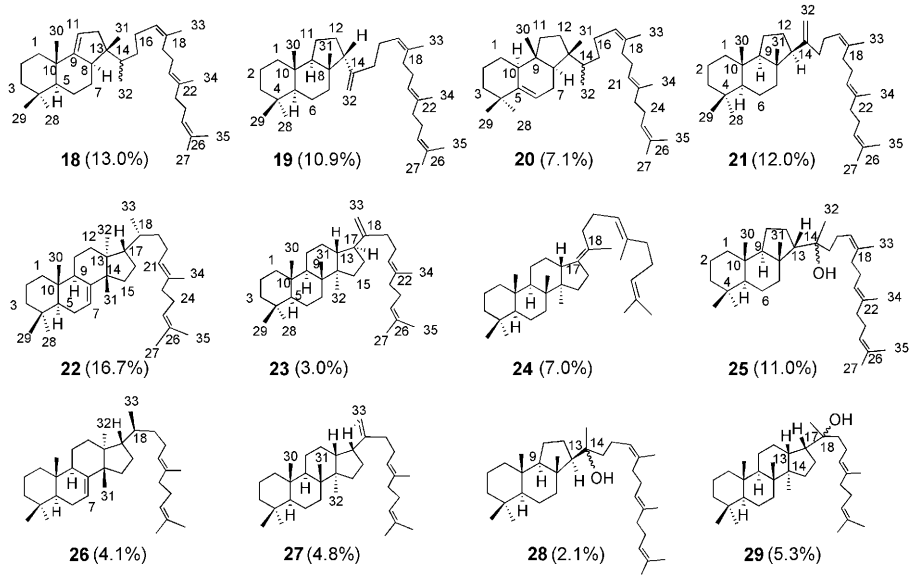
enzymatic products were produced at a high conversion ratio (97 % conversion from **12**), although Abe et al. previously reported that only one product **13** was generated from **12**.[11] The GC and GC–MS profiles of these products were identical to those obtained from *E. coli* (pET26bSHC), which indicates that the extra tag (His × 6) did not affect the polycyclization pathway of **12**. Moreover, the GC patterns from the cell-free homogenates were also identical to those obtained by using purified enzymes. The recombinant enzyme expressed by *E. coli* (pET3aSHC) was purified with a DEAE column according to a previous report,[3a] whereas that expressed by *E. coli* (pET26bSHC) was isolated by using a nickel agarose column (Qiagen).[5] The enzymatic purity was checked by SDS-PAGE (sodium dodexyl sulfate polyacrylamide gel electrophoresis).

Isolation and structures of the products 18–29: Large-scale incubation was carried out to isolate all the enzymatic products. To emulsify analogue **12** (300 mg), its solution was prepared in Triton X-100 (6.0 g) and distilled water (300 mL). The cell-free homogenates of the *E. coli* clone (pET3aSHC) (600 mL) and 0.5 M citrate buffer (900 mL, pH 6.0) were added to this solution. The resulting reaction mixture was incubated at 60 °C for 20 h and then lyophilized and extracted with hexane (100 mL × 3). Thereafter, excess Triton X-100 was removed by column chromatography (a short SiO_2 column was used) with hexane/EtOAc (100:20) as the eluent. The lipophilic materials were analyzed by SiO_2 TLC.

The TLC of the low-polar fraction (performed with hexane) gave three spots, which were named in descending order of their R_f values as Fraction 1, Fraction 2, and Fraction 3. TLC of the high-polar fraction (performed with hexane/EtOAc = 100:5) produced two spots; both of these were named as Fraction 4. Unreacted substrate **12** was found between Fractions 3 and 4 in SiO_2 TLC. Each of the fractions and **12** were separated by SiO_2 column chromatography with hexane as eluent; commercially available SiO_2 was dried in an oven at 200 °C for 2 h prior to use to increase its adsorption ability. Purified Fraction 1 exhibited two peaks in its GC chromatogram, the two compounds (**22** and **26**) being separated by 5 and/or 10 % $AgNO_3/SiO_2$ column chromatography with hexane/EtOAc (100:0–100:1) as eluent. The separated Fraction 2 showed five peaks on its GC trace. Products **18**, **20**, **23**, **24**, and **27** were separable by repeated column chromatography (5 % $AgNO_3/SiO_2$ with hexane/EtOAc as eluent). Product **24** was insoluble in EtOH and therefore it was purified by washing with EtOH. Two products, **19** and **21**, were detected in the purified Fraction 3 by GC. These products were separated by 5 % $AgNO_3/SiO_2$ column chromatography with hexane as eluent. GC analysis revealed that the high-polar Fraction 4 comprised three products: **25**, **28**, and **29**. The complete separation of these products was also achieved by repeated 5 % $AgNO_3/SiO_2$ column chromatography (hexane/EtOAc = 100:0–100:2.5).

Analysis by ^1H and ^{13}C NMR, DEPT, and 2D NMR spectroscopy, including H–H COSY, HOHAHA, NOESY, HMQC, and HMBC, revealed the chemical structures of the enzymatic products **18**–**29** (Scheme 4). Substrate **12** has nine allylic methyl groups. Products **18**–**21**, **25**, and **28** contain four allylic methyl groups that appear in the δ_H range 1.69–1.81 ppm, which indicates that these compounds have a tricyclic skeleton. On the other hand, products **22**–**24**, **26**, **27**, and **29** possess three allylic methyl groups, which suggest a tetracyclic core for these products.

Detailed analysis of the HMBC spectra revealed a planar structure for the enzymatic products **18**–**29** (see the correlation data of HMBC, NOESY, and COSY NMR in the Supporting Information). In **18**, the presence of a double bond between C-9 and C-11 was determined as follows. A clear HMBC cross-peak was observed between Me-30 (δ_H = 1.22 ppm, s, 3H) and C-9 (δ_C = 158.5 ppm, s). The chemical shifts of 12-H (δ_H = 2.09, 2.50 ppm) and the HMBC correlation between Me-31 (δ_H = 0.974 ppm, s, 3H) and C-12 (δ_C = 47.53 ppm, t) indicate the presence of a double bond between C-9 and C-11. Product **18** is a novel compound. Product **19** contains vinyl protons (δ_H = 4.91, 5.17 ppm, both s) that are correlated with C-13 (δ_C = 56.97 ppm, d) in its HMBC spectrum. Furthermore, a strong NOE was observed between Me-31 (δ_H = 1.10 ppm, s, 3H) and 13-H (δ_H = 2.32 ppm, m) of **19**. Thus, in **19**, 13-H is arranged in the β orientation. Product **20** has a novel skeleton. In this product, Me-28 (δ_H = 1.29 ppm, s, 3H) and Me-29 (δ_H = 1.21 ppm, s, 3H) exhibit strong HMBC cross-peaks with C-5 (δ_C = 145.0 ppm, s). Furthermore, Me-30 (δ_H = 1.05 ppm, s, 3H) and Me-31 (δ_H = 1.13 ppm, s, 3H) exhibit clear correlations with C-8 (δ_C = 44.79 ppm, d) and Me-30 shows a strong cross-peak with C-10 (δ_C = 50.85 ppm, d) in the HMBC spectrum. An apparent NOE was found between Me-30 and Me-31. From both the doublet methyl group, Me-32 (δ_H = 1.04 ppm, 3H, d, J = 6.6 Hz) and Me-31, clear HMBC cross-peaks were observed with C-13 (δ_C = 37.30 ppm, s), validating the positions of Me-32 and Me-31 shown in Scheme 4. A strong NOE for 8-H/10-H was found, but no NOEs for 8-H/Me-30 and 8-H/Me-31 were observed, which demonstrates that 8-H and 10-H are oriented in the α disposition. The stereochemistries of **18** and **20** at C-14 were not determined from their NMR spectroscopic data. However, from the point of view of the cyclization mechanism, they possibly have a 14*S* configuration, as discussed below. The carbocyclic skeleton of **21** is identical to that of **19**, but the stereochemistry of **21** at C-13 is opposite to that of **19**: 13α-H for **21** and 13β-H for **19**. For the product **21**, a strong NOE was observed between 9-H and 13-H, which gives 13α-H. Detailed HMBC analysis revealed that the skeleton of **22** is similar to that of euph-7(8)-ene. The double-bond position between C-7 and C-8 was confirmed by a strong HMBC cross-peak between Me-31 (δ_H = 1.18 ppm, s, 3H) and C-8 (δ_C = 146.1 ppm, s). The chemical shift of Me-33 (δ_H =



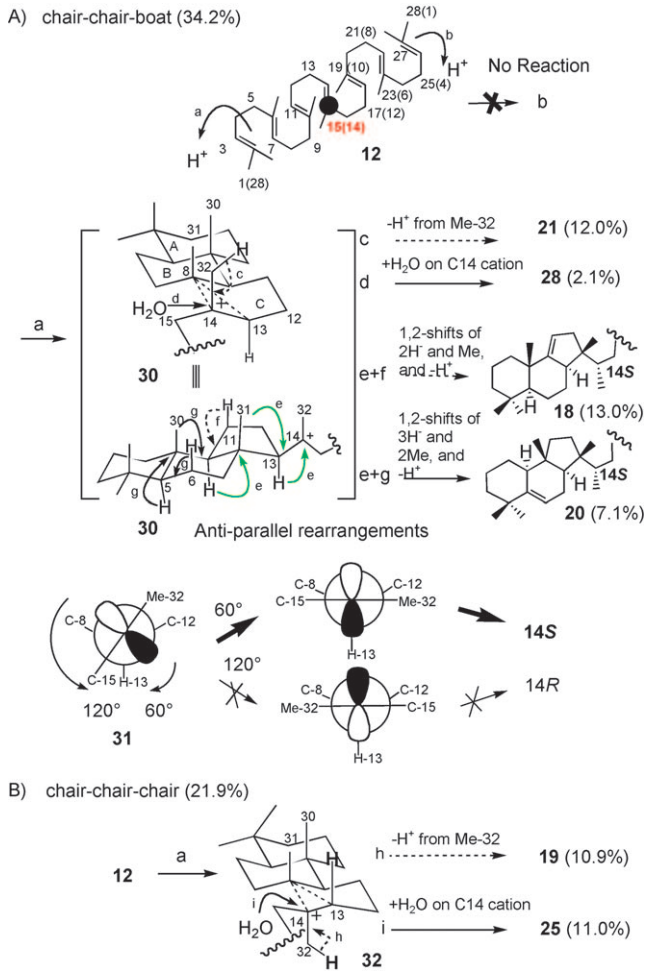
Scheme 4. Structures of the enzymatic products **18**–**29** obtained from **12**. The amount of each product formed is shown in parentheses.

0.848 ppm, d, $J=6.5$ Hz, 3H) in $CDCl_3$ is close to the published value of the corresponding methyl group in euph-7(8)-ene ($\delta_H=0.835$ ppm, d, $J=6.8$ Hz, 3H),[12] which demonstrates an *R* configuration at C-18 in **22**. A detailed NMR analysis for **23** showed that its skeleton is similar to that of dammar-20(21)-ene. A strong NOE between Me-32 ($\delta_H=$ 1.02 ppm, s, 3H) and 17-H ($\delta_H=2.43$ ppm, m) indicates the configuration of 17α-H. Product **24** also has a dammarene-like tetracyclic skeleton, which was revealed by the HMBC spectrum. However, the position of one of the double bonds in **24** is different to that in **23**, and it was found that this double bond is located between C-17 and C-18 on the basis of following finding. The chemical shift of Me-33 ($\delta_H=$ 1.83 ppm, s, 3H) shows that it is an allylic methyl group that has HMBC correlations with C-17 ($\delta_C=137.1$ ppm, s) and C-18 ($\delta_C=126.7$ ppm, s), which indicates that they are sp^2 carbon atoms. The chemical shift values of 13-H ($\delta_H=$ 2.50 ppm, m) and 16-H ($\delta_H=2.26$, 2.37 ppm, both m) further support the finding that C-17 is an sp^2 carbon atom. A strong NOE between Me-33 and 16-H demonstrated that the C-17–C-18 double bond was *Z* configured. Product **25** is a highly polar compound and was isolated from Fraction 4 with a lower R_f value by SiO_2 TLC, as described above. This product has a chemical shift at $\delta_C=75.47$ ppm, which indicates that a hydroxy group is present. Furthermore, Me-32 ($\delta_H=1.31$ ppm, s, 3H) in **25** has HMBC correlations with C-14 ($\delta_C=75.47$ ppm, s) and C-13 ($\delta_C=59.06$ ppm, d), which indicates that the hydroxy group is located at C-14. The 13-H atom is arranged in a β configuration, as determined by a strong NOE between Me-31 ($\delta_H=1.07$ ppm, s, 3H) and 13-H ($\delta_H=1.75$ ppm, m). Product **26** has a skeleton similar to that of **22**, as revealed by detailed NMR analysis. The chemical shift of Me-33 in $CDCl_3$ is 0.886 ppm (d, $J=6.5$ Hz), which is close to that of the corresponding methyl group in tirucall-7(8)-ene ($\delta_H=0.865$ ppm, d, $J=6.7$ Hz, 3H)[12] and indicates an 18*S* configuration, as shown in Scheme 4. The only structural difference between **22** and **26** is found in the stereochemistry at C-18: 18*R* for **22** and 18*S* for **26**. Product **27** has a skeleton similar to that of **23**. A clear NOE was observed between 13-H ($\delta_H=2.03$ ppm, m) and 17-H ($\delta_H=$ 2.75 ppm, m), which indicates a 17β-H configuration. Thus, **27** differs from **23** only in the stereochemistry at C-17. Product **28** is the stereoisomer of **25**; at C-13, the stereochemistry of **28** was opposite to that of **25**. A strong NOE was observed between 13-H ($\delta_H=1.43$ ppm, t, $J=10$ Hz) and 9-H (1.12 ppm, dd, 13.0, 7.0 Hz), which indicates a 13α-H configuration for **28**. The highly polar compound **29**, which contains a hydroxy group ($\delta_C=74.55$ ppm, s), has a tetracyclic skeleton. Furthermore, the absence of an NOE between Me-31 ($\delta_H=1.09$ ppm, s, 3H) and 17-H ($\delta_H=2.10$ ppm, m) of this compound indicates that 17-H has a β orientation.

Mono-, bi-, penta-, and hexacyclic structures were not observed. In contrast, large amounts of tri- and tetracyclic skeletons were produced. The amounts of **18–29** produced were estimated by GC analysis (Figure 1) and are shown in Scheme 4. The ratio of tetracyclic/tricyclic products was 41:56. One may question whether the formation of penta- and hexacyclic products was overlooked in this experiment. However, this can be completely ruled out on the basis of the following three findings. 1) The weights of the partially purified Fractions 1–4 were 57, 98, 65, and 52 mg, respectively, and that of the recovered **12** was 6 mg. Therefore, the total recovered yields are around 93% of the amount of **12** (300 mg) used in the incubation experiment. It is quite usual for complete recovery (≈100%) not to be achieved in the purification process by SiO_2 column chromatography, which indicates that almost all of the enzymatic products were recovered and thus it is unlikely that enzymatic products other than **18–29** were present in the incubation mixture. 2) The R_f values of penta- and hexacyclic compounds on SiO_2 TLC plates must be higher than those of tetracycles **22** and **26** (obtained from the partially purified Fraction 1) according to the fact that the smaller the number of double bonds, the larger the R_f values (see also the Conclusion section),[9] but no spot appeared above Fraction 1. 3) In general, the more rings there are in the cyclized products, the longer the retention times in GC. We conducted the GC analysis for a longer time of 210 min (cf. the time of 100 min shown in Figure 1), but no other peaks appeared in the GC trace after the peak of **29**. These results unambiguously demonstrate that other products, including penta- and hexacyclic compounds, were not overlooked.

Mechanism for the cyclization of 12 into the enzymatic products 18–29: Squalene (**1**) is a symmetrical molecule, but **12** is asymmetric. In our previous studies, we demonstrated that a terminal isopropylidene moiety is indispensable for initiating the polycyclization of **1**.[2,9a] In molecule **12**, an isopropylidene moiety is present at each termini. Thus, two cyclization pathways (paths *a* and *b*) are possible, as shown in Scheme 5. However, all the products **18–29** were produced from path *a* and not from path *b*. As for the carbon numbering, path *a* gives C-15 at the position marked with the closed circle, whereas path *b* affords C-14 at this position (in Scheme 5, the carbon numbering for path *b* is shown in parentheses). We have previously reported that the introduction of a methyl group at C-14 on the squalene backbone interrupts squalene cyclization. This is due to the repulsive interaction between the methyl group introduced at C-14 and the corresponding recognition site in the reaction cavity.[9c,d] As a result, cyclization did not occur by path *b*.

The folding of **12** into a chair-chair-boat conformation gives the cationic intermediate **30**, in which 13-H is arranged in the α disposition (Scheme 5A). Thus, products **21** and **28** with 13α-H were produced from **30**. Proton elimination from Me-32 afforded **21**. Attack by water on the C-14 cation yielded **28**. Products **18** and **20** could also be produced via **30**. The 13α-H atom migrated to the C-14 cation. 1,2-Shifts of Me-31 and 9-H, followed by deprotonation of 11-H (paths *e* and *f*) afforded **18**, with the migrations and deprotonation steps occurring in an antiperiplanar fashion. The successive rearrangements of 13-H, Me-31, 9-H, Me-30, and 5-H, followed by the deprotonation of 6-H, generated **20**. For the cationic intermediate **32**, which is formed by the folding



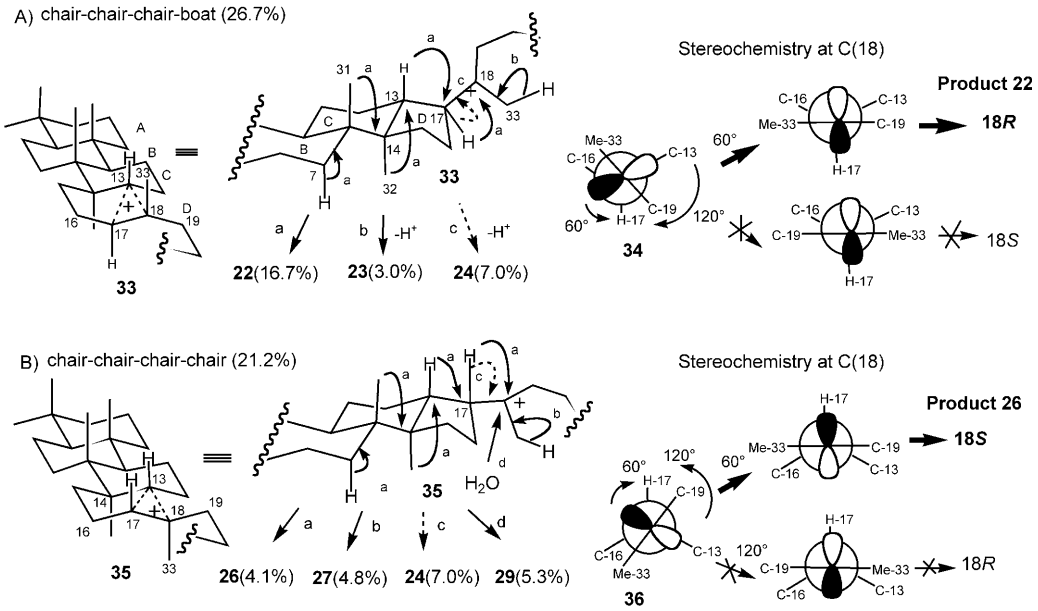
Scheme 5. Mechanisms for the cyclization process and the folding conformations of **12** for the formation of tricyclic products. A) Folding of **12** into a chair-chair-boat conformation (**30**) and mechanisms for the formation of products **18**, **20**, **21**, and **28**. 1,2-Shifts of hydrogen atoms and methyl groups in an antiparallel fashion enable the formation of products **18** and **20**. A Newman projection (**31**) through the C-14–C-13 axis allows us to propose the 14*S* stereochemistry for **18** and **20**. B) Folding of **12** into a chair-chair-chair conformation and mechanisms for the formation of products **19** and **25**.

of **12** into a chair-chair-chair conformation, the migration of Me-31 to C-13 is more unfavorable than in **30** because both Me-31 and 13-H are arranged in the same β orientation and not in an antiparallel fashion, which further supports the finding that **18** and **20** are generated from **30**. The stereochemistry at C-14 of **18** and **20** could not be determined from the NMR data. The Newman projection through the C-13–C-14 axis of **30** is depicted in **31**. A small rotation (ca. 60°) gives the 14*S* configuration, whereas a large rotation (ca. 120°) yields the 14*R* configuration. Because the substrate is tightly constricted by cyclase, the rotation of molecules inside the enzyme cavity of cyclase is restricted.[6,9e] Therefore, it is presumed that products **18** and **20** have the 14*S* configuration. Products **19** and **25** have 13β-H (like **7**), which indicates that **12** was folded into a chair-chair-chair conformation to generate the cationic intermediate **32** (Scheme 5B). Proton elimination from Me-32 yielded **19** and the attack of a water molecule on the C-14 cation afforded **25**.

The analogue **12** was also cyclized to tetracyclic products, which can be classified into two types, the stereochemistries of which are different at C-17, that is, 17α-H or 17β-H. As shown in Scheme 6, the folding of **12** into a chair-chair-chair-boat conformation leads to the formation of the cation **33** with 17α-H (Scheme 6A), whereas organization into a chair-chair-chair-chair conformation gives the cation **35** with 17β-H (Scheme 6B). Deprotonation from Me-33 afforded **23** and **27** (path *b*). Attack by a water molecule on the C-18 cation of **35** gave **29** (path *d*). The elimination of 17-H from **33** and/or **35** leads to the formation of the C-17=C-18 double bond, yielding **24** (path *c*). The production of **24** having *Z* configuration, but not *E* geometry, indicated that the enzymatic reaction proceeded kinetically to form an energetically unfavourable product. Successive migration of hydrides and methyl groups followed by the deprotonation of 7-H gave **22** and **26** (path *a*). The 18*R* configuration in **22** can be attained through a pathway involving only a small rotation (60°) about the C-17–C-18 axis (see Newman projection **34**). As described above, a large rotation (120°), which would lead to **22** with the 18*S* configuration, is prohibited. Similarly, product **26** has the 18*S* configuration due to a small rotation (60°), as depicted in the Newman projection **36**.

Conclusion

In our experiments on the polycyclization of **12** with squalene-hopene cyclase (SHC), significantly large amounts of tri- (56%) and tetracyclic skeletons (41%) were formed. Mono-, bi-, penta-, and hexacyclic scaffolds were not observed. These results indicate that SHC tolerates the elongated C_{35} polyprene **12**. Indeed, only 3% of **12** remained in the incubation mixture. The appendage of an extra C_5 unit on to squalene (C_{30}) prevented the formation of a pentacyclic skeleton such as **2**, which indicates that **12** does not fold correctly in the reaction cavity, although it is partly captured by the cyclase. As a result, aborted cyclization products with tri- and tetracycles were generated. As shown in Scheme 1, the tri- and tetracyclic intermediates **7** and **9** have 13β-H and 17β-H, respectively. The intermediate **32** has 13β-H, like **7**, whereas **30** has 13α-H. The ratio of **30**/**32** was estimated to be around 1.6:1. The tetracyclic intermediate **33** has 17α-H, whereas **35** has 17β-H, like **9**. The ratio of **33**/**35** was 1.26:1. The formation of larger amounts of the false intermediates (13α-H and 17α-H) further supports the conclusion that **12** cannot perfectly fit into the SHC enzyme during the polycyclization reaction, in contrast to **1**. This is because SHC possibly does not have sufficient space to accommodate the entire C_{35} carbon framework. To validate this assumption, X-ray docking studies of **12** with the active sites of the reaction cavity are necessary.



Scheme 6. Mechanisms for the cyclization process and the folding conformations of **12** for the formation of tetracyclic products. A) Folding of **12** into a chair-chair-chair-boat conformation (**33**) and mechanisms for the formation of **22**–**24**. For the formation of **22**, all the hydrogen atoms and methyl groups rearrange in an antiparallel fashion. A Newman projection (**34**) through the C-17–C-18 axis of **33** and a small rotation gives **22** with an 18*R* configuration. B) Folding of **12** into a chair-chair-chair-chair conformation (**35**) and mechanisms for the formation of **24**, **26**, **27**, and **29**. Based on a similar idea to the projection **34**, the Newman projection **36** shows product **26** to have an 18*S* configuration.

Our results from the polycyclization reaction of **12**, which are described in this paper, are significantly different to those reported by Abe et al.[11] Why do our results contradict those of Abe et al.? Our detailed analysis of the HMBC data that appear in the Supporting Information of ref. [11] indicate that the purity of **13** isolated by Abe et al. is not sufficiently high to allow its chemical structure to be determined (see our analysis in the Supporting information). Furthermore, they reported that in SiO_2 TLC the R_f value of the enzymatic product **13** (0.6) was lower than that of the substrate (0.8).[11] This behavior is inconsistent with our previous report[9] according to which cyclized products must have higher positions on a SiO_2 TLC plate than polyolefin substrates, except for hydroxylated products. This is because the number of double bonds in substrates decreases as the number of ring-forming reactions increases (the higher the number of double bonds, the lower the R_f values). The creation of novel compounds with larger cyclic skeletons than pentacycles is fascinating, but our results lead to the conclusion that the generation of the novel hexacyclic terpenoid **13** from **12** by SHC is impossible.

Experimental Section

General analytical methods: NMR spectra were recorded in C_6D_6 on a Bruker DMX 600 or DPX 400 spectrometer. The chemical shifts (δ) are given in ppm relative to the residual solvent peak ($\delta_H = 7.280$ and $\delta_C = 128.0$ ppm) as the internal reference. In the case of $CDCl_3$ solutions, the chemical shifts are given in ppm relative to the solvent peak ($\delta_H = 7.26$ and $\delta_C = 77.0$ ppm). The coupling constants *J* are given in Hz. GC analyses were performed on a Shimadzu GC-8A chromatograph equipped with a flame ionization detector and a DB-1 capillary column (0.53 mm × 30 m). GC–MS spectra were recorded on a JEOL SX 100 spectrometer using the electron impact method at 70 eV and with a DB-1 capillary column (0.32 mm × 30 m). HREIMS was performed by using a direct inlet system. Specific rotation values were measured at 25 °C with a Horiba SEPA-300 polarimeter.

Synthesis of C_{35} substrate 12: The procedure for the synthesis of **12** is described in the Supporting Information.

Incubation conditions and GC analysis: The SHC described here is from *Alicyclobacillus acidocaldarius*.[3] Abe et al. also used the same bacterium species.[11] Standard culture of the cloned *E. coli* and incubation were performed in accord with our published protocols.[3a] The cell-free extract was prepared as follows. The culture of *E. coli* (1 L) encoding the native SHC(pET3a) or SHC(pET26b) (both from Novagen) was harvested by centrifugation. Citrate buffer solution (pH 6.0, 50 mM, 50 mL) was added to the collected pellets and then subjected to ultrasound to disrupt the cells. The supernatant was used for the incubations after removing the cell debris by centrifugation. One mL of the supernatant contains around 200 μg of the pure SHC. The substrate analogue (1 mg) and Triton X-100 (20 mg) were emulsified with distilled water (1.0 mL) and sodium citrate buffer solution (pH 6.0, 0.5 M, 3.0 mL). The cell-free extract (2.0 mL) was added to the solution and then incubated at 60 °C for 12 h. A 15% KOH/MeOH solution (6 mL) was added to terminate the enzyme reaction and then the mixture was heated at 80 °C for 30 min. The lipophilic enzymic products and unreacted substrate analogues were extracted from the incubation mixtures four times with hexane (5 mL) and the quantities of the products and the starting materials were estimated by GC analysis with a DB-1 capillary column (30 m in length, J & W Scientific, USA). GC conditions: injection temp. 290 °C, column temp. 270 °C and carrier pressure 1.0 kg cm^{-2}.

Spectroscopic data for products 18–29

Product 18: ^{1}H NMR (400 MHz, C_6D_6): $\delta = 0.958$ (s, 3H; Me-28), 0.974 (s, 3H; Me-31), 1.016 (s, 3H; Me-29), 1.05 (d, $J = 6.7$ Hz, 3H; Me-32), 1.221 (s, 3H; Me-30), 1.26 (m, 3-H), 1.40 (m, 15-H), 1.46 (m, 5-H), 1.47 (m, 1-H), 1.49 (m, 3-H), 1.50 (m, 2H; 2-H, 6-H), 1.62 (m, 7-H), 1.68 (m, 14-H), 1.695 (s, 3H; Me-35), 1.70 (m, 2H; 2-H, 6-H), 1.725 (s, 3H; Me-34), 1.765 (s, 3H; Me-33), 1.810 (s, 3H; Me-27), 1.85 (m, 2H; 7-H, 15-H),

1.97 (brd, J=12.7 Hz, 1-H), 2.09 (brd, J=15.5 Hz, 12-H), 2.15 (m, 16-H), 2.23 (m, 4H; 19-H, 23-H), 2.30 (m, 4H; 20-H, 24-H), 2.50 (brd, J=15.5 Hz, 12-H), 2.77 (brd, J=10.9 Hz, 8-H), 5.34 (m, 11-H), 5.37 (brt, J=6.8 Hz, 25-H), 5.41 (brt, J=6.8 Hz, 21-H), 5.45 ppm (brt, J=6.8 Hz, 17-H); ^{13}C NMR (100 MHz, C_6D_6): δ=15.64 (q, C-32), 16.11 (q, C-34), 16.14 (q, C-33), 17.09 (q, C-31), 17.72 (q, C-35), 19.58 (t, C-2), 19.72 (t, C-6), 21.64 (q, C-29), 22.46 (t, C-7), 25.26 (q, C-30), 25.83 (q, C-27), 26.99 (t, C-24), 27.05 (t, C-20), 27.22 (t, C-16), 33.10 (q, C-28), 33.11 (t, C-15), 33.50 (s, C-4), 36.64 (s, C-10), 40.20 (t, C-1), 40.20 (t, C-19), 40.37 (t, C-23), 42.85 (t, C-3), 43.96 (d, C-14), 46.03 (d, C-5), 47.53 (t, C-12), 48.48 (s, C-13), 50.28 (d, C-8), 115.9 (s, C-11), 124.8 (d, C-21), 124.9 (d, C-25), 125.5 (d, C-17), 131.0 (s, C-26), 134.8 (s, C-18), 134.9 (s, C-22), 158.5 ppm (s, C-9). The following ^{13}C NMR signals are indistinguishable from each other because they have very similar chemical shifts: C-1/C-19/C-23, C-16/C-20/C-24, C-2/C-6, C-33/C-34. MS (EI): m/z (%): 69 (60), 109 (52), 231 (100), 232 (71), 463 (4), 478 (2) [M^+]; HRMS (EI): m/z: calcd for $C_{35}H_{58}$: 478.4539; found: 478.4551. $[\alpha]_D^{25}=-35.7$ (c=0.24 in $CHCl_3$); oil.

Product 19: ^{1}H NMR (600 MHz, C_6D_6): δ=0.920 (m, 5-H), 0.949 (s, 3H; Me-28), 0.973 (s, 3H; Me-29), 0.997 (s, 3H; Me-30), 1.09 (m, 1-H), 1.101 (s, 3H; Me-31), 1.25 (td, J=13,0, 3.5 Hz, 3-H), 1.45 (m, 2H; 2-H, 6-H), 1.46 (m, 7-H), 1.48 (m, 3-H), 1.51 (m, 11-H), 1.59 (m, 1-H), 1.62 (m, 6-H), 1.64 (m, 2H; 9-H, 11-H), 1.694 (s, 3H; Me-35), 1.72 (m, 7-H), 1.733 (6H, s, Me-33, Me-34), 1.74 (m, 2-H), 1.807 (s, 3H; Me-27), 1.86 (m, 12-H), 2.12 (m, 15-H), 2.16 (m, 12-H), 2.22 (m, 4H; 19-H, 23-H), 2.30 (m, 5H; 20-H, 24-H, 15-H), 2.32 (m, 13-H), 2.43 (m, 2H; 16-H), 4.91 (s, 32-H), 5.17 (s, 32-H), 5.37 (brt, J=6.9 Hz, 25-H), 5.43 ppm (m, 2H; 17-H, 21-H); ^{13}C NMR (150 MHz, C_6D_6): δ=15.80 (q, C-30), 16.12 (q, C-33), 16.13 (q, C-34), 17.74 (q, C-35), 18.82 (t, C-2), 19.62 (t, C-6), 21.05 (t, C-11), 21.61 (q, C-29), 24.99 (q, C-31), 25.85 (q, C-27), 27.07 (t, C-20), 27.23 (t, C-24), 27.39 (t, C-16), 28.16 (t, C-12), 33.13 (s, C-4), 33.61 (q, C-28), 37.04 (s, C-10), 37.08 (t, C-7), 39.82 (t, C-15), 40.16 (t, C-1), 40.21 (t, C-23), 40.73 (t, C-19), 42.66 (t, C-3), 45.85 (s, C-8), 55.83 (d, C-9), 56.97 (d, C-13), 57.24 (d, C-5), 109.3 (t, C-32), 124.8 (d, C-25), 124.9 (d, C-17), 124.9 (d, C-21), 131.1 (s, C-26), 134.9 (s, C-18), 135.1 (s, C-22), 154.6 ppm (s, C-14). The following ^{13}C NMR signals are indistinguishable from each other as they have very similar chemical shifts: C-1/C-19/C-23, C-16/C-20/C-24, C-33/C-34, C-17/C-21/C-25. MS (EI): m/z (%): 69 (100), 95 (32), 123 (20), 191 (49), 231 (15), 463 (4), 478 (5) [M^+]; HRMS (EI): m/z: calcd for $C_{35}H_{58}$: 478.4539; found: 478.4514; $[\alpha]_D^{25}=+7.22$ (c=0.24 in $CHCl_3$); oil.

Product 20: ^{1}H NMR (600 MHz, C_6D_6): δ=1.044 (d, J=6.6 Hz, Me-32), 1.049 (s, 3H; Me-30), 1.133 (s, 3H; Me-31), 1.17 (m, 12-H), 1.18 (m, 1-H), 1.215 (s, 3H; Me-29), 1.293 (s, 3H; Me-28), 1.38 (m, 15-H), 1.39 (m, 3-H), 1.46 (m, 8-H), 1.48 (m, 11-H), 1.52 (m, 3-H), 1.55 (m, 12-H), 1.63 (m, 11-H), 1.63 (m, 2-H), 1.69 (m, 2-H), 1.700 (s, 3H; Me-37), 1.72 (m, 14-H), 1.748 (s, 3H; Me-34), 1.779(s, 3H; Me-33), 1.81 (m, 1-H), 1.815 (s, 3H; Me-27), 2.00 (m, 15-H), 2.03 (m, 2H; 7-H), 2.10 (m, 10-H), 2.12 (m, 2H; 16-H), 2.21–2.26 (m, 4H; 23-H, 19-H), 2.27–2.35 (m, 4H; 20-H, 24-H), 5.37 (brt, J=6.8 Hz, 17-H), 5.37 (brt, J=6.8 Hz, 25-H), 5.45 (t, J=6.9 Hz, 21-H), 5.76 ppm (m, 6-H); ^{13}C NMR (150 MHz, C_6D_6): δ=14.52 (q, C-32), 16.03 (q, C-34), 16.15 (q, C-30), 16.15 (q, C-33), 17.75 (q, C-35), 21.10 (q, C-31), 21.60 (t, C-16), 22.22 (t, C-2), 23.61 (t, C-7), 25.83 (t, C-15), 25.86 (q, C-27), 26.39 (t, C-1), 27.13 (t, C-24), 27.24 (t, C-20), 29.80 (q, C-29), 29.97 (q, C-28), 33.15 (t, C-11), 35.33 (d, C-14), 35.86 (s, C-4), 36.01 (s, C-9), 37.30 (s, C-13), 40.21 (t, C-12, C-23), 40.27 (t, C-19), 41.08 (t, C-3), 44.79 (d, C-8), 50.85 (d, C-10), 117.9 (d, C-6), 124.8 (d, C-21), 124.9 (d, C-25), 125.9 (d, C-17), 131.1 (s, C-26), 134.2 (s, C-18), 135.0 (s, C-22), 145.0 ppm (s, C-5). The following ^{13}C NMR signals are indistinguishable from each other as they have very similar chemical shifts: C-12/C-19/C-23, C-28/C-29, C-20/C-24, C-30/C-33/C-34. MS (EI): m/z (%): 69 (100), 95 (38), 123 (30), 257 (33), 259 (17), 463 (4), 478 (8) [M^+]; HRMS (EI): m/z: calcd for $C_{35}H_{58}$: 478.4539; found: 478.4539; $[\alpha]_D^{25}=+4.55$ (c=0.22 in $CHCl_3$); oil.

Product 21: ^{1}H NMR (600 MHz, C_6D_6): δ=0.848 (s, 3H; Me-31), 0.894 (brd, J=12.1 Hz, 5-H), 0.94 (m, 1-H), 0.963 (s, 3H; Me-30), 0.977 (s, 3H; Me-29), 1.023 (s, 3H; Me-28), 1.28 (m, 9-H), 1.29 (m, 7-H), 1.32 (m, 3-H), 1.45 (m, 2-H), 1.48 (m, 6-H), 1.50 (m, 1-H), 1.54 (m, 3-H), 1.62 (m, 6-H), 1.691 (s, 3H; Me-35), 1.728 (s, 3H; Me-34), 1.75 (m, 2H; 2-H, 11-H), 1.763 (s, 3H; Me-33), 1.804 (s, 3H; Me-27), 1.82 (m, 12-H), 1.92 (m, 2H; 7-H, 11-H), 1.93 (m, 12-H), 2.18 (m, 13-H), 2.2–2.3 (m, 4H; 19-H, 23-H), 2.22 (m, 15-H), 2.30 (m, 5H; 20-H, 24-H, 15-H), 2.41 (m, 2H; 16-H), 5.04 (s, 32-H), 5.21 (s, 32-H), 5.36 (brt, J=6.8 Hz, 25-H), 5.41 (brt, J=6.6 Hz, 21-H), 5.47 ppm (brt, J=6.6 Hz, 17-H); ^{13}C NMR (150 MHz, C_6D_6): δ=15.37 (q, C-31), 15.60 (q, C-30), 16.12 (q, C-34), 16.18 (q, C-33), 17.74 (q, C-35), 18.77 (t, C-2), 19.81 (t, C-6), 21.49 (q, C-29), 25.79 (t, C-11, C-12), 25.86 (q, C-27), 27.07 (t, C-20), 27.23 (t, C-24), 27.55 (t, C-16), 33.16 (s, C-4), 33.73 (q, C-28), 37.37 (s, C-10), 38.13 (t, C-15), 40.18 (t, C-23), 40.20 (t, C-19), 40.24 (t, C-1), 41.45 (t, C-7), 42.89 (t, C-3), 43.98 (s, C-8), 57.55 (d, C-5, C-13), 63.35 (d, C-9), 110.6 (d, C-32), 124.8 (d, C-25), 124.9 (d, C-17), 124.9 (d, C-21), 131.1 (s, C-26), 134.9 (s, C-18), 135.1 (s, C-22), 149.2 ppm (s, C-14). The following ^{13}C NMR signals are indistinguishable from each other as they have very similar chemical shifts: C-1/C-19/C-23, C-20/C-24, C-33/C-34, C-17/C-21/C-25, C-18/C-22. MS (EI): m/z (%): 69 (100), 81 (56), 191 (31), 231 (55), 232 (32), 463 (3), 478 (11) [M^+]; HRMS (EI): m/z: calcd for $C_{35}H_{58}$: 478.4539; found: 478.4456; $[\alpha]_D^{25}=-17.9$ (c=0.54 in $CHCl_3$); oil.

Product 22: ^{1}H NMR (600 MHz, C_6D_6): δ=0.952 (s, 3H; Me-30), 0.961 (s, 3H; Me-28), 1.029 (s, 3H; Me-29), 1.05 (m, 1-H), 1.06 (d, J=6.3 Hz, Me-33), 1.074 (s, 3H; Me-32), 1.18 (s, 3H; Me-31), 1.26 (m, 19-H), 1.29 (m, 3-H), 1.40 (m, 16-H), 1.48 (m, 5-H), 1.52 (m, 2H; 3-H, 11-H), 1.59 (m, 2-H), 1.60 (m, 18-H), 1.62 (m, 15-H), 1.63 (m, 11-H), 1.65 (m, 17-H), 1.687 (s, 3H; Me-35), 1.73 (m, 2-H), 1.74 (m, 1-H), 1.78 (m, 15-H), 1.794 (s, 3H; Me-34), 1.80 (m, 12-H), 1.805 (s, 3H; Me-27), 1.90 (m, 19-H), 1.94 (m, 12-H), 1.97 (m, 6-H), 2.04 (m, 16-H), 2.17 (m, 20-H), 2.23 (m, 6-H), 2.25 (m, 2H; 23-H), 2.31 (m, 20-H), 2.32 (m, 2H; 24-H), 2.42 (brm, 9-H), 5.37 (brt, J=6.8 Hz, 25-H), 5.47 (m, 7-H), 5.48 ppm (m, 21-H); ^{13}C NMR (150 MHz, C_6D_6): δ=13.35 (q, C-30), 16.12 (q, C-34), 17.72 (q, C-35), 18.52 (t, C-2), 18.82 (q, C-33), 19.45 (t, C-11), 21.51 (q, C-29), 22.46 (q, C-32), 24.72 (t, C-6), 25.73 (t, C-20), 25.83 (q, C-27), 27.20 (t, C-24), 27.61 (q, C-31), 28.82 (t, C-16), 33.15 (q, C-28), 33.30 (s, C-4), 34.41 (t, C-12, C-15), 35.42 (s, C-10), 35.61 (t, C-19), 36.10 (d, C-18), 39.31 (t, C-1), 40.25 (t, C-23), 42.72 (t, C-3), 43.88 (s, C-13), 49.49 (d, C-9), 51.69 (d, C-5), 51.74 (s, C-14), 53.72 (d, C-17), 118.5 (d. C7), 124.9 (d, C-25), 125.6 (d, C-21), 131.1 (s, C-26), 134.7 (s, C-22), 146.1 ppm (s, C-8); MS (EI): m/z (%): 57 (100), 69 (72), 97 (47), 463 (32), 478 (7) [M^+]; HRMS (EI): m/z: calcd for $C_{35}H_{58}$: 478.4539; found: 478.4529. $[\alpha]_D^{25}=+21.8$ (c=0.13 in $CHCl_3$); oil.

Product 23: ^{1}H NMR (600 MHz, C_6D_6): δ=0.89 (m, 1-H), 0.91 (brd, J=11.5 Hz, 5-H), 0.974 (s, 3H; Me-30), 0.985 (s, 3H; Me-29), 1.016 (s, 3H; Me-32), 1.029 (s, 3H; Me-28), 1.109 (s, 3H; Me-31), 1.23 (m, 11-H), 1.25 (m, 15-H), 1.27 (m, 3-H), 1.30 (m, 12-H), 1.37 (m, 7-H), 1.47 (m, 3H; 2-H, 6-H, 9-H), 1.50 (m, 3-H), 1.62 (m, 2H; 2-H, 6-H), 1.66 (m, 11-H), 1.69 (m, 16-H), 1.691 (s, 3H; Me-35), 1.72 (m, 7-H), 1.74 (m, 1-H), 1.76 (s, 3H; Me-34), 1.77 (m, 15-H), 1.809 (s, 3H; Me-27), 1.88 (m, 12-H), 1.90 (m, 13-H), 2.10 (m, 16-H), 2.23 (m, 2H; 23-H), 2.27 (m, 19-H), 2.30 (m, 2H; 24-H), 2.43 (m, 3H; 17-H, 20-H), 5.06 (1H, s, 33-H), 5.10 (1H, s, 33-H), 5.37 (brt, J=6.8 Hz, 25-H), 5.47 ppm (brt, J=6.8 Hz, 21-H); ^{13}C NMR (150 MHz, C_6D_6): δ=15.91 (q, C-31), 16.17 (q, 2C; C-32, C-34), 16.42 (q, C-30), 17.73 (q, C-35), 18.97 (t, C-6), 19.07 (t, C-2), 21.59 (q, C-29), 21.79 (t, C-11), 25.48 (t, C-12), 25.84 (q, C-27), 27.16 (t, C-24), 27.44 (t, C-20), 29.38 (t, C-16), 31.71 (t, C-15), 33.52 (s, C-4), 33.65 (q, C-28), 34.72 (t, C-19), 35.77 (t, C-7), 37.76 (s, C-10), 40.18 (t, C-23), 40.91 (t, C-1), 40.96 (s, C-8), 42.41 (t, C-3), 45.67 (d, C-13), 48.29 (d, C-17), 49.77 (s, C-14), 51.43 (d, C-9), 57.30 (d, C-5), 108.2 (t, C-33), 124.9 (d, C-21), 124.9 (d, C-25), 131.1 (s, C-26), 135.1 (s, C-22), 152.5 ppm (s, C-18). The 20-H and 24-H atoms may be exchangeable, as may be the assignments of C-2 and C-6, and that of C-20 and C-24. MS (EI): m/z (%): 69 (100), 95 (85), 191 (72), 221 (72), 261 (40), 355 (48), 368 (27), 429 (35), 478 (15) [M^+]; HRMS (EI): m/z: calcd for $C_{35}H_{58}$: 478.4539; found: 478.4503; $[\alpha]_D^{25}=+30.3$(c=0.20 in $CHCl_3$); oil.

Product 24: ^{1}H NMR (600 MHz, C_6D_6): δ=0.889 (s, 3H; Me-30), 0.896 (m, 1-H), 0.916 (brd, J=11.4 Hz, 5-H), 0.983 (s, 3H; Me-29), 1.020 (s, 3H; Me-32), 1.027 (s, 3H; Me-28), 1.099 (s, 3H; Me-31), 1.24 (m, 15-H), 1.30 (m, 3-H), 1.33 (m, 11-H), 1.43 (m, 7-H), 1.48 (m, 2H; 2-H, 6-H), 1.52 (m, 9-H), 1.53 (m, 3-H), 1.64 (m, 7-H), 1.65 (m, 15-H), 1.67 (m, 4H; 2-H, 6-H, 11-H, 12-H), 1.729 (s, 3H; Me-35), 1.74 (m, 1-H), 1.798 (s, 3H;

Me-34), 1.801 (s, 3H; Me-27), 1.829 (s, 3H; Me-33), 2.26 (m, 3H; 16-H, 23-H), 2.34 (m, 2H; 24-H), 2.37 (m, 16-H), 2.40 (m, 2H; 20-H), 2.43 (m, 2H; 19-H), 2.45 (m, 12-H), 2.50 (m, 13-H), 5.38 (brt, J=6.9 Hz, 25-H), 5.50 ppm (brt, J=7.0 Hz, 21-H); ^{13}C NMR (150 MHz, C_6D_6): δ=15.93 (q, C-31), 16.18 (q, C-34), 16.60 (q, C-30), 16.92 (q, C-32), 17.75 (q, C-35), 18.93 (t, C-2), 19.06 (t, C-6), 21.10 (q, C-33), 21.79 (t, C-11), 25.84 (q, C-27), 27.22 (t, C-24), 27.68 (t, C-12), 27.75 (q, C-29), 28.46 (t, C-20), 30.05 (t, C-16), 30.54 (t, C-15), 33.52 (s, C-4), 33.60 (q, C-28), 34.18 (t, C-19), 35.89 (t, C-7), 37.62 (s, C-10), 40.28 (t, C-23), 40.36 (s, C-8), 40.86 (t, C-1), 42.44 (t, C-3), 47.27 (d, C-13), 50.25 (s, C-14), 51.08 (d, C-9), 57.32 (d, C-5), 124.9 (d, C-25), 125.0 (d, C-21), 126.71 (s, C-18), 131.13 (s, C-26), 134.8 (s, C-22), 137.1 ppm (s, C-17); MS (EI): m/z (%): 69 (100), 81 (53), 135 (37), 137 (47), 191 (69), 341 (52), 463 (13), 478 (22) [M^+]; HRMS (EI): m/z: calcd for $C_{35}H_{58}$: 478.4539; found: 478.4563; $[\alpha]_D^{25}$=+41.7 (c=0.24 in $CHCl_3$); solid.

Product 25: ^{1}H NMR (600 MHz, C_6D_6): δ=0.976 (s, 3H; Me-29), 0.982 (s, 3H; Me-30), 1.005 (s, 3H; Me-28), 1.01 (m, 5-H), 1.04 (m, 1-H), 1.067 (s, 3H; Me-31), 1.28 (ddd, J=12.8, 12.8, 4.1 Hz, 3-H), 1.306 (s, 3H; Me-32), 1.46 (m, 2H; 2-H, 11-H), 1.50 (m, 3-H), 1.52 (m, 2H; 6-H, 9-H), 1.62 (m, 1-H), 1.64 (m, 11-H), 1.68 (m, 3H; 12-H, 15-H), 1.696 (s, 3H; Me-35), 1.72 (m, 6-H), 1.73 (m, 2-H), 1.733 (s, 3H; Me-34), 1.75 (m, 13-H), 1.777 (s, 3H; Me-33), 1.808 (s, 3H; Me-27), 1.90 (m, 7-H), 1.92 (m, 12-H), 2.12 (m, 7-H), 2.22 (m, 4H; 19-H, 23-H), 2.25 (m, 2H; 16-H), 2.29 (m, 4H; 20-H, 24-H), 5.37 (brt, J=6.8 Hz, 25-H), 5.41 (t, J=7.0 Hz, 21-H), 5.42 ppm (t, J=7.0 Hz, 17-H); ^{13}C NMR (150 MHz, C_6D_6): δ=16.13 (q, 2C; C-33, C-34), 16.58 (q, C-30), 17.74 (q, C-35), 18.89 (t, C-2), 20.17 (t, C-6), 21.55 (q, C-29), 21.70 (t, C-11), 23.25 (t, C-16), 24.69 (t, C-12), 25.85 (q, 2C; C-27, C-32), 26.83 (q, C-31), 27.03 (t, C-24), 27.22 (t, C-20), 33.17 (s, C-4), 33.68 (q, C-28), 37.48 (s, C-10), 38.33 (t, C-7), 40.20 (t, 2C; C-19, C-23), 41.12 (t, C-1), 42.67 (weak brt, C-15), 42.69 (t, C-3), 45.02 (s, C-8), 57.12 (d, C-5), 59.06 (d, C-13), 60.39 (d, C-9), 75.47 (s, C-14), 124.7 (d, C-21), 124.9 (d, C-25), 125.4 (d, C-17), 131.1 (s, C-26), 134.95 (s, C-18), 135.06 ppm (s, C-22). The assignments of 11-H and 12-H and those of C11 and C12 may be exchangeable. The carbon signals of C-18 and C-22 are indistinguishable as they have similar chemical shifts. MS (EI): m/z (%): 69 (100), 81 (70), 95 (52), 137 (43), 191 (58), 231 (42), 478 (14) [M^+-H_2O]; HRMS (EI): m/z: calcd for $C_{35}H_{58}$ [M^+-H_2O]: 478.4539; found: 478.4573; $[\alpha]_D^{25}$=+31.5 (c=0.86 in $CHCl_3$); oil.

Product 26: ^{1}H NMR (600 MHz, C_6D_6): δ=0.963 (s, 3H; Me-30), 0.968 (s, 3H; Me-28), 1.03 (s, 3H; Me-29), 1.04 (m, 1-H), 1.059 (s, 3H; Me-32), 1.112 (d, J=6.4 Hz, Me-33), 1.185 (s, 3H; Me-31), 1.30 (m, 2H; 3-H, 19-H), 1.43 (m, 16-H), 1.49 (dd, J=12.1, 5.5 Hz, 5-H), 1.53 (m, 11-H), 1.55 (m, 3-H), 1.58 (m, 18-H), 1.59 (m, 2-H), 1.63 (m, 11-H), 1.64 (m, 17-H), 1.65 (m, 15-H), 1.70 (s, 3H; Me-35), 1.73 (m, 2-H), 1.75 (m, 3H; 1-H, 15-H, 19-H), 1.77 (m, 12-H), 1.79 (s, 3H; Me-34), 1.81 (s, 3H; Me-27), 1.92 (m, 12-H), 2.00 (m, 6-H), 2.11 (m, 16-H), 2.17 (m, 20-H), 2.25 (m, 2H; 23-H), 2.26 (m, 6-H), 2.33 (m, 2H; 24-H), 2.34 (m, 20-H), 2.44 (brm, 9-H), 5.39 (brt, J=6.8 Hz, 25-H), 5.47 ppm (m, 2H; 7-H, 21-H); ^{13}C NMR (150 MHz, C_6D_6): δ=13.36 (q, C-30), 16.11 (q, C-34), 17.73 (q, C-35), 18.49 (t, C-2), 18.65 (q, C-33), 19.44 (t, C-11), 21.51 (q, C-29), 22.26 (q, C-32), 24.70 (t, C-6), 25.42 (t, C-20), 25.85 (q, C-27), 27.23 (t, C-24), 27.57 (q, C-31), 28.63 (t, C-16), 33.16 (q, C-28), 33.29 (s, C-4), 34.30 (t, C-12), 34.47 (t, C-15), 35.39 (s, C-10), 36.34 (d, C-18), 36.63 (t, C-19), 39.27 (t, C-1), 40.24 (t, C-23), 42.69 (t, C-3), 43.83 (s, C-13), 49.50 (d, C-9), 51.59 (s, C-14), 51.66 (d, C-5), 53.43 (d, C-17), 118.5 (d, C-7), 124.9 (d, C-25), 125.6 (d, C-21), 131.1 (s, C-26), 134.7 (s, C-22), 145.1 ppm (s, C-8) ; MS (EI): m/z (%): 69 (73), 221 (34), 281 (18), 285 (16), 355 (28), 429 (20), 463 (100), 478 (10) [M^+]; HRMS (EI): m/z: calcd for $C_{35}H_{58}$: 478.4539; found: 478.4535 ; $[\alpha]_D^{25}$=−38.5 (c=0.18 in $CHCl_3$); oil.

Product 27: ^{1}H NMR (400 MHz, C_6D_6): δ=0.906 (dd, J=12.0, 2.4 Hz, 5-H), 0.92 (m, 1-H), 0.988 (s, 3H; Me-29), 1.002 (s, 3H; Me-30), 1.016 (s, 3H; Me-28), 1.076 (s, 3H; Me-31), 1.129 (s, 3H; Me-32), 1.23 (m, 11-H), 1.25 (m, 3-H), 1.30 (m, 12-H), 1.36 (m, 15-H), 1.40 (m, 7-H), 1.48 (m, 9-H), 1.49 (m, 3-H), 1.50 (m, 2H; 2-H, 6-H), 1.64 (m, 2-H), 1.65 (m, 15-H), 1.66 (m, 11-H), 1.69 (m, 7-H), 1.694 (s, 3H; Me-35), 1.72 (m, 6-H), 1.76 (m, 1-H), 1.760 (s, 3H; Me-34), 1.811 (s, 3H; Me-27), 1.88 (m, 12-H), 1.90 (m, 16-H), 2.01 (m, 16-H), 2.03 (m, 13-H), 2.20 (m, 19-H), 2.23 (m, 2H; 23-H), 2.25–2.35 (m, 4H; 20-H, 24-H), 2.35 (m, 19-H), 2.75 (ddd, J=9.3, 9.3, 9.3 Hz, 17-H), 5.19 (s, 1H; 33-H), 5.21 (s, 1H; 33-H), 5.37 (brt, J=6.8 Hz, 25-H), 5.46 ppm (brt, J=6.8 Hz, 21-H); ^{13}C NMR (100 MHz, C_6D_6): δ=16.14 (q, 2C; C-31, C-34), 16.47 (q, C-30), 17.17 (q, C-32), 17.73 (q, C-35), 19.02 (t, C-2), 19.07 (t, C-6), 21.77 (q, C-29), 22.16 (t, C-11), 25.43 (t, C-12), 25.85 (q, C-27), 27.19 (t, C-24), 27.71 (t, C-20), 28.69 (t, C-16), 33.42 (t, C-15), 33.52 (s, C-4), 33.59 (q, C-28), 35.37 (t, C-7), 37.71 (s, C-10), 39.09 (t, C-19), 40.20 (t, C-23), 40.87 (t, C-1), 41.29 (s, C-8), 42.41 (t, C-3), 44.35 (d, C-17), 44.97 (d, C-13), 50.23 (s, C-14), 51.34 (d, C-9), 57.28 (d, C-5), 109.4 (t, C-33), 124.88 (d, C-25), 124.91 (d, C-21), 131.1 (s, C-26), 135.1 (s, C-22), 152.2 ppm (s, C-18). The assignments of 2-H and 6-H, C-2 and C-6, and C-20 and C-24 may be exchangeable. The carbon signals of C-21 and C-25 are indistinguishable as they have similar chemical shifts. MS (EI): m/z (%): 69 (51), 95 (56), 123 (31), 191 (100), 231 (13), 299 (13), 478 (10) [M^+]; HRMS (EI): m/z: calcd for $C_{35}H_{58}$: 478.4539; found: 478.4510; $[\alpha]_D^{25}$=−13.4 (c=0.26 in $CHCl_3$); oil.

Product 28: ^{1}H NMR (600 MHz, C_6D_6): δ=0.878 (dd, J=12.3, 2.0 Hz, 5-H), 0.95 (ddd, J=13.0, 13.0, 3.3 Hz, 1-H), 0.981 (s, 6H; Me-29, Me-30), 1.032 (s, 3H; Me-28), 1.12 (dd, J=13.0, 7.0 Hz, 9-H), 1.156 (s, 3H; Me-31), 1.21 (ddd, J=12.5, 12.5, 2.8 Hz, 7-H), 1.31 (ddd, J=13.4, 13.4, 3.7 Hz, 3-H), 1.370 (s, 3H; Me-32), 1.43 (t, J=10.0 Hz, 13-H), 1.46 (m, 11-H), 1.50 (m, 2H; 2-H, 6-H), 1.53 (m, 1-H), 1.55(m, 3-H), 1.57 (m, 2H; 15-H), 1.58 (m, 11-H), 1.62 (m, 6-H), 1.699 (s, 3H; Me-35), 1.72 (m, 2-H), 1.743 (s, 3H; Me-33), 1.76 (m, 12-H), 1.778 (s, 3H; Me-34), 1.813 (s, 3H; Me-27), 1.93 (m, 12-H), 2.15 (ddd, J=12.5, 2.8, 2.8 Hz, 7-H), 2.22 (m, 2H; 16-H), 2.24 (m, 4H; 19-H, 23-H), 2.33 (m, 4H; 20-H, 24-H), 5.37 (brt, J=6.7 Hz, 25-H), 5.42 (t, J=6.7 Hz, 17-H), 5.44 ppm (t, J=6.8 Hz, 21-H); ^{13}C NMR (150 MHz, C_6D_6): δ=15.95 (q, C-34), 15.99 (q, C-33), 16.08 (q, C-31), 16.13 (q, C-30), 17.73 (q, C-35), 18.72 (t, C-2), 19.56 (t, C-6), 19.64 (t, C-11), 21.43 (q, C-29), 22.33 (t, C-12), 23.29 (t, C-16), 25.84 (q, C-27), 26.10 (q, C-32), 27.03 (t, C-20), 27.22 (t, C-24), 33.10 (s, C-4), 33.68 (q, C-28), 37.27 (s, C-10), 40.17 (t, C-19), 40.20 (t, C-23), 40.54 (t, C-1), 42.86 (t, C-3), 42.95 (t, C-7), 44.40 (s, C-8), 44.40 (t, C-15), 57.41 (d, C-5), 59.14 (d, C-13), 63.43 (d, C-9), 74.50 (s, C-14), 124.7 (d, C-17), 124.9 (d, C-25), 125.3 (d, C-21), 131.1 (s, C-26), 134.9 (s, C-18), 135.1 ppm (s, C-22). The following ^{13}C NMR signals are indistinguishable from each other as they have very similar chemical shifts: C-3/C-7, C-19/C-23, C-20/C-24, C-6/C-11, C-30/C-31/C-33/C-34. MS (EI): m/z (%): 69 (100), 81 (58), 137 (45), 191 (36), 231 (88), 478 (7), 496 (1) [M^+]; HRMS (EI): m/z: calcd for $C_{35}H_{60}O$: 496.4644; found: 496.4643; $[\alpha]_D^{25}$=+7.12 (c=1.75 in $CHCl_3$); oil.

Product 29: ^{1}H NMR (600 MHz, C_6D_6): δ=0.93 (dd, J=12.0, 2.0 Hz, 5-H), 0.93 (m, 1-H), 0.994 (s, 3H; Me-29), 1.01 (s, 3H; Me-30), 1.024 (s, 3H; Me-28), 1.092 (s, 3H; Me-31), 1.22 (m, 11-H), 1.221 (s, 3H; Me-32), 1.23 (m, 15-H), 1.28 (m, 3-H), 1.309 (s, 3H; Me-33), 1.37 (m, 12-H), 1.40 (m, 2-H), 1.42 (m, 7-H), 1.50 (m, 6-H), 1.52 (m, 2H; 3-H, 9-H), 1.54 (m, 15-H), 1.60 (m, 16-H), 1.63 (m, 3H; 2-H, 19-H), 1.68 (m, 11-H), 1.694 (s, 3H; Me-35), 1.73 (m, 7-H), 1.74 (m, 6-H), 1.76 (m, 12-H), 1.779 (s, 3H; Me-34), 1.78 (m, 1-H), 1.813 (s, 3H; Me-27), 2.07 (m, 16-H), 2.10 (m, 17-H), 2.13 (m, 13-H), 2.22 (m, 2H; 20-H), 2.23 (t, J=7.1 Hz, 23-H), 2.31 (m, 2H; 24-H), 5.36 (brt, J=6.8 Hz, 25-H), 5.42 ppm (t, J=7.1 Hz, 21-H); ^{13}C NMR (150 MHz, C_6D_6): δ=16.12 (q, C-34), 16.18 (q, C-31), 16.52 (q, C-30), 17.17 (q, C-32), 17.76 (q, C-35), 19.13 (t, C-2), 19.17 (t, C-6), 21.79 (q, C-29), 22.89 (t, C-11), 23.48 (t, C-20), 25.87 (q, C-27), 26.45 (t, C-16), 26.76 (t, C-12), 27.15 (t, C-24), 27.71 (q, C-33), 32.79 (t, C-15), 33.57 (q, C-28), 33.63 (s, C-4), 35.75 (t, C-7), 37.72 (s, C-10), 40.20 (t, C-23), 40.89 (t, C-1), 41.20 (s, C-8), 42.50 (t, C-3), 42.69 (t, C-19), 44.10 (d, C-13), 48.43 (d, C-17), 49.49 (s, C-14), 51.15 (d, C-9), 57.35 (d, C-5), 74.55 (s, C-18), 124.9 (d, C-25), 125.4 (d, C-21), 131.2 (s, C-26), 134.9 ppm (t, C-22). The following ^{1}H and ^{13}C NMR signal may be exchangeable: 2-H/6-H, C-2/C-6, C-11/C-12/C-16. MS (EI): m/z (%): 69 (100), 81 (54), 95 (77), 123 (50), 137 (54), 177 (53), 191 (72), 221 (32), 281 (18), 301 (20), 385 (25), 429 (21), 478 (8), 496 (4) [M^+]; HRMS (EI): m/z: calcd for $C_{35}H_{58}$ [M^+-H_2O]: 478.4539; found: 478.4513; $[\alpha]_D^{25}$=+7.12 (c=1.75 in $CHCl_3$); oil.

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