A R T I C L E

Enzymatic cyclization reactions of geraniol, farnesol and geranylgeraniol, and those of truncated squalene analogs having C$_{20}$ and C$_{25}$ by recombinant squalene cyclase†

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The substrate specificity of squalene–hopene cyclase was investigated using the C$_{10}$–C$_{25}$ analogs including naturally occurring substances, *e.g.* geraniol (C$_{10}$), farnesol (C$_{15}$) and geranylgeraniol (C$_{20}$). No cyclization occurred for geraniol, but a significantly high conversion ratio (64%) was observed for farnesol, yielding the cyclic sesquiterpenes consisting of 6/6-fused bicyclic ring systems. Among them, an attractive compound having C$_{30}$ was produced, in the structure of which acyclic the farnesol unit is linked to the bicyclic skeleton through ether linkage. Conversion of geranylgeraniol was low (*ca.* 12%). The squalene analogs having C$_{20}$ and C$_{25}$ also were cyclized in yields of *ca.* 33–36%, but the analogs having the methyl group at C(7) and/or at C(11) underwent no cyclization; the large steric bulk size of C(7)–Me and/or C(11)–Me, which is arranged in α-disposition for all the pre-chair conformation, would have interacted repulsively with the cyclase recognition site near to the C(7) and/or C(11), resulting in no construction of the all-chair conformation inside the reaction cavity. A relatively low yield of geranylgeraniol indicated that a less bulky hydrogen atom must be located at C(14) for the efficient polycyclization reaction. The squalene cyclase shows remarkably broad substrate specificity to accept the truncated analogs having carbon-chain lengths of C$_{15}$–C$_{25}$ in addition to C$_{30}$.

Introduction

The polycyclization of squalene **1** into pentacyclic hopene **2** and hopanol **3** is one of the most complicated biochemical reactions (Scheme 1)[1–4] which is mediated by squalene–hopene cyclases (SHCs) [EC 5.4.99-] from prokaryotic species. The polycyclization reaction proceeds under precise enzymatic control to form five new rings and nine new chiral centers.[1] This polycyclization mechanism is analogous to that catalyzed by eukaryotic oxidosqualene cyclases (OSCs) which lead to lanosterol and numerous plant triterpenes from (3*S*)-2,3-oxidosqualene.[2–4] Investigations of site-directed mutants and substrate analogs [1,2] have led to the proposal that this polycyclization consists of eight reaction steps as shown in Scheme 1: (1) First, cyclization to form A-ring **4** by proton attack on the terminal double bond,[5] (2) second, ring closure to give the B-ring (6/6-fused A/B ring system **5**),[6] (3) third, cyclization to yield a five-membered C-ring (6/6/5-fused A/B/C-tricyclic ring system **6**) by Markovnikov closure, (4) which then undergoes ring expansion to form the six-membered C-ring (6/6/6-fused tricyclic ring system **7**),[7] (5) fifth, cyclization to give the thermodynamically favored five-membered D-ring (6/6/6/5-fused A/B/C/D ring system **8**, 17-*epi*-dammarenyl cation),[8] (6) followed by the second ring enlargement process to form the six-membered D-ring (6/6/6/6-fused A/B/C/D-ring system, prohopanyl cation **9**),[9,10] (7) the last ring closure process to construct the 6/6/6/6/5-fused A/B/C/D/E-ring system (**10**, hopanyl cation),[10] and (8) the final deprotonation reaction to introduce the double bond between C-22 and C-29. This deprotonation reaction occurs exclusively from the (23*Z*)-methyl group.[1,11,12] The polycyclization reaction is featured by the folding of **1** into an all-pre-chair conformation during the multiple reaction steps.

The substrate specificity of the SHC from *A. acidocaldarius* is remarkably broad. (3*R*)- and (3*S*)-oxidosqualenes were converted into 3α- and 3β-hydroxyhopane skeletons, respectively.[12,13] The norsqualenes lacking a methyl group at the terminal position afforded a 6/6/6/6/6-fused pentacyclic ring system (tetrahymanol skeleton) in high yields.[12,14] The C(10)–norsqualene was also
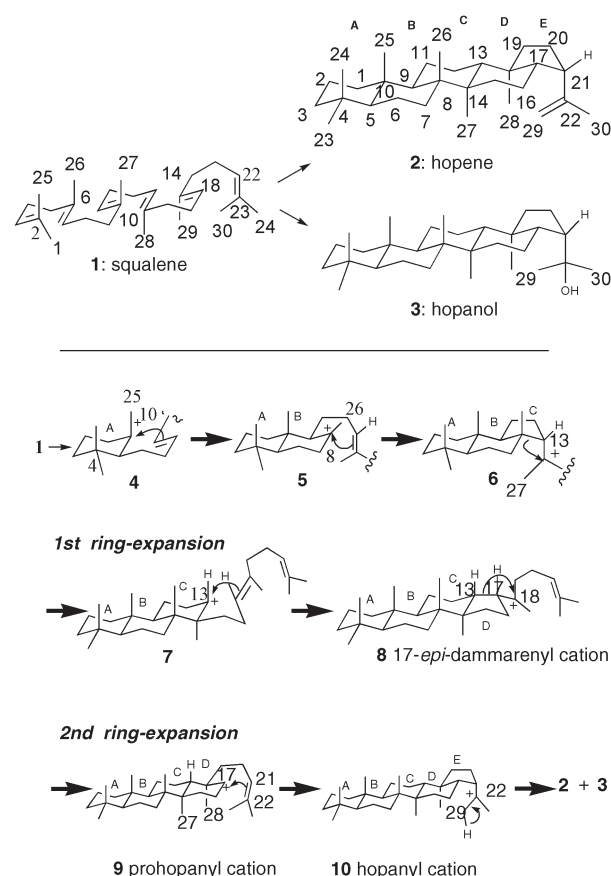
Scheme 1 Cyclization pathway of squalene **1** into hopene **2** and hopanol **3** by squalene–hopene cyclase (SHC).

converted in a high yield into the unprecedented carbocyclic skeleton(s) having 6/5 + 5/5 + (6) ring system(s).[15] On the other hand, the norsqualene lacking the C(15)–Me was converted into hopane and isohopane skeletons.[15] The bisnorsqualene lacking two methyl groups at C(23) was also efficiently converted to the products having 6/6/6/5-fused tetracyclic and 6/6/6/6/6-fused pentacyclic ring

† Electronic supplementary information (ESI) available: additional characterization data for products **21**–**30**. See http://www.rsc.org/suppdata/ob/b4/b407001a/

systems.[12] The terminal methyl groups have a crucial role for the stereochemical control during the polycyclization cascade leading to the hopane skeleton.[12] The C(6)–norsqualene was cyclized into the hopane skeleton in significantly high yields.[16] The regioisomers of **1** with respect to the methyl position were also examined whether they can be accepted as the substrates.[16] The presence of a methyl group at C(7) or C(11) gave no cyclization, but the analog possessing a methyl group at C(18) instead of C(19) of **1** underwent the polycyclization in a quantitative yield.[16] The truncated C_{27}- and C_{22}-analogs having an alcoholic group also were efficiently cyclized into the compounds with $6/6/6/5$ + tetrahydrofuran (THF) ring[1,8] and $6/6/5$ + THF ring,[1,7] respectively, indicating that the carbocationic intermediates **6** and **8** are involved in the polycyclization reaction of **1**. The dihydroxysqualenes, *e.g.* 6,7-erythro-dihydroxysqualene, were converted into the skeleta having $6/6/5$ + THF ring systems.[1] These substrate analogs were used to trap the transient carbocationic intermediates generated during the polycyclization reaction. The cationic intermediates **4** and **5** also have been successfully trapped by other squalene analogs having the highly nucleophilic hydroxyl group(s).[17,18] The enzymatic conversions of the substrate analogs by the SHC were also reported by other workers.[19–24] The C_{31} analogs having a methylidene appendage were examined to show whether they can be converted to the enzymic product(s) or irreversible inhibitors for the SHC activity.[19,22,23] The elongated C_{35}-analog also was accepted as the substrate of the SHC, affording the product having a 6/6/6/6/6/5-fused hexacyclic ring system in a yield of 10%.[24]

The broad substrate specificity of the prokaryotic SHC has prompted us to examine whether the SHC can accept the truncated analogs of **1**. Little is known about the cyclization reaction of the truncated analogs having C_{10}–C_{25}. The following three acyclic isoprenoid compounds were selected: geraniol **11** (C_{10}), farnesol **12** (C_{15}) and geranylgeraniol **13** (C_{20}), which are physiologically important substances frequently encountered in nature. In addition, the chemically synthesized analogs **14**–**16** having C_{20} and C_{25} were also examined.

Structures of these compounds are illustrated in Fig. 1. Very interestingly, farnesol **12** was cyclized in a relatively high conversion yield (64%), but geraniol **11** underwent no cyclization reaction. Geranylgeraniol **13** had a low conversion ratio (12%). Analog **14** had no conversion, but **15** (a regio-isomer with respect to the methyl position) underwent the polycyclization reaction in a yield of 33%, despite **14** and **15** having an identical carbon-length (C_{20}). This finding suggested that the methyl position has a great influence upon the polycyclization cascade. Analog **16** having a longer chain length of C_{25} also could be cyclized (36%). The present investigations give deeper insight into the polycyclization mechanism of **1**, especially regarding the importance of the methyl position and minimum carbon-chain length required for the occurrence of the enzymatic cyclization reaction. Herein we describe the experimental results and discuss the cyclization mechanisms of the truncated analogs.

Results

Syntheses of truncated analogs 14–16

The synthetic methods are shown in Scheme 2. Farnesyl bromide **17** and 1-bromo-3-methyl-but-2-ene **19** were prepared from the corresponding farnesol **12** and 3-methyl-but-2-en-1-ol **18**, respectively, by a treatment of PBr$_3$ in THF.[16] The bromides **17** and **19**, thus prepared, were subjected to the allyl coupling reaction by using pyrrolidine/*n*-BuLi/CuI,[25,26] leading to a mixture of the desired **15** and **1** as major products. To obtain the pure **15**, AgNO$_3$–SiO$_2$ column chromatography was carried out to remove **1**. To prepare **14** and **16**, a mixture of **17** and geranyl bromide **20** in Et$_2$O was added into the metal complex of pyrrolidine/*n*-BuLi/CuI according to the same method as described above. Each of the products was isolated by 5% AgNO$_3$–SiO$_2$ column chromatography in a pure state.

GC analyses of enzymatic products from analogs 11–16

Fig. 2 shows the gas chromatograms of the incubation mixtures prepared by incubating **1** and **11**–**16** with the cell-free homogenates
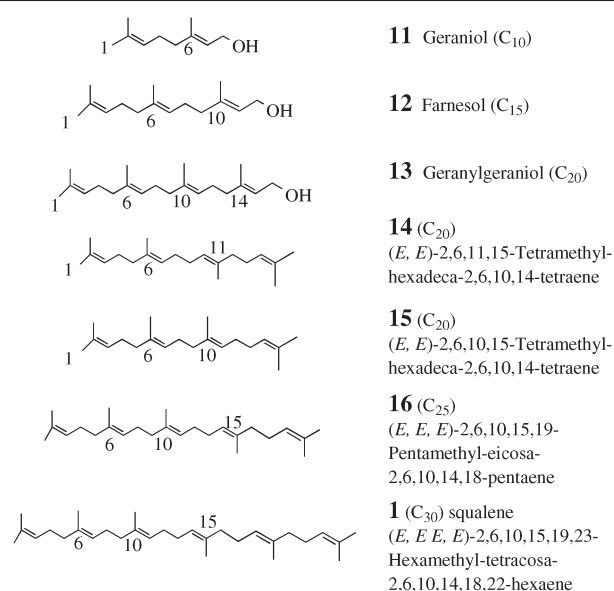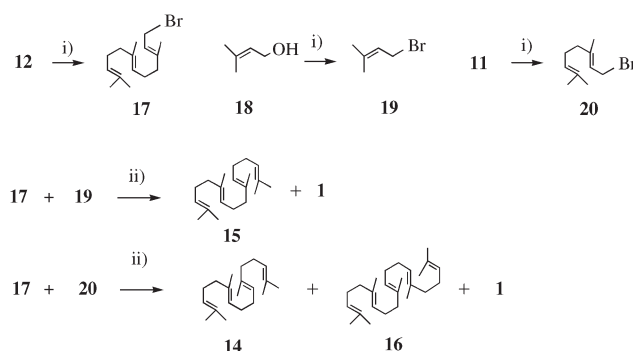

Fig. 1 Structures of compounds employed in this study. The methyl position of **14** is different from that of **15**. Analog **14** has a methyl group at C-11, while **15** at C-10.

Scheme 2 Synthetic scheme of truncated analogs **14**–**16**. Reagents and conditions: (i) PBr$_3$/ THF, 0 °C, (ii) CuI/pyrrolidine/n-BuLi/ Et$_2$O, −38 °C.

of *E. coli* clone encoding the native SHC from *Alicyclobacillus acidocaldarius*.[5,13] Identical incubation conditions were employed to compare the quantities and distribution pattern of enzymic products obtained from each of the substrate analogs. Incubation conditions were as follows: substrate analog 3 μmol, the cell-free extract 1.5 ml as the enzyme source, Triton X-100 20 mg, optimal pH 6.0, optimal temperature 60 °C, incubation time 16 h, total volume 5 ml. To the reaction mixture, was added 5% KOH/MeOH and the products were extracted with hexane. Triton X-100 included in the hexane-extracts was removed with a short SiO$_2$ column chromatography eluting with a mixed solvent of hexane/EtOAc (100 : 20). Fig. 2G shows the product distribution pattern obtained by incubating **1**. Only a small amount of substrate **1** remained in the incubation mixture, indicating almost full conversion of **1** into **2** and **3**. No reaction was observed for **11** having C_{10} (Fig. 2A), the conversion being negligible if present. In contrast, **12** with C_{15} was converted in high yields to four products **21**–**24**, as shown in Fig. 2B. As described below, a strongly high polar product **23** (a diol) was found during the isolation process of the enzymic products by a SiO$_2$ column chromatography. Thus, the incubation mixture was extracted with hexane/EtOAc (50 : 50) instead of hexane usually used. The extract was subjected to the passage through a short SiO$_2$ column by eluting with hexane/EtOAc (100 : 35), but Triton X-100 was in part detected on the gas chromatogram (asterisk symbol in Fig. 2B). Product **24** had a longer retention time. Geranylgeraniol **13** afforded a single product **25** in a yield of 12% (Fig. 2C). No conversion was found for **14** having C_{20}-chain length (Fig. 2D), but the conversion ratio of **15** having the same carbon number as **14** was relatively high (28% for **26** and 5% for **27**, total 33%, Fig. 2E). Analog **15** has a methyl group at position of C-10, while **14** does a methyl group at C-11.

These compounds are the regio-isomers with respect to the methyl position. This finding indicates that the occurrence of the polycyclization reaction depends on the position of the branching methyl group on the linear backbone. Substrate analog **16** having a longer carbon chain (C_{25}) was converted into **28**–**30** in a total yield of 36% (6% for **28**, 4% for **29** and 26% for **30**, Fig. 2F)
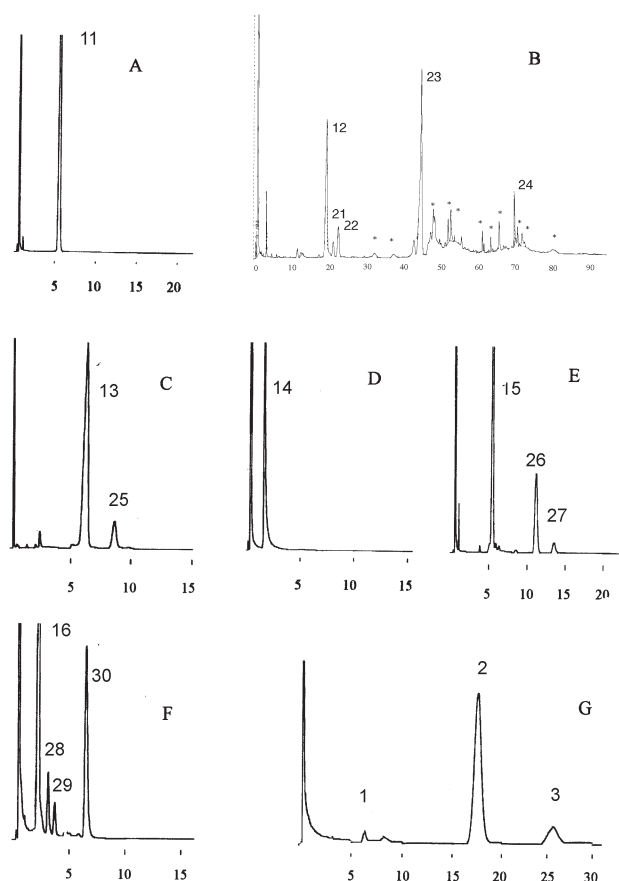

Fig. 2 Gas chromatograms of the reaction mixtures obtained by incubating **11**–**16** and **1** with the wild-type SHC. A: geraniol **11** (C_{10}). B: farnesol **12** (C_{15}). C: geranylgeraniol **13** (C_{20}). D: analog **14** (C_{20}). E: analog **15** (C_{20}). F: analog **16** (C_{25}). G: squalene **1** (C_{30}). Column temperatures: A: 200 °C; B: 150 °C for 0–40 min, and elevated at a rate 5 °C min^{-1} to 290 °C for 40–68 min, and 68–100 min at 290 °C; C, D and E: 250 °C; F and G: 270 °C, N_2 carrier pressure being 1.0 kg cm^{-2} except for B, which was conducted at the pressure of 0.5 kg cm^{-2}. In case of B, Triton X-100 remained in part due to a high polarity of the elution solvent used, the symbol * shows Triton X-100 and impurities.

Isolation and structure determination of enzymatic products 21–24 from farnesol 12

To obtain pure products **21**–**24**, a large-scale incubation was conducted by using 100 mg of **12** and 200 ml of the cell-free homogenates from 4L-culture of the cloned *E. coli.* The lipophilic extract from the incubation mixture was subjected to a SiO_2 column chromatography with a gradient elution using a mixed solvent of hexane and EtOAc (100 : 3 ~ 100 : 30). Products **21**, **22** and **24** were inseparable, but **23** was isolated in a pure state (6.3 mg). A mixture of **21**, **22** and **24** was again subjected to a column chromatography over SiO_2 using hexane/EtOAc (100 : 1.0), giving a pure sample of **24** (8.7 mg), but the separation of **21** and **22** failed. A rechromatography using hexane/EtOAc (100 : 0.5) successfully afforded pure **21** and **22** in the isolation yields of 4.7 mg and 2.0 mg, respectively. The R_f value of product **24** on SiO_2 TLC was 0.40, while those of **21** and **22** were 0.37–0.38 (nearly identical), by developing with hexane/EtOAc (100 : 20). On the other hand, a high polar product **23** had the R_f value of 0.38 for the solvent of hexane/EtOAc (50 : 50). The product distribution ratio was estimated by the GC analysis to be as follows: 3, 7, 45, 9 and 35% for **21**–**24** and the recovered **12**, respectively (Fig. 2B). Structures of these products were

determined by the detailed NMR analyses including DEPT, COSY 45, HOHAHA, NOESY, HMQC and HMBC pulse sequences.

Product **22** had one allylic methyl group (δ_H 1.97, br s, Me-15) and three methyl groups at higher fields (δ_H 0.949, 6H, s, Me-12 and Me-14; δ_H 0.966, 3H, s, Me-13). The clear cross peaks of Me-14 and Me-15 with C-9 (δ_C 57.45, d) were observed in the HMBC spectrum. Furthermore, strong HMBC cross peaks of H-11 (δ_H 3.75, m; δ_H 3.63, m) with C-9 and C-10 (δ_C 36.19, s) were also found. An olefinic proton (δ_H 5.62, br s) had HMBC cross peaks with C-5 (δ_C 50.12, d) and C-9. These NMR data indicated a bicyclic skeleton for **22** (Fig. 3). A strong NOE between H-5 (δ_H 1.25, m) and H-9 (1H, δ_H 1.86, very broad) suggested an α-orientation of H-9. Product **21** had a vinyl proton (δ_H 4.99, s; 4.75, s; δ_C 106.6, t), which had clear HMBC correlations with C-9 (δ_C 59.32, d) and C-7 (δ_C 38.12, t). A clear spin–spin coupling between H-9 (δ_H 1.94, m) and H-11 (δ_H 3.86, dd, *J* 10.8, 3.4; δ_H 3.78, dd, *J* 10.8, 10.8) was observed in the COSY 45 spectrum. These NMR data clarified the double bond position. Product **23** was a very highly polar compound, as described above, and involved no double bond in the ^{13}C NMR. In addition to hydroxymethyl group (2H, δ_H 3.97, d, *J* 6.9, H-11; δ_C 60.9, t), a tertiary alcoholic carbon (δ_C 74.5, s, C-8) was found, thus indicating that **23** was a diol product. A clear HMBC cross peak between Me-15 (δ_H 1.38, s) and C-8 suggested that the hydroxyl group was attached to C-8. A definitive NOE between Me-15 and Me-14 (δ_H 0.70, s) proved the β-arrangement for Me-15. Product **24** had an attractive structure as shown in Fig. 3. The ^{13}C NMR spectrum showed the presence of C_{30}, but EIMS did not give the molecular ion. HRMS (FAB, positive, glycerol) showed that the molecular composition was $C_{30}H_{53}O_2$ (M + H$^+$, observed, 445.4052 requiring 445.4046). The detailed NMR analyses indicated the involvement of the bicyclic skeleton of **23** in **24**. The ^{1}H NMR spectrum showed the presence of three olefinic protons (δ_H 5.56, br t, *J* 6.5; δ_H 5.35, t, *J* 6.8; δ_H 5.37, t, *J* 6.8) and four allyl methyl groups (δ_H 1.70, 6H, s; δ_H 1.72, 3H, s; δ_H 1.81, 3H, s), suggesting that the acyclic farnesyl moiety is involved in **24**. The linkage of the farnesyl residue and the bicyclic skeleton of **23** was established by the clear HMBC cross peak between H-16 (δ_H 3.99, 2H, dd, *J* 6.1, 3.8) and C-8 (δ_C 80.13, s). Thus, the structure of **24** was determined as shown in Fig. 3, which is composed of two farnesol units. A nucleophilic attack of the hydroxyl group of the linear farnesol molecule occurred toward the C-8 cation of the bicyclic skeleton that was in advance generated by the cyclization reaction of the alternative farnesol molecule.

Enzymatic product 25 from geranylgeraniol 13

Sixty mg of geranylgeraniol **13** was incubated with the cell-free extracts (200 mg) under the optimum catalytic conditions. A SiO_2 column chromatography eluting with hexane/EtOAc (100 : 5) gave **25** in a pure state (6.3 mg). Product **25** had one double bond (δ_H 5.63, br s, H-12; δ_C 123.4, d, C-12; δ_C 133.5, s, C-13). The clear HMBC cross peaks of H-15 (δ_H 3.75, dd, *J* 10.8, 3.2; δ_H 3.62, dd, *J* 10.8, 5.6) were found for C-14 (δ_C 58.04, d), C-8 (δ_C 36.34, s) and C-13. The double bond position was further confirmed by the clear HMBC cross peaks of the allyl methyl group (δ_H 1.99, s, Me-20) for C-12 and C-13. The detailed 2D NMR analyses showed that **25** had a 6/6/6-fused tricyclic ring system as shown in Fig. 3.

Enzymatic products 26 and 27 from analog 15 having C_{20}

Analog **15** (82 mg) was incubated for 20 h with the cell-free extracts (250 ml) at the optimum catalytic conditions. After removing the detergent from the hexane-extract, a careful column chromatography over SiO_2 was carried out by eluting with hexane/EtOAc (100 : 3), affording a pure **26** (*ca.* 3 mg), a mixture of **27** and **26** (*ca.* 3 mg), and pure **27** (*ca.* 1 mg) in this elution order, although complete separation was unsuccessful. The R_f values on SiO_2 TLC by developing with hexane/EtOAc (100 : 15) were as follows: 0.93, 0.32 and 0.31 for **15**, **26** and **27**, respectively, suggesting that products **26** and **27** were highly polar. This was further supported by the finding of the tertiary alcoholic carbon (δ_C 73.88, s, for C-14 of **26**; δ_C 72.55, s, for that of **27**) in the ^{13}C NMR spectrum. The methyl

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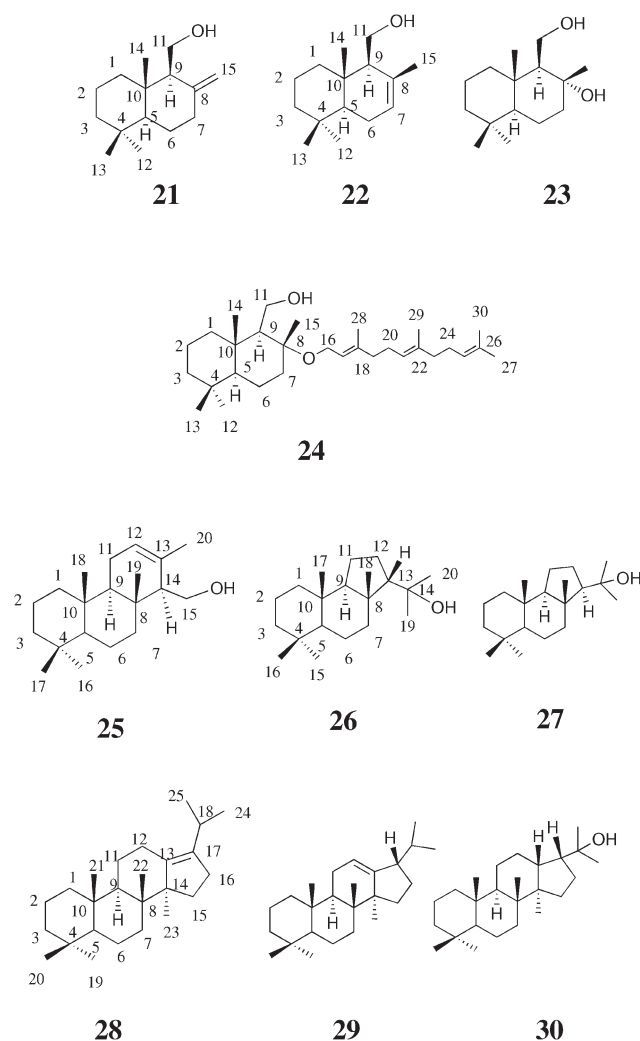**Fig. 3** Enzymic products obtained by incubating truncated analogs **11**–**16** with the wild-type SHC.

protons at C-19 and C-20 of **26** (δ_H 1.22, 3H, s, and δ_H 1.24, 3H, s) and those of **27** (δ_H 1.17, 3H, s; δ_H 1.34, 3H, s) had the clear HMBC cross peaks for C-14, indicating that the hydroxyl groups of **26** and **27** were attached to C-14. No olefinic proton resonance in the ^{1}H NMR and no sp^2 carbon in the ^{13}C NMR spectra suggested that **15** underwent the complete cyclization reaction. The detailed NMR analyses established that **26** and **27** had a tricyclic 6/6/5-fused ring system as shown in Fig. 3. The clear NOEs of Me-18 (δ_H 1.16, s)/Me-19 and Me-18/Me-20 were observed for **27**, but no NOE between them for **26**, indicating that H-13 of **27** had α-configuration, while that of **26** possessed β-orientation. The finding of a strong NOE between H-9 (δ_H 1.07, dd, J 13.2, 7.2) and H-13 (δ_H 1.29, t, J 10.1) for **27** further supported the configuration of 13α-H for **27**.

Enzymatic products 28–30 from analog 16

Fifty mg of substrate analog **16** was incubated for 20 h with the cell-free homogenates (200 ml) from 4L-culture of the wild-type SHC at the optimum catalytic conditions, as described above, and then the reaction mixture was lyophilized. Triton X-100 included in the hexane extract was removed by a passage through a short SiO$_2$ column (Hexane/EtOAc = 100:20). A column chromatography over SiO$_2$ with a gradient elution using hexane to hexane/EtOAc (100:10) afforded **30** in a pure state. A mixture of **28** and **29** was subjected to a 5% AgNO$_3$–SiO$_2$ column chromatography eluting with hexane/EtOAc (100:3), leading to the successful separation of **28** and **29**. Product **28** possessed two doublet methyl groups (δ_H 1.13, 3H, d, J 6.9 and δ_H 1.17, 3H, d, J 6.9 for Me-24 and Me-25), which had the correlations with H-18 (δ_H 2.85, septet, J 6.9) in the COSY 45 spectrum. A tetrasubstituted double bond (δ_C 136.4,

s, C-17; δ_C 138.0, s, C-13) was found in the ^{13}C NMR spectrum. Apparent HMBC cross peaks of H-18/C-13, Me-23 (δ_H 1.30, 3H, s)/C-13 and Me-24(25)/C-17 clarified the double bond position. The detailed NMR analyses established that **28** had a tetracyclic 6/6/6/5-fused ring system. Product **29** also consisted of a tetracyclic 6/6/6/5-fused ring system and had the trisubstituted double bond (δ_H 5.47, m, H-12, δ_C 118.1, d, C-12; δ_C 145.7, s, C-13). The double bond position was determined to be at C12–C13 by the strong HMBC correlations of Me-23, H-12 and H-18 for C-13. H-17 was shown to have β-orientation, since no NOE between H-17 and Me-23 was observed. The 6/6/6/5-fused tetracyclic ring skeleton of **30** was determined by the detailed 2D NMR analyses. The two methyl group protons (δ_H 1.21, s and δ_H 1.27, s; Me-24 and Me-25) had clear HMBC correlations for the alcoholic carbon (δ_C 72.98, s, C-18) and for C-17 (δ_C 50.07, d), indicating that isopropyl alcohol residue was attached to C-17. The HMBC cross peak of Me-23 (δ_H 1.17, 3H, s)/C-13 (δ_C 44.2) and the HMQC spectrum made clear the assignment of H-13 (δ_H 2.09, m). The apparently observed NOEs of Me-22 (δ_H 1.08, 3H, s)/H-13 and H-13/H-17 established β-orientation of H-17.

Enzymatic reactions of geraniol 11 and analog 14

These compounds were subjected to the enzyme reaction by using the amount of the cell-free homogenates five times higher than that of incubation conditions usually used, but no conversion was observed as shown in Fig. 2A and D.

Discussion

Cyclization reactions of geraniol 11, farnesol 12 and geranylgeraniol 13

If **11** (C$_{10}$) is cyclized, the monocyclic skeleton like **4** can be formed as shown in Scheme 3, but no conversion occurred (Fig. 2). On the other hand, a significantly high conversion ratio (64%) was observed for **12** (C$_{15}$) to give four products **21**–**24**, which could be formed from the common bicyclic intermediate **31** (like **5**). The deprotonation from the methyl group at C-8 could give **21** (path *a*). The deprotonation of H-7 gave **22** (path *b*). A nucleophilic attack of a water molecule to the C8-cation of **31** afforded **23** (path *c*). Attack of the hydroxyl group of **12** to the cation **31** led to **24** (path *d*). The significantly high conversion of **12**, but no conversion of **11**, suggested that the carbon number of C$_{15}$ at least is required for starting the cyclization reaction. We have previously reported that the methyl group at C-10 of **1** has a pivotal role in adopting the normal folding of all chair conformation. The C(10)–norsqualene underwent the polycyclization reaction through the unusual folding conformation, affording the novel carbocyclic skeleton(s) having the 6/5 + 5/5/ + (6) ring system.[15] We have proposed that the strong binding of the C(10)–Me of **1** is involved in the cyclase enzyme and suggested that the unusual folding conformation would have occurred due to the trapping of C(15)–Me by the recognition site inherent to C(10)–Me involved in the cyclase. **12** has a methyl group at C-10, therefore **12** could have undergone the cyclization reaction. However, the methyl group is absent in **11**, thus leading to no cyclization. To ascertain this idea, further studies are necessary. **13** (C$_{20}$) also underwent the cyclization reaction, but the yield was low (12%) despite the important C(10)–Me being involved. Intermediate **32** was produced according to Markovnikov closure. The proton elimination at C-12 of **32** gave product **25**. Why was the conversion ratio of **13** low, despite the favorable Markovnikov cation **32** being formed? A bulky methyl group at C-14 of **13** may have repulsively interacted with the enzyme site located near to C-13 of **32**, leading to a decrease in the amount of all the chair conformation required for the formation of **32** (Scheme 3). In addition, the strong 1,3-diaxial interaction between C(8)–Me and C(13)–Me found in **32** may have decreased the formation of **32**. We have previously demonstrated that squalene analog (C$_{30}$), which has the methyl group at C-14 instead of the C(15)–Me of **1**, was cyclized into the 6/6/6-fused tricycle in a similar way as **13**, but the yield (7%) was
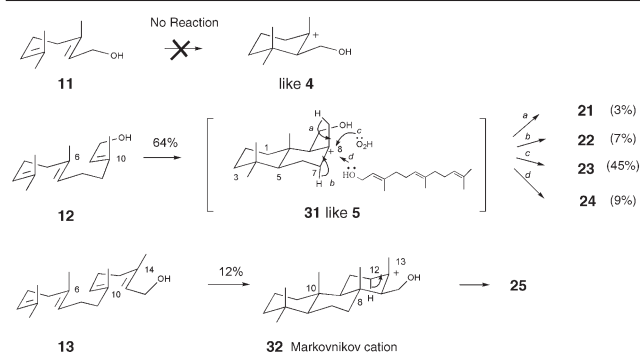
Scheme 3 Cyclization mechanisms of geraniol **11**, farnesol **12** and geranylgeraniol **13** by the SHC.

as low as that of **13**.[16] Thus, it is inferred that the introduction of the methyl group at C-14 of the linear backbone of **1** and the analogs gives a low yield of the cyclization product having the tricyclic ring system

Cyclization reactions of truncated analogs 14–16

In the previous papers,[12,14] we have demonstrated that an isopropyl-idene moiety is required for initiating the polycyclization reaction. Analogs **14**–**16** have this moiety at the both terminal positions, thus the cyclization reaction can start from the both termini (path *a* and *b* of Scheme 4). Analog **14** is a symmetrical molecule, thus the identical folding conformation of chair/chair/chair structure can be adopted during the cyclization reaction as shown in **14a**,**b** (Scheme 4A). Fig.1D shows that no cyclization reaction occurred for **14**. However, analog **15**, the regio-isomer of **14** with respective to the methyl position, could be cyclized into Markovnikov cations **15e** and **15f** *via* the folding conformations **15c** and **15b**, respectively. A water attack to the C-14 cations of **15e** and **15f** gave products **26** and **27** having the 6/6/5-fused tricycle in a yield of 28% and 5%, respectively. The product structures clearly indicated that the polycyclization occurred exclusively *via* path *a* (**15a**), but not *via* path *b* (**15b**). No conversion of **14a**,**b** and **15b** indicated that the introduction of a methyl group at C-7 and/or C-11 prevented the formation of all chair conformation in the reaction cavity. The C(7)–Me or C(11)–Me of **14a**,**b** and **15b** would have repulsively interacted with the binding pocket(s) near to the C-7 or C-11 in the folded conformation, suggesting that the α-face domain of the cyclase accepting the region of C-7 and C-11 is compact. Thus, the steric bulk at C-7 and C-11 of the substrate(s) must be small; a preferred size is hydrogen atom, and a methyl group is too large. Products **26** and **27** differ only in the stereochemistry at C-13; **26** has 13β-H, whereas **27** has the opposite stereochemistry of 13α-H. As shown in Scheme 1, the true intermediate of the 6/6/5-fused tricyclic skeleton **6** has the configuration of 13β-H during the polycyclization cascade of **1**. The product ratio of **26** to **27** was *ca* 85 : 15; a higher production of **26** than that of **27** coincides with the cyclization pathway of **1**, but a relatively high production of **27** (*ca*, 18% of **26**) having the opposite stereochemistry cannot be neglected, suggesting that a further long carbon-chain length may be required for the complete stereochemical control during the polycyclization reaction. Analog **16** having a longer chain (C_{25}) underwent the polycyclization reaction *via* path *a* (**16a**), but not *via* path *b* (**16b**). This finding also indicated that the methyl position is important for the occurrence of polycyclization reaction; introduction of methyl group at C-11 prevented the polycyclization reaction, possibly due to the repulsive interaction between C(11)–Me and the cyclase site located near to the C(11)–Me in the folded all chair conformation. Conformation **16a** led to a Markovnikov cation **16e** having 17β-H *via* the folding of conformation **16c**. This corresponds to **8** formed during the polycyclization of **1**. A water attack to the C-18 cation of **16e** led to the production of **30** (path *c*). Products **28** and **29** could be produced from intermediate **16f** having 17α-H through the folding conformation **16d**. Regarding the formation of **28** and **29**, **16f** may be more preferable than **16e** from the point of view that the deprotonation and 1,2-hydride shift reac-

tions occur in anti-parallel fashion. The 1,2-hydride shift of H-17 to C-18 and the deprotonation of H-13 gave **28** (path *d*). A series of 1,2-hydride shifts of H-17 and H-13 and the deprotonation reactions of axial-oriented H-12 afforded **29** (path *e*). The stereochemistry at C-17 of intermediate **16e** coincides with that of **8** formed during the polycyclization of **1** (see Scheme 1). The structure of **30** having 17β-H unambiguously demonstrated that **30** was produced *via* **16e**, but not *via* **16f**. The product ratio of **30** *via* **16e** to that of (**28** + **29**) *via* **16f** was *ca.* 72 : 28; a large portion of **16** was converted into intermediate **16e** like **8**, but a relatively high production of **16f** (39% of **16e**) indicated that the stereochemical control is not perfect. The results from incubation experiments of **15** and **16** may suggest that longer carbon-chain length ($>C_{25}$) of the substrate, like **1** (C_{30}), is essential to acquiring the complete stereochemical control during the polycyclization reaction. Previously, Corey and Cheng reported the polycyclization cascade of the 2,3-epoxide of **15** by lanosterol synthase; the truncated C_{20}-2,3-oxidosqualene analog was transformed into a 6/6/5-fused tricyclic A/B/C-ring system having a chair/boat structure.[27] All the products possessed the stereo-chemistry of 13α-H, but no product having 13β-H was found in the incubation mixture. It is well known that the 6/6/5-fused tricyclic ring system having 13α-H, but not 13β-H, is formed during the biosynthetic process of lanosterol through a folding conformation of chair/boat/chair structure.[2,3,28–30] Thus, it could be inferred that the stereochemical control by lanosterol synthase is more accurate compared to that by hopene synthase. It should be also noted that the Markovnikov product having 6/6/5-fused tricyclic ring skeleton like **15e** was not trapped during the polycyclization reaction of the longer substrate **16**. Hess suggested by computational studies that the C-ring expansion and D-ring formation occur concomitantly in lanosterol biosynthesis and that the primary role of lanosterol cyclase in the C-ring expansion and D-ring formation is to hold the substrate in the proper conformation inside the reaction cavity for the completion of the cascade.[31] It is anticipated that an increase in the carbon-chain length would give rise to a more precise inter-action between the cyclase enzyme (SHC) and substrate analogs. The more correct conformation given by the longer substrate **16** inside the cavity may have led to feasibility of the ring enlargement process from the five- to the six-membered C ring (**6**→**7**) without violation of Markovnikov's rule.

In the present study, we further demonstrated that truncated analogs (C_{15}–C_{25}) also can be accepted as the substrate of the squalene cyclase. The broad substrate specificity of the squalene cyclase is remarkable. A surprisingly high conversion of farnesol **12** (64%), but no cyclization of geraniol **11** (C_{10}), unequivocally demonstrated that at least C_{15} is necessary for initiating the cycli-zation reaction. An interesting compound **24** was constructed, in which two farnesol molecules are linked. The diol **23**, enzymati-cally prepared with a high yield (45%) and in a single step, may be practically useful as a chiral synthon for the chemical syntheses of natural terpenoid compounds. Acyclic geranylgeraniol **13** (C_{20}) was also cyclized to form 6/6/6-fused tricyclic ring system, but the yield was low. Analog **15** having the same carbon number (C_{20}) as **13** had a higher conversion yield than **13**, indicating that the methyl position of the acyclic isoprenoids is important for the efficient cyclization reaction. The C(14)–Me of **13** may have repulsively interacted with the recognition site located near to C-13 of **32**. The importance of the methyl position was further supported by the findings that conformations **14ab**, **15b** and **16b** underwent no cyclization, that is, a bulky methyl group at C-7 and/or C-11 can-not be tolerated by the cyclase enzyme, due to the repulsive inter-action between α-oriented C(7)–Me or C(11)–Me and the cyclase site(s) located near to their methyl groups. These findings suggest that the α-face domain of the cyclase, which is responsible for the recognition of the C-7 and C-11 positions, is strictly compact, thus cannot accept the large α-oriented methyl groups. This inference is in good agreement with our previous findings that the cycliza-tion reactions of C_{30}-analogs having C(7)–Me or C(11)–Me do not occur.[16] The C_{25}-truncated analog **16** could also undergo the polycyclization to afford a 6/6/6/5-fused tetracycle *via* **16a**. The formation of all the enzymic products found in this study accorded
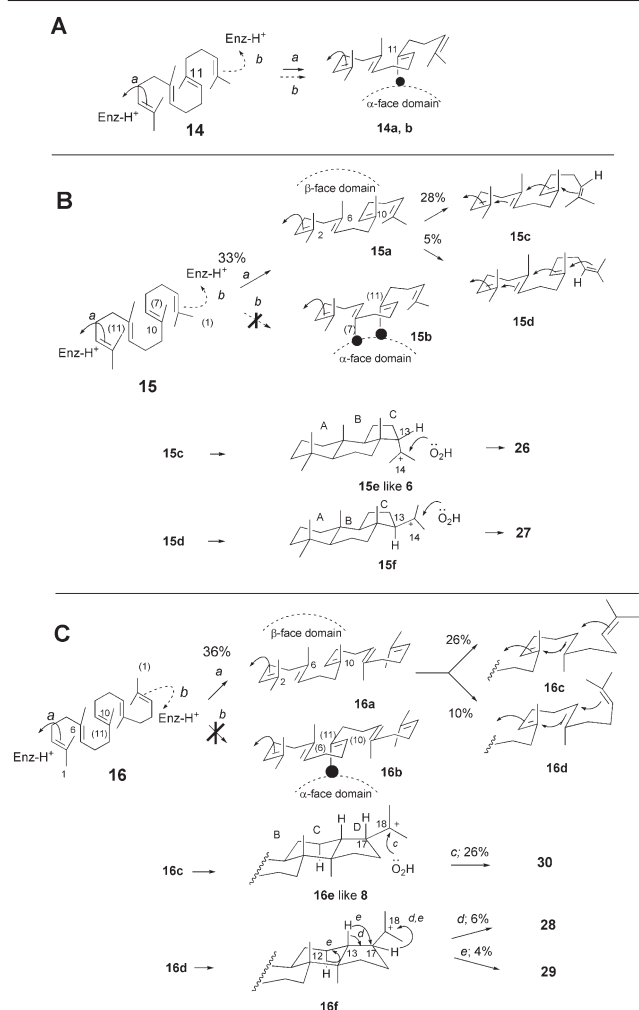
Scheme 4 Cyclization mechanisms of the truncated squalene analogs (C_{20} and C_{25}) by the SHC. A: analog **14** (C_{20}) having the methyl group at C-11. B: analog **15** (C_{20}) having the methyl group at C-10. C: analog **16** (C_{25}). The stereochemistry at C-13 of **15e** (13β-H) is identical to that of **6** formed during the polycyclization reaction of **1**, whereas **15f** has the opposite configuration of 13α-H. **16e** has the same configuration of 17β-H as **8**, but **16f** has 17α-H opposed to **16e**. The symbol of the closed circle indicates that the axial-oriented C(7)–Me or C(11)–Me repulsively interacted with the recognition site(s) involved in the cyclase, due to the introduction of large steric bulk size of the methyl group at this position(s). Possibly, the α-face domain of the cyclase is strictly compact. In contrast, the β-face domain would be less compact, thus can accept the axial-oriented methyl groups at positions of C-2, C-6 and C-10.

with the Markovnikov rule. It should be noted that the carbocationic intermediates **5**, **6** and **8** formed during the polycyclization reaction of **1** were trapped through the present studies using the truncated analogs and that all the truncated analogs (C_{15}–C_{25}) had higher conversion ratios than the elongated C_{35} analog (only 10%).[24] It is unresolved why the conversion ratios of **15** (C_{20}) and **16** (C_{25}) are relatively lower than that of **12** (C_{15}) despite **15** and **16** having longer carbon-chain lengths closer to that of **1** (C_{30}) and why the cyclization of geraniol **11** (C_{10}) was not allowed despite **11** having the minimum carbon-chain length required for the formation of the monocyclic skeleton like **4**? The stereochemical control by the SHC was not perfect for the truncated analogs (**15** and **16**), leading to the false intermediates having the configurations of 13α-H and 17α-H. This finding may suggest that the complete stereochemical control would be attained only by substrate **1** (C_{30}). Further studies on some truncated squalene analogs will provide better understanding for the polycyclization mechanism of the true substrate **1**.

Experimental

Analytical methods

NMR spectra were recorded in C_6D_6 on a Bruker DMX 600 or DPX 400 spectrometer, the chemical shifts being relative to the

solvent peak δ_H 7.280 and δ_C 128.0 ppm as the internal reference for ¹H- and ¹³C NMR spectra, respectively. Some synthetic intermediates were measured in CDCl₃. The chemical shifts in CDCl₃ solution were given according to the internal solvent peaks of δ_H 7.26 and δ_C 77.0 ppm. The coupling constants J are given in Hz. GC analyses were done on a Shimadzu GC-8A chromatograph equipped with a flame ionization detector (DB-1 capillary column (0.53 mm × 30 m). GC-MS spectra were on a JEOL SX 100 spectrometer under electronic impact at 70 eV with a DB-1 capillary column (0.32 mm × 30 m), the oven temperature being elevated according to the methods described in the legend to Fig. 2. HR-EIMS was performed by direct inlet system. Specific rotation values were measured at 25 °C with a Horiba SEPA-300 polarimeter. FABMS (positive) was measured using glycerol matrix.

Incubation conditions

Standard incubation conditions were performed according to the published protocols.[5,13] The cell-free extracts were prepared as follows. 1 L culture of *E. coli* encoding the native SHC was harvested by centrifugation and to the collected pellets was added 50 cm³ of citrate buffer solution (pH 6.0), and then subjected to ultrasonication to disrupt the cells. The supernatant was used for the incubations after removing the cell debris by centrifugation. 1 cm³ of the supernatant contains *ca.* 200 μg of the pure SHC.

Preparation of isoprenyl bromides 17, 19 and 20

Farnesyl bromide **17**, 1-bromo-3-methylbut-2-ene **19** and geranyl bromide **20** were prepared by treatment of the corresponding alcohols farnesol **12**, 3-methylbut-2-en-1-ol and geraniol **11** with PBr₃ in THF at 0 °C for 30 min.[16] The reaction mixture was poured into ice water. The product was extracted with *n*-hexane and dried over anhydrous Na₂SO₄ and used without further purification.

Syntheses of 2,6,11,15-tetramethyl-hexadeca-(6*E*,10*E*)-2,6,10,14-tetraene 14, 2,6,10,15-tetramethyl-hexadeca-(6*E*,10*E*)-2,6,10,14-tetraene 15 and 2,6,10,15,19-pentamethyl-eicosa-(6*E*,10*E*,14*E*)-2,6,10,14,18-pentaene 16

These compounds were synthesized by a coupling reaction of each bromide according to the literature.[25,26] As a typical example, the syntheses of **14** and **16** are described below. To the ethereal solution of pyrrolidine (250 mg in 10 ml), 2.1 ml of *n*-BuLi (1.58 M hexane solution) was added at 0 °C under N₂ atmosphere and stood for 20 min. To the reaction mixture which was further cooled at −38 °C, 250 mg of CuI was added, the color quickly turning into reddish brown, and stirred for 20 min. A mixed solution of **17** (100 mg) and **20** (50 mg) in Et₂O (5 ml) was slowly added and stirred for 60 min. The reaction mixture was poured into brine and the product was extracted with *n*-hexane and purified by the following three steps: SiO₂ column chromatography (*n*-hexane), an open column of reverse-phase (C_{18}) with an eluent of 85% CH₃CN/H₂O and finally with 5% AgNO₃–SiO₂ column chromatography by eluting with a mixture of hexane and EtOAc (100 : 5). Pure **14** and **16** was isolated in yields of 20 mg and 28 mg, respectively. The total yield of **14** and **16** was 26%. NMR data of **14** in C_6D_6: δ_H, 5.44 (2H,br s), 5.36 (br t, *J* 6.8), around 2.2 (12H, m), 1.80 (6H, s), 1.73 (6H, s), 1.68 (6H, s); δ_C, 135.1(s), 131.1(s), 124.9(d), 124.8(d), 40.2 (t), 28.7 (t), 27.2 (t), 25.8 (q), 17.7 (q), 16.1 (q). EIMS; *m/z* (%) 69 (100), 81 (48), 137 (15), 150 (15), 274 (M⁺, 8). NMR data of **16** in C_6D_6: δ_H, around 5.4 (5H, m), around 2.4 (16H, m), 1.84 (6H, s), 1.73 (9H, s), 1.68 (6H, s); δ_C, 135.1 (2C, s), 134.9 (s), 131.0 (2C, s), 124.9 (2C, d), 124.85 (2C, d), 124.8 (d), 40.2 (3C, t), 28.7 (2C, t), 27.2 (t), 27.19 (t), 27.1(t), 25.8 (2C, q), 17.7 (2C, q), 16.1 (2C, q), 16.09(1C, q). EIMS; *m/z* (%), 69 (100), 81 (59), 137 (22), 342 (M⁺, 5). For the preparation of **15**, **19** and **17** were subjected to the allyl coupling reaction. NMR data of **15** in CDCl₃: δ_H, 5.12 (4H, m), around 2.0 (12H, m), 1.69 (6H, s), 1.60(12H, s); δ_C, 135.1 (s), 134.9 (s), 131.4 (s), 131.2 (s), 124.5 (d), 124.4 (d), 124.3 (2C, d), 39.7 (2C, t), 28.4 (t), 28.3 (t), 26.8 (t), 26.6 (t), 25.70 (q), 25.67 (q),

17.68 (q), 17.66 (q), 15.99 (2C, q). EIMS *m/z* (%); 69 (100), 81 (36), 137 (10), 274 (M⁺, 3).

Spectroscopic data of enzymic products 21–30

Product 21. ¹H-NMR (C₆D₆), δ 0.77 (3H, s, Me-14), 0.86 (3H, s, Me-13), 0.91 (3H, s, Me-12), 1.02 (dd, *J* 12.7, 2.4, H-5), 1.10 (m, H-1), 1.17 (m, H-3), 1.30 (m, H-6), 1.42 (m, H-3), 1.47 (m, H-2), 1.58 (m, H-2), 1.61 (m, H-1), 1.66 (br d, *J* 15.0, H-6), 1.94 (m, H-9), 1.95 (br s, H-16), 2.01 (m, H-7), 2.40 (br d, *J* 12.8, H-7), 3.78 (dd, *J* 10.8, 10.8, H-11), 3.86 (dd, *J* 10.8, 3.4, H-11), 4.75 (s, H-15), 4.99 (s, H-15). ¹³C-NMR (C₆D₆), δ 15.36 (C-14), 19.50 (C-2), 21.84 (C-13), 21.84 (C-14), 24.39 (C-6), 33.45 (C-4), 33.68 (C-12), 38.12 (C-7), 39.05 (2 × C, C-1 and C-10), 42.19 (C-3), 55.14 (C-5), 58.73 (C-11), 59.32 (C-9), 106.6 (C-15), 147.9 (C-8). EIMS (%): 69 (25), 81 (23), 109 (100), 124 (42),191 (10), 222 (M⁺, 8). HRMS (EI): calcd. for C₁₅H₂₆O, 222.1984; found 222.1964. [*a*]_D²⁵ = +20.4 (*c* 0.015, EtOH). oil.

Product 22. NMR data (C₆D₆). δ_H 0.95 (6H, s, Me-12 and Me-14), 0.97 (3H, s, Me-13), 1.06 (m, H-1), 1.24 (m, H-3), 1.25 (m, H-5), 1.49 (2H, m, H-2 and H-3), 1.62(m, H-2), 1.86 (very broad, H-9), 1.92 (m, H-6), 1.95 (m, H-1), 1.97 (3H, s, Me-15), 2.04 (m, H-6), 3.63 (m, H-11), 3.75 (m, H-11), 5.62 (br s, H-7). δ_C 14.93 (C-14), 19.14 (C-2), 22.15 (C-13), 22.28 (C-15), 23.96 (C-6), 33.01 (C-4), 33.50 (C-12), 36.19 (C-10), 39.95 (C-1), 42.45 (C-3), 50.12 (C-5), 57.45 (C-9), 60.82 (C-11), 123.6 (C-7), 133.7 (C-8). The assignments of C-13 and C-15 may be exchangeable. EIMS (%): 69 (61), 81 (70), 95 (58), 137 (100), 189 (11), 222 (M⁺, 10). HRMS (EI): calcd. for C₁₅H₂₆O, 222.1984; found 222.1964. [*a*]_D²⁵ = −28.2 (*c* 0.045, EtOH). Mp. 87–89 °C.

Product 23. NMR data (C₆D₆). δ_H 0.70 (3H, s, Me-14), 0.81 (3H, s, Me-13), 0.91 (3H, s, Me-12), 0.936 (dd, *J* 12.3, 1.8, H-5), 0.94 (m, H-6), 1.03 (m, H-2), 1.07 (ddd, *J* 12.9, 12.9, 3.5, H-1), 1.16 (m, H-6), 1.18 (m, H-3), 1.38 (3H,s, Me-15), 1.40 (m, H-3), 1.42 (m, H-2), 1.60 (m, H-7), 1.63 (m, H-9), 1.66 (m, H-1), 1.92 (ddd, *J* 2.9, 6.0,12.6, H-7), 3.97 (d, *J* 6.9, H-11). δ_C 15.99 (C-14), 18.91 (C-2), 21.74 (C-13), 21.74 (C-6), 24.44 (C-15), 33.26 (C-4), 33.61 (C-12), 37.53 (C-10), 40.09 (C-1), 41.92 (C-3), 44.44 (C-7), 55.93 (C-5), 60.59 (C-9), 60.9 (C-11), 74.53 (C-8). EIMS (%): 69 (70), 81 (72), 95 (100), 109 (95), 123 (61), 137 (46), 164 (28), 189 (22), 207 (16), 222 (M⁺–H₂O, 13). HRMS (EI) *m/z* (M⁺–H₂O): calcd. for C₁₅H₂₆O, 222.1984; found 222.1964. [*a*]_D²⁵ = +14.3 (*c* 0.015, EtOH). Mp. 110–114 °C.

Product 24. NMR data. δ_H 0.74 (3H, s, Me-14), 0.83 (3H, s, Me-13), 0.88 (br d, *J* 11.0, H-5), 0.93 (3H, s, Me-12), 1.08 (m, H-1), 1.10 (m, H-6), 1.14 (m, H-3), 1.34 (3H, s, Me-15), 1.37 (m, H-3), 1.39 (m, H-2), 1.55 (m, H-2), 1.55 (m, H-7), 1.56 (m, H-6), 1.70 (6H, s, Me-29 and Me-30), 1.72 (3H, s, Me-28), 1.78 (m, H-1), 1.81 (3H,s, Me-27), 1.82 (m, H-9), 1.88 (m, H-7), 2.12 (2H, br t, *J* 7.5, H-19), 2.22 (dt, *J* 7.2, 7.2, H-23), 2.25 (2H, dt, *J* 7.2, 7.2, H-20), 2.30 (dt, *J* 7.3, 7.3, H-24), 3.99 (2H, dd *J* 6.1, 3.8, H-16), 4.13 (2H, dd, *J* 16.7, 8.6, H-11), 5.35 (t, *J* 6.8, H-21), 5.37 (t, *J* 6.8, H-25), 5.56 (br t, *J* 6.5, H-17). The assignments of Me-27 and Me-30 are interchangeable. δ_C 16.08 (C-29), 16.37 (C-14), 16.41 (C-28), 17.73 (C-30), 18.86 (C-2), 19.66 (C-15), 20.01 (C-6), 21.68 (C-13), 25.84 (C-27), 26.65 (C-20), 27.21 (C-24), 33.25 (C-4), 33.57 (C-12), 37.79 (C-10), 39.14 (C-7), 39.78 (C-19), 40.15 (C-1), 41.90 (C-3), 55.76 (C-5), 56.72 (C-16), 60.11 (C-9), 60.47 (C-11), 80.13 (C-8), 122.21 (C-17), 124.37 (C-21), 124.99 (C-25), 131.04 (C-26), 135.27 (C-22), 139.24 (C-18). The assignments of C-1 and C-23 and those of C-14 and C-28 may be interchangeable. EIMS (%): 80 (82), 91 (100), 135 (48), 159 (21), 186 (24). HRMS (FAB) *m/z* (M⁺ + H): calcd. for C₃₀H₅₃O₂, 445.4046; found 445.4052. [*a*]_D²⁵ −0.31 (*c* 0.04, EtOH); oil.

Product 25. NMR data (C₆D₆). δ_H 0.80 (m, H-1), 0.84 (dd,*J* 12.0, 2.0, H-5), 0.953 (9H, s, Me-17, Me-18 and Me-19), 1.01 (3H, s, Me-16), 1.17 (m, H-9), 1.20(m, H-3), 1.22 (m, H-7), 1.38 (ddd, *J* 13.6,

3.2, H-2), 1.46 (m, H-6), 1.48 (m, H-7), 1.60 (m, H-2), 1.67 (2H, m, H-1 and H-6), 1.98 (2H, m, H-11), 1.99 (3H, s, Me-20), 1.99 (very broad, s, H-14), 2.05 (ddd, *J* 12.8, 3.2, 3.2, H-3), 3.62 (dd, *J* 10.8, 5.6, H-15), 3.75 (dd, *J* 10.8, 3.2, H-15), 5.63 (br s, H-12). δ_C 15.87 (C-19), 15.92 (C-18), 18.85 (C-6), 19.07 (C-2), 21.85 (C-17), 22.14 (C-20), 22.92 (C-11), 33.21 (C-4), 33.35 (C-16), 36.34 (C-8), 37.39 (C-10), 40.07 (C-1), 41.55 (C-3), 42.17 (C-7), 55.04 (C-9), 56.28 (C-5), 58.04 (C-14), 60.74 (C-15), 123.4 (C-12), 133.5 (C-13). The assignments of C-18 and C-19 and those of C-2 and C-6 are interchangeable. EIMS (%): 59 (100), 82(57), 95(48), 123(38), 137(31), 177(21), 191 (71), 219 (25), 234 (24), 259 (14), 274 (M⁺–H₂O, 10). HRMS (EI) *m/z* (M⁺): calcd. for C₂₀H₃₄O, 290.2610; found 290.2615. [*a*]_D²⁵ = 9.23 (*c* 0.051, EtOH); mp. 110–113 °C.

Product 26. NMR data (C₆D₆). δ_H 0.98 (6H, s, Me-16 and Me-17), 0.99 (m, H-5), 1.01(3H, s, Me-15), 1.03 (3H,s, Me-18), 1.05 (m, H-1), 1.22 (3H, s, Me-20), 1.24 (3H, s, Me-19), 1.29(m, H-3), 1.44 (m, H-11), 1.46 (m, H-2), 1.49 (m, H-9), 1.50 (m, H-3), 1.52 (m, H-6), 1.58 (m, H-11), 1.60 (m, H-1), 1.62 (m, H-13), 1.70(m, H-2), 1.71 (m, H-6), 1.82 (2H, m, H-12), 1.90 (m, H-7), 2.12 (br t, *J* 10.4, H-7). The assignments of Me-19 and Me-20 are interchangeable. δ_C 16.52 (C-17), 18.89 (C-2), 20.11 (C-6), 21.54 (C-16), 21.71 (C-11), 25.52 (C-12), 26.82 (C-18), 28.54 (C-20), 30.17 (C-19), 33.18 (C-4), 33.68 (C-15), 37.43 (C-10), 38.09 (C-7), 41.17 (C-1), 42.72 (C-3), 45.20 (C-8), 57.20 (C-5), 59.92 (C-9), 60.74 (C-13), 73.88 (C-14). EIMS (%): 59(100), 95(39), 123(32), 137(27), 191(68), 219(22), 234(24), 274(M⁺–H₂O, 15) [*a*]_D²⁵ = +6.11(*c* 0.01, EtOH). Mp. 115–117 °C.

Product 27. NMR data (C₆D₆). δ_H 0.87 (dd, *J* 12.4, 2.4, H-5), 0.95 (m, H-1), 0.98 (3H, s, Me-16), 0.97 (3H, s, Me-17), 1.03 (3H, s, Me-15), 1.07 (dd, *J* 13.2, 7.2, H-9), 1.16 (3H,s, Me-18), 1.17 (3H, s, Me-20), 1.19 (m, H-7), 1.29 (t, *J* 10.1, H-13), 1.31 (m, H-3), 1.34 (3H, s, Me-19), 1.43 (m, H-6), 1.49 (m, H-11), 1.52 (3H, m, H-1, H-2 and H-3), 1.59 (m, H-6), 1.72 (m, H-12), 1.73 (2H, m, H-2 and H-11), 1.88 (m, H-12), 2.10 (m, H-7). The assignments of Me-19 and Me-20 are interchangeable. δ_C 15.73 (C-18), 15.92 (C-17), 18.72 (C-11), 19.56 (C-2), 19.61 (C-6), 21.43 (C-16), 22.84 (C-12), 30.01 (C-19), 32.06 (C-20), 33.10 (C-4), 33.70 (C-15), 37.24 (C-10), 40.58 (C-1), 42.78 (C-3), 42.88 (C-7), 44.38 (C-8), 57.05 (C-5), 60.92 (C-13), 63.26 (C-9), 72.55 (C-14). The assignments of C-19 and C-20 are interchangeable. EIMS (%): 59 (100), 95 (39), 123 (32), 137 (27), 191 (68), 219 (22), 234 (24), 274 (M⁺–H₂O, 15). HRMS (EI) *m/z* (M⁺–H₂O): calcd. for C₂₀H₃₄, 274.2661; found 274.2626. [*a*]_D²⁵ −7.16 (*c* 0.034, EtOH). Mp. 115–117 °C.

Product 28. NMR data (C₆D₆). δ_H 0.90 (m, H-1), 0.91(m, H-5), 0.96 (3H, s, Me-20), 0.997(3H, s, Me-21), 1.03 (3H, s, Me-19), 1.05 (3H,s, Me-22), 1.13 (d, *J* 6.9, Me-24), 1.17 (d, *J* 6.9, Me-25), 1.28 (m, H-3), 1.30 (3H, s, Me-23), 1.36 (m, H-11), 1.47 (m, H-2), 1.48 (m, H-15), 1.50 (m, H-3), 1.52 (2H, m, H-2 and H-7), 1.58 (m, H-9), 1.65 (m, H-7), 1.66 (m, H-11), 1.67 (H-6), 1.74 (m, H-6), 1.78 (m, H-1), 2.03 (m, H-12), 2.11 (ddd, *J* 10.2, 10.2, 4.9, H-15), 2.34 (m, H-16), 2.47 (m, H-16), 2.56 (m, H-12), 2.85 (septet, *J* 6.9, H-18). The assignments of H-2 and H-6 and those of Me-24 and Me-25 are interchangeable. δ_C 16.67 (C-21), 17.06 (C-22), 18.93 (C-2), 19.07 (C-6), 21.53 (C-24), 21.89 (C-20), 21.89 (C-25), 22.23 (C-11), 23.22 (C-23), 23.26 (C-12), 27.02 (C-18), 29.63 (C-16), 31.13 (C-15), 33.50 (C-4), 33.66 (C-19), 35.63 (C-7), 37.99 (C-10), 40.85 (C-1), 41.98 (C-8), 42.33 (C-3), 52.19 (C-9), 56.73 (C-14), 57.30 (C-5), 136.37 (C-17), 137.99 (C-13). The assignments of C-2 and C-6 and those of C-24 and C-25 are interchangeable. EIMS (%): 69 (58), 67 (81), 95 (89), 121 (91), 161(89), 191 (84), 299 (42), 342 (M⁺, 100). HRMS (EI): calcd. for C₂₅H₄₂, 342.3287; found 342.3278. [*a*]_D²⁵ = −11.7 (*c* 0.01, EtOH).

Product 29. NMR data (C₆D₆). δ_H 0.96 (3H, s, Me-21), 0.97 (3H, s, Me-19), 1.02 (3H, s, Me-22), 1.03 (3H, s, Me-20), 1.03 (m, H-1), 1.04 (3H, d, *J* 6.0, Me-24), 1.08 (3H, d, *J* 6.0, Me-25), 1.17 (3H,s, Me-23), 1.29 (m, H-3), 1.38 (m, H-16), 1.45 (m, H-2), 1.50 (2H,

m, H-3 and H-17), 1.52 (m, H-5), 1.60 (m, H-18), 1.63 (m, H-6), 1.66 (m, H-6), 1.67 (m, H-2), 1.68 (m, H-15), 1.75 (m, H-1), 1.77 (m, H-15), 1.78 (m, H-7), 1.89 (m, H-7), 2.01 (m, H-11), 2.06 (m, H-16), 2.27 (m, H-11), 2.44 (m, H-9), 5.47 (m, H-12). The assignments of Me-24 and Me-25 are interchangeable. δ_C 12.98 (C-21), 18.04 (C-6), 19.06 (C-2), 21.13 (C-20), 21.94 (C-22), 22.11 (C-25), 22.95 (C-24), 24.33 (C-11), 27.20 (C-23), 28.30 (C-16), 31.26 (C-18), 32.79 (C-19), 32.92 (C-4), 33.66 (C-7), 34.09 (C-15), 35.02 (C-10), 38.88 (C-1), 42.30 (C-3), 43.41 (C-14), 49.08 (C-9), 51.17 (C-8), 51.30 (C-5), 54.93 (C-17), 118.08 (C-12), 145.70 (C-13). The assignments of C-20, C-24 and C-25 are interchangeable. EIMS (%): 69 (26), 95 (23), 175 (13), 203 (15), 327 (100), 342 (M$^+$, 11). HRMS (EI): calcd. for $C_{25}H_{42}$, 342.3287; found 342.3278. $[a]_D^{25} = -21.4$ (c 0.036, EtOH).

Product 30. NMR data (C_6D_6). δ_H 0.92(2H, m, H-1 and H-5), 0.99 (3H,s, Me-20), 1.00 (3H,s, Me-21), 1.02 (3H,s, Me-19), 1.08 (3H,s, Me-22), 1.17 (3H,s, Me-23), 1.21 (3H,s, Me-24), 1.23 (m, H-11), 1.24(m, H-15), 1.27 (3H,s, Me-25), 1.39(2H, m, H-3 and H-7), 1.41 (m, H-6), 1.50 (2H, m, H-2 and H-3), 1.51 (m, H-9), 1.52 (m, H-15), 1.58 (m, H-12), 1.67 (3H, m, H-6, H-11 and H-16), 1.73 (3H, m, H-2, H-7 and H-16), 1.75 (m, H-1), 1.98 (ddd, J 9.9, 9.9, 9.9, H-17), 2.09 (m, H-13), 2.10 (m, H-12). The assignments of Me-24 and Me-25 are interchangeable. δ_C 16.16 (C-22), 16.48 (C-21), 17.10 (C-23), 19.12 (C-2), 19.12 (C-6), 21.77 (C-20), 22.86 (C-11), 26.05 (C-12), 27.56 (C-16), 29.74 (C-24), 31.55 (C-25), 32.79 (C-15), 33.54 (C-4), 33.61 (C-19), 35.73 (C-7), 37.73 (C-10), 40.90 (C-1), 41.19 (C-8), 42.49 (C-3), 44.19 (C-13), 49.39 (C-14), 50.07 (C-17), 51.23 (C-9), 57.37 (C-5), 72.98 (C-18). EIMS (%): 95 (42), 191 (100), 302 (33), 342 (M$^+$–H$_2$O,13). The assignments of C-24 and C-25 are interchangeable. HRMS (EI): calcd. for $C_{25}H_{42}$, 342.3287; found 342.3274. $[a]_D^{25} = -3.14$ (c 0.113, EtOH). Mp. 98–100 °C.

Acknowledgements

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