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Enzymic products of the 2,3-oxidosqualene analog having an ethyl residue at 10-position. First trapping of the trimethylcyclohexanone ring by lanosterol synthase

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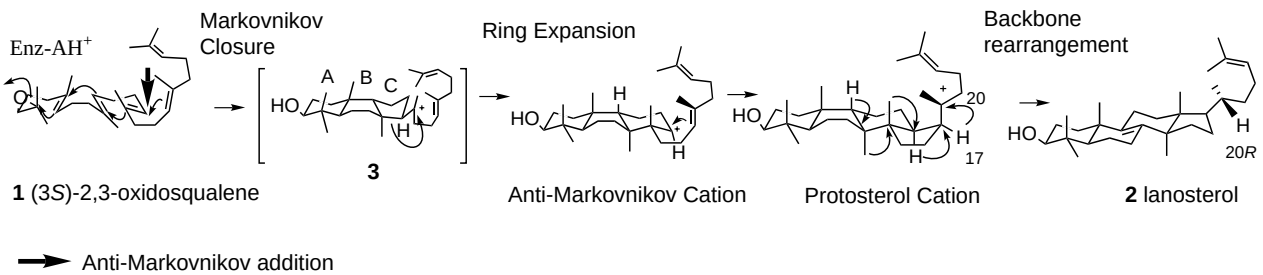
Abstract—Incubation of squalene analog, (3*RS*)-(6*E*,10*E*,14*E*,18*E*)-10-ethyl-2,6,19,23-tetramethyl-2,3-epoxytetracosa-6,10,14,18,22-pentaene with 2,3-oxidosqualene-lanosterol cyclase from pig liver gave four products, consisting of two mono-, one tri- and one tetracyclic lanosterol homolog, suggesting that the steric bulk size at C-10 had greater influence on the polycyclization reaction, compared to that at C-15. The formation of the trimethylcyclohexanone ring by lanosterol synthases has never been reported before. © 2001 Elsevier Science Ltd. All rights reserved.

2,3-Oxidosqualene **1** is cyclized into a variety of triterpene skeletons or lanosterol **2** by eukaryotic cyclases. The polycyclization mechanism is intriguing from the point of view that multiple C–C bond formation occurs under fine stereo- and regio-specificity.[1] It has been believed that **1** is folded in a chair/boat/chair conformation in the enzyme cavity of lanosterol synthase. Numerous studies on substrate analogs by lanosterol synthase have appeared in order to gain insight into the polycyclization mechanism and into the substrate recognition.[1] Among many investigations, special attention has been paid by vanTamlene, Corey and Kyler as to how the cyclization pathway is affected by the substitution of the methyl group at C-10 or at C-15.[2–4] The replacement of the methyl group at C-15 with hydrogen and that of the methyl at C-10 with a vinyl group led to the normal cyclization products.[2,3] However, the analog lacking methyl groups at both C-10 and C-15 afforded an unusual product having the 6,6,5-fused A/B/C tricyclic ring system, which is further linked with a four-membered ring, where the B-ring has a chair structure. This result suggests that the cyclization had proceeded in a pre-organized chair/*chair*/boat (A/B/C) conformation, which is in contrast to the usual folding of **1** into the chair/*boat*/chair conformation by lanosterol synthase. Thus, it has been inferred that the methyl residue at the 10-position is essential for the correct folding of this substrate.[4] They also proposed that the expansion process from a five- to a six-membered ring is involved in the C-ring formation of **2** (Scheme 1), based upon the trapping of the enzymic products having a 6,6,5-fused A(chair)/B(boat)/C-ring system from the truncated C20-analog of **1**. The formation of intermediate **3** having the five-membered C-ring



Scheme 1.

Keywords: lanosterol; 2,3-oxidosqualene; squalene; lanosterol synthase; triterpene cyclase; trimethylcyclohexanone.

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is consistent with Markovnikov's rule.[4] We have reported how the steric bulk size at C-10 and C-15 determines the polycyclization destiny.[5,6] Substitution of the methyl groups of **1** with the slightly bulky ethyl groups at both positions (**4**) halted the polycyclization reaction at the monocyclic ring stage to afford **7** and **14**.[5] The analog **5**, in which the methyl group at C-15 is replaced by an ethyl group, was converted into partially cyclized 6/6/5-fused tricyclic **10** and lanosterol homolog **12**, giving further evidence for the involvement of the ring expansion process.[6] However, it has still remained unclear how the ethyl group substituted only at C-10 (**6**) influences the polycyclization cascade. We describe here the enzymatic products of (±)-**6**, which consist of two monocyclic **8** and **9**, tricyclic **11** and fully cyclized lanosterol homolog **13**. A higher production of prematurely cyclized **8** and **9** indicates that the appropriate bulk size at C-10 is the most critical to the completion of tetracyclic lanosterol formation, compared with that at C-15. Formation of a trimethylcyclohexanone ring by lanosterol cyclase has never been reported before.

The squalene analog (±)-**6** was prepared according to the previously published method.[5–7] The coupling reaction of 3,12-dibromo-10-ethyl-2,6-dimethyl-dodeca-6,10-dien-2-ol with 1-bromo-3,7,11-trimethyl-dodeca-2,6,10-triene, by treatment with cuprous iodide and lithium pyrrolidide, gave the desired (±)-**6**, squalene and 2,3:22,23-dioxidosqualene having ethyl groups at C(10) and C(15) in the yields of 33, 15 and 10%, respectively. These three products were purified by SiO_2 column chromatography. The partially purified enzyme[5,6] was added to the solubilized (±)-**6** (0.5 mg) in a tris buffer (2 ml, pH 7.4) including Triton X-100 (14 mg), and then incubated for 12 h at 37°C. The resulting mixture was lyophilized. The hexane-extract showed three spots on SiO_2 TLC (hexane:EtOAc = 100:10) as the enzymic products, but the GC analysis (30 m DB-1 capillary column) showed the presence of four products. Large-scale incubation of (±)-**6** (26 mg) was carried out to isolate their products, which were purified as follows. An SiO_2 column chromatography by eluting with hexane:EtOAc (100:3) afforded 1 mg of **9** in a pure state. Next, an HPLC (hexane:2-propanol = 100:2) was used to obtain **8** (2 mg) in a pure form. Products **11** and **13** were indistinguishable on SiO_2 TLC, but the separation was done with argentation SiO_2 column (6% $AgNO_3$, hexane:EtOAc = 100:4, isolation yields: 1 mg each). The total conversion from (±)-**6** was ca. 19% from the isolation yields.

Structures of all the products (Fig. 1) were unequivocally determined by detailed analyses of the NMR spectra (DEPT, COSY 45, HOHAHA, NOESY, HMQC and HMBC). Product **8**[8] had four olefinic protons at δ_H 5.43 (3H, m) and 5.37 (1H, t, $J = 6.8$ Hz) along with exomethylene protons [δ_H 5.02 (1H, s) and 4.84 (1H, s)], which were correlated to δ_C 108.6 (t) in the HMQC spectrum. Four allyl methyl residue and one ethyl residue were also found at δ_H 1.80 (3H, s), 1.74 (3H, s), 1.73 (3H, s), 1.68 (3H, s), 2.22 (2H, q, $J = 7.6$ Hz, CH_2CH_3) and 1.14 (3H, t, $J = 7.6$ Hz, CH_2CH_3), suggesting a monocyclic compound for **8**. The ethyl group was shown to be attached to C-9 of **8** by the HMBC correlations from the ethyl protons. The apparent NOE of H-1_{ax} with H-3 showed the stereochemistry (Scheme 2). Product **9**[8] also had four double bonds as well as **8**, proving to be the monocyclic skeleton for **9**. However, no alcoholic carbon signal was detected, but in turn a carbonyl carbon signal (δ_C 211.0 ppm; 1730 cm^{-1} in $CHCl_3$) appeared. Detailed analyses of 2D NMR spectra of **9** revealed the presence of a 2,3,4-trimethylcyclohexanone ring (Scheme 3). The fragment ion of m/z 139 further supported the presence of the trimethylcyclohexanone ring in **9**. As for **11**,[8] the proton signals of two olefins, three allyl methyl and exomethylene groups were found in the ^1H NMR, suggesting a tricyclic system for **11**. Detailed analyses of HMBC and NOESY spectra (Scheme 2) gave the complete structure for **11**. The apparent NOE of H-9 with Me-26 proved β-orientation for H-9, i.e. a boat form

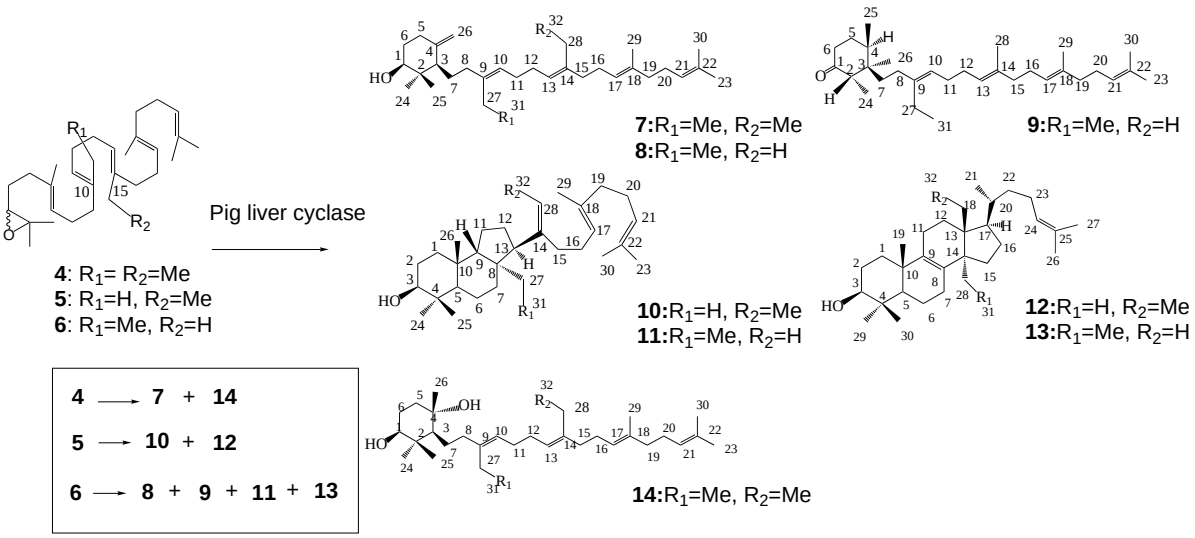
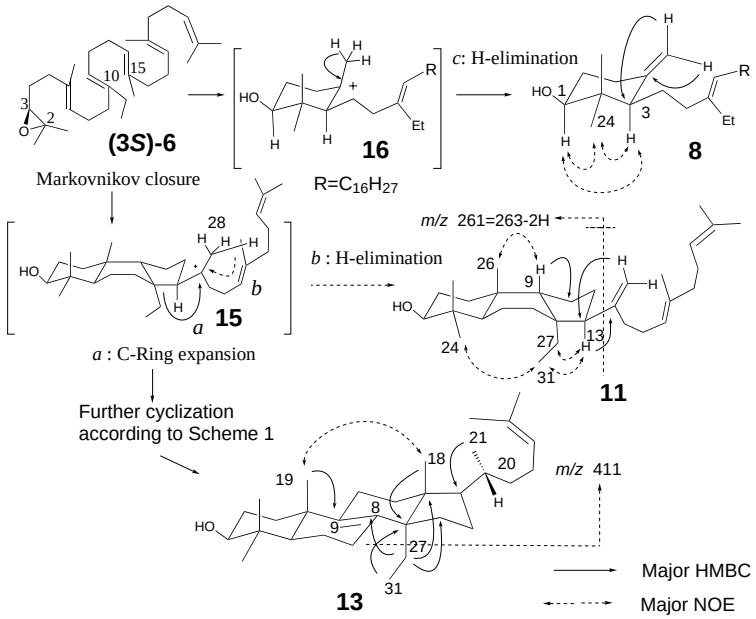
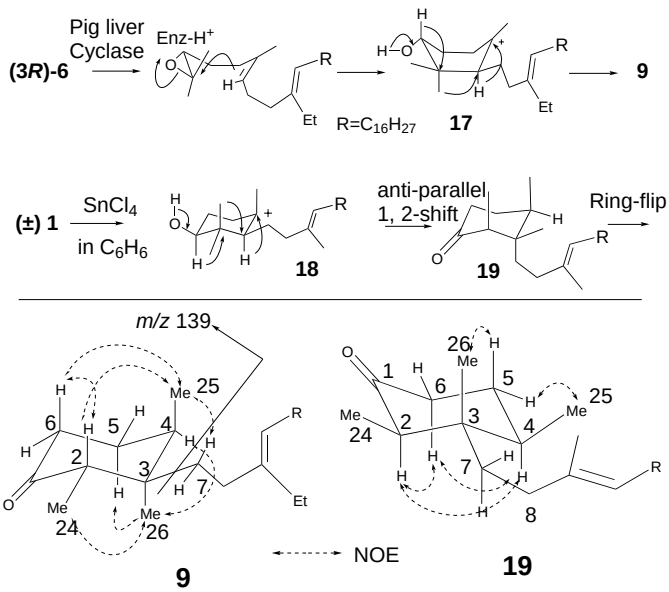


Figure 1. Structures of substrate analogs and the enzymic products by the lanosterol synthase.



Scheme 2.



Scheme 3.

for the B ring and that of H-13 with H-27 also verified β-arrangement for the isoprenoid side chain. The fission ion *m*/*z* 261 (20%) of EIMS further supported the tricyclic ring. ^{1}H and ^{13}C NMR of **13**[8] showed one olefinic proton and three quaternary sp^2 carbons. The HMBC correlation from Me-19 to C-9 and from CH_2-28 to C-8 (Scheme 2) verified the double bond position. Detailed analyses of HMBC and NOESY data established the lanosterol skeleton having ethyl group at C-14. EIMS of **13** showed *m*/*z* 440 (M^+, 8%), 411 (M^+–Et, 100%) and 393 (M^+–Et–H_2O, 35%). The fission patterns are quite similar to those of authentic lanosterol [*m*/*z* 426 (M^+, 60%), 411 (M^+–Me, 100%), 393 (M^+–Me–H_2O, 32%)], further demonstrating a lanosterol homolog for **13**. The stereochemistry at C-20 has remained uncertain, but should have the same *R*-configuration as **2**, based on the reaction mechanism and on the fact that the chemical shift difference for the Me-21 was minimal between **2** and **13**; δ_H of **2** and **13** were 1.15 and 1.14 (d, J=6.3 Hz), and δ_C for C-20 of **2** and **13** were 36.68 and 36.67, respectively, in C_6D_6 solution.[8]

The product distribution of monocyclic (**8** and **9**), tricyclic **11** and fully cyclized **13** was in a ratio of 3:1:1. From **5**, tri-cyclic **10** and completely cyclized **12** were produced in a ratio of 1:2.6,[6] but no monocyclic compound was accumulated. The cavity size or the binding site to accommodate the Me group at C-10 of **1** is possibly more accurate than that at C-15, leading to higher production of monocyclic products from **6**. A looser binding of the ethyl group with the cyclase, due to the slightly bulky size at C-10, would have prevented the completion of the polycyclization reaction, thus resulting in the accumulation of two carbocation intermediates **15** and **16**.[9] Deprotonation from Me-28 of **15** (path b) could give **11**, while an enlargement of the five-membered C-ring of **15** into the six-membered ring (path a), followed by a further cyclization and backbone rearrangement, could give **13** according to Scheme 1. Proton elimination from **16** (path c) could give **8**. Intermediates **15** and **16** could be formed by the cyclization of (3*S*)-**6**, because **8**, **11** and **13** have a β-oriented OH group.

The *Tetrahymena pyriformis* cyclase catalyses the conversion reaction from squalene into pentacyclic tetrahymanol. This cyclase also accepts both enantiomers of (3*R*)- and (3*S*)-**1** to give α- and β-hydroxytetrahymanol, respectively, along with the production of the triterpene having a trimethylcyclohexanone moiety, the monocyclic ketone being produced only from (3*R*)-**1**, but not from (3*S*)-**1**.[10] However, the stereochemistry of

the trimethylcyclohexanone ring has remained unresolved. The chemical shifts of the cyclohexanone ring of **9** were compared with those of **19**[8] having the 2*R*,3*S*,4*R*-stereochemistry,[11] which is available from the chemical synthesis from (±)-**1** by using the Lewis acid $SnCl_4$.[11] A careful comparison of the NOESY spectrum of **9** with that of **19** showed that a strong NOE between Me-25 and H-2 was observed for **9**, whereas there was no NOE between them for **19** (Scheme 3). Detailed NOE analyses allowed us to propose the structure **9** (Fig. 1 and Scheme 3). Compound **19** is produced from (3*S*)-**1** via chair-formed **18**, which is then subjected to the 1,2-rearrangement reactions of the hydride and the methyl in an *antiparallel* concerted manner.[11] On the other hand, it is likely that the formation of **9** proceeds from (3*R*)-**6** via twist boat-formed **17** and that the rearrangement reactions proceed as shown in Scheme 3, taking into consideration the stereochemistry (2*S*,3*R*,4*R*) of the trimethylcyclohexanone ring of **9**. The deprotonation from the O*H* group, formed after the epoxide ring-opening, could give ketone **9**. Formation of **9** led to a surprising and important question, because lanosterol synthase is believed to be active only to (3*S*)-**1**, but inert to (3*R*)-**1**. One possible explanation of this paradox may be that the larger steric bulk size of the ethyl group may have induced incorrect placement of the (3*R*)-epoxide at the catalytic site intrinsic to the (3*S*)-epoxide, where (3*R*)-**6** had been constrained to fold with a boat form in the enzyme cavity. Despite the occurrence of looser binding around the A/B-ring formation site, the cyclase had still a binding ability to (3*S*)-**6** which enabled it to form the chair structure, leading to the production of **8** and to further cyclization to form **11** and **13**; the yield (31%) of **8**, **11** and **13** from (3*S*)-**6** was higher than that (7.7%) of **9** from (3*R*)-**6**.

In conclusion, this is the first report that a triterpene having a trimethylcyclohexanone ring was produced by mammalian cyclase. It is quite interesting from the aspect of molecular evolution that the same cyclohexanone skeleton is also constructed by the squalene cyclase of a protozoa *T. pyriformis*. This supports the idea that triterpenoid cyclases should have evolved from a common ancestor cyclase; that is, a variety of natural triterpene skeletons may have been created by subtle changes in the active sites.[12] This study also gave further evidence that lanosterol is biosynthesized via the ring-expansion process of the five-membered C-ring as shown in Scheme 1. It is noteworthy that the *ethyl group* migrates in a similar way to the methyl of natural **1**. Kyler et al. reported that the vinyl appendage (the same C2 unit as ethyl group) at C-10 had no influence on the cyclization as for Baker's yeast cyclase, leading to the complete polycyclization to give a lanosterol homolog without any abortive cyclization products having been trapped.[2] The specificity for squalene analogs is different between pig liver and Baker's yeast.[5,6]

Acknowledgements

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8. NMR data for **8**, **9**, **11**, **13** and **19**. ^{1}H NMR (600.13 MHz) and ^{13}C NMR (150.9 MHz) in C_6D_6, ppm relative to 7.28 and 128.0 ppm of the solvent peak.
Compound **8**: (analog of Achilleol A), δ_H: 0.92 (3H, s, H-25), 1.13 (3H, s, H-24), 1.14 (3H, t, J=7.6 Hz, H-31), 1.48 (1H, m, H-6ax), 1.68 (3H, s, H-30), 1.72 (1H, m, H-6eq), 1.73 and 1.74 (3H each, s, H-28 and H-29), 1.77 (1H, t, J=6.3 Hz, H-3), 1.80 (3H, s, H-23), 1.85 (2H, bt, J=7.7 Hz), 1.98 (1H, m, H-5ax), 2.02 (1H, m, H-8), 2.22 (2H, q, J=7.6 Hz, H-27), 2.22 (4H, t, J=7.7 Hz, H-15 and 19), 2.30 (8H, m, H-11, 12, 16, 20), 2.38 (1H, m, H-5eq), 2.41 (1H, m, H-8), 3.24 (1H, dd, J=13.8, 6.6 Hz), 4.84 (1H, s, H-26), 5.02 (1H, s, H-26), 5.37 (1H, bt, J=6.8 Hz), 5.43 (3H, m, H-10, 13, 17). δ_C: 13.6 (C-31), 16.1 (C-28 and C-29), 16.2 (C-25), 17.7 (C-30), 23.6 (C-27), 24.7 (C-7), 25.3 (C-23), 26.3 (C-24), 27.1 and 27.2 (C-16 and C-20), 28.4 (C-12), 29.05 (C-11), 32.6 (2C, C-5 and C-6), 35.9 (C-8), 40.2 (C-15 and C-19), 40.6 (C-2), 51.8 (C-3), 76.7 (C-1), 108.6 (C-26), 124.3 (C-10), 124.9 (C-13, 17 and 21), 131.1 (C-22), 134.9 and 135.1 (C-14 and C-18), 141.7 (C-9), 148.0 (C-4). $[\alpha]_D^{25}$ (EtOH) −7.8 (c=0.08).
Compound **9**: δ_H: 0.784 (3H, s, H-26), 0.903 (3H, d, J=7 Hz, H-25), 1.069 (3H, d, J=6.9 Hz, H-24), 1.146 (3H, t, J=7.5, H-31), 1.38 (1H, m, H-5eq), 1.72 (m, H-5 ax), 1.45 (2H, m, H-7), 1.60 (1H, m, H-4), 1.69 (3H, s, H-30), 1.75 and 1.73 (3H each, s, H-28 and H-29), 1.80 (3H, s, H-23), 2.01 (2H, m, H-8), 2.17 (1H, m, H-6ax), 2.20 (2H, q, J=7.5 Hz, H-27), 2.20–2.35 (6H, m, H-11, 12, 15 and 16), 2.25 (1H, m, H-6eq), 2.31 (1H, q, J=6.9 Hz, H-2), 5.36 (1H, bt, J=6.9 Hz, H-21), 5.40 (3H, m, H-10, 13, 17). δ_C: 9.23 (C-24), 13.60 (C-31), 14.05 (C-25), 16.09 and 16.20 (C-28 and -29), 17.71 (C-30), 21.05 (C-26), 24.12 (C-27), 27.11 and 27.22 (C-16 and -20), 28.98 (C-12), 29.45 (C-11), 30.32 (C-8), 34.65 (C-4), 36.25 (C-7), 36.93 (C-6), 42.45 (C-3), 50.07 (C-2), 124.4 (C-10), 124.75, 124.77 and 124.92 (C-10, 13 and 17), 130.88 (C-22), 135.0 and 135.3 (C-14 and 18), 141.85 (C-9) and 211.03 (C-1). Selected δ_H in $CDCl_3$ relative to the solvent peak (7.26

ppm), 0.85 (s, H-26), 1.00 (d, $J=6.7$ Hz, H-25), 1.11 (d, $J=6.9$ Hz, H-24), 0.98 (t, $J=7.6$ Hz, H-31). $[\alpha]_D^{25}$ (EtOH) +1.25 ($c=0.08$). These data are superimposable to those of the trimethylcyclohexanone ring from *T. pyriformis*.[10]

Compound **11**: δ_H: 0.91 (3H, s, Me-25), 1.00 (3H, m, Me-31), 1.05 (3H, s, Me-26), 1.10 (3H, s, Me-24), 1.35 (1H, m, H-27), 1.41 (1H, m, H-1), 1.48 (1H, m, H-1), 1.50 (1H, H-5), 1.55–1.66 (8H, m, H-7, 6, 11 and 2), 1.68 (3H, s, Me-30), 1.69 (1H, m, H-27), 1.74 (3H, s, Me-29), 1.80 (3H, s, H-23), 1.80 (1H, m, H-9), 1.88 (1H, m, H-12), 2.07 (1H, m, H-12), 2.09 (1H, m, H-15), 2.22 (2H, t, $J=7.5$ Hz, H-19), 2.23 (1H, m, H-15), 2.30 (2H, t, $J=7.5$ Hz H-20), 2.32 (1H, m, H-16), 2.43 (1H, m, H-16), 2.51 (1H, dd, $J=9.3$, 3.0 Hz, H-13), 3.14 (1H, dd, $J=11.2$, 5.3 Hz), 4.93 (1H, s, H-28) and 5.17 (1H, s, H-28), 5.36 (1H, t, $J=6.8$, H-21), 5.45 (t, $J=6.7$, H-17). δ_C: 8.9 (C-31), 16.1 (C-25 and C-29), 17.7 (C-30), 19.1 (C-6), 21.0 (C-11), 23.3 (C-26), 25.9 (C-7 and C-23), 27.1 (C-20), 27.2 (C-16), 29.0 (C-12), 29.4 (C-26), 29.6 (C-27), 29.7 (C-2), 35.0 (C-1), 35.7 (C-10), 39.4 (C-15), 39.5 (C-4), 40.2 (C-19), 47.4 (C-5), 49.1 (C-8), 50.8 (C-13), 53.8 (C-9), 79.1 (C-3), 111.0 (C-28), 124.7 and 124.9 (C-17 and C-21), 131.2 (C-22), 135.3 (C-17), 156.0 (C-14). $[\alpha]_D^{25}$ (EtOH) +8.9 ($c=0.09$).

Compound **13**: δ_H: 0.87 (3H, s, H-18), 0.97 (3H, s, H-30), 1.00 (3H, m, H-31), 1.08 (3H, s, H-19), 1.14 (d, $J=6.3$ Hz, H-21), 1.16 (3H, s, H-29), 1.21 (2H, dd, $J=12.8$, 2.0 Hz, H-5 and H-1), 1.30 (1H, m, H-22), 1.42 (1H, m, H-28), 1.53 (3H, m, H-28, 16 and 6), 1.58 (4H, m, H-2 and 15), 1.67 (3H, m, H-17 and 20), 1.72 (3H, H-1, 16 and 22), 1.75 (3H, s, H-27), 1.77 (1H, m, H-12), 1.83 (3H, s, H-26), 1.93 (1H, m, H-12), 2.04 (1H, m, H-7), 2.10 (2H, bt, $J=8$ Hz), 2.15 (1H, m, H-23), 2.20 (1H, m, H-7), 2.33 (1H, m, H-23), 3.16 (1H, dd, $J=10$, 6 Hz, H-3), 5.42 (t, $J=7$ Hz, H-24). δ_C: 10.7 (C-31), 15.7 (C-30), 17.5 (C-18), 17.7 (C-27), 18.5 (C-19), 18.7 (C-16), 18.9 (C-21), 21.2 (C-11), 25.4 (C-23), 25.8 (C-15), 25.8 (C-26), 28.3 (C-2), 28.3 (C-29), 28.5 (C-28), 28.9 (C-6), 30.5 (C-7), 31.5 (C-12), 35.8 (C-1), 36.7 (C-20), 36.8 (C-22), 37.7 (C-10), 39.0 (C-4), 46.8 (C-13), 51.1 (C-5), 51.5 (C-17), 53.7 (C-14), 78.6 (C-3), 125.7 (C-24), 130.8 (C-25), 133.3 (C-8), 136.2 (C-9). $[\alpha]_D^{25}$ (EtOH) +11.4 ($c=0.07$).

Compound **19**: Selected data, δ_H: 0.58 (s, H-26), 0.75 (d, $J=6.7$ Hz, H-25), 1.13 (d, $J=6.7$ Hz, H-24), 1.42 (2H, m, H-7), 1.45 (2H, m, H-5), 1.66 (1H, m, H-4), 1.85 (1H, m, H-8), 1.98 (1H, m, H-8), 2.01 (1H, m, H-6ax), 2.21 (1H, H-4), 2.37 (1H, H-6eq). $[\alpha]_D^{25}$ ($CHCl_3$) −5.4 ($c=0.33$).

9. During the polycyclization reaction, a thermodynamically favored C-8 carbocation intermediate is produced, but no A/B-fused bicyclic product was accumulated,[5,6] suggesting that the lifetime of the bicyclic cation might be significantly short.
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