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Enzymatic Cyclization of 22,23-Dihydro-2,3-oxidosqualene into Euph-7-en-3β-ol and Bacchar-12-en-3β-ol by Recombinant β-Amyrin Synthase

Ikuro Abe,*,† Yuichi Sakano,† Hideya Tanaka,† Weiwei Lou,† Hiroshi Noguchi,† Masaaki Shibuya,§ and Yutaka Ebizuka§

School of Pharmaceutical Sciences and the COE 21 Program, University of Shizuoka, Shizuoka 422-8526, Japan, and Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

Received December 26, 2003; E-mail: abei@ys7.u-shizuoka-ken.ac.jp

Since Ruzicka and co-workers proposed the "biogenetic isoprene rule",[1] the remarkable cyclization of (3*S*)2,3-oxidosqualene (**1**) to β-amyrin (**2**) has fascinated organic chemists for over half a century.[2] β-Amyrin synthase (βAS) (EC 5.4.99.-) is thought to bind the substrate in the *chair*−*chair*−*chair*−*boat* conformation and mediate formation of new carbon−carbon bonds in regio- and stereospecific manner (Scheme 1A).[3] The proton-initiated sequential cyclization first produces the tetracyclic dammarenyl C-20 cation, and the subsequent skeletal rearrangements lead to the pentacyclic oleanyl cation via the baccharenyl and the lupanyl cationic intermediates. Finally, a series of 1,2-hydride shifts with loss of the H-12α proton yields the pentacyclic ring system with the Δ^{12} double bond. βASs from several plants including *Pisum sativum* have been purified; the cDNA has been cloned and functionally expressed in *Saccharomyces cerevisiae*.[4,5] The enzymes show only ca. 20% overall amino acid sequence identity with bacterial squalene:hopene cyclase from *Alicyclobacillus acidocaldarius*, the best characterized squalene cyclizing enzyme with its X-ray crystal structure reported.[6] Recent mutational studies on βAS from *Panax ginseng* have revealed that the active-site residues Y261 and W259 play a critical role for D- and E-ring formation of β-amyrin.[7]

During the cyclization reaction, D-ring formation proceeds through a five-membered ring closure to generate a Markovnikov tertiary cation, which is followed by ring expansion to yield a tetracyclic secondary cation. Formation of the baccharenyl cation thus relieves some ring strain by creating a six-membered D-ring. To further understand the reaction mechanism, here we report enzymatic conversion of 22,23-dihydro-2,3-oxidosqualene (**3**), a substrate analogue lacking the terminal double bond of 2,3-oxidosqualene, therefore making it impossible to form pentacyclic products.[8]

22,23-Dihydro-2,3-oxidosqualene (**3**) was chemically synthesized in racemic form starting from 1,1′,2-trisnorsqualene-3-aldehyde as described before,[9a,10,11] and incubation with recombinant *P. sativum* βAS[5b] resulted in isolation of two products which were completely separated by reverse-phase HPLC.[12] Spectroscopic data (^{1}H and ^{13}C NMR, HMQC, HMBC, and MS) of the major product (3.0 mg, 4% yield) were characteristic of those of tetracyclic triterpene alcohols and showed good accordance with eupha-7,24-dien-3β-ol (butyrospermol)[7b] except the signals due to the terminal double bond, suggesting the structure of euph-7-en-3β-ol (**4**).[13] Confirmation of the structure, including the stereochemistry of C-20, was finally obtained by direct comparison (GC, GC−MS, and ^{1}H NMR) with the chemically synthesized euph-7-en-3β-ol.[14] On the other hand, the minor product (0.7 mg, 1% yield) afforded spectroscopic spectra completely identical with those of bacchar-12-en-3β-ol (**5**),[15] which was also confirmed by direct comparison with an authentic compound.[16]

22,23-Dihydro-2,3-oxidosqualene was thus enzymatically converted to a 4:1 mixture of euph-7-en-3β-ol (**4**) and bacchar-12-en-3β-ol (**5**) (Scheme 1B). The enzyme initiated cyclization of **3** from a *chair*−*chair*−*chair*−*boat* conformation first to generate the tetracyclic dammarenyl C-20 cation with the 17β-side chain. Then, a backbone rearrangement (H-17α→20α , H-13β→17β, CH_3-14α→13α, CH_3-8β→14β) with elimination of H-7α yielded euph-7-en-3β-ol, while D-ring expansion to the baccharenyl cation, and subsequent hydride shift (H-13β→18β) with loss of H-12α as in the case of β-amyrin formation, produced bacchar-12-en-3β-ol.

This is the first demonstration of the enzymatic formation of the baccharene skeleton with a six-membered D-ring. It was remarkable that the D-ring expansion sacrificing a tertiary carbocation for a secondary one took place even in the absence of the terminal double bond.[10] Thus, the enzymatic formation of the anti-Markovnikov six-membered D-ring did not depend on the participation of the terminal π-electrons. In contrast, bacterial squalene cyclases, normally catalyzing formation of pentacyclic triterpenes, have been shown to cyclize 2,3-dihydrosqualene to thermodynamically favored tetracyclic products with a Markovnikov five-membered D-ring; tetrahymanol synthase from *Tetrahymana pyriformis* afforded euph-7-ene, while *A. acidocaldarius* hopene synthase yielded a 1:1 mixture of dammar-13(17)-ene and dammar-12-ene.[10] In addition, it is noteworthy that the cyclization only yielded a product with the Δ^{12} double bond. Since it has been reported that a BF_3-Et_2O-induced backbone rearrangement of 3β,4β-epoxyshionane readily generated bacchar-12-en-3β-ol (**5**),[17] the 1,2-hydride shifts with the elimination of H-12α proton may possibly take place rather spontaneously to form the relatively stable Δ^{12} double bond. In βAS, active-site residues involved in the termination of the cyclization reaction by regiospecific proton abstraction at H-12α have not been identified yet.

In the absence of the terminal double bond, however, most of the reactions were interrupted at the dammarenyl cation, followed by a backbone rearrangement to yield euph-7-en-3β-ol. Here it should be noted that the stereochemistry of the cyclization product was strictly controlled by the enzyme. It is likely that the formation of the C-20*R* configuration from the dammarenyl C-20 cation involves the least motion pathway; i.e. only 60° rotation around the C-17-C-20 bond prior to the proton migration from C-17 to C-20, as in the case of lanosterol formation.[2] Interestingly, as mentioned above, enzymatic cyclization of 2,3-dihydrosqualene into euph-7-ene by *T. pyriformis* tetrahymanol synthase has been reported.[10]

Finally, our result suggests a close relationship between βAS and the triterpene synthases producing eupha-7,24-dien-3β-ol or bacchara-12,21-dien-3β-ol. Only a small modification of the active site would generate the diversity of the cyclization reactions. Indeed, recently it has been demonstrated that W259L mutant of *P. ginseng*

† University of Shizuoka.
§ The University of Tokyo.

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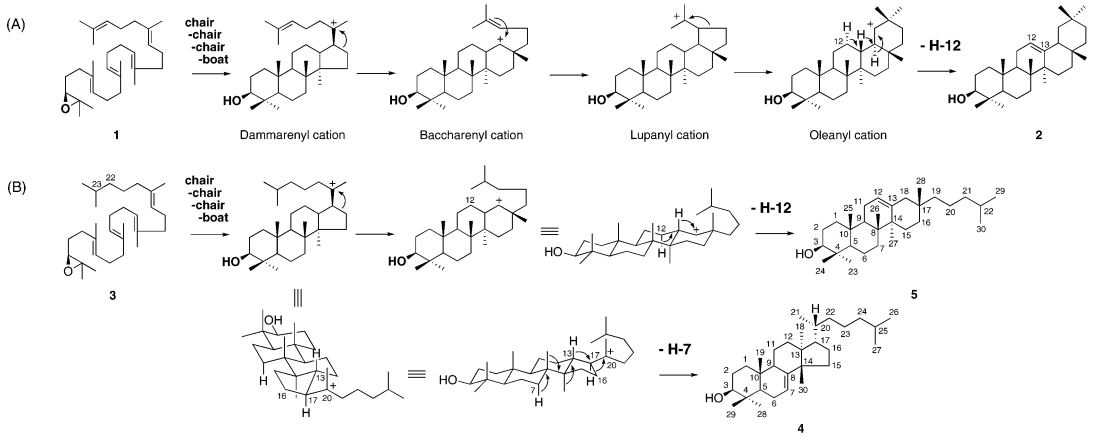
Scheme 1. Enzymatic Formation of (A) β-Amyrin (**2**) and (B) Euph-7-en-3β-ol (**4**) and Bacchar-12-en-3β-ol (**5**) from 22,23-Dihydro-2,3-oxidosqualene (**3**)



βAS yielded eupha-7,24-dien-3β-ol.[7b] Further study of the enzyme reaction by utilizing active-site probes are now in progress in our laboratories.

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Supporting Information Available: Complete set of spectroscopic data of euph-7-en-3β-ol and bacchar-12-en-3β-ol (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.16 (br t, 1H, J = 6.8 Hz), 5.15 (m, 2H), 5.10 (t d, 1H, J = 6.8, 0.8 Hz), 2.70 (t, 1H, J = 6.2 Hz), 2.10 (m, 2H), 2.08 (m, 4H), 2.01−1.99 (m, 8H), 1.93 (m, 2H), 1.67 (m, 2H), 1.62−1.58 (br s, 12H), 1.53 (sept, 1H, J = 6.8 Hz), 1.37 (m, 2H), 1.30 (s, 3H), 1.26 (s, 3H), 1.13 (m, 2H), 0.87 (d, 6H, J = 6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 135.3, 135.1, 135.0, 134.0, 124.9, 124.4, 124.2, 124.0, 64.2, 58.3, 39.9, 39.8, 39.7, 38.6, 36.3, 28.3 (×2), 27.9, 27.5, 26.7, 26.6, 25.7, 24.9, 22.6 (×2), 18.7, 16.0 (×3), 15.9. LRMS (EI): *m*/*z* (% rel int) 428, 410, 81. HRMS (EI): found for $[C_{30}H_{52}O]^+$ 428.3990; calcd 428.4018.

(12) *P. sativum* βAS was expressed in the yeast mutant strain GIL77 (30 L of culture) as described.[5b] The reaction mixture containing **3** (140 mg), 0.45 M sucrose, 1 mM EDTA, 1 mM DTT, and 0.1% Triton X-100 in 650 mL of 0.1 M KPB, pH 7.4 was incubated at 30 °C for 18 h. The incubations were stopped by adding equivalent volume of 20% KOH in 50% aq EtOH, saponified at 30 °C for 24 h, and extracted with 1.3 L of hexane (×3). The combined extracts were evaporated to dryness and separated on SiO_2 column (20% EtOAc/hexane) to yield 21.3 mg of 4,4-dimethylsterol fraction, which was further separated by HPLC (TSKgel Super-ODS, TOSOH; 95% aq CH_3CN; 1.0 mL/min; 40 °C) to give 3.0 mg of **4** and 0.7 mg of **5**, along with 10.5 mg of β-amyrin derived from 2,3-oxidosqualene accumulated in the mutant yeast cells. No other cyclization product was obtained in the reaction mixture, which was confirmed by GC−MS analysis.

(13) ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.26 (dt, 1H, J = 4.0, 2.8 Hz, H-7), 3.24 (dd, 1H, J = 11.0, 4.2 Hz, H-3), 0.97 (s, 6H, Me-28, Me-30), 0.87 (d, 6H, J = 6.8 Hz, Me-26, Me-27), 0.86 (s, 3H, Me-29), 0.83 (d, 3H, J = 6.4 Hz, Me-21), 0.81 (s, 3H, Me-18), 0.75 (s, 3H, Me-19). ^{13}C NMR (100 MHz, $CDCl_3$): δ 145.9 (C-8), 117.8 (C-7), 79.3 (C-3), 53.3 (C-17), 51.3 (C-14), 50.6 (C-5), 48.9 (C-9), 43.5 (C-13), 39.4 (C-24), 39.0 (C-4), 37.2 (C-1), 36.0 (C-20), 35.3 (C-22), 35.0 (C-10), 34.0 (C-15), 33.8 (C-12), 28.5 (C-16), 28.0 (C-25), 27.7 (C-2), 27.6 (C-28), 27.3 (C-30), 24.5 (C-23), 24.0 (C-6), 22.8 (C-26)*, 22.6 (C-27)*, 22.1 (C-18), 18.6 (C-21), 18.2 (C-11), 14.7 (C-29), 13.1 (C-19) (*exchangeable). LRMS (EI; TMS-derivative): *m*/*z* 500, 485, 395. HRMS (EI): found for $[C_{30}H_{52}O]^+$ 428.4047; calcd. 428.4018. $[\alpha]^{29}_D = -12°$ (c = 0.3 in $CHCl_3$).

(14) Hydrogenation of eupha-7,24-dien-3β-ol isolated from Shea butter. As described,[10] in the ^{1}H NMR, 20*R*-Me of euph-7-en-3β-ol, and 20*S*-Me of its (20*S*)-epimer, tirucall-7-en-3β-ol, gave slightly different chemical shifts (δ 0.83 and 0.86, respectively).

(15) ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.21 (t, 1H, J = 2.5 Hz, H-12), 3.20 (dd, 1H, J = 11.0, 5.0 Hz, H-3), 1.05 (s, 3H, Me-27), 1.00 (s, 3H, Me-23), 0.99 (s, 3H, Me-26), 0.96 (s, 3H, Me-25), 0.86 (d, 6H, J = 6.5 Hz, Me-29, Me-30), 0.79 (s, 3H, Me-24), 0.72 (s, 3H, Me-28). ^{13}C NMR (125 MHz, $CDCl_3$): δ 139.6 (C-13), 120.5 (C-12), 79.0 (C-3), 55.4 (C-5), 47.9 (C-9), 45.8 (C-19), 44.7 (C-18), 43.1 (C-14), 40.0 (C-21), 39.0 (C-8), 38.9 (C-1), 38.8 (C-4), 37.0 (C-10), 34.2 (C-17), 34.1 (C-7), 33.4 (C-16), 28.2 (C-23), 27.9 (C-22), 27.3 (C-2), 26.5 (C-15), 23.0 (C-11), 22.7 (C-29)*, 22.7 (C-30)*, 21.8 (C-27), 21.6 (C-28), 21.0 (C-20), 18.3 (C-6), 17.5 (C-26), 16.0 (C-25), 15.7 (C-24) (*exchangeable). LRMS (EI; TMS-derivative): *m*/*z* 500, 485, 395, 280, 279, 220, 190, 135. HRMS (EI): found for $[C_{30}H_{52}O]^+$ 428.4007; calcd. 428.4018. $[\alpha]^{29}_D = +9°$ (c = 0.07 in $CHCl_3$).

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