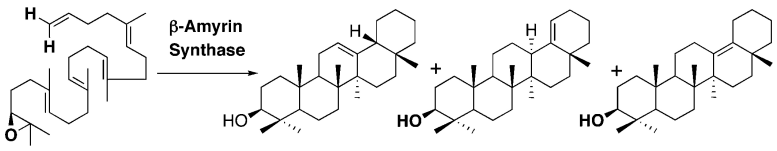
Communication

# Mechanism and Stereochemistry of Enzymatic Cyclization of 24,30-Bisnor-2,3-oxidosqualene by Recombinant β-Amyrin Synthase

Ikuro Abe, Yuichi Sakano, Megumi Sodeyama, Hideya Tanaka,
Hiroshi Noguchi, Masaaki Shibuya, and Yutaka Ebizuka

*J. Am. Chem. Soc.*, **2004**, 126 (22), 6880-6881• DOI: 10.1021/ja0490368 • Publication Date (Web): 15 May 2004






## More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 05/15/2004

# Mechanism and Stereochemistry of Enzymatic Cyclization of 24,30-Bisnor-2,3-oxidosqualene by Recombinant β-Amyrin Synthase

Ikuro Abe,*,† Yuichi Sakano,† Megumi Sodeyama,† Hideya Tanaka,† 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 February 20, 2004; E-mail: abei@ys7.u-shizuoka-ken.ac.jp

β-Amyrin synthase (βAS) (E.C. 5.4.99.) catalyzes the cyclization of (3*S*)-2,3-oxidosqualene (**1a**) to β-amyrin (olean-12-en-3β-ol) (**2**), forming a total of eight chiral centers in a single reaction. The formation of β-amyrin is initiated in the *chair−chair−chair−boat* conformation of **1a**, and the reaction is thought to proceed through a series of rigidly held carbocationic intermediates. During the biosynthesis of β-amyrin, an electrophilic addition of the tetracyclic baccharenyl cation on to the terminal double bond generates the lupanyl tertiary cation with a five-membered E-ring, which is followed by ring expansion to yield the oleanyl secondary cation (Scheme 1). Formation of the oleanyl cation thus relieves ring strain by creating a six-membered E-ring, despite sacrificing a tertiary carbocation for a secondary one. Finally, the oleanyl C-19 cation undergoes sequential 1,2-hydride shifts with elimination of H-12α to yield a 6/6/6/6/6-fused ring system with the $\Delta^{12}$ double bond.[1,2]

Recent advances in enzymology[3] and molecular biology[4] of βAS offered the opportunity to reinvestigate the still cryptic questions regarding the mechanism and stereochemistry of the remarkable ring-forming reaction.[5,6] We report herein enzymatic conversion of 24,30-bisnor-2,3-oxidosqualene (**1b**) by a recombinant βAS from *Pisum sativum*[4b] functionally expressed in yeast. As earlier described by Professor E. J. Corey in 1968, "the omission of these particular carbons from an oxidosqualene-like substrate constitutes a major structural perturbation in the region of the most complex and interesting processes in amyrin biosynthesis, those affecting the development of the D and E rings with their pendant groups".[7] Furthermore, to elucidate the enzyme reaction mechanism, we also carried out synthesis and enzymatic cyclization of $^{13}$C- and $^{2}$H-labeled isotopomers of **1b**.

24,30-Bisnor-2,3-oxidosqualene (**1b**) was chemically synthesized in racemic form starting from 1,1′,2-trisnorsqualene-3-aldehyde.[8] Two isotopomers, [23-$^{13}$C]-**1b** and [23,23-$^{2}$H]-**1b**, were also prepared by Wittig condensation of the aldehyde with $^{13}CH_3PPh_3I$ (99 atom % $^{13}$C) and $C^2H_3PPh_3I$ (95+ atom % $^{2}$H), respectively. Enzyme reaction with the recombinant *P. sativum* βAS resulted in isolation of three cyclization products, which were readily separated by reverse-phase HPLC.[9] Spectroscopic data (MS, $^{1}$H, and $^{13}$C NMR) of the major product (3.8 mg, 19% yield) showed good accordance with those of β-amyrin except the signals due to the terminal methyl groups; the MS spectrum indicated a characteristic parent ion peak at *m*/*z* 190 derived from retro-Diels Alder cleavage of the C-ring, while the $^{1}$H NMR revealed the presence of six methyl singlets (δ 1.15, 1.00, 0.98, 0.94, 0.81, 0.79) and one vinylic proton (δ 5.20, t, $J$ = 2.8 Hz).[10] Further, heteronuclear correlation NMR spectroscopy (HMQC and HMBC) as well as NOEs observed between Me-28/Me-26, Me-28/H-18, and Me-26/Me-25 confirmed the structure of 29,30-bisnor-β-amyrin (**4b**). On the other hand, incubation with [23-$^{13}$C]-**1b** and [23,23-$^{2}$H]-**1b** also afforded $^{13}$C- and $^{2}$H-labeled **4b**, respectively. No significant isotope effects were observed for the yields and patterns of the enzyme reaction products. The NMR spectra of the [$^{13}$C]-**4b** demonstrated that the label appeared at C-19 (δ 34.2), while C-18 (δ 51.8, d, $^{1}J_{CC}$ = 31 Hz) and C-20 (δ 27.4, d, $^{1}J_{CC}$ = 30 Hz) appeared as doublets. Moreover, in the $^{1}$H-decoupled $^{13}$C NMR of the [$^{2}$H]-**4b**, α-deuterium isotope effects were observed for C-19 ($\delta_{C(D)}$ 33.7, t, $^{1}J_{CD}$ = 21 Hz) and C-20 ($\delta_{C(D)}$ 26.8, t, $^{1}J_{CD}$ = 18 Hz), accompanied by β-deuterium-shifted C-18 ($\delta_{C(D)}$ 51.7) and C-21 ($\delta_{C(D)}$ 22.2). These labeling patterns clearly established that the cyclization reaction proceeded through route B in Scheme 1.

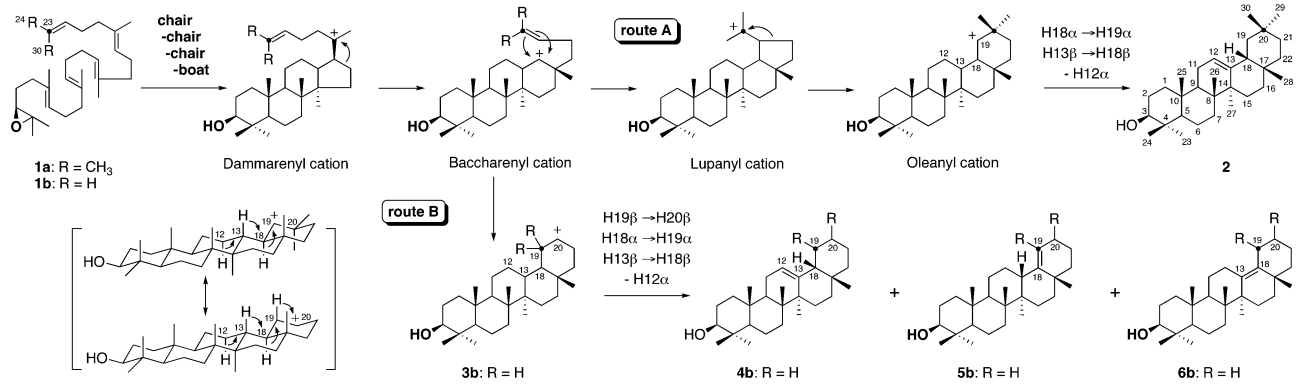
The structure of the second product (1.3 mg, 7% yield) was then determined to be 29,30-bisnorgermanicol (**5b**), a regioisomer with the $\Delta^{18}$ double bond, which was confirmed by the following spectroscopic data ($^{1}$H and $^{13}$C NMR, HMQC, HMBC, NOE, and MS).[11] First, the $^{1}$H NMR showed the presence of six methyl singlets (δ 1.09, 1.04, 0.97, 0.88, 0.77, 0.75) and one vinylic proton (δ 5.15, brs), in good agreement with those of germanicol.[5b] Further, in the NMR spectra of the [$^{13}$C]-**5b**, the label appeared at C-19 (δ 118.5), while C-18 (δ 145.5, d, $^{1}J_{CC}$ = 72 Hz), C-20 (δ 26.5, d, $^{1}J_{CC}$ = 42 Hz), and H-19 (δ 5.15, brd, $^{1}J_{CH}$ = 151 Hz) appeared as doublets. Moreover, in the $^{1}$H-decoupled$^{13}$C NMR spectrum of [$^{2}$H]-**5b**, α-deuterium isotope effects were observed for C-19 ($\delta_{C(D)}$ 118.0, brt, $^{1}J_{CD}$ = 13 Hz) and C-20 ($\delta_{C(D)}$ 25.8, brt, $^{1}J_{CD}$ = 16 Hz), accompanied by β-deuterium-shifted C-18 ($\delta_{C(D)}$ 145.4) and C-21 ($\delta_{C(D)}$ 18.8). In addition, NOEs observed between Me-28/H-13, Me-26/H-13, and Me-26/Me-25 were uniquely consistent with the structure of **5b**. Finally, the structure of the minor product (0.3 mg, 1% yield) was postulated to be 29,30-bisnor-δ-amyrin (**6b**), another regioisomer with the $\Delta^{13(18)}$ double bond, on the basis of its MS and $^{1}$H NMR spectra, which showed good accordance with those of δ-amyrin.[12, 4b]

24,30-Bisnor-2,3-oxidosqualene was thus enzymatically converted to a 3:1:0.2 mixture of 29,30-bisnor-β-amyrin (**4b**), 29,30-bisnorgermanicol (**5b**), and 29,30-bisnor-δ-amyrin (**6b**) by the recombinant *P. sativum* βAS. The efficient formation of **4b** supported the early report by Corey and Gross.[7] Further, the enzyme reactions with [23-$^{13}$C]- and [23,23-$^{2}$H]-labeled isotopomers of **1b** demonstrated that the cyclization of **1b** did not proceed through formation of a bisnorlupanyl *primary* cation with a five-membered E-ring (Scheme 1, route A) (in contrast, the biosynthesis of β-amyrin involves formation of the lupanyl *tertiary* cation), but an electrophilic addition of the tetracyclic C-18 cation on to the terminal double bond directly generated a thermodynamically favored bisnoroleanyl *secondary* cation with a less-strained six-membered E-ring (**3b**) (or its equivalent) (Scheme 1, route B). The E-ring formation of the bisnor products was thus apparently under thermodynamic control. Finally, the enzymatic formation of three

† University of Shizuoka.
‡ University of Tokyo.


10.1021/ja0490368 CCC: $27.50 

***Scheme 1.*** Proposed Mechanism for the Cyclization of 2,3-Oxidosqualene (**1**) (Route A) and 24,30-Bisnor-2,3-oxidosqualene (**1b**) (Route B)




regioisomers with the $\Delta^{12}$ $\Delta^{13(18)}$, and $\Delta^{18}$ double bond suggests lack of strict control on the final rearrangement reactions. It should be noted that formation of **4b** from the bisnoroleanyl C-20 cation (**3b**) requires *three* 1,2-hydride shifts (H-19$\beta$ → 20$\beta$, H-18$\alpha$ → 19$\alpha$, H-13$\beta$ → 18$\beta$), while that of $\beta$-amyrin from the oleanyl C-19 cation involves only *two* 1,2-hydride shifts (H-18$\alpha$ → 19$\alpha$, H-13$\beta$ → 18$\beta$) with elimination of H-12$\alpha$ (Scheme 1). Presumably, the absence of the terminal methyl groups resulted in a structural perturbation in the folding conformation of the E-ring of the intermediate C-20 cation at the active site of the enzyme (in the case of $\beta$-amyrin formation, the oleanyl C-19 cation is folded in all-chair conformation), resulting in the interruption of the sequential rearrangement reactions. To further understand the enzyme reaction mechanism, chemical studies utilizing active site probes in combination with site-directed mutagenesis are in progress.

**Acknowledgment.** This work was supported in part by the COE 21 Program and Grant-in-Aid for Scientific Research (Nos. 14580613, 15101007, and 1531053) from the Ministry of Education, Culture, Sports, Science and Technology, Japan by a Grant-in-Aid from The Mochida Memorial Foundation for Medical and Pharmaceutical Research, and by the Tokyo Biochemical Research Foundation, Japan.

**Supporting Information Available:** Complete set of spectroscopic data of compounds **1b**, **4b**, **5b**, and **6b** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) For recent reviews, see: (a) Abe, I.; Rohmer, M.; Prestwich, G. D. *Chem. Rev.* **1993**, *93*, 2189−2206. (b) Wendt, K. U.; Schulz, G. E.; Corey, E. J.; Liu, D. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 2812−2833. (c) Segura, M. J. R.; Jackson, B. E.; Matsuda, S. P. T. *Nat. Prod. Rep.* **2003**, *20*, 304−317. (d) Xu, R.; Fazio, G. C.; Matsuda, S. P. T. *Phytochemistry* **2004**, *65*, 261−291.

(2) (a) Eschenmoser, A.; Ruzicka, L.; Jeger, O.; Arigoni, D. *Helv. Chim. Acta* **1955**, *38*, 1890−1904. (b) Rees, H. H.; Britton, G.; Goodwin, T. W. *Biochem. J.* **1968**, *106*, 659−665. (c) Barton, D. H. R.; Mellows, G.; Widdowson, D. A.; Wright, J. J. *J. Chem. Soc., C* **1971**, 1142−1148. (d) Seo, S.; Tomita, Y.; Tori, K. *J. Am. Chem. Soc.* **1981**, *103*, 2075−2080. (e) Seo, S.; Yoshimura, Y.; Uomori, A.; Takeda, K.; Seto, H.; Ebizuka, Y.; Sankawa, U. *J. Am. Chem. Soc.* **1988**, *110*, 1740−1745.

(3) For enzyme purification, see: (a) Abe, I.; Sankawa, U.; Ebizuka, Y. *Chem. Pharm. Bull.* **1989**, *37*, 536−538. (b) Abe, I.; Ebizuka, Y.; Seo, S.; Sankawa, U. *FEBS Lett.* **1989**, *249*, 100−104. (c) Abe, I.; Sankawa, U.; Ebizuka, Y. *Chem. Pharm. Bull.* **1992**, *40*, 1755−1760.

(4) For cloning and expression, see: (a) Kushiro, T.; Shibuya, M.; Ebizuka, Y. *Eur. J. Biochem.* **1998**, *256*, 238−244. (b) Morita, M.; Shibuya, M.; Kushiro, T.; Masuda, K.; Ebizuka, Y. *Eur. J. Biochem.* **2000**, *267*, 3453−3460.

(5) (a) Kushiro, T.; Shibuya, M.; Ebizuka, Y. *J. Am. Chem. Soc.* **1999**, *121*, 1208−1216. (b) Kushiro, T.; Shibuya, M.; Masuda, K.; Ebizuka, Y. *J. Am. Chem. Soc.* **2000**, *122*, 6816−6824.

(6) Abe, I.; Sakano, Y.; Tanaka, H.; Lou, W.; Noguchi, H.; Shibuya, M.; Ebizuka, Y. *J. Am. Chem. Soc.* **2004**, *126*, 3426−3427.

(7) For an early report for enzymatic conversion of 24,30-bisnor-2,3-oxidosqualene by $\beta$AS, see: Corey, E. J.; Gross, S. K. *J. Am. Chem. Soc.* **1968**, *90*, 5045−5046. A $^3$H-labeled substrate was incubated with a crude cell-free extract from germinating pea seeds. Recrystallizations of the enzyme reaction product with cold 29,30-bisnor-$\beta$-amyrin were repeated to constant specific activity.

(8) $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 5.80 (ddt, 1H, $J$ = 17.2, 10.4, 6.6 Hz), 5.18−5.11 (m, 4H), 5.00 (br d, 1H, $J$ = 17.4 Hz), 4.93 (br d, 1H, $J$ = 10.1 Hz), 2.70 (t, 1H, $J$ = 6.0 Hz), 2.17−1.97 (m, 8H), 1.69−1.54 (m, 12H), 1.62−1.60 (br s, 12H), 1.30 (s, 3H), 1.26 (s, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 138.8, 135.1, 135.0, 134.4, 134.0, 124.9, 124.6, 124.4, 124.3, 114.2, 64.2, 58.3, 39.7 (×2), 39.0, 36.3, 32.4, 28.2 (×2), 27.5, 26.7, 26.6, 24.9, 18.7, 16.0 (×4). LRMS (EI): *m*/*z* 398, 380, 81. HRMS (EI): found for $[C_{28}H_{46}O]^+$, 398.3554; calcd, 398.3548.

(9) *P. sativum* $\beta$AS was expressed in the yeast mutant strain GIL77 (10 L of culture) as described before.[5] The reaction mixture containing **1b** (40 mg), 0.45 M sucrose, 1 mM EDTA, 1 mM DTT, and 0.1% Triton X-100 in 200 mL of 0.1 M KPB, pH 7.4, was incubated at 30 °C for 18 h. The incubations were stopped by adding equal volume of 20% KOH in 50% aq EtOH, saponified at 30 °C for 24 h, and extracted with 400 mL of hexane (×3). The combined extracts were evaporated to dryness and separated on $SiO_2$ column (20% EtOAc/hexane) to yield 13.2 mg of 4,4-dimethylsterol fraction, which was further separated by HPLC (TSKgel Super-ODS, TOSOH; 95% aq $CH_3CN$; 1.0 mL/min; 32 °C) to give 3.8 mg of **4b**, 1.3 mg of **5b**, and 0.3 mg of **6b**, along with 2.3 mg of $\beta$-amyrin derived from 2,3-oxidosqualene accumulated in the mutant yeast cells.

(10) $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 5.20 (t, 1H, $J$ = 2.8 Hz, H-12), 3.22 (dd, 1H, $J$ = 10.6, 5.0 Hz, H-3), 1.15 (s, 3H, Me-27), 1.00 (s, 3H, Me-23), 0.98 (s, 3H, Me-26), 0.94 (s, 3H, Me-25), 0.81 (s, 3H, Me-28), 0.79 (s, 3H, Me-24). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 145.6 (C-13), 121.6 (C-12), 79.1 (C-3), 55.2 (C-5), 51.8 (C-18), 47.7 (C-9), 41.7 (C-14), 41.6 (C-22), 39.8 (C-8), 38.8 (C-4), 38.6 (C-1), 37.0 (C-10), 34.2 (C-19), 33.1 (C-17), 32.7 (C-7), 28.7 (C-28), 28.1 (C-23), 27.4 (C-20), 27.2 (C-2)*, 27.2 (C-16)*, 26.3 (C-15), 25.9 (C-27), 23.5 (C-11), 22.3 (C-21), 18.4 (C-6), 16.8 (C-26), 15.6 (C-24), 15.5 (C-25) (* = exchangeable). LRMS (EI; TMS derivative): *m*/*z* 470, 455, 380, 365, 279, 190. HRMS (EI): found for $[C_{28}H_{46}O]^+$, 398.3554; calcd, 398.3548. $[\alpha]_D^{28}$ +36° (*c* 0.38, $CHCl_3$).

(11) $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 5.15 (br s, 1H, H-19), 3.21 (dd, 1H, $J$ = 11.5, 5.5 Hz, H-3), 1.09 (s, 3H, Me-26), 1.04 (s, 3H, Me-28), 0.97 (s, 3H, Me-23), 0.88 (s, 3H, Me-25), 0.77 (s, 3H, Me-24), 0.75 (s, 3H, Me-27). $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 145.5 (C-18), 118.5 (C-19), 79.0 (C-3), 55.5 (C-5), 51.2 (C-9), 43.4 (C-14), 40.7 (C-8), 40.6 (C-22), 38.9 (C-1), 38.9 (C-4), 38.8 (C-13), 37.7 (C-16), 37.2 (C-10), 34.6 (C-7), 34.4 (C-17), 28.0 (C-23), 27.4 (C-2)*, 27.4 (C-15)*, 26.5 (C-20), 26.1 (C-12), 25.5 (C-28), 21.2 (C-11), 18.9 (C-21), 18.3 (C-6), 16.7 (C-26), 16.1 (C-25), 15.4 (C-24), 14.7 (C-27) (* = exchangeable). LRMS (EI; TMS derivative): *m*/*z* 470, 380, 365, 341, 279, 231, 201, 190, 176. HRMS (EI): found for $[C_{28}H_{46}O]^+$, 398.3543; calcd, 398.3548. $[\alpha]_D^{29}$ −9° (*c* 0.13, $CHCl_3$).

(12) $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 3.21 (dd, 1H, $J$ = 11.0, 5.0 Hz), 1.08 (s, 3H), 0.99 (s, 3H), 0.97 (s, 3H), 0.88 (s, 3H), 0.86 (s, 3H), 0.77 (s, 3H). LRMS (EI; TMS derivative): *m*/*z* 470, 455, 365, 281, 190, 176, 161. HRMS (EI): found for $[C_{28}H_{46}O]^+$, 398.3560; calcd, 398.3548. $[\alpha]_D^{29}$ −35° (*c* 0.09, $CHCl_3$).

JA0490368