

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

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β -Amyrin synthase (β AS) (E.C. 5.4.99.) catalyzes the cyclization of (3S)-2,3-oxidosqualene (**1a**) to β -amyrin (olean-12-en-3 β -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-12 α to yield a 6/6/6/6-fused ring system with the Δ^{12} double bond.^{1,2}

Recent advances in enzymology³ and molecular biology⁴ of β AS 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 β AS 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 amyirin 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 ¹³C- and ²H-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, [23-¹³C]-**1b** and [23,23-²H]-**1b**, were also prepared by Wittig condensation of the aldehyde with ¹³CH₃PPh₃I (99 atom % ¹³C) and C²H₃PPh₃I (95+ atom % ²H), respectively. Enzyme reaction with the recombinant *P. sativum* β AS resulted in isolation of three cyclization products, which were readily separated by reverse-phase HPLC.⁹ Spectroscopic data (MS, ¹H, and ¹³C 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 ¹H 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 [23-¹³C]-**1b** and [23,23-²H]-**1b** also afforded ¹³C- and ²H-labeled **4b**, respectively. No significant isotope effects were observed for the yields and patterns of the enzyme reaction products. The NMR spectra of the [¹³C]-**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 ¹H-decoupled ¹³C NMR of the [²H]-**4b**, α -deuterium isotope effects were observed for C-19 ($\delta_{\text{C(D)}}$ 33.7, t, ¹*J*_{CD} = 21 Hz) and C-20 ($\delta_{\text{C(D)}}$ 26.8, t, ¹*J*_{CD} = 18 Hz), accompanied by β -deuterium-shifted C-18 ($\delta_{\text{C(D)}}$ 51.7) and C-21 ($\delta_{\text{C(D)}}$ 22.2). These labeling patterns clearly established that the cyclization reaction proceeded through route B in Scheme 1.

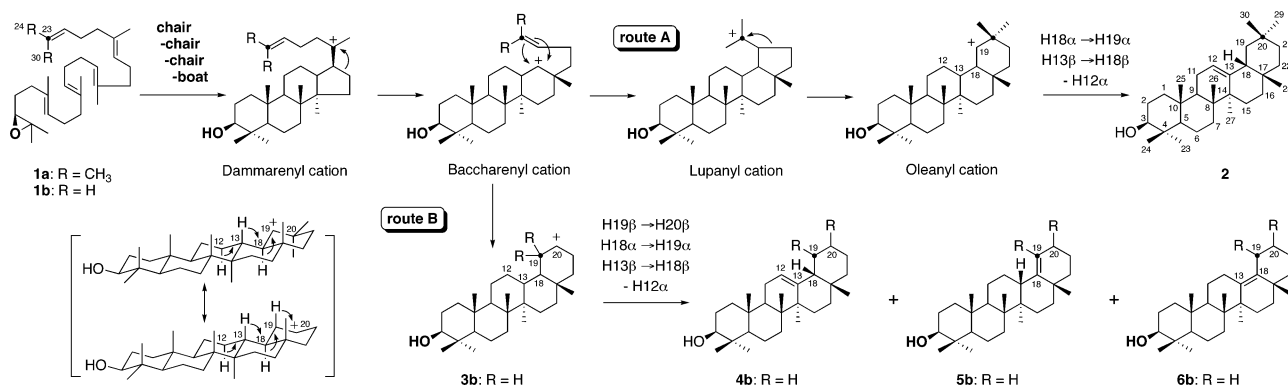
The structure of the second product (1.3 mg, 7% yield) was then determined to be 29,30-bisnorgermanicol (**5b**), a regioisomer with the Δ^{18} double bond, which was confirmed by the following spectroscopic data (¹H and ¹³C NMR, HMQC, HMBC, NOE, and MS).¹¹ First, the ¹H NMR showed the presence of six 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 [¹³C]-**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 ¹H-decoupled ¹³C NMR spectrum of [²H]-**5b**, α -deuterium isotope effects were observed for C-19 ($\delta_{\text{C(D)}}$ 118.0, brt, ¹*J*_{CD} = 13 Hz) and C-20 ($\delta_{\text{C(D)}}$ 25.8, brt, ¹*J*_{CD} = 16 Hz), accompanied by β -deuterium-shifted C-18 ($\delta_{\text{C(D)}}$ 145.4) and C-21- ($\delta_{\text{C(D)}}$ 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 ¹H 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* β AS. The efficient formation of **4b** supported the early report by Corey and Gross.⁷ Further, the enzyme reactions with [23-¹³C]- and [23,23-²H]-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

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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 Δ^{12} , $\Delta^{13(18)}$, and Δ^{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 β $\rightarrow$ 20 β , H-18 α $\rightarrow$ 19 α , H-13 β $\rightarrow$ 18 β), while that of β -amyrin from the oleanyl C-19 cation involves only *two* 1,2-hydride shifts (H-18 α $\rightarrow$ 19 α , H-13 β $\rightarrow$ 18 β) with elimination of H-12 α (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 β -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.

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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>.

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- (8) 1 H NMR (400 MHz, CDCl₃): δ 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, CDCl₃): δ 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 ($\times 2$), 39.0, 36.3, 32.4, 28.2 ($\times 2$), 27.5, 26.7, 26.6, 24.9, 18.7, 16.0 ($\times 4$). LRMS (EI): m/z 398, 380, 81. HRMS (EI): found for [C₂₈H₄₆O]⁺, 398.3554; calcd, 398.3548.
- (9) *P. sativum* β 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 ($\times 3$). The combined extracts were evaporated to dryness and separated on SiO₂ 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 CH₃CN; 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 β -amyrin derived from 2,3-oxidosqualene accumulated in the mutant yeast cells.
- (10) 1 H NMR (400 MHz, CDCl₃): δ 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-29), 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, CDCl₃): δ 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 [C₂₈H₄₆O]⁺, 398.3554; calcd, 398.3548. [α]_D²⁵ +36° (c 0.38, CHCl₃).
- (11) 1 H NMR (500 MHz, CDCl₃): δ 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, CDCl₃): δ 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 [C₂₈H₄₆O]⁺, 398.3543; calcd, 398.3548. [α]_D²⁹ –9° (c 0.13, CHCl₃).
- (12) 1 H NMR (400 MHz, CDCl₃): δ 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 [C₂₈H₄₆O]⁺, 398.3560; calcd, 398.3548. [α]_D²⁹ –35° (c 0.09, CHCl₃).

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