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Stereochemistry of the Cyclization-Rearrangement of (+)-Copalyl Diphosphate to (−)-Abietadiene Catalyzed by Recombinant Abietadiene Synthase from *Abies grandis*

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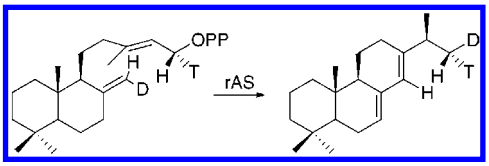
ABSTRACT



Syntheses and enzymatic cyclizations of 8α-hydroxy-17-nor copalyl diphosphate (8a), (15*R*)-[15-$^2H_1$] 8b, and (15*R*,17*E*)-[15-$^3H_1$,17-$^2H_1$] copalyl diphosphate ([^2H,^3H] 2) catalyzed by recombinant abietadiene synthase (rAS) gave 17-nor manoyl oxide (9a), (16*E*)-[16-$^2H_1$] 9b, and (15*S*,16*R*)-[16-$^2H_1$,16-$^3H_1$] abietadiene ([$^2H_1$,$^3H_1$] 4), respectively. These and other results indicate that conversion of CPP (2) to abietadiene (4) occurs by anti S_N' cyclization to a sandaracopimar-15-en-8-yl carbocation intermediate (13$^+$, 13*β*-methyl) followed by hydrogen transfer and methyl migration suprafacially on the *si* face of the vinyl group.

The abietanes comprise a large family of perhydrophenathrene-type diterpenes characterized by an isopropyl group, or its functionalized equivalent, at C13.[1] Familiar examples are abietic acid (**5**) and the isomeric levopimaric, neoabietic, and palustric acids, which are major diterpene constituents of conifer oleoresin.[2] This defensive secretion is produced by pines, firs, spruces, and other conifers as a primary response to wounds caused by physical injuries, insects, large herbivores, and microbial diseases.[3] The derivation of the abietane carbon skeleton from the predicted head-to-tail connectivity of isoprene units led L. Ruzícka to propose that the aberrant isopropyl group might arise by proton-induced rearrangement of a regular isoprenoid precusor, such as pimaric acid.[4] The acid-induced conversion of pimaric and isopimaric acids to abietic acid affords chemical precedent for this biogenetic relationship.[5]

The native enzymes responsible for the cyclization of (*E*,*E*,*E*)-geranylgeranyl diphosphate (**1**) to (−)-abietadiene (**4**) (Scheme 1) were isolated in partially purified form from the stems of both wound-induced grand fir (*Abies grandis*)

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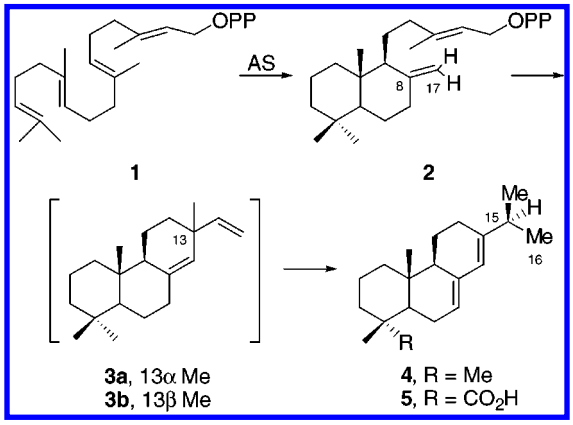
Scheme 1



and lodgepole pine (*Pinus contorta*) saplings.[6] Cytochrome P450 mixed function oxygenases and a dehydrogenase from the same sources, which carry out the sequential oxidation of the C18 methyl group of **4** to produce abietic acid, have also been characterized.[7] The gene encoding abietadiene synthase (AS) from grand fir has been cloned, and the cDNA was functionally expressed as a single polypeptide bearing a putative *N*-terminal plastidial targeting sequence.[8] Recombinant AS (rAS) was heterologously expressed in *E. coli* as a pseudomature enzyme without the *N*-terminal 84 amino acids[9] and used in crude bacterial extracts after lysis and clarification (at 0.1 g wet cell weight/mL buffer). Although the resulting recombinant AS catalyzed the divalent metal ion-dependent cyclizations of both **1** and the predicted bicyclic intermediate (+)-copalyl diphosphate (**2**) to **4**,[9,10] none of the four plausible pimaradiene intermediates was converted into the rearranged diterpene to an appreciable extent under the same assay conditions.[10] Recent deuterium labeling experiments revealed that the cyclization-rearrangement of (+)-CPP to abietadiene occurs with stereospecific "intramolecular" transfer of a hydrogen atom from the 17-*pro-E* position of **2** to the terminal carbon of the side chain, which becomes the *pro-S* methyl of the isopropyl group.[9,10] In this communication, we present evidence indicating a 13β-methyl group in the presumed pimaradiene (**3b**) or pimarenyl carbocation intermediate and a suprafacial relationship between the hydrogen reincorporated at C16 and the subsequent methyl migration to C15.[11]

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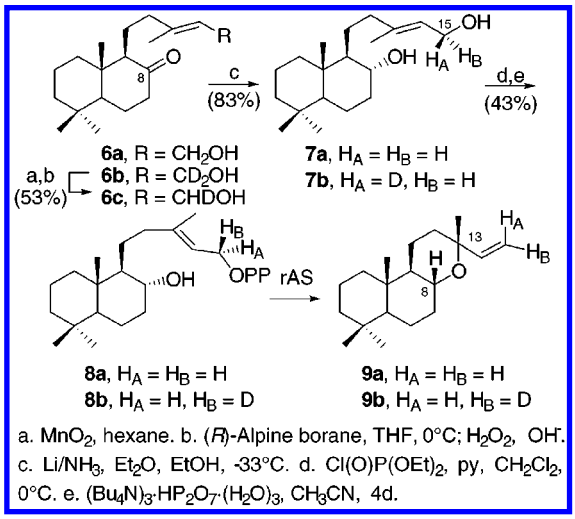
(11) The occurrence of an analogous proton transfer to form the *pro-S* methyl group of a presumptive abietadiene intermediate was inferred from the labeling pattern of ginkgolide A biosynthesized from [6,6,6-$^2H_3$] mevalonate in *Ginkgo biloba* cell cultures: (a) Schwarz, M.; Arigoni, D. In *Isoprenoids Including Carotenoids and Steroids*; Cane, D. E., Ed.; Vol. 2 in *Comprehensive Natural Products Chemistry*; Barton, D., Nakanishi, K., Meth-Cohn, O., Eds.; Elsevier: Oxford, 1999; Chapter 14. (b) Schwarz, M. K. Dissertation 10951, ETH Zürich, Switzerland, 1994.

The coupled cyclization-rearrangement of **2** was intercepted by use of an oxa analogue (**8a**) bearing an 8α-hydroxyl function in place of the exocyclic methylene group (Scheme 2). 8-Oxo-17-nor copalol (**6a**), obtained in three

Scheme 2



steps from methyl copalate,[12] underwent reduction with Li/NH_3 (EtOH, ether, −33 °C) to 8α-hydroxy-17-nor copalol (**7a**, 83%).[13,14] Conversion to the corresponding allylic diphosphate **8a** was accomplished in two steps by phosphorylation [$(EtO)_2P(O)Cl$, py, CH_2Cl_2, 0 °C, 3 h],[15] displacement with pyrophosphate anion [$(Bu_4N)_3HP_2O_7$, CH_3CN, rt, 3.7 d], and purification according to the Poulter methods (66%).[14,16] Incubation of **8a** with rAS[8,10,17] effected clean cyclization to 17-nor manoyl oxide **9a** (yield, ca. 150 μg, 46% by ^1H NMR estimate), the 13β-methyl configuration of which was established by NOE measurements.[14] Acid-catalyzed dehydration (pTsOH·H_2O, ether, rt, 1.5 h) of **7a** afforded a 1.5:1 mixture of **9a** and its 13α-methyl isomer (76%). The formation of 17-nor manoyl oxide in the enzymatic cyclization implicates a β-methyl group at C13

(12) (a) catalytic OsO_4, NMO, acetone; 3:1 mixture of 8,17- and 13,-14-diols (78%); (b) $LiAlH_4$ (or $LiAlD_4$ for **6b**), ether; (c) $NaIO_4$, aqueous acetone (60% for two steps).
(13) All new compounds were characterized at high purity by appropriate ^1H NMR, ^{13}C NMR, and IR spectra. Purity ≥ 95% was established by satisfactory combustion analysis and/or inspection of ^1H and ^{13}C NMR spectra. Detailed experimental procedures and characterization data are provided in the Supporting Information. Key data are presented in the text or in footnote 14.
(14) Key spectral and physical data for selected compounds: **7a**, mp 85−86 °C, ^1H NMR δ 3.41 (td, J = 10.6, 4.9 Hz, 1H, H8); **8a**, ^{31}P NMR δ −7.02, −9.72 (2d, J = 22 Hz); **9a**, NOE irrad at 3.52 obs 0.74 (C20 CH_3, 7.3%), 1.21 (C17 CH_3, 6.7%); **9b**, ^1H NMR δ 5.37 and 6.04 (2d, J = 17.4 Hz, 1H each, CH=CHD).
(15) Ravn, M. M.; Jin, A. Q.; Coates, R. M. *Eur. J. Org. Chem.*, in press.
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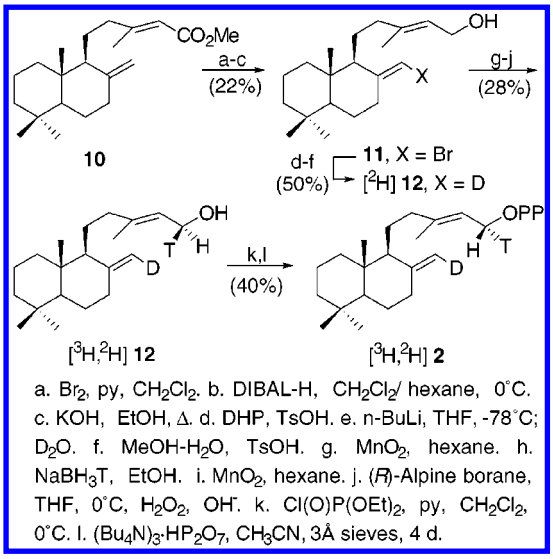
(17) Incubation conditions:[8,10] 30 mM HEPES, pH 7.2, 5.0 mM dithiothreitol, 7.5 mM $MgCl_2$, 20 μM $MnCl_2$, 5% (v/v) glycerol, 80 μM **8a** (0.40 μmol total), 3 × 1 mL rAS cell lysate; 31 °C, 16 h. Purification by pipet chromatography over $MgSO_4$ and silica gel; yield, ca. 150 μg (46%) by ^1H NMR estimate.

of the tricyclic intermediate corresponding to sandaracopimaradiene (**3b**) which in fact is a consistent, albeit very minor, byproduct (ca 2%) that accompanies abietadiene in the extractable products from incubations of **1** with rAS.[8,10]

The stereochemistry of the enzyme-catalyzed S_N' cyclization was determined by deuterium labeling at C15 (Scheme 2). Allylic oxidation (MnO_2, hexane, rt) of **6b** followed by *re*-selective hydride reduction with (*R*)-Alpine borane[18] (THF, H_2O_2, NaOH)[19] and Li/NH_3 reduction gave **7b**. The 15*S* configuration and stereohomogeneity (98% de) of the intermediate keto alcohol **6c** were confirmed by ^{1}H NMR analysis of the corresponding camphanate ester.[20] Conversion to **8b** by diphosphate displacement as described above for unlabeled **7a** was assumed to proceed with complete inversion of configuration, as established for (*S*)-[1-$^2H_1$] geraniol.[15,20] rAS-catalyzed cyclization of **8b** gave rise to **9b**, the 16*E* stereochemistry of which was established by the large coupling ($J = 17$ Hz) between the two vinyl protons.[14] Hence, the stereochemistry of this enzyme-catalyzed S_N' cyclization, and by inference that of **2**, is anti, in agreement with similar S_N' cyclizations that occur in diterpene biosynthesis.[21]

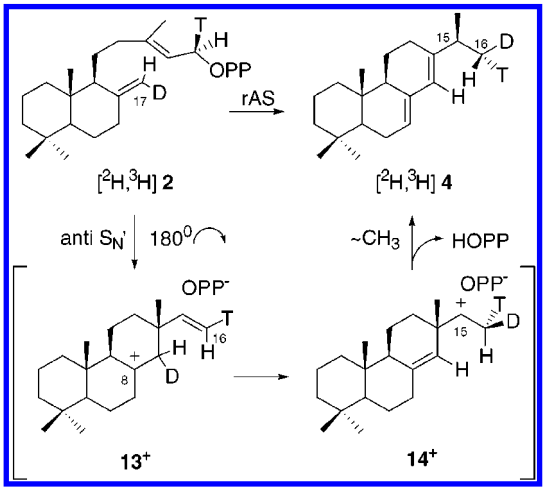
The stereochemistry of the hydrogen reincorporation at C16 during the rearrangement that generates the isopropyl group was elucidated by double isotope labeling (Schemes 3 and 4). (15*R*,17*E*)-[15-$^3H_1$,17-$^3H_1$] **2** was synthesized from

Scheme 3



methyl copalate as shown in Scheme 3. Bromination of the exocyclic double bond followed by diisobutylaluminum

Scheme 4



hydride reduction and dehydrobromination and afforded (17*E*)-17-bromocopalol (**11**). The corresponding tetrahydropyranyl ether was lithiated, deuterated, and hydrolyzed to (17*E*)-[17-$^2H_1$] copalol (**12**). A four-step reaction sequence consisting of MnO_2 oxidation, $NaBH_3T$ reduction, MnO_2 oxidation, and (*R*)-Alpine borane reduction gave (15*S*,17*E*)-[15-$^2H_1$,17-$^3H_1$] copalol ([^{2}H,^{3}H] **12**).[18,19] Conversion to the diethyl phosphate followed by displacement with $(Bu_4N)_3$-P_2O_7H in CH_3CN with inversion of configuration[15] provided (15*R*,17*E*)-[17-$^3H_1$,15-$^2H_1$] **2** (0.13 mCi, 40% radiochemical yield, 21.8 mCi/mmol).

Incubation with rAS[17] and addition of carrier afforded [16-$^2H_1$,16-$^3H_1$] abietadiene (**4**) (1.8 mg, 1.12 μCi/μmol, 1.57×10^8 dpm), which was subjected to Kuhn−Roth oxidation (aq H_2CrO_4, reflux, 16 h)[22] (Scheme 4). The resulting [^{2}H,^{3}H] acetic acid (3.1×10^7 dpm, 20% radiochemical yield, 90% radiochemical purity) was isolated by steam distillation and analyzed by enzymatic chirality assay with malate synthase and fumarase.[23] The *F* values observed, 74.1 ± 2.1 and 74.7 ± 1.6, establish *R* stereochemistry for the chiral acetate, formation of (15*S*,16*R*)-[16-$^2H_1$,16-$^3H_1$] abietadiene (**4**) in the enzymatic reaction, and overall retention of configuration in the replacement of diphosphate of [^{2}H,^{3}H] **2** with deuterium.

Because an anti S_N' cyclization of doubly labeled **2** would generate a vinyl group bearing tritium in the 16*E* position of the pimarenyl intermediate (e.g., **13**$^+$), the deuterium atom from C17 and the methyl group from C13 both migrate to the *si*,*si* face of the vinyl group of **3b**, i.e., a formal suprafacial relationship.[24] This stereochemical finding is at variance with either a concerted pericyclic mechanism

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predicted to be antarafacial on the basis of orbital symmetry considerations[11b] or a synchronous proton addition to C16 and antiperiplanar methyl migration to C15. Consequently a secondary sandaracopimaren-15-yl carbocation (**14**$^+$)/diphosphate anion pair, or alternatively a covalently bonded pimarenyl−enzyme adduct,[25,26] is implicated prior to the methyl group rearrangement and proton elimination steps leading to abietadiene.

The occurrence of bona fide secondary carbocation intermediates in terpene synthase-catalyzed cyclizations and rearrangements is rare, reflecting the higher energy of these species. Examples are found in mechanistic schemes leading to monoterpenes (bornyl diphosphate and fenchol synthases),[27a] sesquiterpenes (pentalenene and trichodiene synthases),[27b] tetracyclic diterpenes (e.g., *ent*-kaurene synthase),[21a] and polycyclic triterpenes (squalene and oxido-squalene synthases).[27c,d] However, in all of these cases the 15 kcal/mol thermodynamic energy barrier between the secondary ion[28] and a preceding tertiary carbocation would be offset by the substantial enthalpic gains associated with cyclizations into C=C double bonds ($\Delta H_{BE} \approx -20$ kcal/mol) or with relief of ring strain (bicyclo[3.1.1]heptane/bicyclo[2.2.1]-heptane, $\Delta\Delta H_{SE} = -19$ kcal/mol),[29] together with the stabilization arising from charge delocalization (bridged ions) and concerted bond-forming reactions.

The thermodynamically uphill isomerization of the tertiary pimarenyl ion **13**$^+$ to the apparently localized secondary ion **14**$^+$ in the absence of an exothermic counterbalance or the possibility of a lower energy concerted mechanism is unprecedented. Although the 15 kcal/mol thermodynamic barrier between tertiary and secondary carbenium ions[28] should be surmountable at room temperature, it nevertheless seems likely that AS participates overtly in catalyzing the process and lowering the thermodynamic deficit. Some important factors to consider in the enzyme-induced destabilization of **13**$^+$/OPP$^-$ and/or stabilization of **14**$^+$/OPP$^-$ carbocation-diphosphate anions are ion pair distances and forces,[30] homoallyl interaction in **14**$^+$, π-complexation with the aromatic rings of amino acid side chains at the active site,[31] interactions with proximal peptide carbonyl dipoles or nucleophilic heteratoms,[32] and electrostatic field gradients. Interestingly, binding of a sandaracopimarenyl amine, mimicing the secondary carbocation intermediate, is greatly enhanced (~1000-fold) by the addition of inorganic diphosphate,[24b] suggesting that AS utilizes the diphosphate anion to stabilize the secondary carbocation intermediate. The X-ray crystallographic structure of rAS and its complexes with pimarenyl ion mimic inhibitors may reveal what specific interactions and mechanisms are involved in the remarkable pimarenyl$^+$−abietadienyl$^+$ rearrangement.

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Supporting Information Available: Experimental procedures and full characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) (a) The relatively efficient enzymatic cyclization of **8a** and the unproductive binding of its 8β-OH isomer ($K_i = 0.18$ μM),[24b] as well as precedent,[21] suggest that the S$_N$′ cyclization probably takes place on the *re*,*re*(α) face of C17 to generate **13**$^+$ with a 14α deuterium substituent. (b) Ravn, M. M.; Coates, R. M.; Peters, R. J.; Croteau, R., unpublished results.

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