Stereospecific intramolecular proton transfer in the cyclization of geranylgeranyl diphosphate to (−)-abietadiene catalyzed by recombinant cyclase from grand fir (*Abies grandis*)

Matthew M. Ravn,[a] Robert M. Coates,*[a] Reinhard Jetter[b] and Rodney B. Croteau[c]

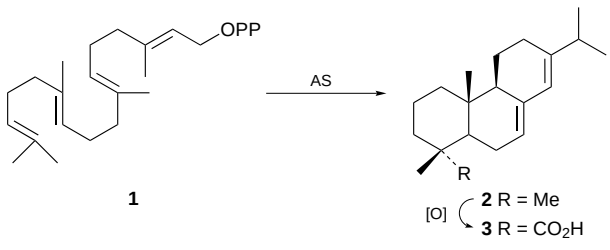
[a] *Department of Chemistry, University of Illinois, 600 S. Mathews Avenue, Urbana, IL 61801, USA*
[b] *Lehrstuhl für Botanik der Universität Würzburg, Mittlerer Dallenbergweg 64, D-97082 Würzburg, Germany*
[c] *Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA*

The cyclization–rearrangement of deuterated geranylgeranyl diphosphate (GGPP) and (+)-copalyl diphosphate (CPP) catalyzed by recombinant (−)-abietadiene synthase from grand fir proceeds with intramolecular proton transfer from C-19 of GGPP, and from the C-17 pro-*E* position of CPP, to form the C-16 *pro-S* methyl group of (−)-abietadiene.

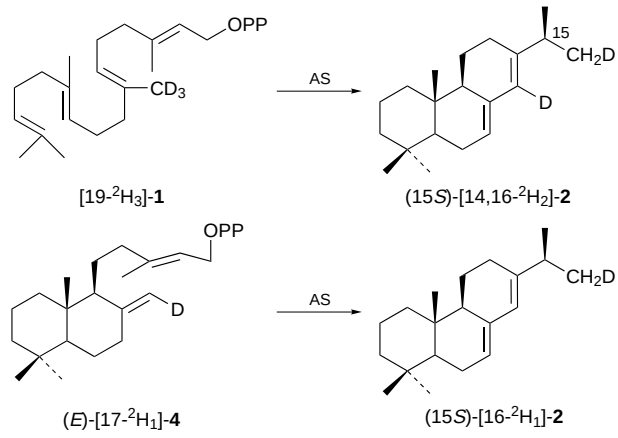
(−)-Abietic acid **3** is a major component of oleoresin exuded by conifer species, including lodgepole pine (*Pinus contorta*) and grand fir (*Abies grandis*),[1] as a defensive secretion against insect and pathogen attack. This diterpenoid resin acid is biosynthesized by cyclization of geranylgeranyl diphosphate (GGPP, **1**) to (−)-abietadiene **2** and sequential oxidations of the C-18-methyl group (Scheme 1).[2] (−)-Abietadiene synthase (AS) was purified and identified as an 80 kDa protein with general characteristics typical for terpenoid cyclases.[3]

AS from grand fir yielded internal amino acid sequence information from which a PCR-based cloning strategy was developed.[4] A 286 base-pair insert was isolated that encoded an 868-amino acid preprotein bearing a putative plastidial transit peptide. The cDNA encoded preprotein was functionally expressed in *Escherichia coli*, thereby confirming that a single enzyme catalyzes this multistep cyclization. Recent experiments with the recombinant enzyme (rAS) demonstrated that (+)-copalyl diphosphate (CPP, **4**) could serve as an efficient alternative precursor of **2**† whereas none of the four pimaradiene isomers tested were converted to abietadiene. Here we report the use of deuterated substrates to examine the mechanism of the rAS cyclization.

The synthesis of the labelled substrates [19-$^2H_3$]-**1** and (17*E*)-[17-$^2H_1$]-**4** followed analogous procedures in the literature. 19-Nor-7-methoxycarbonylgeranylgeranyl benzyl ether prepared by *Z*-selective phosphonate condensation[5] was converted to [19-$^2H_3$]geranylgeraniol by (a) AlD_3; (b) MsCl, Et_3N, CH_2Cl_2; (c) $LiBEt_3D$, THF; (d) Li, NH_3. (17*E*)-[17-$^2H_1$]Copalol was obtained from (17*E*)-17-bromocopalyl THP ether by lithiation, deuterolysis and deprotection.[6] Diphosphate esters were formed by standard methods[7] and were characterized by ^1H and ^{31}P NMR spectra. Incubation of these labelled substrates and [1-$^2H_2$]-**1** with rAS‡ gave (15*S*)-[14,16-$^2H_2$]-, (15*S*)-[16-$^2H_1$]- and (15*S*)-[16-$^2H_2$]-**2** respectively, with one deuterium located exclusively in the upfield methyl doublet of the isopropyl group according to ^1H NMR integrations and broadening from deuterium coupling (Scheme 2).§ No loss of the deuterium transferred to C-16 could be observed by GC–MS (≤1%). The absence of any detectable [$^2H_1$]abietadiene from a cross-over experiment with [19-$^2H_3$]-**1** and unlabelled **1** establishes that proton or deuteron transfer occurs within one catalytic cycle and rules out an enzyme-bound pimara-7,15-diene intermediate.

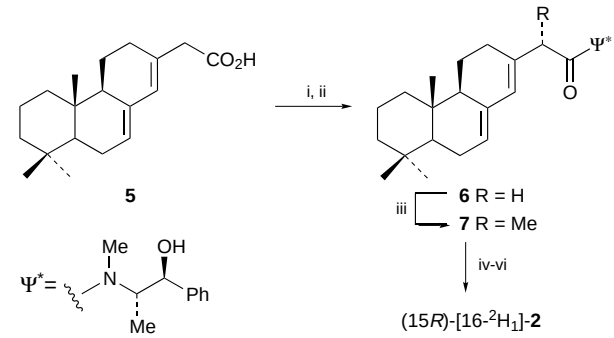


Scheme 1

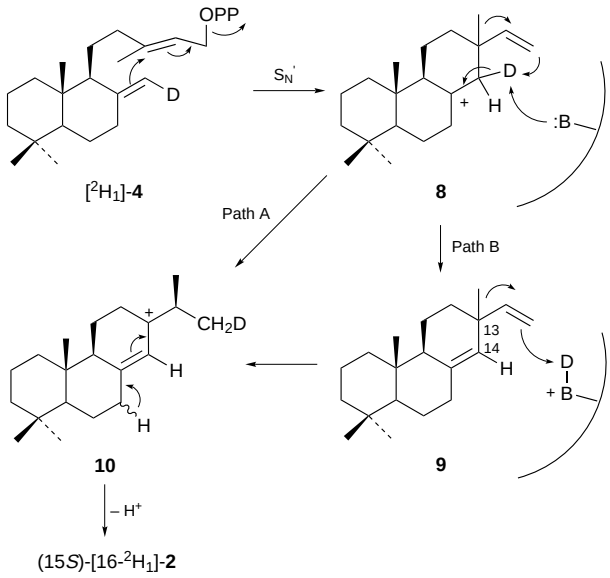


Scheme 2

NMR assignments for the isopropyl methyl groups required unambiguous synthesis of stereospecifically labelled **2** (Scheme 3). Reaction of the tricyclic 8(14)-en-13-one[8] with ethyl lithioacetate·$CeCl_3$ complex provided a mixture of β-hydroxy esters (91%, 3 : 1 α-OH : β-OH) which underwent dehydration (HCl, EtOH, reflux) and saponification to give 7,13-diene acid **5** (65%).¶ Methylation of the ψ-ephedrine amide **6** afforded two diastereomers (94 : 6). The major isomer is assigned the 15*S*



Scheme 3 *Reagents and conditions*: i, $(ClCO)_2$, PhH; ii, (*S*,*S*)-pseudoephedrine, pyridine, THF, 84% from **5**; iii, LDA, LiCl, THF, −78 °C; MeI, −78 °C, 92%; iv, $LiBH_3NH_2$, THF, 73%; v, MsCl, Et_3N, CH_2Cl_2, 0 °C; vi, $LiEt_3BD$, THF, 0 °C, 88% from **7**



Scheme 4

configuration (**7**) based on the established stereodirecting influence of this chiral auxiliary.[9] $LiBH_3NH_2$ reduction,¶ mesylation and $LiBEt_3D$ displacement gave (15*R*)-[16-$^2H_1$]-**2** (64%). The ^1H NMR spectrum shows deuterium coupling and a characteristic upfield isotope shift for the downfield isopropyl methyl group of unlabelled **2** which is therefore assigned the *pro-R* configuration.§ It follows that rAS effects intramolecular proton transfer to the terminal carbon of the vinyl group which becomes the *pro-S* methyl, and that the methyl migration occurs to the *si* face at C-15 of the putative pimara-9(14),15-diene intermediate.

Intramolecular proton transfers to enzyme-bound polyolefin intermediates have been observed previously in pentalenene and taxadiene biosynthesis.[10] The occurrence of an analogous proton transfer to form the *pro-S* methyl of a presumptive abietadiene intermediate was inferred from the labelling pattern of ginkgolide A biosynthesized from [6,6,6-$^2H_3$]mevalonate in *Ginkgo biloba* cell cultures.[11] The same *si* face selectivity for the methyl migration presumed to occur in the biosynthesis of 12-*O*-methylferruginol and cryptotanshinone was deduced from label distributions resulting from incorporation experiments with [*U*-^{13}C]glucose in *Salvia miltiorhiza* cell cultures.[12]

Two plausible mechanisms for this novel cyclization–rearrangement are presented in Scheme 4. S_N' cyclization of **4** analogous to those involved in the biosynthesis of related pimarane and kaurane diterpenes[6,13] gives the pimaren-8-yl carbocation **8** which can undergo either intramolecular C-14-to-C-16 hydrogen transfer (Path A) or enzyme-mediated proton elimination to form a pimara-8(14),15-diene intermediate **9** that re-incorporates the proton at C-16 (Path B). Further research to elucidate the mechanism of the cyclization–rearrangement, the nature of the intermediate(s), and the identity of the participating enzyme base, if any, is underway.

Footnotes and References

* E-mail: coates@aries.scs.uiuc.edu

† R. Jetter and R. Croteau, manuscript in preparation.

‡ Enzyme incubations conditions: HEPES buffer (30 mM, pH 7.2), rAS (100 μl), dithiothreitol (5 mM), $MgCl_2$ (7.5 mM), $MnCl_2$ (20 μM), 5% glycerol (v/v) and GGPP (20 μM) in 1 ml.

§ *Selected ^1H NMR (500 MHz, C_6D_6 J/Hz) data* for isopropyl methyls of (15*S*)-[14,16-$^2H_2$]-**2**: δ_H 1.003 (br d, *J* 6.8, ≈2H, CDH_2), 1.030 (d, *J* 6.8 Hz, 3 H, CH_3). For (15*R*)-[16-$^2H_1$]-**2**: δ_H 1.014 (br d, *J* 6.8, ≈2 H, CDH_2), 1.020 (d, *J* 6.8, 3 H, CH_3). For **2**: δ_H 1.020 (d, *J* 6.9, 3 H, CH_3) and 1.031 (d, *J* 6.9, 3 H, CH_3). Observed deuterium isotope shift $\Delta\delta$ = −0.017 ppm in each case.

¶ *Selected data* for **5** (di-*n*-amylamine salt): mp, 64–65 °C; λ_{max} 244 nm (ε 19 600 M^{-1} cm^{-1}). For **5**: δ_H(500 MHz, $CDCl_3$, *J*/Hz) 5.48 (dt, *J* 5.0, 2.5, 1 H, C-7 =C*H*), 5.90 (s, 1 H, C-14 =C*H*). For (15*S*)-abietadien-16-ol: δ_H(500 MHz, $CDCl_3$, *J*/Hz) 0.99 (d, *J* 7.0, 3 H, CHCH_3), 3.47 (dABq, *J* 7.8, 10.4, $\Delta\nu_{AB}$ 19 Hz, 2 H, CH_2OH).

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