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Mechanism of Abietadiene Synthase Catalysis: Stereochemistry and Stabilization of the Cryptic Pimarenyl Carbocation Intermediates

Matthew M. Ravn,† Reuben J. Peters,‡,§ Robert M. Coates,*,† and Rodney Croteau*,‡

Contribution from the Department of Chemistry, University of Illinois, 600 S. Mathews Avenue, Urbana, Illinois 61801, and Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340

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Abstract: Abietadiene synthase (AS) catalyzes the complex cyclization−rearrangement of (*E*,*E*,*E*)-geranylgeranyl diphosphate (**8**, GGPP) to a mixture of abietadiene (**1a**), double bond isomers **2a**−**4a** and pimaradienes **5a**−**7a** as a key step in the biosynthesis of the abietane resin acid constituents (**1b**−**4b**) of conifer oleoresin. The reaction proceeds at two active sites by way of the intermediate, copalyl diphosphate (**9**). In the second site, a putative tricyclic pimaradiene or pimarenyl(+) carbocation intermediate of undefined C13 stereochemistry and annular double bond position is formed. Three 8-oxy-17-nor analogues of **9** (**17** and **19a,b)** and three isomeric 15,16-bisnorpimarenyl-*N*-methylamines (**26a**−**c**) were synthesized and evaluated as alternative substrates and/or inhibitors for recombinant AS from grand fir. The stereospecific cyclization of 8α-hydroxy-17-nor CPP (**19a**) to 17-normanoyl oxide (**20a**) and the higher inhibitory potency of the norpimarenylamine **26a** (K_i = 0.1 nM) both suggest pimarenyl intermediates having the 13β methyl configuration and 8,14-double bond corresponding to sandaracopimaradiene (**5a**). The 2000-fold stimulation of inhibition by **26a** in the presence of inorganic pyrophosphate indicates an important role for carbocation/OPP anion stabilization of the secondary sandaracopimaren-15-yl(+) ion. The failure of 8β-hydroxy-17-nor CPP (**19b**) to undergo enzymatic cyclization was taken as evidence that **9** is bound with a “coplanar” side chain conformation and that the S_N' cyclization occurs on the 17α face. The routing of the sandarcopimara-15-en-8-yl carbocation toward various diterpenes in biogenetic schemes is attributed to differing conformations of ring C and/or orientations of the C13 vinyl group in the active sites of the corresponding diterpene cyclases.

Introduction

The resin acids are bicyclic and tricyclic diterpene carboxylic acids that occur in conifer oleoresin (pitch).[1] The most abundant of these natural products belong to the abietane family of perhydrophenanthrene diterpenes characterized by an isopropyl substituent at C13, a conjugated diene in the B and/or C rings, and a C18 equatorial carboxyl group, i.e., abietic, levopimaric, neoabietic, and palustric acids (Figure 1, **1b**−**4b**).[1,2] The principal resin acids are usually accompanied by smaller proportions of the pimarane (13α methyl) and isopimarane (13β methyl) isomers differentiated by a quaternary carbon at C13 bearing methyl and vinyl substituents and an annular double bond at the 7,8 or 8,14 positions, as exemplified by sandaracopimaric, pimaric, and isopimaric acids (**5b**−**7b**). The structures of the tricyclic resin acids were determined by classical chemical degradations during the 1880s through 1950,[3] and total and partial syntheses of abietic acid (**1b)** as well as the parent hydrocarbons, sandaracopimaradiene, pimaradiene, and isopimaradiene (**5a**−**7a**), have been reported.[4]

Oleoresin is secreted by conifers in response to insect attack and physical injuries, and this complex mixture of terpenoids serves to protect and seal the injury by oxidative cross-linking of the nonvolatile diterpene components after evaporation of the volatile monoterpene constituents.[1,5] Processed gum rosin, composed primarily of abietic acid, is an abundant chemical commodity having numerous commercial applications.[1,6] The readily isolated abietic and levopimaric acids[7] have been used

* Corresponding authors. R.M.C.: tel. (217) 333-4280; fax (217) 244-8024; E-mail coates@scs.uiuc.edu. R.C.: tel. (509)-335-1790; fax (509) 335-7643; E-mail croteau@wsu.edu.

† University of Illinois.

‡ Washington State University.

§ Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

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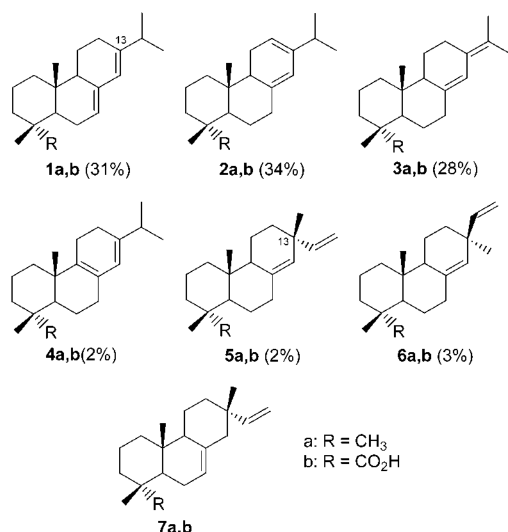


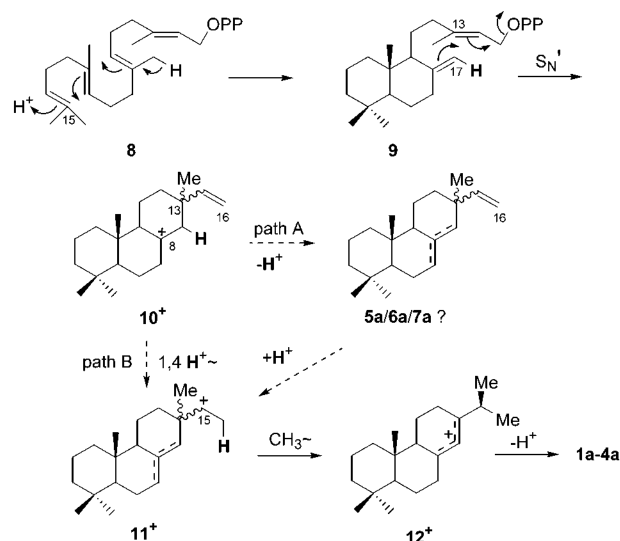
Figure 1. Structures of the common abietane and pimarane types of diterpene hydrocarbons and the corresponding resins acid constituents: abietic, levopimaric, neoabietic, palustric, sandaraopimaric, isopimaric, and pimaric acids (**1b**−**7b**, respectively). The numbers in parenthesis are the relative amounts of each diterpene produced by abietadiene synthase and present as the acids in fresh oleoresin.

extensively as starting materials for synthesis of other diterpenes and various carbocyclic natural products and for chemical investigations.[3,4,8]

Abietadiene synthase[9] is a multiproduct cyclase that generates a mixture of abietadiene (**1a**, 31%), levopimaradiene (**2a**, 34%), neoabietadiene (**3a**, 28%), palustradiene (**4a**, 2%), sandaracopimaradiene (**5a**, 2%), and pimaradiene (**6a**, 3%),[10] a composition similar to that of the corresponding resin acids in fresh grand fir oleoresin.[11] Abietadiene oxidases that convert the abietadiene to abietic acid have been isolated from grand fir and lodgepole pine.[12]

Recombinant abietadiene synthase (rAS) is an operationally soluble, monomeric 84-kDa protein sharing general characteristics with other terpene cyclases of plant origin, including the conserved DDXXD motif in the C-terminal domain together and a functionally important DXDD motif in the N-terminal domain.[13] The structural relationshships among the resin acids led Ruzícka, Eschenmoser, and Heusser to propose their biogenetic origin by the sequence geranylgeraniol → labdanes (manool) → pimaradienes (pimaric acid) → abietadienes (abietic acid).[14] The acid-catalyzed rearrangement of pimaric and isopimaric acids (**6b** and **7b**) to abietic acid afforded relevant chemical precedent for the last step in this prescient biogenetic scheme.[15] The now well-established biosynthesis of *ent*-kaurene

Scheme 1



via *ent*-copalyl diphosphate[16,17] provided analogy for a proposed mechanism for the AS reaction by (1) proton-initiated polyene cyclization of (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP, **8**) to copalyl diphosphate (CPP, **9**), (2) diphosphate ionization and ring closure to an indeterminate pimarenyl(+) or pimaradiene intermediate (**10**$^+$, **5a**, **6a**, or **7a**), and (3) proton-induced rearrangement to abietadiene (**1a**) (Scheme 1).[9] CPP proved to be a competent substrate for rAS, undergoing conversion to the same mixture of abietadiene and pimaradiene isomers **1a**−**6a**.[10a] Steady-state kinetic analysis indicated that the rate-limiting step occurs after formation of CPP because the calculated k_{cat} is 2.2 s^{-1} for either GGPP or CPP, although substrate inhibition (K_i = 5 μM) prevents direct measurement of the rate from GGPP. The catalytic efficiency for CPP ($K_M = 0.4$ μM, $k_{cat}/K_M = 5.5 \times 10^6$ s^{-1} M^{-1}) proved to be 8 times greater than that of GGPP ($K_M = 3$ μM, $k_{cat}/K_M = 7.3 \times 10^5$ s^{-1} M^{-1}) owing to the tighter binding of CPP.[10a,13] The presence of two active sites, one for the conversion of GGPP to CPP and the other for the cyclization-rearrangement of CPP to the abietadiene mixture, has been established by selective inhibition of the first reaction by 15-aza-14,15-dihydroGGPP while retaining the second catalytic activity, as well as by site-directed mutagenesis.[13]

rAS catalyzed cyclizations of the labeled substrates [19,19,19-$^2H_3$]GGPP, (17*E*)-[17-$^2H_1$]CPP, and (15*R*, 17*E*)-[15-$^2H_1$,17-$^3H_1$]CPP revealed that the conversion of CPP to abietadiene occurs with stereospecific intramolecular transfer of the C17 pro-E proton to the C16 *si* face of the vinyl group in the pimaradiene or pimaren-8-yl ion intermediate. This protonation is followed by syn methyl migration to the same vinyl group face (15-*si*), thus creating the characteristic isopropyl group of the abietane products.[18] However, the C13 stereochemistry (α-CH_3 or β-CH_3) and the annular double bond position (Δ-7,8 or Δ-8,14) of the pimarenyl intermediate **11**$^+$ remained uncertain because none of the four plausible pima-

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radiene isomers are converted to abietadiene by AS.[9,10a] The formation of such enzymatically enclosed intermediates in terpene synthase-catalyzed cyclizations (resulting in the failure of exogenous intermediates to participate in the reaction) is a now a well-precedented phenomenon;[17,19] some specific examples are cineole, pentalenene, aristolochene, *epi*-aristolochene, vetispiradiene, and taxadiene synthases.[20]

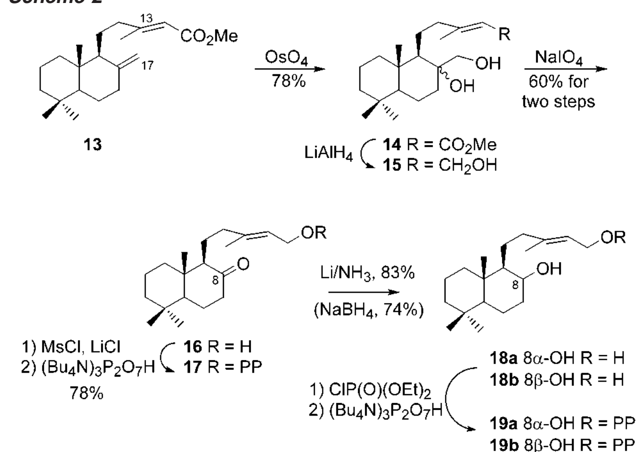
In this paper we report syntheses of three 8-oxy-17-nor analogues of copalyl diphosphate (**9**) and three isomeric pimarenylammonium salts that mimic the putative pimaren-15-yl carbocation **11**$^+$. The AS-catalyzed reactions and/or the inhibitory properties of these and related compounds toward this unusual diterpene synthase shed light on the stereochemistry of the cyclization and rearrangements which generate the abietane structure and on the nature and stabilization of the carbocation intermediates in the mechanism.[21]

8-Oxy 17-Norcopalyl Diphosphate Analogues

Both chemical and biochemical approaches have been utilized to identify transient intermediates in terpene cyclase reactions. The occurrence of an enzymatically enclosed germacrene A intermediate in sesquiterpene synthase-catalyzed cyclizations was verified by site-directed mutagenesis of an active site residue[22a] and by use of 6,7-dihydrofarnesyl diphosphate[22b] that is incapable of undergoing the full cyclization sequence. Support for putative carbocation intermediates in the cyclization−rearrangement cascade effected by oxidosqualene-lanosterol cyclase has been obtained by use of oxa, dehydro, methylidene, and other substrate analogues.[23,24] We considered that 8-oxo and 8-hydroxy 17-nor analogues (Scheme 2; **17**, **19a,b**) of the bicyclic intermediate **9** might serve to reveal the cryptic C13 configuration of the pimarenyl intermediates and the stereochemistry of the S_N' cyclization which takes place at the second AS catalytic site.

The readily available labdane diterpene manool[25] was converted to (13*E*)-methyl copalate (**13**) by oxidative rearrangement with pyridinium chlorochromate (CH_2Cl_2, 80%)[26] to a 2:1 mixture of (13*E*)- and (13*Z*)-copalals, followed by oxidation with MnO_2–NaCN in methanol (93%).[18b,27] Catalytic osmylation of **13** (OsO_4, NMO, acetone, 3 h, 78%)[28] provided a 3:1 mixture

Scheme 2



of the 8,17 and 13,14 regioisomeric diols. Reduction of the mixture of methyl esters ($LiAlH_4$, Et_2O, 0 °C, 10 h) followed by oxidative cleavage ($NaIO_4$, 6:1 acetone−water, 24 h)[18b,29] gave 8-oxo-17-norcopalol (**16**, 60% for two steps) and the chromatographically separable methyl ketone arising from the 13,14-diol. The epimeric 8α- and 8*β*-hydroxy norcopalol analogues were obtained by stereoselective reductions of **16** under dissolving metal conditions (Li/NH_3, EtOH, Et_2O, −33 °C, 83%),[30] or with metal hydride ($NaBH_4$, MeOH, 74%), to give diols **18a,b**, respectively, as crystalline solids. The equatorial and axial positions of the hydroxyl groups in the isomers were established from the ^1H NMR couplings of the H8 protons: **18a**, δ_H 3.41 (td, $J = 10.6$, 4.9 Hz); **18b**, δ_H 3.95(q, $J = 2.6$ Hz).

Despite the possibility of S_N' cyclization with the proximal carbonyl group, phosphorylation of keto alcohol **16** proceeded smoothly under standard conditions by conversion to the allylic chloride (MsCl, LiCl, *s*-collidine, DMF, 0 °C)[31] followed by displacement with tris(tetrabutylammonium) diphosphate (($Bu_4N)_3$-HP_2O_7·$3H_2O$, CH_3CN, 78%)[32] which afforded 8-oxo-17-norcopalyl diphosphate (**17**) as the pure ammonium salt after ion-exchange and cellulose chromatography. In contrast, attempted conversions of diols **18a,b** to the corresponding allylic chlorides by the same procedure resulted only in mixtures of cyclization and addition products. Instead, activation by regioselective phosphorylations ($ClPO(OEt)_2$, py, CH_2Cl_2, 0 °C) followed by slow displacement with tris(tetrabutylammonium) diphosphate at high concentration (CH_3CN, $(NBu_4)_3HP_2O_7$·$3H_2O$, 1 M, 4 d, 3 Å sieves, 68−69% for two steps)[29] provided the primary allylic diphosphates **19a,b** as NH_4^+ salts after ion-exchange and either preparative reverse-phase HPLC or cellulose chromatography.

Chemical cyclizations of the nor-hydroxy analogues **18a,b** were carried out to obtain independently prepared samples of the cyclic ethers and to establish their C13 configurations. Acid-catalyzed cyclization of equatorial diol **18a** (*p*-TsOH·H_2O,

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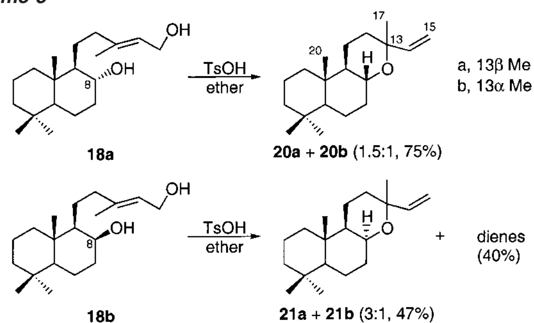
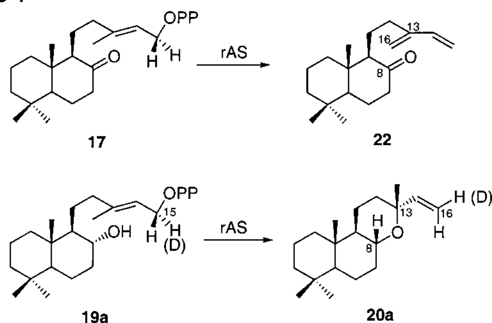
Scheme 3



Scheme 4



Et_2O, 1.5 h, room temperature)[33] gave a 1.5:1 mixture (76%) of inseparable cyclic ethers (13*R*)-**20a** and (13*S*)-**20b** (Scheme 3). NOE experiments with irradiation of both overlapping isomeric H8 methine protons (δ_H 3.52 and 3.54 for major and minor isomers, respectively) showed strong correlations to the C17 (δ_H 1.21, 6.0%) and C20 (δ_H 0.74, 4.3%) methyls for the major isomer and to the C20 (δ_H 0.74, 4.3%) methyl and H15 (δ_H 5.79, 4.8%) vinyl proton for the minor isomer. It follows that the major isomer possesses an axial 13β methyl group (13*R*) and the minor isomer has an equatorial 13α methyl (13*S*). The 13β methyl isomer **20a** proved to be the sole product of the AS-catalyzed cyclization of 8α-hydroxy-17-nor diphosphate, **19a**.

A somewhat slower cyclization of axial diol **18b** under similar conditions (30 °C with TLC monitoring) gave a 3:1 mixture (47%) of cyclic ethers (13*R*)-**21a** and (13*S*)-**21b** and a 5:2.5:1 mixture (41%) of (12*E*)-12,14, (12*Z*)-12,14, and 13(16),14-dienols, respectively, arising from competing 1,4-proton eliminations. NOE experiments on the mixture of cyclic ethers with irradiation of both overlapping isomeric H8 methine protons (δ_H 3.7−3.8) showed strong correlations to the H15 (δ_H 5.76, 10%) vinyl hydrogen for the major isomer (**21a**) and to the H17 methyl (δ_H 1.21, 2.6%) for the minor isomer (**21b**). The slower rate and the lower yield of the cyclic ether products formed from axial diol **18b** are no doubt a response to steric interactions of the axial substiuents on incipient cis-fused C-ring with the C10 angular methyl group.

Incubation of 8-oxo and 8α-hydroxy-17-nor analogues **17** and **19a** with rAS resulted in regiospecific elimination of the former to 17-norlabda-13 (16),14-dien-8-one (**22**, 30% conversion by GC) and stereospecific S_N' cyclization of the latter to 17-normanoyl oxide (13*R*, **20a**, 46% conversion by GC), as the principal products according to GC analyses and NMR spectra (Scheme 4). Dienone **22** was identified by coupling patterns and integrations for the vinyl hydrogens in its ^{1}H NMR spectra as well as its UV spectrum (λ_{max} 226 nm, ethanol) which is similar to that predicted for a 2-alkyl-1,3-butadiene (calcd λ_{max} 222 nm).[34] The structure of the cyclic ether product was confirmed by GC comparisons with the **20a** + **20b** mixture and by ^{1}H NMR spectra and NOE determinations on isomerically pure **20a** from the enzymatic reaction. AS-catalyzed cyclization of (15*R*)-[15-$^2H_1$]**19a**[29] afforded (16*E*)-[16-$^2H_1$]normanoyl oxide (**20a**-d_1) bearing label in the trans position of the vinyl group judging from its ^{1}H NMR spectrum: δ_H 5.37 (d, $J = 17.6$ Hz), 6.04 (d, $J = 17.3$ Hz). In contrast, incubations of the 8β-hydroxy isomer **19b** with rAS under the same conditions gave no significant amounts of extractable products (conversions ≤ 0.2% by GC), despite evidence cited below that the analogue is a strong competitive inhibitor of CPP, exhibiting $K_i = 0.2\ \mu$M, and thus clearly binding at the active site.

Syntheses of Norpimarenylamine Inhibitors

The norpimarenylammonium salts **26a−c** are nitrogen mimics of three of the four possible secondary pimarenyl ions symbolized by intermediate **11**$^+$ shown in Scheme 1. Aza analogues of hypothetical carbocations have often proven to be potent inhibitors of many terpene cyclases as well as tranferases, isomerases, and oxidases associated with isoprenoid biosynthesis.[24,35] It therefore seemed reasonable to expect that the norpimarenylamine isomer having the C13 configuration and double bond position corresponding to the transient carbocation **11**$^+$ would be a more potent inhibitor than its isomers.

The three norpimarenylamines were synthesized by Curtius rearrangements of the corresponding 16-norpimaren-15-oic acids (**24a−c**) and direct hydride reductions of the resulting isocyanates (Scheme 5). A 9:1 mixture of sandaracopimaric and isopimaric acids (**5b** + **7b**) isolated from sandarac resin[33] and pimaric acid (**6b**)[36] were converted in 5 steps to the corresponding pimaradienes, **5a** + **7a** mixture and **6a**, by known procedures.[37] Regioselective dihydroxylation (catalytic OsO_4, NMO, acetone, rt, 88%)[28,38] of the **5a** + **7a** mixture followed by periodate cleavage ($NaIO_4$, acetone−water, rt, 3 h, 90%)[39] and hydride reduction ($LiAlH_4$, ether) furnished the corresponding primary alcohols which proved separable by careful chromatography on silica gel (**23a**, 69%; **23b**, 5%). Jones oxidations (CrO_3, H_2SO_4, acetone) gave the known[40] nor acid **24a** (67%) and its double bond isomer, **24b** (86%). Curtius rearrangement of **24a** with diphenylphosphoryl azide[41] (DPPA, Et_3N, PhH, reflux, 50 min) provided the stable isocyanate **25a** (86%) that was purified by rapid chromatography on silica gel. Reductions of **25a** with $LiAlH_4$ in ether (rt, 2h) and THF (reflux, 2h)

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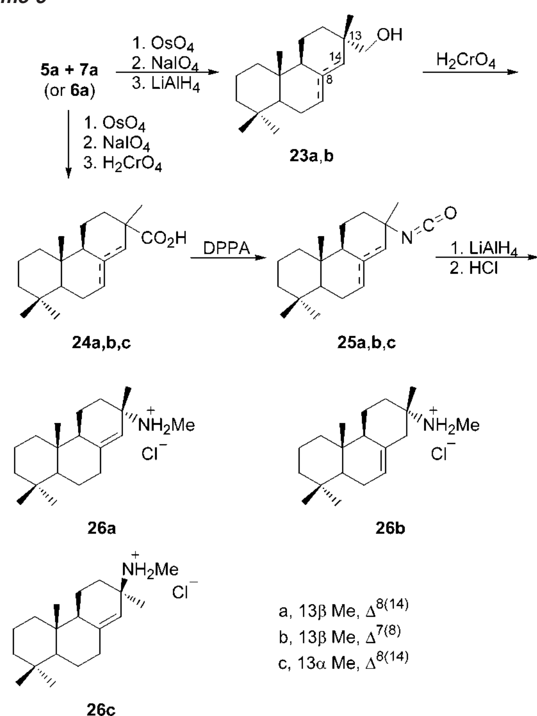
(35) (a) Oehlschlager, A. C.; Angus, R. H.; Pierce, A. M.; Pierce, H. D., Jr.; Srinivasan, R. *Biochemistry* **1984**, *23*, 3582−3589. (b) Reardon, J. E.; Abeles, R. H. *Biochemistry* **1986**, *25*, 5609−5616. (c) Muelbacher, M.; Poulter, C. D. *Biochemistry* **1988**, *27*, 7315−7328. (d) Rahier, A.; Taton, M.; Benveniste, P. *Biochem. Soc. Trans.* **1990**, *18*, 48−52. (e) McGeady, P.; Pyun, H. J.; Coates, R. M.; Croteau, R. *Arch. Biochem. Biophys.* **1992**, *299*, 63−72. (f) Steiger, A.; Pyun, H.-J.; Coates, R. M. *J. Org. Chem.* **1992**, *57*, 3444−3449.
(36) A 10:1 mixture of sandaracopimaric and isopimaric acids (**5a** + **7a**) was isolated according to the procedures of Edwards and Mootoo.[33] It was found later that pure **5a** could be isolated readily by crystallization of the ethanolamine salt. See Supporting Information for details. Pimaric and isopimaric acids were obtained as gifts from Dr. Duane Zinkel at the USDA Forest Products Labratory, Madison, WI.
(37) (a) Coates, R. M.; Kang, H. Y. *J. Org. Chem.* **1987**, *52*, 2065−2074. (b) Chu, M.; Coates, R. M. *J. Org. Chem.* **1992**, *57*, 4590−4597.
(38) For regioselective dihydroxylations of pimaradiene diterpenes, see: (a) Ireland, R. E.; Newbould, J. *J. Org. Chem.* **1963**, *28*, 23−26. (b) Edwards, O. E.; Howe, R. *Can. J. Chem.* **1959**, *37*, 760−774.

Scheme 5



afforded the formamide or the *N*-methylamine, respectively, and the latter was isolated as its hydrochloride salt (**26a**, 56%). Similar procedures sufficed to convert 16-norisopimaren-15-oic acid **24b** and pimara-8(14),15-diene (**6a**) to 15,16-dihydro-15-azaisopimaradiene and the corresponding azapimaradiene as HCl salts **26b,c** in comparable yields.

Inhibition and Kinetic Studies with Recombinant (−)-Abietadiene Synthase

Kinetic analyses of the synthetic substrate analogues and intermediate mimics with rAS were carried out to determine their relative rates and/or affinities for the active site (Table 1). Cyclization rates (k_{cat}) for **17** and **19a** were found to be ~0.03 and ~0.07 s^{-1} (k_{rel} 0.01 and 0.03 to those for GGPP; k_{cat} = 2.2 s^{-1}). Products generated were quantified by GC using geranylgeraniol as an external standard and identified by preparative enzyme assays and comparisons with authentic samples obtained as discussed above. Interestingly, axial nor-hydroxy analogue **19b** did not serve as a substrate for rAS, but instead acted as a competitive inhibitor of the cyclization of [15-^{3}H]-(+)-CPP, exhibiting K_i = 0.2 μM. Both **17** and **19a** also served as effective inhibitors when added immediately prior to radiolabeled substrate.

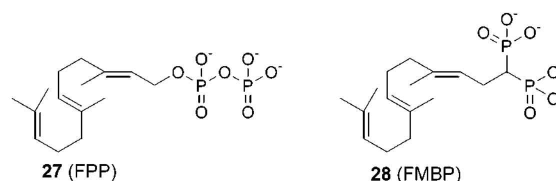
It was expected that the norpimarenylamine isomer corresponding to the cryptic pimarenyl intermediate $\mathbf{11}^+$ would display the highest affinity for rAS and that there might exist synergy between exogenous diphosphate anion (mimicking the ionized diphosphate) and such carbocation mimetic analogues, as previously observed with other terpene synthases.[42] Therefore, the three amines **26a−c** were assayed as inhibitors against rAS with and without the addition of 1 mM inorganic diphosphate (PP_i) (Table 1). As expected from the stereoselective cyclization of **19a** to **20a**, the isomers with the 13β methyl group, **26a,b**, were significantly more potent inhibitors than **26c** with the 13α methyl stereochemistry by 10−15-fold in the absence of inorganic pyrophosphate. The dramatic synergy exhibited by the amine inhibitors in the presence of the additive (30−2000-fold) indicates an enzyme-mediated ion pairing interaction between the pyrophosphate anion and the secondary pimarenyl carbocation.

Table 1. Inhibition and Kinetic Data for Oxy-nor CPP Analogs (**17**, **19a,b**), Norpimarenylamines (**26a−c**), and Truncated Acyclic Substrate Analogs (**27**, **28**)[a]

compd	K_i[b] (nM)	+ 1 mM PP_i K_i[c] (nM)	k_{cat}	product
17	120 ± 10		~0.03	**22**
19a	150 ± 30		~0.07	**20a**
19b	200 ± 50			
26a	240 ± 10	0.13 ± 0.05[d]		
26b	1200 ± 200	2.8 ± 0.2[d]		
26c	11000 ± 700	400 ± 100[d]		
27	8000			
28	6[d]			

[a] Inhibition was determined by utilizing [15-^{3}H]-CPP as the substrate. [b] Competitive inhibition. [c] Uncompetitive inhibition, presumably owing to the previously observed uncompetitive inhibition from divalent metal ion chelation occurring with 1 mM inorganic diphosphate.[10a] [d] Apparent K_i determined by fitting a tight binding inhibitor equation to a Dixon-type experiment.[13]

The inhibition kinetics of two acyclic substrate analogues, (*E,E*)-farnesyl diphosphate (FPP, **27**) and farnesylmethyl-1′,1′-bisphosphonate (FMBP, **28**),[43] as well as the cyclization rate of CPP selectively deuterated in the exocyclic methylene group were also studied to determine the effects of shortening the polyenyl chain, altering the orientation of the polar headgroup, and substituting the heavier isotope for the hydrogen that migrates to C16. The previously reported rAS-catalyzed conversion of FPP to the acylic tetraene (*E*)-β-farnesene most likely occurs by ionization and deprotonation at the CPP cyclization site where diphosphate heterolysis occurs.[10a] FPP proved to be a competitive inhibitor of rAS when added immediately prior to radiolabeled CPP, with K_i = 8 μM. Interestingly, FMBP is a much better inhibitor of rAS with K_i = 6 nM, possibly indicating a twist of the binding domains for the hydrocarbon and diphosphate moieties. Detailed kinetic analysis of the previously characterized (15*R*, 17*E*)-[15-$^3H_1$, 17-$^2H_1$]CPP[18b] revealed a small, but significant, deuterium isotope effect (KIE = 1.4 ± 0.1) on the overall rate, indicating a small degree of C17−H bond-breaking in the transition state or a secondary KIE associated with the $sp^2 \rightarrow sp^3$ rehybridization at C17 in the initiating S_N' cyclization step.



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(41) Ninomiya, K.; Shioiri, T.; Yamada, S. *Tetrahedron* **1974**, *30*, 2151−2157.
(42) (a) Cane, D. E.; Yang, G.; Coates, R. M.; Pyun, H.-J.; Hohn, T. M. *J. Org. Chem.* **1992**, *57*, 3454−3462. (b) Koohang, A.; Coates, R. M.; Owen, D.; Poulter, C. D. *J. Org. Chem.* **1999**, *64*, 6−7. (c) Croteau, R.; Wheeler, C. J.; Aksela, R.; Oehlschlager, A. C. *J. Biol. Chem.* **1986**, *261*, 7257−7263.
(43) Ciosek, C. P., Jr.; Magnin, D. R.; Harrity, T. W.; Logan, J. V.; Dickson, J. K., Jr.; Gordon, E. M.; Hamilton, K. A.; Jolibois, K. G.; Kunselman, L. K.; Lawrence, R. M. *J. Biol. Chem.* **1993**, *268*, 24832−24837.

Discussion

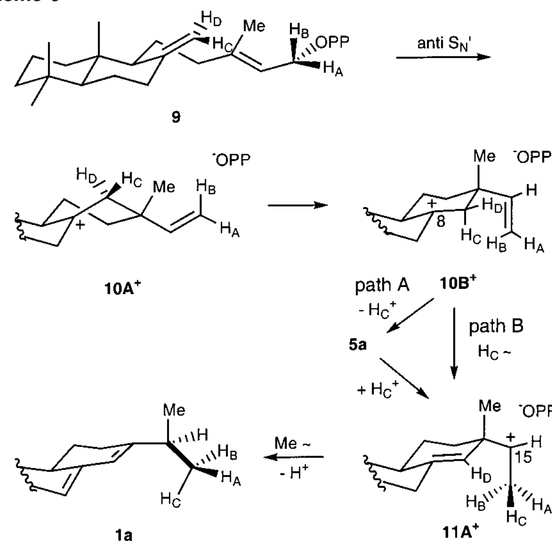
The three 8-oxy analogues (**17**, **19a,b**) of **9** proved to be active as alternative substrates and/or inhibitors of AS and provided useful insights concerning the stereospecifity of the S_N' cyclization step. The slow but stereospecific enzyme-catalyzed cyclization of the 8α-hydroxy analogue to 17-normanoyl oxide (**19a** → **20a**, $k^{19a}/k^{9} = 0.03$) constitutes strong, indirect evidence that pimaren-8-yl intermediate **10**$^+$ in fact has the 13β methyl configuration corresponding to sandaracopimaradiene (**5a**),[44] a minor byproduct formed with the normal substrate. The absence of any 14-oxa-abietene products that would arise by proton transfer from oxygen to C16 and methyl rearrangement is presumably attributable in part to the decreased Bronsted acidity of the oxonium ion intermediate compared to the pimarenyl carbocation **10**$^+$. It seems likely that the 8-oxo analogue **17** undergoes similar ring closure to a cyclic oxocarbenium ion that suffers ring cleavage and regiospecific β-elimination at the methyl group to form dienone **22**.

The AS-catalyzed cyclization of deuterium-labeled (15*R*)-[15-$^2H_1$]-**19a** to (15*E*)-[16-$^2H_1$]-**20a** establishes an anti relationship of the 8α-OH nucleophile and the OPP^- leaving group and, by inference, an anti S_N' cyclization for the normal copalyl PP intermediate **9**. This conclusion conforms with similar outcomes reported for the biosynthesis of normal and enantiomeric diterpenes produced by fungi and higher plants.[45,46] As a consequence, the initially formed pimaren-8-yl(+)/diphosphate-(−) ion pair (**10**$^+$/OPP^-) would have the anion situated on the *si*,*si* face of the C13 vinyl group.

Although the epimeric hydroxy analogue **19b** clearly binds at the copalyl PP cyclization site, as shown by its strong inhibition (K_i^{19b}/K_M^{CPP} = 0.2 μM/0.35 μM = 0.6), this compound with an axial 8β-OH as potential nucleophile fails to undergo enzyme-catalyzed cyclization or elimination. The acid-induced dehydration of the corresponding diol **18b** to a mixture of epimeric cyclic ethers (**21a** + **21b**, 47%, Scheme 3) indicates that the steric interactions arising from the cis B, C ring fusion should not be insurmountable. It seems reasonable to propose that copalyl PP and its 8-oxy-17-nor analogues are bound with the pentenyl diphosphate side chain oriented approximately coplanar to the A and B rings. In this conformation, C13−C17 bond formation would take place on the 17α face of the exocyclic methylene carbon (17 *re*) of **9**, and the 8α-OH group of **19a** would be suitably positioned for S_N' cyclization to normanoyl oxide. In contrast, the axial 8β-hydroxy group of **19b** is too far from C13 to allow cyclization in the same "coplanar" side chain conformation, and the "bent" conformation alternative required for nucleophilic attack on C13 is, according to this interpretation, incompatible with the contours of the copalyl PP binding pocket.

The relative potencies of the three pimarenylamine inhibitors **26a**−**c** (Table 1) are consistent with the 13β methyl configuration of the pimaren-15-yl ion intermediate inferred from the cyclization of **19a** to **20a**.[44] Thus the K_i for **26a** with the 13β methyl substituent is a factor of 46 lower than K_i for its epimer **26c** with the opposite α methyl orientation at C13, and the K_i ratio decreases to 10^{-3} in the presence of 1 mM OPP^-. The smaller but significant potency enhancement of **26a** over its ring B double bond isomer **26b** (5-fold in the absence of OPP^- and 22-fold in the presence of 1 mM OPP^-) indicates a ring C location for the double bond in the pimaren-15-yl(+) ion formed after proton transfer to C16, consistent with previous labeling results.[18] The stimulating effect of inorganic pyrophosphate has been previously observed using aza and sulfonium analogues of tertiary carbocation intermediates in other terpene synthase reactions.[35e,42] However, in these cases, the enhancements in affinity in the presence of OPP^- were only about 1 order of magnitude, considerably less than the 3 orders of magnitude observed here for norpimarenylamine **26a**.

Scheme 6



The results discussed above, together with those from prior deuterium and tritium labeling experiments,[18] support the conformational mechanism illustrated in Scheme 6 for the conversion of **9** to the abietadiene isomers **1a**−**4a** catalyzed by rAS.[47] S_N' cyclization of **9** with the "coplanar" side chain orientation and bonding to the 17α face (17-*re* to 13-*si*) generates the pimar-15-en-8-yl(+) carbocation **10A**$^+$ with the 13β methyl group and ring C initially in a boat conformation. Ring inversion accompanied by ca. 150° rotation of the vinyl group gives rise to conformer **10B**$^+$. Migration of $H14_\alpha$ (H_C) in a stereoelectronically suitable axial orientation to the 16-*si* face of the vinyl group, either intramolecularly or indirectly by transfers to and from the AS active site interior by way of a transient sandaracopimaradiene intermediate (**5a**),[44] leads to secondary pimar-8(14)-en-15-yl(+)/OPP^- ion pair **11A**$^+$. Another −90° rotation about the C13−C15 bond sets the stage

(44) This conclusion is supported by recently published results from mutational analysis of rAS. Mutant N765A proved unable to mediate the intramolecular proton transfer and produced unrearranged sandaracopimaradiene (**5a**) as the sole product. See: Peters, R. J.; Croteau, R. B. *Proc. Acad. Sci. U.S.A.* **2002**, *99*, 580−584.

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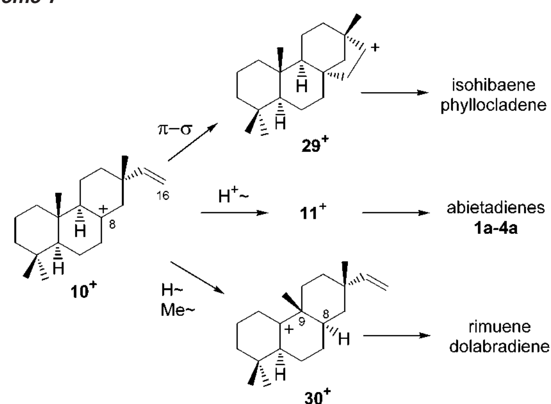
(47) (a) The possibility that the 14 → 16 proton transfer could occur with ring C in a boat conformation (**10A**$^+$) appears unlikely for stereoelectronic reasons. Inspection of a Dreiding model of **10A**$^+$ shows that H_C is aligned orthogonal to the carbocation orbital. (b) A reviewer suggested an alternative mechanism in which the CPP intermediate (**9**) undergoes enzyme-catalyzed allylic rearrangement to its tertiary isomer, epimanoyl diphosphate, a process well precedented in the cyclization mechanisms of monoterpene and sesquiterpene synthases.[19] In this case, rotation of the vinyl group to a cisoid conformation could occur prior to cyclization of the epimanoyl PP intermediate. No evidence is presently available to exclude or support this somewhat more complicated mechanistic alternative.

Scheme 7



for migration of the 13β methyl to the 15-*si* face of the secondary ion to create the isopropyl group; subsequent proton eliminations from the periphery of the resulting allylic abietenyl ion would give rise to the observed **1a**−**4a** isomer mixture.

The suprafacial relationship of the C14−C16 proton transfer and the C13−C15 methyl migration evidently requires separate steps and the obligatory conversion of the more stable tertiary pimaren-8-yl ion **10B**$^+$ to the less stable secondary ion **11A**$^+$ as a discrete intermediate. The small kinetic isotope effect observed when deuterium is transferred is significantly less than the 2−5-fold change expected for a rate-determining primary isotope effect; thus the proton transfer (and the resulting tertiary to secondary carbocation transition) is at most only slightly rate-limiting. The 15 kcal/mol enthalpic difference between tertiary and secondary carbocations[48] might be alleviated significantly by an associated decrease in the separation of the carbocation site and the diphosphate anion in the secondary ion. Measurements with molecular models indicate that the distance between the carbocation sites in **10B**$^+$//OPP$^-$ and **11A**$^+$/OPP$^-$ ion pairs is approximately 3.6−3.7 Å (distance between C8 and C15). The 5 × 10^{-4} decrease in K_i ($\Delta\Delta G \sim 4.5$ kcal/mol) for primarenylamine inhibitor **26a** in the presence of 1 mM diphosphate ion signifies an important role for the leaving group in the transition state composite and is likely a manifestation of ion pairing as a significant stabilizing influence in this seemingly energetically demanding isomerization. This ion pair interaction might also explain the observed suprafacial proton transfer and methyl migration (**10**$^+$ → **12**$^+$ si face selectivity) previously discussed, as it would effectively shield one face of the olefin and enforce a stepwise mechanism. Substitution of the pyrophosphate by the methylbis(phosphonate) group dramatically increases affinity, as indicated by the 3 orders of magnitude increase in affinity of farnesylmethylbis(phosphonate) (**28**, K_i = 6 nM) relative to farnesyl diphosphate (**27**, K_i = 8 μM). The similarity of these effects suggests that the geminal phosphonates in the former may mimic the position of the pyrophosphate anion in the ion-pairing interaction demonstrated by the synergetic binding of **26a** with inorganic pyrophosphate.

The pimaren-8-yl ion **10**$^+$ also figures in biogenetic schemes leading to other types of tricyclic and tetracyclic diterpenes (Scheme 7).[2,17,49] Migration of H9α to C8 is the first step in the sequential backbone rearrangements through **30**$^+$ which give rise to rimuene and dolabradiene. Rotation of the vinyl group and C8−C16 cyclization via **29**$^+$ presumably leads to the bridged tetracyclic diterpenes isohihabene and phyllocladene. It is intriguing to speculate upon the factors responsible for directing this common intermediate toward the structurally different diterpene products. The conformation of ring C, the orientation of the vinyl group, and the position of the diphosphate counterion probably all play critical roles. Maintenance of the boat conformation of **10A**$^+$ together with a 120° rotation of the vinyl group toward C8 allows C8−C16 bonding while avoiding transfer of the orthogonally disposed H14α. However, ring inversion **10A**$^+$ → **10B**$^+$, vinyl group rotation, and diphosphate positioning would prepare the ion to undergo 14−16 proton transfer and methyl rearrangement to the abietadiene family. If both of these outlets are prevented, e.g. by fixing the vinyl group in its initial extended orientation, the H9 to C8 hydride shift may take place to begin the backbone rearrangements to the 9β methyl diterpene family.

Conclusion

The stereospecificity of the AS-catalyzed cyclization of 8α-hydroxy analogue **19a** and the greater potency of pimarenylamine inhibitor **26a** compared to isomers **26b,c** both point to pimarenyl carbocation intermediates **10**$^+$ and **11**$^+$ having 13β methyl stereochemistry.[44] A "coplanar" orientation of the copalyl diphosphate side chain and the consequent 17α-13β (17-*re*-13-*re*) facial specificity in the S_N' cyclization are inferred from the contrary behavior of the epimeric 8α- and 8β-hydroxy-17-nor analogues of the bicyclic intermediate. The important role of ion-pairing interactions in AS catalysis is revealed by a 2000-fold enhancement of binding affinity for aza analogue **26a** in the presence of pyrophosphate anion. A plausible mechanism for conversion of **9** to the abietadiene isomers **1a**−**4a** consists of S_N' cyclization completed with vinyl group rotation and boat−chair inversion, H14α → C16 *si* proton transfer aided by tight ion pairing with the pyrophosphate anion, 13 → 15 methyl migration, and finally proton eliminations. The resulting transformation from a sandaracopimaradiene (or sandaracopimarenyl (+)) structure to the abietadienes brings the isolated double bonds into conjugation, thus enhancing the reactivity of abietic acid and its oleoresin congeners toward atmospheric oxygen for their vital wound-sealing function in vivo.

Experimental Section

Representative preparative procedures and characterization data for 8-oxo-17-norCPP (**17**), for norpimarenylamine **26a**, and for the preparative enzymatic experiments with rAS are given below. General experimental aspects, as well as procedures and characterization data for other compounds, are available in the Supporting Information. Column chromatography was carried out as described by Still et al.[50] using a 50−100 times weight excess of Merck 60 Å, grade 9385, 230−400 mesh silica gel at a pressure of 4−8 psi. The amounts, dimensions, and volumes are specified in the following format: grams of silica gel, column diameter, fraction volume, and void volume. Farnesylmethylbisphosphonate (**28**) was prepared according to a literature procedure.[43]

(48) (a) Bittner, E. W.; Arnett, E. M.; Saunders, M. *J. Am. Chem. Soc.* **1976**, *98*, 3734−3735. (b) Arnett, E. M.; Petro, C. *J. Am. Chem. Soc.* **1978**, *100*, 5408−5416.
(49) Coates, R. M. In *Progress in the Chemistry of Organic Natural Products*; Herz, W., Grisebach, H., Kirby, J. W., Eds.; Springer-Verlag: Vienna, 1976; Vol. 33, pp 73−230.
(50) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923−2925.

(5*S*,9*R*,10*S*,13*E*)-17-Nor-8-oxo-labda-13-en-15-ol (16). Conditions for the catalytic osymylation and periodate cleavage were based on literature procedures.[28,38,39] A solution of methyl copalate **13** (3.60 g, 11.3 mmol), NMO (3.44 g, 29.4 mmol), and two crystals of OsO_4 (ca. 10 mg, ca. 0.039 mmol) in acetone (60 mL) was stirred for 3 h at room temperature. The solvent was removed under reduced pressure, and the residue was purified by column chromatography (150, 44, 25, 0) using 1:1 hexanes−ethyl acetate. The yield of diols as a ca. 3:1 (based on earlier runs) mixture of regioisomers was 3.10 g (78%). A suspension of $LiAlH_4$ (600 mg, 15.8 mmol) in ether (50 mL) was stirred and cooled at 0 °C as a solution of diols (2.29 g, 6.50 mmol) in ether (50 mL) was slowly added. The solution was allowed to warm to room temperature, and after 10 h, excess reagent was hydrolyzed by addition of water (600 μL), 15% NaOH (600 μL), and water (1.8 mL).[51] The solids were filtered through Celite and washed with ether (5 × 100 mL). The combined filtrates were concentrated under reduced pressure to give a mixture of triols which was dissolved in acetone (20 mL). The solution was stirred as $NaIO_4$ (4.15 g, 19.4 mmol) and enough water for complete dissolution were added. After 24 h, the resulting suspension was diluted with water (150 mL) and extracted with ether (3 × 100 mL). The combined ethereal extracts were dried (Na_2SO_4) and concentrated under reduced pressure to give a thin oil. Purification by column chromatography (80, 34, 25, 0) using 4:1 hexanes−ethyl acetate gave keto alcohol **16** (1.14 g, 60%). The bisnor methyl ketone side product arising from cleavage of the 13,14 double bond was tentatively identified by TLC comparisons with a previously prepared sample. Data for **16**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.69, 0.82, 0.94 (3s, 9H, 3CH_3), 1.12 (td, J = 12.6, 4.6 Hz, 1H), 1.21 (qd, J = 13.2, 4.6 Hz, 1H), 1.2−1.4 (m, 2H), 1.42 (dtd, J = 13.0, 3.1, 1.5 Hz, 1H), 1.46 (dd, J = 13.0, 2.6 Hz, 1H), 1.4−1.6 (m, 2H), 1.62 (qd, J = 13.2, 4.9 Hz, 1H), 1.64 (s, 3H, CH_3), 1.7−1.9 (m, 3H), 1.9−2.1 (m, 3H), 2.27 (td, J = 13.2, 7.0 Hz, 1H), 2.38 (ddd, J = 13.2, 4.7, 2.0 Hz, 1H), 4.11 (d, J = 7.0 Hz, 2H, C*H*$_2$OH), 5.34 (t sextet, J = 7.0, 1.3 Hz, 1H, =CH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 14.97 (CH_3), 16.41 (CH_3), 19.26 (CH_2), 19.84 (CH_2), 21.91 (CH_3), 24.35 (CH_2), 33.74 (CH_3), 33.94 (C), 39.13 (CH_2), 39.49 (CH_2), 42.18 (CH_2), 42.91 (CH_2), 42.94 (C), 54.54 (CH), 59.65 (CH_2), 63.64 (CH), 123.78 (CH), 140.32 (C), 212.45 (C); IR (CCl_4) 3622, 3483 (OH), 1710 (C=O) cm^{-1}; $[\alpha]_D^{20}$ −41.1° (*c* 1.27, EtOH); MS (FI) *m*/*z* 294 (M^+ + 2, 7.4), 293 (M^+ + 1, 24.0), 292 (M^+, 100), 291 (4.8), 290 (2.6). Anal. Calcd for $C_{19}H_{32}O_2$: C, 78.03; H, 11.03. Found: C, 77.65; H, 10.81.

(5*S*,9*R*,10*S*,13*E*)-17-Nor-8-oxo-labda-13-en-15-yl Diphosphate, Ammonium Salt (17). Conversion of keto alcohol **16** (100 mg, 0.34 mmol) to the chloride with *s*-collidine (165 μL, 249 mg, 2.05 mmol), LiCl (72 mg, 1.70 mmol), and methanesulfonyl chloride (132 μL, 196 mg, 1.71 mmol) in DMF (3 mL) at 0 °C for 1.75 h followd by displacement of half of the crude chloride with tris(tetrabutylammonium) diphosphate (tribasic) (327 mg, 0.34 mmol) in acetonitrile (2 mL) for 2.3 h gave crude diphosphate **17**.[31,32] Purification as described for 13-aza-13,14-dihydro-CPP[13] by ion exchange chromatography (NH_4^+ form, 2.4 × 10 cm, 8-mL fractions) followed by cellulose chromatography (3 × 18 cm, 8-mL fractions) and lyophilization gave diphosphate **17**: yield 75 mg (78%); ^{1}H NMR (400 MHz, D_2O) δ 0.53, 0.67, 0.78, 1.52 (4s, 12H, 4CH_3), 1.0−1.6 (m, 10H), 1.69 (dt, J = 13.9, 7.8 Hz, 1H), 1.88 (ddd, J = 13.7, 8.3, 6.2 Hz, 1H), 1.98 (ddt, J = 12.7, 9.8, 2.7 Hz, 1H), 2.16 (ddd, J = 12.9, 4.6, 2.2 Hz, 1H), 2.20 (d, J = 13.5 Hz, 1H), 2.38 (td, J = 12.0, 8.3 Hz, 1H), 4.29 (td, J = 6.4, 3.9 Hz, 2H, CH_2OPP), 5.22 (t, J = 6.7 Hz, 1H, =CH); ^{31}P NMR (162 MHz, D_2O) δ −5.84, −9.72 (2d, J = 22.0 Hz).

(5*S*,9*R*,10*S*,13*S*)-15,16-Bisnor-13-isocyanatopimar-8(14)-ene (25a). Conditions for the following reaction were based on those described by Yamada to prepare carbamates except that the alcohol component was omitted to isolate the isocyanate.[41] A solution of acid **24a** (97 mg, 0.33 mmol), triethylamine (279 μL, 203 mg, 2.01 mmol), and diphenylphosphoryl azide (225 μL, 275 mg, 1.00 mmol) in benzene (15 mL) was stirred and heated at reflux for 50 min and then cooled to room temperature. Purification by direct loading of the benzene solution onto a silica gel column (20, 24, 8, 0) followed by elution with 3% ethyl acetate in hexane provided isocyanate **25a** (83 mg, 86%) as a waxy white solid: mp 50−52 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.72, 0.82, 0.87 (3s, 9H, 3CH_3), 0.99 (td, J = 12.6, 4.4 Hz, 1H), 1.02 (dd, J = 12.3, 2.6 Hz, 1H), 1.16 (td, J = 13.0, 4.4 Hz, 1H), 1.27 (qd, J = 12.8, 4.6 Hz, 1H), 1.33 (s, 3H, H13 CH_3), 2.02 (tdt, J = 13.9, 5.9, 1.7 Hz, 1H), 2.23 (ddd, J = 14.3, 4.6, 2.0 Hz, 1H), 5.36 (q, J = 1.3 Hz, 1H, C=CH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 15.01 (CH_3), 19.14 (CH_2), 19.44 (CH_2), 22.31 (CH_3), 22.63 (CH_2), 29.70 (CH_3), 33.56 (C), 33.94 (CH_2), 35.61 (CH_3), 37.96 (CH_2), 38.66 (C), 39.34 (CH_2), 42.24 (CH_2), 50.30 (CH), 54.87 (CH), 57.56 (C), 123.48 (C), 127.29 (CH), 139.78 (C); IR (CCl_4) 2249 (NCO) cm^{-1}. Anal. Calcd for $C_{19}H_{29}NO$: C, 79.39; H, 10.17; N, 4.87. Found: C, 79.21; H, 10.07; N, 4.82.

(5*S*,9*R*,*10S*,13*S*)-*N*-Methyl-15,16-bisnorpimar-8(14)-en-13-amine, Hydrochloride Salt (26a). Conditions for the following reaction were based on a review by Müller.[52] Specific conditions were not given. A solution of isocyanate **25a** (82 mg, 0.29 mmol) in THF (14 mL) was stirred at room temperature as solid $LiAlH_4$ (600 mg, 15.4 mmol) was added in one portion. The solution was refluxed for 2 h until disappearance of both the starting material and the formamide intermediate was observed by TLC. Excess hydride was destroyed by addition of water (0.6 mL), 15% NaOH (0.6 mL), and water (1.8 mL)[51] while cooling in an ice bath. The resulting salts were filtered out and washed with ether (100 mL). The ether filtrate was washed with 1% NaOH (100 mL), and the aqueous layer was extracted with ether (2 × 50 mL). The combined ethereal extracts were extracted with 1% HCl (4 × 50 mL) and water (1 × 25 mL), the combined aqueous layers were basified until strongly basic, and the amine product was extracted with CH_2Cl_2 (5 × 50 mL). The solution was dried (Na_2SO_4) and concentrated under reduced pressure to a white solid (75 mg). The crude amine in ether (1 mL) was converted to the hydrochloride salt with 1 M HCl in ether (0.4 mL). Recrystallization twice from absolute ethanol (<1 mL) gave hydrochloride salt **26a** as a white solid (50 mg, 56%) which did not melt below 200 °C: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.71, 0.82, 0.86 (3s, 9H, 3CH_3), 1.00 (td, J = 12.5, 4.4 Hz, 1H, H12α), 1.03 (dd, J = 12.5, 2.4 Hz, 1H, H5), 1.15 (td, J = 13.0, 4.5 Hz, 1H, H11β), 1.27 (qd, J = 13.0, 4.5

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Hz, 1H, H6β), 1.52 (s, 3H, C13 CH_3), 2.04 (td, $J = 13.8, 5.7$ Hz, 1H, H7α), 2.32 (dd, $J = 14.6, 2.8$ Hz, 1H, H7β), 2.49 (t, $J = 5.5$ Hz, 3H, NCH_3), 5.47 (s, 1H, =CH), 9.34 (br s, 2H, NH_2); ^{13}C NMR (126 MHz, $CDCl_3$) δ 14.89 (CH_3), 18.46 (CH_2), 19.03 (CH_2), 22.31 (CH_3), 22.36 (CH_2), 24.53 (CH_3), 26.32 (CH_3), 30.18 (CH_2), 33.53 (C), 33.89 (CH_3), 35.43 (CH_2), 38.59 (C), 39.31 (CH_2), 42.12 (CH_2), 50.43 (CH), 54.57 (CH), 59.01 (C), 121.18 (C), 145.32 (C); $[\alpha]_D^{20}$ +126.1° (*c* 0.87, EtOH). Anal. Calcd for $C_{19}H_{34}NCl$: C, 73.16; H, 10.99; N, 4.49. Found: C, 73.05; H, 11.16; N, 4.54.

General Aspects for Preparative Incubations with Recombinant Abietadiene Synthase. Conditions for the following enzymatic reactions were based on those described in the literature.[10a,12] All enzymatic reactions were run in Teflon-capped glass tubes. Incubation buffer consisted of 30 mM HEPES (pH 7.2), 5.0 mM dithiothreitol, 7.5 mM $MgCl_2$, 20 μM $MnCl_2$, and 5% (v/v) glycerol. GGPP concentrations ranged from 0.5 to 5 times K_M (ca. 4 μM) with the standard incubations being run at near saturation (20 μM), and temperatures ranged from 31 to 33 °C in a water bath. Radiochemical conversions generally ranged from 2 to 5%, but conversions of 10−20% were occasionally seen. For radiochemical assays, parallel blank runs lacking enzyme gave negligible radioactivity (500 dpm, 0.02%) in the hexane eluants. In larger scale incubations for product isolation and characterization, parallel blank runs lacking enzyme showed no background product formation (0.02%).

Enzymatic products were prepared for NMR analysis by dissolving in C_6D_6 (0.5 mL), concentrating to dryness, and redissolving in C_6D_6 (0.2−0.3 mL). Solvent susceptibility plugs (5 mm GFP No. 99928, obtained from Doty Scientific Inc.) were used to minimize solvent volumes. Acquisition times ranged from 1 to 5 h depending on concentration.

(5*S*,9*R*,10*S*)-17-Norlabda-13(16),14-dien-8-one (22). Enzyme incubations were run as described above using three tubes containing enzyme (recombinant soluble extract, 1 mL each)[10a,13] in buffer (4 mL) with 200 μL of 17-nor-8-oxo-CPP **17** (1 mM, 0.200 μmol, 40 μM final concentration) at 32 °C for 16 h. The organic soluble products were extracted into hexane (2 × 1.5 mL each) and concentrated to 6 mL. The hexane was passed through a pipet column of silica gel followed by a hexane wash (2 mL) and an ether wash (6 mL). GC analysis of the hexane eluents showed only background peaks while analysis of the ether wash showed a single major peak (R_t = 8.7 min, purity by GC 78%). Further purification was accomplished by preparative TLC (6.6 × 1 cm) with 5:1 hexanes−ethyl acetate by scraping and extraction with Et_2O of the appropriate zone (R_f = 0.64, 1 cm band) (yield, ca. 50 μg by ^1H NMR estimate, 30%). Data for **22**: purity by GC = 95%; ^1H NMR (500 MHz, C_6D_6) δ 0.56, 0.64, 0.76 (3s, 9H, 3xCH_3), 1.77 (d, $J = 10.0$ Hz, 1H), 1.87 (td, $J = 12.7, 6.8$ Hz, 1H), 2.0−2.2 (m, 3H), 2.32 (ddd, $J = 13.2, 4.9, 2.2$ Hz, 1H), 2.57 (ddd, $J = 13.4, 10.0, 3.4$ Hz, 1H), 5.02 (s, 1H, =CH_2), 5.05 (s, 1H, =CH_2), 5.10 (d, $J = 11.0$ Hz, 1H, cis CH=CH_2), 5.56 (d, $J = 17.6$ Hz, 1H, trans CH=CH_2), 6.41 (dd, $J = 17.6, 11.0$ Hz, 1H, CH=CH_2); UV−vis (EtOH) λ_{max} = 226 nm; MS (EI) m/z 274 (M^+, 37), 259 (18), 179 (76), 136 (100). The ^1H NMR data for the H14 vinyl peak at δ 6.41 matched those observed in the spectrum of a similar 13(16),14 dienol formed as a minor product in the acid-catalyzed cyclization of diol **18b**. (See Scheme 2 and Supporting Information.)

(5*S*,8*R*,9*R*,10*R*,13*S*)-14-Oxapimara-15-ene (20a). Enzyme incubations were run as described above using three tubes containing enzyme (recombinant soluble extract, 1 mL each)[10a,13] in buffer (4 mL) with 100 μL of 17-nor-8α-hydroxy-CPP (**19a**) (4 mM, 0.400 μmol, 80 μM final concentration) at 31 °C for 16 h. The organic soluble products were extracted with hexane as above and concentrated to 6 mL. The hexane solution was passed through a pipet column of silica gel followed by a hexane wash (2 mL) and an ether wash (6 mL). GC analysis of the hexane eluate showed only background peaks while analysis of the ether fraction showed a single major peak (R_t = 8.3 min, purity by GC 76%): yield ca. 150 μg (46%) and 85% purity by ^1H NMR estimates; ^1H NMR (500 MHz, C_6D_6) δ 0.74, 0.78, 0.81, 1.21 (4s, 12H, 4 × CH_3, C20, C18 or C19, C18 or C19, C17), 3.52 (td, $J = 10.8, 4.9$ Hz, 1H, OCH), 5.01 (dd, $J = 10.7, 1.7$ Hz, 1H, cis CH=CH_2), 5.37 (dd, $J = 17.6, 1.9$ Hz, 1H, trans CH=CH_2), 6.04 (dd, 17.3, 11.0 Hz, 1H, CH=CH_2). The ^1H NMR spectrum and data matched those for the major product (**20a**) resulting from acid-catalyzed cyclization of diol **18a**. The stereochemistry was established by NOE experiments on both the enzymatic product and the **20a** + **20b** mixture generated from the acid-catalyzed cyclization of **18a**: irradiated H8β, observed C10−CH_3 (+7.3%), C13−CH_3 (+6.7%).

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Supporting Information Available: General experimental aspects as well as preparative procedures and characterization data for compounds not given in the Experimental Section above. This material is available free of charge via the Internet at http://pubs.acs.org.

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