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Intermediacy of Eudesmane Cation during Catalysis by Aristolochene Synthase

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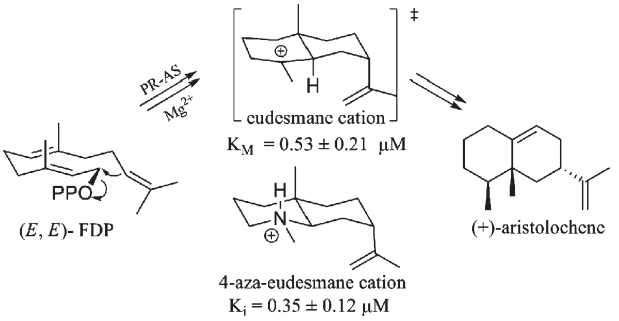

Aristolochene synthase from *Penicillium roqueforti* (PR-AS) catalyzes the formation of the bicyclic sesquiterpene (+)-aristolochene (**5**) from farnesyl diphosphate (**1**, FDP) in two mechanistically distinct cyclization reactions. The first reaction transforms farnesyl diphosphate to the uncharged intermediate (*S*)-(−)-germacrene A (**3**) through a macrocyclization process that links C1 and C10 upon magnesium ion-assisted diphosphate ester activation. In the second reaction mediated by PR-AS, a protonation induced cyclization has been suggested to generate the highly reactive trans-fused eudesmane cation **4** as a consequence of the precise folding of the enzyme-bound germacrene A intermediate. This contribution describes the use of the transition state analogue inhibitor 4-aza-eudesm-11-ene to explore the intermediacy of cation **4** as an on-path intermediate in the biosynthesis of aristolochene. 4-Aza-eudesm-11-ene as the hydrochloride salt **6** was stereospecifically synthesized in seven steps and 37% overall yield starting from chiral enamine **9**. The synthetic sequence featured a highly regio- and stereoselective deracemization reaction of **9** that gave rise to the corresponding Michael adduct in >95% diastereomeric excess as evidenced by optical rotation and NMR measurements. **6** acts as a potent competitive inhibitor of PR-AS (K_i = 0.35 ± 0.12 μM) independent of the presence of diphosphate (K_i = 0.24 ± 0.09 μM). The failure of exogenous PP_i to enhance the binding affinity of **6** for PR-AS could be interpreted against an eudesmyl cation/diphosphate anion pair mechanism as the enzymatic strategy to stabilize the highly reactive eudesmane cation **4**. In addition, these observations seem to rule out simple favorable electrostatic and/or hydrogen bonding interactions between the active site anchored diphosphate ion and the ammonium ion **6** as the binding mode. Ammonium ion **6** seems to act as a genuine mimic of eudesmane cation (**4**) that most likely binds the active site of PR-AS in a productive conformation resembling that adapted by **4** during the PR-AS-catalyzed synthesis of **5**.

Introduction

Recombinant aristolochene synthase from *Penicillium roqueforti* (PR-AS) is a 39 kDa monomeric fungal terpene cyclase that catalyzes the Mg^{2+}-dependent cyclization and rearrangement of farnesyl diphosphate (**1**, FDP) to the bicyclic sesquiterpene (+)-aristolochene (**5**) (Figure 1), the precursor of several sesquiterpenoid mycotoxins including the lethal PR toxin.[1,2] The crystal structure of recombinant

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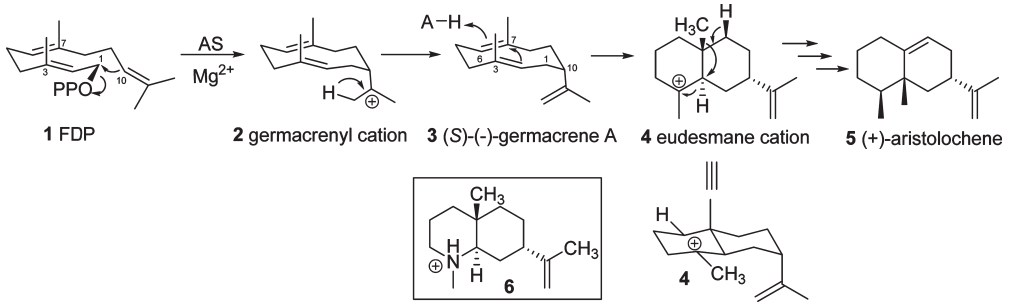

FIGURE 1. Proposed mechanism for the cyclization and rearrangements of FDP to aristolochene (**5**) catalyzed by aristolochene synthase via enzyme-bound germacrene A (**3**) and eudesmane cation (**4**).[5] The structure of the aza-analogue **6** that mimics putative **4** is shown.

PR-AS has been solved at 2.5 Å resolution revealing a typical α-helical class I terpene cyclase fold that contains the two Mg^{2+}-binding motifs found in many class I enzymes at the top of the active site cleft, namely the aspartate-rich DDXX-(D/E) motif, $D^{115}D^{116}VLE$,[119] and the highly conserved NSE triad, $N^{244}DIYS^{248}YDKE^{252}$.[3,4]

The complex cationic reaction mechanism of the PR-AS-catalyzed conversion of FDP (**1**) to aristolochene (**5**) (Figure 1) has been probed previously with deuterium-labeled derivatives and fluorinated analogues of FDP,[6−10] alternative substrates,[11] mechanism based inhibitors,[12] and site-directed mutagenesis experiments.[13−19] These studies provided evidence for two distinct cyclization reactions involving two discrete intermediates, the neutral hydrocarbon (*S*)-(−)-germacrene A (**3**) and the highly reactive eudesmane cation (**4**). Upon binding of three magnesium ions,[3,20,21] diphosphate expulsion from the C1 position of FDP is triggered by the intramolecular attack of the distal C10, C11 π-bond on C1 (Figure 1) in a concerted reaction[22] that occurs with inversion of configuration at C1.[6] Deprotonation of the resulting germacren-11-yl cation (**2**) leads to the flexible (*E*,*E*)-configured (*S*)-(−)-germacrene A as the reactive UU-conformer (14-CH_3 and 15-CH_3 diaxial; Figure 1),[9,23] which 92% of the time remains tightly bound to the active site of the enzyme (~8% of **3** is released by the enzyme as the major side product).[13] Protonation of germacrene A by an as of yet unidentified active site acid[8,20,24] and electron flow from the C2, C3 double bond to C6 leads to the formation of the bicyclic trans-fused eudesmane cation (**4**). Successive 1,2-hydride and methyl migrations followed by loss of the *pro-S* hydrogen from C8[7,8] generate bicyclic (+)-aristolochene (**5**).

In contrast to the mechanism of the formation of germacrene A, which has been studied extensively, the intermediacy of eudesmane cation (**4**) and the mechanism of its formation have received less attention. The existence of the intermediate **4** has been inferred from site-directed mutagenesis experiments, in which replacement of Trp 334, an active site residue ideally placed to stabilize a positive charge on C4 of eudesmane cation, with the aliphatic amino acids Val and Leu led to the accumulation of germacrene A.[16] In addition, the involvement of trans-fused **4** in PR-AS catalysis has also been suggested based on stereochemical results obtained from experiments with deuterated FDP's.[6,7]

Several candidates have been proposed for the acid within the active site that reprotonates germacrene A, including Tyr 92 or a proton shuttle from the solvent to Tyr 92 by way of Arg 200, Asp 203, and Lys 206,[5,13,14] an unprecedented active site oxonium ion,[19] or the diphosphate ion itself.[20] However, there is currently little evidence for any of these proposals. In addition, an alternative mechanism involving protonation of the C6, C7 double bond of germacrene A by intramolecular transfer of a proton from C12[24] has also been excluded experimentally.[8] Hence, the absence of an identified general acid could be interpreted against the existence of the intermediate **4**.

An alternative approach to identify postulated carbocationic intermediates in terpene synthase catalyzed reactions has relied on the use of aza analogues of these reactive and transient cationic species.[25−29] Numerous aza analogues

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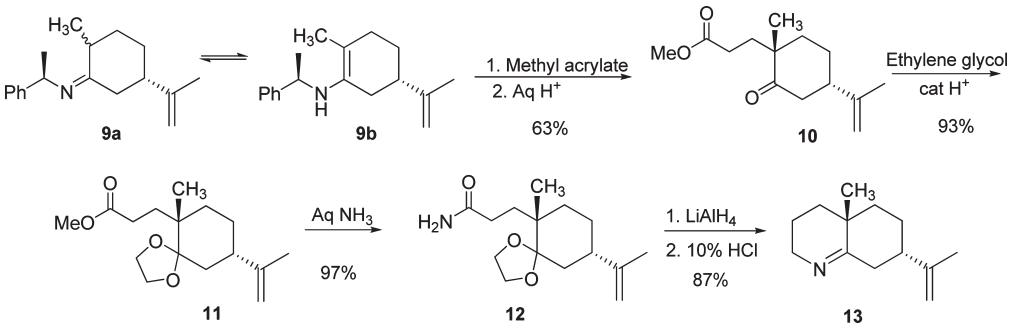
SCHEME 1. Stereospecific Synthesis of Bicyclic Imine 13



have been shown to effectively mimic the topological and electrostatic properties of putative carbocation intermediates, and this strategy has provided potent competitive inhibitors of mono-,[30] sesqui-,[31] and diterpene synthases,[32−35] as well as inhibitors of squalene and oxidosqualene synthases.[28,36−38] In addition, some aza-terpenoids have served as crystallization aids of mono- and sesquiterpene synthases[39,40] to yield valuable structural information.[4]

Here, we report the stereoselective synthesis of the nitrogen-containing eudesmane cation analogue **6**, as well as the kinetic evaluation of this compound as a possible transition state inhibitor of PR-AS in both the absence and presence of exogenous PP_i. Our results support the existence of the on-path intermediate **4** during PR-AS catalysis and shed light on the nature of the stabilization of this important carbocationic intermediate.

Results and Discussion

Synthesis of 4-Aza-eudesm-11-ene (6). The synthetic sequence leading to the bicyclic aza-analogue **6** is outlined in Schemes 1 and 2. Enamine **9b** (prepared in 91% yield from (−)-dehydrocarvone and (*R*)-(+)-1-phenylethylamine)[41] was alkylated with methyl acrylate (THF, room temperature, 7 days) to produce ketone **10**[42,43] in 63% yield as the only regioisomer in an approximately 94% diastereomeric excess (*de*) as evidenced by ^{1}H and ^{13}C NMR spectroscopy.

The optical rotation $[\alpha]^{25}_D$ of −8.1° (*c* 2.0, $CHCl_3$) measured for the Michael adduct **10** was in excellent agreement with the value of +9.0° reported previously for the known enantiomer of **10**, which had been obtained with a *de* of >95%.[42] The high degree of regio- and diastereoselectivity observed during the asymmetric Michael addition involving chiral enamines[44] such as **9b** is well precedented in several synthetic schemes.[45−49] The observed regio- and steroselective induction has been explained previously based on steric and electronic arguments.[50] Primary amide **12** was synthesized in 90% yield over two steps by ketalization of **10** to yield ester **11**[42] followed by reaction with NH_4OH. $LiAlH_4$ reduction of amide **12** to the corresponding primary ketalamine and subsequent exposure of the crude reaction product to aqueous acid afforded the cyclic Schiff base **13** in 87% yield.[46]

The trans-fused decahydroquinoline **14** was obtained in 85% yield by reduction with sodium in ethanol of $\Delta^{1,9}$-octahydroquinoline **13** (Scheme 2).[51,52] ^{1}H and ^{13}C NMR spectroscopic analysis of **14** revealed that it was produced as a single trans-isomer with characteristic ^{1}H NMR signals at δ 3.11 (dd, J = 12.1, 4.6 Hz), 2.68 (td, J = 12.5, 3.1 Hz), 2.44 (dd, J = 12.5, 3.1 Hz), and 0.99 (s), assigned to protons $H3_{eq}$, $H3_{ax}$, H5, and the angular CH_3 at C10, respectively (Scheme 3). These values were in good agreement with those previously reported for the CH_3 at C10 analogue of *trans*-decahydroquinoline at δ 3.06 (d, J = 12.0 Hz, $H3_{eq}$), 2.64 (td, J = 12.0, 3.5 Hz, $H3_{ax}$), 2.21 (H5), and 0.96 (s, 10-Me) ppm,[51] which lacks the isopropylidene substituent at C7. The resonance assigned to the methine H5 of 10-CH_3 *trans*-decahydroquinoline[51] at 2.21 ppm is relatively upfield of the corresponding resonance of H5 at 2.44 ppm in compound **14**. This field shift could be explained by a deshielding effect caused by the 1,3-diaxial interaction between the methine H5 and the isopropylidene group at C7 of **14** (Scheme 3). In addition, the diagnostic ^{13}C NMR signal observed

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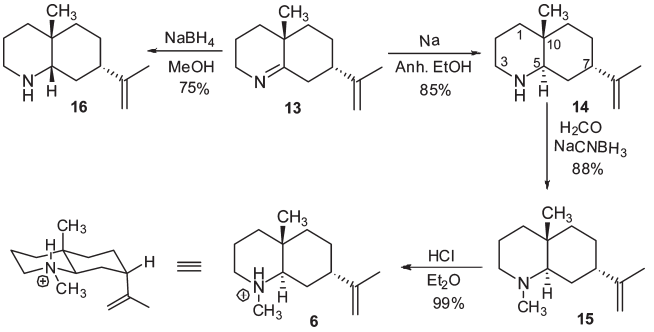
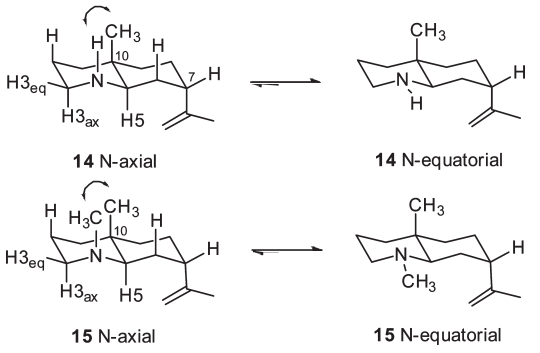
SCHEME 2. Synthesis of Trans-Fused Bicyclic Amine 15, Its Hydrochloride Salt 6, and the *cis*-Fused 4-Aza-decalin 16



SCHEME 3. *syn*-Diaxial Interactions (Double Arrow) in 10-Methyl *trans*-Decahydroquinoline 14 and Its Corresponding *N*-Methyl Derivative (15)



for the CH_3 at C10 of amine **14** at δ_c 15.32 is essentially identical with that previously recorded (15.60 ppm) for the analogous CH_3 group at C10 of *trans*-decahydroquinoline.[52]

Reductive N-alkylation of compound **14** with formaldehyde in the presence $NaBH_3CN$ provided the *N*-methyl *trans*-decahydroquinoline **15** in 88% yield.[53] Exposure of **15** to hydrochloric acid in diethyl ether furnished the corresponding ammonium salt **6** in crystalline form and in essentially quantitative yield. Only the isomer with the methyl group in the equatorial position was detected in ^1H and ^{13}C NMR spectra of *N*-methyl amine **15** (Scheme 3), likely as a consequence of the unfavorable interactions between the two axial methyl groups and two CH_3/H-1,3-*syn*-diaxial interactions in the N-axial conformer of **15**.[54,55] Compound **15** displayed characteristic ^1H NMR signals for $H3_{eq}$, the equatorial *N*-methyl, and the angular CH_3 at C10 at δ_H 2.87, 2.11, and 1.00, respectively, in good accord with the ^1H NMR signals reported for the parent (*N*,10)-dimethyl *trans*-decahydroquinoline (DMDHQ) at 2.87, 2.15, and 0.98 ppm.[54,55] In addition, the bicyclic amine **15** displayed the diagnostic ^{13}C NMR resonance assigned to its N-Me equatorial conformer at 43.03 ppm (43.11 for DMDHQ)[52,54,55] as well as the signal associated with the angular 10-methyl group at 17.11 ppm (17.35 ppm for DMDHQ).[52] The relative stereochemistry of **15** was confirmed by NOE measurements. Saturation of the well-separated signal at δ_H 2.11, corresponding to the *N*-CH_3 protons, led to NOE enhancements of $H3_{eq}$ (7%) and $H5_{ax}$ (3%) at 2.87 and 2.09 ppm, respectively, while no effect was seen on the protons of the methyl group on C10. NOE enhancements were also observed for protons $H3_{ax}$ (18%) and the *N*-CH_3 group (6%), when the broad doublet assigned to $H3_{eq}$ at δ 2.87 was irradiated. The configuration of the methyl at the tertiary C10 position in **15** was therefore unambiguously assigned as anti (trans-fused) relative to H5 based on the absence of NOE enhancement with $H5_{ax}$ when the angular 10-methyl group at 1.00 ppm was irradiated. On the basis of these considerations, it is clear that protonation of amine **15** generates a single diastereomeric hydrochloride, in which the proton on nitrogen adopts the axial position (see *trans*-aza-decalin in Scheme 2 and Figure 2). The equatorial *N*-methyl and trans-fused structure of the ammonium salt **6** displayed in Scheme 2 was further corroborated by the solution of the X-ray crystal structure of this protonated bicyclic amine (Figure 2).[56]

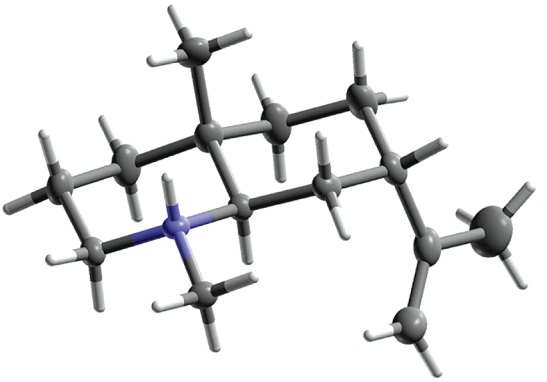


FIGURE 2. ORTEP representation of the chloride salt of 4-aza-eudesm-11-ene analogue **6**.

Notably, the cis-fused decahydroquinoline **16** was obtained exclusively[58,59] from imine **9** by reduction with $NaBH_4$ (Scheme 2).[60,61] The related enzymes tobacco 5-*epi*-aristolochene synthase (TEAS)[62] and henbane premnaspirodiene synthase (HPS)[63] generate their hydrocarbon reaction products via the cis-fused eudesmane cation **17** (Figure 3).[64,65] Hence, our

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(56) $C_{14}H_{26}NCl$, fw = 243.81, T = 150 K, λ = 0.71073 Å, orthorhombic, $P2_12_12_1$, a = 6.7550(1) Å, b = 8.4270(2) Å, c = 25.7000(4) Å, V = 1462.96(5) Å^3, Z = 4, ρ(cal) = 1.107, size = 0.20 × 0.15 × 0.15 mm^3, independent reflections = 3304, final R1 = 0.042, wR2 = 0.097, for $I > 2\sigma(I)$. The data were collected with use of graphite-monochromated Mo Kα (λ = 0.710 73 Å) radiation at 150 K. The structures were solved by direct methods, using SHELXS-96, and refined with all data on F^2 full-matrix least-squares, using SHELXL-97.[57] All non-hydrogen atoms were refined anisotropically. Hydrogen atom positions were located from difference Fourier maps and a riding model with atomic displacement parameters 1.2 times (1.5 times for methyl groups) those of the atom to which they are bonded was used for subsequent refinements.
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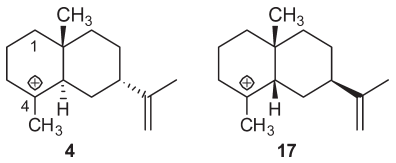

FIGURE 3. Structures of eudesmane cations **4** (trans-fused) and **17** (cis-fused) produced in the active sites of PR-AS and TEAS, respectively.

approach for the synthesis of the aza-analogue **6** can also be used to prepare the *cis*-aza-decalin of **17** from (+)-dihydrocarvone to probe TEAS and HPS mechanisms.

Inhibition of PR-AS by 4-Aza-eudesm-11-ene (6). Incubations of recombinant PR-AS with FDP (**1**) in the presence of Mg^{2+} yielded the expected 92% aristolochene (**4**) accompanied by ~7% germacrene A and a small amount of valencene as determined by GC-MS (see the Supporting Information). Inhibition studies with compound **6** indicated that this ammonium salt acted as a competitive inhibitor with a K_i of $0.35 \pm 0.12\ \mu M$, similar to the Michaelis constant ($K_M = 0.53 \pm 0.21\ \mu M$) determined for the PR-AS-catalyzed conversion of FDP to aristolochene. The similar values for K_i and K_M indicate that the tetrahedral geometry of the ammonium nitrogen relative to the presumably trigonal sp^2 configuration of C4 in eudesmane cation (**4**, Figure 3) did not strongly affect the binding affinity of **6**. The inhibition constant was approximately two times smaller than that measured for 12,13-difluoro FDP.[22] To the best of our knowledge **6** is the tightest binding inhibitor of PR-AS described so far.

Structural studies with soaked cocrystals of the aza-analogues synthesized as presumed mimics of bisabolyl, α-terpenyl, and bornyl cations[39,40] have revealed that these analogues as their protonated amines are sufficiently strong competitive inhibitors of trichodiene synthase and bornyl diphosphate synthase. However, with the exception of 2-azabornane, these protonated aza-terpenes bound to the corresponding $[Mg^{2+}]_3-PP_i$ enzyme complex in what appears to be nonproductive conformations, presumably as a result of favorable electrostatic and hydrogen bonding interactions between the positively charged ammonium nitrogen and the diphosphate moiety.[39,40] In contrast, 2-azabornane, a product-like mimic of bornyl cation that matches the product-like contour of the active site of bornyl diphosphate synthase in its competent closed conformation, was shown to bind the enzyme in a "catalytically productive conformation". Hence, it is likely that the conformation adopted by 2-azabornane in the enzyme resembles that of the actual bornyl cation in solution.[39,66]

The conformational change from the open to the catalytically competent closed conformation of PR-AS is believed to be induced by the formation of a ternary complex involving the binding of three magnesium ions to the Asp-rich and Asn-Ser-Glu (NSE) motifs of AS, as well as to the diphosphate group of the substrate. Hence the tight binding of **6** to PR-AS, even in the absence of inorganic diphosphate, is most likely a consequence of its close structural resemblance to the putative on-pathway carbocationic intermediate **4** rather than a simple consequence of favorable electrostatic interactions with PP_i.[39,40]

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It had previously been observed that the potency of nitrogen-containing and sulfonium ion analogues of presumed carbocationic intermediates in the reactions catalyzed by pinene synthase,[30,67] bornyl diphosphate synthase,[39,67] trichodiene synthase,[31,40] abietadiene synthase,[32,33] ent-kaurene synthase,[34] and squalene synthase[36−38,68] is increased by the addition of inorganic diphosphate (PP_i). These observations have been interpreted to suggest that formation of carbocation−diphosphate ion pair intermediates might be a general strategy used by terpene synthases to stabilize transiently generated carbocations during catalysis. In an attempt to mimic the complex of PR-AS with 4-azaeudesm-11-ene (**6**) in the presence of PP_i, the inhibition constant of **6** for the PR-AS-catalyzed conversion of FDP was also determined in the presence of diphosphate at concentrations of 50, 250, and 500 μM.[69] The K_i value of $0.24 \pm 0.09\ \mu M$ measured in the presence of 250 μM of PP_i was similar to the values (0.35, 0.43, and 0.22 μM) obtained at diphosphate concentrations of 0, 50, and 500 μM, respectively. Clearly the addition of exogenous PP_i did not enhance the binding affinity of this competitive inhibitor, indicating that the stable binding of **4** to the active site of PR-AS was not enhanced by ion pairing with the diphosphate ion. Structural studies with terpene cyclases−substrate analogue cocrystals have also shown that the enzyme-bound PP_i counterions occupy a relative invariant position,[4] and in the case of aristolochene synthase from *A. terreus*,[20] the proximity of the O3 oxygen atom of the $[Mg^{2+}]_3-PP_i$ complex to the C6 and C8 atoms (farnesyl chain) was interpreted to involve the active site anchored PP_i ion as the possible general acid/base responsible for the second cyclization reaction catalyzed by aristolochene synthase.[20]

If these observations hold true, it seems likely that the spatial separation between the newly generated cation at C4 of **4** (Figure 3) and the diphosphate ion could be slightly longer than the distance of a typical covalent C−O bond (1.42 Å), therefore precluding the formation of a presumed tertiary 4-eudesmyl carbocation−OPP_i ion pair. Since the binding affinity of **6** was clearly unenhanced by the addition of exogenous PPi, this observation suggests that the imperfect "puckered" tetrahedral ammonium geometry of **6** does not place the nitrogen atom closer to O3, thus resembling the reactive conformation of a putative unpaired eudesmane cation. The inhibition studies presented here for ammonium ion **6**, showing an insensitivity to PP_i concentrations, indicate therefore that counterion stabilization could be limited to *carbocations* in close proximity to the original PP_i position within the active sites of these terpene cyclases.[34]

Conclusions

The aza-analogue of the putative eudesmane cation (**4**) intermediate during the enzymatic conversion of FDP to aristolochene is a potent competitive inhibitor of PR-AS independent of exogenously added PP_i. These observations indicate that compound **6** acts as an effective transition state inhibitor of PR-AS and further support the intermediacy of **4**

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(69) PP_i on its own is not an inhibitor of PR-AS. For details see ref 1.

as an on-pathway intermediate in PR-AS catalysis. The high potency of this inhibitor in the absence of favorable electrostatic interactions (i.e., in the absence of PP_i) can be ascribed to its actual resemblance to the eudesmane cation and suggests that **6** is likely to bind the active site of PR-AS in a conformation that closely matches that adopted by the actual intermediate **4**.[39,66] Finally, the unusual lack of synergism observed with this compound and inorganic PP_i can rule against the formation of a tightly bound 4-eudesmyl−pyrophosphate ion pair intermediate in the cyclization catalyzed by PR-AS presumably due to substantial spatial separation between cation **4** and the PP_i counterion. Moreover, this suggestion reinforces the central role played by the aromatic active site residue Trp334 in stabilizing the highly reactive eudesmane cation.[16]

Experimental Section

Representative preparative procedures leading to and characterization data for compounds **6**, **10**, **11**, **12**, **13**, **14**, **15**, and **16** are given below.

Methyl 3-((1*S*,4*S*)-1-Methyl-2-oxo-4-(prop-1-en- 2-yl)cyclohexyl)propanoate (10).[42,43] Ketone **10** was synthesized following the method of d'Angelo.[44] Chiral imine (**9**),[41] obtained by condensation of (−)-dehydrocarvone and (*R*)-(+)-*a*-methylbenzylamine, was subjected to asymmetric Michael alkylation with methyl acrylate, followed by removal of the chiral auxiliary to afford ketone **10** in 63% overall yield.[46] $[\alpha]^{25}_{D}$ −8.1 (*c* 2.0, $CHCl_3$) {lit.[42] +9° (for mirror image)}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.77 (br s, 1H), 4.71 (br s, 1H), 3.65 (s, 3H), 2.48 (dd, J = 14.0, 12.5 Hz, 1H), 2.38−2.72 (m, 3H), 2.14 (ddd, J = 14.2, 11.0, 5.1 Hz, 1H), 2.04 (ddd, J = 16.2, 11.0, 5.1 Hz, 1H), 1.87 (dt, J = 13.6, 3.9 Hz, 1H), 1.83−1.75 (m, 2H), 1.73 (s, 3H), 1.70 (ddd, J = 14.2, 11.2, 5.1 Hz, 1H,), 1.55 (ddd, J = 13.6, 11.0, 4.8 Hz, 1H), 1.02 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 214.5, 173.7, 147.3, 110.0, 51.7, 47.3, 46.1, 43.4, 38.3, 32.1, 28.8, 25.9, 21.8, 20.6. The ^{1}H and ^{13}C NMR data are in excellent agreement with those previously reported for *ent*-**10**.[42]

Methyl 3-((1*S*,4*S*)-1-Methyl-2,2-ethylenedioxy-4-(prop-1-en-2-yl)cyclohexyl)propanoate (11).[42] To a stirred solution of ketone **10** (1.25 g, 5.25 mmol) in dry benzene (100 mL) was added ethylene glycol (0.5 mL, 9 mmol) and a catalytic amount of p-TsOH. The mixture was refluxed for 4 h with continuous removal of water, using a Dean−Stark apparatus. The solvents were removed under reduced pressure, and the crude material was purified by flash chromatography on silica gel (eluting with 5% EtOAc−hexane) to give ketal **11** as a clear oil (1.38 g, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.77 (br s, 1H), 4.74 (br s, 1H), 3.96−3.87 (m, 4H), 3.64 (s, 3H), 2.55 (m, 1H), 2.37 (dd, J = 7.9, 3.2 Hz, 2H), 1.65 (s, 3H), 1.60−1.40 (m, 6H), 1.29 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.7, 149.1, 112.5, 109.7, 65.0, 64.7, 51.5, 42.3, 40.6, 35.2, 34.0, 29.2, 29.0, 25.9, 20.9, 19.2. The ^{13}C NMR data are in excellent agreement with those previously reported for *ent*-**11**.[42]

3-((1*S*,4*S*)-1-Methyl-2,2-ethylenedioxy-4-(prop-1-en-2-yl)-cyclohexyl)propanamide (12). To a stirred solution of **11** (1.63 g, 5.77 mmol) in MeOH (120 mL) was added a ca. 35% solution of NH_4OH (80 mL) at room temperature. After 24 h, the MeOH was removed under pressure and the resulting aqueous solution was poured into brine (300 mL), extracted with EtOAc (3 × 100 mL), and dried ($MgSO_4$). Removal of the solvent under reduced pressure gave amide **12** as a white solid (1.49 g, 97%). HR(EI)MS (M^+) found 267.1804, $C_{15}H_{25}NO_3$ requires 267.1834; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.83 (br s, 1H), 5.57 (br s, 1H), 4.67 (br s, 2H), 3.98−3.85 (m, 4H), 2.31−2.11 (m, 3H), 1.91−1.76 (m, 2H), 1.69, (s, 3H), 1.64 (d, J = 12.5 Hz, 1H), 1.60 (dd, J = 4.5, 1.2 Hz, 1H), 1.57−1.46 (m, 3H), 1.27−1.25 (m, 1H), 0.83 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.4, 149.2, 112.8, 108.6, 65.0, 64.8, 42.4, 40.6, 35.2, 34.2, 30.8, 29.6, 25.9, 20.9, 19.3.

(4a*S*,7*S*)-4a-Methyl-7-(prop-1-en-2-yl)-2,3,4,4a,5,6,7,8-octahydroquinoline (13). The bicyclic imine **13** was prepared according to a literature procedure with some modifications.[46] To a stirred solution of amide **12** (1.26 g, 4.72 mmol) in dry THF (100 mL) was added $LiAlH_4$ (1.1 g, 28.95 mmol) at room temperature under N_2. The resulting suspension was refluxed for 1 h and then cooled to room temperature. A 5% solution of NaOH was added slowly to precipitate the aluminum salt byproduct. The white salts were filtered under reduced pressure and washed with additional THF (100 mL). To the resulting THF filtrate was added 10% aqueous HCl (80 mL) and the two-phase mixture was vigorously stirred at room temperature for 18 h. Hexane (150 mL) was added and the two phases were separated. The aqueous layer was washed with hexane (2 × 50 mL), basified with NaOH (pellets), and extracted with Et_2O (2 × 50 mL). The combined ethereal extracts were dried (K_2CO_3) and filtered, and the solvent was removed under reduced pressure to give imine **13** (778 mg, 87%) as an oil. HR(EI)MS (M^+) found 191.1668, $C_{13}H_{21}N$ requires 191.1674; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.93 (br s, 1H), 4.89 (br s, 1H), 3.67 (app ddd, J_{app} = 17.5, 5, 2 Hz, 1H), 3.46−3.38 (m, 1H), 2.66 (app d quintets, J_{app} = 14.5, 3.0 Hz, 1H), 2.60−2.50 (m, 2H), 1.96 (app tdd, J_{app} = 14.5, 5.5, 3.5 Hz, 1H), 1.79−1.66 (m, 2H), 1.72, (s, 3H), 1.61−1.54 (m, 1H), 1.50 (d, J = 4.0 Hz, 1H), 1.47 (br s, 1H), 1.39, (dt, J = 13.5, 4 Hz, 1H), 1.30 (dt, J = 13.5, 4.0 Hz, 1H), 1.19 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.2, 146.7, 112.8, 49.8, 41.5, 38.6, 36.4, 36.1, 36.0, 24.2, 23.4, 22.7, 29.2.

(4a*S*,7*S*,8a*R*)-4a-Methyl-7-(prop-1-en-2-yl)decahydroquinoline (14). The *trans*-decahydroquinoline **14** was prepared according to the procedure of Vierhapper and Eliel.[51,52] To a stirred solution of imine **13** (268 mg, 1.40 mmol) in dry EtOH (13 mL) was added Na (1.0 g, 43.5 mmol) at room temperature under N_2. After 7 h, additional Na (250 mg, 10.9 mmol) was added, and stirring was continued overnight. After 29 h, water (20 mL) was added, and EtOH was removed under reduced pressure. The resulting aqueous layer was acidified with 10% HCl (1 mL) and extracted with Et_2O (3 × 10 mL). The aqueous phase was basified with NaOH (pellets) and extracted with Et_2O (3 × 10 mL). The combined ethereal layers were dried (K_2CO_3) and filtered, and the solvent was removed under reduced pressure. The crude amine was dissolved in CH_2Cl_2 (5 mL) and then loaded onto a basic alumina (Aldrich, 150 mesh, 58 Å) column. Elution with CH_2Cl_2 (removes unreacted starting imine **13**) and then Et_2O gave the bicyclic amine **14** as a clear liquid (230 mg, 85%). HR(EI)MS (M^+) found 193.1831, $C_{13}H_{23}N$ requires 193.1830; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.91 (br s, 1H), 4.86 (br s, 1H), 3.11 (dd, J = 12.1, 4.7 Hz, 1H), 2.68 (dt, J = 12.6, 3.3 Hz, 1H), 2.44 (dd, J = 12.6, 3.3 Hz, 1H), 2.40 (br s, 1H, NH), 1.86−1.70 (m, 4H), 1.75 (s, 3H), 1.57 (dt, J = 13.0, 5.8 Hz, 1H), 1.42 (app br d, J_{app} = 10.5 Hz, 2H), 1.34−1.24 (m, 2H), 1.20−1.11 (m, 2H), 0.99 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 146.9, 110.6, 58.8, 47.6, 40.0, 39.2, 35.7, 34.1, 30.1, 32.1, 22.9, 22.4, 15.3.

(4a*S*,7*S*,8a*R*)-1,4a-Dimethyl-7-(prop-1-en-2-yl)decahydroquinoline (15). *N*-Methyl-*trans*-decahydroquinoline **15** was prepared by using a modification of the procedure of Lee et al.[53] To a stirred solution of amine **14** (70 mg, 0.36 mmol) in MeOH (10 mL) was added formalin solution (2 mL, 37%), acetic acid (1.5 mL, 26.3 mmol), and $NaCNBH_3$ (1.5 g, 23.8 mmol) at room temperature under N_2. After being stirred for 48 h, the reaction mixture was poured into 5% aqueous NaOH (20 mL) and extracted with Et_2O (3 × 15 mL). The combined ethereal layers were dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The crude product was purified by flash-chromatography on silica gel (using a linear gradient from 25% Et_2O in CH_2Cl_2 to 100% Et_2O) to give amine **15** as a liquid (66.3 mg, 88%). HR(EI)MS (M^+) found 207.1902, $C_{14}H_{25}N$ requires

207.1987; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.90 (br s, 1H), 4.84 (br s, 1H), 2.87 (app d quintets, J_{app} = 10.8, 1.8 Hz, 1H), 2.41 (br s, 1H), 2.18 (s, 3H), 2.09 (br d, J = 13.1 Hz, 1H), 1.98 (app ddd, J_{app} = 13.3, 11.0, 3.0 Hz, 1H), 1.87 (qt, J = 13.8, 4.0 Hz, 1H), 1.79−1.73 (m, 2H), 1.74 (s, 3H), 1.64 (dd, J = 12.4, 2.9 Hz, 1H), 1.41 (dt, J = 13.1, 5.6 Hz, 2H), 1.37−1.27 (m, 2H), 1.13 (dt, J = 13.7, 3.4 Hz, 1H), 1.06 (dt, J = 12.8, 4.4 Hz, 1H), 1.00 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 147.0, 66.6, 59.2, 43.1, 40.5, 39.3, 35.9, 34.4, 26.4, 22.8 (2C), 22.0, 17.1.

(4a*S*,7*S*,8a*R*)-1,4a-Dimethyl-7-(prop-1-en-2-yl)decahydroquinolinium Chloride (6). To a stirred solution of amine **15** (66.0 mg, 0.32 mmol) in anhydrous Et_2O (1.2 mL) at 0 °C was added an ethereal solution of HCl (2 M, 160 μL, 0.32 mmol) under N_2. After standing for 1 h, the mixture was filtered to give the hydrochloride salt **6** (77.1 mg, 99%). Mp 215−217 °C; HR(ES)-MS (M^+) found 208.2064, $C_{14}H_{26}N^+$ requires 208.2065; ^{1}H NMR (500 MHz, D_2O) δ 5.01 (br s, 1H), 4.86 (br s, 1H), 3.44 (app dd. J_{app} = 12.7, 4.6 Hz, 1H), 3.03 (dt, J = 13.2, 3.7 Hz, 1H), 2.96 (dd, J = 13.1, 3.1 Hz, 1H), 2.79 (s, 3H), 2.60 (br s, 1H), 2.29 (br d, J = 13.5 Hz, 1H), 1.99 (qt, J = 13.8, 4.4 Hz, 1H), 1.84−1.77 (m, 2H), 1.74 (s, 3H), 1.71 (dt, J = 13.3, 5.5 Hz, 2H), 1.51 (br d, J = 13.5 Hz, 1H), 1.46−1.30 (m, 3H), 1.07 (s, 3H); ^{13}C NMR (125 MHz, D_2O) δ 146.5, 111.2, 67.9, 57.5, 40.6, 38.8, 36.7, 35.4, 34.6, 24.0, 21.8, 21.6, 19.3, 15.1.

(4a*S*,7*S*,8a*S*)-4a-Methyl-7-(prop-1-en-2-yl)decahydroquinoline (16). The bicyclic cis-fused amine **16** was prepared by using a modification of the procedure of Maiti et al.[60] To a stirred solution of imine **13** (18.8 mg, 0.10 mmol) in MeOH (1 mL) at 0 °C was added 1 drop of water followed by $NaBH_4$ (19.0 mg, 0.5 mmol). The reaction mixture was stirred at 0 °C for 5 h and then allowed to warm to room temperature overnight (16 h). Water (3 mL) was added and the resulting solution was stirred at room temperature for 30 min. The solution was then extracted with Et_2O (2 × 5 mL), the combined ethereal extracts were dried ($MgSO_4$) and filtered, and the solvent was evaporated with a stream of nitrogen. The crude amine was then purified by flash chromatography on silica gel with Et_2O and then MeOH as eluting solvents. Fractions having the basic product (MeOH) were combined and the solvent was evaporated with a stream of nitrogen to give a 95:5 mixture of the cis[57] and trans[51,52] decahydroquinolines **16** and **14** (14.3 mg, 75%). HR(EI)MS (M^+) found 193.1822, $C_{13}H_{23}N$ requires 193.1830; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.70 (app br d, J_{app} = 4.6 Hz, 2H), 2.81 (app dt, J_{app} = 13.0, 3.5 Hz, 1H), 2.74 (dd, J = 13, 5.0 Hz, 1H), 2.54 (dd, J = 11.3, 4.1 Hz, 1H), 2.31 (br s, 1H, NH), 1.95−1.83 (m, 3H), 1.78−1.66 (m, 1H), 1.74 (s, 3H), 1.54−1.40 (m, 4H), 1.10 (s, 3H), 0.99 (app br d, J_{app} = 12.4 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 150.2, 108.2, 59.5, 45.0, 40.4, 39.6, 32.3, 32.0, 28.6, 27.3, 26.7, 22.7, 21.2.

Aristolochene Synthase Kinetics. Kinetics assays were carried out according to the standard, linear range, microassay pro cedure developed for limonene and bornyl diphosphate synthases with modifications.[70] This protocol involved the incubation of varying amounts of [1-^{3}H]FDP (specific activity 75 mCi/mmol) at pH 7.5 with a fixed concentration of purified AS (100 nM) in 20 mM Tris buffer containing 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 15% glycerol at pH 7.5. The reactions mixtures containing buffer, FDP, and protein were prepared on ice in a total volume of 250 μL, and were overlaid with 1 mL of HPLC-grade hexane prior to incubation. The assay mixtures were incubated at room temperature (22 °C) for 12 min. The reactions were immediately ice-cooled and quenched by addition of 200 μL of 100 mM EDTA (pH 8.5) and brief vortexing. The hexane overlay and two additional 1 mL hexane extracts were passed through a short pipet column containing silica gel and $MgSO_4$. The column was washed with additional hexane (1 mL) and the combined filtrates were analyzed by liquid scintillation counting with use of 15 mL of scintillation cocktail. Steady state kinetic parameters for wild-type AS were obtained by direct fitting of the data to the Michaelis−Menten equation by nonlinear least-squares regression in conjunction with the graphical procedures developed by Lineweaver−Burk.[71] The calculated values for K_M, k_{cat}, and k_{cat}/K_M of 0.53 ± 0.21 μM, 0.084 s^{-1}, and 11.8 × 10^4 M^{-1} s^{-1}, respectively, are in good agreement with those parameters obtained earlier for aristolochene synthase.[13,19]

Inhibition Studies with PR-AS. The procedure described above to determine the kinetic parameters of PR-AS was followed. Assay mixtures containing buffer, inhibitor, tritiated FDP, and PR-AS (100 nM) were prepared on ice with use of a total volume of 250 μL overlaid with HPLC-grade hexane (1 mL) and incubated at room temperature (22 °C). In some instances, fixed amounts of inorganic PP_i were added to evaluate the effects of this reaction product (PP_i) in the presence of the inhibitor. Kinetic data in the presence or absence of inhibitor with or without PP_i were evaluated as mentioned above. The graphic method of Lineweaver−Burk was used to evaluate the level of inhibition. K_M and K_i were determined by varying [1-^{3}H]FDP concentrations in the presence or absence of fixed amounts of inhibitor with or without fixed concentrations of PP_i.

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Supporting Information Available: Materials, general methods, and instrumentation; protein production; GC-MS spectra; kinetic plots; reproduction of NMR spectra; and X-ray crystallographic data for compound **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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