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Inhibition of (+)-Aristolochene Synthase with Iminium Salts Resembling Eudesmane Cation

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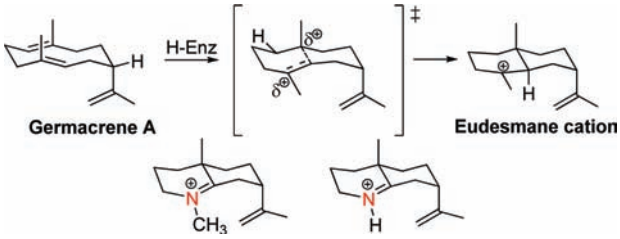
ABSTRACT



Trigonal iminium halides of (4a*S*,7*S*)-1,4a-dimethyl- and (4a*S*,7*S*)-4a-methyl-7-(prop-1-en-2-yl)-2,3,4,4a,5,6,7,8-octahydroquinolinium ions, aimed to mimic transition states associated with the aristolochene synthase-catalyzed cyclization of (−)-germacrene A to eudesmane cation, were evaluated under standard kinetic steady-state conditions. In the presence of inorganic diphosphate, these analogues were shown to competitively inhibit the enzyme, suggesting a stabilizing role for the diphosphate leaving group in this apparently endothermic transformation.

The aristolochene synthases from the fungi *Penicillium roqueforti* (PR-AS)[1a,b] and *Aspergillus terreus* (AT-AS)[1c,d] and the related 5-*epi*-aristoloche synthase (TEAS)[2] from *Nicotiana tabacum* are among the best characterized sesquiterpene synthases.[3] They catalyze the first committed step in the biosyntheis of highly oxygenated mycotoxins including PR toxin[1b,d,e] and plant phytoalexins such as capsidiol.[4]

PR-AS converts the acyclic substrate FDP (**1**) to the eremophilane hydrocarbon (+)-aristolochene (**4**) via a cascade of carbocationic reactions[5] involving ionization of the allylic diphosphate **1**, two cyclization reactions, two sequential 1,2-group migrations (H and Me), and a final regio- and stereospecific loss of the 9Hβ proton (Scheme 1).

The formation of (+)-aristolochene (**4**) by PR-AS is thought to involve highly reactive carbocationic intermediates such as eudesmane cation (**3**) as well as the neutral sesquiterpene (−)-germacrene A (**2**). The transient existence of these two enzyme bound intermediates in aristolochene synthases has been inferred from experiments with mechanism-based inhibitors,[6] substrate analogues[7,8]

(1) (a) Hohn, T. M.; Plattner, R. D. *Arch. Biochem. Biophys.* **1989**, *272*, 137–143. (b) Cane, D. E.; Wu, Z.; Proctor, R. H.; Hohn, T. M. *Arch. Biochem. Biophys.* **1993**, *304*, 415–419. (c) Cane, D. E.; Prabhakaran, P. C.; Salaski, E. J.; Harrison, P. H. M.; Noguchi, H.; Rawlings, B. J. *J. Am. Chem. Soc.* **1989**, *111*, 8914–16. (d) Cane, D. E.; Kang, I. *Arch. Biochem. Biophys.* **2000**, *376*, 354–364. (e) Jelen, H. H. *J. Agric. Food Chem.* **2002**, *50*, 6569–6574.

(2) (a) Vogeli, U.; Freeman, J. W.; Chappell *J. Plant Physiol.* **1990**, *93*, 182–187. (b) Rising, K. A.; Starks, C. M.; Noel, J. P.; Chappell, J. *J. Am. Chem. Soc.* **2000**, *122*, 1861–1866.

(3) Christianson, D. W. *Chem. Rev.* **2006**, *106*, 3412–3442.

(4) (a) Whitehead, I. M.; Threlfall, D. R.; Ewing, D. F. *Phytochemistry* **1989**, *28*, 775–779. (b) Whitehead, I. M.; Ewing, D. F.; Threlfall, D. R.; Cane, D. E.; Prabhakaran, P. C. *Phytochemistry* **1990**, *29*, 479–482.

(5) Cane, D. E. In *Comprehensive Natural Products Chemistry*. *Isoprenoids Including Carotenoids and Steroids*; Barton, D., Nakanishi, K., Meth-Cohn, Eds.; Cane, D. E., Vol. Ed.; Elsevier: Oxford, UK, 1999; Vol. 2, pp 155−200.

(6) Cane, D. E.; Bryant, C. *J. Am. Chem. Soc.* **1994**, *116*, 12063–12064.

(7) Cane, D. E.; Tsantrizos, Y. S. *J. Am. Chem. Soc.* **1996**, *118*, 10037–10040.

(8) (a) Miller, D. J.; Yu, F. L.; Allemann, R. K. *ChemBioChem* **2007**, *8*, 1819–1825. (b) Miller, D. J.; Yu, F. L.; Allemann, R. K *Org. Biomol. Chem.* **2009**, *7*, 962–975.

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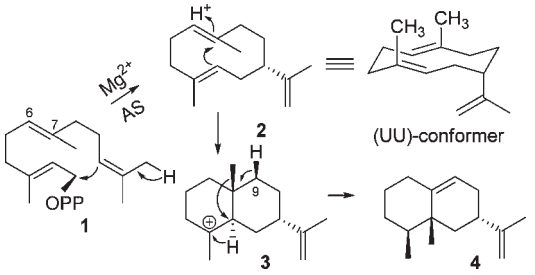
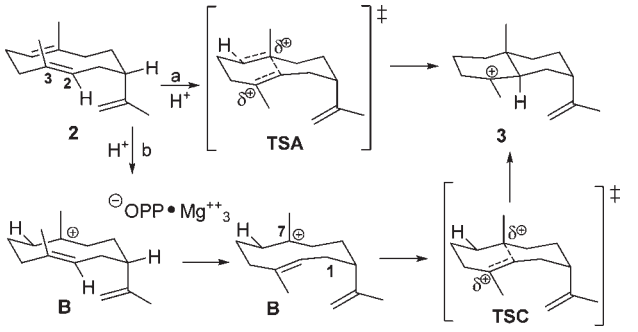
Scheme 1. Biosynthesis of (+)-Aristolochene (**4**)



Scheme 2

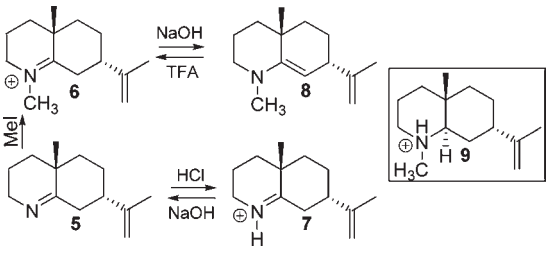


including deuterated forms of FDP (**1**),[1c,9] aristolochene synthase mutants,[10,11] and more recently, with an ammonium ion inhibitor (**9**, Scheme 3) designed to mimic eudesmane cation (**3**).[12a]

One of the key reactions implicated in the biosynthesis of (+)-aristolochene (**4**) is the proton-induced intramolecular cyclization of the flexible hydrocarbon **2** to the highly reactive *trans*-fused bicyclic structure **3** (Schemes 1 and 2). The stereospecificity of this transformation relates to the stable, enzyme-enforced UU-conformation adopted by germacrene A (**2**) in the active site.[13]

Potential mechanisms accounting for the PR-AS-catalyzed π-cyclization of neutral **2** to eudesmane cation (**3**) are outlined in Scheme 2. The concerted pathaway (path a), which goes by way of transition state A (**TSA**), is in agreement with the transannular interactions[14] known to occur between the endocyclic double bonds of *trans*-cyclodeca-

Scheme 3



1,5-dienes (e.g., germacrene A), which likely increase the π-basicity of the C6−C7 double bond of **2**. In addition, experimental and theoretical support for path a stems respectively from the rapid and exclusive formation of *trans*-fused eudesmanes (selinenes) from **2** under acidic biomimetic conditions[15] and recent high level ab initio and DFT calculations, in which no evidence of a discrete protonated form of germacrene A was found.[16] However, studies with fluorinated forms of **1**, namely 2-, 6-, and 14-fluoro-FDP, under PR-AS[8] and TEAS[15a] catalysis seem to suggest the carbocationic path b (Scheme 2). In all cases, the presence of the electron-withdrawing fluorine atom led to the disruption of the catalytic cycle at the germacrene A stage and to the exclusive isolation of fluorinated germacrene As. These latter results could be forwarded in support of either a stepwise process involving a discrete carbocation intermediate (**B**, path b) or a concerted reaction mechamism involving a transition state **TSA** with considerable carbocation character. Following these lines, the possible formation of a stabilized tertiary germacren-7-yl$^+$ cation/diphosphate ion pair[12b] (**B**) represents an attractive mechanistic scenario considering the hypothetical involvement of the synthase-anchored diphosphate group (PP_i) as the general acid that could activate germacrene A toward cyclization.[17] Indeed, based on recent X-ray protein crystallographic studies with PR-AS[18] and AT-AS,[17,19] the transient germacrene-7-yl$^+$ cation **B** would be ideally positioned for ion pairing with the newly generated diphosphate counterion after protonation of **2**.

As a continuation of our ongoing efforts[12] to evaluate aza sesquiterpenes as mimics of presumed carbocations generated during PR-AS catalysis, we reasoned that the sp^2-hybridized iminium ions **6** and **7** (Scheme 3) should resemble a putative transition state **TSC** or its alternative **TSA** (Scheme 2), assuming that the latter has significant carbocationic character. While tetrahedral ammonium

(9) Cane, D. E.; Prabhakaran, P. C.; Oliver, J. S.; Mc Ilwaine, D. B. *J. Am. Chem. Soc.* **1990**, *112*, 3209–3210.

(10) (a) Calvert, M. J.; Ashton, P. R.; Allemann, R. K. *J. Am. Chem. Soc.* **2002**, *124*, 11636–11641. (b) Deligeorgopoulou, A.; Taylor, S. E.; Forcat, S.; Allemann, R. K. *Chem. Commun.* **2003**, 2162–2163. (c) Forcat, S.; Allemann, R. K. *Chem. Commun.* **2004**, 2094–2095.

(11) Felicetti, B.; Cane, D. E. *J. Am. Chem. Soc.* **2004**, *126*, 7212–7221.

(12) (a) Faraldos, J. A.; Kariuki, B.; Allemann, R. K. *J. Org. Chem.* **2010**, *75*, 1119–1125. (b) Faraldos, J. A.; Al-Lami, N.; Allemann R. K. Unpublished results.

(13) Faraldos, J. A.; Wu, S.; Chappell, J.; Coates, R. M. *Tetrahedron* **2007**, *63*, 7733–7742.

(14) Jones, R. V. H.; Sutherland, M. D. *Aust. J. Chem.* **1968**, *21*, 2255–2265.

(15) (a) Faraldos, J. A.; Zhao, Y. X.; O'Maille, P. E.; Noel, J. P.; Coates, R. M. *ChemBioChem* **2007**, *8*, 1826–1833. (b) Adio, A. M.; Paul, C.; Tesso, H.; Kloth, P.; König, W. A. *Tetrahedron: Asymmetry* **2004**, *15*, 1631–1635.

(16) Allemann, R. K.; Young, N. J.; Ma, S.; Truhlar, D. G.; Gao, J. *J. Am. Chem. Soc.* **2007**, *129*, 13008–13013.

(17) Shishova, E. Y.; Di Costanzo, L.; Cane, D. E.; Christianson, D. W. *Biochemistry* **2007**, *46*, 1941–1951.

(18) Caruthers, J. M.; Kang, I.; Rynkiewicz, M. J.; Cane, D. E.; Christianson, D. W. *J. Biol. Chem.* **2000**, *275*, 25533–25539.

(19) Shishova, E. Y.; Yu, F. L.; Miller, D. J.; Faraldos, J. A.; Zhao, Y. X.; Coates, R. M.; Allemann, R. K.; Cane, D. E.; Christianson, D. W. *J. Biol. Chem.* **2008**, *283*, 15431–15439.

analogues of presumed carbocations have been used previously in studies of terpene[12a,20] and sterol[21] biosynthesis, and to a lesser extent in protein crystallographic efforts,[22] to the best of our knowledge the present study constitutes the first example of trigonal nitrogen-containing species used as mechanistic probes in terpene biosynthesis.[23]

Compounds **6** and **7** were easily prepared from imine **5** (Scheme 3), which was available from a previous study.[12a] The iminium salt **6** was obtained as needles from **5** and methyl iodide in 85% yield,[24] while the hydrochloride **7** was quantitatively collected upon exposure of **5** to anhydrous ethereal solutions of hydrogen chloride.

Control experiments demostrate that **6** and **7** were stable in aqueous solutions, despite reports documenting the short lifetime of simple aliphatic iminium ions in water.[25] In fact, iminiun ions **6** and **7** were stable at pD 7 for more than 7 days as judged by ^{1}H NMR measurements (D_2O), in which no hydration or solvolysis products were observed (for details see the Supporting Information). Interestingly, at pH 6.6 (D_2O), no traces of free imine **5** (or the corresponding enamine) were detected in the ^{1}H NMR spectra of **7**, indicating that at pD = 7 compound **7** exists almost exclusively as the protonated salt.[26] On the basis of these observations, **6** and **7** were considered sufficiently stable for evaluation of their inhibitory properties toward PR-AS. As expected, exposure of **6** and **7** to 5% aqueous NaOH afforded the corresponding tertiary enamine **8** and the free imine **5**, respectively, which upon acid treatment (10% aqueous HCl or TFA) reverted to **6** and **7** as the corresponding hydrochlorides or TFA salts.[27]

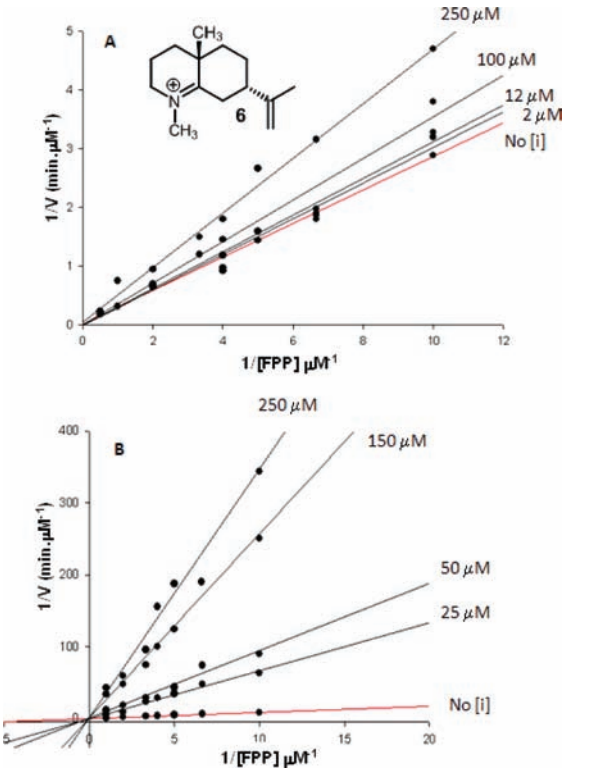


Figure 1. Competitive inhibition of PR-AS with increasing concentrations of iminium ion **6** in (A) the absence and (B) the presence of exogenous PPi (250 μM).

Kinetic evaluation of **6** and **7** with recombinant PR-AS showed that these iminium salts interacted with the synthase rather weakly compared to the tight binding of the natural substrate **1** (K_M = 0.53 ± 0.21 μM).[12a] Although the inhibition studies presented herein clearly indicate that compound **7** acts as a modest competitive inhibitor of PR-AS with a K_i = 28.4 ± 3 μM (see the Supporting Information), the abilities of iminium ion **6** to bind to the enzyme were more difficult to determine due to its elevated inhibition constant (K_i = 219 ± 17 μM, Figure 1, panel A).[28] Intrigued by this unforeseen result and by the possibility that iminium salt **6** could instead act as an alternative nitrogen-containing substrate of PR-AS, a preparative scale incubation with PR-AS was carried out, as described recently.[29] However, GC-MS and ^{1}H NMR (500 MHz) experiments with the crude extracts indicated that compound **6** was not a substrate, despite the inherent

(20) (a) Sandifer, R. M.; Thompson, M. D.; Gaughan, R. G.; Poulter, C. D. *J. Am. Chem. Soc.* **1982**, *104*, 7376–7378. (b) Poulter, C. D. *Acc. Chem. Res.* **1990**, *23*, 70–77. (c) Steiger, A.; Pyun, H. J.; Coates, R. M. *J. Org. Chem.* **1992**, *57*, 3444–3449. (d) McGeady, P.; Pyun, H. J.; Coates, R. M.; Croteau, R. *Arch. Biochem. Biophys.* **1992**, *299*, 63–72. (e) Cane, D. E.; Yang, G. H.; Coates, R. M.; Pyun, H. J.; Hohn, T. M. *J. Org. Chem.* **1992**, *57*, 3454–3462. (f) Koohang, A.; Coates, R. M.; Owen, D.; Poulter, C. D. *J. Org. Chem.* **1999**, *64*, 6–7. (g) Peters, R. J.; Ravn, M. M.; Coates, R. M.; Croteau, R. B. *J. Am. Chem. Soc.* **2001**, *123*, 8974–8978. (h) Ravn, M. M.; Peters, R. J.; Coates, R. M.; Croteau, R. *J. Am. Chem. Soc.* **2002**, *124*, 6998–7006. (i) Roy, A.; Roberts, F. G.; Wilderman, P. R.; Zhou, K.; Peters, R. J.; Coates, R. M. *J. Am. Chem. Soc.* **2007**, *129*, 12453–12460. (j) Mann, F. M.; Prisic, S.; Hu, H.; Xu, M.; Coates, R. M.; Peters, R. J. *J. Chem. Biol.* **2009**, *284*, 23574–23579.

(21) For leading references see: (a) Rahier, A.; Taton, M.; Benveniste, P. In *Inibition of Sterol Biosynthesis in Higher Plants by Analogues of High Energy Carbocationic Intermediates*; Kuhn, P. J. et al. , Eds.; Springer-Verlag: New York, 1990; pp 205−222.3. (b) Abe, I.; Rohmer, M.; Prestwich, G. D. *Chem. Rev.* **1993**, *93*, 2189–2206. (c) Abe, I.; Tomesch, J. C.; Wattanasin, S.; Prestwich, G. D. *Nat. Prod. Rep.* **1994**, *11*, 279–302.

(22) (a) Whittington, D. A.; Wise, M. L.; Urbansky, M.; Coates, R. M.; Croteau, R. B.; Christianson, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15375–15380. (b) Reinert, D. J.; Balliano, G.; Schulz, G. E. *Chem. Biol.* **2004**, *11*, 121–126. (c) Vedula, L. S.; Rynkiewicz, M. J.; Pyun, H.-J.; Coates, R. M.; Cane, D. E.; Christianson, D. W. *Biochemistry* **2005**, *44*, 6153–6163. (d) Koksal, M.; Jin, Y.; Coates, R. M.; Croteau, R.; Chrisianson, D. W. *Nature* **2011**, *469*, 116–120.

(23) For the use of lactams (as dipolar canonical analogous forms of anion-like transition states) in oxidosqualene biosynthesis studies see: (a) Duriatti, A.; Bouvier-Nave, P.; Benveniste, P.; Schuber, F.; Delprimo, L.; Balliano, G.; Cattel, L. *Biochem. Pharmacol.* **1985**, *34*, 2765–2777. (b) Kuhl, K. K.; Anzalone, L.; Arguropoulos, E. D.; Gayen, A. K.; Spencer, T. A. *Bioorg. Chem.* **1989**, *17*, 108–120.

(24) Moynehan, T. M.; Schofield, K.; Jones, R. A. Y.; Katritzky, A. R. *J. Chem. Soc.* **1962**, 2637–2658.

(25) (a) Eldin, S.; Digits, J. A.; Huang, S.-T.; Jencks, W. P. *J. Am. Chem. Soc.* **1995**, *117*, 6631–6632. (b) Eldin, S.; Jencks, W. P. *J. Am. Chem. Soc.* **1995**, *117*, 4851–4857.

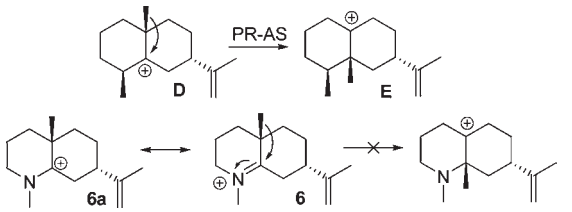
(26) Literature searches with SciFinder turned up a calculated pK_a = 8.06 ± 0.40 for the parent 4a-methyl-2,3,4,4a,5,6,7,8-octahydroquinoline. Clearly the actual pK_a of **7** must be > 8.4

(27) (a) Leonard, N. J.; Steinhardt, C. K.; Lee, C. *J. Org. Chem.* **1962**, *27*, 4027–4031. (b) Leonard, N. J.; Miller, L. A.; Thomas, P. D. *J. Am. Chem. Soc.* **1956**, *78*, 3463–3468. (c) McKenna, J.; Tuelly, A. *J Chem. Soc.* **1960**, 945–952. (d) Walker, G. N.; Alkalay, D. *J. Org. Chem.* **1967**, *32*, 2213–2225.

(28) The K_i value refers to competitive inhibition.

(29) Faraldos, J. A.; O'Maille, P. E.; Dellas, N.; Noel, J. P.; Coates, R. M. *J. Am. Chem. Soc.* **2010**, *132*, 4281–4289.

Scheme 4



electrophilicity of the iminium double bond[30] that could potentially drive a 1,2-Me-shift similar to the one observed with the natural substrate (**D** → **E**, Scheme 4).[31]

Alternatively, the reduced binding affinity [$K_M^{FPP}/K_i^{6} = 2.5 \times 10^{-3}$] measured for the iminium salt **6** might reflect imperfections in its design, given that the Mannich intermediate **6**[30] could also be depicted as the canonical α-amino carbocation **6a** (Scheme 4). While this alteration in the charge configuration of **6** seems unlikely (vide supra), it would place a positive charge on a carbon atom (C2 in the farnesyl chain) that PR-AS does not confront during the native **2** → **3** cyclization. This obervation could potentially explain the weak interaction of **6** (and **7**) with PR-AS.

On the presumption that a more tightly bound active site iminium/diphosphate ion pair could form as a consequence of adding inorganic diphosphate to the reaction media, an additional set of experiments was carried out in the presence of exogenous PP_i (mimicking the competent $[Mg^{2+}]_3$-PP_i enzyme complex).[17,19] In agreement with previous studies with terpene synthases,[20a,d,e,g−i,22,32] the addition of PP_i (250 μM)[33] led to a considerably stronger enzymatic interaction for both inhibitors, with approximately 40- ($K_i = 5.9 \pm 1.2$ μM, Figure 1, Panel B) and 70-fold ($K_i = 0.41 \pm 0.19$ μM, see the Supporting Information) decreases in the inhibition constants of **6** and **7**, respectively.

These results are consistent with the binding and ion pairing of the methylated (**6**) or protonated (**7**) forms of imine **5** at the PR-AS active site as a consequence of favorable electrostatic (**6** and **7**) and/or hydrogen bonding interactions (**7**) between the positively charged iminium nitrogen and an active site bound diphosphate.[22,34] In agreement with our previous NMR experiments, the significant potency enhancement observed for iminium ion **7** in the presence of diphosphate ions seems to rule out the free Schiff base **5** as the reactive species that binds to PR-AS. Moreover, under these experimental conditions, hydrochloride **7** acts as a potent competitive inhibitor of PR-AS, with a binding affinity constant ($K_i = 0.41 \pm 0.19$ μM) almost identical to the Michaelis constant ($K_M^{FDP} = 0.53 \pm 0.21$ μM).[12a]

The approximately 50-fold decrease in K_i for **6** and **7** by the presence of 250 μM diphosphate suggests an active role for the enzyme-bound diphosphate ion on the transition state aggregate/composite, likely as a stabilizing agent for the energetically demanding **2** → **3** cyclization. Thus, these observations toghether with the remarkable insensitivity of the ammonium ion analogue **9** (Scheme 3) of carbocation **3** toward the presence of diphosphate[12a] support an involvement of the diphosphate ion in the protonation step of germacrene A,[17] at the very least by stabilizing a discrete cationic intermediate **B**, or cationic transition states such as **TSA** or **TSC** (Scheme 2) previous to eudesmane cation formation. In addition, the 25-fold tighter binding of ammonium salt **9** ($K_i = 0.24 \pm 0.1$ μM)[12a] to the catalytic site of PR-AS relative to **6** ($K_i = 5.9 \pm 2.2$ μM) could signify that the true transition state involved in the acid-assisted cyclization of **2** to cation **3** is "more product-like" and hence better mimicked by the trans-fused aza-decalin **9** (Scheme 3). This proposal of a product-like transition state to explain the PR-AS-catalyzed formation of eudesmane cation (**3**) will likely satisfy the requirements of a concerted endothermic process (**2** → **3** via **TSA**, path a, Scheme 2). However, it does not fully exclude a partially exothermic stepwise mechanism[35] (**B** → **3** via **TSC**, path b), assuming that the stabilities of the transient germacrene-7-yl$^+$ (**B**) and eudesmene (**3**) cations are otherwise comparable.[36]

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Supporting Information Available. Materials, general methods, and instrumentation; protein production; kinetic plots; and reproduction of NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

(30) (a) Bohme, H.; Vieche, H. G., Eds. *Iminium Salts in Organic Chemistry*, Parts 1 and 2; Willey: New York, 1979. (b) Royer, J.; Bonin, M.; Micouin, L. *Chem. Rev.* **2004**, *104*, 2311–2352. (c) Mayr, H.; Kempf, B.; Ofial, A. R. *Acc. Chem. Res.* **2003**, *36*, 66–77. (d) Arend, M.; Westermann, B.; Risch, N. *Angew. Chem., Int. Ed.* **1998**, *37*, 1044–1070. (e) D'Oca, M. G. M.; Moraes, L. A. B.; Pilli, R. A.; Eberlin, M. N. *J. Org. Chem.* **2001**, *66*, 3854–3864. (f) Mayr, H.; Ofial, A. R. *Tetrahedron Lett.* **1997**, *38*, 3503–3506.

(31) The only Et_2O-extractable product detected by GC-MS and ^1H NMR analyses after basic (5% aqueous NaOH) workup of the enzymatic reaction was enamine **8**. The same product **(8)** was observed in control experiments carried out in the absence of enzyme.

(32) (a) Koohang, A.; Bailey, J. L.; Coates, R M.; Erickson, H. K.; Owen, D.; Poulter, C. D. *J. Org. Chem.* **2010**, *75*, 4769–4777. (b) Poulter, C. D.; Capson, T. L.; Thompson, M. D.; Bard, R. S. *J. Am. Chem. Soc.* **1989**, *111*, 3734–3739. (c) Croteau, R.; Wheeler, C. J.; Aksela, R.; Oehlschlager, A. C. *J. Biol. Chem.* **1986**, *261*, 7257–7263.

(33) It has been shown previously that diphosphate itself does not inhibit the native enzyme from *P. roqueforti*. See ref 1a.

(34) Christianson, D. W. *Curr. Opin. Chem. Biol.* **2008**, *12*, 141–150.

(35) The coversion of a C=C double bond into two C−C single bonds as a consequence of a Markovnikov carbocationic cyclization (e.g., **B** → **3**) is generally exothermic by 20 kcal/mol.

(36) Chappell, J.; Coates, R. M. In *Comprehensive Natural Products II: Chemistry and Biology*; Mander, L., Liu, H.-W., Eds.; Elsevier: Amsterdam, The Netherlands, 2010; Vol. 1, Chapter 16, p 616.