Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 105–107

BIOORGANIC &
MEDICINAL
CHEMISTRY
LETTERS

Epicubenol Synthase. Origin of the Oxygen Atom of a Bacterial Sesquiterpene Alcohol

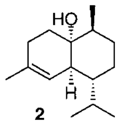
David E. Cane* and Ning Ke

Department of Chemistry, Box H, Brown University, Providence, RI, 02912-9108, USA

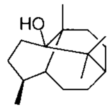
Received 14 October 1999; accepted 8 November 1999

Abstract—Incubation of epicubenol synthase with farnesyl pyrophosphate in the presence of 11.1 atom% $H_2{}^{18}O$ gave epicubenol (**2**) in which the hydroxyl oxygen atom was shown to be derived exclusively from water, as established by GC-selected ion monitoring MS of the derived TMS-epicubenol derivative (**15**). © 2000 Elsevier Science Ltd. All rights reserved.

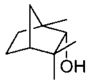
Among the several hundred known cyclic sesquiterpenes and monoterpenes that are formed directly by the enzymatic cyclization of farnesyl diphosphate (**1**) or geranyl diphosphate, the overwhelming majority are hydrocarbons, resulting from deprotonation of a penultimate carbocation intermediate. Although less common, there also numerous examples of cyclic sesquiterpene and monoterpene alcohols whose formation most likely involves the nucleophilic capture of carbocation intermediates by water. Among the terpenoid alcohols, the small number whose formation has been studied at the enzyme level include the sesquiterpenes epicubenol (**2**)[1–3] and patchoulol (**3**),[4,5] the formation of both of which is accompanied by the co-generation of 1 or more sesquiterpene hydrocarbons, and the monoterpene fenchol (**4**).[6,7] In the latter case, the hydroxyl oxygen atom has been explicitly shown to be derived from water.[7] By contrast, the monoterpene alcohols (+)- and (−)-borneol (**5**) are secondary products derived by phosphohydrolase-catalyzed hydrolysis of the corresponding monoterpene synthase cyclization products (+)- and (−)-bornyl diphosphate (**6**),[8,9] respectively, each of which results from recapture of the original ester oxygen atom of the pyrophosphate moiety of the geranyl diphosphate substrate.[10]



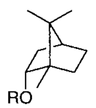
2



3



4



(+)-**5** R=H
6 R=PP

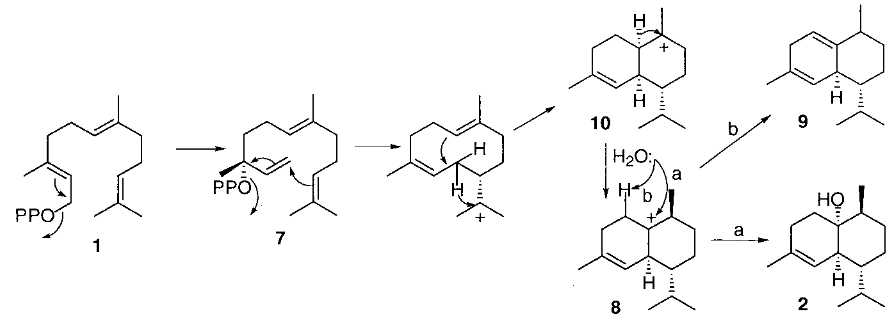
(+)-Epicubenol (**2**), which was first isolated by Gerber from the mycelial extracts of several different *Streptomyces* species,[11] is also produced by the liverwort *Heteroscyphus planus*,[12] while (−)-epicubenol is found in the extracts of various higher plants.[13–15] We have previously reported that a crude enzyme preparation from *Streptomyces* sp. LL-B7 can support the cyclization of farnesyl diphosphate to epicubenol and have presented evidence for a cyclization mechanism initiated by isomerization of farnesyl diphosphate to (3R)-nerolidyl diphosphate (**7**) which then undergoes conversion to an intermediate germacradienyl cation by way of a 1,3-hydride shift.[1–3] A second cyclization followed by a 1,2-hydride shift gives the bicyclic cation **8** which has been proposed to be quenched by capture of water (Scheme 1, pathway a). Independently, Nabeta et al. have purified the corresponding epicubenol synthase from *H. planus* more than 200-fold and have reported analogous mechanistic experiments which also demonstrate the observed 1,2- and 1,3-hydride shifts.[16,17] These researchers also noted the formation of the closely related hydrocarbon cubenene (**9**) in these same incubations. Based on the apparent co-purification of the cubenene and epicubenol synthase activities, they concluded that both products might be formed by the same enzyme, with the ratio of hydrocarbon **9** to alcohol **2** resulting from competition between deprotonation of the common cationic intermediate **8** (Scheme 1, pathway b) and capture of water (Scheme 1, pathway a).

While the direct formation of epicubenol by addition of water to **8** appears to be by far the most reasonable mechanistic possibility, one cannot exclude a priori an alternative pathway involving recapture of the pyrophosphate moiety to give epicubenyl diphosphate (**11**)

---

*Corresponding author. Tel.: +1-401-863-3588; fax: +1-401-863-3556; e-mail: david_cane@brown.edu

0960-894X/00/$ - see front matter © 2000 Elsevier Science Ltd. All rights reserved.
PII: S0960-894X(99)00650-2



Scheme 1.

which would then undergo hydrolytic cleavage, most probably by a separate phosphohydrolase, to give epicubenol (**2**) (Scheme 2). Such a recapture of the pyrophosphate ion, reminiscent of the analogous formation of bornyl diphosphate (**6**) from geranyl diphosphate,[8–10] appears to be geometrically feasible and cannot be ruled out, if only because the epicubenol preparations that have been used for these reactions are not as yet homogeneous. The two mechanistic alternatives can be readily and unambiguously distinguished, however, by determining the origin of the hydroxyl oxygen atom of epicubenol.

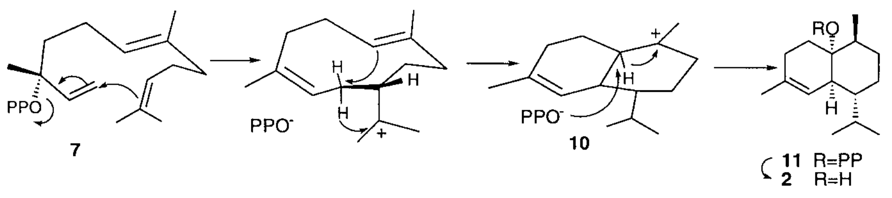
To address this question, partially purified epicubenol synthase[18] (8 mg, spec. act. 0.063 nmol/mg protein•h) was incubated for 12 h at 30 °C with 50 μM farnesyl diphosphate in 8.0 mL of 15 mM Tris buffer, pH 7.8, containing 40 mM $MgCl_2$ and $H_2{}^{18}O$ (11.1 atom%). GC–MS analysis of the concentrated pentane extract confirmed the formation of epicubenol (**2**), along with an apparent sesquiterpene hydrocarbon, *m*/*z* 204, presumed to be cubenene (**9**). On the other hand, as had been previously observed,[3] neither EI nor CI ionization of epicubenol gave a detectable molecular ion, due to the facile dehydration of the tertiary alcohol under the ionization conditions. While this absence of a parent peak had not interfered with earlier experiments involving labeling at alternative sites in epicubenol, analysis of the ^{18}O content of the enzymatically generated epicubenol clearly required that the hydroxyl oxygen atom not be lost during the mass spectrometric analysis. Accordingly, the epicubenol sample was treated with trimethylsilylimidazole[19] and the resulting purified epicubenol TMS ether (**12**) was analyzed by GC–MS and selected ion monitoring of the prominent M–CH_3 (*m*/*z* 279 and 281) and M–C_3H_7 (*m*/*z* 251 and 253) peaks (Scheme 3). As summarized in Table 1, the relative abundances within the *m*/*z* 279:289 and 251:253 pairs indicated an ^{18}O enrichment of 11 atom% for TMS-epicubenol (**12**), completely consistent with the exclusive derivation of the hydroxyl oxygen atom of epicubenol (**2**) from water, as predicted by Scheme 1.

It is interesting to speculate that this same water molecule might also serve as the Lewis base in the competing deprotonation reaction that would lead to the formation

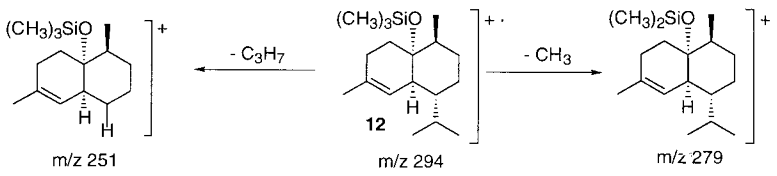
Table 1. Selected ion mass spectrometric analysis of TMS-epicubenol (**12**)

m/*z*	Relative peak intensity ($H_2{}^{16}O$)		Relative peak intensity ($H_2{}^{18}O$)	
	Calculated %[a]	Observed %	Calculated %[b]	Observed %
251	94.6	94.3	84.4	83.2
253	5.4	5.7	15.6	16.8
279	94.2	94.1	84.2	84.3
281	5.8	5.9	15.8	15.7

[a] Calculated from the natural abundance of ^{13}C, ^{29}Si, and ^{30}Si.
[b] Calculated from the experimentally determined natural percentage of M + 2 species and 11.1% ^{18}O content of H_2O.



Scheme 2.



Scheme 3.

of cubenene, especially since the water must be located on the same face of cation **10** as the hydrogen that either undergoes a 1,2-hydride migration to give **8** or is removed as a proton. Indeed, based on crystallographic evidence, a similar role has been proposed for a water molecule in the termination of the cyclization cascade catalyzed by squalene:hopene cyclase that results in the competitive formation of the triterpenes hopene and diplopterol.[20] Further investigations aimed at elucidating the factors controlling the role of the water in epicubenol biosynthesis are in progress.

Acknowledgement

This work was supported by NIH Merit Award grant GM30301.

References and Notes

1. Cane, D. E.; Tandon, M.; Prabhakaran, P. C. *J. Am. Chem. Soc.* **1993**, *115*, 8103–8106.
2. Cane, D. E.; Tandon, M. *Tetrahedron Lett.* **1994**, *35*, 5355–5358.
3. Cane, D. E.; Tandon, M. *J. Am. Chem. Soc.* **1995**, *117*, 5602–5603.
4. Croteau, R.; Munck, S. L.; Akoh; Casimir C.; Fisk, H. J.; Satterwhite, D. M. *Arch. Biochem. Biophys.* **1987**, *256*, 56–68.
5. Munck, S. L.; Croteau, R. *Arch. Biochem. Biophys.* **1990**, *282*, 58–64.
6. Croteau, R.; Satterwhite, D. M.; Wheeler, C. J.; Felton, N. M. *J. Biol. Chem.* **1988**, *263*, 15449–15453.
7. Croteau, R.; Shaskus, J.; Cane, D. E.; Saito, A.; Chang, C. *J. Am. Chem. Soc.* **1984**, *106*, 1142–1143.
8. Wise, M. L.; Savage, T. J.; Katahira, E.; Croteau, R. J. *Biol. Chem.* **1998**, *273*, 14891–14899.
9. Croteau, R.; Felton, N. M.; Wheeler, C. J. *J. Biol. Chem.* **1985**, *260*, 5956–5962.
10. Cane, D. E.; Saito, A.; Croteau, R.; Shaskus, J.; Felton, M. *J. Am. Chem. Soc.* **1982**, *104*, 5831–5833.
11. Gerber, N. *Phytochemistry* **1971**, *10*, 185–189.
12. Nabeta, K.; Katayama, K.; Nakagawara, S.; Katoh, K. *Phytochemistry* **1993**, *32*, 117–122.
13. Ohta, Y.; Hirose, Y. *Tetrahedron Lett.* **1967**, 2073–2075.
14. Tomita, B.; Hirose, Y. *Phytochemistry* **1972**, *11*, 3355–3357.
15. Talvite, A.; Borg-Karlson, A.-K. *Finn. Chem. Lett.* **1979**, 93–96.
16. Nabeta, K.; Fujita, M.; Komuro, K.; Katayama, K.; Takasawa, T. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2065–2070.
17. Nabeta, K.; Kigure, K.; Fujita, M.; Nagoya, T.; Ishikawa, T.; Okuyama, H.; Takesawa, T. *J. Chem. Soc., Perkin Trans. 1* **1995**, 1935–1939.
18. The cell-free extract containing epicubenol synthase was obtained from 1.2 l of a 48-h culture of *Streptomyces* sp. LL-B7 essentially as previously described.[1–3] The protein pellet obtained by 70% ammonium sulfate precipitation was desalted on Sephadex G-25, then applied to a Q-Sepharose anion exchange column and eluted with a linear gradient of 0–500 mM NaCl. The epicubenol synthase activity, assayed with [1-^{3}H]farnesyl diphosphate (69 μCi/μmol), as previously described,[1–3] eluted between 300 and 350 mM NaCl.
19. The enzymatically generated epicubenol was treated with 150 μL trimethylsilylimidazole for 1 h. After quenching with water (1 mL) and extraction with HPLC grade pentane, the pentane extract was purified by passage and through a silica gel column which was eluted with pentane before analysis by GC–MS.
20. Wendt, K. U.; Poralla, K.; Schulz, G. E. *Science* **1997**, *277*, 1811–1815.