# Epicubenol Synthase and the Enzymatic Cyclization of Farnesyl Diphosphate

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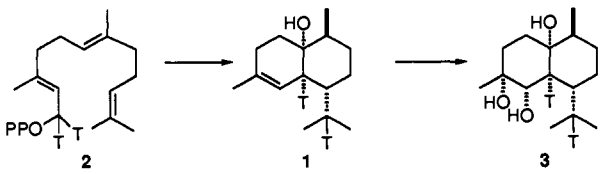
**Abstract:** Incubation of [1-$^3$H]farnesyl diphosphate (**2**) with a cell-free extract obtained from *Streptomyces* sp. LL-B7 gave tritiated epicubenol (**1**), as confirmed by recrystallization of the derived triol **3** to constant activity. Cyclization of [13,13,13-$^2H_3$]farnesyl diphosphate (**2a**) with crude epicubenol synthase gave [13,13,13-$^2H_3$]epicubenol (**1a**), as established by $^2$H NMR analysis. The existence of a 1,3-hydride shift was demonstrated by conversion of [1,1-$^2H_2$]-farnesyl diphosphate (**2b**) to epicubenol (**1b**) which was shown by $^2$H NMR to be labeled with deuterium at C-5 and C-11. These results can be explained by a proposed cyclization mechanism involving the intermediacy of nerolidyl diphosphate (**4**).

The vast majority of the thousands of known sesquiterpenes, comprising some 200 individual cyclic carbon skeletons, have been isolated from eukaryotic organisms, including both marine and terrestrial plants and a wide variety of fungi.[1] By contrast, sesquiterpenoid metabolites are extremely rare among prokaryotes which frequently lack any cyclic higher terpenoids such as cholesterol.[2] An exception to this rule is the formation of the triquinane sesquiterpene pentalenene,[3] the hydrocarbon precursor of the pentalenolactone family of antibiotics,[4] which have been isolated from the culture broths of several known *Streptomycetes*.[5] Some 20 years ago, as part of an extensive search for terpenoid metabolites produced by *Streptomyces*, Gerber reported the isolation from *Streptomyces* sp. LL-B7, sp. LL-B5a, and sp. LL-100-1 (Eren) of the cadinene-type sesquiterpene alcohol (+)-epicubenol (**1**),[6] which was shown by NMR, IR, GC, and polarimetric comparison to be the enantiomer of (–)-epicubenol previously isolated from cubeb oil[7a] and a variety of other plant sources.[7b,c] We now report the isolation from *Streptomyces* sp. LL-B7 of the sesquiterpene cyclase epicubenol synthase, which catalyzes the cyclization of farnesyl diphosphate (FPP, **2**) to **1**, and describe experiments which shed light on the mechanism of this cyclization.

**Scheme I**




## Results

A cell-free extract of *Streptomyces* sp. LL-B7 was prepared from the mycelium obtained from 600 mL of a 42-h fermentation culture by grinding with glass beads. After centrifugation of the broken-cell suspension, the resulting supernatant (240 μg of protein/mL) was used directly for enzymatic cyclizations. The crude cell-free extract (20 mL) was incubated for 120 min at 30 °C in a glass tube with [1-$^3$H]FPP (**2**) (10 μM, 2.9 × 10$^6$ dpm, 72 mCi/mmol) in the presence of 20 mM $MgCl_2$. The reaction was halted by addition of 10 mL of 1 M $Na_2EDTA$, and the mixture was extracted with ether. After addition of 33 mg (0.15 mmol) of (±)-epicubenol as carrier,[8] the organic extract was concentrated and directly treated with $OsO_4$ (Scheme I). The resulting triol **3**[6,9] was readily separated by flash column chromatography from the corresponding heptaols derived from farnesol and nerolidol, which had been generated by competing enzymatic (phosphatase) and $Mg^{+2}$-catalyzed hydrolysis[10] of the substrate FPP. The purified triol **3** was recrystallized to constant mp and specific radioactivity, thereby confirming the identity of

(1) *Encyclopedia of Terpenoids*; Glasby, J. S., Ed.; Wiley: Chichester, England, 1982.

(2) Several examples have been reported of triterpenoids in prokaryotes, e.g. tetrahymanol from *Rhodopseudomonas palustris*: Kleemann, G.; Poralla, K.; Englert, G.; Kjoesen, H.; Liaaen-Jensen, S.; Neunlist, S.; Rohmer, M. *J. Gen. Microbiol.* **1990**, *136*, 2551. The squalene:tetrahymanol cyclase from *Tetrahymena thermophila* has been purified: Saar, J.; Kader, J. C.; Poralla, K.; Ourisson, G. *Biochim. Biophys. Acta* **1991**, *1075*, 93. The squalene: hopene cyclase of *Bacillus acidocaldarius* has been purified, cloned, and expressed: Seckler, B.; Poralla, K. *Biochim. Biophys. Acta* **1986**, *881*, 356. Ochs, D.; Tappe, C. H.; Gaertner, P.; Kellner, R.; Poralla, K. *Eur. J. Biochem.* **1990**, *194*, 75. Ochs, D.; Kaletta, C.; Entian, K. D.; Becksickinger, A.; Poralla, K. *J. Bacteriol.* **1992**, *174*, 298. For a review of polyterpenoid sterol surrogates in prokaryotes, see: Ourisson, G.; Rohmer, M.; Poralla, K. *Annu. Rev. Microbiol.* **1987**, *41*, 301.

(3) Seto, H.; Yonehara, H. *J. Antibiot.* **1980**, *33*, 92.

(4) Cane, D. E.; Tillman, A. M. *J. Am. Chem. Soc.* **1983**, *105*, 122.

(5) (a) Pentalenolactone: English, A. R.; McBride, T. J.; Lynch, J. E. *Antibiot. Annu.* **1957**, 682. Koe, B. K.; Sobin, B. A.; Celmer, W. D. *Antibiot. Annu.* **1957**, 672. Martin, D. G.; Slomp, G.; Mizsak, S.; Duchamp, D. J.; Chidester, C. G. *Tetrahedron Lett.* **1970**, 4901. Takeuchi, S.; Ogawa, Y.; Yonehara, H. *Tetrahedron Lett.* **1969**, 2737. Keller-Schierlein, W.; Lemke, J.; Nyfeler, R.; Zähner, H. *Arch. Mikrobiol.* **1972**, *84*, 301. Nakagawa, A.; Tomoda, H.; Hao, M. V.; Okano, K.; Iwai, Y.; Omura, S. *J. Antibiot.* **1985**, *38*, 111. (b) Pentalenolactone H and pentalenic acid: Seto, H.; Sasaki, T.; Uzawa, J.; Takeuchi, S.; Yonehara, H. *Tetrahedron Lett.* **1978**, 4411. (c) Pentalenolactones H and epi-F: Seto, H.; Sasaki, T.; Yonehara, H.; Uzawa, J. *Tetrahedron Lett.* **1978**, 923. Tillman, A. M.; Cane, D. E. *J. Antibiot.* **1983**, *36*, 170. Seto, H.; Noguchi, H.; Sankawa, U.; Iitaka, Y. *J. Antibiot.* **1984**, *37*, 816. Williard, P. G.; Sohng, J.; Cane, D. E. *J. Antibiot.* **1988**, *41*, 130. (d) Pentalenolactones A, B, D, and F: Cane, D. E.; Sohng, J. K.; Williard, P. G. *J. Org. Chem.* **1992**, *57*, 844. (e) Pentalenolactones O and P: Seto, H.; Sasaki, T.; Yonehara, H.; Takahashi, S.; Takeuchi, M.; Kuwano, H.; Arai, M. *J. Antibiot.* **1984**, *37*, 1076. (f) Deoxypentalenylglucurone: Takahashi, S.; Takeuchi, M.; Arai, M.; Seto, H.; Otake, N. *J. Antibiot.* **1983**, *36*, 226.

(6) Gerber, N. N. *Phytochemistry* **1971**, *10*, 185. Although epicubenol (cadin-4-en-1-ol) was properly identified, the stereochemistry of **1** was incorrectly depicted as that of the isomeric cubenol.

(7) (a) Ohta, Y.; Hirose, Y. *Tetrahedron Lett.* **1967**, 2073. (b) Tomita, B.; Hirose, Y. *Phytochemistry* **1972**, *11*, 3355 and refs 1–6 therein. (c) Talvitie, A.; Borg-Karlson, A.-K. *Finn. Chem. Lett.* **1979**, 93.

(8) Synthetic (±)-epicubenol was prepared using an intramolecular Diels–Alder approach based on that developed by Taber (Taber, D. F.; Gunn, B. P. *J. Am. Chem. Soc.* **1979**, *101*, 3992) for the synthesis of (±)-torreyol and will be reported separately. The synthetic material was identical in spectroscopic properties and chromatographic behavior with an authentic sample obtained from mycelial extracts of *Streptomyces* sp. LL-B7. These results will be reported separately.

(9) The structure and stereochemistry of **3** was unambiguously determined by X-ray crystallography.

(10) Chayet, L.; Rojas, M. C.; Cori, O.; Bunton, C. A.; McKenzie, D. C. *Bioorg. Chem.* **1984**, *12*, 329.

0002-7863/93/1515-8103$04.00/0 

Table I. $^1H$ and $^{13}C$ NMR Assignments ($C_6D_6$) for Epicubenol (1)[a]

| H | δ | C | ppm (m) | H | δ | C | ppm (m) |
|---|---|---|---|---|---|---|---|
| 1 | 1.55 | 1 | 22.42 (t) | 8a | 1.49 | 8 | 31.42 (t) |
| 2a | 1.8 | 2 | 27.06 (t) | 8b | 0.98 | | |
| 2b | 2.05 | | | 9 | 1.62[b] | 9 | 42.46 (d) |
| | | 3 | 133.90 (s) | | | 10 | 72.13 (s) |
| 4 | 5.37 | 4 | 122.57 (d) | 11 | 1.95[b] | 11 | 27.22 (d) |
| 5 | 1.66[b] | 5 | 48.72 (d) | 12 | 0.82 | 12 | 21.80 (q) |
| 6 | 1.12[b] | 6 | 49.63 (d) | 13 | 0.77 | 13 | 15.42 (q) |
| 7a | 0.98 | 7 | 24.48 (t) | 14 | 1.05 | 14 | 15.53 (q) |
| 7b | 1.44 | | | 15 | 1.55 | 15 | 23.55 (q) |

[a] $^1H$ NMR, 400 MHz; $^{13}C$ NMR, 100 MHz. [b] Chemical shifts in $CDCl_3$: H-5, δ 1.68; H-6, δ 1.13; H-9, δ 1.58; H-11, δ 1.94.

the enzymatic cyclization product as epicubenol (**1**). No detectable epicubenol was produced in incubations using boiled controls.

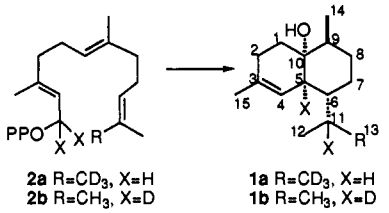
Further exploration of the mechanism of the cyclization reaction using deuterated substrates and $^2H$ NMR analysis required unambiguous assignment of the corresponding $^1H$ NMR resonances of epicubenol.[11] The full $^{13}C$ and $^1H$ NMR assignments obtained on synthetic (±)-epicubenol by a combination of 1D and 2D NMR methods, including $^1H$–$^1H$ COSY, $^1H$–$^{13}C$ HETCOSY, $^1H$–$^{13}C$ long-range HETCOSY, and COLOC NMR, are summarized in Table I. Of particular interest were the assignments of the resonances for the three secondary methyl groups and those of the four methines. Thus H-14 (δ 1.05), which was correlated with C-14 (15.53 ppm), also showed long-range correlations with the signals assigned to C-8, C-9, and C-10. Similarly, the methyl resonances for the isopropyl side chain, H-12 (δ 0.82) and H-13 (δ 0.77), were directly correlated with C-12 (21.80 ppm) and C-13 (15.42 ppm), respectively, and each showed long-range correlations to the corresponding geminal methyl $^{13}C$ resonances. In the COLOC spectrum, C-6 (49.63 ppm) was correlated with both H-12 and H-13. At present, the individual diastereotopic methyl protons have not yet been assigned. H-6 (δ 1.12) was directly correlated with C-6, while the methine carbon C-11 (27.22 ppm) was directly coupled to H-11 (δ 1.95) and showed long-range correlations with H-12 and/or H-13 as well as H-5 (δ 1.66) and H-4 (δ 5.37). H-5 was in turn directly correlated with C-5 (48.72 ppm). H-5, which was correlated to H-4 in the $^1H$–$^1H$ COSY spectrum, also showed a COLOC correlation with the neighboring olefinic carbon C-4. Finally, H-9 (δ 1.62) was directly correlated with C-9 (42.46 ppm).

With the NMR assignments secured, we carried out a preparative-scale incubation of [13,13,13-$^2H_3$]FPP (**2a**) (10 μM), containing [12,13-$^{14}C$]FPP as an internal standard (final specific activity 6.1 nCi/μmol), with crude epicubenol synthase obtained from 600 mL of *Streptomyces* sp. LL-B7 culture. After addition of 3 mg of carrier (±)-epicubenol, the resulting products were rigorously purified by flash column chromatography on $SiO_2$ followed by argentation column chromatography and analyzed by 61.42 MHz $^2H$ NMR spectroscopy. The purified epicubenol (**1a**) (3 mg, 60 nmol of deuterated **1a** before dilution with carrier) displayed a single methyl peak at δ 0.76 ppm, corresponding to deuterium, most probably at C-13 (Scheme II). These results indicate that the cyclization is completely stereospecific and that the individual isopropyl methyl groups retain their identity during the cyclization. Assignment of the overall stereochemistry of the cyclization must await the unambiguous assignment of the individual resonances of the diastereotopic methyl protons.[12]

(11) The 100-MHz $^1H$ NMR spectrum of epicubenol recorded in the presence of Eu(DPM)$_3$ has previously been assigned.[7c]

(12) Because of the small chemical shift difference between H-12 and H-13 (Δδ 0.05 ppm) and the variations in chemical shift between benzene-$d_6$ ($^1H$ NMR) and benzene-$d_0$ (natural abundance) ($^2H$ NMR), we cannot definitively rule out the alternative possibility that D-12 has been labeled. This point is under investigation, along with the unambiguous assignment of the diastereotopic methyl resonances.

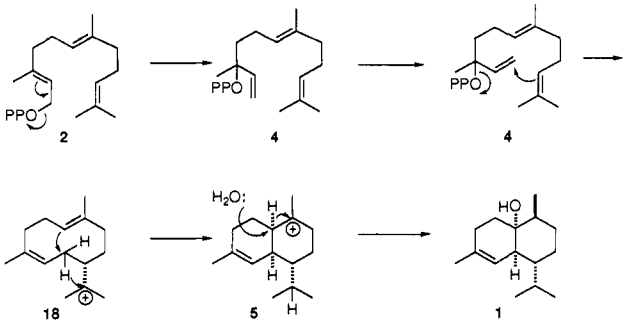
**Scheme II**




**2a** R=$CD_3$, X=H
**2b** R=$CH_3$, X=D

**1a** R=$CD_3$, X=H
**1b** R=$CH_3$, X=D

**Scheme III**




Incubation of [1,1-$^2H_2$]FPP (**2b**) (10 μM) containing [1-$^3H$]-FPP as internal standard (final specific activity 0.41 μCi/μmol) with crude epicubenol synthase gave epicubenol (**1b**) (180 nmol) which displayed two signals in the $^2H$ NMR spectrum at δ 1.62 and 1.93 (Δδ 0.29), corresponding to D-5 and D-11, respectively (Scheme II). In order to further confirm these assignments, the $^2H$ NMR spectrum of **1b** was also recorded in $CHCl_3$, giving rise to signals at δ 1.68 and 1.95 (Δδ 0.27), thereby ruling out the presence of deuterium label at D-9 ($^1H$ NMR δ 1.58; H-9/H-11 Δδ 0.36).

## Discussion

Taken together, these labeling results can be completely accounted for by a cyclization mechanism involving initial rearrangement of FPP to the tertiary allylic isomer, nerolidyl diphosphate (**4**), followed by ionization and electrophilic attack on C-10 of the distal double bond (Scheme III). A 1,3-hydride shift, followed by a second electrophilic cyclization will generate the cadinanyl cation **5**, which, upon 1,2-hydride shift and syn capture of water, will generate epicubenol (**1**). All sesquiterpene synthases reported to date catalyze initial cyclization to six-membered ring (bisabolyl cation (**6**) → trichodiene (**7**) synthase,[13] bergamotene (**8**) synthase[14]), eleven-membered ring (humulyl cation (**9**) → pentalenene (**10**) synthase,[4,15] humulene (**11**) synthase,[16] caryophyllene (**12**) synthase[16]), or *all-trans* ten-membered ring (*trans,trans*-germacradienyl cation (**13**) → aristolochene (**14**),[17] epiaristolochene (**15**),[18] β-selinene (**16**),[19] and patchoulol (**17**) synthase[20] intermediates (Scheme IV). The

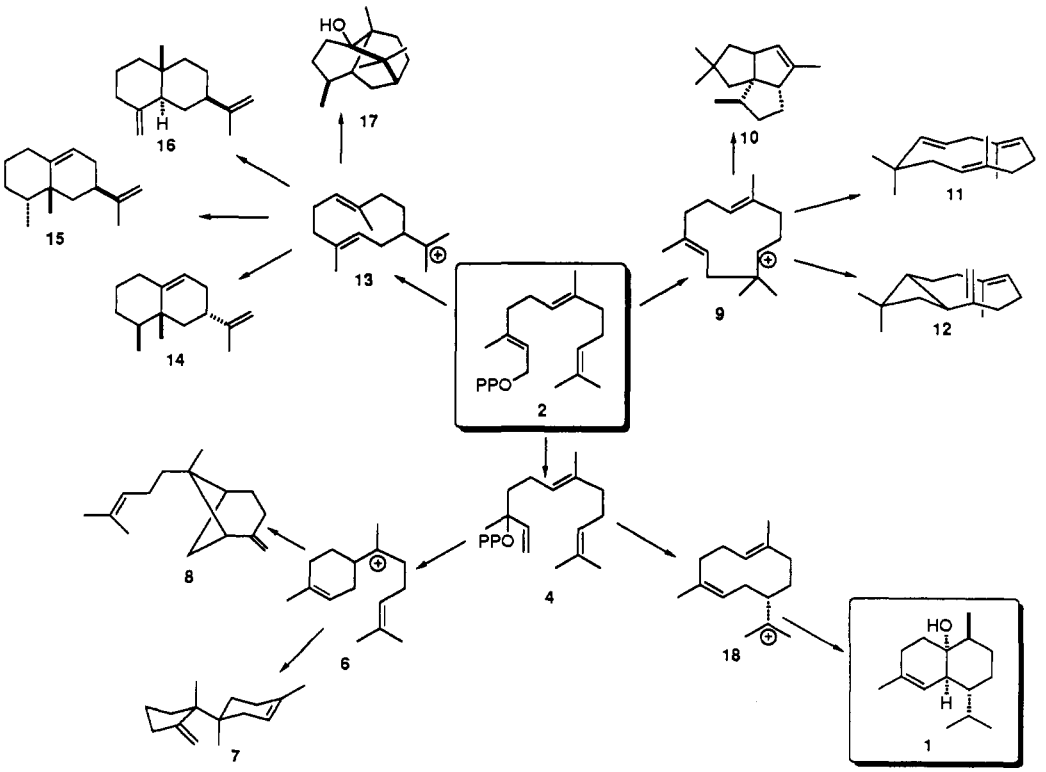
(13) Trichodiene synthase. (a) Mechanism: Cane, D. E.; Swanson, S.; Murthy, P. P. N. *J. Am. Chem. Soc.* **1981**, *103*, 2136. Cane, D. E.; Ha, H.; Pargellis, C.; Waldmeier, F.; Swanson, S.; Murthy, P. P. N. *Bioorg. Chem.* **1985**, *13*, 246. Cane, D. E.; Pawlak, J. L.; Horak, R. M.; Hohn, T. M. *Biochemistry* **1990**, *29*, 5476. (b) Purification: Hohn, T. M.; VanMiddlesworth, F. *Arch. Biochem. Biophys.* **1986**, *251*, 756. (c) Cloning and expression: Hohn, T. M.; Beremand, P. D. *Gene* **1989**, *79*, 131. Hohn, T. M.; Desjardins, A. E. *Mol. Plant-Microbe Int.* **1991**, *5*, 249. Hohn, T. M.; Plattner, R. D. *Arch. Biochem. Biophys.* **1989**, *275*, 92. Cane, D. E.; Wu, Z.; Oliver, J. S.; Hohn, T. M. *Arch. Biochem. Biophys.* **1993**, *300*, 416.

(14) Bergamotene synthase: Cane, D. E.; McIlwaine, D. B.; Harrison, P. H. M. *J. Am. Chem. Soc.* **1989**, *111*, 1152. Cane, D. E.; McIlwaine, D. B.; Oliver, J. S. *J. Am. Chem. Soc.* **1990**, *112*, 1285.

(15) Pentalenene synthase. (a) Mechanism: Cane, D. E.; Abell, C.; Tillman, A. M. *Bioorg. Chem.* **1984**, *12*, 312. Harrison, P. H. M.; Oliver, J. S.; Cane, D. E. *J. Am. Chem. Soc.* **1988**, *110*, 5922. Cane, D. E.; Oliver, J. S.; Harrison, P. H. M.; Abell, C.; Hubbard, B. R.; Kane, C. T.; Lattman, R. *J. Am. Chem. Soc.* **1990**, *112*, 4513. (b) Partial purification: Cane, D. E.; Pargellis, C. *Arch. Biochem. Biophys.* **1987**, *254*, 421–429.

(16) Humulene and caryophyllene synthases: Dehal, S. S.; Croteau, R. *Arch. Biochem. Biophys.* **1988**, *261*, 346.

**Scheme IV**




epicubenol synthase reaction is the first described which must involve a *cis*-germacradienyl cation (**18**) and associated 1,3-hydride shift. Whereas products with *all-trans* geometry can be formed directly from *all-trans*-farnesyl diphosphate, it is predicted that epicubenol, like trichodiene (**7**) and bergamotene (**8**), must be derived through the intermediacy of the tertiary allylic isomer, nerolidyl diphosphate (**4**), already shown to be an intermediate in the enzymatic cyclization of FPP to trichodiene.[21,22] This mechanism is fully consistent with the mechanism of formation of candinane, picrotoxane, and sativan sesquiterpenes which has been extensively investigated at the intact-cell level[23] and discussed in detail by Arigoni.[24] The predicted 1,3-hydride shift to generate the isopropyl side chain is demonstrated here for the first time at the enzyme level for this major class of cadinene metabolites. Further investigations of the mechanism and stereochemical course of epicubenol formation as well as purification of epicubenol synthase are in progress.

(17) Aristolochene synthase. (a) Mechanism: Cane, D. E.; Prabhakaran, P. C.; Oliver, J. S.; McIlwaine, D. B. *J. Am. Chem. Soc.* **1990**, *112*, 3209. (b) Purification: Hohn, T. M.; Plattner, R. D. *Arch. Biochem. Biophys.* **1989**, *272*, 137. (c) Cloning and expression: Proctor, R. H.; Hohn, T. M. *J. Biol. Chem.* **1993**, *268*, 4543. Cane, D. E.; Wu, Z.; Proctor, R. H.; Hohn, T. M. *Arch. Biochem. Biophys.* **1993**, *304*, 415.

(18) Epiaristolochene synthase. (a) Purification: Vögeli, U.; Freeman, J. W.; Chappell, J. *Plant Physiol.* **1990**, *93*, 182. (b) Cloning: Facchini, P. J.; Chappell, J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11088.

(19) β-Selinene synthase: Belingheri, L.; Cartayrade, A.; Pauly, G.; Gleizes, M. *Plant Sci.* **1992**, *84*, 129.

(20) Patchoulol synthase: Munck, S. L.; Croteau, R. *Arch. Biochem. Biophys.* **1990**, *282*, 58.

(21) Cane, D. E.; Ha, H. *J. Am. Chem. Soc.* **1986**, *108*, 3097. Cane, D. E.; Ha, H. *J. Am. Chem. Soc.* **1988**, *110*, 6865.

(22) Cane, D. E. *Acc. Chem. Res.* **1985**, *18*, 220.

(23) (a) Rohr, M. Dissertation ETH (Zürich) No. 5212, 1973. (b) Biollaz, M.; Arigoni, D. *Chem. Commun.* **1969**, 633. Corbella, A.; Gariboldi, P.; Jommi, G. *J. Chem. Soc., Chem. Commun.* **1973**, 729. Corbella, A.; Gariboldi, P.; Jommi, G.; Scolastico, C. *Chem. Commun.* **1969**, 634. Corbella, A.; Gariboldi, P.; Jommi, G. *J. Chem. Soc., Chem. Commun.* **1972**, 600. Turnbull, K. W.; Acklin, W.; Arigoni, D.; Corbella, A.; Gariboldi, P.; Jommi, G. *J. Chem. Soc., Chem. Commun.* **1972**, 598. (c) Dorn, F. Dissertation ETH (Zürich) No. 5554, 1975. Dorn, F.; Bernasconi, P.; Arigoni, D. *Chimia* **1975**, *29*, 24.

(24) Arigoni, D. *Pure Appl. Chem.* **1975**, *41*, 219.

## Experimental Section

**General Procedures.** All reactions requiring nonaqueous conditions were performed in oven-dried glassware under a positive pressure of nitrogen. All solvents were distilled. The term in vacuo refers to the removal of solvents on a rotary evaporator followed by evacuation to constant sample weight (<0.05 mmHg). All the reactions were followed by thin-layer chromatography (TLC) using phosphomolybdic acid for visualization. Commercial TLC plates were Merck 60F-254. Flash column chromatography was performed according to the method of Still[25] on Merck Type 60 silica gel, 230–240 mesh. Melting points (mp) were determined on a Thomas–Hoover apparatus using open capillary tubes and are uncorrected. Liquid scintillation spectrometry was performed on a Beckman LS5801 scintillation counter, using Optifluor scintillation cocktail. Protein concentrations were determined by the Bradford dye-binding assay[26] (Bio-Rad Laboratories) using bovine serum albumin as standard.

**Materials.** [1-$^3$H]Farnesyl diphosphate (72 mCi/mmol),[27] [13,13,-13-$^2H_3$, 12,13-$^{14}$C]FPP,[17] and (1*RS*)-[1-$^3$H, 1,1-$^2H_2$]FPP[15] were prepared as previously described. All other reagents were reagent grade or better. All buffers were prepared with deionized water from a Barnstead Nanopure system.

**NMR Methods.** Nuclear magnetic resonance (NMR) spectra were recorded on Bruker WM-250 or AM-400 WB instruments in the specified deuterated solvent with tetramethylsilane (TMS) as internal standard for $^1$H spectra and $CDCl_3$ or $C_6D_6$ as internal standard for $^{13}$C spectra. Deuterium NMR spectra were recorded at 61.42 MHz in unlocked mode with $^1$H broad-band decoupling; the pulse width was 45° or 90° and acquisition time was 0.75 s. No relaxation delay was used. Natural abundance $CDCl_3$ (δ 7.24) and $C_6D_6$ (δ 7.15) were used as internal standards.

For the 2D $^1$H–$^1$H COSY of epicubenol (**1**), 512 experiments were performed on an AM-400 WB instrument accumulating 8 scans per experiment in a 2K data block over a 2702-Hz sweep width centered at δ 3.38. No relaxation delay was used. The data was zero-filled to 1K in F1 and subjected to Fourier transformation. The spectrum was symmetrized to improve appearance; this did not affect the overall results obtained.

(25) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

(26) Bradford, M. *Anal. Biochem.* **1976**, *72*, 248.

(27) Cane, D. E.; Iyengar, R.; Shiao, M.-S. *J. Am. Chem. Soc.* **1981**, *103*, 914.

For 2D $^1H$–$^{13}C$ heteronuclear shift correlation of epicubenol (**1**), 512 experiments were performed on an AM-400 WB instrument accumulating 88 scans per experiment in an 8K block over a 12 500-Hz sweep width centered at $\delta$ 62.10 (F2). The proton sweep width was 1348 Hz centered at $\delta$ 3.37 (F1). No relaxation delay was used, and the value of $J(^{13}C–^1H)$ selected was 130 Hz. The data was zero-filled to 1K in F1 and subjected to Fourier transformation using sinebell windows in F1 and F2.

For 2D $^1H$–$^{13}C$ long-range heteronuclear shift correlation of epicubenol (**1**), 512 experiments were performed on an AM-400 WB instrument accumulating 96 scans per experiment in an 8K block over a 12 820-Hz sweep width centered at $\delta$ 63.70 (F2). The proton sweep width was 1422 Hz centered at $\delta$ 3.55 (F1). No relaxation delay was used, and the value of $J(^{13}C-^1H)$ used was 130 Hz. The data was zero-filled to 1K in F1 and subjected to Fourier transformation using sinebell windows in F1 and F2.

For COLOC NMR of epicubenol (**1**), 512 experiments were performed on an AM-400 WB instrument accumulating 64 scans per experiment in a 4K block over a 12 820-Hz sweep width centered at $\delta$ 63.68 (F2). The proton sweep width was 1438 Hz centered at $\delta$ 3.59 (F1). No relaxation delay was used, and the value of $J(^{13}C–^1H)$ used was 5.6 Hz. The data was zero-filled to 1K in F1 and subjected to Fourier transformation using sinebell windows in F1 and F2.

**Cultivation of *Streptomyces* sp. LL-B7.** Seed cultures of *Streptomyces* sp. LL-B7, grown for 45 h as previously described,[6] were used to inoculate (10% v/v) a modified fermentation medium consisting of 13.0 g of dextrose, 7.0 g of Lab-Lemco Oxoid Beef Extract, 7.0 g of Bacto Soytone, and 0.1 g of anhydrous $CaCl_2$ in 600 mL of deionized water, pH adjusted to 7.35 before autoclaving.

**Isolation of Epicubenol Synthase.** To prepare a cell-free extract of *Streptomyces* sp. LL-B7, the mycelium from 600 mL of a 42-h fermentation culture was harvested by centrifugation (6100*g*), washed twice with nanopure water and once with breaking buffer (50 mM HEPES, pH 7.45, containing 2.0 mM EDTA, 10 mM 2-mercaptoethanol, and 15% (v/v) glycerol), and suspended in 220 mL of the same buffer. The cells were disrupted by grinding with 120 mL of 0.5-mm glass beads in a jacketed Bead-Beater cell at 4 °C using four 15-s on, 30-s off cycles. The broken-cell suspension was clarified by centrifugation (13 200*g*, 7 min, 4 °C). The resulting supernatant (240 $\mu$g of protein/mL) was used directly for enzymatic cyclization.

**Cyclization of [1-$^3$H]Farnesyl Pyrophosphate (2) to [5,11-$^3H_2$]-Epicubenol.** The crude cell-free extract (20 mL) was incubated for 120 min at 30 °C in a glass tube with [1-$^3$H]FPP (**2**) (10 $\mu$M, $2.9 \times 10^6$ dpm, 72 mCi/mmol) in the presence of 20 mM $MgCl_2$. The reaction was halted by addition of 10 mL of 1 M $Na_2EDTA$, and the mixture was extracted with $5 \times 10$ mL of diethyl ether. To it was added 33 mg (0.15 mmol) of (±)-epicubenol[8] as carrier and the organic extract concentrated in vacuo. The radioactive epicubenol obtained was used for the next reaction without any further purification.

**Epicubene-3,4,10-triol (3).** The above radioactive epicubenol (33 mg, 0.15 mmol) was directly treated with $OsO_4$ (0.038 g, 0.15 mmol) in 1.2 mL of pyridine for 12 h at room temperature. To the mixture was added saturated $NaHSO_3$ solution (5 mL), and the mixture was stirred for 1 h and extracted with diethyl ether ($4 \times 6$ mL). The combined extracts were washed with 5% HCl solution (6 mL), 20% $NaHCO_3$ solution (6 mL), and $H_2O$ (10 mL), dried ($Na_2SO_4$), and concentrated in vacuo. The resulting triol **3** was separated by flash column chromatography ($SiO_2$, 80:20 diethyl ether/pentane) from the corresponding heptaols derived from farnesol and nerolidol, which had been generated by competing enzymatic (phosphatase) and $Mg^{2+}$-catalyzed hydrolysis of the substrate. It yielded 34 mg (0.13 mmol, 90%) of **3** as a pure white solid. The triol **3** was recrystallized five times from diethyl ether/pentane to constant melting point (138 °C) and radioactivity. The average specific activity of the last three crystallizations was 0.68 $\mu$Ci/mmol. 400-MHz $^1H$ NMR ($CDCl_3$) $\delta$ 0.82 (d, $J$ = 6.8 Hz, 3H), 0.90 (d, $J$ = 6.7 Hz, 6H), 1.04 (m, 2H), 1.29 (s, 3H), 1.38 (t, $J$ = 10 Hz, 1H), 1.50–1.65 (m, 6H), 1.87 (d, $J$ = 2.4 Hz, 1H), 1.95–2.05 (m, 2H), 2.82 (s, 1H), 3.19 (s, 1H), 3.76 (d, $J$ = 6.2 Hz, 1H), 4.69 (d, $J$ = 7.1 Hz, 1H); 100-MHz $^{13}C$ NMR ($CDCl_3$) $\delta$ 76.3, 74.8, 70.8, 50.7, 43.8, 42.8, 31.16, 31.05, 26.87, 24.87, 24.61, 24.26, 21.4, 14.63, 14.56; ($NH_4^+$) CIMS [$(M - H_2O + NH_4)^+$] = 256 $m/e$. Anal. Calc for $C_{15}H_{28}O_3$ ($-H_2O - OH$): 221.1905. Found: 221.1913.

**[13,13,13-$^2H_3$, 12,13-$^{14}$C]Epicubenol (1a).** [13,13,13-$^2H_3$, 12,13-$^{14}$C]-FPP (**2a**) (10 $\mu$M, specific activity 6.1 nCi/$\mu$mol) was cyclized to epicubenol (**1a**) by incubation with crude epicubenol synthase (210 mL), obtained from three 600-mL cultures of *Streptomyces* sp. LL-B7, for 2 h at 30 °C in the usual manner. Carrier (±)epicubenol (3 mg, 13.5 $\mu$mol) was added, and the resulting products were rigorously purified by flash column chromatography ($SiO_2$, 15:85 ether/pentane; TLC $SiO_2$, 15:85 ether/pentane; epicubenol (**1a**), $R_F$ 0.35; nerolidol, $R_F$ 0.30; farnesol, $R_F$ 0.15) followed by argentation column chromatography (10% $AgNO_3$ on $SiO_2$, 15:85 ether/pentane). The purified epicubenol (**1a**) (ca. 3 mg) was analyzed by 61.42-MHz $^2H$ NMR spectroscopy (805 dpm $^{14}C$, corresponding to 60 nmol of (3% conversion) deuterated **1a** prior to dilution with inactive carrier): $^2H$ NMR ($C_6H_6$, 61 MHz) $\delta$ 0.76 (D-13), 7.15 ($C_6H_5D$).

**[5,11-$^2H_2$, 5,11-$^3H_2$]Epicubenol (1b).** (1*RS*)-[1-$^3$H, 1,1-$^2H_2$]FPP (**2b**) (10 $\mu$M, specific activity 0.41 $\mu$Ci/$\mu$mol) was cyclized to epicubenol (**1b**) by incubation with epicubenol synthase (210 mL of crude extract) for 2 h at 30 °C in the usual manner. Repeated incubations (total culture volume ca. 7 L) led to the accumulation of ca. 200 nmol of epicubenol (**1b**). Carrier (±)-epicubenol (6 mg, 27 $\mu$mol) was added, and the cyclization product was subjected to flash column chromatography ($SiO_2$, 15:85 ether/pentane) to give pure epicubenol (**1b**) (ca. 6 mg) ($1.62 \times 10^5$ dpm $^3H$, corresponding to 180 nmol (2.2% conversion) of deuterated **1b** before dilution with inactive carrier): $^2H$ NMR ($C_6H_6$, 61.42 MHz) $\delta$ 1.62 (D-5), 1.93 (D-11), 7.15 ($C_6H_5D$); ($CHCl_3$) $\delta$ 1.68 (D-5), 1.95 (D-11), 7.26 ($CDCl_3$).

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**Supplementary Material Available:** 400-MHz $^1H$ NMR spectrum in $CDCl_3$ of synthetic (±)-epicubenol, 61.42-MHz $^2H$ NMR spectrum in $C_6H_6$ of epicubenol derived from [13-$^2H_3$]-FPP, and 61.42-MHz $^2H$ NMR spectra in $CHCl_3$ and $C_6H_6$ of epicubenol derived from [1-$^2H_2$]FPP (4 pages). Ordering information is given on any current masthead page.