Published in final edited form as:

Arch Biochem Biophys. 2007 December 1; 468(1): 140–146.

Inhibition of monoterpene cyclases by inert analogues of geranyl diphosphate and linalyl diphosphate[☆]

Frank Karp^a, Yuxin Zhao^{b,1}, Bindu Santhamma^{b,2}, Bryce Assink^{b,3}, Robert M. Coates^b, and Rodney B. Croteau^{a,*}

^a*Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA*

^b*Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA*

Abstract

The tightly coupled nature of the reaction sequence catalyzed by monoterpene synthases has prevented direct observation of the topologically required isomerization step leading from geranyl diphosphate to the enzyme-bound, tertiary allylic intermediate linalyl diphosphate, which then cyclizes to the various monoterpene skeletons. X-ray crystal structures of these enzymes complexed with suitable analogues of the substrate and intermediate could provide a clearer view of this universal, but cryptic, step of monoterpenoid cyclase catalysis. Toward this end, the functionally inert analogues 2-fluorogeranyl diphosphate, (±)-2-fluorolinalyl diphosphate, and (3R)- and (3S)-homolinalyl diphosphates (2,6-dimethyl-2-vinyl-5-heptenyl diphosphates) were prepared, and compared to the previously described substrate analogue 3-azageranyl diphosphate (3-aza-2,3-dihydrogeranyl diphosphate) as inhibitors and potential crystallization aids with two representative monoterpenoid cyclases, (–)-limonene synthase and (+)-bornyl diphosphate synthase. Although these enantioselective synthases readily distinguished between (3R)- and (3S)-homolinalyl diphosphates, both of which were more effective inhibitors than was 3-azageranyl diphosphate, the fluorinated analogues proved to be the most potent competitive inhibitors and have recently yielded informative liganded structures with limonene synthase.

Keywords

(–)-Limonene synthase; (+)-Bornyl diphosphate synthase; Geranyl diphosphate; Linalyl diphosphate; Fluorogeranyl diphosphate; Fluorolinalyl diphosphate; Azageranyl diphosphate; Homolinalyl diphosphate

Monoterpene synthases (cyclases) catalyze the conversion of geranyl diphosphate (GPP⁴, **1**) to the parent compounds of the various carbon skeletons [1]. The central role of these enzymes

[☆]This investigation was supported by National Institutes of Health Grants GM13956 (to R.M.C.) and GM31354 (to R.B.C.)

*Correspondence to: Rodney B. Croteau, Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA, Tel: 509-335-1790 Fax: 509-335-7643, E-mail: croteau@wsu.edu.

¹Present address: Takeda San Diego Inc., 10410 Science Center Drive, San Diego, CA 92121.

²Present address: Cellular and Structural Biology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229.

³Present address: Albemarle, 1421 Kalamazoo Street, South Haven, MI 49090.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

⁴Abbreviations used: GPP, geranyl diphosphate; LPP, linalyl diphosphate; FGPP, 2-fluorogeranyl diphosphate; FLPP, 2-fluorolinalyl diphosphate; homoLPP, homolinalyl diphosphate (2,6-dimethyl-2-vinyl-5-heptenyl diphosphate); azaGPP, 3-azageranyl diphosphate (3-aza-2,3-dihydrogeranyl diphosphate).

in the origin of the different cyclic monoterpene classes has stimulated considerable interest in the mechanism and stereochemistry of these reactions [2] (Fig. 1) in which the geranyl precursor, because of the topological barrier to direct cyclization imposed by the trans-geometry at C2, must first undergo a preliminary isomerization step to an intermediate competent to cyclize. The mechanism of this coupled isomerization-cyclization reaction (Fig. 1) involves initial, divalent metal ion-assisted, ionization of GPP, with syn-migration of the diphosphate moiety of the ion pair, to provide the enzyme-bound tertiary allylic intermediate linalyl diphosphate (LPP, **2**) [1,2]. In this “ionization-isomerization” step, which removes the topological impediment to cyclization, the first chiral center is introduced at C3 (i.e., either (3R)- or (3S)-LPP (**2a** or **2b**, respectively) is formed, depending on the initial binding conformation of the geranyl substrate). Rotation about C2-C3 and C5-C6 affords the cisoid, anti-endo (helical) conformer of LPP (**2**), which is itself ionized with ensuing C6-C1 cyclization to generate the corresponding monocyclic (4R)- or (4S)- α -terpinyl carbocation:diphosphate anion pair (**3a** or **3b**, respectively). These mechanistic features appear to be common to nearly all monoterpene cyclase transformations, with subsequent steps involving either termination of the reaction by deprotonation or nucleophile capture, or further electrophilic cyclizations via the remaining double bond, hydride shifts, or rearrangements before termination [1–3]. For example, direct deprotonation from the adjacent methyl of the (4S)- α -terpinyl cation (**3b**) yields (–)-limonene (**4**) [4,5], whereas C7-C1 bridging of the (4R)- α -terpinyl intermediate (**3a**) with capture of the resulting cation (**5**) by the paired diphosphate anion generates (+)-bornyl diphosphate (**6**) [6,7].

With this mechanistic model for the multistep isomerization-cyclization reaction to provide an interpretive framework, many monoterpene synthases have been cloned and expressed, and mutagenic studies to effect product outcome have begun to reveal structure-function relationships within the class, and the relationship of the monoterpene synthases to prenyltransferases and other terpenoid synthases [3,8–11]. However, the monoterpene synthases appear to be rather recalcitrant to crystallization and, until recently, the structure of only (+)-bornyl diphosphate synthase had been reported [12]. Bornyl diphosphate synthase is an unusual example of this enzyme type, in that the diphosphate leaving group of the geranyl substrate is recaptured in the final product [13]; most monoterpene cyclization reactions are terminated by water capture, or more commonly by deprotonation, of the final carbocation intermediate [3]. In the case of bornyl diphosphate synthase, structures of complexes with aza-analogues of substrate and carbocation intermediates, as well as with product, were obtained to provide a detailed description of this cyclization cascade [12]; however, the aza-analog of the substrate (azaGPP; 3-aza-2,3-dihydrogeranyl diphosphate) was bound anomalously, perhaps as a consequence of the positive charge, bond distortion, or hydrogen bonding resulting from the N for C substitution [14,15].

There is a need to determine the structures of other types of monoterpene cyclases, with particular reference to substrate binding and the isomerization step common to members of this enzyme class. For this purpose, suitable substrate and intermediate analogues are required to serve as crystallization aids that could provide information about active site interactions. In this paper, we describe the preparation of several unreactive substrate and intermediate analogues (Fig. 2) and their inhibition properties with the representative monoterpene cyclases (+)-bornyl diphosphate synthase from common sage (*Salvia officinalis*) and (–)-limonene synthase from spearmint (*Mentha spicata*). The fluorinated analogues (**7** and **8**) proved to be the most potent competitive inhibitors and have recently yielded informative crystal structures of the corresponding complexes with (–)-limonene synthase [15].

Materials and methods

Synthetic procedures

[1-³H]GPP (56 Ci/mol) was prepared by literature procedures [16], as was 3-azaGPP [17]. 2-FGPP (**7**) [18] and (±)-2-FLPP (**8**) [19] were prepared as outlined in Schemes 1 and 2.

Olefination of 6-methyl-5-hepten-2-one (**12**) with the sodio form of triethyl fluorophosphonoacetate (**13**) [20] in benzene afforded a 1:1 mixture of cis and trans fluoro esters, and reduction of the mixture with LiAlH₄ in THF followed by chromatographic separation gave 2-fluoronerol (**14**, 50%) and 2-fluorogeraniol (**15**, 43%) (Scheme 1) [18]. The configuration of the 2,3-double bond in the isomers was confirmed by correlation of the ¹H and ¹⁹F NMR spectral data with literature values for the fluoro alcohols [18] and with data for the corresponding 2-fluorofarnesol isomers [21].

Conversion of **15** to 2-fluorogeranyl chloride (**17**) (MsCl, LiCl, DMP, 0°C, 98%) [22] followed by S_N2 displacement with tetrabutylammonium diphosphate (CH₃CN, molecular sieves, 24 h) [23] furnished 2-FGPP as its ammonium salt (**7**, 64%) (Scheme 2) after ion exchange chromatography and precipitation and washing with methanol to remove excess inorganic diphosphate. FGPP was characterized by ¹H, ¹⁹F, and ³¹P NMR spectroscopy.

(±)-2-Fluorolinalool (**18**) was obtained from 2-fluorogeraniol (**15**) by conversion to the corresponding methanesulfonate (**16**) with CH₃SO₂Cl (Et₃N, CH₂Cl₂, -10 to 0°C) [24] and solvolysis in 80% aq. acetone (2,4,6-collidine buffer, 60°C) (Scheme 2) [18,25]. The racemic tertiary alcohol (**18**, 42%) was separated from other products by silica gel chromatography and characterized by ¹H, ¹³C and ¹⁹F spectral data.

Phosphorylation of 2-fluorolinalool (**18**) by the Cramer procedure [26–28] as modified by Danilov [29] and Assink [7,30] ((Bu₄N)₂HPO₄, CCl₃CN, CH₃CN, 25°C, 30 min) (Scheme 2) followed by precipitation of inorganic ammonium salts (2% NH₃ in MeOH) and gradient ion exchange chromatography (NH₄HCO₂ in MeOH) on a Dowex 1X8-400 column provided fractions containing monophosphate, diphosphate, and NH₄HCO₂. The ammonium formate was then removed by passage through an Amberlite XAD column with aq. NH₄OH and NH₄OH in methanol as eluents. ³¹P NMR spectra of the eight fractions containing 2-FLPP showed the presence of an impurity tentatively identified as methyl monophosphate ammonium salt (³¹P NMR 3.07 ppm, s; ESI MS 112, CH₃OPO₄H₂⁺). Lyophilization of the later four fractions provided pure FLPP (**8**, 8%), and similar processing of the monophosphate fraction afforded 2-fluorolinalyl monophosphate (**19**, 18%), both of which were characterized by appropriate ¹H, ¹⁹F, and ³¹P NMR spectral data.

(3R)-Homolinalool (**22b**) and (3R)-homoLPP (**9**), as well as the corresponding (S)-enantiomers, were synthesized by Lewis acid-induced rearrangement [31,32] of (2S,3S)-2,3-epoxygeranyl silyl ether (**20b** and **ent-20b**), Wittig methylenation, and phosphorylation (Scheme 3). Asymmetric epoxidation [33] of geraniol afforded (2S,3S)-2,3-epoxygeraniol (**20a**, 94:4 er), and the 2R,3R-antipode (**ent-20a**). The enantiomeric purities were established by conversion to the diastereomeric (S)-camphanate ester ((S)-camphanic chloride, DMAP, pyr, 2h) [34] and ¹H NMR analysis in DMSO-*d*₆. Similar preparation of the (S)-camphanate diastereomers from (±)-epoxygeraniol established that no kinetic resolution occurred under the conditions used. The corresponding t-butyl dimethylsilyl ethers (**20b** and the enantiomer) were formed by reaction with the silyl chloride reagent (3 equiv, 6 equiv imidazole, DMF; ethanolamine; 97 and 98%) [31].

Pinacol-type rearrangement of the epoxy silyl ethers with the bulky organoaluminum Lewis acid reagent, bis(2,6-di-*t*-butyl-4-bromophenoxy)AlCH₃ (1.6 equiv, CH₂Cl₂, -25°C, 3h) [31, 32] gave rise to (S)-aldehyde **21** and the (R)-enantiomer in identical 84% yields after

purification by silica gel chromatography. Methylenation with $\text{CH}_2=\text{PPh}_3$ (ether, -78 to 25°C , 91% and 73%) followed by desilylation with Bu_4NF (2.5 equiv, THF, 42 h) [35] gave (*R*)-homolinalool (**22b**) and its enantiomer, spectrally identical to ($\pm$)-3-homolinalool previously prepared by [2,3] Wittig rearrangement [36]. The stereochemical purities (94:6 and 6:94 er) of the homolinalools were verified by chiral GC analyses.

(3*R*)- and (3*S*)-Homolinalyl diphosphates (**9** and **10**) were prepared by Danilov-Cramer phosphorylations with $(\text{Bu}_4\text{N})\text{H}_2\text{PO}_4$ (5 equiv,) and CCl_3CN (30 equiv, CH_2Cl_2 , 20 min) [7, 29,30]. Removal of inorganic salts and separation of the monophosphates and diphosphates was accomplished as described above for 2-FLPP. The resulting (*R*)- and (*S*)-homoLPPs (**9** and **10**, 28% and 26%) as well as the corresponding (*R*)- and (*S*)-monophosphates (**23** and its enantiomer, 30% and 30%) were characterized by ^1H and ^{31}P NMR spectra.

For further details of the above synthetic and analytical procedures, see Supplemental Information.

Enzyme preparation

The cDNA encoding limonene synthase from spearmint (*Mentha spicata*) [37] was subcloned into the pSBET [38] vector, expressed in *E. coli* and purified (to ~99%) as previously described [39]. This version of the recombinant enzyme was truncated at R58, the approximate cleavage site for the plastid targeting sequence [39]. The cDNA encoding bornyl diphosphate synthase from sage (*Salvia officinalis*) [40] was also subcloned into the pSBET [38] vector, expressed in *E. coli* and purified (to ~98%) as previously described [7,12]. This version of the recombinant enzyme was similarly truncated at E50 to provide a pseudomature form devoid of the plastid transit peptide [9,12].

Inhibition studies

The standard, linear range, micro-assay procedure for limonene synthase and bornyl diphosphate synthase (olefin fraction) involved incubation of [$1\text{-}^3\text{H}$]geranyl diphosphate at pH 7.2 with purified enzyme at 200 ng protein/ml (limonene synthase) and at 3,000 ng protein/ml (bornyl diphosphate synthase; more protein was required because only olefin co-products, formed at a slower rate, were measured) in 50 mM MOPSO buffer containing 10% glycerol, 30 mM MgCl_2 and 2 mM DTT at 31°C for 8 min. Assay mixtures containing buffer, inhibitor, and labeled substrate in a total volume of 100 μl were prepared on ice. Reactions were initiated by the addition of purified enzyme, and the mixtures were immediately overlaid with 1 ml of pentane prior to incubation to trap volatile olefin products. Reactions were terminated by brief vortexing and immediately freezing in a liquid nitrogen bath. The pentane overlay, and two additional 1 ml pentane extracts (on ice) were combined and passed over a short column containing silica gel and MgSO_4 to yield the olefin fraction, which was quantified by liquid scintillation counting. Control experiments (without enzyme) were conducted at each substrate concentration to determine solvolytic background. Protein purity was assessed by SDS-PAGE, and protein concentration was measured by the Bradford assay [41].

Kinetic data, in the presence and absence of inhibitor, were evaluated by computer-assisted, least squares-linear regression analysis in conjunction with the graphical procedures of Lineweaver-Burk [42], Dixon [43], Cornish-Bowden [44] and Hunter-Downs [45] using "Enzyme Kinetics" [46] or "SigmaPlot" [47] software. K_m and K_p values were initially determined by varying substrate concentration in the presence and absence of inhibitor at fixed concentrations, followed by another series of assays in which the concentration of inhibitor was varied while substrate concentrations were held constant. Data for K_m and V_{max} values typically had r -values >0.98 , and the curve fit data had coefficient of determination values (r^2) <0.90 .

Results and discussion

Based on stereochemical reasoning and considerable indirect evidence [1–3], the monoterpene precursor GPP is considered to be cyclized, by the respective monoterpene synthases, to (–)-limonene and (+)-bornyl diphosphate via the corresponding intermediates (3S)-LPP and (3R)-LPP (Fig. 1), each of which can directly serve as an efficient, alternate cyclization substrate [48,49]. Although the isomerization of GPP to LPP is a required step in these monoterpene cyclizations, this essential reaction has never been observed directly because the linalyl intermediate remains bound in the enzyme active site and is cyclized at a faster rate than the coupled isomerization-cyclization from GPP [1–3]. One approach to examining the isomerization step of the coupled reaction sequence would be to obtain crystal structures of monoterpene synthases complexed with suitable geranyl substrate and linalyl intermediate analogues. Until deployment of the inhibitors described in this paper [15], only one crystal structure of a monoterpene cyclase had been solved, that for (+)-bornyl diphosphate synthase, with which complexes with 3-azaGPP and several other aza-analogues were obtained but which revealed limited information about the target isomerization step [12,14].

With the object of obtaining additional, informative crystal structures, several unreactive substrate and intermediate analogues were prepared, including 2-FGPP and (R,S)-2-FLPP in which the electron-withdrawing fluorine substituent suppresses the ionization component of the respective isomerization and cyclization steps, and (3R)- and (3S)-homoLPP in which the methylene insertion effectively prevents diphosphate ester ionization (Fig. 2). The inhibitory properties of these analogues were determined and compared with those of the previously utilized 3-azaGPP [12], both to assess their potential as crystallization aids and to gain mechanistic information, using as model cyclization catalysts recombinant (–)-limonene synthase and (+)-bornyl diphosphate synthase which yield products of the opposite enantiomeric series and represent the extremes of reaction termination chemistry (Fig. 1).

Preliminary evaluation of potency was determined by monitoring inhibition of the conversion of tritium labeled geranyl diphosphate to limonene (limonene synthase) and to the olefinic co-products of bornyl diphosphate synthase which comprise nearly 25% of the total reaction products and consist of (+)-camphene, (+)- α -pinene, (+)-limonene, terpinolene and myrcene [50]. These olefin co-products are much easier to determine than is bornyl diphosphate itself (by hydrolysis and chromatographic purification of the resulting borneol [49]), and have been shown to afford an accurate measure of inhibition, regardless of inhibition type (F. Karp and R.B. Croteau, unpublished). This preliminary assessment indicated large differences in K_i values for the various inhibitors. However, the limitations of the assays, based on extractive isolation of the olefin products and chromatographic separation, resulted in considerable scatter of data points, particularly at higher inhibition levels, and for some inhibitors up to three-fold variation in calculated K_i values from the preliminary data. Therefore, a minimum of three independent assessments of each inhibitor were made by varying inhibitor concentration at fixed substrate concentrations, and by varying substrate concentration at fixed inhibitor concentrations, to allow multiple plotting methods (Lineweaver-Burk, Hunter-Downs, Cornish-Bowden and Dixon plotting) [51] for determining the average and range of K_i values, and the inhibition type.

Table 1 provides a summary of the data, and Figure 3 illustrates a typical data set for inhibition by 2-FGPP as analyzed by Lineweaver-Burk and Dixon plotting. 3-AzaGPP proved to be the weakest of the inhibitors tested, with K_i values for both synthases in excess of 3 mM, and with no consistent inhibition type observed by the various plotting methods employed. Thus, this substrate analogue does not appear to bind particularly well to either enzyme. Nevertheless, 3-azaGPP did afford a crystalline complex with bornyl diphosphate synthase [12], although the

geranyl chain of the analogue was in a conformation inappropriate to proceed to (+)-bornyl diphosphate [14].

(3R)- and (3S)-HomoLPP were reasonably good inhibitors of both enzymes which exhibited predictable enantioselectivities for (3S)-homoLPP ((-)-limonene synthase) and (3R)-homoLPP ((+)-bornyl diphosphate synthase) (Table 1). Thus, (-)-limonene synthase can utilize both (3S)-LPP and (3R)-LPP as alternate substrates in the cyclization to (-)-limonene and (+)-limonene, respectively, with a roughly two-fold preference for (3S)-LPP based on comparison of V/K_m values [48]. Conversely, (+)-bornyl diphosphate synthase can utilize both (3R)-LPP and (3S)-LPP as alternative substrates in the cyclization to (+)-bornyl diphosphate and (-)-bornyl diphosphate, respectively, with a nearly twenty-fold preference for (3R)-LPP based on similar kinetic comparison [49]. Therefore, for both enzymes, the effectiveness of inhibition by (3R)- and (3S)-homoLPP mimics the respective enantioselectivity for (3R)- or (3S)-LPP in the corresponding cyclization reaction. Although the homoLPP enantiomers exhibited competitive inhibition, as might be expected, all showed a significant non-competitive component, as revealed by two of the four data plotting methods, indicative of binding at other locations in addition to the active sites of these enzymes. Thus far, neither (3R)- nor (3S)-homoLPP has yielded a diffraction-grade crystalline complex with any of the several monoterpene synthases tested.

The fluorinated prenyl diphosphates, of the type employed extensively by Poulter and coworkers for evaluation of the prenyltransferase reaction [18,52,53] and (in labeled form) for preliminary testing with several native monoterpene cyclases [19], were effective competitive inhibitors of both limonene synthase and bornyl diphosphate synthase (Table 1). Prior investigations concluded that replacement of the C2 vinyl hydrogen with fluorine, via depletion of electron density in the allylic moiety, significantly suppresses ionization of the corresponding fluoroprenyl diphosphate in both prenyltransferase [52,53] and cyclase [19] catalysis, consistent with the marked rate suppression observed in chemical solvolysis studies with these analogues [18]. This kinetic behavior, coupled to the close resemblance of the fluoro-analogues to the true substrate and intermediate, would predict these analogues to be good competitive inhibitors. Indeed, the preliminary studies using native bornyl diphosphate synthase indicated an approximate K_i for FGPP of 1 μ M (comparable to the K_m for GPP) with a rate suppression in excess of 300-fold (as measured by reduction in the co-produced olefins) [19]. It is also notable that FGPP and FLPP are detectably turned over by limonene synthase to a fluorinated olefin tentatively identified as 2-fluorolimonene [15]. Thus, the fluorinated analogues are not entirely inert, as are azaGPP and homoLPP which yield no detectable enzymatic products. In spite of this, FGPP and FLPP would appear to be most suitable crystallization aids because binding at the active site is assured, the fluorine substituent causes only minor steric and geometric perturbation in the olefin chain [54], and the K_i values are relatively low. It should be noted that FLPP employed here was racemic; thus, K_i values for the respective pure enantiomers are likely to be lower with the corresponding enantioselective enzyme.

The crystal structure of (-)-limonene synthase in two versions liganded to FGPP and FLPP, respectively, has recently been reported [15], and has provided suggestive evidence that GPP is initially bound in an extended conformation which requires multiple repositioning steps to achieve the cyclization-competent form (helical, cisoid) of the LPP intermediate. These fluorinated analogues will likely prove useful in structural studies with other monoterpene synthases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Croteau R. *Chem Rev* 1987;87:929–954.
2. Wise, ML.; Croteau, R. *Comprehensive Natural Products Chemistry: Isoprenoids Including Carotenoids and Steroids*. Cane, DE., editor. 2. Elsevier Science; Oxford: 1999. p. 97–153.
3. Davis EM, Croteau R. *Top Curr Chem* 2000;209:53–95.
4. Pyun HJ, Coates RM, Wagschal KC, McGeady P, Croteau R. *J Org Chem* 1993;58:3998–4009.
5. Coates RM, Elmore CS, Croteau RB, Williams DC, Morimoto H, Williams PG. *J Chem Soc Chem Commun* 1997;1997:2079–2080.
6. Croteau R, Shaskus JJ, Renstrøm B, Felton NM, Cane DE, Saito A, Chang C. *Biochemistry* 1985;24:7077–7085. [PubMed: 4084562]
7. Wise ML, Pyun HJ, Helms G, Assink B, Coates RM, Croteau R. *Tetrahedron* 2001;57:5327–5334.
8. El Tamer MK, Lückner J, Bosch D, Verhoeven HA, Verstappen FWA, Schwab W, van Tunen AJ, Voragen AGJ, de Maagd RA, Bouwmeester HJ. *Arch Biochem Biophys* 2003;411:196–203. [PubMed: 12623068]
9. Peters RJ, Croteau RB. *Arch Biochem Biophys* 2003;417:203–211. [PubMed: 12941302]
10. Katoh S, Hyatt D, Croteau R. *Arch Biochem Biophys* 2004;425:65–76. [PubMed: 15081895]
11. Hyatt DC, Croteau R. *Arch Biochem Biophys* 2005;439:222–233. [PubMed: 15978541]
12. Whittington DA, Wise ML, Urbansky M, Coates RM, Croteau RB, Christianson DW. *Proc Natl Acad Sci USA* 2002;99:15375–15380. [PubMed: 12432096]
13. Cane DE, Saito A, Croteau R, Shaskus J, Felton M. *J Am Chem Soc* 1982;104:5831–5833.
14. Christianson DW. *Chem Rev* 2006;106:3412–3442. [PubMed: 16895335]
15. Hyatt DC, Youn B, Zhao Y, Santhamma B, Coates RM, Croteau RB, Kang CH. *Proc Natl Acad Sci USA* 2007;104:5360–5365. [PubMed: 17372193]
16. Dixit VM, Laskovics FM, Noall WI, Poulter CD. *J Org Chem* 1981;46:1969–1970.
17. Szabo CM, Matsumura Y, Fukura S, Martin MB, Sanders JM, Sengupta S, Cieslak JA, Loftus TC, Lea CR, Lee HJ, Koohang A, Coates RM, Sagami H, Oldfield E. *J Med Chem* 2002;45:2185–2196. [PubMed: 12014956]
18. Poulter CD, Argyle JC, Mash EA. *J Biol Chem* 1978;253:7227–7233. [PubMed: 701246]
19. Croteau R. *Arch Biochem Biophys* 1986;251:777–782. [PubMed: 3800400]
20. Tsai HJ, Thenappan A, Burton DJ. *J Org Chem* 1994;59:7085–7091.
21. Jin Y, Williams DC, Croteau R, Coates RM. *J Am Chem Soc* 2005;127:7834–7842. [PubMed: 15913373]
22. Collington EW, Meyers AI. *J Org Chem* 1971;36:3044–3045.
23. Woodside AB, Huang Z, Poulter CD. *Org Syn Coll Vol* 1993;8:616–620.
24. Crossland RK, Servis KL. *J Org Chem* 1970;35:3195–3196.
25. Bunton CA, Cori O, Hachey D, Leresche JP. *J Org Chem* 1979;44:3238–3244.
26. Cramer F, Rittersdorf WR. *Tetrahedron* 1967;23:3015–3022.
27. Sofer SS, Rilling HC. *J Lipid Res* 1969;10:183–187. [PubMed: 4305712]
28. Fall RR, West CA. *J Biol Chem* 1971;246:6913–6928. [PubMed: 4331199]
29. Danilov LL, Mal'tsev SD, Shibaev VN. *Soviet J Bioorg Chem* 1988;14:712–714.
30. Assink, BK. MS Thesis. University of Illinois; Urbana-Champaign: 1999.
31. Maruoka K, Ooi T, Nagahara S, Yamamoto H. *Tetrahedron* 1991;47:6983–6998.
32. Ooi T, Maruoka K, Yamamoto H. *Org Synth Coll Vol* 1998;9:356–361.
33. Gao Y, Hanson RM, Klunder JM, Ko SY, Masamune H, Sharpless KB. *J Am Chem Soc* 1987;109:5765–5780.
34. Gerlach H. *Helv Chim Acta* 1966;49:2481–2489.
35. Corey EJ, Venkateswarlu A. *J Am Chem Soc* 1972;94:6190–6191.
36. Mulzer J, List B. *Tetrahedron Lett* 1996;37:2403–2404.
37. Colby SM, Alonso WR, Katahira EJ, McGarvey DJ, Croteau R. *J Biol Chem* 1993;268:23016–23024. [PubMed: 8226816]

38. Shenk PM, Baumann S, Mattes R, Steinbiss HH. *BioTechniques* 1995;19:196–200. [PubMed: 8527135]
39. Williams DC, McGarvey DJ, Katahira EJ, Croteau R. *Biochemistry* 1998;37:12213–12220. [PubMed: 9724535]
40. Wise ML, Savage TJ, Katahira E, Croteau R. *J Biol Chem* 1998;273:14891–14899. [PubMed: 9614092]
41. Bradford MM. *Anal Biochem* 1976;72:248–254. [PubMed: 942051]
42. Lineweaver H, Burk D. *J Am Chem Soc* 1934;56:658–666.
43. Dixon M. *Biochem J* 1953;55:170–171. [PubMed: 13093635]
44. Cornish-Bowden, A. *Principles of Enzyme Kinetics*. Butterworths; London: 1976. p. 52-100.
45. Hunter A, Downs CE. *J Biol Chem* 1945;157:427–446.
46. *Enzyme Kinetics*, Trinity Software, v.1.11, Plymouth, NH (1992).
47. *Sigma Plot*, Systat Software, Inc., 9.0, San Jose, CA (2004).
48. Rajaonarivony JIM, Gershenzon J, Croteau R. *Arch Biochem Biophys* 1992;296:49–57. [PubMed: 1605644]
49. Croteau R, Satterwhite DM, Cane DE, Chang CC. *J Biol Chem* 1986;261:13438–13445. [PubMed: 3759972]
50. Schwab W, Williams DC, Davis EM, Croteau R. *Arch Biochem Biophys* 2001;392:123–136. [PubMed: 11469803]
51. Dixon, M.; Webb, EC. *Enzymes*. Academic Press; New York: 1979. p. 332-355.
52. Poulter CD, Satterwhite DM. *Biochemistry* 1977;16:5470–5478. [PubMed: 562673]
53. Poulter CD, Wiggins PL, Le AT. *J Am Chem Soc* 1981;103:3926–3927.
54. O'Hagan D, Rzepa HS. *Chem Commun* 1997:645–652.

Fig. 1.

Stereochemical scheme for the enzymatic conversion of geranyl diphosphate to (-)-limonene and (+)-bornyl diphosphate. Formation of these cyclic products requires preliminary isomerization of geranyl diphosphate to either (3R)- or (3S)-linalyl diphosphate. OPP denotes the diphosphate moiety. Note that the numbering for the linalyl diphosphate skeleton is the same as for geranyl diphosphate.

Fig. 2.
Structures of substrate and intermediate analogues employed with (–)-limonene synthase and (+)-bornyl diphosphate synthase.

Fig. 3.

Inhibition of monoterpene cyclases by FGPP. Lineweaver-Burk (A; $[\text{FGPP}] = 0$ and $100 \mu\text{M}$) and Dixon (B; $[\text{GPP}] = 3$ and $12 \mu\text{M}$) plots illustrating the inhibition of (+)-bornyl diphosphate synthase by FGPP, and Lineweaver-Burk (C; $[\text{FGPP}] = 0$ and $100 \mu\text{M}$) and Dixon (D; $[\text{GPP}] = 3$ and $12 \mu\text{M}$) plots illustrating the inhibition of (-)-limonene synthase by FGPP. Incubation conditions (pH 7.2; $[\text{MgCl}_2] = 30 \mu\text{M}$) and assay procedures are described under Materials and methods.

Scheme 1.
(Ms = SO₂CH₃)

Scheme 2.
 (R = dimethylallyl, Ms = SO_2CH_3)

Scheme 3.(TBS = *t*-BuMe₂Si-, Ar = 2,6-di-*t*-butyl-4-bromophenyl)

Table 1

Inhibition of monoterpene synthases

Enzyme	Inhibitor Average K_i [Range] (μ M)		Inhibition type		AzaGPP
	FGPP	(3R,S)-FLPP	(3R)-HomoLPP	(3S)-HomoLPP	
Limonene synthase	79 [39–122] competitive	71[38–91] competitive	192[82–272] competitive ^a	56[28–152] mixed ^b	>3000 ^c
Bornyl diphosphate synthase	6[3–9] competitive	58[40–75] competitive	214[170–250] competitive ^a	1200[1000–1500] competitive ^a	>3000 ^c

^a Mixed inhibition by Dixon and/or Cornish-Bowden plotting.

^b Significant non-competitive component.

^c No consistent inhibition type observed.