Interception of the Enzymatic Conversion of Farnesyl Diphosphate to 5-Epi-Aristolochene by Using a Fluoro Substrate Analogue: 1-Fluorogermacrene A from (2E,6Z)-6-Fluorofarnesyl Diphosphate**

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Introduction

Terpene synthases catalyze the multistep conversions of their polyisoprenyl substrates into numerous acyclic polyenes and carbocyclic structures through series of alkylations, cyclizations, and rearrangements of carbocations. In some cases, “neutral”, deprotonated intermediates are formed, and these undergo further rounds of transformation upon “activation” through enzyme-directed protonation. Since these intermediates are often not released from the enzyme active sites during the chemical transformation, information about the mechanisms and the nature of the transient intermediates is usually inferred from experiments with labeled substrates, model chemical reactions, and the use of substrate analogues. Examples of cryptic deprotonated intermediates include the tertiary diphosphate (PP), (R)- and (S)-linalyl and (R)- and (S)-nerolidyl PPs, which are converted to cyclic monoterpene and sesquiterpene products, respectively, faster than they are produced by the initial isomerase activity of the cyclases. Neutral intermediates include the macrocyclic sesquiterpenes (R)- and (S)-germacrene A, humulene, and bicyclogermacrene, which all remain as tightly bound intermediates during the respective cyclizations catalyzed by epiaristolochene, aristolochene, pentalenene and germacradienol–germacrene D synthases.

Fluoro analogues have been useful in the study of enzyme mechanisms, and fluoro prenyl derivatives have afforded significant insights concerning putative intermediates in, and mechanisms of, terpene synthase-mediated reactions. While the relatively small size of the fluoro group might not perturb active-site binding extensively, its large inductive effect dramatically alters the apparent stability of allylic carbocations.
that bear a fluorine atom on the central carbon,[8] and seems likely to inactivate fluoro-vinyl double bonds towards protonation or electrophilic alkylation. In fact, 2-fluorogeranyl PP exhibits apparent active-site binding constants with farnesyl PP synthase and several monoterpen synthases similar to those of the natural geranyl substrates.[9–11] However, the rates by which the fluoro analogues are converted to fluoro products by these enzymes[9,11] are suppressed by factors of 10^2 to 10^3. The conversion of geranylgeranyl PP to the tricyclic diterpene taxadiene was intercepted by use of the 6-fluoro substrate variant. The trans ring junction in the fluoro verticilleone products formed by taxadiene synthase provided persuasive evidence for a H10ø configuration in the proposed verticilleone carbocation intermediate in the enzyme-catalyzed mechanism.[12] The pentacyclization of (3S)-2,3-oxidosqualene catalyzed by squa-
mol–hopene cyclase was derailed at the mono- and bicyclic stages when 11- and 14-fluoro analogues were utilized as substrates.[13]

Tobacco 5-epi-aristolochene and henbane premnaspidioidene synthases (TEAS and HPS)[14] catalyze the cyclizations and rearrangements of farnesyl PP (1) to their respective bicyclic ses-
quiterpene hydrocarbon products as key initiating steps in the biosynthesis of the phytalexins, capsidiol, solavetivone, and related metabolites.[15] Considerable evidence supports the for-
mation of an enzyme-bound germacrene A intermediate (2), which undergoes proton-induced cyclization and a 5→4 hydride shift to generate the branch-point eudesmyl carbocation 3b (Scheme 1).[16] Subsequent methyl or methylene migration, diphosphate ionization, and the initial nucleo-
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teration of the resulting germacrene A and the physical characterization of the resultant sesquiterpene derivative.[17] These findings provide further validation for the proposed reaction mechanism proceeding via a tightly bound germacrene A intermediate, and for the use of fluoro-vinyl substitutions to inactivate the double bonds of synthase-bound intermediates towards proto-

Results and Discussion

Synthesis of 6-fluorofarnesyl diphosphate

The synthetic route to 6-fluorofarnesol (10) and its diphosphate derivative (12) outlined in Scheme 2 follows well-prece-
dented methods[12,18] and requires only a brief description. The starting material 2-fluorogeraniol (6)[19] was prepared by olefi-
nation[19a] of 6-methylhept-5-en-2-one with diethyl fluorophos-
phonoacetate[20a] followed by reduction with LiAlH₄ and sepa-
ration of the resulting cis and trans isomers by chromatogra-
phy. Isoprenoid chain extension with acetocetate dianion,
trans-selective enol phosphorylation, methyl cuprate coupling, and ester reduction with iBu₂AlH afforded the known (2E,6Z)-6-
fluorofarnesol[19b] in 50% overall yield by following procedures developed earlier for the synthesis of 6-fluorogeranylgerani-
ol[12] which were based on syntheses reported initially by Sum
and Weiler.[21] 6-Fluorofarnesyl PP was obtained by conversion to the allylic chloride and subsequent S_c2 displacement with tris(tetramethylammonium) pyrophosphate,[22] and was charac-
terized by satisfactory ^1H, ^31P, and ^19F NMR spectra.

Enzymatic cyclization of 6-fluorofarnesyl PP and characteri-

Initial analytical-scale incubations monitored by GC-
MS[21] showed that TEAS catalyzes the cyclization of 6-fluorofarnesyl PP (12) to a single fluoro-substituted product assumed to be 1-fluorogermacrene A (13, Scheme 3) on the basis of its GC and MS behavior. The derived steady-state kinetic parameters and cata-
ytic efficiency of the reaction are reported in Table 1, along with comparable data for the unmodified sub-
strate farnesyl PP (1) under the same conditions. Although the recombinant cyclase exhibits an approxi-
mately twofold higher Michaelis constant (K_m) for the 6-
fluoro substrate, the somewhat faster turnover rate (k_cat) of the analogue results in nearly identical overall catalytic efficiencies (k_cat/K_m) for the two substrates. The kinetic data clearly indicate that the presence of the vinylic fluoro substituent at the C6 position of farnesyl PP has negligible effects on substrate bind-
ing, diphosphate ionization, and the initial nucleo-
philic attack of the C10–C11 double bond.

Preparative-scale incubations of 6-fluorofarnesyl PP NH₄⁺ salt (12) with TEAS performed according to Schenk et al.[16] with modifications [0.125 mg mL⁻¹]
envelope; substrate at 332 μM, buffer 200 mM Tris-HCl (pH 7.5),
40 mM MgCl2 afforded what proved to be pure 13 as a clear
oil in 58% yield following purification by chromatography on
alumina. The optical rotation of the purified fluorogermacrene A (\([\alpha]_0 = -28.1^\circ\) in CDCl3) is opposite in sign to that of the
parent (+)-germacrene A (\([\alpha]_0 = +42.1\) in CCl4). [24]
This clearly indicates that the fluoro substituent on the interaction of the modified sesquiterpene with polarized light. GC analyses (method A, see the Supporting Information) of the pentane/Et2O extracts from the incubation medium showed a single peak (\(t_r = 0.21 \text{ min}\)), albeit with a hump in the base line that is characteristic of (EE)-configured germacrenes owing to their thermal Cope rearrangement to elemenones on the column. [25] In fact, when the injection port temperature was increased to 180°C (method B), a new peak (\(t_r = 16.6 \text{ min}\)) corresponding to 1-fluoroelemenene (14) was observed.

The MS of 1-fluoroelemenene (13) shows a molecular ion peak at \(m/z\) 222 ([M]⁺, 9%, \(C_{13}H_{23}F\)) and an initial loss of 15 \(m/z\) units ([M–CH3]⁺, 43%) followed by four sequential losses of 14 \(m/z\) units. A similar fragmentation pattern has recently been reported for (+)-germacrene A (204 ([M]⁺, 8%) and 189 ([M–CH3]⁺, 37%) . [26] The IR spectrum and data are also very
similar to those observed for germacrene A [26,27] put with an
additional stretching absorption at a 1445 cm⁻¹ diagnostic for the vinylic fluoro group. Also notable was the absence of UV absorption at 350 nm (log ε = 0.3).

500 MHz 1H NMR spectra of 13 were recorded in CDCl3 for
direct comparison with those of (+)-germacrene A. [24] The presence of two distinct conformers of fluorogermacrene A was evident from the extra peaks and integrals observed. At room temperature, the downfield olefinic region showed five broad peaks centered at \(\delta_H = 5.11\) (br s, 1H; H5, 30%), 4.89 (br s, 1H; H5, 70%), 4.68 (s, 1H; H12, 30%), 4.66 (s, 1H; H12, 70%), and 4.58 ppm (s, 1H; H12, 100%). [26] Up field, in addition to a well-defined triplet centered at 2.69 ppm (J = 12.5 Hz) and integrating as 1 hydrogen (100%), five broad CH3 singlets were observed at 1.72 (H13, 100%, both major and minor conformers), 1.61 (H15, 30%, minor conformer), 1.58 (H15, 70%, major conformer), 1.42 (H14, 70%, major conformer) and 1.25 ppm (H14, 30%, minor conformer). The assignments of the six CH3 signals in the spectrum to the UD and UU conformations (13a and 13b, Scheme 4) [26] proposed for the major and minor species present were made by comparison with those recently ascertained for the most abundant conformers (UD and UD) of (+)-germacrene A by using variable-temperature NMR and NOE spectroscopy. [26] In addition, the 470 MHz 19F NMR spectrum of fluorogermacrene A at room temperature showed two well-separated resonances centered at \(\delta_F = -90.4\) (app. asymm. t, 
\(J_{app} = 27.7\) and 21.1 Hz) and \(-111.4\) ppm (d, \(J = 42.8\) Hz) in a 3:7 ratio, assigned to the same UD and UD conformational isomers. The doubled sets of NMR signals in both the 1H and 19F NMR spectra of 13 clearly indicate that this EE-configured cyclodecadiene behaves in solution (25°C) as an equilibrium mixture of two interconvertible conformational isomers in a

![Scheme 3. Cyclization of (E,E)-6-fluorofarnesyl PP (12) to (−)-1-fluoroger-
macrene A (13) catalyzed by TEAS.](image-url)

![Scheme 4. Structures and interconversion of the UD and UD conformers (13a and 13b) assigned to the major and minor species observed in the 1H and 19F NMR spectra of 1-fluoroger-
macrene A (13). The designations “UU” and “UD” refer to the spatial orientation of the two ring methyl groups with respect to the plane of the cyclodecadiene ring. (See ref. [30]).](image-url)
Further evidence for the chemical structure 13 of the fluoro-substituted sesquiterpene product was obtained by a preparative-scale thermal Cope rearrangement in refluxing toluene to 1-fluoro-β-elemene (14) as a single pure stereoisomer in approximately 70% yield after purification by preparative TLC (Scheme 5). The similarity of the conditions required for the thermal [3,3]sigmatropic rearrangements of 1-fluoro-germacrene A and germacrene A (3 h vs. 2 h at 110 °C), the formation of single isomers in both cases, and the similarity of the 1H NMR spectra of the two products were considered good reasons to assign the structure as the 1-fluoro derivative (14) of β-elemene, a sesquiterpene of well-established structure and stereochemistry.[33]

The 5,10-trans diequatorial and 5,7-cis diequatorial orientations of the vinyl and the two isopropenyl substituents predicted for the stable chair conformation of 14 (Scheme 5) were confirmed by the appearance of a doublet of doublets (2.49 ppm, J = 13 and 4 Hz) in the proton NMR spectrum attributable to an axial H5 proton coupled to axial and equatorial protons on the adjacent C6 methylene group.

It is interesting to note that the fluoroelemene product is dextrorotatory ([α]D = +28.0° in CDCl3), and like its (−)-fluorogeramocene A progenitor, is of opposite sign compared to the unfluorinated parent (β-elemene, [α]D = −15.8° in CHCl3).[24] Also noteworthy is the rearrangement of the interconverting conformer mixture 13a + 13b stereospecifically to 1-fluoro-β-elemene 14, and the complete absence of the C5 epimer of 14, even though the latter stereoisomer should be of comparable stability. The stereospecificity of this [3,3]sigmatropic process can be rationalized by the structural similarity of the UU conformer 13a (Scheme 4) to the stable chair conformation of 14 (Scheme 5). Thus, the suprafacial pericyclic transition state with partial bonding between C2/C3 and C10/C5 resembling a strain-free trans-decalin would be readily accessible from 13a. In contrast, a concerted, suprafacial [3,3]-rearrangement of the UD conformer 13b would necessarily produce the C5 epimer of 14 through a highly strained boat/twist-boat conformation of the alternative cis-decalin-like transition state. The lower energy of chair-like transition states in the 1(10)-double bond and blocks the subsequent proton-induced cyclization to the cis-eudesmyl carbocation 16a, hydroxide- and methyl-shift rearrangements to the epi-ermophilenyl ion 16b, and proton elimination from C9.

The isolation of pure 1-fluorogeramocene A[35] as the sole product of TEAS catalysis is consistent with the general mechanism of this class of sesquiterpene synthases.[26b,14b] Initial ionization of the allylic PP to the corresponding allylic cation/PP-Mg anion pair, followed by electrophilic attack at C10 of the isoprenoid chain derived from the fluoro substrate analogue 12 generates the 1-fluoro analogue of the germacren-11-yl carbocation (15, Scheme 6). This carboxonation in turn undergoes rapid TEAS-mediated proton abstraction, presumably from the cis-terminal methyl group,[16] to form the isopropenyl moiety present in fluorogeramocene A. The inability of the enzyme to proceed further through protonation of the 1(10)-double bond,[36] and to generate the 1-fluoro analogue (17) of 5-epi-aristolochene, or other bicyclic fluoro sesquiterpene isomers, as the final sesquiterpene product of the reaction, is attributed to the intrinsic electronegative nature of the fluorine. This inductive effect decreases the π basicity of the fluoro-substituted penta-2,4-dienyl moiety present in fluorogeramocene A.

The stereochemical outcome of the hypothetical TEAS-catalyzed reaction leading to the halogen-free epiaristolochene 4 has been recently reported by Schenk et al.[16] using deuterated farnesyl PPs as substrates. These studies provide strong circumstantial evidence that the active site of TEAS directs the precise folding of the natural farnesyl PP substrate (1) within the protein to guarantee and to secure the UD conformation of the enzyme-bound germacrene A intermediate 2 needed to account for the anti relationship of the CH₃ groups present in 5-epi-aristolochene (4). Accordingly, 1-fluorogeramocene A would be expected to be enzymatically formed in the active site as the product of the hypothetical TEAS-catalyzed formation of 1α-fluoro-5-epiaristolochene (17) via (−)-1-fluorogeramocene A in the UD conformation, its highly unfavorable proton-induced cyclization to the cis-eudesmyl carbocation 16a, hydroxide- and methyl-shift rearrangements to the epi-ermophilenyl ion 16b, and proton elimination from C9.

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less-stable UD conformer 13b, but, once it is released from the enzyme, a rapid rotation of the $\Delta^{10}$-double bond through the ring would establish the observed equilibrium mixture of conformers favoring the more stable UU conformation 13a (Scheme 4).

Acid-catalyzed cyclizations of germacrene A and fluoro-germacrene A

$\alpha$- and $\beta$-Selinenes (19a and 18a), and the tetrasubstituted double-bond isomer selina-4,11-diene ($\alpha$-cyperene, 20a; Scheme 7), are well-known products from the protic cyclization of germacrene A in solution[27] and on silica gel.[37] In some instances, these eudesmane hydrocarbons have been reported as co-products of reactions catalyzed by wild-type TEAS[38] and by mutants of aristolochene synthase.[4,39] To evaluate the effect of the fluoro substituent on the chemically induced cyclization of germacrene A, we characterized and identified by TLC, GC, MS, optical rotation, and spectral properties, and by its thermal rearrangement to 1-fluoro-germacrene A (13) reported in this work provide further evidence for the intervention of germacrene A (2) as an enzy-
Experimental Section

Representative preparative procedures and characterization data for 6-fluorofarnesyl PP and the TEAS incubation product, as well as procedures and characterization data for all other compounds, reproductions of selected NMR spectra, and kinetic graphs are available in the Supporting Information.

(2E,6Z)-6-Fluoro-3,7,11-trimethylundeca-2,6,10-trien-1-ol (10; X = OH): The procedures of Sum and Weiler \(^{21}\) modified by Jin et al. \(^{5,6,16}\) were followed. iBu4AlH (1.0 M in hexane, 790 μL, 0.79 mmol) was added to a cold (−78 °C), stirred solution of fluoro ester 9 (74.0 mg, 0.26 mmol) in toluene (3 mL). After 1 h, MeOH (0.5 mL) was added, and the resulting solution was allowed to warm to room temperature. Sunseted aq. NH4Cl (30 mL) was added, followed by aq. HCl (5 mL, 3 M). The product was extracted with EtO (4 × 50 mL). The combined ethereal extracts were washed with brine (20 mL), dried over MgSO4, and concentrated under reduced pressure to afford essentially pure chloride (27.3 mg) in quantitative yield as a light yellow oil. TLC: \(R_f = 0.32\) (30% EtOAC/hexane); \(^1H NMR\) (400 MHz, CDCl3): \(\delta = 5.43\) (t of sextet, \(J = 7.0, 1.3\) Hz, 1H; vinyl H), 5.10 (brt, \(J = 6.6\) Hz, 1H; vinyl H), 5.11 (t of septet, \(J = 7.3, 1.5\) Hz, 1H; vinyl H), 4.14 (dd, \(J = 7.0, 0.7\) Hz, 2H; CH2OH), 2.26–2.39 (m, 2H; CH2), 2.19 (dd, \(J = 8.5, 6.9\) Hz; CH2), 2.01–2.08 (m, 4H; CH2), 1.69 (s, 3H; CH3), 1.67 (s, 3H; CH3), 1.59 (s, 3H; CH3), 1.55 ppm (d, \(J = 2.7\) Hz; CH3); \(^1C NMR\) (127 MHz, CDCl3): \(\delta = 155.3, 138.9, 131.9, 124.2\) (d, \(J = 7.0\) Hz), 124.1, 111.8 (d, \(J = 17.4\) Hz; CH3), 114.8, 36.6, 29.9 (d, \(J = 7.6\) Hz), 27.6 (d, \(J = 29.6\) Hz), 26.5, 25.9, 17.9, 16.4, 15.7 ppm (d, \(J = 8.2\) Hz); \(^19F NMR\) (376 MHz, CDCl3): \(\delta = -113.2\) ppm (t, \(J = 22.8\) Hz); IR (neat film): \(\nu = 3235\), 2918, 1707, 1671, 1446, 13817, 11885, 11425, 11108, 17965, 10045, 9248, 8299 cm\(^{-1}\).

(2E,6Z)-1-Chloro-6-fluoro-3,7,11-trimethylundeca-2,6,10-triene (11; X = Cl): The procedure of Collington and Meyers \(^{20}\) was followed. Methanesulfonyl chloride (40 mg, 0.36 mmol) was added to a cold (0 °C) solution of anhydrous LiCl (48.0 mg, 1.2 mmol), allylic alcohol 10 (290 μL, 0.12 mmol), and 1-collidine (126.0 mg, 1.10 mmol) in DME (5 mL), and the resulting slurry was stirred at 0 °C for 1.5 h. Water (15 mL) and ether (10 mL) were added, and the organic layer was washed with water (2 × 5 mL). The combined aqueous layers were extracted with ether (3 × 10 mL). The combined ethereal extracts were washed with saturated aq. CuSO4 water, and brine and dried over MgSO4. The solvent was evaporated under reduced pressure to afford essentially pure chloride 11 (31.2 mg) in quantitative yield as a light yellow oil. TLC: \(R_f = 0.81\) (15% EtOAc/hexane); \(^1H NMR\) (400 MHz, CDCl3): \(\delta = 5.48\) (t of sextet, \(J = 7.8, 1.3\) Hz, 1H; vinyl H), 5.13 (t of septet, \(J = 6.8, 1.4\) Hz, 1H; vinyl H), 5.10 (brt, \(J = 7.0, 1.0\) Hz; vinyl H), 4.08 (d, \(J = 7.8\) Hz; 2H; CH2Cl), 2.28–2.39 (m, 2H; 2H; CH2), 2.21 (dd, \(J = 8.0, 6.9\) Hz; 2H; CH2), 2.00–2.04 (m, 4H; 2CH2); 1.74 (d, \(J = 1.4\) Hz; 3H; CH3), 1.68 (s, 3H; CH3), 2.10 (d, \(J = 6.6\) Hz; 3H; CH3); \(^1C NMR\) (127 MHz, CDCl3): \(\delta = 155.3, 138.9, 131.9, 124.2\) (d, \(J = 7.0\) Hz), 124.1, 111.8 (d, \(J = 17.4\) Hz; CH3), 114.8, 36.6, 29.9 ppm (d, \(J = 8.2\) Hz); \(^19F NMR\) (376 MHz, CDCl3): \(\delta = -113.6\) ppm (t, \(J = 22.8\) Hz); IR (neat film): \(\nu = 3232\), 2918, 1707, 1671, 1446, 13817, 11885, 11425, 11108, 17965, 10045, 9248, 8299 cm\(^{-1}\).
aqueous solutions (50 mL each) were prepared. The Tris-HCl solution was adjusted to pH 7.5 by adding powdered NaOH. The pH of the final Tris-HCl/MgCl₂ buffer solution (1:1, 100 mL) was 7.56–7.59. A solution of TEAS (10 mg) in buffer (5 mL) was added to a gently stirred solution of 6-fluorofarnesyl PP (12.0 mg, 0.027 mmol) in buffer (75 mL) to give a final substrate concentration of ca. 350 μM and an enzyme concentration of 0.125 mg mL⁻¹. The solution was cooled and applied directly to a preparative TLC plate. The toluene was evaporated under a stream of N₂. The plate solution was cooled and applied directly to a preparative TLC plate.

**Keywords**: biosynthesis · carboxylations · enzyme catalysis · farnesyl diphosphate · fluoride · terpenoids


[17] The preceding article by Miller, Yu, and Allemann reports the enzymatic cyclization of 2-fluorofarnesyl PP to 5-fluorogermacrene A with recomb. terpene cyclase from Penicillium roqueforti.


[29] The R values in parenthesis refers to the relative populations of each conformer, which were calculated by integration of the individual H NMR signals.

[30] The conformations of flexible germacrenes are denoted as UD, DD, UD and DU. U (up) and D (down) refer to the orientations of C14 and C15.

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[31] (±)-Germacrene A behaves in solution (25 °C) as a 5:3:2 mixture of UU, UD, and DU conformers, respectively. See ref. [24].

[32] N. L. Allinger, J. Am. Chem. Soc. 1977, 99, 8127–8134; the relative steric energies (kcal mol⁻¹) of the four possible conformers of 1-fluorogermacrene A calculated by the Allinger MM2 force-field method were as follows: UU (0.00), UD (0.55), DD (1.85), and DU (1.96).


[35] In the absence of any independent evidence concerning the enantiomeric purity and absolute configuration of the (±)-1-fluorogermacrene A product, we assume that the fluoro analogue has the same 7R stereochemistry of the isopropenyl group as 5-epi-aristolochene (4), since both are produced by TEAS with comparable catalytic efficiencies.


[41] Evidence supporting germacrene A as a neutral intermediate in TEAS and aristolochene synthase catalysis has been provided by Chappell[13] Allemann[4a] and Cane[40] through site-directed mutagenesis.


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