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

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## Abstract

Tobacco 5-epi-aristolochene synthase (TEAS) catalyzes the $Mg^{II}$-dependent cyclizations and rearrangements of (E,E)-farnesyl diphosphate (PP) to the bicyclic sesquiterpene hydrocarbon via a tightly bound (+)-germacrene A as a deprotonated intermediate. With the native enzyme, only a few percent of the putative germacrene A intermediate is released from the active site during the catalytic cycle. 6-Fluorofarnesyl PP was designed and synthesized with the aim of arresting the cyclization-rearrangement mechanism en route to 5-epi-aristolochene. Indeed, incubation of (2*E*, 6*Z*)-6-fluorofarnesyl PP with recombinant TEAS afforded (-)-1-fluororogermacrene A as the sole product in 58% yield. Steady-state kinetic experiments with farnesyl PP and the 6-fluoro analogue showed that the overall catalytic efficiencies ($k_{cat}/K_m$) are essentially the same for both substrates. 1-Fluorogermacrene A was characterized by chromatographic properties (TLC, GC), MS, optical rotation, UV, IR and $^1$H NMR data, and by heat-induced Cope rearrangement to (+)-1-fluoro-β-elemene. $^1$H NMR spectra at room temperature revealed that this (E,E)-configured fluorocyclodecadiene exists in solution as a 7:3 mixture of UU and UD conformers. 1-Fluorogermacrene A underwent trifluoroacetic acid-catalyzed cyclization to give three 1α-fluoroselinene isomers at a rate estimated to be about 1000 times slower than that of the similar cyclization of (+)-germacrene A to the parent selinenes.

### Keywords

biosynthesis; carbocations; enzyme catalysis; farnesyl diphosphate; fluorine; terpenoids

## Introduction

Terpene synthases catalyze the multistep conversions of their polyprenyl substrates into numerous acyclic polyenes and carbocyclic structures through series of alkylations,

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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.[1] 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 diphosphates (PPs), (*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.[2] 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,[3] aristolochene,[4] pentalenene,[5] and germacradienol-germacrene D[6] synthases.

Fluoro analogues have been useful in the study of enzyme mechanisms,[7] 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 monoterpene 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 verticillene products formed by taxadiene synthase provided persuasive evidence for a H10α configuration in the proposed verticillenyl carbocation intermediate in the enzyme-catalyzed mechanism.[12] The pentacyclization of (3*S*)-2,3-oxidosqualene catalyzed by squalene-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 premnaspirodiene synthases (TEAS and HPS)[14] catalyze the cyclizations and rearrangements of farnesyl PP (**1**) to their respective bicyclic sesquiterpene hydrocarbon products as key initiating steps in the biosynthesis of the phytoalexins, capsidiol, solavetivone, and related metabolites.[15] Considerable evidence supports the formation 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 followed by proton transfers give rise to epiaristolochene (**4**) and premnaspirodiene (**5**), respectively. The isolation of germacrene A from incubations of the catalytically impaired mutant form of TEAS, Y520F,[3] as well as the incorporation of a single deuterium at the 1β position of **4** and the retention of the label in **5** from incubations in deuterated media,[16] support the mechanism shown. In this paper, we report the efficient TEAS-catalyzed conversion of (2*E*,6*Z*)-6-fluorofarnesyl PP to (-)-1-fluorogermacrene 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 protonation.

## 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-precedented methods[12,18] and requires only a brief description. The starting material 2-fluorogeraniol (**6**)[19] was prepared by olefination[20a] of 6-methylhept-5-en-2-one with diethyl fluorophosphonoacetate[20b] followed by reduction with $LiAlH_4$ and separation of the resulting *cis* and *trans* isomers by chromatography. Isoprenoid chain extension with acetoacetate dianion, *trans*-selective enol phosphorylation, methyl cuprate coupling, and ester reduction with *i*$Bu_2AlH$ afforded the known (2*E*,6*Z*)-6-fluorofarnesol[19b] in 50% overall yield by following procedures developed earlier for the synthesis of 6-fluorogeranylgeraniol,[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 $S_N2$ displacement with tris(tetrabutylammonium) pyrophosphate,[22] and was characterized by satisfactory $^1H$, $^{31}P$, and $^{19}F$ NMR spectra.

### Enzymatic cyclization of 6-fluorofarnesyl PP and characterization of 1-fluorogermacrene A (13)

Initial analytical-scale incubations monitored by GC-MS[23] 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 catalytic efficiency of the reaction are reported in Table 1, along with comparable data for the unmodified substrate farnesyl PP (**1**) under the same conditions. Although the recombinant cyclase exhibits an approximately 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 binding, diphosphate ionization, and the initial nucleophilic attack of the C10=C11 double bond.

Preparative-scale incubations of 6-fluorofarnesyl PP $NH_4^+$ salt (**12**) with TEAS performed according to Schenk et al.[16] with modifications [0.125 mg $mL^{-1}$ enzyme; substrate at 332 μm, buffer 200 mM Tris·HCl (pH 7.5), 40 mM $MgCl_2$] 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 ($[\alpha]_D$ = -28.1° in $CDCl_3$) is opposite in sign to that of the parent (+)-germacrene A ($[\alpha]_D$ = + 42.1 in $CCl_4$);[24] this clearly indicates a substantial effect of the fluoro substituent on the interaction of the modified sesquiterpene with polarized light. GC analyses (method A, see the Supporting Information) of the pentane/$Et_2O$ extracts from the incubation medium showed a single peak ($t_R$ = 20.1 min), albeit with a hump in the base line that is characteristic of (*E,E*)-configured germacrenes owing to their thermal Cope rearrangement to elemenes 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 min) corresponding to 1-fluoroelemene (**14**) was observed.

The MS of 1-fluorogermacrene A (**13**) shows a molecular ion peak at *m*/*z* 222 ($[M]^+$,9%, $C_{15}H_{23}F$) and an initial loss of 15 *m*/*z* units ($[M\text{-}CH_3]^+$, 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 ($[M\text{-}CH_3]^+$, 37%)).[26] The IR spectrum and data are also very similar to those observed for germacrene A,[26,27] but 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 $\lambda_{max}$ = 210-214 nm for the 1-fluoro sesquiterpene **13**, a spectral property observed for other flexible (*E,E*)-germacrenes that suggests a transannular

interaction between the double bonds;[28] instead a weak absorption appears at $\lambda_{max}$ = 240 nm (log $\varepsilon$=0.3).

500 MHz $^1$H NMR spectra of **13** were recorded in $CDCl_3$ 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 (brs, 1H; H5, 30%), 4.89 (brs, 1H; H5, 70%), 4.68 (s, 1H; H12, 30%), 4.66 (s, 1H; H12, 70%), and 4.58 ppm (s, 1H; H12′, 100%).[29] 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 $CH_3$ 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 $CH_3$ signals in the spectrum to the UU and UD conformations (**13a** and **13b**, Scheme 4)[30] proposed for the major and minor species present were made by comparison with those recently ascertained for the most abundant conformers (UU and UD) of (+)-germacrene A by using variable-temperature NMR and NOE spectroscopy.[24] In addition, the 470 MHz $^{19}$F 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 UU conformational isomers. The doubled sets of NMR signals in both the $^1$H and $^{19}$F NMR spectra of **13** clearly indicate that this *E,E*-configured cyclodecadiene behaves in solution (25 °C) as an equilibrium mixture of two interconvertible conformational isomers in a 7:3 ratio.[31] This experimental result is in excellent agreement with Allinger MM2 calculations,[32] which predict conformers UU (72%) and UD (28%) to be the most stable and therefore the most populated in the ground state at room temperature. (Scheme 4)

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-fluorogermacrene 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 $^1$H 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 conformer of **14** (Scheme 5) were confirmed by the appearance of a doubletof 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 ($[\alpha]_D$=+28.0° in $CDCl_3$), and like its (-)-fluorogermacrene A progenitor, is of opposite sign compared to the unfluorinated parent (β-elemene, $[\alpha]_D$=-15.8° in $CHCl_3$)[24] Also noteworthy is the rearrangement of the inter-converting 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]-rearrangementof 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 [3,3]sigmatropic rearrangements, compared to the boat-like or twisted alternatives, is well established.[34]

The isolation of pure 1-fluorogermacrene A[35] as the sole product of TEAS catalysis is consistent with the general mechanism of this class of sesquiterpene synthases.[2b,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 carbocation in turn undergoes rapid TEAS-mediated proton abstraction, presumably from the *cis*-terminal methyl group,[16] to form the isopropenyl moiety present in fluorogermacrene 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 1(10)-double bond and blocks the subsequent proton-induced cyclization to the 1α*a*-fluoro-*cis*-eudesmyl ion **16a**. The hypothetical TEAS-catalyzed cyclization of the enzyme-bound fluorogermacrene A intermediate en route to 1α-fluoro-epiaristolochene would imply the formation of the fluorocarbocation **16b**, which would also be destabilized by the inductive effect of the adjacent C-F.

The stereochemical outcome of the 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 $CH_3$ groups presentin 5-epi-aristolochene (**4**). Accordingly, 1-fluorogermacrene A would be expected to be enzymatically formed in the active site as the less-stable UD conformer **13b**, but, once it is released from the enzyme, a rapid rotation of the $\Delta^{1(10)}$-double bond through the ring would establish the observed equilibrium mixture of conformers favoring the more stable UU conformation **13a** (Scheme 4).

## Acid-catalyzed cyclizationsof germacrene A and fluorogermacrene A

α- and β-Selinenes (**19a** and **18a**), and the tetrasubstituted double-bond isomer selina-4,11-diene (α-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 cyclizations, we treated (+)-germacrene A (**2**) [24] and (-)-fluorogermacrene A (**13**) with $CF_3CO_2H$ in $CDCl_3$ and monitored the reactions by NMR spectroscopy. Exposure of **2** to 0.1 equiv of $CF_3CO_2H$ in $CDCl_3$ resulted in rapid cyclization to a mixture of **18a**, **19a**, and **20a** in about a 5:2.5:1 ratio, respectively. The progress of the reaction ($t_{1/2}$=2 min, 25 °C) was followed by the gradual replacement of the characteristic H1 and H5 olefinic proton signals (4.78 and 4.51 ppm) of the major UU conformer of germacrene A[24] with the diagnostic angular methyl singlets of **18a** (0.73 ppm), **19a** (0.80 ppm), and **20a** (1.05 ppm).[33a] Although the individual sesquiterpenes were not separated from the resulting three-component mixture, the compounds were readily identified by comparison of the spectral data with the literature values.[33a]

In contrast, 13 was stable under the same acidic conditions (0.1 equiv $CF_3CO_2H$ in $CDCl_3$ at 25 °C) that brought about the rapid cyclization of germacrene A. However, slow cyclization occurred following the gradual addition of 4 equiv of trifluoroacetic acid ($t_{1/2}$~50 min) and led to a 2:2:1 mixture of the corresponding 1-fluoro selinenes **18 b** + **19 b** + **20 b**, which were readily distinguished and quantitated by the sharp signals for their angular methyl groups ($\delta_H$=0.72, 0.75, and 0.99 ppm, respectively). In this case, the fluoro selinene isomers could be separated by preparative TLC on silica gel (61% recovery), and individually characterized by their $^1H$ and $^{19}F$ NMR spectra and by low- and high-resolution EI-MS data. The doublets of triplets observed in the $^{19}F$ NMR spectra of **18b** ($\delta_F$=-191.33, *J*=47.4, 12.4 Hz) and **19b** ($\delta_F$=-183.90, **J**=48.9, 22.9 Hz) arise from axial/axial and axial/equatorial couplings to the protons on C2, in support of the 1α (axial) configurations of the fluoro substituents shown in Scheme 7. The ratio of the half-times and acid-concentration differences indicates that fluorogermacrene A undergoes proton-induced cyclization approximately 1000 times more slowly than its unfluorinated parent under the same conditions. Probably the inductive influence exerted by the fluorine in this case is attenuated somewhat as a consequence of transannular interaction in the transition state for protonation, and the resulting delocalization of positive charge onto C4. Nevertheless the 1000-fold rate retardation would clearly be sufficient to prevent any detectable cyclization by TEAS, and to allow fluorogermacrene A time to diffuse from the active site.

## Conclusions

Synthetic substrate analogues have played a critical role in elucidating the reaction mechanisms of terpene synthases, as exemplified by the use of 1,10-dihydrofarnesyl PP by Cane and Tsantrizos[40] to demonstrate the cryptic germacrene A synthase activity of aristolochene synthase. The synthesis of 6-fluorofarnesyl PP (**12**) and its efficient enzymatic conversion to 1-fluorogermacrene A (**13**) reported in this work provide further evidence for the intervention of germacrene A (**2**) as an enzyme-bound neutral intermediate in the TEAS-catalyzed multistep conversion of farnesyl PP into 5-epi-araistolochene (**4**),[41] and contributes a new chemical tool for mechanistic studies of terpene biosynthesis. Furthermore, steady-state kinetic measurements with both farnesyl PP and its 6-fluoro analogue show that TEAS maintains the same catalytic efficiency ($k_{cat}/K_m$) for both substrates. Thus the =C-H→=C-F alteration at the C6 position of farnesyl PP (**1**) has negligible effects on enzyme binding, substrate orientation, diphosphate ionization, and the initial 1,10 ring closure catalyzed by TEAS.

The product of the abortive mechanism, (-)-1-fluorogermacrene A, was characterized and identified by TLC, GC, MS, optical rotation, and spectral properties, and by its thermal rearrangement to 1-fluoro-β-elemene. $^1H$ NMR spectra of 1-fluorogermacrene A showed that this sesquiterpene analogue exists as a mixture of UU and UD conformers in an approximately 7:3 ratio at room temperature. The more stable conformer (70%) was assigned as the UU (up-up) isomer, in which the two methyl groups and the isopropenyl moiety adopt positions on the top face of the crossed (*E,E*)-configured cyclodecadiene ring, similar to the more stable conformer of the parent germacrene A. Thus, the presence of the 6-fluoro substituent has negligible effects on the conformational properties of the cyclodecadiene ring system, while dramatically altering its reactivity, and thereby provides an effective mechanistic probe for TEAS and related sesquiterpene cyclases.

## Experimental Section

Representative preparative procedures and characterization data for 6-fluorofarnesyl PP and the TEAS incubation product, as well as the methods for kinetic measurements, are given below. General experimental aspects, materials and methods, 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.

### (2*E*,6*Z*)-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.[12,18] were followed. *i*$Bu_2AlH$ (1.0 M in hexane, 790 μL, 0.79 mmol) was added to a cold (-78 °C), stirred solution of fluoroester **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. Saturated aq. $NH_4Cl$ (30 mL) was added, followed by aq. HCl (5 mL, 3 M). The product was extracted with $Et_2O$ (4×50 mL). The combined ethereal extracts were washed with brine (20 mL), dried over $MgSO_4$, and concentrated under reduced pressure. Purification by flash chromatography on silica gel (15% EtOAc/hexane) afforded the known[19b] alcohol **10** (56.0 mg, 88%). TLC: $R_f$=0.32 (30% EtOAc/hexane); $^1$H NMR (400 MHz, $CDCl_3$,): δ=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; $CH_2OH$), 2.26-2.39 (m, 2H; $CH_2$), 2.19 (dd, *J*=8.5, 6.9 Hz, 2H; $CH_2$), 2.01-2.08 (m, 4H; $2CH_2$), 1.69 (s, 3H; $CH_3$), 1.67 (s, 3H; $CH_3$), 1.59 (s, 3H; $CH_3$), 1.55 ppm (d, *J*=2.7 Hz, 3H; $CH_3$); $^{13}$C NMR (127 MHz, $CDCl_3$); δ=155.3, 138.9, 131.9, 124.2 (d, *J*=7.0 Hz), 124.1, 111.8 (d, *J*=17.4 Hz), 59.5, 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*=6.2 Hz); $^{19}$F NMR (376 MHz, $CDCl_3$): δ=-113.2 ppm (t, *J*=22.8 Hz); IR (neat film): ν=3325.9, 2918.9, 1707.0, 1671.2, 1448.6, 1381.7, 1188.5, 1142.5, 1110.8, 1976.5, 1004.5, 924.8, 829.9 $cm^{-1}$.

### (2*E*,6*Z*)-1-Chloro-6-fluoro-3,7,11-trimethylundeca-2,6,10-triene (11; X = Cl)

The procedure of Collington and Meyers[42] 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** (29.0 mg, 0.12 mmol), and *s*-collidine (126.0 mg, 1.10 mmol) in DMF (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. $CuSO_4$, water, and brine and dried over $MgSO_4$. The solventwas 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); $^1$H NMR (400 MHz, $CDCl_3$): δ=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 Hz, 1H; vinyl H), 4.08 (d, *J*=7.8 Hz, 2H; $CH_2Cl$), 2.28-2.39 (m, 2H; $CH_2$), 2.21 (dd, *J*=8.0, 6.9 Hz, 2H; $CH_2$), 2.00-2.10 (m, 4H; $2CH_2$), 1.74 (d, J=1.4 Hz, 3H; $CH_3$), 1.68 (s, 3H; $CH_3$), 1.60 (s, 6H; $CH_3$), 1.56 ppm (d, *J*=2.6 Hz, 3H; $CH_3$); $^{13}$C NMR (127 MHz, $CDCl_3$): δ=155.3, 138.9, 131.9, 124.2 (d, *J*=7.0 Hz), 124.1, 111.8 (d, *J*=17.4 Hz), 41.0, 36.4, 29.9 (d, *J*=7.4 Hz), 27.3 (d, *J*=29.8 Hz), 26.4, 25.8, 17.8, 16.2, 15.7 ppm (d, *J*=6.1 Hz); $^{19}$F NMR (376 MHz, $CDCl_3$): δ=-113.6 ppm (t, *J*=23.0 Hz).

### (2*E*,6*Z*)-6-Fluoro-3,7,11-trimethylundeca-2,6,10-trien-1-yl diphosphate, trisammonium salt (12)

The procedure of Poulter and co-workers[22] was followed. Chloride **11** (25.0 mg, 0.10 mmol) was added to a well-stirred solution of tris(tetrabutylammonium) hydrogen pyrophosphate trihydrate (180.0 mg, 0.18 mmol) in dry acetonitrile (1 mL) containing 3 Å molecular sieves (250 mg). The mixture was allowed to stir at room temperature for 21 h, and the solvent was then removed under reduced pressure by using a 40 °C water bath to yield the $Bu_4N$ salt of diphosphate **12** containing excess $(Bu_4N)_3HOPP$ as yellow oil (182 mg). $^1$H NMR (400 MHz, $CD_3OD$): δ=5.46 (t, *J*=6.2 Hz, 1H), 5.10 (m, 2H; vinyl H), 4.57 (t, *J*=6.5 Hz, 2H; $CH_2OPP$), 3.19-3.29 (m, 24H; $CH_2$), 2.27-2.40 (m, 2H; $CH_2$), 2.12-2.2 (m, 2H; $CH_2$), 2.02-2.08 (m, 4H; $2CH_2$), 1.62-1.72 (m, 24H; $CH_2$), 1.63 (s, 3H; $CH_3$), 1.62 (s,

3H; $CH_3$), 1.60 (s, 3H; $CH_3$), 1.58 (d, *J*=2.8 Hz, 3H; $CH_3$), 1.42 (sextet, *J*=7.4 Hz, 24H; $CH_2$), 1.02 ppm (t, *J*=7.2 Hz, 36H; $CH_3$); $^{31}$P NMR (162 MHz, $CD_3OD$); δ=2.23 (s, inorganic monophosphate), -6.60 (s, PPi), -8.29 (d, *J*=19.6 Hz), -8.77 ppm (d, *J*=19.6 Hz); $^{19}$F NMR (376 MHz, $CD_3OD$): δ=-114.3 ppm (t, *J*=23.3 Hz). The salt was dissolved in ion-exchange buffer (1 mL; 25 mM $NH_4HCO_3$ in 2% propan-2-ol/water, *v*/*v*) and loaded onto a column of BioRad $(NH_4)^+$ cation-exchange resin (ammonium form). The flask was washed with buffer (2×15 mL), and both washes were loaded into the column before elution with ion-exchange buffer (28 mL). Fractions containing diphosphate **12** were combined and lyophilized (18 h) to give a white solid. Solid-liquid extraction with MeOH (3×6 mL) and removal of the solvent under reduced pressure at room temperature gave the trisammonium salt of diphosphate **12** (15.0 mg, 33% overall). $^1$H NMR (400 MHz, $CD_3OD$): δ=5.45 (t, *J*=6.6 Hz, 1H; vinyl H), 5.09 (m, 1H; vinyl H), 4.52 (t, *J*=6.1 Hz, 2H; C$H_2$OPP), 2.27-2.40 (m, 2H; $CH_2$), 2.13-2.19 (m, 2H; $CH_2$), 2.01-2.07 (m, 4H; 2$CH_2$), 1.71 (s, 3H; $CH_3$), 1.66 (s, 3H; $CH_3$), 1.59 (brs, 3H; $CH_3$), 1.57 ppm (d, *J*=2.7 Hz, 3H; $CH_3$); $^{31}$P NMR (162 MHz, $CD_3OD$): δ=8.56 (d, *J*=19.7 Hz), -9.06 ppm (d, *J*=19.5 Hz); $^{19}$F NMR (376 MHz, $CD_3OD$): δ=-114.4 (t, *J*=24.5 Hz). A larger-scale run afforded 112 mg (44%) of diphosphate **12**.

## Steady-state kinetic measurement of TEAS-mediated turnover of 6-fluorofarnesyl PP

Recombinant TEAS was purified for kinetic analyses as previously described.[23] In brief, 500 μL-scale reactions were conducted in triplicate at room temperature and neutral pH with TEAS (15 nm) and variable concentrations of 6-fluorofarnesyl PP (**12**). Reaction products were analyzed on a Hewlett-Packard 6890 gas chromatograph coupled to a 5973 mass-selective detector (MSD) equipped with an HP-5MS capillary column (0.25 mm i.d.×30 m, with 0.25 m Wlm; Agilent Technologies). Product quantification was performed in SIM mode, set to detect ions with *m*/*z* 68, 93, 179, and 220. The GC was operated at a He flow rate of 2 mL $min^{-1}$, and the MSD was operated at 70 eV. Split-less injections (2 μL) were performed with an inlet temperature of 250 °C, a temperature that drives the Cope rearrangement to completion. The GC was programmed with an initial oven temperature of 50 °C (5 min hold), which was then increased by 10 °C $min^{-1}$ up to 180 °C (4 min hold), followed by a 100 °C $min^{-1}$ ramp until 240 °C (1 min hold). A solvent delay of 8.5 min was allowed prior to the acquisition of the MS data. (-)-1-Fluorogermacrene A, detected as (+)-1-fluoro-β-elemene, was quantified by integration of peak areas by using Enhanced Chemstation (version B.01.00, Agilent Technologies). The GC-MS instrument was calibrated with an authentic (+)-1-fluoro-β-elemene standard. Corrected velocity data (Table 1) were fitted to the Michaelis-Menten equation by using GraphPad Prism (version 4.00 for Windows, GraphPad Software).

## Incubation of 6-fluorofarnesyl PP with TEAS

Preparative-scale enzyme incubations were carried out as described by Schenk et al.[16] Tris-HCl (200 mM, pH 5.52-5.60) and $MgCl_2$ (40 mm) stock 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/$MgCl_2$ 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**, 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 final reaction solution (80 mL) was overlaid with pentane (10 mL), sealed with parafilm, and stirred for 13 h at room temperature. The layers were separated, and the remaining aqueous solution was extracted with $Et_2O$ (3×5 mL). The combined extracts were dried ($Na_2SO_4$) and evaporated with a stream of nitrogen to give a clear oil. Purification was carried out by column chromatography on alumina (Brockmann I, standard grade, 150 mesh, 58 Å) with pentane as eluent. Fractions containing product according to TLC ($R_f$=0.68, pentane) were combined and evaporated with a stream of nitrogen to afford 3.4 mg (58%) of 1-fluorogermacrene A

(**13**) as a clear oil. $[\alpha]^{25}_{D}$=-28.1° (*c*=0.43, $CDCl_3$); $^1$H NMR (500 MHz, $CDCl_3$,25 °C): δ=5.11 (brs, 1H; minor conformer, H5), 4.89 (brs, 1H; major conformer, H5), 4.68 (s, 1H; minor conformer, H12), 4.66 (s, 1H; major conformer, H12), 4.58 (s, 1H; both conformers, H12′), 2.69 (t, *J*=12.5 Hz, 1H), 1.72 (d, *J*=1 Hz, 3H; both conformers, H13), 1.61 (brs, 3H; minor conformer, H15), 1.58 (brs, 3H; major conformer, H15), 1.42 (brs, 3H; major conformer, H14), 1.25 ppm (brs, 3H; minor conformer, H14); $^{19}$F NMR (470 MHz, $CDCl_3$, 25 °C): δ=-90.40 (app. asymm. t, $J_{app}$=27.7, 21.1 Hz, minor conformer), -111.37 ppm (d, *J*=42.8 Hz, major conformer); IR (liquid film, $CDCl_3$) ν=2927, 1645, 1445, 1259, 1129, 885, 803 $cm^{-1}$; UV ($CDCl_3$): $\lambda_{max}$ (log ε) 240 nm (0.3); GC $t_R$=20.4 min (method A in the Supporting Information); MS (EI): *m*/*z* (rel. int.): 222.2 (9), 207.2 (43), 193.2 (26), 179.1 (54), 165.1 (22), 147.1 (22), 139.1 (32), 119.1 (46), 107.1 (50), 93.1 (75), 81.1 (50), 68.1 (100).

### Cope rearrangement of 1-fluorogermacrene A to 1-fluoro-β-elemene (14)

A solution of 1-fluorogermacrene A (**13**; 1.7-1.9 mg, 0.009 mmol) in toluene (500 μL) was heated at reflux for 3 h. The solution was cooled and applied directly to a preparative TLC plate. The toluene was evaporated under a stream of $N_2$. The plate was developed by using pentane to give 1-fluoro-β-elemene (**14**; 1.3 mg, 68-76%) as a clear oil. TLC: $R_f$ = 0.79 (pentane); $[\alpha]^{25}_{D}$ = + 28.0° (*c*=0.22, $CDCl_3$); $^1$H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 4.80 (t, *J*=1.5 Hz, 1H; H12), 4.71 (s, 2H; H3), 4.63 (s, 1H; H12′), 4.50 (dd, *J*=20.2, 2.8 Hz, 1H; $H1_{cis}$), 4.29 (dd, *J*=52.5, 3.0 Hz, 1H; $H1_{trans}$), 2.49 (dd, *J*=13.0, 4.0 Hz, 1H; H5), 2.01-1.90 (m, 2H), 1.75 (s, 3H; H13 or H15), 1.73, (s, 3H; H15 or H13), 1.63 (m, 1H) 1.59 (m, 1H), 1.49 (m, 1H), 1.42 (dd, *J*=13.0, 4.0 Hz, 1H), 1.04 ppm (s, 3H; H14); $^{13}$C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 169.2 (C1), 150.1 (C3 or C11), 146.9 (C11 or C3), 112.3 (C4 or C12), 108.4 (C12 or C4), 89.1 (d, *J*=22.1 Hz, C2), 47.1, 45.1, 36.6, 32.3, 26.4, 24.2, 21.1, 16.2, 16.1; $^{19}$F NMR (470 MHz, $CDCl_3$, 25 °C): δ = -104.77 (dd, *J*=54.5, 21.2 Hz); GC $t_R$ = 16.6 min (method B in the Supporting Information). NMR assignments were made based on those reported by Brauchli and Thomas.[43]

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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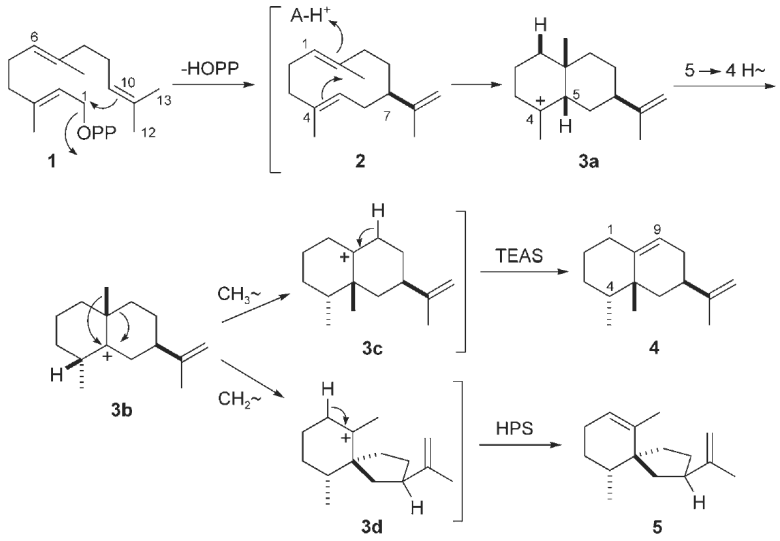
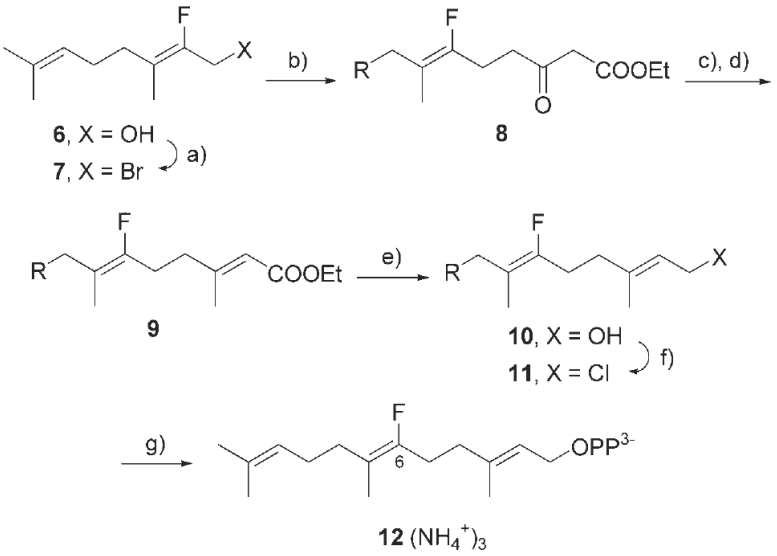


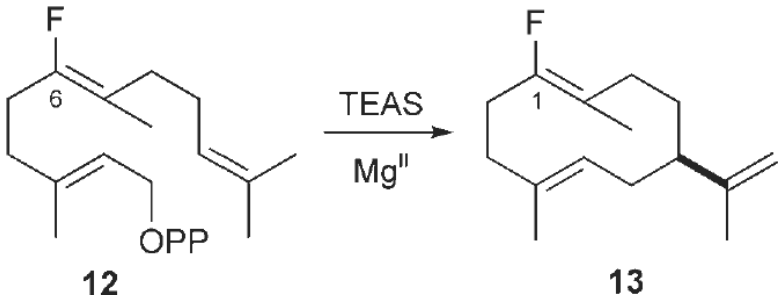

**Scheme 1.**

Mechanisms proposed for the cyclization and rearrangement of farnesyl di-phosphate (**1**) to 5-epi-aristolochene (**4**) and premnaspirodiene (**5**) catalyzed by tobacco 5-epi-aristolochene and henbane premnaspirodiene synthases (TEAS and HPS) proceeding through enzyme-bound germacrene A (**2**) and carbocations **3a-d**.



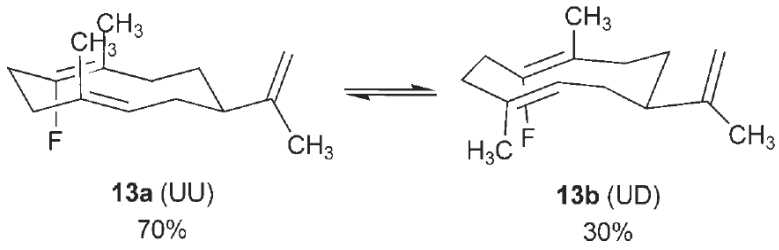

**Scheme 2.**

Synthesis of 6-fluorofarnesyl diphosphate as the trisammonium salt (**12**), R = dimethylallyl. a) $CH_3SO_2Cl$, $Et_3N$, LiBr/THF, -45 to 0 °C, 95 % yield; b) Ethyl acetoacetate, NaH, *n*BuLi/THF, 0 °C, 85 %; c) NaH, $(EtO)_2P(O)Cl/Et_2O$, 0 °C, quant.; d) CuI + MeLi, $Et_2O$, 0 to -78 °C, 70 %; e) *i*$Bu_2AlH$, toluene, -78 °C, 88 %; f) $CH_3SO_2Cl$, s-collidine, LiCl/DMF, 0 °C, quant.; g) $HOPP(NBu_4)_3$, $CH_3CN$, 25 °C, 21 h; ion exchange, 33-44 %.



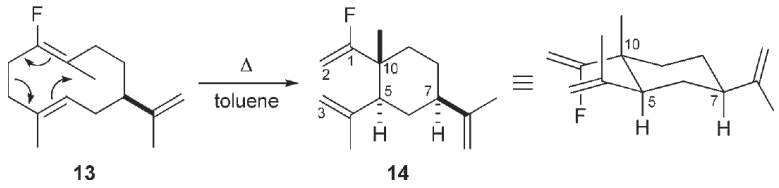

12 13

**Scheme 3.**
Cyclization of (2*E*,6*Z*)-6-fluorofarnesyl PP (**12**) to (-)-1-fluorogermacrene A (**13**) catalyzed by TEAS.




**Scheme 4.**

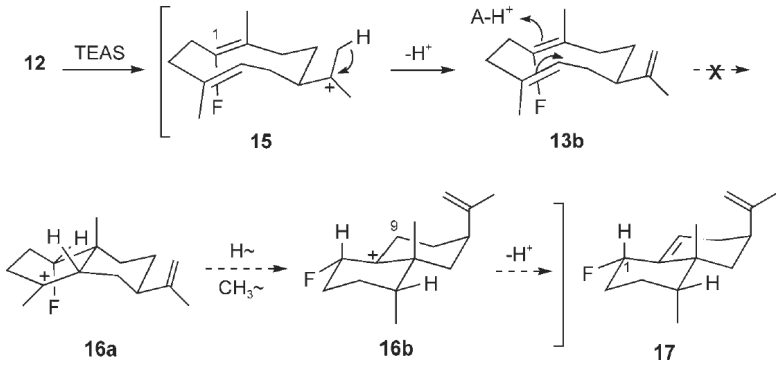
Structures and interconversion of the UU and UD conformers (**13 a** and **13 b**) assigned to the major and minor species observed in the $^{1}H$ and $^{19}F$ NMR spectra of 1-fluorogermacrene A (**13**). The designations “UU” and “UD” refer to the spatial orientation of the two ring methyl groups with respect to the plane of the cyclodecadiene ring (See ref. [30]).




13 14

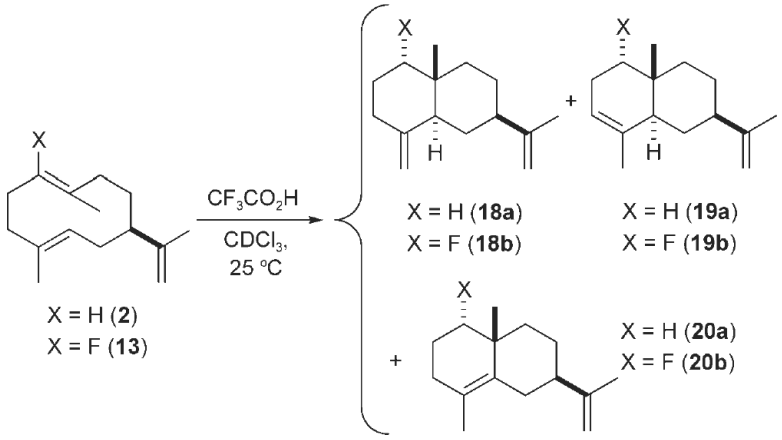
**Scheme 5.**

Cope rearrangementof 1-fluorogermacrene A (**13**) to 1-fluoro-β-elemene (**14**), and the stable chair conformation of the latter.




**Scheme 6.**

Stereochemical outcome of the hypothetical TEAS-catalyzed formation of 1α-fluoro-5-epiaristolochene (**17**) via (-)-1-fluorogermacrene A in the UD conformation, its highly unfavorable proton-induced cyclization to the *cis*-eudesmyl carbocation **16 a**, hydride- and methyl-shift rearrangements to the epi-eremophilenyl ion **16 b**, and proton elimination from C9.




**Scheme 7.**

Chemically induced cyclizations of germacrene A (**2**) and 1-fluorogermacrene A (**13**)to the *trans*-eudesmane sesquiterpenes α- and β-selinenes (**19 a** and **18 a**) and α-cyperene (**20 a**), and the corresponding 1α-fluoro analogues **18 b**, **19 b**, and **20 b** brought about by trifluoroacetic acid catalysis.

**Table 1**

Steady-state kinetic constants for the TEAS-catalyzed reactions of 6-fluorofarnesyl PP (12) and farnesyl PP (1).

| Kinetic constants | 6F-FPP | FPP[a] |
|---|---|---|
| $K_m$ [μm] | 19.67 | 8.40 |
| $k_{cat}$ [$min^{-1}$] | 6.57 | 2.50 |
| $k_{cat}/K_m$ | 0.33 | 0.30 |

[a]Values for farnesyl PP from ref. [23].