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A 1,6-Ring Closure Mechanism for (+)-δ-Cadinene Synthase?

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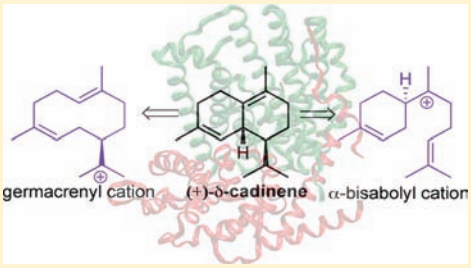
Supporting Information

ABSTRACT: Recombinant (+)-δ-cadinene synthase (DCS) from *Gossypium arboreum* catalyzes the metal-dependent cyclization of (*E*,*E*)-farnesyl diphosphate (FDP) to the cadinane sesquiterpene δ-cadinene, the parent hydrocarbon of cotton phytoalexins such as gossypol. In contrast to some other sesquiterpene cyclases, DCS carries out this transformation with >98% fidelity but, as a consequence, leaves no mechanistic traces of its mode of action. The formation of (+)-δ-cadinene has been shown to occur via the enzyme-bound intermediate (3*R*)-nerolidyl diphosphate (NDP), which in turn has been postulated to be converted to *cis*-germacradienyl cation after a 1,10-cyclization. A subsequent 1,3-hydride shift would then relocate the carbocation within the transient macrocycle to expedite a second cyclization that yields the cadinenyl cation with the correct cis stereochemistry found in (+)-δ-cadinene. An elegant 1,10-mechanistic pathway that avoids the formation of (3*R*)-NDP has also been suggested. In this alternative scenario, the final cadinenyl cation is proposed to be formed through the intermediacy of *trans*, *trans*-germacradienyl cation and germacrene D. In addition, an alternative 1,6-ring closure mechanism via the bisabolyl cation has previously been envisioned. We report here a detailed investigation of the catalytic mechanism of DCS using a variety of mechanistic probes including, among others, deuterated and fluorinated FDPs. Farnesyl diphosphate analogues with fluorine at C2 and C10 acted as inhibitors of DCS, but intriguingly, after prolonged overnight incubations, they yielded 2F-germacrene(s) and a 10F-humulene, respectively. The observed 1,10-, and to a lesser extent, 1,11-cyclization activity of DCS with these fluorinated substrates is consistent with the postulated macrocyclization mechanism(s) en route to (+)-δ-cadinene. On the other hand, mechanistic results from incubations of DCS with 6F-FPP, (2*Z*,6*E*)-FDP, neryl diphosphate, 6,7-dihydro-FDP, and NDP seem to be in better agreement with the potential involvement of the alternative biosynthetic 1,6-ring closure pathway. In particular, the strong inhibition of DCS by 6F-FDP, coupled to the exclusive bisabolyl- and terpinyl-derived product profiles observed for the DCS-catalyzed turnover of (2*Z*,6*E*)-farnesyl and neryl diphosphates, suggested the intermediacy of α-bisabolyl cation. DCS incubations with enantiomerically pure [1-$^2H_1$](1*R*)-FDP revealed that the putative bisabolyl-derived 1,6-pathway proceeds through (3*R*)-nerolidyl diphosphate (NDP), is consistent with previous deuterium-labeling studies, and accounts for the cis stereochemistry characteristic of cadinenyl-derived sesquiterpenes. While the results reported here do not unambiguously rule in favor of 1,6- or 1,10-cyclization, they demonstrate the mechanistic versatility inherent to DCS and highlight the possible existence of multiple mechanistic pathways.



INTRODUCTION

Terpene synthases catalyze complex reaction cascades with high regio- and stereochemical precision involving cyclizations (alkylations), rearrangements, and deprotonations of highly reactive carbocations.[1] Only recently has it become possible to address experimentally the intricate mechanistic details of these reactions through the use of substrate analogues,[2,3] aza-analogues of putative carbocationic intermediates,[4] mutant enzymes,[5] and X-ray crystallography.[6] Fluorine-containing analogues of enzyme substrates have been shown to be instrumental in mechanistic investigations,[7] and in particular, fluoro prenyl derivatives[3] have provided crucial insights regarding the cationic mechanisms of terpene synthases.[8] While the small size of the fluorine atom does not appear to significantly compromise active-site binding,[3d,h,6g,h,8b,d] its intrinsic electronegativity is known to inactivate fluoro-containing double bonds toward protonation and electrophilic alkylation[3c,d,g] and to alter the stability of allylic cation intermediates.[3h,8,9] Fluorine containing prenyl diphosphates have been used to study several terpene synthases including aristolochene synthases from the fungi *Aspergillus terreus* and *Penicillium roqueforti* (AT-AS and PR-AS),[3b,c,g,6h] tobacco 5-*epi*-aristolochene synthase (TEAS),[3d,h] trichodiene synthase (TS),[3e,8e,10] taxadiene synthase,[3a] limonene and (+)-bornyl diphosphate synthases,[3f,6g] and α-pinene synthase.[11]

δ-Cadinene synthase (DCS) from *Gossypium arboreum* is a sesquiterpene cyclase that catalyzes the metal-dependent conversion of farnesyl diphosphate (FDP, **1**) to the bicyclic hydrocarbon (+)-δ-cadinene (**6**) with a specificity of >98% (Scheme 1). This transformation is the first committed step in the biosynthesis of cotton phytoalexins such as gossypol.[12] Similar to all class I terpene cyclases,[6] DCS maintains the characteristic aspartate-rich D^{307}DXXD motif on helix D that

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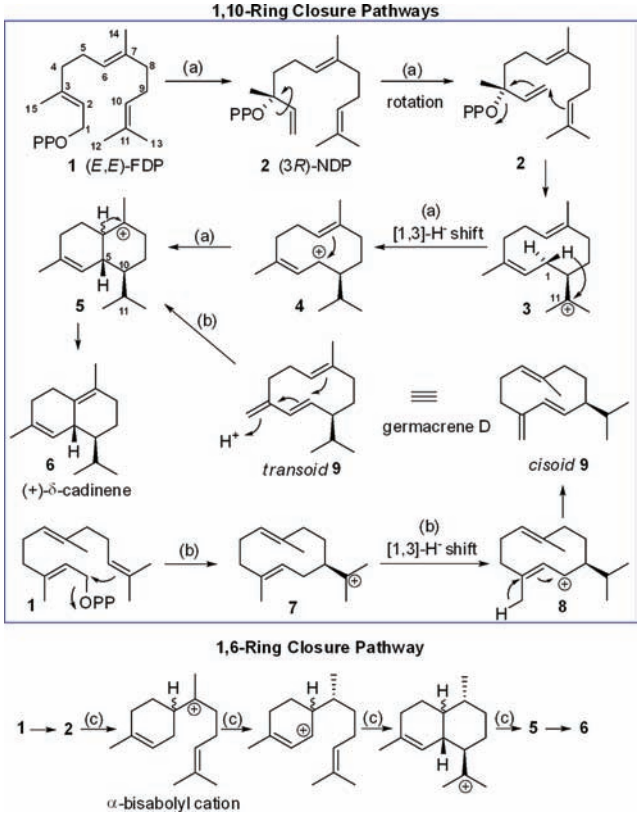
Scheme 1. Reaction Mechanisms Proposed in the Literature for DCS Catalysis[15,20]



chelates two (A and B) of the three Mg^{2+} ions essential for catalysis.[13] However, amino acid sequence alignments as well as X-ray structural analysis[13] have revealed the absence of the conserved second metal-binding "NSE/DTE" motif[6] on helix H. Instead, DCS possesses a second aspartate rich motif (D^{451}DVAE), similar to those found in "isoprenoid chain elongation enzymes" such as farnesyl diphosphate synthase.[14]

Mechanistically, the consensus in the literature[12a,13,15] is in favor of the DCS-catalyzed reaction path a outlined in Scheme 1. This pathway goes through (3*R*)-nerolidyl diphosphate ((3*R*)-NDP, **2**) as an enzyme-bound intermediate that after rotation around the C2,C3 σ-bond, leads to the correct orbital alignment for 1,10-macrocyclization and production of *cis*-germacradienyl cation (**3**).[1a] A subsequent C1 → C11 1,3-hydride shift of the original H-1$_{si}$ of **1**[15c,d] is followed by a 1,6-electrophilic ring closure reaction that generates cadinenyl cation (**5**), from which δ-cadinene (**6**) is formed after proton loss from C6. The tertiary diphosphate **2** has been shown to be a substrate of DCS,[15b,c] and its formation (or that of (2*Z*,6*E*)-FDP, **15**)[16] within the active site of the enzyme has been inferred from [1,2-$^{13}C_2$]acetate as well as [2-^{14}C] and [4-^{14}C]mevalonate labeled feeding experiments.[17,18] In closely related biosynthetic studies, (3*R*)-NDP (**2**) has also been identified as an enzyme-bound intermediate in (+)-1-*epi*-cubenol biosynthesis,[19] and more recently, **2** has been suggested as an intermediate in (−)-δ-cadinene biosynthesis.[15d]

The biosynthesis of cadinane-type sesquiterpenes could also occur without the intervention of enzyme bound NDP (**2**) that ultimately allows the formation of the cis C2,C3-double bond (FDP numbering) present in **6**.[20] In this alternative scenario (path b, Scheme 1), a 1,10-cyclization via the direct displacement of the diphosphate group of **1** yields the transient *trans*, *trans*-germacradienyl cation (**7**), which further undergoes a 1,3-hydride shift followed by proton loss from C15 to generate the neutral hydrocarbon germacrene D (**9**) as the key biosynthetic intermediate.[21] A conformational change of enzyme-bound **9**[20] gives rise to its reactive transoid conformer, which upon proton transfer to the exocyclic double bond and 1,6-ring closure produces the *Z*-configured cation **5**, the final carbocationic intermediate common to pathways (a) and (b). Recently, pathway (b) via germacrene D has been shown to operate in a promiscuous sesquiterpene synthase from *Medicago truncatula* (MtTPS5).[22]

A third mechanistic possibility is also outlined in Scheme 1 (path c). In this pathway, the formation of [5-^{2}H]- and [11-^{2}H]-δ-cadinene from racemic [1-^{2}H]-farnesyl diphosphate was explained by an initial 1,6-electrophilic ring closure that leads to α-bisabolyl cation as the key biogenetic precursor of hydrocarbon **6**.[15b] In support of this proposal, it has been reported previously that NDP (**2**), a reaction intermediate on path a, is converted by DCS to (*E*)-β-farnesene and β-bisabolene in addition to δ-cadinene.[15c] This observation is not easily explained if a 1,10-cyclization mechanism (e.g., path a) is followed. In addition, when [4,4,15,15,15-$^2H_5$]-nerolidyl diphosphate was used, no isotope loss to the solvent was observed,[15b,c] a result that makes unlikely the alternative 1,10-cyclization mechanism[22] via germacrene D (**9**) since it involves protonation/deprotonation at C15.

Here, we describe results obtained through the use of deuterated and fluorinated farnesyl diphosphates as mechanistic probes to examine the proposed DCS catalyzed reaction cascades summarized in Scheme 1. Enzymatic incubations with fluorinated FDP analogues (Figure 1) bearing fluorine at C2

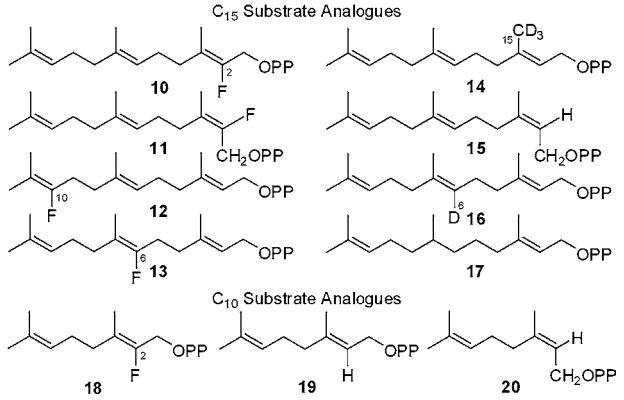


Figure 1. Farnesyl and geranyl diphosphate analogs used in the present study.

(**10** and **11**) and C10 (**12**) led to the formation of 2F-germacrenes (1,10-ring closure) and 10F-humulene (1,11-ring closure) as single products, results that are consistent with a macrocyclization reaction (paths a and b) as the biosynthetic means of (+)-δ-cadinene production. Conversely, however, the strong inhibition of DCS displayed by 6F-FDP (**13**), together with the exclusive bisabolyl- and terpinyl-derived product profiles observed from incubations with (2*Z*,6*E*)-farnesyl (**15**) and neryl (**20**) diphosphates and the absence of germacrene production (i.e., expected 1,10-cyclization activity) upon incubation with 6,7-dihydro-FDP (**17**) are in better agreement with a 1,6-ring closure mechanism (path c) by way of the (6*R*)-α-bisabolyl cation[23] (**37**). While these apparently conflicting

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mechanistic interpretations of the experiments described here do not unambiguously show whether 1,6- or 1,10 cyclization is the pathway followed during the DCS catalyzed turnover of FDP (**1**) to (+)-*δ*-cadinene (**6**), they imply a high degree of mechanistic versatility of DCS and may suggest the existence of multiple pathways to **6**.

RESULTS AND DISCUSSION

Incubation of DCS with (2*Z*,6*E*)-2F-FDP (10), (2*E*,6*E*)-2F-FDP (11), and [15,15,15-$^2H_3$]-FDP (14). The catalytic mechanism of DCS is thought to go by way of (3*R*)-NDP (**2**) involving a coupled ionization-isomerization-1,10-cyclization reaction (**1** → **3**, pathway a, Scheme 1).[15] Hence, the presence of a fluorine atom on C2 should prevent (or slow down), via depletion of electron density,[9] the heterolytic diphosphate ester cleavage reaction that secures the supply of farnesyl cation. Indeed, under steady-state kinetic conditions (2*Z*,6*E*)-2F-FDP (**10**) and (2*E*,6*E*)-2F-FDP (**11**) were found to inhibit the enzyme, albeit with reduced binding affinities (K_i = 70 μM and K_i = 30 μM for **10** and **11**, respectively) when compared to FDP (K_M = 3.2 μM),[13] indicating a rather modest competitive inhibition toward DCS (Supporting Information). Remarkably, the C2-fluorinated 'cis' isomer **11**[3h] binds to the active site of DCS approximately twice as tightly as its corresponding 'trans-configured' analogue **10**, which is structurally a better mimic of all-trans FDP (**1**). These kinetic results together with the better resemblance of diphosphate **15** (and the fluorinated analogue **11**) to the presumed reaction intermediate **2** support the potential intermediacy of (*E*,*Z*)-farnesyl diphosphate (**15**) or its corresponding cation in the biosynthesis of **6**.[17,18,20] This suggestion is in agreement with the observation that (3*R*)-NDP (**2**) binds to DCS with 10-fold greater affinity than FDP (**1**).[15c]

Despite the inhibitory properties of diphosphates **10** and **11** against DCS, GC−MS analyses of individual incubations of **10** and **11** revealed that the enzyme was able to turn over these analogues, giving in both cases single but different fluorinated hydrocarbons characterized by their molecular ions of *m*/*z* 222. The product generated from (2*Z*,6*E*)-2F-FDP (**10**) was readily identified by GC−MS as 2-fluorogermacrene A (**21**, Figure 2),

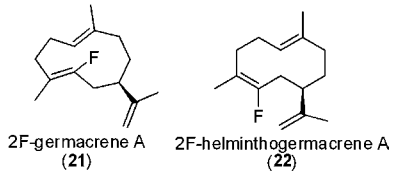

Figure 2. Structures of 2F-germacrene A (**21**) and 2F-helminthogermacrene A (**22**).

a fluorinated cyclodecadiene sesquiterpene isolated during previous mechanistic studies on aristolochene synthase (PR-AS) catalysis (Supporting Information).[3c] For comparison, analytical incubations of (2*E*,6*E*)-2F-FDP (**11**) were carried out with DCS and PR-AS. Similarly, both enzymes were able to turn over **11** to the same product **22** as judged by GC−MS analysis (Supporting Information). Compared to 2-fluorogermacrene A (**21**), the product formed from **11** was characterized by a shorter GC retention time and a much higher reluctance to undergo a thermal Cope rearrangement. On the other hand, **21** and **22** displayed almost identical MS fragmentation patterns. These results, together with ^{1}H- and ^{19}F-NMR spectroscopic analysis, indicated that **22** was indeed 2-fluoro-helminthogermacrene A, the C2,C3 cis isomer of **21**. The ^{1}H NMR spectrum of **22** showed no major changes when a $CDCl_3$ solution of **22** was cooled to −50 °C and then gradually warmed to +50 °C, a result that is in good accordance with the observation that the parent hydrocarbon helminthogermacrene A exists almost exclusively as one conformer at room temperature in C_6D_6.[24] In contrast, *all-trans*-germacrene sesquiterpenes exist as several interconvertible conformational isomers in solution, a dynamic effect that had been observed previously among others[25] for 2F-germacrene A,[3c] 6F-germacrene A,[3d] and (+)-germacrene A.[26]

These results indicate that the fluorinated diphosphates **10** and **11** were able to efficiently suppress the native isomerization of **1** to **2** (path a). Indeed, the DCS-generated 2-fluorogermacrenes **21** and **22** (Figure 2) were shown to preserve the original C2,C3-double bond geometry of their corresponding substrates **10** (trans) and **11** (cis) after the enzymatic 1,10-cyclization reaction. The relative ease with which **21** and **22** are formed is consistent with the possible involvement of the 1,10-macrocyclization(s) pathway assumed to operate in *δ*-cadinene biosynthesis. In particular, the maintenance of the substrate's *Z*/*E*-geometry of the C2,C3 double bond during the enzymatic reaction weighs in favor of the 1,10-pathway (b, Scheme 1) that avoids the formation of a cisoid NDP through the direct generation of *trans*, *trans*-germacrenyl cation[27] (**7**) and then germacrene D as the biosynthetic intermediates.[19,21] In support of pathway b, it could be reasoned that after the enzymatic 1,10-ring closure, the electron-withdrawing effect of the vinylic 2-fluoro substituent might prevent the native C1 → C11 hydride shift (**7** → **8**, Scheme 1) that relocates the positive charge at C1, thus, explaining the observed accumulation of 2F-germacrene A (**21**) or 2F-helminthogermacrene (**22**) after deprotonation. However, since the formation of *δ*-cadinene along pathway b would require protonation of the exocyclic double bond of germacrene D (**9**),[22] the reaction cascade would be expected to show, in addition to proton exchange with the solvent,[2g,i,22] a dependence on pH, as observed for catalysis by TEAS,[28] the maize enzymes TPS6 and TPS11,[2i] the fungal cyclase Cop4,[2p] and some Cop4 mutants.[5o] However, in contrast to Cop4,[2p,5o] the catalytic cycle of DCS was not disrupted by higher values of pH (7.5 → 10.5) and no accumulation of germacrene D was observed (Supporting Information).[29] Moreover, incubations of [15,15,15-$^2H_3$]-FDP (**14**) with DCS in aqueous buffer (H_2O) led to the release of [15,15,15-$^2H_3$]-*δ*-cadinene with the deuterium content intact (Supporting Information). These observations are in agreement with those obtained with [4,4,15,15,15-$^2H_5$]-NDP[15b] and seem to rule out pathway b[20] (Scheme 1).

Incubation of 10F-FDP (12) with DCS. Similar conclusions were reached from experiments with 10F-FDP (**12**). The reduced electron density of the C10,C11-double bond in **12**, coupled to the destabilizing effect of the 10-fluoro-substituent on the developing positive charge on C11 during DCS catalysis, was anticipated to prevent the proposed 1,10-cyclization reaction (Scheme 1). Consequently, the formation of 10F-farnesenes or 10F-bisabolenes arising from the putative (3*R*)-10F-NDP was expected. Diphosphate **12** acted as a competitive inhibitor (K_i = 16.5 μM) of DCS, a result that suggests a potentially high energy barrier for the isomerization of **12** to (3*R*)-10F-NDP during DCS catalysis in support of the DCS-catalyzed 1,10-pathway. DCS was also able to turn over diphosphate **12** yielding a single (>95% by GC−MS)

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fluorinated hydrocarbon (**26**) that failed to undergo thermal Cope rearrangements even at temperatures as high as 250 °C, thus, most likely ruling out a germacrene hydrocarbon as the enzymatic product.[30] The mass spectrum of this fluorinated-hydrocarbon was different from that of an authentic sample of enzymatically generated (*E*)-10F-*β*-farnesene (**25**) (Supporting Information).[31a] In addition, despite the striking similarity between the EI MS spectra of this fluorinated hydrocarbon and (*Z*)-*α*-farnesene (Supporting Information), the chemical generation of an authentic 3:1:1 mixture of 10F-farnesenes[31b] (**23**−**25**, Figure 3) and subsequent GC/MS comparisons with

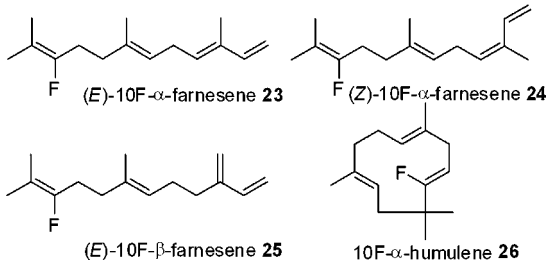


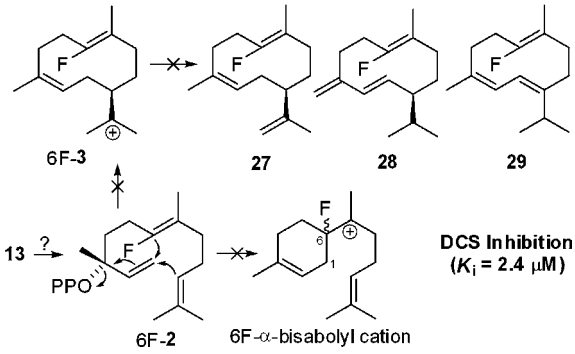
Figure 3. Structures of 10F-farnesenes (**23**−**25**) and 10F-*α*-humulene (**26**).

the DCS-generated product ruled out any of the three possible fluorinated farnesenes. This result indicates that DCS does not seem to convert **12** to its (3*R*)-10F-NDP isomer which likely precludes the enzymatic formation of 1,6-cyclized products from **12**. Diphosphate **12** has been used previously to explore the 1,6-ring closure mechanism catalyzed by trichodiene synthase (TS). Surprisingly, 10F-FDP acted as a potent inhibitor of TS,[8a] thus, suggesting the potential existence of a alternative/degenerate mode of catalysis by TS.

While an unambiguous full structure determination of the product generated from 10F-FDP (**12**) is outstanding, preliminary ^{1}H NMR (500 MHz, $CDCl_3$) spectroscopic data are in agreement with a *α*-10F-humulene hydrocarbon structure (**26**, Figure 3) arising from an unprecedented DCS-mediated 1,11-macrocyclization. This suggestion is supported by the observation of a downfield doublet of triplets signal (J_{H-F} = 40.4 Hz and J_{H-H} = 8.1 Hz) at 5.00 ppm, which strongly resembles the characteristic doublet of triplets absorbance at 5.59 ppm (J_{H-H} = 15.8. Hz, and J_{H-H} = 7.3 Hz) assigned to the H-9 olefinic proton of *α*-humulene.[32] The relative upfield H-9 shift (ca. 0.6 ppm) observed for **26** is consistent with the *γ*-shielding effect of the vinylic fluoro substituent on C9. In contrast to the characteristic ^{19}F-NMR triplet signal (J_{H-F} = 24.6 Hz) at −114.0 ppm of diphosphate **12**, the ^{19}F-NMR (282 MHz) spectrum of the reaction product generated from **12** displayed a doublet (J_{H-F} =41.6. Hz) centered at −119.9 ppm, which further supports the identification of this enzymatic hydrocarbon as 10F-*α*-humulene (**26**) (Supporting Information).

Incubations of 6F-FDP (13) and 6,7-DihydroFDP (17) with DCS. 6-Fluoro-FDP (**13**) has been used previously to probe the catalytic cycles of PR-AS[3c] and TEAS.[3d] The kinetic data obtained for TEAS revealed that the presence of the vinylic fluorine had negligible effects on binding affinity, diphosphate ionization, and the initial 1,10-cyclization reaction.[3d] For DCS, irrespective of which 1,10-mechanism is followed (Scheme 1), the presence of the 6-fluoro substituent should destabilize the transition state(s) leading to cadinenyl cation **5**. Therefore, incubations of DCS with **13** were expected to yield, via 6F-**3**, fluorinated germacrene hydrocarbons (e.g., **27**−**29**, Scheme 2)

Scheme 2. Structures of 6F-Germacrenes (27−29) from a Putative 6F-NDP (6F-2)[a]



[a]The detection of the possible enzymatic release of 6F-**2** in the aqueous phase was not attempted.

through premature deprotonation reactions. Intriguingly, diphosphate **13** was not a substrate of DCS, and even after prolonged enzymatic incubations, no turnover was observed. Steady-state kinetic studies demonstrated that 6F-FDP (**13**) was a potent competitive inhibitor of DCS with an inhibition constant (K_i = 2.4 *μ*M, Supporting Information) comparable to the Michaelis−Menten constant measured for FDP (K_M = 3.2 *μ*M).[13]

Assuming that the C6,C7-double bond of **1** (or **13**) is not likely to participate in the production of (3*R*)-NDP (**2**) (path a, Scheme 1),[15,17,18] the strong inhibition displayed by **13** suggests that the native DCS-catalyzed reaction cascade leading to **6** might proceed through the alternative 1,6-ring closure pathway c (Scheme 1), as 1,10-macrocyclization-derived products would be expected for this analogue from path a.[3c,d] Hence, in this scenario, *δ*-cadinene (**6**) is formed by nucleophilic attack of the central C6,C7-double bond of **2** on C1 to generate the *α*-bisabolyl cation (Scheme 1), which has been reported to be central to other sesquiterpene synthase-catalyzed cyclizations of FDP.[23] The destabilizing effect of the vinylic 6-fluoro substituent toward the proposed electrophilic 1,6-alkylation would explain the inability of DCS to use 6F-FDP (**13**) as a substrate.

The possibility of a 1,6-electrophilic ring closure in DCS catalysis was further supported by incubations with racemic 6,7-dihydroFDP (**17**).[33] Indeed, in agreement with the results previously obtained with the 1,6-cyclase TS,[33a,b] DCS incubations of diphosphate **17** yielded (*Z*)-*α*-6,7-dihydrofarnesene[34] as the main (64%) hydrocarbon. Conversely, 6,7-dihydrogermacrene is the exclusive product detected with the 1,10-cyclase aristolochene synthase from *A. terreus* (AT-AS).[35]

Incubation of DCS with Geranyl (19), Neryl (20), and 2F-Geranyl (18) Diphosphates. It has been documented that sesquiterpene synthases that can isomerize the C2,C3-double bond of (*E*,*E*)-FDP (**1**) often produce cyclic monoterpenes from GDP (**19**).[2h,i,p,36] In contrast, rigorously 'trans' enzymes such as TEAS[2h] or the germacrene A synthase NS1,[2p] produce exclusively acyclic monoterpenes when they encounter **19**. Thus, for 'cis' sesquiterpene cyclases[3h] that utilize a coupled ionization-isomerization-1,6-cyclization mechanism,[23] neryl cation and *α*-terpinyl cation[1b,37] (Scheme 3) can act as good mimics of the corresponding C_{15} *cis*-farnesyl and *α*-bisabolyl

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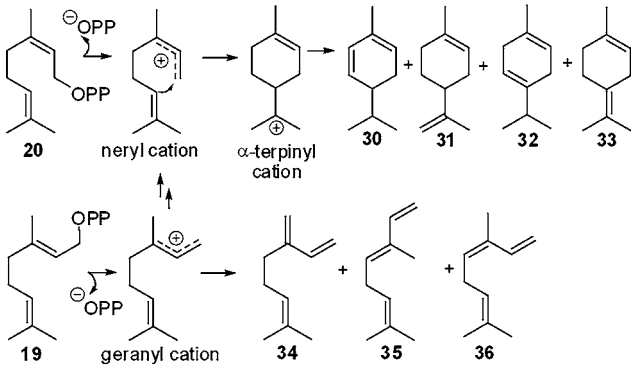
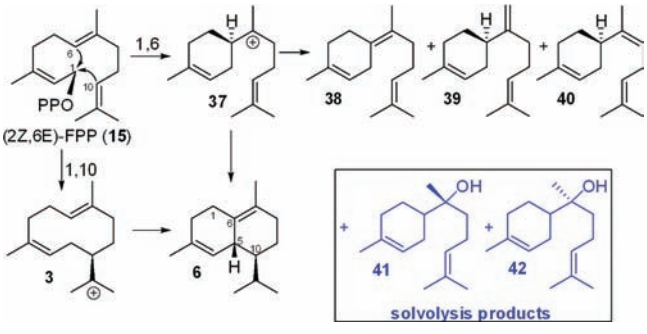
Scheme 3. Monoterpenes Produced by DCS from Geranyl (19) and Neryl (20) Diphosphates



cations, respectively. On the basis of these considerations, and to assess the feasibility of a 1,6-ring closure, the ability of DCS to convert **19** to cyclic monoterpenes was tested. DCS was indeed able to catalyze the conversion of **19** to an approximate 2:3 mixture of cyclic (α-phellandrene (**30**), limonene (**31**), γ-terpinene (**32**), and α-terpinolene (**33**)), and acyclic (myrcene (**34**), (*E*)-β-ocimene (**35**), and (*Z*)-β-ocimene (**36**)) monoterpenes (Supporting Information). Interestingly, neryl diphosphate (**20**) produced only cyclic monoterpenes (Scheme 3)[38] suggesting that the observed acyclic olefins (**34**–**36**, Scheme 3) arise exclusively from the transoid geranyl cation, and that their enzymatic release occurs before isomerization to neryl cation.[1b,37] In accordance with this mechanistic picture, 2-fluorogeranyl diphosphate (**18**) was shown to efficiently prevent the ionization-isomerization step essential for the accumulation of cyclic products; indeed, **18** was a potent competitive inhibitor of the DCS (K_i = 8 μM) (Supporting Information). These results reflect on the ability of DCS to use an isomerization-1,6-cyclization step and sustain a possible reaction via α-bisabolyl cation (Scheme 1) from FDP.

Incubation of DCS with (2*Z*,6*E*)-FDP (15). The possibility of either a 1,10- or a 1,6-ring closure mechanism in DCS catalysis was further evaluated with (2*Z*,6*E*)-FDP (**15**) (Scheme 4). It had been recognized previously that diphosphate **15** could be considered an effective 'preisomerized' form of (*E*,*E*)-FDP (**1**).[2n,3h] Hence, its ionization by DCS should supply an ion pair of cisoid farnesyl cation and diphosphate anion ready for the enzymatic reaction cascade to proceed along path a (Scheme 1). GC–MS analysis of the pentane extractable products from incubations of **15** with DCS showed that the enzyme generated two hydrocarbon products (Supporting Information). The MS fragmentation pattern and GC retention time of the minor product (33%) matched that of δ-cadinene (**6**).[15] To identify the major product (67%), a preparative-scale incubation of **15** with DCS was carried out. The hydrocarbon (50% of total) and alcohol (50% of total) products were initially separated by silica gel column chromatography. Each fraction was then purified further by preparative TLC to yield two hydrocarbon fractions (A and B) and one alcohol fraction. ^{1}H NMR spectroscopic measurements of fraction A revealed the presence of δ-cadinene (**6**) of ca. 85% purity.[15d,39] Fraction B (corresponding to the major GC peak with shorter retention time) was found to be composed of a 3:3:2 mixture of (*Z*)-γ-bisabolene (**38**), β-bisabolene (**39**), and (*Z*)-α-bisabolene (**40**) as judged by ^{1}H NMR spectroscopy and comparison with the literature values previously reported for these compounds.[40,41] In addition, ^{1}H NMR spectroscopy of the alcohol fraction revealed the presence of an inseparable 2:1:1 mixture of nerolidol and the two epimeric α-bisabolols **41** and **42**,[42] which most likely arise from the nonspecific addition of water to α-bisabolyl cation. It is worthy of note that this product distribution (Scheme 4) is remarkably similar to that previously observed from DCS incubations using NDP (**2**) as the substrate.[15c]

Scheme 4. Sesquiterpenes Generated by DCS from (2*Z*,6*E*)-FDP (15)[a]



[a]The (*R*)-configuration of **39**–**40** is assumed based on the intermediacy of a putative (6*R*)-configured α-bisabolyl cation (**37**). Alcohols **41** and **42** are nonenzymatic products.

The accumulation of the bisabolyl-derived olefins **38**–**40**, accounting for ca. 67% (GC–MS) of all cyclic hydrocarbons, is easily explained with an initial DCS-catalyzed 1,6-ring closure reaction that leads to the (6*R*)-α-bisabolyl cation (**37**), which is then either deprotonated to the observed hydrocarbons **38**–**40**, or trapped by a molecule of water to generate the tertiary alcohols **41** and **42** (Scheme 4). However, the observation of considerable amounts of alcohols **41** and **42** from incubation of **15** in the absence of recombinant DCS established, for the most part, their nonenzymatic origin. It is notable that not only **6**, but also the DCS-generated hydrocarbon (*Z*)-γ-bisabolene (**38**) as well as the α-bisabolols are terpene volatiles found in cotton plants.[15c]

Mechanistically, the formation of δ-cadinene (**6**) together with a variety of bisabolyl-derived hydrocarbons (**38**–**40**) supports the possibility of a 1,6-ring closure mechanism. However, the result is intriguing. Either DCS possesses the ability to mediate parallel 1,10- and 1,6-ring closure reactions along energetically similar pathways (Scheme 4), or all cyclic enzymatic products including **6** arise from a predominant conformation of farnesyl cation (**A**, Scheme 5) that allows the 1,6-cyclization mechanism observed with the surrogate diphosphate **15**. The possibility of a DCS-catalyzed 1,6-ring closure pathway has been suggested previously[15b] and the feasibility of the required 1,3-H and 1,5-H shifts involving **37** has been discussed.[43] However, a detailed description of a 1,6-mechanism consistent with both the 5,10-cis stereochemical relationship of cadinenyl-derived hydrocarbons and the deuterium distribution found in **6** using racemic and chiral [1-^{2}H$_1$]-FDPs[15] has not been provided before. In agreement with our proposal (vide infra), Tantillo and Hong have provided computational evidence from gas-phase calculations in support of 1,3- and 1,5-H shifts that could interconnect, via cation **37**, the biosynthesis of amorphadiene (1,6-ring closure) and amorphene (1,10-ring closure) derived sesquiterpenes. These gas-phase calculations energetically favored a 1,6-ring

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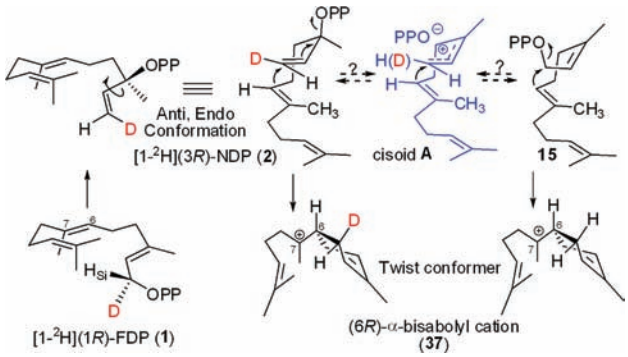
Scheme 5. DCS-Catalyzed Conversion of [1-$^2H_1$](1*R*)-FDP ((1*R*)-1) and 15 to the (6*R*)-α-Bisabolyl Cation (37) via [1-$^2H_1$](3*R*)-NDP (2) or the Corresponding *cis*-Farnesyl Cation/PPO$^-$ Ion Pair



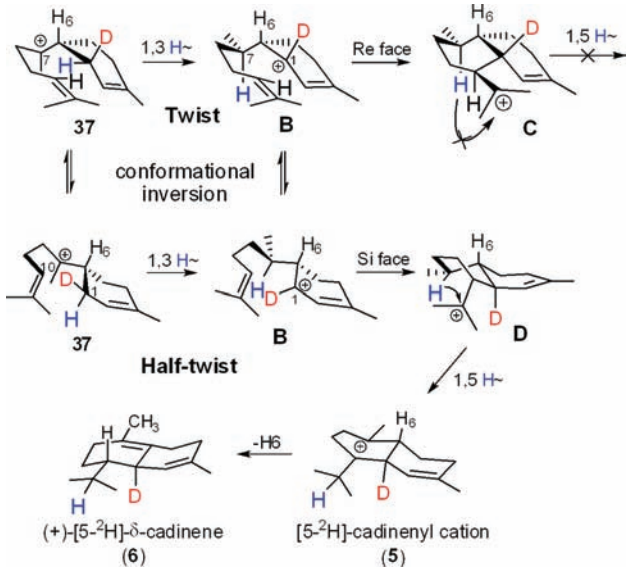
closure rather than the accepted 1,10-cyclization mechanism in amorphene biosynthesis.[44]

Incubation of [6-$^2H_1$]-FDP (16) and [1-$^2H_1$](1*R*)-FDP ((1*R*)-1) with DCS. In the present study, the stereochemical course of the DCS-catalyzed reaction was followed by individual incubations with [6-$^2H_1$]-FDP (**16**) and [1-$^2H_1$](1*R*)-FDP ((1*R*)-**1**), which resulted in the formation of unlabeled **6** and [5-$^2H_1$]-**6**, respectively (Supporting Information), as evidenced by GC/MS analysis. In contrast to what was observed with **6** (base peak at *m*/*z* 161), the GC-mass spectra of the product ([5-$^2H_1$]-**6**) generated from (1*R*)-**1** had a base peak *m*/*z* of 162 (assigned to [M^+-C_3H_7]), thus, indicating that the original H-1$_{Re}$ proton of FDP was retained on C1 during the formation of [5-$^2H_1$]-**6**.[15d] Hence, the loss of an undeuterated isopropyl side chain from [5-$^2H_1$]-**6** confirms the migratory properties of the original H-1$_{Si}$ of diphosphate **1**. The formation of unlabeled **6** from incubations with [6-$^2H_1$]-FDP (**16**) indicates the loss of the H-6 of FDP during catalysis.

While these (and other[15]) limited labeling experiments alone cannot distinguish between a 1,6- and a 1,10-mechanism (Schemes 1, 5 and 6), the experimental observations reported here support an electrophilic 1,6-alkylation reaction as the predominant pathway to (+)-δ-cadinene (**6**).[43] Moreover, the 1,6-ring closure pathway, illustrated in Schemes 5 and 6 with the deuterium labeled substrate (1*R*)-**1** and unlabeled **15**, is in good agreement with previous feeding experiments with [1,2-$^{13}C_2$]-acetate and [2-$^{14}C_1$]- and [4-$^{14}C_1$]-mevalonate in cotton plants, from which a plausible role for diphosphate **15** was inferred,[17,18] and with more recent studies that revealed the (3*R*)-enantiomer of nerolidyl diphosphate (**2**, Scheme 5) as the active substrate of recombinant (+)-DCS.[15c]

Accordingly, we propose that the enzyme-generated tertiary diphosphate **2** (Scheme 5) reacts in the active site pocket of (+)-DCS in the anti, endo conformation typically found in mono- and sesquiterpene cyclases that bring about 1,6-cyclizations.[1a−f,36] Since biomimetic cyclizations of the related linalyl and neryl derivatives are chemically effected mainly from this conformation,[9,45] it seems plausible to suggest that the prenyl chain of **15** adopts a helical orientation similar to that of NDP (**2**), the enzyme bound intermediate generated from FDP. With this chirality, the 6,7-double bond of **2** (and the double bond of **15**) is ideally positioned to effect the presumed anti 1,6-cyclization that ensures the formation of the α-bisabolyl cation (**37**) with the proposed (*R*)-configuration at C6 (Scheme 5). An identical *R*-stereochemistry would be expected

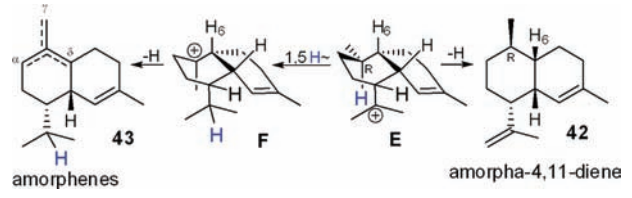
Scheme 6. Conversion of Cation 37 to (+)-6 Involving 1,3- and 1,5-Hydride Shifts of the H-1$_{Si}$ Proton of [1-$^2H_1$](1*R*)-FDP



considering the cisoid farnesyl cation (**A**) as the reactive active site intermediate from which cyclic **37** is formed.

After formation of the (6*R*)-α-bisabolyl cation (**37**), the remaining mechanistic steps to (+)-**6** (Scheme 6) resemble those precedented in amorpha-4,11-diene synthase (AMDS).[2h,46] However, in contrast to AMDS catalysis, MM2 molecular modeling studies indicate that after the unique C1 → C7 hydride shift to the Si face of **37** involving the original H-1$_{Si}$ of FDP,[2h,46] the second cyclization step (**B** → **D**, Scheme 6) requires a conformational ring inversion (twist **B** → half twist **B**) to obtain the exo 1,10-electrophilic cyclization onto the Si face of carbocation **B** that ultimately guarantees the correct 5,10-cis stereochemistry diagnostic of cadinene sesquiterpenes. As shown in Scheme 6, the required conformational change could also occur at the bisabolyl cation (**37**) stage, but since the C1 → C7 hydride shift occurs to the same C7 Si face of cation **37**, the transient 7*R*-stereochemistry displayed by **B** (and **E**, see Scheme 7) would be expected.

Scheme 7. Conversion of Cation E to Amorpha-4,11-diene (42) and Amorphenes (43), respectively[44]



The proposed exo cyclization (Scheme 6) generates the second cyclohexane ring of **D** in a high-energy boat conformation that nevertheless allows the critical 1,5-H shift to cadinenyl cation **5** with concomitant release of strain. Finally, proton loss from C6 of the *trans*-fused cadinenyl cation **5** (Scheme 6) accounts for the formation of (+)-[5-$^2H_1$]-δ-cadinene, or δ-cadinene, when (1*R*)-**1** or [6-$^2H_1$]-FDP (**16**) are used as the respective substrates. It is worth noting that while a similar 1,10 electrophilic cyclization onto the C1 Re face of

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cation **B** (twist **B** → **C**, Scheme 6) could equally explain, via cation **C**, the 5,10-cis stereochemistry present in **6** (Scheme 6), the anti stereochemical relationship between H7 and C11 in **C** precludes the proposed 1,5-hydride shift and hence the installment of the correct deuterium label in **6** observed with *d*-labeled FDPs.[15c,d]

A corollary from this stereochemical analysis stems from the fact that an endo 1,10-cyclization onto the Re-face of carbocation **B** (twist conformer) would give rise to the *cis*-fused amorphenyl cation **E**, the direct precursor of amorpha-4,11-diene **42**.[2h,44,46] Similarly, the high-energy boat conformation of cation **E** (Scheme 7) allows the presumed 1,5-H shift that accounts for amorphene (**43**) biosynthesis.[44]

In summary, the mechanism of the DCS-catalyzed cyclization of FDP (**1**) to (+)-δ-cadinene (**6**) was studied by incubations using a variety of substrate analogues. In agreement with a mechanism via (3*R*)-NDP (**2**),[15c] prenyl diphosphates with vinylic fluoro substituents at C2 (**10**, **11**, and **18**) were shown to act as competitive inhibitors that prevent the initial cationic ionization-isomerization step. Surprisingly, FDP analogues **10** and **11** with fluorine at C2 also acted as substrates of DCS and yielded fluorinated germacrenes arising from 1,10-cyclizations with the stereochemistry of the C2,C3 double bond remaining intact. The isolation of 2F-germacrenes (**21**, **22**), despite inhibition and stereochemical arguments, seems to favor the 1,10-macrocyclization mechanism. Similarly, the inhibition displayed by 10F-FDP (**12**), together with the formation of a 10F-α-humulene (**26**) via a DCS-mediated 1,11-cyclization of **12**, suggests a 1,10-pathway (path a, Scheme 1) as the most plausible biosynthetic pathway. On the other hand, 6-fluoro-FDP (**13**) served as a potent competitive inhibitor of DCS, an observation that is difficult to explain assuming a 1,10-macrocyclization mechanism (Scheme 1). In addition, diphosphate **13** was the only fluorinated C_{15} diphosphate that was not turned over by the enzyme, suggesting an early involvement of the central C6,C7-double bond of **1** during DCS catalysis. Incubations of DCS with (2*Z*,6*E*)-FDP (**15**) produced, in addition to δ-cadinene, a mixture of bisabolenes and bisabolols arising exclusively from a 1,6-ring closure, and resembling the product distribution previously observed when NDP (**2**) was used as the substrate. Taken together, these results are best explained with the involvement of a 1,6-cyclization of (*E*,*E*)-FDP (**1**) in DCS chemistry to generate (6*R*)-bisabolyl cation.[23] Further support for the possible involvement of this mechanism was provided by the observations that C_{10} GDP (**19**) and neryl diphosphate (**20**) generated a variety of 1,6-ring closure products and that 6,7-dihydroFDP (**17**) yielded the acyclic *cis*-6,7-dihydro-α-farnesene as the main product of the enzymatic reaction rather than the expected 6,7-dihydrogermacrene.

CONCLUSION

The results presented here demonstrate for the first time the remarkable mechanistic versatility of DCS that can use an array of distinct cyclizations to generate single reaction products from FDP and its analogues. In addition, the present study supports the possible existence of a 1,6-ring closure mechanism in δ-cadinene biosynthesis[15,43,44] and highlights the high regio- and stereochemical precision of DCS-catalyzed cyclizations. Indeed, with the exception of the inhibitor 6-fluoro-FDP (**13**), from which no products were formed, DCS was able to turn over diphosphates **10**, **11**, and **12**, respectively, to the single fluorinated sesquiterpenes **21**, **22**, and possibly 10F-α-humulene (**26**) via unprecedented 1,10- and 1,11-cyclizations, in which the geometry of the C2,C3 double bond of the starting diphosphates remained unchanged. This observation contrasts with the native cyclization of FDP, in which DCS must convert the initial trans C2,C3 π-bond via NDP (**2**) to cis in δ-cadinene. While a 1,11-cyclization mechanism en route to δ-cadinene seems unlikely, the observation of 1,10-cyclizations from **10** and **11** and the possible dual 1,6- and 1,10-ring closures from **2** and **15** are in agreement with the mechanistic versatility of cadinane-producing sesquiterpene synthases,[43] which do not appear to depend on a common biosynthetic mechanism in spite of their shared ancestral origin.[47]

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, gas chromatograms, mass spectra and/or NMR spectra of key compounds, as well as inhibition kinetics studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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