

Evolutionary and Mechanistic Insights from the Reconstruction of α -Humulene Synthases from a Modern (+)-Germacrene A Synthase

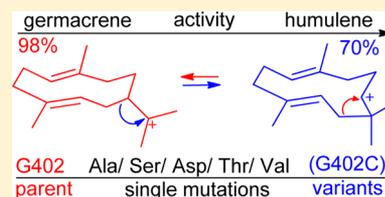
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Supporting Information

ABSTRACT: Germacrene A synthase (GAS) from *Solidago canadensis* catalyzes the conversion of farnesyl diphosphate (FDP) to the plant sesquiterpene (+)-germacrene A. After diphosphate expulsion, farnesyl cation reacts with the distal 10,11-double bond to afford germacrene A (>96%) and <2% α -humulene, which arises from 1,11-cyclization of FDP. The origin of the 1,11-activity of GAS was investigated by amino acid sequence alignments of 1,10- and 1,11-synthases and comparisons of X-ray crystal structures with the homology model of GAS; a triad [Thr 401-Gly 402-Gly 403] that might be responsible for the predominant 1,10-cyclization activity of GAS was identified.

Replacement of Gly 402 with residues of increasing size led to a progressive increase of 1,11-cyclization. The catalytic robustness of these 1,10-/1,11-GAS variants point to Gly 402 as a functional switch of evolutionary significance and suggests that enzymes with strict functionalities have evolved from less specific ancestors through a small number of substitutions. Similar results were obtained with germacrene D synthase (GDS) upon replacement of the homologous active-site residue Gly 404: GDS-G404V generated approximately 20% bicyclogermacrene, a hydrocarbon with a cyclopropane ring that underlines the dual 1,10-/1,11-cyclization activity of this mutant. This suggests that the reaction pathways to germacrenes and humulenes might be connected through a bridged 1,10,11-carbocation intermediate or transition state that resembles bicyclogermacrene. Mechanistic studies using [1-³H₁]-10-fluorofarnesyl diphosphate and deuterium-labeling experiments with [12,13-²H₆]-FDP support a germacrene–humulene rearrangement linking 1,10- and 1,11-pathways. These results support the bioinformatics proposal that modern 1,10-synthases could have evolved from promiscuous 1,11-sesquiterpene synthases.



INTRODUCTION

Class I sesquiterpene synthases catalyze the conversion of the linear substrate (2*E*,6*E*)-farnesyl diphosphate (1, FDP) to all C15 isoprenoid hydrocarbons found in nature.¹ These enzymes mediate a metal-dependent ionization of FDP to generate diphosphate anion and a reactive allylic farnesyl cation (2) which both remain tightly bound to the hydrophobic and largely desolvated active site.² Farnesyl cation (2) then loses a proton to yield linear farnesene hydrocarbons³ or reacts with another double bond in 2 to generate an often tertiary carbocation that is chaperoned by the enzyme toward the product through a well-defined energetic landscape and with extraordinary regio- and stereochemical precision.⁴ Many sesquiterpene synthases serve as templates to restrain FDP (1) in conformations that lead to the formation of a single enantiomerically pure hydrocarbon. Hence, farnesyl cation often adopts only a single productive conformation from which 1,6-, 1,10-, or 1,11-cyclization products are formed.^{1e,5}

Mechanistically, germacrene A synthases⁶ (GASs) are among the simplest class I sesquiterpene cyclases. Without requiring an initial isomerization of *trans*-FDP (1) to the isomeric nerolidyl diphosphate,¹ they catalyze a 1,10-cyclization (Scheme 1) to generate germacren-11-yl cation (3) and then, after deprotonation, germacrene A (4). The large number of known plant

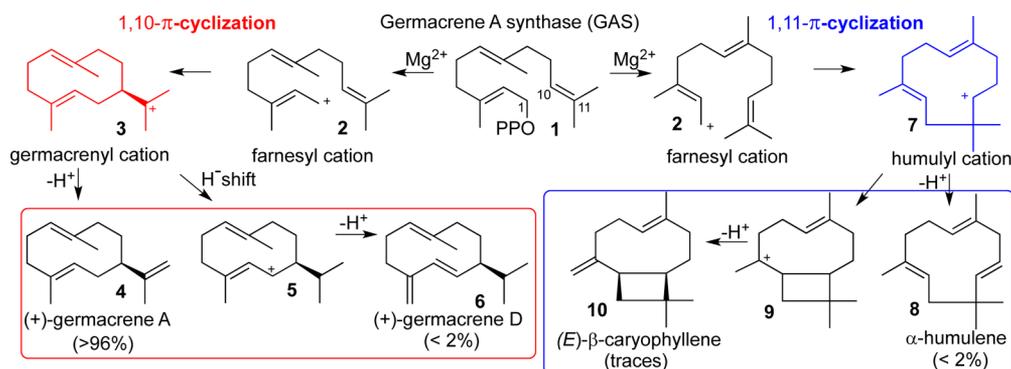
natural products generated by further cyclization and/or oxidation of germacrene A indicates that GASs are widespread.⁷

GAS from *Solidago canadensis*^{6b} produces >96% of (+)-germacrene A,⁸ ~2% of each germacrene D (6) and α -humulene (8), and traces of (*E*)- β -caryophyllene (10). The formation of 1,11-products by GAS is puzzling because germacrene and humulene sesquiterpenes have been proposed to arise through independent biosynthetic pathways catalyzed by distinct 1,10- and 1,11-cyclases.^{1d} Moreover, since the intermediacy of 8 as a neutral GAS-bound precursor of 4 is unlikely,¹ the 1,11-pathway of GAS (Scheme 1) might represent a palimpsest suggestive of the enzyme's evolutionary past.

Recently, phylogenetic analyses of plant sesquiterpene synthases⁹ together with crystallographic data from structural work^{1c} and structural models have led to the suggestion that primordial plant sesquiterpene synthases might have performed only 1,11- and 1,6-cyclizations, while synthases that catalyze 1,10-cyclization pathways have evolved more recently via gene duplication and subsequent mutations.¹⁰ Hence, it might be possible to reconstruct a 1,11-cyclization enzyme from a modern 1,10-sesquiterpene synthase. To explore this possi-

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Scheme 1. *S. canadensis* GAS Turnover of (*E, E*)-FDP (1) to Hydrocarbons 4 and 6 through 1,10-Cyclization or 8 and 10 by 1,11-Cyclization, Respectively^a

^aProducts were identified in GC–MS experiments with authentic standards. Percentages of the total products formed are given in parentheses.

Table 1. Distribution (%) of 1,10- and 1,11-Products and Steady-State Parameters for Native GAS, GDS, and Their Variants with Farnesyl Diphosphate (1)

cyclase	1,10-sesquiterpenes			1,11-sesquiterpenes			total		kinetic parameters		
	4	6	13	8	10	14	1,10-	1,11-	K_M (μM)	k_{cat} (s^{-1})	k_{cat} / K_M ($\text{s}^{-1} \text{mM}^{-1}$)
GAS	96.58	1.67		1.75			98.25	1.75	3.4 ± 0.3	0.043 ± 0.02	12.6 ± 4.8
G402A	54.1	2.6		41.6	1.7		56.7	43.3	8.9 ± 1.3	0.053 ± 0.01	5.9 ± 1.1
G402S	50.7	5.1		42.5	1.7		55.8	44.2	3.3 ± 0.9	0.021 ± 0.01	6.4 ± 1.8
G402D	31.9	18.1	0.5	43.4	2.9	3.2	50.5	49.5	0.5 ± 0.07	0.001 ± 0.01	2.0 ± 0.2
G402C	13.7	14.9	3	62.5	3.8	2.0	31.6	68.4	5.3 ± 1.8	0.030 ± 0.01	5.7 ± 0.02
G402T	11.8	24.0	5.9	51.4	3.7	3.2	41.7	58.3	4.7 ± 0.5	0.054 ± 0.01	11.5 ± 0.02
G402 V	11.0	24.6	6.5	50.7	3.0	4.2	42.1	57.9	1.0 ± 0.2	0.025 ± 0.01	25.0 ± 5.0
G402F, L, I							inactive				
S442C	13.5	2.8	24.3	44.7	1.5	13.2	40.6	59.4	6.3 ± 0.5	0.063 ± 0.01	10.0 ± 0.9
G402C/S442C	1.8	8.4	35.6	38.3	1.5	14.4	45.8	54.2	9.3 ± 2.5	0.068 ± 0.01	7.3 ± 0.2
Average							45.6	54.4	4.9 ± 1	0.039 ± 0.03	7.9 ± 0.1
GDS ^a		98.5		1.5			98.5	1.5	3.6 ± 0.3	0.009 ± 0.002	2.5 ± 0.2
G404T		91.0		4.5	4.5		91.0	9.0	1.1 ± 0.07	0.007 ± 0.001	6.2 ± 0.3
G404 V ^b		70.6		5.6	3.8		70.6	9.4	2.1 ± 0.3	0.001 ± 0.001	0.5 ± 0.03
G404F, L	inactive										

^aValues from ref 12. ^bExcludes ~20% of bicyclogermacrene (12).

bility, we investigated the products generated by GAS and some mutants. GAS is ideal for such an investigation due to its residual 1,11-cyclization activity, its mechanistic simplicity and product specificity, the importance of germacrene A and GAS in sesquiterpene biosynthesis and the good fit of Asteraceae 1,10-cyclases (e.g., GAS) in the recently published phylogenetic study.⁹

Homology models of GAS based on the single X-ray crystal structure of 5-epiaristolochene synthase (TEAS)¹¹ together with multiple sequence alignments of 1,10- and 1,11-plant sesquiterpene synthases were used to identify Gly 402 as part of a conserved motif (Thr 401-Gly 402-Gly 403 in GAS) that could be a mechanistic determinant of 1,10-cyclizations. Indeed, substitution of Gly 402 with residues of increasing size led to the production of significant amounts (44–68%) of α -humulene (8) with often wild-type-like catalytic activity. In addition, mechanistic studies using $[1-^3\text{H}_1]$ -10-fluorofarnesyl diphosphate and the FDP isotopologue ($[12,12,12,13,13,13-^2\text{H}_6]$ -1) led to the identification of a germacrene–humulene rearrangement as the possible mechanistic basis for the observed 1,10- to 1,11-functional switch through single-point mutations of GAS. The reverse, thermodynamically more favorable humulyl to germacrenyl

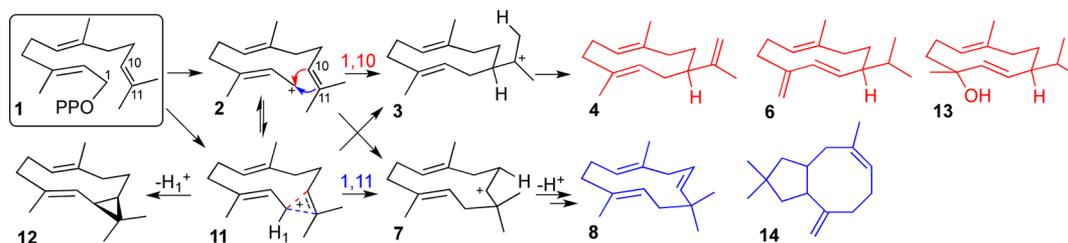
rearrangement, which is found in at least one of the GAS variants, could be an alternative mechanism to direct 1,10-cyclization of FDP catalyzed by sesquiterpene synthases. This mutant GAS might represent an example of a 1,11-ancestor of modern plant 1,10-synthases.

RESULTS AND DISCUSSION

1. Mutagenesis Studies. (+)-Germacrene A and (–)-germacrene D (GDS) synthases from *S. canadensis* were overproduced in *E. coli* and purified as previously described.¹² Combined gas chromatography and mass spectrometry (GC–MS) were used to identify and quantify all enzymatic products (Table 1) by comparison with authentic compounds. Incubations of wild-type GAS and GDS with FDP (1) generated approximately 56:1 and 65:1 mixtures of 1,10- and 1,11-cyclized products (Table 1). The kinetic parameters for turnover of FDP (1) (GAS: $K_M = 3.4 \pm 0.8 \mu\text{M}$, $k_{\text{cat}} = 0.043 \pm 0.02 \text{ s}^{-1}$; GDS: $K_M = 3.6 \pm 0.3 \mu\text{M}$, $k_{\text{cat}} = 0.009 \pm 0.002 \text{ s}^{-1}$) were in good agreement with those reported previously^{6b,12} and similar to those of other plant sesquiterpene synthases.^{3,13}

On the basis of the X-ray single crystal structure of 5-epiaristolochene synthase from *Nicotiana tabacum* (EAS),¹¹ an active site triad, Thr⁴⁰¹-Thr⁴⁰²-Thr⁴⁰³, was proposed to hold

Scheme 2. Cyclization of FDP (1) to Germacrenyl- (3) and Humulyl-Derived (7) Products via Farnesyl Cation (2) or Bridged Carbocation 11 Catalyzed by GAS, GDS, and Their Variants^a



^aCompound 13 and 14 correspond to germacradien-4-ol and (6Z)-asterisca-3(15),6-diene, respectively (Scheme 3). Bicyclogermacrene (12) most likely originates from 11 via proton transfer (H_1).

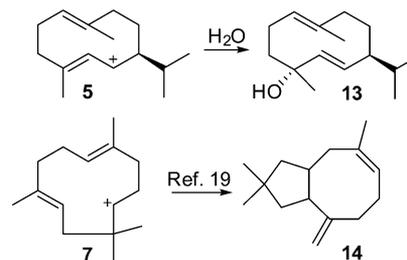
FDP in the conformation necessary for cyclization to germacren-11-yl cation (3) during EAS catalysis.^{7a,11a} Molecular modeling revealed that the triad Thr⁴⁰¹-Gly⁴⁰²-Gly⁴⁰³ could have a similar function in GAS. Extensive amino acid sequence alignments (Supporting Information) indicated that humulene and caryophyllene synthases share a homologous sequence element, Thr/Ser^x-Ser^y-Gly^z, that is ~80% conserved in 1,11-cyclases.¹⁴ Interestingly, 1,11-cyclases do not utilize Gly at their otherwise mostly conserved γ -position. Indeed, only two sequences from *Zingiber* and *Solanum* species, and one from *Medicago* were found to have Ser^y replaced by Ala and Thr, respectively.¹⁵ Therefore, the attempted conversion of GAS to a humulene synthase targeted these substitutions.

Replacements of Gly 402 by Ala or Ser led to the accumulation of α -humulene (8) in substantial amounts (~43%, Table 1), thereby providing strong evidence for the central role of Gly 402 as a structural determinant of fidelity in wild type GAS. These single amino acid replacements most likely lead to an imperfect active site contour that more closely resembles that of a 1,11-synthase, and in which nonselective proton elimination from C9 or C13 enables the simultaneous production of 1,11- and 1,10-products. Alternatively, the products could originate from a common carbonium ion (11), a bridged intermediate arising by anchimeric participation of the distal double bond in diphosphate ionization (i.e., from 1), or by further delocalization of the positive charge in 2 after diphosphate cleavage (Scheme 2).

Notably, replacement of Gly 402 with sterically more demanding amino acids (Asp, Cys) led to a further increase in 1,11-synthase activity, peaking at ~68% in GAS-G402C (Table 1). For GAS-G402D, electrostatic effects may also contribute to the observed product distribution. The wild-type-like catalytic activities of these GAS variants (Table 1), together with their dual germacrene/humulene functionality, is consistent with Gly402 being a plasticity residue or, indeed, a switch of potential evolutionary significance.¹⁶ The 1,11-cyclization activity decreases with additional increasing bulk of residue 402 (58% in GAS-G402T/V) or is abolished altogether (GAS-G402L, GAS-G402I, and GAS-G402F) suggesting that only further modifications outside the active site^{3,17} of GAS-G402C will lead to the specific 1,11-cyclase, from which GAS might have evolved.¹⁸ Indeed, replacement of a second active residue (Ser 442) in GAS-G402C yielded the dual 1,10/1,11-GAS-G402C/S442C that generated almost equal amounts (~50%) of 1,10- and 1,11-products (Table 1). GAS-G402C/S442C is a mixed germacradien-4-ol/ α -humulene sesquiterpene synthase, in which the original germacrene A specificity has been almost entirely lost (98.2%) without detriment to the catalytic activity. Germacradien-4-ol (13) results from reaction

of carbocation (5) with bulk solvent. The third major product (14.4%) produced by the double mutant was tentatively identified as (6Z)-asterisca-3(15),6-diene (14) by MS analysis and comparison of its mass spectrum with those in MassFinder 4.0 (<http://www.massfinder.de/index.htm>) (Scheme 3). This somewhat unusual hydrocarbon (14) most likely arises from humulyl cation (7).¹⁹

Scheme 3. Formation of 13 and 14 from Germacren-1-yl (5) and Humulyl (7) Cations by GAS Variants (See Table 1)



Guided by the homology model of GAS^{11b} (Figure 1), Ser 442 was identified as an active-site residue that could cooperate with Gly 402 in securing the strict 1,10-functionality of the native enzyme. The equivalent residue (Ser 484) in the

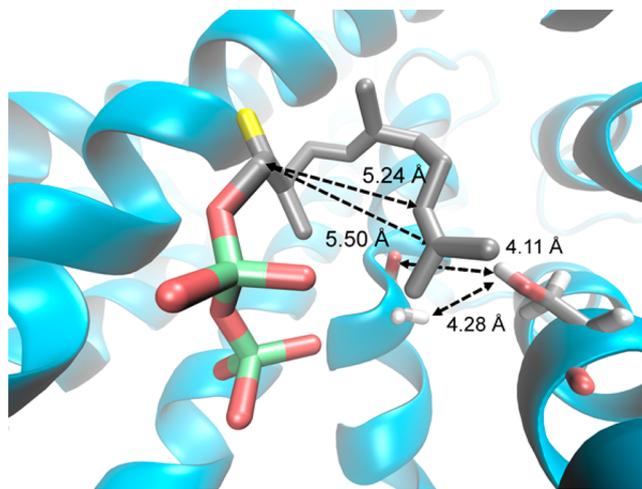


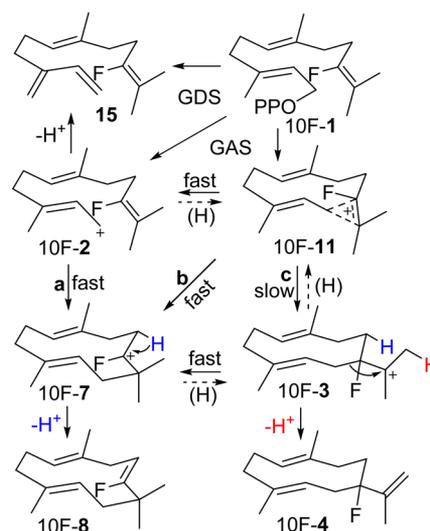
Figure 1. Cartoon representation of the active site of GAS modeled from the X-ray crystal structure of TEAS complexed with the unreactive substrate analogue 2F-farnesyl diphosphate (pdb: 3M01)^{11b} showing the network of possible interactions between Ser442 and Gly402.

promiscuous γ -humulene synthase (gHS) from *Abies grandis*^{7b} was suggested as a plasticity residue crucial for 1,11-cyclization; in particular, gHS variants with the S484C mutation had been shown to be strict (88–93%) 1,11-cyclases.²⁰ In the present case, replacement of Ser 442 with Cys yielded another catalytically robust cyclase (GAS-S442C) that produced almost equal amounts (2:3) of 1,10- and 1,11-products (Table 1). Although additional amino acid replacements in GAS-S442C, GAS-G402C, or GAS-402C/S442C in positions other than 402 and 442 could potentially lead to an enzyme with exclusive 1,11-cyclase activity,¹⁸ the consistent dual 1,10/1,11-activity displayed by all GAS variants (Table 1) suggests that specific 1,10-cyclizing enzymes such as GAS might have evolved from promiscuous ancestors through only a small number of amino acid replacements.^{18b,19} During evolution, gene duplication followed by mutation of at least one gene copy²¹ could have led to structurally stable dual 1,11/1,10-synthases like those described here (Table 1), from which the active site contour of the 1,10-synthase (GAS) was derived through for instance replacement of Cys 402 by Gly or Cys 442 by Ser.²²

It should be noted that the decrease in germacrene A activity parallels an increase in the amount of germacrene D/germacradien-4-ol as the van der Waals volume²³ of residue 402 increases (31.1% in GAS-G402V) (Table 1). Thus, it seems conceivable that germacrene D synthase (GDS) from *S. canadensis*,²⁴ which has a Thr⁴⁰³-Gly⁴⁰⁴-Gly⁴⁰⁵ active site motif identical to that found in GAS (Thr⁴⁰¹-Gly⁴⁰²-Gly⁴⁰³) might have originated from a structurally stable dual 1,10/1,11-synthase utilizing a bulkier valine rather than cysteine at its homologous G404 position. GC-MS analysis of the enzymatic products generated by GDS-G404T revealed high 1,10-germacrene D specificity (90%); further increases in the volume of the side chain of residue 404 (GDS-G404F and GDS-G404L) led, as with GAS, to complete loss of activity (Table 1). Substitution of Gly 404 by Val gave rise to the expected dual 1,10/1,11-cyclase GDS-G404V, which produces, in addition to 8 and 10 (Table 1), considerable quantities of bicyclogermacrene (12) (approximately 20% of the total products) (Scheme 2);²⁵ 12 is a sesquiterpene with an additional cyclopropane ring that bridges the three reactive carbons of FDP (C1, C10, and C11). Mechanistically, 12 could arise by deprotonation at C1 in 3 (1,10-cyclization), 7 (1,11-cyclization), or in the bridged cation 11 (Scheme 2). The detection of bicyclogermacrene as a product generated by GDS-G404V underlines the possibility that carbocation 11 could be an intermediate in both 1,10- and 1,11-synthase-catalyzed cyclization reactions. Moreover, if the intrinsic energetic differences between 11 and germacrenyl (5) and humulyl (7) cations are attenuated in the active site, rapid equilibration of the three species might occur.

2. Mechanistic Studies with 10-Fluorofarnesyl Diphosphate. Although substitutions of hydrogen by fluorine in FDP do not appear to perturb binding to terpene synthases,^{3,11b,26} fluorine-containing double bonds are known to be largely deactivated toward protonation and electrophilic alkylations.^{27,28} Production of acyclic farnesenes, such as the 10F- α -farnesenes or its isomer 10F-(*E*)- β -farnesene (15, Scheme 4) might therefore have been expected from incubation of GAS and GDS¹² with 10-fluorofarnesyl diphosphate (10F-1).^{12,28a} As anticipated, GDS produced the linear farnesene 15 as the predominant (75–80%) enzymatic product (Scheme 4). In contrast, 10F- α -humulene (10F-8)^{28a} was the exclusive product detected in incubations of GAS and 10F-1 (Scheme 4).

Scheme 4. Cyclization of 10F-1 to α -10-Fluorohumulene (10F-8) Following Path a, b (GAS), or c (GAS-G402C)^a



^aCompound 15 (75%) is only detected with GDS,¹² and 10-fluorogermaene A (10F-4) is not a product of GAS, GAS-G402C, or GDS. The terms fast and slow are relative to FDP (1). The dashed arrows indicate potential reversibility with diphosphate 1 (denoted here as H).

This unexpected activity of GAS could be the result of neighboring group participation of the 10,11- π bond during the initial diphosphate ionization step. This plausible cyclization event will bypass formation of the less delocalized carbocation 10F-2 in favor of the bridged carbonium ion 10F-11, and hence will prevent deprotonation by either the diphosphate leaving group,²⁹ or a suitable base. If GAS follows path b (Scheme 4), 1,11-cyclization of 10F-11 will lead to a secondary α -10-fluoro carbocation (10F-7), which is stabilized by additional π -donation relative to 7.^{12,28a} Experimental and computational investigations have previously indicated that the difference in free energy between tertiary carbocations and isomeric protonated cyclopropanes can be sufficiently small to allow rapid equilibration of the two cationic species.³⁰ Hence, a 1,10-cyclization of 10F-11 could also lead to germacrenyl cation 10F-3, which is destabilized by the strong inductive effect of the β -fluoro substituent. A subsequent fast and irreversible ring expansion reaction, likely involving halogen participation, will afford 10F-7 (path c in Scheme 4). An active site base could compete with this rearrangement and generate 10-fluorogermaene A (10F-4). However, this possibility was unambiguously excluded by the failure of the enzymatic product 10F-7 to undergo the thermal Cope rearrangement, typical of all trans-configured germacrenes.⁸

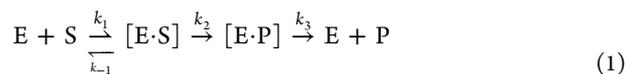
The steady-state kinetic parameters measured for the GAS-catalyzed turnover of 10F-1³¹ indicated that the production of α -10F-humulene (10F-8) is approximately 15-fold less efficient than that of (+)-germacrene A (4) from 1 (Table 2). The 3.5-

Table 2. Steady-State Kinetics Parameters for 1 and 10F-1

enzyme	substrate	K_M (μ M)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1} mM^{-1}$)
GAS	1	3.4 \pm 0.3	0.04 \pm 0.02	12.6 \pm 4.8
	10F-1	11.3 \pm 1.3	0.01 \pm 0.01	0.9 \pm 0.1
G402C	1	5.3 \pm 1.8	0.03 \pm 0.01	5.7 \pm 0.2
	10F-1	1.0 \pm 0.3	0.002 \pm 0.001	2.0 \pm 0.2

fold increase in K_M for 10F-1 (relative to **1**) could reflect structural limitations in the active site of GAS in accommodating 10F-1 in the 1,11-reactive conformation. In contrast, the substantially lower K_M of GAS-G402C indicates the tighter binding of 10F-1 to the active site of this 1,11-cyclase. Indeed, the affinity of GAS-G402C for 10F-1 ultimately translates into a 2-fold increase in its catalytic efficiency relative to GAS. However, the 5-fold increase of the rate of formation of 10F-8 by GAS ($k_{\text{cat}} = 0.01 \text{ s}^{-1}$) relative to GAS-G402C ($k_{\text{cat}} = 0.002 \text{ s}^{-1}$) suggests that these enzymes might follow different reaction mechanisms to turnover 10F-1.

Pre-steady-state and steady-state kinetic experiments with two mechanistically distinct sesquiterpene synthases³² support the minimal catalytic process defined by eq 1, where k_2 and k_3 represent the rates of products synthesized and released from the active site. Pre-steady-state kinetic studies have indicated that binding of S to generate the enzyme–substrate complex ES is rapid and reversible (i.e., $k_{-1} \gg k_2$) and that the enzymatic turnover is often limited by product release. The overall turnover of S is determined by a combination of the rates of the chemical step (k_2) and product release (k_3)



$$k_{\text{cat}}/K_M = k_1 k_2 / (k_{-1} + k_2) \quad (2)$$

where $k_2 \ll k_{-1}$, $k_{\text{cat}}/K_M \sim k_2/K_D$, and $k_{\text{cat}} \sim k_3$ ($K_D = k_{-1}/k_1$).

On the basis of the similar k_{cat} values for turnover of **1** by GAS and GAS-G402C, it may be assumed that this single active-site mutation is unlikely to alter the rate of product release significantly. Hence, when eq 1 is applied to Scheme 4, the differences in overall reaction rates shown in Table 2 can be explained in terms of k_2 . It should be noted that this approximation might not be applicable when rates for two different substrates such as **1** and 10F-1 are compared. Indeed, the 15-fold overall rate attenuation observed during GAS-G402C catalysis with 10F-1 ($k_{\text{cat}} = 0.002 \text{ s}^{-1}$) relative to **1** ($k_{\text{cat}} = 0.03 \text{ s}^{-1}$) is likely the result of effects from the fluoro substituent on k_2 and k_3 . The additional rate retardation factor arising from product release could be attributed in both cases to noncovalent interactions between the 10F-fluoro substituent and active site residues. This proposal is supported by analyses of the X-ray crystal structures of DCS and TEAS with bound 2-fluorofarnesyl diphosphates, which revealed electrostatic interactions between the fluoro substituent and several active site residues including Glu 455 and Asp 451 in DCS or Arg 264 in TEAS.^{13d,11b}

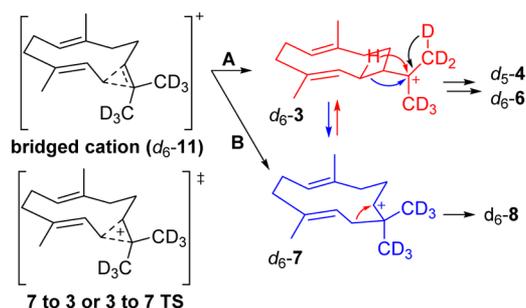
Comparison of the k_{cat} values for 10F-1 (Table 2) suggests that relative to GAS-G402C, GAS is less sensitive to the effects of the fluorine substituent during the irreversible cyclization of 10F-1 to 10F-7 (k_2). Indeed, GAS-G402C appears to follow a pathway that considerably retards the conversion of 10F-7 to 10F-8. Hence, the faster production of carbocation 10F-7 by GAS is consistent with slow and irreversible heterolytic cleavage of the C–O diphosphate ester bond of 10F-1 in the ES complex followed by fast and reversible 1,11-cyclization of 10F-2 to 10F-7 (path a, Scheme 4). Alternatively, relatively more rapid heterolysis of the C–O phosphate ester bond in ES with anchimeric participation of the distal 10,11-double bond may yield the bridged carbocation 10F-11, which subsequently undergoes fast 1,11-cyclization to 10F-7 (path b, Scheme 4). Intriguingly, if these cyclization events (path a or b) reflect the native GAS-catalyzed conversion of FDP (**1**) to germacrene A

(**5**), the exclusive formation of 10F-humulene (10F-8) would necessarily define diphosphate 10F-1 as an abortive substrate analogue^{27,28} of **1**, thus enabling derailment of the catalytic cycle at the stage of humulene cation (**7**). In the present case, the strong destabilizing effect of the fluoro substituent on the transition state between 10F-7 and 10F-3 effectively prevents the natural humulyl-germacrenyl rearrangement. Thus, under kinetic control,³³ the indirect formation of 1,10-cyclized products via 1,11-intermediates might be possible for enzymes effecting catalysis along pathways a or b (Scheme 4). In contrast, the initial formation of a more delocalized carbocation or bridged carbocation intermediate (**11**) (path c) in rapid equilibrium (vide infra) with an isomeric germacrenyl cation (**3**)³⁰ should severely impair, if not abolish, the irreversible branching of **11** → **7** → **3** with the natural substrate **1**.

The slower formation of 10F-8 by GAS-G402C ($k_{\text{cat}} = 0.002 \text{ s}^{-1}$) relative to GAS ($k_{\text{cat}} = 0.01 \text{ s}^{-1}$) could involve formation of the bridged intermediate 10F-11 in equilibrium with the isomeric tertiary carbocation 10F-5³⁰ (path c, Scheme 4), which in turn reduces the concentration of the carbocation(s) committed to 1,11-cyclization. Thus, if a relatively rapid equilibrium is established between 10F-11 and 10F-3, the rate of 1,11-cyclization to 10F-7 is attenuated with respect to the rate of the equilibrating species. The slower formation of 10F-8 is also consistent with a mechanistic scenario in which the protonated cyclopropane 10F-11 undergoes a slow (irreversible) 1,10-cyclization to 10F-3 followed by fast rearrangement to 10F-7. The isomerization of a similar bridged carbocation to a β -fluoro (tertiary) carbocation has been recently proposed during the SAM-dependent C-24-methylation of 26-fluorocycloartenols by a recombinant sterol C-24-methyltransferase from soybean.^{30d}

Although the formation of 1,10-germacrene- and 1,11-humulene-derived sesquiterpenoids is believed to require distinct 1,10- and 1,11-cyclases,^{1d} the construction of highly active dual 1,10/1,11-cyclases through the replacement of a single amino acid (Table 1) points toward a narrow energetic boundary between the 1,10- and 1,11-pathways. Indeed, comparison of the kinetic data obtained for GAS (98% 1,10-specific) and GAS-G402C (70% 1,11-specific) with **1** and 10F-1 suggests a delicate entanglement of 1,10- and 1,11-activities by way of common carbocation(s) that could allow 1,10-products to originate³⁴ from initial 1,11-cyclized precursors (or vice versa)³⁵ via ring contraction/expansion reactions and dual 1,10/1,11-reaction intermediates or transition states.³⁶ Moreover, previous studies with GAS and the hexadeuterated [12,12,12,13,13,13-²H₆]-farnesyl diphosphate analogue *d*₆-**1** (Scheme 5) revealed a considerable change in product distribution (80% **4**, 9% **6**, and 11% **8**) relative to unlabeled **1** (Table 1).^{6b} Since perturbation in product distributions upon isotope substitution generally reflects combined primary and secondary kinetic isotope effects (KIEs) on partitioning steps, this result supports the inherent branching nature of the reaction mechanism of GAS (and GAS-G402C) inferred from our kinetic results with 10F-1 (Scheme 4).

3. Isotopically Sensitive Branching Experiments. Although the actual induced KIE³⁷ on product ratios was not reported in a previous investigation of GAS,^{6b} the drastic increase in the rate of formation of both germacrene D (**6**) and α -humulene (**8**) relative to germacrene A (**4**) seems to indicate that **6**, **8**, and **4** likely originate from a common intermediate, subject to a primary deuterium KIE on the final deprotonation step.

Scheme 5. Mechanistic Possibilities for the GAS-Catalyzed Turnover of [12,13-²H₆]-1 (*d*₆-1) via Intermediate *d*₆-11^a


^aPath A involves a late branching of cation *d*₆-11 toward *d*₆-7. Path B contemplates the reverse humulene–germacrene ring-contraction reaction.

The dual 1,10/1,11-specificity (ca. 1:1) and stability of the GAS variants obtained by single amino acid replacements (Table 1) provides an opportunity to reinvestigate the mechanism of GAS with *d*₆-1 and exploit the phenomenon of isotopically sensitive branching³⁸ by induced KIEs on product ratios. This will provide confirmation of the possible 1,10-origin of the 1,11-linked α -humulene by what appears to be a GAS-catalyzed germacrenyl-humulyl rearrangement. A further objective was to evaluate the possible reversibility of this rearrangement. To this end, [12,13-²H₆]-farnesyl diphosphate (*d*₆-1)³⁹ was synthesized (Supporting Information), analyzed by high-resolution negative ion ES-MS (*m/z* 388 (29.1), 286 (100): 15% *d*₇, 83% *d*₆) and incubated with GAS and selected variants. The resulting enzymatic products were quantified by GC–MS analysis (Table 3). In agreement with the previous

Table 3. Distribution of Enzymatic Products for Incubations of GAS and Selected GAS Variants with FDP (*d*₀-1) and the [12,13]-Hexadeuterated 1 (*d*₆-1) (See Also Table S1, Supporting Information)

enzyme	substrate	4:6	4:8	6:8	4:(6 + 8)	<i>k</i> _H / <i>k</i> _D
GAS	<i>d</i> ₆ -1	11.7	10.9	0.97	5.67	4.98
	<i>d</i> ₀ -1	57.8	55.1	0.95	28.40	
G402S	<i>d</i> ₆ -1	1.91	0.23	0.12	0.21	5.09
	<i>d</i> ₀ -1	9.94	1.19	0.12	1.02	
G402C	<i>d</i> ₆ -1	0.18	0.04	0.28	0.04	4.29
	<i>d</i> ₀ -1	0.86	0.22	0.26	0.16	
G402T	<i>d</i> ₆ -1	0.08	0.05	0.56	0.03	5.01
	<i>d</i> ₀ -1	0.39	0.23	1.48	0.12	
G402C/ S442C	<i>d</i> ₆ -1	-	-	0.31	-	2.69
	<i>d</i> ₀ -1	0.04 ^a	0.03 ^b	0.81	0.02	

^aIncludes 13 (35.6%). ^bIncludes 14 (14.4%). See Table 1

report, when *d*₆-1 was employed as substrate of GAS,^{6b} a considerable decrease (~5-fold) in the germacrene A/humulene (4:8) and the germacrene A/germacrene D (4:6) product ratios was observed. This simultaneous perturbation as consequence of replacing H with D in 1 establishes that the three products (4, 6, and 8) originate from a common intermediate (11). The observed branching toward α -humulene (path A in Scheme 5) translates into an overall deuterium KIE of 4.98 (Table 3) and reflects an interesting example of an enhanced rate for the Wagner-Meerwein rearrangement (3 → 7) resembling that of the (+)- α -pinene synthase catalyzed rearrangement of the tertiary pinyl to the secondary bornyl

cation.⁴⁰ The magnitude of the induced KIE (4.98) is consistent with primary deuterium KIEs on similar *E*₁ proton eliminations catalyzed by terpene synthases^{40–42} and appears to indicate a relatively fast and reversible cyclization of FDP (1) to germacrenyl cation (3) followed by a slow, rate determining deprotonation to 4. Comparable KIEs (4.08–5.46) were obtained with GAS-G402A, GAS-G402S, GAS-G402D, GAS-G402C, GAS-G402T, and GAS-G402 V (Table 3 and Table S1, Supporting Information).

The increased partitioning toward 8 (Table 3) could also be attributed to a secondary β -KIE operating on the alternative and earlier branching of the bridged carbocation 11 to humulene and germacrene products (path B in Scheme 5).⁴² In this scenario, the observed KIEs shown in Table 3 would reflect contributions to the KIE upon branching of 11 (secondary) and/or deprotonation (primary) to 4. Assuming a slow and irreversible early partition of 11 toward the 1,11- and 1,10-cyclized products, the relative proportions of α -humulene (8) and the germacrenes 4 and 6 should be largely unaffected for the nondeuterated (*d*₀-1) and deuterated (*d*₆-1) precursors. Inspection of the product ratios (Table 3 and Table S1, Supporting Information) together with the magnitude of the induced KIEs (4.3–5.5) establish that an early, irreversible branching of cation 11 toward 7 and 3 (path B in Scheme 5) is inconsistent in the present case. In a similar mechanistic study with (–)- α / β -pinene synthase, the identical relative ratios of camphene to the total α - and β -pinenes observed for labeled and unlabeled species supported the early, direct formation of camphene from α -terpenyl cation rather than the late branching mechanism from the pinyl cation.⁴² Likewise, if carbocation 11 cyclizes exclusively to either 3 or 7, and these two species interconvert slowly compared to the rates of their respective conversions to germacrene and humulene products, then the KIE associated with the methyl → methylene *E*₁ elimination should be substantially masked due to the existence of other partially rate-determining steps. Masked primary KIEs on similar deprotonations mediated by terpene synthases yielded KIE values as low as 1.2–1.5.^{38,40} From this analysis, it follows that relatively fast events such as interconversions of humulyl and germacrenyl cations, occurring prior to the slow, rate-determining, and isotope-sensitive deprotonation step, will amplify the effects of the primary KIE on the observed product distribution.

Finally, GAS-G402C/S442C provides an illustration of a secondary β -deuterium KIE on formation of carbocation 3, and hence, the overall 1,10/1,11-product distribution. In contrast to the GAS variants affecting only the Gly402 position, GAS-G402C/S442 effectively suppresses (98.2%) the final deprotonation step to 3 (Tables 1 and 3) with FDP (1). Upon reaction with the isotopologue (*d*₆-1), this mutant yielded an altered dual 1,10/1,11-product profile with intact isotopic content, and in favor of the 1,11-cyclization mode of the enzyme. In this case, the increased branching toward 8, 10, and 14 must be the result of a secondary deuterium KIE acting on a common step. The magnitude of this positive β -deuterium KIE (2.69, i.e., 1.35 per CD₃ group) (Table 3) signifies a rate-limiting cyclization step and is in good agreement with previously reported KIE values on similar processes.^{30c} The increase of 1,11-products observed in this case is consistent with a fast and reversible 1,11-cyclization of 11 (or 2) to humulyl cation (7), followed by a relatively slow, rate-limiting humulyl-germacrenyl ring contraction reaction (Scheme 5). This elegant, yet simple isotope-sensitive rearrangement could represent the molecular

gateway linking ancestral 1,11- and modern 1,10-sesquiterpene synthases in plants.

In summary, the results reported here suggest that the biosynthesis of germacrene and humulene is likely connected by bicyclogermacrene-like bridged 1,10,11-carbocations and transition states (Scheme 5) that link rapidly equilibrating mixtures of germacrene and humulene carbocations. A single amino acid residue (G402 in GAS) appears to act as a functional switch between 1,10- and 1,11-cyclizations supporting the proposal⁹ that modern plant 1,10-cyclases might have evolved from promiscuous 1,11-sesquiterpene synthases. Phylogeny-guided assignments of protein function to gene sequences are notoriously difficult for plant terpene synthases, and our findings may guide future experimental work toward a fuller understanding the evolution of terpene synthases.^{20,22,43,44}

■ ASSOCIATED CONTENT

■ Supporting Information

Synthetic work; GC chromatograms and mass spectra of products produced by GAS, GDS, and mutants from FDP (1), 10F-FDP (10F-1), and [12,13-²H₆]-1; complete chart with product distribution and KIE for most enzymes, homology model of GAS generated from the crystal structures of TEAS and DCS; amino acid sequence alignments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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