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Unearthing the roots of the terpenome

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Although terpenoid synthases catalyze the most complex reactions in biology, these enzymes appear to play little role in the chemistry of catalysis other than to trigger the ionization and chaperone the conformation of flexible isoprenoid substrates and carbocation intermediates through multistep reaction cascades. Fidelity and promiscuity in this chemistry (whether a terpenoid synthase generates one or several products), depends on the permissiveness of the active site template in chaperoning each step of an isoprenoid coupling or cyclization reaction. Structure-guided mutagenesis studies of terpenoid synthases such as farnesyl diphosphate synthase, 5-epi-aristolochene synthase, and γ-humulene synthase suggest that the vast diversity of terpenoid natural products is rooted in the facile evolution of α-helical folds shared by terpenoid synthases in all forms of life.

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Introduction

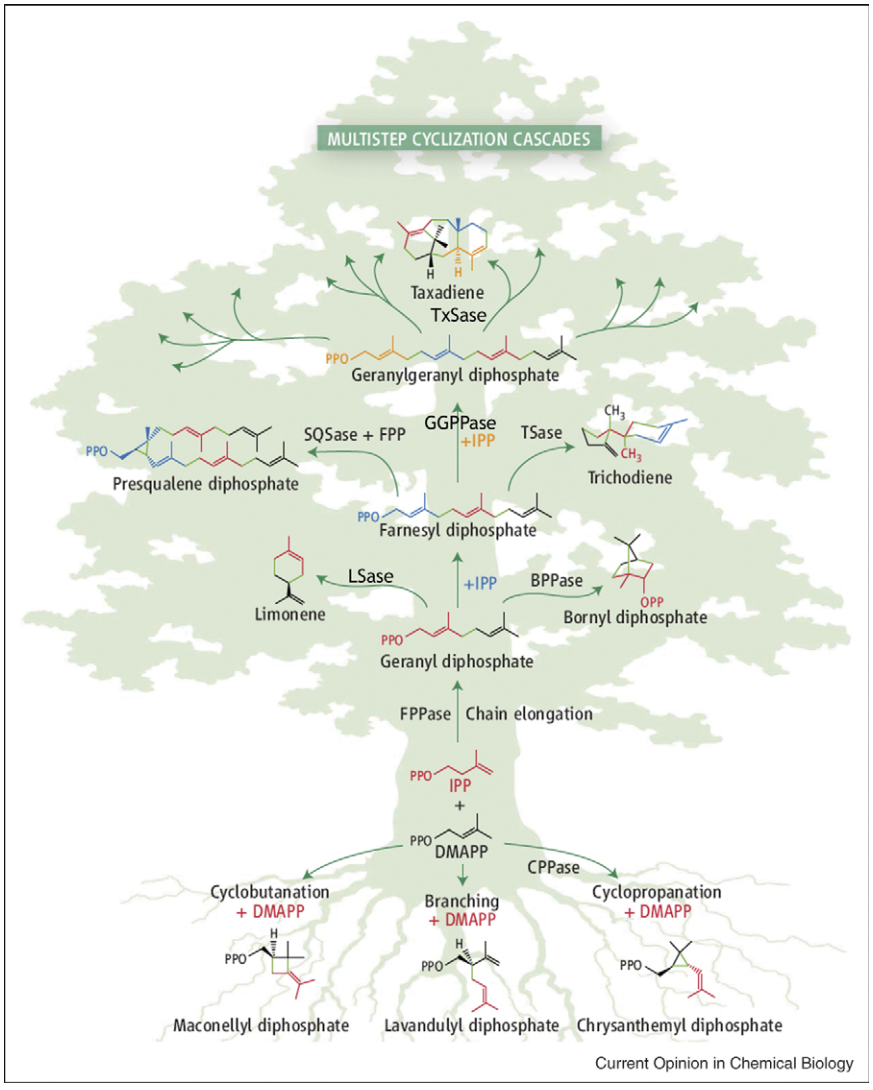
The most structurally and stereochemically diverse family of natural products is that of the terpenoids (a.k.a. isoprenoids), more than 55 000 of which have been identified to date in all life forms. The remarkable diversity of this chemical library — the terpenome — belies its simple roots in the universal 5-carbon precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Figure 1) [1]. These isoprenoids can be coupled in chain elongation reactions to yield increasingly longer polyisoprenoid diphosphates [2,3] which can then be cyclized to generate single-ringed or multiringed products [4–6,7••]. Alternatively, two isoprenoids can be coupled in irregular fashion to yield cyclobutyl, branched, or cyclopropyl products [8].

Isoprenoid coupling and cyclization reactions are catalyzed by terpenoid synthases, a.k.a. terpene synthases or isoprenoid synthases; synthases that catalyze cyclization reactions are also referred to as terpenoid cyclases. The most critical function of a terpenoid synthase is to provide a template that binds the flexible isoprenoid substrate(s) with the proper orientation and conformation so that, upon the generation of a reactive substrate carbocation, a specific trajectory of intermolecular or intramolecular carbon–carbon bond formation is enforced. The template similarly serves as a chaperone in subsequent steps of the reaction cascade, which is ultimately terminated by quenching the final carbocation intermediate through proton elimination or hydroxylation. Curiously, the 'synthetic yield' of the terpenoid synthase reaction can vary dramatically: high fidelity enzymes such as aristolochene synthase from *Aspergillus terreus* generate one exclusive product [9], whereas promiscuous enzymes generate multiple products; for example, γ-humulene synthase generates 52 distinct products of which γ-humulene itself comprises only 28.6% [10]. Biosynthetic promiscuity is a consequence of an imprecise template: a more permissive chaperone allows alternative conformations of substrate(s) or intermediate(s) that allow alternative carbon–carbon bond forming trajectories. In recent years, X-ray crystallographic studies have illuminated the structural basis of fidelity and promiscuity in the coupling and cyclization reactions of terpenoid biosynthesis.

Coupling reactions

The crystal structure of avian farnesyl diphosphate synthase (FPPase) was the first of a terpenoid synthase and revealed a hydrophobic active site nested within an α-helical bundle [11]. Helices D and H contain 'aspartate-rich' motifs DDXX(XX)D that bind a trinuclear magnesium cluster in the crystal structure of the *Escherichia coli* enzyme complexed with IPP and an unreactive analogue of DMAPP (Figure 2a) [12]. In catalysis, the metal cluster triggers DMAPP ionization, which generates an allylic carbocation that alkylates the C3–C4 π bond of IPP to yield a tertiary carbocation at C3. The stereospecific elimination of the C2–H_R proton of the isopentenyl moiety appears to be mediated by the pyrophosphate leaving group [12] to yield geranyl diphosphate (Figure 2a and b) [2,3]. A second chain elongation reaction with geranyl diphosphate thus formed and another molecule of IPP yields farnesyl diphosphate. A similar coupling mechanism is also consistent with recently determined crystal structures of geranylgeranyl diphosphate synthase (GGPPase, which shares the FPPase fold) [13•,14,15]. Comparisons of unliganded and liganded structures of FPPase [12] and GGPPase

Figure 1



Family tree of terpenomic diversity. Examples of monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), and triterpenes (C_{30}) are shown. The reactions indicated are catalyzed by enzymes that share the fold of farnesyl diphosphate synthase. Isoprenoid groups are color-coded to indicate their biosynthetic fates, and newly formed carbon–carbon bonds are green. Abbreviations: BPPase, bornyl diphosphate synthase; DMAPP, dimethylallyl diphosphate; FPPase, farnesyl diphosphate synthase; GGPPase, geranylgeranyl diphosphate synthase; IPP, isopentenyl diphosphate; LSase, limonene synthase; PPO, diphosphate; SQSase, squalene synthase; TSase, trichodiene synthase; TxSase, taxadiene synthase. From Christianson DW: *Science* **316**:60–61 (6 April 2007). Illustration: P Huey. Reprinted with permission from AAAS.

[13•] indicate that ligand binding triggers conformational changes that 'cap' the active site.

That FPPase provides a well-defined template for the isoprenoid-coupling reaction is elegantly demonstrated in mutagenesis studies. For example, the depth of the active site is the primary determinant of product chain length, and FPPase mutants with deeper active sites generate polyisoprenoid diphosphate products containing up to 14 isoprenoid groups [16,17]. Structural comparisons of wild-type and mutant chain elongation enzymes suggest a 'molecular ruler mechanism' in which key residues at the base of the active site are identified as the primary determinants of product chain length [14].

Recently, site-directed mutagenesis has been used to fundamentally alter the isoprenoid-coupling reaction by remolding the template for catalysis. The snowfield sagebrush enzymes, FPPase and chrysanthemyl diphosphate synthase (CPPase), are related by significant (75%) amino acid sequence identity and therefore share homologous structures [18]. However, the active site of FPPase has evolved to optimize the stereoselectivity of the chain elongation reaction and the exclusion of DMAPP from

the IPP-binding site [19], whereas CPPase requires the binding of DMAPP to this site in order to catalyze a cyclopropanation reaction between two molecules of DMAPP (Figure 2b). The preparation of chimeric synthases from FPPase and CPPase remolds the template for substrate binding while preserving residues important for metal binding and substrate ionization in catalysis [20••]. Templates thus remolded are more permissive chaperones that allow the substrates to reorient, thereby allowing alternative trajectories of carbon–carbon bond formation leading to varying mixtures of chain elongation, cyclobutanation, branching, and cyclopropanation products (Figure 2c). Accordingly, naturally occurring enzymes catalyzing these four isoprenoid-coupling reactions probably evolved from an FPPase-like ancestor [20••]. For example, squalene synthase catalyzes a cyclopropanation reaction (Figure 1) and exhibits the FPPase fold despite insignificant amino acid sequence identity [21] (Figure 3a). Clearly, the permissiveness of the biosynthetic chaperone is easily cajoled by mutagenesis in nature or in the laboratory to allow for the generation of new terpenoid products.

Cyclization reactions

As for the isoprenoid-coupling reactions just described, the reaction catalyzed by a metal-dependent terpenoid cyclase is initiated by the formation of a highly reactive carbocation through the metal-triggered departure of the diphosphate group of the substrate, typically geranyl diphosphate, farnesyl diphosphate, or geranylgeranyl diphosphate. In these reactions, however, the initial trajectory of carbon–carbon bond formation is intramolecular rather than intermolecular. Examples of cyclization products are shown in Figure 1.

The first X-ray crystal structures of metal-dependent terpenoid synthases were those of pentalenene synthase [22] and 5-epi-aristolochene synthase [23], which unexpectedly revealed α-bundle folds homologous to the FPPase fold despite insignificant amino acid sequence identity. These enzymes are designated class I terpenoid cyclases [24] and their homologous folds are also exemplified by trichodiene synthase [25] and bornyl diphosphate synthase [26] (Figure 3a). As observed for FPPase [12], the terpenoid cyclases undergo a structural change from an open to a closed active site conformation upon the binding of a complete trinuclear metal cluster and an isoprenoid diphosphate or PP_i moiety [23,25,26,27•,28]. Like FPPase, class I terpenoid cyclases contain an aspartate-rich motif on helix D. However, these cyclases do not contain a second aspartate-rich motif on helix H, but instead contain the signature sequence (**N,D**)D(L,I,V)X(**S,T**)XXX**E** (the 'NSE/DTE motif') in which boldface residues chelate the Mg_B^{2+} ion [23,25,26,27•,28].

The class II terpenoid cyclases squalene-hopene synthase [29] and lanosterol synthase [30] contain two domains, each of which is comprised of a double α-barrel fold topologically unrelated to the α-bundle fold of class I cyclases [24]. The class II cyclases generate the initial substrate carbocation by utilizing a conserved aspartic acid residue to protonate a substrate π-bond or epoxide ring [6]. Interestingly, the class II terpenoid cyclase fold is shared by farnesyl transferase, which catalyzes protein prenylation in certain signal transduction pathways [31], and nisin cyclase, which does not catalyze a terpenoid cyclization but instead catalyzes multiple thioether cyclization reactions in a 34-residue polypeptide [32] (Figure 3b). That the class I α-bundle and class II double α-barrel folds of terpenoid cyclases are conserved in such diverse biosynthetic reactions suggests that these protein folds lend themselves to facile evolution as templates for biological coupling or cyclization reactions [33].

The most recently reported crystal structures of terpenoid cyclases are those of two class I enzymes: limonene synthase, a monoterpene synthase from *M. spicata* (mint) [27•], and aristolochene synthase, a sesquiterpene synthase from *A. terreus* [28]. The crystal structure of limonene synthase reveals a two-domain architecture characteristic of many plant cyclases (Figure 4a) [23,26], in which the C-terminal domain adopts the class I terpenoid synthase fold and the N-terminal domain adopts a fold similar to that of a class II terpenoid synthase [24]. Although the N-terminal domains of these plant cyclases are not known to catalyze cyclization reactions, intron conservation patterns suggest that such two-domain architectures evolved from an ancestral enzyme in which both domains were once catalytically active [34], for example, as found for the diterpene cyclase abietadiene synthase from *A. grandis* [35].

Intriguingly, cocrystallization of limonene synthase with 2-fluorogeranyl diphosphate yields the structure of the complex with 2-fluoro-(3*S*)-linalyl diphosphate, a catalytic intermediate, bound with an extended, nonproductive conformation [27•]. In contrast, cocrystallization with racemic 2-fluorolinalyl diphosphate yields the helical productive conformation required for the cyclization of either enantiomer (though the *S*-enantiomer is preferred). It is possible that the isomerization of geranyl diphosphate to linalyl diphosphate does not require a cyclization-competent substrate conformation, which instead could be achieved after the formation of the linalyl diphosphate intermediate. Nonproductive isoprenoid binding modes observed in the active sites of other class I terpenoid cyclases [23,26,36] suggest that the formation of transient intermediates in terpenoid cyclase-catalyzed reactions is under kinetic rather than thermodynamic control [36].

The binding of isoprenoid substrate and intermediate analogues to bornyl diphosphate synthase similarly reveals nonproductive conformations and orientations,

Figure 2

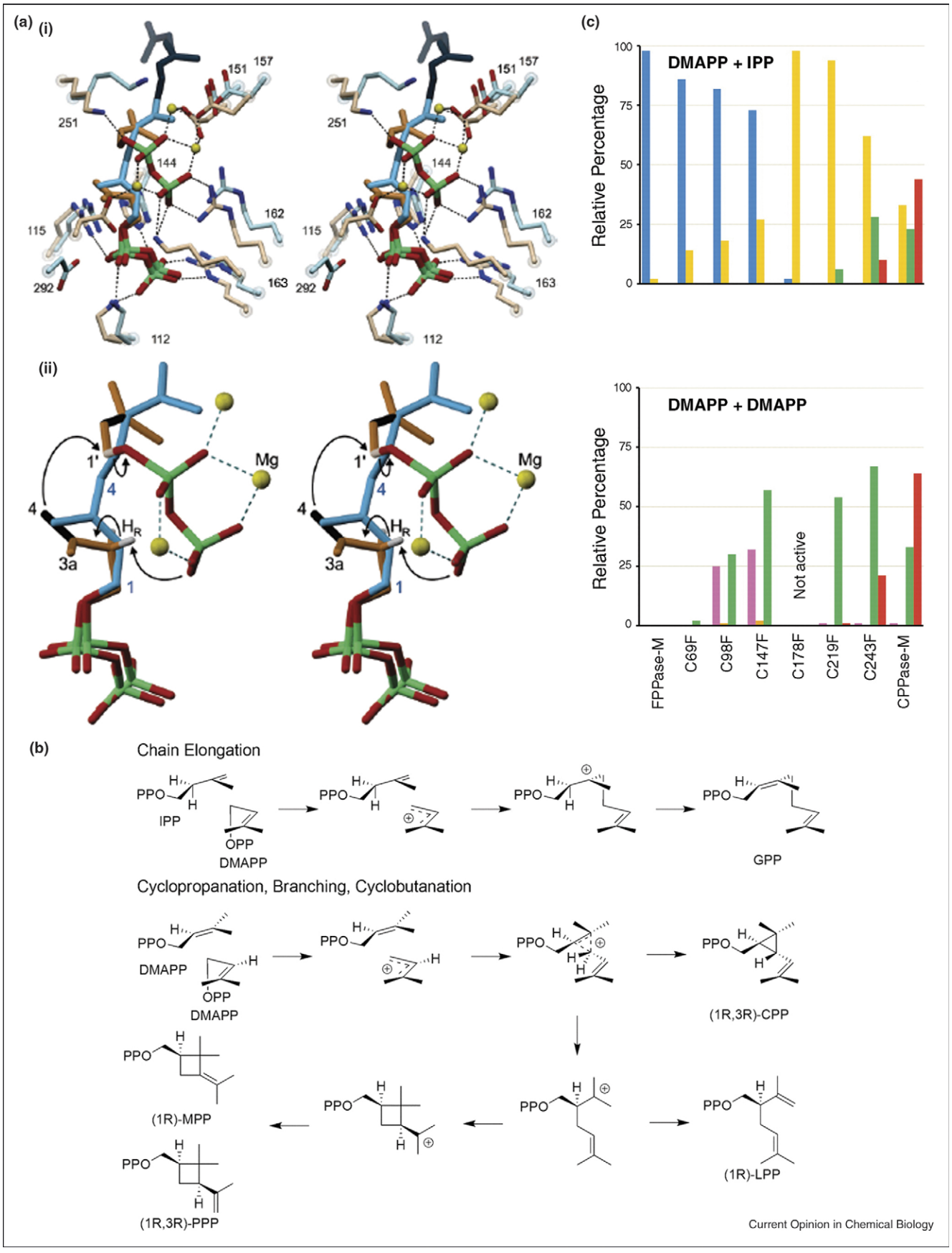

(a)
(i)
151
157
251
144
162
115
292
163
112
(ii)
1'
4
Mg
4
H_R
3a
1
(c)
DMAPP + IPP
Relative Percentage
100
75
50
25
0
DMAPP + DMAPP
Not active
FPPase-M
C69F
C98F
C147F
C178F
C219F
C243F
CPPase-M
(b)
Chain Elongation
PPO
H
IPP
OPP
DMAPP
GPP
Cyclopropanation, Branching, Cyclobutanation
(1R,3R)-CPP
(1R)-MPP
(1R)-LPP
(1R,3R)-PPP
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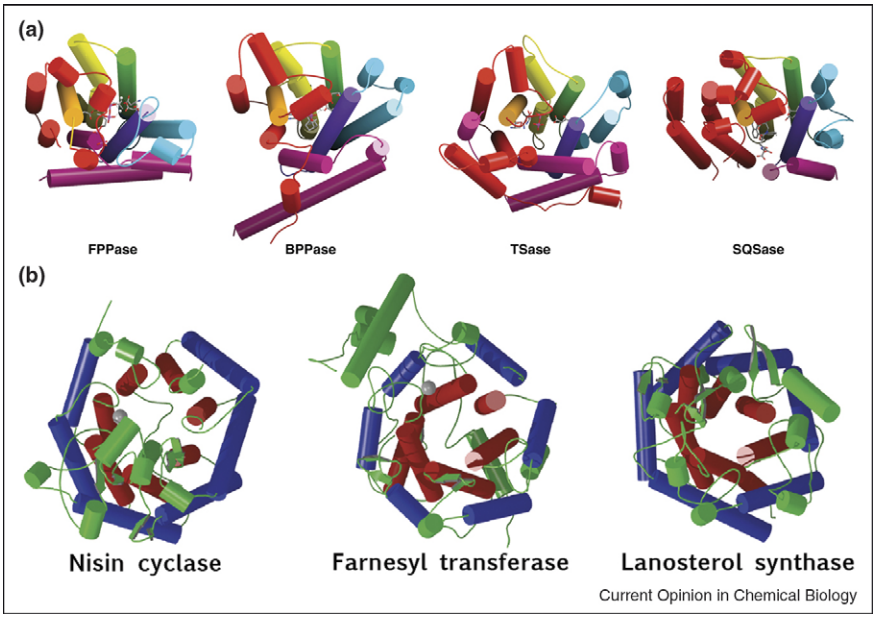
Figure 3



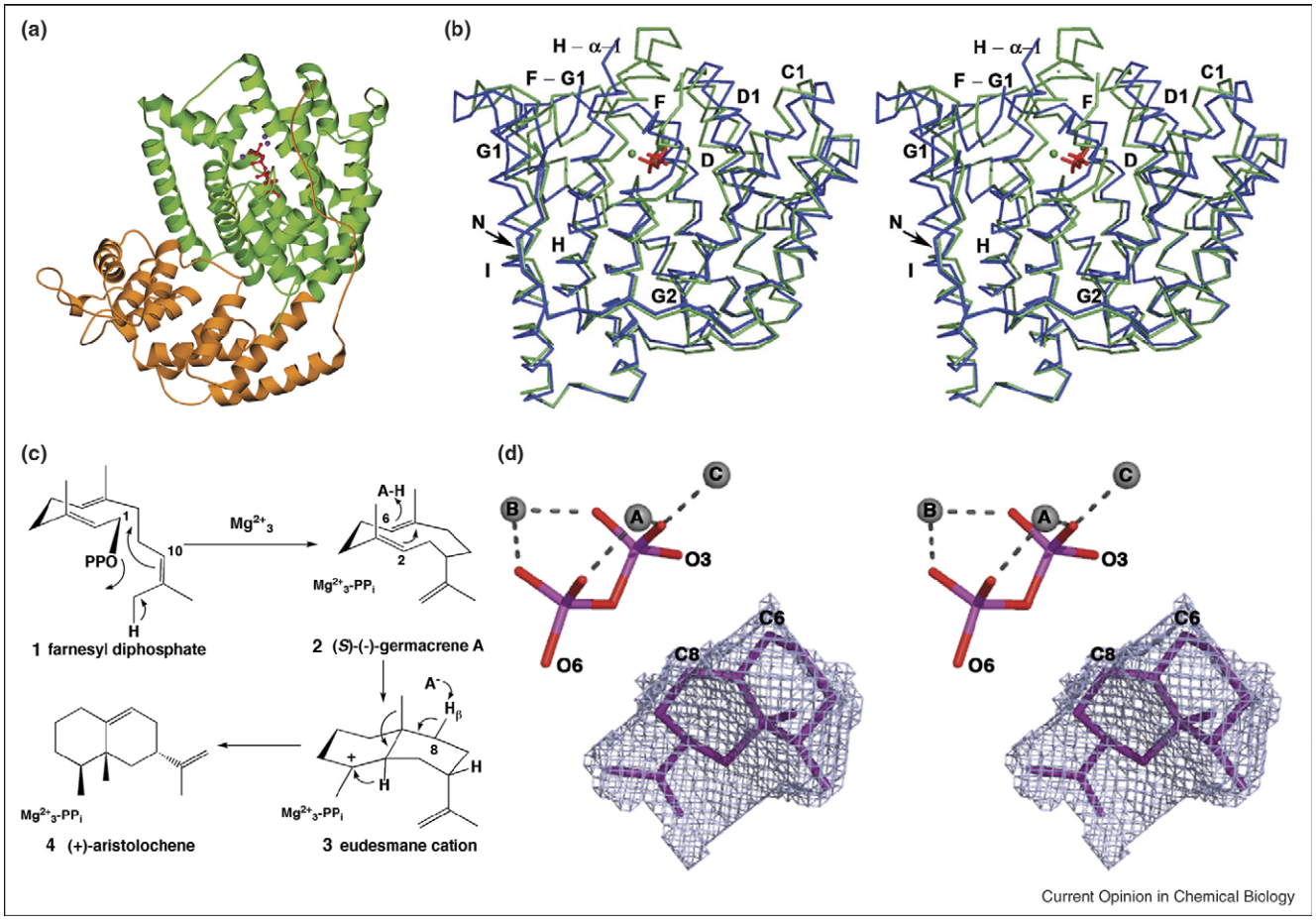
Terpenoid synthase folds. **(a)** The class I terpenoid synthase fold is illustrated by the α-bundle fold of FPPase from *E. coli* [12], colored violet-red to represent the successively larger segments of CPPase substituted for those of FPPase in the CPPase–FPPase chimeras outlined in Figure 2c [20••]. For example, the c69f chimera contains the first 69 residues of CPPase-M (violet segment) and the remaining residues of FPPase-M (blue-red segments), and so forth. The FPPase fold is also shared by the catalytic domains of terpenoid synthases that generate larger and more complex products, such as bornyl diphosphate synthase (BPPase), trichodiene synthase (TSase), and squalene synthase (SQSase); these synthases are color-coded in identical fashion with FPPase to highlight their topological similarities. Reprinted with permission from Ref. [1]. Copyright 2007 American Association for the Advancement of Science. **(b)** The class II terpenoid synthase fold is a double α-barrel conserved in nisin cyclase, farnesyl transferase, and both domains of lanosterol synthase. This fold is topologically distinct from the α-bundle fold of the class I terpenoid synthase. Reprinted from Ref. [33].

except for an aza analogue of the carbocation intermediate that immediately precedes the final product [26]. Apparently, the more product-like the isoprenoid analogue is, the more likely its thermodynamically favorable binding mode observed in the crystal will reflect a catalytically productive binding mode [26]. Accordingly, the active site of a terpenoid cyclase — the template for catalysis — must have a contour that is predominantly product-like. To illustrate, consider the structures of unliganded aristolochene synthase from *A. terreus* and its Mg_3^{2+}–PP_i complex [28], which reveal the 'open'–'closed' conformational transition of the active site required for the cyclization of farnesyl diphosphate (Figure 4b and c). The active site contour in the 'closed' conformation is indeed very product-like (Figure 4d).

Given that terpenoid cyclases can vary in their permissiveness as product-like templates, recent experiments have focused on manipulating the structure of either the enzyme or the substrate to yield alternative products. For example, modest catalytic promiscuity has recently been discovered for trichodiene synthase from *Fusarium*

(Figure 2 Legend) Isoprenoid-coupling reactions. **(a)** (top) Binding conformations of the unreactive DMAPP analogue dimethylallyl-*S*-thiolodiphosphate coordinated to three Mg^{2+} ions (gray-yellow) and isopentenyl diphosphate (IPP) in the active site of farnesyl diphosphate synthase from *E. coli* (beige) [12] superimposed on the binding conformation of geranylgeranyl diphosphate in the active site of geranylgeranyl diphosphate synthase from *S. alba* (blue) [13•]. (Bottom) Stereochemistry of the isoprenoid chain elongation reaction inferred from the superposition above (for clarity, enzyme residues are not shown). The observed binding conformations of DMAPP and IPP suggest that the pyrophosphate product mediates the stereospecific elimination of the C2–H_R proton of the isopentenyl moiety [12]. Reprinted with permission from Ref. [13•]. Copyright 2006 American Chemical Society. **(b)** Isoprenoid-coupling reactions catalyzed by terpenoid synthases. Farnesyl diphosphate synthase (FPPase) catalyzes chain elongation reactions leading to the formation of geranyl diphosphate (GPP) and longer isoprenoid diphosphates. Chrysanthemyl diphosphate synthase catalyzes a cyclopropanation reaction leading to chrysanthemyl diphosphate (CPP). Chimeric synthases constructed from FPPase and CPPase generate the branching product lavandulyl diphosphate (LPP) as well as cyclobutanation products maconellyl diphosphate (MPP) and planococcyl diphosphate (PPP). From Thulasiram HV, Erickson HK, Poulter, CD: *Science* **316**:73–76 (6 April 2007). Reprinted with permission from AAAS. **(c)** Products generated by CPPase-FPPase chimeras when incubated with DMAPP and IPP as indicated: farnesyl diphosphate, blue; geranyl diphosphate, gold; lavandulyl diphosphate, green; chrysanthemyl diphosphate, red; maconellyl diphosphate, mauve; planococcyl diphosphate, orange. Synthase abbreviations are as follows: FPPase-M is G69E/E210Q FPPase and CPPase-M is M98I/E177D/D243A CPPase, constructs prepared to facilitate the preparation of chimeric enzymes; these modified synthases exhibit kinetic parameters comparable to those of the wild-type synthases. Chimeric synthases are indicated by their splice junctions, for example, the c69f synthase contains the first 69 residues of CPPase-M and the remaining residues of FPPase-M (see also Figure 3a). From Thulasiram HV, Erickson HK, Poulter, CD: *Science* **316**:73–76 (6 April 2007). Reprinted with permission from AAAS.

Figure 4

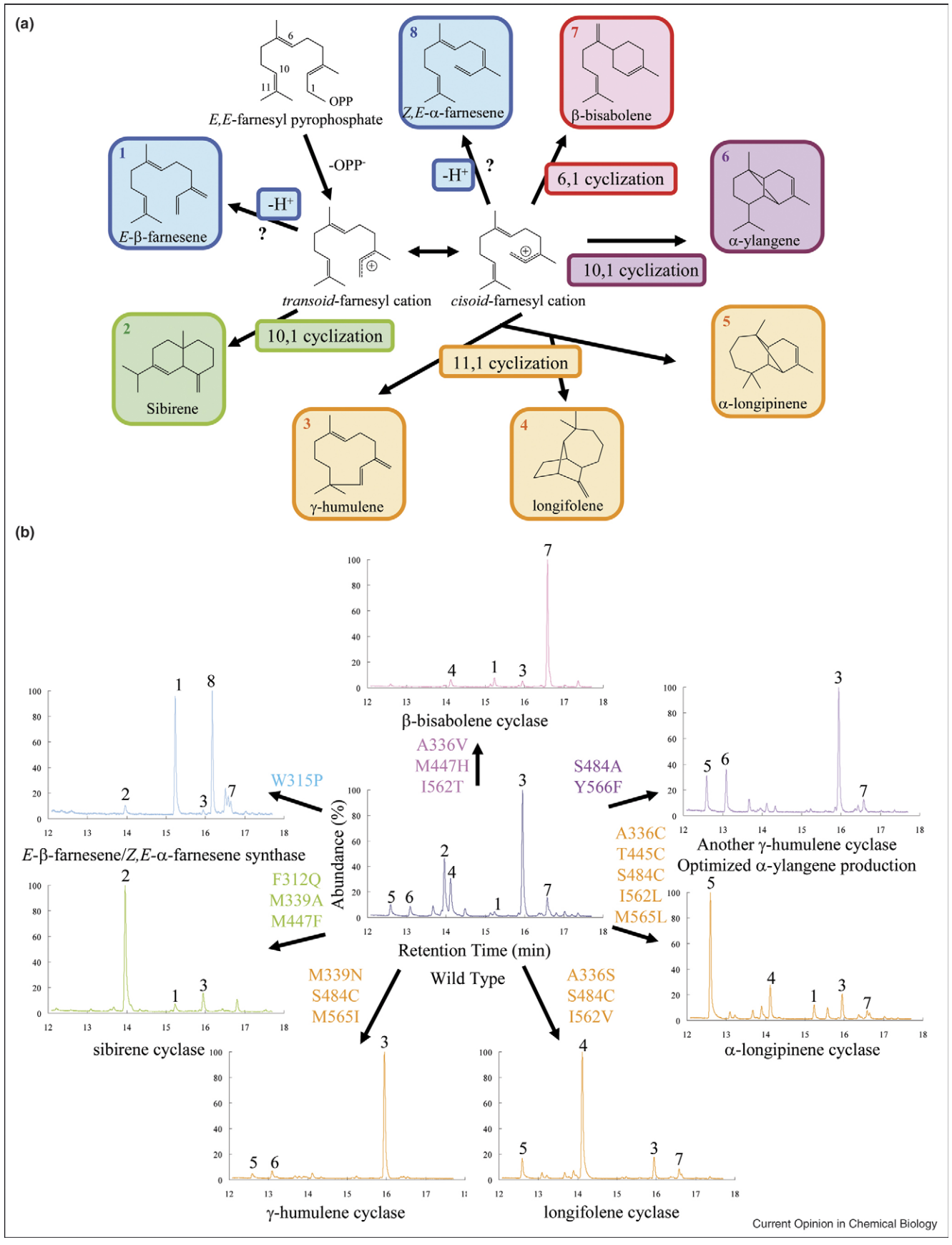


New terpenoid cyclase structures. **(a)** The structure of limonene synthase reveals a two-domain architecture characteristic of plant terpenoid cyclases. 2-Fluorolinalyl diphosphate (red) and three Mn^{2+} ions (purple) bind to the C-terminal catalytic domain, which exhibits the α-bundle fold of a class I terpenoid synthase (green). The N-terminal domain (orange) has no known catalytic function yet exhibits a fold similar to the double α-barrel fold of a class II terpenoid cyclase. Reprinted with permission from Ref. [27•]. Copyright 2007 National Academy of Sciences, USA. **(b)** Stereoview of the superposition of *A. terreus* aristolochene synthase structures in the open, unliganded conformation (blue) and the closed, Mg_3^{2+}–PP_i liganded conformation (green; Mg^{2+} ions are green spheres and the PP_i anion is red). Significant conformational changes are triggered in the indicated helices and loops, the most significant of which is the ligand-induced ordering of the H-α1 loop that caps the active site cleft. Reprinted in part with permission from Ref. [28]. Copyright 2007 American Chemical Society. **(c)** Aristolochene synthase mechanism. Reprinted with permission from Ref. [28]. Copyright 2007 American Chemical Society. **(d)** Stereoview of the enclosed active site surface contour of aristolochene synthase in the Mg_3^{2+}–PP_i complex; protein atoms are omitted for clarity. Aristolochene is modeled within the meshwork surface to illustrate that the three-dimensional contour of the active site is very product-like. Interestingly, the proximity of the PP_i O3 atom to aristolochene C6 and C8 atoms implicates the PP_i anion as a possible general acid/base in the cyclization mechanism. Reprinted with permission from Ref. [28]. Copyright 2007 American Chemical Society.

sporotrichioides, which generates 11% alternative sesquiterpene products; multiple products are also detected when 2-fluorofarnesyl diphosphate and 4-methylfarnesyl diphosphate are used as substrates, and mutagenesis of metal-binding residues alters product ratios [37,38]. In other examples, taxadiene synthase catalyzes the cyclization of 6-fluorogeranylgeranyl diphosphate to generate a mixture of 7-fluoroverticillenes [39], and aristolochene synthase catalyzes the cyclization of 2-fluorofarnesyl diphosphate to form 2-fluorogermacrene A [40]. 5-Epi-aristolochene synthase, for which the stereochemical details of the cyclization mechanism have been recently outlined [41], generates 25 sesquiterpene products [42], and mutagenesis studies targeting evolutionarily variable

(Figure 5 Legend) Re-evolution of terpenoid cyclase specificity. **(a)** Cyclization reactions catalyzed by the promiscuous terpenoid cyclase, γ-humulene synthase; interconversion of the *trans*-farnesyl and *cis*-farnesyl cation occurs through intermediate nerolidyl diphosphate (OPP = diphosphate). **(b)** Saturation mutagenesis of plasticity residues comprising the active site contour of γ-humulene synthase reveals that only 2–5 amino-acid substitutions are required to redirect biosynthesis to form an alternative predominant product as detected using gas chromatography. Product numbers are indicated in (a). Reprinted with permission from Ref. [46••]. Copyright 2006 Nature Publishing Group (http://www.nature.com/nature/).

Figure 5


(a)
E,E-farnesyl pyrophosphate
OPP
-OPP-
-H+
?
E-β-farnesene
Z,E-α-farnesene
β-bisabolene
6,1 cyclization
α-ylangene
10,1 cyclization
transoid-farnesyl cation
cisoid-farnesyl cation
Sibirene
10,1 cyclization
11,1 cyclization
α-longipinene
γ-humulene
longifolene
(b)
β-bisabolene cyclase
A336V
M447H
I562T
W315P
S484A
Y566F
E-β-farnesene/Z,E-α-farnesene synthase
Another γ-humulene cyclase
Optimized α-ylangene production
A336C
T445C
S484C
I562L
M565L
F312Q
M339A
M447F
Abundance (%)
Retention Time (min)
Wild Type
M339N
S484C
M565I
A336S
S484C
I562V
sibirene cyclase
α-longipinene cyclase
γ-humulene cyclase
longifolene cyclase
Current Opinion in Chemical Biology

residues in and surrounding the active site show that product arrays can be altered by design [43••].

Notably, only limited amino acid substitutions are sufficient to remold the active site template and redirect the cyclization cascade catalyzed by a terpenoid synthase. For example, the substitution of a single active site residue converts isokaurene synthase or abietadiene synthase into a specific pimaradiene synthase [44,45], and the biosynthetic array of sesquiterpenes generated by γ-humulene synthase [10] can be refocused to generate alternative predominant products by mutagenesis of only three to five active site residues at a time (Figure 5) [46••]. In exploring the biosynthetic promiscuity of the triterpene cyclase baruol synthase, Matsuda and colleagues advance that mechanistic diversity is the rule for terpenoid cyclases and that cyclization fidelity derives from the efficiency of the template in excluding alternative cyclization pathways [47•].

Conclusions

Product diversity in terpenoid biosynthesis is rooted in the permissiveness of the template that chaperones the orientations and conformations of flexible substrate(s) and intermediate(s) through multistep intermolecular-coupling reactions or intramolecular cyclization reactions. Importantly, promiscuous activities herald the evolutionary potential of terpenoid biosynthesis, because the active site contour serving as the template for catalysis is readily evolvable in nature or in the laboratory to chaperone alternative intramolecular or intermolecular reaction pathways. In class I terpenoid synthases, the binding of substrate(s) and metal ions triggers conformational changes that sequester the active site from bulk solvent [12,13•,23,25,26,27•,28], indicating that the active site contour does not adopt its productive, product-like shape until after the substrate has bound. It is only in this closed enzyme–substrate complex that the synthase triggers the ionization of the substrate diphosphate group, generally regarded as the slowest chemical step of the terpenoid synthase reaction; product release is overall rate determining for synthases that have been subjected to detailed kinetic analysis [48–50]. The conformational change of the class I synthase active site from a closed to an open conformation requires metal ion dissociation and presumably facilitates product release. By contrast, class II synthases appear to contain pre-formed, product-like templates for triterpene cyclization [29,30,51].

Notably, class I and class II terpenoid synthase folds can be combined in multidomain proteins to form larger active site cavities or even bifunctional enzymes. For example, the two class II domains of a triterpene cyclase are joined together in 'face-to-face' fashion to form an active site cleft large enough to accommodate a 30-carbon substrate [29,30]. Although 5-epi-aristolochene synthase contains a catalytically active class I synthase domain and a catalytically inactive class II synthase domain [23], both domains of the related plant cyclase abietadiene synthase are catalytically active and catalyze tandem cyclization reactions [35]. More recently, Cane's laboratory reports that germacradienol-geosmin synthase, a sesquiterpene cyclase from *Streptomyces coelicolor*, is similarly bifunctional, yet in this enzyme both domains adopt the class I terpenoid synthase fold [52••]. Additionally, Toyomasu reports that fusicoccadiene synthase contains two class I terpenoid synthase domains in which the C-terminal domain catalyzes the chain elongation reaction to form geranylgeranyl diphosphate, the cyclization of which is subsequently catalyzed by the N-terminal domain [53•]. Tandem reactions catalyzed by double-domain bifunctional terpenoid synthases may reflect an additional evolutionary strategy for achieving terpenomic diversity.

An emerging view of terpenoid synthase function is that other than triggering ionization of the isoprenoid substrate and serving as a template and chaperone for the coupling or cyclization reaction, the enzyme appears to play a minimal role in the chemistry of catalysis. For example, structural and enzymological studies of certain terpenoid synthases suggest that a π bond or the diphosphate group of the substrate, and not an enzyme-bound residue, serves as a catalytic base [12,28,54]; generally speaking, such regiospecific and stereospecific deprotonations are not well understood and merit further study. Theoretical studies of terpenoid synthase mechanisms further highlight the role of substrate conformation in governing intramolecular proton transfer steps in catalysis and promise to illuminate new features of isoprenoid coupling and cyclization reactions [55]. Thus, it is remarkable that the exquisite structural and stereochemical diversity of the terpenome may well be a consequence of the fact that terpenoid synthases do not excessively intrude on the complex organic transformations occurring in their active sites.

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- •• of outstanding interest

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