doi:10.2533/chimia.2009.340 Chimia 63 (2009) 340–344

Enzymatic C–C-Coupling Prenylation: Bioinformatics – Modelling – Mechanism – Protein-Redesign – Biocatalytic Application

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Abstract: The functional role of isoprenoids and especially enzymatic prenylation in nature and human application is briefly covered, with the focus on bioinformatical, mechanistical and structural aspects of prenyltransferases and terpene synthases. These enzymes are as yet underrepresented but perspectively useful biocatalysts for C–C couplings of aromatic and isoprenoid substrates. Some examples of the successful use in chemoenzymatic synthesis are given including an application for the otherwise difficult synthesis of Kuhistanol A. Computational structure-based site-directed mutagenesis can be used for rational enzyme redesign to obtain altered substrate and product specificities, which is demonstrated for terpene cyclases.

Keywords: Aromatic prenyltransferases · Biocatalysis · Protein-redesign · Substrate specificity · Terpene synthases

1. Introduction

Prenyl-converting or -transferring enzymes are responsible for the formation or modification of naturally occurring isoprenoids (including terpenoids and meroterpenoids). More than 50,000 different compounds have been isolated from fungi, animals, microbial and predominantly plant resources and thus represent the most diverse family of natural products, of which approximately one half are terpenoids (including steroids), the other half are chimeric compounds of moieties from other biosynthetic origins coupled to isoprenoids (meroterpenoids).[1–3] An excellent overview and summary of many pathways and important metabolites is given by Bouvier *et al.*[4] All organisms possess essential isoprenoids. The evolution of the immense metabolic diversity originating from equally diverse isoprenoid converting enzymes is not yet completely understood on the molecular and structural level.

Prenyl-converting enzymes are classified into terpene synthases (cyclases), transferases and hydrolases/isomerases. Transferases can be subdivided in aromatic prenyltransferases, isoprenylpyrophosphate synthases, and protein prenyltransferases (Scheme 1), and other prenyl-converting enzymes not assigned to one of these groups (*e.g.* geranylgeranyl hydrogenase or squalene epoxidase). For all these enzymes, the catalysis mechanism starts with the cleavage of a pyrophosphate leaving group from a prenyl moiety to form an intermediate allylic prenyl cation (Scheme 1, step I). Terpene synthases (cyclases) use this reactive intermediate to perform an intramolecular electrophilic addition to a C=C double bond to form a terpinyl (*e.g.* for monoterpene synthases) or other cyclic cation intermediate to produce a wide variety of terpenoids *e.g.* limonene, pinene, sabinene, camphene, and many others (Scheme 1, step IIa).

Prenyltransferases require a second nucleophilic substrate to which the intermediate isoprenyl cation is transferred (Scheme 1, step IIb). They comprise – among others – oligoprenyl pyrophosphate synthases, protein prenyltransferases, and aromatic prenyl transferases. Aromatic prenyltransferases catalyse the formation of C–C bonds between an aromatic substrate (usually a phenol) and an isoprenoid diphosphate as electrophile (see *e.g.* Scheme 2 for the reaction of UbiA-enzyme). Isoprenyl pyrophosphate synthases are responsible for enzymatic connections (C–C coupling) of two prenyl moieties (either by head to head or head to tail condensation) leading to the elongation of the prenyl chain. In the case of protein prenyltransferases the prenyl cation moiety is usually transferred to the side chain of a C-terminal cysteine by formation of a C–S bond.

1.1 *Importance of Prenylated Metabolites in Nature and Human Use*

Different types of terpenoids serve a number of important physiological and societal functions. Plant terpenoids are classified as primary and secondary metabolites. Isoprenoids as part of the primary metabolism serve basic functions like cell growth modulation and plant elongation. Primary metabolic terpenoids such as steroids are essential for the membrane function and act as hormones and bile acids. Carotenoids are responsible for light harvesting and photoprotection, and ubiquinone, menaquinone and plastoquinone are involved in the electron transport.[1–3,5] Secondary metabolic terpenoids are involved in *e.g.* defense or communication. Their human applications include pharmaceuticals, flavours, fragrances, food supplements, such

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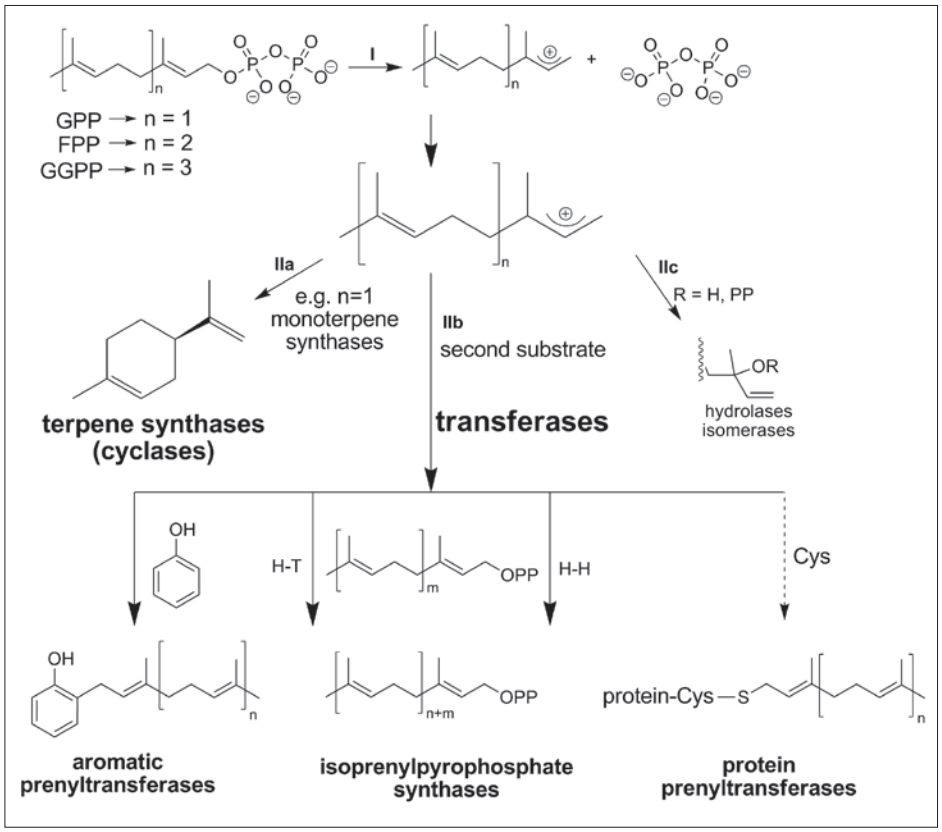


Scheme 1. Classification of enzymes and related general mechanism of prenylations. Except from protein prenyltransferases and hydrolases/isomerases, the three other enzyme families catalyse C–C-coupling prenylation. H–T = head to tail, H–H head to head condensation (*e.g.* squalene synthase with additional removal of OPP, product not shown), PP/OPP = pyrophosphate.

as vitamins or sweeteners. A wide range of terpenoids have shown pharmaceutical activity against human diseases such as cancer.[6,7] The most prominent example is paclitaxel, commonly known under the registered trademark Taxol®. Taxol is a diterpenoid-derived anticancer drug from the bark of the pacific yew tree *Taxus brevifolia*.[8] Another example is the sesquiterpenoid arteminisin from *Artemisia annua* which possesses anti-malaria activity.[9] Also monoterpene rich essential oils, *e.g.* from species of *Mentha*, with (–)-menthol, are important products for the pharmaceutical, agrochemical, food, flavour and fragrance industries.[10,11] In addition to their pharmaceutical use, they can act as attractants, repellents, toxins or antibiotics, or as precursors to such bioactive compounds. They are important for the interaction of sessile plants with other organisms in the context of reproduction, defence, or symbiosis.[12]

Terpenoids are derived from the precursor isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyldiphosphate (DMAPP). Terpenoid building blocks are then formed through condensation of additional IPP moieties by prenylating enzymes and eventually conjugation or cyclisation as the main diversity generating step.[13] The next section will give an overview about diverse prenyl-transferring enzymes.

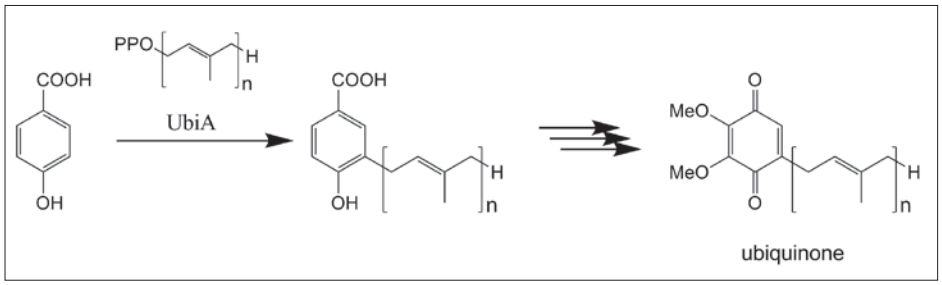
2. Prenyl Transfer to Aromatic Systems – Aromatic Prenyltransferases

2.1 *Bioinformatics*

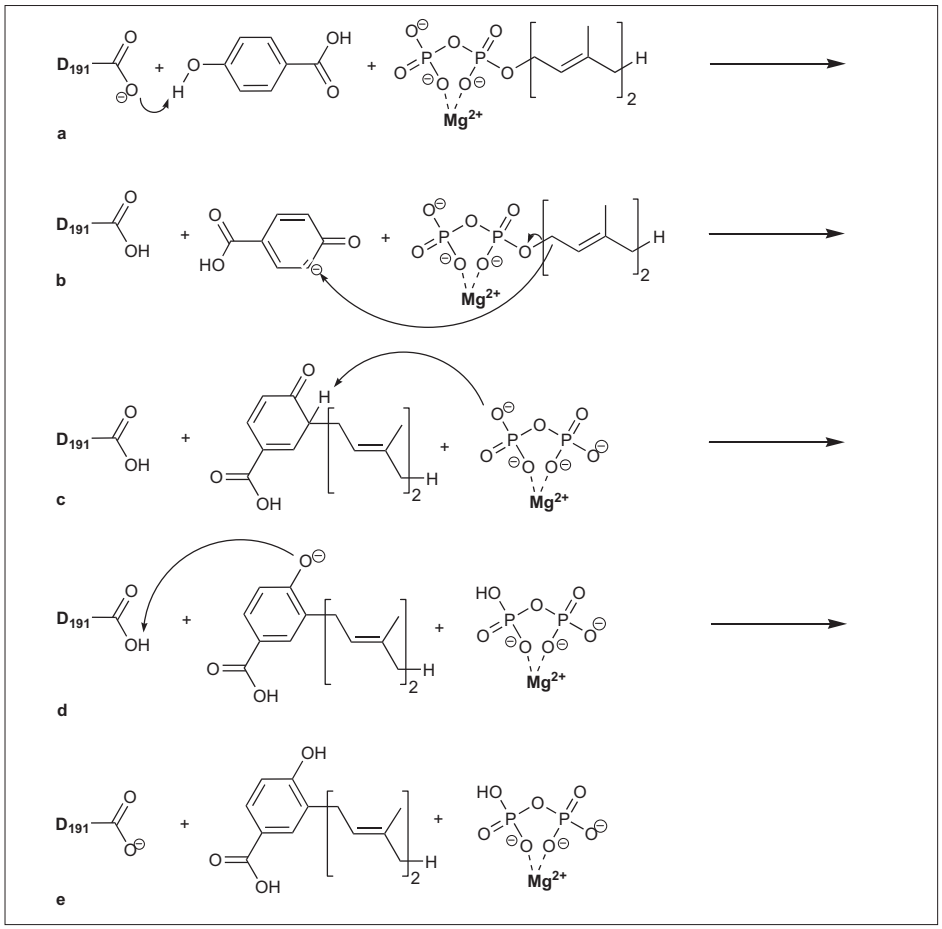
Aromatic prenyltransferases form a heterogenic cluster of enzymes that transfer prenyl residues to C, O, or N atoms of aromatic structures. Often this leads to an increased bioactivity compared to the non-prenylated compounds, most due to an increased affinity to biological membranes.[14] The enzymes are widespread. They can be found in plants, bacteria, and fungi. In recent years, significant progress was made concerning genetics and biochemistry of these enzymes yielding in the finding that not just one type of aromatic prenyltransferases exists. Today three different classes are known that are mainly distinguished by sequence dissimilarity. Enzymes of the group of integral membrane-bound aromatic prenyltransferases were found to be involved in the biosynthesis of lipoquinones (ubiquinone,[15–18] plastoquinone,[19] menaquinone,[20] tocochromanol[19,21]), as well as the biosynthesis of shikonins,[22,23] and sophoraflavones,[24] the latter being of plant secondary metabolism. All these enzymes contain an aspartate-rich motif in their sequence required to bind divalent metal ions, which are necessary for catalytic activity. All members of this class catalyse C-prenylations, *i.e.* they transfer the prenyl residue to a carbon atom of an aromatic substrate. Until now, no X-ray structure is available for this group, but a first homology model could be developed by us recently.[25]

The second group of aromatic prenyltransferases[26] consists of soluble enzymes with no sequence similarity to the enzymes of the first class. So far, four members have been biochemically characterised: CloQ,[27,28] involved in clorobiocin biosynthesis, NphB[29] (naphterpin biosynthesis), Fnq26[30,31] (furanonaphthochinon I biosynthesis) and SCO7190.[29] None of these enzymes contains aspartate-rich motifs, though the activity of NphB depends on Mg^{2+}.[29] The experimental structure of the latter revealed a novel barrel architecture, the ABBA fold. This refers to a cylindrical β-sheet consisting of ten antiparallel β-strands surrounding a solvent-filled core. Four times a sequence of two β-strands follows two α-helices resulting in a $(\alpha\alpha\beta\beta)_4$ structure. The remaining β-strands are appended in a (αββα) structure, to which the term ABBA refers. Because of high sequence similarities, this fold is assumed to be characteristic for the enzymes of this second class. In contrast to the aromatic prenyltransferases mentioned before, Fnq26 was observed to catalyse an allylic (often referred to as 'reverse') prenylation, considering the fact that C(3) rather than C(1) (regular prenylation) of the prenyl residue is connected to the aromatic substrate. All enzymes of the ABBA family add the prenyl chain to a carbon atom of the aromatic structure resulting in a C–C coupling. But Fnq26 and NphB are additionally able to catalyse O-prenylations, whereby an oxygen atom of the aromatic structure serves as prenyl acceptor. The better structural knowledge and their easier accessibility makes this class interesting for future chemoenzymatic synthesis of bioactive molecules.[32]

The third class consists of the soluble fungal indol prenyltransferases.[33] Similar to the described ABBA prenyltransferases they do not contain aspartate-rich motifs and none of them depend on the presence of Mg^{2+}. So far, enzymes involved in three different biosynthetic pathways (ergot alkaloid biosynthesis,[34–41] terrequinone A biosynthesis,[42,43] biosynthesis of indole diterpenes[44,45]) were characterised. The enzymes are able to catalyse C- as well as N-prenylations, that can be regular or reverse. Up to now, no X-ray structure of this class has been successfully determined.



Scheme 2. Biosynthesis of ubiquinones in *E. coli,* (similar in other organisms).



Scheme 3. The essential steps of the catalytic mechanism of UbiA. The formation of the σ-complex (**b** to **c**) is the most critical step and explains the substrate specificities.

Additionally other enzymes (*e.g.* LtxC[46]) are known not to be related to any of the sequences described above; a hint on the existence of further classes of aromatic prenyltransferases.

2.2 *The p-Hydroxybenzoic Acid Oligoprenyltransferase (UbiA) from E. coli*

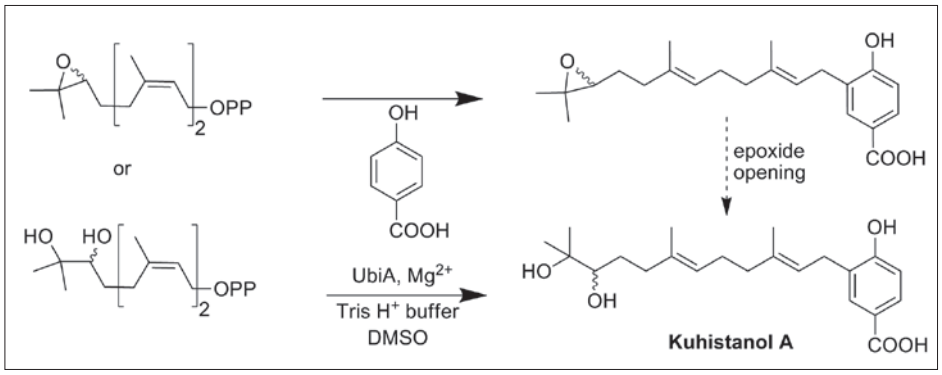
2.2.1 *Characterisation of Substrate Specificity and Application in Chemoenzymatic Synthesis*

The membrane-bound *p*-hydroxybenzoic acid oligoprenyltransferase (UbiA) from *E. coli* consists of 290 amino acid residues encoded by the *ubiA* gene and is known since 1972.[47] The enzyme is involved in the biosynthesis of bactoprenol and of prenylated quinones such as ubiquinone (Scheme 2) required for cell wall biosynthesis and respiration, respectively. *In vivo*, the enzyme transfers diphosphorylated acyclic *trans*-oligoprenyl moieties (diphosphorylated terpene alcohols) to the *meta*-position of *p*-hydroxybenzoic acid (Scheme 2).[48] The enzyme accepts a reasonable number of different aromatic substrates. Thus, it could be shown that not only the natural substrate 4-hydroxybenzoic acid but also 4-aminobenzoic acid, 2,4-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-amino-4-hydroxybenzoic acid, various halogenated 4-hydroxybenzoic acids and some other phenolic acids are accepted by the enzyme, yielding products prenylated in the *meta*-position of the benzoic acid core.[48] This substrate specificity can be explained based on a Friedel-Crafts (S_N1)-like mechanism which was supported by semiempirical quantum mechanical PM3 calculations corresponding to Scheme 3.[49] Substrates have to pass an activation barrier to form the σ-complex which is considerably lower for substrates (~80 kJ/mol) than for non-substrates. Furthermore, the specificity of the enzyme with regard to the accepted and transferred polyprenyl moieties was characterised.[48] In addition to geranylpyrophosphate, geranylgeranylpyrophosphate and octaprenylpyrophosphate several non-natural methallyldiphosphates[47] are also accepted as second substrates. Among these, it was surprising that geranylpyrophosphate was the fastest substrate transferred, whereas the longer ones are relevant for the *in vivo* participation in the ubiquinone biosynthesis and might be expected to be evolutionary more relevant.

Several analogues and mimics of geranyl pyrophosphate have been prepared as probes and for an examination of their ability to inhibit the enzyme.[50,51] For this purpose, cyclopropanated, fluorinated, truncated and diphosphate-substituted candidates were synthesised from geraniol. The effect of these compounds on *UbiA*-prenyltransferase activity varied substantially, ranging from almost full inhibition to, surprisingly, enhanced enzymatic activity at low concentrations by some compounds, with 3-hydroxy-7,11-dimethyldodeca-6,10-dienoic acid being the best inhibitor of those compounds tested.[50]

The rather broad substrate specificity of UbiA allows its use in chemoenzymatic syntheses. Even natural products with oxidised side chains are accessible. Kuhistanol A is a natural constituent of *Ferula* species (Scheme 4). It was first isolated by Y. Takaishi and co-workers in 2000 from the roots of the Uzbekistan plant *Ferula kuhistanica*, which traditionally has been used to treat skin diseases and wounds.[52] Kuhistanol A shows antibacterial activity against methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA).[53]

Kuhistanol A contains a prenylated *p*-hydroxybenzoic acid with hydroxyl groups at C(10') (as a racemic mixture at this position) and C(11'). The simple prenylation of 4-hydroxybenzoates fails by classical methods, *e.g.* Friedel-Crafts conditions produce cyclisation of the isoprenoid moiety, whereas other methods, *e.g.* orthometallation or Pd-coupling methods require extensive protection/deprotection strategies. However, the direct prenylation of *p*-hydroxybenzoic acid could be achieved with *ubiA*-prenyltransferase from *E. coli* (Scheme 4).[48,54,55]



Scheme 4. Biocatalytic steps in the total synthesis of Kuhistanol A.

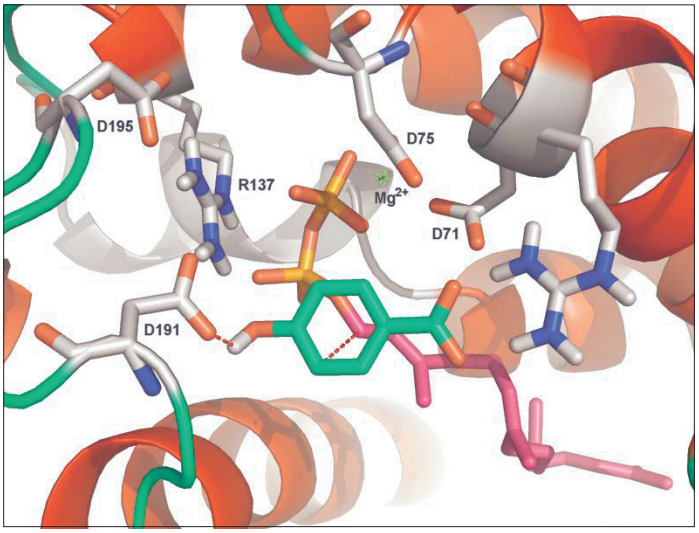


Fig. Model of the active site of UbiA with bound substrates geranylpyrophosphate (magenta) and 4-hydroxy benzoic acid (green), the latter shielding the developing prenyl cation from water. Labeled amino acids were proven by site directed mutagenesis to be essential for the catalysis. Red dashes indicate essential transfers during the catalytic mechanism (Scheme 3).

2.2.2 *Structure–Function Relationships*

UbiA is membrane-bound and so far could not be solubilised without irreversible destruction. Thus, an X-ray structure for UbiA or similar aromatic prenyltransferases has not yet been obtained. Some years ago, we suggested a reaction mechanism (Scheme 3) and a first insecure model of UbiA.[49] Later, a highly relevant 3D-model for UbiA could be developed, based on site-directed mutagenesis of five amino acids and a new structure-based classification of prenylating enzymes (Fig.).[25] This model represents the first structure of an intrinsically membrane-bound prenyltransferase. It explains all the substrate specificities and is in complete agreement with the results of site-directed mutagenesis. The knowledge of the 3D-structure of this enzyme led to the identification of further amino acid residues which are subject for mutations to improve activity and to change the substrate specificity for an improved use in chemoenzymatic synthesis. UbiA represents a unique all α-helix fold for an aromatic prenyltransferase, totally different from the previously mentioned ABBA fold. It shows a considerable fold similarity to the modelling template, the soluble prenyl converting enzyme 5-epi-aristolochene synthase from *Nicotiana tabacum* (pdb-code: 5eau).[56] In the active site, only a few amino acid residues are different, thereby causing a complete switch of the substrate and product specificities between these enzymes. Rational protein redesign with the aim to convert the soluble 5-epi-aristolochene synthase into an aromatic prenyltransferase biocatalyst is currently in progress.

3. Prenyl Transfer to (Internal) Double Bonds – Terpene Synthases

The immense richness of cyclic (and acyclic) plant terpenoids is formed by terpene synthases through a divalent metal ion-assisted (Mg^{2+}, Mn^{2+}) generation of enzyme-bound allylic carbocation intermediates from the prenylpyrophosphate precursors.[57–59] Conserved amino acids are most likely involved in the ionisation within the terpene synthases, for example the DDxxD motif, which serves as an indicator for this enzyme class. The carbocation formed leads to cyclisation reactions followed by internal hydride shifts, methyl migrations, double bond shifts or formations, or more complex Wagner-Meerwein rearrangements before a deprotonation or nucleophile capture terminates the reaction.

Currently, there are only seven X-ray structures of terpene cyclases known, which show a very similar fold.[60]

Two recombinant, stereospecific monoterpene synthases, a (–)-limonene synthase (*CsTPS1*) and a (+)-α-pinene synthase (*CsTPS2*), encoded by *Cannabis sativa* L. cv. 'Skunk' trichome mRNA, have been isolated and characterised.[61] The tertiary structures of these two new monoterpene synthases from *Cannabis sativa*, a (–)-limonene (pdb-code: 2dk0) and (+)-α-pinene synthase were modeled based on homology to a bornyl diphosphase synthase. Docking studies of the substrates geranylpyrophosphate to the active sites and subsequent comparison of amino acid residues led to the identification of three important residues, that seem to govern the different product specificities. Mutations of these residues in the limonene synthase caused not only alteration of the product specificity but in one case also an enhanced catalytic activity of the mutated enzyme in comparison to the wild type.[62] Although increased activity and altered product selectivity did not yet coincide, the experimental results supported the principal correctness of the models. General aspects of the functional role of several amino acid residues in the catalysis can be discussed, including new ones. This will help to achieve an improved redesign in further optimisation rounds.

Received: January 30, 2009

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