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Mechanistic Similarities of Sesquiterpene Cyclases PenA, Omp6/7, and BcBOT2 Are Unraveled by an Unnatural "FPP-Ether" Derivative

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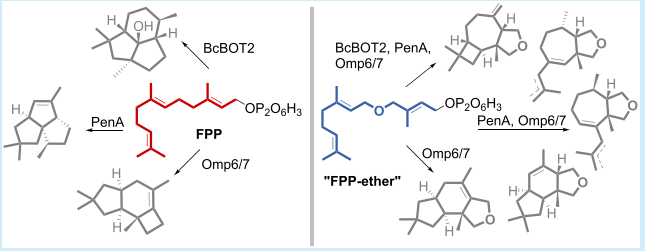
ABSTRACT: The sesquiterpene cyclases pentalenene synthase (PenA) and two Δ^6-protoilludene synthases Omp6 and Omp7 convert a FPP ether into several new tetrahydrofurano terpenoids, one of which is also formed as the main product by the sesquiterpene cyclase BcBOT2. Thus, PenA, Omp6/7, and BcBOT2 follow closely related catalytic pathways and induce similar folding of their diphosphate substrates despite low levels of amino acid sequence similarity. Some of the new terpenoids show pronounced olfactoric properties.

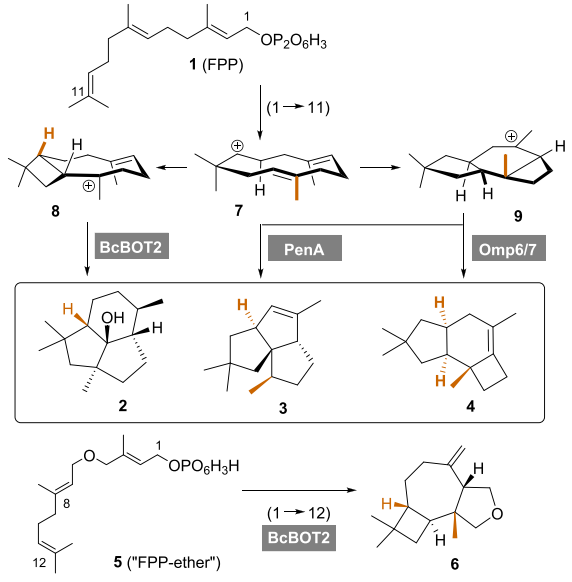


Terpene cyclases (TCs) are the key enzymes that are responsible for the immense diversity of terpenes. Starting from linear, unsaturated, methyl-branched precursors, activated as terminal diphosphate esters, these enzymes provide mono (C10)-, sesqui (C15)-, di (C20)-, and sesterterpenes (C25). Using farnesyl pyrophosphate (FPP, **1**) as a natural substrate, sesquiterpene cyclases (STCs) produce an allyl cationic intermediate that can react further with remotely positioned alkenes to form (oligo)carbocyclic products.[1]

In recent years, efforts have been pursued to validate the substrate specificity of TCs, in particular mono- and sesquiterpene cyclases.[2−4] The introduction of oxygen, sulfur, and halogen atoms into the backbone of FPP **1** represents one type of unnatural FPP derivative of which "FPP-ether" **5** is a noteworthy example (Scheme 1). When this derivative was reacted with presilphiperfolan-8β-ol synthase (BcBOT2), a fungal sesquiterpene cyclase from *Botrytis cinerea*,[5] tricyclic terpenoid **6** was isolated that reveals a significantly altered backbone compared to that of the natural cyclization product presilphiperfolan-8β-ol (**2**).[4a]

Because unnatural FPP derivatives not only open the door to new terpenoid backbones but, in our experience, also provide additional insights into STC mechanisms from a different perspective, the inclusion of additional STCs in such studies could decipher much more general mechanistic commonalities and interrelationships of tricyclic-forming STCs. Thus, in line with this work, we looked for STCs that form other tricyclic terpene backbones via humulyl cation (**7**). Tricyclic pentalenene (**3**) is the product formed by the STC pentalenene synthase (PenA), an enzyme first isolated from *Streptomyces exfoliatus* UC5319 (Scheme 2).[6] Another group of STCs, the protoilludene synthases, is found in various basidiomycetes, including *Omphalotus olearius*, which, in addition to pentalenene (**3**), produces the tricyclic sesqui-

Scheme 1. Natural Cyclization Products 2−4 Formed by the Sesquiterpene Synthases BcBOT2,[5] PenA, and Omp6/7 via Key Intermediate Cations 7−9 (a detailed discussion of the mechanisms is found in the Supporting Information)[6c,9a] and Cyclization Product 6 Derived from "FPP-Ether" 5[a]



[a]Orange-labeled groups serve as guides for the absolute configuration discussed in Schemes 3 and 4.

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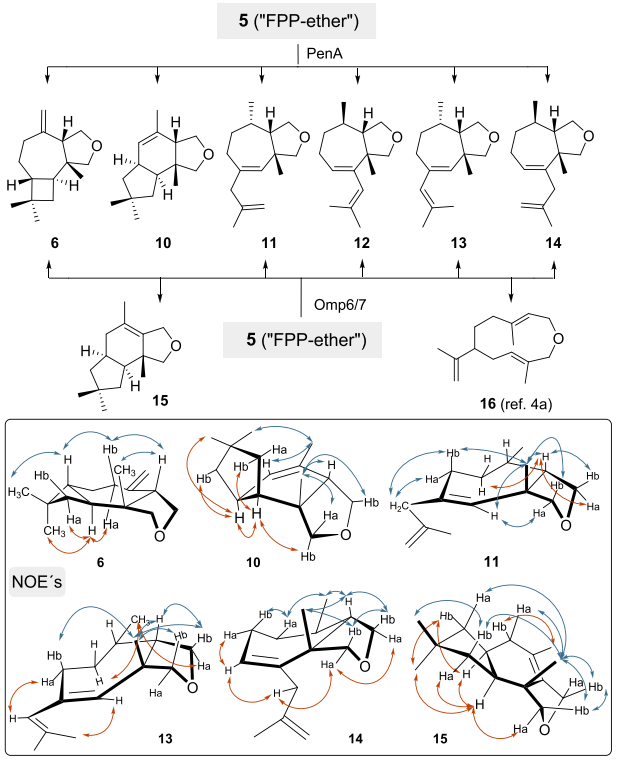
Scheme 2. Formation of Seven Tetrahydrofurano Terpenoids 6 and 10−15 and Macrocycle 16 from "FPP-Ether" 5 and Key NOE−NMR Data for Assignment of the Relative Stereochemistry (further information in Table 1)



terpene Δ^6-protoilludene (**4**). The latter is exclusively produced by the STCs Omp6 and Omp7.[7] Despite the fact that all STCs form annulated tricyclic structures via key intermediate cations **7**−**9**, the products substantially differ in the individual ring sizes and annulation points.

A bioinformatics comparison based on BLAST[8] reveals a 45% similarity of BcBOT2 with PenA or Omp7 with regard to their amino acid sequences. BcBOT2 shares only 42% similar amino acids with Omp7 (see the Supporting Information). Despite these facts, we demonstrate in this work that their catalytic abilities are in fact closely related as unraveled by the use of "FPP-ether" **5**.

For this work, we cloned the STCs PenA,[6] Omp6, and Omp7[7] and expressed them in *Escherichia coli* (see the Supporting Information). *In vitro* enzyme tests to determine enzyme activity and substrate tolerance were performed on a small scale (150 μM, 0.01 mg/mL) using the natural precursor FPP **1** (see the Supporting Information). The key parameters for the three STCs to be optimized were temperature and pH. To investigate possible inhibitory effects or denaturation, substrate and enzyme concentrations were also included in these investigations (see the Supporting Information).

Interestingly, the biotransformations with ether derivative **5**, the synthesis of which has been reported previously,[4a] required an increased temperature (30 °C instead of 10 °C for PenA and 20 °C for Omp7) to achieve better yields for the new unnatural terpenoids. These conditions presumably allow greater conformational flexibility of the protein and easier adaptation of the unnatural FPP substrates.

The semipreparative transformation of ether derivative **5** with PenA yielded six products that could be detected on a $DB5_{HT}$ chromatographic column (Scheme 2). Four of these products were separated and isolated by preparative GC (pGC). Finally, in a second step after the first purification, re-chromatography on a polar WAX column yielded a total of six products instead of four, with products **6** and **10** as well as **12** and **13** co-eluting as pairs on the $DB5_{HT}$ column (Table 1 and Supporting Information).

Table 1. Retention Indices (RIs) on $DB5_{HT}$ and WAX Columns and Percentage Areas *A* (percent) Measured on WAX after pGC

product	RI_{DB5HT}	RI_{WAX}	*A* (%)
6	1586	1871	30.8
10	1586	1884	64.5
11	1593	1908	93.5
12	1638	1958	15.0
13	1638	1985	73.1
14	1649	1994	93.4
15	1600	1909	75.4

The relative stereochemistry of the isolated products was determined with the support of selected one-dimensional (1D) NOE experiments. Because heptafurano terpenoid **12** was isolated only in small amounts as a byproduct of **13**, the assignment of the relative stereochemistry had to be derived from the stereochemistry of product **14** by analogy and the mechanism proposed in Scheme 3.

Considering that protoilludyl cation **8** is an important intermediate on the way to pentalenene (**3**) and Δ^6-protoilludene (**4**), also the STCs Omp6 and Omp7, respectively, were used in *in vitro* tests with FPP **1** as well as FPP ether derivative **5**. Both proteins were active as judged by isolation of Δ^6-protoilludene (**4**) after incubation with FPP **1**. After optimization of the temperature for unnatural substrate **5** on a small scale (see the Supporting Information), several products could be detected. On the basis of the retention indices (GC column $DB5_{HT}$ and WAX) and mass spectra, the formation of terpenoids **6** and **11**−**15** as well as known macrocyclic ether **16**[4a] was confirmed. The analysis was supplemented by GC co-injection experiments with the products isolated from the biotransformation with PenA. However, the main product **15** turned out to be unknown and new. Therefore, the experiment was repeated on a semi-preparative scale (1.5 mM). Omp7 was chosen for this upscaling as it shows a 10-fold higher affinity for its natural substrate, FPP **1**, than Omp6.[9]

The resulting two main products were separated and isolated by pGC. Re-chromatography on a polar WAX column revealed a total of three main products instead of two, with products **6** and an unknown compound co-eluting on the $DB5_{HT}$ column (see the Supporting Information). Extensive use of 1D NOE measurements allowed us to unravel the relative orientation of the methyl group attached to the seven-membered ring. Noteworthy is the opposite orientation of this methyl group in the two product pairs, **11** and **13**, and **12** and **14**. In addition, a *syn* linkage of the furan ring with the cycloheptane ring was found for all products.

Despite the fact that new unnatural sesquiterpenes were generated, the results also provide additional mechanistic insights into the functioning of the three STCs. Our proposed mechanistic sequence for the formation of cycloheptafurans **6** and **11**−**14** is essentially similar to the first steps of the

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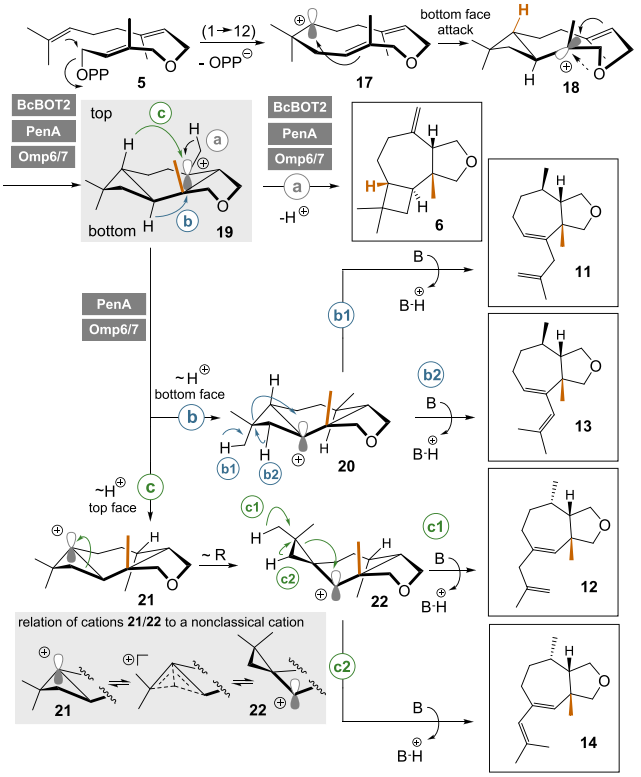
Org. Lett. 2021, 23, 3162−3166

Scheme 3. Mechanistic Considerations about the Formation of Heptafurano Terpenoids 6 and 11−14 Catalyzed by PenA and Omp6/7[a]



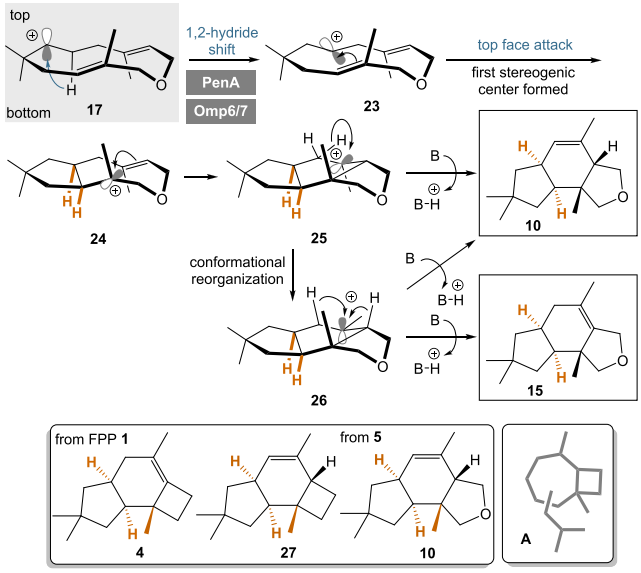
[a]Tetrahydrofurano terpenoid **6** is also formed by BcBOT2; close inspection of the GC-MS spectrum revealed no formation of **11**−**14** (orange-labeled groups serve as guides for the absolute configuration; for additional mechanistic information about PenA and Omp6/7, see chapter 2 of the Supporting Information and literature cited therein).

biotransformation of FPP **1** to presilphiperfolan-8β-ol (**2**), catalyzed by the STC BcBOT2 (Scheme 1). A central role is given to the intermediate cationic cyclobutane **19**, from where three routes (a−c) to furan **6** (route a), to **11** and **13** (route b), and finally to the terpenoids **12** and **14** (route c) can be formulated. The central intermediate **19** is formed after the initial **1** → **12** macrocyclization (**5** → **17**), followed by a second (**17** → **18**) and a third (**18** → **19**) ring closure with **17** being the oxa analogue of humulene cation **7**.

From cation **19** (resembling the oxa analogue of protoilludyl cation **9**), hydrogen abstraction leads directly to tetrahydrofurano-terpene **6** (path a). Alternatively, hydrogen shifts from the two positions between the cyclobutane and cycloheptane rings in **19** lead to carbocations **20** and **21**, respectively. Carbocation **20** (pathway b) collapses via a ring opening of the cyclobutane ring and then yields cycloheptafuran derivatives **11** (pathway b1) and **13** (pathway b2). The second cyclobutyl cation **21** produced via route c can undergo rearrangement to cyclopropyl methyl cation **22** or is present as a nonclassical bicyclobutonium ion with three-center, two-electron bonding.[10] This is the last precursor on the way to regioisomeric cycloheptafuran derivatives **12** (route c1) and **14** (route c2) (Scheme 3). Pathways b1 and b2 as well as c1 and c2 are likely to be mediated by the same basic entity in the active sites of PenA and Omp6/7. A particularly remarkable aspect of our results is the fact that five of the six heptafurano terpenoids **6** and **11**−**14** are generated via a cationic cascade, which is typically catalyzed by BcBOT2 instead of PenA or Omp6/7 (Scheme 1).

Only tricyclic terpenoids **6** and **15** are formed by a series of cationic events similar to the first steps of pentalenene and Δ^6-protoilludene biosyntheses (Scheme 4). The first cyclization

Scheme 4. Mechanistic Considerations about the Formation of Tricyclic Terpenoids 10 and 15 from Humulyl Cation Analogue 17 and Intermediates 23−26[a]



[a]Orange-labeled groups serve as guides for the absolute configuration (see the text). Terpenoids **4** and **27** serve as reference compounds for **10**. Unknown sesquiterpene backbone A relates to new terpenoids **11**−**14**.

product **17** undergoes a cationic shift via an elimination and reprotonation sequence or alternatively a 1,2-hydride shift (**17** → **23** → **24**). Intermediate **24** cyclizes to form cationic tetrahydrofuran **25** and finally after deprotonation to tricyclic tetrahydrofurano terpenoid **10** or conformationally changes via cation **26** to elimination product **15**. However, conformer **26** can also serve as a direct precursor for tetrahydrofurano terpenoid **10**, but the other adjacent diasterotopic proton would have to be abstracted than in the transformation of conformer **25** to **10**.

Our NMR studies provide information about the constitution of the six biotransformation products as well as their relative stereochemistry. Within the framework of these structural elucidations, statements about the absolute stereochemistry remained open. This open point can be solved using known sesquiterpenes such as presilphiperfolan-8β-ol (**2**), pentalenene (**3**), and Δ^6-protoilludene (**4**) as reference points, which is combined with mechanistic considerations. Lead atoms and stereogenic centers in known sesquiterpenes labeled in orange in Schemes 1, 3, and 4 formed during biotransformations and that arrive unchanged in the final product are such reference points. If mechanistic branching occurs due to the use of unnatural FPPs, a stereogenic center formed at an early stage can be taken as a point of reference for assigning the absolute stereochemistry of all chiral centers in the products.

The four STCs BcBOT2, PenA, and Omp6/7 transform "FPP-ether" **5** into the same tricyclic terpenoid **6**, and its

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Org. Lett. 2021, 23, 3162−3166

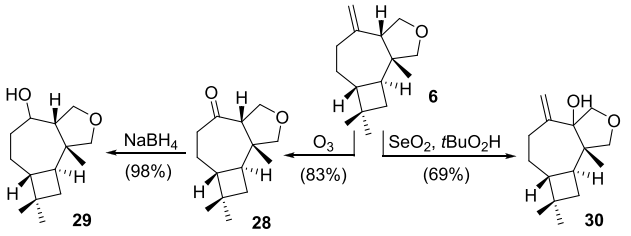
absolute stereochemistry was proposed previously.[4a] The stereogenic center and the H atom labeled in orange, which are formed during cyclobutane and cyclopentane formation, remain unchanged in both mechanistic routes. Both the relative and absolute configuration of presilphiperfolan-8β-ol (**2**) have unequivocally been determined spectroscopically, by derivatization,[11] by X-ray crystallographic analysis of *p*-nitrobenzoate,[12] and more recently by total synthesis.[13] Consequently, the stereogenic center in **6** marked in orange is *R*-configured.

It has also been suggested that the formation of pentalenene (**3**) by PenA takes place via intermediates **8** and **9**;[6,8] the former is structurally closely related to the two conformers **25** and **26** on the way to tetrahydrofurano terpenoids **10** and **15** except for the additional oxygen atom. The orientation of the two hydrogen atoms marked in orange serves as a reference for determining the absolute stereochemistry of terpenoids **10** and **15**, because these two positions are retained after their formation (Scheme 4). One of them is also found in pentalenene (**3**) generated from FPP **1**, and both positions are even retained in Δ^6-protoilludene (**4**) (Scheme 1).

In cycloheptafurans **11**−**14**, the stereogenic center with the angular, orange-labeled methyl group can be named as characteristic. Here, the two natural sesquiterpenes **4** and Δ^7-protoilludene (**27**) can serve as reference substances, which show an upward orientation of the methyl group, which can then also be assumed for cycloheptafurans **11**−**14**. Furthermore, new tetrahydrofurano terpenoid **10** is structurally similar to Δ^7-protoilludene (**27**), in which the cyclobutane ring is complemented by an additional oxygen atom. Interestingly, we could not find any naturally occurring sesquiterpenes with the type A carbon skeleton that would correspond to terpenoids **11**−**14**, so these products truly reveal new modes of action of STCs.

After we were able to produce terpenoid **6** in sufficient quantities by combining chemical synthesis (to **5**) and biotransformation, we expanded the structural diversity of terpenoids with new backbones by semisynthetic transformations (Scheme 5).[14] Thus, ozonolysis in MeOH yielded

Scheme 5. Derivatization of Tetrahydrofurano Terpenoid 6 and Formation of Heptafurano Terpenoids 28−**30**



ketone **28**, a terpenoid that now contains 15 backbone atoms like natural sesquiterpenes but with one carbon atom exchanged by oxygen. The keto group can be reduced with $NaBH_4$ in MeOH to give alcohol **29** as a single diastereomer, as judged by GC-MS. The conformational flexibility of the cycloheptane ring made it difficult to unambiguously determine the configuration of the newly formed stereogenic center.[15] In addition, a Riley oxidation in CH_2Cl_2 gave exclusively allyl alcohol **30**, which remarkably gave the more highly substituted allyl alcohol.

A GC-O (gas chromatography-olfactometry) evaluation was conducted for isolated products **6** and **10**−**15** and semisynthetic derivatives **28**−**30**. This analysis revealed that besides compound **6** that shows a strong ethereal, peppery and camphor odor,[4a] only compound **14** provides a sensory profile in the form of a strong fruity note.

Our results show that the catalytic properties of the STCs BcBOT2, PenA, and Omp 6/7 and the underlying cation cascades are closely related in mechanistic terms despite a low degree of similarity in terms of their amino acid sequences. This observation can be explained by assuming a very similar protein conformation, especially in the three-dimensional lining of the active site. To deepen our understanding of STCs, it will be helpful in the future to also perform co-crystallizations of the enzymes with hydrolytically stable FPP substrates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.1c00882.

Detailed procedures and spectral data (PDF)

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Notes

The authors declare no competing financial interest.

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