

Communication to the Editor

Novel Cyclohexyl Meroterpenes
Produced by Combinatorial BiosynthesisJinyan Jiang,^a Xinyang Li,^a Takahiro Mori,^{a,b,c}
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Received February 7, 2021; accepted February 18, 2021

Structurally diverse fungal meroterpenoids are promising drug seed compounds. To obtain unnatural, novel meroterpene scaffolds, we tested combinatorial biosynthesis by co-expressing functionally distinct terpene cyclase (TPC) genes, *pyr4*, *ascF*, *andB*, or *cdmG*, with the biosynthetic genes for the production of a TPC substrate, (10'*R*)-epoxyfarnesyl-dimethylorsellinic acid-3,5-methyl ester, in *Aspergillus oryzae* NSAR1 as a heterologous host. As a result, all of the tested TPCs afforded the same two novel mono-cyclization products. This study provides important information on the substrate scope of the TPCs, and will contribute to the production of unnatural, novel molecules for future drug discovery.

Key words meroterpenoid; enzyme; biosynthesis; microbial production; drug seed

Introduction

Fungal meroterpenoids exhibit remarkable structural diversity¹⁾ and pharmaceutically important biological activities, as exemplified by the cholesterol-lowering pyripyropene A,²⁾ the immunosuppressant drug mycophenolic acid,³⁾ and the anti-trypanosomal ascofuranone.⁴⁾ The structural diversity is basically attributed to the differences in the core polyketide structure, the prenyl chain length, and the terpene cyclization reactions,⁵⁾ which are determined by polyketide synthase (PKS), prenyltransferase (PT), flavin-dependent monooxygenase (FMO) and terpene cyclase (TPC), respectively.⁵⁾ For example, the TPCs, Trt1, AusL, and Adr1, catalyze the cyclization of a common substrate, (10'*R*)-epoxyfarnesyl-3,5-dimethylorsellinic acid (DMOA) methyl ester (**1**), into preterretonin A, protoaustinoide A, and andratin E, respectively^{6,7)} (Fig. 1). TPC is thus a critical enzyme that constructs the basic molecular scaffolds of fungal meroterpenoids.^{8,9)} However, our knowledge of the substrate scope and reactivity of TPCs with unnatural substrates is rather limited. In this study, we tested a combinatorial biosynthesis by co-expressing the enzymes that produce the TPC substrate **1**, with functionally distinct TPCs with different physiological substrates from **1**, in *Aspergillus oryzae* NSAR1 as a heterologous expression host.¹⁰⁾

Results

Each TPC, Pyr4 from pyripyropene A biosynthesis,¹¹⁾ CdmG from chrodriamanin biosynthesis,¹²⁾ AndB from anditomin biosynthesis,¹³⁾ or AscF from ascochlorin biosynthesis¹⁴⁻¹⁶⁾ (Fig.

S1), was co-expressed with polyketide synthase (AndM),¹³⁾ prenyltransferase (NvfB),¹⁷⁾ methyltransferase (Trt5),⁶⁾ and flavin monooxygenase (Trt8).¹⁸⁾ Interestingly, all of the *A. oryzae* transformants produced the same two products **3** and **4**, along with the diol **2**, which is thought to be formed by an endogenous epoxide hydrolase in the host cell (Fig. 2). From a 10 L DPY culture of *A. oryzae* harboring *andM/nvfB/trt5/trt8/pyr4*, 1.2 mg of **3** and 0.7 mg of **4** were isolated. The high resolution (HR)-MS of **3** was 447.2785 [M-H][−] (Calcd 447.2752 for C₂₆H₃₉O₆), indicating that **3** contains an additional hydroxyl group as compared with **1**. In the NMR data of **3**, we observed the olefinic proton H-2' (4.68 ppm), although the other olefinic proton H-6' in the terpene moiety was absent (Fig. S2, Table S4), which indicated that **3** is a mono-cyclization product. As expected, the correlation spectroscopy (COSY) correlations among H-8', H-9', and H-10', and the heteronuclear multiple bond correlation (HMBC) from H-14' to C-6', C-7', and C-8', from H-13' to C-6', C-12', and C-10', and from H-12' to C-9', C-10', and C-11' established the cyclohexane structure substituted with one hydroxyl group and one methyl group at C-7', one hydroxyl group at C-10', and dimethyl groups at C-11' (Fig. 3). The nuclear Overhauser effects (NOEs) between H-6' and H-10', H-9a' and H-10', H-10' and H-12', H-9b' and H-13', and H-13' and H-14' indicated that the 14'-methyl and H-6' are at the axial position (Fig. 3). Based on the absolute configuration at C-10' in **1**,⁶⁾ those of C-6' and C-7' were deduced to be *R* and *R*, respectively.

The HR-MS of **4** was 429.2653 [M-H][−] (Calcd 429.2646 for C₂₆H₃₇O₅), which is the same molecular formula as **1**. Similar to **3**, the ¹H-NMR spectrum of **4** revealed an olefinic signal for H-2' (4.68 ppm), indicating that **4** is also a mono-cyclization product (Fig. S3, Table S5). The ¹H- and ¹³C-NMR data suggested that **4** possesses an exomethylene group, as indicated by the two singlet signals at 4.49 and 4.82 ppm in the ¹H-NMR data attached to 108.6 ppm in the ¹³C-NMR data. The COSY correlations among H-8', H-9', and H-10', and the HMBC correlations from H-14' to C-6' and C-8', and from H-12' and H-13' to C-6', C-10', and C-11', clearly established the cyclohexane ring substituted with one exomethylene group at C-7', one hydroxyl group at C-10', and geminal methyl groups at C-11' (Fig. 3).

The products **3** and **4** are thought to be generated from a partially cyclized common cationic intermediate (Fig. 4). After the formation of the A ring, the cation at C-7' is quenched by water (path A) or proton elimination from C-14' (path B). The absolute configurations at C-6' and C-7' in **3** are *R*, indicating that the terpene cyclization reaction proceeds with a pre-chair conformation of the A-ring.⁶⁾ It should be noted that the native substrates of all the tested TPCs have a (10'*S*)-epoxyfarnesyl moiety (Fig. S1), which is the opposite configuration from the non-native (10'*R*)-epoxyfarnesyl substrate **1**. Thus, both the opposite stereochemistry of the epoxide ring and the differences in the polyketide core structure of **1** hamper the proper folding of the substrate at the active site of the enzyme, which would result in the incomplete terpene cyclization. Nonetheless, it is noteworthy that all of the tested TPCs yielded the same products **3** and **4**, with identical stereochemistry to the non-native substrate **1**. This incomplete terpene cyclization

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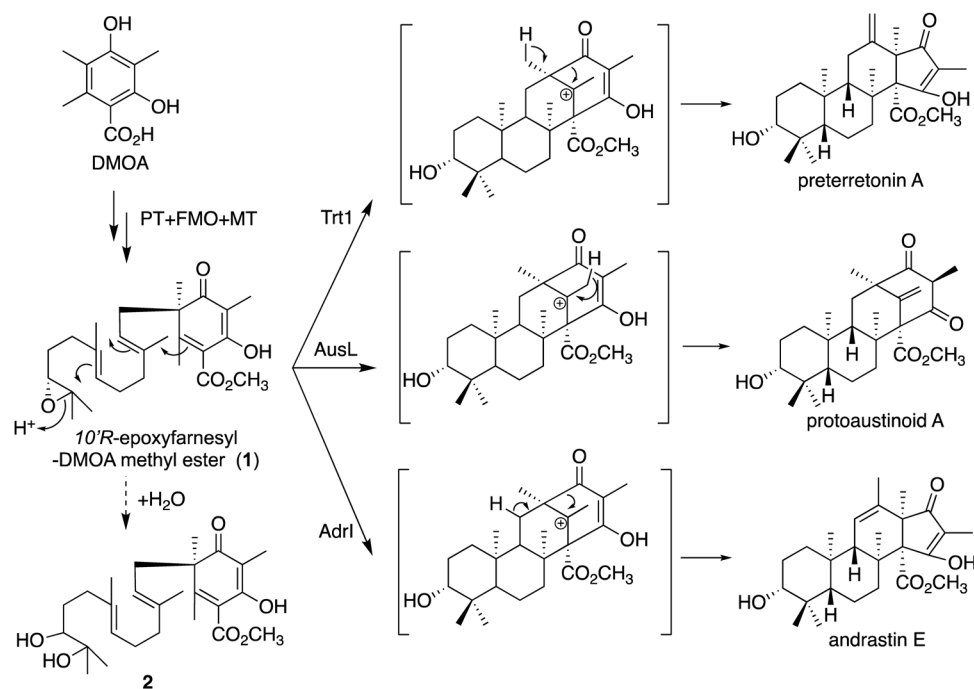


Fig. 1. The Reported Terpene Cyclization Reactions from 10'R-Epoxyfarnesyl-DMOA Methyl Ester (1)

2 is obtained as a shunt product.

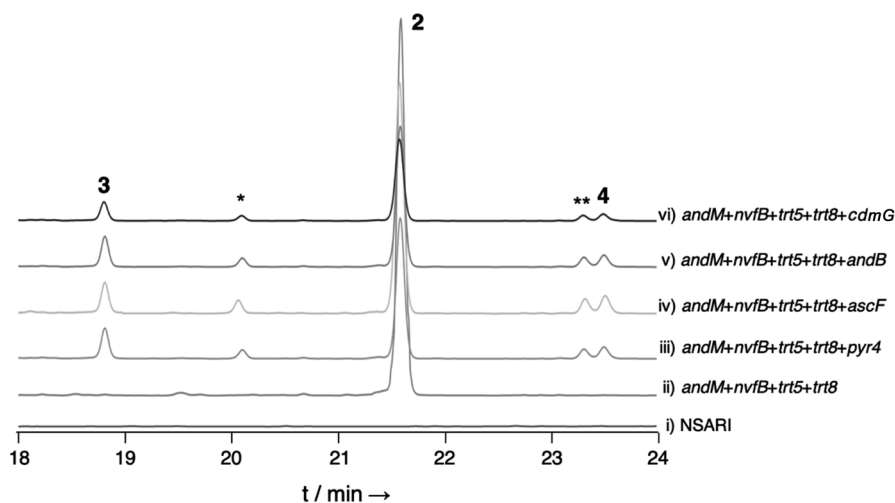


Fig. 2. HPLC Analyses of the Supernatant of Each *A. oryzae* Transformant Culture: i, NSARI; ii, the Transformant Harboring *andM* + *nvfB* + *trt5* + *trt8*; iii, *andM* + *nvfB* + *trt5* + *trt8*, and *pyr4*; iv, *ascF*; v, *andB*; vi, *cdmG*

The chromatograms were monitored at 290 nm. * indicates a mixture of unidentified compounds.

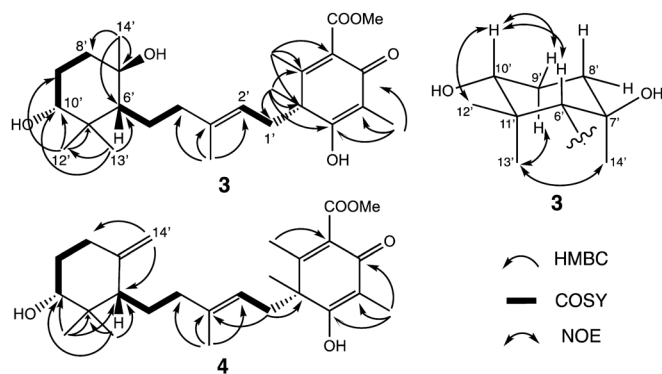


Fig. 3. Key COSY, HMBC, NOE Correlation for Structural Identification

toward unnatural substrate has not been reported in the meroterpenoid TPC reaction.^{8,9} *AscF* produces the monocyclic structure from its native (10'S)-epoxyfarnesyl substrate, but the way of quenching a cation is different from those reported in this study.^{13,14} It would be interesting to compare the substrate-binding manners of these reactions.

Considering that both 3 and 4 share similar bicyclic structures with the reduced nicotinamide adenine dinucleotide (NADH)-fumarate reductase inhibitor sartorypyrone D (notably, the A-ring structure of 4 is identical)¹⁹ and the potential anticancer lead compound ascchlorin^{20–23} (Fig. S4), these novel cyclohexyl meroterpenes could also exhibit prominent biological activities. To increase the product yields for biological tests, further engineering of the TPC enzymes toward the

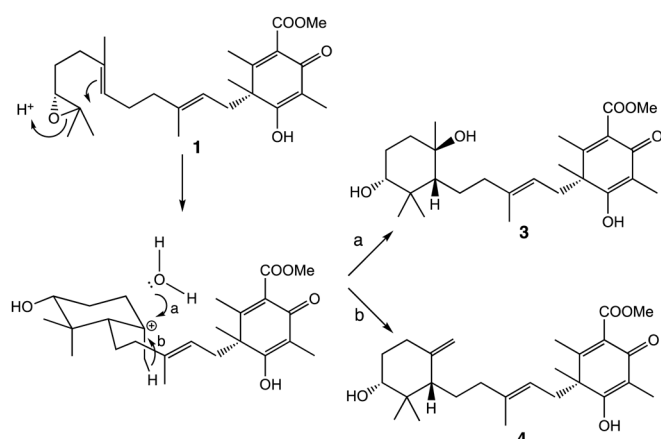


Fig. 4. The Terpene Cyclization Reaction from **1** to **3** and **4**

non-native substrate and optimization of the metabolism in the *A. oryzae* heterologous host are required. This knowledge will pave the way to the construction of practical synthetic biology platforms to produce useful unnatural, novel molecules for future drug discovery.

Acknowledgments This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (JSPS KAKENHI Grant Number JP16H06443, JP17H04763, JP18K19139, JP19H04641, JP19K15703, JP20H00490, JP20KK0173) and JST/NSFC Strategic International Collaborative Research pro-gram Japan–China “Exploitation of the cryptic secondary metabolites from plant microbiome through biological interaction” (JST SICORP Grant Number JPMJSC1701, Japan), the PRESTO program from Japan Science and Technology Agency, Fuji foundation for protein research, Mochida Memorial Foundation for Medical and Pharmaceutical Research, Takeda Science foundation, and Japan Foundation for Applied Enzymology.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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