OL | Organic Letters

Letter

Cite This: Org. Lett. XXXX, XXX, XXX–XXX

pubs.acs.org/OrgLett

The Discovery of Fungal Polyene Macrolides via a Postgenomic Approach Reveals a Polyketide Macrocyclization by *trans*-Acting Thioesterase in Fungi

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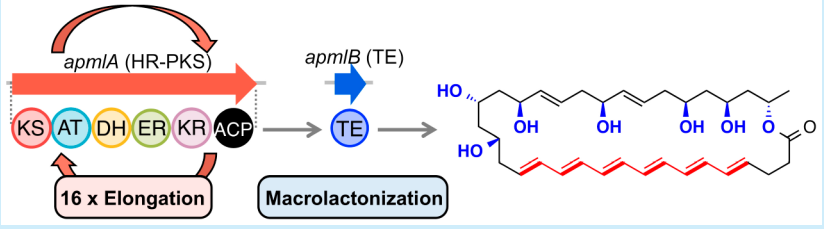
Supporting Information



ABSTRACT: Heterologous expression of a unique biosynthetic gene cluster (BGC) comprising a highly reducing polyketide synthase and stand-alone thioesterase genes in *Aspergillus oryzae* enabled us to isolate a novel 34-membered polyene macrolide, phaeospelide A (**1**). This is the first isolation of a fungal polyene macrolide and the first demonstration of fungal aliphatic macrolide biosynthetic machinery. In addition, sequence similarity network analysis demonstrated the existence of a large number of BGCs for novel fungal macrolides.

Fungi have already provided a wide array of secondary metabolites, and many of these metabolites and their derivatives have made vital contributions to drug development.[1] Fungal secondary metabolites therefore remain an attractive source for drug discovery. Fungi are well-known to harbor a large number of biosynthetic gene clusters (BGCs).[2] Many of them have not yet been developed or linked to their associated natural products. In other words, fungal genomic information contains untapped diverse biosynthetic pathways, and this information is one of the most promising resources for natural product discovery in the postgenomic era.[3]

Synthetic biology methods based on genome mining and heterologous biosynthesis make up a promising approach for translating genomic information into its associated natural products.[4] The vast amount of biosynthetic knowledge that has been accumulated to date facilitates the exploration of BGCs, not only for known pharmaceutically beneficial natural products but also for natural products with novel structures. On the other hand, heterologous biosynthesis allows the production of any natural product, even if it is highly complex, by simply introducing the corresponding biosynthetic genes into a model host.[5] In addition, even biosynthetic genes that are silent in the host organism are available to this method. Therefore, to rationally discover novel natural products and biosynthetic machineries, we have used a synthetic biology method, so-called "postgenomic natural product discovery".

Fungal highly reducing polyketide synthase (HR-PKS) is a multifunctional, iterative type I PKS, which is mainly composed of typical domains, including ketosynthase (KS), acyltransferase (AT), dehydratase (DH), enoyl reductase (ER), ketoreductase (KR), and acyl-carrier protein (ACP) and/or methyl transferase (MT) domains.[6] However, carbon frameworks that differ in terms of chain length and modification pattern are encoded by each gene.[7] These genes are thereby responsible for skeleton formation via diverse biosynthetic pathways, so they have an important role in generating a diversity of natural products. While HR-PKSs are widely distributed among Ascomycota fungi, most natural products produced by HR-PKSs remain undiscovered. We have therefore focused on HR-PKS genes as a preferred landmark in genome mining to find putative BGCs encoding novel natural products and unique biosynthetic machineries.

Received: May 13, 2019

DOI: 10.1021/acs.orglett.9b01674
Org. Lett. XXXX, XXX, XXX–XXX

Here, we demonstrate HR-PKS-guided genome mining and heterologous biosynthesis allow us to discover for the first time fungal polyene macrolides and to reveal a key new offloading system of HR-PKS for fungal aliphatic macrolide formation.

We previously collected insect-associated fungal strains as a unique resource for natural product exploration[8] and obtained their draft genomic information via next-generation sequence analysis. We performed HR-PKS gene-guided genome mining in these sequenced strains and found a simple but unique BGC composed of a HR-PKS (KS-AT-DH-ER-KR-ACP) gene, *apmlA*, and a putative stand-alone thioesterase (TE) gene, *apmlB*, in hairy caterpillar-associated *Arthrinium phaeospermum* (Figure 1A). We then conducted a sequence similarity network

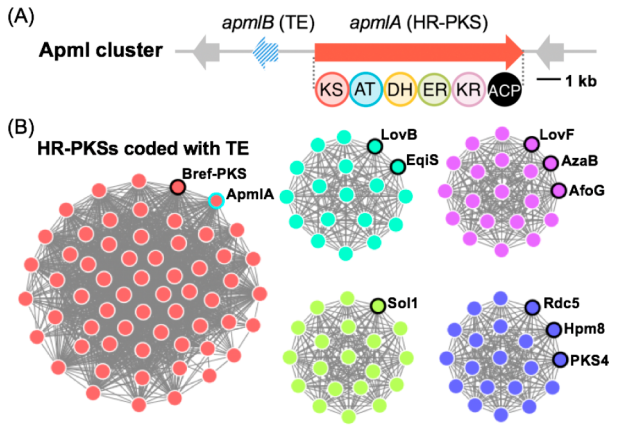


Figure 1. (A) *apml* cluster. (B) Sequence similarity network of the fungal HR-PKS encoded with TE and their characterized representatives with some homologues.

analysis of the HR-PKSs versus functionally characterized or predicted HR-PKSs using the EFI-EST web tool developed by Gerlt and colleagues.[9] Visualization of this data was carried out using Cytoscape software. As a result, HR-PKSs including ApmlA, which are flanked by stand-alone putative TEs, formed a large cluster, which implies that the HR-PKSs work cooperatively with the corresponding TEs in the production of a class of natural products. In the same manner, functionally similar HR-PKSs make clusters like each other (e.g., LovB and EqiS,[10] which construct decalin polyketides; Rdc5, Hpm8, and PKS4,[11] which are involved in the biosynthesis of resorcylic macrolides; and LovF, AzaB, and AfoG,[12] which produce a linear carbon chain that is transferred to another skeleton by an acyltransferase) (Figure 1B). It is notable that an ApmlA cluster in the network analysis contains Bref-PKS, which has been shown to biosynthesize the corresponding polyketide chain of brefeldin A, a 16-membered aliphatic macrolide (Figure 1B and Figure S1), in the presence of a putative TE, Bref-TH, although macrolactonization has not yet been achieved.[13] In the biosynthesis of fungal resorcinolic macrolides like radicicol and bacterial macrolide antibiotics represented by erythromycin, the C-terminal TE domain in each PKS is involved in macrocyclization of the elongated polyketide chain.[11a,14] Considering the biosynthetic manners, BGCs containing an HR-PKS and a stand-alone putative TE may be responsible for the biosynthesis of fungal aliphatic macrolides, an important fungal natural product group, such as cephalosporolides, brefeldin A, berkeleylactones, and rickiols, although there have not been reports of the biosynthesis of these products (Figure S1).[15]

To identify the compound produced by the *apml* cluster and investigate fungal aliphatic macrolide biosynthesis, we conducted heterologous expression of the *apml* cluster in *Aspergillus oryzae* NSAR1,[16] which has been shown to be an excellent host for heterologous biosynthesis of fungal natural products.[5b−d] Initially, we introduced *apmlA* and/or *apmlB* into *A. oryzae* to prepare two transformants: AO-*apmlAB* and AO-*apmlA*. These transformants were cultivated, and the MeOH extracts of their mycelia were analyzed using reverse-phase HPLC (Figure 2A). An *A. oryzae* strain that was

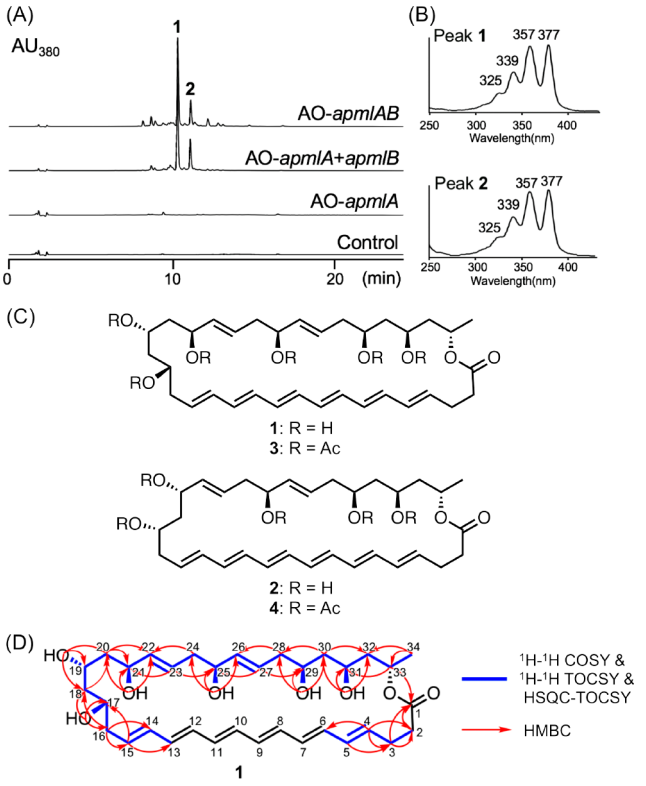


Figure 2. (A) HPLC profiles of the mycelial extracts of the transformants. (B) UV absorption spectra. (C) Structures of **1**−**4**. (D) Two-dimensional NMR correlations of **1**.

transformed using an empty vector was used as the negative control. The HPLC profile of AO-*apmlAB* showed two significant peaks, **1** (major) and **2** (minor), which were absent in the control. Both peaks showed the same UV spectra during diode-array detector (DAD) analysis, whereas different molecular ion peaks were observed via LC-MS analysis at *m*/*z* 609 $[M + Na]^+$ and *m*/*z* 565 $[M + Na]^+$ (panels A and B, respectively, of Figure 2). This suggested that **1** and **2** contained the same polyene structure and that the structure of **2** had a partial structural difference corresponding to 44 Da from **1**. Although no noteworthy peaks appeared for the AO-*apmlA* extract, introduction of *apmlB* into AO-*apmlA* (AO-*apmlA*+*apmlB*) initiated the production of **1** and **2**, revealing that both ApmlA and ApmlB are required for this biosynthesis.

To isolate and determine the structures of **1** and **2**, we performed a scaled-up cultivation of the AO-*apmlAB* transformant and extracted the cultured mycelia with MeOH. Repeated silica gel chromatography facilitated the isolation of **1** (1.1 mg/L), whereas the properties of **2** made it difficult to purify using our equipment. Hence, the structure of **2** was isolated and characterized on the basis of the corresponding

DOI: 10.1021/acs.orglett.9b01674
Org. Lett. XXXX, XXX, XXX−XXX

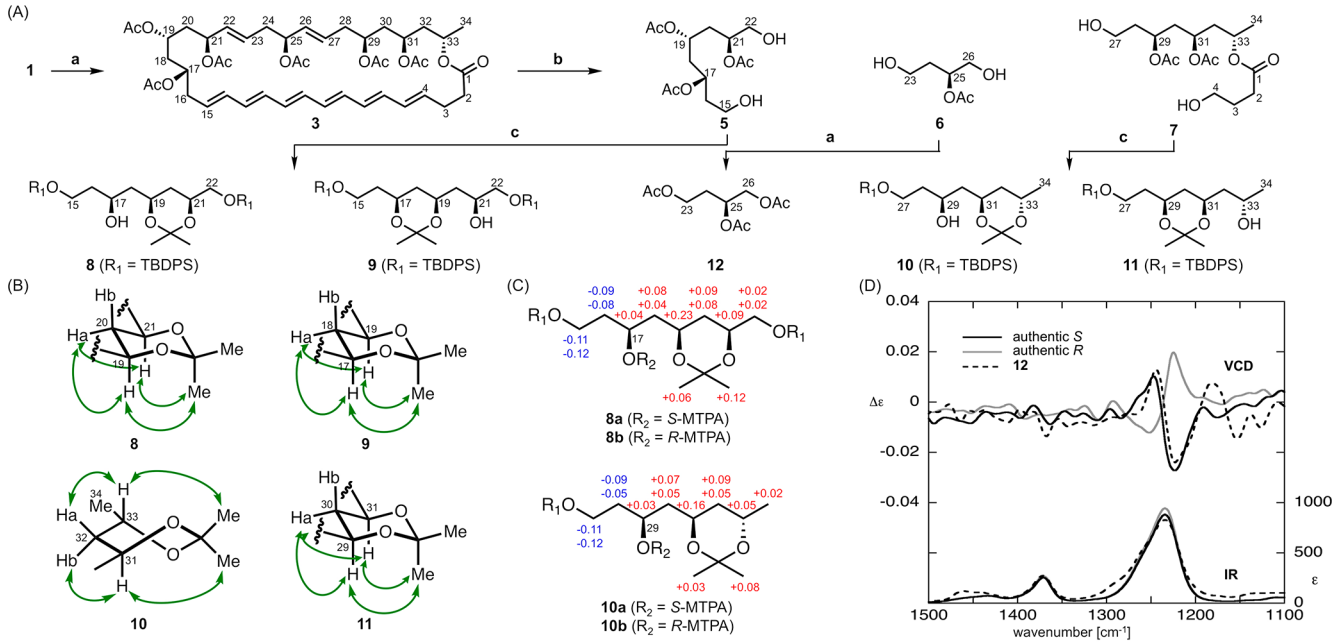


Figure 3. (A) Scheme of fragmentation and derivatization of **1**: (a) Ac_2O, pyridine, rt, overnight; (b) (i) O_3, CH_2Cl_2/MeOH (1:1), −78 °C, 35 min; (ii) $NaBH_4$, 0 °C, 30 min; (c) (i) TBDPSCl, imidazole, DMF, rt, 24 h; (ii) NaOMe, MeOH, rt, 1.5 h; (iii) 2,2-dimethoxypropane, pyridinium *p*-toluenesulfonate, acetone, rt, 1.5 h. (B) Key NOE correlations (green arrow) of acetonides. (C) $\Delta\delta_{H(S-R)}$ values (parts per million) of the MTPA derivatives. (D) VCD and IR spectra of **12** compared with those of (*S*)- and (*R*)-authentic samples (c = 0.06 M for **12** derived from the ozonolysis fragment, or c = 0.15 M for both authentic **12**/$CDCl_3$ solutions, measured at ambient temperature).

acetylated derivative **4** (Figure 2C). The molecular formula of **1** (phaeospelide A) was identified as $C_{34}H_{50}O_8$ on the basis of positive HRESIMS spectra (m/z 609.3366 $[M + Na]^+$) coupled with 34 signals in the ^{13}C NMR spectrum. The four UV absorptions (log ε) at 325 (4.77), 339 (4.75), 357 (4.53), and 377 (4.23) nm and the narrow accumulation of the olefin proton signals (H-7−H-12) within 0.1 ppm (δ_H 6.21−6.31) indicated an all-*trans*-hexaene structure in **1**.[17] The sequential correlations of $^1H-^1H$ COSY and HSQC-TOCSY coupled with the HMBC and HSQC spectral analyses revealed the partial structures of C-13−C-34 and C-2−C-6. The HMBC correlations of H-33/C-1 revealed connectivity between C-1 and C-33 through ester linkage (Figure 2D). The six remaining sp_2 carbons were set between C-6 and C-13 to form the hexaene. The *E* geometries of $\Delta_{22,23}$ and $\Delta_{26,27}$ were characterized by vicinal couplings at $J_{22,23}$ and at $J_{26,27}$ of 15.3 Hz and NOESY correlations of H-21/H-23, H-22/H-24, H-25/H-27, and H-26/H-28 (Figure S2). The assignments of all proton and carbon resonances, listed in Table S1, were achieved on the basis of detailed two-dimensional NMR spectral analyses. Thus, the planar structure of **1**, with 34-membered macrolactone rings and an all-*trans* conjugated hexaene, was elucidated as depicted in Figure 2 (details are provided in the Supporting Information). The locations of all of the olefins and hydroxy groups were consistent with the typical polyketide rule.[6] The spectral analyses of its acetyl derivative **3** supported the planar structure of **1** (Figure S3 and Table S2).

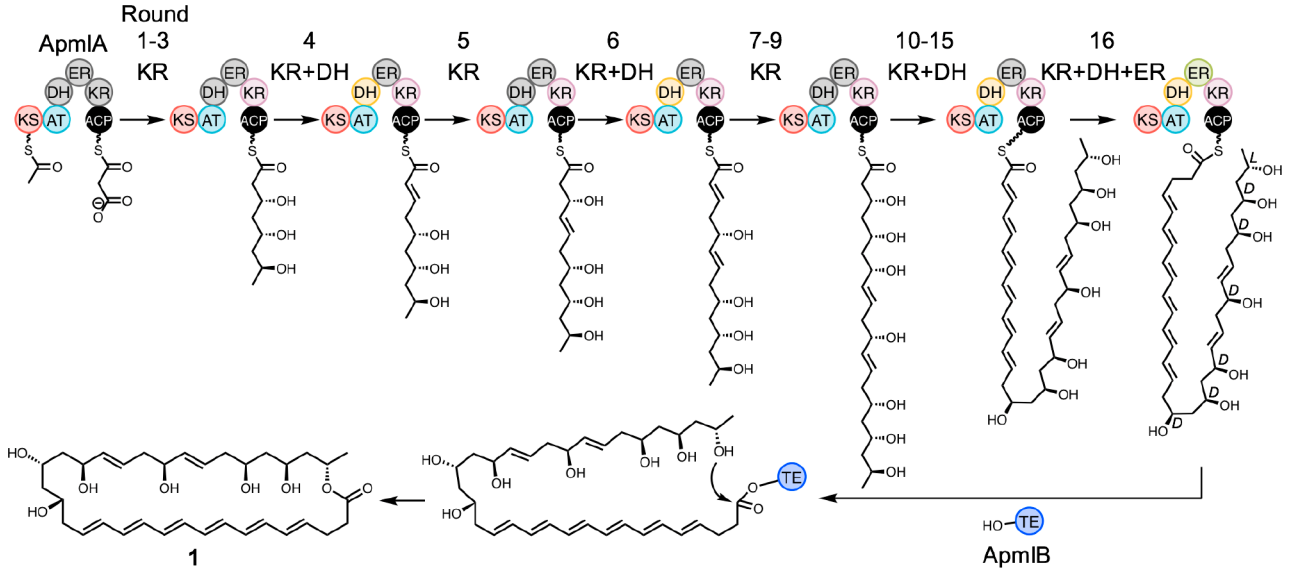
Compound **1** possessed seven chiral centers, at C-17, C-19, C-21, C-25, C-29, C-31, and C-33. To determine all of the absolute configurations, we used a chemical conversion approach. Initially, **1** was acetylated to **3** to improve solubility and stability. Compound **3** was then decomposed via ozonolysis to give three fragments (**5**−**7**). Fragment **6** was derived to give triacetate **12**, whose VCD spectrum was compared with that of authentic (*S*)- or (*R*)-1,2,4-butanetriol, resulting in the identification of a 25*S* configuration in **1** (Figure 3D). Fragment **5** was derived to give acetonides **8** and **9** by three-step reactions (Figure 3A). The NOESY spectral analyses of **8** and **9** showed that both took a chair form, with all oxygen atoms oriented in the same direction (Figure 3B). Similarly, fragment **7** was converted to **10** and **11**. The NOESY spectral analyses of **10** and **11** revealed the following. Compound **11** took a chair form, and its oxygen atoms at C-29 and C-31 were in the *cis* configuration; **10** was in a twist-boat form, and the oxygen atom at C-33 was oriented in the direction opposite to C-31. Finally, the advanced Mosher's method was applied to **8** and **10**, and the absolute stereochemistries at C-17 and C-29 were both determined to be *S*, based on $\Delta\delta_{H(S-R)}$ values (Figure 3C).[18] All of the absolute stereochemistries in **1** were thus identified to be 17*S*,19*S*,21*S*,25*S*,29*S*,31*S*,33*S*.

The planar structure of **4** was determined to be a 32-membered macrolide with the same hexaene motif of **1** via positive HRESIMS spectral and NMR spectral analyses (Figure S4 and Table S3). The detailed structural elucidation is described in the Supporting Information. Compound **2** (phaeospelide B) was thus identified as the deacetylated structure of **4** (Figure 2C). The structural difference between **2** and **1** was only a partial CH_2-CHOH (44 Da) structure: C-16/C-17, C-18/C-19, or C-20/C-21 in **1**, which corresponds to one polyketide elongation cycle. The absolute stereochemistries in **2** were determined to be the same as those in **1**, based on their biosynthetic relevance. We further investigated the production of **1** and **2** in *Ar. phaeospermum*, under several culture conditions. However, HPLC analysis detected no production of **1**, **2**, or their analogues, demonstrating the advantage of this postgenomic approach (Figure S5).

We also screened for antibacterial and antifungal biological activity in **1**, but we have not yet found any such activity.

DOI: 10.1021/acs.orglett.9b01674
Org. Lett. XXXX, XXX, XXX−XXX

Scheme 1. Proposed Biosynthetic Mechanism of 1



On the basis of the structure, including the absolute configuration of the macrolides produced by the *apml* BGC, we have proposed a unique biosynthetic mechanism for generating novel macrolides (Scheme 1). The HR-PKS ApmlA uses acetyl-CoA and malonyl-CoA as its starter and extender units, respectively, and provides the large carbon framework in **1** via 16 cycles of polyketide chain elongation, which is the largest number identified in fungal iterative PKSs thus far.[19] The tailoring module used depends on the extension round. The polyol motif is installed during the first half of the rounds. The absolute configuration of **1** reveals the intriguing function of the KR domain as follows. During round 1, the KR domain reduces β-ketone to an L-oriented hydroxy group, while during later rounds, it provides hydroxy groups in the D-configuration. The biosynthetic aspect of the KR domain is interesting in that it can reduce β-ketone in the L- and D-configurations, according to the situation. The characteristic conjugated hexaene moiety is built during the later rounds (10−15), when the KR and DH domains are at work but ER is off. Phylogenetic analysis of the DH domain of ApmlA shows the similarity with that of Fma-PKS, which biosynthesizes the highly conjugated polyketide side chain of fumagillin,[20] suggesting that a polyene formation is programmed in the DH domain (Figure S6). Finally, the mature ACP-tethered carbon chain is transferred to the serine residue of TE, ApmlB, followed by intramolecular macrolactonization, generating **1**. When one elongation cycle during rounds 7−9 is skipped, compound **2** is biosynthesized instead.

In conclusion, we found a unique BGC comprising an HR-PKS, *apmlA*, and a TE, *apmlB*, in the draft genome of the hairy caterpillar-associated fungus *Ar. phaeospermum*, using HR-PKS-guided genome mining. Heterologous expression of the cluster in *A. oryzae* afforded novel natural products **1** and **2**, which are the first examples of fungal polyene macrolides. We fully elucidated the structure of **1**, including its absolute configuration, via spectral analyses and chemical means, revealing the characteristic 34-membered ring macrolide structure composed of hydrophobic all-*trans*-hexaene opposite a hydrophilic polyol moiety. The structure of **1** also exhibits a unique biosynthetic mechanism, programmed by ApmlA, featuring the largest number of chain elongation cycles among fungal HR-PKSs and the multistereo control of its KR domain. Our analysis results for ApmlA may serve as a clue for understanding the enigmatic "programming" that is encoded in fungal HR-PKS. While HR-PKSs lack a releasing domain, various polyketide chain-release mechanisms exist, such as spontaneous cleavage with pyrone formation,[21] polyketide chain transfer from HR-PKS to nonreducing PKS in asperfuranone biosyntesis,[22] and enzymatic cleavage of the polyketide chain catalyzed by hydrolase in lovastatin biosynthesis.[23] This study is the first demonstration that *trans*-acting TEs catalyze macrolactonization of a polyketide chain on HR-PKS, which is the new offloading system of HR-PKS. Phylogenetic analysis showed a new TE family that may be responsible for fungal macrolide formation (Figure S7). In addition, similar sequence network analysis and phylogenetic analysis using ApmlA as a reference revealed a large family of HR-PKSs that cluster with ApmlB homologues (Figure 1 and Figure S8). In other words, diverse BGCs that are probably involved in aliphatic macrolide biosynthesis are present in various fungal species. Previously, various aliphatic macrolides have been reported from several fungal species, such as *Beauveria bassiana* (cephalosporolides),[24] *Pyrenophora teres* (pyrenolides),[25] *Diplodia sapinea* (diplodialides),[26] *Eupenicillium brefeldianum* (brefeldin A),[13,27] and *Eupenicillium shearii* (eushearilide).[28] Therefore, the BGCs in macrolide producers may correspond to the biosynthesis of previously reported compounds. In contrast, the other BGCs in fungi that have not been reported upon production of macrolides could be a fascinating source of novel fungal aliphatic macrolides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b01674.

Experimental details, figures and additional information, full spectroscopic data, and NMR spectra of new compounds (PDF)

DOI: 10.1021/acs.orglett.9b01674
Org. Lett. XXXX, XXX, XXX−XXX

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Prof. K. Gomi (Tohoku University, Sendai, Japan) and Prof. K. Kitamoto (The University of Tokyo) for providing the expression vectors and the fungal strain. This work was supported by JSPS KAKENHI [Grants 17H05055, 17H05428, and 19H04642 to T.A. from the Japan Society for the Promotion of Science (JSPS)], the Naito Foundation, and the Astellas Foundation for Research on Metabolic Disorders.

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DOI: 10.1021/acs.orglett.9b01674
Org. Lett. XXXX, XXX, XXX−XXX