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Spiroketal Formation and Modification in Avermectin Biosynthesis Involves a Dual Activity of AveC

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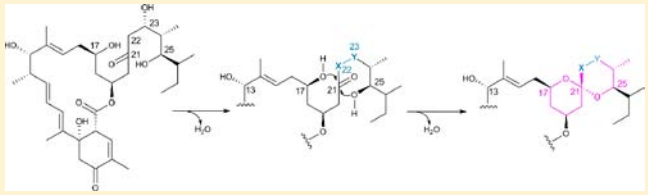
Supporting Information

ABSTRACT: Avermectins (AVEs), which are widely used for the treatment of agricultural parasitic diseases, belong to a family of 6,6-spiroketal moiety-containing, macrolide natural products. AVE biosynthesis is known to employ a type I polyketide synthase (PKS) system to assemble the molecular skeleton for further functionalization. It remains unknown how and when spiroketal formation proceeds, particularly regarding the role of AveC, a unique protein in the pathway that shares no sequence homology to any enzyme of known function. Here, we report the unprecedented, dual function of AveC by correlating its activity with spiroketal formation and modification during the AVE biosynthetic process. The findings in this study were supported by characterizing extremely unstable intermediates, products and their spontaneous derivative products from the simplified chemical profile and by comparative analysis of *in vitro* biotransformations and *in vivo* complementations mediated by AveC and MeiC (the counterpart in biosynthesizing the naturally occurring, AVE-like meilingmycins). AveC catalyzes the stereospecific spiroketalization of a dihydroxy-ketone polyketide intermediate and the optional dehydration to determine the regiospecific saturation characteristics of spiroketal diversity. These reactions take place between the closures of the hexene ring and 16-membered macrolide and the formation of the hexahydrobenzofuran unit. MeiC can replace the spirocyclase activity of AveC, but it lacks the independent dehydratase activity. Elucidation of the generality and specificity of AveC-type proteins allows for the rationalization of previously published results that were not completely understood, suggesting that enzyme-mediated spiroketal formation was initially underestimated, but is, in fact, widespread in nature for the control of stereoselectivity.



INTRODUCTION

Spiroketal natural products have long been appreciated for their remarkable pharmaceutical importance.[1] Among them, the discovery of avermectins (AVEs), along with their naturally occurring analogues, milbemycins (MILs), meilingmycins (MEIs) and nemadectin (NEM), represents one of the most significant developments of antiparasitic agents.[2] These microbial secondary metabolites, which are characterized by a 6,6-spiroketal moiety, share a 16-membered macrolide conjugated with a hexahydrobenzofuran unit (Figure 1). *Streptomyces avermectinius*, isolated from Japan in the 1970s,[3] produces eight members of the AVE family showing the permutation of three structural variations: methoxylation (for "A" series) or hydroxylation (for "B" series) at C5, a double bond between C22−C23 (for "1" series) or its hydrated product at C23 (for "2" series), and substitution with methylpropyl (for "a" series) or isopropyl (for "b" series) at C25. AVEs selectively act on glutamate-gated chloride channels in nematodes to stimulate the release and binding of γ-aminobutyric acid at the nerve endings.[4] This causes an influx of chloride ions into the cells, leading to hyperpolarization and subsequent paralysis of the neuromuscular systems.[5] With the potent anthelmintic activity and relatively low toxicity in humans and animals, AVEs have extremely widespread application for the treatment of agricultural parasitic diseases. Ivermectin, a mixture developed by 22,23-hydrogenation of the B1a and B1b components of AVEs, has proven to be one of the most successful antiparasitic drugs for veterinary and human healthcare during the past 30 years.[2c]

The biosynthetic gene cluster of AVEs has been cloned and characterized (Figure 2A),[6] leading to proposals regarding the AVE-forming chemistry and structural diversity with the combination of previous isotope-labeling[7] and mutagenesis[2b,6b,7e,8] studies. AVE biosynthesis shares a type I polyketide synthase (PKS) system (AveA1-A4) to assemble the macrolide skeleton (Figure 2B).[6d] Oxidative modifications of this macrolide then produce a furan-containing aglycone for further glycosylations. According to the PKS module and domain organization, the resulting polyketide intermediate may bear a key C17, C25-dihydroxy C21-ketone moiety, which could be used to form the 6,6-spiroketal via a C21-carbonyl ketalization-

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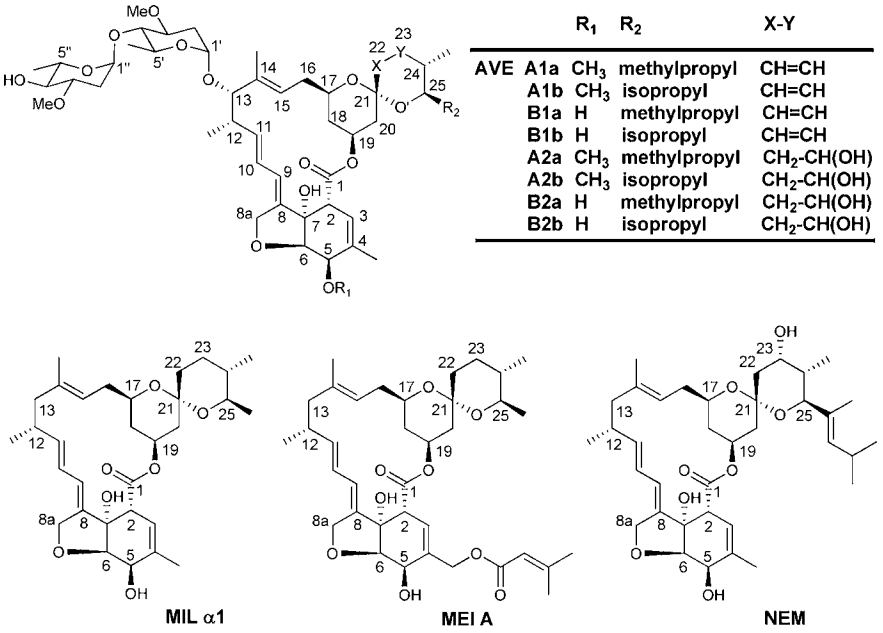

Figure 1. Structures of avermectins (AVEs, eight naturally occurring members), milbemycin (MIL) α1, meilingmycin (MEI) A, and nemadectin (NEM).

based dehydrative cyclization.[2b] Mechanistically, it is distinct from 6,5-spiroketal formation in the biosynthesis of certain polyethers, such as monensins, which depends on a hydroxy-ketone-epoxide motif for epoxide hydrolase-mediated cyclization.[9] The unsolved questions in AVE biosynthesis include: i) whether the dihydroxy-ketone motif exists, and ii) when and how (enzymatically or spontaneously) the reaction takes place. In regard to the diversity of the eight individual members, while the PKS loading module for starter unit choice[10] and the methyltransferase AveD for C5-*O*-methylation[8f] are known to determine the "a" or "b" and the "A" or "B" series, respectively, the dehydratase (DH) domain in module 2 of the PKS AveA1 (AveA1-M2-DH) has long been speculated to be responsible for optional dehydration at C22−C23 (Figure 2B), marking the branching point into the "1" or "2" series. The DH domain contains a mutant active motif, HxxxGxxxxS, which accounts for the partial activity assumed to be modulated by AveC,[2b] a unique protein in the AVE biosynthetic pathway without sequence homology to any enzyme of known function. Although engineering of AveC significantly changed the ratio of the "1" and "2" components,[11] the prediction that modular DH activity needs an AveC-like "*trans*" factor is contradictory to known type I PKS assembly logic.[12] In fact, it later emerged that the AveC counterparts, MelC (53% identity), MeiC (53% identity) and NamC (58% identity) (Figure S1), are present in all biosynthetic pathways of the structurally related compounds, MELs, MEIs and NAM, in which the AveC-relevant dehydration activity is likely unnecessary.[2b] The PKSs for MELs and MEIs have a typical DH-enoylreductase (ER)-ketoreductase (KR) organization to fully saturate the C22−C23 bond,[13] and the PKSs for NAM lack a corresponding DH domain to further process the C23-hydroxyl group (Figure 2C).[14] Therefore, interesting queries arise regarding the action of AveC-type proteins. Do they play a common undiscovered role in AVE-like natural product biosynthesis? Does AveC differ from other counterparts by contributing the specific dehydration activity? In this study, we answer the above lingering questions based on the investigations into AveC, which reveal the unprecedented dual activity of AveC in spiroketal formation and modification in AVE biosynthesis.

RESULTS

Analysis of the Chemical Profile by *aveC* Mutation. We first simplified the chemical profile of *S. avermectinius* for *in vivo* studies. Various media that were previously used to produce AVEs were individually assessed, leading to the selection of a medium, in which *S. avermectinius* mainly produced four "a" components of AVEs, including A1a, A2a, B1a and B2a (Figure 3, I). Under this condition, the loading module of the AVE PKSs may dominantly utilize 2-methylbutyryl (over isobutyryl) as the starter unit for priming polyketide assembly. Furthermore, we inactivated the "A" series-forming gene, *aveD* (encoding a C5-*O*-methyltransferase, Figure 2A), resulting in a single mutant strain VL1001 that only yielded the C5-hydroxyl "B" components, AVE-B1a and AVE-B2a (Figure 3, II). These optimizing efforts abolished (or minimized) the production of the "A" and "b" series of AVEs, providing a condition for VL1001 that primarily produces two products, AVE-B1a and AVE-B2a, which allow us to easily trace the function of AveC.

We then deleted the target gene, *aveC*, in-frame, in VL1001 to make a double mutant strain, VL1002. This strain has the ability to produce a trace of AVE-B2a but completely lost the ability to produce AVE-B1a, as determined by HPLC-MS analysis (Figure 3, III). Intriguingly, we found that at least three pairs of distinct products accumulated. Each pair, **1** and **1′**, **2** and **2′**, or **3** and **3′**, has identical molecular weights (MWs). These findings not only validated the necessity of *aveC* for AVE biosynthesis, but also inspired us to explore the structural consequence of eliminating AveC activity.

Characterization of 1 and 1′ (Spontaneous Double-Dehydration Derivatives from 3 and 3′). We scaled up the fermentation of VL1002 (approximately 100 L culture broths) and began the purification of the stable pair **1** and **1′** for spectroscopic analyses. Compounds **1** and **1′**, which have same molecular formula as $C_{34}H_{48}O_7$ ($[M + Na]^+$ m/z: calcd.

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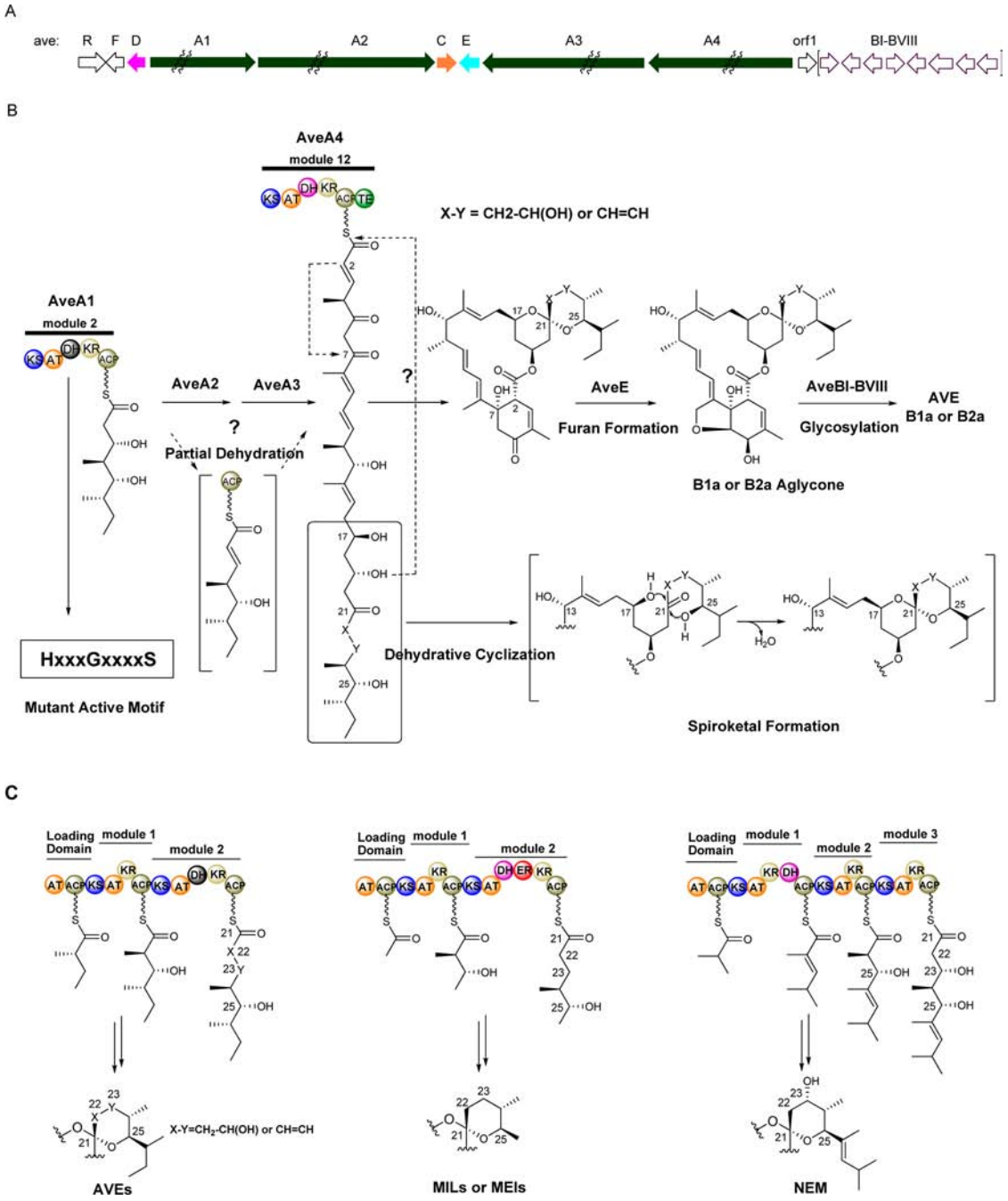

Figure 2. Gene cluster and proposed biosynthetic pathways. (A) Organization of the AVE biosynthetic genes, which encode type I PKSs AveA1-A4 for polyketide skeleton assembly (green), AveC in this study (yellow), AveE for furan formation (blue), AveD for C5-*O*-methylation (pink), and others (white). (B) Exemplified pathway for the AVE components B1a and B2a. The mechanism of dehydrative cyclization for spiroketal formation is shown in the lower bracket. (C) Partial PKSs for assembling the polyketide chains of AVEs, MILs/MEIs, and NEM, and their corresponding spiroketal moieties in the final molecules. The functional domains of selected modules of the PKSs are indicated by color: blue for ketosynthetase (KS), yellow for acyltransferase (AT), black for mutant dehydratase (DH), purple for typical DH, light gray for ketoreductase (KR), dark gray for acyl carrier protein (ACP), red for enoylreductase (ER), and green for thioesterase (TE).

591.3292, found 591.3279), share a furan-lacking, 16-membered macrolide that is similar to the AVE-B2a aglycone in the planar structure. However, they are different from each other, as shown by the opposite orientations of the bonds from C21 to C22 ("α" for **1** and "β" for **1′**) and to O′ ("β" for **1** and "α" for **1′**) (Supplementary Result, Table S3 and Figures S6 and S7). Furthermore, crystallization of these compounds unambiguously confirmed that they are spiroketals, stereoisomeric at C21, "*S*" for **1** and "*R*" for **1′** (Figures 4 and 6, and Supplementary Result). By treatment with formic acid, **1**, possessing a bis-equatorial C−O conformation, can be completely converted into the isomer **1′**, with a bis-diaxial C−O conformation that is same as that of the natural product, AVE-B2a (Figure S17). This stereoisomerization of the spiroketal has been reported in spirofungin synthesis.[15] However, both **1** and **1′** are stable under physiological (pH 7.0−8.0) and the above analytic conditions, indicating that AveC participates in stereospecific spiroketal formation. Given

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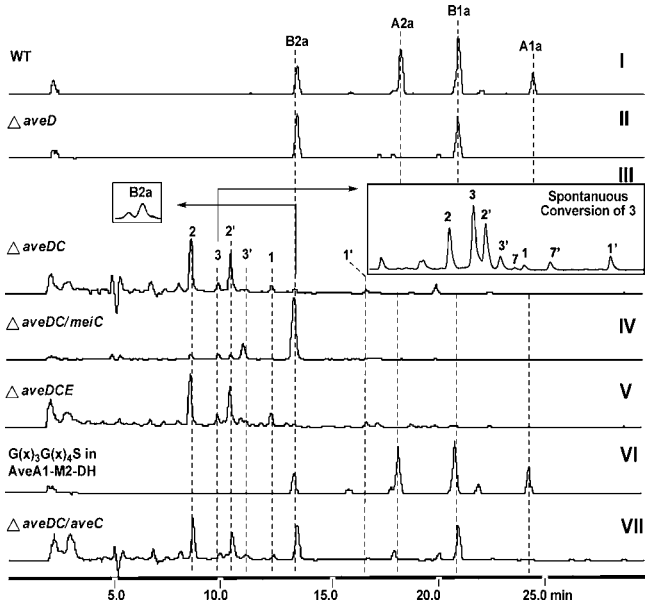


Figure 3. Characterization of AveC/MeiC *in vivo*. Production analysis of *S. avermectinius* and its mutant strains. I, AVE-producing wild type strain; II, VL1001 (Δ*aveD* single mutant); III, VL1002 (Δ*aveDC* double mutant. The charts show the magnified peak corresponding to AVE-B2a (left) and the spontaneously dehydrated products from intermediate **3** (right); IV, VL1003 (VL1002 derivative that carries *meiC in trans*); V, VL1004 (Δ*aveDCE* triple mutant); VI, VL1005 (by mutation of the sequence encoding $H(x)_3G(x)_4S$ into $G(x)_3G(x)_4S$ in AveA1-M2-DH); and VII, VL1006 (VL1002 derivative that carries *aveC in trans*).

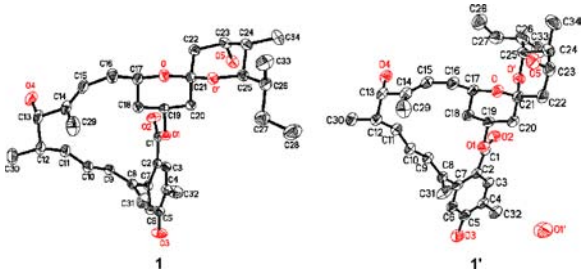
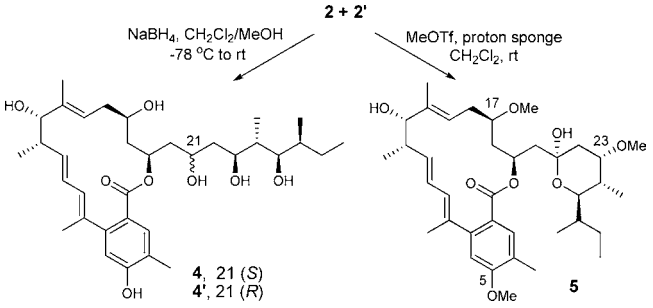


Figure 4. X-ray structures of **1** and **1′**.

that all natural occurring "2" components possess an "*R*" configuration at C21, the spiroketalization of both **1** and **1′** in the absence of AveC could proceed in a spontaneous manner.

Characterization of 2 and 2′ (Spontaneous Single-Dehydration, Major Derivatives from 3 and 3′). We next evaluated the stability of **2** and **2′** ($[M + Na]^+$ *m/z*: calcd. 609.3398 for $C_{34}H_{50}O_8$, found 609.3387 or 609.3389). They can be rapidly converted into each other, and then into **1** and **1′** after longer incubation via the loss of H_2O (Figure 6 and Figure S2). On the basis of fractionation and chemical reduction of **2**, we subsequently confirmed the presence of a C21 carbonyl group in this molecule by characterizing its derivatives, **4** and **4′** ($[M + Na]^+$ *m/z*: calcd. 611.3554 for $C_{34}H_{52}O_8$, found 611.3572 or 611.3358, showing an increase by 2 in MW compared to **2** and **2′**), which contain a triol side chain and are enantiomeric at C21 ("*S*" for **4** and "*R*" for **4′**, Scheme 1, Supplementary Result, Tables S5 and S6 and Figures S9 and S10). The mixture of **2** and **2′**, at a ratio of 2:1, was subjected to NMR analyses, which revealed that they are spiral ring-opened and differ from each other only in C21 hemiketalization

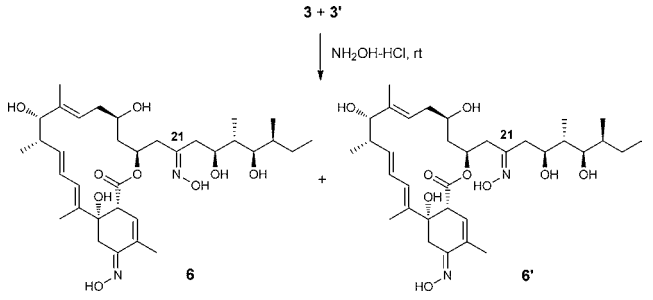
Scheme 1. Chemical Derivatization of 2 and 2′ To Generate 4 and 4′, and 5



through the attack of a hydroxyl, from C25 to afford **2** and from C17 to give **2′** (Supplementary Result, Table S4 and Figure S8). The presence of a hemiketal unit in **2** was also confirmed by characterizing its trimethylated derivative, **5** ($[M + Na]^+$ *m/z*: calcd. 651.3867 for $C_{37}H_{56}O_8$, found 651.3897; Scheme 1, Supplementary Result, Table S7 and Figure S11). These results, for the first time, provided structural evidence of spiroketal formation in AVE biosynthesis, which relies on a dihydroxy-ketone moiety (found in two different hemiketalization forms) for dehydrative cyclization.

Characterization of 3 and 3′ (Intermediates) and 7 and 7′ (Spontaneous Single-Dehydration, Minor Derivatives). Compounds **3** and **3′** ($[M + Na]^+$ *m/z*: calcd. 627.3503 for $C_{34}H_{50}O_8$, found 627.3491 or 627.3493) show an increase by 18 in MW compared to **2** and **2′**. These two compounds can also be converted into each other and after a longer incubation, into the major pair, **2** and **2′** ($-H_2O$), and the minor pair, **7** and **7′** ($-H_2O$), and eventually into **1** and **1′** ($-2H_2O$) (right chart in Figure 3, III). For structural elucidation, we carried out the chemical derivatization of **3** and **3′** using oxime to stabilize their potential keto groups, which may make the compounds extremely unstable. The fraction of **3** or **3′** yielded an identical pair, **6** and **6′**, which have two oximyl groups at C21 and C5 (Scheme 2). Furthermore, **6** and **6′** ([M

Scheme 2. Chemical Derivatization of 3 and 3′ To Generate 6 and 6′



+ Na]$^+$ *m/z*: calcd. 657.3722 for $C_{34}H_{54}N_2O_9$, found 657.3748 or 657.3738; Supplementary Result, Table S8, and Figures S12 and S13) were characterized to be a pair of C21-ketoxime stereoisomers, permitting us to deduce the structures of **3** and **3′** (Figure 6). Similar to **2** and **2′**, **3** and **3′** are isomers, and they possess a spiral ring-opened side chain with different hemiketalization of the C21-keto group. However, distinct from **2** and **2′** in the C2−C7 ring, **3** and **3′** bear a C5-keto-C7-hydroxyl cyclohexene moiety. The latter is consistent with the proposed biosynthetic pathway of AVEs,[2b] in which the linear

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polyketide intermediate may undergo an aldol condensation between C2 and C7 to produce a nonaromatic ring. Therefore, these findings strongly support the idea that **3** and **3′** (as well as the undetectable C21 nonhemiketalized precursor **3″**) could be the real intermediates resulting from *aveC* inactivation. Compounds **3** and **3′** also may undergo a dehydrative cyclization to form the spiroketal moiety, in agreement with our characterization of the corresponding minor stereoisomers **7** and **7′** ($[M + Na]^+$ *m/z*: calcd. 609.3398 for $C_{34}H_{50}O_8$, found 609.3421 or 609.3416; "*S*" for **7** and "*R*" for **7′** at C21, respectively. Supplementary Result, Table S9, and Figures S14 and S15). In the chemical profile obtained from the spontaneous conversion of **3** and **3′** (Figure 6), the ratio between **7** and **7′** was approximately 1:4 as determined by HPLC analysis (right chart in Figure 3, III). Apparently, dehydrative aromatization of the C2−C7 ring is more competitive than dehydrative spiroketalization, leading to generation of **2** and **2′** as the major pair.

Characterization of the Spirocyclase Activity of MeiC. Despite of our extensive attempts, AveC was highly resistant to various methods of expression in different host strains. For assaying spirocyclase activity *in vitro*, we chose MeiC,[13] the homologue of AveC in MEI biosynthesis, because it was detectable in the recombinant *Escherichia coli* strain VL2001 by expression of a maltose-binding protein (MBP)-tagged form (Figure S3). We incubated the **3** and **3′**-containing fraction at 30 °C with the cell free extract of VL2001, which relies on the IPTG induction for MBP-MeiC production, and performed a time course analysis of product yield and substrate consumption (Figure 5, IV and V, and Figure S4). Indeed, **7′**, the C21-(*R*) spiroketal, only appeared in the presence of MBP-MeiC in a relatively rapid, time-dependent manner, while the substrates **3** and **3′** decreased accordingly. Under these enzymatic reaction conditions, the production of spontaneously coupled C21-(*S*) product **7** was not detectable (Figure S4). MBP-MeiC did not accelerate the conversion of **2** and **2′** into **1′**, suggesting that spiroketal formation proceeds in an enzymatic manner and depends on the structure of the C2−C7 ring (Figure 5, VI and VII). According to these data, **7′** can be the only product in the MBP-MeiC-catalyzed reaction. To further ascertain that MeiC functions in AVE biosynthesis, we introduced *meiC* into the *aveC* mutant strain VL1002. In the resulting recombinant strain, VL1003, the production of AVE-B2a was remarkably restored (Figure 3, IV). Therefore, MeiC can substitute for AveC in function, as they both have spirocyclase activity.

Timing of Spiroketal formation in AVE Biosynthesis. Considering our above findings, we reason that AveC, as well as the counterpart MeiC, catalyzes the post-PKS, stereospecific spiroketal formation by converting **3** and **3′** to **7′** (Figure 6). Without the enzymatic activity, the substrates can partially go through a dehydrative cyclization to produce the spiroketal, **7′**, and its isomer, **7**. Only **7′** can be functionalized by the downstream enzymes in the AVE biosynthetic pathway to eventually produce a trace of AVE-B2a, as shown in VL1002 (Figure 3, III, left chart). Spiroketal formation may occur after C2−C7 condensation to afford the hexene moiety and lactonization to close the 16-membered macrolide, but before C6−C8a cyclization to give the hexahydrobenzofuran unit. To support this hypothesis, we constructed a triple mutant strain, VL1004, in which the furan-forming gene, *aveE* (encoding a P450 oxidoreductase), was deleted along with *aveC* and *aveD*. Overall, VL1004 had a chemical profile similar to that of

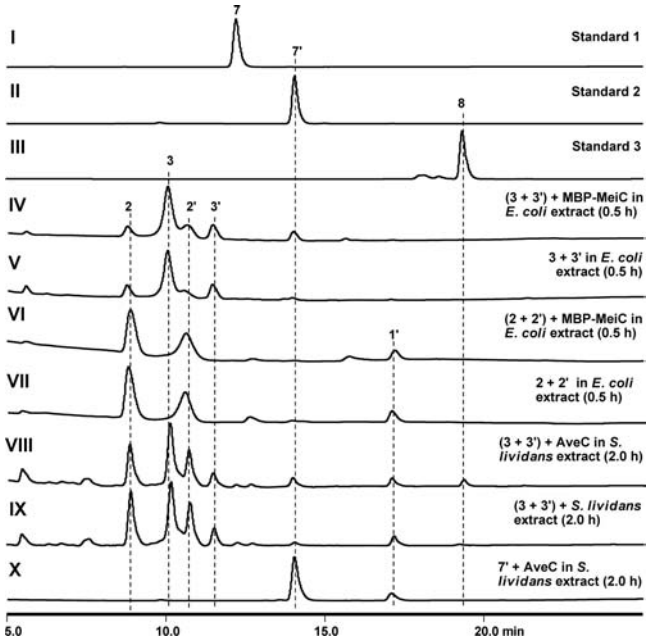

Figure 5. AveC or MeiC-catalyzed *in vitro* biotransformation by using **7** (I), **7′** (II) and **8** (III) as the standards. For MeiC, the intermediates **3** and **3′** were incubated for 0.5 h in the presence of the *E. coli* cell-free extract with (IV) or without (V) MBP-MeiC expression. The same reaction conditions were applied to the conversion of the shunt intermediates **2** and **2′** (VI, with MBP-MeiC expression, and VII, without MBP-MeiC expression). For AveC, **3** and **3′** were incubated for 2.0 h in the presence of the *Streptomyces lividans* cell free extract with (VIII) or without (IX) AveC expression. The same reaction conditions were applied to the conversion of the shunt intermediate **7′** (X).

VL1002 (Figure 3, V), as shown by the product pairs of **1** and **1′**, **2** and **2′**, and **3** and **3′**. However, this strain lost the ability to produce AVE-B2a because ommiting of the AveE-catalyzed furan-forming step in the pathway blocks aglycone formation and thus further tailoring (Figure 2B).

Characterization of the Spirocyclase Activity and Optional Dehydratase Activity of AveC. Intriguingly, the *meiC*-complementing strain, VL1003, lacked the ability to produce AVE-B1a (Figure 3, IV). This result, together with the fact that all the detectable products (e.g., **1** and **1′**, **2** and **2′** and **3** and **3′**) in the *aveC* mutant strain VL1002 contain a C23-hydroxyl characteristic, "2" series-like skeleton, indicated that AveC can be distinct from MeiC and that it plays a role for C22−23 dedydration in determining the "1" and "2" components of AVEs. To support this hypothesis, we first inactivated AveA1-M2-DH by replacing the key active residue His with Gly, as previously reported in functional studies of type I PKS by inactivation of DH domain activity.[16] The resulting mutant strain, VL1005, which has an identical product profile to that of the wild type strain, did not change the ratio of the "1" and "2" components of AVEs (Figure 3, VI). Therefore, this domain is completely inactive, and its involvement in C22−C23 dehydration during the polyketide assembly process can be excluded. Next, we complemented *aveC* into VL1002. In the resulting recombinant strain, VL1006, the production of both AVE-B1a and AVE-B2a was restored as anticipated (Figure 3, VII), indicating that "1" component generation relies on *aveC* expression. Furthermore, the active *aveC*-expressing construct in VL1006 was transferred into *S. lividans*, yielding

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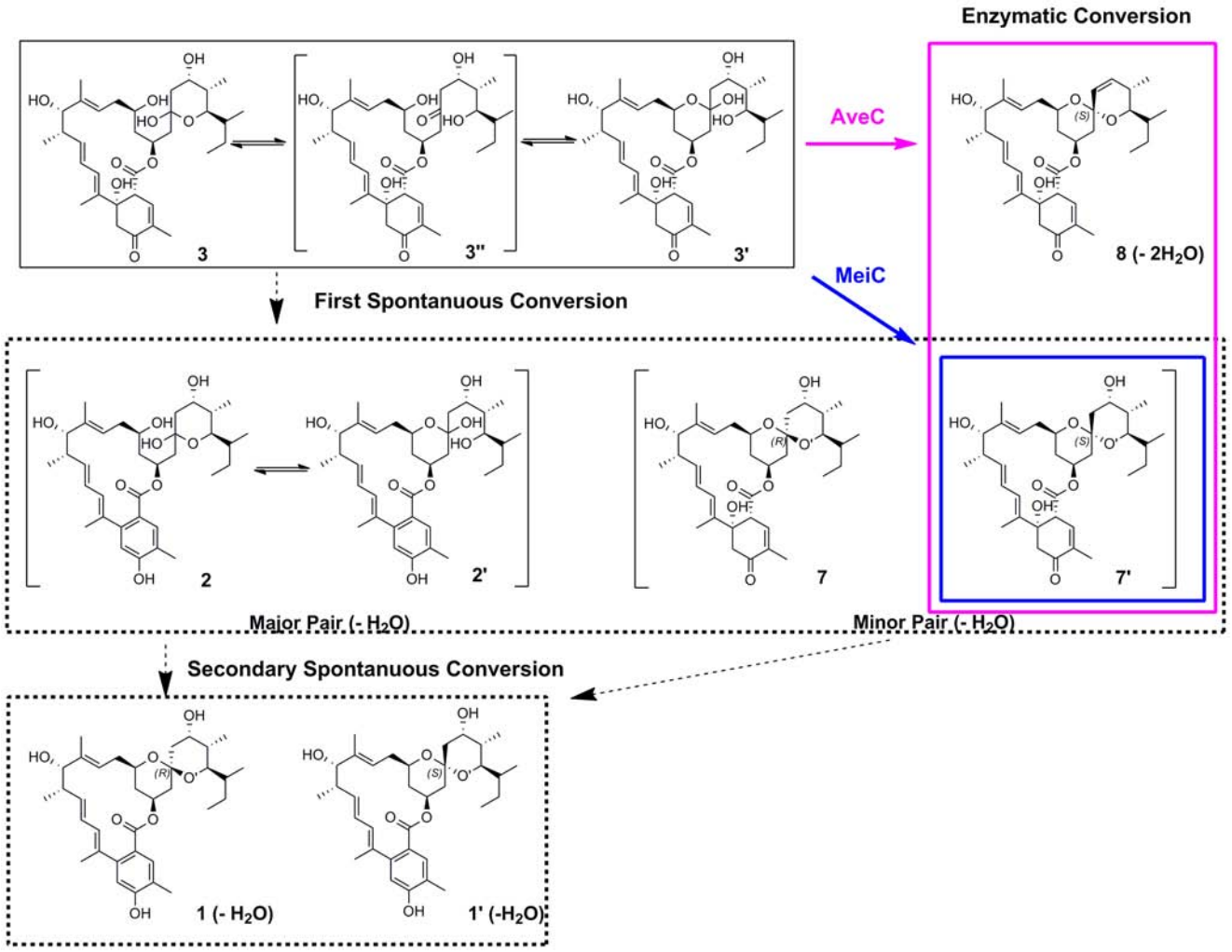

Figure 6. Conversion of the intermediates **3** and **3′**. Whereas the spontaneous way (along with the associated derivative products) is shown in dashed, the enzymatic route is indicated in bold (pink for AveC and blue for MeiC).

VL1007 for AveC production in a *Streptomyces* strain. Similar to the MBP-MeiC extract, the cell free extract of VL1007 produced **7′** in the presence of the substrates **3** and **3′**; however, it was apparently capable of producing an additional new product, **8** ($[M + H]^+$ *m*/*z*: calcd. 569.3456 for $C_{34}H_{48}O_7$, found 569.3473; Figure 5, VIII and IX, and Supplementary Result, Table S10 and Figure S16). This compound ($-2H_2O$ from **3** and **3′**) is identical to a previously characterized intermediate obtained by inactivating *aveE*[8d] (we isolated it from the Δ*aveED* double mutant strain VL1008 as the standard), featuring a dehydrated C22−C23 ene spiroketal moiety in contrast to **7′** (Figure 6). Under the same reaction condition, **7′** cannot be converted into **8** through the loss of H_2O, indicating that C22−C23 dehydration precedes spiroketal formation (Figure 5, X). These findings supported the hypothesis that AveC is the only candidate responsible for C22−C23 dehydration to yield the "1" components. For AveC, the specific dehydratase activity is independent of its spirocyclase function, consistent with previous studies showing that mutation of the AveC sequence can dramatically lower dehydratase activity but maintain spirocyclase activity, primarily producing AVE-B2a over AVE-B1a.[11]

DISCUSSION

There are a number of spiroketal products found in nature including AVEs, for which the spiroketal formation was initially speculated to proceed in a nonenzymatic manner. To date, the biosynthesis of spiroketal natural products remains poorly understood. This is partially because of the difficulty of the identification of catalytic candidates and analysis of unstable intermediates.[17] We have identified AveC as a novel protein by correlating its dual function with spiroketal formation and modification (Figure 6), representing significant progress in the understanding of AVE-like, complex spiroketal natural product biosynthesis. This study utilized the followings: (1) a rational query of the role of AveC in the biosynthesis by comparative analysis with its counterparts; (2) simplification of the chemical profile for gene inactivation-based product tracing; (3) systematic characterization of the extremely unstable intermediates, products and their associated derivative products by coupling chemical derivatization approaches; and (4) comparative *in vitro* biotransformations and *in vivo* complementations for ultimate validation.

AveC has long been considered as a factor to *in trans* activate the corresponding partial dehydratase activity of the AveA1-M2-DH domain of PKSs for optional C22−C23 dehydration in the AVE biosynthetic pathway.[2b] According to the findings in

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this study, it mainly catalyzes the dehydration-coupled, stereospecific spirocyclization of a C17, C25-dihydroxy C21-ketone polyketide intermediate. This paradigm can be shared for all AVE-like natural products, such as MILs, MEIs and NEM, to yield a family characteristic spiroketal. AveC functions after the closures of the hexene moiety and 16-membered macrolide, and before the formation of the hexahydrobenzofuran unit. Spontaneously, the substrates of AveC, **3** and **3′**, prefer to undergo dehydrative aromatization of the C2−C7 ring to afford a major pair of isomers, **2** and **2′**, which are rapidly converted into each other. **3** and **3′** are also able to go through a dehydrative ketalization to form the minor pair, **7** and **7′**, which are spiroketals that are stereoisomeric at C21 and incapable of interconversion (Figure 6). Both isomer pairs can be further transformed into the same stable pair of isomers, **1** and **1′**, through the loss of an additional H_2O.

Intriguingly, sequence analysis revealed that the AveC-type proteins share no sequence similarity to RevJ,[17] the only spirocyclase found in reveromycin biosynthesis for converting a dihydoxy-ketone moiety into 6,6-spiroketal, indicating that the AveC-like proteins are mechanistically similar to but phylogenetically distinct from RevJ. Given the presence of spiroketal natural products in nature that often possess only one configuration at the spiral center, spirocyclases appear to be widely used in spiroketal biosynthesis to control the stereoselectivity. Candidates for spirocyclases, such as AveC and RevJ, may not be easily identified by homology comparison at the sequence level perhaps due to the chemical structure-dependent evolution of spirocyclase activity. Given the extremely unstable nature of the substrate(s) that have the potential to result in two configurations at the spiral center through rapid, spontaneous cyclization, the presence of spirocyclase in the pathway ensures that biosynthesis generates the correct stereoisomer for further functionalization. This is particularly important for AVE biosynthesis because it is highly probable that the intermediates **3** and **3′** can be rapidly degraded to **2** and **2′** via C2−C7 aromatization, which is even much faster than dehydrative spirocyclization to generate **7** and **7′**.

Remarkably, AveC is distinct from all its counterparts (i.e., MelC, MeiC and NamC for other known AVE-like spiroketal natural products), as well as RevJ for reveromycins, because it possesses regiospecific activity for C22−C23 dehydration to replace the completely inactive AveA1-M2-DH domain. This reaction is post-PKS but precedes spiroketal formation, resulting in a modified dihydroxy ketone polyketide intermediate for spirocyclization. The dehydratase activity of AveC could be independent of and compete with its spirocyclase activity *in vivo*. The latter activity tolerates variation of the C22−C23 bond, ultimately leading to simultaneous production of the "1" or "2" series of AVEs. MIL or MEI biosynthesis integrates the KR, ER and cognate DH activities to the PKS module for fully saturating the C22−C23 bond during skeleton assembly process; therefore, these pathways do not require the post-PKS dehydratase activity of MilC or MeiC, suggesting an interesting evolution of AveC-type proteins in microorganisms.

CONCLUSION

We have correlated the function of AveC, a novel protein in the biosynthetic pathway of the potent antiparasitic agents AVEs, to the formation of the characteristic 6,6-spiroketal moiety and its modification. AveC shares a common mechanism with its homologous proteins in the biosyntheses of structurally similar natural products, to catalyze stereospecific spiroketalization of a dihydroxyl-ketone polyketide intermediate. Additionally, it has a unique dehydration activity to competitively act on the substrate, marking the branch point to determine the regiospecific saturation pattern for spiroketal diversity. Characterization of the dual activity of AveC allows for the rationalization of previously published results that were not completely understood, setting the stage for applying AveC chemistry to rational engineering of the pathway to selectively produce single components for pharmaceutical use.

EXPERIMENTAL SECTION

Materials, Bacterial Strains, and Plasmids. Biochemicals and media were purchased from Sinopharm Chemical Reagent Co., Ltd. (China) or Oxoid Ltd. (U.K.) unless otherwise stated. Enzymes were purchased from Takara Biotechnology Co., Ltd., except Taq DNA polymerase, which was purchased from Dingguo Co., Ltd. (China). Chemical reagents were purchased from standard commercial sources. The bacterial strains and plasmids used in this study are listed in Table S1.

DNA Isolation, Manipulation, and Sequencing. DNA isolation and manipulation in *E. coli* or *Streptomyces* strains were carried out according to standard methods.[18] PCR amplifications were carried out on an Authorized Thermal Cycler using either Takara Taq DNA polymerase or PrimeSTAR HS DNA polymerase (Takara Biotechnology Co., Ltd.). Primer synthesis and DNA sequencing were performed at the Shanghai Invitrogen Biotech Co., Ltd. Primers used for the generation of gene deletion cassettes and diagnostic PCR are listed in Table S2. Gene inactivation and complementation were performed using standard procedures.[18] Detailed procedures are described in the Supporting Information. Validations of the genotypes of the mutant strains are shown in Figure S5.

Sequence Analysis. Protein comparison was carried out using available BLAST methods (http://www.ncbi.nlm.nih.gov/blast/). Amino acid sequence alignment was performed by the CLUSTALW method, and the DRAWTREE and DRAWGRAM methods, from BIOLOGY WORKBENCH 3.2 software (http://workbench.sdsc.edu).

Chemical Analysis. The mycelia yielded by 1 mL of *S. avermectinius* culture broth were extracted with 1 mL of methanol. After centrifugation, the supernatant was used for HPLC or LCMS analyses. The HPLC analyses were carried out on an Agilent 1200 HPLC system (Agilent Technologies Inc.) with a Zorbax SB-C_{18} column (4.6 mm × 250 mm, 5 μm) eluting with a flow rate of 1.0 mL·min^{-1} over a 30 min gradient as follows: T = 0 min, 65% B; T = 5 min, 65% B; T = 20 min, 100% B; T = 25 min, 100% B; T = 26 min, 65% B, and T = 30 min, 65% B (phase A, H_2O; phase B, CH_3CN). The column temperature was set to 10 °C, and UV absorbance was detected at 245 nm. HPLC-ESIMS analyses were performed on a Thermo Fisher LTQ Fleet ESIMS spectrometer (Thermo Fisher Scientific Inc.), using the same HPLC condition.

Fermentation, Isolation, Derivatization, and Structural Elucidation of AVE Intermediates, Products, or Associated Derivative Products. Solid MS medium and YEME medium (containing 12.5% sucrose) were used for sporulation and growth, respectively. Selective fermentation was carried out according a previously reported method, with slight modifications.[19] The seed medium was composed of 30 g of corn starch, 4 g of yeast extract, 2 g of soya peptone, and 10 mg of $CoCl_2·6H_2O$/L of H_2O. The fermentation medium was composed of 100 g of corn starch, 10 g of yeast extract, 0.5 g of $K_2HPO_4·3H_2O$, 0.5 g of $MgSO_4·7H_2O$, 4 g of KCl, 10 mg of $CoCl_2·6H_2O$, and 0.8 g of $CaCO_3$/L of H_2O. To produce AVEs or analogues, 1 cm^2 of the sporulated culture of *S. avermectinius* on MS medium was cut, chopped, and inoculated into 40 mL of the seed medium before incubation at 28 °C and 200 rpm for 3 days. Then, 3 mL of the seed culture broth was transferred into 60 mL of the fermentation medium, and it was incubated at 28 °C and 200 rpm for 8 days.

For VL1002, the recombinant strain was successively cultivated to accumulate approximately 100 L of the fermentation broth in total, which was then centrifuged to collect the mycelia. The mycelia were extracted with acetone (10 L × 3 times), and the combined acetone extracts were evaporated under reduced pressure to afford the crude extract (30.5 g). The crude extracts were subjected to separation by silica gel column chromatography (CC) with gradient elution of EtOAc in petroleum ether (0:100 → 100:0, v/v) to produce 35 fractions (Fr. 1−35). Separations were guided by typical UV absorption profiles, which had UV maxima at approximately 245 nm, and mass spectra for the AVE derivatives with the HPLC assay described above.

Detailed procedures for isolation, derivatization and structural elucidation of compounds **1**−**8** are found in the the Supporting Information.

Heterologous Production of MBP-MeiC in *E. coli* and *in Vitro* Biotransformation. A 990 bp DNA fragment encoding MeiC was amplified by PCR using synthesized primers (forward primer: 5′-GAC CCA AGC TTCA ATG ACC GAT CTC GTC GAC GAA GAC-3′, and reverse primer: 5′-GAC CGC TCG AGT TAG TAG CCG GCC GCC GGA C-3′) from *Streptomyces nanchangensis* genomic DNA, which contains the MEI biosynthetic gene cluster. The PCR product was cloned into the HindIII/XhoI site of pQ8, a pET28a derivative with a sequence encoding MBP tag that is inserted into the NdeI/EcoRI sites. In this vector, a His_6 tag and MBP tag were attached at the N-terminus to express *meiC* (768 residues, a molecular weight of 83.5 kDa). The recombinant plasmid, pVL1019, was introduced into the *E. coli* strain Rosetta to give the recombinant *E. coli* strain VL2001. Strain VL2001 was cultivated on Luria−Bertani (LB) agar plates containing 25 mg·L^{-1} chloromycetin and 50 mg·L^{-1} kanamycin overnight at 37 °C. A single colony was inoculated into 5 mL of LB medium containing 25 mg·L^{-1} chloromycetin and 50 mg·L^{-1} kanamycin, and incubated overnight at 37 °C with shaking at 220 rpm. A 5 mL aliquot of the preculture was transferred into 800 mL of LB broth containing 25 mg·L^{-1} chloromycetin and 50 mg·L^{-1} kanamycin, and incubated at 37 °C until the A_{600} was 0.7. After adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM, the culture was incubated at 16 °C for an additional 40 h. The cells were collected by centrifugation at 5000 rpm for 15 min and resuspended in a sonication buffer consisting of 50 mM NaH_2PO_4 (pH 7.5), 300 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 10% glycerol. The cells were sonicated for 20 min on ice, and then the cellular debris was removed by centrifugation at 14 000 rpm for 60 min at 4 °C. The supernatant was filtered through a filter unit and then used as the crude protein extract for *in vitro* enzyme assays.

In vitro MBP-MeiC-catalyzed biotransformation was conducted at 30 °C with 5 μL of **3**-containing solution (approximately 160 μM) or 5 μL of **2** and **2′**-containing solution (approximately 140 μM) in 50 mM Tris-HCl buffer (pH 7.5) by adding 95 μL of the supernatants of crude protein of MBP-MeiC, resulting in a final reaction mixture of 100 μL. Control assays were conducted using the supernatant of the *E. coli* strain, VL2001, without IPTG induction. Reactions were stopped by adding an equal volume of methanol at incubation times of 0.1, 0.5, and 1.0 h. After centrifugation, 30 μL of the reaction supernatant was subjected to HPLC and HPLC-MS analysis.

Heterologous Production of AveC in *S. lividans* and *in Vitro* Biotransformation. pVL1012, which contains *aveC* under the control of the *PermE** promoter, was transferred into *S. lividans* TK24 by conjugation, producing the corresponding recombination strain VL1007 for AveC production. VL1007 was grown in 50 mL of YEME medium at 28 °C for 3 days. The cells were collected by centrifugation, washed twice with 50 mM Tris-HCl buffer (pH 7.5) and resuspended in 5 mL of 50 mM Tris-HCl buffer (pH 7.5). After sonication for 20 min on ice, cellular debris was removed by centrifugation at 14 000 rpm for 60 min at 4 °C. The supernatant was filtered through a filter unite and then used for enzyme assays.

In vitro AveC-catalyzed biotransformation was conducted with 5 μL of **3**-containing solution (approximately 142 μM) or 5 μL of **7′**-containing solution (approximately 170 μM) in 50 mM Tris-HCl buffer (pH 7.5) by adding 95 μL of the supernatant of VL1007 to obtain a final reaction mixture of 100 μL. A control assay was performed by using the supernatant of VL1009, a *S. lividans* derivative carrying the vector pSET152 alone. Reactions were stopped by adding an equal volume of methanol at the incubation time of 2.0 h. After centrifugation, 30 μL of the reaction supernatants were subjected to HPLC and HPLC−MS analysis.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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