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Dissection of Two Acyl-Transfer Reactions Centered on Acyl-S-Carrier Protein Intermediates for Incorporating 5-Chloro-6-methyl-*O*-methylsalicyclic Acid into Chlorothricin

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Glycosylated natural products, which have played critical roles in drug discovery and development, exhibit a remarkable degree of structural diversity; this is partially due to the features of their unusual sugar building blocks. Substitutions of the hydroxyl groups with various functionalities (for example, hydrogen, acyl, amino groups, and acyloxy moieties) on the sugar units often change the chemical properties significantly to reach the full biological activities.[1] Chlorothricin (CHL), produced by *Streptomyces antibioticus*, features a tetronate-containing aglycone (chlorothricolide) that is characteristic of the spirotetronate family.[2] To furnish the structure, this aglycone is decorated by two D-olivoses, the second of which is further O-acylated at the C3′ position and equipped with a 5-chloro-6-methyl-*O*-methylsalicyclic acid (**2**) moiety (Scheme 1). A complete lack of fragment **2** led to a decrease in the antibacterial activity and stability.[3a] Recently we cloned and characterized the CHL biosynthetic gene cluster and proposed that the incorporation of **2** into CHL involves two acyl-transfer steps that depend upon the acyl-S-carrier protein (ACP) intermediates.[3] Here, we delineate this process by in vivo and in vitro studies to identify the pathway-specific enzymes, differentiate the functions of two acyltransferases, and provide insights into the enzymatic mechanisms for biosynthesizing the 5-chloro-6-methyl-*O*-methylsalicyl group of CHL.

Upon bioinformatic analysis,[3a] functional assignments to *chlB1–B6* that constitute a closely linked gene cassette within the *chl* cluster support their roles in supplying the **2** functionality of CHL (Scheme 1 A). The polyketide origin of **2**, which was previously revealed by isotope-labeled experiments,[4] was confirmed by the characterization of ChlB1 as an iterative type I polyketide synthase for 6-methylsalicyclic acid (6-MSA, **1**) biosynthesis via in situ inactivation and heterologous expression.[3] Within the cassette, the prediction that *chlB2* encodes a discrete ACP, and *chlB3* and *chlB6* each encode acyltransferases with high sequence similarity to each other (37% identity), is fully consistent with the predicted biosynthetic pathway: the resulting 6-MSA moiety could be transferred onto ChlB2, which serves as a platform for chlorination and O-methylation (catalyzed by ChlB4 and ChlB5, respectively) before the mature 5-chloro-6-methyl-*O*-methylsalicyl group is incorporated into CHL (Scheme 1 B). To test this hypothesis, the inactivation of *chlB3* was carried out. As expected, the *ΔchlB3* mutant TL1020 shares the same phenotype with the *δchlB6* mutant TL1008 that was previously made;[3a] it produced desmethylsalicyl CHL (DM-CHL), which completely lacked the 5-chloro-6-methyl-*O*-methylsalicyl moiety (Figure 1). To determine the relative timing of these enzyme-catalyzed steps, however, the specific actions of ChlB3 and ChlB6 need to be functionally differentiated.

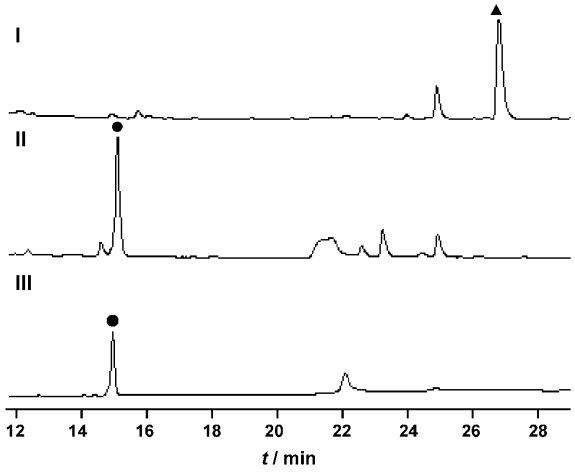

Figure 1. HPLC analysis of the CHL or DM-CHL production in *S. antibioticus* wild-type strain (I), TL1008 (II), and TL1020 (III). ●: DM-CHL, ▲: CHL.

To characterize these enzymes, ChlB2, ChlB3, and ChlB6 were first expressed in *E. coli* BL21(DE3). After the optimization of temperature, inducer concentrations, and induction times, ChlB2 and ChlB3 were expressed in soluble forms and were purified to homogeneity. Incidentally, ChlB6 had to be expressed in another system that included *Pseudomonas putida* KT2440 because it is insoluble in *E. coli*. Finally, the purified His-tagged ChlB2, ChlB3, and ChlB6 were subjected to assays of the acyltransferase activities in vitro. Conversion of ChlB2 from the apo-form to the holo-form was carried out in the presence of the phosphopantetheinyl acyltransferase Sfp and coenzyme A (CoA);[5] this provided the active acceptor for assaying the first acyl-transfer reaction by judging the increase of molecular weight via MALDI-TOF mass spectrometry (MS) analysis (Table 1) and the shift in high-performance liquid chromatography (HPLC) mobility. Initial attempts to use the free acid

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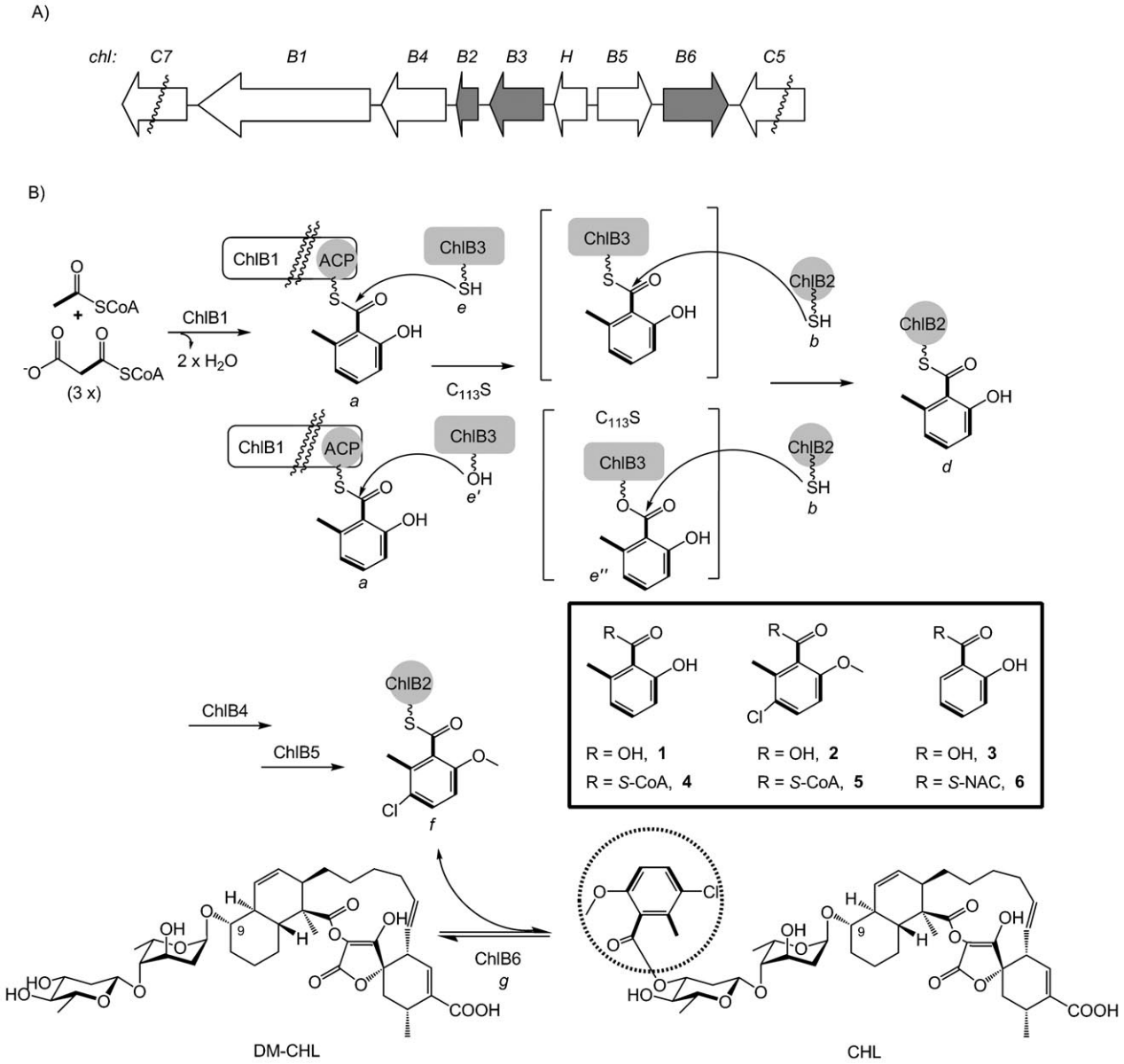

Scheme 1. A) Gene cassette and B) biosynthetic pathway for incorporating 5-chloro-6-methyl-*O*-methylsalicyl group into CHL.

Table 1. MALDI-TOF MS analysis of apo-, holo-, and acyl-*S*-ACPs.

ACPs	ChlB1-ACP $[M+H]^+$		ChlB2 $[M+H]^+$	
	calcd	found	calcd	found
apo-ACP	11655	11655	12351	12349
holo-ACP	11995	11992	12691	12687
6-methylsalicyl-*S*-ACP	12130	12128	12825	12821
5-chloro-6-methyl-*O*-methylsalicyl-*S*-ACP	12178	12177	12874	12873

1 as the substrate failed to detect any activity of ChlB3 and ChlB6; this suggests that the 6-MSA moiety that results from ChlB1 could be directly transferred onto ChlB2, instead of the hydrolysis by water to form **1** by heterologous expression of ChlB1 alone.[3b] Consistent with this result, the finding of detectable salicyl-*S*-ChlB2 in the presence of either ChlB3 or ChlB6 by using the salicyl-*S*-*N*-acetylcysteamine (*S*-NAC) **6** as the surrogate substrate supported their acyl-transfer activities, however, it fell short of distinguishing their substrate specificities; this is probably due to the ignored protein–protein interaction that can occur by using the phosphopantetheinyl arm mimic. To solve this problem, the ChlB1 ACP domain was expressed in *E. coli*, purified, and converted to 6-methylsalicyl-*S*-ChlB1-ACP (***a***) as the acyl group donor by Sfp from synthetic 6-methylsalicyl-*S*-CoA (**4**; Scheme 1B). Indeed, 6-methylsalicyl-*S*-ChlB2 (***d***) was efficiently produced in the presence of ChlB3 (k_{cat} of 1.2 s^{-1} and K_m of 43 μM for 6-methylsalicyl-*S*-ChlB1-ACP), but no reaction occurred if ChlB3 was replaced by ChlB6 (Figure 2A). This result clearly confirmed that ChlB3 functions as the first transferase that is specifically responsible for shuttling of the 6-methylsalicyl group from ChlB1 to ChlB2.

To afford CHL, the ChlB2-tethered acyl group, which may be subsequently chlorinated and O-methylated for the maturation, needs to be appended onto DM-CHL by a regiospecific transferase. To verify this reaction, **2** and its CoA derivative **5** were synthesized, and the 5-chloro-6-methyl-*O*-methylsalicyl group was loaded onto ChlB2 by Sfp, yielding 5-chloro-6-methyl-*O*-methylsalicyl-*S*-ChlB2 (***f***) as the active donor for detecting the second acyl-transfer activity. As shown in Figures 3A and 3B, ChlB6 catalyzed the production of CHL from DM-CHL accordingly, with the deacylation of ***f*** to holo-ChlB2 occurring in a time-dependent manner. Next, the functional specificity of ChlB6 was further confirmed by performing the reverse reaction (Figure 3C and D). By using CHL as the substrate and holo-ChlB2 as the acyl acceptor, DM-CHL was produced accordingly with the appearance of ***f***, but the deacylation of CHL was completely blocked by omitting holo-ChlB2 from the reaction buffer. In contrast, ChlB3 did not catalyze either this forward or reverse reaction under the same conditions. Unambiguously, these findings confirmed ChlB6 as the second transferases for regioselectively appending the 5-chloro-6-methyl-*O*-methylsalicyclic acid (**2**) moiety from ChlB2 onto the end D-olivose unit of DM-CHL to form CHL.

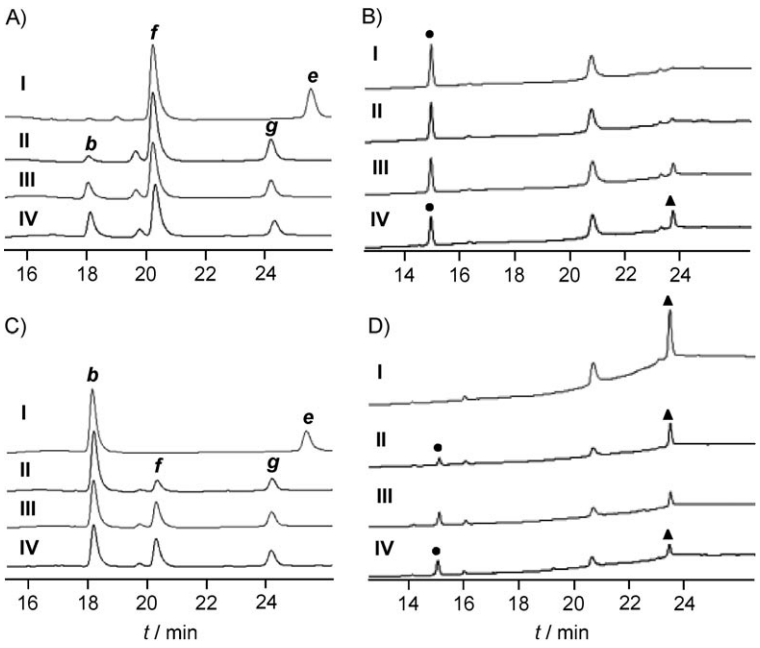

Figure 3. HPLC analysis of 5-chloro-6-methyl-*O*-methylsalicyl shuttling between ChlB2 and CHL in the presence of ChlB3 for 7–8 h (I), and ChlB6 for 1 h (II), 4 h (III) and 7–8 h (IV); A) protein detection and B) chemical detection. The forward attachment of this moiety onto DM-CHL (●) from 5-chloro-6-methyl-O-methylsalicyl-*S*-ChlB2 (*f*) and generation of CHL (▲) and holo-ChlB2 (*b*) is shown; C) protein detection, and D) chemical detection. The reverse deacylation of this moiety from CHL and generation of DM-CHL and 5-chloro-6-methyl-O-methylsalicyl-S-ChlB2 are shown. ChlB6 and ChlB3 are indicated by *g* and *e*, respectively.

Secondary structural prediction suggested that ChlB3 and ChlB6 resemble a family of α,β-hydrolases that contain a key Cys or Ser residue to covalently channel the acyl group shuttling in various reactions (Figure 4A).[6] A few members have been characterized to be the transferases that utilize ACP proteins as the acyl donors or acceptors in nature similar to ChlB3 and ChlB6, including CmaE and SyrC for the aminoacyl group transfer in the biosynthesis of coronamic acid and syringomycin,[7] respectively, and CloN7/CouN7 for the pyrrolylcarbonyl moiety transfer in the biosynthesis of aminocoumarin antibiotics (Figure 4B).[8] Sequence alignment revealed a highly conserved Cys residue in both ChlB3 and ChlB6; this suggests that they fall into the C-type group of this family. To validate its critical role during the acyl transfer process, this residue was replaced by Ala and Ser. Whereas the C113A mutant of ChlB3, and C113A and C113S mutants of ChlB6 were completely inactive, the C113S mutant of ChlB3 exhibited a significantly decreased but detectable acyl-transfer activity (Figure 2B). Remarkably, an apparent intermediate appeared accordingly with the decrease of the substrate ***a*** at the early reaction stage and increase of the product ***d*** at the latter stage. The HPLC fraction containing this intermediate was concentrated and subjected to MALDI-TOF MS analysis, which allowed us to deduce it to be 6-methylsalicyl-*O*-ChlB3 (C113S, ***e***′′, $[M+H]^+$ 38,341±6 found; and 38,310 calculated) by considering the increase of molecular weight from ChlB3 (C113S, ***e***′, $[M+H]^+$ 38,212±6 found; and 38,176 calculated). The difference, 129, was thought to represent the molecular weight of 6-methylsalicyl (134). This result strongly supported the supposition that ChlB3 uses the thiolate side chain as a nucleophile to channel the acyl transfer from ChlB1-ACP to ChlB2 via a two-step process: ChlB3 takes over the resultant 6-methylsalicycloyl group from ChlB1 and then transfers it to

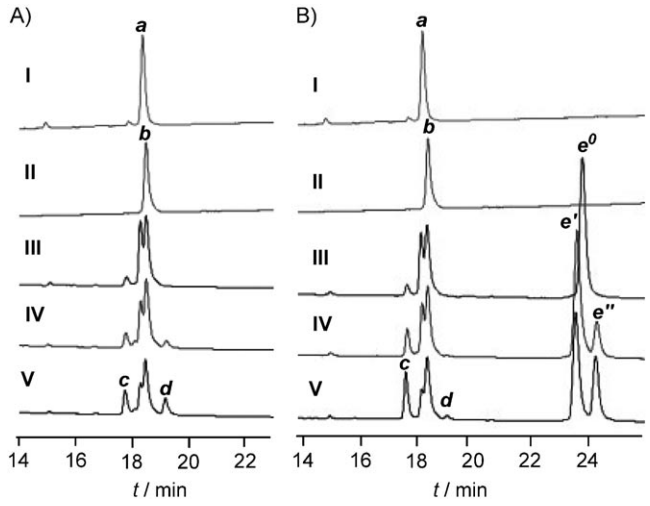

Figure 2. HPLC analysis of the 6-methylsalicyl transfer from ChlB1-ACP to ChlB2. A) conversions of apo-ChlB1-ACP to 6-methylsalicyl-*S*-ChlB1-ACP (*a*; I); apo-ChlB2 to holo-ChlB2 (*b*; II); and 6-methylsalicyl-*S*-ChlB1-ACP to 6-methylsalicyl-*S*-ChlB2 (*d*) and holo-ChlB1-ACP (*c*) in the presence of ChlB6 for 30 min (III), and ChlB3 for 5 min (IV) and 30 min (V), respectively; B) with 6-methylsalicyl-*S*-ChlB1-ACP (I) and holo-ChlB2 (II) as the controls, assays of the 6-methylsalicyl transfer in the presence of the C113A mutant ChlB3 for 1 h (III), and the C113S mutant ChlB3 for 20 min (IV) and 1 h (V), respectively. ChlB3 (C113A), ChlB3 (C113S) and 6-methylsalicyl-*O*-ChlB3 (C113S) are indicated by e^0, *e*′, and *e*′′, respectively.

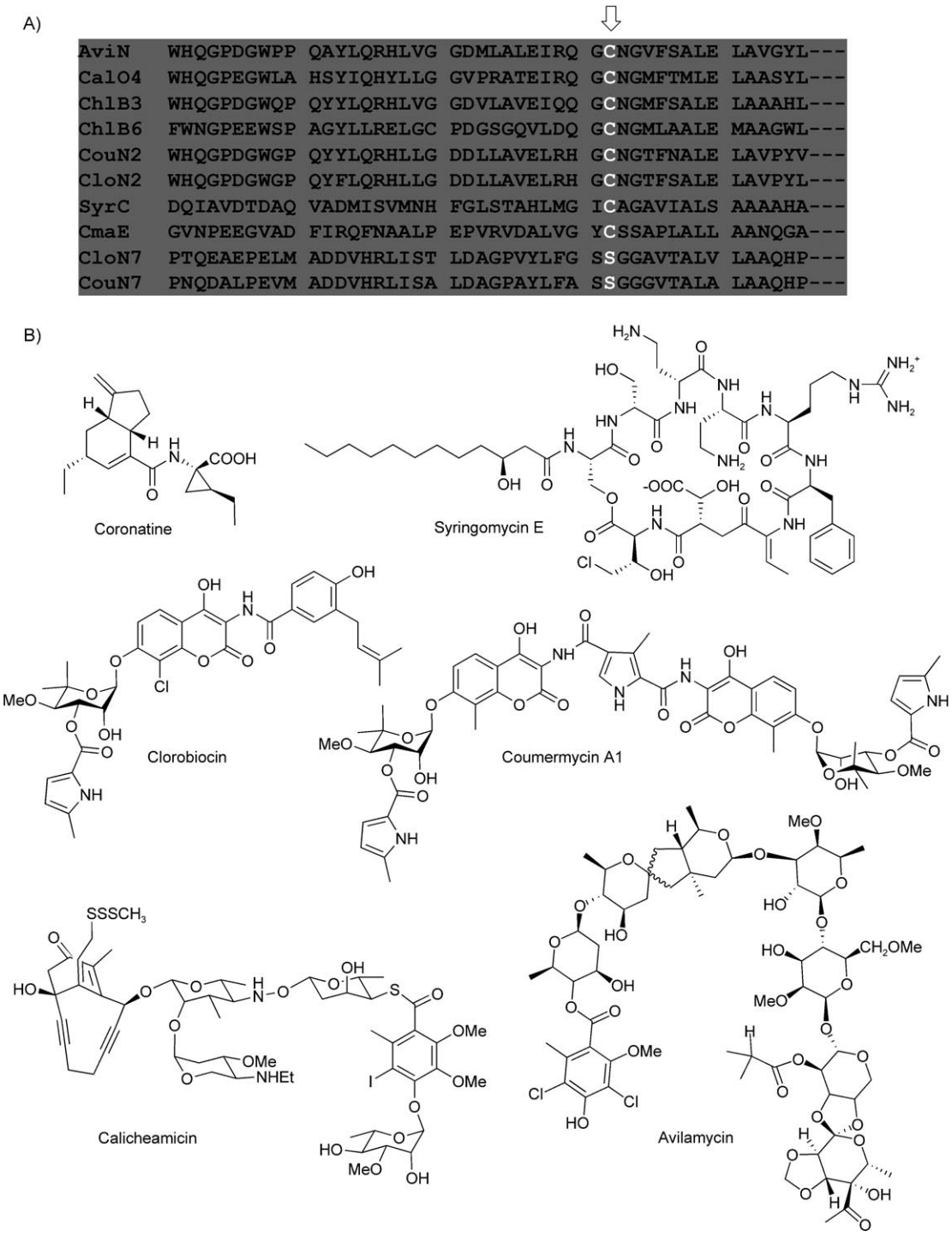

Figure 4. Sequence alignment of selected acyltransferases that belong to the α,β-hydrolase family (A) and relative natural products (B). Arrows indicate the conserved Cys or Ser residue.

ChlB2. The SH functionality can be substituted by an OH group of Ser in a similar nucleophilic manner, although this change dramatically slowed down the second acyl-transfer step from ChlB3 to ChlB2 and made the ChlB3-associated intermediate detectable.

In conclusion, characterization of ChlB1-ACP, ChlB2, ChlB3, and ChlB6 in this study established an ACP-centered strategy that involve two distinct acyl-transfer steps for generation and regioselective attachment of the aromatic moiety **2** onto the sugar unit to furnish CHL. A similar route for sugar acylations could be adopted in other glycosylated natural product biosynthetic machineries, including the carrier protein CloN1 (or CouN1)-associated activities of the acyltransferase pair CloN2/CloN7 (or CouN2/CouN7) for appending the pyrrolylcarbonyl

group to form clorobiocin (or coumermycin, Figure 4B, so far the activities of CloN2 and CouN2 still remain to be proved in vitro).[9] Whereas the covalent tethering of an amino acid onto a discrete carrier protein has been widely speculated as a means of changing the functionalities of the amino acid prior to incorporation,[10] the finding that ChlB2 mediates the aromatic polyketide moiety transfer in the CHL biosynthesis suggests that this strategy could be more common in secondary metabolism: it might be advantageous in optimizing molecular recognition of downstream enzymes (i.e., the halogenase ChlB4 and methyltransferase ChlB5) to the ChlB2-associated intermediate by providing structural features from the ACP protein. Interestingly, the biosynthetic gene clusters of avilamycin (AVI) and calicheamicin (CAL; Figure 4B),[11] both of which contain a sugar-attached, highly modified aromatic moiety of polyketide origin, lack the gene that encodes a discrete ChlB2-like ACP protein. Consistently, only one acyltransferase that exhibits high sequence similarity to ChlB3 and ChlB6 has been found in each pathway (AviN with 49% identity to ChlB3 and 32% identity to ChlB6, and CalO4 with 53% identity to ChlB3 and 37% identity to ChlB6, respectively); this indicates the alterative possibility that the orsellinic acid moiety resulting from the iterative type I PKS (AviM or CalO5) could be directly transferred onto the small molecule, and modifications on this moiety might take place after the sugar acylation. Our experiments differentiate two acyltransferases, confirm the covalent acyltransferase intermediate for acyl group shuttling, set the stage for deciphering the remaining modification steps (chlorination and *O*-methylation), and will eventually contribute to generate new analogues of CHL via combinatorial biosynthesis and chemoenzymatic methods.

Experimental Section

Gene inactivation in *S. antibioticus*: To inactivate *chlB3*, a 2.2 kb XbaI/AatII fragment amplified by PCR by using the following primers 5′-ATA TCT AGA CCC GCG GCG CAA TAC CCG-3′ (the XbaI site is underlined) and 5′-TAT GAC GTC CGT CAC GCT CTC CTG CGC-3′ (AatII site is underlined), and a 1.9 kb KpnI/HindIII fragment amplified by PCR by using the following primers 5′-ATA GGT ACC GCC CTG GTG CCA GGA GTC-3′ (KpnI site is underlined) and 5′-TAT AAG CTT GGA CCA GCA GGT CGA TCC-3′ (HindIII site is underlined) were co-ligated with the 1.5 kb AatII/KpnI fragment that contains the apramycin resistance gene, *aac(3)IV*. The resultant 5.6 kb XbaI/HindIII fragment was cloned into the same site of vector pTL1001 to yield the recombinant construct pTL1050, in which a 732 bp internal fragment of *chlB3* was replaced by *aac(3)IV*.

To inactivate *chlB2*, a 2.0 kb XbaI/AatII fragment was amplified by PCR by using the following primers 5′-ATA TCT AGA CCC GCG GCG CAA TAC CCG-3′(XbaI is site underlined) and 5′-TAT GAC GTC CGG CGA TCC TGA TGA ACT AC-3′ (AatII site is underlined) and a 1.9 kb KpnI/HindIII fragment was amplified by PCR by using the following primers 5′-ATA GGT ACC CCT GAA TGA ACG GGA TGA G-3′ (KpnI site is underlined) and 5′-TAT AAG CTT ACG GCC GCC GCG AGG TAG-3′ (HindIII site is underlined), were co-ligated with the 1.5 kb AatII/KpnI fragment that contains *aac(3)IV*. The resultant 5.4 kb XbaI/HindIII fragment was cloned into the same site of vector pTL1001 to yield the recombinant construct pTL1051, in which a 85 bp internal fragment of *chlB2* was replaced by *aac(3)IV*

Introduction of each recombinant construct into *S. antibioticus* DSM 40725 was carried out by *E. coli–Streptomyces* conjugation by following the procedure described previously.[1] For gene replacement, colonies that were apramycin resistant and thiostrepton sensitive at 37 °C were identified as mutants. The genotype of each mutant strain was confirmed by PCR amplification with the wild-type strain as the control (Figures S1 and S2). Production, isolation, and HPLC analysis of CHL and DM-CHL in *S. antibioticus* were carried out according to the methods described previously.[3]

Protein expression, purification and mutation: By using the cosmid pTL1502 as the template,[3] the DNA fragments encoding individual target proteins were amplified by PCR with PfuUltra™ High-Fidelity DNA Polymerase (Stratagene). The identity of each PCR product was confirmed by sequencing. To express ChlB1-ACP, a 0.3 kb PCR product was obtained by using the primers 5′-TA GAA TTC CAT ATG GCC CCC GAC GAA CTG C-3′ (EcoRI/NdeI site underlined) and 5′-TA TAT AAG CTT TCA GGC CGT TGC CGC CGG-3′ (HindIII site underlined) and then cloned into pSP72 to yield pTL1055. The 0.3 kb NdeI/HindIII fragment was recovered from pTL1055 and ligated into the same site of pSJ8 to make the recombinant construct pTL1056. To express ChlB2, a 0.3 kb PCR product was obtained by using the primers 5′-AT GAA TTC CAT ATG ACA GCA GAA GAA TAC GC-3′ (EcoRI/NdeI site underlined) and 5′-TA TAT AAG CTT CTC GAG GCC GGC GTT CGA GGC G-3′ (XhoI/HindIII site underlined) and then cloned into pSP72 to yield pTL1057. The 0.3 kb NdeI/HindIII fragment was recovered from pTL1057 and ligated into the same site of pET28a to make the recombinant construct pTL1058. To express ChlB3, a 1.0 kb PCR product was obtained by using the primers 5′-AT CTG CAG CAT ATG CGG ACG CCC GAC ATA TTC-3′ (PstI/NdeI site underlined) and 5′-TA TAT AAG CTT CTC GAG GTC GCT CCA GGG AGC C-3′ (XhoI/HindIII site underlined) and then cloned into pSP72, yielding pTL1059. The 1.0 kb NdeI/XhoI fragment was recovered from pTL1059 and ligated into the same site of pET37b, making the recombinant construct pTL1060. To express ChlB6, a 1.1 kb PCR product was obtained by using the primers 5′-AT GAA TTC CAT ATG AAG GTC AGC GGC ATC CAC-3′ (EcoRI/NdeI site underlined) and 5′-TA TAT AAG CTT CTC GAG CGT GTC CCG CTG ATA C-3′ (XhoI/HindIII site underlined) and then cloned into pSP72, yielding pTL1061. The 1.1 kb NdeI/XhoI fragment was recovered from pTL1061 and ligated into the same site of pET37b to yield the recombinant construct pTL1062. To make the construct for expressing ChlB6 in *P. putida* KT2440, The 1.1 kb XbaI/*Avr*II fragment from pTL1062 was cloned into pVLT33 to yield the recombinant construct pTL1063. Whereas the constructs pTL1056, pTL1058, and pTL1060 were introduced respectively into *E. coli* BL21 (DE3) for overproduction of the proteins ChlB1-ACP, ChlB2, and ChlB3, introduction of the construct pTL1063 into *P. putida* KT2440 was carried out by *E. coli–Pseudomonas* conjugation for producing ChlB6. Cells had grown in the LB medium supplemented with 50–100 $\mu g\,mL^{-1}$ of kanamycin at 30–37 °C and 250 rpm until the cell density reached 0.5 at $OD_{600\,nm}$. To induce protein expression, isopropyl-β-D-thiogalactopyranoside (IPTG, 0.01–0.1 mM) was added to the cultures, which were further incubated at 30 °C for 8–24 h. The soluble fractions of overproduced proteins were loaded onto a Ni-NTA resin (Qiagen, Valencia, CA) column for affinity purification. Each purified protein was dialyzed, concentrated with an Amicon Ultra-15 filter, and then stored at −80 °C for in vitro assays. For purification of ChlB1-ACP, TEV protease was added into the dialysis buffer for cleavage of the His-tagged fusion protein MBP, followed by further purification with Ni-NTA affinity chromatography and gel fitration of Superdex 75 10/300 (Amersharm). The resultant ChlB1-ACP protein was then concentrated and stored at −80 °C for in vitro assays.

To mutate ChlB3 and ChlB6, each single amino acid residue exchange was performed by site-specific mutagenesis by using the QuickChange Muti-Site Directed Mutagenesis Kit (Stratagene) according to the manufacture's introductions. With pTL1059 or pTL1061 as the template, the following sets of primers were used for introducing the mutations into *chlB3* or *chlB6*: 5′-GAG ATC CAG CAG GGC GCC AAT GGC ATG TTC AGC-3′ and 5′-GCT GAA CAT GCC ATT GGC GCC CTG CTG GAT CTC-3′ (cordon encoding Ala underlined) for the C113A mutation of ChlB3; 5′-GAG ATC CAG CAG GGC AGC AAT GGC ATG TTC AGC-3′ and 5′-GCT GAA CAT GCC ATT GCT GCC CTG CTG GAT CTC-3′ (cordon encoding Ser underlined) for the C113S mutation of ChlB3; 5′-GTG CTC GAC CAG GGC GCC AAC GGC ATG CTG GCC-3′ and 5′-GGC CAG CAT GCC GTT GGC GCC CTG GTC GAG CAC-3′ (cordon encoding Ala underlined) for the C113A mutation of ChlB6; and 5′-GTG CTC GAC CAG GGC AGC AAC GGC ATG CTG GCC-3′ and 5′-GGC CAG CAT GCC GTT GCT GCC CTG GTC GAG CAC-3′ (cordon encoding Ser underlined) for the C113S mutation of ChlB6. Consequently, the mutated versions of *chlB3* and *chlB6* were confirmed by sequencing and then cloned into pET37a or pVLT33 by using same strategies to make pTL1060 and pTL1063 for native ChlB3 and ChlB6 expression, respectively, yielding pTL1064 for expressing C113A mutant ChlB3, pTL1065 for expressing C113S mutant ChlB3, pTL1066 for expressing C113A mutant ChlB6, or pTL1067 for expressing C113S mutant ChlB6. Protein expression and purification were carried out according to the procedures described above.

Assays of the acyl transfer activities

Transfer of the 6-methylsalicycloyl moiety from ChlB1-ACP to ChlB2: To generate holo-ChlB2, the reaction was carried out in 75 mM MOPS buffer (200 μL; pH 7.5) that contained 10 mM $MgCl_2$, 1 mM Tris-(2-carboxylethyl)-phosphine (TCEP), 450 μM CoA, 5 μM Sfp, and 300 μM apo-ChlB2 at 30 °C for 1 h. To generate 6-methylsalicyl-*S*-ChlB1-ACP, a similar reaction was performed in 75 mM MOPS buffer (100 μL; pH 7.5) with the exception in the following concentrations and components: 150 μM 6-methylsalicyl-*S*-CoA, 2 μM Sfp, and 100 μM apo-ChlB1-ACP. For transferring the 6-MSA moiety, the reaction mixture containing 30 μM 6-methylsalicyl-*S*-ChlB1-ACP and 30 μM holo-ChlB2 was incubated at 30 °C for 1 min, 5 min, 10 min, or 30 min, in the presence of 0.2 μM ChlB3, ChlB6, or mutated protein. To quench the reaction, 10% formic acid (0.25 volumes) was added. Each sample was analyzed by HPLC on a Vydac 218TP54 C18 reversed-phase HPLC column (250×4.6 mm, 5 μm, 300 Å) with the following gradient at room temperature: 0–3 min, 20% B; 3–5 min, 20–35% B; 5–25 min, 35%-55% B; 25–26 min, 55–99% B; 26–29 min, 99% B; and 29–30 min, 99–20% B (buffer A, 0.1% TFA in H_2O; and buffer B, 0.1% TFA in CH_3CN). This was performed at a flow rate of 1 mL min^{-1} with UV detection at 220 nm.

Transfer of the 5-chloro-6-methyl-*O*-methylsalicycloyl group from ChlB2 to DM-CHL: To generate 5-chloro-6-methyl-*O*-methylsalicyl-*S*-ChlB2, the reaction was carried out in 75 mM MOPS buffer (200 μL; pH 7.5) that contained 10 mM $MgCl_2$, 1 mM TCEP, 450 μM 5-chloro-6-methyl-*O*-methylsalicyl-*S*-CoA, 5 μM Sfp, and 300 μM apo-ChlB2 at 30 °C for 1 h. To transfer the 5-chloro-6-methyl-*O*-methylsalicyl group, ChlB3, ChlB6, or mutated protein was added (with the final concentration at 20 μM for the native proteins or 20 μM for the mutant proteins), and the combined reaction mixture was further incubated at 30 °C for 10 min, 30 min, 1 h, 4 h, or 8 h. For protein detection, each 10% formic acid (0.25 volume; 50 μL)-quenched sample was analyzed by HPLC under the same condition described above. For chemical detection, each methanol (2 volumes; 400 μL) quenched sample was analyzed by HPLC on a COSMOSIL 3C18-AR-II reversed-phase HPLC column (250×4.6 mm, 5 μm) with the following gradient at room temperature: 0–5 min, 40% B; 5–25 min, 40%-85% B; and 25–30 min, 80%-40% B (buffer A, 0.1% TFA in H_2O; and buffer B, 0.1% TFA in CH_3CN). This was performed at a flow rate of 1 mL min^{-1} with UV detection at 222 nm.

Deacylation of CHL: Each reaction was carried out in MOPS buffer (75 mM, 100 μL; pH 7.5) that contained $MgCl_2$ (10 mM), TCEP (1 mM, pH 8.0), BSA (1 mM), 5% DMSO, holo-ChlB2 (150 μM) and CHL (150 μM) at 30 °C for 10 min, 30 min, 1 h, 4 h, or 8 h, in the presence of ChlB3 or ChlB6 (20 μM). To quench the reaction, 10% formic acid (0.25 volumes; 25 μL) or methanol (2 volumes; 200 μL) was added. Protein or chemical detection was described above.

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Keywords: acylation · acyltransferase · biosynthesis · chlorothricin · salicyclic acids

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