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Insights into the Functionalization of the Methylsalicyclic Moiety during the Biosynthesis of Chlorothricin by Comparative Kinetic Assays of the Activities of Two KAS III-like Acyltransferases

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Summary of main observation and conclusion Chlorothricin (**CHL**), an archetypal member of the family of spirotetronate antibiotics, possesses a tetronate-containing pentacyclic aglycone that is conjugated with a modified methylsalicyclic acid (**MSA**) moiety through a disaccharide linkage. MSA is a polyketide product assembled by the iterative type I polyketide synthase ChlB1. Incorporation of this pharmaceutically important moiety into **CHL** relies on the activities of two distinct β-Ketoacyl-ACP synthase III (KAS III)-like acyltransferases, ChlB3 and ChlB6, which function together to coordinate the transfer of **MSA** through ChlB2, a discrete acyl carrier protein (ACP). During the maturation of **CHL**, **MSA** needs to be further functionalized by C2-*O*-methylation and C5-chlorination; however, timing of this functionalization process remains poorly understood. In this study, we report comparative kinetic assays of the activities of the two KAS III-like acyltransferases ChlB3 and ChlB6 using substrates that vary in substitution extent and ACP carrier. ChlB3 prefers to transfer the immediately assembled 6-methyl-MSA moiety from ChlB1-ACP to the discrete ACP ChlB2, from which this moiety is preferred to be transferred directly onto the molecule desmethylsalicyl-CHL prior to C2-*O*-methylation and C5-chlorination. Consequently, MSA functionalization appears to occur at the molecule level rather than at the covalently tethered protein level, i.e., ChlB1-ACP or ChlB2. Both ChlB3 and ChlB6 are flexible in substrate tolerance, holding promise for **CHL** engineering-based structural diversity by using variable **MSA** moiety.

Background and Originality Content

During the biosynthesis of bioactive molecules, different starter units and modified units immensely contribute to the structural diversity of those bioactive molecules.[1] Chlorothricin （**CHL**, Scheme 1）, produced by *Streptomyces antibioticus* DSM 40725, belongs to a growing family of spirotetronate antibiotics with broad biological activities by inhibiting pyruvate carboxylase and malate dehydrogenase.[2] **CHL** comprises the tetronate-containing aglycone and two D-Olivose saccharide chains with the 3'-hydroxyl position of the secondary sugar decorated by a 5-chloro-6-methyl-O-methylsalicyclic acid moiety (**1**, Scheme 1). 5-chloro-6-methyl-O-methylsalicyclic acid (**1,** Scheme 1) as a modified unit is crucial to the antibacterial activity and stability of **CHL.**[3] After the biosynthetic gene cluster of **CHL** was cloned and characterized, the gene clusters of a series of compounds with spirotetramate or spirotetronate structure(pyrroindomycins A and B,[4] Abyssomicin C,[5] Quartromicin D,[6] Tetrocarcin A,[7] Kijanimicin,[8] Lobophorins[9]) have been discovered subsequently, leading to discover some novel natural enzymes catalyzing unique reactions.[10]

According to the functional analysis of the gene cluster of **CHL**, ChlB1-ChlB6 were assigned to be responsible for the synthesis of **1**. The iterative type I polyketide synthases (iPKSs) have been showed to synthesize 6-methylsalicyl from one acetyl CoA and three malonyl CoA and install it onto the iterative PKS(iPKS)-ACP domain,[3a, 3c, 11] but 6-methylsalicyl produced by ChlB1 didn't release from pantetheinyl group for the absence of thioesterase (TE) ,[12] Claisen cyclase thioesterase (CLC-TE),[13] or thioester reduction(TR) domain.[14] Conversely two KAS III-like acyltransferases (ChlB3 and ChlB6) centered on the acyl-*S*-carrier protein (ChlB2) intermediates have been verified to be involved in the biosynthesis of **1.**[15]

Our previous biochemical characterization *in vitro* has elucidated that ChlB3 could transfer 6-methylsalicyl group anchored on the ChlB1-ACP to holo-ChlB2, a discrete ACP, resulting 6-methylsalicyl-*S*-ChlB2, and ChlB6 could transfer 5-chloro-6-methylsalicyl loaded on ChlB2 to desmethylsalicyl-**CHL**(**DM-CHL**), producing the final product of **CHL.**[15] The only remaining question was the elusive reaction timing of ChlB4 and ChlB5, which were assumed to be responsible for the C5-chlorination and C2-O-methylation of **1** by bioinformatics analysis. The gene knockout of *chlB4* and *chlB5* led to producing deschloro-chlorothricin (**DCM-CHL**) and **DM-CHL**, respectively, which couldn't provide any clues to the reaction timing of ChlB4 and ChlB5. Theoretically, these two different modifications may occur on 6-methylsalicyl moiety still loaded on the acyl carrier protein of ChlB1-ACP and ChlB2, or ChlB4 and ChlB5 might directly modify 6-methylsalicyl-chlorothricin (**M-CHL**) (Scheme 1).

Which pathway really exists in nature? Due to the failure to get soluble ChlB4 (for chlorination) and ChlB5 (for O-methylation), we will attempt to figure out this puzzle by the kinetic constants of ChlB3 and ChlB6 for different substrates in this report.

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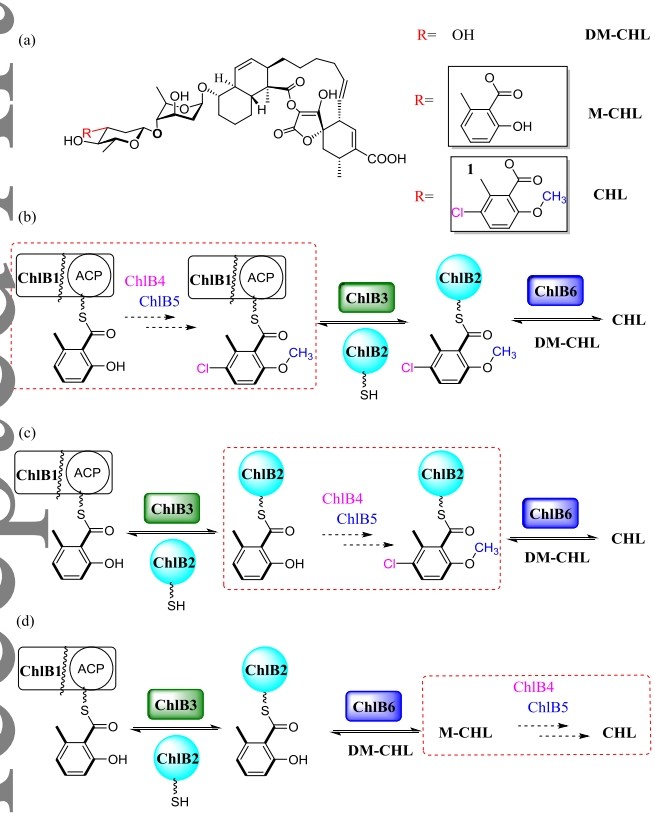
Results and Discussion

Depending on the previous hypothesis, methylation and chlorination could happen on the aromatic acyl group loaded on ChlB1-ACP, ChlB2 or DM-CHL. To detect the kinetic parameters of ChlB3 for different small molecules loaded on ChlB1-ACP, 6-methylsalicyl-S-ChlB1-ACP, 6-methyl-*O*-methylsalicyl-S-ChlB1-ACP and 5-chloro-6-methyl-*O*-methylsalicyl-S-ChlB1-ACP were generated enzymatically by the sfp phosphopantetheinyl transferase (PPTase).[16] Similar to 6-methylsalicyl group, both 5-chloro-6-methyl-*O*-methylsalicyl and 6-methyl-*O*-methylsalicyl groups could be transferred to holo-ChlB2 by ChlB3, and neither of them could be transferred by ChlB6 (Figure 1a, 1b), further confirming the function of ChlB3 and ChlB6. Another two substrates, *O*-methylsalicyl-S-ChlB1-ACP and salicyl-S-ChlB1-ACP which should not exit in *Streptomyces antibioticus* DSM 40725, could also be catalyzed by ChlB3 as we expected (Figure 1c, 1d), indicating the excellent substrate tolerance of ChlB3. All the new modified proteins had an identical retention time to default substrates confirmed by MALDI-TOF-MS (Table S1).



Scheme 1 Possible reaction order of 5-chloro-6-methyl-*O*-methylsalicyclic Acid in Chlorothricin biosynthesis

As mentioned above, many different substrates could be catalyzed by ChlB3 *in vitro*. In fact, only one substrate was preferred or really utilized by the acyltransferase *in vivo*. Our previous data have showed ChlB3 catalyzed the conversion of 6-methylsalicyl-S-ChlB1-ACP to 6-methylsalicyl-S-ChlB2 in a time-dependent reaction with a specific activity [15] of 7.33min^{-1}. With the same experimental conditions, ChlB3 catalyzed the transfer of 6-methyl-*O*-methylsalicyl and 5-chloro-6-methyl-*O*-methylsalicyl groups with the specific activities of 0.006 min^{-1} and 0.008 min^{-1} respectively (Figure 1a, 1b). The obvious difference in specific activities for different substrates proved that 6-methylsalicyl-S-ChlB1-ACP was the optimum substrate of ChlB3. To obtain the accurate enzymatic information of ChlB3, the kinetic parameters of ChlB3 for 6-methylsalicyl-S-ChlB1-ACP and 5-chloro-6-methyl-*O*-methylsalicyl-ChlB1-ACP were subsequently determined (Figure 2 and Table S2). The *K*m values for 6-methylsalicyl-S-ChlB1-ACP and 5-chloro-6-methyl-*O*-methylsalicyl-ChlB1-ACP with fixed concentration of holo-ChlB2 (at 150μM holo-ChlB2) were 0.043 mM and 1.0 mM, and the *k*cat values are about 72.7 min^{-1} and 0.045 min^{-1} respectively. Notably, when the catalytic efficiencies (*k*cat/*K*m) are compared, 6-methylsalicyl-S-ChlB1-ACP as substrate was superior to 5-chloro-6-methyl-O-methylsalicyl-S-Ch1B1-ACP by more than 3,000 times. The kinetical data showed that 6-methylsalicyl was immediately transferred to ChlB2 after synthesis by ChlB1, excluding the possibility that ChlB4 and ChlB5 modified 6-methylsalicyl immediately when it was still loaded on the Ch1B1-ACP (Table S2).

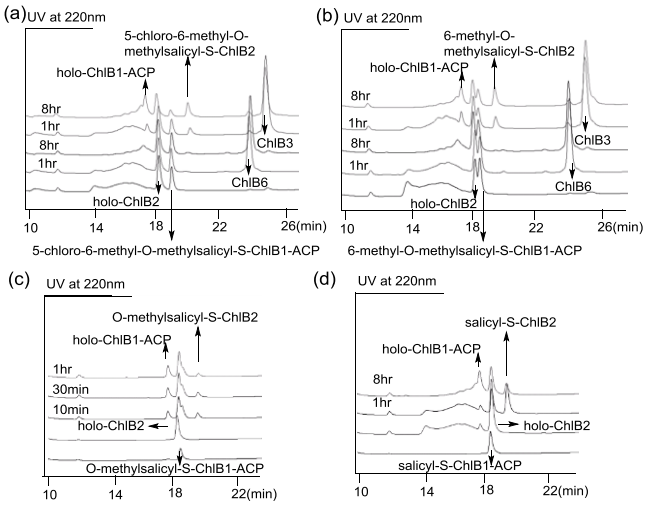


Figure 1 HPLC analysis of acyltransferase activities of ChlB3 and ChlB6 from ChlB1-ACP to holo-ChlB2. a) 5-chloro-6-methyl-*O*-methylsalicyl transfer by ChlB3 and ChlB6 for 1hr and 8hr. b) 6-methyl-*O*-methylsalicyl transfer by ChlB3 and ChlB6 for 1hr and 8hr. c) *O*-methylsalicyl transfer by ChlB3 for 10min, 30min and 1hr. D) Salicyl transfer by ChlB3 for 1hr and 8hr.

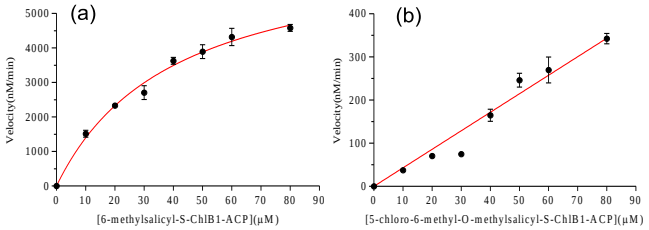


Figure 2 Michaelis-Menten curves of ChlB3 for （a）6-methylsalicyl-S-ChlB1-ACP and （b）5-chloro-6-methyl-*O*-methylsalicyl-S-ChlB1-ACP

Subsequently, we sought to explore the substrate spectrum of ChlB6, which could synthesize CHL by transferring the cargo of 5-chloro-6-methyl-*O*-methylsalicyl on ChlB2 to DM-CHL. Another four substrates, 6-methyl-*O*-methylsalicyl-S-ChlB2, 6-methylsalicyl -S-ChlB2, *O*-methylsalicyl-S-ChlB2 and salicyl-S-ChlB2 were generated enzymatically by the sfp phosphopantetheinyl transferase (PPTase). Interestingly, ChlB6 could transfer each of these acyl groups to **DM-CHL** to produce Deschloro-chlorothricin (**DCM-CHL**), 6-methylsalicyl-chlorothricin (**M-CHL**), O-methylsalicyl -chlorothricin (**MS-CHL**) and salicyl-chlorothricin (**S-CHL**) (Figure 3a-d), indicating that ChlB6 also has excellent substrate tolerance *in vitro*. All the new compounds were confirmed by LC-MS (Table S3).

ChlB6 transferred 6-methylsalicyl groups on ChlB2 with the

highest specificities, i.e. 1.06 min^{-1}, than 5-chloro-6-methyl-*O*-methylsalicyl (0.008 min^{-1}) [15]and 6-methyl-*O*-methylsalicyl (0.0006 min^{-1}) (Figure 3a and 3b), showing that 6-methylsalicyl-S-ChlB2 should be the best substrate of ChlB6. To obtain the accurate enzymatic information, the kinetic parameters of ChlB6 for 6-methylsalicyl-S-ChlB2 and 5-chloro-6-methyl-*O*-methylsalicyl-S-ChlB2 were subsequently determined (Figure 4 and table S4). The *K*m values for 6-methylsalicyl-S-ChlB2 and 5-chloro-6-methyl-*O*-methylsalicyl-S-ChlB2 with fixed concentration of DM-CHL (150μM) are 0.24 mM and 3.8 mM; and the *k*cat Values are 2.79 min^{-1} and 0.12 min^{-1}. The relative specificity constant (*k*cat/*K*m) of 6-methylsalicyl-S-ChlB2 was superior to that of 5-chloro-6-methyl-*O*-methylsalicyl-S-ChlB2 by more than 300 times. The kinetic data demonstrated that 6-methylsalicyl was immediately used to synthesize **M-CHL** after being transferred to ChlB2 by ChlB3, indicating that ChlB4 and ChlB5 only could modify **M-CHL** to form **DCM-CHL** and **CHL**, both of which could be produced in wild-type *Streptomyces antibioticus* DSM 40725.

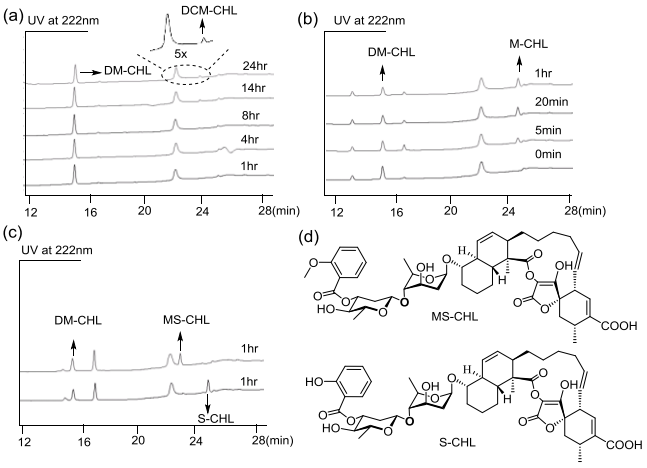


Figure 3 HPLC analysis of acyltransferase activity of ChlB6 from holo-ChlB2 to DM-CHL. (a) 6-methyl-*O*-salicyl transfer by ChlB6 for 1hr, 4hr, 8hr, 14hr and 24hr. (b) 6-methylsalicyl transfer by ChlB6 for 5min, 20min and 1hr. (c) *O*-methylsalicyl and salicyl transfer by ChlB6 for 1hr. (d) The structure of MS-CHL and S-CHL.

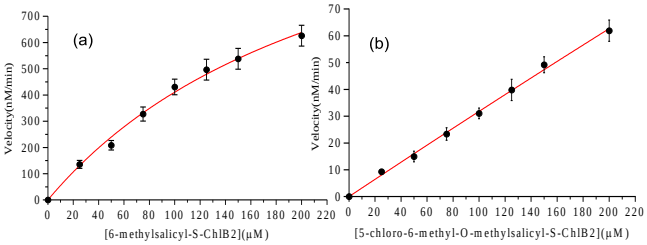


Figure 4 Michaelis-Menten curves of ChlB6 for (a) 6-methylsalicyl-S-ChlB2 and (b) 5-chloro-6-methyl-*O*-methylsalicyl-S-ChlB2.

Many acyltransferases, similar to ChlB3 and ChlB6, have been found in the gene clusters of various natural products (Figure S1), and most of them were KAS III-like acyl transferases responsible for the formation of C-O, C-N and C-S bonds, with the similar mechanism of C-S bond formation catalyzed by FabD, however, KAS III was supposed to catalyze the C-C formation (Figure 5a, 5b). To further obtain the phylogenetic origin of ChlB3 and ChlB6, we compared some representative acyl transferases from the biosynthesis of natural products with *cis*-acyl transferases (*cis*-AT), *trans*-acyl transferases (*trans*-AT) malonyl-CoA–acyl carrier protein transacylases（FabD）, β-Ketoacyl-acyl carrier protein synthase I, II, III (KAS I, KAS II, KAS III, also known as FabB, FabF, FabH) and ketosynthases (KS) in the same phylogenetic tree (Figure 5c). It was obvious that these acyl transferases used in the natural products biosynthesis were divided into two different clades. One clade, including ChlB3 and ChlB6, was phylogenetically related to KAS III which catalyzed claisen condensation, and known as KAS III-like acyl transferase. The other clade, including CouN7[17], CmaE[18], SyrC[19] and MdpB3[20], was in close proximity to malonyl-CoA: [acyl-carrier-protein] S-malonyltransferases (*cis*-AT, FabD and *trans*-AT), and named CouN7-like acyltransferase.

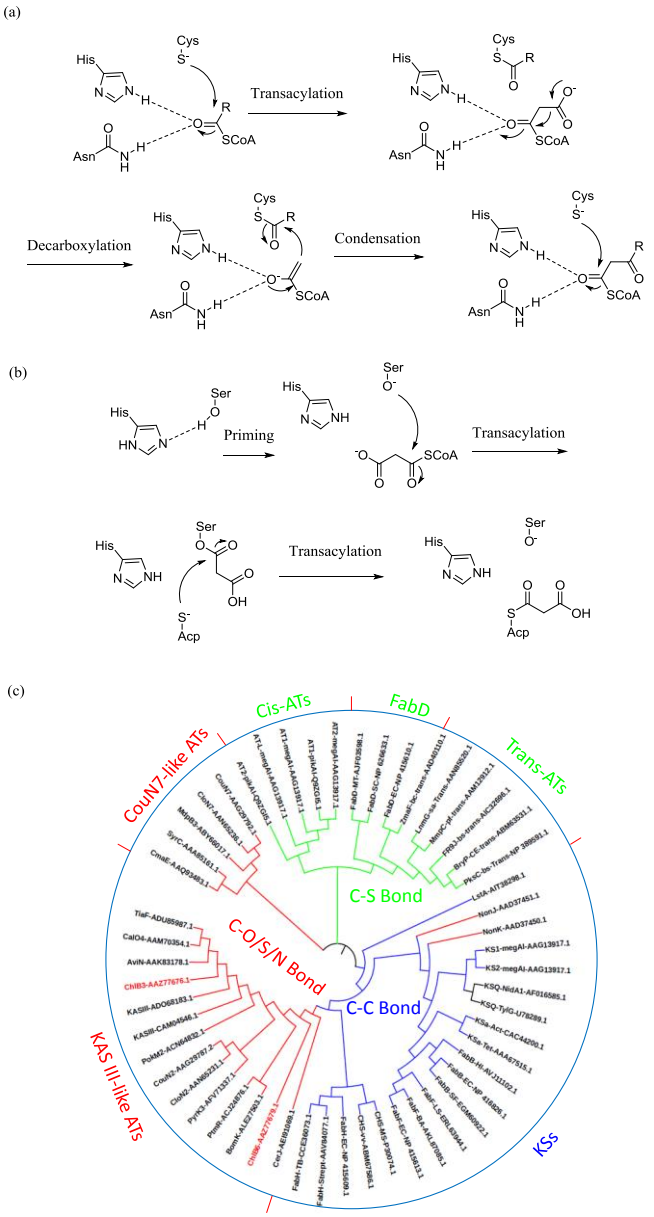


Figure 5 C-C/O/S/N bond formation catalyzed by KSs and ATs. (a)Possible mocecular mechanism of C-C bond formation catalyzed by FabH.(b) Possible mocecular mechanism of C-S bond formation catalyzed by FabD.(c) Phylogenetic tree analysis of ChlB3, ChlB6 with selected KSs and ATs.

ChlB3 and ChlB6 as members of KAS III-like acyltransferases, similar to CerJ[21] with catalytic triad of Cys-His-Asp (Figure S2), used His-Asp to increase the nucleophilic ability of the catalytic Cys residue, and catalyzed transacylation twice to produce the final products. But CouN7-like proteins still need X-ray structure of

this kind of acyl transferase to determine their catalytic mechanism (Figure S3), and only Cys/Ser-His was conserved in the sequence alignment because their protein sequences were obviously different with that of CerJ or FabD. Utilizing the available proteomic information from the UniProt database, we constructed the SSN and GNN of the KAS III-like acyl transferase ChlB3 with the Enzyme Function Initiative-Enzyme Similarity Tool (EFI-EST) and Genome Neighborhood Tool (Figure S4)[22]. More than 1,500 biosynthetic gene clusters including possible KAS III-like acyltransferases were found, and most of the secondary metabolites from these gene clusters have not been excavated yet, form which possible molecular skeleton spanned polyketides, non-ribosomal peptides and terpenes. Therefore, our exploration will provide a reference for predicting the function of these homologous proteins and discovering compounds with novel structures.

Conclusions

According to these data mentioned above, the biosynthesis pathway of **1** should happen in the order showed in Scheme 1d. Firstly ChlB1 synthesizes 6-methylsalicyl loaded on its ACP from one acetyl CoA and three malonyl CoA. Secondly, ChlB3 transfers 6-methylsalicyl from 6-methylsalicyl-ChlB1 to holo-ChlB2. Thirdly, ChlB6 immediately grafts 6-methylsalicyl from holo-ChlB2 to DM-CHL, producing M-CHL. Finally ChlB4 and ChlB5 perform chlorination and *O*-methylation on M-CHL to obtain DCM-CHL or CHL. Manipulation of chain initiation has proven very useful for generating novel products *in vivo*. However, manipulation of the modified units is more powerful in increasing the structural diversity of natural products at the biochemical level *in vitro*. And our findings will provide a unique opportunity to understand other similar functional modified enzymes and contribute to generating new CHL analogs via combinatorial biosynthesis and chemo-enzymatic synthesis.

Experimental

Bacterial Strains, Plasmids, Biochemicals, and chemicals. *E. coli* DH5α was used for general gene clone, and *E. coli* BL21 (DE3) was used for protein expression (Novagen). The Chlorothricin producer, *S. antibioticus* DSM40725 was purchased from DSMZ. Cloning vectors pSP72 (Promega) and expression vectors pET28a, pET37b (Novagen) and pMal-c2X (NEB) were originally from commercial sources. HCoA was from Sigma, and all the other common biochemicals and reagents came from standard commercial sources.

DNA Isolation and Manipulation. DNA isolation and manipulation in *E. coli* and *Streptomyces* were carried out according to standard methods. PCR amplifications were carried out on an Authorized Thermal Cycler (Eppendorf AG) using either Taq DNA polymerase or PfuUltra High-Fidelity DNA polymerase. Primer synthesis and DNA sequencing were performed at the Shanghai Invitrogen Biotech Co., Ltd., and Chinese National Human Genome Center.

Expression and purification of sfp, ChlB1-ACP, ChlB2, ChlB3, and ChlB6. Sfp, ChlB1-ACP, ChlB2, ChlB3 and ChlB6 were expressed and purified as we have reported previously.[15]

Synthesis of CoA derivatives of 5-chloro-6-methyl-O-methylsalicyclic acid and 6-methyl-O-methylsalicyclic acid. CoA derivatives of 5-chloro-6-methyl-O-methylsalicyclic acid and 6-methyl-O-methylsalicyclic acid were synthesized and purified as we reported previously. [15]

Synthesis of CoA derivatives of O-methylsalicyclic acid. 6-methyl-*O*-methylsalicyclic acid or *O*-methylsalicyclic acid (10 μmol, 1.6 eq), PyBOP (10 μmol, 1.6 eq) and K_2CO_3 (25.4 μmol, 4.0 eq) were dissolved in 0.5 mL of freshly distilled THF under argon, and then coenzyme A (6.35 μmol, 1 eq) was dissolved in 0.5 mL of H_2O (O_2 was removed by sonication) and added dropwise to the reaction. After being stirred at room temperature for 2 hr, the reaction mixture was directly purified by RP-HPLC (Venusil XBP-C18 10.0 × 250 mm, 5 μm, 100 Å, 3ml/ml, 0-25 min 20%-60% B, 25-29 min 60% B, 29-30 min 60%-20% B ; buffer A: 10 mM NH_4OAc in H_2O; buffer B: CH_3OH). These fractions containing product were collected, and methanol was removed by evaporation firstly, followed by the removal of water and NH_4OAc by lyophilization. More than 95% CoA was converted into its derivative, and the mass of the product was confirmed by ESI-MS $[M-H]^-$or $[M+H]^+$. For *O*-methylsalicyl-S-CoA, ESI-MS $[M+H]^+$:cald. 902.2, found. 902.3; for 6-methyl-*O*-methylsalicyl-S-CoA, ESI-MS $[M-H]^-$ cald. 914.2, found. 914.4.

Synthesis of CoA derivatives of salicyclic acid. Salicyclic acid (100 μmol, 1.0 eq) in freshly distilled DMF (1 mL) was stirred with PyBOP (1.5 eq), iPr_2EtN (1.5 eq), and thiophenol (3.0 eq) under argon. After being stirred at room temperature for 30 min, the reaction mixture was directly purified by RP- HPLC column (Venusil XBP-C18, 10.0 × 250 mm, 5 μm, 100 Å, 3ml/ml, 0-5 min 5%-70% B, 5-28 min 70%-100% B, 28-30 min 100%-5% B ; buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in CH_3CN). The solvent was removed under reduced pressure to give the product of salicyl-S-phenyl thioester. Then the salicyl-S-phenyl thioester (60.8 μmol, 4.8 eq) and coenzyme A (12.7 μmol, 1.0 eq) were stirred in phosphate buffer (8 mL, 50 mM, pH=8.5) under argon. After being stirred at room temperature for 2 hr, the reaction mixture was directly purified by reverse-phase HPLC (Venusil XBP-C18, 10.0 × 250 mm, 5 μm, 100 Å, 3ml/ml, 0-25 min 20%-60% B, 25-29 min 60% B, 29-30 min 60%-20% B ; buffer A: 10 mM NH_4OAc in H_2O; buffer B: CH_3OH). The fractions containing product were pooled, and methanol was removed by evaporation firstly, followed by the removal of water and NH_4OAc by lyophilization. More than 90% CoA was converted into its derivative, and the mass of the product was confirmed by ESI-MS $[M-H]^-$: cald.888.1, found.888.2.

Characterization of ChlB3 and ChlB6 activities on holo-ChlB2 and small molecule acyl-S-ChlB1-ACP. To generate the small molecule acyl-S-ChlB1-ACP, the reaction was carried out in 75 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP (pH 8.0), 100 μM ChlB1-ACP, 150 μM indicated CoA derivatives, and 2 μM sfp for 1 hr at 30°C. To produce the holo-ChlB2, a similar reaction was performed in 75 mM MOPS (pH 7.5),10 mM $MgCl_2$, 1 mM TCEP (pH 8.0), 300 μM ChlB2, 450 μM CoA, and 5 μM sfp for 1 hr at 30°C. For the transferring of small molecule acyl group, the reaction harboring 75 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP (pH 8.0), 30 μM indicated small molecule acyl-S-ChlB1-ACP and 30 μM holo-ChlB2, was initiated by addition of 0.2 μM ChlB3 or ChlB6, and quenched with 0.25 volume 10% formic acid at indicated time. The quenched reaction was directly analyzed by reverse-phase HPLC (Vydac 218TP54 C18 , 250 × 4.6 mm, 5 μm, 300 Å, 1ml/min, 0-3 min 20% B, 3-5 min 20%-35% B, 5-25min 35%-55% B, 25-26 min 55%-99% B, 26-29 min 99% B, 29-30 min, 99%-20% B; buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in CH_3CN).

Characterization of ChlB3 and ChlB6 activities on desmethylsalicyl CHL (DM-CHL) and small molecule acyl-S-ChlB2. To generate the small molecule acyl-S-ChlB2, the reaction was carried out in 75 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP (pH 8.0), 300 μM apo-ChlB2, 450 μM indicated CoA derivatives, and 5 μM sfp for 1 hr at 30°C. The reaction containing 75 mM MOPS (pH 7.5), 10mM $MgCl_2$, 1 mM TCEP (pH 8.0), 1 mg/mL BSA, 5% DMSO,150 μM small molecule acyl-S-pantetheinyl ChlB2 and 150 μM DM-CHL, was initiated with 20.0 μM ChlB3 or ChlB6, and quenched with 0.25 volume 10 % formic acid or 2 volume methanol at indicated time. The reaction quenched with formic acid was directly analyzed by reverse-phase HPLC (Vydac 218TP54

250 × 4.6mm, 5 μm, 300 Å, the gradient elution condition was as described above). The reaction quenched with methanol was placed at -20°C for 30min, and centrifuged at 12,000 rpm for 20min to remove proteins, followed by the supernatant analysis by reverse-phase HPLC column (COSMOSIL 3C18-AR-II, 250 × 4.6mm, 5 μm, 1ml/min, 0-5 min 40% B, 5-25 min 40%-85% B, 25-30 min 80%-40% B; buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in CH_3CN).

Determination of the kinetic parameters of ChlB3 for the substrates of 6-methylsalicyl-ChlB1-ACP and 5-chloro-6-methyl-*O*-methylsalicyl-S-ChlB1-ACP. To determine the kinetic parameters of ChlB3, reactions were carried out at 30 °C in total volume of 100 μl that contained 50 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP (pH 8.0), 5% DMSO, 150 μM holo-ChlB2, 0.1 μM ChlB3, and various concentrations of 6-methylsalicyl-S-ChlB1-ACP (10, 20, 30, 40, 50, 60, 70, and 80μM) for 3 min. The conversion ratio of 6-methylsalicyl-S-ChlB1-ACP to 6-methylsalicyl-S-ChlB2 was analyzed by RP-HPLC as described above. The initial rate conditions were maintained by computing the substrate conversion at less than 10% on the assumption that these conditions were within the linear range of enzyme concentration and enzyme turnover. The data were fitted with the Michaelis-Menten equation to extract the kinetic constants. While for 5-chloro-6-methyl-*O*- methylsalicyl-S-ChlB1-ACP, reactions were carried out at 30 °C in total volume of 100 μL that contained 50mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP (pH 8.0), 5% DMSO, 150 μM holo-ChlB2, 10μM ChlB3, and various concentrations of 5-chloro-6-methyl-*O*-methylsalicyl-S-ChlB1-ACP (10, 20, 30, 40, 50, 60, 70, and 80μM) for 30 min, and these remaining steps were the same as described above.

Determination of the kinetic parameters of ChlB6 for the substrates of 6-methylsalicyl-S-ChlB2 and 5-chloro-6-methyl-*O*-methylsalicyl-S-ChlB2. To determine the kinetic parameters of ChlB6, reactions were carried out at 30 °C in total volume of 100 μL that contained 50 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1mg/mL BSA, 1 mM TCEP (pH 8.0), 5% DMSO, 250μM DM-CHL, 5μM ChlB6, and indicated concentrations of 6-methylsalicyl-S-ChlB2 (25, 50, 75, 100, 125, 150, and 200μM) for 5 min. The ratio of 6-methylsalicyl-S-ChlB2 to holo-ChlB2 conversion was analyzed by RP-HPLC as described above. The initial rate conditions were maintained by computing the substrate conversion at less than 10% on the assumption that these conditions were within the linear range of enzyme concentration and enzyme turnover. The data were fitted with the Michaelis-Menten equation to extract the kinetic constants. While for 5-chloro-6-methyl-*O*-methylsalicyl-S-ChlB2, reactions were carried out at 30 °C in total volume of 100 μL that contained 50 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1mg/mL BSA, 1 mM TCEP (pH 8.0), 5% DMSO, 250μM DM-CHL, 10μM ChlB6, and various concentrations of 5-chloro-6-methyl-*O*-methylsalicyl-S-ChlB2 (25, 50, 75, 100, 125, 150, and 200μM) for 30 min, and other steps were the same as described above.

Supporting Information

The supporting information for this article is available on the WWW under https://doi.org/10.1002/cjoc.2018xxxxx.

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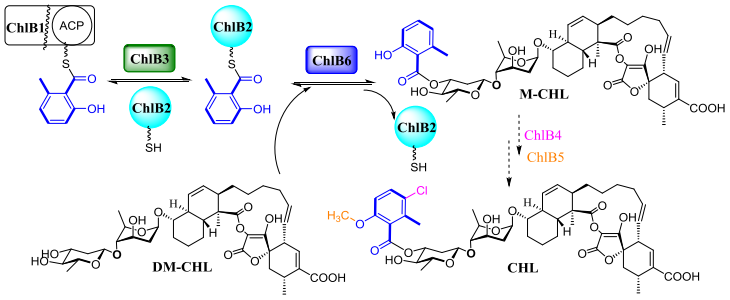

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Speculating the Reaction Timing of 5-chloro-6-methyl-*O*-methylsalicyclic Acid by Kinetic Parameters of Two KAS III-like Acyltransferases for Different Substrates in Chlorothricin Biosynthesis

