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Dissecting Glycosylation Steps in Lobophorin Biosynthesis Implies an Iterative Glycosyltransferase

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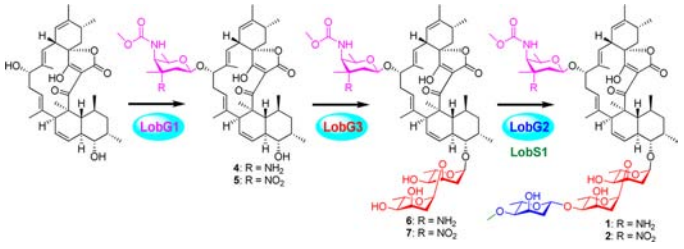
ABSTRACT



The identification of a lobophorin biosynthetic gene cluster from the deep-sea derived *Streptomyces* sp. SCSIO 01127 reveals a paradigm of three glycosyltransferases (GTs) LobG1−LobG3 being responsible for appending four sugars. Characterization of five differentially glycosylated metabolites from three GT gene-inactivation mutants allowed the assignment of GT functions and the implication of LobG3 as an iterative GT to attach two digitoxoses.

Lobophorin (LOB) A (**1**) and B (**2**) (Figure 1) were first discovered from an alga associated actinobacterium[1] and were recently reisolated from the deep-sea derived *Streptomyces* sp. SCSIO 01127.[2] Lobophorins possess a pentacyclic aglycon (also called kijanolide) that features a tetronate moiety *spiro*-linked with a cyclohexene ring and belong to a large class of spirotetronate antibiotics with >60 naturally occurring family members,[3] most of which have both antibacterial and antitumor activities.[3] Well-known examples include kijanimicin (KIJ),[4] tetrocarcin A (TCA),[5] and chlorothricin (CHL) (Figure S1).[6] The potent and diverse biological activities of spirotetronate antibiotics attracted a number of chemical and biosynthetic approaches to generate analogues for structure−activity relationship studies. Few examples were achieved by challenging chemical synthesis due to the highly complex structures of their family members.[7] The recent elucidation of biosynthetic gene clusters[3,8] for CHL, KIJ, TCA, pyrroindomycin, and quartromicin not only reveals a conserved strategy for spirotetronate formation but also provides access to several analogues by pathway engineering.[8a,c] However,

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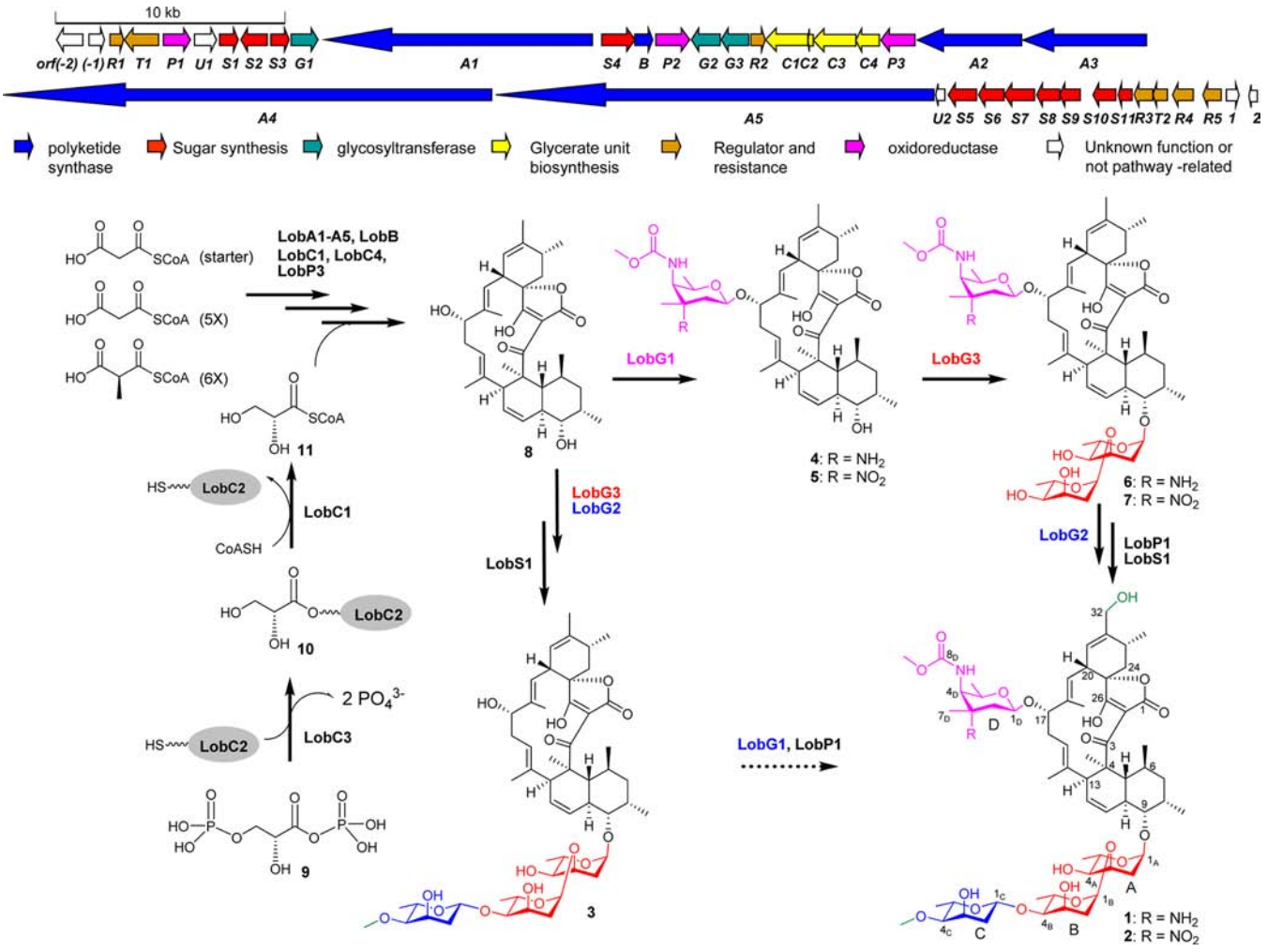

Figure 1. Genetic organization of the *lob* gene cluster in *Streptomyces* sp. SCSIO 01127 and proposed LOB biosynthetic pathway, on glycerol unit formation, glycosylation, hydroxylation, and methylation. The dashed arrow indicates a deduced step. The letters "A−D" denote the four sugar units in LOB. The PKS and sugar pathways were shown in Figure S7 in the Supporting Information.

glycosylations involved in these spirotetronates have not been reported. Structurally, LOB B (**2**) shares the same aglycon as KIJ and differs from KIJ only by lacking an α(1→3) linked and branched L-digitoxose (Figure S1). The biosynthetic pathway for KIJ has been reported; however, the recalcitrance of the producer *Actinomadura kijaniata* SCC 1256 to genetic manipulations impeded the generation of KIJ analogues by pathway engineering.[3] In this study, we reported the identification and characterization of the LOB gene cluster (*lob*) from *Streptomyces* sp. SCSIO 01127 that was amenable to conventional genetic manipulations, thus enabling us to study the LOB biosynthesis at the genetic level and to generate five LOB analogues (**3**−**7**, Figure 1), upon inactivation of three glycosyltransferase (GT)-encoding genes *lobG1*−*G3*. Structure characterization of **3**−**7** allowed us to assign functions to these three GTs and to suggest that LobG1−G3 were sufficient in the attachment of four sugars, probably due to the iterative use of LobG3.

We set out to identify the LOB gene cluster by PCR with degenerate primers NGDH-F/R for NDP-glucose-4,6-dehydratase genes[9] and SOM-F/R for sugar-*O*-methyltransferase genes (Figure S2),[3] together with a pair of specific primers PKSI-F/R targeting LOB polyketide synthase (PKS) genes (Tables S1, S2). This approach allowed us to obtain a 103 005 bp continuous DNA sequence (GenBank accession number KC013978) by the shot-gun sequencing of three overlapping positive cosmids (Figure S3). Bioinformatics analysis identified 40 ORFs (Figure 1), 36 of which

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were assigned to the *lob* gene cluster, by functional comparison of their deduced products to those for KIJ and TCA biosynthesis (Table S3, Figure S4). We hypothesize that LobR1 is related to LOB biosynthesis, because of its high similarity to KijA8, a TetR type regulator embedded in the *kij* cluster.[3] Disruptions of flanking genes, *orf(−1)*, *orf(−2)*, *orf1*/*orf2* (Figure S5), yielded mutants that still produced **1**/**2** at a comparable level to that of the wild type (WT) strain (Figure S6), indicating their nonrelevance to LOB biosynthesis. In contrast, the inactivation of *lobR5*, encoding a LysR family transcriptional regulator, completely abolished LOB production (Figure S6). Taken together, we concluded that the intact *lob* gene cluster spanned from *lobR1* to *lobR5* (Figure 1, Table S3).

Consistent with the high structural similarity of LOB to KIJ and TCA, the *lob* gene cluster displays a genetic organization similar to those of KIJ and TCA (Figure S4), with a more complex regulation network than *kij* and *tca* clusters by having five regulator genes (*lobR1*−*R5*) (Figure 1, Table S3). In contrast, only three are identified in the *kij* cluster (*kijA8*, *kijC5*, and *kijD12*) and two in the *tca* cluster (*tcaR1* and *tcaR2*). LobR1 (77% identity to KijA8), LobR2 (83% identity to KijC5), and LobR3 (75% identity to KijD12) belong to TetR type regulatory proteins. LobR4 shows the highest similarity to TtmF, a putative regulatory protein involved in tautomycin biosynthesis.[10] The LysR family transcriptional regulator LobR5 is demonstrated as a positive regulator (Figure S6). Two genes, *lobT1* (72% identity to KijA5) and *lobT2*, are found in the *lob* cluster, putatively involved in the transportation and resistance of LOB. LobT1 is an EmrB/QacA family drug resistance transporter, and LobT2 is distantly similar to Hoch_4733, a forkhead-associated protein in *Haliangium ochraceum* DSM 14365.

The biosynthesis of LOB aglycon (**8**) was proposed to proceed in the same manner as KIJ aglycon,[3] involving (i) the assembly of a linear polyketide chain by five multifunctional modular type I PKS LobA1−A5 that utilize six malonyl CoA and six methylmalonyl CoA, (ii) a "Diels−Alder"-like intramolecular cycloaddition reaction of the nascent PKS chain, (iii) a subsequent incorporation of the 3-carbon glycerol unit (**11**), and (iv) a final cyclization putatively mediated by the oxidoreductase LobP3 (Figure S7). The tetronate moiety in spirotetronate antibiotics CHL,[8a] KIJ,[3] TCA,[8b] and tetronomycin (TMN)[11] has been confirmed to be derived from glycerol by labeling studies and biochemical characterizations of the involved enzymes. Genetic comparison of the *lob* gene cluster with those of CHL, KIJ, TCA, and TMN identifies four genes, *lobC1* to *lobC4* (Table S3), putatively responsible for the incorporation of the glycerol unit, by analogy to the route (**9**→**10**→**11**) established for TCA and TMN (Figure 1).[8b,11] The incorporation of **11** into **8** may require the ketosynthase III-like protein LobC4. Finally, LobP3, like KijA and TcaE1,[3,8b] may serve as a FAD-dependent oxidoreductase, to furnish the formation of **8** (Figure S7). Consistent with these functional assignments, the LOB productions were abolished in the Δ*lobC1*, Δ*lobC3*, and Δ*lobC4* mutants (Figure S6), confirming their essentiality for LOB biosynthesis.

LOB B (**2**) contains four deoxysugar units (Figure 1), including two L-digitoxoses (sugars A and B), one 4-*O*-methyl-L-digitoxose (sugar C), and one D-kijanose (sugar D). By comparison with those genes in the KIJ pathway, at least 12 *lob* genes are proposed to be involved in deoxysugar biosynthesis, including *lobS1*−*S11* and *lobP2* (Table S3, Figure S7). We propose that the L-digitoxose biosynthesis follows the well established route by Liu and co-workers via in vitro biochemical experiments,[3] requiring six steps: (i) a thymidylylation by LobS9 (KijD5), (ii) a 4,6-dehydration by LobS8 (KijD4), (iii) a 2,3-dehydratase LobS4 (KijB1), (iv) a 3-ketoreduction by LobS10 (KijD10),[12] (v) a 5-epimerization by LobS11 (KijD11), and (vi) a 4-keto-reduction by KijC2. Intriguingly, the 4-keto-reductase KijC2 counterpart was absent in both *lob* and *tca* gene clusters.[3,8b] Finally, LobS1 (84% identity to KijA1), a TylF-family of *O*-methyltransferase,[13] may be involved in forming the 4-*O*-methyl-L-digitoxose. The kijanose biosynthesis has been proposed previously to branch from L-digitoxose at a common intermediate, TDP-2,6-dideoxy-3,4-diketo-D-glucose, for KIJ and TCA (Figure S7),[3,8b] requiring seven steps for further modifications: (i) a 3-aminotransfer by LobS6 (KijD2), (ii) a 3-C-methylation by LobS5 (KijD1), (iii) a nitro-formation by LobS7 (KijD5), (iv) a 4-aminotransfer by LobS2 (KijD7), (v) an *N*-methylation by LobS3 (KijD8), (vi) a putative carboxylation by the P450 enzyme LobP2 (KijB3), and (vii) an *O*-methylation by a not-yet-identified enzyme (Figure S7). In support of this proposed kijanose pathway, functions of TcaB9 (LobS5 homologue) and KijD3 (LobS7 homologue) have been confirmed by biochemical and structural studies.[14] Notably, KijD3 (LobS7) catalyzes a unique sugar amine oxidation reaction, the mechanism of which has been studied in other similar sugar pathways,[15] *e.g.*, in the biosynthesis of calicheamicin,[15b] everninomycin, and rubradirin.[15c] The P450 monooxygease LobP1 shows the highest identity (70%) to KijA3, while no *lobP1* homologues are identified in the *tca* and *chl* clusters, suggesting that LobP1 and KijA3 may be involved in the unique C-32 hydroxylation for LOB and KIJ.

In contradiction to four deoxysugars found in LOB, only three GT-encoding genes, *lobG1*−*G3*, showing a high sequence similarities to various GTs involved in microbial natural products, are identified in the *lob* cluster.

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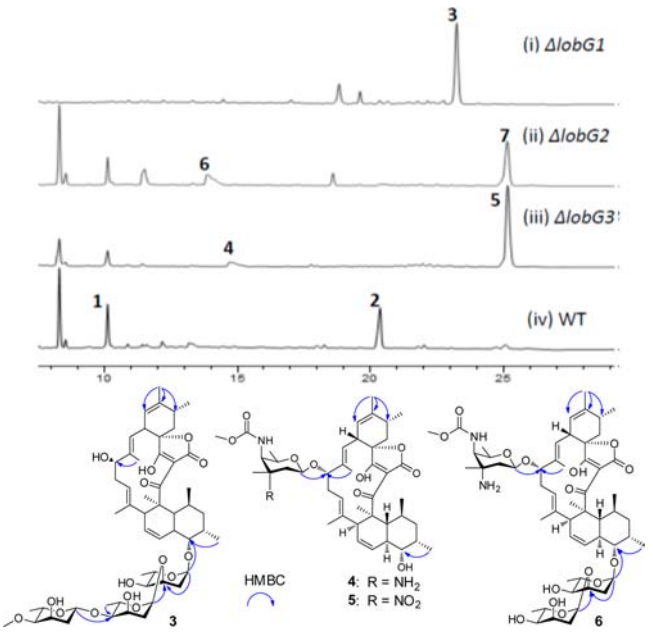

Figure 2. HPLC traces for metabolite profiling of (i) the Δ*lobG1* mutant, (ii) the Δ*lobG2* mutant, (iii) the Δ*lobG3* mutant, and (iv) WT *S.* sp. SCSIO 01127 (**1** was coeluted with an unknown impurity), and key HMBC correlations for compounds **3**–**6**.

Similarly, only four GTs (TcaT1–T4) were found in the *tca* cluster for attaching five deoxysugars in TCA,[8b] different from five GTs (KijA4, KijC1, KijC3, KijC4, and KijD9) in the *kij* cluster for five deoxysugars in KIJ.[3] This suggests that one GT may act iteratively to append two sugar units in both LOB and TCA pathways. To probe their functions, we made inactivation mutants for all three GT genes *lobG1*–*G3* (Figure S5). HPLC analyses revealed that each mutant displayed a metabolite profile distinct from each other and different from WT (Figure 2). Subsequent large scale fermentation led to the isolation of five compounds from these three mutants, **3** (Δ*lobG1*), **4** and **5** (Δ*lobG3*), and **6** and **7** (Δ*lobG2*). Their structures (**3**–**7**, Figure 1) were determined by HRESIMS, 1D and 2D NMR spectroscopic data (see Supporting Information for detailed structure elucidation; Figure 2, Tables S4, Figures S8–S12), identifying **3**–**6** as new LOB analogues and **7** as lobophorin F.[2] The β(1→4) linkage of sugar C in **3** was evident from the coupling constants of the anomeric proton ($J_{1C,\ 2C}$ = 9.5, 2.0 Hz). Antibacterial assays reveal new compounds **3**–**6** compared to **2** exhibit generally less activity against two *Bacillus* strains, and highlight the importance of a nitro group for activity (Table S5). Interestingly, the new derivative **5** that lacks the trisaccharide chain at C-9 showed slightly better activity than **2** against *Staphylococcus aureus* ATCC 29213.

Given the loss of the kijanose moiety in **3** isolated from the Δ*lobG1* mutant, we reason that LobG1 is responsible for appending the unique kijanose (sugar D) in LOB, suggesting that the homologues of LobG1, TcaT2 (76% identity) and KijD9 (72% identity), perform the same function in the TCA and KIJ pathways. Compounds **6** and **7** from the Δ*lobG2* mutant contain only two digitoxoses (sugars A and B) at C-9, suggesting that LobG2 functions to transfer the terminal L-digitoxose (sugar C). Given the highest similarity of LobG2 to KijC3 (80% identity) we suggest KijC3 as the terminal L-digitoxosyltransferase in the KIJ pathway. Interestingly, the terminal digitoxose is attached via a β(1→4) glycosidic linkage, indicating LobG2 as a retaining GT. Notably, **4** and **5** from the Δ*lobG3* mutant contain no digitoxoses at C-9. Although lacking biochemical evidence, we hypothesize that LobG3 is probably responsible for tandemly attaching the first two L-digitoxoses at C-9, thus answering the query that three GTs in the *lob* cluster are found to append four sugars. The iterative use of a single GT for transferring multiple sugars is precedent in natural product pathways,[16] *e.g.*, LanGT1 and LanGT4 in the landomycin pathway,[16a] AveBI in the avermectin pathway,[16b] AknK in the aclacinomycin pathway,[16c] and PnxGT2 in the FD-594 pathway.[16d]

In summary, we have identified the *lob* gene cluster from *Streptomyces* sp. SCSIO 01127 and deciphered the glycosylation steps in LOB biosynthesis (Figure 1). LobG1 transfers kijanose (or its precursor with an amino group replacing a nitro group) to the aglycon (**8**) to afford **5** (or **4**). Subsequently, LobG3 iteratively attaches two L-digitoxoses to **5** (or **4**), yielding **7** (or **6**). Finally, LobG2 catalyzes the attachment of the terminal digitoxose to **7** (or **6**), proceeding with retention of the sugar configuration, to complete the glycosylation steps in the LOB pathway. The 4-*O*-methylation of the terminal digitoxose by LobS1, and the hydroxylation at C-32 by LobP1, may tailor the LOB biosynthesis to produce the final product **2**. Notably, the isolation of **3**–**5** suggests that the catalytic order of LobG1 and LobG3 is uncertain. Conclusively, this study implicates an iterative GT LobG3 and a retaining GT LobG2, both of which are uncommon for natural product biosynthesis, thus warranting further biochemical characterizations.

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Supporting Information Available. Experimental procedures and materials, and characterization data of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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