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Article

Divergence of Classical and C-Ring-Cleaved Angucyclines: Elucidation of Early Tailoring Steps in Lugdunomycin and Thioangucycline Biosynthesis

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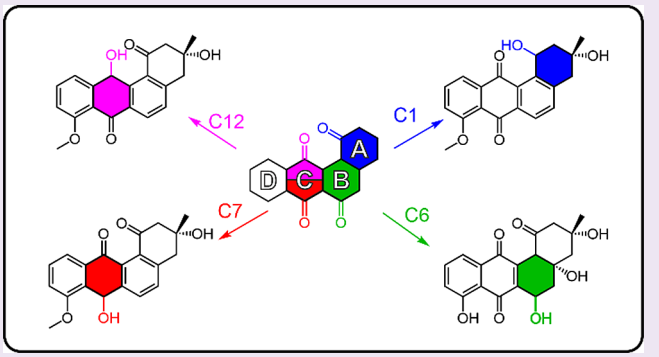
ABSTRACT: Angucyclines are an important group of microbial natural products that display tremendous chemical diversity. Classical angucyclines are composed of a tetracyclic benz[*a*]-anthracene scaffold with one ring attached at an angular orientation. However, in atypical angucyclines, the polyaromatic aglycone is cleaved at A-, B-, or C-rings, leading to structural rearrangements and enabling further chemical variety. Here, we have elucidated the branching points in angucycline biosynthesis leading toward cleavage of the C-ring in lugdunomycin and thioangucycline biosynthesis. We showed that 12-hydroxylation and 6-ketoreduction of UWM6 are shared steps in classical and C-ring-cleaved angucycline pathways, although the bifunctional 6-ketoreductase LugOIIred harbors additional unique 1-ketoreductase activity. We identified formation of the key intermediate 8-*O*-methyltetrangomycin by the LugN methyltransferase as the branching point toward C-ring-cleaved angucyclines. The final common step in lugdunomycin and thioangucycline biosynthesis is quinone reduction, catalyzed by the 7-ketoreductases LugG and TacO, respectively. In turn, the committing step toward thioangucyclines is 12-ketoreduction catalyzed by TacA, for which no orthologous protein exists on the lugdunomycin pathway. Our results confirm that quinone reductions are early tailoring steps and, therefore, may be mechanistically important for subsequent C-ring cleavage. Finally, many of the tailoring enzymes harbored broad substrate promiscuity, which we utilized in combinatorial enzymatic syntheses to generate the angucyclines SM 196 A and hydranthomycin. We propose that enzyme promiscuity and the competition of many of the enzymes for the same substrates lead to a branching biosynthetic network and formation of numerous shunt products typical for angucyclines rather than a canonical linear metabolic pathway.



INTRODUCTION

Angucyclines are a large and diverse group of microbial natural products with important biological activities.[1] Most of these secondary metabolites are produced by *Streptomyces* soil bacteria, and the compounds can be classified into classical and atypical angucyclines based on their biosynthesis.[2] Classical angucyclines and their aglycones, which are termed angucyclinones, are based on a tetracyclic benz[*a*]-anthraquinone carbon frame, in which the A-ring is attached at an angular orientation (Figure 1A),[1] and they include compounds such as landomycin A[3] (**1**), urdamycin M,[4] and gaudimycin C[5] (**2**). Many classical angucyclines, such as **1**, have been noted to harbor prominent anticancer activities.[6] Angucycline landomycin E, a promising anticancer drug, is not a substrate for multidrug resistance efflux pumps.[7]

In addition to classical angucyclines, numerous chemically complex atypical angucyclines, where the angucycline origin is discernible only via analysis of the biosynthetic gene cluster (BGC), have been discovered.[2] A defining feature of atypical angucyclines is the cleavage and subsequent rearrangement of the tetracyclic carbon frame, allowing for further chemical diversity (Figure 1A). The angular A-ring has been opened in compounds such as vineomycin B2[8] and gaudimycin D[9] (**3**). In contrast, C−C bond cleavage of the B-ring followed by more drastic rearrangements, ring contractions, and amino acid incorporation occur in gilvocarcin,[10] kinamycin,[11] and jadomycin A[12] (**4**) biosynthesis, respectively. Finally, in recent years, several metabolites with C−C bond cleavage and modifications to the quinone C-ring, such as lugdunomycin[13] (**5**), oleaceran[14] (**6**, elmonin[15]), and rubiginone H,[16] have

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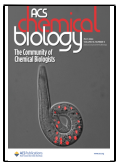

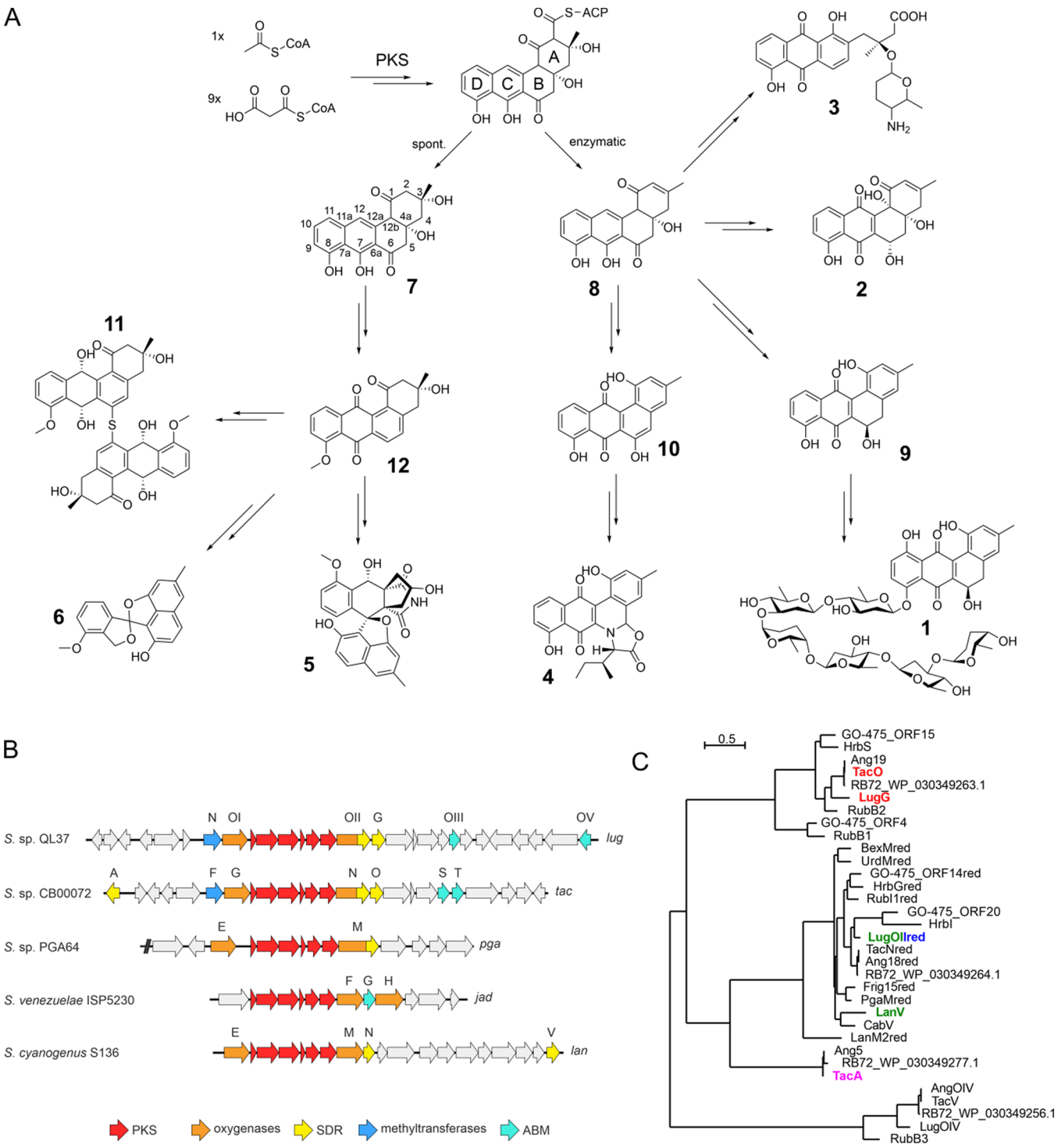

Figure 1. Diversification of angucycline antibiotics. (A) Reaction scheme leading to the formation of the products from classical angucycline pathways landomycin A (**1**) and gaudimycin C (**2**), A-ring-cleaved gaudimycin D (**3**), B-ring-cleaved jadomycin (**4**), C-ring-cleaved lugdunomycin (**5**), oleaceran/elmonin (**6**), and dimerized thioangucycline TAC-A (**11**). (B) Comparison of selected angucycline-producing biosynthetic gene clusters. Legend: *lug*, lugdunomycin; *tac*, thioangucycline; *pga*, gaudimycin; *jad*, jadomycin; *lan*, landomycin. (C) A phylogenetic tree of selected SDR-family enzymes from angucycline BGCs. Investigated 1-, 6-, 7-, and 12-ketoreductases are highlighted in blue, green, red, and magenta, respectively.

been characterized.[17] These findings have revealed that angucyclines form the origin of a large and chemically diverse group of microbial natural products.

Early steps in the biosynthesis of the angucyclinone scaffold have been extensively studied both in vivo[18] and in vitro.[19] Type II polyketide synthases (PKS) utilize one acetyl-CoA and nine malonyl-CoA molecules to produce a highly reactive acyl carrier protein (ACP) tethered linear decaketide, which undergoes stepwise cyclization into an ACP-bound tetracyclic angucyclinone precursor (Figure 1A) catalyzed by angucycline-specific 9-ketoreductases, cyclases, and aromatases. The precursor is finally released from the ACP as one of two key

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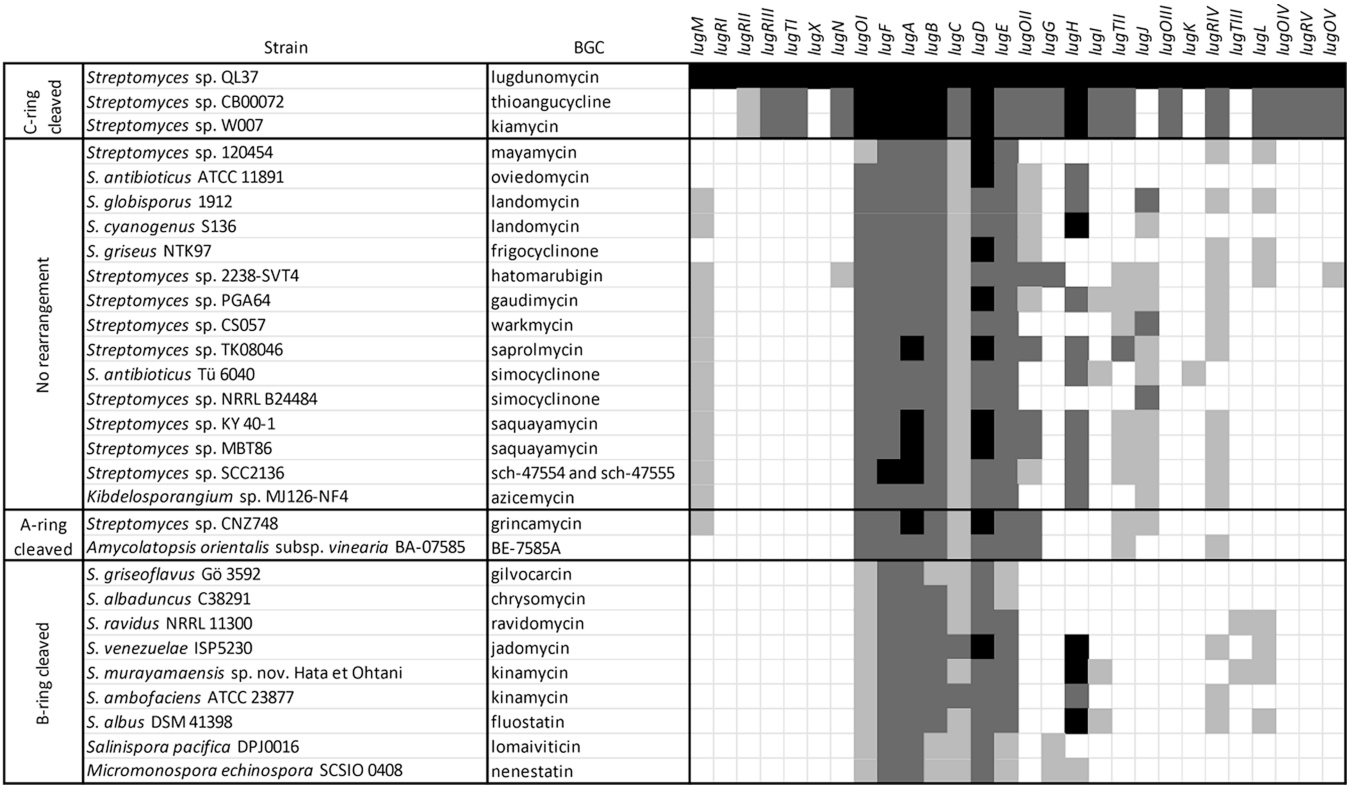
Table 1. Comparison of Lug BGC to Selected Angucycline-Producing BGCs[a]



[a]The presence of genes orthologous to genes from the *lug* cluster is indicated with shades of gray. Light gray indicates a protein sequence identity of 40−60%, dark gray indicates a sequence identity of 60−80%, and black indicates a sequence identity of 80−100%.

intermediates, UWM6 (**7**) or prejadomycin (**8**). The chemical diversity of angucyclines is generated via complex redox reaction cascades that convert the first stable pathway intermediates **7** and **8** to various end products.[1] These reactions are commonly catalyzed by different combinations of enzymes from three families: flavoprotein monooxygenases (FPMO), short-chain alcohol dehydrogenase/reductases (SDR), and antibiotic biosynthesis monooxygenases (ABM). In addition, the functions of various other post-PKS tailoring enzymes, such as methyltransferases and glycosyltransferases, lead to the production of an expansive group of angucycline-type secondary metabolites.

In classical angucyclines, FPMOs such as PgaE or LanE catalyze 12-hydroxylation of **7** or **8**.[20,21] In the landomycin pathway, the biosynthesis continues with 6*R*-stereospecific ketoreduction by the SDR enzyme LanV[22] and 4a/12b-dehydration by LanE to yield 11-deoxylandomycinone[20] (Figure 1A, **9**). In contrast, in gaudimycin biosynthesis, PgaE catalyzes a second hydroxylation at position 12b[23,24] prior to 6S-stereospecific ketoreduction by PgaMred.[5,20,25] The 12b-hydroxylation proceeds via Baeyer−Villiger oxidation,[26] and the lactone ring opening may lead to A-ring-cleaved angucyclines such as **3**.

The biosynthetic pathways toward B-ring-cleaved angucyclines (e.g., **4**) include FPMOs such as GilOI[10] and JadH[20,27] that not only catalyze 12-hydroxylation of **8** but also additionally promote aromatization of A- and B-rings via 4a/12b-dehydration leading to the formation of dehydrorabelomycin (**10**). ABM family enzymes GilOII[10,28] and JadG,[12,28] which require reduced $FMNH_2$ or $FADH_2$ for activity, are responsible for the unique B-ring opening through a C−C bond cleavage.

A key challenge in the elucidation of the biosynthesis of C-ring-cleaved angucyclines such as **5** has been that numerous classical and atypical angucyclines accumulate in cultures of the producing *Streptomyces* strains. For instance, 24 typical and atypical angucyclinones have been discovered from the lugdunomycin producing *Streptomyces* sp. QL37,[13] while 15 angucyclinones, including TAC-A (Figure 1, **11**) and C-ring rearranged **6** (Figure 1), have been reported from the thioangucycline producing *Streptomyces* sp. CB00072. Recent molecular genetic studies have revealed an extended gene set responsible for redox reactions on the lugdunomycin *lug* and thioangucycline *tac*[29] pathways (Figure 1B), but the formation of a series of angucyclinone congeners has prevented unambiguous determination of the biosynthetic steps. In lugdunomycin biosynthesis, the 6-ketoreduction activity of the SDR-family enzyme LugOIIred has been demonstrated in vivo, but remarkably, the enzyme has been shown to additionally catalyze 1-ketoreduction of 8-*O*-methyltetrangomycin (**12**) and 8-*O*-methylrabelomycin (**13**) in vitro.[30] Very recently, the ABM family genes *lugOIII* and *lugOV* have been identified to catalyze 6a/12a epoxidation and implicated in C-ring cleavage, respectively, based on in vivo data.[31] Metabolic in vivo studies into the biosynthesis of thioangucycline have implicated the SDR-family enzymes TacA and TacO to catalyze the reduction of the quinone carbonyl groups at positions 12 and 7, respectively.[29] The co-occurrence of quinone reduction and 6a/12a epoxidation in other C-ring-cleaved angucyclinones such as rubiginone H[16] is also noteworthy. Hence, the reduction of the C-ring has been suggested to be involved in

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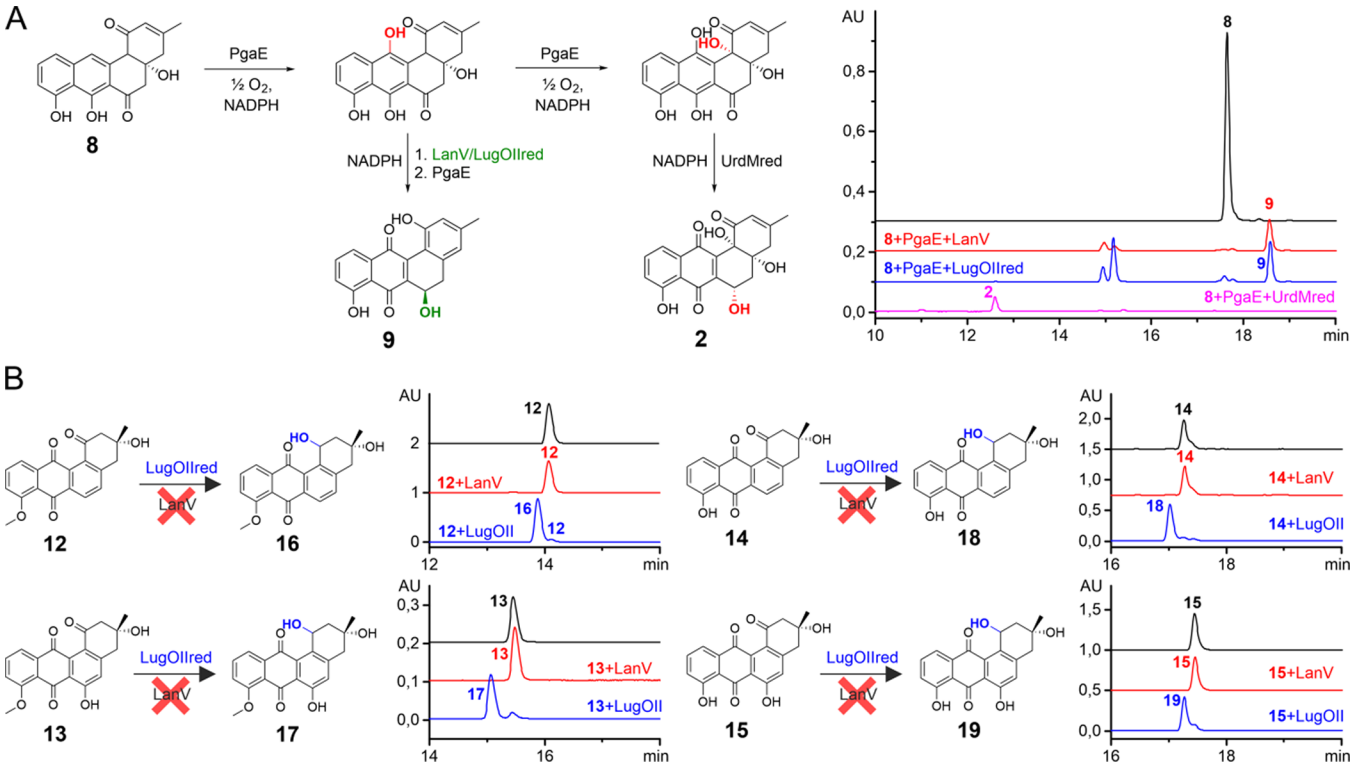

Figure 2. Analyses of 1- and 6-ketoreduction activities of LugOIIred and LanV. (A) Comparative analysis of 6-ketoreduction activity in a coupled assay with PgaE using **8** as a substrate demonstrates that both LugOIIred and LanV catalyze the formation of **9**. This is in contrast to a reaction with PgaE and UrdMred that converts **8** to **2**. (B) Investigation of the substrate promiscuity for 1-ketoreduction using diverse angucyclinone substrates **12**−**15**. LugOIIred converted all substrates to corresponding products **16**−**19**, while no 1-ketoreduction activity was detected for LanV. All HPLC chromatogram traces were recorded at 256 nm.

C-ring cleavage through Grob-type fragmentation of the 6a,12a-epoxide.[29,31−33]

Here, we carried out comparative bioinformatic and biochemical analyses of post-PKS tailoring enzymes from *lug* and *tac* pathways. We demonstrate that early steps in lugdunomycin biosynthesis proceed akin to landomycin biosynthesis until the *lug* pathway diverges through 8-*O*-methylation by LugN. We further detected enzymatic activity for divergent SDR-family ketoreductases that catalyze quinone reduction steps, possibly leading toward C-ring-cleaved angucyclinones. We show that LugG and TacO perform 7-ketoreductions on the *lug* and *tac* pathways, respectively, while TacA catalyzes subsequent 12-ketoreduction in the latter pathway. We further demonstrate that many of the tailoring enzymes compete for the same substrates, which leads to the formation of shunt products and provides an explanation of why strains producing C-ring-cleaved angucyclinones tend to produce libraries of mixed angucyclinones. Our work elucidates the key branching points of C-ring-cleaved angucyclinones and classical angucyclinones.

RESULTS

Comparative Analysis of Gene Sets Involved in Angucycline Tailoring Reactions. To identify genes unique to tailoring reactions on C-ring-cleaved angucyclines, we performed bioinformatic analyses and compared the *lug* BGC to 28 BGCs responsible for the production of known classical and rearranged angucyclines (Table 1). The core genes responsible for synthesis of the angucyclinone carbon scaffold, including minimal polyketide synthase genes *lugABC*, polyketide 9-ketoreductase *lugC*, and angucyclinone-specific aromatase *lugD* and cyclase *lugE*, were conserved and displayed synteny in all BGCs. In addition, all BGCs contained an FPMO similar to that of *lugOI*.

One notable difference was the presence of SDR genes in different numbers and configurations in the majority of classical angucyclines, A-ring and C-ring-cleaved angucyclines, but which were entirely missing from B-ring-cleaved angucyclines (Table 1). Phylogenetic analysis of SDR proteins (Figure 1C) revealed clustering of the bifunctional 6- and 1-ketoreductase LugOIIred together with confirmed 6-ketoreductases (e.g., PgaMred, UrdMred, and LanV). In addition, the *lug* and *tac* BGCs harbored additional SDR-family ketoreductases that seemed to be exclusive for C-ring-cleaved angucyclines (Figure 1B and Table 1). The uncharacterized LugG clustered together with TacO (Figure 1C), which has been implicated in 7-ketoreduction based on molecular genetic studies.[29] Interestingly, hatomarubigin[34] BGC (*hrb*), which reportedly produces nonrearranged angucyclines, encodes HrbS of unknown function, which is highly similar to LugG (65% sequence identity). Additionally, the *tac* BGC codes for another SDR enzyme, TacA, which was characterized as 12-ketoreductase based on knockout studies,[29] but no ortholog for this gene could be identified from *lug* BGC. Finally, *lugOIV* and *tacV*, which show low sequence similarity to the other SDR enzymes, were found to be unique to C-ring-cleaved angucycline BGCs. However, *lugOIV* and *tacV* genes may be redundant, as their deletion did not substantially change the production profiles of the strains.[29,31]

Enzymes of the ABM family, such as LugOIII/TacS and LugOV/TacT, which have been implicated in 6a/12a

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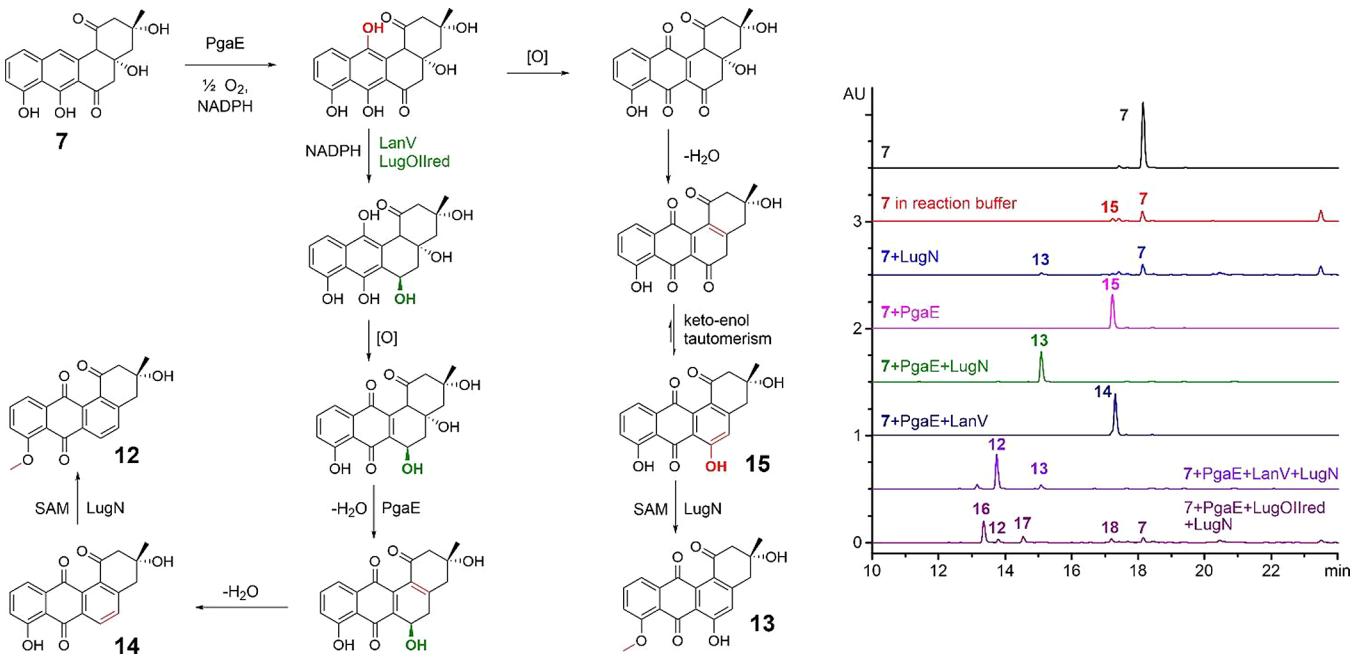

Figure 3. Analysis of the 8-*O*-methylation activity of LugN. Conversion of the substrate **7** into product **13** sequentially by the 12-hydroxylase PgaE and the 8-*O*-methyltransferase LugN. Addition of the 6-ketoreductases LanV or LugOII together with PgaE and LugN directs the transformation of the substrate **7** to **12**. However, the additional 1-ketoreduction activity of LugOII results in the accumulation of **16** as the main product. The HPLC chromatograms were recorded at 256 nm. The structures of **12** and **13** were verified using authentic standards,[28] and compounds **14** and **15** were verified by NMR.

epoxidation and to have a role in C-ring cleavage, were conserved in C-ring-cleaved angucycline pathways. Other proteins of known function included the known B-ring cleavage ABM JadG (Figure 1B) from the jadomycin pathway.[12,28] No homologous proteins were present in classical or A-ring-cleaved angucycline BGCs with the exception of the hatomarubigin BGC[34] encoded HrbF, which has 44% sequence identity with LugOV (Table 1).

Finally, our analysis revealed S-adenosyl-methionine (SAM)-dependent methyltransferases that were found nearly exclusively on C-ring-cleaved angucycline BGCs. Previous metabolic studies have suggested that LugN likely catalyzes 8-*O*-methylation, but the timing of the reaction has not been confirmed in lugdunomycin biosynthesis.[31] In addition, the hatomarubigin pathway encoded a homologous methyltransferase HrbU[34] indicating that the strain appears to have a full set of tailoring genes required for production of C-ring-cleaved angucyclines, even though such metabolites are yet to be discovered.

LugOIIred Catalyzes 6-Ketoreduction Similarly to LanV. Previous metabolic analyses have indicated that LugOIIred harbors 6-ketoreductase activity, but the activity is cryptic in the sense that further tailoring reactions lead to aromatization of the B-ring and loss of the stereocenter at C6.[30] To confirm the stereoselectivity of the 6-ketoreductase activity of LugOIIred, we incubated **8** with the FPMO PgaE from the gaudimycin biosynthetic pathway[24] and LugOIIred in a coupled assay (Figure 2A). LugOI, the putative 12-hydroxylase equivalent of PgaE, could not be produced in soluble form in *Escherichia coli*, but recent in vivo studies have confirmed that *pgaE* is able to complement the *lugOI* deletion mutant of *Streptomyces* sp. QL37, which confirms that the proteins are orthologous, and justifies the replacement of LugOI with PgaE in our assays.[31]

The combination of PgaE and LugOIIred converted **8** into **9** (Figure 2A) similarly to PgaE and LanV,[20] which indicated that LugOIIred is orthologous to LanV in terms of substrate specificity and catalytic activity; both enzymes prefer the 12-hydroxylated derivative of **8** as a substrate and catalyze 6-ketoreduction leading to 6*R* configuration. This was in contrast to UrdMred from the urdamycin pathway, which utilizes a 12,12b-dihydroxylated substrate and converts **8** to **2** with 6*S* configuration in the presence of PgaE (Figure 2A), as we have previously reported.[20]

LanV Does Not Catalyze 1-Ketoreduction Similarly to LugOIIred. The high degree of sequence identity of 66% between LugOIIred and LanV prompted us to probe further the 1-ketoreductase activities reported for LugOIIred. Previously, LugOIIred was shown to catalyze a unique 1-ketoreduction on 8-*O*-methylated **12** and **13**.[30] Here, we wanted to challenge the two enzymes further with various bulkier methylated and nonmethylated substrates to probe their activities and substrate specificities. We incubated **12**, **13**, and their nonmethylated derivatives tetrangomycin (**14**) and rabelomycin (**15**) with either LugOIIred or LanV (Figure 2B). LugOIIred was capable of reducing all of these compounds regardless of the 8-*O*-methylation state of the substrate to **16**–**19**, while none of the substrates were transformed by LanV, confirming that the unique bifunctional activity of LugOIIred is caused by differences in their sequence. The novel reaction products **18** and **19** were identified using mass spectrometry (MS) (Figures S49 and S50), which revealed 2 Da higher masses for the products in comparison to the respective substrates, which is consistent with the 1-ketoreduction activity previously confirmed for **16** and **17**.[27] The possibility of ketoreduction at positions 7 and 12 for **18** and **19** were ruled out based on their UV–vis spectra (Figure S57), which did not

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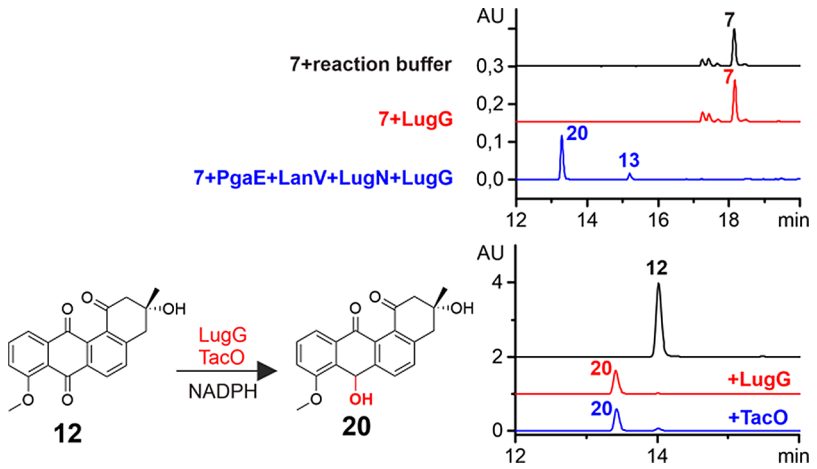

Figure 4. Analysis of the 7-ketoreduction activity of LugG and TacO. LugG does not have enzymatic activity on **7**, but a coupled reaction with PgaE, LanV, LugN, and LugG convert **7** into the product **20**. LugG and TacO are orthologous and convert **12** to **20**. The HPLC chromatogram traces were recorded at 256 nm.

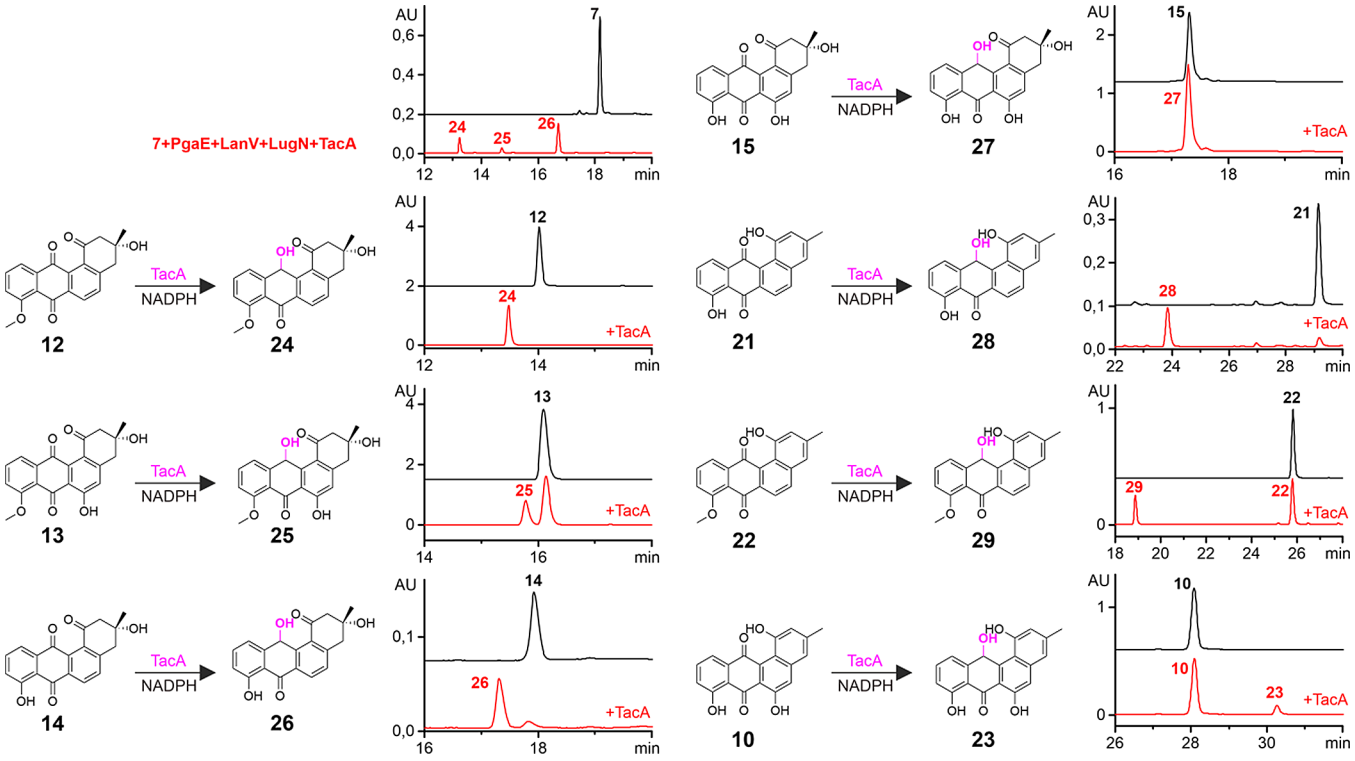

Figure 5. Analysis of the 12-ketoreduction activity of TacA. The substrate **7** is converted to a mixture of products **24**, **25**, and **26** by PgaE, LanV, LugN, and TacA. The 12-ketoreductase TacA harbors broad substrate specificity and can transform various angucyclinone intermediates **12**, **13**, **14**, **15**, **21**, **22**, and **10** to products **24**, **25**, **26**, **27**, **28**, **29**, and **23**, respectively. The structure of **24** was elucidated by NMR spectroscopy, while the structures of **25**, **26**, **27**, **28**, **29**, and **23** were deduced based on the changes in retention time and UV−vis spectra (Figure S41) indicating quinone reduction. The HPLC chromatogram traces were recorded at 256 nm.

show a hypsochromic shift expected for the reduction of a C-ring ketone.

LugN Catalyzes 8-*O*-Methylation after 12-Hydroxylation. A previous metabolic study indicated that 12-hydroxylation, 6-ketoreduction, and 8-*O*-methylation catalyzed by LugOI, LugOIIred, and LugN, respectively, should convert **7** into **12**.[31] To confirm the timing of 8-*O*-methylation and the order of the tailoring reactions, we performed a series of coupled reactions using **7** as a substrate (Figure 3). The incubation of **7** with the SAM-dependent 8-*O*-methyltransferase LugN alone did not lead to enzymatic turnover, except for conversion of trace impurity **15** to methylated derivative **13**. The result was confirmed through production of **15** using the 12-hydroxylase PgaE, which was fully converted to **13** in a coupled assay with PgaE and LugN. To investigate the effect of 6-ketoreduction, PgaE and LanV were utilized to produce **14**, which was readily methylated into **12** by the addition of LugN. If LanV was replaced with LugOIIred, the reaction led to production of additional products **16**−**18** due to the additional 1-ketoreductase activity of LugOIIred (Figure 3). Taken together, the results clarified that 8-*O*-methylation depends on the 12-hydroxylation step but is independent of 6-

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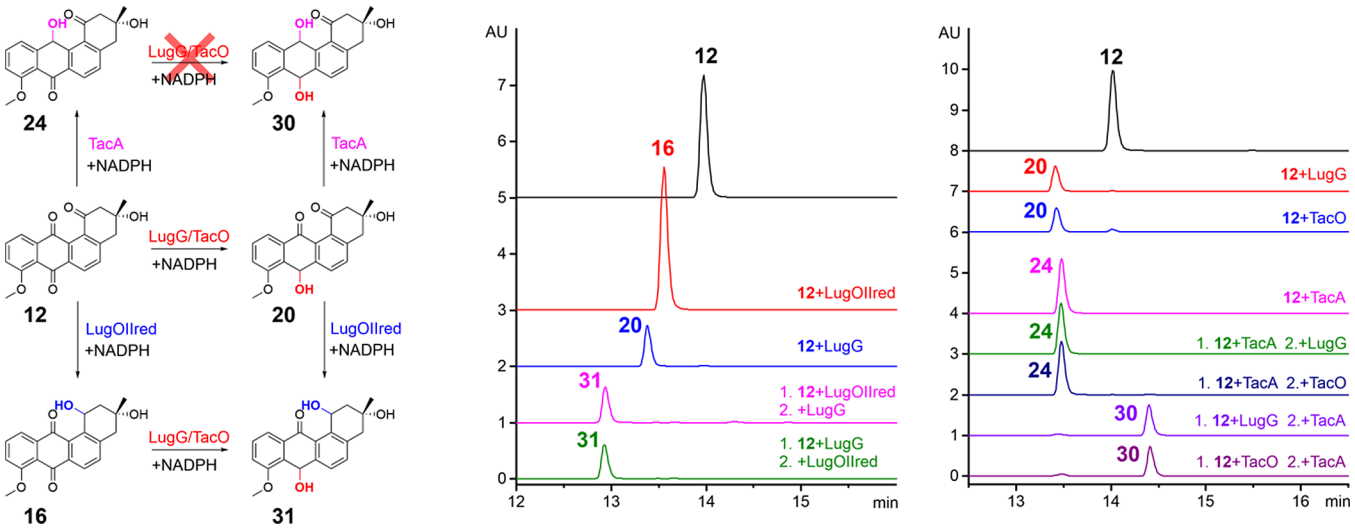

Figure 6. Combinatorial enzymatic reactions for the production of hydranthomycin (**30**) and SM 196 A (**31**). Substrate **12** was converted into **30** and **31** in two-step reactions (labeled with 1. and 2. in the chromatogram traces) with different combinations of ketoreductases LugOIIred, LugG, TacA, and TacO. The HPLC chromatogram traces were recorded at 256 nm.

ketoreduction. The structures of compounds **7**, **12**, and **13** were confirmed with authentic standards. NMR spectra for **12**,[35] **14**,[36,37] and **15**[19] were in agreement with literature (Figures S3−S15). LC-ESI-MS/MS (Figures S46 and S47) for compounds **14** and **15** revealed the expected masses.

LugG Catalyzes Quinone 7-Ketoreduction after 8-*O*-Methylation. We incubated multiple angucyclinone intermediates with LugG in the presence of NADPH to verify the putative 7-ketoreductase function (Figures 4 and S1). LugG converted **12** into a new product 7-deoxo-7-hydroxy-8-*O*-methyltetrangomycin (**20**), which according to LC-ESI-MS/MS analysis has the predicted mass (Figure S51). The same molecule was obtained in a one-pot experiment when **7** was incubated with PgaE, LanV, LugN, and LugG, which led to a near full conversion of **7** into **20** (Figure 4). To elucidate the structure of **20**, chemically pure **12** was incubated with LugG and NADPH in a preparative scale to afford a sufficient quantity of **20** for NMR analysis. The product was verified to be the 7-ketoreduced derivative of **12** based on analysis with 1D and 2D NMR methods (Figures S18−S22 and S41), which unambiguously confirmed that LugG is a 7-ketoreductase. This was mainly deduced based on the disappearance of one carbonyl signal from the ^{13}C NMR spectrum, appearance of the H7 signal in the ^{1}H NMR spectrum, and HMBC correlations of H7/C8 and H6/C7.

To probe the substrate promiscuity of LugG, **7**, **14**, or **15** were incubated with the enzyme, but no new product peaks could be detected (Figure S1). The result indicated that 6-hydroxylation, as well as subsequent 5,6-dehydration and 8-*O*-methylation, are prerequisites for the 7-ketoreductase activity of LugG. The substrate scope of LugG was further probed by incubating the enzyme with **10**, **13**, tetrangulol (**21**), and 8-*O*-methyltetrangulol (**22**) in the presence of NADPH, but none of the compounds were reduced, indicating a rather strict substrate specificity for LugG (Figure S1).

Fully Reduced Quinones Are Formed on the Thioangucycline Pathway by TacO and TacA. Previous metabolic studies have indicated that the SDR-family enzymes TacO and TacA are 7- and 12-ketoreductases, respectively.[29] Incubation of **12** with TacO converted the substrate into 12-deoxo-12-hydroxy-8-*O*-methyltetrangomycin (**20**, Figure 4), which verified that TacO is orthologous to the 7-ketoreductase LugG, as expected based on phylogenetic analysis (Figure 1C) and prior metabolic studies.[29]

To study the 12-ketoreduction reaction in vitro, we assayed TacA with several angucyclinones **10**, **12**, **13**, **14**, **15**, **21**, and **22**, all of which were converted into new products **23**−**29**, respectively (Figure 5). LC-ESI-MS/MS analysis showed that **24** has a predicted mass (Figure S54). The 12-ketoreductase activity was confirmed by scaling up the enzyme reaction, producing **24** for structure elucidation by 1D and 2D NMR analyses (Figures S22−S28 and S41). The data confirmed that one ^{13}C NMR signal for a carbonyl carbon disappeared from **24** in comparison to **12** (Figures S3 and S7), suggesting ketoreduction. Importantly, the regiochemistry of 12-ketoreduction was confirmed from HMBC correlations H11/C12 and H12/C11, as well as from a NOESY signal H11/H12. Recorded NMR spectra were in agreement with previously published.[38] Finally, we performed a one-pot experiment using **7** as a substrate with PgaE, LanV, LugN, and TacA, which led to the formation of the expected product **24** together with two other products **25** and **26** (Figure 5). The accumulation of nonmethylated product **26**, in particular, demonstrated that TacA competes with the 8-*O*-methyltransferase LugN for the substrate **14**.

Combinatorial Enzymatic Synthesis of SM 196 A and Hydranthomycin. Next, we wanted to probe the substrate promiscuity of the various tailoring enzymes and to test if angucyclinones isolated from other *Streptomyces* species could be generated using enzymes from the *lug* and *tac* pathways. Hydranthomycin (7-deoxo-7-hydroxy-12-deoxo-12-hydroxy-8-*O*-methyltetrangomycin, **30**, Figure 6) is a herbicidal antibiotic isolated from *Streptomyces* sp. K93-5305.[39] Initial one-pot reaction with simultaneously added LugG and TacA, or TacO and TacA, together with **12** produced the expected product **30** only in trace amounts. However, stepwise addition of the 7-ketoreductase LugG/TacO and the 12-ketoreductase TacA reduced the quinone fully (Figure 6). First, **12** was incubated with LugG/TacO until the formation of **20** was complete. Subsequent addition of TacA to the reaction mixture led to complete conversion of the product into **30**. In contrast, when LugG or TacO was added to the reaction mixture after

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converting **12** completely into **24** with TacA, no new products were formed, indicating that neither LugG nor TacO has the activity on **24** (Figure 6). The mass of **30** was confirmed using LC-ESI-MS/MS (Figure S55). The enzyme reaction producing **30** was upscaled for structural elucidation through 1D and 2D NMR methods (Figures S29−S34 and S41), which confirmed the structure. The disappearance of two carbonyl signals in the ^{13}C NMR spectrum of **30**, and the appearance of two carbinolic signals H7 and H12 suggested the reduction of two carbonyls. HMBC correlations H6/C7 and H11/C12, and NOESY correlations H7/H8′, H6/H7 and H11/H12 proved that both C-ring quinone carbonyls were reduced, while the C1 carbonyl is not. Recorded NMR spectra for **30** were in good agreement with previously recorded.[29]

SM 196 A (1-deoxo-1-hydroxy-7-deoxo-7-hydroxy-8-*O*-methyltetrangomycin, **31**, Figure 6) is an angucyclinone produced by *Streptomyces* sp. DSM 4769 that displays antibacterial and antiviral bioactivities.[40] Unlike in the case of the formation of **30,** almost complete conversion of **12** into **31** was achieved through sequential 7- and 1-ketoreductions with LugG/TacO and LugOIIred, respectively, regardless of the order of the reactions (Figure 6). The mass of **31** was confirmed using LC-ESI-MS/MS (Figure S56). The enzyme reaction producing **31** was upscaled for structural elucidation through 1D and 2D NMR experiments (Figures S35 and S41), which confirmed the structure. Mainly, only one carbonyl signal was present in the ^{13}C NMR spectrum, and two new carbinolic signals (H7 and H1) appeared in the ^{1}H NMR spectrum, indicating reduction of two ketones. The HMBC spectrum showed intense correlation between H11 and the carbonyl carbon, indicating that the carbonyl is at position 12. The HMBC spectrum showed correlations between H1 and the carbons in the A-ring, as well as correlations H7/C8, H7/C11a, H7/C12a, and H6/C7, indicating that the ketoreductions are at positions 1 and 7.

DISCUSSION

The relatively recent discoveries of C-ring-cleaved angucyclines have further expanded the chemical diversity of this large group of microbial natural products.[1] Studies into the biosynthesis of lugdunomycin and thioangucycline have predominantly focused on molecular genetic studies to date. Here, we carried out biochemical investigations into early tailoring steps of C-ring-cleaved angucyclines and elucidated the branching points of angucycline biosynthetic pathways. We demonstrate that in lugdunomycin biosynthesis, 12-hydroxylation by LugOI and 6-ketoreduction by LugOIIred are the first tailoring steps after the formation of the key common angucyclinone precursor **7** akin to landomycin biosynthesis (Figure 2). Interestingly, the methylation and 1-ketoreduction catalyzed by LugN and LugOIIred, respectively, appear to be highly promiscuous and may occur in any order (Figures 2 and 3). In contrast, the 7-ketoreductase LugG harbors a more stringent substrate specificity and requires 8-*O*-methylated angucyclinones as substrates (Figure 4). These tailoring reactions prime the pathway toward C-ring cleavage, which has been proposed to occur via 6a/12a epoxidation catalyzed by LugOIII and subsequent ring cleavage by LugOV.[28]

Our bioinformatic analysis indicates that *tac* BGC encoded angucyclinone biosynthesis is likely to proceed following a similar paradigm. Orthologous gene products to the lugdunomycin pathway exist for each early tailoring step with a high degree of sequence identity (Table 1). The final shared step in lugdunomycin and thioangucycline biosynthesis appears to be 7-ketoreduction catalyzed by LugG and TacO, respectively. We show that *tac* BGC has an additional 12-ketoreduction catalyzing SDR enzyme TacA, leading to a divergence of the pathways. We show enzymatically that the 7-ketoreductase TacO and the 12-ketoreductase TacA convert **12** into **30**. This study revealed deep insights into the activity and promiscuity of the C-ring quinone reductases. However, further studies assaying these reductases together with LugOIII and LugOV are needed to gain further insight into the possible role of the quinone reduction in the C-ring cleavage.

Earlier metabolic studies have shown that many angucycline pathways produce atypically large libraries of compounds rather than single effector molecules. Here, we demonstrate that a large contributing factor to this phenomenon is the combination of highly promiscuous enzymes and enzymes with a stricter substrate specificity in a single pathway. The promiscuity of the 8-*O*-methyltransferase LugN and the 12-ketoreductase TacA is more of an indication of a branching biosynthetic network, rather than a linear biosynthetic pathway. In contrast, our studies on the SDR family ketoreductases LugG, TacO, and TacA show surprisingly that reaction order is essential in the formation of certain fully reduced products. More specifically, **12** can be converted into hydranthomycin (**30**) by these ketoreductases only if 7-ketoreduction is performed before 12-ketoreduction. This requirement for a specific reaction order ensures that both products may be formed simultaneously. In contrast, the combination of LugOIIred and LugG can convert **12** into **31** in a two-step reaction regardless of the order of ketoreductions. The key question for future studies is to provide biological context to our observations; are many of the angucyclinones produced by these strains redundant shunt products without any biological function, or are these strains producing mixtures of angucyclinones that function synergistically against competing organisms?

MATERIALS AND METHODS

Bioinformatics. For the prediction of the BGCs in the genome sequence of Streptomyces sp. QL37 (NZ_PTJS00000000.1) and other *Streptomyces* strains, the bioinformatic tool antiSMASH 5.0[41] was used. To ascertain the possible role of each gene in *lug*-cluster in the production of C-ring-cleaved angucyclinones, *lug*-cluster was compared with 28 BGCs responsible for the production of known classical and rearranged angucyclines using clinker.[42] A threshold of 40% aa identity between the predicted gene products was used as cutoff for the presence of a gene. Selected angucycline-producing BGCs were compared using NCBI BLAST, and the figure (Figure 1B) was made using EasyFig.[43] Selected SDR-family ketoreductases were aligned with Clustal Omega using Seaview Version 5,[44] and the dendrogram was generated using PhyML[45] with LG on the same software package.

Protein Production and Purification. To obtain protein for the enzymatic experiments, *lug* genes were cloned into pET-28a (+) vector (Novagen), *tacA* into pET-15b, and transformed into *E. coli* BL21(DE3). In addition, *pgaE*,[21] *lanV*,[20] and *UrdMred*[20] previously cloned into pBHBΔ plasmid were transformed into *E. coli* TOP10. The N-terminal His-tagged proteins were expressed in *E. coli* BL21(DE3) and *E. coli* TOP10 cells in a 2xTY medium using L-arabinose induction for pBHBΔ and IPTG induction for pET-28a and pET-15b expression systems. The proteins were purified in a single affinity chromatography step from the cell lysate. Further detail is provided in the Supporting Information text.

Substrate Production, Isolation, and Purification. The substrates required for the enzyme reactions were produced in

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different *Streptomyces lividans* TK24 mutants that we have previously constructed. TK24/pSJ8 and TK24/pSJ6 were used for producing **7** and **8**, respectively, and TK24/pSJ1e for the production of **8** and **10**.[46] Compounds were produced by incubating the producer strains in E1 medium supplemented with LXA-1180 absorbent resin. Metabolites were eluted from the resin with repeated acetone extractions, dried using rotary evaporation, dissolved in methanol, and fractionated with preparative scale reverse phase HPLC. Further details on culture conditions, media composition, and purification are provided in the Supporting Information text.

Enzyme Assays and Product Analysis. Enzyme reactions were carried out in 200 μL of reaction buffer (100 mM phosphate buffer, pH 7.5). The substrates **7**, **8**, and **12**−**15** were dissolved in MeOH, and the substrates **10**, **20**, and **21** were dissolved in DMSO before adding to the reaction buffer. The enzyme concentrations were optimized for each reaction. Either 0.25−1.25 mM NADPH or an NADPH regeneration system was used. Reactions were incubated at 30 °C for 30−120 min, depending on the reaction. The completeness of the reactions was determined spectrophotometrically, as previously.[5] The reaction products were isolated through chloroform extraction and dried and analyzed by HPLC-DAD and LC-MS methods. Some enzyme products were analyzed on high-resolution LC-MS/MS QTOF mass spectrometer as previously described,[31] with the exception that samples were run on both positive and negative ionization modes. Further detail on enzymatic assays and chemical analysis is provided in the Supporting Information text.

Enzymatic and Chemical Production of Tetrangomycin (14), Tetrangulol (21), and 8-*O*-Methyltetrangulol (22). For enzymatic assays, **14** was produced enzymatically from **7** by incubating it with PgaE and LanV. For substrate screening, **14** was converted into **21** as previously described[47] and **12** was converted into **22** as previously described.[40] Further detail is provided in the Supporting Information text.

Enzymatic Production and NMR Analysis of 20, 21, 30, and 31. For NMR structure elucidation, **12** was converted into **20** and **24** with LugG and TacA, respectively. **30** was produced with a two-step enzyme reaction from **12** by first converting it into **20** with LugG and then further converting it into **30** with TacA. **31** was produced with a two-step enzyme reaction from **12** by first converting it into **16** with LugOIIred and then into **31** with LugG. For structure elucidation, **20**, **24**, **30**, and **31** were dissolved in deuterated solvents and analyzed with a 600 MHz NMR spectrometer. The data were processed and analyzed with TOPSPIN (Bruker), and *J*-coupling constants were extracted with ChemAdder (Spin Discoveries Ltd.). Further details on the production and structure elucidation of **20**, **24**, **30**, and **31** are provided in the Supporting Information text and Figures S18−S41.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.4c00082.

Additional experimental details, materials and methods, NMR and MS spectra for major compounds, and UV−vis spectra for all the compounds analyzed with HPLC (PDF)

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Notes

The authors declare no competing financial interest.

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