Hydroxyl regioisomerization of anthracycline catalyzed by a four-enzyme cascade

Zhuang Zhenga,1, Yu-Kang Gonga,b, Qiang Zhoua, Yu Hu, Hong-Min Ma, Yong-Sheng Chen, Yasuhiro Igarashi, Lifeng Pan,1,2 and Gong-Li Tanga,2

Abstract

Natural occurring anthracyclines, usually produced by Streptomyces, belong to a family of aromatic polyketides biosynthesized by type II polyketide synthases (PKSs) (3). During the last 3 decades, biosynthetic studies of this system in bacteria have gained a deeper insight into the anthracycline’s molecular logic and enzymatic mechanism (4–6). In general, the nascent poly-β-ketone generated by minimal PKS undergoes carbon-9 (C-9) reduction first and then cyclizations and dehydrations mediated by cyclases, followed by oxidation of the second ring to afford the anthraquinone portion presented in all anthracyclines. The final hydrolytic release of the anthraquinone produces the nascent anthracycline core, which contains a C-4 hydroxyl group in D-ring (Fig. L4) (3–7). The anthracycline core is then decorated with various tailoring enzymes to generate types of anthracycline antibiotics (8–10). Therefore, most of the anthracyclines possess a C-4 hydroxyl group, and a very few C-4 deoxyanthracyclines have been isolated in nature.

Kosinostatin (KST, 1), a rare C-4 deoxyanthracycline antibiotic produced by marine Micromonospora (M.) sp. TP-A0468, exhibits strong cytotoxicity against various cancer cell lines and an inhibition toward Gram-positive bacteria (11). During our previous studies, the anthracycline intermediate 4, bearing a C-1 hydroxyl group in D-ring, was identified from the kstB1-inactivation mutant strain (Fig. 1B) (12). The high sequence identity between KST gene cluster and other anthracycline gene clusters indicates that KST may generate the C-4 hydroxyl anthracycline as primary anthracycline core; in addition, our labeled acetate feeding experiments further confirmed the prediction that KST has undergone similar assembly process to afford C-4 hydroxyl anthracyline (Fig. 1B) (12). If this is the case, an apparent hydroxyl regioisomerization of anthracycline must be involved in the KST biosynthesis, which is a chemically challenging transformation process. Here, we report a four-enzyme-catalyzed regioslective hydroxylation-dehydration process resulting in the hydroxyl regioisomerization, which is totally distinct from currently known tailoring steps in type II PKS platforms.

Significance

Enzymatic modifications of anthracycline antibiotics are urgently needed in the fields of biosynthesis, biocatalysis, and even medical chemistry. However, neither hydroxyl regioisomerization nor dehydration of anthracycline core was described previously. Here, we discover an unprecedented hydroxyl regioisomerization process in the biosynthesis of a rare carbon-4 deoxyanthracycline, which includes three tailoring steps performed by a four-enzyme cascade: two-component hydroxylases mediated a cryptic hydroxylation, and two NmrA-like short-chain dehydrogenase/reductases catalyzed a reduction-dearomatization followed by a reduction-dehydration process. This study expands the enzymology and chemistry of type II polyketide synthase and provides tools to generate more analogs by engineering or enzymatic semisynthesis.
also encoded by adjacent genes in the aclaromycin and nogalamycin biosynthetic gene clusters, respectively (SI Appendix, Fig. S1). AcER or SnoaL2 was previously discovered to be involved in the production of 1,4-dihydroxylated anthracyclines in vivo; however, in vitro experiment using AcER or SnoaL2 or cell-free lysate showed no bioconversion result (13). In addition, 1,4-dihydroxy-anthracyclines were also isolated from the aclaromycin-producing strain (14). This evidence strongly implies that KstA15 and KstA16 are likely both involved in affording 1,4-dihydroxy-anthracycline, a possible intermediate relating to the bioconversion from 3 to 4 (Fig. 1B). To evaluate their functions, kstA15 and kstA16 were inactivated individually by gene replacement (SI Appendix, Fig. S2). The resultant mutant strains M. TG1711 (ΔkstA15) and M. TG1712 (ΔkstA16) exhibited similar production profiles: both abolished the production of KST and the isomer isoquinocycline (1a) while accumulating two compounds, 3 and 3g (Fig. 2AII and AIV). Evaluations of the NMR and MS spectra (SI Appendix, Note S1 and Note S2) led to the successful identification of their chemical structures, both of which bear a C-4 hydroxyl group in the D-ring while being distinct from each other in the C-7 modification, where 3g is glycosylated with a γ-branched octose (Fig. 2C). The isolation of 3 and 3g, combining with characterized 4 and 4g (Fig. 2AII and C, SI Appendix, Note S4), leads to a confirmation that 3 is biosynthesized by the PKS as the first anthracine core, and subsequently is modified into 4 as second anthracine scaffold by unknown tailoring steps (Fig. 1B). Importantly, KstA15 and KstA16 may perform the first step of modification and synergistically catalyze 1-hydroxylation of 3 to yield 1,4-dihydroxy-anthracycline.

To validate this hypothesis, KstA15 and KstA16 were expressed and purified as His_{6}-tagged proteins (SI Appendix, Fig. S4) and then incubated with 3 and NADH (or NADPH). Subsequent HPLC analysis showed a new peak appears dependent on both enzymes (Fig. 3A). The newly formed compound 5, displaying an obvious red-shift on the UV/Vis spectrometer (Fig. 2B) and increased molecular weight by 16 Da compared with substrate 3, is exactly in agreement with the expected 1,4-dihydroxy-anthracycline product (Fig. 2C). Structural elucidation of 5 provided by preparative enzymatic reactions confirmed 5 to be the 1,4-dihydroxy-anthracycline product (SI Appendix, Note S6). This evidence suggests KstA15/ KstA16 function together to catalyze the C-1 hydroxylation.

To further explore the hydroxylation mechanism, KstA15 and KstA16 were purified under anaerobic conditions and performed the same enzymatic reaction anaerobically. Surprisingly, no production of 5 was observed, but a new compound 3d with a decreased mass of 16 Da appeared, which mainly depended on the
Reduction-Deamortization by KstA11. Our in vitro results unequivocally demonstrated that KstA15/A16 synergistically catalyze hydroxylation of 3 to produce 5, which may serve as an intermediate during the hydroxyl regioisomerization process; thus, a dehydroxylation must be involved in the transformation from 5 to 4. To the best of our knowledge, two enzymatic dehydroxylation pathways of aromatic ring had been reported: Birch-like one electron reduction mechanism, as exemplified by 4-hydroxybenzoyl-CoA reductase, which is a Mo-flavo-Fe/S-dependent protein (16, 17), and reduction-dehydration process-mediated dehydroxylation in the fungal melanin biosynthetic pathway, operated by 1,3,6,8-tetradroxyanthracene reductase, 1,3,8-tridroxyanthracycline reductase, both belonging to the short-chain dehydrogenase/reductase (SDR) superfamily, and scytalone dehydratase (18–20). However, bioinformatic analysis revealed that neither Mo-flavo-Fe/S protein nor scytalone dehydratase homolog has been found in the KST pathway. Although we identified several genes encoding SDR family proteins within the kst cluster, sequential analysis cannot provide any clue about enzymes catalyzing dehydroxylation owing to the reaction flexibility of the SDR family (21). Thus, many efforts have been made to search for gene inactivation mutants producing the same metabolite 5. Fortunately, 5 and its glycosylated relative 5g were both observed from mutant M. TG1713 (kstA11) (Fig. 2AV and C, SI Appendix, Fig. S3), implying that KstA11 may perform the next step of tailoring modification, using 5 as the substrate.

Sequence analysis shows that KstA11 belongs to the NADB-Rossmann superfamily, bearing a conserved NAD(P)H binding motif (GxxGxxG), it also displays a high similarity with the NmrA family transcriptional regulator (22). To characterize the enzymatic function, substrate 5 was incubated with KstA11 purified from Escherichia coli (SI Appendix, Fig. S8) in the presence of NAD(P)H. Interestingly, different products were detected with prolonged reaction time (Fig. 4AI). The intermediate 6a, with a mass increase by 2 Da compared with substrate 5, exhibits different UV/Vis absorption characteristics, and Vismax of 6a falls between 5 and 6b, indicating a middle conjugated degree (Fig. 4E). The final product, 6b, showing a totally different UV/Vis spectral property (Fig. 2B) and the same molecular weight as 6a, is coincident with the dihydro-anthracylene. Structural elucidation of metastable 6b provided by preparative enzymatic reactions confirmed 6b to be the 5,12-dihydroxy-2,3-tetrahydro-1,4-dione product (Fig. 2C, SI Appendix, Note S8). Unfortunately, other enzymatic intermediates are too labile and quickly change into 6b during the extraction process, thereby preventing further purification and structural characterization. Collectively, our enzymatic results preliminarily demonstrated that KstA11 catalyzes the deearomatization of 5 to afford 6b, indicating the dehydroxylation in KST biosynthesis is operated by a reductase-dehydratase pair.

Reduction-Dehydration by KstA10. Previous studies showed that 6b was unstable and could convert into 5 at room temperature spontaneously. After optimizing the handling procedure, we detected the accumulation of 6b as the major metabolite from mutant ΔkstA10 (Fig. 2AVI, SI Appendix, Fig. S3). To verify the function of KstA10, we performed the biochemical assay of purified recombinant protein (SI Appendix, Fig. S10), using 6b as substrate (Fig. 5A). Unexpectedly, the final product 4d, together with a shunt product 4l, could be formed only in the presence of NAD(P)H (Fig. 5BI). Structural elucidation confirmed 4d with the same hydroxyl modification of aromatic ring as 4, but dehydroxylation at C-7 (Fig. 5A, SI Appendix, Note S5). These results preliminarily revealed that KstA10 catalyzes a reduction reaction followed by a dehydration process to rearomatize the D-ring, although less than 10% of 4 was produced after an overnight incubation, and more than 70% of 6b was converted into 5 spontaneously (Fig. 5BII and BIII). Given that 6a is more likely to be adopted by KstA10 than the presence of KstA16 and NAD(P)H (Fig. 3BI–IV). Further structural elucidation by the NMR and MS showed that 3d was a C-7 dehydroxy-anthracylene shunt product (Fig. 3C, SI Appendix, Note S3). In addition, when 3g was incubated with KstA15/KstA16 and NAD(P)H anaerobically, the same enzymatic product 3d was observed through deglycosylation instead of dehydroxylation (Fig. 3BII). We also tested the formation of H2O2 to identify the existence of peroxynthracelene intermediate. Expectedly, H2O2 can be well observed either in the KstA16 or the KstA16/KstA15 coupled reaction system; moreover, the KstA16 reaction system generated more H2O2 than the latter (SI Appendix, Figs. S5 and S6).

When we further investigated the enzymatic mechanism, a similar two-component monooxygenase SnoaW/SnoaL2 involved in nogalamycin biosynthesis was reported, and a reaction model was proposed (15). Our experiment results under anaerobic conditions, together with their proposal, led to replenishing the hydroxylation mechanism; the process resembles the reaction catalyzed by flavoenzymes, where the reduced flavin cofactor activates dioxygen to yield a semiquinone-superoxide radical pair (Fig. 3C). KstA16 first uses NAD(P)H to reduce the quinone moiety of the anthracylene; the reduced substrate then reacts with dioxygen to form highly reactive hydroperoxide intermediate, which then undergoes protonation in the presence of KstA15; the resulting hydroperoxide intermediate undergoes elimination of water to give a ketone followed by a tautomerization to afford the final 1,4-dihydroxylated product. Anaerobically, the resulted anion delocalized into C-7 through resonance over the aromatic ring system, eliminating the hydroxyl of 3 or the glycosyl group of 3g to give 3d (Fig. 3C).

Fig. 3. In vitro biochemical characterization of KstA15/A16-catalyzed hydroxylation. HPLC analysis (UV at 254 nm) of normal enzymatic reaction (A) and anaerobic reaction (B). (C) Proposed enzymatic mechanism.
then we performed consecutive reactions in which the product of KstA11 acts as the substrate for KstA10 (Fig. 5A). Just as expected, more than 90% of substrate 5 was converted into the final product 4 within 1 h (Fig. 5C). Thus, the function of KstA10 as the third tailoring step was fully established in this hydroxyl regioisomerization reaction.

KstA10 is another NmrA-like enzyme belonging to the SDR family with a conserved NAD(P)H binding motif (GxxGxxG) (21, 22). Further analysis indicates that KstA10 is homologous to NAD⁺-dependent nucleotide diphosphate sugar epimerases (23), which act on totally different substrates from that in KST biosynthesis. In addition, scytalone dehydratase in the fungal melanin
biosynthesis is cofactor independent (18, 19). However, evidence obtained from our biochemical assays suggested that the dehydration catalyzed by KstA10 is dependent on NAD(P)H (SI Appendix, Fig. S11). To gain further insights into the enzymatic mechanism, KstA11 and KstA10 assays were conducted in the presence of (S)-[4-2H]NADPH obtained by glucose dehydrogenase from Bacillus megaterium DSM 2004 BmGDH, using [D-1-2H]glucose as the deuterium donor (24, 25), as the proS hydrogen is transferred to the substrate during reaction observed from the cryocystal structure of KstA11 in complex with substrate 5 and NAD\(^+\) (see Structural Insight into the KstA11-Catalyzed Reaction). Expectedly, a 1-Da shifted fragment ion at m/z 416.1103 was detected in a KstA11/BmGDH coupled assay (SI Appendix, Note S9), indicating a deuterium incorporation in 6b; large-scale reactions were then performed to isolate \(^2\)H\(-6b\) and elucidate the exact deuterium incorporation position. A comparison of \(^1^H\)-NMR and \(^1^3^C\)-NMR spectra of \(^2\)H\(-6b\) and 6b showing that C-2 of \(^2\)H\(-6b\) has been split into three peaks (a triplet) and the relative hydrogen peak area has decreased confirmed the deuterium incorporation at C-2 of \(^2\)H\(-6b\) (Fig. 5D and E, SI Appendix, Note S9). A 1-Da shifted fragment ion at m/z 398.0997 and a 2-Da shifted fragment ion at m/z 399.1065 were both observed in the KstA11/ KstA10/BmGDH coupled assay, implying that more than 1 deuterium had been incorporated into 4 (SI Appendix, Note S10). Similarly, we prepared \(^3\)H\(-4\) by large-scale enzymatic reaction and elucidated \(^3\)H\(-4\) by \(^1^H\)-NMR and \(^1^3^C\)-NMR. A head-to-head comparison of \(^1^H\)-NMR and \(^1^3^C\)-NMR spectra of \(^3\)H\(-4\) and 4 showed the peak disappearance of C-4 and relative H-4 of \(^3\)H\(-4\) and a peak attenuation of C-2 and relative H-2 (1/2 peak area), demonstrating the absolute deuterium incorporation at C-4 and partial deuterium incorporation at C-2 in \(^3\)H\(-4\) (Fig. 5D and F, SI Appendix, Note S10). According to the identification of shunt product and deuterium isotope experiments, we proposed that KstA10 functions as a ketoreductase and dehydratase, catalyzing a 1,2-nucleophilic addition, followed by dehydration to aromatize the D-ring. First, KstA10 binds 6a as the real substrate and subsequently isomerizes it into 6b in the active site; next is the nucleophilic addition of proS hydrogen of NADPH to keto-carbonyl of C-4 to form a tetrahedral intermediate. Then the intermediate undergoes elimination of 4-OH, concomitant with hydrogen transferring from 12-OH to carbonyl of C1. Finally, deprotonation of 5-OH accompanied with leaving hydride at C-2 gives the final product 4. In contrast, the formation of 4d may result from deprotonation of 5-OH concomitant with leaving 7-OH, and then deprotonation at C-2 accompanied with protonation of C-7 (Fig. 5D).

Structural Insight into the KstA11-Catalyzed Reaction. To gain mechanistic insight into the unusual reaction catalyzed by KstA11, we determined the crystal structure of KstA11 in complex with substrate 5 and a relevant cofactor NAD\(^+\) to 1.3-Å resolution (SI Appendix, Table S1). In the complex structure, two KstA11 molecules pack together to form a symmetric dimer, and each monomer contains 12α-helixes (α1-α12) and 9 β-strands (β1-β9) and binds with one substrate 5 and one NAD\(^+\) molecule in a 1:1:1 stoichiometry (Fig. 4B). Notably, 5 and NAD\(^+\) directly contact with each other and pack extensively with a solvent-exposed elongated groove located at the middle region of KstA11 (Fig. 4B and C). The specific interactions of KstA11 with substrate 5 and NAD\(^+\) are mainly mediated by hydrophobic contacts and polar interactions (Fig. 4C, SI Appendix, Fig. S7). Specifically, the hydrophobic moiety of 5 packs extensively with the hydrophobic pocket formed by the V114, F126, F150, F155, W158, L171, I246, I252, M255, F256, and F259 residues of KstA11, and the oxygen located at the C-1 position of substrate 5 forms a hydrogen bond with the side chain of Y264 (SI Appendix, Fig. S7A). In parallel, the NAD\(^+\) molecule forms a number of hydrogen bonds with the T14, T16, Q17, D58, T80, K129, and N154 residues, and its aromatic adenine and nicotinamide groups form a cation–π interaction with R37 and a π–π stacking with F151, respectively (SI Appendix, Fig. S7B). Subsequently, mutations that are expected to weaken the interaction between KstA11 and NADH, such as R37E and N154K, or interaction between KstA11 and substrate 5, such as I252Y, all dramatically decrease the catalytic activity (SI Appendix, Figs. S8 and S9). In addition, the conformations of 5 and NAD\(^+\) in the complex are further stabilized by a π–π stacking between 5 and the nicotinamide group of NAD\(^+\), as well as a hydrogen bond between the C-4 oxygen of 5 and the 2'-OH group of NAD\(^+\) (Fig. 4D).

Given the unique chemical structure of 5, the transition state for the KstA11 catalyzed deaminatization reduction of 5 to 6b would be expected to proceed from the 1,4-diketo tautomer of 5 (hereafter referred to as 5a) (Fig. 4F). In the complex, 5a is highly preferred, as the oxygen atoms located at the C-1 and C-4 positions of 5 are well positioned to form two hydrogen bonds with the side chain hydroxyl group of Y264 and the 2'-OH of NAD\(^+\), respectively. Meanwhile, the 2'-OH of NAD\(^+\) also forms a hydrogen bond with K129, which is further hydrogen bonded with the E90 residue to couple with a water molecule (Fig. 4D). Therefore, there is a hydrogen bonding network that proceeds from the water to the C-4 oxygen of substrate 5. On the basis of the aforementioned biochemical and structural data, we proposed an enzymatic mechanism for the KstA11-catalyzed reduction of 5 to 6b (Fig. 4F and G). When substrate 5 and cofactor NADH are in complex with KstA11, the specific interactions among them not only induce the close contact between 5 and NADH but also stabilize the 1,4-diketo 5a. Then, the proS hydrogen of NADH (the hydrogen that is transferred as a hydride to the substrate in the reduction reaction) is directed toward the C-2 position of 5a, as the C-4 of the nicotinamide ring of NAD\(^+\) is highly close to the C-2 carbon of 5 in the complex structure (Fig. 4D). Importantly, aforementioned deuteration-based labeling studies confirmed this type of 1,4-addition reaction (Fig. 5D). Meanwhile, the 2'-OH of NADH, which is fed by the hydrogen bond network that includes K129, E90, and a water molecule, serve as the ultimate proton donor to the C-4 oxygen of 5a to generate the product 6a (Fig. 4G). Finally, 6a is converted to 6b through a chemical rearrangement because of the lower energy level of conjugated system in the latter (Fig. 4F). In line with this proposal, the substitution of the key catalytic K129 residue with Arg essentially abolished the enzymatic activity of KstA11 (SI Appendix, Fig. S9). Interestingly, the E90A mutation has little effect on the enzyme activity, suggesting the K129 may directly couple with a water molecule in the proton relay in this mutant.

Discussion

Combining in vivo genetic characterization and in vitro biochemical studies, we uncovered an unprecedented hydroxyl regioisomerization process operated on the aromatic ring of anthraquinone leading to the 1-hydroxy anthraquinone, an essential intermediate proposed to be a prerequisite for the spirocyclic formation during KST.

Fig. 6. Summary of the hydroxyl regioisomerization performed by KstA15/ A16-KstA11-KstA10.
biosynthesis. This chemically challenging transformation has gone through a hydroxylation and a selective dehydroxylation by two tandem deacetylation and rearomatization processes, which include three tailoring steps acting on the anthracene core: the primary core structure 3 synthesized by the PKS is catalyzed by KstA15/KstA16 to generate a 1,4-dihydroxy intermediate 5; KstA11 performs an asymmetric deacetylation, using 5 as substrate to yield 6a; and 6a can isomerize into 6b in the active site of KstA10, which then undergoes regioselectively reduction-dehydrogenation to afford secondary core structure 4 mediated by KstA10 (Fig. 6).

Significantly, the dehydroxylation operated by KstA11 and KstA10, to the best of our knowledge, is the first enzymatic system realizing the dehydroxylation on the aromatic ring of anthracene. 4-Demethoxydaunorubicin (idarubicin), a synthetic daunorubicin analog, displays superior fat solubility and cellular uptake compared with daunorubicin or doxorubicin, indicating the 4-deoxyanthracenyl core is vital for its antitumor activity (26). Accordingly, it is meaningful to develop an elaborate and versatile system of enzyme-mediated dehydroxylation on the aromatic ring of anthracene. Our results now established feasibly enzyme-mediated dehydroxylation on the aromatic ring of anthracene. It is promising that this dehydroxylation could be used to provide aimed anthracene analogs in the future by protein engineering. In addition, deacetylation or dehydroxylation lend a hand in sense both of chemistry and of biology (16, 17, 27), as the asymmetric hydrogenation of aromatic compounds, which was considered a versatile and practical synthetic strategy method to obtain chiral compound, has been an active and attractive field of methodology research (28, 29). Recently, characterization of the NADPH-dependent reductases involved in fungal 1,8-dihydroxynaphthalene monooxygenase and aflatoxin B1 biosynthesis expanded the substrates to hydroxynaphthoquinones and tricyclic anthrahydroquinones (30–33). The discovery of KstA11-catalyzed regio- and stereoselective hydrogenation of tetracyclic anthracenyls and regioselective dehydroxylation by KstA11/KstA10 couple are remarkable, which indicates that additional enzymes with broad substrate spectrum are waiting to be explored for biocatalysts. Significantly, as a tailoring modification acting on the anthracene core, our results definitely broaden the tailoring spectrum to modify the previously seldom-touched anthracene core to generate more analogs for future cancer drug studies.

Materials and Methods

Materials and methods are summarized in SI Appendix, Materials and Methods. Supporting data are provided in SI Appendix, Figs. S1–S11. X-ray crystallographic data collection, primers, strains and plasmids are summarized in SI Appendix, Tables S1–S3. Physicochemical characterizations of the compounds are provided in SI Appendix, Notes S1–S10.

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