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Nitrososynthase-Triggered Oxidative Carbon–Carbon Bond Cleavage in Baumycin Biosynthesis

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Supporting Information

ABSTRACT: Baumycins are coproduced with the clinically important anticancer secondary metabolites daunorubicin and doxorubicin, which are glycosylated anthracyclines isolated from *Streptomyces peucetius*. The distinguishing feature of baumycins is the presence of an unusual acetal moiety appended to daunosamine, which is hydrolyzed during acidic extraction of daunorubicin from fermentation broth. The structure of the baumycin acetal suggests that it is likely derived from an unknown C3″-methyl deoxysugar cleaved between the C3″ and C4″ positions. This is supported by analysis of the baumycin/daunorubicin biosynthetic gene cluster (*dox*), which also encodes putative proteins consistent with production of an anthracycline dissacharide containing a branched sugar. Notably, the *dnmZ* gene in the *dox* gene cluster possesses high translated sequence similarity to nitrososynthases, which are flavin-dependent amine monooxygenases involved in the four-electron oxidation of amino sugars to nitroso sugars. Herein we demonstrate that DnmZ is an amino sugar nitrososynthase that initiates the conversion of thymidine-5′-diphosphate-L-*epi*-vancosamine to a ring-opened product via a previously uncharacterized retro oxime-aldol reaction.

Daunorubicin (**1**) and its C14-hydroxylated congener doxorubicin (Adriamycin) are archetypal anthracycline natural products that were first entered into clinical practice in 1967 and remain important agents for the treatment of a wide variety of cancers.[1,2] Perhaps less widely appreciated is the fact that these anthracyclines are actually chemical degradation products of a group of more elaborated secondary metabolites, the baumycins (e.g., **2**), which possess distinct, potent, and selective antibiotic and antiproliferative properties (Figure 1).[3,4] The distinguishing feature of baumycins is an unusual acetal moiety of unknown biosynthetic origin linked at the C4′ oxygen of daunosamine. Notably, the baumycin acetal is removed during acidic extraction of the fermentation broth of the producing organism, *Streptomyces peucetius*.[5] The substitution pattern of this six-carbon baumycin acetal suggests that it likely originates from a deoxysugar progenitor via oxidative cleavage of a vicinal diol of the hexose ring. However, the nature of the baumycin sugar precursor and the pathway for its transformation into the acid-labile baumycin acetal has never been addressed.

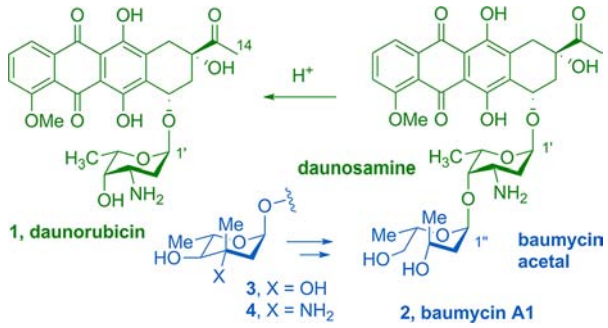

Figure 1. The baumycin family of natural products includes baumycin A1 (**2**), which forms daunorubicin (**1**) upon acid hydrolysis of the acetal moiety (blue in **2**). The structure of the acetal moiety and the *dox* gene cluster suggest that the baumycin acetal is derived from oxidative cleavage of a C3-methyl sugar such as **3** or **4**.

As a result of the clinical importance of doxorubicin, dissecting its biosynthesis in *S. peucetius* has attracted significant attention over the past decades. The identification of the *dox* biosynthetic gene cluster by the Hutchinson group along with targeted gene-disruption experiments have demonstrated that the anthracycline core is the product of a type-II polyketide synthase and various oxidative and methylating tailoring enzymes.[5,6] A glycosyltranferase encoded by *dnmS* appends the 3-amino-2,3,6-trideoxysugar daunosamine to the anthracycline aglycone. An additional glycosyltransferase encoded by *dnrH* has been linked to the formation of acid-sensitive baumycins through gene-inactivation experiments.[7]

We recently characterized *orf36* from the everninomicin gene cluster in *Micromonospora carbonacea* var. *africana*, which encodes a nitrososynthase that catalyzes the oxidation of an amino sugar to its nitroso congener via two consecutive flavin-dependent monooxygenation reactions.[8] Analogous enzymes generating structurally similar nitroso sugars are found in the biosynthetic pathways of rubradirin[9] and kijanimicin.[10] These nitroso sugars then serve as the precursors for nitro sugar moieties in natural products of which they are constituents. Biochemical and X-ray structural studies have demonstrated that these nitrososynthases can utilize $FADH_2$ or $FMNH_2$ and act on anomeric thymidine diphosphate (TDP)-functionalized amino sugar precursors.[11,12] Surprising to us during these endeavors

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was the finding of a putative gene in the *dox* gene cluster, *dnmZ*, with high translated sequence similarity to that of *orf36* (59/70% identity/similarity). This observation was puzzling because daunorubicin and doxorubicin do not possess a nitro(so) sugar. However, analysis of the *dox* gene cluster revealed a number of additional genes that may be involved in the biosynthesis of a C3-methylated TDP-deoxysugar.

This information prompted us to hypothesize that the putative glycosyltransferase DnrH attaches an unidentified C3-methylated deoxysugar (e.g., **3** or **4**, in which X = OH or NH_2, respectively) to **1** and that DnmZ is responsible for the oxidative cleavage reaction converting the putative sugar to the acetal appendage in baumycin A1 (**2**).[13] We initially envisioned that a likely substrate of DnmZ would be TDP-L-mycarose (**3**) (Figure S2a in the Supporting Information), which could be oxidized at the 3-position to form a hydroperoxide intermediate, in analogy with the formation of the *N*-hydroxylamine intermediate from TDP-L-*epi*-vancosamine (**4**) in the first step of the nitrososynthase-catalyzed reaction. The resulting 3′-hydroperoxide-4′-hydroxy compound could then undergo C−C bond cleavage via a Criegee-type pathway,[14] resulting in a dicarbonyl progenitor of the baumycin acetal.

To test this proposal, the genes encoding the DnmZ and DnrH proteins were cloned and overexpressed with N-terminal hexahistidine tags in *Escherichia coli*, and the resulting proteins were purified by Ni affinity chromatography. **3** was prepared in vitro using reconstituted enzymes from the tylosin pathway from *Streptomyces fradiae*.[15] Upon incubation of **3** with DnmZ under typical nitrososynthase reaction conditions, no new products were observed. Furthermore, we also assayed **3** as a substrate for the putative glycosyltransferase DnrH and found no evidence of daunorubicin or doxorubicin glycosylation.

Subsequently, we considered the possibility that the enzymes encoded by the additional sugar biosynthetic genes in the *dox* gene cluster are responsible for producing TDP-L-*epi*-vancosamine itself. Indeed, close inspection of these genes revealed them to be fully consistent with the previously described biosynthesis of TDP-L-*epi*-vancosamine in the chloroeremomicin pathway.[16] Specifically, *dnrX*, *dnmU*, and *dnmV* encode putative proteins with high sequence similarities to a C3-methyltransferase (77%), a C5-epimerase (81%), and a C4-ketoreductase (65%), respectively, in the *epi*-vancosamine pathway (Figure 2). Since the proposed C−C bond cleavage of the retro oxime-aldol reaction occurs at the site of oxidation catalyzed by the nitrososynthase homologue DnmZ, the final product of its reaction with TDP-L-*epi*-vancosamine was expected to be an aldehyde−oxime species (Figure 2).

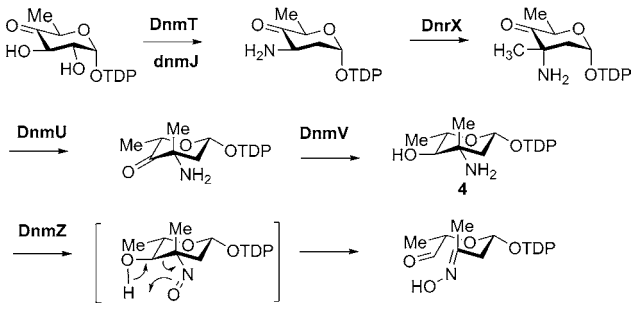

Figure 2. Proposed role for DnmZ nitrososynthase in the baumycin acetal biosynthetic pathway.

Exploration of the chemical precedent for this ring-cleavage transformation revealed two notable entries (Figure 3). First, the

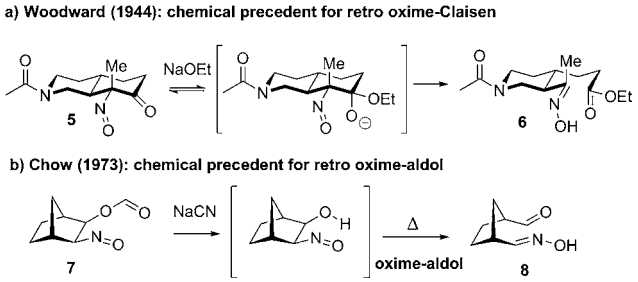

Figure 3. Chemical precedents for nitroso-triggered C−C bond fragmentation.

total synthesis of quinine from 7-hydroxyisoquinoline by Woodward and Doering[17] revealed that a nitroso α-hemiacetal anion of **5** is primed to rapidly cleave to **6** via a retro oxime-Claisen-type reaction. Of close relevance to this work, Chow and co-workers[18] reported that mild nucleophilic hydrolysis of 2-nitroso-3-formyloxybicyclo[2.2.1]heptane (**7**) results in a vicinal hydroxy nitroso species that fragments to form aldehyde−oxime-functionalized cyclopentane **8**. These chemical precedents suggested that a similar fragmentation may affect the key cleavage in the formation of baumycin acetal.

To verify the function of DnmZ, TDP-L-*epi*-vancosamine was generated from TDP-D-glucose using a six-enzyme multistep sequence as described previously.[16] Upon incubation of TDP-L-*epi*-vancosamine with DnmZ, FAD, NADPH, and flavin reductase, we observed the predicted nitroso product of TDP-L-*epi*-vancosamine (m/z 558) and an additional chromatographically distinct compound (m/z 576) corresponding to a hydrated species (Figure 4). The rapid turnover of TDP-L-*epi*-vancosamine suggested that this compound, or a closely related analogue, is the substrate for DnmZ.

These data are consistent with the expectation that the resulting vicinal hydroxy nitroso sugar **9** would undergo cleavage via a retro oxime-aldol reaction to give **10** in accordance with the chemical precedents. Unfortunately, the resulting putative aldehyde−oxime species **10** was found to be highly unstable under reversed-phase and ion-exchange chromatography conditions and thus could not be isolated for direct structural characterization. To provide evidence supporting the structure of **10**, we treated it in situ with different hydrazine reagents, including phenylhydrazine, 2-bromophenylhydrazine, and (carboxymethyl)trimethylammonium chloride hydrazide (Girard's reagent) at the end of the DnmZ reaction.[19] All of the hydrazines reacted smoothly with aldehyde **10** to generate the expected hydrazones, as confirmed by accurate mass determination (Figures 4 and S14). MS/MS analysis of bromophenylhydrazone **12** confirmed the fragmentation of the TDP group as observed in negative-ion mode.

The Girard's hydrazide adduct, **13**, facilitated MS/MS analysis in positive-ion mode, and the fragmentation data were fully consistent with the proposed structure. Finally, we subjected **13** to acid-catalyzed cleavage of the acetals to generate two aldehyde-functionalized fragments, which we captured in situ as the dihydrazides **14** and **15**, whose structures were confirmed by accurate mass determination and MS/MS analysis (Figures 5 and S14). These results strongly suggest that DnmZ is a flavin-dependent monooxygenase that generates a vicinal hydroxy

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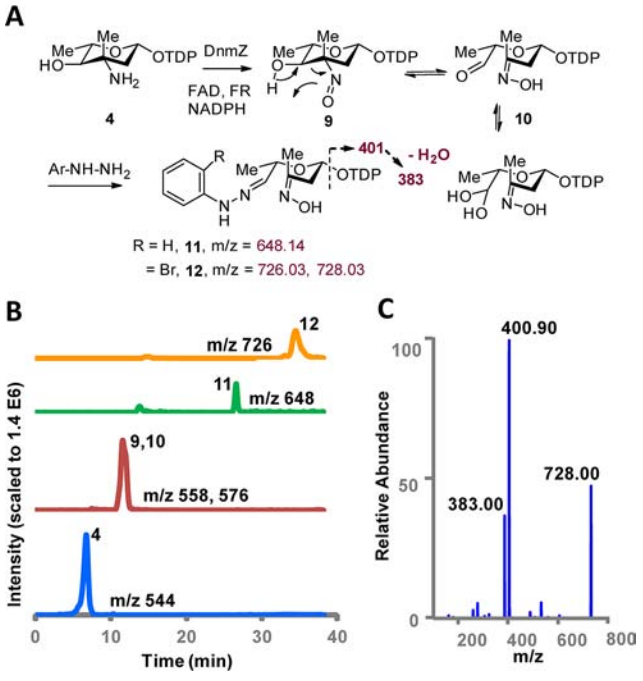

Figure 4. (A) Reaction of TDP-L-*epi*-vancosamine (**4**) with DnmZ, FAD, *Vibrio* flavin reductase (FR), and NADPH and subsequent hydrazone derivatization of the aldehyde−oxime product. (B) Extracted-ion chromatograms of the reaction of TDP-L-*epi*-vancosamine (blue) with DnmZ (red) and separate reactions with added phenyl- and 2-bromophenylhydrazine (green and orange, respectively). (C) MS/MS spectra of hydrazone adduct **12** (selected precursor ion *m*/*z* 728.03).

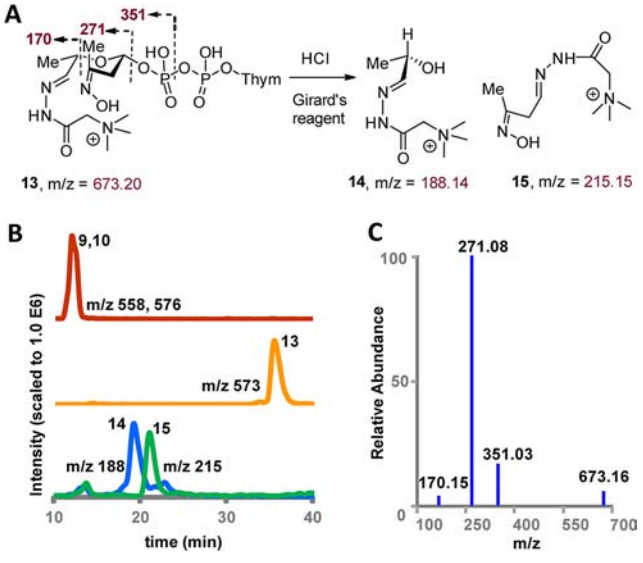

Figure 5. (A) Acid-catalyzed degradation of Girard's adduct **13** derived from aldehyde−oxime **10**. (B) HPLC/MS extracted-ion chromatograms for the reaction of DnmZ (red) with Girard's reagent (orange) and the HCl degradation products **14** (blue) and **15** (green). (C) MS/MS data for **13** (selected precursor ion *m*/*z* 673).

nitroso species, which undergoes a subsequent catalyzed or uncatalyzed retro oxime-aldol cleavage reaction.

The apparent oxime-aldol reaction observed subsequent to the DnmZ-catalyzed nitrososynthase reaction described herein offers some insights into the biosynthesis of the orthoester antibiotic everninomicin, the polyketide rubradirin, and the baumycins.[9,10,20] In our previous studies of nitro sugar formation in the everninomicin and rubradirin pathways, the nitrososynthase activity of ORF36/RubN8 was established using as the substrate TDP-L-*epi*-vancosamine, which is a biosynthetic precursor to the expected substrate, TDP-L-evernosamine (**16**). We observed the hydrated aldehyde−oxime species **10** (*m*/*z* 576) but were unable to account for its structure in the context of nitro sugar formation.[12] The evidence presented herein for the equilibrium between the nitroso and aldehyde−oxime rearrangement products **9** and **10** helps clarify this unresolved question and demonstrates that oxidation of TDP-L-*epi*-vancosamine results in an equilibrium mixture of **9** and **10** for the nitrososynthase in everninomicin biosynthesis. The new DnmZ data suggest that the true substrate of ORF36 is presumably TDP-L-evernosamine, which differs from TDP-L-*epi*-vancosamine by 4-O-methylation (Figure 6) and therefore would be protected from this retro oxime-aldol cleavage reaction.[21]

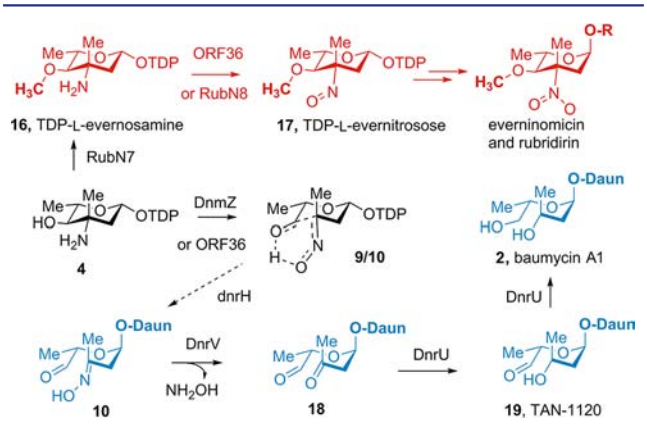

Figure 6. Importance of nitrosylation of TDP-L-*epi*-vancosamine (**4**) to everninomicin/rubradirin and baumycin A1 biosynthesis. The red route shows the late stages of evernitrosose formation, in which methylation blocks C−C bond cleavage. R designates the everninomicin or rubradirin aglycone. The blue route is a possible pathway for the biosynthesis of baumycin A1 (**2**) via the known metabolite TAN-1120 (**19**), which exists in solution as the hemiaminal with daunosamine.

To test this prediction, we next cloned, overexpressed, and purified RubN7, the putative methyltransferase for 4-O-methylation of TDP-L-*epi*-vancosamine in the rubradirin pathway.[9] Upon incubation of TDP-L-*epi*-vancosamine with *S*-adenosyl methionine and RubN7, we observed the formation of TDP-L-evernosamine. Upon further incubation with ORF36/DnmZ, the formation of TDP-L-evernitrosose (**17**) was observed, and turnover in the reaction of DnmZ with TDP-L-evernitrosose was slower than with ORF36 (Figure S8). These results demonstrate that the methylation state of the 4-hydroxyl group of TDP-L-*epi*-vancosamine determines whether this oxidation reaction leads to a nitro sugar or the baumycin acetal pathway.

While our results show that the precursor for the acetal in the baumycin pathway is L-*epi*-vancosamine, questions remain as to the reaction sequence transforming aldehyde−oxime **10** into the fully reduced baumycin acetal. While highly speculative at this point, it is possible to envision a biosynthetic pathway for baumycin biosynthesis that branches from the daunorubicin pathway (Figure 6). Formally, the oxime in **10** must be hydrolyzed to give ketone **18**, and both carbonyls in **18** must be reduced to their corresponding alcohols. It is noteworthy that the potent angiostatic secondary metabolite TAN-1120 (**19**)

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from *Streptomyces triangulatus* is a previously isolated member of the baumycin family in which the aldehyde functionality is not reduced. Instead, it is observed as a hemiaminal with the adjacent daunosamine 3′-amine. The existence of TAN-1120 suggests that it may be a biosynthetic intermediate and that glycosylation precedes final reduction in the baumycin pathway.[3] As revealed by the *dox* gene cluster, the hydrolysis may be catalyzed by DnrV, a putative hydrolase related to the bleomycin resistance determinant, whereas DnrU, a putative aldehyde dehydrogenase, may be responsible for one or more of the requisite carbonyl reductions.

Also remaining to be determined is the timing of glycosylation of the progenitor for the baumycin acetal. Incubation of daunorubicin and TDP-L-*epi*-vancosamine with the putative glycosyltransferase DnrH in the presence and absence of the nitrososynthase DnmZ did not yield any new products. This could be the case because the anthracycline substrate of DnrH is an upstream precursor of daunorubicin (e.g., rhodomycin-D) or the donor acetal is one of the more reduced acetal diphosphates.

In summary, this study demonstrates the unexpected role of the nitrososynthase class of flavin monooxygenases in a previously uninvestigated pathway for deoxysugar C3−C4 bond cleavage biochemistry. The enzyme-catalyzed oxidation of TDP-L-*epi*-vancosamine to its vicinal hydroxy nitroso congener triggers C−C bond cleavage. The overall rearrangement of the hydroxy nitroso sugar to the aldehyde−oxime product is formally that of a retro oxime-aldol transformation. Future studies will ascertain whether the cleavage reaction is enzymatically catalyzed or spontaneous. In either case, from a mechanistic standpoint the cleavage bears a resemblance to that by class-I aldolase enzymes, which activate the retro aldol cleavage via formation of a lysine iminium ion at the 2-keto position to open fructose between C3 and C4.[22] In the retro oxime-aldol mechanism, the polarized nitroso functional group is the surrogate for the iminium group of an aldolase. To the best of our knowledge, this study provides the first evidence of an enzyme that initiates deoxysugar C−C cleavage and expands our understanding into the late steps of baumycin biosynthesis, which is now functionally linked by this study to the biosynthesis of daunorubicin and doxorubicin in *S. peucetius*. From a broader perspective, it is conceivable that oxidation of amino sugars by reactive oxygen species could result in the generation of transient vicinal hydroxy nitroso sugars. On the basis of the results presented here, this would provide a potential mechanism of oxidative deglycosylation of amino sugar functional oligosaccharides via oxidative burst metabolism.

■ ASSOCIATED CONTENT

Supporting Information

Enzymological methods and spectral characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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