Enzymatic Synthesis of the C-Glycosidic Moiety of Nogalamycin R

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Supporting Information

ABSTRACT: Carbohydrate moieties are essential for the biological activity of anthracycline anticancer agents such as nogalamycin, which contains L-nogalose and L-nogalamine units. The former of these is attached through a canonical O-glycosidic linkage, but the latter is connected via an unusual dual linkage composed of C−C and O-glycosidic bonds. In this work, we have utilized enzyme immobilization techniques and synthesized L-rhodosamine-thymidine diphosphate (TDP) from α-D-glucose-1-TDP using seven enzymes. In a second step, we assembled the dual linkage system by attaching the aminosugar to an anthracycline aglycone acceptor using the glycosyl transferase SnogD and the α-ketoglutarate dependent oxygenase SnoK. Furthermore, our work indicates that the auxiliary P450-type protein SnogN facilitating glycosylation is surprisingly associated with attachment of the neutral sugar L-nogalose rather than the aminosugar L-nogalamine in nogalamycin biosynthesis.

Many natural products owe their biological activity to carbohydrate units attached to aglycones.1 Examples of microbial secondary metabolites of medical relevance include the anticancer agent doxorubicin,2 the antibiotic erythromycin,3 and the anthelmintic drug avermectin.4 The most common mode of attachment is via an O-glycosidic linkage, which is unfortunately susceptible to hydrolysis,5 as are carbohydrate units appended through N- or S-glycosylation. Chemically more robust C-glycosylation has been reported in selected instances.6 Of particular interest is nogalamycin (1, Figure 1),7 produced by Streptomyces nogalater ATCC 27451, where the aminosugar nogalamine is attached both via an O-glycosidic linkage and an additional carbon−carbon bond.

The great diversity of naturally occurring carbohydrates has raised considerable interest in their biosynthesis, and several pathways have been elucidated in vitro with purified enzymes.8−13 The most common starting material is α-D-glucose-1-phosphate (2, Scheme 1), which is activated further by nucleotidyl transferases to generate TDP-glucose (3, Scheme 1). Most glycosylated bacterial secondary metabolites are derived from TDP-6-deoxyhexoses, and the second biosynthetic step catalyzed by TDP-D-glucose-4,6-dehydratase is typically also conserved. After formation of TDP-4-keto-6-deoxy-α-D-glucose (4, Scheme 1), the pathways diverge to various C2, C3, or C4 deoxysugars and aminosugars.13,14

The C-glycosidic unit of 1 has raised considerable interest, and the fragment was chemically synthesized soon after the stereochemistry of the natural product was confirmed in 1983.15 However, understanding the biosynthesis and attachment of nogalamine has been more challenging. Characterization of the gene cluster and heterologous expression studies in S. albus led to the isolation of nogalamycin R (5, Figure 1), where L-rhodosamine (4″-epi-2″-deoxy-nogalamine) is attached via the dual linkage system to the anthracycline aglycone.16 Recent studies have verified that the C2−C5″ bond and C4″ epimerization are catalyzed by two related α-ketoglutarate and...
nonheme iron-dependent enzymes SnoK and SnoN, respectively. In this Letter, we report further molecular genetic characterization of the nogalamycin gene cluster and confirm TDP-L-rhodosamine as a true pathway intermediate by enzymatic synthesis.

L-Rhodosamine is a commonly occurring carbohydrate in anthracyclines such as aclacinomycins (6, Figure 1), rhodomyccins (7, Figure 1), cosmomycins, and cytorhodins. To the best of our knowledge, the enzymatic synthesis of TDP-L-rhodosamine (8, Scheme 1) has not been reported, although it has been chemically synthesized from TDP-L-daunosamine via N-dimethylation. The bioinformatic analysis of the nogalamycin gene cluster was complicated by the existence of additional gene products, which have not been experimentally verified, that are involved in the biosynthesis of L-nogalose, the neutral carbohydrate at C7 in 1. The nucleotidyl transferase SnogJ and the 4,6-dehydratase SnogK may be shared in the biosynthesis of the two carbohydrates for generation of the putative last common intermediate 4. We surmised that the next step would be catalyzed by the 2,3-dehydratase SnogH leading to the formation of TDP-3,4-diketo-2,6-dideoxy-α-D-glucose (9, Scheme 1), which would allow transamination by the pyridoxal 5′-phosphate (PLP)-dependent SnogI to generate TDP-3-amino-4-keto-2,3,6-trIDEOXY-α-D-glucose (10, Scheme 1). The pathway could then proceed through 5-epimerization by SnogF (11, Scheme 1) and 4-ketoreduction. Previous models have suggested that the 4-ketoreduction leads to TDP-1-acosamine, which has the same stereoisometry at C4 as the end product L-nogalamine, but we hypothesized that SnogG would catalyze the formation of TDP-1-daunosamine (12, Scheme 1) instead, in a manner similar to daunorubicin and aclacinomycin biosynthesis. Finally, the cluster harbors two homologous genes that code for methyl transferases, SnogX and SnogA (54% sequence identity), that could be responsible for the generation of TDP-L-rhodosamine (8, Scheme 1).

We opted to utilize 3 as the starting material for the synthesis and cloned overexpression constructs for heterologous production of the required proteins in Escherichia coli TOP10. Most of the proteins were cloned from the nogalamycin pathway, but snogK was replaced with the orthologous rmlB originating from E. coli K12. The proteins were produced as N-terminally histidine tagged enzymes, which allowed single-step purification to near homogeneity by affinity chromatography (Figure S1).

We proceeded to perform one-pot enzymatic synthesis for production 8 with the seven enzymes. One of the challenges in multienzyme catalysis is to find conditions where all components are functional. In our case, the solubility of SnogG and SnogA was poor, which led to precipitation of the enzymes over time and, for instance, prevented the use of centrifugal concentrators to reach micromolar protein concentrations. To solve this issue, we immobilized the seven enzymes to TALON affinity beads and conducted the reactions under gentle shaking at 23 °C in a suitable reaction buffer, with 10 mM of 3, 10 μM of SnogH, 30 μM of the other proteins, and 90 μM to 6 mM of the various cofactors and cosubstrates. The benefits of the approach were many-fold, since in addition to improving stability issues, protein immobilization techniques have been shown to enhance overall catalysis by increasing local enzyme concentrations.

Monitoring of the reactions by LC-MS revealed formation of the expected ion 8 ([M−H]−; calcd, 558.1; found, 558.2) as the product. All of the substrate 3 was consumed, but minor quantities of various putative intermediates could be observed (Figure S2). Surprisingly, the presence of both methyl transferases was not essential for the reaction and 8 could be detected in reactions with either SnogX (Figure 2A) or SnogA.
reaction time to 5 h (Figure 2B). The relative activity measurements imply that SnogA is poor in converting monomethylated intermediates to 8. This indicates that the biological role of SnogA in nogalamycin biosynthesis may be to carry out the first methylation reaction, followed by the second methylation step by SnogX, even though both enzymes are capable of catalyzing dimethyllations.

The glycosyl transferase SnogD has been identified to be responsible for the glycosylation at C1,16 and the crystal structure of SnogD has been determined,25 but to date the reaction has only been probed in vitro in the reverse direction. Many glycosyl transferases have been shown to require the aid of P450-like enzymes for catalysis,26 such as AknS/AknT in aclacinomycin biosynthesis, where they are responsible for the transfer of L-rhodosamine to C7 of the aglycone.27 Although the exact function of these auxiliary proteins is unknown, structural studies have indicated their involvement in allosteric activation of glycosyl transferases.26 Generally, these proteins have been implicated to be involved specifically in the transfer of aminosugars.28–31 To investigate whether the corresponding gene snogN is involved in the transfer of 8, we inactivated the gene from the cosmid pSnoaori by RED/ET recombineering in E. coli and introduced the engineered cosmid pSnoΔgN to S. albus by conjugation. Analysis of culture extract revealed surprisingly that the main metabolite produced was nogalamycinone (13, Figure 2B), indicating that SnogN may be working in conjunction with the TDP-1-nogalose transferase SnogE. This would imply that the requirement for auxiliary proteins has more to do with the chemical structure of the aglycone acceptor molecule rather than the donor TDP-aminosugar in nogalamycin biosynthesis. To rule out polar effects, the mutation to snogN was complemented with an intact copy of the gene, which restored production of the double glycosylated metabolite 5 (Figure 2C).

In order to unequivocally confirm the identity of the synthesized nucleotide sugar as 8, we continued the enzymatic synthesis and attached the TDP-carbohydrate to a monoglycosylated nogalamycin scaffold (14, Scheme 1), since double glycosylated nogalamycin standards were available from previous studies.17 The expected product 5 was observed after incubation of the two substrates in the presence of 7 μM of the glycosyl transferase SnogD30 and 2.5 μM of the carbocyclase SnoK,17 α-ketoglutarate, Fe(II) and ascorbate (Figure 2D). The experiment verified the stereochemistry of the attached aminosugar, since the 4″-epimer (15, Figure 1) of 5 has a distinct retention time under our analytical conditions (Figure 2D).17 To the best of our knowledge, this represents the first example of enzymatic synthesis of a TDP-aminosugar and its attachment to a polyketide scaffold.

In summary, this work describes a two-pot reaction of nine enzymes associated with TDP-1-rhodosamine (8) formation and attachment in biosynthesis of the anthracycline nogalamycin. It provides an instructive example of how microbial secondary metabolism pathways have evolved in a circuitous manner; both (i) the initial 4-ketoreduction by SnogG and late rehydroxylation putatively by SnoT are unnecessary from the biological role of SnogA in nogalamycin biosynthesis may be to carry out the first methylation reaction, followed by the second methylation step by SnogX, even though both enzymes are capable of catalyzing dimethyllations.

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### METHODS

**Strain and Culture Conditions.** Escherichia coli TOP10 (Invitrogen) was used as the cloning host. E. coli K12 was used as a
host for engineering the cosmid pSnogaori and ET12567/pUZ8002,52 for intergenic conjugation to *Streptomyces albus.* 53,54 *Streptomyces* cultivations were performed in NoS-soyE1,55 tryptone soya broth (TSB; Oxoid), R2 yeast extract (R2YE), and mannitol soya flour medium (MS). E. coli strains were cultivated in Luria–Bertoli or 2 x yeast extract/tryptone medium (2xTY) and stored at -80 °C. The crude lysate was mixed with TALON Superflow (GE Healthcare) at 4 °C for 30 min, or by omitting SnaGo or SnaX. After immobilizing the enzymes, the master mix [buffer pH 7.5; 50 mM NaPO4·12H2O, 100 mM NaCl, 5% (v/v) glycerol, 90 μM pyridoxal 5′-phosphate (PLP; Sigma-Aldrich), 2 mM S-adenosylmethionine (SAM; Sigma–Aldrich), 6 mM nicotinamide adenine dinucleotide (NAD+, Sigma–Aldrich), 3 mM L-glutamate and 10 mM thymidine-5′-diphospho-β-β-glucose (TDP-β-β-glucose; Carboxylase)] was added and incubated at 23 °C for 2 h with gentle shaking. The reaction mixture was centrifuged at 700g for 10 min at 4 °C, and the supernatant was carefully removed and analyzed by LC-MS (Agilent 1260 Infinity 6120 Quadrupole LC/MS) using the Phenomenex synergy fusion RP (4 μm, 4.6 x 150 mm) column and a gradient from 0.1% formic acid to 100% acetonitrile.

The second reaction was achieved with a reaction containing buffer pH 8, [50 mM NaPO4·12H2O, 100 mM NaCl, 5% (v/v) glycerol], 7 μM SnogD, 2.5 μM SnoK, 6 mM 8 (the concentration was estimated from LC-MS traces, Figure S2), 150 μM 14, 90 μM α-ketoglutarate (αKG), 100 μM Fe(II)SO4, and 200 μM L-ascorbate. The reaction was performed in 200 μL for 3 h at 30 °C with gentle shaking. The reaction was extracted with chloroform and analyzed by HPLC using a reverse phase column (Phenomenex Kinetics, 2.6 μm, 4.6 x 100 mm) using a gradient from 15% acetonitrile with 0.1% formic acid to 100% acetonitrile. The reaction product was compared to authentic standards 5 and 15 obtained from previous studies.16-17 The yield of the reaction was 10.4% as calculated based on integration of HPLC peaks at 256 nm where the absorbance of the substrate and product are comparable.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b00658.

The SDS-PAGE gel of the enzymes used in this study (Figure S1); the purity of 8, shown by LC/MS (Figure S2); the list of the primers used in this study (Table S1) (PDF)

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**Notes**

The authors declare no competing financial interest.

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