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COMMUNICATION

The Rieske Oxygenase SnoT Catalyzes 2′′-Hydroxylation of L-Rhodosamine in Nogalamycin Biosynthesis

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Abstract: Nogalamycin is an anthracycline anti-cancer agent that intercalates into the DNA double helix. The binding is facilitated by two carbohydrate units L-nogalose and L-nogalamine, which interact with the minor and major grooves of DNA, respectively. However, recent investigations have shown that nogalamycin biosynthesis proceeds through attachment of L-rhodosamine (2′′-deoxy-4′′-epi-L-nogalamine) to the aglycone. Here we demonstrate that the Rieske enzyme SnoT catalyzes 2′′-hydroxylation of L-rhodosamine as an initial post-glycosylation step. Furthermore, we establish that the reaction order continues with 2–5′′-carbocyclization and 4′′-epimerization by the non-heme iron and 2-oxoglutarate-dependent enzymes SnoK and SnoN, respectively. These late stage tailoring steps are important for the bioactivity of nogalamycin due to involvement of the 2′′- and 4′′-hydroxyl groups of L-nogalamine in hydrogen bonding interactions with DNA.

Anthracyclines are medically relevant microbial natural products that are widely used in the treatment of cancer.[1] In particular, doxorubicin is a first choice chemotherapy agent for both solid tumors and hematological malignancies.[2] The biological activity of anthracyclines is complex and not fully understood despite 40 years of investigations.[3] Poisoning of topoisomerase II and intercalation to DNA, which cause double-stranded breaks and arrest replication, have been thought to be highly important.[4] Other factors include formation of reactive oxygen species (O_2•−, H_2O_2, and OH•), DNA adduct formation,[5] the ability to evict histones from chromatin,[6] and proteolytic activation of transcription factors.[7]

Anthracyclines consist of an polyphenolic 7,8,9,10-tetrahydro-5,12-naphthacenoquinone aglycone that intercalates to DNA.[1] However, the biological activity is enhanced by carbohydrate units typically positioned at C7 that facilitate DNA binding.[8,9] Nogalamycin (**1**) is an atypical anthracycline that contains two carbohydrate units that can be thought to provide two handles for grasping the DNA molecule.[10] The neutral L-nogalose unit at C7 interacts with the minor groove, whereas the amino sugar L-nogalamine is positioned in the major groove (Figure 1).[11] The interaction of L-nogalamine to DNA is further facilitated through the unusual dual attachment to the anthracycline chromophore via a canonical O-glycosidic bond and an additional C2–C5′′ bond. The latter restricts the rotational freedom of the carbohydrate placing it perpendicular to the aglycone. In a crystal structure of a nogalamycin-oligonucleotide complex, the 2′′-hydroxyl group of L-nogalamine makes a strong 2.5 Å hydrogen bond to a guanine, whereas the 4′′-hydroxyl group forms a 3.4 Å electrostatic interaction with a cytosine on the opposite strand (Figure 1).[10]

The biosynthesis of nogalamycin has been extensively studied in the producing organism *Streptomyces nogalater* ATCC 27451.[12,13,14] The biosynthetic gene cluster encodes more than 30 genes[15] and many steps have been elucidated both *in vivo* and *in vitro*.[16,17,18] These studies have revealed that, surprisingly, the carbohydrate attached at C1 is initially L-rhodosamine,[19] which lacks the 2′′-hydroxyl group and has an opposite stereochemistry at C4′′. Pathway intermediates have been shown to harbor lower biological activity particularly towards topoisomerase II in comparison to **1**,[15] possibly due to non-ideal interactions of the carbohydrates with DNA. In this paper, we present evidence that the 2′′-hydroxyl group is installed by the Rieske protein SnoT as a post-glycosylation event and propose a biosynthetic order for the final steps in nogalamycin biosynthesis during the conversion of L-rhodosamine to L-nogalamine.

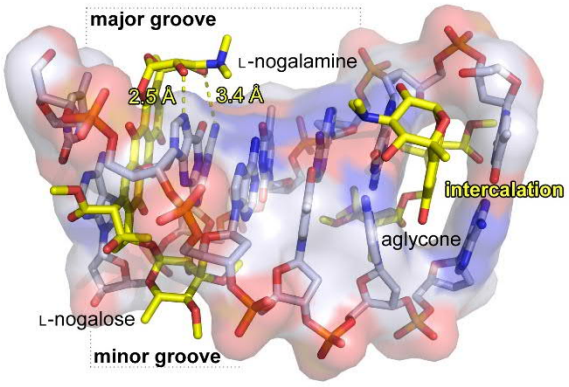

Figure 1. Nogalamycin-oligonucleotide complex[10] demonstrating the importance of the 2′′- and 4′′-hydroxyl groups of L-nogalamine in hydrogen bonding interactions with DNA.

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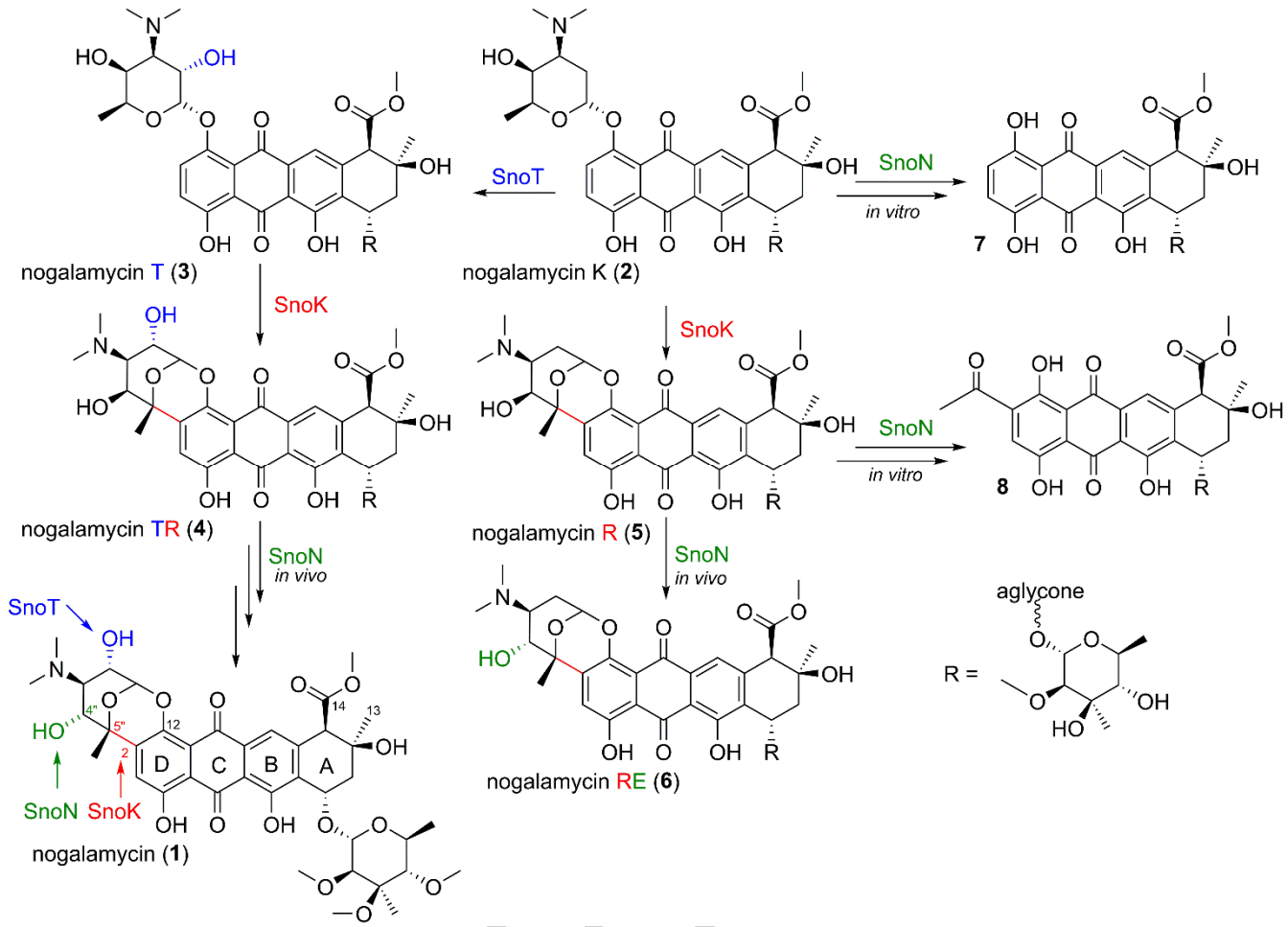

Scheme 1. Possible pathways for late-stage biosynthesis of nogalamycin. This work demonstrates that the initial step is hydroxylation of the C2''-position of L-nogalamine by SnoT, which is followed by SnoK catalysed C2''–C5'' carbocyclization and SnoN catalysed C4'' epimerization. The reactions of SnoK and SnoN have been previously investigated.[16]

Analysis of the nogalamycin biosynthetic gene cluster[15] indicated that only few genes of unknown function, which could potentially catalyze redox chemistry, remained uncharacterized. Our attention was drawn to *snoT*, which encodes a putative Rieske oxygenase, as the likely candidate for 2''-hydroxylation of L-rhodosamine. SnoT harbors 27% sequence identity to the ring oxidizing stachydrine demethylase Stc2, which contains the characteristic [Fe_2S_2] cluster associated with Rieske enzymes and mononuclear iron in the active sites.[20] Structure based sequence alignment (Figure S1) revealed conserved amino acid residues, Cys110, His112, Cys130 and His133 for coordination to the former, and His230, His235 and Asp354 for the latter ligand, in SnoT.

We initiated the study by expressing *snoT* in the heterologous host *Streptomyces albus*/pSnogaori, which contains a cosmid encoding the majority of the nogalamycin pathway responsible for production of **5** and other anthracyclines. Analysis of culture extracts of *S. albus*/pSnogaori+T revealed a new compound with a +16 increase in mass in addition to the other anthracyclines. However, the chromatographic separation proved challenging due to instability issues and the comparable chemical nature of the detected compounds. The novel compound **4** behaved similarly to **5** (Figure S2) with near identical retention times in preparative high-pressure liquid chromatography (HPLC). Limited separation was achieved by two consecutive runs, when collecting only the latter part of the peak. As a result, we focused on the structure elucidation of the amino sugar moiety by proton NMR. 1DTOCSY measurement confirmed the position of the 2''-hydroxyl group by selective irradiation of the anomeric hydrogen to reveal the carbohydrate spin system (Figure S3). Comparative analysis with **5** indicated that the methylene hydrogens (δ 2.23, 2.41) were missing and a downfield hydrogen (δ 3.90) had appeared instead, which was consistent with the attachment of a hydroxyl group. Furthermore, the coupling constant between H2'' and H3'' (*J*=11.0 Hz) indicated towards axial-axial coupling, whereas the coupling constant between H3'' and H4'' was small (*J*=3.1 Hz) revealing axial-equatorial coupling. The data led to the conclusion that the relative stereochemical configuration of C2'' and C4'' are as in **1**[20] and **5**,[15] respectively. The HR-MS analysis was consistent with the structure ESI m/z $[M+H]^+$ obs. 760.2849, calc. 760.2811 (Figure S4). The experiment confirmed unequivocally the function of *snoT*, but the biosynthetic order remained

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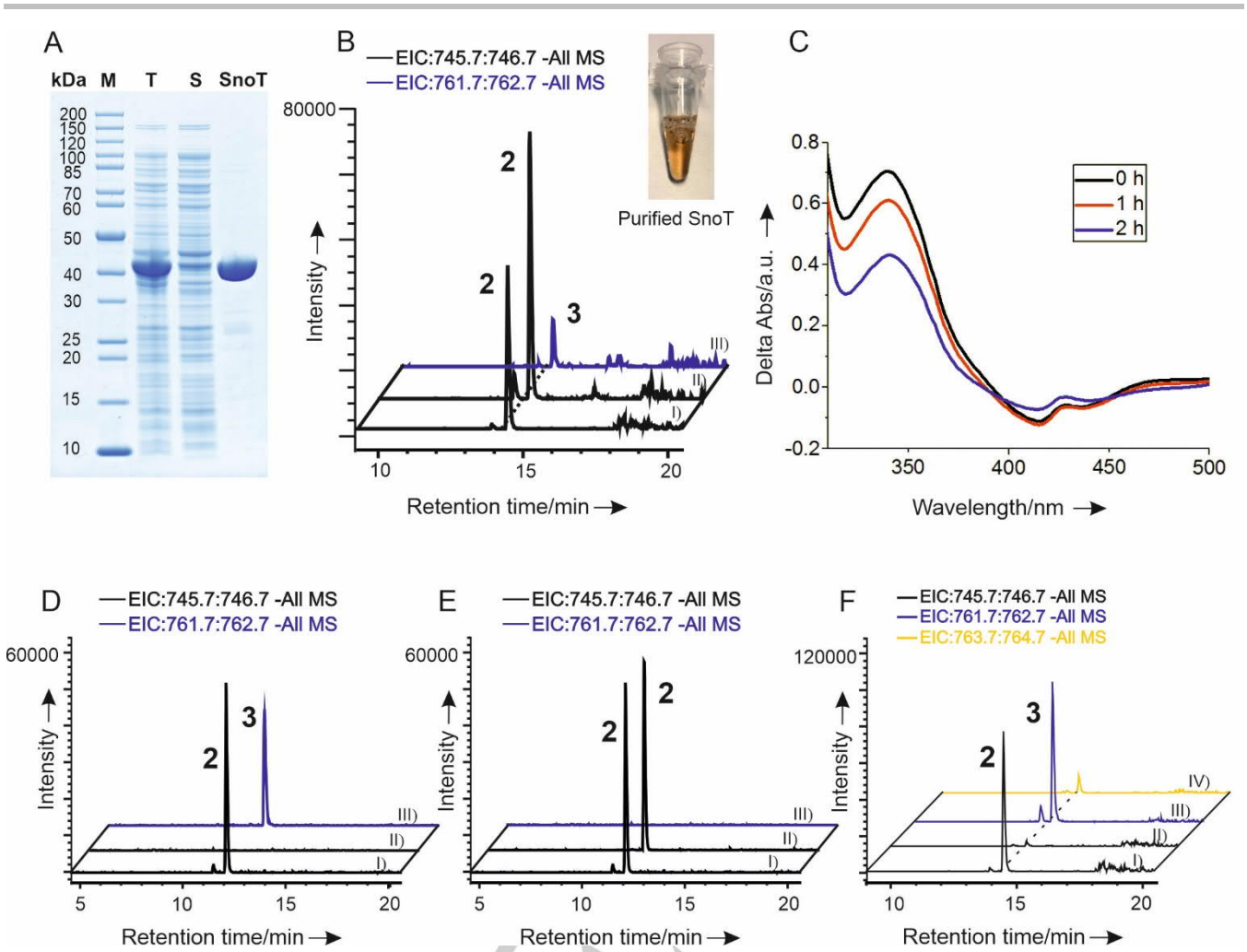

Figure 2. Characterization of the enzymatic activity of SnoT. A) SDS-PAGE of SnoT. Legend: M, marker; T, total cell lysate; S, soluble cell-free lysate. B) Formation of **3** from **2** using purified SnoT and *E. coli* cell-free lysate. C) Differential UV/Vis spectrum of *E. coli* TOP10/pBADT soluble lysate compared to *E. coli* TOP10 lysate showing gradual changes in SnoT. Enzymatic reactions with **2** as a substrate using cell-free lysates of D) *E. coli* TOP10/pBADT expressing *snoT* and E) *E. coli* TOP10 without *snoT*. I) EIC for **2** standard, II) EIC for **2**, III) EIC for **3**. F) Demonstration of incorporation of molecular oxygen to 3 by SnoT cell-free lysate using $^{18}O_2$. I) EIC for **2** standard, II) EIC for **2**, III) EIC for **3**, IV) EIC for incorporation of oxygen-18 to **3**.

inconclusive, since **4** could theoretically be formed either via **3** or **5** (Scheme 1).

To determine the natural substrate of SnoT, we amplified the gene and cloned it to a modified pBAD vector in *Escherichia coli* for production of N-terminally histidine tagged recombinant protein, which was purified to near homogeneity using affinity chromatography (Figure 2A). Rieske oxygenases typically require electron transfer components (e.g. ferredoxin and ferredoxin reductase) for enzymatic activity, but the nogalamycin gene cluster does not encode any candidate gene products for transfer of reducing equivalents from NADPH to SnoT. For this reason, we complemented the SnoT reaction with *E. coli* cell free lysate extracts and could observe the appearance of minor quantities of a compound with a +16 increase in mass to **2** (Figure 2B) by liquid chromatography – mass spectrometry (LC-MS). The experiment indicated that the non-natural electron transfer components from *E. coli* were compatible with SnoT.

We surmised that the low enzymatic activity may have resulted from inactivation of SnoT during the purification process, since iron-sulphur clusters can be rapidly destroyed after few minutes of exposure to aerobic conditions.[22] A time course analysis indicated gradual changes in the spectral properties of the enzyme preparation (Figure 2C), which was consistent with our hypothesis. In order to circumvent this, we proceeded to perform activity assays for the remainder of the study with *E. coli* cell-free lysates without purification of SnoT. The analyses revealed full conversion of **2** to a new peak corresponding to the mass of the expected hydroxylated product **3** in the presence of SnoT (Figure 2D), but not in a control reaction with *E. coli* cell-free lysate alone (Figure 2E). Compounds **5** and **6** were not modified under similar experimental conditions (Figure S5). To demonstrate that SnoT is an oxygenase, we next performed the reaction using $^{18}O_2$, which lead to the appearance of the expected +2 molecular ion of **3** (Figure 2F).

In order to confirm the structure of **3**, large-scale production and purification of **2** from *S. albus*/pSnoΔK, which contains a cosmid from which the carbocyclase gene *snoK* has been deleted,[16] was first carried out. Several reactions with compound **2** and SnoT cell-free lysates were carried out at room temperature to allow the

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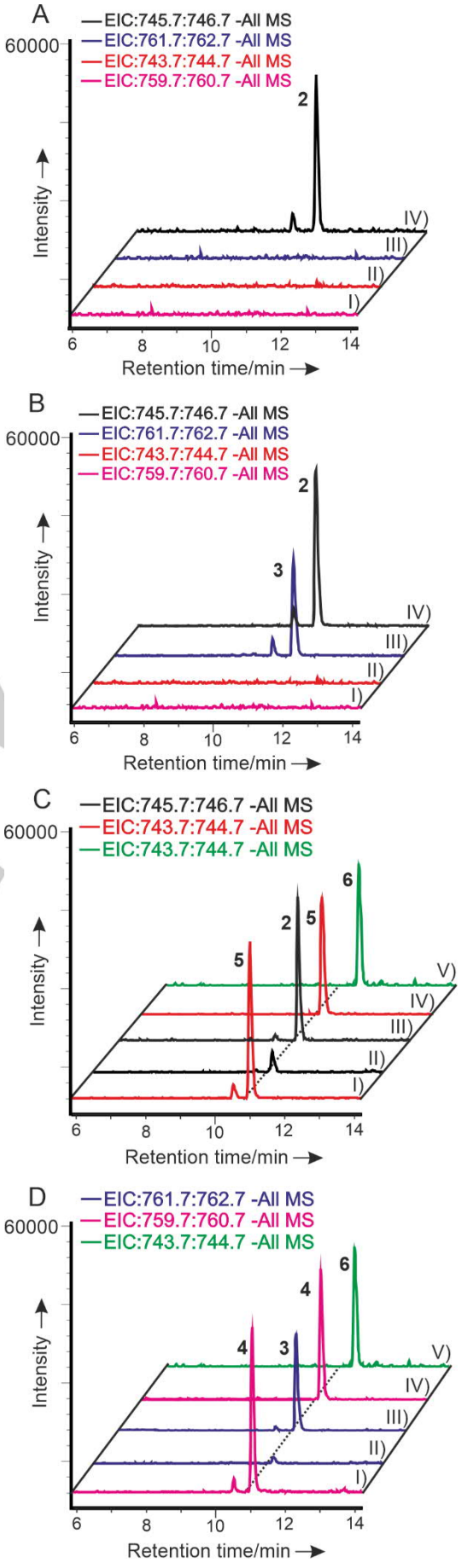
enzymatic modification to occur. The combined reaction was quenched and extracted with chloroform, followed by further purification by LH-20 and preparative HPLC. Structure elucidation by ^{1}H-NMR and COSY, HSQCDE, HMBC and HR-MS (ESI m/z $[M-H]^-$ obs. 760.2833, calc. 760.2822) confirmed that the reaction product was **3** (Table S1, Figure S6–S11). Similarly to **4**, the two methylene hydrogens at position H2´´ (δ 2.24 and 2.53) in **2** had been replaced by a proton downfield (δ 4.38). Furthermore, the C2´´ had also shifted downfield from 27.4 in **2** to 65.4 in **3** as seen from the HSQCDE analysis, which is consistent with the attachment of an OH-group. The coupling constant between H2´´ and H3´´ (*J*=11.2 Hz) indicated again to an axial-axial coupling, concluding that the relative stereochemical configuration at C2´´ was the same as in **4** and **1**.

Deciphering the reaction order in late stages of nogalamycin biosynthesis has been challenging, since many enzymes (e.g. SnoK and SnoN)[16] accept more than one intermediate as substrates. Here we have shown that SnoT was only able to utilize **2** without the additional C2–C5" bond as a substrate, and that 2"-hydroxylation of **5** and **6** did not occur (Figure S5). In contrast, our previous study has demonstrated that also SnoK is able to use **2** as a substrate to produce **5**, which can be further epimerized *in vivo* by SnoN to produce **6** (Scheme 1).[16] Analysis of the SnoN reaction has been particularly challenging, since *in vitro* reactions with **2** and **5** yield degradation products **7** and **8**, respectively.[16]

To investigate if SnoN can epimerize **2** *in vivo*, the strain *S. albus*/pSnoΔK was transformed with the plasmid construct pIJT_LMNT containing both *snoN* and *snoT* (Figure 3A). This new strain *S. albus*/pSnoΔK+NT was cultured for 6 days and LC-MS analysis of the culture extract revealed the presence of **2** and **3** (Figure 3B). Both *snoN* and *snoT* were cloned under the same promoter and the detection of **3** indicated that *snoT* had been expressed. The construct was assembled in an identical manner to the native gene cluster, with *snoT* residing after *snoN*, and therefore the detection of **3** suggested that both genes were successfully transcribed. However, since the epimerization reaction does not alter the mass of the product and the influence of the chemical modification on the retention time is unknown, the experiment did not directly reveal whether *snoN* had been functional. We have previously shown that 4" epimerization does alter the retention time between **5** and **6**, which both contain the additional C2–C5" bond.[16,19] Therefore to verify if SnoN was able to epimerize **2** *in vivo*, the culture extract containing putatively both **2** and **3** was incubated with purified SnoK in the presence of 2-oxoglutarate, Fe(II)SO_4 and L-ascorbate. LC-MS analysis of the reaction products revealed the presence of **5** with no traces of **6** (Figure 3C). This result indicated that SnoN was unable to use **2** and **3** as substrates *in vivo* (Figure 3C and 3D). Further analysis showed that SnoK was able to also cyclize **3** to form **4** (Figure 3D, Scheme 1).

In conclusion, the complicated set of experiments described above revealed that (i) SnoT is only able to catalyze 2"-hydroxylation of compounds that do not contain the C2–C5" bond such as **2** to generate **3**, (ii) SnoK harbors broad substrate promiscuity, but the biological function is likely to be conversion of **3** to **4** and (iii) SnoN has strict preference for intermediates containing the C2–C5" bond such as **4** and **5**, with only the former substrate residing on the pathway towards the end product **1**



(Scheme 1). The findings presented here clarify the last remaining unknown steps in the biosynthesis of **1** and elucidate how the 2′′- and 4′′-hydroxyl groups of L-nogalamine, which are important for DNA interaction, are assembled.

Figure 3. The order of reactions in the final steps of the biosynthesis of nogalamycin catalysed by SnoT, SnoK and SnoN. A) LC-MS analysis of the crude extract from *S. albus*/pSnoΔK showing the presence of **2** (m/z $[M+H]^+$ 746.7). I) Extracted ion chromatogram (EIC) for **4** (m/z $[M+H]^+$ 760.7), II) EIC for **5** (m/z $[M+H]^+$ 744.7), III) EIC for **3** (m/z $[M+H]^+$ 762.7) and IV) EIC for **2**. B) LC-MS analysis of the crude extract from *S. albus*/pSnoΔK+NT revealing the presence of **2** and **3**. I) EIC for **4**, II) EIC for **5**, III) EIC for **3** and IV) EIC for **2**. C) LC-MS analysis of the enzymatic reaction of SnoK using the crude extract from *S. albus*/pSnoΔK+NT as the substrate. The reactions shows the conversion of **2** to **5**. The absence of the epimerization product **6** [16,19] confirmed the inability of SnoN to epimerize **2**. I) Reaction product shown with EIC for **5**, II) Reaction product shown with the EIC for consumed **2**, III) authentic standard **2** IV) authentic standard **5** and V) authentic standard **6** (m/z $[M+H]^+$ 744.7). D) LC-MS analysis of the enzymatic reaction of SnoK using crude extract from *S. albus*/pSnoΔK+NT as the substrate. The results show the cyclization of **3** to **4**. This result confirmed the SnoK can cyclize both compounds **2** and **3** (Scheme 1). I) Reaction product shown with the EIC for **4**, II) Reaction product shown with the EIC for consumed **3**, III) authentic standard **3**, IV) authentic standard for **4** V) authentic standard **6**.

Acknowledgements

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Keywords: anthracycline • hydroxylation • biosynthesis • *Streptomyces* • Rieske enzyme

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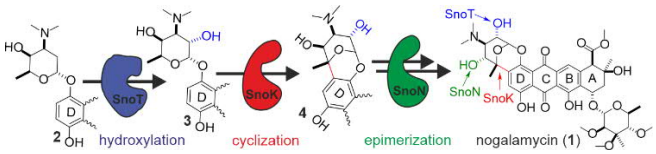

The binding of nogalamycin to the DNA double helix is facilitated by its carbohydrate units. The 2"- and 4"-hydroxyl groups of L-nogalamine are particularly important for the bioactivity due to hydrogen bonding interactions. Here we have elucidated the biosynthetic steps for L-nogalamine formation and show that SnoT installs the 2"-hydroxyl group, followed by 2–5"-carbocyclization and 4"-epimerization by SnoK and SnoN respectively.