ACS chemical biology

Letters

Cite This: ACS Chem. Biol. 2018, 13, 2433−2437

pubs.acs.org/acschemicalbiology

Enzymatic Synthesis of the C-Glycosidic Moiety of Nogalamycin R

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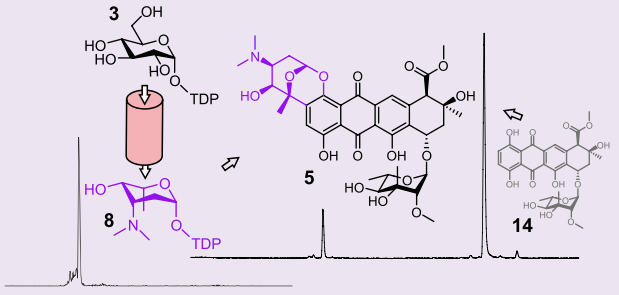
Supporting Information

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

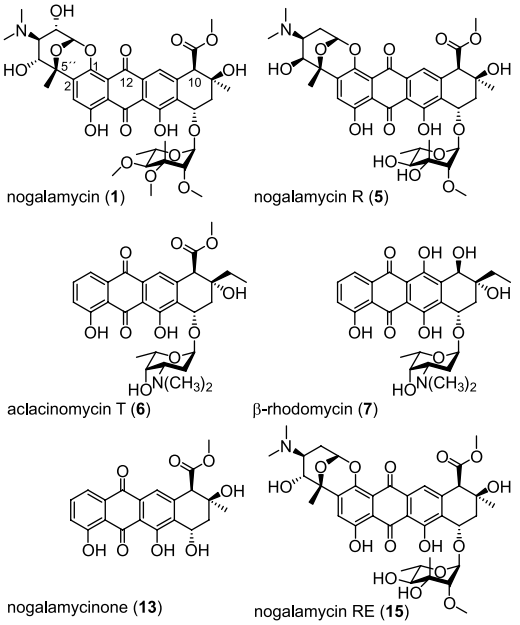
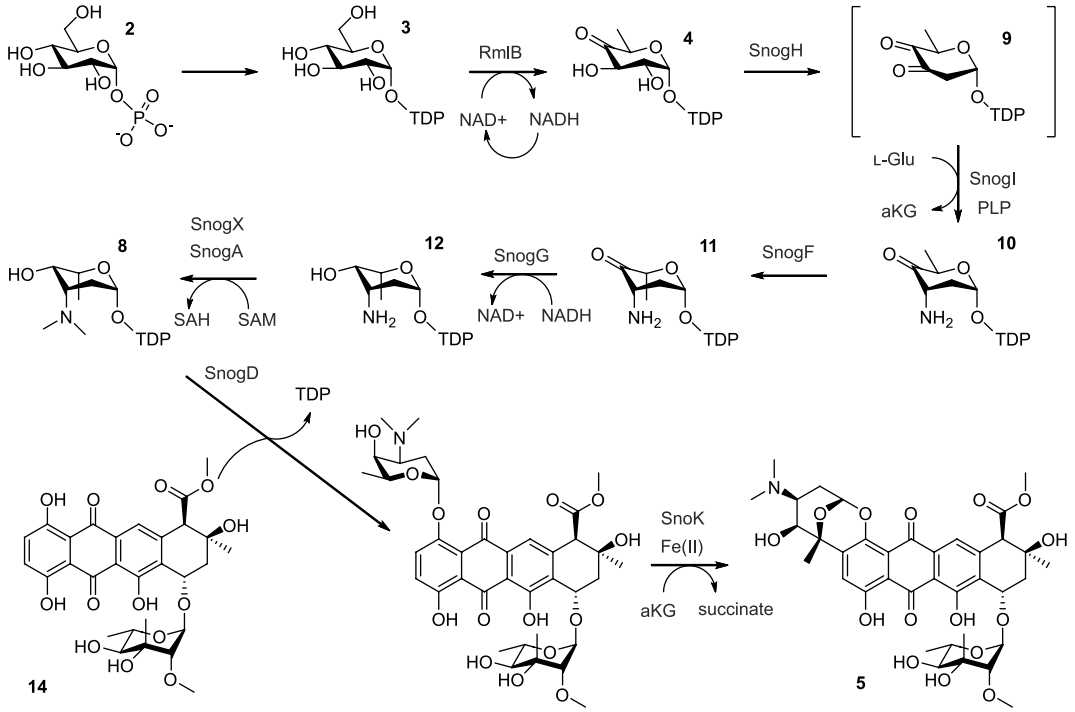


Figure 1. Chemical structures of the anthracyclines nogalamycin (**1**), nogalamycin R (**5**), aclacinomycin T (**6**), β-rhodomycin (**7**), nogalamycinone (**13**), and nogalamycin RE (**15**) relevant to the study.

Received: July 12, 2018
Accepted: August 16, 2018
Published: August 16, 2018

DOI: 10.1021/acschembio.8b00658
ACS Chem. Biol. 2018, 13, 2433−2437

Scheme 1. Model for the Biosynthesis of TDP-L-Rhodosamine and Its Dual Attachment to the Anthracycline Scaffold[a]



[a]TDP, thymidine diphosphate; NAD+, nicotinamide adenine dinucleotide, oxidized; NADH, nicotinamide adenine dinucleotide, reduced; PLP, pyridoxal 5′-phosphate; L-Glu, L-glutamate; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; aKG, α-ketoglutarate.

nonheme iron-dependent enzymes SnoK and SnoN, respectively.[17] 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), rhodomycins (**7**, Figure 1), cosmomycins, and cytorhodins.[18−20] 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.[21] 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-L-acosamine,[22] which has the same stereochemistry at C4 as the end product L-nogalamine, but we hypothesized that SnogG would catalyze the formation of TDP-L-daunosamine (**12**, Scheme 1) instead, in a manner similar to daunorubicin and aclacinomycin biosynthesis.[18] 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.[23] 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.[24]

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

DOI: 10.1021/acschembio.8b00658
ACS Chem. Biol. 2018, 13, 2433−2437

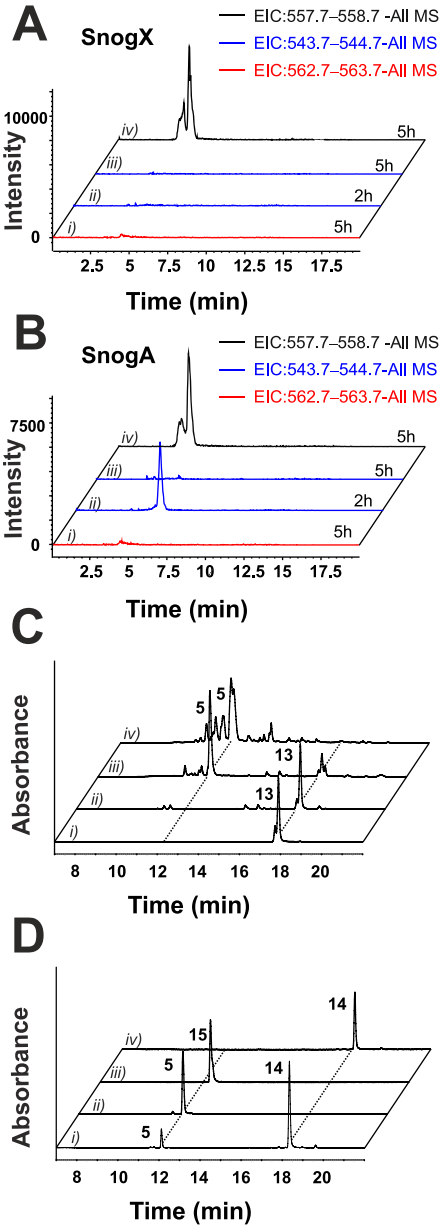


Figure 2. LC-MS and HPLC analysis of *in vitro* and *in vivo* reaction products. (A) LC-MS analysis of the *in vitro* sugar enzymatic formation of TDP-L-rhodosamine using SnogX as the methyltransferase. (*i*) Extracted ion chromatogram (EIC) for the used substrate **3** after a 5 h reaction. (*ii*) EIC for the monomethylated product in a 2 h reaction. (*iii*) EIC for the monomethylated product in a 5 h reaction. (*iv*) EIC for the dimethylated product **8** in a 5 h reaction. (B) LC-MS analysis of the *in vitro* sugar enzymatic formation of TDP-L-rhodosamine using SnogA as the methyltransferase. (*i*) EIC for the used substrate **3** after a 5 h reaction. (*ii*) EIC for the monomethylated product in a 2 h reaction. (*iii*) EIC for the monomethylated product in a 5 h reaction. (*iv*) EIC for the dimethylated product **8** in a 5 h reaction. (C) HPLC analysis of the *in vivo* products shown at 430 nm. (*i*) Standard for **13**. (*ii*) Extract from the strain *S. albus*/pSnoΔgN, (*iii*) *S. albus*/pSnoΔgN + pIJTsyngN, and (*iv*) *S.albus*/pSnogaori and (D) HPLC analysis of the *in vitro* products shown at 430 nm. (*i*) enzymatic synthesis of nogalamycin R (**5**). (*ii*) Standard for **5**. (*iii*) Standard for **15**. (*iv*) Used substrate **14**.

(Figure 2B) alone. After 2 h of incubation, the reaction with SnogX produced solely **8**, whereas a monomethylated intermediate could also be observed with SnogA. However, this intermediate was further converted to **8** after extending the 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 dimethylations.

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 pSnogaori 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-L-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 SnogD[16] 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 represent 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-L-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 stage inversion of stereochemistry at C4″ by SnoN and (ii) the loss of the C2″ hydroxyl group by SnogH and subsequent rehydroxylation putatively by SnoT are unnecessary from the perspective of a synthetic chemist.

METHODS

Strain and Culture Conditions. *Escherichia coli* TOP10 (Invitrogen) was used as the cloning host. *E. coli* K12 was used as a

DOI: 10.1021/acschembio.8b00658
ACS Chem. Biol. 2018, 13, 2433−2437

host for engineering the cosmid pSnogaori and ET12567/pUZ8002[32] for intergeneric conjugation to *Streptomyces albus*.[33] *Streptomyces* cultivations were performed in NoS-soyE1,[16] tryptone soya broth (TSB; Oxoid), R2 yeast extract (R2YE), and mannitol soya flour medium (MS).[32] *E. coli* strains were cultivated in Luria−Bertoli or 2 × yeast extract/tryptone medium (2xTY). Used antibiotics were ampicillin (50 μg mL^{-1}, Sigma-Aldrich), nalidixic acid (500 μg mL^{-1}, Sigma-Aldrich), kanamycin (25 μg mL^{-1}, Sigma-Aldrich), chloramphenicol (25 μg mL^{-1}, Sigma-Aldrich), and apramycin (50 μg−1250 μg mL^{-1}, Sigma-Aldrich).

Cloning and General DNA Techniques. Standard *Streptomyces* techniques were used.[32] Commercial kits were used for recovering DNA from agarose gels and for plasmid isolation (Thermo Scientific and E.Z.N.A, Omega Bio-Tek, respectively). DNA modifying enzymes were purchased from Thermo Scientific. The genes of interest were amplified by PCR using Phusion (New England Biolabs) and pSnogaori[16] or boiled *E. coli* K12 cells as the template. The PCR products were digested and cloned to the modified pBADHisB-plasmid[34] as *Bgl*II−*Eco*RI, *Bgl*II−*Hin*dIII-, or *Pst*I−*Hin*dIII-fragments. The list of primers is given in Table S1. The glycosyl transferase *snog*D was cloned as a truncated version as described earlier.[16] The constructs were verified by sequencing (Eurofins MWG Operon).

The snoΔgN gene inactivation mutant was made by deleting *snogN* (1227 bp) from the cosmid pSnogaori by using the λ Red recombinase system.[35] PCR was performed using DyNAzyme II (New England Biolabs) and pSnogaori as the template and the primers given in Table S1. First, a chloramphenicol resistance gene was introduced instead of the *snogN* gene and subsequently removed by pFLP2.[36] The construct was conjugated to *S.albus* from *E. coli* ET12567/pUZ8002,[32] resulting in the strain *S. albus*/pSnoΔgN. The strain *S. albus*/pSnoΔgN was complemented with a synthetically ordered native *snogN* with additional native promoter sequence upstream of the gene (83 bp) (Geneart). The synthetic gene was cloned to the vector pIJT486,[16] amplified in *S. lividans* TK24 and introduced to *S. albus*/pSnoΔgN by protoplast transformation resulting in strain *S. albus*/pSnoΔgN + pIJTsyngN. The complemented strain was compared to *S. albus*/pSnogaori.[16] The strains were grown for 6 days in NoS-soyE1,[16] after which absorbent (LXA1180, Sunresin, 20 g l^{-1}) was added to the culture. The compounds produced were extracted after 1 day by using methanol. The extract was analyzed by SCL-10Avp HPLC with an SPD-M10Avp diode array detector (Shimadzu) with a reversed-phase column (Phenomenex Kinetics, 2.6 μm, 4.6 × 100 mm) using a gradient from 15% acetonitrile with 0.1% formic acid to 100% acetonitrile. The standard for **13** was obtained from earlier studies.[16]

Protein Production and Purification. The proteins were produced in *E. coli* TOP10 cells as N-terminal histidine tagged recombinant proteins. The cells were grown in 2 L bottles with 500 mL of 2xTY at 30−37 °C under vigorous shaking until the OD_{600nm} reached ∼0.5, after which the cells were induced with 0.02% (w/v) L-arabinose and the growth was carried out at RT for 15−19 h. The cells were collected and suspended in A-buffer [50 mM sodium phosphate, 100 mM NaCl, 5 mM imidazole, 10% (v/v) glycerol]. The cells were lysed by French press (SLM Aminco) or sonication, and the cell debris was removed by centrifugation. The crude lysate was mixed with TALON Superflow (GE Healthcare). After a wash step, the target proteins were eluted from the column with B-buffer (A-buffer with 250 mM imidazole). The proteins were desalted with a PD-10 column (GE Healthcare) and stored at −20 °C in C-buffer [100 mM sodium phosphate, 100 mM NaCl] with glycerol added to 40% (v/v). The proteins SnogG and SnogA were supplemented with 1% of Triton X-100 (v/v) after the lysis of the cells or to C-buffer, respectively. The proteins were analyzed by SDS-PAGE (Figure S1) and photometrically at 280 nm (NanoDrop2000; Thermo Scientific).

Enzymatic Reactions. The reactions were performed in two steps. The first reaction was the synthesis of the dimethylated TDP-L-rhodosamine, while the second step was the attachment of the synthesized TDP-sugar to **14**, obtained from earlier studies,[16] via an O−C and a C−C bond.

The first reaction was performed with 30 μM of RmlB, SnogI, SnogF, SnogG, SnogX, and SnogA and 10 μM of SnogH immobilized in a TALON Superflow (GE Healthcare) at 4 °C for 30 min, or by omitting SnogA or SnogX. After immobilizing the enzymes, the master mix [buffer pH 7.5; 50 mM $Na_3PO_4{\cdot}12H_2O$, 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-α-D-glucose (TDP-α-D-glucose; Carbosynth)] was added and incubated at 23 °C for 2 h with gentle shaking. The reaction mixture was centrifuged at 700*g* for 10 min at 4 °C, and the supernatant was carefully removed and analyzed by LC-MS (Agilent 1260 Infinity 6120 Quadropole LC/MS) using the Phenomenex synergy fusion RP (4 μm, 4.6 × 150 mm) column and a gradient from 0.1% formic acid to 100% acetonitrile.

The second reaction was achieved with a reaction containing buffer at pH 8, [50 mM $Na_3PO_4{\cdot}12H_2O$, 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 (aKG), 100 μM $Fe(II)SO_4$, 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 × 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.

■ ACKNOWLEDGMENTS

The financial support from the Academy of Finland (grant number 285971 to M.M-K.), the Alfred Kordelin Foundation, and the Finnish Cultural Foundation (the Central Foundation and Varsinais−Suomi Regional Funds; to V.S.) is acknowledged. We thank V. Trapp for assistance with protein production and purification.

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DOI: 10.1021/acschembio.8b00658
ACS Chem. Biol. 2018, 13, 2433−2437