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Characterizing Amosamine Biosynthesis in Amicetin Reveals AmiG as a Reversible Retaining Glycosyltransferase

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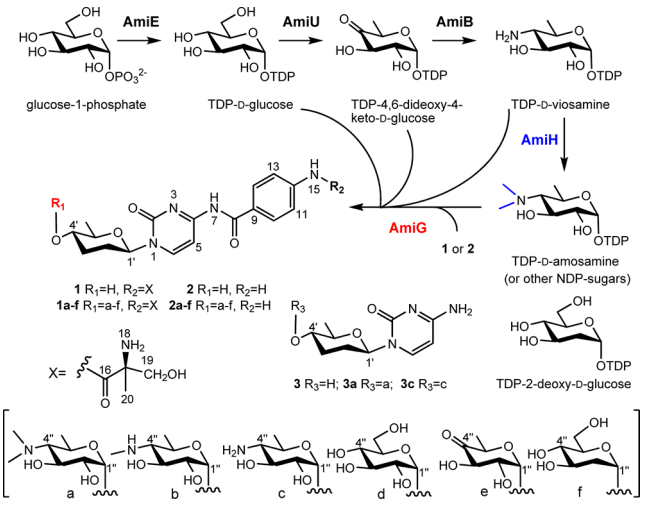
Supporting Information

ABSTRACT: The antibacterial and antiviral agent amicetin is a disaccharide nucleoside antibiotic featuring a unique α-(1→4)-glycoside bond between amosamine and amicetose, characteristic of a retaining glycosylation. In this study, two key steps for amosamine biosynthesis were investigated: the *N*-methyltransferase AmiH was demonstrated to be requisite for the dimethylation in amosamine, and the glycosyltransferase AmiG was shown to be necessary for amosaminylation. Biochemical and kinetic characterization of AmiG revealed for the first time the catalytic reversibility of a retaining glycosyltransferase involved in secondary metabolite biosynthesis. AmiG displayed substrate flexibility by utilizing five additional sugar nucleotides as surrogate donors. AmiG was also amenable to sugar and aglycon exchange reactions. This study indicates that AmiG is a potential catalyst for diversifying nucleoside antibiotics and paves the way for mechanistic studies of a natural-product retaining glycosyltransferase.

Many medicinal natural products are appended with a wide variety of sugar moieties, which are often critical for their biological activity, specificity, and pharmacological properties.[1] Attachments of sugars to natural-product scaffolds are usually catalyzed by glycosyltransferases (GTs), which utilize an activated donor sugar substrate (in most cases a nucleotide sugar) to form a glycosidic bond with either inversion or retention of configuration at the anomeric carbon of the donor substrate.[1] Biochemical characterizations of a number of natural-product inverting GTs have revealed their reaction reversibility and substrate flexibility and have expanded their synthetic utility in glycodiversification of natural products by performing sugar and aglycon exchange reactions.[2] Despite the implication of many retaining GTs in biosynthesis of natural products such as spirotetronates,[3] ansamycins,[4] aminoglycosides,[5] orthosomycins,[6] and nucleoside antibiotics,[7] biochemically well-studied retaining GTs for natural products remain scarce.[5]

Amicetin (**1a**), a disaccharide pyrimidine nucleoside antibiotic with antimicrobial and antiviral activities,[8] was first isolated from *Streptomyces vinaceusdrappus* NRRL 2363,[9] and years later, amicetin analogoues, including bamicetin (**1b**), plicacetin (**2a**), and norplicacetin (**2b**) were also discovered (Scheme 1).[10] The

Scheme 1. Proposed Amosamine Biosynthesis Pathway and Structures of Amicetin (1a) and Related Compounds



most characteristic feature of the amicetin group of antibiotics is the presence of an α-(1→4)-glycoside bond between amosamine and amicetose with retention of configuration at the anomeric carbon of amosamine. This structural feature sets amicetin as a model for studying the biosynthetic mechanism of a retaining GT. We recently cloned and characterized the **1a** biosynthetic gene cluster from *S. vinaceusdrappus* NRRL 2363 and proposed five *ami* gene products for amosamine biosynthesis (Scheme 1),[7] including the functionally characterized nucleotidylyltransferase

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AmiE,[11] the thymidine diphosphate (TDP)-hexose-4,6-dehydratase AmiU, the aminotransferase AmiB, the methyltransferase AmiH, and the GT AmiG. Herein we describe the validation of AmiH as an amosamine *N*-dimethyltransferase and AmiG as a retaining GT tailoring amicetin biosynthesis. Notably, this study demonstrates for the first time the reaction reversibility of a retaining GT involved in secondary metabolite biosynthesis.

BLAST analysis showed that AmiH displays the highest identity (34%) to a bacterial type-12 methyltransferase (GenBank accession number ZP_08874093). AmiH also distantly resembles the *N*-dimethyltransferase EryCVI for TDP-desosamine biosynthesis in erythromycin A,[12] indicating that AmiH functions to dimethylate TDP-D-viosamine (Scheme 1). To confirm the function of AmiH, the *amiH* gene was inactivated to afford the mutant AM1005 (Tables S1 and S2 and Figure S1 in the Supporting Information). HPLC−MS analysis revealed that the AM1005 mutant produced a new metabolite, **1c** (Figure 1). Subsequently, a 10 L-scale fermentation of AM1005

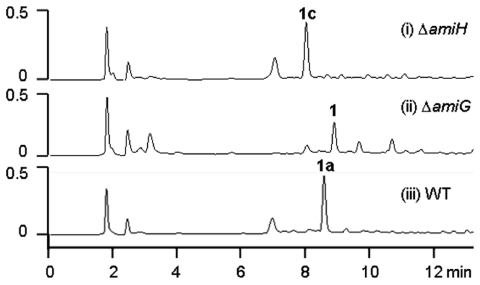


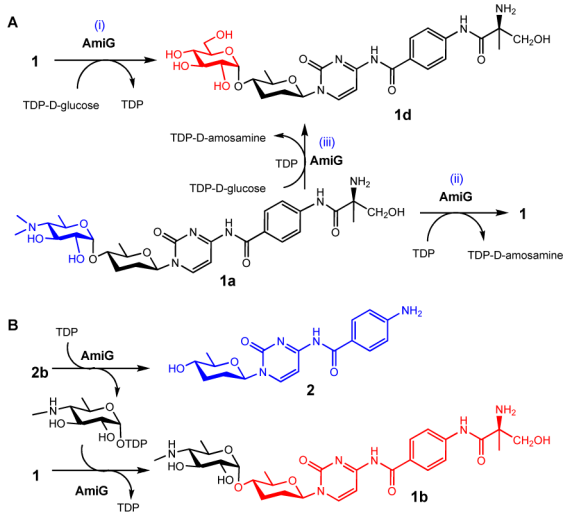
Figure 1. HPLC analysis of metabolite profiles of (i) the Δ*amiH* mutant AM1005, (ii) the Δ*amiG* mutant AM1004, and (iii) the wild-type strain *S. vinaceusdrappus* NRRL 2363.

led to the isolation of three compounds: **1c**, **2c**, and **3c** (Scheme 1). The chemical formula of the main product **1c** was determined to be $C_{27}H_{38}N_6O_9$ by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) (*m*/*z* 591.2773 $[M + H]^+$, calcd 591.2778). On the basis of their 1D and 2D NMR spectra, the only difference between **1c** and **1a** is that the singlet for the two *N*-methyl groups of the dimethylamino substituent at C4″ in **1a** [δ_H 2.64 (6H, s), δ_C 42.4] are absent in **1c** (Tables S3 and S4 and Figures S3 and S4). The chemical formula of the minor compound **2c** was determined to be $C_{23}H_{31}N_5O_7$ by HR-ESI-MS (*m*/*z* 512.2104 $[M + Na]^+$, calcd 512.2121). Comparison of ^{1}H NMR data revealed that **2c** differs from **1c** by the absence of NMR signals for the (+)-α-methylserine moiety in **1c** [δ_H 3.83, 4.13 (2H, ABq, *J* = 11.5 Hz, C19H$_2$); δ_H 1.64 (3H, s, C20H$_3$)] (Tables S3, S4; Figure S5). The other minor compound **3c** has the chemical formula $C_{16}H_{26}N_4O_6$ (HR-ESI-MS *m*/*z* 371.1936, $[M + H]^+$, calcd 371.1930) and was elucidated by NMR analysis (Figure S6 and Table S5) to be didemethylcytosamine (Scheme 1) on the basis of the absence of the two *N*-methyl groups that are present in cytosamine (**3a**) [δ_H 2.77 (6H, s)].[7,13] Generally, N-methylations occurred on the nucleoside diphosphate (NDP)-sugar level prior to glycosyl transfer.[1b] The lack of the two *N*-methyl groups in **1c**, **2c**, and **3c** (Scheme 1) and the failure of the Δ*amiI* mutant to biotransform **1c** into **1a** (Figure S7)[7] established AmiH as the requisite *N*-dimethyltransferase to convert TDP-D-viosamine to TDP-D-amosamine in **1a** biosynthesis.

AmiG is closely related to a number of GTs belonging to the GT1 family but is unique in having a longer C-terminus of about 100 amino acids (Figure S8). The *amiG* inactivation mutant AM1004 (Figure S2) produced a new product distinct from amicetin (Figure 1). This product, **1**, was isolated from a 9 L-scale fermentation and was determined by HR-ESI-MS to have the chemical formula $C_{21}H_{27}N_5O_6$ (*m*/*z* 468.1869 $[M + Na]^+$, calcd 468.1859). Compound **1** differs from **1a** only by the absence of NMR signals for the amosaminyl moiety (Tables S3 and S4 and Figure S9), and the upfield shift of 3.9 ppm for C4′ confirmed the structure of **1** as deamosaminylamicetin (Scheme 1). A minor compound with the chemical formula $C_{10}H_{15}N_3O_3$ (HR-ESI-MS *m*/*z* 226.1193 $[M + H]^+$, calcd 226.1186) was isolated and elucidated as **3** (Scheme 1) on the basis of the absence of amosamine signals that are present in **3a** [e.g., δ_H 4.97 (1H, d, *J* = 3.5 Hz, C1″H)] (Table S5 and Figure S10). These data suggested that AmiG functions as the amosaminyltransferase in **1a** biosynthesis.

Next, we tried to probe the AmiG activity in vitro. The full-length *amiG* gene was amplified by PCR and cloned from the genomic DNA of *S. vinaceusdrappus* NRRL 2363 to yield the expression plasmid pCSG3247 (Table S1). *N*-His$_6$-tagged AmiG was overproduced as a soluble protein in *Escherichia coli* BL21(DE3)/pCSG3247 upon IPTG induction and was purified to near homogeneity by Ni-NTA affinity chromatography (Figure S11). Given the lack of availability of the native donor TDP-D-amosamine, we first tested TDP-D-glucose as a surrogate donor substrate (Scheme 2A, i). To our delight, a new product

Scheme 2. Representative AmiG-Catalyzed Reactions: (A) Forward Reaction (i), Reverse Reaction (ii), Sugar Exchange Reaction (iii); (B) Aglycon Exchange Reaction



was detected upon HPLC−MS analysis (Figure 2, traces i and ii). The new product had a chemical formula of $C_{27}H_{37}N_5O_{11}$ as determined by HR-ESI-MS (*m*/*z* 608.2554 $[M + H]^+$, calcd 608.2568; Figure S12), consistent with the expected product **1d** (Scheme 2A). We then examined the AmiG activity at various pH and temperatures and in the presence of different divalent cations (Figure S13). AmiG functioned at pH 6−9, with the best activity at pH 6.5 in 50 mM MOPS buffer, in contrast to methymycin GT DesVII, which worked best under alkaline conditions.[14] AmiG exhibited activity at 20−40 °C, with the highest turnover at 35 °C. AmiG displayed weak activity without any divalent cations and was still active in the presence of 10 mM EDTA. The AmiG activity was enhanced by the presence of Mg^{2+}, Ca^{2+}, and Mn^{2+}, with optimal activity at >10 mM $MgCl_2$, but was inhibited by other divalent cations (e.g., Co^{2+}, Cu^{2+}, Fe^{2+},

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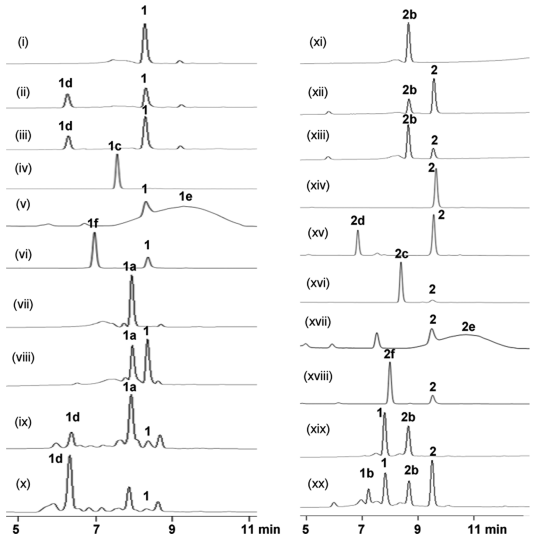

Figure 2. HPLC analysis of representative AmiG assays: (i) **1** + TDP-D-glucose; (ii) **1** + TDP-D-glucose + AmiG; (iii) **1** + UDP-D-glucose (5 mM) + AmiG; (iv) **1** + TDP-D-viosamine + AmiG; (v) **1** + TDP-4,6-dideoxy-4-keto-D-glucose + AmiG; (vi) **1** + TDP-2-deoxy-D-glucose + AmiG; (vii) **1a** + TDP; (viii) **1a** + TDP + AmiG; (ix) **1a** + TDP-D-glucose (5 mM) + AmiG; (x) **1a** + TDP-D-glucose (5 mM) + TDP + AmiG; (xi) **2b** + TDP; (xii) **2b** + TDP (5 mM) + AmiG; (xiii) **2b** + UDP (5 mM) + AmiG; (xiv) **2** + TDP-D-glucose; (xv) **2** + TDP-D-glucose + AmiG; (xvi) **2** + TDP-D-viosamine + AmiG; (xvii) **2** + TDP-4,6-dideoxy-4-keto-D-glucose + AmiG; (xviii) **2** + TDP-2-deoxy-D-glucose + AmiG; (xix) **1** + **2b** + TDP; (xx) **1** + **2b** + TDP + AmiG. Assays were performed in 50 mM MOPS buffer (pH 6.5) at 35 °C for 6 h using 100 μM **1** analogue, 1 mM NDP or NDP-sugar, and 3.3 μM AmiG, unless otherwise stated.

Ni^{2+}, and Zn^{2+}). Under the optimized conditions [50 mM MOPS buffer (pH 6.5) with 10 mM $MgCl_2$ at 35 °C], the sugar nucleotide specificity for AmiG was subsequently probed with 11 other commercially available nucleotide sugars in addition to TDP-D-glucose (Figure S14). AmiG could recognize TDP-D-viosamine, UDP-D-glucose, TDP-4,6-dideoxy-4-keto-D-glucose, and TDP-2-deoxy-D-glucose as donor substrates, converting **1** to **1d**−**1f**, respectively, with the expected molecular masses (Figure 2, traces iii−vi; Figure S15), while conversion of **1** was not observed with TDP-L-rhamnose, ADP-D-glucose, GDP-D-glucose, GDP-mannose, UDP-D-*N*-acetylglucosamine, UDP-D-galactose, or UDP-D-glucuronic acid (Figure S14). Unexpectedly, no conversion of **3** by AmiG was detected using TDP-D-viosamine or TDP-D-glucose as the donor substrate (data not shown), indicating that **3** is not an in vitro acceptor of AmiG. Finally, 1.0 mg of **1d** was isolated, and its structure was characterized by ^{1}H NMR analysis (Figure S12). The presence of an α-(1→4)-glycoside bond between glucose and amicetose in **1d** was self-evident from the coupling constant between the protons on C1″ and C2″ (δ_H 5.05, d, J = 4.0 Hz).

Following established protocols for reverse assays of inverting-GT-catalyzed reactions by the Thorson lab,[2] we found that AmiG could deglycosylate **1a** to afford **1** in the presence of TDP (Figure 2, traces vii and viii), confirming the reaction reversibility of AmiG (Scheme 2A, ii). In a one-pot AmiG-catalyzed sugar exchange reaction carried out by incubating **1a** with TDP-D-glucose (Scheme 2A, iii), the amosamine in **1a** could be directly replaced by glucose to afford **1d** and a minor amount of **1** (Figure 2, trace ix). This sugar exchange reaction was enhanced by the addition of TDP (Figure 2, trace x), probably because the reverse reaction of **1a** was driven by TDP to provide more aglycon **1** for glucosylation as a result of an excess of TDP-D-glucose over TDP in the assay. AmiG could also catalyze the deglycosylation of **2b** to yield **2** (Figure 2, traces xi and xii). UDP was also capable of mediating AmiG reverse catalysis at lower turnover relative to TDP (Figure 2, trace xiii). However, **2b** remained unchanged in AmiG assays with ADP, CDP, and GDP as cosubstrates (data not shown). In the presence of TDP, AmiG could also catalyze the deglycosylation of **2a**, **1c**, and **2c** (Figure S16). However, **3a** and **3c** appeared to be unchanged in AmiG reverse assays (data not shown). Taking advantage of AmiG reverse catalysis, we were able to prepare **2** from **2a** by TDP-mediated deglycosylation. The purified product **2** displayed the chemical formula $C_{17}H_{20}N_4O_4$ as determined by HR-ESI-MS (m/z 345.1557 $[M + H]^+$, calcd 345.1563, Figure S17). The structure of **2** was deduced as deamosaminylplicacetin (Scheme 2) on the basis of its ^{1}H NMR data, which were different from those of **2a** only by the absence of signals for the amosaminyl moiety [δ_H 2.64 (6H, s); δ_H 4.97 (1H, d, J = 3.5 Hz, C1″H)] (Figure S17 and Table S3), and was supported by 2D NMR analyses (Figure S17). AmiG was also able to convert **2** to **2c**−**f** (Figure 2, traces xv−xviii) in the presence of the corresponding sugar donors (Figure S14). The products were characterized to have the expected molecular masses by LC−MS analyses (Figure S15), demonstrating that **2** is another AmiG acceptor.

Subsequently, AmiG was shown to catalyze aglycon exchange reactions (Scheme 2B). For example, by upon coincubation of **2b**, **1**, TDP, and AmiG, the TDP-demethylamosamine generated in situ from **2b** by AmiG reverse catalysis could be transferred to an alternative aglycon **1** to afford **1b** (m/z 605.2942 $[M + H]^+$, calcd for $C_{28}H_{40}N_6O_9^+$ 605.2935; Figure S15) with concomitant formation of the deglycosylated product **2** (Figure 2, traces xix and xx). Similarly, the TDP-D-amosamine generated from **2a** and TDP-D-viosamine generated from **2c** in situ could also be transferred to **1** by AmiG to afford **1a** and **1c**, respectively, in one-pot aglycon exchange assays (Figure S18). In separate sugar exchange assays, **2a** and **2c** could also be converted to **2d** in one-pot sugar exchange reactions, which were enhanced by addition of TDP (Figure S19). These data confirmed that **2** is an alternative acceptor for AmiG. The isolation of **3a** from the Δ*amiF* mutant,[7] where the formation of the amide bond between cytosine and *p*-aminobenzoate was blocked, hinted that the amosaminylation of **3** to produce **3a** should happen in vivo. However, in an aglycon exchange reaction carried out by coincubating **1a**, **3**, and TDP with AmiG, the in situ-generated TDP-D-amosamine was not transferred to **3** to yield **3a** (Figure S20). Incubation of **3** and TDP-D-viosamine with AmiG failed to yield detectable product. These data indicate that **3** is not an in vitro acceptor for AmiG. Although we realized that the labile breakdown of the amide bond between cytosine and *p*-aminobenzoate in amicetin analogues might produce **3c** and **3a** during the isolation process (Figure S21), the production of **3a** by the Δ*amiF* mutant might suggest a substrate discrepancy of AmiG in vivo and in vitro.

Finally, we performed steady-state kinetic characterizations of AmiG-catalyzed reactions. AmiG displayed K_M values of 15.8 μM and 1.99 mM for **1** and TDP-D-viosamine, respectively, and the corresponding k_{cat} values of 1.38 and 1.59 min^{-1} were in close agreement (Table 1 and Figure S22). AmiG showed a k_{cat}/K_M value of 87.4 mM^{-1} min^{-1} toward **1**, a catalytic efficiency comparable to those of teicoplanin GTs.[15] Although AmiG exhibited similar K_M values for **1** and **2** (Table 1 and Figure S22), it appeared that **1** was a favored acceptor over **2** for AmiG on the

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Table 1. AmiG Kinetic Parameters for Different Substrates

Toward **1** and **2** as Acceptors[a]			
acceptor	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})
1	15.8 ± 1.9	1.38 ± 0.08	87.4
2	16.7 ± 2.5	0.84 ± 0.04	50.6
Toward TDP-D-viosamine and TDP-D-glucose as Donors[b]			
donor	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})
TDP-D-viosamine	1.99 ± 0.29	1.59 ± 0.08	0.80
TDP-D-glucose	2.67 ± 0.18	0.56 ± 0.03	0.21

[a]With saturating TDP-D-viosamine (10 mM) as the donor. [b]With saturating **1** (100 μM) as the acceptor.

basis of the k_{cat}/K_M values (Table 1), indicating that the attachment of the terminal (+)-α-methylserine might occur prior to amosamine transfer in **1a** biosynthesis. We also determined the catalytic parameters of AmiG toward the surrogate sugar donor TDP-D-glucose using **1** as a saturating acceptor (Table 1 and Figure S22). Interestingly, AmiG displayed very close K_M values for TDP-D-viosamine (1.99 mM) and TDP-D-glucose (2.67 mM). However, TDP-D-viosamine was apparently a better donor substrate than TDP-D-glucose on the basis of their k_{cat}/K_M values (Table 1). Following established methods,[16] we also determined the equilibrium constant for the AmiG-catalyzed reaction of **1** and TDP-D-viosamine to give **1c** to be K_{eq} = 120 (Figure S23), which differs from the reported values for GtfE (4.5)[2b] and OleD (156).[16] Although GT reverse catalysis displays apparent beauty for sugar nucleotide synthesis,[2g,h] we realize that the making of sugar nucleotides by GT reverse catalysis is yet not practical in some cases, especially when the GT displays a relative large K_{eq}. It turned out to be difficult for us to harvest a large amount of TDP-D-amosamine via the TDP-mediated AmiG reverse reaction using **2a** as a substrate.

In summary, we have validated AmiH as the *N*-methyltransferase and AmiG as the amosaminyltransferase in amicetin biosynthesis. Notably, we have shown that the retaining GT AmiG is capable of reverse catalysis and is amenable to sugar exchange and aglycon exchange reactions in a manner analogous to inverting GTs,[2] despite the distinct catalytic mechanisms of retaining and inverting GTs. Unlike the clearly established S_N2 replacement mechanism of inverting GTs, the catalytic mechanism of retaining GTs remains unclear, with proposals of a double displacement or S_Ni mechanism.[1] In addition, AmiG was demonstrated to have a certain substrate flexibility by utilizing TDP-D-amosamine, TDP-D-demethylamosamine, TDP-D-viosamine, T(U)DP-D-glucose, TDP-4,6-dideoxy-4-keto-D-glucose, and TDP-2-deoxy-D-glucose as sugar donors. Given the kinetic advantage of **1** over **2** in AmiG catalysis, we conclude that amosaminylation by AmiG might tailor **1a** biosynthesis. This study extends the reaction reversibility to a retaining GT and warrants further mechanistic investigations.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization data for new compounds, and determination of AmiG kinetic parameters. 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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