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The in vitro Characterization of Polyene Glycosyltransferases AmphDI and NysDI

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The overproduction, purification, and in vitro characterization of the polyene glycosyltransferases (GTs) AmphDI and NysDI are reported. A novel nucleotidyltransferase mutant (RmlA Q83D) for the chemoenzymatic synthesis of unnatural GDP–sugar donors in conjunction with polyene GT-catalyzed sugar exchange/reverse reactions allowed the donor and acceptor specificities of these novel enzymes to be probed. The evaluation of polyene GT aglycon and GDP–sugar donor specificity revealed some tolerance to aglycon structural diversity, but stringent sugar specificity, and culminated in new polyene analogues in which L-gulose or D-mannose replace the native sugar D-mycosamine.

Introduction

Polyene macrolide antibiotics are a family of diverse natural products primarily produced by *Streptomyces* and closely related bacteria.[1–3] As exemplified by amphotericin B (AmB, **1**), nystatin A1 (**2**), candicidin or FR008 (**3**), pimaricin (**4**), rimocidin (**5**), and filipin III (**6**; Scheme 1), a polyhydroxylated, polyunsaturated macrolactone ring core is the structural signature of family members, and most are decorated with the unique deoxyaminosugar mycosamine.[2] While polyene macrolide antibiotics are most noted for their antifungal properties, these metabolites also display antiviral, antiprotozoal, and even antiprion activity.[4–8] Their primary mechanism of action is derived from the unique interactions that occur between polyene molecules and specific sterol-containing membranes; these interactions generate lethal transmembrane channels. Furthermore, the selectivity of polyene macrolide antibiotics stems from their preference for ergosterol-containing membranes.[3,4,7,8] Remarkably, even after a half century of clinical use of **1**,[4] the development of resistance to polyenes has been sparse.[9] However, the clinical utility of polyenes remains severely restricted by compound insolubility and dose-dependent side effects, most notably nephrotoxicity.[4,5] Thus, the development of formulations and/or analogues to reduce unwanted side effects and/or improve selectivity remains an active area of research.[4,6]

Toward this goal, the most common synthetic strategy for polyene derivatization has relied upon semisynthetic derivatization of the natural product core scaffold carboxyl (e.g., C41 of **1**) and/or the C3′ amine of the appended aminosugar (e.g., mycosamine in **1**).[10–20] In addition to providing analogues with altered antifungal properties, such studies have also challenged the dogma pertaining to the intramolecular interaction between the C3′ protonated amine and C41 carboxylate in channel assemblage.[16,17] As an alternative to synthesis, the genetic loci encoding for pimaricin (**4**),[21,22] nystatin (**2**),[23] AmB (**1**),[24] and candicidin/FR-008 (**3**)[25–27] have been partially or fully characterized.[3,28] This has enabled both the elucidation of key post-PKS modification steps in polyene biosyntheses and the directed engineering of unique polyene analogues.[27–39] The cumulative SAR based upon this diverse array of semisynthetic and engineered polyene derivatives has also clearly illuminated the critical role of the amino-sugar moiety for antifungal activity.[13,15,32]

The growing appreciation of the importance of natural product sugar moieties has spurred the development of methods for natural product glycosylation and glycodiversification—ranging from new synthetic methodologies to enzyme-intensive approaches.[40–42] While a C35-mycarosyl-substituted nystatin analogue with improved antifungal potency exists,[31] there are few reported examples in which the natural polyene mycosamine has been successfully replaced by an alternative sugar.[27,32] In addition, although the functions of enzymes that catalyze the attachment to polyenes (glycosyltransferases or GTs, Scheme 1) have been inferred with in vivo genetic studies, they have evaded in vitro biochemical characterization in part due to the lack of sugar nucleotide substrate availability.[27,32,35,38] Unlike most natural product GTs, which utilize pyrimidine-base sugar nucleotides, bioinformatics and biochemical characterization of the early steps in mycosamine biosynthesis imply that polyene GTs utilize GDP-based sugar nucleotides.[23,38] Herein, we report the first in vitro characterization of two polyene GTs, AmphDI and NysDI. The aglycon and sugar nucleotide substrate specificity of these model polyene GTs were probed with a set of unique GDP-D- and L-sugars. These studies revealed that AmphDI and NysDI have some tolerance

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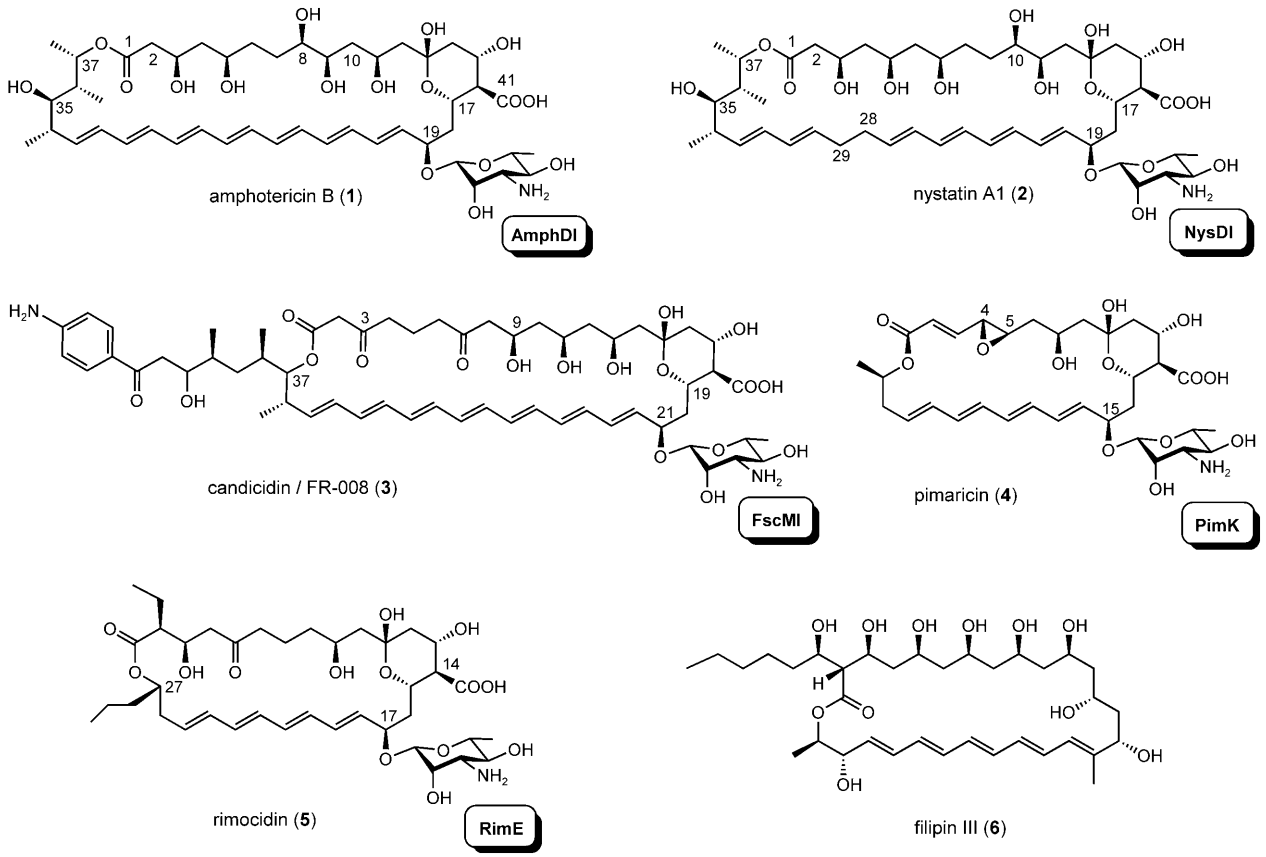


Scheme 1. Naturally occurring polyene macrolides. Amphotericin B (**1**), nystatin A1 (**2**), candicidin/FR-008 (**3**), pimaricin (**4**), rimocidin (**5**), and filipin III (**6**) are all produced by *Streptomyces* strains, and genetic loci for **1**–**5** have been characterized. The corresponding mycosaminyltransferases (AmphDI, NysDI, FscMI, PimK, and RimE, respectively) responsible for glycoside formation are highlighted.

to aglycon structural diversity, but are stringent when it comes to GDP–sugar specificity. This study notably highlights the utility of a recently engineered nucleotidyltransferase variant to synthesize novel GDP–sugars and the application of these reagents,[43,44] in conjunction with the reversibility of GT-catalyzed reactions,[45–49] to study purine sugar nucleotide-dependent GTs.

Results

Over-production and purification of polyene GTs

The polyene GTs (AmphDI, NysDI, FscMI, PimK, and RimE for **1**-**5**, respectively, Scheme 1) share very high sequence (over 65% identity, Figure S1 in the Supporting Information) and functional (mycosaminyl transfer, based upon in vivo gene inactivation)[27,38] similarities. In an effort to study these novel catalysts in vitro, the genes of two polyene GTs, *amphDI* and *nysDI*,[23,24] were PCR amplified from genomic DNA of the amphotericin producer *Streptomyces nodosus* (ATCC 14899) and the nystatin producer *Streptomyces noursei* (ATCC 11455), respectively. Heterologous expression of N-His_6-tagged AmphDI or NysDI in *E. coli* by using a pET28a-based system provided only small amounts of the desired recombinant GTs ($<0.5\ mg\,L^{-1}$ culture under optimized conditions) after affinity chromatography. The alignment of AmphDI and NysDI with three other polyene GTs (PimK,[22] RimE,[50] and FscMI[27]) surprisingly revealed an extended N-terminal sequence that lacks the predicted structure (http://bioinf.cs.ucl.ac.uk/psipred/; Figure S1). Expression of the two truncated GTs, designated AmphDI-T2 and NysDI-T2 (starting from the 'common' methionine residue M21 for AmphDI and M44 for NysDI, Figure S1) under identical conditions led to 10–12 mg of the desired N-His_6-tagged AmphDI-T2 and NysDI-T2 per liter of *E. coli* culture (Figure S2).

Reversibility of polyene GT-catalyzed reactions

Given the difficulty to access polyene GT substrates (both the polyene aglycon acceptor and putative sugar nucleotide donor),[17,51] we first investigated the reversibility of polyene GT-catalyzed reactions as recently described for other natural product GTs.[45–49] Specifically, polyene natural products were incubated with GTs in the presence of NDPs and the loss of mycosamine was assessed by using HPLC (Figures 1A and B). For example, incubation of 20 μM AmB (**1**) with 5 μM AmphDI-T2 revealed a new product only in the presence of 2 mM GDP or dGDP at 30 °C for 6 h (Figure 1C, vi and vii), while no reaction was observed in the absence of NDPs (Figure 1C, i) or enzyme, or in the presence of alternative NDPs (ADP, CDP, UDP, and TDP, Figure 1C, ii–v). LC-MS of the new species was consis-

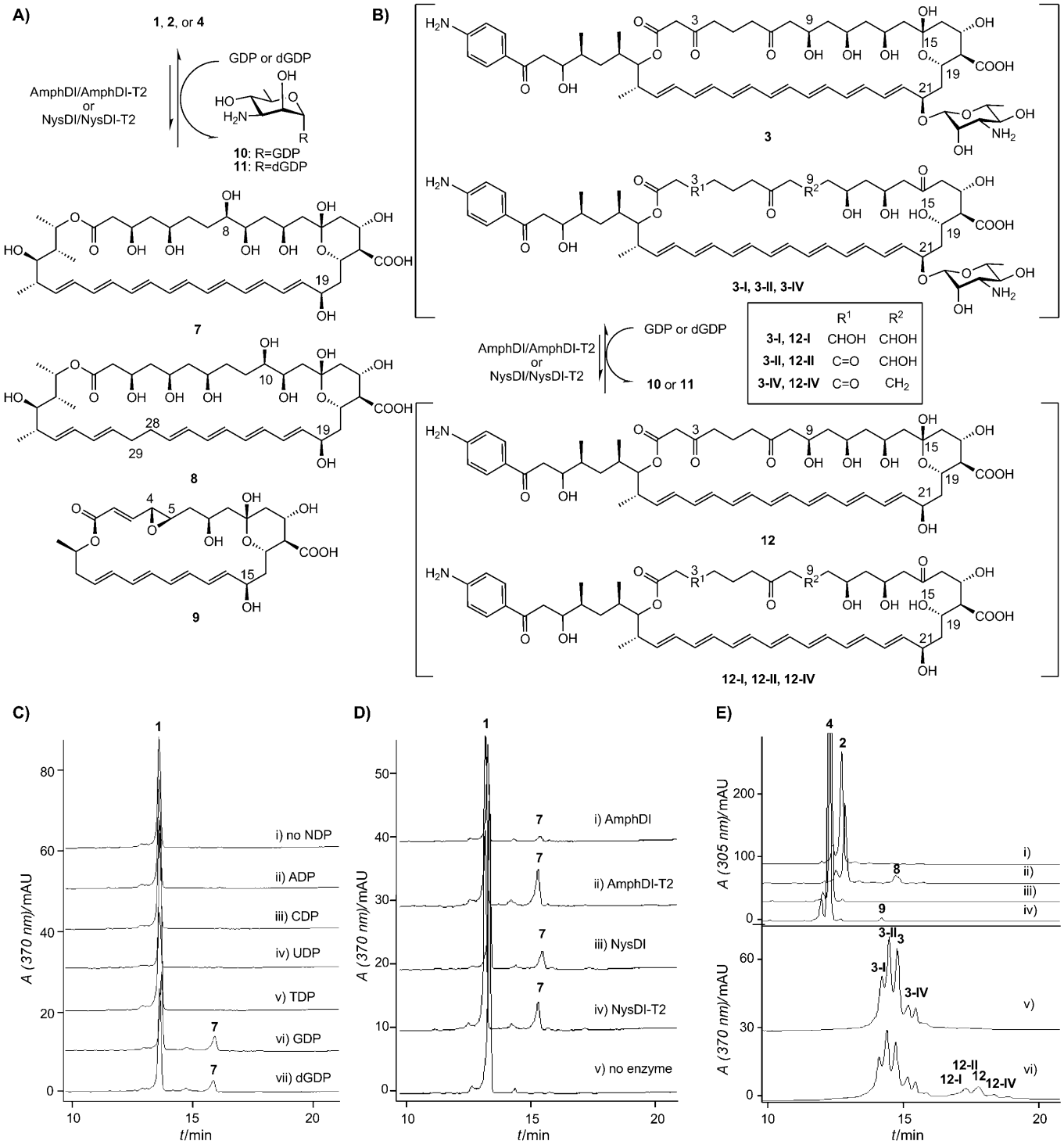


Figure 1. Polyene GT-catalyzed reverse reactions. A) Schematic representation of polyene GT-catalyzed conversion of **1**, **2**, or **4** to deglycosylated products **7**, **8**, or **9**, respectively. B) Schematic representation of polyene GT-catalyzed conversion of candicidin complex (**3-I**, **3-II**, **3**, and **3-IV**) to deglycosylated complex (**12-I**, **12-II**, **12**, and **12-IV**). C) HPLC analyses of AmphDI-T2 NDP-specificity in GT-catalyzed reverse reactions. In this example, AmB (**1**, 20 μM) was incubated with AmphDI-T2 (5 μM): i) without NDP or in the presence of 1 mM, ii) ADP, iii) CDP, iv) UDP, v) TDP, vi) GDP, or vii) dGDP at 30 °C, overnight. D) HPLC analyses of polyene GT-catalyzed reverse reactions with AmB (**1**) and different polyene GTs. For this study, AmB (**1**, 20 μM) was incubated with GDP (1 mM) in the presence of 5 μM: i) AmphDI, ii) AmphDI-T2, iii) NysDI, iv) NysDI-T2, or v) without GT at 30 °C, overnight. E) HPLC analyses of AmphDI-T2 aglycon specificity in GT-catalyzed reverse reactions. In this study, nystatin (**2**, 20 μM), pimaricin (**4**, 50 μM), or candicidin complex (**3-I**, **3-II**, **3**, and **3-IV**, 20 μM) was incubated with GDP (1 mM) in the absence or presence of AmphDI-T2 (5 μM): i) **2**, no enzyme (control), ii) **2**, AmphDI-T2, iii) **4**, no enzyme (control), iv) **4**, AmphDI-T2, v) candicidin complex (**3-I**, **3-II**, **3**, and **3-IV**), no enzyme (control), vi) candicidin complex (**3-I**, **3-II**, **3**, and **3-IV**), AmphDI-T2.

tent with deglycosylated **1** aglycon, amphoteronolide B (**7**, Figure 1A, calcd 778.4; found $[M-H]^-$ 777.5, $[M+Na]^+$ 801.5). Also, consistent with a GT-catalyzed reverse reaction, the parallel formation of GDP-D-mycosamine (**10**) was verified with anion exchange HPLC chromatography and ESI-MS/MS analysis (**10**, calcd 588.1, found $[M-H]^-$ 587.0; Figure S3). In a similar manner, reaction reversibility assessed in the presence of AmphDI, NysDI, and NysDI-T2 revealed an absolute dependence upon GDP/dGDP (Figure 1D, i–iv), and enzyme (Figure 1D, v).

To probe aglycon tolerance, several other polyene macrolides were subjected to the same AmphDI, AmphDI-T2, NysDI, and NysDI-T2 assay conditions. In this study, reaction reversibility was observed with both nystatin (**2**) and, to a lesser extent, pimaricin (**4**), by all four GTs tested (AmphDI, AmphDI-T2, NysDI, and NysDI-T2) only in the presence of GDP or dGDP to provide nystatinolide (**8**, Figure 1A and 1E, calcd 780.4; found $[M-H]^-$ 779.4, $[M+Na]^+$ 803.5) or pimaricin aglycon (**9**, Figure 1A and 1E, iv, calcd 520.2; found $[M-H]^-$ 519.2, $[M+Na]^+$ 543.3), respectively. In a similar manner, reaction reversibility was also established in the presence of GDP by using commercially available candicidin (a complex that consists of at least four major compounds—**3-I**, **3-II**, **3**, and **3-IV**, Figure 1B and 1E, v)[27] to provide the corresponding aglycons (**12-I**, **12-II**, **12**, and **12-IV**, Figure 1B and 1E, vi), the mass ions of which were detectable by LC-MS (**12-I**: calcd 965.5, found $[M-H]^-$ 964.5; **12-II**: calcd 963.5, found $[M-H]^-$ 962.4; **12**: calcd 963.5, found $[M-H]^-$ 962.4; **12-IV**: calcd 947.5, found $[M-H]^-$ 946.3). Finally, the activities of AmphDI, AmphDI-T2, NysDI, and NysDI-T2 toward different polyene macrolides were compared under presteady-state conditions (20 μM polyene glycoside, 0.5 μM GT, 2 mM GDP, 30 °C, up to 1 h). As summarized in Figure S4, the truncated GTs outperformed their original 'extended' counterparts, the magnitude of which varied depending upon the polyene substrate employed.

Synthesis of GDP–sugars

Consistent with previous postulations,[3,38] the specific requirement of GDP (or dGDP) for the reversibility of AmphDI/NysDI-catalyzed reactions is consistent with GDP-mycosamine as the requisite sugar donor. To further probe the sugar nucleotide donor substrate flexibility of polyene GTs, a small set of GDP–sugars was subsequently generated by using both chemical and enzymatic methods (Scheme 2). Together with three commercially available GDP–sugars (GDP-α-D-glucose, **21**; GDP-α-D-mannose, **22**; and GDP-β-L-fucose, **23**) the combination of chemical and enzymatic strategies, which are summarized in the next two paragraphs, provided a set of 21 putative GDP–sugar donors (**17**–**37**, Scheme 2C) for this study.

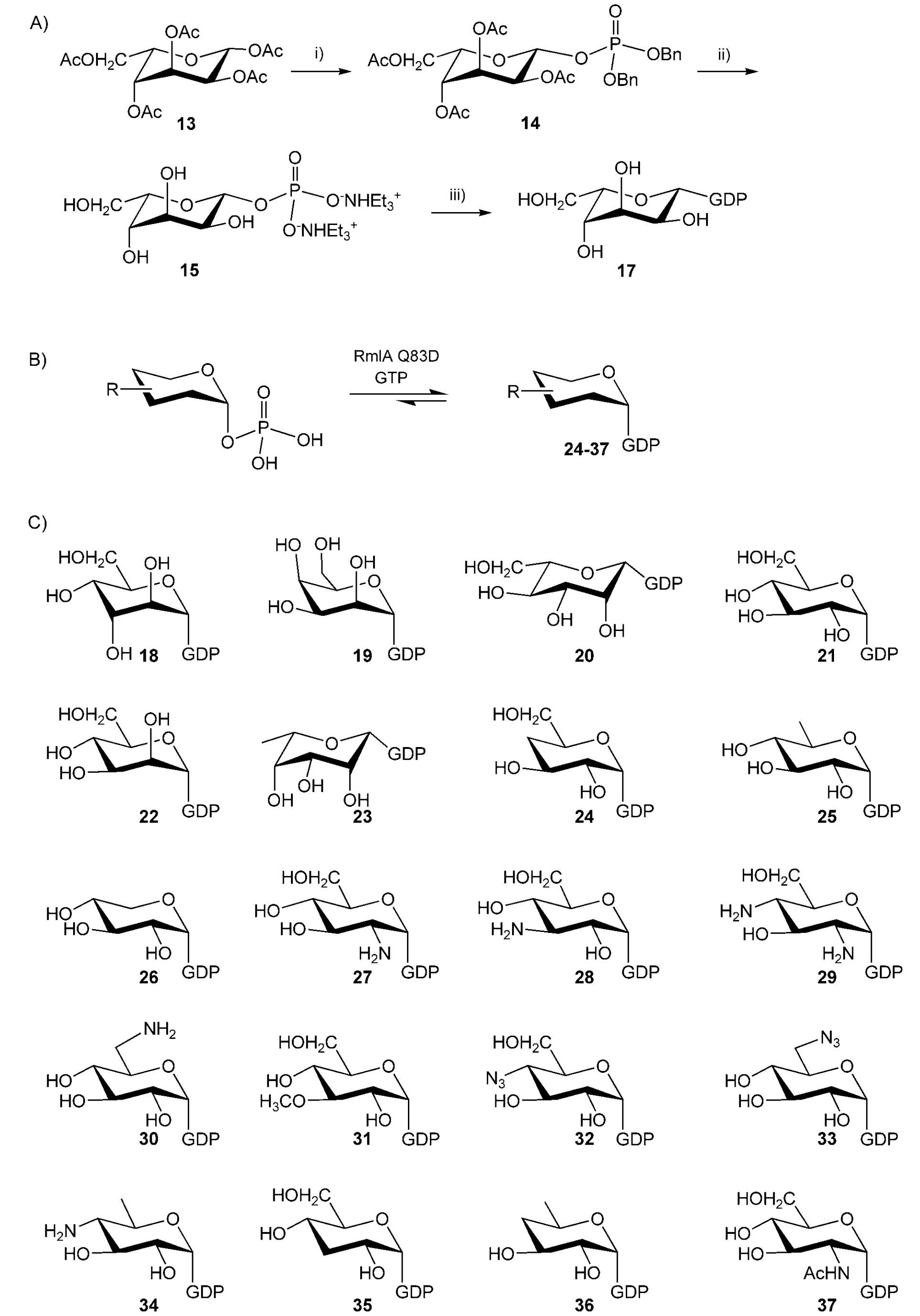
A conventional morpholidate-dependent coupling strategy was applied for the chemical synthesis of several GDP–sugars (Scheme 2A).[52,53] Syntheses of the required α-D-altrose-1-phosphate, α-D-talose-1-phosphate, and β-L-mannose-1-phosphate precursors have been previously reported.[53–57] Following an identical strategy, peracylated β-L-gulose (**13**) was converted to the protected sugar phosphate (**14**) in two steps (55% yield), the deprotection of which led to a triethylammonium sugar phosphate (**15**; 85% yield). Coupling the target sugar-1-phosphates with the guanosine 5′-monophosphomorpholidate (1.6 equiv) provided the desired GDP–sugars (**17**–**20**) in 45–65% yield. Following this route, four sugar nucleotides, GDP-β-L-gulose (**17**), GDP-α-D-altrose (**18**), GDP-α-D-talose (**19**), and GDP-β-L-mannose (**20**) were generated for this study.

A recently described nucleotidyltransferase mutant (RmlA Q83D)[43] with enhanced activity toward GTP was also employed in the synthesis of GDP–sugars for this study (Scheme 2B). Following the established protocol,[43] incubation of sugar 1-phosphates with GTP and purified RmlA Q83D at 37 °C, overnight, provided fourteen additional unique GDP–sugars (Scheme 2C, Figure S5, **24**–**37**). Among them, ten sugar nucleotides (**24**–**32**) were produced in good yields (ranging from 23–96%, Table S1 and Figure S5), while less than 10% conversion was observed for the remaining four sugar-1-phosphates (**33**–**37**, Table S1 and Figure S5). All GDP–sugar nucleotide products were confirmed by LC-MS and ESI MS/MS; spectrometry also confirmed typical secondary fragment ions for $[GDP-H]^-$ (442) and $[GMP-H]^-$ (362) for all reaction products Table S1).

Polyene GT-catalyzed sugar exchange and glycoside formation

GT-mediated sugar exchange enables the exchange of the native sugar within a native natural product glycoside with exogenous carbohydrates, that have been supplied as NDP-sugars.[45,49] Following this same protocol, the donor substrate flexibility of polyene GTs was probed through a sugar-exchange reaction (Figure 2A) by using the putative GDP–sugar donors described in the previous section. Specifically, 20 μM AmB (**1**) and 5 μM AmphDI-T2 were incubated individually with GDP–sugar donors (2 mM for **17**–**23**, 30–300 μM for **24**–**33**, <30 μM for **34**–**37**, Scheme 2C) at 30 °C, overnight. Analysis of the reactions by RP-HPLC revealed new products only in the presence of GDP-α-D-mannose (**22**, Figure 2B, vii) or GDP-β-L-gulose (**17**, Figure 2B, iii), the identities of which were confirmed by LC-MS to be **1a** (calcd 940.5, found 939.4 $[M-H]^-$ and 963.4 $[M+Na]^+$) and **1b** (calcd 940.5, found 939.5 $[M-H]^-$ and 963.5 $[M+Na]^+$; Figure 2A), respectively.

To assess the activity in a more conventional GT-catalyzed assay, a small amount of the acceptor amphoteronolide (**7**, 0.1 mg, 0.128 μmol, 12.8% overall yield), was partially purified from a 10 mL preparative AmphDI-T2 catalyzed reverse reaction (with 20 μM of **1**). This is an advance over chemical routes for the preparation of **7**—for example, a recent chemical approach provided a 10.8% overall yield of **7** in eight steps from **1**.[17] Subsequently, 4 μM of the isolated aglycon (**7**) was incubated with 5 μM AmphDI-T2 and various GDP–sugar donors (2 mM for **17**–**23**, 30–300 μM for **24**–**33**, <30 μM for **34**–**37**). Under these conditions, substantial amounts of **1a** and **1b** (almost 100% conversion, Figure S6) were produced from sugars **17** and **22** while, consistent with 'sugar-exchange' assays, all other donors (**18**–**21**, **23**–**37**) failed to provide glycoside variants. Interestingly, in a prior study, disruption of my-



Scheme 2. Chemical and chemoenzymatic preparation of GDP–sugars. A) The chemical synthesis of GDP-L-β-gulose (**17**). i) Ac_2O/pyridine; HBr/AcOH; $HPO_2(OBn)_2$, CF_3SO_3Ag, $Me_3C_5H_2N/CH_2Cl_2$; ii) H_2/Pd-C; AG 50W-X8 (Et_3NH^+); iii) GDP–morpholidate (**16**) and 1*H*-tetrazole/pyridine. B) The chemoenzymatic synthesis of GDP–sugars. Generally, chemically synthesized sugar-1-phosphate (6 mM) was incubated with GTP (8 mM) in the presence of RmlA mutant Q83D (20 μM). C) GDP–sugars employed in this work. GDP-D-mycosamine (**10**) was generated through reverse GT catalysis, GDP-D-glucose (**18**), GDP-D-mannose (**19**), and GDP-L-fucose (**22**) were commercially available, GDP-L-gulose (**17**), GDP-D-altrose (**20**), GDP-D-talose (**21**), and GDP-L-mannose (**23**) were chemically synthesized (A) and GDP–sugars **24**–**35** were enzymatically synthesized (B).

cosamine biosynthesis in *S. nodosus* led to a minor shunt metabolite with a mass consistent with a hexosyl-amphoteronolide A, which was proposed to be mannosyl- or glucosyl-amphoteronolide.[30] The ability of AmphDI to accept GDP–mannose, but not GDP–Glc, supports the potential in vivo production of mannosyl-amphoteronolide A but refutes the possibility of glucoside formation. When GDP-D-mannose was replaced by TDP-D-mannose (chemoenzymatically prepared from a RmlA reaction)[45,46] in the assay with **7** and AmphDI-T2, no products were detectable, which is consistent with GDP–sugar specificity. In a similar manner, only **17** and **23** were identified as NysDI-T2 donor substrates to afford **1a** and **1b**.

Discussion

Unlike the two-component GTs associated with the biosynthesis of many glycosaminyl-modified polyketides first described by Liu and co-workers,[58–61] AmphD1 and NysD1 do not require an auxiliary protein for in vitro activity. Consistent with prior bioinformatics[3,21–28] and the recent biochemical characterization of a GDP-D-mannose 4,6-dehydratase (NysDIII) encoded by the nystatin biosynthetic gene cluster,[38] this study unequivocally confirms polyene GTs to be (d)GDP–sugar specific. Enabled by a recently reported RmlA mutant engineered to provide the ability to generate a repertoire of unnatural GDP–sugar donors,[43] the highlighted polyene GT-catalyzed sugar exchange and glycoside formation reactions required GDP–sugar donors. While it is typical for the forward and reverse reactions to utilize the same nucleotide,[45–48] one exception now exists; a recent study with the calicheamicin GT CalG3 revealed that nucleotide specificity of the reverse reaction differs from the forward reaction.[49] Attempts toward differentially-glycosylated variants by pathway engineering have led to only a few polyene sugar variations to date, specifically, the replacement of D-mycosamine with 6-deoxy-D-Man, 3-keto-6-deoxy-D-Man polyenes, and an unchar-

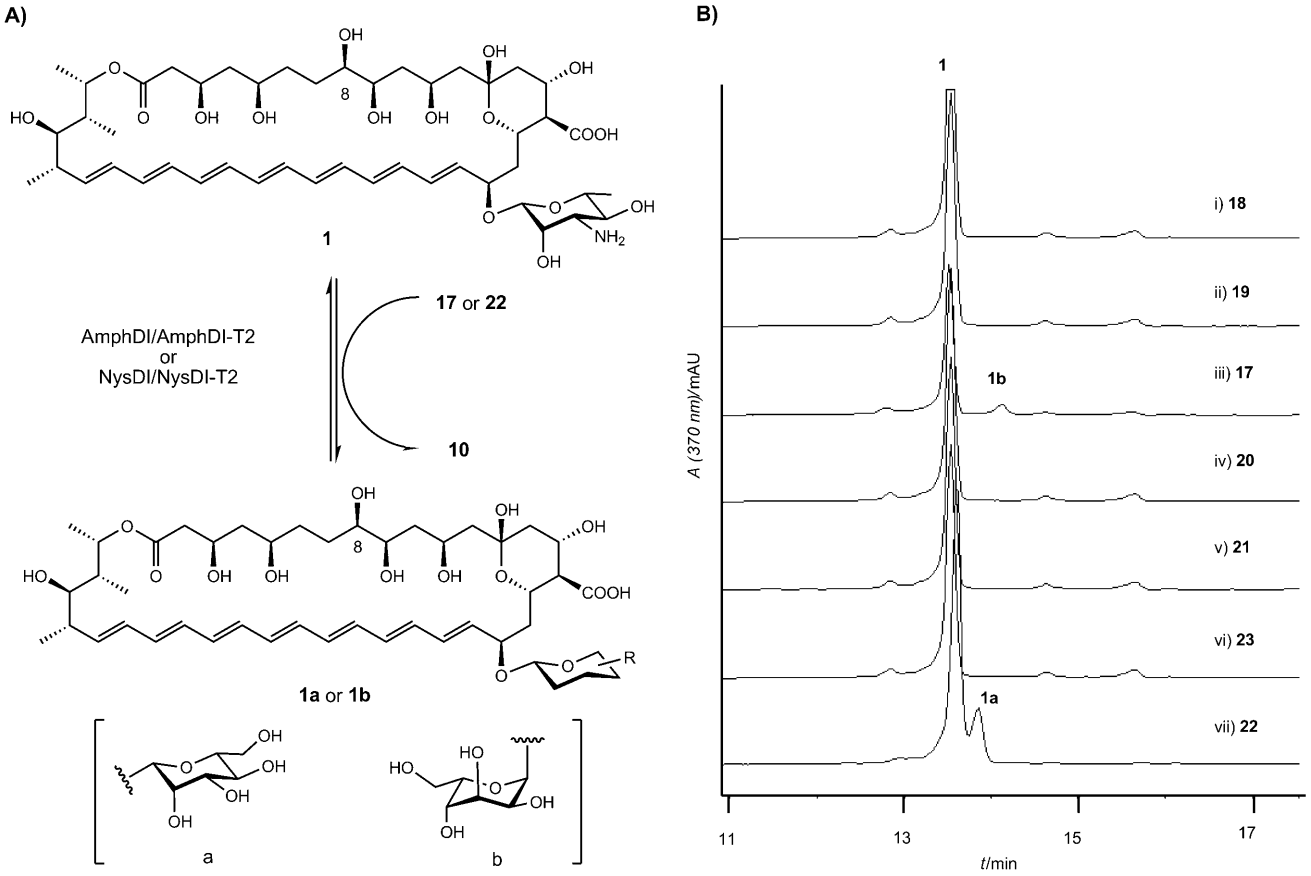


Figure 2. Polyene GT-catalyzed sugar-exchange reactions. A) Schematic representation of polyene GT-catalyzed sugar-exchange reaction. B) HPLC analyses of AmphDI-T2 catalyzed sugar-exchange reactions. In this study, AmB (**1**, 20 μM) was coincubated with AmphDI-T2 (5 μM) in the presence of 1 mM of: i) GDP-α-D-altrose (**18**), ii) GDP-α-D-talose (**19**), iii) GDP-β-L-gulose (**17**), iv) GDP-β-L-mannose (**20**), v) GDP-α-D-glucose (**21**), vi) GDP-β-L-glucose (**23**), vii) GDP-α-D-mannose (**22**).

acterized hexose (putatively Man or Glc).[27,30,32] While the current study led to two new polyene sugar appendages (L-gulose and D-Man), it also suggests that the stringent sugar nucleotide specificity of the polyene GTs might, in part, restrict the generation of variant glycosides by in vivo engineering or in vitro chemoenzymatic methods. However, the recent success of expanding the substrate promiscuity of natural product GTs by directed evolution/engineering could present exciting new avenues to circumvent the sugar nucleotide stringency of polyene GTs and thereby further enhance their synthetic utility.[62,63]

In contrast to their sugar nucleotide stringency, the demonstrated ability of Amph/NysDI to utilize AmB (**1**), nystatin A1 (**2**), candicidin members (Figure 1B), and pimaricin (**4**, Scheme 1), positions these polyene GTs among a growing list of natural product GTs with promiscuity toward aglycon acceptors, exemplified by GTs such as OleD[62–64] or VinC.[47,65,66] Related to this, previous polyene in vivo and in vitro biosynthetic studies established that oxidative tailoring (e.g., 8-hydroxylation by AmphL in **1** and 10-hydroxylation by NysL in **2**) occurs after mycosaminylation,[30,37,38] while the present study revealed hydroxylated aglycons (e.g., **7** and **8**) to be substrates of AmphDI and NysDI in vitro. Thus, the ultimate order of biosynthetic events (hydroxylation and mycosaminylation) in vivo might be dictated by the substrate specificity of the oxidases AmphL and NysL.

Significance

This study extends the fundamental understanding of polyene biosynthesis and the potential for chemoenzymatic diversification of polyene-based antifungal drugs. In the context of biosynthesis, the first in vitro characterization of representative polyene GTs unequivocally confirmed that these enzymes are GDP–sugar dependent and also revealed the correct start codons for the previously identified *amphDI* and *nysDI* genes. In addition, the demonstrated aglycon flexibility of polyene GTs in vitro suggests the order of final tailoring steps implicated from in vivo studies (glycosylation followed by oxidation) must be dictated by oxidase, not GT, specificity. With respect to polyene diversification, this study highlights the utility of the recently engineered nucleotidyltransferase (RmlA) variant to synthesize novel GDP–sugars and the application of these reagents in conjunction with the reversibility of GT-catalyzed

reactions to study purine sugar nucleotide-dependent GTs. By using these reagents, the evaluation of polyene GT aglycon and sugar nucleotide substrate specificity revealed some tolerance to aglycon structural diversity, but stringent sugar specificity, and culminated in new polyene analogues in which L-gulose or D-mannose replace the native sugar D-mycosamine.

Experimental Section

Materials and general methods: *E. coli* DH5α and BL21(DE3) competent cells were purchased from Invitrogen (Carlsbad, CA, USA). *Streptomyces nodosus* ATCC 14899 and *Streptomyces noursei* ATCC 11455 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The pET-28a *E. coli* expression vector was purchased from Novagen (Madison, WI, USA). Primers were ordered from Integrated DNA Technology (Coralville, IA, USA). Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA, USA). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). Candicidin was purchased from U.S. Pharmacopeia (Rockville, MD, USA). Other polyene macrolide antibiotics, such as amphotericin B (**1**), nystatin A1 (**2**), pimaricin (**4**), filipin III (**6**), and sugar nucleotides GDP-D-glucose (**21**), GDP-D-mannose (**22**), and GDP-L-fucose (**23**), were purchased from Sigma.

For chemical syntheses, all moisture-sensitive reactions were performed in flame-dried glassware under an atmosphere of argon. Reactions were generally concentrated under reduced pressure by using a Büchi rotary evaporator at water aspirator pressure ($<$ 20 torr) followed by removal of residual volatile materials under high vacuum (via a standard belt-drive oil pump, $<$ 1 torr). Analytical thin layer chromatography (TLC) was performed with E. Merck TLC plates precoated with silica gel 60 F254 (250 μm thick); column chromatography (FCC) was performed with Silicycle silica gel (40–60 μm, 60 Å pore size). All reagents were purchased from Aldrich (Milwaukee, WI, USA), Sigma (St. Louis, MO, USA), or Fisher Scientific (Pittsburg, PA, USA) and were used without further purification.

Analytical HPLC was performed by using a Varian Prostar 210/216 system connected to a Prostar 330 photodiode array detector (Varian, Walnut Creek, CA, USA). Mass spectra (MS) were obtained by using electrospray ionization with an Agilent 1100 HPLC-MSD SL quadrupole mass spectometer (Agilent Technologies, Palo Alto, CA, USA) connected to a UV/Vis diode array detector. Proton nuclear magnetic resonance (^{1}H NMR) and carbon NMR (^{13}C NMR) spectra were recorded by using Varian ^{UNITY}INOVA 400 MHz and 500 MHz spectrometers in deuterated solvents. Chemical shifts are reported in parts per million (ppm, δ) relative to residual solvent peaks ($CHCl_3$: ^{1}H: δ 7.26, ^{13}C: δ 77.0; H_2O: ^{1}H: δ 4.78).

Cloning, expression, and purification of polyene GTs: Genomic DNA was isolated from the amphotericin producer *S. nodosus* ATCC 14899 and the nystatin producer *S. noursei* ATCC 11455 strains by following a literature procedure.[67] The *amphDI* and *nysDI* genes were amplified from genomic DNA of the corresponding producers with Pfu DNA polymerase, by using the following primer pairs: 5′-CGACTTCATATGGGTGGACGCGAGGCG-3′ (amphDI_F, forward, NdeI) and 5′-GGACATCCTAGATCTCCTCGGTCAGTCGTTTGC-3′ (amphDI_R, reverse, BglII) for *amphDI* (1.45 kb), 5′-GTGCCGGCATATGACCCTTCCTTCCGG-3′ (nysDI_F, forward, NdeI) and 5′-GGGTTTTGGATCCTCCTCGGTCAGTCGGTT-3′ (nysDI_R, reverse, BamHI) for *nysDI* (1.52 kb). PCR products were digested with NdeI/BglII (for *amphDI*) or NdeI/BamHI (for *nysDI*) and ligated into the pET28a expression vector (NdeI/BamHI) to give plasmids pCST551 (NysDI) and pCST571 (AmphDI). For the truncated NysDI, a 1.39 kb *nysDI-T2* DNA fragment was PCR amplified from pCST551 by using the following primer pairs: 5′-GTGTTGCATATGGGCGC-GAATCGGCG-3′ (nysDI-T2_F, forward, NdeI) and 5′-GGGTTTTGGATCCTCCTCGGTCAGTCGGTT-3′ (nysDI-T2_R, reverse, BamHI). Similarly, a truncated 1.39 kb *amphDI-T2* DNA fragment was PCR amplified from pCST571 by using the following primer pairs: 5′-GTGTTGCATATGGGCGCGCACAGG-3′ (amphDI-T2_F, forward, NdeI) and 5′-GGACATCCTAGATCTCCTCGGTCAGTCGTTTGC-3′ (amphDI-T2_R, reverse, BglII). Subsequently, PCR products were digested with NdeI/BamHI (for *nysDI-T2*) or NdeI/BglII (for *amphDI-T2*) and ligated into the pET28a expression vector (NdeI/BamHI) to give plasmids pCST556 (NysDI-T2) and pCST576 (AmphDI-T2).

For AmphDI production, a single transformant of *E. coli* BL21(DE3)/pCST571 was inoculated into LB medium (4 mL) supplemented with kanamycin (50 μg mL^{-1}) and grown by being shaken at 37 °C, overnight. The precultures were used to inoculate LB medium (1 L) containing kanamycin (50 μg mL^{-1}); this culture was grown by being shaken at 18 °C to an OD_{600} of 0.5–0.7. Protein expression was induced with the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.3 mM), followed by growth for an additional 20 h. The cells obtained from 1 L of culture were washed twice with buffer A (20 mM NaH_2PO_4, pH 7.5, 500 mM NaCl, 10 mM imidazole) and resuspended in buffer A (30 mL) supplemented with lysozyme (1 mg mL^{-1}). After a 10 min incubation on ice, the cells were lysed by three rounds of French-press (1200 psi, Thermo IEC), and the insoluble material was removed by centrifugation at 30000 *g* for 1 h (4 °C). The supernatants were loaded onto a HisTrap HT column (1 mL, GE Healthcare) and the N-$(His)_{10}$-tagged AmphDI was eluted with a linear gradient of imidazole (10–500 mM) in buffer A by using a FPLC system (GE Healthcare). The purified protein was desalted through a PD-10 column (GE Healthcare) and stored in a buffer containing Tris-HCl (50 mM, pH 8.0) and glycerol (10%) until use. Protein concentration was measured by using the Bradford assay. The N-$(His)_6$-tagged NysDI, NysDI-T2, and AmphDI-T2 were produced and purified by following the same protocol from *E. coli* BL21(DE3) strains harboring pCST551, pCST556, and pCST576, respectively.

Chemical synthesis of sugar phosphates (Scheme 2 A): The syntheses for the required α-D-altrose-1-phosphate, α-D-talose-1-phosphate, and β-L-mannose-1-phosphate precursors have been previously reported.[54–57]

Dibenzyl-(2,3,4,6-tetra-O-acetyl-β-L-gulopyranosyl)phosphate (***14***): Peracylated β-L-gulose (**13**, 351 mg, 0.9 mmol) was dissolved in acetic acid (2 mL) and 33% HBr in acetic acid (1 mL) was added drop-wise at 0 °C. The reaction was allowed to warm to room temperature by being stirred for 2 h, diluted with cold $CHCl_3$ (100 mL), and washed successively with cold saturated $NaHCO_3$ solution (3× 20 mL), H_2O (20 mL), and brine (20 mL). The organics were dried over anhydrous Na_2SO_4 and concentrated. The crude gulopyranosyl bromide was used directly. A mixture of dibenzyl phosphate (300 mg, 1.08 mmol), silver trifilate (300 mg, 1.17 mmol), 2,4,6-collidine (0.23 mL, 1.74 mmol), and activated 4 Å molecular sieves (400 mg) in dry CH_2Cl_2 (10 mL) was stirred at room temperature under argon in the absence of light for 1 h. The mixture was then cooled to −40 °C and a solution of crude protected pyranosyl bromide in dry CH_2Cl_2 (5 mL) was added drop-wise. The reaction mixture was kept at −40 °C for 2 h and then allowed to warm to room temperature by being stirred, overnight. The filtrate was diluted with CH_2Cl_2 (100 mL) and washed with saturated $CuSO_4$ (2×30 mL), H_2O (20 mL), and brine (20 mL). The organics were dried over

Na_2SO_4, concentrated and purified by silica gel chromatography (hexane/EtOAc, 1:1–1:1.5) to give **14** (300 mg, 55% for two steps). $[\alpha]_D = 1.5$ ($c = 1$, $CHCl_3$); ^{1}H NMR ($CDCl_3$) 7.30–7.22 (m, 10H), 5.59 (dd, $J = 7.2$, 8.3 Hz, 1H), 5.35 (m, 1H), 5.06 (m, 3H), 4.97(d, $J = 7.2$ Hz, 2H), 4.92 (m, 1H), 4.29 (t, $J = 5.7$ Hz, 1H), 4.12 (dd, $J = 11.5$, 5.6 Hz, 1H), 4.03 (dd, $J = 7.3$, 11.5 Hz, 1H), 2.11 (s, 3H), 2.08 (s, 3H), 1.90 (s, 3H), 1.81 (s, 3H); ^{31}P NMR ($CDCl_3$) 2.17; MS: calcd $C_{28}H_{33}O_{13}PNa$ 631.2, found *m/z* 631.3 $[M+Na]$.

*Triethylammonium-(β-L-gulopyranosyl)phosphate (**15**)*: Compound **14** (260 mg, 0.43 mmol) was dissolved in a solution of MeOH (5 mL) and $NaHCO_3$ (1 N, 1.2 mL) and 10% Pd/C was added (90 mg). After being stirred, overnight, at room temperature under a hydrogen atmosphere, (1 atm), the catalyst was removed by filtration and the filtrate concentrated to approximately 3 mL volume. The solution was cooled to 0 °C and NaOH (1 N, 2.5 mL) was added drop-wise while being stirred. The mixture was stirred for an additional 2 h and neutralized with HOAc (1 N). The resulting solution was then submitted to anion-exchange chromatography (Dowex 1×8, 1.2×12 cm) and eluted successively with H_2O (100 mL), NH_4HCO_3 (0.1 M, 100 mL), NH_4HCO_3 (0.2 M, 100 mL), and NH_4HCO_3 (0.3 M, 100 mL). The product-containing fractions, which eluted with NH_4HCO_3 (0.2 M), were pooled and coevaporated with EtOH several times to remove residual NH_4HCO_3. The resulting sugar phosphate sodium salt was transformed into a triethylamine salt by being passed through an AG 50W-X8 cation-exchange column (Et_3NH^+ type, 1.5×10 cm) eluted with ddH_2O. The product-containing fractions (5×20 mL) were pooled and lyophilized to give product (135 mg, 87% yield). ^{1}H NMR (D_2O) 5.16 (t, $J = 7.8$ Hz, 1H), 4.04 (m, 2H), 3.79 (m, 2H), 3.68 (m, 2H), 3.17 (q, $J = 7.2$ Hz, 10H), 1.25 (t, $J = 7.2$ Hz, 15H); ^{13}C NMR (D_2O) 95.72, 74.80, 71.10, 69.50 (×2), 61.64, 46.93, 8.54; ^{31}P NMR (D_2O) 2.64; MS: calcd for $C_6H_{12}O_9P$: 259.0 $[M+H]^+$, found *m/z* 258.7.

Chemical synthesis of GDP–sugars (Scheme 2A): A mixture of triethylammonium sugar phosphate (e.g., **15**) and 4-morpholine-*N,N′*-dicyclohexylcarboxamidinium guanosine 5′-monophosphomorpholidate (1.6 equiv) was coevaporated with dry pyridine (3 mL) three times, after which 1*H*-tetrazole (3 equiv) and dry pyridine (3 mL) were added, and the solution was stirred at room temperature. After three days, the mixture was diluted with water, evaporated, and purified by using a Bio-Gel P-2 column (1.5× 150 cm, 1 mL min^{-1}), eluted with NH_4HCO_3 (0.05 M). The product-containing fractions, which eluted between 160–180 mL, were collected and lyophilized to afford the desired product. The typical yield of this procedure ranged from 46–65%.

*Guanosine 5′-β-L-gulopyranosyl diphosphate (**17**)*: By using the general procedure, triethylammonium-β-L-gulopyranosyl phosphate **15** (50 mg, 0.14 mmol) gave 40 mg of the desired product **17** (46%). ^{1}H NMR (D_2O) 8.10 (s, 1H), 5.93 (dd, 1H, $J = 1.6$, 6.2 Hz), 5.28 (dt, 1H, $J = 1.7$, 8.1 Hz), 4.53 (m, 1H), 4.23 (m, 3H), 4.07 (m, 2H), 3.82 (m, 2H), 3.73 (m, 2H); ^{31}P NMR (D_2O) −12.1, −13.5; ^{13}C NMR (D_2O) 160.0, 155.0, 153.0, 136.7, 115.3, 95.7, 87.9, 84.9, 75.7, 74.8, 71.9, 71.5, 70.1, 69.7, 66.4, 62.3; HRMS: calcd for $C_{16}H_{24}N_5O_{16}P_2$: 604.0693 $[M+H]^+$, found 604.0708.

*Guanosine 5′-α-D-altropyranosyl diphosphate (Scheme 2C, **18**)*: By using the general procedure, triethylammonium-α-D-altropyranosyl phosphate (25 mg, 0.07 mmol) gave 26.5 mg of the desired product **18** (61.5%). ^{1}H NMR (D_2O) 8.09 (s, 1H), 5.91 (d, 1H, $J = 6.2$ Hz), 5.40 (d, 1H, $J = 8.2$ Hz), 4.51 (dd, 1H, $J = 3.3$, 5.1 Hz), 4.33 (m, 1H), 4.20 (dd, 2H, $J = 3.3$, 5.2 Hz), 3.99 (dd, 2H, $J = 2.0$, 4.0 Hz), 3.94 (t, 1H, $J = 3.7$ Hz), 3.83 (m, 2H), 3.74 (dd, 1H, $J = 5.5$, 12.4 Hz); ^{31}P NMR (D_2O) −10.5, −12.9; ^{13}C NMR (D_2O) 159.6, 154.6, 152.4, 138.2, 116.9, 96.9, 87.6, 84.4, 74.5, 71.1, 70.8, 70.6, 66.0, 64.8, 61.6; HRMS: calcd for $C_{16}H_{24}N_5O_{16}P_2$: 604.0693 $[M+H]^+$, found 604.0710.

*Guanosine 5′-α-D-talopyranosyl diphosphate (Scheme 2C, **19**)*: By using the general procedure, triethylammonium-α-D-talopyranosyl phosphate (50 mg, 0.14 mmol) gave 56 mg of the desired product **19** (65%). ^{1}H NMR (D_2O) 8.09 (s, 1H), 5.92 (d, 1H, $J = 6.0$ Hz), 5.60 (d, 1H, $J = 7.9$ Hz), 4.50 (m, 1H), 4.34 (dd, 1H, $J = 2.0$, 3.1 Hz), 4.20 (m, 2H), 4.10 (m, 2H), 3.95 (m, 2H), 3.89 (s, 2H), 3.80 (dd, 1H, $J = 7.7$, 12.0 Hz), 3.72 (dd, 1H, $J = 4.7$, 12.0 Hz); ^{31}P NMR(D_2O) −10.6, 13.0; ^{13}C NMR (D_2O) 158.0, 153.0, 150.9, 136.7, 115.3, 96.1, 86.0, 82.9, 72.9, 71.7, 69.5, 69.5, 68.5, 64.5, 63.8, 60.5; HRMS: calcd for $C_{16}H_{24}N_5O_{16}P_2$ 604.0693 $[M+H]^+$, found 604.0700.

Chemoenzymatic synthesis of GDP–sugars (Scheme 2B): A set of GDP–sugars were generated following protocols previously described for dTDP/UDP-sugars.[54–56,68–70] The reaction was carried out in Tris-HCl buffer (50 mM, pH 8.0) containing $MgCl_2$ (5 mM), inorganic pyrophosphatase (1 U), purified RmlA Q83D (10 μM),[43] sugar-1-phosphate (8 mM), and GTP (6 mM), and incubated at 37 °C for 2 h. The formation of sugar nucleotides (**24**–**37**, Scheme 2C) was analyzed by HPLC with an anion-exchange column (SphereClone SAX, 5 μm, 250×4.60 mm, H_2O with a 0–100% 600 mM ammonium formate gradient over 25 min, 1 mL min^{-1}, A_{254}).

Polyene GT assays

Reverse or sugar-exchange assays with polyene GTs (AmphDI, NysDI, AmphDI-T2, and NysDI-T2): Assays were performed in a total volume of 100 μL containing polyene glycosides (20–50 μM, **1**–**4**) and NDPs (2 mM) or various GDP–sugar donors (2 mM for **17**–**23**, and 30–300 μM for **24**–**33**, less than 30 μM for **34**–**37**, Scheme 2C), and incubated at 30 °C, overnight, in the presence of polyene GTs (5 μM; AmphDI, NysDI, AmphDI-T2, and NysDI-T2) in Tris-HCl buffer (50 mM, pH 8.0) containing MgCl2 (1 mM).

Forward assays: Partially purified acceptor amphoteronolide (**7**, 4 μM) was incubated with AmphDI-T2 (5 μM) and various GDP–sugar donors (2 mM for **17**–**23**, 30–300 μM for **24**–**33**, <30 μM for **34**–**37**).

For all assays, mixtures without polyene GTs served as controls. The reactions were subsequently quenched by the addition of MeOH (100 μL) and were centrifuged to remove proteins. The formation of new products was monitored by reverse phase HPLC (Phenomenex Luna C18, 5 μm, 250×4.6 mm, 0.1% TFA (A) and 10–100% CH_3CN (B) over 30 min, 1 mL min^{-1}, 370 or 305 nm). The conversion rate was calculated by dividing the integrated area of glycosylated product with the sum of integrated area of product and the remaining substrate. The newly-formed products were analyzed by LC-MS (ESI) in negative (−) mode.

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