Chemistry & Biology
Article

Redesign of Polyene Macrolide Glycosylation: Engineered Biosynthesis of 19-(*O*)-Perosaminyl-Amphoteronolide B

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DOI 10.1016/j.chembiol.2010.01.007

SUMMARY

Most polyene macrolide antibiotics are glycosylated with mycosamine (3,6-dideoxy-3-aminomannose). In the amphotericin B producer, *Streptomyces nodosus*, mycosamine biosynthesis begins with AmphDIII-catalyzed conversion of GDP-mannose to GDP-4-keto-6-deoxymannose. This is converted to GDP-3-keto-6-deoxymannose, which is transaminated to GDP-mycosamine by the AmphDII protein. The glycosyltransferase AmphDI transfers mycosamine to amphotericin aglycones (amphoteronolides). The aromatic heptaene perimycin is unusual among polyenes in that the sugar is perosamine (4,6-dideoxy-4-aminomannose), which is synthesized by direct transamination of GDP-4-keto-6-deoxymannose. Here, we use the *Streptomyces aminophilus perDII* perosamine synthase and *perDI* perosaminyltransferase genes to engineer biosynthesis of perosaminyl-amphoteronolide B in *S. nodosus.* Efficient production required a hybrid glycosyltransferase containing an N-terminal region of AmphDI and a C-terminal region of PerDI. This work will assist efforts to generate glycorandomized amphoteronolides for drug discovery.

INTRODUCTION

Many therapeutic agents are derived from polyketide and peptide natural products that contain sugar residues (Weymouth-Wilson, 1997). There is considerable interest in manipulating the sugar moieties of these compounds in order to identify analogs with improved pharmacological properties. Genetic studies on producer organisms have given insights into deoxyhexose biosynthesis and provided glycosyltransferase (GT) catalysts for addition of alternative sugars to aglycones. This work has led to production of new compounds by genetic engineering of producer strains and by in vitro glycosylation techniques (Blanchard and Thorson, 2006; Thibodeaux et al., 2007; Mendez and Salas, 2007). These studies have now been extended to polyene macrolides, antifungal agents that are also active against parasites, enveloped viruses, and prions (Zhang et al., 2008).

Most polyene antibiotics are glycosylated with mycosamine (3, 6-dideoxy-3-amino-D-mannose). The aromatic heptaene perimycin (Figure 1, **1**) is unusual in that it contains perosamine (4, 6-dideoxy-4-amino-D-mannose) (Pawlak et al., 1995). Perosamine also appears in the lipopolysaccharides of several gram-negative pathogens, and its biosynthesis has been reconstituted using enzymes from such organisms (Alberman and Piepersberg, 2001; Zhao et al., 2007). The pathway entails conversion of GDP-mannose to GDP-4-keto-6-deoxymannose followed by transamination (Figure 2). Genes involved in mycosamine biosynthesis have been identified in several polyene-producing streptomycetes (Caffrey et al., 2008). We have focused on *Streptomyces nodosus*, which produces the medically important amphotericin B (Figure 3, **2**) along with a cometabolite, amphotericin A (**3**). The current view of the mycosamine pathway in this organism is shown in Figure 2. The AmphDIII GDP-mannose dehydratase catalyzes formation of GDP-4-keto-6-deoxy mannose that is converted to GDP-3-keto-6-deoxymannose. A transamination catalyzed by the AmphDII protein generates the final activated sugar, GDP-mycosamine. It is unclear how the 3-ketosugar intermediate is formed. No enzyme has yet been identified that might catalyze 3, 4-ketoisomerisation of GDP-4-keto-6-deoxymannose. In the course of biochemical studies on GDP-fucose biosynthesis, Sullivan and co-workers (1998) used *Escherichia coli* and human GDP-mannose 4, 6 dehydratases to generate GDP-4-keto-6-deoxymannose in vitro. They obtained NMR evidence for spontaneous conversion of this intermediate to GDP-3-keto-6-deoxymannose. Thus, it is possible that this step in mycosamine biosynthesis is not enzyme-catalyzed in vivo. Spontaneous 3, 4 ketoisomerisation has also been observed with dTDP-4-keto-6-deoxyglucose, a key intermediate in the biosynthesis of many deoxyhexoses (Naundorf and Klaffke, 1996). However, enzymes that catalyze this reaction have been discovered more recently (Pfoestl et al., 2003). A well-studied example is Tyl1a that operates in the mycaminose biosynthetic pathway in *Streptomyces fradiae*, the tylosin producer (Melançon et al., 2007). This catalyzes interconversion of the 4- and 3-ketosugars via an enediol intermediate (Tello et al., 2008). Although Tyl1a has been extensively characterized, the effects of disruption of the *tyl1a* gene on tylosin glycosylation have not yet been investigated. Indeed, Schell and co-workers have demonstrated biosynthesis of mycaminose in an

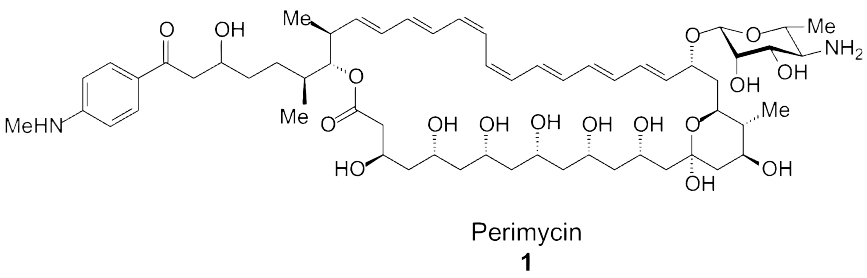


Figure 1. Structure of Perimycin

engineered strain of *Saccharopolyspora erythraea* that has no 3, 4 ketoisomerase (Schell et al., 2008). These considerations suggest that 3, 4 ketoisomerases may be important when it is necessary to divert an NDP-4-keto-6-deoxyhexose away from competing pathways, or when isomerization is accompanied by a switch in alcohol stereochemistry at C-4. Neither of these conditions applies in mycosamine biosynthesis.

The amphotericin aglycones are synthesized by a large modular polyketide synthase (PKS) (Caffrey et al., 2001). The initial products are 8-deoxy-16-descarboxyl-16-methyl-amphoteronolides A and B (**4** and **5**, Figure 3). The AmphN cytochrome P450 oxidises the C-16 methyl group to a carboxyl group to form 8-deoxyamphoteronolides A and B (**6** and **7**). These are mycosaminylated by AmphDI and C-8 hydroxylated by AmphL to give amphotericins A and B (**2** and **3**). Targeted inactivation of *amphN* gives glycosylated amphotericin analogs, in which methyl groups replace the exocyclic carboxyl groups (Carmody et al., 2005). Deletion of both *amphDII* and *amphN* gave analogs **8** and **9** that lack exocyclic carboxyl groups and are glycosylated with a 6-deoxyhexose, presumably a shunt product that results from reduction of the 4-keto or the 3-ketosugar intermediate by an unknown enzyme. The stereochemistry has not yet been determined for C-3 and C-4 of the reduced sugar. These results indicated that AmphDI might have some tolerance toward sugar and aglycone substrates. However, Zhang and co-workers (2008) performed in vitro studies with hexahistidine-tagged forms of AmphDI and NysDI, the mycosaminyl transferase from the nystatin producer *Streptomyces noursei* (Nedal et al., 2007). Both enzymes were found to have narrow sugar substrate specificity, recognizing only GDP-D-mannose and GDP-L-gulose out of a panel of 21 synthetic GDP-sugars (Zhang et al., 2008). GDP-perosamine was not investigated in this study.

Crystal structures have been determined for several plant and bacterial GTs that act on bioactive natural products (Truman et al., 2009). Despite this progress, only a few of these enzymes have been successfully redesigned. Sugar and aglycone substrate specificities have been altered by mutagenesis or by construction of hybrid enzymes. In an early landmark study, Hoffmeister and co-workers (2002) investigated extending glycosyltransferases UrdGT1c and UrdGT1b that incorporate rhodinose and olivose, respectively, as the second and third sugars in the linear trisaccharide chain of urdamycins. Ten amino acids were shown to determine donor and acceptor specificity. Mutagenesis of these residues gave a GT that formed urdamycin P, which has a branched trisaccharide chain. Another important example of a redesigned GT is OleD, which normally catalyzes addition of a glucosyl residue to C2″ of the desosamine sugar of oleandomycin. Williams et al. (2007) performed high-throughput screening to identify four key mutations that increase a weak activity toward small aromatic acceptors. Saturation mutagenesis at three of these positions increased tolerance toward NDP-sugar donors as well as acceptors (Williams et al., 2008a; Williams et al., 2008b). Domain swapping can give a predictable switch in GT substrate specificity. This method has recently been used to construct hybrid GTs that transfer alternative sugars onto deoxystreptamine (Park et al., 2009) and glycopeptide acceptors (Truman et al., 2009). The approach has also expanded the range of flavonoid acceptors used by plant UDP-glucosyltransferases (Hansen et al., 2009). However, many hybrids are nonfunctional, possibly because artificially fused NDP-sugar- and acceptor-binding domains may fail to engage in interactions that are necessary for overall activity.

All of the GT engineering that has been performed so far has been done with enzymes that use dTDP- or UDP-linked sugars. PerDI and AmphDI use GDP-perosamine and GDP-mycosamine, respectively, and are not closely related to any natural product GTs that have been redesigned or structurally characterized. Here, we undertake the first, to our knowledge, in vivo

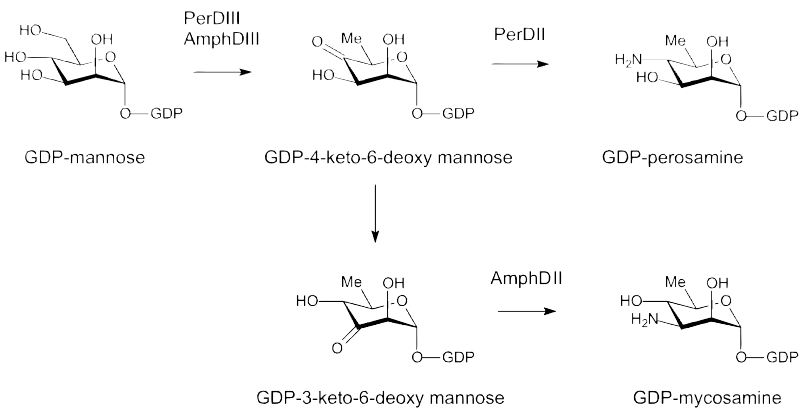
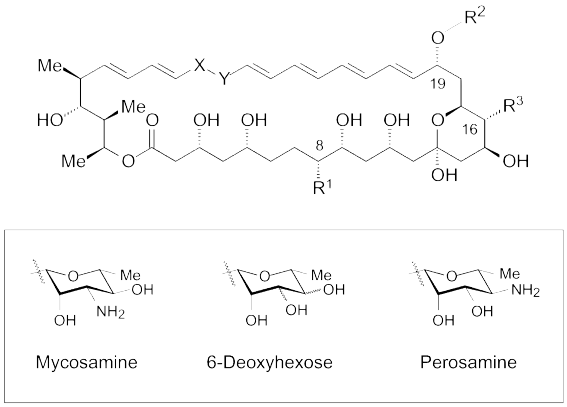


Figure 2. Biosynthetic Pathways to GDP-Perosamine and GDP-Mycosamine in *S. aminophilus* and *S. nodosus*



Compound	R^1	R^2	R^3	X-Y	RMM
2	OH	Mycosamine	COOH	HC=CH	923.5
3	OH	Mycosamine	COOH	H_2C-CH_2	925.5
4	H	H	CH_3	HC=CH	732.5
5	H	H	CH_3	H_2C-CH_2	734.5
6	H	H	COOH	HC=CH	762.5
7	H	H	COOH	H_2C-CH_2	764.5
8	OH	6-Deoxyhexose	CH_3	HC=CH	894.5
9	H	6-Deoxyhexose	CH_3	H_2C-CH_2	880.5
10	OH	6-Deoxyhexose	COOH	HC=CH	924.5
11	OH	Perosamine	COOH	HC=CH	923.5
12	OH	Perosamine	CH_3	HC=CH	893.5

Figure 3. Amphotericins Discussed in This Work

2, amphotericin B; **3**, amphotericin A; **4**, 8-deoxy-16-descarboxyl-16-methyl-amphoteronolide B; **5**, 8-deoxy-16-descarboxyl-16-methyl-amphoteronolide A; **6**, 8-deoxyamphoteronolide B; **7**, 8-deoxyamphoteronolide A; **8**, 16-descarboxyl-16-methyl-19-O-deoxyhexosyl-amphoteronolide B; **9**, 8-deoxy-16-descarboxyl-16-methyl-19-O-deoxyhexosyl-amphoteronolide A; **10**, 19-O-deoxyhexosyl-amphoteronolide B; **11**, 19-O-perosaminyl-amphoteronolide B; **12**, 16-descarboxyl-16-methyl-19-O-perosaminyl-amphoteronolide B.

glycosylation engineering of amphotericin B. The genes for perosamine biosynthesis and attachment were cloned from the perimycin producer *Streptomyces aminophilus*. The PerDI perosaminyl transferase is of interest because it transfers a sugar other than mycosamine to a polyene macrolactone. In principle, introduction of the *perDII* perosamine synthase gene into *S. nodosus* should result in biosynthesis of GDP-perosamine. We aimed to investigate whether AmphDI, PerDI, or engineered GTs could synthesize perosaminyl-amphoteronolides. These studies should assist further attempts to engineer the biosynthesis of amphotericin analogs with altered sugar residues.

RESULTS

Cloning of Perimycin Biosynthetic Genes

A cosmid library was constructed from genomic DNA of *S. aminophilus*. The library was screened with DNA probes derived from amphotericin PKS genes. Hybridizing cosmids were isolated, and restriction fragments were subcloned into pUC118 for limited sequencing. Sequences from the ends of cosmid inserts were used to design oligonucleotide primers so that overlapping clones could be identified by PCR. This approach was used to assemble a set of cosmids representing the entire cluster. The partial sequencing indicated that the organization of genes is similar to that in the candicidin/FR008 cluster (Chen et al., 2003). Homologs of polyene glycosyltransferase and sugar aminotransferase genes were found at one end of the cluster. Complete sequencing of a 4919 bp region (GenBank accession number, GQ380697) revealed *perDI* and *perDII* genes for perosaminyltransferase and perosamine synthase. In the sequenced region, the *perDI* gene was preceded by part of an *N*-methylase gene, whereas *perDII* was followed by a discrete thioesterase (TE) gene and the 5′ end of a *p*-aminobenzoic acid synthase (PABA) gene. *N*-Methylase and PABA synthase enzymes are required for synthesis of the starter unit of the perimycin polyketide. In *Streptomyces* species FR008, the region immediately downstream from the *fscMII* mycosamine synthase gene contains genes for a cytochrome P450 and ferredoxin that catalyze formation of the exocyclic carboxyl group in FR008/candicidin. This late modification does not occur in perimycin biosynthesis, and the region between *perDII* and the discrete TE gene consists of 76 bp of noncoding DNA.

PerDI shows 63–72% sequence identity with mycosaminyl transferases, whereas PerDII shows 73–77% sequence identity with mycosamine synthases (see Figure S1 available online). Some of the differences between PerDII and mycosamine synthases correspond to positions that have been identified as active site residues in perosamine synthases from other bacteria (Cook et al., 2008).

The *perDIII* gene for GDP-mannose 4, 6 dehydratase was found at the opposite end of the cluster. Sequencing of a 3627 bp region (accession number, GQ380698) revealed that PerDIII shows 76–81% sequence identity with GDP-mannose dehydratases that function in GDP-mycosamine biosynthesis (Figure S1). The upstream region contained a gene for a β-glucosidase with strong homology to cellobiose hydrolases. This gene probably marks the end of the cluster because it has no obvious role in perimycin biosynthesis. The downstream DNA contained a 553 bp stretch homologous to the 5′ end of *fscD*, which encodes modules 11 to 16 of the FR008/candicidin PKS. Since our main interest was in perosamine synthesis and attachment, the remainder of the perimycin cluster was not sequenced at this stage.

Inactivation of *amphDII*

Previous work had generated a Δ*amphDII-NM* mutant that produced 16-descarboxyl-16-methyl-19-*O*-deoxyhexosyl amphoteronolide B (**8**) and a tetraene analog (**9**) that is not C-8 hydroxylated (Carmody et al., 2005). This mutant retained a thiostrepton resistance (*tsr*) gene embedded in the chromosome. In this study, it was necessary to generate thiostrepton-sensitive Δ*amphDII* mutants that could be transformed with plasmid constructs that have *tsr* as a selectable marker. A frameshift mutation was introduced into a cloned copy of the *amphDII* gene by end-filling an internal NcoI site (nucleotides 63699–63704; accession number AF357202). A StuI-PstI fragment

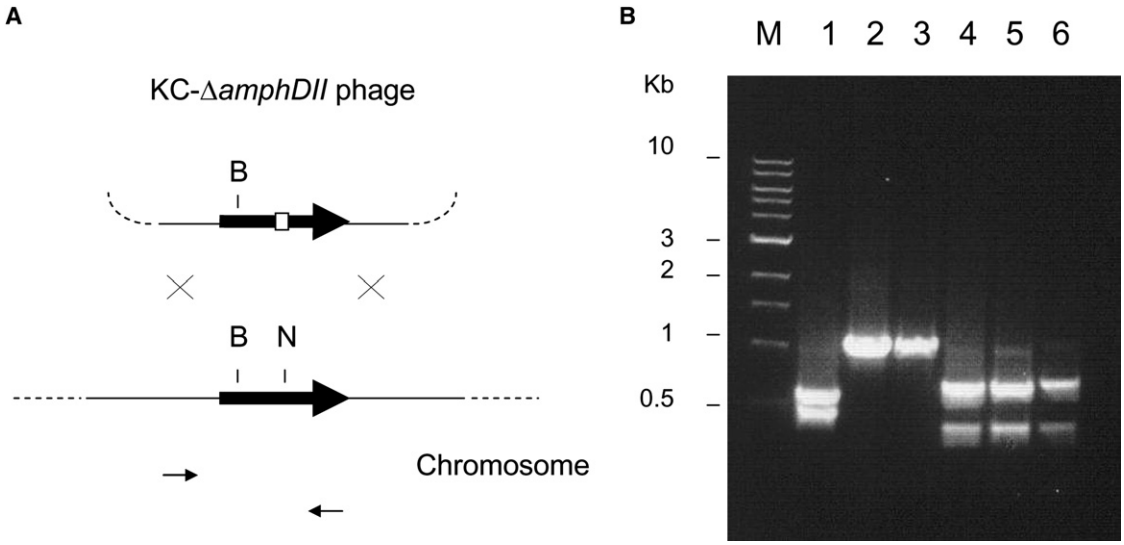


Figure 4. Inactivation of *amphDII*

(A) Replacement of *amphDII* with a mutant version results in loss of a chromosomal NcoI site (N). An adjacent BclI site (B) is unaffected. The small arrows represent the BM1 and BM2 primers used to amplify the *amphDII* region from chromosomal DNA.

(B) Analysis of *amphDII* region in various strains. The *amphDII* region was amplified by PCR and treated with restriction enzymes. The wild-type *amphDII* region contained the expected NcoI site (lane 1) whereas the corresponding sequences from the *amphDII* (lane 2) and *amphDII-NM* (lane 3) mutants did not. All three PCR products were digested by BclI in control digests (lanes 4 to 6).

(nucleotides 62662–65544) containing the mutated *amphDII* sequence was cloned into KC-UCD1 (Carmody et al., 2004). The resulting phage was used to replace the *amphDII* gene in wild-type *S. nodosus* and in the Δ*amphNM* mutant. The genotypes of the resulting Δ*amphDII* and Δ*amphDII-NM* mutants were verified by PCR (Figure 4).

S. nodosus Δ*amphDII* produced polyenes with masses consistent with 6-deoxyhexosyl-amphoteronolide B (**10**), aglycones 8-deoxyamphoteronolides A (**5**) and B (**6**), and amphoteronolide B (Figure S2). The mixture had no detectable antifungal activity at total polyene concentrations as high as 50 μg/ml. This was consistent with absence of the amino sugar. The fact that all polyenes produced by this strain had exocyclic carboxyl groups indicates that the frameshift mutation in *amphDII* does not affect expression of the downstream *amphN* cytochrome P450 gene. *S. nodosus* Δ*amphDII-NM* produced 6-deoxyhexosylated polyenes lacking exocyclic carboxyl groups (**8** and **9**) detected previously by Carmody et al., (2005) as well as aglycones 8-deoxy-16-descarboxyl-16-methyl-amphoteronolides A and B (**4** and **5**).

The *amphDII* gene was amplified and cloned into the expression vector pIAGO (Aguirrezabalaga et al., 2000) under the control of the *ermE* promoter. The resulting pIAGO-*amphDII* construct was transformed into *S. nodosus* Δ*amphDII* and *S. nodosus* Δ*amphDII-NM*. The plasmid-borne *amphDII* gene restored efficient production of active mycosaminylated amphotericins in both strains (data not shown).

Attempts to Engineer the Biosynthesis of Perosaminyl-Amphoteronolides

Previous inactivation of the *amphDIII* gene gave a mutant that synthesized 8-deoxyamphoteronolide A (**7**) as a major product (Byrne et al., 2003). The *perDIII* gene was cloned into pIAGO and transformed into *S. nodosus* Δ*amphDIII*. Analysis of polyene extracts revealed that the resulting transformant produced a mixture of aglycone **7** and glycosylated amphotericins **2** and **3** in approximately equal amounts (data not shown), whereas the host containing the empty vector synthesized aglycone **7** only. The fact that PerDIII can substitute for AmphDIII is consistent with the proposed mycosamine biosynthetic pathway shown in Figure 2. In *S. nodosus*, the PerDII protein should be able to intercept GDP-4-keto-4, 6-dideoxymannose, produced by AmphDIII, and catalyze formation of GDP-perosamine.

We first attempted to replace the *S. nodosus* chromosomal *amphDI-DII* genes with *perDI-DII* genes. This was expected to reveal whether PerDI could transfer perosamine onto amphotericin aglycones. A recombinant phage was constructed to contain the *perDI-DII* genes flanked by sequences upstream and downstream of the *amphDI-DII-NM* region. The *perDI* start codon was precisely located downstream from the *amphDI* promoter and ribosome-binding site. Attempts to perform the gene replacement in *S. nodosus* ΔNM yielded a deletion mutant lacking *amphDI-DII-NM* and containing *perDI-DII* (Figure S3), which had the required genotype. This strain produced low levels of aglycones **4** and **5** but no glycosylated forms (Figure S4). This finding provided evidence that PerDI does not recognize amphoteronolides lacking exocyclic carboxyl groups, even though these aglycones resemble its natural substrate around the glycosylation site. Attempts to obtain a perfect replacement of *amphDI-DII* by *perDI-DII* were therefore discontinued.

The 8-deoxy-16-descarboxyl-16-methyl amphoteronolide B product (**4**) had a lower methanol-solubility than other amphoteronolides and precipitated selectively on partial concentration of crude methanol extracts. It could be purified in 100 mg quantities

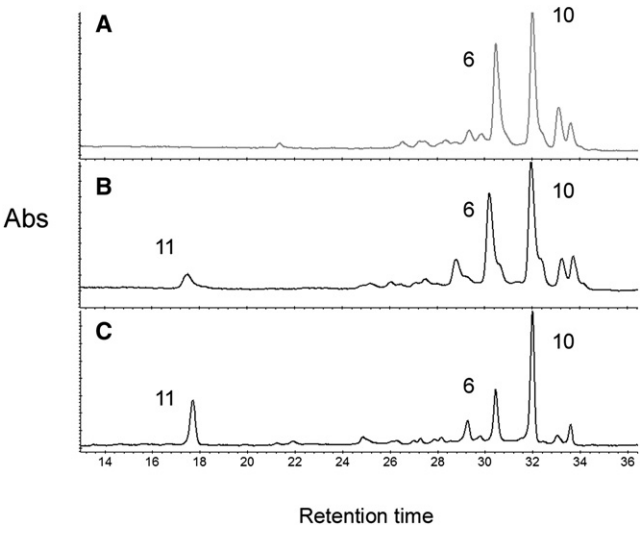


Figure 5. Detection of Perosaminylated Polyene by HPLC

(A) HPLC analysis of polyenes produced by *S. nodosus* Δ*amphDII* transformed with pIAGO.

(B) Polyenes extracted from the same host carrying pIAGO-*perDII*.

(C) Polyenes from the strain carrying pIAGO-*hap2* encoding the Hap2 hybrid GT and PerDII.

from strains containing mutations in *amphNM* and glycosylation genes. Structural analysis of this material by NMR will be described elsewhere. This aglycone may be useful for in vitro studies on polyene glycosylation and exocyclic carboxyl group formation.

The pIAGO-*perDII* plasmid was introduced into *S. nodosus* Δ*amphDII*. Crude polyenes extracted from this transformant showed some antifungal activity. Analysis of the extract by HPLC revealed a low level of a new polyene, in addition to those produced by the host strain containing the empty vector, 8-deoxyamphoteronolide B (**6**) and 6-deoxyhexosyl-amphoteronolide B (**10**) (Figures 5A and 5B). The new compound was a minor component and made up 2% of the total polyene. Further analysis revealed that this compound had a mass appropriate for **11**. The yield of this product was too low to allow further purification and analysis. This result suggested that AmphDI can perosaminylate amphoteronolides, but very inefficiently. The fact that the deoxyhexosylated analog **10** remained as the major product suggests that AmphDI has a preference for GDP-deoxyhexose over GDP-perosamine. Overproduction of AmphDI was not expected to change the proportions of polyene products or to increase the yield of **11**. Attempts were therefore made to construct a hybrid GT.

Assessment of AmphDI-PerDI Hybrid GTs

The GTs involved in natural product glycosylation belong to the GT-B superfamily and have N-terminal and C-terminal domains that function in recognition of acceptor and NDP-sugar donor substrates, respectively. In an effort to increase the yield of the putative perosaminylated analog, hybrid GTs were constructed in which the putative 8-deoxyamphoteronolide-binding N-terminal domain of AmphDI was fused to the putative GDP-perosamine-binding C-terminal domain of PerDI. The internal boundaries of these substrate-binding domains were uncertain, because PerDI and the mycosaminyl transferases are not closely related to natural product GTs that have been characterized more extensively (Liang and Qiao, 2007). It was therefore necessary to identify a functional cross-over point by trial and error. Two hybrid genes were made in initial attempts to design a GT that could efficiently modify the amphotericin aglycone with perosamine. To construct the first of these, the OGTF and GTR1 primers were used to amplify the 5′ region of the *amphDI* gene from the ribosome-binding site to the codons for E191-L192. The GTF1 and OGTR primers were used to amplify the *S. aminophilus* DNA region from the corresponding *perDI* codons for E191-L192 to the end of *perDII*. In both AmphDI and PerDI, E191-L192 are encoded by GAG-CTG codons. In each case, the second codon was altered so as to create a SacI site (GAGCTC). The two products were ligated through their SacI sites to create a hybrid *amphDI-perDI* gene (*hap1*) linked to a *perDII* gene. This DNA was cloned between the BamHI and HindIII sites of pIAGO to form pIAGO-*hap1*.

The second hybrid GT gene (*hap2*) was constructed using primer pairs OGTF plus GTR3 and GTF3 plus OGTR. In this case, the two products were joined through a SacI site introduced to represent GAG-CTC codons for E246-L247 of AmphDI and E244-L245 of PerDI. The second construct was named pIAGO-*hap2*. The cross-over points in Hap1 and Hap2 are highlighted in Figure S1A.

The pIAGO-*hap1* and pIAGO-*hap2* plasmids were introduced into the Δ*amphDII* and Δ*amphDII-NM* mutants. The two constructs differ at only 59 nucleotide positions in a central 165 bp stretch within the GT gene. Thus, the expression levels of the two hybrid genes are expected to be similar. In the Δ*amphDII* strain, pIAGO-*hap1* had the same effect as pIAGO-*perDII* but pIAGO-*hap2* increased the yield of the new analog (**11**) to approximately 25% of the total polyene, and brought about a corresponding decrease in the amount of the aglycone **6** (Figure 5C). This change was caused by the *hap2* gene because the strains are otherwise isogenic. In an additional control experiment, a pIAGO-*perDI-perDII* construct did not elevate production of the new polyene, confirming that PerDI does not recognize amphoteronolides even when expressed from the *ermE* promoter. These results show that Hap2 is a functional hybrid GT in which the amphoteronolide-binding domain of AmphDI has been grafted onto the GDP-perosamine-binding domain of PerDI. Although the two hybrid proteins differ by only 24 amino acids out of 460, Hap2 boosted production of **11**, whereas Hap1 did not. This shows that the choice of cross-over point is critical. It should be noted that the pIAGO vector does not give particularly high expression of cloned genes in *S. nodosus*. None of the GTs (AmphDI, PerDI, Hap1, or Hap2) could be detected as a prominent band when cell lysates of the various transformants were analyzed by SDS-polyacrylamide gel electrophoresis.

The new compound had a retention time different to that of amphotericin B (Figure S5). The mass was that expected for perosaminyl-amphoteronolide B (**11**) ($[M - H]^-$ = 922.5; $[M + Na]^+$ = 946.5) (Figure S6). The yields of the new polyene from *S. nodosus* Δ*amphDII* pIAGO-*hap2* were typically around 40 mg/L. No perosaminylated tetraenes were observed.

Analysis of Perosaminylated Analog by NMR

The analog was purified and examined by proton and COSY NMR. Most resonances are similar to those found in amphotericin B (Figures S7 and S8). The four methyl groups in amphotericin B show NMR resonances in d^6-DMSO as doublets at 0.91, 1.04, 1.11, and 1.13 ppm (McNamara et al., 1998), the last assigned to the mycosaminyl methyl group. NMR analysis of HPLC-purified **11** (~2 mg) in DMSO at 40°C showed four methyl doublets at 0.93, 1.05, 1.13, and 1.17 ppm; this last value has moved downfield, and the resonance was considerably broadened. Addition of an equivalent of methanoic acid to the NMR sample sharpened this doublet, and moved it further downfield to 1.24 ppm, presumably because of the close proximity in space of the perosaminyl's equatorial protonated 4-amino group. The resonance at 2.81 ppm, assigned to the 4′-CH(NH_2) proton, shows two large transdiaxial coupling constants of ~9.5 Hz to 3.37 ppm (5′) and 3.44 (3′). The 2′-proton at 3.70 ppm has a small axial/equatorial coupling constant of 2.7 Hz to the 3′-proton. The 1′-proton has moved from 4.61 in amphotericin B upfield to 4.35 ppm in the perosaminyl analog. Reported NMR analysis of *N*-acetyl perimycin in deuterated pyridine/methanol has coupling constants for 2′ of 0.0 and 3.0 Hz; those for 3′ are 3.0 and 10.0, and those for 4′ are 10.0 and 10.0 (Pawlak et al., 1995).

Perosaminylation of Amphoteronolides Lacking Exocyclic Carboxyl Groups

The pIAGO-*hap2* plasmid was also introduced into the *S. nodosus* Δ*amphDII-NM* strain that produces aglycones lacking exocyclic carboxyl groups. This resulted in formation of a new minor polyene product not produced by the parent strain. Analysis by ESMS indicated that 16-descarboxyl-16-methyl-19-*O*-perosaminyl-amphoteronolide B (**12**) was produced ($[M + H]^+$ = 894.5; $[M - H_2O + H]^+$ = 876.5]; $[M + Na]^+$ = 916.5; formic acid adducts were detected in negative ion mode ($[M + HCOOH]^-$ = 938.5). It was not possible to purify this minor component in quantities sufficient for further analysis. However, it can be concluded that the Hap2 GT is capable of transferring perosaminyl residues to amphoteronolides lacking exocyclic carboxyl groups.

Biological Activities of 19-*O*-Perosaminyl-Amphoteronolide B

Bioassays against *Saccharomyces cerevisiae* indicated that perosaminyl-amphoteronolide B had a minimal inhibitory concentration of 1.9 μg/ml. In parallel control assays, amphotericin B had an MIC of 2.1 μg/ml. The two polyenes also had approximately the same hemolytic activity. The polyene concentrations giving 50% hemolysis (MHC_{50} values) were 3.125 and 3.7 μg/ml for perosaminyl-amphoteronolide B and amphotericin B, respectively, indicating similar potential toxicity. Thus, replacing mycosamine with perosamine may have only a small overall positive effect on these biological activities. However, the chromatographic properties of **11** show that it is slightly more water soluble than amphotericin B.

DISCUSSION

Enzymatic glycorandomization of polyene macrolides will require libraries of NDP-sugar donors and sugar-flexible GTs. Chemical and enzymatic methods have been developed for synthesis of NDP-sugars. These methods have already been used to synthesize 18 different GDP-sugars in vitro. Mendez and Salas, (2007) have assembled plasmid constructs that direct intracellular synthesis of 12 different dTDP-deoxyhexoses. A similar approach might be possible for in vivo generation of up to nine naturally occurring GDP-sugars (D-rhamnose, D-mannose, L-fucose, L-colitose, L-gulose, L-galactose, D-altrose, 6-deoxy-D-talose, and L-fucofuranose). Prospects for generating unnatural NDP-sugars in vivo have also been discussed (Hui et al., 2007; Nic Lochlainn and Caffrey, 2009). Although technologies for generating NDP-sugars are advanced, previous studies indicated that polyene GTs show limited tolerance toward these alternative substrates. To our knowledge, this work takes the first step toward addressing this issue.

Here, our in vivo studies indicate that the PerDI perosaminyl transferase does not act on amphotericin aglycones and that the AmphDI mycosaminyl transferase inefficiently recognizes GDP-perosamine. In the Δ*amphDII* strain containing pIAGO-*perDII*, AmphDI generated small amounts of perosaminyl-amphoteronolide B. This represents the first example of rational engineering of polyene macrolide glycosylation. Introduction of the Hap2 hybrid GT significantly increased the yield of perosaminyl-amphoteronolide B. This shows that the aglycone-specificity of PerDI has been modified. To date, only a few natural product GTs have been redesigned successfully. To our knowledge, this is the first example involving a GT that acts directly on a complex polyketide macrolactone.

The activity of Hap2 indicates that the AmphDI region from residues 1 to 247 functions in aglycone recognition, whereas the PerDI region from residues 248 to 458 is capable of binding GDP-perosamine. This finding is important because it should guide efforts to graft the amphoteronolide-binding domain onto C-terminal domains that recognize dTDP-glucose-derived deoxyhexoses. A greater array of these sugars is available. In addition, GDP-perosamine and GDP-mycosamine binding domains can be fused to N-terminal domains of GTs that recognize other natural product aglycones.

The *S. nodosus* Δ*amphDII* pIAGO-*hap2* transformant retains a functional *amphDI* gene and produces 6-deoxyhexosyl-amphoteronolide B as well as 19-*O*-perosaminyl-amphoteronolide B. Formation of the GDP-deoxyhexose apparently competes with GDP-perosamine biosynthesis in this strain. It is possible that GDP-4-keto-6-deoxymannose undergoes rapid 3, 4-ketoisomerisation and/or reduction. Sequence analysis of the *S. nodosus* genome may help to identify candidate genes for the 3,4 ketoisomerase, if it exists, and the reductase. This will allow deletion of pathways that divert GDP-4-keto-6-deoxymannose away from biosynthesis of alternative GDP-sugars.

Replacing the mycosamine of amphotericin B with perosamine made little difference to antifungal or hemolytic activity. This was not surprising, because previous chemical modification studies have shown that, although a positive charge on the sugar residue is essential for antifungal activity of amphotericin B, its exact location is not critical (Cheron et al., 1988). Recently, a *Streptomyces* species was identified that naturally produces an analog of pimaricin in which mycosamine is replaced by perosamine (Komaki et al., 2009). The perosaminylated analog had a 4-fold decrease in antifungal activity relative to pimaricin. The

Table 1. Oligonucleotides Used in This Study

Oligonucleotide	Sequence 5′ to 3′
PerDIIIF	AAAAGGATCCTGAAAGGGGACCATATGCCAAGCGCGCGCTGATC
PerDIIIR	AAAAAAGCTTCCCTAGTGATGCCGCGGGCTAC
BM1	TTCCAAGCTTCATCACAGGACTCCGTAGAAGGC
BM2	GAAGTCACATATGTCCTATACGTATCCGGTC
PerDIIR	GATCAAGCTTCAGCCCCCGTGCGCTGCGACCGC
PerDIIF	GACCAGATCTTCGGCCTGCCGAGCCTGTCCT
AmphDIIF	TGACAGATCTGACCGAGGAAGTCAAGGATG
AmphDIIR	GATCAAGCTTCATCTCGGTCTCGGCGGTCATCA
GTF1	CAACGAGCTCTTCAAATTCCGCACGCTGGGC
GTF3	GGCGGAGCTCGAGTACCCGCTGGAGATCCCG
GTR3	AGTCGAGCTCCGCCACCGAGTAGCAC
GTR1	GGAAGAGCTCGTTCTCCACCCGCTGG
OGTF	GCATAGATCTCACTTTCCGGAGGGTGTTGTCCATG
OGTR	GATCAAGCTTCAGCCCCCGTGCGCTGCGACCGC

mode of action of pimaricin is different from that of other polyenes (te Welscher et al., 2008). This may explain why moving the sugar amino group has a greater impact on activity in the case of pimaricin. At neutral pH, amphotericin B exists as a zwitterion in which the positive charge on the amino sugar neutralizes the negative charge on the exocyclic carboxyl group. With perosaminyl-amphoteronolide B, increased separation of these charges appears to improve water-solubility slightly.

Although the aminosugar of amphotericin B is important for antifungal activity, preliminary evidence suggests that it may not be essential for other biological effects, such as antiprion activity (Soler et al., 2008). Some of these additional activities might be enhanced by glycosylation engineering. The work reported here will enable the potential of polyene macrolides to be explored more fully.

SIGNIFICANCE

The biosynthesis of 19-*O*-perosaminyl-amphoteronolide B is the first rational redesign of polyene glycosylation by genetic engineering of a producer microorganism. Production of this compound in good yield suggests that sustainable fermentation methods will be able to deliver valuable glycoengineered analogs in quantities sufficient for clinical development. Polyene macrolide GTs and are not closely related to previously characterized GTs and use GDP-sugar donors rather than dTDP- or UDP-linked sugars. The construction of an active AmphDI-PerDI hybrid shows that aglycone- and NDP-sugar-binding domains can be also exchanged between these polyene GTs. The C-terminal regions of these enzymes can now be targeted in attempts to increase sugar flexibility. This work will assist future attempts at glycodiversification of polyene macrolides and other natural products.

EXPERIMENTAL PROCEDURES

DNA Methods

S. aminophilus DSM 40057 was obtained from DSMZ. Total cellular DNA was isolated as described by Hopwood et al. (1985). Chromosomal DNA was partially digested with Sau3A1 and fragments in the size range 30 to 40 kb were purified by sucrose density gradient centrifugation. These fragments were ligated to BamHI-cut pWE15 cosmid vector DNA and packaged into lambda phage particles using a Promega Packagene kit. The library was propagated on *E. coli* XL-Blue MR.

DNA probes were prepared from the amphotericin cluster. The insert from cosmid 4 containing the *amphC* sequence and the insert from cosmid 16 containing the *amphI* sequence were used as PKS probes. Fragments were purified by preparative agarose gel electrophoresis and labeled with digoxygenin-linked dUTP using a Boehringer Mannheim nonradioactive labeling kit. DNA labeling, colony hybridization, and Southern hybridization were performed according to the manufacturer's instructions. Deep Vent DNA polymerase was used in PCRs. DNA sequencing and resequencing was performed by MWG Biotech.

To construct pIAGO-*perDIII*, pIAGO-*perDII*, and pIAGO-*amphDII*, the individual genes were amplified with the forward and reverse primers listed in Table 1 The PCR products were digested with BamHI-HindIII or BglII-HindIII and cloned between the BamHI and HindIII sites of the pIAGO expression plasmid. The pIAGO-*hap1* and pIAGO-*hap2* plasmids were constructed as described in the Results section.

The pIAGO-*perDI-DII* construct was assembled as follows. A plasmid subclone CBY was digested at an EcoRI site just upstream from the *perDI* start codon and ribosome-binding site. The cohesive end was repaired with T4 DNA polymerase. Digestion with HindIII excised a fragment corresponding to nucleotides 484–4919 of the sequenced region (GenBank accession number GQ380697). Ligation between the HindIII and end-repaired BamHI sites of pIAGO positioned the *perDI-DII* region downstream from the *ermE* promoter.

Protoplast transformations and gene replacements were performed as described elsewhere (Carmody et al., 2004; Power et al., 2008).

General Polyene Purification

Polyenes were purified and characterized as described elsewhere (Power et al., 2008). Production cultures were grown in 2-l flasks containing 5 g fructose, 15 g dextrin (Corn, Type II, 15% soluble), 7.5 g soybean flour (Type I), 2.5g $CaCO_3$, 12.5 g Amberlite XAD16 resin, and 250 ml deionized water. Flasks were shaken at 120 rpm (32 mm gyrotary) at 28°C for 3 days. Polyenes were extracted from sedimented mycelia and resin into methanol. Rotary evaporation was used to concentrate the methanol extract to an aqueous residue from which the polyenes precipitated. The precipitate was washed first with water and then with chloroform. Drying gave a yellow powder containing partially purified mixed polyenes.

Large-Scale Purification of 19-*O*-Perosaminyl-Amphoteronolide B (11)

The mycelia and beads from a 7 L culture (28 flasks) were harvested by centrifugation (Sorvall GSA, 10,000 rpm 10 min, 4°C). The combined pellets were

finely dispersed in 7 L methanol and left to soak for 18 hr. The suspension was centrifuged and the supernatant extract was retained. The resulting pellet was re-extracted with a further 7 L methanol. Assay of the combined methanol extracts by UV spectrophotometry (Shimadzu 2401 PC) gave 880 mg heptaenes and 950 mg tetraenes (i.e., 125 mg/L heptaenes and 135 mg/L tetraenes). The extinction coefficient (ε) for monomeric amphotericin B at 405 nm is 1.7×10^5 $M^{-1}cm^{-1}$. For amphotericin A, ε is 0.78×10^5 $M^{-1}cm^{-1}$ at 318 nm.

The total methanolic extract was concentrated in vacuo (below 40°C) until a yellow precipitate formed. This was harvested by centrifugation. The initial precipitate contained 750 mg heptaenes and 400 mg tetraenes. Washing with deionized water (2 × 50ml) gave 700 mg heptaenes and 100 mg tetraenes. After freeze drying, the total dry weight of yellow powder was 1.2 g.

Further purification was performed on a Varian Prostar 210 diode array HPLC with Galaxieworkstation software and UV Diode array detector. The columns (Supelco Ascentis) were C8 reverse phase silica, 5 μm particle size, and 25 cm length. The 4.6 mm diameter analytical column was run at a flow rate of 1 mL/min with sample volumes of 0.02 ml in methanol. A 21.2 mm diameter preparative column was run at a flow rate of 14.8 mL/min with a typical injection volume of 1.0 ml in DMSO. The solvent system was water (A) and methanol containing 0.1% v/v formic acid (B). The gradients were typically 50–90% B over 28 min.

Polyene Analysis

Low-resolution electrospray mass spectrometry was performed on a triple Quadropole Micromass Quattro LC instrument. High-resolution mass spectrometry was performed using a Kratos Concept 1H double-focusing high-resolution sector instrument. FAB (Fast atom bombardment) spectroscopy was performed using 3-nitrobenyl alcohol matrix with HRMS peak matching referenced to stable reference peaks.

NMR spectra were obtained using a Bruker AV500 MHz instrument. A sample of perosaminyl-amphoteronolide B (**11**) was purified twice by HPLC to obtain 2 mg. This was dissolved in d6-DMSO and analyzed at room temperature (298 K) with the water peak suppressed.

Antifungal and haemolytic activities were assayed as described elsewhere (Carmody et al., 2005).

ACCESSION NUMBERS

The sequences of the *perDI-DII* region and the *perDIII* region have been deposited in the GenBank database with accession numbers GQ380697 and GQ380698, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and eight figures and can be found with this article online at doi:10.1016/j.chembiol.2010.01.007.

ACKNOWLEDGMENTS

This work was supported by the European Union (grant GENOVA QLRT-1999-00095 to P.C.), the Irish Higher Education Authority (grant PRTLI cycle 3 to P.C.), and the BBSRC (grant BB/D017270/1 to B.J.R). Some of the work in this publication has emanated from research conducted with the financial support of Science Foundation Ireland under grant number 09/RFP/GEN2132.

Received: September 21, 2009
Revised: December 3, 2009
Accepted: January 11, 2010
Published: February 25, 2010

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