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Engineered biosynthesis and characterisation of disaccharide-modified 8-deoxyamphoteronolides

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Abstract

Several polyene macrolides are potent antifungal agents that have severe side effects. Increased glycosylation of these compounds can improve water-solubility and reduce toxicity. Three extending glycosyltransferases are known to add hexoses to the mycosaminyl sugar residues of polyenes. The *Actinoplanes caeruleus* PegA enzyme catalyses attachment of a D-mannosyl residue in a β 1, 4 linkage to the mycosamine of the aromatic heptaene 67-121A to form 67-121C. NppY from *Pseudonocardia autotrophica* adds an N-acetyl-D-glucosamine to the mycosamine of 10-deoxynystatin. NypY from *Pseudonocardia* sp P1 adds an extra hexose to a nystatin but the identity of the sugar is unknown. Here we express the *nypY* gene in *Streptomyces nodosus amphL* and show that NypY modifies 8-deoxyamphotericins more efficiently than C-8 hydroxylated forms. The modified heptaene was purified and shown to be mannosyl-8-deoxyamphotericin B. This had the same antifungal activity as amphotericin B, but was slightly less haemolytic. Chemical modification of this new disaccharide polyene could give better antifungal antibiotics.

Introduction

Polyene macrolide antibiotics are used for treatment of systemic mycoses and *Leishmania* infections (Lemke et al., 2005). They have many other potential applications (Soler et al., 2008). Polyenes have serious side effects and considerable effort has gone into development of improved analogues by chemical and biological methods (Cereghetti and Carreira, 2006; Caffrey et al., 2016). Most polyenes are modified with a single mycosamine sugar. Chemical studies have shown that glycosylation of the mycosamine amino group can improve water-solubility and pharmacological properties (Cybulska et al., 2000; Szlinder-Richert et al., 2001; Ehrenfreund-Kleinman et al., 2002). We are investigating enzymes that add extra sugars to alternative positions in mycosamine residues of polyenes.

Three naturally occurring polyenes are known to be modified with disaccharides. These are 67-121C from *Actinoplanes caeruleus* DSM43634, NPP (nystatin-like *Pseudonocardia* polyene) from *Pseudonocardia autotrophica*, and a hexosylated nystatin from *Pseudonocardia* sp P1 (Wright et al., 1977; Lee et al., 2012; Barke et al., 2010). The monosaccharide versions of these antibiotics are 67-121A and nystatin A1 (Fig. 1A). The second sugar in 67-121C is D-mannose whereas that in NPP is N-acetyl-D-glucosamine (Fig. 1B). The extra sugar in the *Pseudonocardia* sp P1 polyene is a hexose, possibly mannose or glucose, but its identity has not been determined.

The three polyene extending glycosyl transferases have now been identified, PegA from *A. caeruleus*, NppY from *Ps. autotrophica*, and NypY from *Pseudonocardia* sp. P1. Candidate genes were identified in genome sequences and their functions were confirmed by experimental studies. The *pegA* gene from *A. caeruleus* was expressed in *Streptomyces albidoflavus*, producer of candicidins, aromatic heptaenes similar in structure to 67-121A. This resulted in formation of small amounts of mannosyl candicidins (De Poire et al., 2013). On this evidence, PegA was identified as the extending glycosyltransferase that forms 67-121C (Fig. 1). PegA catalysed little or no glycosylation of amphotericins when expressed in *Streptomyces nodosus* (Stephens et al., 2013). The *Pseudonocardia* sp P1 *nypY* gene was also expressed in *S. albidoflavus* and *S. nodosus*. This led to formation of small amounts of hexosylated candicidins and amphotericins (De

Poire et al., 2013). NypY was then introduced into four mutant *S. nodosus* strains producing 7-oxo-amphotericins, 16-decarboxyl-16-methyl-amphotericins, D-rhamnosyl-amphoteronolides, and D-rhamnosyl-16-decarboxyl-16-methyl-amphoteronolides. Of these, only the 7-oxo analogues were hexosylated. The putative 7-oxo disaccharide analogues were detected by LC-MS but were minor components relative to the normal monosaccharide polyenes. Because of the low yields, none of these new compounds was purified and characterised further (De Poire et al., 2013). Larger amounts of disaccharide-modified polyenes are required for purification, structure analysis, and assessment of biological activities.

The *nypY* gene has been expressed in *Streptomyces noursei*, the commercial source of nystatin A1, but no disaccharide forms were detected (Seipke et al., 2012). This was surprising because this host was expected to synthesise the natural acceptor substrate for NypY. A series of telling experiments was carried out by Kim and co-workers in 2015. The *nppY* gene was identified in the *Ps. autotrophica* genome and its function as an extending glycosyltransferase was confirmed by gene inactivation. The disruption mutants produced 10-deoxynystatin A1. In wild type *Ps. autotrophica* cells, NppY converts this intermediate to 10-deoxyNPP (Fig. 1), which is finally hydroxylated at C-10 by the NppL cytochrome P450 to give NPP. Expression of the *nppY* gene in *S. noursei* also failed to give disaccharide-modified nystatins (Kim et al., 2015). In that host, 10-deoxynystatin is hydroxylated by the NysL cytochrome P450, and this process may prevent addition of the second sugar by heterologous NypY or NppY enzymes. In the biosynthesis of amphotericins A and B, AmphL-mediated hydroxylation occurs at C-8 rather than C-10. This difference may account for the modest activity of NypY towards amphotericins.

In this study we introduced NypY into an *amphL* mutant of *S. nodosus*, which synthesises 8-deoxyamphotericins A and B. These analogues were glycosylated more efficiently than other amphotericins. One of these disaccharide analogues was purified and characterised by NMR, and its antifungal, haemolytic and aggregation properties were assessed.

Materials and Methods

S. nodosus amphL was described previously (Byrne et al., 2003). This is strain IMD2700 in culture collection WDCM227 maintained in University College Dublin. The plasmid pIJ10257-*nypY* was a gift from Ryan Seipke and Matt Hutchings, University of East Anglia. The *nypY* gene has GenBank accession number ADUJ01000856.1 (Barke et al., 2010). The pIJ102567-*nypY* plasmid was originally constructed by engineering the *nypY* gene sequence to contain an *Nde* I site overlapping the start codon and a *Hin* dIII site immediately after the stop codon. The modified sequence was cloned between the *Nde* I and *Hin* dIII sites of plasmid pIJ102567 (Hong et al., 2005; Gregory et al., 2003). The pIJ10257-*nypY* construct was introduced into *S. nodosus amphL* by protoplast transformation (Kieser et al., 2001).

Polyenes were extracted from *S. nodosus* cultures grown on fructose dextrin soya medium containing 5% (w/v) Amberlite XAD16 and 1% (w/v) glycerol (Hutchinson et al., 2010). Thiostrepton and hygromycin were used at final concentrations of 50 μg/ml. Flasks were incubated with shaking for 6 days at 30 ^{o}C and 124 rpm. HPLC was carried out using a Varian ProStar 210 HPLC system with a diode array detector and Galaxie workstation software. Analytical separations were carried out using a Zorbax SB 4.6 x

150 mm C18 column as described previously (De Poire et al., 2013). For LC-MS, quadrupole time of flight mass spectrometry was carried out using a XEVO instrument linked to a Waters Acquity LC system. Solvent A was 1% (v/v) formic acid in water, solvent B was 1% formic acid in acetonitrile. A gradient of 20 to 40% B over 4.25 min was used for separation of heptaenes whereas a gradient of 5 to 100% B over 2.62 min was used for tetraenes. Heptaenes and tetraenes were detected at 405 and 319 nm respectively.

For purification of hexosyl-8-deoxyamphotericin B, *S. nodosus amphL* pIJ10257-*nypY* was grown in 250 ml volumes of production medium in 2-litre trigrooved Erlenmeyer flasks. The total culture volume was 12 litres. After incubation, the thick culture was strained through muslin cloth to separate mycelium, Amberlite XAD16 resin and polyenes from residual liquid medium. The filtered solid material was rinsed in deionised water, and soaked in 1 litre methanol overnight to extract polyenes. The methanol was replaced several times until extracts contained only trace quantities of polyenes, as determined by analytical HPLC. Between 16 and 20 extractions were required to extract all of the polyene, because of the low solubility of 8-deoxyamphotericins. The extracts were combined, and the methanol was removed under vacuum until only residual water remained. This was refrigerated overnight to precipitate polyenes which were collected by centrifugation at 6000 rpm for 15 minutes. To remove lipids, sugars, and other contaminants, the precipitated material was washed by suspension and resedimentation as follows: four times in 30 ml volumes of diethyl ether, once in 30 ml water, and once in ethyl acetate. The partially purified polyenes (around 60 – 70 mg heptaene) were dissolved in 300 ml methanol and filtered through silica to remove impurities that strongly adsorb to chromatography columns. Preparative HPLC was then used to separate the mannosyl-8-deoxyamphotericin B from the much greater quantity of co-produced 8-deoxyamphotericin B and other polyenes. A Supelco Acentis C18 column (21.2 x 250 mm) was used. Solvent A was distilled water, solvent B was 0.1% (v/v) formic acid in methanol. A sample containing 4 mg heptaene in 2 ml methanol was injected for each run. The flow rate was 14.8 ml per min. A long gradient of 50% - 70% B over 40 minutes was required as all polyene components exhibited similar retention times. The peak fractions were immediately neutralised with saturated ammonium carbonate solution to prevent degradation, the methanol was removed under vacuum and the water removed by lyophilisation. Approximately 0.2 mg hexosyl-8-deoxyamphotericin B was obtained per run. Over 100 runs were required to obtain 20 mg purified material. LC-MS analysis of the isolated sample showed a single heptaene with a mass of 1070.5604, corresponding to hexosyl-8-deoxyamphotericin B.

NMR analysis was performed using a Bruker AV500 instrument with methanol-d4 (Cambridge Isotope Laboratories) as a solvent. Circular dichroism spectra were recorded using a Jasco J-810 spectropolarimeter. Polyenes were analysed at a concentration of 10 μM in phosphate buffered saline (PBS) in 2mm cuvettes. A scan of PBS buffer blank was subtracted from polyene spectra.

Antifungal activity was tested using the standard broth dilution test (Pfaller et al., 2002) with *Candida albicans* ATCC10231 as indicator organism. Haemolytic activity was tested against horse erythrocytes as described previously (Carmody et al., 2005).

Results

Engineered biosynthesis of mannosyl-8-deoxyamphotericins

Previous *in vivo* studies indicated that 7-oxoamphotericins were slightly better substrates for NypY than amphotericins A and B (De Poire et al., 2013). This led us to investigate whether NypY might act efficiently on 8-deoxyamphotericins. An *S. nodosus* mutant is available in which the *amphL* gene is inactivated by insertion of a KC515 phage genome (Byrne et al., 2003). This strain produces 8-deoxyamphotericins A and B (Murphy et al., 2010) but contains a thiostrepton resistance gene within the embedded prophage DNA, and cannot be transformed with pIAGO-*nypY* which contains the same *tsr* selectable marker (De Poire et al., 2013). The pIJ10257-*nypY* plasmid also has the *nypY* gene under the control of a strong *ermE* promoter, as well as a hygromycin resistance gene, a phage BT1 integrase gene, and an *attP* attachment site (Hong et al., 2005; Gregory et al., 2003). The chromosome of *S. nodosus* contains an *attB* integration site for this phage (Sweeney et al., 2016). It was found that *S. nodosus* protoplasts could be transformed with pIJ10257-*nypY* with low but reproducible efficiency (around 50 hygromycin-resistant colonies per μg DNA). The presence of the *nypY* gene in genomic DNA was verified by PCR. LC-MS analysis revealed that *S. nodosus* transformed with pIJ10257-*nypY* gave levels of hexosyl-amphotericins A and B similar to those produced by the same strain carrying the *nypY* gene on a replicating plasmid, pIAGO-*nypY* (not shown). A single integrated copy of the gene was as effective as multiple plasmid-borne copies. The pIJ10257-*nypY* plasmid was then introduced into the *amphL* mutant.

Analysis of polyenes extracted from *S. nodosus amphL* pIJ10257-*nypY* revealed extra heptaene and tetraene species not present in control extracts of *S. nodosus amphL*. The masses of the new polyenes were appropriate for hexosylated 8-deoxyamphotericins B and A (Fig. 2 and Fig.s S1 to S4). The hexosyl 8-deoxyamphotericin B ($[M + H]^+$ = 1070.5598) made up approximately 20% of the total heptaene whereas hexosyl amphotericin A ($[M + H]^+$ = 1072.5663) accounted for about 40% of the total tetraene. This suggests that 8-deoxyamphotericins A and B are better substrates for NypY than the C-8 hydroxylated forms. 8-Deoxyamphotericin A is structurally similar to 10-deoxynystatin, suggesting that this is the preferred acceptor for NypY, as is the case for NppY.

Purification of mannosyl-8-deoxyamphotericin B and structural analysis by NMR

S. nodosus amphL pIJ10257-*nypY* was grown on a large scale in production medium and the hexosyl-8-deoxyamphotericin B was extracted and purified by preparative HPLC (Fig. S5). Analysis by LCMS revealed a major heptaene with a mass of 1070.5604 (Fig.s S6 and S7). This material was analysed by NMR. Comparison of the spectrum with that of 8-deoxyamphotericin B shows new peaks indicating the presence of the additional sugar (Fig. S8), and also reveals a small downfield shift of the methyl doublet of the mycosaminyl moiety (Fig. S9). This is consistent with the extra sugar being a D-mannosyl residue in a β-1, 4 linkage to the mycosamine (Fig. 3). This was predicted from the high sequence homology between

NypY and known inverting polyene glycosyltransferases (PegA, AmphDI and NysDI) that use GDP-α-D-mannose or GDP-α-D-mycosamine (Caffrey et al., 2016; Stephens et al., 2013; De Poire et al., 2013).

Biological activities of mannosyl-8-deoxyamphotericin B

Mannosyl 8-deoxyamphotericin B showed approximately the same antifungal activity as amphotericin B. Both compounds showed an MIC of 1.56 μM. The concentrations giving 0%, 50% and 100% haemolysis (MHC_0, MHC_{50} and MHC_{100}) were 0.631, 1.585 and 3.162 μM for amphotericin B and 1.0, 2.512 and 7.943 μM for mannosyl-8-deoxy amphotericin B. Mannosylation appears to give a slight reduction in haemolytic activity.

Monomeric amphotericin B is specific for ergosterol-containing membranes whereas aggregates are also damaging towards cholesterol-containing membranes. Circular dichroism (CD) has been used to assess the aggregation state of amphotericins in aqueous solutions (Cybulska et al., 2000). The non-toxic semisynthetic analogue N-methyl-N-fructosyl-amphotericin B methyl ester (MFAME) shows a different CD spectrum to that of amphotericin B (Cybulska et al., 2000). This results from altered self association. The new disaccharide amphotericin was analysed by CD spectropolarimetry. At concentrations of 10 μM in PBS, amphotericin B and 8-deoxyamphotericin B showed similar spectra with positive bands at 320nm and negative bands at 335 nm. Mannosyl-8-deoxyamphotericin B gave a slightly different spectrum with positive and negative bands at 325 and 350nm (Fig. 4). This suggests that the extra sugar affects the structure of the aggregates formed.

Discussion

It has previously been shown that the AmphDI and NysDI mycosaminyltransferases can use GDP-α-D-mannose and GDP-α-D-mycosamine as donor substrates *in vitro* (Zhang et al., 2008). The high degree of sequence identity between these enzymes and the extending glycosyltransferases suggests that PegA and NypY use GDP-α-D-mannose as activated sugar donor. While N-acetyl-D-glucosamine is activated as a UDP-sugar in many organisms, the PtmE nucleotidyltransferase from *Campylobacter jejuni* has been shown to form GDP-N-acetyl-α-D-glucosamine (Schoenhofen et al., 2009). Either the UDP- or the GDP-linked sugar could be the substrate for NppY. Biochemical experiments are needed but so far it has not been possible to overexpress a polyene extending glycosyltransferase in a functional form. In this work, we show that NypY can catalyse formation of mannosyl-8-deoxy amphotericin B *in vivo*.

With NDP-sugars and deoxysugars, D sugars are α-linked to nucleoside diphosphates and L sugars are β-linked. In natural product biosynthesis, most of the glycosyltransferases that use NDP-sugar donors catalyse reactions that invert the stereochemistry of the anomeric carbon atom, so D sugars are β-linked to acceptors whereas L sugars are α-linked (Rix et al., 2002). In 67-121C, there is a β 1, 4 linkage between the D-mannosyl and D-mycosaminyl residues. This shows that PegA is an inverting glycosyltransferase. The present work shows that NypY catalyses formation of a β 1, 4 linkage and is also inverting.

It has been established that the acceptor substrate for NppY in *Ps. autotrophica* is 10-deoxynystatin (Kim et al., 2015). Furthermore, the *Ps. autotrophica* C-10 hydroxylase (NppL P450) acts efficiently on 10-deoxyNPP but not 10-deoxynystatin (Kim et al., 2016). These findings could explain why expression of NypY or NppY in *S. noursei* did not result in production of disaccharide-modified nystatins (Seipke et al., 2012; Kim et al., 2015). In *S. noursei*, monosaccharide nystatins that have been C-10 hydroxylated by NysL may be poor substrates for the two extending glycosyltransferases. This view is supported by our finding that NypY glycosylates 8-deoxyamphotericins more efficiently than C-8 hydroxylated amphotericins. Since the polyol chain of 67-121C is not hydroxylated by a cytochrome P450, additional mechanisms may ensure that this disaccharide polyene is not exported before the post-polyketide steps are complete. The ABC transporters from *A. caeruleus*, *Ps. autotrophica* and *Pseudonocardia* sp P1 may have a preference for polyenes that are modified with disaccharides rather than monosaccharides. This would allow for co-ordination between late modifications of the macrolactone and export of the final product. Further genetic engineering to exchange transport proteins and P450 enzymes could push the extent of disaccharide formation towards 100%.

With some bioactive natural products, additional sugar residues decrease rather than increase biological activity. OleD- or OleI-catalysed glucosylation of oleandomycin results in inactivation of the antibiotic (Quiros et al., 1998). Glycosylated bacillaenes and geldanamycins are less active than non-glycosylated forms (Nonjuie et al., 2015; Wu et al., 2012). NPP has slightly lower antifungal activity than nystatin A1 but the additional sugar confers an improvement by increasing water-solubility (Lee et al., 2012). As regards antifungal activity and haemolytic activity, mannosyl-8-deoxyamphotericin B shows little difference from amphotericin B and 8-deoxy-amphotericin B. This suggests that the extra β-1, 4 linked sugar *per se* is insufficient to improve the therapeutic index of amphotericin B. With MFAME, the free carboxyl group is converted to a methyl ester, the C-8 hydroxy group is present, the extra fructosyl sugar is attached to the mycosamine amino group, which is also methylated to give a tertiary amine that maintains a net positive charge. All of these structural features contribute to the reduction of toxicity. In aqueous media, the self-association of MFAME differs from that of amphotericin B (Szlinder-Richert et al., 2001). The CD spectra suggest that this is also true of mannosyl-8-deoxyamphotericin B, but further modifications are necessary to afford better antifungal drugs.

In the chemical production of MFAME, imine formation between the mycosamine amino group and glucose is efficient and scalable, and eventually yields N-fructosyl amphotericin B (Cybulska et al., 2000). If the starting materials were biosynthetically produced polyenes with C4′ hexosylated mycosamine residues, then new analogues with branched trisaccharides should be accessible. Production of disaccharide-modified polyenes by fermentation is therefore of interest. However, while 67-121C is abundantly produced by *A. caeruleus*, the yields from *Ps. autotrophica*, *Pseudonocardia* sp P1 and engineered *S. nodosus* strains are low. Further efforts in synthetic biology will be required to produce these compounds in practical quantities.

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Compliance with ethical standards

This study was conducted with the financial support of Science Foundation Ireland, grant number 09/RFP/GEN2132. All authors declare no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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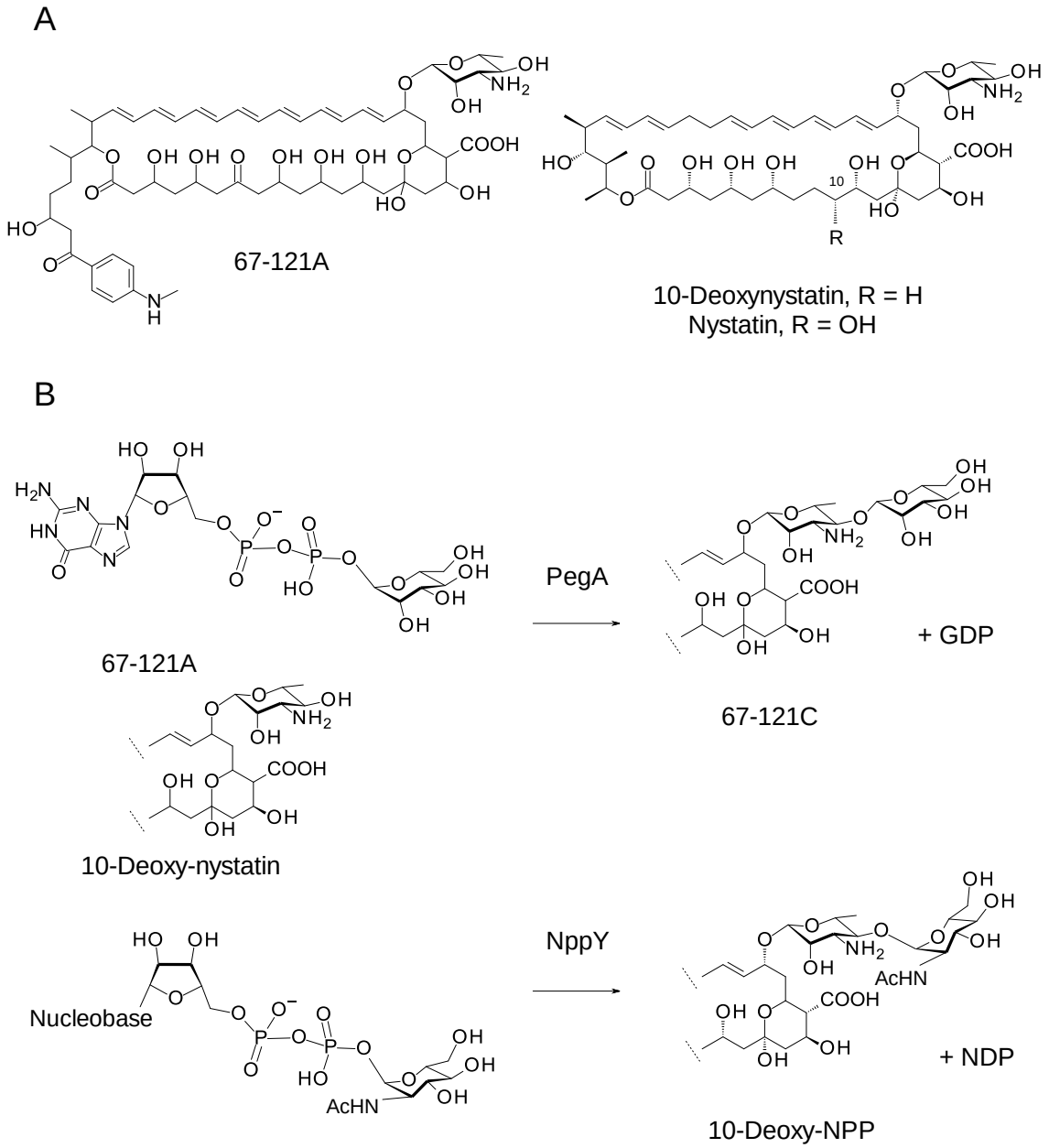
Figure legends

Fig. 1. Naturally occurring disaccharide polyenes. (A) Structures of 67-121A, 10-deoxynystatin and nystatin. (B) PegA catalyses mannosylation of 67-121A and NppY catalyses transfer of N-acetylglucosamine to 10-deoxynystatin. The most likely NDP-sugar donors are GDP-α-D-mannose for PegA, as shown, and UDP- or GDP-linked N-acetyl-α-D-glucosamine for NppY.

Fig. 2. HPLC analysis of heptaenes extracted from *S. nodosus amphL* (A) and *S. nodosus amphL* pIJ10257-*nypY* (B). The hexosylated 8-deoxy amphotericin B peak in panel B is arrowed. The major peak in both chromatograms is 8-deoxyamphotericin B.

Fig 3. *In vivo* mannosylation of 8-deoxyamphotericin B by NypY. The C28-C29 double bond is reduced in 8-deoxyamphotericin A and mannosyl-8-deoxyamphotericin A.

Fig. 4 CD spectra of (A) amphotericin B (B) 8-deoxyamphotericin B, and (C) mannosyl-8 deoxyamphotericin B. Polyenes were at a concentration of 10 μM in PBS.



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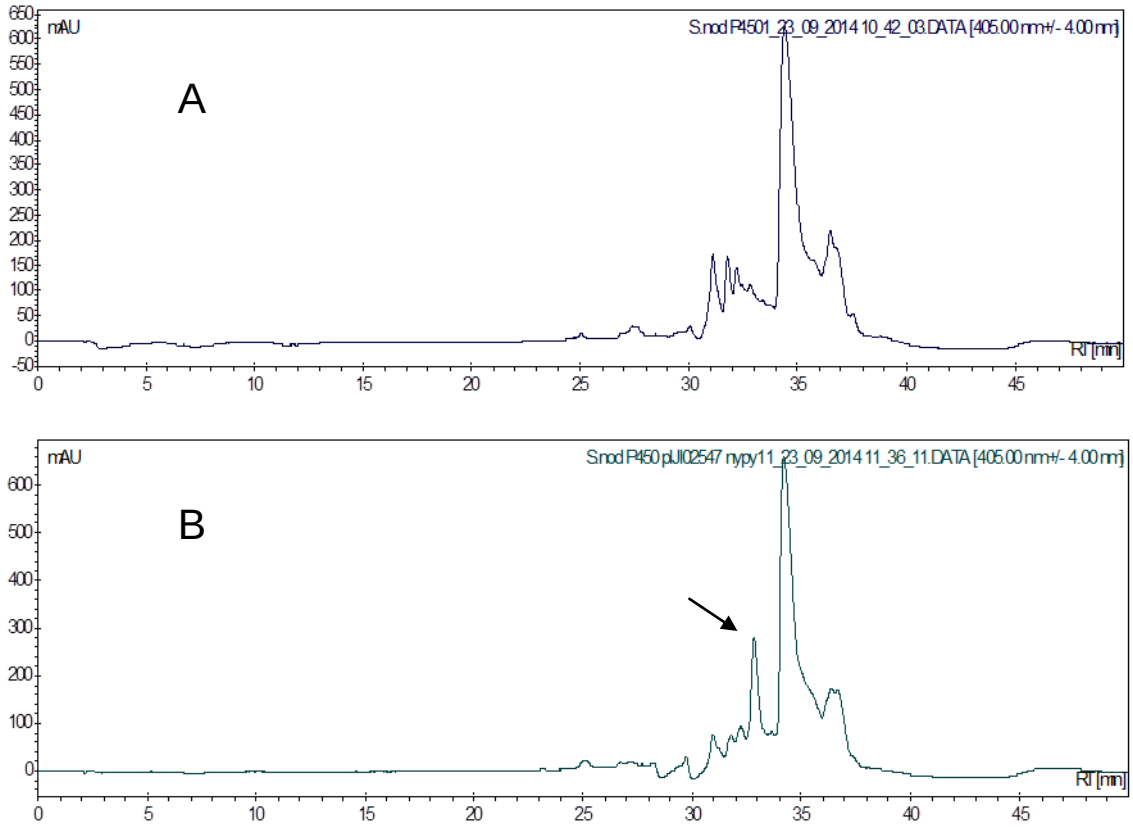

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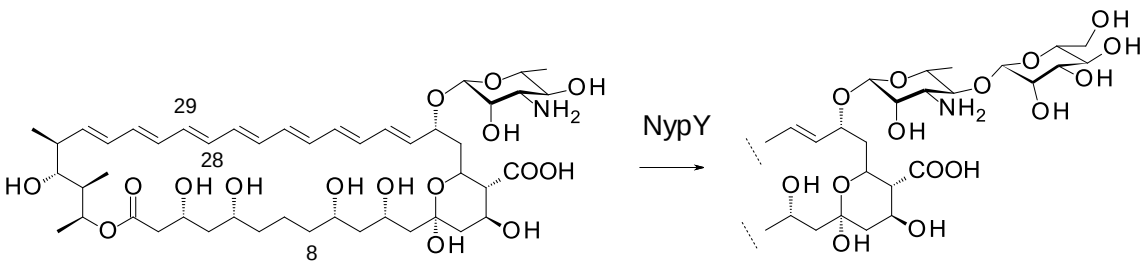

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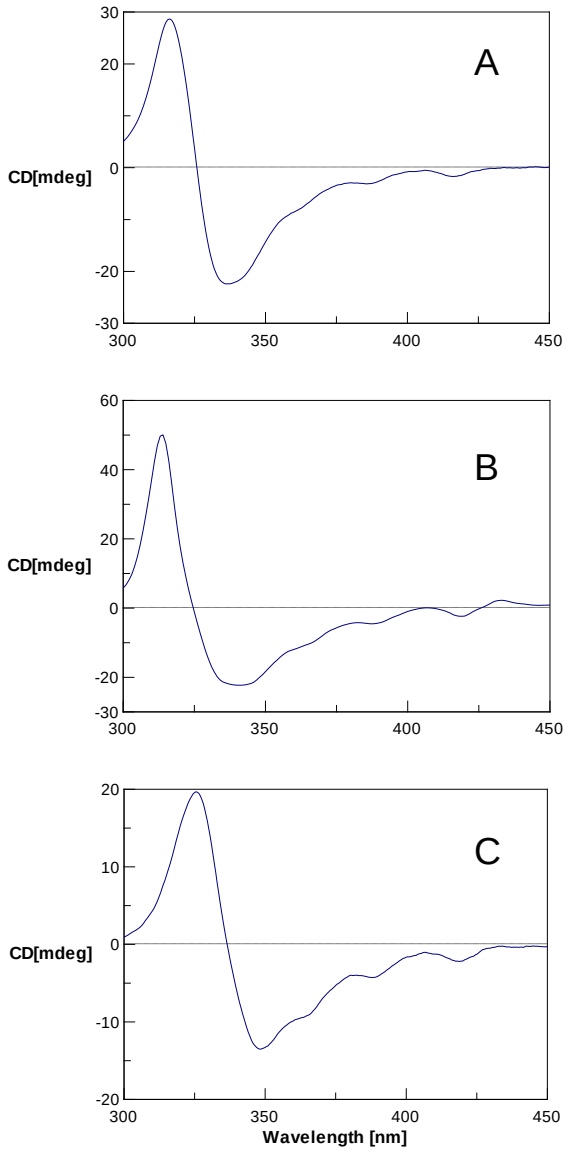

Fig. 4 CD spectra of (A) amphotericin B (B) 8-deoxyamphotericin B, and (C) mannosyl-8-deoxyamphotericin B in phosphate-buffered saline.